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Practical Application of Recombinant Whole-Cell Biocatalysts for the Manufacturing of Pharmaceutical Intermediates Such as Chiral Alcohols

Akinobu Matsuyama,* Hiroaki Yamamoto, and Yoshinori Kobayashi

Tsukuba Research Center, Daicel Chemical Industries, Ltd., 27 Miyukigaoka, Tsukuba, Ibaraki 305-0841, Japan

Abstract:

We have developed efficient biocatalytic processes for the preparation of chiral alcohols, such as (*R*)-1,3-butanediol, ethyl (*S*)-4-chloro-3-hyroxybutanoate, ethyl (*R*)-4-chloro-3-hyroxybutanoate, (*S*)-5-chloro-2-pentanol, (*R*)-5-chloro-2-pentanol, and (*S*)-cyclopropylethanol by stereospecific enzymatic oxidoreduction on a practical level. These chiral alcohols are very important synthons for the synthesis of various pharmaceutical intermediates that lead to antibiotics and inhibitors of HMG-CoA reductase. Here, we present practical applications on biocatalysis using novel recombinant whole-cell biocatalysts that catalyzed enantioselective oxidation and asymmetric reduction with a coenzyme regeneration system.

Introduction

Global sales of single-enantiomer pharmaceutical products are growing at a high rate every year. At Daicel, we are expanding our research, which initially included biocatalysis and chromatographic separation using a simulated moving bed (SMB) in conjunction with multistep organic synthesis, in chiral technologies to solve problems in areas of research, development, and production of pharmaceuticals.[1] Daicel pharmaceutical products are manufactured in cGMP plants in Arai, Japan, on an industrial scale. Biocatalysts are advantageous because environmentally hazardous reagents, solvents, and chemical catalysts can be avoided. Moreover, biocatalysis can be carried out inexpensively and simply with substrates, biocatalysts, and water. We have therefore developed new biocatalytic processes to manufacture optically active compounds with improved efficiency and environmental compatibilities. Our novel biocatalysts include oxidoreductases, aminoacylases, decarboxylases, and transaminases suitable for producing a variety of chiral alcohols and amino acids, which are unique building blocks for pharmaceuticals.[2] We have previously reported on practical applications of biocatalysts for the manufacturing of some chiral alcohols using wild strains.[3,4] Although the activity of these wild strains was useful, it was necessary to construct recombinant biocatalysts to improve the production efficiency since the use of a recombinant biocatalyst was more advantageous than the use of a wild strain for the achievement of high productivity. A recombinant whole-cell biocatalyst is a very convenient, high-performance, and stable source of enzymes. The method is economically advantageous and much less expensive than using purified enzymes. In addition, once the required activity has been found in a wild strain, modern techniques in molecular biology easily allow the expression of the new enzyme in a foreign host with high activity for an industrial process. We therefore attempted to develop the bioprocess using a recombinant microorganism to produce chiral compounds efficiently.[5,6] For instance, many microorganisms and enzymes have been found to catalyze the reduction of ketones to the corresponding chiral alcohols.[7] However, to date, there have been few reports of successful practical applications by asymmetric reduction using a recombinant whole-cell biocatalyst.

This article describes some of our recent developments in preparative asymmetric biotransformation using a recombinant whole-cell biocatalyst.

Possibility of the Application of a Secondary Alcohol Dehydrogenase to a Bioconversion Process. (*R*)-1,3-Butanediol ((*R*)-1,3-BDO) is a starting material of azetidinone derivatives, which are important intermediates in the synthesis of penem and carbapenem antibiotics for industrial use (Figure 1).[8]

We screened the microorganisms for producing (*R*)-1,3-BDO. By screening over 1000 strains, we found that many yeast, fungus, and bacterium strains produced optically active 1,3-BDO from the racemate by enantioselective oxidation of 1,3-BDO to 4-hydroxyl-2-butanone (4H2B). We compared the quantities of (*R*)-1,3-BDO from each strain. The best strain, *Candida parapsilosis* IFO 1396, produced (*R*)-1,3-BDO with 97% ee from the racemate.[3] The (*S*)-1,3-BDO oxidizing enzyme (designated as CpSADH), which could produce (*R*)-1,3-BDO from the racemate, was purified from *C. parapsilosis* IFO 1396 (Figure 2).

* Author for correspondence. E-mail: ak-matsu@daicel.co.jp.

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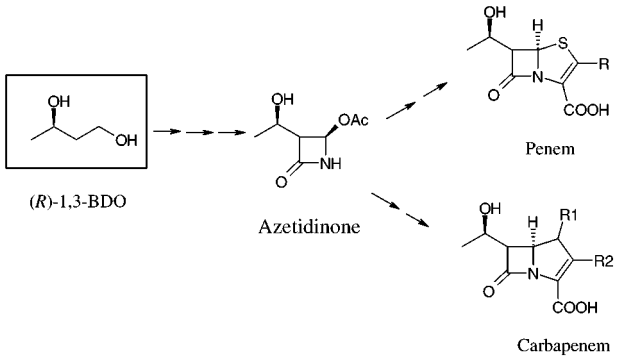

Figure 1. **Industrial uses of (*R*) -1,3-BDO.**

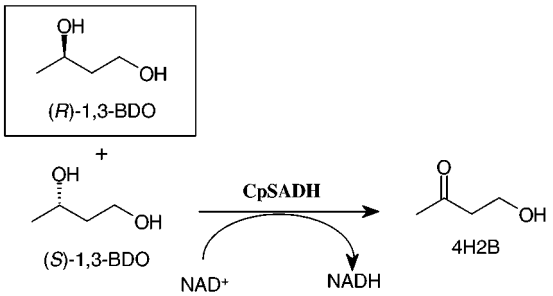

Figure 2. **Enantioselective oxidation of 1,3-BDO by CpSADH.**

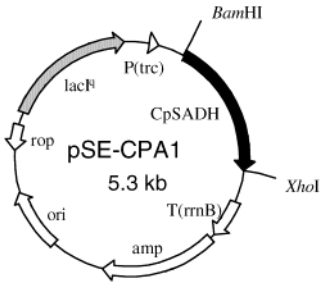

Figure 3. **Construction of pSE-CPA1.**

The CpSDAH enzyme was purified to 5400-fold of the initial activity and characterized in detail. CpSADH catalyzes the NADH-dependent reduction of ketones to the corresponding secondary alcohols. When we began this work, secondary alcohol dehydrogenases specific for (*R*)-2-alcohols, such as (*R*)-2-butanol, had been reported from methylotrophic bacteria and yeasts, but a secondary alcohol dehydrogenase specific for (*S*)-2-alcohols had not been reported. CpSADH catalyzes the enantioselective oxidation of racemic secondary alcohols and the asymmetric reduction of aromatic and aliphatic ketones to their corresponding secondary alcohols with (*S*) selectivity.[5] To construct more efficient whole-cell biocatalysts, we chose to clone this secondary alcohol dehydrogenase gene in an *Escherichia coli* host microorganism. *E. coli* was a suitable host for genetic engineering. Further advantages of an *E. coli* expression system are rapid cell growth and a high expression level of CpSADH. After optimization of a number of parameters, the highest cellular CpSADH activity was obtained with *E. coli* cells containing the construct pSE-CPA1 (Figure 3). The CpSADH activity of a recombinant *E. coli* strain was more than 78 times higher than that of *C. parapsilosis*. The reaction conditions were optimized. A recombinant *E. coli* W3110 strain harboring plasmid pSE-CPA1 produced (*R*)-1,3-BDO (95% ee, 96.8% yield) from the racemate (15% 1,3-BDO) at 30 °C without any need to regenerate NAD^+ from NADH.[6]

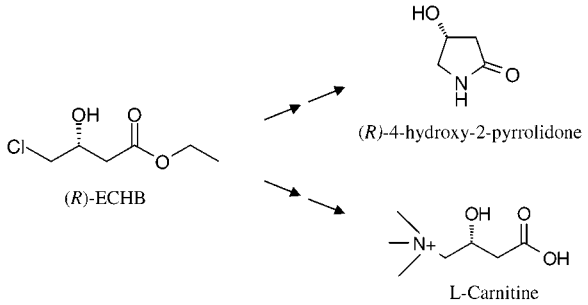

Figure 4. **Industrial uses of (*R*)-ECHB.**

On the other hand, the asymmetrical carbonyl reduction has clear advantages over the kinetic resolution of an equivalent intermediate from the point of view of substrate utilization. The most striking characteristics of CpSADH are its broad substrate specificity and good stereoselectivity. This enzyme has been widely used for the reduction of ketones. The application of this enzyme to the practical synthesis of chiral alcohols was investigated. Ethyl (*R*)-4-chloro-3-hydroxybutanoate ((*R*)-ECHB) is useful for the synthesis of biologically and pharmacologically important materials, such as L-carnitine and (*R*)-4-hydroxy-2-pyrrolidone (Figure 4).[9,10] Several microorganisms and enzymes have been found to catalyze the reduction of ethyl 4-chloroacetoacetate (ECAA) to (*R*)-ECHB.[11] In particular, Kataoka et al.[12] reported the production of (*R*)-ECHB with a molar yield of 94% and an optical purity of 92% ee using *E. coli* cells coexpressing an aldehyde reductase I[13] from *Sporobolomyces salmonicolor* and a glucose dehydrogenase[14] from *Bacillus megaterium* as the catalyst in an *n*-butyl acetate/water biphasic system.

The recombinant *E. coli* expressing CpSADH produced ethyl (*R*)-ECHB from ECAA, which was a derivative of diketen, with 2-propanol (IPA). Under suitable conditions, this recombinant *E. coli* reduces ECAA to ECHB in the (*R*)-configuration at 36.6 g/L and 95.2% yield with 99% ee. Its maximum yield was reached after 14 h of incubation at 15 °C. This asymmetric reduction system did not require an additional NADH-regeneration system, such as a glycolytic pathway or glucose dehydrogenase with a corresponding substrate. CpSADH served as both the synthetic (asymmetric reduction) and regenerating (NADH-regeneration) enzyme. IPA was oxidized to regenerate NADH (Figure 5).[15]

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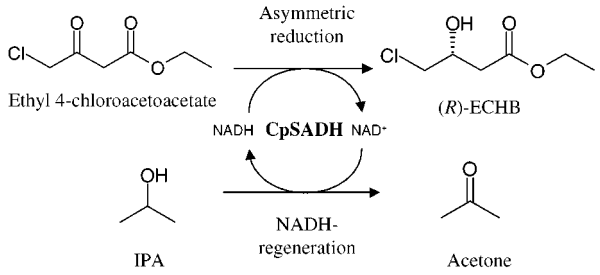

Figure 5. **Preparation of (*R*)-ECHB by CpSADH.**

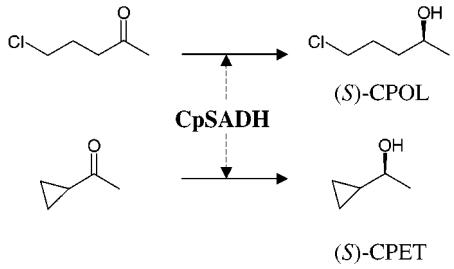

Figure 6. **Preparation of (*S*)-CPOL and (*S*)-CPET by CpSADH.**

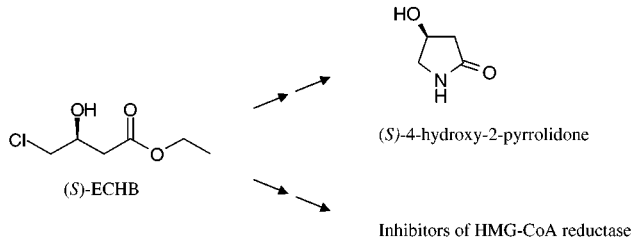

Figure 7. **Industrial uses of (*S*)-ECHB.**

Other promising targets, (*S*)-5-chloro-2-pentanol ((*S*)-CPOL) and (*S*)-cyclopropylethanol ((*S*)-CPET) were also obtained from the corresponding ketones by bioreduction with the same recombinant *E. coli.* (Figure 6). (*S*)-CPOL was produced at 33.8 g/L with 98% ee, and (*S*)-CPET was produced at 18.6 g/L with 98% ee. Thus, CpSADH is very useful to produce a wide range of chiral alcohols.[16]

Asymmetric Reduction with an Enzyme-Coupled Regeneration of the Coenzyme. Ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-ECHB) is known to be a key intermediate of the synthesis of inhibitors of HMG-CoA reductase and antibiotics (Figure 7).[17]

The asymmetric reduction using enantioselective oxidoreductases is a good method for producing (*S*)-ECHB. Several enzymes reducing ECAA to (*S*)-ECHB have been found and purified from *Saccharomyces cerevisiae*,[18] *Geotrichum candidum*,[11b] *Sporobolomyces salmonicolor*,[19] *Candida macedoniensis*,[20] and *Candida magnoliae*.[21]

We found that a novel secondary alcohol dehydrogenase specific for (*R*)-2-alcohols from *Pichia finlandica* catalyzed the asymmetric reduction of ECAA to (*S*)-ECHB with excellent stereoselectivity.

This enzyme (designated as PfODH) has been purified and characterized. PfODH has high specific activity and relatively broad substrate specificity. It catalyzed the NADH-dependent asymmetric reduction of aromatic and aliphatic ketones to their corresponding secondary alcohols with (*R*) selectivity. The gene encoding PfODH was cloned from *Pichia finlandica* and expressed in *E. coli*.[22]

Since CpSADH accepts IPA as a substrate, substrate-coupled regeneration, as well as enzyme-coupled regeneration of the coenzyme, is possible. In contrast, since PfODH barely accepts IPA as a substrate, it was necessary that the cofactor NADH required for the reduction be regenerated by formate dehydrogenase from *Mycobacterium* (FDH). Therefore, we constructed a coexpression plasmid of PfODH and FDH in *E. coli*. Under suitable conditions, the recombinant *E. coli* coexpressing PfODH and FDH reduced ECAA to (*S*)-ECHB at 32.2 g/L and 98.5% yield with 99% ee with cofactor regeneration.

In addition, PfODH produced (*R*)-CPOL from the corresponding ketone (CPON) at 26.1 g/L with 99% ee. Thus, we established biochemical processes using NADH-dependent oxidoreductases in commercial quantities for producing chiral ECHB and CPOL from the corresponding ketones with both configurations (Figure 8).[22]

These inexpensive ketones were synthesized from diketene. Diketene is a very useful compound for the production of various ketones. We produce it in large quantities from acetic acid, which is the most abundant and least expensive chemical product produced by Daicel.

Experimental Section

E. coli cells harboring a high-expression plasmid were grown in 100 mL of a 2× YT medium (Bacto-Tryptone, 20 g/L; Bacto-Yeast extract, 10g/L; NaCl, 10 g/L; pH 7.2) containing ampicillin (50 mg/L) in a 500-ml baffled shake flask at 30 °C on a rotary shaker (140 rpm) to an optical density of 3−4 at 600 nm; after the addition of 2% lactose as an inducer, the culture medium was further shaken for 11 h, and the cells were harvested by centrifugation. Using all of the *E. coli* W3110 living cells harboring the high-expression plasmid, the syntheses of chiral alcohols by asymmetric reduction were investigated without the cofactors. The reduction of ketone was done at 30 °C for 17−48 h in a reaction mixture (25 mL) containing a 200 mM potassium phosphate buffer (pH 6.5), 3−5% (w/v) ketone, cultured cells obtained from 25 mL of the medium, and 5% (w/v) of a compound as an energy source (either IPA or HCOONa) with shaking at 140 strokes per min in a 500-mL Sakaguchi flask. These reactions were done at pH 6.5−7.0. The amounts of ketone and alcohol in the reaction

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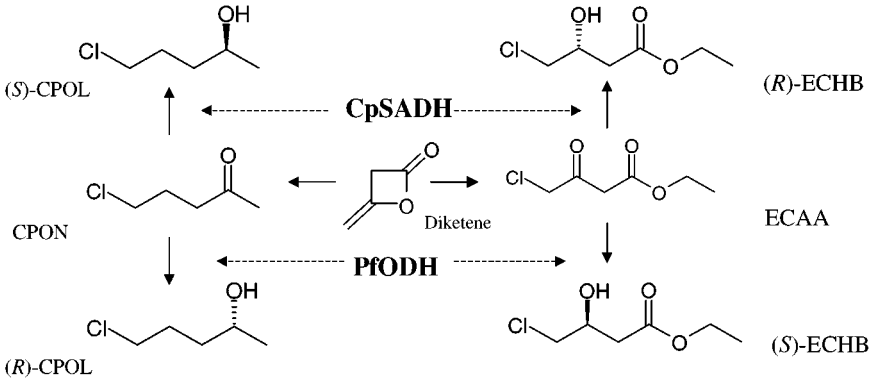

Figure 8. **Preparation of chiral ECHB and CPOL with both configurations.**

mixture were measured by gas chromatography (Shimadzu GC-14A, Kyoto, Japan). The optical purity of alcohol was measured by HPLC with a Chiralcel packed column (4.6 × 250 mm, Daicel Chemical Industries, Ltd., Tokyo, Japan).

Conclusions

In this contribution, we have described the practical applications of novel oxidoreductases to a bioconversion process using recombinant biocatalysts. The genes encoding the oxidoreductases were isolated, and overproduction of the enzymes in *E. coli* was successfully carried out, resulting in much improved biocatalysts, as described above. The modern tools of molecular genetics are currently applied to make enzymes available in larger quantities and at lower costs in recombinant hosts. Thus, by enzymatic conversion of ketones, we supplied various chiral secondary alcohols in both configurations with the above-mentioned biological tools using CpSADH and PfODH properties. We expect that our application of recombinant whole-cell biocatalysts on more advanced intermediates in the synthesis of chiral pharmaceuticals will be expanded.

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