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Immobilized recombinant *Aspergillus oryzae* expressing heterologous lipase: An efficient whole-cell biocatalyst for enantioselective transesterification in non-aqueous medium

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Abstract

Organic esters are used in various industries such as perfumery, flavour and pharmaceutical intermediates. The use of biocatalysts for esterification and transesterification reaction under ambient reaction conditions gives better products for use in flavour and fragrance industries. In the current study, enantioselective transesterification reaction was developed by using recombinant *Aspergillus oryzae* whole-cell biocatalyst expressing lipase-encoding gene from *Candida antarctica*. The recombinant fungal cells were immobilized on Biomass Support Particles (BSPs) to facilitate the reusability of whole-cell biocatalyst. The immobilized CALB expressing whole-cell biocatalyst was used for the optical resolution of (*RS*)-1-phenylethanol by enantioselective transesterification with vinyl acetate as acyl donor. The activity of the whole-cell biocatalyst was optimized and compared with the other whole-biocatalysts *E. coli* displaying CALB, *S. cerevisiae* displaying ROL and *A. oryzae* whole-cell biocatalyst expressing tglA lipase. The initial activity of immobilized CALB expressing *A. oryzae* was at least 15-folds higher than that of *A. oryzae* expressing tglA lipase. The maximum yield of (*R*)-1-phenylethyl acetate reached 88.1% with an enantiomeric excess (ee) of >99% after 3.5 h reaction, while tglA lipase expressing *A. oryzae* showed 90% yield and 95% ee after 48 h. The recombinant *A. oryzae* retained its activity in hexane, heptane, toluene, cyclohexane and octane. Moreover, whole-cell biocatalyst maintained its activity for more than 15 batch reaction cycles. Current study demonstrated the applicability of recombinant whole-cell biocatalyst to bioconversion process in non-aqueous medium.
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Keywords: Lipase; Transesterification; Whole-cell biocatalyst; Immobilization

1. Introduction

Non-aqueous enzymology has several applications in fine chemical, cosmetic and pharmaceutical industries [1]. Organic esters are the most important and versatile components of perfumery, flavour and pharmaceutical industries. Although flavour and fragrance esters are currently produced by chemical synthesis, there is a significant preference for naturally synthesized esters. Moreover, these chemical methods are less effective because of the problems associated with poor selectivity leading to undesirable side reactions, low end product yields and high manufacturing costs. The traditional extractions from plant materials and direct biosynthesis by fermentation are the two natural methods generally used for the production of flavour and fragrances. However, these methods involve the high processing costs and low yields of the desired products and therefore cost-effective methods need to be developed.

Although lipases have been studied for several decades, their usage has been confined mainly to oleo-chemical industries. In organic synthesis, lipase enzymes are playing vital role in catalyzing many reactions including esterifications and transesterifications. Lipases have been employed in the

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transesterification of primary and secondary alcohols for the production of flavour and fragrance esters under the conditions that are milder than those used in the conventional catalysis [2]. Previous studies have demonstrated the production of pharmaceutical intermediates by using lipases in the regio-selective transesterifications such as kinetic resolution of 1-phenylethanol [3] or α-methylene and β-lactams [4]. In particular, lipase B from *Candida antarctica* is the most robust enzyme in organic syntheses with high substrate specificity, catalytic efficiency in the resolution of chiral esters via esterification and transesterification [5], aminolysis, ammonolysis reactions including regio and chemoselectivity [6].

However, the high cost of the immobilized lipases severely limits their use for commercial purposes. In order to overcome this problem, whole-cell biocatalysis in non-conventional media was investigated and immobilization of whole cells on porous Biomass Support Particles (BSPs) made of polyurethane foam has been extensively studied [7,8].

To improve the cost efficiency, the present study attempted to produce flavour and fragrance esters via enantioselective transesterification using recombinant *Aspergillus oryzae* whole-cell biocatalyst expressing lipase B encoding gene from *C. antarctica* developed in our previous study [9]. This paper reports the enantioselective transesterification of vinyl acetate with the (*RS*)-1-phenylethanol, which are commercially valuable in fragrance in industry. Vinyl acetate was used as acyl donor since, vinyl or isopropenyl esters as the acylating agent for transesterification offer an effective solution to overcome equilibrium because the enol co-product is immediately transformed into acetaldehyde or acetone [10,11].

2. Materials and methods

2.1. Materials

(*RS*)-1-Phenylethanol (Wako Pure Chemical Industries, Osaka, Japan) and vinyl acetate (Nacalai Tesque, Kyoto, Japan) were used as reaction substrates in the enantioselective transesterification reaction. Organic solvents were obtained from Nacalai Tesque. (*RS*)-1-Phenylethanol and (*RS*)-1-phenylethyl acetate (Aldrich, Milwaukee, WI, USA) were used as standards for HPLC analysis.

2.2. Strain and cultivation conditions

Recombinant whole-cell biocatalyst expressing *C. antarctica* lipase B gene was obtained by the transformation of *A. oryzae* with pNGA142-PPM-CALB/pNGA142-PPM-tglA plasmids constructed in our previous studies [9,12]. *A. oryzae* transformants were maintained on Czapek-Dox (CD) medium composed of 2% glucose, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.2% KCl, 0.8 M NaCl, 0.05% $MgSO_4{\cdot}7H_2O$ and 1.5% agar. Fungal spores were harvested with 5 ml of 0.01 wt% Tween 80 solution and aseptically inoculated to a 500 ml Sakaguchi flask containing 150 BSPs in 100 ml MP medium (2% maltose, 2% polypepton, 1% KH_2PO_4, 0.2% $NaNO_3$ and 0.05% $MgSO_4{\cdot}7H_2O$), adjusted to pH 6.0 and cultivated at 30 °C in a bioshaker at 150 oscillations/min. The BSPs used for immobilization were 6 mm cubes of reticulated polyurethane foam (Bridge Stone Co. Ltd., Osaka) with a particle voidage of over 97% and a pore size of 50 pores per linear inch. After cultivation for 4 days, the resulting BSPs were collected by filtration, washed twice with distilled water and lyophilized (Free Zone® 2.5 l, Labconco, Kansas, MO, USA) for 24 h.

The biomass concentration within a BSP was measured as follows. Defined number of particles were taken and washed with acetone several times to remove substrate-related compounds and were dried for 24 h at 80 °C. The particles immobilized with fungal mycelium were weighed and treated with aqueous solution of sodium hypochlorite (10%, v/v) to remove biomass. The cleaned particles were rinsed with tap water, dried and reweighed. The amount of biomass was estimated from the difference between the weights. In all the experiments, 10 BSPs containing 50 mg of recombinant *A. oryzae* whole-cell biocatalyst (CALB/tglA) and 50 mg of Novozym 435 were used.

2.3. Enantioselective transesterification

In order to confirm the performance of recombinant *A. oryzae* whole-cell biocatalyst, the optical resolution of (*R*,*S*)-1-phenylethanol was carried out by enantioselective transesterification using vinyl acetate as acyl donor (Fig. 1). Ten BSPs containing 50 mg whole cells of recombinant *A. oryzae* (CALB/tglA) and 50 mg of Novozym 435 were added to the reaction mixture consisting of 30 mg (*RS*)-1-phenylethanol, 21.2 mg vinyl acetate in 3 ml hexane incubated at 30 °C with reciprocal shaking at 150 rpm.

In case of repeated reactions, the reaction conditions were same as mentioned above except for the reaction volume. For repeated reaction, 63.6 mg vinyl acetate and 90 mg (*RS*)-1-PE were dissolved in 9 ml *n*-hexane, and the reaction was started by the addition of 30 BSPs containing the mycelium of recombinant *A. oryzae* expressing the lipase encoding gene from *C. antarctica*.

2.4. HPLC analysis

The yield and enantiomeric excess of the reaction products were determined by HPLC using chiracel OB-H column (Ø

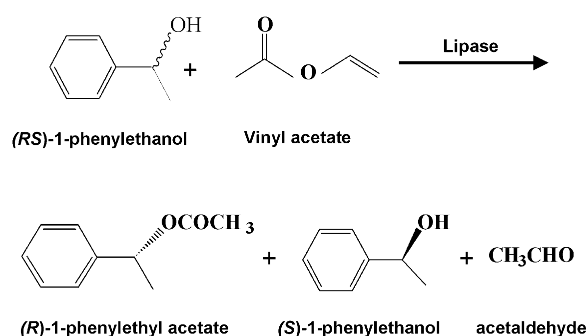

Fig. 1. Schematic representation of optical resolution of (*RS*)-1-phenylethanol to (*R*)-1-phenylethyl acetate catalyzed by lipase enzyme with vinyl acetate as acyl donor.

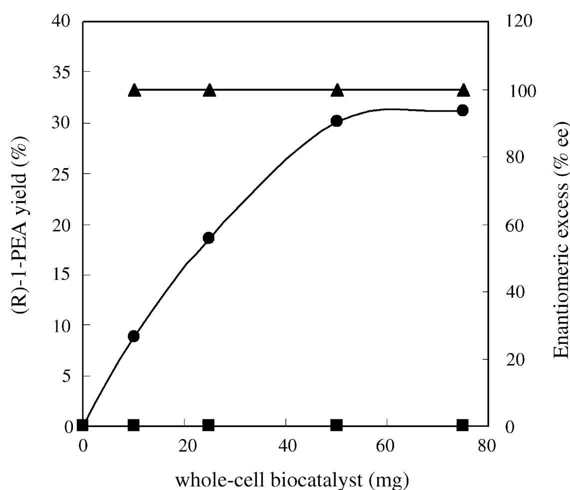

Fig. 2. Effect of amount of whole-cell biocatalyst added to the reaction mixture on reaction yields (%) of (*R*)-1-phenylethyl acetate (*circles*), (*S*)-1-phenylethyl acetate (*squares*) and enantiomeric excess (%ee) (*triangles*) after 30 min reaction at 30 °C. Substrate solution consists of 30 mg (*RS*)-1-phenylethanol, 21.2 mg vinyl acetate in 3 ml hexane.

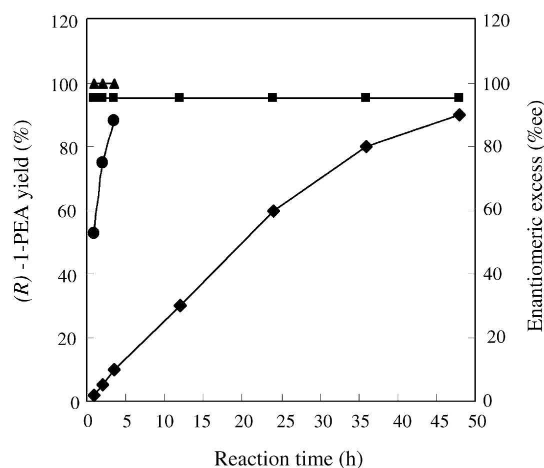

Fig. 3. Time course of (*R*)-1-PEA yields during enantioselective transesterification using the immobilized cells of *A. oryzae*-CALB (circles), *A. oryzae*-tglA (diamonds) and enantiomeric excess (%ee) of *A. oryzae*-CALB (*triangles*) and *A. oryzae*-tglA (*squares*). Reaction was carried at 30 °C by adding 10 BSPs containing 50 mg of recombinant *A. oryzae* (CALB/tglA) in to substrate solution consisting of 30 mg (*RS*)-1-phenylethanol, 21.2 mg vinyl acetate in 3 ml hexane incubated at 30 °C with reciprocal shaking at 150 rpm.

0.46 mm, 25 cm, Diacel Chemical Industries, Tokyo, Japan). Aliquots of 20 μl were withdrawn from the reaction mixture and diluted with 980 μl of the mobile phase of HPLC. After filtration of the resulting solution using Millex-LH (0.45 μm, Millipore, Boston, USA), 5 μl of treated sample was injected and analyzed. The mobile phase was a mixture of hexane/isopropyl alcohol (9:1, v/v) at a flow rate of 0.3 ml/min. Quantification of the reaction products and substrates was performed by measuring absorbance at 210 nm using SPD10A UV detector (Shimadzu, Kyoto, Japan). The experimental data divided by the theoretical yield calculated from the initial substrate quantity were taken as product yield. Enantiomeric excess was calculated as

$$\text{Enantiomeric excess (\%ee)} = \frac{R - S}{R + S} \times 100 \quad (1)$$

3. Result and discussion

3.1. Effect of catalyst amount in BSPs

Fig. 2 shows the effect of the amount of cells in the reaction mixture on yield of enantiomeric products. Higher (*R*)-1-phenylethyl acetate yield was obtained by increasing the amount of cells added to the reaction mixture. However, linearity was not retained above 50 mg of cells (equivalent to 10 BSPs) in the 3 ml reaction mixture. This suggested that the amount of whole-cell biocatalyst added was much greater than the required and external mass transfer resistance had limited the rate of enantioselective transesterification reaction. From this finding, 50 mg of cells were used in the subsequent experiments. (*S*)-PEA was not produced under all conditions while enantiomeric excess remained >99% ee throughout.

3.2. Time course of reaction

Fig. 3 shows the time course of (*R*)-1-PEA production during enantioselective transesterification reaction using recombinant *A. oryzae* expressing CALB gene. After 3.5 h reaction (*R*)-1-PEA yield reached 88.1% with an enantiomeric excess of 99.9% ee and 50 mg of dried cells were used for the reaction which was calculated from the cells isolated from BSPs. The concentrations of (*S*)-1-phenylethanol remained unchanged throughout the reaction, while the control strain *A. oryzae* niaD300 did not catalyze the reaction. The lipase enzyme localized in the cell wall and cell membrane fractions of the recombinant whole-cell biocatalyst is responsible for the catalytic activity [9]. Several previous studies reported the importance of mycelium-bound lipase from a strain of *A. flavus* and *Rhizopus delamer* in catalyzing acidolysis and transesterification reactions, respectively [13,14].

3.3. Effect of various organic solvents

The effect of the nature of the solvent was studied by choosing heptane, hexane, toluene, dichloromethane, tetrahydrofuran (THF), acetonitrile, iso-octane and cyclohexane as the reaction medium for transesterification with whole-cell biocatalyst. As shown in Fig. 4, the recombinant whole-cell biocatalyst could be able to catalyze reaction in most of the hydrophobic solvents used in this experiment. Hexane was found to offer the maximum conversion as compared to other solvents. On the other hand, no reaction was catalyzed in case of water miscible organic solvents. It is known that a minimum quantity of water is essential surrounding the immobilized enzyme for maintaining the enzyme activity. Therefore, hydrophobic solvents are more preferred as compared to hydrophilic once since the latter cause stripping of essential water layer around

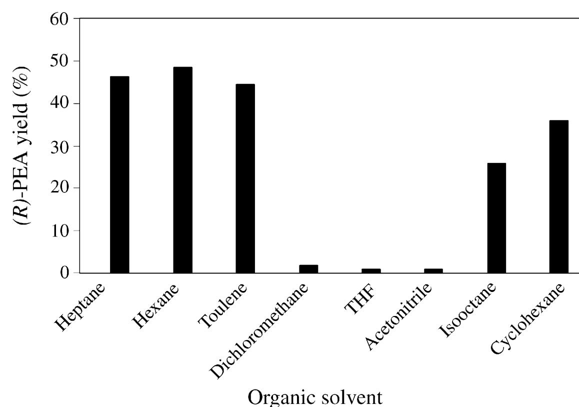

Fig. 4. Effect of organic solvent on the reaction yield of (*R*)-1-phenylethyl acetate (*R*-1-PEA) after 1-h reaction at 30 °C. Substrate solution consists of 30 mg (*RS*)-1-phenylethanol, 21.2 mg vinyl acetate in 3 ml hexane.

the whole-cell biocatalyst, which is necessary for enzyme activity.

3.4. Comparison with different whole-cell biocatalysts

The activity of the whole-cell biocatalyst was optimized and the optimal activity was compared with other fungal, yeast and bacterial whole-cell-biocatalysts (50 mg whole-cell biocatalyst per 3 ml reaction mixture). The initial activity of immobilized CALB expressing *A. oryzae* was at least 15-folds higher than that of recombinant *A. oryzae* expressing triacylglycerol lipase (tglA) [12]. As shown in Fig. 3, the maximum yield of (*R*)-1-phenylethyl acetate reached approximately 90% with an enantiomeric excess (ee) of >99% after 3.5 h reaction, while *A. oryzae* expressing tglA lipase showed 90% yield and 95% ee after 48 h. Even though same expression system was used for tglA and CALB genes [9,12], the difference in the yield of (*R*)-1-PEA is possibly due to the binding of enantiomers to the active site of enzyme. Further work is necessary on structural analysis of tglA lipase to confirm its catalytic activity, which is beyond the scope of present study. In addition, the activity of recombinant whole-cell biocatalyst (CALB) is 10-folds higher than *E. coli* displaying CALB [15] and *S. cerevisiae* displaying *R. oryzae* lipase [16].

3.5. Comparison with commercial enzyme

As shown in Fig. 5, initial transesterification reaction rate of Novozym 435 was three times higher than the whole-cell biocatalyst expressing CALB. In case of Novozym 435, the yield of (*R*)-1-PEA reached 90% within 30 min whereas *A. oryzae* whole-cell biocatalyst took 3.5 h to reach maximum yield of 88.1%. The enantiomeric purity of (*R*)-1-PEA remains same with both the biocatalysts. In both the reactions same amount of Novozym 435 (50 mg) and whole-cell biocatalyst (50 mg) was used. Precise comparison of the highly pure commercial enzyme and whole-cell biocatalyst is not possible since the states of the both enzymes are different.

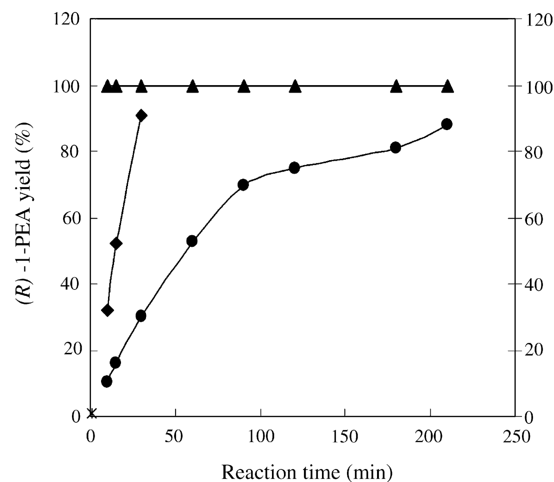

Fig. 5. Comparison of recombinant *A. oryzae* whole-cell biocatalyst expressing lipase B gene from *C. antarctica* and Novozym 435. Reaction yield of (*R*)-1-PEA using whole-cell biocatalyst (*circles*), Novozym 435 (*diamonds*) and enantiomeric excess (*triangles*). Reaction was carried at 30 °C by adding 10 BSPs containing 50 mg of recombinant *A. oryzae* (CALB) or 50 mg Novozym 435 in to substrate solution consisting of 30 mg (*RS*)-1-phenylethanol, 21.2 mg vinyl acetate in 3 ml hexane incubated at 30 °C with reciprocal shaking at 150 rpm.

3.6. Repeated batch reactions

In order to determine the reusability of the recombinant whole-cell biocatalyst, BSPs were filtered off, washed with hexane and reused. It was found (Fig. 6) that transesterification activity remained unaffected even after 15 batch reaction cycles. This confirmed that the by-product acetaldehyde did not deactivate the whole-cell biocatalyst. The commercial enzyme Novozym 435 also remained active during repeated use for 10 reaction cycles and it was difficult to proceed for more repeated batches since the enzyme loss in the filtration (<1 mm diame-

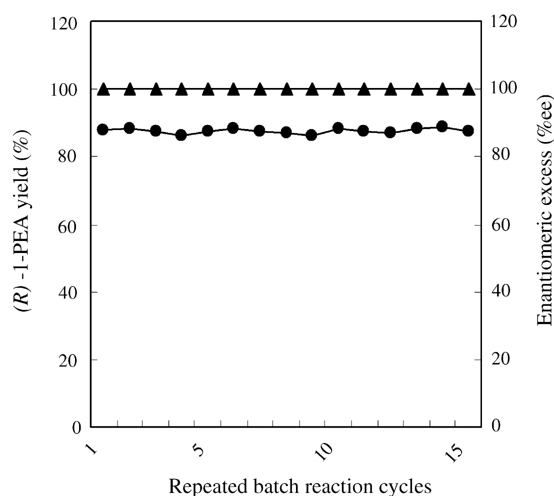

Fig. 6. Effect of repeated use of whole-cell biocatalyst. Reaction yield of (*R*)-1-PEA using whole-cell biocatalyst (*circles*) and enantiomeric excess (*triangles*). Substrate solution consisting of 63.6 mg vinyl acetate and 90 mg (RS)-1-PE was dissolved in 9 ml *n*-hexane, and the reaction was started by the addition of 30 BSPs containing the recombinant *A. oryzae* (CALB). Reaction conditions are same as for the standard reaction.

ter) at the end of each reaction cycle was considerably high. The large size of the BSPs facilitated the easy recovery of biocatalyst from the reaction medium.

4. Conclusion

The kinetic resolution of racemic 1-phenylethanol was successful using recombinant *A. oryzae* whole-cell biocatalyst expressing lipase B gene from *C. antarctica* (CALB). Vinyl acetate was employed as acyl donor by considering the immediate irreversible transformation of enol co-product to acetaldehyde. The results showed that the (*R*)-1-phenylethanol was quantitatively transesterified into (*R*)-1-phenylethyl acetate, leaving behind the (*S*)-1-phenylethanol in the reaction medium. The activity of the recombinant *A. oryzae* expressing CALB gene is higher than the other whole-cell biocatalysts of bacterial, yeast and fungal origin. The recombinant whole-cell biocatalyst is highly selective yielding 88.1% of (*R*)-1-phenylethyl acetate in 3.5 h with a high enantiomeric excess of >99% obtained and activity retained in various hydrophobic solvents. It was demonstrated that *A. oryzae* whole-cell biocatalyst is active for 15 batch reaction cycles. Even though the initial reaction rate of Novozym 435 is higher than the whole-cell biocatalyst, the processing cost and reusability could make the recombinant whole-cell biocatalyst more attractive for the production of commercially valuable esters in bulk quantities and for the transesterification of plant oils.

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