Pseudomonas sp. BUP6 produces a thermotolerant alkaline lipase with *trans*-esterification efficiency in producing biodiesel

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Abstract The present study describes the characteristics of a thermotolerant and alkaline lipase secreted by *Pseudomonas* sp. BUP6, a novel rumen bacterium isolated from Malabari goat, and its *trans*-esterification efficiency in producing biodiesel from used cooking oil (UCO). The extracellular lipase was purified to homogeneity (35.8 times purified with 14.8% yield) employing $(\text{NH}_4)_2\text{SO}_4$ salt precipitation and Sephadex G-100 chromatography. The apparent molecular weight of this lipase on SDS-PAGE was 35 kDa, the identity of which was further confirmed by MALDI-TOF/MS. The purified lipase was found stable at a pH range of 7–9 with the maximum activity (707 U/ml) at pH 8.2; and was active at the temperature ranging from 35 to 50 °C with the optimum at 45 °C (891 U/ml). Triton X-100 and EDTA had no effect on the activity of lipase; whereas SDS, Tween-80 and β -mercaptoethanol inhibited its activity significantly. Moreover, Ca^{2+} (1.0 mM) enhanced the activity of lipase (1428 U/ml) by 206% vis-à-vis initial activity; while Zn^{2+} , Fe^{2+} and Cu^{2+} decreased the activity significantly. Using *para*-nitrophenyl palmitate as substrate, the K_m (11.6 mM) and $V_{\max}$ [668.9 $\mu\text{mol}/(\text{min}/\text{mg})$] of the purified lipase were also determined. Crude lipase was used for analyzing its *trans*-esterification efficiency with used cooking oil and methanol which resulted in the worthy yield of fatty acid methyl esters, FAME (45%) at 37 °C, indicating its prospects in biodiesel industry. Thus, the lipase secreted by the rumen bacterium, *Pseudomonas* sp. BUP6, offers great potentials to be used

in various industries including the production of biodiesel by *trans*-esterification.

Keywords *Pseudomonas* sp. BUP6 · Thermotolerant lipase · *Trans*-esterification · Biodiesel

Introduction

Lipases or triacylglycerol hydrolases (EC 3.1.1.3) are ubiquitous enzymes catalyzing the hydrolysis and synthesis of esters of glycerol and long-chain fatty acids. Lipases are produced by several microorganisms encompassing bacteria, fungi and yeast, coupled with animals and plants. Microbial lipases are widely used in industries, because of their versatile catalytic activities, ease of genetic manipulation and copious yield, in addition to the exponential growth of the lipase producing microorganism in inexpensive media, and the absence of seasonal fluctuations (Benjamin and Pandey 1996). Besides hydrolysis, lipases synthesize esters from glycerol and long-chain fatty acids in low water activity; i.e., lipases can efficiently catalyze esterification, inter-esterification, and *trans*-esterification reactions in non-aqueous media (Aravindan et al. 2007). The versatility in activities and the enantio- and regio-selectivities make lipases a suitable choice of catalyst in many industries involved in organic synthesis, hydrolysis of fats and oils, catalytic resolution of chiral drugs, modification of fats, flavor enhancement and synthesis of fine chemicals (Benjamin and Pandey 1998; Pandey et al. 1999). Many species of bacterial genera such as *Pseudomonas*, *Bacillus*, *Serratia*, *Alcaligenes*, etc., fungi such as *Aspergillus*, *Penicillium* and *Candida* spp. are the best known producers of lipases (Jaeger and Reetz 1998).

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The rumen is the primary seat of fermentation in cattle, where a wide variety of microorganisms reside. Hydrolysis of dietary lipids resulting in the release of free fatty acids is the preliminary process occurring prior to the ruminal biohydrogenation, which is mediated by lipases produced by the rumen microbes (Benjamin et al. 2015). Since lipases are inducible and adaptive in nature, it is expected that the rumen microbial lipases can perform catalysis more or less in the similar manner as the lipases produced by other microorganism, i.e., to thrive in harsh environments such as contaminated soil, industrial wastes, etc. Thus, such ruminal microorganisms can effectively be exploited for various industries beneficial to mankind. Very recently, our group reported *Pseudomonas* sp. BUP6, a novel isolate from the rumen of Malabari goat, capable of producing lipase (Priji et al. 2015).

Biodiesel—the monoalkyl esters (methyl and ethyl esters) of long chain fatty acids—represents a promising alternative to the diminishing reserves of fossil fuel for its use in diesel engines. Biodiesel comes from plant or animal-derived renewable sources and as such, it is biodegradable and less polluting. Enzymatic production of biodiesel has been proposed to overcome the drawbacks of the conventional chemically catalyzed processes (Al-Zuhair et al. 2007). A huge amount of used cooking oil (UCO) generated from food based industries is disposed without any prior treatment causing serious environmental problems. Transformation of the waste oil into biodiesel using lipase highlights exciting opportunities for environmental protection (Kumar and Negi 2015). Upon this background, the present study is focused on the purification of lipase from *Pseudomonas* sp. BUP6 with an emphasis on its characterization, and probable application in the production of biodiesel from UCO.

Materials and methods

Microorganism and production medium

Pseudomonas sp. strain BUP6 (GenBank accession no. KF550910; Microbial Type Culture Collection no. 5925) isolated from the rumen of Malabari goat (Priji et al. 2015) was used for this study. The semi-synthetic medium used in this study contained (g/l, pH 6.9): 5.0 NH_4NO_3 , 4.0 $(\text{NH}_4)_2\text{SO}_4$, 2.0 K_2HPO_4 , 2.0 NaCl , 0.01 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 CaCl_2 , 3.0 yeast extract and 10 ml groundnut oil (supplied as emulsion in distilled water after sonication). The seed culture was prepared by incubating the cells of *Pseudomonas* sp. strain BUP6 in the semi-synthetic medium at 37 °C (200 rpm for 18 h) in a temperature-controlled orbital shaker (in 100 ml Erlenmeyer flasks)

(Scigenics Biotech, India), so as to reach an optical density of 2.5 (λ_{600}).

Assay for lipase activity

The liquid culture was centrifuged at $9400 \times g$ for 10 min at 4 °C, and the clear supernatant so obtained was used as crude lipase. Lipase was assayed by the method described by Gupta et al. (2002) with minor modifications, as described briefly hereunder. For estimating the activity of lipase, 200 μl of crude lipase (supernatant) was added to the assay mixture containing (1.8 ml) NaCl (0.15 M) and Triton X-100 (0.5%) in 0.1 M Tris–HCl, and incubated at 37 °C for 10 min. Subsequently, 20 μl of 50 mM *p*-nitrophenyl palmitate (*p*NPP) in acetonitrile was added, and incubation continued for further 30 min (37 °C). The quantity of *p*-nitrophenol liberated was measured at λ_{405} spectrophotometrically. One unit of lipase activity corresponds to 1 μmol of *p*-nitrophenol liberated per min under the standard assay conditions.

Purification of lipase

Fractionation by $(\text{NH}_4)_2\text{SO}_4$ precipitation

Solid $(\text{NH}_4)_2\text{SO}_4$ salt was slowly added to the crude lipase preparation (i.e., supernatant), so as to reach 20% of saturation and the precipitate was collected by centrifugation ($9400 \times g$ for 10 min at 4 °C). The addition of $(\text{NH}_4)_2\text{SO}_4$ was carried out with continuous stirring, by keeping the solution on ice bath for 1 h in a cold room (4 °C). The supernatant was further precipitated to 20–40, 40–60 and 60–80% of salt saturations, and the precipitate was collected separately in each step. The pellets were re-suspended in 0.1 M Tris–HCl buffer (pH 8.0), subsequently dialyzed (using cellulose membrane tubes) against 0.1 M Tris–HCl buffer (pH 8.0) for 24 h at 4 °C under continuous stirring with two buffer changes in between (Priji et al. 2015). Subsequently, the dialysate was centrifuged ($9400 \times g$ for 10 min at 4 °C), and the supernatant so obtained was subjected to gel permeation chromatography. Protein content and lipase activity of each dialysate fractions were determined using standard methods (Lowry et al. 1951).

Gel permeation chromatography

Among the four dialysate fractions (0–20, 20–40, 40–60 and 60–80%), the fraction which showed the highest specific activity was used for gel permeation chromatography. Glass column (60 $\times$ 2.5 cm) packed with Sephadex G-100 (Sigma Aldrich, USA) was used for the purpose. The Sephadex G-100 column was equilibrated with 0.1 M

Tris–HCl buffer (pH 8.0) and eluted with the same buffer. Fractions of 2.0 ml were collected at every 20 min and assayed for protein content and lipase activity. Fractions with the highest specific activities were pooled and the samples were then stored at 4 °C for further use.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

After each purification step, the purity of lipase was confirmed by SDS-PAGE (4% stacking gel and 12% separating gels), which performed using a vertical mini gel (8 × 7 cm) slab with notched glass plate system. Gels of 1.5 mm thickness were prepared for the entire study. Broad range protein molecular weight marker (Genei, Bangalore) containing myosin (205 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa) aprotinin (6.5 kDa) and insulin (3.5 kDa) was used for determining the molecular weight (MW) of lipase on the gel. After the electrophoresis, the gel was visualized using 0.1% coomassie brilliant blue (CBB) G-250 stain, and photographed.

Mass spectrometric analysis of purified lipase

The prominent lipase band on SDS-PAGE was excised, destained and digested with trypsin. The resulting peptide fragments were desalted and concentrated with pipette tips containing C-18 reverse-phase medium (ZipTip, Millipore). The peptides so obtained were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (BrukerDaltonics, USA) at the mass spectrometry and proteomics core facility available with Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala. The peak list file generated was analyzed by searching the peptide masses against the protein database available with National Centre for Biotechnology Information (NCBI), USA, using the software Mascot (Diaz et al. 2006).

Characterization of lipase

The purified and active fraction of lipase obtained after gel permeation chromatography was used for its characterization studies. To identify the characteristics of lipase, i.e., effects of pH, temperature, substrate (*p*-NPP) concentration, different metal ions (Mg^{2+} , Ni^{2+} , Ca^{2+} , Zn^{2+} , Fe^{3+} and Cu^{2+}), detergents (SDS, Triton X-100, Tween-80) and modifiers [ethylenediaminetetraacetic acid (EDTA), β -mercaptoethanol] on lipase activity were investigated. The effects of these parameters on lipase activity were

expressed in percentage of enhanced activity to its initial activity (purified lipase).

Effect of pH on activity and stability of lipase

The optimum pH for lipase activity was determined by measuring the enzymatic activities in pH varying from 4 to 10 at 37 °C for 30 min of incubation. Stability was measured by pre-incubating lipase at three different pH levels (8, 9 and 10) for 1–5 h, and then the activity was measured. Residual activity was determined in relation to the lipase activity at zero h of pre-incubation.

Effect of temperature on activity and stability of lipase

The optimum temperature for lipase activity was determined by measuring the enzymatic activities in 0.1 M Tris–HCl buffer (pH 8.2) at different temperatures, i.e., 25, 30, 35, 40, 43, 45, 48, 50, 55 and 60 °C for 30 min incubation. Stability was measured by incubating lipase at three different temperatures (40, 45 and 50 °C) for 1–5 h; thereafter, the relative activity was determined at the respective temperatures.

Effect of different detergents and modifiers on lipase activity

For determining the effect of different detergents and modifiers on lipase activity, the purified lipase was incubated with the reaction mixture containing SDS, Tween-80, Triton X-100, EDTA or β -mercaptoethanol at different concentrations of 0.25, 0.5 and 1.0% at 45 °C and pH 8.2 for 30 min; thereafter, the relative activity was determined.

Effect of different metal salts on enzyme activity

Effect of various metal ions on lipase activity was determined by incubating the reaction mixture with different metal salts, i.e., Mg^{2+} , Ni^{2+} , Ca^{2+} , Zn^{2+} , Fe^{3+} and Cu^{2+} to a final concentration of 0.5, 1.0, and 1.5 mM at 45 °C and pH 8.2 for 30 min of incubation.

Enzymatic *trans*-esterification for the production of biodiesel

Trans-esterification of used cooking oil (UCO)

UCO was collected from the nearby restaurant and, prior to use, it was filtered using a muslin cloth so as to remove the solid impurities. Filtered UCO (5 g) and methanol were taken in a two-necked round bottom flask (100 ml) at a molar ratio of 1:12, respectively. Crude lipase of about 750 U (crude supernatant) was added to this mixture, and

Table 1 Summary of enzyme purification

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Crude	263.0	27963.1	106.3	1	100
40–60% (NH ₄) ₂ SO ₄	3.9	6931.4	1796.3	16.9	24.8
Sephadex G-100	1.1	4163.6	3801.4	35.8	14.8

incubated at 37 °C with constant stirring at 200 rpm for 24 h. The progress of the reaction was monitored by withdrawing aliquots (25 µl) of reactants at various time intervals; and mixed with equal volume of heptane. Subsequently, the mixture was centrifuged at 12,000×g for 10 min at 4 °C. The lower glycerol layer was discarded and the upper biodiesel layer was washed with hot water (80 °C) 4–5 times to remove the traces of solvent. Biodiesel samples were dried for 3 h at 110 °C.

Gas chromatography (GC)

The sample was derivatized with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) for analysis by GC. About 1 g of sample or standard was weighed into a vial, 100 µl MSTFA (derivatization reagent) was added and then allowed to stand for 20 min at 24 °C. Afterwards, heptane (4 ml) was added to the mixture and mixed well. The conversion rate of UCO into biodiesel was analyzed by Gas Chromatography (Shimadzu GC 2010Plus, Japan) equipped with the flame ionization detector. The MXT biodiesel column (length 17 m, internal diameter 0.32 mm and 0.10 µm *df*) with nitrogen as carrier gas was (30 ml/min) used for the analysis. The column oven temperature was kept at 55 °C for 1 min and then heated at a rate of 15 °C/min up to 380 °C for 15 min. The standard contained methyl esters of palmitic, stearic, oleic, and linoleic acids (Restek). The methyl esters formed by *trans*-esterification was identified by comparing the peak area of the standard methyl ester of fatty acids at the particular retention time (RT).

Results

Purification of lipase

Of various (NH₄)₂SO₄ fractions of lipase, 40–60% fraction showed the maximum lipase activity, which was 16.9 folds purified with 24.8% yield (Table 1). This fraction was subjected to gel permeation chromatography. Thirty-two fractions were collected at a flow rate of 2 ml/20 min among which 10 and 11 fractions showed the highest specific activity. Thus, Sephadex G-100 chromatography

resulted in 35.8-fold purification of lipase with the yield of 14.8% (Table 1). The lipase active fractions after each purification step were subjected to SDS-PAGE for purity check. The apparent MW of the purified lipase was estimated as 35 kDa (Fig. 1).

Mass spectrometric analysis of purified lipase

The trypsin digested peptides of purified lipase were subjected to mass spectrometric analysis and the resultant array of peptides was compared with the NCBI protein database using the software Mascot (Fig. 2). The peptide sequences showed 63% homology with that of lipase from *Pseudomonas putida* KT2440 (MW 34.2 kDa).

Fig. 1 SDS-PAGE profile of lipase from *Pseudomonas* sp. BUP6. Lane 1 standard protein molecular weight marker; Lane 2 Sephadex G-100 column purified lipase showing apparent molecular weight of 35 kDa; Lane 3 (NH₄)₂SO₄ precipitated fraction; Lane 4 crude enzyme harvested at 37 °C, pH 6.9 and 200 rpm

Fig. 2 Mass spectrum of trypsin-digested peptides of purified lipase from *Pseudomonas* sp. BUP6 with molecular mass 35 kDa

Characterization of purified lipase

Active fraction of lipase obtained by Sephadex G-100 gel permeation chromatography (fraction numbers 10 and 11) was used for the characterization studies.

Effect of pH on lipase activity and stability

Effect of pH on lipase activity was measured at normal assay conditions using 50 mM *p*-NPP as substrate (37 °C, and 30 min) with varying pH. Purified lipase was active at pH range of 7–9 with the optimum activity at pH 8.2, which showed the relative activity of 102% (707.1 ± 9.1 U/ml); but, the activity of lipase was inhibited at acidic pH. The lipase maintained more than 90% of the initial activity at pH 9 stably for 1 h; which clearly indicates its alkaline nature (Fig. 3a, b).

Effect of temperature on lipase activity and stability

Purified lipase was active at a temperature range of 30–50 °C with the optimum at 45 °C (891.4 ± 8.7 U/ml). At optimum temperature (45 °C), lipase showed 128%

activity (compared to initial column purified fraction of lipase) with stability for 4 h. Even at 50 °C, lipase found active for 2 h; thereafter, the activity was declined considerably (Fig. 4c, d).

Effect of detergents and modifiers on lipase activity

SDS and Tween-80 inhibited the activity of lipase to 18.6 and 15.6% of the initial activity, respectively; whereas it was active in the presence of Triton X-100, as under normal assay condition; but lower or higher concentration of Triton X-100 (from 0.5%) inhibited the activity. EDTA exhibited no effect on lipase activity, whereas β -mercaptoethanol almost abolished the activity of lipase, i.e., only a relative activity of 1.2% was retained, which indicated the presence of disulphide bridges in the structure (Fig. 5a).

Effect of metal ions on lipase activity

Lipase activity was enhanced by the addition of Ca^{2+} , Ni^{2+} , and Mg^{2+} ; the maximum activity was obtained in the presence of 1.0 mM concentration of Ca^{2+} (1428 ± 49 U/ml), which was 206% of the initial activity (i.e., relative

Fig. 3 a Effect of pH on lipase activity showing the alkalophilic nature of lipase (pH 7–9). Maximum activity was observed at pH 8.2 (707.1 ± 9 U/ml) with the relative activity of 102%; **b** stability of lipase was analyzed at three pH 8, 9, and 10 among which at the pH 8 and 9, lipase maintained 90% of activity even after 5 h whereas at pH 10, 50% of activity was lost after 1 h of incubation

activity). However, of the other three metal ions tested, Cu^{2+} and Fe^{2+} were found toxic to lipase, as they inhibited the activity of lipase significantly (Fig. 5b).

Optimized reaction conditions

The optimized condition for the maximum activity of lipase produced by *Pseudomonas* sp. BUP6 was 45°C temperature and pH 8.2 in the presence of 1.0 mM Ca^{2+} , at which, the relative activity was enhanced to 206%, compared to the initial lipase activity of column purified fraction.

Analysis of the biodiesel

Crude lipase (supernatant) from *Pseudomonas* sp. BUP6 was used for the production of biodiesel by the *trans*-esterification of UCO with the addition of methanol. The whole process is schematically represented in Fig. 6. Production of biodiesel was found to be 45.36% by maintaining the reaction at 37°C for 3 h, at the expense of 750 U of lipase with oil to methanol ratio of 1:12. Methyl esters of palmitic, stearic, oleic and linoleic acids constituted the biodiesel, and were detected by GC at RTs of 5.4, 6.8, 7.3 and 9.2 min, respectively.

Fig. 4 a Effect of temperature on lipase activity showing its thermotolerant nature. Lipase was found active at temperature ranging from 35 to 50 °C with optimum activity at 45 °C (891.4 ± 9 U/ml) and the relative activity of 128%; **b** stability of lipase was analyzed at three different temperatures, 40, 45 and 50 °C among which at the optimum temperature of 45 °C, lipase was found active for 4 h

Discussion

The present study was focused on the purification, characterization and application potential of lipase produced by *Pseudomonas* sp. BUP6, a rumen bacterium. Purification was carried out by $(\text{NH}_4)_2\text{SO}_4$ salt precipitation and Sephadex G-100 column chromatography. The purified lipase was characterized for its MW, pH, temperature, response to detergents and modifiers, so as to explore its possible applications in the biodiesel industry.

The first objective of this study was to remove as much as unwanted proteins as possible by retaining the lipase active protein unaffected. Generally, precipitation,

ultrafiltration, gel exclusion chromatography were used for the purification of extracellular enzymes (Palekar et al. 2000). The yield and fold of purification are the two critical parameters usually adopted for assessing the purity of an enzyme. Here, the culture supernatant was used as a starting material for the purification of the lipase from *Pseudomonas* sp. BUP6. Ammonium sulphate salt precipitation and sieving through Sephadex G-100 gel resulted in 35.8-fold enrichment of lipase with a yield of 14.8%. Likewise, $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel exclusion chromatography were employed to purify lipase from *P. fragi* and *P. aeruginosa* LST-03 with the yield of 18, 12.6 and 0.96% of the initial activity, respectively (Dandavate

Fig. 5 a Effect of detergents and modifiers on lipase activity. Triton X-100 (0.5%) and EDTA (0.25%) maintained the activity of lipase whereas SDS, Tween-80 and β -mercaptoethanol reduced the activity significantly; **b** effect of metal ions on lipase activity. Ca^{2+} (1.0 mM) enhanced the activity of lipase (1428 ± 48.8 U/ml) by 206% of the initial activity. Mg^{2+} and Ni^{2+} slightly enhanced the lipase activity whereas Zn^{2+} , Fe^{2+} and Cu^{2+} reduced the activity significantly

et al. 2009; Ogino et al. 2007). In this study, the purified fraction of enzyme showed a major band of 35 kDa in SDS-PAGE profile, the identity of which was then confirmed as lipase by MALDI-TOF/MS technique. From the literature, MW of lipase produced by *Pseudomonas* spp. varies from 30 to 95 kDa (Priji et al. 2015). Likewise, the molecular mass of *Pseudomonas* sp. BUP6 lipase agreed closely with that of *Pseudomonas* lipase reported elsewhere (Peng et al. 2010).

Properties of a purified enzyme represent its possible industrial applications. The pH is an important factor affecting the ionization states of amino acids that regulate

the functional structure of an enzyme and hence dictates its overall activity (Sharma et al. 2002). The activity of lipase was analyzed in different buffer systems with pH varying from 4 to 10; among which pH 8.2 was found as the optimum. Lipase from *Pseudomonas* sp. strain BUP6 remained suggestively active at range of pH 8–9 even after 5 h of incubation, indicating that the alkaline condition favors the activity of the enzyme. The result is similar to those lipases reported from other strains of *Pseudomonas* (Kojima and Shimizu 2003; Gaur et al. 2008; Peng et al. 2010). Similarly, lipase withstands a temperature range of 35–50 °C with an optimum at 45 °C. The result obtained is

Fig. 6 Schematic diagram of *trans*-esterification reaction

in accordance with lipases from other *Pseudomonas* spp. such as *P. gessardii*, *P. fluorescens* HU380, *P. fragi* and *P. mendoncina* which were also found to be optimally active within 35–45 °C (Karadzic et al. 2006; Ramani et al. 2010). For the thermostability, the purified lipase retained 100 and 98% of its original activity for 5 h at 30 and 40 °C, respectively (Ramani et al. 2010). Since the lipase from *Pseudomonas* sp. BUP6 maintained more than 80% of its activity at 40 and 45 °C for 5 h, the enzyme can be characterized as thermotolerant alkaline lipase. Thus, this lipase reveals its possibilities of utilization in many industries like detergency and tannery, which demand alkalophilic and thermostable lipases.

Of various metal ions tested, the presence of Ca^{2+} in the reaction mixture significantly stimulated or stabilized the lipase activity, and Mg^{2+} and Ni^{2+} slightly enhanced the activity; while Zn^{2+} , Fe^{2+} and Cu^{2+} inhibited the activity, of which Fe^{2+} showed the strongest inhibitory activity. These effects were at par with that of the lipase reported from *Pseudomonas* sp. AG-8 (Sharma et al. 2001). The inhibitory effects of metal ions could be attributed to the changes in the solubility and behavior of the ionized fatty acids at the interfaces. The possible explanation for the enhanced lipolytic activities of Ca^{2+} is the possession of a calcium binding pocket in bacterial lipases, which strongly stabilizes its activity (Alquati et al. 2002; Schrag et al. 1997). Among various detergents, 0.5% of the Triton X-100 favored lipase activity, but the activity was completely abolished in the presence of SDS and Tween-80. Generally, the response of microbial lipases to the detergents is variable to some extent. However, in accordance

with our results, Castro-Ochoa et al. (2005) also reported the loss of lipolytic activity in the presence of Tween-80 and SDS; however, enhanced effect was observed when incubated with Triton X-100. The presence of β -mercaptoethanol drastically decreased the lipase activity, which indicates the involvement of the disulphide bonds in stabilizing the enzyme. Moreover, the divalent metal chelating agent, EDTA exhibited no effects on lipase activity, which suggests that the enzyme is not a metalloprotein. Similar characteristics were shown by lipases produced by *P. aeruginosa* and *Geobacillus* sp. TW1 (Gaur et al. 2008; Li and Zhang 2005).

The K_m (11.6 mM) and V_{max} (668.9 $\mu\text{mol}/\text{min}/\text{mg}$) values of the lipase produced by *Pseudomonas* sp. strain BUP6 were determined by Michaelis–Menten plot using *p*NPP as substrate. The K_m value is the measure of affinity of enzyme toward the substrate. Low K_m value represents that the enzyme requires only a little quantity of substrate to get saturated. High V_{max} indicates the higher efficiency of the enzyme. i.e., more substrate molecules are converted to product per unit time when the enzyme is fully saturated with the substrate. In general, the K_m values of enzymes vary from 10^{-1} to 10^{-5} M (Fullbrook 1996). Lipase from *P. cepacia* showed the K_m and V_{max} values of 12 mM and 30 $\mu\text{mol}/\text{min}$, respectively, with *p*NPP as substrate (Pencrac'h and Baratti 1996). Lipase from *P. aeruginosa* PsA showed the K_m value of 70.4 mM and V_{max} of 2.24 mmol/(min mg) with *p*NPP as substrate (Gaur et al. 2008). It shows that lipase from *Pseudomonas* sp. BUP6 is more efficient than many other lipases reported from various strains of *Pseudomonas* spp.

Waste cooking oil seems to be a promising feedstock for the production of biodiesel due to its low cost and thereof it reduces the waste disposal and treatment problems associated with many food industries (Kawentar and Budiman 2013). Till date, only a few studies have reported the lipase-mediated *trans*-esterification of used cooking oil (Lam et al. 2010). For instance, Charpe and Rathod (2011) compared the *trans*-esterification efficiency of lipases from *Aspergillus oryzae*, *P. fluorescens*, *P. cepacea*, and *C. rugosa* using waste frying oil and found that *P. fluorescens* lipase exhibited the highest production of fatty acid methyl esters, 63.8% after optimizing the reaction conditions along with the stepwise addition of methanol (Charpe and Rathod 2011). In the present study, only the crude lipase (supernatant) was used without subjecting it to any further purification steps; yet it could *trans*-esterify the free fatty acids upto 45% which can be further enhanced by optimizing the reaction conditions. Thus, from this study, it is evident that the thermotolerant and alkaline lipase from *Pseudomonas* sp. BUP6 is a good candidate for *trans*-esterification reaction.

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Compliance with ethical standards

Conflict of interest The authors also declare that there exist no competing interests.

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