Isolation and Studies on Protease Producing Halophilic Archaea from the Solar Saltpans of Goa

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled, "**Isolation and Studies on Protease Producing Halophilic Archaea from the Solar Saltpans of Goa**" is based on the results of investigations carried out by me in the Master of Science in Microbiology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Bhakti B. Salgaonkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations/experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Isolation and Studies on Protease Producing Halophilic Archaea from the Solar Saltpans of Goa**" is a bonafide work carried out by **Ms. Yuvrani Sunil Halarnkar**, under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of **Masters of Science in Microbiology** in the Microbiology Programme at the School of Biological Sciences and Biotechnology, Goa University.

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Ms. Yuvrani S. Halarnkar

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Abstract

Saline and hypersaline regions make up the largest ecosystem and are widely distributed in various regions of the earth. Several extremophilic microorganisms thrive in these habitats consisting of high salt concentration levels. These microbial populations are capable of surviving in extreme conditions due to the presence of unique enzymes called extremozymes which are functional at harsh environmental conditions like pH, temperature, pressure, water activity, organic solvents. Therefore, they serve as an excellent source for application in industrial processes. In the present study, the two extremely halophilic isolates were screened for determining the hydrolysis of starch, protein, gelatin, CMC, pNPP, chitin, olive oil, coconut oil, tween 80, tween 20, tween 40 and xylan. Both the isolates were capable of hydrolyzing gelatin. Isolate GUYHP1 showed polyhydrolytic activities where protease production was found to be the best amongst all. Optimization studies revealed that the enzyme was active at wide range of NaCl concentrations (15 -30%), substrate concentration (0.1 – 1.5% skimmed milk), pH (5-9), temperature (28- 37°C) with its maximum activity at 25% NaCl, 0.5% skimmed milk, pH 7 and temperature 28% C. Pigment production and protease activity was also determined of the culture in 15%, 20% and 25% NaCl concentration to check the efficacy of enzyme. Morphological analysis, SEM analysis, response to antibiotics were also carried out in order to determine the similarities and differences between Bacteria and Archaea. The results revealed that the two isolates obtained from saltpans of Goa belonged to domain Archaea. Thus, both the isolates (GUYHP1 and GUYHR1) were identified as “Haloarchaea”.

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List of Abbreviations and Symbols

A_W	Water Activity
μL	Microlitre
mL	Millilitre
α	Alpha
β	Beta
kDa	Kilodalton
SDS	Sodium dodecyl sulfate
℃	Degree Celsius
RV	Rodriguez- Valera
NH	Norberg- Hofstein
NTYE	NaCl Tryptone Yeast Extract
NT	NaCl Trisodium Citrate
EHM	Extremely Halophilic Media
MHM	Moderately Halophilic Media
pNPP	para- Nitrophenyl phosphate
w/v	weight/volume
v/v	volume/volume
CMC	Carboxymethyl cellulose
CFS	Cell free supernatant
m g	Milligram
%	Percent
nm	Nanometer
TCA	tricholoro acetic acid
RT	room temperature

Chapter I

Introduction and Literature Review

1. Introduction and Literature Review

1.1. Halophilic microorganisms

Hypersaline environments such as Salt Lakes, Solar Salterns, salt mines are ubiquitous throughout the world. These are the extreme habitats where salinity is much higher or equal to 30% NaCl as compared with that of the sea water (~3.5% NaCl). Halophiles are a group of extremophilic organisms that requires high concentration of NaCl in order to survive and proliferate. These microbial populations adapted to the saline environments are not only compatible with high salinity but also compatible with other extreme conditions prevailing in these habitats such as high or low pH, temperature, and the presence of toxic substances, including heavy metals (Ara et al, 2013). Inspite of such harsh environment, certain microorganisms manage to survive by actively performing their cellular and metabolic processes. These extreme organisms are known as halophiles (salt loving organisms) (Antunes, et al, 2017). Based on the salt tolerance levels required for their growth and metabolic processes, they can be classified as:

(i) Extremely halophilic microorganisms requiring 15-30 % NaCl (~ 2.5-5.2 M NaCl)

(ii) Moderately halophilic microorganisms requiring 3-15 % NaCl (~0.5- 2.5 M NaCl)

(iii) Slightly halophilic microorganisms requiring 1-3% NaCl (0.2- 0.5 M NaCl)

(iv) Halotolerant organisms are those which are able to grow to in the presence and absence of high salt concentrations (Oren et al, 2002)

Halophilic Archaea (haloarchaea) are the extremely halophilic microorganisms belonging to the phylum *Euryarchaeota* in family *Halobacteriaceae* flourishing in environments of high salt concentration primarily in solar salterns and hypersaline lakes. They are exposed to extreme conditions like high temperatures, UV radiations, low water activity (a_w) and low oxygen levels.

The most salt-tolerant microorganisms are included in this family, thus they serve as excellent model organisms for studying molecular basis of high salt adaptation (Oren A, 2002). To the best of my knowledge, as of 2022, haloarchaea altogether consists of 71 different genera containing 275 species (Cui et al, 2021).

Archaea produce potentially useful products such as bacteriorhodopsin, halorhodopsin, extracellular polysaccharides, salt tolerant enzymes, biodegradable poly-b-hydroxyalkanoates (PHAs), ectoin, hydroxyectoine, food additive and site-specific endonuclease (Oren, 2002). Halophilic Archaea are dominantly used in biotechnological fields such as retinal proteins where bacteriorhodopsin/archaeaorhodopsin (27kDa protein acting as a light driven proton pump) from *Halobacterium salinarum* are used for optical biocomputer chips, synthesis of polyhydroxyalkanoates as biodegradable plastics in designing biomedical implants (Trivedi et al, 2011), and the use of gas vesicle genes as delivery vector (DasSarma and Arora,1997) for bioengineering microorganisms to separate into the upper aqueous layer for applications in fermentation and biological control (Elevi et al, 2004). Since they have extraordinary features like protein stability, accumulation of organic salts, presence of extemozymes, they are being widely utilised in all kinds of industrial sectors where extreme conditions prevail (Das et al, 2019).

1.2 Adaptation strategies acquired by Halophilic microorgansims

Halophiles are able to survive in extremely harsh conditions and can actively metabolise in such environments. This is due to certain mechanisms;

- **High Salt in Strategy**: wherein the organisms accumulate inorganic solutes such as K+, Na + and Cl^- in order to be in equilibrium with the environmental osmotic stress induced due to high NaCl concentrations. (Dalmaso et al,2015). Extreme halophiles belonging to the archaeal Halobacteriaceae family such as *H. salinarum, Haloarcula marismortui,*

Halococcus morrhuae; and the bacterial Halanaerbiales family such as *Haloanaerobium praevalens, Haloanaerobium acetoethylicum, H. halobius* maintain their osmotic balance by concentrating K+ inside cells thus raising the concentration of salt inside the cytoplasm (Christian and Waltho 1962, Ventosa et al,1998).

- **Low Salt in Strategy**: Halotolerant and halophilic microorganisms use another strategy by accumulating different osmolytes such as betaine, ectoine which are compatible organic compounds. The chief function of these molecules is to help in osmoprotection against the extracellular salinity without disturbing the normal metabolic processes of the organism and helping in proliferation in hypersaline environment (Quillaguaman et al ,2010).

1.3 Proteins in Extreme Halophiles

Microorganisms inhabiting in hypersaline environments possess specific protein composition helping in sustainability, different from those proteins of organisms inhabiting non-saline environments. These halophilic enzymes have to maintain their catalytic properties at high salt concentrations (Rao et al,1998). At high concentration of salt, water availability is lesser to the protein hence it causes hydrophobic amino acids in a protein to lose its hydration and aggregate (Mevarech et al, 2000). Therefore, high salt concentrations are necessary for strengthening of hydrophobic interactions in a protein. Non halophilic proteins are incapable of functioning at high salt concentrations because the hydrophobic and electrostatic interactions required by them for proper folding and for maintaining stability are greatly altered. This leads to destabilization of the protein causing unfolding and aggregation, ultimately leading to precipitation (Karan et al, 2012). Therefore, high salt concentrations strengthen hydrophobic interactions in a protein. The most striking difference in halophilic and non-halophilic proteins is, the surface of Haloarchaeal proteins are rich in acidic amino acids such as aspartic acid and

glutamic acid unlike the non-haloarchaeal proteins. This is ubiquitous feature which helps in preventing denaturation, aggregation of protein molecules and precipitation in the halophilic protein sequences (Zhang et al, 2013).

1.4 Enzymes from extremely halophilic microorgansims

Enzymes from the extreme halophiles have gained immense attention in the recent years because of their enhanced activity and stability as compared to mesophiles and non-halophilic organisms. Hence, such unusual properties of halophilic enzymes can be utilized in bioremediation processes, harsh industrial processes where the normal enzymes degrade and get denatured easily due to environmental stresses and become inefficient (Mellado and Ventosa, 2003). These enzymes have unique structural and functional properties and are defined as extremozymes, having an increased stability at high temperatures, extreme pH, in the presence of organic solvents and heavy metals and against proteolytic damage. Due to this reason, they are capable of withstanding harsh conditions in industrial processing environments. The process of catalysing a particular reaction of a halophilic enzyme is same as a non-halophilic enzyme. However, differ from them based on the following characteristics;

- Solubility, optimum activity and stability at high salinity
- Resistance to denaturation in extreme environment
- Maintainance of the folded protein structure of enzyme due to presence of salt (Sinha and Khare, 2014)
- Enzymatic reactions take place inspite of low water activity (Madern et al, 2000)
- Presence of large amount of acidic amino acids as compared to the non-halophilic organisms (Delgado- Garcia, 2012)

1.4.1 Extracellular Hydrolytic Enzymes

Extracellular enzymes are those which perform functions by carrying out the hydrolysis of chemical bonds in the substrates composed of complex polysaccharides outside the cells (Ventosa et al, 2005).

1.4.1.1 Amylase (1,4- glucanohydrolases)

Amylases are enzymes involved in the reaction of converting starch into smaller carbohydrates such as disaccharide maltose and and monosaccharide glucose. Starch being a homopolymer is composed of D-glucose units having two subunits;

a. Amylose- linear polymer consisting of α-1,4- linked glucopyranose residues and
b. Amylopectin – branched subunit of starch, consisting of linear glucose units linked by α-1,4- glycosidic bonds. These residues are branched with α -1,6- glycosidic bonds (Sundaram and Murthy, 2014).

1.4.1.2 Cellulase (β-1,4- glucanase)

Cellulose is a homopolymer forming the basic structural component of plant cell wall. It is composed of glucose units linked by β-1,4- glycosidic linkages due to which a compact, crystalline, insoluble is formed. Cellulases are the enzymes degrading cellulose into single glucose units synthesized by plants, fungi, bacteria. They are used for bio fuel production from waste cellulose and also in paper and pulp industry (Sorokin et al, 2015). Cellulases have also been reported to enhance the bleachability of softwood kraft pulp (Singh et al, 2007).

1.4.1.3 Protease (Peptidase)

Proteases, also called peptidases or proteinases, are enzymes that perform proteolysis i.e they break the long chainlike molecules of proteins into shorter fragments (peptides) and free amino acids. Proteolysis is one of the most important biological reactions. It is widely used in industrial processes and applications like detergent, leather, food, pharmaceuticals (Akolkar and Dessai, 2010).

1.4.1.4 Xylanase (endo-1,4-β-xylanase)

Xylan is a major component of plant hemicellulose and as ubiquitous as cellulose. It is a homopolymeric backbone chain of 1,4- linked beta-D-xylopyranose units and short side chains including O-acetyl, alpha-L-arabinofuranosyl and D-glucuronyl or O-methyl-D-glucuronyl residues. Xylanases is involved in the conversion of xylan to D-xylose units (Prakash et., al 2009). It is widely used in paper and pulp industry, food and feed industry and in textile industry (Bharadwaj et al, 2019).

1.4.1.5 Gelatinase

Gelatinase is an enzyme able to carry out hydrolysis of gelatin and other compounds such as collagen, casein and fibrinogen into polypeptides, smaller peptides and individual amino acids that can cross the cell membrane and used by organisms. It is a metalloendopeptidase having applications in treatment of poultry and animal waste, medical applications etc (Balan et al, 2012).

1.4.1.6 Lipase

Lipases are hydrolytic enzymes which is a subclass of enzyme esterases. They act against water insoluble substrates mainly triglycerides hydrolysing to glycerol and smaller free fatty acids. Lipases are widely used as biosurfactantants in food, paper, detergent, paper and pulp industry (Hasan et al, 2006).

1.4.1.7 Esterase

Esterases (EC 3.1.1.x) are the group of hydrolases that catalyzes the cleavage and formation of ester bonds. Esterases split ester molecules into acid and alcohol during a chemical reaction with water. They catalyze the hydrolysis and synthesis of lipids therefore they are widely used as biocatalyst for seperation of enantiomers and do not require co-factors (Godinho et al, 2011)

There are three types of reactions which can be catalysed be esterases: esterification, interesterification and transesterification reactions with very good chemo-, regio-and/or enantioselectivity. Industrial applications include degradation of different materails like cereals, wastes, plastics, and other toxic chemicals, perfume and antioxidant synthesis and in agricultural sectors (Panda and Gowrishankar, 2005).

1.4.1.8 Chitinase

Enzymes capable of degrading chitin to directly to low molecular weight chitooligomers are known as chitinases (E.C 3.2.2.14). They are glycosyl hydrolases with the sizes ranging from 20 kDa to about 90 kDa. (Bhattacharya et al, 2007) Chitin is the second most abundant polysaccharide in nature after cellulose, is found in the exoskeleton of insects, fungi, yeast, and algae, and in the internal structures of other vertebrates. Applications of chitinases inude mosquito control and plant defense systems against chitin-containing pathogens, biocontrol of fungal phytopathogens, single cell protein production (Khan et al, 2015).

1.4.1.9 pNP – phosphatase

This enzyme is an essential enzyme for the cellular functioning having a highly negatively charged protein surface. Phosphatases catalyse the hydrolysis of p NP which liberates inorganic phosphate. They are mainly involved in dimer formation of β structures. Phosphate is an essential component of all cells that must be taken up from the environment (Wende et al, 2010).

1.5 Studies on Protease:

Proteases, also called peptidases or proteinases, are enzymes that perform proteolysis i.e they break the long chainlike molecules of proteins into shorter fragments (peptides) and free amino acids. Proteolysis is one of the most important biological reaction that occurs. Based on their site if action, they are classified as:

- endopeptidases (proteases that cleave peptide bonds within the protein) and
- exopeptidases (proteases that cleave of amino acids from the ends of the protein) (Yegin et al, 2013).

Proteases are considered to be a replacement for the chemicals and an environmentally safe indicator for nature and the surroundings.

1.5.1 Types of Protease: Based upon the stability of pH levels, proteases can be alkaline,neutral, or acidic in nature.

1.5.1.1 Alkaline Proteases

Alkaline pH ranges from 9-11 and, *Bacillus* is an important genus involved in commercial production. Serine protease has been extensively used in detergent industry due to its effectiveness in tolerating highly alkaline conditions. (Gupta et al, 2002). Alkaline serine proteases get easily inactivated by addition of phenyl methane sulfonyl fluoride (PMSF) (Page and Di Cera, 2008). The unique feature of alkaline proteases is that they maintain a constant pH while being exploited for different formulations in pharmaceutical, food and other industries.

1.5.1.2 Acidic Proteases

Acidic proteases are those which maintain and are stable and active the pH between 3.8-5.6 and mostly used in soy sauce and protein hydrosylate. The optimum pH is 3-4 and isoelectric point range is between 3 and 4.5 with a molecular weight of 30–45 kDa (Ravikumar et al, 2012). In comparison with alkaline proteases, these acidic proteases are mostly produced from fungal species like *Aspergillus oryzae, Aspergillus awamori.* Acidic proteases are mainly used in clarification of juices, improving flour texture and tenderizing of muscle fibril. (Zhang et al, 2010).

1.5.1.3 Neutral Proteases

Some proteases can be stable in the range of p H 6-7 i.e they are active at a neutral or weakly acidic or weakly basic pH conditions. They are considered in food industry since have a medium rate of reaction therefore generating less bitterness in food proteins. They have a low thermotolerance during food hydrosylate production therefore controlling neutral protease is an important factor. A divalent metal ion like mettaloprotease is used for the activity of neutral proteases (Woessner et al., 2000). Thermolysin is a neutral protease produced by *B. stearothermophilus* having molecular weight of 34 kDa. It has a single peptide chain without disulfide bridges. This thermolysin neutral protease is very stable with a half-life of 1 h at 80◦C (Fitzgerald et al, 1990; Dawson and Kent, 2000).

1.5.2 Classification of Protease

Based on the mechanism of catalysis, they are classifed as:

serine proteases (EC 3.4.21.x), cysteine or thiol proteases (EC 3.4.22.x), aspartic, carboxyl or acidic proteases (EC 3.4.23.x), glutamic proteases (EC 3.4.23.x), threonine proteases (EC 3.4.25.x), and metalloproteases (EC 3.4.24.x) (Mamo and Assefa, 2018).

1.5.2.1 Serine protease: are those proteases having serine(-OH) at its active site. They are found in exopeptidase, endopeptidase, oligopeptidase, and omegapeptidase group.

1.5.2.2 Cysteine or thiol proteases: are proteases which are active at an intact-SH group in their active site.

1.5.2.3 Carboxyl or aspartic proteases: commonly known as acid proteases are the ones which contain one or more side chain carboxyl groups in their active site.

1.5.2.4 Metalloprotease: requires the presence of tightly bound divalent cations for the catalytic activity.

1.5.2.5 Glutamic protease and Threonine protease: endoproteases newly characterized families of enzymes that require glutamic acid and threonine as its active site, respectively.

1.5.3 Sources of Protease

Proteases are ubiquitous in nature and are necessary for all the biological forms of organisms and their metabolism. Several sources of proteases are existing in nature like plants, animals and microorganisms (Rao et al, 1998). Papain, keratinases and bromelain are the plant proteases. On the other hand, trypsin, pepsin, chymotrypsin, and rennin are animal proteases (Motyan et al, 2013). Trypsin is the main digestive enzyme and is a serine protease that hydrolyses food proteins into smaller units. Pepsin is naturally produced by the intestines essential for the digestion and breaking down of proteins while bromelain and papain are food-derived enzymes from pineapple and papaya, respectively.

Microbial proteases are gaining huge importance in the industrial sector in comparison to that of the enzymes produced by plants and animals. Microbes have a rapid growth rate and require limited space for its cultivation (Souza et al, 2015). The physical and chemical characteristic can be easily manipulated genetically in order to get desired enzymes and cost required for enzyme production is also low (Gupta et al, 2002; Chanalia et al, 2011). Due to the unsuccessful attempt of meeting the desired characteristics for industrial applications, the demand of microbial protease is highly elevated thereby representing the 60% of the total worldwide market of the industrial enzyme.

1.5.4 Functions of Protease

Proteases may be found in intracellularly or extracellularly in cells. Major functions like inactivation and proteolysis of proteins, regulation of synthesis are exhibited by intracellular proteases (Bond and Butler, 1987). Extracellular proteases play crucial role in blood coagulation, cell growth and migration, digestion, tumour growth and metastasis, tissue

arrangement, release of hormones and active peptides from precursor proteins, transportation of secretary proteins across membranes and also involved in action of drug delivery (Leipner and Saller, 2001).

1.5.5 Applications of microbial Proteases:

1.5.5.1 Detergent Industry

Proteases from *Bacillus sp,* such as *Bacillus clausii* and *B. halodurans* have profound application in detergent industry ranging in pH from 9-10 which are highly-alkaline proteases, with greater thermostability (up to 60 ℃). They are mostly serine proteases, with some unique amino acid residue features (Fujinami and Fujisawa, 2010). The amino acids Arg19, Glu271, Thr274, and Arg275 of the commercialized second-generation detergent proteases. The formulations of detergents supplemented with proteases enzyme have been found to be efficient than the conventional detergent technologies for removing proteinaceous stains such as blood, milk, egg, and chocolate and accounts for more than 30% of the world enzyme market (Hamza, 2017).

1.5.5.2 Leather Industry

Alkaline proteases are emerging as important enzymes in the leather industry due its elastolytic and keratinolytic properties. The use of protease has been found to be relevant in the soaking, bating, and dehairing phase of preparing skin and hides. Removal of unwanted pigments by the enzymatic action helps in clean production hides. Proteases active in the pH range of 8-12 and stable at alkaline pH are potential candidates for dehairing of hides. Microbial alkaline proteases have become very popular in leather industries (Khan, 2013).

1.5.5.3. Brewing and Cereal Processing

Proteases play a role in increasing the volume of the filterable extracts and enhances the amount of alpha-amino nitrogen in wort during the mashing stage of brewing and cereal processing. Replacement of the malt protease with industrial proteases, especially neutral proteases from *Bacillus* and also from *Aspergillus* species is necessary as the proportion of malt is decreased or eliminated through use of unmalted cereals (Kotlar et al, 2015).

1.5.5.4 Cheese Making Industry

During cheese production from milk, proteases are added to hydrolyze kappa casein to prevent coagulation by stabilizing micelle formation. Acidic aspartic proteases are utilized as milk-clotting enzymes in cheese making due to their ability to coagulate milk proteins to form curds with associated release of whey (Ward, 2011).

1.5.5.5 Baking Industry

Proteases are used in baking to modify gluten, a protein in wheat with viscoelastic properties that has the ability to expand as bread dough rises during the baking process. Gluten is partially hydrolyzed by a heat-labile fungal protease, such that the enzyme denatures as temperature rises in the early stages of baking. Another application of proteases in baking relates to the modifications linked to flavour and nutritional development. Bacterial neutral proteases may be used for this purpose, for example, in production of biscuits, cookies, and crackers (Naveed et al, 2021).

1.5.5.6 Production of protein hydrosylates

Protein hydrosylates are extensively used as food and feed additive where proteins like soy protein, gelatin, caseins, and whey proteins may be modified using proteases. *Bacillus* alkaline serine proteases are used for preparation of protein hydrolysates. Neutral proteases from *B. licheniformis* are also used for this application. Alkaline proteases from fungi such

as *A. oryzae* and *Rhizopus niveus,* and related strains are more frequently applied (Hamza, 2017).

1.5.5.7 Pharmaceutical Industry

Proteases are used due to its therapeutic properties helpful in treatment against fatal diseases like anticancer, antimicrobial, anti - inflammatory infections and dissolving clots (Srilakshmi et al, 2014). Apart from this, cystic fibrosis, sepsis, digestive disorders, retinal disorder, and many more diseases can also be treated using protease. Asparaginase from *Escherichia coli* play a major role in removing asparagine from the blood in forms of lymphocytic leukemia (Chanalia et al, 2011).

1.5.5.8 Management of Industrial Waste

Proteases are used for managing industrial waste as they are helpful in degradation of waste by transformation to other products or some valuable products (Shankar et., al 2010). In poultry industry, 90% of the protein is found in the feathers as keratin is the main component in this waste as an insoluble form of protein (Deivasigamani and Alagappan, 2008). Decomposition by the pre-treatment with NaOH, mechanical disintegration, and enzymatic hydrolysis helps in solubilizing the feathers. Enzymes from *Bacillus subtilis, Bacillus amyloliquefaciens* along with a disulphide reducing agent such as thioglycolate is useful in hair degradation and are also involved in clearing pipes which are clogged with hair containing deposits (Atalo and Gashe, 1993).

1.5.5.9 Photographic Industry

Alkaline proteases produced from *B. subtilis, Streptomyces avermectnus*, and *Conidiobolus coronatu*s have been successfully reported to recover silver from X-ray and photographic films. These used films have gelatin layer which contain 1.5%-2% of silver by weight.

These enzymes help in decomposing the gelatinous coating on used films from which silver can be recovered (Razzaq, 2017; Shankar et al, 2010).

Table 1: Comparative list of reports on protease producing strains of halophilic Archaea (Castro et., al 2006)

Strain name	Place	NaCl range	Optimum NaCl range	Optimum Temperature	pH	References
Natrococcus occultus	ND		1-2 M	60°C	7-9	Studdert et al., 1997
Natrialba magadii.	ND	1-1.5 M	1.5 M	60°C	8-10	Gimenez et., al 2000
Natrialba hulunbeirensi strain WNHS14	Wadi El Natrun, Egypt	2-3 M	2 M	40°C	9	Ahmed et., al 2021
Halobacterium sp. PB407	ND	3 – 5 M	4 M	37°C	7	Kanlayakrit et., al 2004
Haloarcula sp. TG1	Lake Tuz, Turkey	2-4 M	4 M	50°C	4	Abanoz et al, 2017
Haloarchaeobius sp. FL176	Wuhu, China	3-4 M	3.5 M	40°C	8-9	Zhang et al, 2022
Haloferax mediterranei VKM-B 1538	ND	2-5 M	2	55°C	8–8.5	Stepanov et al., 1992
Natrialba asiatica 172 P1	ND	3-5 M	2	75-80°C	10.7	Kamekura & Seno,1990
Haloferax volcanii	ND		2 M	75°C	7.7- 9.5	Wilson et al,1999
Natronobacterium pharaonis	ND		0.5- 4 M	60°C	10	Stan-Lotter et al,1999
Halobacterium salinarum	ND	3-4 M	4M	ND	8	Norberg & Hofsten ,1969
Haloarcula marismortui	ND		0.5 M KCl	60°C	ND	Franzetti et al, 2002
Halogeometricum borinquense TSS101	Tamilnadu, India	3-5 M	4 M	60 °C	10	Vidysagar et al, 2006

ND: Not Defined; M: Molar; °C: degree celsius

1.5.5.10 Degumming of Silk

A proteinaceous substance, "sericin or silk gum," must be removed by the process of degumming from raw silk in an alkaline solution of soap conventionally. The use of soap is not safe as it carries harmful chemicals. By using alkaline protease, without attacking the fibre, it is a trouble-free method to remove sericin which can then increase the strength of the yarn and does not cause environmental pollution. Hence proteases are greatly used nowadays for degumming of silk (Rajashekhar et al, 2011).

1.5.5.11 Meat Tenderizing

Proteases like papain, bromelain, actinidin are wide utilized in meat tenderizing processes which enhances the flavour of the meat. Meat tenderzation is a progressive process for improving the quality of meat. Proteolytic degradation leads to improved meat tenderness where, Proteases play an important role in degrading the structural proteins in the connective tissues, thus reducing toughness of meat (Arshad et al, 2016).

1.6 Protease from Extremely Halophilic Microorganisms

Mellado et.,al 2005 , studied the moderately halophilic bacterium *Pseudoalteromonas sp*. CP76 and the halophilic archaeon *Natrialba magadii.* Both the culture of the microorganisms produced extracellular proteases. The protease CP1 from bacterium has optimum activity from 0 to 4 M NaCl, along with excellent thermostability at 55°C and activity at alkaline pH values at pH 8.5. Protease from *Natrialba magadii* was having molecular mass of 130 KDa, and requires 1-1.5 M NaCl with optimum pH 8.5 – 10. To check the proteolytic activity of protease CP1, SDS-PAGE zymography copolymerized with gelatin as a substrate was performed.

Abanoz and collegues, 2017 isolated halophilic microorganisms from Çankırı salt mine and Lake Tuz in Turkey inorder to explore versatile protease producers for industrial applications. They also characterized protease enzyme from the best protease producer among

the isolated strains. The highest protease producing strains was found to be of *Haloarcula sp* by 16s RNA analysis. The isolate obtained *Haloarcula sp.* TG1, was found to be 99% identical to *Haloarcula salaria* strain HST01-2R. TG1 protease was fund to be active at high temperature and pH range. The maximum activity was at pH 4.0, 50°C and 4 M NaCl.

Faridah and Suharti, 2021 isolated several halophilic oraganisms capable to producing extracellular protease from Bledug Kuwu,Grobogan ,Central Java . BK1B ans BK1D were the two isolates which were producing highest protease. BK1B was a halotolerent bacterium and BK1D was a moderate halophilic bacterium. Both the isolates worked optimally at pH 6 with temperatures of 42℃ and 45℃ with activity of 2.541 and 3.505 units/ mL.

Kalwasińska et al , 2018 studied the protease production from *Bacillus luteus* H11 isolated from the top layer of the highly saline soda lime. The extracellular serine endoprotease, was remarkably stable in 5M NaCl. The optimum pH and temperature were found to be 10.5 and 45℃ respectively. It had a molecular mass of about 37 kDa and showed activity against azocasein.

Gupta et al, 2015 isolated a novel haloalkaliphilic bacterium strain BNMIITR isolated from soil sample from Sambhar Lake, Rajasthan. Experiment conducted showed that isolate was able to grow in 2–5 M NaCl, pH 6–11 and temperature 18–55 °C, but the optimum growth was found at 3 M NaCl, pH 8–8.5 and 45 °C. After carrying out16s rRNA analysis, the strain was found to be sharing 98% similarity to *Halobiforma lacisalsi* and *Hbf. Haloterrestris*.

According to Kamekura and Sero ,1990 halophilic archaebacterium strain 172 P1 produced three extracellular proteases in media containing 15–27% salts. The serine protease F-11 is a thermophilic and halophilic protease stable at 25% NaCl. The optimum pH was found to be 10.7. The optimal concentration of NaCl required was 10–14% when assayed at 70 °C with azocasein as substrate, though a halophilic characteristic was not distinct at lower temperatures.

Vijayanand et al, 2010 isolated an extremely haliphilic archaeon -Halobacterium sp JS1, from Thalassohaline environment in Tuticorin, Tamilnadu, India. Screening for protease was done using casein plates with 20% NaCl. Based on 16s r RNA sequencing, the isolate was identified. The optimum temperature was found to be 40℃. The maximum growth and proteolytic activity (64 U/ml) were achieved at 4M NaCl.

The studies conducted by Karbalaei-Heidari et al, 2009 shows that protease by moderately halophilic bacterium *Halobacterium karanjensis* strain MA-2 had maximun activity of enzyme was found at pH 8-10, 50-55℃ having molecular weight of 36kDa by SDS-PAGE.

Vidysagar et al, 2009 characterised extremely halophilic protease from halophilic bacterium Chromohalobacter sp. strain TVSP101 from solar salterns. The molecular mass of the protease was 66 kDa determined by using SDS-PAGE with an optimum of 4.5 M NaCl. The optimum temperature and pH for maximum protease activity was found to be 75 ℃ and pH 8.

1.7 Gaps in the Existing Research

Over many decades, extensive research has been carried out on protease produced by Bacteria and Fungi. Till date various studies on archaeal isolates have been conducted and documented, exploring their potential for protease production. However, to the best of my knowledge *Haloferax, Halobacterium, Natrococcus, Natrialba, Haloarcula* are some of the genera from haloarchaea capable of producing protease (Castro et al, 2006). Protease is one such enzyme which is widely used having 60% of the total worldwide market of the industrial enzyme. Carrying out research work from other genera from the family *Halobacteriaceae* can be helpful in discovering more novel sources of proteases thus leading to a better biotechnological future.

Objectives

1. Isolation of halophilic archaea from the salt pans of Goa and screening them for various extracellular hydrolytic activity.
2. Identification of the Potential Protease producing strain and Optimization of parameters for growth and protease production.
3. Partial purification and characterization of protease and determination of the activity.

Chapter II

Materials and Methods

2.Materials & Methods

2.1 Sampling

Sediment and brine samples were collected from saltpan of Nerul, Bardez- Goa on 21st May 2022. Clean zip lock bags were used for storing the sediment samples. Brine samples were collected from different regions of the salt pans. The samples were brought to the lab and processed soon after sampling.

2.2 Cultivation and Maintenance of Halophiles

2.2.1 Media for isolation of halophiles

Rodriguez-Valera (RV) medium containing (g/L) NaCl 234.0; KCl 6.0; $MgSO_4.7H_2O$ 29; $MgCl_2.6H_2O$ 19.5; $CaCl_2.6H_2O$ 1.1; NaBr 0.8; $NaHCO_3$ 0.2; Yeast Extract 5 and Agar 20.0. was used. The pH of the media was adjusted to 7.5-8.0 using 5M NaOH.

2.2.2 Spread Plate Method

Approximately, 50 µL of the brine sample was spread plated on the halophilic media under sterile conditions. Similarly, 1g of sediment sample was diluted in 10ml of 15% NaCl and 15 µL was spread plated. The plates were incubated at room temperature until the pigmented and non-pigmented colonies were observed. The colonies obtained were purified by successive restreaking on halophilic medium till pure cultures obtained.

2.2.3 Selection of extremely halophilic Isolates

Two extremely halophilic isolates were selected based on their size, shape and pigmentation. The isolates were designated as GUYHP1 and GUYHR1 and maintained on medium in which they were isolated. Both the isolates were sub-cultured once a month and incubated at room temperature for further studies.

2.3 Preparation of Starter Culture

Starter culture was prepared by sterilizing 50 ml of RV media in 100 mL of Erlenmeyer flask in absence of agar. Loopful of the culture taken from agar plates were inoculated in the media and the flasks were incubated at 30°C on rotary incubator shaker at 100rpm for 5-6 days.

2.4 Screening of halophilic isolate for extracellular hydrolytic enzymes

The halophilic isolates were screened for production of extracellular hydrolytic enzymes by the method of plate assay. Norberg-Hofstein (NH) media comprising of (g/L) NaCl 200.0, $MgSO_4.6H_2O$ 10.0, KCl 5.0, $CaCl_2.2H_2O$ 0.2, Yeast Extract 1.0, Agar 18.0 substituted with various substrates was used to check the activity of hydrolytic enzymes. The pH of the media was adjusted to 7.5 using 5 M NaOH. 10 µl of log phase culture was spot inoculated on plates and incubated for 10-15 days.

2.4.1 Screening for Amylase:

Screening for amylase was carried out using NH medium agar plates supplemented with 0.2% (w/v) soluble starch as a substrate. Amylolytic activity was detected by flooding the plate with 0.5% KI. Presence of a clear light-yellow zone around colonies against a blue-background indicated hydrolysis of starch (Amoozegar et al, 2003).

2.4.2 Screening for Protease*:*

Proteolytic activity was determined by using 0.5% (w/v) skimmed milk in NH Medium plates. Clear zone around the colonies indicated the production of protease (Pailin et al, 2001).

2.4.3 Screening for Gelatinase:

Gelatinase activity was assessed by using 0.5% (w/v) gelatin in the NH medium and detected by pouring the plates with 15% mercuric chloride acidified with 20% (v/v) concentrated HCl solution. Zone of clearance around colony indicated the presence of gelatinase activity (Balan et al, 2012).

2.4.4 Screening for Esterase:

Screening for esterase activity was detected by using 0.5% (v/v) tween 80, tween 20 and tween 40 as substrate in NH medium. A white precipitate around the colony indicated the presence of esterase activity (Dahiya et al, 2022).

2.4.5 Screening for Cellulase:

Cellulolytic activity was determined by using NH medium supplemented with 0.2% (w/v) Carboxymethyl cellulose. The plates were flooded with 0.1% (w/v) Congo red solution. Zone of clearance around the colony indicated the production of cellulase (Rohban et al, 2009).

2.4.6 Screening for Xylanase:

Xylanase activity was determined on NH medium supplemented with 0.2% (w/v) Xylan and detected by flooding the plates with 0.1% (w/v) Congo red solution. Zone of clearance around the colony indicated the production of xylanase (Rohban et al, 2009).

2.4.7 Screening for Lipase:

Lipolytic activity was assessed by supplementing 0.5% (v/v) of olive oil as substrate in NH medium. A white precipitate around the colony indicated the production of lipase (Gupta et al, 2016).

2.4.8 Screening for Chitin:

Chitinase activity was determined by using 0.5% (w/v) colloidal chitin [Appendix II(31)] as a substrate in NH medium. Cultures were spot inoculated and plates were incubated for 10-15 days. The plates were then flooded with 0.1% (w/v) congo red solution and destained with 5% (w/v) NaCl solution. Zone of clearance around the colony indicated chitinolytic activity (Khan et al, 2015).

2.4.9 Screening for Phosphatase:

Para nitro phenol activity was detected by using NH medium infused with 0.5% (w/v) PNP-phosphate. Zone of clearance around the colony indicated phosphatase activity (Mehta et al, 2001).

2.5 Selection of Potential Isolate and Substrate

Extremely halophilic isolate GUYHP1 was selected for further studies as it showed excellent polyhydrolytic activities with protein hydrolysis being the best.

2.6 Parameters Optimization for Growth and Protease Production

10 µL of the log phase (6 days) culture of isolate GUYHP1 was spot inoculated on NH media containing skimmed milk. The plates were incubated at room temperature for 10-12 days. Zone of clearance around the culture indicated the hydrolysis of protein.

2.6.1 NaCl concentrations

Isolate GUYHP1 was spot inoculated on NH media plates containing various concentrations of NaCl (w/v), i.e., 0%, 5%, 10%, 15%, 20%, 25% and 30%. The NaCl concentration showing the best culture growth along with zone of clearance was determined as optimum NaCl concentration for growth and protease activity.

2.6.2 Skimmed milk concentrations

Isolate GUYHP1 was spot inoculated on NH media plates containing various concentrations of skimmed milk (w/v) i.e., 0.1%, 0.25%, 0.5%, 1.0%, 1.5%, 2.0% and 3%. The skimmed milk concentration showing the best culture growth along with zone of clearance was determined as optimum substrate concentration for growth and protease activity.

2.6.3 Temperature

Isolate GUYHP1 was spot inoculated on NH media plates containing 20% NaCl and 0.5% skimmed milk powder. The plates were incubated at various temperatures such as 4, 28, 37 and 45℃. The temperature showing the best culture growth along with zone of clearance was determined as optimum temperature for growth and protease activity.

2.6.4 pH

Isolate GUYHP1 was spot inoculated on NH media plates containing 20% NaCl, 0.5% skimmed milk powder and a range of pH from acidic to alkaline, i.e., 4, 5, 6, 7, 8, 9 and 10. The pH showing the best culture growth along with zone of clearance was determined as optimum pH for growth and protease activity.

2.7 Partial Purification of Protease

GUYHP1 culture was grown in NH media supplemented with 0.5% skimmed milk solution and was used for protease production studies after 7-8 days of incubation. After production of enzyme, the culture broth was centrifuged at 10,000 rpm (Eppendorf Centrifuge 5804R) for 20 minutes at 4℃. Pre-chilled ethanol was slowly added to the cell free supernatant (CFS) with constant stirring. This mixture of ethanol- CFS was centrifuged at 10,000 rpm for 30 minutes at 4℃ followed by discarding the aqueous layer. The precipitate was dissolved in minimal volume of 0.1M of Phosphate buffer [Appendix II (6)], pH 7.5 and used for further process.

2.7.1 Estimation of Protease Activity on Agar plates

Protease activity was determined using the agar well diffusion method on NH media plates containing 15%, 20% and 25% NaCl with 0.5% skimmed milk. Wells were punched with the help of well-borer (0.6cm in diameter). 50µL of the CFS were inoculated in the wells and incubated at room temperature till it diffused in the plates. Zone of clearance was observed and the highest zone was indicated as optimum protease production.

2.8 Protease assay

Protease assay was determined by the modified method of (Kunitz, 1947). The culture broth was centrifuged at 10000 rpm at 4°C for 10 min. The clear cell-free supernatant (CFS) was used as crude enzyme sample. For the assay, 0.2 ml of the crude extract with 0.5 mL of 1% casein solution (w/v) was mixed with 1 mL of 25 mM Tris HCl (pH 8) and incubated at 37°C in water bath (Bio Technics India) for 15 minutes. 1 mL of trichloroacetic acid was added to it for stopping the reaction and incubated at R.T for 15 minutes. After centrifugation at 10,000 rpm for 10 minutes, 1.7 mL of supernatant with 2 mL Sodium Carbonate (0.2 M) and 1 mL of Folin-Ciocalteau reagent was incubated at R.T in dark for 30 minutes [Appendix II (26- 30)]. Absorbance was noted at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute.

2.8.1 SDS-PAGE and Zymogram

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), a versatile electrophoretic technique for protein separation was performed to check for the presence protease enzyme. It was co-polymerized by using 0.1% gelatin as substrate. Standard molecular markers of pectinase (48 kDa), BSA (bovine serum albumin) (66 kDa), amylase (56 kDa) were used to run in the marker well. The sample well consisted of 40 µL of enzyme (CFS) along with 10 µL sample loading solution [Appendix II (9)]. Subsequently, the gel was run at 100 V for 30-45 minutes using a power pack. This was followed by cutting the gel carefully to divide it into two equal halves, wherein one half was used for staining with the staining solution [Appendix II (10)]. For the other half of the gel, gelatin zymography was performed, as described by Heussen and Dowdle,1980 with some modifications. After electrophoresis, the gel was rinsed in 2.5% Triton X-100 [Appendix II (14)] for 1 h at 25°C to remove SDS and incubated under optimal assay conditions using 20 mM Tris–HCl buffer, pH 8.5 and 50°C [Appendix II (15)] for 30 min to perform the proteolytic activity. The final step was performed

by staining the gel in a solution of 0.5% (w/v) amido black 10B [Appendix II (12)]. To observe the proteolytic activity, gel was destained using destaining solution [Appendix II (13)]. Clear zone against the blue-black background indicated the activity of protease.

2.9 Partial Characterization of the Potential Extremely Halophilic Isolates GUYHP1 and GUYHR1

Morphological and biochemical characterization of extremely halophilic isolate was done using the methods listed below:

2.9.1 Morphological characteristics

The extremely halophilic isolate GUYHP1 and GUYHR1 was streaked on RV media to obtain isolated colonies. Colony size, shape, pigmentation, margin, elevation, consistency and opacity was determined.

2.9.2 Carbohydrate Utilization

For examining the utilization of sugars, 10% stock of 8 different sugars was prepared and autoclaved. Basal media used was NH media along with 0.001% phenol red as an indicator where pH of the media was maintained from 7.2- 7.4. The tubes were autoclaved with 4.5 mL of media along with inverted Durham's tube. 0.5 mL of autoclaved sugar stock and 200 µL of the culture was inoculated and incubated till colour change and gas production was observed.

2.9.3 Response of the isolates to Antibiotics

Various antibiotics are used to differentiate between archaea and bacteria. The domain Archaea differs from domain Bacteria by lacking the presence if peptidoglycan layer. This layer of peptidoglycan is present in bacteria and gets inhibited when treated with antibiotics like penicillin, ampicillin, kanamycin, neomycin, streptomycin. Since Archaea lack peptidoglycan layer, they are resistant to the action of antibiotics. To check this activity, NH medium was supplemented with penicillin (100 IU ml^{-1}) followed by streaking with the culture. The plates

were incubated for 10-12 days at RT. Growth observed indicated that the culture does not belong to the domain bacteria therefore is an archaeal culture.

2.9.4 Light microscopy

Gram staining was performed of the extremely halophilic isolate GUYHP1 and GUYHR1. For this, a clean grease free slide was taken and a smear was made by picking up a single colony from the plate using a sterile loop with 15% NaCl. It was air dried and washed with 2% acetic acid. The smear was stained with Crystal violet (primary stain) for 1 minute. The stain was discarded and flooded again with Gram's Iodine for 30 sec. The smear was rinsed with water followed by decolorization with 90% ethanol for 30 sec. Finally, the smear was stained with safranin (counter stain) for 1 min and air dried. The slide was examined at 100x magnification using the compound microscope (Radical RXL-4).

2.9.3 Scanning Electron Microscope

Field Emission Scanning Electron Microscope (FESEM) analysis of the Halophilic GUYHP1 and GUYHR1 was carried out for studying the structures of the isolates. After the growth of the halophilic culture was observed, with the help of a sterile toothpick, a single colony was picked up from the Petri plate and a smear was made on a clean grease free coverslip with one drop of 15% NaCl. After air drying and heat fixing, coverslip was washed with 2% acetic acid for removing excess salt (Dussault, 1995). 2% glyceraldehyde was used as a fixative for immersing the coverslip and kept overnight. The smear was treated with a series of acetone-water concentration gradient i.e 10%, 30%, 50%, 70%, 90% and kept for 10 min and final 100% gradient concentration was kept was 30 min. The coverslip was then sputter coated with gold film and then viewed under Quanta 250 FEG Scanning Electron Microscope.

2.9.4 Spectrophotometric Analysis of carotenoid pigment from culture GUYHP1 and GUYHR1

For extraction of pigments, centrifugation of cells was carried out at 8000 rpm (Eppendorf Centrifuge 5417R) for 8 mins and the cells were pelleted. To the pellet 0.5 mL methanol was added and vortexed followed by addition of 1 mL of acetone to it making the final ratio of methanol: acetone (2:1) (v/v). It was then vortexed again for the complete extraction of pigments. A UV-Visible spectrophotometer (Analytical Technologies Limited) was used to scan the pigments in the range of 190-800 nm and methanol: acetone (2:1) (v/v) was used as blank. To prevent the photobleaching of pigments, the extracts were collected in tubes wrapped in aluminium foil.

2.9.5 Lipid Analysis

Cell pellets of the 25 mL culture were suspended in 3.75 mL methanol: chloroform (2:1) (v/w) and extracted for 4-6 hours. The supernatant was centrifuged at 10,000 rpm for 20 mins followed by addition of 4.75 mL methanol: chloroform: water (2:1:0.8) (v/v) to the pellet for re-extraction. For achieving phase separation, the supernatants were combined by addition of 2.5 mL of chloroform and methanol each. After centrifugation at 10,000 rpm for 10 mins, the chloroform phase containing the lipids was collected in a clean dry 10 mL glass vial and evaporated by drying. Lipids were redissolved by adding 200 µL of chloroform and spotted on silica gel plates.

2.10. Studies on the isolate GUYHR1

2.112.10.1 Cultivation and Maintenance of extremely halophilic isolate GUYHR1

The isolate GUYHR1 was initially isolated on Rodriguez- Valera media (halophilic media with 25% NaCl) where the culture took approximately 30- 45 days to be fully visible to the naked eye since the colonies appeared pinpoint in shape. When loopful of culture inoculated in liquid

medium, the culture took more than 30 days to fully grow. In order to obtain faster and dense growth, loopful of the fully grown culture was streaked on five different halophilic media that is;

1) NaCl Tryptone Yeast Extract media (NTYE)
2) NaCl Trisodium Citrate media (NT)
3) Moderately Halophilic Media (MHM)
4) Extremely Halophilic Media (EHM)
5) Tomlinson Media

2.10.2 Preparation of Starter Culture

Starter culture was prepared by inoculating loopful of the pure culture from agar plates in 50 ml of NTYE, NT, EHM, MHM and Tomlinson media in 100 mL of Erlenmeyer flask. The flasks were incubated at 30 ℃, 100 rpm on rotary shaker.

2.10.3 Pigment production of the isolate GUYHR1 in different Halophilic media

1.5 mL of the culture from each halophilic media was taken in 2 mL of Eppendorf tubes. Centrifugation of cells was carried out at 8000 rpm for 8 mins and the cells were pelleted. To the pellet 0.5 ml methanol was added and vortexed followed by addition of 1 ml of acetone to it making the final ratio of methanol: acetone [2:1] (v/v). It was then vortexed again for the complete extraction of pigments. A UV-Visible spectrophotometer (Analytical Technologies Limited) was used to scan the pigments in the range of 190-800 nm and methanol: acetone [2: 1] (v/v) was used as blank. To prevent the photobleaching of pigments, the extracts were collected in tubes wrapped in aluminium foil.

Chapter III

Results and Discussion

3. Results and Discussion

3.1 Sampling site and sample collection

In the present study, for isolation of halophilic microorganisms, the solar saltern was selected from Nerul, Bardez Taluka, North-Goa District, Goa (15.5075º N, 73.7843º E) which is located along the west cost of India bordering the Arabian Sea (Fig 3.1A and Fig. 3.1B). Brine and sediment samples were collected from the crystallizer pond of the saltern during the peak salt harvesting period. The sampling was done on 21st May, 2022. The pH of the sample was found to be ~7.0 (Neutral) and temperature was ~35°C.

Fig 3.1 : A) and B) Sampling site: Salt pans of Nerul, Bardez Goa ; C) Collection of the sediment and the brine samples

3.2 Isolation and Purification of Halophilic Microorganisms

The brine and sediments samples collected from solar saltern of Nerul, Goa were spread plated on Rodrigues-Valera (RV) medium (Rodriguez-Valera et al., (1979). White, cream and yellow-coloured colonies were observed after incubating the plates for 4-8 days (Fig 3.2 A and 3.2B). The plates were further incubated and after 20-35 days of incubation,

colonies with red and pink pigmentation started to appear on the plates (Fig 3.3 A and 3.3B). On the basis of the size, colour and pigmentation, the colonies were selected and purified through repeated streaking on the RV medium by quadrant streak technique in order to obtain pure cultures (Fig. 3.4). Two halophilic isolates were obtained in pure form and they were designated as GUYHP1 (Fig. 3.5a) and GUYHR1 (Fig. 3.5b). The isolates were found to be extremely halophilic since they were able to grow on media containing 25% NaCl.

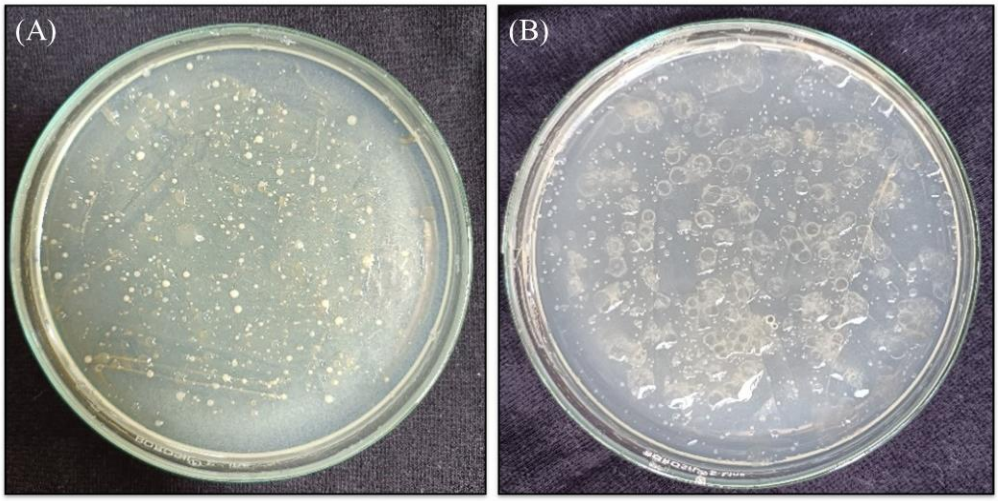

Fig 3.2: Plates inoculated with Brine(A) and sediment (B) sample respectively, after incubating for 8 days.

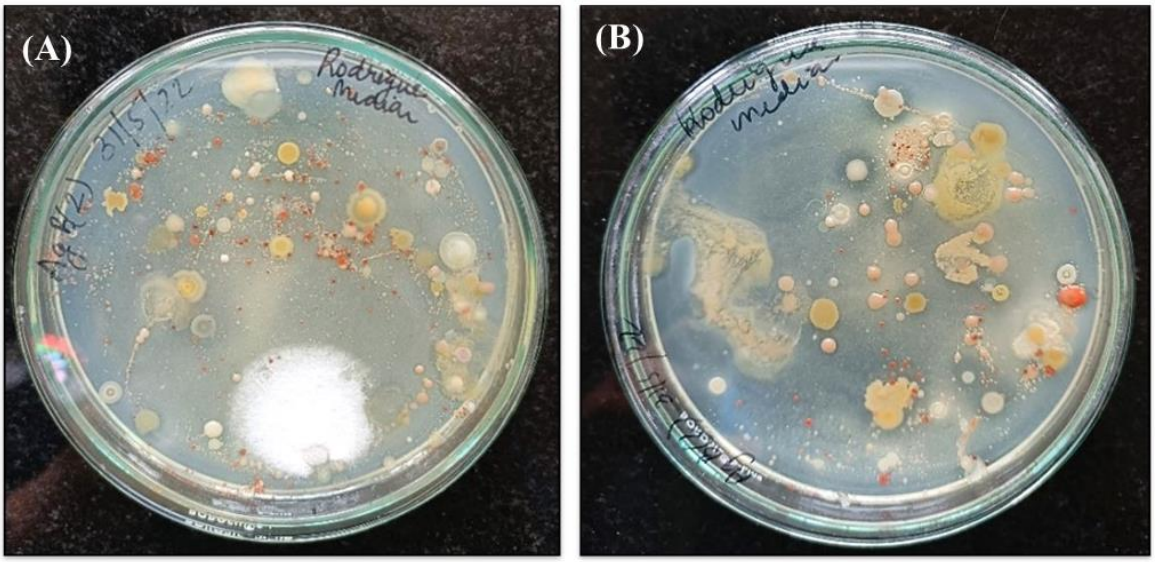

Fig 3.3: Plates inoculated with Brine (A) and sediment(B) sample respectively, after incubating for 45 days.

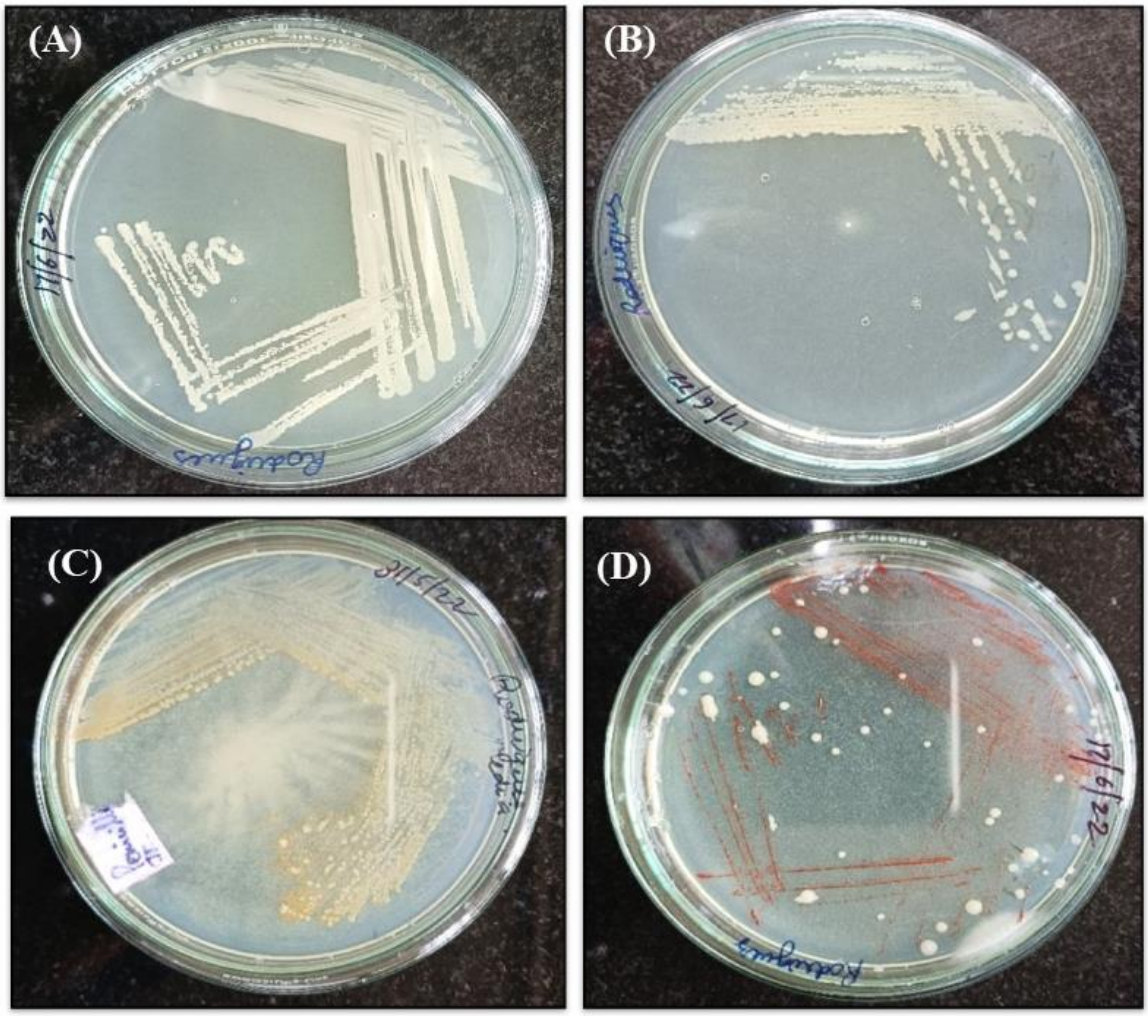

Fig 3.4 (A, B, C): Cultures in progress of purification; **D:** mixed culture

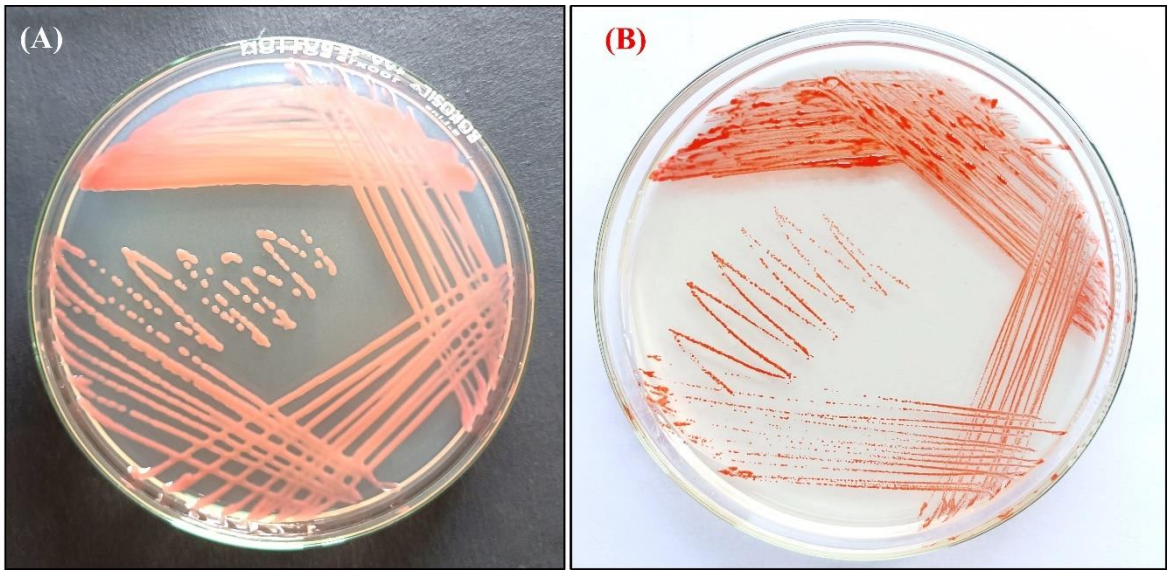

Fig 3.5: Pure cultures on Rodriguez-Valera medium A) GUYHP1 and B) GUYHR1

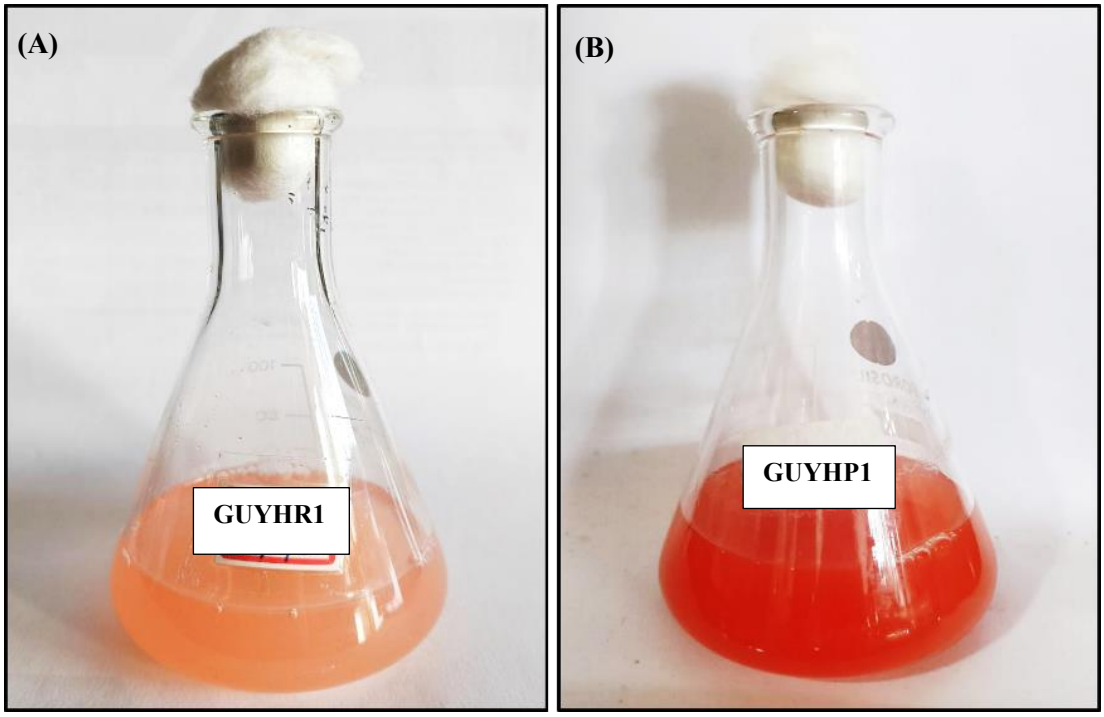

Fig 3.6: Pure cultures in liquid media A) GUYHP1 and B) GUYHR1

3.3 Screening of extremely halophilic isolates for extracellular hydrolytic enzymes

The two halophilic cultures i.e GUYHP1 and GUYHR1 were screened for the production of amylase, protease, esterase, lipase, gelatinase, cellulase, xylanase, laccase, pectinase, phosphatase and chitinase using plate assay method on NH medium containing 20% NaCl (Fig. 3.7 and 3.8). Among the two isolates, gelatinase was produced by both the cultures. However, GUYHP1 was found to be the best producer for gelatinase based on the zone of clearance observed. In addition to gelatinase, GUYHP1 also produced amylase, protease, esterase. Neither of the isolates showed the activity for the rest of enzymes.

The culture GUYHP1 was able to grow on media with all substrate except for pNP phosphate. Similarly, GUYHR1 was unable to grow on medium with substrates like olive oil, coconut oil, pNP phosphate, tween 80, tween 20 and tween 40.

Table 3.1: Extremely halophilic isolates showing extracellular hydrolytic activities in NH media containing 20% NaCl

Enzymes	**Cultures**		***Halogeometricum, Haloferax***	***Halorubrum***
	GUYHP1	GUYHR1	**(Ventosa et al., 2012)**	
Amylase	+ve	-ve	+ve	+ve
Protease	+ve	-ve	+ve	+ve
Gelitinase	+ve	+ve	+ve	+ve
Xylanase	-ve	-ve	ND	+ve
Cellulase	-ve	-ve	ND	+ve
Phosphatase	-ve	-ve	ND	+ve
Esterase (tween 80)	+ve	-ve	+ve	-ve
(tween20)	-ve	-ve	+ve	-ve
(tween40)	+ve	-ve	ND	-ve
Lipase (olive oil)	-ve	-ve	+ve	+ve
(Coconut oil)	-ve	-ve	ND	ND
Chitinase	-ve	-ve	ND	-ve

+ve: Positive, -ve: Negative, ND: Not defined

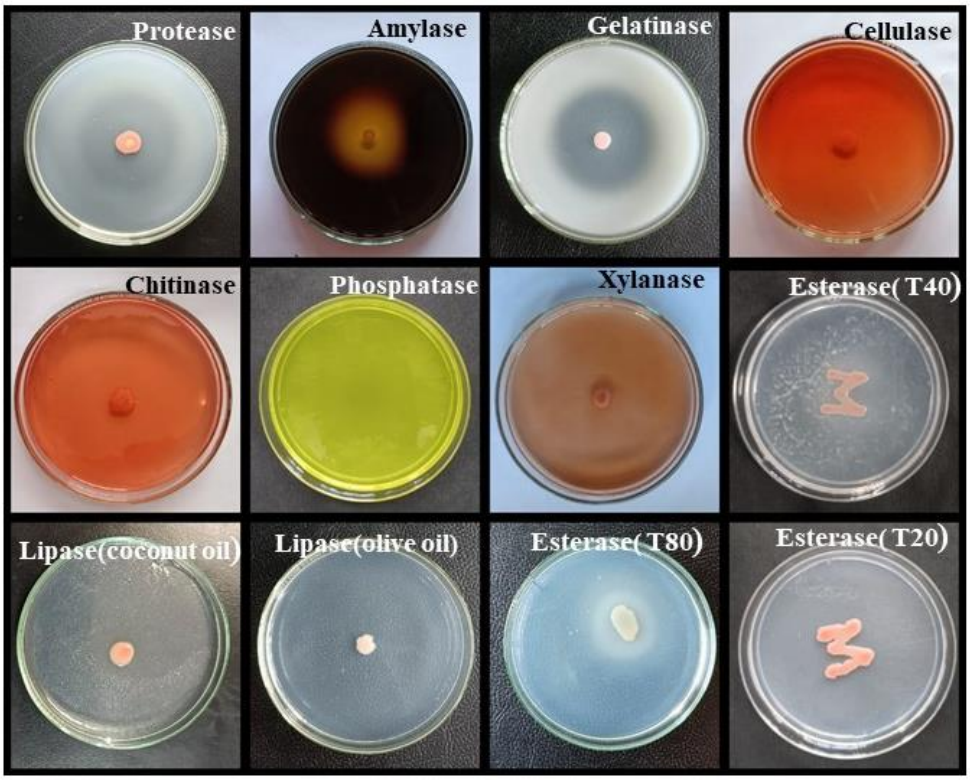

Fig 3.7: Screening of extremely halophilic isolate GUYHP1 for extracellular hydrolytic enzymes

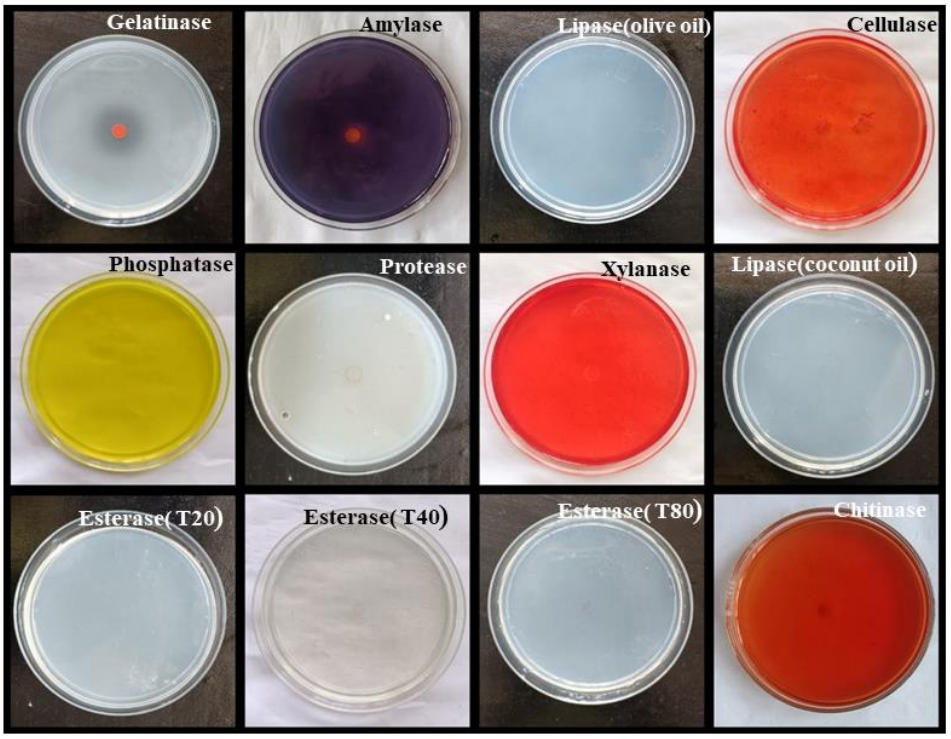

Fig 3.7: Screening of extremely halophilic isolate GUYHR1 for extracellular hydrolytic enzymes

3.4 Selection of the potential isolates:

After screening for the enzymes, GUYHP1 was found to be producing the best amount of protease, as the culture was found to be actively growing and zone of clearance around the colony was observed soon after 3 days of incubation with 0.5% skimmed milk agar plates.

3.4.1 Optimization of culture conditions

Optimization of various parameters for growth was done by growing the isolate GUYHP1 on NH media with varying concentrations of NaCl, skimmed milk and varying the medium pH and temperature of incubation. The protease activity was qualitatively determined by monitoring the zone of clearance around the colony.

Culture GUYHP1 on NH media with varying concentrations of NaCl showed good growth on NaCl ranging from 15%-30%, slight growth on 10% and no growth was seen on 0%, and 5% over a period of 7-15 days of incubation. (Fig 3.9 and 3.10). After 15 days of incubation, the highest protease production was seen at 25% and 30% NaCl. Surprisingly, even after 30 - 40 days of incubation, higher activity of protease could be seen at 15%-30% NaCl plates (Fig 3.11).

On NH media agar plates supplemented with varying concentrations of skimmed milk, protease production from 0.1% - 1.5% was observed. 0.5% of skimmed milk was found to be optimum for growth and protease production as compared to the other concentrations of the substrate. (Fig 3.12)

For checking the optimum pH for growth and enzyme production, GUYHP1 isolate showed activity from pH 5-9. The maximum activity was observed at p H 7-8 (Fig 3.13).

When NH media plates with inoculated culture was incubated at temperature 4°C, 28°C and 37°C. The highest enzyme production was seen at 28°C (Fig 3.14)

Therefore, the maximum activity was at 25% NaCl, 0.5% skimmed milk, pH 7-8 and temperature 28°C.

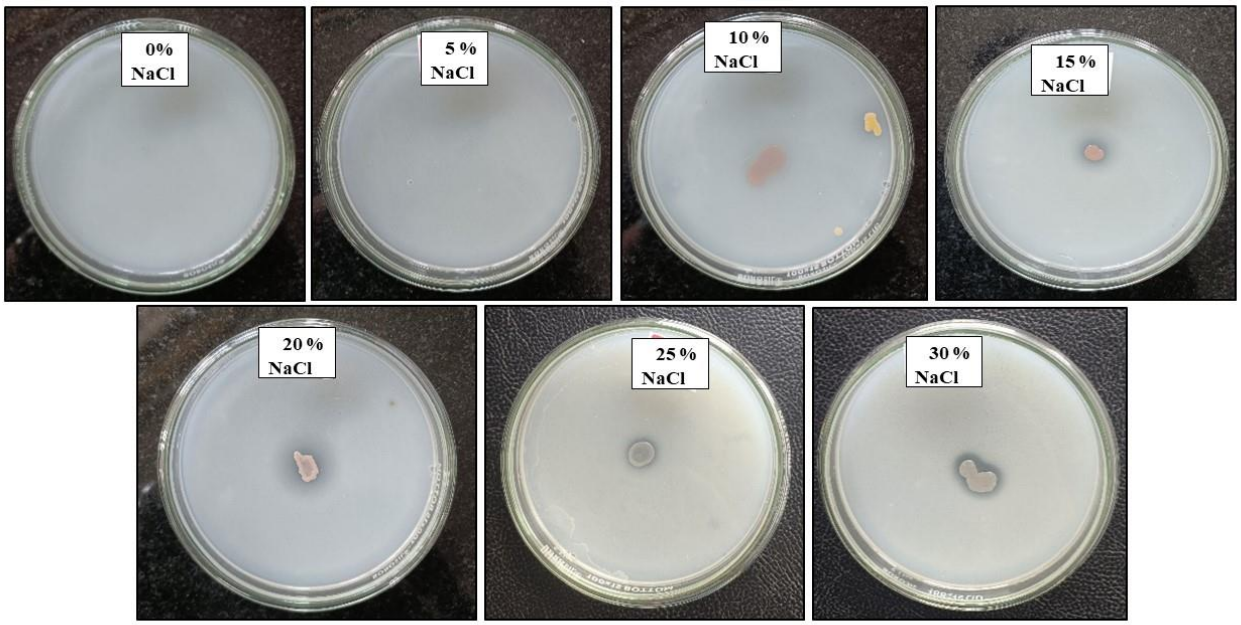

Fig 3.9: Growth and Protease production at different NaCl concentration after 7 days of incubation

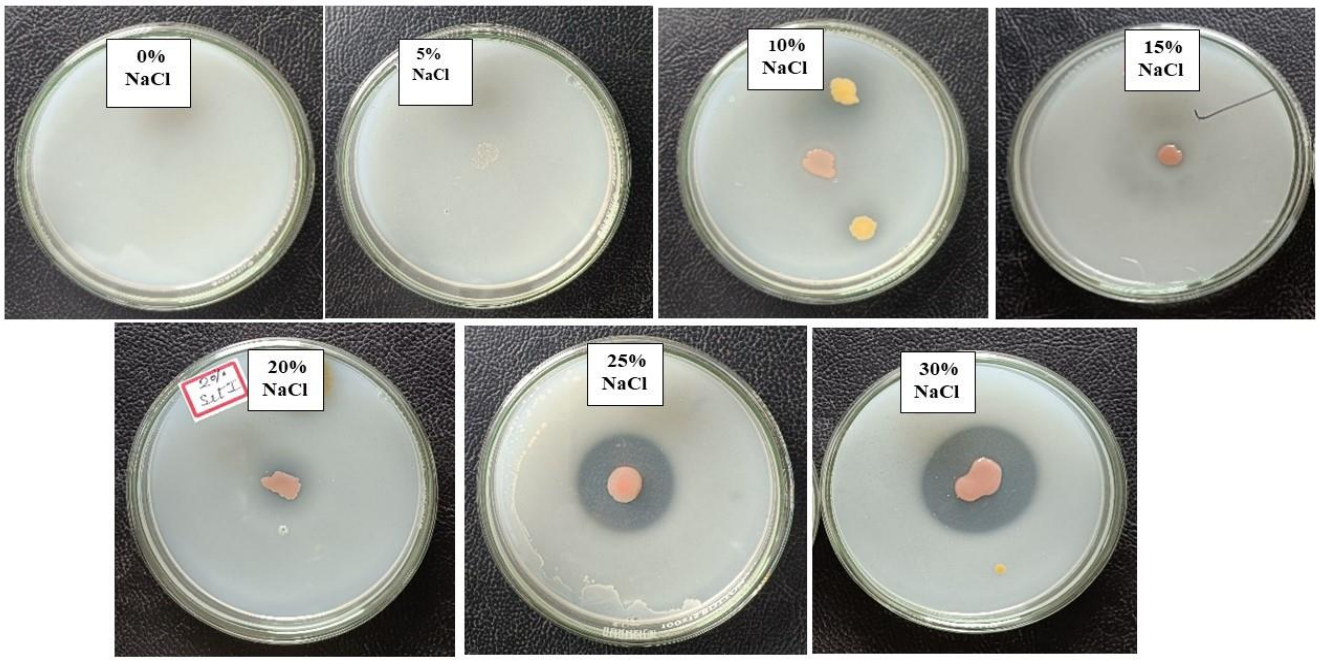

Fig 3.10: Growth and Protease production at different NaCl concentration after 7 days of incubation

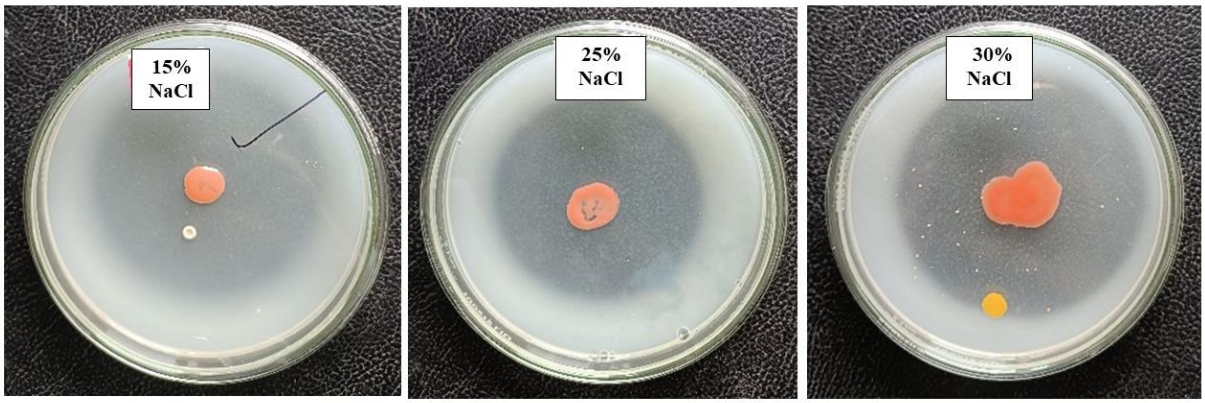

Fig 3.11: Growth and Protease production observed on 15%, 20%, 25% NaCl concentrations after 30 days of incubation time

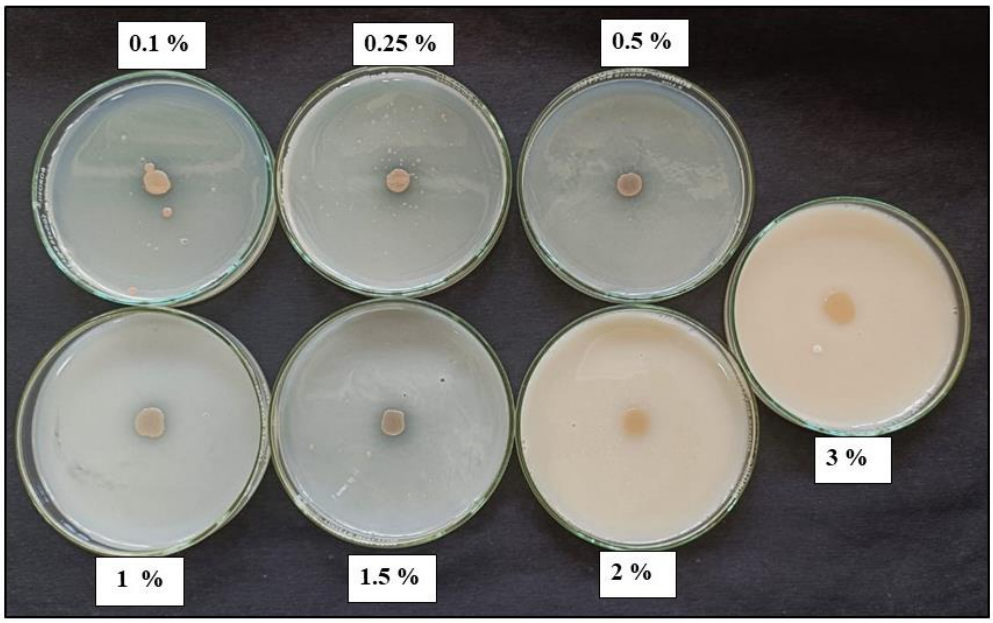

Fig 3.12: Growth and Protease production observed at different substrate concentrations after 10 days

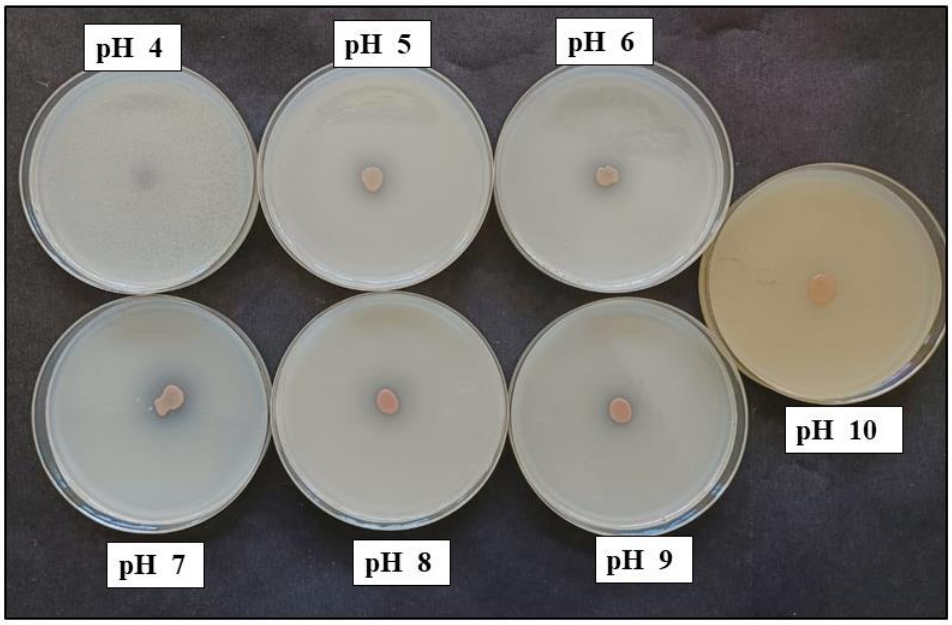

Fig 3.13: Growth and Protease production observed at different pH

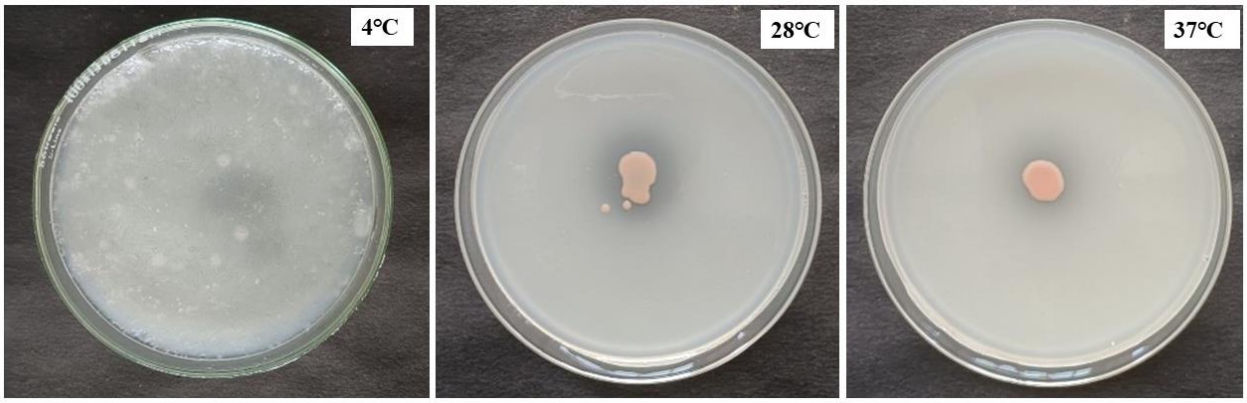

Fig 3.14: Growth and protease production observed at different temperature

3.5 Protease production in varying NaCl concentrations in liquid medium

The growth and enzyme production of the isolate GUYHP1 was checked by inoculating it in NH media with 0.5% skim milk and varying NaCl concentrations (15%, 20% and 25%). Sterile NH media with 0.5% skimmed milk was autoclaved, inoculated with loopful of the culture from the skimmed milk agar plates of different NaCl concentrations and incubated in a rotary shaker 100 rpm at 30°C. After inoculating with

the culture, it was noticed that the colour of the media gradually began to change from white to pale pink. After 15 days of incubation, the colour change of the media was clearly visible indicating the production of protease enzyme along with slight change in the pigmentation. It was noticed that the isolate hydrolyzed the substrate fastest when grown in 25% NaCl concentration due to which the carotenoid production was found to be highest (3.15 A and B)

The difference in the colour of all the media consisting of the isolate along with the substrate could be seen varying from one another.

Fig 3.15 A): Uninoculated autoclaved NH media containing 0.5% skimmed milk with different NaCl concentration; **B)**: Growth of the culture observed after 15 days as change in colour of the media is visible from white to pink in colour due to enzyme production.

3.5.1 Pigment profiling of the culture in different NaCl concentration

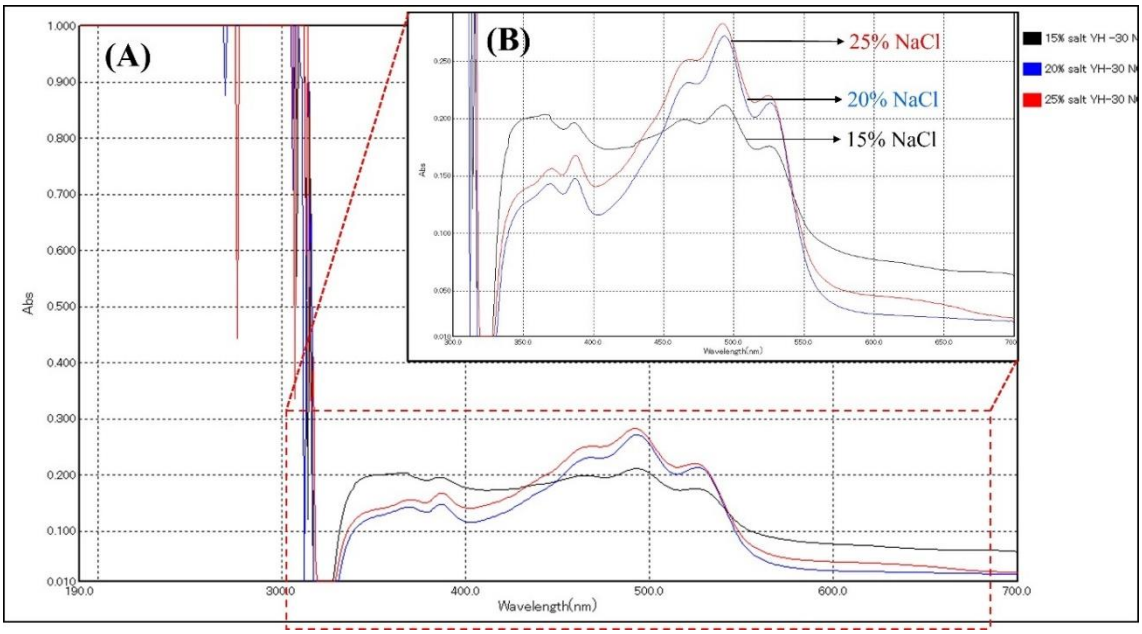

Fig 3.16: UV-Visible spectrophotometer scan of GUYHP1 in different NaCl concentration with 0.5% skimmed milk.

The maximum pigment production was observed by the isolate growing in NH media with 0.5% skimmed milk and 25% NaCl concentration followed by 20% and 15% (Fig 3.16)

The maximum peak of absorption in 25% NaCl concentration was found to be at 492 nm with two shoulder peaks at 469 nm and 524 nm. Similarly, for 20 % NaCl concentration, it was found to be at 493 nm with shoulder peaks at 468 nm and 526 nm. In the lowest NaCl concentration i.e 15% NaCl concentration, the maximum peak was obtained at 493 nm with 465nm and 525 nm as two shoulder peaks.

We can conclude that, higher the concentration of NaCl, more is the pigment production and enzyme activity of the isolate GUYHP1.

3.5.2 Growth curve at different NaCl concentrations

Growth of the isolate GUYHP1 was observed in 15%, 20% and 25% NaCl concentration by checking the turbidity and color change of the media and Absorbance was noted at 600 nm after 7 days of incubation period.

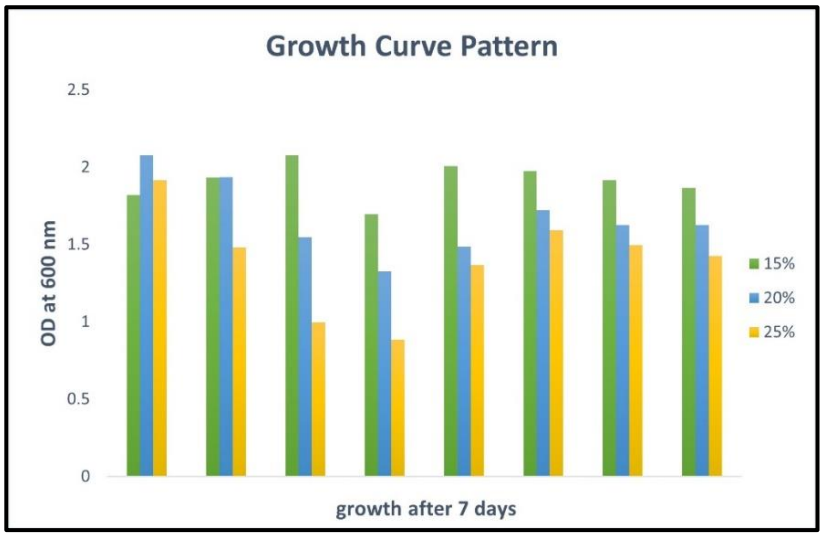

Fig 3.17: Growth curve pattern at 15%, 20% and 25% NaCl concentration

3.5.3 Protease activity at different NaCl concentrations

Protease is an enzyme playing role in breaking peptide bonds and it is necessary to measure and/or compare protease activity. The substrate used for this assay is casein. When casein is digested by the protease, the amino acid tyrosine is released along with other amino acids and peptide fragments. Folin-Ciocalteu reagent (1,2-naphthoquinone-4-sulfonate) which is a mixture of phosphomolybdate and phosphotungstate, reacts with the free tyrosine to give blue colored chromophore which is then measured using a spectrophotometer. The more tyrosine is released, more the chromophores are generated hence stronger is the activity of protease. Absorbance value obtained by the activity of protease is used for comparing to the standard tyrosine curve where, the activity of protease sample can be determined in terms of unit i.e the amount if micromoles of tyrosine equivalent released from casein per minute. One unit of

enzyme activity is defined as the enzyme quantity that liberates 1µg of tyrosine per ml of the reaction mixture per minute. From the test sample, the amount of tyrosine produced can be estimated using the tyrosine standard graph

Protease assay was performed by plotting a standard tyrosine graph to study the enzyme activity of the isolate GUYHP1.

Protease activity of GUYHP1 from different concentrations of NaCl (15%, 20%,25%) was estimated. The enzyme activity of protease enzyme from 15%, 20%,25% NaCl concentration was found to be 0.583, 1.398 and 2.811 units/ mL respectively using the formula;

$$\text{Enzyme activity} = \frac{\text{µmole of tyrosine released x total volume of assay}}{\text{volume of enzyme x time of assay}}$$

3.6 Partial Purification of Protease

3.6.1 Activity of partially purified enzyme on the substrate agar plate

To check the presence of enzyme, 10µL of CFS was inoculated to diffuse on the skimmed milk agar well plates. After incubation period, clear zone indicated presence of protease enzyme. Maximum zone of clearance was observed on the plate with 25% NaCl. This proved that the enzyme requires 25% of NaCl for its activity.

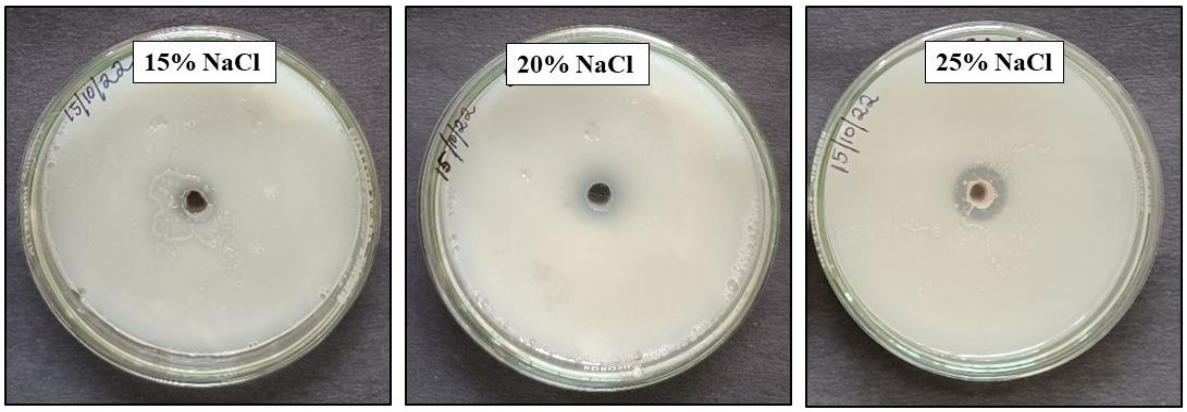

Fig 3.18: Activity of CFS on substrate plates with different NaCl concentration. Zone of clearance is visible due to the presence of enzyme

3.6.2 SDS-PAGE and Zymogram

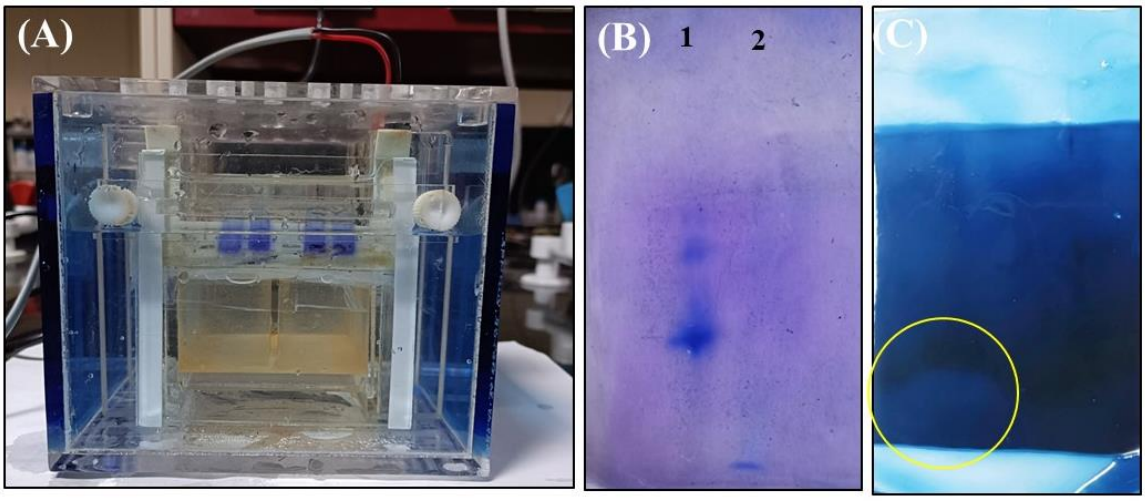

Fig 3.19 (A): SDS-PAGE gel electrophoresis unit; (B): stained gel after electrophoresis; Lane 1: Marker Lane; Lane 2: **Enzyme** band; (C): Gelatin zymography of purified protease; Lane3: zone of clearance observed (marked in yellow)

SDS-PAGE copolymerized with gelatin as a substrate was used for detecting the activity of protease enzyme. After staining the gel, bands were visible in the marker lane. The enzyme band was also obtained in the sample lane (molecular weight of protease is 22kDa). For zymography analysis, zone of clearance against the blue- black background was obtained by destaining the gel after treatment with buffer and amido black 10B.

3.7 Partial Characterization of the Potential Extremely Halophilic Isolate GUYHP1 GUYHR1

3.7.1 Morphological characterization of the isolates

Table 3.2: Colony Characteristics of the halophilic cultures

Isolate	GUYHP1	GUYHR1	*Halogeometricum, Haloferax* (Ventosa et al., 2012)	*Halorubrum*
Media	Rodriguez-Valera	Rodriguez-Valera	Seghal– Gibbons medium	Seghal – Gibbons medium
Time	10- 15 days	30-45 days	7 days	15 days
Temperature	Room Temperature	Room Temperature	40°C	37°C
Colony Size	1mm	Pinpoint	0.5- 1mm	0.5- 1mm
Shape	Circular	Circular	Circular	Circular
Cell Morphology	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic/ rods
Pigmentation	Salmon - Pink	Bright Red	Pink	Red
Elevation	Raised	Raised	convex	Slightly raised
Margin	Entire	Entire	Entire	Entire
Opacity	Opaque	Opaque	-	-
Consistency	Mucoid	Smooth	Mucoid	Smooth

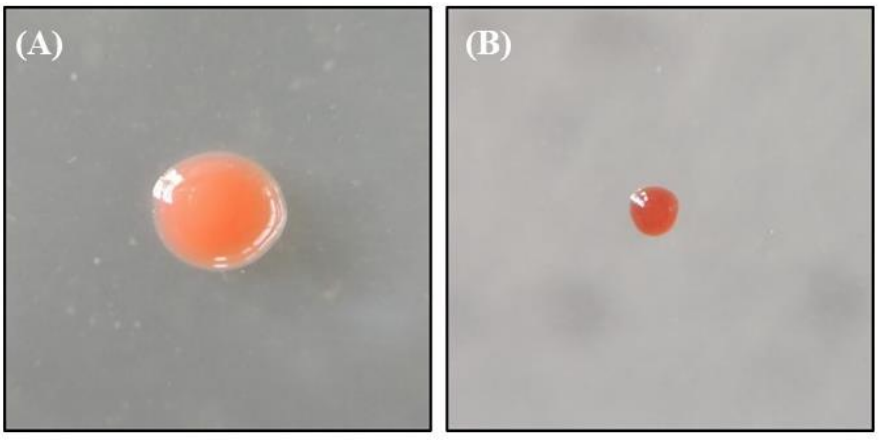

Fig 3.20 A): Single colony picture of isolate GUYHP1; B): Single colony picture of isolate GUYHR1.

3.7.2 Carbohydrate Utilisation

Table 3.3: Utilization of sugars by GUYHP1 and GUYHR1

Sugars	Culture		*Halogeometricum, Haloferax* (Ventosa et al., 2012)	*Halorubrum*
	GUYHP1	GUYHR1		
Glucose	-ve	-ve	+ve	+ve
Galactose	-ve	-ve	-ve	-ve
Sucrose	-ve	-ve	+ve	+ve
Maltose	+ve	+ve	+ve	+ve
Mannose	-ve	-ve	+ve	-ve
Trehalose	-ve	-ve	+ve	ND
Xylose	+ve	+ve	+ve	+ve
Lactose	-ve	-ve	+ve	-ve

+ve: Positive, -ve: Negative, ND- not defined

Isolate GUYHP1 and GUYHR1 could only utilize maltose and xylose as a source of carbon. The rest of the sugars did not serve the isolates as a carbon source.

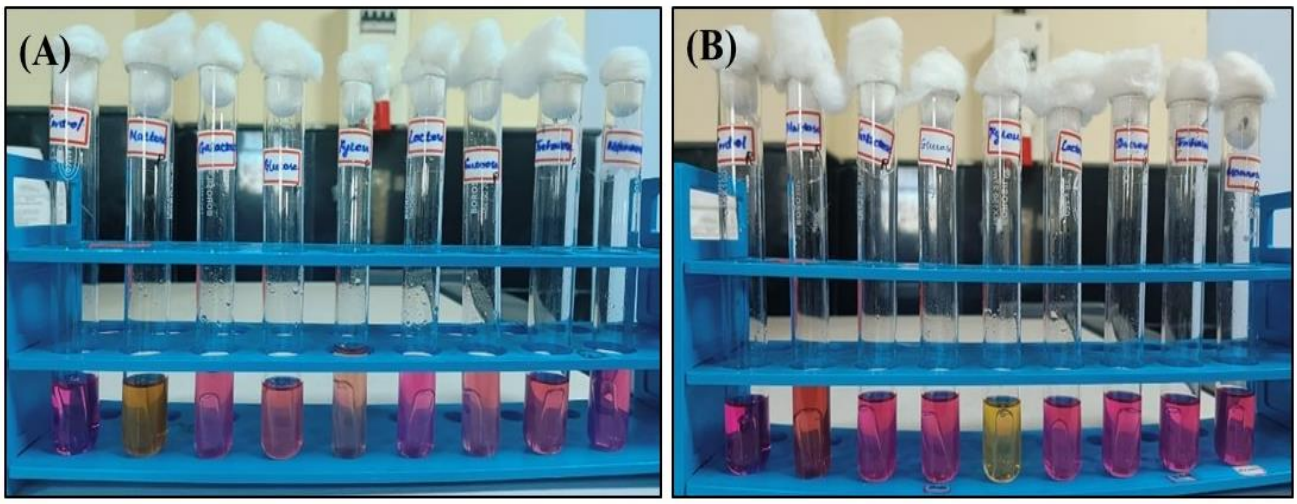

Fig 3.21 A): Sugar utilisation of isolate GUYHP1; B) Sugar utilisation of isolate GUYHR1

3.7.3 Microscopy of the halophilic cultures:

After gram staining, the slides were observed under microscope at 100X magnification where irregular shapes of the cells were visible for both the isolates. No well-defined shape of the organism could be observed (Fig 3.22). Isolates were found to be gram positive.

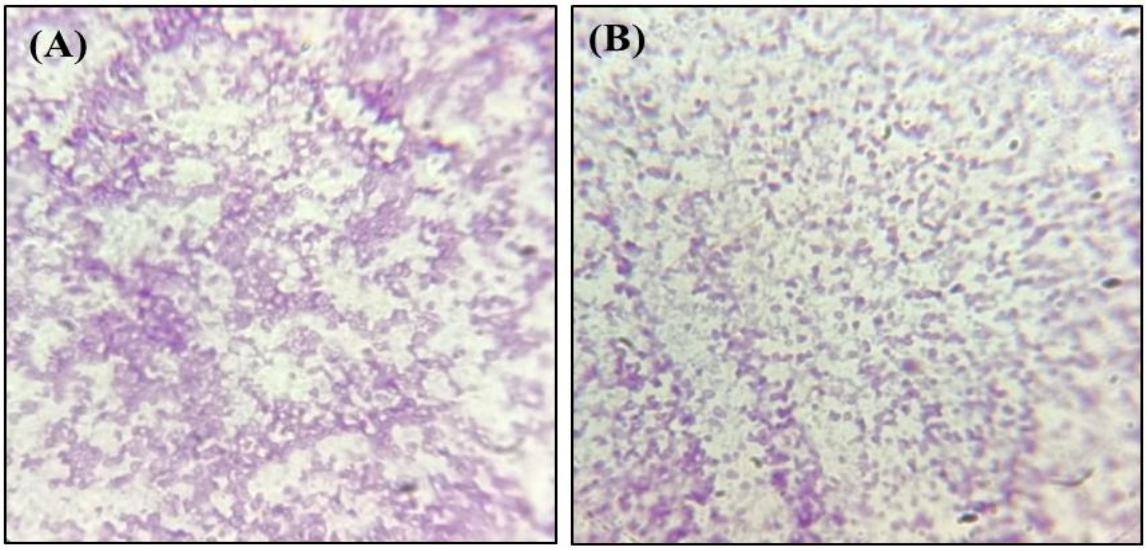

Fig 3.22: Gram staining of A) GUYHR1; B) GUYHP1 observed at 100X

3.7.4 Scanning Electron Microscopy (SEM)

The scanning electron micrographs of the halophilic isolates are presented in Fig 3.23 and Fig 3.24. The isolates GUYHP1 and GUYHR1 were found to be pleomorphic in shape when observed at 60,000X magnification using SEM. The cell surface appeared rough and irregular for both the isolates.

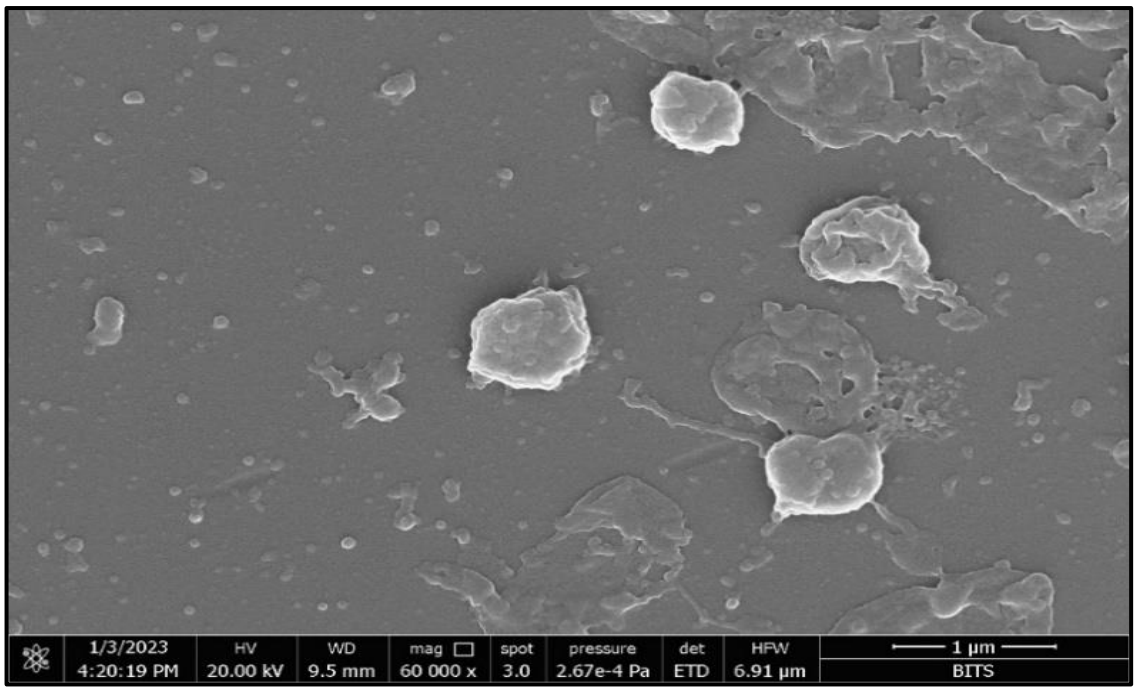

Fig 3.23: Scanning electron micrograph of the extremely halophilic isolate GUYHP1 (pleomorphic cells are visible)

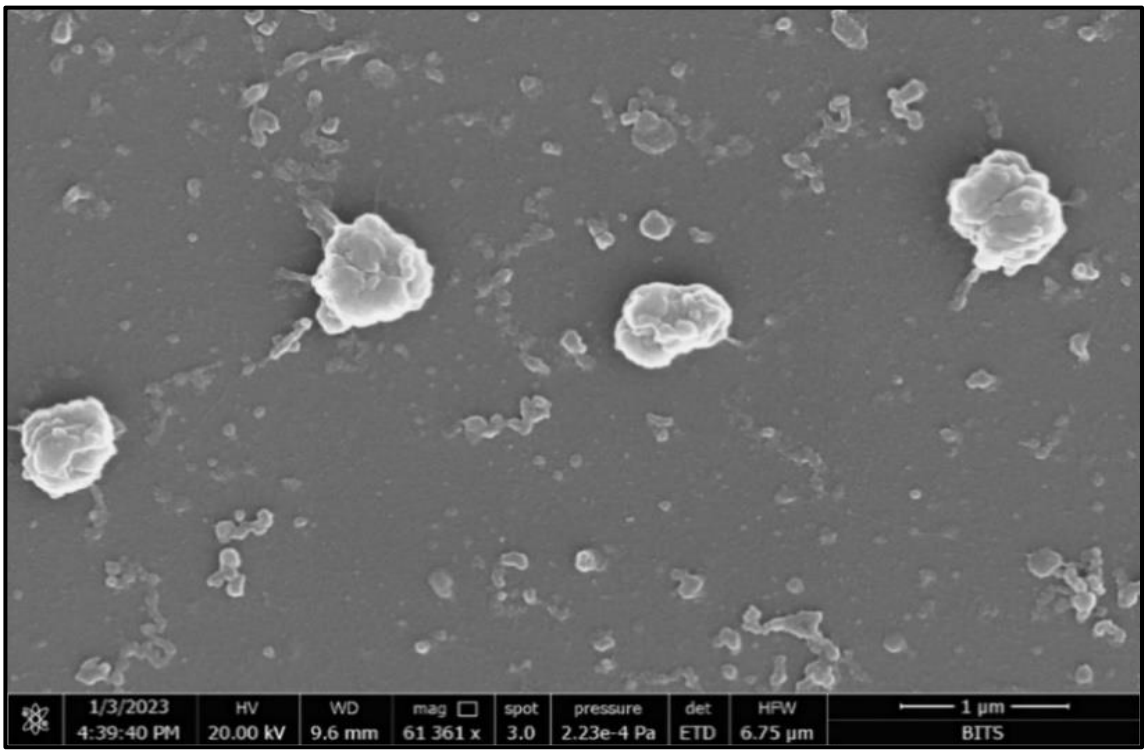

Fig 3.24: Scanning electron micrograph of the extremely halophilic isolate GUYHR1 (pleomorphic cells are visible)

3.7.5 Spectrophotometric Analysis of carotenoid pigment

The pigments in the extract solution showed maximum absorption peak at 494nm with two shoulder peaks and two cis peaks at 371 and 388 nm (Fig 3.25) for isolate GUYHP1 whereas for GUYHR1, the maximum absorbance was observed at 492 nm with 372 and 388 nm as the two cis peaks (Fig 3.26) (Wang et al, 2011).

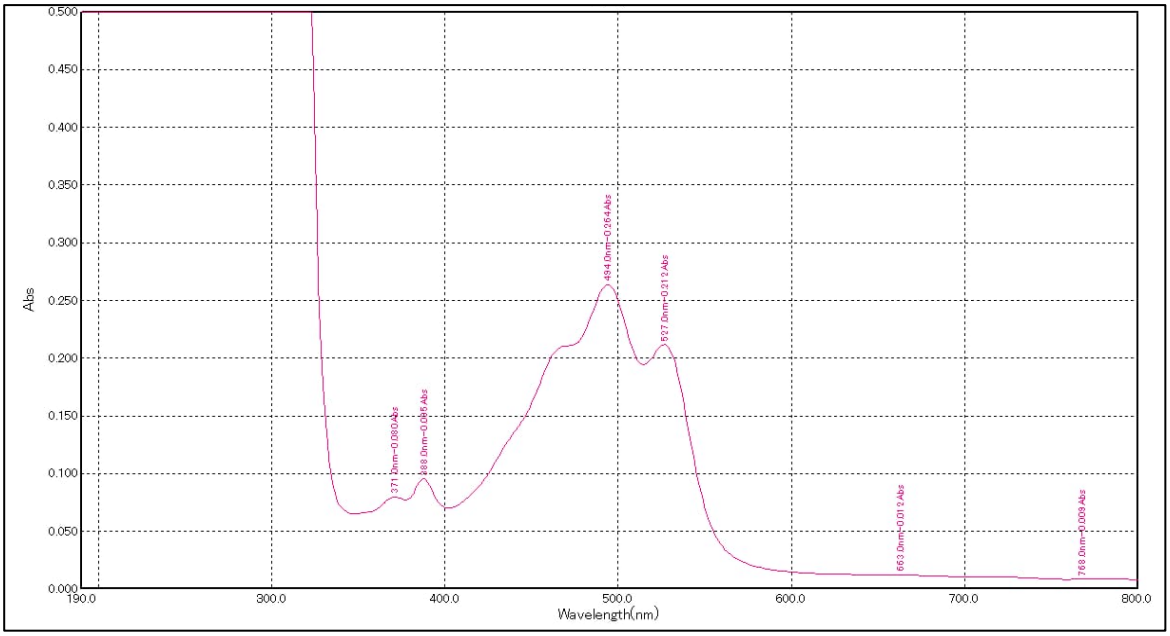

Fig 3.25: Pigment scan of halophilic isolate GUYHP1

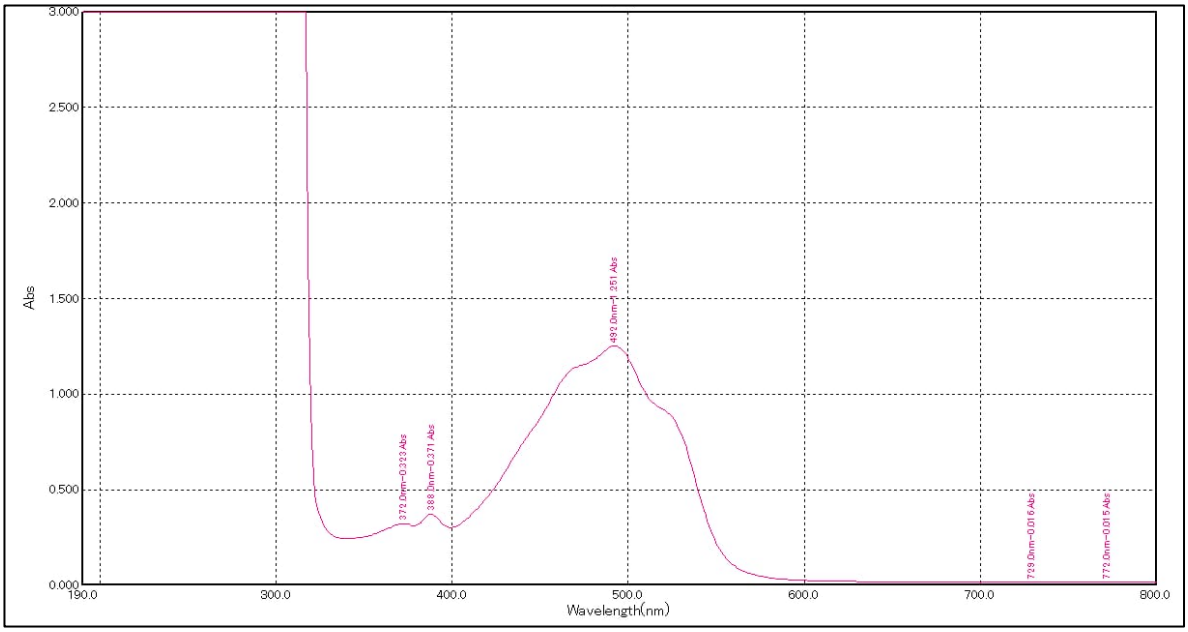

Fig 3.26: Pigment scan of halophilic isolate GUYHR1

3.7.6 Response of isolates to antibiotics

Antibiotics are used for differentiating between the domain Bacteria and Archaea. The cell wall of bacteria is made up of peptidoglycan layer having of N-Acetylglucosamine and N-Acetylmuramic acid which is sensitive to antibiotics. They inhibit the cell wall synthesis in the transpeptidation process. Since the domain Archaea lacks the peptidoglycan layer, they are resistant to antimicrobial agents. Hence, have the ability to grow in the presence of antibiotics. The isolate GUYHP1 was able to grow in the media supplemented with penicillin (Fig 3.27). Hence it belongs to domain "Archaea".

Isolate GUYHR1 was found to be not growing in the presence of penicillin hence it was sensitive to the antibiotic.

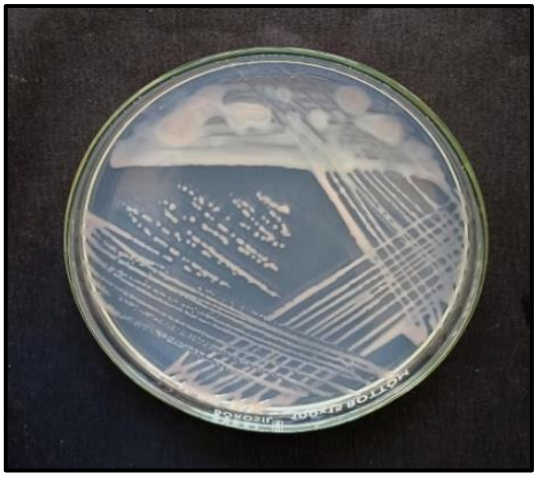

Fig 3.27: GUYHP1 on NH media supplemented with penicillin

3.7.7 Lipid Analysis of GUYHP1 and GUYHR1

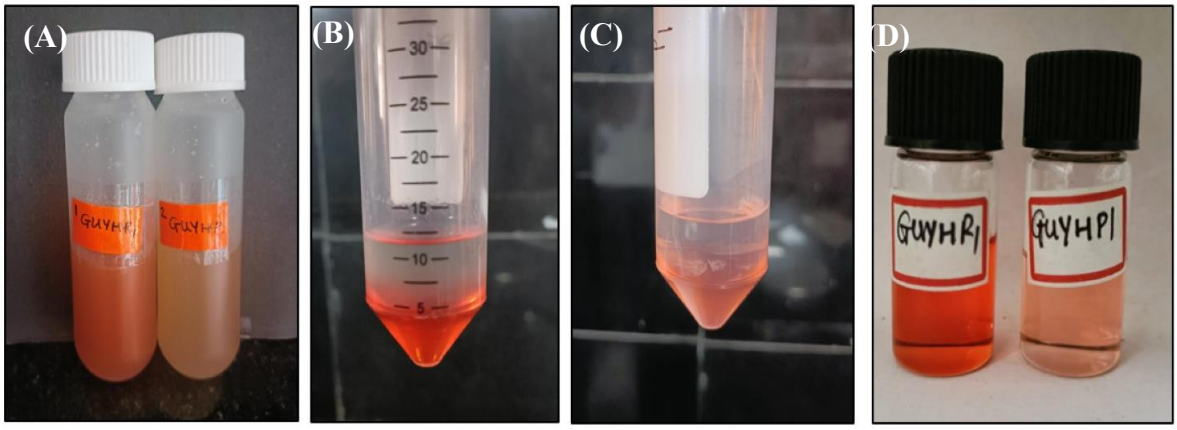

Fig 3.28: A) 25 m L of GUYHP1 and GUYHR1 culture; B) and C) chloroform phase(upper) containing lipids of GUYHR1 and GUYHP1 respectively; D) 10 mL vials containing lipids

Fig 3.29: Lipid analysis carried out of halophilic isolates GUYHP1 and GUYHR1 using chloroform: methanol: water: acetic acid solvent system

After carrying out lipid analysis, it was observed that only carotenoids were separated by running in the solvent system.

3.8 Studies on the isolate GUYHR1:

3.8.1 Isolation of GUYHR1 on different Halophilic media

The isolate GUYHR1 from RV media was streaked on five different halophilic media namely, EHM, NT, NTYE, MHM and Tomlinson's media to check which among the five media the isolate grew fastest and densest. After the incubation period, the isolate was found to be growing on all the media except for MHM but the fastest and dense growth was observed on EHM media. The MHM media is a moderately halophilic media with 178 g/L NaCl concentration in comparison to the other four media having 250 g/L NaCl concentration. This proves that the isolate strictly requires 25% of NaCl to grow (Fig 3.29)

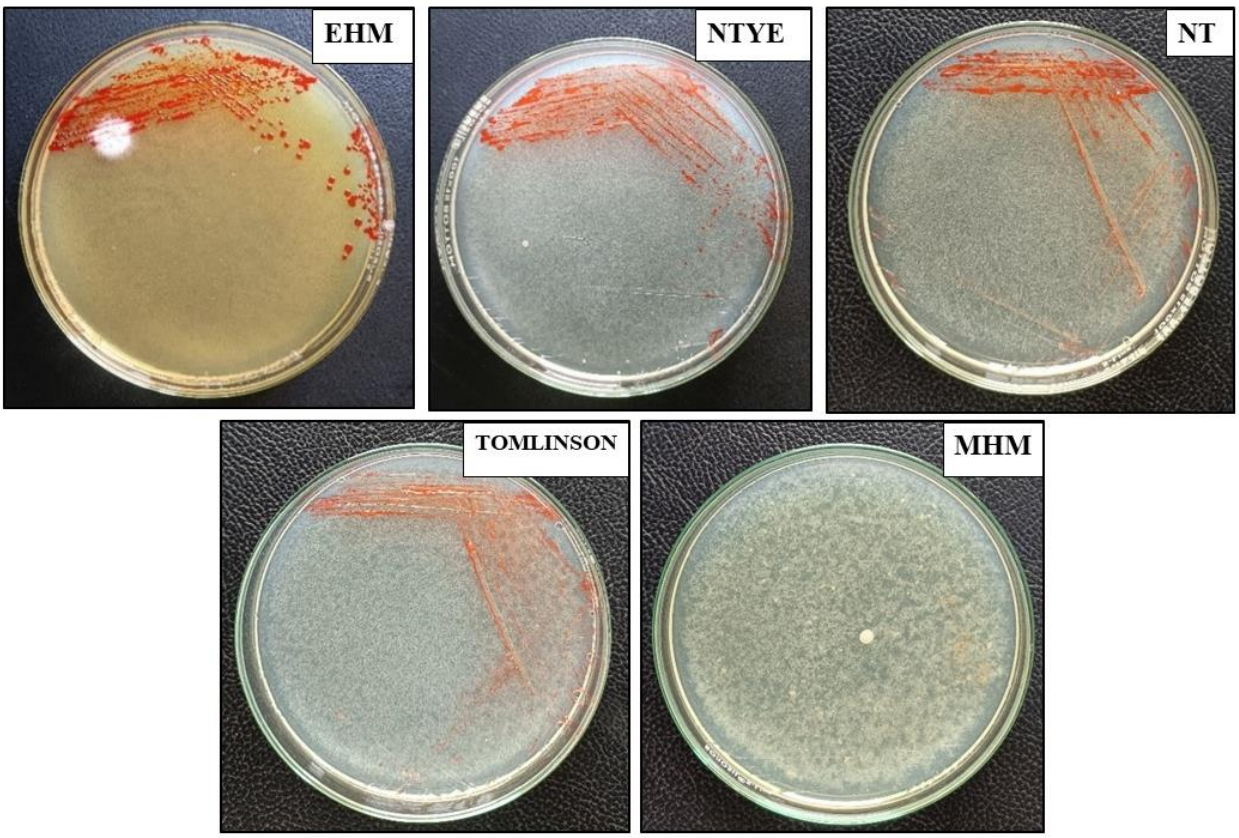

Fig 3.30: GUYHR1 streaked on halophilic media: EHM, MHM, NTYE, NT and Tomlinson media

500 µL of the culture was inoculated from the RV medium in the five different autoclaved halophilic media and incubated in rotary shaker at 30°C. The fastest growth observed was after 12 days in EHM media hence proving that GUYHR1 grows best in EHM media.

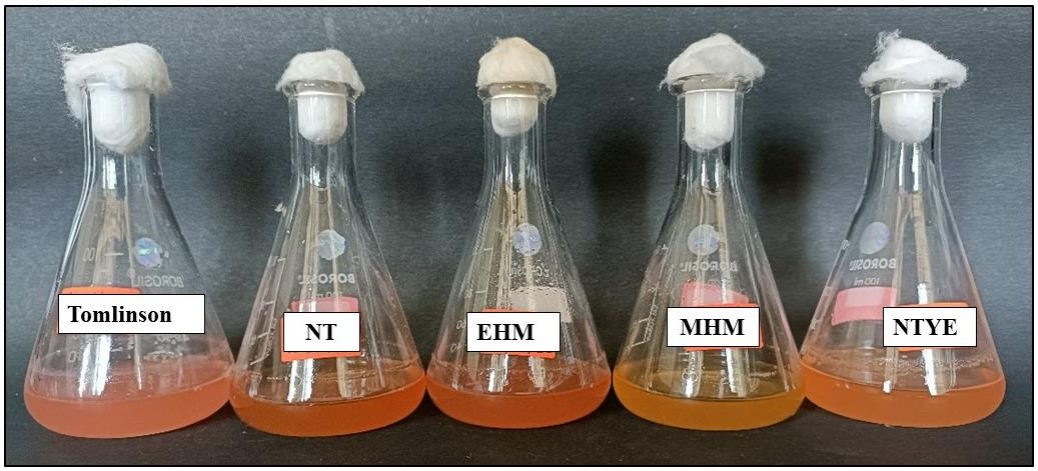

Fig 3.31: GUYHR1 in different Halophilic media

3.8.2 Pigment profiling of GUYHR1 from different halophilic media

From the five different halophilic media, using methanol: chloroform solvent the highest pigment production was seen in EHM media where maximum absorbance was noted at 493 nm followed by Tomlinson media and NT media. Pigment production was found to be absent in MHM media as the culture was not growing in the presence of moderate salt concentration.

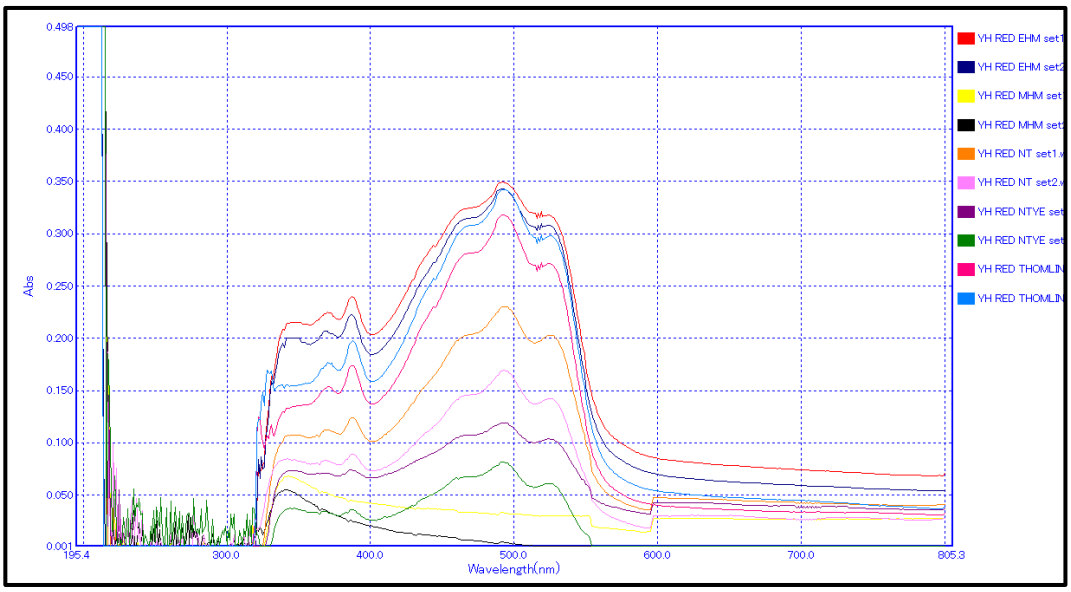

Fig 3.32: Pigment scan of GUYHR1 grown in different Halophilic media

Chapter IV

Summary and Conclusion

Summary of Results and Conclusion

In the present study, two pigmented cultures were isolated from the brine sample obtained from the salt pan of Nerul, Goa. Both the isolates were brightly pigmented and required 25% NaCl for its growth. The isolates were visible after 30-45 days of incubation. The extremely halophilic isolates were screened for hydrolytic enzymes from which both cultures were producing gelatinase. GUYHP1 was found to be producing more than one enzyme i.e protease, amylase, gelatinase and esterase. None of the cultures were found to be producing cellulase, xylanase, chitinase, lipase and phosphatase. Among this, GUYHP1 showed excellent activity for protease as the zone of clearance around the colony could be seen immediately after two days of inoculation.

GUYHP1 was selected as a potential isolate as it showed protein hydrolysis. Different parameters like NaCl concentration, substrate concentration, pH and temperature were used for checking for the optimum growth and production of protease enzyme. By agar plate assay, protease production was seen on 15% to 30% NaCl concentration, 0.1% to 1.5% substrate concentration, pH 5 to 9 and temperature 28°C and 37°C.

Protease production was observed by growing the culture in NH medium with 15%, 20% and 25% NaCl liquid medium. Following which growth and pigment production was found to be highest in 25% NaCl succeeded by 20% and 15% NaCl. The enzyme was partially purified and its presence was confirmed by inoculating on agar well plates consisting of skimmed milk. Maximum zone of clearance was seen on plates containing 25% NaCl concentration followed by 20% and15% NaCl concentration. Enzyme activity was examined by performing protease assay according to Kunitz, 1947. At 25% NaCl concentration, highest enzyme activity was observed. SDS-PAGE and zymography was also carried out and protease activity was detected by the appearance of clear zone against the blue-black background.

Both the halophilic isolates were partially characterised based on morphological characteristics and pigment production. After conducting SEM analysis, the cells were found to be pleomorphic (one cell different from another) in shape. Thus, proving that both the isolates were not bacterial cultures. When streaked on NH medium supplemented with penicillin, GUYHP1 was able to grow therefore indicating antibiotic resistance which is a typical feature of Archaea. However, GUYHR1 was not able to grow in presence of penicillin, therefore it may be sensitive to it.

To conclude, GUYHP1 was found to be an extremely halophilic archaeal isolate obtained from the saltpans of Goa and was exhibiting protease activity at high NaCl concentration of 25% justifying that it is an industrially useful biocatalyst.

Future Prospects

Future Prospects:

1) 16s rRNA gene sequencing of haloarchaeal isolates GUYHP1 and GUYHR1 and phylogenetic tree construction.
2) Analysing the inhibitory effect of chemical reagents on protease activity along with studies on its industrial application.
3) Optimization of parameters like temperature, salt and pH for other enzymes produced by GUYHP1 with detailed characterisation.
4) Purification and molecular weight determination by SDS-PAGE of gelatinase enzyme produced by GUYHR1 could be studied.

Chapter V

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Chapter VI

Appendix

Appendix I: Composition of media

1) RV media

Ingredients	g/L
NaCl	234.0
KCl	6.0
$MgSO_4.7H_2O$	29.0
$MgCl_2.6H_2O$	19.5
$CaCl_2.6H_2O$	1.1
NaBr	0.8
$NaHCO_3$	0.2
Yeast Extract	5
Agar	20.0

2) NH media

Ingredients	g/L
NaCl	200.0
KCl	5.0
$MgSO_4.6H_2O$	10.0
$CaCl_2.2H_2O$	0.2
Yeast Extract	1.0
Agar	18.0

3) EHM media

Ingredients	g/L
NaCl	250.0
KCl	2.0
$MgSO_4.7H_2O$	20.0
$CaCl_2.6H_2O$	0.36
NaBr	0.23
$NaHCO_3$	0.06
Yeast Extract	10.0
Peptone	5.0
$FeCl_3.6H_2O$	0.005
Agar	20.0

4) NTYE media

Ingredients	g/L
NaCl	250.0
$MgSO_4.7H_2O$	20.0
KCl	5.0
Yeast Extract	3.0
Tryptone	5.0
Agar	20.0

5) NT media

Ingredients	g/L
NaCl	250.0
$MgSO_4.7H_2O$	20.0
KCl	2.0
Yeast Extract	10.0
Trisodium Citrate	3.0
Agar	20.0

6) MHM media

Ingredients	g/L
NaCl	178.0
KCl	2.0
$MgSO_4.7H_2O$	1.0
$CaCl_2.6H_2O$	0.36
NaBr	0.23
$NaHCO_3$	0.06
Yeast Extract	10.0
Peptone	5.0
$FeCl_3.6H_2O$	0.005
Agar	20.0

7) Tomlinson media

Ingredients	g/L
NaCl	250.0
MgCl2.6H2O	20.0
KCl	6.0
Yeast Extract	5.0
Peptone	5.0
$CaCl_2.6H_2O$	0.2
Agar	20.0

Note: Media was sterilized by autoclaving at 15 lbs pressure (121℃) for 15 minutes

Appendix II: Stains and Reagents

1) I_2 reagent

I_2	0.3%
KI	0.6%

2) Congo Red (0.1%)

Congo Red	0.1g
Distilled Water	100 mL

3) Mercuric Chloride (15%)

Mercuric Chloride	15 g
Conc. HCl	20 mL
Distilled Water	80 mL

4) NaCl (15%)

NaCl	15 g
Distilled Water	100 mL

5) 5 M NaOH

NaOH pellets	10 g
Distilled Water	100 mL

6) 0.1 M Potassium Phosphate buffer (KPO_4)

A) K_2HPO_4 (1M) - 17.418 g in 100 mL distilled water
B) KH_2PO_4 (1M) - 13.609 g in 100 mL distilled water
71.7 mL of A + 28.3 m L of B = 0.1 M (KPO_4) buffer
pH – 7.2

7) Crystal Violet

Crystal Violet	2 g
95% ethanol	20 mL
1% Ammonium oxalate	80 mL

8) Gram's Iodine

Gram's Iodine	1 g
KI	1 mL
Distilled water	300 mL

(Store in Amber colour bottle)

9) Decolourizer

Ethanol	95 mL
Distilled water	5 mL

10) 2% Acetic acid

Acetic acid	2 mL
Distilled water	98 mL

For SDS-PAGE

11) Resolving gel buffer (1.5 M)

Tris HCl	18.171 g
Distilled Water	100 mL
pH	8.8

12) Stacking gel buffer (1 M)

Tris HCl	12.114 g
Distilled Water	100 mL
pH	6.8

13) Monomer Solution

Acrylamide	29 g
Bis- acrylamide	1 g
Distilled Water	100 mL

14) Running(tank) buffer (1X)

25 mM Tris base	3.02 g
250 mM Gycine	18.7675 g
0.1% SDS	10 mL
Distilled Water	200 mL

15) SDS (10%)

SDS	5 g
Distilled Water	50 mL

16) Bromophenol Blue (1%)

Bromophenol Blue	0.1 g
Distilled Water	10 mL

17) Ammonium Persulfate (10%)

Ammonium Persulfate	0.1 g
Distilled Water	1 mL

18) TEMED (Tetramethylethylenediamine) 4 µL

19) Sample loading buffer(1X)

Stacking gel buffer	1 mL
200 mM β-mercaptoethanol	280 µL
2% SDS	4 mL

0.2% Bromophenol blue	2 mL
10% Glycerol	2 mL

20) Staining solution

Coomasie brilliant blue	0.25 g
Methanol	45 mL
Glacial acetic acid	10 mL
Distilled Water	45 mL

21) Destaining solution

Methanol	45 mL
Glacial acetic acid	10 mL
Distilled Water	45 mL

For gelatin zymography

22) Staining solution

Amido black 10 B	0.5 g
Methanol	30 mL
Glacial acetic acid	10 mL
Distilled Water	60 mL

23) Destaining solution

Methanol	30 mL
Glacial acetic acid	10 mL
Distilled Water	60 mL

24) Triton X-100 (2.5%)

Triton X-100	1.25 mL
Distilled Water	50 mL

25) 20 mM Tris–HCl buffer

Tris HCl	0.3154 g
Distilled water	100 m L
pH 8	

For Protease Assay

26) 1% Casein Solution

Casein	0.1 g
Distilled water	10 mL

27) 25 mM Tris HCl

Tris HCl	0.395 g
Distilled water	100 mL

28) 0.2 M Sodium Carbonate

Sodium Carbonate	0.4236 g
Distilled water	20 mL

29) 10% TCA

TCA	10 mL
Distilled water	90 m L

30) Folin- Ciocalteu Reagent (1:1)

Folin- Ciocalteu Reagent	10 mL
Distilled water	10 mL

31) Preparation of colloidal chitin

- Weigh 20 g of chitin
- Add 350 mL conc HCl
- Keep at 4℃ overnight
- Mixture added to 2 L ice cold 95% ethanol with rapid stirring
- Keep it overnight at -20℃
- Centrifuge at 5000 rpm for 20 mins at 4℃
- Wash with distilled water until pH is 7
- Dried chitin is used for determining chitinase activity

Appendix III: Estimation Methods

1) Standard Graph for Tyrosine (Folin and Ciocalteu, 1927)

Reagents:

i) 500 mM Sodium Carbonate ($NaCO_3$)
5.295in 100 mL distilled water

ii) Folin- Ciocalteu (1:1)
5 mL Folin- Ciocalteu reagent + 5 mL distilled water

iii) Stock Solution: 0.2 mg/m L of Tyrosine

iv) Diluent: distilled water

Protocol:

0.5 mL of stock solution

↓

1.5 mL diluent

↓

Add 5 mL of Sodium Carbonate

↓

Add 1 mL of Folin- Ciocalteu

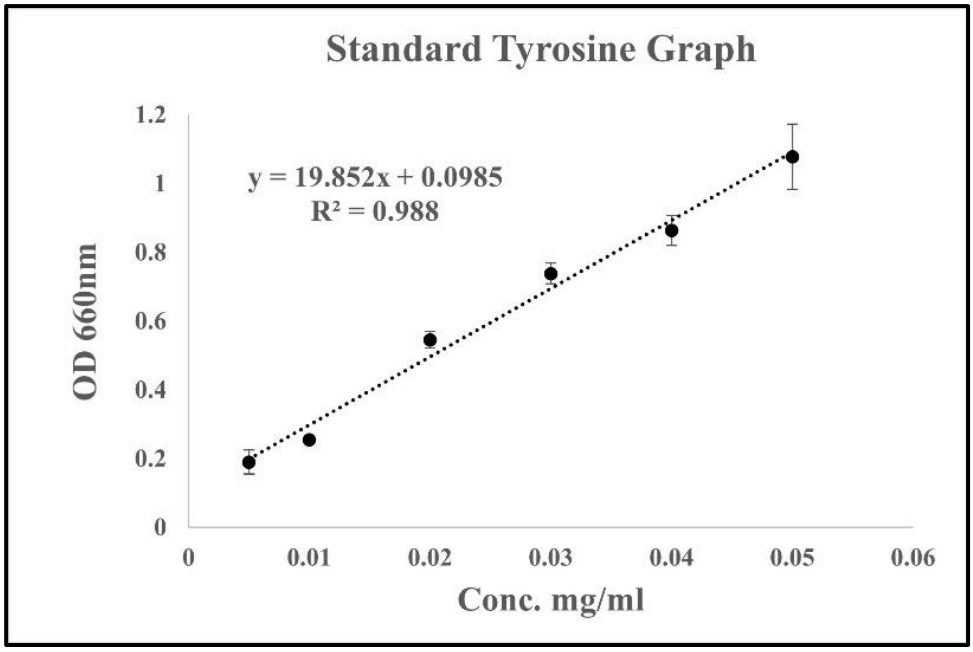
↓

Incubate at 37°C for 30 min

↓

Record absorbance at 660 nm

Standard curve for tyrosine was plotted


Standard Tyrosine Graph
y = 19.852x + 0.0985
R² = 0.988
OD 660nm
1.2
1
0.8
0.6
0.4
0.2
0
0
0.01
0.02
0.03
0.04
0.05
0.06
Conc. mg/ml

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