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Biocatalytic reduction of prochiral aromatic ketones to optically pure alcohols by a coupled enzyme system for cofactor regeneration

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ABSTRACT

A simple, highly efficient, and economical biphasic cell-free system was developed for biocatalytic reduction of prochiral aromatic ketones to furnish enantiopure alcohols. This system is characterized by using endogenous enzymes of the cell-free extract to form enzyme-coupled NADPH recycling system. Besides, it offered much higher productivity than whole cells and greatly simplified the preparation process of biocatalysts in comparison with isolated enzymes. Various prochiral aromatic ketones, especially α-substituted acetophenone derivatives, were reduced to chiral alcohols with excellent enantiomeric excess (ee) and moderate to good yield by this cell-free system.

Alcohol dehydrogenases (ADH), also referred to as carbonyl reductases (CR),[1] are a class of nicotinamide dependent oxidoreductases, which can catalyze the reduction of carbonyl compounds to produce alcohols. Due to their strict recognition of substrate and hence high enantiomeric excess products, ADH/CR are especially suitable for asymmetric synthesis of optically pure alcohols, which are very important chiral building blocks in chemical and pharmaceutical industry.[2] Therefore, biocatalytic carbonyl reduction has been extensively studied in recent years.[3]

Today, whole-cell biocatalysts and isolated enzymes that exhibit ADH/CR activity are predominantly applied in producing enantiopure alcohols through ketone reduction.[4] However, they have their own drawbacks. For whole-cell catalysts, the cellular membrane often retards the entry of substrate into the cellular systems and prevents the product from being released from the cellular system for an easy recovery. Consequently, productivities of whole-cell biocatalysis are severely compromised.[5] For isolated enzymes, costly and time-consuming process of enzyme preparation is the main shortcoming. Also, coenzyme regeneration should be taken into serious consideration since the reactions usually require a stoichiometric amount of expensive coenzyme. As a consequence, several coenzyme recycling systems have been developed, typically substrate-coupled approach and enzyme-coupled approach.[6] Substrate-coupled method can only be accomplished when the enzyme exhibits both reductive and oxidative abilities that are a harsh requirement for a large number of ADH/CR.[7] Enzyme-coupled approach introduces another enzyme, which increases the cost of reaction.[8] Cell-free extract, which contains virtually the functional enzymes inside the cell, was rarely employed as a biocatalyst to conduct asymmetric reduction in recent years,[9] and cell-free extract from recombinant *Escherichia coli,* which is widely used as expression host has hardly been applied to perform carbonyl reduction so far. Herein, we presented a cell-free system by using cell-free extract as a biocatalyst to conduct carbonyl reduction. It should be noted that this method contained an efficient enzyme-coupled system for NADPH regeneration. Besides, this cheap biocatalyst offered much higher productivity than whole cells and greatly simplified the preparation process of biocatalysts in comparison with isolated enzymes.

We have recently demonstrated that *Candida parapsilosis* CCTCC M203011 could achieve enantioselective conversion of racemic 1-phenyl-1,2-ethanediol to produce (*S*)-1-phenyl-1,2-ethanediol (**(*S*)-1b**).[10] In this Letter, we constructed a recombinant *E. coli* BL21(DE3)(pETSCR1) overexpressing NADPH-dependent carbonyl reductase SCR1 (GenBank accession No. FJ939565) which is from *C. parapsilosis* CCTCC M203011. Then the cell-free extract (total soluble protein 5 g/L) from the recombinant *E. coli* was applied for asymmetric reduction of prochiral aromatic ketones.

As expected, cell-free extract alone was inefficacious in asymmetric reduction of α-hydroxyacetophenone (**1a**) to form **(*S*)-1b** (Table 1). Besides, the reactions achieved limited improvement with high ee but low yield when glucose and a low amount of

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Table 1
Discovery of NADPH recycling system

Substrate **1a**[a]	Glucose	NADPH	$NADP^+$	ee[b,c] (%)	Yield[c] (%)
+	–	–	–	–	–
+	+	–	–	>99	8.5
+	–	+	–	>99	5.6
+	–	–	+	>99	4.8
+	+	+	–	>99	92.9
+	+	–	+	>99	93.1

[a] Reaction mixture in 2 mL comprised 1 mL potassium phosphate buffer (pH 6.5, 0.1 M), 1 mL cell-free extract (total protein 5 mg), 40 mM **1a** with or without 10 g/L glucose, 0.1 mM $NADP^+$ and 0.1 mM NADPH.
[b] Sole product **(*S*)-1b**.
[c] Yield and ee were determined by chiral HPLC.

$NADP^+$/NADPH was added separately. However, the complete conversion was unexpectedly achieved with excellent ee and high yield in the presence of both glucose and $NADP^+$/NADPH. Since the molar amount of NADPH was much less than that of substrate and complete conversion was also achieved when NADPH was replaced by $NADP^+$, it can be deduced that NADPH was regenerated during the process. The requirement of glucose in the complete conversion of **1a** suggested that glucose acted as a hydrogen donor to perform the NADPH regeneration. Besides, carbonyl reductase SCR1 had no oxidative activity toward sugars or alcohols (data not shown). Therefore, by utilizing glucose, cell-free extract itself formed an enzyme-coupled NADPH recycling system. In this system, the endogenous enzymes of cell-free extract corresponded to the second enzyme in the classic enzyme-coupled approach. Because $NADP^+$ and NADPH had the same effect on the necessary cofactor regeneration, while $NADP^+$ is more stable and economical than its reductive counterpart, $NADP^+$ was employed as the initial coenzyme in the following experiments. To further confirm the existence of NADPH recycling system, we detected the optical density change at 340 nm in an activity assay system containing only cell-free extract, $NADP^+$ and glucose. The increase of optical density was observed, indicating that the NADPH was generated in the cell-free extract (For NADPH regeneration assay details see the Supplementary data).

Interestingly, there were more than one enzyme, which could achieve NADPH regeneration in this cell-free system. As illustrated in the Figure 1, glucose and trehalose showed the best results (**(*S*)-1b** yield at 93.0% and 92.4%, respectively). Fructose, ethanol, maltose, and lactose also facilitated the conversion to some extent

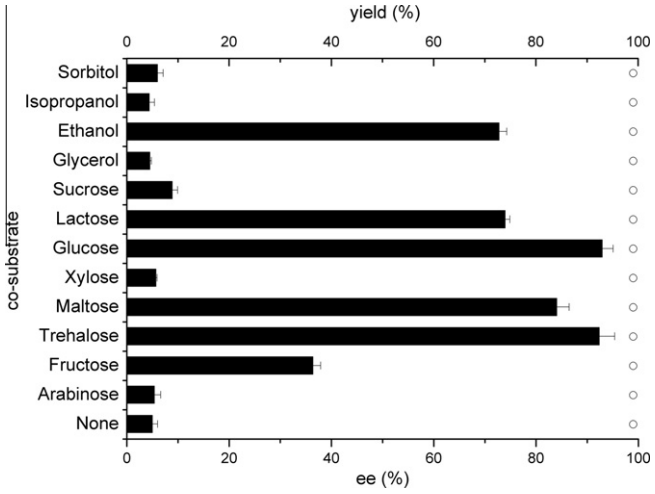

Figure 1. Effect of co-substrate on reduction of **1a** by cell-free system. Co-substrate concentration was 10 g/L. (■) yield, (○) ee.

(where yield ranged from 36.4% to 84.1%). Therefore, by adding several hydrogen donors simultaneously, it is possible that multiple cofactor recycling systems run in parallel and hence greatly enhance the cofactor regeneration. It provided us a potential and powerful alternative to cope with the problem that coenzyme regeneration brings to us. Also, since the coenzyme recycling system was formed by the endogenous enzymes of the cell, *E. coli* BL21(DE3) harboring other ADH/CR also, theoretically, has the potential to form such enzyme-coupled cell-free system. In fact, this system has been successfully used by other ADH/CRs by our group (data not shown).

The expensive coenzymes, such as NADP(H), are the major impediment of the large application of oxidoreductases, therefore, we investigated the requirement of $NADP^+$ for this cell-free system (Table 2). Since the $NADP^+$ and NADPH contained in the cell-free extract show no detectable effect on the reduction (Table 2), we herein defined the total turnover number (TTN) as the molar amount of synthesized product per molar amount of added cofactor. The maximum TTN of 1916 was afforded with 47.9% yield at 0.01 mM $NADP^+$. When 0.02 mM $NADP^+$ was added, however, the cell-free system exhibited high yield (91.3%) and TTN of 1826. Thus, the endogenous enzymes of cell-free extract, which are usually overlooked by most researchers could form an efficient enzyme-coupled NADPH recycling system. Also, this system greatly reduced the costs by avoiding the introduction of another enzyme.

Compared with whole-cell system, this cell-free system performed much higher productivity. As shown in the Figure 2, using the cell-free system, 40 mM **1a** (due to poor solubility of **1a**, no higher concentration was tested for aqueous phase reactions.) was completely converted into the corresponding product of 92.9% yield within 6 h, while whole-cell system required 12 h to accomplish the complete conversion of 10 mM **1a**. The sharp contrast indicated that the substrate/product transportation was greatly retarded by the cellular membrane, but the cell-free system facilitated the interaction between enzyme and substrate/product, resulting in higher productivity.

The majority of ketones are highly hydrophobic, and thus possess poor solubility in aqueous media, which directly leads to low substrate concentrations. We hence developed a water–organic solvent system for asymmetric reduction. Several alkanes, esters, and other reagents were examined as an organic phase and ethyl laurate was finally adopted as an organic phase to conduct further investigation. By using biphasic system, upto 80 mM **1a** was converted into **(*S*)-1b** with 90.1% yield and >99% ee within 12 h, affording a TTN of 3604, while only 10.1% yield was achieved for the aqueous system (Table 3). In comparison with the reported literature involving bioreduction of **1a** to form **(*S*)-1b**,[10,11] this cell-free system exhibited the highest productivity (7.2 mmol/L/h).

In order to examine the generality of the cell-free biphasic system, various aromatic ketones were tested as substrates (Table 3). The range of substrates included α-substituted acetophenone derivatives (**1a**–**9a**), acetophenone (**10a**) and *ortho*-, *meta*-, and

Table 2
Effect of $NADP^+$ concentration on reduction of **1a** by cell-free system

$NADP^+$[a] (mM)	ee[b] (%)	Yield (%)	TTN
0.2	>99	93.2	186
0.1	>99	93.0	372
0.05	>99	92.7	742
0.02	>99	91.3	1826
0.01	>99	47.9	1916
0.005	>99	11.7	936
0	-	-	-

[a] Substrate concentration was 40 mM.
[b] Sole product **(*S*)-1b**.

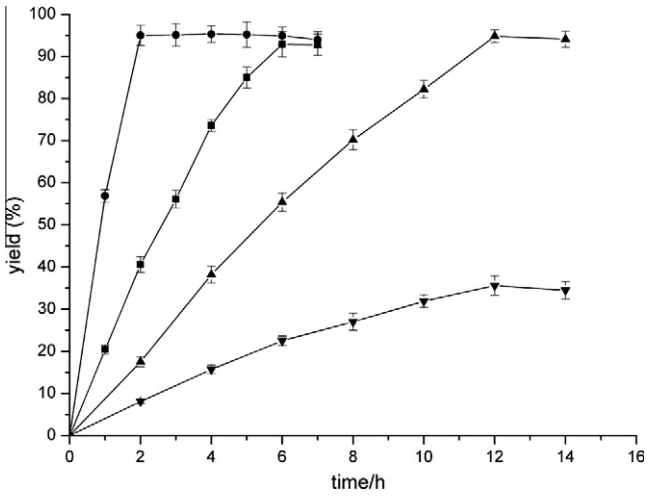

Figure 2. Time course of reduction of **1a**. (●) cell-free system with 10 mM **1a**. (■) cell-free system with 40 mM **1a**. (▲) whole-cell system with 10 mM **1a**. (▼) whole-cell system with 40 mM **1a**.

para-substituted acetophenones (**11a**–**15a**). Among all the aromatic ketones, **1a** was more suitable for this cell-free system mediated asymmetric reduction, giving corresponding chiral alcohol of 90.1% yield in high substrate concentration. Besides, a clear trend was observed that only α-substituted acetophenone derivatives could be reduced to chiral alcohols with excellent yield, indicating substituents at α-carbon, that is, hydroxyl or bromine had a great effect on the enzyme activity by forming hydrogen bond between the active site residues of the enzyme and such electron-withdrawing groups at the α-position of substrates. This was coincided with another enzyme identified from the *C. parapsilosis* CCTCC M203011.[12] In addition, for α-substituted acetophenone derivatives, α-bromo-substitution showed lower yield than α-hydroxy-substitution. We proposed that the hydrogen bond between amino acid of the active site of the enzyme and the substituent at α-carbon played an important role in the reaction, because the results were in conformity with the general rules that the strength of hydrogen bond is in order of hydroxyl > bromine > hydrogen. Although substrates varied, the corresponding alcohols were synthesized with >99% ee in all cases, even on the poor substrates (**10a**, **13a–15a**). The high ee value indicated that the cell-free extract, though complex, produced no side reaction. Besides, the biphasic cell-free system showed much higher yield in comparison with aqueous whole-cell system.

In conclusion, we presented a biphasic cell-free system, which could accomplish asymmetric conversion of various aromatic ketones, involving enzyme-coupled cofactor regeneration. This system exhibited much higher productivity than whole-cell system by breaking the cell membrane barrier. Compared with the isolated enzyme biocatalysts, this system greatly simplified the preparation of biocatalyst, which is usually a costly and time-consuming process. Most importantly, by utilizing the endogenous enzymes of cell-free extract to form coenzyme recycling system, NADPH regeneration could run efficiently without adding extra enzyme. Finally, by introducing ethyl laurate as an organic phase to form a biphasic system, various prochiral aromatic ketones, especially α-substituted acetophenone derivatives, were reduced to chiral alcohols with excellent ee and moderate to good yield. Thus, an enzyme-coupled system for cofactor regeneration has been developed for asymmetric bioreduction. By utilizing the endogenous enzymes that are usually overlooked by most researchers, this system, which is not restricted in carbonyl reductase SCR1, but other ADH/CR, provides us a promising alternative to develop biocatalytic system for fine biochemical synthesis in a simple, economical, and efficient way.

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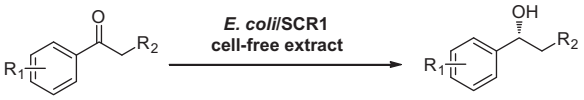
Table 3
Reduction of various aromatic ketones by cell-free system



Aromatic ketone[a]	R_1	R_2	Biphasic cell-free system[b]		Aqueous whole-cell system	
			ee[c] (%)	Yield[c] (%)	ee[c] (%)	Yield[c] (%)
1a[d,e]	H	OH	>99(*S*)	90.1	>99(*S*)	10.1
2a	*m*-Cl	OH	>99(*S*)	90.5	>99(*S*)	7.8
3a	*p*-Cl	OH	>99(*S*)	86.6	>99(*S*)	13.2
4a	*p*-CH_3	OH	>99(*S*)	87.5	>99(*S*)	20.1
5a	*p*-OCH_3	OH	>99(*S*)	91.1	>99(*S*)	22.5
6a	*m*-Cl	Br	>99(*S*)	82.7	>99(*S*)	5.5
7a	*p*-Cl	Br	>99(*S*)	80.4	>99(*S*)	7.7
8a	*p*-CH_3	Br	>99(*S*)	84.6	>99(*S*)	9.7
9a	*p*-OCH_3	Br	>99(*S*)	83.0	>99(*S*)	6.1
10a	H	H	>99(*R*)	5.0	–	–
11a	*o*-Cl	H	–	–	–	–
12a	*m*-Cl	H	–	–	–	–
13a	*p*-Cl	H	>99(*R*)	5.2	–	–
14a	*p*-CH_3	H	>99(*R*)	15.5	–	–
15a	*p*-OCH_3	H	>99(*R*)	11.3	–	–

[a] Substrate concentration: **1a** 80 mM, **2a**–**9a** 25 mM, **10a**–**15a** 10 mM; $NADP^+$ was 0.02 mM; glucose concentration was 10 g/L; reaction time was 6 h.
[b] Ethyl laurate/water = 1:4 (v/v).
[c] Determined by chiral HPLC (**1a**–**10a**, **14a**, **15a**) or GC (**11a**–**13a**).
[d] Glucose concentration was 20 g/L.
[e] Reaction time was 10 h.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.12.069.

References and notes

1. Forrest, G. L.; Gonzalez, B. *Chem. Biol. Interact.* **2000**, *129*, 21–40.
2. (a) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258–268; (b) Schoemaker, H. E.; Mink, D.; Wubbolts, M. G. *Science* **2003**, *299*, 1694–1697; (c) Patel, R. N. *Coord. Chem. Rev.* **2008**, *252*, 659–701; (d) Wolberg, M.; Villela, M.; Bode, S.; Geilenkirchen, P.; Feldmann, R.; Liese, A.; Hummel, W.; Muller, M. *Bioprocess Biosyst. Eng.* **2008**, *31*, 183–191; (e) Ema, T.; Ide, S.; Okita, N.; Sakai, T. *Adv. Synth. Catal.* **2008**, *350*, 2039–2044; (f) Kizaki, N.; Yasohara, Y.; Hasegawa, J.; Wada, M.; Kataoka, M.; Shimizu, S. *Appl. Microbiol. Biotechnol.* **2001**, *55*, 590–595.
3. (a) Moore, J. C.; Pollard, D. J.; Kosjek, B.; Devine, P. N. *Acc. Chem. Res.* **2007**, *40*, 1412–1419; (b) Matsuda, T.; Yamanaka, R.; Nakamura, K. *Tetrahedron: Asymmetry* **2009**, *20*, 513–557; (c) De Wildeman, S. M. A.; Sonke, T.; Schoemaker, H. E.; May, O. *Acc. Chem. Res.* **2007**, *40*, 1260–1266.
4. (a) Goldberg, K.; Schroer, K.; Lutz, S.; Liese, A. *Appl. Microbiol. Biotechnol.* **2007**, *76*, 237–248; (b) Goldberg, K.; Schroer, K.; Lutz, S.; Liese, A. *Appl. Microbiol. Biotechnol.* **2007**, *76*, 249–255.
5. Chen, R. R. Z. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 730–738.
6. Wichmann, R.; Vasic-Racki, D. In *Advances in Biochemical Engineering Biotechnology*; Springer: Berlin, 2005; Vol. 92, pp 225–260.
7. (a) Musa, M. M.; Ziegelmann-Fjeld, K. I.; Vieile, C.; Zeikus, J. G.; Phillips, R. S. *J. Org. Chem.* **2007**, *72*, 30–34; (b) de Gonzalo, G.; Lavandera, I.; Faber, K.; Kroutil, W. *Org. Lett.* **2007**, *9*, 2163–2166; (c) Bisogno, F. R.; Lavandera, I.; Kroutil, W.; Gotor, V. *J. Org. Chem.* **2009**, *74*, 1730–1732.
8. (a) Voss, C. V.; Gruber, C. C.; Faber, K.; Knaus, T.; Macheroux, P.; Kroutil, W. *J. Am. Chem. Soc.* **2008**, *130*, 13969–13972; (b) Zhu, D. M.; Ankati, H.; Mukherjee, C.; Yang, Y.; Biehl, E. R.; Hua, L. *Org. Lett.* **2007**, *9*, 2561–2563; (c) Iyer, R. B.; Bachas, L. G. *J. Mol. Catal. B: Enzym.* **2004**, *28*, 1–5; (d) Hirano, J.; Miyamoto, K.; Ohta, H. *Tetrahedron Lett.* **2008**, *49*, 1217–1219; (e) Mertens, R.; Greiner, L.; van den Ban, E. C. D.; Haaker, H.; Liese, A. *J. Mol. Catal. B: Enzym.* **2003**, *24–25*, 39–52.
9. (a) Utaka, M.; Yano, T.; Ema, T.; Sakai, T. *Chem. Lett.* **1996**, 1079–1080; (b) Ishihara, K.; Higashi, Y.; Nakajima, N.; Utaka, M.; Nakamura, K. *J. Ferment. Bioeng.* **1996**, *81*, 266–268; (c) Keinan, E.; Hafeli, E. K.; Seth, K. K.; Lamed, R. *J. Am. Chem. Soc.* **1986**, *108*, 162–169; (d) Tsuchiya, S.; Miyamoto, K.; Ohta, H. *Biotechnol. Lett.* **1992**, *14*, 1137–1142; (e) Ishihara, K.; Nakajima, N. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 3077–3080; (f) Yang, W.; Xu, J. H.; Pan, J.; Xu, Y.; Wang, Z. L. *Biochem. Eng. J.* **2008**, *42*, 1–5.
10. Nie, Y.; Xu, Y.; Mu, X. Q. *Org. Process Res. Dev.* **2004**, *8*, 246–251.
11. (a) Wei, Z. L.; Lin, G. Q.; Li, Z. Y. *Bioorg. Med. Chem.* **2000**, *8*, 1129–1137; (b) Wei, Z.; Li, Z.; Lin, G. *Tetrahedron* **1998**, *54*, 13059–13072; (c) Tsujigami, T.; Sugai, T.; Ohta, H. *Tetrahedron: Asymmetry* **2001**, *12*, 2543–2549; (d) Hasegawa, J.; Ogura, M.; Tsuda, S.; Maemoto, S.; Kutsuki, H.; Ohashi, T. *Agric. Biol. Chem.* **1990**, *54*, 1819–1827.
12. Nie, Y.; Xu, Y.; Mu, X. Q.; Wang, H. Y.; Yang, M.; Xiao, R. *Appl. Environ. Microbiol.* **2007**, *73*, 3759–3764.