BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

T. Matsumoto · M. Ito · H. Fukuda · A. Kondo

Enantioselective transesterification using lipase-displaying yeast whole-cell biocatalyst

Abstract An enantioselective transesterification in non-aqueous organic solvent was developed by utilizing a lipase-displaying yeast whole cell biocatalyst constructed in our previous study. As a model reaction, optical resolution of (*RS*)-1-phenylethanol, which serves as one of chiral building blocks, was carried out by enantioselective transesterification with vinyl acetate. Recombinant *Rhizopus oryzae* lipase displayed on the yeast cell surface retained its activity in hexane, heptane, cyclohexane and octane. The effective amount of whole-cell biocatalyst in the reaction mixture was 10 mg/ml solvent. In a reaction mixture incubated for 36 h with molecular sieves 4A, the concentration of (*R*)-1-phenylethyl acetate reached 39.8 mM (97.3% yield) with high enantiomeric excess (93.3%ee). In contrast, a reaction mixture incubated without molecular sieves 4A produced little (*R*)- and (*S*)-1-phenylethyl acetate. The results obtained in this study demonstrate the applicability of the lipase-displaying yeast whole cell biocatalyst to bioconversion processes in non-aqueous organic solvents.

T. Matsumoto · H. Fukuda
Division of Molecular Science, Graduate School of Science and Technology, Kobe University,
1–1 Rokkodaicho, Nada-ku,
657–8501 Kobe, Japan

M. Ito
Analysis Service Center, Daicel Chemical Industries ,
Himeji Research Park, 1239 Shinzaike, Aboshi-ku,
671–1283 Hyogo, Japan

A. Kondo (✉)
Department of Chemical Science and Engineering, Faculty of Engineering, Kobe University,
1–1 Rokkodaicho, Nada-ku,
657–8501 Kobe, Japan
e-mail: kondo@cx.kobe-u.ac.jp
Tel.: +81-78-8036196
Fax: +81-78-8036206

Introduction

In recent years, enzymatic transformation of organic compounds has been developed for the preparation of optically active products from racemic substrates (Wong and Whitesides 1994; Otsubo 2000; Berglund 2001). Since enzymes can catalyze a variety of reactions with high specificity and under mild conditions such as low temperature and atmospheric pressure, biotransformation is looked to as a way of resolving the environmental problems involved in chemical procedures (Koeller and Wong 2001). Further, regioselective synthesis is difficult to achieve by chemical procedures, but appears to be amenable to an enzymatic approach.

Lipase-catalyzed biotransformation is one of the most popular and practical of these enzymatic procedures. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze a broad spectrum of reactions, such as hydrolysis of ester bonds and transesterification and ester synthesis, at the interface between substrate and water or in non-aqueous organic solvents (Hiol et al. 2000) and are therefore used in a wide range of industrial processes including food, chemical, pharmaceutical and detergent production. In pharmaceutical production using lipases, previous reports have demonstrated regioselective transesterifications such as kinetic resolution of 1-phenylethanol (Schöfer et al. 2001) or α-methylene β-lactams (Adam et al. 2000) and the enantiodivergent synthesis of the antitumor antibiotic fredericamycin A analog (Akai et al. 2001). In many cases, however, lipases have been used in immobilized form, which is cost- and time-consuming, and can be a barrier to widespread use of enzymatic processes.

To improve cost-efficiency, the present study attempted to achieve enantioselective transesterification using a lipase-displaying yeast in the form of a whole-cell biocatalyst. With the recent development of cell-surface display technology, functional proteins such as enzymes can be genetically immobilized on the yeast cell surface via anchor proteins (Murai et al. 1997). For industrial bioconversion processes, the utilization of surface-dis-

played lipase in the form of whole-cell biocatalysts is more cost-effective and advantageous because the enzymes are displayed on the cell surface spontaneously, regarded as immobilized enzyme, and can be separated easily. We previously reported successful yeast cell-surface display of the active form of ProROL, consisting of pro- and mature-region of *Rhizopus oryzae* lipase (ROL), via the floccu-lation functional domain of Flo1 protein (Flo1p) (Matsu-moto et al. 2002), a yeast flocculin, which is a lectin-like protein and causes cell flocculation by attachment to cell wall mannan (Watari et al. 1994). Truncated Flo1p was fused as an anchor protein to the N-terminus of ProROL. Since this lipase-displaying yeast had been found to efficiently catalyze a methanolysis reaction in a solvent-free and water-containing system (Kaieda et al. 1999), the present study undertook an investigation of the applic-ability of this whole-cell biocatalyst to non-aqueous enantioselective transesterification.

Materials and methods

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 experiment. The reaction solvents hexane, heptane, octane and cyclohexane and the dehydrating reagent molecular sieves 4A (∅2 mm) were obtained from Nacalai Tesque. (*R*)-, (*S*)-1-Phenylethanol (Wako Pure Chemical Industries) and (*RS*)-1-phe-nylethyl acetate (Aldrich, Milwaukee, Wis.) were used as standards in HPLC analysis.

Strains and media

The ProROL-displaying yeast strain was obtained by transformation of *Saccharomyces cerevisiae* MT8–1 (*MATa ade his3 leu2 trp1 ura3*) with plasmid pWIFSProROL, constructed in a previous study (Matsumoto et al. 2002). To prepare the ProROL-displaying yeast, an enriched selective medium (SDC: 0.67% yeast nitrogen base supplemented with appropriate amino acids and nucleotides, 0.5% glucose, and 2% casamino acids) was used.

Preparation of ProROL-displaying yeast

To prepare the ProROL-displaying yeast as a whole-cell biocatalyst, the transformant described above was cultivated in 400 ml SDC medium in a 1-l baffled-flask at 30°C with 150-rpm rotation. After 194-h cultivation, at which point the lipase activity on the yeast transformant peaks (Matsumoto et al. 2002), the cells were collected, washed with distilled water, lyophilized with FreeZone FZ-1 (Labconco, Kansas, Mo.) for 36 h, and sieved to homogenize cell particle size with a 40-mesh sieve.

Measurement of lipase activity on yeast cells

The hydrolytic activity of lipase in culture broth and yeast cells was measured using Lipase Kit S (Dainippon Pharmaceutical, Osaka, Japan) according to the protocol specified by the supplier, and the resulting values were expressed in international units (IU). One unit of lipase activity was defined as the amount of enzyme catalyzing the formation of 1 μmol 2,3-dimercaptopropan-1-ol from 2,3-dimercaptopropan-1-ol tributyl ester per minute. The detailed procedure of lipase activity measurement on the yeast cell surface has been described previously (Matsumoto et al. 2002).

Enantioselective transesterification

In order to confirm the performance of the ProROL-displaying yeast whole-cell biocatalyst, the optical resolution of (*RS*)-1-phenyletha-nol was carried out by enantioselective transesterification with vinyl acetate (Fig. 1). The reaction mixture, consisting (unless otherwise noted) of 30 mg (*RS*)-1-phenylethanol, 21.2 mg vinyl acetate, 30 mg lyophilized cells, 0.3 g molecular sieves 4A and 3 ml heptane was incubated at 30°C with shaking at 150 rpm. The concentration of (*RS*)-1-phenylethanol and vinyl acetate was 80 mM. Aliquots of 50 μl withdrawn and diluted in 950 μl of the mobile phase of HPLC (described below) were used to determine the amount and the enantiomeric excess of the ester formed during the reaction. Solvents for the reaction experiments were dehydrated with molecular sieves 4A for 24 h before use.

High-performance liquid chromatography analysis

In the enantioselective transesterification of (*RS*)-1-phenylethanol, the yield and the enantiomeric excess of the reaction product, 1-phenylethyl acetate, were determined by HPLC using a Chiralcel OB-H column (∅ 0.46 mm, 25 cm, Daicel Chemical Industries, Tokyo, Japan). The mobile phase was a mixture of hexane: isopropanol (90:10, v/v) at a flow rate of 0.3 ml/min on LC10A (Shimadzu, Kyoto, Japan). Quantification of the reaction products and substrates was performed by measuring absorbance at 210 nm

Fig. 1 Schematic representa-tion of optical resolution of (*RS*)-1-phenylethanol catalyzed by lipase
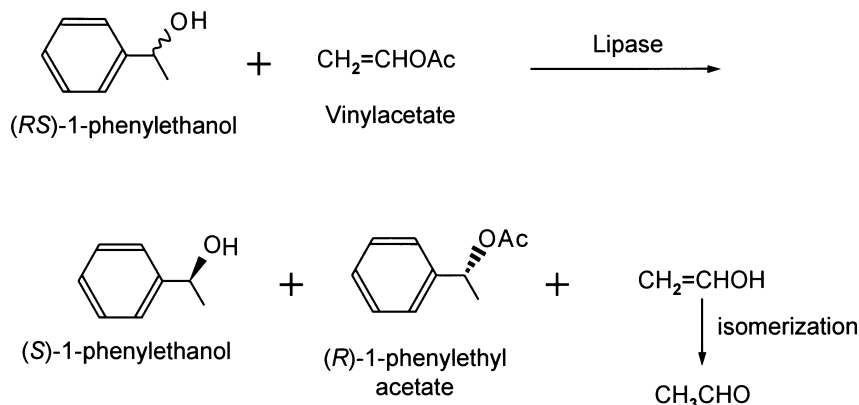
on an SPD10A UV detector (Shimadzu). The retention times for (R)- and (S)-1-phenylethanol, and for (R)- and (S)-1-phenylethyl acetate were 21, 28, 16.5 and 17 min, respectively. Enantiomeric excess (%ee) was calculated as:

$$\text{Enantiomeric excess}(\%ee) = |R - S|/(R + S) \times 100 \qquad (1)$$

where R and S are the concentration of (R)- and (S)-1-phenylethyl acetate, respectively.

Results

Preparation of ProROL-displaying yeast

ProROL-displaying yeast whole-cell biocatalyst was cultivated in SDC medium according to the method reported previously (Matsumoto et al. 2002), and lyophilized for enantioselective transesterification in organic solvent, which was inhibited by the presence of water. Interestingly, lyophilized ProROL-displaying cells exhibited approximately four times higher lipase activity (265 IU/g dry cell weight) than fresh cells (61.3 IU/g dry cell weight). Since this phenomenon was not observed in the case of soluble ROL enzyme (data not shown), this activity enhancement might be unique to the surface-displayed enzyme.

Effect of water content in reaction mixture

In order to investigate the effect of water content in the reaction mixture, transesterification was carried out with and without molecular sieves 4A (Table 1). In the reaction mixture incubated with molecular sieves 4A, the reaction yield of (R)-1-phenylethyl acetate reached 63.2% after a 24-h reaction and enantiomeric excess was 93.3%ee, but without molecular sieves 4A, neither (R)- nor (S)-products were produced in significant quantity.

Activities in various solvents

A comparison of activity on ProROL-displaying yeast in various organic solvents is shown in Table 2. ProROL displayed on the yeast-cell surface could efficiently catalyze an enantioselective reaction in hexane, heptane,

Table 1 Effect of presence of water in reaction mixture on reaction yield and enantiomeric excess (*%ee*). *ProROL* Pro- and mature-region of *Rhizopus oryzae* lipase

Lyophilized yeast cells	ProROL-displaying yeasts	Control yeasts
Molecular sieve	+	−	+
(R)-Product yield (%)[a]	63.2	2.5	0.0
(S)-Product yield (%)[a]	2.2	0.0	0.0
%ee[b]	93.3	99.3	−

[a]After 24-h reaction at 30°C
[b]Enantiomeric excess of (R)-1-phenylethyl acetate produced

Table 2 Comparison of reaction yield and enantiomeric excess in various organic solvents

Solvent	Hexane	Heptane	Octane	Cyclohexane
(R)-Product yield (%)[a]	50.5	63.3	62.0	56.7
(S)-Product yield (%)[a]	1.6	2.2	2.1	1.7
%ee[b]	93.7	93.3	93.3	94.1

[a]After 24-h reaction at 30°C
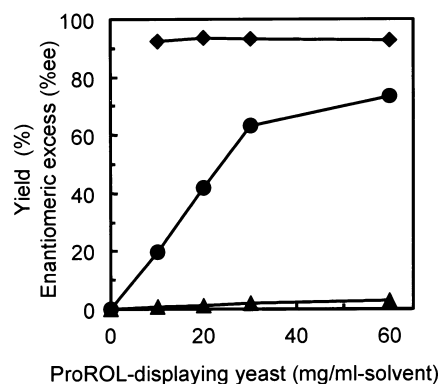[b]Enantiomeric excess of (R)-1-phenylethyl acetate produced

Fig. 2 Effect of amount of whole-cell biocatalyst added to reaction mixture on the reaction yields of (R)-1-phenylethyl acetate (*circles*) and (S)-1-phenylethyl acetate (*triangles*) and enantiomeric excess of (R)-1-phenylethyl acetate (*diamonds*) after 24-h reaction

cyclohexane and octane. Enantioselective transesterification was the most efficiently catalyzed in the case of heptane, although the other solvents also showed good reaction rates. Enantiomeric excess recorded a high level (over 93%ee) in all cases.

Effect of amount of cells in reaction mixture

Figure 2 shows the effect of the amount of cells in the reaction mixture on the reaction yield of each enantiomeric product. A 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 10 mg/ml solvent and the yield seemed to reach a plateau. From this result, 10 mg/ml lipase-displaying yeast cells was used in subsequent experiments. (S)-Product yield remained low under all conditions while enantiomeric excess remained over 90%ee throughout.

Time-course of reaction

Figure 3 shows the time-course of reaction during enantioselective transesterification. When the reaction began, the reaction substrates, (R)-1-phenylethanol and vinyl acetate were immediately consumed and (R)-1-phenylethyl acetate was produced. After 36-h reaction, the concentration of (R)-1-phenylethyl acetate reached
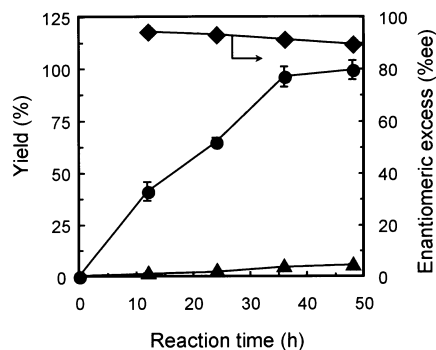
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Fig. 3 Time-course of the reaction yields of (*R*)-1-phenylethyl acetate (*circles*) and (*S*)-1-phenylethyl acetate (*triangles*) and enantiomeric excess of (*R*)-1-phenylethyl acetate (*diamonds*)

39.8 mM, equivalent to 97.3% reaction yield with high enantiomeric excess (93.3%ee). The concentrations of (*S*)-1-phenylethanol and (*S*)-1-phenylethyl acetate meanwhile remained unchanged from the beginning of the reaction (data not shown).

Discussion

As a model reaction for enantioselective transesterification, optical resolution of (*RS*)-1-phenylethanol, which serves as a chiral building blocks, was examined. The substrate reacted in an esterification process using vinyl-acetate as acyl donor (Fig. 1).

In the present study, ProROL displayed on the yeast cell surface could catalyze transesterification only under non-aqueous conditions with dehydration by molecular sieves 4A (Table 1). Kyotani et al. (1988a, 1988b) found that lipase-catalyzed transesterification in organic solvent was inhibited by the presence of water in excess of the amount required to maintain the structure of the lipase molecule in organic solvent. Our result also indicates that water content in the reaction mixture has a critical effect on the transesterification yield. The lyophilization procedure, necessary for removal of water from yeast cells, enhanced the performance of the ProROL-displaying yeast whole-cell biocatalyst. Although the mechanism of this activation was unclear, accessibility between displayed enzyme and substrate might be improved by partial destruction of cell wall components by freezing. Since yeast cells lyophilized for 36 h contain approximately 5 wt% water (data not shown), a dehydrating reagent was needed to catalyze this reaction.

The results shown in Fig. 2 suggest that the effective amount of cells in the reaction mixture is 10 mg/ml solvent. This result suggests that fairly small amount of ProROL-displaying yeast cells, approximately the same as immobilized lipase catalysts previously used by Cardenas et al. (2001) and Salunkhe and Nair (2001), is needed for complete conversion of substrate. In a Michaelis-Menten-type reaction, a higher reaction rate is commonly obtained by increasing the amount of enzyme in soluble form. In the present reaction, however, at amounts greater than

10 mg/ml solvent, it appeared that the interfacial area between the solution and cell surface became rate-limiting, with the presence of excess cells causing a reduction of the solution-cell surface interfacial area.

As shown in Fig. 3, rather higher levels of yield and enantiomeric excess were obtained in the present study than in the reports of Cardenas et al. (2001) (with 240-h incubation, 41% yield, 62%ee) and Schöfer et al. (2000) (72-h, 50% yield, 98%ee). Moreover, native ROL enzyme (F-AP15, Amano Enzyme, Nagoya, Japan) could not catalyze enantioselective transesterification in organic solvent, although rather higher hydrolytic activity (30,000 IU/g) was evident than in ProROL-displaying yeast. From these results, displaying ProROL on yeast cell surfaces by the fusion of truncated Flo1p to its N-terminus might affect the properties of the enzyme molecule. Alternatively, properties of the yeast cell surface, such as hydropathy and charges, might support the accessibility and affinity between enzymes and substrates in organic solvent. One of the advantages of enzyme-displaying whole-cell biocatalysts is that enzymes are immobilized on the yeast cell surface by genetically modified anchor systems, which not only minimizes the risk of inactivation and inhibition, but also improves the performance of displayed enzyme. In addition, this latter features be partially influenced by the function of the pro-sequence of ROL in modulating the folding process, secretion pathway, or thermostability of ROL (Beer et al. 1996).

The ProROL-displaying yeast whole-cell biocatalyst studied here can catalyze enantioselective transesterification in non-aqueous organic solvent, suggesting its potential as a cost-effective whole-cell biocatalyst for applications in pharmaceutical production.

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