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Cloning, expression and characterization of a novel thermal stable and short-chain alcohol tolerant lipase from *Burkholderia cepacia* strain G63

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Abstract

A lipase gene *lip*A and its chaperone gene *lip*B were cloned from *Burkholderia cepacia* strain G63. The *lip*A was composed of 1092 bp, encoding 363 amino acid residues, and the *lip*B composed of 1035 bp, corresponding to 344 amino acid residues. The significant amino acid similarity with *Pseudomonas cepacia* lipase revealed that this enzyme could be classified into the lipolytic subfamily I.2. The *lip*A and *lip*B genes were cloned into pBBR1Tp vector and conjugated into *B. cepacia* strains G63 with the help of pRK2013. The recombinant strain was fermented in 10 l bioreactor and the lipase was purified by a combination of ammonium sulfate fractionation, DEAE ion-exchange chromatography and gel filtration. The purified lipase kept stable at a temperature range of 40–70 °C. After incubated at 70 °C, the optimal temperature of this enzyme, for 10 h it remained 86.1% of its activity. The enzyme was also highly tolerant to a series of organic solution. Incubated in 50% methanol solution up to 48 h, the enzyme still kept 98.3% of its activity. The transesterification activity of soybean oil to fatty acid methyl esters (FAMEs) reached 87.8% after 72 h, indicating that it is a potential biocatalyzer for biodiesel production.

Keywords: Lipase; *Burkholderia cepacia*; Temperature; Organic solvent; Biodiesel

1. Introduction

Lipases (EC 3.1.1.3) are the most important lipolytic enzymes used as industrial biocatalysts for a variety of biotechnological applications [1]. According to the conserved motifs and the biological properties, lipolytic enzymes were classified into eight families [2]. The true lipases were ascribed into family I, which was subgrouped into six subfamilies. Lipases from *Burkholderia* and several species of *Pseudomonas* were ascribed to subfamilies I.2. Lipases in this subfamily are encoded in an operon together with their cognate intra-molecular chaperones, Lifs (lipase specific foldases). Lifs are periplasmic proteins anchored to the inner membrane by a hydrophobic N-terminal domain. They are able to catalyze the folding of lipases into an enzymatically active form, and subsequently translocate them to a secretion multi-protein complex, through which the active lipases are secreted into the culture supernatant [3,4]. Researches showed that Lifs are necessary for high-level expression and efficient secretion of LipA in homologous and heterogenous hosts [5–9].

Although a variety of *Burkholderia* lipase genes have been cloned and expressed in homologous and heterogonous, the high-level production of the active forms in heterogonous as *E. coli* has not yet been achieved. The enzyme proteins are present in the bacterial cytoplasm as inactive inclusion bodies, and the refolding procedure is necessary to get the active enzymes [8]. Lipase expressed in homologous strain exhibited great advantage on the activity and the secretion of lipase [10,11].

The fine characteristics as temperature stability, organic solvent tolerance, and the high activity for various substrates, make *Burkholderia* lipase becoming an excellent biocatalyzer in a broad range of industrial application, including the prospective field as pharmaceutical industry [12], and bioenergy production [13].

With the shortage of petroleum and the increase of the consciousness for environmental protection, biodiesel fuel (fatty acid methyl esters, FAMEs) produced by transesterification oil with short chain alcohol was expected as one of the most important substitute for fossil diesel fuel. So far, the commercial biodiesel has been produced mainly by chemical methods, in

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which process excessive energy requirements and environment pollution are its major drawbacks. Lipases with transesterification capacity in organic solvent are considered as the important biocatalyzer for biodiesel production [1]. Comparing with chemical methods, enzymatic methods simplified the procedure for recovery of FAMEs and the by-product (glycerol), and eliminated environment pollution.

However, the production of biodiesel fuel by enzymatic method has not yet been applied industrially, and the cost of lipase is one of the critical factors. An enzyme with high stable to temperature, tolerant to organic solvent and efficient for biodiesel conversion would be a jealous resource, and the enhancement of the production could greatly deduce the cost of lipase. In this project, the lipase gene (*lip*A) and the lipase specific foldase gene (*lip*B) of *Burkholderia cepacia* strain G63 were cloned and co-expressed in homologous strain. The characteristics of *B. cepacia* G63 lipase, such as temperature stability, organic solution tolerance and transesterification activity were checked to evaluate its potential for the biodiesel production.

2. Materials and methods

2.1. Cloning and expression lipA and lipB gene

A pair of primers P1 (5′-CTT *TCT AGA* CGT GTC TAG TCA GGG CGC AAA C-3′, *Xba*I site) and P2 (5′-CTT *AAG CTT* ACA CAT CAC GCG CGC GGC ATG A-3′, *Hin*dIII site) were used to amplify the entire lipase operon. PCR products were then ligated into pMD18-T simple vector (Takara, Ltd.) and sequenced by ABI 3730 DNA analyzer. The DNA sequence of *lip*A, *lip*B and the lipase operon were deposited in GenBank with the accession number DQ075249 and DQ078752. The amino acid sequence was multi-aligned with the sequence of *P. cepacia* (M58494), *B. glumae* (X70354) and *P. aeruginosa* (D50587) by the software BioEdit, version 7.0.5.

After double digestion with *Hin*dIII and *Kpn*I, the *Hin*dIII–*Kpn*I fragment was inserted into pBBR1Tp (ATCC, USA) digested with same enzymes to yield plasmid pBBR-*lip*AB. With the help of *E. coli* DH5α (pRK2013), pBBR-*lip*AB was transferred into *B. cepacia* strain G63 by three-parental conjugation conducted mainly according to the description of Ditta et al. [14]. Positive clones carrying pBBR-*lip*AB were selected through LB plates with trimethoprim (25 μg/ml) and kanamycin (50 μg/ml). Recombinant strains were established through sequencing of the insert fragment and the lipase activity checked by LB plates containing emulsified olive oil and Rhodamine B.

2.2. Fermentation and lipase purification

Recombinant strain *B. cepacia* G63 (pBBR-*lip*AB) was cultured in a 10 l capacity fermentation vessels. The seed cultures were grown at 30 °C for 12 h on a LB-1 medium (5 g of yeast extract, 10 g peptone, 1 g NaCl per liter, pH 7.0) with 25 μg/ml Tp antibiotics, and inoculated into bioreactor with the ratio 1:100. For the main fermentation, the medium used contained maltose 0.5%, yeast extraction 0.5%, K_2HPO_4 0.2%, $MgSO_4{\cdot}7H_2O$ 0.05%, and emulsified olive oil 1%. The pH of the culture was maintained automatically at 7.0 by using 0.1N NaOH as titrants. Foam was controlled automatically by use of a polyalkylene–silicone mixture. Fully aerobic conditions were maintained by injecting air at a rate of 1 standard liter of air per liter of broth per min (1 vvm) into the region of the impeller that was rotating at about 100 rpm.

Purification of the lipase from the growth medium was performed as following procedures. After centrifuge, 6 l supernatant was collected and condensed into 1 l volume by ultra-filtrating. Then it was subjected to the ammonium sulfate precipitation and the fraction with 30–40% saturated ammonium sulfate was collected and dialyzed. After lyophilization, a part of crude lipase powders were used to catalyze the biodiesel producing, and the remains were subjected to the DEAE ion-exchange resins, and eluted by a linear gradient from 0 to 0.5 M sodium chloride in the buffer. The elution at 0.3 M sodium chloride show lipase activity was collected and condensed, and again chromatographed on the G-75 Sephadex gel. SDS-PAGE used to check the effluent with lipolytic activity of the second Sephadex G75 chromatography.

2.3. Optimum temperature and thermal stability analysis

The optimal temperature for lipase activity was measured by incubating in the temperature range of 30–85 °C in a thermostatically controlled water bath. The thermal stability of lipase was measured by incubating lipase solution at different temperature up to 10 h and the residual activity was measured at 45 °C.

2.4. Organic solution tolerant analysis

The lipase solution was mixed with the same volume of organic solvent to prepare the 50% organic solution, and then the mixtures were incubated at room temperature on the rotator. The residual activity was measure at 45 °C and the organic solution tolerance of enzymes was calculated by comparing the residual activity with the initial activity.

2.5. Lipase activity

Lipase activity was quantified at pH 8.0 by free fatty acid titration with 50 mM NaOH after incubated in a thermostated vessel for 10 min. The assay mixture consisted of 5 ml 50 M Tris–HCl buffer, 50 mM NaCl, 4 ml emulsified olive oil and 1 m1 enzyme solution. The reaction was carried out at 45 °C. One unit (IU) of lipase activity was defined as the amount of enzyme releasing 1 μmol of free fatty acids per minute.

2.6. Transesterification

Eight samples, *Aspergillus niger* A6, *Candida rugosa* lipase AY, *Candida* sp. lipase AYS, *P. cepacia* lipase PS, *P. fluorescens* lipase AK, *Penicillium camembertii* lipase G-50, *Rhizopus niveus* Newlase F, and *Rhizopus oryzae* F-AP15, received from Amano Enzyme Inc., and *B. cepacia* G63 (pBBR-*lip*AB) lipase were used as catalyzers. The enzymatic transesterification reactions were carried out in a 50 ml shaking flask and incubated

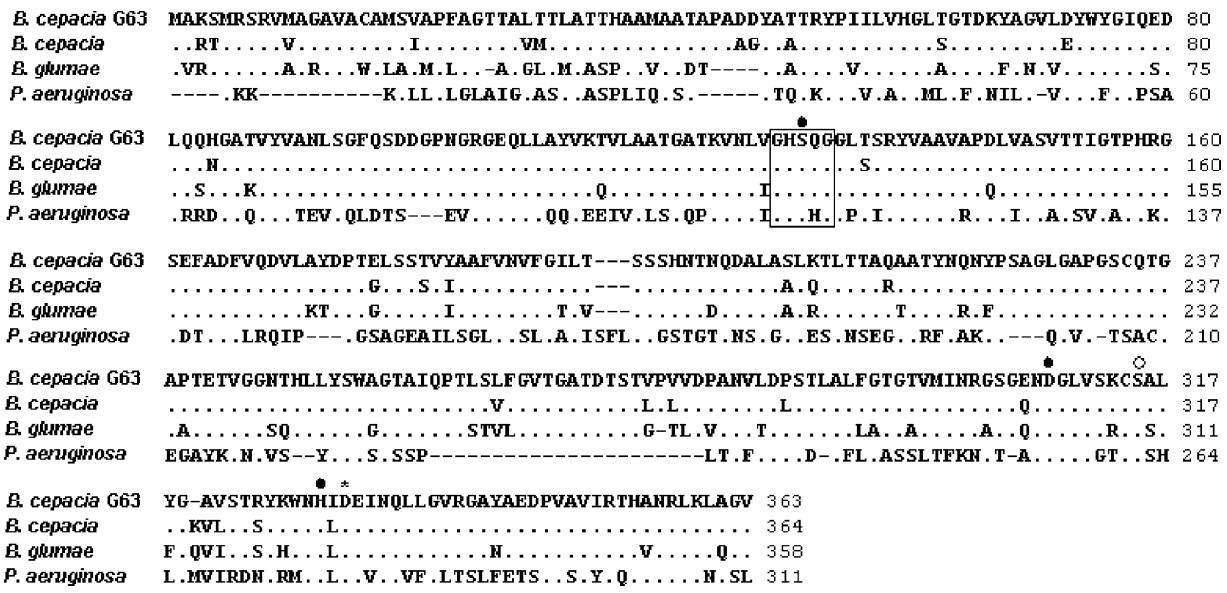

Fig. 1. Alignment of amino acid sequences of G63 lipases and the representatives from *Burkholderia* and *P. aeruginosa*. (●) Amino acid residues belonging to the catalytic triad; (○) cysteine residues forming the disulphide bridge; (*) aspartic residues involved in the Ca^{2+}-binding site.

at 40 °C on a thermostatic rotator with the speed 200 rpm. The reaction mixtures consisted of 4.6 g soybean oil, 0.6 ml methanol (3:1 molar ratio between methanol and oil), 138 mg enzymes and 5% water (based on oil weight).

Fifty microliter samples were taken from the reaction mixture at specified times and centrifuged to obtain the upper layer. 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.

2.7. Gas chromatography and FAMEs quantitative analysis

Quantitative analysis of fat acid methyl esters (FAME) 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 column temperature was increased from 200 to 235 °C at the rate of 3 °C/min, and then keep at 235 °C for 1 min. The temperatures of the injector and detector were set at 230 and 280 °C, respectively.

3. Result and discussion

3.1. Cloning of lipA and lipB of B. cepacia G63

The fragment containing the lipase operon was amplified by PCR with the primer P1 and P2, and then ligated to pMD18-T vector. Nucleotide sequence of the cloned DNA fragment of 2314 bp was deposited to the GenBank with the accession number DQ075249. The open reading frame of *lip*A consisting of 1092 nucleotides and encoding 363 amino acids was found at nucleotide positions 134–1225, and the ORF of *lip*B is composed of 1035 bp, which encoded 344 amino acids, sites at nucleotide positions 1229–2263.

Amino acid sequence of *lip*A was aligned with the sequence from *B. cepacia*, *B. glumae*, and *P. aeruginosa*, of which the X-ray crystal structure were already resolved [15–19]. Sequence similarity between G63 and *B. cepacia*, *B. glumae* and *P. aeruginosa* is 92.0%, 78.2% and 35.6%, respectively. The secondary structure and the activity site of lipase were shown in Fig. 1. The N-terminal signal peptide of precursor protein consists of 44 amino acids residues. The catalytic triad consisted with amino acid Ser87, Asp264 and His285, and the conserved penta-peptide motif consists of Ala-Xaa-Ser-Xaa-Gly containing the Ser87 residue. Distinctly different from *P. aeruginosa* lipase, *Burkholderia* lipase possesses an insertion in the region of 259–280 residues of pre-protein, which forming an anti-parallel double β-strand at the surface of the molecule [16,18]. High similarity of amino acid sequence and secondary structure between G63 lipase and *B. cepacia* lipase indicated that G63 lipase could be ascribed into lipase subfamily I.2 [2], and may also hold similar characteristics with lipase PS, as a prospective biocatalyzer for biodiesel production and organic synthesis [20].

3.2. Expression and purification of lipase

To enhance the production of *B. ecpacia* G63 lipase, *lip*A and *lip*B were co-expressed by high copy vector pBBR1Tp in homologous strain. The recombinant strains of G63 harboring pBBR-*lip*AB was subjected to fermentation in 101 bioreactor and the lipase production reached the maximum 863 IU/ml after 36 h. After a series of purification procedures, the lipase was purified 46.3-fold with an overall yield of 13.6%. Fig. 2 shows the SDS polyacrylamide gel electrophoresis (SDS-PAGE) of the effluent with lipolytic activity of Sepharose G75 chromatography. As shown in Fig. 2, a single protein band of lipase was obtained. From the logarithmic plot of the molecular mass versus the mobility of the standard proteins on SDS-PAGE, the

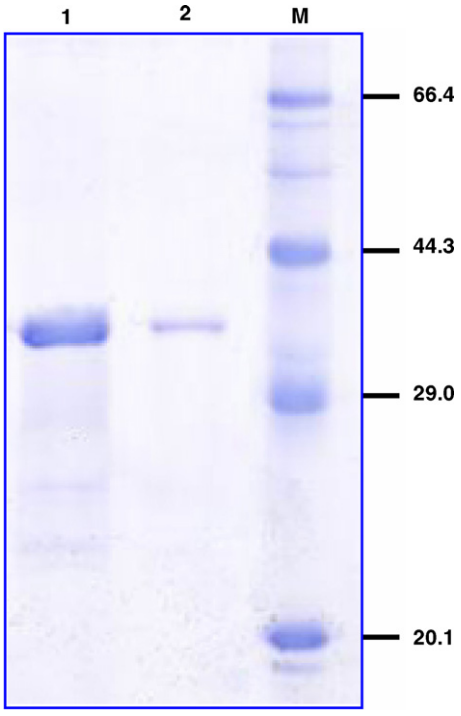

Fig. 2. SDS-PAGE checking the lipase purified by Sephadex G75 gel filtration. M: protein marker; lane 1: 10 μg protein loaded; lane 2: 2 μg protein loaded.

molecular mass of the purified lipase was determined to be 33 kDa, which coincided with the putative molecular mass from the cloned nucleotide sequence.

3.3. Optimal temperature and temperature stability

Higher temperature (>40 °C) was generally used to increase the miscibility and diffusion between different phases, and increase the conversion ratio of oil. In this study, optimal temperature was determined by checking the activity of enzymes incubated in different temperature. The relative activities at various temperatures are shown in Fig. 3, taking the activity at 70 °C as 100%. The maximum activity of lipase G63 and lipase PS were observed at approximately 70 and 50 °C respectively. From 30 to 70 °C, the activity of lipase G63 was increase

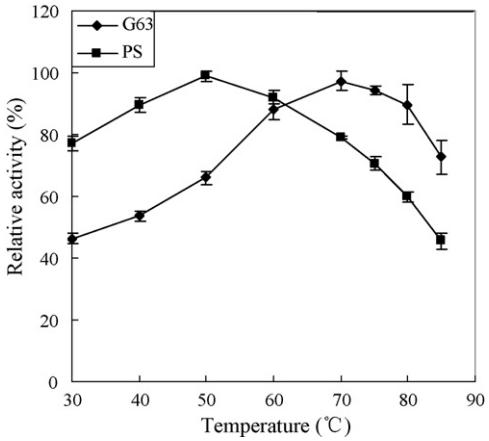

Fig. 3. Activity of *B. cepacia* G63 lipase on different temperature.

from 46.3 ± 1.5% to maximal activity, and the lipase activity decreased to 72.7 ± 5.5% at 85 °C.

Heat stability of lipase G63 and lipase PS were studied by measuring the residual activities after incubation at various temperatures at pH 8.0. Lipase G63 is very stable at a temperature range from 40 to 70 °C. As shown in Fig. 4A and B, lipase preserved 96.2% and 86.1% activity after incubated at 70 °C for 2 h, and 10 h, while lipase PS just preserved 32.0% and 28.4% activity.

3.4. Stability in organic solution

The effect of various alcohols and glycerol on the stability of lipases was studied by incubating enzyme solution in 50% alcohol/water solution at room temperature with shaking. The remaining activities were measured at appropriate time intervals, and the preserved activity of lipases after incubated for 48 h were shown in Fig. 5. There was nearly no effect on the activity of lipase G63 of methanol, ethanol, *tert*-butyl alcohol and glycerol. After incubation in 50% methanol solution for 48 h, there was still 98.34% activity remained. The effect of propylalcohol, isopropyl alcohol and amyl alcohol on the activity of lipase G63 is still limited. Incubated for 48 h, the remaining activity was still 93.5%, 91.7% and 92.5%, respectively. Comparing with lipase G63, the endurance of lipase PS to ethanol, propylalcohol

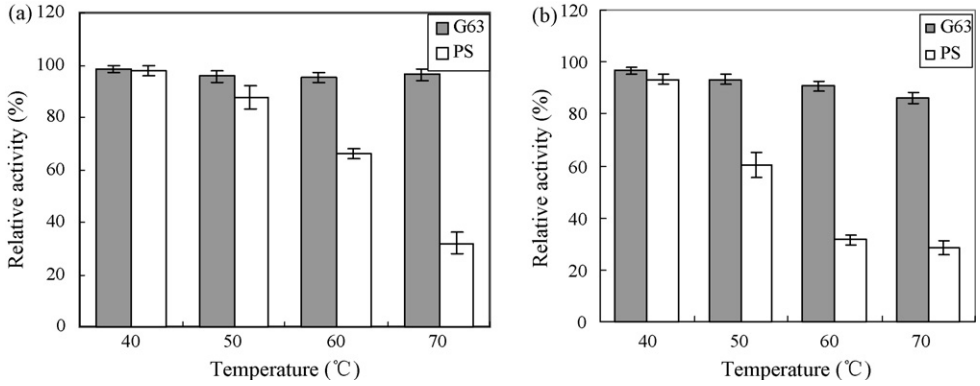

Fig. 4. Temperature stability profile of *B. cepacia* G63 lipase and lipase PS. (A) Relative activity of lipases after incubated at different temperatures for 2 h. (B) Relative activity of lipases after incubated at different temperatures for 10 h.

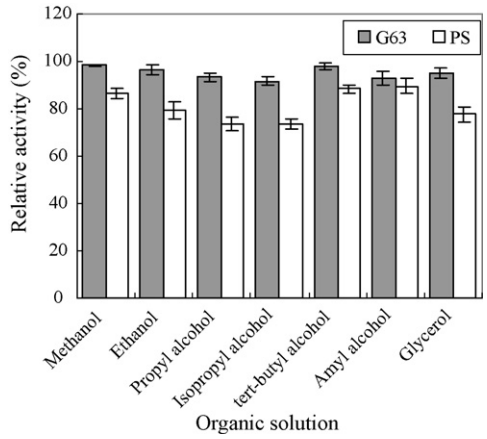

Fig. 5. Residual activity of *B. cepacia* G63 lipase in different organic solutions.

and isopropyl alcohol is weaker, and the remaining activity was 79.3%, 73.5% and 73.6%, respectively.

Organic solvent and the glycerol are also the important factor to affect the activity of lipase. Tested organic short-chain alcohol (C1–C2) were the acyl acceptors in transesterification, and alcohol with chain length of C3–C6 can be used as the solvent to regeneration of the enzymes, or as a solvent to make the reaction mixture become a uniform phase [21,22]. Result of this study showed that G63 lipase is highly tolerant to the organic solvent used in biodiesel preparation.

3.5. Transesterification capacity for biodiesel production

To evaluate the potential of G63 lipase for biodiesel production, eight lipases from Amano Enzyme Inc. were adapted as the control. As the screening experiments were intended for an initial evaluation of the transesterification activity of the lipases, they were conducted under a preliminary set of reaction conditions, which may not have been the optimum set for all the lipases. The conversion ratio of soybean oil to FAMEs is showed in Fig. 6.

B. cepacia G63 lipase and *Pseudomonas cepacia* lipase PS showed the highest activity toward the transesterification of soybean oil with methanol. After 72 h of reaction, the conversion ratio from soybean oil to FAMEs reached 87.8% and 87.4%, respectively. *B. cepacia* lipase PS was an important commercial biocatalyzer in organic synthesis and biodiesel production. In some condition, the transesterification capacity is even better than other commercial lipase as *Thermomyces lanuganosa* and *Candida antarctica*, which were also widely used in biodiesel production [23–26]. Result of this study suggested that G63 lipase is a perspective catalyzer for biodiesel preparation.

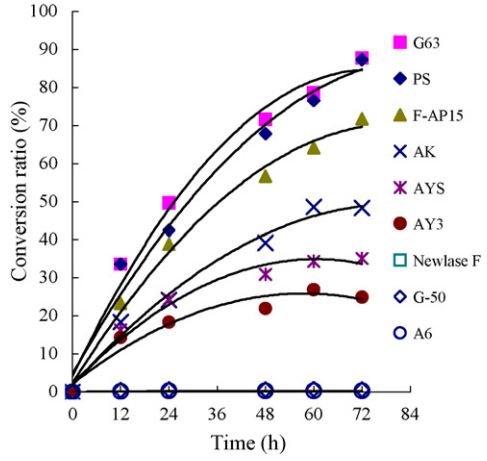

Fig. 6. Biodiesel conversion ratios of different lipases.

The *R. oryzae* lipase F-AP15 ranked in the second place. After reacting for 72 h, the conversion ratio reached 71.7%. Coincide with the researches conducted on a variety of *R. oryzae* lipase formulators [27–29], this result showed that *R. oryzae* lipase F-AP15 would be a nice alternative for transesterification.

P. florescence lipase AK, *C. rugosa* AY, and *Candida* sp. lipase AYS showed limited capacity in this research, and the conversion ratio is 48.2%, 35.1% and 25.0%, respectively. Although *Pseudomonas fluorescens* lipase AK showed high transesterification capacity [30], similar study conducted on the nine lipases from Amano Enzyme Inc. for initial evaluation also showed that the capacity of the lipase AK, AY, and AYS is still limited, while lipase PS from *Pseudomonas cepacia* got the highest yield of alkyl esters [12].

Rhizopus nieveus Newlipase F, *P. camembertii* lipase G-50, and *A. niger* lipase A6 showed very little or no activity for biodiesel production.

4. Conclusions

Although lipase genes from a broad range of microbes have been cloned, lipase resources suitable for biodiesel production and has the prospective for commercial production are still limited. In this study, the *lip*A and *lip*B gene of *B. cepacia* G63 lipase were cloned and expressed homologously. The high sequence similarity with the X-ray crystal structure resolved lipases indicated it could be classified into lipolytic subfamily I.2 according to the classification of Arpigny and Jaeger [2], and the expression of lipase gene in homologous strain supplying an alternative method for large scale production of *B. cepacia* G63 lipase. The characteristics of purified *B. cepacia* G63 lipase, as high temperature stability, organic solution tolerance and transesterification capacity from soybean oil to FAMEs, indicated that it could be a vigorous biocatalyzer in the prospective fields as bioenergy industry, or even in organic synthesis, pharmaceutical industry.

Acknowledgements

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