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# A novel method for enzymatic asymmetric reduction of ketones in a supercritical carbon dioxide/water biphasic system

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ABSTRACT

A novel method to enable asymmetric reduction of ketones by an alcohol dehydrogenase from *Geotrichum candidum* in a supercritical carbon dioxide and water biphasic system is described. The addition of sodium bicarbonate improved the reactivity up to a practical level while retaining excellent enantioselectivity.



Biotransformations in biphasic systems have been investigated widely to improve reaction efficiency and to simplify workup procedure. Recent developments include the application of organic solvent/water, ionic liquid/water, ionic liquid/supercritical carbon dioxide ($scCO_2$), and $scCO_2$/water systems.[1] Using a biphasic system, substrate/product inhibition has been prevented, and workup procedure has been simplified. Among the solvents used in biphasic systems, $scCO_2$ has the benefits of being environmentally benign, non-flammable, of low toxicity, high availability, and ambient critical temperature which make it suitable for biotransformations.[1]

However, the types of enzymes used in $scCO_2$ are very limited, and only lipases have been used widely and successfully.[1] The applicability of other kinds of enzymes such as dehydrogenases needs to be investigated to broaden the variety of organic transformations that can be achieved. Therefore, we have investigated the use of a dehydrogenase for asymmetric reduction in $scCO_2$ and reported previously that asymmetric reduction of ketones was possible when the resting cells of *Geotrichum candidum* NBRC 5767 were used.[2] However, this method still has the drawback that the resting cells of the microbe are difficult to be stored for a long time. The resting cells need to be used immediately after cultivation. The second weak point is that a large amount of biocatalyst is necessary; the ratio of biocatalyst mass to substrate was >100. On the other hand, the use of a crude enzyme preparation (cell dried using acetone) resolves the above-mentioned drawbacks; the biocatalyst can be stored for a long period in a freezer, and the ratio of substrate to biocatalyst was reduced to 1.[3] Therefore, we attempted to use the crude enzyme preparation, an alcohol dehydrogenase, of *G. candidum* NBRC 5767 (APG5) for asymmetric reduction in $scCO_2$/water biphasic system. Contrary to our expectations, without any additive, the reaction hardly proceeded. However, we found that the addition of sodium bicarbonate improved the reactivity and yield of the product up to a practical level.

First, we attempted to react acetophenone with the crude alcohol dehydrogenase of *G. candidum* NBRC 5767 (APG5) in the presence of 2-propanol as the hydrogen source in a biphasic system (2 mL of 0.1 M MES buffer (pH 7.0) and 8 mL of $scCO_2$) at 35 °C as shown in Scheme 1.[4] Disappointingly, the yield was poor (4%). The following reasons can be considered for the low yield.

(1) The high pressure inactivates the enzyme.
(2) Lowered pH of the aqueous layer due to high density of $scCO_2$ inactivates the enzyme.

To examine if the high pressure deactivates the enzyme, a biphasic system composed of supercritical fluoroform ($scCHF_3$) and water under the same reaction conditions was used with the cells and substrate. The reaction in $scCHF_3$ readily proceeded, and the corresponding (*S*)-alcohol was obtained in 57% yield (>99% ee). Therefore, the cause of the low yield of the reaction in $scCO_2$ biphasic system was not the high pressure.

To examine if the lowered pH deactivates the enzyme, the effect of pre-treatment with the supercritical fluid/aqueous buffer biphasic solvent on the enzyme activity was examined. After the pre-treatment, the activity of the enzyme was analyzed by the reaction in aqueous buffer under atmospheric pressure. It was found that the activity was lost completely by the pre-treatment with $scCO_2$ in 8 MPa $scCO_2$/aqueous buffer for 3 h (0% yield). However, pre-treated biocatalyst with $scCHF_3$/aqueous buffer did not lose any activity (76% yield). This strongly suggests that the lowered pH caused by $scCO_2$ irreversibly inactivated the enzyme. Since large changes of pH in the aqueous phase of the $scCO_2$/aqueous buffer biphasic system have been reported,[5] the effect of pH on enzyme

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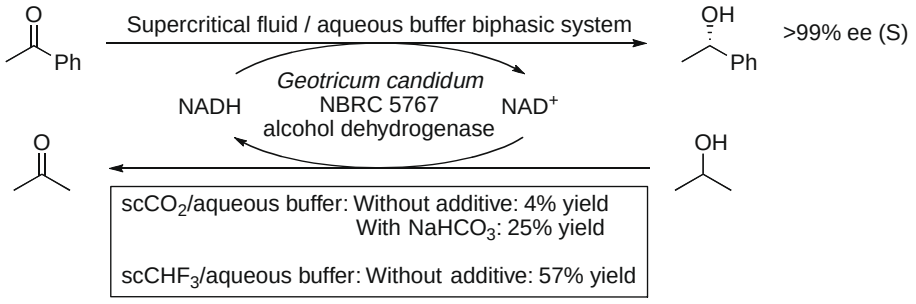


**Scheme 1.**

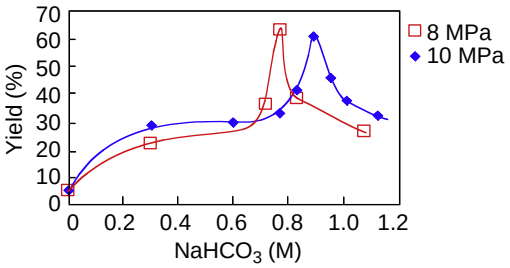


**Figure 1.** Effect of $NaHCO_3$ on the reduction of *o*-fluoroacetophenone in the $scCO_2$/aqueous buffer biphasic system. Reaction conditions: *o*-fluoroacetophenone = 0.082 mmol, $NAD^+$ = 1.3 μmol, $NaHCO_3$ = 0-200 mg; APG5 = 20 mg, MES buffer (0.1 M pH 7.0) = 2 ml, 2-propanol = 2.6 mmol, 8 MPa or 10 MPa $scCO_2$, 5 h, 35 °C.

activity was examined. Indeed, the enzyme was deactivated at a low pH (below 5), and no reaction proceeded under these conditions. Therefore, the inactivation of the biocatalyst was due to the lowered pH of the buffered solution by $scCO_2$.[5] The buffering action of MES buffer was not sufficient for the system.

To overcome the undesired effect of $scCO_2$, we examined the effect of addition of basic salts to the reaction mixture. Among several bases tested,[6] we have found that sodium bicarbonate was the best additive. The addition of 12 mmol of sodium bicarbonate in 2.0 mL of MES buffer (0.1 M, pH 7.0) solution containing 20 mg of enzyme, 0.082 mmol of acetophenone, 2.6 mmol of 2-propanol, and 0.013 mmol of $NAD^+$ gave 25% of the corresponding (*S*)-alcohols (>99% ee) by the biphasic reaction in 8 MPa $scCO_2$ for 3 h. The addition of sodium bicarbonate improved the reactivity from 4% to 25% yield.

Next, the effect of changing the amount of sodium bicarbonate on the reduction of *o*-fluoroacetophenone was examined in the $scCO_2$/aqueous buffer biphasic system at 8 MPa and 10 MPa. As shown in Figure 1, the amount of sodium bicarbonate significantly affected the yields of the reaction. The suitable amounts of sodium bicarbonate at 8 MPa or 10 MPa were 0.77 M or 0.89 M, respectively. The reproducibility of these data was very high. The phenomenon is explained as follows: the amount of sodium bicarbonate necessary to make the reaction mixture pH neutral is very narrow and different at different pressures.

Moreover, the use of sodium bicarbonate enabled the enzymatic asymmetric reduction even in 14 MPa $scCO_2$/aqueous buffer biphasic system (29% yield), whereas the reaction without addition of bicarbonate hardly proceeded (5% yield).

To examine the applicability of the present biphasic system to other substrates, several ketones were used as substrates. These ketones were reduced to the corresponding (*S*)-alcohol in high yields with excellent ee (>99%) as shown in Table 1. The $scCO_2$/aqueous buffer biphasic system was also compared with hexane/aqueous buffer[7] biphasic system. As shown in Table 1, the reaction

**Table 1**
Asymmetric reduction of ketones by dehydrogenase in $scCO_2$/aqueous buffer or hexane/ aqueous buffer

| Reaction conditions Substrates | $scCO_2$/aqueous buffer[a] | | Hexane/ aqueous buffer[b] | |
|---|---|---|---|---|
| | Yield[d] (%) | ee (%) | Yield[d] (%) | ee (%) |
| Acetophenone | 22 | >99 | 21 | >99 |
| *o*-Fluoroacetophenone | 60 | >99 | 49 | >99 |
| *m*-Fluoroacetophenone | 36 | >99 | 42 | >99 |
| *m*-Fluoroacetophenone[c] | 82 | >99 | | |
| *m*-Chloroacetophenone | 50 | >99 | 44 | >99 |
| *m*-Chloroacetophenone[c] | 82 | >99 | | |
| *tert*-Butyl acetoacetate | 37 | >99 | 15 | >99 |
| *tert*-Butyl acetoacetate[c] | 57 | >99 | | |

[a] Reaction conditions: substrate = 0.082 mmol, $NAD^+$ = 1.3 μmol, APG5 = 20 mg, $NaHCO_3$ = 150 mg, 2-propanol = 2.6 mmol, MES buffer (0.1 M, pH 7.0) = 2 mL, 10 MPa $scCO_2$, 5 h, 35 °C.
[b] Reaction conditions: Substrate = 0.082 mmol, $NAD^+$ = 1.3 μmol, APG5 = 20 mg, 2-propanol = 2.6 mmol, MES buffer (0.1 M, pH 7.0) = 2 mL, hexane = 8 ml, 5 h, 35 °C.
[c] Reaction conditions: $NAD^+$ = 6.5 μmol.
[d] Side reactions such as aldol condensation were not observed.

in $scCO_2$ system gave yields that were slightly higher than those obtained in the hexane system when using *o*-fluoroacetophenone and *tert*-butyl acetoacetate as a substrate, and results were similar to those obtained in the hexane system when using other substrates.

Finally, the present biphasic system was applied to the alginate-immobilized dehydrogenase. The asymmetric reduction of *o*-fluoroacetophenone with the immobilized enzyme in $scCO_2$/water biphasic system proceeded, and a 75% yield of the (*S*)-alcohol was obtained in >99% ee.[8]

In conclusion, the addition of sodium bicarbonate enabled the enzymatic asymmetric reduction of various ketones under $scCO_2$/water biphasic conditions of up to a pressure of 14 MPa. Free and immobilized enzyme can be used in the system. We believe that the present method can be applicable to other biocatalytic reactions under $scCO_2$ and will open up a new field for the use of $scCO_2$ for biocatalysis.

## References and notes

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4. The reduction was conducted as described in Ref. 2a except that the crude enzyme was used in place of the resting cells of the microbe and water-adsorbent polymer was not used.
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6. The following basic salts were used: $NaHCO_3$, $CaCO_3$, CaOAc, $KHCO_3$, and $Na_2CO_3$.
7. Asymmetric reduction using resting cells of *Geotrichum candidum* in a hexane/aqueous buffer biphasic system has been reported; Nakamura, K.; Inoue, Y.; Matsuda, T.; Misawa, I. *J. Chem. Soc., Perkin Trans. 1* **1999**, 2397.
8. Conditions: APG5 = 20 mg (immobilized with 0.36 g of calcium alginate); $NAD^+$ = 6.5 μmol; 2-propanol = 2.6 mmol; *o*-fluoroacetophenone = 0.082 mmol; $NaHCO_3$ = 140 mg; MES buffer (0.1 M pH 7.0) = 1.64 mL; $scCO_2$ = 10 MPa; 35 °C, 5 h. The reaction without $NaHCO_3$ afforded only 3% yield of the alcohol.