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LETTER

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Correlation between Intracellular Cofactor Concentrations and Biocatalytic Efficiency: Coexpression of Diketoreductase and Glucose Dehydrogenase for the Preparation of Chiral Diol for Statin Drugs

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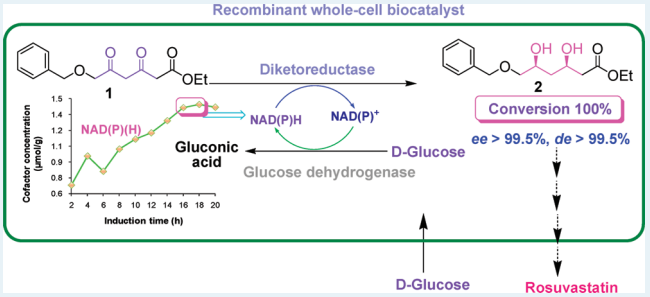
Supporting Information

ABSTRACT: Dynamic change of intracellular nicotinamide cofactor concentrations, the limiting factor for the bioreductions catalyzed by oxidoreductases, was monitored in *Escherichia coli* cells coexpressing diketoreductase and glucose dehydrogenase. On the basis of an unexpected observation, a relationship between catalytic efficiency and cofactor concentrations was established to optimize the process for the preparation of a chiral diol for statin drugs. Consequently, compared to previous reactions by *E. coli* cells expressing diketoreductase alone, exogenous addition of cofactors was completely eliminated to yield an increase of substrate concentration by 15-fold. The present strategy could be employed in the biocatalytic processes catalyzed by nicotinamide-dependent oxidoreductases.



KEYWORDS: diketoreductase, glucose dehydrogenase, nicotinamide cofactor, coexpression, whole-cell biocatalysis, statin drugs

The asymmetric reduction of carbonyl compounds catalyzed by oxidoreductases is an attractive methodology to synthesize optically pure alcohols.[1−4] However, the industrial application of nicotinamide-dependent oxidoreductases has been largely restricted mainly because of the high costs and instability of nicotinamide cofactors, such as NADH and NADPH. Alternatively, whole-cell biocatalysts with coexpression of an oxidoreductase and an NAD(P)H regeneration enzyme has been utilized in various asymmetric bioreductions to eliminate or to reduce the exogenous addition of cofactors on milliliter scales.[5−7] However, in most cases, stoichiometric amounts of expensive cofactors are still required to be added into whole-cell systems to initiate the reactions and to achieve complete conversion of the substrate on larger scales. Gröger and Chamouleau described the "designer cell" of *E. coli* cells coexpressing oxidoreductases and formate dehydrogenase (FDH) or glucose dehydrogenases (GDH) and illustrated the relationship between enzyme activity and substrate conversion, aiming to eliminate the addition of exogenous cofactors.[8,9] Unfortunately, the dynamic change of intracellular cofactor concentrations in the recombinant *E. coli* cells was not taken into consideration in their study. Given that cofactors are constantly biosynthesized and metabolized by different pathways and the intracellular cofactor concentrations may directly correlate with the catalytic efficiency in the presence of enough active enzyme, monitoring the dynamic change of cofactor concentrations inside the cells could be a particularly useful way to ensure the success of a biocatalytic process.

Recently, we cloned a novel NAD(P)H-dependent diketoreductase (DKR) from *Acinetobacter baylyi*, which can stereoselectively reduce ethyl 3,5-diketo-6-benzyloxy hexanoate (**1**) to ethyl *3R,5S*-dihydroxy-6-benzyloxy hexanoate (**2**), a chiral intermediate useful to the synthesis of statin drugs (Scheme 1).[10−12] Previously, several cofactor recycling systems were compared for the efficiency of cofactor regeneration over the course of the bioconversion.[11] However, low substrate concentration and the requirement of exogenous cofactors were the major issues for high costs and process complexity, which hinders the practical application of DKR. Herein, to effectively solve such problems, we constructed a whole-cell biocatalyst by coexpressing DKR and GDH from *Bacillus megaterium*[13] in *E. coli* cells and explored the correlation between intracellular cofactor concentrations and the bioconversion. As a result, under the cooperative guidance of intracellular cofactor concentrations and enzyme activities, we established a more efficient process for the preparation of this important chiral diol to eliminate the addition of exogenous cofactors and to increase the substrate concentration.

We first constructed two coexpression plasmids harboring the *dkr* and *gdh* genes with different orders, respectively, to compare enzyme expressions, activities, and catalytic efficiency. Then, the plasmids, pETDuet-*dkr-gdh* and pETDuet-*gdh-dkr* (Supporting

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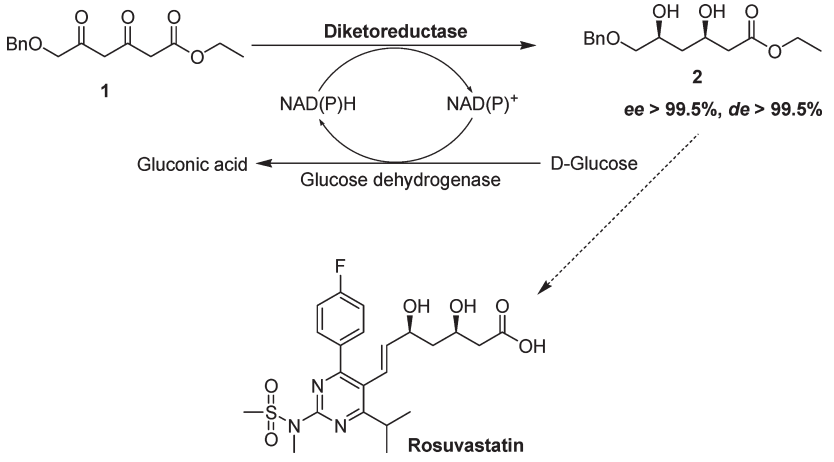
Scheme 1. Bio-Reduction of 1 to 2 by Whole-Cell Biocatalyst Co-Expressing DKR and GDH[a]



[a] Reduction of **1** catalyzed by *E. coli* cells gave *3R,5S*-dihydroxy **2** with *de* and *ee* values both greater than 99.5%.

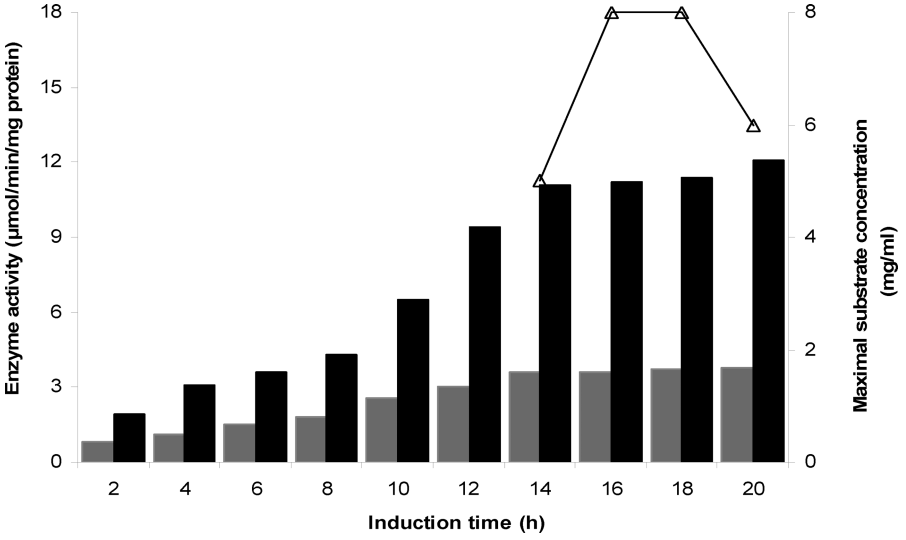


Figure 1. Effects of induction time on enzyme activitiy and substrate concentration. The gray and black columns represent the enzyme activities of DKR and GDH, respectively. Open triangle represents the maximal substrate concentration when the substrate was added at 14, 16, 18, and 20 h.

Information, Figure S1), were transformed into *E. coli* BL21 (DE3) cells for enzyme expression. After optimizing the induction conditions, DKR and GDH in both recombinant *E. coli* strains compatibly achieved the maximal expressions (Supporting Information, Figure S2) and maximal specific activities (Supporting Information, Table S2) at 20 °C and 0.5 mM IPTG. To further select a better recombinant strain with higher catalytic efficiency, both whole-cell biocatalysts after 14 h induction were used to convert substrate **1** varying from 0.5 mg/mL to 12 mg/mL under the same conditions. As shown in Supporting Information, Figure S3, *E. coli* cells harboring pETDuet-*gdh-dkr* exhibited better catalytic efficiency with a complete conversion of **1** at 5 mg/mL, and this strain was used for subsequent studies.

According to the report by Gröger and Chamouleau, both enzyme activity and cell mass are important to the substrate concentration and conversion rate for whole-cell biocatalysis with coexpression of a target enzyme and an NAD(P)H regeneration enzyme.[9] Thus, the timing of adding substrate into the fermentation broth containing the whole-cell biocatalyst would be crucial to affect the catalytic efficiency. Figure 1 indicates that the enzyme activities of DKR and GDH in *E. coli* cells harboring pETDuet-*gdh-dkr* and the cell mass reached a plateau after 14 h induction. When we added the substrate into the fermentation broth at 14 h, substrate concentration of 5 mg/mL was the maximum. According to this fact, we assumed that such a substrate concentration for the whole-cell system would be related to the maximal enzyme activity and cell mass at the point of 14 h induction, and the reactions with substrate concentrations higher than 5 mg/mL would not achieve a completion. Surprisingly, as shown in Figure 1, when the reactions proceeded under the same conditions, complete conversions of **1** to **2** with an increased concentration to 8 mg/mL was constantly achieved despite different timings of adding the substrate, from 14 to 20 h, suggesting that an unexpected discrepancy between optimal induction time and real catalytic efficiency occurred. To explore the causes for this discrepancy, we determined the kinetic behaviors of the dual enzyme system consisting of DKR and GDH to gain insightful information for the complex biocatalysis.

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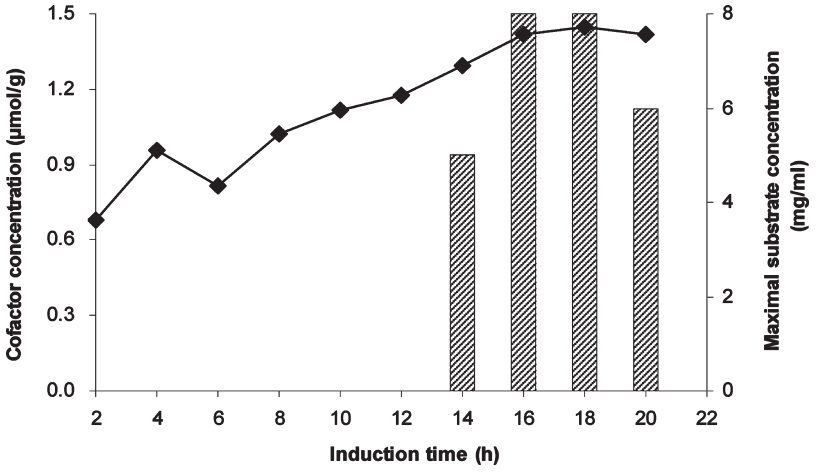

Figure 2. Intracellular cofactor concentrations in recombinant *E. coli* cells coexpressing DKR and GDH.
Closed diamond: total concentration of NAD^+, $NADP^+$, NADH, and NADPH; Column: maximal substrate concentration with complete conversion when substrate **1** was added at 14, 16, 18, and 20 h.

Given that GDH prefers to utilize NADP(H), initial velocities for the bioreduction of substrate **1** were measured with NADPH as the cofactor.[14] As a result, the double reciprocal plots of initial velocity against NADPH and substrate **1** in Supporting Information, Figure S4 and product inhibitions in Supporting Information, Figure S5 indicated that DKR catalyzes a sequential reaction, which fits into the kinetic model of an ordered bi bi mechanism.[15−17] Similarly, the kinetics of glucose oxidation catalyzed by GDH followed an ordered bi bi model according to a previous report.[14] Therefore, the same kinetic characteristics enabled us to synergistically analyze their kinetics during the enzymatic catalysis.

Kinetic equation S1 (Supporting Information, Eq. S1),[15−17] strongly indicated that the concentrations of intracellular cofactors in *E. coli* cells was the rate-limiting factor for the bioreduction of **1** by DKR, leading us to speculate that intracellular cofactor concentration is a direct cause for the discrepancy. To test this hypothesis, the content of reduced and oxidized forms of cofactors[10,13] in the cells were analyzed by RP-HPLC after extraction by reported methods.[18,19] As shown in Figure 2, the concentrations of the four cofactors gradually increased by the induction time, and reached the maximum (1.45 μmol/g) between 16 and 18 h and then decreased slightly after 18 h. This profile was highly consistent with the changes of maximal substrate concentrations with complete conversion, suggesting that, in addition to enzyme activity, cell mass and substrate concentration, the concentration of intracellular cofactors in recombinant *E. coli* cells indeed affects the completion of bioreduction and that maintainance of sufficient amounts of cofactors inside cells is critical to bioconversions. Even though a previous report indicated that product yield is closely related to enzyme activity,[9] our kinetic analysis enabled us to dynamically monitor and predict the progress of bioreductions. Monitoring of intracellular cofactor concentrations could be generally applicable as an approach to ensure the completion of nicotinamide-dependent bioreductions by whole-cell biocatalysts and to eliminate the addition of expensive nicotinamide cofactors.

To examine the strategy of monitoring intracellular cofactor concentrations during the biocatalytic process, we systematically optimized reaction conditions for the bioreduction of **1** by recombinant *E. coli* cells coexpressing DKR and GDH, using a reaction pH of 7.0 (Supporting Information, Figure S6), reaction temperature of 25 °C (Supporting Information, Figure S7a), glucose concentration of 160 mg/mL (28 equiv to substrate) (Supporting Information, Figure S7b) and reaction time of 6 h (Supporting Information, Figure S8). Compared to previous reactions by the recombinant *E. coli* cells expressing DKR alone,[11] the present system increased substrate concentration by 15-fold under the same conditions and completely eliminated the addition of exogenous factors. Thus, by taking the strategy of monitoring the dynamic change of intracellular cofactor concentrations, the efficiency of biocatalytic preparation of the chiral diol for statin side chains was greatly improved in the present study.

In conclusion, we have investigated the dynamic change of intracellular cofactor concentrations in recombinant *E. coli* cells by kinetic analysis and quantitative determination. The correlation between the cofactor concentration and catalytic efficiency was demonstrated by using *E. coli* cells coexpressing DKR and GDH as a whole-cell biocatalyst to prepare a valuable chiral diol. In addition to increased enzyme expression and activity in the recombinant *E. coli* cells, monitoring the dynamic change of intracellular cofactor concentrations has shown great potential to effectively improve the biocatalytic processes catalyzed by oxidoreductases.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details, series of representative tables and figures for detailed descriptions. This material is available free of charge via the Internet at http://pubs.acs.org.

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