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A robust whole-cell biocatalyst that introduces a thermo- and solvent-tolerant lipase into *Aspergillus oryzae* cells: Characterization and application to enzymatic biodiesel production

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ABSTRACT

To develop a robust whole-cell biocatalyst that works well at moderately high temperature (40–50 °C) with organic solvents, a thermostable lipase from *Geobacillus thermocatenulatus* (BTL2) was introduced into an *Aspergillus oryzae* whole-cell biocatalyst. The lipase-hydrolytic activity of the immobilized *A. oryzae* (r-BTL) was highest at 50 °C and was maintained even after an incubation of 24-h at 60 °C. In addition, r-BTL was highly tolerant to 30% (v/v) organic solvents (dimethyl carbonate, ethanol, methanol, 2-propanol or acetone). The attractive characteristics of r-BTL also worked efficiently on palm oil methanolysis, resulting in a nearly 100% conversion at elevated temperature from 40 to 50 °C. Moreover, r-BTL catalyzed methanolysis at a high methanol concentration without a significant loss of lipase activity. In particular, when 2 molar equivalents of methanol were added 2 times, a methyl ester content of more than 90% was achieved; the yield was higher than those of conventional whole-cell biocatalyst and commercial *Candida antarctica* lipase (Novozym 435). On the basis of the results regarding the excellent lipase characteristics and efficient biodiesel production, the developed whole-cell biocatalyst would be a promising biocatalyst in a broad range of applications including biodiesel production.

1. Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) make up a versatile group of enzymes that have the ability to hydrolyze triglycerides at the lipid-water interface. Lipases show wide substrate specificity and they are often used in chemo-, enantio- and stereo-selective reactions of biotechnological importance [1,2].

Although immobilization of lipases may produce some improvements in enzyme features, like stability, activity, selectivity or specificity [3,4], this process has a cost that in certain cases may promote some difficulties to the implementation of the processes. To overcome this drawback, we focused on a whole-cell biocatalyst, which enables the direct use of lipase-producing microorganisms. Among several microorganisms, *Aspergillus oryzae* is a promising host because it has high protein productivity in the expression of heterologous genes using improved promoters [5,6] and can easily be immobilized on porous biomass support particles (BSPs [7]). Thus far, *A. oryzae* strains that have been genetically engineered to express several microbial lipases have been developed for use as whole-cell biocatalysts in biodiesel production and enantioselective transesterification [8–11]. Since practical conditions that promote the reaction include a high temperature and a high concentration of organic solvents, there is a necessity to develop a robust whole-cell biocatalyst. Therefore, introducing robust lipases into *A. oryzae* would further expand the application of this technology.

Lipases from thermophiles often show their extreme stability at elevated temperature and in organic solvents [12]. Thus, they have become objects of special interest for structural investigations and also for industrial applications [13]. The lipase from *Geobacillus thermocatenulatus* (BTL2), isolated by Schmidt-Dannert et al. [14], is an interesting lipase possessing unique structural characteristics. BTL2 comprises 389-amino-acid residues with a molecular mass of 43 kDa, which is relatively larger than those of other microbial lipases [15]. In contrast to most lipases, BTL2 has two lids and a zinc-binding domain that is typically observed in the same family [15]. Moreover, BTL2 shows high stability toward moderately high temperature (40 °C) and organic solvents [14]. Such excellent characteristics of BTL2 have led to extensive studies such as high level expression in *Escherichia coli* [16] and *Pichia pastoris* [17], immobilization on various kinds of supports [18–21], and applications including the kinetic resolution of chiral substrates [22] and aliphatic ester synthesis [20].

In the present study, a recombinant *A. oryzae* whole-cell biocatalyst expressing BTL2 (r-BTL) was developed. The characteristics of r-BTL were evaluated and compared with those of a conventional

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A. oryzae whole-cell biocatalyst expressing *Fusarium heterosporum* lipase (r-FHL), which provides high alkyl ester contents of more than 90% during alcoholysis [23]. In addition, r-BTL was used for biodiesel production from palm oil, which is one of the most abundant, cheapest and available vegetable oils and would be a more sustainable biodiesel-feedstock than rapeseed oil, as shown in life cycle assessments [24,25]. However, because of the low fluidity of palm oil at room temperature, a moderately high temperature is desirable for processing reaction mixtures in transesterification. Given the high thermostability of BTL2, r-BTL was applied to the methanolysis of palm oil at a moderately high temperature.

2. Materials and methods

2.1. Strains and chemicals

A. oryzae niaD300, which is a *niaD* mutant derived from the wild type strain RIB40, was used as a recipient strain for transformation. The *E. coli* strain used for gene manipulation was Nova Blue {*endA1 hsdR17* (r_{K12}^{-} m_{K12}^{+}) *supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB*$^{+}$ *lacI*^{q}*ZΔM15::Tn10 (Tet*^{r}*)*]} (Novagen, Madison, WI, USA). Reticulated polyurethane foam BSPs (Bridgestone Co Ltd., Osaka, Japan) with 6 mm × 6 mm × 3 mm cuboids were used to immobilize *A. oryzae*. Palm oil was obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Construction of lipase expression vectors

The gene encoding BTL2 (Genbank accession number X95309) was isolated from *G. thermocatenulatus* NBRC 15316 by PCR using following two primers; BTL fw-SpeI (5′-ggAAGCTTatgatgaaaggctgccgggtgatggttgtg-3′) and BTL rv-NdeI (5′-ggCATATGtcattaaggccgcaaactcgccaactgctc -3′). The isolated fragment was digested with restriction enzymes *Spe*I and *Nde*I and inserted into the multi-cloning site of pNAN8142 [5,26], and the resultant plasmid named pNAN8142BTL. The construction of pNAN8142FHL for expressing *F. heterosporum* lipase (FHL) was described in our previous paper [9].

2.3. Transformation of A. oryzae

Transformation of *A. oryzae* was carried out according to a method previously described by Gomi et al. [27]. *A. oryzae* protoplasts were prepared using Yatalase (Takara, Shiga, Japan) from mycelia grown at 30 °C for 48 h in dextrin–peptone medium, which consists of 2% dextrin, 1% polypeptone, 0.5% KH_2PO_4, and 0.05% $MgSO_4{\cdot}7H_2O$. The recombinant *A. oryzae* strains carrying pNAN8142BTL and pNAN8142FHL were designated r-BTL and r-FHL, respectively.

2.4. Preparation of Aspergillus oryzae whole-cell biocatalysts

Each member of the recombinant *A. oryzae* strain was grown at 30 °C for 5–6 days on a CD agar plate (2% glucose, 0.1% KH_2PO_4, 0.2% KCl, 0.8 M NaCl, 0.05% $MgSO_4{\cdot}7H_2O$, 1.5% agar and 0.2% $NaNO_3$), and spores were harvested with 5 ml of 0.01% Tween 80. The spore solution was aseptically inoculated into a 500 ml Sakaguchi flask containing 300 BSPs in 100 ml of DP medium (2% glucose, 2% polypeptone, 0.5% KH_2PO_4, 0.1% $NaNO_3$, and 0.05% $MgSO_4{\cdot}7H_2O$) and cultivated at 30 °C on a reciprocal shaker at 150 oscillations per min. The mycelia were immobilized spontaneously within the BSPs by physical adsorption during cultivation. After cultivation for 96 h, the fungal cells immobilized on the BSPs were collected by filtration, washed with distilled water, and lyophilized for 48 h. The lipase-expressing cells thus obtained were used as whole-cell biocatalysts.

2.5. Lipase activity assay

Lipase activity assays of r-BTL and r-FHL under various conditions were performed using p-nitrophenyl butyrate (pNPB) as a chromogenic substrate; 5 μl of pNPB was dissolved with 250 μl of ethanol and then diluted to 50 ml with distilled water. The lipase-hydrolytic activities at various temperatures were determined by an assay at temperature that varied from 30 to 70 °C. The effect of temperature on lipase stability was determined by the lipase activity assay at 40 °C using r-BTL and r-FHL after a 24-h incubation with 0.1 M Tris–HCl buffer (pH 8.0) at temperature that varied from 30 to 80 °C. After incubation, 5% of trichloroacetate was added to the reaction mixture to terminate the reaction. The relative activity was calculated as the ratio of the lipase-hydrolytic activities of r-BTL and r-FHL incubated under each temperature to those at 4 °C.

The effect of the organic solvents on lipase stability was also investigated by the lipase activity assay at 40 °C using r-BTL and r-FHL after a 24 h-incubation with 0.1 M Tris (pH 8) containing 30% (v/v) of each organic solvent (dimethyl carbonate (DMC), ethanol, methanol, 2-propanol, and acetone) at 30 °C. The relative activity was calculated as the ratio of the lipase-hydrolytic activities of r-BTL and r-FHL incubated with the buffer containing each organic solvent to those without each organic solvent.

In the present study, one unit (U) of lipase activity was defined as the amount of enzyme that catalyzes the conversion of 1 μmol of pNPB to p-nitrophenol per minute.

2.6. Methanolysis of palm oil

Methanolysis of palm oil using r-BTL and r-FHL was conducted at temperature that ranged from 40 to 60 °C in a thermo block rotator (NISSIN, Tokyo, Japan) at 35 rpm. The composition of the reaction mixtures were as follows: palm oil 9.63 g, methanol 0.37 g, and distilled water 0.5 g (unless otherwise noted). The reaction mixtures were incubated at the respective temperatures, and the reaction were initiated by adding 100 pieces of either r-BTL or r-FHL. To avoid lipase deactivation by an excess amount of methanol, the methanol (0.37 g) was added stepwise to the reaction mixture at 0, 24, 48 and 72 h, corresponding to the total amount of four molar equivalents of palm oil.

To investigate the effect of the methanol addition pattern on methanolysis, an appropriate amount of methanol was added 1–4 times to adjust the total amount to 4 molar equivalents of palm oil. As described elsewhere, 0.4 g of Novozym 435 (Novozymes, Bagsvaerd, Denmark) was added to each reaction mixture as a reference biocatalyst.

2.7. Gas chromatography analysis

Samples obtained from the reaction mixture were centrifuged at 12,000 rpm for 5 min. The upper oil layer was analyzed using a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) connected to a DB5 capillary column (0.25 mm × 15 m; J&W Scientific, USA). The temperature conditions of the injector and detector were set at 245 °C and 320 °C, respectively. The column temperature was set at 150 °C for 0.5 min, increased to 300 °C at 10 °C/min, and finally maintained at this temperature for 10 min. Tricaprylin served as the internal standard for the quantification of the alkyl esters in the reaction mixture. The detailed procedure for the determination of alkyl ester content was described in a previous paper [28].

3. Results

3.1. Evaluation of the catalytic performance of r-BTL

The gene sequence of BTL2, cloned into pNAN8142 completely corresponded to the GenBank reference sequence (X95309). Among several transformants carrying pNAN8142BTL2, the transformant that provided the highest reaction rate in methanolysis was selected and employed in the subsequent experiments.

To evaluate the catalytic performance of r-BTL, the lipase-hydrolytic activities at high temperature and organic solvent tolerance of r-BTL were investigated and compared with those of r-FHL. The optimum temperature of both immobilized cells was determined by the lipase activity assay at each temperature from 30 to 70 °C (Fig. 1). r-BTL showed a maximum activity of 0.034 U per BSP at 50 °C, while r-FHL showed a maximum activity of 0.010 U per BSP at 40 °C (Fig. 1). From 30 to 50 °C, the lipase activity of r-BTL increased from 0.016 to 0.034 U per BSP. Although the lipase activity gradually decreased at temperature above 60 °C, r-BTL maintained an activity of 0.032 U per BSP even at the highest temperature of 70 °C. The activity of r-BTL in the present temperature range was significantly higher than that of r-FHL. The thermostability

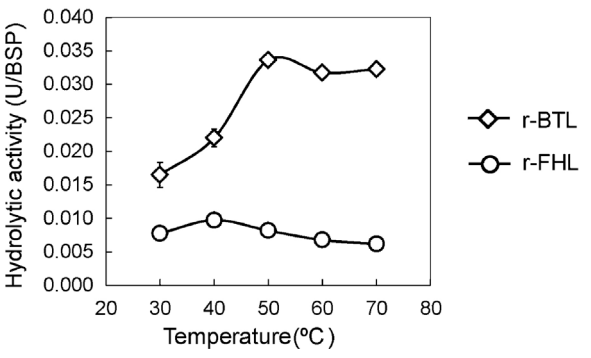

Fig. 1. Effect of temperature on lipase activity of r-BTL (diamond) and r-FHL (circle). The lipase activity was analyzed as described in Section 2.5 in Section 2.