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Production and Optimization of Lipase Enzyme from Mesophiles and Thermophiles

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Abstract

Lipases are enzymes which catalyze the hydrolysis of triacylglycerol to free fatty acids and glycerol. In the present study bacterial cultures were isolated from industrial and oil spilled areas and was screened for Lipase production and activity. Seven bacterial strains were found to have lipolytic ability. These bacterial strains were grown in production media and the lipase enzyme produced was estimated. After optimization of factors like pH, temperature, carbon source, nitrogen and incubation time etc. a maximum lipase enzyme activity of 8U was obtained in the mesophilic bacteria: *P. mirabilis* when sunflower oil was used as a substrate, pH was 6 and temperature was 37°C. *B. coagulans*, another mesophile produced 7.5U of lipase enzyme. Thermophiles: *P. stutzeri,* G. *stearothermophilus* and *B. sporothermodurans* which were isolated, screened for lipase activity and characterized, showed highest lipase activity of 7U at 50°C.

Keywords: Lipase; Lipase activity; Mesophiles; Thermophiles; Optimization

Introduction

Lipases (triacylglycerol lipases EC 3.1.1.3) are enzymes that catalyze the degradation of fats and oils and convert them into fatty acids and glycerol [1,2]. Triacylglycerols are the main substrates for lipases. Lipases are produced by substrates such as natural oils, synthetic triglycerides and esters of fatty acids. Bacterial lipases are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavour enhancement and lipolysis of butter fat and cream [3]. Lipases are used in detergent industry as additives in washing powder, in textile industry to increase the absorbency of the fabrics also for synthesis of biodegradable polymers and for different trans esterification reactions [4-7].

Lipase enzyme has also found use as a catalyst for production of different products used in cosmetic industry in pulp and paper industry in synthesis of biodiesel degreasing of leather and also in pharmaceutical industry [8-11]. Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. Most of the bacterial lipases reported so far are constitutive and are non-specific in their substrate specificity and a few bacterial lipases are thermostable [12]. Among bacteria, *Achromobacter* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Staphylococcus, Serratia* sp. and *Chromobacterium* spp. have been exploited for the production of lipases [13].

In the present study seven lipase producing bacterial cultures were isolated from oil spilled soil and were further used for lipase production. Generally, high productivity of lipase has been achieved by culture medium optimization. Different oil sources, nitrogen sources and physicochemical factors such as pH, temperature and incubation time were studied to check their influence on microbial lipase production. These enzymes are generally produced in presence of oil source with respected to conditions like temperature, pH and incubation times [14].

Material and Methods

Collection of soil sample

The collection of the soil samples was done from industrial and oil spilled industrial areas of Noida. The soil samples were collected in sterile plastic bags and sealed. All samples were labeled with a permanent maker.

Isolation of lipase producing bacterial cultures

The serial dilution of sample soil was done in 5 test tubes and plated on oil containing mineral media and incubated at 37°C for 24 h. After incubation the bacterial colonies were characterized by gram staining and maintained on the nutrient agar slant.

Screening of strains for extracellular lipase

Qualitative analysis of lipase producing micro-organism was carried out by single line streaking of pure culture inoculates obtained through zigzag streaking on mineral lipid emulsion agar media and egg yolk agar media. The plates were incubated at 37°C for 48 h. Lipolytic activity and lipase production was indicated by the formation of zone around the bacterial colonies on mineral lipid emulsion and egg yolk agar plate.

Characterization of lipase producing bacterial cultures

The characterization of bacterial culture was done by biochemical tests. Biochemical test were used for the identification of isolates bacteria culture for lipase enzyme production. Different Biochemical tests were performed to prove the identity of isolated bacterial culture. Urease, carbohydrate, nitrate, SIM medium, IMVIC test, starch, casein hydrolysis test, catalase test, Acetate, high concentration of NaCl test, etc., were used for identification. The bacteria strains were further identified using 16S rRNA gene sequence analysis and BLAST comparison online. The isolated bacteria cultures were inoculated in mineral lipid emulsion broth media. The OD of lipase was taken at 600 nm for 4 days.

Thermophilic characterization: Thermophilic characterizations were analyzed by were culturing the isolated organisms at high temperature (50°C) and the thermophilic property of these isolated strains was established.

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Fermentation (production) media

Fermentation media containing different oil types as carbon source were used for batch fermentation for producing lipase using the isolated bacterial culture. The medium used contained the following composition; Peptone: 5 g/l, KH_2PO_4: 2.5 g/l, Glucose: 1 g/l, Potassium chloride: 0.5 g/l, $MgSO_4$: 0.5 g/l, $CaCl_2$: 0.5 g/l, Oil: 15 ml. The media was autoclaved at 121°C for 15 min. After autoclaving, the isolated bacterial cultures were inoculated in the fermentation Medias. Shake flask culture was carried out in 250 mL Erlenmeyer containing the above media. The flasks were inoculated with 10% seed culture and incubated at 37°C and 220 rpm for a period of 4 Days. The time-course growth curve of isolated organisms was plotted to 8 h after incubation.

Bioreactor culture: Batch fermentation was performed using the above media with different carbon and nitrogen sources. The reactor was inoculated with 10% seed culture in log phase and fermentation was carried out according to type of medium. The lipase activity was measured at different time after incubation.

Lipase activity

After 24 h of inoculation the fermentation broth were taken in falcon tubes. These falcon tubes were centrifuged for 10 minutes at 10000 rpm and the supernatant was then used as the crude enzyme. This crude enzyme was then tested for its enzyme activity by titrating it against 0.05 M NaOH. The amount of NaOH used the amount of acid present in the solution which was directly proportional to the amount of lipase produced [15]. Acid value was calculated by the formula:

$$\mu\text{mol fatty acid/ml } (U) = \frac{(\text{ml NaOH for sample - ml NaOH for blank}) \times N \times 1000}{M}$$

Where:

U=μmol of fatty acid released/ml

N=the normality of the NaOH titrant used (0.05 in this case)

M=Total volume of reaction mixture used

One lipase unit has been defined as the amount of the enzyme that releases one μmol fatty acid per ml under standard assay conditions (U=μmol of fatty acid released/ml) [16].

Optimization for lipase enzyme production

To increase the production and the productivity of the lipase enzyme, different physiological parameters were studied like pH, temperature, carbon source, and incubation time, inoculum, etc. The optimization of lipase production was carried on the variation of physical and chemical parameters of fermentation medium.

Effect of temperature: The effect of temperature was mostly used for the selection of optimum temperature for the production of lipase enzyme. The fermentation media were inoculated and then incubated at different temperature such as 25°C, 37°C and 50°C.

Effect of pH: The fermentation media were prepared having different pH such as 4, 6 and 8 inoculated by the bacterial cultures. The lipase activity was estimated and the optimum pH was selected for maximum lipase enzyme production.

Effect of different oils: Different oils were mostly used as carbon sources. Different oils like mustard oil, soyabean oil and sunflower oil were used in fermentation media. The media was incubated at 37°C and the lipase activity was estimated.

Effect of incubation: Optimization of incubation period was done by the incubation of fermentation media for different times. The fermentation medias were prepared and inoculated with the isolated bacteria in conical flask. The conical flask was incubated for different time intervals like such as 24, 48, 72 and 96 h. The lipase production was determined for all the incubation periods.

Effect of nitrogen sources: The various nitrogen sources including malt extract, peptone, ammonium sulphate, yeast extract, beef extract, meat extract and soyabean extract were supplemented to the production media.

Results

Isolation of lipolytic bacterial cultures from industrial and oil contaminated soils resulted in characterization and identification of 7 cultures. Seven strains were identified by gram staining and purified by sub culturing on agar plates

Qualitative screening for lipase activity

Qualitative analysis of lipase producing micro-organism was carried out by single line streaking on mineral lipid emulsion agar media. The lipase utilizing bacteria were grown on lipid emulsion agar plates. The lipase producers formed a clear zone of hydrolysis on these plates. The cultures from each of the slants were first straight line inoculated on the Egg yolk agar plates. Clear zone was produced in all strains. The isolated bacteria cultures were inoculated in mineral lipid emulsion broth media. The OD of lipase was taken at 600 nm for 4 days. The time-course growth curve of isolated organisms was plotted to 8 h after incubation (Table 1 and Figure 1).

The isolated bacteria cultures were inoculated in mineral lipid emulsion broth media. The OD of bacterial growth was taken at 600 nm every day as shown in Table 2 and Figure 1. After screening of the bacteria cultures, they identification by biochemical tests and sequence analysis.

Depending upon the biochemical tests, sugar utilization results and sequence analysis of the 16S rRNA gene, identification of the unknown bacterial strains was done on basis of similarity index after comparison

Bacterial cultures	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7
1st day	0.18	0.52	0.52	0.43	0.37	0.41	0.53
2nd day	0.61	0.77	1.02	1.04	0.71	0.82	1.02
3rd day	1.14	1.42	1.67	1.81	1.21	1.58	1.67
4th day	1.15	2.24	1.90	2.13	2.02	2.11	2.15

Table 1: OD of growth of bacterial cultures on mineral lipid emulsion broth culture at 600 nm.

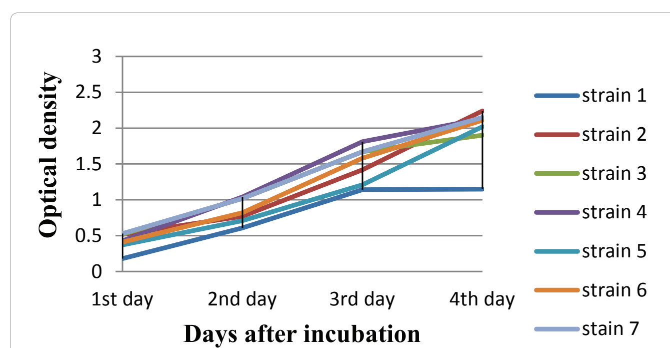

Figure 1: Growth of bacteria culture in mineral lipid emulsion broth media.

S. No.	Type of test	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6	Culture 7
1.	Lactose	-	-	-	-	+	-	-
2.	Fructose	-	+	+	-	+		
3.	Glucose	+	-	-	+	+	+	+
4.	Sucrose	-	+	+	-	+	+	+
5.	Maltose	-	+	+	-	+	+	+
6.	Manitol	-	-	-	-	+	-	-
7.	Glycerol	-	+	-	-	-		
8.	Starch	-	+	-	-	+		
9.	Sorbitol	-	-	-	-	-		
10.	Arabinose	-	-	-	-	+		
11.	Xylose	+	-	-	+	+	-	-
12.	Ribose	-	-	+	-	+		
13.	Citrate test	+	-	-	+	-	-	-
14.	Acetate test	+	-	-	+	-		
15.	MR test	-	+	-	-	+	-	-
16.	VP test	-	-	-	-	-	-	+
17.	Indole test	+	-	+	+	-	-	-
18.	7% NaCl test	-	-	-	-	+	+	+
19.	Casein hydrolysis test	-	+	-	-	-		
20.	Starch hydrolysis test	+	+	+	+	+		
21.	Nitrate test	+	-	+	-	+/-	-	+
22.	Urease test	+	+	-	+	-	-	-
23.	Acetate test	+	-	-	+	-		
24.	Sim media test	+	-	+	-	-		
25.	Catalase test	+	+	+	+	+	+	+
26.	Growth at 45°c	+	+	+	-	+	+	+

Table 2: Biochemical tests results for all the bacterial isolates.

on BLAST. The results showed that strains 2-5 were mesophilic bacteria but strainS1, S6 and S7 were thermophiles.

S1 - *Pseudomonas stutzeri* - 93%

S2 - *Staphylococcus hominis* - 96%

S3 - *Bacillus coagulans* - 95%

S4 - *Proteus mirabilis* - 78%

S5 - *Paenibacillus lactis*- 87%

S6 - *Geobacillus stearothermophilus*- 96%

S7 - *Bacillus sporothermodurans*- 96%

Fermentation media containing different oils as carbon source were used for batch fermentation for producing lipase using the isolated bacterial cultures. The bacterial strains were inoculated in the production media and amount of lipase enzyme produced was calculated using titration. Variations in pH, temperature, carbon source and incubation time resulted in higher production of lipases.

Different oils were mostly used as substrate for enzyme production for e.g. mustard oil, soyabean oil, and sunflower oil. The enzyme units were calculated and the maximum lipase enzyme activity obtained was 7U which was produced when sunflower oil was used as a substrate by *P. mirabilis* on 6th day and *B. coagulans* produced 6.5U. Soyabean oil gave the next highest lipase enzyme activity by *B. coagulans, P. mirabilis* and *S. hominis*. The enzyme activity is shown in Figures 2-4.

The effect of temperature was mostly used for the selection of optimum temperature for the production of lipase enzyme. Different temperature such as 25°C, 37°C and 50°C were used to check the optimum temperature for the lipase production. The maximum lipase enzyme activity was obtained at 37°C and 50°C when the mesophiles and thermophiles were considered separately. The Mesophilic cultures of *B. coagulans, P. mirabilis* and *S. hominis*- gave highest lipase activity (8.5U-7.5U) at 37°C while thermophilic cultures like *P. stutzeri, G. stearothermophilus* and *B. sporothermodurans* gave maximum lipase activity (6.5U) at 50°C as shown in Figure 5. It was surprising to observe that *P. stutzeri* gave similar lipase activity (6.5U) at both 37°C as well as 50°C.

The effect of pH on lipase activity was observed for the selection of optimum pH of fermentation medium for the production of lipase enzyme. The fermentation media having different pH such as 4, 6 and 8 were used. The maximum lipase enzyme activity was produced at pH 6. The maximum lipase enzyme activity obtained was 8.5U on 4th day at pH 6 media by *P. mirabilis* as shown in Figure 6. *B. coagulans* also produced 7.5U of lipase enzyme on the 6th day at pH 6. Thus, the optimum pH for maximum lipase enzyme activity is pH 6. *G. stearothermophilus* and *B. sporothermodurans* produced less enzyme activity as the reaction was carried out at 37°C.

The nitrogen sources were used for lipase production in this study. Among the different nitrogen sources tested, soyabean extract was found to be the best among all the nitrogen sources. The production of lipase activity was 8U as shown in Figure 7; whereas in other nitrogen sources the lipase production was in the range of 2-7U.

Discussion

The present study deals with isolation of new and novel bacterial cultures which have higher production levels for lipases. After screening of soil microbes, 7 strains were identified by gram staining and purified by sub culturing on agar plates & slants.

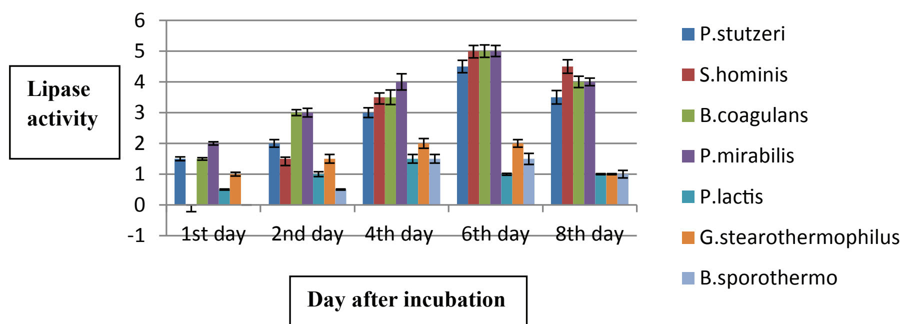

Figure 2: Lipase production with mustard oil as substrate.

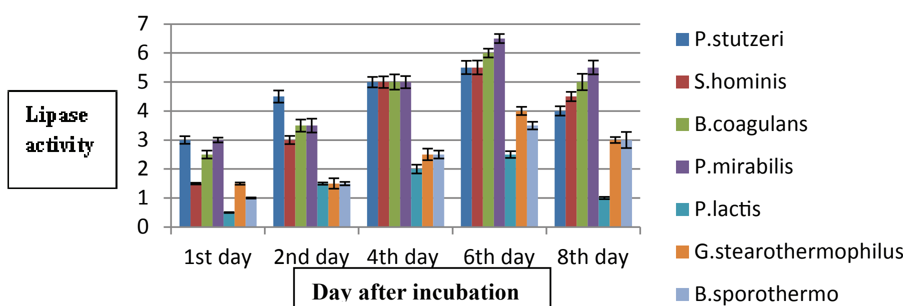

Figure 3: Lipase production with soyabean oil as substrate.

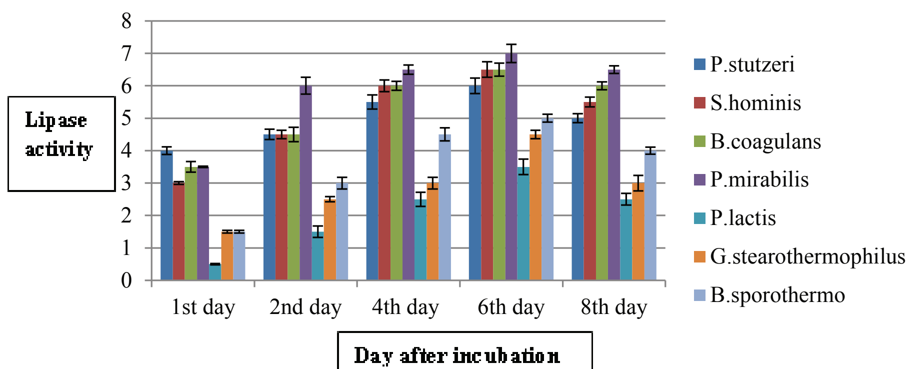

Figure 4: Lipase production with sunflower oil as substrate.

Different Biochemical tests were performed to identify the isolated bacterial cultures. Depending upon the biochemical tests, sugar utilization results and sequence analysis of the 16S rRNA gene. The results showed that: (1) *P. stutzeri* (93%), (2) *S. hominis* (96%), (3) *B. coagulans* (95%), (4) *P. mirabilis* (78%), (5) *P. lactis* (97%), (6) *G. stearothermophilus* (96%) and (7) *B. sporothermodurans* (96%).

P. *stutzeri* and B. *coagulans* have been shown to produce Lipase in other studies also. But in the present study the lipase enzyme activity obtained is very high as compared to other research data. The thermophiles isolated and screened for lipases are unique and novel.

Fermentation media containing different oils were used for batch fermentation for producing lipase using the four isolated bacteria culture from oil spilled soil. The bacterial strain was grown in the production media and amount of lipase produced was calculated using titration. Optimization of pH, temperature, substrate, and incubation, etc., was performed for increasing production of lipase by the isolated bacterial culture. Different oils like mustard oil, soyabean oil and sunflower oil were mostly used as an alternative substrate for enzyme production. The maximum lipase enzyme activity obtained was 7U by *P. mirabilis* on 6th day when sunflower oil was used as a carbon source and 6.5U was produced by *B. coagulans* on 6th day. Soyabean oil gave the next highest amount of lipase enzyme activity.

In another study by Kumar et al., a thermophilic isolate *B. coagulans* BTS-3 produced an extracellular alkaline lipase, the production of which was substantially enhanced when the type of carbon

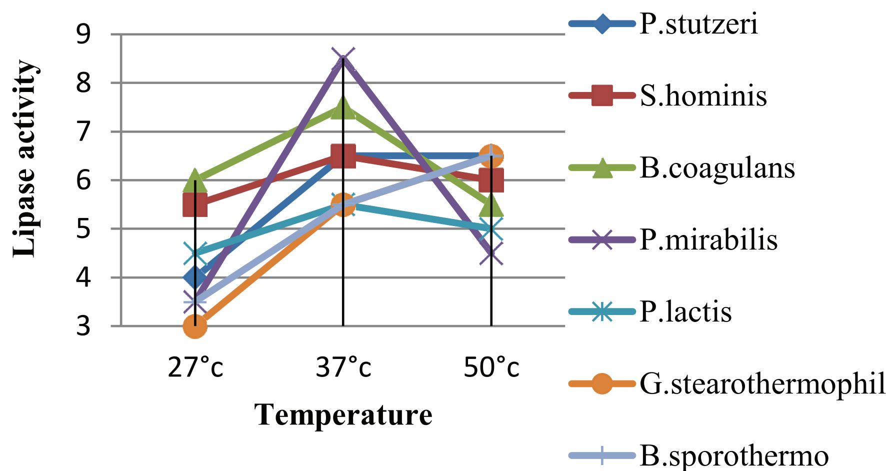

Figure 5: Lipase activity with change in temperature for incubation.

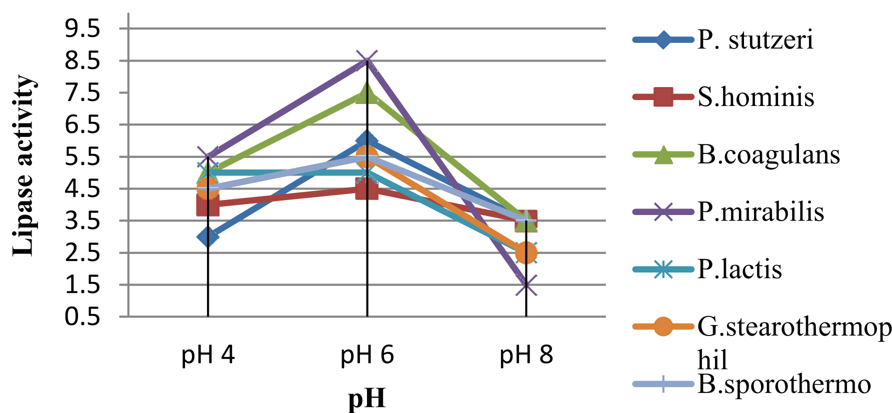

Figure 6: Effect of pH on lipase activity.

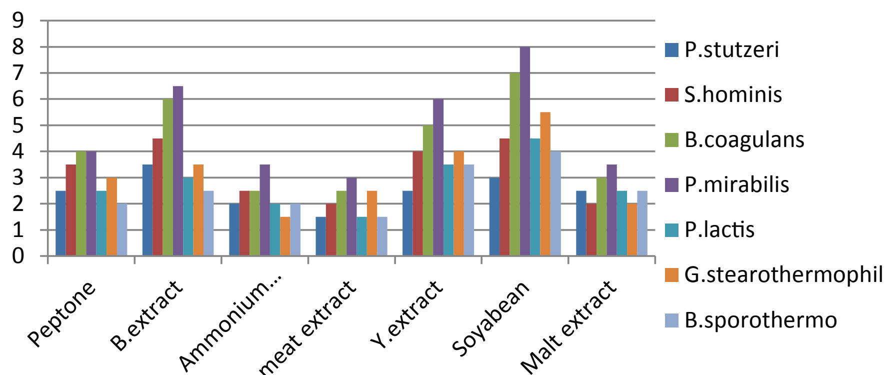

Figure 7: Effect of nitrogen sources on lipase activity.

source, nitrogen source, and the initial pH of culture medium were consecutively optimized [14]. Lipase activity 1.16 U of culture medium was obtained in 48 h at 55°C and pH 8.5 with refined mustard oil as carbon source and a combination of peptone and yeast extract (1:1) as nitrogen sources. Thus, in the present study a production of 7.5U of lipase enzyme activity gives a very high level of production.

In the present study, different temperature such as 25°C, 37°C and 50°C were used to check the optimum temperature for the lipase production by isolated bacteria culture. The maximum lipase enzyme activity obtained was 6.5-8.5U in all fermentation broth culture on 37°C and 50°C.The fermentation media having different pH such as 4, 6, and 8 were used for isolated bacteria culture. The maximum lipase enzyme

activity obtained was at pH 6. The lipase enzyme activity was 7.5-8.5U on 4th-6th day at pH 6 media. Nitrogen source variation was also used for optimization of Lipase activity. Soyabean extract was found to lead to highest lipase activity by *P. mirabilis*.

Another related study by Larbi Daouadji et al. provided an overview about the effect of incubation time, medium pH, temperature, carbon source and nitrogen source for the lipase production was studied [17]. The lipase production they showed were maximum at pH 8, temperature 40°C and incubation time 48 h by the lipase producing bacteria *B. licheniformis*. With a selected carbon source, olive oil and glucose were suitable substrates to maximize lipase production (1.5 U). The optimized concentration of olive oil and glucose was 1% and 1%, respectively. The effect of nitrogen source on lipase production indicated that the yeast extract was suitable substrates for accelerating lipase production (1.47 U).

Another important aspect in the present study was isolation of three thermophiles namely: *G. stearothermophilus, B. sporothermodurans* and *P. stutzeri* which produced around 4U of lipase enzyme activity at 37°C while producing 6.5U at 50°C. These results show that these three bacterial cultures were indeed thermophiles as their optimum temperature for maximum lipase production is 50°C.The amount of lipase activity produce in the present study is comparable and more than all earlier literature and studies.

Conclusion

Our present study provides useful information for the optimization of culture conditions such as pH, temperature, fermentation time, and carbon sources to provide the best lipase production. These results show that lipase producing bacteria are widespread in oil contaminated soil. The sunflower oil is good substrate for lipase production and better than soyabean oil and mustard oil. The optimized growth conditions developed in this study can be used for a large scale in industrial purposes.

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