ISOLATION AND SCREENING OF ENZYMATIC ACTIVITY OF A HALOTOLERANT BLACK YEAST ISOLATED FROM A SALTERN

A Dissertation

Course code and Course Title: MID – Dissertation

Credits: 08

Submitted in partial fulfilment of Master's Degree

Microbiology

by

MS. SIDDHI DEELIP NADODKAR

Roll Number: 21P042014

Under the Supervision of / Mentor

DR. BHAKTI SALGAONKAR

School of biological sciences and biotechnology
M.Sc. Microbiology

Goa University

Date: **April 2023**

Microbiology Programme
School of Biological Sciences & Biotechnology
Goa University, Science Block E,
Taleigao Plateau, Goa - 403206

Examined by:

Seal of the School

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation / Internship report entitled, **"Isolation and screening of enzymatic activity of a halotolerant black yeast isolated from a saltern"** is based on the results of investigations carried out by me in the M.Sc. Microbiology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of Dr. Bhakti 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.

I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Ms. Siddhi Deelip Nadodkar
21P042014
Microbiology programme
School of biological sciences and biotechnology

Date: 17/04/23

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation / internship report **"Isolation and screening of enzymatic activity of a halotolerant black yeast isolated from a saltern"** is a bonafide work carried out by **Ms. Siddhi Deelip Nadodkar** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of **Master in Science** in the Discipline Microbiology at the School of biological Sciences and biotechnology, Goa University.

Dr. Bhakti Salgaonlar
Microbiology programme

Date: 17/09/23

Microbiology Programme
School of Biological Sciences & Biotechnology
Goa University, Science Block E,
Taleigao Plateau, Goa - 403206
School Stamp

Dr. Savita Kerkar
Biotechnology Programme
School of biological sciences and biotechnology
Date: 3/5/23
Place: Goa University

Dean of School of Biological Sciences
& Biotechnology
Goa University, Goa-403206
Office Ph. 8669609246

Acknowledgement

It is my pleasure to convey my gratitude to all those who supported and helped me in many ways during the completion of this dissertation.

I am greatly indebted to my guide ***Dr. Bhakti Salgaonkar****, Assistant professor, microbiology programme, School of biological sciences and Biotechnology, Goa University for her guidance, valuable suggestions, constructive criticism, patience, help and constant encouragement. Her scientific experience as well as effective writing skills and suggestions have influenced me throughout the successful completion of this research investigation.*

I am thankful to ***Dr. Lakshangy Charya****, Programme director of microbiology programme, for providing the infrastructure.*

*I am thankful to P****rof. Sandeep Garg, Dr. Milind Naik, Dr. Trupti Asolkar, Dr. Judith Noronha*** *and* ***Dr. Lata Gawade****, the residing faculty members of Microbiology Programme for their timely help and support.*

With the great sense of gratitude, I would like to thank Research scholars ***Ms. Gandisha Pawar*** *and* ***Mr. Tejas Naik*** *for their constant motivation and help throughout my dissertation period. I would also like to thank my friend* ***Ms. Yuvrani Halarnkar*** *for her support.*

My special thanks to ***Mr. Domingos Dias, Mr. Surendra Velip, Mr. Bhagwant Karpe, Mrs. Robertina Fernandes*** *and the non- teaching staff, Microbiology Programme for their assistance and timely support during the course of this study.*

I thank ***Dr. Durga Prasad Muvva*** *, for his help with the Scanning Electron Microscope at Central Sophisticated Instrumentation Facility, BITS Pillani -Goa.*

Lastly, I thank and dedicate my work to my ***parents*** *who constantly encouraged and motivated me throughout my dissertation period, it wouldn't have been possible without their love and support.*

Ms. Siddhi Deelip Nadodkar

21P042014

Contents

Table of Contents

Abstract

Halophiles are organisms which thrive in high salinity environment like salt pans, salt marshes, salt lakes, saline soils. Apart from high salinity these organisms are found to adapt and actively perform their cellular metabolism in other extreme conditions such as low water activity, high temperature, low oxygen and high radiations. Enzymes produced by such organisms are gaining more industrial importance due to its ability to withstand harsh conditions. In the present study a halotolerant black yeast was isolated from Nerul salt pan. The isolate was examined for its growth on various fungal media showing best growth on Malt extract agar (MEA) medium amongst all the fungal media tested. The culture showed its halotolerant nature when grown on MEA plates containing 0-30%NaCl concentration, as it was able to grow on 0% as well as on media containing upto 25%NaCl concentration showing best growth on 5% NaCl concentration. Screening for the production of extracellular hydrolytic enzymes such as amylase, protease, lipase, esterase, xylanase, cellulase, pectinase, chitinase, phosphatase and gelatinase was carried out. The isolate showed best enzymatic activity for esterase, amylase, pectinase, protease and xylanase enzymes. the study was focused on esterase enzyme as it showed best enzyme activity within less incubation time as compared to other enzymes. The growth optimum studies of esterase enzyme production showed that the production of esterase enzyme was highest at 5% and 10% NaCl concentration showing immense turbidity around the growth of the organism at room temperature which was its preferable enzyme production temperature. Studies on esterase enzyme extracted from the GUSNBS isolate was carried out by partially purifying the enzyme and characterising it using Native PAGE and zymogram analysis. The enzymatic activity of the esterase enzyme was determined by performing enzymatic assay using pNP- acetate as a substrate. The pigment extraction and characterisation using UV Visible spectroscopy and TLC revealed that the pigment was melanin.

List of Tables

List of Figures

List of Abbreviations and Symbols

A_W	Water Activity
µL	Microlitre
mL	Millilitre
α	Alpha
β	Beta
kDa	Kilodalton
°C	Degree Celsius
TM	Tomlinson's Media
MEA	Malt extract Agar
MEB	Malt extract broth
PDA	Potato Dextrose Agar
NH	Norberg- Hofstein
pNPP	para- Nitrophenyl phosphate
pNPA	para-Nitrophenyl acetate
w/v	weight/volume
v/v	volume/volume
CMC	Carboxymethyl cellulose
CFS	Cell free supernatant
m g	Milligram
%	Percent
n m	Nanometer

Chapter I

Introduction and Literature Review

1. Introduction and literature review

1.1 Halophilic microorganisms

High salinity is generally considered as hostile to most of the microorganisms therefore salt is been widely used as a food preservative. Naturally hypersaline environments such as salt lakes, solar salterns, saline soils are widely distributed globally having salinity much higher than that of sea water and almost equal to saturation (more than 30% NaCl concentration) in such harsh conditions also some microorganisms are found flourishing. These microorganisms which can grow in high salinity are termed as Halophiles or salt loving microorganisms. Halophilic microorganisms are not only exposed to high salinity but are also exposed to harmful UV radiations, fluctuating temperatures, pH, and low water availability (*aw*). Inspite of such extreme conditions these microorganisms thrive and actively perform their cellular and metabolic processes (Ventosa et al., 2007)

1.2 Classification of halophilic microorganism

Halophilic microorganisms are classified into 4 categories based on requirement of NaCl for its survival and growth (Kushner and kamekura, 1998).

Table 1.1: Categories of halophilic microorganisms based on their optimum salt requirement

Categories	NaCl requirement Percentage (%)	NaCl requirement Molarity (M)
Slightly halophilic	1 – 3%	0.2 – 0.5M
Moderately halophilic	3 – 15%	0.5 – 2.5M
Extremely halophilic	15 – 30%	Above 2.5 – 5.2
Halotolerant	These are class of microorganisms which does not require salt for its growth but are able to survive in wide range of salinity	

% - percentage, M - molar

1.3. Adaptive strategies

The halophilic microorganisms survive the high salinity stress and its related osmotic and ionic stress. These habitats are characterised by low water index, poor nutrition, which may hamper their metabolism and cell structure. In order to overcome the harsh conditions, microorganisms have implemented various adaptative strategies.

i. **Salt in Strategy** – this is a mechanism in which intracellular salt concentration is maintained equal to the extracellular salt concentration. Osmotic balance is maintained by accumulating high concentration of inorganic ions inside the cell. Potassium and chloride ions are mostly used in raising the salt concentration of the cytoplasm which is absorbed inside the cytoplasm by bacteriorhodopsin and ATP synthase enzyme with the help of transporters (Gunde et al., 2018).

ii. **Salt Out Strategy** – this is also known as compatible solute strategy. In this strategy organic solutes are used to maintain the osmotic balance inside the cell. These osmoprotectants does not affect the activity of the enzyme. Examples include polyols, sugars, glycerol, ectoine etc (corral et al., 2020).

Categories of organic osmolytes (Kanekar et al., 2012)

- Zwitterionic solutes – betaine, ectoine
- Non chargeable solutes – sucrose, trehalose
- Anionic solutes – β- glutamate, hydroxybutyrate

iii. **Photoprotective Pigments –** Halophilic microorganisms produce pigments like carotenoid and melanin which has the ability to screen out the detrimental UV radiations and provides protection against effect of harmful sunlight (Galasso et al., 2017).

1.4. Microorganisms in hypersaline environment

Hypersaline environments are inhabited by microorganisms belonging to all 3 domains of life.

Bacteria: *Halobacillus halophilus*, *Halobacterium karanjensis*

Archaea: *Halobacterium salinarum, Haloferax volcanii*

Eukaryotes: *Hortaea werneckii, Dunaliella salina, Wallemia*

1.4.1. Eukaryotes in Hypersaline Regions

The hypersaline environments were considered to be inhabited by only prokaryotes for a long period of time (Oren, 2002) until 2000, when the first report of the isolation of fungi from solar salterns was reported (Gunde-cimerman et al.,2000). Most of the halophilic fungi are halotolerant in nature, i.e., they do not require NaCl for their growth but are able to survive in wide range of salt concentrations (Plemenitas et al., 2014). The main group of fungi which are considered to be model halophilic and halotolerant organisms include yeast like fungi *Hortaea werneckii, Wallemia ichthyophaga, Debaryomyces hansenii, Aureobasidum pullans,* and *Trimmatostroma salinum* (Gunde-Cimerman et al.,2000)

1.5. Pigment Producing eukaryotes in Saline Regions

Pigments serve as an important adaptive strategy in halophilic microorganisms to tackle the harsh conditions in which halophiles thrive. Pigments protect the microorganism against harmful UV radiations, extreme temperatures, and osmotic stress. Most halophilic microorganisms are found to secrete carotenoid pigment as a stress response to high salinity. But only few halophilic eukaryotes such as *Dunaliella salina* and *Fusarium sp*. (Sakaki et al.,2002) secret carotenoid pigments. However certain rare pigment like blue pigment is found to be secreted by a halophilic fungal species *Periconia* (Cantrell et al., 2006). A yellow

coloured quinone compound is found in *Aspergillus Variecolor* (Wang et al., 2007) but major pigment secreted by halophilic eukaryotes such as yeast and filamentous fungi is melanin.

1.6. Melanin pigment from halophilic yeast

Melanin is a class of high molecular weight metabolites which is synthesised from indolic or phenolic compounds. It is a negatively charged hydrophobic, polymer of 5,6 – indolequinone or 5,6 dihydroxy indole carboxylic acid (Rani et al., 2013). *Horteae werneckii* synthesise DHN (dihydroxynaphthalene) melanin, which can be synthesised under both saline as well as non-saline conditions, but in spite of this, the melanin granules distributed on the outer part of the cell of *Hortaea* are found to be salt based, which leads to reduction of the pore size in the cell wall, thus minimizing the permeability for the main compatible solute glycerol. This mechanism plays a fundamental role in salt tolerance characteristic of black yeast, allowing it to withstand extreme conditions. It possesses protection against oxidative stress, toxic effect of heavy metal and UV rays which can damage human internal tissues. Melanin is found to be medically important, as it shows high antibacterial activity against harmful pathogens like *S. typhi, V. parahaemolyticus,* and *K. pneumonia*. It also possesses anti-inflammatory properties and high immunogenic activity. Due to its heavy metal binding capacity, it is used in bioremediation. Vast range of photoprotective creams, cosmetics and other pharmaceutical products have been produced using melanin, due to its ability to absorb broad spectrum electromagnetic radiations. Extensive applications of melanin around different sectors have led to the growing demand for production of the pigment and microorganisms are often considered as the safe options as they are eco-friendly in nature (Elsayis et al., 2022)

1.7. Enzyme from halophilic yeast

The halophilic enzymes are negatively charged containing more acidic amino acids and have a smaller number of hydrophobic residues on their surface. Due to this, their proteins are able to maintain a functional conformation, reduce surface hydrophobicity and prevent aggregation at the high salt concentration (Slizewska et al., 2022).

The enzymes obtained from halophilic filamentous fungi have gained a lot of potential in industries due to their ability to withstand unfavourable conditions such as:

i) High salt concentration

ii) Presence of organic solvents which normally affect the enzyme activity

iii) Stability and enzyme activity at low water activity

iv) Polythermophilicity

The halophilic eukaryotes mostly produce extracellular enzymes which are easier and efficient to extract in industrial conditions compared to halophilic bacterial enzymes.

Table 1.2: Applications of enzymes produced by halophilic eukaryotes

Enzyme	Halophilic fungi	Application	Reference
A) Food and feed industry			
Protease	*Hortaea werneckii*	Fish sauce production	(Ali et al.,2014)
Pectinase	*Penicillum sp., Aspergillus sp.,*	Processing of fruit juices	(Kutateladze et al, 2009)
β- glucosidase	*Aspergillus sydowii, Phaeothecatriangularis*	Flavour enhancement	(Madhu et al., 2009)
B) Textile industry			
α- amylase	*Trimatostroma salinum*	Detergents	(Primozic et al.,2019)

Protease	*Aureobasidium pullulans*	Detergents	(chi et al., 2007)
Protease	*Aureobasidium pullulans*	Leather tanning	(chi et al., 2007)
C) Paper and pulp industry			
Xylanase	*Halosarpheia fibrosa*	Pulp bleaching in paper production.	(Beg et al., 2001)
D) Renewable energy source			
Cellulase	*Aspergilus flavus, Aspergilus retricus*	Bioethanol production	(Ali et al.,2014)
Xylanase	*Aspergilus gracilis*	Bioethanol production	(Ali et al.,2014)
E) Ecology			
Lipase	*Aspergillus restricus*	bioremediation	(Ali et al.,2014)
Laccase	*Flavodon flavus*	Waste water purification	(Mtui et al., 2008)

1.8. Extracellular hydrolytic enzymes

Extracellular enzymes secreted by microorganisms act as biological catalysts by carrying out the hydrolysis of the chemical bonds in the substrates and thus speeding up biochemical reactions in living organisms. Enzymes have gained commercial importance as they can be extracted from the cell and used to catalyse various industrially important processes.

1.8.1 Amylase (α 1,4- glucanohydrolase)

Amylases are enzymes which hydrolyse the glycosidic bonds in starch molecules, thus converting complex polymers to simple carbohydrates such as disaccharide, maltose, and monosaccharide glucose. Starch is a homopolymer of D-glucose units comprising of two subunits, amylose and amylopectin.

i) Amylose – it is a linear polymer of glucopyranose linked by α-1,4 glycosidic bond.

ii) Amylopectin – it is a branched subunit of starch, having linear glucose residues linked by α-1,4 glycosidic bonds and are branched with α-1,6 glycosidic linkage at every 17- 26 residue (Sundarram and Murthy, 2014).

1.8.1 Cellulase (β-1,4-glucanase)

Cellulose is a homopolymer of glucose units linked together by β-1,4 glycosidic linkages. It is widely distributed around the Earth as it an important structural component of plant cell wall as well as in many forms of algae and oomycetes. The linkages make it compact, crystalline and insoluble therefore making is less susceptible to hydrolysis. Cellulases are complex hydrolytic enzymes which hydrolyses the β-1,4 glycosidic linkages between glucose units. It is industrially important enzyme playing a major role in paper and pulp industry (Rohban et al., 2009).

1.8.3 Protease (Peptidases)

Proteases are the enzyme which catalyses the hydrolysis of peptide linkages found in proteins thus breaking them down into smaller polypeptides or single amino acids. These enzymes are also known as peptidase as it cleaves peptide bonds. Most of the proteases cleave α peptide bonds between the amino acids. They have a wide variety of industrial applications such as in detergent industry, pharmaceutical, leather industry, food industry etc. (Akolkar and Desai, 2010)

1.8.4 Xylanases (endo-1,4, β- xylanase)

Xylan is a structural polysaccharide found in cell wall of land plants constituting 30% of its dry weight. It is a homopolymer made out of β-D-xylopyranose units linked by 1,4 linkages and contains shorts side chains of O-acetyl, α-L-arabinofuranosyl and D-glucouronyl or O-methyl-D-glucuronyl residues. Xylanases catalyses the conversion of Xylan to D-xylose by randomly cleaving the β-1,4 linkages between the xylopyranosyl residues in xylan backbone (Collins et al.,2005)

1.8.5 Gelatinase

Gelatinase is a subtype of enzyme protease known as metallopeptidase which hydrolysis the gelatin and other compunds such as collagen, casein and fibrinogen into smaller peptides or individual amino acids. It is found to be useful in treating poultry and animal waste and also has medical applications. (Hamza, et al., 2006)

1.8.6 Lipases

Lipases are hydrolytic enzymes which acts mainly on water soluble substrates like triglycerides having long chain fatty acids. The hydrolysis of triacylglycerol to glycerol and other small free fatty acids is catalysed by the lipase enzyme. They are mainly used in food, detergent, paper and pulp industry (Hasan, et al., 2006).

1.8.7 Esterase

Esterases hydrolysis the ester bonds into acid and alcohol and are involved in the breakdown of short chain fatty acid triglycerides like tributyrin, ethyl acetate unlike lipases which hydrolyses long chain fatty acids. They have wide variety of application in food industry, pharmaceutical industry and waste water treatment (Dahiya et al.,2022).

1.8.8 Chitinase

Chitin is a polymer of unbranched chains of N- acetylglucosamine sugar linked by β-1,4 glycosidic bonds. This is one of the largest renewable biomass. Chitinase enzyme hydrolyses the β-1,4 glycosidic linkages of chitin to free N-acetylglucosamine residues. Chitinase enzyme hydrolyses the chitin cell wall of insects and is therefore used as insecticide to control the pest (Patil et al., 2015).

1.8.9 Pectinase

Pectin is complex polysaccharide made up of polygalacturonic acid mostly present in the cell wall of higher plants. Pectinases are group of enzymes which degrade the pectin to give free galacturonic acid residues. These are classified based on their mode of action: pectin esterase, pectin hydrolases, and pectin lyases. This enzyme has a wide variety of commercial application such as in fruit and vegetable juice clarification, oil extraction, pulp and paper industry, waste water treatment etc (Sharma et al., 2013).

1.9 Studies on Esterase

Esterases are group of enzymes which catalyses the breakdown of ester bonds leading to formation of acid and alcohol. It preferentially degrades water soluble short chain fatty acid esters. The enzyme acts as an important biocatalyst for biotechnological applications by improving nutraceutical properties of food and flavour enhancement. These enzymes are produced by organisms around all 3 domains of life i.e., Bacteria, Archaea and Eukarya. Certain esterases produced from extremophiles such as thermophiles, halophiles and pyschrophiles have unique properties due to its ability to withstand extreme conditions and therefore have been used in industrial processes which takes place in harsh conditions.

In pharmaceutical industries esterase enzymes are specifically used in the production of optically active pure products. This enzyme acts as a biocatalyst in transesterification, esterification and acidolysis reaction (Dahiya et al., 2022)

1. Transesterification

   R1-COO-R2 + R3-COO-R4 ⟶ R1-COO-R4 + R3-COO-R2

2. Esterification

   R1-COOH + R2-OH ⟶ R1-COO-R2 + H2O

3. ACIDOLYSIS

   R1-COO-R2 +R3-COOH ⟶ R3-COO-R2 + R1-COOH

1.9.1 Sources of esterase

Esterase enzymes are produced by members of all 3 domains of life i.e., Bacteria, Archaea and Eukarya. Esterases produced by microbial sources are gaining more attention due to its low cost of growth and maintenance and ease of genetic modification to get better enzyme yield and specificity. Certain strains of esterase producers are isolated from cheese surfaces (Gandolfi et al., 2000), garbage area containing oil and marine squids (Ranjitha et al., 2009).

1.9.2 Applications of microbially produced Esterase enzyme

i) Pharmaceutical industry

Esterase from *Trichosporon brassicae* is used in the production of a drug Ketoprofen which helps in the reduction of pain and inflammation of arthritis, sunburn and fever. It is also found to relief menstrual pain (Shen et al., 2002)

A commercially available anti- inflammatory drug known as ibuprofen is produced using esterase enzyme extracted from *Pseudomonas sp*. (Kim et al.,2002)

ii) Agriculture industry

The insecticides and nematicides used in the fields contain certain organophosphorus compounds which are found to affect the environment by causing toxicity. Phosphotriesterases from *Alteromonas sp.* are used extensively in detoxifying and degrading such compounds (Horne et al., 2002)

ii) Food and Dairy Industry

Esterases are used in production of wine, beer, fruit juices and alcohol. It is used as catalysts in trans- esterification in order to transform low value fats and oils into more valuable ones.

Esterase has wide applications in dairy industry in enhancing the flavour and aroma of the product. Esterase from *Lactobacillus casei* is used in hydrolysis of milk fat for the purpose of cheese flavour enhancement (Choi and Lee 2001)

iii) Paper and pulp industry

The wood which contains a white sticky liquid made up of triglycerides known as pitch. This affects the machine in the paper industry and also possesses negative effect on the quality of paper. Esterases are therefore used to reduce the pitch in paper industries (Kontkanen et al., 2004).

iv) Detergent and textile industry

The esterase enzyme is added in detergents inorder to clean the leather and helps in bleaching of the fabric (Maugard et al., 2002)

1.10 Report on the study on isolation of halophilic black yeast and characterisation of microbially produced esterase enzymes

Black yeast is a highly melanised halophilic microorganism and is generally considered as the model organism to study the halotolerance in eukaryotes.

Black yeast *H. werneckii* is reported as a causative agent of a superficial mycosis called Tinea nigra. Studies on patients affected with Tinea was carried out by Bonifaz et al (2008) in Mexico which showed that the pathogen affected the palms and soles of humans.

A new strain of black yeast isolated from the Egyptian off shore salt marshes was molecularly identified as *H.werneckii* by ITS1 AND ITS2 gene sequencing by elsayed et al., (2016)

Cabanes et al., (2012) isolated black yeast strain in spain from scuba diving equipments such as from the snorkel and mouthpieces.

According to the reports Hodhod et al., (2020), identified halophilic black yeast strain from intertidal decayed leaves of *Avicennia marina* found on red sea coast of Saudi Arabia

A study was conducted on acute mycosis tinea nigra by Gunde et al., (2002) wherein the yeast culture was isolated from the splenic abscess of two patients suffering from myelomonocytic leukaemia.

According to Gunde et al., it was reported that black yeast *Hortaea werneckii* is polymorphic in nature showing yeast like morphology and then undergoes budding, showing bipolar budding and finally forming pseudomycelial structure.

Chen et al., (2012) isolated black yeast from mangrove plant in *Aegiceras comculatum* and identified it as *H. werneckii* using rDNA sequencing technique. The isolate grown on malt agar and PDA, showed yeast like cells with septate hyphae having bicellular blastospore.

The black yeast isolated by Formoso et al., (2015) from brazil showed amylase, lipase, esterase, and pectinase activity. And was able to grow upto 30% salt concentration.

The sugar utilisation studies reported by Gunde et al., (2000) of *H.werneckii* showed positive utilisation test for glucose, galactose, xylose, arabinose, sucrose, maltose, trehalose and lactose.

Zalar et al., (2019) extensively studied the black yeast and also studied its enzymatic activity which showed positive esterase enzymatic activity.

Halophilic black yeast isolated by Zalar et al., (2002) from the wood immersed in solar salterns of Slovenia, were checked for its enzymatic activity in different NaCl concentration i.e., at 5%, 10% and 17%NaCl concentration. The isolate was reported amylase, esterase, β glucosidase, laccase, lipase, protease and xylanase positive. And the best enzyme activity was found at 5% NaCl concentration.

Esterase enzyme extracted by Meneses et al, (2021) from Aureobasidium pullulans exhibited a molar mass of 50kDa and was found stable at neutral pH and temperature below 30°C. p-nitrophenylcaprylate and p-nitrophenylbutyrate was used as substrate to study enzyme kinetics. SDS- PAGE was Native PAGE was performed and protein was identified based on zymographic technique using chromogenic agar technique.

Zhang et al., (2021) developed a method to detect lipase and esterase activity using zymogram analysis Chromogenic plates containing 1% tributyrin substrate and 0.1% phenol red indicator were spot inoculated with enzyme solution to detect the fatty acid release which decreases the pH of the media hence the phenol red indicator turns yellow. Native PAGE was

performed and the gel was then overlayed with chromogenic agar within 15 days of incubation the activity of the enzyme was detected as yellow colour band.

Table 1.3: Comparative list of reports on esterase producing halophilic eukaryotes.

Sr. No.	Halotolerant eukaryotic microorganism	Place and source of isolation	Isolation media	Enzyme	Reference
1.	*Hortaea werneckii*	Salt marshes, brazil	PDA	Esterase	Formoso et al.,2015
2.	*Wallemia sp.*	Soil of great Sebkha of oran	PDA	Esterase	Chamekh et al.2017
3.	*Penicillium crysogenum*	Meditarrean sea	PDA	Esterase	Boucherit et al., 2022
4.	*Aspergillus caesiellus*	Salt crystals United states of America	PDA	Esterase	Batista- Garcia et al., 2014.
5.	*Hortaea werneckii*	Wood immersed in hypersaline water in Slovenia	ND	Esterase	Zalar et al., 2005
6.	*Trimmatostroma salinum*	Wood immersed in hypersaline water in Slovenia	ND	Esterase	Zalar et al., 2005

1.11. Gaps in the Existing Research

Hydrolytic enzymes are widely derived from plants, animals, bacteria and fungi. But The microbially derived enzymes are more preferred due to its low cost of cultivation and easy manipulation. An extensive study has been done on enzymes produced by microorganisms. The enzymes produced by extremophiles due to its ability to withstand harsh conditions are gaining more industrial importance. Esterase enzymes have wide applications in various field, but these enzymes derived from halotolerant eukaryotes are not much studied and used for industrial purpose. Since halotolerant microorganisms can survive in normal as well as in high salinity conditions Esterase enzyme derived from them are adapted to high salinity conditions and therefore are functional in normal as well high salt concentration. Studying and exploring the enzymes produced from halotolerant eukaryotes with more efficient environmental, industrial and biotechnological applications may lead to discovery of novel halotolerant enzymes.

1.12. Objectives:

1. Isolation and screening of halophilic microorganisms from hypersaline region of Goa for extracellular hydrolytic enzymes
2. Partial characterization of the potential enzyme producing isolate and parameter optimization for growth and enzymes production.
3. Partial purification and characterisation of esterase enzyme.

Chapter II

Materials and Methods

2. Materials and Methods

2.1. Sampling site and sample collection

Halophilic microorganisms thrive in areas having high salinity like salt pan, salt marshes, saline soil etc. In order to isolate halophiles brine samples and sediment samples were collected from a salt pan located at Nerul, Goa. The sediment samples were collected in zip-lock bag while the brine samples were collected in clean bottle. The samples were transported to the lab and were immediately spread plated on the halophilic media.

2.2. Isolation and purification of the halophilic isolate

2.2.1. Media preparation for isolation of halophile

The sample was isolated on an extremely halophilic media known as Tomlinson's medium (TM) having composition (g/L) NaCl – 250.0, KCl -6.0, $MgCl_2.6H_2O$– 20.0, $CaCl_2.6H_20$ – 0.2, yeast extract -5.0, peptone – 5.0 (Tomlinson and Hochstein, 1972) and the pH of the media was adjusted to 7.0 to 7.5 using 1M NaOH.

2.2.2. Spread plate method

Fifty microliters (50µl) of the brine sample and 10µl of sediment sample (1g suspended in 10ml 15% NaCl) was spread plated on the TM agar plate. The plates were incubated at room temperature. After 7 days of incubation transparent colonies were observed on both sediment as well as on brine sample plates. After 30 days of incubation various types of pigmented colonies were observed on the both sediment as well as brine sample plates.

2.2.3. Isolation and Purification of the Halophilic microorganisms

Amongst the various pigmented colonies obtained on the TM agar, the most prominent one was the one having black pigmentation. The black colony observed on the sediment sample plate was carefully isolated using a sterile nichrome loop and streaked on TM agar plate. The isolate was purified by repeated streaking (quadrant streak) of the colony on TM agar plate.

The purified black isolate was designated as GUSNBS. The pure culture was maintained by periodically subculturing it after every 10-20 days.

2.3 Preparation of starter culture

Starter culture of the black Pigmented isolate GUSNBS was prepared by inoculating a loopful of the purified culture from the TM agar plate in 10ml of the sterile TM broth contained in 25ml capacity Erlenmeyer flask. The tubes were incubated at 37°C on rotatory shaker at 100rpm.

2.4 Determining the growth of GUSNBS isolate on fungal media

The GUSNBS isolate was checked for its growth on different fungal media i.e., on Malt extract Agar (MEA) medium [Appendix I (2)], potato dextrose agar (PDA) medium [Appendix I (3)], Sabouraud's agar medium [Appendix I (4)] and Czapek's agar medium [Appendix I (5)]. The fungal media was supplemented with 18% NaCl so as to retain the salt requirement of halophiles. The purified GUSNBS isolate was streaked via. quadrant streak technique on all the above-mentioned fungal media and incubated for 7 days at room temperature to check which media promotes best growth. The growth was monitored every day.

2.5 Understanding carbohydrate utilization and Growth by GUSNBS

The growth of the GUSNBS isolate was examined on various sugar derivative containing plates. Norberg and Hoefstein (NH) medium [Appendix (6)] was used which consists of (g/l) NaCl- 50.0, MgSO4.6H2O- 10.0, KCl- 5.0, CaCl2.2H2O – 0.2, yeast extract- 1.0. The pH of the medium was adjusted to 7.0 to 7.5 using 1M NaOH. The medium was supplemented with various sugars i.e., Sucrose, Glucose, lactose, trehalose, xylose, galactose, mannose and maltose. Ten percent stock of each sugar derivative was prepared and autoclaved for 10 minutes. One percent the autoclaved sugars were added to autoclaved NH medium which was poured into sterile Petri plates. The GUSNBS isolate was streaked via. quadrant streak

technique on the plates using a sterile nichrome loop and the plates were incubated at room temperature for 7 days and observed for growth.

2.6. Study of the tolerance of GUSNBS to NaCl

2.6.1. Growth study on MEA

The GUSNBS isolate was found to show best growth on malt extract agar medium (MEA) plate therefore malt extract agar medium supplemented with different NaCl concentrations were used to check its salt tolerance activity. The GUSNBS culture grown on MEA plate was streaked via quadrant streaking on MEA agar plates containing 0%, 5%, 10%, 15%, 20%, 25% and 30% NaCl (w/v) concentration. The plates were incubated at room temperature for 7 days and visible growth was monitored on day-to-day basis.

2.6.2. Morphological/Colony characteristics on MEA

The halotolerant yeast isolate GUSNBS was streaked on MEA plates containing 0% 5%, 10%, 15%, 20% NaCl concentrations so as to obtain isolated colonies. The size, shape, margin, pigmentation, consistency, elevation and opacity were determined for colonies at each NaCl concentrations.

2.6.3. Cell characteristics on MEA using microscopy

Monochrome staining was performed of the 7day old culture, grown on MEA plate containing 0%, 5%, 10%, 15% and 20% NaCl (w/v) concentrations. Smear of the culture was prepared on a clean grease free slide. The smear was air dried and later heat fixed. Desalting of the smear was done by washing the slide with 2% acetic acid solution and the smear was later stained with crystal violet for 1 minute. Excess stain was drained and the slide was gently rinsed under running tap water, dried and examined under oil immersion objective (100x) of the microscope (Radical microscope).

2.6.4. Scanning Electron Microscopy (SEM)

Field emission scanning electron microscopic (FESEM) analysis of the halotolerant GUSNBS was carried out to analyse the cell structure of the isolate. The culture grown on Malt extract agar (MEA) medium plate (5% NaCl concentration) was used. Smear of the culture was prepared on a clean grease free coverslip, with the help of a sterile nichrome loop. The smear was air dried and then heat fixed. Desalting of the culture smear grown in presence of salt was done using 2% acetic acid solution to remove excess salt. Two percent glutaraldehyde which acts as a fixative was flooded over the coverslip and was allowed to stand overnight (8-10 hours). The smear was treated with a series of acetone gradient ie. 10%, 30%, 50%, 70%, and 90% for 10 minutes each. The smear was lastly exposed to 100% acetone gradient for 30 minutes. Next, the sample was dried, and was sputter coated with gold particles using leica sputter coater device and then viewed under quanta 250 FEG scanning electron microscope (Das et.al. 2019).

2.7. Growth Curve of GUSNBS Isolate

Based on the NaCl tolerance study, it was observed that the GUSNBS culture grew best at 5% NaCl concentration. Therefore, all our further growth experiments were performed using media containing 5% NaCl. For determining the growth curve, starter culture was prepared by inoculating a loopful of the purified culture in 25ml of MEA contained in 50ml capacity Erlenmeyer flask, followed by incubation at 37°C, 100rpm for 4 days. The 0.5% of the starter culture was inoculated in 500ml sterile Malt extract broth (MEB) supplemented with 5% NaCl. The broth was incubated on shaker at 37°C at 100rpm. The growth was determined by periodically withdrawing 2ml of the sample after every 24 hours and measuring the absorbance as optical density (OD) at 600nm using a UV-Visible spectrophotometer (Analytical technology pvt. Limited UV 2080TS) The OD obtained was plotted against time and the various phases of growth of GUSNBS was determined.

2.8. Chemotaxonomic characterisation of GUSNBS isolate

2.8.1. Carbohydrate utilisation test

The sugar fermentation test for the GUSNBS isolate was carried out using NH medium containing 5% NaCl concentration and phenol red indicator. The medium was supplemented with various carbohydrates like Glucose, sucrose, trehalose, ribose, xylose, lactose, galactose, maltose, and mannose. 10% stock of each carbohydrate was prepared and autoclaved for 10 minutes. To sterile media 0.5% of respective sugar derivatives were added. Inverted Durhams tubes were inserted in the tube containing media in order to check gas production. The tubes were inoculated with 100µl of freshly grown culture from MEB containing 5% NaCl concentration and were thereafter incubated at room temperature. The tubes were observed for colour change and gas production in the Durhams tube.

2.8.2. Catalase test

A drop of culture was added to clean grease free slide under sterile conditions. 2-3 drops of hydrogen peroxide were added to the culture drop, formation of effervescence indicates positive catalase test.

2.8.3. Hanging drop method-motility test

On a clean grease free coverslip Vaseline was applied at the corners with the help of a wooden nichrome loop. A drop of culture was added on the coverslip and to it a drop of saline was added and mixed properly. A cavity slide was put on top of the coverslip and was observed under 40X lens of microscope.

2.9. Screening for extracellular hydrolytic enzymes production by GUSNBS isolate

The extracellular enzyme production by the GUSNBS isolate was screened by growing the culture on minimal medium, i.e., Norberg and Hoefstein (NH) medium. The media consist

of (g/l) NaCl- 50.0, $MgSO_4.6H_2O$- 10.0, KCl- 5.0, $CaCl_2.2H_2O$ – 0.2, yeast extract- 1.0. The pH of the medium was adjusted to 7.0 to 7.5 using 1M NaOH. Additionally various substrates were used as sole sources of carbon. The freshly grown culture from MEA plate containing 5% NaCl was inoculated at the centre of the plates.

2.9.1 *Screening for amylase activity*

The GUSNBS culture was screened for amylase activity using starch hydrolysing technique. 0.5% (w/v) of soluble starch was added as substrate in NH medium agar plate. With the help of a sterile nichrome loop the GUSNBS culture was inoculated at the centre of the plate and incubated for 7 days. The detection of amylolytic activity was carried out by flooding the plate with 0.6% KI solution [Appendix II (1)] after 7 days of incubation zone of clearance around the growth of culture against deep blue background indicated the hydrolysis of starch (Das, et al., 2019).

2.9.2. *Screening for cellulase activity*

The screening of cellulose activity was done by addition of 0.5% (w/v) of carboxy methyl cellulose (CMC) as a substrate in NH medium agar plate. With the help of a sterile nichrome loop the GUSNBS culture was inoculated at the centre of the plate. After 7 days of incubation plates were flooded with 0.1% congo red solution [Appendix (2)] (15 minutes) and destained with 1N NaCl. A clear zone around the growth of culture indicated positive test for cellulase activity.

2.9.3. *Screening for Protease activity*

The screening of Proteolytic activity was detected by addition of 0.5% (w/v) skimmed milk as a substrate in NH medium agar plate. With the help of sterile nichrome loop GUSNBS culture was inoculated at the centre of the plate and incubated for 7 days. A zone of clearance around the culture growth against white background indicated positive protease test.

2.9.4. *Screening for Gelatinase activity*

Assession of Gelatinase activity was performed using 0.5% (w/v) gelatin as a substrate in NH medium agar plate. The GUSNBS isolate was inoculated at the centre of the plate using sterile nichrome loop. After 7 days of incubation the plates were flooded with 15% mercuric chloride acidified with 20%(V/V) concentrated HCl [Appendix II (3)]. Zone of clearance around the growth of culture indicated positive test for gelatinase.

2.9.5. *Screening for Xylanase activity*

The xylanase activity was detected by adding 0.5% (w/v) Xylan beechwood in NH medium, With the help of a sterile nichrome loop the GUSNBS culture was inoculated at the centre of the plate. After 7 days of incubation the plates were flooded with 0.1% congo red solution [Appendix II (2)] for 15 minutes thereafter destaining it with 1N NaCl. Clear zone around the growth of the culture against red background indicated positive xylanase activity.

2.9.6. *Screening for Esterase activity*

The detection of extracellular esterase activity was done using 0.1% (v/v) tween 80 as a substrate in NH medium. The GUSNBS culture was inoculated with the help of sterile nichrome loop at the centre of the plate. Following 7 days of incubation a turbid zone of precipitation around the growth of culture indicated positive esterase activity.

2.9.7. *Screening for Lipase activity*

The extracellular lipolytic activity was screened using 0.1% (v/v) olive oil in NH medium plates. With the help of a sterile nichrome loop the GUSNBS culture was inoculated at the centre of the plate and incubated for 7 days. A turbid zone around the growth of the isolate implies positive test for lipase.

2.9.8. *Screening for Pectinase activity*

The screening for pectinase activity was carried out using 0.5% (w/v) pectin as a substrate in NH medium. With the help of a sterile nichrome loop the GUSNBS culture was inoculated at the centre of the plate. After 7 days of incubation period the plates were flooded with 0.6% (w/v) KI solution [Appendix II (1)]. A zone of clearance around the growth of culture was the indication of positive pectinase test (Sharma et al., 2013).

2.9.9. *Screening for Chitinase activity*

The extracellular chitinase activity was detected by adding 0.5% (w/v) colloidal chitin (appendix II) in NH medium. The GUSNBS culture was inoculated with a sterile nichrome ~~loop~~ at the centre of the plate. After 7 days of incubation 0.1% congo red solution [Appendix II (2)] was poured on the plate followed by destaining it using 1N NaCl solution. Zone of clearance around the growth of culture against red background implies chitinase positive (Reetarani et al.,2002)

2.9.10. *Screening for Phosphatase activity*

The phosphatase activity of the culture was screened using NH medium containing 0.5% (w/v) pNP- phosphate. With the help of a sterile nichrome loop the GUSNBS culture was inoculated at the centre of the plate and incubated for 7 days. Zone of clearance around the culture growth indicated positive phosphatase activity.

2.10. Parameter optimization for growth and esterase production

Ten microlitre (10µl) of the culture was spot inoculated on NH medium containing tween 80. The plates were incubated for 7 to 15 days at room temperature for all the parameters expect temperature. Turbid zone around the growth of colony indicated positive esterase activity. The diameter of zone was directly proportional to the amount of hydrolysis.

2.10.1. NaCl concentrations Optimization for Esterase Activity

The NH medium agar plates containing various concentration of NaCl (w/v), i.e., 0%, 5%, 10%, 15%, 20%, 25% and 30% were prepared and 10µl of freshly grown GUSNBS culture was spot inoculated at the centre of the plate. The NaCl concentration showing the best culture growth and zone of precipitation was determined as optimum NaCl concentration for growth and esterase activity.

2.10.2. Temperature Optimization for Esterase Activity

Ten microlitre (10µl) of GUSNBS culture freshly grown in MEB (5% NaCl) was spot inoculated on NH medium agar plates containing 5% NaCl and 0.5% tween 80. The plates were incubated for 7 days at different temperatures such as 4°C, 28°C and 37°C. The temperature showing the best growth and turbid zone around the culture growth was determined as optimum temperature for growth and esterase activity.

2.11. Growth and Esterase production

Five percent of the actively grown culture in MEB was used as starter culture and was inoculated in 250ml NH medium broth contained in 500ml Erlenmeyer flask. It was supplemented with 0.5% tween 80. The flask was incubated at 37°C at 100 rpm. The growth and enzyme production were determined by periodically withdrawing 2ml of the sample after every 24 hours and measuring the absorbance as optical density (OD) at 600nm using a UV-Visible spectrophotometer (Analytical technology Pvt. Ltd. UV 2080TS).

2.12. Partial Purification of Esterase

One month old culture grown in NH medium broth containing 5% NaCl concentration supplemented with 0.5% tween 80 was used for esterase production studies. The culture was centrifuged at 8,000rpm for 20 mins at 4°C. The pellet was discarded and the cell free

supernatant (CFS) was used for esterase enzyme purification. 80%(v/v) Prechilled ethanol was added slowly to the CFS and kept for 2 hours at 4°C. Another set was kept for 2 days at 4°C. Following which the ethanol-CFS mixture was centrifuged at 8,000rpm for 20 mins at 4°C. The supernatant was discarded and the precipitate was dissolved in 5ml 100mM phosphate buffer.

2.13. Activity of Partially purified esterase enzyme

The activity of the partially purified enzyme was determined using chromogenic agar plate method (Zhang et al.,2021). The medium consists of 0.01% phenol red indicator, 1% tributyrate and 0.1% CaCl2. The pH of the medium was adjusted to 7.3 using 1M NaOH. Wells were punched at the centre of the chromogenic plates using sterile corkborer. The partially purified enzyme from both the sets i.e., set 1 with 2 hours of extraction and set 2 with 2 days of extraction, was checked for its enzyme activity by adding it to the well. Another chromogenic plate was also incubated by adding CFS to the well. The yellow zone around red background indicated positive enzyme activity and diameter of the zone was directly proportional to the amount of enzyme activity.

2.14. Estimation of Enzymatic activity of partially purified esterase enzyme using pNP assay

The esterase enzyme estimation was carried out using para nitrophenol acetate as a substrate as described by Yildirim et al.,2009. The culture grown in NH media containing 0.5% tween 80 was taken in clean centrifuge tube and centrifuged at 8000 rpm for 10 minutes. The CFS was used as the source of enzyme. 10mM para nitrophenol acetate solution substrate was dissolved in ethanol and 50mM phosphate buffer in the ratio of 1: 4: 95 (v/v/v). 100µl of the CFS was added to the 1400µl of substrate solution. The mixture was incubated at 55°C for 15 mins using a water bath (Biotechniques India). After incubation the absorbance was measured

at 410nm using a UV visible spectrophotometer (Analytical technology pvt. Limited UV 2080TS).

2.15. Molecular weight determination of Esterase enzyme

2.15.1 Native Polyacrylamide gel electrophoresis (PAGE)

Native PAGE was performed of the partially purified esterase enzyme. The glass plates of the electrophoretic unit were cleaned with 90% ethanol, and sealed together using insulation tape by placing two vertical spacers in between. 1% agarose solution was prepared and used to seal the bottom and sides of the plates so as to prevent leakage of the gel while casting. 10% Resolving gel [Appendix II (6)] was poured into the gap between the sealed glass plates leaving one fourth of space for stacking gel. The solution was carefully overlayed with isoamyl alcohol using a micropipette to prevent oxygen from diffusing into the gel which affects the polymerisation. Ammonium persulphate (APS) and tetramethylethylenediamine (TEMED) present in the buffer bring about the polymerisation process. Once polymerised the isoamyl alcohol overlay was discarded and the top of the gel was washed with distilled water. 6% stacking gel [Appendix II (7)] was prepared and poured onto the surface of polymerised resolving gel. Immediately a clean comb was inserted into the stacking gel solution in order to form wells. Once the stacking gel polymerised, the comb was carefully removed. The insulation tape was removed and the plates were carefully placed and fixed with the help of screws inside the electrophoretic unit. Tris HCl buffer [Appendix II (9)] was poured in the unit so that the gel along with wells submerge completely in the buffer.

2.15.2. Zymogram analysis.

The marker containing 10µl each of the standard protein solution i.e., amylase (56kDa), Bovine serum albumin (BSA) (66 kDa), pectinase (48 kDa), biotin (244.31 Da) was mixed with

20µl sample loading solution [appendix II (12)] and loaded in the marker wells. 30µl of the partially purified esterase enzyme was mixed 20µl of sampling loading solution and was loaded in the sample lane. The unit was covered with lid. The electrophoretic apparatus was connected to power supply unit which was turned on and the electrophoresis was carried out 100v. The electrophoresis was stopped when the tracking dye reached almost end at the bottom of resolving gel. The gel was carefully removed and was cut into two equal halves wherein one half was stained using staining solution [Appendix II (13)] which was destained [the next day using destaining solution [Appendix II (14)] and the other half of the gel was used for zymogram analysis.

The other half of the Native PAGE gel was transferred in a clean petri plate and washed with distilled water until its pH becomes 7-7.3. thereafter molten chromogenic agar (appendix II) containing 0.1%phenol red indicator and 1%tributyrin substrate was overlayed on the gel and was observed for yellow coloured band. The esterase enzyme breaks the tributyrin substrate and releases fatty acids which decreases the pH due to which the phenol red indicator turns yellow (Singh et.al., 2005).

2.16. Extraction and characterisation of pigment

The black pigment from the GUSNBS isolate, was extracted and purified. 1 month old culture grown in MEB containing 5% salt concentration was centrifuged at 8000rpm for 20 minutes the cell pellet was boiled in distilled water for 5 mins and recentrifuged at 8000rpm for for 20 minutes. The pigment was extracted by autoclaving the obtained pellet with 1M NaOH. It was then acidified to pH 2 using concentrated HCl in order to precipitate the melanin. The obtained precipitate was Washed thrice using distilled water. The extracted pigment was dried overnight in dehumidified conditions (Gadd 1982).

2.16.1. UV Visible Spectrophotometry

The extracted pigment was characterised using UV visible spectrometry. The dried pigment was diluted in 1M NaOH and the spectrum at UV- visible range i.e., 180 to 800nm was taken at room temperature using 1cm pathlength quartz cuvettes.

2.16.2. Thin Layer chromatography

Thin layer chromatography of the extracted black pigment was carried out on Silica plates. The chamber was first saturated with the solvent system consisting of n-butanol: water: acetic acid in the ratio of 95:4:1. The pigment dissolved in 1M NaOH was spot inoculated 1.2cm above the bottom of the silica plate and was allowed to dry, The TLC plate was then placed in the saturated solvent chamber and was allowed to run.

Chapter III

Results and Discussion

Results and Discussion

3.1. Sampling site and sample collection

Halophiles thrive in high salinity environments, therefore in order to isolate halophilic microorganisms, samples were collected from a salt pan of Nerul, Bardez Taluka, North Goa District, Goa (having latitude 15.504928 and longitude 73.791708) which is located along the west coast of India bordering the Arabian Sea. The sample were collected on 21st may 2022 (Figure3.1). Brine samples were collected in clean bottles and sediment samples were collected in zip-lock bag. (Figure 3.2) The pH of the salt pan was found to be neutral and the temperature was 37°C.

Fig.3.1. sampling site: Salt pan located at Nerul, Goa

Figure 3.2: Sediment and brine sample collection at Nerul Salt pan

3.2. Isolation and purification of halotolerant yeast

The samples collected from the salt pan were plated on Tomlinson's media (TM) [Appendix I (1)] having 25% NaCl (w/v) concentration. 50µl of the brine sample and 10µl of sediment sample (diluted in 15% NaCl) was spread plated on above mentioned halophilic media and thereafter incubated at room temperature. Initially after 4 days of incubation transparent colonies were observed on the plates. Eventually after 1 month of incubation pigmented colonies appeared. (Figure 3.3) A black coloured colony on the sediment sample plate was observed (Figure 3.4.A) which was isolated and streaked (quadrant streak) on TM and incubated at room temperature. Growth of the isolate was observed after 7 days of incubation but it was a mixed culture (Figure 3.4.B). The isolate was purified by repeated streaking on TM agar plates (3.4.C.) The isolate was then designated as GUSNBS.

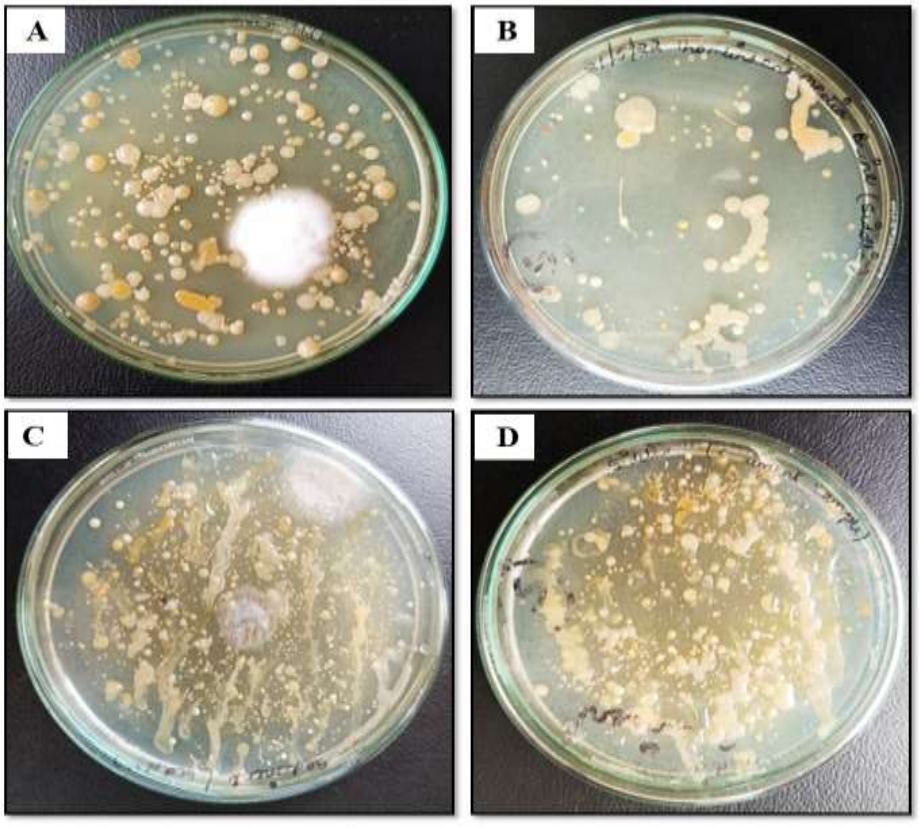


Figure 3.3. Growth of colonies after 1 month of incubation on TM plates inoculated with brine sample (A and B) and sediment sample (C and D).

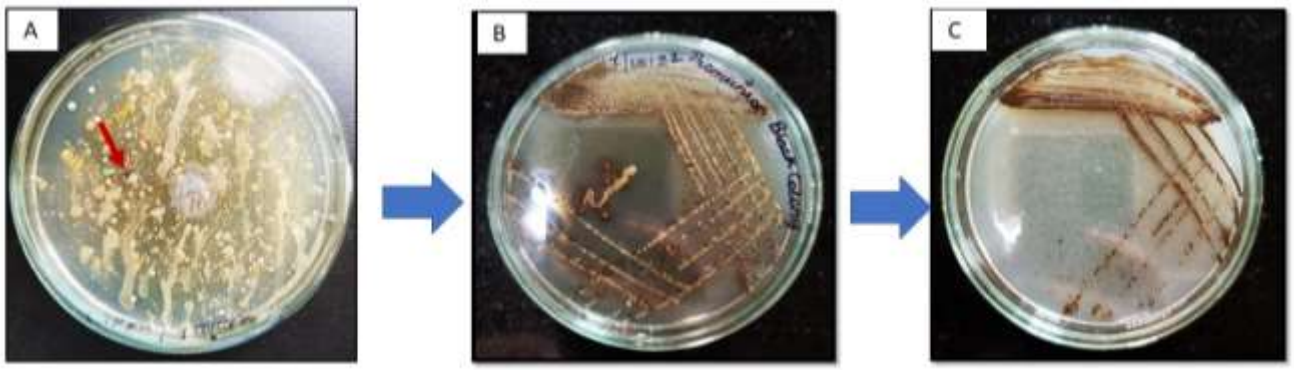


Figure 3.4: Isolation and purification of black coloured colony (A) black coloured colony spotted, (B) isolation of black coloured colony on TM after 7 days of incubation mixed culture obtained, (C) purification of black coloured colony by repeated streaking on TM agar plate.

3.3 Determination of growth of GUSNBS isolate on fungal media

The isolate GUSNBS was examined for its growth on various fungal media i.e., Malt extract agar (MEA), potato dextrose agar (PDA), Czapek's dox agar, Sabouraud's agar, containing 18% NaCl concentration (moderate NaCl concentration). The isolate streaked (quadrant streak method) on the above fungal media showed growth after 7 days of incubation at room temperature. The growth of the isolate was observed on all the mentioned fungal media except on czapek's agar plate, wherein very scanty growth was observed even after 15 days of incubation. while best growth was observed on Malt extract agar plate with bright black pigmented colonies. Therefore, MEA medium was used in the further examination of the isolate (Figure 3.5.).

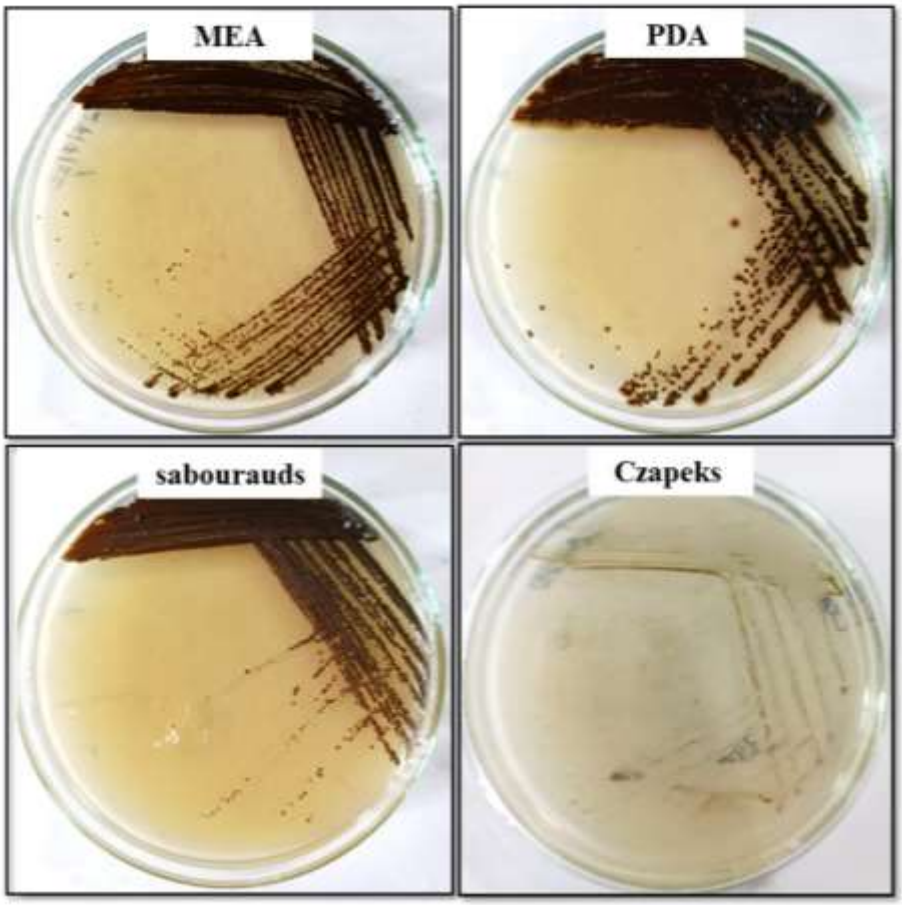


Fig.3.5. Growth of GUSNBS isolate on various fungal media after 7 days of incubation

3.4Understanding carbohydrate utilization and Growth by GUSNBS

It was observed that the growth on Czapek's agar plate was scanty therefore the composition of the media was checked; a striking difference was the carbohydrate source, it was noted that PDA, MEA and Sabouraud's media contained glucose sugar while Czapek's medium contains sucrose. Therefore, the culture was grown on NH medium plates containing different carbohydrate. After 7 days of incubation growth was observed on all the plates containing carbohydrates i.e., Sucrose, Glucose, mannose, lactose, trehalose, galactose, xylose and maltose (figure 3.6) But the growth was less on plates containing sucrose, lactose, galactose plates. Therefore the carbohydrate source is not the reason behind the scanty growth on czapeks medium.

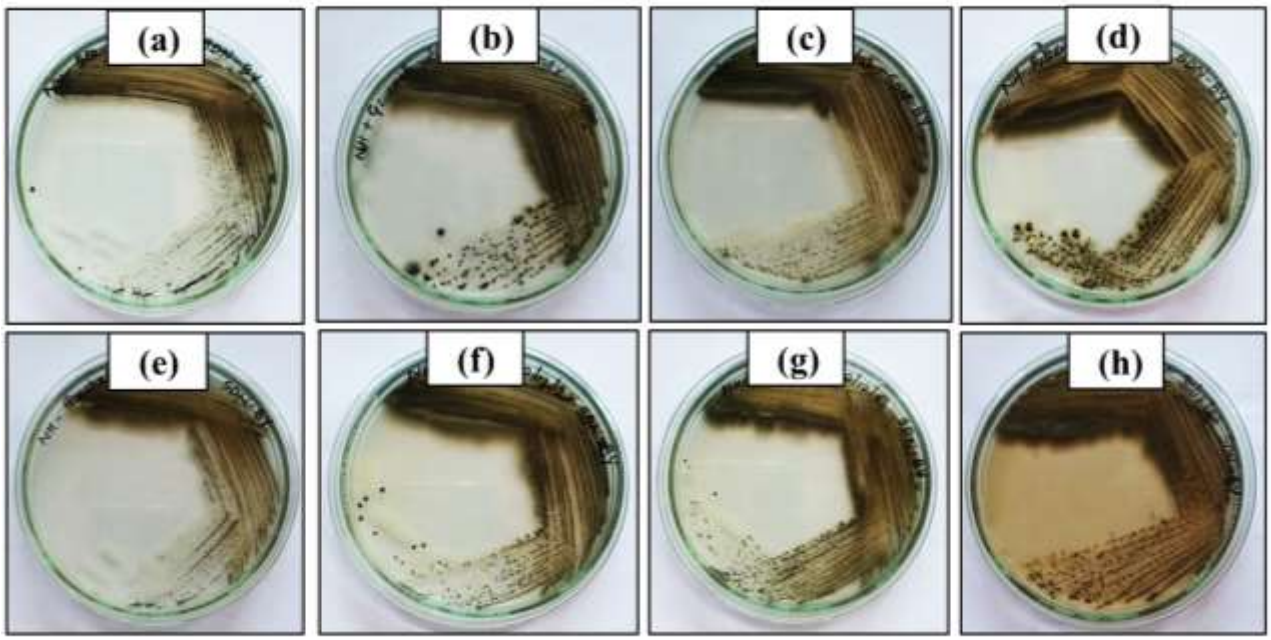


Figure 3.6: Growth of GUSNBS isolate on plate NH media containing various of sugars. (a) sucrose, (b) glucose, (c) mannose, (d) xylose (e) trehalose, (f) galactose, (g)lactose and (h) maltose

3.5. Salt tolerance activity of GUSNBS isolate

The salt tolerance of the GUSNBS isolate was examined on MEA medium plates containing varying NaCl (w/v) concentration i.e., 0%, 5%, 10%, 15%, 20%, 25% and 30%. The culture was found to be halotolerant as it showed growth on 0% NaCl concentration as well as on NaCl concentration upto 25%. Initially salt concentrations of 0% to 10% showed abundant growth with the best growth observed at 5% NaCl Concentration with dark pigmented colonies. It was also observed that, with increase in NaCl concentration the growth of the isolate decreased, with reduced colony size and pigmentation, showing no growth on 30% NaCl concentration after 7 days (Figure 3.7) as well as 15 days of incubation (Figure 3.8).

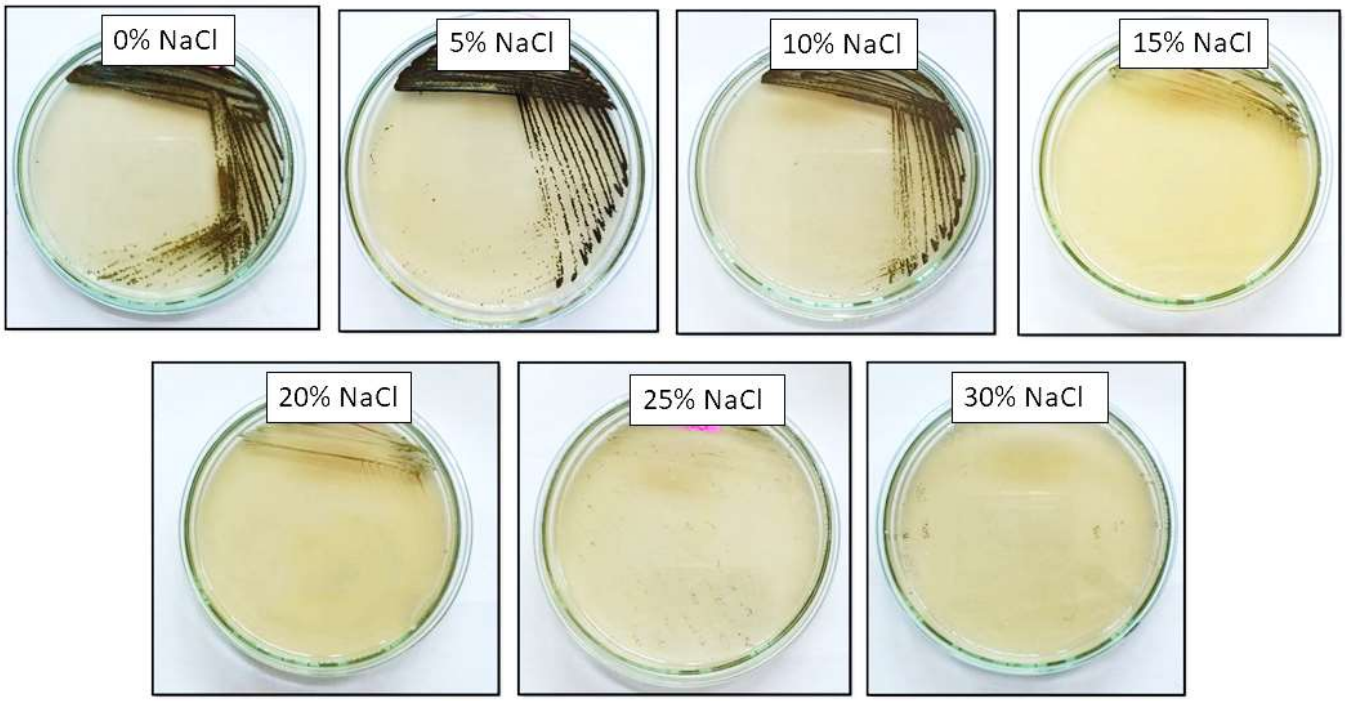


Fig.3.7: Salt tolerance studies of GUSNBS isolate at 0%, 5%, 10%, 15%, 20%, 25% and 30% NaCl concentration after 7 days of incubation.

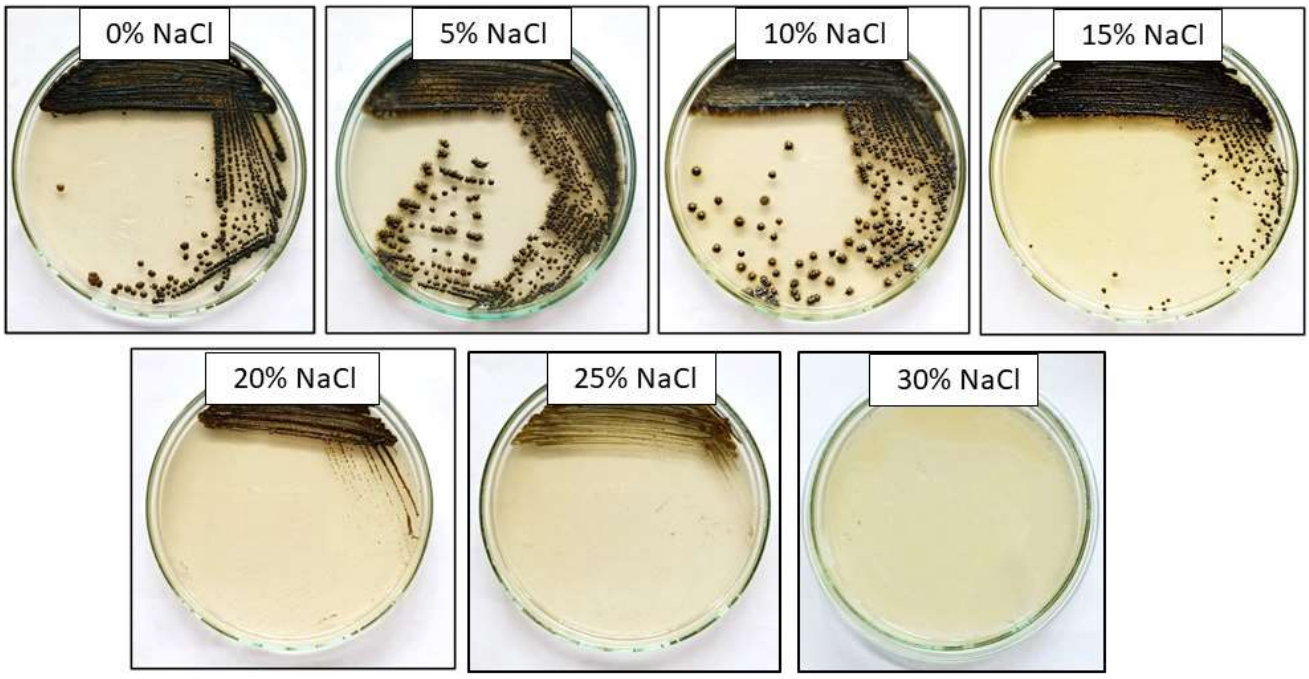


Fig.3.8. Salt tolerance studies of the isolate at 0%, 5%, 10%, 15%, 20%, 25% and 30% NaCl concentration after 15 days of incubation

3.6. Morphological studies of GUSNBS isolate

3.6.1. Colony morphology of GUSNBS isolate

It was observed that the morphology of the GUSNBS isolate varied on each NaCl concentration plate. Initially the colony appeared smooth circular in shape but eventually mycelium like structure appeared from the colony, giving a rhizoid like appearance to the colony. The pigmentation was decreased as the salt concentration increased incidating salt stress. The colony characteristics of the same is noted below.

Table 3.1: Colony morphology of GUSNBS isolate

Colony characteristics	**GUSNBS (0%)**	**GUSNBS (5%)**	**GUSNBS (10%)**	**GUSNBS (15%)**	**GUSNBS (20%)**
Time	15 days	15 days	15 days	15 days	15 days
Temperature	Room temperature	Room temperature	Room temperature	Room temperature	Room temperature
Colony size	6mm	6mm	5mm	3mm	1mm
Colony shape	Rhizoid	Rhizoid	Rhizoid	circular	Circular
Margin	Rhizoid	Rhizoid	Rhizoid	Entire	Entire
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Elevation	Raised	Raised	Raised	Raised	Raised
pigmentation	Black	Black	Black	Black	Brownish
Media	Malt extract medium	Malt extract medium	Malt extract medium	Malt extract medium	Malt extract medium
NaCl concentration	0%	5%	10%	15%	20%

3.6.2. Monochrome staining of GUSNBS isolate

At lower salt concentrations i.e., at 0%, 5%, and 10% oval yeast like cells which were underwent budding were observed. Budding from both the ends of the cells i.e., bipolar budding was seen. Chains of budding cells forming long filamentous pseudomycelium structure indicated polymorphic growth of the isolate. But as the salt concentration increased the cells appeared oval in shape and did not show pseudomycelial growth (figure 3.9.)

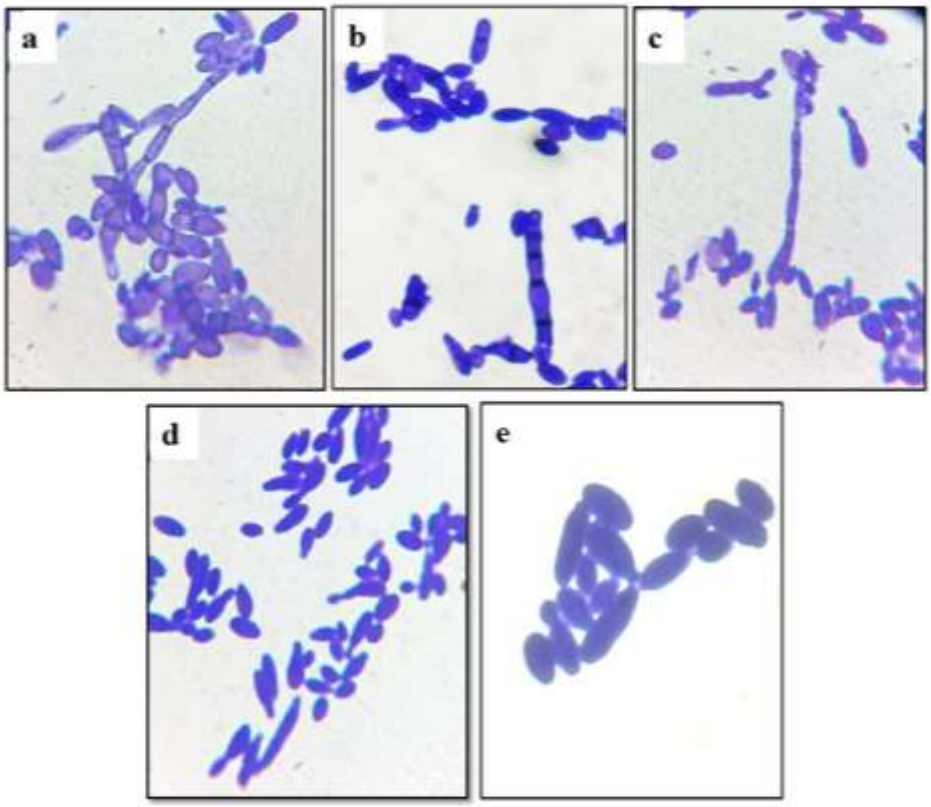


Figure 3.9: Monochrome staining of GUSNBS culture grown on MEA medium containing various NaCl concentration i.e, (a) 0%, (b) 5%, (c) 10%, (d) 15%, and (e) 20%.

3.6.3. FESEM analysis of GUSNBS isolate

The Field emission scanning electron microscopy (FESEM) of the GUSNBS isolate was carried out. Oval yeast like colonies undergoing budding were observed. Bipolar budding cells with side evaginations were noticed. (Figure 3.10.)

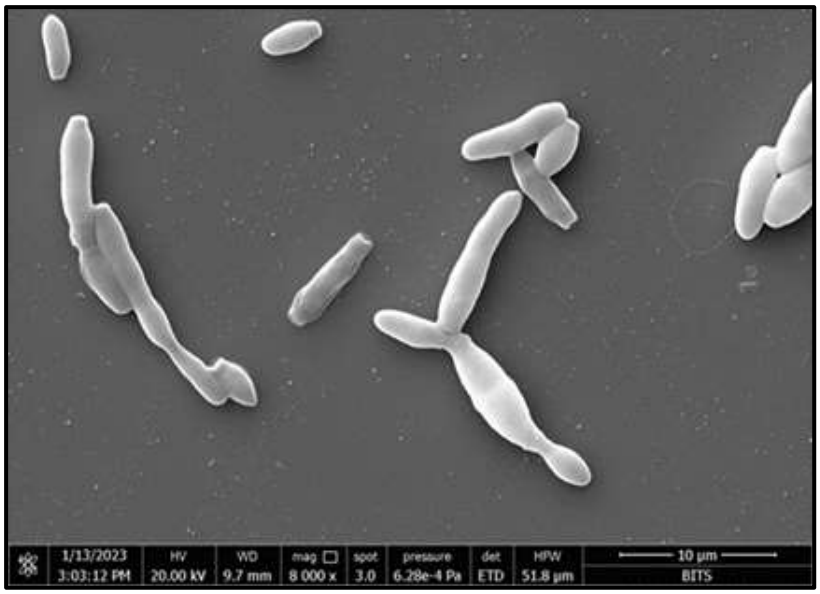


Figure 3.10: FESEM analysis of GUSNBS isolate

3.7. Growth curve of GUSNBS isolate

The GUSNBS culture inoculated in 500ml MEB containing 5% NaCl concentration. After 15 days of inoculation brown turbidity was observed, it showed intense black pigmentation after 30 days of incubation period (Figure 3.11). The growth curve of the same is plotted below (Figure:3.12.)

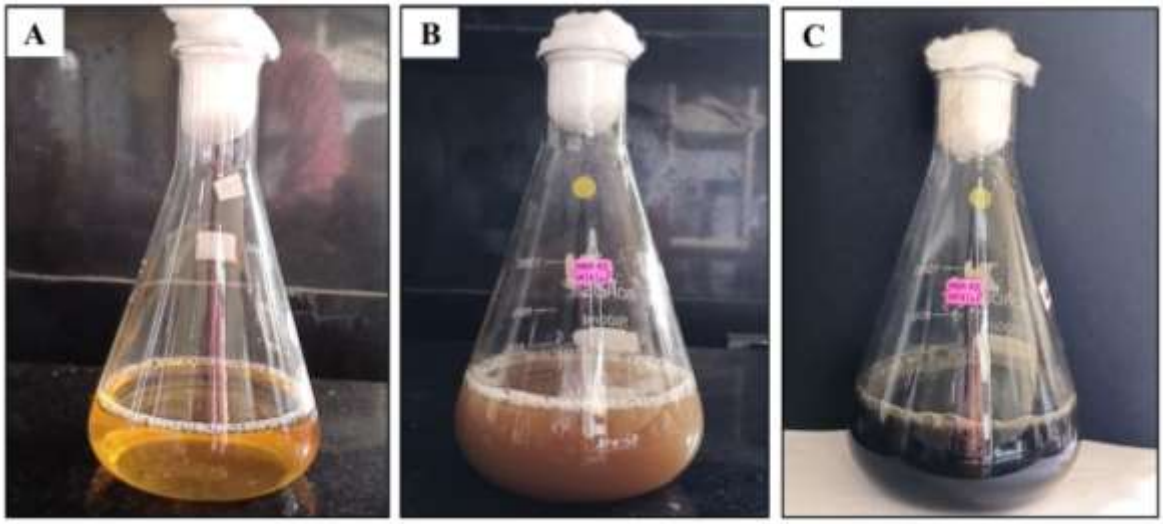


Figure 3.11: Growth of GUSNBS isolate in MEB (Malt extract broth) containing 5% NaCl concentration (A) 0 day of incubation (B) 15 days of incubation and (C) 30 days of incubation.

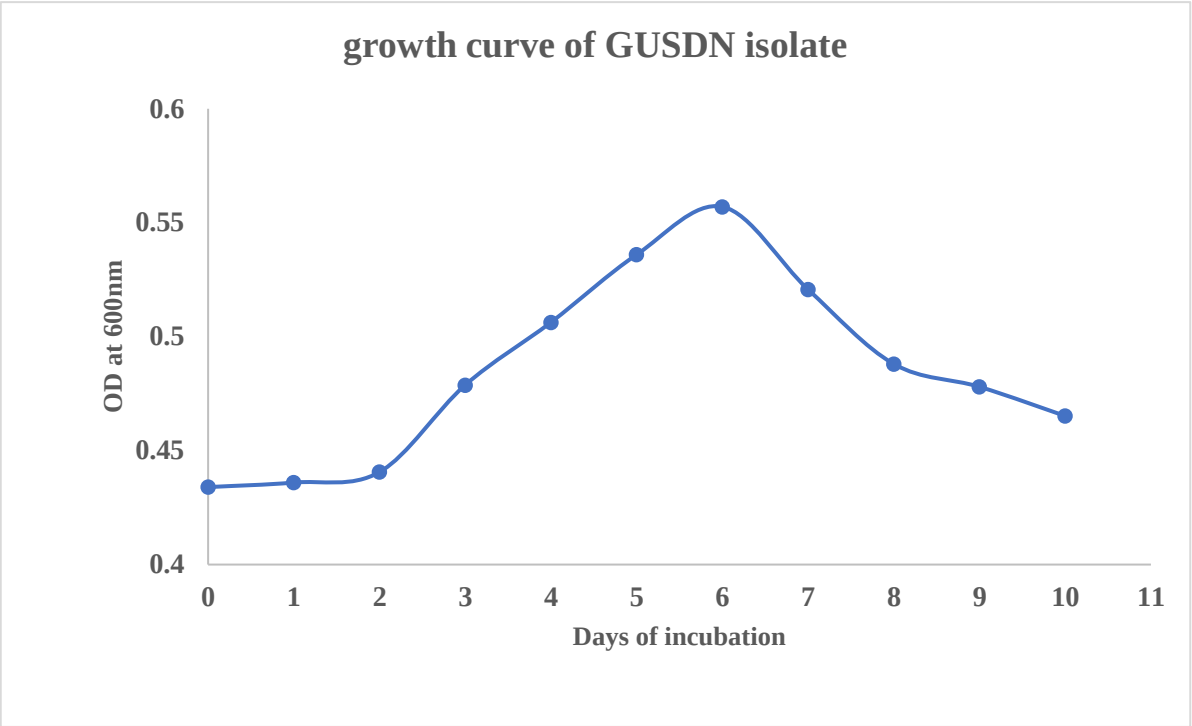


Figure 3.12: Growth curve of GUSNBS isolate when grown in MEB containing 5% NaCl concentration.

3.8. Chemotaxonomic characterisation of GUSNBS isolate

3.8.1. Carbohydrate utilisation test

Eight sources of sugars such as glucose, galactose, lactose, maltose, mannose, trehalose, sucrose and xylose were used to check carbohydrate utilisation. NH medium containing phenol red indicator was used with inverted Durhams tubes. After 7 days of incubation at room temperature colour change was noted, the phenol red indicator in the media turns yellow if acid is produced which is the indication of growth and acid production by the culture. (figure 3.13). The result of the same is tabulated below in table.

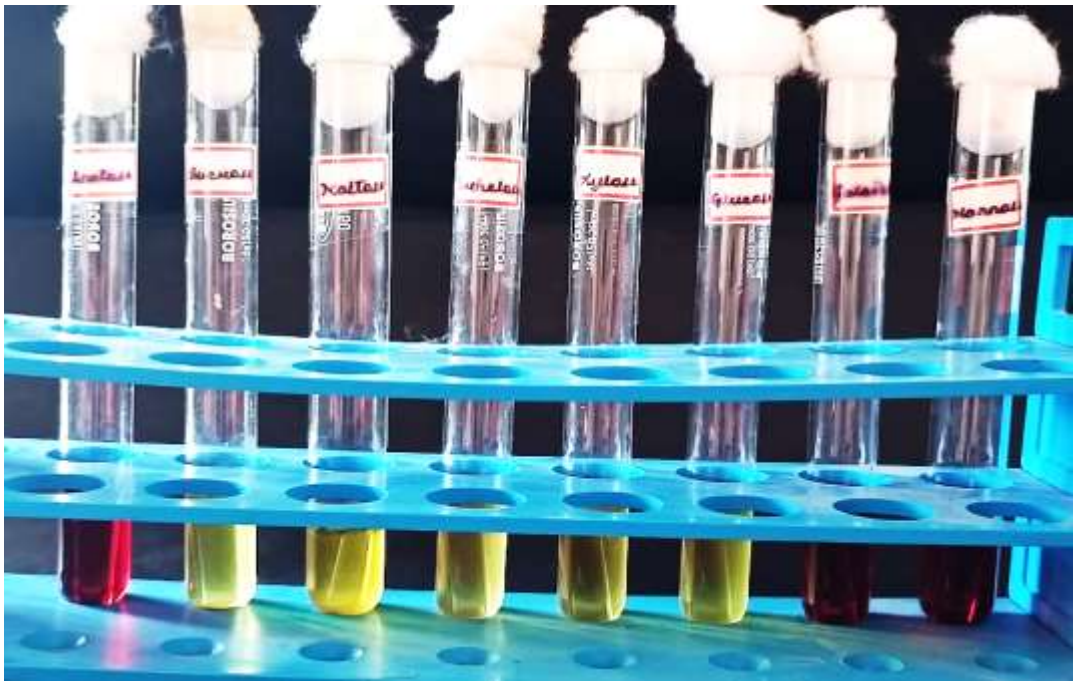


Figure 3.13: Carbohydrate utilisation test for GUSNBS isolate

Table 3.2: The study of utilisation by halotolerant isolate GUSNBS (Zalar et al., 2002)

Sugar utilisation	GUSNBS
Glucose	+
Sucrose	+
Maltose	+
Mannose	+
Trehalose	+
Galactose	-
Lactose	-
Xylose	+

Keys: + indicates growth,

– indicates No growth

3.8.2: Catalase Test

The GUSNBS isolate showed catalase positive test. The catalase test was carried out using 2-3 drops of hydrogen peroxide to a drop of culture, the degradation of hydrogen peroxide to oxygen and water showed effervescence which means catalase positive test.

3.8.3. Hanging drop method

The GUSNBS isolate was found to be non- motile examined by using hanging drop method.

3.9. Screening of the GUSNBS isolate for extracellular hydrolytic enzyme

The black pigmented halotolerant yeast culture GUSNBS was exploited for its potential to produce various extracellular hydrolytic enzymes. The enzymes screened were amylase, xylanase, protease, pectinase, esterase, lipase, cellulase, chitinase, gelatinase, phosphatase (Figure 3.14.). Out of 11 enzymes screened, the isolate showed 5 enzymatic activities which includes amylase, xylanase, protease, pectinase and esterase. The table 3.2. summarises the results of the various hydrolytic enzyme activities.

Table 3.3: extracellular hydrolytic enzyme activity shown by the isolate in NH medium containing 5% NaCl concentration and the following substrates (Gunde et al., 2000).

substrates	Enzyme activity
Amylase (Starch)	+
Xylanase (xylan)	+
Protease (Skimmed milk)	+
Pectinase (Pectin)	+
Esterase (Tween 80)	+
Lipase (Tween 20)	-
Cellulase (CMC)	-
Chitinase (Colloidal chitin)	-
Gelatinase (gelatin)	-
Phosphatase (p-nitrophenyl phosphate)	**No growth**

Keys: + indicates enzymatic activity,- Indicates no enzymatic activity

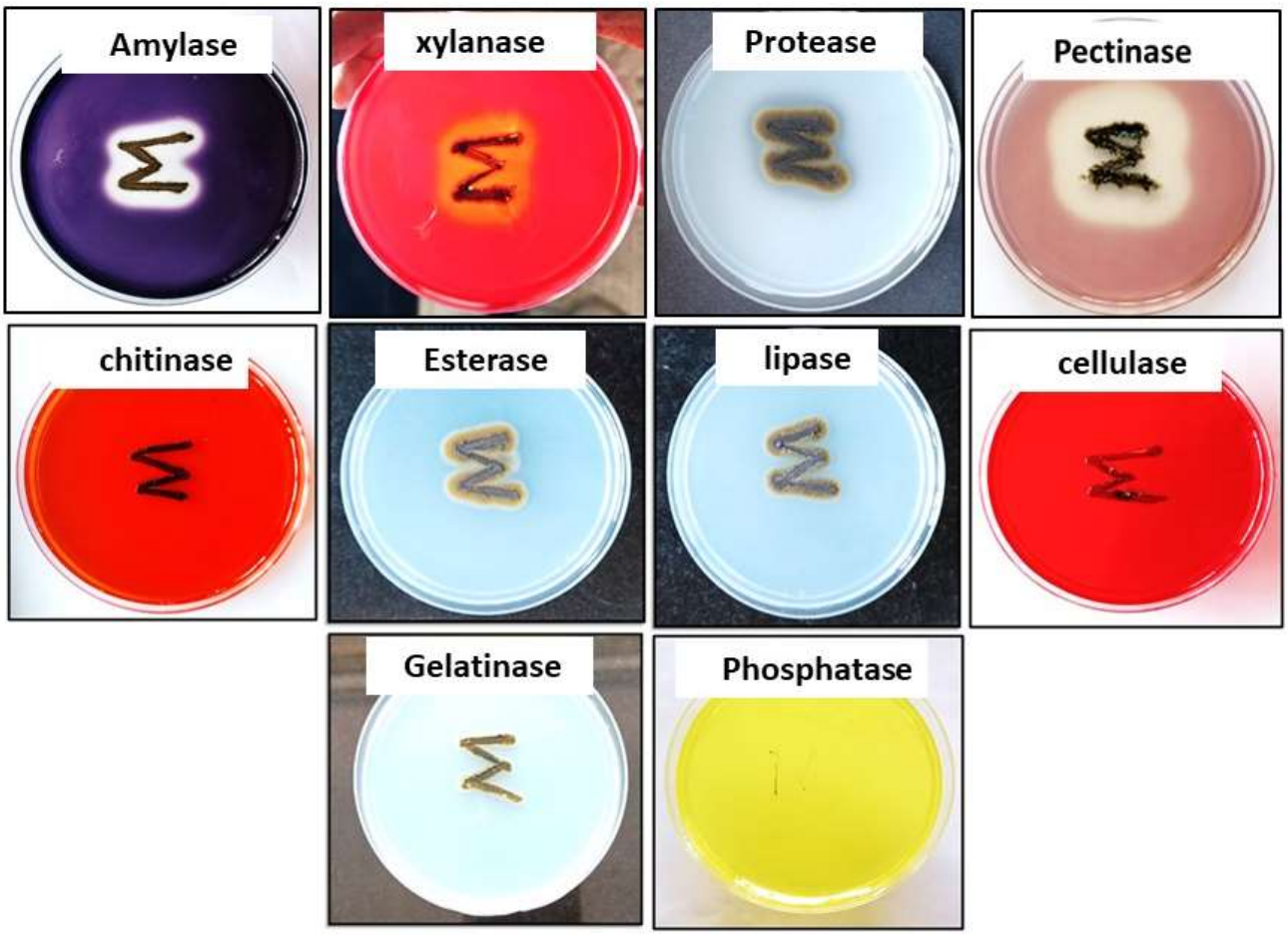


Figure 3.14: Screening of GUSNBS isolate for extracellular enzymatic activities.

Most promising enzyme activity was shown by esterase enzyme in less days of incubation in less days of incubation therefore further studies were conducted on esterase enzyme.

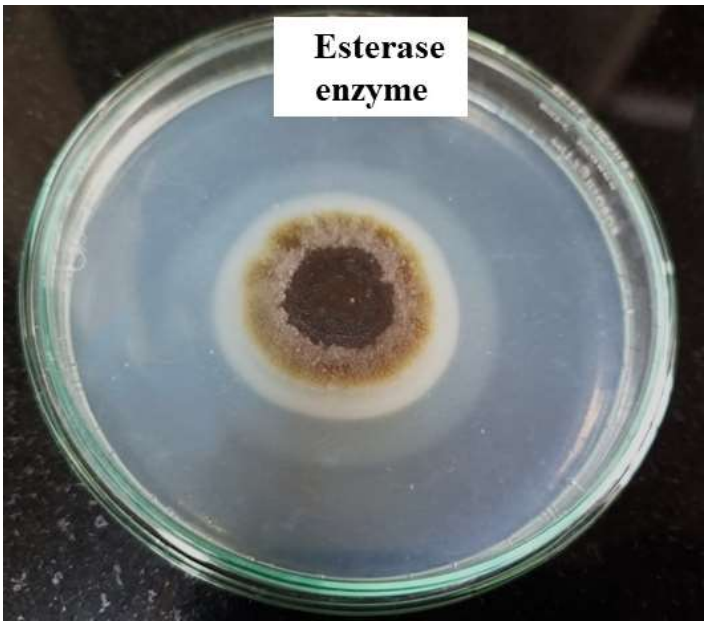


Figure 3.15. Esterase enzyme activity shown by GUSNBS isolate

3.10. Optimisation of esterase activity

The optimum growth conditions such as NaCl concentration, and temperature for production of esterase enzyme was examined.

NH medium supplemented with 0.5% tween 80 containing varying NaCl concentration i.e., 0%, 5%, 10%, 15%, 20% and 25% were used to check at which concentration the enzyme production was maximum (3.16). The diameter of the zone of turbidity around the growth of colony is directly proportional to the enzyme produced, which meant that at 5% the enzyme production was best as the zone of turbidity was bigger and more promising at 5% NaCl concentration. The enzyme production was seen at 0% upto 20% NaCl concentration. The enzyme production on chromogenic agar plates containing varying salt concentration was also observed (3.17) The enzyme production was best at 5% NaCl concentration after 7 days of incubation at room temperature.

The optimum temperature for enzyme production was studied using NH medium agar plate containing 5% NaCl concentration supplemented with 0.5% tween 80 and incubating the plates

at 4°C, 28°C and 37°C for 7 days (3.18). The optimum temperature for esterase activity was found to be 28°C i.e., at room temperature.

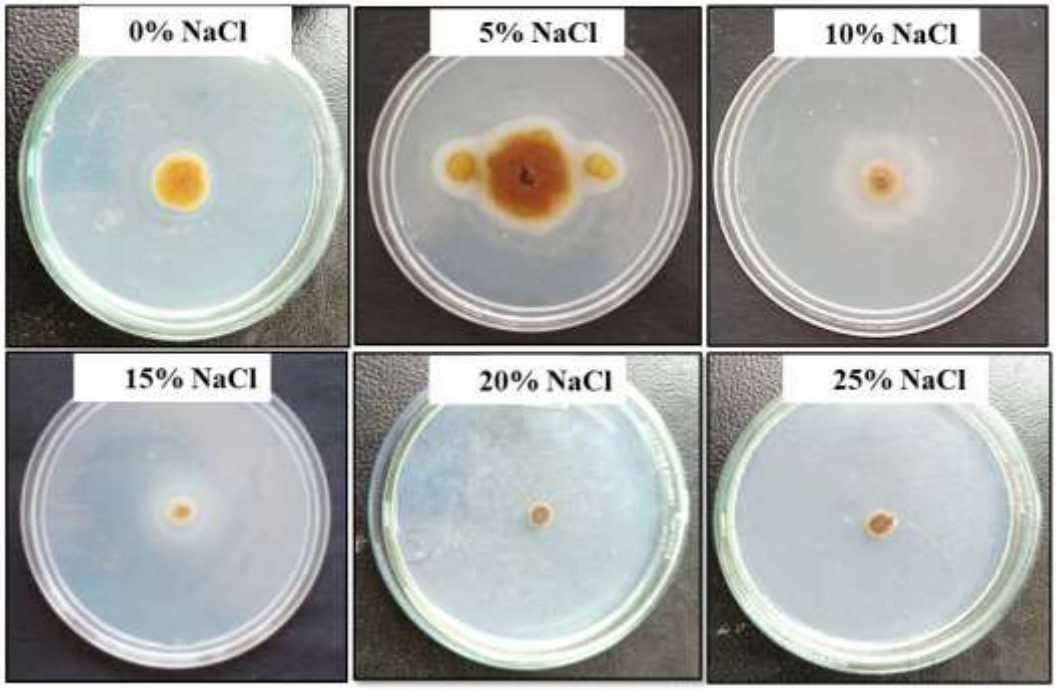


Figure 3.16: NaCl concentration optimization for esterase activity

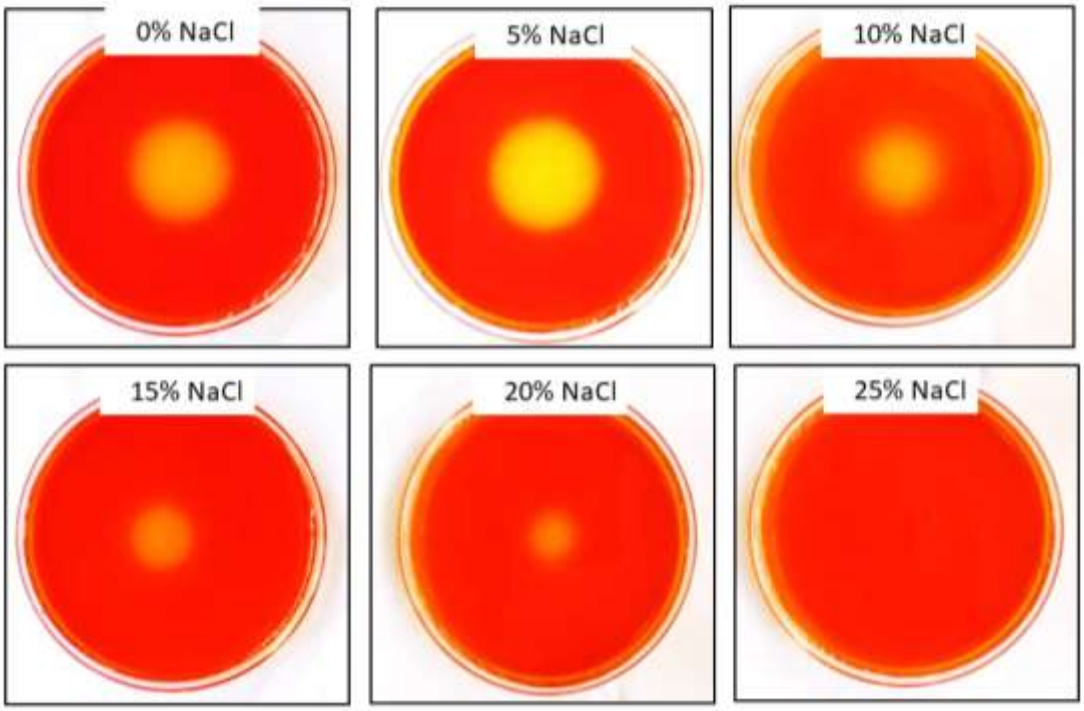


Figure 3.17: NaCl concentration optimisation for esterase using chromogenic agar plate method

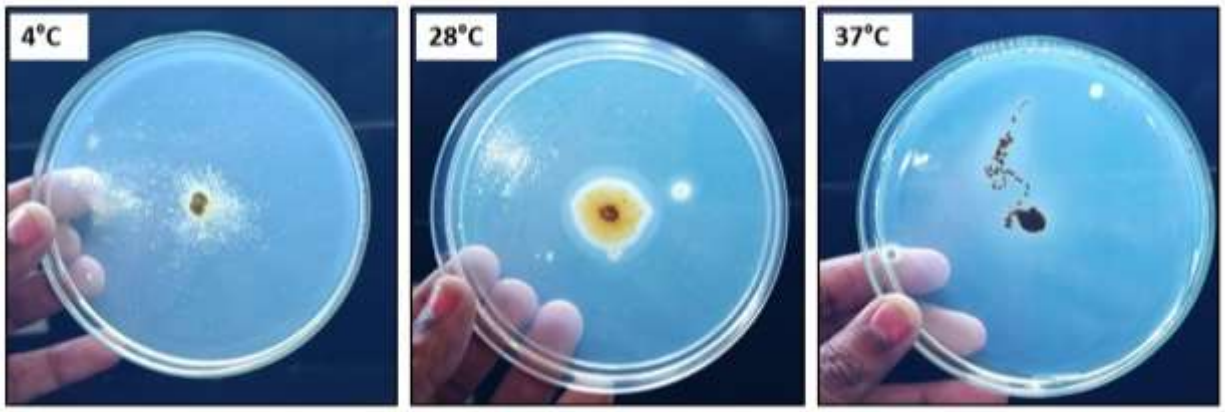


Figure 3.18: The study of optimum temperature requirement for the growth and production of esterase enzyme.

3.11. Growth and esterase production by GUSNBS isolate

The growth and esterase production by the GUSNBS culture in NH broth containing 5% NaCl concentration supplemented with 0.5% tween 80 was examined on daily basis for increasing turbidity (figure 3.19)

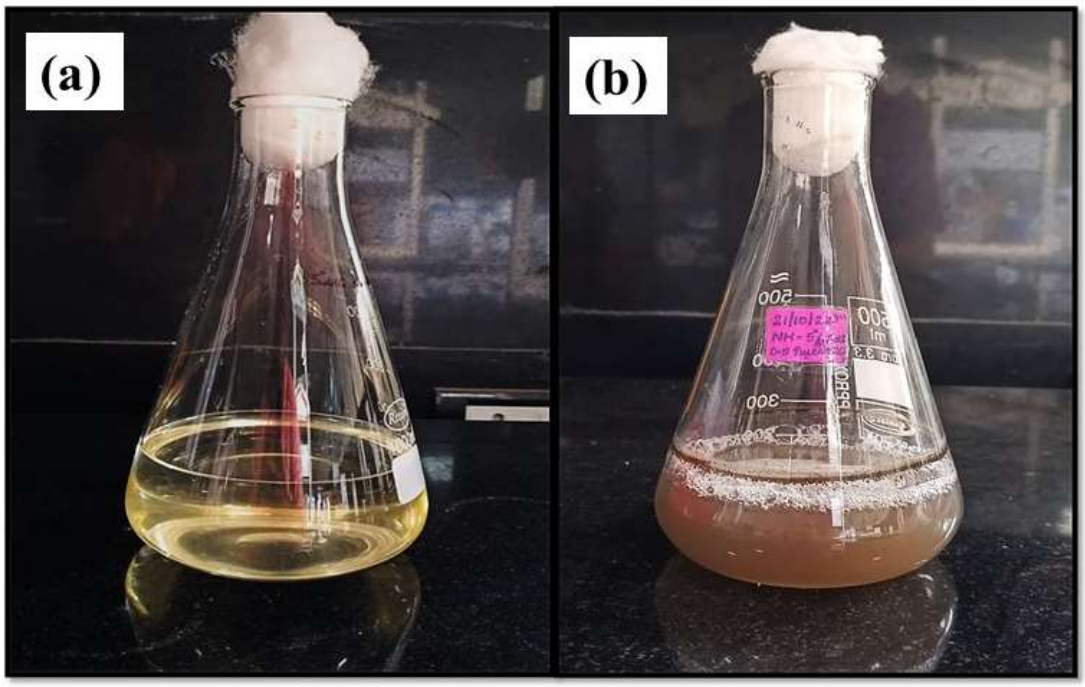


Figure 3.19: Growth of culture in NH media containing 0.5% tween 80 (a) 0day incubation (b) 30 days of incubation.

3.12. Partial purification of Esterase enzyme from GUSNBS isolate

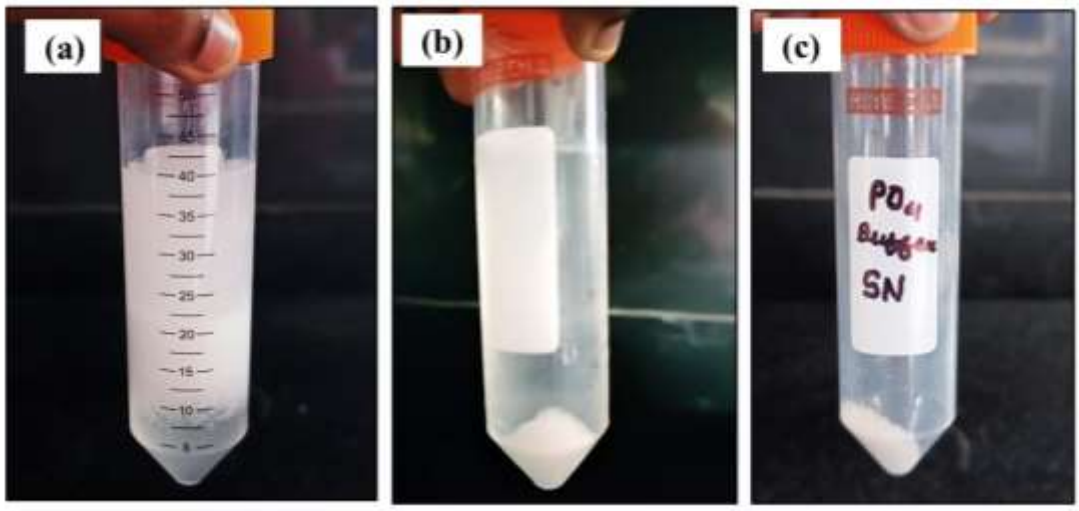


Figure 3.20: partial purification of esterase enzyme using ice cold ethanol extraction method (a) Addition of cold ethanol in CFS, (b) centrifugation of precipitate to extract enzyme (c) enzyme dissolved in phosphate buffer.

3.13. Activity of partially purified esterase enzyme extracted from GUSNBS

The activity of partially purified enzyme was detected on chromogenic agar plate. The chromogenic plate inoculated with CFS showed maximum activity (Figure 3.21 A), followed by the plate without NaCl, inoculated with 2 hours enzymatic extract (Figure 3.21 B) and least enzyme activity was observed on the plate inoculated with 2 days enzyme extract (Figure 3.21C).

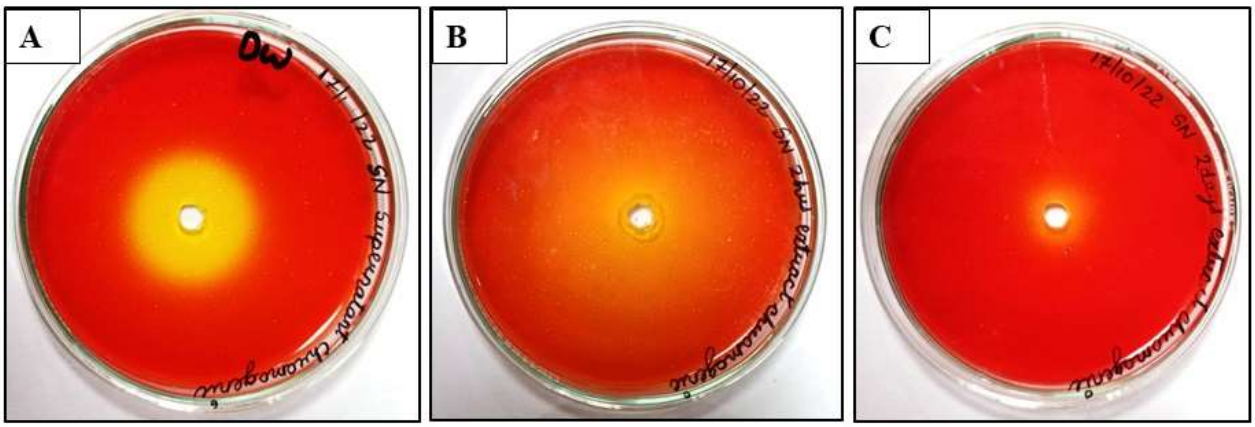


Figure3.21: study of partially purified enzyme activity on chromogenic agar plate (A) plate inoculated with CFS of the 1month old culture grown in NH media supplemented with 0.5% tween 80, (B) plate inoculated With 2 hours of partially purified extract (C) plate inoculated with 2 days of partially purified extract.

3.14. Native PAGE and Zymogram analysis of esterase enzyme

The native PAGE analysis of esterase showed three bands in lane 2 i.e., marker lane and a single band in lane 1 i.e., enzyme lane indicating the esterase enzyme band having ~ 40kDa size (Figure 3.22.a). In zymogram analysis yellow zone in the lane 1 i.e., in enzyme lane (Figure 3.22.b) indicated the presence of esterase enzyme as it showed yellow zone exactly where the band of esterase was seen in the other half of the Native PAGE gel.

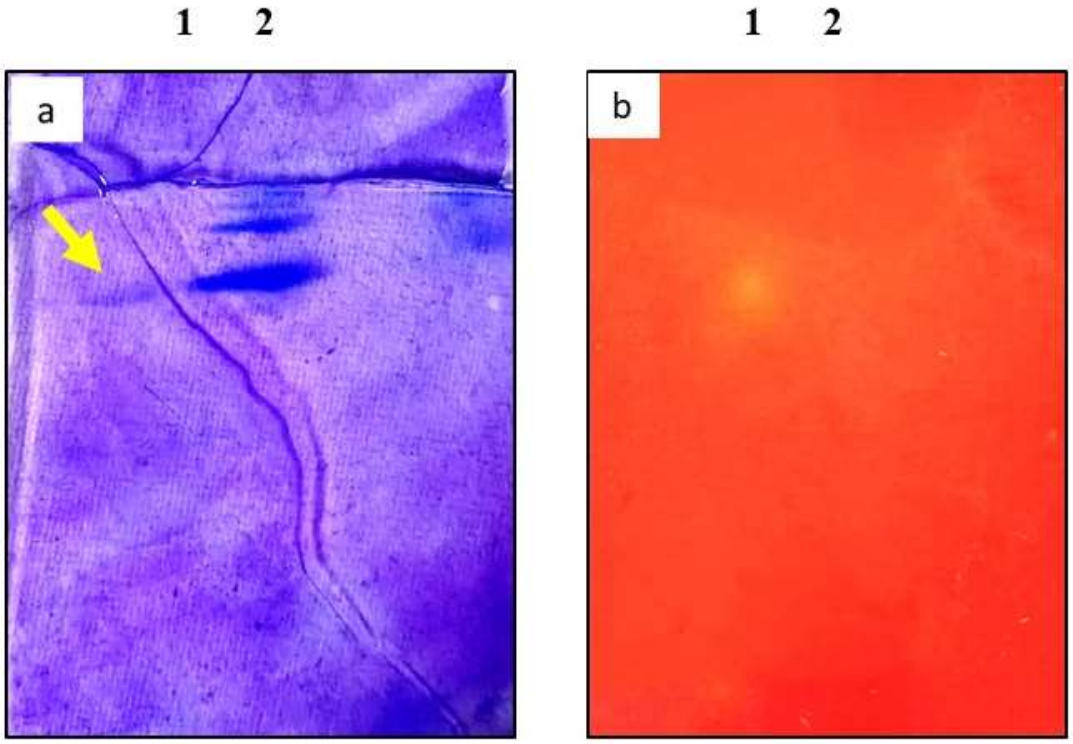


Figure 3.22: a) native PAGE gel after staining 1: enzyme lane, 2: marker lane (b) zymogram analysis using chromogenic molten agar 1: enzyme lane, 2) marker lane

3.15. Estimation of esterase enzyme activity

Esterase activity of the culture GUSNBS was estimated using p-nitrophenol acetate as substrate. One unit of enzyme activity is the amount of protein releasing 1µmole of p-nitrophenol per minute. The enzyme activity of esterase enzyme was found to be 104 units/ml using the formula

$$\text{Enzyme activity} = \frac{\mu\text{mole of paranitro phenol released} \times \text{total volume of assay}}{\text{volume of enzyme} \times \text{time of assay}}$$

3.16. Pigment extraction and characterisation

The brown black pigment from black yeast was extracted. It was insoluble in solvents like methanol and water. The pigment was dissolved in 1M NaOH and further characterised using UV visible spectrometry from 190 to 800nm range. The pigment absorbed highest in the UV range and then drastically declined towards the visible range. The absorption peak was found between range of 220 to 240nm, which is similar to peak obtained by Rani et al., 2013 which is typical to spectral peak of melanin pigment (Rani et al., 2013) and hence lead to the confirmation of the melanin pigment. TLC of the extracted pigment showed rf value of 0.5 using the below formula

$$\text{Rf} = \frac{\textit{Distance travelled by the pigment}}{\textit{Distance travelled by the solvent}}$$

Fig 3.22: a) Pigment extracted from black yeast isolate GUSNBS, b) extracted pigment dissolved in 0.1M NaOH buffer.

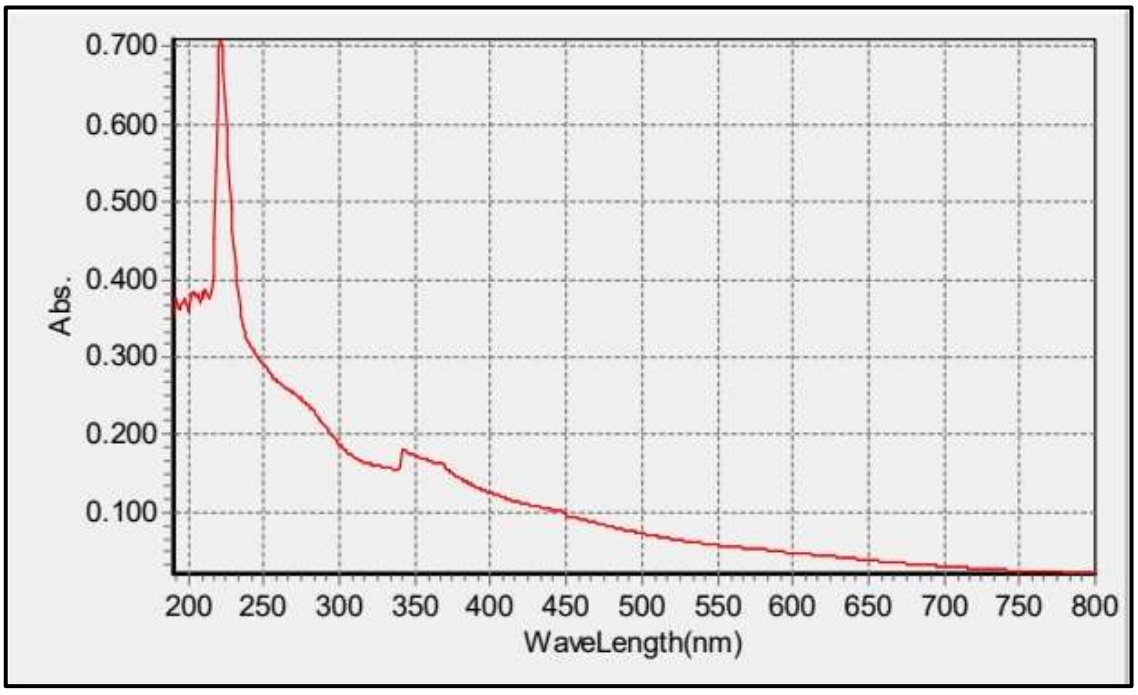


Figure 3.23: UV visible spectrophotometry of extracted pigment: peak showing highest absorption between 200nm to 250nm which is the characteristic of melanin pigment.

Figure 3.24: Thin layer chromatography of the extracted pigment from isolate GUSNBS.

Chapter IV

Summary and Conclusion

Summary of results and conclusion

In recent years, studies on halophilic microorganisms have increased due to its ability to thrive and adapt to various harsh conditions. These organisms are gaining more attention due to its to ability to secret various industrially important metabolites like extremozymes which has the ability to withstand high salt concentration.

In the present study, A halotolerant isolate was successfully isolated from Nerul Salt pan located in Goa. Monochrome staining indicated that the isolated culture was a yeast as it showed blue coloured oval colonies. The GUSNBS isolate was check for its growth on various fungal media i.e., MEA, PDA, Sabouraud's and Czapek's media wherein it was found that the isolate showed best growth on MEA, with scanty growth on Czapeks medium. The halotolerance of the isolate was studied using MEA plates containing varying salt concentration i.e., 0 to 30%. The growth was observed after 7 days of incubation, showing best growth on 5% NaCl concentration. As the salt concentration increased the growth decreased showing no growth on 30% NaCl concentration. The isolate showed polymorphism by showing yeast like colonies on high salt concentration while on low salt concentration the yeast like colonies underwent budding showing bipolar budding and gradually formed pseudo mycelium.

The isolate was screened for potential industrially important enzymes such as amylase, cellulase, protease, xylanase, Gelatinase, esterase, lipase, pectinase, chitinase, and phosphatase. The isolate was found to produce amylase, protease, xylanase, pectinase, esterase, enzymes. It showed excellent esterase activity with less incubation period as compared to other enzymes and therefore was selected for further enzyme studies. The optimization of growth conditions for enzyme production studies showed that the enzyme production was best at 5% NaCl at 37°C. The esterase enzyme was partially purified using ice cold ethanol precipitation method and further characterised using Native PAGE and zymogram analysis technique. The enzymatic

activity for the esterase enzyme was found to be 104 units/ml by performing enzyme assay using Para nitrophenol acetate substrate. The pigment was extracted from the GUSNBS isolate was characterised as melanin by performing UV visible microscopy and TLC (Thin layer chromatography).

To conclude, GUSNBS was a halotolerant isolate having ability to thrive in normal as well as in high salinity conditions. The esterase enzyme produced by this isolate showed excellent enzymatic activity in normal as well as in saline conditions which can serve as an important biocatalysts in industries due to its ability to function in normal as well as in harsh conditions. The photoprotective pigment melanin high significance as antibacterial agent in medicine .

Future Prospects

Future prospects

1. The enzymes produced by the halotolerant isolate can be explored for their potential industrial applications.
2. Study of other industrial important metabolites such as biosurfactants by the isolate can be carried out.
3. The hydrocarbon degrading ability of the isolate can be explored, which has great significance in bioremediation process.
4. The extracted melanin pigment can be checked for its antibacterial and antioxidant activities.

References:

1. Akolkar, A. V., & Desai, A. J. (2010). Catalytic and thermodynamic characterization of protease from Halobacterium sp. SP1 (1). *Research in Microbiology*, *161*(5), 355-362.
2. Ali, I., Siwarungson, N., Punnapayak, H., Lotrakul, P., Prasongsuk, S., Bankeeree, W., & Rakshit, S. K. (2014). Screening of potential biotechnological applications from obligate halophilic fungi, isolated from a man-made solar saltern located in Phetchaburi province, Thailand. *Pak. J. Bot*, *46*(3), 983-988.
3. Batista-García, R. A., Balcazar-Lopez, E., Miranda-Miranda, E., Sanchez-Reyes, A., Cuervo-Soto, L., Aceves-Zamudio, D., ... & Folch-Mallol, J. (2014). Characterization of lignocellulolytic activities from a moderate halophile strain of Aspergillus caesiellus isolated from a sugarcane bagasse fermentation. *PLoS One*, *9*(8), e105893.
4. Beg, Q., Kapoor, M., Mahajan, L., & Hoondal, G. S. (2001). Microbial xylanases and their industrial applications: a review. *Applied microbiology and biotechnology*, *56*, 326-338.
5. Bonifaz, A., Badali, H., De Hoog, G. S., Cruz, M., Araiza, J., Cruz, M. A., ... & Ponce, R. (2008). Tinea nigra by Hortaea werneckii, a report of 22 cases from Mexico. *Studies in Mycology*, *61*, 77-82.
6. Boucherit, Z., Flahaut, S., Djoudi, B., Mouas, T. N., Mechakra, A., & Ameddah, S. (2022). Potential of Halophilic Penicillium chrysogenum Isolated from Algerian Saline Soil to Produce Laccase on Olive Oil Wastes. *Current Microbiology*, *79*(7), 178.
7. Cabañes, F. J., Bragulat, M. R., & Castellá, G. (2012). Hortaea werneckii isolated from silicone scuba diving equipment in Spain. *Medical Mycology*, *50*(8), 852-857.
8. Cantrell, S. A., Casillas-Martínez, L., & Molina, M. (2006). Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. *Mycological research*, *110*(8), 962-970.
9. Chen, J., Xing, X. K., Zhang, L. C., Xing, Y. M., & Guo, S. X. (2012). Identification of Hortaea werneckii isolated from mangrove plant Aegiceras comiculatum based on morphology and rDNA sequences. *Mycopathologia*, *174*, 457-466.
10. Chi, Z., Ma, C., Wang, P., & Li, H. F. (2007). Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast Aureobasidium pullulans. *Bioresource Technology*, *98*(3), 534-538.
11. Choi, Y. J., & Lee, B. (2001). Culture conditions for the production of esterase from Lactobacillus casei CL96. *Bioprocess and Biosystems Engineering*, *24*, 59-63.
12. Collins, T., Gerday, C., & Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS microbiology reviews*, *29*(1), 3-23.
13. Corral, P., Amoozegar, M. A., & Ventosa, A. (2019). Halophiles and their biomolecules: recent advances and future applications in biomedicine. *Marine drugs*, *18*(1), 33.

14. Dahiya, D., & Singh Nigam, P. (2022). Sustainable Biosynthesis of Esterase Enzymes of Desired Characteristics of Catalysis for Pharmaceutical and Food Industry Employing Specific Strains of Microorganisms. *Sustainability*, *14*(14), 8673.
15. Eichlerová, I., Šnajdr, J., & Baldrian, P. (2012). Laccase activity in soils: considerations for the measurement of enzyme activity. *Chemosphere*, *88*(10), 1154-1160.
16. Elsayis, A., Hassan, S. W., Ghanem, K. M., & Khairy, H. (2022). Optimization of melanin pigment production from the halotolerant black yeast Hortaea werneckii AS1 isolated from solar salter in Alexandria. *BMC microbiology*, *22*(1), 92.
17. Galasso, C., Corinaldesi, C., & Sansone, C. (2017). Carotenoids from marine organisms: Biological functions and industrial applications. *Antioxidants*, *6*(4), 96.
18. Gandolfi, R., Gaspari, F., Franzetti, L., & Molinari, F. (2000). Hydrolytic and synthetic activities of esterases and lipases of non-starter bacteria isolated from cheese surface. *Annals of microbiology*, *50*(2), 183-190.
19. Gummadi, S. N., Manoj, N., & Kumar, D. S. (2007). Structural and biochemical properties of pectinases. *Industrial enzymes: structure, function and applications*, 99-115.
20. Gunde-Cimerman, N., Plemenitaš, A., & Oren, A. (2018). Strategies of adaptation of microorganisms of the three domains of life to high salt concentrations. *FEMS microbiology reviews*, *42*(3), 353-375.
21. Gunde-Cimerman, N., Zalar, P., de Hoog, S., & Plemenitaš, A. (2000). Hypersaline waters in salterns–natural ecological niches for halophilic black yeasts. *FEMS Microbiology Ecology*, *32*(3), 235-240.
22. Gunde-Cimerman, N., Zalar, P., de Hoog, S., & Plemenitaš, A. (2000). Hypersaline waters in salterns–natural ecological niches for halophilic black yeasts. *FEMS Microbiology Ecology*, *32*(3), 235-240.
23. Hamza, H. M., Ali, S. M., & Hassan, H. G. (2006). Partial purification of gelatinase enzyme from local isolate of Brevibacillus laterosporus. *National Journal*, *23*, 442.
24. Hasan, F., Shah, A. A., & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial technology*, *39*(2), 235-251.
25. Hodhod, M. S. E. D., Gaafar, A. R. Z., Alshameri, A., Qahtan, A. A., Noor, A., & Abdel-Wahab, M. (2020). Molecular characterization and bioactive potential of newly identified strains of the extremophilic black yeast Hortaea werneckii isolated from Red Sea mangrove. *Biotechnology & Biotechnological Equipment*, *34*(1), 1288-1298.
26. Horne, I., Sutherland, T. D., Harcourt, R. L., Russell, R. J., & Oakeshott, J. G. (2002). Identification of an opd (organophosphate degradation) gene in an Agrobacterium isolate. *Applied and environmental microbiology*, *68*(7), 3371-3376.
27. Jayani, R. S., Saxena, S., & Gupta, R. (2005). Microbial pectinolytic enzymes: a review. *Process Biochemistry*, *40*(9), 2931-2944.

28. Kamekura, M. (1998). Diversity of extremely halophilic bacteria. *Extremophiles*, *2*, 289-295.
29. Kim, Y. H., Cha, C. J., & Cerniglia, C. E. (2002). Purification and characterization of an erythromycin esterase from an erythromycin-resistant Pseudomonas sp. *FEMS microbiology letters*, *210*(2), 239-244.
30. Kontkanen, H., Tenkanen, M., Fagerström, R., & Reinikainen, T. (2004). Characterisation of steryl esterase activities in commercial lipase preparations. *Journal of Biotechnology*, *108*(1), 51-59.
31. Kutateladze, L., Zakariashvili, N., Jobava, M., Urushadze, T., Khvedelidze, R., & Khokhashvili, I. (2009). Selection of microscopic fungi-Pectinase producers. *Bulletin of the Georgian National Academy of Science*, *3*.
32. Madhu, K. M., Beena, P. S., & Chandrasekaran, M. (2009). Extracellular β-glucosidase production by a marine Aspergillus sydowii BTMFS 55 under solid state fermentation using statistical experimental design. *Biotechnology and Bioprocess Engineering*, *14*, 457-466.
33. Meneses, D. P., Paixao, L. M. N., Fonteles, T. V., Gudina, E. J., Rodrigues, L. R., Fernandes, F. A., & Rodrigues, S. (2021). Esterase production by Aureobasidium pullulans URM 7059 in stirred tank and airlift bioreactors using residual biodiesel glycerol as substrate. *Biochemical Engineering Journal*, *168*, 107954.
34. Mtui, G., & Nakamura, Y. (2008). Lignocellulosic enzymes from Flavodon flavus, a fungus isolated from Western Indian Ocean off the coast of Dar es Salaam, Tanzania. *African Journal of Biotechnology*, *7*(17).
35. Ng, A. M. J., Zhang, H., & Nguyen, G. K. T. (2021). Zymography for picogram detection of lipase and esterase activities. *Molecules*, *26*(6), 1542.
36. Oren, A. (2002). Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *Journal of Industrial Microbiology and Biotechnology*, *28*, 56-63.
37. Patil, N. S., & Jadhav, J. P. (2015). Significance of Penicillium ochrochloron chitinase as a biocontrol agent against pest Helicoverpa armigera. *Chemosphere*, *128*, 231-235.
38. Plemenitaš, A., Lenassi, M., Konte, T., Kejžar, A., Zajc, J., Gostinčar, C., & Gunde-Cimerman, N. (2014). Adaptation to high salt concentrations in halotolerant/halophilic fungi: a molecular perspective. *Frontiers in microbiology*, *5*, 199.
39. Primožič, M., Čolnik, M., Knez, Ž., & Leitgeb, M. (2019). Release of Halophilic Extremozymes by Mechanical Cell Disruption. *Acta Chimica Slovenica*, *66*(1), 217-228.
40. Rani, M. H. S., Ramesh, T., Subramanian, J., & Kalaiselvam, M. (2013). Production and characterization of melanin pigment from halophilic black yeast Hortaea werneckii. *Int J Pharma Res Rev*, *2*(8), 9-17.

41. Ranjitha, P., Karthy, E. S., & Mohankumar, A. (2009). Purification and Characterization of the Lipase from Marine Vibrio fischeri. *International Journal of Biology*, *1*(2), 48.
42. Sakaki, H., Kaneno, H., Sumiya, Y., Tsushima, M., Miki, W., Kishimoto, N., ... & Sawabe, A. (2002). A new carotenoid glycosyl ester isolated from a marine microorganism, Fusarium strain T-1. *Journal of natural products*, *65*(11), 1683-1684.
43. Sharma, N., Rathore, M., & Sharma, M. (2013). Microbial pectinase: sources, characterization and applications. *Reviews in Environmental Science and Bio/Technology*, *12*(1), 45-60.
44. Shen, D., Xu, J. H., Wu, H. Y., & Liu, Y. Y. (2002). Significantly improved esterase activity of Trichosporon brassicae cells for ketoprofen resolution by 2-propanol treatment. *Journal of Molecular Catalysis B: Enzymatic*, *18*(4-6), 219-224.
45. Śliżewska, W., Struszczyk-Świta, K., & Marchut-Mikołajczyk, O. (2022). Metabolic Potential of Halophilic Filamentous Fungi—Current Perspective. *International Journal of Molecular Sciences*, *23*(8), 4189.
46. Soliman, H. M., Elsayed, A., Mowafy, A. M., Gebreil, A., & Magdy, N. I. (2017). Molecular Polymorphism and Pleomorphic Pattern of New Strains of Hortaea werneckii Isolated from Salt Marshes of Egypt. *Journal of Environmental Sciences*, *46*(1), 1-10.
47. Sundarram, A., & Murthy, T. P. K. (2014). α-amylase production and applications: a review. *Journal of Applied & Environmental Microbiology*, *2*(4), 166-175.
48. Tomlinson, G. A., & Hochstein, L. I. (1972). Isolation of carbohydrate-metabolizing, extremely halophilic bacteria. Canadian journal of microbiology, 18(5), 698-701.
49. Ventosa, A., Nieto, J. J., & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiology and molecular biology reviews*, *62*(2), 504-544
50. Wang, W. L., Lu, Z. Y., Tao, H. W., Zhu, T. J., Fang, Y. C., Gu, Q. Q., & Zhu, W. M. (2007). Isoechinulin-type alkaloids, variecolorins A–L, from halotolerant Aspergillus variecolor. *The Journal of Natural Products*, *70*(10), 1558-1564.
51. Yildirim, M., Colak, A., Col, M., & Canakci, S. (2009). A new recombinant phosphotriesterase homology protein from Geobacillus caldoxylosilyticus TK4: An extremely thermo-and pH-stable esterase. *Process Biochemistry*, *44*(12), 1366-1373.
52. Zalar, P., Kocuvan, M. A., Plemenitaš, A., & Gunde-Cimerman, N. (2005). Halophilic black yeasts colonize wood immersed in hypersaline water.
53. Zalar, P., Zupančič, J., Gostinčar, C., Zajc, J., de Hoog, G. S., De Leo, F., ... & Gunde-Cimerman, N. (2019). The extremely halotolerant black yeast *Hortaea werneckii*-a model for intraspecific hybridization in clonal fungi. *IMA fungus*, *10*(1), 1-27.

Chapter VI

Appendix

Appendix I: Composition of media

1) Tomlinson Media

Ingredients	g/L
NaCl	250.0
MgCl2.6H2O	20.0
KCl	6.0
Yeast Extract	5.0
Peptone	2.5
$CaCl_2.6H_2O$	0.2
Agar	20.0

Directions: sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

2) MEA

Ingredients	g/L
Malt extract powder	20.0
Glucose	20.0
Peptone	1.0
Agar	20.0

Directions: sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3) PDA

Ingredients	g/L
Infused potatoes	4.0
Dextrose	20.0
Agar	20.0

Directions: sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

4) Sabouraud's Agar

Ingredients	g/L
Dextrose	40.0
Peptone	10.0
Agar	15.0

Directions: sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

5) Czapek's Agar

Ingredients	g/L
Sodium nitrate	2.0
Potassium chloride	0.5
Magnesium glycerol phosphate	0.5
Ferrous Sulfate	0.01
Potassium sulfate	0.35
Sucrose	30.00
Agar	12.00

Directions: sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

6) NH

Ingredients	g/L
NaCl	200.0
KCl	5.0
Yeast extract	1.0
$MgSO_4.7H_20$	10.0
Agar	20.0

Directions: sterilize by autoclaving at 15 lbs pressure (121°C) 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

NATIVE PAGE REAGENTS

6) **Resolving gel buffer (1.5 M)**

Tris HCl	18.171 g
Distilled Water	100 mL
p H	8.8

7) **Stacking gel buffer (1 M)**

Tris HCl	12.114 g
Distilled Water	100 mL
pH	6.8

8) **Monomer Solution**

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

9) Running(tank) buffer (1X)

25 mM Tris base	3.02 g
250 mM Gycine	18.7675 g
Distilled Water	200 mL

10) Bromophenol Blue (1%)

Bromophenol Blue	0.1 g
Distilled Water	10 mL

11) Ammonium Persulfate (10%)

Ammonium Persulfate	0.1 g
Distilled Water	1 mL

12) Sample loading buffer(1X)

Stacking gel buffer	1 mL
0.2% Bromophenol blue	2 mL
10% Glycerol	2 mL

13) Staining solution

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

14) Destaining solution

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

15) Colloidal chitin preparation

20g of chitin

↓

350ml concentrated HCl

↓

Keep overnight at 4°C.

↓

Add 2000ml of 95% ice cold ethanol to the mixture with rapid stirring

↓

Keep it overnight at -35°C

↓

Centrifuge at 5000rpm for 10 mins, and wash neutralize pH

↓

Dry and use

Appendix 3: estimation methods:

I) Protien assay for esterase

Stand curve

Stock solution: pNP para nitro phenyl

10mM pNP solution was made by dissolving 0.013911g og pNP crystals in 10ml of 0.05M tris buffer. The tube was vortex for 30 seconds until the crystals are completely dissolved.

Diluent: 100mM phosphate buffer

Producer:

Conc. of 10mM of pNP were made using tris buffer.

↓

Total volume was made upto 1ml

↓

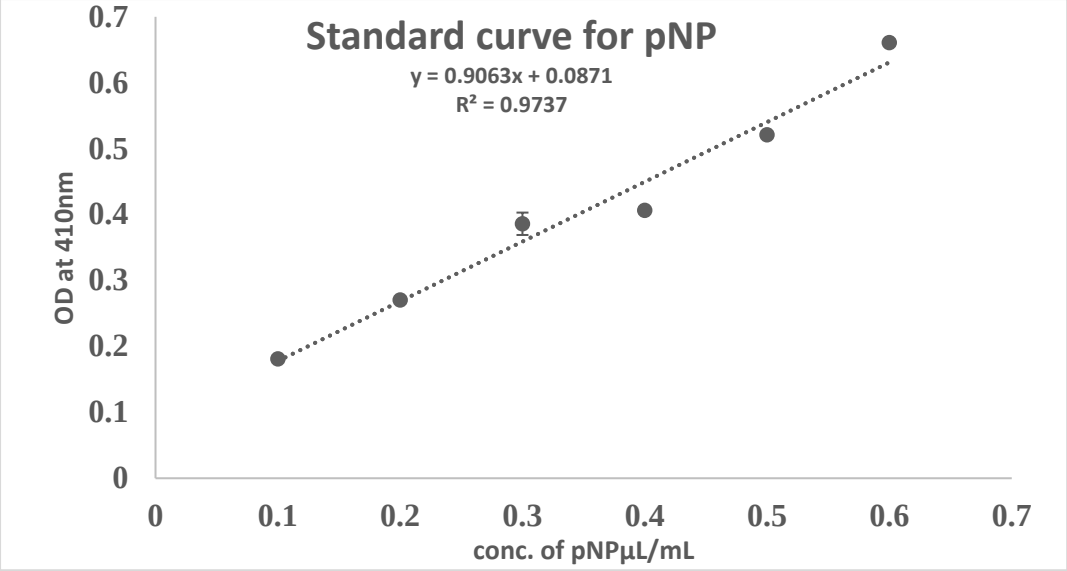
A mixture of 4:95 ethanol: 50mM phosphate buffer

↓

Incubate for 15 minutes at 55°C.

↓

Absorbance at 410 nm was measured



Standard curve for estimation of esterase enzyme activity.

Document Information

Analyzed document	INTRO_METHODS_RESULTS_PLA.docx (D164196833)
Submitted	2023-04-17 12:16:00
Submitted by	Bhakti Salgaonkar
Submitter email	bhakti@unigoa.ac.in
Similarity	0%
Analysis address	bhakti.salgaonkar.unigoa@analysis.urkund.com