PROJECT TITLE

Mining of Extremophilic microbial communities of Himachal Pradesh.

Submitted in fulfillment of the Degree

5 Year Dual Degree Programme B. Tech - M. Tech

June-2017

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT

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CERTIFICATE

This is to certify that the thesis entitled "Mining of Extremophilic microbial communities of Himachal Pradesh" submitted by Mr. Tushar Singh Barwal to Jaypee University of Information Technology Waknaghat in fulfillment of the requirement for the award of the degree of B. Tech – M. Tech dual degree is a record of bona fide research work carried out by him under my guidance and supervision and no part of this work has been submitted for any other degree or diploma.

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Acknowledgement

After a long journey of two years' time has come to look back and remember all those without whom this journey would have been impossible. Nothing worthwhile in life comes without struggle and these are the people, in their own way have helped me to overcome the difficult situations. It's because of these people that I have reached this wonderful destination.

I am indebted to my mentor Dr. Saurabh Bansal for allowing me to work under his guidance. His belief in me strengthened and stabilized me during various phases of this course. It was because of your patience that I could find path out of fog. I also appreciate the untiring patience and efforts during the entire tenure of my project.

I wish to express my deepest sense of gratitude to our current head of department Dr. R.S. Chauhan, for providing all the necessary facilities, and creating an environment which supports research and scientific ventures.

Special thanks to Dr. Gunjan Goel for extending support of his facilities to carry out research and critically review of my work.

I am highly obliged to Mr. Sampan Atri and Miss. Kavita Sharma for his support and guidance at various stages of the study.

I am extremely thankful to non-teaching staff especially Mr. Baleshwar, Mamta mam and Somlata mam who willingly extended their help every time I required.

I am thankful to Jaypee University of Information Technology for funds to carry out my research work.

I am forever thankful to Almighty God.

Tushar Singh Barwal

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CHAPTER-1

Introduction

1.1 Introduction

An extremophile is an organism that thrives under "extreme" conditions. The term frequently refers to prokaryotes and is sometimes used interchangeably with *Archaea*. In this module, however, you will find that extremophiles come in all shapes and sizes, and that our understanding of the phylogenetic diversity of extreme habitats increases daily. The term extremophile is relatively anthropocentric. Most terms used to describe extremophiles are generally straightforward. They are a combination of the suffix *phile*, meaning "lover of," and a prefix specific to their environment. For example, *acidophiles* are organisms that love (*phile*) acid (*acido*).

i. Acidophile: An organism that grows best at acidic (low) pH values.
ii. Alkaliphile: An organism that grows best at high pH values.
iii. Anaerobe: An organism that can grow in the absence of oxygen.
iv. Facultative Anaerobe: An organism that grows in the presence or in the absence of oxygen.
v. Obligate Anaerobe: An organism that cannot grow in the presence of oxygen; the presence of oxygen either inhibits growth or kills the organism.
vi. Endolith: An organism that lives inside rock or in the pores between mineral grains.
vii. Halophile: An organism requiring high concentrations of salt for growth.
viii. Methanogen: An organism that produces methane from the reaction of hydrogen and carbon dioxide, member of the Archaea.
ix. Oligotroph: An organism with optimal growth in nutrient limited conditions.
x. Piezophile (Barophile): An organism that lives optimally at high hydrostatic pressure.
xi. Psychrophile: An organism with optimal growth at temperature 15°C or lower.
xii. Thermophile: An organism with optimal growth at temperature 40°C or higher.
xiii. Hyperthermophile: An organism with optimal growth at temperature 80°C or higher.
xiv. Toxitolerant: An organism able to withstand high levels of damaging elements (e.g., pools of benzene, nuclear waste).
xv. Xerophile: An organism capable of growth at very low water activity.

1.2 Halophiles-

Uses of salt as a preservative predate to the ancient Egyptians and have been used to preserve food for nearly 8000 years. Great Salt Lake, second in salinity only to the warmer Dead Sea, was once considered equally devoid of life. The North arm of Great Salt Lake is saturated with dissolved salts (>30%), yet life has found a way in all such lakes. We now know that hyper saline bodies of water that exceed the modest 3.5 % salt of earth's oceans are populated with rich communities of "halophiles," or salt-lovers. These microbes are in all three of the Domains of life, Archaea, Bacteria, and Eukarya [1], however, eukaryotes are in small numbers. The halophilic microbes are colored with carotenoids compounds in their cell membrane, painting the waters with a pink-orange hue. Some species also have a purple membrane, regions where bacteriorhodopsin (BR) or other rhodopsin-like chromo-proteins reside [2].

In India there are a few locations where you can find rock salt mines one of such location is located in Himachal Pradesh. To our knowledge it is one of the only locations in India where you can find deposit of rock salt and it needs to be explored for the identification of micro flora and look for invaluable genes, enzymes and compounds.

One of the major causes for the study of halophilic organism is to find and understand the biochemical and metabolic pathways involved in extreme condition. The capability of Halophiles to grow under such high salt concentration makes them valuable for future biotechnology pursuits.

Halophiles include Eukarya, bacteria and Archaea at low concentration of salt Eukarya and bacteria are more dominant and as the salt concentration increases the Archaea gets dominant. To prevent loss of water in such a high salt concentration halophilic organism contains some compatible solutes which maintain the osmotic balance. The osmolytes are usually amino acids (example glycine-betain ectoine) or sugar which helps in the maintenance of the balance of water.

The use of enzyme is not new and dates back to the ancient Greeks. All enzymes are proteins with an exception of certain RNA molecules and have diverse functions. In the cellular system the enzymes perform varied functions varying from the maintenance of the cell to the defense of cell against pathogens. Over the past few decades, use of enzymes as a biocatalyst have tremendously contributed to the diverse branches of industries, especially enzymes which are derived from the extremophilic

organism which make them able to rigorous conditions present in most of the industrial processes. Some of the enzymes which are produced by halophilic organism are-

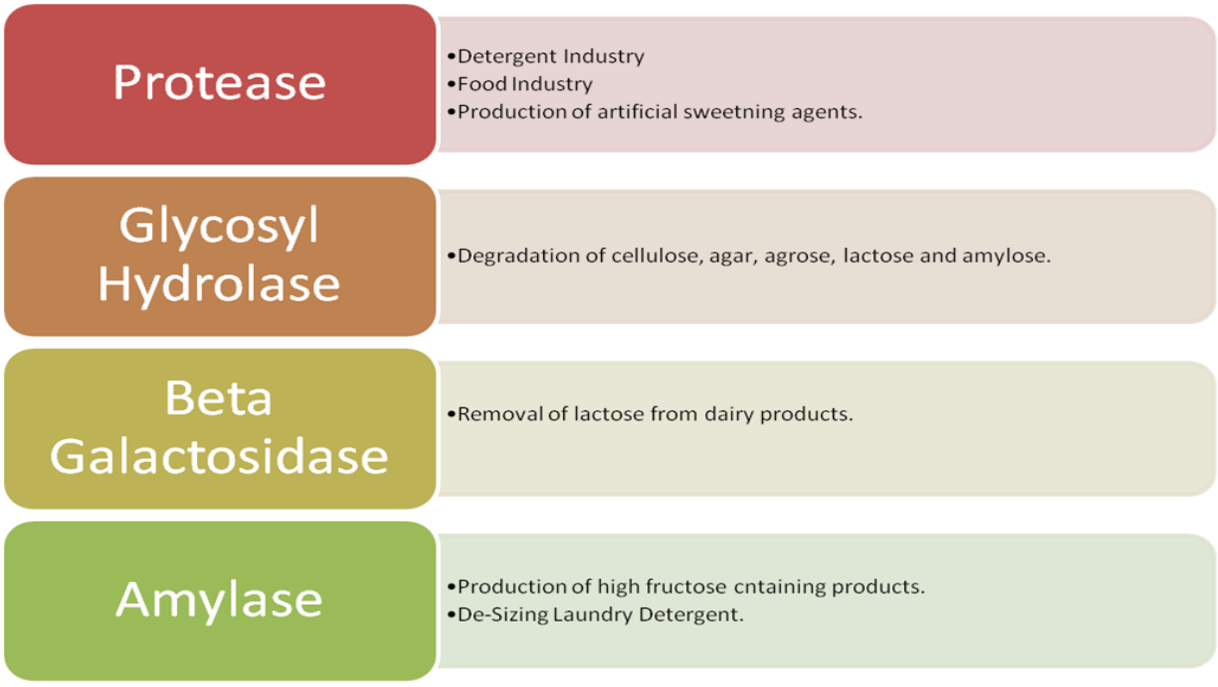

Fig.1.1-Enzymes produced by various Halophilic Organisms.

In order to study the halophilic environment like rock salt mine, the present study was conducted and samples were collected from Rock Salt mine in Drang in Himachal Pradesh. The inhabitants of the rock salt mine were isolated and characterized using both culture and non culture based techniques like PCR and DGGE.

1.3 Objective- Study on Halophilic site in Himachal Pradesh;

A. Characterization of population diversity and strain/species domination.
B. Identify strains that are a key role player in terms of production of various enzymes.

Polymerase chain reaction denaturing gel electrophoresis (PCR DGGE) is a recent technology used in the identification of micro organism isolated from a variety of sources. For the past decade bacterial identification using Molecular methods have become very popular, especially the ones based on sequencing of gene coding for 16s rDNA. Culture independent methods have gained wider acceptance due to the capability to overcome problems associated with selective culturing and isolation of natural

samples. The main reason for employing culture independent methods is the lack of knowledge of real growth conditions in which most bacteria grow.

DGGE is one of the most commonly used culture independent fingerprinting technique. It is based on the separation of PCR product on the size of different sequence. PCR-DGGE is used to access the structure of microbial environment communities in sample without employing any cultivation technique and to determine the community dynamics within change in the natural habitat. [3]

1.4 Rational Behind this study-

1. Subsequent research and future potential application of these strains.
2. Information on characterization of micro-organism isolated from Gumma Salt mines.
3. Exploitation of certain strains for the production of enzyme.

CHAPTER-2

Review of literature

Review of Literature

2.1. Extremophiles: Definition and Terminology

Extremophiles are the organism that perches the extreme ecological niche of our planets, such as intense temperature, pressure, Salt concentration, pH, nutrient concentration, water ability, harmful heavy metals, toxic compounds (organic Solvent) and even places with high level of radiations. Extremophiles can be classified into

I. Halophilic organism, living in presence of high salt concentrations (2.0-30.0%)

II. Thermophilic and hyperthermophilic organism, living at a high temperature (up to 75°C) and very high temperatures (up to 115°C)

III. Acidophile and Alkalophilic, living at extreme acidic and basic environments

IV. Psychrophilic organism, survive and reproduce in extreme cold conditions with a very low temperature (-15°C or lower temperatures)

Apart from the above-mentioned classes the extremophilic organism can be classified into metallophilic organism living in high metal concentration, radiophiles living in high radiation areas, Piezophile, living in a high-pressure environment and organism living in an environment completely devoid of oxygen (Table- 2.1) [5].

The main focus for diverting attention towards research on the topic of extremophiles is based on the strategies employed by these organisms to live and colonise such harsh environments. Halophiles, for example, accumulate certain osmolytes (usually certain amino acids like glycine –betain ectoine, sugars, sucrose and polyols trehalose and glycerol) to maintain proper water level in the cellular system to perform various cellular function [6]. Thermophile, for example, have a special covering around the cell which enables it to live in a very high-temperature environment (55 to 95°C) and carry on with its various biological functions [7].

Extremophilic organism contains various enzymes which confer a high level of flexibility when compared with their non-extremophilic counterpart. High flexibility is achieved by making changes around the active site, this leads to low activation enthalpy, low substrate affinity and high specific activity at a varying degree of temperature [8].

Extremophilic microorganism represents a high commercial and industrial value due to a production of wide variety of biomolecule (example proteins, enzyme, and osmolytes). These organisms have created a multimillion dollar industry. The major sector for the industrial application lies in the agriculture sector and the biomedical sector. As most of the proteins and enzymes are capable of working under very harsh and extreme environments these microbial products tend to find a wide variety of application in the harsh industrial processes. These biomolecules have an advantage of being biodegradable, high specific stability under extreme condition increased amount of product generation, decreased the amount of waste and ability to use cheaper raw materials.

Among all extremophiles thermophiles and hyperthermophiles is the organism which is a most studied organism. Research in extremophiles has unveiled new pathways and biomolecules and creating new questions about the various microbial adaptations to extreme condition and provided with some wide industrial applications. In comparison, other extremophiles like halophiles radiophiles have been studied much less.

Table 2.1 Characteristics of natural extreme environments inhabiting extremophiles.

Stress	Phenotype	Environmental condition	Habitat	Product	Application
Pressure	Piezophile	500.00-1034.00 atm.	Deep marine trenches	Whole Microorganism	Formation of gels and starch granules
High pH	Alkalophile	> 10.0	Soda lakes	Cellulase	Polymer degradation in detergents
Low pH	Acidophile	< 3.0	Sulphide-rich geothermal	Sulfur Oxide	Desulfurization of Coal
Salt	Halophiles	2.0 to 30.0%	Salt Brine , Salterns	Whole Microorganism	Biopolymer
Extremely high	Hyperthermophile	80.00 to 1220.00°C	Submarine Vents	Enzymes	Cellulose and hemicellulose treatment
High Temperature	Thermophile	65.00 to 95.00°C	Terrestrial Hot Springs	Amylase Protease Xylanase	Glucose, Fructose for Sweetener Paper Bleaching Baker Brewing and detergent
Low Temperature	Psychrophile	-12.00 to -4.00 °C	Polar regions, Glaciers, Deep marine trenches	Protease Dehydrogenase Amylase	Cheese maturation, dairy products Biosensor Polymer degradation in case

2.2 Halophilic Microorganisms

2.21 Definition of Halophiles

A halophile is a diverse group of microorganism which adapts to moderate to high degree of salt concentration. A halophilic organism is a group of very diverse organism found in all three domains of life form -Archaea, Bacteria and Eukarya. The natural habitat of halophiles in high salt containing areas like salt or soda lakes, coastal lagoons, salt mines, oceans, and manmade Salterns. Halophilic bacteria reside in a salt concentration of (5-15% or above), whereas the archaea inhabit the very high salinity environment.

Natural habitat conditions of the halotolerant bacteria provide biochemist/physiologist with interesting questions on the strategies employed by these microorganisms to cope up with high osmotic pressure extended by the hypersaline environment. Halophiles are an important organism saline aquatic habitat where it interacts with multicellular eukaryotic species like brine shrimp, brine flies etc. Bacterial halophiles can be classified into five different phyla as shown in Fig 2.1

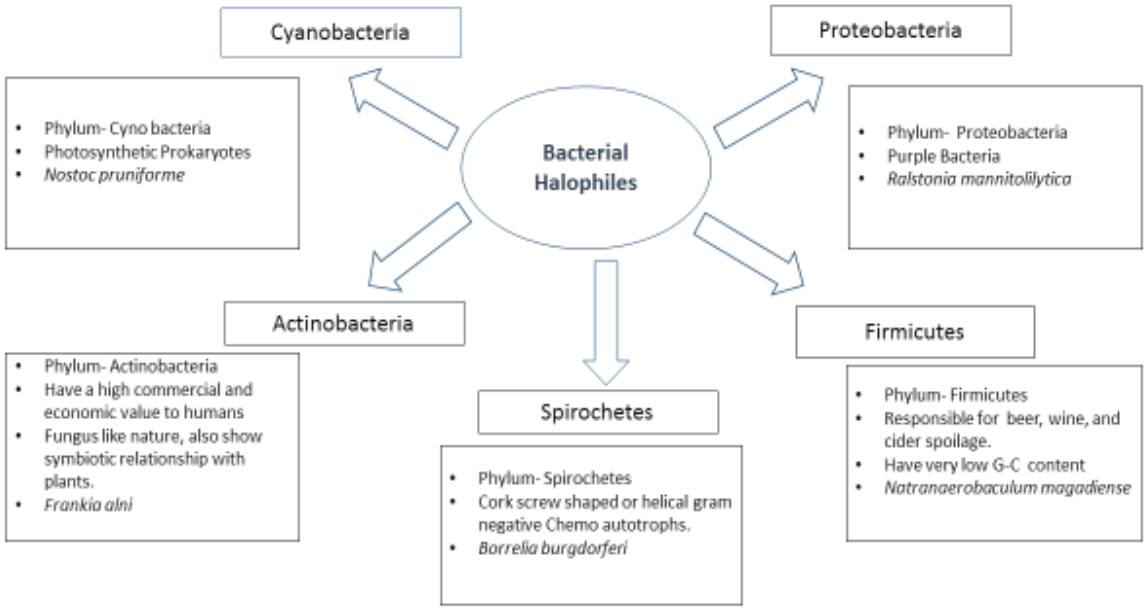

Fig 2.1-Classification of Bacterial halophiles.

Phylogenetic analysis of various halophilic and non-halophilic species has revealed a very high relatedness and many genera family and order have representatives with greatly different salt requirements and tolerance. In the case of Archaea, the most tolerant organism is found in the class Halobacteria [9]. Salt is an integral part of all biological lifeforms, but halophiles are distinguished from their non-halophilic counterparts on the requirement of hypersaline conditions for growth. On the basis of this requirement of salt for growth, halophiles can be classified as slightly halophilic, moderately halophilic and extremely halophilic. Classification on the basis of amount of salt required for the growth of halophilic organism is shown in table 2.2

Table 2.2- Classification of halophiles.

Type	Salt Concentration (M)	Salt Concentration (W/V)	Reference
Slightly Halophilic	0.2-0.85	2-5%	[10]
Moderately Halophilic	0.85-3.4	5-20%	[11]
Extremely Halophilic	3.4-5.1	20-30%	[12]

The halotolerant organism is the one which can grow in an environment of high salinity and in the absence of high salt concentration. Halotolerance has a high evolutionary significance as shown by the concentration of brines during prebiotic evolution at earliest evolutionary times [13]. A thorough knowledge of halotolerant can help in solving human problems like agriculture in arid regions, Xeriscaping aquaculture and remediation of salt affected biotic habitats. The halophilic and halotolerant organism can grow over a wide range of salt concentration depending on the environmental and nutritional conditions. High salt concentration is inhospitable for cells as it causes osmotic imbalance, to protect the cell against such osmotic imbalance halophilic organism employ two major strategies to lower the chemical potential of cell water, this allows the cell to adapt to the external environment these two strategies are “salt in cytoplasm” and “organic-osmolytes mechanism”.

Fig 2.2- Hypersaline habitats: Left: Secovlie Salt works on North Adriatic Sea. Right: Salterns operation near Udupi on the Malabar coast, south India.

2.3 Habitats of Halophiles

Natural Habitats of halophiles vary from Hypersaline environments which are common throughout the world from Hypersaline environments which are common throughout the world to extremely Hypersaline habitat which is very rare. Many halophilic regions lie in the dry and arid areas of the world. The origin of most halophilic habitats lies in the areas where the evaporation of sea water and formation of highly saline brines example great salt lakes and the Dead Sea. Most of these habitats have similar composition as seawater, but the dominant ions present are Na^+, K^+ and Cl^- ions with a pH which varies from a neutral to slightly basic pH. Evaporation of water is the major cause for high ionic composition, because of evaporation-precipitation of salts like gypsum ($CaSO_4.2H_2O$) and other salts take place once their solubility is decreased. The best example for such an environment is the Dead Sea and Lake Baskunchak in Russia. Natural environment combines areas with high pH, Salt concentration and temperature still microbial life has adapted to such harsh environments.

2.3.1 Saline Soils

Salt is a natural component of both soil and water. Soil being a heterogeneous mixture of various compounds contain ions like Na^+, K^+, Ca^{+2} and Cl^-. In saline soil the Na^+ combines with the Cl^- ion present in the soil to form salt. Saline soils tend to be inhabited by halotolerant organism rather than by halophilic strains. Early studies have proved that the inhabitation of saline salt by microorganism is due

to passive inhabitation by the wind [14]. *Bacillus*, *Pseudomonas* and *Alcaligenes* are the most dominant genera present in the soil whereas *Salvinivibrio* is the most dominant genera found in the salterns and salt lakes [15]. Study based on saline based soil habitats are less when compared with other hypersaline environment but isolation of novel strains from Death Valley California, Iraq and Israel have proved that there is still a lot of work to be done in this extremophilic habitat.

2.3.2 Salt Mines

A salt mine is a mine from which halite, commonly known as rock salt is extracted. Halite forms isomeric crystals. These crystals are typically colorless or white but can turn light blue, dark blue, purple, pink, red, orange, yellow or grey depending on the nature of impurity. In (1960) Reiser and Tasch succeeded in isolating diplococci, this organism was able to grow up to 30% (W/V) NaCl and remained unisolated from mine sumps or mine air. The organism was first isolated in Permian salt mine (Kansas, USA). Such similar work was done by (Draganescu, 1990) of the Carpathian area and the Permian rocks in Europe [16], found the genus *Halorubrum* and *Haloaurcula* in England's Winsfort Salt Mine in Cheshire. The Archeal Halophiles were isolated from the Australian Salt mines. Biochemical Studies performed on the organism found an optimal pH of 6.8 to 9.5 in which the organism were able to grow. Various other studies performed on these isolates suggested that they relate to the genus *Halococcus*. Ancient evaporation deposits are found to be inhabited by Halobacteria and some eubacteria. Nearly 47 strains of Bacteria have been isolated from Wins ford Salt mine England [17]. Salt deposits are Scattered evenly around the world, and have been exploited from ancient times by salt mining companies [18] but still very little is known about the micro biota.

2.3.3 Marine Salterns

Marine Salterns are composed of several ponds with a salinity gradient varying from sea water to higher salt concentration. The microbial population varies with change in the environmental conditions. These salterns are sites for commercially operated for producing NaCl. Different marine salterns have been analyzed throughout the world including Spain, Mexico, Santiago, and Newark, California, Italy etc. Major Saline sites studied in India are Sambhar Salt Lake in Rajasthan, Tamil Nadu, Maharashtra, Andhra Pradesh, Orissa and West Bengal. In India there are about 10,000 production site. The main

genera of organism found near Huelva Spain facing the Atlantic Ocean include moderately Halophilic *Deleya, Flavobacterium, Acinetobacter, Haloarucula, Haloaccus* [19]. The red- orange color of the crystallizer ponds is due to the presence of both red halophilic archaea and *Dunaliell*.

2.3.4 The Salt Lakes

These are the land locked bodies that have a concentration of salt and other dissolved minerals significantly higher than most lakes. In most cases the concentration of salt is higher than that of sea water. Certain lakes have a very high concentration of carbonates in them because of which they are called as soda lakes. Most of these salt lakes are colored either red, Pink or bright Red. This coloration is because of the countless micro-organism thriving in this salt water. Some of these environments in which these organisms are found are Great Salt Lake in Utah, Owens Lake in California and the Dead Sea.

2.4 Identification, Characterization of Halophiles

The wide studies carried out on the environment of halophiles have permitted the isolation and taxonomic characterization of large number of halophilic species. In 1935 Hof Inoculated Java mud from various solar soils into a variety of salt concentration media. After a brief inoculation or inoculation tenure red archaeal types and white colonies were isolated including *Bacillus species* which are able to grow in a NaCl concentration of 24%.

In 1992 Liew and Mah reported two halophilic anaerobic bacteria from salterns in California USA. These two anaerobic bacteria were morphologically long, gram negative, motile, flexible rods. The physiological and Biochemical characterization were found to be similar to Haloanaerobic bacteria. Growth of the bacteria was found at a salt concentration of 0.5 to 5 M and a temperature range 23.0-50.0°C. The strains were found to be sensitive to Chloramphenicol but show resistance to penicillin, carbenicilin, D-Cycloserine and tetracycline.

Whole protein and DNA analysis of the samples showed high taxonomical relatedness but a clear distinguishable from other halophilic anaerobic bacteria. The G+C content of the bacteria was found to be 34.8 mol. % which leads to establishment of new species Halo-anaerobacter chitinovorans. Bacterial

Screening from solar slatterns in Sfax (Tunisia) have lead 40 **new** is isolates of halophilic bacteria with an on optimal growth range of 5.0-15% of salt concentration. 16s RNA gene sequencing was done for phylogenetic characterization. Out of the 40 strains 36 strains are said to belong to Gamma-Proteobacteria and 4 strains of Finnicutes. Most of the strains were said to produce large amount of Enzymes like amylase, proteases, phosphatases and DNase.Fig.2.3 shows the phylogenetic tree for the halophilic organism.

2.5 Industrial potential of Halophiles

The applicative potential of Halophiles lies next to only thermophiles. Although at present most application lies in the food (soy and fish sauce) sector, production of beta- carotenoids and aquaculture are a few examples. Many of the application lie under the tanning industry Bio-fuel production and medical industry [20]. Most of the industries tend to release brine into the environment. Halophiles find a wide variety of application in bioremediation of such hyper saline brines.

Halophiles produce wide variety of stable and unique bio-molecules that find useful practical application. Halophiles produce a wide variety of enzyme such as amylase, proteases, lipases and nucleases of high commercial and academic value. These enzymes are capable of functioning under conditions which lead to denaturation and precipitation of most of the non halophilic enzymes [20].

Many novel halophilic bio-molecules have specialized industrial applications for example. Ectoine for cosmetic industry, Glycine, betaine for animal feed additive, pigments for food coloring, compatible solutes for stress resistance. Some other applications are shown below Fig.-2.3. Still there is a lot of untapped potential when it comes to Halophiles.

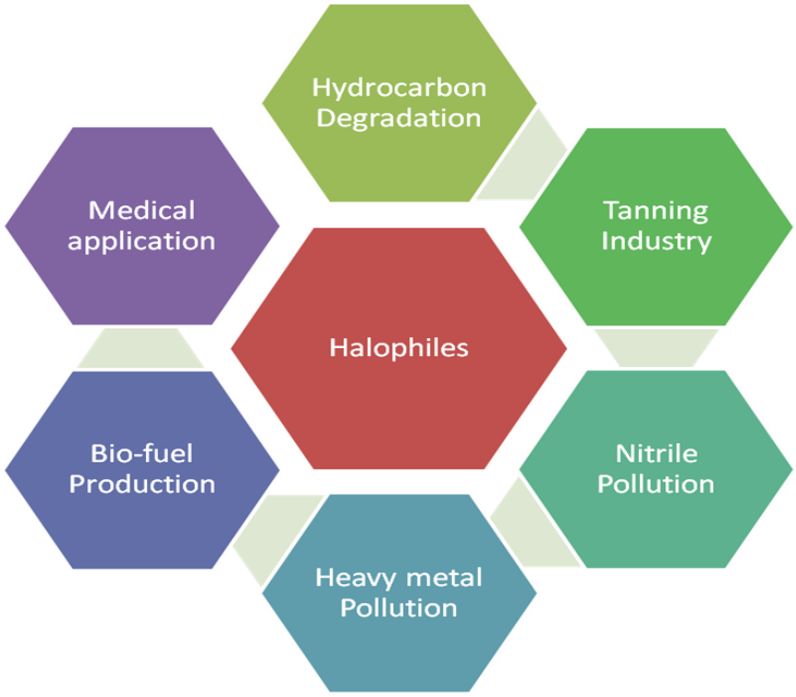

Fig2.3- Industrial application of various Halophiles.

2.6 PCR-DGGE Fingerprinting

Polymerase chain reaction denaturing gradient gel electrophoresis is an applied molecular biological technique used in the identification and monitoring of organism in their natural habitat. PCR-DGGE has become a valid tool to assist traditional m microbiological methods. In the past few years, identification based on sequencing of gene coding for ribosomal 16S rDNA have gained a lot of popularity. Non culture based techniques are gaining a lot of importance due to lack of knowledge of real conditions and difficulty in developing media for cultivating accurately resembling natural conditions. Denaturing gel electrophoresis is unquestionably one of the most widely used culture-independent fingerprinting technique [21].

PCR-DGGE is one of the most widely used technique to study the structure of microbial community in the environment samples without any need for conventional culturing methods. The technique has been widely employed to study microbial makeup, evolutionary relationships and structure of communities residing in rivers, soil, mountains, glaciers, oceans etc.

2.7 Technical aspects-

DGGE is a gel based electrophoresis technique which is employed to detect difference between DNA fragments with a similar length but dissimilar sequences. This is because of the use of denaturing gels which have differential denaturing (melting) profile. Urea is used to produce such denaturing gel solutions with a denaturing gradient consisting of 7M urea and 40% formaldehyde in water.

Gels with different denaturing gradients are prepared and then mixed in order to generate a linear denaturizing gradient. The whole assembly is kept at a temperature within the range of 55 to 65ºC constant throughout the whole process. In a DGGE gel, double stranded DNA fragments are exposed to an increasing concentration of denaturizing agent due to this the DNA melts partially generating melting domains. Formation and the melting temperature of these melting domains is sequence specific. When the melting temperature T_m is achieved the lowest melting domains, part of fragment becomes partially melted creating branched **"breaking molecules"**. The formation of these breaking molecule decreases the mobility of these molecules decreases the mobility of these molecules in acrylamide gel. Due to this reason DNA fragments of same length but different compositions show different response [22].

Most of the times the fragments to be loaded are PCR products, for this reason complete denaturation of whole DNA should be avoided to get good resolution. While performing a PCR GC clams with 30 to 40 nucleotide are added as they insure prevention of complete denaturing of double stranded DNA. Bands in DGGE are visualized using Etbr staining. Most sensitive procedure for staining is silver staining, but the main problem with silver staining is its inability to proceed to hybridization experiment.

SYBER green can also be used for staining DGGE gels. SYBER green does not have any Background staining which allows visualization of very low concentration of DNA. Bio-Rad INGENY and CBS scientific are the main suppliers of DGGE equipments.

2.8 What do we get from DGGE?

PCR-DGGE technique is a highly precise and powerful molecular biology technique to study microbial communities via direct DNA extraction. The environmental samples are subjected to DNA extraction [19]. The DNA sample is then subjected to subsequent PCR amplification of a particular variable DNA region of our interest and obtaining an amplified product.

Most amp icons obtained tend to have same size but have dissimilar sequence combination. In the final result we tend to get a fingerprint that is specific to the analyzed sample the number of bands is relative to number of species present. Most commonly employed target for PCR amplification prior to DGGE is ribosomal DNA.

Targeting the ribosomal DNA helps to uncover the conserved regions of the genome that also include certain variable regions. Most commonly used primers in this case for amplification of variable region are the 16SrDNA for bacteria and 26S rDNA or 18S rDNA for eukaryotic organism [23].

2.9 Outline

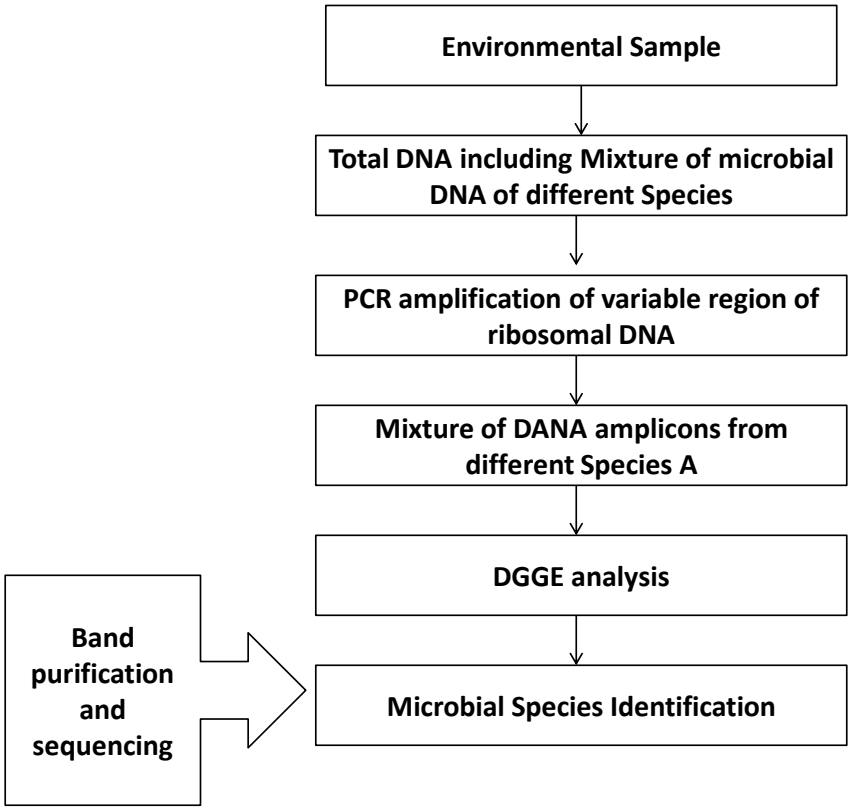

Fig 2.4-Flow diagram of amplification of PCR-DGGE analysis.

2.10 Problems associated with DGGE

All molecular methods are plagued with certain kind of bias, the possible pitfalls of DGGE when applied to environmental samples screening is discussed below. Biases is generally introduced while sampling and sample handling is done, these biases are frequently seen in traditional and molecular methods. All species do not have the same sensitivity to lytic agent this is due to variation in the cell wall composition of different microorganism, this affects any study based on extraction of whole genomic DNA as yield of DNA varies species to species.

Chapter-3

Materials and methods

3.1 Isolation of halophilic microorganism from Gumma Himachal Pradesh

Soil sample was collected in a sterilized reagent bottle from the halophilic site (Gumma, H.P). To 10 gm. of soil sample 10 ml. of saline water was added. 50ml. of LB broth was prepared in 7 flasks each having different concentration of salt (0%, 5%, 10%, 15%, 20%, 25% and 30%) and autoclaved at 121ºC at 15 psi. for 15 minutes. LB agar plates were also prepared for each of the salt concentration. The soil sample was inoculated in 50 ml. LB broth having 0% salt concentration (no addition of salt), and incubated at 37 $^{\circ}$C overnight. Next day 2.5 ml. of the turbid broth (0%) was pipetted to 50 ml. of LB broth having 5% salt concentration and incubated at 37ºC overnight. Also the respected agar plate was streaked from the liquid culture (0% LB broth to 0% Agar plate) and incubated at 37ºC. This process was repeated till 15% of salt concentration (after which there was no growth) Individual colonies were identified and sub cultured to get pure cultures.

3.2 Molecular Identification techniques

3.2.1 Extraction of genomic DNA

Genomic DNA extraction was using soil, water and overnight grown cultures of lab isolates. Pelleting of the cultures and solid debris from soil and water samples were done using centrifugation technique. To these pelleted products 1ml. of extraction buffer was added [M Tris HCl (pH-8), 5M NaCl and 0.5M EDTA] was added. Extraction of genomic DNA was done using slight modification to [Boon et al. 2003] method. Isolates were identified using 16S rDNA gene Sequencing.

3.2.2 PCR product amplification

PCR reaction was carried out with a total volume of 15µL containing 1µL of dH_2O, 1.5µl buffer, 0.3µL of forward and reverse primers and 0.15µL of Taq Polymerase. Lab specific primers were used (Forward Primer PRBA338f: 5'-ACTCCTACGGGAGGCAGCAG-3' and Reverse Primer PRUN518r: 5'-ATT ACC GCG GCT GCT GG-3') and another set of primers with GC clams with (Forward primer PRBA338fGC: 5'-CGC CCG GGC CGC GCC CCG GGC GGG GGGC GGC ACC AG-3' and Reverse primer P518r: 5'-ATT ACC GCG GCT GCT GG-3') as universal primers. Using cyclic reaction with initial denaturation temperature 92ºC for 2 min for PRBA338f and 95ºC for 5 min for PRBA338fGC.With amplification consisting of 35 cycles with denaturation carried out at 92ºC for 30 sec for PRBA338f and 95ºC for 30 sec for PRBA338fGC. Annealing step for the two was carried out at

55ºC for 30 sec for PRBA338 and at 53ºC for 1 min for PRBA338fGC. Final extension was done at 72ºC for 2 min and 10 min for PRBA338 and PRBA338fGC.PCR product was analyzed under UV light in an trans illuminator To visualize the results 100bp ladder was used by Thermos fisher scientific.16S rDNA sequence were analyzed in lab BLAST and clustal omega from the Gene bank database.

3.3 DGGE-Denaturing Gradient Gel Electrophoresis -

Denaturing gradient gel electrophoresis (DGGE) works by applying a small sample of DNA (or RNA) to an electrophoresis gel that contains a denaturing agent. Researchers have found that certain denaturing gels are capable of inducing DNA to melt at various stages. As a result of this melting, the DNA spreads through the gel and can be analyzed for single components, even those as small as 200-700 base pairs.

What is unique about the DGGE technique is that as the DNA is subjected to increasingly extreme denaturing conditions, the melted strands fragment completely into single strands. The process of denaturation on a denaturing gel is very sharp: "Rather than partially melting in a continuous zipper-like manner, most fragments melt in a step-wise process. Discrete portions or domains of the fragment suddenly become single-stranded within a very narrow range of denaturing conditions. This makes it possible to discern differences in DNA sequences or mutations of various genes: sequence differences in fragments of the same length often cause them to partially melt at different positions in the gradient and therefore "stop" at different positions in the gel.

Steps involved in DGGE Gel preparation-

Step1- 60% gel preparation -

Contents	Volume
Acrylamide	5mL
Bis-Acrylamide	1.67mL
Urea	8.33g
Formaldehyde	8mL
TAE (50X)	0.66µL

Step2- 0% gel preparation-

Contents	Volume
Acrylamide	11.25mL
Bis-Acrylamide	3.75mL
TAE(50X)	1.5ml

Step3-Bottom gel-

2ml of 60% gel + 40µL of APS(ammonium per sulphate) +2µLTMED (Tetra methylethylenediamine)

Step4- 20% gel preparation-

5mL of 60% gel + 10 ml of 0% gel +150µL of APS(ammonium per sulphate) + 15 µLTMED (Tetramethylethylenediamine)

Step5- 45% gel preparation

11.25mL of 60% gel + 3.75 ml of 0% gel +150µL of APS(ammonium per sulphate) + 15 µLTMED (Tetramethylethylenediamine)

3.4 Sequencing of DGGE bands

The bands of our interest were excised from the gel using blade and incubated overnight at 4ºC in distilled water to allow DNA diffusion out of the polyacrylamide matrix. The step was subsequently followed by PCR amplification using primes wit and without GC clamps. Once amplified the samples were visualized again in UV light and appropriate volumes were sent for sequencing

Chapter 4

Results

4.1 Isolation of halophilic microorganism from Gumma Himachal Pradesh

Eight different strains were isolated from the rock salt mine, Drang Himachal Pradesh, were cultured on to LB agar and broth at 37ºC As shown in the Fig .The stains were named as 1C to 3C based on the varying concentration they were grown. Nomenclature of bacterial strains- The bacterial strains were named as C (cultivable) from 1C1 to 3C1 in order of their appearance on LB agar Plates supplemented with salt at different concentrations figure 3.1 shows various cultured species.

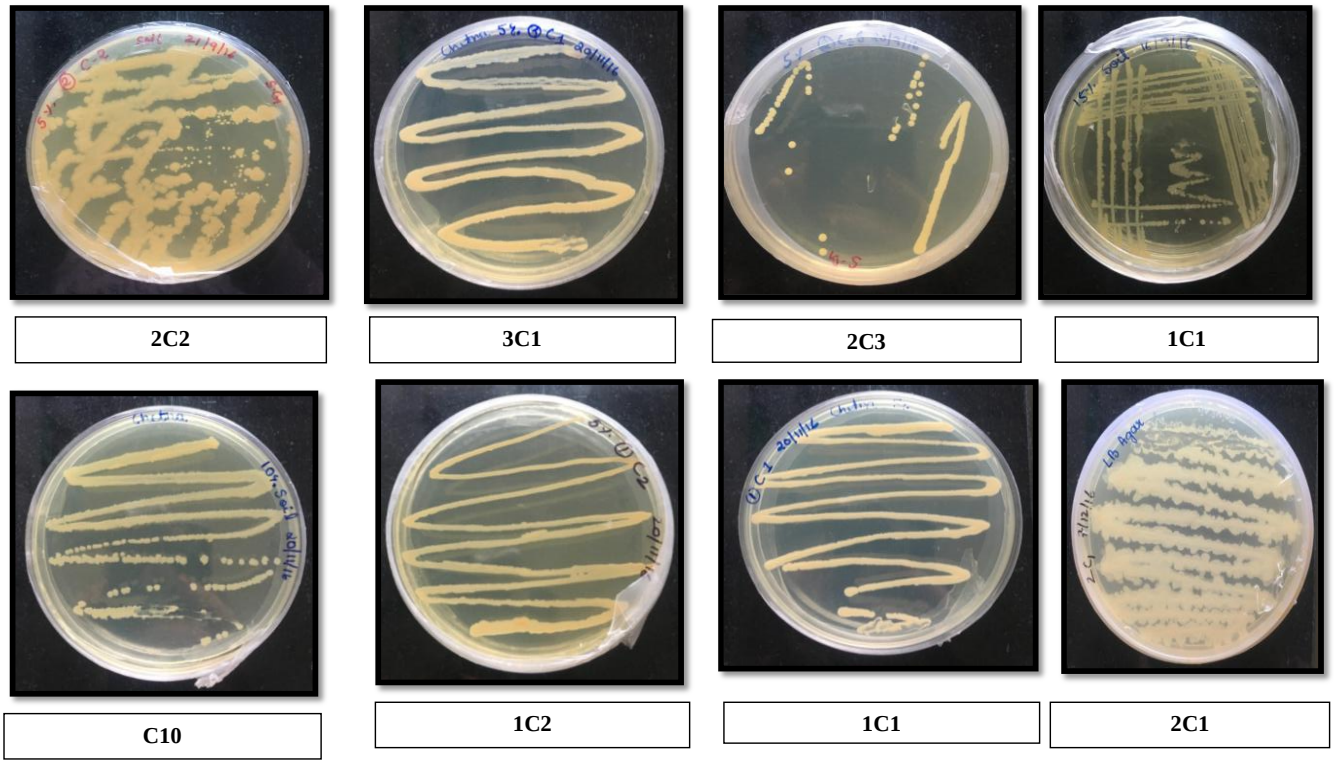

Fig4.1 Cultured isolates from various samples.

Colony	Percentage of Salt	Protease activity
1C1	5% salt conc.	-
1C2	5% salt conc.	-
2C1	5% salt conc.	Present
2C2	5% salt conc.	-
2C3	5% salt conc.	-
3C1	5% salt conc.	Present
E	10% salt conc.	-
F	15% salt conc.	-

Table 4.1- Cultivation of microbes with varying salt concentration and in Proteolytic media.

4.2 Molecular analysis

4.2.1 Extraction of molecular DNA- Extraction of DNA was done using modification to Boon et al method. And the results were visualized under UV light in a transilluminator. Lane A and B contain Bacterial genomic DNA extracted from the water sample where as C, D and E shows the Bacterial genomic DNA extracted from Soil Samples.

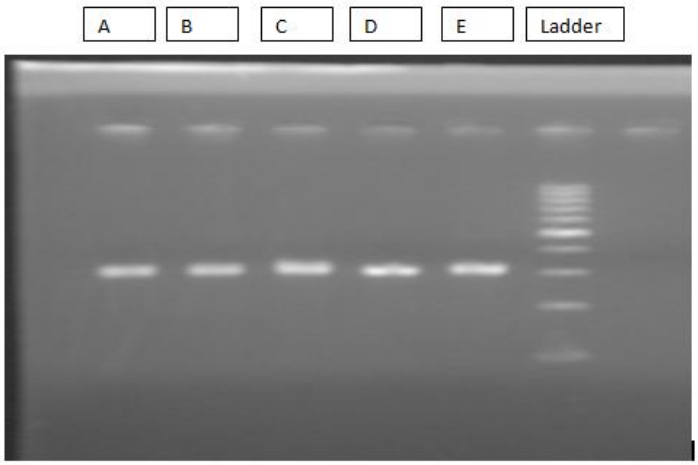

Fig.4.2- Agarose gel containing genomic DNA from different sources.

4.2.2 Amplification of DNA -Genomic DNA was subjected to subsequent PCR Reaction to amplify the 16S rDNA conserved region in the DNA for this Process two sets of primers were used so as to get a very specific amplification. Lane 1 contains the ladder and from lane 2, 3, 4, 5, 6, 7, 8 and 9 contains the

PCR product obtained from the Lab isolated pure colonies obtained via subsequent plating and sub culturing from the salt mines whereas lane marked from A to F contains PCR product extracted directly from the soil and water sample obtained.

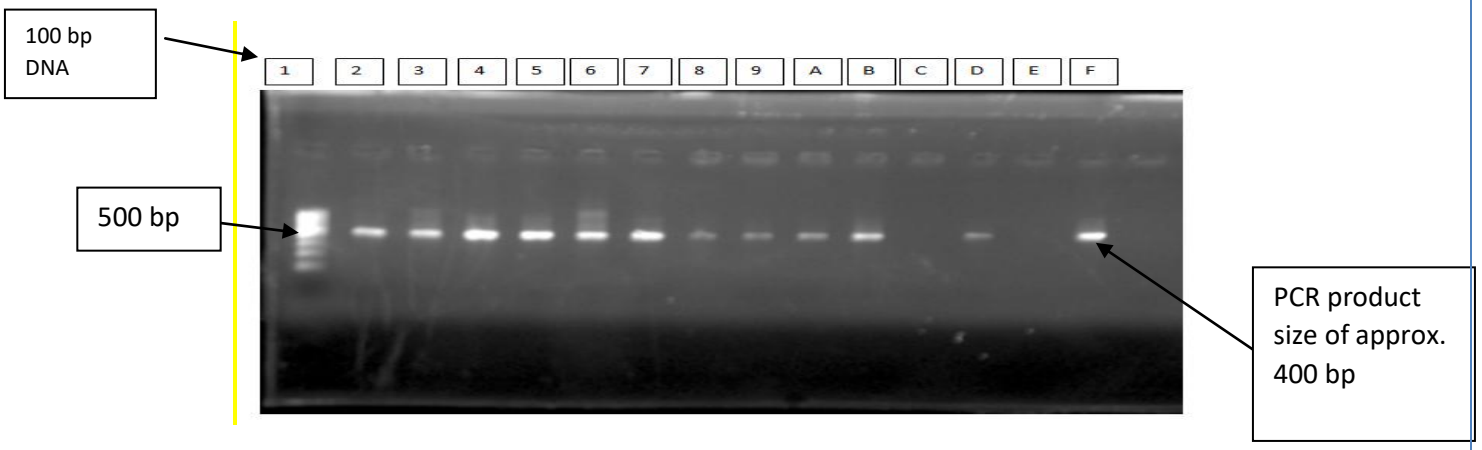

Fig.4.3- Agarose gel containing PCR product from different sources.

4.2.3 DGGE analysis of Halophilic Bacterial Isolates- DGGE analysis of the PCR product shows the that there are different type of microbes present in this site, lane 1, 2, 5, 8 and 9 shows diversity present in the Lab cultures samples whereas lane 3, 7 and 10 shows bacterial isolates from the soil sample and lane 6 and 4 shows bacterial isolates from water sample.

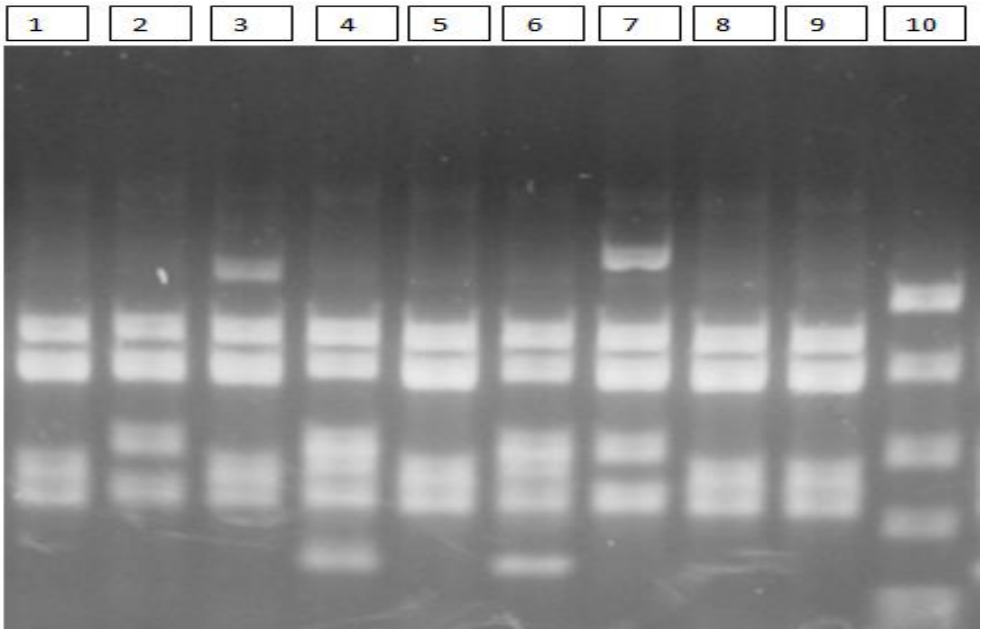

Fig.4.4- DGGE profile of PCR products from different source

Lane Number	Source	Number of organism
1	Pure Colonies	4
2	Pure Colonies	4
3	Soil Sample	5
4	Water Sample	7
5	Pure Colonies	4
6	Water Sample	7
7	Soil Sample	5
8	Pure Colonies	4
9	Pure Colonies	4
10	Soil Sample	5

Table 4.1- Table showing DGGE profile of PCR products.

Chapter 5

Conclusion

Very few studies have been conducted on the culture-independent methods to investigate micro flora in Gumma. The whole composition and functions of the microbial group are largely unknown. Especially after the recent landslides in the research area, no study has been conducted on the effect in the composition of microbial micro flora. The main objective of the study was to analyze the microbial diversity in Gumma. The study supports the fact of availability of large cultural diversity present in the research area. The study also briefly touches the topic of protease production by various lab isolates. Not much can be concluded at the present as the sequencing results are still awaited.

Chapter -5

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