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Purification and characterization of solvent tolerant lipase from *Bacillus* sp. for methyl ester production from algal oil

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Lipase from *Bacillus* sp. isolated from the oil contaminated soil was purified by ammonium sulphate precipitation and ion-exchange chromatography with a 5.1-fold purification and 10.5% yield. SDS-PAGE analysis of the enzyme revealed the molecular mass of 24 kDa. The optimum pH and temperature for lipase activity were 6.5 and 37°C, respectively. The isolated lipase was stimulated by pretreatment with methanol and ethanol as well as by divalent metal ions Ca^{2+}, Mg^{2+} and Mn^{2+}. The enzyme showed high activity towards oleic rich oils. The enzyme immobilized on celite could retain 90% lipase activity after eight cycles. Transesterification of *Botryococcus* sp. oil using the immobilized enzyme for 40 h resulted in 80% yield of fatty acid methyl esters which had good properties for use as biodiesel. Overall results suggested that the solvent tolerant *Bacillus* lipase can be a potential biocatalyst for methyl ester production.

[**Key words:** *Bacillus* sp.; Immobilization; Purification; Solvent tolerant lipase; Transesterification]

Lipases catalyse the hydrolysis of the ester bonds in the molecules of triglycerides, liberating free fatty acids and glycerol under physiological conditions. Lipases are important enzymes for biotechnology due to their ability to catalyse the transesterification reaction which can accept a wide range of acyl groups as their substrates. Lipases also exhibit high tolerance to various environmental factors such as pH, temperature, and organic solvents. Lipases have the potential to meet several needs for a wide range of applications since they are the only enzymes that catalyse the synthesis of esters (1). Lipases have been successfully applied as additives for supplementing digestive enzymes and for laundry formulations. Moreover, they can also be used as catalysts in the food industry as well as the modification of fats and oils (2). Lipases are mainly produced by bacteria, fungi, animals and plants. However, microbial lipases are much more suitable for industrial applications due to their availability. Most bacterial lipases are extracellular with a wide range of temperature and pH operations. *Bacillus* sp. has adapted itself to survive in extreme climatic conditions. A large number of *Bacillus* sp. lipases have been isolated. Nevertheless, most of the studies show only the basic characteristics of the enzymes and very few lipases have been studied in details (3,4). Lipases are also used in the methyl ester production. Methyl ester is produced mainly by chemical methods using alkali and acids as catalysts. Sodium and potassium hydroxide are widely used as alkali catalysts in chemical methods and sulphuric acid is widely used as an acid catalyst. Downstream processing is very difficult when using alkali as catalyst because alkali forms soaps while washing which affects the yield of methyl ester. Furthermore, it is also difficult to separate them from methyl ester. On the other hand, acid catalysts can affect downstream processing leading to the corrosion of the engines. To overcome the problems caused by acid and alkali catalysts, using biocatalysts is the best solution. Various lipases are used as biocatalysts in the production of methyl ester with little or no problems in downstream processing. However, some lipases are liable to inhibition by methanol which strips water from the active site of the lipase. Hence, it is necessary to search for solvent tolerant lipases to overcome the inhibition of the enzyme.

This study focuses on the isolation of a solvent tolerant lipase to catalyse the transesterification. The lipase producing microorganism was isolated from the oil contaminated soil and it was identified as *Bacillus* sp. The purification and characteristics of the lipase from *Bacillus* sp. including its activity after treatment with organic solvents were reported.

MATERIALS AND METHODS

Microorganisms The lipase producing microorganisms were isolated from the soil at the oil production plants located in Thiruvottriyur, Chennai, India. The microbial cultures were enriched by sub-culturing the samples in nutrient broth media containing (w/v) 0.5% olive oil, 0.1% yeast extract, 0.2% NaCl, 0.04% $MgSO_4$, 0.07% $MgCl_2$, 0.05% $CaCl_2$, 0.03% KH_2PO_4, 0.03% K_2HPO_4, and 0.05% $(NH_4)_2SO_4$, pH 7.0. The culture was incubated at 37°C under static conditions for two days.

Screening of lipase producing bacteria The enriched cultures were diluted and incubated in nutrient agar plates (30°C); then they were streaked on to the Rhodamine B-olive oil agar plate containing 28 g/l nutrient agar, 4 g/l sodium chloride, 10 ml Rhodamine B solution (1 mg/ml) and 31.25 ml of olive oil, and incubated at 30°C for two days. While the plates were irradiated with UV light, the presence of orange fluorescent halos was visualized around the colonies. The bacteria showing large fluorescence halos were selected as potential lipase producers. These organisms were isolated and sub-cultured in a nutrient broth containing olive oil (200 mg/l). The strain which showed the maximum lipase activity was identified

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based on cell morphology, Gram staining and biochemical tests as described by Hamid et al. (5).

Lipase production A lipase producing inoculum was prepared by transferring a loopful of fresh culture to a medium followed by incubation at 37°C at 180 rpm overnight. After the incubation, 5 ml of inoculum was inoculated into 100 ml of the basic medium containing (w/v) 1% olive oil, 0.2% $CaCl_2 \cdot 2H_2O$, 0.01% $MgSO_4 \cdot 7H_20$, and 0.04% $FeCl_3 \cdot 6H_2O$. The pH was adjusted to 7.0 with 1 M NaOH (6). The incubations were performed at 37°C with shaking at 180 rpm for two days. The culture was harvested by centrifugation at 8000×*g* for 10 min at 4°C. The supernatants were used for further purification studies and lipase assays.

Purification of the lipase Solid ammonium sulphate was added to the culture supernatant with 80% saturation and kept at 4°C overnight before subjecting to centrifugation at 12,000×*g* for 30 min. The precipitate was dissolved in 250 mM phosphate buffer (pH 7.0) and dialyzed for 24 h at 4°C in the same buffer. The ammonium sulphate precipitated fraction was applied to a phenyl sepharose CL-4B column and pre-equilibrated with 250 mM phosphate buffer (pH 7.0). After washing the column with a gradient of 250-1 mM phosphate buffer (pH 7.0), the unbound proteins were eluted by 1 mM phosphate buffer (pH 7.0). The bound proteins were then eluted by increasing the concentration of NaCl with a linear gradient. Fractions that exhibited lipase activity were pooled and lyophilized for further analysis. The protein concentration was determined by the Lowry method (7) using bovine serum albumin as the standard.

Determination of molecular weight Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (8). The molecular mass of the lipase was estimated by comparing the relative mobilities of different molecular weights as standard markers. Protein bands were visualized by silver staining.

Measurement of lipase activity The enzyme activity was measured by the titrimetric analysis (9). The mixture containing 2.5 ml water, 1 ml 200 mM phosphate buffer (pH 6.5), and 3 ml olive oil was mixed well. The mixture was added with 1 ml of lipase and incubated with shaking at 125 rpm at 37°C for 30 min. The blank was similarly prepared and kept at 4°C to prevent the reaction. After 30 min, 3 ml of ethanol was added to terminate the lipase reaction. The liberated fatty acids were determined by titration against 50 mM NaOH using 4 ml of 0.9% thymolpthalein as an indicator until the end point was observed. One unit of lipase activity was equivalent to 1 μmol of fatty acid released per minute at 37°C.

Effect of pH on the activity of lipase The optimum pH of the purified lipase was determined by incubating the reaction mixture at various pH values ranging from 3 to 9 at 37°C using acetate buffer for pH 3–5, phosphate buffer for pH 6–7, and Tris–HCl buffer for pH 8–9 in the assay buffer. The residual enzyme activity at different pH was measured using the lipase assay as described above.

Effect of temperature on the activity of lipase The optimum temperature of the purified lipase was determined by incubating the reaction mixture at temperatures in the range of 20–60°C at an optimized pH value. The residual enzyme activity was measured by the lipase assay.

Effect of solvents pretreatment on the activity of lipase The effect of organic solvents pretreatment on the enzyme activity was carried out by the addition of 1 ml of methanol, ethanol, acetone, methyl acetate, *t*-butanol, ethyl acetate, benzene, toluene and n-hexane to 3 ml of the diluted enzyme (30 U/ml). The mixture was preincubated at 37°C for 30 min. These solvents pretreated lipases were determined for the residual activity as described above. For the control experiment, 1 ml of distilled water was used instead of organic solvent.

Effect of metal ions on the activity of lipase The effects of different metal ions (at 1 mM), namely Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, K^{+}, and metal chelating agent EDTA on the activity of lipase were investigated by adding the respective metal ions to the reaction mixture and the residual activity was measured as described above.

Substrate specificity The substrate specificity of the isolated lipase towards various oils was tested by using sunflower oil, castor oil, coconut oil, palm oil, waste cooking oil and triolein as substrates to determine the specificity of the isolated lipase and the activity was measured as described above.

Enzyme immobilization The lipase solution used for immobilization contained 220 U/ml of its activity. Celite (SRL, India) was used for the immobilization of the isolated lipase (1 g/5 ml lipase solution). The mixture was stirred for 1 h. The celite immobilized lipase was recovered, dried and stored at 4°C. The activity of the immobilized lipase was 100 U/g.

Operational stability of immobilized lipase To study the operational stability of the enzyme, the immobilized lipase was washed with 50 mM phosphate buffer pH 6.5 after a single reaction and reused to detect the enzyme activity. Each reaction was considered a single cycle and the percent of relative activity was determined by taking the activity of the first cycle as 100%.

Transesterification catalysed by the lipase The *Botryococcus* sp. was obtained from Bengal Aquarium, Ramanathapuram, India. Oil was extracted by soxhlet extraction method using hexane as the solvent and its oil composition is shown in Table 2. The oil was rich in oleic and linoleic acid and this combination is very suitable for biodiesel purpose. The lipase catalysed transesterification was carried out in a glass vial. The reaction mixture contained algal oil (1 g) and methanol with 1:3 molar ratio, water (5 μl), and *t*-butanol (0.75 ml). The reaction was started by adding the immobilized enzyme (8% w/w of oil) and incubated at 37°C for various times with shaking at 150 rpm. The purification of the obtained methyl ester was done as previously described (6). The samples were filtered to remove the immobilized enzyme. The upper layer contained *t*-butanol and methyl ester whereas the lower phase contained glycerol. The upper layer was separated and washed with water to remove traces of methanol and then dehydrated by anhydrous sodium sulphate. After filtration, *t*-butanol was evaporated to obtain the pure methyl ester. The samples obtained were subjected to further analysis to determine the ester content. The biodiesel fuel properties were determined according to the standard tests of ASTM 6751 (American Society for Testing and Materials) and compared to those of diesel fuel.

TABLE 1. Summary of purification procedure from *Bacillus* sp.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield	Purification (fold)
Supernatant of culture	40000	562	71.1	100	1
Ammonium sulphate precipitation	13000	126.3	102.9	32.5	1.44
Phenyl sepharose	4200	11.4	368.4	10.5	5.17

TABLE 2. Oil compositions of *Botrycoccus* sp.

Composition	Fatty acid % (w/w)
Myristic acid	1.6
Palmitic acid	2.2
Stearic acid	4.6
Oleic acid	56.7
Linoleic acid	34.6
Linolenic acid	0.3

Analytical methods The fatty acid composition of the *Botryococcus* sp. oil and methyl ester content in the reaction mixtures were analysed by gas chromatography (Sigma), equipped with an AC30 Carbowax column and a flame ionization detector. Nitrogen was used as a carrier gas whereas hydrogen and oxygen were employed for the purpose of ignition. Methyl heptadecanoate was used as an internal standard. While injecting the samples, the column temperature was kept at 150°C, raised to 240°C at 10°C/min, and maintained for 10 min. The injector and detector port temperatures were set at 250°C (10).

Statistical analysis In the present study, all experiments were carried out in triplicates and the results obtained were analysed for statistical significance ($P < 0.001$) by one way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Among the various isolated strains, the one that showed the maximum lipase activity with a clear zone on Rhodamine B agar plates was isolated. The strain was further analysed by morphological and biochemical tests (Table S1). The result confirms that the isolated organism was *Bacillus* sp. (11). The identified organism was used for further studies.

Purification of lipase The extracellular lipase from the isolated *Bacillus* was purified by ammonium sulphate precipitation and phenyl sepharose hydrophobic interaction chromatography yielding a 5.1 fold purification and 10.5% recovery (Table 1). A single band was obtained for the lipase by SDS-PAGE analysis (Fig. 1, lane 2) indicating the protein content with high level of purity. The molecular mass of the purified lipase was estimated to be 24 kDa which was in agreement with that of the lipase from *Bacillus licheniformis* and *Bacillus subtilis* (12,13) whereas a slightly higher molecular mass of about 25 kDa was reported for *Bacillus* sp. H-257 lipase (14).

Effect of pH The purified lipase exhibited activity over the pH range 4–7 with 100% residual activity observed at pH 6.5 (Fig. 2A). A rapid decline in the enzyme activity was observed on both sides of the pH optimum. The enzyme retained its high activity at pH range of 6–7 and the activity was reduced drastically at extreme pH where about 30% activity was retained at pH 3 and 10. A