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Identification of organic solvent-tolerant lipases from organic solvent-sensitive microorganisms

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ABSTRACT

Organic solvent-tolerant (OST) lipases are becoming increasingly useful in organic synthesis, thereby raising the importance for the discovery of new OST lipases. Until now, the screening method has only focused on pre-selection of OST bacterial strains as microbial resource of OST lipases. With the aim of screening for novel OST lipases from organic solvent sensitive (OSS) microorganisms, we used an established fluorescence-based assay to develop a biphasic reaction for direct functional identification of OST lipases. Using this approach, we have isolated and identified nine OSS bacteria secreting lipases that showed the same level of solvent tolerance of lipases from another 11 OST bacterial strains. Among them, two OST lipases from the OSS bacteria, *Bacillus subtilis* ZJU003 and *Xanthomonas oryzae* ZJU548, showed remarkable stability in 50% (v/v) a variety of hydrophobic organic solvents over an incubation period of 7 days. These results show that OSS microorganisms are a rich microbial resource for OST lipases that can display excellent stability in organic solvents.

1. Introduction

Lipases (triacylglycerol lipases, EC 3.1.1.3) are enzymes that act on ester bonds, catalyzing hydrolysis or ester synthesis in aqueous and organic media [1]. The OST lipases are proven to be excellent biocatalysts for performing various synthetic reactions such as esterification, transesterification and interesterification in organic solvents, such as production of pharmaceuticals and biofuel, under a water-restricted environment [2,3]. Reactions catalyzed by OST lipases in organic solvents offer possibilities for shift of thermodynamic equilibrium toward synthesis, increase of solubility of hydrophobic substrates and control of substrate specificity [4–6]. Despite the advantages for lipase in organic solvent-based systems, they are generally very labile catalysts and easily lose activity in organic solvents [7]. To overcome these limitations, several strategies have been proposed. These include chemical modification of the enzyme, medium engineering, immobilization and genetic engineering technology [5,8]. However, if lipases are naturally stable and exhibit high activity in organic solvents, they can be more conveniently employed. It is therefore important to discover novel OST lipases that have excellent tolerance and activity in the presence of organic solvents.

To date, several OST lipase-producing microorganisms have been identified on the base of the hypothesis that extracellular enzymes secreted by OST microorganisms are stable in the presence of organic solvents [9]. So most of the OST enzymes reported from previous screening studies, such as lipases [5,10,11] and proteases [12,13], are derived from OST microbial strains. These extremophilic microorganisms, competent for growth in the presence of toluene and/or alkanes, mainly belong to *Pseudomonas* sp. [9,11,14–17], *Bacillus* sp. [10,18–20] and *Burkholderia* sp. [4]. However, the scope of that screening method was limited due to the OST microorganisms only representing a small proportion of microorganisms [21,22]. In the last few decades, a large numbers of lipases from OSS microorganisms have been identified, cloned and characterized [23–25]. Despite the majority of lipases from OSS microorganisms displaying low or zero activity in the presence of organic solvents, several lipases were found to be tolerant to organic solvents by enzymatic study [6,26,27]. Therefore, it is possible to identify the OST lipases from the OSS microorganisms.

This paper describes the direct functional screening of new OST lipases from OSS microorganisms. The organic solvent tolerance of the microbial lipases was directly determined by a fluorescence-based microplate assay in a biphasic solvent-buffer system. Nine OST lipases identified from OSS bacteria displayed good stability in 50% (v/v) toluene. Among them, two OST lipases from *Bacillus subtilis* and *Xanthomonas oryzae* showed particularly remarkable stability in 50% (v/v) hydrophobic organic solvents after

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the enzymes were incubated for 7 days. These OSS microorganisms were shown to be promising microbial resources for OST lipases.

2. Materials and methods

2.1. Enzymes and chemicals

4-Methylumbelliferyl oleate (4-MUF-oleate), *p*-nitrophenyl stearate and triolein were purchased from Sigma–Aldrich Co. Ltd. (USA). All other chemicals were of reagent grade and commercially available. Table 1 shows the commercial lipase preparations used in this paper. No pretreatments were applied before use.

2.2. Screening of lipase-producing microorganisms

All the soil samples were collected from fuel-oil-contaminated environments located in Zhejiang province, China. The microorganisms were cultivated by inoculating 0.1 g of each soil sample into 50 mL of enrichment medium (olive oil 10.0 g/L, tween-80 5.0 g/L, $(NH_4)_2SO_4$ 5.0 g/L, KH_2PO_4 3.0 g/L, K_2HPO_4 1.0 g/L and $MgSO_4$ 0.5 g/L; pH 7.2) in 250 mL Erlenmeyer flasks. Incubation was carried out with agitation at 200 rpm and 30 °C for 48 h. Samples from batch cultures were streaked onto olive oil medium plates that contained olive oil 10.0 g/L, gum arabic 10.0 g/L, tryptone 5.0 g/L, yeast extract 3.0 g/L, $(NH_4)_2SO_4$ 5.0 g/L, KH_2PO_4 3.0 g/L, K_2HPO_4 1.0 g/L, $MgSO_4$ 0.5 g/L and bacteriological agar 20.0 g/L at 30 °C for 48 h. The microorganisms forming large transparent zones around the clones on the plates were stored for further screening.

2.3. Selection of the OST lipase producer

The lipase producers isolated from the plates were cultivated in 96-deep-well plates containing 0.5 mL of the olive oil medium in a rotating shaker at 30 °C and 200 rpm for 48 h. Cells and olive oil were removed by centrifugation (4000 × *g*) at 30 °C for 20 min, and resultant clear supernatant (95 μL) was immediately transferred to a 96-well microplate. The supernatant was added with 5 μL of 1.0 M phosphate buffer solution (PBS, pH 7.2) and then mixed with 100 μL toluene. The lipase samples only incubated in buffer under the same conditions were used as the blank reference. After the mixture was incubated at 30 °C and 200 rpm for 24 h, the residual activity of the lipase was directly by fluorescence-based microplate assay.

2.4. Identification of strains by 16S rRNA sequence alignment

The isolated strains were identified by partial 16S rRNA gene alignment. The partial 16S rRNA gene was amplified using genomic DNA of the strain as a template in the polymerase chain reaction (PCR) containing 8f and 1492r primers [28]. The PCR product was examined by electrophoresis and then sequenced. The strain was identified using a BLAST program and sequence alignment based on homology to known 16S rRNA gene sequences in the NCBI database. The 16S rRNA sequences of all strains in this study were deposited in the GenBank database.

2.5. Organic solvent tolerance of microbial strains

The microorganisms were cultured in 250 mL Erlenmeyer flasks containing 50 mL of the olive oil medium. After adding 12.5 mL of organic solvent (benzene, toluene, cyclohexane and *p*-xylene), the flasks were plugged with chloroprene-rubber stoppers to prevent solvent evaporation [9,12]. Incubation was carried out at 30 °C and 200 rpm for 48 h. The microbial growth was determined by measuring the dry cell weight (DCW).

2.6. Effect of organic solvents on lipases from B. subtilis ZJU003 and X. oryzae ZJU548

To study the effect of organic solvents on lipase, strains of *B. subtilis* ZJU003 and *X. oryzae* ZJU548 were cultivated in the olive oil medium at 30 °C and 200 rpm for 48 h. Samples of the cell-free supernatant (3.00 mL) were obtained from the cultivation and mixed with 0.75 or 3.00 mL organic solvents (20% or 50%, v/v, respectively) in screw-capped tubes. The mixtures were incubated at 30 °C with agitation at 200 rpm for 24 h and 7 days. To eliminate the effect of organic solvents in colorimetric method, the organic solvents were removed or diluted before activity determination as follows. The hydrophobic solvents were removed by centrifugation at 4000 × *g* and 4 °C for 20 min. In the case of hydrophilic solvents, the concentration of organic solvent in lipase solution was diluted to 5% (v/v) after incubation. The blank reference was prepared by boiling the solution at 99 °C for 5 min to inactivate the enzyme, and then added 5% (v/v) same type of organic solvent. The residual activity was measured using *p*-nitrophenyl stearate as substrate by the colorimetric method at 30 °C. The tested organic solvents included dimethyl sulfoxide (DMSO), ethanol, acetone, acetonitrile, benzene, toluene, *n*-hexane and *n*-heptane.

2.7. Analytical methods

2.7.1. Fluorescence-based microplate assay

Reactions were initiated by adding 22 μL of 4-MUF-oleate solution (5.0 mM, dissolved in toluene) to a 200 μL mixture of enzyme solution and toluene (to give a final 4-MUF-oleate concentration of 0.5 mM) at 30 °C and 200 rpm. The residual activity of the lipase was determined using a 96-well fluorescence microplate-reader (Thermo Scientific Varioskan Flash, USA) to measure the increases in fluorescence emission at λ_{em} = 460 nm with excitation at λ_{ex} = 360 nm [29]. A unit of lipase activity for 4-MUF-oleate was defined as the amount of enzyme required to release 1.0 μmol of 4-MUF per minute under the conditions described above [29].

2.7.2. Colorimetric method:

Typically, 20 μL *p*-nitrophenyl stearate solution was added to 880 μL of 50 mM PBS (pH 7.2) to give a final concentration of 0.4 mM, and the reaction was started by adding 100 μL lipase solution at 30 °C. The hydrolysis of *p*-nitrophenyl stearate was measured by the production of *p*-nitrophenol at 405 nm using the same microplate-reader [30]. The *p*-nitrophenyl stearate was dissolved in isopropanol at a concentration of 20 mM. One unit of lipase activity for *p*-nitrophenyl stearate was defined as the amount of enzyme required to release 1.0 μmol of *p*-nitrophenol per minute under such conditions [6].

The hydrolysis activity for olive oil was measured by the titrimetric method with few modifications [1]. Lipase activity was measured by the production of free fatty acids which were titrated in a pH-stat titrator (Metrohm, Switzerland) with 0.05 M NaOH for 10 min at the titration end point of pH 8.0. One unit of lipase activity for glyceryl tridecanoate was defined as the amount of lipase that released 1.0 μmol of fatty acid per minute at 30 °C.

3. Results and discussion

3.1. Selection and identification of OST lipase-producing organisms

In order to screen novel OST lipases producers, lipolytic microorganisms have been isolated for further screening process. Cultures of 237 oil-contaminated soil samples using olive oil as the sole source of energy and carbon were incubated at 30 °C and 200 rpm for 48 h. In our study, most of the isolated microorganisms were