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Discovery and characterizaton 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.

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 screenedand sequenced, new lipase genes were discoverd 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 (*Hin*d 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 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). *Hin*d 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 Amerso, 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 *Hin*d 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 *Hin*d 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 Vcm, 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, mannase, 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 secquence of lipase gene *lip-1* has been submmited 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 ddH_2O 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	GGTACCATCGAAGGAAGAATGATGAAAAATAGGAAACGGCAA
Lip-1R	GAGCTCTCAGAACGCCTTTTGCCAGT
Sole F	AGATCTGATGGCTGAGCATTATGGTCAACAAC
Sole R	GAGCTCGTACGTAGAGGTACCCGCAACAGGCTGCTGCGAGAAC

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