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High-level expression and characterization of solvent-tolerant lipase

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In this study, the coding sequence of the lipase from *Proteus* sp. SW1 was optimized via codon optimization and subjected to expression in *Pichia pastoris* GS115. The maximum enzyme yield was 387 mg/L in the supernatants of the shake-flask culture. The purified recombinant lipase exhibited a specific activity of 130 U/mg toward *p*-nitrophenyl Laurate. Its optimum pH and temperature were 8.0 and 40°C, respectively. It was highly stable and even activated in water-miscible solvents, showing over 102% residual activity after 24 h incubation in ethanol, acetone, isopropanol and acetonitrile. In addition, the enzyme showed promoted activity with the increasing concentrations of methanol/ethanol and exhibited the maximum activity at 80%. In a solvent-free system for biodiesel synthesis with a one-step addition of methanol, the recombinant lipase displayed a 87% conversion rate toward palm oil at the high water content of 80%. The highly improved expression level and activity of the recombinant lipase may contribute to enable its commercial-scale production, and the unique properties would make it a particularly promising biocatalyst for biodiesel production in the future.

[**Key words:** Lipase; *Proteus* sp. SW1; Organic solvent tolerance; Biodiesel; Transesterification; *Pichia pastoris*]

Lipases have been widely used as biocatalysts for biotechnological processes by virtue of their enzymatic properties. They are broadly expressed in plants, animals and microorganisms, and those from microorganisms are of particular interest due to their easy production and broad applications in food modification, detergent formulation, fine chemical synthesis and biofuel production (1–3). Microbial lipases are derived from a wide range of microorganisms, including bacteria, yeasts, filamentous fungi and actinomycetes. Of those, *Proteus* derived ones are most interesting due to high enzymatic activity and organic solvent tolerance. For example, the lipases from *Proteus* sp. K107 (4) and *P. Mirabilis* (5) were proved to be promising catalysts for biodiesel production at ambient temperature, and also their crystal structure have been revealed (6,7).

Recently, a lipase, which is encoded by a lipase gene from *Proteus* sp. SW1 and shares 79% identity with the lipases from *Proteus* sp. K107 and *Proteus mirabilis*, was demonstrated to exhibit stability to organic solvents and potential for biodiesel synthesis (8). Unfortunately, the extremely low (1.5 mg/L culture) expression level in *Escherichia coli* (8) may severely hinder its industrial applications. Therefore, efforts should be focused on how to enhance its expression level in heterologous hosts.

Pichia pastoris, a methylotrophic yeast, has become the most frequently used host for heterologous protein production (9). It can grow to very high cell densities and enable the stable, high-level and tightly regulated expression of foreign genes in a secretory fashion, thus simplifying the purification of recombinant proteins.

In the present study, the lipase from *Proteus* sp. SW1 was overexpressed in *P. Pastoris* GS115 and characterized for potential industrial applications. The purified recombinant enzyme exhibited excellent tolerance to the inactivation induced by polar organic solvents and displayed a 87% conversion rate at the high water content of 80% in a solvent-free transesterification system, showing a promising potential as a robust biocatalyst in biodiesel synthesis.

MATERIALS AND METHODS

Strains, vectors, media and reagents *P. pastoris* GS115 and the yeast expression vector pPIC9K were obtained from Invitrogen (USA). *E. coli* XL10-Gold was purchased from Stratagene (USA). Luria–Bertani (LB) medium was prepared for the cultivation of *E. coli* as described in the Manual of Molecular Cloning (10). For yeast culture, Buffered glycerol-complex medium (BMGY), Buffered methanol-complex medium (BMMY) and Minimal dextrose medium (MD) were prepared as specified in the *Pichia* Expression Kit (Invitrogen, USA). Endoglycosidase H (Endo H) was purchased from NEB (USA). ExTaq DNA polymerase, PrimeSTAR DNA polymerase, restriction endonucleases and The pMD18-T vector were purchased from Takara (Tokyo, Japan). *p*-nitrophenol (pNP), *p*-nitrophenol butyrate (pNPB, C4), *p*-nitrophenyl octanoate (pNPO, C8), *p*-nitrophenol laurate (pNPL, C12), *p*-nitrophenol myristate (pNPM, C14), and *p*-nitrophenol palmitate (pNPP, C16) and Methyl heptadecanoate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soybean oil and palm oil were purchased from a local market. All other chemicals used were of analytical grade unless otherwise stated.

Design and synthesis of the lipase gene The coding sequence of the lipase gene (GenBank: ADV92635.1) was optimized according to the codon usage preference of *P. Pastoris* and synthesized by overlapping PCR. The obtained DNA fragment was cloned into pMD18-T vector and confirmed by DNA sequencing (Sangon, China). The resultant plasmid was designated pMD18T-*Rlipase*.

Construction of the expression vector The lipase gene was amplified using the plasmid pMD18T-*Rlipase* as the template. The primers used are as follows: LipF (5′-CG*GAATTC*ATGCCCACTACTTATCCAATTG-3′) and LipR

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(5′-TT*GCGGCCGC*TTA*GTGGTGGTGATGATGATG*CAACTTCTTACTAGCTAAAAAG-3′). The italics in primer sequence indicate the sequence encoding a 6× His tag and the introduced restriction sites of *Eco*RI or *Not*I at the 5′ end of the primers, respectively, are underlined. PCR was performed at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The PCR product cut by *Eco*RI and *Not*I was ligated into the pPIC9K vector (also cut by the same enzymes) in-frame fused with the α-factor (signal peptide) coding sequence, resulting in the recombinant plasmid pPIC9K-*Rlipase*.

Expression of the lipase gene in *P. pastoris* The recombinant plasmid pPIC9K-*Rlipase* was linearized by *Sal*I and transformed into *P. pastoris* GS115 via electroporation (4 kΩ, 50 μF, and 400 V). Transformants were screened on MD plates containing 0.4 mg/L biotin without histidine and identified by PCR using the primer pair LipF and LipR. The recombinant *P. pastoris* GS115 bearing the gene of interest in its genome was inoculated in 100 mL of BMGY medium until the OD600 reached approximately 20. Then all cells were harvested by centrifugation and transferred to 25 mL of BMMY medium. A total of 1% (v/v) methanol was added every 24 h to induce the expression of target protein. Approximately 1 mL of cell culture was withdrawn every 24 h and centrifuged at 6000 ×*g* for 5 min to remove cells. The prepared samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and assay for lipase activity and protein concentration.

Purification of the recombinant lipase After induction with methanol for 168 h, the cell culture was centrifuged at 6000 ×*g* for 5 min. The collected supernatants was dialyzed against a Millipore 10 kDa cut-off membrane at 4°C to remove ions, salts and excessive water. The sample was then subjected to further purification using a His-bind resin and His-bind buffer kit (Novagen, Madison, WI, USA) according to Novagen's instructions.

Assay of lipase activity and protein concentration Lipase activity was measured by a spectrophotometric assay using pNPL as the substrate as previously described (11). Briefly, 100 μL of diluted enzyme solution was mixed with 2.9 mL Tris–HCl buffer (50 mM, pH 8.0) containing 0.5 mM pNPL (the substrate dissolved in isopropanol with a final concentration of 10 mM). After incubation at 40°C for 5 min, the reaction was terminated by adding 500 μL of 10% SDS. The absorbance of the reaction mixture was measured at 410 nm. One unit of lipase activity was defined as the amount of enzyme liberating 1 μmol of pNP per minute under the above conditions. Protein concentration was measured using the Micro-BCA Protein Assay Reagent (Pierce, Rockford, IL, USA).

Western blotting The recombinant lipase was separated on 12% (w/v) polyacrylamide gel and electrotransferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). Western blotting was performed using anti-His-tag monoclonal antibody and blots were developed by enhanced chemiluminescence using SuperSignal West Femo Maximum Sensitivity Substrate (Pierce).

Glycosyl chain detection Glycoprotein staining was performed using the Thermo Scientific Pierce Glycoprotein Staining Kit in accordance with the manufacturer's recommendations. An equal amount of each sample was loaded onto two 12% (w/v) polyacrylamide gels. After electrophoresis, one gel was stained with Coomassie Brilliant Blue G-250, and the other was stained using the Glycoprotein Staining Kit.

Characterization of the recombinant lipase Substrate specificity of the purified lipase was determined by measuring the enzyme's activity according to the standard assay using pNPB, pNPO, pNPL, pNPM, and pNPP as substrates. The optimum temperature and pH of the lipase were monitored by assaying the hydrolytic activity toward pNPL at various temperatures (25°C–55°C) and pH (5–10). The effects of metal cations and detergents on the lipase were investigated after incubation of the lipase in 50 mM Tris–HCl buffer (pH 8.0) at 40°C for 30 min in the presence of certain concentrations of different cations and detergents. The effect was expressed as a relative activity to the control (without metal cations or detergents). To test pH stability and thermostability of the lipase, the enzyme was pre-incubated at certain temperature or pH conditions for 30 min, and the residual activity was then assayed. The residual activity was defined as a percentage of the control (unincubated) values. All values were based on the average of triplicate measurements.

Organic solvent tolerance assay The effects of organic solvents on the lipase was evaluated by incubating the enzyme in various organic solvents (80%, v/v) at 28°C for 3 h or 24 h, and the residual activity was assayed. For methanol/ethanol tolerance studies, the enzyme was incubated in various concentrations of methanol or ethanol at 28°C for 2 h, then the residual activity was tested. For inactivation assay by methanol or ethanol over time, the enzyme was incubated in 80% (v/v) methanol or ethanol at 28°C. At various time points, the aliquots were withdrawn and subjected to assay for the residual activity. The residual activity was defined as a percentage of the control (unincubated) values. All values were based on the average of triplicate measurements.

Transesterification reaction The reaction mixture contains 0.5 g refined soybean oil or palm oil, 156 μL methanol (for palm oil) or 114 μL methanol (for soybean oil), and 120 U lipase in 400 μL Tris–HCl buffer (50 mM, pH 8.0). Reactions were carried out at 37°C for 12 h, 250 rpm with a heating magnetic stirring system.

Thin layer chromatography analysis Samples were taken from the reaction mixture and centrifuged at 12,000 rpm for 5 min. Twenty microliter of the upper layer was mixed with 100 μL hexane, and then 3 μL of the mixture was applied to a silica gel plate. The plate was developed with hexane/ethyl acetate/acetic acid (98: 1.5: 0.5, v/v) and spots were developed with a color reagent of 20% (w/v) phosphotungstic acid in ethanol.

Quantification of fatty acid methyl esters Samples were taken from the reaction mixture and centrifuged at 12,000 rpm for 5 min. Ten microliter of the upper layer was mixed with 990 μL hexane containing 0.5 mg/mL methyl heptadecanoate (internal standard) as the testing sample for gas chromatography (GC) analysis. Briefly, 2 μL sample was injected into Shimadzu GC-2010 Plus system equipped with flame-ionization detector and a capillary column (RtxR-Wax, 30 m × 0.25 mm × 0.25 μm, Shimadzu) for fatty acid methyl esters (FAMEs) analysis. Both the injector and detector temperatures were held at 280°C. The column temperature was increased from 180 to 230°C at 3°C/min, and kept at 230°C for 1 min. The produced FAMEs were identified through comparison with corresponding authentic standards regarding their retention time. Quantitative analysis of FAMEs was performed using internal standard methyl heptadecanoate as reference.

RESULTS

Optimization and synthesis of the lipase gene *P. pastoris* displays a non-random pattern of synonymous codon usage and even some general bias towards a specific subset of codons (12). To enhance the expression level of the lipase gene from *Proteus* sp. SW1 (GenBank: ADV92635.1) in *P. pastoris*, the native, mature lipase gene was optimized and synthesized based on the codon usage bias of *P. pastoris* without changing the encoded amino acid sequence. The re-designed lipase gene was cloned into the pPIC9K vector fused with the mating factor α prepro-leader sequence (MF-α) from *Saccharomyces cerevisiae* and the downstream 6x His tag coding sequence, respectively. The recombinant plasmid was confirmed by DNA sequencing and designated pPIC9K-*Rlipase*.

Expression and purification of the recombinant lipase The pPIC9K-*Rlipase* plasmid was electrotransformed into *P. pastoris* GS115. Positive transformants with halos around the colonies were selected to assess the expression level of the lipase. The transformant with the highest expression level was subjected to shake-flask culture followed with 1% (v/v) methanol induction. The expression of RLipase reached a maximum level of approximately 387 mg/L in the supernatants at 168 h of induction (Fig. 1a). Then the RLipase was purified with a protein concentration of approximately 1040 mg/L after purification. The purified enzyme displayed a specific activity of 130 U/mg towards *p*-nitrophenyl laurate. SDS-PAGE analysis of both supernatants and the purified RLipase revealed the presence of two bands (Fig. 1a and b), and one of which displayed a ~32 kDa of molecular weight, nearly being equal to the calculated molecular weight of this lipase, while the other presented a slightly higher molecular mass of ~36 kDa. Both bands were further verified as the target protein by Western blotting using anti-His-tag monoclonal antibody (Fig. 1c).

Glycosylation analysis of RLipase Through online analysis by Prediction Servers NetNGlyc 1.0 and NetOGlyc 4.0 (http://www.cbs.dtu.dk/services/NetNGlyc/ and http://www.cbs.dtu.dk/services/NetOGlyc/), RLipase was predicted to have one putative N-linked glycosylation site (NVTE) and two putative O-linked glycosylation sites. To investigate whether the variation in the apparent molecular mass of RLipase on SDS-PAGE was due to the glycosylation modification or not, RLipase was treated with Endo H, a glucosaminidase commonly used to identify the composition of the glycan portion of N-glycosylated proteins, followed by staining with the GelCode Glycoprotein Staining Kit to detect the sugar moieties of glycoproteins. As shown in Fig. 2a, both bands on SDS-PAGE appeared as magenta bands after staining, indicating the presence of glycosyl chain in the RLipase molecule. After treatment with Endo H, the molecular mass of RLipase

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