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Criterion 3
Research, Innovations and Extension

3.3.1 Number of research papers published per teacher in the Journals as notified on UGC CARE list during the last five years.

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Data Authentication Declaration

With this declaration, we hereby declare that the information provided in order to obtain NAAC accreditation in this SSR is true to the best of our knowledge. This information is a genuine reflection of our institutional policies and accomplishments and shows our continued dedication to raising the standard of higher education.

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3.3.1 Number of research papers published per teacher in the Journals notified on UGC website during the last five years (2018-19, 2019-20, 2020-21, 2021-22,2022-23)

Sr. No.	Title of paper	Name of the author/s	Department of the teacher	Name of journal	Year of publication	ISSN number	Link to the recognition in UGC enlistment of the Journal /Digital Object		
							Link to website of the Journal	Link to article / paper / abstract of the article	Is it listed in UGC Care list
1	Diversity of Haloalkaliphiles from Hypersaline environment- A Review	Makarand N. Cherekar and Anupama P. Pathak	Biotechnology & Bioinfomatics	Research & Reviews in Biotechnology & Biosciences	2023	2321-8681	https://www.biotechjournal.in/	https://www.biotechjournal.in/images/paper_pdffiles/Div-64e5a9be81652.pdf	UGC Approval No: 63959
2	Cloud Computing - An insight to latest trends and Developments	Rajesh K Sadavarte, Dr. G. D. Kurundkar, Dr. Smita A. Bhopi	Computer Science and IT	International Journal of Scientific Research in Computer Science, Engineering and Information Technology	2022	2456-3307	https://ijsrcseit.com/	https://ijsrcseit.com/PDF.php?pid=CSEIT228227&v=8&i=3&y=2022&m=May-June	UGC Journal No : 64718
3	Deep Learning Convolution Neural Network for Tomato Leaves Disease Detection by Inception	Ms. Swati Waddare Dr. Mr. H. S. Fadewar	Computer Science and IT	The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2022 B. Iyer et al. (Eds.): ICCET 2022, SIST 303, pp. 208–220, 2022.	2022	SIST 303	://doi.org/10.1007/978-981-19-2719-	tps://doi.org/10.1007/978-981-19-2719-5	Yes
4	Computer Vision for Leaf Disease Detection: A Review	Ms. Swati Waddare Dr. Mangesh N. Kothari Dr. H. S. Fadewar	Computer Science and IT	AJOMC Vol. 7 No. 1 (January - March Special Issue - III 2022) updated 25.4.22	2022	Volume 7, Number 1 January-March 2022 ISSN: 2456-8937	https://ajomc.asianpubs.org	https://ajomc.asianpubs.org	UGC CARE APPROVED JOURNAL
5	Extraction And Separation Of Lycopene From Natural Sources Using Chromatographic Technique And Its Application	Abhishek daga, Trupti Tope, Vikas Shukre, Ganesh Kore, Komal Kadam	Biotechnology & Bioinfomatics	International Journal of Creative Research Thoughts (IJCRT)	2021	2320-2882	www.ijcrt.org	ttps://ijcrt.org/papers/IJCRT2104103.pd	ISSN: 2320-2882
6	Extraction and Purification of Curcumin from Turmeric	Makarand N Cherekar Naresh D Joshi and Aditya A Kulkarni	Biotechnology & Bioinfomatics	Asian Journal of Plant Science and Research	2021	ISSN : 2249-7412	https://www.imedpub.com/articles-pdfs/extraction-and-purification-of-curcumin-from-turmeric.pdf	https://www.imedpub.com/articles-pdfs/extraction-and-purification-of-curcumin-from-turmeric.pdf	Scopus Peer Reviewed
7	Production and characterization of a haloalkaline pectinase from Halomonas pantellerinsis strain SSL8 isolated from Sambhar lake, Rajasthan	Makarand N. Cherekar and Anupama P. Pathak	Biotechnology & Bioinfomatics	Current trends in Biotechnology and Pharmacy	2020	Vol. 14 (3) 319-326, July 2020, ISSN 0973-8916	tps://www.abap.co.in/index.php/hon	http://abap.co.in/sites/default/files/CTBP-14-3-319.pdf	Yes
8	Production, Extraction And Uses Of Eco-Enzyme Using Citrus Fruit Waste: Wealth From Waste	Makarand N. Cherekar, Vama Lapsia	Biotechnology & Bioinfomatics	Asian Jr. of Microbiol. Biotech. Env. Sci	2020	ISSN-0972-3005	http://www.envirobiotechjournals.com/journal_details.php?jid=1	http://www.envirobiotechjournals.com/AJMBES/v22i220/AJM-18.pdf	Yes
9	Production, extraction & uses of eco Enzymes using citrus fruit waste - wealth from waste	Vama Lapsia, Makarand N. Cherekar	Biotechnology & Bioinfomatics	Asian Journal of Microbiology, Biotechnology & Environmental Sciences	2020	ISSN: 0972-3005	http://www.envirobiotechjournals.com/journal_details.php?jid=1	http://www.envirobiotechjournals.com/AJMBES/v22i220/AJM-18.pdf	Yes
10	Extraction of Curcumin from Turmeric by using soxhalet unit.	Joshi Naresh Dilip, Kulkarni Aditya Arvindrao, Dr.Cherekar M. N	Biotechnology & Bioinfomatics	ASIO Journal of Microbiology, Food Science & Biotechnological Innovations (ASIO-JMFSBI)	2019	ISSN: 2455-3751	https://www.albertscience.com/home/indexing_of_asio_journals	https://albertscience.com/asset/images/uploads/15701202031157.pdf	Scopus Peer Reviewed
11	Production of Levan, A Potential Low Calorie Sweetener From Low Cost Substrates	Neeta Laddha, Manjusha Chitanand	Biotechnology & Bioinfomatics	Research and Reviews: A journal of Microbiology and Vrology	2019	ISSN:2230-9853	https://medicaljournals.stmjournals.in/index.php/RRJoMV/search/authors/view	https://medicaljournals.stmjournals.in/index.php/RRJoMV/search/authors/view	Yes

Sr. No.	Title of paper	Name of the author/s	Department of the teacher	Name of journal	Year of publica	ISSN number	Link to the recognition in UGC enlistment of the Journal /Digital Object Identifier (doi)		
							Link to website of the Journal	Link to article / paper / abstract of the article	Is it listed in UGC Care list
12	Thermostable pectinase mediated enhanced antimicrobial activity of Emblica Officinalis: A novel application	Mayuri S. Sarsar & Anupama P. Pathak*	Biotechnology & Bioinfomatics	Indian Journal of Geo Marine Sciences	2019	ISSN: 2582-6727 (Online) ISSN: 2582-6506 (Print)	ttp://op.niscair.res.in/index.php/IJM	https://nopr.niscpr.res.in/bitstream/123456789/50469/3/IJMS%2048%289%29%201404-1410.pdf	Yes
13	A Comparative Study of Segmentation Techniques used in Handwritten Documents	Ms. S. A. Bhopi Mr. M. P. Singh	Computer Science and IT	IJFRCSCE Board International Journal of Computer Sciences and Engineering A Unit of Auricle Technologies Pvt. Ltd.	2018	ISSN: 2347-2693	https://www.ijcseonline.org/index.php	https://www.ijcseonline.org/pdf_paper_view.php?paper_id=1869&32-IJCSE-03288.pdf	E-ISSN: 2347-2693
14	Performance analysis of Handwritten Devnagari Character Recognition using Feed Forward , Radial Basis , Elman Back Propagation, and Pattern Recognition Neural Network Model Using Different Feature Extraction Methods .	Ms. S. A. Bhopi Mr. M. P. Singh	Computer Science and IT	IJFRCSCE Board International Journal of Computer Sciences and Engineering A Unit of Auricle Technologies Pvt. Ltd.	2018	ISSN: 2454-4248V	http://www.ijfrcsce.org	http://www.ijfrcsce.org/download/browse/Volume_4/May_18_Volume_4_Issue_5/1528093715_04-06-2018.pdf	ISSN: 2454-4248
15	Feature Extraction Techniques for Marathi Character Classification using Neural Networks Models	Ms. S. A. Bhopi Mr. M. P. Singh	Computer Science and IT	IJFRCSCE Board International Journal of Computer Sciences and Engineering A Unit of Auricle Technologies Pvt. Ltd.	2018	ISSN: 2454-4248V	http://www.iifrcsce.org/	http://www.ijfrcsce.org/download/browse/Volume_4/June_18_Volume_4_Issue_6/1530522616_02-07-2018.pdf	ISSN: 2454-4248
16	Review on Optical Character Recognition of Devanagari Script Using Neural Network	Ms. S. A. Bhopi Mr. M. P. Singh	Computer Science and IT	IJFRCSCE Board International Journal of Computer Sciences and Engineering A Unit of Auricle Technologies Pvt. Ltd.	2018	ISSN: 2454-4249V	http://www.iifrcsce.org/	http://www.ijfrcsce.org/index.php/ijfrcsce/article/view/1333	SSN: 2454-4248

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Diversity of Haloalkaliphiles from Hypersaline environment- A Review

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Abstract:

Haloalkaliphilic bacteria are organisms which thrive in both high salt concentration and high pH habitats, such as soda lakes, soda desserts and saline and alkaline environments. These microorganisms are distributed among diverse groups in the domains bacteria, archaea and eukaryotes. Special mechanisms for adapt to both high pH and high salt concentration make the haloalkaliphiles more attractive for basic type of research and for applications in industrial biotechnology viz the production of valuable enzymes, pigments, compatible solutes, biodegradation and exo-polysaccharides. The aim of this review is to provide diversity of these organisms from such various extreme environments those are distributed worldwide to provide database for discussion to current and future biotechnological and environmental aspects.

Keywards: Diversity, extreme environments, haloalkaliphiles, soda lake, industrial biotechnology

Introduction:

Extreme environments are widely distributed across the world which is exposed to one or more environmental parameters like temperature, salinity, pH, osmolarity or pressure which showing values close to the limit of life. The organisms thrive in such habitat known as extremophiles; in addition, these are normally polyextremophiles which have ability to tolerate two or more extreme conditions, like haloalkaliphiles, halothermophiles and alkalithermophiles. Haloalkaliphiles are organisms that require high salinity (3-30%) and an alkaline pH (pH 9-13) for their growth. Haloalkaliphiles have been reported from a number of environments such as saline-soda lakes, hypersaline alkaline soils, salt mines, marine environments, brine water and salt

marshes (1,2). Haloalkaliphiles usually use small organic molecules like ectoine,

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betaine, proline and intracellular enzymes required maintaining their osmotic balance and pH ranges to survive under extreme saline and alkaline environments (3).

Haloalkaliphiles have a wide range of industrial applications in detergent industry, food stuffs, paper and pulp and pharmaceuticals industry also used in production of pigments, compatible solutes, biofuel, biodegradation and exo-polysaccharides (4,5). The isolation, characterizations, diversity and application of haloalkaliphiles from different environments has been studied by various groups so the aim of this review is to offer a short, but comprehensive report on the diversity of important haloalkaliphilic microbes thriving in extreme environments around the globe.

Historical perspective of Haloalkaliphiles

The term Haloalkaliphile was first used by Soliman and Truper to describe a dual extremophile that possess halophilic and alkaliphilic attributes (6), until the only known examples of such organism belong to the Kingdom Euryarchaeota (7). After Soliman and Truper (1982) Koki Horikoshi also studied the Haloalkaliphiles in detail and described as alkaliphiles consist of two physiological groups of microorganisms, alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 9 or more for their growth, whereas haloalkaliphiles the group of bacteria able to grow under alkaline conditions in the presence of salt require both an alkaline pH (>pH 9) and high salinity (up to 33 % (w/v) NaCl) (2,5).

Haloalkaliphiles are found in both natural and artificial environments in nature, they possess special adaptation mechanisms for survival in highly saline and alkaline pH in such extreme environments which include production of high density branched chains lipids and increased content of cell wall components along with Na+/H+ antiporters as internal pH homeostasis (5).

Studies on ecology, physiology, and taxonomy of haloalkaliphiles revealed an impressive microbial diversity in many saline and alkaline lakes from world. These dual properties made haloalkaliphilic organisms interesting for, basic research and for industrial applications in biotechnology (4,8). Haloalkaliphilic organisms are widely distributed along the phylogenetic tree of life forms, and significant diversity of these microorganisms from major taxonomic groups was reported by various research groups.

Haloalkaliphilic microorganisms have largely been identified and studied from worldwide various locations like Soda Lake, soda desserts, hypersaline and alkaline environments, carbonate springs, salt brines, alkaline soils, Dead Sea, saltern crystallizer ponds and places saturated with respect to sodium chloride. So far, large numbers of hyper saline alkaline environments have been studied to know their chemical composition and ecological diversity of theses extremophiles (9).

The large number of haloalkaliphilic bacterial and archaeal strains depicted wide diversity, as reflected through microbiological, biochemical and molecular approaches (10-13).

During the past years special attention has been focused on the distribution of haloalkaliphiles and their diversity (14,15). Culture dependent and independent methods are usually used to investigate

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biochemical characters, genetic and phylogenetic diversity of haloalkaliphiles from different extreme environments (16). Recently characterization and diversity study from hypersaline lakes of northern Egypt has been carried out by Arayes and their coworker's, they reported massive culturable aerobic haloalkaliphilic diversity from Marsa-Matrouh lake and Al-Hamra lake (17).

In the past few decades soda lakes environments has been studied in detail for existence of extremophiles by various research group. Initially haloalkaliphiles were reported as soda lake inhabitants, a large number of haloalkaliphilic bacteria and archaea were obtained from such environments. Early ecological studies related to haloalkaliphilic microorganisms were reported from the alkaline hypersaline lakes such as Wadi Natrun and Lake Magadii from Kenya (10).

Banda and their research group also studied soda lake Microbial Community with the Effects of Salinity and pH on Diversity and Distribution Pattern in the Brines of soda lake from Badain Jaran Desert, China. Banda and group found significant difference in microbial communities inhabiting the different alkali-saline lakes, both pH and salinity shaped the haloalkaliphilic community in such habitat (18). Sorokin and their research group studied extensively biogeochemical cycling occur in soda lakes and they reported diverse and novel group of haloalkaliphiles from such extreme habitat (19).

One of the noticeable features of such habitats/ lakes is their colour. The water colour of lake may appear from green to red due to the massive blooms of microorganisms (20, 21).

Diversity of Haloalkaliphiles

Oxygenic and anoxygenic phototrophs and heterotrophs

Jannasch (1957) was the first to report about the red colour development in such lakes in which the salt concentration exceeding 200g-1 to mass development of photosynthetic purple bacteria also these bacteria are capable for the sulfate reduction in the bottom sediments and oxidation of the sulfide. He reported *Chromatium* and *Thiospirillum* purple bacteria from brine samples of lake. These lakes are most productive in the world for such organisms (20).

The photosynthetic productivity was mostly due to the dense populations of cyanobacteria. *Spirulina* sp. is usually most dominating amongst such cyanobacteria blooms. Earlier Imhoff and his co-workers carried out detailed study of lake Gabara (91.9g-1 salt) in 1978 and 1979, which is inhabited by *Spirulina* and other cyanobacteria spp. (22, 23).

These cyanobacteria are the principle food of the vast flocks of Lesser Flamingo that inhabit the soda lakes. Other than *Spirulina* few additional cyanobacteria are *Ectothiorhodospira, Halorhodospira and Thiorhodospira* also relate to the primary productivity and these members are acting as a link between the S- and C- cycle of soda lakes (14).

Imhoff and Truper have reported *Ectothiorhodospira halochloris* from the Wadi Natrun Egypt, which appears in green and brown color due to bacteriochlorophyll b production, optimal salinity of lake was reported 14-27% and pH 8.1 to 9.1 (24). Other members of same genus were reported worldwide by different research

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groups which mainly includes (*Ectothiorhodospira vacoulata* reported by) Imhoff et al., in 1981; Kompantseva et al., in 2005; and Upasani, in 2008 (24-26). Additionally red algae like *Dunaliella salina*, *Arthrospira platensis*, *Synechococcus cedrorum*, *Anabaena* sp. are reported from Indian soda lakes (27).

Synechocystis salina, Aphanothece stagnina, Chamaesiphon subglobosus, Rhabdoderma lineare, Synechococcus elongatus, Spirulina fusiformis, Phormidium ambiguum, Phormidium retzii, Oscillatoria splendida, Phormidium foveolarum and *Oscillatoria limnetica* species, all these benthic cyanobacteria were reported by Dubinin and his co workers from Lake Magadi (28).

Other than cyanobacterial population existence of wide aerobic and anaerobic bacteria were reported from extreme hypersaline alkaline environments worldwide by research groups like Duckworth et al. 1996, Imhoff et al., 1979, Woese et al., 1985, Grant et al., 1990, Zavarzin et al., 1999; Mesbah et al., 2007, Sorokin et al., 2002 (10, 23, 29-33).

Aerobic haloalkaliphilic bacteria

Obvious succession of microbial community in hypersaline ecosystem was reveled by observing existence of aerobic bacteria like *Bacillus*, *Halomonas* sp. and anaerobic hydrolytic bacteria *Clostridia* by various research groups. These organisms were reported as consumers of polymers synthesized by cyanobacteria (14, 23).

Grant and colleagues has been done the survey regarding correlation between the aerobic heterotrophic bacteria to cyanobacteria in which bacterial numbers were constant and the dominant types were varied, such type of survey was performed on selective media (30).

Extremely diverse group of aerobic, heterotrophic and organotrophics alkaliphilic and haloalkaliphilic organisms were reported by Duckworth et al. from East African Rift Valley (10), besides comprehensive study made by him and his group to generate a report on Gram-positive bacteria of both the high G+C (Firmicutes) and low G+C (Actinobacteria) lineages from such environments (10). The Gram-positive and Gram negative bacteria reported by Duckworth and his coworkers in this study belonged to *Bacillus* genus and gamma proteobacteria respectively. Similar results were obtained world wide using such types of habitats (29, 34, 35).

It was recorded that, *Bacillus* genus is dominating belonging to phylum Firmicutes and commonly present in such haloalkaline environment.

Haloalkaliphilic *Bacillus* species are the group specifically adapted to grow optimally under moderate halophilic and alkaline conditions, apart from *Haloarchaea*, *Bacillus* species are highly found in such habitat, particularly in saturation ponds, which have 15–25 % salinity. *Bacillus* is the dominant bacterial species in microbial fermentations and important participant in production of various valuble industrial products. Syed Shameer has describd bioprospecting study of haloalkaliphilic *Bacillus* species from solar salterns (36).

The novel haloalkaliphilic species from *Bacilli* taxon were isolated and studied by various research groups. Some of the *Bacillus* members were isolated and grouped into new genera: *Alicyclobacillus, Virgibacillus*,

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Brevibacillus, Aneurinibacillus, Halobacillus, Gracilibacillus and Paenibacillus (37-38).

Similar report showed recently by Arayes and their coworker's, the massive culturable aerobic haloalkaliphilic diversity from Marsa-Matrouh lake and Al-Hamra lakes of northern Egypt, they showed haloalkaliphilic organisms diversity belonging to phylum Firmicutes and the genera like *Alkalibacillus*, *Salinicoccus*, *Gracilibacillus, Thalassobacillus, Halobacillus, Staphylococcus* and *Bacillus* (17).

Large evidence suggests that diverse haloalkaliphilic *Bacillus* species were isolated from various haloalkaline environments from worldwide (34, 36). Recently from Indian soda lakes haloalkaliphilic *Bacilli* diversity was explored by many research groups Tambekar and Dhundale reported the phenotypic analysis of *B. flexus*, *B.cellulosilyticus*, *B. pseudofirmus*, *B. clausii*, *B. krulwichiae*, *B. pumilus*, *B. lehensis*, *B. halodurans*, *B. circulans*, *B. cereus*, *B. agaradhaerens*, *B. sphaericus*, *B. fusiformis*, *B. asahii*, *B.pseudalcalophilus*, *B. okuhidensis*, and *B. gibsonii* (39).

Next to the Gram-positive the majority of Gram negative isolates cultivated belonged to gammaproteobacteria (29). One of the dominant genus Halomonads in gammaproteobacteria was reported from by various groups along with Indian Sambhar and Lonar soda lake (12, 40, 41). East African soda lakes (42), Hypersaline environments of China (43), solar salterns at Tacan in Korea (44) salt lake of Ras Muhammad (45).

These haloalkaliphilic halomonads have attracted researchers due to their special ability of denitrification and oxidation of thiosulfate and sulfide and they are reported by Sorokin et al. (46). Besides gammaproteobacteria the presence of other proteobacteria related to *Pseudomonas spp.*, *Aeromonas spp.* and *vibrio* were also shown by Duckworth and their group (10).

These all lineages were later on reported world wide from different haloalkaline ecosystems such as lake Elmenteita from Kenyan Rift Valley (47), Chaka lake, China (48), soda lakes from Kenyan-Tanzanian Rift valley (49), Former soda Texcoco lake, Mexico (50), Hypersaline Mono lake, California (51, 52).

Methylotrophic haloalkaliphilic bacteria

Soda lake habitats were extensively studied for various haloalkaliphiles, from such environments the methane, methanol and methylamine utilizing organisms also reported by different groups.

Haloalkaliphilic methylotroph *Methylophaga lonarensis* bacterium was isolated from the Lonar soda lake sediments (53) and novel obligately methylotrophic, methane-oxidizing *Methylomicrobium* species was isolatd by Sorokin in 2000 from a highly alkaline environment (54).

These research groups studied such soda lakes of widely different geographical sites and they isolated and reported new haloalkaliphilic species, from the soda lake like Wadi al Natrun (32), Few examples of new haloalkaliphilic species like *Halomonas mongoliensis sp.* nov. and *Halomonas kenyensis sp.* nov., new haloalkaliphilic denitrifiers capable of reducing N_2O reported by Boltianskaia et al. (55), *Alkalilimnicola ehrlichii sp.* nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of

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chemoautotrophic or heterotrophic growth with nitrate or oxygen as the electron acceptor showed by Hoeft and their research group (56), *Salinivibrio sharmensis* a novel haloalkaliphilic bacterium was reported from a saline lake in Ras Mohammed Park (Egypt) (57) and Namsaraev and his group were reported haloalkaliphilic, *Marinospirillum celere* bacterium from Mono Lake (58).

Anaerobic haloalkaliphilic bacteria

Existence of anaerobic haloalkaliphilic bacteria was reported in 1990 however systematic detailed studies were carried out 1996 onwards, Jones and his coworkers were obtained such organisms from extreme hypersaline habitats like Lake Magadi and placed them as a new genus of obligately anaerobic haloalkaliphiles using phylogenetic analysis (20) and in 1999 Zhilina, Zavarzin and his coworkers have reported soda lake anaerobes from a variety of lakes in the former USSR (31, 59).

Considerable diversity of anaerobic haloalkaliphilic organisms was shown from worldwide. The diversity of anaerobic organisms from Egyptian soda lakes of the Wadi al Natrun was shown by Mesbah and colleagues (32). Haloalkaliphilic anaerobic organism like *Alkalilimnicola ehrlichii* was obtained from Mono Lake California, USA by Hoeft et al. in 2007 (56)

Besides soda lakes various types of Tindallia *texcoconensis*, was reported from Texcoco, Mexico Soda lake (60), S*ulfidogens* and *Desulfitispora alkaliphila* species were reported by Sorokin and Muyzer (61).

Besides soda lake various types of haloalkaliphilic anaerobic bacteria were reported from other sources by many research groups like *Halonatronum saccharophilum* bacterium was reported by Zhilina et al. from the coastal lagoon mud of the Lake Magadi (Kenya) (59).

The occurrence of haloalkaliphilic acetogenic bacteria were observed in bottom mud of the Lake Magadi, Kenya, *Natroniella acetigena*, *Thermosyntropha lipolytica, Tindallia californiensis*, *Tindallia magadiensis* were reported from such soda lake environments like Mono Lake (California) by Pikuta (62, 63). Recently *Proteinivorax tanatarense*, was isolated from a decaying algal bloom (64).

Besides soda lakes and soda deserts few natural and artificial ecosystems have been investigated and showed presence of various types of haloalkaliphilic bacteria. *Halomonas campisalis*, denitrifying bacterium was reported from the salt plain of Alkali Lake in Washington State (USA) (65). Arylaliphatic nitriles utilizing haloalkaliphilic *Halomonas nitrilicus* was isolated from soda soils by Chmura and their colleague in 2008 (66).

Recently haloalkaliphilic representatives of nitrifying, sulfur-oxidizing, H_2-oxidizing, carboxydotrophic and fermentative bacteria have recently been isolated from soda lakes and characterized by Sorokin et al., Two novel fermentative anaerobic haloalkaliphiles (*Natranaerovirga pectinivora* and *Natranaerovirga hydrolytica*) from soda lakes can use pectin as substrate either at moderate (*Natronoflexus pectinovorans* from the Bacteriodetes) or high salt concentration (*Natronovirga* from the Clostridiales) showed by Sorokin and his research group (67, 68).

Haloalkaline enzymes producing haloalkaliphiles

Haloalkaliphiles are useful in biotechnology as sources of novel enzymes and proteins, they have developed diverse biochemical,

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structural and physiological modifications which allowing the catalytic synthesis of proteins with interesting physicochemical and structural properties. The haloalkaline enzymes that allows them to be considered as a novel alternative for use in the biotechnological industries because their polyextremophilicity, i.e. they have the capacity to be tolerate a wide range of pH and tolerate high salt concentrations.

Most of the haloalkaliphiles are exhibited protease, lipase, cellulase, amylase, gelatinase, xylanase and catalase activities which reported by many research groups among this protease producing haloalkaliphiles are dominating once. (4, 13, 16, 26, 36, 40, 41, 64).

A few industrial applications of these enzymes have been reported however it is important to investigate applications of this haloalkaline enzymes in more biotechnological processes. Few enzyme producers are Haloalkaliphilic *Nesterenkonia spp*. was reported from various research groups, like Govender and his coworkers in 2009 and Nel and thir colleague in 2011, from Antarctic desert soil and Sun salt pan of Botswana respectively (69, 70).

Other than enzyme producing ability various metabolically active and other important bioactive compounds like compatible solutes, pigments and exo-polysaccharides producing haloalkaliphiles are reported worldwide, few examples of haloalkaliphilic bacteria and archaea producing these industrially important product are listed in Table 1 (71-78).

Also few members of metabolically versatile haloalkaliphiles from halophilic habitats was reported, such as *Nitriliruptor alkaliphilus* and methylotrophic *Methylophaga* sulfidovorans (79-80).

Sulfate-reducing and Sulfur-oxidizing haloalkaliphiles

In recent year's development of various molecular tools for identification have resulted in systematic and detailed investigation of haloalkaline ecosystems. These reports mainly include data based on 16S rRNA identification method, FAME analysis, with these tools scientist have reported diverse haloalkaliphiles and grouped them into different groups.

Sulfate-reducing bacteria is one of the major group from Soda lake habitat which explore and studied by Sorokin and his colleague and they reported novel *Desulfonatronum thioautotrophicum, D. thiosulfatophilum* and *D. magnus*, *Desulfonatronovibrio thiodismutans* and *Desulfononatronospira thiodismutan* and *Desulfonatronobacter acidivorans* and *Desulfobulbus alkaliphilus* from sediments of soda lakes in Kulunda Steppe (Altai, Russia) (46, 81). *Desulfonatronovibrio hydrogenovorans, Desulfonatronum cooperatum* were reported by Zhilina et al and *Desulfonatronum lacustre* and *Desulfonatronum thiodismutans* were showed by Pikuta et al. in 2003 (82,83).

Sulfur-oxidizing *Thioalkalimicrobium cyclicum* and *Thioalkalivibrio jannaschii* were isolated from Mono Lake (California) and *Thioalkalivibrio versutus* was reported from Kenyan Soda lake (33,84). The genera *Thioalkalimicrobium* and *Thioalkalivibrio* were reported from various lakes of the Kenyan Rift Valley (Bogoria, Crater lake Sonachi, Elmenteita, Nakuru and Magadi) and the low-saline Siberian soda lakes (Hadyn, Tsaidam, Low Mukei) (33).

Upasani also showed haloalkaliphilic anoxygenic phototrophic Sulfur-oxidizing

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bacteria in India from Sambhar Soda Lake (27). Saline soda lakes of the Central Asia (*Rhodovulum steppense*) and brackish steppe soda lakes of southern Siberia (*Rhodovulum tesquicola*) also a suitable environment for growth of haloalkaliphilic purple nonsulfur bacteria (85,86).

These all few representative members of diverse and important aerobic and anaerobic, methanogenic, haloalkaline enzyme producing, sulfate-reducing and sulfur-oxidizing haloalkaliphiles from soda lake and extreme habitats rather than these organisms, haloarchaea a distinct group also found in such environment.

Haloalkaliphilic Archaea

The haloalkaliphilic archaea is a distinct physiological group due to their obligate alkaliphily. These extremely halophilic, aerobic archaea placed in the order Halobacteriales, family Halobacteriaceae, and class Haloarchaea that require least 1.5M NaCl for growth (87).

Assessment of a various alkaline and hyper saline lakes from different geological locations of the world, indicated that haloalkaliphilic archaea of the family Halobacteriaceae (so called "halobacteria"), are found in all such lakes and environments (88). Halobacteria are the most dominant microbial population found when hypersaline waters come up to saturation, frequently importing a red coloration to the brines because of C_{50} carotenoids (89).

Currently the classification of this family is mainly based on three taxonomical characters those are 16S rRNA gene sequence, polar lipid composition, and DNA–DNA hybridization (90). Initially Haloarchaea are classified solely on morphological and biochemical criteria, this group initially consists of only two original genera, *Halobacterium* and *Halococcus*, subsequently expanded to six genera (91) but further aerobic, extremely haloarchaea are classified into 28 different genera.

Haloalkaliphilic haloarchaea were assigned firstly in to the genera *Natronobacterium* and *Natrononococcus* described by Tindall et al., followed a study of Lake Magadi in Kenya, after earlier reports of red halophiles at Kenyan and Egyptian alkaline hypersaline sites (6, 92, 93).

Such saline soda lakes support blooms of halobacteria and harbour alkaliphilic representatives of the genera *Natronobacterium* and *Natrononococcus, Natronomonas, Natrialba, Natronorubrum* and *Halorubrum*. Functionally, they have a specific trophic position and flourish on the organic matter concentration arising from evaporation of brine and the death of its microbial population (31). Haloalkaliphilic strains that require high pH, high salt and these organisms also exhibited very low requirements for Mg^{2+}. Several of the haloarchaeal genera referred to earlier exclusively harbor haloalkaliphilic types (often genera with Natrono pre- fixes) (93).

Haloalkaliphilic archaea have been reported from hypersaline alkaline habitats such as soda lakes, soda desserts and soda soils at many different geographical sites by various research groups, for example, Lake Magadi in Kenya (10, 94, 95), the Wadi Natrun in Egypt (6, 96), Owens Lake in California, soda lakes in China, Inner Mongolia, and Tibet (97-102) and from soda lakes of India (27,103).

Novel archaeabacterial diverse strains were isolated and reported by many groups from

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worldwide. *Natronolimnobius baerhuensis* and *Natronolimnobius innermongolicus* and *Natrialba hulunbeirensis* and *Natrialba chahannaoensis* novel haloalkaliphilic archaea were isolated from soda lakes of Inner Mongolia, China (98,101).

Novel extremely haloalkaliphilic members form hypersaline alkaline lakes from Central Asia, Egypt and USA reported by Sorokin et al. Phenotypic and physiological investigation showed clear growth pattern of these members on various substrates. Six extremely euryarchaea reported from such environments were isolated on cellulose as growth substrate and named *cellulotrophic natronoarchaea*, *Natronobiforma cellulositropha* gen. Nov., and extremely haloalkaliphilic members *Natronotalea proteinilytica* gen. nov., sp. nov. and *Longimonas haloalkaliphila* sp. nov., these two proteolytic bacterial strains from the phylum *Rhodothermaeota* were reported from sediments of hypersaline alkaline lakes in Kulunda Steppe (Altai, Russia) by Sorokin and his team in 2017 (104, 105).

Following table shows published haloalkaliphilic archaeal species that have been isolated from soda lakes Table 2.

Recently a novel haloalkaliphilic archaeon *Natronobacterium texcoconense* isolated from soil of the former lake Texcoco in Mexico (106). Thermostable alkaline halophilic-protease producing *Natronolimnobius innermongolicus* WN18 was isolated from Soda lake of Wadi An-Natrun, Egypt by Samy Selim and their coworkers (107).

In the genera *Natronococcus* various species were reported like *Natronococcus jeotgali* (103,108), *Natronococcus occultus* (93,103), *Natronococcus amylolytics* (103,109) and *Natronococcus roseus* (110). Kajale and his research group work on Hypersaline Sambhar Lake for cultivation of diverse microorganisms. Large number of archaea and bacteria were isolated using different cultivation approaches; they reported *Natronococcus* and *Alkalibacillus* as predominant groups in such extreme habitat (111). *Natronorubrum sulfidifaciens*, *Haloterrigena daqingensis* and *Halalkalicoccus tibetensis* an extremely haloalkaliphilic archaeon were reported from saline and alkaline environments of China (102, 112).

Due to the great interest in haloalkaliphilic organisms, all these members of hyper saline and alkaline environments were reported and studied by various researchers.

Conclusion

Extensive number of cultured haloalkaliphiles has been obtained from culture dependent methods which uncovered a much more diverse haloalkaliphiles from various extreme environments from world. Future studies should attempt to isolate diverse members of the uncultured community from such extreme habitats. Using culture independent methods, phylogenic and metagenomics may apply to obtain a diverse range of the haloalkaliphilic organisms from such hypersaline environments. Metagenomics study can be used to explore the overall metabolic capacity of the microbial communities. Several metabolic processes followed by haloalkaliphiles and their byproducts have not yet been detected

Haloalkaliphiles are interesting extremophiles, hypersaline and alkaline environments explored for microbial diverse communities of haloalkaliphiles, this will contributes to our understanding of these ecosystems and can benefit in designing the

applications. These ecosystems represent a valuable source of different industrial compounds with great economical potential and microbial diversity can prove to be a valuable future resource in various industrial and biotechnological processes.

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Table 1: Haloalkaliphiles and their potential role

Microorganism	Product	Reference
Methylophaga lonarensis MPL^T	ectoine, glutamate	Antony et al., 2012 (71)
Desulfonatronospira thiodismutans ASO3-1^T	glycine betaine	Sorokin et al. 2011 (46)
Natronococcus, Natronolimnobius, Halorubrum, Natronomonas	biosurfactants	Selim et al. 2012 (72)

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Thioalkalivibrio versutus	sulfur-oxidizing	Banciu et al. 2004 (84)
Thioalkalimicrobium cyclicum, *Thioalkalivibrio jannaschii*	sulfur-oxidizing	Sorokin et al. 2002 (33)
Halomonas campaniensis MCM B-1027	hydroxybutyrate-co-hydroxyvalerate (PHB-co-PHV) copolymer	Kanekar et al. 2011 (73)
Natrialba magadii ATCC 43099^T	protease	Tindall et al. 1980 (93),Mwatha & Grant 1993(94), Giménez et al. 2000 (74)
Natronolimnobius innermongolicus	protease	Selim et al. 2014 (107)
Nesterenkonia spp	protease, xylanase	Govender et al. 2009 (69), Nel et al. 2011 (70)
Bacillus sp. Vel	protease	Patel et al. 2005 (75)
Clostridium alkalicellulosi DSM 17461^T	cellulose, xylanase	Zhilina et al. 2005 (82)
Desulfonatronum spp	sulfate-reducing	Sorokin et al. 2011 (46)
Desulfonatronovibrio spp		
Thioalkalivibrio versutus strain ALJ 15	Pigments natronochrome and chloronatronochrome	Takaichi et al. 2004 (76)
Halomonas spp.	denitrification	Shapovalova et al. 2008 (78)
Thioalkalivibrio nitratireducens ALEN 2^T	denitrification	Sorokin et al. 2003 (77)
Halomonas nitrilicus sp	biodegradation	Chmura et al. 2008 (66)

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Species	Original location	Reference
Natronococcus occultus	Lake Magadii, Kenya	(Tindall et al. 1984)
Natronococcus amylolyticus	Lake Magadii, Kenya	(Kanai et al. 1995)
Natronobacterium gregoryi	Lake Magadii, Kenya	(Tindall et al. 1984)
Natronomonas phaoronis	Lake Magadii, Kenya	(Tindall et al. 1984) Kamekura et al. 1997
Natrialba magadii	Lake Magadii, Kenya	(Tindall et al. 1984) (Kamekura et al. 1997)
Natrialba hulunbeirensis	Hulunbeir Province, Inner Mongolia	(Xu et al. 2001)
Natrialba chahannaoensis	Lake Chahannor, Inner Mongolia	(Xu et al. 2001)
Natronolimnobius baerhuensis	Lake Baer, Inner Mongolia	(Itoh et al. 2005)
Natronolimnobius innermongolicus	Lake Baer, Inner Mongolia	(Itoh et al. 2005)
Natronorubrum bangense	Bange Lake, Tibet	(Xu et al. 1999)
Natronorubrum tibetense	Bange Lake, Tibet	(Xu et al. 1999)
Halorubrum vacuolatum	Lake Magadii, Kenya	(Mwatha and Grant 1993) (Kamekura et al. 1997)
Halorubrum alkaliphilum	Xinjiang Province, China	(Feng et al. 2005)
Halorubrum luteum	Lake Chahannor, Inner Mongolia	(Hu et al. 2008)
Halorubrum tibetense	Lake Zabuye, Tibet	(Fan et al. 2004)
Halalkalicoccus tibetensis	Lake Zabuye, Tibet	(Xue et al. 2005)
Halobiforma nitratireducens	Lake Chahannor, China	(Hezayen et al. 2002)

Table 2 Soda Lake Haloarchaea (adopted from 'Handbook of Extremophiles')

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Cloud Computing - An insight to latest trends and Developments

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ABSTRACT

Computing technology is rapidly improving and becoming more advanced over time. In order to stay up with the rapid transformation, industry executives pay close attention to the disruptive technologies positioned to deliver value in the cloud. In today's hyperconnected world, staying on top of trends is essential for survival and staying ahead of competitors. Because Cloud Computing has evolved into a large-scale computing system with seamless access to virtually unlimited resources, and various people from various sectors are using Cloud Computing for various reasons, it is now necessary to discuss technology trends and developments in general for Cloud Computing.This paper focuses on the basics of the aspects of understanding of Cloud Computing, as well as the present status, and trends of Cloud Computing. The study also explores the evolving cloud innovation of emerging paradigms likeBlockchain, IoT, AI/AR, Edge, and Green cloud computing, containers, etc. in near future cloud computing systems.

Keywords : Cloud computing trends, cloud computing challenges, Cloud Paradigms and Technologies, IoT, Blockchain, Artificial Intelligence, Hybrid cloud, Serverless, Green cloud computing

I. INTRODUCTION

Cloud computing is a self-service, on-demand Internet infrastructure that allows users to use computer resources from anywhere at any time [1]. It is not a new technology, but rather a new way of providing computer resources. Microsoft Hotmail and Google Docs are two well-known non- healthcare applications, while Microsoft HealthVault and Google Health Platform are two well- known health-care applications [2]. However, as compared to traditional computing, this approach offers three additional benefits: large computer resources available on demand, no upfront commitment from customers, and payment for use on a need-to-know basis [3]. Its uses in industries, businesses, transport, educational, and national security have been documented in several papers, forums, and blogs [4, 5, 6].

In the post-pandemic era, cloud computing will continue to play a key role. According to Cisco, cloud data centres will process 94 percent of workloads and compute instances by 2021, while traditional data centres will process 6 percent. Statista, on the other hand, estimates that the cloud services industry will

be worth around 172.1 billion USD by 2021 [7]. According to the Gartner analysis [8], the pandemic's economic, organisational, and social consequences will continue to serve as a catalyst for digitalisation and cloud service adoption. This is especially true for hybrid workforce use cases including as collaboration, remote work, and innovative digital services.During 2020 and 2021, cloud computing surged as work turned virtual and businesses reacted to the worldwide pandemic by

focusing on the supply of digital services. Rapid adoption and growth are expected to continue in 2022. This Paper mainly focuses on Cloud Computing basics and current trends of cloud computing in section 2 and 3. In addition, section 3 alsodiscusses the latest innovative clouds using Blockchain, IoT, Artificial Intelligence. Section 4 discusses different future innovations like green cloud computing and finishes with conclusion in section 5.

II. Cloud Computing - Insights

In comparison to contemporary versions, early cloud computing offered limited capability. Virtual machines (VMs), also known as virtual private servers, were first introduced, together with supporting capabilities including access key management, block storage devices, and VM snapshots, in the early stages of development.Infrastructure as a service (IaaS) is the most fundamental and general component of the cloud computing architecture. More abstract layers, such as platform as a service (PaaS), software as a service (SaaS), and others, were further developed[1]. Databases, business applications, business analytics, e-commerce, quantum computing, and blockchain systems are just a few of the services and products available in today's cloud systems. Both structured query languages and non-structured query languages are included in databases.Cloud computing data centres contain huge reserves of processing capacity, which means that their benefits lay not only in the variety of services offered, but also in the potential for scaling. Scaling thus is essential not just for mission-critical applications, but also for enabling computationally intensive scientific computations [2, 3].Cloud service providers can offer these scalability options throughout the whole cloud architecture. The ratio between implementation needs and resulting advantages, on the other hand, is determined by the layer of computational abstraction selected [4]:

• Scaling IaaS necessitates the setup of an external load balancer as well as the ability to deploy the scaled application on a generic operating system; nodes can be deployed semi- automatically, but the usage of an external orchestrating tool is desirable.

• Scaling PaaS (e.g., Kubernetes, OpenShift) necessitates the ability to bundle an application into a container(s) as well as the usage of declarative language to specify the cluster and cluster services configuration (i.e. applications).

• Scaling SaaS often necessitates the use of minimal skills or expertise in order to provide flawless outputs, but unable to deploy and perform any customized calculation.

III. Current Trends in Cloud Computing

Cloud computing is expected to expand substantially by 2022. In terms of implementing comprehensive strategies around enterprise-wide cloud migration, the trend will undoubtedly improve. The steady transition from zoom meetings to cloud tool deployment to boost certain functions is inevitable.From altering the global cloud service provider (CSP) industry to organisations shifting from a lift-and-shift strategy to cloud, 2022 will undoubtedly witness a significant increase in enterprises using cloud native technology. To keep up with their competition, businesses are increasingly relying on cloud apps to move and scale. Few of the latest trends are listed here.

3.1 Hybrid cloud

Businesses have generally had two alternatives when shifting to the cloud. They can employ public cloud solutions that are easily available and pay-as-you-go, or more personalised and adaptable private cloud options. Private cloud (in which an enterprise basically has its own cloud and data never has to leave its premises) is also occasionally required for regulatory and security concerns. Today,

major cloud providers like as Microsoft, Amazon, and IBM are boosting the use of "hybrid" models that combine the best of both worlds. Data that must be accessible often and promptly, such as by customers, can be stored on public AWS or Azure servers and accessed via tools, apps, and dashboards.Data that is more sensitive or critical can be maintained on private servers with access controlled and processed using private programmes. A hybrid cloud solution can help by reducing complexity and making the backend stack transparent when it is not needed [5]. Hybrid cloud is clearly the new normal.

3.2 Serverless cloud

The paradigm of serverless computing (FaaS) has recently gained popularity. Serverless computing conceals the execution environment, letting the user to concentrate on the important computations rather than implementation, setup, and scalability [4]. Amazon (AWS Lambda), Microsoft (Azure Functions), and IBM Cloud Functions are among the serverless cloud providers. Also referred as "functions-as-a-service," this concept means that businesses are not constrained to leasing servers or paying for preset amount of storage or bandwidth. It promises a true pay-as-you-go service, with infrastructure that expands discreetly as an application demands. Serverless computing will play a significant role in generating new user experiences that make innovation more accessible throughout the cloud and the overall technology environment [5].

3.3 Artificial Intelligence (AI) in cloud computing

As part of Software-as-a-Service (SaaS) platforms that provide benefits, AI, particularly Machine Learning (ML), may augment existing cloud platforms, data management, and AI tools. Several AI- based platforms, including AWS, Azure, Google Cloud Platform, and IBM Cloud, provide Machine Learning as a Service (MLaaS). Deep learning models for image identification might be incorporated to commercial apps to expedite the development process and customise cloud models[6,7]. Furthermore, AI strives to make IoT and Fog nodes aware of the workload environment and adapt in real time to deliver improved QoS, reduce power consumption, and reduce infrastructure costs. AI includes a wide range of search algorithms, machine learning, reinforcement learning, and planning [8].As a result, the cloud and AI will inevitably revolutionise data storage, automate repetitive processes, and process data across several domains, fostering an atmosphere of efficiency, security, and agility.

3.4 IoT(Internet of Things),Edge Computing and Augmented Reality (AR)

An Internet of Things (IoT) platform is a cloud-enabling platform that interacts with common devices to allow cloud-based applications and services. IoT acts as a mediator, gathering data from various devices through remote device configuration and smart device management. IoT platforms are a cloud computing trend due to their intelligent connectivity. Edge Computing has been considered as a viable approach to address huge computing demands and resource scarcity among mobile users [9, 10]. Edge computing moves application hosting from centralised data centres to the network edge, bringing customers and data generated by applications closer together.Edge computing is seen as one of the primary facilitators of meeting 5G's stringent Key Performance Indicators (KPIs), such as increased mobile broadband, reduced latency, and huge connectivity [11]. Furthermore, advancements in technologies such as 5G and edge computing will

assist the sector in using the AR (Augmented Reality) cloud. 5G, GPS III, and DApps have the potential to take augmented reality to the next level. As the future of augmented reality, the AR Cloud is one of the most promising technologies utilised across sectors such as healthcare, travel, retail, education, real estate, and more. Because of their significant investments in Edge, 5G, and AI/AR, most public cloud providers, including HP, Nvidia, Microsoft, and IBM, have begun shifting workloads [12,13].

3.5 Virtual Cloud Desktops, Development/Database/Desktop as a Service(DaaS) and BlockChain

The software need of a device which is managed by cloud service providers is a virtual cloud desktop. As the computing power is maintained by cloud-based services, the user can begin with a screen and minimal hardware. The virtual cloud desktop reduces the unnecessary expenditures associated with purchasing new hardware. Users must only pay for the real cloud usage. Amazon's Workspaces platform, Microsoft's Windows Virtual Desktop, and Google's Chromebook devices all provide a Desktop-as-a-Service (DaaS) computing architecture. DaaS solutions assist enterprises in reducing desktop support and improving multi-device capabilities.Blockchain is a breakthrough technology with several advantages such as increased data security, decentralisation, improved private key security, microtransactions, and speedier disaster recovery. Blockchain-based cloud storage is gaining popularity because it provides improved protection against fraudsters and hackers. With its interconnected blocks and decentralised design, it combines security and scalability. This technology is useful in cloud storage since it encrypts data stored in the cloud [14,15,16].

IV. What Nextdevelopments in Cloud Computing

4.1 Yotascale

Yotascale is a next-generation computing and autonomous performance monitoring technology designed to eliminate reliance on humans. Yotascale use Artificial Intelligence to make ahead forecasts or choices regarding cloud prices, allowing it to save even more cost. Yotascale can also do real-time analysis to spot anomalous patterns using deep learning approaches (supervised/unsupervised methods or prediction), identify the main cause, and make future forecasts regarding cloud consumption and cost [17].

4.2 Cloud Gaming

Gaming-as-a-service is currently being offered by all of the huge companies. Since 2020, Google, Amazon, and Microsoft have been present in the gaming market, with Sony being the most recent arrival. However, this trend is expected to grow dramatically in the coming year, as cloud technology improves faster than in previous years with the 5G online model. Cloud gaming is definitely showing advantages similar to those of on-demand movie streaming services such as Netflix, Amazon Prime, Hotstar, and so on. Users do not require additional storage space or specialised technology, which has a significant influence on total expenses [18].

4.3 Green Cloud Computing

Green Cloud computing refers to Cloud Computing services' capacity to limit the usage of underutilized resources. Green IT concerns affect both the software stack and the hardware level. A software stack is a collection of applications that work together to generate a result or accomplish a shared purpose. The effective use of computer resources benefits the environment and promotes energy conservation. The use of ready-made computer resources customised to an organization's demands undoubtedly aids in the reduction of power costs. Computers are managed centrally, allowing for greater management of

ancillary expenditures like as energy use and carbon emissions [19,20,21].

4.4 Containers and Kubernetes

Businesses may use containers to create, test, and deploy new apps in a dedicated, cloud-based environment. This allows developers to concentrate on the intricacies of their applications, while IT teams concentrate on deploying and managing solutions as they are produced, making the entire process faster and more efficient. Kubernetes is an open-source container orchestration technology that streamlines the process of delivering and maintaining container-based applications. Aside from automatically scaling apps based on client demand, the software also analyses the performance of new services, allowing firms to solve issues before they arise [22].

V. Conclusion

A commitment to agility and change is required to perfect cloud services. These many trends are intrinsic to the cloud, and they will continue to evolve at a faster rate as cloud usage grows and the cloud is calibrated to give sharper insights. The most anticipated trend will be hybrid cloud computing, with FaaS, AI, and AR gaining traction. Aside from the aforementioned trends, additional notable trends include cloud security, open source, serverless architecture, and the IoT platform. Using a combination of multiple technologies and the cloud to modernise corporate operations can result in significant benefits. Cloud service providers would create more sophisticated tactics to improve data defences, and organisations would incorporate security into their development pipelines and assure cloud-agnostic safeguards across all clouds.

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Deep Learning Convolution Neural Network for Tomato Leaves Disease Detection by Inception

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Abstract. In India, Agriculture is an important sector to improve the economy. It provides over 70% employment overpopulation. So we have to solve their problem through computer-aided systems so that Farmers and Youngsters take an interest in Agriculture and work smartly and without tension. Traditional disease detection was based on feature selection such as color, texture, and shape; these features must be selected for classification, and accuracy was also not high.A Convolution Neural Network (CNN) based method has been proposed here along with Inception V3 for Tomato plant disease detection. It is done by transfer learning technology to retrain tomato disease dataset; an open-source platform is used for the same, which improved accuracy of tomato disease classification without the need of high-end configuration hardware. The accuracy percentage on training is 92.19%, and test accuracy is obtained as 93.03%

Keywords: Convolution Neural Network (CNN) · Deep learning · Image processing · Training set · Test set · InceptionV3

1 Introduction

India's economy mainly depends on agriculture, and it's our duty as Indians to solve their problems through research. The crop production depends on plant growth which mainly depends on the healthiness of leaves. Hence, plant leaf disease detection is crucial in the initial stages and takes essential steps to prevent spreading to other parts of the crop [1].

This work for identifying disease is done manually by farmers, i.e., checking each leaf from the field and using suitable fertilizers and pesticides to avoid their propagation. It requires a lot of effort and expertise for farmers and plant pathologists. Every problem has a solution, so here is one - we can use computer vision to detect plant disease through image processing and cure it at an earlier stage. Tomato leaves are considered for diseases detection in this study because Tomato is primarily used in Indian recipes and is a good source of Vitamin C and A amongst all veggies. Farmers are facing the problem of low production mainly due to leaf diseases. This problem can be solved by using new

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technology to detect leaf disease early and applying fertilizer to increase the production rate. Since the leaf is one of the essential parts of the plant that prepares food by Photosynthesis, leaf disease detection with high accuracy is obligatory to improve production rate[1]. In recent years Image Processing [2], Pattern Recognition [3], Computer Vision [4], Machine Learning [5], Neural Networks [6] have been used for Precision Agriculture. We have taken one step ahead to solve problems using deeplearning [7].

If the leaf disease detection is done in the early stage, it will be helpful for farmers and pathologists to avoid big smash-up by identifying leaf disease in the early stage.

2 Literature Survey

Author Pooja Pawar (2016) worked on more than one crop of different types, and the proposed methodology was Artificial Neural Network [ANN] achieved 80.45% of accuracy; feature selection was difficult using ANN [8].

Mukherjee (2017) used Multi-layer perceptron neural network (MNN) with a Back-propagation classification algorithm for the detection of medical plants like Tulsi and Kalmegh and reported an accuracy of 80% [9].

Many researchers work on image processing, including feature extraction; segmentation deep learning is a hierarchical approach that can study most feature layers exclusive of the segmentation process in leaves [10].

Sigit Widiyanto, Rizqy, and Dini Tri (2019) have proposed a method of CNN on five different types of Tomato Leaf disease identification on 1000 images data [11].

Kalvakolanu Anjaneya Teja (2020) used deep learning CNN to work on 3000 images that achieved 88% accuracy, and the limitation was high-end hardware configuration and a lot of time required for training[12].

The limitations of previous work were the necessity of the use of high-quality images [13], poor recognition rate [14], complicated feature selection [15], and mandatory requirement of high-end hardware configuration [1].

We tried to prevail over a few of the limitations mentioned above, like the requirement of high-end hardware configuration overcome by using low RAM 4.00 GB computer and even on the mobile device it works. Due to the open-source tool Google Colaboratory or 'Colab' there is no need to purchase the software. Using CNN with the Inception model, there is no need for an algorithm for complicated feature selection, classification, etc., which was a tedious task in previous work.Quality images are not required for our work which was also a limitation of previous researchers' work; these problems are overcome in this method.However, it is observed that the execution time for this model is very high. Furthermore, a very high Internet speed and bandwidth are required with cloud. This research paper aimed to evaluate and improve the accuracy of disease detection on extensive data set of about 7000 images with 09 different types of disease of tomato leaf identification using CNN and stated for further improvement.

3 Convolution Neural Network (CNN)

Today, CNN mostly used a deep learning model for image handling jobs like image recognition, classification, etc. [16, 17].

Following layers are used to construct a CNN model.

a. Convolution layers
b. Pooling layers and
c. Fully connected layers
d. Activation function

3.1 Convolution Layer

This layered image is split into a small area (n × n) matrix, weight and bias are applied over it, called filters that are then convoluted with every small section in the input image that yields a feature map. Depth, stride, and padding are the parameters of this layer. Mathematical operations are carried out in this layer to extort the feature map of the input image. The input image is condensed to a smaller size using a kernel. The kernel is shifted on right step by step from starting point of the upper left corner of an image at every step, values in the matrix are multiplied by values in the kernel, and the result is summed, which produces a new matrix with a lesser size than the original image [14, 18, 19].

3.2 Pooling Layers

To reduce the computations, this layer is used. Various types of pooling are Average Pooling, Maxpooling, and stochastic pooling.First, maxpooling maximum value from the stride matrix is chosen, and the average pooling average from stride is chosen in a new matrix [17].

3.3 Fully Connected Layers

Input to fully connected layer is from a max-pooling layer which is flattened. Therefore, the number of parameters is higher than the fully connected layer. Finally, the fully connected layer is connected to the output layer, which is the classifier.

3.4 Activation Function

Various activation functions are Sigmoid, Softmax, Tanh, ReLU, PReLU, LReLU, Swish. These activation functions are used to speed up training; the activation function decides the renovation of the additional weighted input from the node into the activation of the output or node for the input [20].

We have used the Softmax activation function [16]. Applying softmax function accurate class of disease is determined to predict multinomial probability distribution. Therefore, the softmax function is used as an activation function.

$F(x) = \text{Exp}(X_i)/\sum_{j=0}^{n} Exp(Xj)$ where i = 0, 1, 2…….n

Probability = exp (value)/Sum (sum values in list exp (value)) [16].

Steps followed for image classification using Deep learning.

1. Load and preprocess data -

   Train data set
   Test data set

2. Define Model Architecture

   Convolution layer (Inception V3 model)
   The activation function used is softmax

3. Train the model

   Train and validate images using true labels
   Define the number of epochs

4. Evaluate models performance [19] (Fig. 1)

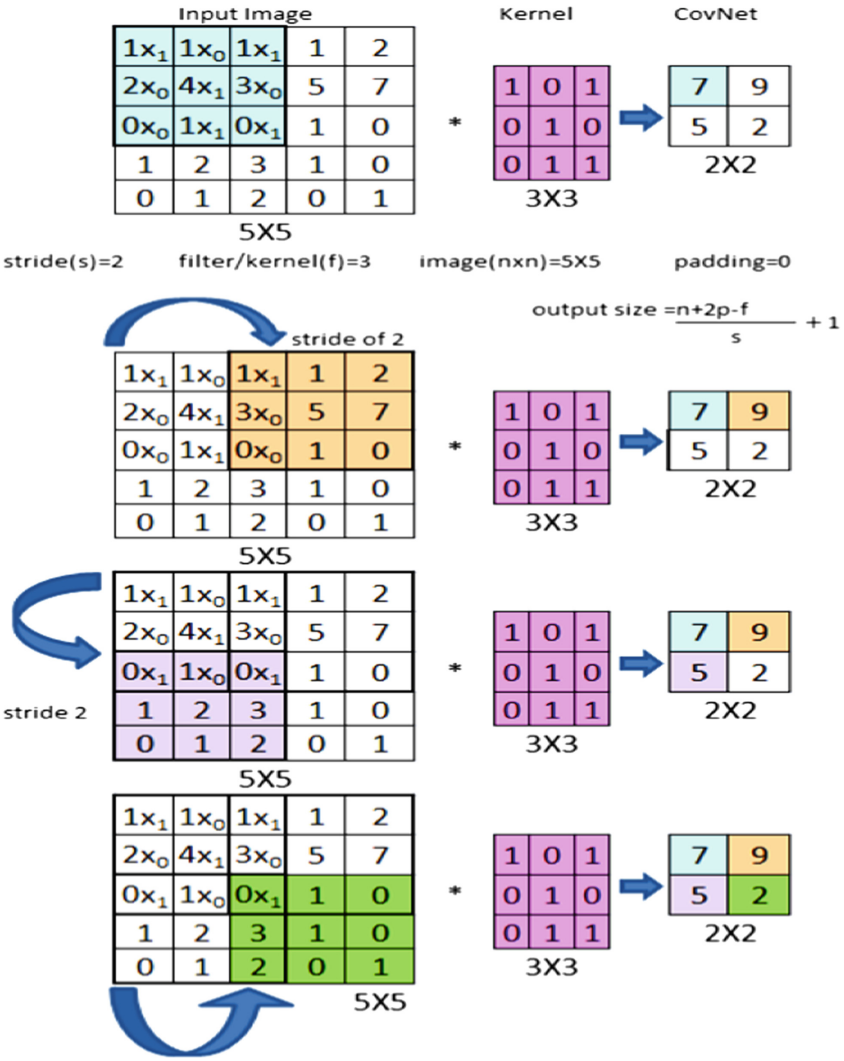

Fig. 1. Convolution operation of 5 × 5 input image and 3 × 3 kernel

Load test data go through preprocessing then predicate class. Following Fig. 2 shows CNN structure [21, 22].

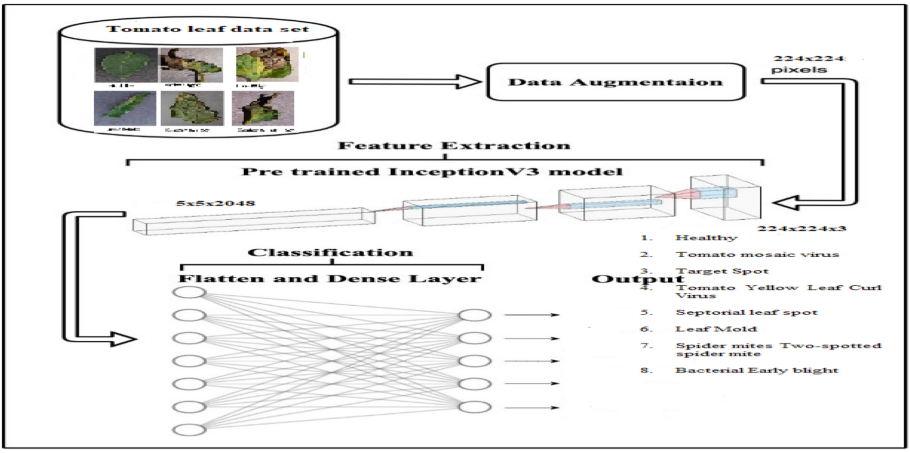

Fig. 2. CNN structure with inception V3 Model

4 Methodology

The proposed plant disease methodology uses 'Google Colab,' an open-source platform for Researchers to work and write code in Python on Machine learning data analysis [23]. There is no need for students or researchers to purchase high-configuration laptops and GPU for data analysis. Instead, one can directly develop the code on mobile or system; only Google chrome and the internet are required. With a registered email id, the researcher can work and store data on the cloud and access it anytime from anywhere [24].

4.1 A Block Diagram

The methodology used in this research is given in the block diagram below (Fig. 3).

4.2 Data Collection/Image Acquisition

To start the research process, data plays a vital role. Without a necessary and adequate data set, it is impossible to move on. To ensure high accuracy large amount of data is collected from Kaggle [25] and GitHub open-source [26, 27]. Some of the images were captured from a camera on-site, from Google. There are no restrictions for image formats, in this study.jpeg,.tiff,.bmp Data set.

Each Training data is saved on Google drive with the file name as 'content/drive/MyDrive/FinalPlantDiseasesDataset/train' and Testing data file as 'content/drive/MyDrive/FinalPlantDiseasesDataset/test' contains ten classes of tomato leaf disease images, including healthy leaf as control.

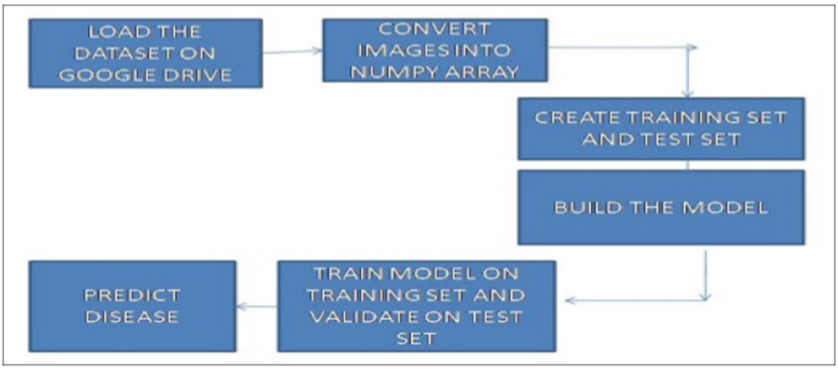

Fig. 3. Block diagram

The training data set contain more than 4500 images, and the testing data set contains more than 2500 images.Nine types of sample tomato disease with one additional healthy class building ten class types are used for this study [27]. Sample images are shown in Fig. 4 [28].

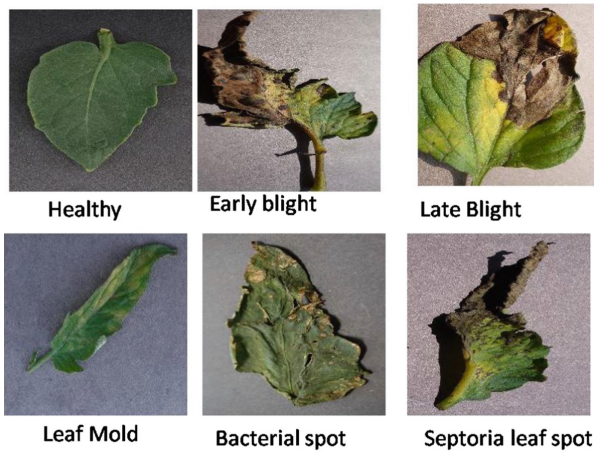

Fig. 4. Tomato Leaf Disease sample images with Healthy Leaf

4.3 Image Preprocessing

More than 7000 images were used in the study, and 2/3rd divided in training and 1/3rd in the Test folder. For different disease types, different folders are used. All images were resized in 224 × 224 pixels without compromising quality [29].

4.4 Model Architecture

Steps to build a model-

Step1. Set up Google Colab
Step2. Import Libraries
Step3. Load and Preprocess data from Google drive
Step4. The validation set created from the training set
Step5. Model Structure definition
Step6. Train Model
Step7. Make Predications

Hyperparameters used for this architecture are given in Table 1.

Table 1. Hyper parameters used for this architecture

Parameter	Value
Target size	224
Batch size	16
Optimizer	Adam
Epochs	30
Activation function	Softmax
Class mode	Categorical

The model has been built with CNN with Inceptionv3 as shown in Adam optimizer is applied for dropping errors and rate loss.The default learning rate is 0.001. It works for a better learning rate. Inception V3 has a pertained model used to increase performance and decrease training time [16, 30].

4.5 Lab Setup

Results were obtained with the help of the experimental setup of the system used shown in Table 2.

Table 2. Lab setup.

System configuration	Details
Operating system	Windows 10 Pro 64 bit
Processor	Intel® core™i3-6006U CPU @2.00 GHz
RAM	4 GB
Software	Google Co laboratory
Tensor flow - GPU	Version 2.5.0

5 Result and Discussion

5.1 Statistical Analysis

The outcome of the training model with Epoch starting from 1 to 30 is shown in Table 3. Gradually the model trained, and the quality of accuracy persistent in getting higher. The concluding training accuracy is 92.19%, and validation accuracy is 93.03%

Total params: 22,314,794
Trainable params: 512,010
Non-trainable params: 21,802,784

Table 3. Details of Training loss, accuracy, validation loss, and accuracy concerning the number of Epoch.

No of Epochs	Training loss	Training accuracy	validation loss	validation accuracy
Epoch 1	7.0335	44.43%	1.4185	86.59%
Epoch 2	3.4036	69.83%	2.1031	83.62%
Epoch 3	2.6164	76.46%	0.5622	95.34%
Epoch 4	2.4756	79.91%	8.8075	68.26%
Epoch 5	2.4606	81.54%	0.7486	94.14%
Epoch 6	2.0918	84.36%	1.4656	90.68%
Epoch 7	2.5728	83.03%	3.1324	85.54%
Epoch 8	2.288	85.16%	3.8267	83.77%
Epoch 9	2.2793	86.10%	2.6473	88.20%
Epoch 10	2.1412	86.22%	1.4505	93.24%
Epoch 11	1.4358	90.00%	3.1428	87.68%
Epoch 12	1.7861	88.33%	2.1814	90.72%
Epoch 13	1.6096	88.61%	2.4543	89.63%
Epoch 14	1.7921	89.14%	5.2493	82.57%
Epoch 15	2.0652	88.67%	3.6557	88.69%
Epoch 16	2.1087	88.49%	0.7143	96.77%
Epoch 17	1.7794	89.55%	5.4706	83.96%
Epoch 18	1.9775	89.68%	3.8199	87.08%
Epoch 19	1.7391	89.76%	1.7775	94.10%
Epoch 20	1.3442	91.46%	0.7848	96.96%
Epoch 21	1.3185	92.40%	2.2148	92.75%
Epoch 22	1.7777	91.01%	0.6835	97.52%

(*continued*)

Table 3. (*continued*)

No of Epochs	Training loss	Training accuracy	validation loss	validation accuracy
Epoch 23	1.4808	91.31%	2.5944	91.10%
Epoch 24	1.9482	90.81%	3.6512	88.66%
Epoch 25	1.2384	92.76%	2.3803	92.94%
Epoch 26	1.1183	93.47%	4.0269	89.52%
Epoch 27	1.8008	91.09%	3.5644	91.36%
Epoch 28	1.483	92.90%	2.4053	93.05%
Epoch 29	1.7185	92.95%	3.02	93.05%
Epoch 30	1.4522	93.03%	2.7343	92.19%

Above table gives in detail the results of each Epoch along with training loss validation loss, and Epochs 30 shows above gives the result of training accuracy as 93.03% and validation accuracy as 92.19%. The training and Validation loss graph is shown in Fig. 5. With several epochs, validation loss decreases, but it's different in the case of training.

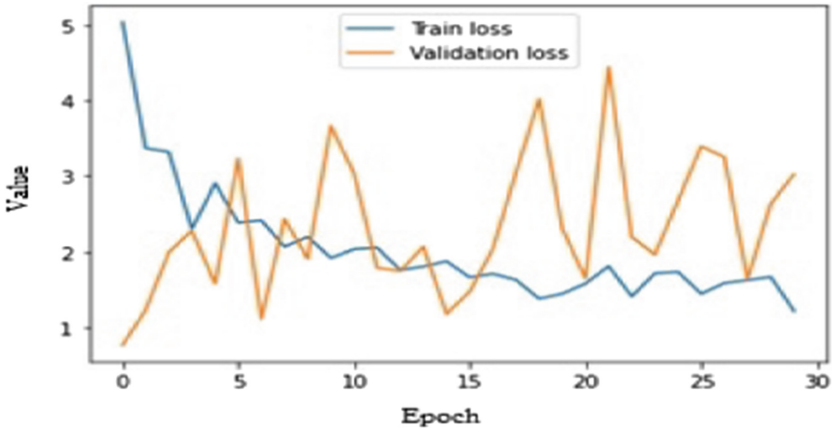

Fig. 5. Training and Validation loss Graph

Following Fig. 6 shows the Accuracy graph of training and validation, accuracy increase with the number of epochs shown in Colab.

Accuracy of training and validation increases gradually with the epochs.During the training, it is observed that the result sometimes depends on the number of epochs; as the number of epochs increases, the accuracy of testing also increases.

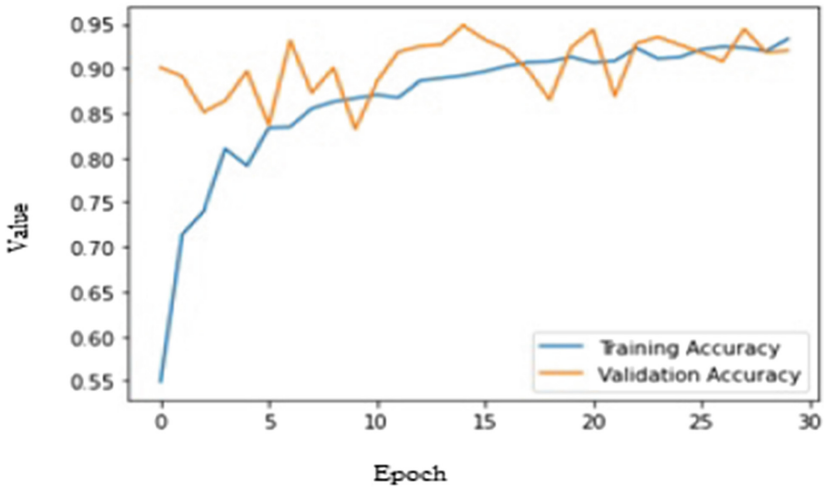

Fig. 6. Training accuracy and Validation Accuracy Graph

The time required for processing is also different on the different systems as well as it also depends on the speed of the internet. Recently advancement in clould computing [31–33] and IoET [34] has been paved the new paradigm in the area of scientific computing. The proposed work can also be extended in this direction.

6 Conclusions

The Proposed training model was successfully implemented using Python language on the open-source platform Colab which doesn't require a high configuration System/Laptop. A considerable amount of data set is required for any training purpose here used Kaggle, Github which is open source. Approximately 7000 images were used for training and validation purposes after the proposed algorithm. We got good results as the final training accuracy is 92.19% and validation accuracy is 93.03%

The Accuracy percentage on the training set is 92.19%, and the Accuracy rate for testing is 93.03%. We raised an innovative system for identifying different nine Tomatoes leaves diseases detection with the accuracy of 93.03%.Still, there is a scope for improvement. It will be helpful for further research in Precision Agriculture, and as a Research Student and Indian Youngster, we can help directly or indirectly to Farmer through such type of research (Table 4).

Table 4. Comparison of our approach with some previous similar works.

First author, Year	Classification algorithms	Accuracy reported	Work on	Limitations	Future direction
Mim, Tahmina Tashrif, 2020 [27]	CNN with ReLu	92.33%	Worked on five different tomato leaf diseases with a training dataset of 5000 images	Training consumes much time and requires high-end hardware configuration	To develop an android based application
Kalvakolanu, Anjaneya Teja,2020 [12]	Deep learning CNN	88.80%	Worked on twelve different tomato leaf diseases with a training dataset of 3000 images	Training consumes much time and requires high-end hardware configuration	To develop an android based application
Our work	Deep learning CNN Inception V3	93.03%	I worked on 10 different classes of tomato leaf diseases with More than 7000 images	Training consumes much time No need for high-end hardware configuration	To reduce training time and work on large data sets

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Computer Vision for Leaf Disease Detection: A Review

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ABSTRACT
Computer vision is one of the leading technologies with wide scope of application. Indian agriculture plays important role in social and financial growth of the nation. Over 70% of Indians directly or indirectly engaged with agricultural based industry. Plant crop diseases are measure threat to this industry causing major loss in production. This review paper is to explore several numbers of researchers working on automated detection of mainly plant leaf disease detection using various techniques from traditional method of image processing, using features like texture, color, shape and Machine Learning (ML) to new holistic approach of Deep learning used for leaf disease detection systems. These are discussed concisely with a review for further studies in sector of Automated Agriculture.

Keywords— ML, Deep learning, ANN, CNN

1 INTRODUCTION

Computer vision is one of recent advances in the field of computer science with wide application. Agriculture sector is prime sector that has wide scope for computer vision Leaf diseases are major threats to plant crops and overall production. India being mainly dependent on agriculture sector for its economy. Early disease detection in the field crop can ease the suitable remedy to get rid of disease and save production loss. Computer vision uses different approaches like machine learning (ML), Artificial intelligence (AI), deep learning (DL) and Convolution Neural Network (CNN). In this paper an attempt is made to take a broad review of contribution of researchers in this sector.

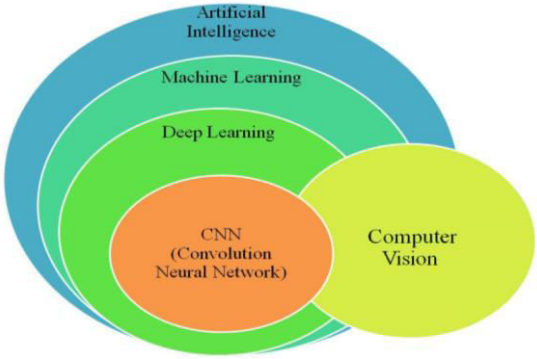


Fig.1 Relationship between Computer vision, Artificial Intelligence, ML, DL and CNN

1.1 Different types of leaf diseases

Leaf is very important part of plant. overall development of plant depends on leaf, if in early stage the leaf disease detection is done then it will be useful for farmer and pathologist to avoid hefty smash up by identifying leaf disease in early stage. Following fig.2 shows different types of diseases.

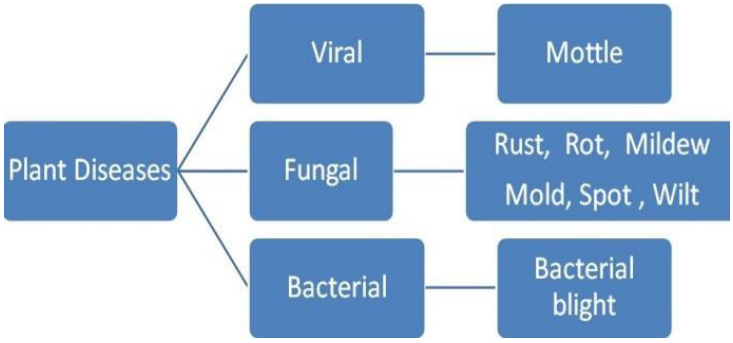


Fig 2.Classification of Leaf Diseases.

These different types of diseases can be recognized by using image processing. Mainly leaf disease classification done according to size, color and shape of leaves.

1.1.1 Viral Diseases

The virus particles are so minute that only an electron microscope can see them. Viruses are obligatory parasite, which means they can only develop and proliferate if they have a living host. Plant viruses require a wound for their initial entry into a plant cell since the membrane is surrounded by a hard cell wall. The organism that causes the wound can sometimes also carry and transmit the virus. In most cases, viruses spread throughout the plant and cause a systemic infection.

Mosaic formation, leaf rolling and curling, spotted leaf, yellowing and vein clearing, dwarfing and stunted growth are some of the symptoms of plant virus diseases

1.1.2 Fungal Diseases

A fungus is a type of eukaryote that digests food from the outside and absorbs nutrients via its cell walls. Fungi are heterotrophic, meaning they get their carbon and energy from other organisms, just like animals. Parasitic fungus obtains their nutrition from living hosts (plants or animals), while saprophytic fungi get their nutrients from dead plants or animals.

Parasitic fungi cause different diseases to plants like rust, smut, early blight late blight, white spots etc

1.1.3 Bacterial Diseases

Bacteria are single-celled microorganisms that are typically 1-2 micro meters in size. Bacteria found on plants can either be beneficial or dangerous. Microbes (called epiphytes) exist on all plant surfaces, and some microbes reside inside the plants (called endophytes). A vast population of bacteria can be seen as aggregates in liquids, biofilms in plants, viscous suspensions that clog plant channels. Bacteria, as plant pathogens, can cause a variety of serious, economically destructive diseases, including wild, spots and blight. [1]

2 LITERATURE REVIEW

2.1 Detection of disease by Machine Learning Approaches

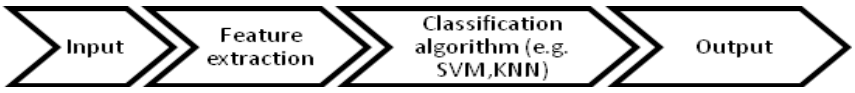
Machine learning means we are training the machine to do some task (image processing) by providing set of trained data [2-6].

Vijay Singh et al. (2017) [7] proposed a genetic algorithm for extricating the highlights like Shape, size and surface of each wheat leaf image. These images of unhealthy wheat leaf would be caught with various shape and size. All the wheat leaf tests were taken as the RGB images.

Award Hollaway (2014) [8] utilized a procedure of histogram evening out to upgrade the differentiation of the images. It gives clear image to natural eyes. It is utilized to accomplish better quality images in dark scale which is utilized in different clinical applications, natural applications, for example, computerized X-beams, wheat leaf disease, plant disease and so on.

J.D. Pujari et.al (2015) [9] worked on fruit crop with k-mean clustering for segmentation and Artificial Neural Network (ANN) for classification got accuracy 90% they also worked on cereal crop used k-mean clustering & foe edge detection used canny edge detector SVM classifier used and got accuracy of 83%.

Machine Learning Approach



Deep Learning Approach

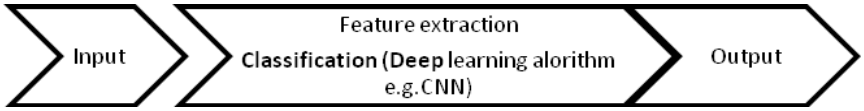


Fig.4 Traditional and deep learning flow for computer vision

2.2 Detection of disease by Deep Learning Approaches

Yang-Yang Zheng et al. (2019) [10] worked on classification and detection of crop for precision agriculture using deep learning classification. They presented classification and detection dataset which consist of large number of image dataset with different number of classes.

Hsieh T.H. and Kiang J.F. (2020) [11] hyper spectral images [HSIs] of agricultural lands were classified by using various versions of Convolution Neural Network (CNN).

Jun Liu and Wang (2021) [12] analysed future trend for identification of plant diseases using deep learning.

Qimei Wang et al. (2021) [13] developed tomato disease detection using object detection and deep convolution neural network. R-CNN with mask and faster these two different methods were used to identify type of tomato disease and to detect infected area of tomato.

Mahmoud A. Alajrami et al. (2020) [14] researchers proposed a system to determine kind of tomato by deep learning (CNN) model with dataset of training and validation got accuracy of 93%.

Karthik R. Menaka R. & Hariharan, M. (2020) [15] It was the first attempt for them to learn custom filters Outcome, there work has important potential for testing COVID-19 X-ray set using CNN.

Agarwal M. et al. (2020) [16] CNN was applied for disease detection and classification of tomato crop proposed models were using VGG16, MobileNet and Inception V3 with accuracy of 91% for 9 classes of diseases.

D. R. Sarvamangala and Raghavendra V. Kulkarni (2020) [17] worked on medical image understanding generally which is done by expert medical professionals. They used CNN for image understanding and observed it is an effect tool for image processing.

Karthik R. et al. (2020) [18] proposed a method for detection of real time tomato disease and pets recognition using GPUs.

Following table summarizes various researches' work on different plant diseases with accuracy.

Table 1. Comparative study of diseases with its accuracy.

First Author , Year	Classification Algorithms	Accuracy	Pros	Cons	Future Direction
Pooja Pawar, 2016 [4]	Artificial Neural Network	80%	Good for more than one crop of different types	Difficult feature selection	To integrate Gabor filter
Mukherjee, 2017[1]	Back propagation Multi layer perceptron Neural Network	80%	Classify medicinal plants Tulsi and Kamlesh based on morphological character.	Task was tougher due to Dithering present at the edges.	To work on other medical plants
Jia Shijie, 2017[19]	VGG 16 + SVM	89%	Regular discovery of tomato pests and disease based on leaf surface	Works only on high quality images only	To work on low quality images
Melike Sardogan, 2018[20]	CNN with Linear Vector Quantization	86%	Worked on four different tomato leaf diseases with training dataset of 500 images	Training consumes much time and requires high end hardware configuration	To improve recognition rate
Robert G. de Luna. 2018[21]	Deep learning	92%	Worked on four different tomato leaf diseases with training dataset of 4923 images	High end hardware configuration	To develop model which requires less time
Azeddine E., 2019[22]	CNN with MobileNets Google Model	90.30%	Worked on ten different tomato leaf diseases with training dataset of 7176 images	Training consumes much time and requires high end hardware configuration	To extend the model for fault diagnosis and improve accuracy

Kumar, Akshay, 2019[23]	CNN with LeNet	91%	Worked on different tomato leaf diseases with training dataset of 14903 images	Training consumes much time and requires high end hardware configuration	To reduce training time
Deepak, Aditi H.2019 [24]	Deep learning CNN with IoT	92%	Worked on three different tomato leaf diseases with training dataset of 200 images	High end Hardware Configuration	To pin point the location of the diseased plant.
Mim, Tahmina Tashrif,2020 [25]	CNN with ReLu	92.33%	Worked on five different tomato leaf diseases with training dataset of 5000 images	Training consumes much time and requires high end hardware configuration	To develop android based application

3 KEY ISSUES AND CHALLENGES

In above table I various image processing techniques are given for leaf disease detection.

Some key issues and challenges are as follow –

To reduce training time

To improve recognition rate

To work on low quality images

To work on large data sets with accuracy

High configuration hardware required.

Along with above issues and challenges there is need for open source software that can be used for image processing. Students who are not capable of purchasing high end configuration system they can also do research from home. Maximum Research was done on image processing using MatLab which is not open source platform. Therefore, there is scope of improvement in the existing further research.

4 CONCLUSION

There is a lot of scope of research in leaf disease detection using Computer Vision and Deep learning. This paper gives an outlook of some of the previous research on Leaf disease detection on different leaves through different algorithm and their results. More focus on Convolutional Neural Network is given because it has breaking results over past decades in various fields such as Image processing using deep learning. It will be helpful for further research.

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EXTRACTION AND SEPARATION OF LYCOPENE FROM NATURAL SOURCES USING CHROMATOGRAPHIC TECHNIQUE AND ITS APPLICATION

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Abstract

Lycopene is a functional component of great dietary importance obtained from many plant sources. In this article, we address the extraction of lycopene using different extraction methods,acetone petroleum ether extraction method, methanol Extraction method acetone and petroleum ether extraction method and hexane Extraction method and screening was carried out by TLC method using standardlycopene .

We also describe the different applications of lycopene hydrogen paraoxide scavenging effect, RNA Damage estimation and lycopene as food colorant

Keywords -.lycopene, Tomato sample, Extraction method, TLC , Applications

INTRODUCTION:

Fruits and Vegetables are main source of natural antioxidant. Antioxidant give protection against harmful free radicals and reduce rate of cancer and heart disease. The most efficient carotenoid antioxidant is Lycopene. Lycopene is one of the carotenoids that naturally occurred in many fruits and vegetables and found to be measured in blood serum. It is mainly found in Tomatoes and other red fruits and vegetables. Lycopene is a pigment principally responsible for the characteristic deep-red color of ripe fruits and vegetables. In the synthesis of Vitamin-A lycopene plays an important role as an intermediate and carotenoid like β-carotene and β- cryptoxent in, influences its development. Lycopene is soluble in fat and synthesized by plant and microorganisms. Lycopene has antioxidant and anti-tumor properties. Regular intake of lycopene containing food reduces the risk of body tumor especially prostate cancer. It also reduces LDL cholesterol and cardiovascular disease. Like essential amino acid, they are not made in the human body and therefore, can only be obtained through diet or supplementation.

Biosynthesis of Lycopene in Tomato: Lycopene biosynthesis in plant cell have been explained by metabolic pathway of carotenoid synthesis in a model plant, Arabidopsis thaliana (1980). The first step specific to the pathway of carotenoid biosynthesis is the production of the symmetrical 40-carbon phytoene from 20-carbon geranyl geranyl pyrophosphate. Phytoene then undergoes a series of four desaturation steps to form a first phytofluene and then, in turn zeta- carotene, neurosporene, and lycopene. Cyclization reaction at each end of lycopene molecule results in the formation of β- carotene, which may they serve as a substrate for production of the xanthophylls (oxygenated carotenoids). Thus, lycopene is a precursor of β- carotene and lutein. Due to common pathway of synthesis of carotenoids, other carotenoids may also be available in small quantities in tomatoes.

Lycopene is synthesized in chromoplast of fruit cells. Most of the cell in the pericarp near the epidermis synthesize higher lycopene levels than the inner tissues of tomatoes (Sharma and Le Maguer, 1999). In tomatoes, full ripening takes place 40-60 days after planting, during which chloroplast change to chromoplast upon synthesis of lycopene.

Biochemistry of Lycopene: Lycopene a carotenoid in the same family as beta-carotene, is what gives tomatoes, pink grapefruit, apricots, red oranges, watermelon, and guava their red color. Lycopene is not merely a pigment, it is a powerful antioxidant that has been shown to neutralize free radicals, especially those derived from oxygen, thereby conferring protection against prostate cancer, breast cancer, atherosclerosis, and associated coronary artery disease. Lycopene's configuration enables it to inactivate free radicals. Lycopene participates in a host of chemical reactions hypothesized to prevent carcinogenesis and atherogenesis by protecting critical cellular biomolecules, including lipids, proteins, and DNA. Lycopene is the most predominant carotenoid in human plasma, present naturally in greater amounts than beta-carotene and other dietary carotenoids. This perhaps indicates its greater biological significance in the human defense system. Its level is affected by several biological and lifestyle factors. Because of its lipophilic nature, lycopene concentrates in low-density and very-low-density lipoprotein fractions of the serum. Lycopene is also found to concentrate in the adrenal, liver, testes, and prostate. However, unlike other carotenoids, lycopene levels in serum or tissues do not correlate well with overall intake of fruits and vegetables.

Lycopene as an Antioxidants Agents: Living tissues have a control mechanism to keep ROS in balance. When ROS are generated in vivo, many antioxidants come into play. Their relative importance depends upon which ROS are generated, how and where they are generated, and which target of damage is considered. Our body defends itself from these phenomena via endogenous antioxidants. However, when endogenous antioxidants become insufficient or imbalanced in defense against oxidants, exogenous antioxidants may help restore the balance. Tomatoes are widely known for their outstanding antioxidant content, including, of course, their often times-rich concentration of lycopene. Researchers have recently found an important connection between lycopene, and its antioxidant properties. Lycopene, a red carotenoid pigment, $C_{40}H_{56}$ found in blood, the reproductive organs, tomatoes and palm oils. It is a Carotenoid without provitamin A activity and present in many fruits and vegetables. It is a red fat-soluble pigment found in certain plants and microorganisms,

where it serves as an accessory light gathering pigment and protect the organisms against the toxic effect of oxygen and light. As an antioxidant its consumption can reduce the risk of some cancers. The FDA has approved Generally Recognized as Safe (GRAS) status to lycopene. Recently the FDA has also given a limited health claim declaration for lycopene, stating "very limited and preliminary scientific research suggests that eating one of the cup of tomatoes and/or tomato sauce a week may reduce the risk of prostate Cancer". Consuming cooked tomato sauces, tomato ketchup, tomato soup, stewed tomatoes and other cooked tomato dishes are excellent sources of lycopene.

MATERIALS AND METHODOLOGY

Isolation of Lycopene by Using Liquid-Liquid Extraction-

Plant Materials

Fresh fruit, ripe fruits All tomato fruits collected from market in Aurangabad. It was identified as a *Lycopersicum esculentum* (Solanaceae)

A. Acetone-Petroleum Ether Extraction Method:

Reagents-

1. Acetone 2. Petroleum ether 3. Anhydrous sodium sulphate 5. 5% sodium sulphate

Two-Three tomato fruit (samples) was taken and pulped well to a smooth consistency in a wearing blender. Weigh 10-15 grams of this pulp. The pulp was extracted repeatedly with acetone by using pestle and mortar or wearing blender until the sample was colorless. Acetone was pooled, extracted and transferred to a separating funnel containing about 20ml of petroleum ether and mixed gently. Added about 20ml of 5% of sodium sulphate solution and shook well in a separating funnel gently. Volume of petroleum ether might be reduced during these processes because of its evaporation. Then 20ml of petroleum ether was added to the separating funnel for clear separation of two layers. Most of the color noticed in the upper petroleum ether layer. Separated the two phases and re-extracted the lower aqueous phase with additional 20ml of petroleum ether until the aqueous phase was colorless. The petroleum ether extract was pooled and washed once with little distilled water. Poured the wash petroleum ether extract containing carotenoids into a brown bottle containing about 10g of anhydrous sodium sulphate. It was kept aside for 30 minutes or more. Decant the petroleum extract into a 100ml volumetric flask through a funnel containing cotton wool washed sodium sulphate slurry with petroleum ether until it was colourless and transferred the washing to the volumetric flask. It was measured at the absorbance in spectrophotometer at 503nm using petroleum ether as a blank.

Fig:3. Acetone Petroleum Ether Extraction Method

B. Methanol Extraction Method:

Reagents-

1.Methanol 2. Carbon tetrachloride 3. Benzene 4. Boiling methanol

Fifty grams of tomato paste was dehydrated by adding 65ml of methanol. This mixture was immediately shaken vigorously to prevent the formation of hard lumps. After two hours, the thick suspension was filtered. The dark red cake had shaken for another 15 min with 75ml mixture of equal volume of methanol and carbon tetrachloride and separated by filtration. The carbon tetrachloride phase had transferred to a separating funnel, added one volume of water and shook well. After phase separation, the carbon tetrachloride phase had evaporated and the residue was diluted with about 2ml of benzene. Using a dropper, 1 ml of boiling methanol was added in portion, the crystals of crude lycopene were appeared immediately and the crystallization was completely by keeping the liquid at room temperature and ice bath, respectively. The crystals were washed 10 times using benzene and boiling methanol. After that measured the absorbance in spectrophotometer at 503nm using methanol as a blank.

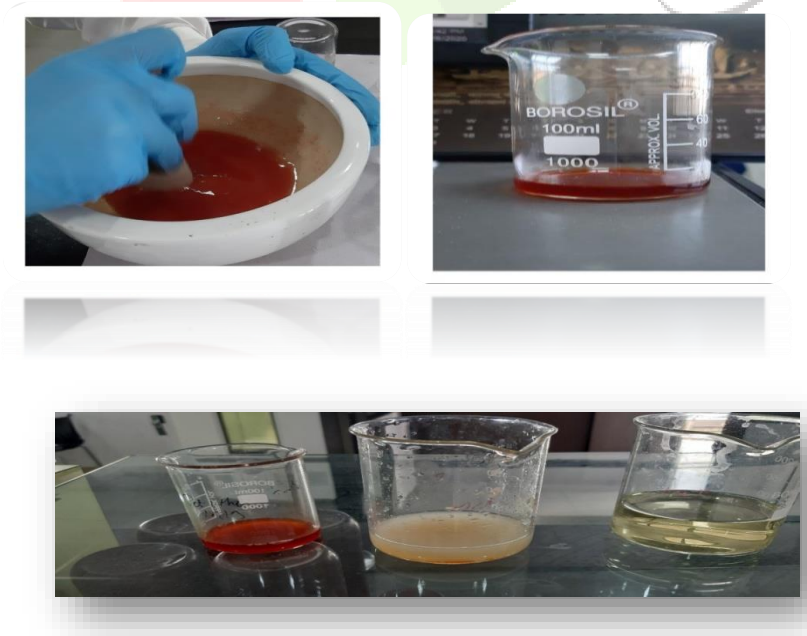


Fig:4. Methanol Extraction Method

C. Acetone-Petroleum Ether Extraction Method:

Reagents-

1.Acetone 2. Petroleum ether 3. Anhydrous sodium sulphate 4. 5% Sodium sulphate 5. Saturated NaCl

Weighted roughly 2 g of tomato paste was placed into a 15 mL centrifuged tube. 4 mL of 50/50 (% volume) mixture of petroleum ether and acetone was added. The centrifuge tube was caped and shaked until the solid becomes fluffy. Then opened the cap and crushed the solid with a spatula. Closed the tube and shook again. Repeated this crushing and shaking two more times. Centrifuge the tube to separate the extract and residues. Transfer the extract (liquid) to a clean centrifuged tube. In the original centrifuged tube, added a new 4 mL of solvent and repeated the entire extraction procedure. The resulting extract was added to the first extract (in the second centrifuged tube). Now washed the mixed extract with saturated NaCl solution (5mL), then with 10% aqueous potassium carbonate (5mL), then with saturated NaCl solution (5mL) again. The organic layer with anhydrous sodium sulphate was dried. Decant the organic layer into a small beaker and concentrated to roughly 0.2 mL by evaporation in the hood (did not applied heat).

D. Hexane Extraction Method-

Reagents-

1.Acetone 2. Hexane 3 Anhydrous Sodium Sulphate 4. 5% Sodium Sulphate 5. Saturated NaCl

Weighted roughly 2.0 g of tomato paste was placed into a 15 mL centrifuged tube. Added 4 mL of a 50/50 (% volume) mixture of Hexane and acetone. Caped the centrifuge tube and shook until the solid becomes fluffy. Opened the cap and crushed the solid with a spatula. Closed the tube and shook again. Repeated this crushing and shaking two more times. Centrifuge the tube to separate the extract and residues. Transfer the extract (liquid) to a clean centrifuged tube. In the original centrifuged tube, added a new 4 mL of solvent and repeated the entire extraction procedure. Added the resulting extract to the first extract (in the second centrifuged tube). Now washed the combined extract with saturated NaCl solution (5mL), then with 10% aqueous potassium carbonate (5mL), then with saturated NaCl solution (5mL) again. Dried the organic layer with anhydrous sodium sulphate. Decant the organic layer into a small beaker and concentrated to roughly 0.2 mL by evaporation in the hood hexane (0.2 mL).

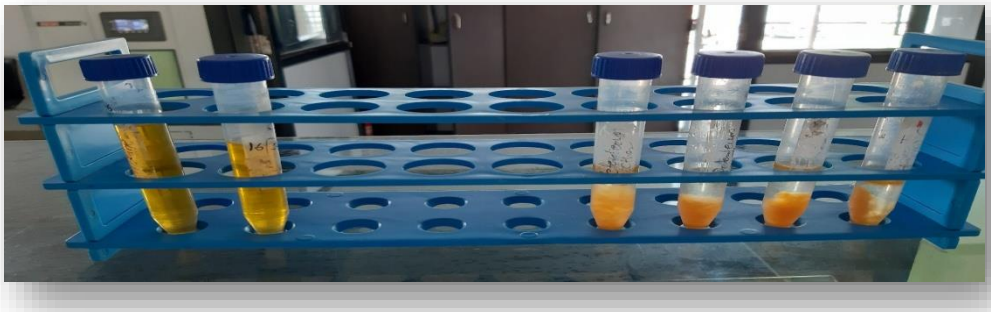
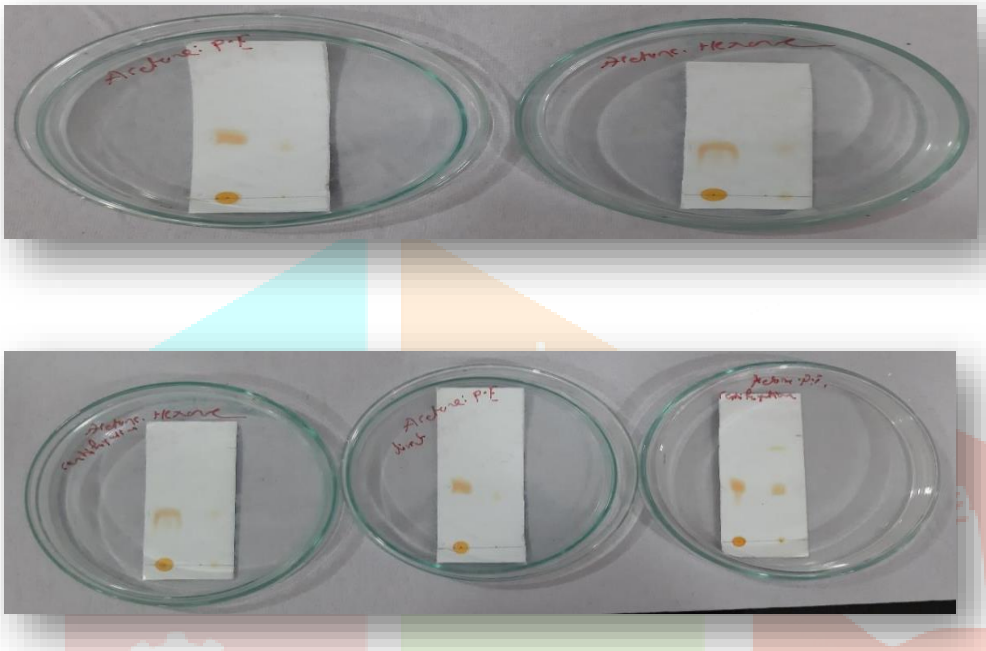


Fig:5. Acetone Petroleum Ether and Hexane Extraction

THIN LAYER CHROMATOPGRAPHY (TLC):

The purified lycopene was subjected to TLC screening method. The TLC was carried out using aluminum sheets (20 × 20cm) pre coated TLC silica gel 60F254 sheets. Three elution solvent systems were selected. (1) petroleum ether; dichloromethane 95:5 (2) 5% methanol in toluene, (3) toluene: hexane 1: 19. The solvent was used and eluted in covered TLC developing tank. Visualization was performed using UV lamp. The Rf solvent was measured;

Rf = **D**istance from origin to component spot (cm) / **D**istance from origin to solvent front (cm).



APPLICATION:

A.Hydrogen Peroxide Scavenging Effects:

The ability of the lycopene to scavenge hydrogen peroxide was assessed by the method of Ruch et al (1998).

Reagents-

1. Phosphate buffer (0.1M, pH 7.4)

2. H_2O_2 (40Mm) in phosphate buffer

1. A solution of H2O2 (40mM) was prepared in phosphate buffer.
2. Lycopene of various concentrations from stock 5mg/ml were added to H2O2 solution (0.6ml) and the total volume was made up to 3 ml.
3. The absorbance of the reaction mixture was recorded at 230nm in a spectrophotometer.
4. The blank solution containing phosphate buffer, without H2O2 was prepared.
5. The extent of H_2O_2 scavenging of the plant extract was calculated as:

Where, A_0 = Absorbance of Control

A_1 = Absorbance in presence of Lycopene

A. Estimation of RNA Damage:

The method described by Chang et al. (2002) was used to assess the RNA damage.

Reagents-

1. **RNA`2. Tris buffer (30mM, Ph 7.4) 3. H_2O_2 (30%) 4. $FeCl_3$ (500M) 5. Agarose (1%) in 1X TAE buffer 6. EtBr (10mg/ml) 7. Gel loading dye (0.25% bromophenol blue, 0.25 % xylene cyanol, 50% glycerol) 8. 50X TAE buffer (Tris base 24.2g, EDTA 18.612g, glacial acetic acid5.7ml, in a total volume of 100ml, pH8.0)**

1. The reaction was carried out in tris buffer (pH 7.4) at 37 C. FeCl3 and H2O2 react with each other resulting in the generation of hydroxyl radicals.
2. Each reaction contained 5µl of tris buffer in RNA (2 µg) and 5µl of tris buffer in lycopene. FeCl3 (5µl) and 10 µl of H2O2 were added to test samples and incubated at 37 C for 15 minutes.
3. To the reaction mixture, 0.06 ml of gel loading was added and electrophoreses in 1% agarose gel containing 3 µl/ml EtBr, at 100V for 15 minutes.
4. Gels were viewed under trans-illuminating UV light and photographed.

B. Lycopene as Food Colorants Against Standard Colour:

Reagents-

1.Lycopene Sample 2. Standard Orange Red colour 3. Sugar Cubes

1. Sugar cube were made by using appropriate concentration of sugar and water.
2. Then by using distilled water the standard color and lycopene sample were diluted according to standard concentration that usually used in foods.
3. By using spreader both sample standard and lycopene was spread on the sugar cubes.
4. Then both sugar cubes were packaged by using aluminum foil and in polythene bags.
5. And then both stored at room temperature.
6. Observed the color efficiency and effect of light on lycopene colour sample against the standard colour.

RESULT:

Extraction Procedure:

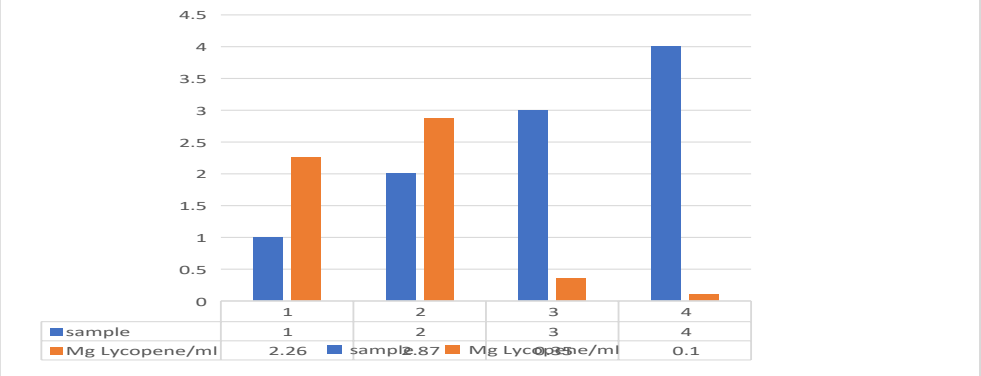
The highest yield of lycopene was found in procedure -2, that use liquid-liquid extraction by using methanol and carbon tetrachloride.

The yield was measured by using standard formulae:

Mg lycopene in 100g sample = 31.206 × Absorbance at 503nm / Weight of Sample.

Table-1: Comparative Yield of Lycopene (Without Any Dilution) Using Four Different Methods

Sr. No.	Sample	Procedure	Mg Lycopene/ml
1	FreshFruits	Acetone: Petroleum Ether	2.26
2	Fresh Fruits	Methanol: Carbon Tetrachloride	2.87
3	Fresh Fruits	Acetone: Petroleum Ether	0.35
4	Fresh Fruits	Hexane: Acetone	0.10



Comparative yield of lycopene using different methods

Thin Layer Chromatography:

Rf = **D**istance from origin to component spot (cm) / **D**istance from origin to solvent run.

Table-2: Comparison of standard sample w.r.t Test (Lycopene)

Sr. No.	Procedure	Rf Value (Standard)	Rf Value (Test)
1	Acetone: Petroleum Ether	0.69	0.59
2	Acetone: Hexane (Centrifugation)	0.51	0.48
3	Acetone: Petroleum Ether (Centrifugation)	0.57	0.51

Table-3: Carotenoid Content in Sample

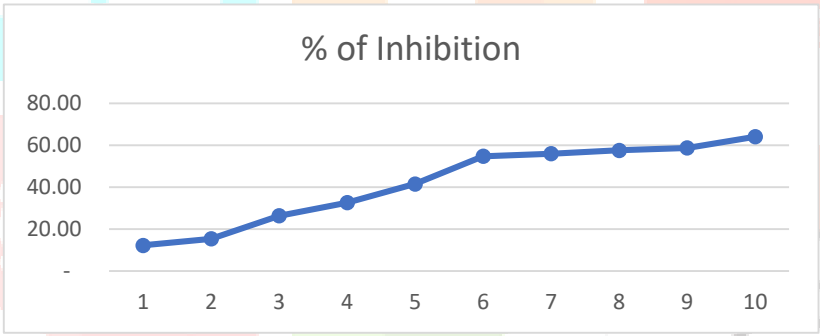
Sr. No.	Procedure	Rf Value
1	Acetone: Petroleum Ether	0.87
2	Acetone: Hexane	0.97
3	Acetone: Petroleum Ether	0.89

Hydrogen Peroxide Scavenging Effects:

The percent of inhibition was increase exponentially by increasing lycopene concentration. The highest percent of inhibition was found to be at 1.0mg/ml of lycopene concentration.

Table-4: Percent of Inhibition (H_2O_2) Against Lycopene Concentration

Sr. No.	Lycopene mg/ml	Phosphate buffer	O.D at 203 nm	% of inhibition
1	0.1	2.9	1.018	12.24
2	0.2	2.8	0.982	15.34
3	0.3	2.7	0.854	26.37
4	0.4	2.6	0.781	32.67
5	0.5	2.5	0.687	41.55
6	0.6	2.4	0.524	54.82
7	0.7	2.3	0.510	56.03
8	0.8	2.2	0.491	57.61
9	0.9	2.1	0.478	58.79
10	1.0	2.0	0.416	64.13



Percent of Inhibition(H_2O_2) Against Lycopene

ESTIMATION OF RNA DAMAGE:

The lycopene's antioxidant property was found to be effective against free radical of H_2O_2 that may be damage RNA. In this First and Third band contain lycopene, hydrogen peroxide and Std. RNA Sample. Second band contain damaged RNA due to Hydrogen Peroxide which has very low frequency compare to control sample.

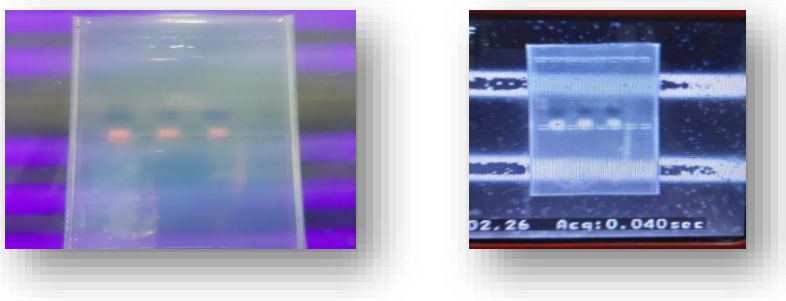


Figure No. 7: Estimation of RNA Damage

Component	Well-1	Well-2	Well-3
Standard RNA	✓	✓	✓
Hydrogen peroxide(H_2O_2)	X	✓	✓
Lycopene	X	X	✓

LYCOPENE AS COLOURING AGENT:

Fig:8. Lycopene as Coloring Agent

Summary:

Lycopene was extracted from tomato paste by simple liquid – liquid extraction using as minimum organic solvent as possible. The main problem was purification of the extract. Four different methods used for the extraction. After that the separation was carried out with the help of Thin Layer Chromatography. Then three types of application carried out. Hydrogen peroxide causes greater oxidation in cellular RNA than in DNA. In these free radicals that are generated in H_2O_2 causes damage the RNA. Lycopene protect the RNA from oxidative insult because lycopene has a great ability scavenging.lycopene has deep red colour and its antioxidants property makes it to use as food colorants. Its shows the good colour efficiency as compare to standard colour that normally use as a food colour.

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Extraction and Purification of Curcumin from Turmeric

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ABSTRACT

Turmeric has long been used as a strong anti-inflammatory in Indian systems of drugs. Traditionally turmeric was called "Indian saffron" because of its deep yellow-orange color and it has been used as a condiment, healing remedy and textile dye. Turmeric is rich in curcuminoids which vary in chemical structures, physico-chemical characteristics. The present work reports on extraction method of curcuminoids using Soxhlet extractor unit. Purification and quantification of curcuminoids was carried out by thin layer chromatography. In the TLC maximum resultant extract (by Hexane) was performed Percentage yield of curcumin was reported 4.09% from Soxhlet extraction method. Different solvent were used for extraction, among them acetone showed maximum yield of each curcuminoids. Separation of curcuminoids was tested in TLC chloroform: methanol at 95:5 showed RF value at 0.59, 0.61, and 0.58 as curcumin, di-methoxy-curcumin, bis de-methoxy-curcumin respectively.

Key words: *Turmeric; Curcuminoids; Soxhlet extractor unit; TLC*

Introduction

Turmeric (*Curcuma longa* l.) is medicinal plant which mostly found in south Asia. Turmeric has long been used as a powerful anti-bacterial, anti-oxidant and anti-inflammatory in Indian systems of medicine. Traditionally turmeric was called "Indian saffron" because of its deep yellow-orange color and it has been used as a condiment, healing remedy and textile dye. Turmeric is rich in curcuminoids which vary in chemical structures, physico-chemical characteristics [1].

Curcumin, demethoxycurcumin and bisdemethoxycurcumin these three compounds are present in turmeric. Commonly these three compounds called as curcuminoid. Curcumin is main compound present in turmeric which gives yellow color to turmeric plant. Curcumin is very expensive and act as anti-cancer agent, which is good supplement to avoid growth of cancerous cells in the body. Asian/Indian farmers can sold curcumin as secondary most valuable product compare to turmeric in the market for farmers curcumin work as Agri-gold. In the present work we focused on extraction purification and quantification of curcuminoids using Soxhlet extractor unit [2].

Materials and Methods

Substrate

Curcuma longa (turmeric) local variety collected from Nanded District. All solvents /chemicals used were of AR grade and obtained from Himedia. The salem, china salem, Krishna, these local variety of turmeric was selected. Fresh rhizomes of turmeric were used for extraction. Collected rhizomes were washed, cut into small pieces and oven dried and powdered for extraction [3].

Method of extraction

Curcuminoids was extracted by using Soxhlet extraction method Fresh rhizomes were cleaned, washed with deionsed water, sliced and dried in the sun for one week and dried again at 50ºC in a hot air oven for six hours. These Dried rhizomes were cut in small pieces, powdered by electronic mill. 6 gm of sample were taken into a thimble and placed in a Soxhlet apparatus; 250 ml of solvent was added and extracted according to their boiling point for seven hours. The solvents used were chloroform (BP=61ºC), methanol (BP=65˚c) and acetone (BP=56.53ºC) [4].

After completion of extraction the dark brown extract was then cooled, concentrated using rotary evaporator. This crude dried extract which was turning black orange in colour. Each raw sample of turmeric was extracted by an equivalent method and yield was calculated [5].

$$\%\ of\ \ curcu\min = \frac{Dry\ wt.of\ \ extracted\ \ curcu\min}{Total\ wt.of\ \ turmeric} \times 100$$

Separation of curcuminoids by TLC

Acetone and Methanol solvent extracts were tested on TLC for presence different curcuminoids. The thin layer chromatography pre-coated silica gel plates were used, these plates were developed using glass beaker, which was pre-saturated with mobile phase for 20 min and each plate was developed up to a height of about 6.8 cm. chloroform: methanol mobile phase was used with the 95:5 composition [6]. After development of chromatogram, plates were removed, dried and spots were analyzed [7].

Results and Discussion

Different collected varieties of turmeric from Nanded district, Maharashtra were used to study of soxhlet extraction method for Curcuminoids from turmeric (Figure 1). After drying Soxhlet extract were weighted and weight percentage of curcuminoids were calculated. In this study we used three local varieties of turmeric, Selam, Kadappa and China selam, for extraction. The dried powder was made and used for Soxhlet extraction method using different solvents, this extract in the form of dark black orange colour obtained once dried (Figures 1 and 2).

Extracted curcuminoids dried and quantified, the percentage of extracted curcuminoids showed in Table 1. Among all varieties of turmeric china selam showed highest curcuminoids that is 4.2% using acetone solvent. Methanol is also good solvent for extraction of curcuminoids Maximum concentration of curcuminoids was obtained in acetone solvent compared to ethanol and methanol.

Dried curcuminoids sample were run on TLC for separation of curcuminoids, which showed three different spots of C, DMC, BDMC (Figure 3). we got RF value at 0.59, 0.61, and 0.58 as curcumin, di-methoxy-curcumin, bis -de-methoxy-curcumin respectively. According to the RF values of standard curcumin we analyzed presence of curcumin in the sample.

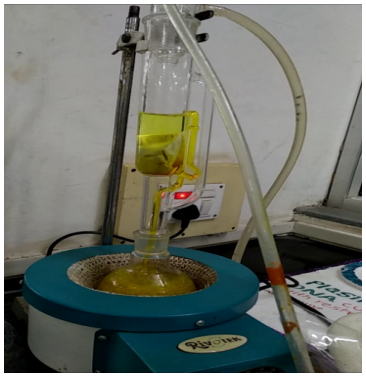

Figure 1: Soxhlet extractor unit.

Figure 2: Extracted curcumin.

Table 1: Extraction of curcuminoids using Soxhlet method.

Variety	Solvent	Curcuminoid (%)
Selam (10 gm)	Ethanol	3.6
	Methanol	3.4
	Acetone	3.9
Kadappa (10 gm)	Ethanol	3.1
	Methanol	3.7
	Acetone	3.8
China selam (10 gm)	Ethanol	3.3
	Methanol	4.0
	Acetone	4.2

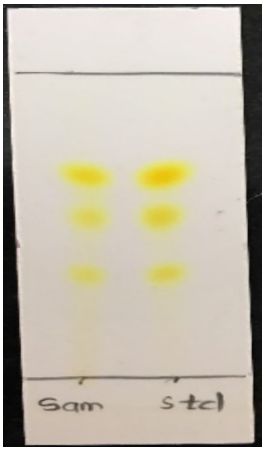

Figure 3: TLC of isolated curcuminoids.

Conclusion

In this study, we extracted natural curcuminoids by soxhlet extractor. We found among all varieties of turmeric china selam showed highest curcuminoids that is 4.2% using acetone solvent. As curcumin is medicinal one and valuable product compare to turmeric in the market for farmers, so it can extract and sold as Agri-gold for farmers. In this present work we found that acetone and china selam gives better results for curcumin extraction. In future further work should carry out to test medicinal properties of curcumin.

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Production and characterization of a haloalkaline pectinase from *Halomonas pantellerinsis* strain SSL8 isolated from Sambhar lake, Rajasthan

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Abstract

Haloalkaliphilic bacterium producing a pectinase was isolated from the Sambhar soda lake, Rajasthan, India. Chemical composition of water sample was analyzed. Pectinase production was studied in submerged fermentation, an appropriate medium for the growth and production was orange peel powder. The bacterium was gram negative and identified as *Halomonas pantellerinsis* strain SSL8 using biochemical tests and 16S rRNA sequencing. It was able to grow and produced pectinase that was stable and active at high pH, temperature and high NaCl concentration. Maximum pectinase production from isolate was observed after 120hr of incubation (0.70U/mL). The maximum pectinase activity was found at 9 pH (0.79U/mL), 40°C Temperature (0.70U/mL) and 10% NaCl concentration (0.85U/mL). Partially purified pectinase enzyme was used for the fruit juice extraction and clarification.

Keywords: Haloalkaliphilic, *Halomonas pantellerinsis,* Sambhar lake, orange peel powder, Pectinase

Introduction

Pectinase constitute a unique group of enzymes which catalyze the degradation of pectic polymers present in the plant cell walls. Pectinases are commercially used in many processes and nearly 25% of the global enzymes sales are attributed to pectinases (1, 2).

In the industrial sector, acidic pectinases are used in the production and clarification of fruit juices, in maceration and solubilization of fruit pulps whereas alkaline pectinases are finding immense use in the degumming of ramie fibers retting of flax, textile processing, coffee and tea fermentations, paper and pulp industry, and in oil extraction (3). Pectinases are produced by many organisms such as bacteria, fungi, yeasts, insects, nematodes, protozoa and plants. A quarter of the global food enzymes sale is met with microbial pectinases. Although the major sources of acidic pectinases are fungi, alkaline pectinases are produced from alkaliphilic bacteria (4)

The present paper describes isolation and identification of a haloalkaliphilic *Halomonas pantellerinsis* strain SSL8 from the hypersaline Sambhar Lake and production of extracellular haloalkaliphilic pectinase from such fruits and vegetable waste to minimize the cost by the selected isolate.

Material and Methods

Site description and sample collection: The Sambhar Lake is the largest inland saline lake located in Thar Desert of Rajasthan, India (26° 52'- 27° 2' N, 74° 53'- 75° 13'E) (Fig. 1). It is an elliptical and shallow lake, with the maximum length of 22.5 km. The width of the lake ranges from 3.2 km to 11.2 km. The total catchments area of the lake is 7560 km^2, most of which lies to

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the north and northeast. The lake has occupied an area of about approximately 225 Sq. Km and average depth of water is about 1 m whereas the maximum depth is about 3m (5, 6, 7). The surface (SU) and Sediment (SD) water samples were collected from four sampling stations located in main lake and salt pans towards Sambhar Lake city. Samples were collected in presterilized bottles in post-monsoon, season. The samples collected from each station were average of ten samples spanning the whole sampling point.

Abiotic characterization of water: The parameters like Temperature, pH were measured at the time of sampling by using Digital Thermometer and Digital pen pH meter respectively. Samples were transported to laboratory in cold box. The samples were filtered and stored in refrigerator during investigation (8).

Various physicochemical parameters were determined for both the samples individually. TS, TDS, TSS were analyzed according to procedures described in APHA. The salinity was measured by using Refractometer (Erma, Tokyo). The dissolved oxygen content (DO) was determined by azide modification method, Biological oxygen demand (BOD) and chemical oxygen demand (COD) were determined by potassium dichromate oxidation method. Chloride was determined by argentometric and sulphate by gravimetric methods. Sodium and potassium were measured directly using the flame photometer (Model Elico CL 361). Carbonates and bicarbonates were measured titrimetrically. Calcium and magnesium were determined by EDTA titrimetric method. Metal ions like Fe, Mn, Zn, As, Cr, Pb, Cu, and Cd were directly analyzed by atomic absorption spectrophotometer. (Model S2 Thermo- USA) (9, 10).

Enrichment, Isolation and cultivations of haloalkaliphiles: The 5ml water and Brine sample was inoculated into nine different media such as Alkaliphilic media at pH- 10.0,[A], Marine agar pH- 10.5 [MA], Nutrient broth at pH- 10.5 [ANA] with 30 % sodium chloride, Halophilic medium [H], modified Horikoshi II medium [H II], Synthetic Sea water medium[S], Alkaline peptone water [AP], Alkaline Bacillus medium (AB) and Tindal's medium [T].. Inoculated media flasks were incubated in shaking incubator for 8 days at 30°C temperature and 150 rpm speed. Incubated samples were further inoculated on respective agar plates. Inoculated agar plates were incubated for 15-20 days at 30°C temperature (11-14).

Screening of isolates for efficient pectinase producer: Isolates were tested qualitatively by growing the culture on modified alkaliphilic pectin agar medium followed by observing zone of hydrolysis around colonies. After incubation the plates were flooded with Iodine-potassium iodide solution (0.3 % iodine, 0.6 % potassium iodide solution) to enhance the clarity of zone. The isolate showing largest zone was selected for further production (2, 15, 16)

Identification of selected strain of the bacterium: Selected isolate was subjected to morphological and Biochemical observation. Sugar utilization pattern of selected isolate was determined using glucose, galactose, mannose, arabinose, fructose, ribose and lactose. Enzyme utilization profile was determined using starch, casein, gelatin, pectin substrates (17).

DNA extraction, amplification and 16S rRNA sequencing: 16S rRNA analysis was performed by extracting DNA of isolates. For DNA extraction isolates were suspended in an extraction buffer (10 mM Tris HCL, pH 8.0; 1 mM EDTA, pH 8.0). Proteinase K solution was added to a final concentration of 100 ug/ml and incubated at 55°C for 2 h with continuous shaking. 0.5 M NaCl was added and incubated at 72°C for 30 min. DNA was extracted by phenol–chloroform extraction. DNA was washed with 70% ethanol and dissolved in Tris–EDTA buffer (pH 8.0). Extracted DNA was analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining (18)

The amplification of 16S rRNA fragments were performed by using (PCR) thermocycler, (Eppendorf) with 530F (52 GTGCCAGC

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AGCCGCGG 32) and 1392R (52 ACGGG CGGTG T GTAC 32) primer pair. The PCR reaction mixture contained 1.5 mM $MgCl_2$, 200 uM dNTP mixture and 0.3 µM of each primer and 1 U of Taq DNA polymerase with a reaction mixture supplied by the manufacturer in a total volume of 100 µl. Reaction mixture was first denatured at 94°C for 3 min, followed denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min. Amplification was completed by a final extension step at 72°C for 7 min reaction was carried out for 30 cycles. PCR products were run on a 1% agarose gel. PCR products were purified by the PEG/NaCl method (19) and directly sequenced using Applied Biosystem model 3730 DNA analyzer (Foster, California, USA). The 16S rRNA sequences were initially analyzed using BLAST program (www.ncbi.nlm.nih.gov/blast/blast.cgi). Multiple sequence alignments of approximately 800 base pair sequences were performed using CLUSTALW2 program version 2.1. Phylogenetic tree was constructed using the neighbor joining method (20). Tree files were generated by PHYLIP and viewed by TREE VIEW program. Bootstrap analysis was applied.

***Pectinase production using synthetic and crude media**:* Pectinase production was carried out submerged fermentation using synthetic and crude media. Presterilized medium containing yeast extract, 1; pectin, 5; KH_2PO_4, 4; NaCl, 200; $MgSO_4.7H_2O$, 1; $MnSO_4$, 0.05; $FeSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 2; NH_4Cl, 2 grams per liter was inoculated and incubated at 30°C temperature and 150 rpm speed at pH 9.

Presterilized crude medium contain 10g orange peel powder mixed with mineral salt solution contain 1% KH_2PO_4, 15% NaCl, 0.1% $MgSO_4.7H_2O$, 0.1% $CaCl_2$ was also inoculated and incubated at same conditions (21, 22).

Assay of pectinase activity: Polygalacturonase activity was determined by quantifying the amount of reducing groups expressed as galacturonic acid units, liberated during the incubation of 1 ml of 1% (w/v) citrus pectin, prepared in 0.2 M phosphate buffer (pH 8.2) with 500 ìl of the enzyme at 37°C for 30 min, by DNSA method. One unit of polygalacturonase activity was defined as the amount of enzyme required to release 1 ìmol of galacturonic acid per minute under standard assay conditions and expressed as units per litre (U/l). Specific activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute per milligram of total enzyme protein and expressed as units per milligram (U/mg) (16, 21, 22).

Partial purification of pectinase: The crude pectinase enzyme supernatant was partially purified using chilled acetone and ammonium sulphate precipitation method. The precipitate was dissolved in 10 ml of 0.2 M phosphate buffer (pH 8.5) and desalting was carried out by dialysis (2).

Determination of protein content: The protein contents of the crude and purified pectinases were determined by the method specified by Lowry *et al.*, using bovine serum albumin as the standard (23).

Effect of pH, temperature, salt and reaction times on pectinase activity : The optimum pH and temperature of the pectinase activity was studied over pH range of 5 to 12 and temperature range from 20 to 50 °C respectively. The effect of various salt concentrations and reaction times on pectinase activity was measured over range of 5 to 30% and 10, 20, 30, 40, and 50 min respectively (2, 24, 25).

Application of pectinase in fruit juice extraction and fruit juice clarification: Fruits (apples) were obtained from a local market Nanded. For the extraction of juice from apple, the apples were chopped into small cubes (3-5mm in size). Ten grams of material were incubated with 1 ml of crude enzymatic extract for 1 h in a shaking water bath with a shaking rate of 100 rpm, at 40 °C. Later the samples were incubated in a boiling water bath for 5 min to inactivate the enzyme. After cooling to room temperature, the juice was filtered by vacuum through filter paper and the volume of juice obtained was measured

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by using 100 mL graduated cylinders. Inactivated enzyme was used as a control and for study the fruit juice clarification, 10 mL portion of juice was taken and centrifuged at 3000 rpm for 10 minutes. The clarity of fruit juice was determined by measuring at 450nm using UV-VIS spectrophotometer (9, 28).

Result and Discussion:

Abiotic characterization of water: Abiotic characterization of Sambhar lake water samples collected in post-monsoon season has yielded diverse results. The colour of post-monsoon water sample was pale green at the time of collection. The typical rotten egg like smell was experienced in the lake atmosphere. The average pH recorded was 10 for water sample.

The Total Solids (TS) and Total dissolved Solids (TDS) were recorded as 131050 mg/l and 88263 mg/l respectively. The Total Solids (TS) and Total dissolved Solids (TDS) recorded in present investigation were higher as compared to the very well studied African soda lake and Kenyan Soda Lake.[22]. Some anionic and cationic concentrations of water were recorded, among all dominating cations and anions were sodium (9930 mg/l) and chloride (7356 mg/l) and the divalent cations Ca^{2+} (1550 mg/l) and Mg^{2+}(1870 mg/l). Carbonates (396 mg/l) and sulphate (9152 mg/l)) anions also recorded in considerable amount.

Also in the water sample the metal concentrations were recorded. The Trace amount of chromium (0.01 mg/l) and arsenic (0.01mg/l) were recorded. Also lead (0.05mg/l), Zinc (0.36 mg/l) and cadmium (0.7mg/l) were present in considerable amount in water sample (27, 28).

Screening of bacterial isolates for Haloalkaliphilic pectinase production: Out of nine broth media used during enrichment the agar media have supported highest diversity and faster growths of haloalkaliphiles were used in further investigation. Out of total 10 isolates which have shown zone of clearance on pectin agar plates, and showing distinct colony characters were selected from alkaliphilc medium (A), synthetic Sea agar (SS), alkaline nutrient agar (ANA) and Marine agar (MA) plates. Small colonies were appeared after incubation of 10 days, further incubation of 10 days have yielded large colonies. Non pigmented and pigmented colonies were observed. Pigmented colonies showed cream, yellow, pink, and red colour pigment. Out of ten morphologically distinct isolates SSL8 rapidly growing extreme haloalkaliphilic strain was selected for further investigation which showing maximum zone of clearance on pectin agar plates. It is motile, cream pigmented, Gram negative, rod shaped bacterium growing at optimum 9 pH, 10% salt concentration and 40°C temperature. It is Catalase and oxidase positive, produces H_2S. The sugars Glucose, Fructose, Mannose, Ribose, Arabinose, Galactose and Lactose are not utilized It is negative for hydrolysis of starch, Casein and Gelatin (14, 28) (Table 1).

Based on morphological, physiological, biochemical characteristic and Phylogenetic analysis of its 16S rRNA gene sequence it was identified as *Halomonas pantellerinsis* strain SSL8 (Fig. 1). The 16S rRNA gene sequence was submitted to NCBI Genbank with accession number KC 434456

Alkaline pectinase production was recorded with two substrates orange peel powder and pectin. Maximum production was observed in growth medium containing orange peel as source of substrate (Fig. 2). Maximum pectinase production from *Halomonas pantellerinsis* strain SSL8 was observed after 120hr or 5 days of incubation (0.70U/mL). A gradual increase in the enzyme level was detected till the 120hr of the fermentation process, whereas, there was a steep decline in the pectinase activity after the 120hr of incubation, as shown in Fig 2. Beyond this period the enzyme production drastically reduced, probably due to the depletion of essential nutrients in the medium and/or accumulation of toxic secondary metabolites.

Effect of temperature: The effect temperature on the alkaline pectinase enzyme production was studied using alkaliphilic medium by conducting

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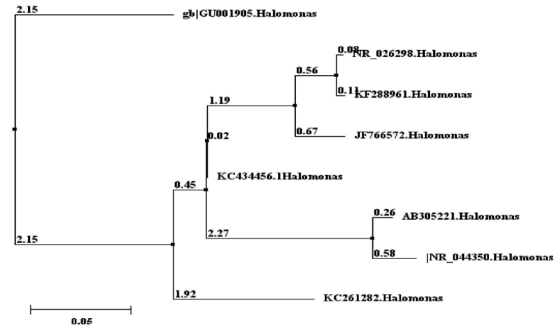

Fig. 1. Phylogenetic tree of isolate SSL8 to other Halomonas.Each number on a branch indicates the bootstrap values. The scale bar indicates 0.05 substitutions per nucleotide position

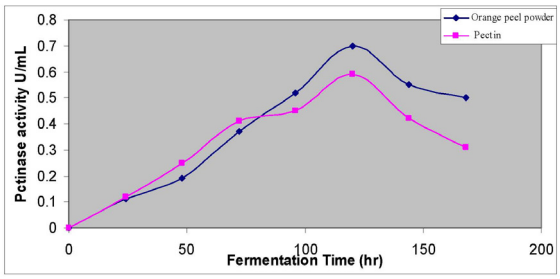

Fig. 2. Effect of fermentation time on pectinase production

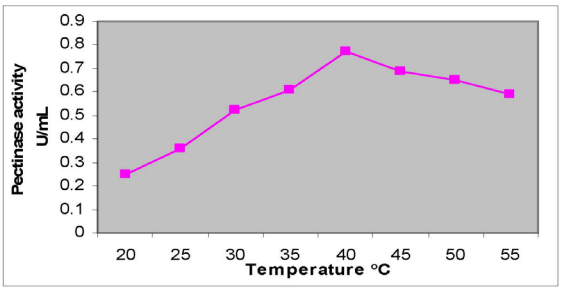

Fig. 3. Effect of Temperature on pectinase Activity

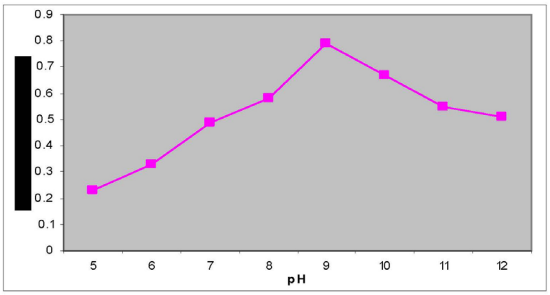

Fig. 4. Effect of pH on pectinase Activity

Table 1. Morphological and Biochemical characterization of Strain SSL8

Characters	SSL8
Morphology	Rod
Gram nature	-
Size (mm)	0.5-2
Colony pigmentation	Cream
Motility	Motile
Oxidase	+
Catalase	+
pH range	08-Nov
Optimum pH	9
Salt range	5-15%
Optimum Salt Concentration	10%
Temperature range (°C)	20-40
Urease	+
Nitrate reduction	+
H2S production	+
Hydrolysis of:	
Casein	-
Gelatin	-
Starch	-
Utilization of:	
Arabinose	-
Fructose	-
D-Glucose	-
D-Galactose	-
Mannose	-
Ribose	-
Lactose	-

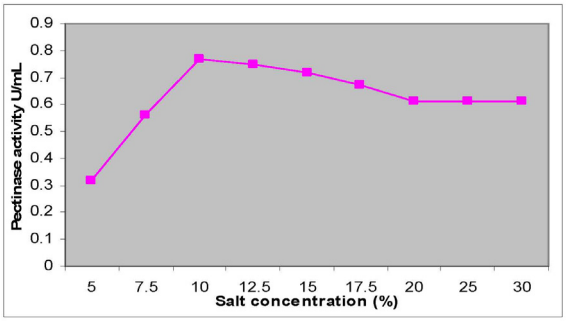

Fig. 5. Effect of Salt concentration on pectinase Activity

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experiments at different temperatures, keeping all other conditions constant for the fermentation. As a temperature increase the pectinase enzyme activity was found to increase and maximum pectinase activity of 0.77 U/ml was found at 40°C (Fig. 3). Further increase in temperature beyond 40°C decreased the pectinase activity till the end of fermentation. Hence optimum temperature was 40°C and was used for further studies. The decrease in enzyme activity at higher temperature may be due to enzyme denaturation.

Effect of pH: The effect of pH on the pectinase production was studied by conducting experiments at different pH (pH range 5, 6, 7, 8, 9, 10, 11, 12) and by keeping temperature at 40 °C. As initial pH was increased from pH 5 to pH 9, the pectinase activity was found to increase. Further increase in initial pH beyond pH 9, the pectinase activity was found to decrease. The decrease in enzyme activity at higher pH may be due to growth and metabolism of organism. A maximum pectinase activity of 0.79U/ml was observed at a fermentation period of 6 days at temperature 40°C and at pH value of 9. Hence optimum pH value was selected as pH 9 (Fig. 4).

***Effect of salt concentration*:** The effect Salt concentration on the alkaline pectinase enzyme production was studied using alkaliphilic medium by varying Salt concentrations (5, 10, 15, 20, 25 and 30% w/v) keeping all other conditions constant. The maximum pectinase activity of 0.85U/ml was found at 10% Salt concentration (Fig. 5).

Pectinase for fruit juice extraction and clarification : The fruit juice extraction by using the pectinase enzyme as well as mixture of other enzymes (cellulose) with pectinase was showed significant results of fruit juice extraction. 31 mL of fruit juice was extracted when apple without peel was treated with pure pectinase and 24 mL juice was extracted when treated with crud pectinase. 17 mL juice was extracted when crude pectinase treatment was given to apple with peel. Whenever the treatment of cellulase on the fruit without peel was given, 26 mL juice was extracted and 19 mL was extracted from apple with peel. (1) 26.5 mL juice was extracted when treatment of crude pectinase and crude cellulose given to the fruit without peel and 19.7 mL was extracted from the same treatment on the fruits with peel. And the apple juice which was extracted by the treatment of crude pectinase has the more clarity as compare to other enzymatic treatment

Conclusions

A haloalkaliphilic bacterial strain isolated from the Sambhar salt lake of India was identified as *Halomonas pantellerinsis* strain SSL8. It produced halo alkaline pectinase that was stable and active at high pH, temperature and high salt concentration. It is showing optimum activity at pH 9, temperature 40 °C and at 10 % salt concentration. The *Halomonas pantellerinsis* strain SSL8 pectinase gives maximum production in fruit juice extraction and also showed good result in fruit juice clarification. Similar study was carried out by Kashyap et al. in 2000 (1).

Considering the high activity and stability in high alkaline pH and temperature, the *Halomonas pantellerinsis* strain SSL8 pectinase may find potential application in the degumming of ramie fibers, retting of flax, textile processing, coffee and tea fermentations, paper and pulp industry, and in oil extraction (25).

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PRODUCTION, EXTRACTION AND USES OF ECO-ENZYME USING CITRUS FRUIT WASTE: WEALTH FROM WASTE

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***Key words**: Ecoenzymes, Flavonoids, Alkaloids, Quinones, protease, lipase*

Abstract–Eco-enzymes is a complex dark brown colour solution produced by fermentation of fruits waste. It has strong sweet and sour fermented scent due to citrus fruit peels. Eco enzyme produced using fruit peels, water and brown sugar in a ratio 3:10:1. After incubation the filtrate was obtained, we found Flavonoids, Alkaloids, Quinones, Saponins as presence of different metabolites. Its IR spectra showed presence of -OH, COOH group. Also, Amylase, protease and lipase were found in the filtrate. We found applicability as floor cleaning, utensils, gardening, etc. the novel approach for recycle and reuse natural waste would help to reduce fruit waste, it is eco-friendly, economical with multipurpose application.

INTRODUCTION

Eco-Enzyme is a kind of organic compound. It is a complex solution produced by fermentation of fresh kitchen waste such as vegetable and fruit peels. It is type of homebrew vinegar, reduced from alcohol by fermentation of kitchen waste as substrate with sugar (Xia Li, hang Wang, 2013). Eco-enzyme can be prepared generally from citrus fruit peels or kitchen waste. Citrus fruit peels are used due to their distinct properties such as fragrance and sharp flavour, source of vitamin C and also rich in medicinal properties along with high acidity value. The sugar which is added is utilized by microbes; due to their metabolism ozone that is derived may kill the bacteria (Pinang)

Eco-enzyme acts as an anti-fungal, anti-bacterial and insecticidal agent. It may also use as cleansing agent. In the present study we work on production of Eco-enzyme from citrus fruit peels and their different applications.

MATERIALS AND METHODS

Citrus peels were collected from fruit vendors, local fruit juice shop, brown sugar was purchased from local grocery (Maharashtra provisions), water used was normal tap water, all the media and chemical used were purchased from Hi-Media (Mumbai), and reagents used were of AR grade.

Production of Eco-Enzyme

The citrus fruit peels (sweet lime, orange, and lemon) were collected washed thoroughly under running tap water and were chopped into small pieces. Then 500 g of brown sugar was added to 5lts of water and stirred until sugar dissolved completely, to it chopped 1.5kg fruit peels were added. The solution was incubated for 3 months in airtight plastic container. Initially the mixture was stirred daily using glass or wooden rod to release the gas formed by fermentation. Further in second month the solution was mixed once in two weeks followed by once in middle of third month.

Enzyme assay

The crude sample was analysed for various enzyme activities including protease, amylase and lipase.

Enzyme assay Protease

Eco-Enzyme crude sample was serially diluted 10^{-1} to 10^{-4} and spread on Skimmed milk agar plates (skim milk powder-28g/L, yeast extract-2.5g/L, dextrose-1g/L, tryptone-5g/L, agar-15 g/L) (with pH 7.0) and incubated at 37 °C for 48 hrs. the positive isolates were further screened for better production of enzyme by assaying the protease activity in liquid culture using casein as substrate at 37 °C

(Hanan S. Alnahdi, 2012).

Enzyme assay Amylase

Eco-Enzyme crude sample was serially diluted 10^{-1} to 10^{-4} and 0.1 mL sample was spread on each starch nutrient agar plates. After incubation for 1-2 days at 37 °C plate was flooded with Gram's iodine and observed for zone of hydrolysis (Ann kipps and P. H. whitehead).

Enzyme assay Lipases

Eco-Enzyme crude sample was serially diluted 10^{-1} to 10^{-4} and spread plated 0.1 mL on Lipid agar (Glucose-1g, tributyrin-4 mL, NaCl-0.5g, NH_4Cl-4.5g, $MgSO_4$–0.5g, D/W-100 mL, agar-agar-2.5g/100mL) and incubated for 37 °C for 24 hrs. The plates were observed for zone of clearance (Suseela Lanka and J. Naveena Lavanya Latha, 2015).

Identification of metabolites

Tests were carried out to confirm the presence of flavonoids, alkaloids, quinones, cardenolides, and saponins.

Alkaline regent test

The 2 mL of sample was treated with few drops of 20% NaOH solution and observed for change in intense yellow colour to colourless solution on addition of dilute HCL.

Wanger's reagent

The 2 mL of Sample was treated with 3-5 drops of Wanger's reagent (1.27g of iodine and 2g of KI in 100 mL of water) and observed for formation of reddish/brown precipitate.

Keller test

5mL of sample was treated with 2 mL of glacial acetic acid in a test tube and few drops of 5% $FeCl_3$ solution was added to it. This was carefully under layered with 1 mL concentrated H_2SO_4, awaited to form brown ring at interface (which is due to deoxy sugars) characteristics of cardenolides.

Ferric chloride test

A 2 mL of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue colour, which shows absence of phenols.

Foam test

2 mL of sample was added in 6 mL of water and was shaken vigorously and observed for formation of foam.

Test for Quinones

2mL of sample was treated with concentrated HCl and observed for formation of yellow precipitate.

Quantification of Acetic acid

The 10 mL of sample was titrated against the titrant 1M NaOH. The few drops of phenolphthalein indicator was added and observed for the formation of pink colour.

Antimicrobial activity

The nutrient agar plates were spread with different microbial suspension (*E.coli, Pseudomonas ssp., Bacillus* spp.) and 1 mL of sample was inoculated using well diffusion method. These plates were incubated for 48 hrs at 32 °C and observed for zone of inhibition.

Effect on plantlet growth

Two containers of soil were taken and labelled as with and without Eco-enzyme, each container added 25 seeds of wheat and 5 mL diluted Eco-enzyme and container kept for plantlet growth.

RESULTS AND DISCUSSION

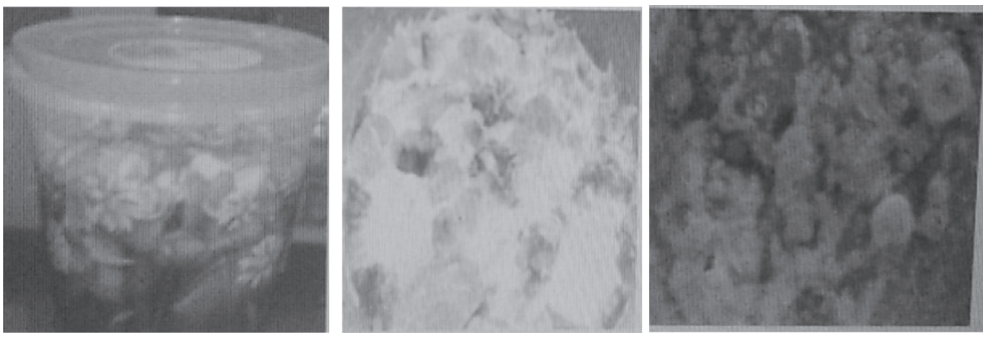
Eco-enzyme solution was produced by using citrus fruit peels. Mostly in this experiment we used citrus fruit peels as in Nanded region they are cultivated on large scale. These citrus fruit peels were collected from various shops of Nanded. The collected peels and juice shop fruit waste survey is follows (Table 1)

Table 1. The survey result of juice shops for peels of citrus fruit (approx. in kg).

Fruit	No. of shops	Fruit waste (kg)	Waste/day
Sweet lime	25	7	175
Oranges	25	6	150
Pineapple	25	4	110
All fruits	25	56	250

After collection of these fruit peels, these were sorted and cleaned and were further used as substrate for Eco-enzyme production. Around 1.5 kg of citrus fruit peels along with brown sugar and water were kept in air tight container and was incubated for 1st cycle of fermentation (Fig 1-a, b, c).

After fermentation of three months, crude



(a) (b) (c)

Fig. 1. Formation of Eco-Enzyme: (a) mixture in air tight container, (b) white layer formed after two weeks, (c) brown crude filtrate after 3 months

solution was filtered and analysed further for presence of different metabolites, enzymes. Based on these tests different application was proposed.

The formation of clear zone around the colonies confirms the production of alkaline protease (Fig. 2a). The zone of hydrolysis and clearance indicates the presence of amylase and lipase enzyme (Fig 2b and 2c).

Further the crude solution was analysed for metabolites presence the intense yellow colour change to colourless solution on addition of dilute HCl acid, indicating Flavonoids presence (Fig 3a). Formation of yellow precipitate was observed, indicating presence of Quinone's (Fig 3b). On shaking vigorously foam formation was observed, indicating Saponins presence (Fig 3c). There was formation of reddish-brown precipitate, indicating Alkaloids presence (Fig 3d). A brown ring at interface shows the presence of deoxy sugar characteristics of cardenolides (Fig 3e).

Antimicrobial activity

The antimicrobial activity was shown against *Pseudomas spp.*, *E.coli*, *Bacillus spp.* Highest antimicrobial activity was shown against *Bacillus spp.* with 18 mm zone of inhibition (Fig 4c). Whereas, 11 mm and 5mm zone of inhibition was shown against *Pseudomas spp.*, *E.coli*, respectively (Fig 4a, 4b)

Quantification of Acetic acid:

The 10 mL Eco-Enzyme contains 0.084 mL of acetic acid which is around 42.25 mL in 500 mL Eco-enzyme thus the pH of Eco-Enzyme is acidic it can be brought to basic upon dilution (Fig 5).

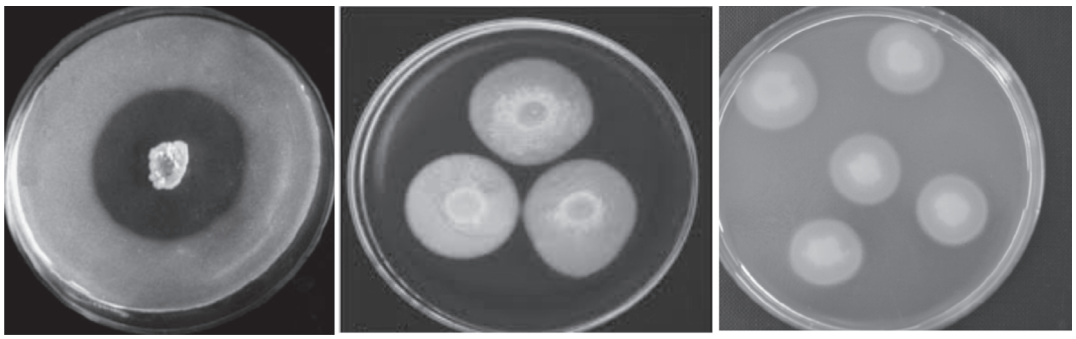
IR Spectra

The crude sample was filtered from membrane filter so that no microbes will enter in liquid. The IR spectra of filtered liquid was performed that represents the presence of -OH and -COOH functional groups (band of 3303.83/cm and the band of 1637.45/cm were observed respectively (Fig 6).

Applications of Eco-enzyme

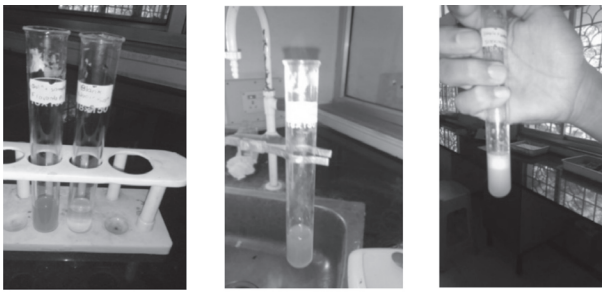
1) Enhanced plant Growth

It was observed that the generation time of seedling was 6 days with Eco-Enzyme while seedlings took 9 days to grow without Eco-Enzyme. Also, seedling

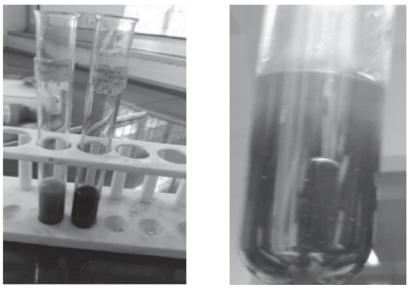


(a) (b) (c)

Fig. 2. Enzyme assay (a) Protease activity, (b) Amylase activity, (c) Lipase activity

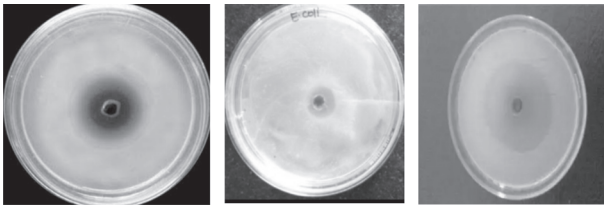


(a) (b) (c)



(d) (e)

Fig. 3. Metabolites: (a) Flavonoids, (b) Quinone's, (c) Saponins, (d) Alkaloids, (e) Cardio glycosides



(a) (b) (c)

Fig. 4. Antimicrobial activity against (a) *Pseudomonas spp*., (b) *E.coli*, (c) *Bacillus spp.*

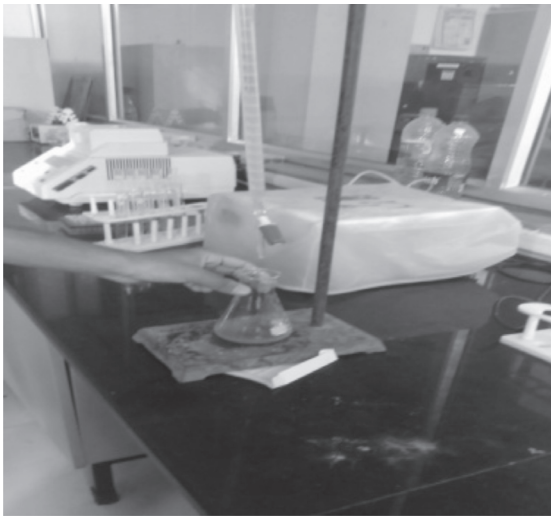


Fig. 5. Formation of light pink colour indicating acetic acid presence.

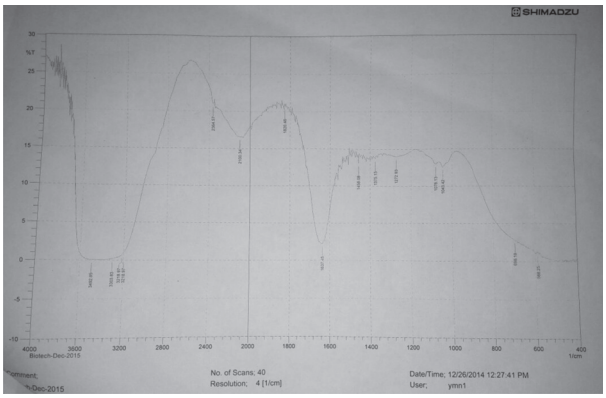


Fig. 6. FTIR spectra of crude filtrate

Table 2. The number of components used in production of Eco-Enzyme

Sr. No.	Components	Cost
1	Brown sugar	50/-
2	Water	0/-
3	Fruit peels	0/-
4	Container	50/-

Table 3. Comparison of Eco-enzyme cost with other available commercial products.

Sr. No.	Commercial products	Market cost/200mL	Eco-Enzyme/ 200 mL
1	Lizol	27/-	10/-
2	Dettol	35/-	10/-
3	Lifeboy	30/-	10/-
4	Vim	18.5/-	10/-

vigour was more with Eco-enzyme than seedling without Eco-enzyme. (Fig 6-a, b, c).

In household application

Eco-Enzyme due to it acidic nature is used in Cleaning of utensils, floor cleaning. Also due to it smell it repels the mosquitos.

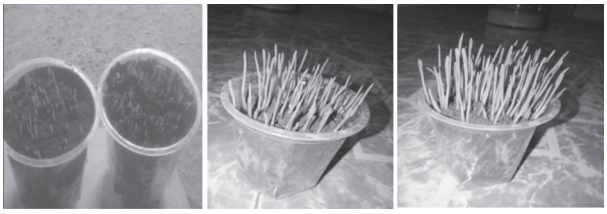
The cost efficiency of Eco-Enzyme

For production of Eco-enzyme citrus fruit peels and other components were used which are available at very cheaper cost (Table 1 and 2).

Whereas, when Eco-enzyme production cost was compared to other commercial products, it was found very cost effective (Table 3).

Eco-Enzyme comparison with commercial cleaning agents

Due to the presence of all-natural raw material



(a) (b) (c)

Fig. 7. (a) Seedling, (b) Seedling + water, (c) Seedling + water + Eco-Enzyme

present in the production it doesn't cause any hazardous or ill effect to environment while the chemical agents get accumulated in the nature and degrade the land over there and also may affect the water bodies over there. It also does not have any toxic effects over to human while few chemical agents do have ill effects. Due to the presence of natural microbes it activates the soil biology and helps in enhancement of plant growth and yield which has vice versa effect by chemical agents. Also, it has efficient commercial cleaning properties, it repels pest like mosquito. It also cheaper in rate, harmless and natural product (Table 4).

(a) (b)

Fig. 9. Application based on pest repellent and cleaning shown by (a) killed mosquito due to Eco-Enzyme and (b) bathroom tiles before and after use of Eco-Enzyme

The dilution amount required for particular application of Eco-Enzyme

As the Eco-Enzyme produced is in acidic nature it has to be diluted before the use for different purposes because the acidic nature may spoil the texture of things on which it is used. The acidic nature can be used for cleaning the floor, toilets, rust patches. But while cleaning the utensils, pets' body, it needs to be diluted as the surface may rust and cause irritation to pets. Further the plants also require the diluted form as many plants needs slight alkaline or neutral pH, also if soil is too much alkaline acidic nature may use to retain the natural pH of soil that is required (Table 5).

Ecological Significance

The produced Eco-enzyme is a multi usage product of kitchen waste which has ecological significant and is eco-friendly.

CONCLUSION

A critical need of the present day for reliable and

Table 4. Comparison of Eco-Enzyme with other chemical agents with respect to different chemical properties.

Sr. No.	Properties	Eco-Enzymes	Chemical agents
1	Production	From natural components	From chemical components
2	Nature	Acidic to alkaline	Most are acidic
3	Nature towards environment	Eco-friendly	Non eco-friendly
4	Cost	cheaper	Costly
5	Degradation	Degradable	Takes much time to degrade
6	Soil biology	Always activates it	Most of the time deactivates soil bilogy
7	Fumes	No production of fumes	Fumes are produced which are harmul
8	Toxic effects	No toxic effects on human	It fumes or other chemical component may be harmful to human

Table 5. Dilution rate for different usage

Sr. No.	Dilution rate	Usage	Application
1	Concentrated enzyme	Toilets, garden pond, water tank	Pour and clean/flush 2-3 times a week
2	10-15 times	Kitchen sink, black mould, pet house, stove, bathroom tiles	Spray occasionally
3	500 times	Drain, pet	Spray occasionally
4	100-1000 times	Purifying air, deodour, pest control	Spray frequently
5	More than 1000 times	Seedling and fertilizer	Spray occasionally

eco-friendly is fulfilled by Eco-Enzyme. Here we had reported the DIY procedure and low drift approach with loads of use for diminishing nourishment waste and basic and noteworthy strides towards lessening in greenhouse gases. In the present review endeavour was made to explore distinctive uses of Eco-Enzyme by estimation of various chemical compounds in it.

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PRODUCTION, EXTRACTION AND USES OF ECO-ENZYME USING CITRUS FRUIT WASTE: WEALTH FROM WASTE

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***Key words**: Ecoenzymes, Flavonoids, Alkaloids, Quinones, protease, lipase*

Abstract–Eco-enzymes is a complex dark brown colour solution produced by fermentation of fruits waste. It has strong sweet and sour fermented scent due to citrus fruit peels. Eco enzyme produced using fruit peels, water and brown sugar in a ratio 3:10:1. After incubation the filtrate was obtained, we found Flavonoids, Alkaloids, Quinones, Saponins as presence of different metabolites. Its IR spectra showed presence of -OH, COOH group. Also, Amylase, protease and lipase were found in the filtrate. We found applicability as floor cleaning, utensils, gardening, etc. the novel approach for recycle and reuse natural waste would help to reduce fruit waste, it is eco-friendly, economical with multipurpose application.

INTRODUCTION

Eco-Enzyme is a kind of organic compound. It is a complex solution produced by fermentation of fresh kitchen waste such as vegetable and fruit peels. It is type of homebrew vinegar, reduced from alcohol by fermentation of kitchen waste as substrate with sugar (Xia Li, hang Wang, 2013). Eco-enzyme can be prepared generally from citrus fruit peels or kitchen waste. Citrus fruit peels are used due to their distinct properties such as fragrance and sharp flavour, source of vitamin C and also rich in medicinal properties along with high acidity value. The sugar which is added is utilized by microbes; due to their metabolism ozone that is derived may kill the bacteria (Pinang)

Eco-enzyme acts as an anti-fungal, anti-bacterial and insecticidal agent. It may also use as cleansing agent. In the present study we work on production of Eco-enzyme from citrus fruit peels and their different applications.

MATERIALS AND METHODS

Citrus peels were collected from fruit vendors, local fruit juice shop, brown sugar was purchased from local grocery (Maharashtra provisions), water used was normal tap water, all the media and chemical used were purchased from Hi-Media (Mumbai), and reagents used were of AR grade.

Production of Eco-Enzyme

The citrus fruit peels (sweet lime, orange, and lemon) were collected washed thoroughly under running tap water and were chopped into small pieces. Then 500 g of brown sugar was added to 5lts of water and stirred until sugar dissolved completely, to it chopped 1.5kg fruit peels were added. The solution was incubated for 3 months in airtight plastic container. Initially the mixture was stirred daily using glass or wooden rod to release the gas formed by fermentation. Further in second month the solution was mixed once in two weeks followed by once in middle of third month.

Enzyme assay

The crude sample was analysed for various enzyme activities including protease, amylase and lipase.

Enzyme assay Protease

Eco-Enzyme crude sample was serially diluted 10^{-1} to 10^{-4} and spread on Skimmed milk agar plates (skim milk powder-28g/L, yeast extract-2.5g/L, dextrose-1g/L, tryptone-5g/L, agar-15 g/L) (with pH 7.0) and incubated at 37 °C for 48 hrs. the positive isolates were further screened for better production of enzyme by assaying the protease activity in liquid culture using casein as substrate at 37 °C

(Hanan S. Alnahdi, 2012).

Enzyme assay Amylase

Eco-Enzyme crude sample was serially diluted 10^{-1} to 10^{-4} and 0.1 mL sample was spread on each starch nutrient agar plates. After incubation for 1-2 days at 37 °C plate was flooded with Gram's iodine and observed for zone of hydrolysis (Ann kipps and P. H. whitehead).

Enzyme assay Lipases

Eco-Enzyme crude sample was serially diluted 10^{-1} to 10^{-4} and spread plated 0.1 mL on Lipid agar (Glucose-1g, tributyrin-4 mL, NaCl-0.5g, NH_4Cl-4.5g, $MgSO_4$–0.5g, D/W-100 mL, agar-agar-2.5g/100mL) and incubated for 37 °C for 24 hrs. The plates were observed for zone of clearance (Suseela Lanka and J. Naveena Lavanya Latha, 2015).

Identification of metabolites

Tests were carried out to confirm the presence of flavonoids, alkaloids, quinones, cardenolides, and saponins.

Alkaline regent test

The 2 mL of sample was treated with few drops of 20% NaOH solution and observed for change in intense yellow colour to colourless solution on addition of dilute HCL.

Wanger's reagent

The 2 mL of Sample was treated with 3-5 drops of Wanger's reagent (1.27g of iodine and 2g of KI in 100 mL of water) and observed for formation of reddish/brown precipitate.

Keller test

5mL of sample was treated with 2 mL of glacial acetic acid in a test tube and few drops of 5% $FeCl_3$ solution was added to it. This was carefully under layered with 1 mL concentrated H_2SO_4, awaited to form brown ring at interface (which is due to deoxy sugars) characteristics of cardenolides.

Ferric chloride test

A 2 mL of the extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue colour, which shows absence of phenols.

Foam test

2 mL of sample was added in 6 mL of water and was shaken vigorously and observed for formation of foam.

Test for Quinones

2mL of sample was treated with concentrated HCl and observed for formation of yellow precipitate.

Quantification of Acetic acid

The 10 mL of sample was titrated against the titrant 1M NaOH. The few drops of phenolphthalein indicator was added and observed for the formation of pink colour.

Antimicrobial activity

The nutrient agar plates were spread with different microbial suspension (*E.coli, Pseudomonas ssp., Bacillus* spp.) and 1 mL of sample was inoculated using well diffusion method. These plates were incubated for 48 hrs at 32 °C and observed for zone of inhibition.

Effect on plantlet growth

Two containers of soil were taken and labelled as with and without Eco-enzyme, each container added 25 seeds of wheat and 5 mL diluted Eco-enzyme and container kept for plantlet growth.

RESULTS AND DISCUSSION

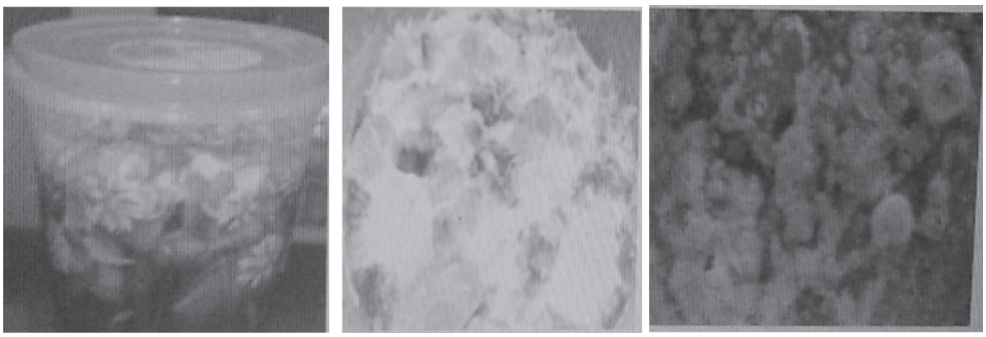
Eco-enzyme solution was produced by using citrus fruit peels. Mostly in this experiment we used citrus fruit peels as in Nanded region they are cultivated on large scale. These citrus fruit peels were collected from various shops of Nanded. The collected peels and juice shop fruit waste survey is follows (Table 1)

Table 1. The survey result of juice shops for peels of citrus fruit (approx. in kg).

Fruit	No. of shops	Fruit waste (kg)	Waste/day
Sweet lime	25	7	175
Oranges	25	6	150
Pineapple	25	4	110
All fruits	25	56	250

After collection of these fruit peels, these were sorted and cleaned and were further used as substrate for Eco-enzyme production. Around 1.5 kg of citrus fruit peels along with brown sugar and water were kept in air tight container and was incubated for 1st cycle of fermentation (Fig 1-a, b, c).

After fermentation of three months, crude



(a) (b) (c)

Fig. 1. Formation of Eco-Enzyme: (a) mixture in air tight container, (b) white layer formed after two weeks, (c) brown crude filtrate after 3 months

solution was filtered and analysed further for presence of different metabolites, enzymes. Based on these tests different application was proposed.

The formation of clear zone around the colonies confirms the production of alkaline protease (Fig. 2a). The zone of hydrolysis and clearance indicates the presence of amylase and lipase enzyme (Fig 2b and 2c).

Further the crude solution was analysed for metabolites presence the intense yellow colour change to colourless solution on addition of dilute HCl acid, indicating Flavonoids presence (Fig 3a). Formation of yellow precipitate was observed, indicating presence of Quinone's (Fig 3b). On shaking vigorously foam formation was observed, indicating Saponins presence (Fig 3c). There was formation of reddish-brown precipitate, indicating Alkaloids presence (Fig 3d). A brown ring at interface shows the presence of deoxy sugar characteristics of cardenolides (Fig 3e).

Antimicrobial activity

The antimicrobial activity was shown against *Pseudomas spp.*, *E.coli*, *Bacillus spp.* Highest antimicrobial activity was shown against *Bacillus spp.* with 18 mm zone of inhibition (Fig 4c). Whereas, 11 mm and 5mm zone of inhibition was shown against *Pseudomas spp.*, *E.coli*, respectively (Fig 4a, 4b)

Quantification of Acetic acid:

The 10 mL Eco-Enzyme contains 0.084 mL of acetic acid which is around 42.25 mL in 500 mL Eco-enzyme thus the pH of Eco-Enzyme is acidic it can be brought to basic upon dilution (Fig 5).

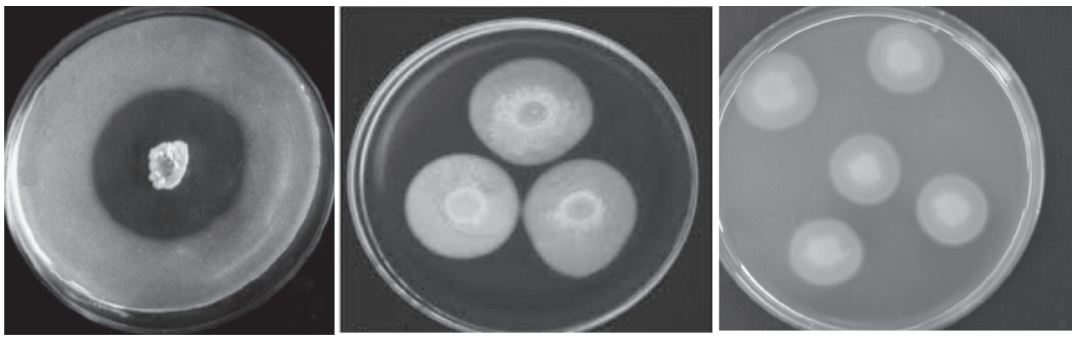
IR Spectra

The crude sample was filtered from membrane filter so that no microbes will enter in liquid. The IR spectra of filtered liquid was performed that represents the presence of -OH and -COOH functional groups (band of 3303.83/cm and the band of 1637.45/cm were observed respectively (Fig 6).

Applications of Eco-enzyme

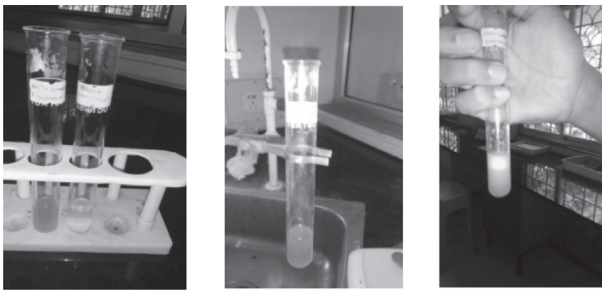
1) Enhanced plant Growth

It was observed that the generation time of seedling was 6 days with Eco-Enzyme while seedlings took 9 days to grow without Eco-Enzyme. Also, seedling



(a) (b) (c)

Fig. 2. Enzyme assay (a) Protease activity, (b) Amylase activity, (c) Lipase activity



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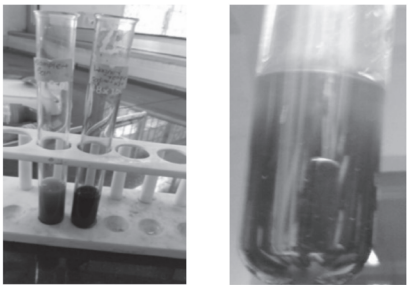
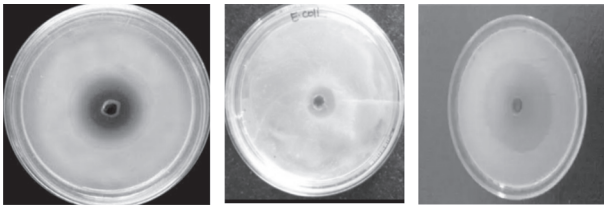


(d) (e)

Fig. 3. Metabolites: (a) Flavonoids, (b) Quinone's, (c) Saponins, (d) Alkaloids, (e) Cardio glycosides



(a) (b) (c)

Fig. 4. Antimicrobial activity against (a) *Pseudomonas spp*., (b) *E.coli*, (c) *Bacillus spp.*

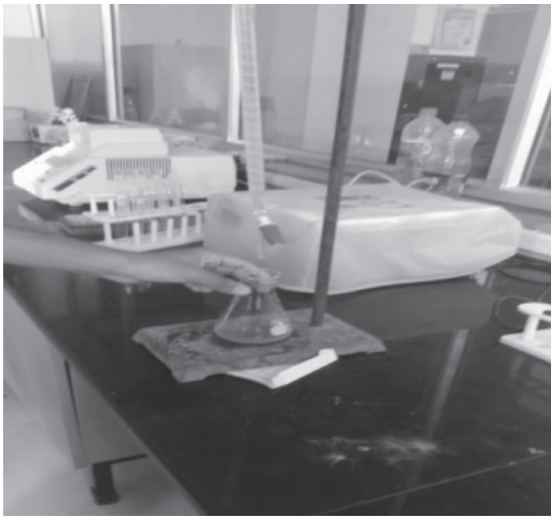


Fig. 5. Formation of light pink colour indicating acetic acid presence.

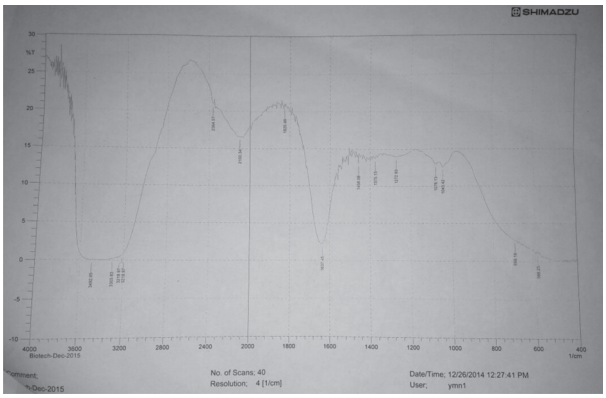


Fig. 6. FTIR spectra of crude filtrate

Table 2. The number of components used in production of Eco-Enzyme

Sr. No.	Components	Cost
1	Brown sugar	50/-
2	Water	0/-
3	Fruit peels	0/-
4	Container	50/-

Table 3. Comparison of Eco-enzyme cost with other available commercial products.

Sr. No.	Commercial products	Market cost/200mL	Eco-Enzyme/ 200 mL
1	Lizol	27/-	10/-
2	Dettol	35/-	10/-
3	Lifeboy	30/-	10/-
4	Vim	18.5/-	10/-

vigour was more with Eco-enzyme than seedling without Eco-enzyme. (Fig 6-a, b, c).

In household application

Eco-Enzyme due to it acidic nature is used in Cleaning of utensils, floor cleaning. Also due to it smell it repels the mosquitos.

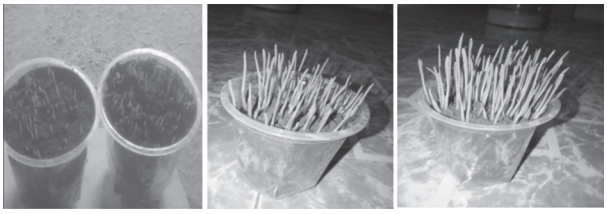
The cost efficiency of Eco-Enzyme

For production of Eco-enzyme citrus fruit peels and other components were used which are available at very cheaper cost (Table 1 and 2).

Whereas, when Eco-enzyme production cost was compared to other commercial products, it was found very cost effective (Table 3).

Eco-Enzyme comparison with commercial cleaning agents

Due to the presence of all-natural raw material



(a) (b) (c)

Fig. 7. (a) Seedling, (b) Seedling + water, (c) Seedling + water + Eco-Enzyme

present in the production it doesn't cause any hazardous or ill effect to environment while the chemical agents get accumulated in the nature and degrade the land over there and also may affect the water bodies over there. It also does not have any toxic effects over to human while few chemical agents do have ill effects. Due to the presence of natural microbes it activates the soil biology and helps in enhancement of plant growth and yield which has vice versa effect by chemical agents. Also, it has efficient commercial cleaning properties, it repels pest like mosquito. It also cheaper in rate, harmless and natural product (Table 4).

(a) (b)

Fig. 9. Application based on pest repellent and cleaning shown by (a) killed mosquito due to Eco-Enzyme and (b) bathroom tiles before and after use of Eco-Enzyme

The dilution amount required for particular application of Eco-Enzyme

As the Eco-Enzyme produced is in acidic nature it has to be diluted before the use for different purposes because the acidic nature may spoil the texture of things on which it is used. The acidic nature can be used for cleaning the floor, toilets, rust patches. But while cleaning the utensils, pets' body, it needs to be diluted as the surface may rust and cause irritation to pets. Further the plants also require the diluted form as many plants needs slight alkaline or neutral pH, also if soil is too much alkaline acidic nature may use to retain the natural pH of soil that is required (Table 5).

Ecological Significance

The produced Eco-enzyme is a multi usage product of kitchen waste which has ecological significant and is eco-friendly.

CONCLUSION

A critical need of the present day for reliable and

Table 4. Comparison of Eco-Enzyme with other chemical agents with respect to different chemical properties.

Sr. No.	Properties	Eco-Enzymes	Chemical agents
1	Production	From natural components	From chemical components
2	Nature	Acidic to alkaline	Most are acidic
3	Nature towards environment	Eco-friendly	Non eco-friendly
4	Cost	cheaper	Costly
5	Degradation	Degradable	Takes much time to degrade
6	Soil biology	Always activates it	Most of the time deactivates soil bilogy
7	Fumes	No production of fumes	Fumes are produced which are harmul
8	Toxic effects	No toxic effects on human	It fumes or other chemical component may be harmful to human

Table 5. Dilution rate for different usage

Sr. No.	Dilution rate	Usage	Application
1	Concentrated enzyme	Toilets, garden pond, water tank	Pour and clean/flush 2-3 times a week
2	10-15 times	Kitchen sink, black mould, pet house, stove, bathroom tiles	Spray occasionally
3	500 times	Drain, pet	Spray occasionally
4	100-1000 times	Purifying air, deodour, pest control	Spray frequently
5	More than 1000 times	Seedling and fertilizer	Spray occasionally

eco-friendly is fulfilled by Eco-Enzyme. Here we had reported the DIY procedure and low drift approach with loads of use for diminishing nourishment waste and basic and noteworthy strides towards lessening in greenhouse gases. In the present review endeavour was made to explore distinctive uses of Eco-Enzyme by estimation of various chemical compounds in it.

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EXTRACTION OF CURCUMIN FROM TURMERIC BY USING SOXHALET UNIT

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ABSTRACT

Economic part of turmeric is rhizomes. Rhizomes are horizontal underground stems. Mostly in India people use turmeric as medicine in Ayurveda as Anti-bacterial, Anti-oxidant, Anti-inflamentary, etc. Curcumin is a colour pigment present in turmeric which gives yellow colour to the turmeric as well as medicinal properties. Curcumin has anti-cancerous medicinal property it has ability to cure the cancer. Hence curcumin can be replaced against expensive medicinal treatment therapy of cancer. Here we are working on extraction of curcumin from turmeric in low cost through biotechnology 3.9% of the curcumin from turmeric extracted using acetone as solvent by soxhlet apparatus method. Indian farmers can sold curcumin as secondary most valuable product compare to turmeric in the market. For Indian farmer curcumin work as an Agri-gold.

Keywords: Turmeric, Rhizomes, Curcumin, anti-cancerous, solvent, low cost, Agri-gold, colour pigment, Soxhlet unit.

INTRODUCTION:

Turmeric plant is mostly found in south asia. Turmeric is medicinal plant. Turmeric is Indian traditional medicinal plant. The scientific name of turmeric is *curcuma longa l.* Turmeric has various medicinal properties such as anti-oxidant, anti-inflamentary, anti-bacterial, etc. Turmeric has yellow colour which obtained due to color pigment curcumin present in turmeric. Curcumin, dimethoxy-curcumin and bisdemethoxycurcumin these three compounds are present in turmeric. Commonly these three compounds called as curcumoid. Curcumin is main compound present in turmeric which gives yellow color to turmeric plant. Curcumin is very expensive but it is anti-cancerous in nature. Curcumin is good supplement to avoid growth of cancerous cells in your body. If we use regularly as a supplement there is no growth of cancerous cells in our body. Cancer cell are present in human body but these are of two types hazardous and non-hazardous. In the region of Nanded district selam, china selam, Krishna, kadappa, etc types of verities of turmeric are present. These verities of turmeric contain 3 % to 4 % curcumin. The aimed of the experiment was to check the presence of curcumin in turmeric by using soxhalet unit [1-7].

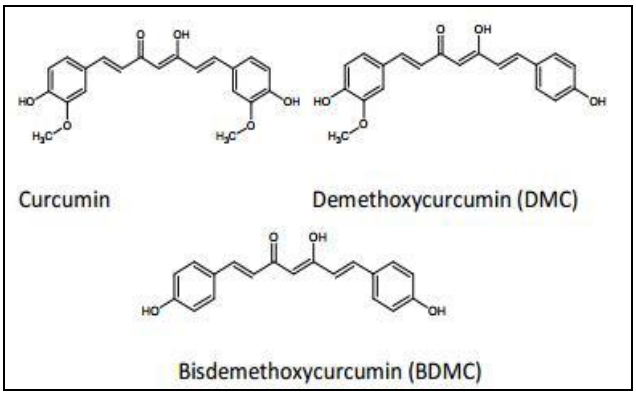


Figure 1: Chemical structure of curcumin and its analogs

SELECTIONOFTURMERIC FOR EXTRACTION:

The salem, china salem, Krishna, these variety of turmeric was selected from the region of Nanded. Fresh rhizomes of turmeric are isolated. Wash the rhizomes and cut into small pieces. Keep this small cut of turmeric in oven for drying at 105°C for 3 hours or in refrigerator. Make fine powder of turmeric for extraction.

Doi: 10.2016-53692176; DOI Link :: http://doi-ds.org/doilink/10.2019-81369613/

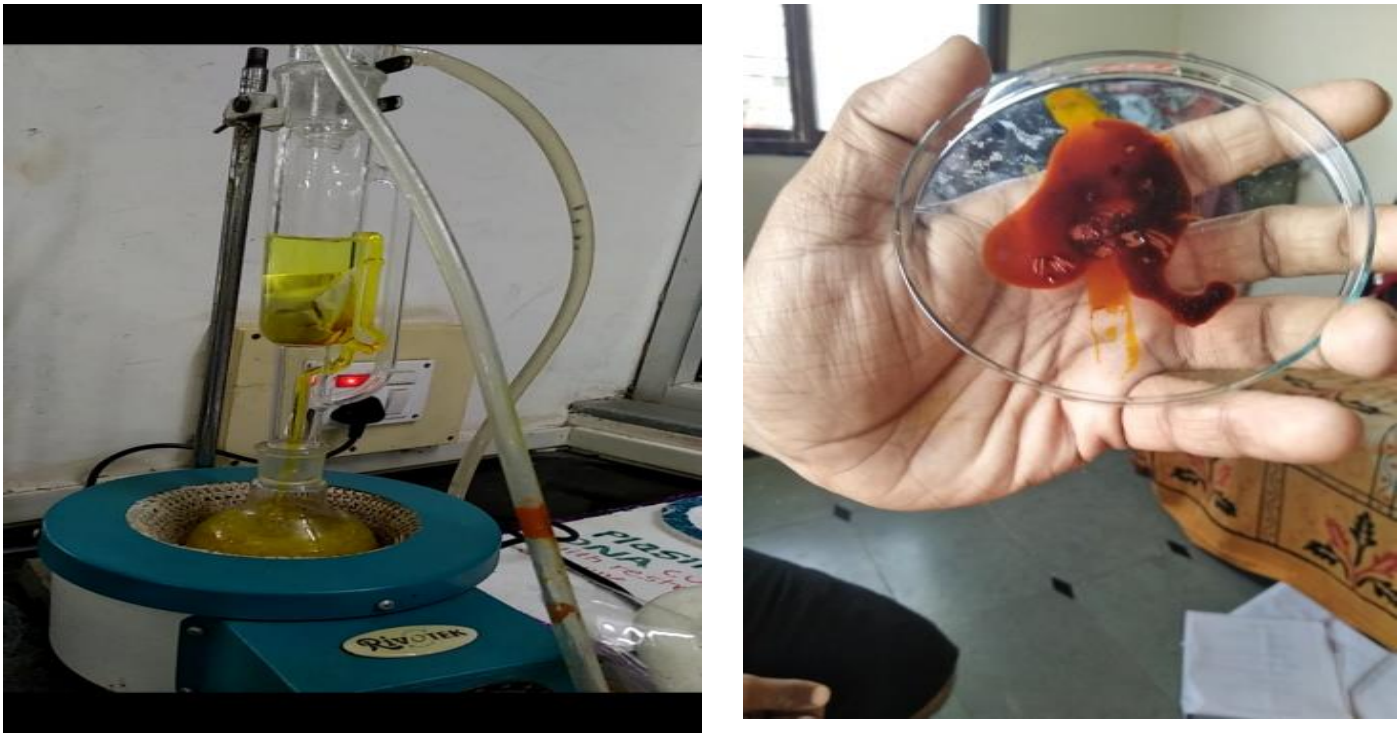
Figure 2: turmeric sample used for experiment

SELECTION OF SOLVENT FOR EXTRACTION

Use organic solvent for extraction because these are volatile in nature they can be evaporating easily. In organic solvent curcumin easily dissolved. Avoid use of water because the solubility of curcumin in water is very low. For 100gm of curcumin extraction 1500ml organic solvent is required.

METHOD FOR EXTRACTION:

Take fleshy rhizomes of turmeric. The rhizomes of turmeric were dried in oven at 105 °C for 3 h. dried rhizome were grinded and obtained uniform powder. The turmeric powder was stored in refrigerator to prevent moisture uptake. 10 g turmeric powder was weighed and embedded in a thimble and kept in the Soxhlet apparatus which was gradually filled with acetone as the extraction solvent. The extraction was carried out at 60 °C within 8 h. After the extraction, the acetone was separated from the extract using rotary evaporator under vacuum at 35°C. The residue was dried and weighed. Presence of Curcumin content was checked by using TLC.



A **B**

Figure 3: A; Soxhalet unit and B; extracted curcumin

DETECTION OF CURCUMIN BY USING THIN LAYER CHROMATOGRAPHY:

Acetone extract were tested in TLC for presence of three curcumoids. The TLC pre-coated silica gel (0.25mm thick) plate were developed using a spreader. Glass tank which was pre-saturated with the mobile phase for 1 hour and each plate was developed to a height of about 10cm. The composition of mobile phase was optimized in ratio 8:2:2. After development plates were removed and dried and spots were observed under UV light.

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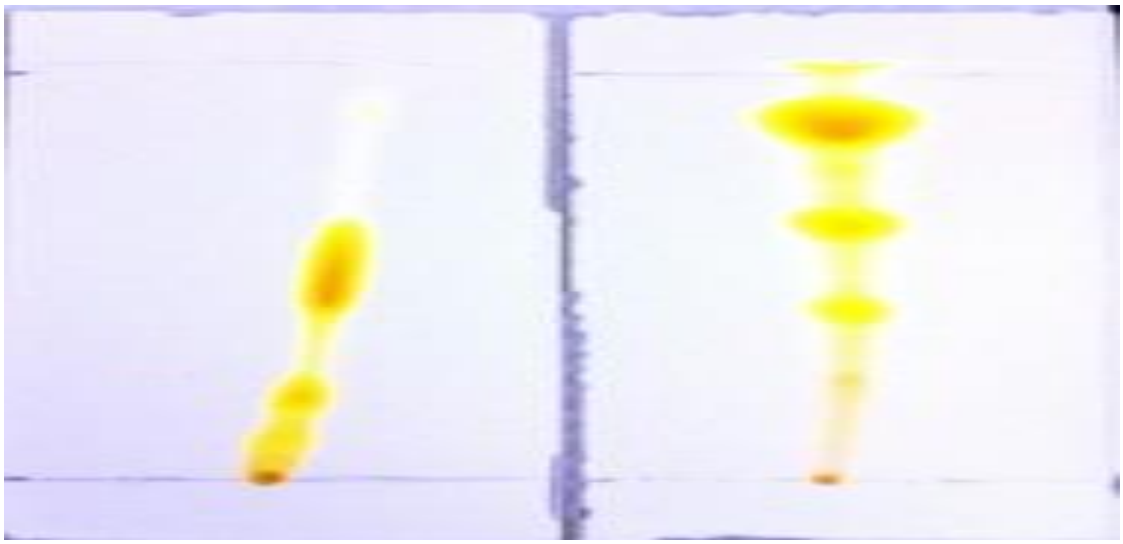

A **B**

Figure 4: TLC of isolated curcuminoids

$RF = \frac{\text{Distance travel by solute}}{\text{Distance travel by solvent}} = \frac{6.8}{9.2} = 0.73$, Therefore the *Rf* value of curcumin is 0.73 approximately.

APPLICATION:

1. **BENIFITS TO COMMON PEOPLE/ PATIENT**
   - Curcumin has anticancer property.
   - Antibacterial, Antioxidant activity
   - Low cost of medicin for cancer treatment.
   - Working of Curcumin in cell

2. **MEDICINAL USE**
   - Anti-cancerous
   - Anti-oxidant
   - Anti-inflammetry
   - Antibiotic
   - Boosts effectiveness of cancer therapies.
   - Curcumin shows antimicrobial activity against bacteria (*streptococcus, staphylococcus, lactobacillus, etc)*
   - Curcumin decreases blood cholesterol [4-7].

EXTRACTED CURCUMIN:

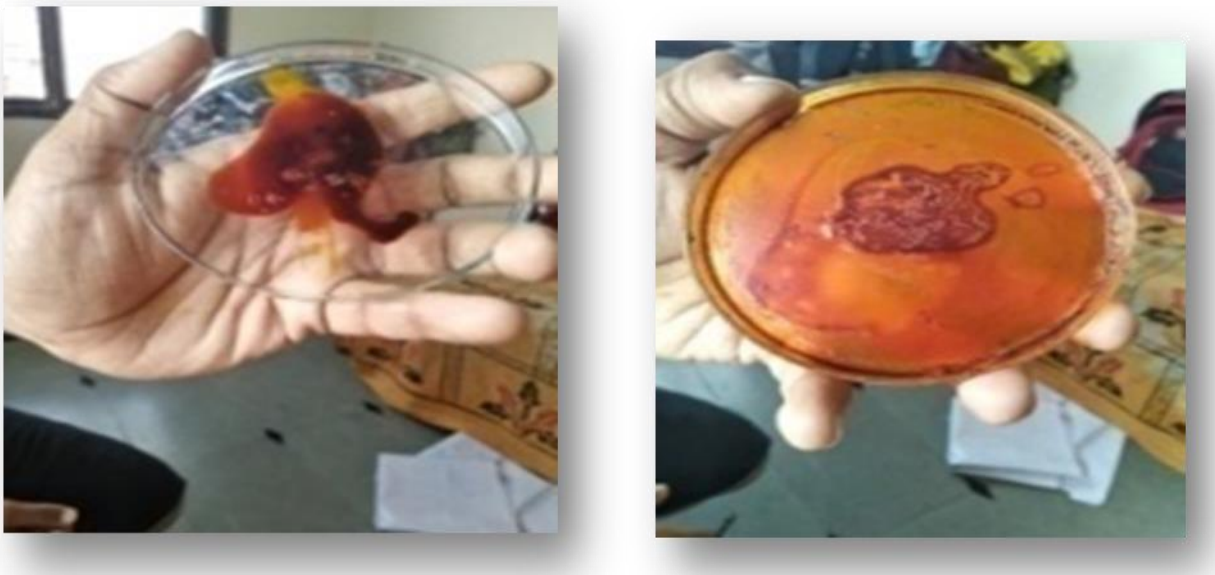

Figure 4: Extracted curcumin

Doi: 10.2016-53692176; DOI Link :: http://doi-ds.org/doilink/10.2019-81369613/

RESULT:

$$\%\ of\ curcumin = \frac{\text{Dry wt. of extracted curcumin}}{\text{total wt. of turmeric}} \times 100$$

Table 1: Extraction of curcuminoid using Soxhlet METHOD

Variety	Solvent	Curcuminoid (%)
Selam (10 gm)	Ethanol	3.6
	Methanol	3.4
	Acetone	3.9
Kadappa (10 gm)	Ethanol	3.1
	Methanol	3.7
	Acetone	3.8
China selam (10gm)	Ethanol	3.3
	Methanol	4.0
	Acetone	4.2

CONCLUSION:

Different solvents with different polarity were used for the extraction of curcumin from turmeric rhizomes. Various solvents were used such as acetone, ethanol, methanol, etc. after concentrating each extract total yield were calculated and determine the percentage of curcumin from each extraction as shown in **table 1**. By this experiment it was concluded that in the turmeric approximately 3 % to 4 % curcumin is present. From the overall result it was observed the more yield of curcumin was obtained in acetone solvent extraction. After extraction yellow coloured extract was obtained, that extract it was called as curcumoid. After the extraction sample turmeric losses their colour and it becomes white in colour.

AKNOLEDGEMENT:

I hereby declared that the total work of extraction of curcumin was done under guidance of Dr. M. N. Cherekar, Head of department of biotechnology and bioinformatics, Assistant prof. Mr. P. N. Narwade, Assistant Prof. Vinay Hibare at Laboratory of Mgm's college of Computer science and Information technology, MGM Campus, Nanded-431605.

RERFRENCE:

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Production of Levansucrase from a Soil Isolate *Bacillus Subtilis* (DQ922949)

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Abstract
Levansucrase is a fructosyl transeferase, which brings hydrolytic breakdown of sucrose to glucose and fructose. The fructose was polymerized to form levan. Levan a biologically active polymer has potential application in the field of food as a low calorie sweetener, cosmetics and in pharmaceuticals as antiviral, anti- diabetic and antitumor agent. In this context the present work brought screening of 11 soil isolates for levan production. Maximum levan producing isolate (25.8g/L) was identified by 16S rRNA as *Bacillus subtilis* (DQ922949). Maximum enzyme activity was found to be 1250 U/ml. The optimum temperature and pH for levansucrase activity were 60^{0}C and 7.0 respectively. Enzyme showed maximum activity at 0.8M sucrose concentration. Different metal ions K^+, Mg^{+2}, Fe^{+3}, Mn^+, $Hg^{+2}$and Ca^{+2} showed inhibitory effect on enzyme activity. The enzyme was partially purified by ammonium sulfate precipitation.
Keywords: Sucrose, Levansucrase, transferase, levan, *Bacillus subtilis* (DQ922949)

Introduction

Levansucrase (**EC 2.4.1.10)** is an extracellular enzyme and produced by both Gram +ve bacteria like *Bacillus sp.* **(Dedonder, 1966**), *Lactobacillus sp* (**Van Hijum *et al.*, 2001**) and Gram –ve bacteria such as *Zymomonas mobilis* (**Dawes *et al.*, 1966**), *Halomonas sp.*(**Poli *et al.*, 2009**), *Erviniamyl ovora* **(Ordax *et al.*, 2010),** *Pseudomonas syringae* (**Visnapuu *et al.*, 2011);** *Streptomyces sp* (**Carlsson *et al.*, 1970**) and *Actinomyces viscosus* (**Pabst *et al.*, 1977**). The enzyme catalyses the synthesis of polysaccharide levan (β-2, 6 linked fructan) from sucrose. Levan is a bioactive polymer with high market value. It has potential application in the field of food industry as viscofier, stabilizer, emulsifier, gelling and binding agent (**Yoo et al, 2004**); in pharmaceuticals as low calorie sweetener, antiviral **(Hermandez *et al.*, 2006),** antidiabetic (**Esawy *et al.*, 2011**) and antitumor (**Dahech I *et al*, 2011**) agent and in cosmetic industry as carrier for fragrances and surface finishing agent (**Beine *et al***. 2008 and **Shih *et al***. 2005). It has prebiotic properties and excellent water holding capacity. Hence microbial levansucrases are of high interest as biocatalyst for catalytic synthesis of levan.

Levansucrase is grouped into glycoside hydrolase 68 family due to its substrate specificity, by the data-base 'carbohydrate-active enzymes' **(Chambert *et al.*, 1976).** It catalyzes two different reactions- hydrolysis of sucrose when water is used as the acceptor and transglycosylation to form fructose polymers releasing glucose **(Ozimic *et al*, 2006**). It has ability to directly use the free energy of cleavage of non activated sucrose to transfer β-D fructosyl units to different acceptors (eg. sucrose, levan) with (β-2, 6) linkage.

The present study is aimed at screening of high levansucrase producer from soil and to study levansucrase activity by optimizing the different parameters like pH, temperature, sucrose concentration and effect of metal ions.

Materials and Methods

Screening of Levansucrase producing bacteria

Soil samples were collected from sugarcane field and the area where jaggery was prepared from sugarcane. Soil samples were inoculated in 5% and 10% sucrose containing medium. The tubes were incubated at room temperature for 24 hrs. A loop full from the tubes showing turbidity was streak inoculated on 10 % sucrose agar plates. Plates were incubated at 30^0C for 24 hrs. Organisms producing mucoid colonies were selected as levansucrase producer. The isolates were sub cultured on nutrient agar slants and incubated at 30^0C for 24 hrs .The slants were maintained and preserved at low temperature and used for further studies.

Levansucrase production

Inoculum preparation

24 hr. old cultures of all the isolates were prepared from preserved slants. A loop full of culture from 24 hr. old nutrient agar slant of each isolate was inoculated in 5 ml of inoculum medium containing (g/L) sucrose 100, yeast extract 2, $(NH_4)_2SO_4$ 1, KH_2PO_4 2, $MgSO_4$ 0.5. The inoculated medium was incubated at 30^0C for 24 hr.

Production medium

5% of inoculum was transferred aseptically to 50ml production medium in 250ml Erlenmeyer flask. The production medium had following composition (g/L) sucrose 100, yeast extract 2, $(NH_4)_2SO_4$ 3, KH_2PO_4 1, $MgSO_4$ 0.6, $MnSo_4$ 0.2.. The pH was adjusted to 7 before autoclaving. The inoculated production medium was incubated at 30^0C for 48 h on rotary shaker at 100rpm.

Extraction of Levansucrase

After incubation period the fermented broth was centrifuged at 10000 rpm for 10min at 4^0C in a cooling centrifuge. The clear supernatant was considered as crude enzyme source and used for levansucrase assay (**Senthikumar and Gunasekaran , 2005).**

Levansucrase assay

The enzyme reaction was initiated by adding 250µl enzyme extract to 250µl of 1M sucrose suspension prepared in 0.2M sodium phosphate buffer **(Senthikumar and Gunasekaran, 2005).** To this 500µl o of sodium phosphate buffer of pH 7 was added. The mixture was incubated at 40^0C for 60min. Two blank assays without the substrate or without the enzyme were prepared in tandem for the trials. All assays were run in triplicate. The concentration of released glucose was measured by dinitrosalicylic acid method **(Somogyi, 1952).**

Levan production

The clear broth obtained was then checked for presence of exopolysaccharide Levan by adding four volumes of chilled aqueous ethanol (70%). The precipitate formed was allowed to settle down at 4^0C for 24hrs. The pellet washed twice with aqueous ethanol and transferred to vial and dried at 60^0C till it was dried. **(MC-Neial and Kristiansen, 1990; Thomas and Reedhamer, 1994).**

Levan detection

2ml of 0.1N HCL was mixed with 2ml of levan solution and boiled at 100^0C for 60min. The hydrolysate obtained was analyzed for fructose and glucose estimation. Fructose estimation was carried by Resorcinol method **(Ashwell , 1957; Vikari and Gisler, 1986)** and glucose was estimated by **Somogyi** method.

TLC for levan

Acid hydrolyzed sample of levan was prepared and used for TLC. Silica Gel plates saturated with solvent 1 Butanol: 2 propanol: water: acetic acid (7:5:4:2) were used. Sample was spotted along with standards, Fructose (Hi media Pvt. Ltd.) and Glucose (Hi media). Plates were sprayed with 5% sulfuric acid in methanol, air dried and then heated at 110^0 C until spots appeared.

Identification of high levan producing strain by 16S rRNA sequencing

The high levan producing strain was identified by 16S rRNA from **ARI Pune**. Genomic DNA of isolate was isolated using Gene Elute Genomic DNA Isolation Kit (**Sigma, USA**) used as template for PCR. Each reaction mixture contained approximately 10ng of DNA; 2.5mM $MgCl_2$; 1x PCR buffer (**Bangalore Genei, Bangalore, India**); 200µM each dCTP, dGTP, dAPT, and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase (**Bangalore Genei, Bangalore, India**) in a final volume of 20µl. FDD2 and RPP2 primers were

used to amplify almost entire 16S rRNA gene, as described previously (**Rawlings 1995**). The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94^0 C for 5 min; 30 cycles of 94^0 C, 60^0C and 72^0 C for 1min each; and final extension at 72^0 C for 10min, and the mixture was held at 4^0 C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%), washed thrice using 70% ethanol and dissolved in Tris-HCL (10mM, pH 8). Samples were run on ABI Prism 3100 Genetic Analyser. The sequencing output was analyzed using the accompanying DNA Sequence Analyzer computer software (**Applied Biosystems**). The sequence was compared with **NCBI GeneBank** entries by using the **BLAST** algorithm.

Optimization of levansucrase activity

Effect of pH on levansucrase activity

Effect of pH on levansucrase activity was determined by incubating 250µl Levansucrase extract suspension to 250µl of 1M sucrose suspension prepared in 0.2M sodium phosphate buffer (**Senthikumar and Gunasekaran, 2005**). To this 500µl of sodium phosphate buffer of pH 4, 5, 6, 7, 8 and 9 were added. All the tubes were incubated at 40^0C for 60min using standard assay conditions.

Effect of temperature on levansucrase activity

Optimum temperature for levansucrase activity was determined by incubating the reaction mixture at different temperatures 10^0C, 20^0C, 30^0C, 40^0C, 50^0C, 60^0C and 70^0C at pH 5 for 60min. in 0.2M sodium phosphate buffer.

Effect of sucrose concentration on levansucrase activity

250µl of enzyme mixture was incubated with different sucrose concentrations ranging from 0.2M, 0.4M, 0.6M, 0.8M, 1.0M, 1.2M and 1.4M in 0.2M sodium phosphate buffer at pH 5, temperature 40^0 C for 60min. The glucose released was then quantified.

Effect of metal ions on levansucrase activity

Enzyme 250µl was incubated with one mmol/l solution of K^+, Mg^{+2}, Fe^{+3}, Mn^+, $Hg^{+2}$and Ca^{+2} in 0.2M sodium phosphate buffer at pH 5 was pre-incubated with the enzyme for 10min. Cations were applied as chloride salts. The reaction was started by addition of buffered sucrose. Control was incubated with distilled water.

Purification of Levansucrase:

Purification was carried by using ammonium sulfate. Different concentrations of ammonium sulfate used were 20%, 35%, 55%, and 70%, 90%. The saturated solution kept overnight at 4^0 C and precipitate obtained was collected by centrifugation at 10000 rpm for 20min.

Estimation of protein content

The protein content of the enzyme was determined by **Folin-Lowry** method (**Lowry, 1951).**

Results and Discussion

In the current study 11 isolates producing mucoid colonies on 10% sucrose agar plates were selected as levansucrase producer. All the isolates were mucoid colony producers and Gram positive in nature. **Vaidya and Prasad (2012**) and **Ghale *et al* (2007)** isolated levan producers reported similar type of colonies on sucrose agar plates. Levan production from all these isolates was carried in levan production medium. After 48hr of incubation at 100rpm, the fermented broth centrifuged and used to precipitate levan. The obtained levan dried at 60^0C till constant weight and measured **(Table-1).** Concentration of levan produced by all the isolates were above 10mg/ml. Isolate SC1, SC3, SC4, SC9, and SC12 isolated from sugarcane field produced 11.5mg/ml, 10mg/ml, 12mg/ml, 10.7mg/ml, and 13.3mg/ml levan while the isolates SJ1, SJ2, SJ3, SJ5, SJ7 and SJ9 obtained from area where jaggery was prepared produced 10.9mg/ml, 11.6 mg/ml, 14mg/ml, 15.4 mg/ml, 12.4 mg/ml and 25.8mg/ml. Levan produced by these isolates was confirmed by fructose and glucose estimation (**Table -1**). High levan producer SJ9 was selected for levansucrase production and identified by 16S rRNA sequencing as *Bacillus subtilis* (DQ922949). **Esawy *et al* (2011)** isolated six strains of *Bacillus subtilis* from honey samples and carried levansucrase production at optimum conditions, the highest levansucrase activity observed was between 62 and 59 U/ml. **Goncalves *et al*** studied levansucrase production from *Bacillus subtilis* Natto CCT7712, and reported enzyme activity 8.53 AU/ml at optimized conditions.

Table: 1 Levan production from screened iosolates

Sr. No.	Isolate	Levan (mg/ml)	Fructose (µg/ml)	Glucose (µg/ml)
1	SC1	11.5.	266	00
2	SC3	10.0	220	00
3	SC4	12.0	285	00
4	SC9	10.7	230	00
5	SC12	13.3	310	00
6	SJ1	10.9	238	00
7	SJ2	11.6	275	00
8	SJ3	14.0	345	00
9	SJ5	15.4	375	00
10	SJ7	12.4	300	00
11	SJ9	25.8	580	00

Each value represents the mean SD of 3 replications

TLC of levan

Sugar components of levan were identified by TLC analysis of levan hydrolyzate. The Rf value of acid hydrolyzed levan from *Bacillus subtilis* (DQ922949) was identical to that of standard fructose (Hi Media Pvt. Ltd.) under laboratory conditions. This result indicated that the obtained compound was levan.

Optimization of levansucrase activity:

Effect of pH on levansucrase activity

Initially at pH 4 the levansucrase activity was lowest as shown in **Fig 1**. Enzyme activity increased steadily at pH 5 and reached to more than 72% of its maximum activity, while at pH 6 activity became more than 85%; the activity reached to highest at pH 7. Beyond pH 8 it reduced markedly. Similar results were reported by **Vinity and Theertha (2012)** who observed maximum levansucrase activity from *Bacillus subtilis* BB04 in pH range of 6-7. Also **Belgith *et al* (2012)** reported optimum pH of 6.5 and **Goncalves *et al* (2013)** reported optimum pH 7.5 for levansucrase from *Bacillus* species. The current study reports activity range of levansucrase at pH (5.5 -7.3) was similar to pH range reported in literature to levansucrase from *Bacillus* species **(Homan *et al,* 2007).** Optimum pH range for most levansucrase studied was between 5.5 and 6.0. **(Yanase *et al,* 1992; Ammar *et al,* 2002)** but the enzyme reported in current study showed activity at broad pH 7 range.

Fig.1

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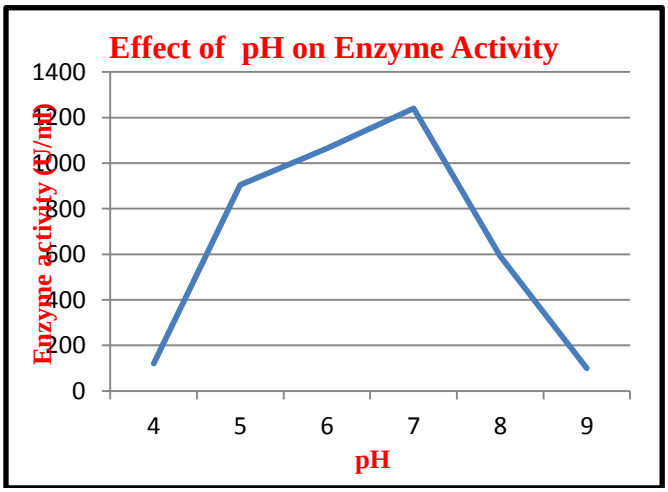

Effect of temperature on levansucrase activity

At 10^0C the activity was very low and reached to 52% of its highest activity at 20^0C. It increased gradually from 40^0C and 50^0C. The activity became highest at 60^0C **(Fig. 2)** and beyond this it falls to 58%. Similar results were reported by **Ammar *et al* (2002)** who found highest levansucrase activity at 60^0C from *Bacillus* sp TH4-2. **Goncalves *et al* (2013)** reported highest levansucrase activity of 5.38U/ml at 50^0C which found much less than the activity reported in this study. The optimum temperature for most of the levansucrase reported ranges between 30^0C to 50^0C, the current study reports enzyme activity range from 40^0C to 60^0C which allows it to work

from mesophilic to thermophilic range. Thus the enzyme works at broader temperature range as compare to other enzymes reported.

Fig.2

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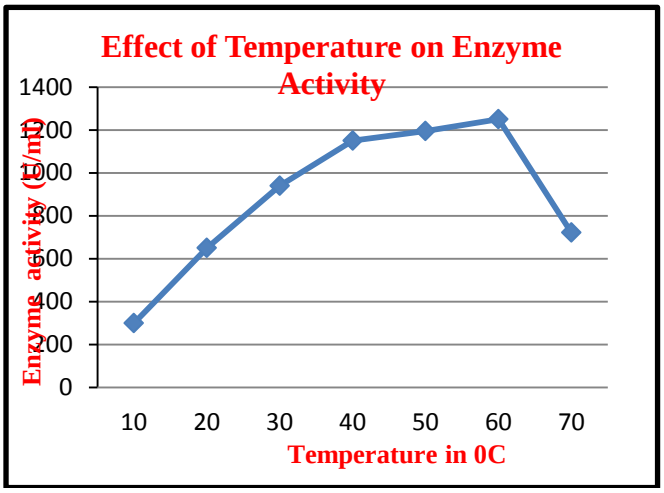
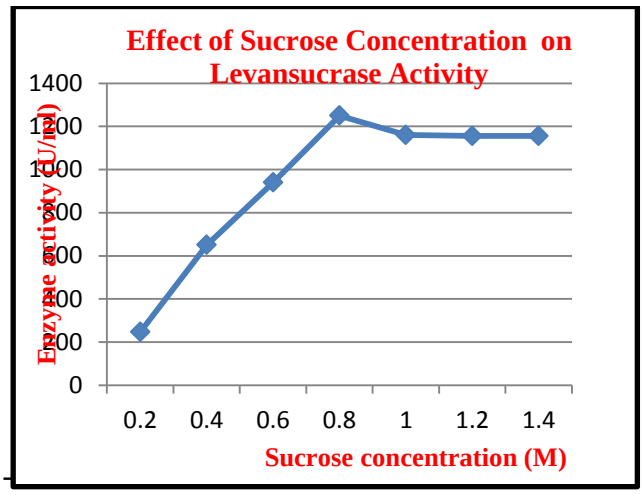


Effect of sucrose concentration on levansucrase activity

At substrate concentration of 0.2M the enzyme activity was 44% of its maximum activity; it became 76% at 0.6M and became highest at 0.8M sucrose. From **(Fig.3)** it is clear that the kinetics of levansucrase from *Bacillus subtilis* (DQ922949) follows **Michaelis - Menten** model. The **Michaelis** constant (Km) for sucrose hydrolyzing activity was determined under optimal conditions and found to be 186mM sucrose. These results was higher than that reported by **Gay *et al* (1983)** and **Homann *et al* (2007)** who studied levansucrase and reported Km values for sucrose hydrolysis was 160mM from *B. subtilis* and 4.0mM for *B. megaterium* respectively.

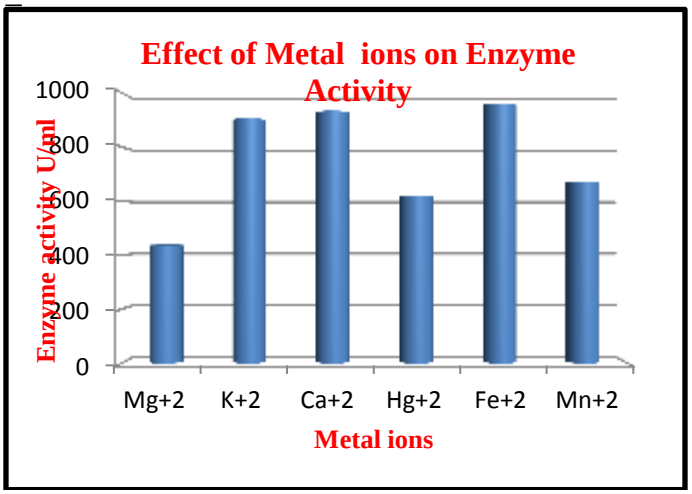
Fig-3



Effect of metal ion on levansucrase activity

As depicted in **(Fig 4)** about 75% enzyme activity was restored with both monovalent cation K^{+} and divalent cations Ca^{+2} and Fe^{+2}. While with Mn^{+2} and Hg^{+2} enzyme levansucrase restored more than 50% of its highest activity. Divalent cation Mg^{+2} inhibited levansucrase activity greatly. However the results reported by **Belgith *et al* (2012)** were different from the results of this study; the levansucrase activity from *Bacillus* species was fore fold times increased with Fe^{+}. **Vinity and Teertha (2012)** reported inhibition of enzyme activity by Fe^{+3}, Mn^{+2}, Ca^{+2} and Hg^{+2} as reported in this study. Thus the literature shows conflicting results regarding the effect of different salts on enzyme activity. This might be due to minor difference in active site composition of enzyme depending on the source.

Fig.4



Partial purification of Levansucrase:

The several protein fractions were obtained after adding ammonium sulfate. Maximum activity of enzyme was obtained in the fraction of 70% ammonium sulfate saturation. The specific activity of levansucrase in fractions obtained after 70% ammonium sulfate saturation was 126.26 U/mg. Hettwer et al (1995) obtained 153 U/mg specific activity for levansucrase obtained from *Pseudomonas syringae.*

Conclusion

Bacillus subtilis (DQ922949) found to be an efficient levansucrase producer. The optimal conditions for enzyme activity were pH 7 and temperature 60^0C with 0.8M sucrose. The enzyme activity found to be 1250 U/ml. Different metal ions like chloride salts of K^+, Mg^{+2}, Fe^{+3}, Mn^+, $Hg^{+2}$and Ca^{+2} showed inhibitory effect on levansucrase activity; Mg^{+2} found more inhibitory while K^+ was least inhibitory. The organism showed efficient levan conversion and the enzyme showed activity in broad temperature and pH range. In addition the levansucrase from *Bacillus subtilis* (DQ922949) can be considered thermostable.

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Thermostable pectinase mediated enhanced antimicrobial activity of *Emblica Officinalis*: A novel application

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Four efficient pectinase producing thermophiles were isolated from Unhala hot water spring (16°35' N, 73°30' E) of Rajapur located in Ratnagiri district of Maharashtra. The isolates were identified as *Bacillus licheniformis, Bacillus cereus, Bacillus Firmus, and Bacillus brevis* using morphological and biochemical techniques. Being an efficient pectinase producer, *Bacillus licheniformis* was selected for pectinase production and further identified using 16s rRNA molecular analysis and sequence was deposited to GenBank nucleotide repository under accession number KT373817. Pectinase produced by *Bacillus licheniformis* using optimized condition was partially purified and molecular weight was determined as 66kda. Characterization of partially purified pectinase was carried out where it showed remarkable pectinolytic activity at 80^{o}C temperature and pH 10.0. The extracted pectinase showed stability in presence of solvents and surfactants such as chloroform, ethanol, butanol, acetone, Tween-80 and Triton X-100 at 1% (v/v) concentration. Enhanced pectinolytic activity was observed in presence of Fe^{3+} ions (10 mM). Increase in antimicrobial activity of amla (*Emblica Officinalis*) was recorded after treatment with partially purified pectinase as a novel application for the first time.

Introduction

Thermophiles are the microorganisms that have been found in extremely hot environment. Such environment is thus of great interest to scientists, as the organisms isolated from such environment are good source of thermostable enzymes which shows most stability under condition of high temperature[1]. They have extremely thermostable cellular components; it gives them unique metabolic capabilities which offer considerable promise for biotechnological applications[2]. Among industrially important enzymes, pectinolytic enzymes have attracted the major attention globally as they have wide application in industrial field, such as food and juice processing, textile processing, degumming of plant fiber, agro waste treatment, oil extraction, paper pulp treatment, waste water treatment, maceration of tea leaves and processing of cotton fabrics[3].

In this context, we have explored an ancient Unhala thermal spring located at Rajapur in Ratnagiri district of Maharashtra state of India (16°35'N Latitude, 73°30' E Longitude) for isolation of thermostable pectinase producer. Various thermostable pectinase producers were isolated and characterized from this spring. Pectinase extracted from isolate was thoroughly assessed for optimization and its application.

Materials and Methods

Water sample of Unhala thermal spring was collected in pre-sterilized plastic container and transported to laboratory within 24 hours. The sample was spread on three different media, namely, nutrient agar, thiosulphate agar, glucose yeast extract (GYE) and incubated at 60 ^{o}C for 24 hours. For further experiments, the medium that showed the highest diversity was used[4, 10]. Amongst four distinct isolates were selected and screened for pectinase production by spot inoculation method on pectin agar plate containing 1% pure pectin, 0.2% KH_2 PO_4, 0.06% K_2HPO_4,$MgSO_4.7H_2O$ by observing zone of clearance[5,6]. Selected isolates were further identified using morphological and biochemical characteristics. Indole, citrate utilization, catalase, oxidase and H_2S production tests were carried out. Hydrolysis of starch, urea, casein, pectin, cellulose and tween 80 was observed. Carbohydrate, vogues-proskauer, and methyl red utilization tests were performed using standard procedures. Appropriate positive and negative controls were used in all these tests. Antibiotic susceptibility was tested by disc diffusion method. Optimization of temperature and pH on growth of organism was determined by varying temperature in the range of 30 to 80^{o}C and with on

increment of 10°C and pH in range of 4 to 10 with increment of 1 unit[7].

RJ1 was further used for pectinase production and identified using 16s rRNA partial gene sequencing method. In this method, DNA was extracted from bacteria Insta crene matrix (Bio-Rad USA) treatment and 16s DNA was amplified into thermo cycler (Applied Bio system, USA) with pair of primers 27F AGA GTT TGA TCM TGGCTCAG,1492RTA CGGYTACCTTGTTACGACTT,[14]785FGGATTA GATACCCTGGTA,907RCCGTCAATTCMTTTRA GTTT[15] .The amplified 16s rDNA PCR product was gel purified with a QIA quick cres extraction kit (Qia-gen, USA) and sequenced in an ABI prism TM377 automated DNA sequencer (Applied Bio system, USA) sequence. Similarly, search was done for 16s r DNA sequence using online search tool called BLAST (http://www.ncbi.nim.nih.gov/blast). Phylogenetic tree was constructed from evolutionary distances using MEGA 6.06 software[8, 9, 10, 11].

Fermentation medium with 1% pure pectin, 0.2% KH_2PO_4, 0.06% K_2HPO_4, $MgSO_4.7H_2O$ was used for production of pectinase. After production, fermented broth was centrifuged at 10,000 rpm and 4 °C for 10 min, 70% ammonium sulphate was added in cell free supernatant for enzyme precipitation. Enzyme precipitate was collected and dialyzed against 0.2 M glycerine-NaOH buffer (pH-10.0) and it was used as partially purified enzyme[12, 13].

Effect of carbon, nitrogen sources, pH, temperature and incubation period on production of pectinase was also assessed. The parameters tested for maximum pectinase production were carbon resources in 0.5% (w/v), such as glucose, lactose, maltose, fructose, sucrose, D-galactose, and D-ribose. Nitrogen sources in 0.5% (w/v) such as peptone, ammonium sulfate, gelatin, ammonium nitrate, urea, potassium nitrate, and sodium nitrate. Phosphorus sources in 0.5% (w/v), such as sodium di-hydrogen phosphate, disodium hydrogen phosphate, potassium hydrogen phosphate, di-ammonium hydrogen phosphate, ammonium hydrogen orthophosphate and some food wastes were used as a substrate sources in 1% (w/v), such as coconut peel, orange peel, banana peel, and pomegranate peel[15].

Molecular weight of partially purified pectinase was determined in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) after comparison with standard broad range protein markers (Merck Biosciences)[16].

Partially purified pectinase was characterized for its catalytic activity at various parameters like temperature ranges of 30°C to 90°C with an increment of 10°C, pH ranges from 4-13 with increments of 1 unit, substrate concentration at 0.5% to 4% with 0.5% increment and incubation time at 30 to 120 min with 30 min interval.

Effect of selected solvents (1%), such as chloroform, ethanol, butanol, hexane, glycerol, benzene, acetone, isopropanol, and other additives, such as Na_2-ethylene diamino tetra acetic acid, sodium dodecyl sulphate, tween 20 and tween 80 was tested on catalytic activity of partially purified pectinase. The effect of various metal ions was also studied on pectinase enzyme activity by using 10 mM metal ions, such as Mg^{2+} ($MgCl_2$), Zn^{2+} ($ZnCl_2$), Fe^{3+} ($FeCl_3$), Cu^{2+} ($CuCl_2$) and Na^+ (NaCl)[7,13,14].

Partially purified thermostable pectinase derived from RJ1 was tested to determine its role in enhancement of antimicrobial activity of amla (*Emblica officinalis*) powder after treatment with it. In this experiment, extract of preheated amla powder (1 g in 50 ml D/W) was treated with 1% of pectinase enzyme for 15 min and 30 min, separately. The antimicrobial activity was assessed against fungi, namely, *Aspergillus niger, Aspergillus flavus, penicillium crysogenum, Aspergillus nodulans, Aspergillus oryzae, and Aspergillus parasiticus* and bacteria, namely, *Bacillus licheniformis, Escherichia coli,* and *Pseudomonas* species. Nutrient agar plates and potato dextrose agar plates were used while cultivating cultures. A spread well plate method was used to observe antimicrobial activity of mixture of amla powder extract and pectinase enzyme. Antimicrobial activities were evaluated by measuring the inhibition zone diameters. Untreated amla powder was used as a control for this experiment.

Results and Discussion

The water sample from Unhala thermal spring was clean, odorless, and alkaline having pH 8 with temperature 44 °C (Fig 1).

Among the three selected media, nutrient agar showed diverse morphotypes with appearance of 72 CFU. Of these four morphological distinct colonies developed zone of clearance around well in pectin agar plate and designated as RJ-1, RJ-2, RJ-3 and RJ-4.

On the basis of morphological characteristics, microscopic features, sugar utilization patterns, enzyme profile, antibiotic sensitivity and physiological features, the isolates were identified as *Bacillus licheniformis, Bacillus cereus, Bacillus Firmus, Bacillus brevis.*

Out of these four, a fast growing and efficient thermostable pectinase producer RJ-1 was selected for further analysis. The phenotypic, biochemical and

physiological characteristics of RJ-1 isolate are shown in Tables 1-4.

The PCR product was gel eluted and sequence data was submitted to NCBI GenBank under the accession number KT373817.

The phylogenetic analysis of 16s rDNA sequence of isolate along with the sequence retrieved from NCBI was carried out with MEGA 6.06 using neighbor joining method `with 1,000 boot strap replicates.The result of phylogenetic analysis showed distinct clustering of the isolate and confirmed the result of sequence similarly analysis (Fig. 2)

Fig. 1 — Unhala hot water spring at Rajapur, Dist. Ratnagiri

RJ-1 exhibited maximum growth and pectinase production at PH-8 (60.18U/ml) and 60 ^{o}C (68.4 U/ml) at 72 hours of incubation with shaking at 120 rpm. Extra molecular pectinase production varied with carbon and nitrogen source. Enhanced growth and production were seen in medium supplemented with orange peel as substrate (62.4 U/ml), fructose as a carbon source(54.6 U/ml), peptone as a nitrogen source (28.9 U/ml) and potassium di-hydrogen phosphate as a phosphorus source (27 U/ml) (Figs 5-10).

Catalytic activity of partially purified pectinase was determined maximum at pH 10 (1.98 U/ml) and 80 ^{o}C temperature (2.28 U/ml), respectively.

Catalytic activities of partially purified pectinase in presence of 10 mM metal ions were recorded where Fe 3^{+} (5.8 U/ml) showed the highest activity.

Substrate concentration (2%) and 60 min of incubation showed maximum catalytic activity (4.68 U/ml and 2.24 U/ml, respectively). The extracted pectinase showed stability in presence of solvents and surfactants, such as chloroform, ethanol, butanol, acetone, Tween-80 and Triton X-100 at 1% (v/v) concentration.

SDS analysis revealed that molecular weight of partially purified RJ-1 pectinase was 66 kDa.

The antimicrobial activity was measured in terms of the diameter of zone of inhibition in cm as given in Table 5 and Figure 3. Pectinase treated *Emblica Officinalis* powder showed maximum zone of inhibition against *E. coli* and *A. Oryzae*. No growth

Table 1 — Morpological characters of bacterial isolates from Unhala thermal spring

Sr.No	Characters	RJ1 *Bacillus licheniformis*	RJ2 *Bacillus cereus*	RJ3 *Bacillus Firmus*	RJ4 *Bacillus brevis*
1	Size	0.5mm	2mm	1mm	2.4mm
2	Shape	Circular	circular	circular	Circular
3	Colour	White	white	white	Shiny
4	Opacity	Opaque	translucent	opaque	Opaque
5	Surface	Smooth	smooth	smooth	Rough
6	Elevation	Raised	flat	raised	Raised
7	Consistency	non sticky	mucoid	non sticky	semi sticky
8	Margin	Entire	entire	entire	Irregular

Table 2 — Microscopic features of bacterial isolates from unhala thermal spring

Sr.No	Characters	RJ1 *Bacillus licheniformis*	RJ2 *Bacillus cereus*	RJ3 *Bacillus Firmus*	RJ4 *Bacillus brevis*
1	Cell length(µm)	2.3	1.8	1.5	2.2
2	Cell Width(µm)	0.5	1	0.5	1
3	Sporulation	†	†	†	†
4	Gram's nature	+ve rod	+ve cocci	+ve cocci	+ve rod
5	Motility	Motile	motile	motile	Motile

Abbreviation: † : Spore former, +ve : Positive

Table 3 — Biochemical characteristics of bacterial isolates from Unhala thermal spring

Sr.No	Characters	RJ1 *Bacillus licheniformis*	RJ2 *Bacillus cereus*	RJ3 *Bacillus Firmus*	RJ4 *Bacillus brevis*
1	H_2S production	−	−	−	−
2	Indole Test	−	−	+	−
3	MR test	+	−	−	−
4	VP test	−	+	−	−
5	Citrate test	−	+	−	+
6	Nitrate reduction	+	−	−	−
7	Esculine hydrolysis	+	+	+	+
	SugarUtilisation				
1	Dextrose	+	−	−	+
2	Fructose	+	+	−	+
3	Lactose	+	−	−	+
4	Sucrose	+	−	+	−
5	mannitol	+	−	+	−
6	Maltose	+	+	+	+
7	Xylose	−	−	−	−
8	Arabinose	−	−	−	−
9	Galactose	+	+	+	−
10	Glycerol	+	−	−	−
11	Cellobiose	+	−	−	−
12	Sorbitol	+	−	−	−
13	Mannose	+	−	−	+
14	Trehalose	+	+	+	+
15	Ribose	+	+	+	+
16	Salicin	+	−	−	−
17	Rhamnose	−	−	−	+
18	Inuline	−	−	−	−
19	Adonitol	−	−	−	−
20	Raffinose	−	−	−	−
	Enzyme Profile				
1	Catalase	+	+	+	+
2	Oxidase	+	+	+	+
3	Protease	−	+	+	+
4	Amylase	+	+	+	+
5	Urease	+	+	+	+
6	Lipase	+	+	+	+
7	Pectinase	+	+	+	+
8	Cellulase	−	−	−	−

Abbreviations: +: Positive test, - : Negative test

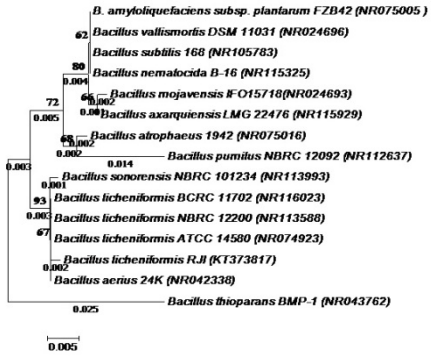

Fig.2 — Phylogenetic tree (evolutionary relationship between selected data)

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 655 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 .06.

Table 4 — Antibiotic susceptibility pattern of bacterial isolates from Unhala thermal spring

Antibiotic discs used	RJ1 *Bacillus licheniformis*	RJ2 *Bacillus cereus*	RJ3 *Bacillus Firmus*	RJ4 *Bacillus brevis*
Ampicillin	R	R	R	1.5
Amikacin	2.4	3.2	1.8	2.1
Cephalothin	1.8	1.1	3.2	1.8
Cephoxitin	2.6	2.8	1.1	2.2
Chloramphenicol	1.9	1.4	2.8	2.4
Ciprofloxacin	2.7	3.1	1.9	3.2
Gentamycin	3.3	1.1	3.1	2.8
Linezoid	1.8	2.7	2.2	1.2
Neomycin	1.1	2.5	2.6	1.6
Norfloxacin	2.1	1.8	1.6	2.5
Penicillin	3.2	1.5	2.8	1.8
Piperacillin	1.2	2.8	1.8	1.4
Polymyxin	1.7	R	R	R
Streptomycin	2.9	2.6	2.4	1.7
Sulphatriad	3	2.8	1.9	2.6
Teicoplanin	2.8	3.4	3.2	2.2
Tetracyclin	1.2	2.3	1.6	2.6
Tobramycin	1.5	1.9	2.1	3.2
Vancomycin	1.8	2.1	1.4	2.2

Zone of inhibition size is given in centimetre.

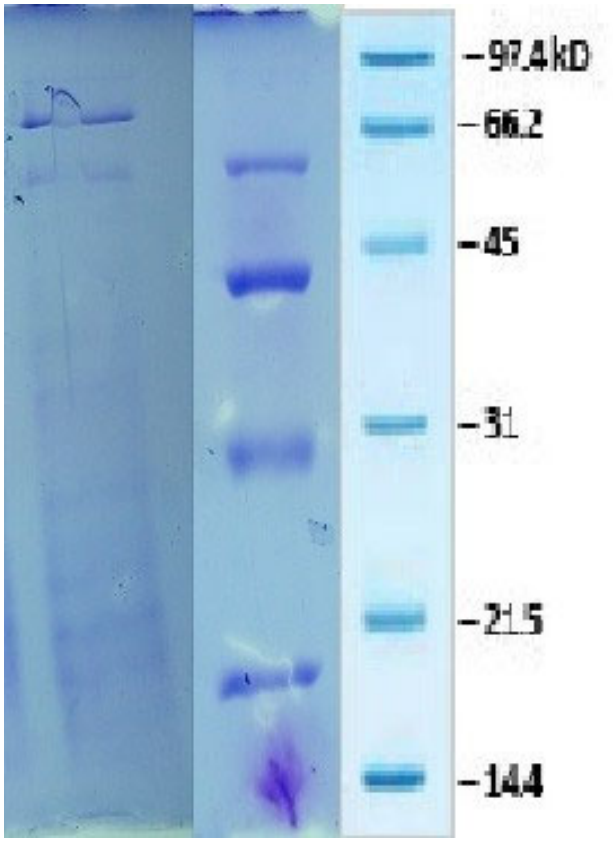

Fig 3 — SDS-PAGE analysis of partially purified pectinase from RJ-1; Lane A: RJ-1 pectinase Lane B: Molecular weight markers (Merck Biosciences, India)

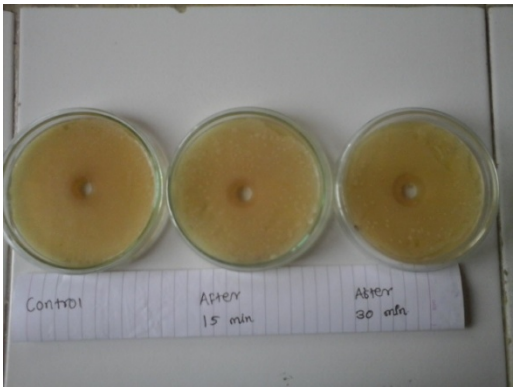

Fig 4 — Antimicrobial activity of *Emblica Officinalis* powder treated with pectinase.

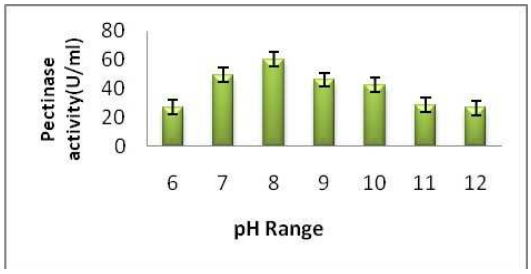

Fig 5 — Effect of pH on alkaline Pectinase

inhibition was observed for *A. flavus* and *A. nodulans*. In present investigation, first time we have recorded enhanced antimicrobial activity of *Emblica Officinalis* when it was assessed with pectinase.

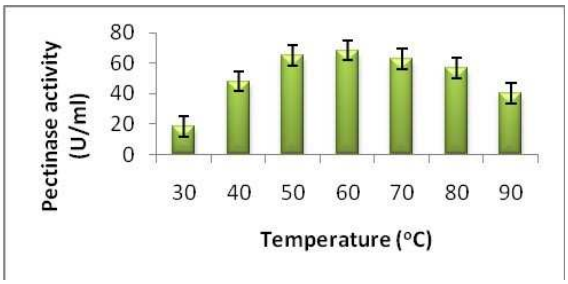

Fig 6 — Effect of temperature on alkaline pectinase production

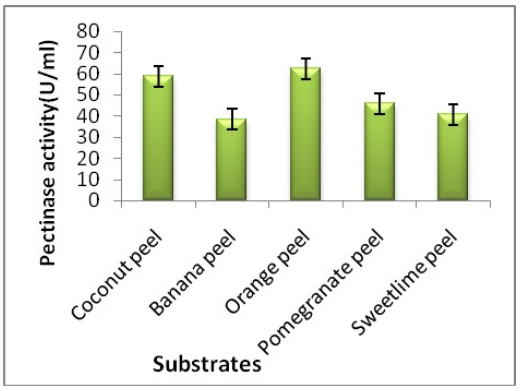

Fig 7 — Effect of substrates on alkaline pectinase

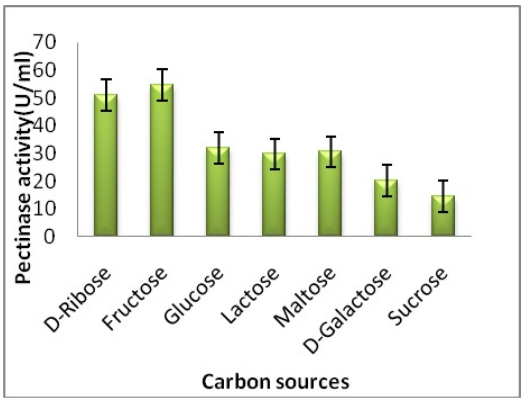

Fig 8 — Effect of carbon sources on alkaline pectinase production

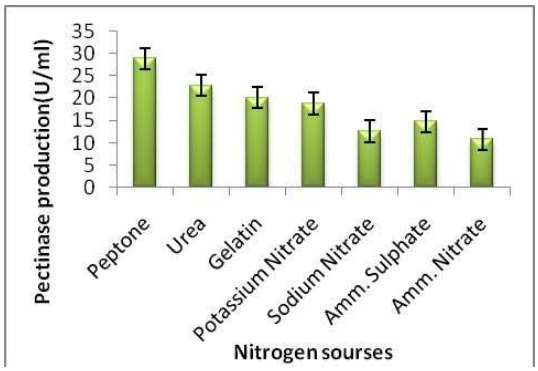

Fig 9 — Effect of nitrogen sources on alkaline

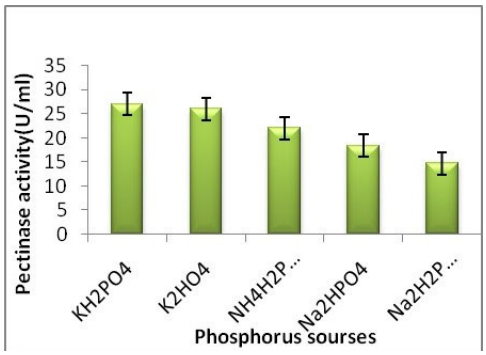

Fig 10 — Effect of phosphorus sources on alkaline pectinase production

Conclusion

An efficient thermostable pectinase producer was isolated from Unhala hot water spring and identified as *Bacillus licheniformis* RJ-1. Remarkable thermostable pectinase production was observed by using inexpensive agricultural residues as macronutrients. The pectinase produced showed stability in the presence of various solvents and surfactants. It has also promoted antimicrobial activity of *Emblica Officinalis*.

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A Comparative Study of Segmentation Techniques used in Handwritten Documents

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***Abstract*—** Handwritten document image segmentation is key step for OCR (Optical Character Recognition) System. It is an important step because inaccurately segmented text lines will cause errors in the recognition stage. The selection of segmentation algorithm being used is the essential factor in deciding the accuracy of the OCR system. Devnagari is the most popular script in India. Devnagari is the script for Sanskrit, Hindi, Marathi, Kashmiri, Sindhi, Bihari, Bhili, Konkani, Bhojpuri and Nepali languages. It has vowels, consonants, vowel modifiers and compound characters, numerals. Optical Character Recognition for Devanagari is highly complex due to its rich set of conjuncts. The nature of handwriting makes the process of text line segmentation very challenging. Several techniques to segment handwriting text line have been proposed in the past. Our purpose is to provide a learning-based approach for segmentation of handwritten document images. This paper presents a quantitative comparison of three algorithms for page segmentation: Projection Profile, Run-length Smearing and Bounding Box along with some morphological operations like erosion, dilation etc. We have implemented these algorithms on our own dataset of handwritten documents. We have experimented and compare the accuracy and results of these methods.

Keywords — OCR, Line and Word Segmentation, Projection Profile, Bounding Box, Run length Smearing

I. INTRODUCTION

In this paper, we compare previous work done on text line segmentation in handwritten documents. There are two types of segmentation bottom-up and top-down. The bottom-up approach use the connected components based methods merge neighboring connected components using simple rules on the geometric relationship between neighboring blocks. Where as in the top-down algorithms projection based methods is used .which is one of the most successful method for machine printed documents since the gap between two neighboring text lines in machine printed documents is typically significant, thus the text lines are easily separable. The projection based methods cannot be directly used in handwritten documents, unless gaps between lines are significant or handwritten lines are straight.

In the segmentation is an image of handwritten document image is decomposed into sub images of lines, words and characters. It is one of the essential steps in an optical character recognition (OCR) system. It is an important step because inaccurately segmented text lines will cause errors in the recognition stage. It makes a major contribution to the error rate of the system.

The rest of the paper is organized as follows. Section II explains the challenges in text line segmentation of handwritten documents. Section III describes some segmentation methods. Section IV provides the related work done in the area of handwritten document image segmentation. Section V presents an extensive performance evaluation and quantitative comparison. Section VI will have the concluding remarks of the study we have done.

II. CHALANES IN SEGMENTATION OF HANWRITTEN DOCUMENT

The variation in the handwriting of each person in handwritten documents makes the segmentation procedure a challenging task. There are many problems encountered in the segmentation procedure of handwritten documents due to skew angles between lines, overlapping words and adjacent text lines touching. The appearance of slant in the text line, punctuation marks and the non-uniform spacing of words are the major difficulties in word segmentation process. Freestyle and unconstrained handwriting text line segmentation is considered a complex and challenging task due to the following characteristics [1]

Fluctuating lines or skew variability [2, 3]. Lines of text in general are not straight. The inter-line distance variability and inconsistent distance between the components may vary due to writer movement. It may be straight, straight by segments,

or curved [1]. According to Okun [4], three types of skew exist in documents:

- A Global skew: all the page blocks have the same orientation
- multiple skew: unaligned paragraphs or slant is different in different blocks of the page such as the FLAUBERT's drafts [2] which contain several blocks of text arranged in a non linear way, and numerous editorial marks such as erasures and word insertion, and
- non uniform text line skew or varying text line slope: slant is different along the same line of text, for example curvilinear text lines.

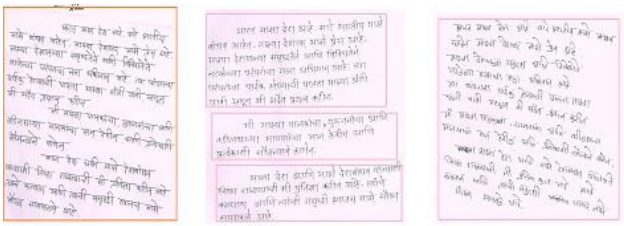

Figure 1. a. Global Skew b. Multiple Skew
c. Non uniform text line Skew

Line proximity i.e. Small gaps between neighboring text lines will cause touching or overlapping of ascenders or descenders. Text lines may be touching or overlapped, when upper-strokes and down-strokes of two consecutive lines are near or touching, or ascenders and descenders of adjacent lines interfere.[6]

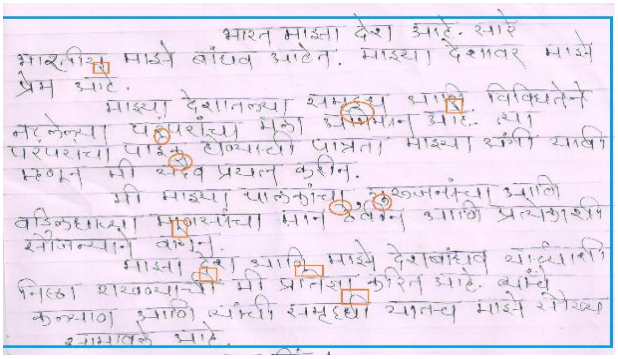

Figure. 2:a. Overlapping components separated (circle) b. touching component separated into two parts (rectangle) in Devnagari Script.

Writing fragmentations ,Characters are made up of more than one connected component. This applies to Indian scripts such as Marathi , hindi, Telugu, Tamil, Bangla, and Malayalam and Arabic writing with massive presence of diacritical points.

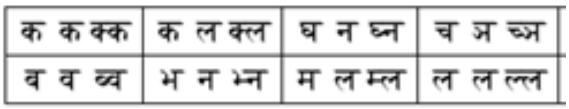

Figure 3 Writing Fragmentation

III. Segmentation Methods

Image segmentation is the division of an image into **regions** or **categories**, which correspond to different objects or parts of objects. *Every* pixel in an image is allocated to one of a number of these categories. A good segmentation is typically one in which:

- pixels in the same category have similar grayscale of multivariate values and form a connected region,
- Neighboring pixels which are in different categories have dissimilar values.

Segmentation algorithms are based on one of two basic proper-ties of intensity values discontinuity and similarity. There are three general approaches to segmentation, termed thresholding, edge-based methods and region-based methods.

A. In thresholding, pixels are allocated to categories according to the range of values in which a pixel lies.
B. In edge-based segmentation, an edge filter is applied to the image, pixels are classified as *edge* or *non-edge* depending on the filter output, and pixels which are not separated by an edge are allocated to the same category
C. In Region-based segmentation algorithms operate iteratively by grouping together pixels which are neighbors and have similar values and splitting groups of pixels which are dissimilar in value.

IV. Related work

Since 1960's character segmentation and recognition is an active field of research. It is still an open problem in the field of pattern recognition and image processing. Text line segmentation can be roughly categorized as bottom-up or top-down.

A top-down page segmentation technique known as the recursive X-Y cut decomposes a document image recursively into a set of rectangular blocks [8]. The connected component based methods merge neighboring connected components using a few simple rules on the geometric relationship between neighboring blocks. Connected component grouping [10] methods are sensitive to topological changes of the connected components, and it is not easy to derive script independent merging rules based on connected components.

Rodolfo P. dos Santos and Gabriela S. Clemente propose an efficient algorithm to segment handwritten text lines[18]. The text line algorithm uses a morphological operator to obtain the features of the images. A sequence of histogram projection and recovery is proposed to obtain the line segmented region of the text. A horizontal histogram projection is performed which results in the text lines positions. A threshold is applied to divide the lines in different regions. To eliminate false lines another threshold is used

Projection based methods may be one of the most successful top-down algorithms for machine printed documents[7].The gap between two neighboring text line is typically significant, the projection of text lines is easily separable in the orthogonal direction. The gaps between two neighboring handwritten lines may not be equal or handwritten lines are not straight [14] these methods cannot be used directly in handwritten documents. Another disadvantage of the top-down approaches is that they cannot easily process complex non-Manhattan layouts.

Arivazhagan, M. (2007) presented a piece-wise projection profile technique to segment a handwritten document into distinct lines of text by obtaining an initial set of candidate lines [7]. The lines traverse around the connected component by associating it to the line above or below.

Text line extraction from unconstrained handwritten documents is a challenge because the text lines are often skewed and curved and the space between lines is not obvious. To solve this problem, Yin & Liu [14] has propose an approach based on minimum spanning tree (MST) clustering with new distance measures.

Louloudis, Gatos, Pratikakis, & Halatsis proposed technique is based on a strategy that consists of distinct steps. The first step includes preprocessing for image enhancement, connected component extraction and average character height estimation [11]. In the second step, a block-based Hough transform is used for the detection of potential text lines.

A top-down page segmentation technique known as the recursive X-Y cut decomposes a document image recursively into a set of rectangular blocks[8] . It uses black pixels instead of using image pixels to achieve improvement in computation.

Two novel approaches to extract text lines and words from handwritten document are presented by Papavassiliou, V., Stafylakis[12]. The Viterbi algorithm is used for line segmentation which is based on locating the optimal succession of text and gap areas within vertical zones along with text-line separator drawing technique. The connected components are assigned to text lines in the end. A gap metric that exploits the objective function of a soft-margin linear SVM that separates successive connected components is used for word segmentation.

V. PERFORMANCE EVALUTION AND COMPARISION

For experimental purpose we have created our own dataset of handwritten characters. We have collected 65 handwritten marathi documents written by different individuals belonging to different categories in a separate sheet without any restrictions. It is done so to collect various samples of handwriting with different writing style, size, width etc. We have digitized the handwritten documents using scanner at 300 DPI in color mode and stored the scanned images in the jpeg format.

Before segmentation we have to perform some preprocessing on every document image as follows

- Convert to black and white
- Remove all object containing fewer than 30 pixels
- Perform morphological operations like erosion and dilation for normalization of image

After preprocessing is over we can apply the image to various segmentation algorithms to verify the results as follows

A projection profile is a histogram giving the number of ON pixels accumulated along parallel lines. By looking for minima in horizontal projection profile of the page, we can separate the lines easily [15].Horizontal projection profile is used for text line segmentation. This approach comprises of two stages - pre processing followed by morphological operations and text line extraction.

The text line algorithm uses a morphological operator to obtain the features of the images. Following, a sequence of histogram projection and recovery is proposed to obtain the line segmented region of the text. First, a Y histogram projection is performed which results in the text lines positions. To divide the lines in different regions a threshold is applied.

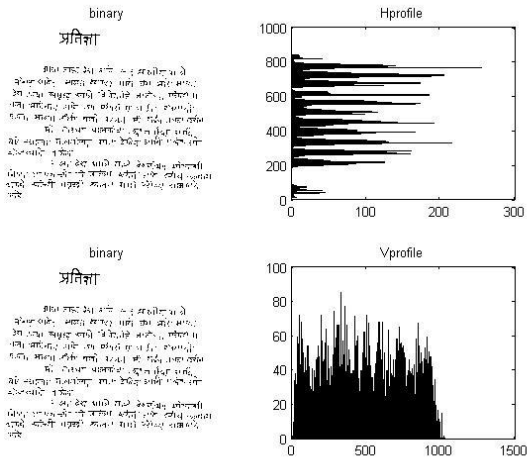

Figure 4 Horizontal and Vertical Projection Profile

In Run **Length Smearing method method [16]**, length of the white run is computed by finding the consecutive white pixels which appears in between two black pixels. We will fill up the white run length into black, when the length of white run is less than five times width of the stroke. Algorithm

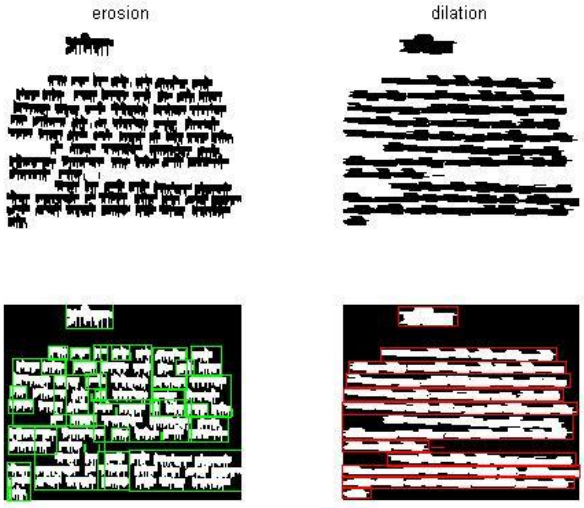

Figure 5. Run length Smearing

A technique based on Bounding Box[16] is used in order to extract individual text line. First the image is converted to gray scale and histogram of that image is plotted. Next find the row containing lesser number of white pixels and identify the measurements of centroids with the region props property. Finally with the help of measurements of centroids individual lines are cropped [16].

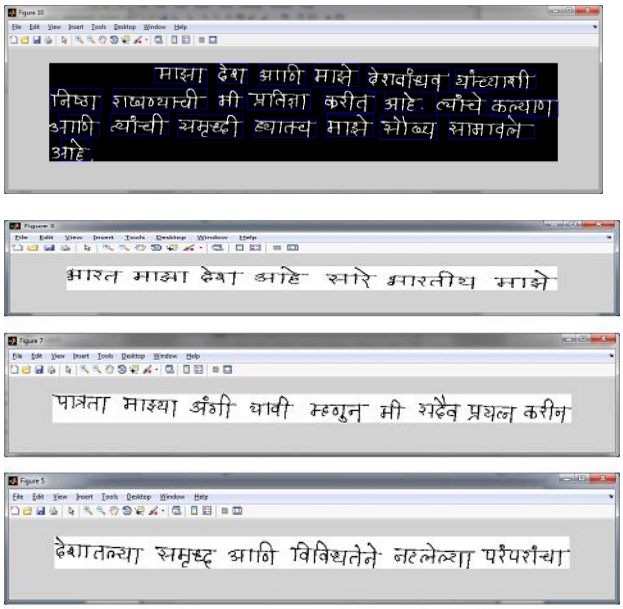

Figure 6. Bounding Box Technique

Using Morphological Operators [17] Erosion and Dilation are the two operators whose combination or series of combination can be applied with different structuring element depending upon the size of the image. In erosion, the input image is eroded with the structuring element to obtain the processed image. The processed image obtained is sharper. The reverse happens in dilation. These operators are usually applied for detecting and removing header line from the word.

Table 1. Segmentation of different handwritten samples

Handwritten Document Sample	Segmentation Methods Accuracy in percentage %					
	Projection Profile	Projection Profile with Morphology	Run Length Smearing method	Run Length Smearing method with morphology	Bounding Box method	Bounding box with morphology
Document 1	93	97	90	90	93	97
Document 2	92	95	91	92	90	95
Document 3	88	92	83	84	88	92
Document 4	86	92	85	88	84	85
Document 5	95	97	92	91	95	97
Document 6	85	88	90	91	85	85

Document 7	**95**	**97**	**91**	**92**	**95**	**97**
Document 8	**87**	**89**	**88**	**89**	**87**	**89**
Document 9	**93**	**96**	**91**	**92**	**93**	**96**
Document 10	**91**	**93**	**88**	**88**	**91**	**93**
Average	**90**	**93**	**89**	**89**	**90**	**91**

Table 2. Results of Segmentation Methods

Segmentation Method	Segmentation Accuracy
Projection Profile	90 %
Projection Profile with Morphology	93 %
Run Length Smearing method	89 %
Run Length Smearing method with morphology	89 %
Bounding Box method	90 %
Bounding box with morphology	91 %

VI. CONCLUDING REMARK

This paper has provided a comparative study of the methods for off-line handwriting text line segmentation previously proposed by researchers. Three different methods like Projection profiles, Run Length smearing method and bounding box methods are used for text line extraction of Handwritten Devnagari Documents. These proposed methods are experimented on our own dataset collected from different writers. We have applied all the methods on 65 handwriiten document samples . Among all other proposed methods Morphological operations with projection profile gives the best segmentation rate of 93% because this method works well for clearly separated lines . But this method cannot divide the touching or overlapping lines and instead it will merge those lines. Skewed or slanted and intersecting lines are main challenges in line segmentation problem . We can combine these methods to get better results of line segmentation in handwritten documents.

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Performance analysis of Handwritten Devnagari Character Recognition using Feed Forward , Radial Basis , Elman Back Propagation, and Pattern Recognition Neural Network Model Using Different Feature Extraction Methods

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***Abstract*—** This paper describes the performance analysis for the four types of neural network with different feature extraction methods for character recognition of hand written devnagari alphabets. We have implemented four types of networks i.e. Feed forward , Radial basis, Elman back propagation and Pattern recognition neural network using three different types of feature extraction methods i.e. pixel value, histogram and blocks mean for each network. These algorithms have been performed better than the conventional approaches of neural network for pattern recognition. It has been analyzed that the Radial Basis neural network performs better compared to other types of networks.

Keywords- *OCR, Devnagari Script, Feature Extraction, Feed forward , Radial basis, Elman Back propagation, Pattern Recognition Neural Network .*

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I. INTRODUCTION

One of the primary way by which computers are able with humanlike abilities is through the use of a neural network. Neural networks are mainly useful for solving problems that cannot be expressed as a series of steps. Pattern recognition is possibly the most frequent use of neural network. The concept of learning in neural networks is used to a large scope in developing an OCR system to recognize characters of various fonts and sizes, and hand written characters. Parallel computational capability of neural network helps to reduce recognition time which is essential in a commercial perspective.

During the past 40 years, significant research effort has been dedicated to OCR for the Devanagari script. R.M.K. Sinha and V. Bansal[1-7] has done widespread work in Devnagari Optical character Recognition (DOCR). Handwritten DOCR is more difficult than English characters due to the complexity of devnagari script i.e. modifiers , compound characters, shape similarity[8]. Artificial neural network (ANN) [9-10] can be used for classification purpose.

Performance of the handwritten Devnagri character recognition system can be analyzed by using various performance measures like mean square error (MSE), percentage error , performance characteristics, recognition percentage etc.[11]. The feature extraction step of optical character recognition is the most important. A poorly chosen set of features will yield poor classification rates by any neural network.[12]
It has been analysed by NilayKarade et al. [13] that the number of hidden layer, number of neurons in hidden layer, validation checks and gradient factors of the neural networks models are taken into consideration during the trainingof a neural network.

The performance of the neural network is much accurate and convergent for the learning with the hybrid evolutionary algorithm[14].The feed forward neural network by using Evolutionary algorithms makes better generalization accuracy in character recognition problems[15].

In this paper we have implemented four different neural network models using three different feature extraction techniques i.e. we have implemented total twelve neural networks using 30 handwriting samples of Marathi alphabets having 44 characters in each sample for training and 5 for testing

II. OPTICAL CHARACTER RECOGNITION (OCR)

Optical character recognition (OCR) is becoming an essential part of document scanners, and is also used normally in banking and postal applications. Printed characters can now be accurately recognized. Optical character recognition is needed when the information should be readable both to humans and to a machine. Optical character recognition is unique because it does not require control of the process that produces the information.

Optical Character Recognition deals with the problem of recognizing optically processed characters. Optical recognition can be performed in two ways either offline or online. In off-line it is performed after the writing or printing has been completed, opposite to this in on-line recognition the computer recognizes the characters as they are drawn. Both hand printed and printed characters may be recognized, but the performance is directly dependent upon the quality of the input documents. Fig.1 shows the different types of character recognition [1] .It is difficult to develop a OCR for unconstrained handwriting. But we can improve the performance of OCR by using the constrained input .The basic steps involved in Optical Character Recognition are

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A. Data Acquisition
B. Preprocessing
C. Segmentation
D. Feature Extraction
E. Classification
F. Post Processing

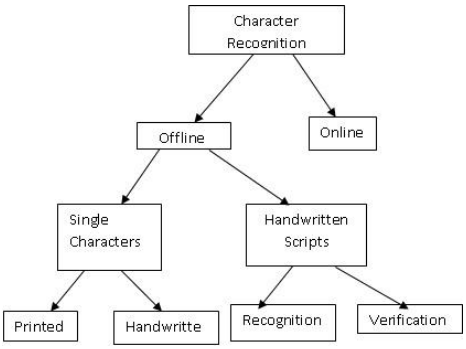

Figure 1. Types of Character Recognition

A. Data Acquisition

It is the first and most important step in OCR In this step we have to collect data i.e. samples of handwritten or printed documents .The collected documents has to be scanned in order digitize the data. The image should have a specific format such as jpeg, bmp etc. The images can be acquired through a scanner, digital camera or any other suitable digital input device. Data samples for the experiment have been collected from different individuals so that more diverse data can be collected.

B. Preprocessing

This step is used to reduce the noise and remove the unwanted data if any. We can also decrease the variation and transforms the data in specific format so that the data can be processed more easily and efficiently .There are various ways through which preprocessing can be done like Binarization, Noise reduction, Stroke width normalization, Skew correction, Slant removal, Filtering, Morphological Operations, Noise Modelling, Skew Normalization, Size Normalization, Contour Smoothing, Compression, Thresholding, Thinning etc

C. Segmentation

It is also an import step in OCR. In this step the document is segmented into Lines, words and the characters. We can to use various segmentation techniques like projection profile, bounding box etc. The accuracy of segmentation affects indirectly on the accuracy of recognition i.e. accuracy of character recognition closely depends upon segmentation. Segmentation promise the effectiveness of classification and recognition.

D. Feature Extraction

Feature extraction means taking out the raw data from the information which is most relevant for classification. There are three types of feature extraction methods a i.e. Statistical, Structural and Global. We can create feature vectors by extracting the significant features from the alphabets. These feature vectors are then used by classifier to recognize the input with target output.

E. Classification

The classification step is the decision making part of the recognition system. There are many existing Classical and soft computing techniques for handwriting classification.

- Classical Techniques: Template matching Statistical techniques Structural techniques
- Soft Computing Techniques: Neural networks (NNs) Fuzzy- logic technique Evolutionary computing techniques

The performance of a classifier depends on various factors like the size of training data set, segmentation technique , types of features etc.

F. Post Processing

In this step the results of classifier are converted into the required format. Post processing can also improve the recognition rate .

III. CHALLENGES IN HANDWRITTEN DEVNAGARI CHARACTER RECOGNITON

Optical Character Recognition (OCR) deals with automatic recognition of characters in a document image. A lot of research is done for English Character Recognition (CR).Various OCR software for English languages are also available. Indian languages are more complicated in terms of structure and computations it is difficult to develop a OCR for them. Devnagari is the most popular script in India. Hindi,Marathi, Nepali etc. languages use devnagari script for writing .Thus, the work on Devnagari script is very useful. The alphabet of the Devnagari script consists of 14 vowels and 33 consonants. The basic characters of Devnagari script are shown in Fig.2.

In Devnagari script a vowel following a consonant takes a modified shape. Depending on the vowel , its modified shape is placed at the left, right (or both) or bottom of the consonant. These are called as modifiers. In character segmentation the modifiers are most difficult to segment . Due to the presence of header line called shirodhara it is difficult to separate the characters in devnagari script. Compound characters can be combinations of two consonants as well as a consonant and a vowel. Recognition of compound characters is also a difficult problem. we have considered only basic characters of Devnagari script.

The complexity of a handwritten character recognition system increases mainly because of various writing styles of different individuals. Most of the errors in such system arise because of the confusion among the similar shaped characters. In Devnagari there are many similar shaped characters. it can be seen that shapes of two or more characters are very similar due to handwritten style of different individual and such shape

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similarity is the main reason of low recognition rate. The modifiers , compound characters and shape similarity are the major reasons of complexity in Devanagri Character Recognition .

Figure 2. Basic Characters of Devnagari Script

IV. ARTIFICIAL NEURAL NETWORKS (ANN)

A neural network can perform computations at a higher rate compared to the classical techniques. It can easily adapt to changes in the data and learn the input signals characteristics, because of its adaptive nature. A neural network is consists of many nodes. Neural network architectures can be classified into two major groups, feed-forward which have no loops and feedback (recurrent) networks in which loops occur because of feedback connections. The most familiar neural networks used in the character recognition are systems are the multilayer perceptron of the feed forward networks and the kohonen's Self Organizing Map (SOM) of the feedback networks(Fig.3).

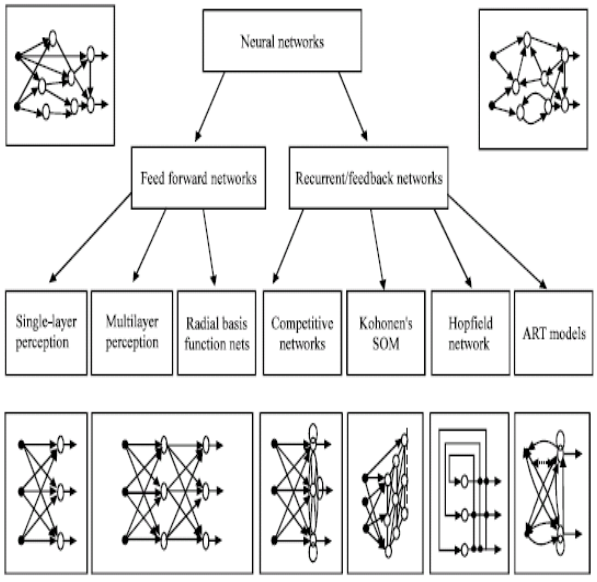

Figure 3. Taxonomy of feed forward and feedback network architectures

A. *Feed Forward Neural Network*

Feed-forward networks have perceptron which are arranged in layers. The first layer takes the inputs and the last layer producing outputs. The middle layers have no connection with the external world, and hence are called hidden layers.
These networks are called feed-forward networks because information is constantly "fed forward" from one layer to the next it is possible because the perceptron in one layer is connected to every perceptron in the next layer i.e. the information flows in only one direction. There is no connection among perceptron in the same layer.
Classification can be done more precisely by varying the number of nodes in the hidden layer, the number of layers, and the number of input and output node. Hence feed-forward networks are commonly used for classification.
Feed-forward networks belongs to the supervised learning, in which pairs of input and output values are fed into the network for many cycles, so that the network learns the relationship between the input and output. Back propagation is the most popular learning technique in feed forward networks. These networks apply a sigmoid function as an activation function.

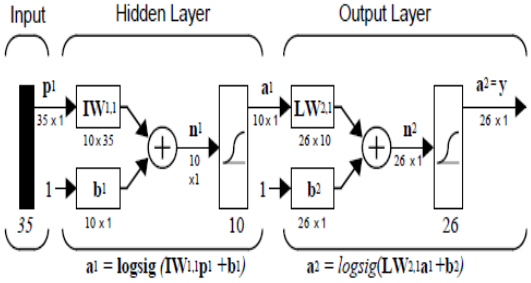

Figure.4 Feedforward NN Architecture

Every time a neural network is trained using input vector of a training sample, the output vector o is compared to the desired value d. The comparison is done by calculating the squared difference of the two.

$$E=(\delta-o)^2 \qquad (1)$$

The value of Error tells us how far we are from the desired value for a particular input. The goal of back propagation is to minimize the sum of error for all the training samples, so that the network behaves in the most desirable way.

$$\text{Minimize} \sum E=(d-o)^2 \qquad (2)$$

Decreasing the value of w in the direction of the gradient leads to the most rapid decrease in error, we update the weight vectors every time a sample is presented using the following formula:

$$W_{new} = W_{old} - n^* (\delta E/\delta W) \qquad (3)$$

where n is the learning rate (a small number ~ 0.1). Using this algorithm, the weight vectors are modified so that the value of error for a particular input sample decreases a little bit every time the sample is presented. When all the samples are presented in turns for many cycles, the sum of error gradually decreases to a minimum value.

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B. *Radial Basis Function Networks*

Radial basis function networks take a different approach to to the design of neural networks than that of Multi Layer Perceptron. They use a curve fitting approach in a high-dimensional space. Learning in this method is equivalent to finding a surface in this hidden space that gives a best fit to the training data. They have a single hidden layer which consists of Radial basis functions which translate the inputs, in a non-linear way to a high-dimensional space. Problems that are not linearly separable in the input space can be found to be linearly separable in the hidden space.

Radial Basis Function (RBF) networks can perform classification. They can be used as function approximators They use a set of radial basis functions, which have the same dimension as the input space. They have a single hidden layer. The solution to the weights can be performed by linear least squares.

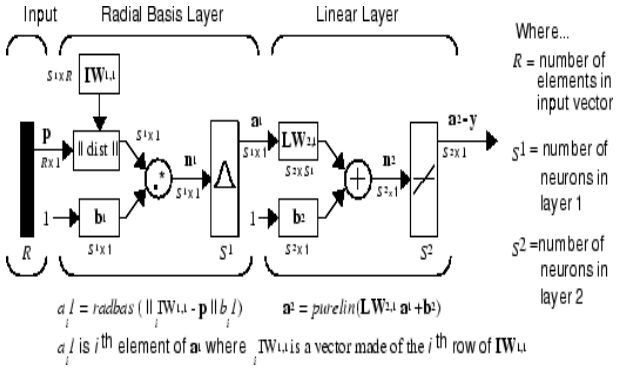

Figure 5. Radial Basis Function Network architecture

Transformation from the input space to the hidden-unit space is nonlinear while the transformation from the hidden-unit space to the output space is linear.Radial basis networks consist of two layers: a hidden radial basis layer of S1 neurons, and an output linear layer of S2neurons.

The || dist || box in this figure accepts the input vector **p** and the input weight matrix $\mathbf{IW}^{1,1}$, and produces a vector having S_1 elements. The elements are the distances between the input vector and vectors $_i\mathbf{IW}^{1,1}$ formed from the rows of the input weight matrix.

The bias vector b1 and the output of || dist || are combined with the MATLAB® operation .* , which does element-by-element multiplication.The output of the first layer for a feedforward network net can be obtained with the following code:

a{1} = radbas(netprod(dist (net.IW{1,1},p) ,net.b{1})) (4)

C. *Elman Neural Network*

The Elman network is a two-layer network with feedback from the first-layer output to the first layer input. This recurrent connection allows the Elman network to both detect and generate time-varying patterns. A two-layer Elman network is shown below(Fig. 6).

The Elman network has tansig neurons in its hidden (recurrent) layer, and purelin neurons in its output layer. This combination is special in that two-layer networks with these transfer functions can approximate any function (with a finite number of discontinuities)

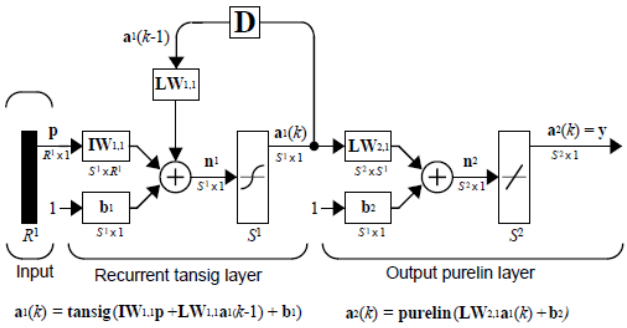

Figure 6. Elman Network Architecture

with arbitrary accuracy. The only requirement is that the hidden layer must have enough neurons. More hidden neurons are needed as the function being fit increases in complexity.

Note that the Elman network differs from conventional two-layer networks in that the first layer has a recurrent connection. The delay in this connection stores values from the previous time step, which can be used in the current time step. Thus, even if two Elman networks, with the same weights and biases, are given identical inputs at a given time step, their outputs can be different due to different feedback states. The network can store information for future reference hence it is able to learn temporal patterns as well as spatial patterns. The Elman network can be trained to respond to, and to generate, both kinds of patterns.

V. PROPOSED OCR SYSTEM

we have designed a form to collect the isolated characters and digits (Fig.7).We have provided printed samples of devnagari alphabets and asked the writers to write the same characters in the blank space provided in front of the printed characters . We have digitized the duly filled forms using scanner at 300 DPI in color mode and stored the scanned images in the jpeg format.

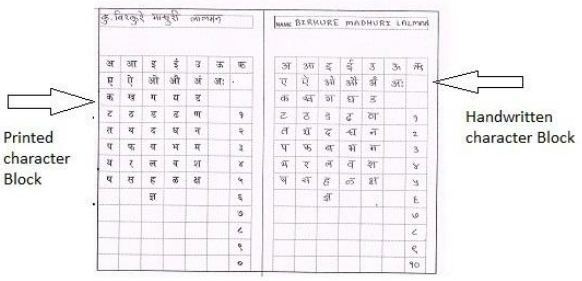

Figure 7. Sample of Handwritten Devnagari Characters

In this paper we have used three feature extraction methods

A. Pixel values
B. Histogram values
C. Block mean values

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A. *Pixel values*

In this method we have used following steps to get the character pattern vector for each characters (digits and alphabets). Binaries the image using Otsu method. Remove noises (impression of other forms, salt and paper noise , corner folding , physically damaged paper , extra lines , stapler pin marks) that might have occurred during the digitization process . Perform the edge detection operation. Perform the hole filling operation to obtain the uniform connected components Perform the labeling operation on the connected components to find the bounding box for each character. Crop the bounding box and resize it into 15 X 15 matrix. (Fig.8) The character pattern is stored in column vector of size 225 X 1.

B. *Histogram values*

For creating a histogram of each character first we need to convert the color image into gray image using rgbtogray function. Then we draw the histogram of each character (Fig.9) and store the histogram count values in a column matrix of 256x1.

C. *Block mean values*

The block mean values can be calculated as follows. Convert the character image into black and white i.e. binary image. Then remove the blank space from left, right, upper and lower side of image .Resize the image into matrix of size 15x15 and after that divide the image into the blocks of 3x3 (Fig.10). Calculate the mean value for each block and Store mean values in to a column matrix of size 25x1 .

After creation of feature vectors we have to create one more file for storing the target values. It will be the six bit representation for each character ex. 000001, 000010, …….. , 101100. A target matrix of 6 X 44. The target matrix contains 44 binary values each having six bits for representation of each character. The input and target required to create a neural network is read from the files and store it in the variables P and T.

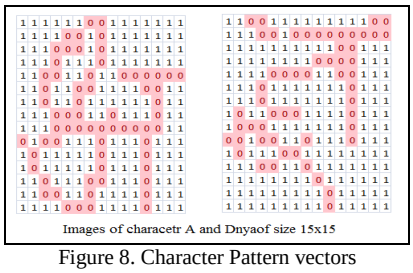

Figure 8. Character Pattern vectors

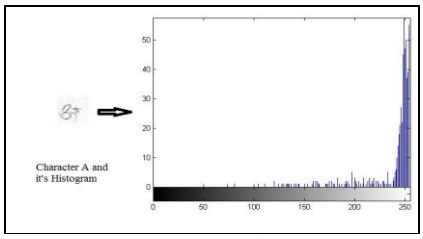

Figure 9. Histogram of Character A

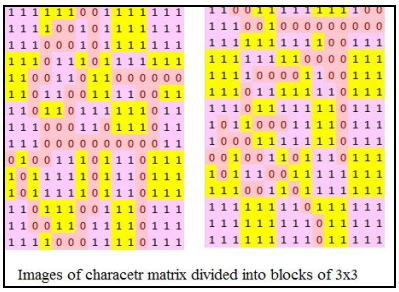

Figure 10. Characters divided into blocks

VI. RESULTS AND DISCUSSION

In this paper we have implemented four types of neural networks i.e. Feed forward , Radial basis, Elman back propagation and Pattern recognition neural network using three different types of feature extraction methods i.e. pixel value, histogram and blocks mean for each network. Totally twelve different networks we have trained using 1320 character samples of handwritten Marathi alphabets collected from 30 different persons and 220 character samples are used for testing purpose .

First we have trained the network then we simulated the network to get the output values and after that we have plotted the regression. The regression values for the four networks i.e. Feed forward , Radial basis, Elman back propagation and Pattern recognition neural network are 0.5 , 1, 0.4, 0.6 respectively . The following figures (Fig.11 to Fig.14) shows the regression plots for all the four types of neural networks i.e. feed forward , Radial Basis, Elman, pattern recognition. which indicates that the performance of Radial basis neural network works is more effective. After getting the regression values we have drawn a bar chart for comparing the performance of these four networks(Fig.15 & Fig.16).

VII. CONCLUSION AND FUTURE SCOPE

In this paper we have done the performance analysis of Feed forward , Radial basis, Elman back propagation and Pattern recognition neural network which clearly mention that the performance of Radial basis neural network is better as compared to other networks . Here we have used three different types of feature extraction methods i.e. pixel value, histogram and blocks mean for each network. We can improve the results by combining these feature extraction methods. We have also not consider the modifiers with the character here so in future we can also use marathi characters with modifiers to train the network

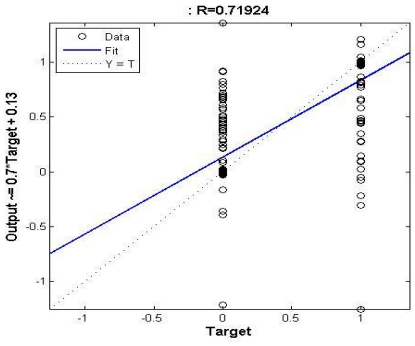

Figure 11. Regression plot for Feedforward Neural Network

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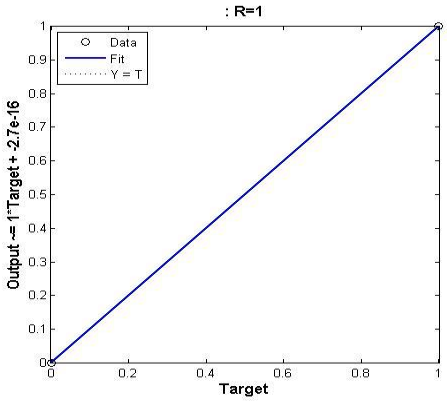

Figure 12. Regression plot for Radial basis Neural Network

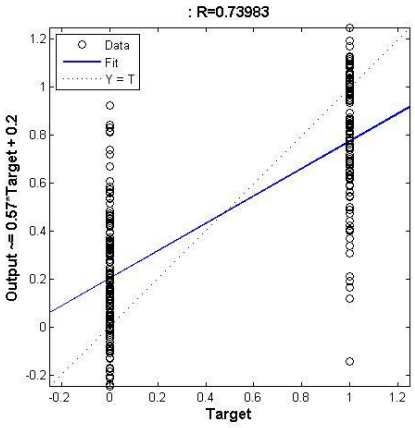

Figure 13. Regression plot for Elman Neural Network

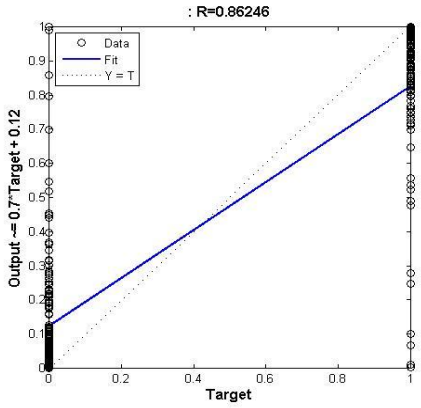

Figure 14. Regression plot for Pattern Recognition Neural Network

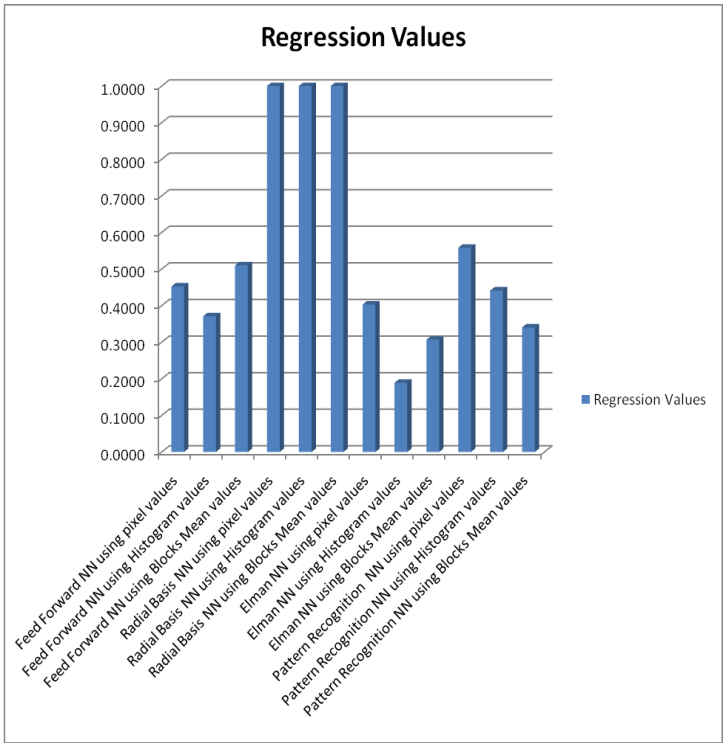

Figure 15. Comparative chart for the Regression values of all the Neural Network methods

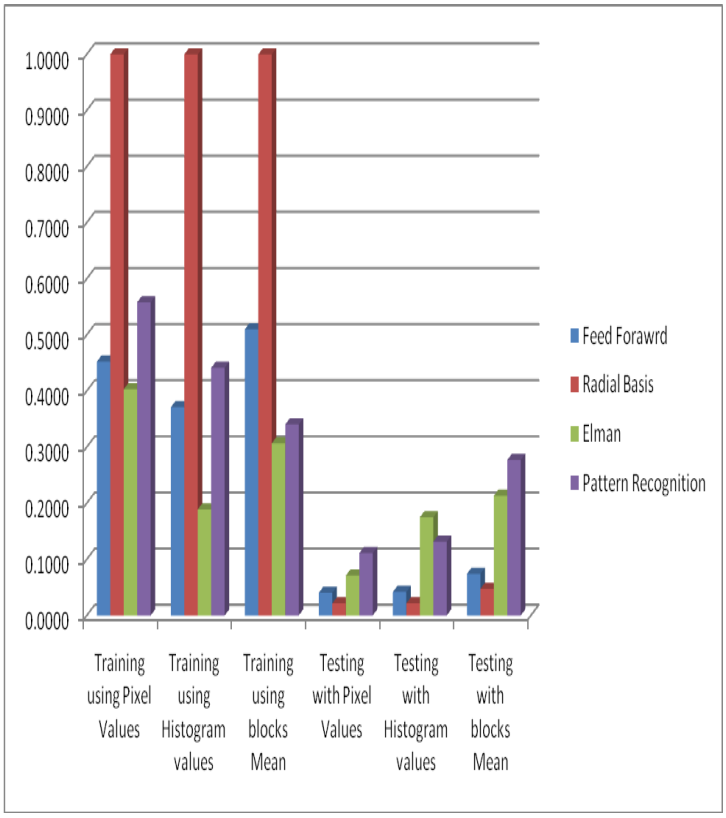

Figure 16. Comparative chart for the feature extraction methods

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Feature Extraction Techniques for Marathi Character Classification using Neural Networks Models

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***Abstract*—** Hand written Marathi Character Recognition is challenges to the researchers due to the complex structure. This paper presents a novel approach for recognition of unconstrained handwritten Marathi characters. The recognition is carried out using multiple feature extraction methods and classification scheme. The initial stages of feature extraction are based upon the pixel value features and the classification of the characters is done according to the structural parameters into 44 classes. The final stage of feature extraction makes use of the zoning features. First Pixel values are used as features and these values are further modified as another set of features. All these features are then applied to neural network for recognition. A separate neural network is built for each type of feature. The average recognition rate is found to be 67.96% , 82.67%,63,46% and 76.46% respectively for feed forward , radial basis , elman and pattern recognition neural networks for handwritten marathi characters.

Keywords- *Marathi handwritten characters recognition, Pixel Value Features, Histogram based features , Zoning based features Artificial neural networks.*

*****

I. INTRODUCTION

Character classification is a form of pattern recognition process. Presence of unwanted objects or disoriented patterns will affect the percentage accuracy. The most basic way of recognizing patterns is through using the probabilistic methods. It is very difficult to achieve 100% accuracy in recognition of handwritten characters. There are verities of writing styles because different peoples will write the same character differently. Handwritten character recognition has many possible application areas in various fields like postal automation, bank automation, form filling etc.

Handwritten character recognition for Indian scripts is quite a challenging task for the researchers because Indian languages are more complicated in terms of structure. Devnagari is the most popular script in India many languages like Hindi, Marathi, Nepali etc. are written in devnagari script .Devnagari script consists of 16 vowels and 36 consonants making 52 alphabets. Marathi is written from left to right. It has no upper and lower case characters. Every character has a horizontal line at the top called as the header line. The header line joins the characters in a word. Vowels are combined with consonants with the help of specific characteristic marks. These marks occur in line, at the top, or at the bottom of a character in a word. It also has complex compound characters in which two or more consonants are combined forming a new special symbol. Compound characters in Marathi script occur more frequently in the script as compared to other languages derived from Devanagari.

OCR work for printed and handwritten characters in various Indian scripts [1-3] is carried out by researchers but major work is found for Bangla [4, 5] and Devanagari. OCR work on printed Devanagari script started in early 1970s. Sinha [6-8] and Mahabala. Sethi and Chatterjee [5] also have done some earlier studies on Devanagari script and presented a Devanagari hand-printed numeral recognition system based on binary decision tree classifier. They used a similar technique for constrained hand-printed Devanagari character recognition. The first complete OCR system development of printed

Devanagari is perhaps due to Palit and Chaudhuri [10] , Pal and Chaudhuri [9]. The method proposed by Pal and Chaudhuri gives about 96% accuracy. Due to the complexities involved with Devanagari script, already existing methods cannot be applied directly with this script. A report on handwritten Devanagari characters was published in 1977 [11]. Kumar & Singh [13] proposed Zernike moments based approach for Devanagari character recognition. The other work on Devanagari character recognition is proposed 64 dimensional chain code features, but still no any standard OCR is available for the same. An excellent survey of the area is given in [15].For recognition of handwritten Devanagari numerals, Ramakrishnan et al. [16] used independent component analysis technique for feature extraction from numeral images. Bajaj et al [14] considered a strategy combining decisions of multiple classifiers. An extensive research on printed Devanagari text was carried out by Veena Bansal and R. M. K. Sinha [12, 13]. Multilayer perceptron was also used for classification by Sandhya Arora et al. [15] for handwritten Devanagari characters.

The rest of the paper is organized as follows: Section II discusses the feature extraction techniques, while section III describes the classification methods. Section IV presents the proposed system. Section V discusses the results obtained iby the proposed system and section VI finally discusses the conclusion and future scope.

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II. FEATURE EXTRACTION TECHNIQUES

Feature extraction is the process of extracting different features from the matrices of digitized characters. The characters are recognized on the basis of these features. Features of a character can be classified into two classes: Global or statistical features and Structural or topological features.

A. Global or Statistical Features

Global features are obtained from the collection of points representing the character matrix. These features can be easily discovered as compared to topological features. Global features are not affected too much by noise or distortions as compared to topological features. A number of techniques are used for feature extraction; some of these are: moments, zoning, projection histograms, n-tuples, crossings and distances.

B. Structural or Topological features

These features are related to the geometry of the character set to be considered. Some of these features are concavities and convexities in the characters, number of end points, no of holes in the characters etc. A lot of research has been done by different researchers to find different structural features. This feature set includes information for a character like location and number of holes in the characters, concavities in the skeletal structure, crossings of strokes, vertical end points of the character and bounding box of the character.

III. CLASSIFICATION METHODS

Classification is another most important component of OCR system. It is basically decides the feature space to which the unknown pattern belongs. Classification is usually done by comparing the feature vectors corresponding to the input character with the representative of each character class. But before doing this the classifier should posses a number of training patterns. A number of classification methods were purposed by different researchers some of these are statistical methods, syntactic methods, template matching, artificial neural networks, kernel methods.

A. Statistical methods

The purpose of the statistical methods is to determine to which category the given pattern belongs. By making observations and measurement processes, a set of numbers is prepared, which is used to prepare a measurement vector. Statistical classifiers are automatically trainable. *k*-NN method compares an unknown pattern to the set of patterns that have been already labeled with class identities in the training stage. A pattern is identified to be of the class of pattern, to which it has the closest distance, where as a bayesian classifier assigns a pattern to a class with the maximum a posteriori probability.

B. Syntactic or structural methods

Syntactic methods are good for classifying hand written texts. This type of classifier, classifies the input patterns on the basis of components of the characters and the relationship among these components. Firstly the primitives of the character are identified and then strings of the primitives are checked on the basis of pre-decided rules. Generally a character is represented as a production rules structure, whose left-hand side represents character labels and whose right-hand side represents string of primitives. The right-hand side of rules is compared to the string of primitives extracted from a word. So classifying a character means finding a path to a leaf.

C. Template matching

This is one of the simplest approaches to patter recognition. In this approach a prototype of the pattern that is to be recognized is available. Now the given pattern that is to be recognized is compared with the stored patterns. The size and style of the patterns is ignored while matching.

D. Artificial neural networks

A neural networks composed of inter connected elements called neurons. A neural network can trained itself automatically on the basis of examples and efficient tools for learning large databases. This approach is non-algorithmic and is trainable. The most commonly used family of neural networks for pattern classification task is the feed-forward network, which includes multilayer perception and Radial Basis Function (RBF) networks. But the limitation of the systems based on neural networks is their poor capability for generalization.

E. Kernel methods

Some of the most important Kernel methods are Support Vector Machines , Kernel Principal Component Analysis (KPCA), Kernel Fisher Discriminant Analysis (KFDA) etc. Support vector machines (SVM) are a group of supervised learning methods that can be applied to classification. In a classification task usually data is divided into training and testing sets. The aim of SVM is to produce a model, which predicts the target values of the test data. Different types of kernel functions of SVM are: Linear kernel, Polynomial kernel, Gaussian Radial Basis Function (RBF) and Sigmoid.

IV. THE PROPOSED SYSTEM

The proposed system is designed to recognize 44 characters of Marathi language. The characters used in the proposed system are shown in Figure1. The recognition system consists of phases like segmentation, pre-possessing, feature extraction, training and testing phase. First of all the handwritten document is segmented into lines. Then the lines are segmented into words, after that words are segmented into characters then pre-processing and normalization is performed on the characters to remove noise and make them identical. We have used three different feature extraction techniques i.e. pixel value features, histogram based features and zoning or blocks based features to get the features of characters. These features are used for both the phases i.e. training and testing the neural network. In the training phase features are extracted from the handwritten normalized characters and used for

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training the neural network. After the training neural network with sufficient samples the weights and biases for each network are saved. In the testing phase characters are extracted from handwritten script and then similar features are extracted from the character again after pre-processing and normalization of the character. The features are applied as inputs to the neural network. The output of the neural network gives the final recognition result. The next section discusses the proposed system in detail. The flow chart of the proposed scheme is described in Figure 2.

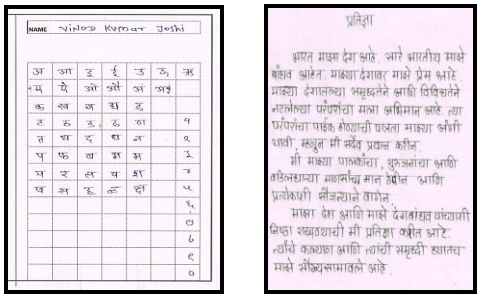

Figure.1 Sample of Handwritten Marathi Characters and Script

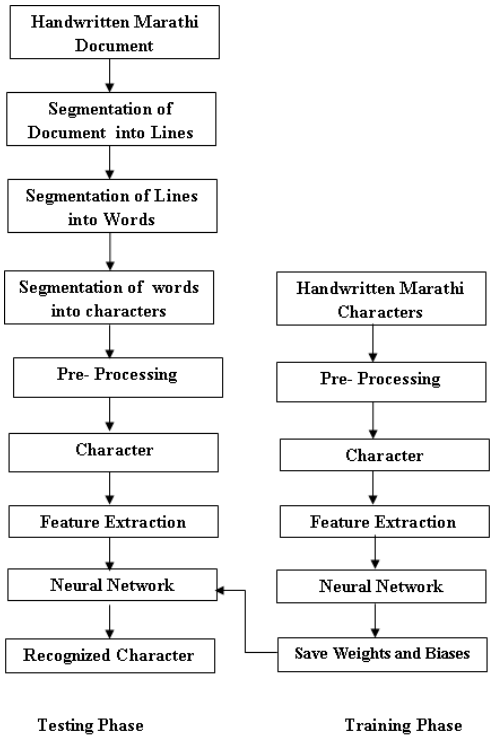

Figure 2. Proposed Character Recognition System

A. *Data collection*

We have collected the samples of handwritten documents from different peoples belonging to different categories in a separate sheet without any restrictions. The database for handwritten marathi documents is created by scanning the handwritten documents at 300 dpi using a flatbed scanner in color mode. The images are stored in jpeg file format. Here we assume that the documents may contain different styles of writing and the documents may be having skewed lines. Multiple, global or non uniform skew may present in the documents. Total 65 samples of handwritten documents having approximately 15 lines , 70 words and 150 characters in each document are scanned resulting into about 9750 different characters samples in the database.

B. *Pre-processing*

Before segmentation we have to perform some preprocessing on every document image. Preprocessing is used to reduce the noise and remove the unwanted data if any. We can also decrease the variation and transforms the data in specific format so that the data can be processed more easily and efficiently. First we need to convert the color document into gray image and then by using the threshold value we can convert it into to black and white. Remove all objects containing less than 30 pixels i.e. remove the unwanted objects or noise included in the image. Perform morphological operations like erosion and dilation of image as the writer may use different pen so thickness of character may be different .In normalization we will convert it into in to characters of single pixel width. We can also remove the skew if it is present in the document.

C. *Segmentation*

The text line segmentation methods can be normally classified into two types bottom-up and top-down. In the bottom-up approach, the neighboring components are grouped using some easy rules depending on the geometric relationship between neighboring blocks. The projection based methods are the top-down algorithms which are one of the most successful methods for machine printed text. The projection based methods are also successful for handwritten text where text lines are straight or easily separable. But due to different writing styles of the people, the text line segmentation is still very challenging. In general, text-line segmentation techniques are script independent. In the proposed system we have used projection based method for segmentation of lines and characters.

In Projection-based methods if an image A is of height H and width W i.e. of H * W size, the projection profile of the image is defined as follows:

$$P(i) = \sum_{j=1,2,\ldots..W} A\,(i,j), i = 1,2,\ldots..H, \quad (1)$$

Vertical Projection: For a binary image of size H * W where H is the height of the image and W is the width of the image, the vertical projection has been defined as

$$VP(j), j = 1,2,\ldots,W \quad (2)$$

This operation counts the total number of black pixels in each vertical column.

Horizontal Projection: For a binary image of size H * W where H is the height of the image and W is the width of the image, the horizontal projection is defined as

$$HP(i), i = 1,2,\ldots,H \quad (3)$$

This operation counts the total number of black pixels in each horizontal row.

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The projection is a one-dimensional signal that denotes the amount of text pixels per row as a result; the lobes (valleys) of the projection correspond to foreground (background) areas of the image. If the text lines have the same skew angle, the amplitude and the frequency of the projection are maximized when the skew of the text is zero. By calculating the projection for each angle and we can estimate the global skew angle according to a selected criterion. A common feature is the overlapping of successive text lines due to the ascenders and/or descenders of some characters. Hence, the formulation of a horizontal line as a separator is often not feasible to overcome this complexity, we can use the projections in order to locate the areas (i.e. the areas between two successive maxima) in which the separators should be allocated [24]. We have to find a path from the left to the right edge in each area, by attempting to move the obstacles.

In the proposed work text is written on plain paper. The lines and words are written in such a way that they do not overlap. We have segmented lines and words using horizontal and vertical projection profiles [21]. Peaks of the horizontal projection profiles separate the lines and in the vertical projection profiles separate the words in the document.
The following five steps are used in Segmentation.

- The line segmentation is based on horizontal histograms of the document. Those rows, for which HP[j] is zero; j = 1, 2, . . . ,H; serve as delimiters between successive text lines.
- The segmentation of the text line into words is based on the vertical projection of the text line. A vertical histogram of the text line is made and white spaces are used as word delimiter. Figure 3.a shows the horizontal and vertical projection profiles of the handwritten script.
- After extracting the sub-images corresponding to words for a text line, we locate the position of the header line of each word. In the horizontal projection of the word image the row containing maximum number of black pixels is considered as the header line.
- The header line can be removed by replacing all black pixels by white pixels.
- To separate character we make vertical projection of the image starting from the header line position to the bottom row of the word image box. The columns that have no black pixels are treated as boundaries for extracting corresponding characters.
- To separate symbols of the top strip we compute the vertical projection of the image starting from the top row of the image to the header line position. The columns that have no black pixels are used as delimiters for extracting top modifier symbol boxes. Figure 3.b shows the segmented words and characters.

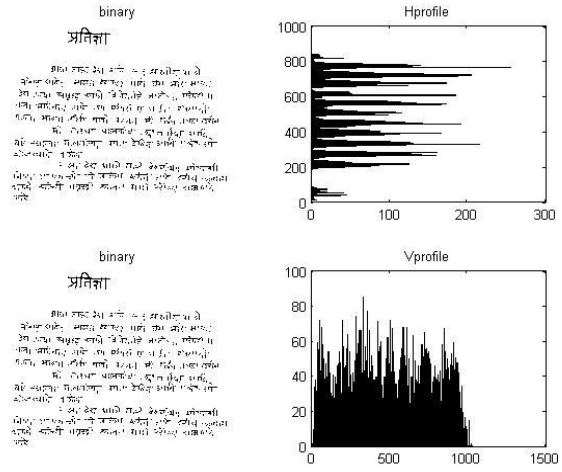

Figure 3.a

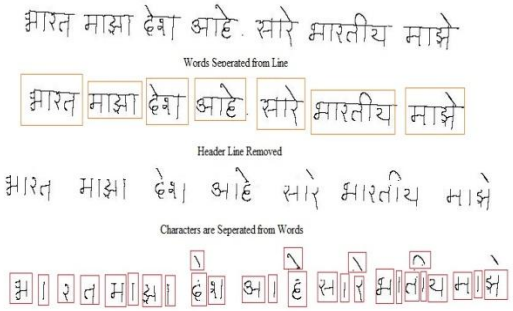

Figure 3.b

Figure 3. (a) Horizontal and Vertical Projection Profile
(b) segmented lines, words and characters

D. Feature extraction

A feature vector is preferred for high recognition results in optical character recognition. The structural feature and gradient based feature perform well in similar shape characters recognition in Devnagari. We considered the performance with features ranging from the simple most features in which each feature vector element was the direct pixel value to more computationally expensive features obtained from projection profiles and zoning features. In further study we may combine the direct pixel, profile feature with the zoning features. In this paper we have used three feature extraction methods i.e. Pixel values features, Histogram based features and zoning/block based features. A brief description of feature extraction is given in this section.

1) Pixel Value Features

In our experiments we have started with a simple feature definition i.e. the pixel value. In Pixel values method the character image is converted to black & white then the noise is removed. The hole filling operation is performed to obtain the uniform character. We formed feature vectors by storing the size normalized two dimensional digit images into one dimensional column feature vectors where each feature element is the pixel value. The image is cropped and resized into 15 X 15 matrixes. Then the character matrix is reshaped into column vector of size 225 X 1.

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2) Histogram Based Features

We have used simple histogram based features, which are easy to extract. Characters can be represented by projecting the pixel gray values. This representation creates one-dimensional signal from a two dimensional image, which can be used to represent the character image Histogram values can be obtained by drawing the histogram of gray character image and store the histogram count values in a column matrix of 256x1.Each feature element represents the histogram value. The histogram feature values range from 0 to 470 which is the gray value of each pixel in the character image. Figure.4 shows histogram of handwritten a character "EE"

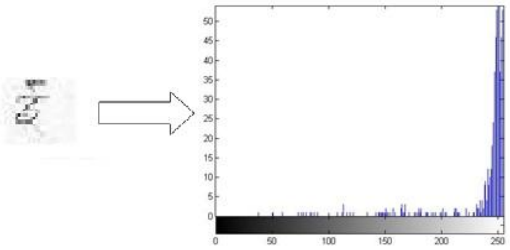

Figure 4. Histogram of character "EE"

3) Zoning /Block Based Features

The frame containing the character is divided into several overlapping or non-overlapping zones. The densities of the points or some features in different regions are analyzed. The image zoning value feature is the average pixel intensity value in a specified region or zone [23]. We defined these zones by dividing the size normalized image into equal number of rows and columns. We divide the character image into the zone size of 3x3 i.e. from the size normalized 15 x 15 character image we have extracted 25 features from 25 zones. We can calculate the mean values as follows. First we have converted the character image into black and white i.e. binary image. Then we have removed the blank space from left, right, upper and lower side of image. After that the images was resized into matrix of size 15x15 and divided into the blocks of 3x3.We have calculated the mean value for each block and then the mean values were stored in to a column matrix of size 25x1. The figure 5 shows the character matrix divide into 3x3 zone size.

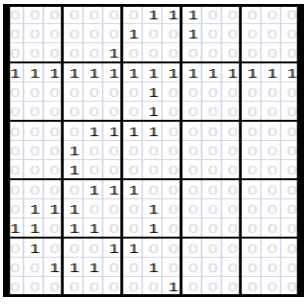

Figure5. Character EE divided into Zone size 3x3

E. Training and Testing of the Neural Network

A neural network is a set of connected input and output units in which each connection has a weight associated with it. The network will adjust the weights during the training phase so it will be able to predict the correct class for the input values. Feed forward neural networks, including multilayer perceptron (MLP), radial basis function (RBF) network, Back Propagation neural network (BPN) etc are used as classifiers in OCR system.

In feed forward architecture, the activations of the input units are set and then propagated through the network until the values of the output units are determined. The network act as a vector valued function it accepts one vector as the input and produce another vector at output.

Radial basis function can be used for approximating functions and recognizing patterns It uses Gaussian activation function. The response of such function is non-negative for all value of x. The function is defined as

$$f(x) = \exp(-x)2 \quad (4)$$

An Elman network is a three-layer network with the addition of a set of "context units". The middle (hidden) layer is connected to these context units fixed with a weight of one. At each time step, the input is feed-forward and a learning rule is applied. The fixed back-connections save a copy of the previous values of the hidden units in the context units.

Elman networks are also known as "simple recurrent networks" (SRN).

we have implemented four types of neural networks i.e. Feed forward , Radial basis, Elman back propagation and Pattern recognition neural network using three different types of feature extraction methods i.e. pixel value features, histogram based features and zone/block based features for each network[20]. The transfer functions TF{i} can be any differentiable transfer function such as TANSIG, LOGSIG, or PURELIN. The training function BTF can be any of the backprop training functions such as TRAINLM, TRAINBFG, TRAINRP, TRAINGD, etc. We have used TANSIG as transfer function and TEAINLM and TRAINGDM as training function.

We have trained totally twelve different types of networks using character samples of handwritten marathi alphabets collected from different persons. The weights and biases for each network are saved after training the neural network with sufficient samples. These already trained networks are then used for classification of the characters which we have separated from the handwritten script in the segmentation step.

First we have trained the network using different features and then we have used the same networks for recognition of the characters which we have separated from the hand written script using segmentation. The network has to simulate to get the output values and after that we have plotted the regression and confusion matrix. The following figures (Fig.7 and Fig.8) shows the performance plots for all the four types of neural networks i.e. feed forward, Radial Basis, Elman, pattern recognition. This indicates that the performance of Radial basis neural network works is more effective. It has been seen from the analysis that the histogram and zoning feature is giving better result compared to other features.

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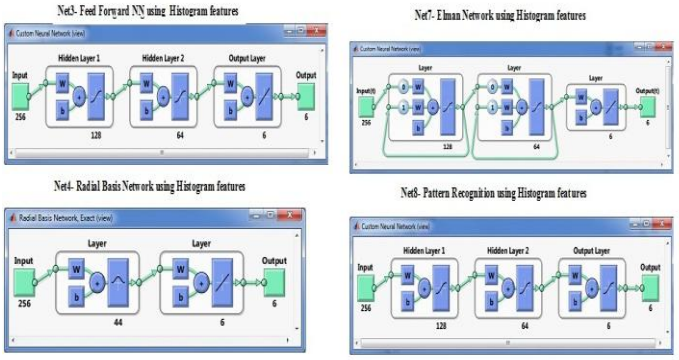

Figure6. Network structure of Feed Forward, Radial Basis, Elman and Pattern Recognition Neural Network

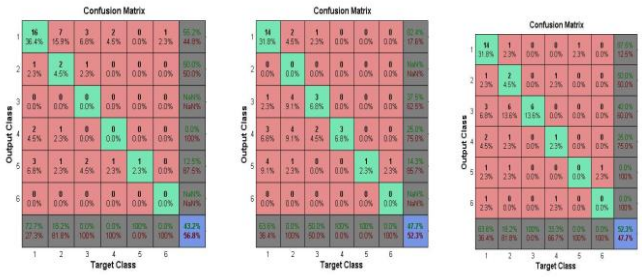

Figure 7. Confusion matrices for different feature extraction techniques

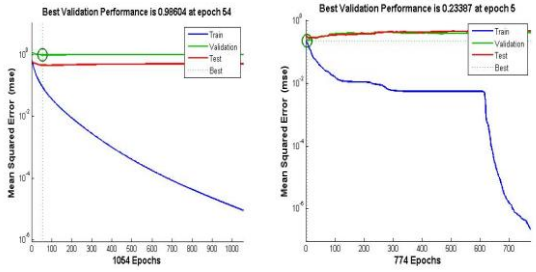

Figure 8. Performance plots for Elman and Pattern Recognition forward Neural Network

V. RESULTS AND DISCUSSION

The dataset of handwritten marathi document is created by collecting the samples from different individuals. About 65 samples of handwritten document are collected containing about more than 9000 character samples. Out of these, two third of the samples i.e. 40 documents were used for training and remaining were used for testing. The handwritten characters may have different shapes as per the writing style of the writer. This may result in classification of the same character to different classes. The characters are then normalized to a size of 15x15 for feature extraction. 225 pixel value features, 256 histogram based features and 25 zone/block based features are used to train the neural network. The inputs to the neural networks are equal to the number of features derived, but the number of hidden neurons depends upon the characters in that class. The performance goal is kept to 0.001 and 5000 epochs are enough to train the network using feed forward algorithm. The feed forward algorithm is the fastest back propagation algorithm. The time required for training the neural networks in each of the 44 classes depends upon the number of characters and the number of samples per character in that class. The testing time is the same irrespective of the class. The proposed system is implemented using MATLAB. The time required to test a character is approximately 0.045 seconds. Table 1 indicates the recognition results for all the three feature extraction techniques. The results show that the zoning based features improve the results considerably for both the training as well as testing samples. We can also improve the results by combining the features and by using different values in zoning based features like sum or mean of the pixels .

Feature Extraction Method/ Neural Network	Feed Forward NN	Radial Basis NN	Elman NN	Pattern Recognition NN
Pixel Value Features	71%	82%	71%	79%
Histogram Based Features	61%	89%	46%	86%
Zoning or Blocks Based Features	72%	77%	74%	64%

Table 1. Results of Three Different Feature Extraction techniques

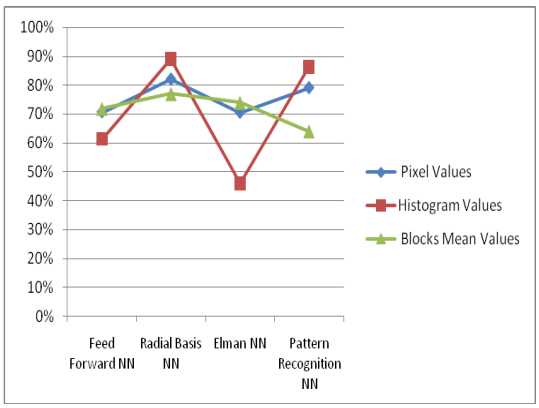

Figure 8. Recognition Results of all four types of Neural Networks

VI. CONCLUSION AND FUTURE SCOPE

In this paper we have tested the four different neural networks which clearly mention that the performance of Radial basis neural networks for recognition of characters separated from handwritten script which is better as compared to other networks. Here we have used three different types of segmentation methods i.e. projection profile, run length smearing and bounding box for. We can improve the results of segmentation by combining these projection profile method with bounding box method. From analysis we can say that zoning or blocks mean are more effective. We have not

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considered the modifiers and compound characters here so in future we can extend the same work for compound characters.

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Review on Optical Character Recognition of Devanagari Script Using Neural Network

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***Abstract*—** During the last decades lot of research work has been done in the field of character recognition on various scripts in various languages. In India peoples are used to speak national language Hindi and spoken by more than 500 million people. Many languages in India, such as Hindi, Marathi and Sanskrit has uses Devanagari as its base script .As compared to English character; Indian script (Devanagri) characters are complicated for recognition. Devnagri script is the basis for many Indian script including Hindi, Sanskrit, Marathi, Kashmiri, and so on. In this paper we present a review of research work that has been done in the field of character recognition in Devanagari script in past.

Keywords*: OCR, Preprocessing, Segmentation, Feature Extraction, ANN.*

*****

I. INTRODUCTION

OCR (Optical Character Recognition) is an emerging field of research in Pattern Recognition. In the world more than 300 million people use Devanagari script. Many languages in India, such as Hindi, Marathi and Sanskrit has uses Devanagari as its base script. Devanagari script has basic set of symbols consists of 34 consonants (or vyanjan) and 18 vowels (or swar)[2].Optical Character Recognition for Devanagari is highly complex. There is one difficulty with the Devanagari script is that a word written in Devanagari can only be pronounced in one way, but not all possible pronunciations can be written perfectly because language is partly phonetic in nature. Optical Character Recognition is a process in which scanned page, a printed document or handwritten document is converted in to ASCII character so that computer can recognize it easily. Due to lot of variations in fonts, size of the written characters; there is difficulty in character recognition. So, to remove difficulties in recognition following stages are used.

There are five major stages in the Character Recognition problem.

1) Scanning
2) Preprocessing
3) Segmentation
4) Feature Extraction
5) Classification
6) Post processing

In preprocessing step noise is removed [2]. After the segmentation of characters artificial neural network (ANN) is used to train the extracted character dataset and it will be then used for classification purpose. Use of artificial neural network techniques improves the performance of character recognition. Type Style and Fonts

Wherever Times is specified, Times Roman or Times New Roman may be used. If neither is available on your word processor, please use the font closest in appearance to Times.

Avoid using bit-mapped fonts if possible. True-Type 1 or Open Type fonts are preferred. Please embed symbol fonts, as well, for math, etc.

II. BLOCK DIAGRAM OF OCR

Any character recognition system goes under following steps, i.e. Image acquisition, Preprocessing, Segmentation, Feature extraction, classification and post processing. Block diagram of general character recognition system is shown in Figure.

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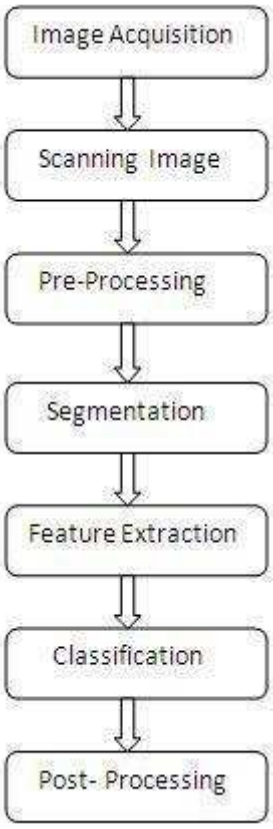

Fig 1. Block diagram of OCR

Following are major stages in the Character Recognition problem.

1) **Scanning:-** First characters written on the hard copy document is get scanned by scanner and then image is converted in to jpg format.
2) **Preprocessing:-**Preprocessing involves following steps
In the proposed OCR system, text is digitized with the help of scanner having resolution between 100 and 600 dpi. The digitized images are usually in gray tone.[1]

Binarization:- This phase consist of the process of converting a gray scale image into binary image by thresholding. Two intensity values are obtained as Black & White.

Size normalization: - As handwritten characters are not uniform in size. So, in order to get characters in uniform size normalization is applied. Each segmented character is normalized in to matrix like 32x32 or 64x64. So, that all characters have same size.

Noise elimination: - In general scanned image may have noise in it. Noise in image is a major problem in character recognition. Due to the presence of noise in the image degrades the quality of image affects on the accuracy in recognition of image. Many filtering techniques are used to remove noise from image. Noise elimination is also called as smoothing. Reduction of noise from the image improves the quality of image. Noise in the image includes distortion, gap in the lines, incomplete corners etc.

Thinning: - The process involves removal of selected foreground pixels from binary image.

3) **Segmentation: -** Segmentation is the process of partitioning an image/document into disjoint and homogenous regions. Segmentation is one of the most important and essential process that that improve the accuracy rate of character recognition system. Devanagari document is partitioned into sequence of lines and words by vertical and horizontal projection respectively. [6].

4) **Feature extraction**:- Feature extraction is defined as extracting the most useful information from the raw data, which minimizes the class pattern variability while enhancing the between class pattern variability [10].Feature extraction is important phase in recognition process and also referred as heart of OCR system. Feature extraction process extracts the most important and relevant shape information present in character.[9].Feature extraction is the special form of Reduction. It reduces the data when input algorithm is very large [8]. Feature extraction methods are broadly classified as Global Transformation and Series Expansion (i.e. Fourier Transforms, Gabor Transform & Wavelets), Statistical Features(i.e. zoning, Projection) and Geometrical and Topological Features (i.e. Extracting and Counting Topological Structures and Coding Graphs & Trees etc) [10]. The methods like histogram of individual characters and GLCM (Gray level co-occurrence matrix) are also considered in feature extraction for character recognition [7].

5) **Classification:** This stage is the decision making step in the optical character recognition system. There are several Classical and soft computing techniques available for handwriting recognition.

Following are the classical techniques used for classification.

a) Template matching: This is one of the basic technique recognition. Matching is used to determine the similarity between two points, curves, or shapes of the same type. In template matching, a 2D shape or a prototype of the pattern to be recognized is available.

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b) Statistical techniques: In this statistical approach, each pattern is represented in terms of d features or measurements and is viewed as a point in a d-dimensional space.

c) Syntactic Approach: In this approach, a formal analogy is drawn between the structure of patterns and the syntax of a language. This approach considers patterns are viewed as sentences belonging to a language and primitives are viewed as the alphabet and the sentences are generated according to a grammar.

6) **Post Processing:** Post-processing stage is the final stage of the proposed recognition system. It prints the corresponding recognized characters in the structured text form.[14][15].

III INTRODUCTION TO MARATHI SCRIPT

Devanagari word is derived from Sanskrit words Deva (god) and Nagari (city) jointly stand for "city of gods" **[4].**

Devanagari was originally developed to write Sanskrit but was later adopted to write many other languages. Base for every Indian script is Devanagari so called mother of all script. It is used to write languages like Hindi, Marathi, Nepali, Bhojpuri, Bhili, Marwari, Magahi, Maithili etc.[5]

The basic characters of Devanagari script consist of 36 consonants (Vyanjan) and 13 Vowels (Swar). Devanagari script has specific composition rules for joining consonants, vowels and modifiers [6].

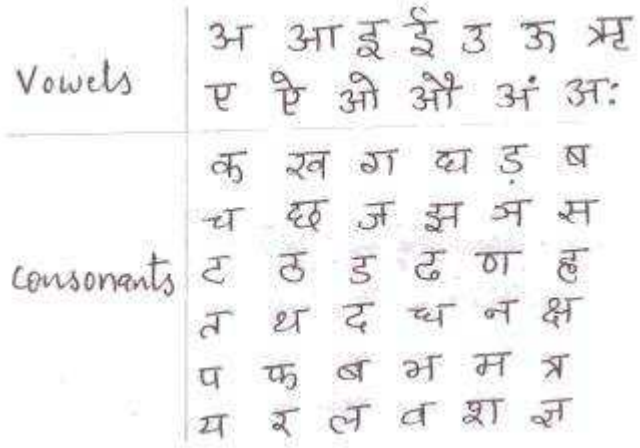

Fig 2: vowels and 36 consonants of Marathi script.

IV ARTIFICIAL NEURAL NETWORKS

A neural network is a powerful data modeling tool to capture and represent complex input/output relationships. Pattern recognition is extremely difficult to automate. As human brain learn and interact with real world object in the same way Artificial Neural Networks (ANN) develops a computational model that behaves same as human brain interacts and learns new things. ANN consists of a number of units called as Neurons with weighted connections and that work parallel. Learning algorithms are adjusting these weights so as to process information. When this neural network is fully trained we will get the information at the output nodes. The most popular algorithms used are Feed Forward Network, Back Propagation Network, and Radial Basis Function etc. The Back Propagation algorithm determines the weight for a multilayer ANN with feed-forward connections. During the learning phase, the computation is done by minimizing a mean square difference between the desired output and the actual output.

Mathematical Model

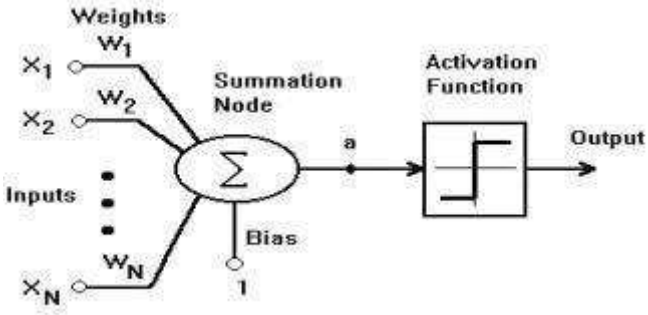

Fig3: Structure of Neural Network

The most common model used in neural network modeling is the multilayer Perceptron (MLP). Supervised learning mode is used in this type of neural network because it requires a desired output in order to learn. This model correctly maps the input to the output. A following fig shows graphical representation of an MLP [11][12].

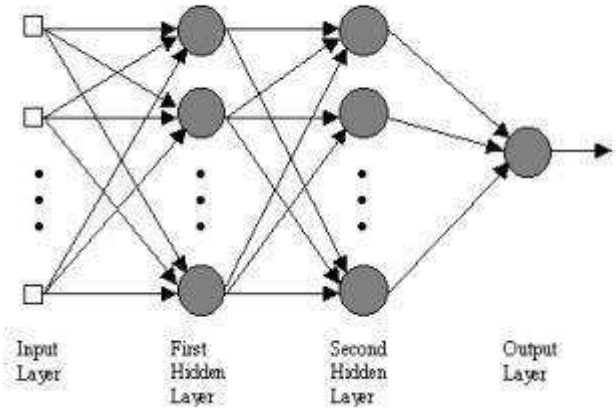

Fig 4:-Block diagram multiplayer Perceptron (MLP).

The inputs are fed into the input layer and get multiplied by interconnection weights as they are passed from the input layer to the first hidden layer. Within the first hidden layer, they get summed then processed by a nonlinear function (usually the hyperbolic tangent). As the processed data

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leaves the first hidden layer, again it gets multiplied by interconnection weights, then summed and processed by the second hidden layer. Finally the data is multiplied by interconnection weights then processed one last time within the output layer to produce the neural network output.[13]

V RELATED WORK

In this paper authors reviewed methods for pattern representation as statistical methods and classification. Among these two approach author pay more detailed attention towards statistical methods approach. Many neural network algorithms are discussed and various approaches like Template Matching, Statistical Approach, and Syntactic Approach and Neural network for optical character recognition system are discussed.[14]

In the paper authors reviewed and presented a method for Devanagari Optical Character Recognition (DOCR). Author reviewed various methods for character recognition system. Two approaches off- line and on-line character recognition techniques have been discussed. But, as compared to on-line more attention is given to off-line character recognition. Various soft computing methods involved and various classification techniques for optical character recognition like Template Matching, Statistical Techniques, and Neural Networks has been discussed.[16].

Neetu Bhatia in his paper presented a detailed review of Optical Character Recognition and proposed various techniques for character recognition system. Handwriting recognition system divided into types as off-line and On-line character recognition. Where off-line handwriting recognition is difficult and involves automatic of text into an image and on-line involves data stream. Usually OCR involves an off-line character recognition process. Which scan and recognize images of the characters? It is translation of images of handwritten character or into machine code without any variation.[17].

In the paper authors had done a brief survey of Devanagari script and different approach used in classifier for Character Recognition. They proposed method for extraction of feature using local intensity distribution of gradient. They created database of handwritten characters. They have used KNN Classifier. For finding similarity in the pattern they proposed Euclidian Distance method based on K-NN Classification.[18]

In the paper author reviewed the existing works in handwritten character recognition based on Evolutionary computing approach. Author discussed about various approaches like Bio-inspired evolutionary algorithms are probabilistic search methods that simulate the natural biological evolution or the behavior of biological entities, fuzzy approach, and genetic approach.[19]

In the paper authors used the Rectangle Histogram Oriented Gradient representation as the basis for extraction of features.

The algorithm operates on per image pixel. They uses dataset of 8000 samples each of 40 basic handwritten Marathi characters. All sample images are normalized to 20 × 20 pixel size. To obtain result they used support Vector Machines (SVM) and feed-forward Artificial Neural Network (FFANN) classification techniques are used to obtain highest accuracy in Marathi character reorganization.[20]

The author discussed the handwriting recognition systems, evolution and progress. The paper focused on Indic scripts like Bangla, Devnagari, Gurumukhi, Kannada, Malayalam, tamil, and Urdu. The paper focused on multitude of feature and classification techniques and explores new opportunities and challenges in imaging sciences [21].

Author proposes new approaches for extracting features in context of Handwritten Marathi numeral recognition. Artificial Network is used for classification. The overall accuracy of recognition of handwritten Devanagari numerals is 99.67% with SVM classifier, 99% with MLP and it is 98.13with GFF [22]

The paper proposed a new shape based technique for recogntion of isolated handwritten Devnagari characters. Using basic structural features like endpoint, cross point, junction points and adaptive thinning algorithm the thinned character is segmented into segments (strokes). The segments of characters are coded using our Average Compressed Direction Code (ACDC) algorithm. The average accuracy of recognition of the proposed system is 86.4%[23].

The paper presented a new approach for extracting features in context of Handwritten Devanagari Vowels recognition. Artificial Network is used for classification technique. The overall accuracy of recognition of handwritten Devanagari Vowels is % with SVM classifier, % with MLP and it is %with GFF[24].

In the paper, authors discussed the characteristics of the some classification methods that have been successfully applied to handwritten Devnagari character recognition and results of SVM and ANNs classification method, applied on Handwritten Devnagari characters. This process extracted features like shadow features, chain code histogram features, view based features and longest run features. These features are then fed to neural classifier and in support vector machine for classification [25].

This paper guides working on the text based image segmentation area. Author first, the need for segmentation and then, the various factors affecting the segmentation process are discussed. Followed by the levels of text segmentation are explored. Advantages and disadvantages of segmentation are also discussed [26].

This paper focuses on extracting structured data from unstructured data using OCR (Optical Character Recognition) and Neural Network. It focuses on recognizing characters of a document, which does script identification from a variety of unstructured printed or handwritten documents. Discrete Cosine transform function is used to obtain data sets for classification and recognition [27].

The paper describes the behaviors of different Models of Neural Network used in OCR. They mainly focused on parameters like number of Hidden Layer, size of Hidden Layer and epochs. They used Multilayer Feed Forward network with Back propagation. In preprocessing segmentation of characters, normalizing of characters and Deskewing obtained by applying basic algorithms. They have used different Models of Neural Network and applied it on the test data to find the accuracy of the respective Neural Network [28].

This paper presents a Complete OCR system for Marathi text newsprint using Minimum distance classifier [29].

An efficient image retrieval technique is proposed, which uses dominant color and texture features of an image. The attempt is made to enhance the existing results by extracting various supportive features like moments invariant, vector Gradient, chain code (freeman chain code) image thinning, structuring the image in box format, noise removal, etc. A performance of approximately 90% correct recognition is achieved [30].

VI CONCLUSION

Character recognition is one of the important applications of pattern recognition. Day by day popularity of OCR is increasing with the advent of fast computers. But still, lot of research work is needed in OCR to handle the complexity and issues in character recognition.

This paper carries out a study handwritten character recognition using Artificial Neural Network. Artificial neural networks are commonly used to perform character recognition due to their high noise tolerance OCR tries to make automation in character recognition to reduce human errors. Artificial neural network is commonly used for training the system. The scanned (input) image is processed and weights are stored and they are used to train data from neural network. Various models like back propagation, multilayer Perceptron used to compare the input image with the trained set to obtain high accuracy in characters recognition.

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