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A rapid and efficient method for directed screening of lipase-producing *Burkholderia cepacia* complex strains with organic solvent tolerance from rhizosphere

Zhengyu Shu,[1,2,3] Ruifeng Lin,[1,2,3] Huan Jiang,[1,2,3] Yanfeng Zhang,[1,2,3] Mingzi Wang,[1,2,3] and Jianzhong Huang[1,2,3,*]

Engineering Research Center of Industrial Microbiology, Ministry of Education, Fujian Normal University, Fuzhou Fujian 350108, PR China[1] *College of Life Sciences, Fujian Normal University, Fuzhou Fujian 350108, PR China*[2] *and Engineering Research Center of Fujian Modern Fermentation Technology, Fuzhou Fujian 350108, PR China*[3]

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Lipase from *Burkholderia cepacia* strain is one of the most versatile biocatalysts and is used widely in many biotechnological application fields including detergent additives, the resolution of racemic compounds, etc. Based on the known whole genomic information of *B. cepacia* strain, both ampicillin and kanamycin were added to the TB-T medium to screen *B. cepacia* complex stains from rhizosphere soil samples. The selected colonies from the modified TB-T medium were then qualitatively determined the ability to produce extracellular lipase on the rhodamine B-olive oil agar plates. A total of 35 lipolytic *pseudo-B. cepacia* complex strains were isolated and the positive rate of lipolytic bacteria was 65%. Among them, 15 *pseudo-B. cepacia* complex strains showed tolerance to benzene, *n*-hexane and *n*-heptane at concentration of 10% (V/V) and were identified by the *recA* gene sequence. All of the 14 lipolytic bacteria were identified as *B. cepacia* complex strains except that the *recA* gene sequence of one lipolytic bacterium, strain ZMB009, was not obtained.

[**Key words:** Directed screening; Lipase; *Burkholderia cepacia* complex; Organic solvent tolerance; Rhizosphere]

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze hydrolysis and synthesis of water-insoluble esters and triglycerides at the interface between the insoluble substrate and water. Except for hydrolysis reactions, lipases can also catalyze transesterification reactions, aminolysis reactions, alcoholysis reactions, etc. These reactions usually proceeded with wide substrate specificity, exquisite regio-, chemo- and/or enantioselectivity, etc., making lipases an important group of biocatalysts in organic chemistry (1). Microbial lipases are often more useful than lipases derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, etc. Most commercially useful lipases are of microbial origin (2).

Organic solvent tolerant bacteria have great potential to catalyze the bitransformations of water-insoluble substances in organic-aqueous biphasic systems (3, 4). Many organic solvent tolerant bacteria, such as *Pseudomonas putida* or closely related *Pseudomonas* sp., *Bacillus* sp., *Rhodococcus* sp., etc. have been isolated form soil, coastal sediment, deep sea, etc. (5). Some organic solvent tolerant bacteria strains can also produce organic solvent-stable extracellular lipase (6–8). In cell surface display technology, the organic solvent-stable lipase can be immobilized on the cell surface of the organic solvent tolerant strain, which would result in an ideal whole-cell biocatalyst.

It is more than 100 years that microbial lipases have been studied and the enzyme characterization of most lipases is known. According to the experiment aim, it is necessary to directly screen a specific lipase-producing strain from environmental samples. In conventional screening method of selective medium for lipase-producing strains, the chance of a specific non-predominant strain is low to be detected because of the gradient dilution process of the environmental samples. Although the metagenomic technology can overcome the above problems, it is tedious and expensive. With more and more microbial whole genomic sequences known, a directed screening method for a specific lipase-producing strain could be established by in-depth analysis of its whole genomic DNA information.

Lipases from *Burkholderia cepacia* strains display high stability in organic solvents, exquisite enantioselectivity, etc. and have been used widely in detergent industry, resolution of racemic mixtures, etc. (9–11). In this article, we took *B. cepacia* complex strains as example to introduce the rapid and efficient method for directed screening of a specific lipase-producing strain from rhizosphere.

MATERIALS AND METHODS

Isolation of *B. cepacia* complex strains Rhizosphere soil samples were collected from farmland at Fuzhou, China. The plants were as follows: *Zea mays*, *Sesamum indicum*, *Vigna sinensis* and *Ipomoea aquatica*, respectively. 5 g rhizosphere soil was placed in a sterile 250 ml flask containing 45 ml 0.9% (W/V) NaCl solution, and the flask was shaken at 250 r/min and 28 °C for 30 min. After 10 min standing at room temperature, 200 μl suspensions were plated on the modified TB-T medium containing the following components (per liter): 20 g agar, 2 g glucose, 1 g L-asparagine, 1 g

* Corresponding author. Tel./fax: +86 591 22868212.
E-mail addresses: hjz@fjnu.edu.cn (J.Z. Huang), shuzhengyu@gmail.com (Z.Y. Shu).

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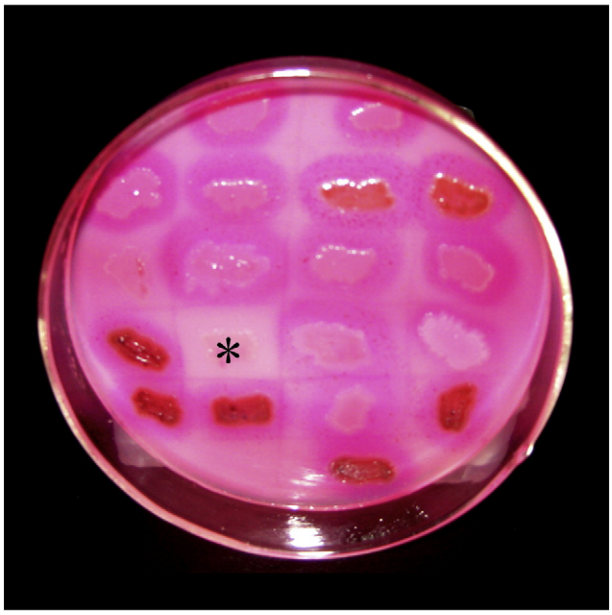

FIG. 1. Production of lipases by *pseudo-B. cepacia* complex strains on the selective screening medium for lipolytic activity. Only one colony marked with * hadn't the activity to hydrolyze olive oil on the olive oil-rhodamine B plate.

$NaHCO_3$, 500 mg KH_2PO_4, 100 mg $MgSO_4.7H_20$, 50 mg trypan blue, 20 mg tetracycline, 60 mg ampenicillin, 50 mg kanamycin, 50 mg nystatin, and the pH was adjusted to 5.5 with 10% phosphoric acid (12). The plates were incubated at 28 °C for 3–4 days. The colonies on the plates were coded as ZMB (*Zea mays*), SIB (*Sesamum indicum*), VSB (*Vigna sinensis*) and IAB (*Ipomoea aquatica*) based on which rhizosphere they came from, followed by a progressive number of isolation.

Secondary screening of pseudo-*B. cepacia* complex strains with lipolytic activity The colonies on the plates with the modified TB-T medium were then streaked onto another selective agar plate for lipolytic bacteria and cultured at 30 °C for 4 days. The selective screening medium for lipolytic bacteria contained the following components (per liter): 1 g sucrose, 1 g yeast extract, 2 g peptone, 1 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_20$, 3 ml olive oil, 3 ml rhodamine B solution (0.1%, W/V). The colonies with orange fluorescent halos around them were purified by repeated streaking on the Luria–Bertani agar plates and the pure culture of the single colony was picked out for lipase production in liquid medium.

Lipase production The pure culture of the single colony was inoculated into Luria–Bertani medium and cultivated on a rotary shaker at 200 r/min and 37 °C for 12 h. 1 ml Luria–Bertani culture broth was then inoculated into 50 ml lipase production medium in 250 ml flask. The lipase production medium consisted of (per liter) 3 g dextrin, 2 g beef extract, 0.75 g $MgSO_4.7H_2O$, 1.4 g K_2HPO_4, and 12.5 ml soybean oil, pH 8.11. The flasks were incubated at 250 r/min and 30 °C for 22 h. The fermentation broth was collected and centrifuged at 8000 r/min for 10 min and the cell-free supernatant was assayed for the lipase activity.

Assay of lipase activity The alkali titrimetric assay was carried out to quantitatively determine the lipase activity using olive oil as substrate (13). The reaction was carried out in 50 mM Tris/HCl buffer (pH 8.0) for 10 min at 60 °C. One unit of lipase activity (U) was defined as the amount of lipase necessary to liberate one μmol fatty acid from olive oil per min under the standard assay conditions.

Tolerance to various organic solvents The pure culture of the single colony was inoculated into Luria–Bertani medium and cultured for 12 h on a rotary shaker at 200 r/min and 37 °C. 50 μl Luria–Bertani culture broth was then inoculated into 4.5 ml Luria–Bertani medium in 30 ml tube. After homogeneity of the bacterial inoculation, 0.5 ml methanol, pentanol, benzene, octanol, hexane and *n*-heptane was added to Luria–Bertani medium, respectively. The tubes were incubated at 250 r/min and 37 °C for 48 h and then stood still for 1 h to separate the culture broth into organic phase and aqueous phase. The OD_{600} of the aqueous phase was determined as the criterion of tolerance to organic solvents.

Identification of the lipolytic pseudo-*B. cepacia* complex strains with broad-spectrum organic solvents tolerance by *recA* gene sequence Genomic DNA extraction was carried out as described by Mahenthiralingam et al. (14). The *recA* gene was amplified by PCR as described by Payne et al. (15). The forward primer and the reverse primer was 5′GAA(G)AAGCAGTTCGGCAA3′ and 5′GAGTCGATGACGATCAT3′, respectively. The PCR amplification protocol consisted of a 5 min denaturation at 95 °C, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and a final hold for an extra 10 min at 72 °C. The PCR products were cloned into pMD18-T vector. The recombination plasmids were transformed into *E. coli* DH5α, and the positive clones were then screened to sequence the *recA* gene.

The nucleotide sequences of the *recA* gene from the following 18 strains have been deposited in GenBank (GenBank accession number shown in brackets): ZYB002 (No. FJ424795), ZMB004 (No. FJ424796), ZMB005 (No. FJ424797), ZMB007-2 (No. FJ424812), ZMB008 (No. FJ424798), ZMB010 (No. FJ424799), SIB001 (No. FJ424800), SIB005 (No. FJ424801), SIB006 (No. FJ424802), SIB010 (No. FJ424810), SIB011 (No. FJ424803), SIB012 (No. FJ424804), SIB013 (No. FJ424805), SIB014 (No. FJ424806), VSB003 (No. FJ424811), VSB006 (No. FJ424807), VSB012 (No. FJ424808), VSB017 (No. FJ424809). The nucleotide sequences of the *recA* gene were aligned using BioEdit software. Phylogenetic and molecular evolutionary analysis were conducted using MEGA version 4.0. The tree was rooted with the *recA* gene of *Pseudomonas cenocepacia* ATTC 17765 (GenBank accession number AF456025).

RESULTS AND DISCUSSION

Modified screening process and method for lipolytic *B. cepacia* complex strains More than 21 *B. cepacia* complex strains whole genomic DNA have been sequenced. Because there is a lipase-encoding sequence on chromosome 2 in all *B. cepacia* complex strains whole genomic DNA, all *B. cepacia* complex strains should have the ability to produce lipase in theoretical analysis. Moreover, there existed the lactamase-encoding sequence and the aminoglycoside phosphotransferase-encoding sequence in all *B. cepacia* complex strains whole genomic DNA, and all *B. cepacia* complex strains also should have the resistance to ampicillin and kanamycin. A modified screening process and method was applied to screen *B. cepacia* complex strains with lipolytic activity.

To improve the screening efficiency for *B. cepacia* complex strains, TB-T medium was modified by the addition of ampicillin and kanamycin, firstly. To determine the effective concentration of ampicillin and kanamycin, *Burkholderia* sp. ZYB002, was selected as the control strain. The reference strain, *Burkholderia* sp. ZYB002, was isolated from the oil-polluted soil sample in traditional olive oil-rhodamine B selective screening medium in our lab. In the preparatory experiment to test the antibiotic tolerance, ampicillin did not show any negative impact on the growth of *Burkholderia* sp. ZYB002 even at the concentration of 600 mg/l. *Burkholderia* sp. ZYB002 strain could normally grow at the low concentration of kanamycin and the growth rate obviously became slow when the concentrations of kanamycin was increased up to 250 mg/l. In this screening process, the concentrations of ampicillin and kanamycin were 60 mg/l and 50 mg/l, respectively.

A selective medium for lipolytic activity was then used as secondary screening medium for *B. cepacia* complex strains with lipolytic activity. In this complex screening process, a high positive rate of lipolytic *B. cepacia* complex strains was obtained. 36% of total colonies screened from the TB-T medium plates showed orange fluorescent halos around them on the selective screening medium for lipolytic bacteria. The proportion rose up to 65% when the modified TB-T medium was used to screen *B. cepacia* complex strains (Fig. 1). The positive rate of lipolytic bacteria in this screening process and method was much higher than that of other screening process and method (16). There existed obvious difference of positive rate of lipolytic *B. cepacia*

TABLE 1. The lipase activity of the fermentation broth from *Burkholderia* sp. ZYB002 and *pseudo-B. cepacia* complex strains

Strain	Lipase activity (U/ml)	Strain	Lipase activity (U/ml)	Strain	Lipase activity (U/ml)
ZYB002	6.9	SIB012	10.1	ZMB003	15.4
SIB001	7.4	SIB013	10.3	ZMB004	12.1
SIB002	15.7	SIB014	11.8	ZMB005	11.1
SIB003	10.4	SIB034	0	ZMB007	1.5
SIB004	15.7	VSB001	11.0	ZMB007-2	10.4
SIB005	35.1	VSB003	13.8	ZMB008	6.4
SIB006	15.8	VSB006	3.1	ZMB009	12.9
SIB007	12.6	VSB012	13.5	ZMB010	9.5
SIB008	6.8	VSB013	13.8	ZMB011	3.8
SIB009	16.5	VSB017	7.0	IAB001	4.4
SIB010	7.7	ZMB001	10.4	IAB003	0
SIB011	11.7	ZMB002	6.2	IAB004	1.8