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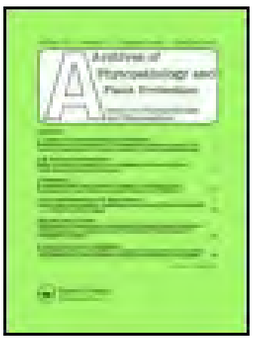

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First report of leaf spot disease caused by Corynespora torulosa MCC-1368 on cotton plant from India

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First report of leaf spot disease caused by *Corynespora torulosa* MCC-1368 on cotton plant from India

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ABSTRACT

In present study, the leaf spot disease of cotton plant emerged in the North Maharashtra region of India was reported. The fungal phytopathogen associated with inducing the leaf spot disease symptoms was isolated and characterised. The isolated fungus was identified as *Corynespora torulosa* (Deposition accession number, MCC-1368; Genbank accession no. MF462072) based on morphological and cultural characteristics and molecular analysis of ITS region. The pathogenicity of fungal phytopathogen was verified by Koch's postulates. To our knowledge, this is the first report of incidence of leaf spot disease caused by *Corynespora torulosa* on cotton plant.

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KEYWORDS
Phytopathogen; leaf spot disease; *Corynespora torulosa*; cotton plant; first report

1. Introduction

Cotton is one of the most preferred and major fibre crop of global significance and cultivated in almost every tropical country. In India, it is the leading fibre crop with the largest cultivation area that constitutes about 41% of the global cotton area (12.846 M ha) (http://cotcorp.gov.in/index.aspx). The cotton is also serves as the most important raw material (holding 59% share) for the Indian textile industry (WWF-India report 2012), which plays a crucial role in Indian economy and growth rate. Cotton production in India is not only associated with the economical growth but also plays important role in sustaining the livelihood of an estimated 5.8 million farmers.

Among the major cotton growing regions of India, North Maharashtra region alone occupies 0.5 M ha (5.23% of total land of India) area under cotton cultivation (Patil and Waykole 2013). In this region, the area under Bt cotton cultivation is around 94.7% of total cotton cultivation. The worldwide cotton production is severely affected by various biotic and abiotic stresses. Among the biotic stresses,

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fungi and bacteria contribute about 11% of total loss of cotton yield (Oerke and Dehne 2004). During the survey of various cotton fields of North Maharashtra region was noticed with leaf spot diseases in cotton cultivation seasons- 2013–2014 to 2014–2015 (June–September). Keeping in view the contribution of cotton in agriculture, its impact on economy and employment and present seedling disease prevalence on cotton crop; the present research is associated with disease incidence, severity of seedling disease, isolation and identification of causative agent and confirmation of pathogenicity by Koch's postulates.

2. Materials and methods

In order to detect the prevalence of disease, a systematic survey was carried out in the cotton fields (Bt RCH-2 variety) of North Maharashtra region between two successive cotton cultivation seasons of year 2013–2014 and 2014–2015 (June–September). The cotton plants were observed with leaf spots of various severities and incidences.

In order to isolate the pathogen associated with leaf spot disease of cotton crop, the leaves with dark brown lesions were collected in sample collection bag (Hi-media) and brought to the laboratory. The surface sterilisation and isolation was performed as described by Dau et al. 2008. The diseased plant leaf samples were washed thoroughly under running tap water to remove the surface contaminants. The small sections (0.5 × 0.5 cm) from the diseased area were cut and surface sterilised with 1% $HgCl_2$ solution for 2 min followed by three-four washes of sterile distilled water and then blotted dry on sterile tissue paper. A symptomless leaf section was taken as a control and surface sterilised. The surface sterilised sections were aseptically placed on Czapek dox agar (CDA) medium supplemented with 1 mg/ml of streptomycin. The plates were incubated in dark condition at 28 °C for six days. The developed fungal colony from the section was further sub-cultured on fresh CDA plate until pure culture of the isolate was obtained.

The morphological feature of the isolated fungal strain was evaluated using slide culture assembly. The morphological features (colony characteristics, mycelial growth pattern, radial growth pattern, spore forms and size of the conidia) were systematically studied as per the key described by Watanabe 2010; Nghia et al. 2008 and Kumar and Singh 2016.The molecular identification of the fungal isolate was carried out at National Centre for Microbial Resource, National Centre for Cell Science, Pune, India. Amplification of ITS region was carried out with universal primers ITS4 and ITS5 according to the protocol of White et al. 1990. The amplified polymerase chain reaction (PCR) products were sequenced using ABI-Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The Basic Local Alignment Search Tool (BLAST) analysis of sequenced data was performed against the non-redundant nucleotide (nr/nt) database and similarity analysis was done using Clustal W. The phylogenetic tree was constructed using MEGA 5.0 software with bootstrap value of more than 50%.

The pathogenicity of the isolate was confirmed by Koch's postulates. The conidial suspension of fungal isolate was prepared by scraping the mycelium to sterile distilled water from 7 day old culture. The spore count of spore suspension was adjusted to 1×10^6 conidia/ml using hemocytometer. The experiments were performed in greenhouse (30/25 °C day/night temperature with a 16 h of photo period). The healthy cotton seedlings (Bt RCH-2 seeds) at the four-leaf stage (18 day older plant) were sprayed with conidial suspension of fungal isolate until the upper and lower leaf surfaces, petioles and stem were uniformly wet. While the control plants were sprayed with sterile distilled water. The vigour index and disease severity index (DSI) of control and test plants were measured as per Islam and Borthakur 2012; Shtienberg 1996; Zhao et al. 2014, respectively. DSI = ∑ (disease severity scale point's × number of plants at each scale point)/(total number of plants surveyed × disease severity scale of the highest scale point observed) × 100.

The experiment was performed in quadruplets. Koch's postulates were satisfied by re-isolating the pathogen from symptomatic test plants.

Analysis of variance (ANOVA) was conducted on the DSI data to determine the overall effect of the isolate and its interactions with cotton plant. The comparisons of the mean DSI data were made using Dunnet's multiple range test ($p < 0.05$).

3. Results

The survey study revealed that the cotton crop was severely affected with leaf spot disease. The leaf spots were irregular in shape, the spots smaller than 10 mm in diameter were tan to black in colour and the spots larger than 20 mm appeared with black borders and yellow halos (Figure 1). The % disease incidence (DI) was recorded for each surveyed region. The disease incidence was observed between

Figure 1. The brown coloured leaf spots on diseased cotton plant observed during survey.

5–36%. Maximum DI was recorded in the month of August when the climatic conditions were congenial for disease development (temperature 22–32 °C, moisture 55–85%).

The leaf spot causing pathogen was isolated from the leaf section of diseased cotton plant and was further sub-cultured and maintained on CDA medium at 28 ± 2 °C. The colony of fungal isolate is slightly polygonal, thick in texture with smooth margin, dark grey on the surface and dark brown on back side (Figure 2). The conidia are long, solitary, straight to slightly curved, obclavate to cylindrical, tapering towards the apex, pale brown, smooth and unbranched (Figure 3). The Table 1 also describes the detail morphological features and cultural characteristics of isolated fungal pathogen. Based on morphological and cultural characteristics, the fungus was identified as *Corynespora*. The BLAST analysis and sequence identity of ITS region by Clustal W identified the fungal isolate as *Corynespora torulosa* (Figure 4). The ITS sequence of *Corynespora torulosa* was successfully deposited to NCBI with Genbank accession number of MF462072. The identified phytopathogenic fungus was deposited at National Centre for Microbial Resource, National Centre for Cell Science, Pune, India with accession number MCC-1368.

In pathogenicity test, after 14 days of inoculation, the tested isolate showed leaf spot symptoms in plants treated with conidial suspension which matched with naturally infected plant symptoms, whereas the control plants were symptomless (Figure 5). There is significant difference in DSI of test plant (44.38) and control plant (1.88). The vigour index of test plant was reduced with 30.84% as compare to the vigour index of control plant (Table 2). The fungal pathogen

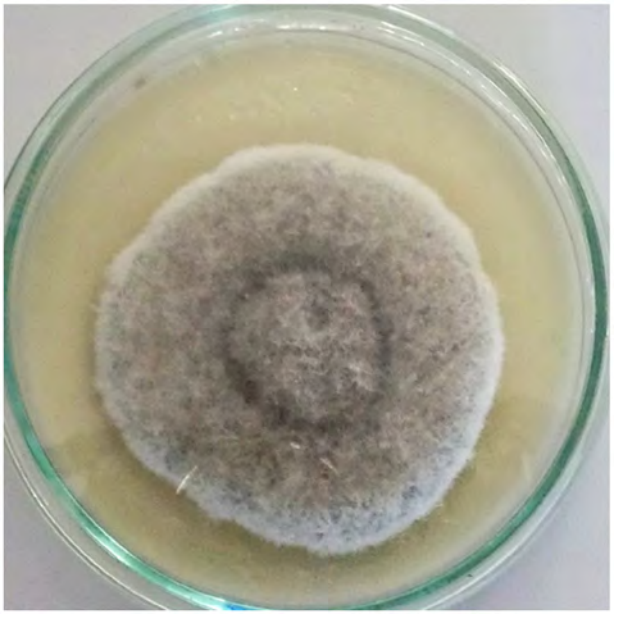

Figure 2. The 6 day old fungal colony of phytopathogen grown on CDA plate incubated at 28 °C.

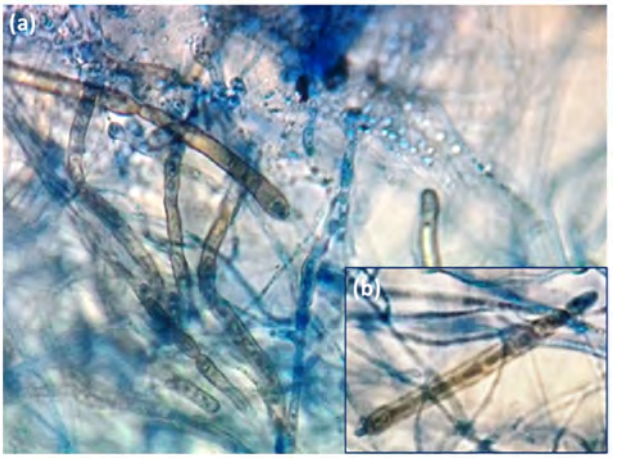

Figure 3. (a) The long thread like septate conidiophore and (b) the smooth and un-branched conidia of *Corynespora torulosa* strain L4 observed under 40× objective lens of compound microscope.

Table 1. Visual growth characteristics of fungal isolate on Czapek dox agar medium after 7 days incubation period at 28 °C.

Characteristics	Description
Growth rate (mm/d)[a]	9 ± 0.28
Colony diameter (mm)[b] Mean ± SD	56.33 ± 0.58
Growth pattern	Thick in texture, polygon in shape, Moderate growth, flat textured, smooth margin
Colony colour	Top – dark grey, Bottom – dark brown
Conidiophore	Long thread like, erect to slightly bent, simple, sparingly septate, non stromatic arising singly from hyphae, branched to unbranched, macronematous
Conidia	Long, solitary, straight to slightly curved, obclavate to cylindrical, tapering towards the apex, pale brown, smooth, unbranched
Conidia size[c]	35–195 × 4–16 µm
Septa	Distoseptate (4–20)
Sporulation[d]	+++

Notes: [a]Growth rate was measured in mm/day, [b]Colony diameter measured at 7th day of incubation, [c]Mean values of length and width of 50 randomly picked conidia ± standard deviation, [d] + Poor sporulation: 1–10 spore/microscopic field (40×), ++ Medium sporulation: 11–50 spores/microscopic field, +++ Good sporulation: More than 100 spores/microscopic field.

was reisolated consistently from the tissues of treated plant, thereby fulfilling the Koch's postulates.

4. Discussion

A number of fungal diseases are prevalent on cotton crop which can cause severe loss of yield. Indian cotton fields are previously reported to be affected with fungal foliar diseases caused by species of *Myrothecium*, *Alternaria*, *Cercospora* and *Curvularia* (AICCIP 2013–2014). Field survey showed that the cultivated cotton crop is sensitive to leaf spot disease and disease severity index was varied from 5 to 36%. There was no any reported incidence of *Corynespora torulosa* on cotton.

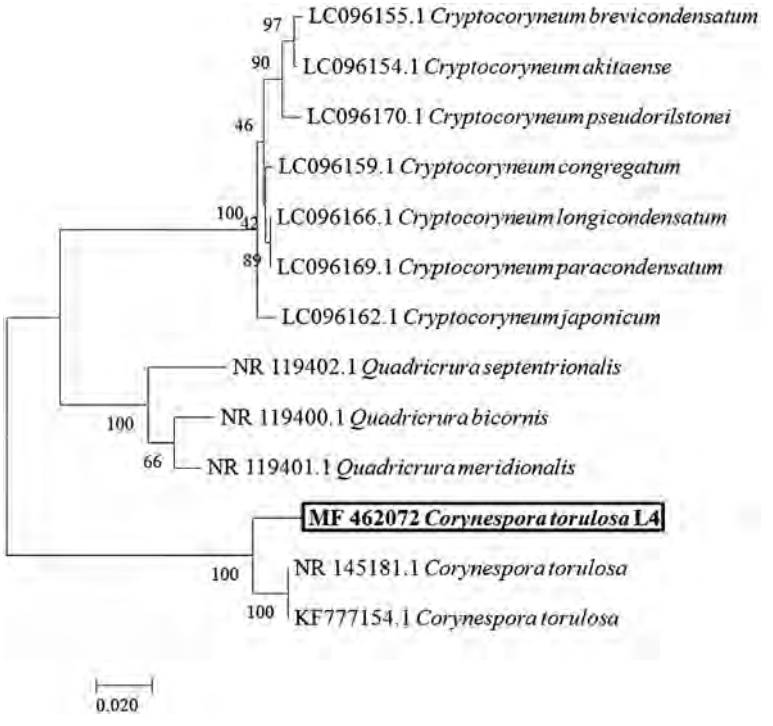

Figure 4. The phylogenetic relationship of fungal isolate *Corynespora torulosa* strain L4 with related fungal strains based on ITS sequences. The phylogenetic tree was constructed using MEGA 5.0 software with bootstrap value of more than 50%.

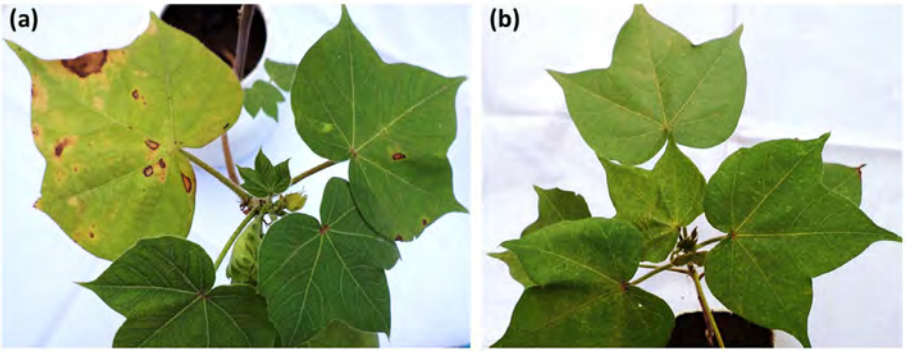

Figure 5. The results of pathogenicity test; (a) test plant with the brown coloured leaf spots and (b) symptomless control plant.

Table 2. DSI and vigour index of test and control plants observed in pathogenicity test.

Cotton plant	DSI	Vigour index	Decrease of vigour index over control (%)
Test	44.38a (1.77)	2924.78a	30.84
Control	1.88b (0.075)	4229.1b	–

Notes: $p < 0.05$ Vs Control after treatment when calculated by one way ANOVA using Dunnet's multiple range test ($p > 0.05$). Values in the same column followed by different lower case letters are significantly different from each others. The numbers in parenthesis describes the average infection scale.

Hence, this is the first report of *Corynespora torulosa* causing leaf spot disease of cotton plant. During literature survey it was observed that, there are no reports of *Corynespora torulosa* as phytopathogen of cotton but the synonyms of this fungus; *Deightoniella torulosa* and *Brachysporium torulosum* (Crous et al. 2013) have been previously reported with other host plants like; banana (Kone et al. 2008) and grande naine (Mora et al. 2013).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Operative utility of salt-stable proteases of halophilic and halotolerant bacteria in the biotechnology sector

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Operative utility of salt-stable proteases of halophilic and halotolerant bacteria in the biotechnology sector

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Abstract

Proteases are universal in existence in all organisms and are needed for metabolic activities of the cell. Also, proteases are the paramount hydrolytic enzymes widely used in industrial sector accounting ~65% of the aggregate worldwide enzyme market. The market revenue of global protease sale is estimated to be $ 2.21 billion by 2021.

Currently, a widening expectation of biotechnology sector necessitates the availability of robust protease with industrially suitable operative features. The commercially-compatible protease should show stability and altered specificity in organic solvents, and be economically as well as ecologically sustainable.

The proteolytic halophilic and halotolerant microorganisms are a novel source of salt-stable proteases. Beside stable in the presence of significant amount of salt, the halo-proteases could occasionally display the polyextremophilic attributes like tolerance to alkaline pH, high temperature, and organic solvent tolerance etc.

This review describes - halophilic/halotolerant proteolytic microorganisms as source of salt-stable protease, mechanisms of high salt tolerance, various physical and nutritional parameters affecting the protease production, purification strategies for protease, characteristics of salt-stable proteases, and commercial significance of salt-stable proteases. The study revises the current status of the research on salt-stable proteases obtained from extremophile with their operative utility in the biotechnology field.

Keywords: Halophiles; Halotolerant; Salt-stable proteases; Organic solvent; Ionic liquids; Detergent industry; Leather industry; Bioremediation; Biomedical industry; Food industry

Content

1. Introduction

Proteases are enzymes having the unique significance in cellular and physiological processes and are synthesized by all organisms. They hydrolyze the protein into soluble peptides/free amino acids. Nearly 2% of the total genome of typical cell encodes for proteases which required for various metabolic and physiological functions [1]. Also, the proteases are extensively used in the industrial sector. Among the industrial enzymes, proteases are the dominant and largest selling industrial biocatalyst [2, 3]. The global market for industrial enzymes is growing at a compounded annual growth rate (CAGR) of 7% from 2015 to 2020 and predicted to occupy $ 6.2 billion by 2020 [4]. The global protease market revenue is estimated to be $ 2.21 billion by 2021 [5]. Among various sources like - animals, plants, and microbes; the microbial sources are preferred for commercial scale protease production. The microbial fermentative enzyme production offers several advantages like - rapid microbial growth, low space needed for cultivation and comparatively easy genetic manipulation protocols [6].

The expanding horizons of biotechnology sector anticipate the availability of robust protease with important features which make it technologically suitable commodity. Besides its ability to function at wide range of temperature and pH; the capability to endure in presence of range of surfactants, soaps, oxidizing agents, bleaches, hydrotropes, stabilizers, brightening agents, builders, organic solvents and salts. This augments the utility of protease in detergent, leather, cosmetics, pharmaceutical, textile, food, waste processing industries. Also, a monetary point of view, the low protease requirement in a typical formulation or biocatalytic reaction further anticipates the availability of robust and functionally efficient protease. The protease from halophilic microorganisms could display superior activity and stability under bizarre conditions like high alkalinity, high temperature, and high salt, organic solvents, ionic liquids, and ultrasonic vibrations.

The salt-stable characteristics adapted by protease could offer the enzyme to remain active in low-water environments and capability to remain functional in organic solvents [7]. The functional ability of an enzyme to work in low water medium/ organic solvents is elucidated as non-aqueous enzymology. The non-aqueous catalysis offers several advantages like (i) stability improvement, (ii) modifications in substrate specificity, (iii) enantiomeric peculiarities, and (iv) elevated product yield [8,9]. The enzymes of halophilic microbe are an appropriate candidate for efficient use in non-aqueous media [10]. From a synthetic point of view protease in anhydrous organic solvents are useful for enzymatic reactions like trans-

esterification, ammonolysis, thiolysis. Such proteases are also useful for stereo-selective, region-selective and chemo-selective changes in a substrate [11].

The protease and protease-blended formulations are favoured in progressing scenario of biotechnological sector. The attributes like - higher reaction rates, operated in ambient reaction conditions, greater reaction specificity and capacity for regulation makes the enzymatic transformation more advantageous compare to chemical processes. The enzymatic protocol also offers environmentally benign set of principles and curtails the usage or genesis of hazardous substances during fine chemical synthesis.

The use of ionic surfactants, amphoteric surfactants, bleaching agents, oxidizing agents, stabilizers, builders, alkalies, brightening agents, ionic liquids, hydrotropes, organic solvents, and occasionally ultrasonic energy in biotechnology sector have generated new challenges on use of enzyme in this field.

In last decades various salt-stable proteases have been documented to be displayed better stability in high salt, alkalinity, wide range of temperature, and are found active in a range of chemicals otherwise behaves as chaotropic agents. Oren has forecasted that the commercial utility of halophilic microbial proteases would depend on the accumulation of scientific knowledge about their enzymology and protein structures [12]; here we reviewed the current trend and utility status of halophilic and halotolerant microbial proteases.

2. Salt dependence of microbes

Salts are ionic compounds consists of metallic 'cation' (positively charged) and non-metal 'anion' (negatively charged). The overall electrical charge on the salt molecule is zero. The cation and anion are strongly bound by ionic bonds. The nature of ionic bond decides the physical and chemical characteristics of salts. The properties of salts include (i) ordered structure – crystal lattice, (ii) strong ionic bonds conferring hardness to salt crystal, (iii) salts have high melting/boiling point, (iv) salts show electrolytic properties; on dissociation in water it forms ions, and (v) salt solution conduct the electricity. The force (F) between two electrical charges in a salt molecule is determined by Coulomb's law:

$$F = \frac{Kq_1q_2}{Dr^2}$$

Where, F, a force between two electrical charges; q_1 and q_2, electrical charges; r, a distance between two ions; D, the electric constant of the medium; and k, a proportionally constant (8.99×10^9 J.m.C^{-2}) [13].

Liquid water and dissolved salts are vital for all forms of life. In water, the salts dissociate as cation and anion and play a significant role in the metabolic process. For example,

the cell membranes of living organisms have pumps to exclude Na^+ and to concentrate K^+ in the cytoplasm.

An aqueous sodium chloride solution (0.85%, w/v) is generally used as saline to hold the microbial culture [14]. The saline provides osmotic protection for typical microbial cells. The excess of salts exerts high osmotic pressure on the cell. An ideal osmotic pressure is accountable for growth and survival of any organism. The most of terrestrial and freshwater-based microbes are unable to survive at high osmotic pressure. They get died or became dormant in high salt containing environs [15].

Also, maintaining osmotic pressure, the salts are physiologically important for the cell to conduct various biochemical reactions. The typical non-halophilic cell consists of various inorganic elements; a majority of them are essential cofactors of several important enzymes [16]. Table 1 summarizes the biological significance of inorganic elements.

The biotopes consisting of saline and hypersaline environments are widely distributed in various geographical locations like saline soils, saline lakes, salt pans/ marshes, sea water, marine ecosystems, salted meat, and salt evaporation pools *etc.* [17-21]. In hypersaline biotopes, evaporation of seawater is accountable for formation of gradients in salinity. Hypersaline environments contain extra salts as compared to seawater (3.5% total dissolved salts). Hypersaline biotopes are grouped into (i) thalossohaline and (ii) athalossohaline. Thalossohaline biotope is hypersaline environment having a similar salt composition to that of seawater. Sodium and chloride are dominant cation and anion, respectively followed by Mg^{2+}, Ca^{2+} (cation), and SO_4^{2-} (anion). The pH of thalossohaline biotope is neutral to slightly alkaline; hypersaline marine lagoons, solar salterns evaporation pond, Great Salt lake, Utah are examples of thalossohaline biotope. Athalossohaline environment contains very different salt composition from that of seawater. It contains a high concentration of carbonate and bicarbonate while chloride and bromide are dominant ions. It contains very trace amount of Mg^{2+}, Ca^{2+}, and SO_4^{2-} . The pH of athalossohaline biotope is 10-11; alkaline soda lakes, Dead Sea are examples of athalossohaline environment.

Various microorganisms were isolated from saline soil, seawater, saline/hypersaline lakes, salt pans, salt marshes, brine springs from underground salt deposits, playas, solar salterns, soda lakes, salt mines, deep-sea sediments, and fermented fish sauces [19,22-24]. These microorganisms have the ability to survive over a wide range of salinities even at saturation concentration of salt (>3 M). The archaea are extreme halophiles which grow in

optimum salt concentration (3.4–5.1 M NaCl) [25]. The salt dependence is also be determined by on physical factors like temperature, pH, and redox potential [26].

Kushner classified the microorganisms depending on their salt requirement. The true halophilic organisms obligatorily require >3 M NaCl for growth while halotolerant organisms are not dependent on salt for growth but are able to tolerate the appreciable salt concentration (<1M NaCl) [27]. The classification of microbes based on their salt concentration requirements is summarized in Fig. 1.

The halophilic and halotolerant microorganisms are well adapted to higher salt concentration; the enzymes produced by them must retain activity in higher salinity and low water activity environs. The halophiles essentially have ability to secret the salt-stabilized enzymes to retain their survival in salinity. Although the intracellular composition (by virtue of compatible solute) is not adapted tolerate the high salt; but outside the cell, it should secrete the high salt-adapted extracellular enzyme. A potential halophilic/halotolerant strain having the ability to secret substantial extracellular hydrolytic enzymes could be further screened as commercial producers and used for the biotechnology industry.

2.1. How cell survive in excess /extreme salt concentration?

Typical cell immersed in a relatively higher salt (hypersaline) containing solution than the cytoplasmic fluid are inescapably lost water and undergo plasmolysis. The effect of surplus salt on typical non-halophilic bacterial cell has been well documented [28,29]. Halophiles prefer the high salt concentration for growth and survival. They possess the unusual adaptive mechanisms as compared to the typical non-halophilic microbes. A perfect model that precisely clarifies that how the cell stabilizes the osmotic pressure in presence of high salt surroundings is still waiting for a precise explanation [30]. Rather than a distinct mechanism, multiple strategies are adopted by halophilic microbes to prevent the loss of cytoplasmic water and to establish osmotic equilibrium across their cell membranes. Several adaptive mechanisms are illustrated as follows.

2.1.1. Salt-in-cytoplasm adaptation

It is a process of accumulation of inorganic salts (e.g. sodium, chlorine and potassium ions) in the cytoplasmic fluid [31]. This involves cations flowing through the membrane into the cytoplasm (Fig. 2). Archaea accumulate intracellular potassium and exclude sodium, whereas bacteria accumulate sodium rather than potassium. As a cell immersed in high salt, the intracellular components (e.g. proteins, nucleic acid, and cofactors) require protection from the denaturing effects of salt [32].

The adaptation mechanism in the halophilic cell requires a higher concentration of salt. In low-salt environs, the lesser concentration of salt or scarcity of salt eliminates the 'shielding effect' of cations and contributes to rapid denaturation of the three-dimensional structure of proteins/enzymes. These observations have been well supported by several research investigators. Salt (particularly of divalent metal) ions autonomously stabilize and regulate the biocatalysis and structural conformation of RNase H1 from *Halobacterium* sp. NRC-1 [33]. The role of divalent Ca^{2+} in preventing denaturation and monovalent Na^{+} in the regulation of enzymatic activity was reported for the protease of *Bacillus* sp. EMB9 [23].

Extremely halophilic archaea and anaerobic halophilic bacteria use salt-in-cytoplasm strategy [34]. The aerobic archaea such as *Halobacterium* sp. [35], and *Natronococcus occultus* grown in salt rich media [36], and the bacterium *Salinibacter ruber* [37] use inorganic salts almost exclusively for osmotic balance.

2.1.2. Compatible-solute adaptation

This adaptive mechanism operates by accumulating a small molecular weight organic solutes in the cell, while excluding the salts from cytoplasm as much as possible, to balance osmotic pressure. These small organic and osmotically active molecules are referred as 'compatible solutes'. These compounds can be synthesized by *de novo* or imported from the surrounding medium (Fig. 3). The generation of compatible solutes within cytoplasm is an energetically expensive process [38]. The compatible solutes also behave as stabilizers for maintaining the active conformation of various macromolecules within a cell to acclimatize the extreme conditions (like heat, cold, desiccation, freezing and excess of salts *etc*). These compatible solutes are polar and dissolvable zwitterions and described as strong water-structure formers. They exert a stabilizing effect without any structural distortion of the protein molecule [34,39-42]. An array of compatible solutes has been recognized from several halophiles (Table 2).

Most halotolerant and moderately halophilic organisms, including the anoxygenic phototrophic bacteria, aerobic heterotrophic bacteria, cyanobacteria, and methanogens, maintain a salt-minimized cytoplasm by accumulating/generating compatible solutes [34].

2.1.3. Salt stable cell surface and membranes

Although inside the cell, cytoplasmic environment is safe from the unfavorable effect of salts by various mechanisms, but externally cell has been enforcedly exposed to high salt concentrations. The cellular membrane of halophilic bacteria elevates relative higher

proportion of anionic phospholipids with increasing salinity as compare to neutral phospholipids [34].

These alterations produce additional surface charges to the membrane and help to maintain the hydration state of the cytoplasmic membrane. Most halophilic archaea possess an S-layer consisting of sulfated glycoproteins, which surrounds the cytoplasmic membrane. The sulfate group imparts a negative charge to S-layer and involved in maintaining the structural integrity in presence of high salt. In addition, archaeal ether lipids are stable in even 5 M salt concentrations as compared to ester lipids found in the membranes of non-halophilic microbes [34].

2.2. Salt and protein interaction

The proteins are soluble in low salt solutions, as the ionic forms of salts get to associate with opposite charges within the protein. This leads to increased hydration of the protein surface. In contrary, the solution containing high salt exerts the increased surface tension of water induces competition between protein and salt ions for hydration. The salts ions responsible for the elimination of an essential layer of water molecules from the protein surface, this leads to denaturation of the protein [30]. Generally, proteins structure destabilized at high salt concentration due to hydrophobic interaction. In presence of higher amount of salt, proteins get altered with respect to (i) solubility, (ii) binding, (iii) stability, (iv) crystallization, (v) protein aggregation and precipitation [43,44]. These properties of proteins are controlled by the ionic interactions of solvent.

In contrary, the presence of salt is an essential to regain the active structural conformation of halophilic proteins/enzymes [45]. The enzymes of halophiles require the presence of high salt concentration (4-5 M) for exhibiting the solubility, stability and catalytic activity [46-48]. Several investigators reported that at low concentrations of salts or in the absence of salts the structural stability and subsequent enzyme activity was lost [45,49].

The halophilic proteins/enzymes were detected to contain (i) relatively higher number of acidic amino acids on the surface, (ii) low lysine, (iii) relatively low hydrophobic center of the protein, and (iv) more salt bridges. Several mechanisms are proposed to know the requirement of a relatively high amount of salt for stability and activity of halophilic proteins.

2.2.1. Proteins have negatively charged protein-surfaces due to acidic amino acids

A halophilic protein possesses negative charge due to hydrated carboxyl groups. The negative charges are shielded by a higher amount of salt, this further arrests unfolding and maintains the solubility of proteins [43,45,50,51] as illustrated in Fig. 4. As compared to

proteins of non-halophilic microorganisms, the proteins of some halophilic microbes comprised of the relatively high proportion of acidic amino acids like (i) serine and threonine [32], aspartic acid and glutamic acid [52,53] and alanine, serine, threonine, and valine [49,54]. Also, a halophilic protein contains a small number of non-polar amino acids like cysteine, isoleucine, leucine, lysine, methionine, phenylalanine, and valine residues [49,52-54].This feature is also correlated with the low pI value (4.2 to 6.8) associated with halophilic proteins [55-57].

These trends were verified by sequence comparison among the protein families. In this view, comparative sequence analysis of the non-halophilic, halophilic, mesophilic and hyperthermophilic glutamate dehydrogenase showed that the halophilic protein comprised of significantly more acidic amino acid residues; with 64% of the total number of charged residues are being either aspartic acid or glutamic acid [58]. The higher proportions of acidic residues and reduced proportions of lysine content have been also detected in elongation factor (EF-Tu) of *Halobacterium marismortui* [59], and halophilic archaeal dihydrofolate reductase [60]. The malate dehydrogenase of *Haloarcula marismortui* contains 20 mol% higher of acidic residues over basic amino acid residues, compared to only 6 mol% excess for a non-halophilic counterpart. The structural analysis confirmed that the acidic residues are located at the surface of the protein and involved to form a strong hydration shell. The crystallographic study based on halophilic and non-halophilic malate dehydrogenase from *Salinibacter ruber* and *Chloroflexus aurantiacus* respectively revealed that acidic amino acids disrupt the pentagonally organized network of water molecules of hydration shell within the halophilic protein [61].

Also, the excess of acidic residues on the enzyme surface confers protection to the enzyme from aggregation in high salt concentration solutions. Paul et al. suggested that the surface also displayed a reduction in surface-exposed lysine residue in halophilic proteins [62]. Oren documented the existence of 'acidic proteomes' in halophiles. The halophilic proteins are soluble in presence of high salt environment while non-halophilic proteins precipitate in such solutions [63]. Based on these studies, the "solvation-stabilization model" [64-66] was proposed which explains the thermodynamic basis and verifies that the protein solubility and stability alteration are correlated.

2.2.2. Conglomeration of slightly hydrophobic groups in higher salinity

As previously discussed, the presence of higher amount of salt in solution induces protein aggregation by enhancing (i) hydrophobic interactions, (ii) increased hydration of ions,

(iii) decreased availability of free water, and (iv) preventing intra- and inter-molecular electrostatic interactions [67,68]. Therefore, the halophilic proteins adapted to preserve the native conformation and remain functional in the presence of high salt content. The halophilic proteins have evolved to require potassium rather than sodium. The potassium ions have an additional advantage of having low water binding nature compared to sodium ions [67].

2.2.3. Presence of spheres of hydration

Halophilic proteins require 'spheres of hydration' to resist the protein-aggregate formation [49,62,69]. Hence, they contain a decreased number of hydrophobic amino acids and increased number of hydrophilic amino acid on their surface [70,71].

An increased amount of smaller hydrophobic amino acids (e.g. glycine, alanine, and valine) compared to larger hydrophobic amino acid is a another adaptive mechanism existed in halophilic enzymes [43,44,49,60,72,73].

It is established from this discussion that halophilic proteins/enzymes are more stable in salt as they (a) have negatively charged protein surfaces offered by acidic amino acids, (b) conglomerate slightly hydrophobic groups in high salt concentrations, and (c) get hydrated at the surface because of carboxylic groups.

On the other side, halotolerant microorganisms grow in low concentration or absence of salt [74]. Thus, their enzyme activity is unaffected by the presence/absence of salts [52]. Hence, they are not salt-dependent in comparison with halophilic proteins/enzymes [32]. On the contrary, the halotolerant microbes use the 'compatible solute strategy' and other mechanisms like amino acid, hydrophobicity, and hydration adaptations are not reported in them [38].

Generally proteins of typical bacteria are soluble in very dilute salt solutions, as the ionic form of salts associate with opposite charge moieties present in protein. The appropriate amount of salt leads to increase the hydration of the surface on protein. The increased salt concentration in solution increases the surface tension of water and creates a struggle between protein and charged salt ions for hydration. Therefore higher amount of salts or presence of heavy metal salts (e.g. Hg^{2+}) contribute to remove the layer of water molecules from the protein surface, and exerts conformational alteration and aggregation of the protein.

In halophilic/halotolerant bacterial proteins, the 'halo-adaptation' is achieved by multiple strategies as previously discussed. The halo-adaptation confers the stability to protein in the presence of higher amount of salts. Also, various studies revealed that the halo-adaptation mechanism not only confers a salt-tolerance to protein but the structural, functional and

physiological adaptation mechanisms confers a novel attribute to protein that - the presence of relatively higher salt is necessary for functioning of these proteins. Therefore, the halo-adapted enzymes catalyze the reaction with higher rate and more efficiency in the presence of higher salt concentration as compare to normal enzymes. Besides the capability to tolerate/ require higher salt concentration, the halo-adaptation mechanism could also confer the stability to these salt-stable proteins/enzymes to retain their active conformation in various catastrophic agents. The traditional expectation from enzyme operated at ambient conditions was broaden up and the catalytic proteins capable to withstand higher amount of salts could be used for catalysing the reactions/bio-transformations in the presence of anionic/amphoteric/non-ionic surfactants, soaps, oxidants, bleaches, hydrotropes, stabilizers, and organic solvents, hence will preferred in modern biotechnology industrial set up.

3. Enzymes of halophilic /halotolerant bacteria

High salt tolerance and a high salt requirement for active conformation of a protein of halophilic microorganisms suggest that they are a better source of salt-stable enzymes and offers biocatalysis in presence of high salt. Fascinatingly, several enzymes from halophilic microorganisms also exhibited a 'polyextremophilic nature' and are remain active/stable in presence of alkaline pH, high temperature and non-aqueous medium besides activity in presence of salt [22,48].

Some of novel attributes of haloenzymes include: (i) activity and stability in high NaCl concentrations, (ii) activity in presence of range of salts, (iii) higher resistance towards denaturation, and (iv) catalysis ability in micro-aqueous or non-aqueous medium [22,43,75]. Halophilic microbes are able to produce some hydrolytic enzymes like amylases, cellulases, DNases, inulinases, lipases, pectinases, proteases, pullulanases, and xylanases [48,76,77]. The stability and activity profiles of these enzymes are varied, making the halophilic enzymes as a valuable commodity in the biotechnology industry. Besides, stability in a wide range of pH and temperature, if any enzyme is stable/active in presence of salt, this novel feature broadens the utility of halophilic hydrolytic enzymes in various applications in biotechnology.

3.1. Protease of halophilic /halotolerant bacteria

Among the various microbial enzymes the protease is a key industrial enzyme accounting for ~60 % of commercially useful enzymes. In the beginning, the industry utilizes the alkaline proteases obtained from alkali-tolerant, mesophilic bacteria. The discovery of extremophilic microorganisms like alkalophile, thermophile, and halophile provide new resource of industrially efficient proteases.

Generally, the proteases (peptide hydrolases) are mainly grouped as exo-proteases and endo-proteases. The exo-proteases act only near the end of a polypeptide chain (proximal to N/C terminus) while endo-proteases cleave internal bonds in polypeptide chains. The protein breakdown into small peptides/free amino acid is mainly mediated by endo-proteases hence have commercial significance. The endo-proteases are further classified into sub-subclasses on the basis of the catalytic mechanism as (i) serine endo-proteases (E.C.3.4.21) have an active centre serine involved in the catalytic process, (ii) cysteine endo-proteases (E.C.3.4.22) have a cysteine in the active centre, (iii) aspartic endo-proteases (E.C.3.4.23) depend on an aspartic acid residue (commonly two) for their catalytic activity, and (iv) metallo endo-proteases (E.C.3.4.24) use a metal ion (often Zn^{2+}, but not always) in the catalytic mechanism. Recently, sub-subclass E.C.3.4.25 has been added for threonine endo-proteases. Majority of extracellular proteases from halophilic and halotolerant bacteria are serine proteases and metalloproteases although rarely cysteine proteases have also been described (Table -8).

3.2. Halophilic protease

Proteolytic enzymes derived from halophilic microbes execute the catalytic function *in vivo* and *in vitro* in presence of 4-5 M NaCl concentration while get inactivated/denatured in absence/low salt concentrations [49]. Majority of these proteases showed optimal activity in the presence of the relatively high concentration of NaCl at pH 5–10 and capable to withstand temperatures from 40 to 75℃.

Majority of halophilic proteases belongs to 'serine protease' group. This group of salt-stable serine/ subtilisin-like serine protease is denoted as 'halolysin'. Such halolysins were detected in *Natrialba asiatica* 172P1 [78], *Haloferax mediterranei* VKMB-1538 [79], *Haloferax mediterranei* R4 [80,81], *Natrialba magadii,* and *Halobacterium* sp. strain NRC-1 [82]. In addition, an unusual extracellular serine protease (130 kDa) was obtained from *Natronococcus occultus* [83].

3.3. Halotolerant protease

Halotolerant proteases are capable to tolerate the high salt concentrations, without salt dependence [43,84]. Majority of extracellular halotolerant proteases are metalloproteases and serine proteases. Although, halotolerant enzymes were commonly produced by halotolerant microorganisms, occasionally halotolerant proteins have been also derived from non-halophiles [25]. The halotolerant proteases are capable to withstand low water availability [85]. The ability to remain active in low water availability (aW) confers the additional attribute to remain active in organic solvents [10,86]. The salt-tolerant enzymes play a key role in

numerous processes that require low to high NaCl concentrations which including various applications in peptide synthesis, detergent formulation, and fish/meat processing industries [25,85].

Table 3 comprises the list of various microbial proteases secreted by halophilic/halotolerant microbes isolated from various ecological biotopes.

3.4. Amino acid composition and salt tolerance of protease

The activity and stability of protease is determined by its definite active conformation oriented by specific physicochemical factors (like pH, temperature, salinity, aqueous /non-aqueous media or presence of activator/inhibitor) available during catalysis reaction. For attaining active conformation, the halophilic enzymes have an improved altered amino acid sequence as compared with typical non-halophilic enzymes. As discussed previously halophilic proteins possesses (i) nearly 20 % more acidic amino acid residues (Aspartic acid and Glutamic acid), localized as clusters on the surface of a protein, (ii) have fewer lysine residues and (iii) increased number of small hydrophobic residues (Alanine and Glycine), and polar residues (Serine and Threonine) in comparison with non-halophilic enzymes. The altered amino acid sequence dispenses several negative charges on halophilic proteins which reduces the hydrophobicity and reduces their propensity towards aggregation at high salt/NaCl. The amino acid composition in many halophilic proteins sequence so evolved that presence of NaCl is essential for their activity.

To analyze the trend of amino acid sequence of salt-stable proteases, the amino acids sequences of comparable length of several halophilic and halotolerant bacteria described in this review are obtained from 'Uniport' a public online database which provide a comprehensive, high-quality and freely accessible resource of protein sequence and functional information. (http://www.uniprot.org). The various amino acid sequences have been aligned by using 'Clustal Omega' software for multiple sequence alignments. Depending on their similarity among the amino acid sequences of serine proteases (Fig. 5) and metallo-protease (Fig. 6) of various halotolerant and halophilic bacteria were represented in tree. The amino acid sequences of both types of proteases from same bacteria were selected for alignment. The amino acid sequence of serine proteases showed 10-85 % similarity while the amino acid sequence of metallo-proteases showed comparatively higher similarity; 12 to 97 %, suggesting that metal dependence/tolerance strategy evolved correspondingly among the selected halotolerant and halophilic bacteria compare to serine protease.

3.5. Protease catalysis mechanism

3.5.1 Serine proteases catalysis mechanism

Serine endo-proteases (E.C.3.4.21) have an active center- serine which involved in the catalytic process. Several proteases isolated from halophilic microbes belongs to the serine proteases. This group of salt-stable serine/ subtilisin-like serine protease is denoted as 'halolysin' and are predomimatly present in halophilic archea. Serine proteases consist of three amino acids – serine (Ser), histidine (His), and aspartic acid (Asp) in their active site. The serine in the active site of protease is more reactive than other serine in the protein. The serine, histidine, and aspartic acid which are present apart from each other in protein sequence and assembled at catalytic site by tertiary structure formation are often referred to as a 'catalytic triad'. The serine of the active site is associated with **His** by hydrogen bond while His is also connected to **Asp** by hydrogen bond. The residues of the catalytic triad form a charge-transfer relay network.

A protease (halolysin) bound to a proteinaceous substrate, then a by covalent catalysis mechanism, **Ser** act on the scissile peptide's carbonyl group of proteinaceous substrate. The serine oxygen nucleophilically attacks the carbonyl carbon of a scissile peptide bond. The hydrogen-bonded His functions as a general base to abstract the serine proton, and the negatively charged Asp stabilizes the positive charge that forms on the His residue. This prevents the development of a very unstable positive charge on the serine hydroxyl and increase its nucleophilicity.

This nucleophilic reaction transfer a proton to the imidazole ring of **His** which leads to formation of the imidazolium ion. This process is assisted by the polarizing effect of the carboxylate ion of **Asp,** which is linked to **His** by hydrogen bond. Overall rearrangement leads to formation of tetrahedral intermediate.

The tetrahedral intermediate further splits to the acyl–enzyme intermediate due to driving force of proton donation from N3 of **His**. This decomposition reaction is supported by the polarizing effect of **Asp** on **His**. The amine leaving group (the new N-terminus of cleaved polypeptide chain) is released and replaced by water. General base catalysis and nucleophilic attack forms second tetrahedral intermediate. The reversal of first step yields the carboxylate product (the new C-terminal portion of the cleaved polypeptide chain), which dissociates from the protease, and regain its initial state. In this process, water is the attacking nucleophile and **Ser** is the leaving group. The preferential binding of the transition state (tetrahedral intermediate) over the enzyme–substrate complex or the acyl–enzyme intermediate is responsible for much of the catalytic efficiency of serine proteases [13].

3.5.2. Metallo-protease catalysis mechanism

Several enzyme requires metal ions either (i) as catalytic centre (e.g. Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, or Co^{2+}) which tightly bound as cofactors to enzyme or (ii) for structural stability to form active conformation (e.g. Na^{+}, K^{+}, or Ca^{2+}). The metal ions like Mg^{2+} and Zn^{2+} may play a role in either structural or catalytic mechanism. Majority of enzymes of halophilic/halotolerant bacteria require presence of Na^{+}, K^{+}, or Ca^{2+} to compensate the excess negative charges oriented due to distinctive amino acid sequence.

The Metallo-proteases (E.C.3.4.24) uses metal ion and glutamic acid residue in catalytic process. The tetrahedral transition state is formed by a penta-coordinated metal ion (usually Zinc) and the catalysis involves direct hydrolysis by water. The metal ion in catalytic center acts as a proton to neutralize negative charge. In metal ion–catalyzed reactions, the metal ion acts like a proton, it neutralizes the negative charge. Actually metal ions are more effective catalysts compare to protons because metal ions can be present in high concentrations at neutral pH at which [H+] = 10^{-7} M. A metal ion's charge make water molecules more acidic to which it bound as compare to free H_2O. Hence, it is a source of nucleophilic OH^{-} ions even below neutral pH [13].

Cysteine endo-protease and aspartic endo-protease are rarely documented in halophilic and halotolerant bacteria. Cysteine endo- protease possesses cysteine, histidine, and aspartate/aspargine in the active site and involves formation of covalent acyl enzyme intermediate. The aspartic endo- protease consists of a catalytic triad aspartate, tyrosine and aspartate and mechanism involves direct hydrolysis by water.

4. Fermentative production of salt-stable proteases

The fermentative protease production by halophilic/halotolerant bacteria depends on various factors like pH, temperature, salinity, inoculum level, agitation/aeration, incubation period, carbon, and nitrogen sources. The optimization of these factors is primary step to developing industrial-scale fermentative production with economical protease yield.

The nutritional and physical factors are optimized using (i) classical; one variable at a time approach (OVAT) and (ii) software assisted; response surface methodology (RSM). The traditional one-at-a-time strategy for optimization of protease production is criticized as a time-consuming process, requires an undefined number of experiments and can't provide the data of interaction among the all experimental variables [87,88]. Hence, rational and time-saving response surface methodology provides a suitable alternative for optimization of protease production by halophilic microorganisms. Both approaches were effectively used by various

researchers, also prior to experimental designing for response surface method; several experiments are necessarily based on one variable at a time approach. Table 4 summarizes the RSM strategy used by various researchers to improve the protease production.

4.1. Optimization of physical factors

4.1.1. Influence of initial pH of the medium

The pH of medium is a crucial factor for achieving desired yield of protease. The media pH affects (i) the nutrient availability, (ii) metabolic responses, (iii) genetic regulatory mechanism(s), (iv) product/by-product formation, and (v) alteration in diffusional barrier of cell membranes [19,89]. The optimum pH required for protease production by various halophilic/halotolerant microbes is in the range of 7-11 [19,20,90-95]. In addition, pH adjustment is also important to maintain the active conformation and stability of protease.

The presence of relatively high amount of salt in fermentation media is obvious to cultivate halophilic/halotolerant microbe. Addition of salts not contributes in pH alteration, while presence of extremely high concentration of salt imparts slight alkalinity to the medium. The presence of salt regulates the ionic strength of solution and may alter the behavior of media components. It is best strategy to sterilize the salt solution separately as their higher concentration may inactivates/react with other media components to form unusual compounds which will alerts the media pH and/or partially inhibits the growth of microorganisms.

4.1.2. Effect of incubation temperature on protease production

Generally, incubation temperature is an essential physical factor, significantly modifies the synthesis and secretion of protease. The cultivation temperature affects the energy metabolism and oxygen uptake [96], protein synthesis [97], the viscosity/permeability of cell membrane [98] and normal cell physiology [99]. For halophilic microorganisms, the optimum temperature is in the range of 30-50℃.

A higher optimal temperature of 55℃ was also recorded for salt-stable alkaline protease production from *Bacillus* species [93,94,100], while optimum temperature 28℃ is responsible for effective protease production from *Actinopolyspora* sp. VITSDK2 [92].

In a large-scale fermentation process, particularly operated at >40℃, it leads to considerable water loss due to evaporation. The slight decrease in water could alter the ionic strength of fermentation media as it contains a comparatively high amount of salt. The evaporation from submerged cultured halophile/halotolerant bacterial process in shake-flasks at elevated temperatures can alter the salinity of growth medium and excessive loss of water leads to salt precipitation [101]. Hence, during scale-up and commercial scale fermentative

protease production using halophilic/halotolerant bacteria, the water losses should be controlled carefully.

4.1.3. Effect of inoculum size on protease production

Generally the growth rate of halophilic microbe is slow as compare to non-halophile; even the growth of halotolerant microbe in media of high salinity is often slow [101]. If the large scale fermentative production of protease initiated with too small inoculum then it extends for weeks, which is unreasonable as per economic point of view as the fermentation cycle should complete within few days (ideally 3) rather than weeks. Also, the protease synthesis is component of primary metabolism; hence too heavy inoculum could not yield the economically viable amount of protease despite of the good growth.

Based on these factors the inoculum size should be optimized; as appropriate size inoculum plays a key role in the growth and protease secretion. Mostly, the inoculum size 1 to 3% was found suitable for extracellular protease production by halophilic/halotolerant microorganisms. Optimum inoculum level is an essential factor for maximal enzyme secretion because low enzyme production is observed at low inoculum level while inhibition of protease production is recorded at high inoculum level [102].

4.2. Optimization of nutritional factors

4.2.1. Carbon source

Incorporation of suitable carbon source, such as glucose [91], D-galactose [92], glycerol [103], maltose [104], mannitol [105], and starch [106] have improved the protease production from halophilic/halotolerant microbes. Several reports have documented that nitrogen sources like shrimp shell powder, crab shell powder, and oyster shell powder fulfills the demand of carbon sources. These are therefore inexpensive and non-conventional carbon sources [94,100,107,108].

4.2.2. Nitrogen source

The presence of nitrogen source is an essential factor for the growth of heterotrophic halophilic/halotolerant microbes [19,109]. Yeast extract and/or peptone [90,104,105,110], casaminoacids [91], casein [92,111], and beef extract [106] are some of the nitrogen sources used for the production of protease from halophilic/halotolerant microbes.

As described previously, low-cost nitrogen sources such shrimp shell powder and crab shell powder are used in the production of protease from *Bacillus* species [93,94]. The nitrogen sources should be used in specified concentration otherwise an excess incorporation leads to feedback repression of protease production [112].

A list of optimum conditions required for the enzyme production from several halophilic/halotolerant microorganisms is summarized in Table 5.

Optimal production of any biological material needs efficient process development policies which are cheap, feasible, and robust. The process development is the key step in large-scale production of a product including the enzymes like proteases. However, enhanced protease production can be achieved by employing the best combination of essential media ingredients like carbon and nitrogen sources and, metal ions. The yeast extract and skimmed milk have been well documented as good carbon as well as nitrogen source respectively for the production of protease from haloarchaeal microorganisms [113,114].

4.2.3. Salt

Salts are an essential component to cultivate the halophilic bacteria. The salinity of medium determines the polar lipid composition of the cell membranes of halophilic bacteria. An increased salt concentration induces the change in the membrane lipids and could alter the extracellular enzyme secretion and growth rate of bacteria [115].

Also, the salt is needed to stabilize and promote the enzyme activity of halophilic bacteria. The presence of suitable amount of salt in media confers the specific ionic strength which further augments the electrostatic coupling between salts and enzyme, and subsequently confines the enzyme aggregate formations. The degree of stabilization of enzyme/proteins of halophilic/halotolerant bacteria is determined by the protein–salt ratio [116]. Besides affecting the bacterial growth, the protease secretion and stabilization is also influenced by salts by modulating the synthesis of compatible solutes [117] which are further offer stability to salt-stable enzyme. Although the mechanism is not elucidated yet, the elevated concentration of NaCl offers protection to the hydrophobic amino acids and improves enzyme activity and thermal tolerance to protease [116-118]. However, protective effect of NaCl was not apparent with regard to the enzyme's thermostability of protease from haloalkalophilic *Bacillus pseudofirmus* [119].

The proteases obtained from *Halobacterium* sp. strain HP25, *Bacillus subtilis* strain BLK-1.5, *Halobacillus* sp. CJ4, and *Salinicoccus* sp. UN-12 showed maximal protease production in the presence of 4.3 M, 1.2 M, 2 M, and 0.35 M NaCl, respectively [95,120-122]. These wide differences in protease production from halophilic/halotolerant microbes might be due to the salt tolerance of species, composition of growth medium, variation in media ingredient behavior in presence of varied salt concentration and enzyme secretion influenced by salt dependent altered membrane composition.

The presence of salts like Ca^{2+} and K^{+} in cultivation media of halophilic/halotolerant microorganisms is found significant as it offers the thermostability to an enzyme [116,122]. Similarly, the calcium ion is recognized to enhance the protease production in *Halogeometricum* sp. TSS101 [113], *Jeotgalicoccus* sp. [19], *Bacillus* sp. NPST-AK15 [20], and *Virgibacillus halodenitrificans* RSK CAS1 [111]. The range of typical salts and minerals concentration required for the growth of non-halophile, halotolerant, and halophile [101,123] is compared in Table 6.

Very less literature is available about large scale fermentative industrial protease production as the use of halophilic/halotolerant bacteria at industrial scale is not completely developed field. Based on above discussion the clear advantages of halophilic/halotolerant bacteria as protease source includes (i) The marine/saline low cost proteinaceous byproducts/ waste could be used as production media/media component, (ii) the hard water could serve the purpose to cultivate the halophilic/halotolerant bacteria on large scale wherever no alternative options are available, (iii) contamination probabilities will greatly reduce as the production media constitute relatively higher amount of salt which selectively promote the growth of only desired inoculated bacterium, (iv) appropriate protein-salt ratio or ionic strength maintenance during termination of fermentation cycle might contribute to stabilize the protease.

The process of fermentative protease production by employing halophilic/halotolerant bacteria also have several constrains like (i) use of high quality, corrosion resistant fermenters as excess of salts enhance the corrosion progression, (ii) individual sterilization of media component; in presence of high temperature during sterilization the reducing sugars and amino acids could react with abundant salts present in media leads to form several growth/enzyme inhibitors.

5. Protease purification strategies

Various techniques have been used to recover the extracellular protease from a fermented broth consisting of a heterogeneous mixture of proteins/enzymes. The purification of halophilic proteins is a challenging because of excess salts in production media. The presence of salts is like necessary evil as it required for maintaining the structural integrity of halophilic proteins throughout the purification. In general, halophilic proteins have acidic surfaces and the associated negative electrostatic forces may potentially provide a stabilizing force; these proteins may unfold at low salt concentrations due to the repulsive force exerted by the acidic residues. Many halophilic proteins also have a low thermal stability in absence of

appropriate proportion of salt [124]. Overall it is accepted fact that the enzymes of halophiles are unstable in low concentrations/absence of salts [43].

For the suitable choice of purification technique, the general literature available to purify the usual enzyme/proteins not gives any clue; the ammonium sulfate precipitation many times yields the precipitate with a considerable loss of protease activity. The high ionic strength of salt containing culture broth upsets protein purification protocols based electrophoresis and chromatography principles by employing traditional gels/media [125]. Hence in succeeding purification steps, amount of NaCl in buffers should be less than 2.5 M to make the enzyme purification fruitful by using these media.

Halophilic proteins suspended in desired salt-buffer solution have high electrical conductivity, which further made the electrophoretic separation more challenging [35]. Also, crystallization of halophilic proteins in high salt concentration solution is difficult task [124].

5.1. Precipitation and enzyme concentration

In order to purify extracellular proteins/enzymes, the first step is a separation of cellular biomass either by filtration or centrifugation. The filtrate/supernatant of centrifuged suspension consists of all secreted proteins. Various strategies were used for concentration of extracellular proteases like (i) salting out using ammonium sulfate, (ii) solvent extraction by using acetone [126] and ethanol, and (iii) ultra-filtration [127,128].

Among these strategies salting out and solvent extraction are most commonly use. Alkaline protease from halotolerant *Jeotgalicoccus* sp. and *Bacillus* sp. NPST-AK15 were concentrated by employing 80% (w/v) ammonium sulfate respectively [19,20], while alkaline serine protease from a *Bacillus pseudofirmus* precipitated at 70% (w/v) ammonium sulfate [119]. Similarly, 50-70% (w/v) ammonium sulfate precipitates the alkaline protease of *Streptomyces pseudogrisiolus* NRC-15 [129] while 60-80% (w/v) ammonium sulfate was required to precipitate the alkaline protease of halo-tolerant *Bacillus aquimaris* VITP4 [130].

The presence of excess salt in culture broth offers a challenge to obtain the ammonium sulfate precipitate of protein; an alternative approach of the organic solvent was attempted by several researchers. In this view, ethanol and acetone precipitation method has been reported for halo archaeal protease [113,131-133].

Even at large-scale the biotechnological industries prefer acetone precipitation, as it could be recovered from the batch fractionation [2]. Various acetone proportions were suggested for precipitation of salt-stable proteins. A chilled acetone (50-80%; v/v) was used to precipitate the protease from *Salinivibrio* sp. strain AF-2004 [134], and *Bacillus iranensis* [21],

while 80% (v/v) acetone precipitation was reported for proteases of *Halobacillus karajensis* and *Salinivibrio* sp. strain MS-7 [133,135]. An ice-cold ethanol was also attempted to achieve initial precipitation of *Alkalibacillus* sp. NM-Fa4 protease [136].

Instead of salt/solvent precipitation method, an ultrafiltration technique is a suitable alternative to achieve protein precipitation without inducing any denaturation effect on proteins. The dilute protein solution obtained during chromatographic separation could also be concentrated using ultrafiltration [137]. The salt-stable alkaline proteases from *Halobacillus karajensis* [135], *Virgibacillus* sp. EMB13 [105], *Bacillus pseudofirmus* [119], *Halobacterium* sp. strain HP25 [120], *Marinomonas arctica* PT-1 [138] and *Bacillus iranensis* (X5B) [21] were precipitated by employing ultrafiltration technique.

5.2. Aqueous two-phase extraction systems

Aqueous two-phase extraction using polymer/s and a salt is the suitable method used to recover enzymes [139], the use of environment-friendly polymers in this method is an additional benefit over organic solvent extraction. For the purification of various proteases by employing aqueous two-phase method the mixture of (i) polyethylene glycol (PEG) and dextran, (ii) polyethylene glycol (PEG) and salts such as H_3PO_4, $MgSO_4$ were suggested [140-142].

The success of aqueous two-phase extraction method depends on various factors like (i) molecular weight of polymer, (ii) hydrophobicity of polymer, (iii) concentration of polymer, (iv) nature of salt-multivalent/polyvalent and (v) temperature of two-phase system [143]. The polymer-polymer phase split into separate phases with lower polymer concentration at lower temperatures, while polymer-salt phase necessitates high polymer concentrations at lower temperatures. However, the expense of this approach limits its approval as compared to traditional methods [144].

5.3. Chromatographic purification

Multi-step chromatographic techniques are employed for the purification of the enzyme after precipitation/ultrafiltration of halophilic proteins. Chromatographic techniques like gel filtration, ion exchange, isoelectric precipitation, affinity, hydrophobic interaction, HPLC *etc*. results in purer fractions of proteins with increased specific activity.

Ion-exchange chromatography is frequently used to purify non-halophilic proteins. However, in case of halophilic proteins, the eluting buffer should consist of relatively higher salt concentration to retain the native/active conformation. The use of a high salt eluting buffer is a constraint during ion-exchange chromatography which demands the dilution of protease

containing fermented broth. The dilution imparts additional constraint as it makes the purification process cumbersome and at low salt concentration, the conformation of halophilic protein may denature. The alternative methods like (i) gel filtration, (ii) affinity chromatography, (iii) hydrophobic chromatography by employing gradient of ammonium sulfate on carboxymethyl cellulose or Sepharose 4B columns, and (iv) hydroxylapatite chromatography by using high salt buffer medium were suggested to purify halophilic proteins [45]. In the initial-to-middle steps of enzyme purification the chromatographic separation by employing new, rigid and cross-linked agarose gels like - biogel, superose, sephadex, sephacryl, sepharose, toyopearl and superdex were also suggested. Table 7 summarizes the various purification approaches employed for purification of salt-stable proteases from halophilic/halotolerant microbes, by monitoring the protease activity, estimation of total protein content and SDS-PAGE electrophoresis.

The presence of relatively higher amount of salt is obligatory during purification of salt-stable protease to sustain the stability or to extend the thermal denaturation. Currently various hurdles are associated with purification of salt-stable proteases like (i) intricate enzyme recovery protocol are required to tackle the high amount of salt in fermentation broth, (ii) high ionic strength of fermentation broth limits the choices of purification gel/media, (iii) salt-stable proteases suspended in desired salt-buffer solution have high electrical conductivity and make electrophoretic separation more challenging, and (iv) crystallization of halophilic proteins suspended in high salt concentration solution is difficult task.

Advances in biotechnology offered improved versions and accessible options to purify the salt-stable proteases. Acetone precipitation, aqueous two-phase extraction, hydoxylapatite chromatography columns, and immobilized metal ion affinity chromatography (IMAC) could improve the efficiency of enzyme recovery. Ample scope is available to design the novel, salt-stable, rigid and stable gel/matrix for halo-enzyme purification.

6. Biochemical properties of salt-stable protease

Several halophilic/halotolerant microbes have been studied for protease production and used for various possible industrial applications based on their unique properties and have been studied extensively (Table 8). The diverged range of proteases produced by halophilic/halotolerant microbes includes serine protease, metallo protease, serine-metallo protease, and cysteine protease. The proteases from these microbes showed unique biochemical properties of industrial significance.

6.1. pH

The proteases obtained from halophilic/halotolerant microorganisms are functional in wide array of pH and are usually active in the range of 8-11 [20,95,120,122,138,145,146]. Even proteases with higher optimum pH (range pH 9-11) are also reported [147]. The salt-stable proteases with optimal catalytic activity at acidic pH are rarely reported [148].

6.2. Temperature

Proteases from halophilic/halotolerant microorganism are active in a broad range (30 to 80℃), while the protease with higher optimal temperature showing maximum activity at 90℃ is reported from *Oceanobacillus iheyensis* [147]. The presence of salt enhances thermostability of purified protease [116]. Likewise, PEG and Ca^{2+} also play important role in enhancing the thermostability of enzymes [116,149].

Thermodynamic properties of the salt-stable protease have been rarely described in the literature. A deactivation rate of the purified protease of *Nocardiopsis alba* OK-5 in presence of various concentration of NaCl and compatible solutes was reported with an explanation based on thermodynamic data [150]. Also, a thermostable protease with optimum activity in 2 M NaCl was reported from haloalkaliphilic *Oceanobacillus* sp; the protease has unaltered enzyme activity at optimum temperature (50℃) in presence of organic solvent iso-octane (30%, v/v) [151].

Raval et al. have coupled the temperature profile of Ve2-20-91protease obtained from *Bacillus pseudofirmus* with the salt [119]. The optimum temperature of the protease was reported to be shifted from 50 to 80℃ vis-à-vis increasing NaCl concentrations (0–3 M). This observation was also confirmed by studying K_{cat} values determined at various temperatures and different salt concentrations.

6.3. Salts and metal ions

The catalytic activity and stability of enzymes of halophile require the presence of specific salts (NaCl/KCl) [152]. These salts should essentially present to adjust the ionic strength of the solution and for maintaining the active conformation of protease. Also, catalytic activity and stability of enzymes were depend on the concentration of these salts; halophilic proteins were active and stable at ~ 4 M NaCl, with optimum range 1-2 M [116,132,153]. The halophilic proteins were inactivated/denatured at <1 M NaCl and completely lost the activity in the absence of salt.

The alkaline protease of the haloalkaliphilic *Bacillus* sp. was active in the range 0.2-0.5 M NaCl and activity gets lowered with the further increase of NaCl [116,147,153,154]. Protease from halotolerant *Bacillus cereus* SIU1 showed enzyme activity in 0 – 2 M NaCl

[152]. Likewise, purified protease from *Jeotgalicoccus* sp. retained >98% activity in NaCl (0–1.5 M), revealed the halotolerant nature of enzyme [19]. Table 9 summarizes the salt requirement for catalytic activity of salt-stable proteases derived from halophilic/halotolerant microorganisms

Besides NaCl, generally other metal ions (Ca^{2+}, and Mg^{2+}) also significantly affect the catalytic efficiency and stability of salt-stable proteases. Metal ions behave as 'electrophilic catalysts' during reactions [130]. Among the various metal ions, Ca^{2+} was responsible for stimulation of protease activity and prevention of autolysis [103,155,156]. Several reports were documented on an enhancement of thermal stability of halophilic proteases in the presence of Ca^{2+} [20,121,122,157].

Moreover, divalent metal ions like Mg^{2+}, Cu^{2+}, Ba^{2+}, Co^{2+}, Fe^{3+}, Zn^{2+}, and Mn^{2+} are contributed in improving the activity of extracellular proteases obtained from various halophilic/halotolerant microorganisms [21,107,121,158-160].

6.4. Molecular weight

The molecular weights of salt-stable proteases obtained from halophilic/halotolerant microbes have been reported in the range 20-71 kDa [91,122,133,138,161]. However, high molecular weight halophilic proteases with 100 kDa from *Halobacillus thailandensis* sp. nov was also documented [162]. A molecular mass of proteases of various halophilic/halotolerant microbes is listed in Table 8.

6.5. Substrate

Various reports documented that the proteases from halophilic/halotolerant microbes have the capability to hydrolyze the range of natural and artificial substrates *viz.* casein, gelatin, bovine serum albumin, hemoglobin, peptone, tryptone, yeast extract, wheat gluten, azocasein, casamino acids, skim milk, N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (Suc-AAPF-pNA) and Nα-Benzoyl-D,L-arginine p-nitroanilide hydrochloride (BApNA).

The presence of higher amount of salt could also affect the structural conformation of proteinaceous substrate commonly used in the enzyme assay, which further modulates the enzyme catalysis rate. The presence of NaCl (1.5%) alters the secondary structure of gelatin by disrupting (i) molecular order of lower helix, (ii) electrostatic forces, (iii) salt bridges, and (iv) gel strength and textural properties; and forms random coil/disordered structure of gelatin [163,164].

The suitable substrates are used to determine the enzyme unit of commercial salt-stable proteases. Table 10 summarizes the substrate specificity of proteases of halophilic/halotolerant microbes against various natural proteinaceous substrates.

Kinetic parameters like K_M, K_{cat}, E_a, and V_{Max} of enzymes are important factors in scale up and economics of biotechnological process based on utility of the enzymes. Several complexes as well as synthetic chromogenic substrates are used for evaluation of the kinetic properties of proteases of halophilic/halotolerant microbes. The kinetic parameters of various proteases obtained from halophilic/halotolerant bacterial strains are summarized in Table 8.

The halophilic/halotolerant bacteria are now well recognized source of diverged range of kinetically efficient proteases (molecular mass 20-71 kDa) capable of functioning in wide array of physicochemical factors. Various salt-stable proteases were secreted in substantial amount and were documented to active and stable in (i) extensive range of pH (8 to 11), (ii) temperature (30-90℃), (iii) non- or micro-aqueous systems by virtue of their stability in repertoire of organic solvents, (iv) broad range of salinity (0-4 M NaCl); with obligate or facultative necessity of salt, (v) wide range of surfactants, bleaching agents, oxidizing agents, stabilizers, builders, brightening agents, ionic liquids, and hydrotropes. These unique biochemical characteristics of the salt-stable enzymes have industrial significance which offers to scale up the massive and economical use of salt-stable enzymes in biotechnological sector.

7. Operative utility of salt-stable proteases in the biotechnology sector

Proteases are most versatile and unique biotech commodity for analytical and industrial claims in detergent, pharmaceutical, leather, silk, bakery, fish/soy sauce fermentation, soy processing, feather processing, brewery, meat tenderization, waste treatment and management, silver recovery, anti-biofilm agents, sub-culturing of cell lines, and peptide synthesis [19,109,145,165-169]. Proteases from halophilic/halotolerant microorganisms offer major biotechnological benefits over non-halophilic proteases owing to their catalytic stability and efficiency at high salinity and other unusual conditions. The salt-stable proteases could be utilized in biotechnology sector with the broader perspective.

7.1. Detergent and textile industry

7.1.1. Protease in detergent formulation

Proteases are commonly used in household detergent formulations since 1913 [170]. It specifically added in these formulations to remove the natural proteinaceous stains like blood, coffee, egg yolk albumin, grass, sweat, sauces, grease/dirty motor oil, spinach, milk, tea, and keratin [23,91,130,160,169,171]. Protease-based detergent formulations demonstrated better

cleansing properties with an environment-friendly approach as compared to traditional synthetic detergents.

The enzyme-detergent formulation consists (w/v): enzymes (protease, lipase, amylase, and cellulase), 0.004%; ionic surfactants, 0.03%; non-ionic surfactants, 0.02%; and bleaching and oxidizing agents, 0.14% [172]. Besides this, the detergent industry utilizes various chemical ingredients to make commercially efficient detergent formulation. Table 11 summarizes the repertoire of ingredients and their role in the detergent formulation.

The proteases thus should be robust to remain stable in the bleaching and oxidizing reactions happening during fabric washing. The performance of protease in detergent formulation depends on various imperative factors like (i) the stability of protease in ionic and non-ionic detergents, oxidizing, bleaching agents, and harsh detergent formulation ingredients, (ii) tolerance to high pH values (9-11), (iii) to withstand activity in temperature range between 30-60℃, (iv) compatibility with total dissolved solids of water and occasionally high salt concentration (NaCl and $CaCl_2$), and (v) stain degradation and removal potential [19,169,173-175].

Stability profile of several salt-stable proteases obtained from halophilic/halotolerant microorganisms in various detergent formulation ingredients is summarized in Table 12. In commercial detergent formulations and dishwashing solution, detergent compatible proteases were used in granular or stabilized liquid suspension form [176]. However, a granulated form of enzymes has limited shelf life as compared to liquid formulations; as they are directly exposed to bleaching agents for a longer period. Hence, a highly stable enzymes are necessary for tablet/granulated forms. On the other hand, auto-proteolysis is a major problem for stabilization of proteases in liquid-formulations [177-179]. Moreover, the concentration and nature of the surfactants used in the liquid-detergent formulation have a significant influence on stability during the storage [178].

New proteases with novel catalytic attributes were always preferred to improve the wash performance of currently used enzyme-based detergent formulations. The catalytic activity at high salt concentration is the desirable assurance of protease used in detergent, as NaCl is a key ingredient in the granulation process prior to addition of the protease in the detergent formulation [174]. In addition, calcium salts were commonly used to stabilize the enzymes in the liquid detergent formulation [180], the tolerance to calcium ions might contribute to extending the shelf life of protease in the detergent formulation [19]. Additionally, saline water/salty water from several Geo-climatic environments and canal command area is

available for washing purpose, where detergents show ineffective cleaning performance. Mokashe et al. documented the use of salt-stable alkaline protease of *Jeotgalicoccus* sp. in detergents may possibly improve the washing efficiency of detergent in saline water [19].

Currently, importance of detergent-friendly biocatalyst is exponentially growing for detergent formulators owing to (i) better cleaning potential, (ii) less efforts, (iii) rapidity of cleaning performance, (iv) environment-friendly approach, (v) restoration of fabric structure, (vi) ambient washing temperature, (vii) less time of agitation upon prior soaking, (viii) an increased washing efficiency and less alkaline nature, (ix) reduced water consumption, and (x) readily biodegradable nature [109]. The compatibility and stability must be evaluated in detergents for longer periods. Also, the proteases having isoelectric point equal to formulated detergent pH were found more compatible with enzyme-detergent formulation utility [6]. According to Banik and Prakash the protease should be compatible and remain active for at least 60-90 min in detergent solution; as this specific time required for cloth washing by either manually or machine [181].

Natural proteases from halophiles are used in a formulation of detergents [12,182]. Various powdered detergents such as Rin, Surf Excel, Henko, Super Wheel, Wheel, Tide, Tide Plus, Nirma, Ghari, Henko, Technobright, and Ariel were employed to evaluate the stability and compatibility of various proteases of halophilic/halotolerant microbes by several researchers. Ibrahim et al. documented a protease of *Bacillus* sp. NPST-AK15 which remained stable in various laundry detergents (Persil, Arial, Tide, Bonux, X-Tra, REX, OMO, and Ayam) for 1 h at 40℃ and have retained ~75–100% activity in presence of these commercial detergents [20].

Wu et al. documented a significant compatibility of protease obtained from marine bacterium *Pseudoalteromonas* sp. 129-1 with liquid detergents (1%) such as Ariel, Liby Tide, OMO, and Walch [145]. Also the proteases of *Bacillus* sp. EMB9 [23], *Geomicrobium* sp. EMB2 [183] or *Virgibacillus* sp. EMB13 [105] were confirmed as detergent compatible enzymes. An alkaline protease of *Bacillus circulans* MTCC 7942 [169] and *Salinicoccus* sp. UN-12 [122] were documented as an effective bio-additive for detergent formulations and were effective in hard water.

Ultrasound aided washing machines were proposed as a new technological alternative for the removal of tenacious dirt/stains from clothes, dishes, medical device. An ultrasound intensified detergent compatible proteases from *Bacillus circulans* MTCC 7942 and *Salinicoccus* sp. UN-12 were reported [122,184].

7.1.2. Protease for improving washing performance

Use of detergents reduces the time and energy required for cleaning by manual and automated washing [185]. In enzyme-detergent formualtion, the detergent compatible enzyme contributes to further improvement and acceleration of the washing performance. In this direction, various techniques are used to quantify the effectiveness of washing. The various approaches includes - (i) visual analysis, (ii) turbidity measurement, (iii) weight loss measurement, (iv) reflectance of light measurement, and (v) densitometric scanning. Various methods employed for evaluation of washing performance by detergent compatible proteases obtained from halophilic/halotolerant microbes are summarized in Table 13.

7.1.2.1. Visual Analysis

Washing performance is evaluated by visual investigation after deliberate staining, drying, washing, and drying of cloth. In order to enhance fixation of stain, several researchers have dried the stained cloth after soaking it in 2% (v/v) formaldehyde for 30 min. A white cotton cloth is usually needed to compare the contrast developed by stains, prior and after washing. Generally, blood is used as proteinaceous dirt/stain; alternatively, egg yolk albumin, grass, sweat, sauces, and milk are also used as proteinaceous dirt/stain. The stained white cloth is subjected to washing at different conditions like presence/absence of (i) detergent, (ii) protease, and (iii) protease–detergent formulation [23,91,100,103,105,111,130,169].

7.1.2.2. Turbidity measurement

The fine level analysis of washing performance is measured by evaluating the turbidity. After staining and subsequent drying, the stained cotton clothes are washed at different conditions like presence/absence of detergent, protease, and protease–detergent formulation and the turbidity of a soaked solution is analyzed regularly using turbiditimeter. The turbidity is correlated with stain removal by different detergent or detergent-enzyme formulations. The washing performance of protease-detergent mixture and protease from *Alcaligenes* sp. (MTCC 9730) was evaluated by this approach [186].

7.1.2.3. Weight loss measurement

The efficiency of washing performance was also investigated by weight loss method. The white cotton cloth was soaked in goat blood for 1 hr at 100℃. The fixed stained on the cotton cloth was measured initially. The stained clothes were washed at different conditions like in presence/absence of detergent alone and detergent and enzyme mixture for 15 min at 60℃. After washing, the cotton clothes were rinsed in water and dried (4 hr at 80℃). The cleaned cotton clothes were finally determined for weight loss. The washing performance is

calculated as the percent of the blood stain removed. The washing competence of protease from *Trichoderma estonicum* was evaluated by employing weight loss measurement technique. A percent blood stain removed was 59.7% after washing in the protease-detergent mixture and percent blood stain removed was 55.1% in detergent alone [187].

7.1.2.4. Reflectance of light measurement

A more sophisticated instrumental method - reflectance light measurement was suggested for evaluating the washing performance in recent years. This approach needs a special instrument – a reflectance spectrophotometer. The spectrophotometer measures the reflectance light in terms of K/S value; where K and S are adsorption and scattering coefficient, respectively. The whiteness of the stained cloth is inversely proportional to K/S.

Sinha and Khare [105] documented the washing performance of a protease obtained from *Virgibacillus* sp. EMB13 by measuring the reflectance of the cloth by a GretagMacbeth color matching spectrophotometer. Similarly, Jain et al. also documented the washing performance of halo-alkaline protease obtained from *Bacillus* sp. SM2014 by measuring the reflectance of stained clothes using UV/Vis spectrophotometer (Shimadzu, Japan) in the range of wavelength from 300–800 nm in reflectance mode [91]. Recently, Mokashe et al. reported the washing performance of detergent compatible protease obtained from *Salinicoccus* sp. UN-12 by measuring the reflectance of the washed cloth using UV/VIS-reflectance grade spectrophotometer (Datacolor-600) [122].

7.1.2.5. Densitometric scanning

The washing performance of stained clothes could be visualized by densitometric scanning. The cleansing efficiency of *Microbacterium luteolum* protease in a combination of a detergent was studied by this approach [188].

7.2. Leather industry

In the twenty-first century, the majority of a leather manufactures utilize the principles of tanning based on received wisdom and experience rather the scientific understanding [189]. In leather industry, the journey of animal hide to quality leather is passes through various steps including - (i) curing and preservation of hides and skins, (ii) soaking, (iii) dehairing, (iv) liming, (v) deliming, (vi) bating, (vii) pickling, (viii) tanning, (ix) dyeing, (x) fatliquoring, and (xi) drying. Traditionally in all steps, the leather industry utilizes various toxic and environment polluting chemicals like lime and sodium sulpfide. These processes also contributes to the enormous upsurge of BOD and COD in leather industry effluents.

Currently, the technology development in tanning and leather industry is fuelled by the constant reappraisal of enzymology. Use of enzyme in the leather industry can lead to new thinking, new developments and more profitability in an eco-friendly sustainable approach.

The enzymatic leather processing could be a suitable, eco-friendly approach over traditional lime-sulpfide process [190-193]. Proteases are suitably used in nearly all process of leather industry which includes (i) dehairing, (ii) dewooling of leather, and (iii) improvement in the quality of leather. The leather processed by employing the protease is cleaner, softer with stronger surface and with fewer spots [2]. The positive attributes of enzymatic leather processing are (i) reduction/total exclusion of lime and sulpfide use, (ii) negligible hide quality loss, and (iii) to recover intact hairs as a byproduct (which may be used for other applications like the manufacturing of synthetic fibres, biogas and a foaming agent).

Enzymatic dehairing contribute in the reduction of total solids and COD by 20%, and 45% respectively [189,194,195]. The proteolytic leather processing in alkaline condition is precise and easy as the alkaline proteases hydrolyze the swollen hair root and hair follicle. The alkaline condition contributes in swelling of the hair roots, and the succeeding protease catalysis of hair follicle protein easily removes the hair without hydrolysis of it [167,196-198].

Throughout the leather making inclusion of various chemical/factor is required to boost the process, these includes (i) NaCl (6-15%), (ii) biocides (0.1-1%), (iii) surfactants (0.25%), (iv) lime (3%), and (v) pH (9-11). During soaking and other related procedures, 6-15% NaCl is used to achieve appropriate swelling and texturing of a hide [189]. Also, it is needed to arrest the bacterial activity by using biocides. The use of salt-stable protease in overall leather processing contributes to replace/reduce the use of eco-burden chemicals, and reduction in process time. The enzymatic soaking reduces 45% time assisted with surfactants. Also, any salt-stable protease exhibits the elastolytic activity could be useful to process leather with additional outcomes like (i) leather is more uniformly flat, (ii) the skin is thinner with greater surface area (as the elastin is degraded), (iii) strength per unit thickness is increased, and (iv) leather become less stretchy.

The alkaline protease obtained from halophilic/halotolerant *Bacillus alkalitelluris* TWI3, *Bacillus tequilensis* P15 and *Vibrio alginolyticus* were effectively used for leather processing [160,199,200].

7.3. Biomedical industry

7.3.1. Proteases as antimicrobial agent

Currently, the rapid, widespread and exponential occurrences of antibiotic-resistant pathogenic microbes are emerging globally; threatening the efficacy of currently available antibiotics. The antibiotic resistance disaster has been due to irrational use of antibiotics and lack of new drug development [201-206]. In this view, novel treatment options by green approach or use of a novel antimicrobial agent is currently one of the most prioritized areas in medical biotechnology research.

The Enzybiotics consist of enzyme-antibiotic conjugates are useful in bacterial and fungal infections treatment [175]; this emphasizes the utility of enzyme as an important pharmaceutical choice. Even the purified microbial proteases were tested as antimicrobial agents. Alkaline proteases produced by halotolerant *Bacillus aquimaris* VITP4 exhibited the antimicrobial activity against pathogens - *Escherichia coli*, *Pediococcus* sp, *Pseudomonas* sp, *Yersinia enterocolytica* and *Candida albicans*, *Candida famata* and *Neurospora crassa* [90].

7.3.2. Proteases in tissue culturing and cell lines

During animal cell culturing, the trypsin is commonly used for the detachment and passaging of the adherent cell culture. The trypsin obtained from mammalian systems (e.g. porcine derived trypsin) may lead the viral contamination (e.g. porcine parvoviruses). Also, the trypsin forms aggregate in the cell suspension. Any salt-stable protease physiologically functional in wide range of buffers/salts and having the same substantiality could be a better alternative for trypsin. Also based on production economics and contamination problem; the microbial salt-stable proteases are a better option [207]. Proteases from marine isolates *Bacillus pumilus* MTCC 7514 [208], *Vibrio* sp. V26 [209] were found effective and proposed for application in tissue culturing and maintaining of cell lines.

7.4. Food industry

7.4.1. Protease in fish sauce fermentation

Fish sauce is a conventional, salty condiment, rich in essential amino acids, vitamin B_{12}, and minerals [210]. The fish sauce fermenting organisms should possess the capability to secret protease and active in a high concentration of salt. Therefore, proteolytic halophilic/halotolerant bacteria are desirable in fish sauce fermentation. Proteases from *Halobacillus* sp. SR5-3 [211], *Halobacterium* sp. SP(1) [212] were documented for their applications in an acceleration of fish sauce fermentation.

Similarly, the salt-stable proteases are efficiently explored for preparation of salty, proteinaceous fish- and meat-based products. The production of soy sauce by employing salt-stable protease was also documented [22].

7.4.2. Protease in meat tenderization

Meat (pork, beef, lamb, goat, poultry) is a considered as a nutritionally rich food for the human diet, which provides nutritional protein, essential amino acids, vitamin B_{12}, phosphorus, iron, and zinc [213-215]. Meat tenderness is means of toughness or resistance to cut and it is an important sensory quality which determines the quality and cost of meat [216].

Currently, repertoires of chemical and physical methods are available for improving tenderness. The use of exogenous proteases preferred for its efficiency and specificity required for meat tenderization. Several proteases from the plant, bacterial, and fungal sources having the potential to alter the structure of the connective tissue and myofibrils were documented for meat tenderization and allied applications. Protease from *Aspergillus oryzae* and *Bacillus* sp. are capable to tenderize the meat after 24 h at 4℃ [217,218]. Mukhopadhyay and Chakrabarti [219], documented that alkaline protease of *Bacillus subtilis* AKP(N) has the capability to tenderize the chicken meat sample rapidly (within 4 h). Malathi et al. reported the meat tenderization potential of alkaline protease obtained from *Vibrio alginolyticus* [200].

7.4.3. Biscuit and dough

The salt-stable proteases can be effectively used in dispensation of dough flour for getting less viscoelastic properties required for preparation of biscuit, wafer, and pizza [220]. It is possible to obtain the desired dough rheological properties by using flours in combination with salt-stable protease.

7.5. Technical biotechnology industry

7.5.1. Protease in photographic industries

Proteases are suitably used for hydrolysis of gelatin layer and recovery of silver from used/waste X-ray films. The silver content of X-ray film is 1.5-2%, w/w [221]. Although, it could be recovered by expensive, tedious and environmentally unsafe chemical methods like incineration of the films, oxidation of the metallic silver, and chemical stripping of the gelatin–silver layer [222]. Alternatively, the silver recovery by proteases is effective, economic and eco-friendly. Additionally, proteolytic recovery ensures the recycling of polyester film [223].

The significance of silver recovery using proteolytic reaction was documented by several researchers. For this purpose, the protease of moderate halophile *Bacillus* sp. EMB9 [224], and alkaline protease of *Bacillus licheniformis* K-3 [225] were efficiently used which is responsible to hydrolyze the gelatin layer within 8 and 20 min, respectively. The presence of NaCl (1.5%) affects the structural conformation of gelatin; by distorting the structure in presence of salt it makes gelatin more susceptible to catalyze by protease [163,164].

7.5.2. Immobilization of salt-stable protease

The immobilized protease displayed better enzymatic and kinetic properties. It also ensures the reusability of proteases [226]. The thermally stable immobilized preparation was able to successfully hydrolyze whey proteins at high temperature with a high degree of hydrolysis. For immobilization of proteases, various supports were used which includes carbohydrate polymers [227,228], polyvinyl alcohol beads [229] and microbial polysaccharides [230]. Freshly the attempts to immobilize the protease using nanoparticles like TiO2, magnetic, bimetallic Ag-Au, and silica have also been fruitful [231-233]. Sinha and Khare [234] studied the possibility of silica nanoparticles as an effective enzyme support for halophilic *Bacillus* sp. EMB9 protease to prepare an active, stable, recyclable enzyme formulation.

The halophilic proteases are highly salt-stable, additionally if any salt-stable enzyme if having stability in higher temperatures, chemical reagents, detergents, chaotropic agents, organic solvents, and extreme pH values could offer better outcomes on nanoparticle immobilization [30,183].

7.5.3. Protease as anti-fouling coating agents

Antifouling coatings are used to control biofouling. The biofouling is the accumulation and adhesion of organisms (bacteria, fungi, algae, and corals) on any surface in the aqueous/marine environment. The cleaning of biofouling from water intakes, piping systems, and seawater based cooling devices imparts the huge economic burden. In this view, antifouling paints containing tributyltin (TBT) were widely used to control biofouling [235,236], but the TBT is toxic and not effective against non-marine organisms involved in biofouling and ban by many countries. Currently, copper paints [237]; biocides like dichloroflumid, zine, zineb irgarol 1051 diuron, chlorothalonil, pyridine, and pyrithione [238,239] are compromised options for antifouling. The natural products having lesser environment damaging effects could be a better alternative [236].

In this view, proteases of halophilic microbe are a natural, economic, nontoxic and effective alternative for antifouling coatings as compared to toxic chemicals. Proteolytic enzymes derived from halophilic microorganisms demonstrated a significant catalytic activity at various bizarre environs like high salt, high pH, high temperature, and organic solvent tolerance/stability. Likewise, Akolkar et al. predicted the use of organic solvent tolerant protease from *Halobacterium* sp. SP1(1) as a better alternative for tin- and copper-based antifouling coating agents [166]. Moreover, Peres et al. documented the performance of papain

based antifouling coatings in the natural marine environment with environment-friendly approach [240].

More investigations will be desirable to understand the role of salt-stable proteases in the development of eco-friendly antifouling coating strategies.

7.5.4. Proteases as anti-biofilm agents

Bacterial biofilm is formed as a result of reversible adherence of bacterial community and it is embedded in a sticky polymeric matrix comprised of polysaccharides, proteins, and nucleic acids [241]. Bacterial biofilm naturally develops in various environments and nosocomial infection. Also, the biofilms are commonly detected on equipment in contact with natural water (pipelines vessels, aquaculture equipment, cooling water system, marine sensors, cables, fishing nets, and the pillars of bridges, and oil platforms). The artificial bacterial biofilms are useful in food, pulp and paper, textiles industry and wastewater treatment. However, control of biofilm is an important aspect, in order to avoid harmful effect by the biofilm; it is necessary to search eco-friendly anti-biofilm agents to fight with biofouling.

The proteolytic enzymes have the ability to dissociate proteinaceous polymeric matrix present within interconnecting bridge of cells in biofilms [242,243]. The cell to cell adhesion within biofilm could weaken/strengthened depending on the type/concentration of proteases [244]. Although various proteases are documented as anti-biofilm agents [245,246], very few are reported from halophilic/halotolerant microbes. Wu et al. documented an alkaline protease with effective anti-biofilm activity obtained from marine bacterium *Pseudoalteromonas* sp. 129-1 [145].

7.6. Waste treatment

Salt-stable proteases found several applications for treatment and utilization of waste with the environmentally-friendly approach. These proteases are accountable for efficient treatment/mineralization of household, fish, seafood/marine food industrial waste. Fish processing industry produces above 60% byproducts like head part, skin, trimmings, fins, frames, viscera and roes as a waste while only 40% part of the product is suitable for human consumption [247]. Such leftover fish waste creates environmental pollution threat worldwide. However, these fish wastes comprised of a considerable quantity of proteinaceous material, which could be converted into a value-added products like animal feed, fish meal, and fertilizer [248].

Annamalai et al. documented a production of halo-stable proteases from halophilic bacterial strains using marine waste (crab shell, shrimp shell, and squid pen powder)

[93,94,100]. Similarly, Maruthiah et al. reported a protease production using marine shell waste from marine bacterium *Bacillus* sp. APCMST-RS3 [107]. The protease was found effective for deproteinization of the shrimp shell waste; the deproteinized shrimp shell hydrolysates also exhibited appreciable antioxidant activity.

7.7. Bioremediation

The liquid waste generated by leather, marine food industries is hypersaline; occasionally industrial waste is saline and/or contains the organic solvent [249]. The pre-treatment of such saline and hypersaline waste is environmentally necessary to protect the water quality and aquatic life. Currently, chemical methods are only approach to treat the hypersaline effluents, as the biological approach is unsuccessful due to high salt concentration and alkaline pH of waste [249]. Availability of salt-stable protease could compensate the need of robust biocatalytic agent for bioremediation of salty marshes and industrial wastewater containing hazardous pollutants and organic solvents.

The protease of *Alkalibacillus* sp. NM-Da2 was available as an efficient and eco-friendly catalytic substitute for treatment of hypersaline wastewater [250].

7.8. Organic solvent tolerance of salt-stable proteases

Generally, organic solvents are accountable for enzyme denaturation and stripped of the essential water layer [251-253], which alters the structural stability and reduces the catalytic activity of an enzyme. The halophilic/halotolerant enzymes are catalytically functional under extremely high salt concentration and they have been reported to be stable in medium with low available water activity (a_w) [254]. The ability to remain functional in low water activity confers them a potential to remain active in non-aqueous conditions in presence of several organic solvent. Hence, the halophilic enzymes could efficiently use for biocatalysis in the presence of an organic solvents [255].

Traditionally, proteases are used to hydrolyse the protein into peptides/amino acids (catabolic reaction), but if any protease retains its activity in non-aqueous or semi-aqueous medium (eg. organic solvents or altered water content) could perform multifaceted catalysis like it may (i) improve the exquisite catalytic properties, (ii) catalyze reversible reaction and involved in peptide bonds synthesis (anabolic reaction), (iii) alters the substrate specificity with regio- and enantio-selectivity and involved in novel biotransformations and contemporary organic synthesis. This open ups the investigation of proteases at industrial scale for peptide and fine chemical synthesis [256]. Besides this, the use of organic solvents as medium for enzymatic reaction offers a number of advantages over traditional aqueous enzymology, which

includes (i) elevated solubility of hydrophobic substrates, (ii) alteration in hydrolytic equilibrium in presence of reduced water activity, and (iii) lower the chances of microbial contamination in the reaction mixture [86].

The solvent tolerant enzyme does not require special stabilizing methods like immobilization [257], chemical modification [258] and entrapment of enzyme in reversed micelles [259] due to natural stability of these enzymes against organic solvents and which comes to be very useful biocatalysts for non-aqueous enzymology [255,260].

In last decade, very few reports were available on solvent stable halophilic proteases *viz*. *Salinivibrio* sp. strain AF-2004 [134], *Halobacterium salinarum* [261], *Halobacterium halobium* [262], *Proteobacterium* [263], and *Natrialba magdii* [264]; the number of publications on solvent stable proteases of halophilic/halotolerant microorganisms were enormously increased in recent years. Table 14 summarizes the list of organic solvent stable proteases of halophilic/halotolerant microorganisms.

7.9. Stability of salt-stable proteases in ionic liquids

Ionic liquids are heat-stable liquids with low vapor pressure. They are favored alternates for hazardous organic solvents, as ionic liquids are considered as a possible green media for non-aqueous enzymatic catalysis [133,265]. Besides regio- and enantio-selectivity, the ionic liquids have also additional attributes like alterable polarity, hydrophobicity, and solvent miscibility [266,267]. The stable enzyme showing activity in ionic liquids could be used in non-aqueous enzymology with wider applicability spectrum [269]. The utility of ionic liquids in non-aqueous enzymology with added additional benefits as compared to organic solvents and opened the new avenue in biochemical engineering.

The activity of the protease from a halophilic bacterium *Salinivibrio* sp. strain MS-7 in imidazolium-based ionic liquids was studied [133]. Recently, Mokashe et al. reported the ionic liquid (imidazolium) stable protease obtained from S*alinicoccus* sp. UN-12 [122]. Similarly, ionic liquid-tolerant *Bacillus amyloliquefaciens* CMW1 and its salt-stable protease [269] and the influence of ionic liquids on hydrolysis of casein by a proteolytic lumbrokinase enzyme were described [270].

8. Conclusions

Currently, the technology expansion in biotech-industry is powered by the continual reassessment of various developments in enzymology. The utility of enzyme in the widening industrial sector can lead to new thinking, new developments and more profitability in an eco-friendly sustainable approach. Microbial proteases play a significant role in industrial

processes. The availability of proteolytic enzymes from extremophilic microorganisms fueled the utility of proteases. A diversity of proteases from various bacteria provides the basis for their newer applications in various industrial sectors. The salt-stable proteases obtained from the halophilic and halotolerant microorganisms represents the unique and novel alternative for biotechnology industries in several claims. As reviewed in this study, the salt-stable protease obtained from halophilic/halotolerant bacteria are demonstrated to be functioning in wide array of physicochemical factors. The robustness of these protease against various factors includes – activity and stability in presence of broad range of salt/s, wide range of pH and temperature, non-aqueous systems, surfactants, bleaching agents, oxidizing agents, stabilizers, builders, brightening agents, ionic liquids, and hydrotropes as analyzed by several researchers.

The article reviews the current usefulness and future trends of salt-stable proteases from halophilic/halotolerant bacteria in detergent and textile industry, improving washing performance in hard water, in leather industry, in biomedical industry as antimicrobial agent, tissue culturing and cell lines protocols, for improving quality of foods (fish sauce, meat, biscuits and dough), anti-fouling coating, anti-biofilm formation, waste treatment and bioremediation, and fine chemical synthesis in non-aqueous systems (like organic solvents, ionic liquids or excess salts). Authors believed that even several applications have been currently undisclosed and could be explored in future with further understanding of protease structural behavior and ever-flourishing expansions in applied technology field.

Although the current operative utility of salt-stable protease is based on available intrinsic robustness of them but there is ample scope for structural and functional improvement of salt-stable protease by various approaches like physical/chemical modification, immobilization, protein engineering and recombinant DNA perspective in order to design novel variants of these enzymes that will be custom-made to precise applications and apt for industrial set up. The utility of novel salt-stable protease from halophilic or halotolerant extremophilic microorganisms with suitable industrial applications is incessant voyage.

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Figure captions

Fig. 1. Classification of microorganisms based on their salt requirements

Fig. 2. Adaptive strategy of halophiles in saline/hypersaline environment by salt in cytoplasm mechanism

Fig. 3. Adaptive strategy of halophilic/halotolerant microorganisms in saline environment by compatible solute mechanism

Fig. 4. Salt stability of halophilic proteins. **(A)** Enzymes/proteins having negative charges on protein surfaces conferred by acidic amino acids (denatured conformation); **(B)** The negative charges on protein surfaces neutralized it by cation; e.g. Na (active conformation).

Fig 5. The amino acid sequences of serine proteases of various halotolerant and halophilic bacteria were represented in tree depending on their similarity among amino acids determined by Clustal omega software.

Fig 6. The amino acid sequences of metallo-proteases of various halotolerant and halophilic bacteria were represented in tree depending on their similarity among amino acids determined by Clustal omega software.

Tables

Table 1

Biological significance of various inorganic elements

Element (symbol)	Biological importance
Calcium (Ca)	Important ion for movement of molecules inside the cell acts as messenger ion, a constituent of bacterial endospore (calcium dipocolinic acid)
Potassium (K)	Most abundant cation inside the cell
Sodium (Na)	Most plentiful cation outside the cell
Chloride (Cl)	Most plentiful anion outside the cell
Magnesium (Mg)	Needed for proper functioning of many enzymes; component of chlorophyll
Iron (Fe)	Essential component of various molecules and is associated with electron transport chain
Boron (B)	Acts as a cell signaling molecule, involved in electron transfer and redox sensing
Copper (Cu)	Essential component of many metallo enzymes and some naturally occurring pigments
Phosphorous (P)	Required for phosphorylation associated with metabolic interactions
Zinc (Zn)	Cofactor of alcohol dehydrogenase and carboxyl peptidase
Magnesium (Mg)	Cofactor of phosphohydrolases
Manganese (Mn)	Cofactor of arginase
Iron (Fe)	Component of cytochrome and ferredoxin
Copper (Cu)	Cofactor of cytochrome oxidase
Potassium (K)	Cofactor of pyruvate kinase
Sodium (Na)	Cofactor of ATPase
Molybdenum (Mo)	Cofactor of nitrate reductase
Selenium (Se)	Cofactor of glutathione peroxidase
Nickel (Ni)	Cofactor of urease

Table 2

Compatible solutes secreted by halophilic microorganisms [34]

Class of compatible solute	**Specific compatible solute**	**Microorganism**
Polyoles	Glycerol and arabite Glucosyl-glycerol	Algae, yeast, fungi Cyanobacteria
Betaines	Glycine betaine	*Actinopolyspora halophile*, anoxygenic phototrophic bacteria, cyanobacteria, and methanogens
Amino acids	Dimethylglycine Proline α-glutamine β-glutamine	*Methanohalophilus* sp. *Bacillus* spp. and other *Firmicutes* Corynebacteria *Methanohalophilus* sp Marine cyanobacteria
Dimethylsulfoniopropionate Glutamine amides	N-α-carbomoyl-glutamine amide N-α-acetyl-glutaminyl-glutamine amide	*Ectothiorhodospira marismortui* Anoxygenic phototrophic proteobacteria, *Rhizobium meliloti*, and *Pseudomonas* spp.
Acetylated diamino acids	N-δ-acetyl-ornithine and N-ε-acetyl-α-lysine N-ε-acetyl-α-lysine	*Bacillus* spp. and *Sporosarcina halophila,* Methanogens
Ectoines	Ectoine and hydroxyectoine	Proteobacteria, *Brevibacteria*, gram-positive cocci, and *Bacillus* spp.

Table 3
Protease producing halophilic/halotolerant microorganisms isolated from various ecological biotopes

Microbial strain	Ecological biotopes	Halophilic /halotolerant microorganism	NaCl optimum	NaCl range	Reference
Halobiforma sp. strain BNMIITR	Soil sample Sambhar lake, Rajasthan, northern India.	Haloalkaliphilic bacterium	17.4% (3 M)	11.6-29% (2-5 M)	[18]
Jeotgalicoccus sp.	Saline soil, Bharwade, Shirpur, India	Halotolerant	5.8% (1 M)	0-11.6% (0-2 M)	[19]
Bacillus iranensis strain X5B	Saline mud, hypersaline lake, Aran-Bidgol, Iran	Moderately halophilic	5-7.5% (0.87-1.3 M)	2.5-15% (0.44-2.6 M)	[21]
Bacillus sp. EMB9	Solvent contaminated soil, New Delhi, India	Moderately halophilic	NS	NS	[23]
Pseudoalteromonas sp. strain CP76	Water and sediment of solar salterns and hypersaline soils, Isla Cristina (Huelva, Spain)	Moderately halophilic	NS	NS	[76]
Actinopolyspora sp. VITSDK2	Marine sediments, Marakkanam coast, Bay of Bengal, Southern India	Halophilic	NS	6-18% (1.035-3.11 M)	[92]
Bacillus firmus CAS 7	Marine sediments of Parangipettai, Tamilnadu, India	Halophilic	NS	NS	[93]
Bacillus alveayuensis CAS 5	Marine sediments of Parangipettai, Tamilnadu, India	Halophilic	NS	NS	[94]
Bacillus subtilis strain BLK-1.5	Soil samples, salt mines, Karak, Pakistan	Halotolerant	7% (1.21 M)	2-12% (0.35-2.07 M)	[95]
Bacillus halodurans CAS 6	Marine sediments of Parangipettai, Tamilnadu, India	Halophilic	NS	NS	[100]
Micrococcus sp. VKMM 037	Effluent waste, caustic soda factory, Arumuganeri, Tuticorin district, Tamil Nadu, India	Haloalkaliphilic	NS	NS	[103]
Salinivibrio sp. strain MS-7	Maharlu salt lake, south of Iran	Moderately halophilic	10% (1.75 M)	5-20% (0.87-3.45 M)	[104]
Virgibacillus sp. EMB13	Seawater sample, Somnath Coast of Gujarat, India	Moderately halophilic	NS	NS	[105]
Halomonas meridiana	Mucus of *Acropora* sp., South east coast, India	Moderately halophilic	NS	NS	[106]

Microbial strain	Ecological biotopes	Halophilic /halotolerant microorganism	NaCl optimum	NaCl range	Reference
Bacillus sp. APCMST-RS3	Marine sediment samples, Rajakkamangalam estuary, Kanyakumari, Tamil Nadu, India	Halophilic	NS	NS	[107]
Bacillus sp. APCMST-RS7	Marine sediment samples, Rajakkamangalam estuary, Kanyakumari, Tamil Nadu, India	Halophilic	NS	NS	[108]
Bacillus sp. strain NPST-AK15	Sediment and water samples, Wadi El-Natrun valley hypersaline soda lakes, northern Egypt	Halotolerant	0-5% (0.87 M)	0-20% (0-3.45 M)	[110]
Virgibacillus halodenitrificans RSK CAS1	Marine ascidian *P. mammillata,* Andaman and Nicobar Islands, India	Halophilic	NS	NS	[111]
Halogeometricum sp. TSS101	Solar evaporated salt pond of Tuticorin, coastal area of Tamilnadu, India	Extremely halophilic archaeon	15-20% (2.6 - 3.45 M)	8-25% (1.38 – 4.35 M)	[113]
Bacillus pseudofirmus	Seawater near the Veraval coast lighthouse, Gujarat, India	Moderately haloalkaliphilic bacterium	NS	NS	[119]
Halobacterium sp. strain HP25	Brine, multicolor solar salt pond water, saline soil, saline mud and raw salt, Emisal salt, Lake Qarun, Fayoum, Egypt	Extremely halophilic	30% (5.18 M)	20-30% (3.45-5.18 M)	[120]
Halobacillus sp. CJ4	Soil of Chott Eldjerid hypersaline lake, Tunisia	Moderately halophilic	5% (0.87 M)	0-24% (4.15 M)	[121]
Salinicoccus sp. UN-12	Saline soil, Shirpur, India	Halotolerant	6% (1.035 M)	0-16% (0-2.76 M)	[122]
Alkalibacillus sp. NM-Fa4	Alkaline, hypersaline lakes of the Wadi An Natrun, Egypt	Halophilic	9.86% (1.7 M)	5.22-19.72% (0.9-3.4 M)	[136]
Halorubrum ezzemoulense strain ETR14	Water and sediment samples, solar saltern of Sfax, Tunisia	Halophilic	10-20% (1.75-3.45 M)	5-30% (0.87 -5.18 M)	[146]
Oceanobacillus iheyensis O.M.$A_1$8	Salt-enriched soil, salt panes, Okha, Gujarat, India	Haloalkaliphilic	15% (2.6 M)	5 -20% (0.87-3.45 M)	[147]

Microbial strain	Ecological biotopes	Halophilic /halotolerant microorganism	NaCl optimum	NaCl range	Reference
Haloalkaliphilic bacterium O.M.E$_1$2	Salt-enriched soil, salt panes, Okha, Gujarat, India	Haloalkaliphilic	10% (1.75 M)	5 -20% (0.87-3.45 M)	[147]
Nocardiopsis alba OK-5	Salt enriched soil Okha, along the coastal region of Gujarat, India	Halotolerant	NS	NS	[150]
Oceanobacillus sp.	Salt enriched soil Jodiya, Western Coast of Gujarat, India	Haloalkaliphilic	NS	NS	[151]
Virgibacillus sp. SK33	1 month-old fish sauce	Moderately halophile	5% (0.87 M)	0-25% (0 - 4.35 M)	[158]
Virgibacillus halodenitrificans SK1-3-7	Fish sauce fermentation	Moderately Halophilic	NS	NS	[159]
Halobacillus sp. LY6	Saline soil, Yuncheng, China	Moderately halophilic	12% (2.07 M)	0 -20% (0-3.45 M)	[161]
Halobacterium sp. SP1(1)	Brine samples, salt pans, Kandla, Gujarat, India	Halophilic	25% (4.35 M)	20-30% (3.45 - 5.2 M)	[166]
Bacillus halodurans Strain US193	Salted compost	Halotolerant	0%	0.12.5% (2.16 M)	[171]
Alkalibacillus sp. NM-Da2	Mixed water–sediment samples, lakes of the Wadi An Natrun, North–Western Egypt	Halophilic	NS	NS	[250]
Geomicrobium sp. EMB2	Sambhar Salt Lake, India	Moderately halophile		5 -20% (0.87-3.45 M)	[256]
Bacillus aquimaris VITP4	Kumta coastal area, Karnataka, India	Halotolerant	1-3% (0.18 – 0.52 M)	0-15% (0 - 2.6 M)	[276]
Salicola sp. IC10	Hypersaline habitats, South Spain	Halophilic	15-20% (2.6 - 3.45 M)	15-30% (2.6 – 5.2 M)	[277]
Virgibacillus marismortui	Pla-ra, fermented fish, Thailand	Halotolerant	15% (2.6 M)	0-20% (0 - 3.45 M)	[281]
Halobacillus sp. SCSIO 20089	Marine sediment South China Sea	Halophilic	NS	NS	[283]
Salimicrobium halophilum strain LY20	Saline soil, Yuncheng, China	Moderately halophilic	10% (1.75 M)	0.5-25% (0.087-4.35 M)	[284]
Bacillus subtilis JM-3	Anchovy sauce naturally fermented	Halophilic	NS	NS	[285]

NS: Not specified

Table 4

Statistical methods used to improve the protease production from halophilic/halotolerant microbes

Microorganism	Design	Software	Fold yield	Reference
Virgibacillus halodenitrificans RSK CAS1	RSM	Minitab 16	NS	[111]
Bacillus sp. EMB9	PBD, CCD	Design expert software	2.5	[224]
Geomicrobium sp. EMB2	PBD, CCD	Design Expert software	20	[256]
Bacillus acquimaris VITP4	PBD, CCD	Graphpad Prism 5 and Design Expert Software	1.5	[279]
Pontibacillus sp. SY–8	PBD, RSM	Design Expert software	3.5	[280]

NS: Not specified

Table 5
Optimal fermentation parameters for the production of salt-stable proteases from halophilic/halotolerant microbes

Bacteria	Carbon source	Nitrogen source	Salt	Inoculum	pH	Temperature (^{0}C)	Agitation (rpm)	Incubation period (h)	Yield U/ml	Reference
Jeotgalicoccus sp.	None	Peptone	NaCl, $CaCl_2$, KH_2PO_4, $MgSO_4$	NS	10	37	100	40	256	[19]
Bacillus sp. SM2014	Glucose	Casamino acids	NaCl	NS	7	37	150	120	NS	[91]
Bacillus firmus CAS 7	Shrimp and crab shell powder	Shrimp and crab shell powder	NaCl, K_2HPO_4, $MgSO_4$,	1%	9	55	150	60	2478	[93]
Bacillus alveayuensis CAS 5	Shrimp shell powder	None	NaCl, K_2HPO_4, $MgSO_4$,	1%	9	55	150	60	2872	[94]
Bacillus subtilis strain BLK-1.5	NS	NS	NaCl	NS	10	37	NS	NS	NS	[95]
Bacillus halodurans CAS6	Shrimp shell powder	None	NaCl, K_2HPO_4, $MgSO_4$,	1%	9	50	150	60	3413	[100]
Micrococcus sp. VKMM 037	Glycerol	Peptone	NaCl	NS	10	40	250	NS	NS	[103]
Salinivibrio sp. strain MS-7	Maltose	Peptone, Yeast extract	NaCl, $MgSO_4$, $CaCl_2$, $MgCl_2$	NS	8	30	180	72	494	[104]
Virgibacillus sp. EMB13	Mannitol	Yeast extract, peptone	NaCl, KCl, $CaCl_2$, $MgCl_2$	4%	8	30	150	24	270	[105]
Halomonas meridiana RA001	Starch	Beef extract	K_2HPO_4, $MgSO_4$, $FeSO_4$	10%	7	37	140	NS	NS	[106]
Bacillus sp. APCMST-RS3	Shrimp & Oyster shell powder	None	NaCl	NS	NS	50	150	48	4000	[107]
Bacillus sp. NPST-AK15	Fructose	Yeast extract	NaCl, $CaCl_2$, $BaCl_2$	NS	11	40	200	48	1263	[110]

Bacteria	Carbon source	Nitrogen source	Salt	Inoculum	pH	Temperature (^{0}C)	Agitation (rpm)	Incubation period (h)	Yield U/ml	Reference
Virgibacillus halodenitrificans RSK CAS1	Shrimp shell powder	Casein	NaCl, $MgSO_4$	1%	7	40	125	48	1461	[111]
Salinicoccus sp. UN-12	Glucose	Soyabean meal	NaCl	NS	7.5	30	100	--	--	[122]
Geomicrobium sp. EMB2	None	Casamino acids, yeast extract	NaCl, $MgCl_2$	3%	8.5	30	150	96	721	[256]

Table 6

Salts and minerals requirement for the growth of non-halophile, halotolerant, and halophile

Component	Halophile (g/l)	Halotolerant (g/l)	Non-halophile(g/l)
NaCl	125-250	50-100	5-8
KH_2PO_4	-	0.01-0.28	1.0-4.0
$MgSO_4.7H_2O$	10.0-29.0	0.1-20.0	0.25-0.30
KCl	2.0-6.0	0.02-2.0	0.5-12.0
$CaCO_3$	-	-	5.0-17.0
$FeSO_4.4H_2O$	-	-	0.01-0.1
$ZnSO_4.8H_2O$	-	-	0.1-1.0
$MnSO_4.H_2O$	-	-	0.01-0.1
$CuSO_4.5H_2O$	-	-	0.003-0.01
$Na_2MoO_4.2H_2O$	-	-	0.01-0.1
Na citrate	3	3	-
K_2SO_4	0-5	-	-
K_2HPO_4	0-0.5	0.5-7.5	-
$(NH_4)_2SO_4$	0-1	0-2	-
$MgCl_2.6H_2O$	20-50	-	-
$CaCl_2.6H_2O$	0.2-1.1	0-0.36	-
NaBr	0-0.8	0-0.23	-
$NaHCO_3$	0-0.2	0-0.06	-

Table 7
Various strategies applied for purification of salt-stable proteases from halophilic/halotolerant microorganisms

Microbial source	Purification methods	Purification Fold	Recovery (%)	MW (kDa)	Reference
Jeotgalicoccus sp.	Culture supernatant	1	100	51	[19]
	$(NH_4)_2SO_4$ precipitate	10	76		
	Sephadex G-100	36	51		
	DEAE-Cellulose	39	38		
Bacillus sp. NPST-AK15	Cell free supernatant	1	100	32	[20]
	$(NH_4)_2SO_4$ precipitate	1.6	66.2		
	DEAE-Sephadex G-50	15.6	18.6		
	Sephadex G-50	16	18.3		
Bacillus iranensis (X5B)	Crude	1	100	48-50	[21]
	Acetone precipitation	1.29	72		
	Ultrafiltration	4.34	61		
	Carboxymethyl (CM)	45.19	55		
Bacillus sp. EMB9	Culture supernatant	1	100	29	[23]
	$(NH_4)_2SO_4$ precipitate	15.2	7.6		
	CM-cellulose	488.9	2.8		
Pseudoalteromonas sp. strain CP76	Culture supernatant	1	100	38	[76]
	Q-Sepharose ion exchange	21.4	11.3		
	Superdex 200	64.8	2.9		
Bacillus aquimaris VITP4	Culture supernatant	1	-	34	[90]
	Membrane filtration	19	-		
	$(NH_4)_2SO_4$ precipitate	23	-		
	Dialysis	32	-		
Bacillus sp. SM2014	Culture supernatant	1	100	71	[91]
	$(NH_4)_2SO_4$ precipitate	5.49	84.08		
	Dialysis	19.97	76.33		
	Sephadex G-100	64.11	54.4		

Microbial source	Purification methods	Purification Fold	Recovery (%)	MW (kDa)	Reference
Bacillus firmus CAS 7	Culture supernatant	1	100	21	[93]
	$(NH_4)_2SO_4$ precipitate	1.1	38.6		
	DEAE-cellulose	1.7	18.7		
	Sephadex G-50	2.6	12.3		
Bacillus alveayuensis CAS 5	Culture supernatant	1	100	33	[94]
	$(NH_4)_2SO_4$ precipitate	2.15	46.48		
	DEAE-cellulose	6.29	15.88		
	Sephadex G-75	7.77	12.86		
Bacillus halodurans CAS6	Culture supernatant	1	100	28	[100]
	$(NH_4)_2SO_4$ precipitate	1.92	51.93		
	DEAE-cellulose	5.57	17.93		
	Sephadex G-50	7.96	12.54		
Micrococcus sp. VKMM 037	Culture supernatant	1	100	41	[103]
	$(NH_4)_2SO_4$ precipitate	2.30	55		
	Q-Sepharose	9.43	21		
Virgibacillus sp. EMB13	Crude	1	100	49 & 54	[105]
	Ultrafiltration	2.1	33		
	DEAE-cellulose	2.5	5.6		
	Sephadex G-75	4.2	3.1		
Bacillus sp. APCMST-RS3	Culture filtrate	1	100	40	[107]
	$(NH_4)_2SO_4$ precipitate	3.48	53.22		
	Gel filtration (G-75)	8.49	22.66		
Bacillus sp. APCMST-RS7	Culture filtrate	1	100	32	[108]
	$(NH_4)_2SO_4$ precipitate	2.93	35.91		
	DEAE-Sepharose	6.60	24.30		
Virgibacillus halodenitrificans RSK CAS1	Culture supernatant	1	100	21	[111]
	$(NH_4)_2SO_4$ precipitate	2.13	52.47		
	DEAE-Cellulose	5.97	21.702		

Microbial source	Purification methods	Purification Fold	Recovery (%)	MW (kDa)	Reference
	Sephadex G-75	8.7	15.33		
Bacillus pseudofirmus	Culture supernatant	1	100	37.2	[119]
	$(NH_4)_2SO_4$ precipitate	5.88	37.45		
	Ultrafiltration	7.95	12.48		
	Phenyl Sepharose	15.64	15.77		
Halobacterium sp. strain HP25	Culture supernatant	1.4	100	21	[120]
	Ultrafiltration (Amikon)	14	87		
	Dialysis	18	64		
	Superdex 200 HR	167	31		
Salinicoccus sp. UN-12	Culture supernatant	1	100	63	[122]
	$(NH_4)_2SO_4$ precipitate	5	56		
	DEAE-Cellulose	11	19		
	Sephadex G-100	17	4		
Salinivibrio sp. strain MS-7	Culture supernatant	1	100	21	[133]
	80% acetone precipitate	3.2	68		
	DEAE-cellulose	3.6	55.6		
Alkalibacillus sp. NM-Fa4	Culture supernatant	1	100	19.7	[136]
	Ethanol precipitate	4.5	48.4		
	Q-Sepharose	9.8	15.5		
Marinomonas arctica PT-1	Cell culture supernatant	1	-	63	[138]
	$(NH_4)_2SO_4$ precipitate	7	-		
	Amicon ultra filters	21	-		
	DEAE-Sepharose chromatofocusing	42.7	-		
Pseudoalteromonas sp. 129-1	Culture supernatant	1	100	35	[145]
	$(NH_4)_2SO_4$ precipitate	2.8	72		
	Anion exchange	6.5	43		
	Superdex gel-filtration	14.95	21		
Oceanobacillus sp.	Culture supernatant	1	100	30	[151]

Microbial source	Purification methods	Purification Fold	Recovery (%)	MW (kDa)	Reference
	$(NH_4)_2SO_4$ precipitate	3.62	35		
	Phenyl Sepharose 6FF HIC	27.83	28		
Virgibacillus halodenitrificans SK1-3-7	Culture supernatant	1	100	20 & 36	[159]
	$(NH_4)_2SO_4$ precipitate	2.2	76		
	Phenyl-Sepharose	3.7	31		
	DEAE-Sephacel	9.4	20		
	Source Q	14.1	5		
Halobacillus sp. LY6	Culture supernatant	1	100	69	[161]
	$(NH_4)_2SO_4$ precipitate	-	-		
	Sephacryl S-100	23.6	18.1		
Bacillus halodurans US193	Crude extract	1	100	37	[171]
	$(NH_4)_2SO_4$ precipitate	3	54		
	FPLC (Anion exchange)	4.9	21		
Alkalibacillus sp. NM-Da2	Culture supernatant	1	100	35	[250]
	Ethanol precipitate	5.5	39.9		
	Q-Sepharose	13.3	11.3		
Bacillus safensis S406	Culture filtrate	1	100	29	[278]
	$(NH_4)_2SO_4$ precipitate	3	81.5		
	Sephadex G-75	4.67	43.76		
	Mono-Q	8.18	35.51		
	Ultrafiltration	12.70	20.29		
Chromohalobacter sp. TVSP101	Culture supernatant	1	100	66	[282]
	Protein concentrator Nalgene (50kDa)	12	77		
	Ethanol precipitate	16	60		
	Phenylsepharose 6B column	30	55		
	Gel permeation G-100	180	22		
Halobacterium sp. SP1(1)	Culture supernatant	1	100	42.1	[286]
	10 kDa concentrate	2.25	89.34		

Microbial source	Purification methods	Purification Fold	Recovery (%)	MW (kDa)	Reference
	Sephacryl S-200	111.254	17.41		

Table 8
Biochemical properties of salt-stable proteases obtained from halophilic/halotolerant microbes

Bacteria	Optimum pH	Optimum Temperature (℃)	Protease type	Inhibitor	MW (kDa)	K_m, V_{max}, and k_{cat}	Other activity	Reference
Halobiforma sp. strain BNMIITR	9	55	Serine	PMSF	NS	NS	NS	[18]
Jeotgalicoccus sp.	10	48	Metallo	EDTA	51	NS	Enhanced activity in presence of rhamnolipid & cyclodextrin	[19]
Bacillus sp. NPST-AK15	10.5	60	Serine	PMSF	32	K_m - 2.5 mg/ml, V_{max} - 42.5 µM/min/mg, and k_{cat} - 392.46 × 10^3/min	Detergent-, solvent- and salt-compatible thermoactive alkaline protease	[20]
Bacillus iranensis (X5B)	9.5	35	Serine	PMSF	48-50	K_m - 0.126 mM and V_{max} - 0.523 mM/min, k_{cat} - 3.284 × $10^{-2}s^{-1}$	Moderately halophilic and can activated by metals especially Ca^{2+}	[21]
Bacillus sp. EMB9	9	60	Serine	PMSF	29	K_m - 2.22 mg/ml, V_{max} - 1111.11 U/ml	Stable in hydrophilic and hydrophobic organic solvents	[23]
Pseudoalteromonas sp. strain CP76	8.5	55	Metallo	EDTA	38	NS	NS	[76]
Bacillus aquimaris VITP4	8	40	Metallo	EDTA	34	NS	Antimicrobial activity against common bacterial and fungal pathogens	[90]
Bacillus sp. SM2014	10	60	Serine	PMSF	71	K_m - 0.57 mg/ml and V_{max} – 445.23 U/ml	Stable in presence of various surfactants, bleach, oxidizing agents, solvents	[91]
Actinopolyspora sp. VITSDK2	10	60	Serine	PMSF, DFP	22	NS	Protease retains 96% activity after 10 days incubation at RT	[92]
Bacillus firmus CAS 7	11	70	Metallo	EDTA, 1,10 Phenanthroline	21	NS	Quite stable in the presence of anionic, non-ionic surfactants and organic solvents	[93]
Bacillus alveayuensis CAS 5	9	50	Metallo	EDTA	33	NS	Highly stable in presence of ionic, non-ionic surfactants and commercial detergents	[94]

Bacteria	Optimum pH	Optimum Temperature (℃)	Protease type	Inhibitor	MW (kDa)	K_m, V_{max}, and k_{cat}	Other activity	Reference
Bacillus subtilis BLK-1.5	10	50	NS	NS	38	NS	NS	[95]
Bacillus halodurans CAS6	10	70	Metallo	EDTA	28	NS	Potential additive for detergent and antioxidant synthesis	[100]
Micrococcus sp. VKMM 037	10	40	Serine	PMSF	41	NS	Stable/only marginally inhibited in presence of various organic solvents, surfactants and reducing agents	[103]
Virgibacillus sp. EMB13	7.5	50	Metallo	EDTA, 1,10 Phenanthroline	49, 54	K_m - 7.5 mg/ml, V_{max} - 156.25 µ/min/ml	Remarkable stability in presence of salt and solvents	[105]
Bacillus sp. APCMST-RS3	9	60	Serine	PMSF	40	K_m - 0.6666 g/l, V_{max} - 1111.11 U/ml	Detergent stable, stain removal and deproteinization with proven antioxidant potential	[107]
Bacillus sp. APCMST-RS7	8	50	Serine	PMSF	32	K_m - 0.0002 g/l and V_{max} -1428.57 U/ml	Bacterium effectively deproteinized shrimp shell waste with maximum protease activity	[108]
Virgibacillus halodenitrificans RSK CAS1	9	50	Metallo	EDTA	21	NS	Significant stability at higher levels of salt, metal ions, organic solvents and commercial detergents	[111]
Haloalkaliphilic bacterium	11	37	Serine	PMSF	30–32	K_m - 0.153 mg/100 ml, V_{max} - 454 U/ml	SDS and Triton X-100 stable	[118]
Bacillus pseudofirmus	10	50	Serine	PMSF	37.2	K_m - 0.08 mg/ml, V_{max} - 6.346 µM/min, k_{cat} - $3.99\times10^{-2}S^{-1}$	Stable in various surfactants and detergents	[119]
Halobacterium sp. strain HP25	8	60	Serine	PMSF	21	K_m - 523 µg/ml, V_{max} - 2500 µg/min/ml	Stability in presence of organic solvents and laundry detergents	[120]

Bacteria	Optimum pH	Optimum Temperature (℃)	Protease type	Inhibitor	MW (kDa)	K_m, V_{max}, and k_{cat}	Other activity	Reference
Halobacillus sp. CJ4	9	45	Serine	PMSF	NS	NS	Highly stable in organic solvents and detergent additives	[121]
Salinicoccus sp. UN-12	8.5	55	Serine	PMSF	63	NS	Ultrasound active, stable in ionic liquids	[122]
Streptomyces pseudogrisiolus NRC-15	9.5	50	Serine	PMSF	20	K_m - 0.25, V_{max} - 72.8 μ/mg	Laundry detergent additive to improve washing performance	[129]
Bacillus aquimaris VITP4 Strain	8	40	Serine	PMSF	34.7	K_m - 0.817 mg/ml/min, V_{max} - 0.472 mg/ml, k_{cat} - 2.31 S^{-1}	Surfactant stable and detergent compatibility	[130]
Salinivibrio sp. strain MS-7	8	50	Serine Metallo	PMSF, Pefabloc SC, chymostatin, EDTA	21	K_m - 1.14 mg/ml, V_{max} - 130 U/mg	Outstanding activity in organic solvents and imidazolium-based ionic liquids	[133]
Alkalibacillus sp. NM-Fa4	9.5	45	Cysteine	IAA	19.7	K_m - 1.3 mg/ml, V_{max} - 1111 mg/min/ml	Stability towards SDS, H_2O_2, urea, and retained most of its activity in presence of Tween 80	[136]
Marinomonas arctica PT-1	8	37	Serine	PMSF	63	Skim milk K_m - 1.16×10^{-3} mg/ml V_{max} - 7×10^{-5} U, and Gelatin K_m - 7.60×10^{-4} mg/ml and V_{max} - 0.2×10^{-3} U	NS	[138]
Pseudoalteromonas sp. 129-1	8	50	Serine	PMSF	35	NS	Valuable additive in laundry detergent and non-toxic anti-biofilm agent	[145]
Halorubrum ezzemoulense strain ETR14	9	60	NS	NS	NS	NS	Good temperature, pH, and salinity tolerance	[146]
Oceanobacillus iheyensis O.M.$A_1$8	11	50	NS	NS	35	NS	NS	[147]

Bacteria	Optimum pH	Optimum Temperature (℃)	Protease type	Inhibitor	MW (kDa)	K_m, V_{max}, and k_{cat}	Other activity	Reference
Haloalkaliphilic bacterium O.M.E$_1$2	11	50	NS	NS	29	NS	NS	[147]
Nocardiopsis alba OK-5	10-11	70	Serine	PMSF	20	K_m - 0.50 mg/ml and V_{max} - 3634.12 U/min	Extreme resistance against urea denaturation, oxidizing and reducing agents and surfactants	[150]
Oceanobacillus sp.	10	50	NS	NS	30	NS	Enzyme retained 70% activity in 10% (v/v) isooctane	[151]
Virgibacillus halodenitrificans SK1-3-7	9	60	Serine	PMSF	20 and 36	NS	High stability towards organic solvents, surfactants, and oxidizing agents	[159]
Halobacillus sp. LY6	10	40	Metallo	EDTA	69	NS	Stable in presence of various metal ions	[161]
Bacillus halodurans US193	10	70	Serine	PMSF	37	NS	Bio-additive in detergent industry	[171]
Alkalibacillus sp. NM-Da2	9	56	Serine	PMSF	35	NS	Degrade the dissolved protein in synthetic sea water system	[250]
B. safensis S406	11	60	Serine	PMSF	29	K_m - 0.48 mM and V_{max} -52.63× 10^{-3} U/mg	Deproteinization of shrimp waste	[278]
Halobacillus sp. SCSIO 20089	8	30	Metallo (thermolysin-like)	EDTA	35	NS	Unique cold-active thermolysin-like protease with potential in both basic research and industrial application	[283]
Salimicrobium halophilum strain LY20	10	80	Serine	PMSF	30	NS	Stable in presence of surfactants and water soluble organic solvents	[284]
Halobacterium sp. SP1(1)	8	37-40	Serine	PMSF	42.1	K_m - 0.262 mg/ml and V_{max} - 40.984 U/ml in presence of 2 M NaCl	Fish sauce fermentation, possible application as a antifouling coating agent alternative	[286]

Table 9
Salt requirement for the catalytic activity of salt-stable proteases obtained from halophilic/halotolerant microorganisms

Bacteria	Optimum salt (range)	Reference
Halobiforma sp. BNMIITR	NS	[18]
Jeotgalicoccus sp.	0 (0 - 1.5 M)	[19]
Bacillus sp. NPST-AK15	0.26 M (0 - 3.5 M)	[20]
Bacillus iranensis X5B	0.98 M (0 - 3 M)	[21]
Bacillus sp. EMB9	0.18 M	[23]
Pseudoalteromonas sp. strain CP76	0-1 M (0 - 4 M)	[76]
Bacillus aquimaris VITP4	0 M (0 - 4 M)	[90]
Bacillus sp. SM2014	2.6 M (0 - 5.2 M)	[91]
Actinopolyspora sp. VITSDK2	NS	[92]
Bacillus firmus CAS 7	5.2 M (0 - 6.05 M)	[93]
Bacillus alveayuensis CAS 5	6.05 M (0 - 6.05 M)	[94]
Bacillus subtilis BLK-1.5	0.35 M (0.18 - 0.87 M)	[95]
Bacillus halodurans CAS6	5.2 M (0 - 5.2 M)	[100]
Micrococcus sp. VKMM 037	0.85 M (0 - 3.42 M)	[103]
Virgibacillus sp. EMB13	0.18 M (0 - 2.6 M)	[105]
Bacillus sp. APCMST-RS3	2.5 M (0.5 - 3.5 M)	[107]
Bacillus sp. APCMST-RS7	1.5 M (0.5 - 2.5 M)	[108]
Virgibacillus halodenitrificans RSK CAS1	4.31 M (0 - 6.9 M)	[111]
Haloalkaliphilic bacterium	0.035 M (0 - 0.18 M)	[118]
Halobacterium sp. strain HP25	3 M (5 - 4.31 M)	[120]
Halobacillus sp. CJ4	0.4 M (0 - 5 M)	[121]
Salinicoccus sp. UN-12	0 (0 – 4 M)	[122]
Salinivibrio sp. strain MS-7	0-1 M (0 - 3 M)	[133]
Alkalibacillus sp. NM-Fa4	1 M (0 - 3.5 M)	[136]
Marinomonas arctica PT-1	NS	[138]
Pseudoalteromonas sp. 129-1	0.87 M (0 - 5.2 M)	[145]
Halorubrum ezzemoulense strain ETR14	1.73 M (1.73 - 4.31 M)	[146]
Oceanobacillus iheyensis O.M.$A_1$8	NS	[147]
Haloalkaliphilic bacterium O.M.$E_1$2	NS	[147]
Nocardiopsis alba OK-5	4 M (0 - 4 M)	[150]
Oceanobacillus sp.	2 M (1 - 4 M)	[151]
Virgibacillus halodenitrificans SK1-3-7	0.5 M (0 - 4 M)	[159]
Halobacillus sp. LY6	1.73 M (0 - 4.31 M)	[161]
Bacillus halodurans US193	0.25 M (0 - 2 M)	[171]
Alkalibacillus sp. NM-Da2	2.7 M (0 - 3.1 M)	[250]
Halobacillus sp. SCSIO 20089	NS	[283]
Salimicrobium halophilum strain LY20	2.16 M (0 -3.4 M)	[284]

NS: Not specified

Table 10
Substrate specificity of salt-stable proteases obtained from halophilic/halotolerant microbes

Microorganism	**Substrate specificity**	**Reference**
Jeotgalicoccus sp.	Casein>BSA>hemoglobin>gelatin	[19]
Bacillus sp. strain NPST-AK15	Casein> gelatin>BSA	[20]
Actinopolyspora sp. VITSDK2	Casein>gelatin>BSA	[92]
Bacillus firmus CAS 7	Casein>haemoglobin>gelatin> azocasein >egg albumin>BSA	[93]
Bacillus alveayuensis CAS 5	Casein>gelatin>hemoglobin> azocasein>BSA	[94]
Bacillus halodurans CAS6	Casein>gelatin>azocasein>egg albumin>hemoglobin>BSA	[100]
Virgibacillus sp. EMB13	Casein>hemoglobin>BSA>gelatin	[105]
Bacillus sp. APCMST-RS3	Casein> gelatin>BSA	[107]
Bacillus sp. APCMST-RS7	Casein> gelatin>BSA	[108]
Virgibacillus halodenitrificans RSK CAS1	Casein>hemoglobin>gelatin> azocasein>egg albumin>BSA	[111]
Salinicoccus sp. UN-12	Casein > gelatin > haemoglobin >BSA >keratin	[122]
Bacillus aquimaris VITP4	Casein>gelatin>BSA>egg albumin	[130]
Alkalibacillus sp. NM-Fa4	Casein>peptone> skim milk>BSA>wheat gluten>gelatin	[136]
Marinomonas arctica PT-1	Skim milk>gelatin>casein	[138]
Alkalibacillus sp. NM-Da2	Yeast extract> casein>tryptone>peptone> gelatin>BSA>wheat gluten>skim milk>hemoglobin	[250]
Salicola sp. IC10	Egg albumin>gelatin>BSA>casein	[277]
Halobacillus sp. SCSIO 20089	Casein>gelatin>BSA>hemoglobin> collagen>elastin>feather	[283]

Table 11

Role of various detergent formulation ingredients in detergent industry

Detergent formulation component	Importance in detergent formulation	Reference
SDS	Ionic surfactant and surface active agent	[112]
Hydrogen peroxide	Acts as a bleaching agent and starting material for oxygen-based bleach systems	[112]
Sodium perborate	Serves as a source of active oxygen in many detergent formulations	[112]
Disodium cocoamphodiacetate	Foam booster, viscosity builder, and conditioning agent	[122]
Sodium chloride	Principal component in the granulation process during formulation of detergent	[174]
Ethanol, Glycerol, Methanol	Used as solubilizers and co-solvent for preparation of detergent formulation	[275]
Cyclodextrin	Freshening agent or used for removal of malodor	[271,272]
Hydrotropes	Prevent gelation process in both concentrated and diluted detergent systems, for enhancing the solubility of detergent in product. Examples- Sodium xylene sulfonate and Sodium cumene sulfonate	[273,274]

Table 12
Stability and compatibility of various salt-stable proteases of halophilic/halotolerant microbes in detergents and detergent ingredients

Microorganism	Detergent formulation ingredients				Commercial detergents	Reference
	Ionic surfactants	**Nonionic surfactants**	**Oxidizing/ bleaching/ perturbing agents**	**Bio-surfactant/Hydrotropes/ other detergent formulation component**		
Halobiforma sp. strain BNMIITR	SDS 0.5% - 45%, 1% - 60%; CTAB 0.5% - 90%, 1% - 80%	Tween 80 0.5% - 120% 1% - 100%; Triton X-100 0.5% - 90%, 1% - 80%; Tween 20 0.5% - 102%, 1% - 120%	NS	NS	NS	[18]
Jeotgalicoccus sp.	SDS 1% - 33.7%	1% Triton X-100 - 58.1%, 1% Tween 80 - 74.7%	H_2O_2 1% -105.2%, Sodium perborate 1% - 81.5%	1% Rhamnolipid - 121.6%, 1% Cyclodextrin - 112.8%	NS	[19]
Bacillus sp. strain NPST-AK15	SDS 0.1% - 66.3%, 0.5% - 41.2%; CTAB 0.5% - 51.5%, 1% - 35.5%, 5% - 22.3%	Triton X-100 0.5% - 82.5%, 1% - 77.1%, 5% - 68%; Tween 80 0.5% - 104.2%; 1% - 102.3%, 5% - 85.6%; Brij™ 93 0.5% - 91.3%, 1% - 89.5%, 5% - 92.2%	H_2O_2 0.5% -102.5%, 1% - 102.3%, 5% - 93.7%	NS	Persil - 80%, Tide - 78%, Bonux - 62%, X-Tra - 80%, REX - 85%, Ayam - 90%, Arial - 36.2%, OMO - 45.9%	[20]
Bacillus firmus CAS 7	SDS 1% - 78.33%, 10% - 70.33%	Tween 80 1% - 88%, 10% - 72.33%; Triton X-100 1% - 75.67%, 10% - 70.33%	Sodium perborate 1% - 80.33%, 10% - 74.67%	NS	NS	[93]

Microorganism	Detergent formulation ingredients				Commercial detergents	Reference
	Ionic surfactants	Nonionic surfactants	Oxidizing/ bleaching/ perturbing agents	Bio-surfactant/Hydrotropes/ other detergent formulation component		
Bacillus alveayuensis CAS 5	SDS 1% - 66%	Tween 80 1% - 90.33%, Triton X-100 1% - 78.33%; Sodium deoxycholate 1% - 69.33%	NS	NS	Rin - 80.33%; Ariel - 72.48%; Henko - 80.26%; Tide - 79.38%	[94]
Bacillus halodurans CAS6	SDS 1% - 82.34%	Tween 80 1% - 94.33%, Triton X-100 1% - 72.67%; Sodium deoxycholate 85.67%	NS	NS	Rin - 88.34%, Ariel - 79.00%, Henko - 85.67%, Tide - 76.67%	[100]
Bacillus sp. APCMST-RS3	SDS 5 mM - 121%	5 mM Tween 20 - 135.65%, 5 mM Tween 40 - 129.67%, 5 mM Tween 60 - 125.21%, 5 mM Tween 80 - 119.65%, 5 mM Triton X-100 - 63.38%	NS	NS	Surf excel - 111.11%, Ariel - 129.66%, Tid - 124.58%, Rin - 99.65%, Technobright - 94.87%, Henko - 98.65%	[107]
Bacillus sp. APCMST-RS7	SDS 5 mM - 28.75%	5 mM Tween 20 - 95.04%, 5 mM Tween 40 - 114.52%, 5 mM Tween 60 - 95.61%, 5 mM Tween 80 - 90.69%, 5 mM Triton X-100 - 29.78%	NS	NS	Surf excel - 126.24%, Ariel - 138.03%, Tide - 131.48%, Rin - 111.13%, Technobright - 117.05%, Henko - 119%	[108]
Virgibacillus halodenitrificans RSK CAS1	SDS 1% - 59.21%, 10% - 51.10%	Triton X-100 1% - 65.17%, 10% - 57.41%; Tween 80 1% - 70.41%,	NS	NS	Rin – 74.20%, Ariel – 76.41%, Henko – 71.12%, Tide - 67%	[111]

Microorganism	Detergent formulation ingredients				Commercial detergents	Reference
	Ionic surfactants	**Nonionic surfactants**	**Oxidizing/ bleaching/ perturbing agents**	**Bio-surfactant/Hydrotropes/ other detergent formulation component**		
		10% - 61.26%; Sodium deoxycholate 1% - 79.31%, 10% - 70.25%				
Halobacterium sp. strain HP25	SDS 0.1% - 5%	NS	Urea 8 M - 30%	NS	Ariel - 93%, Persil - 91%	[120]
Halobacillus sp. CJ4	SDS 1% - 87.7%, CTAB 25 mM - 89.6%	10% Tween 20 - 86.9%, 10% Tween 40 - 97.5%, 10%Tween 80 - 98.4%, 10% Triton X-100 - 90.7%	H_2O_2 1% -50.4%	Na_2CO_3 100 mM - 100%	NS	[121]
Salinicoccus sp. UN-12	SDS 1% - 130.52%, CTAB 1%- 55.03%	1% Triton X-100 – 47.30%, 1% Tween 80 – 112.62%, 1% Tween 20 – 103.82%	H_2O_2 1% - 100.77%, Sodium perborate 1% - 99.41%	1% Cyclodextrin - 112.8% Disodium cocoamphodiacetate, Sodium xylene sulfonate (%),Sodium cumene sulfonate (%)	Nirma - 95.3% Rin - 92.8% Surf Excel - 92.1% Tide - 94.1% Ghari - 92.4%	[122]
Bacillus aquimaris VITP4	SDS 1 mM - 98.45%, 5 mM - 90.91%; CTAB 1 mM -100%, 5 mM - 87.91%	Triton X-100 1% - 100%, 5% - 93.03%; Tween 80 1% - 80.89%, 5% - 69.17%; Tween 20 1% - 95.89%, 5% - 82.12%	SDS 1 mM – 95.34%, 5 mM – 86.41%	NS	Surf Excel - 64.85%, Ariel - 82.14%, Tide - 97.90%, Rin - 75.89%, Ujala - 88.08%, Henko - 91.07%, Ponvandu - 84.81%, Arasan - 99.12%, Surf - 81.05%, Tide plus - 93.01%, Wheel - 76.31%, Mr. White - 46.04%	[130]
Salinivibrio sp. strain MS-7	SDS 1% -10.5%	0.1% Triton X-100 - 94.7%; 1% Tween 20 - 100%, 5% Tween 20 - 91%; 1% Tween 80 - 100%,	H_2O_2 1% - 75.4%	NS	NS	[133]

Microorganism	Detergent formulation ingredients				Commercial detergents	Reference
	Ionic surfactants	**Nonionic surfactants**	**Oxidizing/ bleaching/ perturbing agents**	**Bio-surfactant/Hydrotropes/ other detergent formulation component**		
		5% Tween 80 - 83%				
Alkalibacillus sp. NM-Fa4	SDS- 1% - 166%, 5% - 197%	Triton X-100 1% -112%, 5%-No activity; Tween 80 1% - 94%, 5% - 55%; Tween 20 1% - 53%, 5% - 44%	H_2O_2 1% -112%, 5% -134%; Urea 2 M - 100% 4 M - 144%	NS	NS	[136]
Pseudoalteromonas sp. 129-1	SDS 0.5% - 85.2%	1% Triton X-100 - 98.2%, 1% Tween 80 - 98.4%	H_2O_2 1% -112%	NS	Liquid detergents OMO - 91.2%, Ariel - 87.7%, Tide - 92.1%, Walch - 96.8%, Liby - 91.4%	[145]
Virgibacillus halodenitrificans SK1-3-7	SDS 1.5% - 93%	10% Tween 80 - 93%, 10% Tween 40 - 94%, 10% Triton X-100 - 67%	H_2O_2 1.5% - 98%	NS	NS	[159]
Bacillus halodurans US193	SDS 1% - 52.4%, CTAB 25 mM - 60%	10% Tween 20 - 70.9%, 10% Tween 40 - 85.5%, 10% Tween 80 - 90.4%, 10% Triton X-100 - 80.7%	H_2O_2 1% - 30.4%	$Na_2 CO_3$ 100 mM - 119.6%	Liquid detergent Detch - 97.59%, Judy - 96.44% Solid detergents Skip -100%, Ariel - 95.49%	[171]
Alkalibacillus sp. NM-Da2	SDS 1% - 65%, 5% - 57%	Tween 20 1% - 4%, 5% - 56%, Tween 80 1% - 81%, 5% - 44%, Triton X-100 1% - 21%,	H_2O_2 1% - 46%, 5% - 124%	NS	NS	[250]

Microorganism	Detergent formulation ingredients				Commercial detergents	Reference
	Ionic surfactants	**Nonionic surfactants**	**Oxidizing/ bleaching/ perturbing agents**	**Bio-surfactant/Hydrotropes/ other detergent formulation component**		
		5% - 0%				
B.safensis S406	SDS 0.1% - 87.83%, 0.5% - 51.01%, 1% - 34.45%	Tween 20 1% - 138%, 5% - 115%, Tween 80 1% - 121%, 5% - 102%, Triton X-100 1% - 123%, 5% - 109%	H_2O_2 1% - 97.31%, 5% - 58.24% Sodium perborate 1% - 99% 2% - 71%	NS	Solid detergents Dixan Nadhif Ariel New Det OMO Liquid detergents Lav+ Textile Carrefour Ariel	[278]
Salimicrobium halophilum strain LY20	SDS 10 mM - 85.1%	1% Tween 80 - 98.9%, 1% Triton X-100 - 97.2%	NS	NS	NS	[284]

Table 13
Methods employed for evaluation of washing performance by detergent compatible proteases from halophilic/halotolerant microbes

Microorganism	Stain removed	Washing evaluation method	Reference
Bacillus sp. EMB9	Blood	Visual method, reflectance, and whiteness index measurement method	[23]
Bacillus sp. SM2014	Blood, Tomato sauce, Turmeric	Visual method, reflectance, and whiteness index measurement method	[91]
Bacillus alveayuensis CAS 5	Blood	Visual method	[94]
Bacillus halodurans CAS6	Blood	Visual method	[100]
Micrococcus sp. VKMM 037	Blood	Visual method	[103]
Virgibacillus sp. EMB13	Blood	Visual method, reflectance, and whiteness index measurement method	[105]
Virgibacillus halodenitrificans RSK CAS1	Blood	Visual method	[111]
Salinicoccus sp. UN-12	Blood , Egg yolk albumin, Grass, Tomato sauce	Visual method, reflectance method	[122]
Bacillus aquimaris VITP4	Blood, Tea, and Coffee	Visual method	[130]
Bacillus halodurans US193	Blood	Visual method	[171]
Bacillus tequilensis P15	Blood	Visual method	[199]
Vibrio alginolyticus	Blood	Visual method	[200]

Table 14
Organic solvent stability of various salt-stable proteases obtained from halophilic/halotolerant microbes and their possible applications

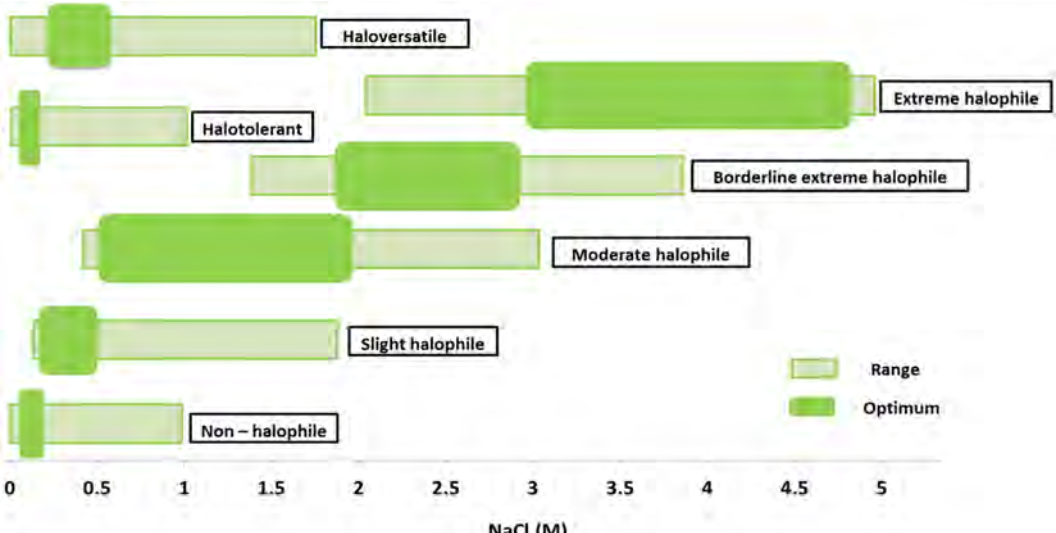
Bacteria	Organic solvent	Applications	Reference
Bacillus sp. strain NPST-AK15	Acetone, benzene, chloroform, dimethyl ether, diethyl ether, isopropanol, ethanol, methanol, and toluene	Non-aqueous organic synthesis	[20]
Bacillus iranensis strain X5B	Butanol, chloroform ethanol, hexane, and methanol	Peptide synthesis in non-aqueous media	[21]
Bacillus sp. EMB9	Benzene, butanol cyclohexane, decane, dodecane, ethanol, heptane, methanol, octane propanol, and toluene	Non-aqueous organic synthesis	[23]
Bacillus sp. SM2014	Acetone, benzene, butanol, DMSO, decane, hexane, heptane, isooctane, toluene, xylene	Peptide synthesis	[91]
Bacillus firmus CAS 7	Acetonitrile, benzene, ethanol, ethyl ether hexane, isopropanol, and methanol	Non-aqueous organic synthesis	[93]
Bacillus alveayuensis CAS 5	Acetonitrile, benzene, ethanol, ethyl ether hexane, isopropanol, and methanol,	Non-aqueous organic synthesis	[94]
Bacillus halodurans CAS6	Benzene, hexane, isopropanol and xylene	Non-aqueous organic synthesis	[100]
Micrococcus sp. VKMM 037	Acetone, butanol, ethanol, ethylene glycol, and DMSO	Non-aqueous organic synthesis	[103]
Virgibacillus sp. EMB13	Acetonitrile, benzyl alcohol, butanol, cyclohexane, decane, dodecane, ethanol, hexane, heptane, methanol, octane, propanol, and toluene	Non-aqueous organic synthesis	[105]
Bacillus sp. APCMST-RS3	Acetone, butanol, benzene, chloroform, ethanol, hexane, methanol, 2- propanol, petroleum ether and xylene	Non-aqueous organic synthesis	[107]
Bacillus sp. APCMST- RS7	Acetone, benzene, chloroform, DMSO, ethanol, hexane, and methanol	Non-aqueous organic synthesis	[108]
Virgibacillus halodenitrificans RSK CAS1	Acetonitrile, benzene ethanol, ethyl ether, hexane, isopropanol and methanol	Non-aqueous organic synthesis	[111]
Halobacterium sp. strain HP25	Butanol, ethanol, hexane methanol, and propanol	Organic solvent based enzymatic synthesis	[120]
Halobacillus sp. CJ4	Acetonitrile, benzene, chloroform, cyclohexane, DMSO, ether, isopropanol, methanol, and toluene	Organic synthesis, peptide synthesis, and enzymatic esterification of oligosaccharides	[121]
Salinicoccus sp. UN-12	Acetonitrile, DMSO, glycerol, ethanol, hexane, iso-octane, methanol, n-hexadecane, toluene	Biocatalytic agent for non-aqueous environ reactions and detergent formulation industry	[122]
Salinivibrio sp. strain MS-7	Chloroform, DMSO, ethanol, ethyl acetate, *n*-hexane, and toluene	Non-aqueous organic synthesis	[133]

Bacteria	Organic solvent	Applications	Reference
Alkalibacillus sp. NM-Fa4	Benzene, butanol, chloroform, ethanol, hexane, isopropanol, and methanol	Non-aqueous organic synthesis	[136]
Pseudoalteromonas sp. 129-1	Chloroform, DMSO, ethanol, ethyl acetate, isopropanol, isoamyl alcohol, hexane, and methanol, xylene	Peptide synthesis	[145]
Virgibacillus halodenitrificans SK1-3-7	Acetonitrile, butanol, DMSO, ethanol, ethyl acetate and 2-propanol	Biocatalyst in aqueous-organic solvent systems	[159]
Bacillus halo-durans US193	Acetonitrile, DSMO and methanol	Peptide synthesis	[171]
Alkalibacillus sp. NM-Da2	Benzene, isopropanol and ethanol	Bioremediation of saline soils and treatment industrial wastewater	[250]
Geomicrobium sp. EMB2	Benzene, butanol, cyclohexane, *n*-decane, *n*-dodecane, ethanol, heptane, isooctane, and toluene	Non-aqueous organic synthesis	[256]
Salimicrobium halophilum strain LY20	Acetone, acetonitrile, benzene, dodecane, DMF, DMSO, ethanol, hexane, heptane, isooctane and methanol	Non-aqueous organic synthesis	[284]

Highlights

Manuscript is based on following aspects of bacterial salt-stable proteases

- Salt dependence of microbes
- Enzymes of halophilic /halotolerant bacteria
- Optimization of salt-stable protease production
- Protease purification strategies
- Biochemical properties of salt-stable protease
- Operative utility of salt-stable proteases in the biotechnology sector


Haloversatile
Extreme halophile
Halotolerant
Borderline extreme halophile
Moderate halophile
Slight halophile
Range
Optimum
Non – halophile
0
0.5
1
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2
2.5
3
3.5
4
4.5
5
NaCl (M)

Figure 1

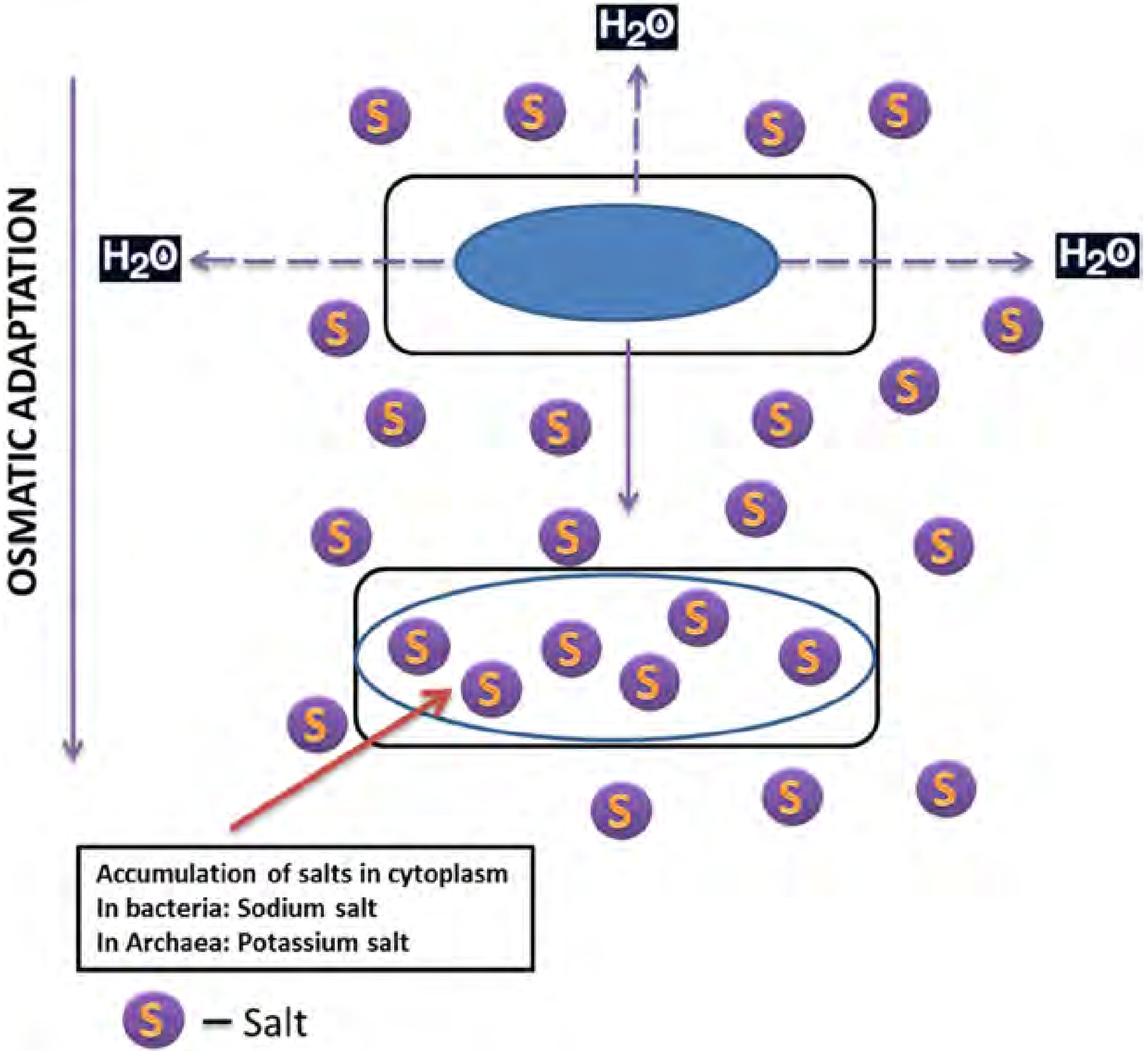

OSMATIC ADAPTATION
H2O
H2O
H2O
Accumulation of salts in cytoplasm
In bacteria: Sodium salt
In Archaea: Potassium salt
S – Salt

Figure 2

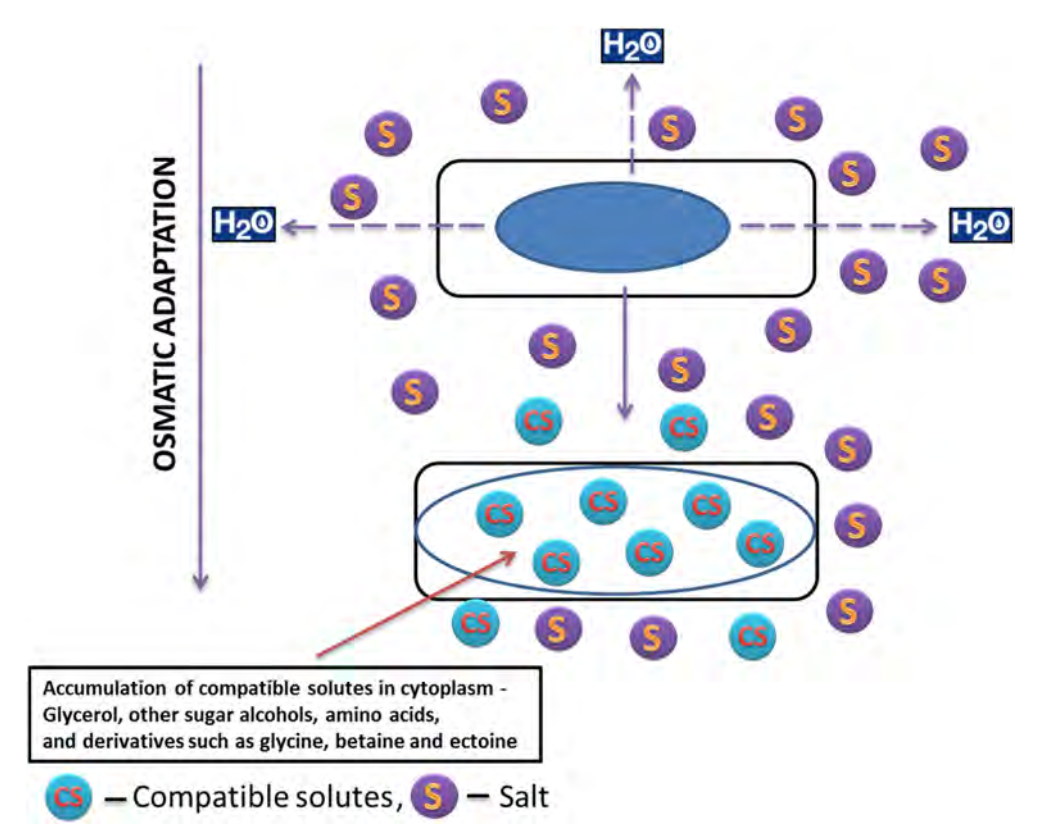
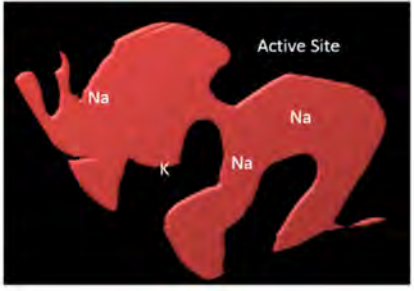

OSMATIC ADAPTATION
H2O
H2O
H2O
S
CS
Accumulation of compatible solutes in cytoplasm - Glycerol, other sugar alcohols, amino acids, and derivatives such as glycine, betaine and ectoine
CS – Compatible solutes, S – Salt

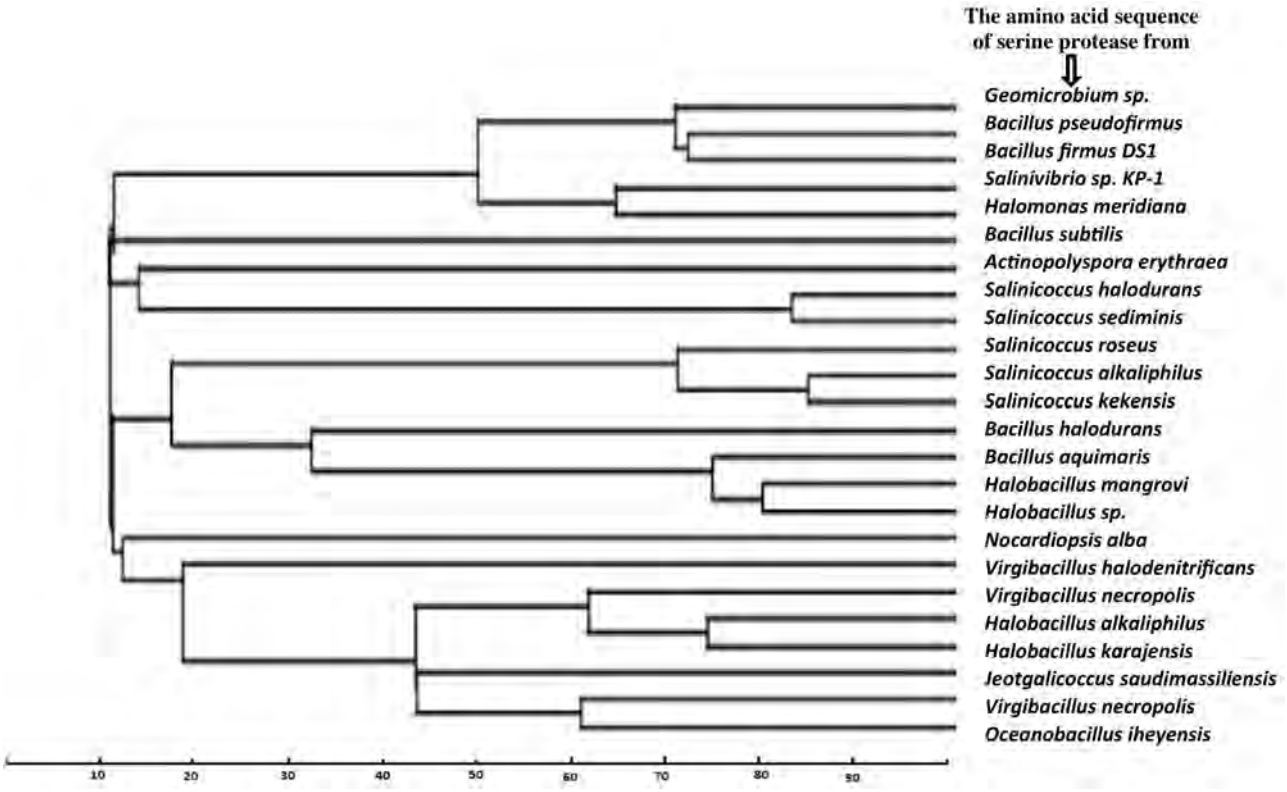
Figure 3

(A) Denatured conformation



(B) Active conformation

Figure 4


The amino acid sequence of serine protease from
Geomicrobium sp.
Bacillus pseudofirmus
Bacillus firmus DS1
Salinivibrio sp. KP-1
Halomonas meridiana
Bacillus subtilis
Actinopolyspora erythraea
Salinicoccus halodurans
Salinicoccus sediminis
Salinicoccus roseus
Salinicoccus alkaliphilus
Salinicoccus kekensis
Bacillus halodurans
Bacillus aquimaris
Halobacillus mangrovi
Halobacillus sp.
Nocardiopsis alba
Virgibacillus halodenitrificans
Virgibacillus necropolis
Halobacillus alkaliphilus
Halobacillus karajensis
Jeotgalicoccus saudimassiliensis
Virgibacillus necropolis
Oceanobacillus iheyensis
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80
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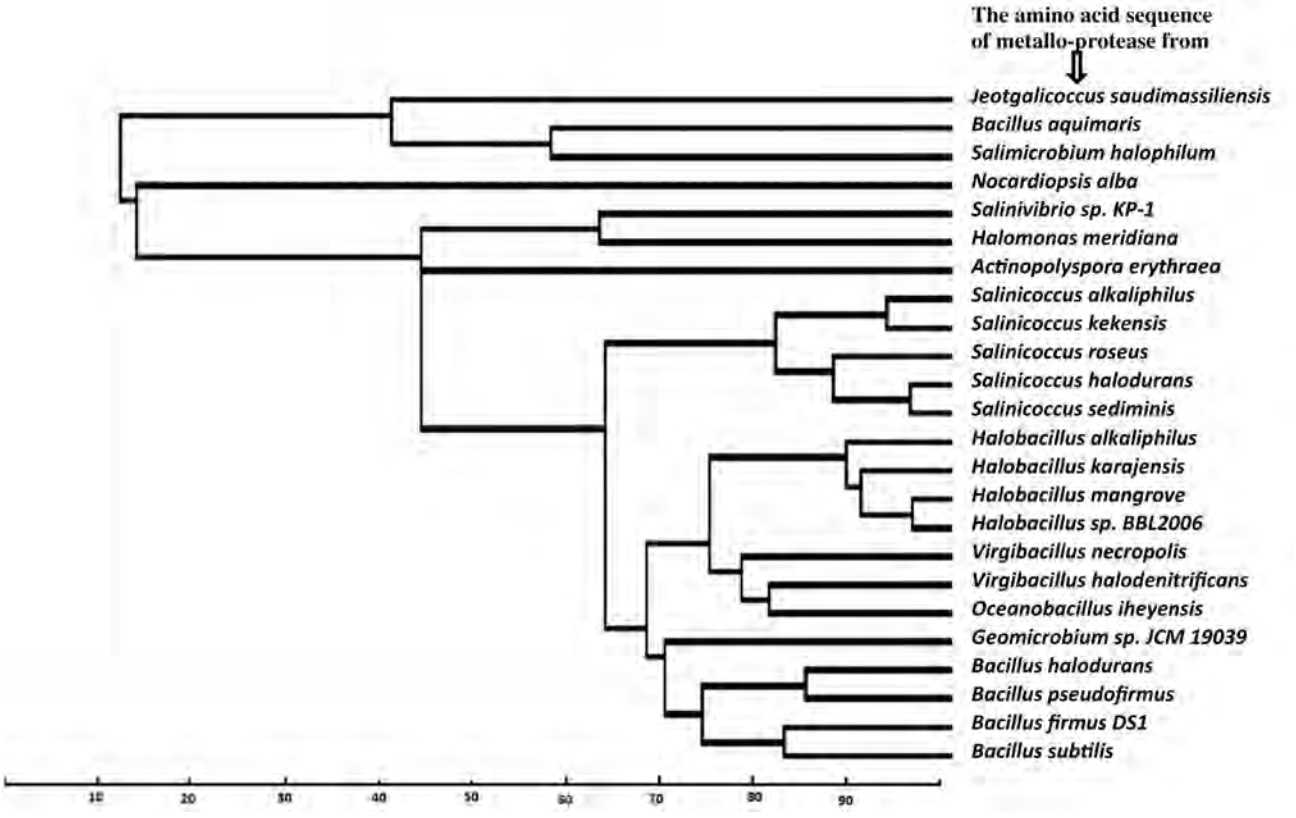
Figure 5


The amino acid sequence
of metallo-protease from
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Bacillus aquimaris
Salimicrobium halophilum
Nocardiopsis alba
Salinivibrio sp. KP-1
Halomonas meridiana
Actinopolyspora erythraea
Salinicoccus alkaliphilus
Salinicoccus kekensis
Salinicoccus roseus
Salinicoccus halodurans
Salinicoccus sediminis
Halobacillus alkaliphilus
Halobacillus karajensis
Halobacillus mangrove
Halobacillus sp. BBL2006
Virgibacillus necropolis
Virgibacillus halodenitrificans
Oceanobacillus iheyensis
Geomicrobium sp. JCM 19039
Bacillus halodurans
Bacillus pseudofirmus
Bacillus firmus DS1
Bacillus subtilis
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20
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40
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60
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80
90

Figure 6

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Production optimisation and characterisation of extracellular protease secreted by newly isolated *Bacillus subtilis* AU-2 strain obtained from *Tribolium castaneum* gut

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Bacillus subtilis
Response surface methodology
Food-grade protease

ABSTRACT

A novel protease secreting bacterial strain was isolated from the gut of *Tribolium castaneum* residing in stored *Glycine* max (soybean) seeds. The strain was identified as *Bacillus subtilis* AU-2. A response surface approach using Plakett-Burman Design (PBD) was used for the screening and a Central Composite Design (CCD) was used for the optimization of production parameters. An elevated protease production (580 U/mL) was achieved in optimised medium comprised of (g/L) soy bean meal, 10 g; glucose, 7.5 g; KH_2PO_4, 1.0 g; $CaCl_2$, 1.0 g and pH, 7.0 by using 3.5% inoculum at 37 temperature in agitated (60 rpm) batch culture after 48 h.

The three step enzyme purification achieved 26.81 fold purified protease which yielded a specific activity of 22773 U/mg and 34% recovery. The purified extracellular protease of *B. subtilis* AU-2 has molecular mass 38 kDa. The protease was documented as metallo-protease having optimum pH 7.5 and temperature 40. The purified protease showed appreciable endurance in presence of sodium dodecyl sulphate, Triton X-100, hydrogen peroxide and sodium per-borate. The protease efficiently digests proteinaceous substrates like casein, soybean flour, bovine serum albumin, haemoglobin, and egg albumin. The protease of newly isolated *B. subtilis* is now available for various food applications like soybean hydrolysate preparation, meat tenderisation, casein lysate preparation, milk clotting, and food waste treatment.

1. Introduction

Microbial proteases are prevalent enzyme for biotechnology sector, comprise of 60% utility and are largest selling biocatalyst for industrial applications (Li et al., 2013; Mokashe et al., 2018). The scientific understanding of enzymology has promoted the utility of this age-old food commodity in the current context of food applications. The enormous biodiversity of microorganisms advocates the search for new proteases and continuous innovation in existing knowledge of protease enzymology. Bacterial proteases exists in massive range of variants and specificity (Mokashe et al., 2018) and have been appreciated for their utilisation profile in food sectors like meat tenderisation, milk clotting, protein hydrolysate preparation, bioactive peptide synthesis, and allied food manufacturing process (Contesini et al., 2018). The safety of the enzyme secreting strain should be the principal attention in proposing usefulness of enzyme for food application (Pariza and Johnson, 2001).

The widening applications of proteases are dependent on bio-prospecting for efficient protease secreting source. The insects are the most diverse and adapted to varied ecological niches; they harbours ~10 times more microbes in their gut than total cells of the insect (Rajagopal, 2009). The insect gut possesses enormously diverse microbiota which consists commensal, parasite and mutualistic microbe (Mrazek et al., 2008). The studies on insect-microbes symbiosis revealed that the insect do not possess the several enzymes to digests the nutrients and they depend on their gut microbiota for utilization of polymeric nutrients (Douglas, 2013). The dependence of several insects on microbes for lignocellulose utilization is well documented (Ni and Tokuda, 2013). Also, the gut microbiota has improved digestion efficacy in several insects (Santo Domingo et al., 1998; Broderick et al., 2004).

The present study is based on the assumption that the legume pod borer insect might harbour the proteolytic commensal bacteria which could secrete the efficient protease of industrial use. The legume seeds (*family* Fabaceae) consist of abundance of nutritious proteins (~40%)

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1878-8181/

(Baudoin and Maquet, 1999). Majority of insects are reported to reside and live by causing damage to seeds in storage period. The proteinaceous seed kernel consumed by insects residing in seed metabolizes it to easily absorbable fundamental units by taking advantage of gut microflora (Priya et al., 2012). This study revealed the presence of novel protease secreting bacterium in the gut of insect *Tribolium castaneum* collected from stored soybean seeds. The aim of the research work is statistical production optimisation and characterisation of extracellular protease secreted by newly isolated *Bacillus subtilis* AU-2. The protease of newly isolated bacterial strain is suggested for commercial food applications viz. (i) soybean hydrolysate preparation, (ii) meat tenderisation, (iii) casein lysate preparation, (iv) milk clotting, and (v) food waste treatment.

2. Materials and methods

2.1. Chemicals and media

All chemicals and media were purchased from HiMedia, SD Fine (India), Sigma-Aldrich (USA).

2.2. Insect collection

Live insects were collected from six month naturally stored *Glycine* max seeds (Nandurbar, India). The collected insects were released in glass jar with a one inch layer of dried plaster of Paris at bottom. The containers were placed in individual plastic bags with sterile moist cotton to provide necessary humidity for transportation to the laboratory. The insects were sterilized by exposing to UV light for 1 min, to reduce external bacterial contamination.

Fifteen live insects randomly collected were rinsed with sterile distilled water 3 times. Then each insect was rinsed in 70% ethanol for 2 min, followed by three quick rinses of sterile phosphate-buffered saline (PBS) having composition, NaCl, 0.88 g; KCl, 0.02 g; Na_2HPO_4, 0.144 g; KH_2PO_4, 0.024 g in 100 mL distilled H_2O; pH 7.0 (1 ×). The insect were ice-anesthetized and then peripheral, anterior, and posterior parts were detached with sterile forceps, and the gut fluid was obtained in sterile PBS (1 ×).

2.3. Identification of insect

The morphological features of insect were documented and used for identification. Also, a phylogenetic trait was analysed by studying mitochondrial cytochrome oxidase gene of insect. The phylogenetic study was determined at National Centre for Microbial Resource (NCMR), Pune.

For this, adult insect collected was ground into fine powder by using a mortar and pestle. The total DNA was extracted from the insect by using the protocol of DNase easy Blood & Tissue Kit (Qiagen, Hilden, Germany). The quality of the extracted DNA samples were patterned on 0.8% agarose gel, and DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Nano Drop technologies, Willing Minton, USA). The extracted DNA samples were stored at −20°Cuntil further processing. The cytochrome oxidase (COI) gene sequence was amplified using primers, (COIf: 5′- GGTCAACAAATCAT AAAGATATTGG -3′ and COIr: 5′-TAAACTTCAGGGTGACCAAAAAA TCA-3′) (Folmer et al., 1994). The amplified products were directly sequenced using the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit on a 3730xl Genetic Analyser (Applied BioSystems). The sequence data obtained was assembled and analysed using DNA sequence assembling software ChromasPro. The similarity search of newly generated COI gene sequences was performed on the NCBI database.

The COI sequences were aligned using Clustal X version 2.0 (Larkin et al., 2007), and the same sequences were grouped under one haplotype. The phylogenetic analyses based on the neighbor-joining (NJ) and maximum likelihood (ML) methods were performed using MEGA 5.05 (Tamura et al., 2011). The sequence was published on GenBank by assigning the accession number.

2.4. Isolation of bacteria from insect gut

The insect gut homogenate was quickly inoculated in Erlenmeyer flask containing enrichment medium - soybean meal broth comprising of (g/L) - soybean meal powder, 10; yeast extract, 1.0; and glucose, 1.0. The inoculated medium was incubated at 37°Cfor 72 h incubation on rotary shaker (100 rpm). After 72 h incubation, 0.1 mL aliquot of culture broth was spread on sterile skim milk agar medium containing (g/L)- skim milk powder, 28; yeast extract, 2.5; glucose, 1.0; agar, 30 and incubated at 37°C for 48 h. Among various isolated bacterial strains, the AU-2 strain exhibiting zone of proteolysis was selected for further identification.

2.4.1. Identification of bacterial strain

Various morphological, cultural, biochemical and ribotyping characteristics were examined for identification of isolated strain. The phylogenetic taxonomic depiction was determined on the basis of nucleotide sequences at National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. For the phylogenetic identification, the total genomic DNA of bacterial strain AU-2 was obtained as per Sambrook et al. (1989). Subsequently, the 16S rRNA gene was amplified from the total chromosomal DNA using universal eubacteria specific primer 16 F27 (5′-CCA GAGTTTGATCMTGGCTCA-3′) and 16 R1525 × P (5′TTC TGC AGTCTAGAAGGAGGTGWTCCAGCC-3′) by PCR. The reaction mixture (25 µL) consists of 10 × buffer (2.5 µL), 2 mM dNTP (2.5 µL), 10 pMol/l 16F27 (1.25 µL), 10 pMol 17R1525XP (1.25 µL), 10U Taq DNA polymerase (0.2 µL), template DNA (2 µL) and water (15.3 µL). The PCR amplification were attuned by setting the cycles - (i) denaturation at 95°C for 1 min, (ii) annealing at 55°C for 1 min, (iii) extension of annealing at 55°Cfor 1 min and (iv) final extension at 72°C for 10 min. The PCR was operated for 35 cycles on PCR cycler (Applied Biosystem PCR system). The amplified DNA was further purified with PEG-NaCl and incubated for 10 min at 37°C. The precipitate was collected by centrifugation at 16000 × *g* for 15 min at 4°C. The pellet was washed twice with 70% ethanol, dried under vacuum, re-suspended in distilled water at concentration of > 0.1 pmol/µL and the purified DNA was sequenced using BIGDYE terminator kit 3.1 CABI PerkinElmer, USA. The sequencing reactions were run on ABI-PRISM 31D automated sequencer (Model 3730; Applied Biosystem, USA).

The 16S rRNA nucleotide sequence obtained was aligned by BLAST analysis at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequence of AU-2 strain has been submitted to the GenBank database and assigned with accession number. For establishing phylogenetic relationship with other bacteria, currently accessible sequences at NCBI were used and performed multiple sequence alignment by using BLASTN 2.2.20 + .

2.5. Protease assay

The protease activity was assessed by spectrophotometric method described by Nakanishi et al. (1974). For this, 1.0 mL suitably diluted protease was allowed to react with 1.0 mL of buffered substrate. The buffered substrate consists of 0.65%, w/v casein prepared in 0.2 M Phosphate buffer having pH 7.5. The reaction mixture was kept at 40 °C. After 10.0 min the catalytic reaction was stopped by 3.0 mL chilled Trichloro-acetic acid (TCA) reagent. The released fragmented peptides were spectrophotometrically (UV-VIS 1700, Shimadzu, Japan) measured at 275 nm by using tyrosine as the standard. At pH 7.5 and temperature 40°C, the amount of protease required to produce the peptide-fragment equivalent to 1.0 µg tyrosine per min per mL is considered as one unit (U) of protease.

2.6. Growth of bacteria

The bacterial growth was determined in terms of cell density. For this a biomass was harvested by centrifugation (12000 × *g* for 10 min at 4°C) and washed thrice with cold distilled water. The washed bacterial biomass was vacuum dried at room temperature till a constant weight was attained.

2.7. Optimization physical and chemical parameter for protease production

Based on literature survey several provisional experiments were conducted based on one variable at a time (OVAT) approach to resolve the range of crucial growth factors like pH, temperature, agitation and inoculum. Also, the suitable carbon source and nitrogen source for protease production were screened. For this a basal medium consists of (g/L) KH_2PO_4, 1 g; $CaCl_2$, 1 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; pH, 7.0 was supplemented with 1% w/v carbon source (starch, glucose, fructose, maltose, xylose, sucrose and molasses) individually. A control set without any carbon source was also kept. Also, various nitrogen sources (beef extract, bovine serum albumin, casein hydrolysate, casein, gelatin, peptone, skim milk, soy meal, and yeast extract)were supplemented (1%, w/v) individually in basal medium consists of (g/L) glucose, 1; KH_2PO_4, 1 g; $CaCl_2$, 1 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; pH, 7.0. All inoculated flasks were incubated at 37°Cunder agitation (100 rpm) and the protease yield after 48 h.

2.8. Statistical optimization of protease production media

To evaluate the optimum parameter for maximum growth and protease production a software-aided statistical approach was used. The Plackett–Burman design (PBD) was employed for preliminary screening of factors. The screened and potentially influencing factors were further optimised by response surface methodology using Central Composite Design (CCD) for determining the optimum values of individual factors.

2.8.1. Plackett–Burman design (PBD)

Based on these trial experiment seven factors *viz*. pH, agitation, glucose, soybean meal, KH_2PO_4, $CaCl_2$ and inoculum subjected for screening for their influence on growth and protease production. A two level (low and high) of seven factors was tested by this approach viz. pH (6, 8); agitation (0, 120 rpm); glucose (0, 1.5 g/L); soybean meal (0.5, 1.5 g/L); KH_2PO_4 (0.1, 0.5 g/L); $CaCl_2$ (0.1, 0.5 g/L) and inoculum (1, 6 mL). Total twelve experiments designed by the Plackett-Burman method were performed in single block as described in Table 1. The protease activity and growth (responses) were evaluated and documented. These responses were analysed for getting regression equations.

2.8.2. Central composite design (CCD)

The five factors pH (6.0–8.0), agitation (0–120 rpm), glucose (0–1.5 g/L), soybean meal (0 to 5 g/L), and inoculum (1–6 ml) exhibited their significant influence on protease production were further subjected to a 2^n factorial Central Composite Design (CCD) for optimizing their values. The CCD suggested a set of 32 experiments which were accordingly performed (Table 2). Growth and protease production were documented as response. The response data were investigated by the Minitab software, the 3D contour plots generated to analyse the interaction among screened factors. Validity of chosen quadratic model predicted by Minitab software was confirmed experimentally.

2.9. Purification of protease

The *B. subtilis* strain AU-2 was cultured in optimised medium, the cell broth was centrifuged (12000 × *g*, 10 min, 4 °C). The cell-free supernatant was sequentially precipitated with increasing concentration of ammonium sulphate. The protein-precipitate recovered using ammonium sulphate (75% w/v) was dialyzed (dialysis membrane number 110, HiMedia, Mumbai) using phosphate buffer (pH 7.5). The proteins were further fractionated by using Sephadex-G-75 (Sigma, USA) and DEAE- cellulose (HiMedia, Mumbai, India) as described previously (Patil et al., 2016). All purification steps were executed at 4°C. The molecular weight and homogeneity of purified protease were evaluated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (12.0%, SDS-PAGE) as per protocol of Laemmli (1970), the protein bands were visualised by Coomassie brilliant blue R-250 staining. The molecular mass of the protease was determined by comparing its electrophoretic mobility with commercial protein markers (Standard medium range protein marker, Genei, Bangalore, India).

2.10. Effect of temperature and pH on protease activity and stability

The effect of temperature on activity and stability of purified protease of *B. subtilis* AU-2 was evaluated at different temperatures (20–70 °C). Also, the thermal stability of enzyme was evaluated by incubating the protease at various temperature (25 to 75°C) for 30 and 60 min. The protease assay was performed at 40°C. Similarly, the effect of pH on purified protease on activity and stability was assessed over a wide pH range (6.0–10.0) using various buffers at 40°C. An aliquot of the purified protease of *B. subtilis* AU-2 and substrate was prepared with various buffers *viz*.- 0.2 M glycine-HCl buffer (pH 2.0); 0.2 M acetate–sodium acetate buffer (pH 3.0–4.0); 0.2 M monobasic–dibasic sodium phosphate buffer (pH 6.0–8.0); and 0.2 M carbonate–bicarbonate buffer (pH 9.0–10.0). For evaluating the stability at various pH, the purified protease aliquot were prepared in various buffers (as described above); for an hour and protease assay was performed at 40°C with

Table 1
Plackett-Burman (2-level factorial) experimental design for screening of various parameters; growth and protease yield of respective experiments using *Bacillus subtilis* AU-2.

Expt. No.	Glucose (%, w/v)	pH	KH_2PO_4 (%,w/v)	Soybean meal (%, w/v)	Agitation (rpm)	$CaCl_2$ (%, w/v)	Inoculum (%, w/v)	Protease activity (U/mL)	Growth Dry weight (mg/mL)
1	1.5	6	0.5	1.5	0	0.5	1	5.42 ± 0.69	0.09 ± 0.01
2	0.0	8	0.1	0.5	0	0.5	6	34.94 ± 1.30	0.32 ± 0.07
3	0.0	6	0.1	0.5	0	0.1	1	8.43 ± 1.78	0.1 ± 0.05
4	1.5	8	0.5	0.5	120	0.5	1	198.19 ± 7.62	1.12 ± 0.10
5	1.5	6	0.5	0.5	0	0.1	6	79.52 ± 4.12	0.61 ± 0.05
6	1.5	6	0.1	0.5	120	0.5	6	13.86 ± 2.01	0.71 ± 0.10
7	0.0	6	0.5	1.5	120	0.1	6	27.71 ± 3.59	0.38 ± 0.10
8	0.0	8	0.5	0.5	120	0.1	1	42.77 ± 1.90	0.41 ± 0.04
9	1.5	8	0.1	1.5	0	0.1	1	450.00 ± 6.12	2.04 ± 0.07
10	1.5	8	0.1	1.5	120	0.1	6	505.42 ± 5.52	2.18 ± 0.14
11	0.0	6	0.1	1.5	120	0.5	1	6.02 ± 0.98	0.1 ± 0.03
12	0.0	8	0.5	1.5	0	0.5	6	182.53 ± 4.52	1.22 ± 0.16

Table 2
Central composite design and respective responses (growth and protease yield) using *Bacillus subtilis* AU-2.

Expt. No.	Glucose (%,w/v)	pH	Soybean meal (%,w/v)	Agitation (rpm)	Inoculum (%,w/v)	Protease activity (U/mL)	Growth (Dry weight) (mg/mL)
1	0.75	7	0	60	3.5	274.10 ± 6.30	1.15 ± 0.05
2	1.5	8	0.5	120	1	340.96 ± 2.50	1.15 ± 0.02
3	1.5	6	1.5	120	1	162.65 ± 1.80	0.34 ± 0.04
4	0	8	0.5	120	6	314.46 ± 1.40	1.03 ± 0.03
5	1.5	8	1.5	0	1	460.84 ± 3.60	1.35 ± 0.05
6	0.75	9	1	60	3.5	151.20 ± 1.20	0.41 ± 0.04
7	0.75	7	1	−60	3.5	97.59 ± 1.40	0.94 ± 0.05
8	0.75	7	1	60	3.5	491.57 ± 4.50	1.23 ± 0.03
9	1.5	6	1.5	0	6	203.01 ± 2.30	1.43 ± 0.02
10	0	6	1.5	120	6	10.84 ± 1.10	0.14 ± 0.01
11	0.75	7	2	60	3.5	550.00 ± 4.80	1.09 ± 0.01
12	0.75	7	1	60	3.5	492.77 ± 4.50	1.23 ± 0.02
13	1.5	6	0.5	120	6	392.17 ± 3.50	0.84 ± 0.03
14	0	8	0.5	0	1	153.01 ± 2.20	0.61 ± 0.04
15	0.75	7	1	60	3.5	498.80 ± 3.40	1.24 ± 0.04
16	0.75	7	1	60	−1.5	0 ± 0	0 ± 0
17	1.5	6	0.5	0	1	257.83 ± 2.11	1.11 ± 0.01
18	0	8	1.5	120	1	334.94 ± 1.20	1.54 ± 0.02
19	0.75	7	1	60	3.5	501.20 ± 4.30	1.22 ± 0.04
20	0	6	0.5	120	1	5.42 ± 0.80	0.1 ± 0.00
21	0	6	0.5	0	6	3.01 ± 0.00	1.3 ± 0.01
22	0	6	1.5	0	1	5.42 ± 0.80	0.11 ± 0.02
23	2.25	7	1	60	3.5	124.10 ± 1.40	0.4 ± 0.03
24	−0.75	7	1	60	3.5	0 ± 0	0 ± 0
25	1.5	8	0.5	0	6	280.12 ± 2.40	0.7 ± 0.01
26	0	8	1.5	0	6	368.07 ± 1.10	1.3 ± 0.02
27	1.5	8	1.5	120	6	568.07 ± 1.20	2.3 ± 0.03
28	0.75	7	1	180	3.5	535.54 ± 2.30	1.21 ± 0.02
29	0.75	7	1	60	3.5	492.17 ± 3.50	1.29 ± 0.02
30	0.75	7	1	60	8.5	356.63 ± 3.40	1.5 ± 0.01
31	0.75	5	1	60	3.5	0 ± 0	0 ± 0
32	0.75	7	1	60	3.5	498.80 ± 4.50	1.25 ± 0.02

buffered casein substrate (pH-7.5).

2.11. Effect of inhibitors on protease activity

The interaction of protease with repertoire of well-known protease inhibitor like phenyl methyl sulfonyl fluoride (PMSF), *N*-ethylmaleimide, ethylenediaminetetraacetic acid (EDTA) and 1, 10 phenanthroline gives clue about type of protease. For this, the enzyme aliquots were prepared in buffer containing respective protease inhibitor (5 mM), and pre-incubated for 1 h at 27 °C. The enduring protease activities were assessed and equated with a control.

2.12. Effect of metal ions on protease activity

The effect of metal ions on protease activity was evaluated. In this experiment, purified protease was pre-incubated independently with 5mMchloride salt of respective metals ($BaCl_2$, $CaCl_2$, $CdCl_2$, $CuCl_2$, $FeCl_2$, $HgCl_2$, KCl, LiCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$, $SnCl_2$, and $ZnCl_2$) for 1 h at 27°C. The influenced protease activity was assessed and comparing it with a control.

2.13. Effect of surfactants and oxidizing agents on protease

The activity of purified protease was also analysed in the presence of various surfactants, and oxidizing agents (1%, w/v). For this, the aliquot of purified enzyme was pre-incubated with anionic detergent (Sodium dodecyl sulphate), non-ionic detergents (Triton X-100, and Tween-20) and oxidizing agents (Hydrogen peroxide and sodium perborate) at 27°Cfor 1 h, and subsequently assayed for the residual protease activity.

2.14. Substrate utilization profile

The ability of protease to act against the range of natural substrates, like bovine serum albumin (BSA), casein, egg yolk albumin, gelatin, haemoglobin, and soybean flour was evaluated. For this, 1.0 mL of each substrate (2%), prepared in phosphate buffer (pH 7.5) was mixed with purified protease and incubated at 40°C for 10 min. One unit (U) of protease activity was defined as that amount of enzyme required to produce peptides equivalent to 1.0 µg tyrosine in the filtrate per minute per millilitre at pH 7.5 and 40 °C.

2.15. Statistical analysis

The reported enzyme units were the average value of results of three independent experiments. All the data are expressed as average ± standard deviation (SD). Statistical analysis was performed using Microsoft Excel. The production media and physical factors optimisation were achieved by response surface approach. A software package Minitab® 18.1 (Trial version) was used for the design of screening and optimisation experiments, data analysis, quadratic model building, response surface and 3-D contour plots generation to understand the interaction of different variables.

3. Results and discussion

3.1. Insect

The insect was collected from stored *Glycine* max seeds. It was identified as *Tribolium castaneum* (Coleoptera: Tenebrionidae) on the basis of the morphological features using the taxonomic key suggested by Bousquet (1990). The identification was further confirmed by analysing the phylogenetic attributes of insect. The partial DNA sequence of mitochondrial cytochrome oxidase (COI) gene of studied insect was

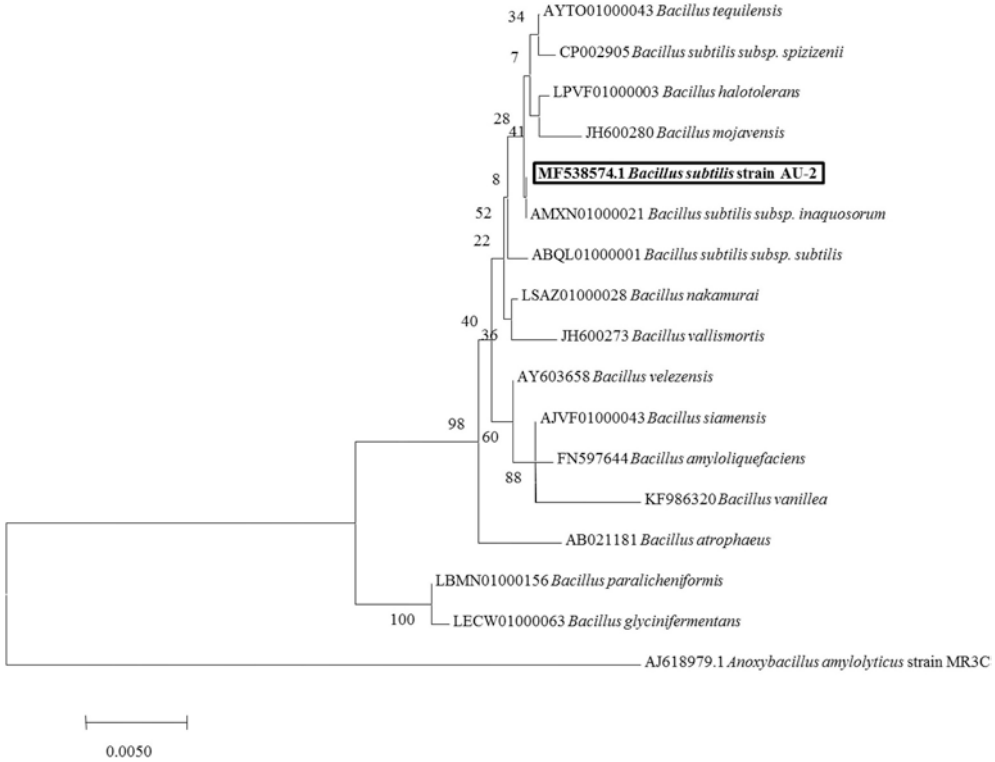

Fig. 1. Phylogenetic relationship of newly isolated strain *Bacillus subtilis* AU-2.

used for identification and the sequence had been submitted to GenBank databases with accession number: MH442992.

The *Tribolium castaneum,* also known as red flour beetle commonly is a tropical beetle pest of stored grains and pulses (Hagstrum and Subramanyam, 2009). The presence of *Tribolium castaneum* insect on stored soybean was also reported by LeCato and McCray (1973). These insects digestive system is adapted consume proteinaceous diet obtained from *Glycine* max seeds. Based on assumption that the gut microbiome of *Tribolium castaneum* (residing in *Glycine* max seeds)*;* could contain an efficient protease producing bacterial strain, further experimentation was lineated.

3.2. Microorganism

On basis of morphological, cultural, biochemical characteristics (Table S1) and phylogenetic analysis, the efficient protease secreting bacterial strain AU-2 was identified as *Bacillus subtilis.* The partial DNA sequence of 16S rRNA gene (1479 bp) of AU-2 strain was documented with accession number - MF538574.1 at NCBI database. This partial DNA sequence was also compared with related strains to establish the phylogenetic relationship with correlated bacterial strains (Fig. 1).

The present investigation has revealed the presence of protease secreting *Bacillus subtilis* in gut of *Tribolium castaneum*. The *Bacillus subtilis* is present as commensal in *Tribolium castaneum* gut was also documented previously (Kumari et al., 2011). The *B. subtilis* has been reviewed by the Food and Drug Administration (Centre for Veterinary Medicine) and included it in list of Direct-Fed microorganisms (US Food and Drug Administration, 1999). Also, *B. subtilis* was reported to be used for preparation of East Asian fermented foods –natto (Hosoi and Kiuchi, 2003). The Hong et al. (2009) documented that *B. subtilis* had adapted to survive as human gut commensals and mediate important roles like biofilm formation, and antimicrobial secretion. Likewise, the quorum sensing molecules (QSMs) secreted by *B. subtilis* strain JH 642 are found to be play a substantial role in safeguarding the intestinal health (Fujiya et al., 2007). The probiotic preparation supplemented with *Bacillus subtilis* are commercially available in several countries which includes - BioPlus-2B® (Denmark), Anaban™, and Biosporin® (Europe) (Elshaghabee et al., 2017). Moreover, *B. subtilis* strains have been conferred GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (Contesini et al., 2018).

Hence, by considering the safe nature of this insect gut commensal strain, the protease obtained from it could offer potential application in food industry. The food manufacturers are inclined towards safe and mild enzymatic methods to replace traditionally used strong acid hydrolysis.

3.3. Statistical optimization of protease production media and physical factors

The suitability of any protease secreting strain for industrial application depends on enzyme yield. The protease production and growth is depends on the media composition and physical factors. In current investigation, total seven factors (glucose, pH, KH_2PO_4, soybean meal, agitation, $CaCl_2$, and inoculum size) were selected for primary screening by Plackett–Burman design (PBD). All the experimental sets were incubated for 48 h, as it yields highest enzyme and further prolonged incubation not contribute to increase protease yield. The experimental PBD design with respective responses (protease activity and growth) is summarised in Table 1. Based on the regression coefficient values of responses; Glucose (79.2); pH (106.0); Potassium dihydrogen phosphate (−40.2); Soybean meal (66.7); Agitation (2.8); Calcium chloride (−56.1); and Inoculum (11.1); further five factors which showed positive influence on protease production were further selected for central composite design (CCD) of response surface method. The K_2HPO_4 and $CaCl_2$ showed a minimum effect were taken in low-level in subsequent experimentation.

By using various levels of glucose, pH, soy bean meal, agitation, and inoculum; the CCD designed experiments were accomplished consequently (Table 2). The responses (growth and protease activity) were inputted to Minitab software to generate the quadratic regression equations and 3D contour plots.

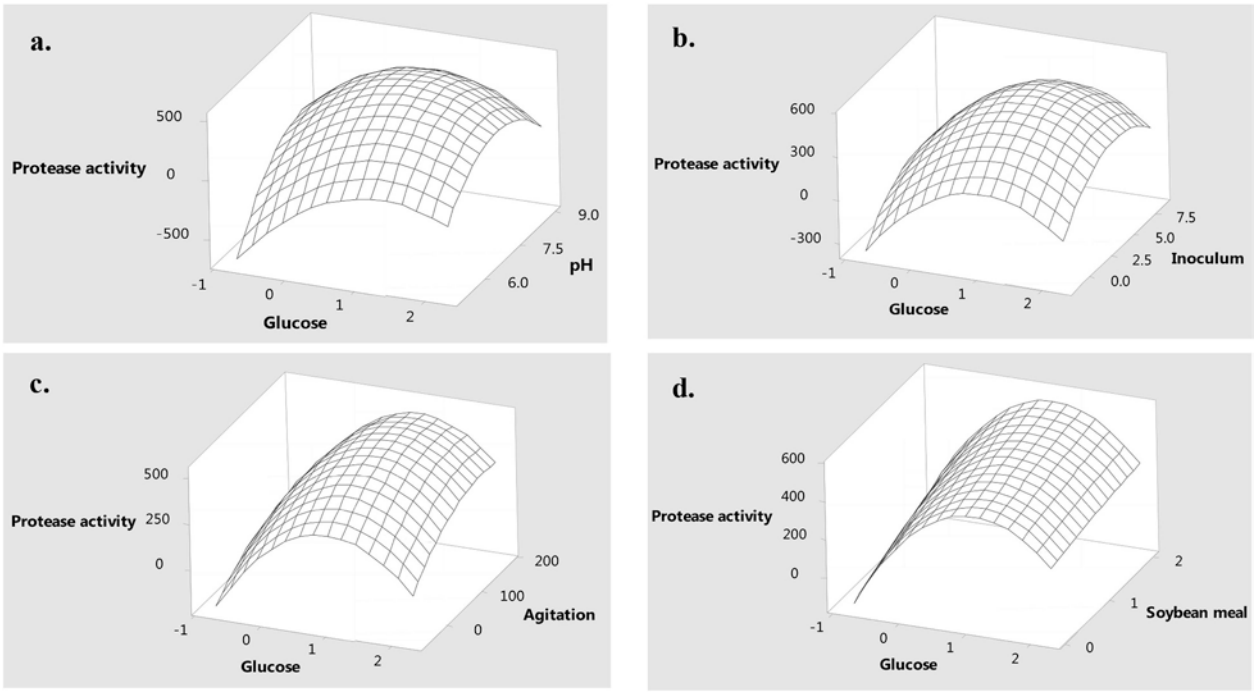

Fig. 2. The interacting prominent effects of optimised parameters **(a)** glucose and pH; **(b)** inoculum and glucose; **(c)** glucose and agitation; and **(d)** glucose and soy bean meal on protease production by *Bacillus subtilis* AU-2.

3.3.1. Quadratic regression equation

To get 3D contours graphs, the protease activity was analysed by taking two factors at a time and other three at a fixed level. Fig. 2 shows the various interacting effects of glucose, soybean meal, pH, agitation, and inoculum. The resulted protease production based on their mutual interaction is depicted as response surface plots, which shows the combined effects of these factors. Each 3D contours graph generated is almost convex contour plot, which designates that there were precise optimum levels.

Fig. 2a shows that increasing the concentration of glucose and pH leads to elevate protease production by *B. subtilis* AU-2, while further raise (i) beyond 1% glucose and, (ii) pH towards alkaline range has lowered the protease production. The *B. subtilis* AU-2 strain grew in pH range of 6.0 to 9.0; while the maximum protease production was detected at pH 7.0.

Also, the protease production was promoted with increase of agitation rate and inoculum level up to optimum frontier, while further boost of these factors has lowered the protease production by *B. subtilis* AU-2, as shown Fig. 2b and c. The protease production by altering the glucose concentration with specific bio-inoculum load and efficient utilization of available oxygen was assessed by this interaction.

As shown in Fig. 2d, the presence of organic nitrogen source – soybean alone was not adequate to maximize the protease production; addition of carbon source like glucose was essential for the improving the protease production of the strain. Although, the glucose promoted protease production up to 1% concentration further increase in glucose level has lowered the protease yield. Similarly, protease production was detected to be suppressed by glucose beyond 1.0% concentration in almost all interactions – glucose-pH, glucose-inoculum, and glucose-agitation (Fig. 2a, b and 2c); this emphasizes that excess glucose beyond the optimum concentration might repressed the protease production or altered the osmoregulation which further adversely affected the growth of strain and protease secretion. The soybean meal showed vis-à-vis response on protease production and even 2% soybean meal was endured by the strain. For large scale commercial enzyme production, the use of cost-effective media is vital. The ability to utilize soya bean by the *B. subtilis* AU-2 is a positive gain as the soybean meal is a comparatively cheap and easily obtainable media component, its industrial appropriateness for economical protease production at large scale is an encouraging factor.

The optimum values of selected factors for maximum protease production were calculated using the quadratic regression equation.

Protease activity = 4167 + 639 Glucose + 1192 pH + 65.5 Inoculum - 649 Soybean meal + 0.19 Agitation - 159.2 Glucose*Glucose - 86.1 pH*pH - 9.68 Inoculum*Inoculum - 8.1 Soybean meal*Soybean meal - 0.00718 Agitation*Agitation - 42.6 Glucose*pH + 0.8 Glucose*Inoculum - 20.0 Glucose*Soybean meal + 0.175 Glucose*Agitation + 1.6 pH*Inoculum + 115.0 pH*Soybean meal + 0.203 pH*Agitation - 2.4 Inoculum*Soybean meal + 0.194 Inoculum*Agitation - 0.67 Soybean meal*Agitation

The optimized medium evaluated using response surface approach consists (g/L) - soy bean meal, 10 g; glucose, 7.5 g; KH_2PO_4, 1 g; $CaCl_2$, 1 g and the pH should be 7.0. The batch culture consists of the optimized medium inoculated with 3.5% inoculum and agitated at 60 rpm at 37°C yielded 580 U/mL after 48 h. Further incubation has not contributed to increase the protease yield and leads to slight decline in protease level. Hence the incubation period for optimized protease production was kept 48 h. Protease activity obtained from fermentation medium using OVAT approach was 158 U/mL. Hence, the systematic response surface approach has enhanced to 4.0 fold improvements as compare to the production using basal medium. Relatively higher fold (7.0-fold) enhancement in enzyme production was achieved using response surface approach from *Bacillus subtilis* FBL-1, which yielded 578 U/mL protease in optimized medium (Kim et al., 2016).

3.4. Purification of protease

Extensively purified protease has been a prerequisite to characterise the enzyme for studying the biochemical profile and to confirm the suitability of enzyme to use in food biotechnology. Various approaches have been utilised for purification of extracellular proteases secreted by *Bacillus* strains (Mokashe et al., 2018); in this study the first step of purification was concentration of proteins by ammonium sulphate

Table 3
Purification summary for protease obtained from *Bacillus subtilis* AU-2.

Purification steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crud broth	309.8 ± 10.7	264257 ± 19514	853 ± 66	100 ± 0	1.00 ± 0.00
$(NH_4)_2SO_4$ precipitation (75%)	229.3 ± 15.8	208635 ± 13092	908 ± 7	79 ± 1	1.07 ± 0.09
Ion exchange-DEAE-Cellulose	16.9 ± 1.5	131225 ± 3931	7782 ± 623	50 ± 2	9.16 ± 1.06
Gel filtration-Sephadex G-75	3.8 ± 0.02	88675 ± 1870	22773 ± 361	34 ± 2	26.81 ± 2.47

precipitation method. Subsequently, chromatographic separation was carried by using DEAE-cellulose and Sephadex G-75 columns. The sequential three step purification scheme yielded 26.81-fold purification with a specific activity of 22773 U/mg and 34% recovery (Table 3). The purification results were consistent with the purification strategy employed previously for isolation of proteases from various strains of *Bacillus subtilis*. A 24% recovery and 27.63 fold purified protease with 11.33 U/mg specific activity (Maruthiah et al., 2013) and 17.87% recovery and 10 fold purified protease with 87.79 U/mg specific activity (Sathishkumar et al., 2015) were obtained from *Bacillus subtilis* AP-MSU 6 and *Bacillus subtilis* GA CAS8 respectively.

The purified protease of *B. subtilis* AU-2 was detected as a single band on polyacrylamide gel. The molecular mass of extracellular protease of *B. subtilis* AU-2 was estimated as 38 kDa by SDS-PAGE (Fig. 3). The documented results are in consistence with recently reported 37.6 kDa metallo-protease from *Bacillus subtilis* (Si et al., 2018). The molecular weight of the purified proteases of different strains of *Bacillus subtilis* was determined as 44 kDa (Yang et al., 2000) and 41 kDa (Sathishkumar et al., 2015). In contrary, several reports described low molecular-weight extracellular proteases (15–28 kDa) from different strains of *Bacillus subtilis* (Adinarayana et al., 2003; Kim and Kim, 2005; Maruthiah et al., 2013; Rehman et al., 2017).

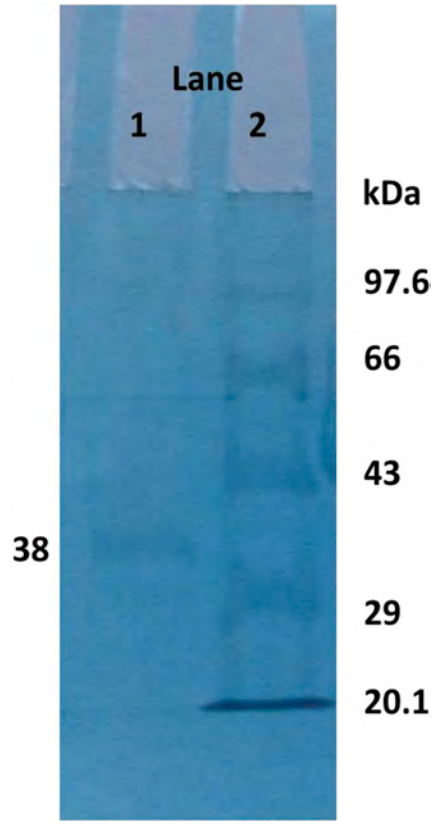

Fig. 3. Electrophoretic separation (SDS-PAGE) of purified protease of *Bacillus subtilis* AU-2; Lane 1: purified protease, and Lane 2: standard molecular weight markers.

The purified protease of *B. subtilis* AU-2 also confirmed to be homogenous by using gel electrophoresis was further subjected for biochemical characterization. The biochemical characterization of the protease is an authoritative obligation for newly reported enzyme as the applicability enzyme in food applications depends on various factors like sensitivity to pH, temperature, and ability to remain active and stable in presence of several surfactants, salts, and oxidising agents.

3.5. Effect of pH on protease activity and stability

Most of the proteases obtained from *Bacillus sp.* are alkaline proteases (Maruthiah et al., 2013; Si et al., 2018) with exception of protease of *Bacillus subtilis* KT004404 which has optimum pH 6.0 (Rehman et al., 2017). The protease of *B. subtilis* AU-2 studied in this investigation was active in the broad pH range of 6.0–10.0 with optimum activity at pH 7.5. It was remain active and stable at neutral pH while also showed appreciable stability in alkaline pH range compare to acidic pH (Fig. 4). Henceforth, the protease produced by *B. subtilis* AU-2 is a neutral protease like extracellular protease secreted by *B. subtilis* Y-108 (Yang et al., 2000).

Several food preparation are processed using a neutral protease hence protease from *B. subtilis* AU-2 could be utilized like commercially available Neutrase® in food industry. The ability of protease active at neutral to slightly alkaline pH range offers its potential to apply as key commodity for fish protein hydrolysate preparation.

3.6. Effect of temperature on protease activity and stability

Purified protease of *B. subtilis* AU-2 has showed activity in broad temperature range of 20–60°Cwith the optimum temperature 40 °C(Fig. 5a). The protease get totally inactivated at 70°C. Also, thermal endurance was determined after 30 and 60 min at various temperature 25–65°C (Fig. 5b); the enzyme has retained 74% and 62% activity after heating at 55°C for 30 and 60 min respectively. The enzyme get rapidly inactivated beyond 55°C. These results are in accordance with

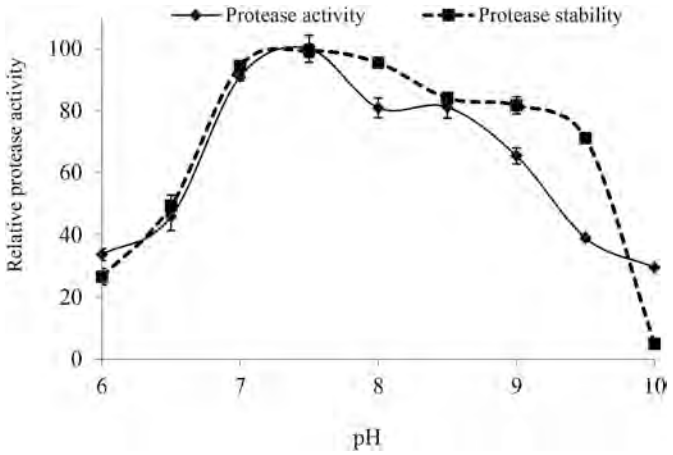

Fig. 4. Effect of pH on enzyme activity and stability of purified protease obtained from *Bacillus subtilis* AU-2.

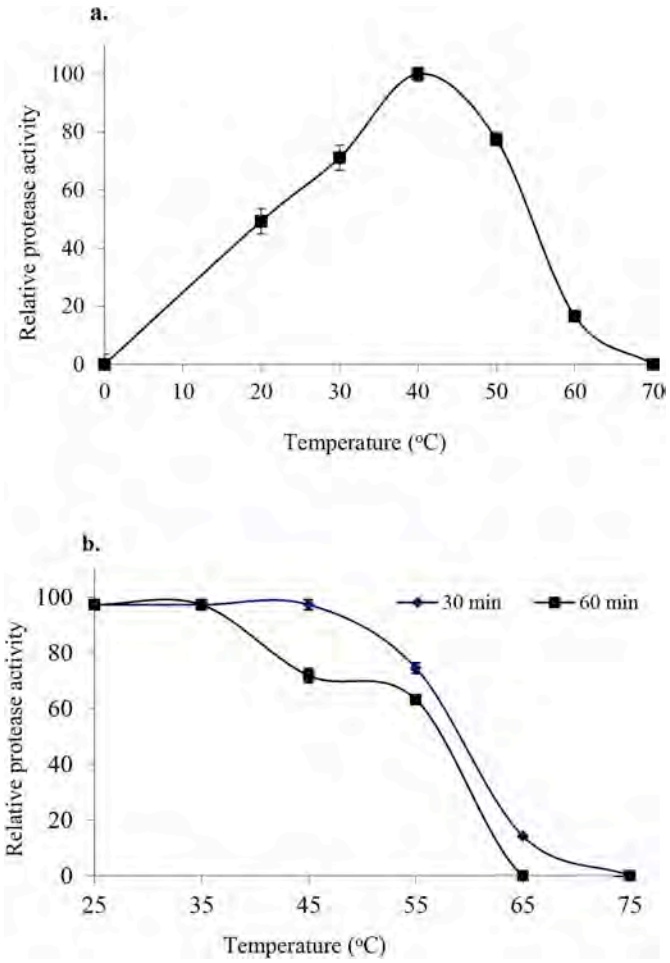

Fig. 5. (A) Effect of temperature on enzyme activity of purified protease obtained from *Bacillus subtilis* AU-2; **(B)** Thermal stability evaluated at various temperature after 30 and 60 min.

extracellular protease of *Bacillus subtilis* AP-MSU6 (Maruthiah et al., 2013). Previously, thermo-stable proteases having optimum temperature 50-55°Cwere reported from *Bacillus subtilis* Y-108 (Yang et al., 2000) and *Bacillus subtilis* KT004404 (Rehman et al., 2017).

3.7. Effect of inhibitors on protease

The effect of various inhibitors on protease activity is summarized in Table 4. The enzyme was inhibited by EDTA, and 1, 10 phenanthroline suggesting that the protease of *B. subtilis* AU-2 is the metalloprotease. Similarly, metallo-proteases were reported from *Bacillus subtilis* KT004404 (Rehman et al., 2017), *Bacillus subtilis* Y108 (Yang et al., 2000; Si et al., 2018). In addition, serine proteases form *Bacillus subtilis* PE-11 (Adinarayana et al., 2003) and *Bacillus subtilis* DR8806 (Farhadian et al., 2015) have also been described.

3.8. Effect of surfactant and oxidizing agents on protease

The effect of various chemicals such as surfactants, and oxidising agent on protease activity was examined. The protease showed appreciable endurance (80 to 96%) in presence of Tween-20, SDS, Triton X-100, H_2O_2 and sodium per-borate) as summarised in Table 4. Comparable outcomes were documented for metallo-protease obtained from *Bacillus subtilis* FBL-1 (Kim et al., 2016).

Food grade hydrogen peroxide (H_2O_2) used as a strong oxidising agent and effective antimicrobial agent in food industry. The hydrogen peroxide is used to improve or preserve the quality of margarine, cheese, instant tea, wine, or juice, corn syrup, and starch. These foods are treated with 0.05 to 1.5% (v/v) hydrogen peroxide. The capability to withstand active conformation in presence of H_2O_2 by protease of *B. subtilis* AU-2 could advises its utility in food industry.

Table 4
Effect of various activators/inhibitors on the purified protease of *Bacillus subtilis* AU-2.

Inhibitors/activators	Relative protease activity (Mean ± SD)
Protease inhibitors	
Control	100.36 ± 0.75
PMSF (5 mM)	98.77 ± 2.47
N-ethylmleimide (5 mM)	94.06 ± 4.49
1, 10 Phenanthroline (5 mM)	10.51 ± 1.36
EDTA (1 mM)	19.55 ± 1.50
EDTA (5 mM)	16.22 ± 1.03
Surfactant and oxidising agents (1%)	
Triton X-100	83.42 ± 1.21
Sodium dodecyl sulfate (SDS)	85.45 ± 3.62
Tween 20	77.55 ± 1.80
Hydrogen peroxide (H_2O_2)	93.63 ± 1.78
Sodium per-borate	96.81 ± 0.55
Metal ions (5 mM)	
$BaCl_2$	96.09 ± 3.60
$CaCl_2$	112.24 ± 1.95
$CdCl_2$	53.73 ± 6.65
$CuCl_2$	69.51 ± 1.52
$FeCl_2$	105.36 ± 2.30
$HgCl_2$	0.29 ± 0.33
KCl	107.82 ± 1.40
LiCl	95.66 ± 2.84
$MgCl_2$	110.35 ± 1.70
$MnCl_2$	117.02 ± 3.14
NaCl	91.46 ± 0.38
$NiCl_2$	69.73 ± 1.93
$SnCl_2$	90.95 ± 2.89
$ZnCl_2$	105.07 ± 1.33

3.9. Effect of metal ions on protease

The relative activity of protease was conserved in presence of Ba^{2+}, Li^+, Na^+, and K^+, (5 mM) salts. The protease activity was elevated in the presence of Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} as compare to control; however the enzyme was significantly get inhibited by Sn^{2+} Cd^{2+}, Cu^{2+}, Hg^{2+}, and Ni^{2+}. The effect of metal ions on protease activity of *B. subtilis* AU-2 was shown in Table 4.

Similar effect of metal ions on protease activity has been testified earlier. Metal ions like Fe^{2+}, Hg^{2+},and Cu^{2+} have lowered the activity; while Co^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} have enhanced the activity of protease obtained from *Bacillus subtilis* KT004404 (Rehman et al., 2017). Also, protease from *Bacillus subtilis* DR8806 showed enhanced activity in presence of metal ions like Ca^{2+}, K^+, Mg^{2+}, Fe^{2+} while the activity was lowered in the presence of Hg^{2+}, Ba^{2+}, and Cu^{2+} (Farhadian et al., 2015).

3.10. Substrate specificity of protease

The protease of *B. subtilis* AU-2 has ability to digest several proteinaceous substrates. The purified protease was tested with different natural proteinaceous substrates like bovine serum albumin, casein, egg albumin, gelatin, haemoglobin, and soybean flour. The purified enzyme has substrate utilisation profile as - casein (277 ± 12 U/mL); > soybean flour (250 ± 3 U/mL); > BSA (219 ± 3 U/mL); > haemoglobin (216 ± 9 U/mL) > egg albumin (210 ± 7 U/mL) > gelatin (0.0 U/mL). The gelatin was very slowly degraded by this enzyme even after 10 h incubation it showed poor proteolysis (16.6 U/mL). The results were in consistent with recent study about *B. subtilis* which reported the protease which unable to digest gelatine (Si et al., 2018).

The capability of this protease to utilize wide range of proteinaceous food substances like casein, egg albumin, haemoglobin, and soybean flour emphasizes its utility in enzymatic protein lysate preparation from milk, egg, meat, soybean flour. Although, the enzyme showed poor activity against gelatin, it could be a positive gain for specific proteolysis without impairing gelatin present in food as gelling agent. The gelatin used in wide range of food products as gelling agent due to its elasticity and clarity (Saha and Bhattacharya, 2010). The protease of *B. subtilis* AU-2 hence used for precise proteolysis application without upsetting the consistency of gelatin-stabilised food preparation.

4. Conclusion

The *Bacillus subtilis* AU-2 strain investigated in this study is an insect commensal and could be suitably used in food sector for food grade protease production like other *Bacillus subtilis* strains reported earlier. The improved production of protease by *B. subtilis* AU-2 in medium containing soybean meal (nitrogen source) revealed that the strain has been well adapted to utilise the soy bean seed proteins. The protease of *B. subtilis* AU-2 was found active in ambient physical parameters (pH, temperature) emphasises its utility in food applications. The metal ion reliant feature of metallo-protease from *B. subtilis* AU-2 and its capability to remain active in presence of various inorganic salts also provides assurance of activity and/or stability of this biocatalyst in the presence of various salts usually present in food.

The most significant findings includes the capability to catalyse various proteinaceous substrates like casein, egg albumin, haemoglobin, and soy bean flour, this accentuates its utility in enzymatic protein lysate preparation from milk, egg, meat, and soybean flour. The poor enzymatic activity of this protease against gelatin could be precisely utilize for achieving organized protein digestion without impairing the gelatin content which further assists to maintain consistency of gelatin-stabilised food preparation. Also, the capability to withstand H_2O_2 by protease of *B. subtilis* AU-2 advises its utility in improving/digesting the peptide contents of H_2O_2-preserved proteinaceous food. For food applications, the safety of the production strain is the most crucial factor; hence the protease of newly isolated insect commensal *Bacillus subtilis* AU-2 could be effectively used in food sector. The noteworthy outcomes of food related applications were also investigated which will be documented in subsequent study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101122.

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SHORT COMMUNICATION

Incidence of leaf spot disease on cotton caused by *Curvularia verruculosa* and role of its hydrolytic enzymes in pathogenesis

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Abstract The finding described in this study is the first report of leaf spot disease of cotton caused by *Curvularia verruculosa* surveyed in the state of Maharashtra (India). The isolated phytopathogenic fungal strain was identified using morphological characteristics and molecular identification of ITS gene sequence (MF784436) and D1D2 region of LSU gene (KY978073). The ability of fungal strain to secrete hydrolytic enzymes viz., pectinase, xylanase, protease, cellulase and lipase was detected. The secretion profile of hydrolytic enzymes by *C. verruculosa* was also examined in planta and in vitro. The secretion of cellulase, xylanase and protease was found to be inducible on cotton-stalk powder containing media; while secretion of pectinase and lipase was constitutive in glucose containing medium. The hydrolytic enzymes secretion during etiological progression of disease was detected on cotton leaves at regular interval of 24 h up to 10 days. A significant correlation ($P < 0.05$) was observed between hydrolytic enzymes secretion and disease severity index. The increased level of hydrolytic enzymes in infected plant sample indicates their role in disease progression. The newly documented fungal phytopathogen *Curvularia verruculosa* was deposited at National Fungal Culture Collection of India, Pune with accession number of NFCCI-4119.

Keywords *Curvularia verruculosa* · Transgenic Bt cotton · Leaf spot disease · Phytopathogen

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India is the second largest cotton producer in the world (22% of the global cotton production) with largest cultivation area (30% of global cotton area) among the major cotton growing countries (WWF-India report 2012).

The cotton crop is prone to several fungal, bacterial and viral diseases; among these infections the fungal infections are predominant on cotton and cause the major yield loss (Bell 1999). In India, the various fungal diseases reported on cotton includes fusarium wilt, verticillium wilt, grey mildew, alternaria leaf spot, myrothecium leaf spot, root rot and anthracnose (AICCIP 2005–06, 2013–14). The disease establishment by phytopathogenic fungi utilizes different mechanisms like synthesis and liberation of cell wall degrading hydrolytic enzymes, toxin production and synthesis of metabolites which interfere with the normal growth of host plant. The hydrolytic enzymes are foremost virulence factors of the fungal phytopathogen for disease establishment (Gour and Dube 1975; Have et al. 2002; Kikot et al. 2009; Bellincampi et al. 2014; Kubicek et al. 2014). The present study focused on isolation of fungal phytopathogen associated with the infected cotton plant leaves and detection of its enzymatic virulence factors.

In the cotton cropping seasons of 2013–14 and 2014–15 (June–September), the cotton plants in Maharashtra (India) were surveyed. The plants were observed with leaf spots that started as minute, irregular in shape (5–18 mm in diameter) with chlorotic area around the spots. However, the mature spots turned into necrotic lesion with a halo center inducing leaf chlorosis and premature senescence. The estimated disease incidence and disease severity index (DSI) were 36 and 49.38% respectively. The maximum disease incidence was recorded in the month of August. The disease prone climatic conditions in this month (temperature, 25–32 °C; humidity, 55–90%) could have supported the fungal infection.

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The infected leaves with prominent brown lesions surrounded with chlorotic region (Fig. 1a) were collected from different cotton growing fields in the surveyed area. The fungus was isolated from the section of diseased leaf on Czapek Dox Agar (CDA) media containing streptomycin (50 µg/ml). The morphological identification was carried out by the key described by Webster and Weber (2007) and Watanabe (2010). The fungus attained 90 mm wide colony on CDA in 6 days at 28 °C. The colony appeared as greenish gray on CDA, having pale colored fimbriate margin with grayish black reverse. The colony had circular, flat, filamentous appearance with slightly hairy, spreading and aerial mycelia with abundant sporulation. The hyphae were septate, branched, sub-hyline to brown in color. The conidiophore aroused singly, simple to rarely branched, macronematous, septate, sub-hyline to brown in color (Fig. 1b) on CDA. The conidia developed 17–38 µm long and 10–17 µm wide, with 2–4 cells broadly ellipsoidal, straight to slightly curved with one of the central cell being darker and larger, second and third cells appeared brown to pale brown while apical and basal cells were sub-hyaline, without hilum basally on slide culture assembly.

The morphology of the fungus is similar with that described for genus *Curvularia* (Kusai et al. 2016). The molecular characterization of isolated fungal culture was carried out at National Fungal Culture Collection of India (NFCCI-ARI) Pune, India. The ITS gene was amplified with fungal universal primers ITS-4 and ITS-5. The D1D2 region of LSU-rDNA gene was amplified by using primers LROR and LR5. The sequencing PCR was set up with ABI-Big Dye terminator v 3.1 cycle sequencing kit. The BLAST analysis and sequence identity of ITS and D1D2 region by Clustal W identified the fungal isolate as *Curvularia verruculosa*. The sequences of ITS and D1D2 region of LSU-rDNA genes of *C. verruculosa* were deposited to NCBI with Genbank accession numbers MF784436 and KY978073 respectively. This newly isolated fungal phytopathogen—*Curvularia verruculosa* was deposited at National Fungal Culture Collection of India, Agharkar Research Institute (NFCCI-ARI) Pune, India with deposition accession number of NFCCI-4119.

The pathogenicity of isolated fungus was assayed by spraying the spore suspension (1×10^6 spores ml^{-1}) on healthy plants at the four-leaf stage (18 days older plant). The control plants were treated with sterile distilled water. All the tested cotton plant leaves developed typical disease symptoms after 10–12 days of inoculation while control plants were symptomless. The vigor index and disease severity index (DSI) of control and test plants were measured as per Islam and Borthakur (2012) and Zhao et al. (2014) respectively. The DSI of test plants was 30 and a decrease of 29.75% in vigor index over control plant was also observed. The fungus was re-isolated from the leaf spots of treated cotton plants as an endorsement of Koch's postulates.

Hydrolytic enzymes are considered as most commonly produced than any other virulence factors by the fungal pathogens for disease establishment. The role of hydrolytic enzymes in penetration and break down of cuticular, cellulosic and pectic layers of plant cell wall is well documented (Have et al. 2002; Bellincampi et al. 2014; Kubicek et al. 2014). The detection of extracellular hydrolytic enzyme (viz. pectinase, xylanase, protease, cellulase and lipase) secretion by *C. verruculosa* was detected on solid media containing respective substrates. The zones of hydrolysis were produced around the fungal colony as a result of secretion of hydrolytic enzymes. The activities of hydrolytic enzymes were also determined in basal liquid media [g/l; $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01] supplemented with 1% cotton stalk powder and 1% glucose. The secretion of pectinase, xylanase, protease, cellulase and lipase in basal liquid medium (supplemented with 1% cotton stalk powder) was recorded

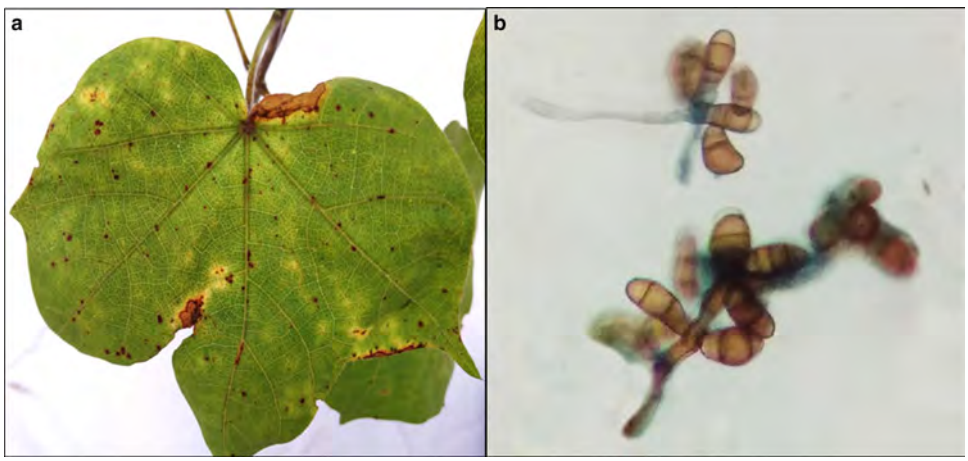

Fig. 1 **a** Symptomatic cotton leaf, **b** conidiophore of *C. verruculosa*

as 1.46, 2.91, 2.18, 0.74 and 3.14 U/ml respectively (Fig. 2). The activity of pectinase and lipase in liquid medium supplemented with 1% glucose powder was 1.03 and 0.82 U/ml respectively, whereas the activity of xylanase, cellulase and protease was not detected in the glucose containing liquid medium (Fig. 2). These results indicated that the expression of cellulase, xylanase and protease was inducible while the expression of pectinase and lipase was constitutive in *Curvularia verruculosa*. According to the study of Kapat et al. (1998), the constitutive as well as induced type of enzyme secretion is important for disease development. The constitutive enzyme secretion during early phase of infection may help the pathogen for faster penetration.

The secretion of hydrolytic enzymes during pathogenesis was analyzed on cotton leaves after the regular interval of 24 h up to 10 days post inoculation (Fig. 2). The secretion of pectinase was found maximum (0.91 U/ml) among the studied enzymes. The secretion of pectinase was observed from 1st day of inoculation; the level of enzyme secretion was continuously increased and found maximum at 8th day of inoculation. The xylanase secretion in cotton leaf tissues was negligible at 1st day of inoculation but rose progressively and found maximum at 8th day. The considerable level of lipase was detected at 1st day of inoculation and a linear increase in activity was noticed till 6th day of inoculation, later on, a gradual decrease in activity was noticed. Cellulase secretion was found maximum at 6th day with a gradual increase in activity, right from 1st day of inoculation. The protease was least secreted enzyme among all the hydrolytic enzymes. The secretion of protease was observed from 2nd day of inoculation and found maximum at 7th day.

The hydrolytic enzyme secretion and disease development was correlated by analyzing the enzyme secretion and disease severity index (leaf area covered by disease) over 10 days of incubation. The enzyme secretion potential and DSI values were found to be elevated with increase in period of incubation (Fig. 2). The correlation between hydrolytic enzyme (pectinase, xylanase, protease, cellulase and lipase) production and disease rating scale was studied by Pearson Correlation Coefficient ($P < 0.05$). A

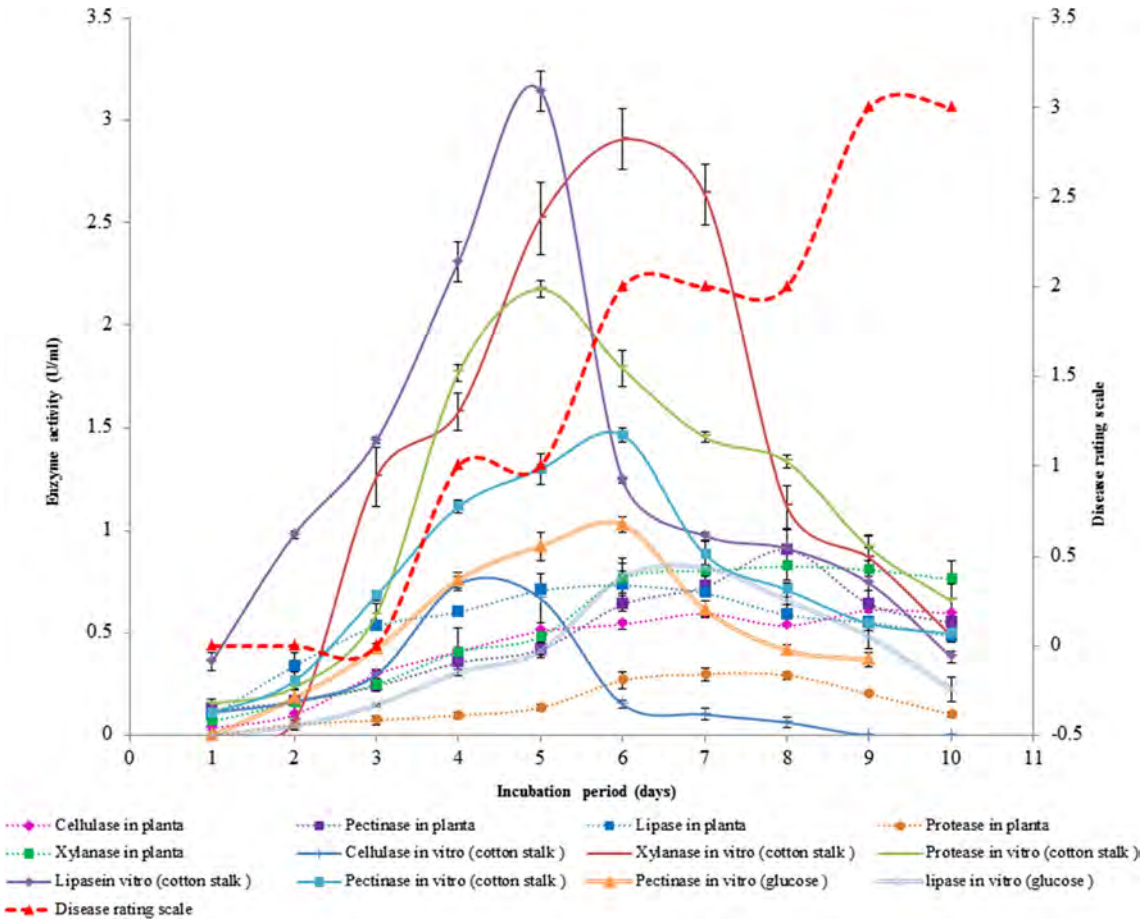

Fig. 2 In vitro secretion of hydrolytic enzymes by *C. verruculosa* in basal liquid medium containing cotton stalk powder/glucose as a carbon source and In planta secretion of hydrolytic enzymes by *C. verruculosa* during pathogenesis and its correlation with disease rating scale

significant correlation between hydrolytic enzymes (pectinase, xylanase, protease, cellulase and lipase) secretion and DSI was observed ($P < 0.05$). The presence of significant correlation between enzyme production and disease development indicated that, the hydrolytic enzymes act as an arsenal for penetration and colonization of phytopathogenic fungus within the host, which in turn develops disease. It was also observed that extracellular hydrolytic enzyme secretion pattern greatly differs during in planta and in vitro condition. This may be due to some inducing factors present in plant as well as some plant defense reactions and pH of cell sap.

In the present study, the phytopathogen associated with leaf spot disease of cotton was isolated and identified as *Curvularia verruculosa*. Although, *C. verruculosa* has been previously reported as a causative agent of leaf spot disease of Typha (Tandon and Bilgrami 1962), Sorghum, Triticum, Oryza (Sivanesan 1987) and Cynodon (Huang et al. 2005), the incidence of *C. verruculosa* infection to cotton plant has not been reported. Hence, to the best of our knowledge, this is the first report of *C. verruculosa* causing leaf spot disease of cotton. Moreover, this is the first approach to study in planta and in vitro secretion of hydrolytic enzymes by *C. verruculosa* on host plant.

Though the leaf spot disease caused by *Curvularia* sp. is concerned as minor disease, the outcomes of disease survey and pathogenicity test indicated that, *C. verruculosa* can cause serious outbreaks in the state of Maharashtra, which may lead to severe loss of cotton production under unpredictable global climatic vicissitudes. The findings of this study could be helpful for development of potential control measures and management of the newly isolated fungus in India. In addition to this, the studied enzymatic virulence factors may be appropriate targets for antifungal therapy.

Acknowledgements The authors are thankful to Dr. S. K. Singh, National Fungal Culture Collection of India, Agharkar Research Institute (NFCCI-ARI) Pune, India for molecular identification and deposition of fungal strain.

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MICROPROPAGATION

Quercetin and silver nitrate modulate organogenesis in *Carissa carandas* (L.)

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Abstract
An *in vitro* organogenesis protocol for *Carissa carandas* L. was developed using an auxin transport inhibitor (quercetin) and silver nitrate ($AgNO_3$), an inhibitor of ethylene action, in association with cytokinins in the culture medium. This protocol produced the maximum number of shoots from aseptic seedling-derived shoot apex explants of *C. carandas.* The highest rate of shoot multiplication was recorded on MS medium containing 2.0 mg L^{-1} 6-benzylaminopurine; 0.5 mg L^{-1} kinetin, and 0.75 mg L^{-1} quercetin at after 4 wk of culture. Similar results were obtained when MS medium fortified with 2.0 mg L^{-1} BAP, 0.5 mg L^{-1} kinetin, and 1.5 mg L^{-1} $AgNO_3$ was used. However, successful rooting was achieved on quarter strength MS medium with 0.5 mg L^{-1} indole-3-acetic acid. In this study, an inhibitor of auxin transport and ethylene action maximized shoot multiplication in medium fortified with cytokinins. The established rapid micropropagation method could be used to conserve elite genotypes of *C. carandas*.

Keywords *C. carandas* · Quercetin · $AgNO_3$ · Cytokinins

Carissa carandas (L.), an important shrub of the Apocynaceae family, usually grows in hilly or forest areas. It is native and common throughout India, Java, Malaysia, Myanmar, Pakistan, and Sri Lanka (Motwani *et al.* 2012). The plant produces berry-size fruits, which are traditionally used in the treatment of malaria, diarrhea, and skin infections. The roots are used as a stomachic and antihelminthic, the stem is used to strengthen tendons, and leaves are used as a remedy for fever (Kirtikar and Basu 1998). The plant extracts and the isolated single compounds have been reported to have several pharmacological activities such as antioxidant, anti-inflammatory, and cytotoxic activity (Begum *et al.* 2013; Galipalli *et al.* 2015; Bhadane and Patil 2017). A wider group of bioactive metabolites such as triterpenes, sesquiterpene, flavonoids, and phenolics have been reported in different parts of *C. carandas* (Naim *et al.* 1988; Itankar *et al.* 2011; Patil *et al.* 2012). It is also reported to be a potential source of anthocyanin, which is used to color food products (Iyer and Dubash 1993).

The variety of medicinal applications of this plant has led to overexploitation, which has endangered its existence. Thus, for the preservation of elite genotypes of this plant, conservation efforts are urgently needed. Earlier studies reported vegetative propagation methods such as cutting, grafting, and air layering as conservation strategies for *C. carandas* (Misra 2007). Previous studies on air layering of *C. carandas* reported problems with callus formation instead of root formation from the explant and requires elevated amounts (5.0 to 20.0 g L^{-1}) of plant growth hormones (Raut *et al.* 2015). Moreover, these strategies are rarely used because of the long time required for conservation. Besides vegetative propagation, seed sowing in soil is used as an alternative method for conservation. However, seeds of *C. carandas* are basically recalcitrant in nature with short viability, and therefore storage of these seeds is not possible. It has been reported that with a long period of preservation (up to 60 d), seeds lost their viability and had a 20% germination rate (Vanajalatha 2013). It appears that relevant research has not been done on these conventional methods for propagating *C. carandas.*

There has been renewed interest and progress in plant tissue culture for *in vitro* propagation and mass multiplication of elite, rare, and endangered plants with important medicinal

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properties (Arora and Bhojwani 1989). Attempts have been made for *in vitro* propagation of *C. carandas* using nodal shoot tip cultures from mature plants. These studies revealed successful propagation of *C. carandas* in a culture medium containing auxins, cytokinins (Rai and Misra 2005; Hasmah *et al.* 2013), and adenine sulfate at varying concentrations (Imran *et al.* 2012).

Quercetin belongs to the flavonoid group and seems to have a synergistic effect with cytokinins on shoot multiplication. It has been reported that flavonoids are auxin transport inhibitors and that they are responsible for *in vivo* inhibition of auxin synthesis in plants (Brown *et al.* 2001), in a similar fashion as has been reported for synthetic auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) and 1-naphthylphthalamic acid (NPA), which inhibit auxin transport and promote shoot multiplication of *in vitro* cultured plants (Singh and Syamal 2000; Lall *et al.* 2005). Jacobs and Rubery (1988) reported that quercetin acts as a competitive inhibitor of NPA and binds at auxin binding sites available in membrane receptors of plant cells. This mechanism has been found to disable auxin efflux to maximize shoot proliferation by inhibiting auxin transport. Therefore, quercetin could be used as a natural and effective source to improve the shoot multiplication of *in vitro* cultured plants.

In addition, there is increasing interest in the use of $AgNO_3$ for shoot multiplication of *in vitro* cultured plants. It acts as a source of nitrate in the tissue culture medium and favors the shoot multiplication process. It has also been reported to be an inhibitor of ethylene action in *in vitro* cultures.

The present study reports a novel *in vitro* propagation protocol using quercetin and $AgNO_3$, as inhibitors of auxin transport and ethylene action in the culture medium, respectively. These results could be useful for *in vitro* propagation of elite plants such as *C. carandas.*

All of the chemicals and the plant growth regulators used were purchased from Hi-Media®, Mumbai, India. Seeds from ripened fruits of *C. carandas* were collected during the summer from Aner River Forest, Dhule, India (400 m AMSL, 21.26°N, 75.11°E). All seeds were surface sterilized inside a LAF (laminar air flow) cabinet as described in the method of Bhadane and Patil (2016). Seeds were sterilized using a 0.1% (*v/v*) solution of commercial bleach Lizol® containing 4% (*w/v*) benzalkonium chloride (Reckitt Benckiser, Gurgaon, India) for 5 min, and rinsed with sterile distilled water

Table 1 Effect of 6-benzylaminopurine (BAP) and kinetin with quercetin on shoot multiplication of *Carissa carandas* after 4 wk of culture

BAP (mg L^{-1})	Kinetin (mg L^{-1})	Quercetin (mg L^{-1})	Mean number of shoot per explant	Mean length (cm) of shoot per explant
1.0	–	–	0 ± 0.00o	0.00 ± 0.00m
2.0	–	–	2 ± 1.51n	1.20 ± 1.05kl
3.0	–	–	3 ± 1.35mn	1.39 ± 0.53jkl
4.0	–	–	3 ± 0.67lm	1.64 ± 0.21ij
1.0	0.5	–	2 ± 1.54mn	1.08 ± 0.74l
2.0	0.5	–	4 ± 1.14kl	1.45 ± 0.24ijk
3.0	0.5	–	5 ± 1.30jk	1.58 ± 0.19ij
4.0	0.5	–	5 ± 1.47jk	1.73 ± 0.16hij
1.0	–	0.75	6 ± 1.08ij	1.81 ± 0.30hi
2.0	–	0.75	7 ± 1.87hi	2.18 ± 0.21fg
3.0	–	0.75	7 ± 1.14hi	2.27 ± 0.21 fg
4.0	–	0.75	8 ± 1.04gh	2.40 ± 0.28ef
1.0	–	1.5	9 ± 1.03fg	2.03 ± 0.17gh
2.0	–	1.5	10 ± 1.70cde	2.45 ± 0.23def
3.0	–	1.5	10 ± 1.37def	2.18 ± 0.27fg
4.0	–	1.5	9 ± 1.40efg	2.54 ± 0.28cdef
1.0	0.5	0.75	11 ± 2.31cd	2.81 ± 0.22bcd
2.0	0.5	0.75	15 ± 1.62a	3.24 ± 0.28a
3.0	0.5	0.75	11 ± 2.37cd	2.76 ± 0.30bcd
4.0	0.5	0.75	12 ± 1.96c	2.90 ± 0.28bc
1.0	0.5	1.5	11 ± 1.79cd	2.74 ± 0.25bcde
2.0	0.5	1.5	13 ± 1.56b	2.92 ± 0.21ab
3.0	0.5	1.5	11 ± 1.79cd	2.77 ± 0.13bcd
4.0	0.5	1.5	11 ± 1.75cd	2.82 ± 0.30 bcd

The values correspond to the means ± standard deviation of 10 replicates. Means in each *column* followed by the same *letter* were not statistically different according to DMRT (Duncan's multiple range test) at $p < 0.05$

followed by treatment with 70% (*v/v*) ethanol for 30 s, then rinsed with sterile distilled water three times. After drying the seeds for 5 min on sterile Whatman® filter paper no.1 (Sigma-Aldrich®, St. Louis, MO), they were inoculated onto half-strength MS medium (Murashige and Skoog 1962). The pH of the medium was adjusted to 5.8 (before autoclaving) with 0.1 N HCl or 0.1 N NaOH using a digital pH meter (Aquasol, Rakiro Biotech, Mumbai, India) and gelled with 0.8% (*w/v*) bacteriological agar (Hi-Media®) for aseptic seed germination. Shoot apices of 6-wk-old seedlings were used as a source of explants. MS medium with varying concentrations and combinations of 6-benzylaminopurine (BAP), kinetin, $AgNO_3$, and quercetin were used as the shoot multiplication medium. All media were autoclaved at 121 °C and 108 kPa pressure for 20 min. 6-benzylaminopurine, kinetin, $AgNO_3$, and quercetin were added to the medium after autoclaving when the medium was cooled to 55 °C.

Shoot multiplication studies were conducted using MS medium containing (i) 1.0, 2.0, 3.0, or 4.0 mg L^{-1} BAP, either alone or in combination with 0.5 mg L^{-1} kinetin, (ii) BAP alone or in combination with kinetin plus 0.75 or 1.5 mg L^{-1} quercetin, and (iii) BAP alone or in combination with kinetin plus 1.0 or 1.5 mg L^{-1} $AgNO_3$.

After aseptic inoculation of explants in a LAF cabinet, they were kept in a culture room under controlled conditions of 25 ± 2 °C, 55 ± 5% relative humidity, and a 16-h photoperiod with photon flux of approximately 40 μmol m^{-2} s^{-1} provided by white fluorescent light (Philips, Kolkata, India). Data on the total number and length (cm) of the shoots were recorded.

Microshoots (> 2 cm long) obtained from the shoot multiplication study were used for *in vitro* rooting. Prior to cutting shoots from the callus clumps, they were gently stirred in sterile double-distilled water to remove any excess agar. Explants were further blotted to remove traces of water. These shoots were then cultured on half strength and quarter strength MS medium fortified with 0.25, 0.5, 1.0, or 2.0 mg L^{-1} indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) alone. After 4 wk of culture, data on root formation from each explant was recorded.

Shoots with well-developed roots were removed from the culture vessels, and roots were washed with sterile distilled water, and transferred to a 100-mL glass beaker containing a mixture of autoclaved garden soil and vermicompost (1:1

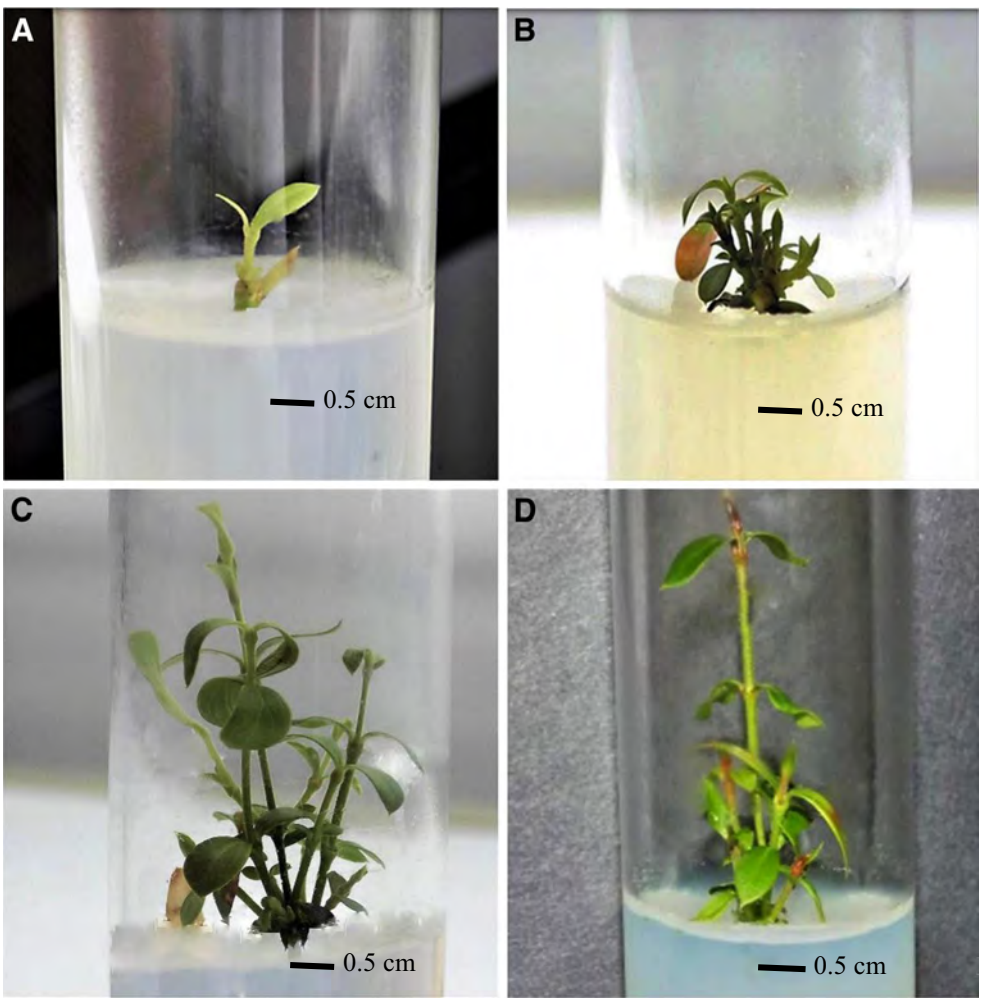

Fig. 1 Growth response of *Carissa carandas* shoot tips on an MS medium (Murashige and Skoog 1962) protocol (*A*) day 1; (*B*) with 3.0 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} kinetin; (*C*) 2.0 mg L^{-1} BAP, 0.5 mg L^{-1} kinetin and 0.75 mg L^{-1} quercetin; (*D*) with 2.0 mg L^{-1} BAP, 0.5 mg L^{-1} kinetin and 1.5 mg L^{-1} $AgNO_3$ after 4 wk.

Table 2 Effect of 6-benzylaminopurine (BAP) and kinetin with $AgNO_3$ on shoot multiplication in *Carissa carandas* after 4 wk of culture

BAP (mg L^{-1})	Kinetin (mg L^{-1})	$AgNO_3$ (mg L^{-1})	Mean number of shoots per explant	Mean length (cm) of shootlet per explant
1.0	–	–	0 ± 0.00q	0.00 ± 0.00k
2.0	–	–	2 ± 1.51p	1.20 ± 1.05ij
3.0	–	–	3 ± 1.35nop	1.39 ± 0.53hij
4.0	–	–	3 ± 0.67mno	1.64 ± 0.21fgh
1.0	0.5	–	2 ± 1.54op	1.08 ± 0.74j
2.0	0.5	–	4 ± 1.14klm	1.45 ± 0.24ghi
3.0	0.5	–	5 ± 1.30jkl	1.58 ± 0.19fgh
4.0	0.5	–	5 ± 1.47ijk	1.73 ± 0.16efgh
1.0	–	1.0	4 ± 0.90lmn	1.86 ± 0.14cdef
2.0	–	1.0	4 ± 0.77klm	2.04 ± 0.26 bcde
3.0	–	1.0	6 ± 1.00hij	2.08 ± 0.31bcde
4.0	–	1.0	6 ± 1.43fghi	2.14 ± 0.23bcd
1.0	–	1.5	6 ± 1.52ghij	1.80 ± 0.18defg
2.0	–	1.5	6 ± 1.49fghi	2.19 ± 0.27bc
3.0	–	1.5	7 ± 1.70 cdefg	2.11 ± 0.18bcd
4.0	–	1.5	7 ± 1.37defg	2.21 ± 0.19bc
1.0	0.5	1.0	7 ± 1.76efgh	2.05 ± 0.22bcde
2.0	0.5	1.0	9 ± 0.85abc	2.30 ± 0.22b
3.0	0.5	1.0	7 ± 1.17cdefg	2.17 ± 0.27bc
4.0	0.5	1.0	8 ± 1.35cdef	2.15 ± 0.27bcd
1.0	0.5	1.5	8 ± 1.56bcde	2.09 ± 0.24bcd
2.0	0.5	1.5	10 ± 1.95a	3.08 ± 0.18a
3.0	0.5	1.5	8 ± 1.07abcd	2.23 ± 0.14bc
4.0	0.5	1.5	9 ± 1.56ab	2.22 ± 0.14 bc

The values correspond to means ± standard deviation of 10 replicates. Means in each *column* followed by the same *letter* were not statistically different according to DMRT (Duncan's multiple range test) at $p < 0.05$

ratio) for acclimatization. The plantlets were irrigated with 1/10 diluted MS salt solution without sucrose every 24 h. The beakers containing plantlets were covered with polyethylene bags to maintain proper humidity. The acclimatized plantlets were transferred to 10-cm^3 plastic pots containing natural soil, covered with polyethylene bags and placed in a shed house for secondary hardening at 28 ± 2 °C and a 12-h light cycle using natural light.

Experiments were conducted in a randomized block design, and the results were presented as mean ± SD of ten replicates. Data from shoot multiplication and *in vitro* rooting were subjected to analysis of variance (ANOVA) to determine

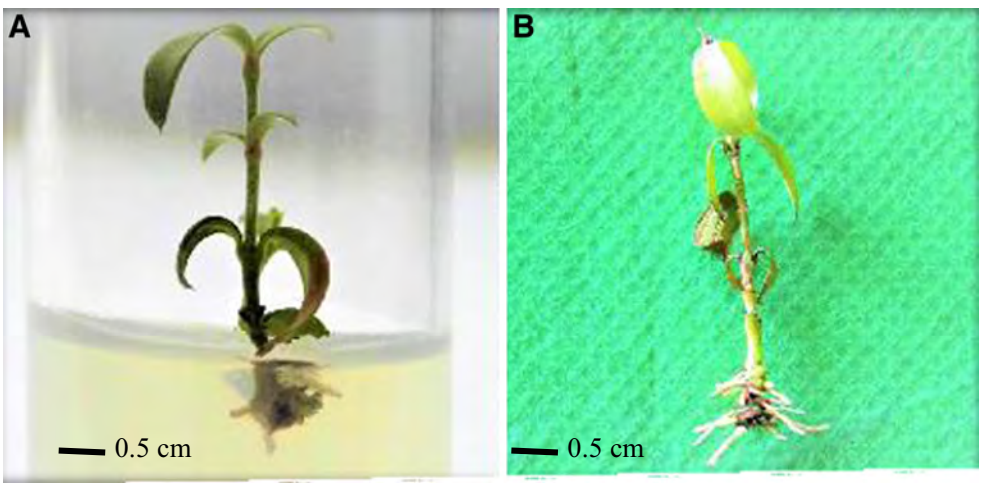

Fig. 2 *In vitro Carissa carandas* root induction of cultured shoots. (*A*) Root induction of the shoots cultured on quarter-strength MS medium (Murashige and Skoog 1962) containing 0.5 mg L^{-1} indole-3-acetic acid. (*B*) A shootlet with multiple roots after 4 wk of culture.

Table 3 Effect of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) on *in vitro* rooting of *Carissa carandas* shoots in quarter strength MS medium (Murashige and Skoog 1962) after 4 wk of culture

IAA (mg L^{-1})	IBA (mg L^{-1})	Mean number of roots per explant	Mean length (cm) of roots per explant
0.25	–	0 ± 0.00c	0.00 ± 0.00b
0.5	–	8 ± 3.16a	1.21 ± 0.65a
1.0	–	4 ± 1.35b	0.89 ± 0.36a
2.0	–	3 ± 0.67b	0.90 ± 0.17a
–	0.25	0 ± 0.00b	0.00 ± 0.00a
–	0.5	1 ± 0.92ab	0.47 ± 0.72a
–	1.0	1 ± 1.08a	0.49 ± 0.60a
–	2.0	Callusing	Callusing

The values correspond to means ± standard deviation of 10 replicates. Means of each auxin treatment within *column* followed by same *letter* were not statistically differ according to DMRT (Duncan's multiple range test) at $p < 0.05$

the significant variations. The data processing was completed using software XLSTAT version 16 (Addinsoft, New York, NY), and the means were compared according to Duncan's multiple range test (DMRT) at $p \leq 0.05$.

Shoot development was observed at the cut edges of aseptically derived explants (shoot apices) on the 13th d of incubation on MS medium fortified with 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} kinetin. On this medium, the average number of shoots was 5 ± 1.30 with a recorded length of 1.73 ± 0.16 cm (Table 1). Figures 1*A*, *B* reveal growth of explants in the culture medium on the 1st and 13th day. Addition of quercetin to cytokinin-containing medium resulted in a significant increase in the number of shoots per explant and shoot length (Table 1). The results demonstrated that 2.0 mg L^{-1} BAP in combination with 0.5 mg L^{-1} kinetin and 0.75 mg L^{-1} quercetin induced significant ($p < 0.05$) shoot multiplication and growth in *C. carandas* (Fig. 1; Table 1).

Similar results were obtained when $AgNO_3$ in combination with BAP and kinetin was used in the culture medium for shoot multiplication. A significant increase ($p < 0.05$) in shoot numbers (10 ± 1.95) and shoot length per explant was observed in MS medium containing 2.0 mg L^{-1} BAP, 0.5 mg L^{-1}, kinetin, and 1.5 mg L^{-1} AgNO3 after 4 wk of incubation (Table 2; Fig. 1*D*). Moreover, shoot multiplication in culture medium with BAP alone and in combination with $AgNO_3$ was found to be significantly lower than in medium with a combination of BAP, kinetin, and $AgNO_3$ at the tested concentrations.

Well-developed shoots were transferred to MS medium containing different concentrations of IAA and IBA. It was found that quarter strength MS medium induced rooting at all the concentrations of IAA and IBA tested, except at 0.25 mg L^{-1}. The primordial tiny roots on the shoots formed during the 2nd and 3rd wk of incubation (Fig. 2*A*). Of the concentrations of IAA tested, the maximum number of roots (8 ± 3.16) per shoot, and their average length (1.21 ± 0.65 cm) after incubation for 4 wk was observed at 0.5 mg L^{-1} ($p < 0.05$) (Fig. 2*B*). IBA, however, could induce rooting on developed shoots, but the response was low compared to IAA. Roots formed in quarter strength MS medium with 1.0 mg L^{-1} IBA were found to be much smaller (0.49 ± 0.60 cm) and fewer in number (only one per shootlet; Table 3). The entire process of hardening took 8 wk, and all the plantlets showed satisfactory growth during this period (Fig. 3). The survival frequency of plantlets during hardening was about 70%.

It is a well-known fact that cytokinins are involved in bud breakage, cell division, and shoot initiation and multiplication from explants (George *et al.* 2008; Mazri 2015). The present results on the use of quercetin are in agreement with the earlier research of Krishnan and Siril (2015), in which quercetin was used in combination with kinetin and BAP for shoot multiplication of *Oldenlandia umbellata*. Silver nitrate in the medium was believed to induce a less-oxidized cellular environment and upregulation of cytokinin biosynthetic genes, which favored shoot multiplication (Paladi *et al.* 2017). The increase in the number of shoots in the present study may also possibly be attributed to the effects of quercetin and silver nitrate.

Fig. 3 Acclimatized and established *Carissa carandas* plantlets in plastic pots containing natural soil after 8 wk.

The earlier studies on successful micropropagation of *C. carandas* have shown the impacts of different concentrations and combinations of phytohormones. Hasmah *et al.* (2013) reported 5.5 shoots per explants, with an average shoot length of 3.3 cm, on MS medium containing 4 mg L^{-1} BAP. Earlier, Rai and Misra (2005) reported 12.54 shoots per explant with a height of 4.39 cm in MS medium containing 13.32 μM BAP. On the other hand, Imran *et al.* (2012) employed a combination of 1.5 mg L^{-1} BAP, 1.0 mg L^{-1} kinetin, 1.0 mg L^{-1} TDZ, and 15 mg L^{-1} adenine sulfate in MS medium and obtained 27 shoots per explant. Apparently, the use of inhibitors ($AgNO_3$ and quercetin) in the culture medium resulted in improved organogenesis and shoot multiplication in the present study and is a novel result for *C. carandas*.

In the micropropagation of *Wrightia tinctoria*, higher concentrations of the medium constituents and combinations of auxins resulted in the cultured shoots forming callus tissue, instead of root formation (Purohit and Kukda 2004). In another study, rooting of *C. carandas* was achieved on half-strength MS medium (Rai and Misra 2005). In the present study, root induction on cultured shoots was successfully established in quarter-strength MS medium supplemented with lower concentrations of auxins without callus formation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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Review

Factors influencing the gut microbiome in children: from infancy to childhood

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The human microbiota plays a crucial role in educating the immune system and influencing host health right since birth. Various maternal factors along with the vertical microbial transfer from the mother, as well as the horizontal environmental transmission and internal factors relating to the infant, play a crucial role in modulating the gut microbiota. The early life microflora is highly unstable and undergoes dynamic changes during the first few years, converging towards a more stabilized adult microbiota by co-evolving with the host by the age of 3–4 years. Microbiota studies have underlined the role of dysbiosis in developing several metabolic disorders like obesity, diabetes and immune-related disorders like asthma, to name a few. Thus, understanding early life microbial composition and various factors affecting the microbial community will provide a platform for developing strategies/techniques to maintain host health by restoring gut microbial flora. This review focuses on the factors that affect the microbial composition of the foetus *in utero*, during birth, infancy through childhood.

Keywords. Birth mode; infants; maternal health; microbiome

1. Introduction

The study of the human gut microbiome has gained importance in past years due to the important contribution of microbes in host health, stimulating immune system development, host nutrient metabolism, as well as promoting differentiation of mucosal structure and function. These actions of the microbiota have a widespread impact beyond the gastrointestinal tract (Tremaroli 2012; Doré *et al.* 2013; Cénit *et al.* 2014). The gastrointestinal tract (GIT) is known to be the most densely populated anatomical site forming a 'mini-ecosystem' (Tojo *et al.* 2014). Thus, characterizing the gut microbiota is one of the most important aspects of understanding the host microbiome relation. The flexibility of the human gut microbiome is a characteristic feature as, despite the daily oral intake of particular food, the composition of the microbiota and the metagenome remains essentially unaffected. Influences on the microbiota are evident across the human lifespan and depend on various internal and external factors (Ottman *et al.* 2012; Odamaki *et al.* 2016; Korpela and de Vos 2018).

Post birth, the composition of the microbiota is initially derived from colonization by the early settlers to which a baby is exposed in their environment, and during delivery, which, along with other factors, such as diet and medications, substantially affect the entry of subsequent microbial species into the suitable micro-environments in the host. Before birth, the foetus is highly protected and isolated by the mother with everything being filtered by the mother's organs before reaching the baby *in utero* (Perez-Muñoz *et al.* 2017). At birth, the immune system of a foetus is thus not well educated, which is partially beneficial as the foetus

cannot mount a severe reaction against the maternal antigens (Yan *et al.* 2004; Al-Hertani *et al.* 2007; Gervassi and Horton 2014). Studies suggest that the GIT microbiota acts as a major source of antigens including peptidoglycan, lipoproteins, lipopolysaccharides and flagellin. All of these antigens shape, activate and educate the innate and adaptive immune systems (Schwandner *et al.* 1999; Wang *et al.* 2001; Patten and Collett 2013). Thus, an individual requires a stable microbial composition (microscopic composition), along with a suitable macro environment (as the bodily/surrounding environment affects the microbial diversity), for normal metabolic functioning of the microbiota.

While a dysbiotic microbial composition can disturb the biological functions in the host, a healthy microbial community is needed for maintaining sound health. The interaction between the microbiome and host is most crucial during the early lifetime as critical changes in the abundance and composition of the microbiome prevail in early life, which becomes more or less stable and remains throughout a lifetime, thus dictating the health of future adult life (Neu 2015). During the early years of life, the intestinal microbiome is relatively dynamic, and these initial dwellers have a key impact on the host health throughout life (Scholtens *et al.* 2012; Tanaka and Nakayama 2017). Thus, it is important to understand the factors that influence and modify the microbiome at various stages of life for an individual, with an emphasis on early life. This review focuses on the various factors that affect the microbiota of children from infancy to childhood.

2. Phase 1: Foetal stage

2.1 *Maternal diet during pregnancy*

The bacterial colonization of the neonatal gut begins when *in utero* and the maternal intestinal flora is a major source of healthy microbiota for the infant, which persists during the early weeks of life (Vaishampayan *et al.* 2010). Gut microbial composition varies with the diet and health status of the host, and these factors during pregnancy can affect the maternal gut microbiota, which in turn can affect the infant *in utero* and even post birth (figure 1). Previous studies have not only shown a clear association between diet and gut

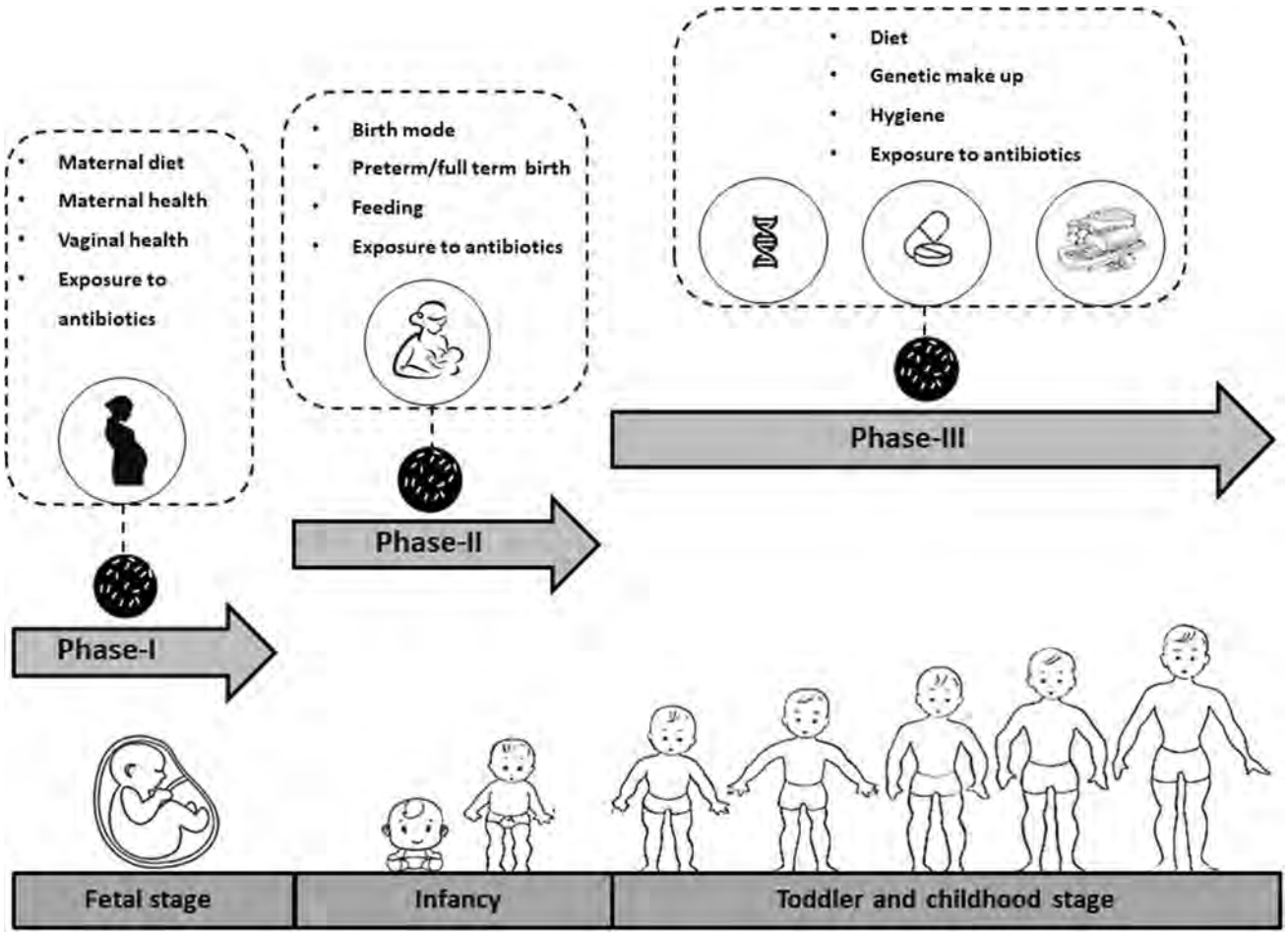

Figure 1. Overview of the factors influencing the gut microbiome at different stages from phases 1 to 3 (foetal stage, infancy, toddler and childhood stage).

microbiome but have effectively proved the importance of maternal dietary modulations in influencing the changes in the gut microbiome (Chu *et al.* 2016; Lundgren *et al.* 2018).

Recently, Kristen Meyer *et al.* using two dietary treatments in a longitudinal study reported that modifying the maternal diet (like changing fat to carbohydrate intake or changing consumption of specific sugars) is associated with significant alterations in the milk microbial composition and human milk oligosaccharide (HMO) composition (Meyer *et al.* 2017). The breast milk microbiome and HMOs come in direct contact with the breastfeeding infants and thus can modify their gut microbial composition (Jost *et al.* 2015). The results thus suggest a possible role of maternal food habits during and post pregnancy on infant gut microflora development (Meyer *et al.* 2017).

In another study in mice, Gohir *et al.* (2015) looked into how the maternal diet (before and during pregnancy) affects the gut microbial composition. In case of high-fat fed female mice, the authors observed significant changes in the gut microbiota composition later in pregnancy, compared to those fed on a normal chow diet. Mothers fed with a high-fat diet before and during pregnancy had higher levels of *Akkermansia* and *Bifidobacterium*. These changes in the microbial composition can alter the abundance of genes that favour various metabolic processes during pregnancy (Gohir *et al.* 2015) and thus can affect the inceptive microbial composition of the infant. Further, Gibson *et al.* demonstrated that exposure to specific food items, such as fish oil (PUFA rich diet) to rat dams, resulted in a decrease in microbial richness and altered intestinal microbial composition (Gibson *et al.* 2015). The authors also observed that offspring born to mothers consuming fish oil showed an abundance of taxa of opportunistic pathogens like *Bilophila wadsworthia*, *Enterococcus faecium* and *Bacteroides fragilis* in their gut which can lead to altered immune response (Gibson *et al.* 2015). Also, studies have shown that risks for spontaneous preterm delivery (Myhre *et al.* 2011) and childhood allergic diseases (Bertelsen *et al.* 2014) are reduced by habitual maternal intake of probiotic-containing food.

A recent study in *Macaca fuscata*, a primate model, revealed that maternal consumption of high-fat diet during pregnancy or post birth results in dysbiosis of the neonatal intestinal microbiome (Ma *et al.* 2014). They observed the dominance of *Bacteroidetes* and the absence of *Spirochetes* in the gut of high-fat diet fed dams. These shifts were accompanied by a decrease of *Treponema* and increased the abundance of *Prevotella* (Ma *et al.* 2014). Similar to the results obtained in primate studies, Chu *et al.* (2016) demonstrated that in humans too, a high-fat maternal diet alters the neonatal gut microbiome independent of the maternal body mass index. They observed that the infant gut microbial composition varied by maternal diet during pregnancy with *Bacteroides* levels reduced in infants born to mothers consuming a high-fat diet during pregnancy (Chu *et al.* 2016). Another less studied factor is alcohol consumption during pregnancy and its ill effects on faecal microbiome development. Alcohol consumption during pregnancy has been associated with various disorders (Ouellette *et al.* 1977) in the neonate and preterm birth (Miyake *et al.* 2014). It is well known that alcohol consumption alters the gut microbial composition (Dubinkina *et al.* 2017) even during pregnancy (Labrecque *et al.* 2016). These changes in the maternal gut microbial composition can affect the initial infant gut colonization, making it more prone to infections and diseases later in life. All in all, these studies emphasize that the maternal diet during pregnancy strongly influences the infant gut microbiota development.

2.2 *Maternal health during pregnancy*

Mothers share their microbes and metabolites with the foetus *in utero*, during delivery and lactation, and thus the maternal health during pregnancy affects the development of the foetus. The commensal microbial community in the human gastrointestinal tract plays crucial roles in the immune response and metabolic homeostasis, metabolic adaptations and, thus, in normal pregnancy (DiGiulio *et al.* 2015; Gomez-Arango *et al.* 2016; Smid *et al.* 2018).

Studies have shown that offspring of mothers suffering from any type of diabetes or having high BMI (overweight/obese) are at risk of developing diabetes and obesity in later life (Weiss *et al.* 2000; Whitaker 2004; Pirkola *et al.* 2010; Deierlein *et al.* 2011; Mehta *et al.* 2012; Gaillard *et al.* 2013). It is hypothesized that maternal diabetes and obesity influence the offspring's risk for developing chronic metabolic diseases, through changes in the maternal microbial composition during pregnancy and lactation (Singh *et al.* 2017). These alterations in microbial composition can have a negative impact on maternal and offspring health, by altering host metabolic pathways due to its effect on the abundance of genes that favour these metabolic pathways (Collado *et al.* 2010; Gaillard *et al.* 2013; Galley *et al.* 2014; Gallardo *et al.* 2015; Gohir *et al.* 2015; Hussen *et al.* 2015).

Direct studies trying to find the link between maternal health and infants have found that the microbial composition of infants born to obese mothers is distinct as compared to those born to lean mothers (Collado *et al.* 2010; Galley *et al.* 2014) and the offspring are associated with increased oxidative stress (Gallardo *et al.* 2015). Collado *et al.* analysed the gut microbial composition by fluorescent *in situ* hybridization with flow cytometry (FCM-FISH) and by quantitative real-time polymerase chain reaction (qPCR) in overweight and normal-weight pregnant women and observed that the infant's faecal microbial community was relatable to maternal weight gain during pregnancy. The authors found significant alterations in the gut microbial taxa in the two groups, of which *Bacteroides* and *Staphylococcus* were distinctly higher in overweight and obese women (Collado *et al.* 2008).

In another study, Collado *et al.* observed that high weight and BMI of mothers were associated with increased *Bacteroides*, *Clostridium* and *Staphylococcus* levels and decreased the abundance of the *Bifidobacterium* group. The presence of *Akkermansia muciniphila*, *Staphylococcus* and *Clostridium difficile* groups was decreased in infants born to mothers in the normal BMI range during pregnancy (Collado *et al.* 2010). Another study involved analysing the composition of the gut microbiota in 50 pregnant women in Spain by qPCR; they observed that Enterobacteriaceae*, Escherichia coli and Staphylococcus* numbers were increased, but *Bifidobacterium* and *Bacteroides* numbers were significantly reduced in overweight and obese pregnant women, which is contradictory with the previous study (Santacruz *et al.* 2010). Another study demonstrated that the microbiome associated with obese/overweight mothers leads to offspring with higher chances of obesity at early age. They reported that this can be extrapolated from the early life microbial composition of infants which can act as an indicator of obesity development in later life (Stanislawski *et al.* 2018).

Inflammation of the placenta and changes in feto-placental functions are seen as some of the adverse effects associated with maternal obesity or excess gestational weight gain (Challier *et al.* 2008; Kaphingst *et al.* 2008; Ditchfield *et al.* 2015). Though there is no definitive evidence supporting this hypothesis yet, new evidence from studies suggests that dysbiosis of the placental microbiota may be responsible for complications during pregnancy (Prince *et al.* 2016). The altered placental microbiome could be associated with ascending vaginal infections or oral commensal bacteria (Fardini *et al.* 2010). Out of the several factors that can affect the placental microbiota, gestational diabetes mellitus (GDM) is one. But only a few studies have investigated the effect of GDM on the placental microbiota (Bassols *et al.* 2016; Zheng *et al.* 2017). The authors in these studies confirmed the hypothesis that the placenta microbial composition in women diagnosed with GDM was different from that of normoglycaemic women. They observed a decrease in the abundance of the order *Pseudomonadales* and the genus *Acinetobacteria* in GDM women (Bassols *et al.* 2016) along with a rise in *Proteobacteria* levels, and a decrease in *Bacteroidetes* and *Firmicutes* in women with GDM (Zheng *et al.* 2017). Kumar *et al.* showed that neonates born to females with GDM are at high risk of developing atopic dermatitis and risk of allergen sensitization (Kumar *et al.* 2009), further associating GDM with childhood disorders.

To understand the effect of maternal diabetes on the microbiota of infants, a study analysed the meconium of neonate subjects born to mothers with diabetes before pregnancy and gestational diabetes and observed that their gut microbial composition grouped differently from those born to mothers without diabetes (Hu *et al.* 2013). Specifically, the meconium samples from the diabetes group had enriched levels of Bacteroidetes, members of the Lachnospiraceae family and Parabacteriodes genera, with lower levels of Proteobacteria. These alterations in the gut microbial composition were not affected by delivery mode, indicating the chances that maternal diabetic condition may have an impact on the foetal microbial composition, which in turn may affect the foetal health (Hu *et al.* 2013). It can be concluded from the above-mentioned studies that the main patterns seen in mothers suffering from obesity are high levels of *Bacteroides*, *Staphylococcus* and Enterobacteriaceae, with high levels of *Proteobacteria* and lower levels of *Acinetobacter*, *Bacteroides* and *Firmicutes* seen in mothers showing GDM. These changes predispose the offspring to higher chances of developing metabolic syndromes during childhood due to the dysbiotic microbial composition. Due to the higher chances of morbidity in offspring born to mothers with GDM or obesity, research is now focused on the effects of probiotics on pregnant women with GDM and obesity. Studies have shown that administration of probiotics to mothers with GDM results in better glycaemic control (Laitinen *et al.* 2009; Karamali *et al.* 2016), cholesterol levels, reduced insulin resistance and weight gain (Dolatkhah *et al.* 2015; Jafarnejad *et al.* 2016; Karamali *et al.* 2016) and reduced the risk of GDM (Laitinen *et al.* 2009; Luoto *et al.* 2010). Though there are studies which demonstrate no significant effects of probiotics during GDM and obesity in pregnancy (Lindsay *et al.* 2014, 2015; Zheng *et al.* 2018), none report any harmful effects.

It has also now been demonstrated by several studies that the baby *in utero* is not in a sterile environment and the amniotic fluid surrounding the foetus has its own unique microflora. Recent studies have observed higher microbial diversity and reduced alpha diversity in amniotic fluid samples associated with preterm deliveries (DiGiulio *et al.* 2008; Urushiyama *et al.* 2017). Another study by 16S rRNA gene sequencing identified *S. sanguinegens* and *F. nucleatum* in amniotic fluid as causes of preterm birth (Young-Ah *et al.* 2016). These studies indicate that maternal health during pregnancy must be closely monitored as any complications during pregnancy affect both the maternal and foetal health.

2.3 *Vaginal health*

The vaginal microbiome plays a possible role in the health of the mother and the newborn due to its direct contact with the foetus, and its possible role in colonizing the placenta. Vaginal dysbiosis during pregnancy is observed to be associated with negative reproductive outcomes, risk of postabortal infection (Larsson *et al*. 1992), early (Donders *et al.* 2000) and late miscarriage (Hay *et al.* 1994; Llahí-Camp *et al.* 1996) and premature rupture of foetal membrane and preterm birth (Hillier *et al.* 1995; Flynn *et al.* 1999; MacIntyre *et al.* 2015; Brown *et al.* 2018). The vaginal microbial composition of healthy mothers consists of members of the orders *Lactobacilliales*, *Clostridiales*, *Bacteroidales* and *Actinomycetales* (O'Hanlon *et al.* 2013).

To understand the role of vaginal health during pregnancy and its effect on infant health, Aagaard *et al.* studied the vaginal microbial composition of 24 healthy pregnant subjects. The authors observed that both richness and diversity were reduced during pregnancy with *Lactobacillus* being the dominant species observed (Aagaard *et al.* 2012). Romero *et al.* similarly reported the dominance of *Lactobacillus* spp. in healthy pregnant women in the first-ever longitudinal study of vaginal microbiota during pregnancy and also described increased stability of vaginal microbiota in pregnant woman compared to non-pregnant women of reproductive age (Romero *et al.* 2014). Similarly, Walther-António *et al.* studied the vaginal microbiota of 12 women during their healthy pregnancy at equal intervals of eight weeks in a longitudinal study and observed low microbiome diversity accompanied with high stability and dominance by *Lactobacillus* spp. (Walther-António *et al.* 2014). In another longitudinal study, MacIntyre *et al.* analysed the vaginal microbiota of 42 subjects during healthy pregnancy and the post-partum period. The authors reported that while the post-partum vaginal microbiome is not *Lactobacillus* dominant, it is more rich and diverse than the vaginal microbiota of ongoing pregnancies (MacIntyre *et al.* 2015). A study performed recently including 492 subjects confirmed the previous results and observed that the vaginal microbiome of women with healthy ongoing pregnancies has a relatively lower richness and diversity, along with a high abundance of *Lactobacillus* with a lower prevalence of *Mycoplasma* and *Ureaplasma*, which is otherwise related to preterm birth and low birthweight (Freitas *et al.* 2017), thus building our understanding of the vaginal microbiome in pregnancy. Other studies have suggested that the place of birth, that is either hospital or home, can also have an impact on the vaginal microbiota, which in turn can affect the infant gut microbial diversity which may last for long periods. They reported higher levels of *Clostridium* (Van Nimwegen *et al.* 2011; Combellick *et al.* 2018) and Enterobacteriaceae family (Combellick *et al.* 2018) in infants born in hospital as compared to those born at home. Nimwegen *et al.* reported that Vaginal home delivery was associated with a decreased risk of eczema, sensitization to food allergens and asthma (Van Nimwegen *et al.* 2011). Lactobacillus species (i.e., L. iners, L. crispatus, L. jensenii and L. gasseri) maintain vaginal health by maintaining a low pH and inhibiting the growth of pathogens (due to lactic acid production by fermentation of the available glycogen) and by producing a protein called bacteriocin that can actively kill unwanted bacteria (O'Hanlon *et al.* 2013). In a meta-analysis study, it was observed that bacterial vaginosis caused by the alteration in bacterial composition doubles the risk of preterm delivery and preterm labour, also predisposing the mother to higher chances of miscarriages and infection (Leitich and Kiss 2007). A large population-based study indicates that women with improper blood glucose levels have higher chances of vulvovaginal candidiasis infections as compared to those with controlled glucose levels (Faraji 2012; Sharma and Solanki 2014). Additionally, a few studies also report that the risk of vaginal mycoses in pregnant women with improper glucose levels is nearly two times higher (Nowakowska *et al.* 2004) compared to that in a pregnant woman with a controlled glucose level (Nowakowska *et al.* 2004; Lukic *et al.* 2017).

These studies indicate that the vaginal discharge during pregnancies (especially with GDM) must be checked for vaginosis and baseline data of the vaginal microbial composition and its abundance during pregnancy must be established to prevent pregnancy complications.

2.4 *Maternal exposure to antibiotics*

Exposure to antibiotic therapy and its modulatory effects on the human microbiome can begin *in utero* and continue throughout critical growth and development stages. Tanaka *et al.* 2009 demonstrated that alterations in the gut microbiota of infants whose mothers were treated with antibiotics were found to be similar to the alterations seen in infants treated with antibiotics, highlighting the influence of maternal medications on infant health (Tanaka *et al.* 2009). For example, it was observed in one of the previous studies that in mice, prenatal antibiotics reduces the diversity and structure of the microbiota in offspring (Tormo-Badia *et al.* 2014).

A recent study involving 36 overweight pregnant women studied the effect of the use of two intrapartum antibiotics Cephazolin and Benzylpenicillin by mothers on their infant's oral and gut microbial composition. They observed a high abundance of the family Streptococcaceae, Gemellaceae and Lactobacillales in infants born to mothers who were not exposed to intrapartum antibiotics. Along with this, families belonging to phylum *Proteobacteria* were found to be abundant in infants exposed to intrapartum antibiotics, a pattern often regarded as a signature of dysbiosis and inflammation (Gomez-Arango *et al.* 2017). Another study observed the effect of maternal antibiotic consumption on mothers and their nursing infant's gut microbiome. In this longitudinal study, breast milk and infant stool samples were collected at six time points from birth to one-month post-antibiotic initiation. It was reported that the relative richness of Bifidobacteria and Veillonella lowered after antibiotic treatment in most of the infant gut samples affecting the early colonizers of the infant gut (Rachel Rock *et al.* 2017).

A study by Gonzalez-Perez *et al.* 2016 demonstrated that in mothers treated with antibiotics during pregnancy and lactation, there were profound alterations in the composition of the gut microbiota in mothers and infants. *Streptococcus* spp. dominated the GIT microbiota of treated mothers, whereas *Enterococcus faecalis* predominated within the infant gut (Gonzalez-Perez *et al.* 2016). Another study demonstrated that the use of broad-spectrum antibiotics by pregnant mothers and infants at an early age can cause a shift in the gut microbial composition and may increase the

chances of development of colitis in susceptible offspring by affecting a critical stage of their microbial and immune development (Miyoshi *et al.* 2017). These findings point to the fact that uptake of antibiotics by mothers can affect the infant gut microbiome, which in turn can affect the infant's health and development and thus must be well observed and monitored.

3. Phase 2: Early infancy

3.1 *Mode of delivery*

The gut microbiome undergoes co-evolution with the host itself being influenced by various factors. The mode of delivery has a crucial impact on the type of microbiota ingested by the infant during birth. The delivery mode impact persists for months, and perhaps longer, after the birth as it contributes to microbiota development which can affect the normal physiological processes and disease development (Salminen *et al.* 2004; Dominguez-bello *et al.* 2010; Lorenza *et al.* 2014; Kumbhare *et al.* 2017). If a baby is normally delivered (vaginal delivery), the neonate comes in contact with the vaginal and the gut microbiome of the mother. The major microbiota colonizing the infant's gut is thus similar to the composition of the vaginal microbiome with a minor component being from the surrounding environment. On the other hand, a newborn delivered by caesarean section does not come in contact with the mother's vaginal microbiome, and the major component of the infant gut microbiome in this case is contributed by the nosocomial surrounding and the mother's skin microbiome. Recent studies reported a relatively increased risk of asthma (Chu *et al.* 2017b), obesity (Kuhle and Woolcott 2017; Rutayisire *et al.* 2016b), coeliac disease (Mårild *et al.* 2012) and type 1 diabetes (Cardwell *et al.* 2008; Adlercreutz *et al.* 2015) in children born via C-section, along with a lower frequency of atopic sensitization and allergy development in the vaginally delivered infants (Eggesbø *et al.* 2003; Negele *et al.* 2004; Bager *et al.* 2008; Huurre *et al.* 2008).

In particular, to determine whether the mode of delivery has an impact on the microbiome composition of the infant, researchers have studied the gut microbial diversity of infants from their birth to an age of 5–7 years as after this age an adult microbiome is established which remains throughout life. Earlier studies, such as those performed by Grolund in 1999, showed that the gut microbiome composition of infants delivered by caesarean delivery (CD) was significantly different when compared to vaginal delivery (VD) babies (Gronlund 1999). Vaginally delivered newborns exhibit bacterial taxa composed of various genera including Lactobacillus, Prevotella, Escherichia, Bacteroides, Bifidobacterium and Streptococcus spp. (Penders *et al.* 2006; Huurre *et al.* 2008; Dominguez-bello *et al.* 2010; Fallani *et al.* 2010; Azad *et al.* 2013; Liu *et al.* 2015). Another study by Biasucci *et al.* demonstrated that the gut microbiota of the caesarean delivery infants was less diverse than the microbiota of vaginally delivered infants, which may have long-term effects on the health of the infants (Biasucci *et al.* 2008), such as stronger immunological response (Huurre *et al.* 2008). In particular, it was demonstrated that CD delivered infants had minor amounts of *Bifidobacteria* (; Chen *et al.* 2007; Huurre *et al.* 2008; Dominguez-bello *et al.* 2010; Azad *et al.* 2013; Rutayisire et al. 2016a) and *Escherichia-Shigella* and absence of *Bacteroides* (Fallani *et al.* 2010; Song *et al.* 2013; Jakobsson *et al.* 2014), while VD delivered infants were characterized by *Bifidobacteria* (Hansen *et al.* 2015; Pandey *et al.* 2012), predominantly *B. longum* and *B. catenulatum* species (Gronlund 1999; Biasucci *et al.* 2008; Dogra *et al.* 2015) with higher amounts of *Klebsiella* in CD delivered infants (Dogra *et al.* 2015). Chu *et al.* 2017a, b and Dominguez-bello *et al.* 2010 showed that the differences in the intestinal gut microbiota of neonates were significant and seemed to be driven by the mode of delivery, demonstrating an increased association of *Propionibacterium*, *Corynebacterium* and *Streptococcus* with caesarean-born neonates resembling the skin surface microbiota, whereas *Lactobacillus* and *Prevotella* were observed to be associated with vaginally delivered neonates, which was more similar to their mother's vaginal microbiota.

It is also demonstrated that the mode of delivery and feeding habits have combined effects on the infant's gut microbiome (Song *et al.* 2013). It was found that *Bifidobacteria*, which is the dominant bacteria in VD delivered infants, has several beneficial effects for the infant's health, the growth of which in turn is affected by several stimulating factors present in human milk (Sela *et al.* 2008; Thurl *et al.* 2010). It is generally observed that caesarean section born infants have delayed colonization of *Bifidobacteria* and *Bacteroides* with over-representation of *Clostridium* and *Staphylococcus* and the Enterobacteriaceae family. To overcome this dysbiotic state and mimic the flora of vaginal birth infants in caesarean section infants, a new technique called vaginal swab seeding was introduced. The technique involves modulating the microbiota of caesarean section infants by swabbing the infants with mother's vaginal microbiome during birth. The first paper published by Dominguez-Bello and colleagues on vaginal seeding demonstrated that such swabs could restore the microbial composition of caesarean born infants, though partially, in a manner which was similar to the vaginal infant's microbiome (Dominguez-bello *et al.* 2016). This approach may not help to restore the exact microbial composition but will help in increasing microbial diversity and reduce the risk of immune-related disorders, but more studies on larger cohorts and longitudinal follow-up for longer durations are required to confirm the benefits.

3.1.1 *The preterm and full-term birth:* The pattern of gut microbial colonization in premature infants in an intensive care setting varies when compared with that of healthy, term and breastfed infants (Penders *et al.* 2006; Arboleya *et al.*

2012; Hill *et al.* 2017; Itani *et al.* 2017). Preterm infants (usually having very low birthweight) are at a downside in case of harbouring a well-developed set of gut microbiota. These observations can be attributed to several factors such as mode of delivery (mostly caesarean section). Thus, they never come in contact with the vaginal microbiome of the mother; another reason is the very low birthweight of the infants, which calls for special care to be taken, as a result of which they are usually not breastfed and are fed by other sterile means parenterally, with delayed complete nutritional feeding.

Furthermore, preterm infants are at a higher risk of infections due to living in an intensive care unit with a high bacterial load and frequent exposure to antibiotics, along with a delay in exposure to mother's skin and breast milk microbiome. These factors are together responsible for the reduced gut microbial diversity in preterm infants with increased colonization by pathogenic microorganisms.

In preterm infants with a gestational age of <33 weeks, the intestinal microbiota had reduced bacterial diversity (Gewolb *et al.* 1999; Rougé *et al.* 2010; Moles *et al.* 2013). Arboleya *et al.* (Arboleya *et al.* 2012) studied the gut microbial colonization in preterm and full-term infants and reported that preterm infants showed high levels of facultative anaerobes such as *Enterococcus*, *Enterobacter* and *Lactobacillus*, and lower levels of strict anaerobes like *Bifidobacterium, Bacteroides* and *Atopobium* when compared with term infants (Schwiertz *et al.* 2003; Magne *et al.* 2006; Arboleya *et al.* 2012). These results were similar to those reported by Magne *et al.* 2006 and Schwiertz *et al.* 2003 also reported higher levels of *Enterococcus*, *Staphylococcus* genera and the Enterobacteriaceae family. Also, it is observed that *Proteobacteria* and *Firmicutes* are among the phyla that dominate in PT infants when compared to FT infants (Embleton *et al.* 2017; Hill *et al.* 2017). Preterm infants thus show a perturbed early microbial composition compared to full-term infants, which increases their chances of developing immune system disorders, making them more prone to infections due to inadequate immune maturity linked to the dysbiotic microbiota.

3.2 *Feeding*

Human milk, mostly the first dietary exposure to the neonate, is the best link between the mother and the infant. It has a complex and dynamic composition which is very different when compared to the formula-based products in all aspects including nutritional value and its composition, such as the presence of certain growth factors and enzymes (Guaraldi and Salvatori 2012; Scholtens *et al.* 2012). These bioactive compounds (like human milk oligosaccharides), which are present in human milk, are beneficial to infants as they not only help better development but also strengthen the immune system of the newborn (Xiao *et al.* 2017), provide protection against allergies (Oddy 2017) and may also offer protection from coeliac disease (Akobeng 2005; de Palma *et al.* 2012), obesity (Miralles *et al.* 2006), type-2-diabetes (Pettitt and Knowler 1998; Pereira *et al.* 2014), diarrhoea (Strand *et al.* 2012) and many other metabolic disorders (Horta *et al.* 2007; Hoddinott *et al.* 2008; Walker 2010; Zivkovic *et al.* 2011). The WHO recommends that an infant must be breastfed for at least the first six months of life, following which introduction of solid foods should be done. Though a single component of breast milk does not influence the infant gut microbiota, there is evidence that human milk oligosaccharides (HMOs) play a significant role by stimulating the growth of *Bifidobacteria* and *Bacteroides* (Boehm and Moro 2008; Marcobal and Sonnenburg 2012). Human milk oligosaccharides modulate the health of infants by their actions such as prebiotic effect, modulating innate immune responses and intestinal cell responses and anti-inflammatory effects (Boehm and Moro 2008; Kuntz *et al.* 2008; Thurl *et al.* 2010). It is also demonstrated that human milk contains certain proteins and stimulation factors which enhance the growth of beneficial bacteria in the infant's gut which further help in the breakdown of complex oligosaccharides present in human milk (Thurl *et al.* 2010; Marcobal and Sonnenburg 2012).

To settle the question of whether the feeding habits have an impact on the gut microbial diversity of infants, many researchers have tried to study the faecal microbiota of infants fed with different feeding habits. Based on their study, Tannock demonstrated that there are differences in bacterial groups present in human milk and formula food (Tannock 1994). Cong *et al.* examined the relationship of feeding types and the infant gut microbiome; Breast milk feeding was found to be associated with a higher diversity of the infant's gut microbiome compared to non-breast milk feeding (Cong *et al.* 2016). Further, it has also been reported that in breastfed infants, *Bifidobacterium* species, specifically *B. breve*, *B. longum*, *B. dentium*, *B. infantis* and *B. pseudocatenulatum*, are the most prevalent *Actinobacteria* (Harmsen *et al.* 2000; Jost *et al.* 2012; Song *et al.* 2013; Bäckhed *et al.* 2015; Stewart *et al.* 2018). Also, *Firmicutes* phylum is constituted primarily of Lactic acid bacteria such as *Lactobacillus*, *Enterococcus* as well as *Clostridium* species (Harmsen *et al.* 2000; Bergström *et al.* 2014). Breastfed and vaginally delivered term infants show lower levels of C. difficile and E. coli and higher levels of Bifidobacterium spp., which are beneficial for infant health (Penders *et al.* 2006).

Based on the influence of feeding habits on the gut microbial composition of infants, a few studies are found to be contradictory and suggest that there are no significant differences in the bacterial composition of breast and formula-fed infants (Penders *et al.* 2006; Adlerberth and Wold 2009). However, most of the studies support the finding that *Clostridium* and the *Streptococcus* species, *Bacillus subtilis*, *Bacteroides*, *Escherichia coli* (Benno 1984; Fanaro *et al.* 2003; Penders *et al.* 2006; Adlerberth and Wold 2009; Fallani *et al.* 2010; Bergström *et al.* 2014; Timmerman *et al.*

2017) and *Enterococcus* (Fanaro *et al.* 2003; Adlerberth and Wold 2009), in the formula-fed infants were significantly higher than those in the breastfed infants. Further studies have shown that maternal health during lactation affects the milk microbiome. Since milk is a direct and major source of microbiome for the offspring, any changes in the milk microbiome can directly modulate the infant's gut microbiome which can have detrimental effects on the offspring's health. Milk from obese mothers has been found to contain a different and less diverse bacterial community compared with milk microbiota from normal-weight mothers (Cabrera-rubio 2012). It was observed that *Bifidobacterium* levels were reduced and those of *Staphylococcus* were increased in the milk samples of obese mothers as compared to the normal-weight mothers (Collado *et al.* 2012). Huurre and co-workers demonstrated lower levels of *Bifidobacterium* spp. in infants at an early age who were shown to mount a stronger humoral immune response, suggesting the vulnerability of the gut barrier (Huurre *et al.* 2008). Another recent study has demonstrated that feeding habits may interact with other factors such as child race/ethnicity to affect the infant gut microbial composition (Savage *et al.* 2018). Yet another study suggests that there is a complementarity relationship between breast milk composition and the associated microbiome, wherein bacteria producing specific amino acids present in reduced amounts in breast milk complement the composition, thus maintaining infant protein balance (Baumann-Dudenhoeffer *et al.* 2018).

3.3 *Use of antibiotics in neonates*

The use of antibiotics causes changes in the gut microbial composition by inhibiting the growth or killing of both beneficial and pathogenic species, thereby allowing the overgrowth of strains resistant to antibiotics, making the individual more susceptible to infections. These changes in the composition of gut microbiota caused by antibiotics can last for weeks to several months. The use of broad-spectrum prophylactic antibiotics in preterm or low birthweight infants is a very common practice. Because of the high susceptibility to infections in newborns with low birthweight/preterm and the difficulty in diagnosing infections in preterm infants, antibiotics are the most commonly prescribed class of medications in the neonatal intensive care unit (NICU) (Clark 2006; Patel *et al.* 2009). Consequently, this intervention reduces the diversity of gut flora (Fricke 2014) and delays the colonization of commensal flora and thus affects the host metabolic activity (Zhu *et al.* 2017). Of interest is the fact that studies have shown that the use of antibiotics and its prolonged exposure results in alterations in gut microbial ecology (Neu 2015; Cong *et al.* 2016), increased risk of inflammatory bowel disease in childhood (Hviid *et al.* 2011; Mårild *et al.* 2013) and can lead to antibiotic-associated diarrhoeas (AAD) due to nosocomial pathogens (Song *et al.* 2008). These negative outcomes are often associated with pathogens such as Klebsiella pneumoniae and Clostridium difficile (Young and Schmidt 2004; Song *et al.* 2008), which can lead to the development of Clostridium difficile associated diarrhoea (Beaugerie *et al.* 2003). Studies have also linked increased antibiotic exposure and decreased microbial diversity to increased risk for necrotizing enterocolitis (NEC) in premature infants (Alexander *et al.* 2011; Brower-Sinning *et al.* 2014; Hourigan *et al.* 2016). Gibson *et al.* observed the antibiotic resistome of premature infants in response to varying antibiotic exposures. They reported that the preterm gut microbiome has relatively reduced species richness; resistome analysis showed that meropenem, ticarcillin-clavulanate and cefotaxime treatments led to decreased species richness, while gentamicin and vancomycin had variable effects on species richness, highlighting the varying effects of antibiotic classes (Gibson *et al.* 2016). It was reported in another study that preterm infants who were exposed to antibiotic treatment for more than 5 days are associated with low bacterial diversity and an increased risk of sepsis (primarily caused by group B Streptococcus), necrotizing enterocolitis and death (Kuppala *et al.* 2011; Greenwood *et al.* 2014). Tanaka *et al.* 2009 and Fouhy *et al.* 2012 studied the microbiota of neonates treated with antibiotics in the early days of life and reported similar results. They observed that antibiotic exposure reduced the diversity of the infant's gut microbiota as well as altered its composition, with a decrease in levels of Bifidobacterium and rise in *Proteobacteria* levels. They observed that though the levels of gut flora bounced back by the study's end, the species diversity did not (Fouhy *et al.* 2012). Also, it has been observed that antibiotic resistance genes are present in infants at an age as early as two months, even though they were not subjected to antibiotic treatment (Zhang *et al.* 2011). Some possible reason for the same can be the vertical transmission of antibiotic-resistant organisms from the mother's milk, GIT or even from hospital environments where a high level of antimicrobial resistant organisms are observed (Leta *et al.* 2016), thus making the infant's gut a potential source of AMR genes. It is observed that antibiotic uptake during early life increases the risk of overweight and obesity in children in later life (Ajslev *et al.* 2011; Trasande *et al.* 2013; Bailey *et al.* 2018). Similar results about higher chances of adiposity due to early antibiotic exposure were reported by studies performed in mice models (Cho *et al.* 2012). Apart from obesity, the early use of antibiotics is also associated with increased risk of allergy development (Mckeever *et al.* 2002; Johnson *et al.* 2005). These studies suggest that the merits of administering broad-spectrum antibiotics in infants should be reassessed and other narrow-spectrum antibiotics must be sought for use for the shortest period possible.

4. Phase 3: Childhood

4.1 *Diet*

The beginning of weaning for infants marks the steady and slow approach of their gut microbial composition to that of

adults with some major shifts in taxonomic groups, and an increase in gut microbial diversity, thus pointing to the role diet plays in modulating the microbial community (Stark and Lee 1982; Koenig *et al.* 2011; Bäckhed *et al.* 2015; Stewart *et al.* 2018). Turnbaugh *et al.* performed experiments in germ-free mice by colonizing them with human microbial communities and observed that the bacterial community that colonizes initially is unstable and can be altered by diet even within a single day (Turnbaugh *et al.* 2009b). The introduction of solid food to the breastfed infant causes an increase in *Enterobacteria* and *Enterococci*, along with colonization by Bacteroides spp., Clostridium and Streptococcus. In contrast, formula-fed infants do not display such a sharp transition of gastrointestinal flora on the introduction of outside solid food (Stark and Lee 1982). This is because, since birth, formula-fed infants are exposed to a diet which includes certain complex compounds, thus establishing the microbial community to support the food habits.

The type of diet further affects the community structure of the gut microbiota. Carlotta De Filippo and colleagues analysed the faecal microbiota of European children (EU) and children from a rural African village consuming a western diet and a diet rich in fibre, respectively. They observed that children with the high fibre diet displayed a considerable increase in the *Bacteroidetes* level with a decrease in the level of *Firmicutes*. These results were accompanied by higher short-chain fatty acid levels in the rural diet of children than in the EU children with an abundance of a distinctive set of bacteria from the genus Prevotella and Xylanibacter, known to be responsible for fermenting cellulose and xylan to liberate energy (De Filippo *et al.* 2010).

Further, microbes in the distal gut contribute to host health through the biosynthesis of vitamins and essential amino acids, as well as the generation of important metabolic by-products from dietary components left undigested by the small intestine and producing SCFAs (Whitt and Demoss 1975; Walker *et al.* 1998; Metges *et al.* 1999; Magnúsdóttir *et al.* 2015). The type of SCFA and microbial species dominance depends majorly on the type of substrate available. For example, high levels of the *Bacteroidetes* phylum are seen in subjects consuming a high fibre and polysaccharide diet, while high levels of *Firmicutes* are seen in high-fat diet consumers (De Filippo *et al.* 2010; David *et al.* 2014; De Filippis *et al.* 2016). Changes in the gut microbial composition are also seen when the diet is switched from meat-based to a vegetarian diet (David *et al.* 2014; Gomez *et al.* 2016). It was reported that children consuming a more rural and less westernized diet had a gut microbial community structure that differed from those consuming a westernized diet. In a study that compared the gut microbiota of Bangladeshi and US children, it was observed that the Bangladeshi children who consumed a carbohydrate and rice-rich diet with bread and lentils and rarely any meat reported a dominance of *Prevotella* in these children when compared to the US children whose diet included animal meat and proteins, showing a dominance of Bacteroides (Lin *et al.* 2013). Another study reported that in children from Leyte, the occurrence of a particular type of microbial community was linked to a diet-dependent nutrient bias. In particular, the high-fat westernized diet of children from Ormoc was found to have association with a lower abundance of Prevotellaceae (P-type) microbiota, while the children from Baybay who consumed a carbohydrate-rich diet showed lower Bacteroidaceae and higher P-type microbiota, suggesting that the altered microbial composition could be a reason for obesity in children from Ormoc (Nakayama *et al.* 2017).

Bergstrom *et al.* assessed the faecal microbiota composition of 330 subjects and observed that changes in the gut microbiota occurred post weaning, from age 9 to 18 months; these changes include decrease in levels of *Lactobacilli*, *Bifidobacteria* and Enterobacteriaceae which dominate breast milk microbiota with an increase in *Clostridium* and *Bacteroides* spp. (Bergström *et al.* 2014).

The importance of the gut microbiome in the carbohydrate metabolism is seen in a case of dysbiosis caused by disruption of the mucous membrane, such as in the case of IBD or Crohn's disease, which results in a shift of the gut microbiome from obligatory aerobes to facultative aerobes and a reduction in the metabolism of carbohydrates to produce SCFAs, thus leading to intestinal inflammation (Morgan *et al.* 2012). Overall, shifts in the diet can modulate the bacterial composition which leads to the prevalence of microbes possessing genes responsible for the metabolism of various compounds (Gritz and Bhandari 2015).

4.2 *Exposure to antibiotics during childhood*

Antibiotic therapies worsen the situation by negatively influencing the already unstable gut microbiota in the subjects under medication. In addition, these alterations in microbial compositions can remain for long periods of time, spanning months and even years with partial or complete recovery (De La Cochetière *et al.* 2005; Huse *et al.* 2008; Dethlefsen and Relman 2011; Panda *et al.* 2014; Rashid *et al.* 2015). Use of broad-spectrum antibiotics allows growth of the pathogen *Clostridium difficile* which results in infections and severe diarrhoea (Stevens *et al.* 2011; Brown *et al.* 2013). Moran Yassour and colleagues investigated the effects of multiple antibiotic treatments in the first three years of life and found results very similar to those seen in adult studies. They measured the diversity and richness of the microbiota at the species and strains level and reported that antibiotic-treated children had less diverse and more unstable gut microbial composition compared to untreated children (Yassour *et al.* 2016). Another study on a cohort of 142 Finnish children aged 2–7 years reported that the use of macrolide was associated with enduring changes in microbial composition and reduced diversity. Certain variations, such as the abundance of *Bifidobacterium* and *Bacteroides*

and antibiotic resistance, were restored within a year after the antibiotic course. However, the abundances of certain other genera and diversity did not recover for about a couple of years after the course. Also, it was noted that penicillin use did not have as severe an influence on the microbiota, suggesting the varying influence of distinct antibiotics (Korpela *et al.* 2016).

Antibiotic resistance is a serious issue worldwide due to its effect on the resistome profile of the microbiome (Jernberg *et al.* 2007; Bengtsson-Palme *et al.* 2015), which is growing due to the ever-increasing use of antibiotics, as well as the slow-moving drug development due to economic and regulatory challenges (Piddock 2012). Multidrug-resistant (MDR) bacterial infections are rising rapidly in US children and causing longer hospital stays, according to a new study in the Journal of the Pediatric Infectious Diseases Society. In another study involving a culture-based method, the authors demonstrated that even healthy untreated children from various cities across three continents acted as reservoirs of multidrug-resistant genes, implying that healthy isolates from certain regions can also act as a source of MDR genes (Lester *et al.* 1990). A study in Bangladesh reported the increasing cases of MDR in children. Using a culture-based technique in 15 children, the authors observed that the gut of toddlers in Bangladesh acts as a reservoir for MDR bacteria of the family Enterobacteriaceae, with many bacteria containing plasmids with antibiotic resistance genes (Monira *et al.* 2017). Thus, it is now important to illustrate the ill effects of antibiotics abuse to prevent self-prescription and to spread awareness and implement the use of antibiotic stewardship programmes which will help curb the spread of antibiotic resistance in the population and reduce the spread of infections.

4.3 *Hygiene and microflora hypothesis*

In 1976, Gerrard *et al.* studied a relationship between the occurrence of allergy, atopic disease and stated, 'atopic disease is the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria, and helminths' (Gerrard *et al.* 1976). Later, on similar lines, Strachan proposed the hygiene hypothesis suggesting that a lack of exposure to germs and infections, with an extremely clean environment during early childhood, may not be able to challenge the immune system enough to gain memory and be prepared for future infections, acting as a risk for developing childhood diseases. Strachan proposed that this inadequate development of the immune system in children may pose a threat to children's health by elevating the risk of allergies and other immune hypersensitivities in life. This exposure to a wide variety of germs in early life was linked to unhygienic contact with siblings or play areas (Strachan 1989). This theory was aided by the results of a study in a large cohort of children where the occurrence of hay fever was found to be inversely proportional to family size (Strachan *et al.* 1996). Since then, the theory has been supported by results from many studies stating that early-life exposure to germs through contact with pets or older siblings, as well as a larger family size, enhances the rate of maturation of the microbiome (Stewart *et al.* 2018) and acts as a shield against the risk of developing allergic disease and asthma (Ball *et al.* 2000). In contrast, certain studies suggest that exposure to cats, in particular, may lower the risk of allergic disease or asthma development but exposure to dogs may increase the risk (Takkouche *et al.* 2008), while some suggest the opposite (Hugg *et al.* 2008).

Gary Huffnagle proposes that the real reason for this relation between certain immune diseases and environmental pressure is that the western lifestyle limits microbial exposure to a great extent and thus modifies our gastrointestinal microflora, leading to establishment of tolerance, allergies and other inflammatory diseases, an idea called the 'microflora hypothesis' (Huffnagle and Noverr 2005). Bisgaard *et al.* studied children (n = 411) at high risk of developing asthma and reported that reduced microbial richness was inversely related with the possibility of allergic sensitization, peripheral blood eosinophils and allergic rhinitis in the first few years of life (Bisgaard *et al.* 2011).

In another study, microbial diversity and composition were studied in children at the age of 2 from two different regions. It was observed that infants with allergic diseases had decreased levels of *Lactobacilli* and *Bacteroides* and higher levels of *Staphylococcus aureus* with higher proportions of aerobic microbes as compared to non-allergic children (Björkstén *et al.* 1999). Overall, it can be hypothesized that sequential changes in lifestyle can lead to changes in the gut microbial composition, which in turn can affect the maturation of the infant immune system and increase the risk of allergic disease development.

4.4 *Host genetics and gut microbiome during childhood*

Host genetic interactions act as a part of a complex network of factors affecting the microbial composition. The answer to how host genetics and environmental exposure modify the gut microbiota has been obtained through targeted and candidate gene approach studies.

Evidence for the influence of genetic make-up on microbial composition is provided by many studies reporting the link between the absence of FUT2 gene and alteration in gut microbial composition. The FUT2 gene encodes an enzyme α-1,2-fucosyltransferase responsible for the expression of ABO histo-blood-group antigens on mucosal surfaces (Kudo *et al.* 1996; Koda *et al.* 2000). Due to their enzyme secreting status, individuals who carry one or both the alleles are known as 'secretors,' whereas those possessing nonsense mutations without the enzyme are known as 'non-secretors'. Studies have reported that the Non-secretors are at a

disadvantage and are more prone to inflammatory disorders such as coeliac and Crohn's disease (McGovern *et al.* 2010; Forni *et al.* 2014), which may be possibly due to modifications in the gut microbiota (Rausch *et al.* 2011; Tong *et al.* 2014). In humans, twin pairs make it easier to study genotype changes and its health influences; thus, most of the studies providing such valuable information are based on twin pair studies.

Stewart and colleagues used temporal temperature gradient gel electrophoresis (TTGE) to understand the link between host genetics and microbial composition in children. While studying the Eubacterial population, the authors observed that the degree of similarity in the bacterial community was higher in identical compared with non-identical twins and was lowest in the unrelated control group (Stewart *et al.* 2005). Authors in another study further observed the role of several functional genomic variations linked to IBD in the genes NOD2, CARD9, ATG16L1, IRGM and FUT2. Analysis revealed that in healthy controls a higher genetic risk of IBD was associated with a decrease in the genus *Roseburia* of the phylum *Firmicutes* (Imhann *et al.* 2016).

In a recent study, Kumbhare *et al.* showed that the gut bacterial community structure in 13–14-year-old Indian and Finnish children differs significantly. Specifically, the Finnish children possessed higher Blautia and Bifidobacterium, while genera Prevotella and Megasphaera were predominant in the Indian children. The study also demonstrated a strong influence of FUT2 and birth mode variants on specific gut bacterial taxa, the influence of which was noticed to differ between the two populations under study (Kumbhare *et al.* 2017).

Apart from the studies performed in children, many studies have been performed in adults (Zoetendal *et al.* 2001; Turnbaugh *et al.* 2009a; Goodrich *et al.* 2014) involving gene targeted approaches which provide clear evidence that the host genetic make-up influences the gut microbial composition. Elaborate studies in human children are thus needed to provide a detailed understanding of the relationship between gut microbiota and host genotype from the initial stages; thus, it can act as a guide for microbiota targeted approaches.

5. Conclusion

A significant revolution in the sequencing technologies in the past decade has enabled researchers to decipher the 'host-microbe' interplay in a more robust and cost-effective way than earlier. This has enriched our understanding of the role of microbiota in human health and disease development, proving microbiota to be a major influencer of host health. The research studies discussed in this review have furthered our understanding of how the factors influence or are associated with immune and physiological development, the mechanisms of which are now known to be mediated through the microbiome. Thus, studies in this direction are needed to delineate the core microbial composition during early age and understand the role of the microbiome in host health development.

Understanding the changes in the gut microbial community right from the early stages may advance our knowledge of dysbiosis associated with the development of allergies, gastrointestinal diseases and metabolic disorders such as obesity and diabetes during childhood. These efforts will lead to the development of disease-specific biomarkers that can be used potentially for diagnostics and eventually to design treatment strategies.

Additionally, it is now evident from many studies that exploring the diversity of host-associated microbes will provide only a partial picture of the actual host-microbiome interactions; thus, use of multi-omics approaches will help us determine the functionality of these gut residents and provide deeper insights into the complex interactions. This integrated approach will eventually help in designing holistic treatment regimens, especially in the early stage of life, to improve both maternal and child health.

Considering the recent advancements in microbiome studies, the therapeutics for modulating the microbiome for health has now moved from being 'community medicine' to 'personalized medicine', thus posing a serious need to carry out comprehensive studies to understand the individual responses to the interventions carried out worldwide. These efforts will help in creating a personalized intervention, especially in mothers and infants, to modulate the microbiome and thus improve health.

It is to be noted that this review makes an attempt to summarize the current understanding based on the studies published till the time of constituting this article, and the authors sincerely apologize if not all the recent studies have been cited in this article.

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WILEY

REVIEW

Ethnopharmacology, phytochemistry, and biotechnological advances of family Apocynaceae: A review

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The family Apocynaceae is one of the largest and important families in angiosperm. Several members of this family have medicinal properties and have been in the treatment of various ailments. Most of them are consumed as food by tribal people whereas a few plants are used as source of poison. Members of family Apocynaceae are rich in alkaloids, terpenoids, steroids, flavonoids, glycosides, simple phenols, lactones, and hydrocarbons. Other compounds such as sterols, lignans, sugars, lignans, and lactones have been isolated and systematically studied. Few studies have reported antioxidant, anti-inflammatory, antimicrobial, and cytotoxic activities of crude extracts as well as single compound(s) isolated from various members of the family Apocynaceae. *Holarrhena antidysenterica*, *Rauvolfia serpentina*, *Carissa carandas*, and *Tabernaemontana divaricata* are the extensively studied plants in this family. The present review provides a detailed outlook on ethnopharmacology, phytochemistry, and biological activities of selected members of this family. Moreover, it also covers the biotechnological advances used for large-scale production of bioactive compounds of therapeutic interest along with plant tissue culture-based approaches for conservation of this medicinally valuable family.

KEYWORDS

Apocynaceae, alkaloids, terpenoids, steroids, flavonoids, anti-inflammatory, cytotoxic, conservation

1 | INTRODUCTION

Apocynaceae is one of the largest families in the plant kingdom. Robert Brown (1810) separated Asclepediaceae from Apocynaceae for a practical reason. Due to the taxonomic conflict between the families Asclepediaceae and Apocynaceae, there is variation in exactly how many species or genera belong to Apocynaceae family. Endress, Liede-Schumann, and Meve (2014) reported a revised and recently updated classification for the Apocynaceae family. According to this updated classification, family Apocynaceae consists of 424 genera with more than 4,600 species distributed in five subfamilies such as Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae, and Asclepiadoideae.

The plants of family Apocynaceae are native throughout India, Pakistan, China, Bangladesh, and Sri Lanka (Mahmood, Meer, Munir, Nazar, & Naveed, 2011). Apocynaceae members are the shrubs, woody, or herbaceous plants containing milky latex. Most of the plants of this family are rich in alkaloids and have immense medicinal importance. Several species are also widely grown ornamentally. Some members of Apocynaceae are consumed by people in the rural area as a food and some others are used as a poison (Aiyambo, 2010). The plants of Apocynaceae have been used worldwide for medicinal applications, and hence, they are included in different traditional systems of medicine such as Indian, Chinese, and Thai (Bhat, Hegde, & Hegde, 2012). In the present review, we have made an attempt to provide information about habitat, medicinal uses, phytochemistry, and pharmacology of some widely studied species of family Apocynaceae. The recent biotechnological advances to produce bioactive compounds of plant origin are also highlighted. Thus, there are significant possibilities of finding the novel bioactive compounds with medicinal and pharmacological properties from various members of the family, and it will yield to new sources of lead compounds for future applications.

2 | HABITAT

Apocynaceae plants show naturalized distribution, and birds and human activities have led to their dispersal. The members of this family are distributed in the tropical, subtropical, and temperate zones. The plants of this family are mainly trees and shrubs; however, subshrubs and, rarely, herbs are also found. *Alstonia scholaris* (R. Br.) is distributed throughout the tropics and rainforests of West

and Central Africa (Oze, Nwanjo, & Onyeze, 2007). *Carissa carandas* (Linn.) is native and common throughout India, Sri Lanka, Java, Malaysia, Myanmar, and Pakistan (Motwani et al., 2012). *Catharanthus roseus* (Linn.) was originally native from Madagascar island and widely cultivated for hundreds of years and can now be found growing wild in most warm regions of the world (Aslam et al., 2010). *Holarrhena antidysenterica* (Wall) is found throughout India up to the altitude of 4,000 ft. It is especially abundant in the sub-Himalayan tract (Shah et al., 2010). *Nerium oleander* is an ornamental tree with 10–12 ft in height planted in gardens for its fragment and beautiful flowers. This plant is native from North Africa and Southeast Asia and grows in subtropical regions (Dolgova & Ignateva, 1963). *Rauvolfia serpentina* (Benth. ex Kurtz) is widely distributed in Asian countries, Burma, and Thailand. In India, it abundantly grows in Punjab, Sikkim, Bhutan, and in sub-Himalayan tract up to altitude of about 4,500 ft (Vakil, 1955). *Tabernaemontana divaricata* (Linn.) is found in the Konkan, Western Ghats in Malabar, through North India up to an elevation of 3,000 ft (Muzaffar & Daulatabadi, 1986). *Thevetia peruviana* (SCHUM), commonly known as yellow oleander, is distributed in tropical and subtropical regions (Langford & Boor, 1996). *Wrightia tinctoria* (R. Br.) is distributed globally and is also abundantly found in India where it is commonly known as Indrajav or jaundice curative tree particularly in South India (Ramchandra, Basheermiya, Krupadanam, & Srimannarayana, 1993).

3 | ETHNOMEDICAL USES OF SOME MEMBERS OF FAMILY APOCYNACEAE

Plants from Apocynaceae family are known globally to possess broad-spectrum ethnomedicinal uses. Table 1 summarizes the ethnomedicinal importance of various plants of family Apocynaceae. Some important members of this family, namely, *Catharanthus roseus*, *Alstonia scholaris*, *Rauvolfia serpentina*, *Holarrhena antidysenterica*, *Nerium indicum*, and *Thevetia peruviana* have been documented in books of Ayurveda, Siddha, Unani, and Homeopathy. The plants and their parts described in this section are used in indigenous systems of medicines for the treatment of various diseases and disorders.

Carissa carandas produces berry-sized fruits that are traditionally used as stomachic, antidiarrheal, and antihelminthic; stem is used to strengthen tendons, fruits are used in skin infections, and leaves as a remedy for fever (Kirtikar & Basu, 1998). *Tabernaemontana divaricata* has been used for the treatment of fever, pain, and dysentery (Boonyaratanakornkit & Supawita, 2005). It is also known to treat various disorders such as asthma, diarrhea, epilepsy, eye infection, fever, fractures, headache, inflammation, leprosy, rheumatic pain, ulceration, and vomiting. It has been used as antihelminthic; antihypertensive; aphrodisiac; diuretic; a remedy against poison; and tonic for brain, liver, and spleen (Warrier & Nambiar, 1993). *Alstonia boonei* stem bark is utilized for treating febrile illness, painful urination, rheumatic conditions and jaundice, malaria, fever, and intestinal helminthes (Bello et al., 2009; Majekodunmi & Odeku, 2009; Olajide et al., 2000). *Catharanthus roseus* (Linn.) is used for the treatment of diabetes, fever, malaria, throat infections, and chest complaints. It is also used for the regulation of menstrual cycles (Santhi, Muthulakshmi, Amutha, & Karpakaselvi, 2016). The parts of *Holarrhena antidysenterica* have been used in the treatment of dysentery and diarrhea. In the Ayurvedic and Unani system of medicine, it is used as antihelmintic and antidiarrheal and is also used to treat skin diseases (Shah et al., 2010). *Nerium oleander* has been traditionally attributed with several medicinal properties such as kustha or leprosy, valipalita or aging (Charak), upadamsha or syphilis (Sushruta), indralupta or alopecia (Vagbahtt), and netra-kopa or conjunctivitis (Chakara dutta; Banerjee, Vasu, Pancholi, Rajani, & Nivsarkar, 2011).

4 | PHYTOCHEMISTRY

Extensive work has been carried out on phytochemical aspects of many members of family Apocynaceae. A number of spectroscopic and chromatographic methods are used for structural elucidation of the metabolites. A detailed account of phytoconstituents isolated from various parts of Apocynaceae plants is summarized in Table 2.

4.1 | Alkaloids

Apocynaceae is well known as alkaloid-rich family, and several species have earlier been evaluated for the presence of different types of alkaloids. The types of alkaloids isolated and reported from different members are indole, iboga, and vinca alkaloids (Figure 1). Arambewela and Ranatunge (1991) evaluated 11 indole alkaloids including voacangine, voacristine, vobasine, and tabernaemontanine **(1–4)** from different extracts of *Tabernaemontana divaricata* leaves, flowers, and stem bark. The structural identification was elucidated by infrared (IR) and nuclear magnetic resonance (NMR) spectral data. Further, crude extracts comprising these alkaloids were shown to have antibacterial potential. Kam, Loh, and Wei (1993) isolated conophylline **(5)** and conophyllidine **(6)** as two new dimeric indole alkaloids with a yield of 3.5 mg/kg from it. Recently, Kam, Pang, Choo, and Komiyama (2004) evaluated a total of 42 different types of bioactive alkaloids from *Tabernaemontana divaricata* with new ibogan (Conolabines A and B; **15**) and vallesamine alkaloid **(16)** derivatives. Low et al. (2010) reported Lirofoline B **(7)** as a new pentacyclic indole alkaloid in its stem bark with a yield of 3.2 mg/kg. Another important member of this family, *Catharanthus roseus*, is reported to possess more than 130 alkaloids in different parts. Five monoterpene indole alkaloids (vinblastine, serpentine, vincristine ajmalicine, and 3′,4′-anhydrovinblastine; **8–12**) of *Catharanthus roseus* are reported to have therapeutic properties (Oudin, Courtois, Rideau, & Clastre, 2007). The activities shown by these compounds are anticancer, antineoplastic, and anti-hypertensive (Van der Heijden, Jacobs, Snoeijer, Hallard, & Verpoorte, 2004). Further, Svoboda, Neuss, and Gorman (1959) reported vinca alkaloids leurosine and vincaleukoblastine **(17, 18)** with anticancer activity against P-1534 leukaemia in mice model. A monoterpenoid indole alkaloid picrinine **(13)**, as well as E and Z-alstoscholarine (**14**), were reported in *Alstonia scholaris* (Xiang-Hai, Ya-Ping, Tao, & Xiao-Dong, 2008).

These alkaloids have 19 or 18 carbon atoms in their structure and are previously reported for anticancer and antifertility potential

TABLE 1 Worldwide ethnomedicinal uses of selected members of Apocynaceae

Ailment	Plant species	Country/province	Part/type of remedy used	References
Stomachaches	*Wrightia tinctoria*	Tamil Nadu	Seed juice taken orally	Muthu, Ayyanar, Raja, & Ignacimuthu, 2006
	Carrisa edulis	Kenya	Root and stem bark decoction is taken orally	Gakuya, Itonga, Mbaria, Muthee, & Musau, 2013
Diarrhea	*Holarrhena antidysenterica*	India	Fruits	Kaul & Atal, 1983
	Alstonia scholaris	Indonesia	Bark preparation used for treatment	Wiart, 2006
Fever	*Wrightia tinctoria*	India	Fruits	Muthu et al., 2006
	Thevetia neriifolia	West Nigeria	Leaves preparation is used to control fever	Ayoola et al., 2008
	Voacanga Africana			
	Tabernaemontana divaricata	Bangladesh	Leaf infusion taken orally	Rahmatullah et al., 2009
Skin infections	*Wrightia tinctoria*	India	Oil from this plant used as antidandruff agent.	Muthu et al., 2006
	Rauwolfia tetraphylla	Tamilnadu	Paste of whole plant powder and castor oil is applied on skin	
	Geissospermum sericeum	Brazil	Preparation from stem bark.	Steele, Veitch, Kite, Simmonds, & Warhurst, 2002
	Nerium indicum	Bangladesh	Crushed leaves are applied on affected area	Rahmatullah et al., 2009
		Rajasthan, India	Paste of leaves and root oil applied on infected area of ringworm.	Upadhyay, Roy, & Kumar, 2007
Dysentery	*Holarrhena antidysenterica*	Bangladesh	Powdered leaves extracted in boiled water to obtain juice which is taken orally.	Rahmatullah et al., 2009
Toothache	*Voacanga Africana*	West Nigeria	stem	Ayoola et al., 2008
	Alstonia scholaris	Burma	Latex used for relief from toothache	Wiart, 2006
Wound healing	*Acokanthera schimperi*	Zegie Peninsula	Preparation of crushed fresh leaves applied on wound.	Teklehaymanot & Giday, 2007
	Holarrhena antidysenterica	Gujrat, India	Latex of plant applied on wound	Kaul & Atal, 1983
Malaria	*Carrisa edulis*	Kenya	Root and stem bark preparation	Gakuya et al., 2013
Rheumatism	*Holarrhena antidysenterica*	Gujrat, India	Stem bark plaster used	Kaul & Atal, 1983
	Carissa carandas	India	Root and root bark are effective in rheumatism.	Upadhyay et al., 2007
Asthma	*Alstonia scholaris*	India	Flowers used in respiratory trouble.	Guha Bakshi, Sensarma, & Pal, 1999
Birth control	*Nerium indicum*	Rajasthan, India	Roots preparation is abortifacient upon local application as well as internal administration	Upadhyay et al., 2007
Women complaints	*Holarrhena pubescens*	Bangladesh	Bark of this plant used to control vaginal discharge.	Hossan et al., 2010
	Alstonia scholaris	Eastern Himalaya, India	Leaves and stem bark preparation used in menstrual disorders.	Kala, 2005
Lactation	*Alstonia scholaris*	Vietnam	Decoction of leaves given orally.	Wiart, 2006
		Assam, India	Fresh bark decoction mixed with milk and consumed orally to increase lactation and decrease viscosity of breast milk	Sharma & Sharma, 2010
Pediatric care	*Holarrhena antidysenterica*	Gujrat, India	Seed preparation given orally to children's	Kaul & Atal, 1983
Snake bite	*Carissa carandas*	India	Leaf extract is given orally.	Desai, 1975

(Continues)

TABLE 1 (Continued)

Ailment	Plant species	Country/province	Part/type of remedy used	References
	Rauwolfia serpentina	Karnataka, India	Squeezed roots tied on bitten area.	Prakasha, Krishnappa, Krishnamurthy, & Poornima, 2010
	Alstonia scholaris	Karnataka, India	Stem bark crushed and given orally for chewing.	Prakasha et al., 2010
	Tabernaemontana divaricata	Karnataka, India	Root paste mixed with buttermilk and given orally.	Prakasha et al., 2010
Urinary tract infection	*Hemidesmus indicus*	Bangladesh	leaves	Hossan et al., 2010
Premature ejaculation	*Carissa spinarum*	Ethiopia	Drink tea prepared by decoction of unripen fruit and body wash with the infusion	Belayneh & Bussa, 2014
Mouth, throat, and coughing problems	*Acokanthera schimperi*	Ethiopia	Gargling to rinse throat using preparation of stem and leaves. Effective in tonsillitis.	Belayneh & Bussa, 2014
	Alstonia scholaris	Bangladesh	Leaves paste taken orally with honey.	Rahmatullah et al., 2009
Cardiac disorders	*Thevetia neriifolia* *Voacanga Africana* *Stropanthus hispidus*	West Nigeria	Stem bark and root bark preparations are used as cardiotonic.	Ayoola et al., 2008
Measles	*Thevetia neriifolia*	West Nigeria		Ayoola et al., 2008
Diabetes	*Catharanthus roseus*	Tamil Nadu, India	A mixture of whole plant powder and cow's milk taken orally.	Muthu et al., 2006
		South Africa	Leaves are boiled to prepare infusion and taken orally.	Erasto, Adebola, Grierson, & Afolayan, 2005
	Plumeria obtuse	Limpopo, South Africa	Leaves boiled in water and filtered extract is taken orally for three times in a day.	Siddiqui, Ilyas, Rasheed, & Begum, 2004
Ear pain	*Nerium oleander*	Tamil Nadu	Boiled stem bark juice mixed with equal amount of ginger oil and 1-2 drops were poured in ear	Muthu et al., 2006
Sexually transmitted infections	*Catharanthus roseus*	Limpopo, South Africa	Roots are boiled in water and warm preparation administered anally to control Gonorrhoea infection	Pereira et al., 2010
	Alstonia scholaris	India	Dried stem bark powder mixed with Cow's milk taken orally for 3-7 days to control Gonorrhoea infection	Sahu, 2011
Appetizer	*Carissa carandas*	Bangladesh	Ripened fruits act as an appetite stimulant. Syrup prepared from a decoction of leaves and taken orally.	Rahmatullah et al., 2009

TABLE 2 List of phytochemicals isolated from selected members of Apocynaceae family

Phytochemical class/subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
Alkaloids						
Indole alkaloid	1	Voacangine	73255	*Tabernaemontana divaricata*	Flowers	Arambewela & Ranatunge, 1991
	2	Voacristine	196982	*Tabernaemontana divaricata*	Leaves, flowers	Arambewela & Ranatunge, 1991
	3	Vobasine	5374722	*Tabernaemontana divaricata*	Stem bark	Arambewela & Ranatunge, 1991
	4	Tabernaemontanine	45268651	*Tabernaemontana divaricata*	Stem bark	Arambewela & Ranatunge, 1991
	5	Conophylline	15226696	*Tabernaemontana divaricata*	Leaves	Kam et al., 1993
	6	Conophyllidine	44566831	*Tabernaemontana divaricata*	Leaves	Kam et al., 1993
Pentacyclic indole alkaloid	7	Lirofolines	46184733	*Tabernaemontana divaricata*	Stem bark	Low et al., 2010
Terpenoidal indole alkaloid	8	Vinblastine	241903	*Catharanthus roseus*	Whole plant	Heijden et al., 2004
	9	Serpentine	73391	*Catharanthus roseus*	Leaves, Roots	Heijden et al., 2004
	10	Vincristine	5978	*Catharanthus roseus*	Whole plant, leaf	Heijden et al., 2004
	11	Ajmalicine	441975	*Catharanthus roseus*	Whole plant, Leaf, Flower, Roots	Heijden et al., 2004
	12	3',4'-anhydrovinblastine	443324	*Catharanthus roseus*	Leaves, shoot	Heijden et al., 2004
	13	Picrinine	46229104	*Alstonia scholaris*	Leaves	
	14	E and Z- alstoscholarine	16215339 and 16215340	*Alstonia scholaris*	Leaves	Xiang-Hai et al., 2008
Ibogan alkaloid	15	Conolabine A and B	-	*Tabernaemontana divaricata*	Stem bark	Kam et al., 2004
	16	Vallesamine	13783712	*Tabernaemontana divaricata*	Stem bark	Kam et al., 2004
Vinca alkaloids	17	Leurosine	442111	*Catharanthus roseus*	Whole plant	Svoboda et al., 1959
	18	Vincaleukoblastine	241902	*Catharanthus roseus*	Whole plant	Svoboda et al., 1959
Steroidal alkaloid	19	Regholarrhenine	101529337	*Holarrhena antidysenterica*	Stem bark	Bhutani, Ali, Sharma, Vaid, & Gupta, 1988
	20	Holadysenterine	16742955	*Holarrhena antidysenterica*	Seeds	Kumar, Singh, Bhandari, Gupta, & Kaul, 2007
	21	Conessine	441082	*Holarrhena antidysenterica*	Seeds	Kumar et al., 2007
	22	Isoconessimine	11772257	*Holarrhena antidysenterica*	Seeds	Kumar et al., 2007
	23	Kurchessine	442979	*Holarrhena antidysenterica*	Seeds	Kumar et al., 2007
Flavonoids						
	24	Rutin	5280805	*Wrightia tinctoria* , *Wrightia tomentosa*, *Wrightia coccinea*	Fresh leaves	Muruganandam, Bhattacharya, & Ghosal, 2000
	25	Epicatechin	72276	*Carissa carandas*	Fruits	Patil et al., 2012
	26	Quercetin	5280343	*Carissa carandas*	Fruits	Patil et al., 2012

(Continues)

TABLE 2 (Continued)

Phytochemical class/subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
				Alstonia scholaris	Leaves	Hui, Sun, Zhu, Guo, & Rao, 2009
				Tabernaemontana divaricata	Leaves	Jain, Sharma, Ghule, Jain, & Jain, 2013
	27	Kaemferol	5483905	*Carissa carandas*	Fruits	Patil et al., 2012
				Alstonia scholaris	Leaves	Hui et al., 2009
	28	Isorhamnetin	5281654	*Alstonia scholaris*	Leaves	Hui et al., 2009
	29	Quercetin 3-0-beta-D-galactopyranoside	-	*Alstonia scholaris*	Leaves	Hui et al., 2009
	30	Kaemferol 3-0-beta-D-galactopyranoside	-	*Alstonia scholaris*	Leaves	Hui et al., 2009
	31	Isorhamnetin 3-0-beta-D- galactopyranoside	-	*Alstonia scholaris*	Leaves	Hui et al., 2009
	32	Calycosin	5280448	*Tabernaemontana divaricata*	Root and Stem	Liang et al., 2006
	33	Formononetin	5280378	*Tabernaemontana divaricata*	Root and Stem	Liang et al., 2006
	34	Farnisin	5378220	*Tabernaemontana divaricata*	Root and Stem	Liang et al., 2006
	35	Ellagic acid	5281855	*Tabernaemontana divaricata*	Leaves	Jain et al., 2013
				Carissa carandas	Fruits	Patil et al., 2012
	36	Hyperoside	5281643	*Tabernaemontana divaricata*	Leaves	Jain et al., 2013
Terpenes						
Monoterpenes	37	3-carene	26049	*Carissa carandas*	Flowers	Zaki, El-Tohamy, Hashem, & Abd El-Fattah, 1981
	38	Camphene	6616	*Carissa carandas*	Flowers	Zaki et al., 1981
	39	Menthol	16666	*Carissa carandas*	Flowers	Zaki et al., 1981
	40	ρ-Cymene	7463	*Carissa carandas*	Flowers	Zaki et al., 1981
	41	α-Terpineol	17100	*Carissa carandas*	Flowers	Zaki et al., 1981
	42	Piperitone	6987	*Carissa carandas*	Flowers	Zaki et al., 1981
	43	Neryl acetate	1549025	*Carissa carandas*	Flowers	Zaki et al., 1981
	44	Geranyl acetate	1549026	*Carissa carandas*	Flowers	Zaki et al., 1981
	45	β-Ionone	638014	*Carissa carandas*	Flowers	Zaki et al., 1981
Sesquiterpenes	46	Carindone	101316738	*Carissa carandas*	Root	Singh & Rastogi, 1972
	47	(+)-Carrisone	5086419	*Carissa carandas*	Root	Singh & Rastogi, 1972
	48	Nerolidol	5284507	*Carissa carandas*	Flowers	Zaki et al., 1981
	49	Farnesol	445070	*Carissa carandas*	Flowers	Zaki et al., 1981
Triterpenes	50	Lupeol	259846	*Carissa carandas*	Roots	Pakrashi, Datta, & Ghosh-Dastidar, 1968
				Holarrhena antidysenterica	Seeds	Bhattachartya, Tarafdar, & Saha, 2009
	51	Carrisic acid	-	*Carissa carandas*	Leaves	Naim, Khan, & Nizami, 1988
	52	Carrisol	-	*Carissa carandas*	Fruits	Naim, Khan, & Nizami, 1985
	53	Betulinic acid	64971	*Carissa carandas*	Leaves	Begum, Syed, Siddiqui, Sattar, & Choudhary, 2013
				Nerium oleander	leaves	Siddiqui, Hafeez, Begum, & Siddiqui, 1988; Fu et al., 2005
					Seeds	Bhattachartya et al., 2009

(Continues)

TABLE 2 (Continued)

Phytochemical class/subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
				Holarrhena antidysenterica		
	54	16β-hydroxybetulinic acid	-	*Carissa carandas*	Root	Singh & Uppal, 2015
	55	Ursolic acid		*Carissa carandas*	Roots	Pakrashi et al., 1968
			64945	*Nerium oleander*	leaves	Siddiqui et al., 1988; Fu et al., 2005
	56	α-Amyrin	73170	*Carissa carandas*	Root	Singh & Uppal, 2015
				Tabernaemontana divaricata	Root and stem	Liang et al., 2006
	57	α-Amyrin acetate	92842	*Tabernaemontana divaricata*	Root and stem	Liang et al., 2006
	58	β-Amyrin	73145	*Wrightia tinctoria*	Seed pods	Ramchandra et al., 1993
	59	Betulin	72326	*Nerium oleander*	leaves	Siddiqui et al., 1988; Fu et al., 2005
	60	Oleanderol	-	*Nerium oleander*	leaves	Siddiqui et al., 1988
	61	Oleandric acid	-	*Nerium oleander*	leaves	Fu et al., 2005
	62	Oleanerolide	11113483	*Nerium oleander*	leaves	Fu et al., 2005
	63	Epoxydammarane 3β, 25-diol	-	*Nerium oleander*	leaves	Fu et al., 2005
	64	Wrightial	-	*Wrightia tinctoria*	Seed pods	Ramchandra et al., 1993
	65	Taraxasterol acetate	13889352	*Tabernaemontana divaricata*	Root and stem	Liang et al., 2006
	66	Alstonic acid A and B	91895416 and 91895417	*Alstonia scholaris*	Leaves	Wang, Ren, & Liu, 2009
	67	2,3-Secofernane	-	*Alstonia scholaris*	Leaves	Wang et al., 2009
	68	*Cis* and *Trans* Karenin	6440661 and 101921671	*Nerium oleander*	Leaves	Siddiqui, Begum, Siddiqui, & Lichter, 1995
	69	3β,27-dihydroxy-urs-18-en-13,28-olid	-	*Nerium oleander*	Uncrushed leaves	Begum, Sultana, & Siddiqui, 1997
	70	3β,22α,28-trihydroxy-25-nor-lup-1(10), 20(29)-dien-2-one	-	*Nerium oleander*	Uncrushed leaves	Begum et al., 1997
	71	Oleanolic acid	10494	*Carissa carandas*	Root	Itankar et al., 2011
				Nerium oleander	leaves	Siddiqui et al., 1988
	72	Betulinaldehyde	99615	*Holarrhena antidysenterica*	Seeds	Bhattachartya et al., 2009
Isohopane triterpenoid	73	Carandinol	102202376	*Carissa carandas*	Leaves	Begum et al., 2013
Glycosides						
Cardiac glycosides	74	Kaneroside	-	*Nerium oleander*	Leaves	Siddiqui, Hafeez, Begum, & Siddiqui, 1986
	75	Neriumoside	-	*Nerium oleander*	Leaves	Siddiqui et al., 1986
	76	Oleandrin	11541511	*Nerium indicum*	Fresh leaves	Dai, Wang, Dong, Hu, & Nan, 2011
	77	Neriantin	12313293	*Nerium indicum*	Fresh leaves	Dai et al., 2011
	78	Adynerin	441840	*Nerium indicum*	Fresh leaves	Dai et al., 2011
	79	Deacetyloeandrin	37401	*Nerium indicum*	Fresh leaves	Dai et al., 2011
	80	Odoroside-H	205840	*Carissa carandas*	Roots	Hegde, Thakker, & Joshi, 2009
				Nerium oleander	Leaves	Begum, Siddiqui, Sultana, Zia, & Suria, 1999
	81	Rhamnoside	-	*Carissa carandas*	Roots	Hegde et al., 2009
	82	Rhamnoglycoside	-	*Carissa carandas*	Roots	Hegde et al., 2009
	83	Evomoside	-	*Carissa carandas*	Roots	Hegde et al., 2009
	84	Thevetin	159331	*Thevetia peruviana*	Seeds	Kohls, Scholz-Böttcher, Teske, Zark, & Rullkötter, 2012

(Continues)

TABLE 2 (Continued)

Phytochemical class/subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
	85	Acetylthevetin	90479481	*Thevetia peruviana*	Seeds	Kohls et al., 2012
Cardenolides	86	Neridiginoside	-	*Nerium oleander*	Leaves	Begum et al., 1999
	87	Nerizoside	10816404	*Nerium oleander*	Leaves	Begum et al., 1999
	88	Neritaloside	44566654	*Nerium oleander*	Leaves	Begum et al., 1999
	89	Neriifolin	441867	*Thevetia peruviana*	Stem bark	Miyagawa, Ohtsuki, Koyano, Kowithayakorn, & Ishibashi, 2009
	90	Peruvoside	12314120	*Thevetia peruviana*	Stem bark	Miyagawa et al., 2009
	91	Thevefolin	44537896	*Thevetia peruviana*	Stem bark	Miyagawa et al., 2009
	92	(20S)-18,20 epoxydigitoxigeninR-L-thvetoside	-	*Thevetia peruviana*	Stem bark	Miyagawa et al., 2009
Terpenoids glycosides (Sesquiterpene glucoside)	93	Carandoside	44179851	*Carissa carandas*	Stem	Wangteeraprasert & Likhitwitayawuid, 2009
(Anthocynidin glycosides)	94	7-O-Methylcyanidin3-robinobioside	-	*Catharanthus roseus*	Flowers	Toki et al., 2008
	95	Rosinidin 3-robinobioside	-	*Catharanthus roseus*	Flowers	Toki et al., 2008
(Secoiridoid glycoside)	96	Alstonoside	-	*Alstonia scholaris*	Stem	Thomas, Kanaujia, Ghosh, Duggar, & Katiyar, 2008
Flavonoid glycosides (Isoflavone glycosides)	97	Formononetin 7-O-β-D-apiofuranosyl-(1-6)-β-D-glucopyranoside	-	*Alstonia scholaris*	Stem	Thomas et al., 2008
	98	Biochanin A 7-O-β-D-apiofuranosyl-(1-6)-β-D-glucopyranoside	-	*Alstonia scholaris*	Stem	Thomas et al., 2008
(Flavonol glycosides)	99	Syringetin-3-O-robinobioside	-	*Catharanthus roseus*	Stem	Brun, Dijoux, David, & Mariotte, 1999
	100	Quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)	-	*Catharanthus roseus*	Stem, Leaves, Seed, Petals	Ferreres et al., 2008
	101	Quercetin -3-*O*-(2,6-di-*O*-rhamnosyl-glucoside)	-	*Catharanthus roseus*	Petals	Ferreres et al., 2008
	102	Kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)	-	*Catharanthus roseus*	Stem, leaves, seed, petals	Ferreres et al., 2008
	103	kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside)	-	*Catharanthus roseus*	Stem, Seed, Petals	Ferreres et al., 2008
	104	Isorhamnetin-3-*O*-(2,6-di-*O*-rhamnosylgalactoside)	-	*Catharanthus roseus*	Stem, Leaves	Ferreres et al., 2008
	105	Isorhamnetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside)	-	*Catharanthus roseus*	Seeds	Ferreres et al., 2008
	106	Quercetin 3-O-{β-D-glucopyranosyl-(1-2)-[α-L-rhamnopyranosyl-(1-6)]-β-D-galactopyranoside}	-	*Thevetia peruviana*	Leaves	Tewtrakul, Nakamura, Hattori, Fujiwara, & Supavita, 2002
(Flavanone glycosides)	107	(2S)-5-O-β-D-glucopyranosyl-7,4'-dihydroxy-3',5'-dimethoxyflavanone	-	*Thevetia peruviana*	Leaves	Tewtrakul et al., 2002

(Continues)

TABLE 2 (Continued)

Phytochemical class/subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
	108	(2R)-5-O-β-D-glucopyranosyl-7,4'-dihydroxy-3',5'-dimethoxyflavanone	-	*Thevetia peruviana*	Leaves	Tewtrakul et al., 2002
5. Sterols						
	109	Stigmasterol	5280794	*Alstonia scholaris*	Root, Root bark	Adotey, Adukpo, Opoku Boahen, & Armah, 2012
				Wrightia tinctoria	Stem	Ramchandra et al., 1993; Jain & Bari, 2010
				Carissa carandas	Roots	Galipalli, Patel, Prasanna, & Bhutani, 2015
	110	β-sitosterol	222284	*Alstonia scholaris*	Stem	Adotey et al., 2012
				Wrightia tinctoria	Stem	Ramchandra et al., 1993
				Carissa carandas	Roots	Pakrashi et al., 1968 ; Bhaskar & Balakrishnan, 2015
	111	Campesterol	173183	*Alstonia scholaris*	Root, Root bark	Adotey et al., 2012
				Wrightia tinctoria	Stem	Ramchandra et al., 1993; Jain & Bari, 2010
	112	14α-methylzymosterol	-	*Wrightia tinctoria*	Seeds	Akihisa, Ahmad, Singh, Tamura, & Matsumoto, 1988
	113	Desmosterol	439577	*Wrightia tinctoria*	Seeds	Akihisa et al., 1988
	114	Clerosterol	5283638	*Wrightia tinctoria*	Seeds	Akihisa et al., 1988
	115	24-methylene-25-methylcholesterol	5283645	*Wrightia tinctoria*	Seeds	Akihisa et al., 1988
	116	24-dehydropollinastanol	-	*Wrightia tinctoria*	Seeds	Akihisa et al., 1988
	117	7-dihydrositosterol	5283634	*Rauvlfia serpentine*	Roots	Karmakar & Chakraborty, 1983
	118	Sitosterol glucoside	70699351	*Carissa carandas*	Roots	Hegde, Satyanarayana, & Joshi, 2012
	119	Cholest-5-en-3- β-ol	-	*Carissa carandas*	Roots	Balakrishnan, Balasubaramaniam, Sangameswaran, & Bhaskar, 2011
	120	20-Hydoxypregnan-18-oic acid	-	*Carissa carandas*	Leaves	Bhadane & Patil, 2017
	121	Sitosta-5,23-dien-3β-ol	-	*Holarrhena antidysenterica*	Stem bark	Usmani, 1995
Simple phenolic compounds						
	122	Syringic acid	10742	*Carissa carandas*	Fruits	Patil et al., 2012
	123	Vanillic acid	8468	*Carissa carandas*	Fruits	Patil et al., 2012
				Catharanthus roseus	Leaves	Proestos, Chorianopoulos, Nychas, & Komaitis, 2005
	124	ρ-coumaric acid	637542	*Carissa carandas*	Fruits	Patil et al., 2012
	125	Caffeic acid	689043	*Carissa carandas*	Fruits	Patil et al., 2012
	126	Chlorogenic acid	1794427	*Carissa carandas*	Fruits	Patil et al., 2012
				Catharanthus roseus	Leaves	Proestos et al., 2005
	127	Scopoletin	5280460	*Carissa carandas*	Root	Galipalli et al., 2015
	128	Gallic acid	370	*Catharanthus roseus*	Leaves	Proestos et al., 2005
	129	Hydroxytyrosol	82755	*Catharanthus roseus*	Leaves	Proestos et al., 2005
	130	Ferulic acid	445858	*Catharanthus roseus*	Leaves	Choi et al., 2004
	131	3-O-Caffeoylquinic acid (3-CQA)	-	*Catharanthus roseus*	Leaves	Ferreres et al., 2011; Wong, Lim, Ling, & Chan, 2014
				Nerium oleander	Leaves	Wong et al., 2014
	132	4-O-Caffeoylquinic acid (4-CQA)	-	*Catharanthus roseus*	Leaves	Ferreres et al., 2011; Wong et al., 2014

(Continues)

TABLE 2 (Continued)

Phytochemical class/subclass	Sr. no.	Name of compound	PubChem CID	Name of plant species	Source	References
				Nerium oleander	Leaves	Wong et al., 2014
	133	5-O-Caffeoylquinic acid (5-CQA)	-	*Catharanthus roseus*	Leaves	Ferreres et al., 2011; Wong et al., 2014
				Nerium oleander	Leaves	Wong et al., 2014
Lignans						
	134	Carinol	-	*Carissa carandas*	Roots	Pal, Kulshreshtha, & Rastogi, 1975
	135	(6R,7S,8S)-7a-[(b-glucopyranosyl) oxy]lyoniresinol	-	*Carissa carandas*	Stem	Wangteeraprasert & Likhitwitayawuid, 2009
	136	carissanol	340931	*Carissa carandas*	Stem	Wangteeraprasert & Likhitwitayawuid, 2009
	137	(-)-nortrachelogenin	394846	*Carissa carandas*	Stem	Wangteeraprasert & Likhitwitayawuid, 2009
	138	syringaresinol 4-O-b-glucopyranoside		*Vinca major*	Leaves	Sohretoglu, Masullo, Piacente, & Kirmizibekmez, 2013
Others						
	139	Eicosanoic acid	10467	*Carissa carandas*	Seeds	Shrivastava & Bakodia, 1979
				Tabernaemontana divaricata	Flower	Ali Khan, Mat Jais, & Afreen, 2013
	140	Hexadecanoic acid, 15-methyl ester	985	*Carissa carandas*	Seeds	Shrivastava & Bakodia, 1979
				Wrightia tinctoria	Flower	Ramalakshmi, Edaydulla, Ramesh, & Muthuchelian, 2012
				Catharanthus roseus	Leaves	Brun, Bessière, Dijoux-Franca, David, & Mariotte, 2001
				Tabernaemontana divaricata	Flower	Ali Khan et al., 2013
				Holarrhena antidysenterica	Flower	Paramanantham & Murugesan, 2014
	141	Octadecanoic acid,9Z,12Z-octadecadienoic acid	-	*Carissa carandas*	Seeds	Shrivastava & Bakodia, 1979
				Tabernaemontana divaricata	Flower	Ali Khan et al., 2013
	142	9Z-octadecenoic acid	445639	*Carissa carandas*	Seeds	Shrivastava & Bakodia, 1979
	143	Pentadecanoic acid ester	38762	*Wrightia tinctoria*	Flower	Ramalakshmi et al., 2012
				Catharanthus roseus	Leaves	Brun et al., 2001
	144	Caproic acid	8892	*Catharanthus roseus*	Leaves	Brun et al., 2001
	145	Capric acid	-	*Catharanthus roseus*	Leaves	Brun et al., 2001
	146	Caprylic acid	379	*Catharanthus roseus*	Leaves	Brun et al., 2001
	147	Nonanoic acid	8158	*Catharanthus roseus*	Leaves	Brun et al., 2001
	148	Tridecanoic acid	12530	*Catharanthus roseus*	Leaves	Brun et al., 2001
	149	Arabinogalactun	24847856	*Nerium oleander*	Leaves	Dong & Fang, 2001
	150	L-Ascorbic acid	54670067	*Carissa carandas*	Fruits	Zaki et al., 1981; Patil et al., 2012
	151	Dihydrojasmone	62378	*Carissa carandas*	Flowers	Rastogi, Vohra, Rastogi, & Dhar, 1966
	152	6-Decaprenylphenol	-	*Carissa carandas*	Roots	Zaki et al., 1981; Patil et al., 2012
	153	4-Amino-1-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1,4-dideoxy-b-D-glucopyranuronic acid	-	*Carissa carandas*	Roots	Zaki et al., 1981; Patil et al., 2012
	154	Vitamin E	14985	*Tabernaemontana divaricata*	Flower	Ali Khan et al., 2013

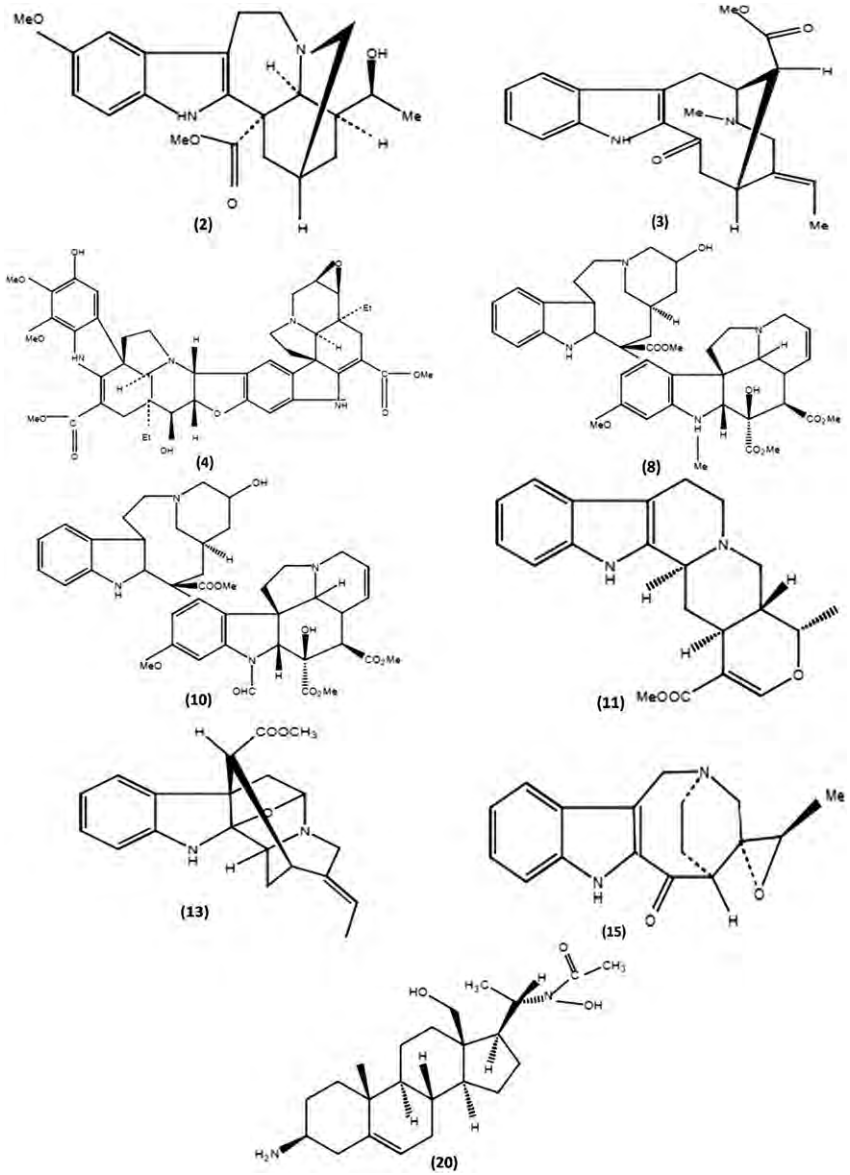

FIGURE 1 Chemical structure of alkaloids isolated from Apocynaceae plants

(Jagetia & Baliga, 2006; Kamarajan, Sekar, Mathuram, & Govindasamy, 1991). Besides indole, ibogan, and terpenoidal alkaloids, Bhutani et al. (1988) reported steroidal alkaloid regholarrhenine **(19)** and its derivatives from *Holarrhena antidysenterica* with a total yield of alkaloids 0.44%. Recently, a new steroidal alkaloid holadysenterine **(20)** was isolated from *Holarrhena antidysenterica* with a few previously known steroidal alkaloids such as conessine, isoconessimine, and kurchessine (**21**–**23**; Kumar et al., 2007). The identification and structure elucidation of these compounds were mostly done by 1D- and 2D-NMR and high-resolution mass spectrometry techniques.

4.2 | Flavonoids

Many members of family Apocynaceae are reported to possess flavonoids, but their contents were proportionally lower than alkaloid content. Muruganandam et al. (2000) isolated rutin as a major constituent from fresh leaves of three different species of *Wrightia* genus. In another study, Patil et al. (2012) reported rutin, epicatechin, quercetin, and kaemferol **(24**–**27)** in *Carissa carandas* fruits as principal flavonoids (total flavonoid content 4.8 mg/100 g). Similarly, quercetin, kaemferol, and isorhamnetin **(28)** were also reported in leaves of *Alstonia scholaris* with newly isolated 3-0-beta-D-galactopyranoside derivatives of quercetin, kaemferol, and isorhamnetin (**29**–**31**; Hui et al., 2009). Root and stem extracts of *Tabernaemontana divaricata* were also found to contain novel flavonoids, namely, calycosin **(32)**, formononetin **(33)**, and farnisin (**34**; Liang et al., 2006). Thereafter, Jain et al. (2013) isolated three flavonoids (ellagic acid, hyperoside [**35**, **36**] and quercetin) from *Tabernaemontana divaricata* and characterized them by thin-layer chromatography (TLC) analyses. All these flavonoids showed anti-inflammatory potential against croton oil-induced edema in an albino mouse model with IC_{50} of 500 μg/ml. Figure 2 shows isolated flavonoids from Apocynaceae plants.

4.3 | Terpenes

Like alkaloids, the abundance of terpenes and their derivatives has been reported in many members of family Apocynaceae. *Carissa* genus

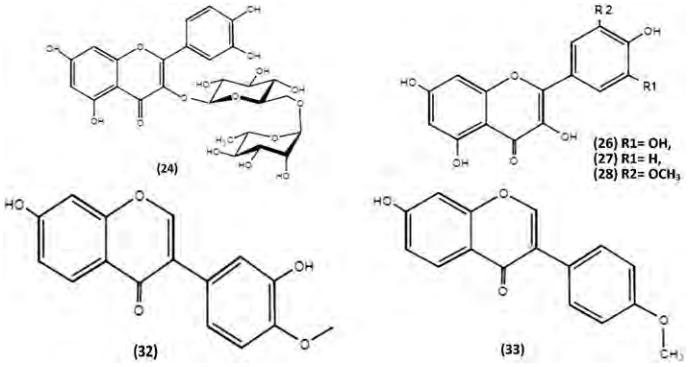

FIGURE 2 Chemical structure of flavonoids isolated from Apocynaceae plants

of this family contains comparatively higher amount of terpenoids (mono, sesqui, and tri terpenoids). In the systematic review of Kaunda and Zhang (2017), 22 triterpenes, 18 monoterpenes, and 16 sesquiterpenes from different species of *Carissa* genus were reported. Among all, maximum terpenoids were reported in *Carissa carandas*. The monoterpenoids were found at maximum abundance in flowers of *Carissa carandas* (**37**–**45**; Zaki et al., 1981). Sesquiterpenes **(46**–**49)** were found to be uniformly distributed in roots and flowers (Singh & Rastogi, 1972) whereas triterpenoids **(50**–**56)** were found evenly distributed in leaves, roots, and fruits (Arif & Fareed, 2011; Naim et al., 1985 & 1988; Pakrashi et al., 1968; Singh & Uppal, 2015). The sesquiterpene–carrisone, triterpene–lupeol, and oleanolic acid **(71)** were found to exhibit anti-inflammatory activity against proinflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α, and interlukin-1b (Itankar et al., 2011). On the other hand, carandinol **(73)**, the first isohopane-type triterpenoid from *Carissa carandas* leaves, had significant cytotoxic effect against HeLa, 3T3, and PC-3 cancerous cell lines (Begum et al., 2013).

Another genus *Nerium* of this family also contains different types of triterpenoids. Leaves of *Nerium oleander* are the richest source of triterpenoids. Siddiqui et al. (1988) isolated oleanderol **(60)** with other known triterpenoids such as betulinic acid, betulin **(59)**, oleanolic acid, and ursolic acid. Later on, *cis*-karenin and *trans*-karenin were isolated by Siddiqui et al. (1995) as cytotoxic pentacyclic triterpenoids. These compounds were novel and found to contain moieties of ursane and caumaric acid. Begum et al. (1997) isolated two new triterpenoids **(69**–**70)** from fresh uncrushed leaves of *Nerium oleander* and structures of the probable compounds were elucidated by one- and two-dimensional NMR spectroscopic studies. Recently, a total of 15 triterpenoids including previously known and newly isolated oleandric acid and oleanerolide **(61**–**62)** from *Nerium oleander* were evaluated for their anti-inflammatory and anticancer potential (Fu et al., 2005). Among them, oleandric acid, ursolic acid, betulinic acid, betulin, and derivatives of epoxydammarane 3β,25-diol showed significant anticancer activity against lung carcinoma cell line HepG2.

Wrightia tinctoria of this family contains Wrightial **(64)**—a new triterpenoid along with known compound β-amyrin (**58**; Ramchandra et al., 1993). α-amyrin, α-amyrin acetate **(57)**, and taraxasterol acetate **(65)** were isolated from the roots and stem of *T. divaricata* (Liang et al., 2006). Alstonic acids A and B as well as 2,3-secofernane **(66**–**67)** triterpenoids were reported from the leaves of *Alstonia scholaris* (Wang et al., 2009). Colorless crystals of betulinaldehyde **(72)** and lupeol, as well as white crystals of betulinic acid, were isolated from *Holarrhena antidysenterica* seeds (Bhattachartya et al., 2009). The molecular characterization of triterpenoid synthesis in *Catharanthus roseus* was studied by Huang et al. (2012), and they reported that cuticular wax layer of *Catharanthus roseus* leaf is the site for ursolic and oleanolic acid synthesis. These triterpenoids seem to have antimicrobial, anti-inflammatory, and antitumor activity. Recently, El-Kashef, Hamed, Khalil, and Kamel (2015) reported more than 95 common terpenoids from different plants of 36 genera of family Apocynaceae. Figure 3 represents structures of selected terpenes isolated from Apocynaceae plants.

4.4 | Glycosides

Glycosides are the group of phenolic compounds present only in certain plant species. The cardiac glycoside is a major class of glycosides and widely used in the treatment of heart failure. In all, genera *Thevetia* and *Nerium* are reported to possess the highest glycosides (Figure 4). Siddiqui et al. (1986) isolated two cardiac glycosides, namely, kaneroside and neriumoside **(74, 75)**, from the leaves of *Nerium oleander*. Thereafter, cardiac glycosides **(76**–**79)** from *N. indicum* were studied for molluscicidal activity against *Pomacea canaliculata* (Dai et al., 2011). The activity shown by all tested cardiac glycosides (LC_{50} at 3.71 mg/L) was found to be lower than standard metaldehyde (3.88 mg/L). Cardiac glycosides were also isolated from the roots of *Carissa carandas* and characterized spectroscopically (FT-IR, ^{1}H-NMR, and ^{13}C-NMR) as odoroside H, rhamnoside, rhamnoglycoside, and evomoside (**80**–**83**; Hegde et al., 2009). Similarly, thevetin and acetylthevetin **(84, 85)** were isolated from the seeds of *Thevetia peruviana* (Kohls et al., 2012). Cardenolides, an important class of cardiac glycosides were found to be present in *Nerium oleander* and *Thevetia peruviana*. The isolated cardenolides of *Nerium oleander* leaves were odoroside-H, neridiginoside (75 mg/g), nerizoside, and neritaloside **(80, 86**–**88)** reported for central nervous system-depressant activity on mice (Begum et al., 1999). However, neriifolin, peruvoside, thevefolin, and (20S)-18,20-epoxydigitoxigenin R-L-thvetoside **(89**–**92)** are the cardenolides of *T. peruviana*. All these cardenolides were screened for TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) resistance-overcoming activity. Among them, thevefolin was reported to be effective for TRAIL resistance in human gastric adenocarcinoma cells (Miyagawa et al., 2009).

Unlike cardiac glycosides and cardenolides, other important types of glycosides such as flavonoid glycosides and terpenoids glycosides

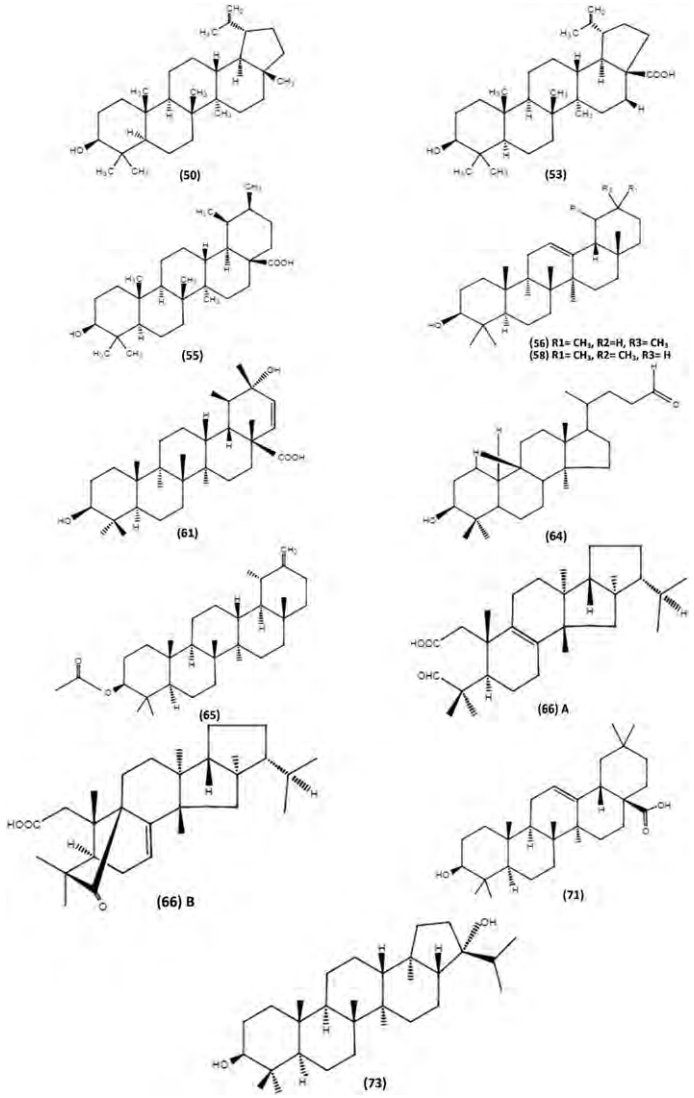

FIGURE 3 Chemical structure of terpenes isolated from Apocynaceae plants

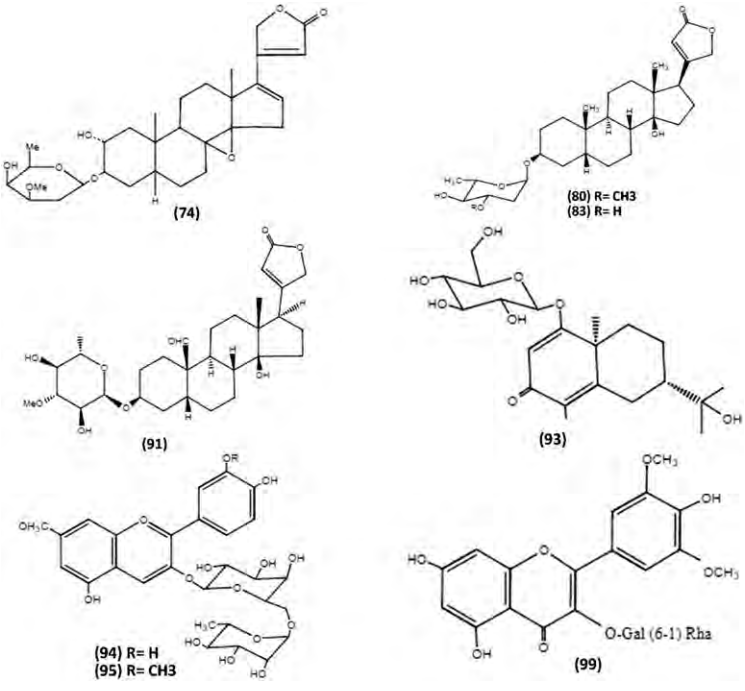

FIGURE 4 Chemical structures of glycosides isolated from Apocynaceae plants

are also reported in different species of family Apocynaceae. Sesquiterpene glucoside, that is, carandoside **(93)** was isolated from *Carissa carandas* stem (Wangteeraprasert & Likhitwitayawuid, 2009). Anthocynidin glycosides **(94, 95)** were isolated from orange-red flowers of *Catharanthus roseus* (Toki et al., 2008). Secoiridoid glycoside **(96)** and isoflavone glycosides **(97, 98)** were isolated from *Alstonia scholaris* (Thomas et al., 2008). Brun et al. (1999) isolated syringetin-3-O-robinobioside as a flavonol glycoside **(99)** from the stem of *Catharanthus roseus*. Later, antioxidative flavonol glycosides (glucoside and galactoside of kaempferol, quercetin, and isorhamnetin; **100**–**105**) were isolated from different parts of *Catharanthus roseus* (Ferreres et al., 2008). All these glycosides have marked DPPH, superoxide, and NO radical scavenging potential with maximum protective activity against NO (IC_{50} 232 μg/ml). Another flavonol **(106)** and flavanone glycosides **(107, 108)** were isolated from leaves of *Thevetia peruviana* and reported for their anti-HIV-1 reverse transcriptase (RT) and anti-HIV-1 integrase activities (Tewtrakul et al., 2002).

4.5 | Sterols

Sterols, also referred as phytosterols or steroid alcohols, are present with potential bioactivity in several Apocynaceae plants. Bioactivities shown by sterols include hepatoprotective, anti-inflammatory, and antihyperlipidemic activities (Al-Youssef & Hassan, 2014). Among all members, *Wrightia tinctoria* and *Alstonia scholaris* are quite rich with different kinds of phytosterols (Figure 5). Stigmasterol, β-sitosterol, and campesterol **(109**–**111)** are commonly present in different parts of these plants (Adotey et al., 2012). Jain and Bari (2010) isolated stigmasterol and campesterol from *Wrightia tinctoria* stem extract, and structural elucidation was carried out using UV-Visible, FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectrophotometric analyses. Besides, a gas chromatography-mass spectrometry (GC-MS) analysis was also carried out to study percent abundance of reported compounds. In an earlier study, some uncommon sterols **(112**–**116)** from unsaponifiable lipids of *Wrightia tinctoria* seeds are reported (Akihisa et al., 1988). Another singular sterol, that is, 7-dihydrositosterol **(117)** was isolated from *Rauvolfia serpentina* roots (Karmakar & Chakraborty, 1983). Sitosta-5,23-dien-3β-ol **(118)** was isolated from the stem bark of *Holarrhena antidysenterica* (Usmani, 1995). Moreover, β-sitosterol, stigmasterol, sitosterol glucoside **(119)**, and cholest-5-en-3-β-ol **(120)** were previously isolated from *Carissa carandas* roots by various researchers (Balakrishnan et al., 2011; Bhaskar & Balakrishnan, 2015; Hegde et al., 2012). The stigmasterol from *Carissa carandas* was reported for marked anti-inflammatory activity (Galipalli et al., 2015). Recently, Bhadane and Patil (2017) investigated antioxidative and anti-inflammatory potential of methanolic extracts of *Carissa carandas* leaves by activity-guided fractionation and 20-hydroxypreganan-18-oic acid **(121)** was reported as a potential antioxidative and anti-inflammatory steroid with t EC_{50} values 546.4 and 498.5 μg/ml, respectively, for DPPH and OH^{-} radical scavenging activities.

4.6 | Simple phenolic compounds

Simple phenolic compounds are mostly derivatives of hydroxybenzoic and hydroxycinnamic acid, which contain C6C1 and C6C3 skeleton with carboxyl group attached to aromatic ring (Dewick, 2002; Figure 6). They have been studied for potential bioactivities in some members of family Apocynaceae (Bhaskar & Balakrishnan, 2015). In a systematic review of Kaunda and Zhang (2017), a total of 16 simple phenolic compounds from different species of *Carissa* genus were reported. Among them, simple phenolics such as syringic acid, vanillic acid, *p*-coumaric acid, caffeic acid, and chlorogenic acid **(122**–**126)** were isolated from *Carissa carandas* fruits (Patil et al., 2012). Scopoletin **(127)** was isolated from the roots of the same plant with anti-inflammatory activity and chlorogenic acid was reported for preventing Type-II diabetes mellitus (Begum et al., 1999). Chlorogenic acid, as well as vanillic acid, along with gallic acid, hydroxytyrosol, and ferulic acid **(128-130)**, were also isolated from *Catharanthus roseus* leaves (Choi et al., 2004; Proestos et al., 2005). Further, three different derivatives of caffeoylquinic acid **(131-133)** were isolated from *Catharanthus roseus* leaf vacuoles and studied for their localized interaction with Class III peroxidases. All these phenolics act as a substrate for peroxidase and showed higher H_2O_2 scavenging up to 9 mM/s

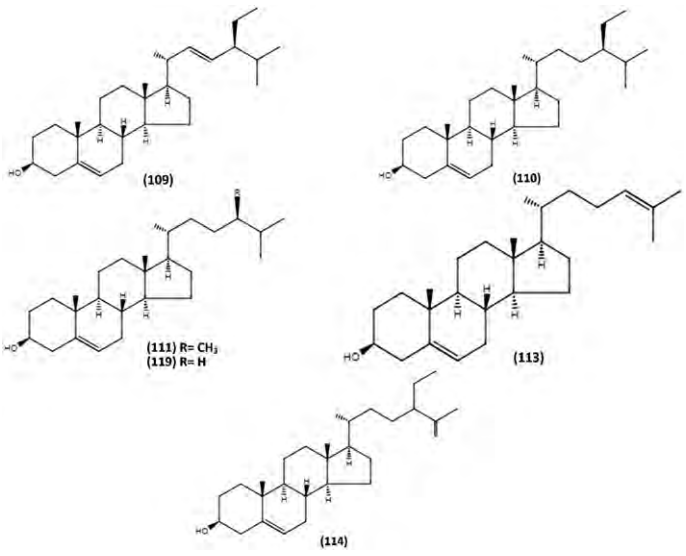

FIGURE 5 Chemical structure of sterols isolated from Apocynaceae plants

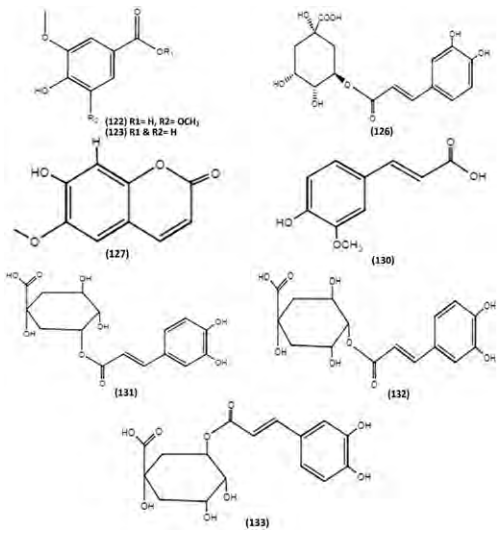

FIGURE 6 Simple phenolic compounds isolated from Apocynaceae plants

(Ferreres et al., 2011). The same compounds were isolated by Wong et al. (2014) from six different plants of Apocynaceae family including *Nerium oleander* and *Catharanthus roseus*. Coumarins were reported in abundance in the preliminary phytochemical analysis of ethanolic extracts of *Wrightia tinctoria* and *Nerium oleander* flowers (Joselin, Brintha, Florence, & Jeeva, 2012).

4.7 | Lignans

A few reports are available on lignans from some members of family Apocynaceae. Kaunda and Zhang (2017) reported a number of lignans from *Carissa edulis* and *Carissa spinarum*. An earlier study on isolation of lignan from root extract of *Carissa carandas* reveled carinol **(134)** as phenolic lignan (Pal et al., 1975). Thereafter, three new lignans, namely, (6R,7S,8S)-7a-[(b-glucopyranosyl) oxy] lyoniresinol, carissanol, and (-)-nortrachelogenin (**135**–**137**; Figure 7) were isolated from *Carissa carandas* stem (Jagetia & Baliga, 2006). Recently, syringaresinol 4-O-b-glucopyranoside **(138)** was isolated from *Vinca major* (Sohretoglu et al., 2013).

4.8 | Others

In addition to these major phytoconstituents, many Apocynaceae plants also possess other compounds with diverse biological activities.

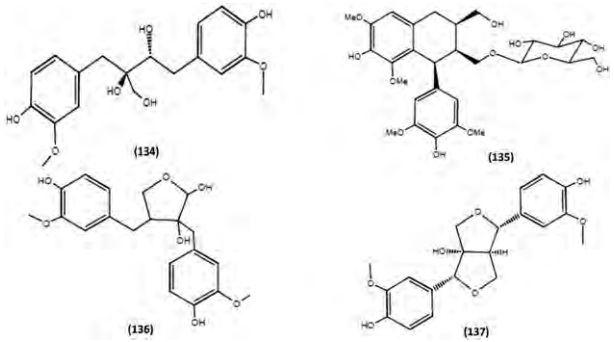

FIGURE 7 Chemical structure of lignans isolated from Apocynaceae plants

These mainly include fatty acids and esters, hydrocarbons, vitamins, and glycoproteins. A wide range of fatty acids and esters were reported in Apocynaceae plants. Shrivastava and Bakodia (1979) isolated eicosanoic acid, hexadecanoic acid, octadecanoic acid, 9Z,12Z-octadecadienoic acid, and 9Z-octadecenoic acid **(139**–**142)** from the seed oil of *C. carandas*. Fatty acids such as pentadecanoic acid ester **(143)** along with hexadecanoic acid, 15-methyl ester isolated from *Wrightia tinctoria* flower extract and exhibited antioxidant and antimicrobial activities (Ramalakshmi et al., 2012). A total of 17 fatty acids were isolated from *Catharanthus roseus* leaves and analyzed by GC-MS analysis (Brun et al., 2001). Among all these fatty acids, some of them were common in *Tabernaemontana divaricata*, *Holarrhena antidysenterica*, *Carissa carandas*, and *Wrightia tinctoria*, whereas compounds **(144**–**148)** were found to be uncommon (Ali Khan et al., 2013; Paramanantham & Murugesan, 2014). Besides fatty acid esters, a new arabinogalactum **(149)** was isolated from leaves of *Nerium oleander*, which acts as immunologically important glycoprotein (Dong & Fang, 2001); L-ascorbic acid (Rastogi et al., 1966), dihydrojasmone, and two other compounds (**150**–**153**; Patil et al., 2012) were isolated from different parts of *C. carandas*. Vitamin E **(154)** was reported in *Tabernaemontana divaricata* flowers (Ali Khan et al., 2013).

5 | PHARMACOLOGICAL PROPERTIES

Ethnomedicinal uses and traditional knowledge of Apocynaceae plants have resulted in the extensive evaluation of pharmacological properties of many plants of this family. The crude extracts, as well as the isolated compounds, are known to possess a wide range of bioactivities such as antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, and cytotoxic. Despite the fact that many species of this family are known for ethnomedicinal potential, very few of them have been systematically studied and evaluated pharmacologically. The ensuing paragraphs and Table 3 provide a detailed outlook of biological activities exhibited by plant extracts, fractions, and single isolated compounds.

5.1 | Antioxidant activity

Phenolics are abundantly present in plants of the family Apocynaceae. The distinctive characteristics of phenolic compounds make them as potential reducing agents, metal chelators, and free radical quenchers (Rice-Evans, Miller, & Paganga, 1996). Various methods have been used to study in vitro antioxidative potential of plant extracts (Krishnaiah, Sarbatly, & Nithyanandam, 2011). Though there are several reports on in vitro antioxidant activity, reports of in vivo study are very few. The antioxidant activity of *Nerium oleander* flowers methanol extract was evaluated by DPPH, reducing power, ABTS, hydroxyl radical scavenging, and other three methods (Singhal & Gupta, 2012). Among all these assays, the test extracts strongly scavenged the DPPH radicals with IC_{50} 193.37 μg/ml, which was found comparable to BHT (189.57 μg/ml). Using the same methods, the antioxidative potential of *Wrightia tinctoria* was also evaluated and the ethanolic flower extract showed maximum scavenging potential against DPPH radicals with IC_{50} of 43.16 μg/ml (Ramalakshmi et al., 2012). Another member of the

TABLE 3 Biological and pharmacological properties of Apocynaceae members

Plant and plant part	Biological activity	Extract/compound	Effective concentration/dose	Study model	References
Alstonia scholaris					
Flowers	Antioxidant (DPPH)	Methanolic and aqueous extract	IC_{50} 34.2 and 50 μg/ml	In vitro	James et al., 2011
Leaves	Anticancer	80% methanolic extract	4% crude extract	In vitro	Surendren et al., 2012
	Anti-HIV	Methanol and butanol extract	10 and 15 μg/ml, respectively	In vitro (using CEM-GFP cell lines infected with HIV-1NL4.3)	Sabde et al., 2011
	Antiarthritic	Ethanolic extract	400 mg/kg	In vivo (Freund's adjuvant induced rat)	
Stem bark	Cardioprotective	Aqueous extract	300 mg/kg	In vivo (Streptozotocin induced rat)	Bandawane et al., 2011
	Antidiabetic	Aqueous extract	300 mg/kg	In vivo (Streptozotocin induced rat)	Bandawane et al., 2011
	Anti-inflammatory	Methanolic extract	400 mg/kg	In vivo (Carrageenan induced rat paw)	Subraya & Gupta, 2012
Carissa carandas					
Leaves	Antioxidant (DPPH and OH^- radical scavenging)	Methanolic extract	EC_{50} 546 and 498 μg/ml	In vitro	Bhadane & Patil, 2017
	Anti-inflammatory	Methanolic extract	1.3 mg/ml	In vitro	Bhadane & Patil, 2017
	Cytotoxic	Carandinol from ethyl acetate fraction of methanolic extract	IC_{50} 6.87 μg/ml	In vitro (Using HeLa cell line)	Begum et al., 2013
	Hepatoprotective	Methanol and acetone (3:1) extract	100 mg/kg	In vivo (CCl_4 induced liver injury in albino rat)	Bhati et al., 2014
Roots	Anti-inflammatory	Carissone and Scopoletin from methanolic extract	IC_{50} 20.1 and 24.6 μg/ml, respectively	In vitro (Using J774A.1 cell lines)	Galipalli et al., 2015
	Hepatoprotective	Ethanolic extract	400 mg/kg	In vivo (CCl_4 and Paracetamol induced liver injury in albino rat)	Hegde et al., 2009
Catharanthus roseus					
Leaves	Hepatoprotective	Ethanolic extract	500 mg/kg	In vivo (Simvastatin induced albino rat)	Ahmed & Srinivasa Rao, 2013
	Antimalarial	Ethanolic extract	IC_{50} 49.63 μg/ml	In vitro (Antiplasmodial against *Plasmodium falciparum*)	Ravikumar et al., 2012
	Anti- HIV	Methanolic extract	2.5 μg/ml	In vitro (Using CEM-GFP cell lines infected with HIV-1NL4.3)	Sabde et al., 2011
	Antibacterial	Ethanolic extract	5 mg/ml	In vitro	Govindasamy & Srinivasan, 2012
Whole plant	Cytotoxic	Leurosine (Indole alkaloid) from 95% ethanolic extract	IC_{50} 0.73 μM	In vitro (Using MDA-MB-231 human breast cancer cell lines)	Wang et al., 2012
Holarrhena antidysenterica					
Leaves	Antioxidant (Superoxide and OH^- radical scavenging)	Methanolic extract	IC_{50} 500 and 285 μg/ml, respectively	In vitro	Ganapathy et al., 2011
Seeds	Antidiabetic	Ethanolic extract	600 mg/kg	In vivo (Streptozotocin induced rat)	Keshri et al., 2012
	Antiurolithic	70% ethanolic extract	IC_{50} 0.27 mg/ml	In vitro (Inhibition of CaOx crystal aggregation)	Khan et al., 2012
			100 mg/kg	In vivo (CaOx induced urolithiasis rat)	
Nerium oleander					
Leaves	Cardioprotective		300 mg/kg	In vivo (Using guinea pigs)	Botelho et al., 2017

(Continues)

TABLE 3 (Continued)

Plant and plant part	Biological activity	Extract/compound	Effective concentration/dose	Study model	References
		Oleandrin from methanolic extract	0.3 mg/ml	In vitro (Using ventricular myocytes of mice)	
Flower	Antioxidant (DPPH, ABTS, Superoxide and OH^- radical scavenging)	Methanolic extract	IC_{50} 193, 156, 210, and 211 μg/ml, respectively	In vitro	Singhal & Gupta, 2012
	Hepatoprotective	Methanolic extract	400 mg/kg	In vivo (CCl_4 induced liver injury in rats)	Singhal & Gupta, 2012
	Anticonvulsant	50% hydroalcoholic extract	200 mg/kg	In vivo (Pentylenetetrazol induced convulsion in Swiss albino mice)	Singhal & Gupta, 2012
Leaves	Anticandida	Methanolic extract	MIC 1 mg/ml	In vitro	Wankhede et al., 2013
Rauvolfia serpentina					
Roots	Anti-HIV	Methanolic extract	10 μg/ml	In vitro (Using CEM-GFP cell lines infected with HIV-1NL4.3)	Sabde et al., 2011
Seeds	Antimalarial	Petroleum ether extract	100 ppm	In vivo (Mosquito larvicidal)	Das & Chandra, 2012
	Cytotoxic	Reserpine (Indole alkaloid)	IC_{50} 14.52 μM	In vitro (Drug resistant tumour cell lines)	Abdelfatah & Efferth, 2015
Leaves	Antidiarrheal	Methanolic extract	400 mg/kg	In vivo (Castor oil induced diarrhea in mice)	Ezeigbo et al., 2012
Tabernaemontana divaricata					
Leaves	Antioxidant (Metal chelating, OH^-, DPPH, and superoxide radical scavenging)	Ethanolic extract	68% metal chelation by extract (IC_{50} 5.3, 5.2, and 5.8 mg/ml for radical scavenging)	In vitro	Anbukkarasi et al., 2016
	Anti-inflammatory	Hexane of methanolic extract	0.7 $\mu g \cdot cm^{-2}$	In vivo (Croton oil induced edema in albino mice)	Jain et al., 2013
	Anticandida	Methanolic extract	MIC 1 mg/ml	In vitro	Wankhede et al., 2013
	Antidiarrheal	Hydroalcoholic and aqueous extract	300 mg/kg	In vivo (Castor oil induced diarrhea in rats)	Raj et al., 2013
Flower	Antidiabetic	Methanolic extract	300 mg/kg	In vivo (Alloxan induced mice)	Rahman et al., 2011
	Gastroprotective	Methanolic extract	500 mg/kg	In vivo (Aspirin and ethanol induced gastric ulcer in rats)	Ali Khan et al., 2013
	Antifertility and Abortifacient activity	Methanolic extract	500 mg/kg	In vivo (Using female wistar rats)	Mukhram et al., 2012
Whole plant	Hepatoprotective	Ethanolic extract	400 mg/kg	In vivo (DEN and Fe NTA induced albino rats)	Poornima et al., 2014
	Anticonvulsant	Ethanolic extract	400 mg/kg	In vivo (Maximal electroshock, pentylenetetrazol , isoniazid induced convulsion in albino mice)	Basavaraj et al., 2011

(Continues)

TABLE 3 (Continued)

Plant and plant part	Biological activity	Extract/compound	Effective concentration/dose	Study model	References
Thevetia peruviana					
Leaves	Wound healing	Hexane extract	200 mg/kg	In vivo (Dead space wound rat model)	Rahman et al., 2017
Fruits	Wound healing Anticancer	Water extract Methanolic extract	200 mg/kg 100 mg/kg	In vivo (Dead space wound rat model) In vivo (EAC cell line in albino mice)	Rahman et al., 2017 Haldar et al., 2015
Seeds	Antimalarial	Ethanolic extract	IC_{50} 58.83 μg/ml	In vitro (Antiplasmodial against *Plasmodium falciparum*)	Ravikumar et al., 2012
Flowers	Gastroprotective	linalool and 1,8-cineole from volatile oil	33 and 36 mg/kg, respectively	In vivo (Ethanol induced gastric ulcer in mice)	Kumar et al., 2015
Whole plant	Anticancer	Cardenolides from methanolic extract	200 μg/ml	In vitro (Against Hep G_2, HL-60 and PC 62 cancerous cells)	Barbon et al., 2012
Wrightia tinctoria					
Leaves	Antimalarial (Larvicidal)	Petroleum ether and aqueous extract	LC_{50} 0.37 and 0.11%	In vitro (Against filarial larvae *Culex quinquefasciatus*)	Sakthivadivel et al., 2014
Fruit	Antimalarial (Larvicidal) Antidiabetic	Petroleum ether and aqueous extract Methanolic and ethyl acetate extract	LC_{50} 1.31 and 0.09% 300 mg/kg	In vitro (Against filarial larvae *Culex quinquefasciatus*) In vivo (Alloxan induced mice)	Sakthivadivel et al., 2014 Rani et al., 2012
Flower	Antioxidant (Metal chelating, OH^- and DPPH radical scavenging)	Ethanolic extract	IC_{50} 43.16 μg/ml	In vitro	Ramalakshmi et al., 2012
Hancornia speciosa					
Leaves	Anti-inflammatory and wound healing	Ethanolic extract of *H. speciosa* leaves and its isolated compounds	25 μg/ml	In vitro wound healing and cell migration and proliferation assay	Geller et al., 2015
Gymnema sylvestre					
Leaves	Immunomodulatory property	Isolated compound (gymnemic acid)	0.1–20 μg/ml	In vitro as well as in vivo in rat models	Singh et al., 2016

family, *Tabernaemontana divaricata*, was also studied for its antioxidant activity using metal chelating, DPPH, and superoxide radical scavenging methods (Anbukkarasi, Thomas, Sundararajan, & Geraldine, 2016). Qualitative and quantitative DPPH scavenging assays were studied to determine the antioxidative potential of *Tabernaemontana divaricata* leaves extract (Rumzhum, Rahman, & Kazal, 2012). Recently, Bhadane and Patil (2017) evaluated antioxidant activity of methanolic leaves extract and column eluted steroidal fraction of *Carissa carandas* by TLC bioautography method. The results indicated a yellow colored DPPH radical scavenging band by the antioxidant compound on TLC plates against the purple background. *Holarrhena antidysenterica* showed significant ROS scavenging potential and the activity was attributed to the higher total phenolic content of plant extract (Ganapathy, Ramachandra, & Rai, 2011). James et al. (2011) studied the antioxidative potential of *Alstonia scholaris* flower extract, and the reported activity was found higher than fruit extract. Further, significant DNA damage inhibition and antioxidative potential of *Carissa carandas* were studied by Verma, Shrivastava, and Kumar (2015).

5.2 | Anti-inflammatory/analgesic activity

Inflammation and pain are the prime signs of acute and chronic conditions in various diseases. NSAIDs (nonsteroidal anti-inflammatory drugs) are commonly used to treat inflammatory conditions; however, their prolonged use has adverse side effects. Several plants of family Apocynaceae are known for the in vitro and in vivo anti-inflammatory activity. Arulmozhi, Mazumder, Sathiyanarayanan, and Thakurdesai (2012) showed the potent anti-inflammatory activity of dichloromethane fraction from ethanolic extract *Alstonia scholaris* leaves. Subraya and Gupta (2012) studied in vivo anti-inflammatory activity of *Alstonia scholaris* stem bark methanol extract in rat paw edema models. The reported activity of the extract was found comparable to indomethacin (10 mg/kg) with paw edema inhibition of 64.86% and 67.29%, respectively, in carrageenan-induced rat paw edema models. Similarly, Jain et al. (2013) reported significant anti-inflammatory activity of different fractions of *Tabernaemontana divaricata* leaves extracts against croton oil-induced edema in male albino mice model. The activity of hexane and methanolic fractions was found comparatively better than indomethacin with IC_{50} less than 500 μg/cm^2. Purified bioactive compounds such as lupeol, stigmasterol, oleanolic acid, carissone, and scopoletin from *Carissa carandas* roots showed inhibitory potential against proinflammatory mediators (Galipalli et al., 2015). Among them, scopoletin and carissone showed significant inhibition of NO with IC_{50} 24.6 and 20.1 μg/ml, respectively. Recently, in vitro anti-inflammatory activity of steroidal derivative from *Carissa carandas* leaves was studied by erythrocyte membrane stabilizing potential and the activity was found comparable to diclofenac sodium at 3.5 mg/ml (Bhadane & Patil, 2017).

5.3 | Anticancer/cytotoxic activity

Conventional anticancer drugs exert a cytotoxic effect by inhibiting the cell division. However, several side effects on normal cells have restricted the use of many anticancer drugs. Plant-derived compounds are less toxic and have lesser or no adverse effects and, therefore, are preferred in cancer treatment all over the world. A number of in vivo and in vitro studies have been carried out by various researchers to evaluate anticancer and cytotoxic potential of Apocynaceae plants. Among them, the potent cytotoxic activity of methanolic extract of *Tabernaemontana divaricata* leaves was reported by Rumzhum et al. (2012) using brine shrimp lethality bioassay. Wang et al. (2012) reported cytotoxic activity of eight different indole alkaloids from *Catharanthus roseus* against human breast cancer cells (MDA-MB-231) using MTT assay. Among them, leurosine showed higher inhibition of cancer cells with IC_{50} 0.72 μM, which is comparable to standard vinblastine (IC_{50} 0.67 μM). Siddiqui et al. (2012) reported cytotoxic compounds (Odorosides A and B, oleandrin, adynerin, and doxorubicin) from *Nerium oleander* leaves showing growth-inhibiting activities against four human cancer cell lines, namely, MCF-7 (breast), SF-268 (central nervous system), HT-144 (skin), and NCI-H 460 (lung). In vitro anticancer effect of *Alstonia scholaris* was studied on mammary gland carcinoma cells using cytosolic marker enzymes. It revealed cytotoxic as well as the antiproliferative effect of plant extract on tumor cells (Surendren, Jayanthi, & Smitha, 2012). Thereafter, Begum et al. (2013) reported carandinol, a first isohopane triterpene from *Carissa carandas* leaves with a potent cytotoxic activity against cancer cell lines. In another study, methanolic extract of *Thevetia peruviana* fruit extract (50 and 100 mg/kg) showed a significant antitumor activity against Ehrlich's ascites carcinoma cell line in albino mice (Haldar, Karmakar, Chakraborty, Ahmad, & Haldar, 2015). Cardenolides from *Thevetia peruviana* have been reported as a potent anticancer agent with growth inhibition against Hep G_2, HL-60, and PC 62 cancerous cells. Among them, maximum inhibition was recorded against HL-60 cells at a dose of 200 μg/ml (Barbon, da Silva, Sampaio, & Baldo, 2012). Similarly, reserpine, an indole alkaloid from *Rauvolfia serpentina*, was reported as a potent cytotoxic compound against drug-resistant tumor cells and it was concluded that the reserpine could be used in cancer therapy (Abdelfatah & Efferth, 2015).

5.4 | Cardioprotective activity

Cardiac glycosides and cardenolides discussed in the earlier section were referred to be potential cardioprotective agents in various heart-related disorders. *Nerium oleander* and *Thevetia peruviana* are the most important members of this family used in the treatment of cardiovascular complications. The antiatherogenic potential of the *Alstonia scholaris* stem bark extract was evaluated by Bandawane, Juvekar, and Juvekar (2011), and it was observed that low-density lipoprotein (LDL)-cholesterol lowering potential of the extract possibly may be involved in the prevention of cardiovascular complications. Gayathri et al. (2011) studied the cardioprotective effect of *Nerium oleander* flower extract on experimental rats by inducing myocardial oxidative stress using isoproterenol and concluded that tested extract was highly effective in cardioprotection by improving the antioxidative defense during myocardial necrosis. In another study, methanolic extract of *Rauvolfia serpentina* roots provided dual protection, that is, antidiabetic and cardioprotective to the alloxan-induced diabetic mice

(Azmi & Qureshi, 2012). In this study, the extract was found to significantly ($p < .05$) lower total cholesterol, LDL, and very LDL level in the experimental animals.

5.5 | Antidiabetic activity

A number of plants of family Apocynaceae are known to exhibit antidiabetic activity. The antidiabetic effect of the extract could be due to the improvement in the utilization of glucose by peripheral tissues, due to the increased sensitivity of target tissues towards insulin, or due to the better glucose metabolism. The bark of *Alstonia scholaris* exhibited potential antidiabetic effect in rats, which demonstrated its usefulness in long-term complications associated with diabetes mellitus (Bandawane et al., 2011). The ethanolic extract of *Holarrhena antidysenterica* seeds showed antihyperglycemic activity by lowering serum glucose level in diabetic albino rats and significantly increased glucose tolerance. It also prevented weight loss in diabetic rats and corrected biochemical parameters (Keshri, Chandra, & Sharma, 2012). In another study, Rahman, Islam, Ali, Islam, and Hossain (2011) found that methanolic extract of flowers of *Tabernaemontana divaricata* has considerable effect in lowering fasting blood glucose level in alloxan-induced diabetic mice and it was comparable to metformin at the plant extract dose of 300-mg/kg body weight experimental rats. The methanolic extract and partially purified ethyl acetate fraction of *Carissa carandas* unripe fruit significantly lowered the blood glucose level in the experimental animals. Both, the extract and fraction at 400 mg/kg, when given orally to alloxan induced experimental rats, showed potent antidiabetic activity (Itankar et al., 2011). Similarly, significant antidiabetic activity of *Wrightia tinctoria* fruit extracts at 300 mg/kg in alloxan-induced rat model was reported by Rani, Pippalla, Mohan, and Gangaraju (2012).

5.6 | Antimalarial activity

The use of plant metabolites for the treatment of malaria is well documented in the traditional system of medicine. The first antimalarial drug, quinine, was isolated from the bark of *Cinchona* species. Several alkaloids with potent antimalarial activity have been reported in members of family Apocynaceae (Miettinen et al., 2014). Recently, antiplasmodial activity of alkaloids as well as phenolics and flavonoids of 10 different genera of Apocynaceae were systematically reviewed by Chan, Wong, and Chan (2016). Iyiola, Tijani, and Lateef (2011) investigated in vivo antiplasmodial activity of ethanolic extract of *Alstonia boonei* stem bark in *Plasmodium berghei* NK-65-infected mice. Significant ($p < .05$) and a dose-dependent antiplasmodial activity were observed in early infection and curative stages when the extract was administered orally. In another study, Ravikumar, Inbaneson, and Suganthi (2012) reported in vitro antiplasmodial activity of *Catharanthus roseus* and *Thevetia peruviana* against *Plasmodium falciparum*. Of the two plants, *Catharanthus roseus* showed powerful antiplasmodial activity (IC_{50} 49.63 μg/ml). Mosquitos are the vectors of malaria, and hence, mosquito larvicidal activity is also considered as a preventive measure in combating it. In this direction, mosquito larvicidal activity of *Rauvolfia serpentina* seed extracts was reported by Das and Chandra (2012). The higher mortality of mosquito larvae was recorded with petroleum ether extract at 100-ppm level after 24 hr. Later on, the larvicidal activity of *Wrightia tinctoria* fruit and leaves extracts were reported against malaria, filaria, and chikungunya causing *Culex quinquefasciatus* and anopheles vectors (Sakthivadivel et al., 2014).

5.7 | Anti-HIV activity

HIV (human immunodeficiency virus) causes AIDS (acquired immune deficiency syndrome), which leads to generalized immunosuppression. The plants with anti-HIV activity possess phytochemicals as potent immunomodulators. Several investigations have reported antiviral activities of many members of family Apocynaceae. Recently, oleandrin from *Nerium oleander* was reported for its anti-HIV activity (Singh et al., 2013). In vitro assay was performed to evaluate anti-HIV potential by infecting HIV viruses to the HeLa CD4-LTR/β-gal human cell lines. These infected cell lines were treated with oleandrin and Azidothymidine (AZT) and incubated further for 2 hr, and the level of viral infection was measured by assaying RT activity. The results revealed that oleandrin reduced the amount of envelope proteins of virus whereas AZT inhibited viral replication in infected cells by reducing RT activity. In another systematic study, moderate anti-HIV-1 activity (40%-60% inhibition) of alkaloid-rich extracts of *Catharanthus roseus* and *Alstonia scholaris* leaves, as well as from *Rauvolfia serpentina* roots, was reported (Sabde et al., 2011). Betulinic acid (Moghaddam, Ahmad, & Samzadeh-Kermani, 2012) and ellagic acid (Narayan & Rai, 2016) from different plant sources were reported for their anti-HIV potential. The other members of family Apocynaceae such as *Carissa carandas*, *Nerium oleander*, *Holarrhena antidysenterica*, and *Tabernaemontana divaricata* are known to contain betulinic acid and ellagic acid as a major phytoconstituents. However, their anti-HIV activity is not yet established.

5.8 | Gastroprotective activity

Gastric and duodenal ulcers are the most prevalent gastrointestinal disorders all over the world. There is an urgent need to develop a well-targeted therapeutic strategy for the treatment of these ulcers. *Tabernaemontana divaricata* was earlier reported to be effective in gastroprotective complaints. In vivo studies carried out by pyloric ligation (Khan, 2011) as well as aspirin and ethanol (Ali Khan et al., 2013) induced gastric ulceration in rat models. In both studies, methanolic extract of *Tabernaemontana divaricata* flowers significantly ($p < .05$) reduced ulcer index, total acidities, and volume of gastric juice. The results were found comparable with standards omeprazole (8 mg/kg) and misoprostol in both the studies. The antiulcer activity of alcoholic extract of *Carissa carandas* roots was studied in acetic acid-induced chronic gastric ulcer in a female rat model (Merai & Jadhav, 2014). This study revealed that alcoholic extract more significantly ($p < .001$) reduced ulcer index and resulting effect was similar to the effect of Ranitidine at concentration 500 mg/kg. Besides ulcer index, other parameters such as free acidity, total acidity, mucin, and pepsin content as well as total protein content were also evaluated in control, extract, and standard treated groups. The gastroprotective effect of *Thevetia peruviana* volatile oil (linalool and 1,8-cineole as principal

constituents) was evaluated in mice (Kumar, Shukla, Shukla, & Alok, 2015). Oral as well as inhaled (for 60 min) volatile oil (100 µl/kg) showed significant activity against ethanol-induced gastric ulcers but did not show a protective effect in indomethacin-induced mice model.

5.9 | Hepatoprotective activity

Hepatoprotective activity of *Nerium oleander* flower (Singhal & Gupta, 2012) and *Carissa carandas* leaves (Bhati, Shukla, & Sharma, 2014) was evaluated in carbon tetrachloride (CCl_4)-induced hepatotoxicity or liver injury in albino rats. In both the studies, hepatoprotective activity was evaluated by histopathological observations and by measuring total bilirubin, serum glutamate pyruvate transaminase and serum glutamate oxaloacetate transaminase. Similarly, reduced level of bilirubin, serum glutamate pyruvate transaminase, and serum glutamate oxaloacetate transaminase also revealed potent hepatoprotective activity. Besides that, a root extract of *Carissa carandas* was used as hepatoprotective medicine by tribal people of the Western Ghat and was validated by Hegde et al. (2009). Similarly, the hepatoprotective activity of *Catharanthus roseus* leaves extract was reported in simvastatin-induced hepatotoxicity in rats (Ahmed & Srinivasa Rao, 2013). Moreover, the hepatoprotective activity of ethanolic extract of the whole plant of *Tabernaemontana divaricata* was reported in DEN as well as Fe NTA-induced liver injury in Wistar albino rats (Poornima, Chella Perumal, & Gopalakrishnan, 2014).

5.10 | Antimicrobial activity

Antimicrobials are the compounds that inhibit the growth of microorganisms even at low concentrations. Antimicrobial compounds are secondary metabolites synthesized by microorganisms themselves or by plants. A wide range of metabolites such as alkaloids, terpenoids, phenolics, steroids, and flavonoids are known to have antimicrobial activity (Ncube, Afolayan, & Okoh, 2008). Different extracts of *Tabernaemontana divaricata* leaves were studied for antibacterial potential against bovine mastitis-causing bacteria isolated from clinical cases of particular disease (Gopinath, 2012). Isolated strains were *Streptococcus agalactiae*, *Streptococcus uberis*, *Escherichia coli*, and coagulase-negative *Staphylococcus aureus*. Among all these strains, *Staphylococcus aureus* was found most susceptible to methanolic extract with zone of inhibition of 26 mm, and the least response was shown by *Streptococcus agalactiae* with zone of inhibition of 18 mm. Besides that, ethanolic extract of *Wrightia tinctoria* flowers (at 250 µg/ml) showed broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacterial strains (Ramalakshmi et al., 2012). Maximum zone of inhibition (14 mm) was recorded against *Staphylococcus aureus* whereas the least response was shown by *Klebsiella pnuemoniae*. The GC-MS analyses of this extract revealed hexadecanoic acid as a major component. Similarly, the antibacterial potential of different parts (leaves, stem, roots, and flowers) of *Catharanthus roseus* was evaluated against common human pathogens (Govindasamy & Srinivasan, 2012). Ethanolic extract of *Catharanthus roseus* leaves showed excellent antibacterial activity against tested pathogens. However, the antifungal activity of *Tabernaemontana divaricata*, *Catharanthus roseus*, and *Nerium oleander* was reported only against *Candida albicans* (Wankhede, Routh, Rajput, & Karuppayil, 2013). In this study, the Minimum Inhibitory Concentration (MIC) of ethyl acetate extracts of *Tabernaemontana divaricata*, *Nerium oleander* and methanolic extract of *Catharanthus roseus* was found to be 1 mg/ml. The Minimum Fungicidal Concentration (MFC) of different solvent extracts of *Nerium oleander* was recorded as 8 and 16 mg/ml. Further, this study concluded that *Nerium oleander* could be used topically in vaginal candidiasis or other Candida infections.

5.11 | Other effects

Arulmozhi, Mazumder, Sathiyanarayanan, and Ashok (2011) studied the in vivo antiarthritic activity of ethanolic extract of *Alstonia scholaris* in Freund's adjuvant-induced arthritis in a rat model. The levels of glutathione, glutathione peroxidase, superoxide dismutase, malonaldehyde, and myeloperoxide from synovial tissue of a sacrificed animal after 28 days were found to be elevated. On the other hand, the myeloperoxidase arthritic index, gastric lesions indices, and gastric juice secretion were found to be significantly decreased.

The antidiarrheal potential of *Rauvolfia serpentina* (Ezeigbo et al., 2012) and *Tabernaemontana divaricata* (Raj, Balasubramaniam, & Nadeem, 2013) was evaluated by castor oil-induced diarrhea in mice and rat, respectively. The extracts of both the plants showed a significant dose-dependent antidiarrheal activity. All doses of extracts (100–400 mg/kg) of *Rauvolfia serpentina* showed comparable antidiarrheal activity with standard drug atropine sulphate (3 mg/kg) with a reduction in intestinal weight and fluid volume. Similarly, *Tabernaemontana divaricata* extract doses (100–300 mg/kg) resulted in significantly ($p < .05$) reducing the wet feces in rats comparable with standard loperamide (5 mg/kg).

Tabernaemontana divaricata was further found to have anticonvulsant (Basavaraj, Shivakumar, Shivakumar, & Manjunath, 2011) and antifertility activity (Mukhram, Shivakumar, Viswanatha, & Rajesh, 2012). Anticonvulsant activity was analysed by estimating gamma-aminobutyric acid level in various convulsion (maximal electroshock, pentalenetetrazol, strychnine, picrotoxin, and isoniazid) induced albino mice. This study concluded that alcoholic extract of *Tabernaemontana divaricata* flowers had potent anticonvulsant activity at various doses (100, 200, and 400 mg/kg). The resulting activity of reduced duration of tonic extensor phase in tested animals was comparable to standard diazepam at 5 mg/kg. An increased gamma-aminobutyric acid level in serum was also reported after treatment with extract and gabapentin at 20 mg/kg. In another study, different extracts of *Tabernaemontana divaricata* flowers were found to have antifertility activity. The methanolic extract of flowers at 500-mg/kg body weight showed a significant estrogenic as well as the early abortifacient activity in female rat model. Similarly, the anticonvulsant activity was also reported in *Tabernaemontana divaricata* and in *Nerium oleander* (Singhal & Gupta, 2011). In this study, hydroalcoholic extract (100 and 200 mg/kg) of *Nerium oleander* flower significantly reduced ($p < .01$) spontaneous locomotors activity with protection against electroshock-induced convulsions.

The in vivo and in vitro studies were conducted to prove antiurolithic activity of crude extract of *Holarrhena antidysenterica* seeds (Khan, Khan, & Gilani, 2012). In vivo assay was studied using

ethylene glycol-induced calcium oxalate (CaOx) urolithiasis rat model and in vitro assay by inhibition of CaOx crystal aggregation potential. The in vitro study showed that crude extract did not show nucleation but had the ability to inhibit CaOx crystal aggregation similar to potassium citrate (a well-known CaOx crystallization inhibitor). Similarly, results of an in vivo study showed inhibition of calcium oxalate monohydrate crystal formation in urine. These results suggested that the crude extract of *Holarrhena antidysenterica* seeds has potential to reduce the risk of stone disease.

Of late, hexane and water extracts of *Thevetia peruviana* leaves and fruits respectively were evaluated for their wound healing potential (Rahman, Rahman, Haris, & Mahmood, 2017). The activity was studied on incision, excision, and using dead space wound rat models. Both the extracts showed the highest wound breaking strength on skin collagen tissue development as well as early epithelization. This wound healing potential was also proved by enhanced antioxidant activity and histological data on more collagen formation.

5.12 | Toxicity studies

Despite ever-increasing interest in the medicinal uses of Apocynaceae plants, only a few reports are available on toxicological aspects of these plants. Some members of this family are reported for pediatric poisoning, but reports on serious poisonings are very rare (Saravanapavananthan & Ganeshamoorthy, 1988). Besides that, some of the plants are reported for emetic potential and have antitoxins against snake poisoning. The plants with poisonous effects have already been documented in the U.S. FDA poisonous plant database (www.accessdata.fda.gov/scripts/) as well as documented in different books and research articles (Campbell, 1983; Eddleston & Persson, 2003). According to Eddleston et al. (1999), seeds of *Thevetia peruviana* cause dizziness, vomiting, and cardiac dysrhythmias upon ingestion. Scientific investigations suggested that in vivo rat models could tolerate 2,000-mg/kg body weight dose of *Nerium oleander* extract after oral administration during 14 days of observation (Singhal & Gupta, 2011). Similarly, no mortality as well as no adverse effect have been observed throughout 14 days observation with a dose of 2,500-mg/kg body weight of *Wrightia tinctoria* extract that was administered in ICR mice (Jain, Bari, & Surana, 2011). In another study, ethanol, methanol, and chloroform extracts of *Carissa carandas* were studied for acute oral toxicity and none of them showed any sign of toxicity during observation period (Alam, Shahriar, & Bhuiyan, 2014). A range of 0.1-, 0.5-, and 1-g/kg body weight dose of *Catharanthus roseus* extract was given orally up to 14 days to the Sprague Dowley rats (Kevin, Hussin, Zhari, & Chin, 2012). The serum biochemistry, as well as liver and kidney functions, were determined. After 14 days, no significant changes were observed in body weight as well as organ weight, serum biochemistry, and liver and kidney functions. Prior to determining the antifertility potential of *Tabernaemontana divaricata*, methanolic and aqueous extracts were evaluated for their acute oral toxicity in Swiss and Wistar albino mice (Mukhram et al., 2012). No morbidity and mortality were shown by both animal models upon administration of 2,000-mg/kg body weight dose of methanolic and aqueous extracts. Acute toxicity of different fractions and methanolic extract of *Carissa carandas* fruits was evaluated to prove its nontoxic nature. But all extracts and fractions of *Carissa carandas* showed decreased blood glucose level in oral glucose tolerance test (Itankar et al., 2011).

6 | BIOTECHNOLOGICAL STRATEGIES USED FOR PRODUCTION OF BIOACTIVE METABOLITES

Plant secondary metabolites have widely been used as drugs, pigments, food supplements, fragrance, and biopesticides. However, indiscriminate use and overharvesting have threatened many medicinally valuable plant species. Therefore, there is a need to explore alternative approaches to obtain commercially/medicinally important metabolites of plant origin in a sustainable manner. Various biotechnological tools have been used for the large-scale and sustainable production of bioactive compounds. Several members of Apocynaceae have been studied for in vitro propagation, hairy root culture using tools and techniques of plant tissue culture (Ref) Callus and cell suspension culture are widely used for the production of bioactive metabolites similar to native plant. Of late, *Agrobacterium rhizogenes*-mediated transformation is largely explored for bioactive metabolite production using hairy root culture.

6.1 | Callus and cell suspension culture

Callus is an unorganized and proliferative mass of cells growing on artificially prepared solid medium. In vitro callus induction can be achieved by supplying essential nutrients such as carbon and nitrogen source, vitamins, minerals, and phytohormones in the medium (Perez-Jimenez, López-Soto, & Cos-Terrer, 2013). Callus consists of varying cell lines that differ in secondary metabolite production. Some cell lines show intracellular metabolite synthesis, and some others show extracellular. Hence, callus tissue upon extraction in suitable solvent releases several bioactive metabolites (Karuppusamy, 2009). Screening of maximum bioactive metabolite producing as well as genetically stable cell line from callus is therefore most important. If such screened cells are used as inoculum for cell suspension culture, it gives increased production of the desired compound. The cell suspension culture is nothing but growth of in vitro grown friable callus in moving liquid culture medium. Cell suspension culture improves nutrient utilization ability of plant cells as compared to callus culture, and it also yields maximum cell biomass (Raharjo, Eucharia, Chang, & Verpoorte, 2010). In this system, stable cell lines from callus show luxuriant growth with increased secondary metabolite production. The metabolite yield can be further improved by precursor feeding or elicitors treatment. Elicitation triggers bioactive metabolite production by applying chemical or physical stress in moving liquid cultures. Nowadays, elicitation treatment is found to be effective for the increased production of such metabolites that are usually not produced by plant cells.

In vitro grown callus of *Holarrhena antidysenterica* was reported to possess bioactive metabolites like the host plant. Phytochemicals such as steroids were isolated from callus of this plant, and callus extract was reported for antimicrobial activity against *Staphylococcus aureus*, *Salmonella typhimurium*, and *Escherichia coli* (Mahato, Mehta,

& Roy, 2013). Bioactive glycosides such as Thevetin B, peruvoside, digitoxigenin, and neriifolin were qualitatively and quantitatively determined by high-performance liquid chromatography from the callus of *Thevetia peruviana* (Taha, Farag, Shams, Abdel-Azim, & Seif El-Nasr, 2011). Cell suspension culture strategies have also been employed for the large-scale production of secondary metabolites. A number of reports are available on cell suspension culture of *Catharanthus roseus* and *Tabernaemontana divaricata* for the production of alkaloids. Stafford, Smith, and Fowler (1985) studied the regulation of growth and alkaloid synthesis in suspension culture of *Catharanthus roseus* up to 45 days and reported increased biomass with alkaloid content. Indole, as well as terpenoidal indole alkaloid synthesis, was also reported in cell suspension culture of *Tabernaemontana divaricata* (Dagnino, Schripsema, & Verpoorte, 1993; Van der Heijden, Lamping, Out, Wijnsma, & Verpoorte, 1987). The alkaloid-producing cell lines were further reported for biotransformation of indole alkaloids when fed in suspension culture. Panda, Mishra, and Bisaria (1992) reported maximum alkaloid production (0.66 g/100 g cell biomass) in *Holarrhena antidysenterica* cell suspension culture. Of late, several researchers studied the effect of precursors and elicitors to enhance secondary metabolite production in cell suspension culture. El-Sayed and Verpoorte (2002) studied the effect of precursor feeding on alkaloid accumulation in *Catharanthus roseus* cell suspension culture. They reported increased methyl jasmonate accumulation in culture upon feeding with tryptamine and loganine. On the other hand, elicitation with methyl jasmonate in cell suspension culture of *Thevetia peruviana* enhanced peruvoside (cardenolide) production (Zabala, Angarita, Restrepo, Caicedo, & Perea, 2010). The highest oleandrin (antitumor metabolite) production was reported with *Nerium oleander* suspension culture in a medium fortified with progesterone and cholesterol (Ibrahim et al., 2009). In another study, oleandrin production was achieved using the combined mechanism of *Agrobacterium tumefaciens* mediated transformation and fungal elicitation in *Nerium oleander* cell suspension culture (Ibrahim et al., 2007). Arias, Zapata, Rojano, and Arias (2016) studied the effect of different light wavelengths on cell biomass accumulation, phenolic content, and antioxidant activity. The results of this finding showed that phenolic content and antioxidant capacities of light-treated cultures were lower than that of culture treated in dark. However, total phenols were found between 7.21 to 9.46 mg gallic acid equivalent (GAE)/g of cell biomass extract for all light conditions.

6.2 | Hairy root culture

In recent decades, hairy root culture has received renewed interest all over the world. Secondary metabolite production using hairy roots attributed to expression of tDNA from Ri plasmid of *Agrobacterium rhizogenes*. Growth capacities of hairy roots are due to the lateral root formation from the first day to 1 week (Maldonado-Mendoza, Ayora-Talavera, & Loyola-Vargas, 1993). Sometimes, exogenously supplied auxin in trace amount is reported to increase both elongation and lateral branching (Hashimoto, Yukimune, & Yamada, 1986). Several researchers have reported long-term genetic stability of hairy roots during culture (Baiza, Quiroz-Moreno, Ruíz, & Loyola-Vargas, 1999). In addition to growth, hairy root culture has showed unique secondary metabolite production abilities, which may not always be the same as that of plant roots (Parr & Hamill, 1987). Besides that, hairy root culture can produce secondary metabolites that parallel with root growth. Hence, it is possible to continuously obtain secondary metabolites during hairy root culture, which is not possible with cell suspension cultures (Holmes, Li, Green, Ford-Lloyd, & Thomas, 1997).

Hairy root culture has been successfully established for many medicinally important plants. Among selected members of Apocynaceae family, *Catharanthus roseus* and *Rauvolfia serpentina* are only reported for *Agrobacterium rhizhogenes*-mediated gene transfer in hairy root culture. Several reports are available on hairy root culture of *Catharanthus roseus* and *Rauvolfia serpentina* for the production of alkaloids. Bhadra, Vani, and Shanks (1993) reported indole alkaloid producing hairy roots from seedling of *Catharanthus roseus* through infection of *Agrobaccterium rhizogenes* strain 15834. The same strain as well as strain A4 was used to produce alkaloids from hairy roots of *Rauvolfia serpentina*. Thereafter, inventions on hairy root culture from both the plants are continuously on. Sim, Chang, Liu, and Jung (1994) reported production of catharanthine and ajmalicine (67 and 30.2 mg/L) from fungal elicited and *A. rhizogenes* (15834) infected hairy roots. The same compounds were also isolated from 2-year-old transformed hairy roots of *Catharanthus roseus*. The infection and elicitation respectively were carried out using *Agrobacterium rhizogenes* strain 1855 and methyl jasmonate, fungal homogenates of *Aspergillus sp*. and *Trichoderma sp*. Later, production of catharanthine using NO elicitation (Zhou, Zhu, Shao, Wu, & Tang, 2010) and terpenoidal indole alkaloids (ajmalicine and serpentine) have also been reported from hairy roots of *Catharanthus roseus* (Batra, Dutta, Singh, Kumar, & Sen, 2004). Three new sarpagine group monoterpene alkaloids were isolated from hairy root culture of *Rauvolfia serpentina* (Sheludko, Gerasimenko, Kolshorn, & Stöckigt, 2002). Recently, Hanafy, Matter, Asker, and Rady (2016) reported maximum accumulation of vinblastine, vincristine, and catharanthine in hairy root culture of *Catharanthus roseus* in full strength liquid MS medium. Besides, a potential antimicrobial activity of these isolated compounds was reported against tested Gram-positive bacteria, fungi, and yeast.

6.3 | Plant endophytes

Endophytes are the microorganisms that colonize inside the host plant tissues without causing any harm to them (Bacon & White, 2000). Endophytes mostly establish a symbiotic relationship with their plant host, but some act as parasites, and these complexities are varied among hosts and microbes (Verma, Kharwar, & Strobel, 2009). During their association with the host plants, they produce similar phytochemicals as that of their host plants (Puri, Verma, Amna, Qazi, & Spiteller, 2005). Hence, there are huge possibilities to explore endophytic microorganism for production of bioactives in a sustainable manner. Sustainable secondary metabolites production can be achieved after successful isolation of endophytic microorganism followed by its mass cultivation.

A plethora of secondary metabolites have been isolated from endophytic microorganisms. Among these, fungi are the most widely explored group of endophytes. Hundley (2005) isolated epoxycytochalasin derivatives and xylobovide from endophytic fungi (genus *Xylaria*) of *Alstonia scholaris*. The isolated compounds were reported for in vitro cytotoxic activity against HeLa cancer cells. The anticancer metabolite vincristine and vinblastine were isolated from *Fusarium oxysporum*, an endophytic fungus of *Catharanthus roseus* (Kumar, Patil, Rajamohanan, & Ahmad, 2013). The culture filtrate (1 L) yielded 67 and 76 μg vincristine and vinblastine, respectively. Besides that, endophytic *Penicillium sp*., *Colletotrichum gloeosporioides*, and *Aspergillus awamori* have been isolated from *Rauvolfia serpentina* (Nath, Chattopadhyay, & Joshi, 2015). The extract obtained from culture filtrates of these endophytes revealed the dominant presence of phenolic compounds, steroids, and flavonoids. Further, these extracts exhibited strong antimicrobial, antioxidant, and hypocholesterolemic activities. Huang, Cai, Hyde, Corke, and Sun (2007) isolated more than 15 endophytic fungi from *Nerium oleander*, and the culture filtrate was studied for antioxidant activity. Among all these isolates, *Chaetomium sp*. contained the maximum phenolics and showed strong antioxidant activity. Further, *Carissa carandas* is another potential member of Apocynaceae family, which is reported for the presence of various bioactive compounds in different parts. A total of 126 endophytic fungi from 12 different genera were isolated from the leaves of *Carissa carandas* (Tenguria, Firodiya, & Khan, 2012), but bioactive secondary metabolites from these fungi are yet to be described.

7 | MICROPROPAGATION: A STRATEGY FOR CONSERVATION OF APOCYNACEAE PLANTS

Tissue culture is based on the theory of totipotent ability of the plant cell to regenerate the entire plant from explant tissue. Nowadays, medicinal and aromatic plants are the main objects for in vitro propagation and large-scale production of secondary metabolites with pharmaceutical, food, and cosmetic applications (Karuppusamy, 2009). Due to overutilization of medicinal and aromatic plants for commercial purpose, many species are already extinct and remaining are under the endangered category. Micropropagation is considered as the best solution for the conservation of elite genotype of plants. Successful shoot tip/meristem culture, lateral bud culture, and somatic embryogenesis can lead to conservation of plant genotypes. The formation of bud from explant of tobacco led to the discovery of kinetin and many functional cytokinins (Miller & Skoog, 1953). Somatic embryogenesis for carrot explant using coconut milk as a source of nutrient was reported (Steward, 1958). Single cell by somatic embryogenesis has potential to regenerate entire plant due to the totipotent ability of cell. Embryogenesis and organogenesis from pollen culture of datura anthers were studied by Forster, Heberle-Bors, Kasha, and Touraev (2007).

Due to overexploitation, many species of family Apocynaceae are on the verge of extinction and conservation efforts are urgently needed. Clonal propagation has already been used for conservation of a few plants. The plants such as *Holarrhena antidysenterica* and *Carissa carandas* bear various bioactivities, and they are slow growers, and seeds of these plants have limited viability and poor germinability. The seeds of *Carissa carandas* are quite delicate and need to be sown immediately after they are picked up from the fruit. The seeds of these plants also cannot be preserved for a longer duration. Attempts of preservation led to loss of their germination ability. In vitro propagation of *Holarrhena antidysenterica* using plant growth regulators in solid MS medium was also reported (Kumar, Sharma, & Agrawal, 2005; Mallikarjuna & Rajendrudu, 2007). Plant growth regulators (PGRs) employed were 6-Benzylaminopurine (BAP), kinetin, 1-Naphthaleneacetic acid (NAA), Thidiazuron (TDZ), and adenine sulphate for multiple shoot induction from nodal cuttings of the wild plant. Similar attempts have been made for the propagation of *Carissa carandas* using shoot apex and nodal part (Hasmah, Bhatt, & Keng, 2013; Imran, Begum, Sujatha, & Mallaiah, 2012; Rai & Misra, 2005). Some reports are available on in vitro propagation of *Rauvolfia serpentina* (Sarker, Islam, Islam, Hoque, & Joarder, 1996) and *Nerium oleander* (Botelho et al., 2017; Vila et al., 2010). Among them, *Nerium oleander* was propagated for ornamental purpose because some members of this family are garden plants. More combinations of PGRs at increased concentrations were used in earlier studies for in vitro propagation, which ultimately enhanced the production cost. Hence, there is a need to use alternate and economical source of PGRs to reduce production cost as well as to get maximum shootlet production in lesser time.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research Article

Microbial transformation of crop residues into a nutritionally enriched substrate and its potential application in livestock feed

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Abstract
Bioconversion of three different agro-residues (groundnut shells, pigeon pea husk and wheat straw) was studied using endophytic fungi with a view to increasing the nutritive value and to evaluate its feasibility as poultry feed. An endophytic fungal isolate obtained from *Celastrus paniculatus,* effectively biotransformed selected agro-residues in solid state fermentation. After 21 days incubation, isolate CPL-1significantly altered the nutritional values of all tested agro-residues. Cellulose, hemicellulose, and lignin content were significantly reduced ($P < 0.05$) whereas, total carbohydrates were significantly increased in the biotransformed waste as compared to untreated residues. Of the three agro-residues studied, the groundnut shells were found to have maximum carbohydrate content (13.92 ± 0.7 g/100 g) after the treatment. Similarly, the total crude protein and total nitrogen contents of the treated waste were also significantly improved ($P < 0.05$) as a function of treatment with the isolate CPL-1 with their highest contents (24.95 ± 1.4 and 15.53 ± 1.2 g/100 g, respectively) recorded in the treated groundnut shells. The isolate CPL-1 was identified as *Colletotrichum* spp. based on the morphology. The tannins and phytate contents were found to be significantly lower ($P < 0.05$) in the processed wastes. Application of treated agro-residues in poultry diets revealed that the biotransformed groundnut shells and pigeon pea waste can be added up to 20 and 10%, respectively to the commercial poultry diet used in the study without any adverse effects. The results showed that the treated residues of groundnut shells can be used as a partial substitute to the conventional poultry diets as they are rich in enzyme phytase and other nutrients and have good digestibility.

Keywords Bioconversion · Agro-residues · Endophytic fungi · Crude protein · Poultry diet

1 Introduction

Feed is the most important factor in the poultry business which constitutes around 70% of the total production cost [1]. A number of ingredients are used to formulate the poultry diet. It mainly uses maize and soybean meal as the carbon and protein source, respectively. Several countries use other grains such as wheat, sorghum, canola and sunflower meal as well as animal-derived protein ingredients like fish and meat meal [2]. Mineral supplements play a vital role in the development of poultry. Poultry, being the monogastric animal, cannot fully assimilate the inorganic supplements which are provided in the form of calcium and phosphorus supplements that includes dicalcium phosphate, rock phosphate and bone meal [3]. The increasing cost and decreasing feed production are the major hurdles in the progress of poultry industry in the developing countries. Moreover, diversion of food grains such as maize and sorghum from feed market to ethanol production has dramatically increased their cost globally [4, 5]. Therefore, there is the urgent need to use alternative feedstuffs and look into the possibilities of bioconversion of agro-residues into feed in a sustainable way. Globally, 140 billion metric tons of lignocellulose biomass

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is generated every year from agriculture. India, being the agro-based country, the huge amount agro-wastes has raised the challenge to dispose or reuse this biomass. Ministry of New and Renewable Energy (MNRE 2009), Govt. of India estimated that about 500 Mt of crop residue is generated every year. These wastes are usually destroyed by burning that create environmental pollution [6]. Cellulose, hemicellulose, and lignin are the major polymers present in agro-residues which are used as a cheaper feed source for ruminants. However, digestibility of these polymers is the major concerns for their utilization as feed especially in a monogastric animal such as poultry. Thus, bioconversion of these complex polymers into readily available simple sugars and amino acids using endophytic fungi can significantly increase the digestibility and palatability of the agro- residues. Moreover, utilization of such waste for poultry feed cannot only reduce feed cost of the poultry farming but also reduce the environmental pollution. The conversion of lignocellulosic biomass into digestible carbohydrates is biologically feasible and can be easily adopted for bioconversion of agricultural wastes. The world over, attempts have been made for bioconversion of agricultural residues into value-added products such as pulp, animal feed, poultry feed and biofertilizer through the action proteolytic and lignin-degrading enzyme secreting microorganisms such as bacteria and fungi [7]. Although significant scientific work has been undertaken in this direction, no efforts made to develop and scale up the process for the production of valuable end products such as poultry feed using the endophytic fungi. Endophytic microorganisms multiply inside the living tissues of the plant without causing any negative effect on their host. Biotechnological potential of a number of endophytic fungi is well established [8]. In the present work, we made an attempt to isolate, identify and evaluate bioconversion potential of phytase producing endophytic fungal isolates obtained from *Celastrus paniculatus*. The study also evaluated the nutritional quality of the biotransformed residue and evaluated its suitability as substitute for commercially used poultry feed in broiler chicken.

2 Materials and methods

2.1 Source of endophytic fungi for bioconversion studies

Endophytic fungi were isolated from the symptomless parts of *C. paniculatus* like leaves and twigs. Plant specimens were collected from Toranmal forest, MS, India (1115 m AMSL, 21.26° N, 74.09° E and 21.26° N, 27° E) India. The mature and healthy plant specimens were brought safely to the laboratory and processed immediately to reduce the risk of contamination. Plant material was thoroughly washed with running tap water and air dried in laminar flow hood. Washed tissues of leaves, bark, and twigs were surface sterilized using 0.5% sodium hypochlorite (vol/vol) for 5 min followed by 70% ethanol (vol/vol) for 3 min, and finally rinsed with sterile distilled water 5 times. After surface sterilization, the samples were air dried under laminar flow hood. The samples were cut into small pieces (approximately 0.5 cm × 0.5 cm) with the sterile scalpel. A piece of inner tissue (0.5 cm × 0.5 cm) was placed aseptically on the surface of sterile water agar plates and incubated at 25 °C for 12 h dark and light cycle until fungal hyphae emerge out of plant tissue. After incubation of 10 to 15 days, fungal hyphae were observed. Individual hyphal tips were removed from the plate and aseptically transferred onto fresh sterile potato dextrose agar (PDA) medium supplemented with chloramphenicol (40 µg/mL) to avoid bacterial contamination [9]. Plates were incubated for 1 to 2 weeks at 25 °C to obtain a pure culture of fungi. Isolated endophytic fungal cultures were designated a code based on the plant sample from where they were isolated. All the isolates were maintained by subculturing at monthly intervals and stock cultures were preserved at 4 °C. From actively growing a stock culture, sub-culturing were made on fresh slants and after 7 to 8 days of incubation at 25 °C these were used as starting a culture for identification as well as for bioconversion studies.

2.2 Screening for phytase production

The isolated strains of the endophytic fungi were screened for their ability to produce phytase. The screening was carried out by growing the isolated fungi on agar plates containing (g/L) Dextrose 12; NH_4NO_3 4.0; $MgSO_4{\cdot}7H_2O$ 0.5; KCl 0.5; $MnSO_4{\cdot}7H_2O$ 0.01; $FeSO_4{\cdot}7H_2O$ 0.03 and calcium phytate 5 as a source of phosphorus (pH 5.5, after sterilization). The isolates producing clear halos were selected as phytase producers. The isolates showing halos in the primary screening were subjected to secondary screening in a shake flask culture. The sporal suspension was obtained by scrubbing the pure culture with 5 mL of a sterile aqueous solution of 2% tween 80. Inoculum was developed by inoculating 5 mL spore suspension (approximately 10^6 spores/mL) in 250 mL Erlenmeyer flask containing 50 mL seed medium containing (g/L) glucose 20; NH_4NO_3 5.0; $MgSO_4{\cdot}7H_2O$ 0.5; KCl 0.5; $MnSO_4{\cdot}7H_2O$ 0.01 $FeSO_4{\cdot}7H_2O$ 0.03 and calcium phytate 5. After the development of sufficient biomass, 10% of inoculum was transferred to production medium with the same composition. The pH of the media was maintained at 5.5 (after sterilization). The fermentations were carried out at 30 °C and 150 rpm on a rotary shaker (Steelmate Novatech, Pune, India) in a 500 mL shake flask containing 150 mL of production medium.

At the end of the process, fungal biomass separated by centrifugation, dried and the clear supernatant was used for determining the enzyme activity.

2.3 Bioconversion studies of various agro-wastes using phytase positive endophytic isolates in solid state fermentation (SSF)

Different agricultural wastes were collected from nearby farms of Shirpur city. The agricultural wastes selected for the study included wheat bran, pigeon pea husk and shells of groundnut. The good quality, low-density agro-residues of the selected crops were washed thoroughly with boiling water; sun dried and pulverized using to obtain particles of 4 to 5 cm size. Flat glass bottles (18 cm × 7.5 cm) with a volume of 350 mL were filled with 15 to 20 g of respective agro-residue and sterile distilled water was added to achieve different moisture levels (50%, 60%, 70% and 80%). Large scale bioconversion studies were carried out using shallow bamboo trays. The baskets were filled with pre-washed; sun dried and pulverized agro-residues of the selected crops. The solid matrix was supplemented with 2% ammonium nitrate and glucose as nitrogen and carbon source, respectively. Spore suspension (10^8 spores per gram of solid matrix) of the bioassay positive test fungi was inoculated aseptically onto the surface of the solid matrix. The bottles were incubated at 30 °C for 3 weeks and the growth of fungi was monitored. After 3 weeks, the bioprocess was terminated and the nutritional parameters of the processed material were determined.

2.4 Determination of nutritive value of agro-residues before and after bioconversion

Cellulose, hemicellulose, and lignin were determined as per the methods described by Huang et al. [25]. Total N was determined by the Kjeldahl's assay. Total protein content was determined by precipitation of washed sample with trichloracetic acid (TCA) followed by the Kjeldahl's assay. The nitrogen (N) content was calculated from the titer value and the crude protein was obtained by multiplying the N content by a factor (N × 6.25). A blank determination was carried out simultaneously. Tannins were determined as per the previously reported spectrophotometric method [10]. The phytate content was measured by ferric chloride assay. Standard ferric chloride precipitates phytate in acidic conditions [11]. The mineral (Ca, Fe, Cu, Zn, Mg, and Co) contents were determined on aliquots of the solutions of the ash by established atomic absorption spectrophotometer procedures using a Perkin-Elmer atomic absorption spectrophotometer (model 372), while Na and K were analyzed with a flame photometer. Dry weight (DW) was determined gravimetrically by drying at 105 °C to a constant weight. Total carbohydrates and total fats were measured as per the methods of analysis of the AOAC International [12].

2.5 Animal feed, feed preparation using fermented biomass and poultry trials

A commercially available broiler feed (SKM Animal Feeds & Foods Limited, TN, and India) was purchased from the local market and used as a starter and basal diet for broiler chickens. Broiler Starter-Crumble Broiler was used as the basal diet for chicks of 0 to 4 weeks of age. Whereas, Broiler Finisher-Mash Broiler was used in the young chickens from 4 to 6 weeks as per the manufacturer's recommendations. The test diets (starter and finisher) were prepared by supplementing the standard basal diet with 5% and 20% (wt/wt) of fermented biomass. The poultry trials were conducted using 600 one-day-old white broiler chicks up to 6 weeks of age at Wathoda Poultry Farm, Shirpur, MS, India. The birds were divided into 4 groups ($n = 100$). Group A birds were fed standard diets supplemented with 5% fermented biomass. Groups B and C chickens were fed the basal diet plus 10% and 20% fermented biomass, respectively. Group D served as a standard control which received normal basal and finisher diet as per manufacturer's recommendations. Birds were housed in the poultry farm and were exposed to light 24 h a day. The feed and water were provided ad libitum and the animals were vaccinated as per the schedule.

3 Results

3.1 Endophytes isolation and preliminary screening

Total 18 strains of endophytic fungi were isolated from the various parts of *C. paniculatus*. The isolates were designated by different codes and were maintained at 4 °C. All the isolates were subjected for preliminary screening to evaluate their phytase activity and bioconversion potential in solid state bioprocess. Based on their potential in the screening experiments, 2 isolates were selected for bioconversion studies.

The phytase activity screening using calcium phosphate containing agar plates relies on the formation of clear halos as an indicator of phytase activity [13, 14]. According to the phytase screening test, one of the isolate-CPL-1, identified as *Colletotrichum* spp. produced extracellular phytase resulting in the formation of the clear zone on a calcium phytate-containing agar plate. Another isolate obtained from the same plant, designated as CPBA-2 and identified as *Togninia* spp. did not showed a significant zone of clearance though it showed considerable

bioconversion potential. The phytase production ability of CPL-1 was also checked in liquid media. The growth of isolate-CPL-1 in phytate-containing media was found to be comparable to a negative control lacking phytate in the cultivation media. The results demonstrated that isolate-CPL-1had the ability of extracellular production of phytase when cultivated in submerged cultures. The phytase activity of isolate-CPL-1 was found to be 0.89 and 0.24 U/mL.

Figure 1 represents the colonial morphology and microscopic structure of sporangium of the two selected isolates. Isolate CPL-1 and CPBA-2, obtained from a leaf and bark tissue of *C. paniculatas* were identified as *Colletotrichum* spp. and *Togninia* spp. respectively based on their morphological characters. Therefore, based on the results of the screening studies, both the isolates-*Colletotrichum* spp. and *Togninia* spp. were selected due to stronger degradation ability of cellulosic and hemicellulose waste. Both the strains grew on tested raw agro-wastes such as wheat straw, groundnut shells, and pigeon pea waste when cultivated in SSF for 3 weeks and significantly transformed the waste residue (Fig. 2). It was observed that the moisture content of the matrix has a profound effect on the growth of the test fungi and the bioconversion of residue. The maximum growth was observed at 70% moisture level and the lowest at 50% for both the isolates. The insufficient moisture level may alter the physical properties of the solid substrate thereby affecting the growth of mycelia [15]. On the other hand, a higher than optimum moisture level reduces oxygen transfer and impedes fungal growth [16]. Both the endophytic isolates could produce large amounts of extracellular cellulase and hemicellulase in the SSF process that resulted in excellent growth on cellulosic residue.

3.2 Nutritive value of bioprocessed residues

The proximate composition of the agro-residues before and after fermentation using *Colletotrichum* spp. and *Togninia* spp. is shown in Tables 1 and 2, respectively. The results reveal that *Colletotrichum* spp. have better bioconversion activities as compared to *Togninia* spp. *Colletotrichum* spp. mediated process significantly altered the nutritional values of all tested wastes. Cellulose, hemicellulose, and lignin content were significantly reduced in the bio-transformed waste as compared to untreated residues. On the other hand, total carbohydrates were significantly increased all treated waste with groundnut being the best with maximum g carbohydrates (13.92 ± 0.7 g/100). Total crude protein and N content of the treated waste were significantly improved. Crude protein and N content were the highest (24.95 ± 1.4 and 15.53 ± 1.2, respectively) in the bio-transformed groundnut shells using *Colletotrichum*

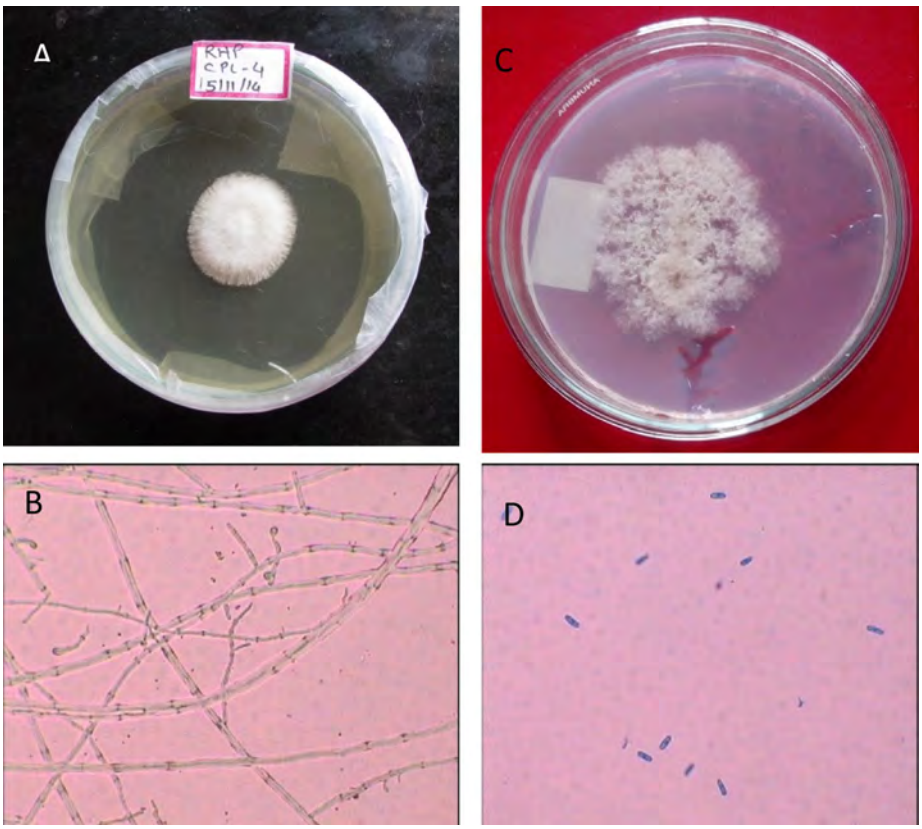

Fig. 1 Isolate CPL-1 *Colletotrichum* spp. (**a**) and structure of hyphae (**b**). Isolate CPBA-2 *Togninia* spp. (**c**) and structure of sporangium (**d**)

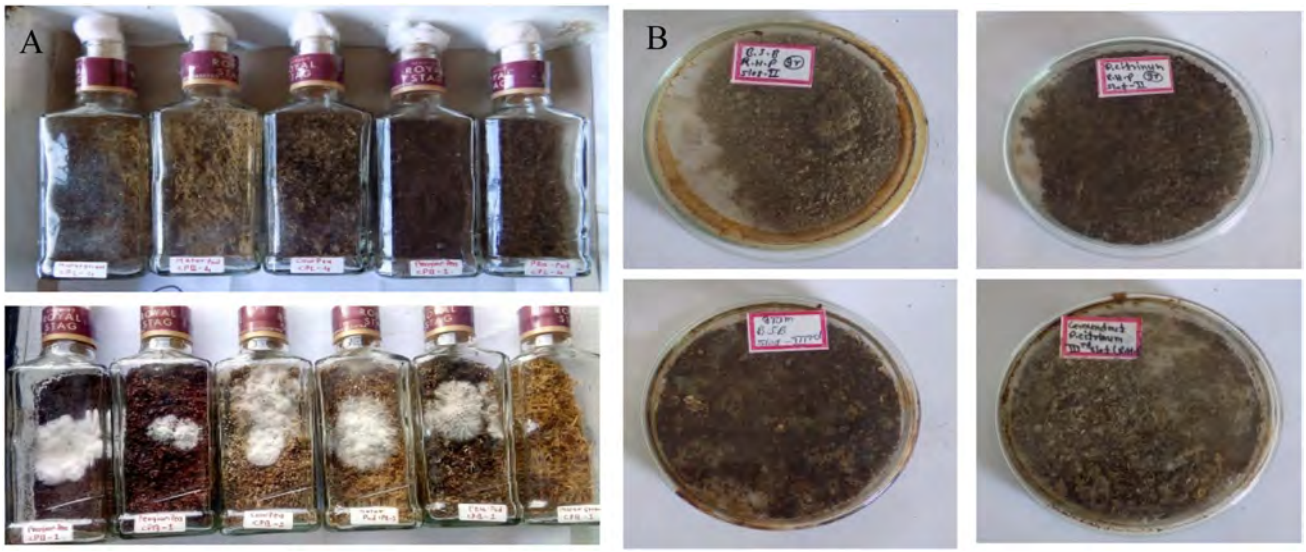

Fig. 2 Growth of endophytic isolates in various agro-wastes in solid state fermentation (**a**). The agro-residue after bioconversion (**b**)

Table 1 Proximate composition of the treated and untreated wastes on dry matter basis (g/100 g) using isolates CPL-1 *Colletotrichum* spp

Parameter	Unfermented waste			Fermented waste		
	Wheat	Pigeon pea	Groundnut	Wheat	Pigeon pea	Groundnut
Cellulose	34.80 ± 1.2	22.65 ± 1.4	64.5 ± 2.5	20.2 ± 1.1	13.23 ± 0.8	30.2 ± 2.1
Hemicellulose	22.36 ± 0.9	18.20 ± 0.9	11.2 ± 0.5	12.2 ± 0.8	9.90 ± 0.6	8.2 ± 0.4
Lignin	15.58 ± 0.7	10.23 ± 0.5	12.2 ± 0.4	10.23 ± 0.5	8.2 ± 0.4	8.2 ± 0.5
Crude protein	3.45 ± 0.2	8.41 ± 0.5	4.35 ± 0.2	10.20 ± 0.8	16.41 ± 1.2	24.95 ± 1.4
Total carbohydrates	1.32 ± 0.1	7.32 ± 0.3	7.32 ± 0.3	10.22 ± 0.6	11.32 ± 0.7	13.92 ± 0.7
Nitrogen, %	6.23 ± 0.2	8.21 ± 0.2	7.23 ± 0.3	14.20 ± 0.9	13.21 ± 1.1	15.53 ± 1.2
Total fat	2.22 ± 0.2	6.22 ± 0.2	9.32 ± 0.4	6.20 ± 0.2	9.22 ± 0.24	8.32 ± 0.8
Ash	5.23 ± 0.2	1.95 ± 0.2	2.01 ± 0.1	5.2 ± 0.2	2.95 ± 0.32	3.06 ± 0.7
Tannin and phytate content, µg/100 g of waste						
Tannins	702 ± 10.1	665 ± 09.1	454 ± 08.1	502 ± 11.1	589 ± 09.9	314 ± 07.1
Phytate	200 ± 07.1	611 ± 10.5	465 ± 09.1	175 ± 05.1	298 ± 05.5	303 ± 08.3

Values are means of 3 replicates ± SD. Statistical significance between treated and untreated waste was evaluated using Student's *t* test. $P < 0.05$ was considered the statistically significant difference between treated and untreated waste

spp. The tannins and phytate content was also found to be altered significantly when the waste was subjected for bioconversion using *Colletotrichum* spp. (Table 1). The phytate is responsible for chelating divalent cations such as Ca, Fe, Mg, and Zn, which leads to a dietary deficiency of these important minerals and ultimately retards the growth of animals. The phytate content of the fermented sample was reduced significantly as a result of microbial bioconversion.

Tannins impart a dark color to the waste whereas the phytate is known for its anti-nutrient effect as they chelate various metal ions such as Ca^{+2}, Mg^{+2}, Fe^{+2}, and Zn^{+2}. Moreover, poultry birds are unable to metabolize the phytate and therefore it has to be supplied in the form of inorganic phosphate through diet to avoid phosphate deficiency in these animals. Therefore, phosphate utilization in these animals can be improved using the feed rich with microbial phytase. The endophytic fungi in our studies produced phytase thereby reducing the phytate content and ultimately reduced its anti-nutrient effects.

3.3 Poultry trials using supplementation of bio-transformed waste

Based on the obtained results of the nutritional evaluation, groundnut shell waste was found to have a high nutritional value and hence the treated groundnut shells waste was selected for poultry trials. The suitability of the

treated groundnut waste as feed was evaluated on broiler chickens (Fig. 3). The commercial poultry diet was supplemented with 5%, 10% and 20% of fermented biomass and its effect on body weight; feed conversion ratio and feed consumption behavior of broiler chickens were evaluated.

The data on the effect of a diet supplemented with 5%, 10% and 20% of fermented biomass on body weight of the chickens is shown in Table 3. No significant difference ($P < 0.05$) was observed on the body weight of test groups animals fed with different test diets. The highest body weight (1930.3 ± 5.89 g) was achieved when the broilers were fed with feed supplemented with 20% fermented biomass. The body weight gain at the end of the study was even better (1920.3 ± 6.41 g) than the test group fed with the standard diet. The pattern of weight gain of all test groups (groups A, B, and C) was

Table 2 Proximate composition of the fermented ground shells wastes on dry matter basis (g/100 g) isolate CPBA-2 *Togninia* spp.

Parameter	Unfermented waste			Fermented waste		
	Wheat	Pigeon pea	Groundnut	Wheat	Pigeon pea	Groundnut
Cellulose	34.80 ± 1.2	22.65 ± 1.4	64.5 ± 2.5	29.2 ± 1.6	19.21 ± 0.9	41.2 ± 3.1
Hemicellulose	22.36 ± 0.9	18.20 ± 0.9	13.2 ± 0.6	13.2 ± 0.7	10.90 ± 0.7	7.2 ± 0.4
Lignin	15.58 ± 0.7	10.23 ± 0.5	12.2 ± 0.4	11.23 ± 0.5	7.9 ± 0.4	8.8 ± 0.5
Crude protein	3.45 ± 0.2	8.41 ± 0.5	4.35 ± 0.2	6.45 ± 0.4	15.42 ± 1.2	09.20 ± 0.5
Total carbohydrates	1.32 ± 0.1	7.32 ± 0.3	7.32 ± 0.3	11.22 ± 0.8	10..32 ± 0.7	10.42 ± 0.9
Nitrogen, %	6.23 ± 0.2	8.21 ± 0.2	7.23 ± 0.3	10.32 ± 1.2	11.21 ± 1.2	10.29 ± 0.7
Total fat	2.22 ± 0.2	6.22 ± 0.2	9.32 ± 0.4	5.20 ± 0.4	7.21 ± 0.25	6.32 ± 0.9
Ash	5.23 ± 0.2	1.95 ± 0.2	2.01 ± 0.1	1.2 ± 0.8	2.56 ± 0.40	2.26 ± 0.8
Tannin and phytate content, µg/100 g of waste						
Tannins	702 ± 10.1	665 ± 09.1	454 ± 08.1	658 ± 10.8	611 ± 09.1	398 ± 07.1
Phytate	200 ± 07.1	611 ± 10.5	465 ± 09.1	195 ± 07.1	597 ± 11.8	445 ± 09.8

Values are means of 3 replicates ± SD. Statistical significance between treated and untreated waste was evaluated using Student's *t* test. $P < 0.05$ was considered the statistically significant difference between treated and untreated waste

Fig. 3 **a** Large-scale bioconversion of groundnut waste using in bamboo trays; **b** bioprocessed waste

Table 3 Body weights (g) of broiler poultry fed with 5%, 10% and 20% (w/w) fermented biomass

Age of birds	Groups			
	A	B	C	D (control)
24 h old	37.8 ± 2.09	38.2 ± 1.09	37.8 ± 2.09	37.2 ± 2.12
1st week	150.5 ± 3.14	120.5 ± 2.14	150.5 ± 3.14	151.5 ± 3.22
2nd week	385.6 ± 3.28	312.6 ± 3.18	385.6 ± 3.28	395.6 ± 4.21
3rd week	751.3 ± 4.23	690.3 ± 4.33	751.3 ± 4.23	745.3 ± 4.89
4th week	1002 ± 4.55	1012 ± 4.15	1002 ± 4.55	1020.5 ± 5.55
5th week	1590.5 ± 5.96	1568.5 ± 5.23	1590.5 ± 5.96	1690.5 ± 6.33
6th week	1910.3 ± 5.89	1901.3 ± 6.78	1930.3 ± 5.89	1920.3 ± 6.41

Values are means of three replicates ± SD

also found normal and it was consistent with the standard group (group D).

The feed consumption profile of the broiler chickens fed with 5%, 10% and 20% (wt/wt) of bioprocessed material is represented in Table 4. No significant difference was observed in feed consumption in the groups given different dietary treatment. Feed consumption was slightly decreased as the percentage of fermented biomass concentration is increased. The lowest feed consumption (3529.92 ± 4.55) was noticed when the finisher diet supplemented with 20% bioprocessed waste (Table 5). Moreover, the feed conversion rate was found to be improved when the animals were fed diets supplemented with 15% of bio-transformed agro-wastes. No significant difference was observed in the feed conversion rate of the test birds at the tested percentage.

Table 5 Feed conversion ratio of broiler fed with 5%, 10% and 20% (wt/wt) fermented biomass

Age of birds	Groups			
	A	B	C	D (control)
1st week	1.09	1.09	1.07	1.07
2nd week	1.19	1.25	1.19	1.14
3rd week	1.40	1.49	1.38	1.44
4th week	1.67	1.61	1.56	1.63
5th week	1.89	1.83	1.78	1.77
6th week	1.98	1.95	1.89	2.60

4 Discussion

Use of cheaper agro-residues as a feedstuff in the poultry diet has the potential not only to support the poultry business economically but it will also enhance the environmental sustainability. A number of agro-wastes have been evaluated for their suitability as poultry feed with varying success [17]. Portsmouth et al. [18] obtained satisfactory growth of chickens fed on diets containing 2.5% feather meal but failed with 5%, while Thomas et al. [19] obtained satisfactory performance with 7% feather meal. The processed material of groundnut in our study showed excellent palatability in broiler chickens. The proximate chemical analyses data reveals that the nutritive value of groundnut waste is improved after fermentation using *Colletotrichum* spp.

The mycelial growth and enzyme action during the SSF break down the complex polysaccharides into simple hexoses which are less complex structures and easily degradable [20]. Therefore, bioprocessed groundnut shells have improved the nutritional value of the test diet, suggesting that they can be potentially be used for supplementing the poultry feed.

The crude protein content found to be doubled in the treated waste. The increase in crude protein may be due to release mycelial proteins of the fungi or the degradation of complex polysaccharides to form single cell protein (SCP) by the growing fungus during the fermentation process [21]. Secretion of extracellular enzymes such as pectinases, xylanases, cellulases and amylases by the fungus for utilization of the complex polysaccharides may responsible for increased protein content of the bioprocessed material [22–24]. The decrease in tannin and phytate content of the waste material may be due to phytase producing ability of the test fungi.

Total carbohydrates were also found to be increased indicating the release of hexoses as a function of production of extracellular protease by the *Togninia* spp. No significant decrease was reported in the tannins and phytate content in all three treated residues. The findings of the study demonstrated the economic and environmentally benign bioconversion of solid agro-residues into economically important products such as poultry feed using the endophytic microorganisms in SSF. Endophytic fungi used in the present study effectively transformed the cellulose and lignin-rich waste into simple hexoses. It appears that biological transformation of agro-waste using the endophytic fungi is economically feasible and can be easily adopted for bioconversion of agricultural wastes.

India is the agriculture based country where disposal of agro-wastes is the huge challenge. Thus, utilization of such

Table 4 Feed consumption of broiler fed with 5%, 10% and 20% (w/w) fermented biomass

Age of birds	Groups			
	A	B	C	D (control)
1st week	181.23 ± 3.47	180.21 ± 3.19	179.37 ± 2.81	185.04 ± 3.23
2nd week	512.12 ± 2.23	520.06 ± 3.55	562.02 ± 3.60	538.35 ± 3.17
3rd week	1010.33 ± 4.89	1133.42 ± 4.19	1120.53 ± 3.97	1042.51 ± 4.30
4th week	1789.71 ± 3.92	1791.37 ± 4.51	1793.26 ± 4.39	1855.17 ± 3.98
5th week	2867.09 ± 3.95	2799.23 ± 3.49	2857.77 ± 4.59	2902.06 ± 3.74
6th week	3586.21 ± 4.42	3632.21 ± 3.35	3529.92 ± 4.55	3556.24 ± 3.21

Values are the mean SD ± of 10 observations

waste by bioconversion can be a sustainable approach because the recycling and reduction of waste can reduce environmental pollution as well as it will reduce our dependence on conventional food grains as animal feedstuff. Although significant scientific work has been undertaken in this direction [17, 26] there have been no efforts made to develop and scale up the process for the production of valuable end products such as poultry feed using endophytic fungi. Findings of this study are a step forward in commercial exploration of endophytic fungi.

5 Conclusions

Fungal bioconversion of agro-residues improves their nutritional and energy profile. Both the endophytic fungi are able to transform the selected agro-residues into the digestible hexoses and significantly improved the protein and nitrogen content. The bio-transformed groundnut shell waste has highest total proteins, total carbohydrates, and digestible fats. Moreover, the phytate and tannin content was significantly lowered. Thus, the bioprocessed agro-residue using endophytic *Colletotrichum* spp. can be used as a partial substitute for energy and protein in poultry diet, especially in the countries where these products are grown and are widely available. The inclusion percentage of the supplement in this study will significantly reduce the use of conventional feed ingredients in poultry diet and ultimately reduce the cost of poultry diet. However, better understanding and more study on palatability, digestibility and feed intake are needed for increasing the inclusion percentage of bio-transformed waste into poultry diet is needed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experiments were carried out in compliance with the ethical and scientific standards for research on humans and animal subjects (Research Policy Document, UNN, 2017). All the methods were performed according to the guidelines of animal experimentation as approved by institutional animal ethics committee.

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Data Article

Data on the inhibitory effect of endophytic fungi of traditional medicinal plants against pancreatic lipase (PL)

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ABSTRACT

This article describes isolation and pancreatic lipase (PL) inhibitory potential of 18 endophytic fungi isolated from the various parts of six indigenous medicinal plants. PL catalyzes absorption and hydrolysis of triglycerides into di-glycerides into mono-glycerides and free fatty acids. PL inhibitors are well-known for the disruption of pancreatic lipase activity. The quest for novel pancreatic lipase inhibitors is crucially important owing to their therapeutic potential in the treatment of obesity and related chronic diseases. The present dataset provides information about the presence of endophytic fungi in the internal tissues of selected plants and the PL inhibitory potential of their metabolites using bioassay based screening. Absence of the yellow zone surrounding the standard Orlistat and test extract indicated PL inhibition due to the cumulative effect of metabolites present in the extract. The data suggests that TLC bio-autographic method is simple, rapid and reproducible and therefore it could be effectively used for high throughput screening of PL inhibitors from natural sources.

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Specifications Table

Subject area	Biology
More specific subject area	Secondary metabolites and enzyme inhibition
Type of data	Tables and figures
How data was acquired	TLC Bio-autography on silica gel 60 F_{254} 25 × 25 cm plates (Merck, Germany) using Spraylin automatic sample applicator (Aetron, India).
Data format	Analysed
Experimental factors	The endophytic fungi were isolated from the differnet tissues of various indigenous medicinal plants and cultivated at shake flask. Metabolites were extracted using ethyl acetate.
Experimental features	Inhibitory effects of extracts of endophytic fungi against PL
Data source location	North Maharashtra, MS, India. (21.26°N and 75.11°E). Data analysis: Shirpur, MS, India
Data accessibility	The data is available with this article.

Value of Data

- The dataset can be useful in the field of rapid throughput screening of fungal extracts for the presence of enzyme inhibitors.
- The data provides *in vitro* validation for significant PL inhibitory effect, which is an important target for the development of anti-obesity lead compounds.
- This data may suggest further studies on the detailed characterization of PL inhibitors from endophytic fungi for possible future therapeutic application.

1. Data

The data summarized in Table 1 lists the percent inhibition of PL by inhibitors present in crude extract of endophytic fungi isolated from their host plant. A total of eighteen fungal endophytes were isolated from the different parts of the selected plants. Data shown in Fig. 1 represents inhibition of PL by extracts of endophytes using phenol red olive oil agar method. Complete absence of yellow halo surrounding standard, Orlistat well and extract of CLL-2 showed complete inhibition of pancreatic lipase by the metabolites present in the test extract. However, partial inhibition was seen in the well containing the extract CLL-1. Tube assay in Fig. 2 showed a dose dependent PL inhibition by the selected extract. Color intensity in the tube 2–6 in (Fig. 2) changed from pink to red as function of inhibition of PL. Data shown in Fig. 3 represents TLC bio-autography based screening of fungal extract for PL inhibition. In order to confirm the effectiveness of enzyme assay, extract of endophytes was screened for pancreatic lipase inhibition by modifying TLC bio-autographic method. This modified method is rapid and a large number of samples can be screened in a single TLC plate in a shorter duration [1,2]. Change in the colour of the plate from red to orange yellow indicated PL activity in the control while absence of yellow halo indicates PL inhibition (Fig. 3).

2. Experimental design, materials and methods

2.1. Microorganisms and preparation of extract

Endophytic fungi were isolated from various tissues of the selected plants as per standard isolation method [3]. After isolation, pure culture of each isolate was maintained on sterile potato dextrose agar (PDA) medium as a stock culture at 4 °C.

The isolated endophytic fungi were grown on agar plugs [4]. After visible growth, the agar plugs were crushed and metabolite extraction was carried out using ethyl acetate. All the crude extracts of each isolated fungi were screened out for qualitative inhibition of pancreatic lipase by various methods.

2.2. PL inhibition assay: chromogenic olive oil plate method

The ethyl acetate extract of the test endophytes was screened for pancreatic lipase inhibitory activity by chromogenic olive oil plate method. This method is based on change in the color of media due

Table 1
Source of endophytic fungi and their lipase inhibitory potential.

Sr. No.	Host plant	Tissue	Isolated endophytic fungi	Lipase inhibition	% inhibition
1	*Citrus lemon*	Leaves	**CLL1**	+++	**100**
2		Leaves	CLL2	+	50
3		Leaves	CLL3	+	50
4		Bark	CLB1	+	50
5		Bark	CLB2	−	Nil
6	*Nerium oleander*	Leaves	NOL1	+	40
7		Bark	NOB1	−	Nil
8	*Withania somnifera*	Leaves	WSL1	−	Nil
9		Leaves	WSL2	−	Nil
10		Bark	**WSB1**	+++	**100**
11		Bark	WSB2	−	Nil
12	*Aloe vera*	Leaves	AVL1	+	40
13		Bark	AVB1	−	Nil
14		Bark	AVB2	−	Nil
15	*Punica granatum*	Leaves	PGL1	−	Nil
16		Bark	PGB1	−	Nil
17	*Catharanthus roseus*	Leaves	CRL1	−	Nil
18		Bark	CRB1	++	60
19	**Orlistat standard** (120 mg/ml)			+++	**100**

+++: Complete inhibition, ++: Moderate Inhibition, +: Low inhibition, -: No inhibition. The values are average of triplicate samples (n = 3).
The isolates designated with bold font show 100% inhibition of the PL. It for the purpose of readers' attention only.

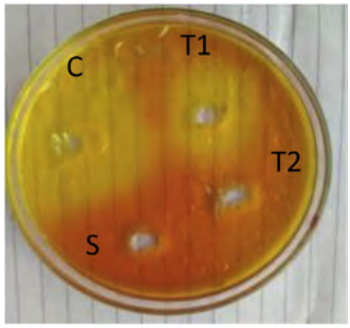

Fig. 1. Olive oil plate method for screening of endophytes, C: control; T1: test extract of CLL1; T2: test extract of CLL-2; S: Standard Orlistat.

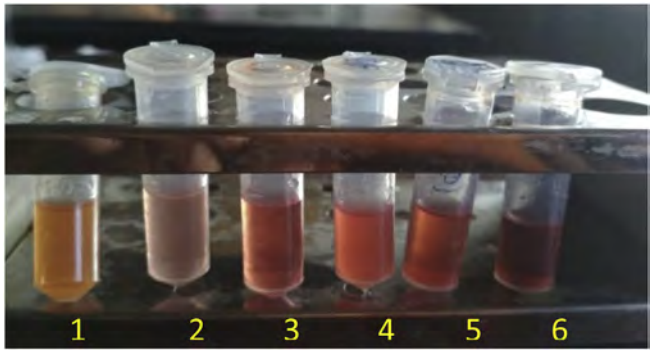

Fig. 2. Lipase assay in tube: 1: control, 2–6: crude extract of endophyte

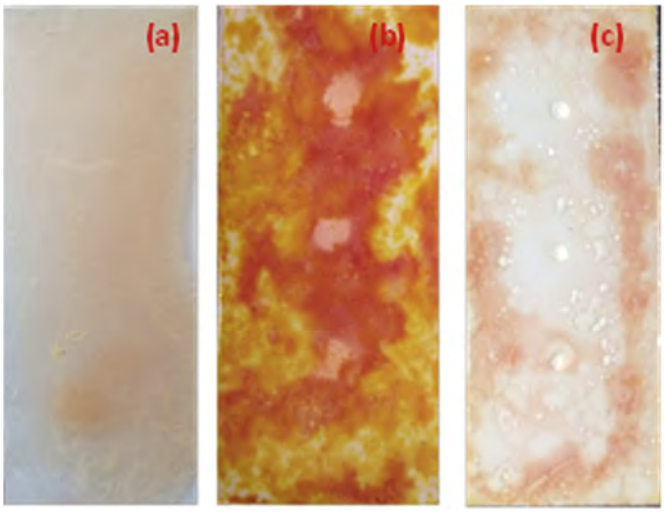

Fig. 3. TLC Bio-autography. (a) Control. (b) Test extract of endophytes. (c) Standard Orlistat.

to acid production as result of enzyme activity [5]. Plates of Olive oil agar were prepared and wells were punched. Then, 100 μL of preincubated master-mix of porcine pancreatic lipase (PPL, 50 μL) and extract (50 μL) of each endophyte was loaded in a well. The control well comprised of PPL preincubated with distilled water instead of culture extract. Orlistat (120 mg/mL) were preincubated with PPL and used as standard inhibitor of PPL. All plates were incubated at 37 °C for 24 hr.

2.3. Lipase inhibition assay using tube method

PPL enzyme (100 μL, 10 mg/mL) in phosphate buffer (pH 7.4) was preincubated with same volume of the test extract and the Orlistat standard (1–10 mg/mL) separately. Distilled water instead of test extract or Orlistat was used as control. All tubes were incubated at 37 °C for 20 min. After incubation, 100 μL of substrate prepared in distilled water (pH 7.4) was added in each tube and again incubated at 37 °C for 20 min. After incubation, phenol red indicator (10 μL) was added to each tube and the tubes were observed for the change in color. Assay was performed in triplicates.

2.4. TLC bio-autography

The aluminum coated TLC (silica gel F_{254} 25 × 25 cm) sheets were used for TLC bio-autography. The data was obtained by spotting test extracts and standard PL inhibitor, Orlistat (100, 50 and 10 μg/mL) onto a separate TLC plate (2.5 × 10 cm). Ethyl acetate was spotted on another TLC sheet and used as control plate. After complete drying of spots, Whatmann filter paper was impregnated with PPL solution (10 mg/mL) in phosphate buffer (pH 7.4) was overlaid on the all spotted plates. All the plates were incubated at 37 °C for 15 min for enzyme-inhibitor interactions. Substrate agar solution with few drops of phenol red indicator was poured on the all plates after the filter paper was peeled off [6,7]. Thereafter, all the plates were incubated at 37 °C for 20 min for enzyme substrate reactions.

2.5. Data analysis

Data reveals formation of yellow halo due to the enzyme (PL) activity; whereas absence of yellow halo surrounding the standard and test extract of endophytic fungus showed complete inhibition of PL by the metabolites present in the test extract. A dose dependent inhibition of PL was observed in tube assay. TLC bio-autographic data confirmed the presence of PL inhibitory metabolites in the test extract.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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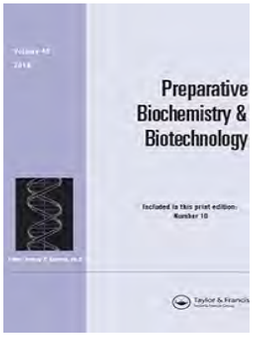

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Steroidal fraction of *Carissa carandas* L. inhibits microbial hyaluronidase activity by mixed inhibition mechanism

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Steroidal fraction of *Carissa carandas* L. inhibits microbial hyaluronidase activity by mixed inhibition mechanism

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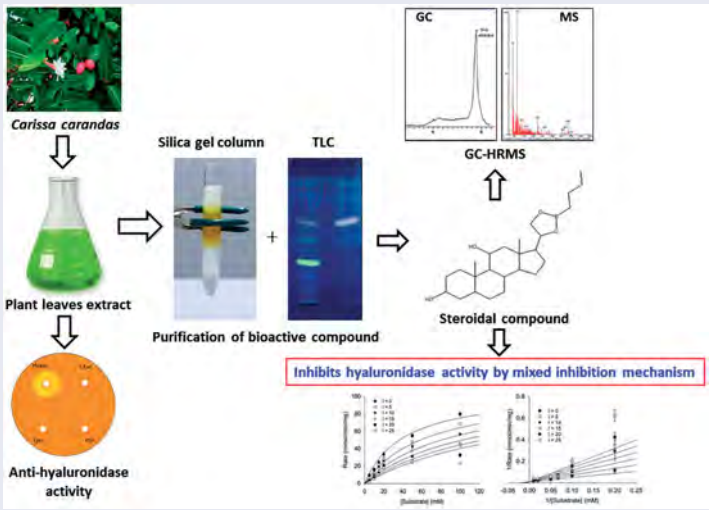
ABSTRACT

Hyaluronidase (hyase) is a hyaluronic acid (HA) depolymerizing enzyme produced by many pathogenic bacteria as a virulence factor to establish and spread infections. Present studies established that a steroidal fraction (SF) isolated from leaves of *Carissa carandas* act as a strong hyase inhibitor. The kinetic parameters involved in the inhibition of hyase by purified SF were studied and compared with standard hyase inhibitor quercetin. The purified SF showed the highest inhibition with an IC50 of 5.19 mM in comparison with a standard inhibitor, quercetin (IC50 8.63 mM). The inhibition constant (Ki) of purified SF determined by Dixon plot was 8.32 mM, which was significantly lower than that of quercetin standard. The kinetic behavior of enzyme hyase revealed to be more complex than classical competitive and uncompetitive inhibition where inhibitor affects both Km and Vmax. The inhibitor (I) favored the binding to the enzyme–substrate (ES) complex where Km value appeared to decrease (Kmapp $<$ Km). The inhibitor also leads to decrease in the apparent maximum velocity of the enzyme–substrate reaction (Vmaxapp $<$ Vmax). These results signpost toward mixed nature of inhibition of enzyme hyase by purified SF. Anti-hyaluronidase activity by a bioactive metabolite from *C. carandas* has not been reported so far and has high therapeutic potential against spread of pathogen and its toxins in the host.

KEYWORDS

Carissa carandas; hyaluronidase inhibitor; inhibition constant; mixed inhibition; steroidal fraction

GRAPHICAL ABSTRACT



Introduction

Microbial hyaluronidases (hyase, EC 4.2.2.1) are the glycosidase enzymes capable of depolymerizing hyaluronic acid (HA) present in the extracellular matrix. Hyases degrade HA by cleaving the β-1, 4-glycosidic bond between N-acetyl-D-glucosamine and D-glucuronic acid to produce disaccharide units.[1] A wide variety of microorganisms produce enzymes capable of depolymerizing HA. They are ubiquitously distributed in nature and are found in mammals, invertebrate animals, animal venoms, pathogenic fungi, bacteria and also bacteriophages.[2]

In humans, a ground substance present in connective tissues provides a line of defense against pathogenic bacteria. The viscous nature of such a substance usually acts as a

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/lpbb.

barrier for penetration of pathogens and their extracellular products; however, some bacteria have adapted ways to weaken the limits of connective tissues. Many pathogenic bacteria are able to establish and spread infections at the mucosal or skin surface by producing the enzyme hyase as their potential virulence factor.[3] This potent virulence factor of pathogen plays a pivotal role in several life- threatening diseases, such as gangrene, meningitis, synovitis, hyperplasia, nephritis, mycoplasmosis, periodontal disease, mastitis, pneumonia, septicemia, syphilis, toxic shock syndrome, and wound infections.[4–8] HA is a major constituent of most connective tissues, particularly in the skin, hyase acts as an important weapon of pathogens facilitating their spread from an initial site of infection. While the resulting disaccharides serve as an important energy source for the pathogenic bacteria to grow and spread the infection through tissues.[9,10] Moreover, the polymer HA plays an important role in immune system functions.[11] The breakdown of HA by the hyases of pathogenic organisms may lead to the modulation of immune system, facilitating the growth of pathogens in the host.[3]

The microorganisms capable of producing enzyme hyase include various species of *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Propionibacterium,* and *Clostridium*.[12–15] While it has also been reported in different species of *Candida*, including *C. albicans, C. krusei, C. tropicalis, C. parapsilosis,* and *C. guillermondii*.[16] The fungal strains of *Penicillium* sp. (*P. funiculosum, P. purpurogenum*),[17] *Streptomyces* sp., *Pseudozyma aphidis*, *Cryptococcus laurentii*[18] were reported for the production of enzyme hyase. The invertebrates like leeches (*Hirudo medicinalis, Nephelopsis obscura, Desserobdella picta* and *Glossiphonia complanata*),[19] spiders (*Vitalius dubius, Loxosceles intermedia* and *Hippasa partita*),[20,21] social wasp (*Polybia paulista*),[22] honey bees (*Apis dorsata* and *Apis mellifera*),[23] scorpions (*Palamneus gravimanus* and *Buthus martensi*)[24,25] also produce hyase. The causative agent of syphilis - *Treponema pallidum and Treponema pertenue* are also reported to produce hyase.[26] The veterinary pathogens, *Streptococcus uberis* and *Streptococcus dysgalactiae* that cause mastitis have also shown the synthesis of hyaluronidase.[27–29] Recently, hyases were reported as important virulence factors of Group B *Streptococcus* (GBS) involved in ascending vaginal infections in pregnant women leading to increased fetal injuries, preterm birth defects, and fetal demise.[30,31]

In view of combating such life-threatening bacterial infections, there is an urgent need to develop exceptional therapeutic strategies against bacterial infections.[32] Anti-virulence therapeutic agents could be developed against bacterial infections; since HA is a major constituent explicitly targeted by hyase.[3,10,33–35]

In the present study, the plant *Carissa carandas* (Family: *Apocynaceae*) was used since it is a common drought-tolerant plant found in Indian subcontinent. A bioactive metabolite from its leaves was isolated, purified, and characterized by thin layer chromatography (TLC) and gas chromatography high-resolution mass spectroscopy (GC-HRMS) analysis as a steroidal compound. The crude extract and purified steroidal fraction of *C. carandas* was proved to be a strong hyase inhibitor in qualitative and quantitative hyase inhibition assay. The kinetic parameters of hyase in presence and absence of inhibitor were studied and the mechanism of inhibition of enzyme by purified steroidal fraction was also elucidated. Many plant derived natural bioactive compounds were proved to be a potent hyase inhibitors, which includes alkaloids,[36] flavones and flavone analogs,[37,38] terpenes,[39] saponins,[40] and polyphenols.[41] The shrub *C. carandas* was reported to have various pharmacological activities such as, anti-pyretic, analgesic, anti-rheumatic, anti-convulsant, anti-inflammatory, anti-helminthic, anti-diabetic, anti-diarrheal, anorexia, astringent, and appetizer,[42–44] however, its anti-hyaluronidase activity remains unexplored.

Experimental

Chemicals and reagents

Sodium salt of hyaluronic acid from *Streptococcus equi* as a substrate and other chemicals like Gram's iodine (HiMedia, Mumbai, India), quercetin (Sigma-Aldrich, Bangalore, India) were used. While solvents used were of analytical grade and purchased from Merck, Mumbai. The enzyme hyaluronidase was prepared from a newly isolated bacterial strain *Brevibacterium halotolerans* DC1 (unpublished data). This enzyme was purified by using ammonium sulfate (70%) precipitation, ion exchange chromatography (DEAE-cellulose) and gel filtration chromatography (Sephadex G-100).

Plant material and extraction

The leaves of *Carissa carandas* were collected from Aner reservoir forest region (21.26° N, 75.11° E), North Maharashtra, India. The whole plant and its leaves were identified and authenticated by an expert plant taxonomist, and a voucher specimen (RCP-11/2016) was deposited at the Department of Botany, R. C. Patel ACS College, Shirpur. The leaves were thoroughly washed with water, shade dried and ground to make a fine powder. The polarity based extraction of powdered plant material was carried out by soxhlet apparatus using organic solvents with increasing polarity that included hexane, ethyl acetate, and methanol.[45] A 100 g of powdered plant material was soaked for 48 hr in each solvent sequentially at room temperature. The supernatant was recovered, and respective solvents were added twice to the residue. After extraction procedure, the solvent was evaporated and the residue was concentrated under reduced pressure using rotary vacuum evaporator (Equitron, Mumbai, India).[46] A gummy solid of dark brownish colored residue obtained after evaporation was stored in a refrigerator at 4 °C until further use.

Plate assay for the screening of hyaluronidase inhibitor

The plate assay of Patil and Chaudhari [47] with slight modifications was used for the screening of hyase inhibitor from a crude methanolic extract of leaves of *C. carandas*.

In brief, the plate contained a 20 ml of medium comprised of polymer substrate hyaluronic acid (0.4 mg/mL) and agarose (1%) as a solidifying agent. The medium was sterilized and poured into Petri plate up to a depth of 3–4 mm. Upon solidification, four equidistant wells of 5 mm diameter were punched with the help of sterile cork borer and emptied by suction to which 50 µL of assay mixture containing 30 µL enzyme hyaluronidase (1 mg/mL), and 20 µL of test extract was aseptically dispensed. The negative control was comprised of 30 µL of enzyme hyaluronidase and 20 μL of sterile saline while quercetin was served as a positive control.[36,48] The plate was then incubated for 1 hr at 37 °C. After incubation, the plate was flooded with Gram's iodine for 1 min to visualize clear zones around the wells.

Purification and characterization of hyaluronidase inhibitor

The crude semisolid extract (5 mg) obtained after extraction was dissolved in 10 mL methanol and applied on precoated silica gel TLC plate (Silica gel F 254, Merck, Darmstadt, Germany) using Spraylin-V automated sample applicator (Aetron, Mumbai, India). The TLC plate was developed in pre-saturated chamber containing Toluene: Methanol (8:1) as a mobile phase. After development, the plate was air dried and derivatized using 10% ethanolic sulfuric acid as a spraying reagent[49] and followed by heating at 110 °C for 10 min to develop characteristic color spots.

The crude methanolic extract of *C. carandas* was subjected for activity guided fractionation using silica gel (60–120 mesh size) column chromatography.[50] The dried crude extract (2 g) was dissolved in 8 mL methanol and adsorbed on to the silica gel. The slurry was air dried and applied on packed silica gel column (30 cm ×1.8 cm) and eluted with mobile phase Toluene: Methanol (8:1). The fractions of 5 mL each were collected and checked by TLC for homogeneity in same mobile phase and fractions bearing similar R_f values were pooled together and allowed to form crystals for further characterization.

Hyaluronidase inhibition assay

Hyaluronidase activity was determined by a turbidometric method of Di Ferrante[51] with some modifications. In this assay, inhibition of hyaluronidase by plant extracts and quercetin standard was indicated by a slower loss of absorbance compared with hyaluronidase alone.

In brief, a crude methanolic extract, purified fraction, standard hyase inhibitor quercetin, stock solutions of HA (0.5 mg/mL) and hyaluronidase (1 mg/mL) were prepared in acetate buffer (0.2 M sodium acetate-acetic acid, pH 6.0, containing 0.15 M NaCl). The assay mixture comprised of acetate buffer, 200 µg hyaluronic acid, 30 µg hyaluronidase, and 0–70 µL of diluted crude methanolic extract or purified fraction as an inhibitor to a final volume of 0.5 mL. The mixtures were incubated at 37 °C for 15 min, and the reaction was stopped with the addition of 2 mL of cetyl trimethyl ammonium bromide (CTAB) (2.5%) in NaOH (2%, pH 12.5). All assays were performed in triplicate. After 10 min, the A_{400} of each sample was measured against a blank containing 0.5 mL acetate buffer and 2 mL CTAB. The unit of enzyme activity was expressed as the quantity of enzyme which produces a 50% reduction of the turbidity given by the initial quantity of the substrate.

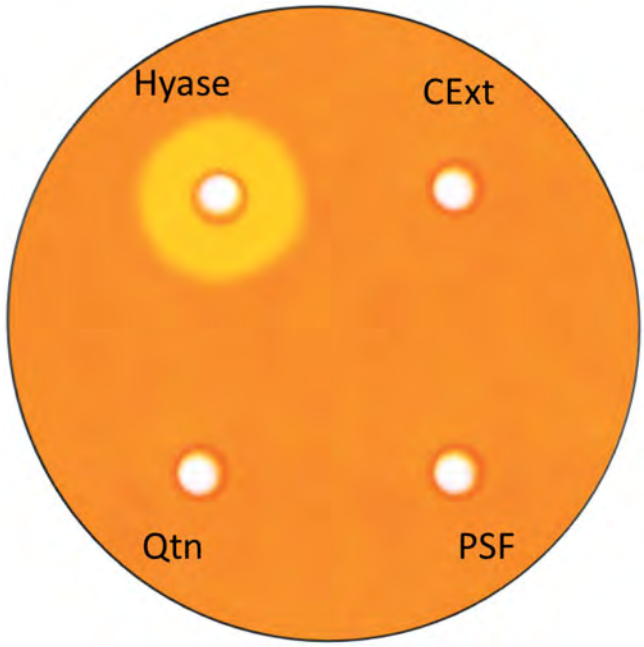

Figure 1. Plate assay for the screening of hyaluronidase inhibitor from *C. carandas* leaves extract (Hyase: Hyaluronidase enzyme; CExt: Crude methanolic extract of *C. carandas* leaves; PSF: Purified steroidal fraction; and Qtn: Quercetin as a standard hyaluronidase inhibitor).

Determination of kinetic parameters

The kinetic profile of hyaluronidase inhibition by using crude extract, purified fraction of *C. carandas* leaves and standard inhibitor quercetin was determined. The initial rate of reaction was calculated by using Michaelis–Menten equation, where velocity was plotted against substrate concentration.[52] The substrate concentration range was used as 5, 10, 15, 20, 50 and 100 mM. The Michaelis-Menten constant (Km) and maximum velocity (Vmax) of enzyme-substrate reaction were determined by using Lineweaver–Burk double reciprocal plot.[53] Inhibition constant (Ki) for *C. carandas* crude extract, purified fraction and quercetin standard were determined using Dixon plot (1/V versus [I]).[54] The inhibition behavior was determined by using Dixon plot in conjunction with Lineweaver-Burk plot.[55,56] A turn over number (Kcat) of an enzyme was determined in presence and absence of inhibitor.[57–59] The IC50 value was determined for crude methanolic extract, purified steroidal fraction and quercetin. The assay mixture containing an enzyme (300 µL), the substrate (300 µL) and inhibitor (1 µL) was incubated at 37 °C for 10 min. The reaction was terminated by using CTAB and the enzyme activity was determined by the turbidometric method.[51] The log (inhibitor) versus response curve model was used for data analysis.

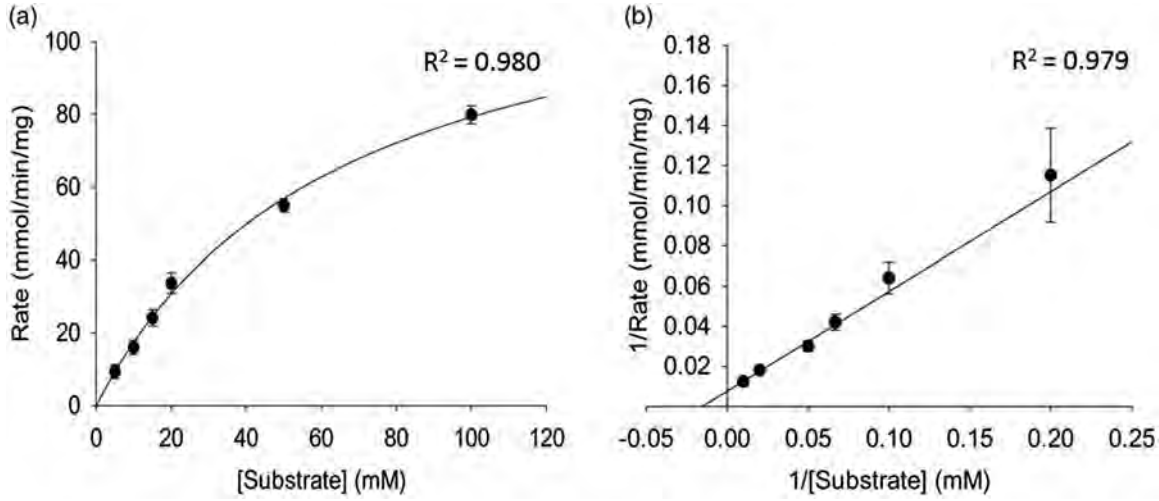

Figure 2. Michaelis-Menten plot (A) and Lineweaver-Burk double reciprocal plot (B) for enzyme hyaluronidase in absence of inhibitor.

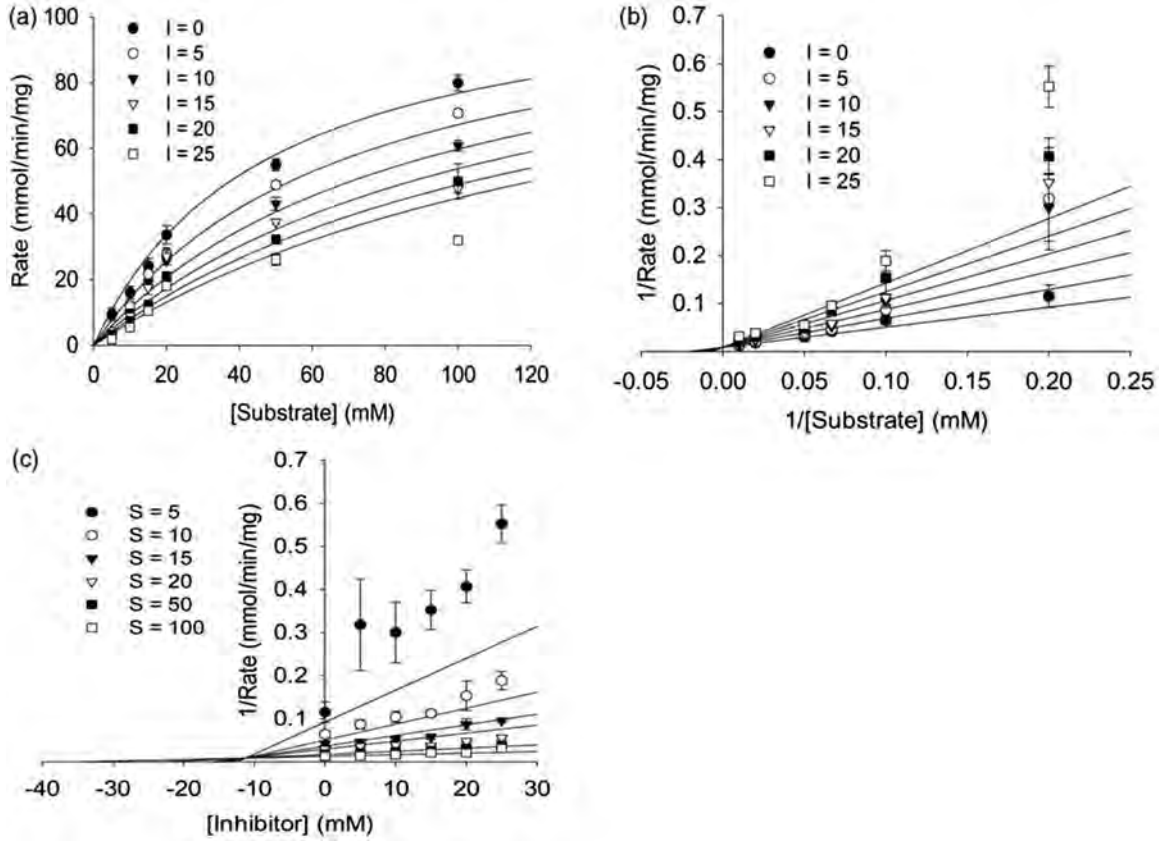

Figure 3. Mixed inhibition of enzyme hyaluronidase by crude methanolic extract of *C. carandas*, (A) Michaelis–Menten plot, (B) Lineweaver–Burk double reciprocal plot, and (C) Dixon plot, where I: Inhibitor and S: Substrate.

Gas chromatography high-resolution mass spectroscopy (GC-HRMS) analysis

The purified fraction of *C. carandas* leaves extract near to homogeneity showed a strong hyase inhibition activity which was further subjected to GC-HRMS analyses for the identification of bioactive compounds. GC-HRMS was performed by using Agilent 7890-5 GC (Santa Clara, USA) having HP-5 fused silica capillary column (30 m × 0.20 mm) with the phase thickness of 0.25 μm. It is coupled with Jeol Accu TOF GCV mass spectrometer with flame ionization detector (FID). The inlet temperature was 250 °C while injection volume was 10 μL. The injection was carried out in a split mode at the split ratio of 10:100 with the gas flow rate of 1 mL/min. Identification of purified fraction was done by comparing the mass spectrum with those present in the NIST 05 database.

Statistical analyses

All the experiments were carried out in triplicate and the results presented as mean ± SD. The kinetic data and the

Table 1. Kinetic parameters of hyase in presence and absence of inhibitors.

			With inhibitor		
			C. carandas		
Sr. No.	Parameters	Without inhibitor	Crude methanolic extract	Purified steroidal fraction	Standard hyase inhibitor (Quercetin)
1.	Vmax (mM/min/mg)	130.9 ± 9.18	NA	NA	NA
2.	Km (mM)	65.1 ± 8.55	NA	NA	NA
3.	$Vmax_{app}$ (mM/min/mg)	NA	113.11 ± 5.81	106.34 ± 6.56	120.18 ± 5.29
4.	Km_{app} (mM)	NA	47.21 ± 5.20	41.17 ± 5.67	52.79 ± 4.81
5.	Ki (mM)	NA	11.27 ± 1.17	8.32 ± 1.01	12.65 ± 1.08
6.	IC 50 (mM)	NA	12.87 ± 2.23	5.19 ± 2.14	8.63 ± 2.43
7.	Kcat sec^{-1}	0.654	0.632	0.597	0.636
8.	Inhibition type	NA	Mixed	Mixed	Mixed

Data presented as mean ± SD in three separate experiments. NA = Not applicable.

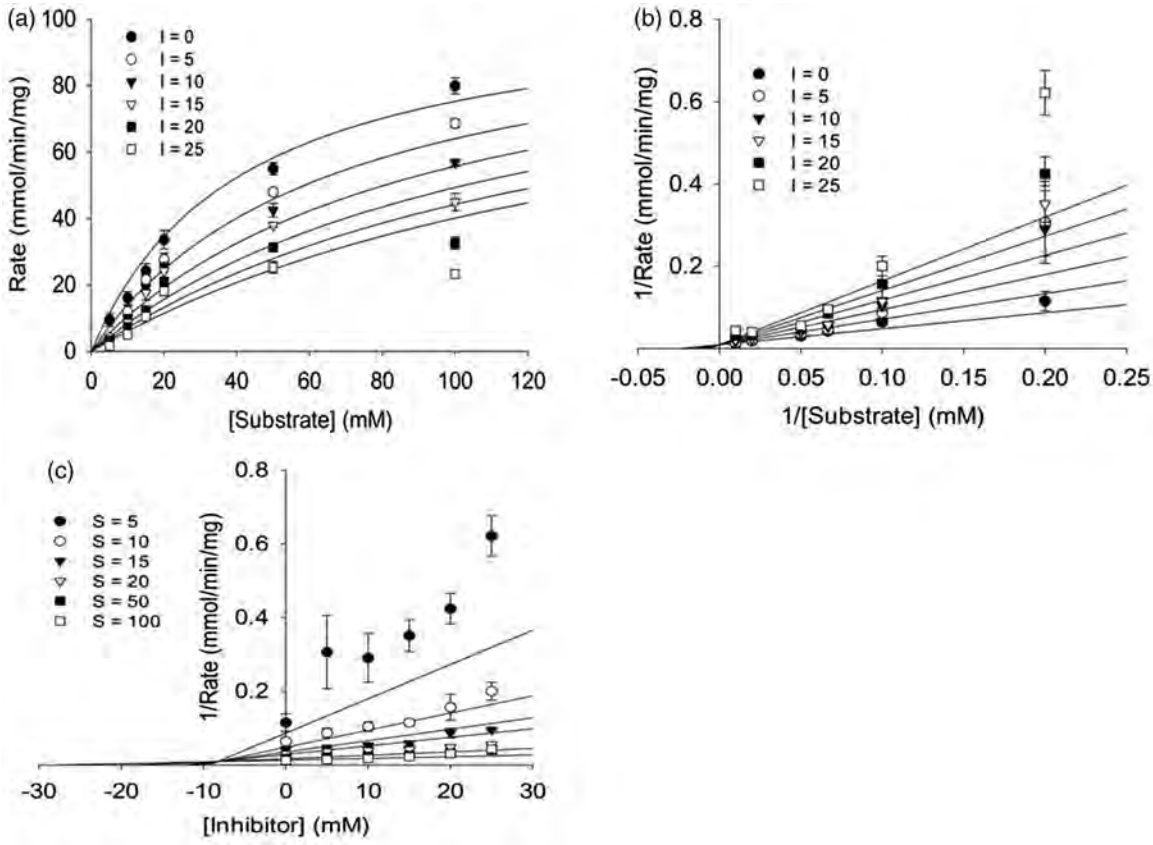

Figure 4. Mixed inhibition of enzyme hyaluronidase by purified steroidal fraction of *C. carandas*, (A) Michaelis–Menten plot, (B) Lineweaver–Burk double reciprocal plot and (C) Dixon plot, where I: Inhibitor and S: Substrate.

graphs were analyzed using Graph Pad Prism 5 (San Diego, USA) and Sigma Plot 12.0 software (Bangalore, India). However, the kinetic parameters of hyase inhibition were obtained by nonlinear regression analysis using the same softwares.

Results and discussion

Plate assay for the screening of hyaluronidase inhibitor

In plate assay, the distinct zone of clearance (23 ± 0.9 mm) was appeared around the negative control well of enzyme hyase, whereas the zones of clearance failed to appear around the wells filled with crude extract of *C. carandas* leaves, steroidal fraction purified from it and quercetin standard indicative of inhibition of hyase activity (Fig.1). The assay involved the radial diffusion of an enzyme sample in agarose, hydrolyzing the substrate hyaluronic acid blended with it. The undigested hyaluronic acid forms a dark yellow colored complex with Gram's iodine causing the appearance of a zone of clearance due to the depolymerization of substrate HA. In negative control well, hyase depolymerized substrate HA in medium making it unavailable to form a dark yellow colored complex with Gram's iodine and form a zone of clearance. This indicated that the crude extract of *C. carandas* leaves, steroidal fraction purified from it and quercetin showed inhibition of enzyme hyase.

Determination of kinetic parameters

The kinetic parameters of hyaluronidase catalyzed reaction in presence and absence of inhibitor were determined by

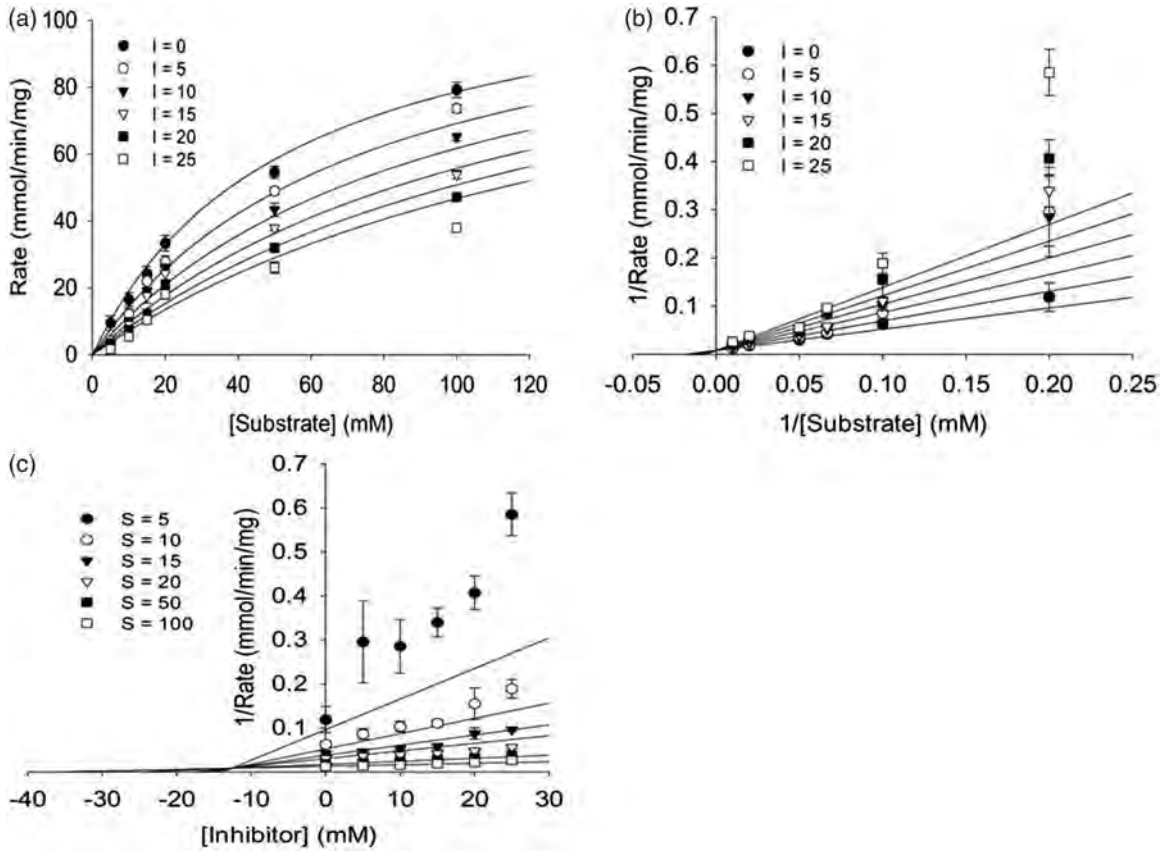

Figure 5. Mixed inhibition of enzyme hyaluronidase by standard inhibitor quercetin, (A) Michaelis–Menten plot, (B) Lineweaver–Burk double reciprocal plot and (C) Dixon plot, where I: Inhibitor and S: Substrate.

using single substrate - single inhibitor approach. The order of reaction was studied by plotting the substrate concentration [S] against the rate of reaction (V) through Michaelis–Menten equation (Fig. 2A). The Km and Vmax values in the absence of inhibitor were calculated as 65.1 ± 8.55 mM and 130.9 ± 9.18 mM/min/mg respectively by using Lineweaver–Burk plot (Fig. 2B). While, in the presence of purified steroidal fraction as an inhibitor, the apparent Km (Km_{app}) and apparent Vmax ($Vmax_{app}$) were decreased to 41.17 ± 5.67 mM and 106.34 ± 6.56 mM/min/mg respectively (Fig. 4A and B; Table 1). The turn over number of the enzyme (Kcat sec^{-1}) in absence of inhibitor was calculated as 0.654 sec^{-1} (Table 1), which decreased in presence of crude as well as a purified form of inhibitor. The inhibition constant (Ki) of the enzyme with the purified steroidal fraction as an inhibitor was observed as 8.32 ± 1.01 mM (Fig. 4C). Similarly, the crude extract of *C. carandas* leaves and quercetin standard showed a decrease in Km_{app} and $Vmax_{app}$ (Fig. 3 and 5; Table 1). In all the above results, the interaction between the inhibitor and enzyme seems to be more complex than classical competitive and uncompetitive inhibition where inhibitor affects both Km and Vmax. The inhibitor (I) favored the binding to the enzyme-substrate (ES) complex where Km value appeared to decrease (Kmapp < Km). The inhibitor also leads to a decrease in the apparent maximum velocity of the enzyme-substrate reaction (Vmaxapp < Vmax) which is in tune with previous

Figure 6. TLC profile of crude extract (Lane-1) and purified steroidal fraction (Lane-2) of *C. carandas*.

assumptions of Storey.[60] These entire results signpost towards the mixed nature of inhibition of enzyme hyase by the inhibitor molecule.[54,61] The purified steroidal fraction exhibited an IC50 of 5.19 ± 2.14 mM, which showed strong and better inhibition potential than positive control, quercetin (IC50 of 8.63 ± 2.43 mM) and crude extract (IC50 of 12.87 ± 2.23 mM) (Table 1). The inhibition constants (Ki) of crude extract, purified steroidal fraction and the standard inhibitor quercetin were found

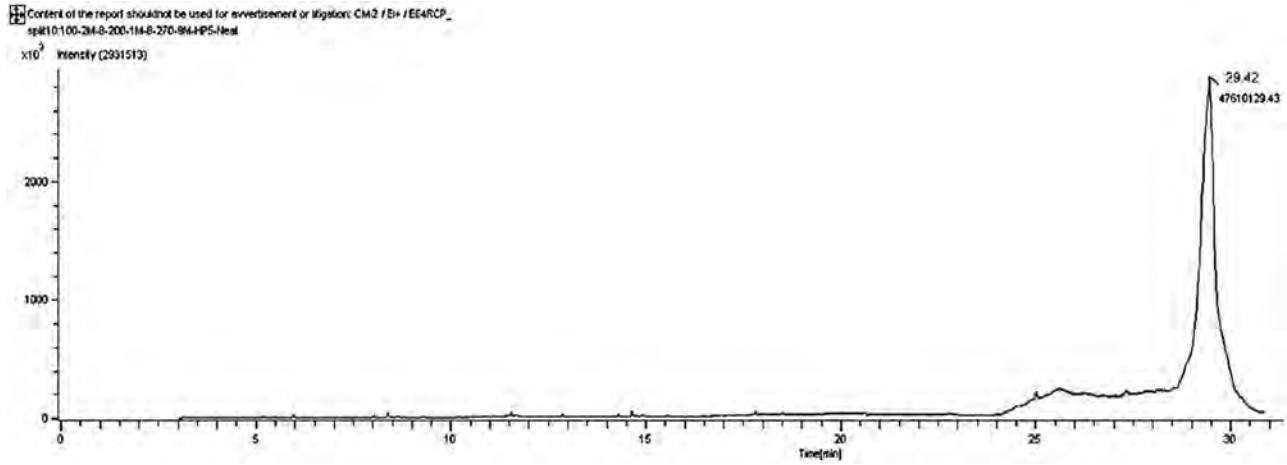

Figure 7. Gas chromatogram of the purified steroidal fraction of *C. carandas*.

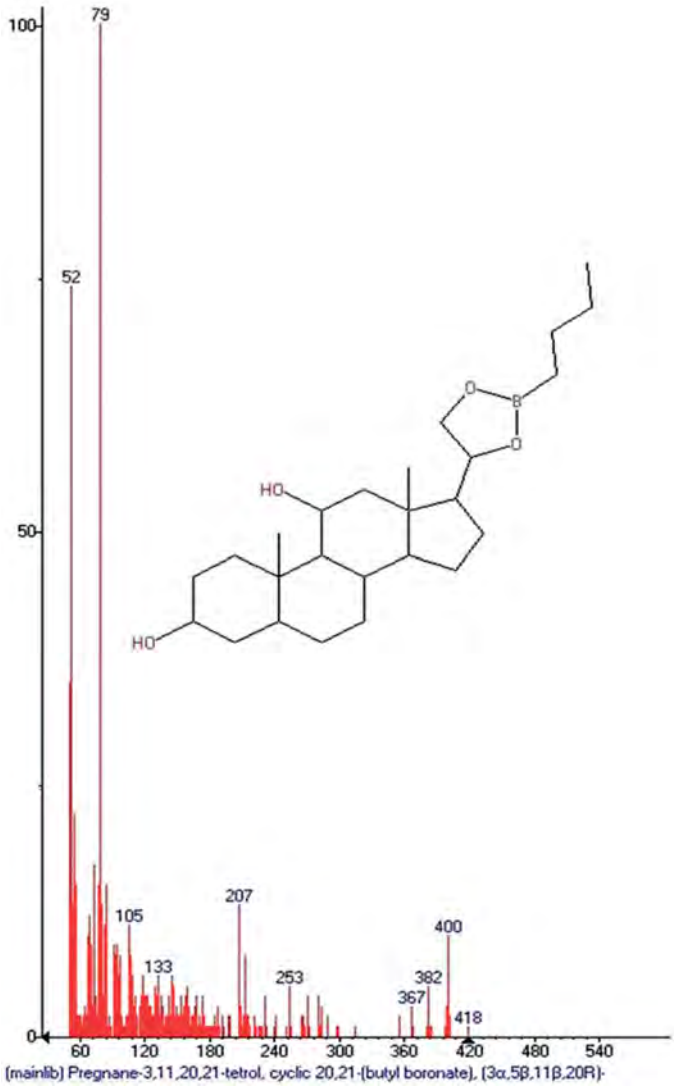

Figure 8. Mass spectrum analysis of the purified steroidal fraction [Pregnane-3,11,20,21-tetrol, cyclic 20,21-(butyl boronate), (3α, 5β, 11β, 20R)] of *C. carandas*.

to be 11.27 ± 1.17, 8.32 ± 1.01 and 12.65 ± 1.08 mM respectively. Moreover, the values of turnover number (Kcat) were significantly reduced (0.597 sec^{-1}) in presence of purified steroidal fraction as an inhibitor.

Characterization of hyaluronidase inhibitor

Various mobile phases were tested on TLC for the separation of bioactive metabolites from a crude extract of *C. carandas* leaves. The best separation was achieved with Toluene: Methanol (8:1) and 10% ethanolic sulfuric acid as a spraying reagent (Fig. 6).

In total 74 fractions of 5 mL each were eluted in the silica gel column chromatography, the eluted fractions were checked for the presence of single band on TLC plate. The fractions 28–43 that showed single blue colored fluorescence (R_f - 0.62) were pooled and used for the hyase inhibition activity. The fraction numbers 1–27 and 44–74 were also tested for hyase inhibition activity, but they did not show hyase inhibition potential. While the fraction number 28–43 showed the strong hyase inhibition activity and hence further characterized on TLC. On TLC it showed blue colored characteristic fluorescence with 10% ethanolic sulfuric acid as a spraying reagent (Fig. 6).

The gas chromatogram of the purified fraction of *C. carandas* revealed a single major peak (RT 29.42 min) (Fig. 7). The mass spectroscopic analyses followed by library search revealed a steroidal compound namely Pregnane-3, 11, 20, 21-tetrol, cyclic 20, 21-(butyl boronate), (3α, 5β, 11β, 20R) [$C_{25}H_{43}BO_4$] (Fig. 8). On the basis of results of TLC, gas chromatogram and mass spectroscopic analysis, it was concluded that the purified fraction of *C. carandas* leaves has steroidal nature.[62]

Conclusions

The results of the present study concluded that the purified steroidal fraction from *C. carandas* leaves extract has potential hyase inhibition activity. The hyase inhibitor was purified by silica gel column chromatography by using Toluene: Methanol (8:1) as a mobile phase. The kinetic parameters for the inhibition of the hyase by crude extract and purified steroidal fraction revealed the mixed mechanism of inhibition; since inhibitor affects both Km and Vmax of the reaction. The bioactive compound namely Pregnane-3, 11, 20, 21-tetrol, cyclic 20, 21-(butyl boronate), (3α, 5β, 11β, 20R) [$C_{25}H_{43}BO_4$] was identified as the main constituent by GC-

HRMS. This natural and plant-derived potent hyase inhibitor can serve as an important pharmacological and therapeutic tool. This could be effectively employed in the development of an anti-hyaluronidase therapeutic agent that can specifically target the pathogen by inhibiting the activity of hyase, which is a potent virulence factor of pathogens. This hyase inhibitor from *C. carandas* can also be employed in the development of anti-venom preparations; since hyase is an important constituent of venom that allows the venom components to spread.

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वस्तु आणि सेवा कर विधेयक २०१७ : एक दृष्टिक्षेप

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शिरपूर जि.धुळे

गोषवारा :-

बऱ्याच काळापासुन प्रलंबित असलेले व अतिशय महत्वाचे असे वस्तू व सेवा कर विधेयक हे राज्यसभेत पारित झाल्यानंतर १ जुलै २०१७ पासून GST कायद्याची अमंलबजावणी झालेली आहे. GST मुळे आता अप्रत्यक्षकरप्रणाली मध्येसुधारणाहोणार आहे व देशाच्या अर्थव्यवस्थेवर त्याचा अनुकूल परिणाम घडून येईल. कारण GST मुळे आता वस्तू व सेवांवर अनेककर न देता एकचकर नागरिकांना द्यावा लागणार आहे. GST ही एकत्रित कर प्रणाली असल्यानेकेंद्रसरकारचे ७तर राज्य सरकारच्या ११ प्रकारच्या विविध करांची जागा आता GST घेईल. आता केंद्रसरकारच्या सेंट्रलजीएसटी (GST)तर राज्याचा स्टेटजीएसटी(GST) अशा प्रकारेकरांची वसूली केली जाणार आहे. GST मुळे राष्ट्रीय उत्पन्नात वाढ होऊन भारतीय अर्थव्यवस्था प्रगतिपथावर जाईल.

प्रस्तावना :-

शासनाला देशाचेसंरक्षण व देशातील लोकांसाटीकल्याणकारी योजना राबविण्यासाटी रक्कम खर्च करावी लागते.ही रक्कम विविध करांची आकारणीकरूनमिळवावी लागते. परंतु भारतातील करचनाही अत्यंत गुंतागुंतीची असल्याकारणाने एकाच वस्तूची वेगवेगळ्या राज्यात बऱ्याच वेळेस वेगवेगळी किंमत आकारली जाते आणि त्यात उपभोक्त्याला जास्त किंमत मोजावी लागत असे व त्यातूनत्याचे शोषणहोई. यावर सक्षम उपाय म्हणून एकत्रकर सर्व देशात असावा हे विचारात घेऊन २०११ साली घटनेत १२२ वी दुरूस्तीकरून सर्वमतानें ८ ऑगस्ट २०१६ रोजी वस्तू व सेवा कराला लोकसभेत मंजूरी मिळाली व त्यानंतर १३ एप्रिल २०१७ मध्ये राज्यसभेत मंजूरी मिळाल्यानंतर राष्ट्रपतीनी या विधेयकाला संमती दिली.

संशोधकाने या लेखात GST मधीलतरतूदी, करांचे दर, GST प्रणालीचे लाभ, करांच्या टप्प्यात झालेले बदल इत्यादी बाबींचा विचार केलेला आहे.मात्र हे पाहण्याअगोंदर GST ची व्याख्या पाहणे उचित ठरेल.

GST हा वस्तू आणि सेवांवरील प्रत्येक टप्प्यावर मूल्यवृध्दीहोणाराकर असून त्यात उत्पादक अथवा सेवा पुरविणाऱ्यांपासून किरकोळ विक्रेत्यांपर्यंतची एकसाकळी आहे.मात्र कराचा भार अंतिम उपभोक्त्याला सहन करावा लागतो.

वस्तू व सेवा कर भारतातील अप्रत्यक्षकराच्या क्षेत्रातीलसुधारणांचे लक्षणीय पाऊल आहे.केंद्र व राज्य सरकारच्या अनेककरांच्या एकत्रिकरणामुळे ग्राहकांवरील २५% ते ३०% एकढेकरांचे ओझेकमीहोईल.

संशोधनाची उद्दिष्टे :-

संशोधकानेसदर विषयाच्या विवेचनासाठी पुढील उद्दिष्ट्ये ठेवली आहेत.

१) वस्तू आणि सेवा कर GST च्या तरतुदींचा अभ्यास करणे.

२) जनतेवरील करांच्या भाराचा व एकत्रीत करप्रणालीच्या लाभाचा अभ्यास करणे.

संशोधनाची पध्दत :-

या संशोधनाकरीता दुय्यम साधन सामुग्रीचा वापर करण्यात आला असूनसंशोधकाने विविध पुस्तके, मासिके, वर्तमानपत्रे, इंटरनेटवरील ब्लाग इत्यादीची मदत घेतली आहे.

विवेचन :-

भारतात वस्तू व सेवा कराची GST संकल्पना सर्वप्रथम डॉ.विजय केळकर यांच्या अध्यक्षतेखाली २००५ मध्ये नेमण्यात आलेल्या टास्क फोर्स ने आपल्या अहवालात मांडलीहोती. त्यानंतरजवळपास १५ वर्ष या वस्तू व सेवा करावर विचार मंथन सुरूहोते.मात्र जगात सर्वप्रथम १९५४ मध्ये फ्रान्स मध्ये GST ची अंमलबजावणी झालेली आहे.त्यानंतर १४० देशांमध्ये आता हाकर स्विकारला गेला आहे. GST हीकर प्रणाली तिच्या पारदर्शकतेमूळे प्रशासकीय अंमलबजावणीत सोपी असते. व्यापारात वाढ, कर आधाराचा विस्तार व कर अनुपालनत्यामुळेकेंद्र व राज्य सरकारच्या महसूलातही वाढ होऊन आर्थिक प्रगतीस प्रोत्साहन मिळू शकते. GST करकायद्यात वस्तुची निर्मिती किंवा उत्पादनापासूनते अंतिम उपभोक्त्यापर्यंत प्रत्येक टप्प्यावर अदा केलेल्या कराची जमा रक्कमसमायोजित करता येईल. तात्पर्य फक्त वर्धित मुल्यावर कर

ओझेसहन करावे लागेल.

GST अदाकरण्याचे दायित्व :-

GST कर प्रणाली अंतर्गत मालाचा पुरवठा आणि सेवापूर्ती करणाऱ्या व्यक्तिला कर भरावा लागेल. जेव्हा करपात्र व्यक्ति, उलाढालीची रूपये २० लाखाचीरसीमित मर्यादा पारकरत, असेल तेव्हा त्या व्यक्तिवर कर भरण्याची जबाबदारी येते. (ईशान्यकडील राज्य वगळता)

GST मध्येअंतर्भूतहोणारेकर:-

अ) केंद्रसरकारद्वारे आकारले जाणारेकर

१) केंद्रिय उत्पादन शुल्क
२) अतिरिक्त उत्पादन शुल्क
३) सेवा कर
४) औषधी व सौदर्य प्रसाधनावरील कर
५) अतिरिक्तसीमा शुल्क
६) केंद्रिय विक्री कर
७) विशेष अतिरिक्तसीमा शुल्क

ब) राज्य सरकारद्वारा आकारले जाणारेकर

१) राज्य विक्री कर
२) मनोरंजनकर
३) खरेदीकर
४) लॉटरी व जुगार यावरील कर
५) चैनीच्या वस्तूवरील कर
६) सट्टे बाजारावरील कर
७) जकात आणि प्रवेश कर
८) जाहिरातीवरील कर
९) वस्तू आणि सेवांच्या पुरवठ्याशी संबंधित अधिभार व इतकर

GST कक्षेबाहेर ठेवण्यात आलेलेकर :-

GST आल्यानंतर व्यापारी वर्गाला लागू होणारे खालील कर तसेच चालू राहतील. व्यवसाय कर, मुद्रांक शुल्क, मालमत्ता कर, वाहतूक कर, आयातीवर बेसिक कस्टम ड्युटी इत्यादीकर भरावे लागतीलते टाळता येणार नाही.

सद्यस्थितीत GST कर नसलेल्या वस्तु व सेवा :-

सार्वजनिक हिताच्या दृष्टीनेकेंद्र किंवा राज्य सरकार GST शिफारशी नुसार वस्तू किंवा सेवा दोन्ही पुरवठादारांना GST कर आकारणीतून अटींच्या अधीन राहूनसंपूर्णपणे किंवा अंशत: सुट देऊ शकते.

१) मानवी उपभोगासाठी लागणारे मद्यार्क व निकामपुरवठा GST कक्षेच्या बाहेर ठेवलेले आहे. या वस्तूंवर GST आकारला जाणार नाही. या शिवाय पाच पेट्रोलियम उत्पादनांवर सुध्दा सद्यस्थितीला GST आकारला जाणार नाही.

२) शिक्षण, आरोग्य, सेवा आणि धार्मिक यात्रेला GST च्या कक्षेच्या बाहेर ठेवण्यात आलेले आहे.

३) SEBI व IRDA ची सेवा, रेल्वेद्वारे मालाची वाहतूक, फळ भाज्यांची पॅकेजिंग, कचरा व्यवस्थापन, शाळेत मीड डे मील आणि डांन्स, संगित या सारख्या प्रशिक्षण सेवांवर सद्यस्थितीत GST कर आकारला जाणार नाही.

शासनामार्फत पुरवल्या जाणाऱ्या सेवा सुविधांवर कराची आकारणी :-

शासनामार्त पुरवल्या जाणाऱ्या सोयी GST कक्षेच्या बाहेर ठेवल्या आहेत. मात्र काही सेवा करपात्र आहेत अशा सेवा पुढीलप्रमाणे

१) पोस्टखात्याची स्पिड पोस्ट, एक्सप्रेस पार्सल, आयुर्विमा सेवा

२) विमान, जहाजाच्या आत अगर बाहेर तसेच विमानतळ, बंदराशी संबंधित दिल्या जाणाऱ्या सेवा

३) प्रवासी अथवा मालवाहतूक सेवा इत्यादी.

GST कर रचनेत ऑक्टोंबर १७ मध्ये झालेले बदल :-

GST ची आसाममधील गुवाहाटी येथे झालेल्या परिषेदत विविध वस्तूवरील करांचे दर पूर्वीपेक्षा कमीकरण्याचा निर्णय घेण्यात आला.त्यानुसार आता GST मुळे २११ वस्तू स्वस्त झाल्या.मात्र या कराच्या रचनेत' केलेल्या बदलामुळे केंद्रसरकारच्या उत्पन्नात रूपये२०,००० करोडची घटहोईल असा अंदाज व्यक्त केला आहे.कमी केलेल्या करांच्या वस्तू पुढीलप्रमाणे

१) सिमेंट, पेंट, वॉशिंग मशिन, वॉश बेसिन इत्यादी प्रकारच्या ५० वस्तूवरच आता २८% GST आकारण्यात येईल.

२) स्टेशनरी, घड्याळ, सुटकेस, वॉल पेपर्स, चटया, ग्रेनाईट, प्लायवूड, शॅम्पू, आफ्टर शेविंग क्रीम, मेकअपचेसाहित्य इत्यादी १७८ वस्तूवरील करांचा दर २८% वरून कमीहोऊन १८% करण्यात आला.

३) फ्लोरिंग, मार्बल, सावण इत्यादी १३ प्रकारच्या करांचे दर १८% वरून कमीहोऊन १२% करण्यात आला.

४) AC हॉटेल मधील जेवण, मिठाई प्रकारच्या ६ वस्तूवरील करांचा दर १८% वरून ५% करण्यात आला.

५) ८ वस्तूंवरील करांचा दर १२% वरून कमीहोऊन ५% करण्यात आला.

६) ६ वस्तूंवरील करांचा दर ५% वरून कमीहोऊन ०%

करण्यात आला.

GST करामुळेहोणारे लाभ :-

१) कर आकारण्याच्या व भरण्याच्या पध्दतीतसहजता आणि सुलभता येईल.

२) कराची चोरी किंवा कर न भरणे कमीहोईल.

३) संपूर्ण देशात एकाचकिंमतीला वस्तू व सेवा खरेदीकरता येईल.

४) संपूर्ण व्यवहार संगणीकृत पध्दतीने ऑनलाईनहोणार असल्यामुळे पारदर्शक व जलदगतीनेहोतील.

५) भारताच्या निर्यातीत १० ते १५% नी वाढ होईल.

६) चांगली उत्पादने जी देशाच्या एका भागात मिळतातती सर्वत्र मिळायला लागतीलत्यामुळेग्राहकांना वस्तूंची निवड करण्याचे पर्याय उपलब्ध होतील.

निष्कर्ष :-

वरील विवेचनावरून असे म्हणता येईलकी GST मुळेसंपूर्ण उद्योग जगताचे चित्र बदलेल. उद्योगांनागती पकडण्यासाटीही योग्यसंधी आहे. उपभोक्त्याच्या दृष्टीने विचार करता वस्तू आणि सेवावरील करांचे ओझेकमीहोणेहा सर्वात मोठा फायदा असेल. GST मुळे उत्पादन खर्च कमीहोऊन भारतीय उत्पादन देशात व परदेशात स्पर्धा करण्याससक्षम बनतील व स्थुल देशांतर्गत उत्पादन (GDP) मध्ये १ ते १.५% नी वाढ होईल. आज देशात दिवसेंदिवस वस्तू आणि सेवांच्या उत्पादनात वाढ होत आहे व सद्यस्थितीतकरप्रणालीत असलेल्या विविध करांच्या संख्यमुळेकरसंकलनाच्या खर्चात वाढ होतहोती. यासाठी अतिशय सोपी व पारदर्शी असलेल्या कर प्रणालीची आवश्यकताहोती जी या GST च्या अंमलबजावणीमुळे शक्य होईल.

संदर्भ :-

१) प्रा. प्रविण कामथे, प्रा.मेघना पाटील (२०१७) जीएसटी वस्तू आणि सेवा करकायदा एक परिचय, साई ज्योती पब्लिकेशन्स, नागपूर

२) दिपक पाटील (२०१७) व्हिजन रिसर्च - ए पिअर रिव्हूड नॅशनलरिसर्चजर्नल

३) प्रा.डॉ.राजेंद्र रसाळ, सार्वजनिक आय-व्यय, सक्सेस पब्लिकेशन पुणे

४) रंजना कोसंबे, भारतीय अर्थव्यवस्था, भगीरथ प्रकाशन, पुणे

५) योजना मासिक, नोव्हेंबर २०१६

६) दैनिक महाराष्ट्र टाईम्स, जुलै २०१७

७) अर्थसंवाद मासिकचे विविध अंक

८) www.wikipidia

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गडचिरोली जिल्हयात आदिवासी विकास महामंडळाव्दारे राबविण्यात आलेल्या आदिवासी विकास योजनांचे मुल्यमापन

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प्रस्तावना

आदिवासी विकास महामंडळ राबवित असणा—या योजनांचे गडचिरोली सारख्या अतिदुर्गम भागात अनन्य साधारण महत्व आहे. गेल्या काही वर्षात शासनाच्या भूमिकेत आमुलाग्र बदल झाला असून ग्रामीण भागातील विविध विकास कार्यक्रमावरील खर्चाचे प्रमाण कमी झाले आहे. आदिवासींचे वास्तव्य असलेल्या अतिदुर्गम भागामध्ये शासनाला विकासात आदिवासी जमातींना सामावून घेण्याच्या दृष्टीने आदिवासी विकास महामंडळाच्या योजना महत्वपूर्ण भूमिका वठवू शकते. आदिवासी विकास महामंडळाज्या योजना राबवित असते अशा योजनांचे मूल्यमापन करणे आवश्यक असते. या दृष्टीने राबविण्यात येणा—या विकास योजनांचे मुल्यमापन करण्यात आले आहे.

१.एकाधिकार खरेदी योजना :—

सन १९७७—७८ मध्ये महराष्ट्रातील आदिवासी उपयोजना क्षेत्रात ६ तालुक्यात प्रायोगिक तत्वावर सुरु करण्यात आली या एकाधिकार खरेदी योजनांमुळे

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भारतातील भिल्ल जमात : एक अभ्यास

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सारांश :-

भारतात विविध जाती, धर्म, पंथ, बोलीभाषा असून देखील जगात भारताने आपले वेगळेपण टिकवून ठेवले आहे. भारतात काही जमाती प्राचीन काळापासून अस्तित्वात असल्याच्या दिसून येतात. अशा ज्या जमाती पूर्वीपासून अस्तित्वात आहेत. त्यांनाच मूळ निवासी असे म्हणतात. भारतीय राज्यघटनेनुसार राष्ट्रपतींनी घोषित केलेल्या जमातींना अनुसूचित जमाती असे संबोधले जाते. भारतात अनुसूचित जमातीत एकूण ४७ जातींचा समावेश केलेला आहे. यापैकी भिल्ल एक जमात असून लोकसंख्येने सर्वात जास्त आहे. भिल्ल जमात महाराष्ट्र, गुजरात, मध्ये प्रदेश, राजस्थान या राज्यांमध्ये नदी-नाले, डोंगरी भागात मोठ्या प्रमाणात वास्तव्य करतांना दिसून येते.

प्रस्तावना:-

भिल्ल जमात आदिवासी जमातीपैकी एक मुख्य जमात म्हणून ओळखली जाते. भिल्ल जमातीची लोकसंख्या आदिवासी जमातीपैकी मोठ्या प्रमाणावर दिसून येते. भारतातील आंध्रप्रदेश, गुजरात, राजस्थान मध्ये प्रदेश, महाराष्ट्र व त्रिपुरा या राज्यांमध्ये भिल्ल जमात मोठ्या प्रमाणात दिसून येते. अरवली पर्वत, विंध्य व सातपुडा पर्वत रांगेतील दाट जंगल व डोंगरी भाग हे त्यांचे राहण्याचे मुख्य ठिकाण दिसून येते. सातपुडा प्रदेश हा प्रामुख्याने भिल्ल प्रदेश म्हणून ओळखला जातो. या पर्वतातील तापी नदी व नर्मदा नदी खोऱ्यातील प्रदेश 'भिलवाडा' म्हणून ओळखला जातो.२ महाराष्ट्र राज्याचा विचार केल्यास नंदुरबार व धुळे जिल्ह्यात भिल्ल जमात अधिक दिसून येते. मध्ये प्रदेश मधील नेमाड जिल्हा, गुजरात मधील सुरत व भडोच जिल्हा तसेच महाराष्ट्रातील जळगाव जिल्ह्यातील पर्वत रांगामध्ये आदिवासी भिल्ल मोठ्या प्रमाणात आढळून येतात.

संशोधनाचे उद्देश:-

१)आदिवासी भिल्ल जमातीचा चिकीत्सक अभ्यास करणे.
२)भिल्ल जमातीच्या उत्पतीवावत अभ्यास करणे.
३)भिल्ल शब्दचा अर्थ शोधने.
४)भिल्ल जमातीतील पोटजातींचा अभ्यास करणे.

संशोधनाचे गृहीतकृत्य:-

१)भिल्ल जमात ही एक आदिवासी जमात आहे.
२)या जमातीचे अस्तित्व प्राचीन काळापासून असल्याचे दिसून येते.
३)भिल्ल जमातीचे राहण्याचे मुख्य ठिकाण जंगलातील डोंगरी भाग , नदी नाले इत्यादी आहे.
४)भिल्ल जमातीची स्वतःची बोलीभाषा आहे.
५)भिल्ल जमातीच्या वऱ्याच पोटजाती जाती असून सण-उत्सव, रूढी, परंपरा यात साम्य आहे.

संशोधन पद्धती :-

प्रस्तुत संशोधनात संशोधकाने माहितीचे संकलन करण्यासाठी प्राथमिक व दुय्यम साधन सामुग्रीचा उपयोग केलेला आहे. प्राथमिक स्रोतांअंतर्गत निरीक्षण परीक्षण पद्धतीचा वापर केला आहे. निरीक्षण परीक्षण पद्धतीसाठी मुलाखत अनुसुचीचा देखील वापर केलेला आहे. तसेच दुय्यम साधन सामुग्रीचा वापर करीत

असतांना अप्रकाशित पीएचडी प्रबंध, संदर्भ ग्रंथ, वार्षिक अंक, मासिके व वर्तमान पत्रे, तसेच विविध शासकीय अहवाल इत्यादींचा उपयोग केलेला आहे.

भिल्ल शब्दचा अर्थ:-

भिल्ल प्राचीन काळात मध्येपुर्वेतून आलेल्या प्रोट्रो-ऑस्ट्रलाईड लोकांचे वंशज होय. असे मत मानववंश शास्त्रांचे मत आहे. भिल्ल लोकांचा उल्लेख प्राचीन संस्कृत व नंतरच्या अपभ्रंश वाङ्मयात आलेला आहे. मात्र भिल्ल शब्दचा अर्थ रानटी जमात असा घेतलेला दिसून येतो. उत्तर भारतातील तमिळ कवींनी या रानटी आदिवासींना उद्देशून 'विल्लुवर' (धनुर्धर) असा शब्द प्रयोग केला आहे. हे शब्द सांप्रतच्या भिल्लांच्या पुर्वाजाविषयी वापरलेले असावे. असा केंब्रीज इतिहासकारांचा मत आहे. 'विल्लू' किंवा 'बिल्लू' या शब्दाचा अर्थ द्रविडीयन भाषेत धनुष्य असा होतो. धनुष्य हे भिलांचे शस्त्र आहे. तसेच मध्ये भारतातील रानटी लोकांचा वैदिक वाङ्मयात 'निषाद' असा उल्लेख केलेला आहे. भिल्ल शब्दाचा उल्लेख वेगवेगळ्या कालखंडात आढळून येते.[३]

भिल्ल शब्दची उत्पती :-

भिल्ल शब्द संस्कृत भाषेतील 'भिल्ल' या शब्दाचे तदभाव रूप आहे. जो संस्कृत मधील 'भिल–विल–भेदणे' या धातूपासून वनला आहे. 'भिल्ल' म्हणजे 'धनुर्धारी' 'बिल' म्हणजे 'धनुष्य'किंवा 'बिल्लावर' या शब्दाचा अपभ्रंश रूप 'भिल्ल' होय. महाभारतातील यादव कुळाशी संबंधित हरिवंश पुराणामध्ये भिल्लांचे अनेक प्रकारचे संदर्भ आढळतात. थोडक्यात भिल्ल जमात इतकी प्राचीन आहे की, ई.स.पु. ५०० मध्ये ही याची नोंद केली आहे.[४]

भिल्लांच्या उत्पत्तीविषयी आख्यायिका :-

भिल्ल जमातीच्या उत्पत्तीबावत अनेक संदर्भ मिळतात. पहिला संदर्भ म्हणजे जेंव्हा प्रभू रामचंद्र दंडकारण्यात आले तेंव्हा त्यांना शबरी भिल्लीण भेटली. त्याकाळात या प्रदेशात राहणाऱ्या लोकांना 'शवर' 'किरात', 'निषाद' अशा नावांनी संबोधले जात असे. थोडक्यात ह्या सातपुड्यातील लोक म्हणजेच भिल्ल होय.

दुसरा संदर्भ असा दिला गेला आहे की, महादेवाची मानवपत्नी पहाडकन्या हिला अनेक पुत्र झाली. त्या पुत्रापैकी एका बसक्या नाकाच्या व काळ्यारंगाच्या मुलाने महादेवाचे नांदी मारून टाकले. याच राग महादेवाला आले व महादेवाने त्या मुलाला जंगलात सोडून देण्यास सांगितले. तो मुलगा म्हणजे भिल्ल समाजाचे मूळ संस्थापक होय.

भिलांच्या पोटजाती किंवा प्रकार :-

१) पावरा भिल्ल :-

खानदेशातील अनुसूचित जमातीमधील 'पावरा' ही भिल्ल जमात आहे. या जमातीतील पावरा भिल्ल उंच, धडधाकट, गोरे व नाकी डोळा सरस आहेत. त्यांची तुलना नेहमी राजपुतांशी केली जाते. डॉ. धनंजय चौधरी यांच्या अभ्यासानुसार खानदेशातील पावरा स्वतःला राजपूत समजतात. समाजात पावरा जमातीची वस्ती मुख्यतः नंदुरबार जिल्ह्यातील अक्राणी, शहादा, तळोदा, तर धुळे जिल्ह्यातील शिरपूर तालुक्यात व जळगाव जिल्ह्यातील चोपडा, रावेर आणि यावल तालुक्यात आढळते. पावरा आदिवासी जमात सातपुड्याच्या भिल्ल आणि आदिवासी जमातीपेक्षा सर्वस्वी वेगळी व स्वतंत्रपणे वावरणारी आगळीवेगळी जमात आहे. पावर भिल्लांचे मूळ ठिकाण पावागड हे आहे. म्हणून पवारा भिल्ल पवागडाहून आलेले असावे. काही पवार भिल्ल 'मथवा' संस्थानातून आल्याचे सांगतात. म्हणून स्थानिक लोक 'माथवाडी' म्हणून ओळखले जातात. भिलाटीचे भिल्ल व पवारा भिल्ल दोहांमध्ये स्थान, राहणीमान, भाषा इत्यादी बावतीत फरक दिसून येते.

२) कोकणा भिल्ल :-

कोकणा महाराष्ट्रातील एक प्रमुख आदिवासी जमात असून १९८१ च्या जनगणनेनुसार त्यांची लोकसंख्या ३,५२,९३२ इतकी आहे. ही जमात ठाणे, नाशिक, धुळे, नंदुरबार या जिल्ह्यामध्ये वास्तव्य करीत आहे. कोकण हे कोकणा जमातीचे मूळ वस्तीस्थान आहे. कोकणा जमात ही राज्यातील स्थानिक जमात असून

समुद्र किनारा आणि सयाद्री पर्वत यामधील पट्ट्यात वसलेली आहेत. कोकणा भिल्ल भिलाटीतील भिल्ल पेक्षा वेगळे आहेत. सामाजिक, सांस्कृतिक, आर्थिक, धार्मिक व शैक्षणिक बाबतीत दोघांमध्ये फरक दिसून यतो.

३) तडवी भिल्ल:-

तडवी भिल्लांच्या उत्पातीविषयी अनेकांनी आपले मते मांडलेली आहे. 'तरवी' हा अरबी शब्द व त्याचा अर्थ 'मार्गदर्शक' किंवा 'वाटाड्या' असा आहे. 'भिल्लराज' या हिंदी नाटकानुसार 'तरवी' हा अरबी शब्द असून तलवार चालविणारा असा त्याचा अर्थ होतो. उर्दू शब्द 'तरवी' वरून त्याचे तडवी नाव पडले. तडवी ही आदिवासी भिल्लांमधीलच धर्मपरीवर्तीत जमात आहे. त्यापैकी काही तडवी भिल्ल इस्लाम धर्मीय आहेत. तडवी म्हणजे वाटून मुसलमान झालेला भिल्ल असे महाराष्ट्र शब्दकोशात विभाग-४ मध्ये आढलळते. भिल्लांच्या मुख्यशाखेपासून फुटून 'तड' म्हणजे दूर झालेल्या लोकांना 'तडवी' असे म्हणतात.

तडवी भिल्लात वतनदार व सुखवस्तू असे दोन भेद आहेत. तडवी भिल्लांनी औरंगजेबाच्या कारकिर्दीत (१६५८ ते १७०७) मुसलमान धर्माची दीक्षा घेतली असावी किंवा मुसलमानांचे काही संस्कार घेतलेले दिसून येतात. तडवी समाज दिवाळी, आखाजी (अक्षय्य तृतीया), होळी, पोळा हे सन साजरे करतात.

भिलाटीतील भिल्ल मात्र तडवीपेक्षा दिसायला वेगळे आहेत.

४) गावित तथा मावची :-

खानदेशातील गावित, गामित ही एक आदिवासी भिल्ल जमातीपैकी आहे. १९८१च्या जनगणनेनुसार गावित भिल्लांची लोकसंख्या ११०८२८ होती. धुळे जिल्ह्यात गावित, मावची भिल्ल एकवटलेले दिसून येतात. आदिवासी मावची भाषा बोलणारे गावित, वळवी, वसावे, पाडवी, नाईक व देसाई इत्यादी उपप्रकार मावची भिल्लांचे आहेत. धनंजय चौधरी यांच्या अध्ययनानुसार गावित भिल्लांपेक्षा भिलाटीवस्तीतील भिल्लांमध्ये सामाजिक, आर्थिक, राजकीय व शैक्षणिक प्रगती कमी प्रमाणात दिसून येते.

५) नायकडा :-

नायकडा ही एक लाहनशी अनुसूचित जमात आहे. १९७१ च्या जनगणनेनुसार महाराष्ट्र राज्यातील लोकसंख्या ९३१५ तर धुळे जिल्यात ५४८७ इतकी होती. नायकडा याचा अर्थ 'नायक' असा होतो. त्यांना नाईक म्हणून देखील ओळखतात. चोलीवाला नायका, कपाडिया नायका, मोठा नायका आणि नाना नायका या त्यांच्या पोटजमाती आहे. वहुतेक सर्वे नायकडा लोक आपली मातृभाषा भिल्लीच बोलतात.

६) धानका:-

खानदेशातील आदिवासी भिल्ल जमातीत धानका ही एक जमात आहे. ही जमात धुळे जिल्यात ४४०११ व जळगाव जिल्यात ११०२९ या दोन जिल्यात आढळते. धानका जमातीत तडवी, तटरिया वळवी या उपगटांचा समावेश होतो. हे लोक धुळे जिल्यातील अक्कलकुवा, नवापुर, तळोदा, नंदुरबार व जळगाव जिल्ह्यांच्या सातपुडा पर्वत रांगात व लगतच्या गुजरात राज्यात राहतात. सामाजिकदृष्ट्या ते स्वतःला बाकीच्या भिल्लांपेक्षा वेगळे समजतात. परंतु भिल्लांशी त्यांचे संबंध नाकारीत नाही. धानका स्वतःला तडवी धानका व तेटारिया धानका या नावांनीही ओळखले जातात. भिलाटीतील भिल्ल जमातीचा विचार केल्यास धानका भिल्ल अनेक बावतीत भिलाटीतील भिल्लांपेक्षा वेगळे आहेत.

७) लाड्या भिल्ल :-

खानदेशातील आदिवासी भिल्ल जमातीतील 'लाड्या भिल्ल' ही एक जमात आहे. ताडोदा तालुक्याच्या पश्चिम भाग व आक्कल्कूवाच्या खालचा भाग या भौगोलिक प्रदेशात राहणाऱ्या पाडवी , वळवी ,वसावा, वसावे, गावित वैगरे कुळातील भिल्लांना तळोदा तालुक्याच्या पूर्व भागातील व सातपुड्यातील भिल्ल 'लाडक्या' किंवा लाड्या भिल्ल' या नावाने ओळखले जातात. हा भिल्ल गट वळवी, वसावे, वसावा, यांच्यापैकीच आहे. परंतु त्याचा हा एक प्रादेशिक गट समजला जातो.

८) मथवाडया भिल्ल :-

खानेशातील भिल्ल जमातीतील मथवाडया भिल्ल ही एक जमात आहे. ही जमात 'नर्मदा जवळील मथवाडा मूळ रहिवासी असल्याने त्यांना मथावाडी म्हणून ओळखले जाते. मथावाडी लोकांना लंगोटे , माथावाडे असे देखील संबोधले जाते.

९) बोंडे गवाल:

खानदेशातील भिल्ल जमातीपैकी बोंडे गावाल ही एक जमात आहे. हा भिल्ल गट लोकसंख्येने फार कमी आहे. बोंडे भिल्ल आदिवासी देवदेवतांचे मुखवटे बांधून कथा सांगत गावोगाव नाचत फिरतात. गावात गुरे चारण्याचे काम करतात व मयताच्या उत्तरक्रियेच्या वेळी वाद्य वाजवितात. लग्न कार्यात हे लोक वाजंत्रीचे काम करतात. भिलाटीतील भिल्ल मात्र या भिल्लांपेक्षा वेगळे आहेत.

१०) मेवासी भिल्ल :-

खानदेशातील जुन्या मेवासी इस्टेट मध्ये राहणाऱ्या भिल्लांना परदेशीकेतुसार मेवासी भिल्ल म्हणून ओळखले जातात. हे गट भिन्न नावांनी ओळखले जात असले तरी त्यांची भाषा, पेहराव, संस्कृती, राहन-सहन, चालीरीती, रूढी, परंपरा, धार्मिक आणि सांस्कृतिक विधी सारख्याच आहेत.

खानदेशातील भिलाटीतील भिल्लांमध्ये काही चालीरीती सारख्या असल्या तरी यापेक्षा अनेक बाबतीत हा समाज वेगळा दिसतो.

११) डांगी भिल्ल:-

आदिवासी जमातीतील डांगी भिल्ल ही एक जमात आहे. गुजरात राज्यातील हवा डांगचा प्रदेशात डांग भिल्लांची वस्ती असावी. या परिसरात शबरी नावाची भिल्लीण राहत होती. या भागातील भिल्लांमध्ये कलाचे दारिद्र्य व लोकसंख्या वाढ दिसून येते. खानदेशातील भिलाटी वस्तीत राहणारा भिल्लांचे साल्हेर, सुल्हेर, साक्री इत्यादी . भिल्ल जमातीशी काही बाबतीत साम्य दिसून येते.

१२) राठवा भिल्ल:-

आदिवासी भिल्लामध्ये राठवा भिल्ल ही एक जमात दिसून येते. राठवा बाबत असे मत आहे हे नायकडामधील आडनाव आहे. त्यांच्या पेक्षा आपण उच्च आहोत असे ते मानतात. ते अंशतः भिल्ल असल्याचे दिसतात. आपला धर्म हिंदू असल्याचे ते सांगतात. हनुमान , अंबाबाई यांची उपासना ते करतात. लमाणी आणि बंजारी मातृ भाषा असल्याचे ते सांगतात. नंदुरबार जिल्ह्यातील नवापुर तालुक्यातील खांडबारा येथे कै. चामुंडालाल सी. राठवा हे आदिवासी भिल्ल समाजाचे निवृत्त शिक्षक व गाढे अभ्यासक होते.

१३) चोप्रा किंवा चोवरा :-

आदिवासी भिल्ल जमातीतील चोप्रा किंवा चोधरा ही एक भिल्ल जमात आहे. ही जमात मूळ गुजरात राज्यातील जमात होय. मुसलमानी आक्रमणानंतर ते दक्षिणकडे सरकले. पावागड हे त्यांचे मूळ ठिकाण होय. कोकणा, नाईक, गमित, मावची इत्यादी जमातीपेक्षा ते आपणास श्रेष्ठ समजतात. त्यांच्यात भारतीय, चंद्रताला , चोकापूर, टाकारीया, बाळबाड, सातला मोटा ,नाना खोंडा, वामनिया, मरेल, कनावी, राजपूत व रावलीया इत्यादी कुळे आहेत.हे कुल मात्र भिलाटी वस्तीत राहणाऱ्या भिल्लांमध्ये दिसून येत नाही.

१४) भिलाटीतील भिल्ल:-

खानदेशातील इतर आदिवासी भिल्ल जमातीपेक्षा भिलाटीतील भिल्ल जमात सामाजिक, सांस्कृतिक, आर्थिक, व शैक्षणिक बाबतीत वेगळी आहे. ज्याना खानदेशात कोटील, कटोनि, वरडे,कोटले , भिलाला अशा अनेक नावांनी देखील ओळखले जातात. जळगाव जिल्ह्यात कोटील भिल्लांचा एक प्रकार अस्तित्वात आहे. कोटीली ही भिल्लांची एक पोटजमात असून सातपुड्याच्या पायथ्याशी वसलेली गावे वगळता तालुयात गावोगावी ही जमात विखुरलेली आहे. कोट त्यांच्या वस्त्यांना ;भिलाटी' म्हणतात . कोटल्यांची भिलाटी किंवा वस्ती जमीनदार, बागायतदार , गुजर पाटील, कुणबी समाजाच्या आसपास आढळते. 'गाव तिथे भिलाटी' असा वाक्प्रचार कदाचित रूढ झालेला आहे. भिलाटी तील भिल्ल कामाच्या शोधात गावाकडे स्थलांतरित झाले असावे.[५]

निष्कर्ष:-

१)भारतीय राज्यघटनेनुसार भिल्ल एक आदिवासी जमात आहे.
२)भिल्ल जमात प्राचीन काळापासून अस्तित्वात असल्याचे विविध संदर्भ दिसून येतात.
३)भिल्ल जमात आजही नदी-नाले, जंगल, डोंगरी भागात, गावाबाहेर किंवा गावालगत वस्तू करून राहत असल्याचे दिसून येतात.
४)भिल्ल जमातीची स्वतःची बोलीभाषा दिसून येते.

५)भिल्ल जमातीत विविध पोटजामती असुन आचर-विचार, संस्कृती, वोलीभापा यात वराच साम्य दिसून येतो.

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4. एक समाजशास्त्रीय अभ्यास'. अप्रकाशित पीएचडी प्रबंध, डॉ.बाबा साहेब आंबेडकर मराठवाडा विद्यापीठ औरंगावाद.पृ.क्र.५५ ते ५६ धनंजय रमाकांत चौधरी-(२०११) "खानदेशातील आदिवासी जमातीतील भिल्लांच्या सामाजिकप्रगतीचा इतिहास" अप्रकाशित पीएच.डी प्रबंध, उ.म.वी. जळगाव.

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A Geographical Analysis of Organic Fertilizers Application in Shirpur Tehsil of Maharashtra (MS), India

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Sanjaykumar N Patil [***]

Abstract

Dhule district of Maharashtra state comprises four tehsils viz. Dhule, Shirpur, Sakri, and Shindkheda. The soil quality and productivity are being affected badly in the southern parts of Shirpur tehsil. It is necessary to evaluate the adverse impacts of chemical fertilizers in the Shirpur tehsil. The present study is organized in order to find out the farmers attitude towards organic fertilizer application in the Shirpur. 598 farmers from 12 villages of four tehsils were questioned for their perspective towards organic and chemical fertilizer application in the farm. The research outcome reveals different attitudes for organic fertilizer application and benefits in different groups i.e. education levels of farmers, farm size, and villages. The large farm size farmers and educated farmers are more in numbers compared to small size farmers and illiterate farmers. Therefore an attempt has been made to find out the attitude of farmers towards utilization of organic fertilizer in the farms for sustainable farming.

Keywords:

Agriculture;
Fertilizers;
Organic;
Productivity;
Soil;
farm size etc.

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1. Introduction

The soil quality and productivity are being badly affected by the overconsumption of chemical fertilizers in the southern part of Shirpur tehsil. The soil and environment protection are one of the basic principles of the organic farming and it advocates the natural ways of improvement in the environment. In the organic farming the synthetic fertilizers, herbicides, and medicines are hardly applied [12]. The concept of organic agriculture builds on the efficient use of locally available resources, and the use of technologies like soil fertility management, the closing of nutrient cycles, control of pests and diseases by means of natural antagonists.

From the mixed farming point of view, it is necessary to investigate the opportunities and limitations of stockless organic farming with regard to both agro-ecological, economic stability, and sustainability of farming systems [2]. The mixed farming concept opens up new ways of achieving sustainable development [11]. The share of corn, sunflower, and rape in crop mix the investment in precision farming is paid off after six years under Hungarian conditions [4]. The implementation of precision farming promotes the rational application of chemicals but requires capital investment. According to K. Takács-György, the reduction of

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chemical use and environmental load in agriculture is increasingly desired. Developed countries promote the minimum use of pesticides and farmers have to change traditional practices accordingly. Organic agriculture has the potential to improve the soil fertility, biodiversity conservation, and sustainable agricultural production. It also improves agronomic and economic performance to yield more stable tropical ecosystems, especially in risk-prone tropical ecosystems. Organic farming is having good potential to achieve better food quality and food security [5]. Chemical farming or conventional agriculture often creates an unstable ecosystem in which the potential for maximum yield is inevitably associated with risks due to ecosystem instability [10].

Farmers' groups are increasingly adopting organic techniques as a method of improving productivity and food security in these systems. However, no systematic attempt has hitherto been made to track the extent to which these approaches are being employed, or their effectiveness compared to other approaches, in meeting economic, social and environmental objectives [8], [11]. An important issue with the development of organic farming is tillage, tillage intensity in particular. Despite the suggestions of mentors of the organic farming theory and of farmers associations to reduce tillage intensity, the majority of organic farmers still apply deep inversion tillage with a plough [3],[6], [9].

1.1 Location and Extent of Study Region

Shirpur tehsil is located in Dhule district of the northern part of Maharashtra state. Dhule district comprises four tehsils. The Shirpur tehsil has covered an area of 804.02 sq. km. Shirpur tehsil is lying between $21^{0}11'$ north and $21^{0}38'$ north latitudes and $74^{0}41'$ east and $75^{0}11'$ east longitudes.

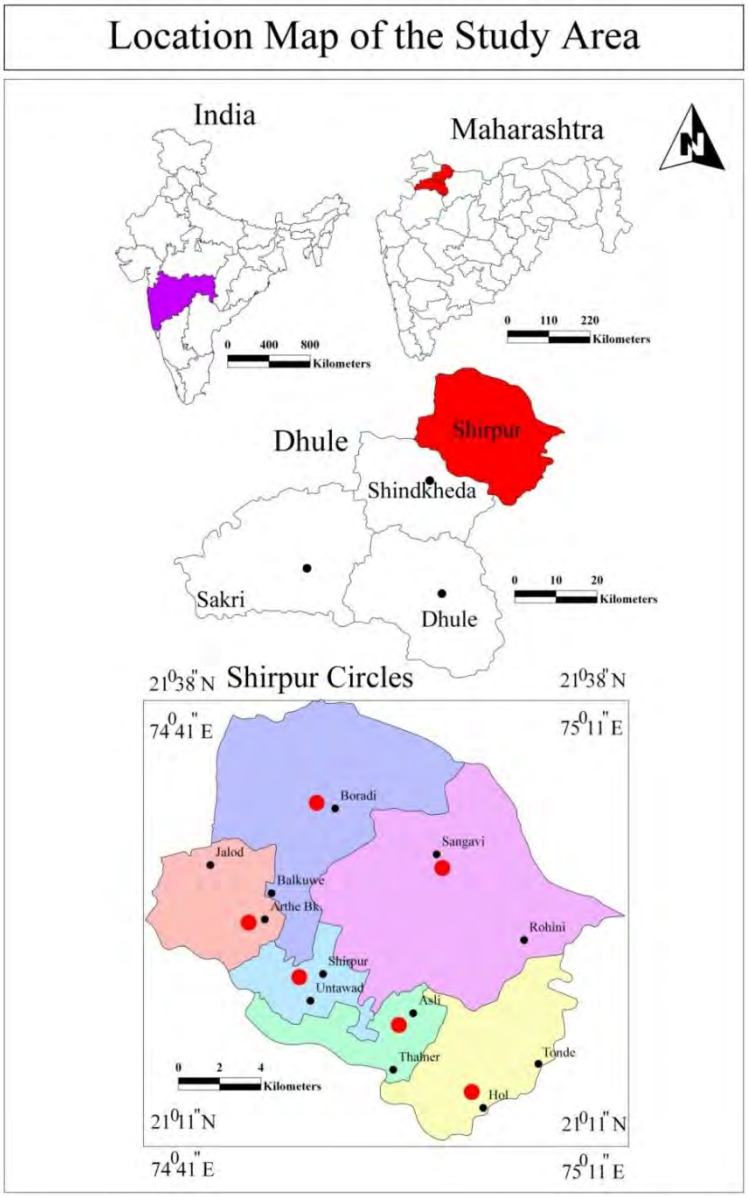

Figure 1 Location Map

2. Research Method

2.1 Source of Data

The relevant primary data are obtained from the respondents (farmers) by administering a well-structured interview schedule. The researcher has made a visit to each and every household and relevant data are obtained from them by establishing a good rapport with them.

The interview questionnaire covers topics such as irrigation, use of inorganic, organic inputs, attitudes of the farmers and their awareness on sustainable development practices. In addition, Focus Group Discussions was held with the farmers to gain insights and obtain more qualitative data. Official statistical data were obtained through secondary sources such as official records and census reports.

2.2 Data Analysis

The important independent variable for the analysis of the data is farm size, respondent's education level and size of the village. The collected data are classified and tabulated. The chi-square test is applied to examine the association between socioeconomic status of farmers and their awareness and adoption of organic farming practices. Further percentages and averages are applied depending on the requirement of the situation.

2.3 Methodology

The primary data was collected through questionnaire and interactions with farmers (respondents) of four villages in Shirpur tehsil. The data was processed for estimation of organic fertilizer application level in the study area. Then Chi-square test has been used to find out the correlation of organic fertilizers application to farm size and education level of farmers.

2.4 Sampling Locations

Shirpur tehsil of Dhule district is focused on the present study. The tehsil is predominantly an agrarian region. Agricultural is done in all villages of the tehsil. Shirpur tehsil has six circles Arthe, Shirpur, Thalner, and Holnanthe are agriculturally developed in terms of yield potential and irrigational facilities. Boradi and Sangavi are agriculturally backward because most of the areas of it consist by Satpura mountain ranges.

The size of the study sample is limited to two villages of each circle the tehsil and ten percent of the farmers from the sample villages. The study sample villages and the respondents (farmers) were drawn by adopting Multi-Stage Stratified Random Sampling Technique to represent marginal, small, medium and large farmers. Accordingly, a survey of twelve villages in Shirpur tahsil named Boradi, Balkuwe, Sangavi, Rohini, Arthe Bk, Jalod, Shirpur, Untawad, Thalner, Asli, Hol, and Tonde was undertaken and a total of 598 farmers were studied.

2.5 Sampling Procedure

In the first phase, the researcher has selected one developed village one backward village from each circle of the study region.

According to the Table. 1 in the second phase is the selection of sample villages from each circle. There are six blocks or revenue circles in Shirpur tehsil. Shirpur tehsil has 147 revenue villages. Thus, a total of 12 villages are selected from six blocks representing 10% of the farmers in each village.

Tables and Figures are presented center, as shown below and cited in the manuscript.

Table 1. In the third stage involves the selection of farmers from study villages.

Sr. No.	Circle / Block	Sampling Village	No. of farmers	10 % Selected Farmers
01	Boradi	Boradi	611	61
		Balkuwe	528	53
02	Sangavi	Sangavi	749	75
		Rohini	688	69
03	Arthe	Arthe	376	38
		Jalod	274	27
04	Shirpur	Shirpur	1034	103
		Untawad	236	24
05	Thalner	Thalner	699	70
		Asli	356	36
06	Holnanthe	Hol	165	17
		Tonde	251	25
Total			**5967**	**598**

Source: Collected and Tabulated by Researcher

From each village 10% of the farmers are selected as sample, thus totally 598 farmers are selected from six blocks of Shirpur tehsil.

3. Results and Analysis

3.1 Application Level of Organic Fertilizers

The application of organic inputs (fertilizers) is discussed in relation to use of green manure, compost, ash and animal dung. Data presented in Table. 2 indicate the farm size wise respondent's application of organic fertilizers.

It is noticed that out of the total 598 respondents 11.37% farmers used bio-fertilizers for cultivation. More than 70 % of the respondents of Arthe, Thalner, Hol and Tonde Village apply cow dung and ash as bio-fertilizer for cultivation. Out of 598 respondents, 23.58 % of them use chemical fertilizer for cultivation. 65.05% farmers in the Shirpur tehsil are utilizing both bio and chemical fertilizers. This level of application is prominent among the farmers of Shirpur, Boradi, Hol, Asli, Jalod and Balkuwe villages.

Table 2. Village wise number of consumers of organic fertilizers and chemical fertilizer

Villages	Bio-fertilizers	Chemical Fertilizers	Both	Total
Boradi	4 (6.56)	17 (27.87)	40 (65.57)	61
Balkuwe	5 (9.43)	15 (28.30)	33 (62.27)	53
Sangavi	11 (14.67)	16 (21.33)	48 (64.00)	75
Rohini	11 (15.94)	14 (20.29)	44 (63.77)	69
Arthe	5 (13.16)	6 (15.79)	27 (71.05)	38
Jalod	3 (11.11)	7 (25.93)	17 (62.96)	27
Shirpur	11 (10.68)	31 (30.10)	61 (59.22)	103
Untawad	3 (12.50)	5 (20.83)	16 (66.67)	24
Thalner	6 (8.57)	14 (20.00)	50 (71.43)	70
Asli	5 (13.89)	8 (22.22)	23 (63.89)	36
Hol	1 (5.88)	4 (23.53)	12 (70.59)	17
Tonde	3 (12.00)	4 (16.00)	18 (72.00)	25
Total	**68 (11.37)**	**141 (23.58)**	**389 (65.05)**	**598**

Source: Collected and Tabulated by Researcher

Chi square value = 12*

df =22

* = Significant at 5 percent level= 33.92

The chi-square test is applied for to verify the significance. The computed chi-square value is 12 which is smaller than its tabulated value at 5 percent (33.92) level of significance. Hence, there is a significant difference among the farmers of different villages with respect to their choice of crop cultivation. It implies a homogeneous trend among the farmers of the study villages. Most of the farmers in Shirpur tahsil used organic as well as chemical fertilizers because of boi fertilizers like oil cake, green manure, compost, ashes and animal dung easily available in villages.

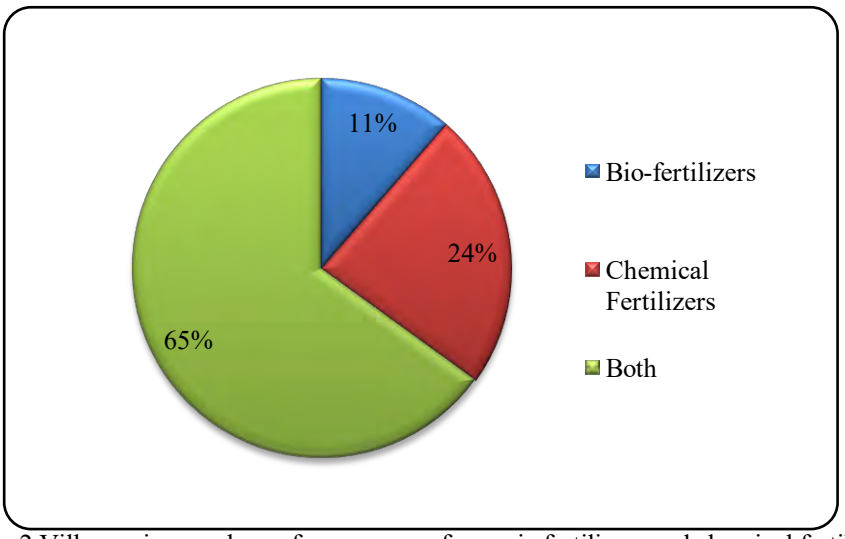

Figure 2 Village wise numbers of consumers of organic fertilizers and chemical fertilizer

Moreover, 65.05 percent of the farmers apply bio-fertilizer as well as chemical fertilizers for cultivation. This level of mixed application of fertilizers is reported by more than a half of the respondents of all sampled villages.

Table 3. Farm size wise Respondents' Application Level of Organic Fertilizers

Farm Size	Bio-fertilizers	Chemical Fertilizers	Both	Total
Marginal	31 (40.79)	18 (23.68)	27 (35.53)	76
Small	25 (9.47)	87 (32.95)	152 (57.58)	264
Medium	8 (4.23)	27 (14.29)	154 (81.48)	189
Large	4 (5.80)	9 (13.04)	56 (81.16)	69
Total	**68 (11.37)**	**141 (23.58)**	**389 (65.05)**	**598**

Source: Collected and Tabulated by Researcher

Chi-square value = 112
df = 6
* = Significant at 1 percent level = 16.81

Table 3 presents data on the farm size wise respondents' application level of bio-fertilizers for crop cultivation. A half of the large and medium farmers (50%) apply mixed fertilizers for cultivation. A more than half of the small farmers (57.58%) also used mix fertilizer and 40.79 % of marginal farmers giving preference to the use of biofertilizer.

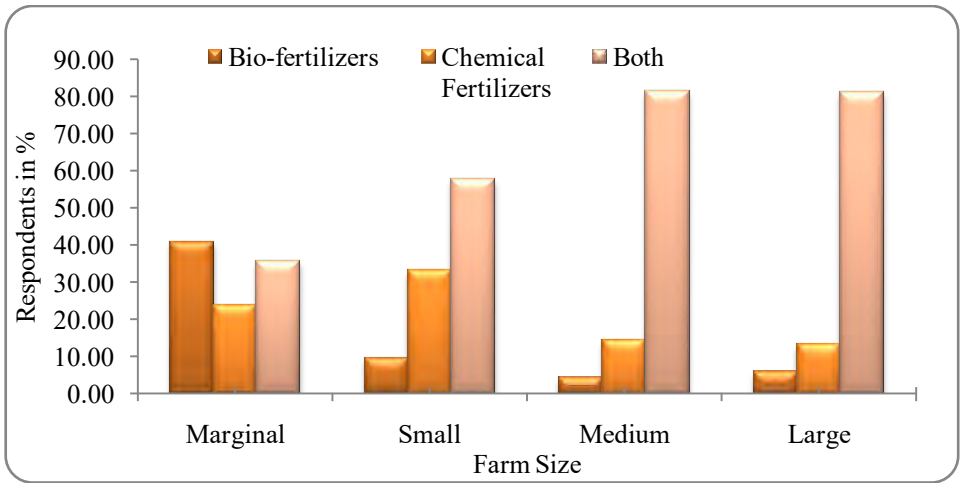

Figure 3 Farm size wise Respondents' Application Level of Organic Fertilizers

The data analysis from this study shows, the bio-fertilizers are mostly used by the medium and small-scale farmers and the multiple fertilizers is the applied by the medium and large farmers in the study region.

The chi-square test reveals positive association. The computed chi-square value is 112 which is greater than the tabulated value (16.81) at 1 percent level of significance. Hence, the difference in farm size is statistically significant with respect to farmers' application level of cow dung and ash as bio-fertilizer. A similar result has been observed with respect to the application of oil cake as bio-fertilizer and also green manure and compost as bio-fertilizer.

The marginal farmers apply more quantity of cow dung and ash as bio-fertilizer per hectare cropped area than others. This is due to easy availability in their farms as bio-waste. The medium farmers and large farmers apply more quantity of oil cake and green manure as bio-fertilizer compared to others.

Table 4. Education wise Respondents Application Level of Organic Fertilizers

Education	Bio-fertilizers	Chemical Fertilizers	Both	Total
Illiterate	27 (17.76)	49 (32.24)	76 (50.00)	152
Primary	20 (11.05)	50 (27.62)	111 (61.33)	181
Secondary	10 (5.35)	26 (13.90)	151 (80.75)	187
Degree	11 (14.10)	16 (20.51)	51 (65.38)	78
Total	**68 (11.37)**	**141 (23.58)**	**389 (65.05)**	**598**

Source: Collected and Tabulated by Researcher

Chi-square value = 39*
df = 6
* = Significant at 1 percent level= 16.81

The computed chi-square value for table 4 is 39 which is greater than its tabulated value (16.81) at 1 percent level of significance. Hence, the difference in educational status is statistically identified as significant with respect to farmers' choice of fertilizers selection.

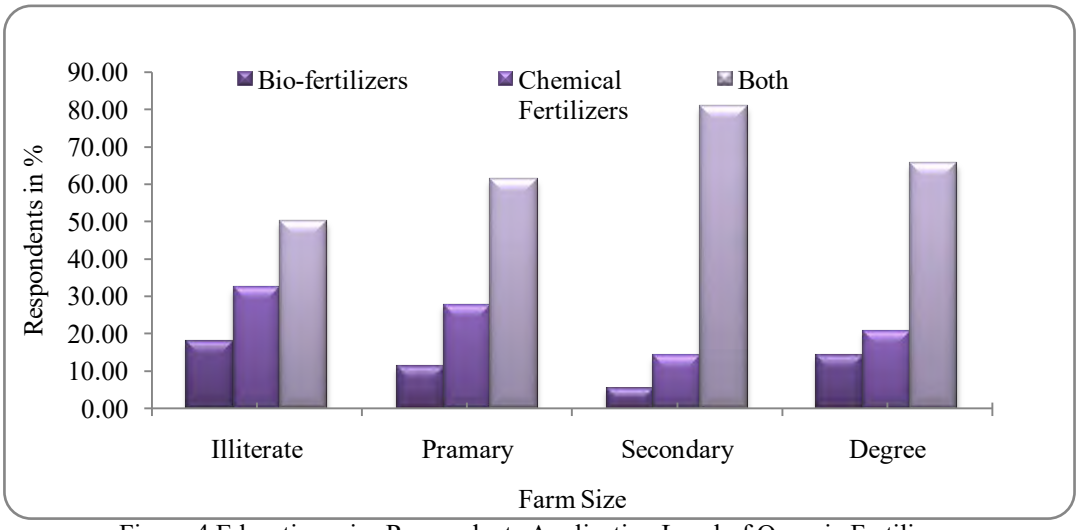

Figure 4 Education wise Respondents Application Level of Organic Fertilizers

Table 5. Village Wise Respondents' Views on Advantages of Bio-fertilizers

Village	Advantages of Bio-fertilizers				Total
	Production of Nutritious food	Free from disease	Eco-friendly method	All	
Boradi	23 (52.27)	3 (6.82)	4 (9.09)	14 (31.82)	44
Balkuwe	16 (42.11)	3 (7.89)	4 (10.53)	15 (39.47)	38
Sangavi	25 (42.37)	8 (13.57)	5 (8.47)	21 (35.59)	59
Rohini	25 (45.45)	4 (7.28)	3 (5.45)	23 (41.82)	55
Arthe	16 (50.00)	3 (9.38)	4 (12.50)	9 (28.12)	32
Jalod	11 (55.00)	2 (10.00)	1 (5.00)	6 (30.00)	20
Shirpur	25 (34.72)	10 (13.89)	8 (11.11)	29 (40.28)	72
Untawad	12 (63.16)	1 (5.26)	2 (10.53)	4 (21.05)	19
Thalner	17 (30.36)	7 (12.50)	9 (16.07)	23 (41.07)	56
Asli	13 (46.43)	3 (10.71)	2 (7.14)	10 (35.72)	28
Hol	6 (46.15)	2 (15.38)	1 (7.69)	4 (30.77)	13
Tonde	11 (52.38)	3 (14.29)	2 (9.52)	5 (23.81)	21
Total	**200 (43.76)**	**49 (10.72)**	**45 (9.85)**	**163 (35.67)**	**457**

Source: Collected and Tabulated by Researcher

Chi-square value = 20*
df = 33
* = Significant at 1 percent level = 16.81

The degree of freedom is more than 30, therefore, the significance of chi-square value is 20 which is greater than the calculated value (16.81) 1 percent level of significance. Hence, the difference among the villages is statistically identified as significant with respect to respondents' views on advantages of the use of bio-fertilizers for cultivation and maintenance the fertility of the land.

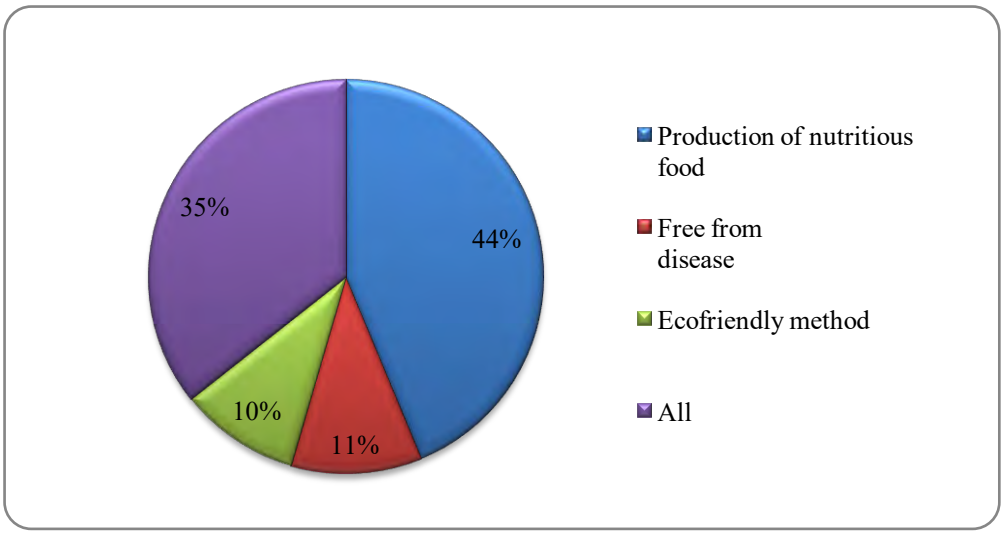

Figure 5 Village Wise Respondents' Views on Advantages of Bio-fertilizers

3.2 Views on Bio-fertilizers

Data presented in Table 5 reveals the village-wise respondents' views on advantages of applying bio-fertilizers. Out of total respondents, 457 respondents having a positive view on advantages of bio-fertilizer application for crop cultivation. Out of total respondents (457), some are used only organic fertilizers for cultivation while some are used mixed fertilizers (organic and chemical).

Out of the total 457 respondents, 43.76 % of them hold the view that through the application of bio-fertilizers, they can produce food with high nutritious value. More than 52 % respondents from Untawad, Jalod, Boradi, and Tonde believe that the application of bio-fertilizer leads high crop yield with good nutrition values.

10.72 % of total and 15.38 % respondents of the Hol village think that bio-fertilizers can produce disease-free food.

9.85 % of the total and more than 11% of Thalner, Arthe and Shirpur villagers feel the advantage of applying bio-fertilizers in terms of eco-friendly method of cultivation than other advantages.

Moreover, 35.67% of the respondents believe the multiple advantages of applying bio-fertilizers, such as the production of production of disease-free food, food raised through bio-fertilizers give more strength and stamina to consumers. The majority of the respondents of Rohini village (41.52%) Thalner village (41.07%) Shirpur village (40.28%), Balkuwe village (39.47%) and Asli village (35.72%) prefer all multiple advantages of applying bio-fertilizers to raise their crops.

Table 6. Farm size wise Respondents' Views on Advantages of Bio-fertilizers

Farm Size	**Advantages of Bio-fertilizers**				**Total**
	Production of nutritious food	**Free from disease**	**Eco-friendly method**	**All**	
Marginal	41 (70.69)	4 (6.90)	7 (12.07)	6 (10.34)	58
Small	91 (51.41)	32 (18.08)	15 (8.47)	39 (22.03)	177
Medium	51 (31.48)	8 (4.94)	16 (9.88)	87 (53.70)	162
Large	17 (28.33)	5 (8.33)	7 (11.67)	31 (51.67)	60
Total	**200 (43.76)**	**49 (10.72)**	**45 (9.85)**	**163 (35.67)**	**457**

Source: Collected and Tabulated by Researcher

Chi-square value = 75*
df = 9
= Significant at 1 percent level=21.66

Data presented in Table 6 indicates the farm size wise respondents views on advantages of applying bio-fertilizers to raise their crops. It could be noted that a considerable majority of the marginal farmers (70.69 %) prefer the advantage of applying bio-fertilizers in terms of possession more strength and stamina by consuming food raised through bio-fertilizers and 18.08% of the small farmers say it as the production of disease-free food. A considerable majority of the medium farmers (53.70 %) and large farmers (51.67 %) opine the multiple advantages of applying bio-fertilizer to raise their crops, such as production of nutritious food, disease-free food, food raised through bio-fertilizers gives more strength and stamina to consuming human beings and animals and it is an eco-friendly method.

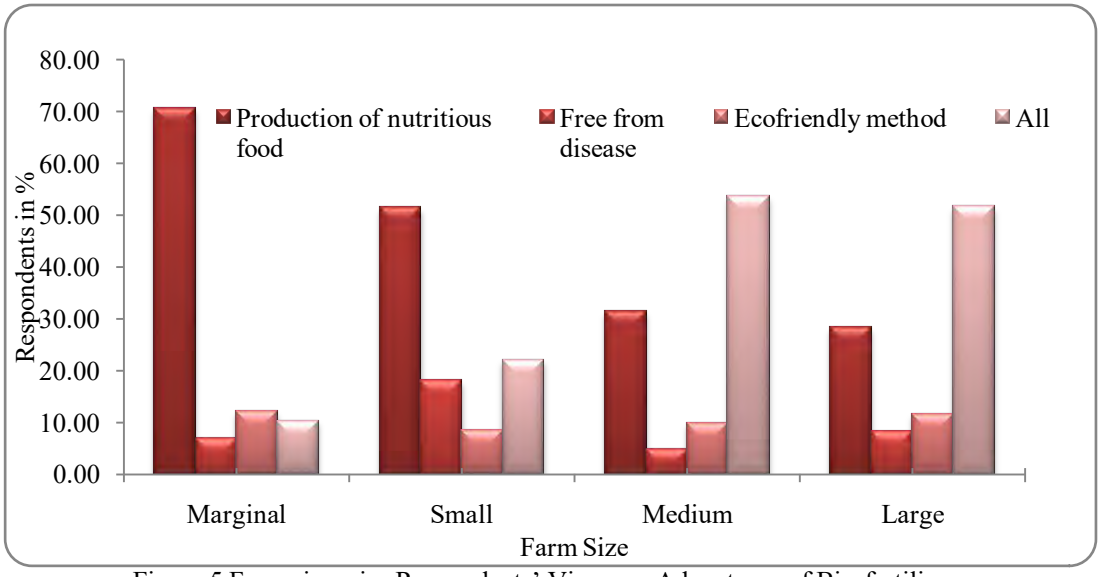

Figure 5 Farm size wise Respondents' Views on Advantages of Bio-fertilizers

The computed chi-square value for table 6 is 75 which is greater than its tabulated value at 1 percent (21.66) level of significance. Hence, the difference in farm size is statistically found to be significant with respect to respondents' views on advantages of applying bio-fertilizers to raise their crops. A similar result has been observed with respect to the application of chemical fertilizers. It is obvious from the above analysis that the medium and large farmers mainly refer the multiple advantages of applying bio-fertilizers and chemical fertilizers. Whereas the majority of the small farmers and marginal farmers highlight the individual advantage of applying bio-fertilizers and also chemical fertilizers.

Table 7. Education-wise Respondents' Views on Advantages of Bio-fertilizers

Education	**Advantages of Bio-fertilizers**				**Total**
	Production of Nutritious food	**Free from disease**	**Eco-friendly method**	**All**	
Illiterate	73 (70.87)	9 (8.74)	7 (6.80)	14 (13.59)	103
Primary	81 (61.83)	19 (14.50)	6 (4.58)	25 (19.08)	131
Secondary	28 (17.39)	17 (10.56)	26 (16.15)	90 (55.90)	161
Degree	18 (29.03)	4 (6.45)	6 (9.68)	34 (54.84)	62
Total	**200 (43.76)**	**49 (10.72)**	**45 (9.85)**	**163 (35.67)**	**457**

Source: Collected and Tabulated by Researcher

Chi-square value = 199*
df = 9
* = Significant at 1 percent level= 21.66

Table 7 presents data on the education level and respondents' views on advantages of applying bio-fertilizers and chemical fertilizers. It is noted that the majority of the illiterate respondents prefer the bio-fertilizers for advantages of high-quality food for humans and animals. According to primary educated respondents group, the bio-fertilized food and crops are more nutritious.

The majority of the (16.15 %) secondary level educated farmers prefer bio-fertilizer due to its eco-friendly nature.

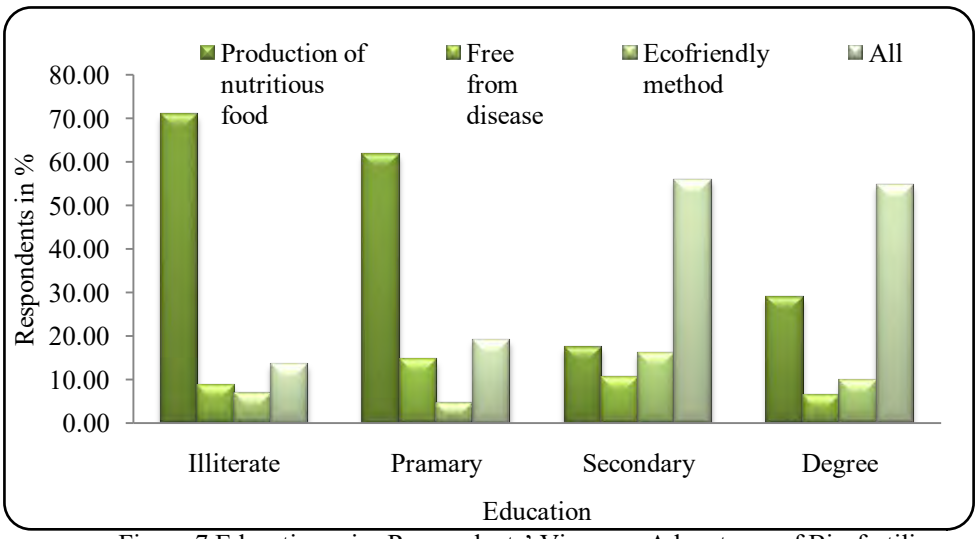

Figure 7 Education-wise Respondents' Views on Advantages of Bio-fertilizers

More than a half of the degree (54.84 %) and secondary (55.90 %) level educated farmers to feel that the multiple advantages of applying bio-fertilizers, such as the production of nutritious food, disease-free food production, food that gives more strength and stamina and as an eco-friendly method.

The computed chi-square value for table 7 is 199 which is greater than its tabulated value at 1 percent level (21.66) of significance. Hence, the difference in educational status is found to be statistically identified as significant with respect to respondents' views on advantages of applying bio-fertilizers to raise their crops. A similar result has been observed with respect to the application of chemical fertilizers.

The above analysis reveals that degree level educated farmers to realize more on multiple advantages of applying bio-fertilizers rather than an individual advantage. It is pointed out that the majority of the illiterate and primary level educated farmers to perceive mainly on the individual advantage of either applying bio-fertilizers or chemical fertilizers to raise the crops.

3.3 Discussion

Many researchers have highlighted the importance of the application of bio-fertilizers in cultivation. They have realized the problems associated with the application of chemical fertilizers particularly it affects the health of the soil and human beings. Hence, there is a need to review works in this regard [7]. have pointed out the implications of integrated nutrient management in terms of organic manures, green manures, crop inoculates. They have reported that this type of nutrient management in agriculture reduces pollution and maintains soil productivity and agricultural output [1]. have analyzed the components of the agricultural system and identified the conflict between organic and chemical agriculture. Further, they came to a conclusion that application of composting as the best strategy to increase the agricultural production. Food and fertilizer technology center (1995) has identified the evils of application of weedicides to destroy the weeds in the cropped area. Application of bio-fertilizers good for soil health and better way for deduction of input cost of cultivation. Government and NGO's should be promoted and motivate framers for use of bio-fertilizers.

4. Conclusion

From the complete analysis, it can be concluded that there are different attitudes regarding the use of organic fertilizers and benefits of its application according to farm size and education level of farmers. Large farm size farmers and educated farmers used more organic fertilizers compared to small size farmers and illiterate farmers. Some educated large and medium land size holding farmers adopted both fertilizers but small land size holding farmers mostly used chemical fertilizers because they trying for maximum production from a small piece of land. Bio-fertilizers and chemical fertilizers both are highly used by educated medium farm size farmers. The degree level educated farmers are more conscious about multiple advantages of applying bio-fertilizers rather than a single advantage. Small farm size farmers focus on maximum production from available small agricultural land and hence they do not pay any attention to the benefits of bio-fertilizers. The education wise result reveals that the degree level educated farmers are more aware of the multiple advantages of applying bio-fertilizers and the negative impacts of chemical fertilizers. The majority of the illiterate and primary level educated farmers to concentrate mainly on the individual advantage of either bio-fertilizers or chemical fertilizers application.

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ग्राम समस्या निराकरणात महात्मा गांधीजींचे योगदान

– प्रा. डॉ. रमाकांत अंबादास चौधरी
इतिहास विभाग
आर. सी. पटेल महाविद्यालय शिरपूर

खेड्याकडे चला – असा संदेश राष्ट्रपिता महात्मा गांधी यांनी स्वातंत्र्य लढ्याच्या वेळीस हिंदुस्थानी जनतेला दिला होता. ग्रामीण भागातील व्यक्ती आर्थिकदृष्ट्या स्वावलंबी होऊन खेडी स्वयंपूर्ण व्हावीत असा त्यांचा उद्देश होता. विसाव्या शतकातच तंत्रज्ञानातील क्रांतीला सुरुवात झाली आणि या क्रांतीने शहरांना आपल्या कवेत घेतले. उंच-उंच वाढणाऱ्या इमारती, आरशालाही लाजवतील असे चकाचक रस्ते, वाहतुक सुविधेतील विस्मयजनक बदल, उच्च तंत्रज्ञानावर चालणारे कारखाने, करमणुकीच्या नवनवीन साधनांमध्ये दिसेंदिवस पडणारी भर, जागतिकीकरणाचा रेटायामुळे वसुधैव कुटुंबकम् या कल्पनेला बळ मिळत जग फारच छोटे होत गेले. या छोटेखानी जगात मग खेडी आणि शहरे यांच्यातील अंतर आणखीच कमी होत गेले आणि शहरांतून खेड्याकडे वळण्याऐवजी माणसांची पावले शहरांकडे वळू लागली. परिणामी गावांचा विकास खुंटला व गाव अधिक अविकसित झाली.

औद्योगिक क्रांतीचे अपत्य म्हणजे नागरीकरण. हे नागरीकरण मानवी जीवनाला शापग्रस्त ठरण्याची भीती निर्माण झाली आहे. औद्योगिक क्रांतीमुळे पारंपरिक हस्तोउद्योगांचा ऱ्हास, कृषी क्षेत्रात घडून आलेला तांत्रीक बदल, शिक्षणाचा प्रचार आणि प्रसार इत्यादी विविध कारणांमुळे खेड्यातील माणूस उदरनिर्वाहासाठी प्रचंड वेगाने शहरी भागाकडे स्थलांतरीत होऊ लागला आहे. त्यामुळे शहरी लोकसंख्या भयानक प्रमाणात वाढू लागली आहे. या अतिरिक्त लोकसंख्यामुळे बेकारी, दारिद्र्य, अन्नटंचाई, गुन्हेगारी, वेश्याव्यवसास, ,आरोग्याच्या समस्या, अन्न-वस्त्र-निवारा यासारख्या प्रश्नांमुळे नागरी जीवन अक्षरक्ष: घुसळून निघत आहे.

तंत्रज्ञानातील या बदलाने माणसांच्या राहणीमानात देखील लक्षणीय बदल घडवले. शहरांमध्ये रोजगाराच्या संधीमध्ये वाढ झाली. रोटी, कपडा और मकान या मूलभूत गरजांपैकी रोटीची हमी देणारे ठिकाण म्हणून आता शहरांकडे पाहिले जात आहे. औद्योगिकीरणामुळे तसेच सेवाक्षेत्रांचा विस्तारामुळे शहरांमध्ये मोठ्या प्रमाणात रोजगार निर्मिती होत आहे. यातच या शहरांकडे शासनाने देखील विशेष लक्ष पुरवण्यास सुरुवात केल्यामुळे रोजगार मिळवून देणारे हक्काचे ठिकाण अशी शहरांची नवी ओळख बनत चालली आहे.

उद्देश:–

१) ग्रामसमस्यांवरील उपाययोजना महात्मा गांधीजींच्या विचारसाधनातून शोधणे.
२) महात्मा गांधीजींच्या ग्रामस्वराज्याची संकल्पना समजून घेणे.
३) भारतातील ग्रामसमस्यांवर प्रकाश टाकणे.

संशोधन पध्दती:–

प्रस्तुत संशोधनासाठी इतिहासाच्या सर्व साधारण संशोधन पध्दतीचा वापर करतांना आंतरविद्याशाखीय संशोधन पध्दतीचाही उपयोग केला आहे.प्रस्तुत विषयाकरीता महात्मा गांधीसंबंधीत विविध संदर्भग्रंथ, पुस्तके या साधनांचे संकलन करुन त्या साधनांची चिकित्सा करुन त्याची उपयुक्तता प्रस्तुत विषयाकरीता ठरविली आहे.

भारतातील खेड्यासंबंधी गांधीजींचे निरीक्षण:–

भारत म्हणजे खेड्यांचा देश. गांधीजींच्या मते भारताची खरी संस्कृती जर कोठे नांदत असेल तर ती खेड्यात. खऱ्या भारताचे चित्र शहरात नसून ते खेड्यात आहे. गांधीजींच्या मते, हिंदुस्थान हा काही थोड्या शहरात आढळणार नाही, तर तो ७ लाख खेड्यात आढळेल. गांधीजी असेही म्हणत की, कला, संस्कृती फक्त खेड्यात नांदते. भारतातील खेड्यासंबंधी गांधीजींनी केलेले निरीक्षण सूक्ष्म वस्तुनिष्ठ होते.

१) भारतीय खेड्यातील लोकांमध्ये प्रचंड अंधश्रध्दा दिसून येतात.
२) भारतीय खेड्यातील नदी, तलाव, नाल्यातील पाण्याच्या गैरवापरामुळे पाणी दुषीत झालेले आढळते.
३) भारतीय खेड्यात शौचालये नसल्यामुळे गावात दुर्गंधी पसरलेली असते.
४) भारतीय खेड्यातील रस्ते खराब व स्वच्छ नसतात.
५) भारतीय खेड्यातील लोक आरोग्याच्या समस्याबाबत जादुटोणा, मंत्रतंत्राचा वापर करतात.
६) भारतीय खेड्यातील लोकांमध्ये व्यसनांचे प्रमाण अधिक आहे.
७) भारतीय खेड्यातील लोकांना वर्षातील चार महिने काम नसल्यामुळे त्या काळात इतर आर्थिक स्त्रोत नाहीत.
८) गावात लघुउद्योगांचे प्रमाण नगण्य आहे.
९) गावात कृषीयुक्त भूमीच्या तुकडीकरणामुळे शेती लाभदायक नाही.
१०) गावात जातपात, वंशभेद, कटाक्षाने पाळले जातात.
११) गावातील लोकांचे शहरीभागाकडे स्थलांतरीत होण्याचे प्रमाण वाढले आहे.

ग्राम स्वराज्य:–

भारताचा सर्वांगिण विकास करावयाचा असेल तर अगोदर भारतातील खेड्यांचा विकास झाला पाहिजे. खेड्यांचा विकास म्हणजेच संपूर्ण भारताचा विकास असे गांधीजींना वाटते. भारतातील खेडी विकसीत करण्यासाठीच्या उपाययोजना त्यांनी आपल्या ग्रामस्वराज्य संकल्पनेत मांडल्या आहेत. स्वराज्य हा शब्दाची फोड

स्व+राज्य अशी केली जाते. स्वराज्य या शब्दाचा अर्थ आपले स्वत:चे राज्य असा होतो. स्वराज्य हा शब्द वैदिक साहित्यात स्वराज्याच्या संदर्भात आत्मानुशासन तथा आत्मसंयम या अर्थाने प्रयुक्त झाला आहे. स्वत:च्या अधीन, आपल्या नियंत्रणात असलेले राज्य म्हणजे स्वराज्य. अधीन या शब्दाचा इंग्रजी शब्द इंडिपेण्डस समानार्थी असून देखील भिन्न स्वरुपाचा अर्थ आढळतो. इंडिपेण्डस शब्दाचा अर्थ मर्यादांनी मुक्त, निरंकुश स्वातंत्र्य किंवा स्वच्छता असा होतो. यावरुन असे म्हणता येईल की, इंडिपेण्डस या शब्दात स्वराज्य या शब्दाचे पावित्र्य दिसत नाही.

स्वराज्याची व्याख्या करतांना महात्मा गांधीजी म्हणतात की, लोकसंमतीनुसार चालणारे शासन म्हणजे स्वराज्य होय. लोकसंमत शासनात लोकांनी आपल्या मताधिकाराचा हक्क बजावून लोकनियुक्त प्रतिनिधींच्याद्वारे शासनाचा कारभार चालवावा. गांधीजी म्हणतात की, मूठभर लोकांच्याद्वारे स्थापन झालेली सत्ता म्हणजे खरे स्वराज्य नव्हे. सत्ता विभागली पाहिजे त्या करीता महात्मा गांधीजींनी विकेंद्रीकरणाचे तत्व स्विकारले दिसते. विकेंद्रीकरणाच्या तत्वानुसार राजकीय सत्ता गावापर्यंत पोहचल्याशिवाय गावांचा सर्वांगिण विकास होवू शकणार नाही असे गांधीजी मानत. देशातील ग्राम हा घटक अतिशय महत्वाचा मानून ग्राम जेव्हा स्वयंपूर्ण, आत्मनिर्भर होतील तेव्हाच खऱ्या अर्थाने ग्रामस्वराज्य निर्माण होईल. प्रत्येक गावाने आपल्या प्राथमिक गरजा भागविल्या पाहिजेत. त्यासाठी गावातील ग्रामोद्योगांचे पुनर्जिवन करणे काळाची गरज आहे.

आत्म निर्भर गावं

गांधीजींच्या मतानुसार ज्या दिवसापासून शहरे परकीय बाजारपेठेची केंद्रे बनली त्या दिवसापासून भारताच्या दारिद्र्याला सुरुवात झाली. गांधीजी शेतकऱ्यांच्या, मजुरांच्या हक्कामध्ये समर्थन करतांना म्हणतात की, कृषीवर शेतकरी व मजुरांची मालकी असावी की शहरात राहणाऱ्या जमीनदारांची.शेतकरी व मजुरांनी आपल्या हक्कासाठी एकत्रित येऊन संगठित हासणे अत्यावश्यक आहे. मात्र ह्या संघटनांचा उद्देश विरोध नसून समन्वय असला पाहिजे. गाव मजबूत, समृध्द तथा बलवान झाला पाहिजे. याबाबत गांधीजी म्हणतात- शेतकऱ्यांजवळ राजकीय सत्तेबरोबर इतर सत्ताही त्यांच्या हातात असली पाहिजे.

गांधीजींच्या मतानुसार शहरांचा विकास हे शोषणाचे प्रतिक आहे. कारण शहरे गावातील संपत्तीचं अपहरण करते. जसे अन्नधान्य, भाजीपाला, दुध, कच्चामाल इत्यादीचा प्रवाह सतत शहरांकडे होत असतो. यामुळे गावांचा नाश तथा पतन होत आहे. गावांचे शोषण गांधीजींच्या शब्दात एक प्रकारची हिंसा आहे. या हिंसेमुळे सर्व जगाचा सर्वनाश होईल. शहरे ग्रामीण अर्थव्यवस्थेवर आधरित परजीवी आहे. म्हणून गावांचा नाश म्हणजेच शहरे नाश होण्याचे महत्वपूर्ण कारण ठरेल. म्हणूनच गांधी म्हणतात की जर गावांचा नाश झाला तर भारताचा विनाश होईल.

गांधीजींच्या ग्रामस्वराज्यातील गाव आत्मनिर्भर असेल. प्रत्येक गाव आपल्या गरजांच्या परिपूर्तिसाठी इतरांवर अवलंबून नसतील. गावातील सर्व कार्य सहयोग नीतिवर आधारीत राहील. गावात नाट्यशाळा, शाळा, सभा-भवन, विहिरी, तलाव इत्यादींची सुविधा असतील. या सुविधांची व्यवस्था परस्पर सहयोग आणि समानतेच्या आधारावर असतील. या समाज वर्गात अस्पृयता, जातीभेदाचे पंख गळून पडतील. महात्मा गांधीजींच्या स्वप्नातील गाव हे प्रचलित गावांपेक्षा भिन्न असतील. म्हणून स्वयंपूर्ण ठरेल अशा गावातील लोकांच्या गरजा गावातच पूर्ण झाल्या पाहिजेत. सरकारने गावातील लोकांना त्यांना लागणारी खादी स्वत:च तयार करा असा संदेश दिला पाहिजे. बैल घाण्याचे तेल, पेंड, हातसडीचा तांदूळ, डाळी, मध, खेळणी, चटया, साण, हातकागद इत्यादी वस्तू गावातच तयार होवू शकतात. त्या तयार झाल्या तर मरणावस्थेत असलेल्या गावांना संजिवनी प्राप्त होईल. गांधीजी असे म्हणत की, खादी हा मध्यवर्ती सूर्य असून तिच्या भोवती इतर ग्रामोद्योग अनेक ग्रहांप्रमाणे फिरतात. त्यांना स्वतंत्र अस्तित्व नाही. शहरातील कारखान्यात तयार झालेल्या वस्तूंऐवजी गावातील तयार झालेल्या वस्तू वापरुन उद्योगांचे, हस्तउद्योगांचे पुनरुज्जीवन केले नाही तर गावांना आपण त्यांचे योग्य स्थान कधीच देणार नाही. खादी मेली तर गाव ही मरतील. खादी हा दारिद्र्यावरील रामबाण उपाय आहे असे गांधीजी मानत असत.

गांधीजींच्या मतानुसार प्रत्येक गावाने आपल्या शेती तथा उद्योगात परिवर्तन करावे लागेल. प्रत्येक गावाने आपलया आवश्यकतेनुसार अन्नधान्य, कपड्यासांठी कापसाचे उत्पादन घेतील. अशा गावात पशुंसाठी चरण्यासाठी जमीन सुरक्षित ठेवली जाईल. गावातील लोकांच्या मनोरंजनासाठी व खेळ खेळण्यासाठी मैदानांची व्यवस्था केली जाईल. शेती, पशुपालन, मैदाने, सडक इत्यादीसाठी वापरलेल्या जमीनी व्यतिरिक्त इतर जमीनींवर नगदी पीके घेऊ शकतात. परंतु गावाने अफू, गांजा, भांग, अफिमया मादक पीकांची उत्पादन घेऊ नये. दैनंदिन जीवनात उपयोगी पडणाऱ्या वस्तूंची निर्मितीचे उत्पादन ग्रामवासी ग्राम उद्योगाच्या माध्यमातून करतील. परंतु यासाठी विदेशी तंत्रज्ञानाची आवश्यकता नाही. हे ग्राम उद्योग दुसऱ्यांना लुटण्यासाठी नसावेत. ग्राम उद्योगांचा विकास म्हणजे भारताचा विकास जर ग्राम उद्योगांचा ऱ्हास झाला तर भारतातील सात लाख गावांचा सर्वनाश होईल असे महात्मा गांधीजींनी भाकीत केले आहे.

निष्कर्ष:-

१) भारताचा सर्वांगिण विकास करावयाचा असेल तर अगोदर भारतातील खेड्यांचा विकास झाला पाहिजे. खेड्यांचा विकास म्हणजेच संपूर्ण भारताचा विकास असे गांधीजींना वाटते.

२) प्रत्येक गावाने आपल्या प्राथमिक गरजा भागविल्या पाहिजेत. त्यासाठी गावातील ग्रामोद्योगांचे पुनर्जिवन करणे काळाची गरज आहे.

३) गांधीजींच्या मतानुसार प्रत्येक गावाने आपल्या शेती तथा उद्योगात परिवर्तन करावे लागेल. शेती, पशुपालन, मैदाने, सडक इत्यादीसाठी वापरलेल्या जमीनी व्यतिरिक्त इतर जमीनी व कागदी पीके घेऊ शकतात. परंतू गावाने अफू, गांजा, भांग, अफिमया मादक पीकांची उत्पादन घेऊ नये. दैनंदिन जीवनात

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४) गांधीजींच्या मतानुसार शहरांचा विकास हे शोषणाचे प्रतिक आहे. कारण शहरे गावातील संपत्तीचं अपहरण करते. जसे अन्नधान्य, भाजीपाला, दुध, कच्चामाल इत्यादीचा प्रवाह सतत शहरांकडे होत असतो. यामुळे गावांचा नाश तथा पतन होत आहे.

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आंतरराष्ट्रीय उत्तर-दक्षिण परिवहन कॉरीडॉरचे ऐतिहासिक अध्ययन

प्रा. डॉ. रमाकांत अंबादास चौधरी
इतिहास विभाग,
आर.सी.पटेल कला, वाणिज्य व विज्ञान महाविद्यालय
शिरपूर जि. धुळे

प्रस्तावना

जगपातळीवर कोणत्याही सेवा पुर्विणाज्या व्यवस्थेसाठी प्राकृतिक अंतर आणि वेळ या दोन मोठ्या समस्या टरल्या आहेत. यावर उपाययोजना केली तर जग वैश्विक खेडे होण्याऐवजी वैश्विक केंद्रबिंदू बनु शकते.

सध्या जगात CPEK बद्दल चर्चा होत आहे. CPEK च्या माध्यमातून चीन सुपरपावर बनण्याचे स्वप्न रंगवत आहे. CPEK च्या माध्यमातून चीन भारतावर आंतरराष्ट्रीय दबाव आणण्याचा प्रयत्न करीत आहे. या CPEK च्या अहंकारातून चीन भारताला म्हणाला होता की, एकतर CPEK मध्ये सहभागी व्हा अन्यथा आमचा दबाव सहन करत रहा. पण भारताने One belt one road ला प्रखर विरोध करून INSTC प्रोजेक्ट ला सुरूवात केली.

INSTC प्रोजेक्ट हा ७२०० कि.मी. जर्मनी, समुद्र आणि रेल्वे मार्गयुक्त कोरीडोर आहे. या प्रोजेक्टमुळे वेळ (४०%) आणि खर्चात (३०%) बचत होणार आहे. भारत, इराण आणि रशिया यांच्यातल्या व्यापाराला चालना देण्यासाठी INSTC प्रोजेक्ट सुरू केला जात आहे.

INSTC प्रोजेक्ट मध्ये भारत, इराण, रशिया, अझरबैजान, कजाकिस्तान, आर्मेनिया, बेलारूस, तजाकिस्तान, किर्गीस्तान, ओमान, सिरीया, तुर्की, युक्रेन इत्यादी १३ देश सहभागी होत आहे. तसेच तुर्कमेनिस्तान या प्रोजेक्ट मध्ये सहभागी नाही परंतु या प्रोजेक्ट मध्ये महत्वाची भूमिका बजावत आहे. मुंबई, मास्को, बाकू बंदर, बंदर-ए-अब्बाज, बंदर-ए-अन्झाली, अस्त्राखान बंदर यांच्यात व्यापार वृध्दी करणे हा INSTC प्रोजेक्टचा उद्देश आहे. INSTC प्रोजेक्टमुळे भारताला प्रचंड फायदा होणार आहे. भारतीय उत्पादने मध्य आशिया, रशिया आणि युरोपात निर्यात केली जातील.

उद्दिष्टे

१. भारताचे विस्तारीत शेजारी राष्ट्रसोबत आंतरराष्ट्रीय उत्तर-दक्षिण वाहतूक कॉरिडोरवर व्यापारी माल आणि प्रवासी वाहतूक करण्यासाठी वाहतूक संबंधाची प्रभावीता वाढविणे.

२. भारताचे विस्तारीत शेजारी राष्ट्रसोबत जलमार्ग, भुमार्ग, रेल्वे मार्ग वाहतुकीद्वारे आंतरराष्ट्रीय बाजारपेठांपर्यंत प्रोत्साहन देणे.

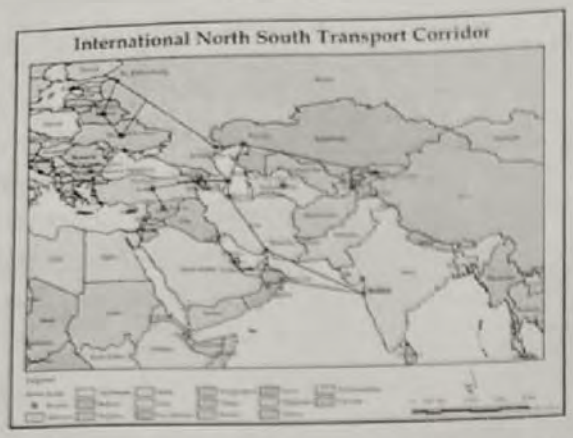
३. भारताचे विस्तारीत शेजारी राष्ट्रसोबत प्रवासी आणि व्यापारी मालाच्या आंतरराष्ट्रीय वाहतुकीची संख्या वाढविण्यास मदत करणे.

४. भारताचे विस्तारीत शेजारी राष्ट्रसोबत प्रवास, सुरक्षेची तसेच पर्यावरणीय संरक्षणाची सुरक्षा प्रदान करणे.

International North South Transport Corridor. हा एक मल्टिमोडल ट्रेड रूट आहे. गेल्या महिन्यात या रूटचे ड्राय रन केले आणि ते यशस्वीही ठरले. या ड्रायरनमध्ये तीन महत्वाचे देश सामिल झाले होते. यात भारत इराण आणि रशिया यांनी मिळून ड्रायरन केले. हा प्रोजक्ट इंडिया रशिया कोरिडोर या नावानेही प्रसिध्द आहे. आयएनएसटीसी हा रूट ७२०० कि.मी चा असून तो जलमार्ग, भुमार्ग आणि रेल्वेमार्गाने कनेक्ट केलेला आहे. या प्रोजक्टमध्ये एकूण १३ देश सामिल झाले आहेत. नकाशा १ च्या माध्यमातुन या रूटचा मार्ग समजुन घेऊ. भारतातील मुंबईतील न्हावाशेवा बंदरातून इराणच्या बंदर-ए-अब्बाजला कनेक्ट होईल. इराण बंदर ए अब्बाज ते इराणच्या उत्तरेतील बंदर ए अन्झाली भूमार्ग कनेक्ट होईल. बंदर-ए-अन्झाली ते रशियाच्या अस्त्राखान बंदरला कनेक्ट होईल. अस्त्राखान बंदर ते रशियाच्या सेंट पिटर्सबर्ग रेल्वे मार्गे कनेक्ट केले जाईल. दुसज्या मॅपच्या मधुन डिटेल समजून घेऊ ही जी ब्लयु लाईन आहे ती सुवेज कॅनल रूट आहे. आणि जी रेड लाईन आहे ती आयएनएसटीसी रूटची आहे. या दोघात तुलना केली तर अंतर जवळ जवळ निम्यावर आलेले दिसते. नॉटीकल माईलच्या भाषेत सांगावयाचे झाल्यास सुवेज कॅनलचा रूट हा ८५०० नॉटीकल माईल आहे आणि आयएनएसटीसीचा रूट केवळ ४५०० नॉटिकल माईल आहे. म्हणजेच हा रूट ३०% स्वस्त आणि ४०% शॉर्टर आणि सेफ आहे. कारण सुवेज कॅनल रूट हा नेहमी कटकटीचा, वर्दळ असलेला आणि समुद्री चाच्याचा नेहमी धोका असणारा रूट आहे. या रूटच्या माध्यमातुन भारतीय उत्पादने जलदगतीने युरोशियात पोहचण्यास मदत होणार आहे. इंडियन स्पायसी, हनी, कॉटन आणि अल्फायन्जो मैंगो इत्यादी वस्तुची

मागणी युरोशियात प्रचंड आहे.

नकाशा क्रं. १



नकाशा क्रमांक १ नुसार भारतातील मुंबईतील न्हावाशेवा बंदरातून इराणच्या बंदर-ए-अब्बाजला समुद्रमार्गाने जोडला जाईल. इराणच्या बंदर-ए-अब्बाज ते इराणच्या दक्षिणेकडील बंदर-ए-अन्झाली भूमार्गाने जोडला जाईल. बंदर-ए-अन्झाली ते रशियाच्या अस्त्राखान बंदराला समुद्रमार्गाने जोडला जाईल. रशियाच्या अस्त्राखान बंदर ते सेंट पिट्सबर्ग रेल्वमार्गाने कनेक्ट केला जाईल. नंतरच्या काळात सेंट पिट्सबर्गहून बाल्टीक समुद्रातून फिनलॅण्ड, ग्रीनलॅण्ड ला कनेक्ट होता येईल.

नकाशा क्रं. २

नकाशा क्रमांक २ मध्ये दर्शविलेली निळ्या रंगाची रेषा ही ती सुवेज कॅनल रूट आहे. याची तुलना लाल रंगाची रेषा असलेला मार्गाने केली तर जवळ जवळ ५०% अंतर कमी दर्शवितो. या नवीन मार्गामुळे भारताला रशिया, मध्यआशिया आणि युरोपला कनेक्ट होणे सोयीस्कर होणार आहे.

International North South Transport Corridor हा मार्ग पुढील प्रमाणे कार्यरत होणार आहे. या कॉरिडोर मध्ये एकूण १३ देश एकमेकांना जोडले जाणार आहे.

१. भारताच्या मुंबई बेट ते इराणच्या बंदर-ए-अब्बाज व इराणच्या बंदर-ए-अन्झाली ते अझरबेझानच्या बाकु बंदरापर्यंत, बाकु बंदर ते आर्मेनिया, आर्मेनिया ते टर्की, टर्की ते सिरीया.

२. भारताच्या मुंबई बेट ते इराणच्या बंदर-ए-अब्बाज व इराणच्या बंदर-ए-अन्झाली ते रशियातील अस्त्राखान बंदर पर्यंत, रशिया ते युक्रेन, रशिया ते बेलारूस, बेलारूस ते युक्रेन

३. भारताच्या मुंबई बेट ते इराणच्या बंदर-ए-अब्बाज व इराणच्या बंदर-ए-अन्झाली ते कझाकीस्तानच्या अक्ताऐव बंदरापर्यंत, कजाकिस्तान ते किर्गीस्तान, किर्गीस्तान ते तजाकिस्तान,

४. भारताच्या मुंबई बेट ते ओमनच्या मस्कत बंदरापर्यंत अशा मार्गाद्वारे एकूण १३ देश International North South Transport Corridor ने एकमेकांना जोडले जाणार आहे. या कॉरिडोरमुळे या १३ सभासद राष्ट्रांमध्ये व्यापारवाढीला गती मिळेल यात शंका नाही.

INSTC चा फायदा

१. या प्रकल्पाचा प्राथमिक उद्देशानुसार सध्या वापरल्या जाणाऱ्या पारंपारिक मार्गावरील वेळा आणि पैशाच्या दृष्टीने खर्च कमी होणार आहे.

२. भारत, इराण, मध्य आशिया आणि रशिया यांच्यात व्यापारी मार्गात सुधारणा होऊन त्यांच्यात द्विपक्षीय व्यापाराला गती प्राप्त होईल.

३. फेडरेशन ऑफ फॉरवर्डर्स असोशिएशन इन इंडियाच्या www.fifai.org या संस्थेने केलेल्या अभ्यासात असे आढळते की, पारंपरिक मार्गापेक्षा ३० % स्वस्त आणि ४०% वेळेची बचत होणार आहे.

४. अभ्यासकांचा अंदाज आहे की आय.एन.एस.टी.सी. मुळे मुंबई मॉस्को, तेहरान, बाकू बंदर इत्यादी प्रमुख शहरामधील व्यापारिक संपर्क वाढण्याची दाट शक्यता आहे.

INSTC चे महत्व

युरेशिया आणि आफ्रिका खंडाला हार्ट ऑफ वर्ल्ड असेही म्हणतात. कारण या खंडांनी पृथ्वीवरील ७५ % भूमी व्यापलेली आहे. या खंडामध्ये ८० % ह्युमन रिसोर्स अव्हेलेबरल आहे. या दोन खंडांना जुने जग म्हणुनही ओळखले जाते. याबाबत माजी ब्रिटिश सैन्य अधिकारी अल्फ्रेड मॅनिडर असे म्हणतो की, "जुन्या जगताचा जो सत्ताधिश तोच संपूर्ण जगाचा अधिपती ठरेल." या त्याच्या विधानावरून या खंडांना महत्व प्राप्त होते. अमेरिकन नेव्ही ऑफिसर अल्फ्रेड महान म्हणतो की, "Sea is the great highway" त्याच्या मते, अखिल जगताशी संपर्क साधण्यासाठी समुद्रमार्गच प्रभावी मार्ग आहे. परंतु आजच्या काळात त्याचा हा सिध्दांत कालबाह्य ठरला आहे. कारण अखिल जगताशी संपर्क साधण्यासाठी केवळ

समुद्रमार्गानेच संपर्क साधता येत नाही. कारण लॅण्ड लॉक देशांसाठी हे प्रमेय कुचकामी ठरते. आज समुद्रमार्गाबरोबरच भूमार्ग, वायुमार्गही महत्वाची भूमिका बजावतांना दिसतात. INSTC हा कॉरिडोर केवळ समुद्रमार्गानेच नव्हे तर भूमार्ग, रेल्वेमार्गानेही एकूण १३ देश एकमेकांजवळ आले आहेत.

INSTC चा मार्ग हा काही नवीन नाही. हा मार्ग प्राचीन व्यापारी मार्ग म्हणूनही ओळखला जातो. मध्यकाळात प्रशिया म्हणजे आजचा इराण या ठिकाणी १५०७ ते १७०९ या काळात सफाविद घराण्याची सत्ता होती. या काळात १००० भारतीय व्यापाऱ्यांचे या ठिकाणी वास्तव्य होते असा उल्लेख इतिहासात आढळतो. असे काय झाले की INSTC सध्या खूप चर्चेत आला. खरे तर इ.स. २००० मध्ये भारत, इराण व रशिया यांच्यात कॉरिडोर संबंधी चर्चा झाली. इ.स. २००२ मध्ये या तिन्ही देशांमध्ये चर्चा होऊन या तीन देशात करार झाला. परंतु इराणच्या अण्वस्त्र कार्यक्रमामुळे अमेरिकेने इराणवर आर्थिक निर्बंध टाकले. तसेच अमेरिका व रशियाच्या कटूसंबंधामुळे हा प्रकल्प रखडला गेला. इ.स. २०१५ मध्ये अमेरिकेचे अध्यक्ष बराक ओबामा यांनी इराणवरचे आर्थिक निर्बंध हटविले. इ.स. २०१६ मध्ये भारताने रशिया व इराणसोबत चर्चा करून पुन्हा या प्रकल्पाला चालना दिली. इ.स. २०१७ मध्ये या प्रकल्पामध्ये एकूण १३ देश सामील झाले. सध्या या प्रकल्पाचे ड्राय रन यशस्वीपणे पार पाडले आहे. प्रत्यक्षात हा प्रकल्प इ.स. २०१८ मध्ये सुरू होणार आहे.

निष्कर्ष

१. भारत, रशिया आणि इराण यांनी एक समान उद्दिष्ट साधण्यासाठी ७२०० किलोमिटर लांब आणि जलमार्ग आधारीत बहुउद्देशीय वाहतुकीचे उद्दिष्ट असलेल्या महत्वाकांक्षी इंटरनॅशनल नॉर्थ-साऊथ ट्रान्सपोर्ट कॉरिडोर ची स्थापना केली. भारत, इराण आणि रशिया या प्राचीन व्यापारी मार्गाचे पुनरूज्जीवन करून इंटरनॅशनल नॉर्थ-साऊथ ट्रान्सपोर्ट कॉरिडोर हा व्यापारी मार्ग हिंदी महासागर आणि पर्शियन खाडीला आणि कॅस्पियन समुद्राशी जोडतो. त्यामुळे भारत आणि रशिया यांच्यात इराणमार्गे एक व्यापारी मार्ग उपलब्ध झाला आहे.

२. इंटरनॅशनल नॉर्थ-साऊथ ट्रान्सपोर्ट कॉरिडोर करारावर सप्टेंबर २००० मध्ये तिन्ही राष्ट्रांनी चर्चा करून २००२ मध्ये अंमलबजावणी केली. नंतरच्या काळात १० अन्य देश या प्रकल्पात सामील झाले. त्यात अर्मेनिया, अझरबैजान, बेलारूस, कझाकिस्तान, किर्गीस्तान, ओमान, सीरीया, तजाकिस्तान, तुर्की, युक्रेन आणि बल्गेरिया (निरीक्षक सभासद) इत्यादी राष्ट्र या प्रकल्पात सामील झाले.

३. इंटरनॅशनल नॉर्थ-साऊथ ट्रान्सपोर्ट कॉरिडोर मुळे भारत आणि रशिया या दोन्ही देशामधील द्विपक्षीय व्यापाराला गती प्राप्त होईल. रशियाचे अध्यक्ष व्लादिमिर पुतीन यांच्या नवी दिल्लीला भेट दिल्यावर डिसेंबर २०१४ मध्ये झालेल्या "ड्रिझबा-दोस्ती" या विधानामुळे २०२० पर्यंत द्विपक्षीय व्यवसायासाठी ३० अब्ज डॉलर्स लक्ष निश्चित केले आहे. ब्रिक्स समिट २०१६ मध्ये दोन्ही पक्षांच्या भेटीत संयुक्त भारत-रशियन निवेदनात म्हटले आहे की, "द्विपक्षीय व्यापाराच्या वाढीचा संबंध येतो तेव्हा आंतरकेंद्रीय तत्वाचे महत्व ओळखणे, दोन्ही बाजूंनी इंटरनॅशनल नॉर्थ-साऊथ ट्रान्सपोर्ट कॉरिडोरच्या अंमलबजावणीसाठी अधिक लक्ष देण्याचे स्वागत केले आहे जे या क्षेत्रातील आर्थिक एकात्मता वाढविण्यास कारणीभूत ठरतील."

४. भारताच्या विदेश व्यापार धोरण (२०१५-२०२०) मध्य आशियातील भारताच्या व्यापाराचे आणि गुंतवणुकीच्या लिंकचा विस्तार करण्यासाठी वाहतूक कॉरिडोरचे महत्व अधोरेखीत करते.

५. भारत सध्या मध्य आशिया, रशिया आणि इतर युरोपियन राष्ट्राकडे व्यापार करण्यासाठी पारंपरिक व्यापारी मार्गावर अवलंबून आहे जो अतिशय महाग, जास्त वेळ घेणारा व असुरक्षित आहे.

६. इंटरनॅशनल नॉर्थ-साऊथ ट्रान्सपोर्ट कॉरिडोर प्रकल्प हा कमी खर्चीक, वेळेची बचत करणारा व सुरक्षित मार्ग आहे. या प्रकल्पामुळे भारताचा व्यापारी मालाची निर्यात भारताच्या विस्तारी शेजारी राष्ट्रासोबत होईल त्यामुळे भारताची आर्थिक स्थिती बळकट, सुदृढ होईल यात शंका नाही.

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३. अ.वि.भागवत - राजकोय भूगोल, नरेंद्र प्रकाशन, पुणे

४. अ.वि.भागवत - भूराजनिती, मुरलीधर प्रकाशन, पुणे

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लिलाव मांडला जाणार आहे.

११. सामाजिक न्यायाची कल्पना खाजगी करणामुळे बाजुला पडते.

१२. गुणवत्तेला वाव न राहता पैसा हाच महत्वाचा घटक होईल.

सारांश :

भारताच्या उच्च शिक्षणात खाजगीकरणामुळे ग्रामीण भागात व्यावसायिक व इतर प्रकारचे शिक्षण देणारी महाविद्यालये स्थापन झालेली नाहीत. तसेच प्रादेशिक शैक्षणिक असमतोल मागासलेल्या भागात मोठया प्रमाणात दिसुन येतो. ग्रामीण भागातील आर्थिकदृष्टया दुर्बल घटकांना वाढणारी फिस, देणग्या यामुळे उच्च शिक्षण घेणे न परवडणारे आहे. त्यामुळे गुणवत्ता असूनही पैशाच्या अभावामुळे हा वर्ग उच्च शिक्षणापासुन वंचित राहील्यामुळे नैराश्य, न्युनगंड, आत्महत्या, मानसिक दुर्बल्य या सामाजिक समस्या निर्माण होऊ शकतात.

एकंदरीत शिक्षण हे उन्नत जीवन सामर्थ्य देणारे रसायन आहे. त्यामुळे शिक्षण ही राष्ट्राची मोठी गुंतवणूक आहे. व्यक्ती समाज आणि राष्ट्रे यांच्या विकासाची संजीवनी आहे. थोडक्यात शिक्षणाचे खाजगीकरण थांबवून दुर्बल घटकांना प्रवाहात आणून सामाजिक न्याय प्रस्थापित करणे गरजेचे आहे.

शिफारशी:

१. शिक्षणाचे खाजगीकरण थांबवावे. त्यामुळे गरीब व दुर्बल घटकांना उच्च शिक्षण घेवून राहणीमान उंचावता येईल.

२. मानवी विकास निर्देशांक उंचावण्यासाठी उच्च शिक्षणास गुणवत्ता वस्तुंचा दर्जा देवून त्यांची अंदाजपत्रकीय तरतुद करावी.

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मठांचे मराठा स्त्रियांच्या जीवनातील स्थान व धार्मिक महत्त्व

प्रा.डॉ. रमेश धनराज जाधव

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शिरपुर जि. धुळे

प्रस्तावना :

अमरकोात दिलेल्या व्याख्येनुसार 'मठ' म्हणजे 'विद्यार्थ्यांचे निवासाचे स्थान होय'. सामान्यत: ज्या ठिकाणी ब्रह्मचारी, साधू, भिक्षू, संन्याशी व बैरागी इत्यादी प्रकारच्या स्त्रिया व पुरु ा यांचे निवासस्थान असते त्यास मठ असे संबोधले जाते. संस्कृत मधील मठ या धातूपासून मठ हे नाव बनले आहे. धर्म, मठवासी व्यक्तींचा निवास हे धर्म, संप्रदाय, कालखंड आणि उद्देश यानुसार तात्पूरता किंवा कायमस्वरुपी असा दोन्ही प्रकारचा असल्याचे दिसून येते. सर्वसाधारणपणे धार्मिक उपासनेत जीवन व्यतीत करणे, त्याचप्रमाणे गूठवाद आणि संन्यास यांच्याकडे वळण्याची मानवाची नैसर्गिक प्रवृत्ती मठसंस्थेला जन्म देत असते. लौकिक समाजात राहून आपले अंतिम कल्याण साधणार नाही. असे ज्यांना वाटते त्या व्यक्ती मठ संस्थेकडे आकर्ा ित होतात. भारतीय लोकांच्या जीवनामध्ये मोक्षाला फार महत्व असल्यामुळे प्राचीन काळापासून भारतीयांनी मठसंस्थेला अभिप्रेत असलेले आश्रमव्यवस्था स्विकारलेली दिसून येते.

वि ायविवेचन :

मठांचा उल्लेख प्राचीन ग्रंथांतून आढळून येतो. मंदिरांचा उपयोग देवतेची पूजाअर्चा व आराधने साठी केला जातो. परंतु मठ हे धर्म अथवा संप्रदायाच्या अनुयायासाठी असते.[१] देवस्थान अथवा मठांना महारा ट्रातील स्त्रियांच्या सामाजिक आणि धार्मिक जीवनात महत्त्वाचे स्थान होते. त्याचप्रमाणे मठांबद्दल स्त्रियांच्या मनात अतिशय आदर व पूज्यभाव होता.

१८ व्या व १९ व्या शतकात मठांना महत्त्वाचे स्थान होते. मठात देवदेवतेची पूजा अर्चा, किर्तन धर्मिक ग्रंथाची पारायणे चालत असत. अशा मठां मध्ये भाविकांची राहण्याची व भोजनाची जेवणाची

१०. राजर्षी शाहू महाराजांचे जाहीरनामे, आदेश व हुकुम

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इतिहास विभाग प्रमुख, आर.सी.पटेल कला, वाणिज्य व विज्ञान महाविद्यालय, शिरपूर, जि. धुळे.

इ.स. १८८४ - १८९५ या काळात कोल्हापूर संस्थानचा कारभार राजर्षी शाहूंच्या राज्याभिषेकापर्यंत दिवाण, सरसुभे व सरन्यायाधिश यांचे एक ॲडमिनिस्ट्रेटिव्ह कौन्सील पहात होते. छत्रपती शाहू महाराजांनी कोल्हापूर संस्थानचा कारभार इ.स. १८९५ मध्ये हाती घेतल्यानंतर ते बरखास्त केले आणि आपणास राज्यकारभारात मदत करण्यासाठी एक 'हुजूर कार्यालय' निर्माण केले आणि 'हुजूर चिटणीस' या पदाधिकाऱ्यास या कार्यालयाचा प्रमुख म्हणून नियूक्ती केली.[१] राजर्षी शाहू महाराजांच्या राज्यकारभाराची एकूण तीन मुख्य खाती होती. त्यात प्रामुख्याने जनरल, दुसरे मुलकी आणि तिसरे न्यायखाते याचा समावेश होता. जनरल खात्याचा प्रमुख 'दिवाण' होता. मुलकी खात्याच्या प्रमुख 'सरसुभा' व न्यायखात्याचा प्रमुखास 'सरन्यायाधीश' असे म्हणत.[२]

शाहू महाराजांचा जन्म २६ जून १८७४ रोजी कागल येथील घाटगे घराण्यात झाला. त्याचे मूळ गाव यशवंत होते. त्यांच्या वडीलांचे नाव जयसिंगराव घाटगे आणि आईचे नांव राधाबाई होते. कोल्हापूर संस्थानचे राजे शिवाजी महाराज चौथे यांच्या निधनानंतर त्यांच्या पत्नी आनंदीबाई यांनी यशवंतरावास १७ मार्च १८८४ रोजी दत्तक घेतले व शाहू हे नाव ठेवले. २ एप्रिल १८९४ रोजी त्यांचा राज्यारोहण सोहळा झाला. त्यांनी कोल्हापूर संस्थानची सुत्रे होती घेतल्यानंतर आपल्या संस्थानात प्राथमिक शिक्षण, जातीभेद निवारण, अस्पृश्यकता निवारण अशा प्रकारच्या अनेक सुधारणा केल्या. त्या सुधारणा करण्यासाठी त्यांनी अनेक जाहीरनामे, आदेश हूकूम काढले.

- **मोडीऐवजी बालबोधीचा वापर**

करवीर इलाख्यातील दप्तर हे मोडी लिपीत असल्याने साधारण शिकलेल्या माणसास अर्ज वाचण्यात अडचणी येतात व त्यामुळे वेळ वाया जातो. म्हणून हुजूरकडे येणारे सर्व अर्ज बालबोध लिपीत असले पाहिजे असे नियम करण्यात आला. यामुळे जनतेची बरीच सोय होते असेही नजरेस आले. म्हणून इ. स. १९१७ च्या गणेशचतूर्थीपासून यापुढील सर्व पत्रव्यवहार, नोंदण्या, ताकीदा, ठराव, जबान्या , बारनिश्या, नगदी हिशेब व पावत्या त्याचप्रमाणे खाजगीकडील सर्व वह्या व सर्व दप्तराचे लिहणे. बालबोधीत असावे अशा प्रकारचे आज्ञा करण्यात आली.[३]

- **राजकीय भाग घेण्यास मनाई**

'लोकप्रतिनिधी सभा' या नावाची संघटना इ. स. १९०६ मध्ये कोल्हापूर येथे स्थापन करण्यात आली. या सभेत पास झालेले ठराव या सभेमार्फत छत्रपती शाहू महाराजांकडे पाठविले. त्यानुसार कोणत्याही सरकारी नोकराने स्वतः प्रत्यक्ष अगर

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पश्चिम खान्देशातील भूमिगत चळवळ - एक दृष्टीक्षेप

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प्रस्तावना

दि. ७ ऑगस्ट १९४२ रोजी मुंबई येथे अखिल भारतीय कॉंग्रेस महासमितीचे अधिवेशन सुरु झाले आणि दुसऱ्या दिवशी म्हणजे ८ ऑगस्ट १९४२ रोजी 'चलेजावचा' ऐतिहासिक ठराव या अधिवेशनात पास करण्यात आला. ब्रिटीश शासनाने म. गांधीजीसह अनेक नेत्यांना भारत स्वरक्षण कायद्याखाली दि. ९ ऑगस्ट १९४२ रोजी पहाटे अटक करुन तुरुंगात पाठविले. त्याचबरोबर स्वरक्षण कायद्याखाली प्रत्येक प्रांतातील व जिल्ह्यातील राष्ट्रीय कॉंग्रेसच्या नेत्यांना, कार्यकर्त्यांना अटक करुन क्रिमिनियल अॅक्ट अन्वये राष्ट्रीय कॉंग्रेसच्या कमिट्या बेकायदेशिर ठरविण्यात आल्या त्याचप्रमाणे भारत स्वरक्षण कायद्यानुसार प्रातांत मिरवणूका व जाहिर सभांवर बंदी घालण्यात आली. त्यामुळे सर्व देशभर सहभागी लोकांची धरपकड, खटले व शिक्षा यांचे सत्र सुरु झाले. त्यामुळे सर्वत्र या घटनेचा निषेध करण्यात आला. त्यात प्रामुख्याने महाराष्ट्रात सातारा, सोलापूर, पुणे, ठाणे, नगर, पूर्व खान्देश व पश्चिम खान्देश व इतर शहरांमध्ये व्यापाऱ्यांनी दुकाने बंद ठेवून कामगारांनी कामे बंद करुन हरताळ पुकारला.

चलेजाव चळवळीचे स्वरुप काय असावे यासंबंधी मार्गदर्शन करण्यास नेत्यांना सवडच मिळाली नाही. ब्रिटीश सरकारच्या दडपशाहीचे ते शिकार बनले. काही कॉंग्रेस कार्यकर्ते भूमिगत झाले. ब्रिटीश सरकार विरुद्ध सुरु केलेल्या लढा यशस्वी करण्यासाठी खेडोपाडी जावून प्रचार करणे आवश्यक होते. त्या प्रचारासाठी कॉंग्रेसचे कार्यकर्ते पोलीसांची नजर त्यांच्यावर पडू नये म्हणून रात्रीच्या वेळी खेडोपाडी जावू लागले. दिवसा शेतात, जंगलात आणि तळघरात लपून राहत असत किंवा वेशांतर करुन माहिती मिळवीत असत अशा प्रकारे चलेजाव चळवळ यशस्वी करण्यासाठी कॉंग्रेसचे कार्यकर्ते भूमिगत राहू लागले आणि यातूनच भूमिगत चळवळीचा उदय झाला.

उद्देश

१) धुळे जिल्ह्यातील भूमिगत चळवळीची माहिती मिळविणे.

२) भूमिगत चळवळीत कोणकोणत्या कार्यकर्त्यांनी कशा प्रकारे सहभाग घेतला याची माहिती मिळविणे.

संशोधन पद्धती

या विषयासाठी विश्लेषणात्मक संशोधन पद्धतीचा अवलंब केला असून हा विषय पूर्ण करण्यासाठी अप्रकाशित लघुशोध प्रबंध व उपलब्ध दुय्यम साधनांचा उपयोग करण्यात आलेला आपणास दिसून येईल.

विषय विवेचन

जयप्रकाश नारायण यांनी भूमिगत चळवळीचे नेतृत्व केले. कॉंग्रेसचे बरेचसे कार्यकर्ते भूमिगत झाले होते. या काळात जनतेच्या मनात ब्रिटीश शासनाच्या दडपशाही विरुद्ध, अधिकाऱ्याविरुद्धा असंतोष निर्माण झाला होता. मुंबई येथे गेलेले पश्चिम खानदेशचे नेते श्री बाळभाई मेहता मुंबईहून हे सुरतमार्गे विरदेल स्टेशनवर उतरले आणि दलवाडे येथील प्रमुख कार्यकर्ते देवमणदादा यांच्या शेतात रात्री पश्चिम खानदेशातील प्रमुख कार्यकर्त्यांची सभा घेतली. या सभेत चलेगाव चळवळीचा संदर्भात कॉंग्रेसचे धोरण आणि सरकारची निती यावर विचार विनिमय झाला. परंतु उघड बंडात मोडतोडीचे कार्यक्रम येतात का? याबाबत मतभेद झाले. त्यामुळे एक प्रश्नावली तयार करण्यात आली आणि म. गांधीजीचे शिष्य श्री. किशोरीलाल भाई मुश्रीवाला यांचे मार्गदर्शन घेण्याचे ठरले. त्यानुसार श्री. विष्णूभाऊ पाटील सेवाग्राम येथे गेले आणि चरखा संघाचे अध्यक्ष श्री. जाजूंजीच्या बंगल्यावर १६ ऑगस्ट १९४२ रोजी काही प्रमुख नेत्यांची सभा घेण्यात आली या सभेस श्री. विष्णूभाऊ उपस्थित होते. या सभेत विचार विनिमय होवून कार्यक्रमाचा आराखडा तयार करण्यात आला. त्यानूसार -

१) भूमिगत राहून चळवळ चालविणे.

२) रेल्वे रुळ उखडणे आणि

३) दूरध्वनीच्या तारा तोडणे

इत्यादी कार्यक्रमांना मान्यता देण्यात आला आणि त्यानुसार भूमिगत चळवळ सुरु झाली.

भूमिगत चळवळीची जबाबदारी

श्री. बाळूभाई मेहतांच्या मार्गदर्शनाखाली धुळे जिल्ह्यात दौरे सुरु झाले. श्री. विष्णुभाऊ पाटील यांच्यावर धुळे, शिंदखेडा, शिरपूर व साक्री भागाची जबाबदारी दिली. तर शहादा, तळोदा, नवापूर, नंदूरबार आणि धडगाव या भागाची जबाबदारी श्री. नानासाहेब ठकार

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माझे गाव मोहाडी – एक दृष्टीक्षेप

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एम.ए. (इतिहास), डॉ. बाबासाहेब आंबेडकर मराठवाडा विद्यापीठ, औरंगाबाद

सारांश

सुमारे ४५०-५०० वर्षापूर्वी मोहाडी या गावात तिरमले पाटील यांचे वास्तव्य होते. त्या काळात येथे गोसावी समाजाची वस्ती असावी, परंतु रामसिंग, जामसिंग आणि शामसिंग हे राजूपत बांधव मोहाडीला आले असता संघर्ष झाला. त्या संघर्षात तिरमले पाटील यांचा पराभव आणि रामसिंग राजपूत यांचे मोहाडीवर वर्चस्व प्रस्थापित. त्यानंतर धनसिंग नाईक (जाधव) हे मोहाडीला आले त्यांना काही काळांनंतर लखा नाईक (पवार) यांचे आजोबा यांना मोहाडीला आणले. नारायण सिरसाठ(महार) मोहाडीला आले. काही कालावधीनंतर गणपत महाजन मोहाडीला आले. त्यानंतर मथुरेहुन मौजीराम गोपाल आले.

मोहाडी गावात जूनी दोन हनुमानाची मंदीरे असून, ज्वालामुखी माता मंदीर, मोती माता मंदीर, गुनाबाबू मंदीर, सती मंदीर, धोंडामाय मंदीर, बाळू महाराज मंदीर, मरीमाय मंदीर त्याचप्रमाणे तीन समाध्या जीर्ण अवस्थेत आहे. या गावात विविध सण उत्सव परपंरागत पद्धतीने साजरा केले जातात. कानबाईचा उत्सव, दसरा, दिवाळी, होळी, गोटपूजा, गोवर्धन पूजा इत्यादी बंजारा समाजाची परंपरागत असलेली जात पंचायतीचे महत्त्व कमी झाले असले तरी काही प्रमाणात तिचे महत्त्व आजही आहे. तांड्यात असणारे नायक आणि कारभारी यांना विशीष्ट प्रसंगी आजही मानसन्मान दिसून येतो. या गावार वारकरी संप्रदायाचा प्रभाव असल्यामुळे खूप काळापासून सप्त्याचा कार्यक्रम आयोजित केला जातो. या गावातील फार थोड्याप्रमाणावर लोक नोकरीला आहेत.

या गावात बहुते शेतकरी हे अल्पभूधारक आहेत. बंजारा समाज आपली उपजीवीका भागविण्यासाठी ऊसतोडीचे काम करतो.

प्रस्तावना

राष्ट्रीय इतिहासाच्या लिखाणाबरोबर विशेषत: २० व्या शतकात गेल्या ८०-९० वर्षात प्राचीन अवशेष, शिलालेख, स्थापत्य अवशेष आणि त्यांचा शोध जगभर घेतला जावू लागला आणि त्यातूनच स्थानिक इतिहास उजेडात आला.१

स्थानिक इतिहासाचे प्रथम उगमस्थान युरोपात झाला. नार्वे, फिनलॅन्ड आणि इंग्लंड या देशामध्ये स्थानिक इतिहास लेखनास सुरुवात झाली आणि हळूहळू आशिया खंडातील देशामध्ये स्थानिक इतिहास लेखनाची भावना वाढीस लागली. जागतिक पातळीवर सर्वात जास्त स्थानिक इतिहासाचे लेखन झालेले दिसते.२

नार्वे लोकल स्टडी जनरल त्यानंतर फिनलॅन्ड मध्ये स्थानिक इतिहासाला जास्त महत्व आहे. स्थानिक इतिहासामध्ये जिल्ह्याचा इतिहास देखील समावेश करण्यात आला. महत्त्वाची गोष्ट म्हणजे राजकीय इतिहासाची मांडणी न करता सामाजिक परिस्थितीचा अभ्यास करतांना भौगोलिक परिस्थितीचा त्याच्यावर कसा परिणाम झाला आहे. या सर्व गोष्टी अभ्यासल्या जावू लागल्या.३

जपान या देशातही स्थानिक इतिहास लेखन २० व्या शतकात रुढ झाले. चिन या देशातही लहान खेड्यातील भागात बखरकार होवून गेले. संशोधनाच्या प्रसिद्धीसाठी Bulleting of Chaina" "Local History and Thoracal" यातून प्रसिद्धी दिली.

इंग्लंडमध्येही १९६२ मध्ये लेस्टन या विद्यापीठाने स्थानिक इतिहासावरील लेख एकत्रित करुन School of Local History and Historian" या नावाने प्रसिद्ध केले.४

अमेरिकेतही १९७० नंतर याद्वारे लहान लहान शहरांचा ( ) इतिहास उजेडात आणला.५

महाराष्ट्रामध्ये नवनवीन विद्यापीठांच्या निर्मीतीमुळे स्थानिक इतिहास उजेडात आला.

मी ज्या गावात जन्मलो, राहतो ते गाव कसे विकसित झाले किंवा प्राचीन काळात माझे गाव कसे होते याची भावना निर्माण होऊन त्यातूनच स्थानिक इतिहासाला चालना मिळाली आणि यातूनच आपल्या गावाचा इतिहास आपण मांडावा ही संधी ह्या परिषदेमुळे म्हणजेच डॉ. प्रशांत देशमुख यांनी उपलब्ध करुन दिली. ६

उद्देश

१) मोहाडी या गावांतील विविध समाजाची माहिती मिळविणे.
२) मोहाडी या गावातील जून्या मंदीराची व समाध्या यांची माहिती मिळविणे.
३) साजरा होणाऱ्या सण आणि उत्सवाची माहिती मिळविणे.
४) गावातील नोकरदार वर्गांची माहिती मिळविणे.

संशोधन पद्धती

या शोधनिबंधाकरिता विश्लेषणात्मक पद्धतीचा वापर केला असून उपलब्ध असणारी मंदीरे, समाध्या व मुलाखती व प्रत्यक्ष पाहणी द्वारे शोध निबंध पूर्ण करण्याचा प्रयत्न केलेला आहे.

विषय विवेचन

मोहाडी हे गाव २००७१ उत्तर अक्षववृत्त व ७५०४२ पूर्व रेखावृत्त असून हे गाव पाचोरा तालूक्यातील असून जि. जळगांव आहे. हे गाव पाचोरा-लोहारा या रस्त्यावर असून साजगावपासून उत्तरेला २ कि.मी. अंतरावर आहे या गावाचे नाव मोहाडी यावरून पडले असावे की, मोठ्या प्रमाणावर महुचे झाडे या परिसरात होती यावरुन या गावास मोहाडी असे नाव पडले असावे. आजही या परिसरात बरीच महूची झाडे आहेत.

मोहाडी या गावात प्रामुख्याने राजपूत, माळी, बंजारा, महार आणि गोपाळ समाजाची लोक वास्तव्यास आहेत. थोडक्यात त्यांचा पूर्व इतिहासाची माहितीचा आढावा घेण्याचा प्रयत्न केलेला आहे, त्याचप्रमाणे मांग समाजाची दोन घरे व एक भिल समाजाचे घर आहे.

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१२. शाहू महाराजांची सामाजिक व राजकीय सुधारणा : एक दृष्टीक्षेप

प्रा. रामसिंग राज्या वसावे
राज्यशास्त्र विभाग, आर. सी. पटेल कला, वाणिज्य व विज्ञान महाविद्यालय, शिरपूर, जि. धूळे.

प्रस्तावना

स्वातंत्र्यपूर्व भारतातील सामाजिक परिस्थितीत महत्वपूर्ण बदल अथवा परिवर्तन घडवून आणण्यात शाहू महाराजांची भूमिका महत्वपूर्ण राहिली आहे. म्हणूनच सामाजिक व राजकीय क्षेत्रात त्यांनी केलेल्या कार्यावरून भारताच्या इतिहासात एक महान समाजसुधारक महत्वाचे स्थान प्राप्त झाले आहे. राजश्री शाहू महाराजांचे प्रत्येक कार्य प्रगमनशिल (पुरोगामी) विचारांशी निगडीत होते. त्यांनी स्त्रीयांची शिक्षण, सामाजिक समता, सत्यशोधक समाज आदि विविध दृष्टीकोनातून व त्यांच्या वैचारिक भूमिकेतून प्रत्येक पातळीवर समाजाला सहभागी करतांना आढळून येते. त्यांच्या या भूमिकेतूनच भारतीय लोकांच्या मानसिकेताला आकार मिळाला आहे. महात्मा फुलेंपासून सुरू झालेल्या क्रांतीकारक सुधारणा शाहू महाराजांनी सत्यशोधक चळवळीच्या माध्यमातून पुढे चालविल्या आहे. शाहू महाराजांना तळागाळातील लोकांच्या समस्याविषयी जाणीव होती. शिक्षणाच्या अभावामुळे समाजात अज्ञान, अंधश्रध्दा, जातीयता, अस्पृश्यता व कामगारांच्या समस्या निर्माण झाली आहे याची त्यांना जाणीव होती. म्हणूनच शाहू महाराजांनी १८८४ ते १९२२ या कालखंडात सामाजिक परिस्थितीत महत्वपूर्ण बदल घडवून आणले. त्यांनी आपल्या कार्याने व वैचारिकतेने समाजाला एक कृतीप्रवण व शास्त्रीयवृत्ती बौध्दीक क्षमता या विविधांगानी बदल घडवून आणला. त्यांचे हे कार्य केवळ कोल्हापुरक्षेत्र मर्यादित नसून संपूर्ण महाराष्ट्रभर आहे. शाहू महाराजांनी मागासवर्गीय, स्त्री, पुरूष, मजुर यांना समाजाच्या मुख्य प्रवाहात आणून सन्मानाने व अभिमानांने जीवन जगण्याचा अधिकार मिळवून दिला. छत्रपती शाहू महाराजांच्या अशा प्रकारच्या सामाजिक व राजकीय विकास कार्याची विश्लेषण प्रस्तूत शोधनिबंधात करण्यात आला आहे.

शोधनिबंधाची उद्दिष्टे

१) छत्रपती शाहू महाराजांचे ब्राम्हणेत्तर चळवळीतील कार्य अभ्यासणे

२) छत्रपती शाहू महाराजांचे लोकशाही विचाराचे विश्लेषण करणे

गृहितके

१) सामाजिक , शैक्षणिक , आर्थिक व राजकीय क्षेत्रात आमुलाग्र बदल घडवून आणण्यासाठी शाहू महाराजांचे विचार महत्त्वाचे आहे.

२) आज संपूर्ण देशाला बलशाली करण्यासाठी शाहू महाराजांचे विचार मोलाचे आहे.

संशोधन पध्दती

प्रस्तुत संशोधनासाठी ग्रंथालय व दुय्यम साधनांचा वापर करण्यात आलेला आहे. उपलब्ध माहितीच्या आधारे विषयांची मांडणी वर्णात्मक व विश्लेषणात्मक पध्दतीचा वापर केला आहे.

सामाजिक स्थिती

राजश्री शाहू महाराज राजा होण्यापूर्वी कोल्हापूरात मराठ्यांची सत्ता अस्तित्वात होती. परंतु प्रत्यक्षात ही सत्ता युरोपीयन , पारशी आणि ब्राम्हण या तीन वर्गाच्या हाती सर्व कार्यांवर नियंत्रण ठेवण्याची जबाबदारी होती. जेव्हा शाहू महाराजांना राजा म्हणून घोषीत करण्यात आले तेव्हापासून युरोपीयन व पारशी लोकांना सामान्य लोकांचा दर्जा देण्यात आला. ब्राम्हण वर्गाचा दर्जा हा वरच्या पातळीवरच होता. परंतु वेदोक्त प्रकरणानंतर त्यांनी ब्राम्हणांचे वर्चस्व कमी केले. हजारो वर्षापासून ब्राम्हणांकडून दलीत पददलीत, बहुजन समाजाला जी वागणूक दिली जात होती ती सामाजिक प्रथा शाहू महाराजांनी बंद केली आणि आत्मविश्वास , स्वआदर विसरलेल्या बहूजन समाजाला स्वत:प्रती उत्तरदायी बनविण्याचे कार्य शाहू महाराजांनी केले. 'माझं हिंदूस्थान ' या पुस्तकात ब्राम्हण राजवटीविषयी वर्णन करतांना डब्ल्य.जे. विल्कीन्स म्हणतात की , 'संपूर्ण जग हे दैवी सत्तेखाली आहे , देव हा मंत्र सत्तेखाली आहे. तर मंत्र हे ब्राम्हण वर्चस्वाखाली आहे. त्यासाठी ब्राम्हण हाच आमचा देव आहे.' (Jadhav pp १६) ही समाजाची वास्तविक स्थिती होती. धर्माच्या नावाखाली बहुजन समाजाचा अज्ञानाचा फायदा घेऊन सामाजिक शोषण करणे हे धर्मोपदेशक वर्गाचे डावपेच शाहू महाराजांनी ओळखले होते. त्यासाठी शाहू महाराजांनी हिंदू-धर्मातील सामाजिक विषमतेविरूध्द क्रांतीकारक निर्णय घेतला.समाजात समाजिक परिवर्तन घडवून आणण्यासाठी ब्राम्हण विरूध्द ब्राम्हणत्तेर चळवळीला सुरूवात झाली.

शाहू महाराज : ब्राम्हणेत्तर चळवळ

राजश्री शाहू महाराज हे एक ब्राम्हणत्तेर चळवळीतील विचारशील व कृतीशील नेतृत्व होते. त्यांनी जनतेला आणि कनिष्ठ वर्गातील समुदायाला राजकीय सत्तेत समान सहभागांची संधी उपलब्ध करून दिली. ब्राम्हणत्तेरांना सुरक्षित राजकीय अधिकार देण्याच्या उद्देशाने १९१६ मध्ये निपाणी येथे 'दि डेक्कन रयत असोशिएशन' ची स्थापना केली. त्यासाठी महाराष्ट्रभर दौरे करून लोकांच्या सभा आयोजित करून ब्राम्हणत्तेर चळवळीचा प्रचार केला. ब्राम्हणेत्तर चळवळी संबंधी ते म्हणतात की, 'महाराष्ट्रातील अस्पृश्यासह तमाम मागासलेल्या बहुजन समाजाचा उद्धार हे माझे पवित्र जीवन कार्य आहे. सरकार मला पध्युत करण्याच्या बुरखेबाज धमक्या देत आहे. तुम्ही पद्च्युत करण्यापूर्वीच मीच आत्मसंतोषतने गादीचा राजीनामा देईल पण एकदा हातात घेतलेली ब्राम्हणत्तेराची चळवळ प्राण जाईतोवर नाही.' (P.B.Salunkhe PP ४५८)

शाहू महाराजांना वेदोक्त प्रकारणामुळे फार मोठा संघर्ष करावा लागला. वेदाच्या वादळाला तोंड देण्यासाठी महाराष्ट्रीयन सामाजिक जीवनाला सत्यशोधक चळवळीचा आधार जनतेला उपलब्ध करून दिला. शाहू महाराजांनी स्वत: ब्राम्हणत्तेर चळवळीचे नेतृत्व केले. त्यासाठी त्यांना त्यांच्या विरोधात ब्राम्हणांनी बदनामीचा प्रसार केला. कोल्हापुरला सभा

आयोजित करून शाहू महाराज हे मराठा व कनिष्ठ जातीच्या लोकांना पाठिंबा देत असल्याचा आरोप करण्यात आला. ब्राम्हणेत्तर चळवळ ही वेदोक्तवादामुळे कोल्हापूर येथे सुरू झाली. ब्राम्हणेत्तराचा आत्मा हा महात्मा फुलेंच्या शिक्षण व सत्यशोधक समाजातून विकसित आला आहे. त्याबद्दल छत्रपती शाहूच्या मतानुसार ब्राम्हणाकडून सामाजिक भेदभाव व मानहानीच्या परिस्थितीमुळे ब्राम्हणेत्तर समाजातील प्रबोधन या पवित्र कार्याला सुरूवात झाली आहे. हे लक्षात घेण्यासारखे आहे. त्यामुळे ब्राम्हणेत्तरांचे भविष्य उज्ज्वल आहे. यावर त्यांचा पक्का विश्वास होता. छत्रपती शाहू महाराज आणि त्यांना मदत करणारे प्रबोधन ठाकरे, श्रीपतराव शिंदे, वालचंद कोठारी, आणि जो खरा आणि प्रामाणिक देशभक्त व समाजसुधारक ब्राम्हणेत्तर चळवळीत काम करीत होते. त्यांना ते विशेषकरून मार्गदर्शन करीत होते.

राजश्री शाहू महाराज : लोकशाही विचारप्रणाली

राजश्री शाहू महाराज लोकशाही विचार प्रणालीचे पुरस्कर्ते होते. कोल्हापूर संस्थानातील लोकांच्या अधिकारांचे संरक्षण करतांना त्यांनी लोकशाही मूल्यांचा स्वीकार केला होता. शाहू महाराजांनी १८९४ मध्ये कोल्हापूर संस्थानाची राजकीय सूत्रे स्वीकारली आणि सर्वप्रथम कायद्याने वेठबिगारी पध्दत बंद केली. मागासलेल्या वर्गांसाठी नोकरीत पन्नास टक्के आरक्षणाची तरतुद केली. आपल्या संस्थानात अस्पृश्यता निवारणाचा कायदा सर्वप्रथम शाहू महाराजांनी केला. १९१८ मध्ये कुलकर्णी वतने आणि बलुतेदारी पध्दत बंद केली. समाजातील वाईट प्रथा बंद करण्यासाठी सर्वप्रथम शाहू महाराजांनी पुढाकार घेतला आणि डॉ.बाबासाहेब आंबेडकर यांनी गुलामगिरीची संबंधीत असलेली महारवतेने नष्ट करण्यासाठी १९२८ या कालावधीत मुंबई कॉन्सीलमध्ये खुप प्रयत्न केले. परंतु त्यांना यश आले नाही. शेवटी स्वातंत्र्यानंतर भारतीय संविधनाने कायद्याने महारवतने कायदा रद्द केला होता. १९५५ मध्ये अस्पृश्यता निवारण कायदा केला. समाजातील सर्वांचे हित व्हावे अशी महाराजांची मनातील इच्छा होती. हे त्यांच्या २ एप्रिल १८८४ च्या आदेशातून स्पष्ट होते.

सर्व प्रजा सतत तृप्त राहून सुखी असावी त्यांच्या कल्यणात सतत वृध्दी व्हावी व आमचे संस्थानाची हरएक प्रकारे भरभराट होत राहावी अशी आमची उत्कट इच्छा आहे. हा आमचा हेतू परिपर्ण करण्यात आमच्या पदराचे सर्व लहान थोर जहांगिरदार सरदार मानकरी इमानदार कामगार , व्यापारी आदि तमाम प्रजाजन शुध्द अंत करणापासून मोठया राजनिष्ठेने आम्हास मदत करतील , अशी आमची पूर्ण आशा आहे. ही आमची कारकीर्द दीर्घकाळ चालवून सफल करावी. अशी मी जगतपित्यांची परमात्म्याची एक भावाने प्रार्थना करतो. (Ahire D.C.PP १२९)

शाहू महाराजांनी समग्रक्रांतीचे प्रारूप महाराष्ट्रासमोर ठेवले होते. तत्कालीन जाती व धर्म व्यवस्थेच्या विरोधात जाऊन अस्पृश्यता निवारण , मोफत व सक्तीचे शिक्षण , विद्यार्थी वस्तीगृहाची योजना सुरू करून सामान्य नागरिकांना शिक्षणांची दालने खुली केली. शाहू महाराजांनी गरीब लोकासाठी जिनिंग आणि व्हिविंग मिल शाहूपुरी व्यापारपेठ आणि गुळाची निर्मिती , शेतकऱ्यांसाठी सहकारी संस्था 'किंग एडवर्ड ॲग्री कल्चरल इन्स्टियुट' , राधानगरी धरण आदि अनेक प्रकल्प सुरू करून हरित क्रांतीकडे वाटचाल केली. त्यातून कृषिक्षेत्रात आधुनिकीकरण करून शेतकऱ्यांमध्ये विश्वास निर्माण केला. आधुनिक तंत्रज्ञ

पिकांच्या वापरामुळे पिकांच्या उत्पन्नात वाढ व फायदा देणारे अनेक प्रयोग केले. त्याचबरोबर चित्रकला , संगित , रंगभूषी अशा कलांना दिलेला आश्रय यावरून त्यांचा उदात्त दृष्टीकोन दिसून येतो. त्यांनी गरीबांसाठी घरे बांधून राहण्यासाठी उपलब्ध करून दिली त्याचबरोबर नोकरी दिली यावरून शाहू महाराज हे समग्र समाज क्रांतीचा प्रेरणा होता ही स्पष्ट होते.

निष्कर्ष

१) राजश्री शाहू महाराजांनी लागू केलेले आरक्षण तत्व भारतीय संविधनात समाविष्ट झालेले दिसून येते.

२) शाहू महाराजांनी बहुजन समाजावर स्वअत्विची जाणीव जागृती निर्माण केल्याचे दिसून येते.

३) शाहू महाराजांनी बहुजन समाजाला राजकीय अधिकार मिळवून देण्यात महत्वाची भूमिका बजविल्याची दिसून येते.

४) ब्राम्हणेत्तर चळवळीवर शाहूंचा कार्याचा आणि स्फुर्तीदायक व्यक्तीमत्वाचा बराच परिणाम होऊन मागासवर्गातील तरूणांनी अनेक ठिकाणी वस्तीगृह आणि शाळा सुरू करण्याचे कार्ये हाती घेतले.

५) लोकशाही विकासाच्या दृष्टीने ग्रामपंचायतीची स्थापन करून आरोग्य , पाणी पुरवठा व शिक्षण यासारखे सत्ता ग्रामपंचायतीना दिली. लोकांच्या हाती पूर्ण स्वातंत्र्यांचे हक्क हे लोकशाहीच्या दृष्टीने चालविली चळवळ आढळून येते.

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'गुर्जर बोलीतील उखाण्यांचे स्वरुपव वाङ्मयीन विशेष '

डॉ. सुधाकर सीताराम चौधरी
मराठी विभाग,
आर.सी. पटेल कला, वाणिज्य व विज्ञान महाविद्यालय
शिरपूर जि.धुळे

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प्रास्ताविक :-

लोकवाङ्मयाचा एक महत्वाचा घटक म्हणजे उखाणा होय. मौखिक परंपरेतुन उखाणेहेसंक्रातहोतहोत जिंवत राहीलेले दिसतात. उखाण्यांची फार मोठी प्राचीन परंपरा दिसते. ह्या उखाण्यांमधून जसे 'ज्ञानाचे दर्शन' होते, तसेचहे उखाणे मनोरंजनही करतात. उखाणा हा सर्वदूर सर्व भाषांमध्ये आढळून येतो. उखाण्यामधील शब्द दैनंदिन व्यवहारातील साधेसोपे पण अर्थगर्भ स्वरुपाचे असतात. उखाण्यामधून जीवनाचे शाश्वत तत्वचिंतन मांडलेले असते. विनोद, थट्टा, मस्करी करुन मोठा गर्भीत अर्थ त्यातुन मांडलेला असतो.

गुर्जर बोलीतील उखाण्यांचे स्वरुप :

गुर्जर बोलीतील उखाण्यांचे दोन प्रकार आढळतात.

१) गुर्जर बोलीतील -प्रश्नोत्तर रुपी उखाणे

२) गुर्जर बोलीतील - प्रश्नविरहीत उखाणे (नाव घेण्याचे उखाणे)

१) प्रश्नोत्तर रुपी उखाणे :-

या प्रश्नोत्तर रुपी उखाण्याचे पुन्हा दोन उपप्रकार पडतात -

अ) प्रत्यक्ष प्रश्न विचारणारे उखाणे :- या प्रत्यक्ष प्रश्न विचारणाऱ्या उखाण्यात हायहाय करु काय? असा प्रश्नार्थी रोख असतो.

ब) प्रश्न विचारण्याचा भाव निर्माण करणारे उखाणे :- या उखाण्यात प्रत्यक्ष प्रश्न न विचारता केवळ प्रश्न विचारण्याचा भाव निर्माण केलेला असतो. उदा. धरी धोपटी दिंधू उपटी.

प्रत्यक्ष प्रश्न विचारणाऱ्या उखाण्यात खालील उखाण्यांचा समावेश करता येतो.

१. हाय-हाय-करु काय (बर्फ)
२. गुडघा एवढो हत्ती (अळूची पाने)

या उखाण्यांचे निरीक्षण केले असता असे निदर्शनास येते की, या उखाण्यांची रचना प्रश्नस्वरुपाची असते. या उखाण्यांच्या शेवटी प्रश्नार्थक सर्वनाम आलेले आहे. याखेरीज प्रत्यक्षात प्रश्न न विचारता केवळ प्रश्न विचारल्याचा भाव निर्माण करणारेते उखाणे पुढीलप्रमाणे :

१. लाल पालखी हिरवो दांडो
तिनमा बसे बोडके रांडो (लाल मिरची)
२. एवढूसू पोरं वरथून खास, खालेहागसं (जाते)
३. हरीण पयस |
खाले दूध गयस (जाते, घटा)
४. काकून दोन कान, काकान काई नयी (कढई)
५. धल्लान लोंडके न धल्लीन चोंडके (भिकबाई)

अशाप्रकारे विविध विषयावरील औसुक्यपूर्ण, रंजकयुक्त व ज्ञानाला, तर्कशक्तीला आवाहन करणारे उखाणे यात येतात.

२) प्रश्नविरहीत उखाणे : (नांव घेण्याचे उखाणे) : या नाव घेण्याच्या उखाण्यांमध्ये लग्नप्रसंगी घ्यावयाच्या उखाण्यांचा समावेश प्रामुख्यानेहोतो. वधु-वरांना लग्नप्रसंगी उखाण्यातून नाव घेण्याचा आग्रह केला जातो. नाव घेतांना वधू-वर लज्जेने चूरहोतात. त्यांचा गोंधळ उडतोते बावरतात. त्यांचीही तारांबळ पाहणे उपस्थितांना गमतीशीर वाटते. म्हणून त्यांना आग्रह धरला जातो. उखाण्यांना मानवी भावजीवनात अढळ स्थान आहे. गुर्जरी उखाणेही याला अपवाद नाहीत. गुर्जर बोलीत पुढीलप्रमाणे प्रश्नविरहीत (नाव घेण्याचे) उखाणे आढळतात.

१) वावरमा काम करान लागस ऊन,
मी त स् XXX नीसून.

२)उभी व्हती माडीवर, ऊन पडी साडीवर
तथई आये जोरी, मोती घे पोरी
XXX नाही घरीतर मोल कोण करी.

वरील दोनही प्रकारची उखाणे गुर्जरांमध्ये प्रचलित आहेत. विशेषत: लग्नप्रसंगी नाव घेण्याचे उखाणे म्हटले जातात. तर अन्य फावल्या वेळी तर्कबुद्धी शोधण्यासाठी प्रश्नोत्तररुपी उखाणे म्हटले जातात.

उखाण्यांचा आशय व अभिव्यक्ती विचार :

गुर्जरी उखाण्यांचा आशय साधा, सरळ व सोपा आहे. त्यांच्या उखाण्यात प्रतीक योजना असलीतरी उत्तर मिळाल्यावर प्रतीकांमधील गूढसहज आकळते. प्रश्नोत्तररूपी उखाणे बुचकळ्यात टाकणारे प्रश्न विचारतात, तर्कबुद्धीला आवाहन करतात. उखाण्यातील सर्व रुपके दैनंदिन जीवनातील असलीतरी त्यांच्यातीलसंगतीतात्काळ लक्षात येतेच असे नाही. रंजन व्हावे विशेषत : बुद्धीचे रंजन व्हावे, हा

प्रधान हेतू या उखाण्यांचा असल्याने त्यांच्यात प्रतीक योजना अपरिहार्यतेतून. 'कळीची फणी भाराने वाकते' हसूत लक्षात घेऊन 'खो-खो खोकली मान वाकडी', 'चौदा पिल्लांची माय मवाळी' हा उखाणा योजला जातो. आकाराने लहान असलेला विंचू माऊच्या माणसाल्लाहेरण करतो. म्हणून 'एवढीशी दवी चूर-चूर करे', 'पल्ला बाणानं हेरण करे' हा उखाणा तयारहोतो. जीभेतील लाळ समाजाच्या भुंकण्यावरून 'एवढूस गडू भुईसा दडू' हा उखाणा तयारहोतो. ताऱ्यातून निघणाऱ्या पिठावरून 'एवढूस पोर वरपून खास, खानेहाराम' हा उखाणा तयारहोतो. हे सर्व उखाणेतर्कसंगती लावण्याचा आग्रह धरतात. व्यक्तीचा चतुःरस्त्रपणा तपासतात. व्यक्तीचे अनुभवकौशल्य, बुद्धीकौशल्य तपासून पाहतात. यामुळेही उखाणे रंजक व प्रबोधकही होतात. नाव घेण्याच्या उखाण्यात वस्तुतः तसे अर्थही नसतात. केवळ प्रिय व्यक्तिच्या नावाच्या योजनेसाठी ओढून-ताणून यमक योजले जातात. त्यांच्यात अंतर्गत सु-संगती असतेच असे नाही.

उखाण्यांचे वाङ्मयीन विशेष :-

१) तर्कशक्तीला आव्हान :

व्यक्तीची तर्कबुद्धी, व्यवहारज्ञान, बुद्धीकौशल्य, स्मरणशक्ती, चतुःरस्त्रपणा, आकलनक्षमता इत्यादी बुद्धीजन्य कसोट्यांवर उखाण्यांचा भर असतो. नांव घेण्याच्या उखाण्यात केवळ रंजन असतेतेथे अशा कसोट्यांची अपेक्षा नसते. मात्र प्रश्नोत्तरकी उखाणे व्यक्तीच्या बुद्धीजन्य कौशल्याची कसोटी पाहतात.

१) हरीण पयस खाले दूध गयस

२) धरी धोपटी, दीधू उपटी

३) कायाहाडका, धव्वी मांस, जो नई वयखू तिनो सत्यानास

४) खो-खो खोकली मान वाकडी, चौदा पिल्लांची मान वाकडी.

जात्यातून पडणारे पिठ प्रत्येकाने पाहिलेले असते. परंतुत्यासाठीहरिण आणि दुधही रुपक योजना मात्र व्यक्तीच्या तर्कबुद्धीला आवाहन करते. हरीण म्हणजे जात्याचे पाळ व त्यातून गळणारे पिठ म्हणजे दूध. अशीसुसंगती लावून 'जाते' असे उत्तर देणारा चतुःरस्त्र बुद्धीमान ठरतो. घडाच्या भाराने वाकलेल्या कळीच्या झाडाला वाकडी मान असणाऱ्या म्हातारीचे रुपक योजल्याने तिचीसुसंगती कळीच्या फणीशी लावणारा तर्कबहाद्दर ठरतो. सिताफळाच्या बरच्या बीटनाला काळीहाडे हे रुपक उलगडण्यासाठी चतुःरस्त्र बुद्धीमतेचीच गरज असते. अशा प्रकारे प्रश्नोत्तर स्वरुप उखाणे मानवी तर्कबुद्धीला आव्हान करतात. रंजनासोबतच बुद्धिकौशल्य, आकलन कौशल्य, स्मरण कौशल्य वृद्धींगत करण्याचे अप्रत्यक्ष कार्यही उखाणे करीत असतात.

२) अलंकार :

पूर्वीच्या उखाण्यातून प्रामुख्याने पुढील प्रकारे अलंकार योजले जातात.

१) रूपक अलंकार

२) यमक अलंकार

३) अनुप्रास अलंकार

१) रूपक अलंकार- पूर्वी सांगितल्या उखाण्यात पुढील रूपक अलंकाराची उदाहरणेआहेत.

अशा विविध रूपक अलंकार युक्त उखाण्यांतून, वर्णनातून, व्यक्ती, पदार्थ यांच्या गुणवर्णनी विविधतेत रूपक शोधून काढण्यात तर्कबुद्धीला आव्हान असते.

२) यमक अलंकार :

प्रश्नोत्तरकी उखाण्यात मुख्य यमक योजना आढळते.

उदा. १) चाल चालखी, हिरवी दांडी,

तिनसा वर्षे वोढके गेंडी.

२) हरीण पयस खाले दूध गयस

या अनुक्रमे दांडी-गेंडी, पयस-गयस, खोड-गोड, दांडी-सोंडी, गडू-दडू साम्यातून यमक योजना आढळते.

३) अनुप्रास अलंकार :

प्रश्नोत्तर रूपी उखाण्यात अनुप्रास अलंकारही आढळतो. त्याची उदा. ते पूर्वीप्रमाणे-

उदा. १) हायहाय, कर काय,

राजा सीमान देवू कार!

२) काकून दीन कान, काकानं काटे नही

वरील सर्व उखाण्यात अनुक्रमे 'क' , 'ध', 'च', 'य', 'ड' यांसारख्या वर्णांची पुनःरुक्ती आलेली आहे. म्हणून येथे अनुप्रास अलंकाराचा आढळहोतो.

नांव घेण्याच्या उखाण्यासध्येसुद्धा यमक, अनुप्रास अलंकाराचा आढळहोतो.

नांव घेण्याच्या उखाण्यांचा हेतू रंजन असल्यामुळे त्यांच्या लयबद्धतेकडे व यमक योजनेकडे जास्त लक्ष दिलेले आढळते.

१. चाचरमा काम करान लागम ऊन,

मी त म XXX नीमून.

वरील उखाण्यात अनुक्रमे 'ऊन-सून' अशा प्रकारची यमक योजना आढळते.

यमक अलंकाराखेरीज या उखाण्यात अनुप्रास अलंकारही आढळतो. वरील उखाण्यात अनुक्रमे 'न', 'र', 'श', 'ड', 'ट', 'त' या वर्णांची पुनःरुक्ती आलेली आहे. त्यातून अनुप्रास अलंकार साध्य

झालेला आहे. आणि त्यामुळेहे सर्व उखाणे कर्णमधूर झालेले आहेत.

४) रसचर्चा :

प्रश्नोत्ररुपी उखाण्यात सर्वसाधारणपणे रुपक योजना असल्याने काहीसा गूढभाव निर्माण होतो. श्रोत्याची जिज्ञासा ताणली जाते. बुद्धीगम्य आनंद मिळतो. उत्तराच्या पूर्तीनंतर आल्हाद मिळतो.

गुडघा ऐवढो हत्ती, तिनोसुपडा येवढो कान
जिक मारु उखानू का उपाडूतारो कान.

या उखाण्यातून जिज्ञासा ताणली जाते.

घरी धोपटी दीधो उपटी

या उखाण्याचे उत्तर मिळाल्यावर बिभत्स रस निर्माण होतो. ' चायी चोयी निट कर' या उखाण्यातूनहास्य रसाची निर्मितीहोते. नांव घेण्याच्या उखाण्यांमध्येश्रृंगाररस केंद्रस्थानी असतो.

१) उभी होती माडीवर, ऊन पडी साडीवर
तथई आये जोरी मोती घे पोरी

५) रंजकता : प्रश्नस्वरुप उखाणे रंजकतेच्या दृष्टिनेसमृद्ध आहेत. त्याची काही उखाणे पुढीलप्रमाणे-

उदा. १) लाल पालखी, हिरवो दांडो,
तिनमा बसे बोडके रांडो.

२) धव्वी बाटली, तिन हिरवू बुच्चन

आभाळाला खांब नसतो, घोड्याला शिंग नसते, नदीला झाकण नराते, तळहाताला केस नसतो, जिभेला हाड नसते. हीसाधीच पण आश्चर्यकारक उत्तरे ऐकूनश्रोत्यांचे रंजनहोते. रुपक उलगडण्याच्या बुद्धीजन्य प्रयत्नातूनहीश्रोत्यांचे रंजन साध्य केले जाते. नांव घेण्याची उखाणेहीतरप्रामुख्याने रंजनासाठीच घेतली जातात. सलज्य भावनेने भांबावलेले नवपरिणीत वधूवर जेव्हा पहिल्यांदाच एकमेकांचे नाव उच्चारतात तेव्हा सर्वांचेच रंजनहोते. म्हणून उखाणेहा लोकवाङ्मय प्रकार रंजनप्रधान प्रकारठरतो.

निष्कर्ष :

१) उखाण्यांना मोठी परंपरा लाभलेली दिसते. प्राचीनसंस्कृत भाषेपासून उखाण्यांचीसमृद्ध परंपरा चालत आलेली दिसते. हे उखाणे मौखिक परंपरेने आलेले दिसतात.

२) उखाण्यात बुद्धीकौशल्य, रंजन, चातुर्य, पांडित्य दर्शन, निपुणता, बहुश्रुतता वगैरे वैशिष्ट्ये आढळतात.

३) उखाण्यातून सण, उत्सव, परंपरा, चालिरीती, संकेत, श्रद्धा रुढीचे प्रतिबिंब पडलेले आढळून येते.

४) उखाण्यांना लोकजीवनात भावनिक महत्व आहे. काही विधी तर उखाण्यांशिवाय पूर्णचहोत नाही. विशेषत: लग्नात तर या नाव घेण्याच्या उखाण्यांना विशेष महत्व आहे.

५) गुर्जरी बोलीतील उखाण्यांचा आशय साधा सरळ व सोपा असला तरी त्यात प्रतीक व रुपक योजना आढळते. प्रत्येक उखाण्यांत प्रतीक आहे. रुपके ही दररोजच्या व्यवहारातील आहेत.

६) गुर्जरी बोलीतील उखाण्यांतून व्यक्तीची तर्कबुद्धी, व्यवहारज्ञान, बुद्धीकौशल्य, स्मरणशक्ती, चतःरस्त्रपणा, आकलन क्षमतातपासली जाते.

७) गुर्जरी बोलीतील उखाण्यात अलंकारांचा व रसांचासहज वापर केलेला दिसतो.

अशाप्रकारे गुर्जर बोलीतील उखाण्यांचा शास्त्रीय अभ्यास करुन निश्चित स्वरुपाच निष्कर्ष मांडता येतात.

संदर्भ :-

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मराठी विभागप्रमुख,
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खान्देशात अहिराणी ही बोली बोलली जात असल्यामुळे या भागाला 'अहिराण पट्टी' असेही म्हटले जाते. 'अहिराणी' म्हणजे 'आभीर वाणी'. प्राचिन आभीर लोकसमूहाची भाषा म्हणजे अहिराणी. आभीर म्हणजे अहिर आणि अहिर म्हणजे गवळी होय. म्हणून खान्देशात पूर्वी गवळी राजाची वा कानडांची सत्ता होती असे म्हटले जाते.

अहिराणी बोली म्हणजे अभीर लोकांची बोली होय. अभीरांची बोली अभिराणी. अभीरचा अपभ्रंश अहिर आणि या अभिराणीचा अपभ्रंश अहिराणी असा आहे. भारतीय संस्कृती कोशामध्ये अहिर ही एक जात () आहे. हे अहिर लोक बिहार, ओडीसा, उत्तरप्रदेश, राजस्थान, पंजाब, सौराष्ट्र व खान्देशच्या परिसरात पसरलेले आहेत. अहिर हा अभीर या संस्कृत शब्दाचा अपभ्रंश आहे.

१९ व्या शतकामधील प्रसिद्ध संस्कृत पंडीत कवी श्री. तारानाथ तर्क वाचस्पती यांनी 'वाचस्पत्यम्' हा ग्रंथ लिहिला. त्यात त्यांनी अभीर या शब्दाची 'अभि + ईर' अशी व्युत्पत्ती सांगितलेली आहे. 'आभीर म्हणजे निर्भय, नीडर, जो भीरु नाही तो'. असा अर्थ सांगितलेला आहे. आभीर या शब्दाचा अर्थ निडर, निर्भय, अभीरु म्हणजे न घाबरणारा असा होतो. यावरुन अभिर लोकांच्या गुणवैशिष्ट्यांवर प्रकाश पडतो. आभिर लोक हे गुरे पाळतात, त्यासाठी त्यांना जंगल व वनांमध्ये भटकावे लागते. गाई – गुरांना घेऊन अभीर लोक हे दक्षिणेकडे तामिळनाडू, पूर्वेला बंगाल पर्यंत पोहोचलेले दिसतात. यावरुन अभीर लोक हे निर्भयतेने स्थलांतर करतात असे आपल्याला म्हणता येते.

श्री. एल. ब्राईट यांच्या मते 'आभीर' हे 'इभरी' या हिब्रू शब्दाचे रुप आहे, तर श्री. व्ही. कनकसभाई आपल्या 'दि तामिल्स एटीन हंड्रेड इयर्स अॅगो' या ग्रंथात 'आभीर' हा शब्द 'आयर' या तमिळ भाषेतील शब्दाचे संस्कृत रुप आहे असे म्हणतात. थोडक्यात 'आभिर' हे एका मोठ्या व शक्तीशाली अशा लोकांच्या समूहाच्या वंशाचे नाव आहे. महाभारत काळापासून ते गुप्तकाळापर्यंत त्यांना आभीर म्हणूनच ओळखले जात होते. इ. स. १५० मध्ये भारतामध्ये आलेला प्रख्यात भूगोल तज्ज्ञ टॉलेमीनेही त्याच्या देशाला 'अबिरिया' असे म्हटले आहे. 'आभीरांचा देश तो अबिरिया'.

'आभीर' लोकसमूहाची ओळख इ. स. ५ वे शतक म्हणजेच गुप्तकाळापर्यंत 'आभीर' या शब्दानेच होत होती. पण साधारण इ. स. ५५० मध्ये अमरसिंहाने 'अमरकोश' तयार केला. यात त्याने 'आभीर – अहिर – गवळी' असा अर्थ दिला. म्हणजेच 'आभीर' या मुळ शब्दाचे प्राकृतातील वा लोकभाषेतील 'अहिर' हे रुप अमरसिंहाच्या काळात रुढ झालेले दिसते. यापुढे 'आभिर' या शब्दाऐवजी 'अहिर' हा शब्द प्रचारात येऊन पुढे रुढही झाला व त्यापुढे आणखी 'अहिर' म्हणजेच 'गवळी' हा अर्थही 'अहिर' या शब्दाचा रुढ झालेला दिसतो.

आभीर लोकांचा मुख्य व्यवसाय गोपालन हा होता. गोपालन करण्यासाठी या लोकांना नेहमी भटकंती करावी लागायची. हे आभीर लोक आर्य लोकांप्रमाणेच अफगाणिस्थानमार्गे भारतात आले व भारतात आल्यानंतर या आभीर लोकांनी पंजाबमध्ये आपली पहिली वसाहत वसविली.

पतंजलीने आपल्या 'शुद्राभिरम्' या ग्रंथामध्ये आभिरांचा उल्लेख केलेला आहे. पतंजलीचा कालखंड हा इ. स. पूर्व दुसरे शतक असा मानला जातो. म्हणजेच आभीर हा लोकसमूह इ. स. दुस–या शतकापासून भारतात वास्तव्यास होता असे दिसते.

आभीर लोकसमूह हा आशियाकडून सिंधू नदीच्या खो–यामधून भारतात आला. आभीर लोकांना आर्य म्हटले जाते. इ. स. पूर्व पहिल्या अथवा दुस–या शतकात आभिर लोकांचे भारतात आगमन झाले असावे. अहिरांना बंगालमध्ये 'गोआला' व मध्यप्रदेशात 'गवळी' किंवा 'ग्वाला' असे म्हणतात. अहिर हे कृष्णभक्त आहेत.

आभीर लोक प्राचिन काळापासून खान्देशात वास्तव्य करीत असल्याचे पुरावे शिलालेख व प्राचिन ग्रंथामध्ये सापडतात. रामायण, महाभारतासारख्या ग्रंथामध्येही या आभीर लोकांचे उल्लेख आलेले दिसतात. या आभीर लोकांवर आर्य लोकांच्या जीवनपद्धतीचा प्रभाव पडलेला दिसतो. खान्देशात आजही अहिरे शिंपी, अहिरे ब्राह्मण, अहिर सोनार अशा जाती व अहिरराव, अहिरे अशी आडनावे दिसतात. प्राचिन काळी हे सर्व अभीर होते. अभीर हे मुळचे भारतीय की भारताबाहेरुन आले याबद्दल इतिहास संशोधकात व अभ्यासकात एकवाक्यता दिसत नाही. आभीर लोक हे भारताबाहेरुन आलेले आहेत असे मत प्रसिद्ध इतिहास संशोधक वेबर, टॉलेमी व डॉ. भांडारकर मांडतात तर अभीर हे भारतीयच आहेत असे मत डॉ. श्रीधर व्यं. केतकर, डॉ. सरकार, डॉ. सिद्धेश्वर शास्त्री मांडतांना दिसतात.

अभीर ही एक प्राचिन भारतीय जमात असून या अभीरांचा तपशीलवार, तर्कशुद्ध व सुसंगत असा इतिहास आजतरी उपलब्ध नाही. अभीर, अहिर लोकांचा प्रदेश खान्देश हा होय. या विषयी डॉ. श्रीधर व्यं. केतकर यांनी आपल्या 'महाराष्ट्रीय ज्ञानकोश' या ग्रंथाच्या खंड ७

व ८ मध्ये माहिती दिलेली आहे. डब्ल्यू क्रूक (१९७४) यांनी आपल्या 'दि कास्ट अॅण्ड ट्राईबज् ऑफ नॉर्थ वेस्टर्न इंडिया' च्या खंड १ ला व आर. इ. इन्थोव्हेन यांनी आपल्या 'दि ट्राईबज अॅण्ड कॉस्ट ऑफ बॉम्बे' या ग्रंथांच्या खंड १ मध्ये व डॉ. भांडारकरांच्या 'दक्षिणेचा इतिहास' या ग्रंथात अहिर, अभीर लोकांविषयी माहिती आलेली आढळते.

अभीर, अहिर हे आर्य की अनार्य याविषयी विद्वान संशोधकात एकमत आढळत नाही. रामायण, महाभारत आणि पुराणांमध्ये अभिर लोकांचा उल्लेख येतो. महाभारत व पुराणात या अभिर लोकांना शुद्र म्हटलेले आहे. महाभारतात सभापर्व अध्याय ३२, श्लोक १० मध्ये अभिर लोकांचा उल्लेख येतो. याचबरोबर बृहतसंहितेमध्ये दक्षिणभागाकडील एक देश म्हणून या भागाचा उल्लेख आलेला दिसतो.

श्री. भा. रं. कुलकर्णी हे अहिर संस्कृती ही आर्यसंस्कृती असल्याचे सांगतात तर श्री. दा. गो. बोरसे हे अहिरांना चंद्रवंशीय यादव आर्य आहेत असे म्हणतात. श्री. चांदोरकर हे अहिरांना यदुवंशीय म्हणतात व अहिर लोकांचे शुद्ध अहिर व शुह अहिर असे दोन भेद करतात. थोडक्यात श्री. चांदोरकर, श्री. भा. रं. कुलकर्णी यांना अहिर, अभिर लोक हे आर्यवंशीय वाटतात. जॉर्ज ग्रिर्असनने 'अभिर हे भिल्ल आहेत' असे मत मांडलेले आहे. भिल्ल हे अभिरापासून आलेले आहेत असे ते म्हणतात. मनुस्मृतीकार मनुने अभीर हे 'अंबुष्ट स्त्री व ब्राह्मण' यांची संतती होय असे मत व्यक्त करुन अभिरांची गणणा शुद्रांमध्ये केलेली दिसते.

खान्देशमध्ये अभिरांचे वास्तव्य होते व कालांतराने या अभिर लोकांचे नाव अहिर झालेले दिसते. या अभिर लोकांची बोली ही 'आभिरी' वा 'अहिराणी' होय. अहिराणी ही एक स्वतंत्र बोली आहे. या अहिराणी बोलीवर गुजरात राज्यातील गुजराथी या भाषेचा मोठा प्रभाव व परिणाम झालेला आहे. अहिराणी या बोलीवर गुजराथी या भाषेबरोबरच मराठी, नेमाडी, व हिंदुस्थानी या भाषांचा मोठा प्रभाव झालेला दिसतो. अहिराणी बोली बोलणारे अभिर लोक हे स्थलांतर करुन खान्देशमध्ये स्थिरावलेले आहेत. हे लोक पंजाबमार्गे स्थायिक झालेले आहेत. या स्थलांतरामुळे त्या - त्या ठिकाणच्या प्रादेशिक भाषेचा, बोलींचा परिणाम, प्रभाव व भाषेचे मिश्रण या लोकांच्या बोलीत झालेले आढळते. या अहिराणी बोलीवर या भाषांप्रमाणेच मागधी, अर्धमागधी, शौरसेनी, लाटी, महाराष्ट्री, पैशाची अशा प्राकृत भाषांचाही परिणाम झालेला दिसतो. खान्देशचा भौगोलिकलेच्या संदर्भात विचार केला तर या अहिराणी बोलीवर शेजारील विदर्भाकडील वैदर्भी, मध्यप्रदेशाकडील हिंदी, महाराष्ट्रातील मराठी, गुजरात राज्यातील गुजराती तसेच खान्देशमधील भिल्ल लोकसमुदायाच्या 'भिल्ली' या बोलीभाषेचा संकर व मोठा प्रभाव झालेला आढळून येतो.

अहिराणी ही अभिर वा अहिर लोकांची बोली म्हणून ओळखली जाते. खान्देशवर अभिर लोकांचे राज्य होते. खान्देशातील लोकांचा मुख्य व्यवसाय शेती हा आहे. खान्देशात जळगांव, धुळे व नंदुरबार या तीन जिल्ह्यांचा समावेश होतो. पण अहिराणी बोलीसंदर्भात मात्र या तीन जिल्ह्यांबरोबर औरंगाबाद जिल्ह्यामधील सोयगांव, कन्नडचा भाग व नाशिक जिल्ह्यामधील मालेगांव, कळवण, सटाणा तालुक्यातील काही गावांचा समावेश होतो. खान्देशी बोली ही प्रामुख्याने सातपुड्याचा पर्वत, अजिंठ्याचा डोंगर, सह्याद्री पर्वताच्या रांगा, वाघूर नदी व चांदवड यांच्यामध्ये बोलली जाते. खान्देशात या खान्देशी बोलीबरोबरच इतर अनेक स्थानपरत्त्वे भिन्न-भिन्न बोली बोलल्या जातात.

अहिराणी बोली - अहिर हे लोक / जात - जातीची बोली
अहिर जातीची बोली

खान्देशी बोली - खान्देश हे प्रदेश वाचक नाव / नाम
खान्देश - भू भाग - व्यापक / परिसर मोठा त्यामुळे इतर अनेक बोली, प्रदेशपरत्त्वे इतर बोली समाविष्ठ आहेत.

धुळ्याकडील तळोदे, शहादे या भागामध्ये गुजर या जातीच्या लोकांचे प्राबल्य मोठे असल्याने तिकडे 'गुर्जरी बोली' बोलली जाते. नंदुरबार जिल्ह्यात नवापुर, धडगांव, अक्कलकूवा वगैरे भागात पावरा या वन्य जातीच्या लोकांची संख्या ही मोठ्या प्रमाणावर आढळून येते. त्यामुळे या भागात ही लोक आपल्या 'पावरी' या बोलीतून आपला संपर्कव्यवहार करीत असतात. शहादा, नवापुर हे तालुके गुजरात राज्याजवळ असल्याने व या ठिकाणच्या लोकांचा संपर्कव्यवहार हा गुजरात राज्याशी मोठ्या प्रमाणावर असल्याने या लोकांच्या बोलीवर 'गुजराथी' या भाषेचा मोठा प्रभाव पडलेला दिसून येतो. नाशिक जिल्ह्यामधील चांदोर डोंगराच्या उत्तरेकडील भागाला 'अहिराणपट्टी' असे म्हटले जाते. या भागामध्ये खान्देशी ही बोली बोलली जाते. या भागात सटाणा, कळवण, चांदवड, मनमाड, नांदगांव, मालेगांव या तालुक्यातील खान्देशला लागून असणा-या बहुसंख्य गावांचा समावेश होतो. औरंगाबाद जिल्ह्यामधील सोयगांव तसेच अजिंठ्याच्या डोंगरांना लागून असलेला कन्नड तालुक्यामधील बरेचसे लोक खान्देशी बोली बोलत असतात. सातपुड्याच्या पर्वत रांगेत येणारे पण मध्यप्रदेशात मोडणारे शहापुर, इच्छापुर, वलवाडी, ब-हाणपुर या भागातही मुख्यत्त्वे करुन खान्देशी बोली बोलली जाते. खान्देशातील बोली ही खान्देशी बोली म्हणून ओळखली जात असली तरी या खान्देशातील बोलीवर ती भाषा बोलणा-या विविध जाती - जमाती व शेजारील प्रदेशात बोलल्या जाणा-या इतर बोली वा भाषांचा प्रभाव व परिणाम झालेला आढळतो. या प्रभावातूनच मुळ भाषेपेक्षा भिन्न अशी दुसरी स्वतंत्र बोली आकाराला आलेली दिसते. नंदुरबारच्या परिसरात नंदुरबारी, गुर्जर समाजाची वा जातीची गुर्जरी बोली, धेडगुर्जरी बोली, बागलाणच्या

परिसरात बागलाणी, महार जातीची महाराऊ, लेवापाटीदार समाजाची लेवापाटीदारी, लाडसिक्की समाजाची लाडशिक्की अशा बोली आकाराला आलेल्या दिसतात. भौगोलिक वा प्रादेशिक रचनेनुसार खाल्यांगी (पूर्व), वरल्यांगी (पश्चिम), तप्तांगी (उत्तर दिशा), डोंगरांगी (दक्षिण) अशा नवीन बोली आकाराला आलेल्या दिसतात.

खान्देशी आणि अहिराणी हे शब्द साधारणत: एकाच अर्थाने वापरले जातात, पण त्यामध्ये सुक्ष्म भेद आहे. खान्देशी हा शब्द प्रदेशवाचक शब्द आहे. खान्देशी बोली ही संकल्पना अहिराणी बोलीपेक्षा व्यापक संकल्पना आहे. अहिराणी बोली ही संकल्पना जात वा बोलीवाचक संकल्पना आहे. अहिर लोकसमूदाय हा बाहेरुन आलेला लोकसमूदाय आहे व या लोकांनी खान्देशावर आपली सत्ता प्रस्थापित केल्यामुळे साहजिकच त्यांची बोली ही इतर बोलीपेक्षा लवकर प्रतिष्ठित पावली. अहिर लोकांकडे सत्ता असल्यामुळे त्यांनी खान्देशातील काळ्या कसदार जमिनीवर शेती करायला सुरवात केली. इतर जातीतील लोकांनी अहिरांच्या संपर्कामध्ये राहून त्यांची बोली, भाषा, संस्कृती, रुढी इ. अनेक गोष्टींचे अनुकरण केले. या अनुकरणामुळे या लोकांच्या मुळ बोलीवर अहिराणीचा मोठा प्रभाव पडला. वेगवेगळ्या जाती – जमातीतील लोकांनी या भौगोलिक परिसरातील आपल्या बोलीला अहिराणी असे नाव न म्हणता 'खान्देशी' हे प्रदेशवाचक वा प्रादेशिक नाव स्विकारले. खान्देशची बोली ती खान्देशी. अहिराणी बोली या संकल्पनेपेक्षा खान्देशी बोली ही संकल्पना व्यापक, मोठी व सर्वसमावेशक आहे.

खान्देशी बोलीचे क्षेत्र व प्रकार

डॉ. रमेश सुर्यवंशी यांची खान्देशातील खान्देशी या बोलीचा प्रदेश व सामाजिक अंगांनी अभ्यास करुन दोन प्रकारे वर्गीकरण केलेले आहे.

खान्देशी – प्रादेशिक प्रभेद	**सामाजिक प्र–भेद**
बागलाणी (नैऋत्य)	अहिराणी
तप्तांगी (उत्तर)	लेवापाटीदार
डोंगरांगी (दक्षिण)	गुजरी
वरल्ह्यांगी (पश्चिम)	लाडसिक्की
खालल्यांगी (पूर्वदिशा)	पावरी
नंदुरबारी (नंदुरबार परिसर)	काटोनी
	तडवी
	परदेशी
	घाटोळी
	दखनी
	महाराऊ

खान्देशातील सर्वच जाती – जमातीची लोक दैनंदिन व्यवहारासाठी अहिराणी बोलीचा वापर करीत असतात. खान्देशात अनेक जाती – जमातीची लोक गुण्या – गोविंदाने राहत असतात व आपला पारंपारिक व्यवसाय करीत असतात. प्रत्येक जातीची स्वत:ची अशी खास बोली आहे. या स्वत:च्या जातीच्या बोलीवर परिसरातील अहिराणी या बोलीचा मोठा प्रभाव पडलेला आढळतो. या खान्देशी बोलीच्या कक्षेत अनेक जाती – जमातीनुरुप लोक व त्यांची बोली येते. या बोलीच्या कक्षेत येणा–या बोली म्हणजे – लेवापाटीदार जातीची लेवापाटीदारी बोली, गुर्जर जातीची गुर्जरी बोली, लाडशाखीय वाणी लोकांची लाडशिक्की, पावरा जमातीची पावरी बोली, अहिर लोकांची अहिराणी, परदेशी लोकांची परदेशी, महार लोकांची महाराऊ बोली इ. होय.

तापीनदीच्या परिसरामध्ये बोलल्या जाणा–या खान्देशी बोलीची विशिष्ट अशी हेल वा ढब आढळून येते. या ठिकाणच्या बोलीत ज्, च्, झ्, श्, छ् या व्यंजनांचे उच्चारण पार्श्व आहे. अशा स्वतंत्र वैशिष्ट्यामुळे ही बोली खास खान्देशी बोली म्हणून ओळखली जाते. अजिंठ्याच्या डोंगररांगांच्या भू – प्रदेशावर खान्देशी बोली बोलणारे लोक च्, ज्, छ्, झ् या व्यंजनांचे उच्चारण दन्त्य करतात. अशा रीतीने खान्देशी या बोलीत भू – प्रदेश, जाती – जातीनुसार वेगवेगळे फरक वा बदल होतांना आढळतात.

खान्देशातील लोकांची अहिराणी ही बोलीभाषा आहे. या अहिराणीचे भाषिक क्षेत्र खान्देश हे होय. खान्देशमध्ये वेगवेगळ्या जाती – जमातीची लोक वास्तव्य करुन राहतात. ब–याच जातीच्या स्वतंत्र अशा खास बोली अस्तित्त्वात आहेत. या जातीतील लोक इतरांशी बोलतांना अहिराणी बोलीतून बोलतात. पण यांची अहिराणी बोली ही भिन्न स्वरूपाची दिसते. अहिराणी बोलीचे स्थान, जात, व्यक्तीपरत्वे काही भेद पडलेले आहेत. यांनाच अहिराणीच्या उपबोली म्हणता येईल. त्याचे वर्गीकरण पुढीलप्रमाणे करता येते.

अहिराणी बोलीच्या उपबोली

उपबोली	प्रभावक्षेत्र
मध्यवर्ती अहिराणी	धुळे, अमळनेर, शिंदखेडा
बागलाणी बोली	कळवण, नांदगाव, सटाणा, बागलाण, निफाड, चांदवड
लाडशिक्की बोली	खान्देश परीसरातील लाडशाखीय वाणी समाज बांधव
नंदुरबारी बोली	नंदुरबार, तळोदा, शहादा, दोंडाईचा, नवापुर, अक्कलकुवा
डांगी बोली	डांगचा परीसर
नेमाडी बोली	चारण वंजारी समाज बांधव
पावरी बोली / भिल्ली बोली	आदिवासी व भिल्ल समाजबांधव
भावसारी रंगारी बोली	खान्देशातील भावसार समाजबांधव
लेवापाटीदारी बोली	यावल, भुसावळ, सावदा, फैजपुर, जळगांव, मुक्ताईनगर
गुर्जरी बोली	शहादा, नंदुरबार, शिरपुर, चोपडा परिसरातील गुजर समाजबांधव
तडवी बोली	तडवी समाजबांधव

निष्कर्ष

१. अहिराणी म्हणजे अभीर लोकांची बोली होय. अभिरांची बोली 'अभिराणी' अभीरचा अपभ्रंश 'अहिर' आणि अभिराणीचा अपभ्रंश 'अहिराणी' असा झालेला दिसतो. हे अहिर लोक या प्रदेशात बिहार, ओडिसा, उत्तरप्रदेश, राजस्थान, पंजाब, सौराष्ट्र या भागातून प्राचिन काळी स्थलांतर करुन आलेले दिसतात.

२. अभिर लोक हे स्थलांतर करुन आल्यामुळे त्यांचा अनेक भू-प्रदेशांशी प्रत्यक्ष संबंध आलेला दिसतो. त्यामुळे या अहिराणी बोलीवर मागधी, अर्धमागधी, शौरसेनी, लाटी, महाराष्ट्री, पैशाची अशा प्राकृत भाषांचा परिणाम व प्रभाव झालेला दिसतो. याचबरोबर खान्देशला जवळचे असणारे गुजरात राज्य व तेथील लोकांची भाषा गुजराथीचाही मोठा प्रभाव अहिराणी बोलीवर दिसतो. मराठी, नेमाडी व हिंदुस्थानी भाषांचाही मोठा प्रभाव व परिणाम अहिराणीवर जाणवतो.

३. खान्देशी आणि आहिराणी हे शब्द ब-याचदा एकाच अर्थाने वापरले जातात पण त्यांच्यामध्ये सूक्ष्म भेद आहे. खान्देशी हा शब्द प्रदेशवाचक शब्द आहे व खान्देशी बोली ही संकल्पना अहिराणी बोलीपेक्षा व्यापक संकल्पना आहे. अहिराणी बोली ही संकल्पना जात वा बोलीवाचक संकल्पना आहे.

४. खान्देश हा अठरापगड जाती - धर्मांनी वसलेला समृद्ध व शांत प्रदेश आहे. या खान्देशातील बोलीवर ती भाषा बोलणा-या विविध जाती - जमाती व शेजारील प्रदेशात बोलल्या जाणा-या इतर बोली - भाषांचा प्रभाव व परिणाम झालेला आढळतो. या प्रभावातूनच मुळ भाषेपेक्षा भिन्न अशी दुसरी स्वतंत्र बोली आकाराला आलेली दिसते. नंदुरबारच्या परिसरात नंदुरबारी, गुर्जर समाजाची वा जातीची गुर्जरी बोली, बागलाणच्या परिसरात बागलाणी बोली, महार जातीची महाराऊ, लेवापाटीदार समाजाची लेवापाटीदारी, लाडसिक्की समाजाची लाडशिक्की अशा बोली आकाराला आलेल्या दिसतात.

५. खान्देशच्या भौगोलिक व प्रादेशिक रचनेनुसारही काही बोली आकाराला आलेल्या दिसतात. त्यात खाल्यांगी (पूर्व), वरल्यांगी (पश्चिम), तप्तांगी (उत्तरदिशा), डोंगरांगी (दक्षिण) अशा विस्तीर्ण प्रादेशिक भू - भागासंदर्भात बोली आकाराला आलेल्या दिसतात.

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१) राजवाडे संशोधन मंडळ ग्रंथमाला - ऐतिहासिक लेखचर्चा भाग ४ था, ग्रंथ २६ वा, स. न. १९५४ - खान्देश या नावाचे मुळ लेखक - मो. रा. जोशी
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Tagore's Sublime Views on Life in Poetry

Abstract: Rabindranath Tagore, a universally acknowledged poet, loved life and he found it worth celebrating. Though he is considered as a mystic-philosopher, he is basically a poet who passionately adores life with all its beauty and oddity. He accepts life as a gift of divine and for him divine is not separated from the life and nature. Rather he reflects the philosophy of Upanishads which relates that Brahma reflects in the forms of living and non-living in the world. Love for life is a motif of his poetry.

Key Words: Celebration, Death, Galvanizing, Life, Sublimity

Rabindranath Tagore is primarily well known among non-Bengali readers for his devotional poems in Gitanjali. Rather his other poetic anthologies such as Crossing, Lover's Gift, Poems, Child, Fruit Gathering, The Crescent Moon and Gardener are equally great. The anthologies sometimes surpass Gitanjali so far as variety, melody, diction and liveliness are concerned. These anthologies display poet's passion for life. The poet loves one and all without condition and without restriction. His love for life, his bonding with the fellow beings, his deep insight into the world of child and his empathy of lovers get reflected in these anthologies. Even Gitanjali, though popular for mystic and devotional element, consists some of poems displaying his urge for life.

Rabindranath Tagore's views towards life are completely fresh and anew. He passionately and intensely met the life. He was not a poet sitting in ivory tower and

writing of the fantasy. In fact, he lived his life fully and undergone through all the unavoidable circumstances. He encountered joys and grieves. He rejoiced births and endured the grief of deaths of near and dear ones. He lived in the metro of that time, Calcutta among the elites and lived on the bank of Padma among the peasants. He travelled enormously in the country and abroad. He saw the various hues and shades of life. Direct contact with fellow beings and nature; and the influence of folk songs like Baul songs deepened his understanding of life. His curiosity of life was never diminished. Asit Bandyopadhyay while commenting on Tagore's Prabhatsangit writes about him in an essay Rabindranath Tagore: Poet and Dramatist: "The poet no longer wanted to lead a lonely and desolate life in the languor of the evening; he wanted to meet the world and embrace it with love and joy". Prabhatsangit is one of the earlier poetic anthologies of the poet written I Bengali. In it, the poet says: "I do not know how my heart opened up today/To greet the entire world in a loving embrace".

His affinity with the world finds expression in his poems. In Chabi O Gan he paints the pictures of joys and sorrows of daily life. In, Kadi O Komal he deifies the earth and celebrates the earthy existence: "I do not want to die in this beautiful world/ I want to live in the midst of men".

In his later life when he had gone to Lake Balaton in Hungary for the rest after an illness, he was invited to plant a tree in the neighboring garden. He planted a tree and wrote a few lines:

When I am no longer

On this earth,

My tree will let the ever renewed

Leaves of thy spring

Murmur to the wayfarer-

The poet did love while he lived…

Tagore's affection for life is pure, genuine and intense. His commitment to life is unparalleled. For him, life is carnival. In Poem 16 in Gitanjali, Tagore describes how he lived and loved the life:

I have had my invitation to this world's

Festival, and thus my life has been blessed.

My eyes have seen and my ears have heard.

It was part at this feast to play upon my instrument,

And I have done all I could.

The poet realizes that the life which came to his share is absolutely lovely. Life is beautiful in itself and moreover his love makes the life more beautiful. Consequently he sings in Gitanjali:

WHEN I go from hence let this be my parting word, that what I have seen is unsurpassable.

I have tasted of the hidden honey of this lotus that expands on the ocean of light, and thus am I blessed – let this be my parting word.

In this playhouse of infinite forms I have had my play and here have I caught sight of him that is formless. (Poem No .96)

Rabindranath Tagore is very fortunate since the good and kind people came into his life. He acquired knowledge from them, he acquired wisdom from them, and he shared the sense and sensibility with them. Moreover he received an appreciative attitude from them. In a small poem he says:

Thy gift of the earliest flower came

to me this morning, and came

the faint tuning of thy light.

I am a bee that has wallowed in the

heart of thy golden dawn,

My wings are radiant with its pollen.

I have found my place in the feast of

songs in thy April, and I am freed

of my fetters like the morning of

its mist in a mere play. (Poem 41 Crossing)

Rabindranath was a staunch pioneer of freedom. He believed that a person could live his life copiously and spontaneously only when he is free from all sort of bondages. He solicits for the freedom in the poem 42 in Crossing:

Free me as free are the birds of the

wilds, the wanderers of unseen

paths.

Free me as free are the deluge of rain,

and as the storm that shakes its

locks and rushes on to its un-

known end.

Free me as free is the forest fire, as is

the thunder that laughs aloud

and hurls defiance to darkness.

Rabindranath reveals the secret how he loved the life. He lived the life passionately and experienced its essence with his senses. In another poem, he says that total acceptance of life makes one to accept the death as the death itself is an offshoot of the life itself:

I have kissed this world with my eyes and my limbs;

I have wrapt it within my heart in numberless folds;

I have flooded its days and nights with thoughts till the world

And my life have grown one,--

And I love my life because I love the light

of the sky so enwoven with me.

If to leave this world be as real as to love it—

Then there must be a meaning in the meeting

and the parting of life.

If that love were deceived in death,

Then the canker of this deceit would eat into all things,

And the stars would shrivel and grow black. (Poem 53 Fruit –Gathering).

In poem number 67 in Poems he rejoices the life:

I have seen, have heard, have lived;

In the depth of the known have felt

the truth that exceeds all knowledge

Which fills my heart with wonder and

I sing. (12-16)

Rabindranath had seen the deaths of his near ones and he had undergone through the agony of inevitable and everlasting agony. Even the sorrowful experiences made him to realize the beauty of life which is consequent result of shortness of the life. For him, life of a person may end but the human life is ever flowing:

NONE lives for ever, brother, and nothing lasts for long.

Keep that in mind and rejoice.

Our life is not the one old burden,

our path is not the one long journey.

One sole poet has not to sing one aged song.

The flower fades and dies; but he who wears the flower has not to mourn for it for ever.

Brother, keep that in mind and rejoice. (Poem No. 68 The Gardener)

The poet celebrates the life and loves it but at the cost of nothing. He finds that the life is not only worth loving and but worth worshipping also. In Poem 2 of The Gardener, he proclaims that he is a poet of life and he sings the songs of life. Nothing can attract him as the life itself. The world beyond has no charm for him as he finds the pleasure and grief, hope and disappointment worth celebrating:

"I am ever as young or as old as the youngest and the oldest of this

village." "Some have smiles, sweet and simple, and some a sly

twinkle in their eyes."

"Some have tears that well up in the day light, and others tears that

are hidden in the gloom."

"They all have need for me, and I have no time to brood

over the after-life." "I am of an age with each, what matter

if my hair turns grey?"

The poet does not mind of mourning but he does not wish to squander his life only in mourning:

"It is sweet to sit in a corner to muse and write in rhymes that

you are all my world.

It is heroic to hug one's sorrow and determine not to be

consoled.

But a fresh face peeps across my door and raises its eyes to my eyes.

I cannot but wipe away my tears and change the tune of my song.

For time is short." (17-25 Poem 46 The Gardener)

Rabindranath Tagore's love for the life does not come as a mere theme in his poetry, but it is the essence of his solicitous contemplation. As a leaf of a tree is green in and out, as a droplet of water is wet in and out, so is his love for life. He loved as he lived and he lived as he loved. Loving the life is his religion. In Poem 75 in The Gardener, he narrates a story an ascetic who renounces his home, family, wife and child. The ascetic calls them as delusion and discards all in exploration of God, while Tagore considers them as forms of the divine. On the ascetic's decision of leaving the home, the God sighs and protests. The poet's God does not wish the sacrifice of life on the part of devotee rather He wishes people to accept the life as a divine gift:

AT midnight the would-be ascetic announced:

"This is the time to give up my home and seek for God. Ah, who has held me so long in delusion here?"

God whispered, "I," but the ears of the man were stopped.

With a baby asleep at her breast lay his wife, peacefully sleeping on one side of the bed. The man said, "Who are ye that have fooled me so long?"

The voice said again, "They are God," but he
heard it not. The baby cried out in its dream,
nestling close to its mother.

God commanded, "Stop, fool, leave not thy home," but still he heard not.

God sighed and complained, "Why does my servant wander to seek me, forsaking me?"

The life has showed the poet how to live and the great souls he met during his life time endowed him the love and joy. The poet is grateful to them:

Blessings have I own in this life

of the Beautiful.

In the vessel of men's affection I taste

His own divine nectar.

Sorrow, hard to bear,

has shown me the unhurt,

unconquered soul.

On the day when felt death's

impending shadow,

fear's defeat has not been mine.

The great ones of the Earth

have not deprived me of their touch,

their undying words have stored

in my heart.

Grace I had from god of life;

this memory let me leave

in grateful words.(Poem No.120 Poems)

The poetry of Rabindranath has an inherited quality of sublimity. His love for life and his contemplation over it makes the readers enthralled. Moreover, his poetry shapes the views of the readers and makes them to love and cherish the life. The greatness of a poet lies in the transforming and galvanizing capacity of his poetry and it is certain that the poetry of Rabindranath retains this quality.

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शरद जोशी के साहित्य में शैक्षणिक व्यंग्य

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वर्तमान हिन्दी में 'व्यंग्य' शब्द को अंग्रेजी में 'सटायर' शब्द का पर्याय माना जाता है। साहित्य की अन्य विधा के समान 'व्यंग्य' विधा भी पाश्चात्य साहित्य की देन मानी जाती है। आज व्यंग्य साहित्यकार भी देशों और भाषाओं में महत्वपूर्ण स्थान है। 'सटायर' शब्द लैटिन शब्द 'satura' जिसका अर्थ 'गडबड' से विकसित हुआ है सैतुरा शब्द के दो रूप मिलते है। पुरातन काल में 'सैतुरा' शब्द 'परनिंदा' का अर्थ में प्रयुक्त होता था, इस ऐतिहासिक अर्थ की छाया वर्तमान 'सटायर' शब्द पर भी पडी है। सटायर में केवल परनिंदा नही होता है। कुछ बातों में हेराफेर भी माना जाता है।

हिन्दी भाषा एवं साहित्य में 'व्यंग्य' शब्द संस्कृत भाषा का शब्द है, जो 'वि' उपसर्ग एवं 'व्यत' प्रलय से 'अज्' धातु के योग से बनता है। व्यंग्य शब्द का प्रयोग शब्द शक्तियों में भी मिलता है।

भारतीय समाज विविधता से भरा हुआ है। जितनी विविधताएँ देखी जाती है, उतनी ही, समस्याएँ एवं असंगतियाँ भी दिखाई देती है। राजनीतिक, सामाजिक, सांस्कृतिक एवं धार्मिकता के साथ-साथ शिक्षा के क्षेत्र में बडे पैनामें विसंगतियाँ तथा असंगतियाँ भी देखने को मिलती है। शिक्षा का क्षेत्र भी समाज का एक अभिन्न अंग है। शिक्षा, व्यक्ति, समाज एवं राष्ट्र की प्रथम अनिवार्यता होती है। राष्ट्र को सुयोग्य शिक्षित नागरिक प्रदान करना ही शिक्षा का व्यापक उद्देश्य है। आजादी के संघर्ष काल में शांतिनिकेतन, काशी विश्वविद्यालय गुरूकुल, जामिया-मिलिया जैसी राष्ट्रप्रेमी संस्थानों की स्थापना की गई थी। समय के बदलने के साथ ही मानों उनकी मूल भावना विदा हो गई। स्वाधीन भारत के निर्माताओं ने शिक्षा पर ध्यान दिया। डॉ.राधाकृष्णन की अध्यक्षता में 'राधाकृष्णन आयोग' की स्थापना के साथ शिक्षा सुधार की दिशा में प्रयत्न प्रारंभ हुआ। अनेक कमीशन हमारे सामने आए किन्तु कोई ठोस निष्कर्ष प्राप्त नही हो सके।

विविध क्षेत्रों की विसंगतियों की तरह शिक्षा का क्षेत्र भी अध:पतन, अव्यवस्था, अनैतिकता और अराजकता आदि दुर्गुणों से अछूता न रह सका। शिक्षा जैसे पवित्र क्षेत्रों में भी कई प्रकार की विसंगतियाँ देखने को मिलती है। शरद जोशी ने शिक्षा क्षेत्रों में आयी विसंगतियों की सूक्ष्म जाँच-पडताल कर विसंगतियों पर तीव्र आघात किये है।

वर्तमान शिक्षा व्यवस्था में शिक्षा संस्थान की बाढ सी आ गई है। रोज नये-नये स्कूल तथा कॉलेज खुल रहे है, सरकार की ओर से आर्थिक सहायता न भी मिले, इन्हें कोई कठिनाई नहीं होती। संस्था को चलना एक उत्तम व्यवसाय (धंदा) हो गया है। उनका एकमात्र उद्देश्य है आर्थिक लाभ। अब संस्था विद्या का केंद्र न रहकर दुकान बन गए है। आजादी के बाद शिक्षा संस्थान की बाजारू प्रवृत्ति पर शरद जोशी ने व्यंग्य के बाण चलाये है। अपनी एक रचना 'उत्तम शिक्षा की व्यवस्था शीघ्र प्रवेश ले' में भारतीय शिक्षा व्यवस्था और संस्थानों की स्थिति पर प्रकाश डालते हुए लिखते है - "सभी सदस्य चाहते है कि उनके अपने मकान खडे हो और महाविद्यालय के भवन का काम लग जाये, सब को ईंट, लेबर की सुविधा हो जायें"[१]

शिक्षा संस्थान के व्यवस्थाओं पर तथा उनके स्वार्थी प्रवृत्ति पर जोशी ने कसकर व्यंग्य किये है। शिक्षा व्यवस्थापन के सदस्य शैक्षणिक संस्था के कार्य और प्रशासन में आवश्यकता से अधिक रूचि दिखाते है। वे जो चाहते हैं वही होता है। शरद जोशी ने शिक्षा संस्थान में काम करने वाले अध्यापकों की मजबूरी एवं विवशता को प्रस्तुत करते हुए लिखते है - "शिक्षा मंत्री की पुत्री का विवाह था। भूगोल के अध्यापक जमीन समतल करने में लगे थे। हिन्दी के अध्यापक सुंदर अक्षरों में निमंत्रण पत्र लिख रहे थे। गृहविज्ञान की अध्यापिका मिठाइयाँ बना रही थी। वाणिज्य के अध्यापक आलू-बैंगन खरीद कर ला रहे थे। इतिहास के प्रोफेसर शामियाना तनवाकर कालीन बिछा रहे थे। सभी व्यस्त थे।"[२]

शरद जोशी ने शिक्षा संस्थान को अपने फर्म की शाखा समझने वाले तथा कर्मचारियों को मुनीम समझनेवाले शिक्षा व्यवस्थापकों पर व्यंग्य किये है। एक ओर वर्तमान सरकार शिक्षा सुधार के लिए नये-नये कमीशन का गठन कर करोडो रूपये पानी में बह रही है लेकिन सरकार अभी तक शिक्षा की प्राथमिक आवश्यकता भी पुरी नहीं कर पायी है। निष्कर्ष रूप में कहा जा सकता है कि शरद जोशी ने शिक्षा संस्थान में चल रही हर छोटी बडी विसंगति का पर्दापाश कर उस पर तीव्र आघात किये है। शिक्षा संस्थान के भवन निर्माण,

नियुक्तियाँ, कर्मचारियों को अपनी निजी संपत्ति समझने वाले व्यवस्थापकों पर कुठाराघात किया है।

आज पाठ्यक्रमों की जटिलता और विषय विविधता के बाद भी मुख्य उद्देश्य मात्र उपाधि प्राप्त करना रह गया है। अगर नौकरीओं के लिए उपाधि की अनिवार्यता न होती तो सभी शिक्षा संस्थानों को ताले लग जाते। उपाधि प्राप्त करना ही जब लक्ष्य बन गया है, तो पढने की झंझट कौन करता। इसी असंगति पर व्यंग्य करते हुए जोशी लिखते है - "प्रथम श्रेणी प्राप्त करने के लिए अब किताबों का ढेर लगाना बेवकूफी है। परीक्षा के बाद जरा-सी भाग दौड करने से ही प्रथम श्रेणी प्राप्त होती है।"[३]

वर्तमान शिक्षा के गिरते स्वर, परीक्षानुमा पाठ्यक्रम आदि विसंगतियों को उजागर कर शिक्षा क्षेत्र का कच्चा चिठ्ठा जोशी ने प्रस्तुत किया है। जोशी लिखते है कि "दुनिया की सारी जरूरी बाते छात्रों के पाठ्यक्रम में ठूँस देनी चाहिए।"[४] विषयों का विस्तार और किताबी बोझ के साथ पाठ्यक्रमों की निस्सारता को भी शरद जोशी ने व्यंग्य का विषय बनाया है। वे अपनी एक रचना 'घास छीलने का पाठ्यक्रम' में प्रचलित पाठ्यक्रमों की व्यर्थता का उपहास करते है। आजादी के बाद हर क्षेत्र में आरक्षण नीति का बिगुल बज रहा है जोशी इस आरक्षण नीति पर व्यंग्य करते हुए लिखते है - "प्रतिष्ठित महाविद्यालयों में डोनेशन और रिश्वत के दम पर या तो रईस बच्चे प्रवेश पाते है या सरकारी निगमों के अंतर्गत कम नंबर लाने के बावजूद हरिजन छात्र। दोनों आजादी के बाद पनपी दो अनैतिक धाराओं के प्रतीक है।"[५]

शरद जोशी ने इस आरक्षण नीति का तीव्र शब्दों में आघात किया है। जो नीतियाँ बच्चों में लापरवाही और निराशा का जीवन जीने के लिए विवश करती है, उनके स्वप्नों को नष्ट-भ्रष्ठ करती है, ऐसी राजनीति से प्रेरित आरक्षण नीति पर जोशी ने व्यंग्य किये है।

वर्तमान शिक्षा प्रणाली में परीक्षा महज एक औपचारिकता बन गयी है। परीक्षा के पूर्व प्रश्नपत्र की खोज, परीक्षा में नकल, परीक्षा के बाद उत्तर पत्रिका की खोज इसी बात को लेकर शरद जोशी ने व्यंग्य किये है। वे लिखते है - "कुछ लडके आज भी ऐसे है जिनकी नानी मरती है कि हमारी पीढी के कुछ नमूने आज भी पाये जाते है। आज कई स्तर है, जिन से लडका पास होता था, हो सकता है। एक तो यह कि पढ लिया जाये। परीक्षा पास करने का यह पुराना स्टाइल है और कुछ प्रतिशत छात्र इसे भी आज अपनाते है।"[६]

शिक्षा प्रणाली में छात्रों के साथ-साथ अध्यापक भी महत्वपूर्ण अंग है। समाज अध्यापकों की ओर आदर्श की नजरों से देखता है। लेकिन वास्तविकता यह है कि समाज में अन्य अधिकारीयों की प्रतिष्ठा से अधिक प्रतिष्ठा अध्यापकों की होती है। आज अध्यापक, छात्र, प्राचार्य, व्यवस्था, प्रशासन आदि के चक्रव्यूह में घुटन महसूस कर रहा है। आज अध्यापकों की आर्थिक दशा अत्यंत शोचनीय है। महिनों वेतन न मिलना, ऊँची रकम पर दस्तखत कर कम तनखा देना, डोनेशन माँगना आदि के जाल में वह चक्कर खाता हुआ नजर आता है। एक ओर समाज और सरकार द्वारा शिक्षकों को सम्मान देने की बात की जाती है, दूसरी ओर उन्हें रोजी-रोटी की चिंता से मुक्त भी नही किया जाता। शिक्षक नाम ही आज निरीहता का पर्याय बन गया है। बेचारा मास्टर कहकर उसे संबोधित किया जाता है। जहाँ तक अध्यापक का ऐसा दयनीय चित्र है, वही दूसरी ओर वह योग्यता में ज्ञान में, अध्ययन में आलसी होता जा रहा है। शरद जोशी अध्यापक के अनुकूल तथा प्रतिकूल बर्ताव पर बडे तीखे शब्दों में व्यंग्य करते हुए लिखते है - "प्राध्यापक पढने-पढाने के बजाय गुटबाजी और पॉलिटिक्स में रूचि लेने लग गये है। छात्रों को भडकाकर स्ट्राईक लगाना, सस्ती लोकप्रियता हासिल करने के लिए नोटस लिख देना, परीक्षा में पूछे जाने वाले प्रश्न बताना, ट्युशन लेना, कक्षा में न पढाना आदि विकृतियाँ आज आम बातें है जो व्यंग्यकारों की कलम से छूटी नही है।"[७]

अध्यापकों की दयनीय स्थिति पर प्रकाश डालते हुए शरद जोशी एक रचना में लिखते है - "प्रोफेसर सहयोग नही करेगा, तो लडके हल्ला मचायेंगे। विधायक को पता लगेगा कि क्षेत्र के युवकों की शैक्षणिक प्रगति में अमुख प्रोफेसर पर्याप्त सहयोग नहीं कर रहा है तो वे शिक्षा मंत्री से कहकर उसका तबादला करवा देंगे। पेपर आऊट करवाना, नकल होने देना और मार्क्स बढवाना, ये सब प्राध्यापकों को आवश्यक दायित्व है।"८जोशी ने शिक्षा संस्थान में अध्यापकों की दयनीय स्थिति का चित्रण कर प्रचलित शिक्षा व्यवस्था पर प्रहार किया है।

आज की शिक्षा प्रणाली में गुरू-शिष्य परम्परा खंडित होती नजर आ रही है। एक काल ऐसा था कि गुरू को ईश्वर मानने की परम्परा भारत में थी परंतु आज की शिक्षा पध्दती में गुरू-शिष्य परम्परा का स्थान गौण हो गया है। आज 'गुरू देवो भव' के बजाय 'शिष्य देवो भव' की स्थिति उत्पन्न हो गयी है। अक्सर अध्यापकों का मजाक उडाना, उनकी नकल करना, उनका अनादर करना जैसी विकृतियाँ तेजी से बढ गयी है। दूसरी तरफ अध्यापक भी अपनी कार्य शैली, आचरण के कारण छात्रों की आलोचना का विषय बन गये है। ज्ञान का अभाव, आचरण और स्वार्थ ने अध्यापक की गुरू-

महिमा को घटाया है। शरद जोशी अध्यापक के आचरण की सूक्ष्म जाँच पडताल कर उस पर प्रहार करते है।

शिक्षा क्षेत्र में फैली असंगतियों में अनुसंधान के गिरते स्तर को भी शरद जोशी ने व्यंग्य का विषय बनाया है और चिंता प्रकट की है। एक ओर महाविद्यालय, विश्वविद्यालय की नौकरियों में अनुसंधान डिग्री आवश्यक मानी गयी। जिसके कारण शोध कार्य का स्तर गिरता चला गया। आज अनुसंधान की संख्या इतनी बढ गई है कि डाक्टरेट हास्य का विषय बनकर रह गया है। जोशी अपनी एक रचना 'प्रभ हमें डाक्टरेट से बचां' में व्यंग्य करते हुए कहा है कि - "मुझे डर लगता है विश्वविद्यालय वालों से कहीं वे मुझे ऑनरेरी डाक्टरेट न दे दें। इस मौसम में उन्हें किसी ऐसे शख्स की तलाश रहती है, जिससे वे मानद पी-एचडी. या डाक्टरेट दे सकें।"[९]

आज डाक्टरेट प्राप्त करने के बजाय लोग उस से दूर भागने लगे है। उन्होंने अनुसंधान क्षेत्र में व्याप्त असंगतियों पर व्यंग्य किये है। आज विश्वविद्यालयों में होने वाले अनुसंधान कार्य पर कुठाराघात करते हुए लिखते है शोध के लिए किसी भी ऐसे गधे को पकडा लिया जाता है, कारण क्या तो - "बडा कागज रंगा है इसने। हो जाये साले पर एक पी-एच.डी उसका गला दबा दिया जाता है और विश्वविद्यालय का हेड ऑफ डिपार्टमेंट, पोस्टमार्टम करता है, शैली पर पाश्चात्य प्रभाव है, भाषा मुहावरेदार है, हास्य में व्यंग्य का। व्यंग्य में हास्य का पुट है, भाषा में ओज नही, प्रसाद गुण है और पी-एच.डी. होने पर मुर्दा इतिहास को सौप दिया जाता है।"[१०]

अत: हम कह सकते है कि शरद जोशी ने शिक्षा क्षेत्र के लेखन, पटन एवं अध्यापन से संबंधित सभी स्थितियों में व्याप्त विरोधाभास को व्यंग्य का विषय बना कर प्रस्तुत किया है। राजनीति के समान शिक्षा क्षेत्र भी विकृतियों का अड्डा बना हुआ है। शिक्षा संस्थान चलाना एक धंदा बन गया है। अँगूठा लगानेवाले अक्षर शत्रु शिक्षा संस्था के संचालक है। विश्वविद्यालय राजनीति और कर्म से पलायन का आदर्श बन गया है। अंग्रेजों द्वारा निर्धारित शिक्षा पद्धति में अंशमात्र परिवर्तन करके चलायी जा रही है। शिक्षा क्षेत्र में फैली हर छोटी बडी विसंगति को अजागर कर उसमें नैतिक सुधार लाने का उद्देश शरद जोशी का रहा है।

संदर्भ

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९) शरद जोशी - झरना नीम: शाश्वत चोख, द्वितीय संस्करण २००५, पृ.३०

१०) शरद जोशी - झरना नीम: शाश्वत चोख, द्वितीय संस्करण २००५, पृ.५५

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साहित्य के क्षेत्र में राजाराम मोहनराय का योगदान

– डॉ. सुनील मुरलीधर पाटिल
हिन्दी विभागाध्यक्ष,
आर.सी.पटेल कला, वाणिज्य एवं विज्ञान महाविद्यालय, शिरपूर जिला धुलियाँ

साहित्य का सृजन विभिन्न भाषाओं में की जाती है। साहित्य का अर्थ मानव कल्याण के लिए लिखी हुई रचना साहित्य को प्रमुख रूप से दो भागों में विभाजित किया गया है। जिनमें पहला रससाहित्य और दूसरा ज्ञान साहित्य है। साहित्य में उन रचनाओं का लिया जाता है जिसमें मानव के दय पर प्रभाव डालती है। उसमें विचारों को जन्म देती है। काव्य, उपन्यास, कहानी, एकांकी, नाटक, जीवननियाँ, संस्मरण एवं समीक्षाएँ यात्रा वर्णन यह सब रसात्मक साहित्य के अंग है। साहित्य के अंतर्गत इन्ही का समावेश होता है। मनुष्य के दय के साथ रसात्मक साहित्य का स्त्रोत बना रहता है। जब दय मेंविशेष भाव को तीव्र गति से संचार हो, तो वह कभी कभी गीत, कहानी, नाटक तथा अन्य किसी विधा के रूप में फूट पडता है। भावों का प्रवाह जितना तीव्र होगा, रचना उतनी ही सशक्त रूप लेगी। काव्य को साहित्य का शिखर कहा जाता है। आचार्य मम्मट ने काव्य के बारें में कहा है कि यश, धन, व्यवहार, ज्ञान, अनार्थ निवारण, आनंद और कान्ता के समान मधुर तथा कोमल उपदेश की प्राप्ति के लिए काव्य की सृष्टि होती है।

ज्ञान साहित्य का एक प्रमुख अंग है। यह साहित्य प्रकृति एवं समाज के बारें में जानकारी देता है। सामाजिकी, मानविकी एवं विज्ञान आदि विषयों का विवरण ज्ञान साहित्य के अंतर्गत आता है। वह साहित्य मनुष्य के मस्तिष्क से, बुद्धिसे संबंध रखता है। यथार्थ और इस तथ्यों का चित्रण इस साहित्य में होता है। ज्ञान साहित्य से मनुष्य अपनी सामाजिक, आर्थिक, राजनीतिक एवं मनोवैज्ञानिक समस्याएँ समझने और सुलझाने का प्रयत्न करता है।

संस्कृत एवं अन्य भाषाओं में रस साहित्य का प्रचुर मात्रा में सृजन हुआ। भारतीय साहित्य के इतिहास दृष्टि से प्रमुख समस्या थी। उन्नीसवीं शताब्दी तक ज्ञान साहित्य की उपेक्षा हुई। भारत की लगभग सभी भाषाओं में अभिव्यक्ति का माध्यम पद्य था। गद्य की ओर किसी का ध्यान नहीं गया। गद्य का सृजन पाश्चात्य शिक्षा के प्रभाव से हुआ। बंगाली भाषा में राजाराम मोहनराय की भूमिका महत्वपूर्ण रही है।

भारत का इतिहास काफी पुराना है। प्राचीन युग में कोई न कोई सभ्यता फलती-फूलती रही है। भारत की भौगोलिक एवं आर्थिक स्थिति कुछ ऐसी रही है कि इतिहास के आरंभिक काल से विभिन्न वंशों तथा विभिन्न सामाजिक स्थितिवाली जातियों के लोग यहाँ आते रहे है। भारत में नाना प्रकार की बोलियाँ बोली जाएँ और उनमें अनेक प्रकार की विभिन्नता हो। भारत में १७९ भाषाएँ और ५४४ बोलियाँ बोली जाती है। भाषा चार परिवार समूहों में वर्गकृत किया है। जिन्हें आर्य, द्रविड, एशियाई एवं बर्मा।

भारत के भाषागत विकास की दृष्टि से दो भाषा परिवार सबसे अधिक महत्वपूर्ण है - भारतीय आर्य और द्रविड। इस समय द्रविड भाषाएँ दक्षिण की चार भाषाएँ है। तमिल, तेलगु, मलयालम और कन्नड। इन में तमिल भाषा की साहित्यिक परम्परा प्राचीन है। दक्षिण भारत की चारों भाषाओं में संस्कृत के शब्द बडी संख्या में मिलते है।

वर्तमान भाषाओं का स्वरूप प्रमुख रूप से पश्चिमी प्रभाव का प्रतिफल है। अंग्रेजी साहित्य के माध्यम से भारतीयों का यूरोप की विचारधारा से परिचय हुआ और आधुनिकभारतीय भाषाओं के साहित्यों को नई दिशा दी। सबसे पहले बंगाल में शुरू हुआ। फलस्वरूप साहित्य की नई विधाओं कहानी, नाटक, निबंध और उपन्यास का जन्म हुआ। काव्य में अतुकांत कविता का श्रीगणेशा भी इसी संपर्क का फल था। बंगाली भाषा के नए साहित्य प्रतिमान भारत की अन्य भाषाओं में अपनाए गए। शरदचंद्र चटर्जी, बंकिमचंद्र चटर्जी और रवीन्द्रनाथ टैगोर की रचनाओं का भारत की सभी भाषाओं में अनुवाद हुआ। इस युग के सभी साहित्यकारों के शिल्प तथा भाव संपदा पर अपनी छाप छोडी है।

१९ वीं सदी के पुर्व बंगाली भाषा में गद्य की रचना नहीं हुई थी। १७ वीं सदी के अंत में और १८ वी सदी के आरंभ में कुछ पुर्तगालि ईसाइयों ने बंगाली भाषा में गद्य रचना की थी। ईस्ट इंडिया कम्पनी को कुछ पत्र और दस्तावेज बंगाली गद्य में लिखे थे। इन कागजों की भाषा साहित्यिक सौष्ठव नहीं है। वह बोलचाल की भाषा है, उसमें फारसी और अरबी शब्दों की भरमार है और उसकी शैली ज्ञानी बोझिल है कि आज शिक्षित बंगाली वर्ग उसे सरलता से अध्ययन नही कर सकता।

कलकत्ता में फोर्ट विलीयम कालिज की स्थापना सन १८०० में की गयी तथा इसी के साथ-साथ बंगाली गद्य साहित्य का इतिहास आरंभ होता है। भारत की विभिन्न भाषाओं की शिक्षा देने के लिए कालिज की स्थापना ईस्ट इंडिया कंपनी के अधिकारीयों की थी। इस कालिज में एक बंगाली विभाग था जिसके अध्यक्ष विलियम केरी नामक मिशनरी थे। इस विभाग में आठ अन्य अध्यापक थे। अध्यापकों को प्रोत्साहित करने के लिए बंगाली में पाठ्यपुस्तकों की रचना की।१ दस वर्षो में अनेक पाठ्य पुस्तकों की रचना की। कालिज के बाहर के अनेक विद्वानोंने भी इस ज्ञान-यज्ञ में अपना-अपना योगदान दिया। जिनमें रामरामबसु, मृत्युंजय विद्यालंकार और राजीव लोचन मुखोपाध्याय का नाम प्रमुख है। इन रचनाकारों में मृत्युंजय विद्यालंकार सबसे प्रमुख थे। १८०१ से १८१३ के बीच उनकी चार किताबें प्रकाशित हुई। उन्हें बंगाली गद्य का जनक कहा जा सकता है।

राजाराम मोहनराय की पहली बंगाली किताब १८१५ में प्रकाशित हुई थी। उसके बाद भी उन्होंने बंगाली में कई किताबों की

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रचना की। उन किताबों और ग्रंथों में उनके द्वारा किए गए मुंडक, मांडूक्यकेन और ईश आदि उपनिषदों के अनुवाद मुख्य है।२

राजाराम मोहनराय ने बंगाली पत्र-पत्रिकाओं में भी सामायिक और ज्ञान-विज्ञान के विषयों पर अनेक लेख लिखे। इससे बंगाली गद्य सृजन हुआ। इस काल की अधिकतर बंगाली रचनाएँ अंग्रेजी, संस्कृत और फारसी किताबों का अनुवाद था। फोर्ट विलियम केरी ने १८०१ में बंगाली भाषा का व्याकरण तथा १८१५ में बंगाली-अंग्रेजी कोश का प्रकाशन किया। इतिहासमाला किताब का प्रकाशन भी किया। इसमें सहज सरल शैली में कहानियाँ कही गई है।

बंगाली पत्र-पत्रिकाओं ने बंगाली गद्य का विकास में महत्वपूर्ण भूमिका निभाई। १८१८ में बंगाली गजेटी, दिग्दर्शन और समाचार दर्पण पत्रों का आरंभ हुआ। इनमें साप्ताहिक समाचार दर्पण प्रमुख था। इन में जन्म, विवाह और मृत्यु के समाचार, नए-नए अविष्कार और नई-नई मशीनों एवं औद्योगिक विकास से संबंधित समाचार विशिष्ट विद्वानों द्वारा किताबों का परिचय कराना, न्यायधीशों, कलेक्टरों एवं अधिकारियों की नियुक्ति कराना, भारत और युरोप में व्यापार समाचार आदि की जानकारी दी जाती थी। बंगाली की सात पत्रिकाओं का प्रकाशन १८२० से १८५० के मध्य में हुआ। इनमें संवाद कौमुदी के संपादक राजाराम मोहनराय ने अंग्रेजी में बंगाल हेराल्ड और बंगाल में बंगदूत की नींव डाली। उन्नीसवीं सदी के अंगडाई लेते हुए बंगाल की तस्वीर हमारे सामने आ जाती है। उस समय के बंगाली समाज की सारी शक्तियाँ और सीमाएँ पाठक की आँखों के सामने स्पष्ट हो जाती है। इससे बंगाली भाषा की शब्द संपदा बढी। बंगाली भाषा संस्कृत के पाश से मुक्त हुई। ईश्वरचंद्र विद्यासागर, माइकेल, मधुसूदन दत्त, बंकिमचंद्र चटर्जी और सबसे बढकर रवीन्द्रनाथ टैगोर जैसे साहित्य साधकों ने बंगाली भाषा को सूक्ष्म से सूक्ष्म भावों की अभिव्यक्ति का सशक्त माध्यम बना दिया।३बंकिमचंद्र के उपन्यासों में देशभक्ति की प्रचंड भावना थी और भारत का राष्ट्रगीत वंदेमातरम, उनके आनंदमठ उपन्यास में आया है। उन्होंने भारत माता की देवी के रूप में आराधना की है। जिस की मुक्ति के लिए उसके पुत्रों को अपना सर्वस्व बलिदान करने के लिए तैयार रहना चाहिए। वंदेमातरम गीत क्रान्तिकारीयों तथा राष्ट्रवादियों के लिए पवित्र मंत्र बन गया था। जिसे गाते-गाते उन्होंने छातियों पर गोलियाँ सही और कई वीरों को फासी की सजा दे दी गयी।

बंगला व्याकरण लिखने का सर्वप्रथम प्रयास राजाराम मोहनराय ने किया। इंग्लैण्ड जाने से पहले राजाराम मोहनराय ने बंगला भाषा का व्याकरण (गौडीय व्याकरण) लिखा। व्याकरण की आवश्यकता और उद्देश्य से लेकर अन्तयानुप्राश तक को विषय उन अध्यायों में आते है। पहली बार एक कठिन विषय बंगाली भाषा में अनुवाद के लिए राजाराम मोहनराय द्वारा लिया गया और उनका वेदान्त तथा उपनिषद का अनुवाद विषय की सूत्रपरकता को ध्यान में रखते हुए, एक अंश गद्य रूप में आया। उनकी विश्लेषण क्षमता अत्याधिक तीव्र थी उनके लेखन में मुहावरों का भी यथास्थान प्रयोग मिलता है। वेदान्त की बंगाली भाषा में अनुवाद करते समय राजाराम मोहनराय ने विशेष सतर्कता से काम लिया। व्याकरण प्रयोग से राजाराम मोहनराय सिद्धहस्त थे। उन्होंने बंगाल तथा संस्कृत रचना में वैज्ञानिक दृष्टिकोण अपनाया तथा अध्यात्मिक और दार्शनिक विषयों पर बहस के विलक्षण कार्य चुना। जबकि दूसरों ने प्रसिद्ध ऐतिहासिक कहानी, चरित्र-चित्रण और पौराणिक कथाओं पर लिखा जो कि तुलना में साधारण थे। राजाराम मोहनराय को प्रथम बंगाली गद्य साहित्य लेखक होने का अद्वितीय सम्मान प्राप्त है। वह पहले ऐसे व्यक्ति है जिन्होंने बंगाली गद्य को उचा तथा दार्शनिक विषयों को अभिव्यक्ति का माध्यम बनाया।४ समकालीन बंगाली भाषा और साहित्य की स्थिति को ध्यान में रखा जाय तो यह एक महान उपलब्धि है और इसने बंगाली भाषा और साहित्य के भविष्य में व्यक्त महान संभावनाओं को प्रदर्शित करती है।

काव्य, उपन्यासों एवं नाटकों के अलावा साहित्य की अन्य विधाओं में भी पर्याप्त उन्नति हुई। धार्मिक, दार्शनिक एवं नैतिक साहित्य प्रचुर मात्रा में रचा गया। तत्वबोधिनी पत्रिका ने टैगोर, विद्यासागर, बसु, केशवचंद्र सेन और स्वामी विवेकानंद ने बंगाली तथा अंग्रेजी दोनों भाषाओं में विपुल धार्मिक तथा दार्शनिक साहित्य की रचना की। राजेंद्रलाल मिश्र द्वारा संपादित विविधार्थ संग्रह बंकीमचंद्र द्वारा बंगदर्शन, द्विजेंद्रनाथ टैगोर एवं स्वर्ण कुमारी द्वारा भारती ने बंगाली भाषा को अनेक नए लेखक प्रदान किए। दैनिक पत्रों में हितवारी और बंगवासी के नाम स्मरणीय है।

बंगाली में अनेक श्रेष्ठ जीवनियाँ एवं आत्मकथाएँ की भी बंगाली भाषा में रचना की गयी। राजनारायण बसु, देवेंद्रनाथ टैगोर तथा विश्वनाथ शास्त्री ने अपनी आत्मकथाएँ लिखी। राजाराम मोहनराय चटोपाध्याय, जोगीन्द्रनाथ बसु, मधुसूदन दत्त और बिहारी लाल सरकार, विद्यासागर, रवींद्रनाथ टैगोर बंगाली साहित्य के कीर्तीमान है। उन्होंने साहित्य का प्राय सभी विधाओं में अपनी प्रतिभा का परिचय दिया है।

संदर्भ

१. राष्ट्रीय जीवन चरित्र निर्माण ग्रंथावली - वर्मा एवं यदुवंशी, खंड ६, पृ.२१०
२. राष्ट्रीय जीवन चरित्र निर्माण ग्रंथावली - वर्मा एवं यदुवंशी, खंड ६, पृ.२११
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समकालीन हिंदी गजलकार जहीर कुरेशी के गजलों में नारी विमर्श

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हिन्दी विभागाध्यक्ष
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शिरपूर जिला धुलियाँ

वर्तमान समाज में हिन्दी कविता का जितना महत्व है, उतना ही महत्व गजल कृतियों को भी है। हिन्दी काव्य संसार में गजल का विकास तेजी से हो रहा है। गजल मुख्यत: उर्दु-अरब की चर्चित विधा रही है। एक जमाना था जब आशिक और माशूका की मोहब्बत को गजल कहा जाता था। समकालीन हिन्दी गजल साहित्य क्षेत्र में अपनी स्वतंत्र उपस्थिति को दर्ज कराने में सक्षम रही है। आज उसे एक लोकप्रिय काव्यविधा माना जाने लगा है। गजल के संबंध में पुष्पा राही का कहना है कि, "आज हिन्दी में गजल की बढती लोकप्रियता एवं दिन-प्रतिदिन समृद्ध हो रही, इसकी परम्परा का एक उल्लेखनीय कारण यह है कि समसामायिक हिन्दी कवि इसे एक मात्र उधार ली गई विधा के रूप में नही, वरन् आत्मिभिव्यक्ति के एक सशक्त, समानुकूल माध्यम के रूप में बरत रहे हैं। वे गजल को हिन्दी की प्रकृति के अनुरूप नया मुहावरा, अलंकार एवं चरित्र देने में संलग्न है।"१

हिन्दी गजल की भाव-धारा निरंतर प्रवाहित रही है। यह भाव-धारा कभी क्षीण तो कभी तीव्रगति से प्रवाहित होती आयी। हमे यह अंगीकार करना ही होगा की हिन्दी गजल की अक्षुण्णता ही उसकी शक्ति रही, नियति रही है, प्रकृति रही है। इसमें निरंतरता है, भाव-प्रवणता है, वैचारिक मंथन है, तीव्रता है, माधुर्य है, निजी संवेदना है, आत्मीयता है। इसमें गजलकारों के भावों एवं विचारों का सहज स्फुरण है। एहसास की गहराई गजल का प्राण है। गजलकार एहसास की गहराई को जितनी शिद्दत से महसूस करता है गजल का प्रवाह और प्रभाव उतना ही सटीक होता है। गजल के श्रवण, पठन में लय, संगीतात्मकता एहसासों की गहराई पर निर्भर करता है। कुरेशी की गजलें इस कसौटी पर खरी उतरती है।

समकालीन हिन्दी गजलकारों में जहीर कुरेशी का नाम बडा महत्वपूर्ण है। उन्होंने अपनी गजलों में आम-आदमी के जीवन से जुडी समस्याओं का अंकन करने की बडी ईमानदारी की है। इस में भी उन्होंने नारी शोषण का यथार्थ चित्रण किया है। मानव मूल्यों का संस्कार करनेवाले जहीर कुरेशी हिन्दी साहित्य में एक सशक्त गजलकार के रूप में अपने को स्थापित कर चुके है। पुरूष प्रधान समाज में नारी शोषित है। वह धार्मिक, सामाजिक, रीतिरिवाजों, परम्पराओं प्रथाओं से मुक्ति पाना चाहती है। कुरेशी ने अपनी गजलों में नारी जीवन की वेदना को सशक्त अभिव्यक्ति दी है। नारी को देवी का स्थान दिया जाने पर भी उसका हर तरह से शोषण होना दुर्भाग्यपूर्ण है। वैश्वीकरण के युग में उपजी भोगवादी लालसा में नारी टूट रही है। व्यवस्था के हाथों का खिलौना बनकर भी समाज के मन में उसके प्रति कोई सहानूभूति नही जगती। नारीअपहरण, उसका यौन शोषण, पैसों के लालच में अपने ही करीबी रिश्तेदार उसे बेच देते है, उसका जबरदस्ती अनमेल विवाह कर देना बेहद दुखदायक है। विधवा बनने की आशंका भी बनी रहती है। देहज जैसा बुरा रिवाज उसका दम घोट देता है। किसी जमाने में सती होकर जलनेवाली नारी आज देहज के कारण पति के हाथों जीवित जलाई जा रही है। इतना सब कुछ उसके साथ होने के बावजूद नारी अपनी व्यथा किसी को नही कहती है। कुरेशी कहते है कि,

"कल सती होकर जली थी, आज पति के हाथ,
बन गई जीवित जलाने की प्रथा औरत।"२

जहीर कुरेशी यह जानते है कि भारतीय नारी अपनी लक्ष्मण रेखा कभी नही लाँघती। यदि कोई नारी विचलन का शिकार होती भी है, तो उसे कितनी जबरदस्त मानसिक तैयारी करनी पडती है। घर, परिवार, रिश्ते-नाते समाज आदि तमाम बंधनों के विचारों का उसके मन में तुफान निर्माण हो जाता है। काफी सोच-विचार कर, मानसिक रूप से तैयार होकर वह इस निर्णय तक पहुँची है -

"जब भी औरत ने अपनी सीमा-रेखा को पार किया
पार-गमन से पहले, खुद को कितने दिन तैयार किया।"३

जहीर कुरेशी ने मानसिक द्वंद्व में घिरी, लिंगभेद के कारण होनेवाले अत्याचारों को सहनेवाली नारी पुरूष वर्ग के खिलवाड से अपने स्व को दास्ता के आँचल में छिपाए रखनेवाली, दुख को मन में दबाए रख होठों पर मुस्कराहट लिए जीनेवाली नारी की घुटन को कुछ यूँ अभिव्यक्त किया है -

"आँखों में आँसू का झरना, अधरों पे मुस्कानें है,
औरत ने इस द्वंद्व युद्ध में खुद को लहू-लुहान किया।"४

आज के शहरी परिवेश में लडकियों को शिक्षा और नौकरी के नाम पर जो खुला वातावरण प्राप्त है, उसे लडकियाँ उस खुलेपन के अंतर्गत अपनी देह के जादू को 'चेक' की तरह भुनाने पर आमादा है। वे अपनी असली उडान भूल जाती है। उसकी आजादी को स्वेच्छाचार में बदल जाती है। जिसके बाद उसे आत्मग्लानी होती है। जहीर कुरेशी इस विसंगति पर भी कहते है कि -

"जो अपनी देह को, यौवन को खुलकर खर्च करती थी
वो लडकी अपनी पे कल पछताने वाली है।"५

नारी जीवन की पीडा का एक और नया रूप जहीर कुरेशी ने चित्रित किया है। इन्सान स्वार्थ में इतना अंध हो जाता है कि उसकी सुझ-बूझ भी शायद नष्ट हो जाती है। वह इतने नीचले स्तर पर गिर जाता है कि वह अपने स्वार्थ को पाने के लिए अपनी पत्नी को दूसरे सामने परोसता है। यह व्यक्ति के नैतिक पतन की अंतिम अवस्था है।

"महत्वकांक्षी पति के इशारे पर
गई थी कल भी वो 'साहब' के बिस्तर तक।"६

आज समाज के सामने यह सवाल है नारी संख्या की कमी समाज में दिन ब दिन पुरूष की तलाश में नारी की संख्या का प्रतिशत कम हो रहा है। लिंग निर्धारण परीक्षण कर नारी को कोख में ही कत्ल किया जा रहा है। लडकी भ्रुणहत्या देश की ज्वलंत समस्या है। कुरेशी कहते है कि -

लिंग निर्धारण समस्या हो गई
कोख में ही कत्ल कन्या हो गई
लोग कर पाए नही खुल कर विरोध
सिर्फ अखबारों में निंदा हो गई।"७

भौतिकवादी युग में कुछ युवतियों का अधेडों से विवाह करने में अपना भविष्य सुरक्षित समझती है, जिनके पास अपार धन है। आयु का अंतर अब विवाह संबंधों में आडे नही आता। आज के समाज के इस कटु सत्य पर भी जहीर कुरेशी अपने शेर के माध्यम से कहते है कि -

"किसी बरगद की बाहों में पहुँचकर
लता पर पेड की छत बन गई है।"८

इक्कीसवीं सदी के सोच तक आते-आते महानगरीय आधुनिकाएँ अपनी देह से आजाद हो रही है। वह वस्तुएँ बेचने के नये फंडों के तहत अपने देह का खुला प्रदर्शन कर रही है। बहुराष्ट्रीय कंपनियों के नये नये 'प्रॉडक्ट' को 'लॉन्च' कराने हेतु वह रैम्प पर थिरक रही है। रैम्प पर कॅटवॉक करती 'मॉडल्स' की मानसिकता पर जहीर कुरेशी कहते है कि -

"अति पारदर्शी वस्त्रों की फैशन-परेड में
हाथों से तन छिपाना, जरूरी नही लगा।"९

भारतीय समाज में लडकी को बोझ माननेवालों की कमी नही है। देहज देकर भी लडकी को ससुराल में अपनापन, स्नेह मिलेगा यह जरूरी नही है। परिवार में बेटी के साथ क्या होगा इस भय से परेशान पिता को बेटी के भविष्य की चिंता सताती रहती है। वह यह भी मान लेता है कि उसे पति से पत्नी के सारे सुख मिल भी जाए लेकिन बराबरी की जगह नही मिलती। भौतिक सुखो के अलावा भी जीवन होता है, जिसमें बराबर का दर्जा, समानतापूर्ण व्यवहार महत्वपूर्ण होता है। कुरेशी कहते है कि -

"लाख रूपए में खरीदा था पिता ने मेरा वर
वो मेरा सर्वांग होकर भी, पराया है बहुत।"१०

अनैतिकता के कारण राह भटक चुकी नारी को कदम कदम पर समाज की घिनौने नजरों का, तीखी बातों का सामना करना पडता है। उसकी मनोव्यथा को सिवाय उसके कोई नही जानता पाप-पुण्य की भावना से भी वह परे हो जाती है। पति की स्वार्थ सिद्धी और अनैतिक सुख के लिए वह अपना शरीर तक दाँव पर लगा देती है। अपनी व्यक्तिगत खुशियों की मोहताज बन जाती है। नारी मन की पीडा, ऊब, घुटन, अकेलेपन की भावना को उसके मन की तहों में उतकर जाना होगा। कुरेशी लिखते है कि -

"अनैतिकता के चश्में को बदलकर देखना होगा,
गलत राहों पे वो कैसे गई, ये सोचना होगा।"११

निष्कर्षत: कहा जा सकता है कि जहीर कुरेशी नारी जीवन के प्रति अत्यंत संवेदनशील है। पुरूष प्रधान संस्कृति में शोषित नारी की घुटन, कुंठा, अकेलेपन तथा दयनीय स्थिति की भावना को मर्मस्पर्शी अभिव्यक्ति दी है। नारी पर होनेवाले अत्याचार, लैंगिक शोषण, पुरूषों का दृष्टिकोन, अस्तित्व बोध, देहज-प्रथा, वेश्या समस्या, देह प्रदर्शन, बाजारवाद की प्रथा, कन्या भ्रुणहत्या आदि बातों का चित्रण कुरेशी जी ने अपनी गजलों में किया है। नारी की मनोव्यथा-वेदना उनकी गजलों का केंद्र बिंदु बना है। उनकी गजलों में नारी जीवन के विविध रूपों और उसके उत्पीडन देखने को मिलता है।

अन्य गजलकारों की गजलों में नारी का विस्तार से चित्रण नही हुआ है जितना कुरेशी जी के गजलों में नारी का चित्रण मिलता है। नारी का अन्तर्द्वंद्व उसके मन की गुत्थिया, घुटन-टूटन, मजबूरी की छटपटाहट उसके सामाजिक प्रश्न, उसकी मनोवृत्ति की गहराई तक गये है। नारी की विवशता और उत्पीडन को बडे मार्मिकता से चित्रण किया है। उनकी गजलों में नारी के प्रति आस्था, सहानुभूति एवं मानवीयता का दर्शन स्पष्ट होता है।

संदर्भ सूची

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मंगलेश डबराल की कविताओं में सामाजिक चेतना

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शिरपूर, जिला धुलियाँ

****

समाज एक विशाल शब्द है। परिवार से मानव समूह तक समाज के विविध रूप है। व्यक्तियों के समूह से समाज होता है, जिनका उद्देश्य स्थायी और स्पष्ट होते है। मनुष्य एक समाजप्रिय प्राणी है। अकेलापन को त्यागकर परिवार जीवन को अपनाया है। उसके बाद उसमें समाज भावना का विकास हुआ है। समाज विशेष उद्देश्य से बनी एक संस्था है। जिसका उद्देश्य व्यक्ति समाज की रक्षा करना, उनका अन्नयन और हित करना है। व्यक्ति समाज से शक्ति और प्रेरणा प्राप्त करना है। कवि मनुष्य से अधिक जागृत होता है। वह समाज अंगो को अपनी रचना का विषय बनता है। कवि पीडा, अवसाद, निराशा, घुटन आदि का अनुभव करता है। वह अकेले ही नहीं अपितु सम्पूर्ण समाज का करता है। कवि भी समाज का एक हिस्सा है।

मनुष्य सामाजिक प्राणी है। समाज के बिना मनुष्य जी नही सकता। समाज के साथ अटूट संबंध है। कवि का भी संबंध समाज के साथ अटूट है। कवि समाज में रहकर समाज का मूल्यांकन करता है। कवि का दायित्व है कि उसने समाज को जागृत करना चाहिए।

मंगलेश डबरल की काव्य यात्रा प्रमूख रूप से सामाजिक विसंगतियों से आरंभ होती है। इसमें जनता की बेचैनी, दुःख, दर्द, पूँजीवादी विसंगतियाँ तथा मध्यमवर्गीय जीवन की सच्चाइयाँ आदि आते है। सन १९८० के बाद उभरने वाली पीढी में मंगलेश एक ऐसे युवा कवि है जिनके यहाँ अपने समय की आम बात से हटकर अभिव्यक्ति की नैतिकता धीरे-धीरे विकसित होती दिखाई दे रही है। उनकी रचनाएँ अपने अनुभव पर आधारित होने के कारण उनमें जीवंतता झलकती है। मंगलेश वर्तमान शोषण मूलक व्यवस्था के

खिलाफ आक्रोश, नफरत और उसे उलट कर एक नयी मानवीय व्यवस्था का निर्माण करना चाहते है। मंगलेश समाज में जो अत्याचार, अन्याय, शोषण, बाजारवाद, कुंठा एवं हिंसात्मक कृत्य आदि का चित्रण किया है। आज समाज काफी बदल चुका है। जीवन में तनाव बढ रहा है। आम आदमी का जीवन कठिन बन रहा है। पुरी दुनिया में बाजारवाद हावी हो गया है। मनुष्य का न सम्मान है, न उसके रिश्तों का। इस बात पर मंगलेश खेद जताते है। समाज को जगाने का काम भी करता है।

मंगलेश डबराल की कविताओं का यथार्थता एक मुख्य पहलू है। पहाड से भिन्न परिवेश, जिनमें शहर, महानगर और जीवन के विभिन्न पक्षों का समावेश है। उनकी कविताओं का महत्वपूर्ण संसार है - संवेदना और दृष्टि दोनों को विस्तार दिया है। मंगलेश की कविता में सामाजिक यथार्थ का जीवंत अभिव्यक्ति हुई है। इसमें सामाजिक क्रान्ति की चिंगारी बहुत तीक्ष्ण ढंग से उजागर होती है। 'सफेद दीवार' का एक उदाहरण उल्लेखनीय है।

"शहर की सबसे लंबी सबसे सफेद दीवार, खाली है इस वक्त
इस पर लिख जा सकती है कोई कविता, कल सुबह के लिए कोई
संदेश इस पर दर्ज किया जा सकता है, अगली लडाई का ऐलान।"[१]

जिंदगी का सरलीकरण मंगलेश की कविता में नहीं है। यथार्थ जीवन को शांत और संयत स्थिति में बिना हैरत और अफसोस को ढालना बहुत बडी तपस्या है। यथार्थ को किसी प्रकार अंगीकार करना है। इसे देख सकते है -

"कुछ दिन मन में विद्रोह होता है घुमड न रहती है
कोई दुख देखकर नीची कर लेनी होती है निगाह।"

मंगलेश डबराल में नारी जाति के प्रति गहरी आस्था है। भीतर के दुःख को करूणा की आवाज से पुकारते है। नारियाँ - तारे के प्रकाश की तरह, तुम्हारे भीतर लडकी और अंधा आदमी आदि कविताओं में भारतीय नारी की संघर्ष भरी कहानियों को जीवनशक्ति से पकडने का सार्थक प्रयत्न किया है। भारतीय समाज में नारी को भोग की वस्तु समझकर उसके साथ गलत व्यवहार किया जाता है। आदर्श रखकर पतिव्रत धर्म का उपदेश देकर समाज ने उसे खूब छला है। जीवन में बार-बार छली जाने के बावजूद भी नारी अंततः प्रेम में बसा हुआ घर चाहती है।

"एक आँख से हँसती एक से रोती हुई
वह फिर से आ पहुँचती है पुरूष के सामने
जैसे उसका कुछ न छीना गया है
जैसे वह उसी तरह करती आ रही हो प्रेमा।"[२]

मंगलेश ने औरत के भीतर का दर्द को पहचाना है। उस का बिखरा हुआ घर, बहता हुआ जल और खाये बिना खाये बिना खाना का खत्म होना, 'प्रेम करती स्त्री' कविता में उभरा है।

"प्रेम करती स्त्री, ठगी जाती है रोज
उसे पता नही चलता बाहर क्या हो रहा है
कौन ठग रहा है कौन है खलनायक
पता नही चलता कहाँ से शुरू हुई कहानी।"[३]

काव्य में प्रेम के लिए महत्वपूर्ण स्थान है। मंगलेश की प्रारंभिक कविताएँ रोमान्टिक परिवेश को लेकर हमारे सामने आयी है। प्रेम का रंगीन चित्र, प्रेम का मधुर मोहकमय रूप काव्य में हमे मुग्ध कर लेता है। मंगलेश की कविता बेचैनी, छटपुराहट और मानवीय पीडा का स्वर देती है। वह प्रेम है जो मंगलेश को बाँधता है। चीजों को फिर से अनुभव करते उन्हें बदलते हुए देखने और उनमें रहने के लिए उकसाता है। इतना सब होते हुए भी दुःख को दुःख न होकर अवसाद में बदलता है। मंगलेश प्रेम कविता में कवि के गरिमायी व्यक्तित्व की पहचान कराते है। प्रेम करना, या निभाना, कहने या सुनने भर की बात नहीं है। प्रेम यह शब्द इतना वजनदार है कि जिसे कमजोर उठा नहीं सकता है।

"प्रेम एक भारी पत्थर है
कैसे उठेगा तुझ जैसे कमजोर से।"[४]

मंगलेश की कविता 'प्रेम सफलता की कुंजी' में सफल प्रेम की चर्चा हुई है। मंगलेश प्रेम और सफलता का संबंध एक दूसरे से जोड देते है।

"प्रेम में उन्हें मिली सफलता
उन्होंने सफलता से किया प्रेमा।"[५]

कविता की दुनिया में मंगलेश ने प्रेम की गरम आहट को गुम नहीं होने दिया। बल्कि उसे जीवन संघर्ष की धधकती आग के भीतर काफी जगह दी है। मनुष्य में प्रेम करने की आग जलती रहे। मंगलेश उन कवियों में नही है जो प्रेम करने के लिए जीवन भर खाक छानते है।

मंगलेश की दृष्टि में जीवन के प्रति गहरी आस्था है। जीवन को सम्पूर्ण गहराई के साथ अपनाते है। इसलिए उनके पहले काव्य संग्रह 'पहाड पर लालटेन' से लेकर 'लेखक की रोटी' तक में जीवन का विस्तार मिलता है। आदमी के भीतरी बाहरी निराशा, समझौता और संघर्ष के बावजूद जो इच्छाएँ सपनों और उम्मीदों से भर जीवन जो जीवन के प्रति गहरी आस्था उत्पन्न करते है उसके बारे में वे कहते है कि

"बाहर एक बाँसुरी सुनाई देती है, एक और बाँसुरी है
जो तुम्हारे भीतर बजती है, और सुनाई नहीं देती।[६]

समकालीन कविता में समसमायिक मानव चेतना पर दबाव पडने के कारण आस्था-अनास्था अनेक रूपों में रूपायिकता होती है। कभी-कभी इन दबावों के कारण ही, विसंगतियों के कारण ही व्यक्ति के मन में अनास्था अपने आप जागृत होती है, तो कभी-कभी जीवन के प्रति आस्थावादी स्वर भी जागृत होता है। जीवन के लिए आस्थामय संघर्ष अर्थात आगे बढना चाहते है। इस प्रकार मंगलेश की कविताएँ एक नयी उम्मीद, नये विश्वास, नयी उमंग, नयी आस्था और नयी जिजीविषा के साथ पाठकों को उद्रेलित करती हुई सामने आती है। जीवन के प्रति जिजीविषा भाव मंगलेश में वर्तमान है। ध्वस्त नैतिक मूल्यों एवं आस्थाओं के बीच राह निकालने का, अपने पथ के निर्माण का संकल्प यहाँ हम देख सकते है। यही संकल्प मनुष्य को महान बनता है।

कवि शोषित-पीडित व्यक्ति को अपनी कविताओं में स्थान देते है। 'लालटेन' संघर्ष का प्रतीक है। कवि ने शोषक के खिलाफ लडने की चेतावनी दी है। देश की प्रगति के लिए शोषण का खुलकर विरोध करना चाहिए। कवि 'लालटेन' के माध्यम से लोगों को मन में मुक्ति के संघर्ष की बीज बोना चाहा। आम आदमी के जीवन से शोषण का अंधकार हमेशा के लिए खत्म कर देना चाहता है। अन्याय के विरूद्ध में प्रतिशोध की आवाज बनकर खडी है। लेकिन यह आवाज उग्र और आक्रमक नही है, बल्कि इस का स्वरशांत और धीमा किंतु देर तक गूँजती रहती है। मंगलेश शोषण और अन्याय से उपर उठकर नये मानव को जीवन देने की चेष्टा करते है। अपनी कविता द्वारा स्वस्थ एवं सुंदर सहज जीवन का भोग करने का निमंत्रण देता है। पहाडी आँचलों में भोले भाले मनुष्य का समाज कई स्तरों पर शोषण का शिकार है। इसका चित्रण मंगलेश ने बडे मर्मस्पर्शी ढंग से अंकित करते है।

"उनकी धमनियों में गूँजती भुख
खोजती है अपना गुस्सा और अपना प्रेम
उनके रोओं से उडती है बारूद।"[७]

मंगलेश की कविताओं में निम्न मध्यमवर्गीय का चित्रण भी किया है। उन्होंने पहाडी प्रदेश के समाज को अपनी कविताओं को केंद्र बनाया। यह पहाडी प्रदेश मंगलेश की रोम-रोम में बसा हुआ है। वहाँ के जीवन से इनका रचनात्मक जुडाव है। उच्च वर्ग जो शोषक है। लोगों का शोषण करते है। मध्यम वर्ग किसान और मजदूरों का है। इनका जीवन तो कष्ट से भरा है और वे जिंदगीभर सिर्फ परिश्रम करते है। मंगलेश मध्यमवीय चेतना के कवि है। कवि यह कहते है कि शक्ति के बल पर इंसान कुछ भी कर सकता है। उसका उद्देश्य एक मात्र है वह है सफलता। इस सफलता के लिए वह शक्ति का सहारा लेता है।

"सीढियाँ चढते है उपर से नीचे देखते है।
जहाँ लोग एक दूसरे को धक्का देते बढ रहे है।"[८]

मंगलेश मध्यमवर्गीय भावभूमि के क्रियाशील रचनाकार है। उन्होंने अपनी कविता में एक रचनाकार की पीडा को व्यक्त किया है। जो अपनी रोजी-रोटी छोडकर गाँव से शहर चला आया है। जीवन की अभावग्रस्त स्थितियों में उन्हें शहर की संवेदनहीनता के साथ जीवन से समझौता करने के लिए मजबूर करती है। पेट की भूख ही वह बेबसी है, जिसने अपनों को पटक दिया है। जहाँ हम नि:शब्द रोटिया कुरते है और शहर में आत्म निर्वासन की स्थिति झेलते खुद को असुरक्षित असहाय महसूस करते है और कहते है -

"क्या करू कहाँ जाँऊ किस रास्ते पर
किस दोस्त के यहाँ क्या कहूँ
कुछ करने से पहले सारा कुछ कह दूँगा एक बारा।"[९]

अज्ञेय जी की प्रसिद्ध पंक्तियाँ है - "दु:ख सब को माँजता है।" मंगलेश की कविताओं के लिए यह उक्ती लागू होती है। मंगलेश में करूणा दु:ख दर्द एवं अवसाद का बडा जोर है। मंगलेश की कविता हमारे मन में गहरे अवसाद को जन्म देती है। वह अवसाद आदमी को अकेला कर देता है। जो बुरी तरह टूट गया है, थक गया है। एक अकेली लडाई में कुछ भी न बदलने के कारण निराशा और अवसाद में डूबे हुए आदमी को अपनी कविताओं में बताते हुए मंगलेश ने किसी का सहारा नही लिया। उनका 'थकान' कविता इस दृष्टि से महत्वपूर्ण है -

"रात जब नि:शब्द, हमारी छाती पर झुकी होती है
काले रंग की थकान बिस्तर पर चढती है
माँस पेशियों के मोड लांघती है।"[१०]

उदासी और अवसाद का स्थायी भाव मंगलेश की कविता में होने के कारण एक जीवन के लिए संरचना पत्थरों की कहानी और शुरूआत जैसी कविताएँ है, जिनमें अतीतोन्मुख अवसाद है। मंगलेश अपनी कविता में लिखते है कि, "मै घर का रास्ता भूलना नही चाहता" अपनी उदासी के लिए क्षमा माँगना नही चाहता, पर सच तो यह है कि घर की और लौटना अब उतना आसान नही रह गया है। इस परिवर्तन का एक आत्मस्वीकार भी है और वह अलग-अलग कविताओं में भिन्न परिस्थितियों की ओर जाता है। कवि कहते है -

"हमें भी मिली छोटी सी एक जगह
थोडी सी हवा एक बिस्तर
मुसीबते याद रखने के लिए

एक डायरी।"[११]

मंगलेश की कविता रास्तों की खोज में संघर्षरत है। सच्चाई यह है कि वे मनुष्य के दुःख बोध और उसकी संघर्ष चेतना के अनन्य कवि है। कवि संघर्ष की जिंदगी जी रहा है। लेकिन कवि हिम्मत नही हारता। उनकी कवितओं में जीवन की जटिलता को ठोने की ताकत है। उनका अनुभव जानकारी ही कविता की ताकत को बढाती है। 'मुक्ति' कविता में कवि पहाड के संघर्षशील व्यक्तियों से स्वयं को जोडते है। उनकी दिनचर्या में मुक्ति-प्रयासों का साक्षी बनकर उनके बीच होने का एहसास कराते है।

"अपने मरे हुए बच्चों की खोज में,
मैने उन्हे एक जंगल से निकलकर
दूसरे जंगल में जाते हुए देखा है।
मैने उन्हें बेशुमार पौधों की तरह नाचते देखा है,
इस रक्त रंजित रेगिस्तान में।"[१२]

मंगलेश आशावादी कवि है, निराशा रचनात्मक, प्रेरक तथा उम्मीद पैदा करनेवाली है। कवि निराशा को फैशन के रूप में अपनाते है। वहाँ कवि उसे एक प्रेरक भूमिका में प्रदान करते है। उसे उम्मीद के स्त्रोत में बदल देते है। उनकी कविता में दुःख कही करूणा, कही विद्रोह और कही जीवन के लिए कोमलता का स्पेस चुनती है।

"मैं दुःख हूँ मुझमें एक धीमी काँपती हुई रोशनी है।"[१३]

मंगलेश की कविताओं में संबंधों की दुनिया का मानवीय चेहरा उपस्थित है। जहाँ तमाम अनुभवों के साथ बूढे पिता है, उदास माँ है, संघर्ष में साथ देने वाली पत्नी भी है, स्कूल जाते बच्चे भी है और इन आत्मीय लोगों के बीच हत्यारे भी है। कवि मंगलेश कविता के मानवीय पहलू से जुडे है। आगे बढकर कवि कहते है कि पिता जब थक जाते है तो उन्हें बेटे के सहारे की आवश्यकता महसूस होने लगती है। अपने से दूर चले गये बेटे के लौटने की प्रतीक्षा उन्हें अपने अंतिम दिनों में होती है।

"अंधेरे में पिता माँगते है थोडी सी उम्मीद
परदेश गये बेटे के लौटने की।"[१४]

मंगलेश बताना चाहते है कि यह भूमंडलीकरण राष्ट्रीय षडयंत्र के बिना संभव नही है। भूमंडलीकरण ने सब कुछ को बाजार बना दिया है। आज का बाजार पहले के बाजार से भिन्न है। बाजार कविता आज के उपभोगतावादी मूल्यों पर एक सधा हुआ प्रहार करती है। विज्ञापनों की चकाचौंध में विवेक का सहज प्रकाश डूबता जा रहा है। बाजार, उपभोक्तावाद, शोषण, अत्याचार की इस दुनिया के बीच मंगलेश 'मनुष्यता' को बचाना चाहते है।

"यह ऐसा समय है
जब कोई हो सकता है, अंधा, लंगडा, बहरा, बेघर, पगला।"[१५]

कवि मंगलेश जीवन में आनेवाले पहाड जैसी समस्याओं को हल करने की कोशिश अपनी कविता में करते है। तमाम उदासियाँ और असफलताओं के बावजूद भी कवि घर का रास्ता नही भूलना चाहता। आदमी की उदासी घर की चाह और संघर्ष की थकान मूलत: जिंदगी के ही हिस्से है।

हम यह कह सकते है कि सामाजिक प्रक्रिया की चपेट में आकर कुचल दिए गए लोगों को सम्मान और गौरव का एहसास कराने वाली इन कविताओं में मंगलेश डबराल का सर्वाधिक सार्थक कवि-कर्म प्रकट हुआ है। जमीन से जुडे हुए कवि, गाँव की, समाज की और देश की रोजमर्या समस्याओं से भलि-भाँति परिचित है। मंगलेश की कविता यथार्थ है, मौलिक है, सहज है और जन जीवन से जुडी हुई है। इस प्रकार कवि के सोच का संसार बहुत व्यापक है।

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समकालीन हिन्दी महिला उपन्यासों में नारी–विमर्श

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कला व विज्ञान महाविद्यालय दोंडाईचा

आधुनिक युग में सबसे अधिक चर्चा जिस विषय की हो रही वो है, किसान, किन्नर, वृद्ध, पर्यावरण, दलित आदिवासी एवं नारी विमर्श आदि की चर्चा हो रही है। मनुस्मृति में नारी के संबंध में एक उक्ति मिलती है–"यत्र नार्यस्तु पूज्यन्ते रमन्ते तत्रदेवता" अर्थात जहाँ नारियों की पूजा होती है, वहाँ देवताओं का निवास रहता है। इस कथन से पता चलता है कि समाज को नारी का अनादर नही करना चाहिए। बल्कि आदर करने के लिए प्रेरणा करना चाहिए। जहाँ नारी का सम्मान एवं आदर किया जाता है। वहाँ सुख, समृद्ध एवं शांति रहती है। समाज रूपी रथ के दो पहिये नारी और पुरूष है। इन में एक भी पहिया निकल जाये या तो छोटा–बडा कर दिया जायेगा तो समाजरूपी रथ नही चल पायेगा।

हिन्दी साहित्य की प्रत्येक विधाओं में नारी विमर्श की विचारधारों को प्रतिबिंबित किया है। नारी जीवन की यथार्थमय सामाजिक दशा का चित्र खीचा है। नारी के जन्म पूर्व से लेकर असकी अंत्योंष्टि तक की गाथा को सार्थक चित्रण किया है। पुरूष प्रधान संस्कृति में नारी कोसमता की भावभूमि पर अधिष्ठापित कराना एक बडा चुनौतिपूर्ण कार्य रहा है। 'साकेत' काव्य में कवि मैथिलीशरण गुप्तजीने नारी जीवन की करूण कथा कही है –

"अबला जीवन हाय तुम्हारी करूण कहानी
आँचल में है दूध और आखों में पानी।"[१]

नारी विमर्श को डॉ.अर्जुन चव्हाण ने इस अवधारणा को स्पष्ट करते हुए कहा है – "हिन्दी उपन्यासों में संदर्भ में वस्तुत: नारी विमर्श समकालीन विचार चिंतन है। समकालीन हिन्दी उपन्यासों में नारी विमर्श की अपूर्व पहल नजर आती है। यह सही है कि नारी जीवन हिन्दी के आरंभिक उपन्यासों का भी केंद्रीय विषय रहा है। चाहे 'भाग्यवती' हो, चाहे देवरानी जेठानी की कहानी नारी जीवन का चित्रण दोनों का मूल विषय रहा है। इन सभी में नारी की या तो जीवन गाथा केंद्र में रही है या उसकी व्यथा–कथा अपनी अपनी अस्मिता, आत्मचेतना और अस्तित्व बोध के प्रति नारी की सजगता असल में स्वांतत्र्योत्तर कालीन उपन्यासों में ही उभरने लगी। समकालीनअर्थात सन १९६० के बाद के उपन्यासों में यह उत्तरोत्तर चेतना स्त्री विमर्श की मुख्य शक्ति है, जो समकालीन हिन्दी उपन्यासों में प्रर्याप्त मात्रा में परिलक्षित है।"[२]

नारी विमर्श परम्परागत भारतीय नारी संहिता के विरूद्ध एक गंभीर बहस है। साथ ही नारी की सार्थक पहचान बनाने का संघर्ष भी। इन्ही केंद्र बिंदू को लेकर हिन्दी उपन्यासकारों ने नारी जीवन की व्यथा को समाज के सामने प्रस्तुत किया है। जिसके फलस्वरूप हिन्दी साहित्य में उषाप्रियंवदा, कृष्णा सोबती, मृदुला गर्ग, सुर्यबाला, नासिरा शर्मा, प्रभा खेतान, अलका सरावती, चित्रा मुद्गल, ममता कालिया, क्षिति शर्मा, अनामिका ऋता शुक्ला, दीप्ति खंडेलवाल, कांता भारती, मैत्रेयी पुष्पा आदि महिला लेखिकाओं ने नारी विमर्श के आंदोलन को धार दी।

नारी विमर्श ने हिन्दी उपन्यासों में नारी को देखने–समझने की नई दृष्टि दी है। केवल नारी की पुरूषों से बराबरी का मुद्दा ही महत्वपूर्ण नही है। समाज के हर क्षेत्र में अपनी अस्मिता, स्वातंत्रता और निजता

अधिकार के लिए लडना नारी विमर्श का बडा लक्ष्य है। विगत तीन–चार दशकों में हिन्दी उपन्यासों में नारी विमर्श को लेकर बहुत कुछ लिखा गया है। नारी कभी माँ, बेटी, पत्नी एवं बहनआदि अनेक रूपों में विद्यमान रही है। नारी से ही नरशक्तिमान हुआ है। नारी नर के के जीवन की पोषण एवं प्रगति करती रही है। ममता, त्याग, करूणा की मूर्ति, कोमलता एवं वात्सल्य आदि अनेक उपमा नारी को दी जाती है। वास्तविक रूप से नारी कोई पहचान नही है। वह अपनी पहचान की तलाश में सदैव रही पर उसकी उपेक्षा ही हुई।

ममता कालिया के उपन्यास में नारी का परिवर्तित रूप देखने को मिलता है। रूढियों से जुझती नारी अपनी आजादी के लिए जुझती नारी अपने संघर्ष में कभी सफल–असफल होती नारी का चित्रण देखने को मिलता है। 'नरक दर नरक' उपन्यास की नायिका उषा प्रेम की जटिलता का अनुमान करके ही चौकनी रही थी। इतनी चौकन्नी होने के बावजूद पता नही कैसे जगन और उषा के बीच प्रेम घुसपैठ कर गया। तब रात के वक्त दोनों अपने–अपने अकेलेपन से पैदा हुई जरूरतो के मारे हुए थे। ममता के उपन्यासों में नारी की विवाहपूर्व विभिन्न संवेदनाओं की यथार्थ एवं मार्मिक अभिव्यक्ति हुई है। प्रेम की तरलता का एहसास ही भिन्न होता है। प्रेम की इसी ज्वाला में जगन ने लिप्ट में उषा की आँखे चुमली। लिप्ट के साथ ही उषा का दिल भी तेजी से नीचे उतरा फिर धीरे–धीरे स्थिर हो गया, एकदम जड, भय और बौखलाहट में"[३]

प्रभा खेतान का 'छिन्नमस्ती' उपन्यास नारी यातना, मुक्ति एवं विद्रोह को प्रस्तुत करनेवाला उपन्यास है। उपन्यास की अधिकाश नारी दर्द को एवं पीडा को अपने अंदर समेटे हुए है। दाई माँ उपन्यास के चुपचाप देखती है। जब असहाय हो जाता है तब माँ चली जाती है। छोटी माँ के आँसू नीना में विद्रोह भर देते है। प्रिया की माँ शोषक और शोषित दोनों रूपों में प्राप्त होती है। उपन्यास की नायिका प्रिया को विद्रोह बनानेवाली घटनाओं की श्रृंखला बडी होती है। वह विसंगत परिस्थितियों में भी छिन्नमस्त बनकर अपना मार्ग स्वयं चुनती है। उसमें जीने की तीव्र जिजविषा है।

मृदुला गर्ग के प्रायःसभी उपन्यासों में नारी विमर्श के विभिन्न रूपों को देखा जा सकता है। 'कंठगुलाब' तो नारी विमर्श का सशक्त उपन्यास है। अत्याचारी पति लकवे से अपंग होते ही नमिता उसकी उपेक्षा एवं तिरस्कार करती है। यह प्रतिशोध मुख्यतः असीमा के चरित्र मेंसबसे अधिक दिखाई देता है। छोटी उम्र में हीवह सबकुछ जान लेती है। दुनिया में रहने के लिए होम साईस नहीं कराटेकी जरूरत है। कराटे सीखना मोक्ष पाने के समान है। वह नमिता के पति पर अपने कराटे के दॉवपेच कुछ ऐसे आजमाती है कि कुछ देर बाद ही उसे लकवा हो जाता है। असीमा को नारी का शक्तिरूप ही पसंद है। वह अपनी लडाई स्वयं लडना जानती है। "उसमें प्रतिशोध इतना तेज है किवह अपना नाम सीमा से बदलकर असीमा रख लेती है। अपने पिता को वह हरामी नंबर एक और भाई को हरामी नंबर दो कहती है।"[४]

मैत्रेय पुष्पा का 'चाक' उपन्यास एक विद्रोह गाथा है। अंतरपुर की औरतों को पुरूषीय अहं, शील और सतीत्व की रक्षा के नाम पर बलि चढा दी जाती है। सीता की तरह भूमि प्रवेश करती है। इस गॉव के अनेक इतिहास के दस्तावेज दर्ज है। करमवीर की पत्नी साधजी और हुकूमकौर की बहू तथा डोरिया और थानसिंह की भाभी रेशम अपने पतिकी मृत्यू के पाच महिने पश्चात गर्भवती रह जाती है। अपने द्वारा उठाया गया कदम उसे गलत नहीं लगता। वह अपनी सास से जिस प्रकार बाते करती है, उससे उसकी विद्रोही प्रवृत्ति सामने आती है। पत्नी के मृत्यु के बाद पति दूसरी शादी कर सकता है और पत्नी मात्र जीवनभर उसके दुःख में डूबी रहे, यह उसे स्वीकार नही है। वह मनुष्य होने के कारण देह की जो आवश्यकता है, उसे पुरा करना जरूरी समझती है वह अपनी सास से कहती है – "माईयों! तुम मेरे पीछे क्यों पड पड गई हो। मेरे चालचलन की

झंडी पहराना जरूरी है। बिरथा ही छानबीन करने में लगी हो। आज को तुम्हारा बेटा मेरे जगह होता तो पूछती किंतु किसके संग सोया था? अब उसकी बॉह गहले । मेरे मरे पीछे तेरही तक का भी सबर न करता और ले आता दूसरी। तुम खुश हो रही होती कि पूत की उजडी जिंदगी बस गई। पर मेरा फजीता करने पर तुली हो।"[५]

'अनारो' यह मंजुल भगत की प्रौढतम् कृति है। लेखिकाने मध्यमवर्ग एवं निम्न वर्ग की सारी भयावह सच्चाईयों को उजागर करने का प्रयास किया है। अनारों इस उपन्यास की प्रधान नायिका है जो इमानदार, स्वाभिमानी, रूढिप्रिय, आत्मनिर्भर एवं समाज भीत नारी है। महानगरीय झुग्गी कालोनी में रहकर वह कोठियों में काम करती है। इसका पति नंदलाल आलसी, कामचोर, चरित्रहीन तथा कर्तव्यहीन है। परिणाम स्वरूप घर गृहस्थी का सारा भार अनारों पर आता है। वह कभी घर से भाग जाता है। अब भी घर आता है तो शराब के नशे में डूबा रहता है। घर आने पर अनारों को पिटता है। वह नंदलाल के प्रेमिका छबीली से संबंध होने की बात को जानते हुए भी कभी उससे रूष्ट नही होती। नंदलाल घर से भागता है वह उतनी ही तत्परता से उसकी राह देखती है। कठोर जुबानवाली अनारों हृदय की अति कोमल तथा संवेदनशील है। उसके पति ने दुःख के अलावा कुछ नही दिया फिरभी वह पति को ही परमेश्वर मानती है।

भारतीय नारी का सच्चा प्रेम आज के पुरूषों को दिखाई नही पडता है। मंजुल भगत का 'टूटा हुआ इंद्रधनुष' उपन्यास में प्रेम का भिन्न रूप को दर्शाया गया है। 'शोभना' इसउपन्यास की नायिका है। जो प्रेम को वासना से मुक्त रखकर उसे एक आदर्श तथा उदात्त रूप देना चाहती है। इस भय से कही कुरूप को सत्य माननेवाला यह संसार उसके प्रेम को भी यथार्थ की शिला पर पटक पटककर नीरस न बना दे। वह उसे एक ऐसा स्वप्निल रूप दे देती है जो केवल आत्मिक तथा भावनात्मक स्तर पर ही जिया जा सकता है। मनीष शोभना का प्रेमी है। वह प्रेम को यथार्थ मानता है। और उसे ज्ञात है कि हर यथार्थ की एक मांग होती है। जो उसे देनी पडती है। शोभना के अनुसार प्रेम की क्षणिक अनुभूति भीउतनी ही सत्य है चाहे वह कितनी भी नश्वर क्यों न हो। इसी कारण वह मनीष से विवाह नही करना चाहती। वह मनीष से कहती है – "मनीष यदि दिनरात तुम्हारी नाक के सामने कोई मखमली सुंगधित लाल गुलाबों का गुलदस्ता थामकर कहे, तुम आठो पहर उन्हें ही सूंघते और देखते रहो, तो बताओ तुम कर सकोगे? प्यार भी ऐसा ही है।"[६] यहॉ पर शोभना अपने प्रेमी को पति नही बनाना चाहती और कहती है कि –"तुम्हारे और मेरे बीच में जो भी है, मै उसे रिश्ता नही बनाना चाहती रिश्ते उब जाया करते है।"[७] शोभना की दृष्टि में शरीर की वास्तविकता केवल रोग और मृत्यु नही अपितु जन्म और यौवन भी है। वह मनीष से इसलिए विवाह नहीं करना चाहती कि आठों पहर जीवन से जुझने के लिए मनीष की आवश्यकता नही है।

भारतीय नारी का सामाजिक एवं आर्थिक विषमताओं से उत्पन्न मानसिक यंत्रणा का बडा सजीव चित्रण उषा प्रियवंदा का 'पचपन खम्भे लाल दीवारे' उपन्यास में हुआ है। सुषमा उपन्यास की नायिका है। यह उपन्यास अंतर्मुखी, कुंठित स्वतंत्रता और कर्तव्य के बीच छटपटाती भीरू सुषमा की करूण कहानी है। सुषमा का चरित्र एक चेतना के रूप में ही लेखिका ने उद्घाटित किया है। मॉ उसे केवल पैसा कमाने का साधन मानती है। उसके मन को नही जानती। इसी से सुषमा विद्रोही बन जाती है। वह अपनी मॉ से कहती है – "जरा अपने दिल के अंदर झॉककर देखो कि तुमने मेरे लिए क्या किया है। मेरा आराम से रहना ही तुम्हे खटकता है। तुम शादी तय करो नीरू की मै अपने सारे गहने कपडे उठाकर दे डालूंगी। यही तो तुम चाहती है।"[८] चारित्र्यहीनता के डर से वह अच्छे वेतनवाली नौकरी भी छोड देती है। वह एक स्वाभिमानी होने के

कारण नौकरी के क्षेत्र में पवित्र रहती है। इसके माध्यम से उषा प्रियवंदा जी ने महिला वर्ग जागृत किया है कि पैसे के लिए सामाजिक वासना का शिकार मत हो जाओ।

आज की नारी नौकरी व्यवसाय में अग्रेसर होकर अपने आत्मसम्मान को पहचानने लगी है। नारी विमर्श को समर्थन देनेवाली नारीवादी लेखिका प्रभा खेतान लिखती है कि – "स्त्री की मुक्ति उसके आर्थिक दृष्टि से स्वावलंबी होने में है।"[९] उनके छिन्नमस्ता उपन्यास की नायिका प्रिया नौकरी के लिए पति से संघर्ष करती है। और प्रतिभासंपन्न सशक्त संकल्प से उँची स्थान पर पहूँचती हैं। उसकी उन्नति पति को बर्दास्त होती नही है। मालती आदवानी के बारे में कहती है – "प्रिया उर्जावान बनकर मनुष्य बनकर पारिवारिक दमन शोषण के विरूद्ध स्वयं आवाज उठाती हैं दूसरों को भी प्रेरित करती है।"[१०] प्रिया की तरह वंशदा भी ब्युटी क्लिनिक चलाती है। नीना भी प्रिया के साथ काम करती है।

'अकेला पलाश' उपन्यास की नायिका 'तहमीना' नौकरी के बहाने घर से दूर रहती है। दरसल घर मे पति से यौन तश्पजी में वह असंतुष्ट है। लेखिका स्पष्ट रूप में लिखती है – "पति भी उदासीनता से व्यथित होकर नौकरी करने को बाध्य होती है।"[११] यह उपन्यास नारी के अंतरंग में छूपी कई रहस्य को खूला करने का प्रयास करता है।

भारतीय नारियों का आर्थिक रूप से शोषण का शिकार बनाया जाता है। रूपया न कमाने के कारण वह पति के द्वारा चुपचाप अत्याचार को सहती है। विधवा नारियों की हालात तो बहुती खराब है। शिवानी का 'भैरवी' नामक उपन्यास में विधवा राजेश्वरी का चित्रण है। राजेश्वरी के पति के मृत्यु के बाद समाज कार्य में कार्यरत रहती है। उसके चित्रण के द्वारा लेखिका अन्य विधवा के जीवन को नया संदेश देती है। उसका वर्णन करते लेखिका लिखती है – "विधवा होने पर अपने ही साहस से अपने पैरो पर खडी थी। वैधव्यरिपु को उन्होंने पुरी शक्ति लगाकर धकेला था, संपूर्ण रूप मे पराजित और धराशाई कर दिया। एम.ए.एल.टी. कर अब प्रधानाध्यापिका थी।"[१२]

उपर्युक्त हिन्दी महिला उपन्यासों के अध्ययन से हम यह कह सकते है कि प्राचीन काल में नारी को जितने अत्याचार सहना पडा उतना ही अत्याचार, अन्याय, पीडा एवं दर्द आज की नारी को सहना पड रहा है। इसका महत्वपूर्ण कारण यही है कि नारी शिक्षित हुई है। परवह स्वयं अर्थोजन कर रही है। विधवा या तलाक पीडित होकर भी आज वह खुद को संभाल रही है। अनिष्ठ प्रथाओं, रूढिओं एवं अंधश्रद्धा का विरोध कर रही है। आज वह एक आदर्श और सत्वशील रूप में समाजमुख हो रही है। नये विचारधारा से प्रभावित होकर भी भारतीय नारी सजग बन रही है। फिर भी समाज में नारी की वास्तविकता स्थिति से पाठकों का परिचय करना अवश्य समझते है। नारी को सिर्फ पूजनीय ही नही बनाया बल्कि सच्चाई को धरातलपर खडा करने का कार्य किया है। नारी के विविध रूपों को अद्भूत संगम समकालीन महिला लेखिकाओं ने उपन्यास साहित्य में मौलिक काम किया है। नारी विमर्श के हर पहलुओं को सूक्ष्मतासे अवलोकन, परीक्षण एवं चित्रण हिन्दी उपन्यासओं में किया है।

संदर्भ

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रिसर्च जर्नी

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समकालीन हिन्दी कहानियों में दलित विमर्श

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साहित्य की कोई भी विधा उपने आप में किसी न किसी विमर्ष को लेकर चलती है। चाहे वह नारी, दलित, किसान, आदिवासी, मजदूर एवं पर्यावरण या अन्य किसी से संबंधित विमर्ष हो सकता है। साहित्य का सृर्जन मनुश्य की मानसिक, भावनिक एवं सामाजिक जरुरतों की सहज उत्पत्ति है। इसीलिए उसमें मनुश्य की भावनाएं, विचार, व्यवस्था से अपेक्षाएं, समाज के साथ संबंध, उत्तरदयित्व समाज के लिए प्रेरक विचारों की अभिव्यक्ति होती है। साहित्य समाज निर्माण कार्य का महत्वपूर्ण अंग है।

दलित षब्द की व्युत्पत्ति संस्कृत भाशा की 'दल' धातू से हुई है जिसका अर्थ खंडित होना, कटना एवं अविकसित होता है। प्राचीन भारतीय साहित्य में षूद्र, दास, दस्यु, चांजल, अस्पृष्य एवं अंत्यज षब्दों का प्रयोग मिलता है। जो 'दलित' षब्द के प्राचीन रुप है। सन १९६० के बाद मराठी दलित साहित्य की तर्ज पर हिन्दी दलित में भी दलित साहित्य लिखा जाने लगा।

आधुनिक काल का हिन्दी कथा साहित्य अपने समय के समस्याओं को उजागर करने में सक्षम रहा है। दलित विचारधाराओं की अभिव्यक्ति तो साहित्य के माध्यम से हो रही है किन्तु वास्तविक जीवन में इसका कोई प्रभाव नही हुआ। हमारा देष लोकतंत्र के बुनियाद पर खडा है। लोकतंत्र के बल कागज और कलम का सेतु मात्र बना है। भारत लोकलंत्र देष है। यहाँ हजारों समस्याओं के कारण मानवीयता केवल दिखावा, ढोंग एवं नाटक लगती है। भारत हो या विष्व के कोई भी राश्ट्र जो वर्णभेद या वर्गभेद के कारण विशता मूलक समाज को जन्म देते है। संसार की प्रक्रिया में कई वर्ग उपेक्षित भूखमरी अथवा षोशण, अत्याचार, बलात्कार आदि के षिकार बन रहे है।

हिन्दी साहित्य की 'कहानी' सबसे लोकप्रिय विधा है। वर्तमान साहित्य में प्रकापित कहानियों में समकालीन हिन्दी कहानी की नब्ज को टटोला जा सकता है। सच तो यह है कि आज की कहानी में नारी पीडा समाज की संवेदना, ग्रामीण परिवेष आदिवासीयों का दुःख एवं दलितों का दुखभरा चित्रण दिखाई देता है। नये रचनाकारों के माध्यम से सामाजिक, आर्थिक, सांस्कृतिक, ऐतिहासिक स्थितियों पर तीव्र गति से लेखन कार्य हो रहा है। मानव ही कहानी का मुख्य विशय वस्तू है। सभी विमर्ष से आगे निकलकर समसामायिक प्रष्नों की अजुगूँज है और जीवंत प्रष्नों के जबाब खोजने का प्रयास कर रही है।

दलित विमर्ष पर कहानियों में दलितों के विद्रोह एवं ओक्रोष जीवन-संघर्श, मनुवादी मानसिकता का विरोध, प्रतिकूल नाटकीय स्थिति में जीवन जीने की विवषता और मानवीय अधिकारों के प्रति सजगता आदि को अभिव्यक्त किया गया है। सामाजिक विशमता एवं विसंगियों का विरोध यह दलित आत्म सम्मान की रक्षा का है, जिससे उन्हें सदियों से वंचित रखा गया है, वर्ण व्यवस्था के नाम पर भी उनके साथ जातिगत, धार्मिक एवं सामाजिक भेदभाव किया गया है, तो कभी उन्हें मूलभूत अधिकारों से वंचित रखा गया है। इसी सच्चाई को दलित कहानियों में उजागर किया है।

ओमप्रकाष वाल्मीकी की 'समाज' कहानी में हिंदू समाज व्यवस्था में व्याप्त अस्पृष्यता को दर्षाया गया है। कहानी के दलित गायक हरीष के सवर्ण मित्र कमल उपाध्याय को दलित समझकर अपमानित होना पडता है। अपने दलित मित्र की षादी में उपस्थित रहने पर सुबह चाय पीने के लिए वह चाय की दुकान पर पहूँचता है। चायवाला पहले उसे थोडा इंतजार करने को कहता है, परंतु जैसे ही उसे पता चलता है कि वह एक दलित बारात में आया है, तो उसके तेवर बदल जाते है। कमल उसके बदले व्यवहार को समझ नही पाता। वो पैसे देने की बात करता है तब चायवाला कठोरता से कहता है -वो पैसे षहर में जाकर दिखाना। दो पैसे हो या जेब में तो सारी दुनिया सिर पे ढाये घूमोकू कहीं और जाके पियो।१ स्पश्ट है की आज भी गॉवों में दलितों के साथ छूआछूत का कैसा व्यवहार होता रहा है।

मोहनदास नौषराय की 'अपना गाँव' कहानी दलितों पर अत्याचार करने का अधिकार गाँव के सवर्ण समाज को परम्परागत रुप में विरोध करने की हिम्मत किसी में नहीं।

जयप्रकाष कर्दम की 'लाठी' कहानी सवर्ण द्वारा दलितों को पिटने की परम्परा को लेकर आती है। फग्गन चाहकर भी बदनी से बदला नही ले सकता। हरिसिंह की कमर में लगी लाठी के दर्द को घर के सभी सदस्य अपने अंतर महसूस करते है, किंन्तु कुछ कर नहीं पाते। उनकी विवषता उनके दलित होने में पिघल जाती है।

डॉ. विवेकीराय की 'बडा आदमी' कहानी में भी इसी तथ्य की पृश्टि है। यह कहानी बडे आदमी के छोटेपन की है। बडे आदमी के उस बडेपन का पर्दाफाष करती है, जैसे उसने गरीब का पेट काटकर उसकी खडी फसलों को उजाडकर गाय, गोरु, खेतीपर कैंजा करके उसे बेघर करके पाया है। ऐसा आदमी बडा आदम कहलाता है। सामाजिक यथार्थ के विद्रुप चित्रण के साथ ही कहानी के षीर्शक में सार्थक व्यंग्य छिपा है।

हिन्दी की दलित विमर्ष परक कहानियों में दलितों की पीडा एवं वेदना को खुलकर अभिव्यक्ति हुई है। समकालीन परिप्रेक्ष्य में दलितों की उन्नति के प्रयत्नों को अनदेखा नही किया जा सकता है। भारत के कई भू-भागों में आज भी दलितों को दर्दनाक स्थिति से गुजरना पड रहा है, जिसे कहानीकार अपने लेखन में दर्षाता है। सूरपाल चौहान की 'टिल्लू का पौता' कहानी छुआछुत के भेदभाव में दलितों को किस प्रकार प्रताडिया किया जा रहा है, इसे स्पश्ट करती है। कहानी का नौकरपेषा नायक हरिसिंह छुट्टी लेकर सहपरिवार विवाह के लिए अपने गाँव सोनपूर चला जाता है। गाँव पैदल चलते समय प्यास लगने पर बुढे सुवर्ण के कुएँ के पास पहुँचकर उससे अनुमति लेकर बाल्टी से रस्सी बाँधकर पानी निकालने की कोषिष करता है। तब बुढा सुवर्ण किसान कहता है - अरे रुको कौन गाँव जा रहे हो? वे भंगानिया, नेक पीछे हट को पानी पी यह षहर ना है गाँव है मारे लाठियों के कमर तोड दी जाएगी। सारे (साले) भंगिया-भंगिया चमारा के षहर के जाके नए,-नार लत्ता (कपडे) पहर के गाँव में आ जाते है। कछु (कुछ) पतौन चलतु कि जो भंगिय चमार के है कि नाय (नही)२ स्पश्ट है कि आजादी के सत्तर सालों बाद भी दलित आजाद नहीं है। उन्हें अभी भी देहातों में सुवर्णो के कुएँ पर पानी भरने का अधिकार नहीं है।

जातिय षोशण के संदर्भ में दयानंद बटोही की 'सुरंग' कहानी में दिखाई देता है। इसमें दलित छात्र को सवर्ण प्राध्यापक पी-एच.डी. करने से इनकार कर देते है। तब नायक का यह कथन सारी व्यवस्था से प्रष्न है डॉ. साहब आप भूल जाये कि मै हरिजन हूँ। गुलाम हूँ। पराधीनता की जंजीर टूटती है।

आज तक आप जैसे तानशाहों ने हम लोगों को अंधेरे को अंधेरे में रखा ही अब मै पूछता हूँ - आज क्यों रिसर्च करने देंगे।३

दलित विमर्ष परक हिन्दी कहानियों में यौन षोशण का चित्रण हुआ है। इसमें तथाकथित सवर्ण पुरुशों द्वारा दलित नारीयों का यौन षोशण निच्चित ही एक ओर समाज का घिनौना रुपर दर्षाता है, तो दूसरी ओर दलित अस्मिता को तोडनेवाले समाज के विरोध में दलितों में जागृती कर उन्हें यौन षोशण से मुक्ति दिलाने का काम करता है।

प्रेम कपाडिया की 'हरिजन' कहानी धर्म के आड में हो रहे दलित नारियों के षोशण को उजागर करती है। इसमें देवदासी प्रथा के नाम पर हो रहे दलित कुमारियों के षोशण की व्यथा-कथा है। कहानी की देवदासी परबतिया के बेटे - प्रेम द्वारा अपने पिता के नाम के बारे में पुछने पर उसका कथन है -बेटाकू ये हामरी बदनसीबी कि तेरे बाप का पक्का पता नहीकू जो औरत रोज नये मर्द के साथ सोती हो उसके बच्चे के बाप का नाम कैसे पता चल सकता है।४

रत्नकुमार सांभरिया की 'षर्त' कहानी में दलित पानाराम की बेटी पर सरपंच ठाकूर का बेटा बलात्कार करता है। कावेरी की 'सुमंगली' कहानी की अनाथ लडकी सुगिमा को ठेकेदार ने बारह वर्श की आयु में ही औरत बना दिया और कई वर्शो तक उसके षरीर का षोशण करता रहा। सी.बी.भारती की 'भूख' कहानी में दो बच्चे की माँ किसनी माय के आने पर असका बाप किरतो उसे गाँव के दिकू बाबू को पाँच सौ रुपयों में बेच देता है। जो उसे हमेषा के लिए नौकरानी और रखैल बनाकर रख देता है। यहाँ बाप अपनी बेटी को बेचकर पेट की भूख मिटाता है और दिकू बाबू उसे खरीद कर अपने षरीर की।

हिन्दी दलित कहानियों में धार्मिक स्थितियों का प्रतिरोध अत्यंत आक्रोषपूर्ण रुप में करती है। विषेशत: दलित कहानीकारों ने अपनी कहानियों में विशमतामूलक अर्थव्यवस्था का मानवीयता के धरातल पर विरोध किया है। ओमप्रकाष वाल्मीकी की 'सपना' कहानी का नायक गौतम एक प्रामाणिक और स्वाभिमानी पात्र है, जो गाँव के सभी लोगों के साथ मिलकर मंदिर का निर्माण करता है। दलितों द्वारा मंदिर निर्माण में विषेष प्रयत्नों के बाद जब मंदिर बन कर तैयार हो जाता है, तो अनुश्ठान के वक्त उसकी जाति आडे आ जाती है, और उनका स्थान जाति के अनुसार जुते-चप्पलों की जगह के आसपास निर्धारित किया जाता है। मंदिर तथा धर्म व्यवस्था में दलितों की हैसियत क्या होती है, इस तथ्य को उजागर किया है। नायक अपने अपमान को सह नहीं सकता और दंगा-फसाद कर समाज के सामने अपने हक की माँग करता है।

इस प्रकार देश में व्याप्त दलितों के मंदिर प्रवेष निशेध की समस्या को उठाया है। 'भय' कहानी में दलितों में धर्म के नाम पर प्रचलित अंधश्राधा को उजागर किया है। कहानी का मुख्य पात्र दिनेष अंधविष्वास में डुबकर भगवान को बलि देने हेतु सुअर के बच्चे की हिंसा करता है मनही मन घुटन में संत्रस्त होकर अंतत: पागल हो जाता है।

'भूमिधर' कहानी इस दलित यथार्थ साक्षात्कारी है कि समाज में षोशक वर्ग ही षक्तिषाली है। वह जब चाहे किसी भयानक अजगर की भाँति गरीबों को निगल सकता है। उसकी वक्र भृकुटि के प्रकोप से कोई और भदई जैसे लोगों के जीवन की षांति, व्यवस्था, सुख, सुरक्षा जब चाहे तब नश्ट की जा सकती है। दलितों के आर्थिक विकास में वर्ण व्यवस्था का विरोध सूरजपाल चौहान की 'साजिष' में दिखाई देता है। कहानी का पढा-लिखा नत्थू अपना व्यवसाय छोडकर बैंक से लोन लेकर मेटाडोर खरीदना चाहता है, तब मैनजेर सलाह देता है कि ट्रान्सपोर्ट के काम में कई प्रकार के लफडे है, फिर तुझे इस काम अनुभव नहीं है। यह काम तो बडे-बडे व्यापारियों का है।५ स्पश्ट है कि वर्णवादी व्यवस्था के हिमायती यही चाहते है कि दलितों का विकास न हो, वे अपने पारंपारिक व्यवसाय में जुडे रहे।

टोमप्रकाष वाल्मीकी की 'पच्चीस चौका डेढ सौ' कहानी दलितों की आर्थिक स्थिति के प्रतिरोध को दर्षाती है, जिसमे गाँव के द्वारा दलितो का कैसे चौधरी के आर्थिक षोशण हो रहा था स्पश्ट होता है। चौधरी दलित-गरिबों के पास से धन लुटने के लिए उन्हें पहले सूद पर रुपये देता और सौ रुपयों के डेढ सौ रुपये वसूल करता है। कहानी का नायक सुदिप पढ-लिखकर पिता को समझाना चाहता है कि पच्चीस चौका सौ होता है, डेढ सौ नही। इसीप्रकार 'अम्मा', 'खानाबदोष' कहानी में दलितों के आर्थिक षोशण को उजागर किया है।

हिन्दी दलित कहानियों में दलित अपने अधिकारों के प्रति सजग होकर उनमें परिवर्तन को दर्षाता है। सूरजपाल चौहान 'परिवर्तन की बात' कहानी में गाँव का रघु ठाकुर मरी हुई गाय को उठाने के लिए किसन के साथ जबरदस्ती करता है। तब वह नकारते हुए कहता है कि ठाकुर साहब अब समय बदल रहा है। क्या आप नहीं चाहते कि समय के साथ हम भी बदले।६ नैमिषराय की 'आवाजे' कहानी में सदियों से अन्याय-अत्याचार सहनेवाले में हत्तर समाज के लोग ठाकूर अवतारसिंह के खिलाफ विद्रोह करते है - हम जूठन न लेंगे और न गन्दगी साफ करेंगे।७ यह निष्चय ही समाज परिवर्तन की ओर ले जानेवाला है।

ओमप्रकाष वाल्मीकी की 'चिडीमार' एक सषक्त कहानी है। एक भंगी समाज से आयी युवती का सरकारी नौकरी करते हुए ऑफिस से लेकर रोड-रोमियों का घर तक पीछा करना, जातिवाचक षब्द से चिल्लाना वर्तमान समय के यथार्थ को प्रसतुत करता है।

दलितों पर होनेवाले अन्याय, अत्याचारों के प्रतिषोध की मानसिकता को दलित कहानियों में उभार गया है। सवर्णो द्वारा बलात्कार एवं देह का षिशण आदि का कहानियों के पात्र खुलकर विरोध कर प्रतिषोध की मानसिकता मे दिखाई देते है। डॉ. कुसुम वियोगी की कहानी 'अंतिम बयान' की दलित नायिका अतरो भी स्वयं पर गाँव के मुखिया के बेटे राजेंद्र द्वारा बलात्कार के इरादे से किये गये निंदनीय व्यवहार का प्रतिषोध वह राजेंद्र का षिष काटकर लेती है। स्पश्ट है कि दलित समाज की नारियों में अपने षोशण को लेकर प्रतिषोध का भाव पनपता है।

रत्नकुमार सांभरिया की 'क्षितिज' कहानी की रेवती ससुराल में जमीनदार की हवसका षिकार होते समय अपनी इज्जत की रक्षा हेतु जमीनदार नानकसिंह की नाक पर खुरपु का वारकर उसकी नाक काँट देती है। ओप्रकाष वाल्मीकी की 'मुंबई कांड' कहानी में दलितों के प्रतिषोध का मानवीय रुप दिखाई देता है। कहानी के सुमेर को मुंबई में अम्बेडकर की मूर्ति को अपमानित करना तथा उनके समर्थकों पर गोली चलाना आदि घटनाएँ अखेरती है। वह इसे अपमान समझता है और इसका बदला गाँधीजी की मूर्ति को जूतों का हार पहनाकर लेना चाहता है, पर वह ऐसा नही करता। उसे वास्तविकता का पता चलने पर उसकी मानसिकता बदल जाती है। कुछ गाँधी को बापू कहते है और कुछ आम्बेडकर को बाबा कहते है। वहाँ बाबा कहनेवाले मारे गये, यहाँ बापू कहनेवाले मारे जायेंगे।८

समकालीन दलित विमर्ष का अध्ययन करते हुए दलित तमाम यातनाओं, कश्टों, प्रताडनाओं एवं उपेक्षाओं को भोगे हुए यथार्थ के आधार पर प्रामाणिक एवं मार्मिक अभिव्यक्ति मिली है। हिन्दी का दलित विमर्ष परक कहानी - साहित्य आज भी प्रासंगिक है। इसमें चित्रित दलितों का सामाजिक, धार्मिक एवं राजनैतिक परिवेष तमाम विसंगतियों और विशमताओं को चित्रित करता है। हिन्दी दलित कहानी दलितों की पीडा, वेदना की अभिव्यक्ति सक्षमता से करती है। इसके अलावा दलित विमर्ष के रुप में आर्थिक और धार्मिक विशमताओं का प्रतिषोध, यौन षोशण, प्रतिषोध की मानसिकता,

अधिकारों के प्रति सजगता एवं प्रामाणिक अनुभवों की यथार्थ अभिव्यक्ति जैसे कई आयाम सामने आते है।

संदर्भ सूची

१) ओमप्रकाष वाल्मीकी - सलाम, पृ.१२
२) सूरजपाल चौहान - हैरी कब आयेगा ?, पृ.२६
३) दयानंद बटोही - सुरंग, पृ. १०९
४) समस
५) सूरजपाल चौहान -साजिष, पृ.११३
६) सूरजपाल चौहान - हैरि कब आयेगा ?, पृ.२०
७) मोहनदास नैमिषराय - आवाजे, पृ.६
८) ओमप्रकाष वाल्मीकी - मुंबईकांड (घुसपैठिये), पृ.४०

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४. लोकमाता अहिल्याबाई होळकर (गौरवग्रंथ) - प्रा. एकनाथ बोडखे, विद्यावार्ता प्रकाशन, लातूर पृ. १६२

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७. किता, पृ. १७३ ते १७४.

८. दै. लोकमत - ३१ मे २०१६ पृ. १८

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११. किता, ११७

१२. अहिल्याबाई होळकर - विमल खडसेकर, राजहंस प्रकाशन, पुणे पृ. १४५

१३. लोकमाता अहिल्याबाई होळकर (गौरवग्रंथ) - संपादक डॉ.व नवीद शिंदे, स्नेहवर्धन प्रकाशन पुणे २०१४ पृ. १२

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स्वातंत्र्य युध्दातील खानदेशातील आदिवासी योध्दा व संघर्ष

प्रा. डॉ. आर. एस. पवार
मोतीप्रगतशाळ विभाग प्रमुख,
आर.सी.पटेल महाविद्यालय शिरपूर जि. धुळे

प्रस्तावना :-

१९४७ ला भारताला स्वातंत्र्य मिळाले ते केवळ स्वातंत्र्य लढा देवूनच. इंग्रजांना चले जाव चा नारा केवळ पंडित नेहरू स्वातंत्र्य सेनानींनी दिला असे नव्हे तर खानदेशात खानकोपऱ्यात आपली जीवन व्यतीत करून त्यांनी आपली संस्कृती व सामाजिक चाल आणि राष्ट्रप्रेमाची भावना जोपासली होती. त्यांच्यातल्या स्वाभिमानी स्वभावामुळे त्यांनी इंग्रजी सत्तेच्या विरोधात बंडाचे निशाण उभारले. ब्रिटीश सत्तेविरुध्द शस्त्र उगारण्याचे पहिले कार्य महान आदिवासी योध्दांनी केले. हिंदुस्थानावर ज्या ज्या वेळी आक्रमणे झालीत त्यांना प्रतिकार करण्याची क्षमता केवळ आदिवासी योध्यांमध्ये होती. त्यांची अतुलनिय कामगिरीचा इतिहास दुर्लक्षित झालेला दिसतो. तसेच त्यांच्या कार्याचा इतिहासावर म्हणावा प्रकाश टाकलेला दिसत नाही. भारतीय स्वातंत्र्य चळवळीच्या इतिहासात आदिवासी जमातीचे योगदान महत्वपूर्ण आहे. प्रस्तुत शोध निबंधात महान आदिवासी योद्धयांचा व त्यांच्या प्रमुख लढ्यांचा इतिहास मांडण्याचा प्रयत्न केलेला आहे.

उद्देश :-

१. स्वातंत्र्य संग्रामातील आदिवासी महान योद्धांचे इंग्रजविरुध्द उठावातील योगदानाचा अभ्यास करणे.

२. स्वातंत्र्य संग्रामातील प्रमुख आदिवासी महान योद्धांचा पराक्रमाचा परिचय करणे.

३. स्वातंत्र्य लढ्यातील खानदेशातील प्रमुख लढ्यांचा अभ्यास करणे.

४. इंग्रजविरुध्द आदिवासी लढाई संघाचा अभ्यास करणे.

संशोधन पध्दत :-

प्रस्तुत शोध निबंधात प्राथमिक व दुय्यम साधनांचा संदर्भ

घेतला असून ऐतिहासिक ग्रंथ, संशोधनपत्रिका यातून माहिती संकलन करून प्रस्तुत शोध निबंध अभ्यासपूर्ण विश्लेषणात्मक मांडण्याचा प्रयत्न केला आहे.

विषय विवेचन :-

१८१८ साली पेशवाईचा शेवट होऊन खानदेशसह महाराष्ट्रात इंग्रजी सत्ता प्रस्थापित झाली. भारतीय स्वातंत्र्याच्या चळवळीचे पर्व विलक्षण तेजेस्वी आहे, देशावर प्रेम आणि आत्मसमर्पण करणा-यांचे योगदान फार मोठे आहे. महाराष्ट्रात जे आदिवासी बंडखोर होते त्यात खानदेशातील भिल्ल प्रमुख होते. इंग्रजी राजवट विरुध्द १८१८ ते १८५७ च्या स्वातंत्र्य युध्दात भिल्लांनी ब्रिटीशांच्या अन्यायी राजवटीविरूध्द उठाव केला. उत्तरेला सातपुड्या पर्यंत दक्षिणेस सातमाळा, चांदोर, अजिंठा पर्वतरांगेपर्यंत पसरलेला प्रदेश म्हणजे खानदेश होय. खानदेशातील जमातीत भिल्लांची संख्या मोठी होती. त्यांचे अधिष्ठान रानात, डोंगरकपारीत असून सह्याद्री ते सातपुडा पर्वतांची साखळीचे दुवे आदिवासी भिल्ल होय. इंग्रजांचा सततचा होणाज्या अन्यायामुळे भिल्ल जमात फारच हवालदिल झाली. अन्याय करणा-या गो-यांना इंगा दाखविण्याचे त्यांनी ठरविले. यासाठी सातपुडा, सातमाळा आणि अजिंठ्याच्या कुशीत वावरणारे सर्व आदिवासी भिल्ल एकत्र झाले. तलवारी आणि बनाट्या घेऊन स्वातंत्र्य युध्दात अन्यायाविरूध्द उठावात सहभागी झाले.

खानदेशातील स्वातंत्र्याचा लढा :-

पेशव्यांचा प्रदेश चार विभागात विभागून प्रत्येक विभागावर स्वतंत्र इंग्रज अधिकारी नेमले. अहमदनगर, पुणे, धारवड, व खानदेश. खानदेश विभागावर कॅप्टन ब्रिग्ज यांची नेमणुक केली. त्यावेळी खानदेशात आदिवासींनी धुमाकूळ घातला होता. इंग्रजी सत्तेविरूध्द त्यांनी बंड पुकारले होते. खानदेशात आदिवासी भिल्लांची संख्या फार मोठी होती.

आदिवासींचा भूप्रदेश खानदेश :-

महाराष्ट्राच्या उत्तरेला सातपुडा पर्वत, दक्षिणेस सातमाळा, चांदोर, अजिंठा पर्वत रांगेपर्यत पूर्वेस हाती टेकड्या व पश्चिमेस सह्याद्री डोंगराच्या रांगापर्यंत पसरलेला हा भूप्रदेश होता. चिंचोळी पट्टीचा तो प्रदेश खानदेश म्हणून ओळखला जातो. ब्रिटीश साम्राज्यकाळात जळगांव व धुळे मिळून खानदेश एकच जिल्हा होता. पुढे पूर्व खानदेश व पश्चिम खानदेश असे दोन भाग करण्यात आले. खानदेश या नावाचे मूळ शोधण्याचा आजवर बराच प्रयत्न झाला. खांडनवन, कन्हदेश, स्कंददेश, खाणदेश, खानाचा देश अशी विविध नावांची उत्पत्ती संशोधकांनी केली. इ.स.१३ व्या शतकापासून या भूप्रदेशावर मोगलांची सत्ता रूढ झाली. गुजराथच्या राजाने या भूप्रदेशावर प्रभुत्व असणा-या फारूखी घराण्यातील खान ही संज्ञा दिली. तेव्हापासून या भूप्रदेशाला खानदेश हे नाव प्राप्त झाले. याच खानदेशात वास्तव करणाज्या आदिवासी जमातींचा उल्लेख स्वातंत्र्य लढ्यातील त्यांचे योगदान महत्वपूर्ण आहे.

खानदेशातील प्रमुख आदिवासी जमाती :-

महाराष्ट्रात जे आदिवासी बंडखोर होते त्यात खानदेशातील भिल्ल, पुणे सातारा जिल्ह्यातील रामोशी ही मुख्य जमात होती. इंग्रजांनी खानदेशावर इ.स.१८१८ मध्ये ताबा मिळविला तेव्हा भिल्लांनी उठाव करण्यास सुरूवात केली. त्यांचे लढाईचे तंत्र गनिमी कावा सारखे होते. सपाटीवरच्या प्रदेशावर येऊन लुटालुट करणे, सक्तीने पैसा वसूल करणे, गुरे पळवून नेणे, प्रवाश्यांना लुटणे व पसार होणे असे लढाईचे तंत्र अवलंबिले. ब-याचदा इंग्रजांनी त्यांच्याशी सलोखा करण्याचा प्रयत्न केला. परंतु अन्याय व अत्याचार विरूध्द आदिवासींनी आपले बंड सुरूच ठेवले. १८५७ च्या स्वातंत्र युध्दात भिल्लांनी खानदेश, नाशिक व अंबापाणी अशा अनेक ठिकाणी इंग्रजांशी संघर्ष केला.

स्वातंत्र्य लढ्यातील खानदेशाचे महान योध्दे व त्यांचे उठाव :-

सातपुड्याच्या कुशित राहणा-या आदिवासींचे जीवन अतिशय साधे व आनंदीत होते. जंगल हा त्यांचा पोटाचा मुख्य आधार होता आणि तेच त्यांच्याकडून हिरावून घेण्याचा प्रयत्न केला. १८१९ मध्ये सर्व आदिवासी भिल्लांनी सर्व बाजूंनी उठाव केला. डोंगरी भागातील ठाणी आदिवासी बंडखोरांनी काबीज केली व आपल्या सोबतींना पठारावर पाठवून लुट करणे हे तंत्र त्यांनी वापरले. या विरोधात इंग्रजांनी कडक पावले उचलली. काही ठाणी इंग्रजांनी ताब्यात घेतल्या. बज्याच शुर आदिवासींना फाशी देण्यात आली. परिणामी आदिवासी भिल्ल अधिक क्रोधीत झाले. परिसरातील सर्व एकत्रित येऊन इंग्रजांचा अंमल असलेल्या खेड्यात लुटमार, दरोडे, खुन अशी दहशत निर्माण केली. स्वतंत्र टोळ्या इंग्रज राजवटीविरूध्द उभ्या राहिल्यात. अमळनेर उपविभागातील पारोळा गावातील उठाव कॅप्टन ब्रिग्ज च्या हत्येचा प्रयत्न झाला. यात दशरथ बांड, शेख दुल्ला यांनी सहकार्य केले. इंग्रजाविरूध्द या स्वातंत्र्य लढ्यात नेतृत्व करणारे टोळ्यांचे प्रमुख हिज्या नाईक, हरिया भिल्ल, गुमानसिंग, भिमा नाईक, उमेड वसवा, झुंझार नाईक, भागोजी नाईक, देवाजी नाईक, तंट्या भिल्ल, बिरसा मुंडा, तानका भिल्ल, खा-या नाईक इत्यादी प्रमुख आदिवासी योध्दे होते.

महान योध्दा उमाजी नाईक :-

उमाजी नाईक याची स्वातंत्र्य लढ्यात महत्वपूर्ण भूमिका मानली जाते. इंग्रज सत्तेला हैराण करून सोडणा-या उमाजी नाईकचा बंदोबस्त करण्यासाठी इंग्रजच नाही तर इंग्रजांच्या गुलामगिरीत पंत, साचिव, निंबाळकर, पटवर्धन, जहागिरदार, सावकार, पाटील आणि

इंग्रजांनी खास पुना हॉर्सेची स्थापना करून उमाजी नाईकला पकडण्याचा जाहिरनामा रॉबर्टसनने काढला. त्या जाहिरनाम्याविरुध्द उमाजीने स्वत:ला राजा घोषित इंग्रजांना शह दिला. जाहिरनाम्याप्रमाणे खानदेशवासीयांनी त्यांना साथ दिली असती तर स्वातंत्र्य आधीच मिळाले असते.

महान योध्दा भागोजी नाईक :-

भागोजी नाईकाचा जन्म भिल्ल या आदिवासी जमातीत झाला. शेळ्यामेंढ्या चारणे हा त्यांचा मुख्य व्यवसाय होता. १८५७ च्या स्वातंत्र्य लढ्यात त्यांची महत्वाची भूमिका होती. इंग्रजांची खास नजर त्यांच्यावर होती. कारण इंग्रज सत्तेला हादरा देणारे व सळो की पळो करणारे भागोजी नाईक यांचे लढण्याचे तंत्र फारच निराळे होते. जंगलाची माहिती असल्याने उठाव करून ते पसार होत असत. १८ फेब्रुवारी १८५८ रोजी येवला येथील किल्ल्यात भागोजी नाईकाच्या टोळीवर मेजर पॅटिजर व कॅप्टन नस्सल यांनी अचानक हल्ला केला. या लढाईत भागोजींनी चाळीस इंग्रजांना ठार करून स्वत:ची सुटका करून घेतली. पुढे आपले संघटन वाढवत इंग्रजांना विरोध करीत राहिले.

महान योध्दा तंट्या भिल :-

स्वातंत्र्य लढ्यातील महान योध्दा म्हणून तंट्या भिल यांचे नाव घेतले जाते. तंट्या भिल जातीने गुन्हेगार मानला जात असे परंतु माणूस म्हणून आदर्श पुरूष होता. लहानपणापासूनच भाला फेकणे, धनुष्य बाण चालविणे, कुश्ती या गोष्टी शिकत होता. संरजामशाहीत तंट्याचे शेत गेल्याने संरजामशाहीविरूध्द सतत लढत राहिला.

महान योध्दा बिरसा मुंडा :-

बिरसा मुंडा यांनी कायदेभंगाची चळवळ उभारून आदिवासी समाज शोषण मुक्त करण्याचा प्रयत्न केला. राजकीय आंदोलन चालवून आदिवासींचे पारंपरिक हक्क, सांस्कृतिक अधिकार या विरूध्द उठाव केला. १८९५ मध्ये शेतसारा न भरण्याचे व कायदेभंगाचे आव्हान केले, त्यात त्यांना २० वर्षाचा तुरूंगवास झाला. पुढे इंग्रजांनी त्यांना मुक्त केले. जून १९०० मध्ये त्यांचा मृत्यू झाला.

महान योध्दा खा-या नाईक :-

खानदेशात खा-या नाईक यांनी इंग्रजी सत्तेला मोठे आव्हान केले होते. त्या काळात त्यांचे नाव घेण्यासही लोक घाबरत. त्यांचा जोडीदार भिमा नाईक या दोघांनी मिळून १५०० भिल्लांची फौज उभी केली. नोव्हेंबर १८५७ मध्ये कलेक्टर साहेबांचा मुक्काम होता. त्यांच्या शेजारील गावे लूटून कलेक्टरला शह दिला. खा-या व भिमा नाईक यांनी इंदोरहून मुंबईकडे इंग्रजांचा बैलगाडीतून चाललेला खजिना मोठ्या हिमतीने लुटला. इंग्रजांविरूध्द या दोन महान योध्द्यांनी स्वातंत्र्यासाठी शौर्य दाखविले.

महान योध्दा हरिया भिल :-

आदिवासींच्या उठावांपैकी अत्यंत परिणामकारक संघर्ष म्हणजे १८२२ मध्ये हरिया भिल्ल याने खानदेशात ब्रिटीशांविरूध्द बंड केले. त्याच्या विरोधात कॅप्टन रॉबिनसन यांची नियुक्ती करण्यात आली. हरिया भिल्ल याला शोधण्याचा इंग्रजांनी खुप प्रयत्न केला परंतु हरिया इंग्रजांच्या हाती लागला नाही. सातपुड्याच्या पर्वतरांगामध्ये पसार होवून गुप्तपणे नेतृत्व करीत राहिला.

महान योध्दा गुमान नाईक :-

१८२४ मध्ये गुमानसिंग नाईक हा थाळनेर परिसरातील सातपुड्याच्या पर्वतरांगामध्ये लुटमार करीत होता. त्याचा उपद्रव कमी व्हावा यासाठी ले. ॲड्रान, ले. लिग्वीस्टन यांची नियुक्ती करण्यात आली. थाळनेर परिसरात जवळपास ३५ घरे जाळून टाकली. चार पुरूष दोन स्त्रिया ठार झाल्या याचा परिणाम इंग्रज सरकारवर झाला. शांतता प्रस्थापित करण्यासाठी इंग्रजांनी बराच प्रयत्न केला. परंतु त्याचा फारसा परिणाम झाला नाही.

११ एप्रिल १८५७ चा खा-या नाईक व इंग्रज संघर्ष :-

आदिवासी भिल्लांचा उठावाचे स्वरूप दिवसेंदिवस वाढत होते. इंग्रज पलटणी हैरान झाल्यात. भिल्ल त्यांना पूरन उरलेत. खानदेशातील शिरपूर पासून २४ कि.मी. अंतरावर असलेल्या अंबापाणी येथे असलेल्या भिल्ल व ब्रिटीश सैन्यात भिषण लढाई झाली. आदिवासींचे नेतृत्व खा-या नाईक करीत होता. सोबत दौलतसिंग, महादेव नाईक, भिमा नाईक, हनुमंतराव नाईक हे मुख्य होते. अक्राणीमहाल भागात ब्रिटीशांविरुध्द असलेला काळूबाबा खा-या नाईकला येवून मिळाला. ११ एप्रिल १८५७ रोजी मेजर इव्हासन्स कॅप्टन बर्च, ले. वेसवी यांनी भिल्लांवर हल्ला केला. त्यात ६५ भिल्ल शहिद झाले, १७० जखमी झाले. कॅप्टन बर्च, ले.वेसवी जखमी झालेत. या लढाईत एक ब्रिटीश अधिकारी मारला गेला. या लढाईनंतर ड्रम ट्रायल खटला होवून ६२ आरोपींपैकी ५७ आरोपींना गोळ्या घालून ठार मारण्यात आले. या घटनेचा बदला नगरच्या भिल्लांनी घेतला. नांदगावच्या आसपास ते एकत्रित झाले ही बातमी ले.स्टुअर्टला कळताच ३०० सैनिकांची पलटण ४०० भिल्ल सैनिक अशी भिडत झाली. भिल्लांनी नदी किना-याने लगतच्या दाट झाडीचा आश्रय घेतला. दोघी बाजूंनी हल्ला सुरू झाला. भिल्लांनी इंग्रजांवर अक्षरश: आग ओकायला सुरूवात केली. त्यांच्या आगीच्या मा-याने कॅप्टन मॉन्टेगोमेरी जखमी झाला. कॅप्टन स्टुअर्ट मरण पावला. ५० इंग्रज सैनिक जखमी झाले. त्यात कॅप्टन चेंबरलेस, कॅप्टन डॉक्सीन यांचा समावेश होता. या लढाईत २५ भिल्ल शहिद झाले. या लढाईत इंग्रजांना विजय मिळाला असला तरी आदिवासी भिल्लांचा उठाव, संघर्ष सुरूच होता.

१७ नोव्हेंबर १८५७ ची खजिना लूट :-

१७ नोव्हेंबर १८५७ पर्यंत खा-या नाईक व भिमा नाईक यांनी १५०० लोकांची टोळी तयार केली. त्यावेळच्या खानदेशच्या हद्दीपासून ३० मैलावर उत्तरेस होळकरांच्या हद्दीतील जामली चौकीजवळ इंदोरहून मुंबईकडे जाणारा सात लाखाचा खजिना बैलगाड्यातून जात होता तो त्यांनी लुटला. या घटनेचा इंग्रज सत्तेला जबरदस्त आघात झाला. त्यानंतर सेंधवा घाटात टेलीफोनच्या तारा तोडल्या, पोस्ट ऑफिस लुटले, अफुने भरलेली सात बैलगाड्या लुटल्या. २९ ऑक्टोबर १८५७ रोजी शिरपूरवर हल्ला केला. ही घटना म्हणजे इंग्रज सत्तेच्या विरूध्द मोठे बंडच होते. कारण कॅप्टन बर्च हे या विभागाचे नेतृत्व करीत होते.

झुंझार नाईक व इंगज संघर्ष :-

खानदेशात चिखली नावाची दोन संस्थाने होती. एक गंगथा व दुसरी शहादा चिखली. झुंझार नाईक हा शहादा चिखलीच्या जहांगिरदारात एक बलाढ्य सरदार होता. तुकोजी होळकरांपासून चिखलीच्या जहांगिरदारचे होळकरांबरोबर संबंध होते. होळकरांबरोबर मोहिमेवर जात असतांना राणा भिकाजीने झुंझार नाईकचा वध केला त्याचा मुलगा देवाजी नाईक याने आपल्या पित्याचा बदला घेण्यासाठी स्वतंत्र टोळी जमविली व अक्राणीवर हल्ला केला. अक्राणी किल्ल्यावर लुटालुट करून जाळपोळ केली. आपल्या पित्याचा वध करणा-या भिकाजीचा त्याने वध केला. पुढे पेशवाई जावून इंग्रज सत्तेवर आले. कॅप्टन ब्रिग्ज यांनी देवाजी नाईकची पराक्रमी वृत्तीचा दखल घेत चिखली संस्थानची जहांगिरी त्याला दिली व आपल्या सैन्यात 'क' श्रेणीच्या लष्करी सेवेत सामावून घेतले. पुढे १८१८ च्या सुमारास जहांगिरदारच्या सनदांचा तपास करण्यासाठी पाडळदा येथे ब्रिटीश अधिकारी आले. देवाजी नाईकची सनदची मागणी करताच ही माझी सनद आहे म्हणून ब्रिटीश अधिका-यावर तलवार उगारली. पुढे इंग्रजांनी पकडून धुळे येथील तुरूंगात डांबले.

निष्कर्ष :-

१. खानदेशातील आदिवासी भिल्ल जमात मुळातच पराक्रमी व स्वाभिमानी तसेच राष्ट्रप्रेमी होती. त्यांनी इंग्रज जुलमी सत्तेचा विरोध केला. टिकटिकाणी बंड करून इंग्रजांना हैराण केले.

२. स्वातंत्र्य लढ्यात केवळ खानदेशातील नव्हे तर महाराष्ट्रभर आदिवासीची संघटना होती. त्यांनी टिकटिकाणी टोळ्या टोळ्यांनी इंग्रजांविरूध्द उठाव करून संघर्ष केला.

३. सातपुडा पर्वत रांगेत वास्तव्य करणारे व ज्यांची पोट त्या वन्य जंगलावर आधारीत होती. अशा जंगलावर अमंल बसवून ते नष्ट करण्याचा प्रयत्न स्थानिक सावकार, जमिनदार, व्यापारी यांना इंगजांनी संरक्षण देवून त्यांच्या विरोधात कार्यवाही केल्यात. त्याचा बदला म्हणून येथील आदिवासी भिल्लांनी स्थानिक जमिनदार व इंग्रजांविरूध्द बंड पुकारले. लुटमार करणे, खुन करणे, टोळ्या टोळ्यांनी संघर्ष करणे आदिवासी भिल्लांना पर्याय नव्हता.

४. आदिवासी भिल्ल जमातीचा लढा केवळ इंग्रज सत्तेविरूध्द होता. स्थानिक जनतेला त्यांनी त्रास दिला नाही.

सारांश :-

इंग्रज सत्तेत आल्यावर खानदेशातील आदिवासींच्या जंगल जमीन या मुख्य उदरनिर्वाहाच्या साधनावर कडक नियम व कायदे लावले त्यांना जंगलातून हाकलण्याचा प्रयत्न केला. म्हणून आदिवासींनी इंग्रजांविरूध्द बंड पुकारले. खानदेशचा भूप्रदेश आणि तेथील आदिवासी जमात मुळातच लढाऊवृत्तीची असल्याने इंग्रजांना त्यांच्या विरूध्द फार कौशल्य पणाला लावावे लागले. स्वातंत्र्य युध्दात आदिवासीची मोलाची कामगिरी दिसते. त्यांनी इंग्रजांना हैराण करून सोडले. त्यांच्या या बलिदानाची नोंद इतिहासात फार कमी आढळते. याचे मुख्य कारण या आदिवासी जमातीत शिक्षणाचा अभाव होता. शिक्षणाची गंगा त्यांच्याकडे जाई पर्यंत उशिर झाला. परंतु त्यांच्या बलिदानाची, त्यागाची व लढाऊ बाण्याची या खानदेशातील सातपुड्याच्या पर्वतरांगेत सदैव गुंजत आहे. त्यांच्या शौर्यगाथा उभा खानदेश कधीही विसरू शकणार नाही.

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