Appl Biochem Biotechnol (2009) 159:355–365
DOI 10.1007/s12010-008-8419-5

Cloning and Expression of *Pseudomonas fluorescens* 26-2 Lipase Gene in *Pichia pastoris* and Characterizing for Transesterification

Jiangke Yang · Bo Zhang · Yunjun Yan

Received: 28 August 2008 / Accepted: 27 October 2008 /
Published online: 13 November 2008

Abstract *Pseudomonas* lipases are important biocatalysts widely used in a variety of industrial fields. An extracellular lipase gene *lip*A with 1,854-bp open reading frame was cloned from *Pseudomonas fluorescens* 26-2. The multialignment assay of the putative amino acid and the secondary structure prediction revealed this enzyme could be classified into the lipolytic subfamily I.3 and secreted via adenosine-triphosphate-binding cassette pathway. The *lip*A gene was integrated into *Pichia pastoris* GS115, and the methanol-inducible recombinants with Mut^S and Mut^+ phenotypes were acquired. The characteristics and the transesterification capacity shown by this enzyme suggested it is a useful biocatalyst for biodiesel preparation.

Keywords *Pseudomonas fluorescens* · Lipase · *Pichia pastoris* · Transesterification · Biodiesel

Introduction

Pseudomonas lipases are important biocatalysts widely used in a variety of industrial fields including detergent additives, fats or oils processing, chiral resolution [1], and pharmaceuticals preparation [2]. *Pseudomonas fluorescens* lipase, in particular, has enzymatic advantage in chiral resolution of racemic mixtures and biodiesel production [3–5].

Pseudomonas lipases are generally classified into two types. One is represented by lipases of *Pseudomonas aeruginosa* and *Pseudomonas glumae* (*Burkholderia glumae*). The active expression of these enzymes must be helped by the lipase-specific foldase (Lif proteins). This type of enzymes contains a typical signal peptide and is secreted through secreton-mediated pathway. Another type is represented by lipases from *P. fluorescens* and *Serratia marcescens*. Lipases of this type lack a typical N-terminal signal sequence but

J. Yang · B. Zhang · Y. Yan (✉)
Key Laboratory of Molecular Bio-physics, College of Life Science and Technology,
Huazhong University of Science and Technology, Wuhan 430074, China
e-mail: yanyunjun@tom.com

contain a C-terminal-targeting signal. They are secreted by the adenosine triphosphate (ATP)-binding cassette (ABC) protein-mediated exporters, which consist of three cell envelope proteins, an inner membrane ATPase, a membrane fusion protein, and an outer membrane protein [6, 7].

Efficiently expressed recombinant strains of industrial enzymes are regarded as prerequisite for the commercial production. To keep the consistency of the inner environment, reported lipase genes from *P. fluorescens* [8], *Pseudomonas alcaligenes* [9], and *Pseudomonas cepacia* [10] were generally expressed in original or homological hosts, but their expression is difficult to reach an intended level. Expression in *Escherichia coli* supplied an alternative to reach a high-level expression, but the proteins usually accumulate in the cells as an inactive inclusion body. To get the active lipases, a complicated *in vitro* refolding procedure is required [11].

Pichia pastoris is a robust expression system. Generally, the foreign gene can be stably integrated into the genome by the single crossover which happened in the region of His4 or AOXl to generate the recombinants with Mut^+ phenotype (normally utilize methanol). It can also replace the AOX1 gene between the end sequence of 5-AOX1 and 3-AOX1 to bring the Mut^S phenotype (methanol utilize slowly). So far, several lipase genes from human, filamentous fungi, yeast, and bacteria have been cloned and expressed [12–17]. But compared with the effective expression of lipase from other organism, the expression of *Pseudomonas* lipases in yeast is rarely reported [13].

Biodiesel fuel (fatty acid methyl esters, FAMEs) produced by transesterifying oil with short-chain alcohol was expected as a substitute for fossil diesel fuel. So far, the commercial biodiesel has been produced mainly by chemical methods, in which process excessive energy requirements and environment pollution are its major drawbacks. Compared with chemical methods, enzymatic methods catalyzed by lipases have the advantages of mild reaction conditions, simplifying the procedure for the recovery of FAMEs and the by-product (glycerol) and non-polluting emission. An enzyme efficient for biodiesel conversion would be a voluble resource, and the enhancement of the production could greatly deduce the cost of lipase.

In this study, a lipase gene (*lip*A) of *P. fluorescens* 26-2 was cloned and integrated into *P. pastoris* genome via two strategies to generate recombinants with Mut^+ and Mut^S phenotypes. The lipase was methanol-induced expressed, and the characteristics and transesterification capacity of the enzyme were also determined to evaluate its potential for biodiesel preparation.

Materials and Methods

Bacterial Strains

Strain *P. fluorescens* 26-2 was isolated from oil-contaminated soil and identified by 16S rRNA sequence (GenBank Accession number DQ993350). *E. coli* XL1-Blue and *P. pastoris* GS115 (Invitrogen) were used as recipients for plasmid transformation and the expression of the foreign gene.

Culture Mediums

The minimal dextrose medium (MD) consisting of 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 2% dextrose was used for yeast recombinants selection, and the minimal methanol

mediums (MS) consisting of 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 0.5% methanol were used to determine the phenotypes of recombinants. The buffered glycerol complex medium (BMGY) consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol was used for yeast cells growth, and the buffered methanol complex medium (BMMY) consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 0.5% methanol was used for lipase-inducible expression.

Phylogeny Analysis

The sequences were aligned by the program of Clustal W Multiple Alignment of BioEdit sequence alignment editor software package (North Carolina State University). Aligned sequences were analyzed by the molecular evolutionary genetics analysis (MEGA2) software, version 2.1, which was also used to produce a phylogenetic dendrogram reflecting the evolutionary relationship between the isolates and the reference strains by the neighbor-joining method according to the Kimura two-parameter model [18].

Gene Cloning and Plasmid Construction

Lipase gene was amplified by polymerase chain reaction (PCR) with primers pFS1 (5′-CT *GAATTC* ATG GGT GTA TAC GAC TAC A-3′, *Eco*RI site) and pFAn1 (5′-CTT *GCGGCCGC* TCA GGC GAT CAC AAT TCC GT-3′, *Not*I site) which were designed based on the conserved upstream and downstream sequence of lipase gene of *P. fluorescens* Pf0-1 (GenBank accession number: CP000094). The PCR fragment was cloned into vector pMD18-T (Takara) to get pMD-*lip*A and then the double strands of *lip*A were sequenced by Sangon Ltd., Shanghai. The sequence of *P. fluorescens* lipase gene was deposited into GenBank with the accession number DQ789596. Sequences were multialigned by ClusterW program, and the secondary structure of the lipase was predicted by the PredictProtein program [19]. To clone into pPIC9K, PCR fragments were digested with *Eco*RI and *Not*I and then inserted into pPIC9K to get plasmid pPIC9K-*lip*A.

Transformation and Selection of the Recombinants

Enzyme *Sac*I was used to linearize the plasmid pPIC-*lip*A for the single crossover to generate the phenotype Mut^+, and *Bgl*II was used to generate the methanol utilization slow phenotype (Mut^S). About 6 μg of linearized DNA was mixed with 80 μl of competent cells, and the electroporation was conducted on Gene Pulser (Bio-rad) according to the suggestion of the manufacturer for fungus. Positive clones was initially selected by MD plates and then checked by PCR. The Mut^+ and Mut^S phenotypes were determined by the growth rate of the recombinants on MS and MD media.

Fermentation and Lipase Purification

A single colony of recombinants were picked into 50-ml BMGY mediums and grown at 28–30 °C in a shaking incubator (250–300 rpm) until culture reaches an OD_{600}=3–6. The cells were harvested and transferred into 50-ml BMMY mediums to make the suspension with OD_{600}=1.0. The expression of the lipase was induced by methanol with a final concentration of 0.5% for 5 days, and the same amount of methanol was added each 36 h. The lipase activity was checked at interval time.

Purification of the lipase was conducted mainly according to the description of Yang et al. [10]. For lipase powder prepared to catalyze the biodiesel production, the fermentation was centrifuged and the supernatant was subjected to the ammonium sulfate precipitation and the fraction with 20–40% saturated ammonium sulfate was collected and dialyzed. After lyophilization, lipase powders were kept for transesterification.

Characterization of Lipase

Lipase activity was quantified at pH 7.5 by free fatty acid titration with 50 mM NaOH after being incubated in a thermostated vessel for 10 min. The assay mixture consisted of 5-ml 50-mM Tris–HCl buffer, 50 mM NaCl, 4-ml emulsified olive oil, and 1-ml enzyme solution. One unit (U) of the activity was defined as the amount of enzyme liberating 1 μmol of fatty acid per minute at 45 °C.

The effect of metal ion and metal chelant on the lipase was conducted by incubating the lipases in the 5-mmol/l metal ion (Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}) and EDTA solution for 30 min and then subjected to the lipase activity assay.

Transesterification and Gas Chromatography Analysis

The procedures of transesterification and gas chromatography analysis were described by Yang et al. [10]. Briefly, transesterification reactions were carried out in a 50-ml shaking flask and incubated at 40 °C on a thermostatic rotator with the speed 200 rpm/min. The reaction mixtures consisted of 4.6 g soybean oil, 0.6 ml methanol (3:1 molar ratio between methanol and oil), 138 mg enzyme, and 5% water (based on oil weight). Five microliters of the upper layer and 300 μl of 1.4 mM heptadecanoic acid methyl ester, served as the internal standard, were mixed thoroughly for gas chromatography analysis. Quantitative analysis of FAMEs was performed on a GC-14B gas chromatograph (Shimadzu Corp., Kyoto) connected to a capillary column (30 m×0.25 mm×0.25 μm, INNOWAX, Angellym, USA). The conversion ratio (%) of soybean oil to biodiesel was calculated by dividing the actual amount of FAMEs with that when all the oil has been conversed.

Results

Strain Identification

The taxonomy status of the lipase production strain was identified by the phylogeny analysis based on 16S rRNA sequence (Fig. 1a). Isolated strain is closely related to *P. fluorescens* Pf0-1, and the sequence similarity between them is up to 99%. Considering that *P. fluorescens*, *Pseudomonas putida*, and *Pseudomonas syringae* possibly belong to a phylogenetic complex, a phylogeny tree was constructed based on the amino acid sequence of the cloned lipase and reference strains (Fig. 1b). As shown by Fig. 1, the isolated strain was phylogenetically ascribed to *P. fluorescens*.

Lipase Gene Cloning

The sequence of the cloned lipase gene (*lip*A) was submitted to the GenBank with the accession number DQ789596, and the length of open reading frame (ORF) is 1,854 bp, encoding 617 amino acids. The protein contains a conserved penta-peptide motif (Gly-Xaa-

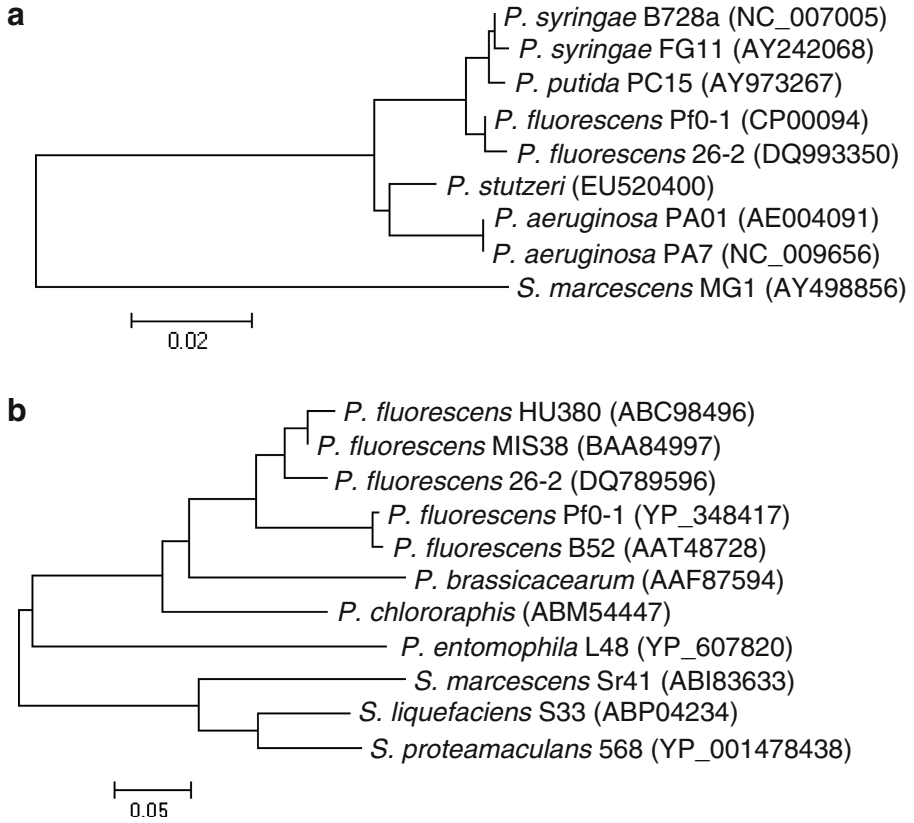

Fig. 1 Phylogenetic relationship of the isolated *P. fluorescens* 26-2 and the representatives of related bacteria. Kimura-2 distances were derived from a distance matrix to construct an optimal unrooted tree using the neighbor-joining method. *Numbers* in *parentheses* are the accession numbers of the sequences used. **a** The tree constructed basing on 16S rRNA sequence of strains and **b** constructed on the amino acid sequences of lipase gene

Ser-Xaa-Gly). Secondary structure prediction showed that the enzyme lacked a typical N-terminal signal sequence but contained a C-terminal targeting signal, which was essential for the secretion of the protein by an ABC exporter (Fig. 2). A Gly-rich domain with four GGXGXD motifs is closely related to the targeting signal. These repeats play a critical role in the secretion of some polypeptide passengers [20]. The high similarity between the 26-2 and the identified lipase of *P. fluorescens* HU380 [21] and *S. marcescens* Sr41 [22] showed that cloned lipase belonged to the lipase family I.3.

Expression of Lipase in *P. pastoris*

Two strategies were used to generate the recombinants with Mut^+ and Mut^S phenotypes (Fig. 3a,b). According to the phenotypes of the recombinants on the tributyrin–MS plates, the lipase was secretly expressed, and the secreted expression capacity of the Mut^+ cells

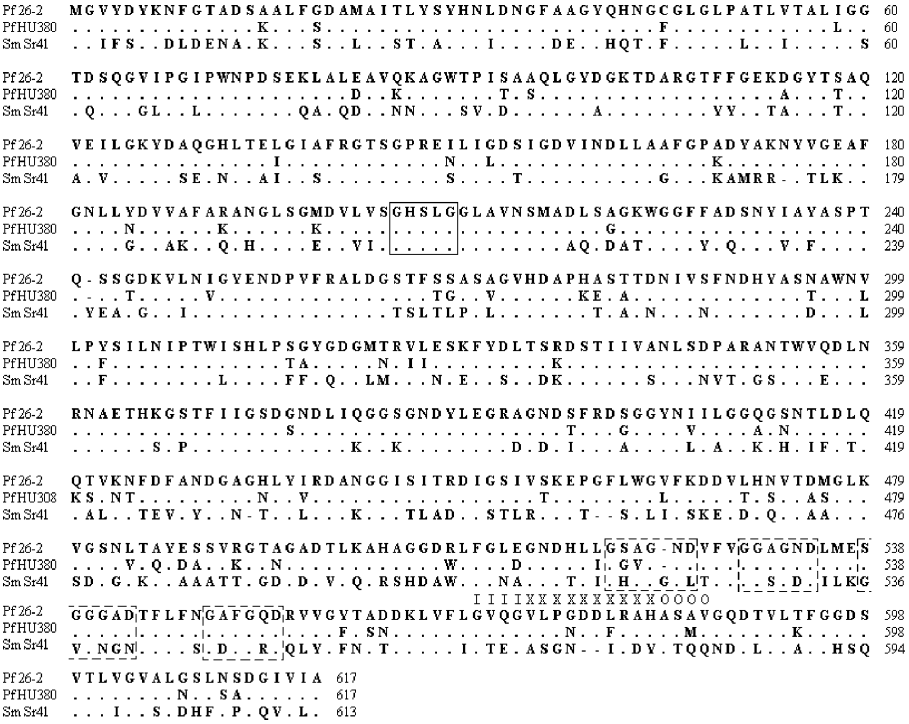

Fig. 2 Multialignment of putative amino acid sequence of lipase from *P. fluorescens* 26-2, *P. fluorescens* HU380, and *S. marcescens* Sr41. *Solid boxes* represent conserved penta-peptide motif, and the *dashed box* represents the four putative glycine-rich boxes. *O*: Outside helix cap; *X*: Central transmembrane helix segment; *I*: Inside helix cap

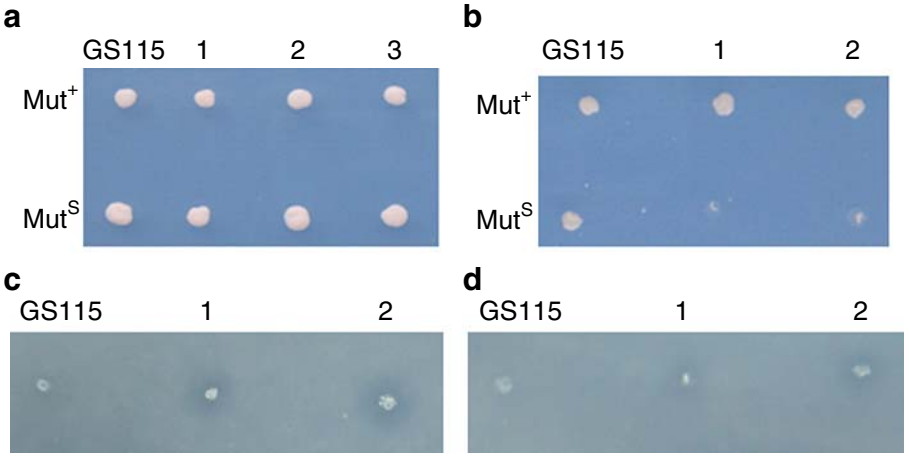

Fig. 3 Phenotypes of the recombinants. **a** Recombinants with Mut^S and Mut$^+$ phenotype growth on the MD plate; **b** Recombinants with Mut^S and Mut$^+$ phenotype growth on the MS plate; **c** Mut$^+$ recombinant growth on tributyrin–MS plate; **d** Mut^S recombinants growth on tributyrin–MS plate

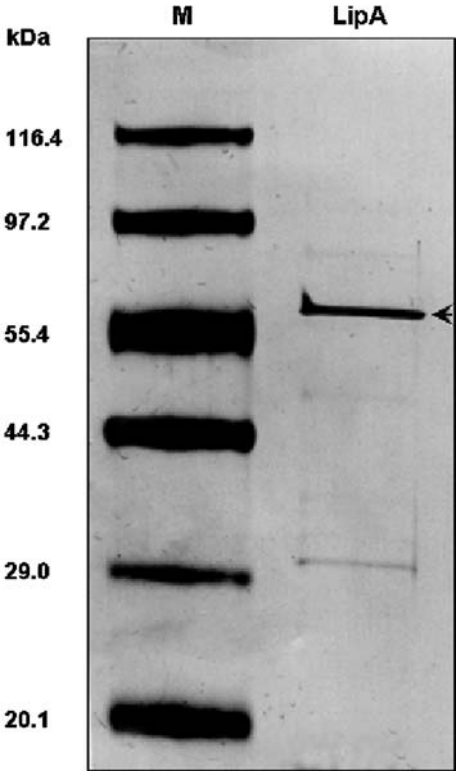

Fig. 4 SDS-PAGE of purified *P. fluorescens* 26-2 lipase. The band of lipase is indicated by the *arrow*

were better than the Mut^S cells (Fig. 3c,d). After inducible expression, the lipase was purified and the size determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was about 63 kDa (Fig. 4). According to the lipase production curve (Fig. 5), the maximal activity of Mut^+ recombinants could reach 20.3 IU/ml after methanol-inducible expression for 72 h, while the Mut^S recombinants just reached 5.6 IU/ml.

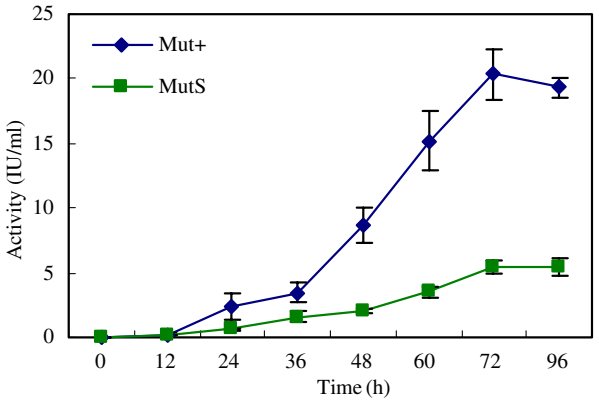

Fig. 5 Lipase production curve of the recombinants

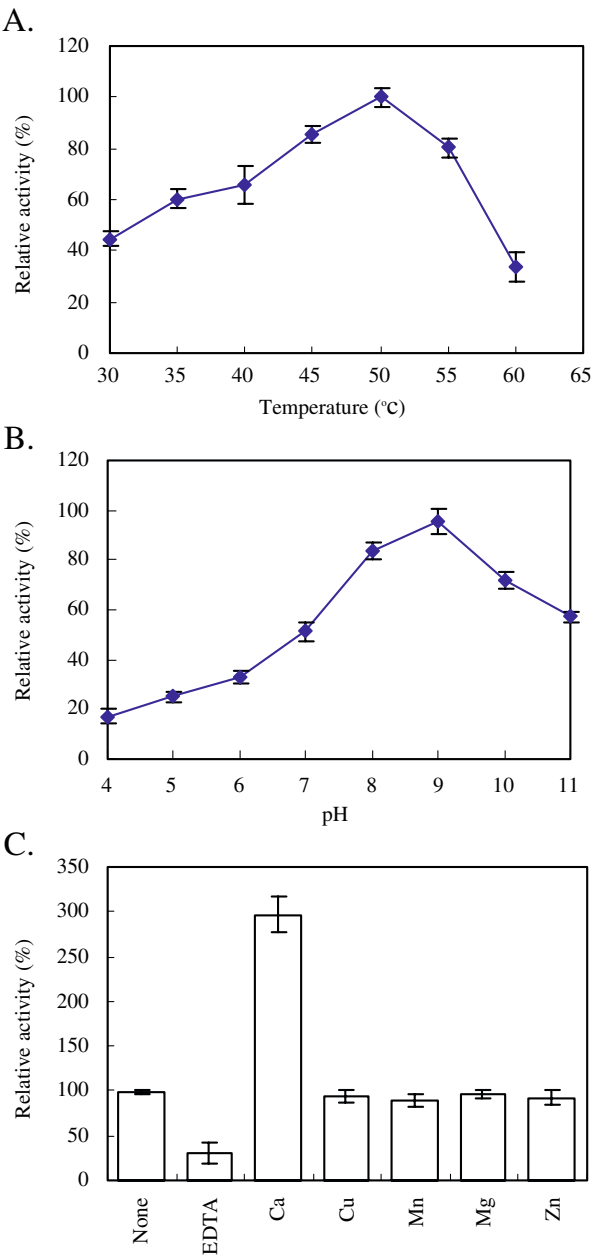

Fig. 6 Activity of *P. fluorescens* lipase under different conditions. **a** Relative activity of lipase in different temperature; **b** Relative activity of lipase in different pH; **c** Effect of various metal ions or metal chelate on lipase activity

Characterization of the Lipase

The optimal temperature, pH, and effect of ion and metal chelates on the activity of the purified lipase were determined. As shown by Fig. 6, the optimal temperature of lipase is 50 °C. When temperature varied from 45 to 55 °C, the enzyme still keeps 80% relative activity (Fig. 6a). Purified lipase showed better activity in alkaline solution, and the optimal

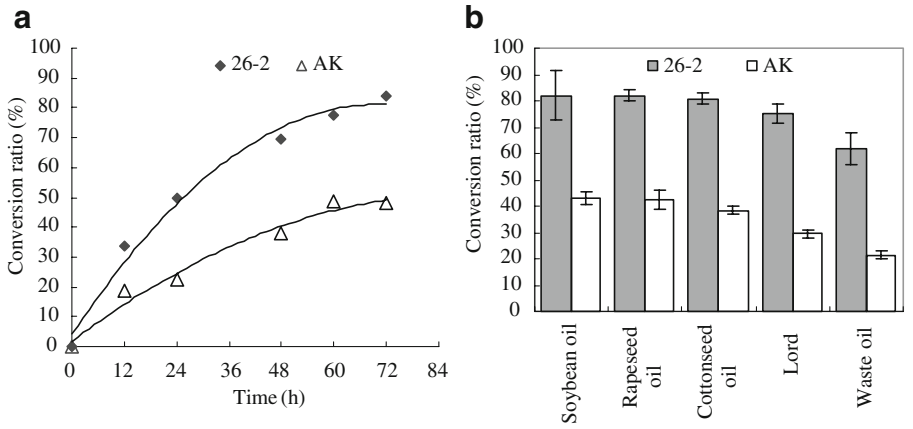

Fig. 7 Biodiesel conversion ratios of *P. fluorescens* 26-2 lipase and lipase AK. **a** Conversion curve of lipases with soybean oil as substrate. **b** Conversion ratio of lipase with different source of oil after 72-h reaction

solution pH for the enzyme is pH 9.0, when the solution becomes acidic, the activity will be seriously inhibited (Fig. 6b).

As reported before, metal ion and ion chelates could affect the activity of lipase [23, 24]. In this study, ion Ca^{2+} could significantly enhance the activity of the lipase up to 2.8-fold, while EDTA could dramatically reduce the activity (Fig. 6c). This indicated that cloned lipase is putatively similar with other *Pseudomonas* lipase [25] holding the Ca^{2+}-binding site in the active domain. Ion Cu^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} have weak effects on the lipase activity.

Transesterification Capacity

Transesterification capacity of *P. fluorescens* 26-2 lipase was evaluate though biodiesel preparation mixture, and the lipase AK from *P. fluorescens* (Amano) was used as a control. The conversion ratio of soybean oil to FAMEs is shown on Fig. 7. The capacity of *P. fluorescens* 26-2 is significantly higher than lipase AK. After 72 h of reaction, the conversion ratio from soybean oil to FAMEs reached 83.8%, while the conversion ratio of lipase AK was 48.2% (Fig. 7a).

A series of plant and animal source oil was used to check the substrate adaptability of lipases (Fig. 7b). Lipase of *P. fluorescens* 26-2 showed nice adaptability to the tested oil, and the conversion ratio was up to 80%, even for the free-fatty-acid-rich waste oil; the conversion ratio still reached 65%, while the conversion capacity of lipase AK is very

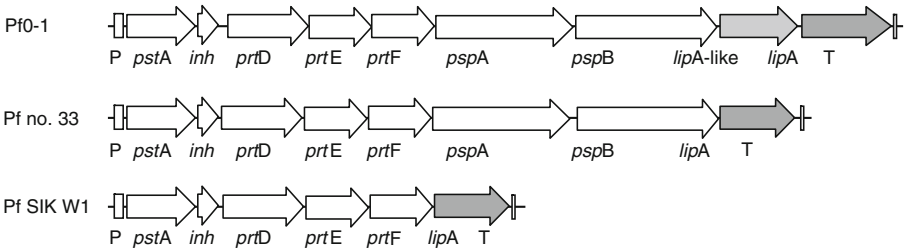

Fig. 8 Schematic of the organization of the lipase operon from different *P. fluorescens* strains

limited. Result of this study suggested that lipase tested is a perspective biocatalyst for biodiesel preparation.

Discussion

The size and organization of *P. fluorescens* lipase genes in genome have abundant diversity [26–28]. Typical lipase operon consists of lipase gene and the ABC secret components, aprD, aprE, and aprF [26]. The genome organization analysis on the lipase operon of *P. fluorescens* no. 33 showed that, except the secret components, the operon still contains the serine proteinases, pspA and pspB [29]. For the high sequence similarity (84%) between cloned lipase gene and the counterpart of *P. fluorescens* Pf 0-1, we analyzed the genome organization of lipase operon in Pf 0-1 genome. We found that, except the components similar with the lipase operon of the *P. fluorescens* no. 33, it contains another ORF with the size 1,689 bp. Putative amino acid analysis revealed that this enzyme contains a conserved Lip3 domain (Fig. 8); thus this means that two types of lipase 3 may exist in the same operon, and further functional analysis will elucidate this organization form.

Lipase-catalyzed biodiesel fuel preparation is regarded as a clean and green technique. An enzyme efficient for biodiesel conversion would be a valuable resource, and the enhancement of the production could greatly reduce the cost of lipase. Generally, enzyme with different organism source has its special characteristics. Previous studies showed that enzymes even come from the same species of *P. fluorescens* which have different transesterification capacities [30, 31]. In this study, biodiesel conversion rate of lipase of *P. fluorescens* 26-2 reached 83.8%, significantly higher than the commercial lipase AK (48.2%), to which a similar conversion ratio was also observed [5, 10].

In this study, a novel *P. fluorescens* lipase belonging to the lipase family I.3 was cloned. The secreted expression of the cloned lipase gene in *P. pastoris* settled a foundation to construct high-level expression recombinants suitable for large-scale preparation. The characteristics of the lipase such as high optimal temperature and transesterification capacity from soybean oil to FAMEs indicated that it could be a biocatalyst in the prospective bioenergy industry.

Acknowledgements We thank S. Zou for her technical assistance. This work was granted by the Chinese High-Tech Development Program (2006AA020203, 2007AA05Z417).

References

1. Tanaka, M., Demizu, Y., Nagano, M., Hama, M., Yoshida, Y., Kurihara, M., et al. (2007). *The Journal of Organic Chemistry*, *72*, 7750–7756. doi:10.1021/jo701317d.
2. Gotor-Fernández, V., Brieva, R., & Gotor, V. (2006). *Journal of Molecular Catalysis. B, Enzymatic*, *40*, 111–120. doi:10.1016/j.molcatb.2006.02.010.
3. Luo, Y., Zheng, Y., Jiang, Z., Ma, Y., & Wei, D. (2006). *Applied Microbiology and Biotechnology*, *73*, 349–355. doi:10.1007/s00253-006-0478-3.
4. Moreira, A. B. R., Perez, V. H., Zanin, G. M., & de Castro, H. F. (2007). *Energy & Fuels*, *21*, 3689–3694. doi:10.1021/ef700399b.
5. Zhao, X., El-Zahab, B., Brosnahan, R., Perry, J., & Wang, P. (2007). *Applied Biochemistry and Biotechnology*, *143*, 236–243. doi:10.1007/s12010-007-8043-9.
6. Arpigny, J. L., & Jaeger, K. E. (1999). *The Biochemical Journal*, *343*, 177–183. doi:10.1042/0264-6021:3430177.
7. Rosenau, F., & Jaeger, K. E. (2000). *Biochimie*, *82*, 1023–1032. doi:10.1016/S0300-9084(00)01182-2.

8. Ahn, J. H., Pan, J. G., & Rhee, A. J. S. (2001). *Applied and Environmental Microbiology*, *67*, 5506–5511. doi:10.1128/AEM.67.12.5506-5511.2001.
9. Gerritse, G., Hommes, R. W., & Quax, W. J. (1998). *Applied and Environmental Microbiology*, *64*, 2644–2651.
10. Yang, J., Guo, D., & Yan, Y. (2007). *Journal of Molecular Catalysis. B, Enzymatic*, *45*, 91–96. doi:10.1016/j.molcatb.2006.12.007.
11. Quyen, D. T., Schmidt-Dannert, C., & Schmid, R. D. (1999). *Applied and Environmental Microbiology*, *65*, 787–794.
12. Quyen, D. T., Schmidt-Dannert, C., & Schmid, R. D. (2003). *Protein Expression and Purification*, *28*, 102–110. doi:10.1016/S1046-5928(02)00679-4.
13. Jiang, Z., Zheng, Y., Luo, Y., Wang, G., Wang, H., Ma, Y., et al. (2005). *Molecular Biotechnology*, *31*, 95–102. doi:10.1385/MB:31:2:095.
14. Aloulou, A., Grandval, P., De Caro, J., De Caro, A., & Carriere, F. (2006). *Protein Expression and Purification*, *47*, 415–421. doi:10.1016/j.pep.2006.01.004.
15. Fernandez, L., Perez-Victoria, I., Zafra, A., Benitez, P. L., Morales, J. C., Velasco, J., et al. (2006). *Protein Expression and Purification*, *49*, 256–264. doi:10.1016/j.pep.2006.06.015.
16. Pfeffer, J., Richter, S., Nieveler, J., Hansen, C. E., Rhlid, R. B., Schmid, R. D., et al. (2006). *Applied Microbiology and Biotechnology*, *72*, 931–938. doi:10.1007/s00253-006-0400-z.
17. Zhao, W., Wang, J., Deng, R., & Wang, X. (2008). *Journal of Industrial Microbiology & Biotechnology*, *35*, 189–195. doi:10.1007/s10295-007-0283-8.
18. Kimura, M. (1980). *Journal of Molecular Evolution*, *16*, 111–120. doi:10.1007/BF01731581.
19. Rost, B., Yachdav, G., & Liu, J. (2004). *Nucleic Acids Research*, *32*, W321–W326. doi:10.1093/nar/gkh377.
20. Létoffé, S., & Wandersman, C. (1992). *Journal of Bacteriology*, *174*, 4920–4927.
21. Kojima, Y., Kobayashi, M., & Shimizu, S. (2003). *Journal of Bioscience and Bioengineering*, *96*, 242–249.
22. Akatsuka, H., Kawai, E., Omori, K., & Shibatani, T. (1995). *Journal of Bacteriology*, *177*, 6381–6389.
23. Kimura, H., Futami, Y., Tarui, S., & Shinomiya, T. (1982). *Journal of Biochemistry*, *92*, 1243–1251.
24. Xu, T. W., Xu, J. H., Yu, W., & Zhong, J. H. (2006). *Biotechnology Journal*, *1*, 1293–1301. doi:10.1002/biot.200600135.
25. Nardini, M., Lang, D. A., Liebeton, K., Jaeger, K. E., & Dijkstra, B. W. (2000). *The Journal of Biological Chemistry*, *275*, 31219–31225. doi:10.1074/jbc.M003903200.
26. Chung, G. H., Lee, Y. P., Jeohn, G. H., Yoo, O. J., & Rhee, J. S. (1991). *Agricultural and Biological Chemistry*, *55*, 2359–2365.
27. Kumura, H., Hirose, S., Sakurai, H., Mikawa, K., Tomita, F., & Shimazaki, K. (1998). *Bioscience, Biotechnology, and Biochemistry*, *62*, 2233–2235. doi:10.1271/bbb.62.2233.
28. Amada, K., Haruki, M., Imanaka, T., Morikawa, M., & Kanaya, S. (2000). *Biochimica et Biophysica Acta*, *1478*, 201–210.
29. Kawai, E., Idei, A., Kumura, H., Shimazaki, K., Akatsuka, H., & Omori, K. (1999). *Biochimica et Biophysica Acta*, *1446*, 377–382.
30. Luo, Y., Zheng, Y., Jiang, Z., Ma, Y., & Wei, D. (2006). *Applied Microbiology and Biotechnology*, *73*, 349–355. doi:10.1007/s00253-006-0478-3.
31. Devanesan, M. G., Viruthagiri, T., & Sugumar, N. (2007). *African Journal of Biotechnology*, *6*, 2497–2501.