

## Full Length Research Paper

# Optimized production of lipase from *Bacillus subtilis* PCSIRNL-39

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Lipases catalyze the hydrolysis of long chain triglycerides. Microbial lipases are receiving much attention because of their industrial potential in the chemical, pharmaceutical, medical, cosmetic, biosurfactant synthesis, leather industries, mutation, agrochemicals and paper manufacturing industries. This article presents the isolation of maximum lipase producing bacteria and the optimization of different conditions for the maximum production of lipase. *Bacillus subtilis* PCSIRNL-39 shows maximum production of lipase at 45°C with pH 7 using nitrogen source peptone and carbon source sucrose. *B. subtilis* PCSIRNL-39 showed the best production at 5% inoculum size, while Ca<sup>2+</sup> and Mg<sup>2+</sup> were found best stimulator for enzyme production during the study. Tween 20 and 80 enhanced better lipase production than other surfactant. The kinetic parameters of V<sub>max</sub> and K<sub>m</sub> for the lipase were measured to be 101 μM/min.mL and 7.6 mg, respectively.

**Key words:** *Bacillus subtilis*, microbial lipase, production, optimization.

## INTRODUCTION

Lipases are enzymes that catalyze lipids into fats and glycerols. This reaction acts on oil and water interface (Reis et al., 2008). Lipases are involved in certain reactions like aminolysis in organic solvents, transesterification and esterification. Lipases can be obtained from animals, plants and microbes.

The lipase can be produced from microorganisms through the process of fermentation. According to Vakhlu and Kour (2006), lipases are also extracted from plants and animals. Lipases achieved from microbial sources are more stable than that extracted from plants and animals. Bacteria like *Pseudomonas* spp.,

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*Staphylococcus* spp., *Chromobacterium* spp., *Alcaligeannes* spp., *Achromobacter* spp. and *Bacillus* are commercially used for lipase production.

Bacterial lipases can be obtained by submerged fermentation (SmF) as well as solid-state fermentation (SSF). Due to easy production along with inexpensive techniques of fermentation and low energy consumption, the lipases obtained from microbes (fungi, yeast and bacteria) are given preference for industrial application. However, SSF is the most appropriate process due to its various benefits and bioconversion parameters. Lipase production by SSF using olive oil cake and sugar cane bagasse are very cost effective (Cordova et al., 1998) and in this case, oil cakes show best production (Ramachandran et al., 2007).

The most useful lipase producer genus used in industry is *Bacillus* (Jaeger et al., 1994). They are gram positive endospore formers which produce large variety of enzymes, and are considered as strong extra cellular lipase producer. They demonstrate the ability to grow in various ranges of temperature, pH and salt concentration. Due to its stability, the lipases have immense industrial importance.

Recently microbial lipases are used as biosensor. The principle of this biosensor is using lipases to produce glycerol from triacylglycerol, measuring the release of glycerol and quantifying the non-esterified fatty acids. This principle is used to diagnose cardiovascular disorders.

Lipases were produced from locally isolated *Bacillus* species, by using different agro-industrial wastes through solid state fermentation (Mazhar et al., 2016b). This study was designed to investigate physico-chemical parameters for optimized lipase production in cost free medium.

## MATERIAL AND METHODS

### Microorganism and media

Microorganism culture PCSIRNL-39, used in the study was obtained from Molecular Biology Laboratory at FBRC, LLC Lahore, Pakistan. PCSIRNL-39 was previously isolated from soil and biochemically characterized as *Bacillus* spp. Production medium used for submerged fermentation, had glucose 5 g/l, peptone 5 g/l, yeast extract 5 g/l, olive oil 5%, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5 g/l and NaCl 3 gm/l (Mazhar et al., 2016 a,b).

### Genotypic characterization

#### Preparation of genomic DNA

For the purpose of preparing genomic DNA, Ausubel et al. (1994) method was slightly modified. Single colony from pure culture of PCSIRNL-39 was inoculated into LB broth and grown for 24 h at 37°C in water bath shaker.

Overnight grown culture was centrifuged to pellet the cells for 5 min at 8000 rpm. After discarding, the supernatant pellet was resuspended in 567 µl of 1xTE buffer. The addition of 3 µl of 20 mg/ml proteinase K and 30 µl of 10% SDS was made afterwards.

The sample was incubated at 37°C for 1 h.

Thorough mixing in Eppendorf tubes, 100 µl of CTAB/NaCl solution (0.7 M NaCl, 10% N-acetyl-N, N, N-trimethyl ammonium bromide) was added. Incubation was performed at 65°C for 10 min after mixing. One equal volume of chloroform/isoamyl (24:1) was used to perform the chloroform extraction.

Centrifugation of samples started at 10,000 rpm for 5 min after adding equal volume of chloroform/isoamyl. Chloroform extraction was repeated after transferring the aqueous phase into a new Eppendorf tube. 0.6 volume of isopropanol was added in order to obtain the nucleic acid precipitates. Genomic DNA was pelleted down by centrifugation at 10,000 rpm for 10 min. Pellet was washed with 500 µl 70% ethanol, dried at 37°C for 10 min and dissolved in 200 µl of TE buffer.

### Amplification of 16S rDNA region

Amplification of 16S rDNA region was performed through polymerase chain reaction (PCR). PCR mix (24 µl) was prepared by adding MgCl<sub>2</sub> (25 mM) 1.5 µl, forward and reverse primer (100 µM) each 0.5 µl, sterile deionized water 14.7 µl, dNTP mix (10 mM each) 4 µl, Taq buffer (10X) 2.5 µl, Taq DNA Polymerase (5 U/µl) 0.3 µl in 0.2 ml PCR tube and 1 µl of genomic DNA was transferred.

PCR sample was centrifuged for 2 to 3 s after mixing gently. All steps were performed on ice and the tubes were placed into Mini Cyclor PCR System. The following amplification procedure was adopted.

Step 1: Initial Denaturation: 94°C for 5 min

Step 2: Denaturation: 94°C for 1 min

Step 3: Annealing: 52°C for 1 min

Step 4: Elongation: 72°C for 1 min

Step 5: Extension: 72°C for 10 min final

The following DNA primers were used in this experiment:

Forward primer: 5'-AAACTYAAAKGAATTGACGG-3'

Reverse primer: 5'-ACGGGCGGTGTGTRC-3'

Y = C/T; K = G/T; R = A/G

The forward primer was complementary to the upstream of 16S rDNA and the reverse was complementary to the upstream sequences of 23S rRNA gene sequences.

### Electrophoresis of amplified 16S rDNA fragments

Agarose gel (0.8%) was used to check the amplification products. 0.8 g of agarose was boiled in 1x TAE buffer (100 ml). This solution was cooled to 40°C and 5 µl of ethidium bromide solution (10 mg/ml) was added. The combs were placed in gel casting stand after pouring the agarose solution to it.

When the gel was set after 20 min, combs were removed and the casting tray was then placed into tank. 1x TAE was added to gel until the surface of the gel was raised by 2 to 3 mm. 1 µl of 6x gel loading buffer was missed with 5 µl of the PCR product. Starting from the second well, the samples were loaded into the agarose gel wells, 3 µl of DNA molecular weight marker was loaded in first well afterward.

Electrophoresis was performed at 100 V for 2.5 h and the PCR products were observed on UV illuminator. Gene clean kit was used to purify the PCR product. Dideoxy chain termination method was used to sequence both strands of PCR product. Blast program was used to make comparison between 16 rRNA gene sequence with NCBI/EZtaxon/Ribosomal Database Project (RPD)/ EMBL nucleotide sequence databases.

### Parameters for optimization of lipase production

Different parameters were used to optimize the lipase production such as pH of the medium, incubation time, substrate selection and effect of moisture content of the substrate. Lipase activity was measured according to the procedure of Mazhar et al. (2016a).

#### Effect of pH

Different pH level of the production medium was set ranging from 4.0 to 9.0, using 0.1 N NaOH and 0.1 N HCl. The medium was autoclaved at 15 lbs pressure and 121°C for 20 min.

#### Effect of temperature

Erlenmeyer flasks (250 ml) were filled with 50 ml of selected production medium and sterilized. The flasks were incubated at 20, 25, 30, 35, 40, 45, 50, 55 and 60°C after inoculation for 72 h.

#### Optimization of inoculum size

Inoculum size was optimized by inoculating the production broth, using 24 h seed culture of varying size 4, 5, 6, 7, 8, 9, and 10% (v/v). Estimation of growth in batch culture and lipase activity was made.

#### Different carbon source

For determination of the effect of carbon sources (5 g/l) on growth of lipase *Bacillus* sp., sucrose, glucose, maltose, starch, fructose, lactose and mannitol were used as basal carbon sources. Basal medium was used for assaying and determination of lipase activity.

#### Different nitrogen sources

Different organic nitrogen sources were also used for optimization. Ammonium sulphate, tryptone, yeast extracts, beef extracts soya bean meal and different combination of these sources were used as nitrogen sources.

#### Incubation period

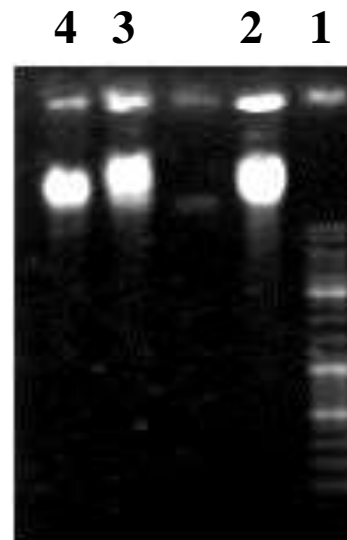
*Bacillus* sp. was grown in agro industrial waste, containing salt media at 45°C in an incubator. It was assayed for lipase activity after various incubation times, that is, from 24 to 120 h.

#### Effect of surfactant on lipase production

The effect of each of the selected surfactant Sodium dodecyl sulphate, viz., Tween-20, Triton X-100 and Tween-80 was separately evaluated, by incorporating (1%; v/v) the surfactant to the production broth which was inoculated with 5% (v/v) inoculums.

#### Effect of metal ions

Different metal ions such as 1 mM of CaCl<sub>2</sub>, HCl, MgSO<sub>4</sub>, NaCl, MnSO<sub>4</sub>, ZnSO<sub>4</sub>, Fe<sub>2</sub>SO<sub>4</sub>, K<sub>2</sub>PO<sub>4</sub> and CuSO<sub>4</sub> were supplement separately, in the medium. Optimal conditions were used for incubation, and determination of lipase production was done.



**Figure 1.** Isolated genomic DNA of bacterial strains: Line 1: DNA marker Ferment as Cat # SM0331; Line 3: DNA of PCSIRNL-39.

#### Cumulative effect of optimized components

The optimized broth containing 2.5% mustard oil cake and 2.5 (%; w/v) bagasse, in minimal media was calibrated to a final pH of 7.0, to determine cumulative effect of all the selected components on lipase production by *Bacillus subtilis* after 72 h at 45°C.

## RESULTS

### Genotypic characterization

The genomic DNA of PCSIRNL-39 was isolated as shown in Figure 1. Amplification of 16S rRNA region mostly yielded one distinct DNA fragment, of approximately 500 bp in length (Figure 2).

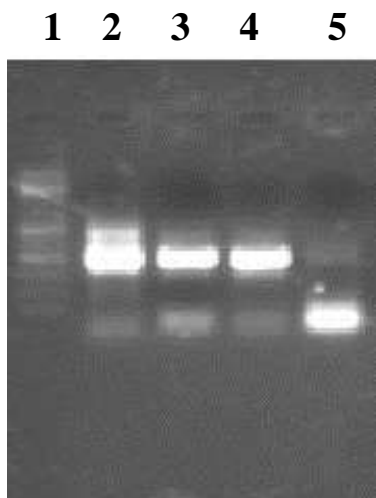
The amplification product was cleaned with the help of gene clean kit. The size and concentration of purified gene product was determined on agarose gel (Figure 3). Sequencing of the PCR product was preceded by the use of CAMB (Centre for Applied Molecular Biology) sequencing facility.

The sequences obtained were compared with the GenBank database, using the BLASTN tool. PCSIRNL-39 showed 99% homology with published sequences of *B. subtilis*. Sequence for the distinct rDNA fragment was submitted to Gen Bank and accession number, assigned as KT374117.

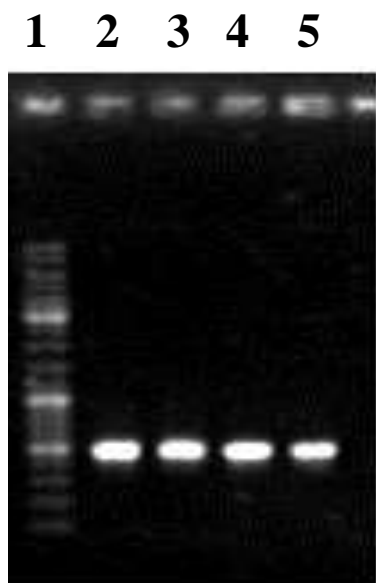
### Optimization of parameters

#### Effect of temperature

The effect of the incubation temperature on the



**Figure 2.** Amplification products of 16S rDNA region of isolated strains: Line 1: DNA Marker Fermentas Cat # SM0331; Line 2, 3, 4: Amplification product 500 bp; Line 5: Control.



**Figure 3.** Gene clean product after amplification Line 1: DNA Marker; Fermentas Cat # SM0331; Line 2: PCSIRNL-39.

production of extracellular lipases by *B. subtilis* was investigated. Lipase activity at different incubation temperature such as 25, 30, 35, 40, 45, 50 and 55°C were observed. The maximum lipase production for PCSIRNL-39 was observed at 45°C, 47.50 U/ml. A sharp decrease in lipase production was, however, observed at

50 to 55°C, as shown in Figure 4.

#### **Effect of pH**

The effect of pH on the production of extracellular lipase by *B. subtilis* was observed. Lipase activity at different pH such as 4, 5, 6, 7, 8 and 9 were determined. Results inferred that, neutral pH favored the production of extracellular lipase (Figure 5).

#### **Effect of inoculum size**

Figure 6 showed that at the inoculum size of 5% the lipase activity was 80.63 U/ml; further increase will cause lipase production to decrease at a high speed.

#### **Effect of carbon source**

Starch, glucose, fructose, sucrose, lactose, maltose and manitol were used as carbon source. PCSIRNL-39 showed best results with sucrose and fructose 40.67 and 31.70 U/ml, respectively (Figure 7).

#### **Effect of nitrogen source**

The bacterial strain PCSIRNL-39 expressed best lipase production with peptone and yeast extract 50.70 and 45.87 U/ml, respectively (Figure 8). In the presence of inorganic nitrogen [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>Cl, and NH<sub>4</sub>NO<sub>3</sub>], the best lipase production was observed with NH<sub>4</sub>NO<sub>3</sub> (45 U/ml). The bacterial strain PCSIRNL-39 showed the least lipase activity, however, with trypton (16.37 U/ml) among all nitrogen sources investigated.

#### **Effect of incubation time**

After inoculation, the fermentation media was incubated for different time periods like 24, 48, 72, 96 and 120 h. Experiment revealed that best incubation time for lipase production was 72 h from bacterial strain PCSIRNL 39 used in this experiment (Figure 9).

#### **Effect of metal ions**

As reported from this study on microbial lipases, concentration as low as 1 mM of some metal ions, can affect the production of lipase by *Bacillus* sp. Effect of various metals (Mg<sup>2+</sup>, Na<sup>+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup>, Ag<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>, Ca<sup>2+</sup> and K<sup>+</sup>) were determined.

In Figure 10, PCSIRNL-39 showed maximum lipase production with Mg<sup>2+</sup> and Ca<sup>2+</sup> while Ag<sup>2+</sup> and Cu<sup>2+</sup> acted as inhibitors for lipase production.

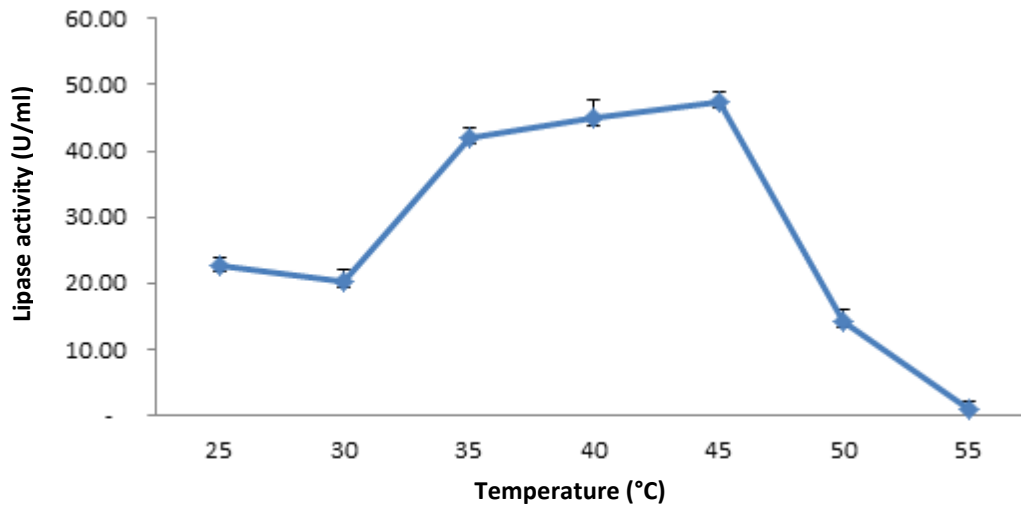


Figure 4. Optimization of temperature for lipase production.

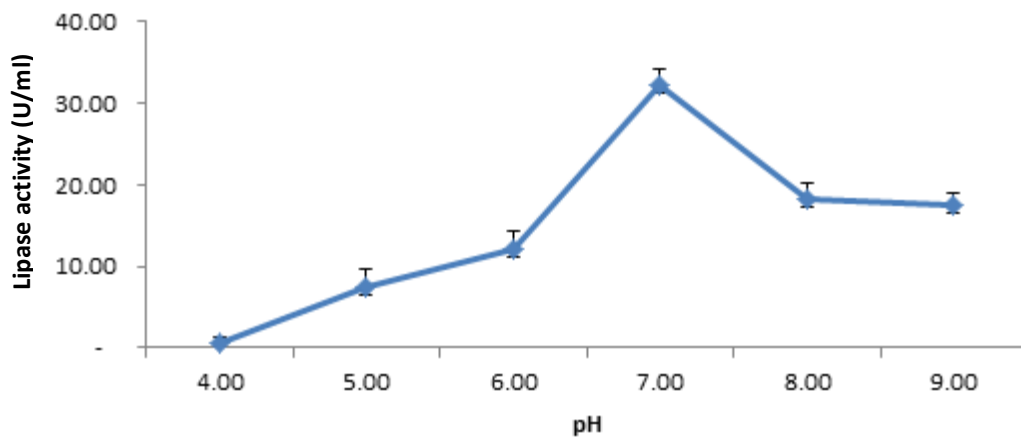


Figure 5. Optimization of pH for lipase production.

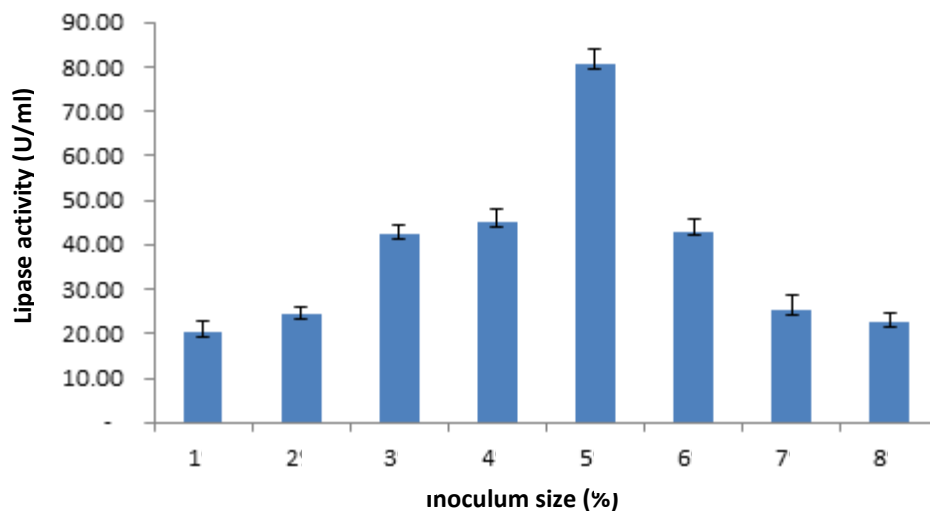


Figure 6. Optimization of inoculum size for lipase production.

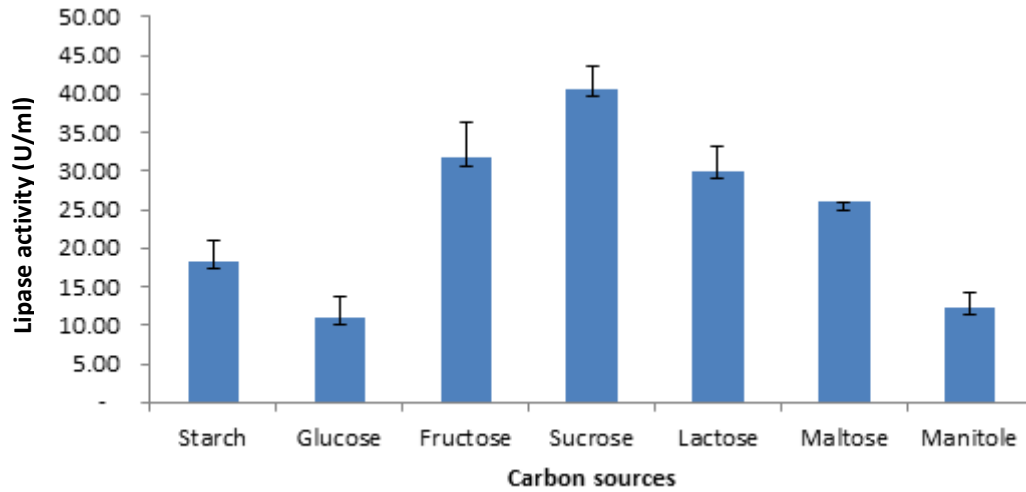


Figure 7. Effect of different carbon sources on lipase production.

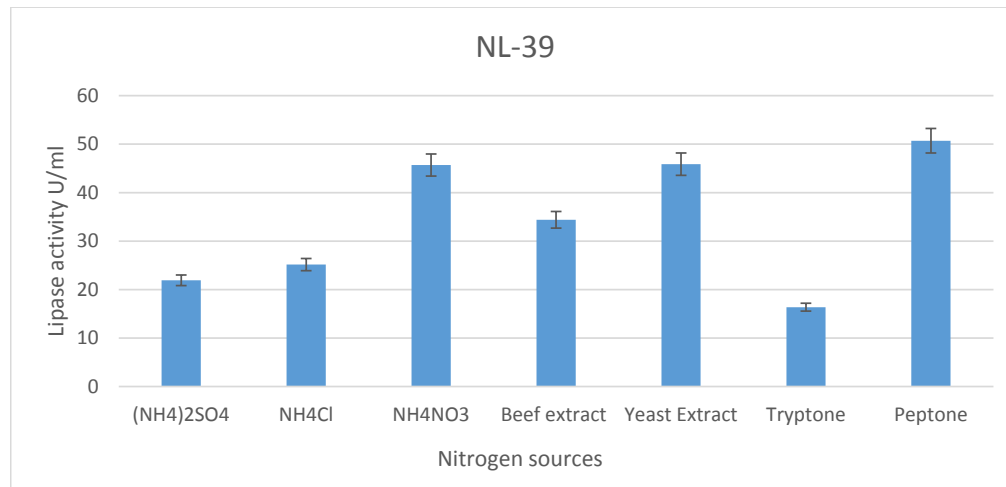


Figure 8. Effect of various nitrogen sources on lipase production.

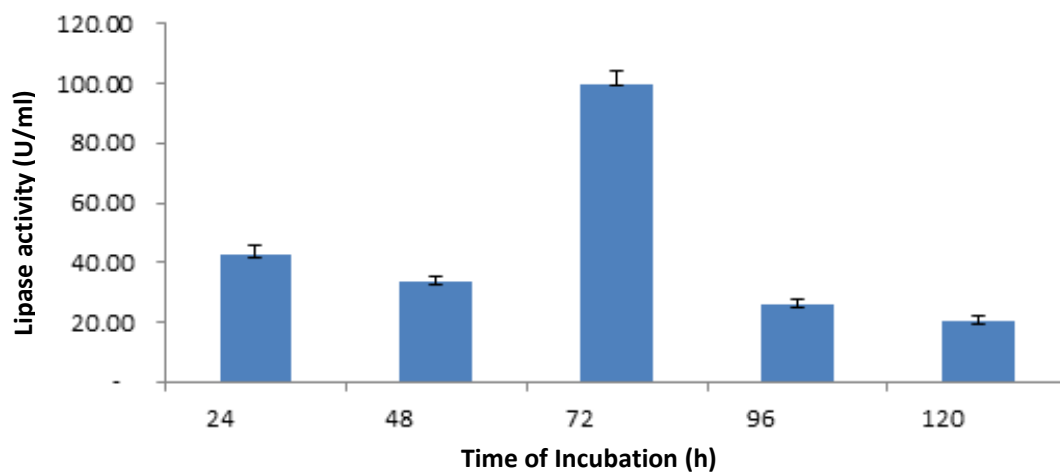


Figure 9. Effect of incubation time for lipase production.

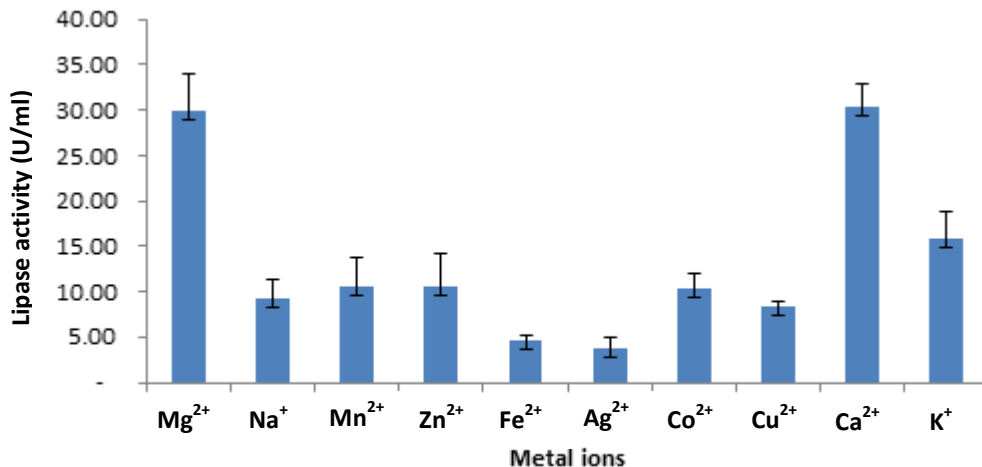


Figure 10. Optimization of metal ions for lipase production.

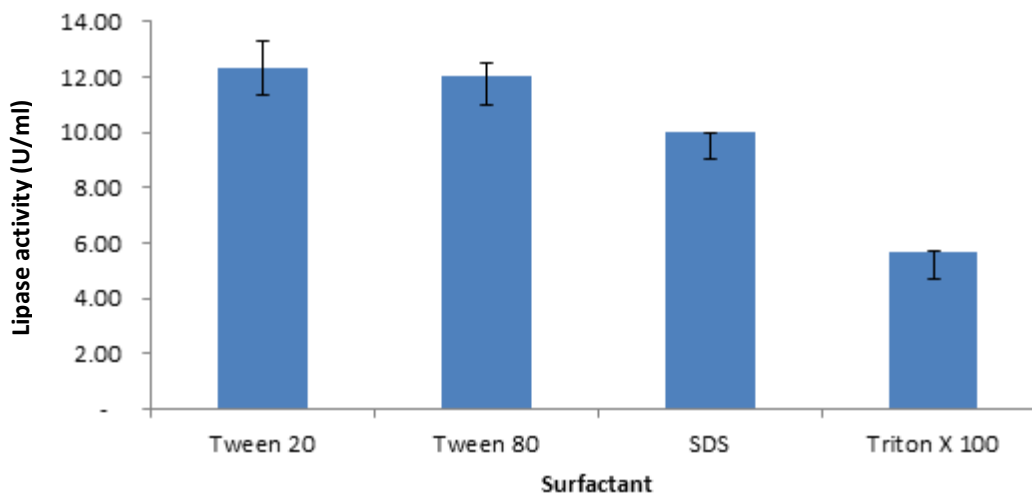


Figure 11. Optimization of detergent for lipase production.

### Effect of detergents

Detergents like SDS, Tween 80, Tween 20 and triton 100 inhibit lipase production as illustrated in Figure 11.

### Enzymatic activity

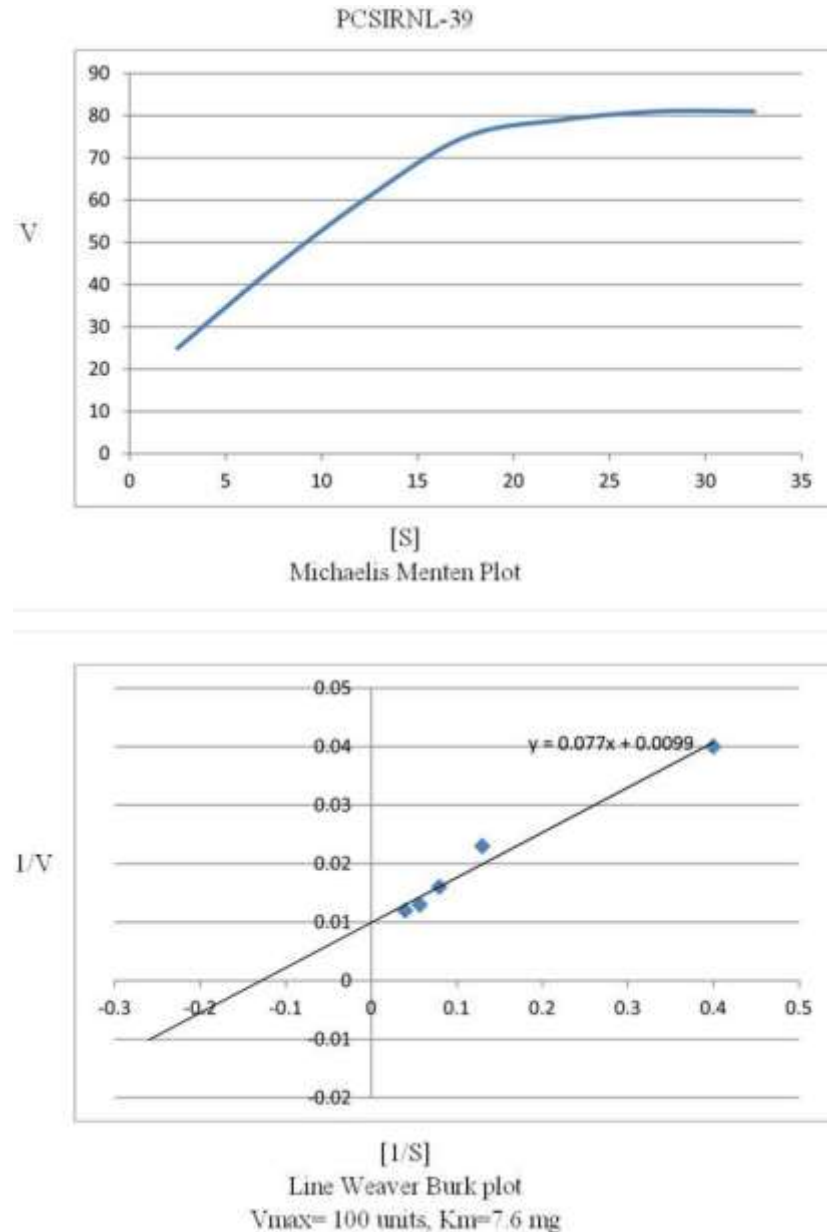
Lipase activity assay was carried out with help of Yamada et al. (1962) method. Lipase enzyme produced from PCSIR NL39 exhibited  $V_{max}$  and  $K_m$  of 101  $\mu\text{mol}/\text{min}/\text{mL}$  and 7.6 mg, respectively (Figure 12).

## DISCUSSION

Lipase constitutes a major group of biocatalysts, which

have immense biotechnological applications. During the study, a lipase producing isolate PCSIRNL-39 was screened and identified as *B. subtilis* using 16S rDNA gene sequence analysis. 16S rDNA sequence obtained was aligned with Genbank sequences, using the BlastN program. This revealed a close relatedness to *B. subtilis* with 99% similarity, with an existing database. The identification of lipase producing *Bacillus* sp. by biochemical and 16S rDNA sequence analysis was done by different scientists (Akanbi et al., 2010; Kanimozhi and Perinbam, 2010; Prasad and Manjunath, 2012).

The bacterial ability to produce lipases is dependent on medium composition and various environmental factors such as temperature, pH, incubation time, etc. that greatly influenced the lipase synthesis and its activity, optimization of medium composition and other parameters for lipase production, which was performed in



**Figure 12.**  $V_{max}$  and  $K_m$  calculations of lipase enzyme.

this study.

The study revealed that the best carbon sources for lipase production were fructose and sucrose. In addition to bagasse, during this study, whereas mannitol and glucose showed relatively low lipase production, the results are similar to Alabras et al. (2017). On the other hand, the type of nitrogen source in the medium also influences the lipase production (Ghosh et al., 1996). De-Almeida et al. (2016) has earlier shown lipase production by various substrates individually, and after mixing with oils like olive oil. In the present experiment, the best nitrogen sources were peptone and yeast extract, similar to the results shown by Hasan et al. (2001). Basically,

organic nitrogen such as peptone and yeast extract, have preferably been used as nitrogen source for lipase production by various *Bacillus* spp. (viz. *Bacillus* strain A30-1, *B. alcalophilus*, *B. Licheniformis* strain H1), different pseudomonas (viz. *Pseudomonas* sp., *P. fragi*, *P. fluorescens* BW 96CC) and *Staphylococcus haemolyticus* (Oh et al., 1999; Ghanem et al., 2000; Lanser et al., 2002; Sharma et al., 2002), while tryptone and yeast extract have been used for *S. haemolyticus* L62 (Oh et al., 1999).

In case of the present research,  $NH_4NO_3$  as inorganic nitrogen source also showed maximum activity of 45 U/ml. Ammonium chloride and ammonium sulphate had



22.33 and 25.17 U/ml enzymatic activity, respectively. Songs et al. (2001) reported that *Candida rugosa* produced optimum lipase when ammonium nitrate is used as nitrogen source. According to the work reported by Markossian et al. (2000), *B. thermoleovorans* showed maximum activity when yeast extract is used as nitrogen source. If the yeast extract is replaced by ammonium sulphate, no activity will be observed (Pimentel et al., 1994). Vmax and Km of PCSIRNL-39 lipase were measured to be 101  $\mu\text{mol}/\text{min}/\text{mL}$  and 7.61 mg, whereas Vasiee et al. (2016) reported Vmax and Km value of 0.367  $\mu\text{M}/\text{min}/\text{mL}$  and 5.3 mM, respectively.

Incubation periods ranging from a particular hour to several hours, was found to be the best for maximum lipase production, by bacteria. In this study, all *Bacillus* sp. showed maximum lipase production at 72 h and similar results were examined by Sarkar et al. (1998). In contrast, 12 h was the optimum incubation period for *A. calcoaceticus* and *Bacillus* sp. (Mahler et al., 2000) and 16 h the optimum incubation period for *B. thermocatenuatus* (Dannert et al., 1997). However, the maximum lipase activity was shown to be 72 and 96 h in the case of *P. fragi* and *P. fluorescens*, respectively (Pabai et al., 1996; Dong et al., 1999).

Optimum temperature for lipase production was observed with respect to optimum temperature for growth. In the case of *B. subtilis* PCSIRNL-39 investigated in this study, the optimum temperature was 45°C which is in agreement with the result of Alabras et al. (2017).

At times, the production of lipase is inhibited by calcium. In our study,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  enhanced lipase production in contrast with Patkar and Bjorkling (1994) who showed the inhibitory effects of  $\text{Zn}^{2+}$  and  $\text{Mg}^{2+}$ .

## Conclusion

The above study describes the optimized culture conditions such as temperature, pH, incubation time, metal ions, carbon and nitrogen sources for lipase production from newly isolated *B. subtilis* strain PCSIRNL-39, especially in an inexpensive medium. Lipases, produced from our isolate can be used as biode detergent and for bioremediation of waste water.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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