# *Chapter 3*
# *Application of PS11 cells for biodiesel production*

## 3.1 Introduction

Lipases produced from solvent degrading bacterial strains have enormous biotechnological potential in the field of biodiesel production due to their stability in organic solvents. 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, a diesel fuel produced from vegetable oils or animal fat is gainingmore importance as an alternative fuel. It has several advantages over diesel fuel, like produces less smoke and particulates, has higher octane numbers, emission of lower carbon monoxide and hydrocarbon, and is biodegradable. Alkali catalysts such as metal alkoxides and hydroxides are widely used for large scale biodiesel production processes (Barnwal and Sharma, 2005). However, the process exhibits certain drawbacks, such as, costly product recovery, generation of environment polluting alkali effluents. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology. They have the ability to perform catalysis at extremes of temperature, pH, and in organic solvents withchemo-, regio-, and enantioselectivity.They can catalyze different reactions including hydrolysis of triglycerides, trans-esterification and chiral synthesis of esters under natural conditions. The lipase catalyzedtrans-esterification of fatty acid is thus an alternate approach to the alkali catalyzed trans-esterification in production of biodiesel fuel. Lipases from *Photobacterium lipolyticum* (Yang *et al.*, 2009), *Pseudomonas aeruginosa* (Ji *et al.*, 2010), *Ralstonia sp.* CS274 (Yoo *et al.*, 2011) have previously been reported to catalyze trans-esterification reactions. However, lipase being costlier than the alkaline catalysts has limited the commercialization of this technique. Moreover, the enzyme can be inactivated during trans-esterification reaction utilizing methanol or ethanol as acyl acceptor. The cost of enzyme catalyzed biodiesel production can be reduced by reusing the enzyme for successive cycles of production (Neilson *et al.*, 2008).Organic solvent tolerant property of lipases, especially tolerating methanol and ethanol, can be exploited for this purpose. Hence, efforts to screen naturally evolved lipases having self-possessed organic solvent tolerance property is highly sought.

As *Geobacillus stearothermophilus* PS11 exhibited different solvent degradation capabilities, it was further employed for the production of solvent tolerant lipase. Therefore, this chapter deals with production, optimization and characterization of lipase produced by petroleum hydrocarbon degrading strain of *Geobacillus stearothermophilus* PS11. The organic solvent tolerance property of PS11 lipase was studied here. Application of solvent tolerant PS11 lipase in enzymatic transesterification of vegetable oil for biodiesel production in presence of high methanol concentration was also studied in this chapter.

## 3.2 Materials and methods

### *3.2.1 Determination of lipase production by Geobacillus stearothermophilus PS11*

The petroleum hydrocarbon degrading strain of *Geobacillus stearothermophilus* PS11 **(accession no KC311354)** was used to determine its capability in lipase production in this study. The strain was inoculated in nutrient agar supplemented with 1% tributyrin. It was incubated for 48 h at 50 °C. The lipolytic activity was confirmed by the formation of a clear zone around the colonies.

### *3.2.2 Production media and enzyme preparation and measurement of bacterial growth*

Growth and lipase production by *G.stearothermophilus* PS11 was carried out in basal production medium (LPM-1) composed of (g/l): peptone, 5; beef extract, 3; sodium chloride, 2; Tween-80, 5; and olive oil, 10; pH 8. The autoclaved medium was inoculated at 1% (v/v) overnight grown bacterial culture and incubated at 37°C under shaking at 120 rpm for 96 h. After every 6 h cells were removed by centrifugation and the supernatant was desalted and used for measurement of lipase activity.

Growth was followed by recording absorbance at 660 nm. For dry cell mass measurement, 1.0 ml culture broth was centrifuged at 10000 rpm at 4°C for 10 min to pellet down the cell mass. The pellet was washed twice with distilled water and dried at 105°C till constant mass was achieved.

### *3.2.3 Lipase assay*

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 10μM pNPPin 50 mM Tris HCl (pH 9) and 0.1 ml enzyme preparation. Reaction mixture was incubation at 60°C for 10 minutes followed by addition of 200 μl of 1 M calcium chloride, which precipitates free fatty acids. The precipitate was pelleted by centrifugation at 6000 rpm for 10 mins. Lipase activity was measured spectrophotometrically in the supernatant by increase in absorption at 410 nm due to the release of p-nitrophenol (pNP) from pNPP. One lipase unit (EU) is defined as the

amount of enzyme that liberated 1 µmol p-nitrophenol minute$^{-1}$ under the assay conditions.

### *3.2.4 Optimization of PS11 lipase production by OVAT method*

For optimization of lipase production by *G. stearothermophilus* PS11, the effect of various process variables such as nitrogen source, carbon source, substrate specificity, agitation speed and cultivation time were studied using OVAT method. Each variable was optimized and was incorporated further in the subsequent experiments.

For these, the bacterial cells were grown for 96 h at 37 °C under shaking condition. The cells were removed by centrifugation at 10,000 rpm for 10 min and the supernatants were used for measurement of lipase activity at each step.

Lipase production was studied in presence of different organic and inorganic nitrogen sources such as peptone, yeast extract, tryptophan, sodium nitrate and soyabean meal at a concentration of 1% (w/v). To determine the effect of substrate on enzyme production, olive oil in the LPM-1 was replaced with soybean oil, sunflower oil, ricebran oil and coconut oil, each at 1% (w/v). The production medium was supplemented with different carbon source such as glucose, sucrose, galactose, starch and lactose at 1% (w/v). The agitation speed for enzyme production was further optimized by agitating the culture at variable speed ranging from 100 – 180 rpm for 96 h at 37 °C. Finally, for determining the cultivation time for highest lipase production, the bacterial culture samples were withdrawn at 24 h intervals for a period of 96 h and enzymatic activity was monitored in the culture supernatant.

### *3.2.5 Purification of PS11 lipase and determination of molecular weight*

The bacterial isolate was cultured in LPM-1 for 72 h at 37°C. Bacterial cells were pelleted by centrifugation at 5000 rpm for 10 min. The supernatant obtained after centrifugation was subjected to 0-40% ammonium sulphate precipitation. 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 in same buffer. The desalted enzyme preparation was loaded on to DEAE-sephacel column pre-equilibrated with 50 mM Tris HCl buffer (pH 9.0) and

eluted with a linear gradient of 0- 0.50 M NaCl in the same buffer. The active fractions were collected, lyophilized and applied on Sephadex G-75 column. The active fractions were pooled and subsequently used for characterization of the enzyme. Molecular weight of PS11 lipase was detected on 15% SDS-polyacrylamide gel electrophoresis (8). Low molecular weight protein standard ranging 20 to 97 KDa was used as marker.

### *3.2.6 Characterization of purified PS11 lipase*

#### *3.2.6.1. Effect of pH on activity and stability of PS11 lipase*

The pH optimum was determined by measuring the enzyme activity at pH 4-12 under standard condition. The pH stability of the enzyme was determined by pre-incubating the purified enzyme preparation with different buffers in pH range from 4.0 to 12.0 at 4°Cfor 2 h followed by monitoring the lipase activity. Buffer systems (50 mM) used were acetate-HCl (pH 4.0–5.0), sodium hydrogen phosphate–NaOH (pH 6.0–7.0), Tris-HCl buffer (pH 8.0–9.0) and glycine-NaOH buffer (pH 10.0–12.0).

#### *3.2.6.2. Effect of temperature on activity and 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 pre-incubating the enzyme at different temperatures from 10 to 80°C for 2 h followed by determination of enzymatic activity.

#### *3.2.6.3. Effect of metal ions and chemical reagents on PS 11 lipase activity*

The effect of various metal ions ($Ca^{2+}$, $Na^{+}$, $Mn^{2+}$, $Cu^{2+}$, $Fe^{2+}$ and $Zn^{2+}$), enzyme inhibitors (EDTA), reducing agent (mercaptoethanol), and oxidizing agents (hydrogen peroxide) on purified lipase activity was determined at the final concentration of 1 mM and 5 mM. For this the enzyme was pre-incubated with either of these chemicals for 1 h at 4°C. The lipase activity in absence of metal ion or chemical reagent served as control (100% activity).

### *3.2.6.4. Effect of detergents on lipase stability*

The effect of various detergents (1%, v/v), including Triton X-100, Tween-20, Tween-80, Tween 60, N-laurylsarcosine, SDS and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (CHAPS) on the lipase activity was investigated by p-NPP method. Lipase activity measured in absence of detergent was considered as 100%.

### *3.2.6.5 Effect of organic solvent on lipase stability*

In order to study the effect of organic solvents on enzyme stability, suitably diluted lipase (250 EU) 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 in a shaking incubator (180 rpm) at 4°C. The lipase activity was measured 48h later using p-NPP method. The organic solvents used were hexane, xylene, toluene, benzene, dichloromethane, diethylether, ethylacetate, isopraponal, acetone, ethanol, acetonitrile, methanol and dimethyl sulfoxide (DMSO). The lipase activity without organic solvent was considered as 100%.

Among the organic solvents, methanol is widely used as acyl acceptor in enzymatic trans-esterification of vegetable oils and animal fats. Hence, to determine the stability properties of the enzyme in such media, lipase preparation (250 EU) was incubated with different concentration of methanol ranging from 5 to 50% (v/v) and residual activity was examined after 24 h later.

### *3.2.7 Application of lipase PS11 in biodiesel production*

#### *3.2.7.1. Sample preparation*

Enzymatic trans-esterification of commercial sunflower and olive oil in methanol was performed according to the method described by Yang *et al.* (3) with certain modifications. At first, a mixture of sunflower oil or olive oil (4 ml) and methanol (0.50 ml) were prepared. To this mixture, enzyme solution (250 EU) was added. Trans-esterification reaction was conducted for 48 h at 30 °C with rotary shaking (200 rpm).

### *3.2.7.2. Determination of biodiesel production by TLC method*

100 µl sample was withdrawn at 6h intervals and mixed with 200µl of hexane. The mixture is centrifuged and 5 µl of the separated upper layer was applied to TLC plate. The plate was developed in the chamber containing a solvent mixture of hexane, acetone, and acetic acid (95:4:1) and was air dried. The plate was then soaked in potassium permanganate solution (0.5%, w/v) and the biodiesel spot were visualized. Fatty acid methyl ester was used as reference biodiesel.

### *3.2.7.3. Determination of biodiesel production by gas chromatography*

Fatty acid methyl esters (FAMEs) of biodiesel were analyzed using a gas chromatographer (GC-2014,Shimadzu, Kyoto, Japan) which was equipped with a Stabilwaxcolumn and flame ionization detector (FID). The GC was calibratedby methyl oleate, methyl linoleate, and methyl palmitateunder various concentrations. During the analysis, the temperatureof the injector and FID were controlled at 250 °C. Thetemperature of the column was raised programmatically. Thetemperature was initially maintained at 150° C for 2 min, andwas further raised to 250° C by a rate of 10° C min/1. Finally,the temperature of the column was maintained at 250 °C for 5 min.

### 3.3 Results

#### 3.3.1 Kinetics of lipase production by PS11 cells

*Geobacillus stearothermophilus* PS11 isolated from soil sample collected from a solvent extraction unit produced extracellular lipase.Time course of cell growth and lipase production by*G. stearothermophilus* PS11 in production medium are shown in Fig 1. The bacteria exhibited a minimal lag phase. The exponential phase lasted up to 18 h and the stationary phase continued till 90 h. Lipase production started during the exponential growth phase but gradually attained its peak at the late stationary phase of growth.

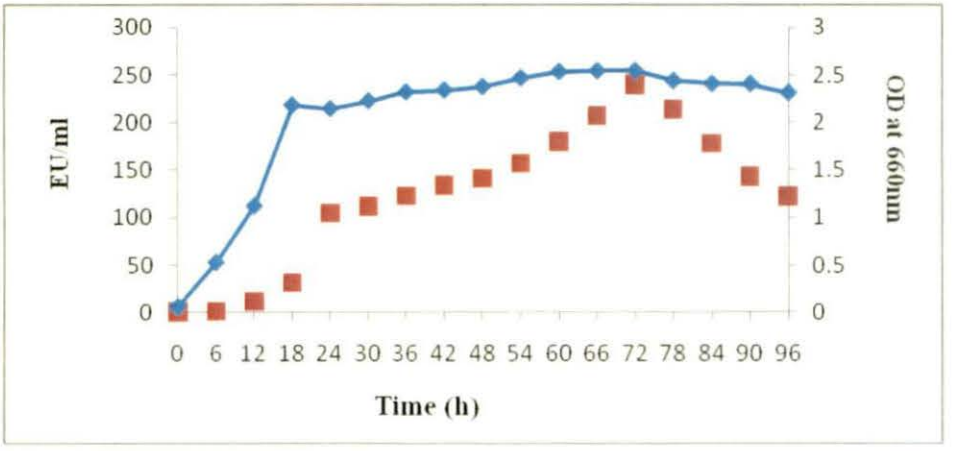



**Fig 1**.Determination of bacterial growth ( ◆) and enzyme ( ■ ) production.

#### 3.3.2 Optimization of PS11 lipase production by OVAT method

The effective parameters for lipase production was optimized by studying the effect of various process variables, like nitrogen source, carbon source, substrate, agitation speed and cultivation time on enzymatic activity. The results of optimization are summarized in Table-1. Optimization of suitable nitrogen source for enzymeproduction is very important as nitrogen is the precursorfor protein synthesis; besides, it can influence the pH of themedium which in turn may affect the activity and stability ofenzyme. Among various nitrogen sources examined, peptone showed maximum enzyme activity of 310 EU/ml (Table1).

**Table 1: Optimization of PS11 lipase production by OVAT method**

| N source | *EU/ml | C source | *EU/ml | Substrate specificity | EU/ml | Agitation | EU/ml | time | EU/ml |
|---|---|---|---|---|---|---|---|---|---|
| Tryptone | 123±2.12 | Glucose | 367.5±5.19 | soyabean oil | 226± 2.18 | 100 rpm | 246±1.2 | 12h | 422±1.56 |
| Yeast extract | 200.5±3.45 | Sucrose | 288.12±2.1 | sunflower oil | 275±2.88 | 120rpm | 312±3.4 | 24h | 567±3.2 |
| Peptone | 310±1.23 | Galactose | 109.45± 5.67 | ricebran oil | 370±1.56 | 140rpm | 368±2.13 | 48h | 620±2.4 |
| Soybean meal | 187±3.12 | Starch | 187±3.7 | coconut oil | 312.5±4.1 | 150rpm | 420±4.71 | 72h | 765±2.16 |
| Sodium nitrate | 156±4.45 | Lactose | 276±3.1 | olive oil | 250±3.86 | 180rpm | 387±2.3 | 96h | 4.1±2.17 |

*EU means one lipase unit is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol minute$^{-1}$ under the assay conditions.

The effect of various carbon sources such as glucose, sucrose, lactose, galactose and starch, on enzyme production was determined. Glucose supported the highest production of lipase (367.5 EU/ml). Among the various lipid sources tested for lipase production, rice bran oil (1% v/v) was the best inducer (390 EU/ml), followed by coconut, sunflower, olive and soybean oil. Lipase production by *G. stearothermophillus* PS11 was influenced by agitation rates with highest activity (420 EU/ml) at 150 rpm. For determining the incubation time for maximum lipaseproduction all the parameters for highest activity were taken into consideration. PS11 strain exhibited optimum lipase production of 765 EU/ ml at 72 h incubation during stationary phrase.

### 3.3.3 Purification of PS11 lipase and determination of molecular weight

The PS11lipase was purified from the extracellular medium by $(NH_4)_2SO_4$ precipitation, anion exchange chromatography and gel filtration. The bacterial culture was grown for 72h in LPM-1 at 37°C. The supernatant obtained after centrifugation of culture was subjected to $(NH_4)_2SO_4$ precipitation and about 47% of the enzyme was recovered in 0-40% fraction. The dialysed enzyme preparation was subjected to DEAE-sephacel chromatography. The active fractions were pooled, concentrated and subjected

to gel filtration chromatography, which resulted in increase in specific activity of enzyme to 90.58 and recovery of the enzyme was 22.67% (Table-2).

**Table 2:** Purification table of lipase produced 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 |

*EU means one lipase unit is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol minute$^{-1}$ under the assay conditions.

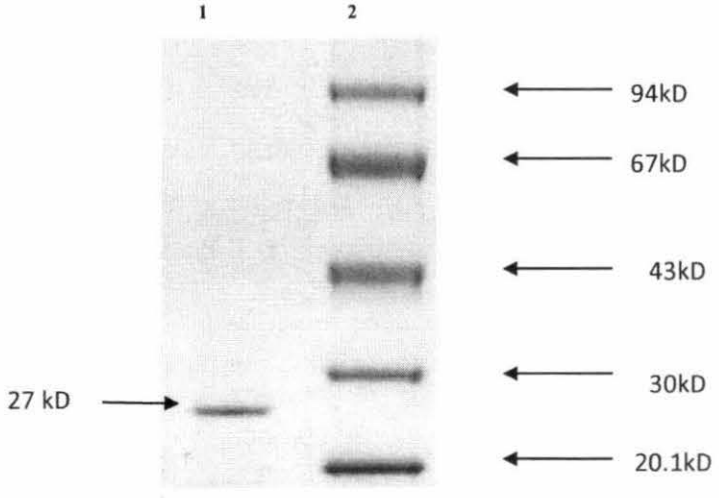



**Fig 2.**SDS-PAGE of purified lipase from *Geobacillus stearothermophilus* PS 11.Lane a: purified lipase from *Geobacillus stearothermophilus* PS 11. Lane b: protein standard: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soyabean trypsin inhibitor (20.1 kDa).

The SDS-PAGE analysis of purified enzyme (Fig 2) revealed the presence of a prominent protein band of approximately 27 kD, indicating that the enzyme was probably a single chain protein or a homomultimeric protein of 27 kD subunits.

### 3.3.4 Effect of pH and temperature on activity and stability of PS11 lipase

The purified enzyme was active in the pH range 4-12 with pH optimum at 9. The effect of pH on the stability of lipase was tested by incubating the enzymes over a range of pH values. The enzyme was alkalostable showing stability over a pH from 8 to 11 (Fig 3). The enzyme could not retain its activity in acidic pH.

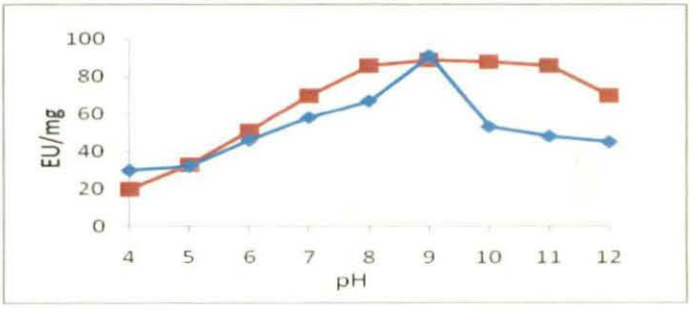



**Fig 3.** Determination of optimum pH (♦) and pH stability (■) of PS11 lipase

The optimum temperature for enzyme was 50°C and a sharp decline in enzymatic activity was noted with in temperature above the optimum value.For determination of thermal stability, the purified enzyme was pre-incubated at 10° to 80° C for 2h and then assayed for enzymatic activity.

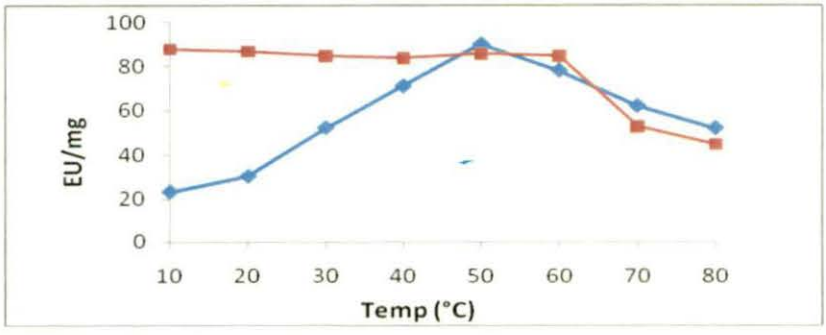



**Fig 4.** Determination of optimum temperature (◆) and temperature stability (■) of PS11 lipase.

The results in figure 4 indicates that PS11 lipase was highly thermostable as it retained its activity between 10-60°C while the thermal stability profile at higher temperatures showed that the enzyme PS11 is not stable above 60°C.

### 3.3.5 Effect of metal ions and chemical reagents on PS 11 lipase activity

The impact of various metal ions on the lipolytic activity of *G. stearothermophilus* PS11 is shown in Table 3. Almost 20% lipase activity was inhibited by $Fe^{2+}$, $Mn^{2+}$whereas $Cu^{2+}$, and $Zn^{2+}$ inhibited almost 40%of its activity respectively.

**Table 3:** Effect of different chemicals on lipase stability

| Chemicals | Relative activity (%) | |
|---|---|---|
| | 1mM | 5mM |
| None | 100 | 100 |
| $FeSO_4$ | 89 ± 4.1 | 80 ± 4.1 |
| $MnSO_4$ | 94 ± 2.7 | 84 ± 2.7 |
| NaCl | 112 ± 1.3 | 107 ± 1.3 |
| $CuSO_4$ | 80 ± 2.4 | 68 ± 2.4 |
| $ZnSO_4$ | 67 ± 1.2 | 60 ± 1.2 |
| $CaCl_2$ | 115 ± 3.5 | 105 ± 3.5 |
| EDTA | 100 ± 1.9 | 100 ± 1.9 |
| $H_2O_2$ | 86 ± 2.1 | 80 ± 2.1 |
| Mercapthoethanol | 105 ± 1.3 | 105 ± 1.3 |

The lipase was pre-incubated with the chemicals for 1 h at 50 °Cbefore measuring the enzyme activity.
The presented results are the average of three repeated experiment with±standard deviation from the mean

An increase in activity by 10% was observed in presence of $Na^+$ and $Ca^{2+}$. Interestingly, EDTA, a metal ion chelator didn't alter the enzymatic activity indicating that functioning of enzyme did not have absolute requirement of metal ion(s).

### 3.3.6 Effect of detergents on lipase stability

The influence of various detergents on PS11 lipase was also determined and the results are shown in Table- 4. PS11 lipase activity was increased by Triton-X 100, Tween-80, Tween-60and Tween-20 with maximum enhancement of 168% in presence of Triton-X-100 (Table 4).Decrease in lipase activity was noted in presence of anionic detergents such as N-laurylsarcosine (78%), SDS (69%) with maximum reduction in presence of CHAPS (65%).

**Table 4:** Effect of different detergentson lipase stability

| Detergents | Relative activity (%) |
|---|---|
| None | 100 |
| N-laurylsarcosine | 78 ± 2.1 |
| SDS | 69 ± 1.8 |
| CHAPS | 65 ± 2.1 |
| Tween 20 | 116 ± 0.7 |
| Tween 60 | 129 ± 2.7 |
| Tween 80 | 134 ± 1.9 |
| Triton X 100 | 168 ± 1.0 |

### 3.3.7 Effect of solvents on lipase stability

The organic solvent tolerance capability of the enzyme was investigated to exploit its application in enzymatic biodiesel production. To determine the organic solvent tolerant property, the purified PS11lipase was pre-incubated with organic solvents having log*P* value ranging between -0.76 to 3.60, for 48 h under shaking, followed by determination of enzymatic activity. The lipase was stable and restored almost 90% of its activity when p-xylene, benzene, toluene, hexane and methanol (25% v/v each) were added to the purified enzyme.The residual activity was 76% in presence of ethanol (Table-5).Acetic acid was more harmful than other organic solvents as PS11 lipase could restore only 18% of its activity. This may due to the acidity inactivating the lipase

**Table 5:** Effect of different organic solvent on lipase stability

| Organic solvents | Log *P* | Relative activity (%) |
|---|---|---|
| Control | — | 100 |
| Methanol | -0.76 | 90 |
| Acetonitrile | -0.03 | 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 |

25% (v/v) of organic solvents were added to the enzyme solution andincubated for 48 h in a rotary shaker (180 rpm) at 50° C.

As methanol is one of the widely used media for biodiesel production, stability of PS11 lipase in presence of 25% (v/v) methanol led us to further investigate its tolerance level at different concentration of methanol.

**Table 6:** Effect of methanol on the stability of PS11lipase

| MeOH % (v/v) | Relative activity (%) |
|---|---|
| None | 100 |
| 5 | 123 ± 3.2 |
| 10 | 120 ± 2.8 |
| 20 | 111 ± 1.5 |
| 30 | 87 ± 1.2 |
| 40 | 72 ± 1.2 |
| 50 | 30 ± 0.4 |

Different concentration of methanol were added to enzymes solution and incubated for 24 h in a rotary shaker (180 rpm) at 50° C

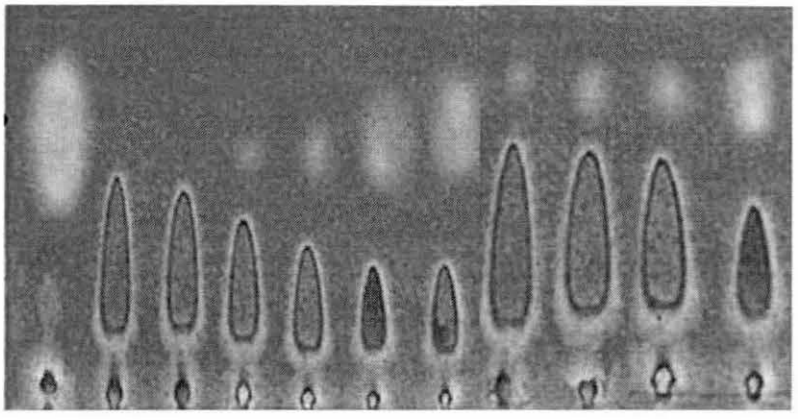
It was observed that PS11 lipase retained 87% and 72% of its activity when grown in presence of 30% and 40% (v/v) methanol respectively (Table 6.).

### 3.3.8 Application of lipase PS11 in biodiesel production

*3.3.8.1. Determination of biodiesel production by TLC method*

PS11 catalyzed the transesterification reaction of both sunflower oil and olive oil in methanol (Fig 5), though it preferred olive oil over sunflower oil. Biodiesel production reached its maximum at around 24 h and 36 h in the case of olive oil and sunflower oil respectively.

Olive oil Sunflower oil



BD NC 0h 6h 12h 24h 36h 6h 12h 24h 36h

**Fig 5.** Application of the purified lipase in biodiesel production. TLC analysis of PS11 lipase catalyzed biodiesel (BD) production using methanol and sunflower oil /olive oil. BD corresponds to reference and produced biodiesel. Negative control (NC) corresponds to the absence of PS11 lipase in the reaction medium.

*3.3.8.2. Determination of biodiesel production by gas chromatography*

Chromatogram of standard biodiesel (Fig6a) showed that there were three fatty acid methyl esters.The main fatty acid methyl esters of biodiesel include palmitic acid methyl ester, oleic acid methyl ester and linoleic acid methyl ester. These components made up more than 90% of the total biodiesel.Presence of these three main fatty acid methyl esters was also noted in PS11 lipase catalyzed trans esterification of sulflower oil in presence of methanol. GC analysis was a served as a

conclusive proof for biodiesel production by PS11.

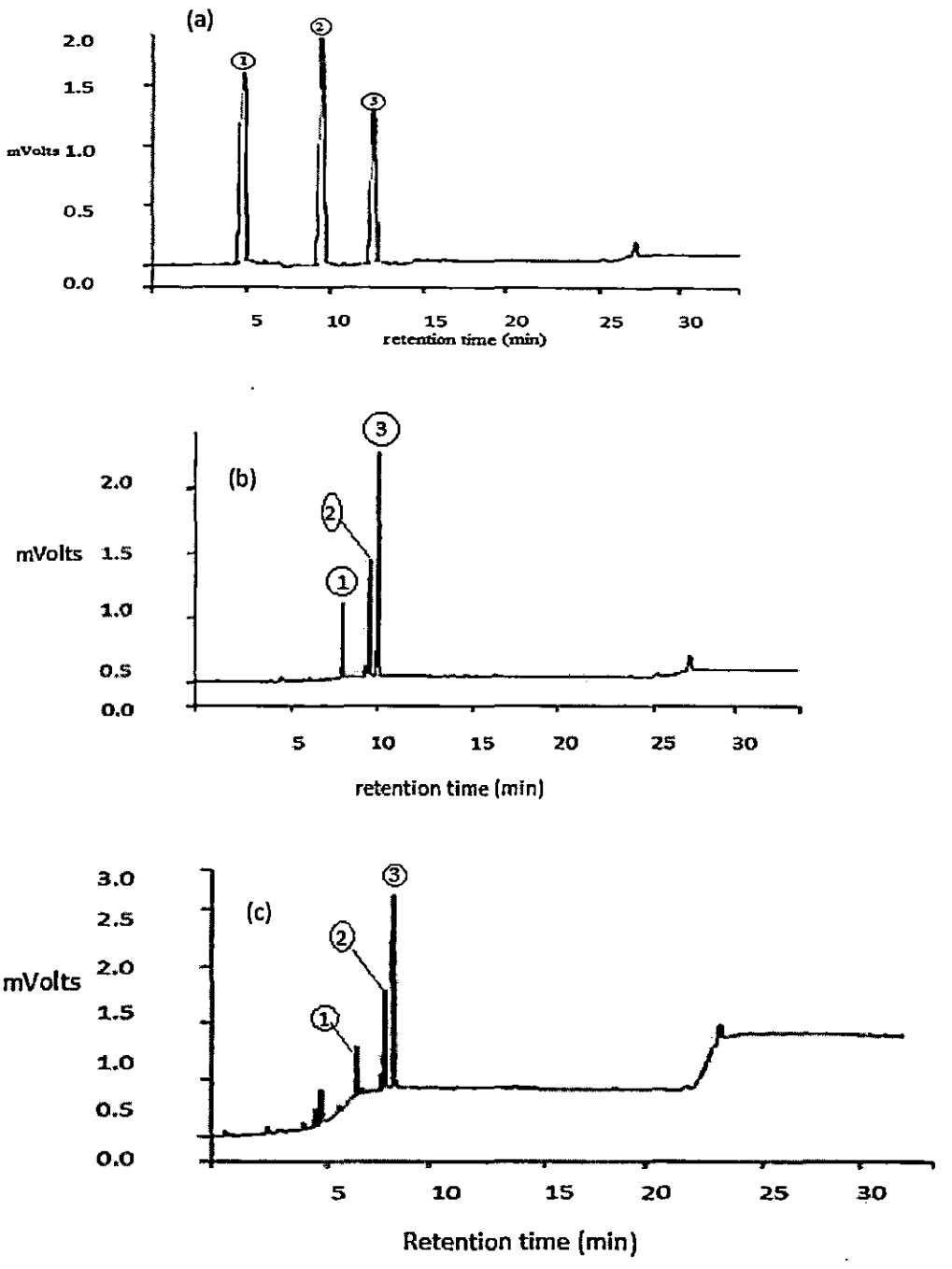



**Fig 6:**Gas chromatographic pattern of biodiesel from sunflower oil. Standard sunflower oil (a), Peak1. free fatty acid of palmitic acid, 2. free fatty acid of oleic acid and 3. free fatty acid of linoleic acid. Standard biodiesel (b), Biodiesel catalyzed by PS11 lipase mediated transesterification of sunflower oil (c ).Peak1. palmitic acid methyl ester, 2. oleic acid methyl ester and 3. linoleic acid methyl ester.

## 3.4 Discussion

Microbial lipases have enormous biotechnological potential in biodiesel production due to their stability in organic solvents, broad substrate specificity and cofactor independent catalysis. Biodiesel is defined as monoalkyl ester of fatty acids from vegetable oil and produced by enzyme mediated catalytical transesterification with petrochemically derived methanol. Fang *et al.,* (2006) reported the presence of more lipaseproducingisolates in the grease-polluted soilcompared to the soilcollected from other places because there was more lipidic content in those soil samples to be utilized by microorganisms. Similarly, as the isolated strain *G. stearothermophilus* PS11 was isolated from petroleum polluted site hence it exhibited lipase production by producing clear zone of hydrolysis in tributyrin agar plate. Thus, PS11 was further employed for the production of lipase that can mediate the trans esterification of vegetable oil in presence of methanol.

Lipase production by PS11 cells was noticed during its late exponential phase but gradually enzyme production increased in stationary phase of growth. Similar finding was reported by Abada et al. (2008). The possible reason might be related to the recent finding of lipase produced by*Streptomyces rimosus* GDS(L).Vujaklija *et al* (2003) proposed a unique feature among prokaryotes in using triacylglycerols as storage compounds or as possible carbon sources for antibiotic synthesis in stationary phase. Horinouchi *et al.* (2002) proposed that hydrolysing enzymes are required for the stationary phase of bacterial growth.

Production of lipase was optimized using one variable at a time (OVAT) method. Nitrogen sources have varied effect on lipase production and sources like peptone have been reported to augment lipase production (Gunasekaran *et al.*, 2006). Peptone served as the best nitrogen source for PS11 lipase production. Similarly, *Bacillus* sp and *Pseudomonads* produced maximum lipase activity in presence of organic nitrogen sources like peptone and yeast extract (Sirisha *et al.*, 2012). Carbon source is one of the essential constituent of the microbial fermentation medium that affected the overall cellular growth and metabolism. Carbon source are reported as to inducelipases.In this study, glucose supported the highest production of lipase. The result 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. However, in some studies glucose has been found to cause repression of lipase production (Gupta *et al.,* 2004). Decrease of lipase activity in presence of other carbon source could be due to their catabolic repression in the medium (Kiran *et al.,* 2008). The expression of lipases was markedly influenced by lipid sources due to their substrate inducible nature. Rice bran oil served as the best substrate for PS11 lipase production. Earlier soybean and mustard oils are reported to support maximum lipase production in bacterial isolates (Kumar *et al.,* 2005). The high content of fatty acids specifically PUFA in rice bran oil (38% monounsaturated, 37% polyunsaturated and 25% saturated) might be associated with high level of lipase synthesis.

Microorganisms 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 has been found to 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 (Randa *et al.,* 2009). Highest lipase production by PS11 was influenced at the agitation rate of 150 rpm. At this speed, the aeration of the culture medium was increased, and this further led to a sufficient supply of dissolved oxygen in the media. The production of lipase was found to decrease when shaken at 180 rpm. Higher agitation rates could increase the oxygen pressure of the system but did not bring about the increase in production, probably because at a high agitation rate, the structure of enzyme would be altered. However, lowering the aeration rate could cause a reduction in the lipase yields. This indicates that a reduction in oxygen supply is an important limiting factor for lipase synthesis (Abusham *et al.,* 2009).

The present isolated strain PS11 exhibited optimum lipase production in 72 hours. The results of several other studies have shown the optimum incubation period for lipase production to be 12 to 24 h (Dharmsthiti *et al.,* 1998) which was in contrast with our results. However it was noted by Kumar *et al.,* (2012) 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.* The production of enzyme in the late phase was also reported by others (Joseph *et al.,* 2006; Wang *et al.,* 2009). Most

*Bacillus* species required more than 2 days for the maximum lipase activity to occur (Kiran *et al.*, 2008).

The PS11lipase was purified from the extracellular medium by ammonium sulfate precipitation, anion exchange chromatography and gel filtration method. 8.04 fold purification of PS11 lipase with a yield of 22.67% was obtained through the mentioned purification processes. Ji *et al.* (2010) reported 4.3 fold purification of an organic solvent tolerant lipase from *P. aeruginosa* LX1 with specific activity of 156 U/mg. SDS-PAGE analysis of purified lipase revealed the presence of a prominent protein band of approximately 27 kD, indicating that the enzyme was probably a single chain protein or a homomultimeric protein of 27 kD subunits. It is almost similar to that of lipase (27.5 kDa) from *S. rimosus* (Abrami *et al.*, 1999) but lower than that of lipase (50 kDa) from *Streptomyces cinnamomeus* (Sommer *et al.*, 1997).

pH acts as an important parameter affecting the activity ofenzymes due to its effect on the structure of proteins. Lipase purified from PS11 was active at an optimum pH 10 and the enzyme was alkalostable showing stability over a pH from 8 to 11. Lipase isolated from *S. rimosus* R6-554W (Abrami *et al.*, 1999) and *S. fradiae* var. k11 (Zhang *et al.*, 2008) also showed activity over a wide range of pH from 4 to10. In contrast, thelipase purified from *Geotrichum* sp. SYBC exhibited stability at acidic pH from 3 to 6 (Cai *et al.*, 2009).

To investigate the effect of temperature, lipase activity was determined at different temperature ranging from 10 to 80°C. PS11 lipase was thermostable as it retained its activity at 60°C. Gradual decrease in the enzyme activity was noted with gradual increase of temperature. It is a well-known fact that the protein conformation is changed or degraded at higher temperatures, and hence decrease in the lipase activity was noted at higher temperatures. An extracellular SCO7513 lipase from *S. coelicolor* A3 (Barnwal and Sharma, 2005) with optimal temperature at 55°C was previously reported by Cote and Shareck (2007). Lipase from *P. aeruginosa* LX1 also showed almost the same thermostable profile retaining 100% activity at 50°C. (Ji *et al.*,2010).

Effects of inhibitors on enzyme activity primarily give an insight into the nature of an enzyme, its cofactor requirements, and the nature of the active center (Sigma and Mooser, 1975). Interestingly, EDTA, a metal ion chelator didn't alter the

enzymatic activity of PS11 lipase indicating that functioning of enzyme did not require metal cofactor. Our results are in agreement with Saeed *et al.* (2005) and Lin and Ko (2005) who also showed that activity of lipases produced from *Bacillus*sp. and *Pseudomonas* sp. were not affected by EDTA. PS11 lipase activity was inhibited by $H_2O_2$ whereas β mercaptoethanol showed stimulating effect on the enzyme. Dheeman *et al.* (2011) and Soliman et al. (2007) also observed the similar effect of mercaptoethanol on lipase from *Amycolatopis mediterranei* and *Geobacillus thermoleovorans*, respectively.This increase in lipase activity in presence of β mercaptoethanol could be explained as inhibition of oxidation of sulfhydryl groups, in lipase (Gupta *et al.*, 2004).

PS11 lipase activity was increased by Tween-80, Tween-60and Tween-20 with maximum enhancement of 168% in presence of Triton-X-100. Similar report of lipase purified from *Streptomyces* sp. CS133 was activated byTriton X-100, Tween-80 and Tween-20 (Mander *et al.*, 2012). Catalytic activity of lipases is governed by interfacial activation, a property observed when the lipid substrate starts to form an emulsion thereby presenting an interface for the enzyme to act. Addition of a detergent decreases the surface tension between the organic and aqueous phase present in the reaction mixture and enhances the rate of emulsification. The non-ionic detergents weaken hydrophobic interaction within the protein resulting in disaggregation and thus stimulating the enzyme activity. The enhanced PS11lipase activity by tweens could be due to their effectas both inducers because of their chemical similarity to the natural substrates and surfactants, stimulating the enzyme release as suggested by Epsinosa *et al.* (1990). On the other hand, PS11 lipase activity was found to decrease in presence of anionic detergents (SDS, N-lauryl sarcosine) and zwitterionic CHAPS. The anionic detergents may inactivate protein by acting upon the disulphide linkages resulting in decline in activity.

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 greater is the polarity, the lower the log *P* value and the greater the toxicity of solvent. In this study, various low polarity, water immiscible solvents (high log *P*) such as *p*-xylene, *n*-hexane, toluene and benzene were tested. In addition, high polarity, water-miscible solvents, low log *P* (methanol, ethanol,

acetonitrile and acetic acid) were also investigated. The stability of the PS11 lipase in organic solvents did not follow the log *P* trends. It is well known that water acts as a lubricant that affords a high conformational flexibility 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. Lipase production was enhanced in presence of solvents having higher log *P*-value. The lipase was stable and restored almost 90% of its activity when p-xylene, benzene, toluene, hexane and methanol (25% v/v each) were added to the purified enzyme. The residual activity was 76% in presence of ethanol (Table-5). Acetic acid was more harmful than otherorganic solvents as PS11 lipase could restore only 18% of its activity. This may due to the acidity inactivating the 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).

Stability of PS11 lipase in presence of methanol led to explore the tolerance profile of the enzyme in varied concentration of methanol as it is one of the widely used media for biodiesel production. PS11 lipase retained 87% and 72% of its activity when grown in presence of 30% and 40% (v/v) methanol respectively 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 for 15 days at 30°C and 160 rpm (Fang *et al.*, 2006). In contrast, lipase isolated from *Photobacterium lipolyticum* for biodiesel production lost its stability even at very low concentration (10% v/v) of methanol (Yang *et al.*, 2009). High stability PS11 lipase even in presence of higher concentration of methanol (30- 50% v/v) makes it a promising tool in the field of biodiesel production.

Enzymatic transesterification of sunflower oil by PS11 lipase in presence of methanol was carried out to check the ability of lipase for biodiesel production. Commonly, the time period required by various microbial lipases to catalyzetrans-esterification reaction varies in the range 5–72 h (Bajaj *et al.*, 2010). However, the reaction time for PS11 lipase was significantly lesser than the time reported by

Kumari *et al.* (2009) and Yang *et al.* (2009) of 60 h and 72 h, respectively, for transesterification of jatropha and soybean oil in methanol using a lipase extracted from *Enterobacter aerogenes* and *P. fluorescens*, respectively