Purification and characterization of alkaline lipase production by *Pseudomonas aeruginosa* HFE733 and application for biodegradation in food wastewater treatment

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ABSTRACT

An alkaline lipase strain of *Pseudomonas aeruginosa* HFE733 was isolated from soil samples of domestic waste. The alkaline lipase from *P. aeruginosa* HFE733 was purified and characterized. The enzyme was purified 9.97-fold by means of ammonium sulphate precipitation, Sephadex G-25 and diethylaminoethyl (DEAE) cellulose chromatography. Purified alkaline lipase protein was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the molecular mass was 51.0 kDa. The enzyme exhibited maximum activity at 40 °C and pH 8.5. The enzyme is stable at pH 7.0–8.5. It was remarkably activated by some metal ions and chemical reagents, such as Fe³⁺, Al³⁺, β-mercaptoethanol, cysteine, DL-dithiothreitol (DTT), Tween 80 and Triton X-100, but suppressed by sodium dodecyl sulfate (SDS) at 10 mmol/L. The *P. aeruginosa* HFE733 strain and lipase exhibited remarkable potential for biodegradation of oil and organics (measured as chemical oxygen demand (COD)). We demonstrated that it can be used for biodegradation of food wastewater from restaurants.

KEYWORDS

*Pseudomonas aeruginosa*; alkaline lipase; purification; characterization; application

Introduction

Lipases known as triacylglycerol acylhydrolases (E.C. 3.1.1.3) are ubiquitous carboxylic ester hydrolases that can catalyze hydrolysis of the long chain triglycerides to fatty acids, diacylglycerol, monoacylglycerol and glycerol [1]. However, in non-aqueous medium, lipases synthesise esters from glycerol and long-chain fatty acids [2]. Lipases are largely produced from bacterial strains like *Pseudomonas alcaligenes* [3], *Pseudomonas aeruginosa* [4–7], *Pseudomonas fragi* [8], *Bacillus subtilis* [9] etc. and fungi like *Penicillium expansum* [10], *Trichoderma* [11], *Penicillium chrysogenum* [12], *Aspergillus* [13] etc. Besides hydrolysis activity, lipases also display interesterification, esterification, aminolysis and alcoholysis activity [14], which has contributed to their wide use in pharmaceuticals, paper manufacture, textile, food, detergents and cosmetics manufacture industries [15,16].

In recent years, more and more food wastewater is discharged in China, causing increasingly serious environmental pollution. Oil films on water surfaces prevent the diffusion of oxygen from air into water, which leads to the death of many aquatic species. Biodegradation of lipid-rich wastes has long been carried out [17,18]. Furthermore, many reports have described the use of lipolytic enzymes in wastewater treatment [4,19]. Microbial strains and lipases could have an impact on reducing the fats and oil content in food wastewater, and may help in reducing severe pollution problems caused by wastewater.

In this study, we report the isolation of an alkaline lipase strain of *Pseudomonas aeruginosa* HFE733 from soil samples of domestic waste. We purified and characterised the alkaline lipase from *P. aeruginosa* HFE733, and applied *P. aeruginosa* HFE733 strain and its lipase for treatment of restaurant wastewater.

Materials and methods

Chemicals

Ethylene diamine tetraacetic acid (EDTA), DL-dithiothreitol (DTT), glutathione (GSH) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Microorganism

Lipase-producing strains were isolated from soil samples from sites of stacking domestic waste. The microorganisms were grown on Gauze-agar (peptone 10 g/L, yeast
Production of lipase

The optimized fermentation medium was composed of: olive oil 5.92 mL/L, yeast extract 34 g/L, cane sugar 12.5 g/L, copper sulfate 0.4 g/L and manganese sulfate 0.2 g/L. The pH of the medium was adjusted to 7.0. Erlenmeyer flasks (250 mL) containing 50 mL sterile culture medium were inoculated with 5 mL inoculums (6.2 × 10⁸ CFU/mL). The flasks were incubated at 30 °C for 60 h on an orbital shaker. The microbial suspension was centrifuged at 6000 g for 10 min, and the clear supernatant was assayed for lipase activity.

Lipase activity assay

Lipase activity was determined by an olive oil emulsion method [20]. The substrate was prepared by mixing 50 mL of olive oil with 150 mL of polyvinyl alcohol solution (2%, w/v) to obtain emulsion. The reaction mixture consisting of 5.0 mL of phosphate buffer (0.25 mol/L, pH 7.5), 4 mL of the substrate emulsion above and a 1.0 mL crude enzyme extract was incubated for 15 min at 40°C in a vessel. After 15 min, the reaction was stopped, 15 mL 95 wt% ethanol reagent was added, and the amount of free fatty acids released in the reaction was titrated with 0.05 mol/L of sodium hydroxide solution in the presence of phenolphthalein indicator. In addition, a blank experiment for comparison without adding crude enzyme was carried out by the above-described assay procedure. One unit (1 U) of lipase activity is defined as the amount of enzyme required to release 1 μmol free fatty acids per minute under the assay conditions. The relative activity presented in the figures is the rate (%) of enzyme activity at other test conditions relative to the highest enzyme activity at one test condition.

Purification and determination of the molecular weight of lipase

The culture supernatant was subjected to 80% saturation of ammonium sulphate and after overnight preservation at 4 °C, the precipitated enzyme solution was separated by centrifugation (9408 g, 15 min), and then was dissolved in phosphate buffer (0.25 mol/L, pH 7.5). Then the enzyme was desalinated at 0.5 mL/min with phosphate buffer (0.25 mol/L, pH 7.5) by a Sephadex G-25 column (0.9 × 30 cm). The enzyme was concentrated by ultrafiltration membrane (Millipore, USA) using centrifuge (6000 g, 25 min). Finally, the partially purified enzyme was passed through a diethylaminoethyl (DEAE) cellulose column (3 × 20 cm) and active enzyme was eluted by Tris-HCl (pH 8.5) with a gradient of NaCl (0.1–0.6 mol/L) at a flow rate of 2 mL/min. The fractions with enzyme activity were collected, concentrated by lyophilization and used for further experiments.

The enzyme from the DEAE cellulose column was loaded onto sodium dodecyl sulfate polyacrylamide gel (12%) electrophoresis (SDS-PAGE) following the method of Laemmli [21]. The molecular weight of the lipase was determined by comparing its mobility with the medium range marker proteins (14.3–97.2 kDa). Protein concentration was measured following the method of Lowry et al. [22].

Effects of temperature on enzyme activity and stability

The effect of temperature on lipase activity was determined by performing an enzymatic hydrolysis reaction for 15 min at various incubation temperatures (30, 35, 40, 45, 50 and 55 °C). To assay the thermostability, the residual enzyme activities were measured after pre-incubating the enzyme at different temperature (30, 40, 50, 60 and 70 °C) for 1, 2, 3 and 4 h without substrate.

Effects of pH on enzyme activity and stability

The optimal pH of lipase was determined by performing reactions in 50 mmol/L sodium acetate–acetic acid (pH 5.0–5.5), phosphate buffer (pH 6.0–7.5), Tris-HCl (pH 8.0–8.5) and glycine-NaOH (pH 9.0–10.0), respectively. The effect of pH on lipase activity was investigated by performing enzymatic hydrolysis reactions at various pH levels (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0) for 15 min. Stability of the enzyme at different pH was studied by incubating the enzyme (without substrate) at different pH ranging from 5.0 to 10.0 for up to 4 h at 30 °C. Then its activity was measured.

Effect of metal ions and chemical reagents on lipase activity

The effects of metal ions on enzyme activity were determined by assaying the residual activity after the enzyme was incubated with 1 and 10 mmol/L of various metal ions: Na⁺ (NaCl), K⁺ (KCl), Mg²⁺ (MgCl₂), Ca²⁺ (CaCl₂), Mn²⁺ (MnSO₄·H₂O), Fe²⁺ (FeCl₂), Co²⁺ (CoCl₂), Cu²⁺ (CuSO₄·5H₂O), Zn²⁺ (ZnCl₂), Ba²⁺ (BaCl₂), Li⁺ (Li₂SO₄), Fe³⁺ (FeCl₃), Pb²⁺ (Pb(NO₃)₂) and Al³⁺ (Al₂(SO₄)₃·16H₂O). No metal ion was used in the control sample solution. Some
of the modifying reagents and amino acids (EDTA, DTT, \(\beta\)-mercaptoethanol, cysteine, GSH, SDS, Tween 20, Tween 80 and Triton X-100) were also tested to study their effect on lipase activity.

**Biodegradation in food wastewater treatment**

The food wastewater was collected from a restaurant in Wuhan China. In this experiment, 10% (v/v) fermentation broth (including cells and enzymes) was added to food wastewater and was cultured for 30 °C at 180 rpm. The oil concentration and chemical oxygen demand (COD) were determined, to analyze the degradation ability. Two control experiments were performed, in which no fermentation broth was added to restaurant wastewater: control 1, with incubation at 30 °C and 180 rpm; control 2, with stewing at 30 °C.

Oil concentration was determined according to the gravimetric method [23]. Chemical oxygen demand (COD) was determined by dichromate titration [23]. Sampling was carried out every 24 h. All the experiments were performed in triplicate, and the average was calculated.

**Data analysis**

The data are presented as mean values from triplicate experiments with standard deviation (±SD). Data analysis was performed using Origin 8.0.

**Results and discussion**

**Microorganisms**

One isolated strain of HFE733 showed the maximum lipase activity of 9.27 U/mL. Strain HFE733 was identified and deposited by the China Center for Type Culture Collection (CCTCC, Wuhan, China) with a culture collection number of CCTCC M 2015 735. 16S rRNA were sequenced and subjected to phylogenetic analysis to further identify strain HFE733. Nucleotide-nucleotide BLAST in NCBI results showed that strain HFE733 has more than 99% sequence similarity with the first hit of *Pseudomonas aeruginosa*. Based on the 16SrRNA gene sequence, a phylogenetic tree was made (Figure 1), and it showed 99.58% sequence similarity to *P. aeruginosa* (BAMAO1000316). According to the results reported above, it is reasonable to identify strain HFE733 as *P. aeruginosa*.

**Purification of the lipase from *P. aeruginosa* HFE733**

Purification of the enzyme was performed through the following steps: ammonium sulphate precipitation (Figure 2), a Sephadex G-25 column (Figure 3) and DEAE cellulose column chromatography (Figure 4). All purification steps are summarized (Table 1), which shows 9.97-fold purification with a recovery of 31.54% and specific activity of 79.25 U/mg protein.

The SDS-PAGE experiments showed that the enzyme reaches electrophoresis type of purity with a molecular mass of approximately 51.0 kDa (Figure 5) calculated using Syngene G: BOX (USA). This result is similar to the molecular mass reported for lipase from other *P. aeruginosa* strains, such as *P. aeruginosa* LX1 (56 kDa) [5] and *P. aeruginosas* an-ai (54 kDa) [6].

**Effects of temperature on enzyme activity and stability**

We studied the effect of temperature on lipase activity over the range 30–55 °C (Figure 6(a)). The lipase exhibited the highest activity at 40 °C, which was similar to the corresponding enzymes from *Burkholderia* sp. ZYB002 [24] and *P. aeruginosa* LX1 [5]. The purified lipase was stable between 30 and 40 °C, retaining approximately 70% of its activity, but the thermal stability of the enzyme declined at 50–70 °C (Figure 6(b)).

**Effects of pH on enzyme activity and stability**

The effect of pH on lipase activity is shown in Figure 6(c). The lipase was active at pH 6.0–9.5 and exhibited the highest activity at pH 8.5, indicating that this lipase was an alkaline enzyme, and was stable at pH 7.0–8.5. After incubation in buffers within a pH range of 5.0–10.0 for 4 h, the enzyme retained more than 70% of its activity (Figure 6(d)). In consistence with the present study, the activity of lipase produced by *Halobacillus* sp. AP-MSU 8 [25] and *Xanthomonas oryzae* pv. oryzae YB103 [26] was reported to reach a maximum at pH 9.0. Alkaline lipases are now popularly explored for a large variety of industrial purposes.

**Effect of metal ions and chemical reagents on lipase activity**

Among the different metal ions studied for their influence on alkaline lipase activity, the activity of the lipase was strongly influenced by the presence of metal ions (Table 2). The enzyme showed the maximum stimulation by Fe³⁺, similar to the lipase from *Bacillus licheniformis* MTCC 2465 [27]. The enzyme activity was also strongly stimulated by Al³⁺. However, the lipase activity was inhibited by the other tested metal ions (Table 2). The inhibitory nature of metals has been thought to be due to interaction of methal ions with the charged side chain...
groups of surface amino acids, thus influencing the conformation and stability of the enzyme [28].

The effects of inhibitors and surfactants on lipase activity are presented in Table 3. The enzyme was activated by β-mercaptoethanol and cysteine. This may be explained by β-mercaptoethanol counteracting the oxidative effect of the S–S bond formed between two cysteine residues [29]. The addition of DTT also had a
strong stimulation on enzyme activity, indicating that the thiol group could be essential for the activity of this enzyme. The lipase was stable in the presence of EDTA, suggesting that no metals are in or close to the active site of the enzyme. The addition of GSH resulted in non-remarkable stimulation or inhibition, suggesting that the sulfhydryl group of the enzyme is stable.

Surfactants are known to reduce the interfacial tension between oil and water and to increase the lipid–water interface area, which enhances the rate of lipase catalyzed reactions in return [7,30]. However, different surfactants had different effects on lipase activity. In this study, the lipase was inhibited by the non-ionic surfactants such as Tween 80 and Triton X-100, similar to the corresponding enzymes from *Pseudomonas* [31] and *Pseudomonas cepacia* [32]. The strong inhibition by 10 mmol/L SDS could be due to local conformation changes in the active site of the enzyme molecule, resulting in inhibition, partial reversible unfolding and subsequent inactivation.

Table 1. Purification of lipase from *Pseudomonas aeruginosa* HFE733.

| Purification steps | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification fold |
|--------------------|--------------------|--------------------|--------------------------|-----------|-------------------|
| Crude extract      | 200.94             | 1598.00            | 7.95                     | 100.00    | 1.00              |
| (NH₄)₂SO₄          | 64.40              | 1226.00            | 19.04                    | 76.72     | 2.39              |
| Sephadex G-25      | 20.16              | 1184.00            | 58.58                    | 74.09     | 7.37              |
| Diethylaminoethyl (DEAE) DE-52 | 6.36 | 504.00 | 79.25 | 31.54 | 9.97 |

Figure 4. DEAE cellulose chromatography elution from Sephadex G-25 chromatography.

Figure 3. Sephadex G-25 chromatography elution from ammonium sulphate precipitation.

Figure 2. Ammonium sulphate precipitation from the clear supernatant.

Figure 5. SDS–PAGE of purified lipase from *Pseudomonas aeruginosa* HFE733. Lane M, protein size marker; Lane 1, crude enzyme; Lane 2, enzyme purified after 80% ammonium sulphate precipitation; Lane 3, active fraction of Sephadex G-25 column; Lane 4, active fraction of DEAE cellulose anion-exchange column.
As a next step in our study, we evaluated the effect of the \textit{P. aeruginosa} HFE733 fermentation broth in oil-rich restaurant wastewater treatment. The results are shown in Figure 7(a) and (b). It was found that the initial oil content and COD in the food wastewater were 4296.00 mg/L and 11449.42 mg O$_2$/L, respectively. After 6 days of treatment, they decreased to 195.79 mg/L and 1243.97 mg O$_2$/L, respectively, whereas control 1 had 3179.06 mg/L and 4798.93 mg O$_2$/L, respectively, and control 2 had 3825.72 mg/L and 7460.28 mg O$_2$/L, respectively. After 6 days of treatment, the oil content

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
Additives & 1 mmol/L & 10 mmol/L \\
\hline
NaCl & 62.18 $\pm$ 0.00 & 63.64 $\pm$ 2.93 \\
KCl & 70.74 $\pm$ 0.00 & 80.40 $\pm$ 2.77 \\
MgCl$_2$ & 73.52 $\pm$ 3.07 & 101.17 $\pm$ 1.32 \\
CaCl$_2$ & 80.69 $\pm$ 3.66 & 115.9 $\pm$ 2.41 \\
MnSO$_4$·H$_2$O & 68.18 $\pm$ 0.07 & 78.79 $\pm$ 1.97 \\
FeCl$_3$ & 74.62 $\pm$ 1.68 & 119.53 $\pm$ 1.22 \\
CoCl$_2$ & 48.43 $\pm$ 4.03 & 50.69 $\pm$ 4.19 \\
CuSO$_4$·5H$_2$O & 48.79 $\pm$ 2.64 & 67.52 $\pm$ 1.76 \\
ZnCl$_2$ & 68.76 $\pm$ 3.44 & 104.17 $\pm$ 2.87 \\
BaCl$_2$ & 73.52 $\pm$ 3.29 & 92.17 $\pm$ 1.32 \\
Li$_2$SO$_4$ & 92.17 $\pm$ 2.71 & 85.44 $\pm$ 1.83 \\
FeCl$_3$ & 104.10 $\pm$ 1.24 & 161.45 $\pm$ 1.19 \\
Pb(NO$_3$)$_2$ & 79.74 $\pm$ 4.61 & 113.97 $\pm$ 3.83 \\
Al$_2$(SO$_4$)$_3$·16H$_2$O & 80.25 $\pm$ 2.63 & 143.16 $\pm$ 1.68 \\
Control & 100 $\pm$ 1.24 & 100 $\pm$ 1.24 \\
\hline
\end{tabular}
\caption{Effect of metal ions on activity of lipase from \textit{Pseudomonas aeruginosa} HFE733.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
Additives & 1 mmol/L & 10 mmol/L \\
\hline
EDTA & 77.99 $\pm$ 2.94 & 94.21 $\pm$ 2.42 \\
DTT & 126.23 $\pm$ 1.44 & 125.28 $\pm$ 1.97 \\
$\beta$-Mercaptoethanol & 89.14 $\pm$ 3.73 & 126.57 $\pm$ 5.64 \\
Cysteine & 110.06 $\pm$ 4.44 & 138.36 $\pm$ 1.28 \\
GSH & 101.01 $\pm$ 2.56 & 95.28 $\pm$ 3.06 \\
SDS & 112.58 $\pm$ 2.71 & 85.44 $\pm$ 1.83 \\
Tweeze 20 & 104.10 $\pm$ 1.24 & 161.45 $\pm$ 1.19 \\
Tweeze 80 & 79.74 $\pm$ 4.61 & 113.97 $\pm$ 3.83 \\
Al$_2$(SO$_4$)$_3$·16H$_2$O & 80.25 $\pm$ 2.63 & 143.16 $\pm$ 1.68 \\
Control & 100 $\pm$ 1.24 & 100 $\pm$ 1.24 \\
\hline
\end{tabular}
\caption{Effect of various reagents on the activity of lipase from \textit{Pseudomonas aeruginosa} HFE733.}
\end{table}


Biodegradation in food wastewater treatment

As a next step in our study, we evaluated the effect of the \textit{P. aeruginosa} HFE733 fermentation broth in oil-rich restaurant wastewater treatment. The results are shown in Figure 7(a) and (b). It was found that the initial oil content and COD in the food wastewater were 4296.00 mg/L and 11449.42 mg O$_2$/L, respectively. After 6 days of treatment, they decreased to 195.79 mg/L and 1243.97 mg O$_2$/L, respectively, whereas control 1 had 3179.06 mg/L and 4798.93 mg O$_2$/L, respectively, and control 2 had 3825.72 mg/L and 7460.28 mg O$_2$/L, respectively. After 6 days of treatment, the oil content...
decreased by 95.44% and COD, by 89.14%. These results suggested that the *P. aeruginosa* HFE733 fermentation broth was effective for oil removal and COD decrease, and can be used for food wastewater treatment. Similar studies have been carried out with other lipase-producing strains, such as *P. aeruginosa* SL-72 [33] and *Aspergillus awamori* BTMFW032 [34]. The crude lipase of *P. aeruginosa* SL-72 was added to wastewater contaminated with crude oil, resulting in degradation of 82.83% of the oil content and 86.39% reduction of COD after 7 days of treatment, slightly lower than the effect of the HFE733 fermentation broth. The lipase from *Aspergillus awamori* BTMFW032 was evaluated for the treatment of hospital wastewater containing ayurvedic oil; 91.4% of the oil was degraded, indicating a good degradation effect after the treatment.

The oil removal and COD decrease in control 1 and control 2 may be due to the role of indigenous microorganisms. The fermentation broth contains strains and lipases, which may co-act to remove the oil and decrease the COD value. The alkaline lipase from *P. aeruginosa* HFE733 can hydrolyse fats and oils into glycerol and fatty acids, and glycerol and fatty acids can be utilized by *P. aeruginosa* HFE733 strain. We are now studying this interaction further to explore optimal pretreatment conditions.

**Conclusions**

In this study, to avoid the excess wastewater pre-treatment process and high cost, the *P. aeruginosa* HFE733 strain and lipase were added to lipid-rich food wastewater. We observed remarkable oil removal and COD decrease effects, showing important application potential. The purified lipase from *P. aeruginosa* HFE733 was an extracellular enzyme with a molecular mass of 51.0 kDa. The enzyme exhibited maximum activity at 40 °C and pH 8.5, and was stable over range of pH values (7.0–8.5). The purified enzyme was remarkably activated by several metal ions and chemical reagents, such as Fe3⁺, Al3⁺, β-mercaptoethanol, cysteine, DTT, Tween 80 and Triton X-100, but was suppressed in the presence of 10 mmol/L SDS. Further, the *P. aeruginosa* HFE733 strain and lipase showed they have promising potential for biodegradation as part of food wastewater treatment.

**Disclosure statement**

The authors declare that they have no competing interests.

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