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Role of Systematic Foliar Micro-Morphology in Identification of Grasses

S. Pathak

Dept. of Botany, Mahadevananda Mahavidyalaya, Barrackpore, North 24-Parganas 700120.
Corresponding author : spathak.bsi@gmail.com

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ABSTRACT

The grass family is one of the largest of all plant families. Extensive survey and micro-morphological study was conducted to explore the diversity and generate a documentation of the family Poaceae (grasses) in north eastern parts of India. The region forms a significant segment of both the Himalayan and the Indo-Burma biodiversity hotspots. Although, floral morphological characters are noteworthy in the identification of all levels of taxonomic ranks as families, tribes, genera, species etc.; but members of the family Poaceae do not flower for a greater part of their life span; therefore foliar epidermal studies can be valuable and worthy in their categorization, completing their systematic position and illustrating their phylogenetic relationship among various taxa. This study can be considered subsequent to cytological enumerations. Systematic variation of foliar epidermis with the aid of Scanning Electron Microscope (SEM) pertains to the epidermal cell size, stomatal types, shape of silica bodies, micro-hairs, macro-hairs, subsidiary cells, prickles, hooks, papillae, short and long cells etc. on abaxial surface. Twenty (20) species of family Poaceae collected from the different localities of north east India were examined under Scanning Electron Microscope (SEM) for detailed micro-morphological variations. The species under investigation were selected on the basis of rarity in distribution in north east India. The present paper encompasses a detailed micro-morphological study along with illustrated key and vivid photographs to delimit allied species unfeasible to separate morphologically and proves to be supportive in determining the taxonomic as well as phylogenetic position of intimately related and complicated taxa.

Key words: Foliar, Identification, Grasses, Morphology, Systematic.

INTRODUCTION

Morphological characters play significant role in the identification of all levels of taxonomic ranks as families, tribes, genera, species etc. The family Poaceae (Gramineae) commonly known as 'grasses', comprises about 700 genera, 11,000 species distributed worldwide (Ullah et al., 2015; Chen & Peterson, 2006; Christenhusz & Byng, 2016). The foliar epidermal studies are important for categorization, delimitation and sorting out the evolutionary and phylogenetic problems in plants (Stace, 1984; Chaudhri et al., 2014; Bibi, 2015). Grasses do not flower for a greater part of their life span, therefore foliar epidermal studies are useful in completing their systematic positions and phylogenetic relationships among various taxa

(Ortunez, 2010; Yanis, 2010; Khan, 2017; Yan et al., 2018) and secondly it is significant after cytological studies (Goel et al., 2011; Nazir et al., 2013; Ullah et al., 2015). Heywood (1971) drew attention towards the microscopic studies in concluding systematic disparity among closely related and alike species (Metcalf, 1960; Stebbins & Khush, 1961; Chaudhri et al., 2014).

Stomatal characters are vital to delineate the diverse taxa in terms of phylogenetic and taxonomic considerations (Shaikh & Pötdar, 2018; Soreng et al., 2015). Angiosperm leaves are the most suited and preferable for this purpose (Watson, 1990). Information on foliar micro-morphology can shed more light on structural features and their possible functional

attributes (Perveen, 2006; Desai et al., 2013). Several authors recognized the importance of these stomatal features in recognizing the different taxa (Tateoka, 1959; Stebbins & Khush, 1961; Amarasinghe, 1988). It is well established that foliar anatomy and epidermal features are very important in grass systematics and has been utilized for characterization of broad groups, within sub-families and tribes (Redmann, 1985). Furthermore, the study is imperative to correlate between taxa and intimately associated genera (Vicari, 2002; Wu & Ge, 2012).

The foliar epidermis of grasses is constructed of two distinct types of cells; the long, rectangular cells with blunt ends arranged parallel with the long axis of the leaf and elongate horizontally, constitute slightly less than half of the total epidermal cells and are frequently referred as 'fundamental elements', 'undifferentiated cells', 'ordinary epidermal cells' or simply the 'long cells' (Ellis, 1979). Besides, the other type of cells are the 'short cells' which are cube to rectangular in shape with blunt ends formed as the product of asymmetric division of intercalary meristem which give rise to all cells except the long cells (Kaufman, 1970). The long and short cells together constitute the 'costal (C)' and 'inter costal (IC)' region (Acedo, 2001). These cells produce cork-silica cell (Sb), three types of prickles (Pr), macro hairs (Ma), which may be unicellular or 2-celled with basal cell (Bc) and apical cell (Ac), micro hairs (Mi), stomata (St) with prominent to blur subsidiary cells (Sc). Scanning Electron Microscopic (SEM) studies related to the morphology of the abaxial surface of the leaves of 20 species collected from the different areas of north east India has been worked out.

MATERIAL AND METHODS

North East India (262,179 km² area including 8 states) forms a significant part of both the Himalayan and the Indo-Burma biodiversity hotspots. SEM studies of the abaxial surface of the leaves of 20 species were carried out. The species were selected on the basis of their scarcity of distribution in this study area along with those species having difficulty in identification with allied taxa. All the samples were taken from live specimens collected during

the survey and are housed at CAL herbarium. Collection and preservation of specimens were done following Smith (1971), Jain and Rao (1977), Van Balgooy (1987), Rao and Sharma (1990) and Singh & Subramaniam (2008). Specimens were morphologically studied under Olympus SZ2-ILST Microscope and Nikon Stereo Zoom Microscope and live photographs were taken with 'Canon Power Shot SX50HS (50X optical zoom) camera'. Literature consultation were done following standard Floras, monographs and revisions (Bor 1940, 1960; Sreekumar and Nair 1991; Shukla 1996; Moulik 1997; Veldkamp 1999; Kabeer and Nair 2009), studying authentic herbarium specimens housed at ASSAM, ARUN, CAL (herbaria acronyms according to Thiers 2015) and matching with digital images of specimens including type materials available at different herbarium databases (<https://science.mnhn.fr>, <http://apps.kew.org/herbcat>, <https://plants.jstor.org>, http://linneanonline.org/linnaean_herbarium.html). For SEM study, the specimens were mounted on 'metallic stubs' with the help of 'double coated scotch tape' and then dried in the 'desiccator' without the use of any chemicals (Jeffree & Read (1991); Hartola, 2015; Butterfield 2017; Mohammed & Abdullah, 2018). The stubs were coated with gold ion in the 'Gold Coater' (Karnovsky, 1965; Baghaei, 2007; Murphy, 2010; Butterfield, 2017) and scanned at different magnifications in the 'HITACHI-S530 Scanning Electron Microscope, Japan', housed in the University of Burdwan.

RESULT

Characterization of the abaxial epidermis of leaf:

1. *Briza media*: Costal (C) and inter costal (IC) region distinct; IC region formed of thick walled, long cylindrical to tubular cells; prickle (Pr) 2-celled, basal cell (Bc) cylindrical, apical cell (Ac) acute, distributed only in the IC region; stomata (St) not frequent. S. Pathak 48474 (CAL). (Pl. 1. figs. A & B).
2. *Bromus catharticus*: Costal (C) and inter costal (IC) region distinct; IC zone formed of tubular, cylindrical cells; silica bodies (Sb) rectangular, arranged in parallel rows; macro

- hairs (Ma) unicellular, base of the hairs round to bilobed; stomata (St) scarce. *S. Pathak* 48570 (CAL). (Pl. 1. figs. C & D).
3. *Calamagrostis emodensis*: Costal (C) and inter costal (IC) region indistinct; prickles (Pr) present, bicellular, basal cell (Bc) cylindrical, apical cell (Ac) sharply acute, arranged in rows in the costal region; stomata (St) scanty. *S. Pathak* 48584 (CAL) (Pl. 1. figs. E & F).
 4. *Capillipedium parviflorum* (R. Br.) Stapf: Costal (C) and inter costal (IC) region almost indistinct; tubular, hypha-like deposition seen; silica bodies (Sb) globular, arranged irregularly all over the surface; stomata (St) arranged in rows; subsidiary cells (Sc) distinct, lanceolate with blunt ends. *S. Pathak* 48566 (CAL). (Pl. 1. figs. G & H).
 5. *Cenchrus prieurii* (Kunth) Mair: Costal (C) and inter costal (IC) region distinct; IC region made of narrowly, cylindrical cells with wavy walls; silica bodies (Sb) obscure; prickles (Pr) sharp, present at the edge of the IC region; stomata (St) shrouded deep within the costal region; guard cells swollen, helps in reducing transpiration; subsidiary cells (Sc) unclear. *S. Pathak* 48521 (CAL) (Pl. 1. figs. I & J).
 6. *Cynodon barberi* Rang. & Tadul.: Costal (C) and inter costal (IC) region distinct; silica bodies (Sb) prominently butterfly shaped; macro hairs (Ma) unicellular, present in the costal region; stomata (St) present in 2 to 3 rows in the costal area; subsidiary cells (Sc) indistinct. *S. Pathak* 48543 (CAL) (Pl. 2. figs. A & B).
 7. *Enteropogon dolichostachyus* (Lag.) Keng: Costal (C) and inter costal (IC) region almost distinguishable; silica bodies (Sb) numerous, prominently club shaped, present throughout the entire surface; prickles (Pr) unicellular, frequent, present at the edges of inter costal zone; micro (Mi) and macro hairs (Ma) not seen; stomata (St) deep seated within the silica bodies, exhibiting xerophytic character. *S. Pathak* 48504 (CAL). (Pl. 2. figs. C & D).
 8. *Eulalia quadrinervis* (Hack.) Kuntze: Costal (C) and inter costal (IC) region indistinct; inter costal (IC) region made of rectangular cells, arranged in rows with prominently sinuous wall; silica bodies (Sb) prominent, ovoid to globose, scattered individually or in clusters of 2 to 4; stomata (St) arranged in rows separated by 1 to 2 epidermal cells; subsidiary cells (Sc) distinct. *S. Pathak* 48554 (CAL). (Pl. 2. figs. E & F).
 9. *Holcus lanatus*: Costal (C) and inter costal (IC) region prominent; silica bodies (Sb) prominent, ovoid, restricted within the inter costal region; macro hairs (Ma) present, having prominently bulbous base and curved to hooked apices, distribution restricted in the costal region; stomata (St) arranged in rows in the costal area, separated by a single epidermal cell; subsidiary cells (Sc) distinct. *S. Pathak* 48474 (CAL). (Pl. 2. figs. G & H).
 10. *Iseilema antheophoroides* Hack.: Costal (C) and inter costal (IC) region distinct; cells of C area cylindrical, walls almost sinuous; silica bodies (Sb) markedly globose, arranged in rows in the costal area, also noticed to be present at both ends of stomata; micro hairs (Mi) bicellular, present costally; stomata (St) arranged in rows in the costal area, separated by a single epidermal cell; subsidiary cells (Sc) distinct. *S. Pathak* 48471 (CAL). (Pl. 2. figs. I & J).
 11. *Leptochloa chinensis* (L.) Nees: Costal (C) and inter costal (IC) region distinct; IC region narrow, cells of the this area are regular, cylindrical, walls definitely sinuous; silica bodies (Sb) club shaped, irregularly scattered generally in the costal area; prickles (Pr) sharp, found at the edge of IC region; stomata (St) arranged in a single row in the costal area; subsidiary cells (Sc) distinct. *S. Pathak* 48421 (CAL). (Pl. 3. figs. A & B).
 12. *Leptochloa panicea* (Retz.) Ohwi: Costal (C) and inter costal (IC) region markedly distinct; silica bodies (Sb) prominently butterfly shaped, arranged in rows in the intercostal region; prickles (Pr) present, minute, with sharp pointed apices, in the inter costal region; macro hairs (Ma) present, unicellular; stomata (St) present in 2 to 3 rows

- in the costal area; subsidiary cells (Sc) indistinct. *S. Pathak* 48409 (CAL). (Pl. 3. figs. C & D).
13. *Miscanthus nepalensis* (Trin.) Hack.: Costal (C) and inter costal (IC) region almost distinct; cells of the costal region cylindrical, rectangular; silica bodies (Sb) distinctly irregular shaped, scattered; stomata (St) present along the edge of IC region; subsidiary cells (Sc) indistinct. *S. Pathak* 48401 (CAL). (Pl. 3. figs. E & F).
 14. *Ophiuros exaltatus* (L.) Kuntze: Costal (C) and inter costal (IC) region not prominently distinct; silica bodies (Sb) few in number, butterfly shaped, arranged in rows in the IC region; stomata (St) present in rows in the costal area; subsidiary cells (Sc) distinct. *S. Pathak* 48454 (CAL). (Pl. 3. figs. G & H).
 15. *Phalaris arundinacea* L.: Costal (C) and inter costal (IC) region distinct; silica bodies (Sb) minute, irregular, restricted in IC zone; prickles (Pr) present, unicellular, arranged in rows in the IC region, apex sharp pointed, base prominently forked; stomata (St) scarce. *S. Pathak* 48470 (CAL). (Pl. 3. figs. I & J).
 16. *Sacciolepis indica* (L.) Chase: Costal (C) and inter costal (IC) region markedly distinct; IC region narrow, cells with wavy walls; silica bodies (Sb) faintly butterfly shaped, arranged in rows in the costal region; prickles (Pr) present, unicellular with curved tip in the IC region; stomata (St) present costal area; subsidiary cells (Sc) linear. *S. Pathak* 48522 (CAL). (Pl. 4. figs. A & B).
 17. *Schizachyrium delavayi* (Hack.) Bor: Costal (C) and inter costal (IC) region faintly separable; cells cylindrical, with prominent undulated wall; silica bodies (Sb) butterfly shaped, arranged in rows in the costal region; prickles (Pr) present, 2-celled, basal cell (Bc) cylindrical, terminal cell (Tc) with acute, sharp apex; stomata (St) present, with indistinct subsidiary cells (Sc). *S. Pathak* 48454 (CAL). (Pl. 4. figs. C & D).
 18. *Sporobolus coromandelianus* (Retz.) Kunth: Costal (C) and inter costal (IC) region markedly distinguishable; prominent tubular structures present at the junction of C and IC region; silica bodies (Sb) irregular, scattered; hairs and prickles (Pr) not seen; stomata (St) present in rows of 1 to 2 in the IC zone; subsidiary cells (Sc) distinct. *S. Pathak* 48412 (CAL). (Pl. 4. figs. E & F).
 19. *Stenotaphrum dimidiatum* (L.) Brongn.: Costal (C) and inter costal (IC) region markedly distinguishable; C region made of square to rectangular rows of cells; IC narrow, made of irregular to rectangular cells; silica bodies (Sb) irregular, scattered; hairs and prickles (Pr) not seen; stomata (St) present in costal area; subsidiary cells (Sc) distinct. *S. Pathak* 48432 (CAL). (Pl. 4. figs. G & H).
 20. *Vulpia bromoides* (L.) Gray: Costal (C) and inter costal (IC) region distinct; IC made of rectangular cells with prominently undulated wall; silica bodies (Sb) irregular, scattered in IC; prickles (Pr) present at the edge of IC, with sharp, curved apices; threadlike deposition seen all over the entire surface; stomata (St) scarce. *S. Pathak* 48424 (CAL). (Pl. 4. figs. I & J).
 11. *Leptochloa chinensis* (L.) Nees: Costal (C) and inter costal (IC) region distinct; IC region narrow, cells of the this area are regular, cylindrical, walls definitely sinuous; silica bodies (Sb) club shaped, irregularly scattered generally in the costal area; prickles (Pr) sharp, found at the edge of IC region; stomata (St) arranged in a single row in the costal area; subsidiary cells (Sc) distinct. *S. Pathak* 48421 (CAL). (Pl. 3. figs. A & B).
 12. *Leptochloa panicea* (Retz.) Ohwi: Costal (C) and inter costal (IC) region markedly distinct; silica bodies (Sb) prominently butterfly shaped, arranged in rows in the intercostal region; prickles (Pr) present, minute, with sharp pointed apices, in the inter costal region; macro hairs (Ma) present, unicellular; stomata (St) present in 2 to 3 rows in the costal area; subsidiary cells (Sc) indistinct. *S. Pathak* 48409 (CAL). (Pl. 3. figs. C & D).

13. *Miscanthus nepalensis* (Trin.) Hack.: Costal (C) and inter costal (IC) region almost distinct; cells of the costal region cylindrical, rectangular; silica bodies (Sb) distinctly irregular shaped, scattered; stomata (St) present along the edge of IC region; subsidiary cells (Sc) indistinct. S. Pathak 48401 (CAL). (Pl. 3. figs. E & F).
14. *Ophiuros exaltatus* (L.) Kuntze: Costal (C) and inter costal (IC) region not prominently distinct; silica bodies (Sb) few in number, butterfly shaped, arranged in rows in the IC region; stomata (St) present in rows in the costal area; subsidiary cells (Sc) distinct. S. Pathak 48454 (CAL). (Pl. 3. figs. G & H).
15. *Phalaris arundinacea* L.: Costal (C) and inter costal (IC) region distinct; silica bodies (Sb) minute, irregular, restricted in IC zone; prickles (Pr) present, unicellular, arranged in rows in the IC region, apex sharp pointed, base prominently forked; stomata (St) scarce. S. Pathak 48470 (CAL). (Pl. 3. figs. I & J).
16. *Sacciolepis indica* (L.) Chase: Costal (C) and inter costal (IC) region markedly distinct; IC region narrow, cells with wavy walls; silica bodies (Sb) faintly butterfly shaped, arranged in rows in the costal region; prickles (Pr) present, unicellular with curved tip in the IC region; stomata (St) present costal area; subsidiary cells (Sc) linear. S. Pathak 48522 (CAL). (Pl. 4. figs. A & B).
17. *Schizachyrium delavayi* (Hack.) Bor: Costal (C) and inter costal (IC) region faintly separable; cells cylindrical, with prominent undulated wall; silica bodies (Sb) butterfly shaped, arranged in rows in the costal region; prickles (Pr) present, 2-celled, basal cell (Bc) cylindrical, terminal cell (Tc) with acute, sharp apex; stomata (St) present, with indistinct subsidiary cells (Sc). S. Pathak 48454 (CAL). (Pl. 4. figs. C & D).
18. *Sporobolus coromandelianus* (Retz.) Kunth: Costal (C) and inter costal (IC) region markedly distinguishable; prominent tubular structures present at the junction of C and IC region; silica bodies (Sb) irregular, scattered; hairs and prickles (Pr) not seen; stomata (St) present in rows of 1 to 2 in the IC zone; subsidiary cells (Sc) distinct. S. Pathak 48412 (CAL). (Pl. 4. figs. E & F).
19. *Stenotaphrum dimidiatum* (L.) Brongn.: Costal (C) and inter costal (IC) region markedly distinguishable; C region made of square to rectangular rows of cells; IC narrow, made of irregular to rectangular cells; silica bodies (Sb) irregular, scattered; hairs and prickles (Pr) not seen; stomata (St) present in costal area; subsidiary cells (Sc) distinct. S. Pathak 48432 (CAL). (Pl. 4. figs. G & H).
20. *Vulpia bromoides* (L.) Gray: Costal (C) and inter costal (IC) region distinct; IC made of rectangular cells with prominently undulated wall; silica bodies (Sb) irregular, scattered in IC; prickles (Pr) present at the edge of IC, with sharp, curved apices; threadlike deposition seen all over the entire surface; stomata (St) scarce. S. Pathak 48424 (CAL). (Pl. 4. figs. I & J).

KEY TO THE SPECIES BASED ON SEM CHARACTERS

- 1a. Costal and intercostals region distinct 2
- 1b. Costal and intercostals region indistinct 15
- 2a. Prickles present 3
- 2b. Prickles absent 4
- 3a. Prickle unicellular
Enteropogon dolichostachyus
- 3b. Prickle 2-celled
Briza media
- 4a. Silica bodies prominent 5
- 4b. Silica bodies obscure
Cenchrus prieurii
- 5a. Silica bodies ovoid, rectangular to irregular, scattered, rarely in rows 6
- 5b. Silica bodies butterfly shaped, arranged in rows 14
- 6a. Marco hairs present, unicellular 7

- 6b. Macro hairs absent9
- 7a. Base of macro hair bilobed
Bromus catharticus
- 7b. Base of macro hair not lobed8
- 8a. Base of the macro hair prominently bulbous
Holcus lanatus
- 8b. Base of the macro hair not bulbous
Cynodon barberi
- 9a. Thread like deposition present all over the entire surface.....
Vulpia bromoides
- 9b. Thread like deposition absent10
- 10a. Walls of the cells of the costal or inter costal regions definitely sinuous.....11
- 10b. Walls of the cells of the costal or inter costal regions straight.....5
- 11a. Silica bodies markedly present at both tips of the stomata.....
Iseilema antheophoroides
- 11b. Silica bodies present scattered irregularly.....12
- 12a. Silica bodies globose to club shaped
Leptochloa chinensis
- 12b. Silica bodies irregular in shape
Miscanthus nepalensis
- 13a. Tubular cells present at the junction of costal and inter costal region.....
Sporobolus coromandelianus
- 13b. Tubular cells absent
Stenotaphrum dimidiatum
- 14a. Subsidiary cells distinct
Ophiuros exaltatus
- 14b. Subsidiary cells indistinct
Leptochloa panicea
- 15a. Tubular, network like deposition present in the entire surface.....
Capillipedium parviflorum
- 15b. Tubular, network like deposition absent16
- 16a. Walls of the cells of the costal or inter costal regions definitely sinuous.....17
- 16b. Walls of the cells of the costal or inter costal regions straight.....19
- 17a. Silica bodies found in clusters.....
Eulalia quadrinervis
- 17b. Silica bodies individually present18
- 18a. Prickles unicellular
Sacciolepis indica
- 18b. Prickles 2- celled
Schizachyrium delavayi
- 19a. Base of prickles prominently forked
Phalaris arundinacea
- 19b. Base of prickles not forked.....
Calamagrostis emodensis

DISCUSSION

Grasses are considered one of the major prevalent plant type which plays a precious role as source of food and energy for all sorts of wildlife and organics. Leaves of grasses have been analyzed traditionally under optical microscope. But recently the use of SEM in the study of leaf epidermal surface sturdily supports their taxonomic implications, which can portray structures more visibly, revealing distinct aspects of critical features. The present investigation is aimed at studying their foliar morphology under SEM which exposed several interesting facts as, the morphological characters of the stomata of each species under analysis, which are vital to correlate and demarcate among the different taxa in terms of phylogenetic and taxonomic point of view when reproductive features are unavailable. The epidermal cells, their wall traits, nature and distribution of macro and micro hairs, along with stomatal features are suitably applied here which have separated the species, effectively and accurately, applying which a key has been prepared. Furthermore, the study contributed in thriving and lucratively establishing a relationship among distant taxa and closely associated ones, facilitating the identification of the species and its derivatives.

CONCLUSION

Consequently, it can be evidently accomplished that, such exercise of examination of foliar stomata should be considered helpful in determining the taxonomic as well as phylogenetic position of intimately related and complicated taxa. The pragmatic micro-morphological characters proved to be vital in identification and delimitation of closely related taxa which has been segregated with the aid of 'taxonomic keys'. Furthermore, the study was very helpful in detection and classification of taxa at the species levels where reproductive features lacked. Consequently, it can be evidently accomplished that, such exercise of examination of foliar micro-morphological features should be considered supportive in determining the taxonomic as well as phylogenetic position of intimately related and complicated taxa.

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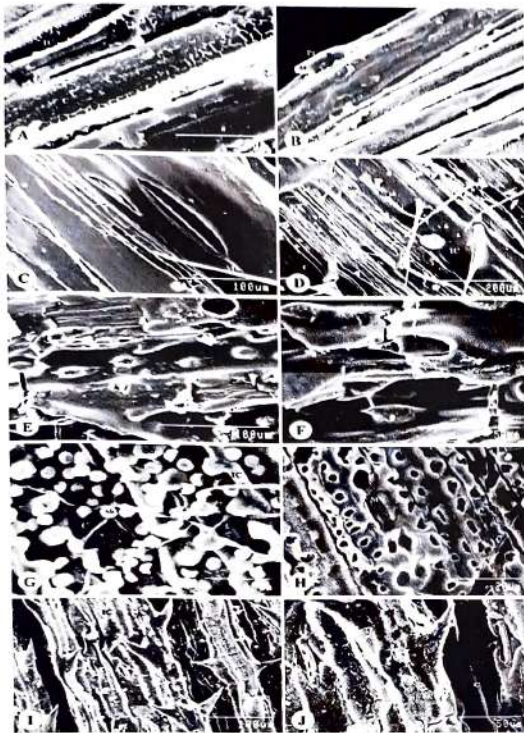


Plate 1. Scanning Electron Micrographs of abaxial leaf surfaces. A & B: *Briza media*; C & D: *Bromus catharticus*; E & F: *Calamagrostis emodensis*; G & H: *Capillipedium parviflorum*; I & J: *Cenchrus piceurii*. [Costal region (C); Inter costal region (IC); Silica body (Sb); Micro hairs (Mi); Macro hairs (Ma); Prickle (Pr); Basal cell (Bc); Apical cell (Ac); Stomata (St)].



Plate 2. Scanning Electron Micrographs of abaxial leaf surfaces. A & B: *Cynodon dactylon*; C & D: *Eriopogon dolichostachys*; E & F: *Eriopogon quadrinervis*; G & H: *Holcus lanatus*; I & J: *Ischaemum anthephoroides*. [Costal region (C); Inter costal region (IC); Silica body (Sb); Micro hairs (Mi); Macro hairs (Ma); Prickle (Pr); Basal cell (Bc); Apical cell (Ac); Stomata (St)].

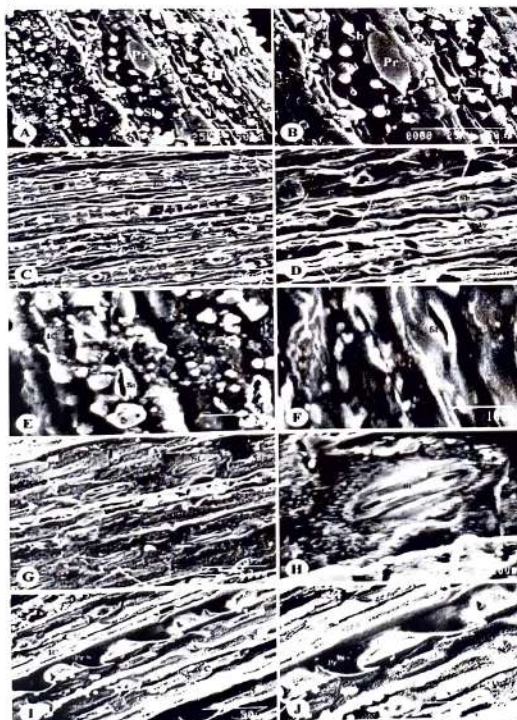


Plate 3. Scanning Electron Micrographs of abaxial leaf surfaces. A & B: *Leptochloa chinensis*; C & D: *Leptochloa panicea*; E & F: *Miscanthus nepalensis*; G & H: *Ophturus exaltatus*; I & J: *Phalaris arundinacea*. [Costal region (C); Inter costal region (IC); Silica body (Sb); Micro hairs (Mi); Macro hairs (Ma); Prickle (Pr); Basal cell (Bc); Apical cell (Ac); Stomata (St)].

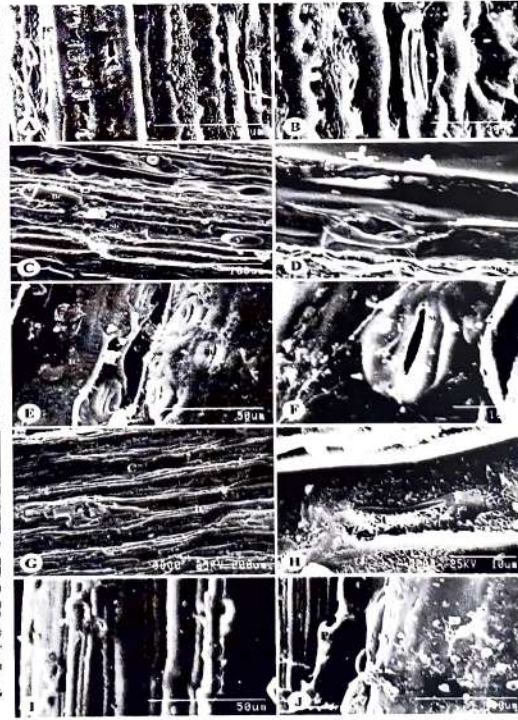


Plate 4. Scanning Electron Micrographs of abaxial leaf surfaces. A & B: *Saccolopha indica*; C & D: *Schizochytrium dictyosporum*; E & F: *Sporobolus coromandelianus*; G & H: *Stenotaphrum dimidiatum*; I & J: *Vulpia bromoides*. [Costal region (C); Inter costal region (IC); Silica body (Sb); Micro hairs (Mi); Macro hairs (Ma); Prickle (Pr); Basal cell (Bc); Apical cell (Ac); Stomata (St)].

Review Article

Recycling and reuse of azo dye polluted water for agriculture through bioremediation-A microbial perspective

Shivani M. Yagnik¹, Vikram H. Raval⁴, Ramesh K. Kothari³, Charmy R. Kothari^{2*}

¹Department of Microbiology, Christ College, Rajkot-360005

²Department of Biotechnology, Christ College, Rajkot-360005

³Department of Biosciences, Saurashtra University, Rajkot-360005

⁴ Department of Microbiology & Biotechnology, Gujarat University, Ahmedabad-380009

Email: dr.charmykothari@gmail.com

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ABSTRACT

Recycling water for irrigation is necessity of the day. Industrial effluents are huge in amount so its recycling becomes countable. Textile industries are one of the main domains of whole industrial sector. The textile hubs are with increasing risk of dye contaminated effluent pollution to the water reservoirs. This is a direct risk to ecosystem through crops. Textile effluents should be treated before expelling to environment; there are many methods amongst which bioremediation through bacteria are reported most sustainable, non-toxic and cheaper. Studies reveals about azo dye degradation and detoxification of such effluents through bacterial bioremediation. It's a need to gaze on the issue as a need of the day; as through water and crops such pollutants may bio-magnify and can have hazardous effects.

Key words: Water recycling, Bioremediation, Irrigation, Textile industry, Azo dye

Since the beginning of the human civilisation, people have been using colours. Mauveine, the first synthetic dye was discovered by Perkin who patented his invention and set up a production line in 1856 (Hunger *et al.*, 2002). In the beginning of 20th century, synthetic dyestuff had almost supplanted natural dye (Van der Zee and Villaverde, 2005). In the textile industry, azo dyes are the largest group of dyes, constituting 60-70% of all dyes produced (Carliellet *al.*, 1995). The effluents of such industries are polluting water reservoirs and through that crops are affected (Khan and Malik, 2018). Today agriculture has different challenges. Water crisis and interruptions in rain water due to climate change affects agriculture yields (Hassan *et al.*, 2013). Water recycling can become the answer to these problems; hence studies towards textile industrial water bioremediation are needful.

Textile Industries- A reservoirs of Azo dyes

The textile industries use various dyes in greater

extent in processing units which are liberated directly in natural water bodies as an industrial effluent. The textile industry is one of the largest industries which are significantly contributing economies globally. The textile manufacturing process has thumping consumption of potable water that generates significant amount of effluent with complex pollutants and their mixture viz. heavy metals, chlorinated compounds, dyes and dyestuffs (Islam and Mostafa, 2018). Toxic industrial effluents and textile dyes pose a potential threat to both the environment as well as human kind (Chu and Phang, 2019; Yagnik 2012). Hence, it is essential to remediate such pollutants to protect the environment.

Dyes and Azo Dye

Dye is any coloured substance with affinity towards the substrate it is applied onto. Majority of dyes are water soluble and used as aqueous solution which sometimes requires a mordant that intensify onto the substrate (Purwar,

2016). Azo dyes are widely used on industries, it is have an azo bond ($R_1-N=N-R_2$). Azo dyes are scarcely biodegradable organic compounds due to their high stability to light and resistant to microbial attack. These dye are resistant to conventional biodegradation, however under anaerobic condition; they have been associated with the generation of metabolites. According to Saratale and coworkers there are some species of microorganisms that under certain environmental condition, are able to completely mineralize several type of azo dye (Saratale *et al.*, 2011). Azo dyes have high water solubility. This property makes dye a potent toxic consumable of biotic component upon expelled to environment through textile industries (Talarposhtiet *et al.*, 2001). Majorly such pollutants are least biodegradable and get incorporated to food chain through crops. Even studies reports that water holding capacity of agricultural soil decreases due to irrigation activities carried out using untreated effluents. Thus raises a major concern for recycling of dye polluted water (Rawat *et al.*, 2016).

Biological Methods of textile effluent treatment

A major paradigm shift in environmental science research is seen and microbial clean-up is the front runner and is adjudged as Bioremediation. It is a general approach for microbes to get acclimatizes to the surrounding including toxic wastes. In due course of time, newer strains may develop by genetic exchange methods which resist the toxic and harmful substances in environment, utilize it as substrate and eventually degrade them and render them non-toxic several such reports regarding biotransformation reactions are very well documented (Saratale *et al.*, 2011). Degradative biotransformation which includes various enzymes of microbial origin such as tyrosinase (Zhang and Flurkey, 1997), lignin peroxidase, laccase (Hatvani and Mecs, 2001), aminopyrine N-demethylase, NADH-DCIP reductase (Bhosale *et al.*, 2006), hexane oxidase are well reported in the past researches (Saratale *et al.*, 2011).

Microbial transformation or bioremediation of textile dyes using enzymatic treatment confer advantages as it is cost effective, eco-friendly, follows complete degradation, reduces the amount of sludge produced and recycle water

used in treatment processes (Saratale *et al.*, 2011). A diverse group of microbes have been tried and tested for their capacities to decolorize a range of dyes which includes bacteria (Jadhav *et al.*, 2010). Bacterial cells represent an inexpensive and promising tool for the removal of various azo dyes. Bacteria play an important role in transforming toxic compound into non-toxic or less toxic and degrading.

Biodegradation of Dye by Bacteria

There are presence of different chromophore groups like azo, triphenyl methane, phthalocyanine etc. are responsible for their structural diversity (Bakshiet *et al.*, 1999). Azo dyes are electron-deficient xenobiotic compounds because they possess the azo and sulfonic electron-withdrawing group and are there for recalcitrant in nature however, many microorganisms are complete enough in biodegrading these dyes. Substantial work has been done and it is a proven phenomenon that majority of mono and di-azo dyes can be degraded by anaerobe, facultative anaerobes and certain aerobic group of bacteria. These bacterial groups adopt a robust mechanism of reductive cleavage of azo bonds via azo reductase enzyme. Under anaerobic conditions the resultant product is a colourless solution of hazardous aromatic amines (Van der Zee and Villaverde, 2005). Reactive sulphonated dyes are very soluble by structure but not all are used by textile fibres during the dyeing process and there for, are discharged as effluents from dyeing and printing units. Sulfonilic acid groups that are introduced to increase the water solubility of the dye and the azo group confer resistance to microbial attack, making them recalcitrant to oxidative decolorization (Coughlin *et al.*, 1999). There are some reports on aerobic desulfonation of sulphonated aromatic compounds but most of the sulphonated azo dyes are handily biodegraded under aerobic condition. Aerobic condition is required for complete degradation of the toxic intermediate compounds into harmless products (Kalme *et al.*, 2007). The Table 1 summarizes the details of bacterial isolates varied environmental conditions of temperature, pH along with their treatment methodology that are used for decolourization of azo dyes.

Table 1: Dye decolorisation studies by bacteria in recent past (2014-2020)

Organism	Dye and CI number	Type of dye & Molecular weight(g/mol)	Time in hrs	% removal	Temp.& pH	Mechanism	Approach/ Technique	References
<i>Bacillus halodurans</i> MTCC 865	Acid Black-24 26370	Azo; 731.7	6	90%	37°C 9.0	Biodegradation	Alkaline aerobic treatment	Prasad and Rao (2014)
<i>Acinetobacter baumannii</i> YNWH 226	Congo red 22120	Azo; 696.7	16	99.1	37°C 7.0	Biodegradation	RSM based aerobic treatment	Ning <i>et al.</i> , (2014)
<i>Geobacilluscatenulatus</i> MS5	Congo Red 22120	Azo; 696.7	32	99	30-80°C 4.6	Enzymatic	Thermostable Laccase	Verma and Shirkot (2014)
<i>Proteus. vulgaris</i> <i>P. mirabilis</i>	Various mono azo and anthroquinone dyes	NA	12	>70 for all dyes individually	37°C 7.0	Biodegradation	Azo-reductase and consortia	Parmar and Shukla (2015)
<i>Ochrobactrum sp.</i> , <i>P. aeruginosa</i> & <i>Providencia vermicola</i>	Textile Effluent NA	NA	16	>95 for consortia	37°C 7.0	Biodegradation Enzymatic	Aerobic treatment Consortia (coculture)	Vijayalakshmi and Muthukumar (2015)
<i>Bacillus sp.</i>	Acid red 14900, Remazol black 20505, Reactive blue 61200	Azo; 879.9; 991.8; 626.5	48	100	35°C 5.0-7.0	Biodegradation Enzymatic	Aerobic treatment Consortia	Dwivedi and Tomar (2017)
<i>Bacillus fermus</i>	Acid Blue-9 17560	Azo sodium salt 792.9	72	97	37°C NA	Enzymatic	Aerobic treatment	Neethaet <i>al.</i> , (2018)
<i>Arthrobacter soli</i> BS5	Reactive black 5 20505	Di-Azo 991.8	120	98	37°C 5.0-9.0	Enzymatic	NA	Khan and Malik (2018)
<i>Bacillus sp.</i> CF96	Indigo carmine Acid Blue 74	Azo 466.4	NA	90	60°C 8.0	Enzymatic	Laccase	Javadzadeh and Asoodeh (2019)
<i>Shewanella sp.</i> NIAB-BM15	Red-S3B 53000	Azo 1136.32	4	100	37°C NA	Enzymatic	NA	Shakeel <i>et al.</i> , (2020)
<i>Pseudomonas stutzeri</i> , AK6	Acid Blue 113 18777	Di-azo 681.7	96	86.2	37°C NA	Enzymatic	Azoreductase and laccase	Joshi <i>et al.</i> , (2020)

Bacterial remediation of textile effluent and Agriculture

Water crisis for drinking and agriculture purpose creates a demand for reuse of water; this makes industrial effluent treatment a must phenomenon in cheaper and sustainable way, which can be promising by bacterial remediation (Kabra et al., 2012). A large amount of textile industrial effluent can be treated by potential dye decolorizing bacteria and such treated and detoxified effluent can be used for agriculture usages, this can fill the gap between demand and crisis of usable water (Islam and Mustafa, 2018).

Conclusion

Disposal of wastewater effluent generated by dye industries into environment without proper treatment negatively affect the soil and water and affects crops through them. This demands to invent sustainable approach to remediate the hazardous chemical compounds present in the textile effluent. Biological wastewater treatments through bacterial strains with capabilities of dye degradation and turning it to non-toxic component are studied for reuse of such water for irrigation. In this regard, use of these bacteria can enhance the process efficiency of degradation which is economically feasible, ecofriendly and easy to handle. This can lead a chain of recycle and better reuse of water, even agriculture which is facing a cut through of water crisis for irrigation can be made possible to decrease the dependency on rain water or other water resources in the area near textile industrial hubs.

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Computational analysis and physico chemical characterization of phospholipase A2 protein in various poisonous snakes

Nandini N Trivedi¹, Jeel A Ladhani¹ and Pratibha Parihar^{*2}

¹Research Student

²Assistant professor in Bioinformatics

Department of Biotechnology, Genetics and Bioinformatics, N.V. Patel college of Pure and Applied Sciences, CVMU university, Vallabh Vidyanagar-388120, Gujarat, India.

*Corresponding author email address: pratibhaparihar@gmail.com

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ABSTRACT

Snake venoms are known for their extensive potential in clinical and medical sciences due to the presence of pharmacologically active proteins. The present work included the *insilico* study of phospholipase A2 protein sequence and structure from twenty different venomous snake species. These are predictions of various physical and chemical properties, domain scanning, and structural analysis. The average molecular weights of protein found in different snake species were calculated as 15.229 kDa, most of the proteins were basic and thermostable. Prot Comp 9.0 tool predicts the location of phospholipase A2 protein in the extracellular region or plasma membrane. CYS_REC tool recognizes 7-16 cysteine residues in phospholipase A2 protein sequences and a probable disulfide bond ranges from 4-7. The clustering algorithm in the phylogenetic tree grouped all the species in 3 clusters and showed that the PLA2 of Inland Taipan and Coastal taipan are the most recent and closest neighbors in the cluster and are found distant from the rest of the snake species.

Key words: Venom, phospholipase A2, physico chemical properties, phylogenetic tree

Venom is a poisonous or sometimes lethal secretion containing zootoxins secreted by the fangs of an animal. It assists defense against terrorizations by immobilization and digestion of prey (Bauchot, 1994). The production of zootoxins is mainly due to the modification of parotid salivary glands which are encapsulated in a muscular sheath generally situated on the sides of the head, below, and behind the eye.

Venoms are composed of around twenty different compounds, mostly proteins (enzymes), polypeptides, lipids, steroids, amino polysaccharides, amines, quinines, neurotransmitters (Bottrall et al, 2010) which helps in the digestion of prey. These compounds play a vital role in various biochemical functions such as the transmission of nervous or muscular impulses, coagulation of blood, regulation of blood pressure. It can also be used as a defense me-

chanism and as a pharmacological or diagnostic tool (Bauchot, 1994). Different poisonous snake venoms are rich in pharmacologically active proteins such as proteolytic enzymes (metalloproteases and serine proteases), cysteine-rich secretory proteins, arginine esterase, thrombin-like enzymes, Kunitz peptides, short neurotoxin, long neurotoxin, hyaluronidase, phospholipase A2, three-finger toxins, acetylcholinesterase, nucleases (RNase, DNase, and Phosphodiesterase), and L-amino-acid oxidase (Kang et al. 2011; Modah et al. 2016). These components bind or interfere with the affinity and mechanism of a large number of targets such as ion channels or membrane receptors, and hemostatic pathways (Beraud et al. 2011; Tasoulis et al. 2017). The venoms present in snakes, spiders, honey bees, wasps, reptiles, scorpions, cone snails, and amphibians may

beuseful in the medical and biotechnological or clinical potential. Phospholipase A2 (PLA2s) are enzymes that break down and release fatty acids or phospholipids and thus produce glycerol and phospholipid. These enzymes perform many pharmacological activities such as cardiotoxic, neurotoxic, anticoagulant, myotoxic, and hemorrhagic effects.

The enzyme is rich in α -helix. There are six residues in the active region: His-48, Asp-49, Tyr-28, Gly-30, Gly-32, and Asp-99. During the catalytic activity of PLA2s, the conserved residue, Asp49, plays an important role in coordinating Ca^{2+} , thus helping in intermediate transition state stabilization. Thus, for the catalytic activity, Ca^{2+} acts as a co-factor (Ojeda *et al.* 2017).

In the present work, we studied twenty various venomous species sequences with the help of bioinformatics tools and available databases. It involved retrieval of sequences, physical and chemical properties prediction, domain scanning, and structural analysis. Further, the findings could be useful for designing various peptide-based drugs for the treatment of the chronic disorder.

Materials and Methods Sequence retrieval

Twenty snakes were selected based on their poisonous property and the availability of their protein sequences in the database. The UniProt database (<https://www.uniprot.org/>) was used to retrieve their protein sequences. The sequences were saved in the FASTA file along with their accession IDs given in table 1.

Physicochemical Analysis

The physicochemical properties of protein like aliphatic index (AI), amino acid composition, atomic composition, estimated half-life, extinction coefficient (EC), grand average of hydropathicity (GRAVY), instability index (II), molecular weight (MW), theoretical isoelectric point (pI), were calculated with the Prot Param tool (<https://www.expasy.org/>) (Artimo *et al.*, 2012).

Domain Analysis

As reviewed by El-Gebali *et al.*, 2019, Pfam is a

curated database of classification of protein families and domains. It is used further for the multiple sequence alignment, identification of distant homology, and annotation of protein sequences generated by profile hidden Markov models (HMM).

Secondary Structure Prediction and analysis

The Self Optimized Prediction method With Alignment (SOPMA) (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) (Geourjon *et al.*, 1995) tool was used to predict the secondary structures of all the protein sequences. It uses the homolog method to form homologous secondary structures. The accuracy of SOPMA is 69.5%. It gives detailed information for all three states of secondary structure (α -helix, β -sheet, and coil) in a whole sequence. CYS_REC (<http://linux1.softberry.com/berry.phtml/topic>) tool was used to predict the disulfide bond patterns (SS) and linkages.

Homology modeling and validation:

SWISS MODEL SERVER was used to model the structures of all the species and the predicted structures were validated using the PROCHECK tool. Homology modeling methods is a highly reliable and accurate method to build models for evolutionarily related proteins using the homologous experimental protein structures.

Phylogenetics analysis

The phylogenetic tree was constructed for Phospholipase A2 toxin of all snake species to study the evolutionary history of snake venom proteome. Distance score was calculated using the Kimura 2-parameter model and clustering was done using the neighbor-joining (NJ) method using the software package CLC genomic workbench.

Result and analysis Retrieval of sequences:

All the sequences were downloaded from the UniProtKB protein database and were saved in FASTA format in the notepad. The sequence length was found to range between 118-152 aa and the average length 137 aa. The detailed information of accession number, sequence length, and protein name are shown in table 1.

Table 3 Relationship between productivity and agricultural inputs

Speciesnames	UniprotKBID	Length (inaa)
<i>Acanthophsantarcticus</i> (DEATHADDER)	P81236	119
<i>Notechisscutatus</i> (MainlandTigersnake)	P00609	119
<i>Oxyuranusmicrolepidotus</i> (InlandTaipan)	Q45Z42	146
<i>Pseudonajatextilis</i> (Easternbrownsnake)	P23026	145
<i>OphiophagusHannah</i> (KingCobra)	Q9DF33	146
<i>Oxyuranusscutellatus</i> (Coastaltaipan)	Q45Z47	146
<i>Dispholidustypus</i> (BoomsLang)	I0BWR7	150
<i>Agkistrodonpiscivorus</i> (Easterncottonmouth)	P51972	123
<i>Echiscarinatus</i> (Sawscaledviper)	Q7T3S7	136
<i>Daboiasiamensis</i> (EasternRussell'sviper)	Q7T2R1	138
<i>Najasumatrana</i> (Malayanspittingcobra)	Q92084	146
<i>Many-bandedkrait</i> (Bungarusmulticinctus)	P00617	147
<i>Bungaruscaeruleus</i> (Indiankrait)	Q9DF52	152
<i>Crotalusdurissusterrificus</i> (SouthAmericanrattlesnake)	P62022	138
<i>Enhydrinaschistose</i> (Beakedseasnake)	P00610	119
<i>Najaatra</i> (Chinesecobra)	P00598	146
<i>Bitisgabonica</i> (Gaboenviper)	P00620	118
<i>Agkistrodoncontortrixlaticinctus</i> (Broad-bandedcopperhead)	P49121	137
<i>Bothropsasper</i> (Fer-de-lance)	P20474	138
<i>Laticaudaculubrine</i> (Yellow-lippedseakrait)	P10116	118

Physiochemical Analysis

The various physical and chemical parameters for Phospholipase A2 are shown in Table 2. The molecular weight for various venomous snake Phospholipase A2 protein is ~12-16 kDa and the average molecular weight is 15.229 kDa. At the isoelectric point (pI), proteins are stable and compact. The computed pI value of most of Phospholipase A2 protein sequences in the poisonous snake was found to be pI > 7 indicating their basic nature while some of the viper, cobra, and krait species show a slightly acidic nature of Phospholipase A2 protein. The utility of calculation of computed isoelectric point (pI) of a protein is the identification of charged nature of the protein, analyzing antibody behavior and in the purification of the proteins (Gasteiger et al., 2005). The extinction coefficient (EC) of Phospholipase A2 protein at 280nm ranges from 12,000 to 38,000 M⁻¹cm⁻¹ for the concentration of Cys, Trp, and Tyr. The highest EC value was found in South American rattle snake and Eastern brown snake which indicates the

presence of a high concentration of Trp, Tyr, and Cys while the lowest EC value was found in the Yellow-lipped seakrait. The computed EC values are useful in the concentration of protein, quantitative analysis of protein-ligand, and protein-protein interactions (Gill and Von Hippel, 1989). Instability index (II) is a measure of the in vitro stability of proteins. The instability index value less than 40 is considered as a stable protein while a higher than 40 value shows unstable protein in *in-vitro* condition. Phospholipase A2 sequences showed high stability for most snake species. The instability index value is related to in vivo half-life of the protein. Unstable protein with more than 40 instability index value shows an in vivo half-life of fewer than 5 hours, whereas *in-vitro* stable protein shows an in vivo half-life of more than 16 hours. Protein Aliphatic Index is the relative amount of aliphatic side chains present in the protein-like Alanine, Valine, Leucine, and Isoleucine. The higher value of the Aliphatic Index shows higher thermal stability of globular proteins. Aliphatic

amino acids showed a higher hydrophobic nature of amino acids. The Aliphatic Index value ranges between 66.5 to 84.33 for cytotoxins protein. 37.2-71.1 The higher value of the aliphatic index shows the thermostability of protein over a wide temperature range. Many-banded krait and Indian krait showed the highest Aliphatic Index. The presence of both hydrophobic and hydrophilic (charged) residues within protein shows the amphipathic nature of cytotoxins found in Indian krait. It is an important criterion for a molecule for biological membrane perturbation. The Grand Average of Hydropathicity (GRAVY) (Kyte and Doolittle, 1982) index indicates the solubility of the proteins. The positive value of GRAVY indicates the hydrophobic nature of protein while the negative value shows the polar nature of the protein. The result shows proteins have negative GRAVY values for all the snake species.

M.W.-molecular weight, pI- theoretical pI, EC- extinction coefficient, estimated half-life, II- instability index, A1- aliphatic index, grand average of hydropathicity (GRAVY), AA- amino acid Domain analysis

Proteins are made of one or many motifs or functional and/or structural regions that may associate with a diverse range of protein families, termed as domains. Phospholipase A2 has the domain Phospholipase A2 in all the species. The clan shows four families PLAG12, Phospholip_A2_1,

Phospholip_A2_2 and Phospholip_A2_3. Phospholipases A2 (PLA2s) are enzymes with EC number as 3.1.1.4 which hydrolyse the fatty acid between the second fatty acid "tail" and the glycerol molecule. PLA2 is stimulated by melittin present in the venom of the snake. In our analysis, the domains of all the above species were predicted from the Pfam database and the data are shown in

the table below.

Secondary structure prediction:

The secondary structure element like an alpha helix, extended strand, and random coil of all the protein sequences was predicted by the SOPMA tool. The result shows that most of the regions in the sequences were occupied by random coils than an alpha helix, extended strand, and beta-turn. Phospholipase A2 showed a higher percentage of the alpha helix which is highest in Indian krait and Broad-banded copperhead. High

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Homo logy Modeling

The 3D structures of all the species were modelled by using SWISS MODEL server given in Fig.1 and predicted models were validated using PROCHECK tool. The template and organism name with its identity scores are shown in table 6. Most of the structures showed high homology with the template structure. The Ramachandran plots core was obtained for all the species was found to be above 85% for all the structures. The Generated models were visualized in RasMol as shown in table 6.

(July 2021)

COMPUTATIONAL ANALYSIS AND PHYSICO CHEMICAL

TABLE 2: Physio chemical analysis of PHOSPHOLIPASE A2

Species name	M.W. (kDa)	pI	AI	II	Half life(in hours)	EC	Freq.ofapolar AA	Freq.ofpolar AA	Highfreq. AA	lowfreq. AA	Gravy
Deathadder	12.8	8.55	44.3	34.80 Stable	11	25,590	0.420	0.361	(C),(G)	(H)	-0.428
MainlandTigersnake	13.67	8.56	42.68	50.38 unstable	1.4	25,020	0.353	0.361	(C)	(M), (W)	-0.720
Inland Taipan	16.32	8.8	61.50	48.56 Unstable	30	22,520	0.432	0.336	(C)	(W)	-0.286
Easternbrownsnake	16.68	9.04	59.86	34.44 Stable	30	38,450	0.414	0.359	(Y)	(M)	-0.388
kingCobra	15.90	4.86	68.83	36.31 Stable	30	22,520	0.425	0.390	(C)	(M), (W)	-0.132
Coastal taipan	16.10	8.81	64.86	41.69 Unstable	30	21,240	0.445	0.322	(C)	(W)	-0.275
Boomslang	16.34	8.65	69.66	38.34 Stable	30	23,860	0.447	0.353	(C), (L)	(M)	-0.156
Easterncottonmouth	13.98	8.92	42.03	24.49 Stable	1.4	29,430	0.366	0.366	(K)	(H)	-0.658
saw scaledviper	15.5	5.44	64.48	38.71 Stable	30	36,400	0.404	0.331	(C)	(H)	-0.393
eastern Russell's viper	15.39	4.92	60.07	32.19 Stable	30	23,800	0.413	0.377	(C)	(P), (W)	-0.162
Malayan spitting cobra	16.18	7.34	62.94	34.69 Stable	30	29,490	0.438	0.377	(A), (C)	(E), (H), (W)	-0.269
Many-bandedkrait	16.22	8.05	71.76	21.08 Stable	30	19,330	0.442	0.327	(C)	(W)	-0.183
Indiankrait	15.76	5.42	68.69	23.53 Stable	30	18,110	0.428	0.428	(C)	(W)	-0.079
SouthAmerican rattlesnake	15.90	8.98	60.79	48.43 Unstable	30	37,680	0.435	0.312	(C)	(H), (Q)	-0.260
Beakedseasnake	13.44	8.54	45.96	43.01 Unstable	1.4	16,200	0.328	0.395	(C)	(W)	-0.716
Chinese cobra	16.01	6.34	62.26	33.22 Stable	30	29,490	0.445	0.384	(A), (C)	(H)	-0.208
Gaboon viper	13.37	5.29	37.20	32.82 Stable	1.1	24,900	0.339	0.424	(G)	(H), (L), (P), (W)	-0.696
Broad-bandedcopperhead	15.77	9.15	66.93	26.34 Stable	30	28,150	0.372	0.321	(K)	(Q)	-0.482
Fer-de-lance	15.75	8.98	63.62	38.94 Stable	30	25,020	0.420	0.304	(C),(K)	(H)	-0.329
Yellow-lipped seakrait	13.35	8.72	46.35	42.38 Unstable	1.4	12,360	0.373	0.398	(C)	(W)	-0.483

Table 3: Pfam results

Speciesname	Domain	Start	End	Accession	Score	E-value	Description
DeathAdder	Phospholip_A2_1	2	110	PF00068.20	120.0	6.6E-35	PhospholipaseA2
Mainland Tiger snake	Phospholip_A2_1	2	110	PF00068.20	110.5	5.8E-32	PhospholipaseA2
InlandTaipan	Phospholip_A2_1	29	137	PF00068.20	112.7	1.3E-32	PhospholipaseA2
Eastern brown snake	Phospholip_A2_1	29	136	PF00068.20	104.6	4E-30	PhospholipaseA2
KingCobra	Phospholip_A2_1	29	138	PF00068.20	119.1	1.3E-34	PhospholipaseA2
Coastaltaipan	Phospholip_A2_1	29	137	PF00068.20	115.7	1.5E-33	PhospholipaseA2
Boomslang	Phospholip_A2_1	25	130	PF00068.20	119.4	1.1E-34	PhospholipaseA2
Eastern cottonmouth	Phospholip_A2_1	2	108	PF00068.20	111.8	2.3E-32	PhospholipaseA2
SawScaledviper	Phospholip_A2_1	18	124	PF00068.20	113.4	7.7E-33	PhospholipaseA2
EasternRussell's viper	Phospholip_A2_1	18	124	PF00068.20	109.1	1.7E-31	PhospholipaseA2
Malayanspitting cobra	Phospholip_A2_1	29	137	PF00068.20	117.3	4.7E-34	PhospholipaseA2
Many-banded krait	Phospholip_A2_1	29	138	PF00068.20	104.8	3.5E-30	PhospholipaseA2
Indiankrait	Phospholip_A2_1	29	136	PF00068.20	120.9	3.5E-35	PhospholipaseA2
SouthAmerican rattlesnake	Phospholip_A2_1	18	124	PF00068.20	109.0	1.7E-31	PhospholipaseA2
Beaked seasnake	Phospholip_A2_1	2	110	PF00068.20	108.2	3.1E-31	PhospholipaseA2
Chinesecobra	Phospholip_A2_1	29	137	PF00068.20	118.8	1.6E-34	PhospholipaseA2
Gaboonviper	Phospholip_A2_1	2	105	PF00068.20	102.6	1.7E-29	PhospholipaseA2
Broad-banded copperhead	Phospholip_A2_1	18	124	PF00068.20	104.7	3.7E-30	PhospholipaseA2
Fer-de-lance	Phospholip_A2_1	18	124	PF00068.20	110.4	6.4E-32	PhospholipaseA2
Yellow-lipped seakrait	Phospholip_A2_1	2	109	PF00068.20	107.7	4.4E-31	PhospholipaseA2

Table 4: Result of secondary structure of all the species predicted

Speciesname	Alphahelix(Hh)	Extendedstrand(Ee)	Randomcoil(Cc)
Deathadder	29.41%	19.33%	49.58%
Mainlandtigersnake	33.61%	15.97%	48.74%
InlandTaipan	33.61%	11.64%	44.52%
Easternbrownsnake	39.04%	11.64%	44.52%
kingCobra	31.51%	11.64%	44.52%
Coastaltaipan	39.73%	10.27%	48.63%
Boomslang	39.33%	12.00%	44.67%
Easterncottonmouth	35.77%	16.26%	46.34%
SawScaledviper	36.96%	14.49%	43.48%
EasternRussell'sviper	32.19%	15.75%	48.63%
Malayanspittingcobra	31.97%	14.97%	48.98%
Many-bandedkrait	35.17%	17.24%	46.21%
Indiankrait	44.93%	13.77%	39.86%
SouthAmericanrattlesnake	44.93%	13.77%	39.86%
Beaked seasnake	36.13%	15.97%	41.18%
Chinesecobra	31.51%	16.44%	50.00%
Gaboonviper	33.05%	18.64%	45.76%
Broad-bandedcopperhead	45.26%	16.79%	32.12%
Fer-de-lance	42.03%	15.94%	37.68%
Yellow-lippedseakrait	36.44%	15.25%	44.92%

Table 5: Location of disulphidebridgein the Phospholipase A2 structure

Species Name	Positions of cysteinesresidues	Probablepattern of disulphide bond formation
Deathadder	1127 29 44 45 51 60 71 78 85 90 9299118	11-71,29-45,44-99,51-90,60-85,78-92
MainlandTigersnake	1127 29 44 45 51 60 71 78 85 90 9299118	27-60,29-45,44-99,51-92,71-85,78-90
InlandTaipan	1338 54 56 71 72 78 88 99106112 117119127146	54-88,56-72,71-106,78-112,99-117
Easternbrownsnake	1338 54 56 71 72 78 87 98105111 116118125144	54-111,56-72,71-125,78-118,87-98,105-116
kingCobra	1338 54 56 71 72 78 88 99106113 118120130149	54-106,56-72,71-130,78-99,88-113,118-120
Coastaltaipan	1338 54 56 71 72 78 88 99106112 117119126145	38-106,56-72,71-126,78-119,88-112,99-117
Boomslang	616 48 50 65 66 71 72 81 99105 110112119137144	48-144,50-66,65-119,71-105,72-81,99-110, 112-137
Easterncottonmouth	2628 43 44 49 50 57 75 81 86 88 95116123	26-116,28-44,43-95,50-88,57-81,75-86
sawscaled viper	4244 59 60 65 66 73 91 97102104 111129136	42-129,44-60,59-111,66-104,73-97,91-102
easternRussell'sviper	1042 44 59 60 65 66 73 91 97102 104111131138	42-131,44-60,59-111,66-104,73-97,91-102
Malayanspittingcobra	1338 53 55 70 71 77 87 98105112 117119126145	38-98,55-71,70-126,77-119,87-112,105-117
Many-bandedkrait	1342 54 56 71 72 78 88106113118 120127146	42-54,56-72,71-127,78-120,88-113,106-118
Indiankrait	1338 52 54 69 70 76 86 97104111 116118125144	52-97,54-70,69-125,76-118,86-111,104-116
SouthAmericanrattlesnake	4244 59 60 65 66 73 91 97102104 111131138	42-97,44-60,59-111,66-104,73-131,91-102
Beaked seasnake	1127 29 44 45 51 60 71 78 85 90 9299118	11-71,29-45,44-99,51-92,60-85,78-90
Chinesecobra	1338 53 55 70 71 77 87 98105112 117119126145	38-98,55-71,70-126,77-119,87-112,105-117
Gaboonviper	2426 41 42 47 48 73 83 85 92112 118	24-112,26-42,41-92,48-85,73-83
Broad-bandedcopperhead	4244 59 60 65 66 73 91 97102104 111131137	44-60,59-111,65-131,66-104,73-97,91-102
Fer-de-lance	4244 59 60 65 66 73 91 97102104 111131138	42-131,44-60,59-111,66-104,73-97,91-102
Yellow-lippedseakrait	11272944455160	11-51,27-44,29-45

Secondary structure prediction:

The secondary structure element like an alpha helix, extended strand, and random coil of all the protein sequences was predicted by the SOPMA tool. The result shows that most of the regions in the sequences were occupied by random coils than an alpha helix, extended strand, and beta-turn. Phospholipase A2 showed

a higher percentage of the alpha helix which is highest in Indian krait and Broad-banded copperhead. High frequency of cysteines present in all protein sequences high stability of proteins by disulfide bonds. Disulphide bonds are detected by software CYS_REC (refer to table 5) which is used for terminating the function all in kraits.

Table 6: 3-Dstructure detail for Phospholipase A2 for all the snakes pecies.

SPECIESNAME	TEMPLATE AND ORGANISMNAME	TEMPLATEIDENTITY
Acanthophsiantarcticus (DeathAdder)	5p2p.1.A	54.70%
Notechisscutatuscutatus(MainlandTigersnake)	2not.2.A	100%
Oxyuranusmicrolepidotus(InlandTaipan)	1psh.1.A	55.93%
Pseudonajatextilis(Easternbrownsnake)	2not.1.A	65.25%
OphiophagusHannah(kingCobra)	1gp7.2.A	80.82%
Oxyuranusscutellatuscutellatus(Coastaltaipan)	3v9m.1.A	68.64%
Dispholidustypus (boomsnake)	5wzs.1.A	57.02%
Agkistrodonpiscivoruspiscivorus(Easterncottonmouth)	5wzs.1.A	57.02%
Echiscarinatus(SawScaled Viper)	1oz6.1.A	100%
Daboiasiamensis(EasternRussell'sviper)	1oqs.1.A	89.34%
Najasumatrana (Malayanspittingcobra)	1yxh.1.A	88.10%
Bungarusmulticinctus (Many-bandedkrait)	1bun.1.A	99.17%
Bungaruscaeruleus (Indiankrait)	1dpy.1.A	91.53%
Crotalusdurissusterrificus (SouthAmericanrattlesnake)	6tmy.2.C	99.18%
Enhydrinaschistose (Beakedseasnake)	2not.2.A	78.15%
Najaatra (Chinesecobra)	1yxh.1.A	90.48%
Bitisgabonica (Gabooviper)	1vpi.1.A	54.28%
Agkistrodoncontortrixlaticinctus(Broad-bandedcopperhead)	1s8g.1.A	100%
Bothropsasper(Fer-de-lance)	5tfv.1.A	99.18%
Laticaudacolubrine(Yellow-lippedseakrait)	2not.2.A	66.10%

Fig.1:3 Dstructure of PhospholipaseA2 for all the snakes pecies

1.DEATHADDER



2. MainlandTigersnake



3. Inland Taipan



4.Easternbrownsnake



5. kingcobra



6. Coastaltaipan



7. Boomslang



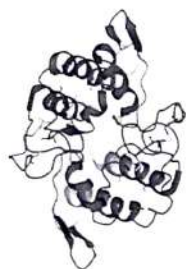
8. Easterncottonmouth



9. Sawscaledviper



10. EasternRussell'sviper



11. Malayan spitting cobra



12. Many-bandedkrait



13. Indiankrait



14. S. American rattle snake



15. Beaked sea snake



16.Chinesecobra



17. Gaboonviper



18. Broad-bandedcopperhead

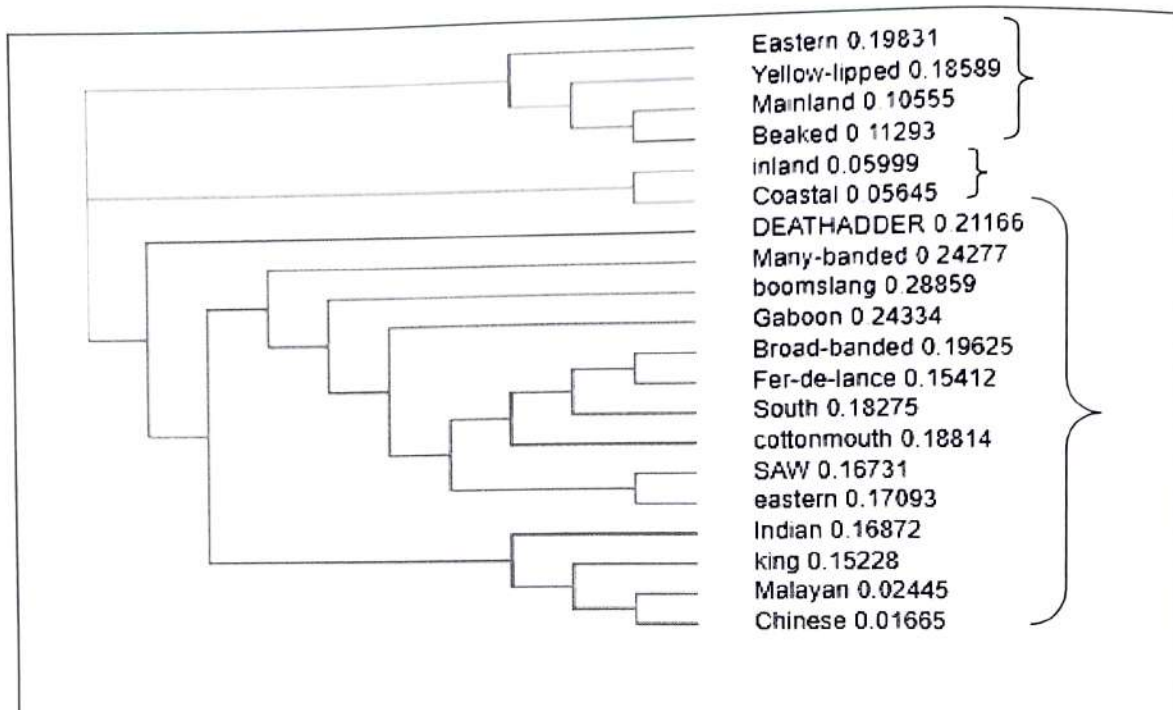


19. Fer-de-lance



20. Yellow-lippedseakrait





Conclusion

Primary structure analysis revealed that Phospholipase A2 protein of most of venomous snake has an average molecular weight 15.229 kDa, basic and hydrophobic in nature. The secondary structure analysis confirmed random coils dominated followed by an alpha helix and beta strand. The presence of disulfide bridges further established stability of protein and thus aiding in formulating their uses. Further, phylogenetics study shows that the evolution is the major factor that plays in the physico chemical properties of protein in the snake species. A phenotype of any organisms influence from both its genotype and environmental condition. Present analysis revealed the toxic phospholipase A2 in present in different organisms were more or less related to one another and may have originated from the same ancestor.

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Efficient dye decolorization by *Virgibacillus marismortui* B6 and its impact on seed germination

Shivani M. Yagnik¹, Vikram H. Raval², Ramesh K. Kothari³, Charmy R. Kothari^{4*}

¹ Department of Microbiology, Christ College, Rajkot-360005, Gujarat, India

² Department of Microbiology and Biotechnology, University School of Sciences, Gujarat University, Ahmedabad-380009, India

³ Department of Biosciences, Saurashtra University, Rajkot-380009, Gujarat, India.

⁴ Department of Biotechnology, Christ College, Rajkot-360005, Gujarat, India

*Corresponding Author email: dr.charmykothari@gmail.com

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ABSTRACT

Globally water pollution is a menace and treatment of industrial discharge is a mammoth task. Microbial sources have long been employed for bioremediation of the majority of such hazardous and toxic waste and are probably the only answer to serious environmental issues in years to come. To assess textile dye decolorization 33 bacterial isolates were screened against 4 different textile dyes and effluents. Isolates that showed remarkable ability to decolorize Reactive black B5 dyewere studied for morphological features, biochemical characters, and molecular identification by 16SrDNA sequencing. A critical parameter of assessing the toxic effects on seed germination was studied using wheat (*Triticum aestivum*) and green gram (*Phaseolus mungo*). The present study is aimed at developing a cost-effective and eco-friendly method of textile waste treatment. Affirmative effects of bacteria treated effluents on seed germination indicate futuristic bioremediation processes to be developed and its further implications in agriculture.

Key words: Dye decolorizing bacteria, *Virgibacillusmarismortui*, azo dyes, seed germination, phytotoxicity

Introduction

Synthetic dyes give fabulous results of shades and finishing this makes its use un-avoidable in the era as compared to natural dye. Textile industries are the major ones to use dyes and even have a great part to expel dye polluted water to the environment. This directly affects the consumption of usable water, issues like water crisis and pollution to resources, as the textile industry mainly uses azo dyes which are very toxic to the life forms (Mahmood et al., 2015; Moawad et al., 2003). The serious problem with azo dyes is their degradability as they are recalcitrant, and if degradable it is partial degradation or secreting more toxic end

products.(Lellis et al., 2019). The water thrown out of dyeing industries need to get pre-treatments to reduce the toxicity levels of end products and decrease the percentage of non-biodegradable components of dye from water disposed or reused in agriculture (David Noel and Rajan 2015; Bardi and Marzona 2013).Physical and chemical processes are available wastewater treatments, but they are cost expensive, low degradation levels, and high toxic by-product production.(Shakeel et al., 2020). The alternative way of wastewater treatment before draining in main water resources is biodegradation using microorganisms, which has shown a cost-effective and eco-friendly approach

to rectify the problem (Parmar and Shukla 2015; Saratale *et al.*, 2011). The only disadvantage is the speed or rate of reactions as the dye-containing wastewater discarding values are rapidly increasing and the speed of biodegradation of dye is very slow. (Shukat *et al.*, 2019)

The improvisation of biodegradation speed is by the addition of specific potential microbial cultures to the wastewater plant to overcome the basic problem of slow bio-degradation process rate. The indigenous species show more potency for the biodegradation work rather than the potent species of other areas (Nasrin *et al.*, 2019; Neetha *et al.*, 2018). To achieve this, there is a need to isolate the potential microbial species from the region of industrial wastewater of dyeing or printing companies. As indigenous species show more potency for biological degradation. Hence it is required to isolate the microbial species from the area of disposing for the pre-treatment of dye-containing wastewater (Khan and Malik 2018).

Materials and Methods

Description of the collected sample

Textile dyes Reactive black B55 (RbB5, di-azo), Reactive red 120 (RR120, di-azo), Reactive Orange 16 (RO16, mono-azo), and Reactive Yellow 145 (RY145, mono-azo) were procured from one of the cotton saree printing industry with common effluent treatment plant (CETP) of Jetpur, Gujarat, India with 21.7547° N latitude and 70.6180° E longitude coordinates, wastewater sample was collected with random sampling technique, transported to the laboratory and preserved at 4° C until further process.

Isolation and Screening of dye decolorizing bacteria from the wastewater effluent

A 10% (v/v) industrial effluent was inoculated in 200ml of Nutrient broth containing 500ml Erlenmeyer flask, incubated at room temperature for 48h. 10% (v/v) of this preincubated nutrient broth was inoculated in 200ml fresh, Sterile Bushnell and Haas medium (BHM) containing glucose (0.1% w/v) and peptone (0.1% w/v) with

250 ppm of all four dyes mentioned in the description of collected sample in 500ml flask and was incubated at room temperature for 72h. Using serial dilution technique, the enriched BHM media broth was used as samples were spread onto BHM agar plates with varied dye concentrations of 100- 500ppm of all four dyes followed by incubation at 37°±2°C temperature for 24-48 h. Morphologically different isolates were selected and named as B1-B6, R1-R8, Y1-Y12, G1-G7 and were preserved on nutrient agar slants at 4°±2°C.

Screening of Dye decolorizing bacteria

A) Primary screening

Inquest to isolate a potent bacterial isolate with the ability to decolorize and degrade azo dye, 33 morphologically different bacterial species were primarily screened against each dye with 500ppm concentration. 50 ml BHM with (1% w/v) glucose and peptone was inoculated with the 1% (v/v) of the activated culture in a 150 ml flask. Experimental sets were incubated at room temperature at static conditions. Samples were collected at 0h, 6h, 12h, and 24h respectively. The inoculated broth was then centrifuged, and supernatants were examined spectroscopically on a respective wavelength of individual dyes using a UV-Visible spectrophotometer (Shimadzu, Japan). Liquid broth without inoculation was used as a control. Percentage of decolorization was calculated with the following formula (Khan and Malik 2018):

$$\% \text{decolorization} = \left[\frac{(\text{O.D. of control sample} - \text{O.D. of the treated sample})}{\text{O.D. of Control}} \right] \times 100$$

B) Secondary screening

Based on results of primary screening, 4 bacterial isolates viz. R1, B2, B4, and B6 were selected further on their efficiency and rate of decolorization of various textile dyes. The efficiency was assessed as a percentage of decolorization. These four isolates are further studied for their dye decolorizing potential with a set of an experiment consisting of 200ml BHM media containing (0.1 % w/v) glucose, peptone, and all four dyes (RbB5, RR160, RO16, RY145)

with 500ppm concentration in 500ml flasks. Media consisting of all four dyes were inoculated with the primarily screened isolates (R1, B2, B4, and B6) with 5%(v/v) inoculum in each flask and were incubated at room temperature for 96h. An experimental set for consortia analysis with each organism in equal inoculum size (1.25%v/v) was incubated against mixed dye-containing broth. Samples were collected, centrifuged and supernatants were examined using a UV-Visible spectrophotometer at 12h of intervals. Percentage decolorization was calculated for analysis.

All the experiments were performed in triplicate and results were statistically analyzed. The media used were procured from Hi-Media Laboratories, Mumbai, India.

Sugar utilization, Biochemical and Molecular identification of potential isolates

Biochemical characterization is one of the basic techniques for the identification of microorganisms. The selected 33 isolates were examined for various biochemical tests viz. Catalase test, Oxidase test, Methyl Red test, Voges Proskauer test, Citrate Utilisation, Indole Production, Urease Production, Hydrolysis of Casein, Gelatine, Starch, Sugar fermentation: Glucose, Xylose, Maltose, Mannitol, Fructose, Sucrose, Trehalose, Galactose, Lactose, Inositol, Sorbitol, Dextrose, Arabinose, Mannose, Cellobiose, Raffinose, Rhamnose, H₂S Production test, and Nitrate reduction Test.

Molecular identification

The potential 4 isolates were selected and identified by molecular 16S rRNA gene amplification method using 27 F5' (AGAGT TGA TCMTGGCTC A G) 3' and 1492R5' (TACGGYTACCTTGTTACG ACTT) 3' primers for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30µl reaction mixture by using an EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2 minutes, 35 cycles of 95°C for 1 minutes, 55°C, and 72°C for 1min each were performed, finishing with a 10-min step at 72°C.

After successful gene amplification, the PCR products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). A sequencing reaction was performed using a PRISM Big-Dye Terminator v3.1 Cycle sequencing kit. Sequencing primers used were 785F 5'(GGATTAGATACCCTGGTA) 3' and 907R 5'(CCGTCAATTCMTTTRAGTTT) 3'. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5min, followed by 5min on ice, and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequence so obtained was phylogenetically analyzed using BLASTn (<http://www.ncbi.nlm.nih.gov/blast>) tool (Altschul et al., 1990). The sequence obtained was submitted to the NCBI domain for the required accession numbers.

Seed germination test

The phytotoxic effects were assessed to adjudge the toxicological aspect of dye decolorization if any, by an organism and its further reuse for irrigation. The effect of Reactive black B55 and Reactive Orange 16 dyes and their metabolites on germination and growth of these 2 types of economically important crops were studied. In this study, the effect of two different dyes Reactive black B5 and Reactive Orange was evaluated on germination of seeds of 2 crops: Monocot-Wheat (*Triticum aestivum*) and 2) Dicot-Green gram (*Phaseolus mungo*). For the two plant varieties mentioned above, 06 seeds of each were taken with 3 different samples pre-treatment viz. seeds+d/w, seeds+synthetic dye effluent, and seeds+ bacteria treated effluent. These were then placed on Whatman filter paper in a sterilized Petri plate. The regular watering was done by simply wetting the filter paper with 5ml of respective pre-treatment every 12 h continuously for 7 days. All the plates were incubated at room temperature (28±2°C). Six seeds three of each crop plant were soaked and treated with 3 different solutions (distilled water, synthetic dye

effluent, synthetic dye effluent treated with bacterial isolates) were placed on a Whatman filter paper in the sterilized Petri plate. The filter papers were wetted with 5 ml of distilled water (control) or with various concentrations of dye in distilled water every 12 hour. All dishes were kept at room temperature (28 ± 2 C) for a period of 7 days. At the end of the germination experiment, the root and shoot length were measured in cm/plant. The results were recorded upto the 9th day of growth and then compared.

Results and Discussion

Morphological and Microscopic Characteristics of the selected bacterial isolates

Several dye tolerant bacterial isolates were selected based on different colony characteristics and microscopic examinations summarized in Table 1. Amongst all 33 bacterial isolates selected, 25 isolates—selected, 25 were Gram-positive (04 rod-shaped; 21 cocci), while 08 were Gram-negative (06 rod-shaped; 02 cocci). Colony characters viz. colony size shape, margin, elevation, texture, opacity, and pigmentation were recorded for all these isolates. It was observed that 08 of the isolates were showing irregular colony shape, the remaining 25 had round to oval-shaped colonies on nutrient agar plates. Moreover, 07 of the isolates had raised colonies, while 05 others have slightly raised colonies and remaining 11 had flat colonies appearance. When the margin was studying it was noted that 10 of the isolates had an uneven margin while the remaining 31 were with an even margin.

The pigmentation of the isolated colonies was studied. A total of 09 isolates showed slightly yellow to brown colored pigmented colonies, while the remaining 24 were either white or off-white. Only 02 isolates had a mucoid appearance while the other 31 showed smooth texture. Lastly, it was observed that 22 isolates showed opaque while 11 had translucent colonies. All the isolates were preserved in Nutrient agar slants and 40% (v/v) glycerol vials under refrigeration for

further screening of their dye decolorizing potential.

Primary and Secondary Screening of dye decolourizing bacterial isolates

The efficient dye decolorization needs a faster dye decolorization rate, to select such isolate screening was performed first with four textile dyes against each bacterial isolate. Bacterial isolates B6, B4, B2, and R1 show a high percentage of decolorization than others. All four isolates can decolorize all four treated dyes of 500ppm concentration in 24 hrs. Dye RbB5 is decolorized at 96.3% by B6 isolate with the highest efficiency shown, followed by R1 at 94.5 %, B2 at 74.0%, and B4 at 42.1%. Dye RY145 is decolorized at 89.0% by B2, 83.4% by R1, 80.8% by B6, and 44.2% by B4. Dye RO16 shown an 85.9% to 82.6% range of decolorization by these four isolates. Dye RR120 is decolorized at 84.4% by B2 with highest % decolorization followed by B6 with 82.7%, R1 with 56.9%, and B4 with 53.3 % decolorization (data not shown). Other isolates also show decolorization of these dyes but with relatively slow rates. The spectral analysis studies of treated dyes with 33 isolates scrutinized four isolates B6, B4, B2, and R1 with potency to decolorize azo-dyes RbB5, RY145, RO16, and RR120 at 500ppm in 24hrs.

Textile industrial effluents are majorly having high concentrations of dyes, sometimes it reaches 1200ppm. Even effluent contains multiple dyes at times, to screen the multiple dye decolorizing property, secondary screening was performed using mixed dye (500ppm each) with four selected potential dye decolorizing isolates and their consortium, observed for 96hrs. At the end of 48 hrs, the consortia gave the highest percentage of decolorization with 61.61% followed by B6 with 50%, B2 34.82, R1 26.79%, and B4 with least 24.11% of decolorization. After 96hrs the spectral analysis shows the best result amongst the above five experimental sets with isolate B6 giving 89.29% decolorization, followed by consortia 71.96%, B2 60.71%, R1 51.79%, and B2 50%. The screening results of mixed dye decolorization

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shows isolate B6 with the efficient decolorization ability (Figure 1).

Sugar utilization of dye decolourizing bacterial isolates

The ability of all the bacterial isolates to ferment various sugars was assessed. It was observed that they were able to utilize and ferment sugars in the order mentioned below Glucose = Dextrose

= Mannitol = Sucrose = Trehalose = Galactose = Inositol = Sorbitol = Mannose = Cellobiose > Maltose > Xylose = Lactose = Rhamnose > Raffinose > Fructose = Arabinose. For two sugars, Fructose and Arabinose it was a general observation that all the isolated bacteria showed the least preference for these two sugars (data not shown).

Table 1: Cell and colony morphology of dye decolorizing bacteria

Sr. No.	Isolate	Gram Reaction	Cell Shape	Colony Size	Colony Shape	Margin	Elevation	Texture	Opacity	Pigment
1	B1	+ve	Rod	Medium	oval	even	flat	smooth	opaque	white
2	B2	-ve	Rod	medium	Irregular	Uneven	raised	smooth	Translucent	pale yellow
3	B3	-ve	Rod	small	round	even	flat	smooth	opaque	yellow
4	B4	-ve	Rod	medium	round	Uneven	raised	mucoid	opaque	white
5	B5	-ve	Cocci	Small	round	even	raised	smooth	Translucent	brownish-yellow
6	B6	+ve	Rod	medium	Irregular	uneven	flat	mucoid	translucent	creamy white
7	R1	-ve	Rod	medium	Irregular	uneven	raised	smooth	translucent	pale yellow
8	R2	+ve	Rod	Medium	round	uneven	flat	smooth	opaque	white
9	R3	+ve	Rod	Medium	round	even	flat	smooth	opaque	white
10	R4	+ve	Rod	large	oval	even	flat	mucoid	opaque	off white
11	R5	+ve	Rod	Medium	oval	even	raised	smooth	opaque	white
12	R6	-ve	Rod	Medium	oval	even	flat	smooth	translucent	white
13	R7	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
14	R8	+ve	Cocci	Small	round	even	raised	smooth	translucent	pale yellow
15	Y1	+ve	Cocci	Small	round	even	raised	smooth	translucent	brownish-yellow
16	Y2	+ve	Rod	Medium	Irregular	uneven	flat	smooth	opaque	off white
17	Y3	-ve	Cocci	Small	round	even	raised	smooth	translucent	yellow
18	Y4	+ve	Rod	Medium	Irregular	uneven	flat	smooth	opaque	off white
19	Y5	+ve	Cocci	Small	round	even	raised	smooth	translucent	pale yellow
20	Y6	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
21	Y7	+ve	Rod	Medium	oval	even	flat	smooth	opaque	white
22	Y8	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
23	Y9	+ve	Cocci	Small	round	even	raised	smooth	translucent	brownish-yellow
24	Y10	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
25	Y11	+ve	Rod	Medium	irregular	uneven	flat	smooth	opaque	off white

25	Y11	+ve	Rod	Medium	irregular	uneven	flat	smooth	opaque	off white
26	Y12	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
27	G1	+ve	Rod	Medium	oval	even	flat	buttery	opaque	dirty white
28	G2	-ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
29	G3	+ve	Rod	Medium	irregular	uneven	flat	smooth	opaque	off white
30	G4	-ve	Cocci	Small	round	even	raised	smooth	translucent	brownish-yellow
31	G5	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
32	G6	+ve	Rod	Medium	oval	even	raised	smooth	opaque	white
33	G7	+ve	Rod	Medium	irregular	uneven	flat	smooth	opaque	off white

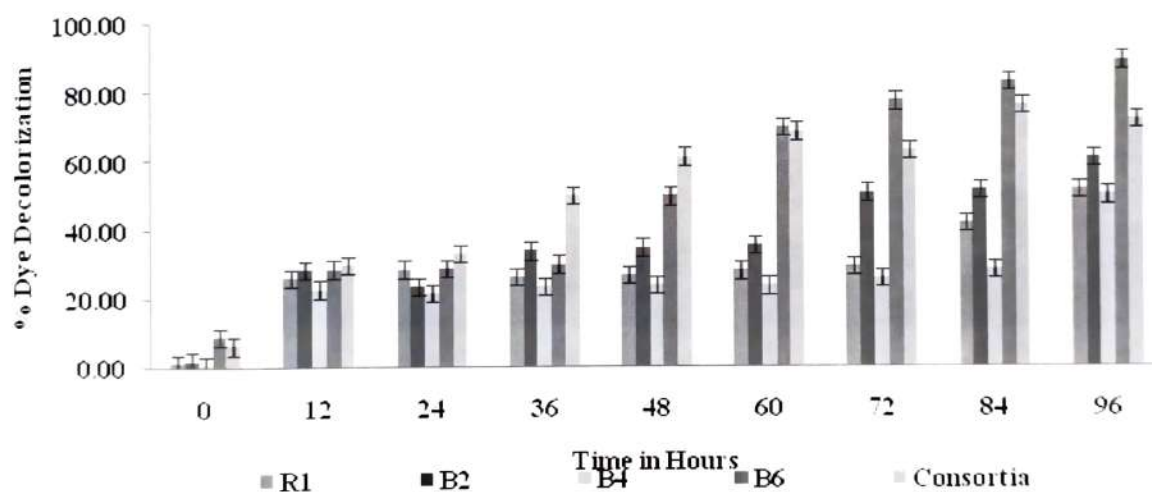


Figure 1: Secondary screening results of selected 4 bacterial isolates and their consortia against mixed dye

Biochemical and Molecular Characterization of the dye decolorizing bacteria

Biochemical characterization of all 33 isolates was studied. The majority of selected cultures showed positive results for the catalase and oxidase test. 06 cultures were showed positive results for citrate utilization, while all others were either negative or showed no detectable results. All of the isolates were positive for Indole production. All isolates enriched and obtained from yellow dye were showing positive

results for urease production while all other isolates were urease negative. For nitrate utilization, 21 cultures showed positive results while the remaining 12 were negative. When utilization of complex sources of carbon was assessed it was observed that as high as 26 bacterial isolates were able to utilize starch, 17 were able to hydrolyze casein and only 11 isolates were able to utilize gelatine. For H_2S production also it was observed that only 11 isolates were able to do so. A detailed result is summarized in Table 2.

Four bacterial isolates that were specifically selected from a secondary screening of dye

decolorization and had enormous potential to degrade various dyes selected in the present study were identified by 16S rDNA gene homology analysis. As mentioned in materials and methods, the ~1500bp 16S rDNA gene was amplified by using universal primers. The sequence data were further analyzed for finding the closest homologs for the microbe by comparing gene sequences with reference strains. The nucleotide sequence for all four isolates has been submitted to the GenBank database with accession numbers MF772441-MF772444. Based on nucleotides homology and phylogenetic analysis the microbes were identified. Two of the isolates B2 and R1 were identified and had the closest homology with *Enterobacter cloacae*, isolate B4 as *Leclerciaadecarboxylata* and isolate B6 as *Virgibacillusmarismortui*. Phylogenetic trees were constructed for all isolates using Neighbour Joining Method with 0.75 maximum sequence difference using an online tool from NCBI nucleotide BLAST tool (Figure 2).

Seed Germination test

In this study, it was found that the seeds germination of wheat, as well as mung, was significantly affected by the textile dyes and effluent (Nasrin et al., 2019; Moawad et al., 2003). However, post-treatment of the effluent with potential dye decolorizing bacterial isolate, the Figure 1: Secondary screening results of selected 4 bacterial isolates and their consortia against mixed dye

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seeds which indicates that such bacterial remediation approach can be useful in the recycling effluents and other industrial wastewaters (Nasrin *et al.*, 2019). Additionally, it is a good approach for a sustainable environment and for the betterment of irrigation in developing countries where the majority of agriculture is

dependent on rain (Vora *et al.*, 2007). For such areas where water scarcity is substantial and alternative sources of water for irrigation are sought, there such a bioremediation approach mediated by bacterial treatment would render the effluents detoxified and nullify the toxic effects of textile dyes (Khan and Malik 2018).

Table 2 Biochemical characterization of selected dye decolorizing bacteria

Sr. No.	Isolate	Catalase	Oxidase	Methyl Red	Voges Proskauer	Citrate Utilisation	Indole Production	Urease Production	Casein	Gelatine	Starch	H ₂ S Production	Nitrate reduction
1	B1	+	+	+	-	-	+	-	-	-	+	+	+
2	B2	+	+	+	-	-	+	-	+	-	+	-	+
3	B3	+	-	-	-	+	+	-	-	-	+	-	+
4	B4	+	+	+	-	+	+	-	+	+	+	+	+
5	B5	-	+	-	-	-	+	-	+	-	+	-	+
6	B6	+	+	-	-	-	+	-	+	+	+	+	+
7	R1	+	+	+	-	-	+	-	-	-	+	+	+
8	R2	+	+	+	-	+	+	-	+	-	+	+	+
9	R3	+	-	+	+	-	+	-	-	-	-	+	+
10	R4	+	+	+	-	-	+	-	-	-	-	+	-
11	R5	+	-	+	-	-	+	-	-	+	+	+	-
12	R6	-	+	-	-	-	+	-	+	+	+	+	-
13	R7	+	+	-	-	-	+	-	-	-	+	+	+
14	R8	+	+	-	-	-	+	-	-	-	-	+	+
15	Y1	+	+	-	+	-	+	+	-	-	+	-	+
16	Y2	+	+	-	+	-	+	+	+	+	+	-	-
17	Y3	+	+	+	+	-	+	+	-	+	+	-	-
18	Y4	+	+	-	+	-	+	+	+	+	-	-	+
19	Y5	+	+	+	+	-	+	+	-	+	+	-	-
20	Y6	+	+	+	-	-	+	+	+	-	+	-	-
21	Y7	+	+	+	-	-	+	+	+	-	+	-	-
22	Y8	+	+	-	-	-	+	+	+	-	+	-	+
23	Y9	+	+	+	-	-	+	+	-	-	+	-	+
24	Y10	+	+	+	+	-	+	+	+	-	-	-	+
25	Y11	+	+	+	-	+	+	+	+	+	+	-	-
26	Y12	+	+	+	-	-	+	+	+	+	-	-	-
27	G1	-	-	-	-	-	+	-	+	-	+	-	+
28	G2	+	+	+	-	nd	+	-	+	-	+	-	+
29	G3	+	-	nd	-	nd	+	-	-	-	+	-	+
30	G4	+	+	+	-	nd	+	-	-	-	-	-	+
31	G5	-	+	nd	-	+	+	-	-	-	+	-	+
32	G6	-	-	nd	-	nd	+	-	+	+	+	-	-
33	G7	-	-	nd	-	nd	+	-	-	-	+	-	-

+: A positive result -: A negative result nd: results not detected

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EFFICIENT DYE DECOLORIZATION

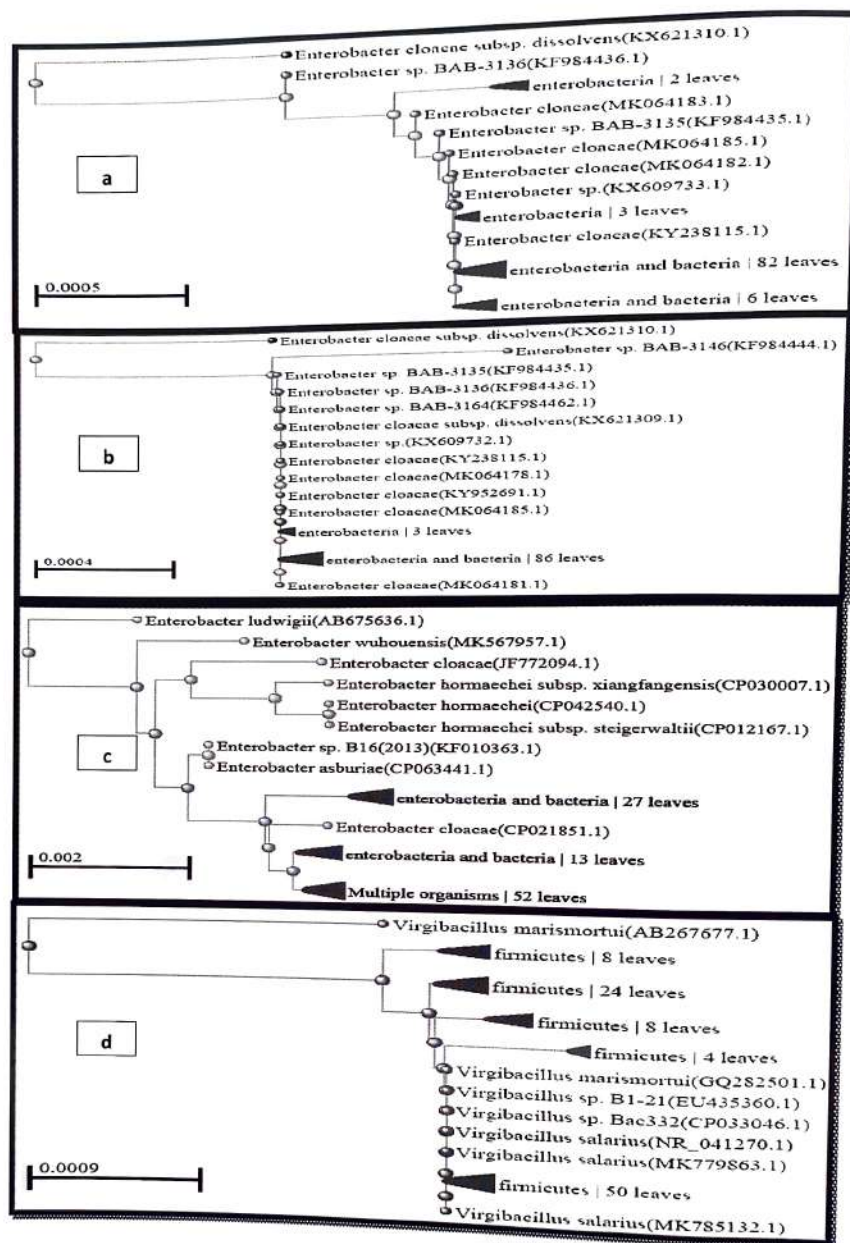


Figure 2: Phylogenetic analysis for (a) *Enterobacter cloacae* B2 (b) *Enterobacter cloacae* R1 (c) *Leclerciaadecarboxylata* B4 (d) *Virgibacillusmarismortui* B6

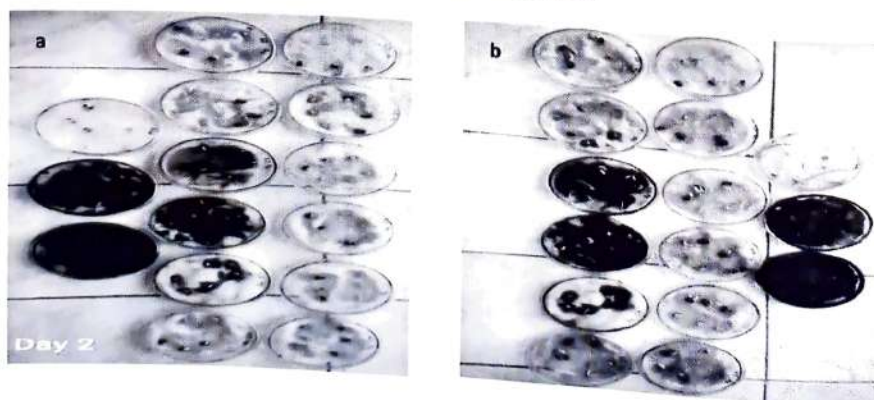


Figure 3: Effect of dyes (effluent) and bacteria treated effluent on germination of seeds 2nd day (a) L-R Lane 1 Control with distilled water and dyes, Lane 2 Seeds treated with Reactive black B5, Lane 3 Seeds treated with Reactive Orange, and 9th day (b) L-R Lane 1 Seeds treated with Reactive black B5, Lane 2 Seeds treated with Reactive Orange, Lane 3 Control with distilled water and dyes.

Conclusion:

The study on azo dye decolorization and seed germination test for treated dye sample conclude that it's promising for recycling of azo dye polluted water to detoxify it with bacterial isolate B6 identified as *Virgibacillus marismortui*, and can be used further for irrigation with supportive studies. This shows more than 90% of decolorization at 500ppm of dye concentrations which makes it useful for industrial waste treatments as their dye concentrations expelled are in such range. The non-phytotoxic nature of the treated dye sample makes it more encouraging to be used for bioremediation.

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Role of Amul on Milk Producers with Special Reference to Anand District In Gujarat

Alpeshkumar D Patel¹ and Dr. R. D Modi²

*Assistant Professor, Shri V.Z. Patel Commerce College, Anand, Gujarat-388001(India)

** Principal, C.P.Patel and F.H.Shah Commerce College, Anand, Gujarat-388001(India)

Corresponding Author E-mail ID: adpatel75@gmail.com

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ABSTRACT

Animal husbandry activity in India is an early occupation established in rural conditions. The dairy co-operative sector in India has made a significant contribution in creating employment opportunities for the people and providing food security in addition to providing supplementary income in rural areas. The main objective of this research paper is to study the role of AMUL on milk producers in Anand district. It is also necessary to analyze the contribution of AMUL in terms of benefits to milk producers through suggestions for overcoming barriers in various development programs for Amul milk producer development and access to milk producers. Data have been collected by primary and secondary data for this study. A sample size of 160 respondents was selected for this study. The ex-post facto research design of social science was used for the research. The study used statistical measures such as frequency, percentage, arithmetic mean and correlation method. This study highlights the positive role of AMUL on milk producers.

Key words: Milk Union, Milk Producers, Dairy Co-operative, Dairy Development

INTRODUCTION

India's dairy co-operative structure has made a major contribution in increasing milk production. In the year 2017-18, the milk production was about 176.3 million tonnes, which was 17 million tonnes in the year 1951. At present the daily consumption of milk in the country is 375 gm / day. Thus, India's co-operative dairy sector has made a significant contribution in creating employment opportunities for rural people and providing food security in addition to increasing the income of small and marginal farmers and landless laborers in rural India.

HISTORY OF AMUL

The Anand Milk Union Limited (AMUL) is an Indian dairy Co-operative, located at Anand in the

state of Gujarat, formed in 1948, it is a co-operative brand managed by a co-operative structure, the Gujarat co-operative Milk Marketing Federation Ltd., which today is jointly owned by 3.6 million milk producers in Gujarat. AMUL sprung from the seeds sown in the black soil of Charotar, an area in the Kaira district of Gujarat, as a dairy co-operative movement to empower the milk producers. The Taste of India, a brand so distinctively Indian has been a part of our lives for nearly seven decades now and still is able to touch a chord in our hearts. As a brand AMUL has grown from being merely a differentiating factor to protect the interests of producers and consumers. AMUL inspired 'Operation Flood' and heralded the 'White Revolution' in India. It began with only two village

level dairy co-operatives and 250 liters of milk per day, nothing but ooze compared to the flood it has become today. AMUL distributes over a million liters of milk per day, it also collects and processes various milk products, during the peak, on behalf of more than a thousand village individually owned by half a million milk producers members. AMUL too has become a symbol of the aspirations of millions of milk producers.

DEVELOPMENT PROGRAMS IMPLEMENTED BY AMUL FOR THE DEVELOPMENT OF MILK PRODUCERS:

Amul has implemented various developmental programs for milk producing members such as collecting from milk producers twice a day, paying regular amount to milk suppliers, collecting milk and delivering it to district milk union, providing quality balanced animal feed, innovative fodder breeding. , Animal health care service, artificial insemination service to milch cattle, distribution of profits to shareholders, contribution to rural socio-economic development, periodic animal insurance, health insurance and some other promotional developmental activities in the interest of milk producers.

LITERATURE REVIEW

G.Panchbhai, G. and et al. (2017) This study is mainly aimed at finding the relationship between the socio-demographic profile of dairy farmers and the scientific methods of animal husbandry they have adopted through the use of correlation analysis. In the present study he has used the ex-post facto research method of the social sciences. There is positive significant relation with extant of adoption dairy animal management practices.

Kumar, Nishi and Saha (2011) concluded that milk producer's satisfaction with dairy co-operative societies. Involvement of organisational, potential of market and motivation of economic condition was found to have strongly influence on the satisfaction level of milk producers.

Mattigatti R. and et al. (1990) analyzed Karnataka State Co-operative Milk Producers Federation Limited and its impact on dairy development. He has discussed regarding different issues of co-operative Milk Producers Federation Limited. He studied the role of milk producer's co-operative societies and their impact on dairy activities in Dharwad district, Karnataka.

Panchbhai, G. and et al. (2017) researcher the main objective of the study was to asses limitation faced by dairy farmers. The research study was conducted at Shivamrut Dudh Utpadak Sahakari Snagh Maryadit, Solapur in Akhuj district of Maharashtra state. The results of the research show that ticks in the sheds of milch animals, milk production is low, animals need to be fed large quantities of animal feed, need to focus on high cost of cross breed, dairy milk farmers face shortcomings.

Prasad, A. and Maheswari, M. (2018) in this study attempted to women empowerment through dairy development and co-operative organization with also promoting their awareness of its empowerment. Co-operative plan improve social advancement through economic growth. Sum up of the study is that positive relationship between the socio-economic and supporting background and aware the index of women through co-operative dairy and alter their mindset towards earning and empowerment.

STATEMENT OF PROBLEM

Many review of the existing literature indicates that there is no inclusive study on Aanad Milk Producers Union Limited (AMUL). It is a self-effacing attempt to study the different issues of AnandMilk Producers Union Limited (AMUL). Therefore current study was planned with objective for: To study the profiles of the milk producers, To study the awareness and attitude of milk producers about different development programmes of AMUL and To study the constraints faced and suggestions by milk producers in terms of getting benefits of different development programmes conducted by AMUL.

Materials and Method:

Research Design: As the study on descriptive research design has been used in order to describe the milk producer's role of AMUL.

Area of Study: The study has been conducted in Anand District, Gujarat.

Sample Size: The sample size purposively selected by the researcher, in this study is 160 milk producers.

Sampling Technique: The researchers choose purposively sampling method to collecting data.

Sources of Data: This research study is based on primary data as well as secondary data to accomplish objectives of the study.

Primary Data: The primary data for the study has been collected from milk producers through personal interview of questionnaire. The questioner is designed in a systematic manner

covering adequate and relevant questions which is useful study. The sample size of 160 milk producers' respondents purposively has been selected for the study.

Secondary Data: The secondary data collected through various books, journals, research articles, News Paper articles and annual report from AMUL, NDDB, and GMMF.

Data Collection: In the research study the researcher has used the self-structured questionnaire and interview method for collecting relevant information.

Data Analysis: After data collection were classified, tabulated, analyzed and interpreted to formulate the findings significant. The statistical measures such as frequency and percentage, arithmetic mean and correlations were used in the study.

DATA INTERPRETATION**Table: 1: Socio-Demographic Profile of the Milk Producers**

	Frequency	Percent
Age of the Respondents		
Young age group (up to 35 years)	29	18.12
Middle age group (between 36 to 50 years)	97	60.63
Old age group (above to 50 years)	34	21.25
Education Level of the Respondents		
Illiterate	00	00.00
Primary Education	10	06.25
Secondary Education	40	25.00
Higher Secondary Education	78	48.75
Graduation and above	32	20.00
Gender		
Male	115	71.88
Female	45	28.12
Family Types		
Nuclear Family	32	20.00
Joint Family	128	80.00
Family Size of Respondents (in Numbers)		
Small (Up to 4 members)	26	16.25
Medium (5 to 8 members)	60	37.50
Large (9 and above members)	14	8.75
Marital Status		
Married	123	76.88
Unmarried	27	16.87
Widow	04	2.50
Divorced	06	03.75

Occupation		
Farming + Animal Husbandry	125	78.12
Animal Husbandry + Job	12	7.50
Animal Husbandry + Business	23	14.38
Annual Income		
Very low (up to ₹ 50,000)	49	30.62
Low (₹ 50,00 to 1,00,000)	21	13.12
Medium (₹ 1,00,00 to 1,50,000)	50	31.25
High (₹ 1,50,000 to 2,00,000)	28	17.50
Very High (Above ₹ 2,00,000)	12	7.50
Social Participation		
Membership in one organization	13	8.13
Membership in two organization	48	30.00
Membership in more than two organization	91	56.87
Membership along with position holding	8	05.00
Land Holding (in acre)		
Marginal (Up to 1.00)	17	15.62
Small (1.01 to 2.00)	26	21.87
Medium (2.01 to 4.00)	69	48.75
Large (Above 4.00)	13	13.76
Herd Size		
Small (Up to 2 animals)	131	81.87
Medium (3 to 5) animals	25	15.67
High (6 and above five Animals)	04	02.50
Experience		
Very low (Up to 6 Years)	12	7.50
Low (7 to 612 Years)	20	12.50
Medium (13 to 18 Years)	39	24.38
Very high (25 to 30 Years)	89	55.62

Source: Field Survey figures in the percentage

ATTITUDE OF MILK PRODUCERS TOWARDS DIFFERENT DEVELOPMENT PROGRAMS OF AMUL

Table 2: Distribution of the milk producers according to their attitude of milkproducers towards different development programmes of AMUL (n=160)

No.	Attitude	Number	Per cent
1	Very Low (Up to 32.40)	05	3.12
2	Low (32.41 to 46.80)	10	6.25
3	Medium (46.81 to 61.20)	31	19.38
4	High (61.21 to 75.60)	68	42.50
5	Very High (75.61 to 90.00)	46	28.75
Total		160	100.00

Source: Field Survey figures in the percentage

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ROLE OF AMUL ON MILK PRODUCERS WITH SPECIAL

The present data in Table 2 clarify shows that slightly more than two-fifth (42.50 %) of the milk producers have high level of attitude followed by 28.75 %, 19.38 % and 6.25 % of the milk producers were with very high, medium and low level of attitude towards different development programmes of AMUL. Only 3.12 % of them have

very low level of attitude towards different development programmes of AMUL.

From the above results, it can be concluded that slightly less than three-fourth (71.25 %) of the milk producers have high and very high level of attitude towards different development programmes of AMUL.

AWARENESS OF MILK PRODUCERS REGARDING DIFFERENT DEVELOPMENT PROGRAMS OF AMUL

Table 3: Awareness of milk producers regarding different development programmes of AMUL (n=160)

No.	Awareness	Number	Per cent
1	Very Low (Up to 3.40)	03	1.87
2	Low (3.41 to 6.80)	10	06.25
3	Medium (6.81 to 10.20)	96	60.00
4	High (10.21 to 13.60)	45	28.13
5	Very High (13.61 to 17.00)	06	03.75
Total		160	100.00

Source: Field Survey figures in the percentage

The present data in Table 3 clearly shows that exactly three-fifth (60.00 %) of the milk producers have medium level of awareness followed by 28.13 %, 6.25 % and 03.75 % of the milk producers were with high, low and very high level of awareness regarding different development programmes of AMUL. Only 01.87 % of them have very low level of awareness regarding

different development programmes of AMUL.

From the above results, it can be concluded that slightly more than four-fifth (88.13 %) of the milk producers have medium to high level of awareness regarding different development programmes of AMUL. Milk producers are more or less benefited through the different development programmes of AMUL.

RELATIONSHIP BETWEEN ROLE OF PROFILES OF MILK PRODUCERS AND DIFFERENT DEVELOPMENT PROGRAMS

Age and Different Development Programmes of AMUL

The data illustrated in Table 4 shows that there was negative and non significant correlation ($r = -0.208$) between age of milk producers and different development programmes.

Hence, the null hypothesis that "there is no relationship between age of milk producers and different development programmes" was accepted. It reflects that age of milk producers did not influence on milk producers through different

development programmes significantly.

Education and Different Development Programmes of AMUL

The data presented in Table 4 indicates that there was positive and highly significant correlation ($r = 0.420^{**}$) between education of milk producers and different development programmes.

Thus, the null hypothesis that "there is no relationship between education of milk producers and different development programmes" was rejected.

Table 4: Relationship between Role of Profiles of Milk Producers and Different development programs (n=160)

Sr. No.	Independent variables	Correlation coefficient (<i>r</i> ' value)
1	Age	-0.208
2	Education	-0.420**
3	Experience	0.385**
4	Type of family	0.116
5	Social participation	0.334**
6	Occupation	0.185**
7	Size of land holding	0.113
8	Herd size	0.367**
9	Annual family income	0.162**
10	Attitude towards different development programmes of AMUL	0.423**
11	Awareness regarding different development programmes of AMUL	0.383**

* = Significant at 5% level of probability, ** = Significant at 1% level of probability, NS = Non significant at 0.05 level, Source: Computed from field data

The above finding indicates that education played significant role in increasing different development programmes.

The probable reason might be the higher literacy level of milk producers provides opportunity for greater exposure to the print media and better ability to understand complicated matter regarding various development programmes of AMUL.

Type of Family and Different Development Programmes of AMUL

The data presented in Table 4 illustrates that there be positive and non significant correlation ($r = 0.116$) among type of family of milk producers and overall extent of role of AMUL in terms of giving benefits to milk producers through different development programmes.

Thus, the null hypothesis that "there is no relationship between type of family of milk producers and different development programmes" was accepted. Thus, it can be said that type of family of milk producers had not any significant influence in increasing different development programmes.

Social Participation and Different Development Programmes of AMUL

The data presented in Table 4 illustrates that there be positive and highly significant correlation ($r = 0.334^{**}$) among social participation of milk producers and overall extent of contribution of AMUL in terms of giving benefits to milk producers through different development programmes.

Thus, the null hypothesis that "there is no relationship between social participation of milk

producers and different development programmes" was rejected.

Thus, it can be said that social participation of milk producers had significant influence in increasing different development programmes.

Higher education and active participation in social organizations might have provided the milk producers more opportunity and exposure to share their ideas, information and knowledge regarding benefits obtained for milk producers from different development programmes of AMUL.

Land Holding and Different Development Programmes of AMUL

It is apparent from the data depicted in the Table 4 that there was positive and non-significant correlation ($r = 0.113$) among land holding of milk producers and overall extent of contribution of AMUL in terms of giving benefits to milk producers through different development programmes.

Hence, the null hypothesis that 'there is no relationship between land holding of milk producers and overall extent of contribution of AMUL in terms of giving benefits to milk producers through different development programmes' was accepted.

Thus, it can be inferred that farm size of milk producers had no significant influence on their different development programmes.

Herd Size and Different Development Programmes of AMUL

The data presented in Table 4 makes it clear that occupation of the milk producers had positive and highly significant correlation ($r = 0.367^{**}$) with overall extent of contribution of AMUL in terms of giving benefits to milk producers through different development programmes.

Hence, the null hypothesis that "there is no relationship between herd size of milk producers and different development programmes" was rejected.

Hence, it may be concluded that herd size of respondents have played a relevant role in shaping different development programmes.

Occupation and Different Development Programmes of AMUL

The data presented in Table 4 makes it clear that occupation of the milk producers had positive and highly significant correlation ($r = 0.185^{**}$) with overall extent of contribution of AMUL in terms of giving benefits to milk producers through different development programmes.

Hence, the null hypothesis that "there is no relationship between occupation of milk producers and different development programmes" was rejected.

Hence, it may be concluded that occupation of respondents had played a relevant role in shaping different development programmes.

Annual Income and Different Development Programmes of AMUL

The data presented in Table 4 reveals that correlation between annual income and their level of overall extent of contribution of AMUL of milk producers about AMUL was positive and highly significant ($r = 0.162^{**}$)

Hence, the null hypothesis that 'there is no relationship between annual income of milk producers and overall extent of contribution of AMUL in terms of giving benefits to milk producers through different development programmes' was rejected.

From above results, it can be inferred that higher annual income played significant role in increasing level of overall extent of contribution of AMUL of milk producers about AMUL.

Higher annual income might have motivated the milk producers to participate and involve in development programmes of AMUL which might have further resulted in getting benefits out of them.

CONSTRAINTS FACED BY MILK PRODUCERS

Table 5: Constraints faced by Milk Producers in terms of getting benefits from different development programmes of AMUL

No.	Constraints	Mean	Rank
1	Lack of awareness about development programmes of AMUL as well as benefits getting out of them	0.68	X
2	High registration fee and other fees for participating in development programmes of AMUL	0.80	IV
3	Lack of training facilities	0.69	IX
4	Unavailability of emergency veterinary services	0.84	III
5	Lack of improved equipments for milk collection	0.73	VII
6	Low procurement price	0.85	II
7	Delay in getting sale proceeds	0.89	I
8	Lack of timely collection of milk	0.78	V
9	To many formalities	0.77	VI
10	Lack of market for value added products	0.72	VIII

Source: Field Survey

It can be seen from the Table 5 that main constraints faced by milk producers in terms of accomplishment benefits from different development programmes of AMUL in descending order of rank were; delay in getting sale proceeds (I), low procurement price (II), unavailability of emergency veterinary services (III), high registration fee and other fees for participating in development programmes of AMUL (IV), lack of timely collection of milk (V), too many formalities (VI), lack of improved equipments for milk collection (VII), lack of market for value added products (VIII), lack of training facilities (IX), and lack of awareness about development programmes of AMUL as well as benefits getting out of them (X).

It can be seen from the Table 6 that major constraints faced by milk producers in terms of getting benefits from different development programmes of AMUL in descending order of rank

were; timely sale proceeds (I), procurement price should be increased (II), provision for emergency veterinary services (III), registration fee and other fees for participating in development programmes of AMUL should be reduced (IV), timely collection of milk (V), avoidance of too many formalities (VI), there should be improved equipments for milk collection (VII), provide good market facilities for value added Products (VIII), timely training to milk producers (IX) and create awareness about development programmes of AMUL as well as benefits getting out of them (X).

MAJOR FINDINGS

- A great majority (81.88%) of the milk producers belonged to middle to old age group.
- Majority (80.00 %) of the milk producers had joint family obligation.

SGGESTIONS BY MILK PRODUCERS

Table 6: Suggestions to overcome the constraints faced by Milk Producers in adoption

No.	Suggestions	Mean	Rank
1	Create awareness about development programmes of AMUL as well as benefits getting out of them.	0.74	X
2	Registration fee and other fees for participating in development programmes of AMUL should be reduced.	0.87	IV
3	Timely training to milk producers.	0.75	IX
4	Provision for emergency veterinary services.	0.89	III
5	There should be improved equipments for milk collection.	0.80	VII
6	Procurement price should be increased.	0.91	II
7	Timely sale proceeds.	0.92	I
8	Timely collection of milk.	0.85	V
9	Avoidance of too many formalities.	0.83	VI
10	Provide good market facilities for value added products.	0.79	VIII

Source: Field Survey

- A great majority (89.00 %) of the milk producers had high to very high level of experience in animal husbandry activities.
- A great majority (86.87 %) of the milk producers had good social participation either through two or more than two organizations.
- Slightly less than three-fourth (70.62 %) of the milk producers belonged to small to medium landholding.
- Majority (81.87 %) of the milk producers possessed one to two milch animals
- Slightly more than three-fourth (78.12 %) of the milk producers were depending on farming + animal husbandry and farming.
- Slightly less than three-fourth (71.25 %) of the milk producers have medium to high and very high

level of annual income.

- Slightly less than three-fourth (71.25 %) of the milk producers has high with very high level of attitude towards different development programmes of AMUL.
- Slightly more than four-fifth (88.13 %) of the milk producers have medium to high level of awareness regarding different development programmes of AMUL.
- Slightly less than three-fourth (71.87 %) of the milk producers have medium to high level of different development programmes.

CONCLUSIONS

In conclusion, animal husbandry is an important part of the Indian agro-based economy; It also provides employment opportunities to the poor and vulnerable sections of the rural society. India's dairy industry is developing on a

cooperative basis based on the Amul model. To further improve this, a joint partnership of Amul and milk producers will have to bring about a change in knowledge and technology which will have a direct impact on the animal husbandry practices adopted by milk producers. Sources of education and information among dairy farmers are also likely to be effective in improving dairy farming in rural areas.

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Heterobeltiosis for Seed Yield in Sesame (*Sesamum indicum* L.)

Dr. Sukla Das

Chief Volunteer (West Bengal), BLESS India
E-mail: drshukladas055@gmail.com

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ABSTRACT

Heterobeltiosis for 1000 seed weight and seed yield/plant were evaluated in 45 double cross combinations in sesame derived from a selective mating of 15 F_1 's of an 8 x 8 diallel cross (excluding reciprocals) for purpose of selection of high yielding hybrids which would give desirable recombinants in segregating generations. Heterobeltiosis was computed on the basis of superiority of hybrid for 1000 seed weight and seed yield/plant. Most of the double cross combinations had highly significant positive heterobeltiosis for 1000 seed weight and seed yield/plant. It was observed that 21 double cross combinations have shown highly significant positive heterobeltiosis for both characters, though estimated economic heterosis was negative for 1000 seed weight. High values of heterobeltiosis in (B 14 x T 12) x (R 9 x MT 67-52) and (B 14 x T 12) x (B 9 x R 9) indicate feasibility of exploitation of hybrid vigour for seed yield.

Key words: Heterobeltiosis, 1000 seed weight, seed yield/plant, double cross combinations, sesame.

INTRODUCTION

Sesame (*Sesamum indicum* L.) is one of the most important ancient oilseed crops (Bedigian and Harlan, 1986). Despite high nutritional value and oil quality, research on sesame has been scarce (Bedigian, 2003). Average productivity of this important oilseed crop in India is only 453 kg ha⁻¹, which is far below the average productivity in China (1127 kg ha⁻¹) and Egypt (1211 kg ha⁻¹). The exploitation of hybrid vigour appears to be one of the alternatives for making further breakthrough in seed yield. The degree of economic heterosis should be measured as superiority, if any, of F_1 hybrid over the best commercial variety released so far for cultivation. This estimate, in real sense, decides whether the hybrid is worth exploiting or not. Hybrid vigour which has been exploited commercially in cross-pollinated crops like maize, sunflower and pearl

millet, could also play an important role in the improvement of yield of several self-pollinated crops in which heterotic effects are pronounced. For the feasibility of developing commercially exploitable hybrids, more information on the desirable parental combination exhibiting high degree of heterotic response is inevitable. The present investigation was, therefore, conducted to know the extent of heterosis for 1000 seed weight and seed yield/plant in the hybrids which can be directly used for cultivation or further selection to get desirable recombinant/s in segregating generations so that high-yielding genotypes of good promise could be evolved.

MATERIALS AND METHODS

The varieties B 67, B 14, B 9, R 9, MT 67-52, T 12, RT 54 and TC 25 which were chosen on the basis of their diversity in seed yield were crossed in a 8 x 8 diallel design (excluding reciprocals) during

kharif season in first year at Bidhan Chandra Krishi Viswavidyalaya Farm, Kalyani. The 28 hybrids along with their 8 parents were grown in a randomized block design with 3 replications during pre-kharif seasons of second and third year. One row of 3 metres length was allotted for each hybrid and parent with a spacing of 50 cm x 8 cm between the rows and the plants in a row. Observations of ten competitive plants of each row from each replication were recorded for yield and yield contributing characters. A selective mating with 15 F₁'s : (1) – B 67 x B 14, (2) – B 67 x B 9, (3) – B 67 x R 9, (4) – B 67 x MT 67-52, (5) – B 67 x T 12, (6) – B 14 x B 9, (7) – B 14 x R 9, (8) – B 14 x MT 67-52, (9) – B 14 x T 12, (10) – B 9 x R 9, (11) – B 9 x MT 67-52, (12) – B 9 x T 12, (13) – R 9 x MT 67-52, (14) – R 9 x T 12 and (15) – MT 67-52 x T 12 was conducted during pre-kharif season of third year based on the combining ability of the first season's set of F₁s. The double hybrid generation comprising of 45 cross-combinations was raised during kharif season of the same year in a randomized block design with 3 replications. One row of 3 metres length was allotted for each double hybrid with 50 cm spacing between the rows and 8 cm between plants in a row. Observations of ten competitive plants of each row were recorded for 1000 seed weight and seed yield/plant. Heterobeltiosis for 1000 seed weight and seed yield/plant was calculated from the mean difference of F₁ and best parent. Percent heterobeltiosis was estimated as

$$\frac{\bar{F}_1 - \bar{BP}}{\bar{BP}} \times 100$$

where BP was the hybrid which was comparatively superior for 1000 seed weight and seed yield/plant.

RESULTS AND DISCUSSION

Earlier study heterobeltiosis for oil content of the 15 F₁ hybrids by Das and Samanta (1989) showed negative values concluding that heterosis breeding for increasing oil content is not desirable. Estimates of combining ability,

however, indicated the influence of both additive and non-additive gene actions for 1000 seed weight and seed yield/plant (Das and Sen, 1989). In this context, it would be worthwhile to mention that Shrivastava and Singh (1981) also found more negative than positive heterosis for seed yield with high estimates of specific combining ability than the corresponding estimates for general combining ability indicating the preponderance of non-additive gene expression. Godawat and Gupta (1985) also reported that dominance was important for seed yield. However, heterosis in seed yield observed by him was attributed to heterosis for plant height and number of capsules/plant. The strategy for improvement of these two characters for increasing seed yield will be further selections for desirable recombinants from the progenies raised by crossing the comparatively superior hybrids. With this in view, the 15 hybrids referred above were therefore used in this experiment to develop the double hybrid generation of 45 cross-combinations for the study of heterobeltiosis for 1000 seed weight and seed yield/plant.

Table-1 shows highly significant positive heterobeltiosis in 23 cross combinations and significant positive heterobeltiosis in 3 cross combinations for 1000 seed weight. On the contrary, 40 cross combinations show highly significant positive heterobeltiosis for seed yield/plant. It is evident from the table that most of the cross combinations showing highly significant positive heterobeltiosis for 1000 seed weight also exhibit highly significant positive heterobeltiosis for seed yield/plant. In this connection, it would be worthwhile to mention the work of Chavan *et al.* (1982) who obtained significant positive heterosis for capsules/plant, number of days to maturity and seed yield/plant. Likewise, analysis of data from a 4 x 4 complete diallel revealed that positive heterosis over the midparental value was most pronounced for branches/plant, capsules/plant, seeds/capsule and seed yield/plant.

CONCLUSION

In view of the present finding, it is worthwhile to mention that since appreciable economic heterosis is noted, at least for seed yield/plant in the double cross combination i.e., (B 14 x T 12) x (R 9 x MT 67-52) and (B 14 x T 12) x (B 9 x R 9), these could be the source of hybrid seed of economic value for seed yield. The high heterotic response for both characters in 22 double cross combinations may principally be due to accumulation of favourable additive genes and complementary and duplicate epistasis which may be fixable. Such cross combinations are also expected to throw more superior segregants owing to their balanced homeostatic system than their respective superior parents and may be improved further by mass selection followed by recurrent method of breeding.

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Table 1- Heterobeltiosis for 1000 seed weight and seed yield/plant in double hybrid cross combinations

Sl. No.	Double cross combinations	1000 Seed weight	Seed yield/plant
1.	(B 67 x B 14) x (B 9 x R 9)	-3.062	21.099**
2.	(B 67 x B 14) x (B 9 x MT 67-52)	7.612**	2.155**
3.	(B 67 x B 14) x (B 9 x T 12)	10.303**	17.856**
4.	(B 67 x B 14) x (R 9 x MT 67-52)	-0.151	19.199**
5.	(B 67 x B 14) x (R 9 x T 12)	1.570	21.538**
6.	(B 67 x B 14) x (MT 67-52 x T 12)	5.716**	-15.844
7.	(B 67 x B 9) x (B 14 x R 9)	11.425**	32.533**
8.	(B 67 x B 9) x (B 14 x MT 67-52)	11.445**	29.765**
9.	(B 67 x B 9) x (B 14 x T 12)	4.853**	34.655**
10.	(B 67 x B 9) x (R 9 x MT 67-52)	5.777**	35.345**
11.	(B 67 x B 9) x (R 9 x T 12)	4.460**	26.821**
12.	(B 67 x B 9) x (MT 67-52 x T 12)	5.367**	-25.842
13.	(B 67 x R 9) x (B 14 x B 9)	-0.522	30.171**
14.	(B 67 x R 9) x (B 14 x MT 67-52)	12.166**	34.889**
15.	(B 67 x R 9) x (B 14 x T 12)	1.972*	35.945**
16.	(B 67 x R 9) x (B 9 x MT 67-52)	7.520**	5.594**
17.	(B 67 x R 9) x (B 9 x T 12)	4.568**	19.403**
18.	(B 67 x R 9) x (MT 67-52 x T 12)	-0.508	6.364**
19.	(B 67 x MT 67-52) x (B 14 x B 9)	-1.658	6.364**
20.	(B 67 x MT 67-52) x (B 14 x R 9)	6.113**	23.148**
21.	(B 67 x MT 67-52) x (B 14 x T 12)	4.352**	48.001**
22.	(B 67 x MT 67-52) x (B 9 x R 9)	-13.717	46.448**
23.	(B 67 x MT 67-52) x (B 9 x T 12)	7.846**	42.472**
24.	(B 67 x MT 67-52) x (R 9 x T 12)	4.931**	33.675**
25.	(B 67 x T 12) x (B 14 x B 9)	6.572**	38.210**
26.	(B 67 x T 12) x (B 14 x R 9)	11.929**	41.047**
27.	(B 67 x T 12) x (B 14 x MT 67-52)	7.900**	40.429**
28.	(B 67 x T 12) x (B 9 x R 9)	-16.718	40.503**
29.	(B 67 x T 12) x (B 9 x MT 67-52)	-14.499	24.267**
30.	(B 67 x T 12) x (R 9 x MT 67-52)	2.874**	38.697**
31.	(B 14 x B 9) x (R 9 x MT 67-52)	-0.393	36.600**
32.	(B 14 x B 9) x (R 9 x T 12)	-1.505	33.382**
33.	(B 14 x B 9) x (MT 67-52 x T 12)	4.177**	-26.362
34.	(B 14 x R 9) x (B 9 x MT 67-52)	-8.303	21.356**
35.	(B 14 x R 9) x (B 9 x T 12)	-10.030	39.335**
36.	(B 14 x R 9) x (MT 67-52 x T 12)	7.367**	-27.228
37.	(B 14 x MT 67-52) x (B 9 x R 9)	-18.187	43.491**
38.	(B 14 x MT 67-52) x (B 9 x T 12)	-7.469	43.669**
39.	(B 14 x MT 67-52) x (R 9 x T 12)	7.789**	35.865**
40.	(B 14 x T 12) x (B 9 x R 9)	-12.982	61.424**
41.	(B 14 x T 12) x (B 9 x MT 67-52)	1.722*	26.166**
42.	(B 14 x T 12) x (R 9 x MT 67-52)	-2.571	62.183**
43.	(B 9 x R 9) x (MT 67-52 x T 12)	-1.408	-25.275
44.	(B 9 x MT 67-52) x (R 9 x T 12)	2.104*	18.233**
45.	(B 9 x T 12) x (R 9 x MT 67-52)	1.059	39.591

* Significant at 5 % level of probability

** Significant at 1 % level of probability

Electronic-National Agriculture Market (e-NAM): A Paradigm Shift in Agricultural Marketing

NITISH KUMAR SINGH and PROF. M. V. ALAGAWADI²

¹Research Scholar, Department of Business Studies, School of Business Studies (SBS),
Central University Karnataka, Kalaburagi (CUK), Kalaburagi, Karnataka.

²Professor and Research Guide, Department of Business Studies, SBSCentral University
Karnataka (CUK), Kalaburagi, Karnataka.

corresponding author E-mail: nitish.singh2012@gmail.com

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ABSTRACT

This study aims to get insight into agricultural marketing reforms that have evolved post-independence in India, especially through e-NAM (electronic-National Agriculture Market). The study is conceptual and primarily based on literature reviews on agricultural marketing developments and reforms in India. There is a wide variation in agricultural growth across different states in India as per the acceptance of the APMC Model Act, 2003 by the States. Few reforms brought in by the government with the e-NAM, Agricultural Produce and Livestock Marketing (Promotion and Facilitation) Act, 2017 and Model Contract Farming Act, 2018 to furnish the interest of buyer and producers. With the advancement of technology, e-platforms have transformed the scenario of agricultural marketing in India. This article helps to comprehend the scope of digital marketing in the agriculture sector to resolve the inherent problems present in agricultural marketing. The study suggests that the current scenario of farmers and traders in India through a working model of e-NAM and the benefits of the electronic platform have helped them more effectively in a digital way than conventional agricultural marketing.

Key words: Agricultural marketing, Agricultural Produce and Livestock Marketing (Promotion and Facilitation) Act 2017, Model Contract Farming Act 2018, Electronic platform, electronic-National Agricultural marketing (e-NAM).

INTRODUCTION

In this changing world, digitalisation has evolved as a prime reason for the paradigm shift in agri-business. Digital media has revolutionised the core part of marketing. Digital platforms have created an opportunity for all to develop a trusted and transparent medium. The new age mantra is "Think Locally, Act Globally" (Devine-Wright, 2013; cited in Ishak & Balikisu, 2015) and in developed countries like Japan, USA we can see that implementation of the same. In a developing country like India, the same mantra has been

applied in every corporate to focus on more exports. As India is an agrarian country and its economy is primarily dependent on the agriculture sector, this sector has occupied approximately sixty per cent of the workforce and continues to be the primary source of farmers' income (Chengappa, 2018). In the transition phase of commercialisation, the farmer has faced many problems and constraints in selling the produce.

Marketing of agricultural produce can be done in many ways, but one cost-effective and

transparent strategy could be Digital Marketing (Singh, 2017). The use of digital technologies to market the product is termed digital marketing (DM). After the independence, the situation of farmers in India has become vulnerable. The annual growth rate was 2.7 per cent, and later on, in the Nineties, it slackened to 1.8 per cent (Singh, 2004:37). The farmer is at the end of the ladder in availing of trade benefits, and nobody cares about his well-being. Many reforms have been done to change this situation, but the agriculture sector's invariable development has led to uneven crop production and productivity across the regions. The government's appropriate policy and programme execution have provided the base for the emerging platforms with new technology to achieve massive production and distribution.

Internet technology has played a vital role in transforming the agriculture sector from purchasing seeds to selling the harvested crops, providing market information and weather information, decreasing production costs, and getting the returns for their produce (Gupta and Sharma, 2018). Though technology is available, farmers face unprecedented challenges and inherent problems in agribusiness in rural India (Singh, 2004). The unregulated and unorganised agricultural mandis have forced farmers to sell their produce to intermediaries. In the Indian agricultural market, intermediaries had dominated because there was a lack of infrastructure, no proper transportation facilities, absence of warehousing facilities and unpredictable weather (Gupta and Sharma, 2018). In recent years, the Government of India (GOI) has taken many initiatives to uplift farmers and stop the exploitation of farmers by intermediaries.

The Agricultural Produce Marketing Committee (APMC) act has been adopted by 24 states and two union territories (as per the State-wise status of marketing reforms on the GOI website till 2016) to derive advantages of market reforms. After establishing the model APMC act, it enables the

farmers to get the market exposure, facility of the warehouse, direct purchase Centre and development of agricultural markets in the country (Gupta and Sharma, 2018). Presently, e-platform has changed the scenario of agricultural marketing in India. With the advent of technology and ease of adapting it, farmers and consumers have started adopting online platforms in agribusiness in India.

This research article explains the scope of e-platform in the agriculture sector to solve inherent problems in agricultural marketing in rural India. The paper is organised into four sections. First section deals with the introduction of agriculture marketing and its overview. The second section explains the post-independence market reforms through model acts and the current Indian Agriculture Market scenario under the material and methods. The following section discusses e-NAM for agricultural marketing, its process of working and the benefits. Finally, managerial implication and concluding remarks made in the last section.

MATERIAL AND METHODS

The study is primarily based on literature reviews on agricultural marketing developments and reforms that occurred in India. The authors have explained the significant role of agriculture marketing reforms in enhancing farmers' socio-economic conditions with the advent of the Agricultural Produce Marketing (Development and Regulation) Act, 2003, APMC Model Rules 2007, Agricultural Produce and Livestock Marketing (Promotion and Regulation) Facilitation) Act, 2017 and model Agricultural Produce and Livestock Contract Farming and Services (Promotion and Facilitation) Act, 2018. Various literature was gathered and reviewed from government published reports, agriculture journals, newspapers and websites of different institutions, and online published articles related to agricultural marketing.

This paper aims to get insight into agriculture marketing reforms that have evolved post-

independence in India and describe the present scenario of agricultural market transformation through an online trading portal, i.e., "e-NAM". To understand the present scenario of agricultural marketing, the authors have summarised the following government contribution towards agricultural marketing reforms.

History of Agricultural Marketing in India

In India, an agricultural marketing programme was present approximately a hundred years ago in the British era. The previous studies explain that the first farm produce in India was raw cotton and bought by Manchester (UK) textile mills at reasonable prices. In 1886, the first regulated market ("Karanja") was inaugurated under Hyderabad Residency Order, and afterwards, in 1887 first law enacted "Berar Cotton and Grain Market Act of 1887". It gave the authority to British Resident to supervise the regulated markets and select any place in their district as a market place for buy and sale of agricultural produce. Then in later years, this Act was the base for other models established in other provinces of India. In 1928, the Royal Commission on Agriculture had recommended some critical changes in the regulation of marketing and trade practices and the establishment of market yards in regulated markets. However, till independence, no significant progress has been seen in agriculture marketing. The significant reforms came into existence after the independence of India. More than half of the total population is dependent on agriculture in India, and most of them fall under the marginal and small farmers' category (Chand, 2004). These farmers have faced issues and challenges related to a storage problem, supply of inputs and services, and agricultural marketing (Gupta and Sharma, 2018).

Agriculture always has been acted as a vital sector of the Indian economy. After post-independence, agriculture growth and development were considered the well-being of farmers, employment opportunities, and resource bases for other allied industries. The

farmers' community had resources for production, but the issue was ambiguous marketing facilities and ill infrastructure. Therefore, the government established policies and regulations to provide a better agricultural marketing system and incentive prices to motivate farmers to cope with this issue.

Government Intervention in Agricultural Market Reforms

At the time of independence, Mandis were 268, and after the APMC act, it went around 6300 Mandis in India in 2007 (Chauhan, 2008); presently, the number has increased to more than 6700 (Report of NIAM, 2017). The government has intervened persistently in agriculture policy implication, market infrastructure and regulatory measures of agricultural marketing. From time to time, the government has changed several regulations and policies in the agriculture marketing system after encountering various defects like farmers had to face the high cost of marketing, getting undue low prices, transportation problem, insufficient marketing yard and others.

In the 1960s, most States enacted Agricultural Produce Markets Regulation (APMR) Acts and started to operationalise (Mehta, 2006; cited in Minten *et al.*, 2012). Agricultural Produce Market Committee (APMC) Act was established under the Agricultural Produce Marketing Act (APMA). The APMC Act was passed originally in 1954 to establish physical infrastructure in the market, form farmer-led management committees, create a systematic sale in the market, and regulate the charges borne by farmers. Organised agricultural marketing came into the picture through the *Agricultural Produce Market Committee* who framed new policies and enacted them with enforcement (Minten *et al.*, 2012).

The National Commission on Agriculture has defined agricultural marketing as a "process that starts with a decision to produce saleable farm products that also includes pre-and post-harvest operations, assembling, grading, storage,

transportation and distribution." In the 1970s, the advent of the green revolution has shown the friendly policy environment to help farmers grow. Several marketing committees are formed and started serving the interest of the farming community. By 1978, the majority of states had enacted APMA legislation (Mehta and Sharma, 2017). Several problems in this system have been uncovered throughout time. Like farmers are obligated to sell their produce at state-owned mandis under certain State Acts, and those who want to sell their commodities in the APMC mandis must pay a market fee. Farmers must also arrange for their produce to be carried from their farms to the nearest mandi, incurring transportation and fuel expenditures. Several intermediaries were involved in moving the produce from farm to shop and receives a portion of the overall price as a commission (Bisen and Kumar, 2018). It leads to a high price for the consumers subsequently. The Indian farmers were predominantly small and marginal with the trivial marketable surplus and limited their bargaining power in front of intermediaries (Mehta and Sharma, 2018). Both farmers and consumers were unable to do anything as all the control was in the hand of middlemen. The government was also under a financial crisis and colossal debt.

In the 1990s, the era of liberalisation, privatisation and globalisation has emerged to rescue the country from a grave financial crisis. The policyholders, bureaucrats and marketing institution started losing their control as they started focusing on the self-sufficiency of the food problem. However, these things had worsened farmers' situation as they started seeking help from intermediaries for credit requirement. The control of trading went directly to intermediaries, and they moulded the marketing policies as per their benefits. As per the World Bank Report, the estimated number of Indian agricultural workers 25.7 per cent would fall by 2050 from 58.2 per cent total workforce in 2001 (Economic Times, 2018). Due to this, many problems have emerged

and a dire need for reforms in agriculture marketing to save farmers.

In 2003, the union government made the first attempt at agricultural market reform to create a Model Agricultural Produce Marketing Committee (APMC) Act. The government has modified the preceding APMC Act and has released the Model APMC Act to be enacted by the states. In compliance with the Model Act 2003, the state government has updated the APMC Act according to the regional and local requirements of the states. The most significant revisions to the Model Act were that direct purchases from farmers were permitted, contract farming was encouraged and allowed, and market yards' monopoly has broken (MoAFW, 2000). Although the purpose of these Acts was essentially the same, namely to regulate the trading practices, to enhance market efficiency with the reduction of market charges, protect producer-seller interests and most importantly, eliminate unnecessary intermediaries, many of these Acts differ even in meaningful content (MoAFW, 2003). After years of struggle, agricultural marketing progress has been delayed and diverse; the Model APMC Act, 2003 accepted at the state level with partial and few rejections. Only sixteen states have modified their statutes thus far, and some States do not have the APMC (MoAFW, 2003). In 2007, the Ministry of Agriculture drafted Model APMC Rules and distributed them to States and U.Ts to make it easier to change the existing rules. Only six states have announced to amend the rules.

Need of Next Level of Reforms

The limitations of the existing agricultural marketing system created the need for the next level of reforms in this sector. The APMCs are unable to provide minimum support prices assured by the state. As per Gulati, 2009 in the last five-seven years, there is a wide variation in agricultural growth across different states in India. The marketing laws are monitored and regulated by the APMC of the state Government. At one end of the spectrum, there are states like

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(July 2021)

"Gujarat that are showing strong growth of 8-10 per cent per annum in agriculture, while at the other end are states like Uttar Pradesh, West Bengal, etc., that are growing barely at 1-2.5 per cent per annum". As per the Ministry of Agriculture and Farmers Welfare (MoAFW), influential areas of reforms to pursue in the marketing of agricultural produce were:

- (i) Direct purchase of produce by exporters or retailers or buyers from farmers.
- (ii) A non-market committee member could control the private market yard.
- (iii) Single license for trade throughout the state.
- (iv) Eligibility for Contract farming to promote employment among small and marginal farmers.
- (v) Facility for e-Trading or e-auction with a single fee.

These all areas in the marketing of agriculture have forced the government to come up with a new platform of marketing for farmers and traders. The Economic Survey 2014-15 recommended constructing a national agricultural marketing electronic platform for farmers to market their produce across the country and receive remunerative prices (PIB, 2016). In April 2016, the central government inaugurated the e-National Agricultural Market (e-NAM) in eight states and districts, bringing wholesale mandis in these areas together to establish an online agricultural trading platform. The new, improved physical markets attracted and benefited the farmers and traders by providing remunerative prices and eliminating middlemen (Reddy, 2018). Technology has played a vital role to increase food production in India, regardless of the limited source of output.

Apart from technology-driven marketing, the government also tried to cater the contract farming or service agencies. The APMC markets are not functioning effectively because most of the times small and marginal farmers are not

preferred by the traders or contract buying agencies (Singh, 2013). The contract farming (CF) agencies were allowed, and the government also encouraged the CF practices as prescribed in Model APMC Act, 2003. The contracted produce created confusion and loss to traders and commission agents as produce was not coming to the APMC mandi and directly taken by the buyers from the farm field. So ultimately, it would not pass through the mandi agents, which will suffer their businesses in terms of commission. Therefore, traders and commissions agents agitated and appealed to remove it from the APMC domain. It was also observed that CF was paying market fees and other levies charges to the APMC without taking any services rendered by them. Therefore, the Committee of State Ministers on Agricultural Reforms recommended putting CF out of the ambit of APMC (MoAFW, 2003). Henceforth, for the betterment of the farmers, traders, commission agents, and CF, the Union Ministry of Agriculture and Farmers Welfare (MoAFW) has brought out another new model act.

The Agricultural Produce and Livestock Marketing (Promotion and Facilitation) Act, 2017 (APLMA, 2017), is apparently more in sync with the present times. The APLM Act, 2017 had communicated a significant policy change and suggested that there is no need for regulations in markets anymore and farmers are free to sell their produce. The APLMA 2017 report describes that Model Act, 2003 provisions were retained in APLM Act, 2017 except some, namely-CF will not come under the purview of APMC; delineated market area for marketing of agricultural produce, introduced e-trading and Farmer Producer Company (FPC); created farmer consumer yards, market sub-yards and special commodity markets for private parties and APMC, and lastly clubbed farm produce and livestock into one legislation. This model act was introduced to improve marketing efficiency and advance realisation for all stakeholders. The amendments to the Agricultural Produce Market Committee

Acts and the Essential Commodities Act (GOI, 1955) remove interstate transportation restrictions on agricultural produce and promote and attract private sector participation in agriculture commerce (MoAFW, 2017). This model Act also emphasised private-public partnerships at the APMC level for the first time in history.

Parallel to this, the government cannot find the right solution for resolving the disputes arising out from the contracts, production uncertainties, and market unpredictability. To overcome this, the government has been making comprehensive interventions to de-risk the agricultural produce at various stages and efficient monetisation of the produce with the help of promotion of Contract Farming and Services Contract. Taking advice from the past, Punjab was the only state that has formulated a Contract Farming Act, 2013 but never operationalised it (Singh, 2013a). In Union Budget 2017-18, Model Contract Farming Act was announced as an exclusive model law. The guiding factor in formulating this law was to protect and promote the interests of small and marginal farmers in particular. To safeguard the buyer and producer with legal support Model CF Act reduces market volatility and a farmer's market risk. After few drafts and constructive suggestions and consultation from the stakeholders, the Ministry of Agriculture and Farmers Welfare (MoAFW) introduced a model Agricultural Produce and Livestock Contract Farming and Services (Promotion and Facilitation) Act, 2018 (hereafter APLCFSA, 2018). The salient features of Model Contract Farming Act, 2018 were explained below:

- a) This model Act comprehensively includes all categories of horticultural crops, livestock, dairy, poultry and fishery.
- b) It guides the contracting parties on the pre-agreed price mentioned in the contract. This will benefit both in case of violent movement upside or downside vis-à-vis pre-agreed price as a win-win framework.

- c) Limits of stockholding of agricultural produce exempted upto 6 months of their trade requirement for the buyer.
- d) Apart from the contract relating to market services, the producer can get support from the buyer for improving more services relating to pre-production, production and post-production.
- e) Right or title ownership of the producer's land cannot be transferred in any case.
- f) The dispute redressal mechanism should implement at block, district or regional-level state authorities and not with an APMC.
- g) Every agreement should be registered with a Registering and Agreement Recording Committee.
- h) The contract buyers need not pay the market fee and commission charges, i.e., 5 to 10 percent as they remain outside the purview of APMC.

Contract farming aims to provide farmers with greater price realisation, but few areas weren't covered effectively in this Act and the previous model acts. With the few limitations of the present agricultural marketing system, the Indian government has strapped to focus on an electronic marketing platform to eliminate the present constraints of the agricultural marketing system.

The primary constraints are explained briefly below:

Markets were fragmented within a state: The markets (Mandis) are established by the APMC Act in different regions to cover the whole state, but multiple trading licenses are compulsory, and market fees have to pay at different locations. Apart from this, handling charges and market accessibility obstruct selling off produce.

- (i) Infrastructure amenities are not available: The farmers need a cold storage facility, quality checking labs, auction platforms and weighing bridges. These facilities are not available in all the Mandis. Previous studies show that farmers do not have these

infrastructure amenities to market their produce.

- (i) Market fees and commission agent tax: Whenever the farmer sells his produce, he has to pay market entry fees, weighing charges and market fees. Market committee and commission agents have authorised for taking taxes from the produce sold. These taxes and fees make the profit significantly less, and the farmer does not get any profit margin after his all efforts.
- (ii) Asymmetric price of commodities: The trader generally offers less than the price quoted by the government news media. So, farmers did not have any other option other than selling it.
- (iii) High Intermediation cost: Ministry of Agriculture (MoA) indicates that in 2004 the share of producers differs for all commodities from "56 to 89 per cent for paddy", "77 to 88 per cent for wheat", "72 to 86 per cent for coarse grains" and "79 to 86 per cent for pulses", "40 to 85 per cent in oilseeds" and "32 to 68 per cent in case of fruits, vegetables and flowers". It has created a need for reform in agricultural marketing channels.
- (iv) After post-harvest: ICAR (2015) reported no other options available after post-harvest losses of food grain commodities ranging from 4 to 12 per cent and total losses estimated around "Rs 92,651 crores at an average price value of 2014".
- (v) The non-existence of Credit facility: There is a huge need for a credit facility for an Indian farmer just after harvesting crops so that they can wait for the right time for the right price of that produce.

In e-NAM, there are many facilities and benefits provided to farmers, which fill the void of the present agricultural marketing system. The experts also suggested focusing more on e-trading and creating awareness regarding the e-National Agricultural Market (e-NAM) to join and sell their produce across the states. Hence, e-

NAM will be going to act as a paradigm shift in Indian agricultural marketing.

ELECTRONIC NATIONAL AGRICULTURAL MARKET(e-NAM)

During the last fifty years, a detailed assessment of India's agricultural marketing system revealed several issues linked to controlled marketplaces (Acharya, 2004). The present scenario of agricultural marketing in India will improve with the help of technology and the internet. The above-mentioned works of literature have been meticulously compiled to present an image of agricultural market reforms and development that are being implemented to strengthen the spirit of farmers and various strategies to eliminate deficiencies in the agricultural marketing system with the introduction of e-platforms, particularly in the context of e-NAM. This amplifying process needs to be embraced with robust infrastructure, warehousing and logistics facility, and an efficient marketing network (Saxena, *et al.*, 2017).

The Union Government took the initiative to encourage farmers in many states and UTs of India to market their produce through an electronic platform for agriculture marketing, i.e., e-NAM that is the replica of Karnataka Model; ReMS (Rashtriye e-Marketing Services). The motive of e-NAM is to recreate a similar model for trade in agricultural marketing to support farmers and traders. ReMS has created a benchmark for the whole nation and every state has to implement this type of initiative for the welfare of the farmers and traders. However, as per the findings (Reddy, 2018), the initial phase of e-NAM hasn't picked up in terms of market arrivals and bidding activity.

Under the Digital India flagship programme, Electronic National Agricultural Market (e-NAM) system was introduced in 2016 for farmers and traders to market their crops online across India. The e-NAM system and platform are operationalised and maintained by Small Farmers' Agribusiness Consortium (SFAC) with Strategic Partner viz. M/s Nagarjuna Fertilizer

and Chemicals Limited. SFAC is a registered society of the Department of Agriculture, Cooperation & Farmers' Welfare (DAC&FW) under the Ministry of Agriculture and Farmers' Welfare (MoA & FW) (source: <http://www.enam.gov.in>). The e-NAM system was launched on 14th April 2016 with initial acceptance of 21 Mandis across eight states in 24 commodities on a pilot basis (Press Information Bureau, 2016). In 2018, 585 Mandis integrated with e-NAM in 16 states and 2 UTs and 2021, 1000 mandis have joined across the country from 18 states and 3 Uts (<http://www.enam.gov.in>)

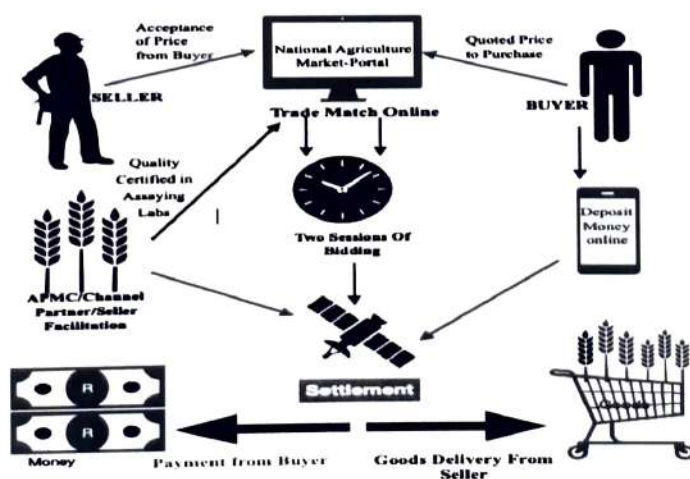
The MoAFW proposed broad objectives for the e-NAMs as follows:

- (i) "Transparent sale transactions and price discovery",
- (ii) "Liberal licensing of traders/buyers and commission agents by state authorities",
- (iii) "Harmonisation of quality standards of agricultural produce and provision for assaying",
- (iv) "Single point levy of market fees",
- (v) "Provision of scientific techniques such as soil testing laboratories."

The e-NAM platform that can be accessed online by different stakeholders would help network all physical APMC Mandis. This platform has increased choices to sell the agricultural produce by farmers, lower intermediation costs,

reduce market fragmentation and lower price for the final consumer. There are three main stakeholders in the e-NAM system-Farmers, Traders, and Buyers/exporters. The working model of Mandis, training manuals, guidelines for using e-NAM application and registration and operational guidelines for different stakeholders are given online. To understand the e-NAM process or working model, the author has administered at one of the APMC mandis where an e-NAM model is functioning. There are many APMCs where e-NAM is not implemented, and it would be implemented phase-wise. The GOI provides funds to each APMCs to install software, machinery, assaying labs, skilled persons for a year, and building infrastructure.

Process of e-NAM: To understand the process of e-NAM, authors have visited one of the e-NAM enabled mandi, which is very well established and equipped with all the equipment and machinery. The function of the e-NAM will be the same throughout the country, but it can be different only as per the APMC size and cropping pattern. The e-NAM Centre opens in the early morning with two online sessions. First, it starts from 8 am to 12 pm and later from 1 pm to 5 pm on working days. The farmers who entered the gate with their produce would carry the Lot receipt (Entry receipt). The receipt includes the details of the farmer, contact number, Commission Agents



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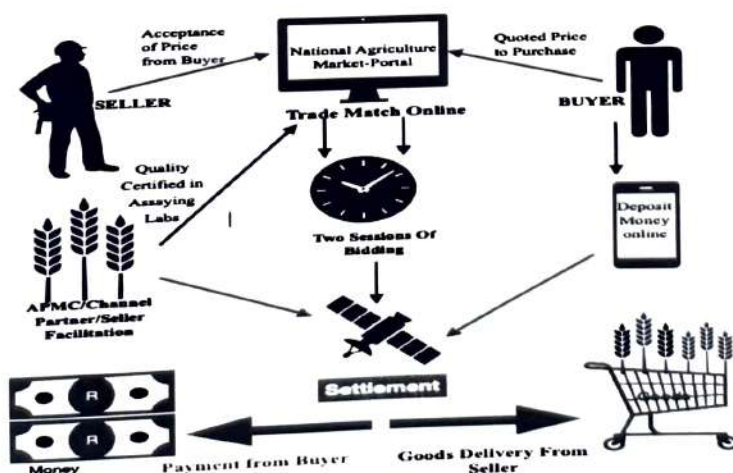


Figure 1: A working model of e-NAM

Source: Author compilation (<http://sfacindia.com>)

name, commodity name, trader company name, no. of bags of commodity, and approximate quantity of a commodity. After this, one person from the e-NAM assaying team will collect the sample of a commodity for quality check and grading and then it will have assigned with assaying parameter. Then, this commodity will be e-auction online through the e-NAM portal and buyers can bid the commodity and quantity available on the e-platform. The result of the e-auction will be declared after the session end, and winners get the text message and mail for confirmation. After the confirmation, a farmer will get the money within 48hrs of time of the transaction and sometimes cheques. The farmer will get the money into his/her account and with prescribed details like the buyer's name, the total amount paid by the other party, and the quantity bought. The farmer can demand more price of their produce if he is not satisfied with the e-auctioned value. Thus, the transaction of money and commodity will be transparent and smooth.

The vision of implementing the e-NAM system is to provide higher returns to traders and farmers, reduce transaction cost to buyers/exporters, encourage value chain model and logistics of commodities, uniform market prices and motivate for warehousing. The e-NAM portal provides a one-stop solution for all the APMC related information and services, including details of all tradable commodities, buy & sell trade offers, update current commodity prices, and response to trade offers and e-tenders.

Benefits of Implementing E-NAM

After implementing e-NAM in the first phase, the response was outstanding, and the number of Mandis joined were more than expected. Presently a total of 1000 Mandis are enrolled into e-NAM, but 585 were till March 2019 and in the next phase, another 415 Mandis joined the platform in May 2020. There are many advantages of using the e-NAM platform that can

be encashed by farmers and traders in the marketing of the crop. The benefits of the implementation of e-NAM in agricultural marketing for stakeholders are listed below as per the e-NAM portal:

- i. *Transparent and Cashless Transaction:* The farmers and traders can sell their commodities online and get money through online banking. Online it can be seen the amount credited and who is the buyer.
- ii. *Real-Time Price discovery of commodity:* This facilitates the farmers and traders to know the commodity prices. When selling the commodity, they can check the current price of that commodity and sell at par prices.
- iii. *No middleman Interference:* The main aim of the e-NAM scheme is to transfer the direct benefit to the farmers and traders for the crop sold. The government was successful in doing the same and thereby eliminated the middlemen interference.
- iv. *Guarantee of payment:* Earlier, it was impossible to get the payment at the same time of selling and farmers have to wait for weeks and months for their own money. In this scheme, farmers and traders are confident of getting money in stipulated time.
- v. *Certified Quality check, logistic and warehousing Facility:* The certification of the commodity was not possible for all traders and farmers; only those who have a good relationship with the manager can only get the certificates. The logistics and warehousing were also the same, and small farmers and traders have to wait for their turn or else they have to sell without this facility.
- i. *Ease of Access to the market:* Reaching to the market has become more accessible, and most of them are selling their produce to Mandis directly. This facility has helped them to sell their produce on the same day.

- ii. Strong and effective supply chain: The supply chain has become more robust and efficient for all commodities. The process of arrival of commodities, procurement of products and logistics are maintained very well.
- iii. Elimination of Transaction cost: Earlier, farmers have to pay transaction cost and market fees for entering each time to the individual mandi, but e-NAM has eliminated that market fee and transaction cost for each time.

MANAGERIAL IMPLICATIONS

Agricultural market changes in India are a pressing necessity of the hour, and e-NAM can shift global and local markets. e-NAM will be impactful if every state implements it in their APMCs. Not only farmers, traders and consumers will get the benefit, but the APMCs will also receive the benefits like reduction in human resources requirement, an automated record of every trade and financial transaction, complete information about the trade for the past and present-day, month and year (Sekhar, et al., 2018). The study findings reveal that e-NAM will also help policymakers and business owners analyse the price trends, arrival quantity, and other trading activities in a nutshell (Singh and Alagawadi, 2021; Reddy, 2018). As per NSSO Report 2012, the number of farmers declined at an annual rate of 1.8% to 14.6 crores in 2011-12 from 16.6 crores in 2004-05, and if the decline rate persists (assuming), then in 2021-22, the number of farmers would be below 12 crores (Saxena et al., 2017). Therefore, it can be understood as agricultural marketing in the country has achieved limited success in marketing practices. It needs to strengthen the extension services and efficient marketing system, which can only be achieved through electronic-National Agricultural Marketing.

CONCLUSION

More than half of the population is dependent on agriculture, and most farmers, i.e., approximately

75%, are small and marginal farmers. The researcher focuses on shifting conventional agricultural marketing to e-platform marketing in India. Though government intervened in agriculture marketing reforms in enhancing farmers' socio-economic conditions with the advent of the Agricultural Produce Marketing (Development and Regulation) Act, 2003, APMC Model Rules 2007, Agricultural Produce and Livestock Marketing (Promotion and Regulation) Facilitation Act, 2017 and model Agricultural Produce and Livestock Contract Farming and Services (Promotion and Facilitation) Act, 2018, but few things were not covered by all, leading to the importance of e-trading. To gain competitive advantage, farmers and traders' prerequisite is to use internet technologies. The need of the hour is to bring better price realisation and motivate the farmers for higher growth of output to raise production and production. The systematic and organised reforms of the agricultural market have established an electronic-National Agricultural Market to support the farmers in a more effective way to meet the market demand. The transparent payment, elimination of middlemen, one license, guarantee of payment in the stipulated time, provision for e-trading, facility of assaying labs, reductions of transaction cost, freedom of selling to anywhere in India, etc., have made the paradigm shift in agricultural marketing. After implementing e-NAM, disruption of the market resulted in a safeguard of farmers and trader's economic growth. The government support towards the well-being of farmers needs to be applauded and precursor to future reforms in the agricultural marketing system. This study is limited to theoretical aspects of e-NAM function and its benefits. For further study, one may assess the effective operations and benefits gained by stake holders.

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Effect of Shifting Cultivation on Soil health and Microbial diversity in the Mulshi Taluka in Pune District

Vasudevan Patturajan¹, Dr. Ravindra G. Jaybhaye², Dr. Yogesh Shouche³

1- Dr. D. Y. Patil Arts, Commerce, Science College, Pimpri, Pune-411018, 2- Department of Geography, Savitribai Phule Pune University, Pune-411007, 3- National Centre for Cell Science, Pune University campus, Pune-411007
Corresponding author email: vasudevanpatturajan63@gmail.com

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ABSTRACT

Shifting cultivation is an ancient agricultural form still practiced in different parts of the world. The present study aimed to find out the impact of shifting cultivation on soil bacterial communities and soil quality in the Mulshi taluka of Pune district. 16 soil samples, from 4 villages of the taluka were collected. The physicochemical analysis of these soils was carried out. Bacteria were isolated, characterised and partially identified from these soil samples. The soil samples included the crop (millet) harvest soil and fallow land soil.

The bacterial isolates showed a strong positive relation with organic carbon, nitrogen and clay and a negative relation with specific density of the soil. Total 939 bacterial isolates that belonged to 12 different bacterial genera were isolated. All properties of the soil were found to be significantly influenced by the fallow age.

Key words: Shifting cultivation, physico-chemical, microbial, millets, fallow land

INTRODUCTION

Mulshi is situated in the southwest of the Pune district, having a latitude of 18.30 N and a longitude of 73.30 E. The rainfall of more than 2000 millimeters is received in Mulshi. The west part of taluka (about 15.09 %) is covered by forest. Population density is low in this region and has 60 to 70 percent cultivators.

Millets is the common term used for many small-seeded annual grass grain crops. They are produced in fields with less fertile soil and low rainfall conditions. Nutritionally, Millets are superior to rice and wheat, hence considered as a cheaper source of proteins, minerals, and vitamins for all the poor. Also, since they are practically deprived of grain storage pests, they have indefinite storage life (Sampath *et al.*, 1986). In India, 4.5 percent of the cultivated area is occupied by Millets. They are restricted to large

areas of drylands and hilly tracks. Millet can adapt to a wide variety of soil types from very less fertile to highly fertile soil. They can tolerate some level of alkalinity (Joshi & Agnihotri, 1984).

The appearance, fertility, and chemical characteristics of the soil vary with respect to the minerals and plant materials that form them and keep transforming. Most of the reactions involve water, mineral, and biological factors. The soil quality is influenced by its physicochemical properties (Brady & Weil, 2008). Several properties of the soil work hand in hand to maintain equilibrium in the soil environment. This balance is very much necessary for the sustainability of all forms of life.

Micro organisms are highly responsible for the increase in biomass production and maintaining the ecosystem through their decomposition activity thereby affecting soil fertility. Their

performances lead to various vital ecosystem processes like nutrient cycling and availability, productivity, plant growth, suppression of root diseases, and soil structure (Jesus *et al.*, 2010). This naturally occurring microbial community inhabits the plant rhizosphere and effectively promotes plant growth through various interactions (Saharan & Nehra, 2011). This microbial population is influenced by varied factors that lead to the shift in their communities. One of them is shifting cultivation method, which leads to a decrease in the microbial population and diversity.

Shifting cultivation was widely practiced in the Western Ghats, extending further north along its forested heights till the southwest extension of Aravalis in the Dungarpur district. In this system, the natural vegetation is cut down and burned as a method of clearing the land for cultivation, and then, when the plot becomes infertile, the farmer moves to a new fresh part and does the same again. The cultivators shift from one place to other for the cultivation of the land and they came back on the same land when land regained fertility after about 20 to 30 years.

The clearance of jungles is the prerequisite of shifting cultivation. The felling of trees and clearing of bushes, however, accelerates soil erosion and accentuates variability of rainfall which may lead either to draughts or floods. The overall impact is the decline in soil fertility. The ecosystems lose their salient characteristics. Soil degradation is caused by various reasons like salt accumulation, soil erosion, depletion of organic matter and nutrients. As the cycle of shifting cultivation becomes shorter which is 4-5 years, the biomass that depends on the humus of the soil, declines, and the biodiversity is considerably reduced (Rasul & Thapa, 2003). An increase in human population density and livelihood security demand has forced the people in this region to practice the most nonviable type of cultivation in which the fallow period is shortened along with the conversion of forest to permanent agricultural expansions (Yadav, 2013).

The following of land following shifting cultivation helps in soil moisture conservation, enrichment of soil texture and structure, the addition of potassium to soil during burning, increase in soil pH, increase in soil microbial biomass, restriction of pests and pathogens, development of good canopy, no capital investment except labor and seeds, and above all, the outcome is organic produce free from fertilizers or pesticides (Kesavan & Swaminathan, 2006).

Unfortunately, these parameters and their effects on shifting cultivation with respect to the accumulation of forest biomass, nutrient cycling, and the role of microbes that varies with fallow length are still poorly understood. Therefore the need of the hour is to search for sustainable ways of cultivation that can transform shifting cultivation system and have fewer environmental consequences. Understanding the plant-soil-microbe interactions thus can help to find a solution for transforming the shifting cultivation system. The main objective of this research was to study the effect of shifting cultivation systems on microbial diversity and thus in return on soil fertility.

MATERIALS AND METHODS

Soil sample collection

16 soil samples from 4 villages of the Mulshitaluka were collected between February 2014 and April 2014 after harvesting the cultivated crop and also from fallow land. The sample was collected by laying 5 quadrants of 1m by 1m, within the 2 to 4 acres of the shifting cultivation plots identified in the villages. 200g soil per quadrant was collected from 1cm to 9cm depth and was pooled together in 5kg sterile polythene bags and transported at room temperature to the laboratory within 24 hours. On arrival at the laboratory, the soil samples were stored at 4°C, the soil samples were processed within 3 to 4 days. To determine all the parameters for each soil sample, a portion of the soil was air-dried and sieved through a 2 mm sieve and then was diluted in water.

Analysis of physicochemical properties of soil
The pH was measured with the help of a pH meter. An Electrical Conductivity meter was used to measure Electrical Conductivity (EC) (Jackson, 1973). The oxidation capacity of organic carbon in presence of potassium dichromate and concentrated sulphuric acid and then subsequent spectrophotometric estimation of the oxidized product yielded the amount of organic carbon. This was estimated by taking the optical density at 660nm then quantified against a standard graph of sucrose and determined in terms of percentage (%) in soil (Jackson, 1973).

The available phosphorus from soil was reacted with sodium bicarbonate (pH 8.5) and conc. Sulphuric acid, and the subsequent optical density was estimated at 880nm (Jackson, 1973). The available potash was extracted from soil in soluble form in ammonium acetate solution and estimated by flame photometer method (Jackson, 1973). The soil CaCO_3 was mixed with 0.5N HCl, whereby CO_2 was released, and the excess HCl was neutralized by 0.5N NaOH titration and the amount of CaCO_3 was estimated from this (Rowell, 1994).

Total cation (Ca + Mg) was estimated according to Tucker & Kurtz, (1961), using Na EDTA and complexometric titration. Soil Calcium was quantified based on the stable complex it forms with EDTA in alkaline condition (pH 12), and a purple-blue colour was formed in presence of calcon indicator. This indicator was pink in colour when EDTA was free.

The soil available sodium was estimated by first extracting it with the solution of sodium or ammonium acetate and then estimating the soil sodium by flame photometer (Havre, 1961).

The soil moisture was estimated by drying method (Reynolds, 1970). Briefly, 20g soil was weighed in an aluminium crucible and kept it in a dry oven at 105°C for 6h to 8h. After which the crucible and soil sample was weighed again and the difference in the weight gives the soil moisture content in percentage.

The soil texture was determined by the International Pipette method as described by Gee & Bauder, 1986. Briefly, 50 g of air dry soil that was passed through 2 mm sieve, was taken in 500 ml bottle. 100 ml dispersion solution (33 g of Sodium Hexametaphosphate and 7 g of Sodium carbonate per litre of distilled water) was added to it and the bottle was shaken at regular intervals for half an hour in order to obtain a homogeneous solution. This was then transferred to 1000 ml glass measuring cylinder and the volume was brought to 1000 ml by adding water. This was again shaken for 30sec. Then according to the International approved system, first pipetting was done with 50 ml pipette at 10 cm depth and 50 ml sucked solution was transferred in 60 ml china dish. Mixtures of clay and silt particles were present in it. Second pipetting was done with 50 ml pipette at 10 cm depth. Sucked 50 ml, which contained clay particles, was then transferred in 60 ml china dish. Next, the remaining soil solution was passed through 0.02 mm sieve and the material was washed through the sieve using jet of water. Sand particles that remained on the sieve were collected in china dish. Material collected in all the three china dishes was dried overnight in oven at 105°C. Then it was cooled in desiccators and weighed immediately. The weight of fine sand was determined by deducting the weight of clay, silt and coarse sand particle from 100.

Water Holding Capacity measured by Keen-Raczkowski-Box Method (Keen & Raczkowski, 1921). Briefly, air-dried soil was crushed and passed through a 2mm sieve. The internal perforated floor of the Keen box was covered by Whatman filter paper No.1. The weight of the box with filter paper was noted as (a). The sieved soil was added to the box in small amount with gentle tapping after every addition so as to fill the box uniformly. The top of the soil was made plane and the Keen box containing soil was placed in the water tray for saturation overnight. Next day, the box was removed and wiped dry from outside. The Keen box with wet soil was weighed again

and the weight was noted as (b). The box was then placed in oven at 105°C to dry the soil to a constant weight. After cooling the box in a dessicator, its weight was noted as (c). The water holding capacity of the soil was then calculated using the following formula.

$$\text{Saturation Moisture \%} = \frac{(c-a) - (b-a)}{(b-a)} \times 100$$

Isolation and identification of bacteria

The microbial community analysis was done by total viable count of soil bacteria using serial dilution of the soil. 1g of soil was mixed in 100ml saline and serially diluted up to 10^{-10} and then 0.1ml of each dilution was spread on sterile nutrient agar plate in triplicates. These plates were incubated at room temperature for 24 to 72 hours. The subsequent bacterial colonies developing on the plates were counted and the colony forming units (cfu) per ml of soil sample was estimated. The isolated bacterial colonies growing on these plates were visually identified and picked up daily, the colony morphology recorded and the bacterial colonies were subject to Gram staining, Spore staining, capsule staining and biochemical identification was done up to genus level as per Bergey's manual of Determinative Bacteriology, 9th edition.

Statistical analysis

Statistical analyses were performed using SPSS software version 21. The correlation between all the soil parameters tested in this study was generated. Using R software version 3.6.1, Heat maps were developed for this correlation analysis data generated from SPSS.

Based on the data obtained richness, Simpson's diversity and evenness indexes were calculated. The results were submitted to principal component analysis (PCA) to determine the common relationships between parameters.

RESULTS AND DISCUSSION

Physicochemical analysis

16 soil samples of the shifting cultivation plots from 4 villages were collected and the

physicochemical analysis was performed for each sample. The average and standard deviation of each parameter were statistically calculated and were considered for further analysis. The results obtained are compiled and the average data of each parameter including the fallow period is presented below in the table. These results were then utilized to interpret the effect of shifting cultivation on the fertility of the soil. Elevation of the village location was also considered as one of the parameters that may affect soil fertility with respect to shifting cultivation. Also, the microorganisms were isolated from each soil sample to study the same effect and in turn the soil fertility.

The soil analysis of Mulshitaluka shows that the pH of this soil is strongly positively related to available phosphates, calcium, magnesium, calcium carbonate, and moisture whereas neutral to the carbon and nitrogen content. Electric conductivity was shown to be neutral to sand and moisture. Carbon was strongly positively related to the nitrogen content similarly available phosphorus was related to Ca, Mg, moisture, apparent density, and strongly negatively related to potash, but was neutral to the water holding capacity, porosity, and clay parameters. In the same way, calcium was strongly positively related to Mg, moisture, apparent density but neutral to water holding capacity. Magnesium showed a strong positive relation with moisture, apparent density whereas CaCO_3 was neutral to a specific density. It was also seen that sand was strongly negatively related to silt and water holding capacity but clay was strongly positively related to water holding capacity (WH) and silt was neutral to both densities. Lastly, it was observed that water holding capacity was strongly negatively related to apparent density.

When TVC was considered there was a strong positive correlation with clay and negative relation with specific density but was neutral to pH of the soil. These results are observed in Figure 1.

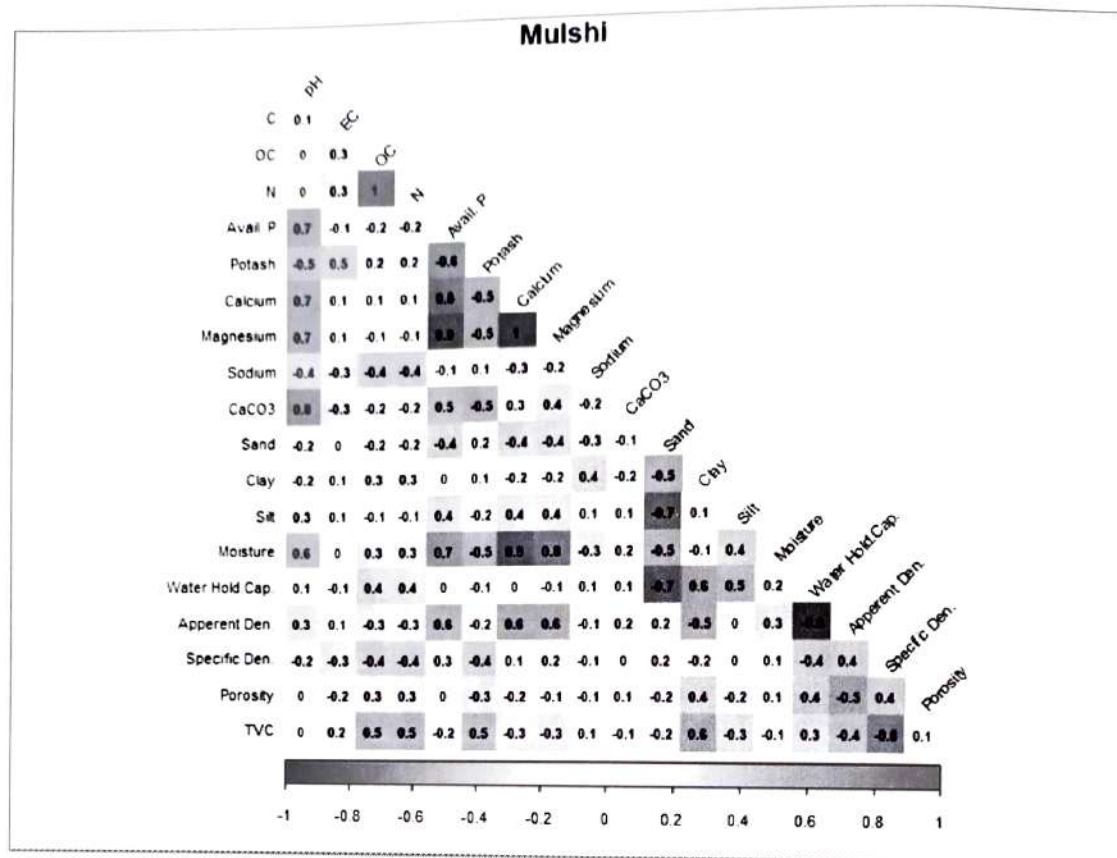


Figure 1: Pearson Correlation coefficient among soil parameters of Mulshi Soil; the values indicated in red are a negative correlation, and those in blue are a positive correlation; the number of degrees of freedom (df) = 1, Strong positive correlation (above +0.6), strong negative correlation (above -0.6) and neutral relation (zero).

In Mulshitaluka, a positive correlation is seen between organic carbon, nitrogen, potash, sodium, and clay with the bacterial count, whereas a negative relationship exists with calcium, magnesium, silt, and soil specific density and the bacterial count.

Mulshitaluka also showed a similar pattern during the SCS, where, there was a decrease in the values of pH, phosphorus, potash, calcium, magnesium, sodium, and sand, and an increase in the values of organic carbon, nitrogen, clay, silt, and WHC.

Microbial analysis

All samples, which were analyzed, contained various bacteria in varying population numbers and thus are dominant in the soil. In the soil samples studied here, they seem to be unevenly spread. Any type of soil shows the presence of bacteria but their population number varies and is dependent on the soil texture and organic substrate in the soil. The bacterial population is highly influenced by pH, temperature, humidity, agriculture practice, fertilizers, pesticide, and the addition of organic matter (Rao, 1994).

TVC of 939×10^6 cfu/ml was determined for the 16 samples collected from 4 villages of Mulshi Taluka. Total 12 different bacterial genera isolated from all the 16 soil samples were *Actinobacteria*, *Frankia*, *Jonesia*, *Bacteriodes*, *Acetobacter*, *Beijerinckia*, *Staphylococcus*, *Streptomyces*, *Nocardia*, *Pseudomonas*, *Agrobacterium*, and *Nitrobacter* in the increasing order of the number of each genus isolated from the soil (Figure 2).

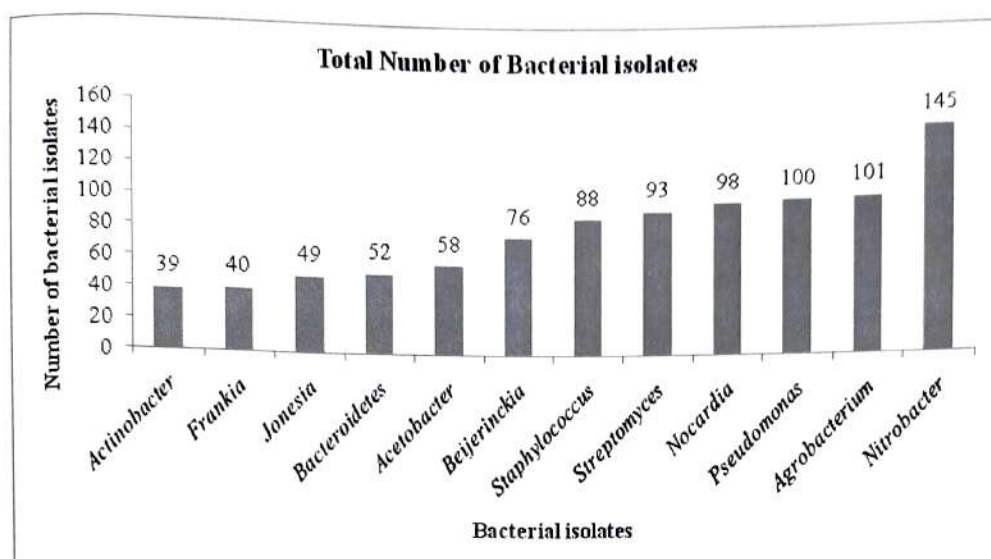


Figure 2: Number of each bacterial isolate from all the samples

Simpson's diversity index (SDI)

It was observed that the SDI was close to 1 which states that the diversity is high in all the soil studied here (Table 1).

Bacterial Isolates	Total number	Bacterial Isolates	Total number
<i>Actinobacter</i>	39	<i>Staphylococcus</i>	88
<i>Frankia</i>	40	<i>Streptomyces</i>	93
<i>Jonesia</i>	49	<i>Nocardia</i>	98
<i>Bacteroidetes</i>	52	<i>Pseudomonas</i>	100
<i>Acetobacter</i>	58	<i>Agrobacterium</i>	101
<i>Beijerinckia</i>	76	<i>Nitrobacter</i>	145
Total	939		
Simpson	0.904064	1/D	1.10612

Richness

The number of species per sample is a measure of richness. The more species present in a sample, the 'richer' the sample. Accordingly, the soil of Mulshi showed the presence of 12 different isolates (Figure 2).

Evenness

Evenness is a measure of the relative abundance of the different species making up the richness of an area. If a community is dominated by one or two species, it is considered to be less diverse than the one in which several different species

have a similar abundance. Thus, all samples have unevenness because the total numbers of individuals in the sample are quite unevenly distributed between their respective isolates, dominated by *Nitrobacter* isolate.

As species richness and evenness increase, diversity also increases. Simpson's Diversity Index takes into account both richness and evenness to measure diversity. This complement (1-D) represents the probability that two individuals randomly selected from a sample will belong to different species. Thus high diversity is observed in all the soils.

Table 2: Physicochemical analysis of soil samples

Crop /Fallow	Village	pH	EC	OC	N	Avail. P	Potash	Calcium	Magnesium	Sodium	CaCO ₃	Sand	Clay	Silt	Moisture	Water Hold. Cap.	Apparent Den.	Specific Den.	Porosity	TV C((x10 ⁶ cfu/ml)
Nachani	Shirkewadi	6.5	0.1	0.3	22.4	12.06	12.88	2.3	20.87	1.78	9.5	12.79	30.18	33.52	9.75	55.15	1.31	2.32	60.76	8
Khursani	Shirkewadi	5.93	0.12	1.65	11.55	6.01	26.51	1.95	12.27	0.96	4.75	9.66	29.64	35.45	9.78	67.05	1.04	2.07	62.26	15
Fallow	Shirkewadi	6.58	0.13	0.9	48.3	11.84	14.25	2.95	24.96	1.22	6.38	10.34	29.89	37.2	11.76	54.5	1.3	2.03	57.71	69
Nachani	Varak	5.83	0.11	0.75	52.5	5.67	35.69	1.3	9.41	0.67	4.5	27.7	28.02	23.17	8.81	48.47	1.09	2.19	62.6	32
Khursni/Hulg	Varak	5.98	0.2	0.53	37.1	5.78	47.42	1.1	7.77	0.83	4.25	20.27	28.56	25.1	7.76	49.6	1.11	1.93	59.24	41
Sawa	Varak	5.73	0.12	1.28	89.6	7.23	47.42	1.1	7.77	0.83	4.25	21.7	25.84	24.52	8.8	49.4	1.13	2.12	61.04	63
Nachani	Varak	5.58	0.19	1.82	12.74	7.23	24.92	1.15	7.36	0.78	3.75	10.95	35.15	28.06	9.85	73.83	0.96	2.23	65.48	83
Khursani	Varak	5.77	0.15	0.9	41.3	6.28	41.71	1.5	8.18	0.72	4.8	22.07	24.19	31.3	8.71	52.39	1.21	1.95	50.26	30
Fallow	Varak	6.16	0.18	2.46	17.22	6.01	63.53	1.5	8.18	1.26	5.25	16.19	36.19	23.44	8.83	64.26	1.03	1.56	58.68	141
Khursani	Nav	5.94	0.08	0.3	21.0	6.23	33.48	6.5	4.5	1.09	8	26.34	28.42	18.27	7.23	43.75	1.17	2.24	61.87	63
Vara	Nav	6.04	0.02	0.6	18.2	6.56	32.74	7.5	4.5	4.02	7.5	11.7	33.92	39.88	7.67	80.28	0.86	1.78	61.97	71
Fallow	Nav	5.55	0.05	0.3	37.1	6.9	32.21	8	6.14	6	3.3	14.1	37.97	25.63	7.72	63.27	0.97	2.03	61.39	99
Nachani	Tamhini	5.51	0.09	0.5	35.0	6.78	32.0	1.1	9.86	5.93	2.75	20.64	31.1	22.76	8.17	39.67	1.25	2.12	57.22	45
Khursani	Tamhini	5.61	0.19	0.9	63.0	6.9	47.52	1.5	8.59	0.66	2.63	19.82	34.3	37.31	8.02	53.35	1.15	2.21	58.02	27
Vara	Tamhini	5.64	0.15	0.54	37.8	6.9	67.06	1.5	8.18	2.66	4	13.65	31.55	28.02	7.85	55.37	1.15	2.07	57.49	99
Fallow	Tamhini	5.51	0.13	0.2	22.4	6.78	61.89	1.1	9.41	6.54	3.5	11.54	31.95	35.52	8.31	57.85	1.15	2.17	59.17	53

Table 3: Number of Bacterial genus isolated from each soil sample

Crop / Fallow	Villages	<i>Acetobacter</i>	<i>Frankia</i>	<i>Pseudomonas</i>	<i>Staphylococcus</i>	<i>Beijerinckia</i>	<i>Jonessia</i>	<i>Bacteriodes</i>	<i>Nitrobacter</i>	<i>Agrobacterium</i>	<i>Nocardia</i>	<i>Streptomyces</i>	<i>Actinobacteria</i>	TVC ((x10 ⁶ cfu/ml)
Fallow w	Shirkewadi	3	2	4	2	9	7	7	6	6	9	8	6	69
Nachani	Shirkewadi	0	0	1	1	0	0	0	3	1	1	1	0	8
Khuras	Shirkewadi	1	0	2	1	0	1	1	4	2	2	1	0	15
Nachani	Varak	3	1	3	3	1	3	2	6	3	4	3	0	32
Khur/Hul	Varak	2	3	8	7	4	0	3	3	4	3	2	2	41
Saw a	Varak	4	2	9	11	6	4	2	8	6	6	3	2	63
Nachani	Varak	8	2	8	7	3	3	4	14	9	10	6	9	83
Khur	Varak	2	0	1	1	0	1	0	11	4	4	6	0	30
Fallow w	Varak	8	5	14	9	14	7	9	21	16	18	14	6	141
Khur	Nave	4	3	12	8	5	3	3	10	7	3	4	1	63
Varai	Nave	3	4	4	6	11	3	4	8	6	9	11	2	71
Fallow w	Nave	4	4	11	12	6	11	6	17	11	9	7	1	99
Nachani	Tamhini	2	4	8	4	3	2	2	6	5	4	4	1	45
Khur	Tamhini	0	0	0	1	1	0	1	4	6	6	8	0	27
Varai	Tamhini	9	6	9	6	6	4	5	16	11	8	12	7	99
Fallow w	Tamhini	5	4	6	9	7	0	3	8	4	2	3	2	53

*Note: Fallow soil data is highlighted in grey

The nitrogen content of fallow lands was significantly high in the Varak and Nave as compared to the crop harvested soil. The other two fallow lands from Tamhini and Shirkewadi did not show higher nitrogen content than the crop harvested soil and the reason could be the length of the fallow period. Clay also increased in all the fallow lands but the sand level decreased. The same is observed in Table 2. Thus fallowing such lands for more than 7 to 8 years would bring the

soil parameters back to normal. In the case of Tamhini village, the fallow soil parameters and the TVC showed some deviation in their results. The TVC of fallow soil was less than crop harvested soil. The reason could be the fallowing period of the land which might be less than two years.

Tamhini and Nave villages showed the maximum presence of all the isolates in the soils collected after harvesting Varai, while Varak village soil collected after harvesting Nachani exhibited the

maximum presence of all isolates as seen in Table 3. This shows that crop cultivation did not influence the number and diversity of the bacteria in that soil.

Correlation analysis

A Pearson correlation coefficient was computed to assess the relationship between the various soil parameters and bacterial genus isolated from these soils.

In Mulshitaluka, a positive correlation is seen

between organic carbon, nitrogen, potash, sodium and clay with the bacterial count, whereas a negative relation exists with calcium, magnesium, silt, and soil specific density and the bacterial count. The millet crops grown during this time were Nachani (*Eleusine corocana* or African millet), Varai (*Panicum miliaceum*, or proso millet) Sawa (*Echinochloa frumentacea* or Indian Barnyard millet) /Khurasani (*Hyocymus niger*, Niger seed) and fallowed.

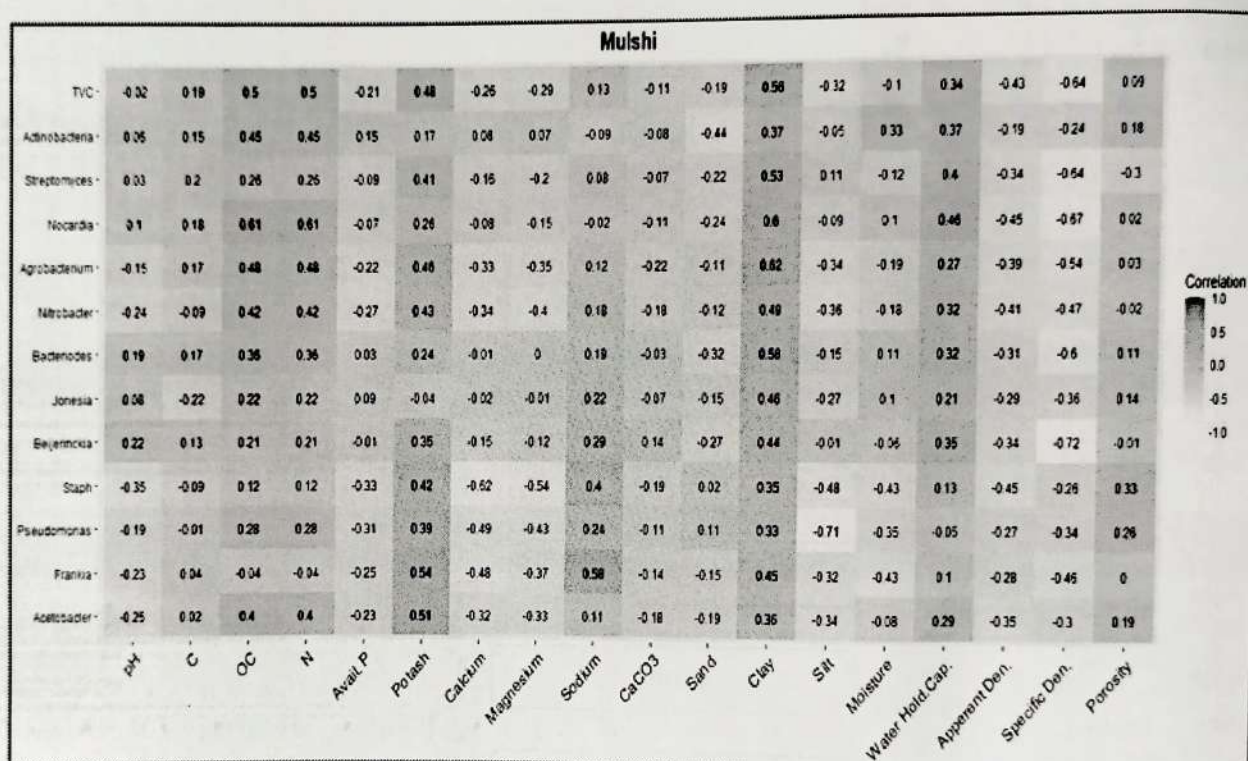


Figure 3: Correlation Analysis of Mulshi soil parameters with bacterial isolates

All the isolates showed a positive correlation with clay. Similarly, all were positively related to organic carbon and nitrogen except *Frankia*, potash except *Jonesia*, and water holding capacity except *Pseudomonas*. All the isolates showed a negative correlation with specific and apparent density. In the same manner, they all were negatively related to Ca and Mg except *Actinobacter*, *CaCO₃* except *Beijerinckia*, and silt except *Streptomyces* (Figure 3).

Acetobacter, *Staphylococcus*, *Streptomyces*, and *Frankia* showed a strong positive relation with potash while *Frankia*, *Jonesia*, *Bacteriodes*, *Nitrobacter*, *Agrobacter*, *Nocardia*, and *Streptococcus* showed a strong positive relation with clay. *Nitrobacter*, *Agrobacter*, *Nocardia*, and *Actinobacter* exhibited a strong positive correlation with organic carbon and nitrogen (Figure 3), the obvious reason for this could be the ability to fix nitrogen.

Frankia, *Beijerinckia*, *Bacteriodes*, *Nitrobacter*, *Agrobacter*, and *Nocardia* were strongly negatively related to specific density whereas

Frankia, *Pseudomonas*, and *Staphylococcus* showed a strong negative relation with Ca (Figure 3).

Principal component analysis

Agricultural data from different villages were subjected to principal component analysis (PCA) to find having similar overall patterns in the reduced multivariate data space without loss of information due to dimensionality reduction. Varimax rotated first three PCs were used to make a three-dimensional scatter plot. Variables that will tend to cluster together in the 2D scatter

plot will have similarities in the discriminatory power of the variables included in the study. All the statistical analysis was done in SPSS version 21.

In the Mulshi taluka it is observed that in Component 1 CaCO_3 (0.436), Avail P (0.582), Mg (0.543), pH (0.449), and Ca (0.511) has a similar effect; whereas in component 2, it is observed that Potash has (0.019) dissimilarity effect compared to other parameters in the study (Figure 4).

Component Plot in Rotated Space

Region: Mulshi

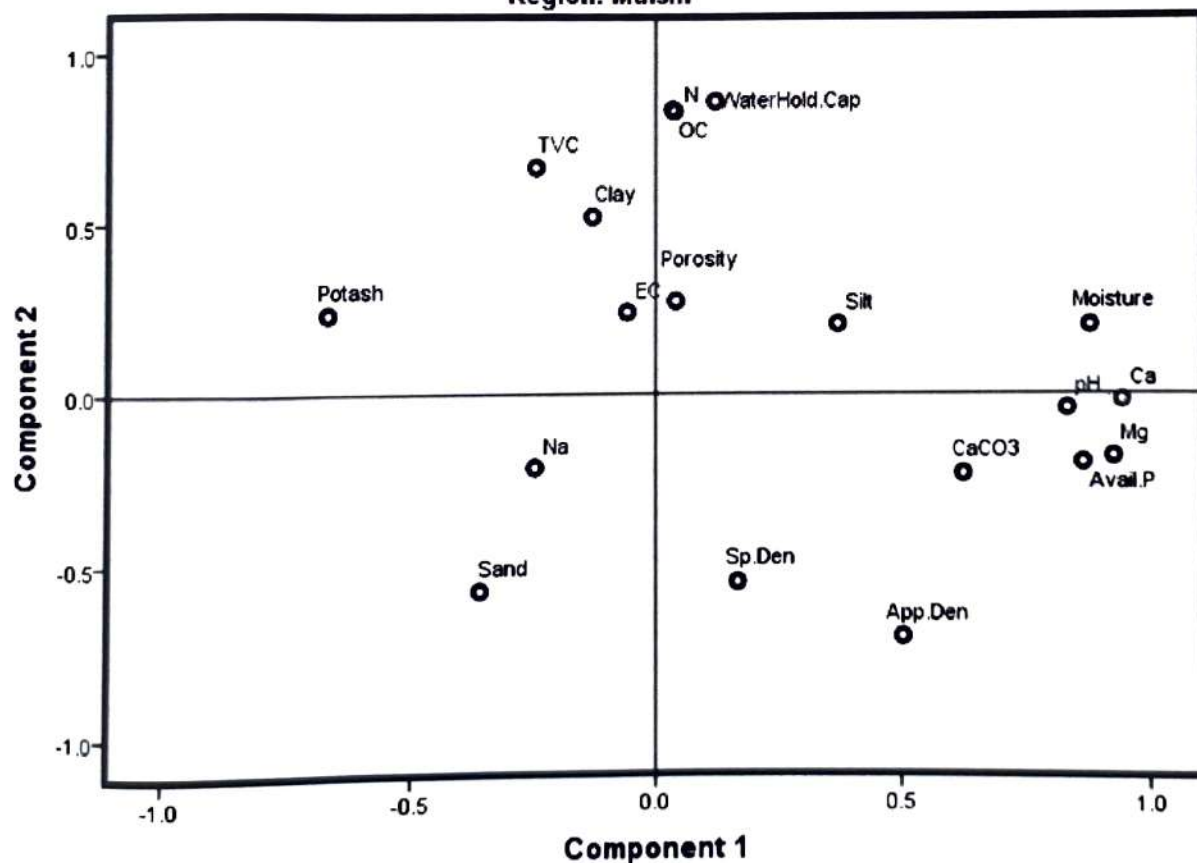


Figure 4: PCA for Mulshi

The genus *Beijerinckia* was one bacterial genus isolated from Mulshi soil, which generally, is encountered in wastewater, forest soil, and compost. It is a free-living aerobic chemoheterotrophic nonsymbiotic bacterium. They show the ability to fix atmospheric nitrogen. Their cell morphology and some physiological characteristics differ from other nitrogen-fixing bacteria. When tropical soil was analyzed about 48% of samples were positive for *Beijerinckia* (Becking, 1961a). This microorganism adapts to limiting conditions due to a strong correlation between the mineral requirements and the mineral composition of the soils (Becking, 1961b). The pH of Mulshi soil was in the range of 5.5 to 6.3 which may have supported the growth of this microorganism. Also, there are high chances of sewage water being used for cultivation. Thus, *Beijerinckia*, which is known to be found in wastewater and grows at acidic pH was isolated from this soil. Alagawadi *et al.*, (2014) has also reported the isolation of 65 strains of *Beijerinckia* from the soil samples of different forest ecosystems of Western Ghats, Karnataka.

Bacillus was not isolated from Mulshi soil for some unknown reasons. The absence of the class of Bacilli in grassland soils was noted by another study (Masse *et al.*, 2017) and it coincides with our study. This may be due to different soil types, pH, and carbon content (Hansel *et al.*, 2008).

The genus *Nitrobacter* which can oxidize nitrite to nitrate is found in freshwater, seawater, and soil and is the only nitrite oxidizer present there (Takahashi *et al.*, 2020). Members of this genus are chemo-organotroph as well as chemolithotroph. They grow well at a temperature range of 32 to 49°C and at a pH range of 6 to 7.5. All the soils in this study had a pH range of 5.5 to 6.3 and thus this bacterium was found in all soils. *Pseudomonas* is one of the most abundant genera in rhizosphere soil (Kumar *et al.*, 2016). They are also known to play a crucial role in plant growth by altering plant phytohormone

concentrations (Leveau&Lindow, 2005; Shiley, 2020). In this accordance, *Pseudomonas* was isolated from all soil in relatively high abundance. *Nocardia* species are aerobic relatively slow-growing Actinomycetes (Brown-Elliott *et al.*, 2006). They are found in soil, sludge, contaminated water, soils, plants, spoiled material. It is found widely in dry locations, especially during dust storms (Sazak *et al.*, 2012). The abundance of *Nocardia* spp. isolates from the soil are affected by pH, soil type, climate, temperature (Arends *et al.*, 2011). In the current study, its presence was noted in all soils mainly due to the temperature and the dry climate in these regions.

Agriculture is the major factor influencing the soil environment and its microbiome (Nannipieri *et al.*, 2003). But the farmers in these regions did not use any fertilizer though they practiced shifting cultivation. Thus, to an extent, this aspect could not explain the variability of the recorded results. A few of them are pH, soil texture, and metal content. Even temperature, precipitation, and other climatic events may influence the soil microorganism community (Li *et al.*, 2017; Maphuhla *et al.*, 2020). In this research, the soils were collected from the lands where only rainfed crops were cultivated were exposed to similar weather conditions. Therefore, the climatic conditions cannot explain the differences between the examined soils. The maintenance of ecosystem functions even under stressful conditions is enabled by large microbial diversity (Wertz *et al.*, 2007). On the contrary, in soils with low microbial diversity, even a little loss of species may expose the environment to functional changes (Wagg *et al.*, 2014). In the discussed soils, differences in the activity and structure of the microbes inhabiting them may be important during fallow period.

The length of fallow period is important for soil fertility restoration and improvement of soil properties. In the fallow period, the land is left free or fallow for few seasons. This allows the land to naturally regain nutrients. Microbes decompose

dead plants, animal waste and other organic material that gets collected in the land. Thus, the soil regains the lost nutrients (Kozak & Pudalko, 2021). In the initial part of fallow, the vegetation grows quickly, from the root systems of the previous crop which is already present there and also from the new seedlings. Nutrients from the surface soil and subsoil are used and stored in the vegetation. These are then partly returned to the soil through litter and rain wash from the aboveground vegetation. Then the litter decays rapidly due to the intense biotic activity at the soil surface and is converted into soil organic matter. During this time, leaching and erosion are decreased due to ground cover and rooting mass. Humus and litter layer grow until equilibrium between build-up and rate of oxidation is reached (Nye & Greenland, 1960; Styger & Fernandes, 2006). The fallow land in this study was abandoned for more than four years which lead to the increase in the favourable soil parameters and in the microbial population.

Although the fallow cycle has shown to increase soil erosion and disturb the watershed area, a usual practice of *Alnus nepalensis* plantation along with crops in Jhum would serve to complete the need for nitrogen (N) (Rathore *et al.*, 2010).

10 years of revegetation with the nurse plant *Alnus nepalensis* showed that the levels of soil-available N and K were increased and the growth of microorganisms was also promoted (He *et al.*, 2013). *Alnus nepalensis* (Alder) based fallow Jhum land system was found better as it was detected that the soil organic matter (3.20%) was significantly increased which contributed to soil fertility and better nutrient cycling (Mishra *et al.*, 2018). The use of the mixed cropping method in this cultivation system not only increases the soil fertility but also protects the crops from insect and pest attacks (Ramakrishnan, 1984; Lizarazo *et al.*, 2020). Thus deliberate plantation of trees like *Alnus nepalensis* and mixed cropping could help in improving soil health.

CONCLUSION

The results demonstrate significant differences between the soils studied, both regarding physicochemical and biological parameters. Based on both these parameters it can be concluded that the length of the fallow period influences soil health. The TVC obtained in the fallow soils was significantly higher than the crop harvested soils. Thus the land management systems should take actions to satisfy the needs of the cultivators by providing various alternatives for their sustainability.

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Conflicts of interest

The authors declare no conflict of interest.

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Development of Nutritious Rusk by Incorporation of Millets

Ameena Sabeer¹ and Dr. Betty Rani Isaac²

M.Sc. Student¹ Associate Professor² Department of Home Science, St. Teresa's College,
Ernakulam, Kerala- 682035

E-mailID: bettyraniisaac@teresas.ac.in

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ABSTRACT

Millets are highly nutritious, non glutinous and non-acid forming foods. Hence they are soothing and easy to digest. Millets contain about 8 per cent protein and 4 per cent fat. They are rich source of vitamins and minerals. Millets are especially rich in calcium. The dietary carbohydrate content of millets is also relatively high. Antinutritional factors such as phytate and polyphenols are also present in millets but they are mostly confined to the seed coat and the milled millets are generally free from the anti-nutritional factors.

Millets have many nutraceutical properties that are helpful to prevent many health problems such as lowering blood pressure, risk of heart disease, prevention of cancer and cardiovascular diseases, decreasing tumor cases etc. Other health benefits are increasing the time span of gastric emptying, provides roughage to gastro intestine. The objective of this study was to develop a nutritious multi millet rusk. The millets incorporated were kodo (*Paspalum scrobiculatum*), barnyard (*Echinochloa esculenta*) and little millet (*Panicum sumatrense*). Four formulations were used (50:50, 70:30, 100: 100) and rusk made with 100% wheat flour was used as control. The products were analyzed for sensory, nutritional evaluation and keeping quality. Results of the sensory evaluation showed that a combination of whole wheat flour and millet mix at 50:50 as the best treatment through. Assessment of nutrient composition showed that multi grain rusk were low in fat and high in dietary fiber and protein compared to the control rusk. The shelf life of the product was studied by packing in polypropylene foil, polyethylene terephthalate and glass containers. The parameter used for shelf life study included moisture, peroxide value, sensory evaluation and microbial analysis.

Key words: Millets, rusk, fiber, shelf-life study.

Kodo, Barnyard and little millets are highly nutritious. The millet grains are rich in non-starchy polysaccharides and dietary fibre which are beneficial for diabetics as it slowly release glucose to the blood. It contains very healthy polyunsaturated fat. By consuming millets on a regular basis, a diabetic can reduce spikes in blood glucose levels, which otherwise leads to numerous complications. Three main types of millet included in the present study are Kodo, Barnyard and Little millet. Rusk is ideal snack for

anyone owing to convenience and compactness. They differ from other bakery products in that it is less sweet and less costly, long storage life and free from microbial spoilage.

Rusk is less costly and has long shelf life. Rusk is enjoyed by many including diabetics during tea time. It is easy to store compared to other snacks. Millets are less popular in our society as people are unaware of the nutritive value of it and due to the lack of knowledge in preparing recipes out of it. Healthy baked foods

are an easy way to reach the consumers and popularise these nutritious grains. Addition of multigrain to bakery products increases the nutritive value of the product and adds variety to our diet.

Overall the global burden of diabetes has increased significantly in recent decades and will continue to soar in the next few decades (Lin *et al.*, 2020). Globally, the number of people with diabetes mellitus has quadrupled in the past three decades, and diabetes mellitus is the ninth major cause of death. About 1 in 11 adults Worldwide now have diabetes mellitus, 90% of whom have type 2 diabetes mellitus (Yan *et al.*, 2018). A healthful eating pattern, regular physical activity, and often pharmacotherapy are key components of diabetes management. Dietary treatment is the basis for treatment of diabetes. Nutritional management help in optimizing glycaemic control and reduce risk factors and other complications. (Vessby *et al.*, 2011).

Millets are a group of highly variable small seeded grasses, widely grown around the world for fodder and human food. They are one of the oldest foods known to humans and are rich source of vitamins and minerals. Millets are especially rich in calcium. Millets have many nutraceutical properties that are helpful to prevent many health problems (Kumaret *al.*, 2010 and Saritha and Singh, 2016).

Bakery, a crucial segment of food processing industry, is one of the oldest and traditional activities in India. A Rusk is a hard, dry snack or a twice-baked bread. The word 'rusk' when used in this specification, includes any cooked product of grain-based dough, whether it has been raised with yeast or not (William, 1976).

According to IIMR (2016), increasing millet consumption through new product development and promotion could be a good strategy for enhancing nutritional and health status of community. Hence developing technology that makes millet based value added products as convenient to make and easy to access at reasonable prices will find great demand in the

market particularly in urban places where there is growing requirement for nutritious foods. Keeping this in view the present study was conducted to develop multi millet rusk by incorporating three different millets.

OBJECTIVES

1. To develop and standardise multi millet rusk by using mix of three different flours viz, kodo, barnyard and little millet in different proportion with wheat flour
2. To evaluate the acceptability of the developed product by sensory evaluation
3. To assess the nutritional composition of the product
4. To ascertain the shelf life of the product

METHODOLOGY

The present study entitled "Development of nutritious rusk by incorporation of millets

" was undertaken to make rusk using multi millets such as kodo, barnyard and little millet and to evaluate their nutritional, organoleptic, and microbiological characteristics and to determine the keeping quality of the developed products. The methods adopted for the study are given under the following headings:

Selection of raw ingredients

The main ingredient in rusk i.e. refined flour was replaced with whole grains such as wheat and millets. Three types of millets selected were;

1. Kodo millet (*Paspalum scrobiculatum*)
2. Barnyard millet (*Echinochloa esculenta*)
3. Little millet (*Panicum sumatrense*)

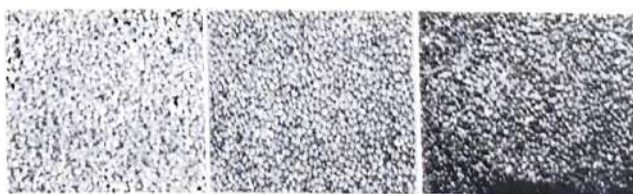


Plate -1
Kodo millet

Plate -2
Barnyard millet

Plate -3
Little millet

(*Paspalum scrobiculatum*) (*Echinochloa esculenta*) (*Panicum sumatrense*)

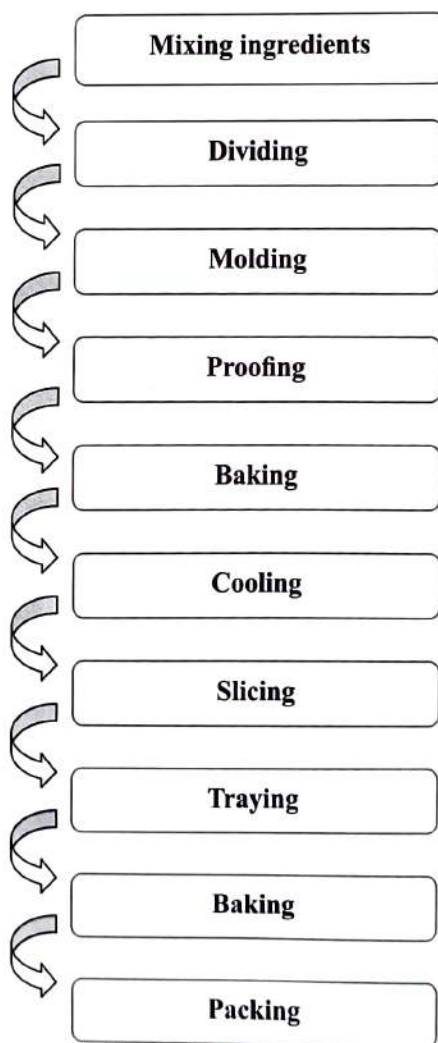
Other ingredients used are whole wheat flour, oil, yeast, salt, water. All the ingredients were collected from online shopping and local market.

Method followed for preparation of rusk

Rusks were prepared using method as described by Yaseen (2000), with some modifications.

Usually, rusk is prepared from maida with added yeast, shortenings, water, sugar and salt. In the present study standardized recipe was altered by adding whole wheat flour and different types of millets such as kodo millet, barnyard millet, and little millet in the proportions given below in the title formulation of the recipe

Flow diagram for steps involved in preparation of rusk



Formulation of recipe

To formulate the recipe wheat flour and mix of three millets were taken in different proportions such as:

1. 100:0
2. 50:50
3. 30:70
4. 0:100

Preparation of Rusk

Ingredients	Amount (g)
Flour	72
Sugar	8
Fat	20
Yeast	a pinch
Salt	a pinch

Procedure

Rusk is a yeast leavened crisp product, which has been baked twice. Yeast and water were mixed in a bowl and kept it for 30 min then sugar was added for activation of yeast. Flour and other ingredients except fat were added to the yeast water solution. Adequate amount of water is added until the dough has reached its required consistency. Fat was added in last. Mixing was carried out for 10-12 min and proofing for 45 minutes is done where the dough is allowed to rest and rise a final time before baking and then baking. Baking was done at 200 degree C for 15 minutes and the loaf was left to cool overnight. The next day, the loaf was sliced and then baked a second time at 160 degree C for 20 minutes and finally the prepared rusks were left for cooling and then packed in polyethylene packs and stored at room temperature for further evaluation.

Sensory evaluation

Sensory evaluation of rusk was carried out using a nine (9) point Hedonic scale with a range of 1 indicating an extremely disliked and 9 indicating an extremely liked sample.

The samples were evaluated for its sensory attributes such as odor, flavor, texture, appearance and overall acceptability by the panel consisted of 10 subjects. The sample with highest mean score for different characteristics was chosen as the most preferred one. Rusk prepared with 100% wheat was taken as the control.

Computation of nutritive values

Nutritive values of each ingredient used for the preparation of rusk were calculated using the food composition tables (IFCT, 2017). The nutritive value of the major ingredients like kodo, barnyard, and little millet were calculated using the food composition table by Indian institute of millet research (IIMR, 2016).

Shelf life study

The selected rusks were stored in polypropylene foil, glass jars and plastic container (polyethylene terephthalate) and kept at an ambient room temperature for 30 days. The stored rusks were then analysed for chemical parameters such as moisture content and peroxide value, microbiological analysis for the presence of yeast and mould and organoleptic evaluation for appearance, taste, texture, flavor, odour and overall acceptability. The shelf life study was done for a period of 30 days.

Packages used for the storage of rusk

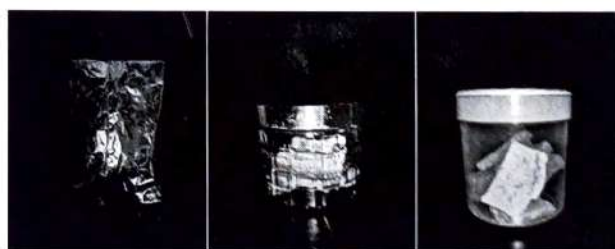


Plate -4
Polypropylene foil

Plate -5
Glass jars

Plate -6
Polyethylene
terephthalate
container

Data tabulation and interpretation

All the data were collected regarding the development of multi millet rusk such as physical characteristics, sensory qualities, nutritional composition were tabulated and interpreted using percentage analysis

FINDINGS AND DISCUSSION

Preparation of rusk

The rusk was prepared by the method described by Yaseen (2000). All the ingredients were

collected from the local markets. Four The different formulations are given in the table formulations were prepared including the control. given below:

Table- 1 Different formulations used for the preparation of rusk

Sl.no	Treatment	Wheat flour:Millet mix proportion	Whole wheat flour (g)	Kodo (g)	Barnyard (g)	Little millet (g)
1	Control (T ⁰)	100: 0	300	-	-	-
2	Recipe 1 (T ¹)	50:50	150	50	50	50
3	Recipe2 (T ²)	30:70	90	70	70	70
4	Recipe 3 (T ³)	0:100	-	100	100	100



Plate -7 control (T⁰) (100:0)

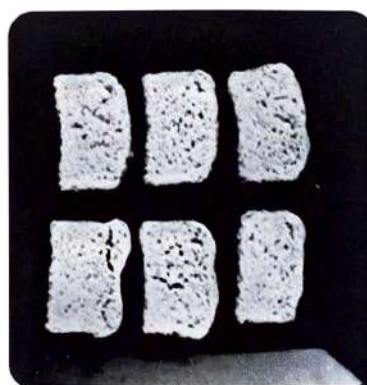


Plate-8 recipe (T¹) (50:50)



Plate -9 recipe (T²) (30:70)



Plate -10 recipe (T³) (0:100)

Sensory evaluation

The multi millet rusk standardized in the present study was assessed organoleptically by a panel of

10 judges using a score card on a 9 point hedonic scale and compared with control rusks^{T°} (rusk made with 100% wheat flour).

Table- 2 Mean organoleptic scores of prepared rusks

SL.NO	PARAMETERS	CONTROL(T°) W: M (100:0)	(T ¹) W: M (50:50)	(T ²) W: M (30:70)	(T ³) W: M (0:100)
1	Appearance	9	9	8.6	6.5
2	Taste	9	7.7	6	7
3	Odor	8	8.5	8	6
4	Flavor	8	7.4	7	6.5
5	Texture	9	8.2	6	5.3
6	Overall acceptability	9	8.5	7	6.4
7	Mean value	8.6	8.2	7.1	6.2
8	Percentage	95.5	91	78.8	68.8

W (Whole wheat Flour) : M (Millet Mix)

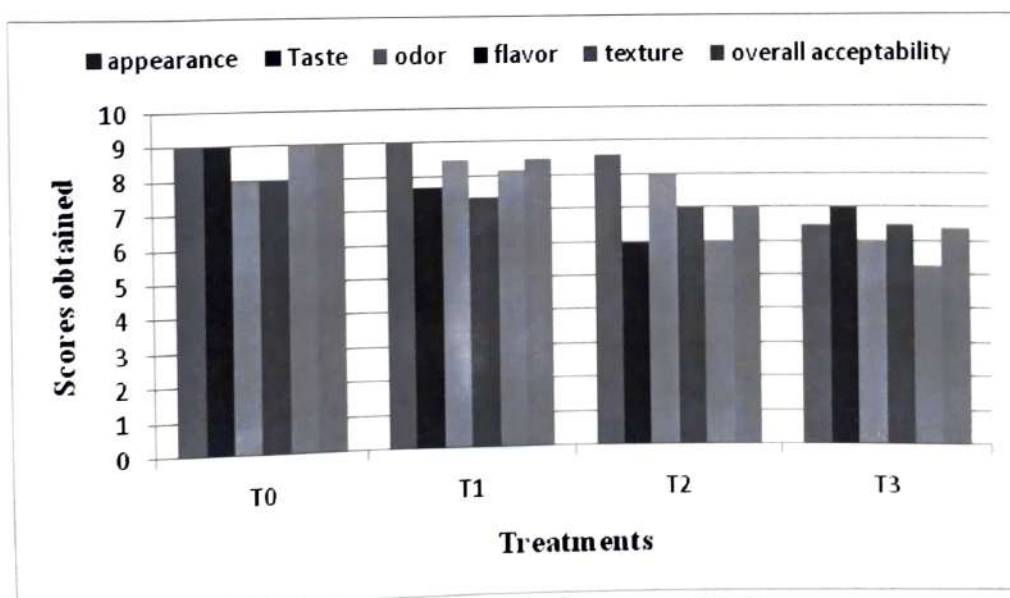


Figure - 1 Sensory evaluation scores of rusk

It was found that rusk with 50% wheat and 50% millet flour (kodo, barnyard, little) was highly accepted as the best by the panel members. Based on the maximum scores obtained for overall acceptability, rusk with 50% wheat flour and 50% millet flour (kodo, barnyard, little) is selected for detailed study. The selected recipe was further studied for nutrient composition and keeping quality

Nutritive value

Based on the results of the sensory evaluation rusk sample T¹(W:M 50:50) was selected as the best one. Nutritive values of each ingredient used for the preparation of rusk were calculated using the food composition tables (IFCT, 2017 and IIMR, 2016) from which nutritive value per 100g was obtained.

Table -3 Nutrient composition of the Rusk T¹(W:M 50:50) per 100g

Ingredients	Amount (g)	Nutrient content						
		CHO(g)	Energy(Kcal)	Protein (g)	Fat (g)	Fibre (g)	Iron (mg)	Calcium (mg)
Kodo millet	12	7.9	39.72	1.07	0.168	1.08	0.28	1.83
Barnyard millet	12	7.8	36.84	1.26	0.26	1.2	0.6	2.4
Little millet	12	7.8	41.52	1.07	0.30	0.9	0.15	1.92
Wheat flour	36	23.1	115.41	3.81	0.55	3.96	1.48	11.14
Ghee	20	-	180	-	20	-	-	-
Sugar	8	8	32	-	-	-	-	-
Total	100	54.6	445.49	7.21	21.27	7.14	2.51	17.29

From the above table, it is clear that the rusk of 100 grams provides 445.49 Kilo calories of energy, 7.21 grams of protein, 54.6 grams of carbohydrate, 21.27grams of fat, 7.14 grams of fibre, 17.29 mg calcium, and 2.51 mg iron. Thus, the rusk provides sufficient dietary fibre and protein needed for the diabetic patient.

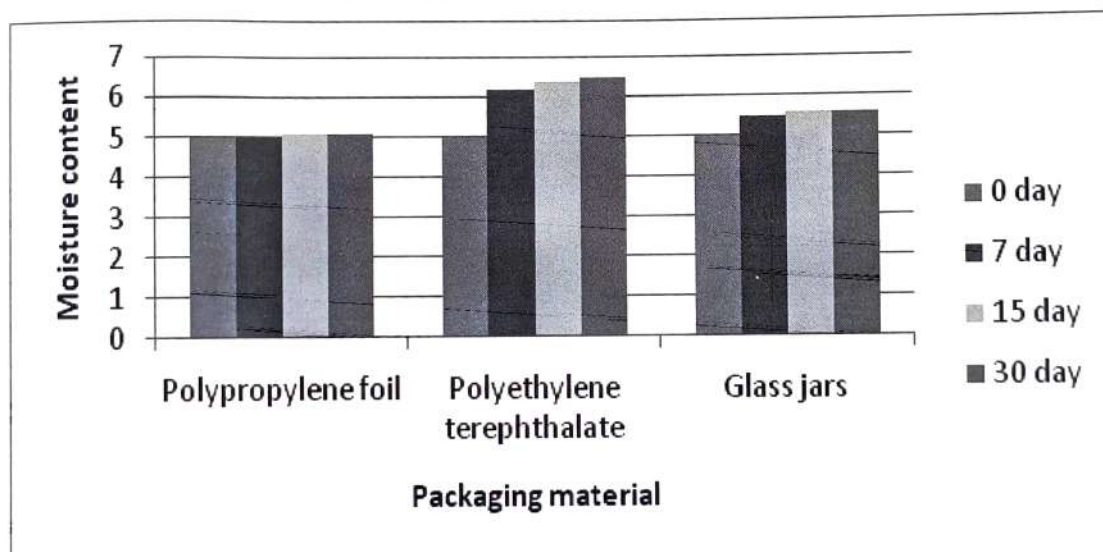
Shelf life study

Based on the results of the sensory evaluation

rusk sample T¹(W:M 50:50) was selected and shelf life study was conducted. The selected rusks were stored in polypropylene foil, glass jars and plastic container (polyethylene terephthalate) and kept at an ambient room temperature for 30 days. These stored rusks were then analysed for chemical parameters such as moisture content and peroxide value, microbiological analysis for yeast and mould and organoleptic evaluation.

Table- 4 Moisture content and peroxide value of the stored Rusk T¹ (50:50)

Duration	Types of packaging	Moisture content (%)	Peroxide value
0 days	Polypropylene foil	5	0
	Polyethylene terephthalate	5	0
	Glass jars	5	0
7 days	Polypropylene foil	5	0
	Polyethylene terephthalate	6.2	0
	Glass jars	5.5	0
15 days	Polypropylene foil	5.1	0
	Polyethylene terephthalate	6.4	0
	Glass jars	5.6	0
30 days	Polypropylene foil	5.1	0
	Polyethylene terephthalate	6.5	0
	Glass jars	5.6	0

**Figure -3 Moisture content of stored rusk**

Moisture content in the rusk stored in polyethylene terephthalate was increased from 5% to 6.5% after 30 days of storage. Whereas, the moisture content of the rusks stored in glass jar were increased to 5.6%. Storage in polypropylene foil was found to be the best, as it had only 5.1% moisture after 30 days storage time. As per BIS specification (IS 8555-2013), the estimated value for moisture content of rusk is

up to 10 percent. Moisture content of the samples were found to be within the permitted limit.

The peroxide value of the stored rusk in different packaging material was found to be zero which means that the product has not undergone any oxidation within the 30 days of storage.

Sensory evaluation of the rusk after storage

Sensory evaluation of the rusk plays an important

role in the acceptability and shelf stability of products. The sensory evaluation of the rusk stored in different packages were periodically evaluated with respect to the sensory parameters using a score card on 9 point Hedonic scale by a selected panel of judges.

Table -5 Sensory evaluation scores of the stored ruskT¹ (50:50)

Duration	Types of packaging material	Sensory parameters							
		Appearance	Taste	Texture	Flavor	Odor	Overall acceptability	Mean score	Percentage
0 days	Polypropylene foil	9	7.7	8.5	7.4	8.2	8.5	8.2	91
	Polyethylene terephthalate	9	7.7	8.5	7.4	8.2	8.5	8.2	91
	Glass jars	9	7.7	8.5	7.4	8.2	8.5	8.2	91
7 day	Polypropylene foil	9	7.7	8.5	7.4	8.2	8.5	8.2	91
	Polyethylene terephthalate	9	7	8	7	8	8	7.8	86.6
	Glass jars	9	7.2	7.8	7	8	8	7.8	86.6
15 days	Polypropylene foil	9	7	7	7	8	8	7.6	84.4
	Polyethylene terephthalate	9	7	7	7	7	8	7.5	83.3
	Glass jars	8	7	7.5	7	7	8	7.4	82.2
30 days	Polypropylene foil	9	7	7	7	7	8	7.5	83.3
	Polyethylene terephthalate	8	7	7	6	7	8	7.1	78.8
	Glass jars	8	7	7	6	7	8	7.1	78.8

Sensory scores obtained for rusk was found to decrease as the storage period increases. Samples in different packages retained sensory qualities (80% score) after 15 days of storage. After 30 days of storage rusks stored in the sealed polypropylene foil scored above 80%. The other two samples also maintained average score i.e. >75%.

Microbial analysis

In order to ascertain the keeping quality, microbial analysis of the rusk sample T¹ (W:M 50:50) in different packages for the detection of yeast and mould growth was conducted at the end of 30 days.

Table-6 Microbial load of stored multi millet ruskT¹ (50:50)

Storage Duration	Types of packaging	Yeast and mould count (cfu/g)	Permitted level(cfu/gm)
30 days	Polypropylene foil	13	<1.0 × 10 ⁶
	Polyethylene terephthalate	12	
	Glass jars	13	

From the above table it is clear that the yeast and mould growth in polypropylene foil and glass containers were 13 cfu/g and in poly ethylene terephthalate was found to be 12cfu/g. All the three samples stored in different containers were found to be microbiologically safe after 30 days of storage.

SUMMARY, CONCLUSION AND IMPLICATIONS

From the above study it can be summarised that ready eat to eat snack (rusk) prepared with wheat flour and multi millets namely kodo, barnyard, little millet for diabetic subjects was highly acceptable. Based on the results of the sensory evaluation rusk sample T¹(W:M 50:50) was further studied and was found to be, nutritious, microbiologically safe and these samples could be safely stored for 30 days. Due to the nutritional benefits along with fiber content multi millets if made available in ready-to-eat form or convenient foods can be consumed as health and nutritional foods. Even though the consumption of millet as direct food is declining in India, preference for processed foods is surprisingly picking up in urban areas.

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Significance of Seaweed Liquid Fertilizer (SLF) on Seedling Growth of *Solanum lycopersicum* L. & *Abelmoschus esculentus* (L.) Moench

Vivek S* & Palanisamy M

Botanical Survey of India, Southern Regional Centre, Coimbatore.
Corresponding author: vivekprasanthnss@gmail.com

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ABSTRACT

An experiment was conducted in the laboratory of Botanical survey of India, southern regional centre to investigate the effect of Seaweed Liquid Fertilizer from *Gracilaria tenuistipitatum* C.F.Chang&B.M.Xia on seed germination and seedling growth of tomato and bendhi. The concentrations of SLF were 10%to 100% and of water respectively along with a control where only water was used to treatseeds by soaking for 12 hours in prior to sowing on saturated tissue paper in petri-dishes. Theexperiment was laid out following RCBD design where each treatment replicated three times.Germination percentage of all the seeds treated with SLF of above-mentioned concentrations was significantly higher over control. Seed germination was enhanced significantly in tomato and bendhi at 50% of SLF.

Key words: Seaweed Liquid Fertilizer,Effect, Plant growth,*A. esculentus*,*S. lycopersicum*

Introduction

Agriculture success mainly depends on a Germination of seeds, plant growth, soil quality, climate and well balanced fertilizer application. Low germination percentage, slow rate and lacking in seedling growth are common problem in vegetable crops. Tomato (*Solanum lycopersicum*) and Okra (*Abelmoschus esculentus*) are one of the important vegetable crops grown throughout the tropical and subtropical parts of the world either as the sole crop orintercrop with maize or another (Emuh et al., 2006). These crops play an important role in the human diet by supplying carbohydrate, protein, fats, minerals and vitamins that are usually deficient in the staple food (El-Kader et al., 2010). The problem of seedling establishment is found in both crops of tomato and okra suffers differently due to lack ofgermination and/or delay in germination of seeds and some soil borne diseases which affect growth and development of crop plants. Due to these reasons farmers are using heavy doses of inorganic fertilizers to fulfil the problem. There are many chemical methods available to regulate this disease. An intensive use of these chemicals has increased crop yields but depletes the soil micro-environment and

causes soil and water pollution. (Pimentel, et al.,1995). Application of suitable organic plant growth regulators like SLF is more convenient aswell as economic as it needs a very small in quantity. Application of PGR does not requireany special technique or expensive procedure. Seaweed fertilizers are favoured not only due to their elements such as nitrogen, phosphorus and potash content, but also presence of traceelements and metabolites similar to plant growth regulators. Liquid fertilizers derived from seaweeds found to be superior to chemical fertilizers because of high level organic matter,micro and macro components, vitamins, fatty acids and growth regulators (Slvaraj et al., 2004). The effects of *Gracilaria tenuistipitatum* seaweed on germination and growth of tomato and okra are not reported. Hence the present research was planned to investigate the effect of SLF on seed germination of those crops and to observe the effective result in different concentrations

MATERIALS AND METHOD

Collection of seaweeds

Seaweeds were collected from two districts of Andhra Pradesh east coast of India. The

intertidal region of this coast is dominated by eroded rocks favours for the growth diverse marine macro algal species. The collected seaweeds were washed with seawater to remove the foreign particles, sand particles and epiphytes. Morphologically distinct thalli of algae were placed separately in polythenebags and brought to the laboratory. Samples were washed thoroughly using tap water to remove the salt on the surface. The wet seaweed samples were shade dried for 3 days. After shade drying, seaweed was cut into small pieces and powdered. The SLF was prepared from this as per the method of Bhsole *et al.*, (1975).

Preparation of Seaweed Liquid Fertilizer

The 1kg powder of each seaweed sample was mixed with water (20 L) in the proportion of 1:20. Then it was boiled for 1 h. The mixture was cooled and filtered with muslin cloth Bhsole *et al.*, (1975). The filtrate was designated as stock solution and was used to prepare various concentrations (10%, 25%, 50%, 75% and 100%) of extract by mixing appropriate proportion of Seaweed Liquid Fertilizer with sterilized distilled water.

Selection of seeds

Healthy seeds of *Solanum lycopersicum* and *Abelmoschus esculentus* were collected from Department of Vegetable crops, Tamil Nadu Agriculture University, Coimbatore. The seeds were soaked in different concentrations of different Seaweed Liquid Fertilizers up to 24 hour and then used for germination study by paper towel method.

Paper towel method

In paper towel method, different concentrations (10%, 25%, 50%, 75%, and 100%) of Seaweed Liquid Fertilizer of *Gracilaria tenuistipitata* were used as base for the treatment of seeds of *Solanum lycopersicum* and *Abelmoschus esculentus*. The controls were maintained by using distilled water. In paper towel method, 10 treated seeds were placed in tissue paper by maintaining appropriate distance, fold the paper at four sites properly and kept in the water to maintaining moisture content. After 14 days, germination percentage, seedling length and seed vigour index was measured under room temperature in natural condition.

Vigour index

Seed vigour is an important quality parameter which needs to be determining to addition germination. Vigour index of seeds calculated by using Abdul Baki and Anderson (1973) formula.

$$\text{Vigour index} = \text{Germination (\%)} \times \text{seedling length (cm)}.$$

Statistical analysis

Data was analyzed statistically using ANOVAs. All the measurements were triplicates

Results Discussion

Data obtained on various growth parameters in *Solanum lycopersicum* and *Abelmoschus esculentus* treated with different concentration of SLF are given as follow.

Effect on seed germination

Germination percentages of tomato and bhendi seeds were affected differently by SLF. In bhendi, SLF enhanced germination percentage over control. At 50% concentration, SLF have the highest percentage of seed germination of 96 ± 2.22 after 12 hours, respectively. Lower concentration of SLF also enhanced seed germination, which was significantly higher than that of 100% concentrations of SLF. Among the different concentration of SLF, the medium concentration (50%) gave better performance than that of lower (10%) and higher concentration (100%). The seed germination percentage in control was 80 ± 2.24 after 12 hours, which was the lowest one. Thus, the highest germination percentage was obtained in tomato by treating with 50% concentration of SLF. (Fig 1&2)

Seed germination of tomato, a day neutral crop was completed within 14 days after sowing. After 12 hours, 98 ± 2.0 and 82 ± 1.24 seeds were germinated in 50% concentration of SLF and control, respectively. Second highest seed germination was obtained at lower concentration of SLF (25%). Among the five concentrations 50% of SLF gave better performance than other concentrations. Similar trend in seed germination was observed after 14 days after sowing. Rate of germination from starting was highly steady in tomato compared to other crop seeds in the present study. PGRs of present study were

enhanced seed germination significantly, over control after 12 hours. (Fig 1&2)

Germination of bhendi seeds (a day neutral plant) was started from 0 hour and ended at 12 hours after sowing. At beginning, rate of germination was medium compared to tomato. Tomato was steady, but bhendi was slow. This two day neutral plant, tomato behaved different to that of bhendi. Moreover, SLF found more suitable in enhancing seed germination. However, bhendi and tomato preferred comparatively higher concentration of SLF. The present result agreed to the earlier research in Okra (Selvaanathi and Beulah Jerlin 2020) and tomato (Castor et al., 1987). The germination enhancing potential of seaweeds might be attributed to the presence of gibberellin in seaweed liquid fertilizer (Jennings and Tulloch, 1965; Anantharaj and Venkatesalu, 2001; Rayorath et al., 2008).

Seedling stage is the most sensitive stage in the life cycle of plant and hence it is Susceptible to physical and chemical adversities. The application of different levels of SLF increased the growth and development pattern of seedlings growth. Seedlings which are treated with SLF become suppressed and inhibited when compared to the seedlings which are treated with water. This may be due to the metabolic activity (Sobithabai et al., 2005)

Effect on shoot length

Results showed that PGRs present in the SLF had stimulatory effect on shoot length of bhendi all along the growth period. The highest shoot length (21.12 ± 0.21 cm and 17.48 ± 0.31) was observed at the concentration of 50 and 75% of SLF at 14 DAS respectively. The shoot length was gradually increased with proceeding of time after sowing. Up to 14 DAS, the shoot length was similarly increased by different concentrations of SLF and the values were significantly higher over control. In tomato, the data on shoot length showed that the SLF produced the highest shoot length and these were 8.16 ± 0.24 cm and 7.26 ± 0.20 cm at 50% and 75% of concentration 14 DAS respectively. The minimum shoot length (4.94 ± 0.28 cm) was recorded in control condition at 14 DAS. SLF similarly enhanced shoot growth in both seeds of tomato and bhendi, which was significantly higher over control. Similar

observations were made in *Cajanus cajan* (Mohan et al., 1994) Jothinayagi and Anbazhan (2009), Sethi and Adhikary (2009) and Hernández-Herrera et al., 2014.

Effect on root length

Root length of bhendi significantly varied from control due to application of SLF. At different concentration and -14 DAS, root lengths were higher at 50% concentration followed by lower concentration of same PGR. However, the lowest root length was obtained in control condition. In tomato, SLF at 50% concentration enhanced root length significantly higher over other treatments. After 14 DAS, the highest root length was 4.24 ± 0.11 cm at 50% concentration followed by 3.44 ± 0.18 cm at 10% concentration. In control, the lowest length of roots was observed. The results obtained in the present experiment support the findings of Bhore et al. (1999) in tomato.

CONCLUSION

In conclusion, this study shows that liquid seaweed extracts from *Gracilaria tenuistipitatum* (50%) were more effective at stimulating the growth of both seedlings, and therefore, they are potential candidates for the production of effective bio stimulants. Surprisingly, the extracts of both species showed better results when they were applied at lower concentrations than more concentrated extracts. SLF may provide a powerful and environmentally friendly approach to nutrient management. This study provides valuable information on the identification and utilization of seaweed resources for agriculture. The presence of inorganic minerals in SLF makes them an excellent choice as organic fertilizers. The practice of applying eco-friendly seaweed extract can be recommended to growers to help attain better germination and growth of other crops. Future studies on specific mechanisms attributed to plant growth are required to determine the potential of *Gracilaria tenuistipitatum* as commercial growth bio stimulants which can be promoted as eco-friendly bio fertilizers.

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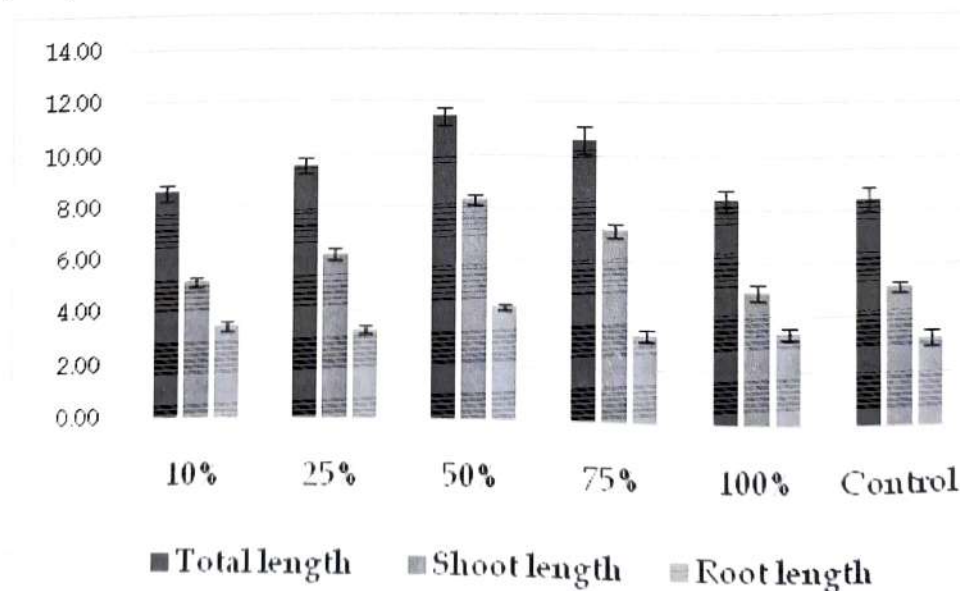


Fig 1.Effect of different concentrations of SLF treatment on the growth parameters of *Solanum lycopersicum*

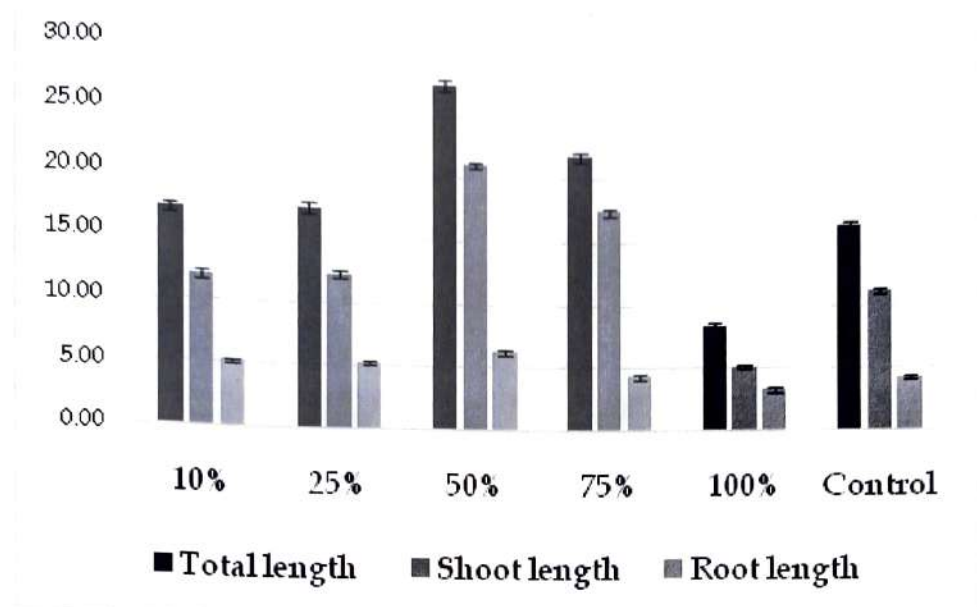


Fig 2. Effect of different concentrations of SLF treatment on the growth parameters of *Abelmoschus esculentus*

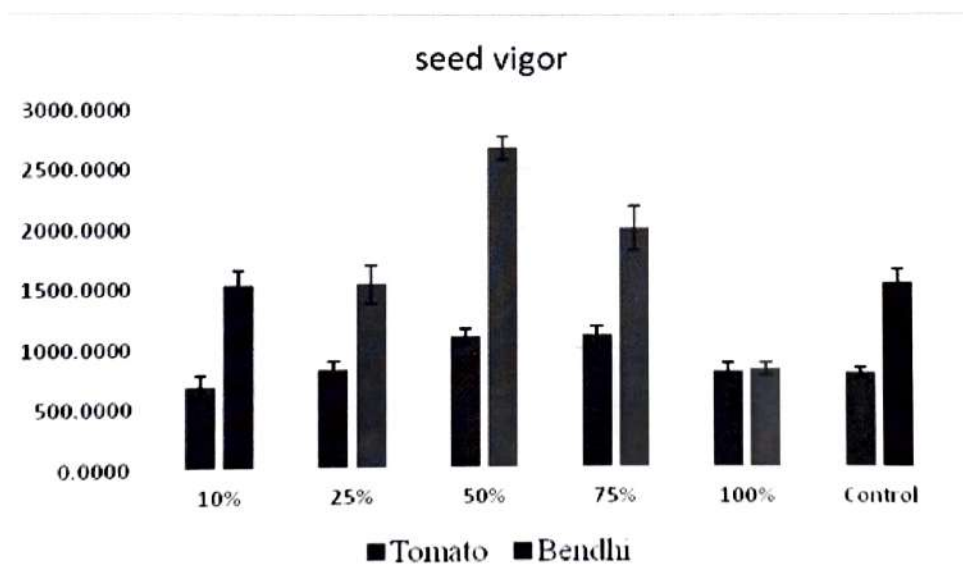


Fig 3. Effect of different concentrations of SLF treatment on the seed vigor of *Solanum lycopersicum* and *Abelmoschus esculentus*

Phytochemical Screening and GC-MS Analysis of *Clausena dentata* (Willd.) Roem

S. Manikandan¹, G. Priya², E. Rajesh³ and T. Sekar^{4*}

^{1,2,3}Post Graduate & Research Department of Botany, Pachaiyappa's College, Chennai – 600030, Tamil Nadu, India

⁴Department of Biotechnology, College of Science and Humanities, SRM Institute of Science & Technology, Ramapuram, Chennai – 600089. Tamil Nadu, India

*Corresponding author: E-mail: tsekar_bot@yahoo.com

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ABSTRACT

The present study is aimed to screen phytochemicals present in *Clausena dentata*. Methanol is used as a solvent system and leaves are considered for this study. Secondary metabolites are chemical compounds responsible for all functions in plants. Leaves of *Clausena dentata* studied for the presence of steroid, terpenoid, flavonoid, coumarins, carbohydrates, saponins, tannins, cardiac glycosides, phenols and alkaloids. The present study showed that steroid, flavonoid, carbohydrates, tannins, cardiac glycosides, phenols and alkaloids were found to be present whereas coumarins and saponins was absent. The present study also deals with GC-MS analysis which results in observation of 36 peaks with different retention time, peak area and peak height. Each peak represents particular chemical compounds present in this studied plant.

Key words: *Clausena dentata*, Methanol, Phytochemicals, Secondary metabolites, GC-MS analysis,

INTRODUCTION

Clausena dentata (Willd.) Roem is a small tree, which belongs to the family Rutaceae. The essential oil and its components of leaf act as mosquito repellent and displayed larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus* larvae (Rajkumar et al., 2010). These plants are aromatic trees, shrubs, and a few herbs and are distributed throughout the humid and temperate regions of the world. The leaf juice is applied to the injured eye in Tamil Nadu (Apparananthamet al., 1986). The stem bark of *Clausena dentata* is used in veterinary medicine for the treatment of wounds and sprains (Chopra et al., 1956). Even though *C. dentata* has a lot of potential medical uses, the study of pharmacological properties is very scarce

(Subba Rao et al., 1976). The enzymatic antioxidants are produced itself in our body, whereas most of non-enzymatic antioxidants are obtained from either natural plants or synthetics which are used for the treatment for various diseases (Lee et al., 2004). A number of plants belong to the family Rutaceae are of medicinally valuable and furnish several drugs and pharmaceutical products.

Phytochemicals are chemical compounds which are used traditionally in various medicinal preparations to cure various ailments of human beings. They are of plants origin and may be primary metabolites or secondary metabolites. Naturally, many plants are found to possess various secondary metabolites, like alkaloids, tannins, glycosides, volatile oils and contain

minerals and vitamins, which possess different therapeutic uses (Wang *et al.*, 2005). Fiorentino *et al.*, (2007) the phytochemical studies have revealed that the leaf extract contains phenolic antioxidant compounds, such as flavonoids, phenolic glycosides, tannins, gallic acid derivatives, anthocyanins. Gas chromatography-mass spectroscopy (GC-MS) is a simultaneously analytical technique used for the identify compounds in traditional medicines and medicinal plants. GC-MS studies in recent years have been increasingly useful for the analysis of medicinal plants as this technique has proved to be an important method for the analysis of non-polar components and volatile essential oil, lipids, fatty acids (Jie *et al.*, 1988) and alkaloids (Betz *et al.*, 1997). Considering the importance of the plant, the present study was undertaken to study its secondary metabolite constitution and GC-MS analysis in leaves of *Clausena dentata*.

MATERIALS AND METHODS

PLANT COLLECTION AND EXTRACTION

The fresh leaves of *Clausena dentata* were collected from Pachaimalai hill, Salem District, Tamil Nadu, India. The healthy leaves were washed thoroughly in tap water and also rinsed using distilled water and then air dried under shade in room temperature for two weeks. The dried leaves were grinded and fine powdered by using electric mixer for extraction. Two fifty grams (250g) of powdered plant materials were soaked in 750ml of methanol and incubated for 48hrs. The soaked material were filtered by whatman filter paper no.1 for the extraction. The filtrate was concentrated using a vacuum rotary evaporator (Superfit-ROTAVAP, India). The percentage yield of extracts was calculated (Rashmi Tambe *et al.*, 2014).

PHYTOCHEMICAL EVALUATION

It is important to screen and analyze bioactive components, not only for the quality control of crude drugs, but also for the elucidation of their therapeutic mechanisms (Harborne 1994). Qualitative phytochemical screening of leaf

extract with methanol was performed as per the protocols stated by Kokate (1996), Trease *et al.*, (1989), Harborne (1998), Sofowora, (1993), Mace *et al.*, (1963). The extract profile established was performing for different phytochemical screening tests for composition of chemical. The extract using standard methods for identification of steroid, terpenoid, flavonoid, coumarins, carbohydrates, saponins, tannins, cardiac glycosides, phenols and alkaloids.

1. Test for sterols

Libermann-burchard Test: To 2ml of plant extract add 1ml of acetic acid. This mixture is cooled and few drops of concentrated sulphuric acid were added. Colour change from violet to blue or bluish green indicates the presence of sterols.

2. Test for carbohydrates

Fehling's Test: Take 1ml of plant extract and add few drops of diluted Hydrochloric acid. Heat the content in the water bath. After few minutes, add 6-7 drops of Fehlings A and B solution. Red colour precipitate obtained which shows the presence of carbohydrates.

3. Test for Cardiac Glycosides

Take 1ml of plant extract add 2ml of glacial acetic acid and 2 drops of 5% ferric chloride solution. Then it was treated with 1ml of Concentrated Sulphuric acid. Formation of Brown ring at the interface indicates the presence of Cardiac Glycosides.

4. Test for Flavonoids

Take 2ml of plant extract add few drops of 10% sodium hydroxide solution. Intense yellow colour formed and it turned to colourless on addition of few drops of concentrated sulphuric acid which indicates the presence of flavonoids.

5. Test for Terpenoids

Take 2ml of plant extract add 2ml of chloroform and 3ml of concentrated sulphuric acid. This form a layer and formation of reddish brown colour indicates the presence of

terpenoids.

6. Test for Tannins

Take 1.0ml of extract 1.0ml of 5% ferric chloride was added. A blue colour indicates condensed tannins green colour indicates the presence of tannins.

7. Test for Alkaloids

To 2ml of leaf extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

8. Test for Coumarins

To 1mL of Leaf extract, 1mL of 10% NaOH was added. Formation of yellow colour indicates presence of coumarins.

9. Test for Phenols

To 1ml of the leaf extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols.

10. Test for Saponins

Foam test: To the 2.0ml of extract 5ml of distilled water is added and shake vigorously for a stable persistent froth.

PREPARATION OF SAMPLE FOR GC-MS ANALYSIS

About 30gm of *Clausena dentata* shade dried plant leaf was soaked in 95% methanol for 12hrs. The extract was then filtered through whattman filter paper No. 1 along with 2 gm sodium sulphate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper was made wet with 95% methanol along with sodium sulphate. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phyto-components in the plant material. 2 μ l of this solution was employed for GC-MS analysis (Merlin et al., 2009).

GAS CHROMATOGRAPHY - MASS SPECTROMETRY ANALYSIS

GC-MS analysis was carried out by using Agilent 7890B, Gas chromatography associated to 5977A Mass Spectrometer. Column HP-5MS fused capillary column (30 x 250 μ m x 0.25 μ m) composed of 5% Phenyl Methyl siloxane. Helium (99.999%) was used as carrier gas at a constant flow of 1ml per min and injection volume of 2 μ l was adopted (split less mode). The injected sample was detected by Turbo mass gold detector with the aid of Turbo mass 5.2 software. During 36 minute GC extraction process, the oven temperature was programmed from of 110°C with an increase of 10°C/min up to 200°C, then 5°C/min up to 280°C (9 minutes hold). The injector temperature was set at 250°C (mass analyzer). Mass spectra were taken at 70eV and fragments from 45-450 Da. The MS detection was completed in 36 minutes. The detection employed the NIST (National Institute of Standards and Technology). Compounds identification: Identification was based on the molecular structure, molecular mass and calculated fragment.

RESULTS AND DISCUSSION PHYTOCHEMICAL ANALYSIS

The phytochemical screening of leaf extract of *Clausena dentata* was carried out to study the presence or absence of various secondary metabolites as shown in Table 1. Methanol was used as a solvent system. The present study showed that steroid, flavonoid, carbohydrates, tannins, cardiac glycosides and phenols were found to be present whereas Coumarins and saponins were absent. According to Pandith et al., (2014), phytochemicals are chemical compounds which have complimentary and overlapping mechanism of action in the body including antioxidant effects modulation of detoxification enzymes, stimulation of the immune system and modulation of hormone metabolism. They are responsible for many activities such as antioxidant, antifungal,

antibacterial, antitumour, antidiabetic etc., reagents for medicinal uses (Krishnarajuet al.,1995).

Table 1. PHYTOCHEMICAL ANALYSIS OF METHANOLIC LEAF EXTRACT OF *CLAUSENA DENTATA*

S.No	Phytochemical Constituents	<i>Clausena dentata</i>
		Leaf
1.	Steroid	+
2.	Terpenoid	+
3.	Flavonoid	+
4.	Coumarins	-
5.	Carbohydrates	+
6.	Saponins	-
7.	Tannin	+
8.	Cardiac Glycosides	+
9.	Phenol	+
10.	Alkaloid	+

GC-MS ANALYSIS

GC-MS analysis of methanolic leaf extract of *Clausena dentata* shows 36 peaks which indicate the presence of 36 major secondary metabolites. The result revealed the presence of 36 different chemical constituents with peak area percentage were 1H-Pyrrole, 1-methyl – (2.31%), Levoglucosenone – (0.09%), 2-t-Butyl-5-propyl-[1,3]dioxolan-4-one – (0.85%), L-Proline, 1-methyl-, methyl ester – (0.21%), cis-Linaloloxide – (0.14%), 2-Furanmethanol, 5-ethenyltetrahydro- $\alpha,\alpha,5$ -trimethyl-, cis – (0.07%), 2,5-Dimethyl-4-hydroxy-3(2H)-furanone – (0.15%), 1-4-Hydroxylysine lactone – (1.72%), Dodecane – (0.56%), Benzaldehyde, 4-(1-methylethyl) – (0.70%), 4-Hydroxy-2-methylpyrrolidine-2-carboxylic acid – (0.77%), 2-Methoxy-4-vinylphenol – (0.57%), Pyrrolidine, 1,1'-methylenebis – (11.60%), Caryophyllene – (0.38%), Piperidine, 1-nitroso – (2.38%), 3',5'-Dimethoxyacetophenone – (0.54%), N-Caryophyllene oxide – (0.14%), N-(Cyclohexyl)succinimide – (0.30%), 4-(6,6-Dimethyl-2-methylenecyclohex-3-enylidene)pentan-2-ol – (0.22%), Tetradecanoic acid – (0.26%), 3,7,11,15-Tetramethyl-2-

hexadecen-1-ol – (0.28%), Hexadecanoic acid, methyl ester – (0.28%), Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester – (0.27%), n-Hexadecanoic acid – (6.81%), 2-Allyl-4-methylphenol – (16.69%), 10,13-Octadecadiynoic acid, methyl ester – (16.69%), 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z) – (0.45%), Phytol – (3.15%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z) – (16.69%), 3,7,7-Trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene – (0.33%), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester – (0.10%), Diisooctyl phthalate – (1.65%), Squalene – (2.15%), 1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E) – (0.18%), Neodiosgenin(3 β ,25S) acetate – (0.24%), Vitamin E – (9.92%)(Table 2). The GC-MS spectrum confirm the presence of 36 components with the retention time 2.69, 4.52, 6.03, 6.93, 7.46, 7.72, 7.89, 8.21, 9.27, 9.49, 10.30, 11.44, 12.13, 12.64, 13.29, 14.70, 14.83, 15.05, 15.82, 16.98, 17.45, 18.36, 18.67, 19.21, 19.60, 20.08, 20.16, 20.32, 20.98, 22.21, 24.00, 24.17, 27.64, 29.76, 33.28 and 36.08, respectively (**Fig. 1**). Hymavathiet *al.*, (2009) carried out a bioassay

guided fractionation and chemical investigation of the stem bark of *Premna tomentosa* and reported the presence of four icetexane diterpenes (icetexatriene-10,11,12,16-tetrol, 8,11,13 icetexatriene-10,11,16-triol, 8,11,13 icetexatriene-7,10,11,16-tetrol and 7,10-epoxy-8,11,13 icetexatriene-11,12,16-triol) and other known compounds such as coniferaldehyde, syringaldehyde, luteol, betulin, and 2-(4-methoxyphenyl)-2-butanone.

Similar types of compounds were identified among the thirty six compounds of this present study. Sermakian *et al.*, (2012) identified 17 compounds with 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- as the major compounds in the methanol extract of leaves of *Cassia italica*. The compounds were found to have anti-inflammatory, cancer preventive, insectifuge, hypocholesterolemic, antihistaminic, nematocidal, hepatoprotective, anticoronary properties

anteczemic, antiacne. GC-MS analysis of ethanol extract of *Caesalpinia sappan* revealed the presence of 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-, n-Hexadecanoic acid, 1,2-Benzenedicarboxylic acid and di-isooctyl ester (Sarumathy *et al.*, 2011).

Rajkumar and Jabanesen, (2010) The leaf essential oil of the *Clausena dentata* revealed presence of 14 major compounds was biofloratriene, sabinene, β -bisabolol and borneol. Parasuraman *et al.*, (2009) Octadecanoic acid and n-Hexadecanoic acid were identified in the leaves of *Cleistanthus collinus*. Praveen kumar *et al.*, (2010) Hexadecanoic acid, Phyto, 9, 12 – Octadecadienoic acid, n-hexadecanoic acid, Squalene and 9, 12, 15-Octadecatrienoic acid was identified in the ethanol leaf extract of *Vitex negundo*.

Table 2. IDENTIFICATION OF COMPOUNDS FROM METHANOLIC LEAF EXTRACT OF *CLAUSENA DENTATA* BY GC-MS ANALYSIS

S.No	RT (min)	Name of Compounds	Molecular Formula	Molecular Weight	Peak Height	Peak Area %
1.	2.69	1H-Pyrrole, 1-methyl	C ₅ H ₇ N	81	7757003	2.31
2.	4.52	Levogluconone	C ₆ H ₆ O ₃	126	912877	0.09
3.	6.03	2-t-Butyl-5-propyl-[1,3]dioxolan-4-one	C ₁₀ H ₁₈ O ₃	186	3607323	0.85
4.	6.93	L-Proline, 1-methyl-, methyl ester	C ₇ H ₁₃ NO ₂	143	3823232	0.21
5.	7.46	cis-Linaloloxide	C ₁₀ H ₁₈ O ₂	170	2779879	0.14
6.	7.72	2-Furanmethanol, 5-ethenyltetrahydro $\alpha,\alpha,5$ -trimethyl-, cis	C ₁₀ H ₁₈ O ₂	170	1698163	0.07
7.	7.89	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	C ₆ H ₈ O ₃	128	1630109	0.15
8.	8.21	1-4-Hydroxylysine lactone	C ₆ H ₁₂ N ₂ O ₂	144	31338046	1.72
9.	9.27	Dodecane	C ₁₂ H ₂₆	170	13485501	0.56
10.	9.49	Benzaldehyde, 4-(1-methylethyl)	C ₁₀ H ₁₂ O	148	25773320	0.70
11.	10.30	4-Hydroxy-2-methylpyrrolidine-2-carboxylic acid	C ₆ H ₁₁ NO ₃	145	12762893	0.77
12.	11.44	2-Methoxy-4-vinylphenol	C ₉ H ₁₀ O ₂	150	11964512	0.57
13.	12.13	Pyrrolidine, 1,1'-methylenebis	C ₉ H ₁₈ N ₂	154	57403450	11.60
14.	12.64	Caryophyllene	C ₁₅ H ₂₄	204	15464808	0.38

15.	13.29	Piperidine, 1-nitroso	C ₅ H ₁₀ N ₂ O	114	20357370	2.38
16.	14.70	3',5'-Dimethoxyacetophenone	C ₁₀ H ₁₂ O ₃	180	14121173	0.54
17.	14.83	Caryophyllene oxide	C ₁₅ H ₂₄ O	220	6976303	0.14
18.	15.05	N-(Cyclohexyl)succinimide	C ₁₀ H ₁₅ NO ₂	181	10490545	0.30
19.	15.82	4-(6,6-Dimethyl-2-methylenecyclohex-3-enylidene)pentan-2-ol	C ₁₄ H ₂₂ O	206	7974338	0.22
20.	16.98	Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	228	8415588	0.26
21.	17.45	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	296	15443732	0.28
22.	18.36	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270	15953448	0.28
23.	18.67	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	C ₁₈ H ₂₈ O ₃	292	7516696	0.27
24.	19.21	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	124675075	6.81
25.	19.60	2-Allyl-4-methylphenol	C ₁₀ H ₁₂ O	148	380069483	16.69
26.	20.08	10,13-Octadecadiynoic acid, methyl ester	C ₁₉ H ₃₀ O ₂	290	376215835	16.69
27.	20.16	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	C ₁₉ H ₃₂ O ₂	292	31001337	0.45
28.	20.32	Phytol	C ₂₀ H ₄₀ O	296	184997280	3.15
29.	20.98	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	C ₁₈ H ₃₀ O ₂	278	274028700	16.69
30.	22.21	3,7,7-Trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene	C ₁₄ H ₂₀ O	204	16350664	0.33
31.	24.00	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄	330	3204032	0.10
32.	24.17	Diisooctyl phthalate	C ₂₄ H ₃₈ O ₄	390	75581899	1.65
33.	27.64	Squalene	C ₃₀ H ₅₀	410	62444741	2.15
34.	29.76	1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E)-	C ₂₀ H ₃₄ O	290	4199517	0.18
35.	33.28	Neodiosgenin(3 β ,25S) acetate	C ₂₉ H ₄₄ O ₄	456	4130301	0.24
36.	36.08	Vitamin E	C ₂₉ H ₅₀ O ₂	430	88934792	9.92

CONCLUSION

In the present study, methanolic leaves extract of *C. dentata* showed more number of secondary metabolites such as steroid, flavonoid, carbohydrates, tannins, cardiac glycosides and phenols were present. Coumarins and saponins were found to be absent. Gas chromatography and mass spectroscopy analysis showed the existence of 36 phytochemical constituents which contribute the activities like anti-inflammatory, anticancer, hypercholesterolemia and other activities. GC-MS report can be used as drug detection and biochemical markers in the pharmaceutical industries to recognize the authentic plants and separate from its adulterants. Further study is required for possible development of novel drugs using some of the bioactive compounds found in *C. dentata*.

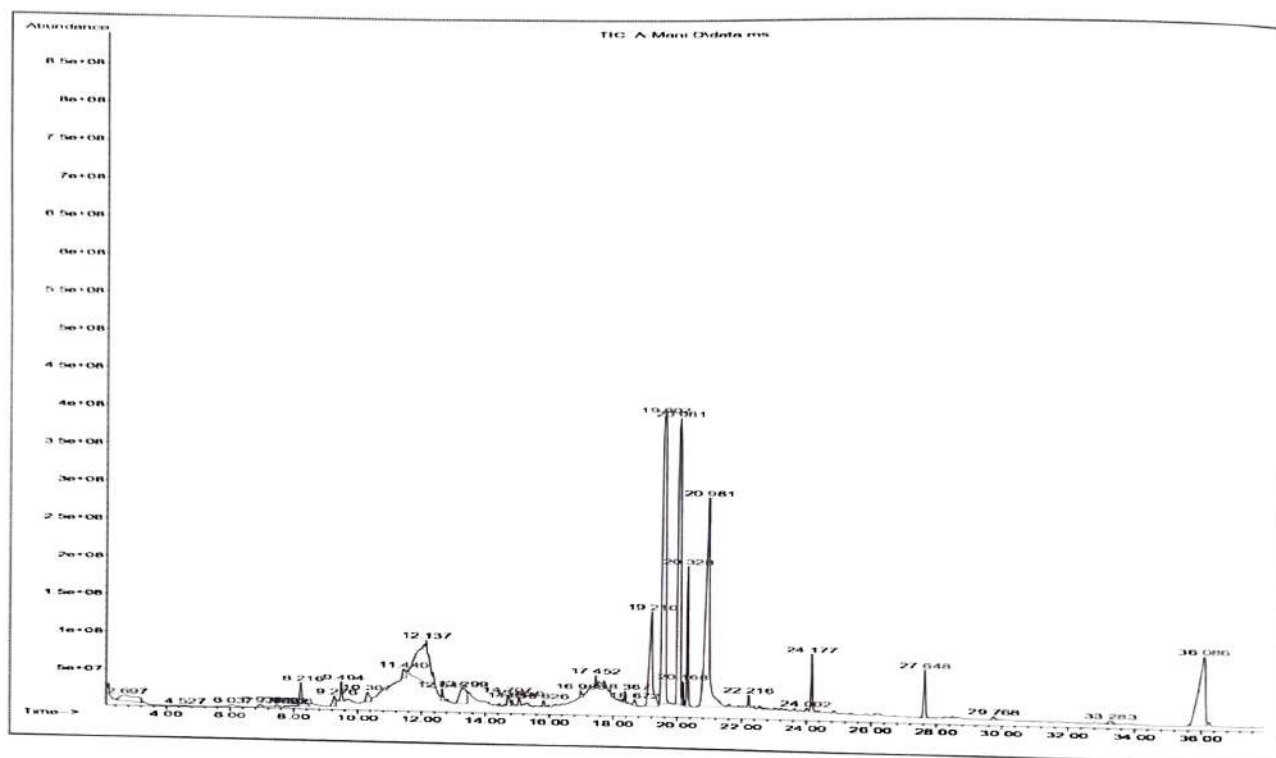
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Fig 1. CHROMATOGRAM SHOWING RETENTION TIME OF COMPOUNDS FOUND IN LEAVES OF *CLAUSENA DENTATA*



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