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Enhanced Production of ε-Caprolactone by Coexpression of Bacterial Hemoglobin Gene in Recombinant *Escherichia coli* Expressing Cyclohexanone Monooxygenase Gene

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Baeyer-Villiger (BV) oxidation of cyclohexanone to ε-caprolactone in a microbial system expressing cyclohexanone monooxygenase (CHMO) can be influenced by not only the efficient regeneration of NADPH but also a sufficient supply of oxygen. In this study, the bacterial hemoglobin gene from *Vitreoscilla stercoraria* (*vhb*) was introduced into the recombinant *Escherichia coli* expressing CHMO to investigate the effects of an oxygen-carrying protein on microbial BV oxidation of cyclohexanone. Coexpression of Vhb allowed the recombinant *E. coli* strain to produce a maximum ε-caprolactone concentration of 15.7 g/l in a fed-batch BV oxidation of cyclohexanone, which corresponded to a 43% improvement compared with the control strain expressing CHMO only under the same conditions.

Keywords: Cyclohexanone monooxygenase, ε-caprolactone, bacterial hemoglobin, *Escherichia coli*

Compared with traditional chemical processes, enzymatic biotransformations offer milder and safer conditions and have been successfully employed in the production of many valuable chemicals [4, 5]. However, one of the obstacles in the broader use of enzymatic biotransformations is the requirement of cofactors such as FADH and NAD(P)H by cofactor-dependent enzymes, which necessitate recycling of these cofactors for continuous production of the target molecules [5, 28]. To circumvent this problem, whole-cell-based biotransformations have been considered as more feasible processes, since the cofactors can be replenished by intracellular carbohydrate or amino acid metabolism [5, 22].

Baeyer-Villiger (BV) oxidation, an oxidation reaction of cyclic ketones to corresponding lactones, has received considerable attention, since it can be useful for synthesizing value-added fine chemicals [12, 21, 26]. However, traditional chemical BV oxidation works with unstable and toxic reactants under harsh conditions, and generally tends to lack stereospecificity [12, 21]. The identification of various BV monooxygenases (BVMOs) and their application in the production of optically pure lactones have been recently reported [12, 21, 26]. Among them, cyclohexanone monooxygenase (CHMO; E.C. 1.14.13.22) from *Acinetobacter calcoaceticus* NCIMB 9871 is the best-characterized BVMO that converts cyclohexanone to ε-caprolactone [12, 25]. Since CHMO can convert a broad range of non-chiral substrates to chiral products, numerous attempts to use CHMO for the production of a variety of fine chemicals have been reported [10, 12, 21]. However, CHMO requires NADPH as a cofactor, which in the absence of an efficient recycling system cannot be regenerated from NADP, thus effectively stopping the reaction [25, 26].

In order to increase the productivity of biological BV oxidation, microbial systems expressing CHMO have been developed and the oxidation conditions were optimized, which includes active CHMO expression, employment of most suitable hosts, and additional expression of NADPH regenerating enzymes [3, 19, 20, 25, 26]. In particular, a number of metabolic engineering approaches have been focused on increasing the intracellular availability of NADPH in order to improve the overall productivity of microbial BV oxidation [3, 19, 20, 26]. However, there is another potentially limiting factor for microbial BV oxidation, the supplementation of oxygen, because CHMO requires oxygen

as a substrate for oxidation [10]. In addition, organic solvents such as cyclohexanone and ε-caprolactone are known to be accumulated in the cell membrane, potentially causing a loss of membrane function and limiting the transfer of substrates (cyclohexanone and oxygen) into the cell, thus reducing the productivity of microbial BV oxidation [10, 11, 20, 22]. Consequently, supplementation of the oxygen-carrying machinery is necessary to sustain the intracellular BV oxidation.

Escherichia coli is known to have a native flavohemoglobin (flavoHb) encoded by the *hmp* gene [8, 9]. However, the primary function of flavoHb is the conversion of nitric oxide (NO) to nitrate, protecting the cell against NO-induced oxidative stress, even though flavoHb was reported to be involved in oxygen sensing [7–9]. On the other hand, the hemoglobin from *Vitreoscilla* (Vhb) is a well-known bacterial oxygen carrier capable of binding oxygen and delivering it to the respiratory apparatus, which allows obligate aerobic *Vitreoscilla* to survive under the oxygen-limited conditions [13, 16, 23]. Expression of Vhb in heterologous hosts such as *E. coli* and *Bacillus subtilis* enhanced the microbial growth and formation of target product through the stimulation of oxygen-dependent metabolism under oxygen-limited conditions [1, 14, 15, 18, 24]. Expression of Vhb in various eukaryotic systems was also effective in improving the growth and viability of the cells [6, 27]. In addition, Vhb expressed in *E. coli* was predominantly located in the cytoplasmic area adjacent to the cell membrane, which would allow it to deliver the oxygen to the intracellular oxygen-dependent apparatus immediately after trapping it from the extracellular environment [17, 23]. These observations suggested that an improvement of the BV oxidation in the recombinant *E. coli* overexpressing CHMO might be achieved by simultaneous overexpression of Vhb. Hence, this study examined the feasibility of improving the ε-caprolactone production by introducing Vhb into CHMO-expressing recombinant *E. coli*.

To express CHMO in *E. coli*, plasmid pMM4 [12] carrying the *chnB* gene encoding CHMO from *A. calcoaceticus* NCIMB 9871 was used. Plasmid pMM4, a derivative of pET-22b(+) (Merck Biosciences, Darmstadt, Germany), was kindly donated by Professor Jon Stewart (University of Florida, USA). The structural *vhb* gene was obtained by PCR using primers vhb-F (5′-AATTCATATGTTATTCAACCGCTTG AGCGT-3′) and vhb-R (5′-AGGCGAATTCCTTTAATA AGGAGATATGCGTGTTA-3′) from plasmid pBARVhb that was kindly donated by Professor Gie-Taek Chun (Kangwon National University, Republic of Korea). The amplified *vhb* gene fragment was digested with *Eco*RI and *Nde*I and then inserted into the pACYCDuet-1 plasmid (p15A replicon, Cm^r; Merck Biosciences) to construct pMJ001.

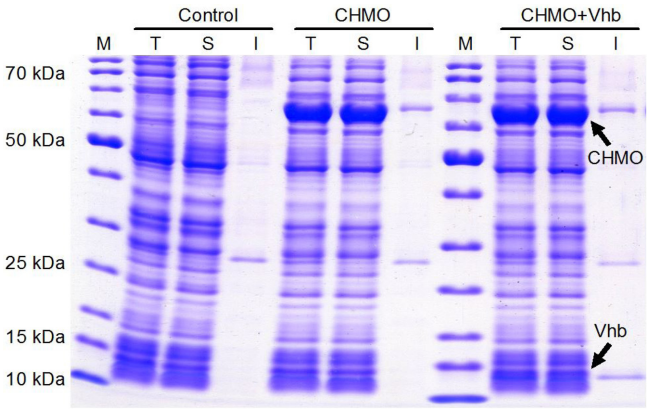

Fig. 1. SDS-PAGE analysis of the crude cell extract of recombinant *E. coli* BL21(DE3) strains harboring pET-22b(+) + pACYCDuet-1 (control), pMM4 + pACYCDuet-1 (CHMO), and pMM4 + pMJ001 (CHMO+Vhb).
Cells were harvested after 4 h of 0.1 mM IPTG induction (LB medium, 25°C). T, S, and I denote total, soluble, and insoluble protein fractions, respectively. The arrows indicate the corresponding protein bands with the estimated molecular mass of CHMO and Vhb. Lane M indicates size markers.

The expression pattern of Vhb was investigated in a batch culture of recombinant *E. coli* BL21 (DE3) (Invitrogen, USA) harboring plasmids pMM4 and pMJ001. Previously established optimum conditions for maximizing CHMO expression (induction with 0.01 mM IPTG in Luria-Bertani (LB) medium at 25°C for 4 h; [20]) were used to express both Vhb and CHMO expression. To assess the expression of Vhb and CHMO, crude *E. coli* extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 1, the expression of Vhb (estimated molecular mass of ~14 kDa) was observed along with strong expression of CHMO (~61 kDa). Although a prominent band corresponding to Vhb was observed in the insoluble fraction, the band of active Vhb was also observed in the soluble fraction. The CHMO activity of the CHMO-expressing strain (359 ± 66 U/g cellular protein) was similar to that of the CHMO/Vhb-coexpressing strain (303 ± 49 U/g cellular protein), indicating that CHMO expression was not significantly affected by its coexpression with Vhb.

To check whether Vhb expression would change the profiles of microbial BV oxidation activity at various substrate concentrations, the whole-cell CHMO activity assay was performed with the CHMO/Vhb-coexpressing strain and various concentrations of cyclohexanone, according to the

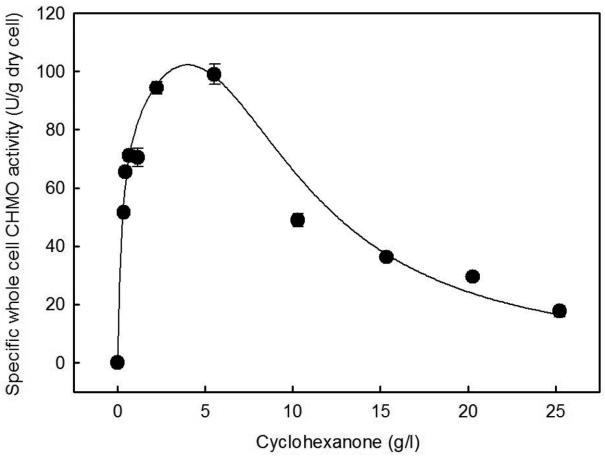

Fig. 2. Effects of cyclohexanone concentration on whole-cell CHMO activity of the recombinant *E. coli* BL21(DE3) expressing CHMO and Vhb.

method suggested in our previous study [20]. As shown in Fig. 2, the maximum whole-cell CHMO activity of 99.0 ± 3.5 U/g dry cells was achieved at a cyclohexanone concentration of 6 g/l in the reaction buffer (50 mM sodium–potassium phosphate, 5 g/l glucose, pH 7.0); this activity was slightly higher than that of a CHMO-expressing strain in our previous study (90 U/g dry cells) [20], possibly due to the expression of Vhb. However, the whole-cell CHMO activity was significantly reduced at excessive cyclohexanone concentrations (>10 g/l), in line with our previous report for the CHMO-expressing strain [20].

The effect of Vhb coexpression on BV oxidation of cyclohexanone was evaluated further in a fed-batch experiment using the CHMO/Vhb-coexpressing strain under the same culture conditions as in our previous report [20] and a cyclohexanone concentration of <6 g/l. Fig. 3A shows the profiles of ε-caprolactone production and intracellular CHMO activity of the CHMO/Vhb-coexpressing strain. After BV oxidation was initiated at 22.5 h of fed-batch culture, a continuous increase in ε-caprolactone concentration was observed. Despite a reduction in cell growth observed when the ε-caprolactone concentration reached 7 g/l, product formation was still maintained even at ε-caprolactone concentrations of >11 g/l, which is in contrast to the results for the control CHMO-expressing strain [20]. With Vhb coexpression, 15.7 g/l of ε-caprolactone was produced at the end of oxidation; that is, a 43% increase in comparison with the control strain expressing CHMO alone [20].

In our previous study, concentrations of ε-caprolactone higher than 11 g/l strongly suppressed the production of soluble CHMO, causing an accumulation of inactive CHMO

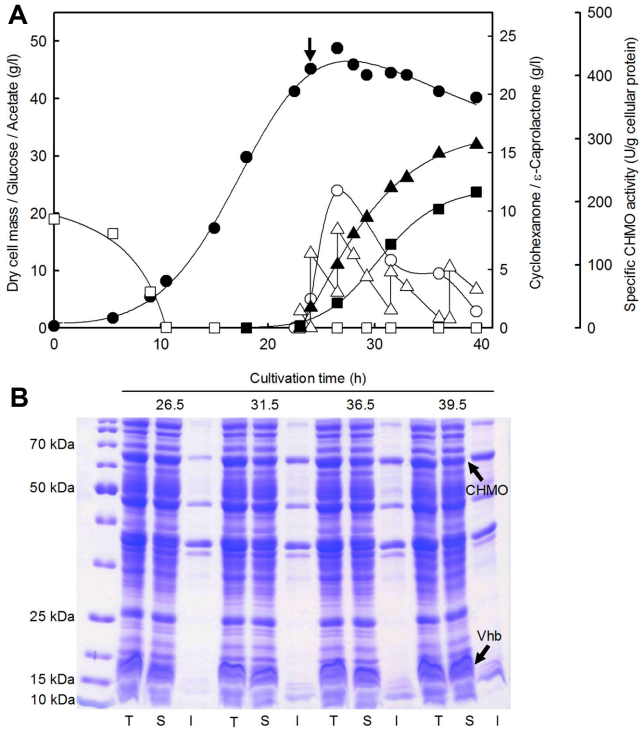

Fig. 3. Profiles of ε-caprolactone production and intracellular CHMO activity (**A**) and CHMO and Vhb expression pattern (**B**) in fed-batch BV oxidation of cyclohexanone in the recombinant *E. coli* BL21(DE3) expressing CHMO and Vhb.
Symbols denote as follows: dry cell mass (closed circle); specific CHMO activity (open circle); glucose (open square); acetate (closed square); cyclohexanone (open triangle); and ε-caprolactone (closed triangle). The arrow in panel A indicates the addition of 0.15 mM IPTG. In panel B, lane M indicates size markers. T, S, and I denote total, soluble and insoluble proteins fraction, respectively. The arrows indicate the corresponding protein bands with the estimated molecular mass of CHMO and Vhb.

in inclusion bodies [20]. To test whether the increased ε-caprolactone production by the CHMO/Vhb-coexpressing strain was due to changes in protein expression, the profiles of intracellular CHMO activity were assessed. The profiles of CHMO and Vhb expression were also analyzed by SDS-PAGE. Interestingly, the profile of CHMO expression observed in fed-batch BV oxidation was similar for the CHMO/Vhb-coexpressing strain and for the CHMO-expressing strain in our previous study [20]. After ε-caprolactone reached 12 g/l (at 31.5 h of culture), a significant reduction in intracellular CHMO activity was observed (from 218 ± 7.9 U/g cellular protein at 26.5 h to 107 ± 7.8 U/g cellular protein at 31.5 h; *i.e.*, a reduction of ~50%; Fig. 3A). A considerable amount of CHMO was insoluble, along with an increase in insoluble Vhb (Fig. 3B).

Table 1. Summary of ε-caprolactone production in fed-batch cultivation of recombinant *E. coli*.

Plasmids	Dry cell mass (g/l)	ε-Caprolactone concentration (g/l)	ε-Caprolactone productivity[a] (g/l·h)	Acetic acid concentration (g/l)
pMM4[b]	38.7	11.0	0.82	29.7
pMM4 + pMWzwf[b]	42.0	15.3	0.94	12.4
pMM4 + pMJ001	40.2	15.7	0.95	23.7

[a]Productivity was estimated during the ε-caprolactone production period after IPTG induction.

[b]The results from fed-batch BV oxidation of the control strain (recombinant *E. coli* BL21(DE3) overexpressing CHMO only) and the G6PDH-overexpressing strain (recombinant *E. coli* BL21(DE3) overexpressing CHMO and G6PDH) were cited from our previous study [20].

The enhanced production of ε-caprolactone (up to 15.7 g/l) is possibly due to the more efficient oxygen supply because of Vhb expression and not due to an increase in CHMO stability. The results of fed-batch BV oxidation are summarized in Table 1.

Based on the results of fed-batch BV oxidation, we propose that microbial BV oxidation can be developed without engineering a metabolic pathway for NADPH regeneration, because Vhb overexpression and the overexpression of native glucose-6-phosphate dehydrogenase (G6PDH), an NADPH regenerator [20], similarly increased the ε-caprolactone production. Acetate formation by CHMO/Vhb-coexpressing *E. coli* was rather higher than that by *E. coli* expressing G6PDH: whereas G6PDH overexpression led to a 60% reduction in acetate formation (12.4 g/l) in comparison with the control (29.7 g/l) [20], Vhb overexpression resulted in only a 20% reduction (to 23.7 g/l). These results indicate that acetate formation is strongly and inversely related to the regeneration of NADPH. Acetate formation is related to the intracellular redox balance of pyridine nucleotides, including NADH and NADPH [2, 19]. No substantial enhancement of cell growth by coexpression of CHMO and Vhb was observed in the current study. Whereas G6PDH overexpression resulted in a 9% increase in the final cell concentration in comparison with the control culture [20], the overexpression of Vhb resulted in only a 4% increase (Table 1). The enhancement of cell growth at high concentrations of organic solvents would likely require the overexpression of enzymes involved in detoxification and homeostasis, as opposed to the overexpression of a protein involved in oxygen delivery. In addition, the production of soluble CHMO was not sustained by Vhb, because considerable amounts of both CHMO and Vhb became insoluble when the ε-caprolactone concentration exceeded 12 g/l. The overexpression of Vhb also did not significantly affect the expression of CHMO; however, it may have increased the intracellular oxygen levels and thus led to an increase in ε-caprolactone production. Despite the enhanced ε-caprolactone production, CHMO stability and cell growth were not improved by Vhb expression, suggesting that other strategies to improve the production of soluble recombinant proteins and to tolerate organic solvents should be considered in order to develop a more resilient *E. coli* strain for microbial BV oxidation.

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