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Simultaneous Biocatalyst Production and Baeyer-Villiger Oxidation for Bioconversion of Cyclohexanone by Recombinant *Escherichia coli* Expressing Cyclohexanone Monooxygenase

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Abstract

Cyclohexanone monooxygenase (CHMO) catalyzing Baeyer-Villiger oxidation converts cyclic ketones into optically pure lactones, which have been used as building blocks in organic synthesis. A recombinant *Escherichia coli* BL21(DE3)/pMM4 expressing CHMO originated from *Acinetobacter* sp. NCIB 9871 was used to produce ε-caprolactone through a simultaneous biocatalyst production and Baeyer-Villiger oxidation (SPO) process. A fed-batch process was designed to obtain high cell density for improving production of ε-caprolactone. The fed-batch SPO process gave the best results, 10.2 g/L of ε-caprolactone and 0.34 g/(L · h) of productivity, corresponding to a 10.5- and 3.4-fold enhancement compared with those of the batch SPO, respectively.

Index Entries: Simultaneous biocatalyst production and Baeyer-Villiger oxidation; cyclohexanone monooxygenase; *Escherichia coli;* fed-batch process; ε-caprolactone.

Introduction

The Baeyer-Villiger (BV) reaction is an oxidative transformation of an acyclic ketone to an ester or a cyclic ketone to a lactone *(1).* For more than 100 yr, stereo- and enantioselective BV oxidation has been a key synthetic operation in organic synthesis because products such as homochiral ε-caprolactones have been widely used as building blocks in organic and polymer syntheses *(2).* Particularly, ε-caprolactone is a com-

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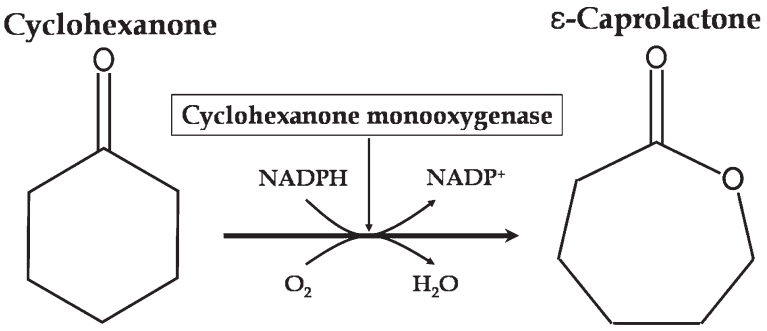

Fig. 1. BV oxidation of cyclohexanone by CHMO.

mercial intermediate useful for tailoring polyols and hydroxyfunctional polymer resins with its enhanced flexibility. Conventional chemical processes, using oxidants such as peroxyacid and transition metal catalyst, had low stereoselectivity, causing high recovery cost and environmental pollution *(3,4).* By contrast, biologic processes involving enzymatic BV oxidation are able to produce an optically active form owing to its regio- and stereoselectivity *(5).*

Cyclohexanone monooxygenase (CHMO) isolated from *Acinetobacter* sp. NCIB 9871 belongs to one group of BV monooxygenase. It was characterized as a 59-kDa monomeric FAD- and NADPH-dependent oxidoreductase *(6,7).* As a result of its several advantages, such as the ability to oxygenate a wide range of ketones, broad substrate tolerance, and high enantioselectivity, the use of CHMO in organic synthesis to produce chiral synthons has industrial interests *(5–8).* Because reducing powers have to be supplied to the biologic oxidation process with CHMO (Fig. 1), strategies of whole-cell biotransformation are generally attempted. Several expression systems for the production of soluble CHMO in recombinant *Escherichia coli* were constructed to come up with the best expression system. Inducible systems with *araBAD* and *T7* promoters in recombinant *E. coli* were employed to express CHMO stably up to 3.5 U/mL of activity and to produce ε-caprolactone from cyclohexanone at 0.79 g/(L · h) of productivity. In this sense, typical processes of biotransformation were designed to divide biocatalyst production with microbial BV oxidation *(9–17).* Separation of CHMO production from its reaction might lead to the complicated and high-cost steps for collecting the biocatalyst from culture broth, preparing bioconversion reactors, and cofactor recycling.

In the present study, a biotransformation process of simultaneous biocatalyst production and Baeyer-Villiger oxidation (SPO) was devised to synthesize ε-caprolactone from cyclohexanone by recombinant *E. coli* expressing CHMO in batch and fed-batch fermentations. In the SPO process, fermentation of microbial biocatalyst with CHMO activity was accompanied by bioconversion of cyclohexanone into ε-caprolactone in a single bioreactor.

Materials and Methods

Bacterial Strain and Plasmid

E. coli BL21(DE3) [F^-, *ompT*, r_B^-, m_B^-] was used as a host for expressing CHMO of *Acinetobacter* sp. NCIB 9871. Plasmid pMM4 containing the *chnB* gene encoding CHMO under the *T7* promoter was donated by Prof. Jon D. Stewart of the University of Florida *(17).*

Culture Conditions

Luria-Bertani (LB) medium (10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of sodium chloride) and defined medium containing 20 g/L of glucose *(18,19)* were used for cultivation of *E. coli* BL21(DE3)/pMM4. Batch fermentation was carried out in a 500-mL baffled flask containing 200 mL of LB medium in a shaking incubator (HK-S125C; Hankook Mechanics, Hwaseong, Korea) at 25 and 30°C and 250 rpm. After the optical density at 600 nm reached 1, different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) were added to the culture broth to determine an optimal concentration of IPTG for soluble expression of CHMO.

Simultaneous Biocatalyst Production and BV Oxidation

An SPO process was carried out in a 2.5-L benchtop fermentor (KoBiotech, Incheon, Korea) with a 1-L working volume. A batch type of SPO was carried out in LB medium at 25°C, 700 rpm, and 1 vvm. The acidity of the broth was controlled at pH 7.0 by the addition of 2 *N* HCl or 2 *N* NaOH. After 0.01 m*M* IPTG induction when the OD at 600 nm reached 1, pure cyclohexanone was added at 1 g/L or cyclohexanone solution (25 g/L aqueous solution) was fed continuously at a rate of 0.15 g/(L · h). A fed-batch type of SPO was performed in the defined medium at 25°C. To increase cell mass, a pH-stat feeding strategy was employed *(19).* After glucose was depleted and the pH rose to a value greater than its setpoint, feeding solution composed of 800 g/L of glucose and 20 g/L of $MgSO_4 \cdot 7H_2O$ was fed automatically into the bioreactor. The pH was adjusted to 6.8 by the addition of 28% ammonia water. Agitation speed was 1000 rpm and gradually increased to 1300 rpm to prevent the limitation of dissolved oxygen tension, and aeration rate was maintained at 1 vvm. At the late exponential phase, 1.5 mL of 0.1 *M* IPTG was added and pure cyclohexanone was fed stepwise to control its concentration under 1.5 or 3 g/L.

Dry Cell Mass, Glucose and Acetic Acid Concentrations

OD was measured with a spectrophotometer (UltroSpec 2000, Pharmacia Biotech, NJ) at 600 nm and multiplied with 9 predetermined conversion factor of 0.36. Glucose was analyzed with a glucose kit (Youngdong Pharmaceutical, Yongin, Korea). The concentration of acetic acid was

measured by high-performance liquid chromatography (Younglin, Seoul, Korea) equipped with an Aminex HPX-87H column (Bio-Rad, Richmond, CA) heated at 60°C. A mobile phase consisted of 5 m*M* H_2SO_4 solution. Detection was carried out with a reflective index detector (Knauer, Berlin, Germany).

CHMO Activity Assay and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

CHMO activity was determined with a modified method described in a previous article *(16).* Endogenous NADPH oxidation before substrate addition was measured at 30°C for 2 min, and then cyclohexanone (25 m*M*) was added to the assay mixture. The NADPH oxidation rate was monitored at 30°C for 10 min to estimate CHMO activity. Specific activity was defined as the amount of enzyme able to oxidize 1 μmol of NADPH/(min·g of cellular protein). Protein concentration was determined using a protein assay kit (Bio-Rad). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out according to the method described by Laemmli *(20).*

Assay of Cyclohexanone and e-Caprolactone

A sample was prepared by mixing 500 μL of fermentation broth with the same volume of ethyl acetate solution containing 1 g/L of methyl benzoate as internal standard. After vortexing and centrifuging, the organic phase was collected. Solvents were extracted twice using the same procedure. A sample of the organic phase was injected into an M600D gas chromatograph (Younglin) fitted with a 15-m SPB-5 column (Supelco, Bellfonte, PA). The temperature of the column was programmed as follows: 50°C for 2 min, from 50 to 215°C at 15°C/min, and 215°C for 5 min.

Results

Optimization of CHMO Expression Condition

It has been well known that overexpression of a heterologous gene in recombinant *E. coli* frequently leads to formation of inclusion bodies. Additionally, control of fermentation conditions would diminish protein aggregation. Culture conditions of IPTG concentrations of 0.01, 0.1, and 1.0 m*M* and temperatures at 25 and 30°C were applied to obtain soluble and biologically active CHMO in batch fermentation with LB medium. As shown in Fig. 2, 25°C and 0.01 m*M* IPTG were chosen as the best tested conditions for CHMO expression, at which maximum specific activity was 290 U/g of cellular protein. After 9 h of fermentation, proteins expressed by *E. coli* BL21(DE3)/pMM4 were analyzed with SDS-PAGE. Except for the case at 25°C and 0.01 m*M* IPTG, inclusion bodies were produced. Their amounts were about half those of total cellular proteins. The thickest band of soluble CHMO was observed at 25°C and 0.01 m*M* IPTG,

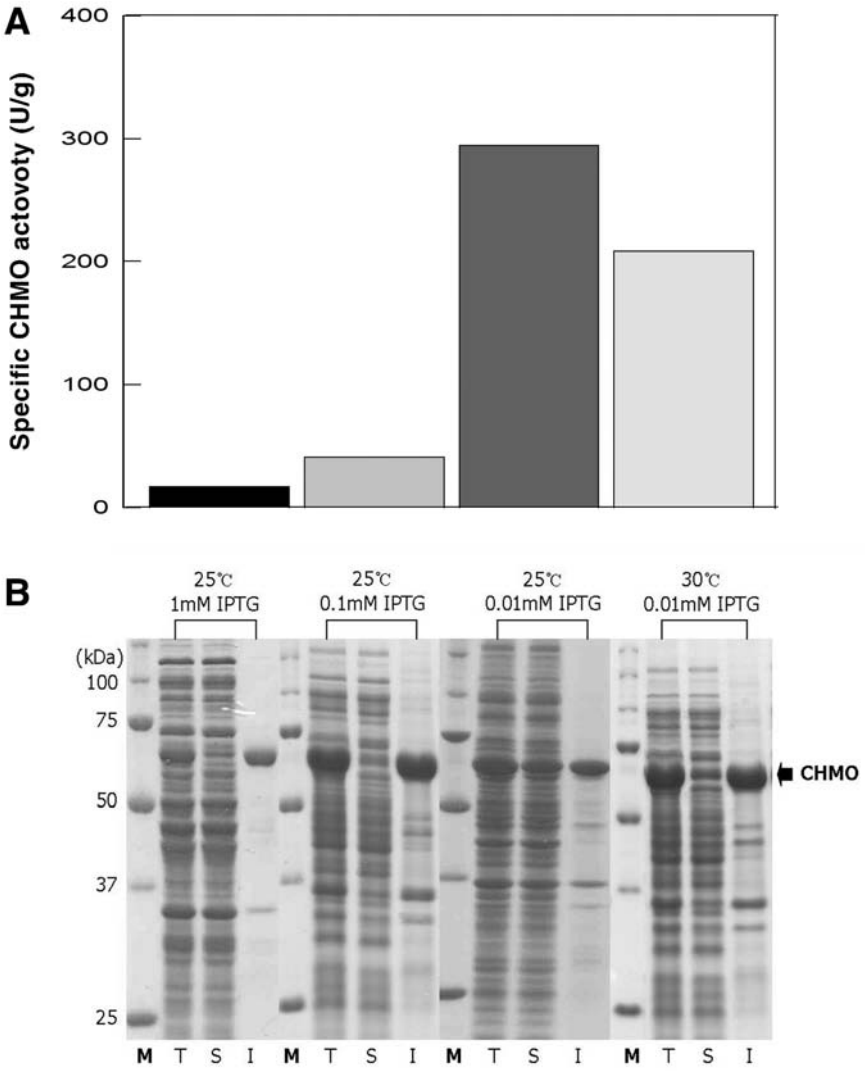

Fig. 2. Activity assay and SDS-PAGE analysis of CHMO expression in *E. coli* BL21(DE3)/pMM4 at various IPTG induction concentrations and temperatures. Samples for assay were collected after 6 h of induction in batch fermentation with LB medium. **(A)** Specific activity of CHMO; **(B)** SDS-PAGE. The arrow indicates CHMO. T, total cell lysate; S, soluble fraction; I, insoluble fraction; M, size marker.

and CHMO activity was maintained for about 9 h. However, inclusion bodies rapidly accumulated as cell growth entered the stationary phase (data not shown).

Batch Process of SPO

To operate simultaneously the growth of a recombinant *E. coli* with CHMO activity and BV oxidation by whole *E. coli* cells, IPTG induction for CHMO expression was accompanied by the addition of cyclohexanone in

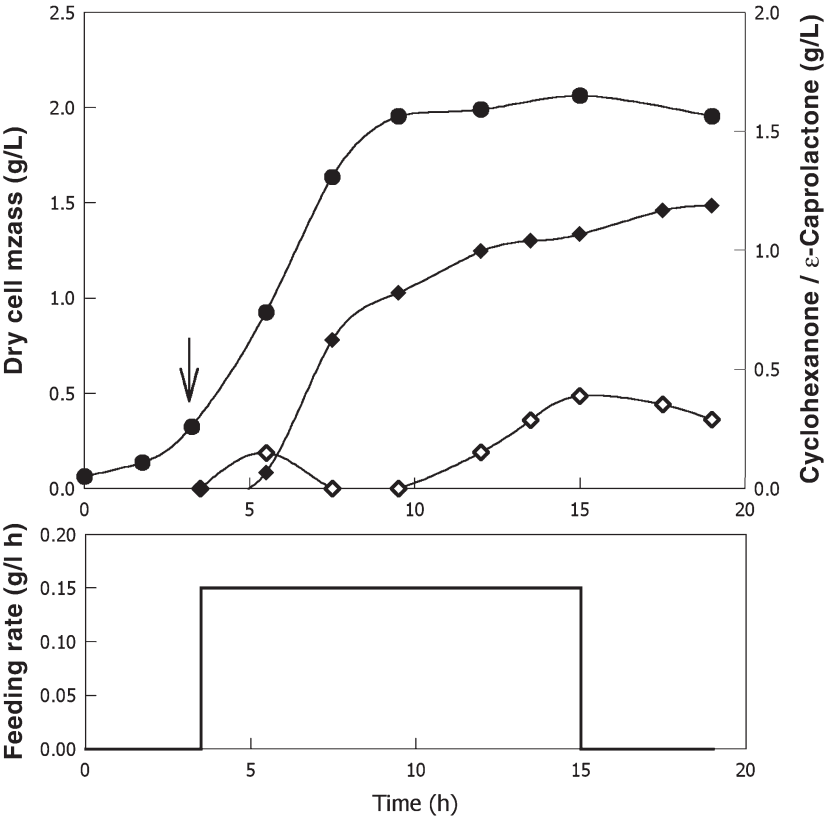

Fig. 3. Batch type of SPO process with continuous cyclohexanone feeding at rate of 0.15 g/(L · h): (●) dry cell mass; (◇) cyclohexanone concentration; (◆) ε-caprolactone concentration; (—) feed rate. The arrow indicates IPTG induction.

batch fermentation. Supplementation of 0.01 m*M* IPTG and 1 g/L of cyclohexanone resulted in 2.06 g/L of dry cell mass, 0.97 g/L of ε-caprolactone, and 0.10 g/(L · h) of productivity. To minimize an inhibitory effect of cyclohexanone and to improve the production of ε-caprolactone, a minimal feeding strategy of cyclohexanone was introduced. Figure 3 shows profiles of a batch type of SPO with continuous feeding of cyclohexanone at a rate of 0.15 g/(L · h). Cyclohexanone concentration was controlled under 0.4 g/L. BV oxidation rate decreased when cell growth entered the stationary phase, and 0.29 g/L of cyclohexanone remained unused. The SPO process with batch-type cell growth and continuous feeding of cyclohexanone resulted in 1.95 g/L of dry cell mass and 1.19 g/L of ε-caprolactone. These results showed that cyclohexanone oxidation was maintained during growth conditions. To improve the production of ε-caprolactone by keeping up the cyclohexanone oxidation rate, a fed-batch type of SPO using a high-cell-density culture technique was introduced.

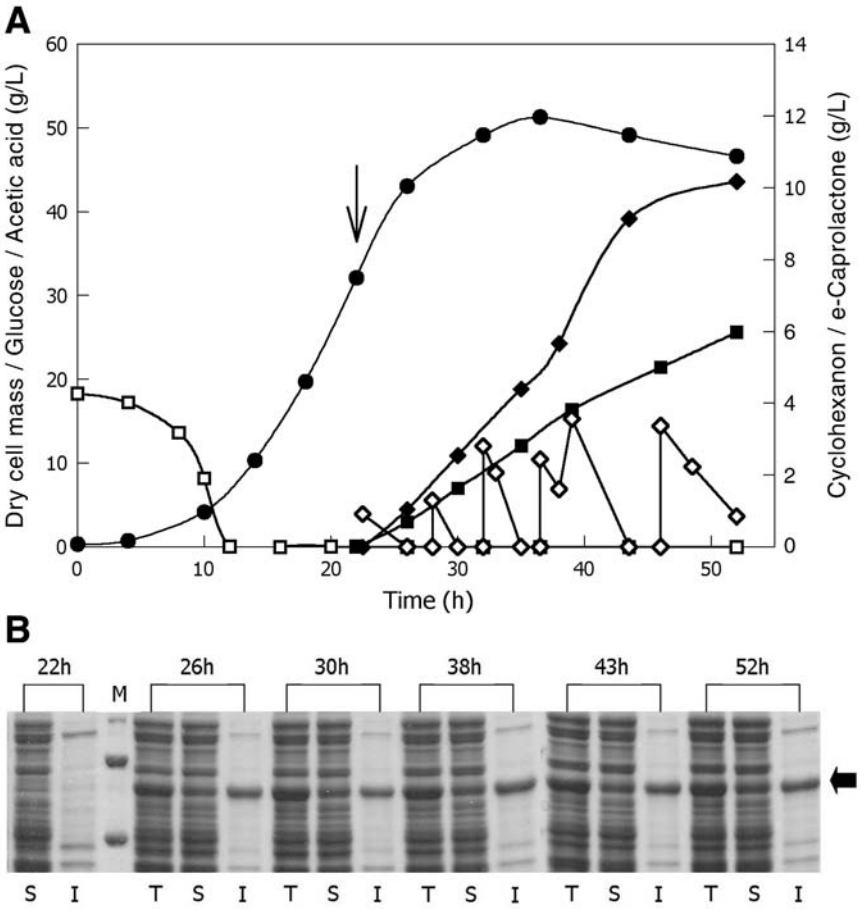

Fig. 4. Fed-batch type of SPO process. Cyclohexanone was fed stepwise and controlled under 3 g/L. **(A)** Profile of growth and cyclohexanone oxidation: (●) dry cell mass; (□) glucose concentration; (■) acetic acid concentration; (◇) cyclohexanone concentration; (◆) ε-caprolactone concentration. The arrow indicates IPTG induction. **(B)** SDS-PAGE analysis of CHMO expression patterns: T, total cell lysate; S, soluble fraction; I, insoluble fraction; M, size marker. The arrow indicates CHMO.

Fed-Batch Process of SPO

A fed-batch type of the SPO process was designed to obtain a high concentration of the recombinant *E. coli* cells with CHMO activity and to improve production of ε-caprolactone. Two fed-batch types of SPO controlling cyclohexanone concentration under 1.5 and 3 g/L were carried out in order to reduce toxic effects by cyclohexanone on cell growth. First, a feeding strategy of pH-stat led to the exponential growth of *E. coli*. After IPTG induction at the late exponential phase, pure cyclohexanone was added to a final concentration of 1 g/L for adaptation of the cells to the organic compound. After all of the initially added cyclohexanone was con-

sumed, cyclohexanone was fed stepwise to control the concentration under 1.5 g/L with this SPO process, 41.60 g/L of dry cell mass, 5.61 g/L of ε-caprolactone, and 0.12 g/(L · h) of productivity were obtained.

Another fed-batch type of SPO with controlling cyclohexanone under 3 g/L is shown in Fig. 4A. The strategy of cell growth, CHMO induction, and initial addition of cyclohexanone were the same as for the previous fed-batch SPO. All of the cyclohexanone initially added was consumed within 4 h. Additional feeding of cyclohexanone and supplementation of glucose maintained the BV oxidation reaction for 30 h. With this SPO process, 10.17 g/L of ε-caprolactone and 0.34 g/(L · h) of overall volumetric productivity were obtained. After induction at the late exponential phase of 22 h, CHMO was expressed in soluble form and maintained throughout the fed-batch fermentation (Fig. 4B).

Discussion

Batch and fed-batch types of the SPO process successfully produced active CHMO and synthesized ε-caprolactone from cyclohexanone. The active form of CHMO was obtained by induction at 0.01 m*M* IPTG and 25°C. This indicated that control of the expression rate probably gave a proper condition for correct folding of CHMO. The formation of CHMO inclusion bodies at high temperature and IPTG concentration was probably owing to the fact that a high protein synthesis rate driven by the strong *T7* promoter rendered the nascent CHMO polypeptides trapped in an incorrectly folded state *(16). E. coli* strains are highly sensitive to organic solvents. It has been believed that the organic solvents break down microbial membranes, leading to cell death *(21,22).* In batch SPO processes, a minimal feeding strategy of cyclohexanone, which was introduced to avoid a metabolic burden imposed on cell growth by cyclohexanone, had similar results compared with the initial addition strategy. As shown in Fig. 3, BV oxidation rate decreased and, hence, cyclohexanone accumulated after the exponential phase. This was probably owing to the fact that depletion of an energy source at the stationary phase limited the supply of NADPH into the BV oxidation reaction.

To overcome insufficient supplementation of NADPH and to increase ε-caprolactone productivity, the SPO process with a high-cell-density culture technique was attempted. At high-cell-density conditions, the oxygen uptake rate was maximized. This state allowed CHMO to be expressed in soluble form, as done by Doig et al. *(10).* SDS-PAGE analysis, shown in Fig. 4B, confirmed the soluble expression of CHMO throughout the fed-batch operation. Even though intrinsic solvent tolerance of *E. coli* was not altered in the fed-batch SPO, high concentrations of the *E. coli* cells expressing CHMO permitted the oxidation reaction at high cyclohexanone and ε-caprolactone concentrations. A similar result showed that high concentra-

Table 1
Summarized Results of ε-Caprolactone Production in Batch and Fed-Batch Types of SPO Process

Type of SPO	Dry cell mass(g/L)	ε-Caprolactone concentration (g/L)	ε-Caprolactone productivity (g/[L · h])[a]	Conversion yield (m*M*/m*M*)
Batch				
Initial addition	2.06	0.97	0.10	0.90
Continuous feeding	1.95	1.19	0.08	0.71
Fed batch				
Controlling under 1.5 g/L	41.60	5.61	0.12	0.67
Controlling under 3 g/L	46.62	10.17	0.34	0.73
Typical biotransformation[b]	7.83	7.90	0.79	0.88

[a]Productivity was calculated at the stage of ε-caprolactone production.
[b]These values were obtained from ref. *15.*

tions of a biocatalyst also improved the oxidation rate of bicyclo[3.2.0]hept-2-en-6-one and final product concentration *(9).* The process parameters for ε-caprolactone production are summarized in Table 1. Two batch types with initial dumping and continuous feeding of cyclohexanone gave similar results in ε-caprolactone production. A high density of the recombinant *E. coli* with CHMO activity was obtained by the fed-batch SPO process and led to a significant enhancement in ε-caprolactone synthesis. Final ε-caprolactone concentration and productivity in the fed-batch SPO controlling cyclohexanone concentration under 3 g/L were 10.5 and 3.4 times higher than those of the batch SPO. The considerable improvement in BV oxidation of the fed-batch SPO process might be attributed to the sufficient supply of NADPH and oxygen by glucose feeding and aeration.

To assess the performance of the SPO process, the key experimental results were compared with those reported elsewhere *(15).* As shown in Table 1, the final concentration of ε-caprolactone of the fed-batch SPO corresponded to a 1.3-fold increase relative to the previous biotransformation, but the productivity of the SPO process needs to be improved. The SPO process could be implemented further by enforcing key metabolic pathways such as cofactor regeneration.

Acknowledgments

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