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Full Length Research Paper

Isolation and characterization of lipase-producing *Bacillus* strains from oil mill waste

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***Bacillus* strains (B1 - B5) producing extra cellular lipase were isolated from the soil sample of coconut oil industry. The strains were identified by morphological and biochemical characters. Growth of the organisms and lipase production were measured with varying pH (4 - 9) temperature (27, 37 and 47ºC) and various substrate concentrations. The result indicated that the lipase production varied between *Bacillus* strains and also between varying parameters tested. The maximum lipase production was recorded at pH 7 during 24 h of the culture period by *Bacillus* strain B5. Among the substrates tested, coconut oil at a concentration of 0.5% was found to enhance the lipase production in the same *Bacillus* strain B5. Further the effect of medium temperature indicated that the production of lipase was maximum at 37ºC. Statistical analysis revealed that the variation in lipase production was highly significant between bacterial strains than the independent influence of pH, substrates concentration and medium temperature.**

Key words: *Bacillus*, lipase, substrates.

INTRODUCTION

Lipids constitute a large part of the earth's biomass and lipolytic enzymes play an important role. Lipases and esterase have been recognized as very useful biocatalyst. They are the major industrial enzymes extensively used in pharmaceuticals, textiles, food, medical, and chemical industries (Salleh et al., 1999). They catalyse at lipid-water interfaces involving interfacial adsorption and subsequent catalysis. They are hydrolases acting on carboxyl ester bonds. 3-D structures of lipases show a hydrolase's fold as well as a nucleophilic elbow, where the catalytic action of serine is located (Ollis et al., 1992; Cygler et al., 1993). Industrial scale extraction of lipases is carried out in bacteria, fungi, actinomycetes and cultures of plant and animal cells. Among them, microbes are metabolically versatile and hence have advantage in many industrial processes leading to the development of microbial biotechnology. Microbial lipases and phospholipases can be used to modify naturally occurring lipids. Thus lipases produced by *Candida* and *Mucor* sp. have been applied to increase the content of n-3 polyunsaturated fatty acids (PUFA) (Ako et al., 1995; Heraldson et al., 1995), archidonic acid (Shimada et al., 1995) and triglycerides for use as health products (Henderson et al., 1998). However there is still more scope for finding more lipases with novel and specific properties through screening by using tributyrin agar and specific plate among plate assay (Rohit et al., 2001). Considering the importance of lipase enzyme, lipase producing *Bacillus* sp. have been characterized and optimized in the present study.

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MATERIALS AND METHODS

Sample collection

For the present study, soil sample was aseptically collected from oil-spilled areas of the coconut oil extracting industry in a sterile container for the isolation of lipase producing organisms under laboratory condition.

Isolation of lipolytic microbes

To begin with, lipolytic microbes were isolated from the collected soil sample. For this, 1.0 g of soil was dissolved in 100 ml of distilled water. Then it was serially diluted (10^{-1} to 10^{-5}) and the diluted samples were plated on nutrient agar for total viable count. Then the dominant organisms were isolated and individually streaked on tributyrin (Hi media 071) agar plates and the formation of halo zone around the colony on tributyrin agar was considered as the positive colony

Identification

The isolated dominant organisms were identified as *Bacillus* sp. based on morphological, biochemical and physiological characters according to Bergey's manual of determinative bacteriology (Holt et al., 1996) and further confirmative test was made on MYP broth.

Further the enrichment culture technique enabled to isolate stains with lipolytic activity in tributyrin media plates. In 24 isolates were collected from the soil sample and among them five isolates (B1 to B5) showed high lipolytic activity. The lipolytic microbes were further screened and characterized by their features and reactions, then identified as gram positive, rod shaped motile organisms (Table 1). Finally the morphological and biochemical tests indicated that the suspected were *Bacilllus* sp. Thus five different (B1 to B5) bacillus strains were identified

Optimization parameters

Varying the following parameters one at a time with optimization of fermentation media. The parameters varied were i. incubation period, ii. temperature, iii. pH and iv. lipid substrates. The influence of growth period on biomass and lipase production of *Bacillus sps* (B1 – B5) was assessed by culturing it on production media for different time duration (24, 48 and 72 h). The effect of medium pH on lipase production was assessed at different pH ranged from 4 to 9 by culturing the isolated *Bacillus sps* (B1 – B5) on medium with different temperatures (27, 37 and 47°C) was also assessed.

Lipid optimization by substrates

By using different substrates sources such as olive oil, coconut oil and sunflower oil, their effect on lipase production by the selected *Bacillus* sp. was assessed at optimum pH (pH 7) and temperature (37°C). Further the influence of substrate concentration (0.5 to 2.5%) on lipase production was also assessed on the optimized substrate, which maximized the lipase production.

Enzyme assay

Isolated *Bacillus sps* (B1 – B5) were assayed for extra cellular lipase production using titrimetric method (Sadasivam and Manickam, 1996).

Lipase activity

One unit of lipase activity was defined as the amount of enzyme releasing one mole of free fatty acid in one minute under standard assay condition.

$$\text{Lipase activity (µg/ml/min)} = \frac{\text{Volume of alkali consumed x Normality of NaOH}}{\text{Time of incubation x Volume of enzyme solution}}$$

Table 1. Biochemical identification of lipase-producing *Bacillus* strains from oil mill waste.

Test	Results
Catalase	+
V-P	+
Starch	+
Casein	+
Gelatin	+
Citrate	+
Nitrate	+
Acid production	+
Glucose	+
Arabinose	+
Xylose	+
Mannitol	+
MYP	Yellow

Statistical analysis

The results obtained were subjected to relevant statistical analysis described by Zar (1974).

RESULT

Enrichment culture technique enabled the isolation of strains with lipolytic activity in tributyrin media plates. In total, 28 isolates were collected from the soil sample and among them; five isolates (B1 to B5) showed high lipolytic activity. The lipolytic microbes were further screened and characterized by their features and reactions and then identified as Gram positive, rod shaped motile organisms (Table 1). Finally the morphological and biochemical test indicated that the suspected organisms were *Bacillus* sp.

The efficiency of lipolytic *Bacillus sps*. (B1 - B5) was assayed with different substrates like coconut oil, sunflower oil, olive oil and also at varied medium pH, temperatures and substrate concentration at different time intervals. Among the tested substrates, *Bacillus* sp. showed maximum activity (0.0029 µg/ml/min) in coconut oil at pH 7 (Figure 1). Among the *Bacillus* sp. tested, maximum lipase activity was achieved by the following *Bacillus* sp. B1, B4 and B5. Two way ANOVA test indicated that the influence of various *Bacillus* strains on lipase production was statistically more significant ($P < 0.01$) than the independent influence ($P > 0.05$) of substrates

The results on the effect of medium pH on the tested sun flower oil indicated that the lipase production were maximum (0.026 µg/ml/min) at pH 7. In low (4, 5 and 6) and also at high (8 and 9) medium pH, the lipase activity was less for all the tested *Bacillus* strains. Similar to that of recorded in the first experiment, here also the *Bacillus* strain B5 showed maximum lipase production at the opti-

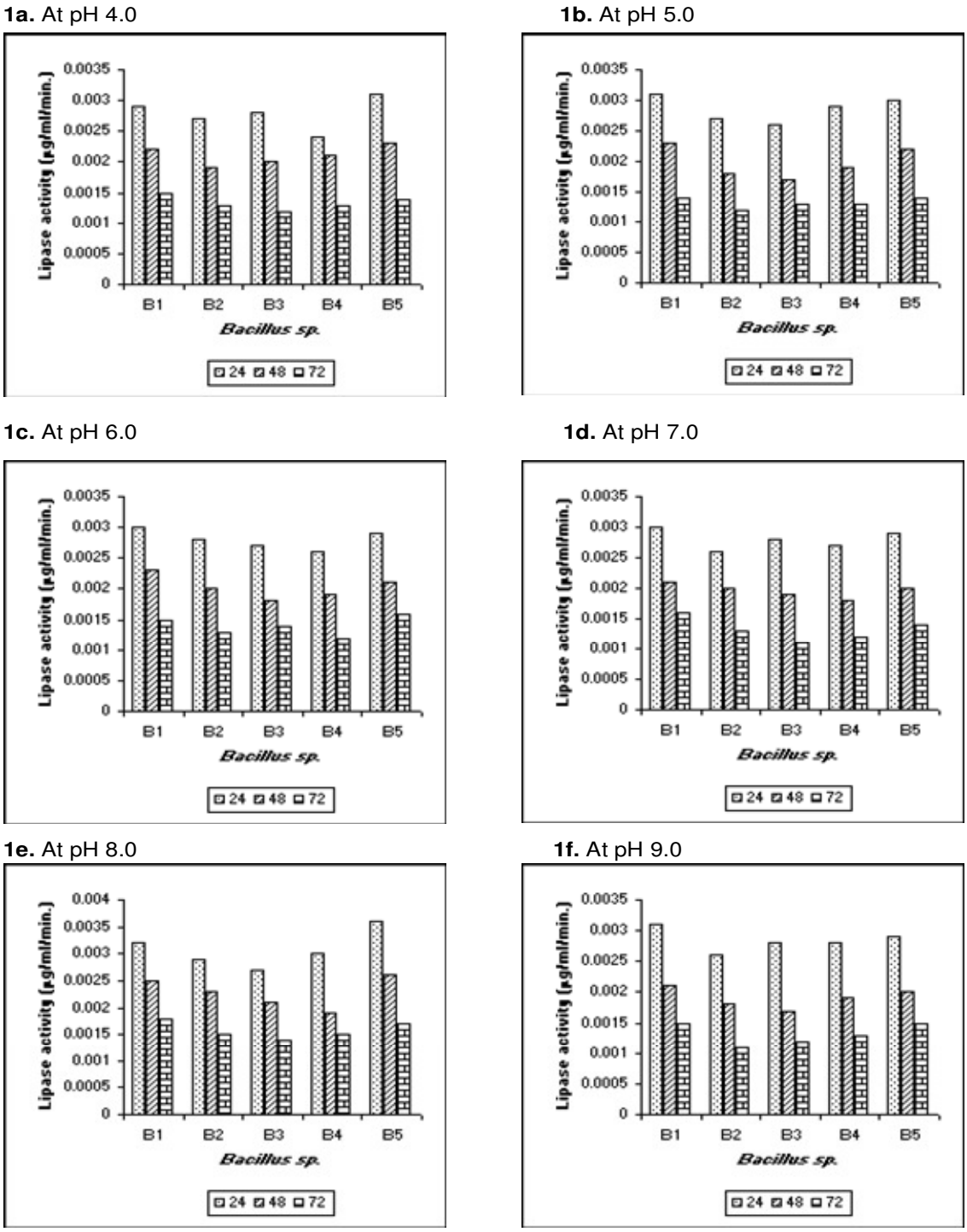


Figure 1. Effect of coconut oil on lipase activity of *Bacillus* sp. (B1 - B5) cultured for different time intervals (24 – 72 h) at different medium pH 4 – 9.

mum medium pH (7.0), (Table 2) (Figure 2) Two-way analysis of variance for the data on lipase production indicated that the influence of different *Bacillus* strains was statistically more significant ($P < 0.01$) than the independent influence ($P < 0.05$) of medium pH.

The result of the effect of medium pH on the tested olive oil indicated that the lipase production was maximum (0.026 µg/ml/min) at pH 7. In low 4, 5, and also at high 8 and 9 medium pH the lipase activity was less for all the tested bacillus strains similar to that recorded in the first experiment has also the bacillus strain B5 showed maximum lipase production at the optimum me-

Table 2. Summary of two-way analysis of variance for the data on lipase production by *Bacillus* strains as a function of substrate, medium pH, substrate concentration and medium temperature.

Factor	Variance	DF	MS	F	P-value
A. Substrate					
A1) Coconut oil	Variance due to Bacillus strain	4	1.99E-07	8.73	< 0.01
	Variance due to medium pH	5	6.23E-08	2.76	< 0.05
A2) Sunflower oil	Variance due to Bacillus strain	4	1.99E-07	16.16	< 0.01
	Variance due to medium pH	5	5.09E-08	4.15	< 0.05
A3) Olive oil	Variance due to Bacillus strain	4	2.88E-07	13.94	< 0.01
	Variance due to medium pH	5	2.06E-08	9.344	< 0.10
B. Substrate concentration	Variance due to Bacillus strain	4	1.99E-07	16.20	< 0.01
	Variance due to substrate concentration	5	5.09E-08	4.15	< 0.05
C. Medium Temperature	Variance due to Bacillus strain	4		8.73	< 0.01
	Variance due to medium temperature	5		2.77	< 0.05

P < 0.05 is statistically significant.

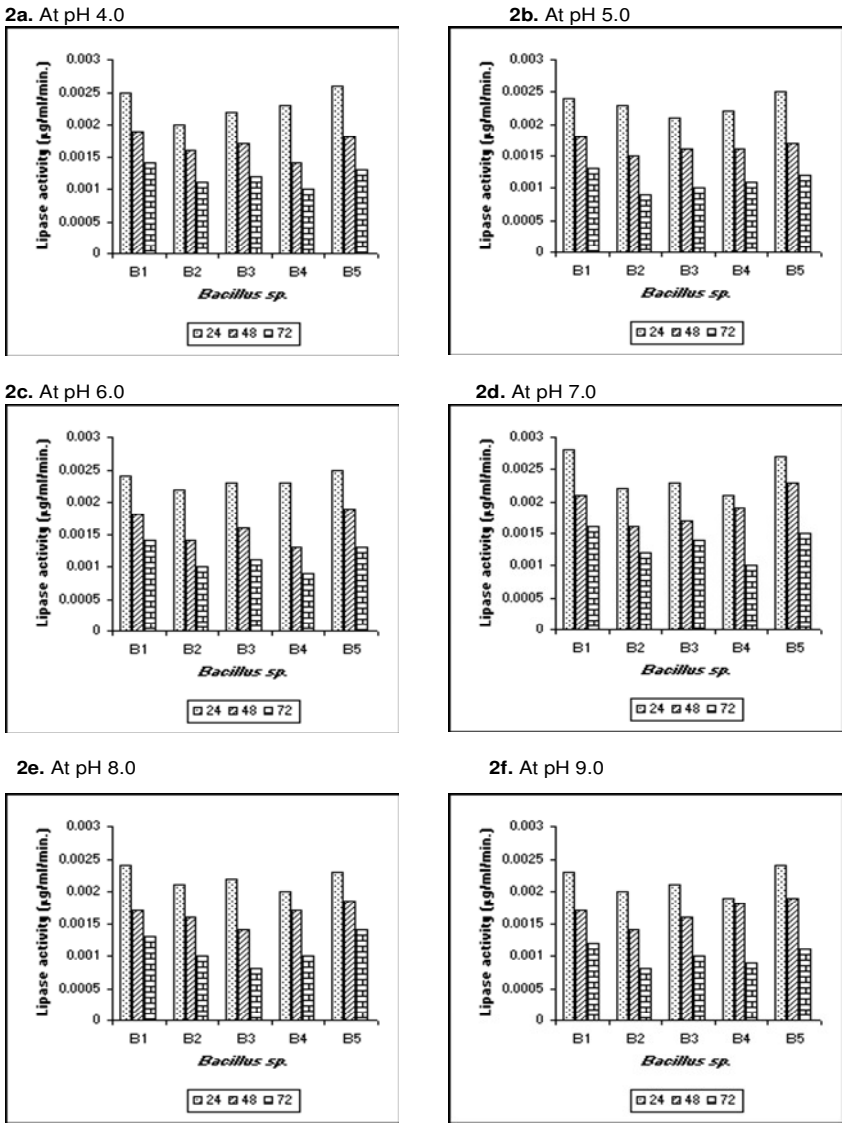


Figure 2. Effect of sunflower oil on lipase activity of *Bacillus* sp. (B1 - B5) cultured for different time intervals (24 – 72 h) at different medium pH 4 – 9.

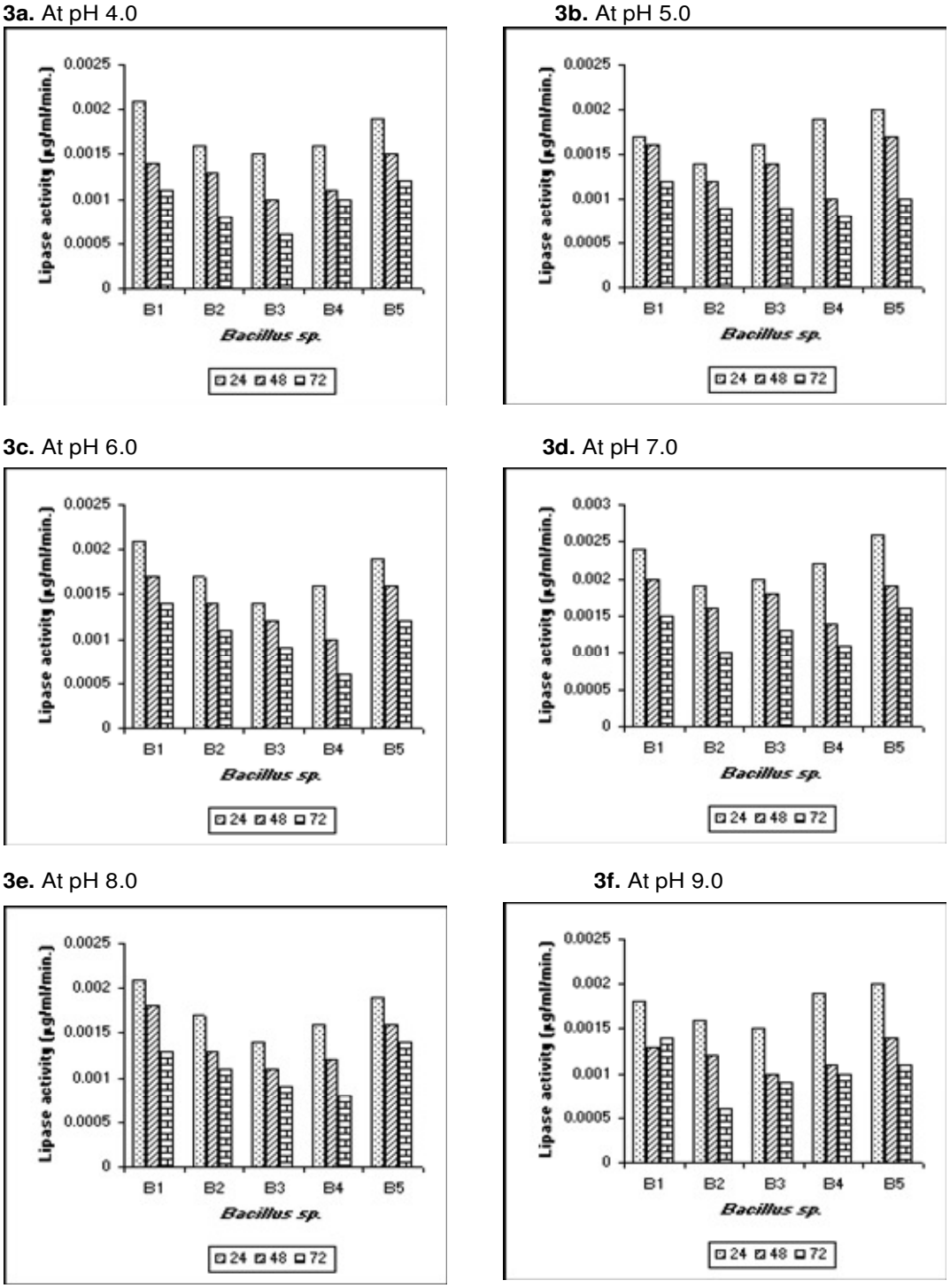
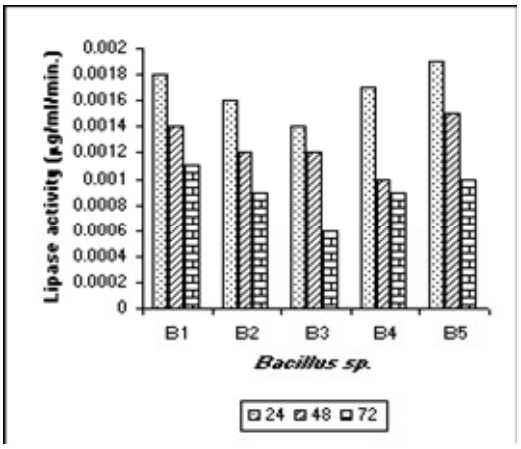


Figure 3. Effect of olive oil on lipase activity of *Bacillus* spp. (B1 - B5) cultured for different time intervals (24 - 72 h) at different medium pH 4 – 9.

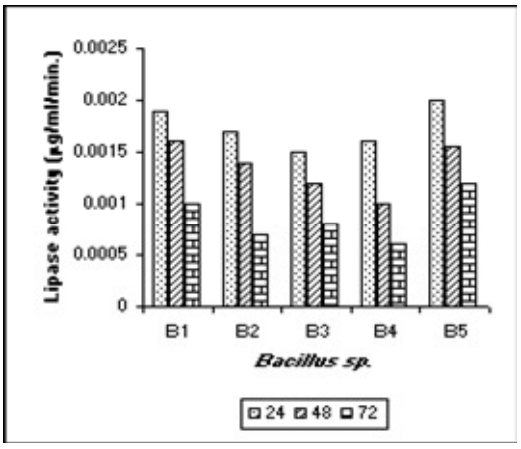
dium pH 7 in Figure 3. Two-way analysis of variance for the data on lipase production indicated that the influence of different *Bacillus* strains was statistically more significant ($P < 0.01$) than the independent influence ($P < 0.05$) of medium pH.

The lipase activity of *Bacillus* strains was high (0.0029 µg/ml/min) when grown at the medium temperature of 37°C at the optimum pH of 7.0. But it was less in both low (27°C) and high (47°C) medium temperatures. At the tested pH, all the selected strains established a steady

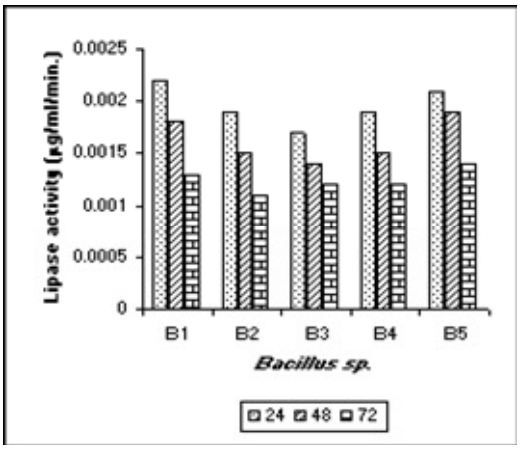
4a. At 0.5% concentration



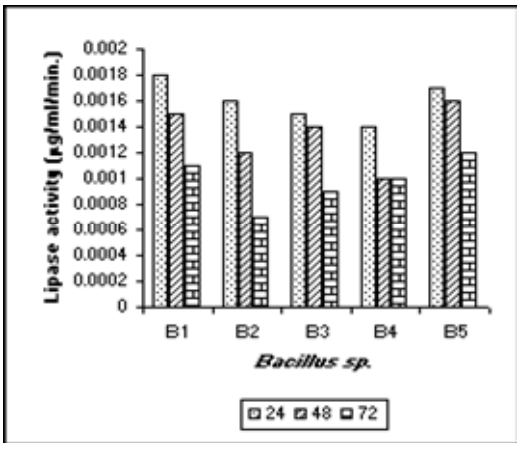
4b. At 1.0% concentration



4c. At 1.5% concentration



4d. At 2.0% concentration



4e. At 2.5% concentration

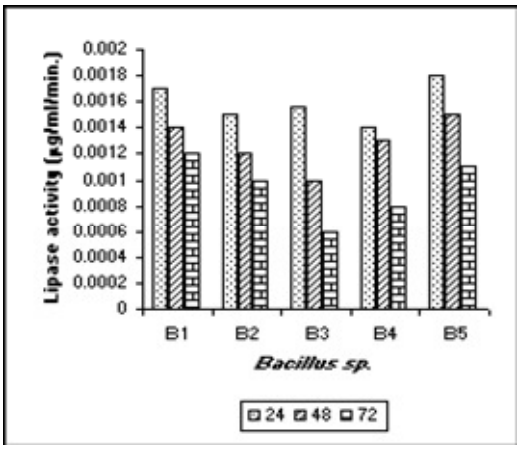
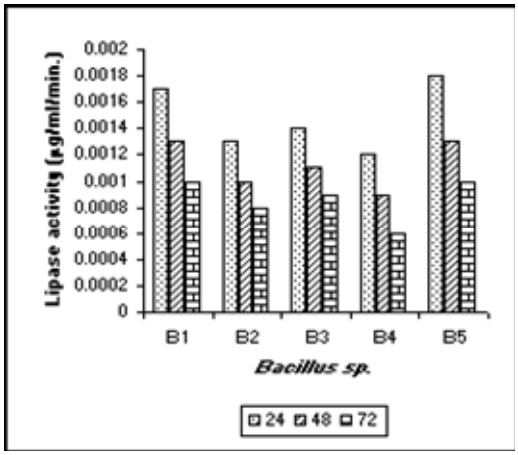


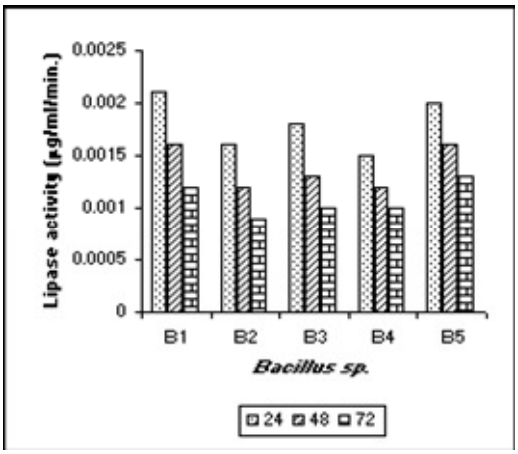
Figure 4. Effect of different concentrations (0.5 - 2.5%) of olive oil on lipase activity of *Bacillus* strains (B1 - B5) cultured for different time intervals (24 – 72 h).

decline in lipase production with increasing culture period from 24 to 72 h. This was the case for all the substrate tested in the present study. Irrespective of the substrate tested, maximum lipase activity was recorded during 24 h of culture period (Figure 4). Results on the effect of substrate (olive oil) concentration indicated that the lipase activity was maximum (0.0022 ug\ml\min) for *Bacillus* sp. in (Figure 5) B1 1.5% concentration. In other tested con-

5a. At 27^0C



5b. At 37^0C



5c. At 47^0C

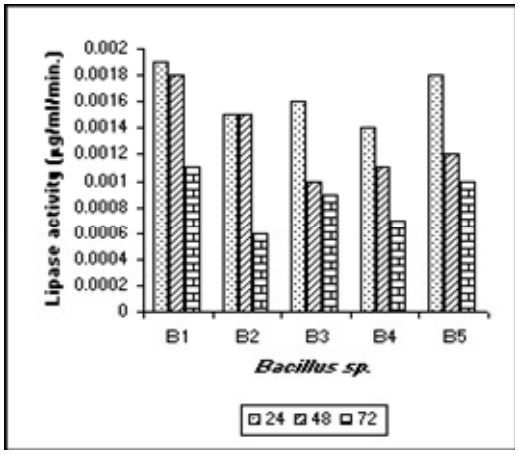


Figure 5. Effect of optimised olive oil on lipase activity of *Bacillus* strains (B1 - B5) cultured for different time intervals (24 - 72 h) at different temperature (27 - 47°C).

centrations, the lipase activity was low. Further in all the tested concentration, the other *Bacillus* strains displayed low lipase activity, when compared to activity registered by *Bacillus* strain B1 ($P < 0.01$).

DISCUSSION

In the present study, the lipase producing bacterial strains were isolated from coconut oil mill soil and identified as *Bacillus* sp. (B1 to B5). Among the different substrates tested, olive oil was found to be suitable for enhancing the lipase production by the isolated and screened *Bacillus* strains and the maximum lipase activity (0.0039 µg/ml/min) was recorded by *Bacillus* sp. 5 (B5). Furthermore, irrespective of the substrates tested, the lipase activity was maximum at pH 7.0. In low and high medium pH tested, the lipase activity was less. This result is in consistence with the earlier report of Achamma et al. (2003). They inferred that the lipase activity of *Bacillus* spp. was maximum at pH 7 during the 24 h of culture period. In the present study, in all the tested substrates and also in all the media pH, the tested *Bacillus* strains showed maximum activity during 24 h of the culture period. On further increase in culture period to 72 h, the lipase activity decreased. High levels of lipase activity were also obtained in *Bacillus* strains when olive oil was used as the substrate. Rohit et al. (2001) reported that the lipase production was more when vegetable oil, olive oil, soya bean oil, sunflower oil and gingelly oil were used as the carbon source.

In the present study, the influence of medium temperature indicated that the lipase production by the isolated strains was higher (0.001 to 0.0021 µg/ml/min) at 37°C when compared to those at 27 and 47°C. Here also maximum activity was displayed by *Bacillus* sps 5 (B5). Walavalkar and Bapat (2001) have reported that, the lipase activity of *staphylococcus* sp. was maximum at 37°C.

Lakshmi et al. (1999) reported that the production of lipase was high in medium added with vegetable oil than the medium added with glucose. In contradiction, Banerjiee 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 both lipase and esterase levels. They also further inferred that, if olive oil was used as the only carbon source for growth, the enzyme activities of *Candida guilliermondii* and yeast sp. showed a four to five fold increases. As reported by Nakashima et al. (1988), the presence of olive oil as growth medium greatly enhanced the lipase activity of *Bacillus* strain 5(B1) in the present study.

Fadiloglu band Erkmen (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 ($P < 0.01$) decreased the lipase activity and biomass content. They also reported that organic nitrogen sources were found to increase lipase synthesis by *Candida rugosa* grown in the presence of olive oil.

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