Bioconversion of fish waste by halophilic microorganisms having chitinolytic and proteolytic activity

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By

MR. SAINIL VINOD NAIK

Seat number: 22P0420016

ABC ID: 225274461746

PRN: 201906021

Under the Supervision of

DR. BHAKTI SALGAONKAR

School of Biological Sciences and Biotechnology

Microbiology Programme

GOA UNIVERSITY
गोय विद्यापीठ

Goa University

Date: April 2024

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

Seal of the School

Examined by:

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Bioconversion of fish waste by halophilic microorganisms having chitinolytic and proteolytic activity"** is based on the results of investigations carried out by me in the Microbiology Programme at the School of Biological Sciences and Biotechnology, Goa University under the Supervision 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.

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Seat no: 22P0420016

Date: 08/04/2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Bioconversion of fish waste by halophilic microorganisms having chitinolytic and proteolytic activity"** is a bona fide work carried out by **Mr. Sainil Vinod Naik** under my supervision 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 Salgaonkar
Microbiology Discipline

Date: April 2024

Dean

Dr. Bernard Rodrigues

School of Biological Sciences and Biotechnology

School/Dept stamp

Dean of School of Biological Sciences & Biotechnology Goa University, Goa-403206

Date: 8/4/24

Place: Goa University

PREFACE

This study addresses the pressing issue of fish waste management, particularly focusing on crustacean waste in coastal regions like Goa, India. With fish curry rice being a staple food in the region, fishing is a primary occupation, leading to significant fish waste generation. Crustacean waste, rich in chitin and protein, poses a challenge due to its slow degradation and environmental impact. Current treatments, including physicochemical methods, have drawbacks such as harmful gas emissions. Hence, the utilization of halophilic microorganisms for waste processing emerges as a promising solution. This study aims to explore the biomineralization and bioremediation potential of halophilic microorganisms in degrading crustacean waste effectively. Through this study, we endeavour to contribute to sustainable waste management practices and mitigate environmental contamination in coastal areas.

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

Mr. Sainil Vinod Naik

22P0420016

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ABBREVIATIONS USED

Entity	Abbreviation
Alpha	α
Ammonium persulphate	APS
Beta	β
Cell free supernatant	CFS
Centimetre	cm
3,5-dinitrosalicylic acid	DNSA
Degree Celsius	°C
extremely Halophilic medium	EHM
Enzymatic index	EI
Fourier-transformed infrared spectra analysis	FTIR
Gross Domestic Product	GDP
Grams	g
Kilodalton	kDa
Litters	L
Microlitre	µL
Millilitre	mL
Milligram	mg
Molar	M
modified Moderately Halophilic Medium	MHM
NaCl tryptone yeast extract	NTYE
Norberg- Hofstein	NH
N-acetylglucosamine	NAG
Percent	%

Polyacrylamide gel electrophoresis	PAGE
Revolutions per minute	rpm
Shrimp waste control	SWC
Shrimp waste test	SWT
Scanning Electron Microscopy	SEM
Tetramethyl ethylenediamine	TEMED
Ultra violet	UV
volume/volume	v/v
Water Activity	A_w
weight/volume	w/v

ABSTRACT

A huge quantity of seafood waste is generated from fish processing plants, fish markets and seafood restaurants on daily basis. Crustacean waste is one of the major concerns as it is difficult to degrade and remains in environment causing pollution. Halophiles are organisms which thrive in high salinity environment like salt pans, salt marshes, salt lakes, saline soils. In the present study halophilic microorganisms were isolated from the Shiroda salt pan. Twelve halophilic isolates were screened for fish degrading enzymes such as chitinase, protease, gelatinase and lipase by plate assay method. Out of which five isolates were found to have chitinolytic activity. GUSN1 isolate was found to have best chitinolytic activity and was selected for further investigation. Salt tolerance study showed best growth at 15% NaCl concentration. Gram staining confirmed that GUSN1 isolate exhibited cocci shaped morphology and stained gram-negative Studies on chitinase enzyme extracted from the GUSN1 isolate was carried out by partially purifying the enzyme and characterising it using Native PAGE and zymogram analysis. The enzymatic activity of the chitinase enzyme was determined by performing enzymatic assay using colloidal chitin as a substrate. Submerged fermentation was carried out to determine the ability to degrade shrimp shell waste. GUSN1 showed significant degradation of shrimp shell waste in the NH broth, which was confirmed by FESEM and FTIR analysis. Release of N-acetyl glucosamine, % degradation and % demineralization was determined to check the efficiency of shrimp shell degradation.

CHAPTER I

INTRODUCTION

INTRODUCTION

1.1. Background

Goa, being the costal state of India, the staple food of people is fish curry rice. Hence one of the primary occupations of the locals is fishing. Fish consumption leads to generation of fish waste which includes the fish processed thresh as well as the non-eatable catch. One of the major concerns of the fish waste is the Crustacean waste which is difficult to degrade and remains in the environment for long as compared to the proteinaceous counterpart. A total of 12–14 million tons of crustacean debris are produced worldwide, with 60,000–80,0000 tons coming from India alone (Yadav et al. 2019). This Crustacean waste is composed majorly of chitin, protein, and other minerals such as calcium carbonate in minimal amount. This waste when disposed untreated leads to foul smell and propagation of pathogenic microorganisms resulting in soil and water contamination, ultimately leading to disturbance in the ecosystem. crustacean waste is primarily treated using two methods, the physicochemical techniques and the biological method using microorganisms. The chemical treatment releases harmful gases causing global warming that leads to environmental problems. Therefor processing this waste using halophilic microorganisms is of primary concern. Furthermore, an in-depth study of the complete degradation of fish scale, prawn shell, and crab shell employing halophilic microorganisms has not yet been carried out. Biomineralization and bioremediation of crustacean waste using halophilic microorganisms having potential of producing chitinolytic and proteolytic activity will be the target of this study. Employing halophilic microorganisms has added advantage the crustacean waste as it is marine originated and therefor employing salt loving organisms will treat this waste best as compared to its non-halophilic mesophilic counterparts.

1.1 Aims and Objectives

i. Isolation and screening of halophilic microorganism from hypersaline region for extracellular hydrolytic enzymes (chitinase, protease, gelatinase).

ii. Partial characterization of promising enzyme producing halophilic isolate and partial purification a of the promising enzyme.

iii. To determine the ability of the halophilic isolate to degrade fish waste.

1.2 Hypothesis/Research Questions

In Goa, majority of the population consume fish this leads to the generation of fish waste on daily basis. Crustacean waste is one of the major concerns which contains chitin, protein and calcium carbonate as major components. This waste can therefore be bioremediated using microorganisms having potential to produce proteolytic and chitinolytic activities so as to manage sea food waste to reduce environmental contamination.

1.3 Scope

i. **Application in Fish Waste Management and Bioremediation**: By employing halophilic microorganisms' ability to degrade shrimp shell waste could offer environmentally friendly solutions for the disposal of seafood processing by-products.

ii. **Application of seafood waste for bioethanol production:** seafood waste can be biodegraded for sustainable production of biofuel without harming the environment

iii. **Medical applications:** The by-products of chitin degradation such as N-acetylglucosamine, Chito oligosaccharides, glucosamine and chitosan can be used in human medicines as drug against inflammations.

CHAPTER II

LITERATURE REVIEW

LITERATURE REVIEW

2.1.Fish industry in India

India's coastline spans more than 8000 kilometres, with a small number of islands included in its borders. Nine coastal states and over 60 districts make up the coast line. one third of India’s population relies on marine resources with a substantial fine fish and shellfish intake (Senapati and Gupta, 2014). According to Saha et al., 2021 In India, the average yearly per capita fish consumption is between 5 and 10 kilograms, with approximately 56% of the population being fish consumers. India is the third largest fish producer in the world contributing to 1% of its Gross Domestic Product (GDP) (Government of India, 2019 a) with a total production of 13.7 million metric tones as of 2018-19 (Government of India, 2019 b).

2.1.1. Fish industry in Goa

Goa which is situated on the central west coast of India and has a costal length of 104 Kms (1.28% of Indian coast line) and comprises of two costal districts namely, South Goa and North Goa (Lekshmi et al., 2020). Goa is well known across the globe for its hospitality and fish curry rice. The fried fish, fish curry and rice are the staple food of majority of Goans with annual average per capita consumption of fish being 15-17 kg (Parab, 2021). As of 2018 the marine fish landings of Goa was 0.59 lakh tonnes which contribute to about 1.85% of the total marine fish landings India (Ravikanth and Kumar, 2015). This marine fish landing recorded about 550 different types of fish and shellfish (Shreekant and Mujawar 2021).

2.2. Source of seafood waste generation in Goa

Goa being a famous tourist destination where seafood industry drives the hotel industry the demand for seafood has increased significantly which has caused a significant increase in

seafood waste generation. During seafood processing mostly the meat is utilized while head, tail, shells, fines, skins and bones are thrown as waste (pal et al., 2014). In general, seafood waste is generated from different parts i.e. commercial local fish markets, fish processing plants, restaurants, households, fish shores, fishing at the sea and aquaculture activities (Ravanipour et al.,2021). The major fish landing sites of goa are depicted in Fig.1. Atlas Fisheries private Limited located in Old Goa is one of the major fish processing plants in Goa.

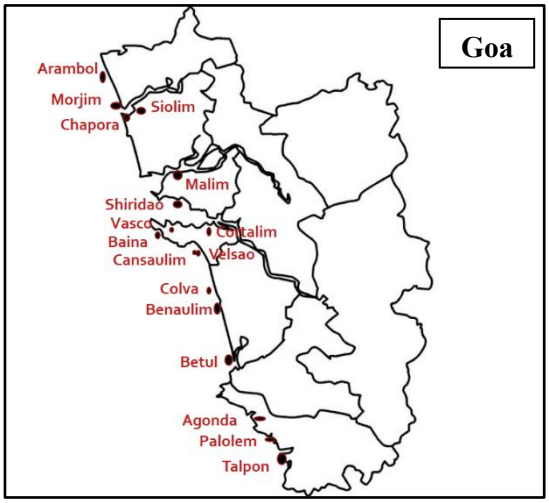

Fig.1: Major fish landing sites of Goa (Shreekanth et al., 2015).

Table.1: fish processing plants in Goa

Company	Location	Purpose
Atlas Fisheries Pvt. Ltd	Old Goa, Panaji, North Goa	Manufacture and export of frozen fish.
National Agro Farms	Ponda, North Goa	Manufacture and supply of prawns.
Export Trade centre	Salem, Bicholim, North Goa	Export of dry fish.
Ulka Seafoods Pvt. Ltd	Goa Velha, North Goa	Export of sea foods

2.3. Impact of seafood waste

Commercial processing of fishery results in the generation of huge amounts of waste which include both solid wastes along with wastewater which are unutilized and are either carried by municipalities which most of them are buried in landfills or dumped near shore causing environmental pollution (Bozzano and Sarda., 2002). This causes fisheries waste to accumulate over time, polluting coastal and marine ecosystems with unpleasant scents which attract flies creating unhygienic atmosphere and the release of biogenic amines that harm marine life (Xu et al., 2008). The seafood waste discarded near the coastline can have a negative impact on the aesthetic values of the beaches for marine tourism visitors (Mcllgorm et al., 2022). This causes decline in the tourist comfort value of beaches affects resort perceptions and influences vacation destination selection, which may result in a loss of revenue for the tourism industry of Goa.

2.4.Characterization of fish waste

The process of processing fish involves eliminating its internal organs, heads, scales, and bones. The seafood waste can broadly be classified as fine fish waste and crustacean waste. From the findings of (Younes & Rinaudo, 2015) fish scale consists of protein, calcium phosphate, calcium carbonate, magnesium carbonate, chitin, and pigments. When these crustaceans are processed, their exoskeletons are removed and often disposed of as waste. Worldwide, 12–14 million tons of waste from crustaceans' shells are produced each year; of this waste, approximately 3/4 of the total dry weight is made up of the exoskeleton, which is produced during peeling and processing (Yadav et al. 2019). Only in India approximately 60,000-80,0000 tons of chitinous waste are produced by crustacean shell waste. These waste products from crustaceans are diverse in nature with major component being chitin, a naturally

occurring polymer giving shells their tensile strength. Table 2 summarises the composition of crustacean shells. Pigments and a trace amount of lipids are also present in the shells (Pal et al., 2021). Cho et al., 1998 reported that with varying species and seasons the composition of the crustacean shells also varies.

Table.2: Composition of crustacean shells.

source	% Protein	% Ash	% Chitin	% moisture
Shrimp shells	32.7	32.6	36.4	45.6
Crab shells	34	42.2	23.7	-
Mussel shells	9.9	23.2	23.5	-

2.4.1. Chitin

Chitin a major component of crustacean shells is a natural polysaccharide composed of β-1,4-Nacetylglucosamine (Fig.2). The crystalline, stable, rigid and water-insoluble structure of chitin is a result of the N-acetylglucosamine polymer chain, that contains hydrogen bonds between molecules (Dliyauddine et al., 2020). Chitin is ranked second in abundance in nature after cellulose and is synthesized by enormous number of living organisms (Younes & Rinaudo., 2015).

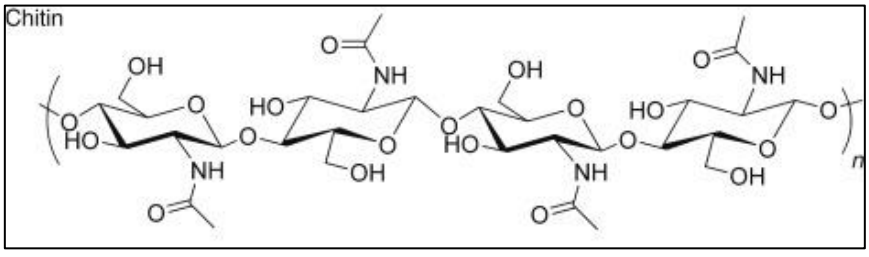

Fig.2: Structure of chitin.

2.5. Microbial degradation of fish waste

The environment, public health and economy can all be at risk from the inappropriate and unregulated disposal of seafood waste, thus measuring and managing it should be of the utmost importance. (Ebenezer et al., 2020). In fish processing industries chemical methods such as acid or alkali treatment and heat treatment are used to degrade seafood waste. The products released after such treatment are hazardous and can cause environmental damage (Arvanitoyannis and kassaveti., 2008). Chemical treatment releases harmful gases causing global warming in addition to this it has several other disadvantages as it requires high concentration of acids and bases and high energy (Saini et al.,2020). To overcome the hazards from chemical treatment an alternative method is the use of microorganisms or their enzymes to treat the crustacean shell waste. When compared to physicochemical techniques, bioremediation is preferred because it is more cost-effective, secure, environmentally friendly, and capable of recovering the most amount of pigment, lipids, chitin, chitosan, Chito oligosaccharides, and protein hydrolysate which can be utilized to make high-quality products (Cheba et al., 2018). Therefore, bioremediated of crustacean waste using microorganisms having potential of producing chitinolytic and proteolytic activity will be sustainable method to treat seafood waste (Samant et al., 2019).

Table.3: Microorganisms used in fish waste degradation

Microorganisms	**Type of fish waste**	**Enzyme activity**	**reference**
Bacillus cereus and *Exiguobacterium acetylicum*	Shrimp shell	proteolytic activity	Sorokulova., et al 2009
Serratia marcescens FS-3 strain	Crab shell	protease activity	Jo et al., 2008

Streptomyces anulatus CS242	Shrimp shells	Chitinase activity	Mander et al., 2016
Pseudomonas fluorescens	Shrimp shells	chitinase	Alhasawi and Appanna., 2017
Paenibacillus sp. AD	Shrimp waste	chitinase	Kumar et al., 2018
Lactobacillus paracasei and *Serratia marcescens* FS-3 strain	Chemically demineralized red crab shells	Protease activity	Jung et al., 2006
Lactobacillus plantarum and *Pseudomonas aeruginosa*	Crab shell and shrimp waste	Protease activity	Pal et al., 2014
Brevibacterium iodinum, Bacillus sp. and *Vibri*o sp	Crab shell, prawn shell and fish scale	Protease, cellulase and chitinase activity	Samant et al., 2019

2.6.Halophilic microorganisms used in fish waste degradation

2.6.1. Halophilic microorganism

Natural hypersaline environments, like salt lakes, solar salterns, and saline soils, are found all over the world. They have salinities that are significantly higher than those of seawater and nearly reach saturation (more than 30% NaCl concentration). Despite these extreme conditions, some microorganisms have been found to thrive in these environments. These microbes are known as halophiles or salt-loving microorganisms, because they can thrive in high salinities. Halophilic microorganisms are also exposed to o harmful UV radiations, fluctuating temperatures, pH, and low water availability (aw) (Ventosa et al., 1998). Halophilic microorganisms are classified into 4 groups based on requirement of NaCl for its survival and growth. Microorganisms from all three domains of life inhabit hypersaline environments.

Table.4: Categories of halophilic microorganisms based on their optimum salt requirement

Categories	**NaCl requirement Molarity (M)**
Slightly halophilic	0.2-0.5 M
Moderately halophilic	0.5-2.5 M
Extremely halophilic	2.5-5.2 M
halotolerant	These are class of microorganisms which does not require salt for its growth but are able to survive in wide range of salinity

2.6.2. Extremozymes from halophiles

The halophilic enzymes have less hydrophobic residues on their surface, are negatively charged, and contain a higher concentration of acidic amino acids. As a result, at high salt concentrations, their proteins are able to maintain a functional arrangement decrease surface hydrophobicity, and avoid aggregation (Slizewska et al., 2022). The enzymes obtained from halophiles have gained a lot of potential in industries and in other aspects due to their capacity to endure adverse conditions.

2.6.2. (a) Chitinase

Chitinase enzyme was first observed by Bernard. Chitinase enzymes belong to glycosyl hydrolases which hydrolyse the chitin into its small monomer unit's N-acetyl – D-glucosamine by breaking the glycosidic bonds (Fukamizo.,2000). Chitinolytic enzymes are broadly classified into two main classes: i) endo-chitinase (E. C. 3.2.1.14) that act on internal bonds in

a chitin chain; ii) exo-chitinase that act on chitin chain on non-reducing site (Saini et al.,2020). Exochitinase are further subdivided into two categories: chitobiosidase (E.C. 3.2.1.29) that cleaves alternate β-1,4 glycosyl linkage in chitin from the non-reducing end and produces chitobiose as the end product; β-(1, 4)-N-acetyl glucosaminidase (E.C 3.2.1.30) β-(1,4)-N-acetyl glucosaminidase cleaves β-1,4 glycosyl linkage in chitin from non-reducing end producing Gl_CNA_C (Dukariya and Kumar., 2020). There are only few halophilic organisms reported to produce chitinase. *Halomicrobium* sp. isolated from hypersaline lake that produces salt tolerant chitinase (Sorokin et al., 2015). *Aspergillus awamori* producing halotolerant chitinase was reported by Esawy et al. (2016).

Table.5: list of chitinase producing microorganisms

Microorganism		**Source of isolation**	**References**
Fungi	*Aspergillus terreus*	Marine water	Das et al., 2019
	Aspergillus awamori	Honey	Esawy et al., 2016
Bacteria	*Bacillus pumilus* JUBCH08	Beach sediment	Bhattacharya et al., 2016
	Paenibacillus elgii HOA73	Field soil	Kim et al., 2017
	Enterobacter sp.	Fresh water sediment	Tran et al., 2018
Archaea	*Pyrococcus furiosus*	Lake	Gong et al., 2018

	Halomicrobium sp.	Hypersaline lake	Sorokin et al., 2015
	Thermococcus kodakarenis KOD1	Volcanic area	Tanaka et al., 2004

2.6.2. (b) Protease

Proteases are the type of enzyme that breaks down proteins into smaller polypeptides or single amino acids by hydrolysing the peptide bonds present in proteins. Because they cleave peptide bonds, these enzymes can also be referred to as peptidases. The majority of proteases break the α peptide bonds that connect the amino acids (Akolkar and Desai, 2010).

2.6.2. (c) Gelatinase

Gelatinase is a metallopeptidase subtype of protease that hydrolyses gelatine and other proteins like collagen, fibrinogen, and casein into smaller peptides or individual amino acids. It has been proven to have medical uses in addition to being beneficial in treating animal and poultry waste (Hamza, et al., 2006).

2.6.2. (d) Lipases

Hydrolytic enzymes known as lipases primarily function on substrates that are soluble in water, such as triglycerides that include long-chain fatty acids. The lipase enzyme catalyses the hydrolysis of triacylglycerol to glycerol and other minor free fatty acids. Their primary applications are in the food, detergent, paper, pulp industries and can be used to treat fish waste (Hasan, et al., 2006)

CHAPTER III

METHODOLOGY

METHODOLOGY

3.1. 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, samples of sediment and brine were collected from the solar salterns of Shiroda, located in the state of Maharashtra- India (having latitude 15.7714418 and longitude 73.6712731) during the pre-monsoon summer. Samples of brine and sediment from salterns were retrieved from three distinct saltpans using clean bottles and zip-lock bags respectively. The pH and temperature of the samples was determined at the time of sampling.

3.2.Media and growth conditions

Four different media were used for selecting extremely halophilic microorganisms, 1. extremely Halophilic medium (EHM) having composition (g/L) NaCl 250.0; $MgSO_4.7H_2O$ 20.0; $CaCl_2$ $2H_2O$ 0.36; KCl 2.0; $NaHCO_3$ 0.06; NaBr 0.23; peptone 5.0; yeast extract 10.0; $FeCl_3.6H_2O$ in traces, 2. modified Moderately Halophilic Medium (MHM) having composition (g/L) NaCl 178.0; $MgSO_4.7H_2O$ 1.0; $CaCl_2$ $2.H_2O$ 2.0; KCl 2.0; $NaHCO_3$ 0.06; NaBr 0.23; peptone 5.0; yeast extract 10.0; $FeCl_3.6H_2O$ in traces, (Mani et al., 2012; Salgaonkar et al., 2019) 3. NaCl tryptone yeast extract (NTYE) medium having composition (g/L) NaCl 250.0 $MgSO_4.7H_2O$ 20.0; Yeast Extract 3.0; Tryptone 5.0; KCl 5.0; Agar 20.0, 4. NT medium having composition (g/L) NaCl 250.0; $MgSO_4.7H_2O$ 20.0; Yeast Extract 10.0; Tri-Sodium Citrate 3.0; KCl 2.0; Agar 20.0 with pH adjusted to 7.0 to 7.5 using 1M NaOH (Bragança and Furtado, 2009; Elevi et al., 2004). Self-sealing bags were used to incubate the plates for 35-40 days at room temperature until various shades of pigmented colonies appeared.

3.3.Isolation and Purification of the Halophilic microorganisms

Two strategies were used to isolate halophilic microorganisms: direct plating and enrichment method.

Direct plating: A loopful of sediment sample and 50 µl of brine sample was directly surface spread plated on the appropriate halophilic medium.

Enrichment technique: 1 g of sediment sample was aseptically introduced in to 50 ml of liquid minimal medium, i.e., Norberg and Hoefstein (NH) [Appendix I (1)] medium having 18% NaCl concentration, supplemented with fish waste and prawn shell waste as a sole source of carbon, following incubation for up to 15-30 days at room temperature. Next, 50 µl aliquots were spread plated on the appropriate halophilic medium. Plates were then incubated at room temperature for 30 to 45 days in self-sealing bags until the arrival of colonies (Salgaonkar and Rodrigues, 2019). Individual colonies were selected based on their pigmentation, size, shape, texture, margin and were transferred to fresh media plates. The cultures were purified by repeated streaking on the respective media using quadrant streak technique. The isolates were stored at room temperature and were sub cultured after every 45 days.

3.4.Screening for Crustacean waste degrading enzymes

Twelve isolates were screened for Crustacean waste degrading enzymes through a rapid plate assay method using Norberg and Hoefstein (NH) [Appendix I (1)] medium having 18% NaCl concentration. 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 based on the enzyme of intrest. Zone diameter was measured, and enzymatic index (EI) [(total diameter−colony diameter)/ colony diameter] was calculated (Vakkachan et al., 2023).

3.4.1. Screening for Chitinase activity

3.4.1.(a) Colloidal chitin preparation

10 g of chitin flakes were crushed and added to 150 ml of concentrated HCL and incubated at 4°C overnight. This mixture was then added to 1 L of ice-cold 95% ethanol with constant stirring and kept overnight at -35°C. This was followed by centrifugation at 5000 rpm for 20 minutes. The precipitate obtained was washed repeatedly with distilled water until its pH was 7 (Wiwat et al., 1999).

3.4.1.(b) Chitinase activity

The extracellular chitinase activity was detected by adding 0.5% (w/v) colloidal chitin in NH medium [Appendix I (1)]. A sterile nichrome loop was used to inoculate the isolates at the centre of the plate. Following a 15-day incubation period, the plates were flooded with 0.3% I_2-0.6% KI (w/v) KI solution [Appendix II (1)]. Formation of clear zone around the colony was the indication of positive chitinase test (Verma1 and Garg1, 2018).

3.4.2. Gelatinase activity

Assession of Gelatinase activity was performed using 0.5% (w/v) gelatin as a substrate in NH medium. The isolates were inoculated at the centre of the plate using sterile nichrome loop. Following a 15-day incubation period, the plates were flooded with 15% mercuric chloride acidified with 20%(v/v) concentrated HCl [Appendix II (3)]. A clearance zone around the colony indicated positive test for gelatinase (Menasria et al., 2018).

3.4.3. Protease activity

The screening of Proteolytic activity was done by supplementing of 0.5% (w/v) skimmed milk as a substrate in NH medium. With the help of sterile nichrome loop

cultures were inoculated on the plate. A clearance zone encircling the culture against white background indicated positive protease test (Rathakrishnan and Gopalan., 2022)

3.4.4. Lipase activity

The extracellular lipolytic activity was screened using 0.5% (v/v) olive oil in NH medium [Appendix I (1)]. With the help of a sterile nichrome loop the isolates were inoculated and incubated for 15 days. A turbid zone of precipitation around the growth of the isolate implied positive test for lipase (Mustafa and kaur, 2009).

3.5. Morphological characterization of the halophilic isolate GUSN1

3.5.1. Colony characteristics

To get isolated colonies, the halophilic isolate GUSN1 was surface streaked on MHM solid agar medium. Size, margin, shape, consistency, coloration, elevation, pigmentation and opacity of the colonies were measured.

3.5.2. Cell characteristics using microscopy

Smear of actively growing GUSN1 culture was prepared on clean grease free glass slide, air dried and heat-fixed. Desalting of the smear was done by washing the slide with 2% acetic acid solution followed by staining the smear with primary stain, crystal violet. The stain was then discarded and the smear was covered with Gram's iodine. The smear was rinsed with water and decolorized with 70% ethanol. Finally, it was counter stained with safranine, rinsed with water and dried. The slide was then examined under oil immersion objective (100 X) of the phase contrast microscope (AXIOM CL20) **(**Dussault, 1955).

3.6. Study of the tolerance of GUSN1 to NaCl

MHM agar medium [Appendix I (3)] supplemented with different NaCl concentrations were used to check the salt tolerance capacity of the GUSN1 isolate. The GUSN1 culture grown on MHM medium was streaked using quadrant streaking on MHM agar plates

containing 5%, 10%, 15%, 20% and 25% NaCl (w/v) concentration. The plates were incubated at room temperature for 15 days and visible growth was monitored.

3.7.Studies on the best fish waste degrading enzyme

3.7.1. Preparation of starter culture

Starter culture of the GUSN11 isolate was prepared by inoculating a loopful of the purified culture from the MHM agar plate in 25 ml of the sterile MHM broth contained in 50 ml capacity Erlenmeyer flask and was incubated at 37°C on shaker incubator at 100rpm.

3.7.2. Studies on growth and chitinolytic activity

Five percent of the actively grown GUSN1 culture in MHM was used as starter culture and was inoculated in 250 ml NH medium broth contained in 500 ml Erlenmeyer flask. It was supplemented with 0.5% (w/v) colloidal chitin. The flask was incubated at 37°C at 100 rpm for 25-30 days. After the growth of the culture, the culture was centrifuged at 8,000rpm for 20 mins at 4°C. The pellet was discarded and the cell free supernatant (CFS) was obtained. The chitinolytic activity of the CFS was measured using similar technique as Ramirez et al. (2004). 1:1 mixture (v/v) of CFS and 10% (w/v) colloidal chitin in 50mM tris HCL buffer pH 7 [Appendix II (6)] was incubated for 1 h at 45°C. Enzyme control with 1:1 mixture (v/v) of CFS and tris HCL buffer; substrate control with 1:1 mixture (v/v) of 10% (w/v) colloidal chitin in 50mM tris HCL buffer and tris HCL buffer were incubated for 1 h at 45°C with the test. The reaction was stopped by the addition of 1 ml 1% NaOH. The concentration of reaction products was determined by 3,5-dinitrosalicylic acid (DNSA) assay, with NAG as a reference compound and the absorbance was measured at 540 nm. The chitinase activity was defined as the amount of enzyme required to produce 1 μmol N-acetylglucosamine per hour per millilitre of crude enzyme extract.

3.7.3. Partial purification of chitinase enzyme and its activity

The GUSN1 culture grown in NH media broth supplemented with 0.5% colloidal chitin was used for partial purification of the chitinase enzyme. 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 chitinase enzyme purification. 80%(v/v) Prechilled ethanol was added slowly to the CFS and kept for 2 hours at 4°C. Following which the ethanol-CFS mixture was centrifuged at 10,000rpm for 30 mins at 4°C. The supernatant was discarded and the precipitate was dissolved in 5 ml 50 mM tris HCL buffer (pH 7.5) and this was used as chitinase crude extract (Salgaonkar et al., 2019). The activity of the partially purified chitinase enzymes was determined as mentioned in 3.7.2 by replacing the CFS with the chitinase crude extract.

3.8 Native-Polyacrylamide gel electrophoresis (PAGE)

3.8.1. Preparation of the gel

Native PAGE was performed of the CFS and partially purified chitinase 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 (7)] 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) [Appendix II (12)] and N,N,N',N'-Tetramethyl ethylenediamine (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 (8)] 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 (10)] was poured in the unit so that the gel along with wells submerge completely in the buffer (Nowakowski et al.,2014).

3.8.2. Running of the gel for protein separation

The marker containing 10 µl each of the standard protein solution i.e., amylase (56kDa), Bovine serum albumin (BSA) (66 kDa), biotin (244.31 Da) was mixed with 20 µl sample loading solution [Appendix II (13)] and loaded in the marker wells. 30 µl of the partially purified chitinase enzyme was mixed with 20 µl of sampling loading solution and was loaded in the enzyme lane. 30 µl of the CFS was mixed with 20 µl of sampling loading solution and was loaded in the CFS 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 at 100v.

3.8.3. Staining of the gel

The electrophoretic run was stopped when the tracking dye reached almost 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 (14)] which was destained the next day using destaining solution [Appendix II (15)]. 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. 1% agar supplemented with 1% colloidal chitin in distilled water was prepared to detect the enzyme activity by zymogram analysis. The

colloidal chitin agar was overlayed onto the polyacrylamide gel in a petri plate followed by incubation at 37°C overnight. Zone of hydrolysis exhibiting chitinolytic activity were visualized by flooding the plate with 0.3% I_2-0.6% KI (w/v) KI solution (Ramirez et al., 2004).

3.9.Biomineralization of Fish waste using GUSN1 isolate

3.9.1. Procurement of the fish waste

Fresh samples of crustacean shell waste of *Fenneropenaeus indicus* and other finfish waste were collected from the peeling sheds of a commercial seafood market in Mapusa, Goa. The samples were washed thoroughly with tap water and stored at -20°C until used.

3.9.2. Proximate composition of shrimp shell

3.9.2.(a) Moisture content

Standard techniques were used to determine the moisture content (AOAC, 2003). In a dry, spotless crucible, 1 g of the material was precisely weighed. For 10 to 12 hours, the crucible was kept in an oven between 100 and 105°C until a steady weight was achieved. After 30 minutes of cooling in the desiccator, the dried sample's final weight was noted. The following formula was used to get the moisture content percentage:

$$\text{Moisture (\%)} = \frac{\text{weight original sample} - \text{weight of dried sample}}{\text{weight of original sampele}} \times 100$$

3.9.2.(b) Crude ash content

The ash content was measured using the standard AOAC (AOAC 2000) procedures, which involved heating an empty, clean crucible to 600°C for one hour, cooling it in a desiccator, and then weighing it (W1). One gram of sample was added to the crucible (W2). After that, the crucible was kept in a muffle furnace for three hours at 550°C. The

crucible was cooled and weighed (W3) (Rao et al., 2000). The following formula was used to get the percent of ash:

$$\text{Ash (\%)} = \frac{\text{difference in weight of ash}}{\text{weight of sampele}} \times 100$$

Difference in weight of ash = W3 – W1

3.9.3. Degradation of the fish waste through fermentation strategy

Submerged fermentation was carried out in 500ml flask containing 250ml minimal medium, i.e., Norberg and Hoefstein (NH) medium [Appendix I (1)]. The experiment was carried out in two sets; set A and set B with each set having 10% of (1) crustacean shell waste, (SW) (2) fish waste (FS), (3) crustacean shell waste plus fish waste (SW+FW), (4) control (only media). After sterilization 10% of the OD (OD600 = 0.4) GUSN1 culture inoculum was added to set A and was incubated for 20 days at 37 °C. Set B was kept as control without adding culture.

3.10 Evaluation of fish waste degradation potential of GUSN1 isolate

3.10.1. Monitoring release of N-acetyl D-glucosamine

The fish waste degradation potential of GUSN1 isolate was evaluated by monitoring the release of f N-acetyl D-glucosamine (Tasun et al. 1970). Two ml of the supernatant was centrifuged at 10,000 rpm for 10 minutes from the fermentation flasks after 15, 20 and 25 days. The pellet was discarded and one ml of the supernatant was transferred into fresh test tubes. To each tube one ml of DNSA reagent [Appendix II (16)] was added and were kept in boiling water bath for 5 minutes. 10 ml of distilled water was added to the tubes after colling and absorbance was taken at 540nm keeping distilled water as blank. The

concentration of the released f N-acetyl D-glucosamine was calculated from the standard DNSA graph using NAG as reference [Appendix III (1)].

3.10.2. Percent degradation of fish waste

To determine the percent degradation of the fish waste due to GUSN1 isolate the fermentation content was filtered through a muslin cloth at the end of incubation. The residue obtained was washed thoroughly using distilled water and dried at 80 °C overnight. The weight of the residue after drying was noted (Vakkachan et al., 2023). The percent degradation was then calculated through the equation:

$$\%\ \text{degradation} = \frac{\text{mass of original sample} - \text{mass of residue}}{\text{mass of original sampele}} \times 100$$

3.10.3. study of utilization of fish waste as substrate by GUSN1 culture

The growth of GUSN1 isolate using fish waste as the sole source of carbon 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 growth of GUSN1 was determined.

3.11Structural analysis of shrimp waste after treatment with GUSN1

3.11.1. Scanning Electron Microscopy (SEM)

Field emission scanning electron microscopic (FESEM) analysis was used to confirm the effect of GUSN1 on shrimp shell waste degradation. Samples of shrimp flakes of the degradation experiments (SWT) and the control conditions (SWC) were used. 100 mg of shrimp flakes were placed inside a clean 2 ml Eppendorf tubes. Desalting of the shrimp

flakes which was present in media containing salt was done using 2% acetic acid solution to remove excess salt (Dussault, 1955). 1 ml of Two percent glutaraldehyde which acts as a fixative was added and kept at room temperature for 12 hours. The samples were then dehydrated with an ascending series of acetone gradient ie. 10%, 30%, 50%, 70%, and 90% for 10 minutes each. The shrimp flakes were 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)

3.11.2. Fourier-transformed infrared spectra analysis (FTIR)

Samples of shrimp flakes of the degradation experiments (SWT) and the control conditions (SWC) were placed inside a clean 2 ml Eppendorf tubes and was washed thoroughly with distilled water. After drying the samples were embedded in KBr disk with Perkin Elmer-hydraulic press and subjected for FTIR with a Perkin Elmer-spectrum 400 FT-IR/FT-FIR spectrometer at room temperature. The graph was plotted with the help of the origin software (Kumar et al., 2018)

3.11.3. proximate composition

The moisture content and the crude ash of the shrimp flakes after treatment with GUSN1 (SWT) was determined as mentioned in section 3.9.2.(a) and 3.9.2.(b).

3.11.4. Demineralization (DM)

Demineralization (DM) was expressed as percentage and computed by the following formula as described by Ghorbel- Bellaaj et al 2012.

$$\mathrm{DM\%} = \frac{[(\mathrm{AO} \times \mathrm{O})] - (\mathrm{AR} \times \mathrm{R})]}{(\mathrm{AO} \times \mathrm{O})} \times 100$$

where AO and AR were the ash concentrations (g/g) before and after fermentation and O and R were the mass (g) of the original sample and the residue, respectively).

CHAPTER IV

ANALYSIS AND CONCLUSIONS

ANALYSIS AND CONCLUSION

4.1.Sampling site and sample collection

Samples were collected from a salt pan of Shiroda, Vengurla Taluka, Sindhudurg District, Maharashtra (latitude 15.7714418 and longitude 73.6712731) which is located along the west coast of India bordering the Arabian Sea (Fig:1). The sample were collected on 14th may 2023. Brine samples were collected in clean bottles and sediment samples were collected in zip-lock bag. (Fig:2) The pH of the salt pan was found to be alkaline (8) and the temperature was 42°C.

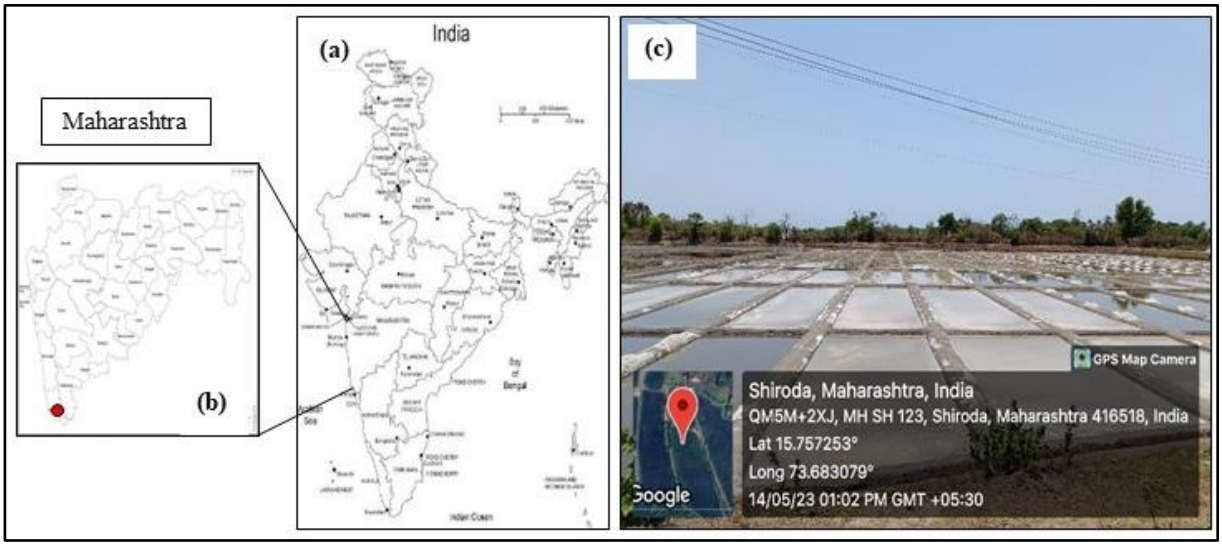

Fig.1: Sampling site: (a) map of India; (b) state of Maharashtra; (c) the Salt pan located at Shiroda, Maharashtra (highlighted in red dot).

Fig. 2: sample collection at the solar salterns of Shiroda.

4.2.Isolation of the Halophilic microorganisms

50µl of the brine sample and loopful of sediment sample was spread plated on NTYE, NT, MHM and EHM. The plates were put in self-sealing zip lock bags and thereafter incubated at room temperature. Over the course of five days of incubation, no growth was seen. However, after 15 days of incubation, a few non-pigmented colonies and colonies with a slight red pigmentation emerged. Table 1 represents the CFU/mL after 5 days, 15 days, and 35 days of incubation. The colonies with weak pigmentation became strongly pigmented after the plates were further incubated up to a period of 45 days (Fig:3). Some of the visually distinct cultures were purified as depicted in Fig: 4. The isolates were named as GUSN series which represents **G**oa **U**niversity **S**ainil **N**aik.

The enrichment samples were plated out and incubated for a period of 45 days and visually distinct cultures were purified by repeated streaking (Fig: 5). The isolates obtained were ES1, ES2, ES3, ES4, ES5, and ES6 were isolated and maintained in NH media supplemented with specific substrate. Table: 2 shows the details of each isolate with respect to the respective enrichment source, growth media, pigmentation and colony morphology.

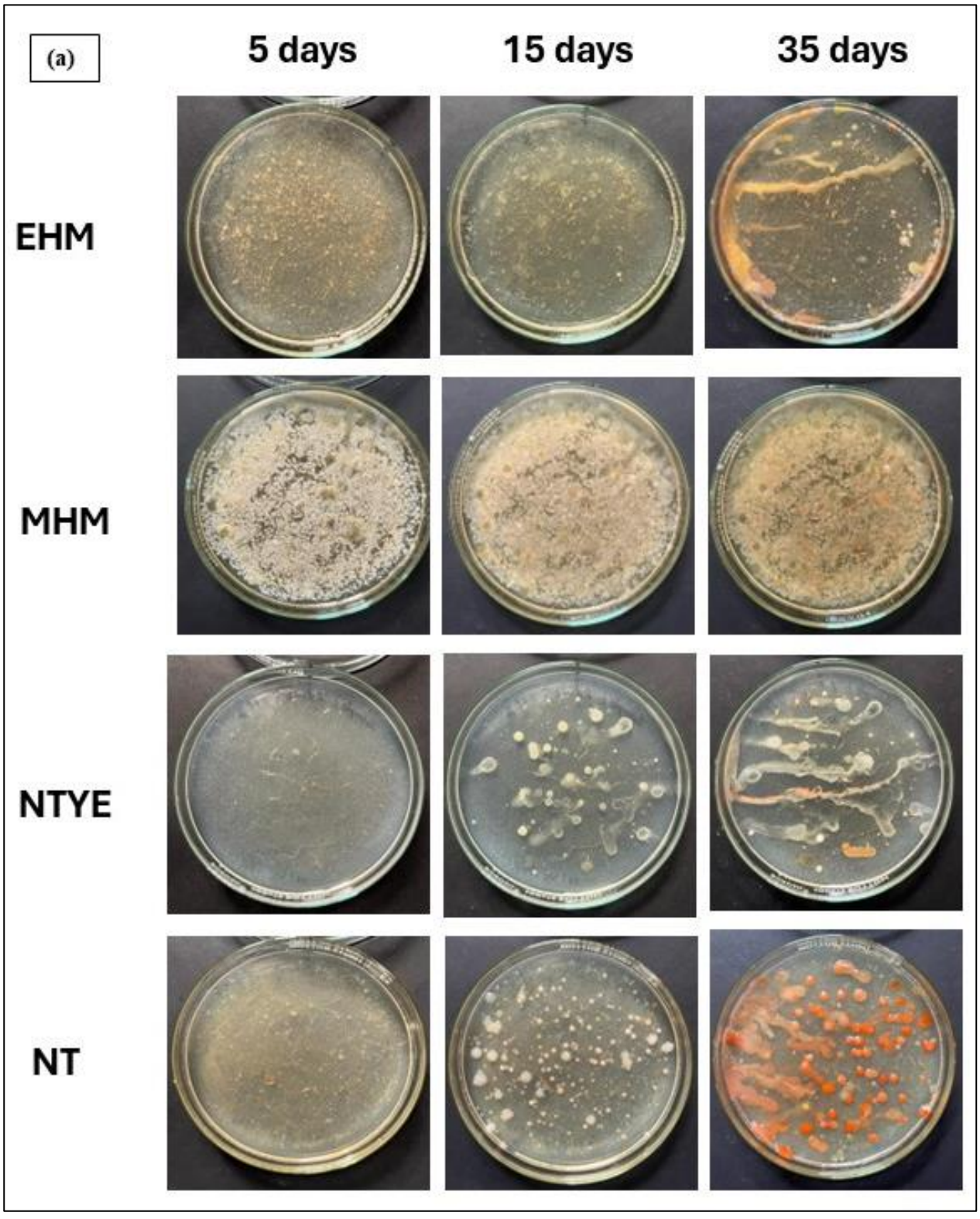

Fig. 3(a): Colonies of halophilic microorganisms obtained from Sediment sample of Shiroda salt pan after incubation for various time interval.

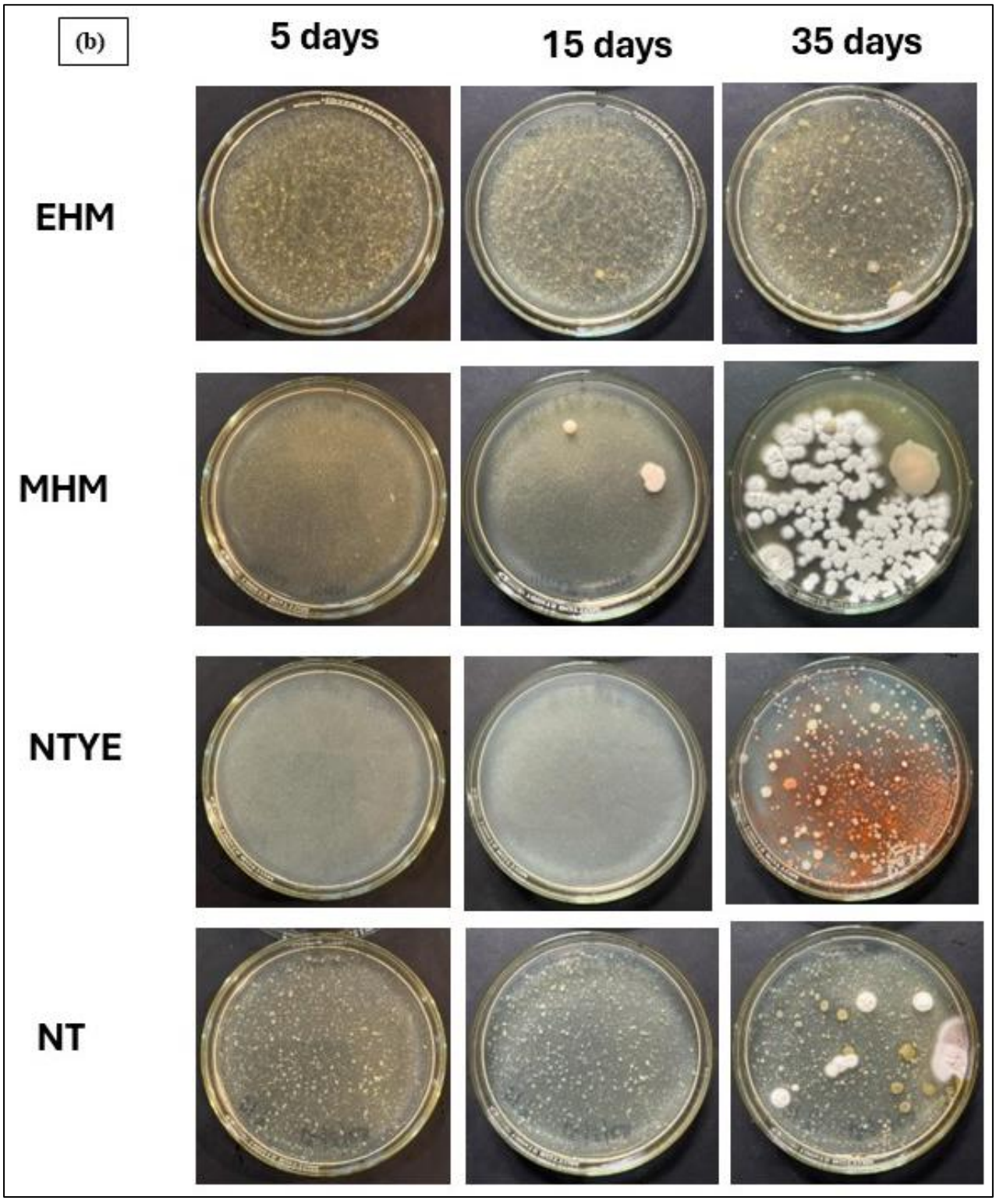

Fig. 3(b): Colonies of halophilic microorganisms obtained from Brine sample of Shiroda salt pan after incubation for various time interval.

Table 1: CFU/mL after 5 days, 15 days, and 35 days of incubation of brine and sediment sample obtained from Shiroda salt pan.

Days of incubation	5 days	15 days	35 days
	Viable cell count (CFU/ml) or (CFU/gm)		
Brine sample			
EHM	-	2×10^{1}	1.4×10^{2}
MHM	2×10^{1}	2×10^{2}	TNTC
NTYE	-	-	2.9×10^{3}
NT	-	-	7×10^{2}
Sediment sample			
EHM	1.2×10^{2}	1.8×10^{2}	1.4×10^{3}
MHM	TNTC	TNTC	TNTC
NTYE	2×10^{1}	6.8×10^{2}	2.4×10^{3}
NT	-	2.6×10^{3}	4.1×10^{3}

Key: - TNTC: too numerous to count; - no growth

Fig 4: Some of the purified halophilic cultures obtained from Shiroda salt pan.

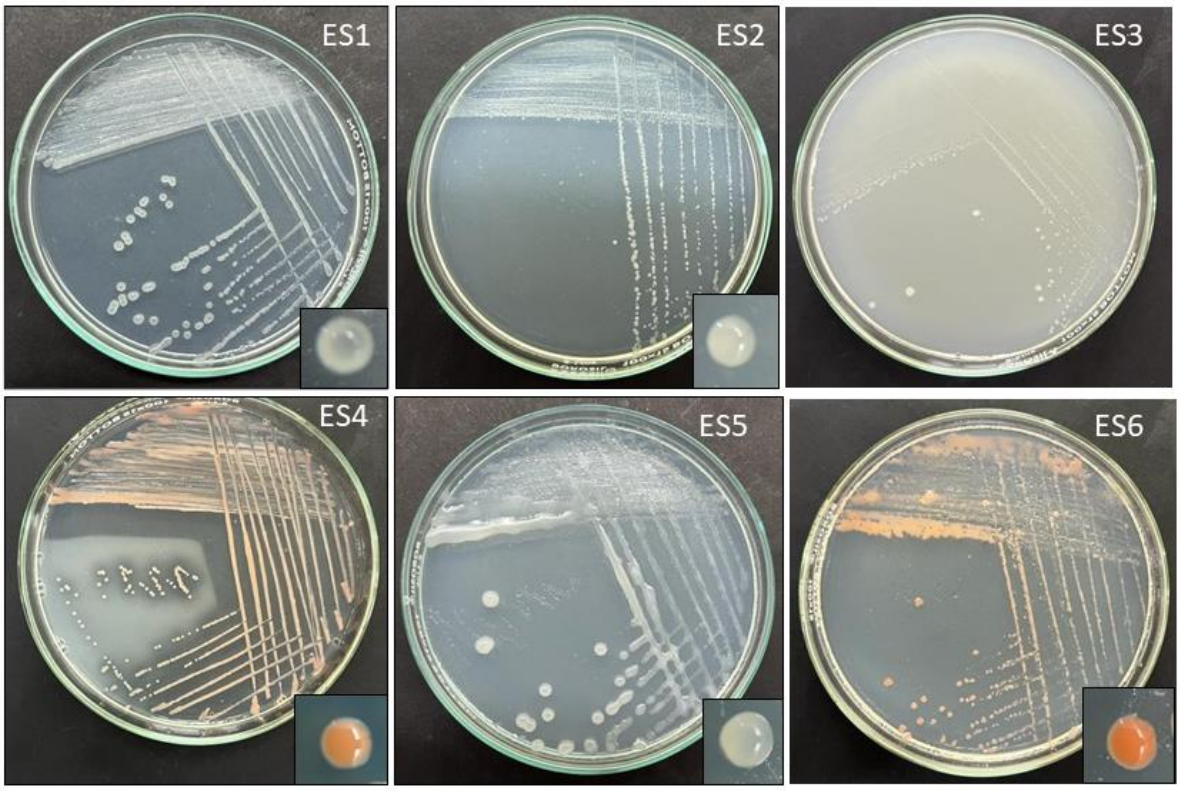

Fig: 5 Pure cultures of the halophilic microbial isolates obtained from enrichment sample.

Table. 2: Colony characteristics of halophilic microbial isolates and their enrichment source.

Isolate	Enrichment source	Growth media	Pigmentation	Colony morphology
ES1	Coconut oil cake	NH + colloidal chitin	Cream	Circular
ES2	Prawn shell	NH + colloidal chitin	Cream	Circular
ES3	Prawn shell	NH + skimmed milk	Cream	Circular
ES4	Sugar cane bagasse	NH + skimmed milk	Light orange	Circular
ES5	Fish waste	NH + olive oil	Cream	Circular
ES6	Sugar cane bagasse	NH + olive oil	Light orange	Circular

NH: Norberg & Hofestens

4.3.Screening of isolates for Crustacean waste degrading enzymes

The crustacean waste comprises of mainly 20%-40% chitin, 30%-40% protein, 30%-50% calcium carbonate and calcium phosphate (Samant et al., 2019). Twelve halophilic microbial cultures were screened for the production of chitinase, gelatinase, lipase and

protease using plate assay method. Table: 2 summarises the results of the Crustacean waste degrading enzymes of the Twelve isolates. The producers of chitinase were ES4, ES7, GUSN1, GUSN2 and GUSN4. Out of these, GUSN1 was found to be the best producers as indicated by the zone of clearance around the colony after adding iodine solution. The primary component of the crustacean shell waste is chitin, so choosing the right chitinase producing culture is an important step in the biomineralization of crustacean shell waste (Sabry 1992). The enzymatic index of the chitinase produced by GUSN1 is found to be 3.5 as shown in table: 4, which is higher than as previously described chitinase produced by *Priestia megaterium* (EI=2.2), *Bacillus subtilis* (EI=2.04) and *Bacillus amyloliquefaciens* (EI=2) (Vakkachan et al., 2023). The chitinolytic index of chitinase enzyme produced by *Bacillus cereus* strain MHS (EI=1.06), *Bacillus thuringiensis* strain YWC2-8 (EI=1.05), *Stenotrophomonas maltophilia* (EI=1.22) and *Enterobacter cloacae* strain 34978 (EI=3.4) as previously reported by Puspita et al., (2017) was lower than the enzymatic index of chitinase enzyme produced by GUSN1. The enzymatic index of protease and gelatinase produced by GUSN1 isolate was found to be 2 and 2.1 respectively. The GUSN1 culture was used as promising candidate for the crustacean waste degradation experiment because of its high chitinase producing potential and its co-production ability to produce chitinase, protease and gelatinase enzyme (fig: 6).

Table.3: Extracellular hydrolytic enzyme activity shown by the twelve isolates in NH medium containing 17% NaCl concentration and the following substrates.

Isolate	Extracellular hydrolytic enzymes (Substrate)			
	Chitinase (colloidal chitin)	Gelatinase (gelatin)	Protease (skimmed milk)	Lipase (olive oil)
ES1	-	-	-	-
ES2	-	-	-	-
ES3	-	-	-	-
ES4	+ (EI=3.1)	+ (EI=1.7)	+ (EI=1.8)	-
ES5	-	-	-	-
ES6	-	-	-	-
ES7	+ (EI=2.3)	-	+ (EI=1.4)	-
GUSN1	+ (EI=3.5)	+ (EI=2.1)	+ (EI=2)	-
GUSN2	+ (EI=2.1)	+ (EI=1.1)	-	-
GUSN3	-	-	-	-
GUSN4	+ (EI=2.4)	-	-	-

Keys: + indicates enzymatic activity, - Indicates no enzymatic activity, EI: enzymatic index, ES: enrichment sample,

Table.4. Enzymatic index of the enzymes produced by GUSN1.

Enzyme	Culture grown (diameter in cm)	Zone of clearance (diameter in cm)	Enzymatic index (EI)
Chitinase	1	4.5	3.5
gelatinase	0.8	2.5	2.1
protease	0.9	2.7	2

Key: cm: centimetre; EI: enzymatic index

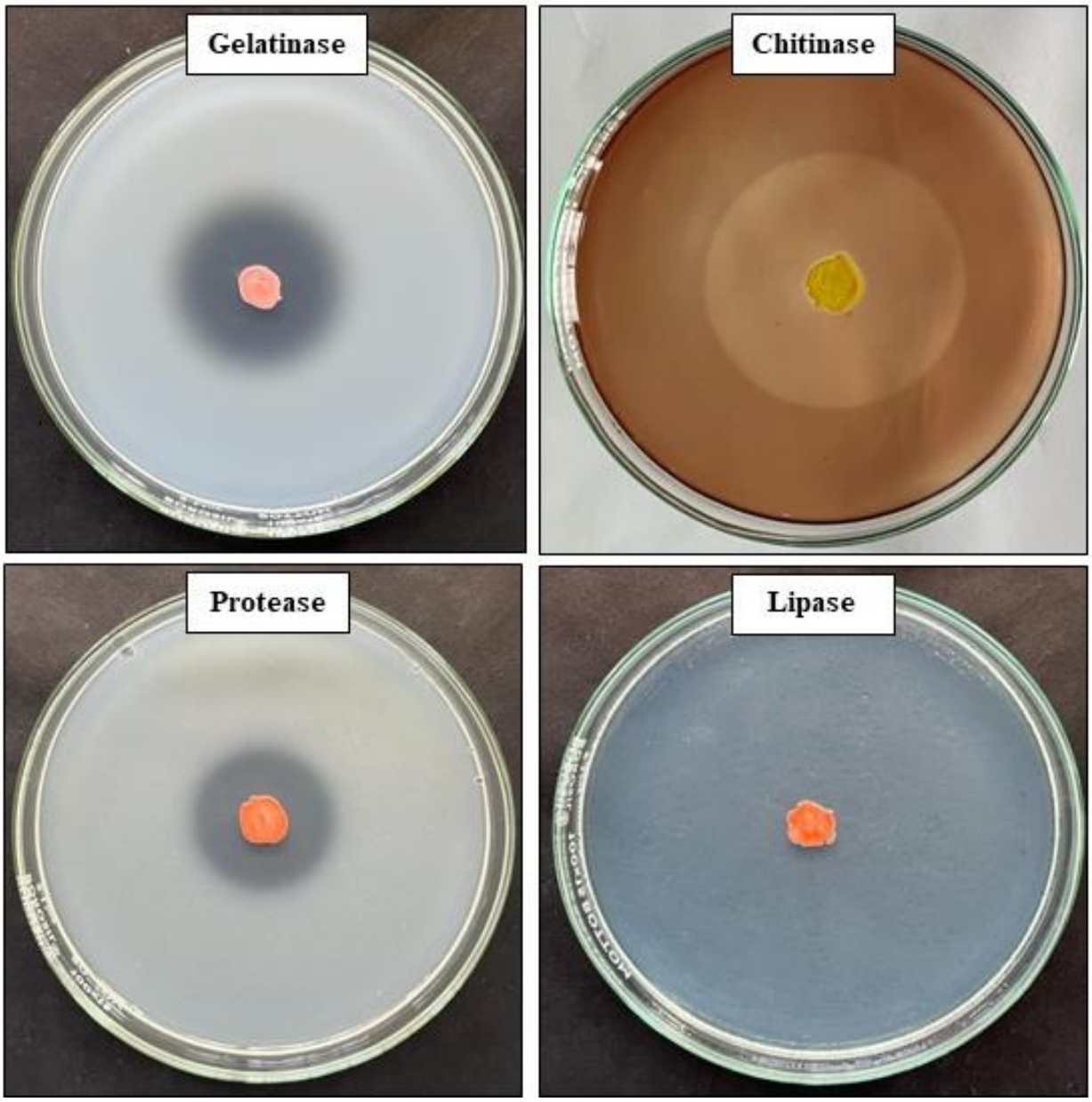

Fig. 6. Screening of GUSN1 culture for crustacean waste degrading enzymatic activity.

4.4.Morphological studies of GUSN1 isolate

4.4.1. Colony morphology of GUSN1 isolate

The morphology of the colonies was studied on MHM medium after two weeks of incubation. On the medium, the colonies (~0.3 mm) were circular, opaque, entire, smooth-edged, and red-pigmented (Fig: 7).

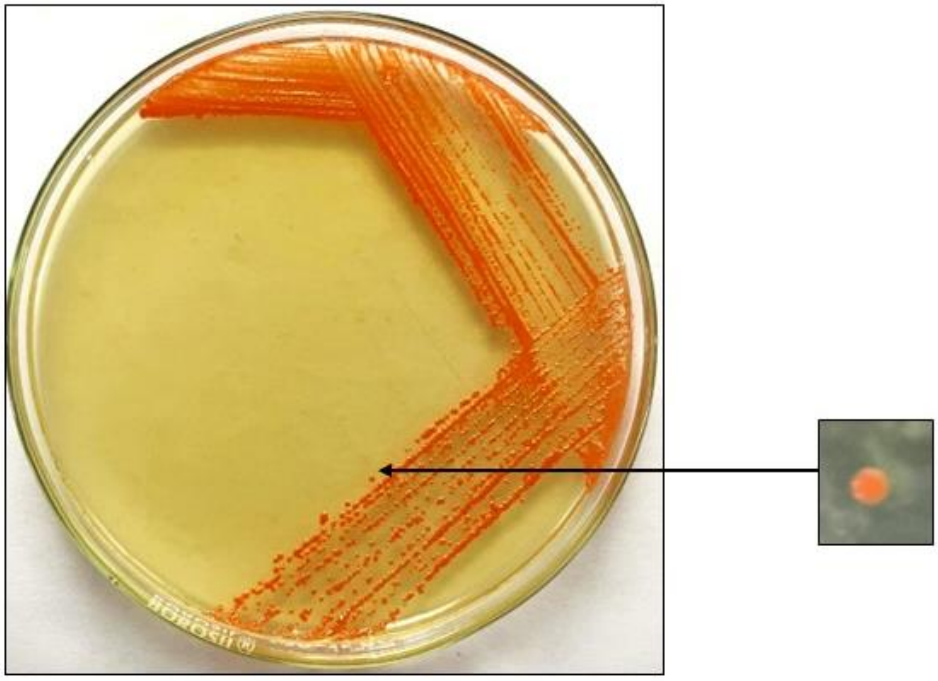

Fig.7: colony morphology of GUSN1 on MHM media.

4.4.2. Cell characteristics using microscopy

When examined under a phase contrast microscope (1000× magnification), the GUSN1 isolate showed signs of cocci shape and stained Gram negative (fig: 8).

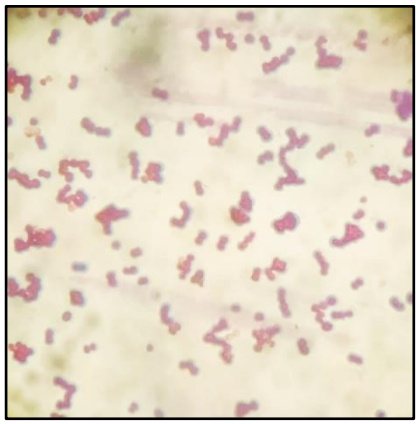

Fig. 8: Gram staining of GUSN1 culture grown on MHM medium.

4.5.Salt tolerance activity of GUSN1 isolate

The salt tolerance of the GUSN1 isolate was examined on MHM medium containing varying NaCl (w/v) concentration i.e.,5%, 10%, 15%, 20% and 25%. The culture was found

to be halotolerant as it showed growth on 10% NaCl concentration as well as on NaCl concentration up to 25%. salt concentrations of 15% to 25% showed abundant growth with the best growth observed at 15% NaCl Concentration with dark pigmented colonies (Fig: 9). It was also observed that, the growth of the isolate decreased with the decrease in NaCl concentration.

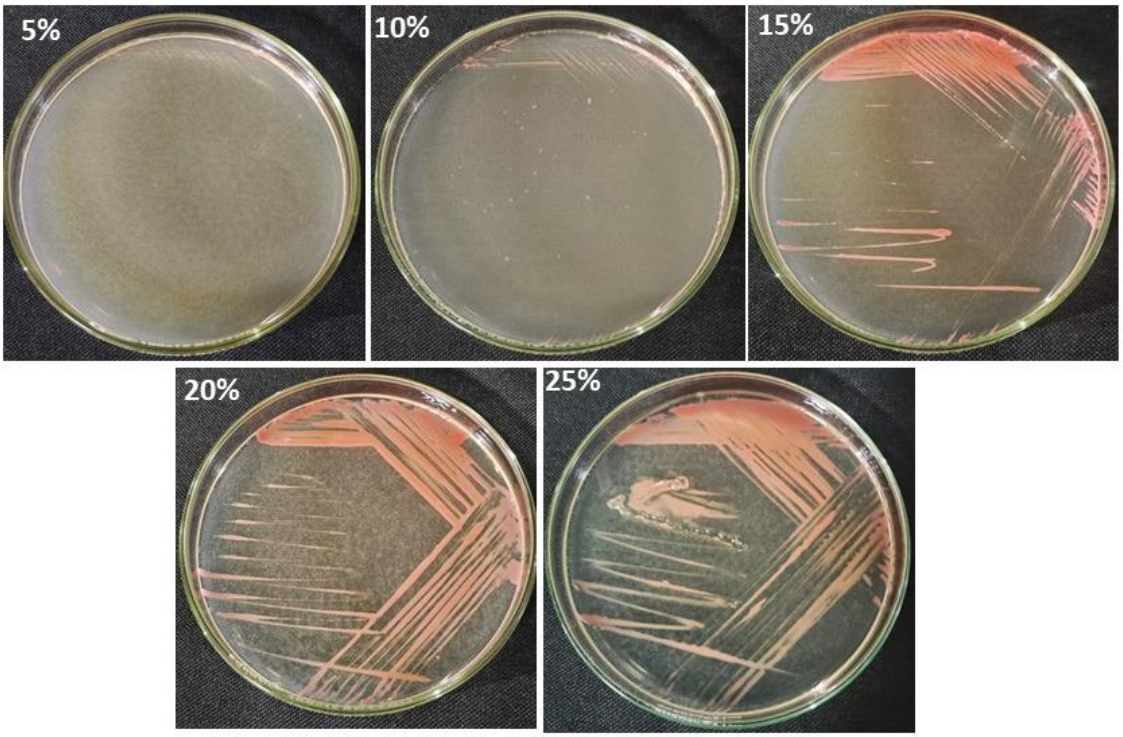

Fig. 9: Salt tolerance studies of GUSN1 isolate at 5%, 10%, 15%, 20% and 25% NaCl concentration after 15 days of incubation.

4.6.Studies on chitinase enzyme produced by GUSN1isolate

4.6.1. Growth of GUSN1 and chitinolytic activity of CFS

The GUSN1 culture inoculated in 25ml MHM containing 17% NaCl concentration. After 15 days of inoculation orange turbidity was observed (Fig:10 B). The chitinolytic activity of CFS of GUSN isolate at 17% salt concentration was found to be 5.613 U/ml which was similar to that of *Virgibacillus marismortui* M3-23 produced chitinase enzyme (Essghaier et al., 2011). These findings attest to the enzyme's halotolerant properties. The chitinase-producing *Planococcus rifitoensis*, as previously reported by

Essghaier et al. (2010) can produce in the absence of NaCl but its enzyme activity declined with increasing salinity. The activity of such enzymes at high salinity can be used in the biomineralization of fish waste as it has high salt concentration.

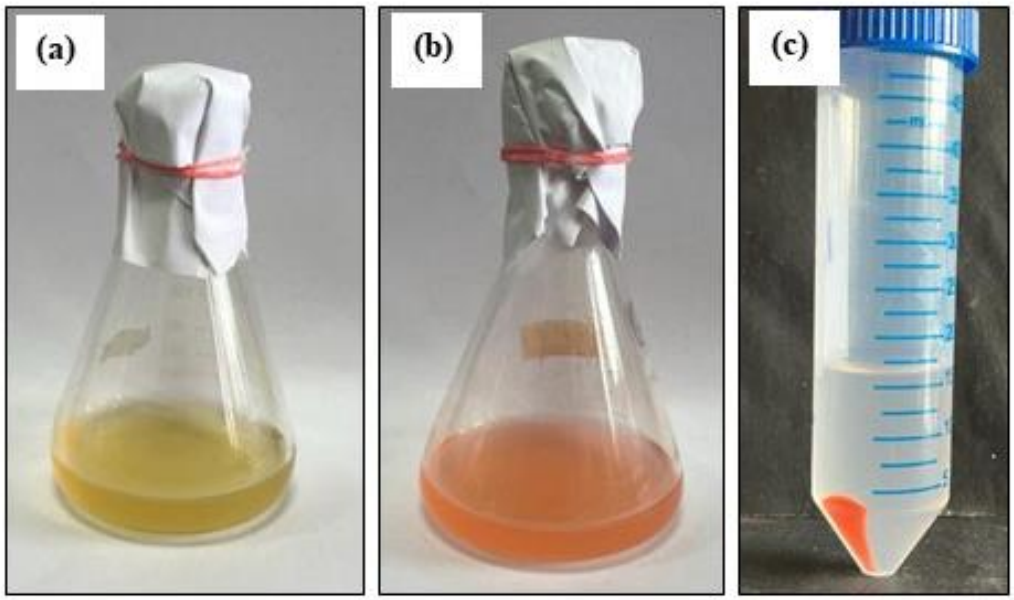

Fig.10: Growth of GUSN1 isolate in MHM broth containing 17% NaCl concentration (A) 0 day of incubation; (B) 15 days of incubation; (C) CFS obtained from GUSN1.

4.6.2. Partial purification of chitinase enzyme and its activity

The GUSN1 culture inoculated in 250ml NH containing 17% NaCl concentration supplemented with 0.5% colloidal chitin as a sole source of carbon for the production of chitinase enzyme (Fig:11 a). The chitinase enzyme was partially purified using the ice-cold ethanol extraction method (Fig: 11). The enzyme activity of the partially purified chitinase enzyme from GUSN1 isolate at 17% salt concentration was found to be 2.35 U/ml.

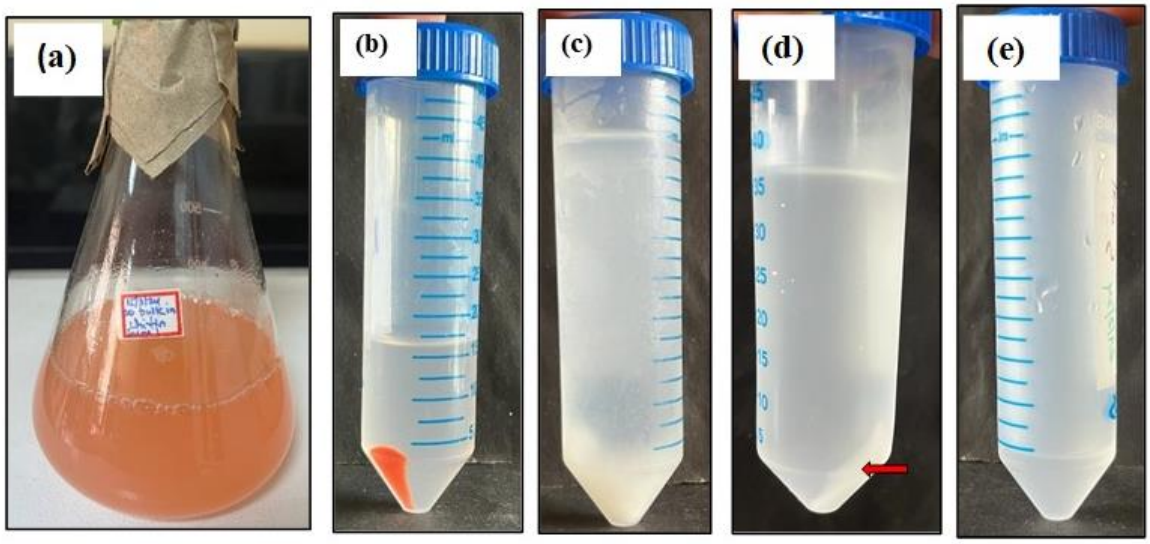

Fig. 11: Partial purification of chitinase enzyme using ice cold ethanol extraction method (a) GUSN1 isolate grown in Nh media containing colloidal chitin, (b) CFS obtained by centrifugation, (c) Addition of cold ethanol in CFS, (d) centrifugation of precipitate to extract enzyme (e) enzyme dissolved in tris HCL buffer.

4.7.Native PAGE and Zymogram analysis of chitinase enzyme

The native PAGE analysis of chitinase showed three bands in lane 3 i.e., marker lane (Fig: 12. a). In zymogram analysis zone of clearance in the lane 3 i.e., in enzyme lane (Fig: 12. b) after flooding with iodine indicated the presence of chitinolytic activity in the CFS.

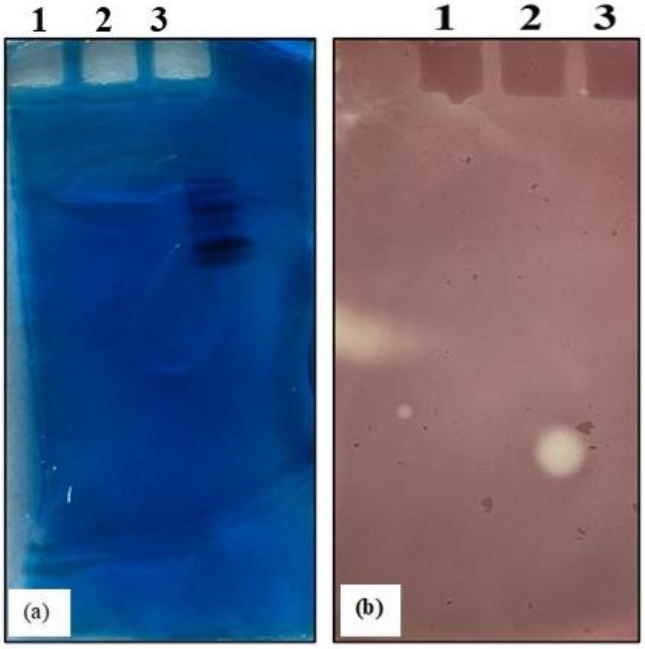

Figure. 12: a) Native-PAGE gel after staining 1: CFS lane, 2: enzyme lane, 3: marker lane (b) zymogram analysis using chromogenic molten agar 1: marker lane, 2: enzyme lane, 3: CFS lane.

4.8. Biomineralization of fish waste using GUSN1 isolate

4.8.1. Procurement of the fish waste

The seafood industries generate a large quantities of waste like shrimp shells, prawn waste, fish waste and the management of this massive waste is a challenge to the seafood processing industry's sustainability (Kumar et al. 2018). Fig. 13.a indicates the seafood waste generated at the local fish market located in Mapusa, Bardez taluka, Goa (15.5882° N, 73.8130° E). For this study crustacean shell waste of *Fenneropenaeus indicus* and other finfish was collected from Mapusa fish market (Fig 13.b).

Fig. 13: (a) fish waste generated at Mapusa fish market. (b) fish waste collected at Mapusa fish market.

4.8.2. Proximate composition of shrimp shell

Understanding the raw materials approximate composition is the first stage ithe biomineralization process. While the species, group, season, and several other factors affect the composition of Shrimp shell waste, as noted by Gorbel-Bellaaj et al., (2012). Moisture content and crude ash content of the shrimp shell used in the study was determined. The emergence of grey-white ash signifies the complete oxidation of all organic materials inside the sample (Fig: 14. b). The results obtained (Fig: 14. c) were within the range in the earlier reports of crustacean shell composition (Sathyaruban et al., 2019).

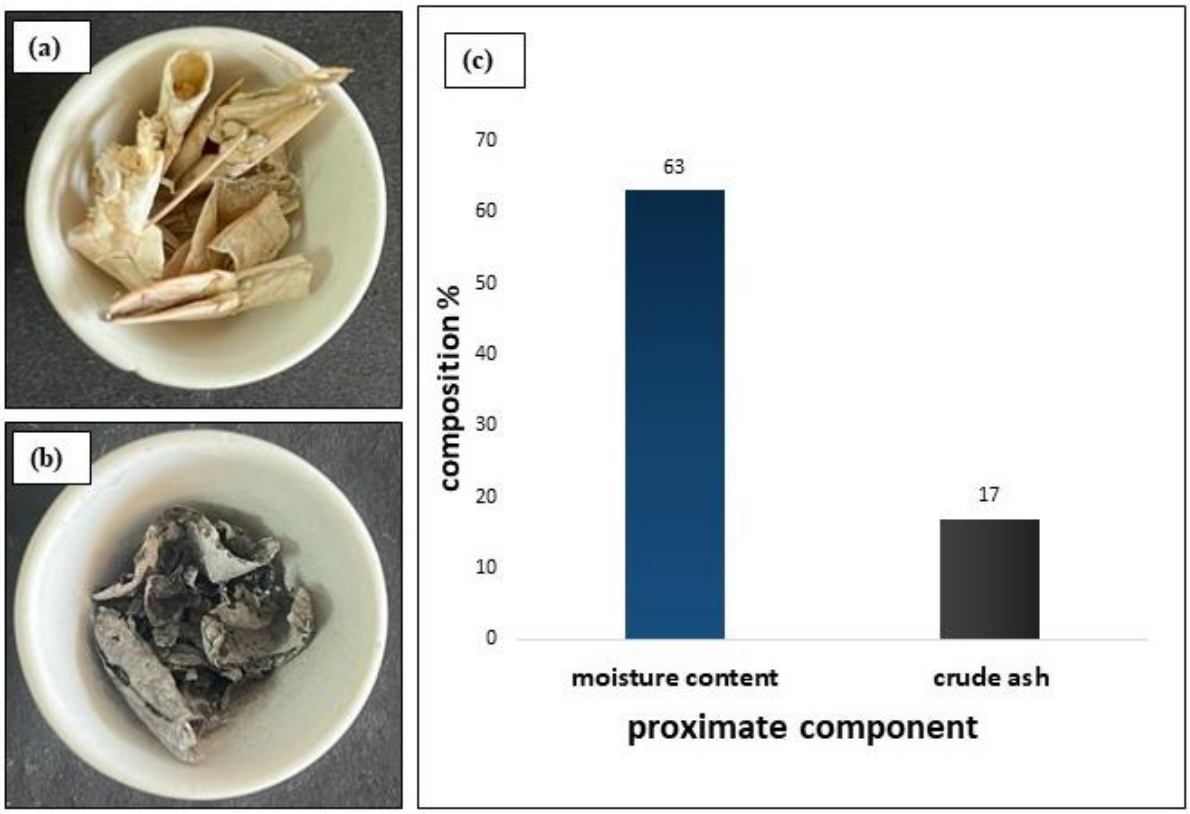

Fig. 14: (a) Shrimp shells after oven drying at 105°C; (b) Shrimp shells after heating at 550°C; (c) Proximate composition of shrimp shell.

4.8.3. Degradation of fish waste through fermentation strategy

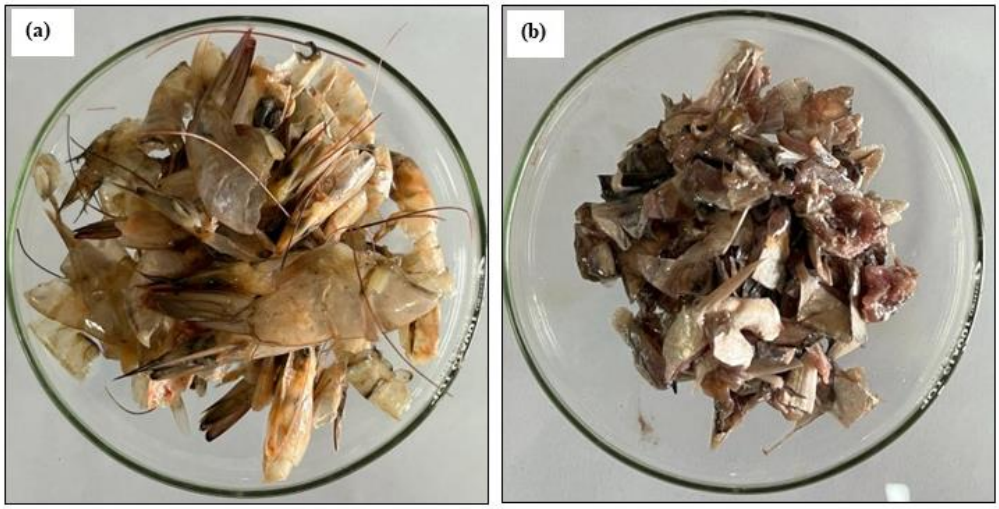

Fig. 15: Fish waste used in study: (a) Shrimp shell waste; (b) finfish waste.

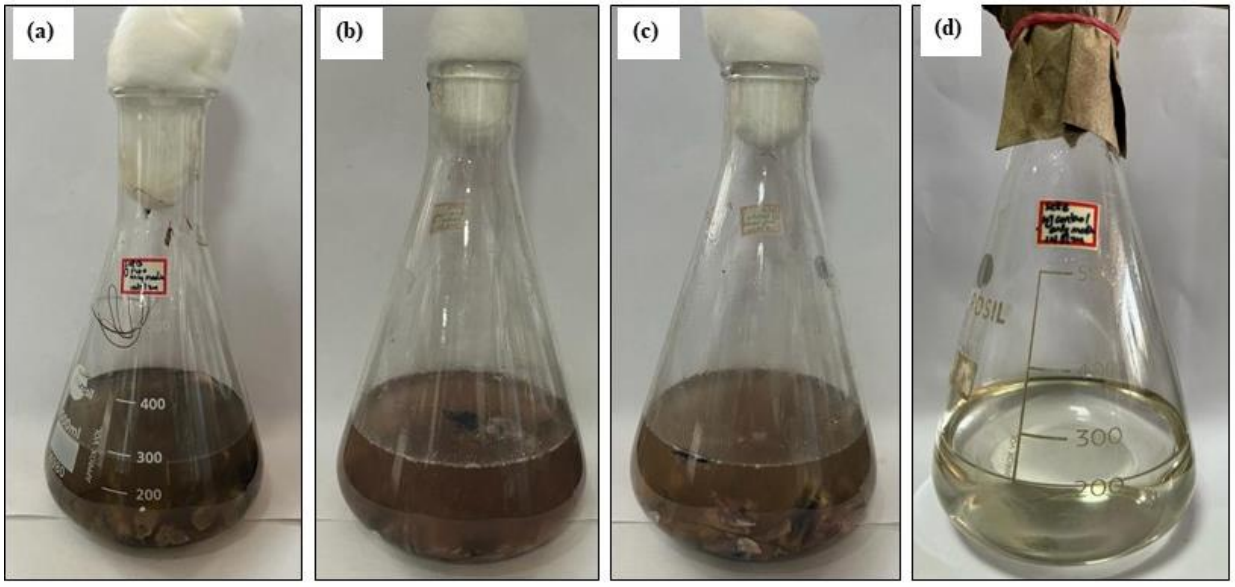

Fig. 16: Set A, NH media inoculated with GUSN1 culture containing: (a) shrimp shell waste (SWT); (b) Finfish waste; (c) Shrimp shell waste + finfish waste; (d) control.

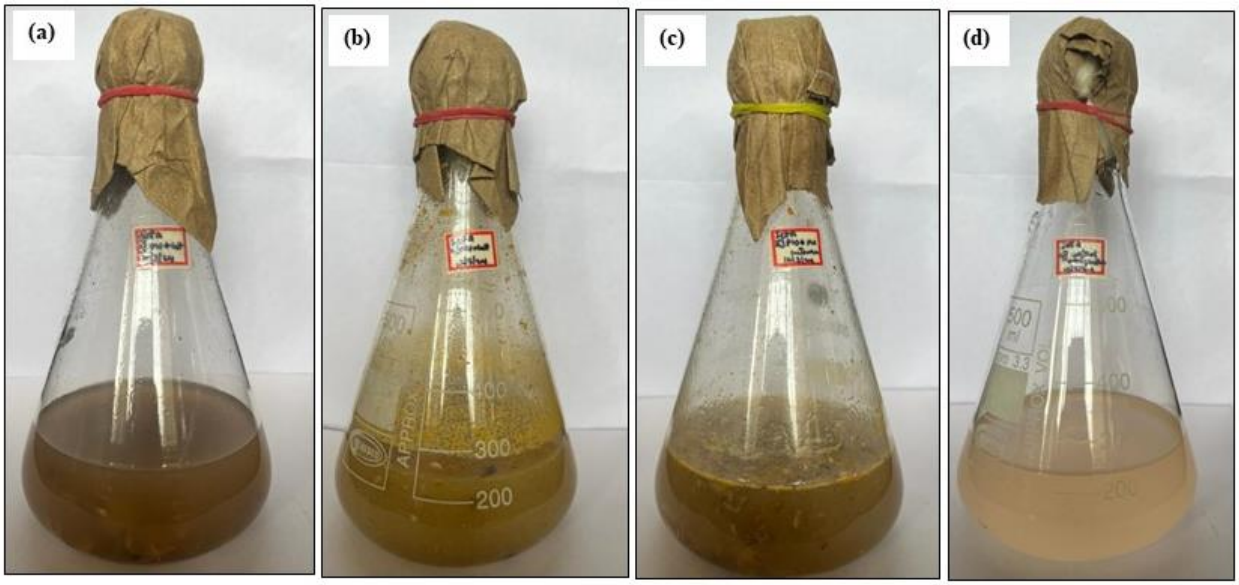

Fig. 17: Set A, after 20 days treatment with GUSN1 culture containing: (a) shrimp shell waste (SWT); (b) Finfish waste; (c) Shrimp shell waste + finfish waste; (d) control.

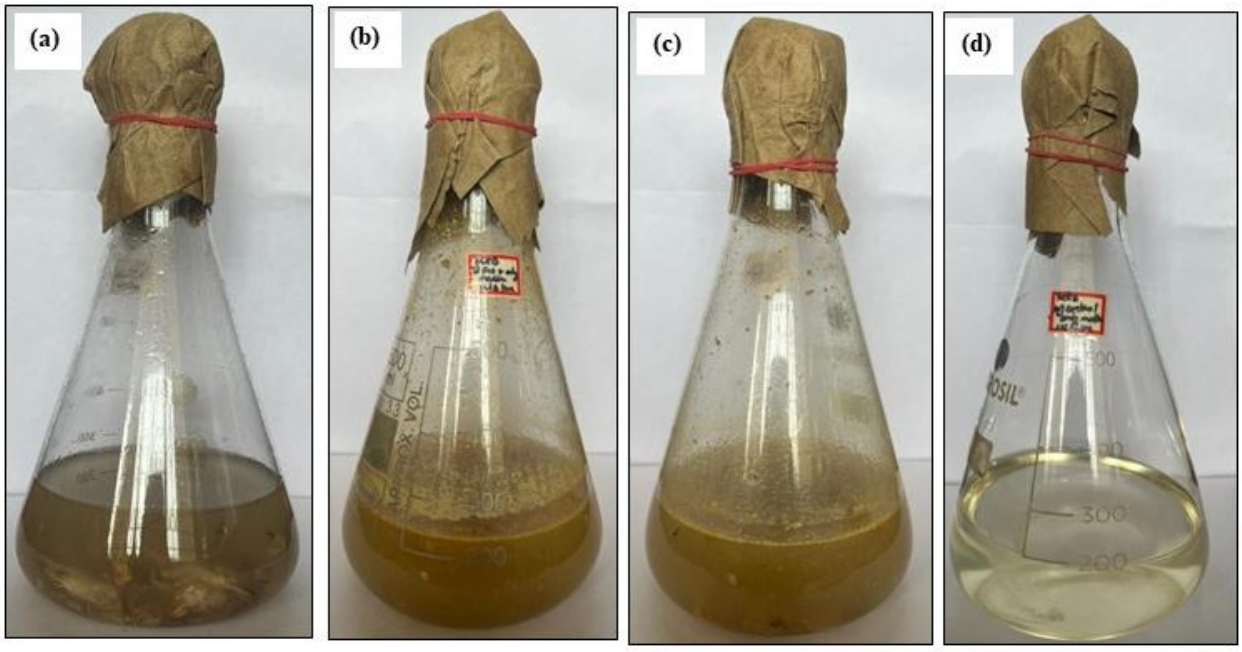

Fig.18: Set B, NH media without GUSN1 culture (control) after 20 days of incubation: (a) shrimp shell waste (SWC); (b) Finfish waste; (c) Shrimp shell waste + finfish waste; (d) control.

4.9.Evaluation of fish waste degradation potential of GUSN1 isolate

4.9.1. Estimation of N-acetyl D-glucosamine sugar

The analysis of the NAG release data (Fig: 19) revealed that there was an increase in NAG release in SWT compared to SWC as the incubation period increased. This indicate that the enzymes liberated by the GUSN1 isolate catalysed biodegradation of macromolecules of shells i.e. chitin into its derivative products i.e. N-acetyl D-glucosamine which are eventually utilized by the cell for growth and reproduction (Pal at el., 2021).

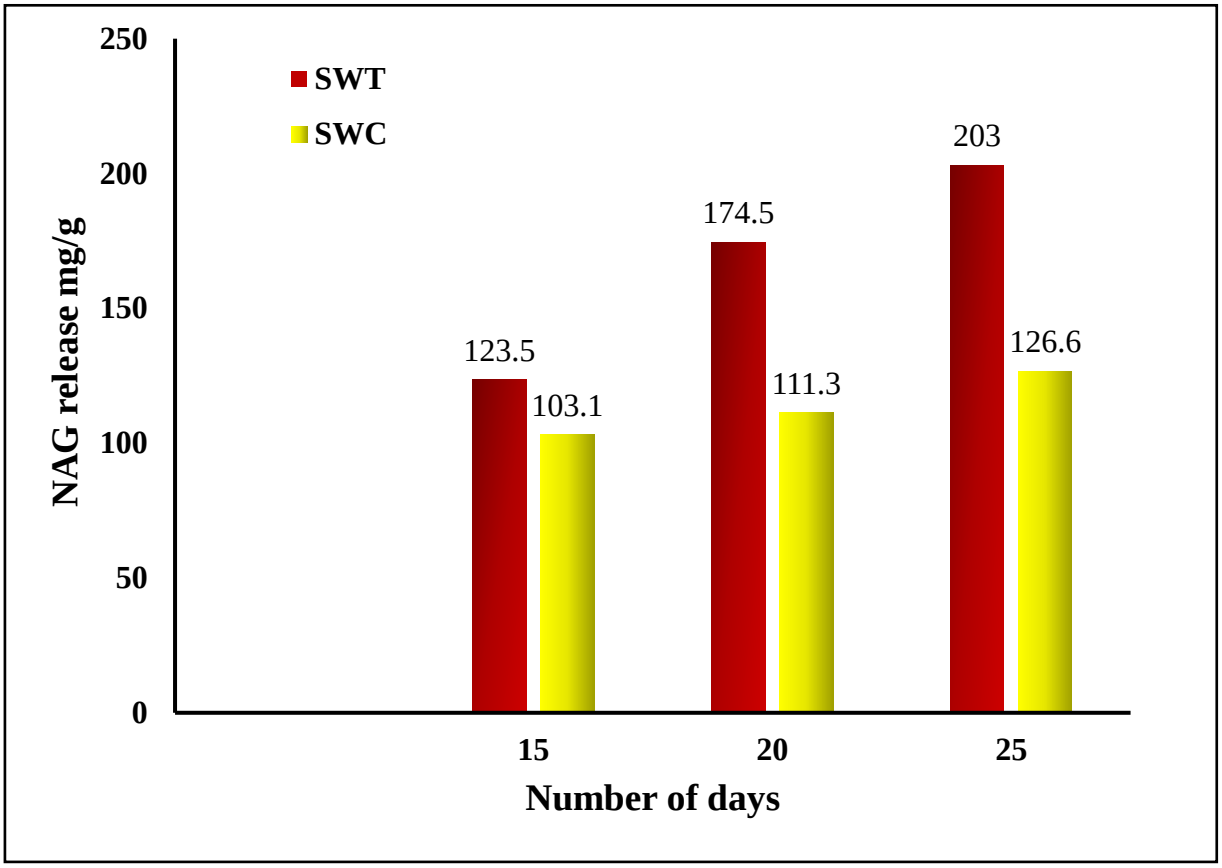

Fig. 19: Release of N-acetyl glucosamine in the culture supernatant during the degradation of shrimp waste in SWT and SWC.

4.9.2. Percent degradation

The percentage degradation of shrimp shell waste after treatment with GUSN1 was found to be 65.7 % which was higher as compared to the weight loss of shrimp shell waste after treatment with *Alcaligenes denitrificans, Bacillus amyloliquefaciens, B. megaterium, B.subtilis, Azotobacter chroococcum and Pseudomonas flurescens,* as previously described by Sabry (1992).

4.9.3. Study of utilization of fish waste as substrate by GUSN1 culture

The absorbance at 600 nm of set A i.e. fish waste inoculated with GUSN culture and set B i.e. fish waste without addition of GUSN1 culture is depicted in Fig: 20. The significant increase in absorbance in set A inoculated with GUSN1 isolate as compared to that of absorbance in set B indicates the growth of culture by utilizing fish waste as a substrate for growth. GUSN1 isolate inoculated in shrimp shell waste (SW) showed the best difference in absorbance signifying that the GUSN1 isolate can utilize shrimp shell waste as a substrate for growth.

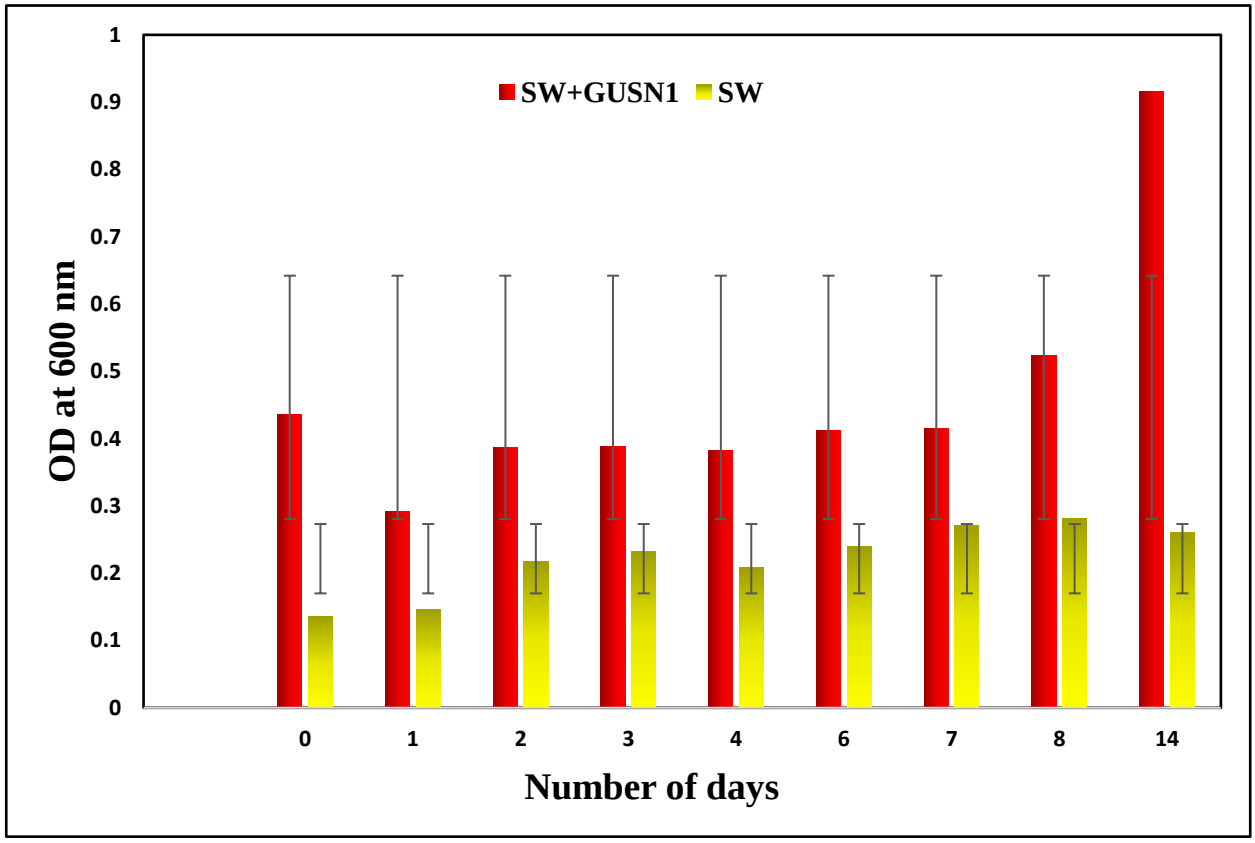

Fig. 20 (a): Growth of GUSN1 using Shrimp shell waste as a substrate, absorbance at 600 nm of shrimp waste inoculated with GUSN1 isolate (SW+GUSN1) and a control without addition of GUSN1 (SW).

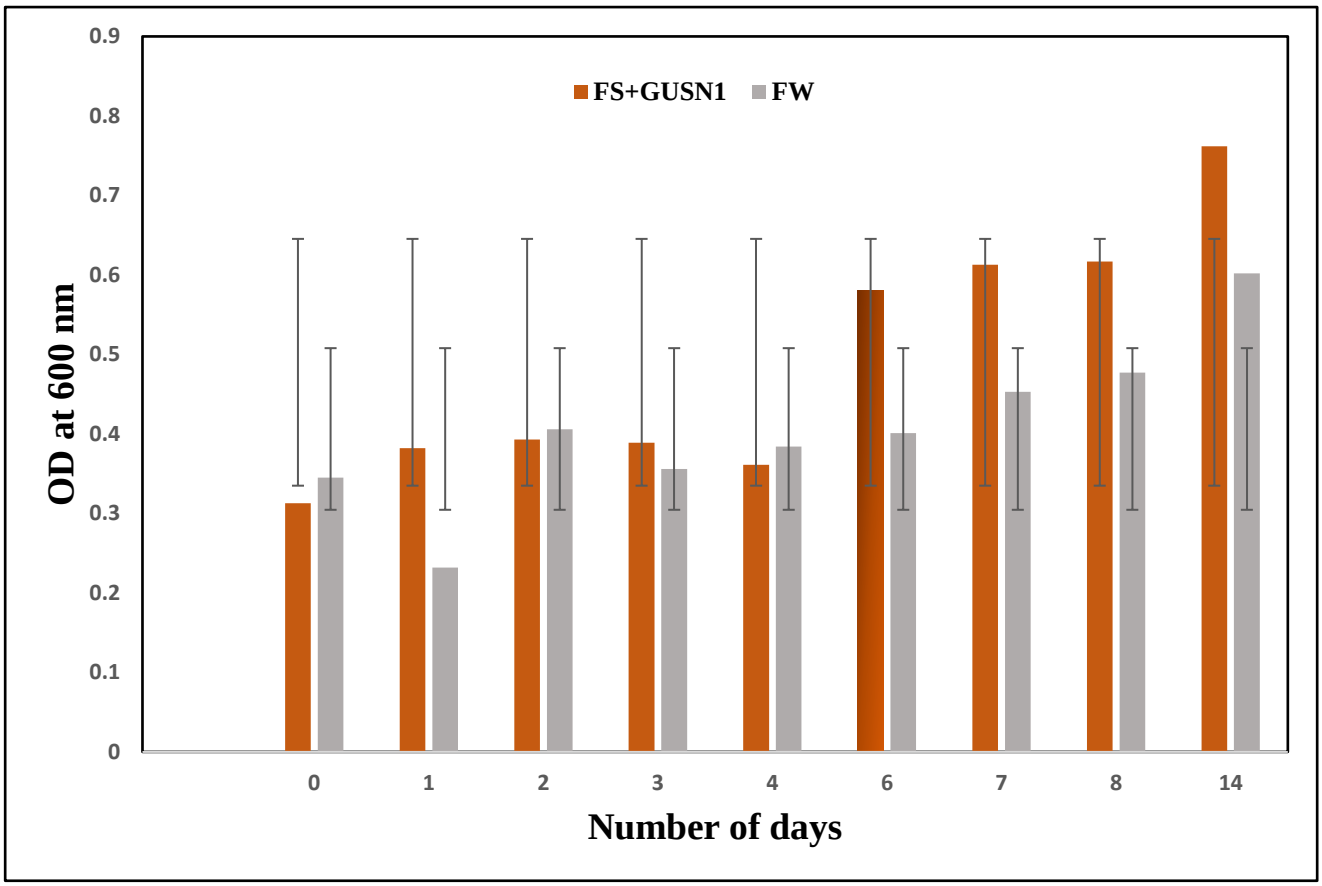

Fig. 20 (b): Growth of GUSN1 using finfish waste as a substrate, absorbance at 600 nm of finfish waste inoculated with GUSN1 isolate (FW+GUSN1) and a control without addition of GUSN1 (FW).

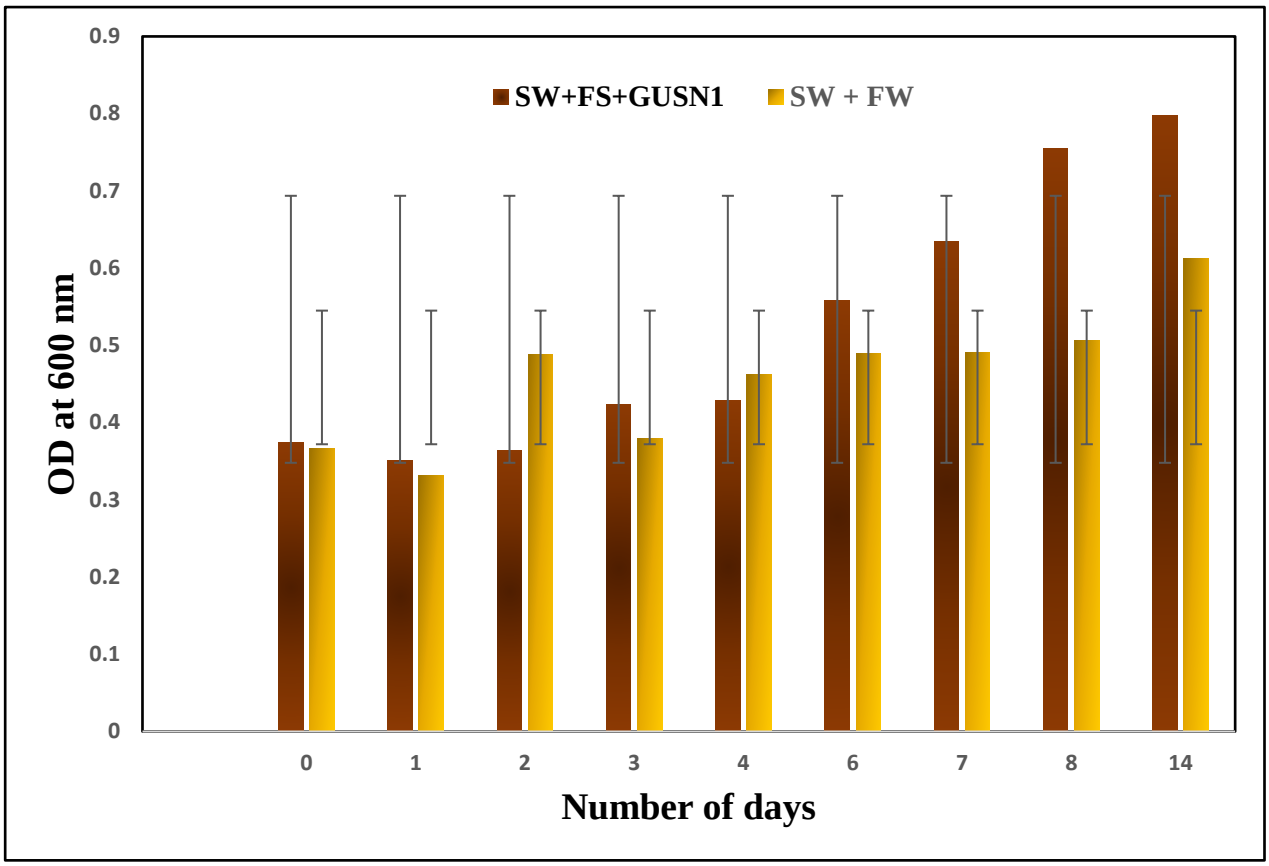

Fig. 20 (c): Growth of GUSN1 using shrimp shell waste plus finfish waste as a substrate, absorbance at 600 nm of shrimp shell waste plus finfish waste inoculated with GUSN1 isolate (SW+FW+GUSN) and a control without addition of GUSN1 (SW+FW).

4.10. Structural analysis of shrimp waste after treatment with GUSN1

4.10.1. Scanning Electron Microscopy (SEM)

The field emission scanning electron microscopy (FESEM) of the untreaded shrimp flakes and shrimp flakes treated with the GUSN1 was carried out. The SEM of the shrimp shell after fermentation revealed marked morphological changes. The shrimp shell of the control condition showed almost a smooth surface (Fig: 21.A), whereas after fermentation shrimp flakes became cracked and a number of pores appeared (Fig: 21.B), indicating shrimp shell degradation by the microorganism produced enzyme during the treatment. Similar changes were noted following the bacterial treatment of shrimp SW with *Penicillium* sp. LYG0704 (Lee

et al., 2009), *Aeromonas hydrophila* SBK1 (Halder et al., 2013), and *Paenibacillus* sp. AD (Kumar et al. 2018). These results indicate that the GUSN1 culture was very effective in shrimp shell waste degradation.

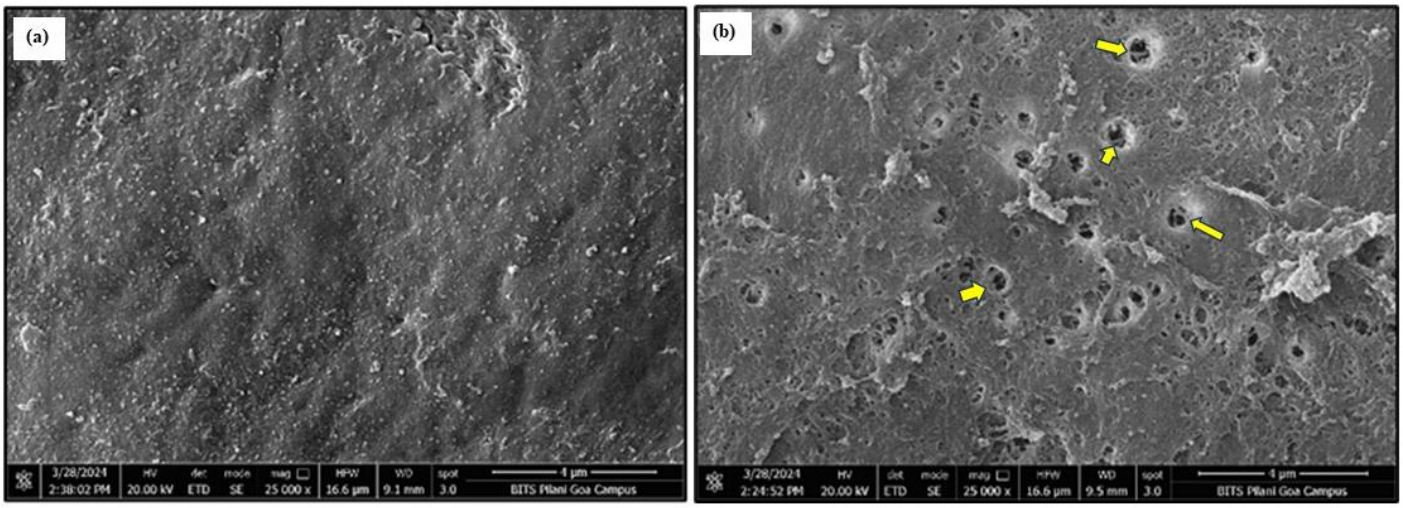

Fig.21: Scanning electron micrographs of (A) Untreated shrimp flakes (SWC); (B) shrimp flake treated with GUSN1 (SWT).

10.2. FTIR analysis

Table 5 provides an overview of the several bonds found in chitin along with the spectral bands that correspond to them. The FTIR spectra of untreated shrimp flakes (SNC), shrimp flakes treated with GUSN1 (SNT) are shown in Fig: 22. The peaks seen in the untreated shrimp flakes (SNC) at 3275 cm−1, 1634 cm−1, cm−1516, and 1403 cm−1 are characteristic of chitin. In treated shrimp flakes (SNT), aromatic skeleton vibrations inside plane deformation caused a band shift to 1411 cm−1 and an increase in peak size at 1403 cm−1, which corresponds to the OCH3 group. The intensity of the other peaks likewise changed. These alterations all point to the chitinaceous material's breakdown which are similar to the findings of Kumar et.al (2018).

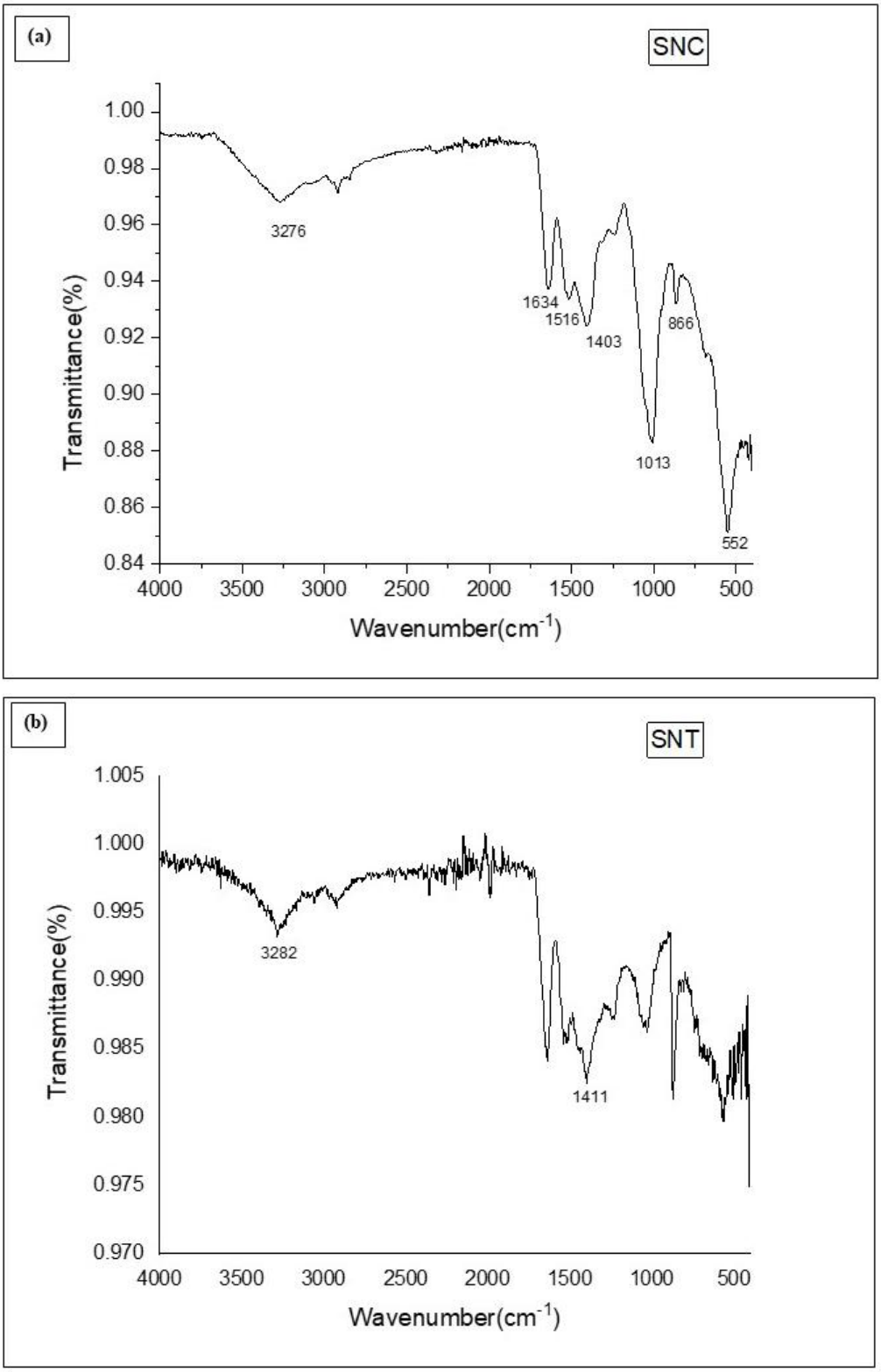

Fig. 22: FTIR spectra (a) Untreated shrimp flakes (SWC); (b) shrimp flake treated with GUSN1 (SWT).

Table. 5: Wavelength of the bands of stand chitin obtained by the FT-IR (Ca´rdenas et.al 2004).

Vibration mode	Std. chitin (cm-1)
OH stretching	3462
NH stretching	3107
Symmetric CH3 stretching and asymmetric CH2 stretching	2925
Amide I band	1647
Amide II band	1560
CH2 bending and CH3 deformation	1419
Amide III band and CH2 wagging	1318
Asymmetric bridge O2 stretching	1150
C-O stretching	1020
CH3 wagging along chain	953

4.10.3. Proximate composition of shrimp shells treated with GUSN1 isolate

The moisture content and the crude ash of the shrimp flake treated with GUSN1 (SWT) are depicted in fig: 23. The crude ash content of the shrimp shells treated with GUSN1 isolate decreased by 7% as compared to the crude ash content of untreated shrimp flakes.

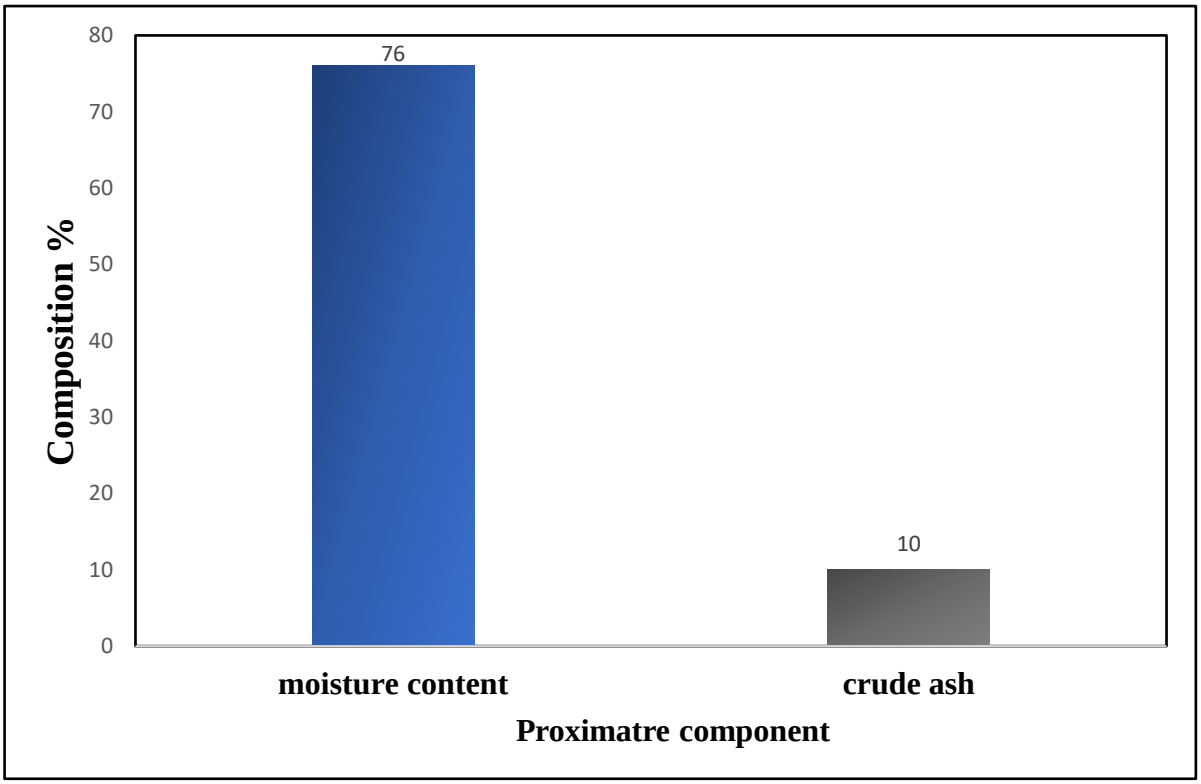

Fig.23: moisture content and crude ash content of Untreated shrimp flakes (SWC) and shrimp flake treated with GUSN1 (SWT).

4.10.4. Demineralization

The demineralization efficiency of the shrimp shell waste treated with GUSN1 isolate was found to be 93.08% which was much higher as compared to *Bacillus cereus* SV1 having demineralization efficiency of 67.15% and *Bacillus licheniformis* RP1 having demineralization efficiency of 59.4 as previously reported by Ghorbel- Bellaaj et al., (2012). Furthermore, Vakkachan et al., (2023) did not achieve a demineralization efficiency of more than 80% using the bacterial isolates *Priestia megaterium, Bacillus subtilis, Bacillus amyloliquefaciens* while GUSN1 isolate gave a significantly high demineralization efficiency of 93.8%.

4.11. Conclusion

In recent years, studies on halophilic microorganisms have gained much interest 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 extremozymes which has the ability to withstand high salt concentration. Solar salterns are great resources for researching halophilic microorganism.

In the present study, we have successfully isolated halophilic microorganism producing extremozymes. Brine and sediment samples were collected from solar salterns located at Shiroda, Maharashtra. The obtained samples were spread plated on four different halophilic media and simultaneously the samples were enriched with fish waste for the isolation of halophilic microorganisms. Twelve isolates were screened for potential crustacean waste degrading enzymes such as chitinase, protease, gelatinase and lipase. Five isolates were found positive for chitinase activity out of which GUSN1 showed best chitinolytic activity. GUSN1 isolate was used for further studies due to its high chitinolytic activity and co-production ability to produce chitinase, protease and gelatinase.

The halotolerance of GUSN1 was studied suing MHM plates containing varying salt concentration i.e., 5 to 25%. Best growth was on 15% and 20% NaCl concentration while there was negligible growth on 5% NaCl concentration indicating the extremely halophilic nature of the isolate. Gram staining indicated GUSN1 being cocci shaped and stained gram negative. GUSN1 showed excellent chitinase activity and being the main enzyme for crustacean waste degradation it was selected for further enzyme studies. The chitinase enzyme was partially purified using ice cold ethanol precipitation method and further characterised using Native PAGE and zymogram analysis technique. The enzymatic activity of the chitinase enzyme was found to be 2.35 U/mL.

There are clearly insufficient affordable options for the usage of shell waste in the sea food sectors. Biodegradation of crustacean waste and its use for producing valuable products have acquired interest. For this study, fish waste was collected from the peeling sheds of a commercial seafood market in Mapusa and fermentation strategy was employed to determine the potential of GUSN1 isolate to degrade fish waste. Proximate analysis of the shrimp shell revealed 63% moisture content and 17% crude ash content. The degradation of fish waste was confirmed through estimation of release of N-acetyl glucosamine, percent degradation, demineralization and residue analysis that included FESEM and FTIR. The percent degradation of shrimp shell waste was found to be 65.7% whereas the demineralization efficiency was found to be 93.08%. FESEM and FTIR results showed the effective degradation of the shrimp shell waste after treatment with the GUSN1 isolate.

To conclude, GUSN1 was a halophilic isolate having ability to thrive in high salinity conditions. The ability of the isolate to co-produce chitinase, protease and gelatinase enzymes can serve as an excellent potential candidate for the biomineralization and bioremediation of the crustacean waste.

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Appendix I: Composition of media

APPENDIX I: COMPOSITION OF MEDIA

1. NH Media (Norberg and Hofstein)

Ingredients	Grams/litre
NaCl	200.0
$MgCl_2.6H_2O$	10.0
KCl	1 5.0
Yeast Extract	1.0
Agar	20.0

pH 7.0 (Adjust using 1M KOH)

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

2. extremely Halophilic medium (EHM)

Ingredients	Grams/litre
NaCl	250.0
$MgCl_2.6H_2O$	20.0
$CaCl_{2.}H_2O$	0.36
$NaHCO_3$	0.06
KCl	2.0
NaBr	0.23
peptone	5.0
Yeast Extract	10.0
$FeCl_{3.}\ 6H_2O$	trace
Agar	20.0

pH 7.0 (Adjust using 1M KOH)

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

3. **Modified Moderately Halophilic Medium (MHM)**

Ingredients	Grams/litre
NaCl	178.0
$MgCl_2.6H_2O$	1.0
$CaCl_2.H_2O$	2.0
$NaHCO_3$	0.06
KCl	2.0
NaBr	0.23
peptone	5.0
Yeast Extract	10.0
$FeCl_3.6H_2O$	trace
Agar	20.0

pH 7.0 (Adjust using 1M KOH)

Directions: sterilize by autoclaving at 15 lbs pressure (121℃) for 15 minutes

4. **NaCl tryptone yeast extract (NTYE)**

Ingredients	Grams/litre
NaCl	250.0
$MgCl_2.6H_2O$	20.0
KCl	5.0
Tryptone	5
Yeast Extract	3.0
Agar	20

pH 7.0 (Adjust using 1M KOH)

5. **NT**

Ingredients	**Grams/litre**
NaCl	250.0
$MgCl_2.6H_2O$	20.0
KCl	2.0
Tris-sodium citrate	3.0
Yeast Extract	10.0
Agar	20

pH 7.0 (Adjust using 1M KOH)

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

APPENDIX II: STAINS AND REAGENTS

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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. 50 mM Tris HCL buffer

Tris HCL	7.88g
Distilled water	1000 mL

NATIVE PAGE REAGENTS

7. Resolving gel buffer (1.5 M)

Tris HCl	18.171 g
Distilled Water	100 mL
p H	8.8

8. Stacking gel buffer (1 M)

Tris HCl	12.114 g
Distilled Water	100 mL
pH	6.8

9. Monomer Solution

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

10. Running(tank) buffer (1X)

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

11. Bromophenol Blue (1%)

Bromophenol Blue	0.1 g
Distilled Water	10 mL

12. Ammonium Persulfate (10%)

Ammonium Persulfate 0.1 g

Distilled Water 1 mL

13. Sample loading buffer(1X)

Stacking gel buffer 1 mL

0.2% Bromophenol blue 2 mL

10% Glycerol 2 mL

14. Staining solution

Coomasie brilliant blue 0.25 g

Methanol 45 mL

Glacial acetic acid 10 mL

Distilled Water 45 mL

15. Destaining solution

Methanol 45 mL

Glacial acetic acid 10 mL

Distilled Water 45 mL

16. DNSA reagent

i. Sodium potassium tartarate 60g

Distilled water 100 mL

ii. DNSA 2g

2M NaOH 40 mL

APPENDIX III: STANDARD ASSAY

APPENDIX III: STANDARD ASSAY

1. Sugars assay for chitinase

Standard curve of N-acetyl glucosamine

Stock: 10mg/ml NAG

Diluent: distilled water

Reagent: DNSA reagent

Procedure:

Prepare dilutions of standard sugar solution with distilled water

↓

Add one mL of DNSA reagent to one mL of sugar solution mixture

↓

Place the tubes in boiling water bath for 5 minutes, cool

↓

Add 10mL distilled water to all the tubes

↓

Absorbance at 560 nm was measured