Production of Lipase Enzyme using Seaweed Associated Bacteria for its Application in Destaining

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I hereby declare that the data presented in this Dissertation entitled "Production of Lipase Enzyme using Seaweed Associated Bacteria for its Application in Destaining" is based on the results of investigations carried out by me in the Microbiology Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of Dr. Lakshangy Charya 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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PREFACE

In the realm of fabric care and sustainability, exploring the potential of lipase enzymes in destaining fabric marks a significant stride towards eco-friendly cleaning solutions. Lipase enzymes, naturally occurring proteins, possess remarkable abilities to break down fatty residues, making them promising candidates for efficient and environmentally conscious fabric destaining. This preface delves into the mechanisms, applications, and ecological implications of utilizing lipase enzymes in fabric care, shedding light on a burgeoning frontier in the quest for greener textile maintenance practices.The main aim of this study is to use microbial origin enzymes as a alternative for harmful chemicals used in the destaining process.

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ABBREVIATION USED

Entity	Abbreviation
Artificial seawater	ASW
Carboxymethyl cellulose	CMC
Modified Artificial Seawater	MASW
Microliter	µl
Millimeter	ml
Percentage	%
Sodium Chloride	NaCl
Zobelle Marine Agar	ZMA
Zobelle Marine broth	ZMB

ABSTRACT

Lipases are hydrolytic enzymes having wide applications. This study emphasized on lipase production by seaweed associated bacteria and their application in destaining and deinking of fabric stains.

Two seaweeds associated bacterial isolates P1 and S-T-2 obtained from Padiana and Sargassum seaweed, elaborated considerable amount of lipase activity in the presence of Tween 20 and Tween 80 substrate in Modified artificial seawater media. Lipase produced by P1 and S-T-2 had NaCl concentration and temperature optima of 1.5%,6.0 and 280C respectively. The Bacterial isolates elaborated lipase activity in media supplemented with coconut and palm oil, indicating the use of coconut and palm oil cakes generated as waste after oil extraction can be used as substrate for bulk production of lipase enzyme. The lipase enzyme produced by P1 and S-T-2 were able to remove oil and ink from the prestained fabric therefore S-T-2 was the better as compared to P1 as showed more destaining and deinking activity.

Keywords: Seaweed associated bacteria, Lipase. Oil hydrolysis, destaining, deinking

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Seaweeds, also known as marine macroalgae, are multicellular, sessile, photosynthetic eukaryotes unlike plants they do not have specialized tissues like roots as well as vascular structures (Suhelen *et al.*,2013). They vary greatly in size – unicellular of 3–10 μm to giant kelps up to 70 m long and growing at up to 50 cm per day.

The main source of production in the marine environment is seaweed. Since they have been establishing the foundation of the aquatic food chain, marine algae are important to the ecosystem and are required by nearly all aquatic organisms (Huynh and serediak, 2006).

They are also well-known environmental stress indicators that are impacted by factors like temperature stress, desiccation, and competition with coastal animals, all of which have a significant impact on seaweed distribution (Juhmani *et al.*,2020)

In Asian countries, they are eaten as food because they are also a storehouse and producer of a variety of secondary metabolites, such as gums and resins, which are important in industry and are a highly rich source of carbohydrates, proteins, and fatty acids and also produces various polymers such as Fucoidan, laminarin, alginates etc by diverse groups of microorganisms (Salehi *et al.*,2019). The nutrient composition of different seaweeds is given in the **Table 2.2**

(Mathew *et al.*,2018) (Mišurcová,2011).

Environmental concerns associated with detergent production include the negative impact of chemical compounds on aquatic life and ecosystems. Phosphate containing detergents can react adversely in water, creating nutrients that stimulate the growth of the algae, depleting oxygen and ruining the ecosystem. This industry is under pressure to develop eco-friendly products, reduce energy consumption and navigate regulatory challenges to ensure competitiveness and sustainability. The seaweed associated bacteria produce a variety of

polymer degradating enzymes such as agarase, amylase, cellulase, protease, esterase,lipase,chitinase,alginate lyase carrageenase, pectinase,pulluan hydrolase,β-glucanase,laminarase.Theses have wide application in industries (Jonnadula &Ghadi,2011;Naik *et al.*,2019).

Since the seaweeds are easily available, we are attempting to isolate lipase/esterase producing bacteria with the seaweed associated to check the efficiency in stain removal from the fabric.

1.2 AIM AND OBJECTIVE

Aim: To produce lipase enzyme using seaweed associated bacteria and determine its application in destaining process.

Objective

1.Isolation of seaweed associated bacteria.

2.Screening of seaweed associated bacteria for producing enzymes.

3.Effect of pH, temperature and NaCl concentration on lipase producing from selected bacterial isolates.

4.Application of Lipase enzyme

1.3 HYPOTHESIS

The hypothesis is that lipase enzymes produced by seaweed associated bacteria can effectively degrade lipid-based stains due to their ability to break down lipids found in stains. This could potentially lead to the development of environmentally friendly and efficient destaining agents for various applications, such as laundry detergents, cleaning products, or stain removal in industrial processes.

1.4 SCOPE

Lipases are one of the widely used biocatalyst due to their wide range of application. Lipase is produced from various sources such as plants, animals, bacteria fungi and yeast. Since the production of lipase enzyme is limited in the plant and animals. There will be opportunities to use microbial lipase as the production is greater and less space is required and the less energy cost process is required. Microbial lipase has wide range of application in various industries

1.Paper and pulp industry: The presence of lipases improves paper brightness and reduces pollution in waste waters. They are employed in the management of pitch and in the elimination of triglycerides and waxes from pulp made for the paper industry. 2. Detergent Industry: Because lipases may degrade oil deposits and fatty stains, they are used in the detergent business. They are essential to enzyme-based laundry detergents and stain removers because they increase cleaning processes' effectiveness.

3.Waste Treatment: Lipase are used in waste treatment to degrade the oils, fats and grease present in the industrial effluent and thus minimize the pollution.

4.Food industry: Lipase enzymes are used in the modification of oil and fats, production of various fatty acid esters. Which helps in enhancement of flavour in the dairy products, cheese ripening etc.

5.Pharmaceutical Industry: Modifying the pharmacokinetics of the drug by altering their stability and bioavailability.

CHAPTER 2: LITERATURE REVIEW

2.1 Seaweeds

Marine macroalgae are can be divided into three groups based on coloration and taxonomic classification: Rhodophyta (red), Phaeophyta (brown), and Chlorophyta (green). The classification is mainly based on the pigment produce, polysaccharide as it reserves and the nature of the cell wall (Salehi *et al.*, 2019) as summarised in the **Table 2.1**

Table 2.1: Classification of Seaweeds

Criteria	**Chlorophyceae**	**Rhodophyceae**	**Phaeophycean**
PIGMENT			
1.Cholorophyll	Chl a, b	Chl a	Chl a, c
2.Carotenoids	beta-carotene, lutein violaxanthin, zeaxanthin neoxanthin	Beta-carotene, lutein, zeaxanthin	beta-carotene, fucoxanthin violaxanthin
3.Phycobiliproteins		Phycocyanin, Phycoerythrin	
POLYSACCHARIDES			
1.Storage	Starch	Floridean starch	Laminarin
2.Structural (cell wall)	Ulva, Xylan, Mallan, Cellulose	Carrageenan, Agar Xylan Mannan cellulose	Alginate, Fucoidan cellulose
Examples	*Ulva intestinalis, Ulva linza, Ulva rigida ,Codium bursa , Cladophora rupestri, Parsiolla carophyla*	*Chondrus crispus, Gelidium pusillum, Gracilaria gracillu,s Palmaria palmata, Rhodochorton purpureum*	*Fucus serratus, Laminaria hyperborean,Laminaria digitata, Padina pavonia Petalonia fascia*

Table 2.2: The nutrient composition of three groups of seaweeds.

Nutrient Composition		**Chlorophyceae**	**Rhodophyceae**	**Phaeophyceae**
1.Proteins		10-30%	15-20%	5-16%
2. Lipids		alpha-linolenic acid, cholesterol, methylene cholesterol and β-sitosterol	eicosapentanoic acid, docosahexaenoic acid, desmosterol, cholesterol, sitosterol, fucosterol and chalinasterol	eicosapentanoic acid, docosahexaenoic acid, fucosterol, desmosterol,
3.Minerals		Calcium, iodine, iron, potassium, phosphorus, manganese		
4.Vitamins	**Water - soluble**	Vitamin B12, Vitamin C	Vitamin B, Riboflavin, Thiamine	Vitamin B Riboflavin, Thiamine, Vitamin C,
	Fat soluble	Vitamin E, β-carotene, lutein, violaxanthin zeaxanthin neoxanthin	Vitamin E, β-carotene, lutein, zeaxanthin	β-carotene, fucoxanthin, violaxanthin
5.Dietary fibers	**Water-soluble**	Starch in the chloroplast	Floridean starch granules outside plastids	Laminarin as oil Droplets
	Water-Insoluble	Ulvan, Xylan Mannan, Cellulose	Carrageenan, Agar, Xylan Mannan, Cellulose	Alginate, Fucoidan Cellulose

2.2 Bacterial -Seaweed Interaction

Large amount of variety of complex extracellular compounds including water soluble polysaccharides (Glucose, mannose, galactose, rhamnose, xylose) which are release in the surrounding of the seaweeds. These polysaccharides are used by the bacteria to colonize on the surface of the seaweed. The colonizing bacteria uses these polysaccharides as a source of carbon and helps them to further colonize the host. (Lahaye & Axelos, 1993; Armstrong *et al.,* 2001; Steinberg *et al.,* 2002; Lane & Kubanek, 2008).

2.2.1 Seaweed associated bacteria

The field of researching bacterial communities linked to seaweeds is still in its early stages, and there is not enough information available to compare bacterial communities linked to various seaweeds in a meaningful way Taylor *et al*., (2004,2005).

The bacterial populations linked to seaweed and saltwater display unique features. These communities are formed on the macroalgal surface through mechanisms of selection, and the random colonization of different bacteria is responsible for the significant variety seen in these communities. Furthermore, studies have shown that several species of marine macroalgae growing in the same ecological niche have diverse bacterial communities (Lachnit *et al*., 2009). Conversely, bacterial communities belonging to separate species that shared an ecological niche showed some degree of resemblance to those belonging to the same macroalgal species but residing in distinct environmental settings (Lachnit *et al*., 2009; Nylund *et al*., 2010).

Marine microorganisms are new and rich sources of physiologically active chemicals. From marine species, over 18,000 natural products have been identified, and hundreds of new compounds are discovered every year. This number is increasing swiftly. Untapped marine

microorganisms represent a substantial untapped source of novel metabolites. Marine bacteria can be found in sediments, saltwater, and marine microorganisms.

The seaweed surface secretes a range of organic chemicals that act as nutrients for bacterial growth and the formation of microbial biofilms, in addition to offering a surface that is suitable for microbial settling. Seaweed's surface is home to a diverse range of microorganisms, including diatoms, bacteria, fungi, spores, protozoa, and larvae of marine invertebrates. These populations are highly dynamic and complicated. Among them, bacteria are present in the cytoplasm of living host cells (e.g., Herbaspirillumsp in Caulerpataxifolia) and on the surface of seaweed. They regulate numerous stages of the life cycle of eukaryotic creatures (Duan *et al*., 1995).

2.3 Microbial Enzymes

Enzymes are classified as biocatalysts with unique properties that generate cell metabolic processes to promote biochemical reactions in biological systems (Nigam, 2013).

2.4 Lipase

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are serine hydrolases that catalyze the hydrolysis of water-insoluble triglycerides at an oil-water interface, producing diacylglycerol, monoacylglycerol, free fatty acid, and glycerol. Enzymes can speed chemical reactions in immiscible or anhydrous solvents, including aminolysis, alcoholysis, acidolysis, esterification, and transesterification (Joseph *et al*., 2018) as shown in the **Figure 2.1.**

Lipases are serine hydrolases and contain consenses sequence G-X1-S-X2-G as the catalytic moiety where G= Glycine, S= Serine, Xl-histidine and X2= glutamic or Aspartic acid (Svedson, 1994)

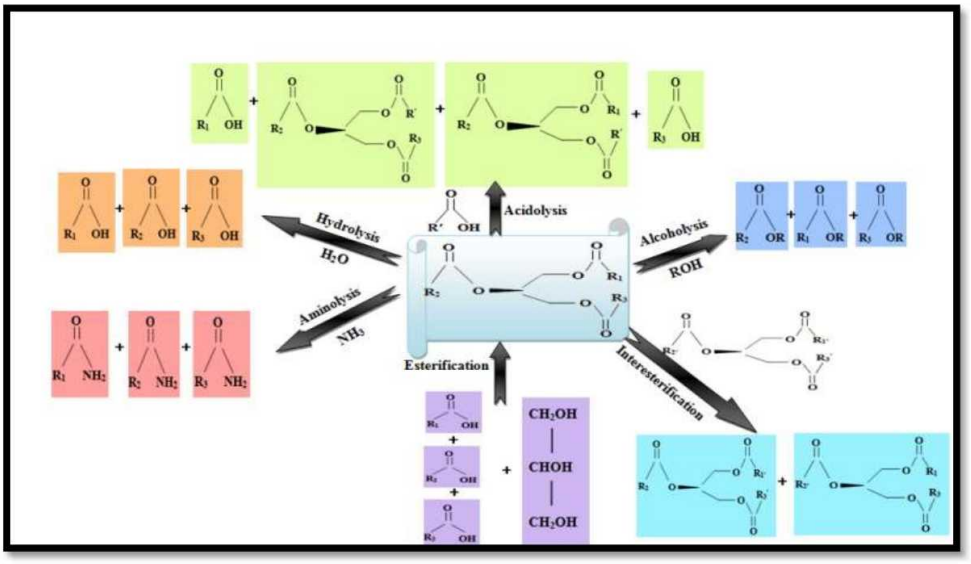


Figure 2.1: Lipase-catalyzed esterification, hydrolysis, aminolysis, and transesterification (Fatima et al., 2021)

Lipases are useful biocatalysts because of their broad substrate selectivity, strong regio- and/or stereo selectivity during catalysed processes, high stability in organic solvents, and moderate catalysis. The lipases in their family are among the hydrolases that function on carboxylic ester linkages. In the body, lipases hydrolyze triglycerides to create glycerol, fatty acids, diglycerides, and monoglycerides. In addition to their natural ability to hydrolyze carboxylic ester bonds, lipases can catalyze the processes of esterification, interesterification, and transesterification in nonaqueous environments (Snellman *et al.*, 2002).

2.5 Properties of Lipase

Lipases can catalyze synthetic reactions including esterification and transesterification in the form of acidolysis, alcoholysis, and interesterification in the presence of modest amounts

of water in addition to their traditional ability to catalyze hydrolytic reactions Unlike other enzymes, oil-water or air-water interfaces activate the lipases (Shimada *et al.,* 2005; Villeneuve *et al.,* 2000). Divakar and Manohar (2007) grouped the reactions catalyzed by lipases into three important types:

2.5.1 Hydrolysis:

This occur in aqueous conditions where ester bond cleavage is the predominant reaction due to high water content. Fatty acids, diglycerides, and other products are now produced using this process. Monoglycerides, dairy product flavourings, and laundry and household detergents.

2.5.2.Esterification

A high yield of the esterified products is achieved under controlled circumstances; this reaction happens under low water conditions, e.g., in almost anhydrous solvents. Making oleic acid esters from primary and secondary aliphatic and terpenic alcohols is one of the most prevalent examples. Geranyl and menthyl esters are formed from butyric acid, geranol, or lauric acid, and menthol, among other substances (Marlot *et al.,* 1985).

2.5.3. Transesterification

This is the exchange of an acid moiety between two or more compounds (the reaction is called interesterification if the acyl donor is an ester, and is known as acidolysis if the acyl donor is a free acid; in alcoholysis, the nucleophile alcohol functions as an acyl acceptor) (Macrae, 1985).

2.6 Structure of Lipase

X-ray crystallography has helped identify the structure of numerous lipases. Certain common features have evolved as a result of these studies.

These proteins belong to the α/β hydrolase fold family, with a core of parallel β strands and α helices. They have a highly conserved sequence of five amino acids. Gly-X-Ser-X-Gly. This pentapeptide sequence creates a unique β-turn-α motif known as the 'nucleophilic elbow' (Nardini & Dijkstra 1999; Ollis *et al*., 1992). The active, nucleophilic serine residue is located at a hairpin bend between the β strand and α helix.

Lipases use a catalytic triad of serine, histidine, and aspartic acid/glutamic acid as their active site .The active sites of proteases and lipases share several chemical similarities. The two are structurally distinct, with the hydroxyl group of the serine residue orientated differently.

2.7 Mode of Action

In the field of scientific research, chemical analyses, industrial catalysis etc, enzymes are extensively applied (Sharma *et al*., 2001b). Among the known enzymes, lipases have exceptional characteristics which can carry out reactions at the interface flanked by the aqueous and non-aqueous media. This is due to their aptitude to utilize relatively a broad spectrum of substrates, showing high stability towards extreme temperature and pH and also with certain organic solvents, do not require cofactors. Due to three-dimensional structure of various lipases, all have been classified as serine hydrolases (Winkler *et al*., 1990; Jaeger *et al*., 1993). The reason for this is because of the active site that composed of the catalytic triad Ser-Asp (Glu)-His is similar to serine proteases (Derewenda and Sharp, 1993; Brumlik and Buckley, 1996lipases are also used to catalyze the transesterification reaction using alcohol together with fats, oils and free fatty acids to fabricate alkyl esters. In the figure 2.2, Triglyceride conversion by using lipase enzyme Due to the wide-ranging significance, lipases therefore show as an important topic of severe study (Bornscheuer, 2000). Lipase research is alerted mainly on structural depiction, general depiction of performance

(Bornscheuer, 2000) and industrial uses. Lipases are the enzymes that belong to the class of serine hydrolases that do not require any cofactor. The substrates of lipases are triacylglycerols that have low solubility in water. These enzymes catalyse the hydrolysis of ester bonds under natural conditions between an insoluble substrate and aqueous phase in which the enzyme get dissolved (Saxena *et al*., 1999).The lipases act on fats and oils, and further hydrolyse into the substituted glycerides and fatty acids, and on total hydrolysis into glycerol and fatty acids (Bjorkling *et al*., 1991; Ghosh *et al*., 1996).

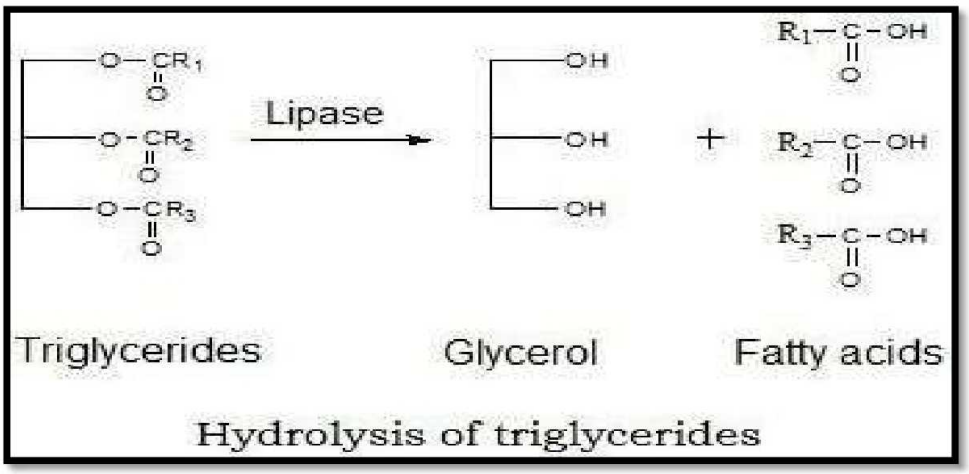


Figure 2.2: Triglyceride conversion by using lipase enzyme

(Bornscheuer, 2000).

2.8 Types of lipase based on function

Lipases can be divided into different groups based on their specificity, making it important to evaluate their industrial uses (Sarmah *et al*., 2018). (Verma et al.,2021) define four major categories of lipases based on their specificity. They are divided into four major types:

1) regioselective (2) substrate-specific (3) nonspecific (4) enantioselective

Table 2.3: Types of Lipases based on Function

Sr. No	Types	Application	References
1	**Regioselective** Regioselectivity refers to the ability to distinguish between primary (sn-1,3) and secondary (sn-2), ester functionalities in triacylglycerol molecules. **i)sn-1,3 specific (1,3-regiospecific)** They catalyze the hydrolysis of triacylglycerol at the C1 and C3 sites of triglycerides, resulting in free fatty acids, 2-monoacylglycerols, and 1,2 or 2,3 diacylglycerols. **(ii) sn-2 specific (2- regiospecific)** These enzymes selectively remove fatty acids from the second carbon position of a triacylglycerol molecule. The sn-2 specificity is unusual and can hydrolyze oleic and linoleic acids from the sn-2 location of triglycerides	This is essential for generating structured lipids and their applications in chemical and pharmaceutical industries, as well as analytical purposes. oil modification, for biodiesel production processing and food and dairy products, Pharmaceutical, organic synthesis, polymer synthesis and agrochemicals.	(Macrae and Hammond, 1985; Tong *et al.*,2016) (Verma *et al.*,2021)

2	**Substrate-specific** They work on a specific substrate in raw materials to accelerate product synthesis. i)Fatty acid specific lipases ii)Alcohol specific lipases	Biodiesel Production Production of high purity diacylglycerols	(Sarmah *et al.*,2018)
3	**Non-specific lipases** This class of lipase are robust and have ability to act on multiple substrates as lipase (generally catalyze the hydrolysis of triacylglycerols into free fatty acids and glycerol with mono- and di-acylglycerols).	cosmetic industry to biodiesel production	(Carvalho *et al.,* 2015; Sarmah *et al.*, 2018; Verma *et al.*, 2021)
4	**Enantioselective lipases** This class of lipase can hydrolyze one of the isomers of racemic.	Hydrolysis of menthol benzoate to cosmetic/food product. Synthesis of isomeric compounds. Transesterification of secondary alcohols to pharmaceutical products	(Li et al. 2018; Samarh *et al.* 2018; Verma *et al.* 2021)

2.9 Sources of lipase

Lipases are extracted from several sources in nature. In nature, microbial lipases are ubiquitous. Because they are more readily available, have better stability, and are less expensive to produce than plant and animal lipases, they have significant commercial value. Marine microflora is capable of producing enzymes and protein-active chemicals. Most lipases are extracellular enzymes produced through fungus and bacteria (Boutaiba *et al.* 2006). Lipase-producing microorganisms have been isolated from food deterioration, where they cause dairy products like cheese to lose their flavor, or from sewage and olive oil, landfills, contaminated butter wastewater, soil samples polluted with petroleum, and oil mill effluent (Casas-Godoy *et al.* 2018).

Table 2.4.1: Lipase Producing Gram positive bacteria (Sharma *et al.,* 2001., Perez *et al.*, 2003)

Source	Genus	Species
Bacteria(Gram-positive)	Bacillus	*B. megaterium* *B. cereus* *B. stearothermophilus* *B. subtilis* *Recombinant B. subtilis 168* *B. brevis* *B. thermocatenulatus* *Bacillus sp. IHI-91* *Bacillus strain WAI 28A5 Bacillus sp.* *B. coagulans* *B. acidocaldarius* *Bacillus sp. RS-12*

		B. thermoleovorans ID-1 Bacillus sp. J33
	Staphylococcus	*S. canosus*
		S. aureus
		S. hyicus
		S. epidermidis
		S. warneri
	Lactobacillus	*Lactobacillus delbruckii sub sp. bulgaricus*
		Lactobacillus sp
	Streptococcus	*Streptococcus lactis*
	Micrococcus	*Micrococcus freudenreichii*
	Propionibaclerium	*M. luteus*
		Propionibacterium acne
	Burkholderia	*P. granulosum*
		Burkholderia sp.
		B. glumae

Table 2.4.2: Lipase producing Gram negative bacteria

Source	Genus	Species
Bacteria(Gram negative)	Pseudomonas	*P. aeruginosa* *P. fragi* *P. mendocina* *P. putida 3SK* *P. cepacia* *P. fluorescens* *P. aeruginosa KKA-5* *P. pseudoalcaligenes F-11 Pseudomonas sp.* *P. fluorescens MFO Pseudomonas sp. KWI56*

Table 2.4.3: Lipase producing Fungi and yeast

Source	Genus	Species
	Chromobacterium	*C. viscosum*
	Acetobacter	*A. pseudoalcaligenes* *A. radioresistens*
	Aeromonas	*A. hydrophila* *A. sorbia LP004*
Fungi	Rhizopus	*R. delemar* *R. oryzae* *R. arrhizus* *R. nigricans* *R. nodosus* *R. microsporous* *R. chinensis* *R. japonicus* *R. niveus*
	Aspergillus	*A. flavus* *A. niger* *A. fumigatus* *A. oryzae* *A. carneus* *A. repens* *A. nidulans*
	Penicillium	*P. cyclopium* *P. citrinum* *P. roqueforti* *P. fumiculosum* *Penicillium sp.*

Source	Genus	Species
		P. camambertii
		P. wortmanii
	Mucor	*Mucor miehei*
		M. javanicus
		M circinelloides
		M. hiemalis
		M. racemosus
	Ashbya	*Ashbya gossypii*
	Geotrichum	*G. candidum*
	Beauveria	*Beauveria bassiana*
	Humicola	*H. lanuginosa*
	Rhizomucor	*R. miehei*
	Fusarium	*Fusarium oxysporum*
		F. heterosporum
Yeasts	Candida	*C. rugosa*
		C. tropicalis
		C. antarctica
		C. cylindracea
		C. parapsilosis
		C. deformans
		C. curvata
		C. valida
	Rhodotonila	*R. glutinis*
		R. pilimornae
	Pichia	*P. bispora*
		P. maxicana
		P. sivicola
		P. xylosa
		P. burlonii
	Saccharomyces	*S. lipolytica*
		S. crataegenesis

Table 2.4.4: Lipase producing Actinomycetes and Haloarchea

Source	Genus	Species
	Torulospofa Trichosporon	*Torulospora globora* *Trichosporon asteroids*
Actinomycetes	Streptomyces	*Streptomyccs fradiae NCIB 8233* *Streptomyccs sp. PCB27* *Streptomyccs sp. CCM 33* *S. coelicolor* *S. cinnamomeus*
Haloarcheae	Halomonas Marinobacter	*Halomonas salina, Halomonas campisalis* *Marinobacter lipolyticus SM19*

2.5 Application of the Lipase Enzyme

Lipases have emerged as an important biocatalyst in biomedical applications. Lipases can be used as diagnostic tools, as their presence can specify a disease or infection. Lipases catalyze synthetic reactions that lead to production of life-saving drugs. These can be also used as for the treatment of high cholesterol levels and digestive aids (Hasan *et al*., 2006).

2.5.1 Lipases in Processing of fat and oil

Modification of fats and oil is one of the main areas in food processing industry that wants novel economic and green technologies. Fats and oils are crucial ingredients of foods. Lipases even allow developing the characteristics of lipids by changing the location of fatty acid chains in the glyceride and exchanging one or more of these with new ones. Due to this, a relatively cheap and less needed lipid can be developed to a higher value fat. The removal of phospholipids in vegetable oils (de-gumming) using highly selective microbial phospholipases is also a recently developed environmentally friendly process (Clausen K, 2001). There are many studies on the hydrolysis of fats and oil by lipases used either in the pure form, in the immobilized form or in the cell bound form (Ghosh *et al*., 1996).

2.5.1 Lipases used in Food Industry

In biotechnology field, there are different industrial applications that result in biotech products that are used in day-to-day life. Lipases have immense application in food industry such as in cheese ripening, flavor development and EMC technology (Saxena R.K. *et al.*, 1999). Lipases are used ex situ to produce flavors, and to modify the structure by inter- or transesterification, in order to obtain products of increased nutritional value, or suitable for parental feeding. It is also used in food to adapt flavor by synthesis of esters of short chain fatty 42 acids and alcohols, which are known flavor and fragrance compounds (Macedo G.A *et al.*, 2003).

2.5.2 Lipases in Detergents

Lipase has also used in detergent making which is now ordinary in developed countries, where more than half of all detergents presently available containing enzymes. Currently laundry detergents are becoming more famous due to their increasing use in washing machine, where it shows softness, resiliency to fabrics, antistaticness, dispersible in water and gentle to eyes and skins. There are various different brands or types of laundry detergents, and usually they claim some unique qualities (Bajpai D, 2007). There has been a fabulous raise in the implication of the biotechnological applications of lipases since the last two decades where they exhibit incredible usefulness in catalytic performance. The most recent tendency in detergent industry is towards inferior wash temperatures which not only save energy, but also assist to retain the texture and quality of fabrics (Weerasooriya M.K.B. and Kumarasinghe A.A.N, 2012). Detergent industries are the primary clients of enzymes, in terms of both volume and worth. The use of enzymes in detergents formulations enhances the detergents capability to eliminate tough stains and building the detergent environmentally secure. Nowadays, numerous laundry detergent products have cocktails of

enzymes including lipases, amylases, cellulases and proteases (Kumarasinghe A.A.N, 2012).

2.5.3 Lipase in pulp and paper industry

Lipases in Paper and Pulp Industry Lipases presence decreases the pollution in waste waters and increases whiteness of the paper. They are used for control of pitch, in removal of waxes and triglycerides from pulp produced by paper industries. The pitch control method was developed by Nippon paper industries in Japan from Candida rugosa to hydrolyze 90% of the 17 triglycerides (Jaeger and Reetz, 1998). They also help in the removal of lipid stains during paper recycling and to avoid the formation of sticky materials (Guncheva and Zhiryakova, 2011; Dube *et al*., 2008).

2.5.4 Lipases in Oleochemical Industry

The current trend in the oleochemical industry includes the use of immobilized lipases to progress various reactions using mixed substrates. Due to which immobilized enzyme is used which ensures highest amount of productivity as well as running of the processes. The extent for the application of lipases in the oleochemical industry is huge as it saves energy and minimizes thermal removal during hydrolysis, alcoholysis and glycerolysis.

2.5.5 Lipase Bioremediation and Environmental Processes

Lipases are used in degradation of organic debris, treatment of residual water rich in oil and sewage treatment from a wide-ranging industry (Hasan *et al.,* 2006). Moreover, they are useful in the treatment of biofilm deposits and oil contaminated soils (Demarche *et al.,* 2011; Gea *et al*., 2007)

2.5.6 Lipases used in Cosmetics and perfumery

Lipases have an important application in cosmetics and perfumeries as it shows activities in surfactants and in aroma production. Retinoids have a major industrial potential in cosmetics

and pharmaceuticals such as skin care products. Water-soluble retinol derivatives were prepared by catalytic reaction of immobilized lipase.

2.5.7 Lipases in Tea Processing

The quality of black tea is dependent largely on the dehydration, mechanical breaking, and enzymatic fermentation to which tea shoots are subjected. During manufacture of black tea, enzymatic breakdown of membrane lipids initiate the formation of volatile products with characteristic flavor properties, emphasize the importance of lipid in flavor development. Lipase 44 obtained by Rhizomucor miehei enhances the stage of polyunsaturated fatty acid found by minimizing in total lipid content

2.5.8 Lipase in biomedical application

Lipases have emerged as an important biocatalyst in biomedical applications. Lipases can be used as diagnostic tools, as their presence can specify a disease or infection. Lipases catalyze synthetic reactions that lead to production of life-saving drugs. These can be also used as for the treatment of high cholesterol levels and digestive aids (Hasan *et al*., 2006).

2.5.9 Lipases used as Biosensors

An emerging field is the use of microbial lipase as biosensors which can be chemical or electronic in nature. A crucial use of lipases is identification of lipids used for clinical purpose. The basic theory is to use a lipase to produce glycerol from triacylglycerol and to determine the quantity of the released glycerol or the non-esterified fatty acid by the chemical and enzymatic method. This principal diagnoses the patients with complaints of cardiovascular and enables the physicians precisely to Non-specific lipase, obtained from C. rugosa with high specific activity has been chosen to permit fast release of glycerol. C. rugosa lipase biosensor, which helps to conjugates to bio-recognition group in DNA, has been developed as probe.

2.5.10 Lipases used in Medical Applications

Lipases isolated from the wax moth were obtained to have a bacteriocidal reaction on Mycobacterium tuberculosis (MBT) H37Rv. This basic study may be regarded as part of global unselected screening of biological and other materials for determining new promising sources of drugs. Lipase from Candida rugosa has been used to synthesize Iovastatin, which is a drug that has lower serum cholesterol level. The asymmetric hydrolysis of 3-phenylglycidic acid ester which is a crucial intermediate in the synthesis of diltiazem hydrochloride that is a widely used coronary vasodilator.

2.5.11 Lipases in Energy Industry

Lipases are used in transesterification of animal/vegetable oils in production of biodiesel, biokerosene and lubricants from renewable sources (Jaeger and Eggert, 2002). In addition the additives produced from these enzymes are also essential in decreasing the viscosity of biodiesel. Biodiesel fuel originates from renewable natural resources and concomitantly reduces sulfur oxide production.

Table 2.5 Major application of microbial Lipases

(Vulfson, 1994; Sharma *et al.*, 2001)

Sr.No	**Industry**	**Product of Application**	**Action**
01	Dairy Food	Development of flavoring agent in milk cheese and butter	Hydrolysis of milk fat, cheese ripening, modification of butter fat
02	Bakery Foods	Shelf-life prolongation	Flavour improvement
03	Food dressings	Mayonnaise dressing and whippings.	Quality improvement
04	Health food	Health foods	Transesterification
05	Fish and Meat	Meat and fish product fat removal	Removal of fat
06	Laundry	Cleaning cloths	Reducing stain
07	Cosmetics	Skin and sun-tan creams, bath oil etc	Esterification
08	Surfactants	Polyglycerol and carbohydrates fatty acid esters used industrial detergents and as emulsifiers in food formulation such as ice-cream	Production of lysophospholipids
09	Fuel industry	Biodiesel production	Transesterification
10	Pharmaceutical	Produce various intermediates used in manufacture of medicine.	Expolyester alcohol hydrolysus
11	Pollution control	To remove hard stains and hydrolyzed oil and grease	Hydrolysis and transesterification of oils and grease

CHAPTER 3: METHODOLOGY

3.1Sample collection

Seaweed and water samples were collected from Vagator beach. The seaweed and water samples were collected in zip lock bags and bottles respectively. The water samples were checked for the physicochemical parameters such as salinity, pH and temperature. The seaweed samples were used for isolating seaweed associated bacteria.

3.2 Isolation of seaweed associated bacteria

The seaweed samples were rinsed using filter sterilized sea water and were made into pieces using sterile forceps. The enrichment for the isolation of seaweed associated bacteria was done in two ways (1) Direct plating wherein, the seaweed pieces were placed on Zobelle marine agar (ZMA), (2) Enrichment in broth, the seaweed pieces were inoculated in Zobelle Marine broth. The plates and flasks were incubated at 28ºC for 24 hours. After incubation, from the plate, the bacterial cultures growing at the edge of the seaweed were streaked on ZMA. From the cultures grown in flask serial dilutions were carried out up-to 10^{-4} and the 10^{-2}, 10^{-3}, 10^{-4} dilutions were spread plated on ZMA. Streaked and spread plates were incubated at room temperature for 24 hours. The methodology was taken from Tawara *et al.,* (2015) used for the isolation of seaweed associated bacteria

3.3Purification and maintenance

The colonies obtained were purified by streaking on ZMA and maintained

3.4 Screening of enzymes

The purified bacterial isolates were screened for the following enzymes:

3.4.1a. Screening of agarase enzyme

The ability of bacterial isolates to produce agarase was checked by spot inoculating the isolates on Artificial Seawater (ASW) Agar containing agar as the sole source of carbon. The plates were incubated at room temperature for 36-48 hours. After incubation the plates were flooded with 5% Lugol's Iodine and were observed for zones of clearance.

This methodology is taken from Naik *et al*., (2018), the best isolate was determined as the one showing maximum zone of clearance on addition of Lugol's iodine

3.4.1b Screening of protease enzyme

The ability of bacterial isolates to produce protease was checked by spot inoculating the isolates on ASW agar containing 1% skimmed milk. The plates were incubated at room temperature for 24 hours. After incubation the plates were observed for zones of clearance. This methodology was taken from Sneha et al., (2014) for isolation of protease producing bacteria from marine environment,

3.4.1c Screening of amylase enzyme

The ability of bacterial isolates to produce amylase was checked by spot inoculating the isolates on ASW agar medium containing 1% soluble starch. The plates were incubated at room temperature for 24-48 hrs. After incubation the plates were flooded with 1 % Gram's Iodine and were observed for zones of clearance. This methodology was taken from Naik *et*

al., (2018), wherein amylase activity of seaweed associated bacteria was checked by growing the culture in seawater broth containing starch as the carbon source.

3.4.1d Screening of lipase enzyme

The ability of bacterial isolates to produce lipase was checked out by spot inoculating the isolates on ASW agar medium containing 1% Tween 80. The plates were incubated at room temperature for 24 hours. After incubation, the plates were observed for white precipitate around the colonies. This methodology was taken from L. Ramnath *et al.*, (2017)

3.4.1e Screening of esterase enzyme

The ability of bacterial isolates to produce esterase was checked by spot inoculating the isolates on ASW agar medium containing 1% Tween 20. The plates were incubated at room temperature for 24 hours. After incubation, the plates were observed for precipitate around the colonies. This methodology was taken from L. Ramnath *et al.*, (2017).

As explained by L. Ramnath et. al. (2017), Tween 80 consists of esters of oleic acid and hence is degraded by lipase and tween 20 consists of esters of lower chain fatty acids and is degraded by esterase enzyme. The principle is based on calcium precipitation, wherein the fatty acids released bind to the calcium present in the medium and forms an insoluble precipitate as observed around the colony.

3.4.1f Screening of alginate lyase enzyme

The ability of bacterial isolates to produce alginate lyase was checked by spot inoculating the isolates on sodium-alginate agar containing 0.5% sodium alginate. The plates were incubated at room temperature for 24 hours. After incubation, the plates were observed for

zones around the colonies. The methodology was taken from Wang *et al*., (2017), for the screening of alginate lyase producing bacteria

3.4.1g Screening of cellulase enzyme

The ability of bacterial isolates to produce cellulase was checked by spot inoculating the isolates on ASW agar medium containing 1% carboxy-methyl cellulose. The plates were incubated at room temperature for 24-48 hours. After incubation, the plates were flooded with 1% Congo red for 15 minutes and then decolorized with 1% NaCl for 15 minutes. These steps were repeated three times to remove excess unbound Congo red and the zones of clearance around the colonies were observed. This methodology was given by Naik *et al* (2018).

3.4.1h Screening of pectinase enzyme

The ability of bacterial isolates to produce pectinase was checked by spot inoculating the isolates on ASW agar medium containing 0.5% pectin. The plates were incubated at room temperature for 24 hours. After incubation, the plates were observed for clear zones of hydrolysis around the colonies. This methodology was given by T. Fouzia *et al*., (2018).

3.5 Biochemical characterization

The selected bacterial isolates were tested for the following biochemical characteristics

Gram character

3.5a Gram staining

Smear of bacterial isolates was prepared on clean grease free slides, air dried and heat fixed. The heat fixed smear was flooded with Gram's Crystal violet for 1 minute. The side was drained, washed with water and flooded with Gram's iodine for 1 minute. The slide was washed with 70% ethanol for 10-20 secs until the colour of crystal violet comes out. The slide was flood with safranin for 1 min. It was then washed briefly, drained, air dried and

observed under oil immersion lens. The Gram character and morphology of the bacterial isolates were noted down.

3.6 Effect of NaCl concentration on lipase production

The ability of bacterial isolates to produce lipase was checked by spot inoculating the isolates on the Modified ASW media containing 1.8g/L calcium chloride and varying NaCl concentration as 0.5%,1%,1.5%, 2%,3%,4% and 5% with two different substrates Tween20 and Tween80. The plates were incubated at room temperature for 24 hours. After incubation the plates were observed for clear zone.

3.7 Effect of temperature on lipase production

The ability of bacterial isolates to produce lipase was checked by spot inoculating the isolates on the Modified ASW media with two different substrates Tween 20 and Tween 80. The plates were incubated at 15^0C,R.T,37^oC ,45^oC. After incubation the plates were observed for clear zone.

3.8 Effect of pH on lipase production

The ability of bacterial isolates to produce lipase was checked by spot inoculating the isolates on the modified ASW media with different pH as 4,5,6,7 and 8 for two different substrates Tween 20 and Tween 80. The plates were incubated at room temperature for 24 hours. After incubation the plates were observed for clear zone.

3.9 Effect of different substrate on lipase production

The ability of bacterial isolates to produce lipase was checked by spot inoculating the isolates on the ASW media containing for different commercial oils as substrates such as coconut oil, groundnut oil, sunflower oil , mustard oil and palm oil. The plates were

incubated at room temperature for 24 hours. After incubation the plates were observed for clear zone.

3.10Application of Lipase enzyme

Lipase is an extracellular enzyme. The cell free culture broth of P1 and S-T-2 bacterial isolates was used to check the application of lipase enzyme for stain removal and deinking.

Fabric cloths pre-stained with pickle oil (200µl) and kept for the overnight to dry, similarly fabric marked with the blue ball point pen were used for deinking. The stained cloths were taken in the separate petri plates. The bacterial culture was grown in the modified ASW broth and kept on the shaker for 24 hrs. The culture was taken in the 50 ml of falcon tubes & centrifuged for the 10,000 rpm for 10 mins. The supernatant was filter sterilized using 0.45-micron nitro cellulose filter paper under sterile condition.

The filtrate was used to check the stain removal ability. A control was set up by putting the pickle oil-stained cloth in the 10 ml of MASW media broth without culture inoculation. Test was carried out by pouring 10 ml of filtrate on the stained fabric in a sterile condition. The plates were observed after every 1 hour and results were noted by visual examination.

CHAPTER 4:
ANALYSIS AND CONCLUSION

4.1 Seaweed sample collection

Seaweed samples were collected from Vagator beach, the sampling site is shown in **Figure 4.1**. The physicochemical parameters were noted and are mentioned in the **Table 4.1**. Five different types of seaweeds samples were collected and identified tentatively as a) *Gelidium* sp. (b*) Sargassum* sp.(c) *Gracilaria* sp. (d) *Pdiana* sp. (e) *Stoechospermum* sp**.** by visualizing their tentative morphological characteristics. *The* seaweed samples are shown in **Figure 4.2**

Fig 4.1: Seaweed sampling at Vagator beach

Table 4.1: Physicochemical Parameters of water sample collected from the sampling sites

Physicochemical parameters	**Result**

Salinity	4%
pH	8.0
Temperature	31°C

Figure 4.2: Seaweed samples collected and tetatively identified (a) *Gelidium* sp. (b*) Sargassum* sp:(c) *Gracilaria* sp (d) *Padiana* sp (e*) Stoechospermum* sp

4.2 Isolation of seaweed associated bacteria

Seaweed associated bacteria were isolated by direct plating and enrichment method **Figure 4.3.**

A total of 15 morphologically distinct bacteria were isolated from 5 different seaweeds samples were summarized in the **Table 4.2**.

(a) (b) (c) (d)

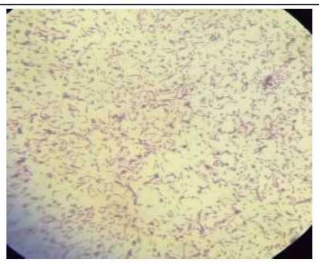
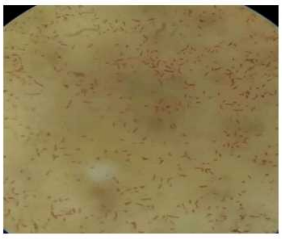
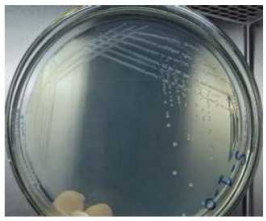
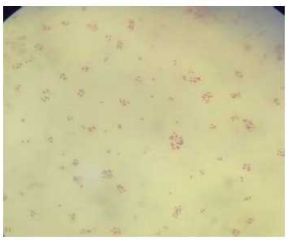
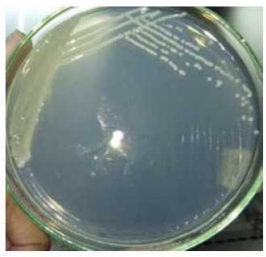
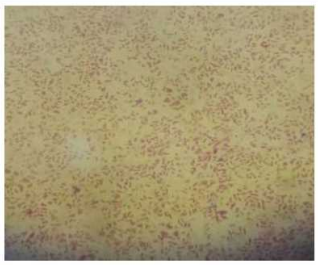
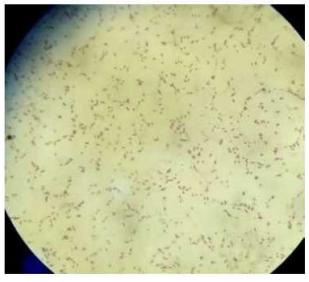
Figure 4.3 Isolation of seaweed associated bacteria (a)Enrichment in ZMB (b) Direct plating on ZMA (c)Isolated colonies obtained on ZMA (d)Purified bacterial colonies on ZMA

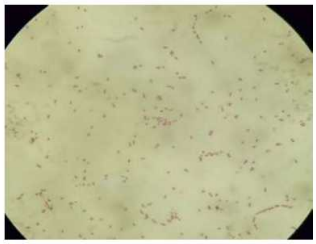
Table 4.2 List of seaweed associated bacteria obtained from Vagator beach

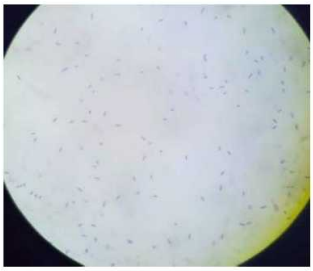
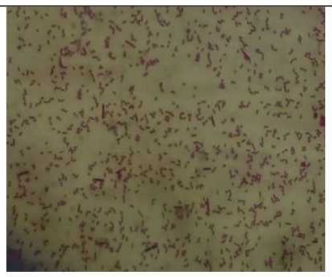
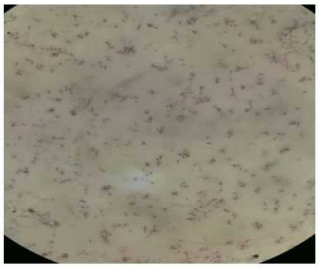
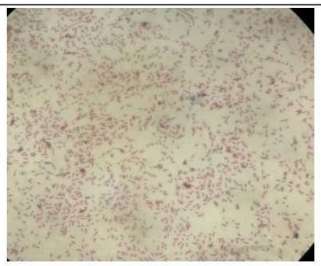
Seaweed Samples	Bacterial Isolates	Culture code
Gelidium sp	2	G1, G2
Sargassum sp	4	S-1-0, S (0), S-T-2, S(T)
Gracilaria Sp	2	GC1, GC2
Padiana sp	1	P1
Stoechospermum sp	6	SM1, SM2, SM3, SM4, SM5, SM6

4.4 Purification and maintenance

The purified colonies of bacterial isolates and their Gram character was shown in the **Figure 4.4.**The colony characterization of bacterial isolates is summarized in the **Table 4.3**

Culture Code	Culture Plate	Gram staining	Gram Character
G1			Gram positive pleomorphic
G2			Gram positive Short rods
S-1-0			Gram negative cocci
S (0)			Gram negative Short rods
S-T-2			Gram positive Short rods

S(T)			Gram negative Short rods

SM1			Gram positive Long thin rods
SM2			Gram negative Short rods
SM3			Gram negative Short rods
SM4			Gram negative Short rods

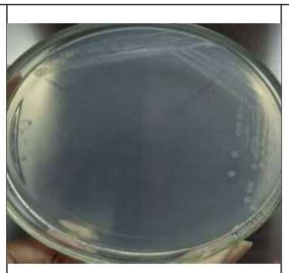
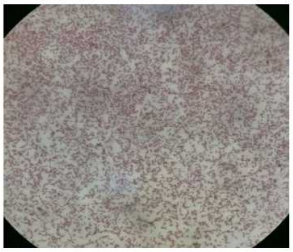
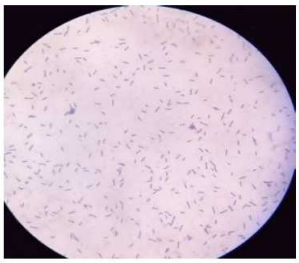
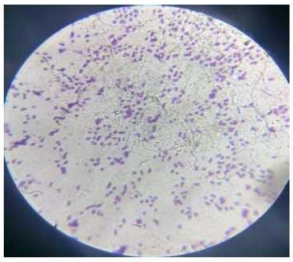
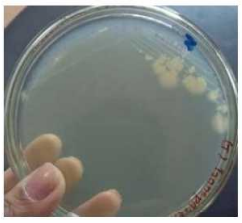
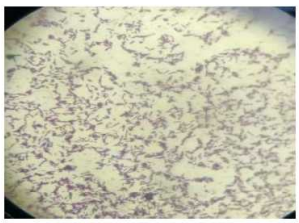
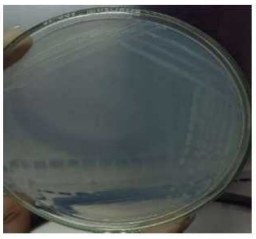
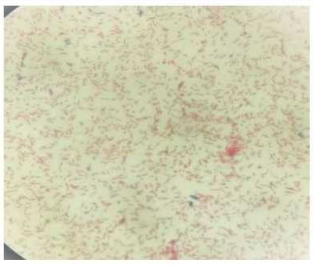
SM5			Gram negative Short rods
SM6			Gram positive Long rods
GC1			Gram positive cocci
GC2			Gram positive long rods
P1			Gram negative Short rods

Figure 4.4: The purified colonies and gram staining of bacterial isolates

Table 4.3 Colony characteristics of 15 seaweed associated bacterial isolates

Colony code	G1	G2	S-1-0	S (0)	S-T-2	S(T)	GC1	GC2	SM1	SM2	SM3	SM4	SM5	SM6	P1
Media	ZMA	NA	ZMA	ZMA	ZMA	ZMA	NA	NA	ZMA	ZMA	ZMA	NA	NA	NA	NA
Temperature	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C	24 °C
Time	R.T	R.T	R.T	R.T	R.T	R.T	R.T	R.T	R.T	R.T	R.T	R.T	R.T	R.T	R.T
Shape	circular	Circular	circular	Circular	Circular	Circular	round	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Size	0.1 cm	Small	0.2cm	0.2 cm	0.1	0.1	0.1	0.1	0.1 cm	0.2cm	Small	0.1 cm	Pinpoint	Small	Small
Colour	Off-white	White	Creamy	Pale yellow	white	Creamy	Whitish	White	Off white	Creamy	White	White	White	White	Creamy
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	convex	convex	Raised	Raised	Convex	Convex	Convex	Convex	Flat	Flat	Convex	Flat	Flat	Flat	Convex
Surface texture	smooth	Mucoid	Mucoid	Mucoid	Mucoid	Smooth	Smooth	Smooth	Mucoid	Smooth	Mucoid	Smooth	Smooth	Smooth	Smooth
Consistency	mucoid	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Mucoid	Butyrous	Butyrous	Butyrous	Mucoid	Butyrous
Opacity	opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Transparent	Opaque	Transparent	Opaque
Gram Character	Gram +ve pleomorphic	Gram +ve Short rods	Gram -ve cocci	Gram -ve Short rods	Gram +ve Short rods	Gram -ve short rods	Gram +ve cocci	Gram +ve Long rods	Gram +ve Long thin rods	Gram -ve Short rods	Gram -ve Short rods	Gram -ve Short rods	Gram -ve short rods	Gram +ve Long rods	Gram -ve Short rods

4.3 Screening of Seaweed associated bacterial isolates for enzyme production

The 15 isolates obtained from seaweed samples were screened for 7 different enzymes; Protease, amylase,esterase,lipase cellulose, pectinase and alginate lyase. Most of the bacterial isolates were positive for amylase, protease,lipase and agarase.The isolates showing positive results for four enzymes are summarized Table 4.4 and shown in the Fig 4.5

Table 4.4 Bacterial isolates showing positive results for the following enzymes

Culture Code	Amylase	Protease	Lipase	Agarase
G1	+	+	+	+
G2	+	+	+	+
S-1-O	-	-	-	-
S (0)	+	+	+	+
S-T-2	+	+	-	+
S(T)	+	+	-	+
GC1	+	-	-	+
GC2	+	+	+	+
SM1	-	-	-	-
SM2	-	-	+	-
SM3	-	+	-	-
SM4	+	+	-	+
SM5	+	+	+	+
SM6	+	+	-	+
P1	+	+	+	+

Six bacterial isolates namely G1, G2, S(O), GC2, SM5 and P1 showed enzyme production i.e amylase, protease, lipase and agarase. Other bacterial isolates namely S-T-2, S(T), GC1, SM2 SM3, SM4 and SM6 showed variable results for the four enzymes. Bacterial isolates S-1-0 and SM1 were negative for all the enzymes tested.

Bacterial isolates G1,G2,S(O), GC2,SM2,SM5,P1shows precipitation around the colony due to the ca +2 ions present in the media forms the insoluble ca+ salts around the colony. The zone of the hydrolysis formed around the colony due to the hydrolysis of lipids takes place.Sachan &Shewta (2018) reported Pseudomonas aeruginosa JCM 5962(T) which was isolated from the soil of the sugarcane field. Lee et al.,2015 reported that Bacillus species

isolated from the oil contaminated area showed clear zone around the colony when Tween 20 used as substrate due to the complete degradation of the salts of the fatty acids.

In this study we were interested in the seaweed associated bacteria producing lipase enzyme and since seven bacterial isolates were showing positive results for lipase production they were used for the further studies. These isolates showed luxuriant grow on the ZMA therefore lipase activity was checked on the modified ASW media amended with Tween 20 and Tween 80. Clear zone was observed around the colony as shown in the **Figure 4.6**

4.4 Lipase production under different growth conditions

Based on result for screening the bacterial isolates for lipase production on MASW supplemented with Tween 20 and Tween 80, Two isolates namely P1 and S-T-2 were selected for further studies.

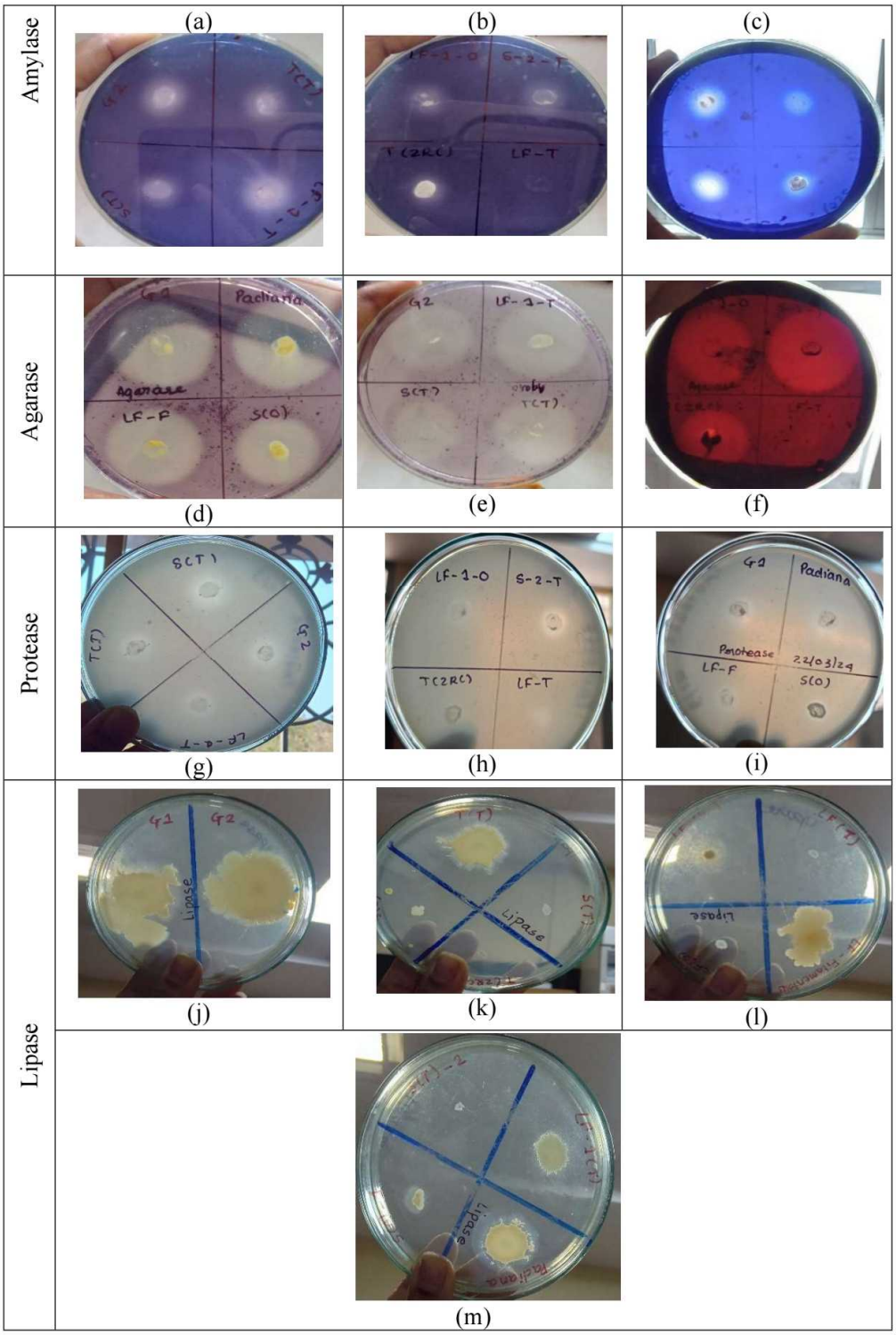


Figure 4.5: Screening of Enzymes

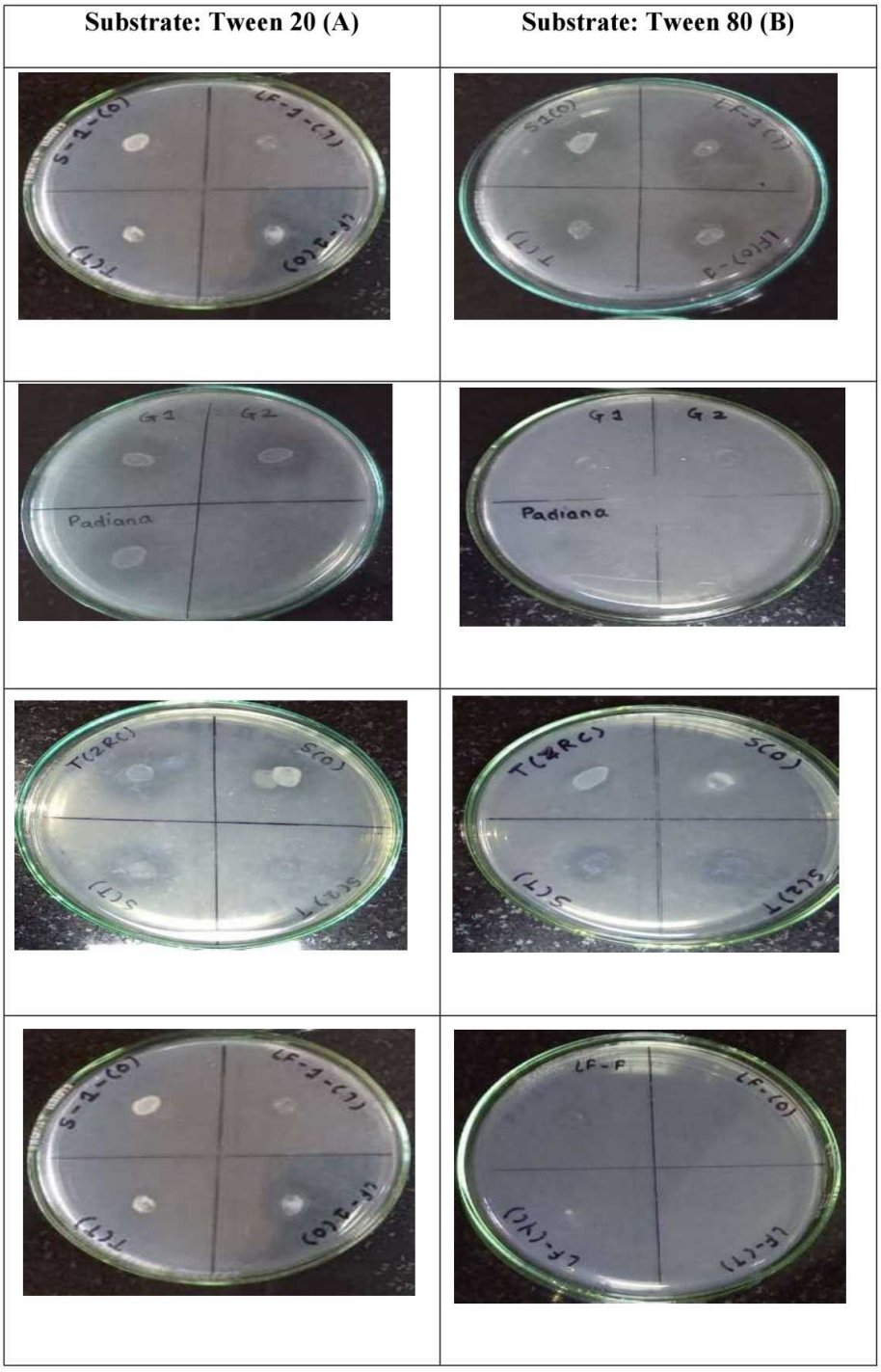


Figure 4.6: Production of Lipase enzyme by seaweed associated bacteria on 2 different substrates (A) Tween 20 (B) Tween 80

4.5 Effect of varying NaCl Concentration on lipase activity for selected bacterial isolates

Bacterial isolates P1 and S-T-2 were checked for lipase production in MASW media with varying concentration upto 15% NaCl concentration. Both the isolates showed growth upto 15% NaCl concentration. The enzyme production was seen upto 8 %. At 8%,9% and 15 % NaCl concentration there was slight growth as shown in the **Figure 4.7**. To record the optimum NaCl concentration required for lipase production by these bacterial isolates, we did another set of experiment ,where we used lower NaCl concentration upto 5% .It was observed that the optimum NaCl concentration was 1.5% as shown in the **Figure 4.8.1** .The zone of hydrolysis was maximum at this percentage as shown in the figure 4.8 .As reported by Saha et al.(2020) *Bacillus* sp.,*Vibrio* sp., *Chrysobacterium* sp.,isolated from seaweed *Agarophyton vermicullophylum* could tolerate salinity of 1.65 %,whereas, among the *Gammaproteobacteria* ,*Pseudomonas*,could tolerate salinity levels of 0.85%

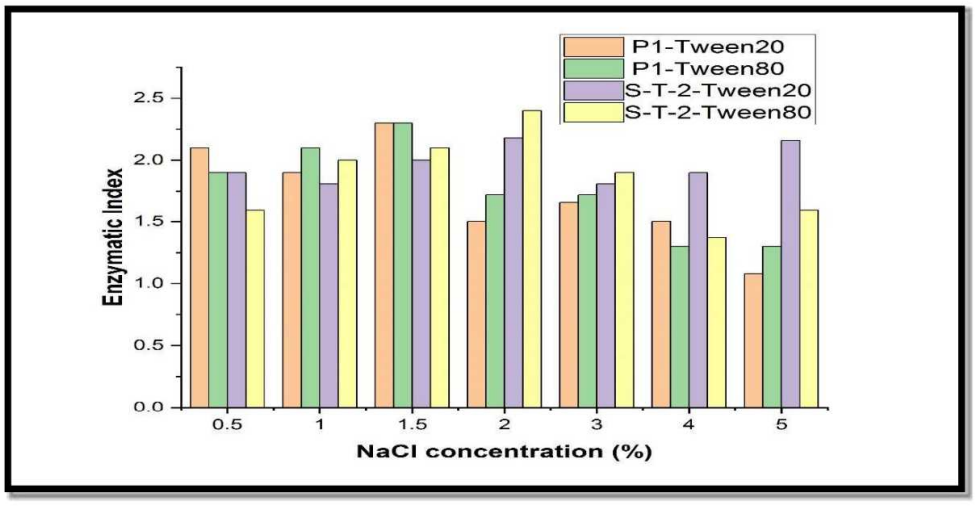


Figure 4.8.1: Graph showing effect of NaCl concentration upto 5 %

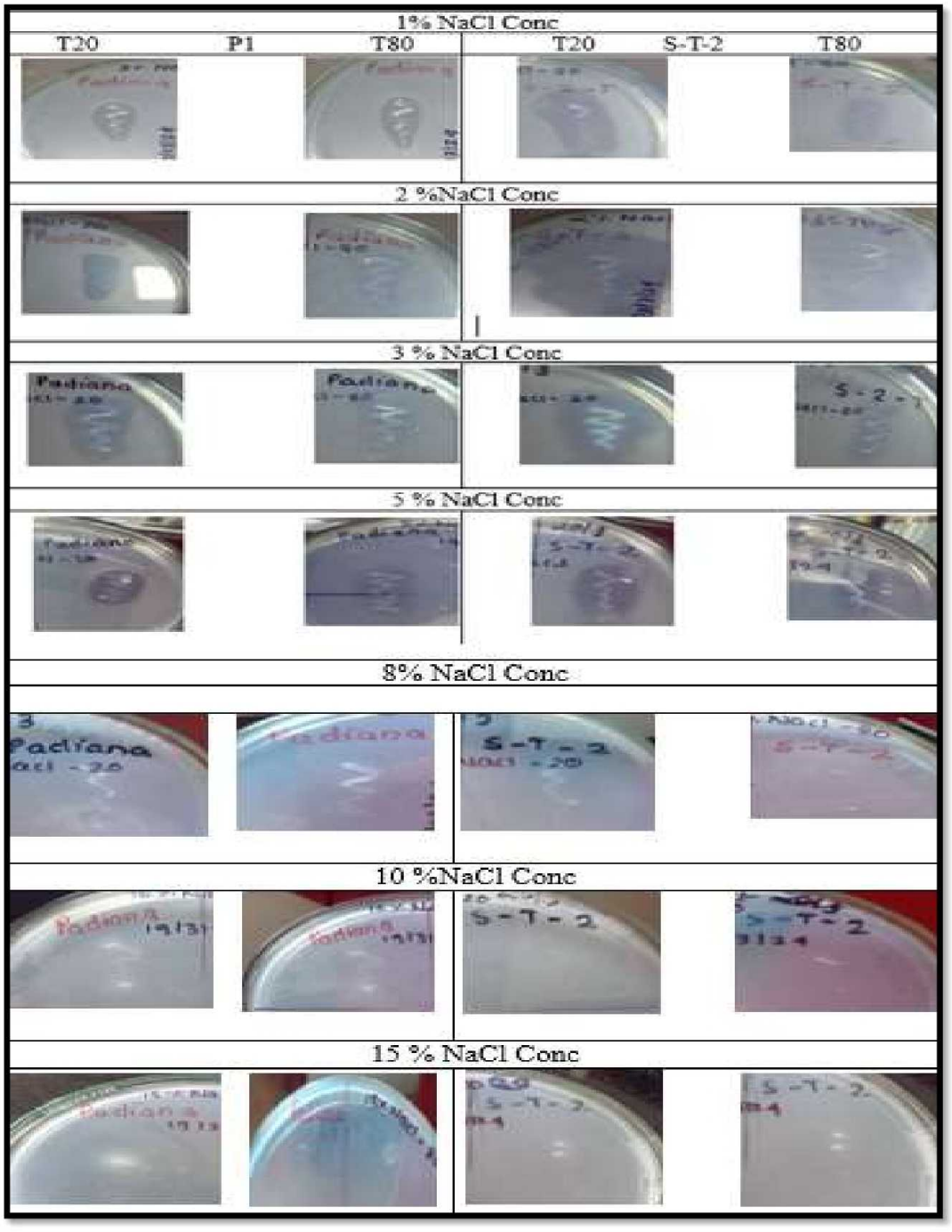


Figure 4.7: Effect of NaCl concentration upto 15%

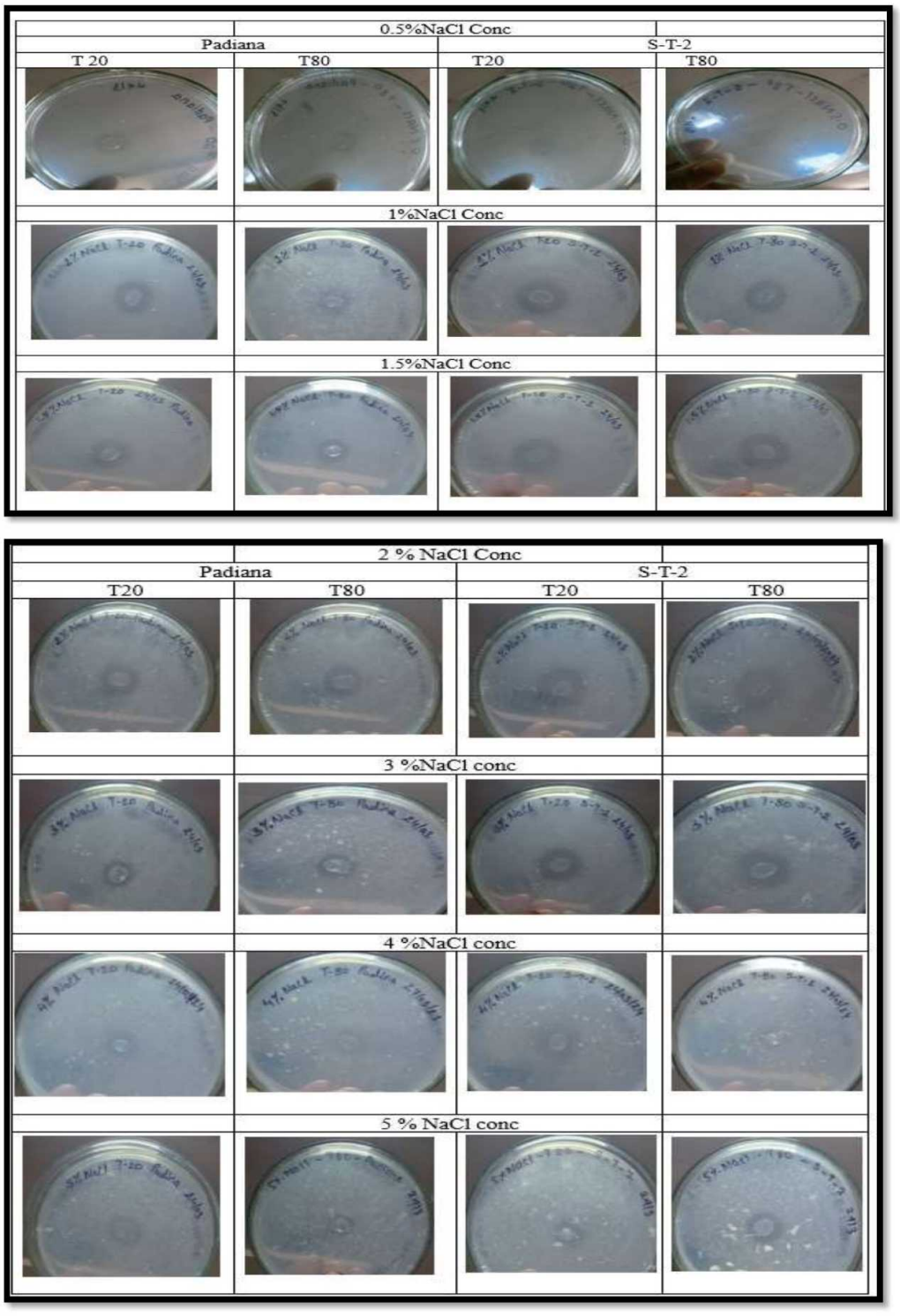


Figure 4.8: Effect of NaCl concentration upto 5 %

4.6 Effect of Different Temperature on Lipase activity

The maximum activity was observed for the bacterial isolates i.e. P1 and S-T-2 as 24 ^{0}C as shown in the **Figure 4.9 and Figure 4.9.1.** It is revealed that the isolate Bacillus cereus.A25 isolated from coconut oil contaminated area shows a maximum lipase activity (380U/mL) at temperature 40 ºC(Mohan *et al.*, 2010)

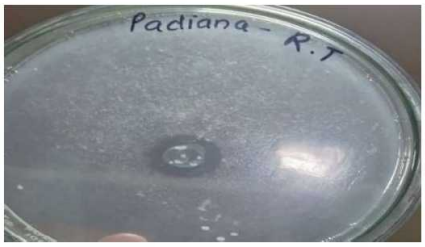
P1	S-T-2
15^{0}C	
	
Room temperature	
	
37 ^{0}C	
	

Figure 4.9: Effect of temperature on lipase production

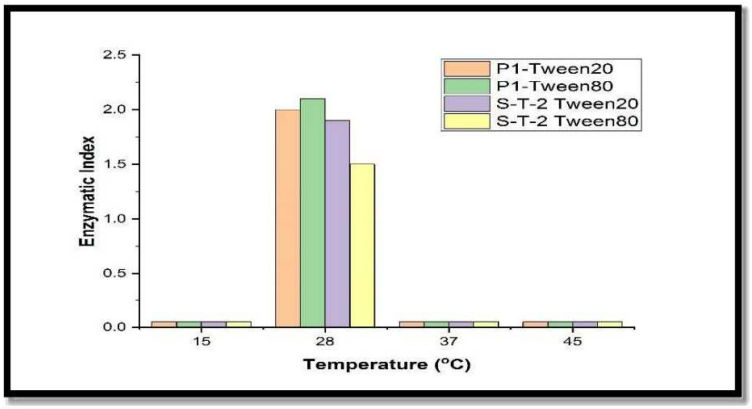


Figure 4.9.1: Graph showing optimum temperature on lipase production

4.7 Effect of varying pH on Lipase activity for selected Bacterial Isolates

Bacterial isolates were checked for the enzyme production in the MASW media having the pH from the 5 to 8. It was observed that all the pH values showed growth and enzyme production. Based on the zone of hydrolysis it is observed that both culture P1 and S-T-2 showed highest enzymatic index at pH 6 indicating the optimum pH for the growth and enzyme production as shown in the **Figure 4.10 and Figure 4.10.1.** It was reported that lipase activity of *Bacillus cereus* which was isolated from oil contaminated area was maximum at pH 7.0 and the optimum pH ranges for lipase production was at pH 7 for *Penicillium chrysogenum* (Ferrer *et al.,* 2000). In another study, there is a strong influence of pH on lipase enzyme production with the maximum activity at pH 8.0 (Manoj *et al.,* 2010).

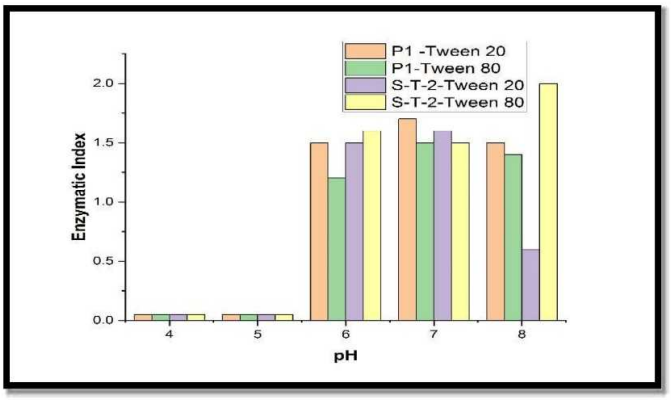


Figure 4.10.1: Graph showing optimum pH on lipase production

4.7 Effect of different substrates on Lipase Activity

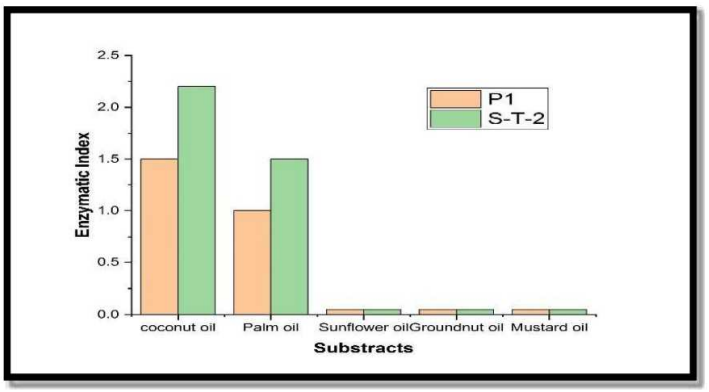


Figure 4.11.1: Graph showing different substrates on lipase production

Bacterial isolates P1 and S-T-2 showed lipase activity growth on media supplemented when coconut oil and Palm oil were used as substrates. Bacterial isolate S-T-2 show maximum lipase activity in coconut oil as compared to P1 isolate. The enzymatic index for S-T-2 was 2.2 and P1 it was 1.5 respectively when the coconut oil used as substrate. Both the isolates were not showing zone of clearance when sunflower oil,groundnut oil, mustard oil as substrates. As reported by Sachan and Shweta (2018)The lipolytic activity for coconut,sesame and flex oil cakes were observed after 72 hrs incubation and mustard oilcake shows after 48hrs of incubation whereas groundnut oilcakes fermentation media did not induce significant lipase activity.

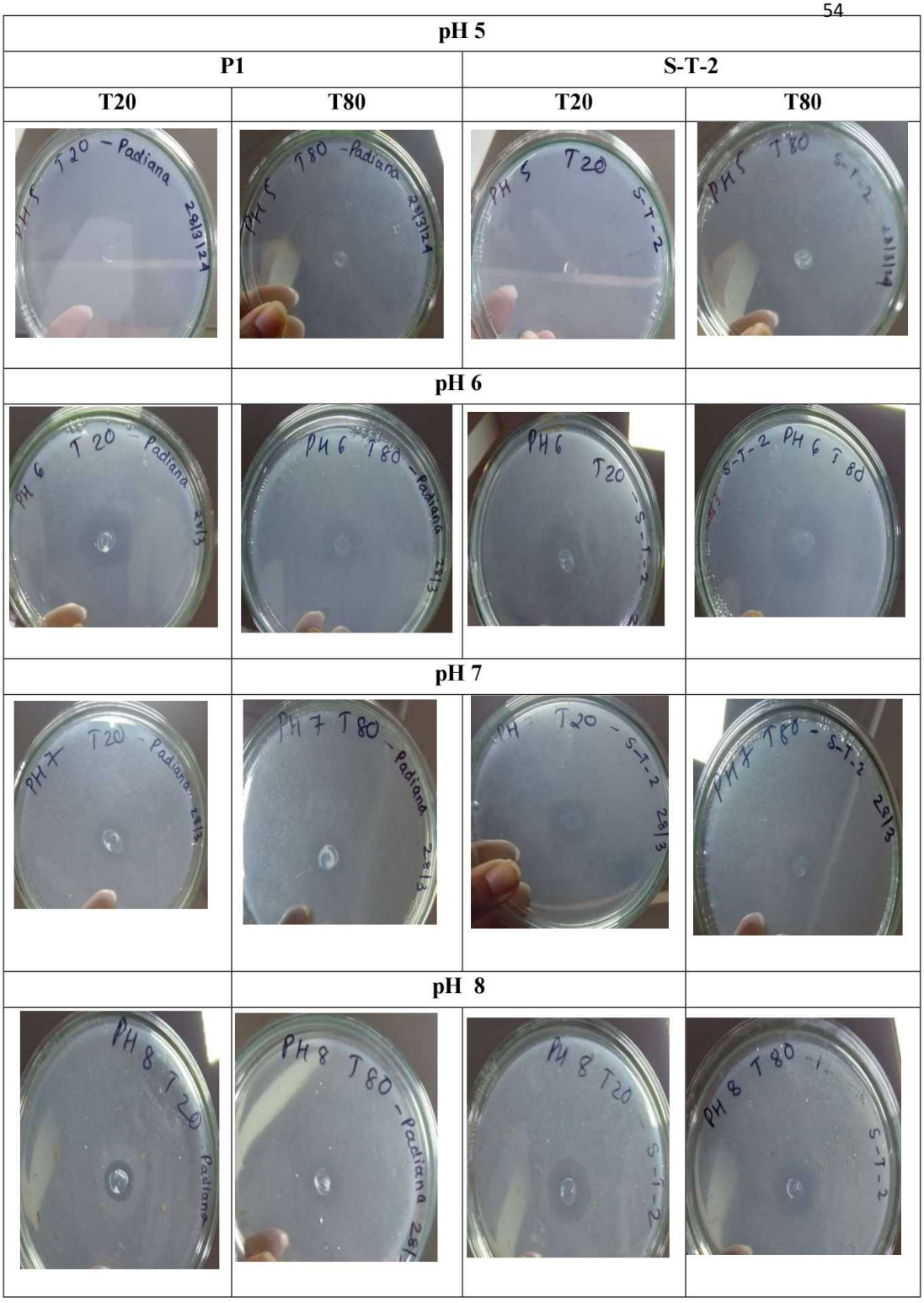


Figure 4.10: Effect of varying pH on lipase production

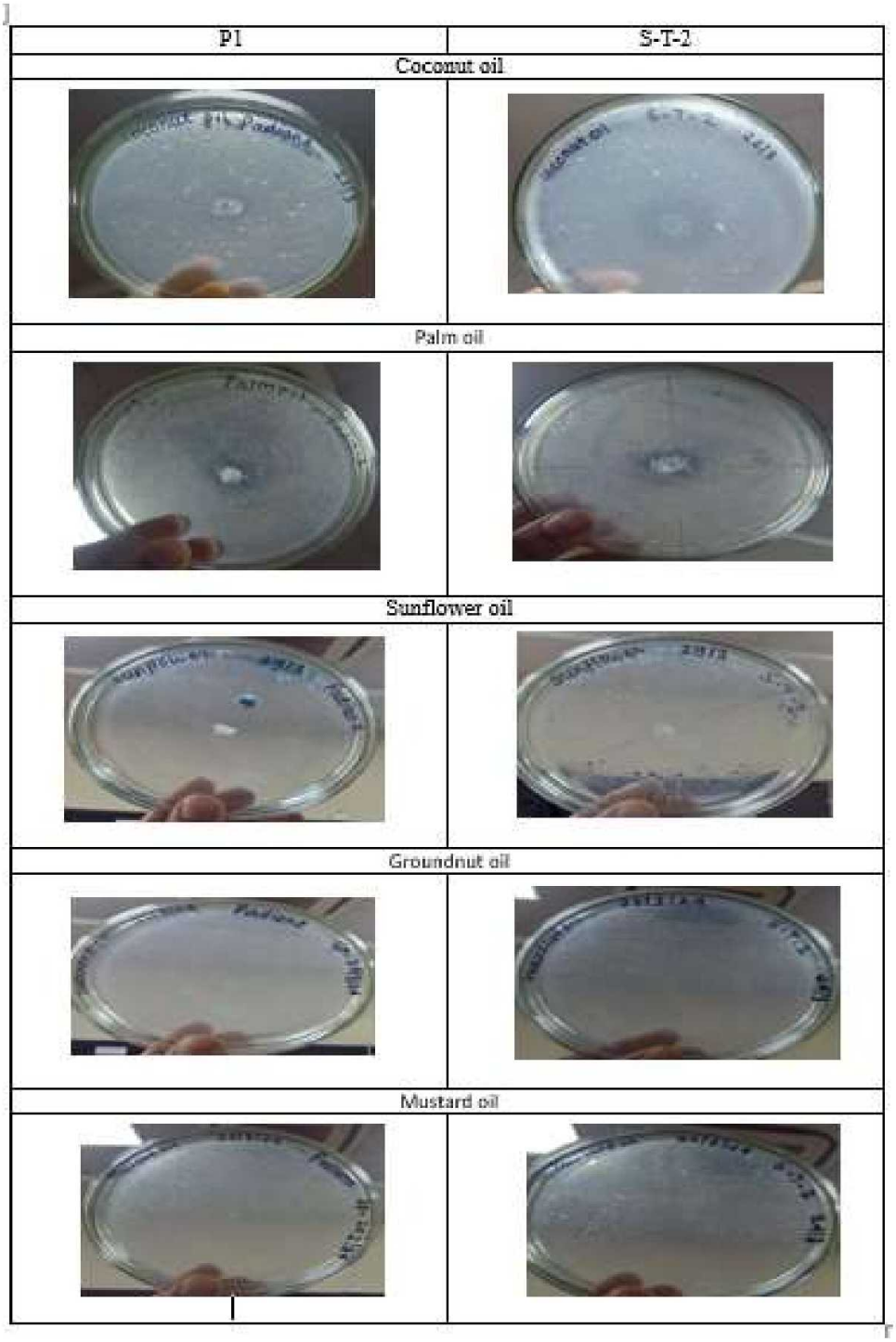


Figure 4.11: Effect of different substrate on Lipase production

4.8 Application of Lipase enzyme in pickle oil Degradation

Visually it was observed that as the time of incubation increased more and more oil droplets left the fabric and came into supernatant. It was recorded that the Bacterial isolates P1 was showing more oil droplets left from the cloth rather than Bacterial isolate S-T-2. For the deinking experiment the supernatant was collected after 4 hrs and appeared to be blue as compare to the control. In this experiment bacterial isolate P1was showing potential production of lipase enzyme as compared to isolate S-T-2 as shown in the **Figure 4.12.**

As reported by Chandran and Sreecha,2020 isolated Bacillus *cereus* A.25 from the soil contaminated with coconut oil showed stability in pH and temperature which played a major role in destaining, dehairing and in waste water treatment because 92 % reduction in oil and grease content by the treatment of lipase indicates that there is a scope for this enzyme in the treatment of oil effluents and bioremediation. Based on the applications, lipase enzyme showed better results in destaining ability compared to others. This proved helpful for manufacturing a washing soap product named swadeshi that showed 95% destaining ability of pickle oil-stained fabric cloth.

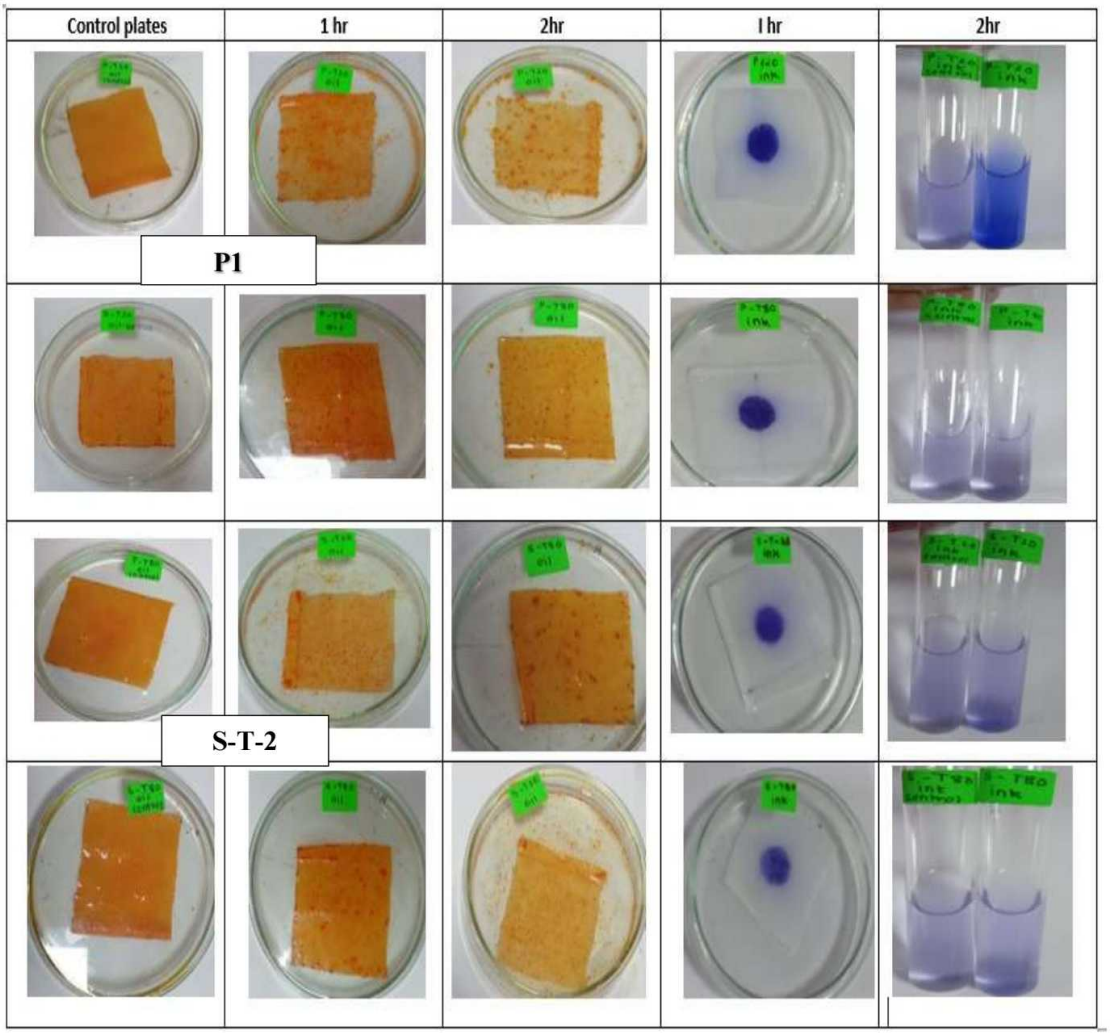


Figure 4.12: Application of Lipase enzyme in destaining Process

CONCLUSION

Seaweeds isolated from the Vagator beach were identified as *Gelidium* sp., *Sargassum* sp,*Gracilaria* sp ,*Padiana* sp and *Stoechospermum* sp.15 seaweed associated bacteria were isolated and screened for production of different enzymes.In this stucdy more emphasis were laid on elaborating maximum production produced by seaweed associated bacteria.Out of 15, 2 bacterial isolates P1and S-T-2 were selected and effect of varying NaCl concentration ,pH ,temperature was checked for enzyme production using two different substrates Tween 20 and Tween 80.Both the bacterial isolates P1and S-T-2 were checked for hydrolysis of different oils.So that oil cakes obtained after oil extraction can be utilized for the bulk production of lipase enzyme.The ability of the two isolates was checked for removal of oil and ink from the prestain fabric.It is observed that lipase enzyme produced by bacterial isolates S-T-2 showed bettere destaining and deinking activity.

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Appendix I: Composition of Media

Zobelle Marine Agar (ZMA)

Composition	g/L
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15

Distilled water	1000ml
pH	7.2

(ZMB) Zobelle Marine Broth

Composition	g/L
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Distilled water	1000ml
Final pH (at 25°C)	7.6±0.2

Modified artificial seawatwer media

Composition	g/L
NaCl	24.60
$MgSo4.7H_2O.$	6.290
$CaCl_2.2H_2O$	1.800
KCl	0.670
$NaHCo_3$	0.180

$MnCl_2.H_20$	4.660
Peptone	1.00
Agar	15g
Distilled water	1000ml
pH	7.2

NA (Nutrient Agar)

Composition	g/L
Peptone	50g
Beef extract	10g
NaCl	5g
Agar	15g
Distilled water	1000ml
pH	7.2

(ASW)Artificial seawater broth

Composition	g/L
NaCl	24.600
KCl	0.670
$CaCl_2.2H_2O$	1.360
$MgSo4.7H_2O.$	6.290
$MnCl_2.H_20$	4.66g
$NaHCo_3$	0.180g
pH	7.5
Distilled water	1000ml

Artificial sweater Agar

Composition	g/L
NaCl	24.600
KCl	0.670

$CaCl_2.2H_2O$	1.360
$MgSo4.7H_2O.$	6.290
$MnCl_2.H_20$	4.66g
$NaHCo_3$	0.180g
Agar	15g
pH	7.5
Distilled water	1000ml

Nutrient broth

Composition	g/L
Peptone	50g
Beef extract	10g
NaCl	5g
Distilled water	1000ml
pH	7

APPENDIX II: COMPOSITION OF REAGENT

Saline

Composition	g/L
NaCl	8.5g
Distilled water	1000

Lugol's Iodine

Composition	g/100ml
Potassium iodide	10g
Iodine	5g
Distilled water	100ml

Gram's Iodine

Composition	g/300ml
Potassium Iodide	2g
Iodine	1g
Distilled water	300ml

1% Congo Red

Composition	g/300
Congo red	1g
Distilled water	50ml
Ethanol	50ml

1 M NaCL

Composition	g/L
NaCl	58.44g
Distilled water	1000ml