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**Biodegradation of waste greases and biochemical properties of a novel lipase
from *Pseudomonas synxantha* PS1**

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Abstract

A lipase-producing bacterial strain was isolated from oilwell produced water in Shengli oilfield (Shandong province, China) and identified as *Pseudomonas synxantha* by 16S rDNA sequence analysis (named *Pseudomonas synxantha* PS1). The strain PS1 showed maximum lipase activity of 10.8 U/ml after cultured for 48 h at 30°C, with lactose (4 g/l) as carbon source, tryptone (8 g/l) as nitrogen source, olive oil (0.5%, v/v) as inductor and the initial pH 8.0. Meanwhile, the lipase gene from *Pseudomonas synxantha* PS1 was cloned and expressed in *Escherichia coli* BL21 with the vector pET28a. The novel gene (*lipPS1*) has an open reading frame of 1425 bp, and encodes a 474-amino-acid lipase (LipPS1) sharing the most identity 87% to the lipase in *Pseudomonas fluorescens*. LipPS1 preferably acted on substrates with a long chain (C10–C18) of fatty acids. The optimum pH and temperature of the recombinant enzyme were 8.0 and 40°C towards the optimum substrate p-nitrophenyl palmitate respectively. The LipPS1 showed remarkable stability under alkaline conditions and was stable at pH 7.0–10.0 (retaining more than 60% activity). From the organic solvents tests, the lipase was activated by 15% (v/v) methanol (112%), ethanol (127%) and n-butyl alcohol (116%). LipPS1 presented strong biodegradability of waste grease, 93% waste grease was hydrolyzed into fatty acid after 12 h at 30°C. This is the first report of the lipase activity and lipase gene obtained from *Pseudomonas synxantha* (including wild strain and recombinant strain) and the recombinant LipPS1 with the detailed enzymatic properties. Also the preliminary study of the waste greases biodegradability shows the potential value in industry applications.

Keywords: Characterization; *Pseudomonas synxantha*; Lipase; Biodegradation

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are a class of enzymes that can catalyze the hydrolysis of long-chain triacylglycerols into fatty acids and glycerol. They are widely distributed in bacteria, yeasts, fungi, plants and animals (Pahoja and Sethar 2002). The lipases are widely used in various industries like food, dairy, chemical, textile, pharmaceutical, cosmetic, detergent production, and especially in biodiesel production and synthesis of new polymeric materials (Gupta et al. 2004). As containing so many applications, novel enzyme with distinct features such as thermostable, alkaline, high activities and stabilities in organic solvents is of interest for industrial applications (Fang et al. 2006).

Lipases from *Pseudomonas* species have attracted more attention since they have the potential application in detergent and chemistry industries. Although many *Pseudomonas* lipases genes from *Pseudomonas cepacia* (Dhake et al. 2013), *Pseudomonas aeruginosa* (Bose and Keharia 2013; Grbavc'ic et al. 2015), *Pseudomonas resinovorans* (Lee et al. 2012), *Pseudomonas stutzeri* (Maraite et al. 2013) and *pseudomonas fluorescence* (Hakiminia et al. 2013) have been cloned and sequenced, there is no report of *P. synxantha* lipase including wild strain lipase activity, lipase gene cloning from *P. synxantha* and recombinant strain. Recently, the increased waste oils and greases in waste water polluted the environment seriously (Kumar et al. 2012). Biodegradation and synthesis of biodiesel, two main waste grease processing solutions were recognized so far. Waste greases used for biodiesel production attracted people's attention and has been studied extensively (Tan et al. 2010; Luna et al. 2014). However, oils used for biodiesel production required pure enough. In other words, oils for transesterification could not contain water. But waste greases in these environments like rivers, lakes, ocean and sewer, were not suitable for synthesis of biodiesel. One reason was oil content was low, and the other was oil-water separation needing a large economic expenditure. So biodegradation became the effective treatment method of waste oil in the environment (Sangeetha et al. 2011). Lipase provided an excellent hydrolysis activity towards long-chain waste greases and played an important role in promoting the degradation of waste greases.

Previously, we have obtained a lipolytic strain which was identified as *Pseudomonas synxantha* (named *Pseudomonas synxantha* PS1). In this study, a novel lipase gene (*lipPS1*) was obtained from *P. synxantha* and the in-vivo functional expression of the *lipPS1* was successfully achieved. The enzyme characterizations including the enzyme activity/stability, optimum temperature,

optimum pH and conditions for lipase production were also described. This first report lipase from *Pseudomonas synxantha* would contribute to *Pseudomonas synxantha* strain research significantly. As showing high biodegradability towards waste greases, this alkaline-adapted lipase (LipPS1) showed potential value in industrial applications according to the enzyme characterizations especially in the detergent and environment industry.

Materials and Methods

Chemicals, strain screening and culture conditions

PrimeSTAR HS DNA Polymerase, LA Taq, T4 DNA ligase, DNA marker and restriction enzymes were purchased from TaKaRa Biotechnology Corporation (Otsu, Japan). Protein marker was purchased from MBI Fermentas (Vilnius, Lithuania). Isopropyl- β -D-thiogalactopyranoside (IPTG), ampicillin and kanamycin were purchased from Amresco (Shanghai Genebase Co., Ltd, China). DNA Mini kit and Plasmid Mini Prepare kit were purchased from Axygen Biosciences (Union City, CA, USA). *Escherichia coli* DH5 α (Invitrogen) and plasmid pMD19-T (TaKaRa) were used for gene cloning and sequencing. Plasmid pET-28a (Novagen) was the vector used to construct the protein expression plasmid in *E. coli* BL21 (DE3). The substrates p-nitrophenyl esters were bought from Sigma. Yeast extract and tryptone were obtained from Shanghai Sangon Biotechnology Co. Ltd (Shanghai, China). All other analytical chemicals were purchased at local markets.

In this work, enrichment culture technique was applied. LB medium added with 1% olive oil emulsion was used to isolate potential bacterial strains. One ml sample (oil well produced water) was added to a flask with 100 ml sterile distilled water. With activating on a rotary for 40 min, 0.1ml activation culture broth was inoculated into 100 ml enriched medium. Then the medium was shaken at 120 rpm, 30°C for 48 h. After several rounds of enrichment, inocula was serially diluted and plated onto Rhodamine B agar plates (Cai et al. 2014). The microbes showing obviously hydrolysis circle were isolated, purified and transferred to maintenance slants. The strain (PS1) showing the highest ratios of hydrolysis circles was selected and identified by 16S rDNA (GenBank accession numbers: KM232508) sequence analysis. Multiple sequence alignments were performed using DNAMAN and CLUSTAL W. The unrooted phylogenetic tree of 16S rDNA comparison was constructed using the MEGA program. The gene of 16S rDNA was amplified and sequenced using primers universal for the majority of prokaryotes (forward primer 5'-CCTACGGGAGGCAGCAG-3',

98 reverse primer 5'-ACGGGCGGTGTGTAC-3').

99 **Optimization of culture medium composition**

100 In an effort to optimize the medium components, the single-factor experiments method of adding different carbon sources,
101 nitrogen sources and greases in *P. synxantha* PS1 culture medium was used. Various carbon sources, nitrogen sources and greases
102 which were considered to increase extracellular lipase production by some microorganisms were used for lipase production
103 (Rajendran and Thangavelu 2007). Basal medium was composed of 1.0g/l NaCl, 0.5g/l NaH₂PO₄, 1.0g/l NH₄NO₃ and 0.5g/l KH₂PO₄.
104 The experiment was performed to verify the statistical results of optimized value of different factors and the medium was prepared
105 with these factors as constituents. The effects of the different carbon sources were studied as follows (4 g l⁻¹): lactose, maltose, starch,
106 sucrose and glucose. The effects of the different nitrogen sources were studied as follows (8 g l⁻¹) casein, tryptone, yeast extract,
107 soybean flour and peanut powder. To examine the effect of greases, five different greases 0.5% (v/v) (sesame oil, coconut oil,
108 sunflower oil, palm oil and olive oil) were compared in the lipase production. The *P. synxantha* PS1 was cultivated in 250 ml flasks
109 containing 50 ml of culture medium at 200 rpm and 30°C. After incubation the content of flask was centrifuged at 5000 g and the
110 supernatant was used as crude enzyme extract (Joshi et al. 2006).

111 **Gene cloning and lipPS1 analysis**

112 Based on the information of *Pseudomonas* spp. lipases in GenBank, degenerate primers (forward primer DP-*lip*PS1-F:
113 5'-TCSGGVCCCTCGGGAAA-3', DP-*lip*PS1-R: 5'-GTRTYGCCCTTRTGCG-3') for the lipase gene sequence were designed.
114 According to NCBI search, the following gene sequences of predicted lipase from *Pseudomonas* spp. were analyzed to design
115 degenerate primers: *P. fluorescens* LBUM223 (CP011117), *Pseudomonas* sp. KB700A (AB063391), *Pseudomonas simiae* WCS417
116 (CP007637), *Pseudomonas poae* RE1-1-14 (CP004045), *Pseudomonas fluorescens* PCL1751 (CP010896). The PCR amplification
117 was enforced with thermostable polymerase LA Taq in the GC buffer. The scheme of amplification included 30 cycles with the
118 following conditions: DNA denaturation at 94°C for 30 s, annealing of primers at 52°C for 30 s, and elongation at 72°C for 40 s. The
119 targeted fragment with approximate length as we anticipated was sequenced in two directions in BGI (China).

120 Then, genomic walking and nested PCR were employed to obtain the complete lipase sequence (CDS sequence) using TaKaRa

LA PCR™ *in vitro* Cloning Kit (TaKaRa). Target DNA upstream and downstream sequence of lipase was specifically amplified. Through sequences splicing and ORF sequences searching (NCBI ORF Finder tool <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), the complete lipase sequence (*lipPS1*) was obtained and primers LipPS1-F/LipPS1-R (forward primer *lipPS1*-F: 5'-CGGAATTCATGGGTGTGTTTACTA-3', reverse primer *lipPS1*-R: 5'- CCAAGCTTTTAACTGATGACAATCCCC-3', underlined nucleotides indicate restriction enzyme sites BamHI/XhoI) for the complete lipase sequence were designed. The complete *lipPS1* sequence (Genbank accession number: KT313129) was amplified by PCR with the following scheme: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension of 8 min at 72°C. The PCR products were cloned into pMD-19T simple vector after recovering by DNA gel extraction kit (Axygen, China), and then transferred into *E. coli* DH5a. The open reading frame (ORF) was predicted using the NCBI ORF Finder tool (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The signal peptide was predicted by the SignalP 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). Enzyme Mw and pI were predicted using the ExPASy proteomic server program Compute pI/Mw (http://web.expasy.org/compute_pi/). Meanwhile, as no report of *P. synxantha* lipase in previous research, the three-dimensional structure of LipPS1 was predicted by the SWISS-MODEL server and the protein structure was viewed by PdbViewer.

Functional expression of lipase in *E. coli*

Plasmid pET-28a was used for gene expression in *E. coli*. After digestion by BamHI/XhoI, the *lipPS1* gene was reclaimed and connected with pET-28a vector, which were digested by the same restriction endonuclease. The recombinant plasmid pET-28-*lipPS1* was transformed into *E. coli* BL21 (DE3). The recombinant lipase strain was incubated in 5 ml Luria-Bertani (LB) medium containing kanamycin (50 µg ml⁻¹) at 37°C for 12 h and then transferred into 50 ml LB medium for propagation. IPTG (0.1 mM) was added to the medium until the absorbance at 600 nm was 0.6. With the cultivation condition at 20°C for 18 h, the crude enzyme was collected by ultrasonic broken after the strains were harvested by centrifugation (12,000 × g, 10 min) (Cai et al. 2014).

Purification of recombinant lipase and SDS-PAGE analysis

After induction by IPTG, cells were separated by centrifugation, dispersed in sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) sample buffer and heated to 100°C. Finally, proteins were separated by SDS-PAGE (12.5% acrylamide/bis-acrylamide) and stained with coomassie blue.

The previous articles described the methods for heterologous protein expression and Ni-NTA purification procedures in our group. After lysed by sonication, the crude enzyme was passed through a 0.22 µm filter and then applied to a Ni-NTA sperflow column (1 ml, Qiagen). After equilibrated with the lysis buffer (NPI 10: 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0), the column was subsequently washed with 10 ml of wash buffer (NPI 20: 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) to remove the impurity protein. The fusion protein (His-tagged LipPS1) was eluted with a linear gradient of washing buffer (from NPI 50 to NPI 250). The eluted protein was desalted and concentrated by ultrafiltration using a 50 ml Amicon Ultra Centrifugal Filter Device with a molecular weight cut-off of 10 kDa (Millipore, USA). The purified enzyme was resuspended in sodium phosphate buffer (pH 7.0) containing 20% glycerol and stored at -40°C. The crude extract and the pure enzyme were analyzed by SDS-PAGE. All purification steps were carried out at 4°C. Protein concentration was determined by the Bradford method with bovine serum albumin (BSA) as the standard.

Assay of lipase activity

Enzyme activities of LipPS1 solution were assayed by measuring the absorbance at 405 nm of liberated p-nitrophenol. The reaction mixture (0.5 ml) contained 50 µl of 4-nitrophenyl palmitate solution (final concentration of 25 mM in the solution of isopropanol and dimethyl sulfoxide with volume ratio 3:1) as the substrate, 440 µl of lipase assay buffer (50 mM Glycine-NaOH buffer, pH 9) and 10 µl of appropriately diluted enzyme sample. 1 U is defined as the amount of enzyme releasing 1 µmol p-nitrophenol per min under the assay conditions.

Characterization of lipase

The lipase activity of the enzyme sample without any processing was considered as control (100%). Each experiment was repeated three times and each experiment included three replicates. The average values of triplicate measurements were used as each activity value. All values are means± SD from three independent experiments (repeats with SD of ≤ 5%). The ability of the purified enzyme to hydrolyze various substrates p-nitrophenyl esters (C2-C18) was examined at 40°C and pH 8.0.

The pH optimum for the enzyme activity was studied over a range from pH 2-10 for 5 min (40°C). The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 12 h and incubated at 40°C. The following buffer systems were used: pH 2.0-3.0 with 50 mM glycine-HCl, pH 4.0-5.0 with 100 mM citric acid-sodium citrate, pH 6.0-7.0 with 200 mM sodium phosphate, pH 8.0 with 50 mM Tris-HCl, 9.0-10.0 with 50 mM glycine-NaOH and pH 11.0 with 50 mM sodium hydrogen phosphate-NaOH. The temperature optimum for the enzyme activity was assayed at 10-50°C (pH 9.0). The thermal stability of LipPS1 was evaluated by assaying its residual activity after incubation of the enzyme at various temperatures for 12 h in sodium phosphate buffer (pH 9.0). The effects of metal ions on the lipase activity were determined with various metal ions (1 and 8 mM) such as Fe^{2+} , Mg^{2+} , Ca^{2+} , Cu^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+} and Mn^{2+} (pH 9.0). Meanwhile, effects of surfactants (Tween 20, Tween 80, TritonX-100, sodium dodecyl sulfate (SDS), EDTA and DTT at the concentration of 1.0 mM) and various organic solvents (Methanol, Ethanol, Acetonitrile, Benzene, n-hexane, Chloroform, DMSO, Acetone, n-propanol and Isopropanol) (15% (v/v) and 25% (v/v)) were measured using the spectrophotometric assay as above. The reaction mixtures containing the enzyme sample were incubated at 40°C for 60 min in 50 mM glycine-NaOH buffer, pH 9.0. The enzyme sample without any additives was considered as control (100%).

Biodegradation of waste grease

The waste greases contains plant oil and animal oil from the kitchen liquid waste was applied in this study. The reaction mixture containing 5.0 ml liquid waste (containing 150 mg waste oil) and 3.0 mg purified enzyme. After shaking with 150 rpm at a certain temperature for 15 h, fatty acid products were detected in the samples. Liquid waste without enzyme was as control. To confirm a suitable temperature for the lipase-catalyzed biodegradation of waste grease, a series of temperature (20°C, 30°C, 40°C and 50°C) were tested.

Results

Identification of bacteria and phenotypic characterization

With the progress of the above research, 1046 bp of 16S rDNA (GenBank accession numbers: KM232508) fragment in strain PS1 was finally obtained. Based on the 16S rRNA analyses, strain PS1 was identified as *Pseudomonas* spp. In the phylogenetic tree,

the 16S rDNA sequence of strain PS1 displayed the similarity of 97-99% to the other *Pseudomonas* sp. (including *P. cedrina*, *P. azotoformans*, *P. fluorescens*, *P. gessardii*, *P. reactans* and *P. synxantha*) (**Fig. 1a**) and showed the most homologous with *P. synxantha*. The results of 16S rDNA analysis and lipase gene analysis allowed the strain PS1 to be ascribed to the species *P. synxantha*. Other analyses were done to further support the isolate as a *P. synxantha* strain. In other research, we cloned a thermostable carboxylesterase (EstPS1) from *Pseudomonas synxantha* PS1. The GenBank accession number for the *Pseudomonas synxantha* PS1 carboxylesterase gene is KT070707. Homology analysis revealed that EstPS1 in *Pseudomonas synxantha* shared the most identity 98% with the whole-genome shotgun contigs (WGS) of *P. synxantha* BG33R, 88% with carboxylesterase EstA in *Pseudomonas fluorescens* A506 (GI: 387159426) and 84% identical to the carboxylesterase in *Pseudomonas poae* RE*1-1-14 (GI: 445198867). This provided the further evidence that the new selected strain belong to *P. synxantha*. Transparent zone method proved that the strain could produce lipase, for an obvious circle was detected on the screening plate (**Fig. 1b**). Meanwhile, as no report of this lipase in previous research, phenotype info of *P. synxantha* PS1 was shown in **Fig. 1c**.

Nutritional factors and environmental conditions on lipase production

In this experiment, lactose was found to be the superior carbon source (lipase activity 6.53 ± 0.32 U ml⁻¹). Except starch, remarkable lipase activity was obtained with maltose, sucrose and glucose. Many inorganic nitrogen sources had an effect on lipase production (Dutta and Ray 2009), and we also found nitrogen sources exhibited high enhancement on lipase production in this study. Nitrogen sources like soybean flour and peanut powder exhibited high lipase production of 5.83 ± 0.78 and 5.64 ± 0.6 U ml⁻¹ respectively (**Fig. 2a**). Tryptone led to the highest activity of 7.8 ± 0.52 U ml⁻¹, and casein or yeast extract was found to be unsuitable as nitrogen source in this study.

Lipase production was carried out using different grease as induction nutrition with lactose as carbon source and tryptone as nitrogen source, other conditions remaining the same. Lipase inductor and time course of fermentation had a significant effect on maximum production of lipase (Lotti et al. 2001; Kulkarni and Gadre 2002). Maximum lipase activity 10.8 ± 0.52 U ml⁻¹ was observed at 48 h of olive oil (0.5%, v/v) (**Fig. 2b**). Palm oil, sunflower oil, coconut oil and sesame oil led to almost the same lipase production at 48 h, they were found to be 9.16 ± 0.35 , 8.98 ± 0.47 , 8.65 ± 0.29 and 8.42 ± 0.55 U ml⁻¹. In conclusion, the strain PS1

showed maximum lipase activity of 10.8 U/ml after cultured for 48 h at 30°C, with lactose (4 g/l) as carbon source, tryptone (8 g/l) as nitrogen source, olive oil (0.5%, v/v) as inductor and the initial pH 8.0.

Homology analysis of LipPS1

A 650-bp fragment was amplified from the genomic DNA of *P. synxantha* PS1 by using the degenerate primers DP-*lip*PS1-F/DP-*lip*PS1-R (**Fig. 1d**). Sequence analysis indicated that it shared the most identity with a lipase from *P. fluorescens* GI: 489309678. By nested-PCR and chromosome walking, about 2 kb DNA fragment was cloned and sequenced, which contains a full-length ORF consisting of 1425 bp. The GenBank accession number of *lip*PS1 is KT313129. Sequence analysis revealed that the sequence of the *lip*PS1 ORF encoding a polypeptide of 474 amino acids uses ATG as the start codon. The G+C content (%) of the *lip*PS1 is 59.2%.

Using the neighbor-joining method (CLUSTAL W), the *lip*PS1 amino acid in *P. synxantha* PS1 was aligned with the other confirmed lipase from other *Pseudomonas* sp. (**Fig. 3a**). Homology analysis revealed that LipPS1 in *P. synxantha* shared the most identity 87% to the lipase in *P. fluorescens* GI: 489309678 (87% to *P. simiae* GI: 835531504, 86% to *P. tolaasii* GI:515538732, 85% to *P. veronii* GI:817110927, 51% to *P. lundensis* GI:860320366, 23% to *P. chlororaphis* GI:565887480, 35% to *Pseudomonas* sp. WCS374, 58% to *P. moraviensis* GI:566144243, 55% to *P. fuscovaginae* GI:498134231, 56% to *P. kilonensis* GI:802904114 and 39% to *Pseudomonas* sp. PH1b GI:640705641. Phylogenetic tree based on the protein sequences of LipPS1 and relating species. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches (**Fig. 3b**). Also, LipPS1 contains a single catalytic domain of the alpha/beta hydrolase family and belongs to the family of triacylglycerol lipase (EC 3.1.1.3). The molecular weight of LipPS1 was estimated to be 49.8 kDa, and the pI value was calculated to be 4.60 by the ExPASy compute pI/Mw program algorithm. Visual inspection of the alignment revealed conservation of aa in regions associated with catalysis and stabilization of the protein, e.g. the catalytic triad Ser²⁰⁷, Asp²⁵⁵, and His³¹³. The conserved region, Gla-Xxx-Ser-Xxx-Gly (the feature of the lipase sequences from *Pseudomonas* sp.) is boxed (Angkawidjaja et al. 2007) (**Fig. 3**).

Expression and purification of the recombinant enzyme in *E. coli*

Recombinant strain (BL21-pET-28-*lip*PS1) was grown to saturation in LB medium supplemented with appropriate antibiotic to

express the recombinant protein. The optimum induction temperature was 20°C, as shown in **Fig. 4a**, the SDS-PAGE results showed that the recombinant protein appeared as the soluble protein. Through Ni-NTA purification procedures, the purified LipPS1 migrate a single band on SDS-PAGE with an apparent molecular mass of about 50 kDa, which was identical to the calculated value. The recombinant strain was spreading to the screening plate to confirm the expression, and very strong transparent circle was detected after cultured at 37°C for 4h (**Fig. 4b**). The purified enzyme migrated on SDS-PAGE as a single band with an apparent molecular mass of about 50.0 kDa (**Fig. 4a**). The results indicated that Ni-NTA affinity chromatography was an appropriate method for this protein purification.

Substrate specificity analysis

The activities of the enzyme towards various p-nitrophenyl esters were investigated (**Fig. 4c**). With respect to the fatty acid specificity, the enzyme exhibited high hydrolyzed activity on long chain fatty acids (C10-C18). Lipase showed highest activity towards 4-nitrophenyl palmitate among the substrates examined. The enzyme specificity towards lipids with fatty acid residues of C10-C16 chain length strongly suggest that the enzyme used in this study was a true lipase.

Optimum pH and pH stability

The LipPS1 exhibited higher activities over a pH range of 6-10, among which the highest specific enzyme activity was at pH 8.0 (**Fig. 5a**). The activity of LipPS1 decreased significantly below pH 6 and about 65% of the maximal activity at pH 6. Stability of the lipase was investigated in buffer solutions over the pH range of 4-10. **Fig. 5b** showed that the lipase is an alkaline pH stability enzyme which was most stable at pH 6-10, among which the highest stability was at pH 7 (retaining 86% activity). This was very similar to protease-free lipase from *Pseudomonas* sp. (Kulkarni and Gadre 2002).

Optimum temperature and thermal stability

Over a temperature range of 20-60°C, LipPS1 showed more than 67% of the highest activity. Among which the highest specific enzyme activity was at 40°C (**Fig. 5c**). The lipase derived from *P. gessardii*, *P. fluorescens*, *P. fragi* and *P. mendocina* were found to be optimally active within 35-45°C (Ramani and Sekaran 2012). Residual activities were determined with standard assay conditions. The lipase was entirely stable at 10°C-60°C and more than 47% of its activity was retained after 12 h in 50°C (**Fig. 5d**).

A lipase from *P. fluorescens* has a similar temperature optimum and stability (Kulkarni and Gadre 2002).

Stability in organic solvents and metal ions

From the organic solvents tests, the enzyme is fairly stable in organic solvents (**Table 1**). Many reported lipase from *Pseudomonas* sp. exhibited stability in some organic solvents, such as *P. aeruginosa* CS-2 (Peng et al. 2010) and *P. cepacia* (Dhake et al. 2013). Also we found that the lipase was activated by 15% (v/v) methanol (112%), ethanol (127%) and n-butyl alcohol (116%). Except benzene had an obvious inhibition, the other organic solvents led to slightly influence on lipase activity.

At low concentration (2 mM), Ca^{2+} , Fe^{2+} , Cu^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+} partly inhibited the enzyme activity (**Table 2**). However, at high concentration (10 mM), Cu^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+} showed significantly inhibited effects especially for Mn^{2+} (only 13% of the highest activity). In contrast, Mg^{2+} enhanced the enzyme activity of 110% (2 mM) and 106% (10 mM).

Biodegradation of waste grease

As shown in **Fig. 6a**, lipPS1 presented strong biodegradability of waste grease. Waste grease at the topside of the reaction mixture was hydrolyzed nearly completely. Total theoretical amount of fatty acid was defined as 100%. Waste grease degradation efficiency was determined through fatty acid production rate. In biodegradation of waste oil (**Fig. 6b**), two influence factors of the recombinant LipPS1 on the reaction was investigated including temperature and reaction time. The results indicated that the optimal temperature of LipPS1 biodegradation reaction was 30°C, and 93% waste grease was hydrolyzed into fatty acid. Even high temperature (50°C) could accelerate the reaction rate, but the final conversion was lower. Conversion of the biodegradation at 20°C was 86% after 15h, which was higher than 83% at 50°C under the same condition.

Nucleotide sequence accession numbers

The GenBank accession numbers for the *Pseudomonas synxantha* PS1 16S rDNA gene is KM232508, and KT313129 for *lipPS1* gene.

Discussion

Lipase is an important enzyme which was used especially in lipid hydrolysis, biodiesel production and synthesis of new

polymeric materials (Sangeetha et al. 2011). Selecting new bacterial strains or improving bacterial strains is a prerequisite and effective solution in industrial applications and will be important for maximal lipase production. *Pseudomonas* species are well known for their ability to produce and secrete a large number of useful extracellular enzymes. Cloning the novel lipase gene with distinct features, especially from easily grown bacterium, is of interest for industrial applications. Although many lipases from *Pseudomonas* sp. have been intensively investigated (Gupta et al. 2004), there was no reports of lipase gene expression coding from *Pseudomonas synxantha*. In this work, the novel recombinant enzyme LipPS1 with detailed enzymatic properties and biodegradation of waste grease had preferable research significance.

Protein sequence alignment showed that LipPS1 belongs to lipase family I.3. Homology analysis revealed that LipPS1 in *Pseudomonas synxantha* shared the 50-90% identity with predicted triacylglycerol lipase in *Pseudomonas* sp.. The homology results reveal the remarkable research significance of the novel recombinant enzyme including enzyme characterizations. The LipPS1 was stable at pH 6.0-10.0 compared with that of *Pseudomonas aeruginosa* SL-72 (pH 7.0-8.0) (Verma et al. 2012), *Pseudomonas fluorescens* (pH 7.0) (Panizza et al. 2013), *Pseudomonas cepacia* (pH 7.0) (Li et al. 2015) *Pseudomonas gessardii* (pH 5.0) (Ramani et al. 2010) and *Pseudomonas stutzeri* PS59 (pH 8.5) (Li et al. 2014). The optimum temperature (40°C) of the recombinant lipase is similar to lipase from *P. gessardii*, *P. fluorescens*, *P. fragi* and *P. mendoncina*, which were found to be optimally active within 35-45°C (Ramani and Sekaran 2012).

In this study, various metal ions (Ca^{2+} , Fe^{2+} , Mg^{2+} , Cu^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+}) were used to test the influences on the enzyme activity. All the tested metal ions (1 mM) were found to inhibit the enzyme activity except Mg^{2+} . However, among the reported lipases, the influences of the metal ions on enzyme activities are quite different. Many lipases were stimulated by metal ions like Ca^{2+} , such as *Pseudomonas aeruginosa* lipase (1 mM, 116%) (Benattouche 2012), *Bacillus cereus* C7 lipase (1 mM, 200%) (Dutta and Ray 2009) and *Bacillus subtilis* lipase (10 mM, 116%) (Ma et al. 2006). On the other hand, the activity of *Bacillus pumilus* lipase B26 (Ca^{2+} -independent lipase) was almost constant at a wide range of Ca^{2+} concentration (Kim et al. 2002). The protein structure that lipases do not contain calcium binding sites may provide the presence of this reason. Log *P* is a measure of the polarity of an organic solvent, which is defined as the logarithm of its partition coefficient in a standard n-octane/water two phase

system. Laane et al. (1987) concluded that the solvent parameter that correlates best with enzyme activity was log *P*. In this study, various solvents with different log *P* were used to test the enzyme stability (Table 1). The results indicated that LipPS1 was highly denatured in hydrophobic solvents such as Benzene or n-hexane but was fairly stable in alkanes and short chain (C1-C4) alcohols. LipPS1 revealed high hydrolysis activity of waste oils in aqueous phase as a result of its stability in aqueous solvent,

Fats and oils are the major wastes generated from food processing industries, dairy industries, kitchen activities, bakeries and beverages industries etc. and are released into running water or sewages without prior treatment which causes destruction not just to the environment but also to the flora and fauna. Many lipases have been studied in various literature reports in relation to their biodegradation of fats and oils. A lipase produced by *Penicillium chrysogenum* SNP5 was employed for the bioremediation of used cooking oil (Kumar et al. 2012). Two lipases derived from *Bacillus subtilis* and *Bacillus pumilus* showed high biodegradability towards African locust bean oil (Ouoba et al. 2003). *Aspergillus* and *Penicillium* lipases are able to convert waste cooking olive oil into lipid-rich biomass (Papanikolaou et al. 2011), which could be used as an excellent food or feed nutritional supplement. To shorten lipase-catalyzed reaction time and reduce enzyme loss, a Recirculating Packed Bed Reactor was used for production of pinolenic acid concentrate from pine nut oil (Zhao et al. 2012). Even so, there was no reports of lipase from *Pseudomonas synxantha* lipase been studied. In this study, we found that the enzyme was considerable stable in short chain (C1-C4) hydrophilic alcohols. For *Pseudomonas synxantha* PS1 isolated from oil well produced water in Shengli oilfield, these may be the reasons of high conversion in biodegradation of waste grease.

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Table 1 Effect of various organic solvents (Methanol, Ethanol, Acetonitrile, N-butyl alcohol, Are propanol, n-hexane, Isopropanol, DMSO, Acetone and Benzene) (15% (v/v) and 25% (v/v)) on the recombinant lipase activity. The enzyme activity was measured using the spectrophotometric assay as above at 40°C (pH 8.0).

Organic solvent	Relative activity (%)	
	15%	25%
Control	100	100
Methanol	112	87
Ethanol	127	88
Acetonitrile	95	78
N-butyl alcohol	116	69
Are propanol	96	50
n-hexane	83	74
Isopropanol	95	61
DMSO	93	85
Acetone	92	88
Benzene	63	46

The data represent the mean of three experimental repeats with SD of $\leq 5\%$.

Table 2 Effect of metal ions on the lipase activity was determined with various metal ions (2 and 10 mM) such as Ca^{2+} , Fe^{2+} , Mg^{2+} , Cu^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+} (pH 8.0). The reaction condition without metal ions was as control.

Metal ions	Relative activity (%)	
	2 mM	10 mM
Control	100	100
Ca^{2+}	92	62
Fe^{2+}	80	53
Mg^{2+}	110	106
Cu^{2+}	87	35
Mn^{2+}	90	13
Co^{2+}	67	24
Ni^{2+}	97	31
Zn^{2+}	83	28

The data represent the mean of three experimental repeats with SD of $\leq 5\%$.

Legends to Figures

Fig. 1 (a) Rooted phylogenetic tree of isolate PS1 to other *Pseudomonas* species. Phylogenetic tree was constructed based on comparison of 16S rDNA sequences. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. (b) Transparent circle of wild bacteria on the screening plate. (c) Phenotype info of *Pseudomonas synxantha* PS1. (d) Degenerate primers design.

Fig. 2 (a) Lipase production by *Pseudomonas synxantha* using various carbon sources (4 g l⁻¹) and nitrogen sources (8 g l⁻¹) in the medium. (b) Lipase production of *Pseudomonas synxantha* obtained in different grease condition with different fermentation time. (■) sesame oil (●) coconut oil (▲) sunflower oil (▼) palm oil (◄) olive oil.

Fig. 3 Protein structure of LipPS1. (a) Conserved sequence alignment of LipPS1. The structures are denoted as follows: ▲, the catalytic site (Ser207, Asp255 and His313). The reserved amino acid motif GX SXG is boxed. (b) Phylogenetic tree based on the protein sequences of LipPS1 and relating species. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. (c) The three-dimensional structure of this enzyme was predicted by the SWISS-MODEL server. (d) The catalytic triad Ser²⁰⁷, Asp²⁵⁵, and His³¹³ residues were seen in the regions (in green).

Fig. 4 Recombinant lipase analysis. (a) SDS-PAGE analysis of the recombinant lipase and purified protein with an induction temperature of 20oC and an induction time of 18 h. *lane* M: standard marker proteins; *lane* 1: supernatant of recombinant bacteria lysate. (20°C); *lane* 2: supernatant of empty pET28a lysate; *lane* 3: purified recombinant protein. (b) Transparent circle of recombinant bacteria on the testing plate. (c) Relative activity on different substrates.

Fig. 5 Characterization of LipPS1. (a) The pH optimum of the recombinant lipase activity was studied over a range from pH 2-10 for 5 min (40°C). The following buffer systems were used: pH 4-5 with 100 mM citric acid-sodium

citrate, pH 6-7 with 200 mM sodium phosphate, pH 8.0 with 50 mM Tris-HCl, 9-10 with 50 mM glycine-NaOH.

(b) The pH stability of the recombinant lipase activity was determined by incubating the enzyme in different buffers for 12 h and incubated at 40°C. (c) The temperature optimum for the enzyme activity was assayed at 10-60°C (pH 8.0). (d) The thermal stability of LipPS1 was evaluated by assaying its residual activity after incubation of the enzyme at various temperatures for 12 h in sodium phosphate buffer (pH 8.0).

Fig. 6 Biodegradation of LipPS1. (a) Phenomenon of biodegradation of waste oil. (b) Yield of fatty acid from waste oil biodegradation at various temperatures with different time. (■) 20°C (▲) 30°C (▼) 40°C (◄)50°C.

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Fig. 1 (a) Rooted phylogenetic tree of isolate PS1 to other *Pseudomonas* species. Phylogenetic tree was constructed based on comparison of 16S rDNA sequences. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. (b) Transparent circle of wild bacteria on the screening plate. (c) Phenotype info of *Pseudomonas synxantha* PS1. (d) Degenerate primers design. 235x137mm (150 x 150 DPI)

Fig. 2 (a) Lipase production by *Pseudomonas synxantha* using various carbon sources (4 g l⁻¹) and nitrogen sources (8 g l⁻¹) in the medium. (b) Lipase production of *Pseudomonas synxantha* obtained in different grease condition with different fermentation time. (■) sesame oil (●) coconut oil (▲) sunflower oil (▼) palm oil (◄) olive oil.

227x160mm (220 x 220 DPI)

Fig. 3 Protein structure of LipPS1. (a) Conserved sequence alignment of LipPS1. The structures are denoted as follows: ▲, the catalytic site (Ser207, Asp255 and His313). The reserved amino acid motif GXSGX is boxed. (b) Phylogenetic tree based on the protein sequences of LipPS1 and relating species. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. (c) The three-dimensional structure of this enzyme was predicted by the SWISS-MODEL server. (d) The catalytic triad Ser207, Asp255, and His313 residues were seen in the regions (in green). 201x140mm (300 x 300 DPI)

Fig. 4 Recombinant lipase analysis. (a) SDS-PAGE analysis of the recombinant lipase and purified protein with an induction temperature of 20°C and an induction time of 18 h. lane M: standard marker proteins; lane 1: supernatant of recombinant bacteria lysate. (20°C); lane 2: supernatant of empty pET28a lysate; lane 3: purified recombinant protein. (b) Transparent circle of recombinant bacteria on the testing plate. (c) Relative activity on different substrates.
203x142mm (300 x 300 DPI)

Fig. 5 Characterization of LipPS1. (a) The pH optimum of the recombinant lipase activity was studied over a range from pH 2-10 for 5 min (40°C). The following buffer systems were used: pH 4-5 with 100 mM citric acid-sodium citrate, pH 6-7 with 200 mM sodium phosphate, pH 8.0 with 50 mM Tris-HCl, 9-10 with 50 mM glycine-NaOH. (b) The pH stability of the recombinant lipase activity was determined by incubating the enzyme in different buffers for 12 h and incubated at 40°C. (c) The temperature optimum for the enzyme activity was assayed at 10-60°C (pH 8.0). (d) The thermal stability of LipPS1 was evaluated by assaying its residual activity after incubation of the enzyme at various temperatures for 12 h in sodium phosphate buffer (pH 8.0).

106x80mm (600 x 600 DPI)

Fig. 6 Biodegradation of LipPS1. (a) Phenomenon of biodegradation of waste oil. (b) Yield of fatty acid from waste oil biodegradation at various temperatures with different time. (■) 20°C (▲) 30°C (▼) 40°C (◆) 50°C. 203x142mm (300 x 300 DPI)