ISSN: 2348-8948
Vol: 2; Issue: 11

RESEARCH ARTICLE

OPTIMIZATION OF LIPASE PRODUCTION IN SUBMERGED FERMENTATION BY MARINE *STAPHYLOCOCCUS SAPROPHYTICUS* USING FACTORIAL DESIGN EXPERIMENT

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Date Received:	Date of Accepted:	Date Published:
04-Oct-2014	28-Oct-2014	11-Nov-2014

Abstract:
In the present study, an attempt has been made to investigate the isolation of lipolytic bacteria from the gastrointestinal tract of marine fish *Sardinella longiceps* and optimization of lipase production by the isolated bacterial strain. The marine fish *Sardinella longiceps* was chosen for the present study and from the gut of the fish, a maximum lipase producing strain was identified as *S. saprophytics* through 16s rRNA sequencing analysis. The influence of triglycerides on lipase production indicated that the tested gingerly oil gave maximum lipase production and its optimized concentration of lipase, Sorbitol, NaCl and peptone production was 1% , 2.0% , 4% and 2.5%. Statistical optimization with Factorial Design showed that Peptone, Sorbitol, NaCl and MgSo4 were the highly influenced factors for lipase production. Gingerly oil and Tween 40 have negative effect on lipase production. However, the commercial exploitation of lipases is still in its infancy, due to the economics of lipase industry.

Keywords: *Sardinella longiceps,* Peptone, Sorbitol, NaCl, Gingerly oil, 16s rRNA sequence

Introduction

Lipases are an important group of biotechnologically relevant enzymes because of their catalytic activity in both aqueous and nonaqueous media. They have diverse applications in a wide variety of industries as well as biotechnological fields such as detergent, oleochemical, organic synthesis, food, dairy, fat and oil modification, pharmaceutical industries etc. (Gupta *et al,* 2004). Lipases are used as additives in detergent formulations, in cleaning solutions and in waste treatment cocktails for downstream industrial processes and for domestic use also (Lai *et al.,* 2007). There are many reasons explaining this interest in lipases. First, they not only catalyze hydrolysis but also reverse reactions such as esterification (Singh, *et al.,* 2006) and transesterification (Gaur *et al*., 2008). Secondly, they usually retain their structure and activity in organic solvents. Thirdly, they have several advantages over chemical catalysts substrate specificity, lower temperature and pressure requirements. Lipase production depends on several process variables such as, substrate concentration, inoculum level and inducer concentration.

Lipases occur in animals, plants and microorganisms. Microbial lipases have a broad spectrum of industrial applications as they are more stable when compared with plant and animal lipases and they can be obtained cheaply. Some of the commercially important lipase producing fungi are: *Mucor miehei, Aspergillus nigei, A. terreus,, Penicillium* sp. and other *Aspergillus* sp (Ankit *et al.,* 2011). The yeasts include Saccharomyces *lipolytica, S. crataegenesis, Rhodotorula glutinis, Pichia bispora, P. maxicana* and *P. sivicola* (Karadzic et al., 2006). Lipases are also reported in halophilic species such as *Marinobacter lipolyticus* and *Natronococcus* sp (Bhatnagar *et al,* 2005. Boutaiba *et al,* 2006).

Thermostable enzymes are particularly attractive for industrial applications because of their high activities at the elevated temperatures and stabilities in organic solvents (Mobarak-Oamsari *et al*., 2011; Gupta *et al,* 2003) and a very important issue for developing better lipase biocatalyst (Gupta *et al,* 2003). In review works on lipase assays conclude that specific screening methods for lipases are still needed (Georgina and Marty, 2007).

Physico-chemical parameters such as the composition of production medium, the carbon and nitrogen sources, minerals and trace metals, pH, temperature and aeration. (Gupta *et al.,* 2004; Venkata Ratna Ravi Kumar *et al.,* 2011).). Lipase activity and its production depend upon the composition of the fermentation medium (Gilbert *et al,* 1991; Henriette *et al,* 1993 **;** Usama *et al*., 2009) The mechanism of formation and export of the lipase requires lipase translation across the inner and outer membrane. Solid state culture is generally defined as the growth of microorganisms as solid materials in the absence or near absence of free water (Vuddaraju *et al*., 2010). Organic solvents are generally known to have detrimental effect on microorganisms and the enzyme it produces, but enzymes are often denatured and inactivated by organic solvents. Various methods are currently available for the determination of lipase activity usually involving hydrolysis (Sirisha *et al*., 2010)

2. MATERIALS AND METHODS

2.1. Bacterium and lipase activity

The bacterium used in this study was isolated from the gut of marine fish *Sardinella logiceps* collected from Colachal coast of Kanyakumari District, Tamilnadu, India. It produced a clear zone when streaked on sprit blue agar supplied with 1% tributyerin after 48 h of incubation. Based on morphological, physiological and biochemical characteristics, the lipase positive colony was identified as *Staphylococcus saprophyticus* by using 16s rRNA gene sequencing and also by following standard keys of Bergey's Manual of Determinative Bacteriology. The phylogenetic relationship of *Staphylococcus* sp wiih other closely related species are represented in (Fig -1).

2.2. Medium optimization

The medium optimization experiment was with cultivating the bacterium in enrichment medium containing beef extract (0.3 %), peptone (1.0 %), NaCI (2%) and glucose (0.5 %) at pH 7 for 24 h and then 10 % of enriched culture was inoculated in 250 ml flask containing 45 ml basal medium (g/L) - Glucose - 10 g; Yeast extract - 1 g; Peptone - 10 g; K2HP04 -1 g; MgS04 -0.5g and NaCI - 10 g; at pH 7. The culture was then incubated in a shaker incubator (at -150 rpm) for 2 days at 32°C. The cells were then harvested by centrifugation at 10000 rpm for 15 min and the supernatant was further used for lipase assay. In this study totally 16 experiments were generated with 6 experimental factors.

3. RESULTS

3.1. Media optimization for lipase production

The effects of nutritional factors such as triglycerides, carbon & nitrogen sources, surfactants, NaCI, metal ions, and various organic solvents on lipase production were studied. The physical parameters such as pH were also studied. All experiments were carried out in triplicates, and average values were presented.

3.2.1. Effect of different triglycerides on lipase production

There were 8 different lipid sources were screened to test their influence on lipase production and the results are given in Fig 2. The results showed that all the oils have high influence either positively or negatively on lipase production by this bacterium. Among this, maximum lipase production (51.91 U/ml) with high specific activity (2.9 U/mg) was achieved in gingerly oil supplemented medium when compared with control (26.87 U/ml; and 1.09 U/mg) respectively. It showed that cod liver oil (32.97 U/ml; and 2.74 U/mg) and palm oil (28.01 U/ml; and 1.50 U/mg) also have some positive effect on inducing lipase synthesis. The others have negative effect and reduced the lipase production.

3.2.1.1. Influence of different concentrations of gingerlly oil on lipase production.

Among all the tested triglycerides, the lipase production was high in gingerly oil supplied medium, therefore, it was tested by varying concentrations to determines it's optimum level. The results showed that, there was a positive linear increase in lipase production with respect to the increasing in concentration of gingerly oil from 0.2 to 1.0 % and maximum production attained at 1% (57.07 U/ml; 0.80 U/mg). Further increase in concentration above 1.0% of gingerly oil resulted in decreasing level of lipase production (Fig-2a).

3.2.2. Effect of various carbon sources on lipase production

The effect of different carbon sources on lipase production was studied by culturing the organism in individually prepared basal medium supplied with 10 different carbon sources and it was compared with control medium without any carbon sources. Among the carbon sources tested, sorbitol (33.26 U/ml; 0.49 U/mg) has the highest influence on maximum lipase production. Maltose (26.53 U/ml; 0.77 U/mg), lactose (26.76 U/ml: 0.56 U/mg) and xylose (25.28 U/ml; 1.21 U/mg) were also supported the lipase production in positive way but the effect is less and xylose produced maximum amount of protein specific lipase production (1.21 U/mg) specific activity due to high total protein production. (Fig-3).

3.2.2.1. Influence of different concentrations of sorbitol on lipase production

The effect of carbon sources revealed that sorbitol was the best source to induce the lipase synthesis as much. So various concentrations of sorbitol which influence the lipase synthesis were tested (Fig- 3a). Among the different concentrations of sorbitol tested, 2.0% was the optimum concentration to produce maximum lipase activity (51.01 U/ml lipase productions; 0.51 U/mg specific lipase activities). Concentration above this level, the lipase production decreased.

3.2.3. Effect of different nitrogen sources on lipase production

Fig. 4 provides the effect of various nitrogen sources on lipase production by S. *saprophytics* cultured in 9 different sources. Among these sources high lipase production was observed in organic nitrogen source-peptone supplied medium (40.34 U/ml lipase production; 0.39 U/mg specific lipase production) Next to peptone, ammonium sulphate was observed as a good lipase producer with good specific lipase production (29.78 U/ml; 1.17 U/mg). In the present study, yeast extract and gelatin were also observed as good lipase producers (28.45 and 28.84 U/ml respectively). Here the organic nitrogen source ammonium nitrate (18.27 U/ml) indicated relatively low lipase production when compared to other nitrogen sources and control (19.13 U/ml; 0.30 U/mg)

Influence of different concentrations of peptone on lipase production

Since the lipase production was maximum at peptone medium, therefore different concentrations of peptone was tested for lipase production (Fig - 4a). Among the different concentrations of peptone, 2.5% was found to be optimum to produce highest amount of lipase (102.63 U/ml; 1.29 U/mg) with highest protein specific activity. Further increasing concentration of peptone above 2.5%, the lipase production decreased.

3.2.4. Effect of different surfactants on lipase production

The effect of different surfactants on production of lipase by *S.saprophytics* is given in Fig-5. It was studied by culturing of individually prepared medium with 6 different surfactants and without surfactant (control). The results showed that all the tested surfactants have strong effect on lipase production. Among the tested surfactants Tween 40 was found to be produced maximum lipase (77.7 U/ml) with highest specific activity 1.21 U/mg. The other surfactants that have positive effect on lipase production were Tween 60 (67.91 U/ml; 1.11 U/mg) and Poly Ethylene Glycol (49.05 U/ml; 0.88 U/mg). In this study the surfactants like triton X 100, Tween 20 and SDS have negative effect on lipase production and they drastically reduced the lipase production over the control(44.10 U/ml).

Influence of different concentrations of Tween 40 on lipase production

Since the lipase production was maximum at Tween 40 supplied medium, different concentrations (0.1 -1.0%) of Tween 40 were tested for lipase production. Among the different concentrations of Tween 40 tested 0.4%> was found to be an optimum to produce high amount of lipase (183.33 U/ml) with high specific activity (4.25 U/mg). The concentration beyond 0.4%> decreased the enzyme production (Fig - 5a).

3.2.5. Effect of various concentrations of sodium chloride on lipase production

The effect of different concentrations of sodium chloride on lipase production by the test organism was studied. Among the tested concentrations 4 % NaCI was the optimum to produce maximum lipase and protein specific lipase (109.43 U/ml; 2.52 U/mg). In this study there was a gradual increase in lipase production was observed in NaCI concentration from 1 to 4%. But above this optimum level, the enzyme production was decreased. At the tested higher concentration of 10% the lipase production was only 36.65%> of the highest activity observed in optimum 4% NaCI (Fig - 6)

3.2.6. Effect of various metal ions on lipase production

There were 9 different metal ions screened to test their influence on lipase production and the results are given in Fig-7. The results showed that all the metal ions have high influence on lipase production either positively or negatively. Among the metal ions tested, positive effect on lipase production was observed in magnesium sulphate and magnesium chloride supplemented media (54.28 U/ml; 0.95U/mg and 54.16 U/ml; 1.50 U/mg). But in the control media without the supplementation of metal ion produced 45.60 U/ml lipase. Concerning the other trace elements, the lipase production was hugely reduced. In all the tested metal ions, EDTA (22.68 U/ml) and zinc shlphate (22.46 U/ml) affects the lipase production in higher extent.

3.2.7. Effect of different organic solvents on lipase production

Fig-8 provides the effect of various organic solvents on lipase production by *S. saprophytics* cultured in different sources such as tetra decane, octane, nonane, dodecane, hexadecane, petrol, diesel. Kerosene and control media. The results showed that this bacterium effectively utilized all the organic solvents for maximum lipase production over the control (86.84 U/ml; 3.83 U/mg) and no decrease in lipase production was observed in these supplied organic solvents. Among these sources, high lipase production was observed in kerosene (219.37 U/ml; 4.29 U/mg). Lower lipase production was observed in dodecane (103.20 U/ml; 3.59 U/mg) but it was 16% higher than the control. The other best organic solvent observed in this study is octane (155.76 U/ml) but it was only 71% of the highest production observed in kerosene.

3.2.8. Effect of initial pH on lipase production

Effect of initial pH on lipase production resulted that this bacterium prefers alkaline pH for optimum lipase production and it was higher in pH 9.0 (39.2 U/ml; 0.79 U/mg). The highly acid pH significantly reduced the lipase production. At pH 3.0 the lipase production was 3 fold decreased (11.5 U/ml; 0.5 U/mg). There was a gradual increase in lipase production was observed when the pH raised from acid to alkaline range. Moreover at the highest alkaline pH (10 to 12) the lipase production drastically reduced (Fig-9).

3.3. Statistical Optimization

In the present study Factorial Design was used to identify the medium components having greatest effect on lipase production (Table -1). The experiments were conducted by using 6 factors for 16 trials with two levels of concentration for each variable corresponding to lipase production (Table -2). The statistical analysis with MIN1TAB 15.1 statistical package clearly showed that considerable variation from 66.02 U/ml to 116.62 U/ml of lipase production corresponding to the variation in medium composition.

The main effects of examined factors on lipase production were calculated and are presented in Table 3. The t-test for any individual effect allows an evaluation of the probability of finding the observed effects purely by chance. The results showed that Peptone. Sorbitol, NaCI and MgSo4 have positive effect on lipase production 'which has positive t-value. Among the nutrients screened Gingerly oil and Tween 40 have negative effect on lipase production. In this work, variables with confidence levels greater than 90% were considered as significant. Among the positive nutrients NaCI was proved to have the most profound influence on lipase production (p=0.001). The effect of peptone is also very high (p=0.036). These two have high effect on lipase production and these two occupies first two positions in this experiment. Table- 4 shows the Analysis of Variance (ANOVA) for linear model on effect of independent variables on lipase production. The effect of significant and most important variables on lipase production is given by the model in equation. Lipase (U/ml) = 52.5 + 10.9 A + 7.47 B + 15.4 C- 19.3 D- 13.1 E + 43.1 F. Where A, B, C, D, E and F respectively for sorbitol, gelatin, gingerly oil, NaCl, tween 40 and MgSo4.

4. DISCUSSION

Gingili oil is so obvious triglyceride that induces the lipase production was previously proved in *Serratia rubidaea* (Immanuel *et al,* 2008). In contrast to the present study Gulati *et al.* (2005) also observed that lipase production by *Fusarium globulosum* was high in neem oil supplied media and in this study neem oil was observed as an inhibitor for lipase synthesis by this bacterium. The effect of supplementary carbon sources on lipase production showed that sorbitol produced maximum lipase than other tested carbon sources and control. It was previously confirmed that sorbitol plays a vital role in the stability of lipase produced by *Baciillus stearothermophilus* (Sasmita Sabat *et al*., 2012). The present study supports the previous studies of Abdul Rahman *et ai* (2006) on the positive effect of sorbitol on lipase production by *Pseudomonas* sp. respectively. Also Dutta and Ray (2009) observed good lipase production in sorbitol supplied medium by *Bacillus cereus* C7.

Lipase production by *Serratia marcescens* is a good evidence for the positive effect of peptone in lipase production (Gao *et al.* 2004). Ellaiah (2004). reported that Production of lipase by immobilized cells of *Aspergillus niger* . Gulati *et al,* (2005) also observed that lipase production by *Fusarium globulosum* was highly induced by peptone. NaCI for maximizing the lipase production. In the same way lipase production by *Salinivibrio* sp. strain SA-2 (Amoozegar *et al,* 2008) and *Halomonas campisalis* MCM B-365 optimally need 4% (1M) and 6% (1.5M) NaCl respectively. Belarbi *et al*., (2000), reported that lipase production by *Salinivibrio* sp.

The organic solvents used in this study are usually rich in carbon and this result inferred that this bacterium may utilize the organic solvents as nutrient source for specific synthesis oflipase. Similar observations were made in *Burkholderia glumae* (Boekema *et al,* 2007) and *P. pseudomallei* lipase production induced by n-hexadecane.

CONCLUSION

Based on the results, the NaCI played a key role in lipase production by this species. Here both the linear and quadratic effect of NaCI was found to be significant on lipase production (Camacho *et al,* 2009). Lipase production by *Serratia marcescens* is a good evidence for the positive effect of peptone on lipase production (Gao *et al,* 2004). In the present study, two nutrients which have considerable effect on lipase production are Sorbitol and MgSo4. It was previously confirmed that sorbitol plays a vital role in the stability of lipase produced by *Baciillus stearothermophilus* (Sasmita Sabat *et al*., 2012).

Table 1: Percentage levels of nutritional factors selected for factorial design

Nutritional factors	Low level (-1) (%)	High Level (+)(%)
Sorbitol (A)	0.5	2.0
Peptone (B)	0.5	2.5
Gingelly Oil (C)	0.4	1.0
NaCl (D)	2.0	4.0
Tween 40 (E)	0.2	0.8
$MgSo_4$ (F)	0.1	0.3

Table 2: Factorial design matrix for the evaluation of selected nutrients on lipase production by *Staphylococcus saprophyticus*

Run	A	B	C	D	E	F
1	+	-	+	+	-	-
2	+	+	-	-	-	+
3	-	-	+	-	+	+
4	-	-	-	+	-	+
5	+	-	-	+	+	+
6	-	-	+	+	+	-
7	+	-	+	-	-	+
8	+	-	-	-	+	-
9	+	+	-	+	-	-
10	-	+	-	+	+	-
11	-	+	+	-	-	-
12	+	+	+	-	+	-
13	+	+	+	+	+	+
14	-	-	-	-	-	-
15	-	+	+	+	-	+
16	-	+	-	-	+	+

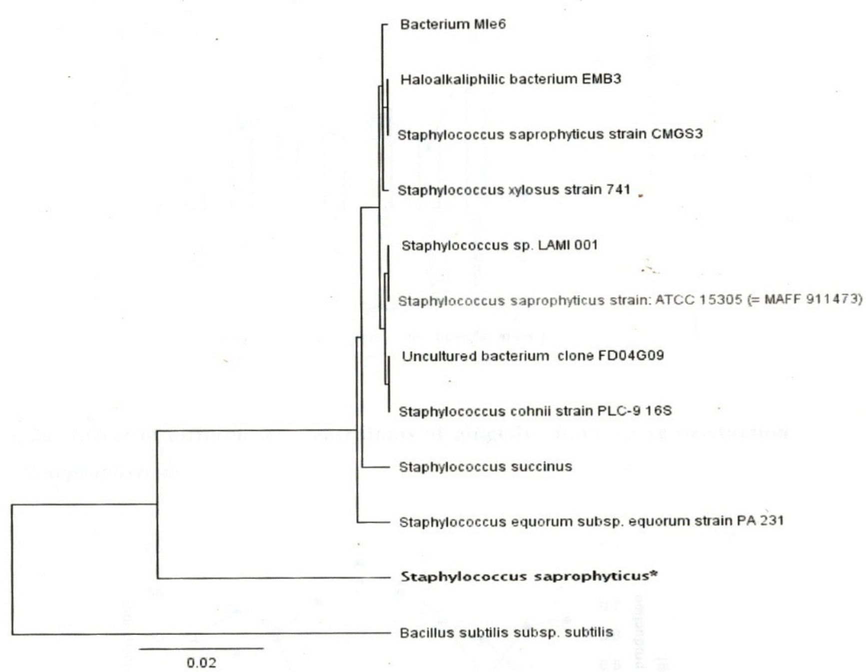
Table : 3 Estimated effect and coeffricient for lipase production from Factorial design

Factors	Effect	Coefficient	SE Coefficient	t-value	P-value
Constant		87.18	11.00	4.77	0.001
Peptone	10.882	5.44	4.40	2.47	0.036
Sorbitol	5.603	2.80	5.87	1.27	0.236
NaCl	23.08	11.54	2.93	5.24	0.001
Gingelly Oil	-5.777	-2.88	14.70	-1.31	0.223
Tween 40	-2.613	-1.306	22.09	-0.59	0.568
MgSo4	-4.313	44.09	44.09	0.98	0.354

Table : 4 Analysis of variance (Anova) for linear model on effect of independent variables on Lipase production

Source	DF	SS	MS	F	P
Regression	6	2965.68	492.28	6.36	0.007*
Residual Error	9	699.89	77.77		
Total	15	3665.57			

Fig 1. The phylogenetic relationship of *Staphylococcus saprophyticus* with other closely related species



* - Isolate used in this study

Fig 2. Effect of different triglycerides on lipase production by *S. saprophyticus*

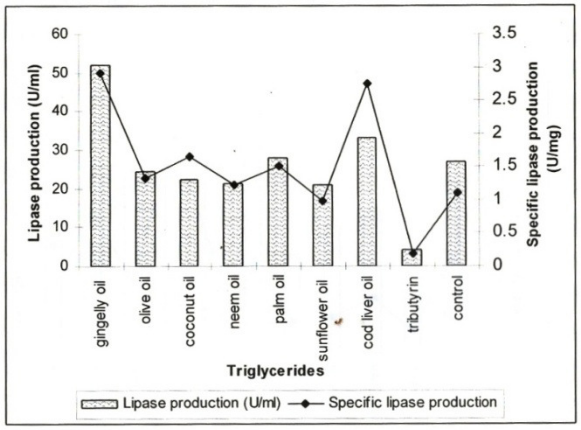


Fig 2a. Effect of different concentrations of gingelly oil on lipase production by *S. saprophyticus*

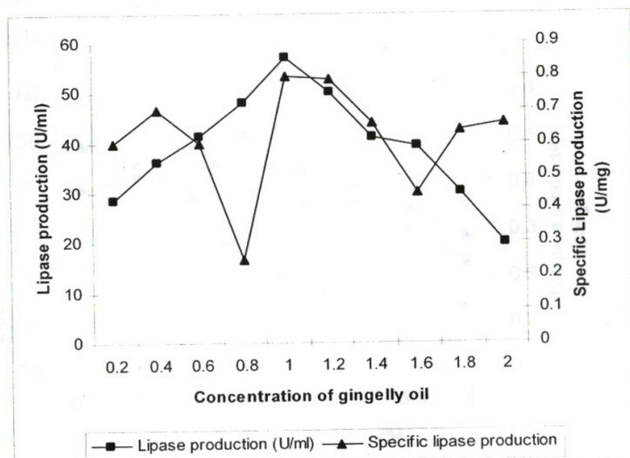


Fig 3. Effect of different carbon sources on lipase production by *saprophyticus*

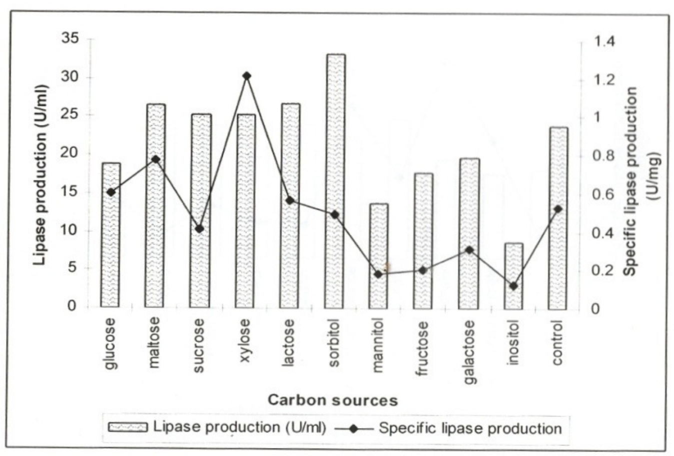


Fig 3a. Effect of different concentrations of sorbitol on lipase production by *S. saprophyticus*

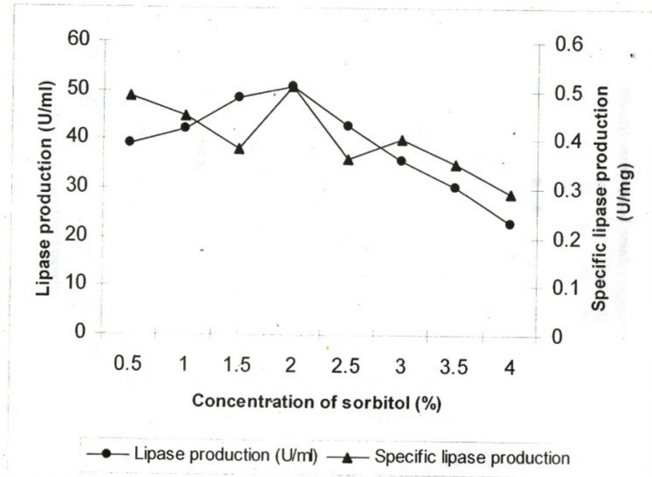


Fig 4. Effect of different nitrogen sources on lipase production by *S. saprophyticus*

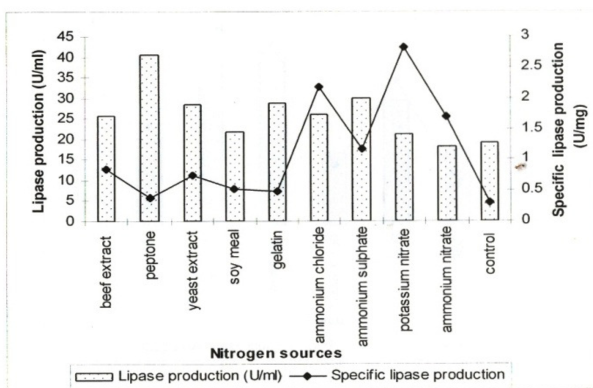


Fig 4a. Effect of different concentrations of peptone on lipase production by *S. saprophyticus*

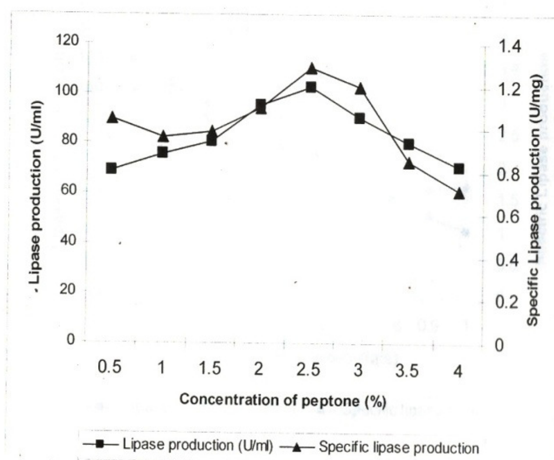


Fig 5.Effect of different surfactants on lipase production by *S. saprophyticus*

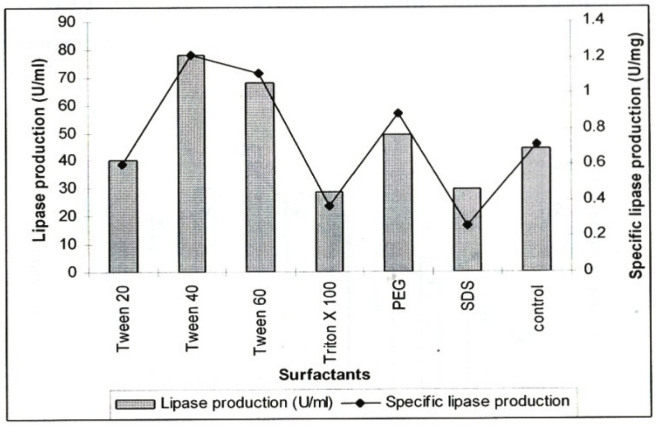


Fig 5a.Effect of different concentrations of Tween 40 on lipase production by by *S. saprophyticus*

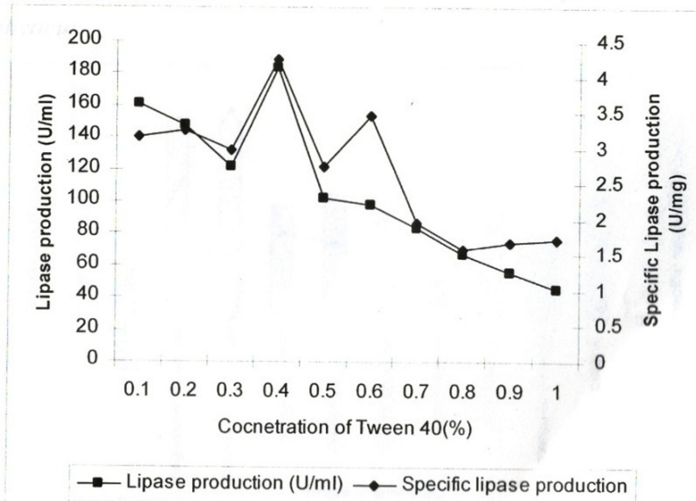


Fig.6 Effect of various concentrations of sodium chloride on lipase production by *S. saprophyticus*

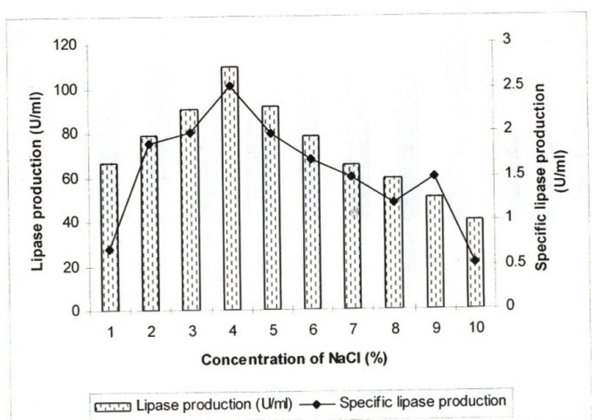


Fig. 7 Effect of various trace elements (metal ions) on lipase production by *S. saprophyticus*

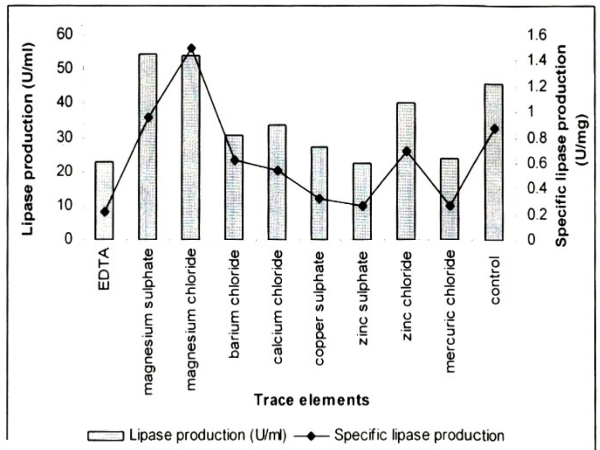


Fig. 8 Effect of different organic solvents on lipase production by *S. saprophyticus*

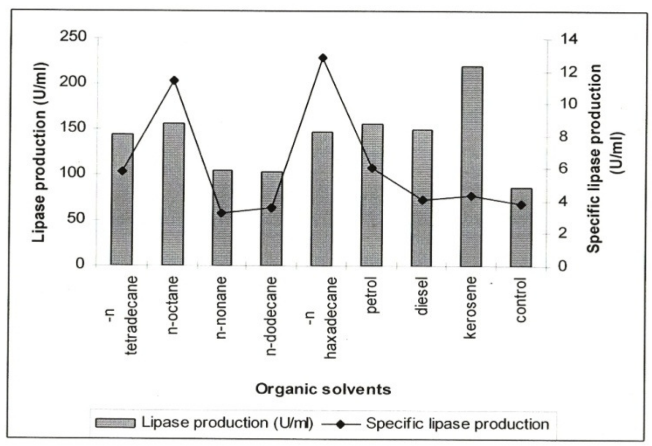
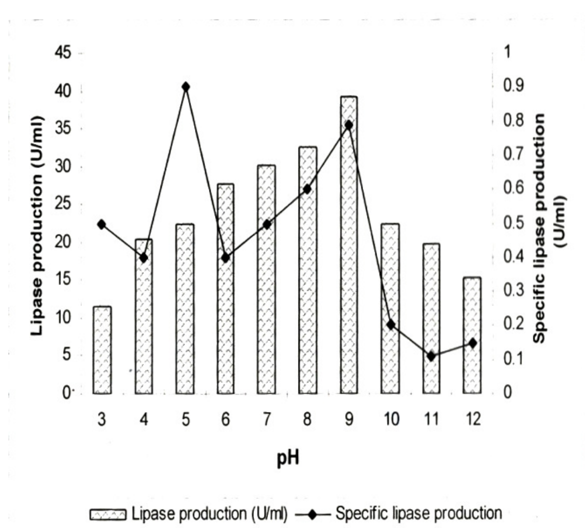


Fig. 9 Effect of initial medium pH on lipase production by *S. saprophyticus*



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