OPTIMIZATION OF LIPASE ENZYME FROM NOVEL STAPHYLOCOCCUS SAPROPHYTICUS BACTERIA FROM MARINE SOIL

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*Abstract:*Lipasesare widely usedin various industries such as food, detergent, pharmed and also as biocatalysts in industrial processes such as esterification, biosurfactant production and bioremediation. Lipases are produced by life forms of various origin from both terrestrial and aquatic sources.There is a need for continuous research and development to produce more efficient lipases. The marine environment is a suitable source for novel lipases, owing to their unique characteristics, allowing them to function in harsh environments. The quantity and efficiency of lipase produced can be influenced by various parameters such as pH, composition of culture medium and temperature. In this paper, *Staphylococcus saprophyticus* bacteria was isolated from marine soil and screened for lipase activity. Parameters like pH, substrate, incubation time, metal ions, carbon source and nitrogen sources, detergents were used for optimization. Among these parameters, maximum lipase produced with pH 7 as parameter was found to be 6.82U/ml.

Index terms: lipase, Marine organism, *Staphylococcus saprophyticus*, optimization,

I. INTRODUCTION

The marine ecosystem is the largest ecosystem on planet Earth, housing millions of species of organisms. In spite of the marine ecosystem containing most of the microorganisms, these ecosystems remain largely unexplored, especially taxa such as protists (Sogin M.L et al., 2006). Marine soil is known to be a good source of novel enzyme producing bacteria as they are rich in nutrients and thus, there is high proliferation of microorganismsthat have shown to be highly active in nutrient recycling. Many marine microorganisms are involved degrading of organic matter(bioremediation)through specific pathways which is one of their unique characteristics. Marine microorganisms are mostly found to be extremophiles. They have high range of enzymatic activity and capable of catalyzing various biochemical reactions using their unique enzymes. For a long time, enzyme extraction and production were from terrestrial sources but now marine microbes are widely used because quantities and efficiency of the enzymes produced were more and satisfactory. Enzymes obtained from marine sources have shown to possess superior characteristics such as production of larger quantities of enzymes in a shorter time frame, higher stability in terms of temperature (especially those obtained from microbes in deep sea thermal vents) and also increased catalyzing activity. Thebacteria *Staphylococcus saprophyticus* is a gram-positive coccus belonging to the coagulase-negative genus Staphylococcus. They are commonly seen in urinary tract infections. Lipids represents 3% to 55% of organic matter in aquatic environment (Jeffrey LM.,1966;Ogura N et al., 1975) and lipids are hydrolyzed by lipases produced by heterotrophic bacteria. Lipases are the third largest used enzyme group in industries after proteases (approx. 60%) and carbohydrase. Lipases are exploited as cheap and versatile catalysts to degrade lipid in more modern applications. Lipases catalyze the hydrolysis of ester bond of triglycerides to glycerol and free acids and they are also valuable biocatalysts. Lipases are also efficient in various other reactions such as esterification, transesterification, biosurfactant production and aminolysis. Lipases also find enormous applications in the food production, biodegradation of plastic and processing industries. In the dairy sector, lipases are used to enhance the flavor and time of ripening of cheese (C. Zhang et al., 2012; I.V. Wolf et al., 2009). In bakeries, lipase is used to improve the texture and flavor of goods, making them soft and increasing shelf life (F. Hasan et al., 2006; R. Aravindan et al., 2007). Lipases are also used to improve the nutritional content of fish and vegetable oils by incorporating EPA and DHA (V. Venugopal, 2008). The lipase enzyme is also used to produce pure enantiomers by resolution of racemic mixtures in the pharmaceutical and medical industries (S.L. Smitha et al., 2014; K. De Godoy Daiha et al., 2015; Carvalho et al., 2015). Lipases also find extensive usage in the detergent industry as lipases are used to remove lipid/fat stains (C. Zhang et al., 2012; F. Hasan et al., 2006; L.P. Parra et al., 2008), in paper manufacturing as lipase can break down triglycerides, removing the pitch from wood (M Chandrasekaran et al., G. Kaur et al., 2016;A. Gutiérrez et al., 2009; L. Ramnath et al., 2017; A. Gutiérrez et al., 2010; S. Ugras et al., 2017; J. Charoenpanich et al., 2011; S. Apa et al., 2015) and also in the synthesis of biodiesel, which follows transesterification reactions (H. Su et al., 2016; D. Yuan et al., 2016; A. Ghaly et al.,

2010; X.Wang et al., 2017;D. Surendhiran et al., 2015; Y. Louhasakul et al., 2016; M.R.Z. Passarini et al., 2013). They are also employed in the production of biodiesel, oil degradation, effluent treatment and production of biodegradable polymers (Hasan et al 2006). There is intense global interest in lipase production but marine microbial lipases remain unexploited yet.

II. MATERIALS AND METHODS

1. Sampling site:-

Marine soil from Kollam Beach (Kerala) was collected from the shore in sterile polythene bags, which contained some amount of seawater to maintain the moisture content.

2. Screening of the microbes for lipase activity:-

The soil sample was serially diluted to 10^{-3} and the dilutions were spread plated on Tributyrin Agar medium. The plates were then incubated for 48hours at 37°C. The colony that showed the zone was taken and pure culture was made on Nutrient agar plates by quadrant streaking, and incubated overnight. The bacterial characterization was done using Bergey's manual.

4. Optimization:-

Optimization was carried out on the following parameters:

i. pH

600ml of the lipase production medium (peptone-6g, $NH_4H_2PO_4$ 0.6g, NaCl-1.5g, $MgSO_4.7H_20$-0.24g, $CaCl_2.2H_2O$-0.24g) was transferred 100ml each to six conical flasks with one as a control. The pH was adjusted in each flask in the range from 5 to 9. It was then inoculated with 5ml of the supernatant bacterial broth and kept for incubation in the shaker at a temperature of about 30°C. Readings were taken from the 0^{th} hour to 96^{th} hour. From this, the ideal pH was found for lipase production.

ii. Substrates

600ml of lipase production medium were transferred to six conical flasks, 100ml each. Different substrates were used which includes palm oil, coconut oil, olive oil, glycerol and sunflower oil. 2ml of each of these substrates were transferred to corresponding conical flasks and then inoculated with 5ml of supernatant bacterial broth. It was incubated in the shaker at a temperature of 30°C. Readings from taken from the 0^{th} hour to 96^{th} hour. From this, the ideal substrate was found for lipase production.

By taking the readings from the 0^{th} hour to 96^{th} hour, it showed that the maximum lipase production happened at ideal conditions of incubation time 96hour.

iii. Carbon source

300ml of the production media was transferred to six conical flasks, 50ml each with 1ml of ideal substrate and adjusted to ideal pH. To each of the flask 0.5g of different carbon sources were added which includes: Dextrose, Mannitol, Sucrose, Lactose and Starch, and one was kept as control.The media was then inoculated with the bacterial broth culture and kept for incubation in the shaker at a temperature of 35°c for 96 hours. Readings were taken at the 96^{th} hour.

iv. Nitrogen Source

300ml of the production media was transferred to six conical flasks, 50ml each with 1ml of ideal substrate and adjusted to ideal pH. To each of the flask 0.5g of different nitrogen sources were added which includes: peptone, malt, beef extract, yeast extract and meat extract and one was kept as control. The media wasinoculated with bacterial broth and kept for incubation in the shaker at a temperature of 40°C for 96hours. Readings were taken at the 96^{th} hour.

v. Metal Ions

300ml of the production media was transferred to six conical flasks, 50ml each with 1ml of ideal substrate and adjusted to ideal pH. To each of the flask, 0.5g of metal salts were added which includes Calcium chloride, Mercuric chloride, Potassium chloride, Sodium chloride and Magnesium chloride, and one was kept as control. The media was inoculated with bacterial broth and kept for incubation in the shaker at a temperature of 45°C for 96hours. Readings were taken at the 96^{th} hour.

vi. Detergents

300ml of the production media was transferred to six conical flasks, 50ml each with 1ml ideal substrate and adjusted to the ideal pH. To each of the flask, 0.5g of different detergents were added which includes GUM, Poly Ethylene Glycol, Sodium Dodecyl Sulphate, Triton X and Tween 20, and one was kept as control. The media was inoculated with bacterial broth and kept for incubation in the shaker at a temperature of 50°C for 96hours. Readings were taken at the 96^{th} hour.

5. Lipase activity assay:-

The crude enzyme was centrifuged and 1ml of the supernatant was taken in a test tube. 1ml of reaction buffer (500µl Triton X and 500µl HCl at pH 9). The mixture was then incubated at 25°C for 5 minutes. After that, 40µl of pNPP was added. The mixture was emulsified for 2 minutes and readings were taken using UV- Vis spectrophotometric analysis.

Lipase Enzyme Activity was found out by the equation:

Enzyme Activity = $A \times V/t \times e \times v$

Where A is the Absorbance; V is the total volume of mixture; t is the incubation time, e is the extinction coefficient and v is the volume of enzyme used.

III. RESULTS AND DISCUSSION

Sample collection

The bacteria isolated from the marine soil sample was found to be *Staphylococcus saprophyticus*.

Screening of Lipolytic bacteria:-

In the present study, lipase producing bacteria were identified by culturing them on Tributyrin agar medium and the one showing a degradation zone was chosen. The bacterial specimen was found to be Gram positive.In a similar study, (P. Ranjitha et al., 2009) lipase producing bacteria were identified by culturing the bacteria on a slightly modified Zobell 2216E, Spirit blue A agar and Rhodamine B agar. (Loreto P. Parra et al., 2015) screened the lipolytic bacteria by culturing the microorganisms on marine agar media which contains spirit blue agar along with lipase reagent.

Biochemical characterization

In the present study, *S. saprophyticus* showed positive for Catalase test and negative for Mannitol fermentation test.
Pseudomonas sp. was used in another study (G.Seghal Kiran et al., 2008) for lipase production. Insimilar studies, *Burkholderia anthina*(Dayong Jin et al.,2012) was used for lipase production and psychrotrophic Acinetobacter sp. CR9 (Ramesh Chand Kasana et al., 2008) was also studied for its lipase production.

Optimization

Optimization for the various parameters were performed (Table 1- Table 6)and maximum activity was found to be at a pH of 7, Olive oil as substrate, Maltose as the Carbon source, Yeast extract as Nitrogen source, KCl as the metal ion source and GUM as the detergent source.Maximum lipase activity for *S. saprophyticus* was observed after 96 hours, by using olive oil as the substrate at pH 7. In a study using *Pseudomonas aeruginosa* (Nilkamal Mahanta et.al., 2007),similar results were obtained for the parameters used incubation time of 96 hours, pH 7, maltose as the best Carbon source and $NaNo_3$ as the best nitrogen source.Another study carried out using *Aeromonas* sp.EBB-1for similar parameters (Azita Navvabi et.al., 2018). In another study involving various parameters using *Streptomyces* sp, ideal pH was 6(Dongming Lan et.al., 2016).

FiguresandTables

Table 1: Effect of pH on lipase activity

pH	Control		5		6		7		8		9	
Hour(s)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)
0	107.328	0.335	116.008	0.370	84.931	0.245	92.101	0.273	89.720	0.264	101.381	0.311
24	-	-	108.630		156.031		106.372		196.426		204.411	
48	-	-	284.702	1.049	332.155	1.240	358.526	1.346	310.529	1.153	349.798	1.311
72	-	-	223.620	0.803	219.908	0.788	229.206	0.825	238.772	0.864	283.566	1.044
96	-	-	248.072	0.901	240.477	0.871	437.498	1.664	405.088	1.533	338.425	1.265

Table 2: Effect of different substrates on lipase activity

Substrat	Control	Glycerol	Coconut oil	Palm oil	Olive oil	Sunflower oil

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Hour(s)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)
0	105.868	0.329	116.956	0.374	90.264	0.266	105.710	0.340	93.876	0.281	109.254	0.266
24	-	-	112.708		110.717		96.978		126.823		112.696	
48	-	-	126.037	0.410	192.171	0.676	199.514	0.706	170.289	0.588	198.350	0.701
72	-	-	124.171	0.403	145.799	0.490	72.631	0.195	107.646	0.336	112.223	0.354
96	-	-	176.026	0.611	155.014	0.527	154.398	0.524	203.947	0.724	167.938	0.579

Table 3: Effect of different Carbon sources on lipase activity at 96th hour

Carbon sources	Control		Sucrose		Lactose		Mannitol		Maltose		Dextrose	
Hour(s)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)
96	62.355	0.154	104.646	0.324	138.475	0.460	173.998	0.683	213.539	0.762	177.625	0.618

Table 4:Effect of different Nitrogen sources on lipase activity at 96th hour

Nitrogen sources	Control		Meat extract		Peptone		Yeast extract		Beef extract		Malt extract	
Hour(s)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)
96	200.439	0.709	270.070	0.748	159.361	0.544	259.676	0.948	225.293	0.810	230.31	0.366

Table 5: Effect of Metal ions on lipase activity

Metal ions	Control		$CaCl_2$		$NaCl_2$		$MgCl_2$		KCl		$HgCl_2$	
Hour(s)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)
96	200.443	0.710	223.580	0.803	246.49	0.895	261.509	0.955	282.259	1.039	217.927	0.780

Table 6: Effect of Detergents (surfactants) on lipase activity

Surfactants	Control		GUM		PEG		SDS		Trinton X		Tween 20	
Hour(s)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)	Concentration (µg/ml)	Absorbance (400 nm)
96	146.180	0.491	276.685	1.016	161.294	0.552	180.400	0.629	163.333	0.560	188.536	0.662

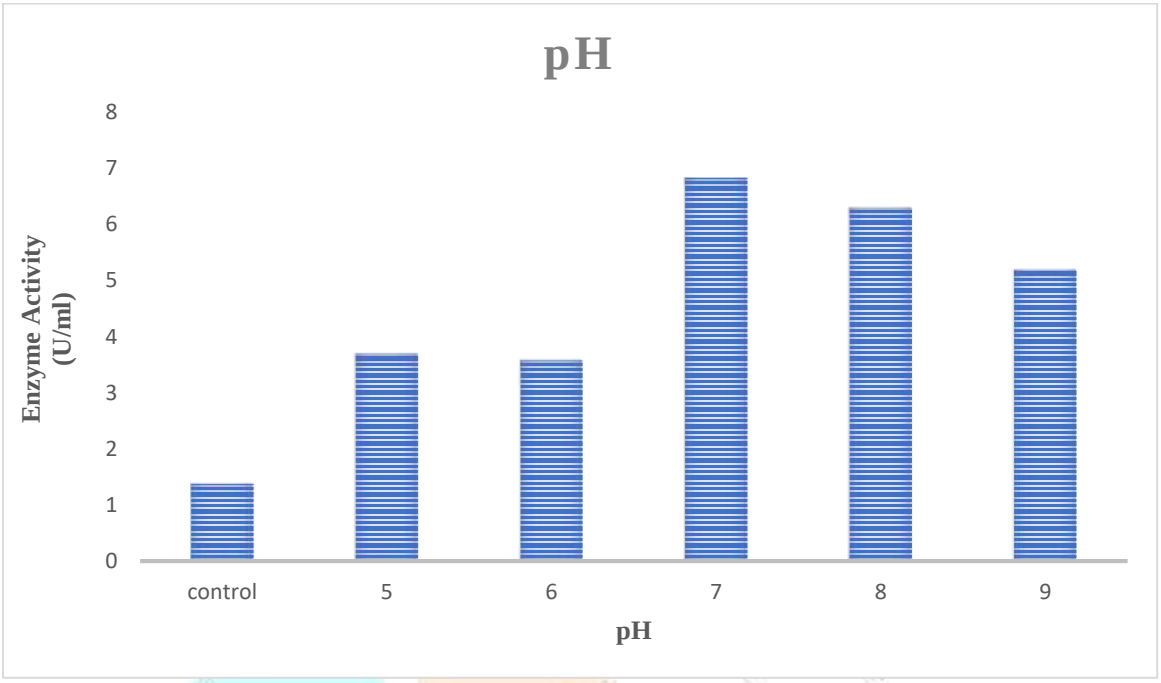


Figure 1

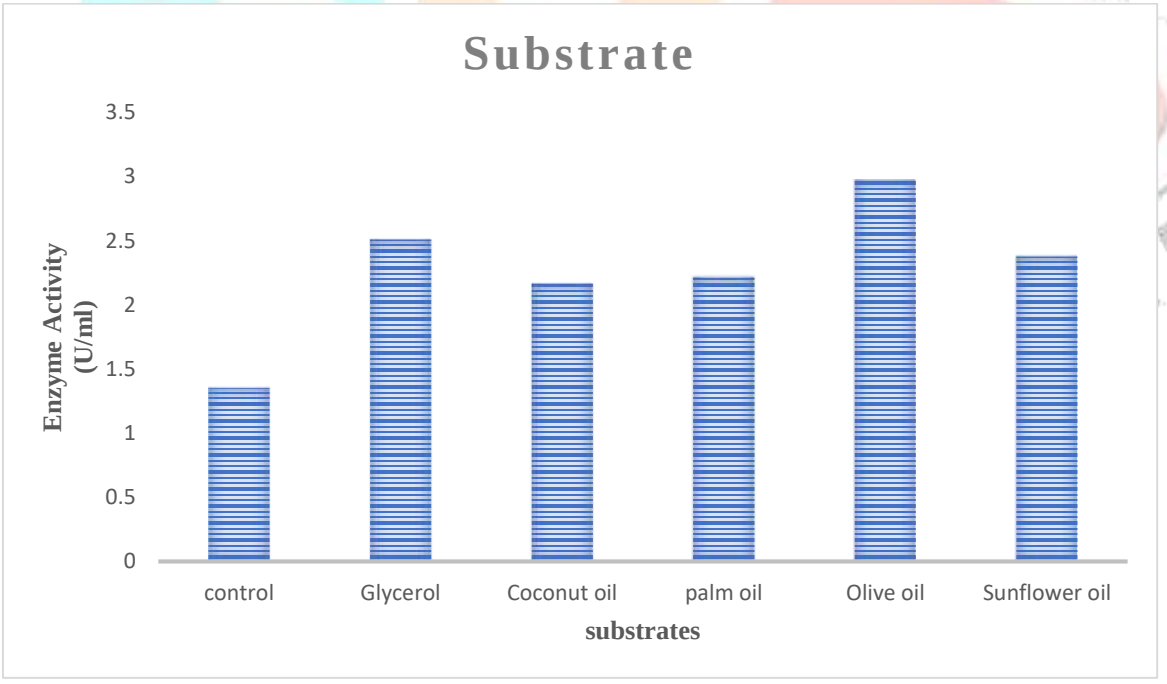
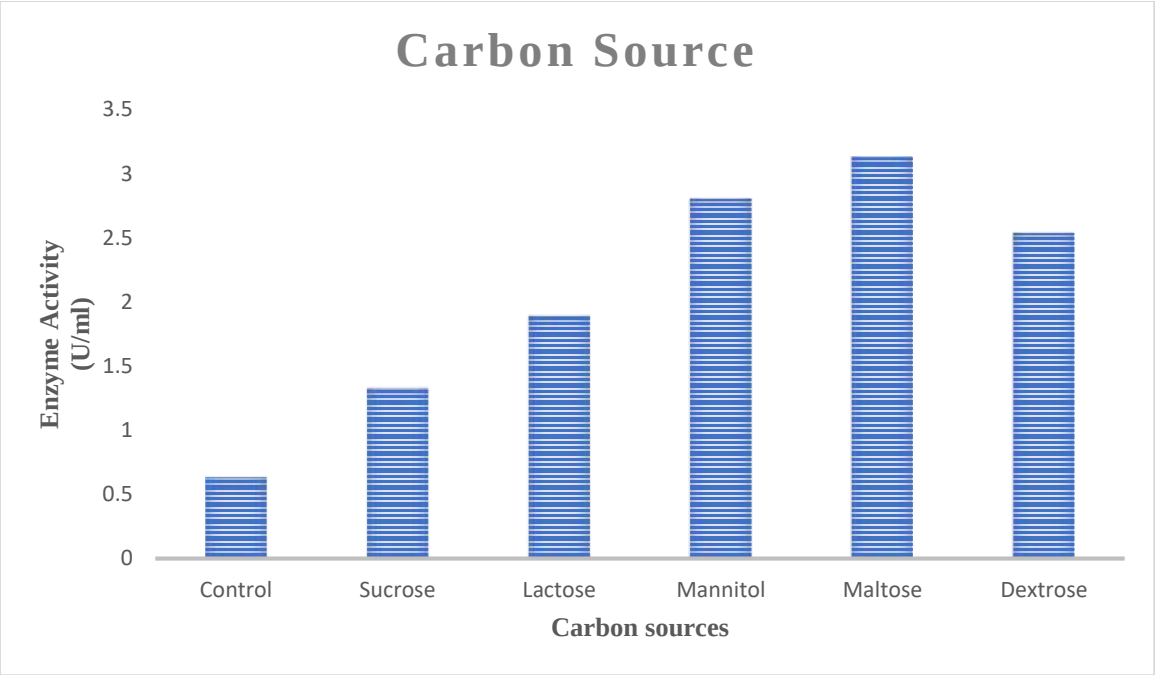


Figure 2



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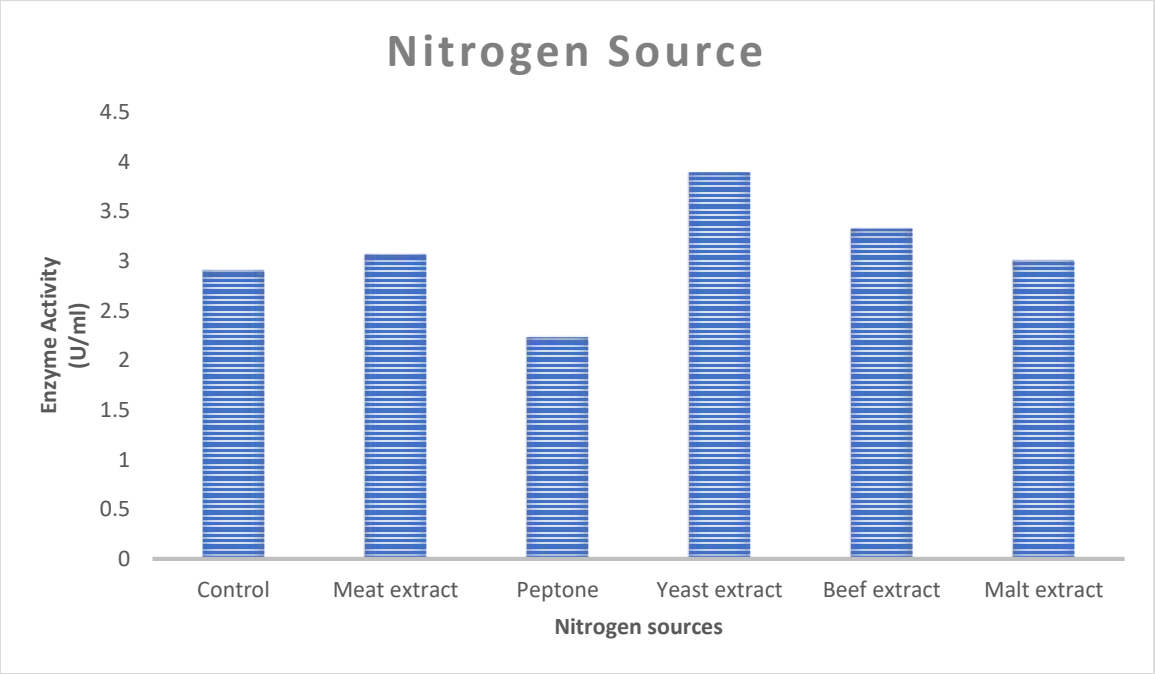

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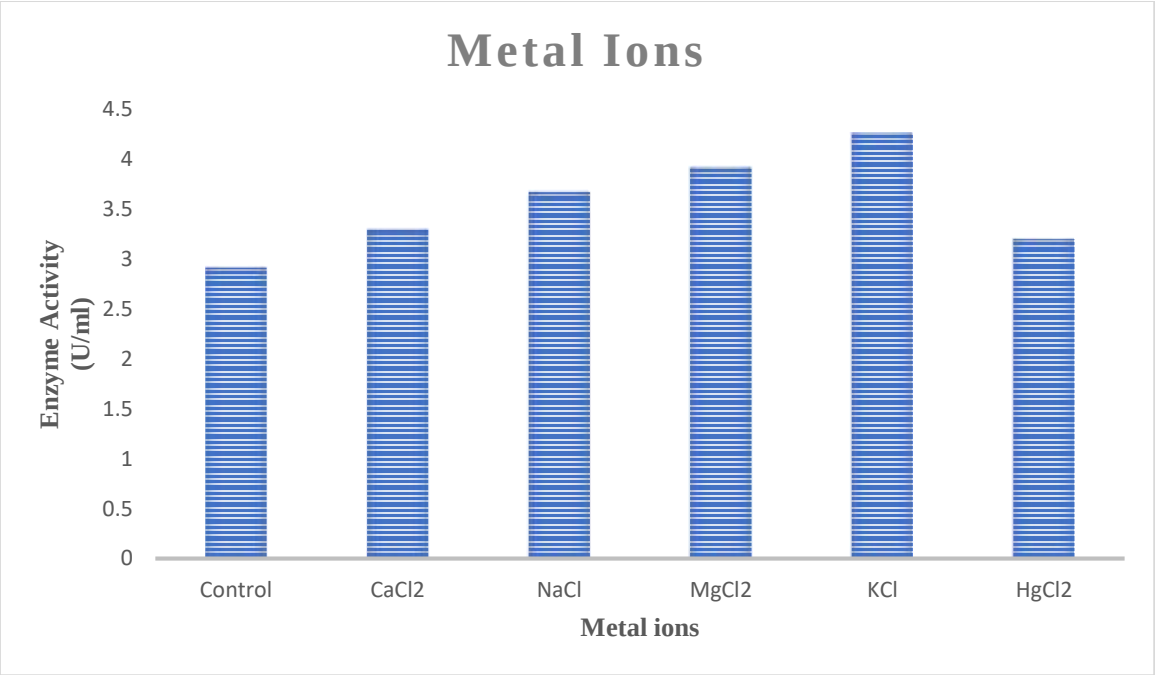

Figure 5

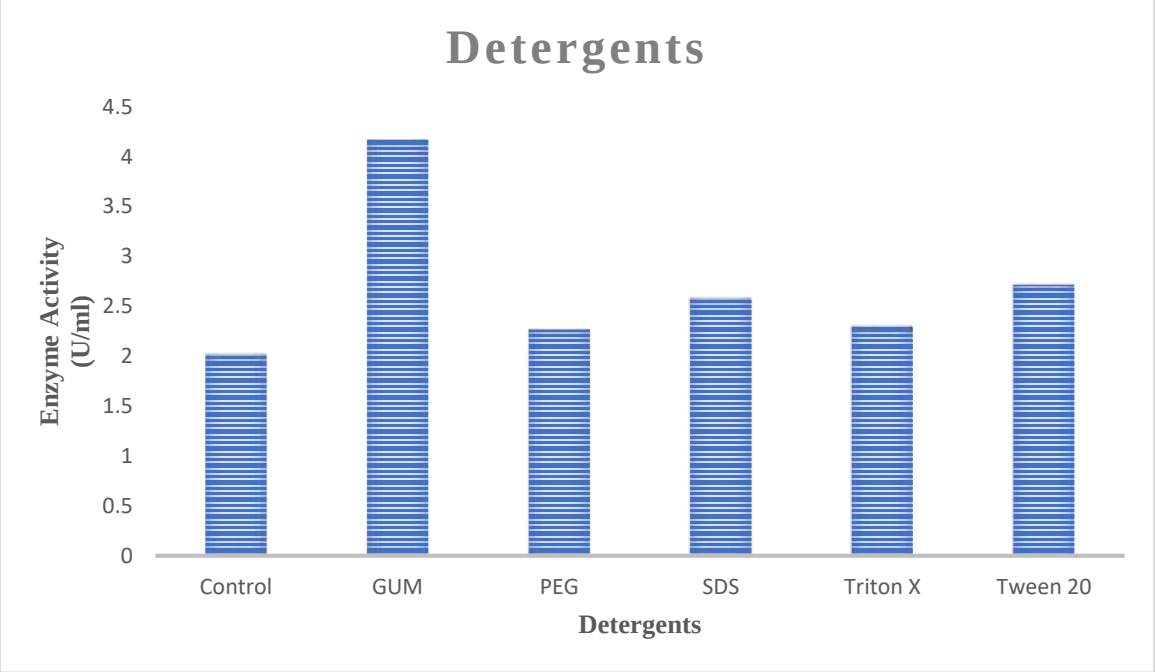

Figure 6

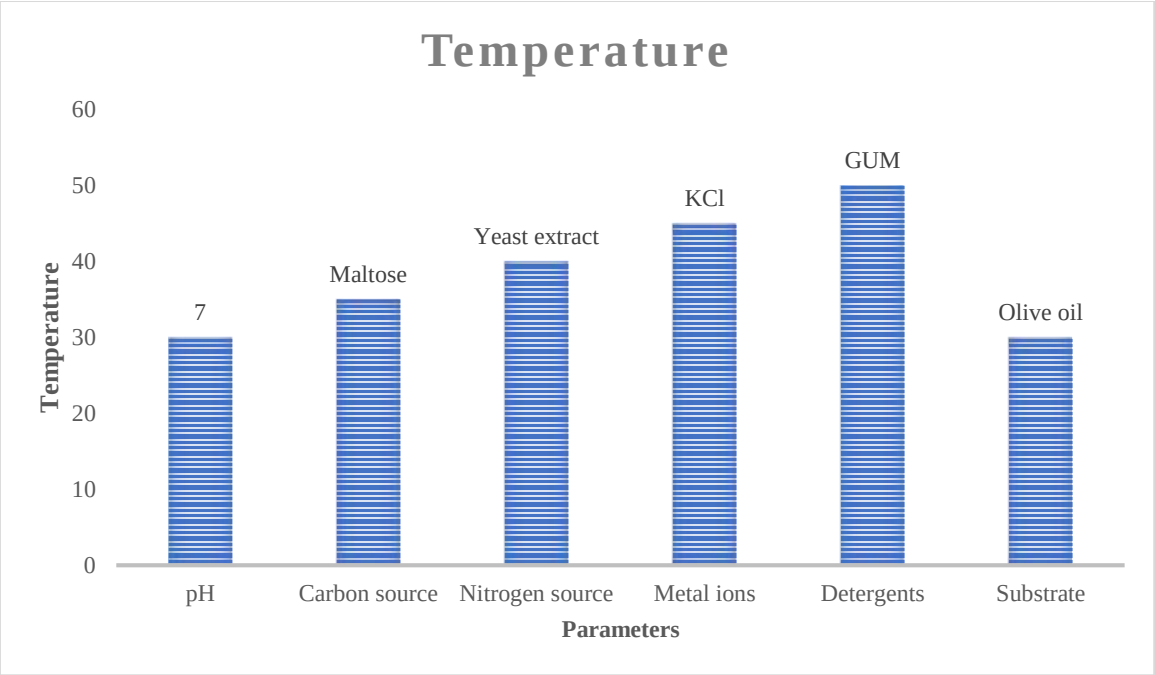

Figure 7

IV. CONCLUSION

In the present study, marine soil was used as the sample for bacterial isolation to produce lipase. The soil sample was serially diluted and the dilutions were plated on Tributyrin agar plates which screens the lipase producing bacteria and the bacterial colonies which shows degradation zone was chosen for further study. The bacteria was characterized according to Bergey's manual and was found to be *Staphylococcus saprophyticus*. This bacteria was used for lipase production under certain parameters like pH, substrate, carbon source, nitrogen source, metal ions, surfactants, incubation period etc. It was found that the maximum lipase was produced at 96 hours in pH 7, olive oil as ideal substrate, maltose as ideal carbon source, yeast extract as ideal nitrogen source, potassium as ideal metal ion and GUM as ideal detergent for lipase production. Among all the above results, maximum lipase was produced at a pH 7 and was found to be 6.82U/ml.

V. ACKNOWLEDGMENT

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