



Discovery and characterization of a novel lipase with transesterification activity from hot spring metagenomic library



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ABSTRACT

A new gene encoding a lipase (designated as *Lip-1*) was identified from a metagenomic bacterial artificial chromosome (BAC) library prepared from a concentrated water sample collected from a hot spring field in Niujie, Eryuan of Yunnan province in China. The open reading frame of this gene encoded 622 amino acid residues. It was cloned, fused with the oleosin gene and over expressed in *Escherichia coli* to prepare immobilized lipase artificial oil body AOB-sole-lip-1. The monomeric Sole-lip-1 fusion protein presented a molecular mass of 102.4 kDa. Enzyme assays using olive oil and methanol as the substrates in petroleum ether confirmed its transesterification activity. Hexadecanoic acid methyl ester, 8,11-Octadecadienoic acid methyl ester, 8-Octadecenoic acid methyl ester, and Octadecanoic acid methyl ester were detected. It showed favorable transesterification activity with optimal temperature 45 °C. Besides, the maximal biodiesel yield was obtained when the petroleum ether system as the organic solvent and the substrate methanol in 350 mmol/L (at a molar ratio of methanol of 10.5:1) and the water content was 1%. In light of these advantages, this lipase presents a promising resource for biodiesel production.

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1. Introduction

As society advances, its needs for fuel are increasing. Because of depletion of world petroleum reserves and increasing environmental concerns, biodiesel, a renewable and biodegradable fuel, is an ideal resource to replace fossil fuel [1]. Recently, the enzymatic transesterification mediated by lipase has drawn more and more attention in biodiesel production because this method has the advantages of requiring simple equipment, operating in mild reaction conditions, and being environmentally friendly [2,3].

Lipases, also known as triacyl glycerol acyl hydrolases, can catalyze the hydrolysis of triglycerides, diglycerides, and monoglycerides to free fatty acids and glycerol. They are also able to carry out synthetic reactions such as esterification and transesterification under thermodynamically favorable conditions such as low water content [4]. Lipase-mediated enzymatic transesterification can facilitate biodiesel production. Because of their site selectivity, chemical selectivity, substrate selectivity, and lower toxicity, lipases are promising enzymes for use in detergents,

food processing, organic cosmetics, and the pharmaceutical industry [5]. Currently, many microorganisms (including bacteria, yeasts and fungi) are considered as potential extracellular lipase-producing strains. Although lipases exist widely in higher plants and animals, microbial lipases are getting more attention due to their abundant gene resources, high stability, and broad substrate specificity [6].

The temperature in hot springs is usually over the limit of eukaryotic life (near to 60 °C), which limits the microbial life to Bacteria and Archaea (and their viruses). Thermostable enzymes from thermophilic microorganisms are important biocatalysts for industrial and biotechnological purposes, given that they can work at high temperatures in which mesophilic enzymes would be denatured [7]. Thermophilic enzymes were primarily screened in a culture-based manner, but metagenomics of hot springs facilitate the search of new biocatalysts by functional screening for the desired activity or by shotgun sequencing and the search for the target enzyme in metagenomic libraries [8]. Compared to the traditional screening and cultivating method, extracting total DNA directly from an environmental sample, then constructing a metagenomic library and sequencing the DNA via a high-throughput method can produce more comprehensive data about the microbial resources in that environment [9].

The Eryuan Niujie hot spring, located in Eryuan Niujie village of Yunnan province (latitude 26.6040', longitude 99.5649'), is a

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neutral hot spring (water temperature 58 °C, pH 7.0) with high calcium and fluoride content. Because of long-term traditional slaughter of livestock in the surrounding area, the waste of these livestock normally flow into the spring, so the fat content here is very rich. This activity among local residents has become a form of continuous selective pressure on microorganisms to develop organic matter degradation enzymes, and the hot spring has become a new source of thermal stable and organic solvent tolerant enzymes.

In this study, Eryuan Niujie hot spring metagenomic libraries were constructed, positive clones with lipase activity were screened and sequenced, new lipase genes were discovered and recombinant expressed. Finally, a new lipase with high tolerance for the production of biodiesel was identified and characterized.

2. Materials and methods

2.1. Materials

Eryuan Niujie hot spring microorganisms: Isolated from Eryuan Niujie hot spring (latitude 26.6040', longitude 99.5649', water temperature 58 °C, pH 7.0). Hot spring water samples were filtrated by a 20 µm nylon mesh, then concentrated into a sterile centrifuge tube with a 0.22 µm membrane from Millipore, sealed and transported (4 °C) into the lab and stored at –80 °C.

Strains and plasmids: CopyControl pCC1BAC (*Hind* III) Cloning-Ready Vector and TransforMax™ EPI300™ Electrocompetent *E. coli* cell were purchased from Epicentre (Madison, WI). Cloning strain Mach1-T1 was purchased from Bomaide Gene Technology (Beijing, China). BL21(DE3) was purchased from Invitrogen (Shanghai, China). Cloning vector T-Vector pMD19 (Simple) was purchased from TaKaRa Biotechnology (Dalian, China). pET32a(+) was purchased from Invitrogen (Shanghai, China).

Main reagent: TransStart FastPfu DNA Polymerase and Taq DNA polymerase were purchased from Transgen Biotech (Beijing, China). QuickCut™ *Bgl* II, QuickCut™ *Kpn* I and QuickCut™ *Sac* I, QuickCut™ *Not* I, DNA Ligation Kit Ver.2.1 were purchased from TaKaRa Biotechnology (Dalian, China). *Hind* III was purchased from New England Biolabs. E.Z.N.A.™ Plasmid Mini Kit I, E.Z.N.A.™ Gel Extraction Kit were purchased from Omega. Protease K was purchased from Merck, lysozyme was purchased from Amersco, low melting point agarose and pulsed field gel electrophoresis agarose were purchased from Omega. Mid Range I PFG Marker was purchased from NEB(Beijing,China). All chemicals were of the purest available grade.

Instruments: Pulsed field gel electrophoresis Mapper III (Bio-Rad, America), Gene Pulser II (Bio-Rad, America), GC/MS-QP2010 gas chromatography mass spectrometry(Shimadzu, Japan).

2.2. Methods

2.2.1. Construction of the bacterial artificial chromosome (BAC) library

About 20 Mb of mixed microbial DNA was extracted directly from microbes in the Eryuan Niujie hot spring of Yunnan Province according to Rondon's method [10]. Briefly, a cell pellet embedded in low-melting-point agarose was immersed in a lysis solution with *Hind* III for 20 min at 37 °C. To construct the BAC library, the agarose plug with DNA was subjected to pulsed-field gel electrophoresis using the CHEF Mapper System (Bio-Rad, Hercules, California), and 50- to 100-kb fragments were recovered from the gel by electroelution. A sample (100 ng) of the *Hind* III-digested DNA fragment was ligated into copy-control plasmid pCC1BAC Cloning-Ready Vector (25 ng; Epicentre, Madison, WI), which had been cleaved with *Hind* III, according to the manufacturer's instructions. The ligation mixture (2 µL) was electroporated into *Escherichia coli* EPI300 electro-competent cells (20 µL, Epicentre)

using the GenePulser Xcell (Bio-Rad) as described previously. The transformed cells were immediately inoculated into ice-cold SOC medium (0.5 mL) and allowed to recover at 37 °C for 1 h before plating. After incubation at 37 °C for 16 h, white colonies were picked using the QPix2 XT robotic colony-picking workstation (Genetix, New Milton, Hampshire, UK) and inoculated into 384-well microtiter plates containing LB medium with 12.5 mg/L chloramphenicol and 10% (v/v) glycerol. The clones were stored at –80 °C.

2.2.2. Identification and evaluation of BAC library

Clones which were randomly selected from the BAC library were inoculated into LB medium (containing 12.5 mg/mL chloramphenicol) at 37 °C overnight. Plasmid DNA was extracted according to Sambrook's method [11]. After digestion with *Not* I, the sizes of the inserted fragments were analyzed by pulsed field gel electrophoresis (5 V/cm, 5–15 s at 120 °C, 13 h at 12.5 °C).

2.2.3. Screening of the BAC clones based on enzyme activities

A total of 68,352 clones of the BAC library were chosen randomly from 178 384-well microtiter plates. The clones preserved in 384-well microtiter plates were photocopied to large Petri dishes containing LB with chloromphenicol and various substrates corresponding to screening amylase, cellulase, man-nase, xylanase, protease, and lipase activities at 37 °C. After 24 h, colonies surrounded by a transparent area, indicating breakdown of the tributyrin, were positive for lipase activity.

2.2.4. Sequencing of lipase positive plasmids

The BAC plasmids from lipase-positive clones and other organic matter hydrolases were extracted using a Qiagen Large-Construct kit (Qiagen, Hilden, Germany). The 49 mixed positive plasmids with organic matter hydrolysis activity were linearized and ligated to vectors containing Barcode. Then the vectors were analyzed by pyrosequencing with a 454 Life Sciences Genome Sequencer GS FLX Titanium (Rohe, USA). The ORF was analyzed using a Meta Gene Annotator and the domain analysis was carried out with HMMER3.0 in the Pfam24.0 database. Then the ORF was categorized and annotated using blast in KEGG and COG databases.

2.2.5. PCR amplification of the target gene

The primers were designed by genomic sequencing results to clone the lipase gene. The sequence of lipase gene *lip-1* has been submitted into the NCBI (National Center for Biotechnology Information) bank(accession No. KX057486). According to the known sequence of GenBank in NCBI the primers were designed to clone the oleosin protein gene(accession No. EU234463). All the primers were synthesized by Sangon Biotech (Shanghai, China) and are summarized in Table 1. Gene sequencing was accomplished by Qingke Biotechnology (Beijing, China).

The polymerase chain reaction (PCR) reaction system amplifying the genes *lip-1* and *sole* contained FastPfu DNA polymerase 1 µL, 5 × buffer 10 µL, dNTPs 5 µL, primer F and R 1 µL each, template DNA 1 µL, and ddH₂O 31 µL. The reaction condition was carried out with 5 min initial denaturation at 94 °C, followed by 30 cycles: 94 °C for 30 s, 52 °C/56 °C for 30 s, 72 °C for 2 min, and a final

Table 1
Primers used in this paper.

Primer name	Nucleotide sequence ^a (5'–3')
Lip-1F	<u>GGTACCATCGAAGGAAGAATGATGAAAAATAGGAAACGGCAA</u>
Lip-1R	GAGCTCTCAGAACGCCITTTGCCAGT
Sole F	AGATCTGATGGCTGAGCATTATGTCACAAC
Sole R	GAGCTCGTACGTAGAGGTACCCGCAACAGGCTGCTGCGAGAAC

a: The linker in Lip-1F is underlined.

extension of 10 min. The PCR products were incubated with polyadenylic acid and Taq DNA polymerase at 72 °C for 30 min. The final products were purified using a gel extraction kit and then were cloned into the pMD19-T, and sequenced.

2.2.6. Construction of fusion expression plasmids

After digestion with *Bgl* II/*Sac* I, the oleosin gene *sole* was purified from the vector pMD19-T-*sole* after being excised from the gel and was then cloned into pET32a(+). After digestion with *Kpn* I/*Sac* I, the novel lipase gene *lip-1* was purified from the vector pMD19-T-*lip-1* after being excised from the gel and was then fused to the C-terminus of the oleosin gene by a linker polypeptide, the fusion expression vector pET32a(+)-*sole-lip-1* was constructed.

2.2.7. Expression of recombinant proteins

The recombinant plasmid pET32a(+)-*sole-lip-1* was transformed into *E. coli* BL21 (DE3). The transformed cells were cultured in LB broth containing 100 mg/mL ampicillin at 37 °C until the OD₆₀₀ reached 0.6, then induced by IPTG with a final concentration of 1.0 mmol/L at 28 °C for 6 h. 1 mL induced cells with the final OD₆₀₀ reached 1.5 were harvested by centrifuging at 5000 g for 5 min at 4 °C.

2.2.8. Immobilization of lipase

After centrifugation, 1 mL of the induced cells with the final OD₆₀₀ reached 1.5 was resuspended in 1 mL sodium phosphate buffer (10 mM, pH7.5) containing 300 µg lecithin and 30 µL olive oil. The resulting mixture was subjected to sonication with an Ultrasonic Processor VCX760 at an amplitude of 30% for 20 s, and then cooled on ice for 5 min. The same process was repeated twice and the oil bodies above were collected by centrifugation at 12,000g for 2 min, and then washed with 1 mL sodium phosphate buffer (10 mM, pH7.5), to immobilize lipase AOB-*sole-lip-1*.

2.2.9. Transesterification and GC–MS analyses [12]

The general transesterification reaction was processed in a stoppered test tube under the following conditions: AOB-*sole-lip-1* was collected and resuspended in 6 mL petroleum ether containing 200 µL olive oil and 0.6 mmol methanol at 37 °C with shaking at 180 r/min for 30 h. All analyses were performed using a GCMS-QP2010 coupled with an Rtx-5MS column (30-m length, 0.25-mm diameter, 0.25-µm film thickness; Agilent, Folsom, USA). The injection port was maintained at 250 °C. The oven program was as follows: starting at 60 °C and heating at 5 °C/min to 280 °C, maintained for 6 min. Helium (grade 5) was used as carrier gas at a pressure of 57.1 kPa [13–16].

2.2.10. Optimum reaction temperature

Since it is one of the most important factors influencing enzyme activity, the effect of temperature on transesterification was analyzed. In this study, we used temperatures of 25 °C, 30 °C, 37 °C, 40 °C, 45 °C, and 55 °C for 30 h in the general transesterification reaction system. The resulting supernatants were analyzed by GC–MS. All other conditions were the same as for general transesterification, and each experiment was repeated 3 times to minimize experimental errors.

2.2.11. Optimum solvent systems

To demonstrate the effect of the solvent system on transesterification, based on a previous study in our lab and by other researchers [12,17], n-hexane, tert-butyl tert-butyl alcohol, petroleum ether, tert-amyl alcohol, n-hexane/tert-amyl alcohol (4:1), and n-hexane/tert-butyl alcohol (4:1) were used. The resulting supernatants were analyzed by GC–MS. All other conditions were

the same as for general transesterification, and each experiment was repeated 3 times to minimize experimental errors.

2.2.12. Methanol tolerance analysis

Different amounts of methanol were added to the transesterification reaction system to reach final concentrations of 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, and 500 mM. The resulting supernatants were analyzed by GC–MS. All other conditions were the same as those for general transesterification, and each experiment was repeated 3 times to minimize experimental errors.

2.2.13. Water content analysis

AOB-*sole-lip-1* was used to catalyze transesterification reactions at various water contents in the reaction system. The final water contents that were tested were 0%, 1%, 2%, 3%, 4%, 5%, and 6%. The rate of transesterification was analyzed by GC–MS. As described above, all other conditions were the same as for general transesterification, and each experiment was repeated 3 times.

3. Results

3.1. Construction of BAC library

The Eryuan Niujie hot spring genomic BAC metagenomic library contains about 88,000 recombinant clones and the average insert fragment was 50 kb, with a library capacity of 4.4G bp (Fig. 1).

3.2. Screening results of positive clones with lipase activity from the BAC library

A total of 68,352 positive clones preserved in a 384-well microtiter plate were photocopied to the LB medium containing 1% tributyrin to screen for clones positive for lipase activity. At first we identified 10 positive clones, and after secondary screening 4 strains which could produce obvious transparency in the surrounding medium were obtained. (Fig. 2).

Then 49 BAC plasmids with lipase activity or other organic matter degradation activities include amylase, cellulase, protease, mannanase and xylanase were extracted and sequenced, 250,061 reads were obtained and the total length of the sequence was 96.5 Mbp and the average read length was 386 bp. After splicing by Newbler (version 2.3), we obtained 672 contig, the total length was 249,576 bp and the average length was ~4 Kbp. Finally we obtained 38 full-length hydrolase genes which contained 8 esterase genes (Table 2). Eight genes exist in four strains which could produce obvious transparency in Fig. 2. And *lip-1* was in Z1. Compared to the other 7 esterase genes, *lip-1* has both the lowest gene and the lowest amino acid identity, therefore we recognized it as a novel gene and chose it for further investigation.

3.3. Expression of lipase fusion protein

pET32a(+)-*sole-lip-1* was overexpressed in *E. coli* BL21(DE3). After 6 h of induction with IPTG at 28 °C, the proteins from induced and non-induced cells containing the vector pET32a(+) only and the expression vector pET32a(+)-*sole-lip-1* were analyzed by SDS-PAGE. A clear band corresponding to *Sole-lip-1* fusion protein at 102.4 kDa was predominately found in cell lysate (Fig. 3).

3.4. Transesterification activity of *sole-lip-1*

The transesterification activity of the immobilized enzyme AOB-*sole-lip-1* and the blank control were analyzed by GC–MS

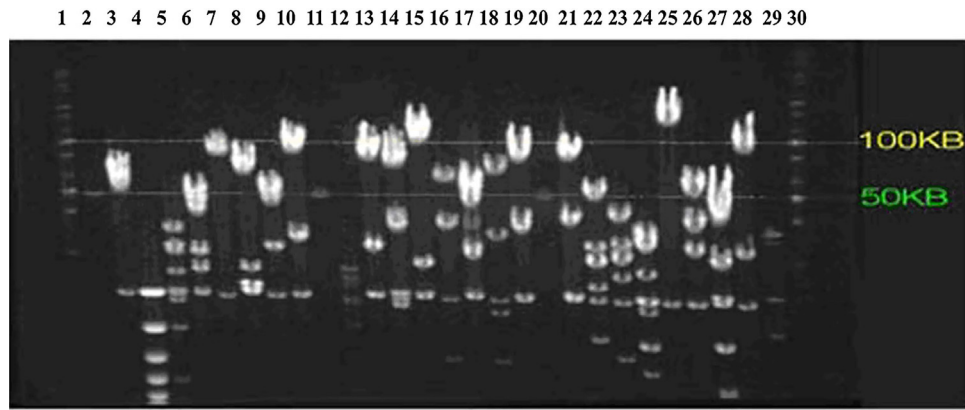


Fig. 1. Random analysis of Eryuan Niujie hot spring BAC metagenomic library. Lane 1, 30: MidRange I PFG Marker (NEB); lanes 2–29: recombinant plasmids digested with NotI

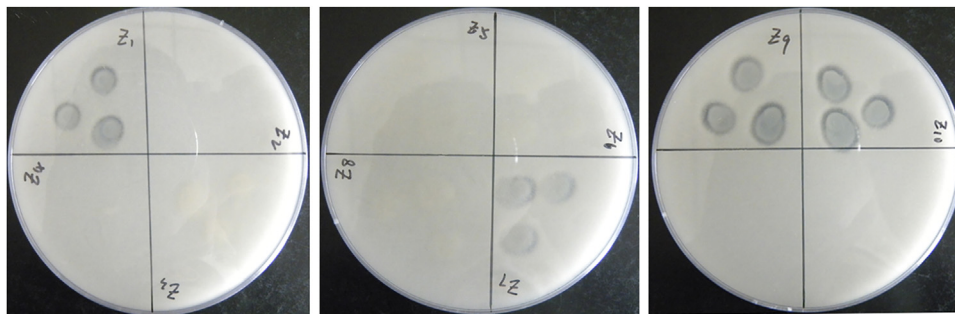


Fig. 2. Screening of the BAC clones. The screening of the BAC library by the LB solid plate containing 12.5 mg/L chloramphenicol and 1% tributyrin.

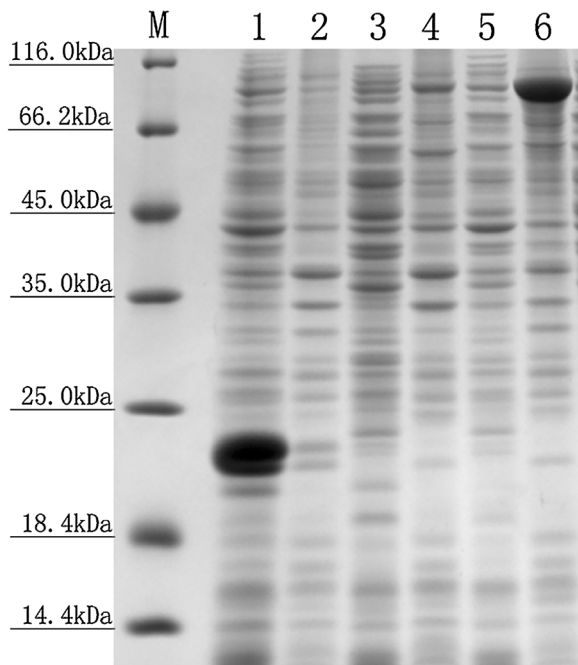


Fig. 3. The results of fusion protein expression. M: Unstained Protein MW Marker; 1: cells with pET32a(+) only induced by IPTG (supernatant); 2: cells with pET32a(+) only induced by IPTG (inclusion bodies); 3: cells with pET32a(+)-sole-lip-1 non-induced by IPTG (supernatant); 4: cells with pET32a(+)-sole-lip-1 non-induced by IPTG (inclusion bodies); 5: cells with pET32a(+)-sole-lip-1 induced by IPTG (supernatant); 6: cells with pET32a(+)-sole-lip-1 induced by IPTG (inclusion bodies).

(Fig. 4). Hexadecanoic acid methyl ester (the peak appearing at 30.167 min), 8,11-Octadecadienoic acid methyl ester (the peak appearing at 33.581 min), 8-Octadecenoic acid methyl ester (the peak appearing at 33.690 min), and Octadecanoic acid methyl ester (the peak appearing at 34.172 min) were both detected in the standard sample containing 4 fatty acid methyl esters (Fig. 4A) and the sample contained immobilized enzyme AOB-sole-lip-1 (Fig. 4B). While small amount of esters Hexadecanoic acid methyl ester (30.241 min), 8-Octadecenoic acid methyl ester (33.763 min) were also detected in the blank control, that could be explained by the fact that excessive methanol can facilitate transesterification to some extent even without catalyst (Fig. 4C). The peak appearing at 31 min represents an impurity.

3.5. Enzymatic properties of lipase transesterification

3.5.1. Optimum reaction temperature

The effect of temperatures ranging from 25 to 55 °C on lip-1 transesterification activity for biodiesel production is shown in Fig. 4. Results showed (Fig. 5) that there was a rise in the biodiesel yield catalyzed by AOB-sole-lip-1 when the temperature was gradually increased from 25 to 45 °C and the maximal biodiesel yield was obtained at 45 °C, but further increments in temperature to 55 °C caused a decrease in the biodiesel yield.

3.5.2. Optimum solvent systems

Compared with the n-hexane solvent system, the biodiesel yield in t-Butanol, t-Pentanol, n-hexane/t-Butanol (4:1) and n-hexane/t-Pentanol (4:1) systems was lower. While the petroleum ether system was the best, the biodiesel yield in this system was 1.44-fold that of the n-hexane solvent system (Fig. 6).

Table 2

The blast result of the lipase sequences from Eryuan hot spring.

Lipase name	Gene Length	Gene Identity%	AA Length	AA Identity%	Predicted function
Lip-1	1869	0	622	36	Lipase/esterase
Lip-2	945	77	314	79	Alpha-beta hydrolase family esterase
Lip-3	900	79	299	60	patatin like esterase
Lip-4	756	0	251	48	GDSL family lipase
Lip-5	732	75	243	53	Esterase/lipase/thioesterase family active site
Lip-6	741	94	246	99	Glycerophosphoryl diester esterase
Lip-7	810	74	269	63	diguanylate phosphodiesterase
Lip-8	876	0	291	99	Predicted esterase of the alpha-beta hydrolase superfamily

3.5.3. Methanol tolerance analysis

Methanol is toxic to enzymes, and most enzymes are inhibited or even inactivated in an environment containing methanol. However, methanol is one of the substrates for transesterification,

so the methanol tolerance of the lipases was very important [18]. In our work, the methanol concentration was varied from 50 mmol to 500 mmol to investigate its effect on the biodiesel yield. As can be seen from Fig. 7, the maximal biodiesel yield was obtained at

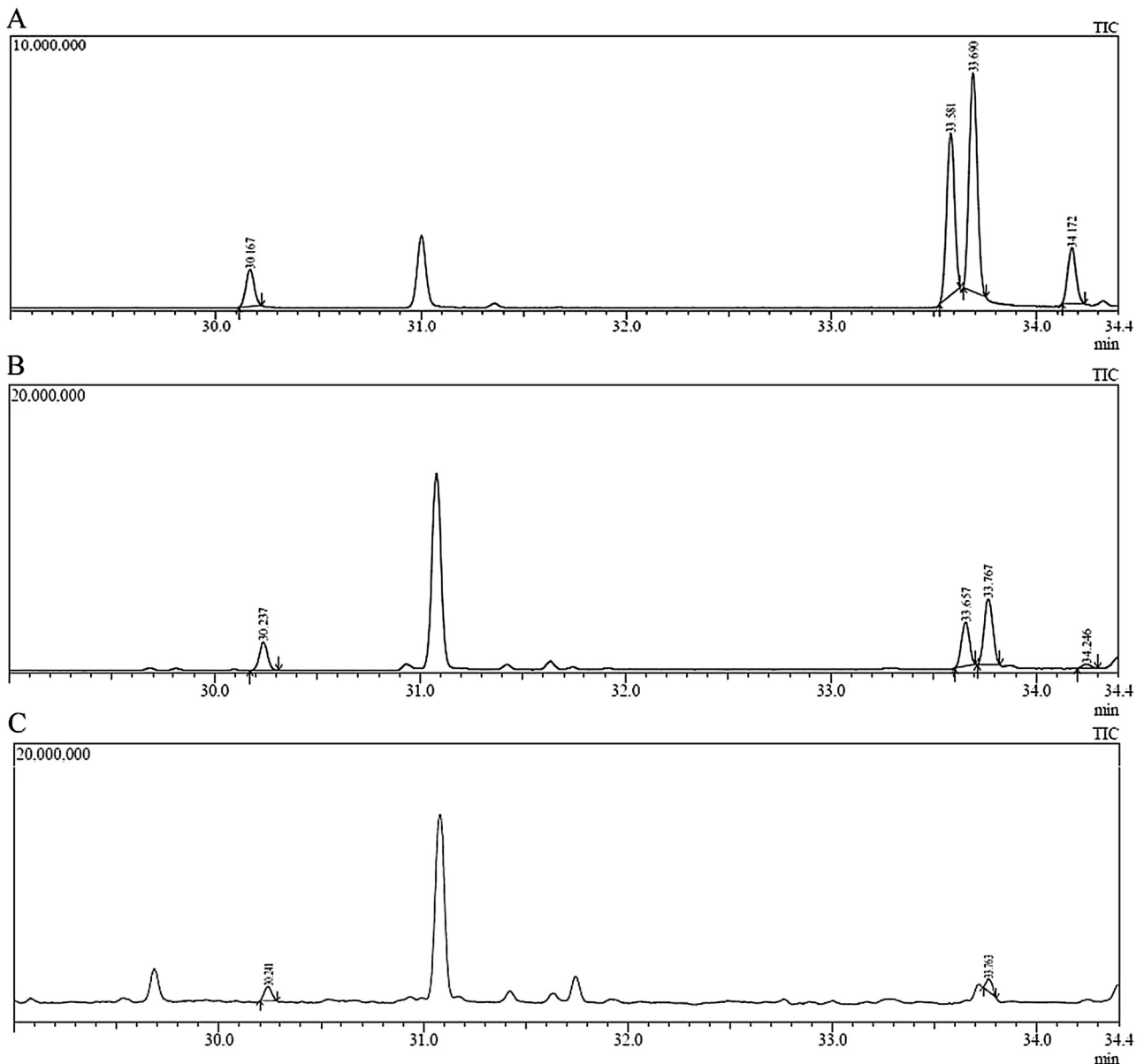


Fig. 4. The results of transesterification on GC-MS. A: The standard sample; B: Transesterification results of AOB-sole-lip-1; C: Transesterification results of blank control.

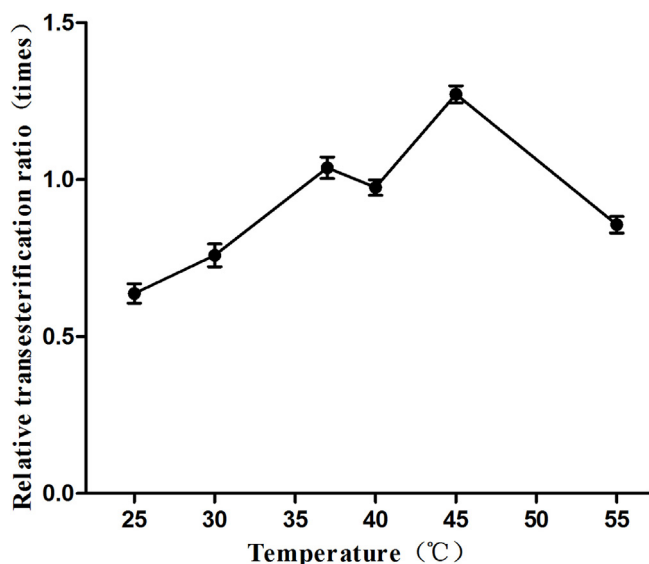


Fig. 5. The reaction temperature of lipase lip-1. (The reaction parameters: 3.5:1 molar ratio methanol to oil, 1% (w/w) water, 200 rpm for 30 h in petroleum ether system.).

350 mmol/L (at a molar ratio of methanol of 10.5:1), which was higher than the molar ratio of methanol to oil in the complete transesterification reaction (3:1).

3.5.4. Water content analysis

Compared with the system without water, the biodiesel yield was higher in the system with water, because the transesterification of lipase occurs in a biphasic system [19]. When the water content was below 5% there was only a small amount of fatty acids, whereas when the water content reached 6%, the amount of fatty acids rapidly increased, because excess water may stimulate the competitive hydrolysis reaction. When water content was 1%, the relative enzymatic activity reached its peak value, after which it decreased with increasing water content (Fig. 8).

4. Discussion

Extremophiles are microbes which can grow and play special roles in certain extreme environments. Consequently, enzymes with unusual biological functions isolated from extremophiles can

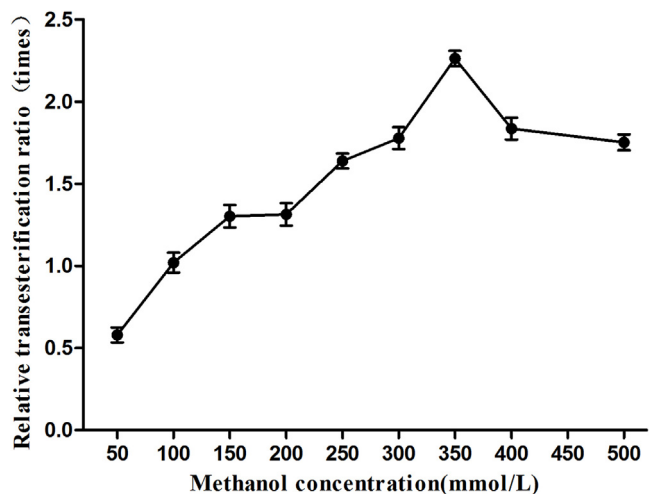


Fig. 7. The methanol tolerance of lipase lip-1. (The reaction parameters: 1% (w/w) water, 45 °C, 200 rpm for 30 h in petroleum ether system.).

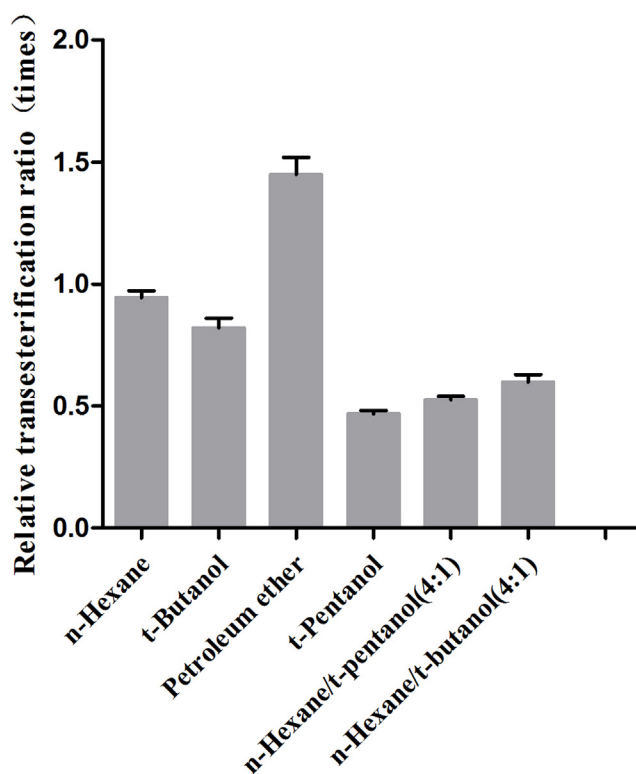


Fig. 6. The organic solvents tolerance of lipase lip-1. (The reaction parameters: 3.5:1 molar ratio methanol to oil, 1% (w/w) water, 45 °C, 200 rpm for 30 h).

be utilized in specific industrial applications [20]. For a long time, functional genes were screened using traditional separation methods; However, about 99% of microorganisms in the natural environment cannot be obtained by traditional isolation and culture methods [21]. Recently, a metagenomic approach has complemented the culture-dependent method which avoids the cultivation of diverse microorganisms from environmental samples and involves directly obtaining the global genomes of the organisms [22]. This approach greatly expands the usable scope of the microbial resources, and makes possible the acquisition of a large number of uncultured microbial resources from the environment. In this study, a BAC library was constructed with environmental DNA extracted from a hot spring. There were 8 esterase/lipase genes obtained from the BAC library and 3 of them were novel genes. We choose lip-1 for further investigation because of its lowest gene and the amino acid identity.

The *lip-1* gene is expressed with the *sole* gene in a prokaryotic expression system. From Fig. 3 we can see the fusion protein was expressed in inclusion bodies. This is because of the strong hydrophobicity of the oleosin, therefore all the fusion protein was aggregated and expressed in the form of inclusion bodies. Here we used artificial oil body technology to refolding the fusion protein. Under ultrasonic oscillation, oil bodies can be disintegrated and reconstructed spontaneously. This characteristic of oil bodies allows oleosin-tagged lipase to be anchored to the surface of artificial oil bodies (AOBs), because of the intense hydrophobic character of oleosin and the hydrophilic character of lipase [12].

Lipase-catalyzed biodiesel preparation is considered as a clean and green technique [23]. The enzymatic properties of new lipase for biodiesel production would be a valuable resource. In this study, the transesterification activity of the immobilized enzyme AOB-sole-lip-1 was analyzed. The optimum temperature, organic solvent type, methanol concentration, and water content were tested. Optimal conditions for production of biodiesel were: 45 °C,

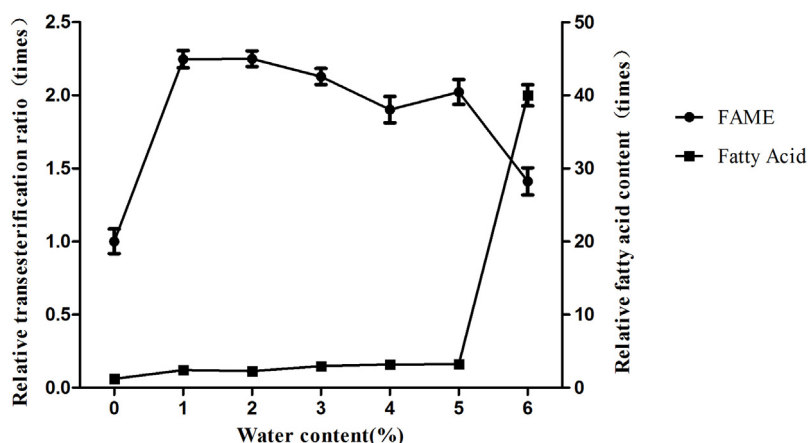


Fig. 8. The water concentration tolerance of lipase lip-1. (The reaction parameters: 3.5:1 molar ratio methanol to oil, 200 rpm for 30 h in petroleum ether system).

in a petroleum ether system, with 350 mmol methanol and 1% water content. Notably, the methanol toxicity test suggested that AOB-sole-lip-1 presented transesterification activity in a high methanol concentration (350 mmol/L) environment without significant change.

5. Conclusion

In conclusion, a novel lipase gene was cloned from the BAC library of a hot spring. The lipase gene was expressed in the prokaryotic expression system of *E. coli* BL21(DE3) and renatured using artificial oil body technology. Enzymatic property analysis showed this lipase has high tolerance to methanol. Because of the low cost of the preparation process, high stability, and methanol tolerance, this novel lipase gene *lip-1* has great prospects for biodiesel production.

Compliance with ethical standards

The authors have no conflict of interest.

This article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.btre.2016.12.007>.

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