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Research Article

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Isolation, Purification, and Characterization of Solvent-Tolerant Lipase from *Geobacillus stearothermophilus* PS11

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Abstract

An organic solvent-stable lipase secreted by *Geobacillus stearothermophilus* PS11, a solvent-tolerant strain, was purified and characterised. Purification raised the specific activity 8.04-fold, with 22.6% of the activity recovered. The purified enzyme had a molecular weight of roughly 27.5 kDa. It held its activity well in the presence of organic solvents such as toluene, benzene, propanol and methanol, retaining 745 EU/ml even after 72 h of incubation. Activity peaked at 764 EU/ml at 50 °C and pH 10.0, and the enzyme remained stable across a broad temperature range of 10 °C to 60 °C. Given this tolerance to solvents, heat and alkaline conditions, the lipase shows promise as a useful biocatalyst for the biodiesel industry.

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Introduction

Diesel fuel underpins the industrial economy of developing countries. As industrialisation has spread worldwide, the world now faces two linked problems: the depletion of fossil fuels and the degradation of the environment (Akhter 2022). Researchers are therefore looking for renewable, environmentally friendly substitutes for petroleum diesel. One such substitute is biodiesel, which is made from vegetable oils or animal fats. Compared with conventional diesel, it emits less smoke and fewer particulates, has a higher cetane number, and releases less carbon monoxide and fewer unburned hydrocarbons. It is also biodegradable and nontoxic, and it improves the lubricity of low-sulfur diesel. Given these benefits, biodiesel is attracting growing interest as an alternative fuel.

Lipases are the key enzymes in biodiesel production (Cersino 2023). Methanol and ethanol, however, inactivate many lipases, and only those that tolerate organic solvents, especially these two alcohols, can markedly improve biodiesel yields (Cersino 2023). Few bacteria are known to produce solvent-tolerant lipases, and most of the strains reported so far belong to the genus *Pseudomonas* and were isolated from soil or marine samples [5]. We previously isolated a solvent-tolerant strain, *Bacillus thermophilus* PS11 (now classified as *Geobacillus stearothermophilus*), which grows in the presence of a wide range of solvents. Here we show that this strain produces a lipase that remains stable in solvents such as toluene, n-octanol, propanol, methanol and benzene. This finding could open simpler, new routes for synthetic processes and, in turn, for alternative biodiesel production.

Materials and Methods

Isolation of lipase-producing, solvent-tolerant bacterial strains

This study used the solvent-tolerant strain *Geobacillus stearothermophilus* PS11 (accession no. KC311354), which had been isolated from soil. The strain was streaked onto nutrient agar containing 1% tributyrin and incubated at 37 °C for 48 h. Lipolytic activity was confirmed by a clear zone of hydrolysis around the colonies.

Measurement of bacterial growth

An overnight culture of strain PS11 was inoculated into nutrient broth overlaid with 5% tributyrin and incubated at 37 °C with shaking at 140 rpm. Growth and dry cell mass were determined following the method of Foronda 2025.

Production medium and enzyme preparation

A 1% (v/v) inoculum from an overnight nutrient broth seed culture was transferred into the basal production medium (LPM-1), which contained (g l^{-1}): peptone 5, beef extract 3, sodium chloride 2, Tween 80 5 and olive oil 10, with the pH adjusted to 8. The cultures were incubated at 37 °C with shaking at 140 rpm for 96 h. Every 24 h, the cells were removed by centrifugation, and the supernatant was desalted and used to measure lipase activity.

Lipase assay and protein determination

Lipase activity was assayed by following the hydrolysis of p-nitrophenyl palmitate (pNPP). The 2.5 ml reaction mixture contained 2.4 ml of 50 mM Tris-HCl (pH 9) with pNPP and 0.1 ml of concentrated enzyme preparation. After incubation at 60 °C for 10 min, 200 µl of 1 M calcium chloride was added to precipitate the free fatty acids. The p-nitrophenol (pNP) released from pNPP was quantified from the increase in absorbance at 410 nm. One unit of lipase activity was defined as the amount of enzyme that liberated 1 µmol of p-nitrophenol per minute under these assay conditions.

Optimization of production media

Process parameters and media composition are among the most important levers for boosting lipase production to meet industrial demand. Optimization was therefore carried out by varying several growth parameters. For each experiment, the cells were grown for 96 h at 37 °C with shaking. They were then removed by centrifugation, and the supernatants were used to measure lipase activity. The parameters tested for maximal lipase yield were incubation time (24, 48, 72 and 96 h) and agitation speed (100-200 rpm). To assess the effect of the oil substrate, the medium was supplemented with 1% (v/v) rice bran, sunflower, soybean, olive or mustard oil. The effects of carbon sources (1% w/v; glucose, sucrose, lactose and maltose) and nitrogen sources (1% w/v; soybean meal, sodium nitrate, peptone, beef extract and yeast extract) on lipase production were also examined. At every step,

lipase activity was measured under standard assay conditions to determine the optimal yield.

Purification and molecular weight determination of the lipase

The isolate was grown in LPM-1 at 37 °C for 72 h. The cells were removed by centrifugation at 5000 rpm for 10 min at 4 °C in a refrigerated centrifuge. Solid ammonium sulphate (0-40% saturation) was added to the supernatant, and the mixture was held at 4 °C for 24 h to precipitate the enzyme. The precipitate was collected by centrifugation at 10,000 rpm for 40 min and redissolved in 50 mM Tris-HCl buffer (pH 9.0). It was then desalted by dialysis against the same buffer, using a cellulose dialysis bag with a 3500 Da cut-off. The desalted preparation was loaded onto a DEAE-Sephacel column, and the active fractions were pooled, lyophilised and applied to a Sephadex G-75 column. The active fractions from this step were used to determine the molecular weight. Protein concentration (mg ml^{-1}) was measured by the method of Lowry et al. (1951).

The molecular weight of the lipase was estimated by electrophoresis on a 15% polyacrylamide gel in a Bio-Rad mini-gel system. A low-molecular-weight protein marker (20-97 kDa) served as the standard.

Effect of organic solvents on lipase stability

To test stability in organic solvents, the purified lipase was suitably diluted in 50 mM Tris-HCl (pH 9.0) and mixed with each solvent to a final concentration of 25% (v/v). The mixtures were incubated at 4 °C for 48 h in a shaking incubator at 180 rpm, and the residual activity was then measured by the pNPP method. The solvents tested were hexane, xylene, toluene, benzene, dichloromethane, diethyl ether, ethyl acetate, isopropanol, acetone, acetonitrile, methanol and dimethyl sulfoxide (DMSO). The activity of the enzyme without any solvent was taken as 100%.

Effect of pH on activity and stability of PS11 lipase

The optimum pH of the purified lipase was determined by assaying activity in different buffers over a pH range of 4.0-12.0 under standard assay conditions. For pH stability, the enzyme was preincubated in the same buffers (pH 4.0-12.0) at 4 °C for 2 h, and the remaining activity was then assayed. All buffers were used at 50 mM: acetate-HCl (pH 4.0-5.0), sodium hydrogen phosphate-NaOH (pH 6.0-7.0), Tris-HCl (pH 7.0-9.0), disodium hydrogen orthophosphate-NaOH (pH 9.0-11.0) and glycine-NaOH (pH 10.0-12.0).

Effect of temperature on activity and stability of PS11 lipase

The effect of temperature on the purified lipase was determined by incubating the reaction mixture at temperatures from 10 °C to 80 °C under standard assay conditions. To assess thermal stability, the enzyme was preincubated at temperatures from 10 °C to 80 °C for 2 h, and the residual lipase activity was then measured.

Results and Discussion

Bacterial growth and lipase production

The time course of cell growth and lipase production by PS11 in the production medium is shown in Figure 1. The culture showed only a short lag phase. The exponential phase lasted up to 18 h, and the stationary phase continued until 90 h. Biomass declined slightly after 96 h, probably because nutrients had run out. This pattern is similar to that reported by Nurhasanah (2022).

Lipase was first detected in the early stationary phase (24 h) and reached its maximum of 760 EU/ml in the late stationary phase (72 h). This suggests that the extracellular lipase behaves as a secondary metabolite. Activity then fell gradually after 72 h, so 72 h was kept as the incubation period in all later experiments.

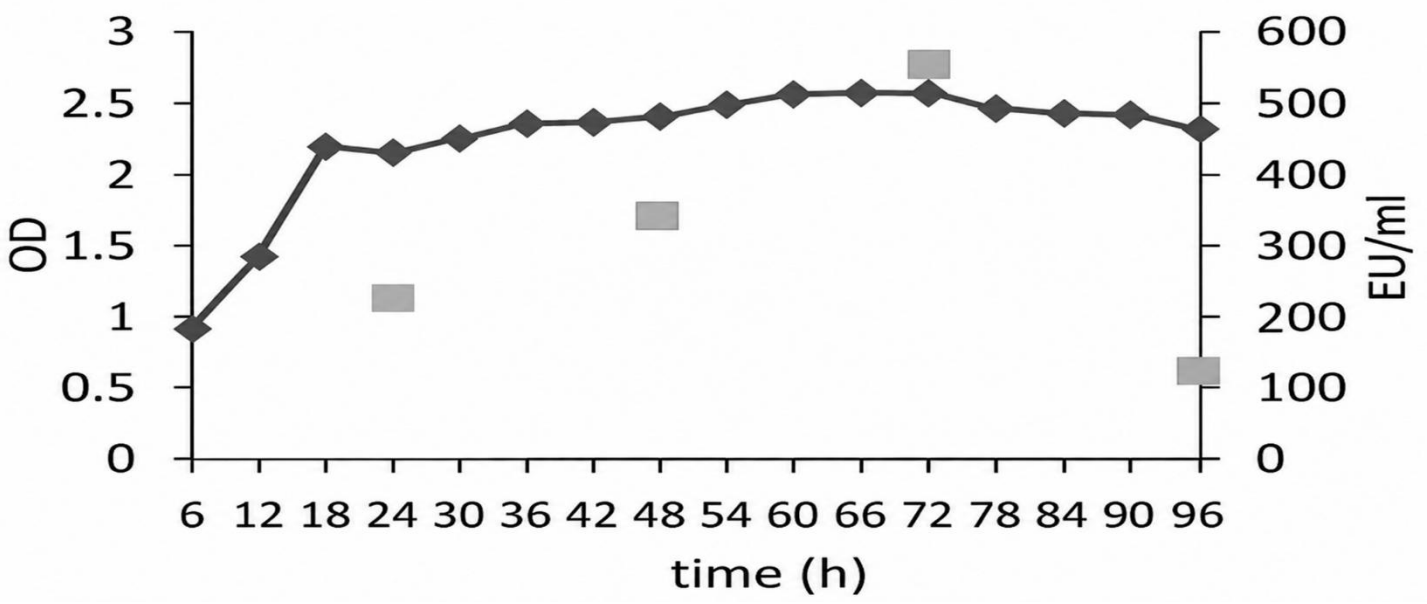


Figure 1 Determination of bacterial growth (◆) and enzyme (▬) production

Optimization of lipase production media

Several nitrogen sources were tested for their effect on lipase synthesis. Peptone gave the highest activity (310 EU/ml; Figure 2a), in line with the report by Mustafa (2023) that peptone enhances lipase production.

Because microorganisms differ in the carbon source they need for maximal lipase output, different carbon sources were also compared. Glucose was the best (367.5 EU/ml; Figure 2b). Glucose is known to repress lipase production in some organisms, but in PS11 it acted as an inducer. This agrees with Shakir et al. (2026) and Tan et al. (2023), who also obtained maximum lipase production in glucose-containing medium.

Lipidic substrates appear to be essential for a high lipase yield. Of the oils tested, rice bran oil supported the best production by PS11 (370 EU/ml; Figure 2c). Its fatty acid profile, which is rich in unsaturated fatty acids (about 38% monounsaturated, 37% polyunsaturated and 25% saturated), may favour high lipase synthesis.

Lipase was produced throughout the 96 h incubation, with the highest level at 72 h (765 EU/ml) during the stationary phase. Xia et al. (2024) reported optimum production at much shorter times, between 12 and 24 h, which differs from our findings. By contrast, Yao et al. (2021) found that *Bacillus cereus* strains reached high biomass at 48 h and peak lipase activity at 72 h, and most *Bacillus* species need more than two days for maximal lipase activity (Zafar et al. 2024).

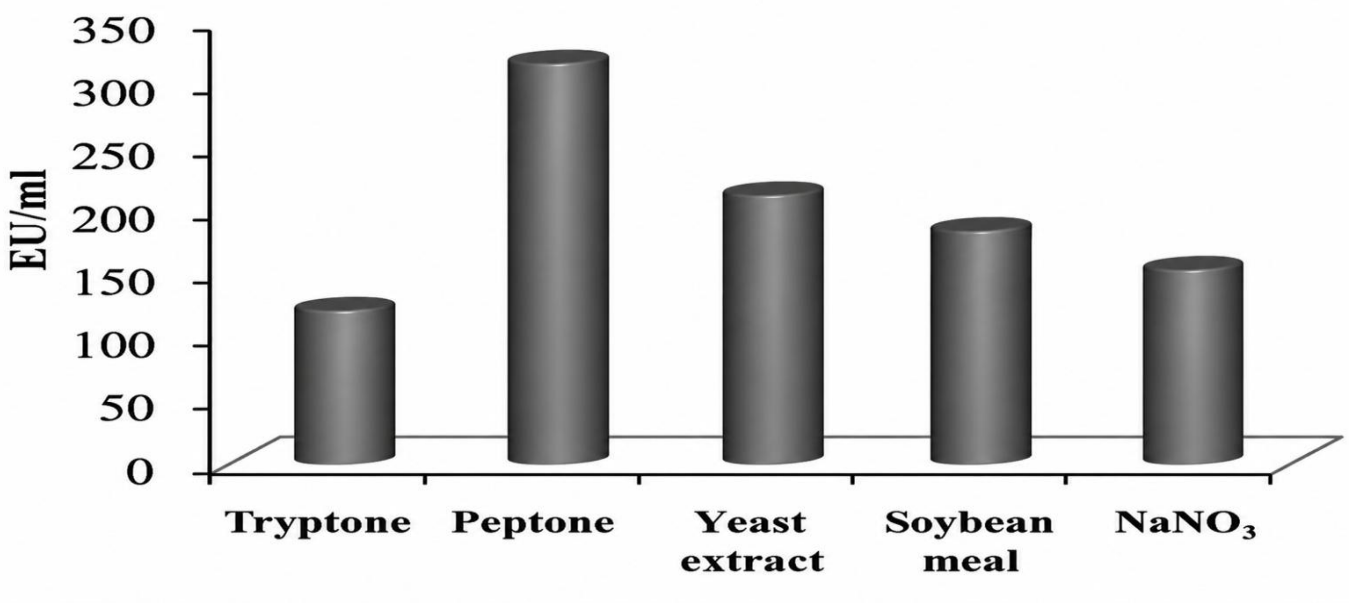


Figure 2(a)

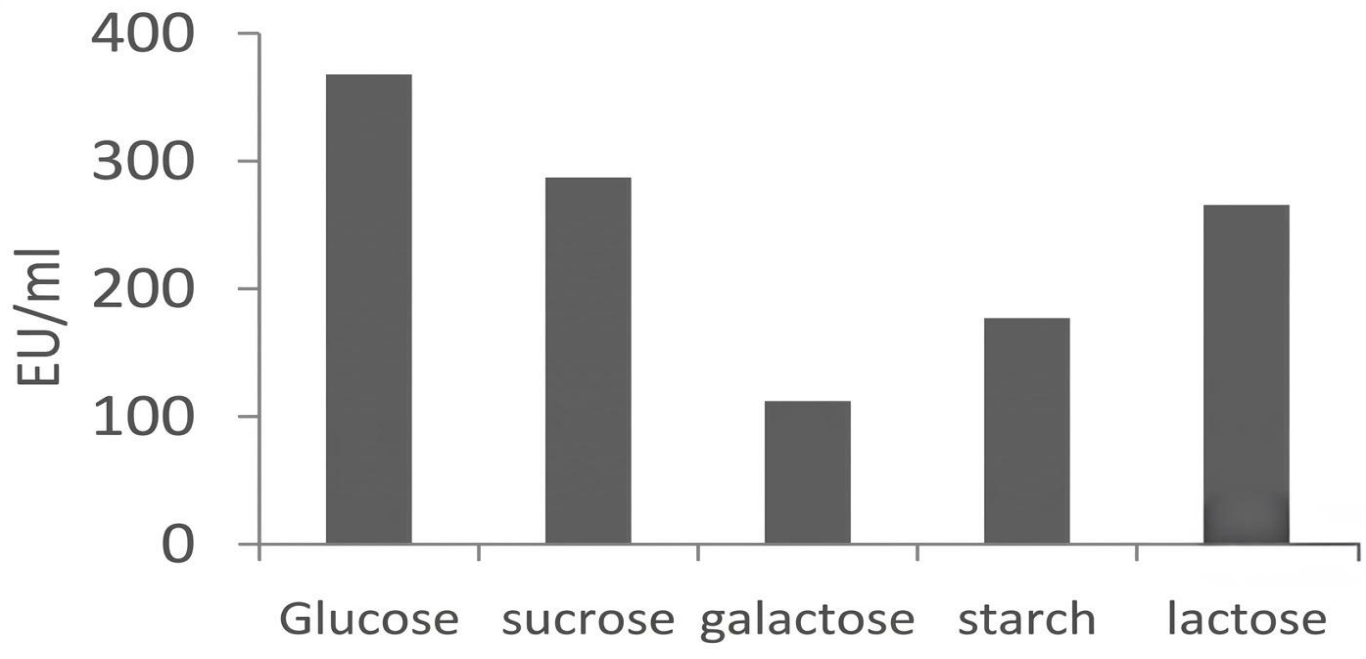


Figure 2(b)

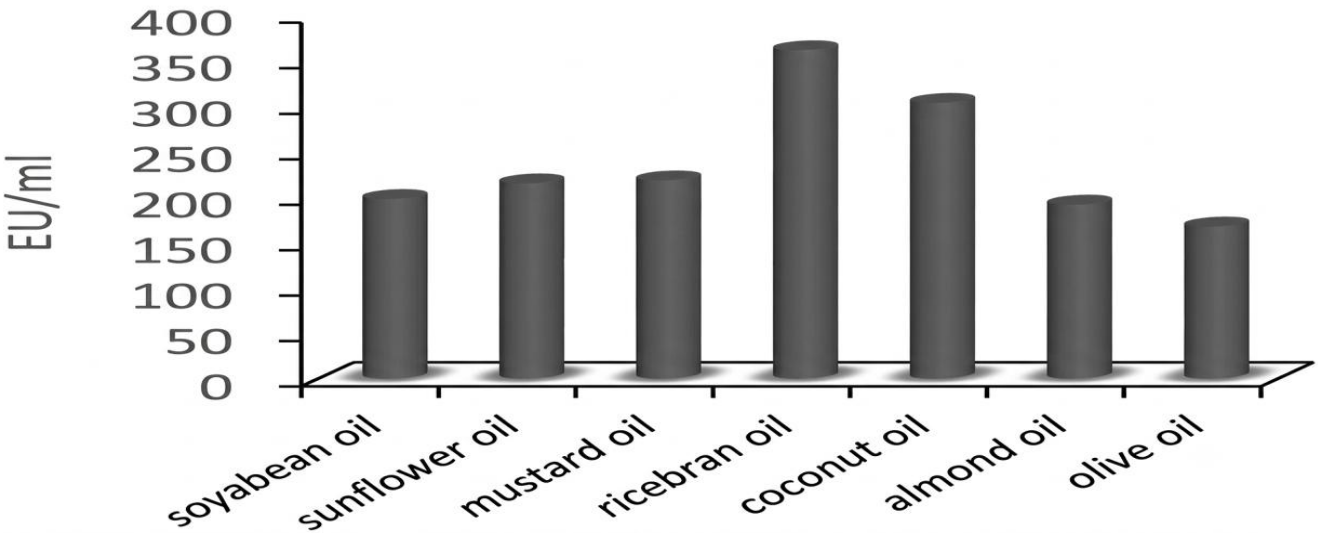


Figure 2(c)

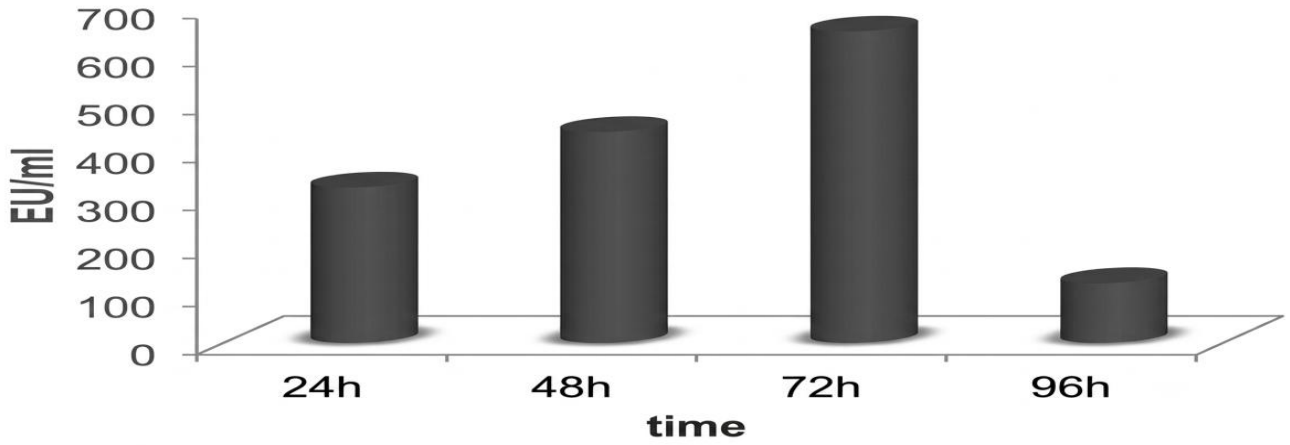


Figure 2(d)

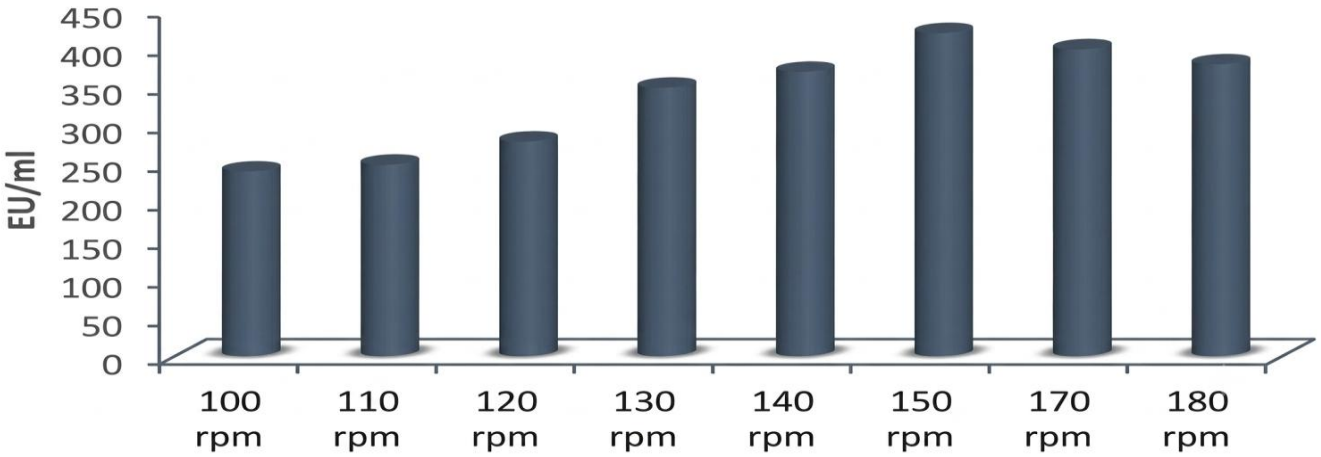


Figure 2(e)

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

Agitation rate affected both lipase yield and cell growth. Lipase production rose with agitation speed and peaked at 150 rpm (420 EU/ml), most likely because the medium received enough dissolved oxygen. Higher speeds reduced production (Figure 2e), possibly because intense shear alters the structure of the enzyme. Microorganisms differ in their oxygen requirements, since oxygen serves as the terminal electron acceptor in oxidative reactions that supply energy for cellular activity. Agitation speed also determines how well the flask or bioreactor is mixed, the oxygen transfer rate, the contact area between the phases, how well the carbon source disperses, and how available the nutrients are.

Purification and molecular weight determination

The lipase was purified from the extracellular medium by ammonium sulfate precipitation followed by two column chromatography steps. A large share of the activity was lost after precipitation, since less than 50% of the precipitated lipase could be redissolved. The three-step protocol (ammonium sulfate precipitation, ion-exchange chromatography and gel filtration) gave an 8.04-fold purification with a 22.6% recovery (Table 1). SDS-PAGE showed that the purified enzyme has a molecular mass of 27.5 kDa (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

Several organic solvent-stable lipases have been reported so far. The toxicity of a solvent is usually expressed by its log P value, the partition coefficient of the solvent between octanol and water. The effect of different solvents on the purified lipase is shown in Table 2, and the solvents chosen covered a wide range of log P values, from low to high. The lipase was stable and retained almost 90% of its activity after exposure to p-xylene, benzene, toluene and hexane (25% v/v each) for 2 h. In methanol and ethanol, the residual activity fell to 42% and 46%, respectively. A similar pattern was seen with the lipase of *Staphylococcus saprophyticus* M36, which kept 32% and 36% of its activity in methanol and ethanol (25% v/v) after 15 days at 30 °C and 160 rpm (Mustafa et al. 2023).

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

The stability of PS11 lipase did not follow the log P trend. In general, the more polar a solvent is, the lower its log P and the more toxic it is to enzymes. Water acts as a lubricant that gives enzyme molecules their conformational flexibility, and polar solvents tend to strip this essential water from the protein, which can change its conformation and reduce stability. Even so, the solvents affected PS11 lipase in different ways, and it is well known that the effect of organic solvents differs from one lipase to another (Mustafa et al. 2023). Zafar et al. (2024) likewise found no clear relationship between a solvent's solubility in water and lipase stability.

Effect of pH on activity and stability of PS11 lipase

PS11 was inoculated into the lipase production medium, and the pH was varied over a wide range (4-12). Maximum lipase activity of 417.5 EU/ml was observed at pH 10 (Figure 4), with activity rising steadily from pH 5 to this peak. The pH of the production medium plays a critical role in the physiological performance of the bacterial cell and in the transport of nutrients across the cell membrane, and therefore in maximising enzyme production. Xia (2024) likewise reported that culture pH strongly affects many enzymatic processes and the transport of components across the cell membrane. To assess pH stability, the enzyme was incubated over a range of pH values. It remained stable at high alkaline pH, which is an important attribute for industrial use.

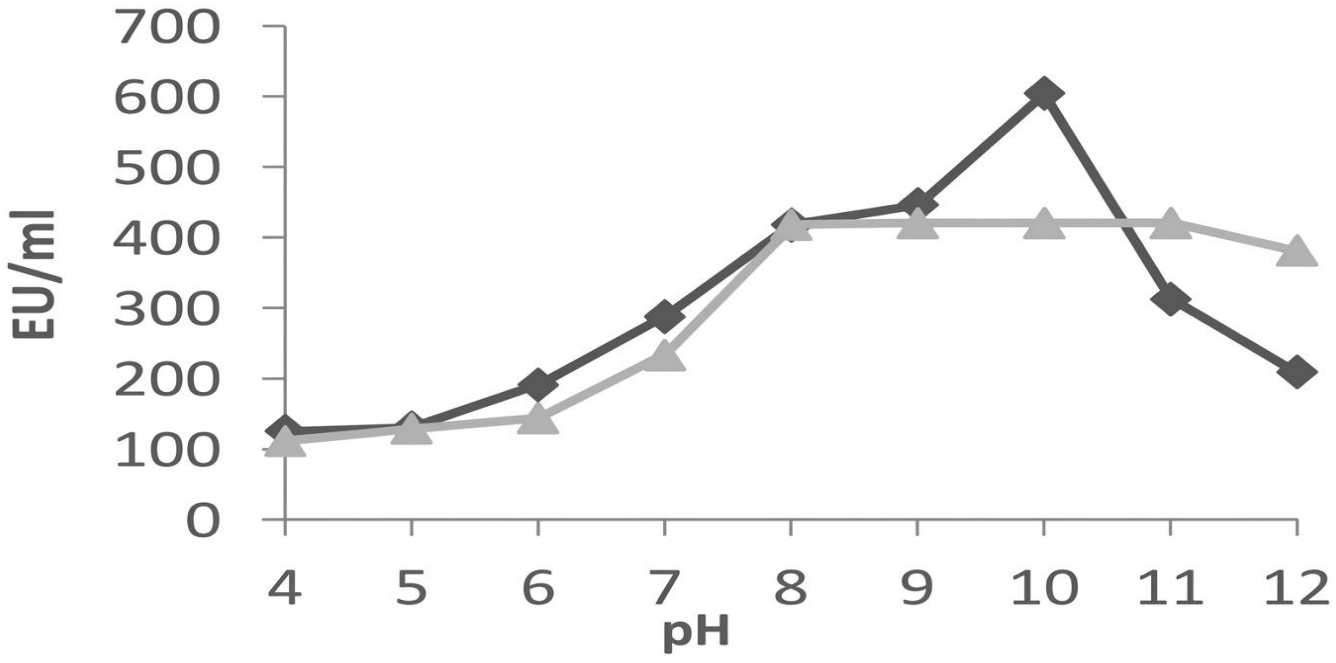


Figure 4 Determination of optimum pH (◆) and pH stability (▲) of PS11 lipase

Effect of temperature on activity and stability of PS11 lipase

Temperature is a critical parameter that must be controlled, and the best value usually differs from one organism to another. The optimum temperature for lipase production was 50 °C, although activity was also good at 40 °C. Activity then declined gradually and reached a minimum at 70 °C (Figure 5).

Thermal stability was tested by exposing the enzyme to different temperatures for 2 h. PS11 lipase showed its highest activity at 50 °C and stayed stable from 10 °C to 60 °C. Activity fell further as the temperature rose to 70 °C, showing that the enzyme is not stable at high temperatures.

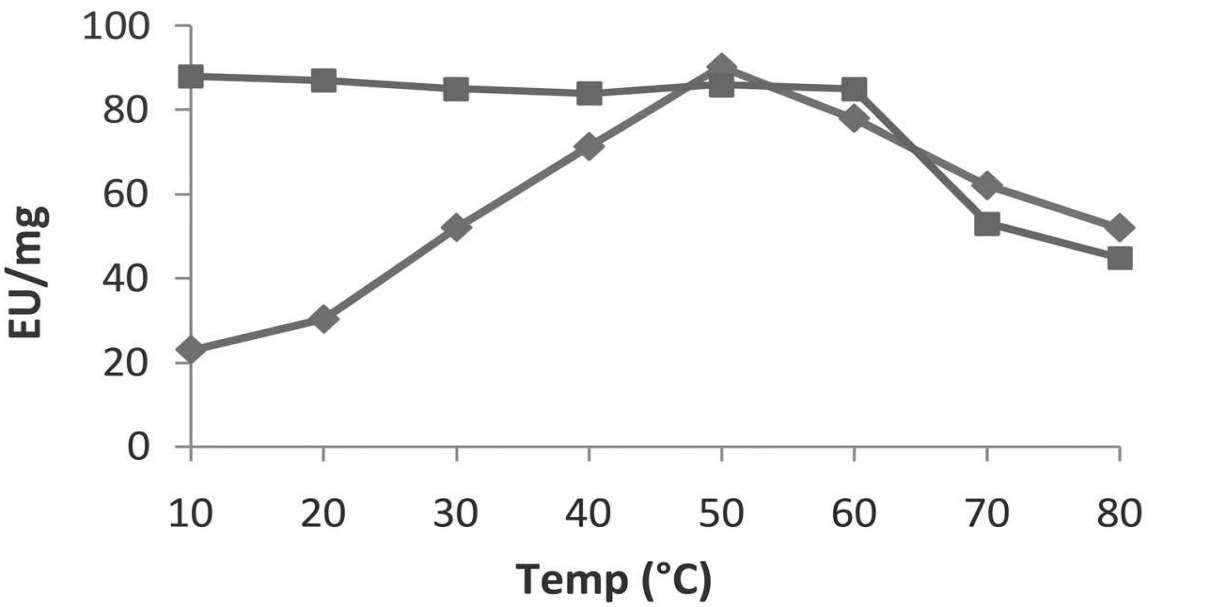


Figure 5 Determination of optimum temperature (◆) and temperature stability (■) of PS11 lipase

Foronda et al. (2025) reported a link between enzyme synthesis and energy metabolism in bacteria, regulated by temperature and oxygen uptake. For extracellular enzymes, temperature appears to affect secretion, possibly by altering the physical properties of the cell membrane. Protein conformation is also known to change or break down at higher temperatures, which explains the loss of lipase activity at elevated temperatures.

Conclusion

A major obstacle to lipase-catalysed biodiesel production is that the enzyme loses much of its activity in organic solvents, particularly methanol and ethanol. In this study, a solvent-tolerant lipase of 27.5 kDa was purified from *Geobacillus stearothermophilus* PS11. Its stability in the presence of methanol and ethanol makes it a promising candidate for biodiesel production.

Author contributions

AKS: Data curation, Project administration, Conceptualization, Visualization, Writing – original draft, Supervision, Validation, Writing – review & editing, Investigation, Software, Formal analysis, Methodology, Resources.

Ethical Permissions

Not Applicable

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Conflict of interest

The authors declare that the research was carried out without any commercial or financial relationship that could be interpreted as a potential conflict of interest.

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