

Full Length Research Paper

# Optimization of medium components and operating conditions for the production of solvent-tolerant lipase by *Bacillus sphaericus* MTCC 7542

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The production of lipase by *Bacillus sphaericus* MTCC 7542 growing on basal media with various carbon, nitrogen sources and environmental conditions was studied. Studies were undertaken to improve lipase production. Olive oil, Tween 80, rice bran oil, sunflower oil, soybean oil, palm oil and groundnut oil were tested as different lipid sources in this medium, with olive oil at 1% giving a lipolytic activity of 0.19 U/ml after 48 h, which was the highest yield obtained in this study. The effect of carbon source was studied by adding glucose, fructose, sucrose, lactose, starch and cellulose to a medium containing potassium nitrate and other minerals maintained as constant. The best yield of 0.20 U/ml after 48 h was obtained with the medium supplemented with 0.2% of glucose. The effect of nitrogen source was studied by adding peptone, yeast extract, tryptone, gelatin, casein, ammonium chloride, urea, ammonium sulphate, ammonium nitrate and ammonium acetate. The maximum yield of 0.21 U/ml after 48 h was obtained with the medium supplemented with yeast extract and ammonium chloride. Enzyme production was best at 7.5 pH, within a range tested from 6.5 to 8. The maximum enzyme was obtained at 35°C within a range tested from 25 to 40°C. These results are promising because this strain produces lipase in an inexpensive medium and we succeeded in increasing the lipolytic activity 3-fold over the initial values obtained with the non-optimized medium.

**Key words:** *Bacillus sphaericus* MTCC 7542, basal media, olive oil, lipolytic activity, ammonium chloride, mineral salts.

## INTRODUCTION

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3.) are one of the most important classes of industrial enzymes. They hydrolyze esters preferentially at the interface between lipid and water in heterogeneous systems. Lipases are used in the production of detergents, cosmetics, pharmaceuticals, flavor enhancers and foods (Falch, 1991; Saad, 1995; Macrae et al., 1990; Ghosh et

al., 1996; Downey, 1980; Revah and Lebeault, 1989; Gandhi, 1997). Fundamental studies on polymerizations revealed some remarkable capabilities of lipases for polymerization chemistry. The polymerization and transesterification studies generally demand the presence of organic solvents and high temperature. Microbial lipases that can function as catalysts in non-aqueous solvents offer new possibilities such as shifting of the thermodynamic equilibria in favor of synthesis, enabling the use of hydro-phobic substrates, controlling substrate specificity by solvent engineering and improving thermal stability of the enzymes (Koops et al., 1999). Apart from all the advantages, major limitation in carrying out the reaction under water-restricted environment is the tendency of organic solvents to strip water molecules from enzyme surface, especially

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**Abbreviations:** OST, Organic solvent tolerant; 4-NPP, 4-nitro phenol palmitate; NaCl, sodium chloride; MTCC, microbial type culture collection; U/ml, units per milliliter.

into the active site leaving the enzyme inactive (Yang et al. 2004). To overcome these limitations, several strategies like chemical modification of amino acids on enzyme, surface (Desantis and Jones, 1999), protein engineering (Magnusson et al., 2005), medium engineering (Laane, 1987), use of ionic liquids (supercritical fluids) (Katalin et al., 2002) and colyophilization with non-buffer salts (Mine et al., 2003) for enhancing enzyme activity and stability have been demonstrated. Alternately, it has been proposed that instead of modifying enzyme for increasing solvent stability, it would be more desirable to screen for naturally evolved solvent tolerant enzymes for application in non-aqueous enzymatic synthesis.

There are many reports of purification and characterization of microbial lipases but very few reports are available on screening of lipase for its solvent tolerant ability (Rahman et al., 2006; Salameh and Wiegel, 2007; Nawani and Kaur, 2000). Solvent stable lipases have been reported from solvent tolerant *Pseudomonas* and *Bacillus* sp. (Isken et al., 1998; Baharum et al., 2003; Hun et al., 2003; Gaur et al., 2008). Enzymes from these microbes are attuned to work under solvent rich environment, thus exhibit solvent stability. Organic solvent tolerant lipases are required in biotechnological applications, especially in the production of biopolymeric materials, biodiesel and in the synthesis of fine chemicals (Sulong et al., 2006). This report describes the nutritional factors and environmental condition effecting the lipase production and stability of the crude enzyme in various organic solvents.

## MATERIALS AND METHODS

*Bacillus sphaericus* MTCC 7542 was obtained from the Microbial Type Culture Collection and gene bank (MTCC), Institute of Microbial Technology, Chandigarh, India. The stock culture was maintained using a nutrient agar medium containing [g/l]: peptone 10.0; beef extract 10.0; sodium chloride 5.0; and agar 12.0 at 4°C. The media components were obtained from Hi Media Laboratories (Mumbai, India). All other chemicals used in this study are of analytical grade.

### Organic solvents tolerance of *B. sphaericus* MTCC 7542

Solvent tolerance was determined as plate overlay solvent tolerance as described by Ogino et al. (1994). Five microliter of an overnight culture was spotted on an NM plate. Plates were kept open approximately for 20 min until the drops were dry, and after that solvents like methanol, ethanol, propanol, butanol, acetone, acetonitrile, toluene, hexane and benzene solvents were poured onto the plates. The plates were left in the screwed jars to avoid evaporation of solvents. Colonies were examined for solvent tolerance after incubation at 35°C for 24 h.

### Tbriutyryn agar plate assay

*B. sphaericus* MTCC 7542 was screened for its lipolytic activity in Luria-Bertani (LB) agar plate containing 1.5% tributyrin at 30°C for two days. After two days incubation at 4°C was done to bright clear

zone on the plate (Suoniemi and Tynkkynen, 2002).

## Lipase production

*B. sphaericus* MTCC 7542 was grown in a basal medium containing  $\text{KH}_2\text{PO}_4$  (1.0 g/l);  $\text{K}_2\text{HPO}_4$  (1.0 g/l); yeast extract (3.0 g/l); NaCl (0.1 g/l);  $\text{CaCl}_2$  (1.0 g/l); olive oil (10 ml/l) at pH 7.0 and 30°C. Then, the culture was cultivated under the conditions described earlier. Five milliliter of the culture were taken at the intervals of 12 h, the culture was centrifuged at 10,000 rpm, 4°C for 20 min and the supernatant obtained was used as the crude lipase for determination of lipase activity.

## Enzyme assay

Lipase activity was determined by following the method of Kilcawley et al., 2002. In brief, a stock solution (50 mM) of 4-nitrophenyl palmitate (4-NPP) was prepared in 1:1 of acetonitrile and n-butanol. The reaction mixture contained 20 µl of 4-NPP stock solution, 200 µl of cell-free culture supernatant (crude lipase), and 1.8 ml of Tris-buffer (0.1M, pH 8.0). The reaction mixture was incubated at 35°C for 10 min. The amount of liberated 4-nitrophenol (pNP) was recorded at 400 nm. The concentration of 4-nitrophenol released was determined from a standard curve of 4-nitrophenol (2–20 µg/ml in 0.1M Tris-HCl buffer, pH 8.0). One unit of lipase activity was defined as micromoles of 4-nitrophenol released from 4-NPP per ml per min under standard assay conditions.

## Optimization experiments

Various parameters were studied in order to achieve maximum enzyme production. Lipase was produced by growing the culture in a basal medium containing different lipid sources (1.0% v/v) such as olive oil, Tween 80, rice bran oil, sunflower, soybean oil, palm oil, and groundnut oil. The effect of lipid concentration on lipase production was studied with the addition of various concentrations (0.5 - 2.0% v/v) of olive oil. The compositions of other nutrients were maintained as constant. Effect of various carbon sources (0.2% w/v): glucose, fructose, sucrose, lactose, starch and cellulose on lipase production was studied. Then, the effect of different concentrations of glucose (0.10 - 0.25% w/v) on lipase production was studied. The effect of various organic nitrogen sources (0.3% w/v) like peptone, yeast extract, tryptone, gelatin and casein, and inorganic sources (0.2% w/v) like ammonium chloride, urea, ammonium sulphate, ammonium nitrate and ammonium acetate was studied. Effect of incubation temperature on lipase production was analyzed with a range from 25 to 40°C with 5°C interval. The production medium pH was adjusted between (6-8) to study the effect of pH on enzyme production and.

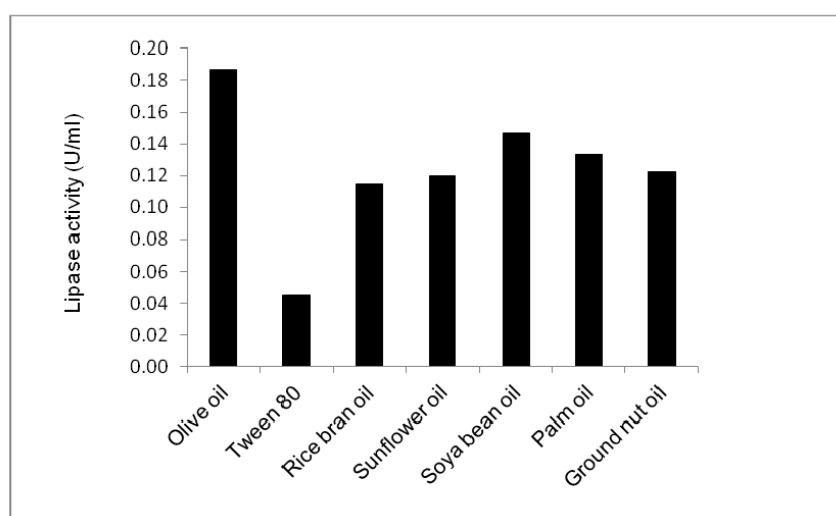
## RESULTS AND DISCUSSION

### Organic solvents tolerance of *Bacillus sphaericus* MTCC 7542

The effects of organic solvents of *Bacillus sphaericus* MTCC 7542 were investigated by plate spreading methods (Table 1). From the experimental observation *Bacillus sphaericus* MTCC 7542 was able to grow in all organic solvents except benzene, hexane and toluene. The bacillus culture exhibits the growth in the hydrophilic solvents having low log P values compared to the hydrophobic solvents having high log P values.

**Table 1.** Organic solvent tolerance of *B. sphaericus* MTCC 7542.

S/N	Solvent	Log P Value	Colony
1	Methanol	-0.764	+++
2	Ethanol	-0.235	+++
3	Propanol	0.074	+++
4	Butanol	0.8	+
5	Acetone	-0.208	+++
6	Acetonitrile	-0.394	+++
7	Hexane	3.9	—
8	Benzene	2.13	—
9	Toluene	2.69	—

**Figure 1.** Effect of different substrate on lipase production by *Bacillus sphaericus* MTCC 7542.

### Effect of different lipid sources on lipase production

Some natural fats or oils have been used as inducers for enzyme fermentation (Hiol et al., 1999). Amongst the various carbon sources tested for lipase production, olive oil (1%, v/v) was the best inducer followed by soybean oil and palm oil (Figure 1). Earlier, soybean oil was reported as the best lipid source for the enzyme production by a bacterial isolate (Gupta et al., 2004). Lipases are generally produced using lipidic sources such as oils and fats with various nitrogen source (Gupta et al., 2004). *Cryptococcus* sp. used sardine oil in preference to olive oil as a the production of lipase (Kamini et al., 2000).

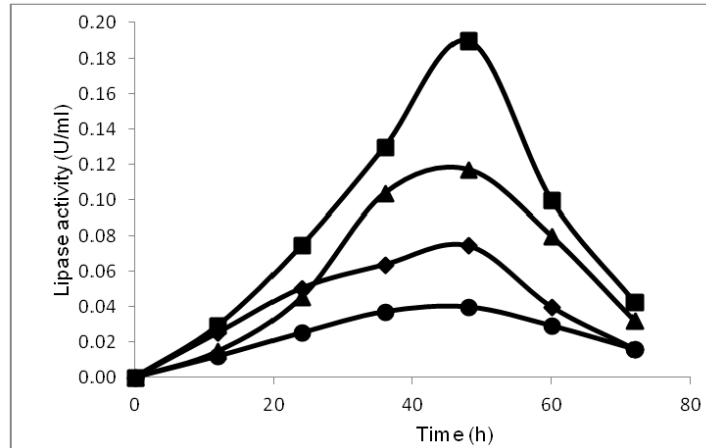
### Effect of the lipid source concentration on lipase production

The effect of the concentration of the lipid source on lipase production was studied with the addition of

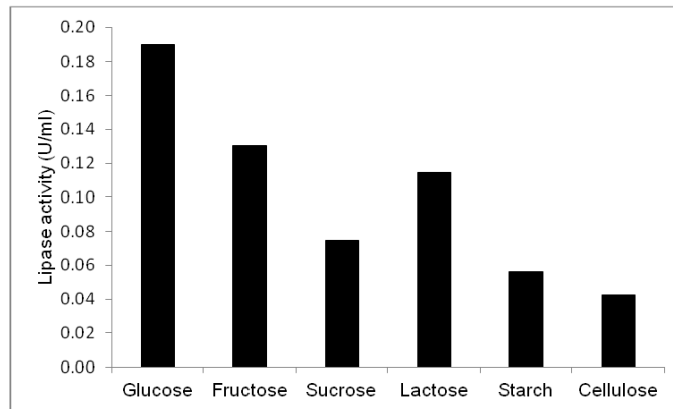
different concentrations (0.5, 1.0, 1.5 and 2.0 %) of olive oil. The concentrations of other nutrient were maintained constant. The olive oil concentration had a strong influence on the production of lipase by *Bacillus sphaericus* MTCC 7542, as shown in Figure 2; The maximum lipase activities were obtained in 1.0 to 1.5% of olive oil used as lipolytic source. The higher oil concentrations could be affect oxygen transfer in the production medium.

### Effect of carbon on lipase production

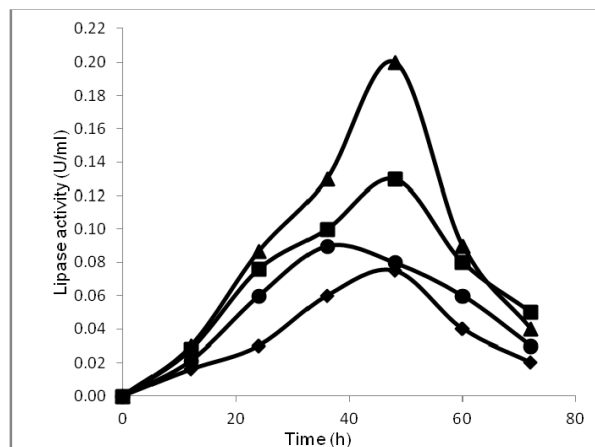
Carbon is a main component of cells and the influences of carbons are shown in Figure 3. Among the six carbohydrates, glucose was the optimal carbohydrate for the lipase production. The glucose concentration had a strong influence on the production of lipase by *Bacillus sphaericus* MTCC 7542, as given in Figure 4; with an increase in glucose concentration up to 0.2%, and then slowly decrease in the lipolytic activity.



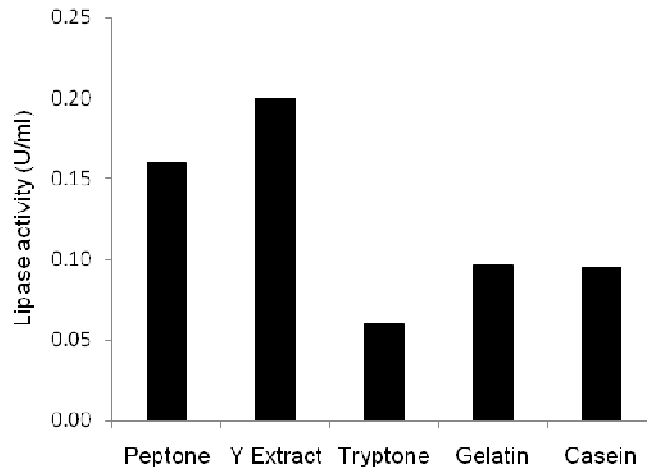
**Figure 2.** Effect of the concentration of olive oil on lipase production by *Bacillus sphaericus* MTCC 7542. Concentration of olive oil 0.5% (♦); 1% (■); 1.5% (▲); 2% (●).



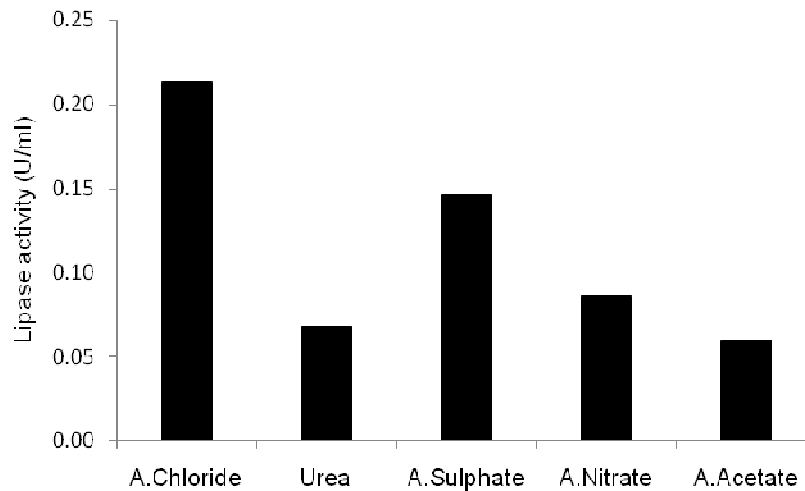
**Figure 3.** Effect of carbon source on lipase production by *Bacillus sphaericus* MTCC 7542.



**Figure 4.** Effect of the concentration of glucose on the production of lipase by *Bacillus sphaericus* MTCC 7542. Concentration of glucose 0.1% (♦); 0.15% (■); 0.2% (▲); 0.25% (●).



**Figure 5.** Effect of organic nitrogen source on lipase production by *B. sphaericus* MTCC 7542. Y Extract, yeast extract.



**Figure 6.** Effect of inorganic nitrogen source on lipase production by *B. sphaericus* MTCC 7542. A. Chloride, ammonium chloride; A. Sulphate, ammonium sulphate; A. Nitrate, ammonium nitrate; A. Acetate, ammonium acetate.

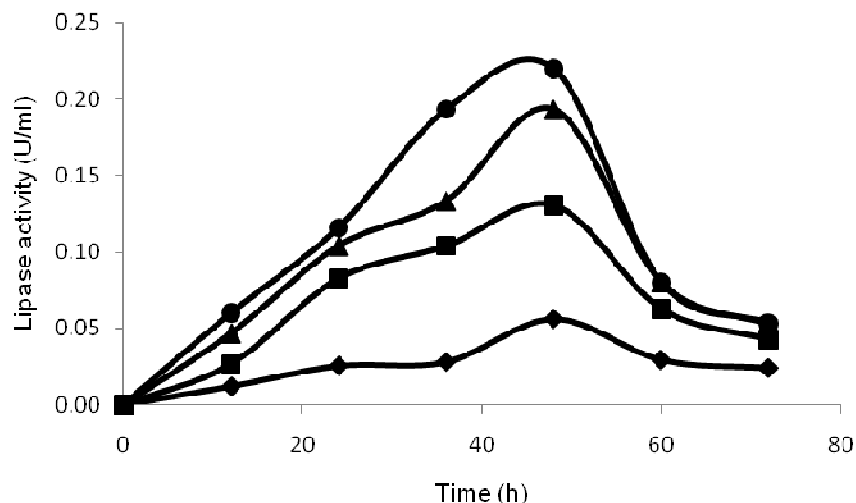
### Effect of nitrogen source on lipase production

Nitrogen sources, including organic nitrogen and inorganic nitrogen sources play an important role in the synthesis of enzymes because inorganic nitrogen sources can be used quickly, while organic nitrogen sources can provide many cell growth factors and amino acids, which are required for cell metabolism and enzyme synthesis. Therefore, both nitrogen are used in lipase production. The influences of organic nitrogen sources was lipase production shown in Figure 5. Yeast extract had the showed maximum enzyme activity among the six organic nitrogen sources used. For various inorganic nitrogen sources tested for lipase production, 0.2% of

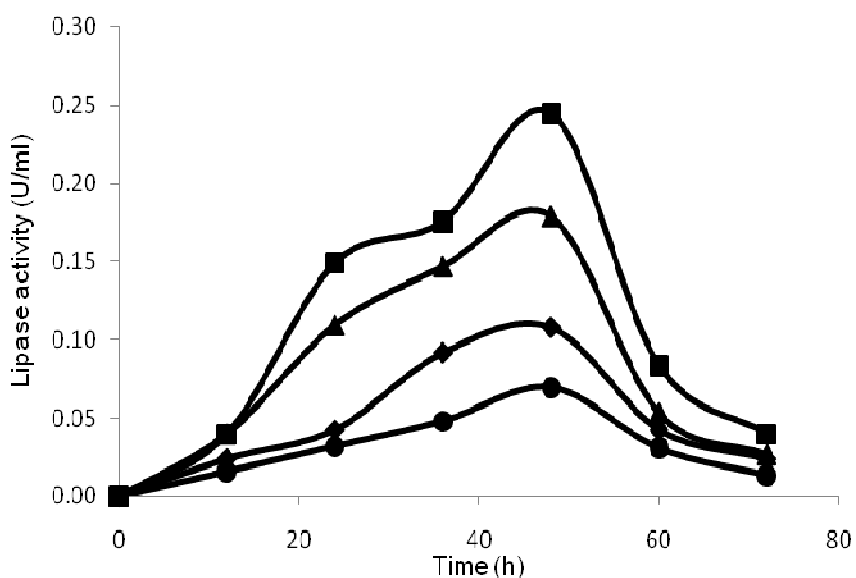
ammonium chloride was the best source followed by ammonium sulphate and ammonium nitrate as shown in Figure 6.

### Optimization of pH and temperature on lipase production

The pH of the culture is one of the most important environmental parameters affecting microbial cell growth and biochemical metabolism. The lipase production increased dramatically up to 7.5 and dropped significantly at pH 8. The optimal pH for lipase activity was pH 7.5 with a 1.5 fold higher than the pH 6 (Figure 7), indicating



**Figure 7.** Effect of pH on lipase production by *B. sphaericus* MTCC 7542. Initial pH of medium: 6.5 (♦); 7 (▲); 7.5 (●); 8 (■).



**Figure 8.** Effect of temperature on lipase production by *B. sphaericus* MTCC 7542. Cultivation temperature: 25°C (♦); 30°C (■); 35°C (▲); 40°C (●).

that the extracellular lipase was alkaline in nature. The optimal incubation temperature for lipase activity was determined to be 30°C when tested under the range of 25 to 40°C (Figure 8). The lipase production at 30°C was almost twofold higher than that at 25 and 40°C.

## Conclusion

In this study, organic-stable lipase from the organic solvent tolerant strain *Bacillus sphaericus* MTCC 7542 was found to display extreme stability in the presence of various hydrophilic organic solvents than hydrophobic

solvents *Bacillus sphaericus* MTCC 7542 produced maximum amount of lipase activity after 48 h of incubation at 30°C and pH 7.5. Maximum lipase activity was with glucose as the carbon source and with olive oil as lipidic source, yeast extract and ammonium chloride together as the nitrogen source.

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