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Asymmetric reduction of ketones by *Geotrichum candidum*: immobilization and application to reactions using supercritical carbon dioxide

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ABSTRACT

The enantioselectivity for the reduction of ketones by *Geotrichum candidum* NBRC 5767 was improved upon immobilization of the whole cell onto an ion exchange resin with polyallylamine. Furthermore, immobilization of the cell enhanced the stability of the enzyme and enabled a continuous-flow reaction under normal aqueous conditions. The biocatalyst was also applied to the reaction in supercritical carbon dioxide.

1. Introduction

Immobilization of an enzyme is one of the most effective methods for improving stability and ease of handling and recycling; various methods have thus been reported.[1] As a type of enzyme that has been immobilized, hydrolase accounts for the majority, as represented by the commercial lipases for organic synthesis such as Novozym435 and PS-C. Compared with hydrolases, only a few oxidoreductases are immobilized[2] in spite of the fact that oxidoreductases are important for the asymmetric reduction of ketones,[3] and so the development of immobilized oxidoreductases such as dehydrogenases is necessary.

One of the reports for the immobilization of an alcohol dehydrogenase is that of the *Geotrichum candidum* cell on a water absorbing polymer.[4] Reactions using the biocatalyst in an organic solvent,[4a] supercritical carbon dioxide ($scCO_2$),[4b,c] and an ionic liquid[4d] have been explored. However, the immobilization method is associated with only a weak physical bonding force between the cell and polymer, thus the immobilized cell can be used only in non-aqueous media. A stronger bonding method is necessary to further improve the enzyme performance in aqueous, as well as non-aqueous media. Therefore, in this report, the whole cell of *G. candidum* NBRC 5767 was immobilized onto an ion exchange resin with polyallylamine,[5] and reactions using the biocatalyst in aqueous and $scCO_2$ solvents are examined. For the reaction conducted in an aqueous buffer, immobilization improved the enantioselectivity and stability. The immobilization also made a continuous-flow reaction feasible. The biocatalyst was also applied to the reaction in $scCO_2$, and recycling of the catalyst accompanying $scCO_2$ depressurization as well as continuous-flow reaction was possible for the first time for an alcohol dehydrogenase.

2. Results and discussion

2.1. Immobilization of the whole cell

The whole cell of *G. candidum* was immobilized onto an ion exchange resin with the aid of polyallylamine.[5] This immobilization method was applied because it was suitable for the reaction in $scCO_2$ for a decarboxylase-catalyzed carboxylation.[6] Moreover, this immobilization method has the advantage of a low diffusion barrier inside the gel layer around the enzyme, and stable operation was achieved for L-asparagic acid synthesis.[5]

2.2. Reaction in aqueous solution

The asymmetric reduction of acetophenone **1** was conducted in order to investigate the performance of the immobilized cell using 2-propanol as a hydrogen source and NAD^+ as a coenzyme in MES buffer (pH 7.0, 0.1 M). As shown in Scheme 1, (*S*)-1-phenylethanol was obtained in high yield using the immobilized cell as well as free cell. Surprisingly, the enantioselectivity increased from 90% to >99% by the immobilization.

Next, the effect of kind of buffer on the reduction of **1** was investigated. Using Tris, HEPES, and KPB (pH 7.0, 0.1 M), similar results were obtained (Tris-free cell: yield 80%, ee 93%, immobilized cell: yield 77%, ee >99%; HEPES-free cell: yield 76%, ee 90%, immobilized cell: yield 78%, ee >99%; KPB-free cell: yield 75%, ee 89%, immobilized cell: yield 72%, ee >99%).

The effect of pH on the reduction of **1** was also examined. When the free cell was used, yield decreased at pH 5 and 10 (pH 5: yield

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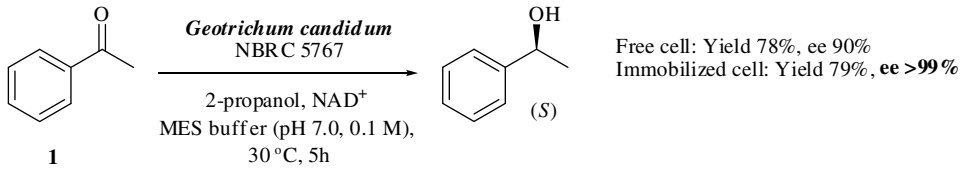


Scheme 1.

63, ee 94%, pH 6: yield 75%, ee 89%, pH 7: yield 78, ee 90%, pH 8: yield 67%, ee 93%, pH 9: yield 74%, ee 92%, pH 10: yield 59%, ee 90%). However, when using the immobilized cell, high yields were obtained at the pH 5–10 (yield 85–89%, ee >99%). This result shows the higher stability of the enzyme in the immobilized cell than that in the free cell at pH 5 and 10.

To examine the effect of temperature, the reduction of **1** was conducted at 30, 40, 50, and 60 °C. The yield is shown in Figure 1. At 30 °C and 40 °C, yields that were obtained using the free cell were similar to those obtained using the immobilized cell. However, at 50 °C and 60 °C, better yields were obtained when using immobilized cells. These results also support the observation that the enzyme in the immobilized cell has higher stability than that in the free cell.

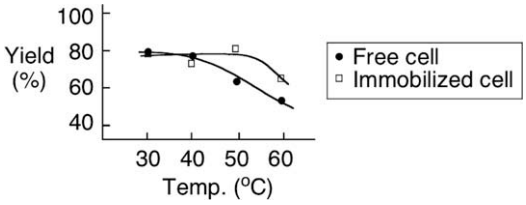
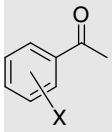
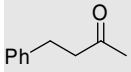
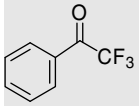
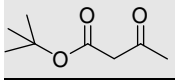


Figure 1. Effect of temperature on the reduction of **1** by the free or immobilized cell of *G. candidum*.

Various ketones were reduced by the immobilized cell. The results are shown in Table 1. Excellent enantioselectivities and high to moderate yield were obtained.

Table 1
Reduction of various ketones by the immobilized cell in MES buffer

Substrate		Yield (%)	ee (%)
	X = H **1**	81	>99 (S)
	o-F **2**	87	>99 (S)
	p-F **3**	27	>99 (S)
	o-Me **4**	50	>99 (S)
	**5**	49	>99 (S)
	**6**[a]	78	>99 (S)
	**7**	87	>99 (S)

Reaction conditions are described in the Section 4.
[a] $NADP^+$, 5 mg, was added instead of NAD^+, 5 mg.

The feasibility of recycling was examined by conducting the reduction of **1** in MES buffer, repeatedly. As shown in Table 2, it could be recycled for four times without significant loss in yield. The enantioselectivity was excellent even after recycling for four times. This proves that the present immobilization method bonds the cell and carrier strongly, although the reaction in water was not possible with the immobilization on water absorbing polymer reported before due to the lack of a strong bonding force.[4]

Table 2
Recycling of the immobilized cell for the reduction of **1** in MES buffer

Run	Yield (%)	ee (%)
1st	48	>99 (*S*)
2nd	61	>99 (*S*)
3rd	65	>99 (*S*)
4th	66	>99 (*S*)
5th	44	>99 (*S*)

Acetophenone (85.6 μmol) was converted as described in the Section 4. After the extraction of product, the mixture was kept under reduced pressure (50 mmHg) at rt for 30 min and under 4 mmHg for 15 min, and then to carry out next reaction, MES buffer (3 mL), 2-propanol (1.3 mmol), NAD^+ (5 mg) and acetophenone (85.6 μmol) were added.

The continuous-flow reduction of **1** in MES buffer was then conducted. The yield was about 60% after 4 h as shown in Table 3, showing the high stability of the enzyme in the immobilized cell as expected from the result of stability and recycling experiments.

Table 3
Continuous reduction of **1** by the immobilized cell in MES buffer

Recovering time (h)	Yield (%)
0–1	57
1–2	54
2–3	62
3–4	56

Reaction conditions are described in the Section 4.

2.3. Reaction in $scCO_2$

The immobilized cell was used for the reaction in $scCO_2$. First, the time-course for the reduction of **1** was examined. The yield increased according to the time (Fig. 2), which shows that the biocatalyst is surely active in the $scCO_2$ Table 4.

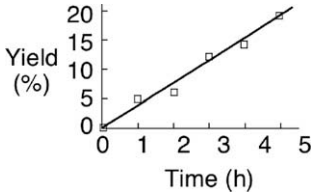


Figure 2. Time-course of the reduction of **1** by the immobilized cell in $scCO_2$.

The effect of temperature and pressure on the reduction of **1** by the immobilized cell in $scCO_2$ was examined because the properties of $scCO_2$ change according to the temperature and pressure.[7] Similar results were obtained for the reaction at the pressure ranging from 8 MPa to 11 MPa (8 MPa: Yield 13%, ee >99%, 9 MPa: Yield 14%, ee >99%, 10 MPa: Yield 19%, ee >99%, 11 MPa: Yield 16%, ee >99%). On the other hand, temperature had a profound effect on

Table 4
Reduction of various ketones by the immobilized cell in $scCO_2$

Substrate	Yield (%)	ee (%)
1	18	>99 (*S*)
2	20	>99 (*S*)
3	0	—
4	<1	—
5	12	>99 (*S*)
6[a]	4	>99 (*S*)
7	8	>99 (*S*)

Reaction conditions are described in the Section 4.
[a] $NADP^+$, 5 mg, was added instead of NAD^+, 5 mg.

the reaction. At 35 °C and 40 °C, the reaction proceeded and gave similar results (35 °C: Yield 20%, ee >99%, 40 °C: Yield 27%, ee >99%). However, at 50 °C and 60 °C, the reaction did not proceed at all. This is probably due to the low solubility of the substrate at the low density of CO_2 at higher temperatures.

Next, various ketones were reduced by the immobilized cell in $scCO_2$, and excellent enantioselectivities were obtained, although the yields were poor, and it was necessary to improve this.

The feasibility of recycling was examined using *o*-fluoroacetophenone **2** as a substrate. After the reaction, the $scCO_2$ pressure was decreased, and the product was extracted. Then, the catalyst was kept under reduced pressure (50 mmHg) at rt for 30 min and under 4 mmHg for 15 min to use it in the next cycle of reaction. As shown in Table 5, it could be recycled up to two times, although the yield decreased. This is the first report for the recycling of alcohol dehydrogenase used for reaction in $scCO_2$.[8]

Table 5
Recycling of the immobilized cell for the reduction of **2** in $scCO_2$

Run	Yield (%)	ee (%)
1st	20	>99 (*S*)
2nd	9	>99 (*S*)
3rd	7	>99 (*S*)
4th	0	—

Compound **2** (1 μL) was converted at 35 °C, 10 MPa for 5 h in $scCO_2$ by immobilized cell (0.5 g). After the extraction of the product, mixture was kept under reduced pressure (50 mmHg) at rt for 30 min and under 4 mmHg for 15 min, and then to carry out next reaction, MES buffer (0.75 mL), 2-propanol (0.65 mmol), NAD^+ (5 mg), and **2** (1 μL) were added followed by pressurization with CO_2.

Furthermore, the immobilized cell could be used in the continuous-flow reaction. This is the first complete flow system using alcohol dehydrogenase and $scCO_2$.[9]

3. Conclusions

The whole cell of *G. candidum* was immobilized onto an ion exchange resin with polyallylamine. The immobilization improved the enantioselectivity and stability, as shown by the reaction conducted in an MES buffer. The immobilization also enabled the continuous-flow reaction and recycling of the catalyst. The biocatalyst was then applied to the reaction in $scCO_2$, and various ketones were reduced with high enantioselectivities. The recycling of the catalysts accompanying the $scCO_2$ depressurization and continuous-flow reaction were possible for the first time for alcohol dehydrogenase using $scCO_2$.

4. Experimental

4.1. General

The whole cell of *G. candidum* NBRC 5767 was obtained by cultivation according to the literature.[10] The ion exchange polymer [IRA-96SB (Organo Co.)] was kindly supplied by Nacalai Tesque, Inc., and was used after drying with vacuum pump. Polyallylamine (PAS-880 (Nitto Boseki Co.)) was purchased from Nacalai Tesque, Inc. and used without further purification. Other chemicals were purchased from Nacalai Tesque, Inc., Wako Pure Chemical Industries, Ltd, and Aldrich Chemical Co. and used without further purification. Gas chromatographic analyses were performed using chiral GC-columns (CP-Chirasil-Dex-CB (Varian, 0.32 mm × 0.25 μm×50 m) or CP-cyclodextrin-B-2,3,6-M-19 (Varian, 0.25 mm × 0.25 μm × 25 m)) equipped on the Shimadzu GC-14A or GC-14B with C-R8A. The column temperatures and retention time of the substrates and products for the reactions of **1**–**7** are as follows, respectively. Compound **1**: 120 °C, 8.3 min, (*R*) 12.0 min, (*S*) 12.6 min; **2**: 135 °C, 4.8 min, (*R*) 7.6 min, (*S*) 7.9 min; **3**: 130 °C, 6.5 min, (*R*) 10.0 min, (*S*) 10.6 min; **4**: 140 °C, 6.4 min, (*R*) 10.5 min, (*S*) 11.4 min; **5**: 140 °C, 18.7 min, (*S*) 26.3 min, (*R*) 26.8 min; **6**: 135 °C, 3.1 min, (*S*) 14.4 min, (*R*) 14.7 min; and **7**: 115 °C, 16.2 min, (*S*) 18.9 min, (*R*) 19.5 min.

4.2. Immobilization of the cell

The whole cell of *G. candidum* was immobilized according to the literature method[5] as follows. To a solution of polyallylamine (PAS-880, 2.9 g) in a potassium phosphate buffer (pH 7.0, 0.1 M, 5.8 g), whole cell (6.5 g wet wt) was mixed, after which ion exchange polymer (IRA-96SB, 48 mL) was mixed and dried for 2 h with a fan and for 3 h under reduced pressure. The resulting immobilized cell (14.1 g dry wt) was used for the reaction or stored at −30 °C.

4.3. Batch reaction in an aqueous solution

NAD^+ (5 mg) was suspended in an MES buffer (0.1 M, pH 7.0, 3.0 mL). The substrate (10 μL) and 2-propanol (1.3 mmol) were added to the suspension, followed by the free cell (0.25 g) or immobilized cell (0.50 g). The reaction mixture was stirred at 130 rpm for 5 h at 30 °C, extracted with ether (5 mL × 4), and analyzed by GC using undecane as an internal standard. The absolute configuration of the product was determined by comparison of the GC retention times with those of authentic samples.

4.4. Flow reaction in aqueous solution

The immobilized cell (2.5 g) was packed in an up-flow tubular reactor (1/2 inch × 10 mm × 135 mm),[11] and substrate solution (**1** (0.011 M), 2-propanol (12.72 M), and NAD^+ (1.2×10^{-5} M) in an MES buffer (0.10 M, pH 7.0)) was pumped (JASCO, PU-2085) at the rate of 0.15 mL/min. The system reached equilibrium after 3 h, and then the product was analyzed by GC using undecane as an internal standard.

4.5. Experimental apparatus for $scCO_2$ reactions

The apparatus consists of a CO_2 gas cylinder, pump (Jasco SCF-Get), manometer (Taiatsu Techno, Co., Osaka, 15 MP an 25 MPa), stainless steel pressure-resistant vessel (Taiatsu Techno, Co., Osaka, TVS-N2 type, 10 mL), stop valve (Swagelok, SS3NBS4), water bath, and magnetic stirrer (Koike, HE-16GA).

4.6. Reaction in $scCO_2$

At first, NAD^+ (5 mg) was added to the MES buffer (0.1 M, pH 7.0, 0.75 mL) in a pressure-resistant vessel followed by the immobilized cell (0.50 g). Substrate (0.008 mmol) and 2-propanol (0.65 mmol) were added to a small glass tube, and the glass tube was placed in pressure-resistant vessel, so that the substrate did not come into contact with the catalyst before adding CO_2. Then,

the vessel was placed in the water bath at 35 °C and pressurized with CO_2 to 10 MP. The reaction mixture was stirred with magnetic stirrer for 5 h at 35 °C, cooled to 0 °C for 10 min, depressurized, extracted with ether (5 mL × 3), and analyzed by GC using undecane as an internal standard.

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8. Recycling of the immobilized *G. candidum* on water absorbing polymer without decreasing the $scCO_2$ pressure in a semi-flow reaction was reported in Ref. 4c, where the substrate was injected repeatedly through an HPLC injection valve to the biocatalyst kept in $scCO_2$.
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