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REGULAR ARTICLE

Purification and characterization of solvent stable lipase from a solvent tolerant strain of *Geobacillus stearothermophilus* PS 11

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ABSTRACT

An extracellular organic solvent stable lipase produced by solvent tolerant strain of *Geobacillus stearothermophilus* PS11 was purified and characterised. The overall purification was 8.04 fold with a yield of 22.6%. The molecular weight of purified lipase was approximately 27.5 kDa. The purified lipase activity was stable (745 EU/ml at 72h incubation) in presence of toluene, benzene, propanol, methanol etc. The enzyme activity was maximum (764 EU/ml) when assayed under optimum temperature and pH of 50°C and 10.0, respectively. The enzyme showed stability at a wide range of temperature from 10°C to 60°C. This solvent stable lipase can be a novel tool for biodiesel industry.

Keywords: Solvent stable, lipases, *Geobacillus stearothermophilus*

INTRODUCTION

Diesel fuels are the key to the industrial economy of a developing country. With the industrialization bloom in the whole globe, the world is confronted with the twin crises of fossil fuel depletion and environmental degradation **Van. (2005)**. In order to resolve these problems, researchers are interested to find an alternative environmental friendly reproducible diesel fuel. Biodiesel is an alternative diesel fuel that is produced from vegetable oils or animal fats. It have the following advantages over diesel fuel: produce less smoke and particulates, have higher cetane numbers, produce lower carbon monoxide and hydrocarbon emissions, are biodegradable, and nontoxic engine lubricity to low sulfur diesel fuels. With this huge range of advantages, it is gaining more and more importance as an alternative fuel. Lipase is the key enzyme used to produce biodiesel **(Marchetti *et al.* 2005, Barnwal and Sharma 2005)**. Only organic solvent tolerant lipase, especially tolerating methanol and ethanol, can distinctly improve the production of biodiesel **(Kaieda *et al.* 2001)**. Till date, the number of bacteria producing organic solvent tolerant lipase is limited, and most of the isolated organic solvent-tolerant strains belong to the genera are *Pseudomonas* isolated from soil or marine samples [5]. We have previously isolated a solvent tolerant strain of *Bacillus thermophilus* PS11 (now known as *Geobacillus stearthermophilus*) that could grow in presence of wide range of solvents. In this work, we report that the bacterial strain is capable of producing lipase that is stable in presence of various solvents such as toluene, n-octanol, propanol, methanol, benzene etc. This will open novel and simpler routes for the synthetic processes and in turn pave a route to alternative biodiesel production.

MATERIAL AND METHODS

Isolation of lipase producing solvent tolerant bacterial strains

Solvent tolerant strain of *Geobacillus stearothermophilus* PS11 (accession no KC311354), previously isolated from soil is used in this study **Sarkar and Ghosh (2012)**. It was plated on nutrient agar supplemented with 1% tributyrin. After 48 h of incubation at 37°C, the lipolytic activity was confirmed by the formation of a clear zone around the colonies.

Measurement of bacterial growth

Overnight grown culture of PS11 strain was inoculated in nutrient broth overlaid with 5% tributyrin and incubated at 37 °C under shaking condition at 140 rpm. Growth and dry cell mass of the isolate was determined according to the process described by **Sarkar and Ghosh (2012)**.

Production media and enzyme preparation

A 1% (v/v) bacterial suspension was transferred from an overnight nutrient broth seed culture to the basal production medium (LPM-1) composed of (g l^{-1}): peptone 5, beef extract 3, sodium chloride 2, tween 80 5 and olive oil 10, pH 8. Bacterial cells were grown at 37 °C under shaking condition at 140 rpm for 96 h. After every 24 h cells were removed by centrifugation and the supernatant was desalted and used for measurement of lipase activity.

Lipase assay and protein content determination

Lipase activity was measured on the basis of hydrolysis of p-nitro phenyl palmitate (pNPP). A 2.5 ml of the assay mixture consisted of, 2.4 ml of 50 mM Tris HCl (pH 9) with pNPP and 0.1 ml concentrated enzyme preparation. Incubation of the reaction mixture at 60°C for 10 minutes was followed by addition of 200 µl of 1 M calcium chloride. The precipitation of

free fatty acids with calcium chloride was used to detect lipase activity. Release of p-nitrophenol (pNP) from pNPP was measured as the increase in absorption at 410 nm. One lipase unit is defined as the amount of enzyme that liberated 1 µM p-nitro phenol per minute under the assay conditions.

Optimization of production media

Optimization of process parameters and manipulation of media composition are the most important techniques used for the overproduction of lipase to meet industrial demands. Optimization was carried out through modification of several growth parameters. For these, the bacterial cells were grown for 96 h at 37 °C under shaking condition. The cells were removed by centrifugation and the supernatants were used for measurement of lipase activity. The various parameters optimized for obtaining maximal lipase yield were, incubation time (24, 48, 72 and 96 h), agitation speed (100 – 200 rpm). Substrate specificity was determined using 1% (v/v) rice bran oil, sunflower oil, soybean oil, olive oil, and mustard oil. Further the effect of various carbon sources (1% w/v) such as glucose, sucrose, lactose, maltose, and nitrogen sources (1% w/v), that is, soybean meal, sodium nitrate, peptone, beef extract, yeast extract were also examined for production of lipase. For each step lipase activity was assayed under standard condition to know the optimal yield.

Purification and Molecular weight of crude enzyme

The bacterial isolate was cultured in LPM-1 for 72 h at 37°C. Bacterial cells were removed by using cooling centrifuge (4°C) at 5000 rpm for 10 min. The enzyme was precipitated by adding solid ammonium sulphate (0-40%) at 4°C for 24 h. The precipitate was collected by centrifugation at 10,000 rpm for 40 min and dissolved in 50 mM Tris HCl buffer (pH 9.0). It was then desalted by dialysis with a cellulose dialysis bag (3500 Da pore-size) in same buffer. The desalted enzyme preparation was loaded on to DEAE-sephacel column. The active fractions were collected, lyophilized and applied on sephadex G75 column. The active fractions were used to determine the molecular weight. Protein content (mg ml -1) was determined by Lowry method **Lowry *et al.* (1951).**
Molecular weight of lipase was detected on 15% polyacrylamide slab gel using mini gel system Bio-Rad. Low molecular weight protein standard ranged from 20-97 KD was used to determine the molecular weight of the purified lipase.

Effect of organic solvent on lipase stability

In order to study the effect of organic solvents on enzyme stability, suitably diluted purified lipase in 50 mM Tris–HCl (pH 9.0) was mixed with different organic solvents to yield a final concentration of (25%, v/v) and then the mixture was incubated on a shaking incubator (180 rpm) at 4 °C for 48 h. The residual lipase activity was measured using p-NPP method. The used organic solvents were hexane, xylene, toluene, benzene, dichloromethane, diethylether, ethylacetate, isopraponal, acetone, acetonitrile, methanol and dimethyl sulfoxide (DMSO). The initial lipase activity (without containing organic solvents) was considered to be 100%.

Effect of pH on activity and pH stability of PS11 lipase

The optimum pH of purified lipase was determined by incubating it with various buffers at a pH range of (4.0–12.0) and lipase activity was determined under standard assay conditions. The pH stability of the enzyme was characterized by preincubating the purified enzyme with different buffers at pH range from 4.0 to 12.0 at 4 °C for 2 h and assayed for lipase activity. Buffer systems were used at a concentration of 50 mM: acetate–HCl buffer (pH 4.0–5.0), sodium hydrogen phosphate–NaOH buffer (pH 6.0–7.0), Tris–HCl buffer (pH 7.0–9.0), disodium hydrogen orthophosphate–NaOH buffer (pH 9.0–11.0), and glycine–NaOH buffer (pH 10.0–12.0).

Effect of temperature on activity and temperature stability of PS11 lipase

The effect of temperature on purified lipase was determined by incubating the reaction mixture at a temperature range of 10°-80° C under standard assay conditions. The thermostability of the enzyme was determined by incubating the enzyme at different temperatures from 10° to 80° C for 2 h and lipase activity was determined.

RESULTS AND DISCUSSION

Measurement of bacterial growth and lipase production

Time course of cell growth and lipase production by PS11 in production medium are shown in figure 1. The bacteria exhibited a minimal lag phrase. The exponential phase lasted up to 18 h and the stationary phase continued till 90 h. A slight decline in biomass was noted after 96 h probably due to nutrient depletion. The findings are similar with results reported by **Abada (2008).**

Lipase production was noted from the early stationary phase (24h) but the production attained maximum level (760 EU/ml) during the late stationary phase (72h) of the microorganism suggesting that the extracellular lipase is a secondary metabolite. Furthermore, the enzyme activity gradually decreased after 72h. Therefore, the optimum incubation periods was maintained throughout the studies.

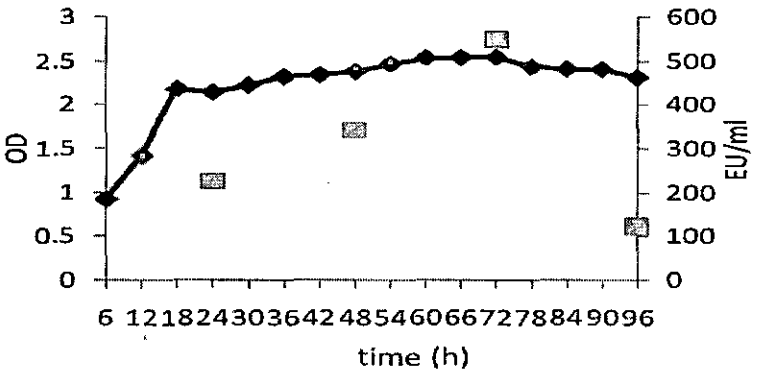

Figure 1 Determination of bacterial growth (◆) and enzyme (□) production.

Optimization of lipase production media

Different sources of nitrogen were tested in order to determine their influence on the synthesis of lipase. Peptone showed maximum enzyme activity (310 EU/ml) as shown in figure 2a. **Gunasekaran and Das (2004)** has also reported about peptone to augment lipase production

Since each microorganism requires a different carbon source to produce lipase at its maximum level, the influence of different carbon sources was tested. Glucose (367.5 EU/ml) was the best carbon source (figure 2b). In some cases glucose has been found to cause repression of lipase production but the case was opposite for PS11 where glucose acted as an inducer. The present study is in agreement with the findings of (**Lakshmi *et al.* 1999** and **Banerjee *et al.* 1985**) who reported maximum production of lipase in medium containing glucose.

Lipidic sources seem to be essential for obtaining a high lipase yield. Among the various lipidic carbon source tested rice bran oil (370 EU/ml) best supported lipase production by PS11 (figure 2c.). The high content of fatty acids specifically PUFA in rice bran oil (38% monounsaturated, 37% polyunsaturated, and 25% saturated) might support high level of lipase synthesis.

The production of lipase was observed till 96h of incubation and it was highest at 72h (765 EU/ml) during stationary phrase. The results of several other studies have shown the optimum lipase production at varying time period between 12 to 24 h **Dharmsthiti *et al.* (1998)** which was in contrast with our results. However it was noted by **Kumar *et al.* (2005)** that a high biomass was obtained at 48 h of incubation and high lipase activity was found in 72 h of incubation time in strains of *Bacillus cereus.* Most *Bacillus* species required more than 2 days for the maximum lipase activity to occur (**Joseph *et al.* 2006, Wang *et al.* 2009**).

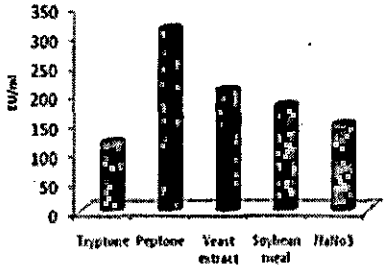

Figure 2(a)

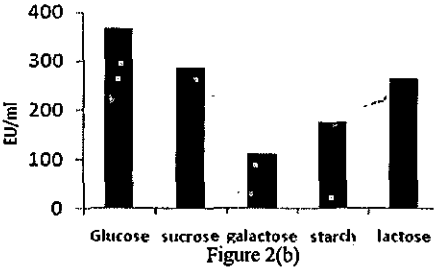

Figure 2(b)

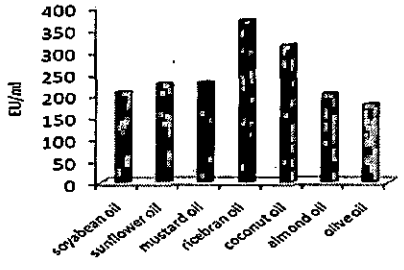

Figure 2(c)

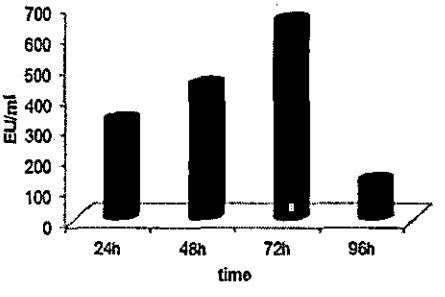

Figure 2(d)

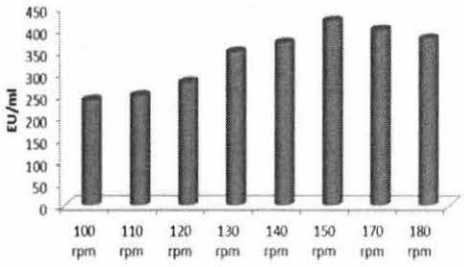

Figure 2(e)

Figure 2 Optimization of lipase production. a. nitrogen source, b. carbon source, c. substrate, d. incubation time, e. agitation rate

Agitation rates influenced the lipase yield and cell growth. Lipase production increased with increase in agitation speed and reached a maximum at 150 rpm (420 EU/ml) due to sufficient supply of dissolved oxygen in the media. Further increase in agitation lowered the production of lipase (figure 2e) probably because at a high agitation rate, the structure of enzyme would be altered Micro-organisms vary in their oxygen requirements as oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed influence the extent of mixing in the shake flasks or the bioreactor, oxygen transfer rate, surface area of contact with the media components, dispersability of the carbon source and the nutrient availability.

Purification and molecular weight determination

Lipase was purified from the extracellular medium by ammonium sulfate precipitation followed by different column chromatography. A large amount of lipase was lost after ammonium sulfate precipitation; less than 5O% of the precipitated lipase was resolubilized. Our three step purification protocol to purify the lipase enzyme from PS11 involving ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography resulted in 8.04 fold purified lipase with 22.6% recovery rate (Tab 1) and the characterizations of the purified enzyme revealed a molecular mass of 27.5 kDa in SDS-PAGE (Figure 3).

Table 1 Purification of lipase from PS11 strain

Step	Total EU	Total Protein(mg)	Specific activity (EU/mg)	Purification fold	Yield (%)
Crude	323.60	28.72	11.26	1	100
Ammonium sulphate	152.60	2.90	52.62	4.67	47.00
DEAE-sephacel	83.60	1.46	56.96	5.05	25.83
Sephadex G-75	73.37	0.81	90.58	8.04	22.67

Figure 3 SDS-PAGE of purified lipase from *Geobacillus stearothermophilus* PS 11. Lane a: purified lipase from *Geobacillus stearothermophilus* PS 11. Lane b: protein standard : phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soyabean trypsin inhibitor (20.1 kDa).

Organic solvent stability of lipase

To date, some organic solvent stable lipases have been reported. The toxicity of the organic solvents is usually measured by log P value (the partition coefficient of the given solvent in an equimolar mixture of octanol and water). The effect of different organic solvents on purified lipase is shown in Table 2. In this study, organic solvents with various log P value ranging from low to high were selected. The lipase was stable and restored almost 90% of its activity when p-xylene, benzene, toluene, and hexane (25% v/v each) were added to the purified enzyme for 2h at C and 1 0 rpm. In presence of methanol and ethanol, the residual activity was 42% and 46%, respectively.

Table 2 Effect of different organic solvent on lipase stability

Organic solvents	Log P	Relative activity (%) at concentration of 25% (v/v)
Control	—	100
Methanol	-0.76	90
Acetonitrile	-0.33	30
Ethanol	-0.24	76
Acetic acid	-0.23	18
Benzene	2.0	81
Toluene	2.5	92
P-xylene	3.1	91
n-Hexane	3.1	80
Hexane	3.6	86

25% (v/v) of organic solvents were added to the enzyme solution and incubated for 48 h in a rotary shaker (180 rpm) at 4 °C.

A similar result was shown by the lipase from *Staphylococcus saprophyticus* M36; the residual activity was 32% and 36%, respectively, when methanol and ethanol (25% v/v) were added to the enzyme up to for 15 days at 30 C and 160 rpm **Fang *et al.* (2006)**.

The stability of the PS11 lipase in organic solvents did not follow the log *P* trends. The greater is the polarity, the lower the log P value and the greater the toxicity of solvent. It is well known that water acts as a lubricant that affords a high conformational fexibility to enzyme molecules. If one follows the trends of log*P*, the lower the log *P* values, the less hydrophobic the solvent, so the enzyme is less stable and there may be change in the conformation of the enzyme molecules. However, different organic solvents showed different tolerance profiles to the PS11 lipase. It is well-known that the effect of organic solvents on enzyme activity differs from lipase to lipase **Sugihara *et al.* (1992)**. There was no clear correlation between the solubility of an organic solvent in water and stability of lipase in its presence **Ogino *et al.* (2000)**.

Effect of pH on activity and pH stability of PS11 lipase

PS11 was inoculated in the lipase production medium and incubated at a wide range of pH (4- 12). At pH 10, maximum lipase activity of 417.5 EU/ml was observed (Figure 4.).The enzyme activity gradually increased from pH 5 to its maximum at pH 10.

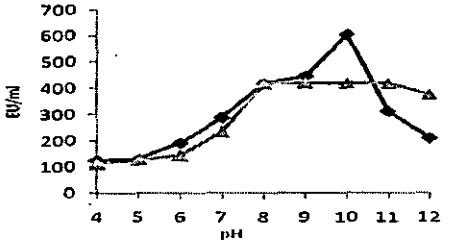

Figure 4 Determination of optimum pH (◈) and pH stability (△) of PS11 lipase

The pH of the production medium plays a critical role for the optimal physiological performances of the bacterial cell and the transport of various nutrient components across the cell membrane aiming at maximizing the enzyme production. **Moon and Parulekar (1991)** reported that the pH of culture has been shown to strongly affect many enzymatic processes and transportation of various components across the cell membrane. The effect of pH on the stability of lipase was tested by incubating the enzymes over a range of pH values. The enzyme also exhibited stability in high alkaline pH which is an important industrial attribute.

Effect of temperature on activity and temperature stability of PS11 lipase

Temperature is a critical parameter which needs to be controlled and this usually varies from organism to another. The optimum temperature for the lipase production was 50°C, although at 40°C the enzyme activity was good. The lipase activity gradually decreased and reached a minimum at 70^0 C (Figure 5).

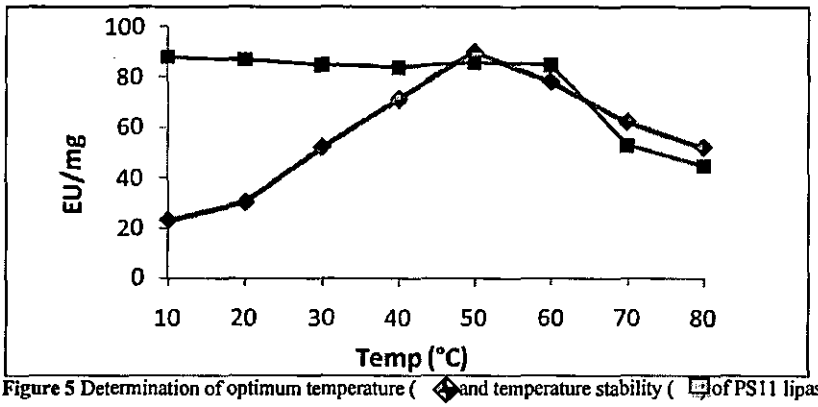

Figure 5 Determination of optimum temperature (◆) and temperature stability (□) of PS11 lipase

The enzyme stability was tested by exposing the enzyme to different temperatures for 2 h. PS 11 showed highest activity at 50°C and its activity remained stable from 10°C to 60°C. The activity further declined as the temperature increased to 70°C showing that the enzyme was not stable at high temperatures. The studies by **Frankena *et al.* (1986)** showed that there was a link between enzyme synthesis and energy metabolism in bacteria, and this was controlled by the temperature and oxygen uptake. As for the extra-cellular enzymes, temperature was found to influence their secretion, possibly by changing the physical properties of the cell membrane. It is a well-known fact that the protein conformation is changed or degraded at higher temperatures, and hence a decrease in the lipase activity was noted at higher temperatures.

CONCLUSION

The major challenge of biodiesel catalyzed by lipase is that its activity significantly reduces in presence of organic solvents, especially methanol and ethanol. Therefore, in this study the solvent tolerant lipase of 27.5kDa was purified from *Geobacillus stearothermophilus* PS11 that could be very useful for biodiesel production as it is stable in presence of methanol and ethanol.

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