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Content

Characterization of a novel alkaline lipase from *Pseudomonas* sp. HCU2-9 for detergent formulation

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Abstract
A novel alkaline lipase was purified and characterized from *Pseudomonas* sp. HCU2-9. The lipase was purified to homogeneity using ammonium sulphate precipitation and gel filtration chromatography. The molecular weight of lipase was approximately 45 kDa by SDS-PAGE. The lipase showed maximal activity at pH 9 and 60°C, respectively. The enzyme was stable in the pH range 5-9 for 30 min and at 45-55°C for 60 min. Higher activity was observed in the presence of Ca^{2+}, Mg^{2+}, Mn^{2+} ions, Tween 80 and Triton X-100 while Co^{2+}, Cu^{2+}, Zn^{2+} ions, DTT and PMSF showed inhibitory effect. The enzyme hydrolyzed both synthetic and natural triglycerides with maximum activity for tripalmitin and palm oil, respectively. It also hydrolyzed esters of *p*-nitrophenol with highest activity for *p*-nitrophenyl palmitate. Organic solvents were found to have little effect on the activity of the lipase. The enzyme showed stability towards selected commercial detergents, oxidizing agents and protease. The remarkable resistance capability of the lipase makes it a potential additive for better detergent formulation.

Keywords: characterization, detergent, lipase, oxidizing agent, purification

Introduction

Lipases (acylglycerol acylhydrolase, EC 3.1.1.3) are an important group of biotechnologically valuable enzymes. Although lipases have been found in many species of animals, plants, bacteria, yeast and fungi. The enzymes from microorganisms are the most interesting because of their potential applications in various industries such as dairy, food, pharmaceutical, paper, biodiesel, textile, cosmetic industries and chemical industries (Saxena et al., 1999; Jaeger and Eggert, 2002). As for industrial enzymology, lipases are commonly included in detergents (Liu et al., 2009). As a detergent additive, the increasing usage of alkaline lipase is mainly due to its affiliation with the nonphosphate detergents. At low temperature (especially below 40°C), capability of removing fat-containing dirt is very poor if the alkaline lipase become nonfunctional. Ideally, alkaline lipase in a detergent should have high activity and stability over a broad range of temperature and pH. Moreover, alkaline lipases should be also compatible with different components in a detergent including surfactants, metal ions, oxidants and proteases (Wang et al., 1995).

A considerable number of bacterial lipases have been commercially produced. Of these, the important ones are *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas* (Jaeger et al., 1994; Palekar and Vasudevan, 2000). As for industrial enzymology, lipases are commonly included in detergents. For this reason, increasing numbers of lipases have been exploited by researchers in the detergent industry for example, *B. subtilis* DR8806 and *P. stutzeri* PS59 (Shirin et al., 2013; Li et al., 2014). However, very few information were available on the compatibility of the lipases with detergent ingredients (Mohamed et al., 2000; Liu et al., 2009)

In present study, a numbered of lipase-producing bacteria in soil were isolated and optimized the production of lipase by using the waste of industrial vegetable oil as a carbon source. After optimization of culture conditions, the enzyme was purified by using ammonium sulphate precipitation and gel filtration chromatography. The lipase from the novel isolated bacterium was also characterized and evaluated as an additive for detergent formulation.

Materials and methods

Microorganism

A bacterial strain was isolated from an oil-contaminated soil that showed the highest lipolytic activity. The initial assay to detect lipolytic activity was done on lipase test medium (Tween 80 agar) containing, per litre of distilled water, 15 g agar, 10 g peptone, 5 g NaCl, 0.10 g $CaCl_2.2H_2O$ and 10 ml Tween 80. The lipolytic activity was indicated by the zone of white sediment after it had been incubated for 24 h at 37°C. Then, agar well diffusion was done on lipase test medium (Tween 80 agar) and tributyrin agar plates containing, per litre of distilled water, 15 g agar and 15 ml tributyrin. The lipolytic activity was indicated by the clear zone after it had been incubated for 24 h at 37°C. Bacterial isolate was completely identified and characterized based on morphological and biochemical characteristics.

Enzyme production

The strain was inoculated into 25 ml of basal medium containing, per litre of distilled water, 0.2% (w/v) glucose, 0.5% (w/v) tryptone, 0.01% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) K_2HPO_4 supplemented with 2% (v/v) olive oil [partially modified basal medium of Silva et al. (2005)] in 125-ml Erlenmeyer flasks incubated on rotary shaker at 200 rpm for 18-24 h at room temperature (27-30^{o}C). At which time it was approximately with 0.08-0.1 of OD_{600}, 0.5 ml of culture was inoculated into 250 ml of basal medium supplemented with 2% (v/v) wastewater from palm oil refining process in 500-ml Erlenmeyer flasks incubated on rotary shaker at 200 rpm for 24 h at room temperature (27-30^{o}C).

Purification of lipase

In the first step of purification, ammonium sulphate precipitation was carried out overnight at 4^{o}C at final concentration of 80% saturation. Following centrifugation at 13,200g for 40 min at 4^{o}C, the pellet was dissolved in 50 mM sodium phosphate with 0.15 M NaCl buffer pH 7.0 and dialyzed against distilled water containing 1 mM EDTA. The dialyzed enzyme was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate its extent of purity. Gel filtration was used for a subsequently purification step. The dialyzed enzyme was applied onto a pre-equilibrated HiPrep 16/60 Sephacryl S-100 column (Pharmacia, Sweden). The purification was operated at the flow rate of 0.1 ml/min. Fractions (0.5 ml) were collected automatically by AKTA FPLC collector (Amersham Biosciences, Germany). Fractions with lipase activity were dialyzed extensively and run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate lipase purity. Protein concentration and lipase activity were also assayed.

Lipase assay

Lipase activity was measured spectrophotometrically using *p*-nitrophenyl palmitate (*p*NPP) (Sigma) as the substrate. The assay mixture consisted of 0.1 ml of sample mixed with 0.9 ml of substrate solution containing 10 mg of *p*NPP dissolved in 1 ml propan-2-ol diluted in 9 ml of 50 mM Tris-HCl pH 8.0 containing 40 mg of Triton X-100 and 10 mg of gum arabic. The assay mixture was incubated at 37°C for 30 min and the *p*-nitrophenol released was measured at 410 nm. One unit of activity was defined as the amount of enzyme that liberated 1 nanomole of *p*-nitrophenol per min under the assay conditions (Maia et al., 2001).

Protein measurement

The protein concentration of enzyme preparations was measured according to the method described by Bradford (1976) with bovine serum albumin as the standard.

Gel electrophoresis and activity detection

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemli (1970). After the electrophoresis, the method of Lima et al. (2004) was used to detect lipolytic activity.

Characterization of the purified lipase

Effect of pH on activity and stability

Activity assays were done using the *p*NPP method at 37°C in assay mixtures buffered to various pH values. The following buffers were used to study the effect of pH and type of buffer on activity and stability of purified lipase: citrate-phosphate pH 5-6; phosphate pH 7; Tris-HCl pH 8; glycine-NaOH pH 9-10, all at 50 mM. Stability assays were done by incubating the purified lipase at 37°C for 30 min in buffers of different pH values (citrate-phosphate pH 5-6; phosphate pH 7; Tris-HCl pH 8; glycine-NaOH pH 9-10, all at 50 mM). The residual lipase activity was measured at 37°C using 50 mM Tris-HCl buffer pH 8.0 by the *p*NPP method.

Effect of temperature on activity and stability

Activity assays were done using the *p*NPP method with 50 mM Tris-HCl buffer pH 8.0, at 40, 45, 50, 55 and 60°C. Stability assays were done by incubating purified lipase at 40, 45, 50, 55 and 60°C for 60 min prior to the activity assay. Residual lipase activity was measured at 37°C using 50 mM Tris-HCl buffer pH 8.0 by the *p*NPP method.

Effect of various substances on purified lipase activity

The purified lipase was incubated in the presence of various substances including metal ions (1 mM), surfactants (0.1%, w/v), emulsifier (0.1%, w/v), inhibitor (1 mM) and oxidizing agents (0.1%, w/v) at 37°C for 30 min. Residual lipase activity was then measured using the *p*NPP method.

Evaluation of purified lipase as an additive for detergent formulation

The purified lipase was biochemically characterized for its potential usage in the detergent industry and all experiments were done in triplicate. The effects of various commercial detergents (1%, w/v) on lipase activity were examined by incubating the purified lipase at pH 9 at 50°C for 60 min. Residual lipase activity was measured at 37°C using 50 mM Tris-HCl buffer pH 8.0 by the *p*NPP method.

Effect of various organic solvents on purified lipase stability

The stability of purified lipase in organic solvents was investigated (Ogino et al., 2000) by appropriately mixing 3 ml of purified lipase and 1 ml of solvent in crew cap vials to obtain a final solvent concentration of 25% (v/v). The solution was incubated in shaker (150 rpm) at 37°C for 0-4 h and residual lipase activity was measured in the aqueous phase.

Substrate specificity of purified lipase

The purified lipase was used to study its ability on hydrolyze *p*NP esters and triacylglycerides. In case of *p*NP esters (*p*-nitrophenyl caprate, laurate and palmitate), the experiments were determined colourimetrically by the *p*NPP method. For triacylglycerides (tributyrin, trilaurin, tripalmitin and triolein) and oils (coconut oil, corn oil, olive oil, palm oil, soybean oil and sunflower oil), the experiments were determined by colorimetric method for determination of free fatty acids.

Statistical analysis

An analysis of variance (ANOVA) was used to compare the results. A post-hoc test was conducted to determined differences among treatment means using Turkey HSD following a check for homogeneity of variance by the Levene test. Means with $P < 0.05$ were considered to be significantly different

Results and discussion

Lipolytic activity

Sixty-five strains of bacteria were screened with respect to production of lipase. Detection of lipolytic activity on lipase test medium was carried in a preliminary study, followed in order by agar well diffusion by using tributyrin agar and lipase activity was then measured using the *p*NPP method.

Consequently, a strain HCU2-9 was isolated as the best producer and finally identified as *Pseudomonas* sp. HCU2-9 by conventional biochemical methods.

Purification of lipase

The purification scheme is summarized in Table 1. The enzyme was purified 36.3-fold with 38.9% recovery. The purity was confirmed by the presence of a single band on SDS-PAGE suggesting that *Pseudomonas* sp. HCU2-9 secretes only one extracellular lipase. The molecular weight of the purified lipase was estimated to be 45 kDa by SDS-PAGE and zymogram assay.

The purification of an extracellular lipase from *Pseudomonas* sp. HCU2-9 using a two-step procedure was similar to previous reports on lipase purification from other by conventional precipitation and chromatography. Examples include purification of lipase from *Pseudomonas* sp. DMVR46 (Patel et al., 2014) and *P. aeruginosa* AAU2 (Bose and Keharia, 2013).

Table 1: Summary of lipase purification from *Pseudomonas* sp. HCU2-9

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude lipase	2540	252	10.1	100	1
Ammonium sulfate precipitation	1623	59.6	27.2	63.9	2.7
Sephacryl S-100 HR column	989	2.7	366.3	38.9	36.3

Characterization of purified lipase

Effect of pH on the activity and stability of purified lipase

Lipase activity of *Pseudomonas* sp. HCU2-9 was maximal at pH 9 (Table 2). Even at more acidic pH values, lipase activity was observed with value at pH 5 being 89.16% of the maximum activity. The lipase was stable between pH 5 and pH 9 after incubation for 30 min at 37°C (Table 2). Nevertheless, this is similar to other bacterial lipases. Results were reported by Sarkar et al. (2012) for lipase from *Staphylococcus aureus* with maximum activity and stability at pH 8 and pH 9, respectively. Li et al. (2014) also reported the lipase from *P. stutzeri* PS59 with maximum activity at pH 8.5. Since the lipase from *Pseudomonas* sp. HCU2-9 was found to be active and stable over a wide pH range of 5 to 9 which indicates its potential applicability in the laundry industry.

Table 2: Effect of pH on the activity and stability of purified lipase from *Pseudomonas* sp. HCU2-9

pH	Relative activity (%)	
	Lipase activity	Lipase stability
5	89.16 ± 0.24	90.12 ± 0.24
6	94.08 ± 0.11	92.56 ± 0.11
7	97.45 ± 0.06	95.40 ± 0.15
8	98.05 ± 0.18	97.19 ± 0.04
9	100.00 ± 0.16	100.00 ± 0.09
10	87.30 ± 0.02	88.22 ± 0.12

Effect of temperature on the activity and stability of purified lipase

The optimal temperature for lipase activity was 60°C (Table 3). Results show that the enzyme could hydrolyzed (*p*NPP) at 40°C (approximately 85% relative activity) and the activity gradually increased with increasing temperature until it reached the optimum at 60°C. The optimal temperature of 60°C was similar to that of lipase from *Aeromonas caviae* AU04 (Velu et al., 2012). The purified lipase from *Pseudomonas* sp. HCU2-9 was stable from 45 to 55°C (Table 3) and it retained 94.25% of it activity for 60 min at 60°C.

Table 3: Effect of temperature on the activity and stability of purified lipase from *Pseudomonas* sp. HCU2-9

Temperature (°C)	Relative activity (%)	
	Lipase activity	Lipase stability
40	85.12 ± 0.35	90.48 ± 0.10
45	89.08 ± 0.15	100.01 ± 0.06
50	94.16 ± 0.24	98.09 ± 0.13
55	96.25 ± 0.19	97.05 ± 0.04
60	100.00 ± 0.05	94.25 ± 0.11

Effect of various substances on purified lipase stability

It was observed that DTT and PMSF strongly inhibited lipase activity (Table 4). On the other hand, lipase activity was stimulated by Ca^{2+}, Mg^{2+}, Mn^{2+} ions, surfactants and emulsifier. Moreover, the enzyme exhibited significant stability in the presence of commercial detergents and oxidizing agents. Stimulation of *Pseudomonas* sp. HCU2-9 lipase by Ca^{2+}, Mg^{2+}, Mn^{2+} ions had some similarity to the results of Sarkar et al. (2012) and Li et al. (2014). For inhibition of lipase of *Pseudomonas* sp. HCU2-9 by DTT indicated that the presence of a sulphur containing animo acid(s) at its active site (Lima et al., 2004) and inactivation of the lipase by PMSF may be caused by modification of an essential serine residue that plays a key role in the catalytic mechanism (van Oort., 1989). The enzyme exhibited significant stability in the presence of commercial detergents and oxidizing agents. Results suggest that purified lipase of *Pseudomonas* sp. HCU2-9 can be considered as a potential candidate to be used as in biotechnology and essentially for further commercial utilization as a additive in detergent formulations.

Table 4: Effect of various substances on the activity of *Pseudomonas* sp. HCU2-9 lipase

Compounds	Relative activity (%)[a]
Control[b]	100.00 ± 0.04
Metal ions (1 mM)	
$AlCl_3$	98.36 ± 0.32
$BaCl_2$	95.11 ± 0.22
$CaCl_2$	150.08 ± 0.12
$CuSO_4$	35.13 ± 0.29
$CoCl_2$	44.47 ± 0.18
$MgCl_2$	158.25 ± 0.25
$MnCl_2$	142.16 ± 0.65
$ZnCl_2$	67.30 ± 0.02
Surfactants (0.1%, w/v)	
Tween 20	125.25 ± 0.23
Tween 80	132.13 ± 0.09
Triton X-100	135.11 ± 0.18
SDS	120.05 ± 0.06
Sodium cholate	113.10 ± 0.17
Emulsifier (0.1%, w/v)	
Gum arabic	123.12 ± 0.49
Gelatin	128.16 ± 0.14
Inhibitor (1 mM)	
β-mercaptoethanol	58.13 ± 0.16
EDTA	98.02 ± 0.18
DTT	20.14 ± 0.15
PMSF	18.06 ± 0.11

Compounds	Relative activity (%)[a]
Oxidizing agents (0.1%, w/v)	
Hydrogen peroxide	99.26 ± 0.28
Sodium hypochlorite	98.11 ± 0.26
Detergents (1%, w/v)	
Breeze power	95.78 ± 0.32
Pao silver nano	91.22 ± 0.89
SA8 premium	69.45 ± 0.13
Breeze excel liquid	89.15 ± 0.75
Hygiene Laundry Detergent	92.23 ± 0.26
Fineline machine wash detergent	83.12 ± 0.33
Enzyme (0.05 mg/ml)	
Protease	89.12 ± 0.22

[a] The relative activity was calculated by using the control as a reference (100%).
[b] Control was represented the assay mixture without the addition of any substance.

Effect of various organic solvents on purified lipase stability

Effect of various organic solvents on the stability of *Pseudomonas* sp. HCU2-9 lipase are shown in Table 5. The enzyme was found to be quite stable and active in most of the organic solvents. The highest stability was achieved in hexane and isopropanol with the relative lipase activity of 124.29% and 114.08%, respectively after 4 h. The activation of lipase could be explained by the interaction of organic solvents with hydrophobic amino residues present in the lid that covers the catalytic site of the purified lipase, thereby maintaining the lipase in its open conformation (Singh and Banerjee, 2007)

Table 5: Effect of various organic solvents on the stability of purified lipase from *Pseudomonas* sp. HCU2-9

Organic solvents (25%, v/v)	Relative activity (%)[a]
Control[b]	100.00 ± 0.04
Acetone	84.28 ± 0.50
Chloroform	89.18 ± 0.36
Ethanol	87.15 ± 0.45
Hexane	124.29 ± 0.35
Isopropanol	114.08 ± 0.29
Methanol	91.13 ± 0.19
Toluene	92.17 ± 0.08

[a] The relative activity was calculated by using the control as a reference (100%).
[b] Control was represented the assay mixture without the addition of any organic solvent.

Substrate specificity of purified lipase

The hydrolytic rate of the lipase towards various synthetic triglycerides and *p*-nitrophenyl esters was examined. As shown in Table 6, the enzyme hydrolyzed all synthetic triglycerides and maximum activity was obtained with tripalmitin. Activity on *p*-nitrophenyl palmitate (*p*NPP) was 10-fold higher than that on *p*-nitrophenyl caprate (*p*NPCA). Similar results were proposed by Saxena et al. (2003). It could be confirmed that this enzyme has greater specificity for long-chained triacylglycerols. The enzyme also hydrolyzed all vegetable oils, with the highest activity obtained using palm oil.

Table 6: Specificity of purified lipase from *Pseudomonas* sp. HCU2-9 in respect of various lipids

Substrate	Relative activity (%)[a]
Triacylglycerols[b]	100.00 ± 0.04
Tributyrin	75.19 ± 0.32
Trilaurin	78.15 ± 0.04
Tripalmitin	100.00 ± 0.08
Triolein	95.33 ± 0.27
p-nitrophenylesters[c]	
p-nitrophenyl caprate (*p*NPCA)	12.11 ± 0.32
p-nitrophenyl laurate (*p*NPL)	45.03 ± 0.21
p-nitrophenyl palmitate (*p*NPP)	100.00 ± 0.04
Oils[d]	
Coconut oil	85.42 ± 0.19
Corn oil	79.23 ± 0.05
Olive oil	95.63 ± 0.16
Palm oil	100.00 ± 0.03
Soybean oil	95.04 ± 0.09
Sunflower oil	75.15 ± 0.11

[a] The relative activity was calculated by using the tripalmitin, *p*-nitrophenyl palmitate and palm oil as the reference (100%).
[b] Activity toward tripalmitin was accepted as 100%
[c] Activity toward *p*NPP was accepted as 100%
[d] Activity toward palm oil was accepted as 100%

Conclusions

In conclusion, a novel alkaline lipase from *Pseudomonas* sp. HCU2-9 was purified and characterized. The lipase has an optimal pH and temperature of pH 9 and 60°C, respectively. Moreover, the analysis of the stability of the enzyme in the presence of various metal ions, surfactants, emulsifier, oxidizing agents, commercial detergents, organic solvents and protease, as well as its broad substrate specificity, demonstrates that the enzyme exhibits potential as a commercial additive in detergents. Further research is needed to study the mechanism of the high-washing performance of the *Pseudomonas* sp. HCU2-9 lipase.

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