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Isolation and Identification of Lipid Degrading Micro-Organisms, Optimization of Medium and Partial Purification of the Lipase Enzyme

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Abstract: **Lipases (E.C. 3.1.1.3) are a class of serine hydrolase which belongs to the α/β hydrolases super family. Triacylglycerol acyl esters hydrolase are carboxyl esterase that catalyses both hydrolysis and synthesis of ester formed from glycerol. Lipase enzyme that catalyzes the hydrolysis of stored triacylglycerol releasing di and monoglycerol.**

The project deals with the screening of lipase producing organisms. Using Staphylococcus aureus isolated from oil spilled soil, the production of lipase was attempted along with its purification and characterization studies. When different carbon and nitrogen sources are supplemented in the culture medium glycerol, starch, peptone, potassium nitrate found to be the best. When cultivated at optimal temperature and pH of 37°C and 7.5 the lipase produced maximum lipase activity. The organic nitrogen source (peptone) was found to be best lipase enhancing element. The enzyme was partially purified using 60% ammonium sulphate precipitation.

Keywords: **Lipase, *Staphylococcus aureus*, fermentation, optimization.**

I. INTRODUCTION

Enzymes have been used by man since biblical time, either as vegetable rich in enzyme or as microorganism and their product (in brewing processes, in baking and in the production of alcohol). Modern enzyme technology really began in 1874 by Danish chemist Christian Hansen. It is quite recently that industrial importance of enzymes was realized. Earlier enzymatic processes, particularly fermentation was the focus of numerous studies in the 19th century and many valuable discoveries in this field were made. Today enzymes are major contributors to clean industrial product and processes. Enzymes show numerous advantages over chemical technology as far as their specificity, efficiency and compatibility with environment is concerned [1].

The lipid constitutes a very important heterogeneous group of organic substances in plant and animal tissue and related either actually or potentially to the fatty acid.

Chemically they are various types of esters of different alcohol. In addition to fatty acid and alcohol some of the lipid may contain phosphoric acid, nitrogenous base and carbohydrate. Biologically lipids are a chemically diverse group of compound. According to Bloor, lipids are compound having the following characteristics:

1) Insoluble in water

2) Solubility in one or more organic solvents such as ether,chloroform,benzene,acetone etc. so called fat solvents[2].

Fat and oil is the major or principle stored form of energy in many organisms. Phospholipids and sterols are major structural element of biological membrane. Other lipids although present in relatively small quantities play crucial role as enzyme cofactor, electron carrier, light absorbing pigment, hydrophobic anchors for proteins, chaperon to help membrane protein fold, emulsifying agent in the digestive track, hormone and intracellular messenger [3].

Biomedical Importance:

Lipids are important dietary constituents and acts as fuel in the body. It yield more energy per gram (9.5 cal/gm as compared to carbohydrate 4 cal/gm).

Lipid may exert an insulating effect in the body while lipids around internal organs like kidney may provide padding and protect the organ. Lipid supply so called essential fatty acid (EFA) which cannot be synthesized in the body and are required in the diet for normal health and growth. The nervous system is particularly rich in lipids. Some vitamins like A, D, E, K are fat soluble [2].

Micro-Organisms Producing Lipase:

Amongst lipase producing organisms *Bacillus, Candida, Penicillium*, Pseudomonas, *Rhizomucor* and *Rhizopus* spp. are the outstanding ones [6]. Bacterial lipases are glycoprotein but some extracellular bacterial lipases are lipoprotein. Most of the bacterial lipase reported so far are constitutive and are nonspecific in their substrate specificity and few bacterial lipase are thermostable but mostly they require some form of oil, fatty acid, fatty acid alcohol or fatty acid esters for induction [7],[8]. Among bacteria A*chromobacter, Alcaligens, Arthrobacter, Pseudomonas, Staphylococcus and Chromobacterium* spp.

Other Lipase Producing Sources:

Pancreatic lipase of porcine origin is one of the earliest recognized lipase and is still the best known lipase. Plant lipases are not used commercially. The animal and microbial lipases are used extensively. The most important source of animal lipase is the pancreas of cattle, sheep, hog and pig. The disadvantage with pancreatic lipase is that they cannot be used in the processing of vegetarian or kosher food. Also that these extract contain component which have undesirable effect. The pig pancreatic extract contain trypsin which produces bitter tasting amino acids. They are also likely to contain residual animal viruses hormone etc. so, microbes are major source of lipase production industrially which also produces 100 or so enzymes [1].

However, enormous interest in lipase were increasing consequently, there is steadily increasing demand to identify and characterize new lipase from different habitat (air, soil, water). The knowledge of lipolytic enzymes in industrial applications is increasing at rapid and exciting rate.

II. MATERIALS AND METHODS

Sample Collection:

For present study, soil sample was collected from oil-spilled areas of the oil extracting companies in a flask for the isolation of lipase producing organism under laboratory condition.

Isolation of Lipolytic Microbes:

Lipolytic microbes were isolated from the collected soil sample for this; 1gm of soil was dissolved in 100 ml of distilled water. Then it was serially diluted (10^{-1} to 10^{-5}) and the diluted sample were plated on nutrient agar (NA) for viable count .Then the dominant organism were isolated and individually streaked on NA supplemented with egg yolk . The formation of halo zone around the colony on NA supplemented with egg yolk was considered as positive colony.

Characterization of Isolates:

The isolated dominant organisms were identified based on morphological, biochemical characters according to Bergey's Manual of Determinative Bacteriology. The identified microorganisms were assessed for lipolytic activity in the culture medium containing yeast extract 0.3%, peptone 0.5% and olive oil 3ml/ 100ml at pH 7 and 37°C.

Lipase Production:

The bacterial culture was grown in 250 ml Erlenmeyer flask containing 50 ml of fermentation media inoculated with 1% (v/v) (0.5 OD_{600}) of cell suspension and incubated at 37^0C on a rotary shaker (120 rpm) for 48 hrs.

Determination of Enzyme Activity:

Extracellular lipase activity was measured in culture supernatant after centrifugation (8000 rpm for 20 min). Using olive oil emulsion as a substrate (Titrimetric method). For each test run, a blank control was separately performed in each case. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µmol of equivalent fatty acid / (ml.min) under the standard assay condition [14].

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Determination of Protien Concentration:

The protein concentration was determined according to Lowry method using Bovine serum albumin as a standard (Lowry et al., 1951).

III. OPTIMIZATION PARAMETERS

Varying the following parameter one at a time with optimization of fermentation media.

The parameters varied were;

1] Incubation period **-** The effect of incubation period on lipase production was determined by Incubating the culture flask at different incubation period 24,48 and 72 hrs.

2] Temperature **-** The effect of temperature (27^0C, 37^0C, 47^0) on lipase activity was assessed.

3] pH **-**The effect of pH on lipase production was studied by incubating the culture flask at different pH viz.5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0

4] Lipid substrate- The increase in lipase production was checked by using different lipid substrate Like Sunflower, Soybean, Coconut and Olive Oil.

5] Optimized lipid **-** After screening, the maximum lipase- yielding substrate was then taken for Concentrations optimization of various concentrations (0.5, 1, 1.5, 2, 2.5 ml/50ml).

6] Carbon sources **-** The effect of carbon sources on lipase production was determined by using Five different carbon sources namely Xylose, Starch, Glycerol, Mannitol ,Glucose. They were tested at the concentration of 1% (w/v).

7] Nitrogen Sources **-** To test the effect of nitrogen sources on lipase production,five different Such as nitrogen sources Phenylalanine,Tryptophan,Ammonium Nitrate, Potassium nitrate, Peptone. They were tested at the concentration of 2% (w/v).

IV. PARTIAL PURIFICATION OF LIPASE

1) Ammonium Sulphate Fractionation:

The chart as mentioned by Dixon & Webb et al, 1964 was applied to calculate the solid ammonium sulphate to be added to achieve any given concentration of the cell free filtrate under investigation.

In order to partially purify the lipase enzyme, the filtrate was treated with 20,40,60,80, & 100% saturation. For each Ammonium sulphate concentrated both the enzyme activity & protein content was measured & then the specific activity for each fraction can be calculated.

V. RESULT AND DISCUSSION

Preliminary study:

The samples were collected from oil spilled areas are streaked on NA supplemented with egg yolk the isolate showing the clear zone (lipolytic activity) around the bacterial growth in the plates is an indication for the production of extracellular lipase [11].

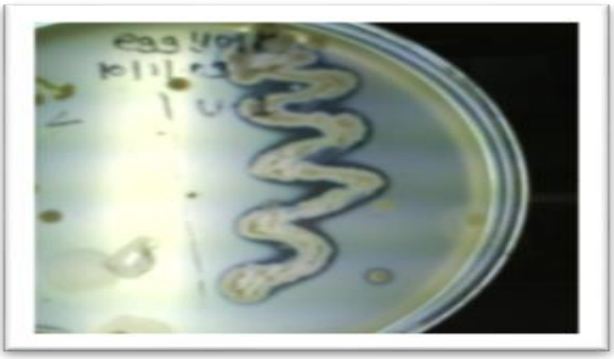

Figure: Strain A

Figure: Strain B

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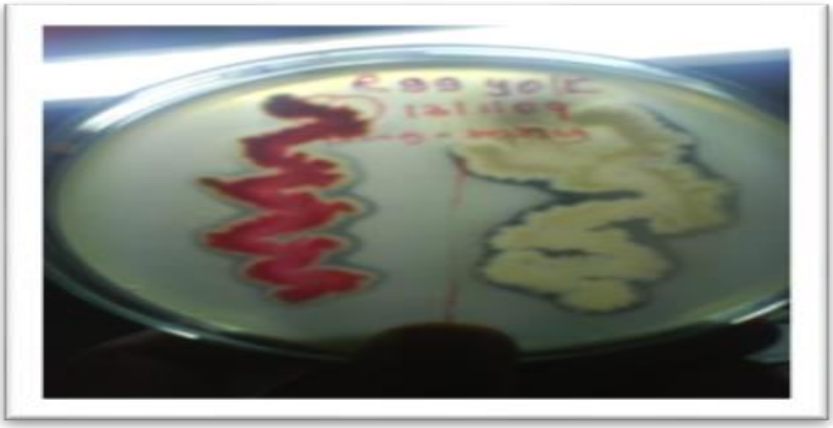

Figure: Strain C

Based on the cell shape, cell arrangement, relation to oxygen, morphological and biochemical test the strains were identified according to Bergey's Manual of Determinative Bacteriology. The morphological character of the strain A, B, C shows in following table.

TABLE 1: Morphological characters of the isolated strains

Characters	**Strain A**	**Strain B**	**Strain C**
Size	1µm	1.5µm	1-1.5µm
Shape	Spherical	Circular	Circular
Colour	White, creamy	Golden yellow	Red
Opacity	Opaque	Opaque	Opaque
Margin	Regular	Circular	Circular
Gram character	Gram positive cocci,	Gram negative rod	Gram negative rod
Motility	Non motile	Motile	Motile

TABLE 2: Biochemical tests for identification of microorganisms

Tests	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Serratia marcescens***
Glucose	+	+	+
Mannitol	+	+	+
Sucrose	Ab	-	+
Catalase	+	+	Ab
Oxidase	-	+	-
TSI slop	Ab	R	Ab

(+: Positive, -: Negative, R: Red, NA: Ab- Absent)

From the above morphological and biochemical characters the isolated organisms were identified as:

(A) *Staphylococcus aureus*

(B) *Pseudomonas aeruginosa*

(C) *Serratia marcescens*

An isolate showing the highest hydrolysis of olive oil (substrate) was chosen as a candidate for further characterization. Nutrient broth supplemented with olive oil shows higher activity after 48 hrs of incubation period at 37^0C at pH 7.5 for 120 rpm by *Staphylococcus aureus.*

So, further characterization studies were done on *Staphylococcus aureus.* (Table 3).

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TABLE 3: Lipolytic activities of the isolated strain

Sr. no	Isolates
1	***Staphylococcus aureus***
2	*Pseudomonas aeruginosa*
3	*Serratia marcescens*

OPTIMIZATION PARAMETERS:

1] Effect of incubation period on lipase production:

The effect of incubation period on lipase production by *staphylococcus aureus* indicated linear increase from 2.4 to 4.10 U/ ml corresponding to the increase of incubation period from 24 hrs to 48 hrs. At 72 hrs of the incubation period the lipase production decreased to 2.60 U/ml. When inoculated 1% (v/v) inoculum size at pH 7 and temperature 37^0C in shaker incubator for 120 rpm.

TABLE 4: Effect of incubation period on lipase production

Sr. no.	Incubation period (hrs)	Enzyme activity (U/ml)
1	24	2.40
2	**48**	**4.10**
3	72	3.60

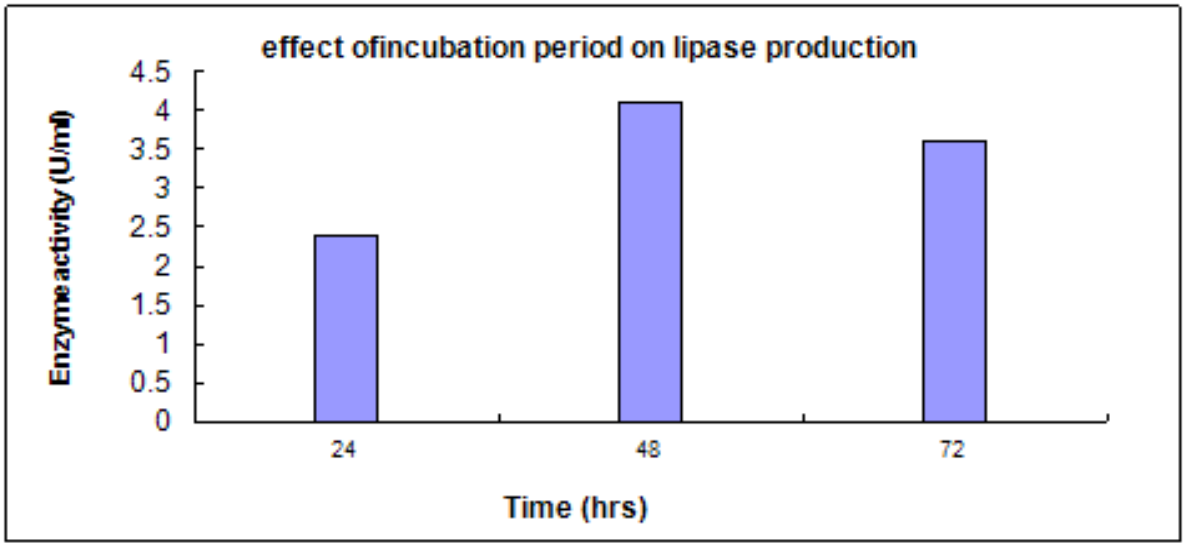

Figure:1 Effect of incubation period on lipase production

2] Effect of temperature on lipase production:

Experiment on the effect of temperature indicated that the lipase production of the *staphylococcus aureus* was maximum 4.90 U/ml at the optimum temperature of 37^0c but below 27^0c and above 47 ^{0}cthe lipase production recorded very low (3.30 U/ml to 3.10 U/ml) respectively.

TABLE 5: Effect of temperature on lipase production

Sr.No.	Temp. (°C)	Enzyme Activity(U/ml)
1	27°C	3.30
2	**37°C**	**4.90**
3	47°C	3.10

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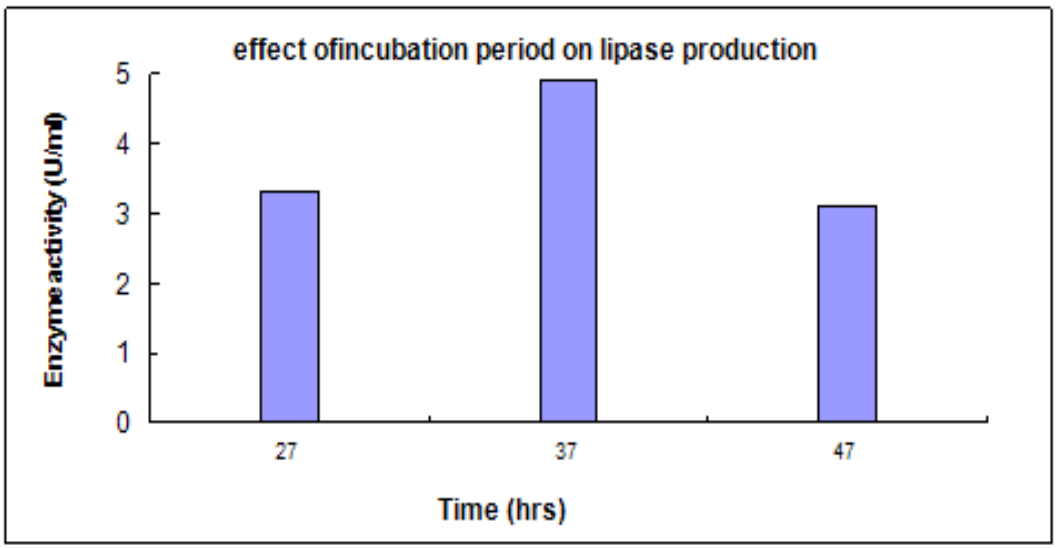

Figure: 2 Effect of temperature on lipase production

3] Effect of pH on lipase production:

The effect of pH of the medium on the lipase production by *staphylococcus aureus* indicated a linear increase from 1.2 to 7.2 U/ml corresponding to the increase of pH from 5.5 to 7.5.

At the pH of the 9 lipase production decreased (1.90 U/ml).

TABLE 6: Effect of pH on lipase production

Sr.No	pH	Enzyme activity (U/ml)
1	5.5	1.20
2	6.0	2.20
3	6.5	4.70
4	7.0	5.10
5	**7.5**	**7.20**
6	8.0	3.90
7	8.5	2.10
8	9.0	1.90

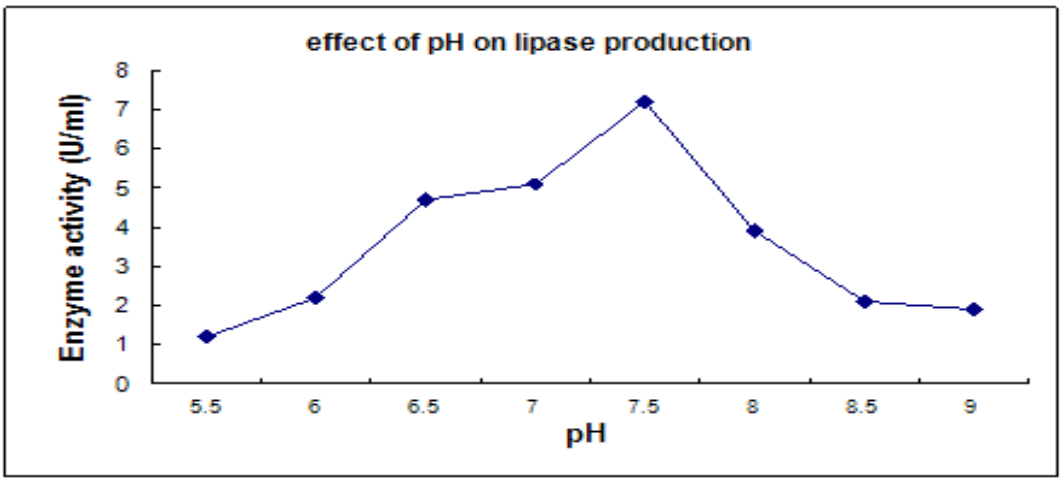

Figure: 3 Effect of pH on lipase production

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4] Effect of different substrate on lipase production:

Lipid induced lipase production by *staphylococcus aureus* was confirmed by the addition of lipid emulsion at 1.5 ml /50ml to the culture medium.

On the basis of the activity, the olive oil was found to be suitable for maximize the lipase production. (6.50 U/ml). This is because triglyceride is important substrate for lipase production. As they can act as an inducer as well as inhibitor. In the present study, all the tested triglycerides were found to induce the lipase synthesis with different level of enzyme production.

TABLE: 7: Effect of Triglycerides on lipase production

Sr. No.	Triglycerides (3%)	Enzyme activity (U/ml)
1	**Sunflower**	**4.10**
2	**Soybean Oil**	**3.00**
3	**Coconut Oil**	**2.90**
4	**Olive Oil**	**6.50**

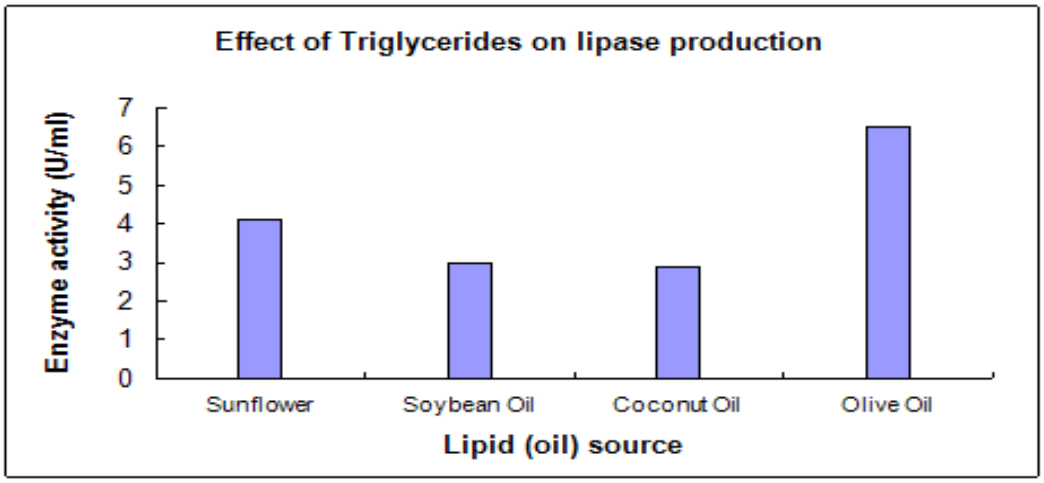

Figure:4 Effect of different substrate on lipase production

5] Optimization of lipid substrate (olive Oil) at different concentration:

The effect of olive Oil at different concentration 1.0, 2.0, 3.0, 4.0 & 5.0 % v/v of the medium on lipase production. By *staphylococcus aureus* indicated a linear increase from 2.85 U/ml to 6.45 U/ml corresponding to increase in concentration from 0.5 to 1.5 ml/50 ml.

At the concentration of 2.0 to 2.5 ml/50ml the lipase production was decreased

TABLE 8: Effect of substrate (olive Oil) at different concentration

Sr. no	Different concentrations of Olive oil (% v/v)	Enzyme activity (U/ml)
1	1.0	2.85
2	2.0	4.70
3	3.0	**6.45**
4	4.0	5.20
5	5.0	2.70

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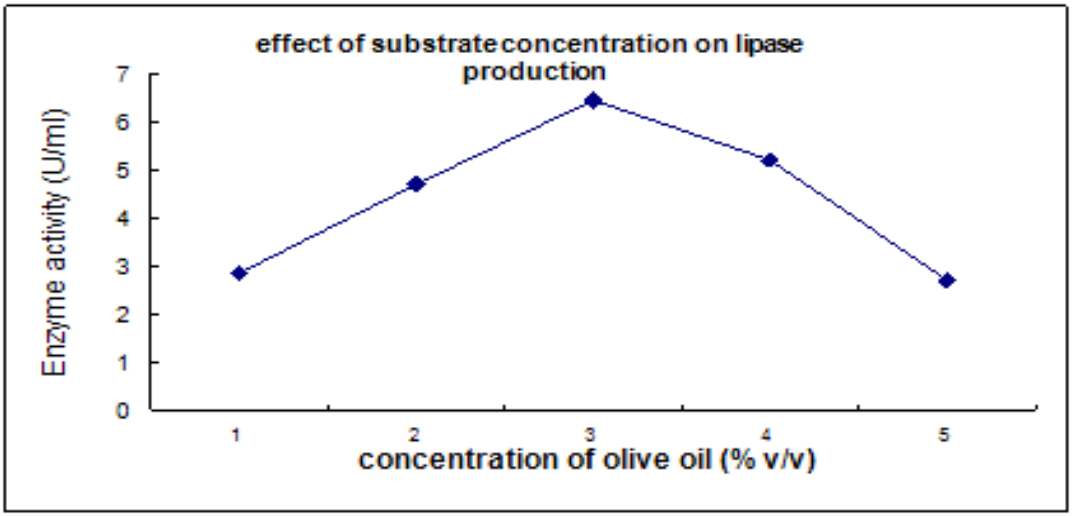

Figure :5 Effect of substrate (olive Oil) at different concentration

6] Effect of different carbon sources on lipase production:

A rang of different carbon sources mainly carbohydrate were screened for their efficiency to support lipase production by *staphylococcus aureus* at the fixed concentration of (0.5 gm/50ml) 10 gm/lit. On the basis of lipase activity, it was concluded that the medium containing glycerol was more suitable for maximum lipase (4.50 U/ml) production than other carbon sources.

TABLE 9: Effect of different carbon sources on lipase production

Sr. No	Carbon Source (1%)	Enzyme activity (U/ml)
1	Xylose	1.80
2	**Glycerol**	**4.50**
3	Starch	3.80
4	Mannitol	2.80
5	Glucose	2.90

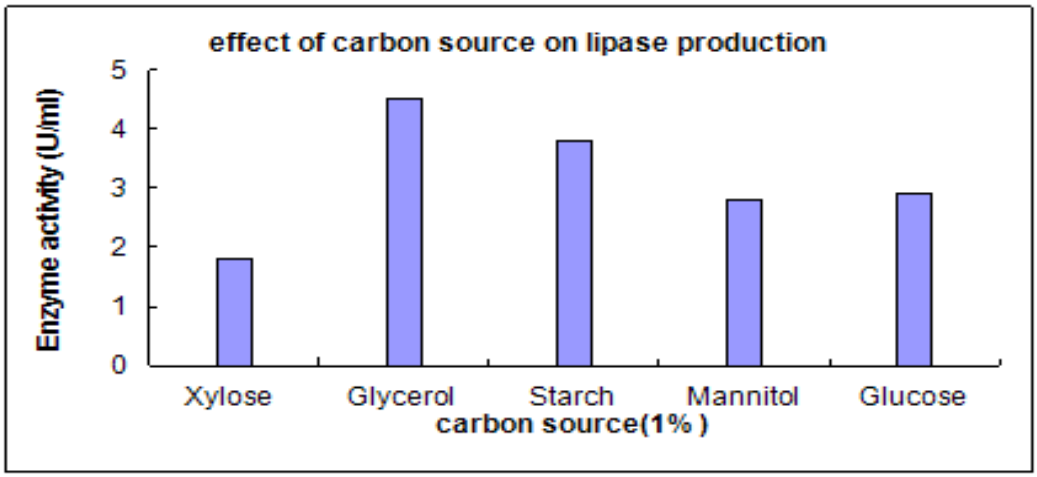

Figure: 6 Effect of different carbon sources on lipase production

7] Effect of different Nitrogen sources on lipase production:

A range of different nitrogen sources mainly Ammonium nitrate, Potassium nitrate, Phenylalanine, Tryptophan, Peptone were screened at a fixed concentration of 2.0% (W/v). The lipase production was greatly influenced by the studied nitrogen sources.

Among these nitrogen sources Peptone produced maximum lipase (6.80 U/ml) compared to others.

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TABLE 10: Effect of different Nitrogen sources on lipase production

Sr. no	Nitrogen source (2%)	Enzyme activity (U/ml)
1	Phenylalanine	2.30
2	**Peptone**	**6.80**
3	Ammonium nitrate	3.65
4	Potassium nitrate	2.00
5	Tryptophan	2.90

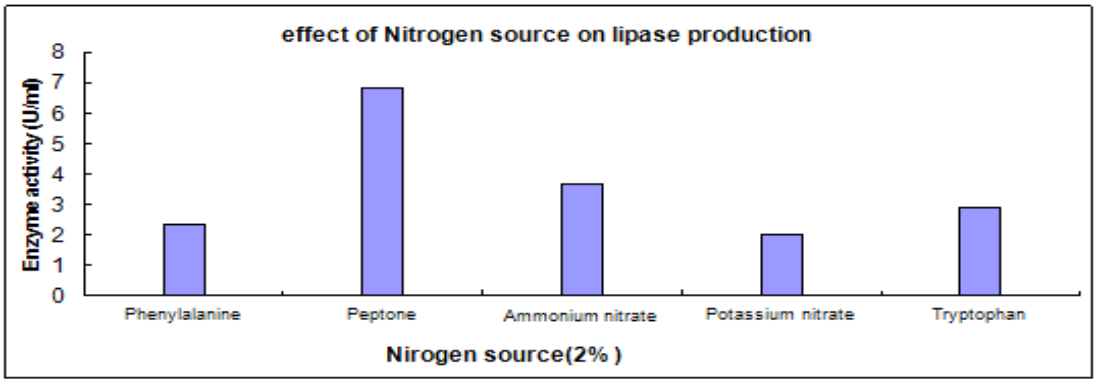

Figure: 7 Effect of different Nitrogen sources on lipase production

PARTIAL PURIFICATION OF LIPASE:

Ammonium sulphate precipitation:

The test strain was first enriched on the enrichment medium containing yeast extract 0.3 %, peptone 0.5 %, olive oil 3%, Glycerol 1%, Peptone 2% at pH 7.5 and then cultured in nutrient-optimized experimental medium at room temperature for 2 days by reciprocal shaking. Then, after 2 days of growth, the culture filtrate was collected by centrifugation.

In order to partially purify the lipase enzyme, the filtrated was treated with 20, 40, 60, 80 & 100% saturation present result shows that Ammonium sulphate was the best proportion, providing a higher enzyme activity. 76 % yield was obtain by using 60 % saturation concentration with 1.14 fold purification.

TABLE 11: Purification table of lipase

	Unit activity (U/ml)	**Total protein (mg)**	**Specific activity (U/mg)**	**Fold purification**	**Yield (%)**
Original broth	7.50	0.006	$1.250x10^3$	1	100
Ammonium sulphate precipitation (60%)	5.70	0.004	$1.425x10^3$	1.14	76%

VI. DISCUSSION

In the present study, the lipase producing bacterial strains were isolated from oil spilled areas and identified as *Staphylococcus aureus*. Among the different substrate tested, olive oil was found to be suitable for enhancing the lipase production by the isolated and screened Staphylococcus *aureus* strain. Rohit et al. (2001) reported that the lipase production was more when vegetable oil, olive oil, soyabean oil, sunflower oil and gingili oil were used as carbon source. As reported by Nakashima et al. (1988)the presence of olive oil as growth medium greatly enhanced the lipase activity of bacillus strain. Also some grey S*treptomyces* were found to be the best lipase producers using 1% olive and palm oil, Nasser et al. (2001).

Furthermore, irrespective of the substrate tested, the lipase activity was maximum at pH 7.5, in low and high pH tested , the lipase activity was less. This result is in consistence with the earlier report of Achamma et al. (2003) and mates and Sunakevitz (1972).

In the present study, the influence of medium temperature indicated that the lipase production by the isolated strains was higher at 37^0 C when compared to those at 27^0 C and 47^0C. Which is consistence with the earlier reported by Walavalkar and Bapat (2001). Also Selva mohan and Alavesam (2008) show highest lipase activity of isolated bacillus spp. at 37 ^{0}C. Immanuel and Essakkiraj (2007) shows lipase production by *Serratia marcescens* was higher at cultivation temperature of 25^0C compared to 30 and 35 ^{0}C. While *Pseudomonas aeruginosa* MB shows higher lipase production at 30^0C as reported by Marcin and Katz (1993).

In the present study, in all the tested substrate and also in all the media pH the tested *staphylococcu*s strain show maximum activity during 48 hrs. Of the culture Period. On further increase in culture Period to 72 hrs. The lipase activity was decreased. Nasser et al.(2001) reported that lipase production by *Staphylococcus* spp . Was greater at 48 hrs. Of incubation period.

The effect of different carbon sources on lipase activity shows glycerol and glucose decreases the lipase activity, which is consistence with the earlier reported by Mates and Sudakevitz (1972). Lakshmi et al. reported that the production of lipase was high in medium added with vegetable oil than the medium added with glucose. In contradiction Bannerjee et al. (1985) reported that some microorganisms showed higher activities when grown in medium containing glucose. Novotny et al. (1988) reported that olive oil in combination with glucose increases lipase activity and in most cases and also the presence of olive oil, together with glucose or glycerol in the medium significantly decreased the both lipase and esterase level. They also inferred that if the olive oil was used as the only carbon source for growth, the enzyme activities of *Candida guillermondii* and yeast spp. showed a four to five fold increases. This result shows that glycerol act as inducer as well as inhibitor for lipase production.

The effect of nitrogen sources on lipase production shows organic nitrogen (peptone) shows highest lipase activity as compared to other nitrogen sources Fadilog and Erkman (2002) also reported that olive oil in combination with other nitrogen sources enhanced the lipase production , but the presence of carbon source in the olive oil significantly decreased the lipase activity and biomass content. They also reported that organic nitrogen sources were found to increase lipase synthesis by C*andida rugosa* grown in the presence of olive oil. Lima et al. reported that lipase production by *Pseudomonas aurantiogriseum* was high when using inorganic nitrogen sources , but organic nitrogen sources displayed more lipase production as compared to use of two organic nitrogen sources.

VII. CONCLUSION

The present study revealed that extracellular lipase production by *Staphylococcus aureus* isolated from oil spilled areas was found to be accelerated at optimized culture conditions such as medium pH, temperature and various substrate concentrations. From the result it can be concluded that the medium pH of 7.5 and temperature of 37^0C were optimum for maximizing lipase production by *Staphylococcus aureus*. The assessment of various substrates for optimizing the production of lipase by *Staphylococcus aureus* it could be concluded that the optimum substrate contained: olive oil emulsion 30gm/lit, peptone 20gm/lit, glycerol 10ml/lit. The lipase produced by *Staphylococcus aureus* was partially purified by using ammonium sulphate precipitation method at 60% saturation. Therefore, the *Staphylococcus aureus* is a potential strain for lipase production in industries.

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