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Effects of NADH kinase on NADPH-dependent biotransformation processes in *Escherichia coli*

Won-Heong Lee · Jin-Woo Kim · Eun-Hee Park · Nam Soo Han · Myoung-Dong Kim · Jin-Ho Seo

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Abstract Sufficient supply of NADPH is one of the most important factors affecting the productivity of biotransformation processes. In this study, construction of an efficient NADPH-regenerating system was attempted using direct phosphorylation of NADH by NADH kinase (Pos5p) from *Saccharomyces cerevisiae* for producing guanosine diphosphate (GDP)-L-fucose and ε-caprolactone in recombinant *Escherichia coli*. Expression of Pos5p in a fed-batch culture of recombinant *E. coli* producing GDP-L-fucose resulted in a maximum GDP-L-fucose concentration of 291.5 mg/l, which corresponded to a 51 % enhancement compared with the control strain. In a fed-batch Baeyer–Villiger (BV) oxidation of cyclohexanone using recombinant *E. coli* expressing Pos5p, a maximum ε-caprolactone concentration of 21.6 g/l was obtained, which corresponded to a 96 % enhancement compared with the control strain. Such an increase might be due to the enhanced availability of NADPH in recombinant *E. coli* expressing Pos5p. These results suggested that efficient regeneration of NADPH was possible by functional expression of Pos5p in recombinant *E. coli*, which can be applied to other NADPH-dependent biotransformation processes in *E. coli*.

W.-H. Lee · J.-W. Kim · J.-H. Seo (✉)
Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea
e-mail: jhseo94@snu.ac.kr

E.-H. Park · M.-D. Kim (✉)
Department of Food Science and Biotechnology, Kangwon National University, Chuncheon 200-701, Korea
e-mail: mdkim@kangwon.ac.kr

N. S. Han
Department of Food Science and Technology, Chungbuk National University, Cheongju 361-763, Korea

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Introduction

Biotransformation processes have grown increasingly as an alternative way to traditional chemical processes for production of a variety of nutraceuticals and fine chemicals because it has big potentials as a more selective, safer, and cleaner process (Drepper et al. 2006; Duetz et al. 2001). Especially, whole-cell based biotransformation can be more feasible than enzyme-based biotransformation since most of biological processes are composed of complex metabolic pathways, which include cofactor dependent reactions such as NADPH (Duetz et al. 2001; Zhao and van der Donk 2003). In addition, the use of whole cells can be more economical and stable than the use of purified enzymes in terms of cost-saving and biocatalyst stability (Duetz et al. 2001; Park 2007). However, the whole-cell based system has problems with limitation of productivity caused by depletion of cofactors and reduction of their regeneration. Therefore, it is essential to use appropriate cofactor regeneration systems for improving the productivity of target compounds (Duetz et al. 2001; Park 2007; Zhao and van der Donk 2003).

NADPH is an important reducing agent for biosynthesis of cellular components and defense systems against various oxidative stresses (Sauer et al. 2004). It is known that NADPH is involved in more than 100 reactions inside the cell and regenerated through several reactions in the carbon and amino acid metabolisms. Especially in *Escherichia coli*, the oxidative pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA), and transhydrogenase systems are known to be major sources of NADPH regeneration. It was reported that PPP, TCA, and transhydrogenation contributed

35–45 %, 20–25 %, and 30–45 % of NADPH which is required for biosynthesis during aerobic cell growth, respectively (Sauer et al. 2004).

Hence, several enzymes involved in the above pathways have been frequently used as a NADPH regenerator for NADPH-dependent biotransformation processes. Overexpression of glucose-6-phosphate dehydrogenase (G6PDH) in PPP could have been a representative example for NADPH regeneration in biotransformation processes (Kwon et al. 2006; Lim et al. 2002). But one carbon molecule should be removed by decarboxylation of hexose sugars in the oxidative branch of PPP, which may reduce product yield (Lee et al. 2009a; Lee et al. 2010). Even though overexpression of pyridine nucleotide transhydrogenase (UdhA and PntAB) can be another way for NADPH regeneration (Kabus et al. 2007; Sanchez et al. 2006), overexpression of those enzymes has sometimes shown an undesirable reverse reaction such as transfer of hydrogen from NADPH to NAD^+ instead of from NADH to $NADP^+$, due to the fact that those enzymes are involved in maintaining the chemical equilibrium between pyridine nucleotides pool (Anderlund et al. 1999; Nissen et al. 2001). Generally, an excess amount of NADH is generated by glycolysis when the cell is growing on glucose, suggesting that direct conversion of NADH to NADPH could increase the intracellular NADPH availability and thereby improve the productivity in biotransformation processes. However, *E. coli* does not have a native enzyme catalyzing direct conversion of NADH to NADPH. It has only NAD kinase that is known to convert NAD^+ to $NADP^+$ (Kawai et al. 2001), which suggests that a new enzyme should be considered for direct phosphorylation of NADH in biotransformation processes using *E. coli*.

We have attempted to improve the productivity of NADPH-dependent biotransformation processes using recombinant *E. coli* systems able to produce GDP-L-fucose and ε-caprolactone as model systems. GDP-L-fucose is an essential nucleotide sugar for production of functional carbohydrates with biological activities (Han et al. 2012). Baeyer–Villiger (BV) oxidation of cyclohexanone to ε-caprolactone catalyzed by cyclohexanone monooxygenase (CHMO, EC 1.14.13.22) is known to be very useful for synthesizing value-added fine chemicals (Leisch et al. 2011). As illustrated in Fig. 1a, b, both GDP-L-fucose biosynthesis and production of ε-caprolactone from cyclohexanone require NADPH as a cofactor (Han et al. 2012; Leisch et al. 2011). Previously, a considerable improvement in the productivity of above the biotransformation processes was achieved through additional overexpression of endogenous NADPH-regenerating G6PDH in *E. coli* by 21 % for GDP-L-fucose and 39 % for ε-caprolactone, compared with the cases without G6PDH overexpression (Lee et al. 2011; Lee et al. 2007).

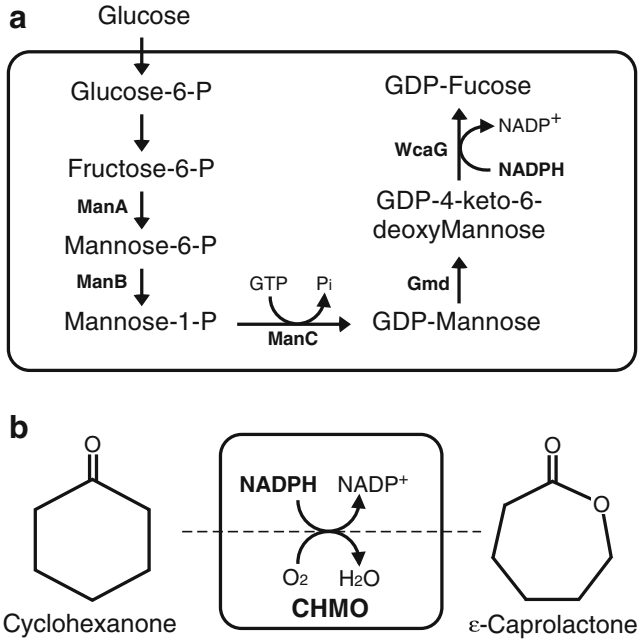

Fig. 1 The metabolic pathway for GDP-L-fucose biosynthesis in recombinant *E. coli* (**a**) and Baeyer–Villiger (BV) oxidation of cyclohexanone by recombinant *E. coli* overexpressing cyclohexanone monooxygenase (**b**). The names of enzymes are abbreviated as follows; ManA, mannose 6-phosphate isomerase; ManB, phosphomannomutase; ManC, mannose 1-phosphate guanylyltransferase; Gmd, GDP-D-mannose-4,6-dehydratase; WcaG, GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase; CHMO, cyclohexanone monooxygenase. Pi and GTP denote phosphate and guanosine 5′-triphosphate

In this study, the possibility for improving the productivity of GDP-L-fucose and ε-caprolactone production was explored by expression of NADH kinase (Pos5p, EC 2.7.1.86) catalyzing direct conversion of NADH to NADPH. Since Pos5p is known to be the main source for mitochondrial NADPH regeneration in *Saccharomyces cerevisiae* (Outten and Culotta 2003; Strand et al. 2003), the effects of Pos5p expression on GDP-L-fucose production and ε-caprolactone production in recombinant *E. coli* was assessed in a series of fed-batch fermentations.

Materials and methods

Strains and plasmids

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for genetic manipulation. *E. coli* BL21star(DE3) and BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) were used as host for GDP-L-fucose biosynthesis and BV oxidation of cyclohexanone, respectively. Plasmid pmBCGW (pBR322 replicon, IPTG inducible, Amp^r) was previously constructed for

overexpression of GDP-L-fucose biosynthetic enzymes including ManB, ManC, Gmd, and WcaG (Lee et al. 2009b). Plasmid pMM4, which harbors the *chnB* gene encoding CHMO from *A. calcoaceticus* NCIMB 9871 (Walton and Stewart 2004), was kindly donated by Professor Jon Stewart (University of Florida, FL, USA). The *POS5* gene was obtained by PCR using the genomic DNA of the *S. cerevisiae* CEN.PK2-1D strain as template. PCR primers POS5_F (5′-GGAATTCCATATGAGTACGTTGGATTCACATTCC-3′) and POS5_R (5′-CCGCTCGAGTTAATCATTAT CAGTCTGTCT-3′) were used for amplifying the *POS5* gene. To obtain the *POS5* gene without the mitochondrial targeting sequence (Strand et al. 2003), a PCR primer was designed to exclude the coding sequence for the first 17 amino acid residues. The amplified *POS5* gene fragment was cloned into plasmid ACYCDuet-1 (p15A replicon, IPTG inducible, Cm^r, Merck Biosciences, Darmstadt, Germany) after digestion with *Nde*I and *Xho*I. The resulting plasmid was named as pHpos5 and confirmed by DNA sequencing.

Culture conditions

Luria–Bertain (LB) medium (0.5 % (*w*/*v*) yeast extract, 1 % (*w*/*v*) tryptone, and 1 % (*w*/*v*) NaCl) with appropriate concentrations of antibiotics was used for plasmid preparation and seed cultivation.

For GDP-L-fucose biosynthesis, fed-batch fermentation was performed in a 2.5-l bioreactor containing 1.0 l defined medium at 25 °C (Lee et al. 2011). After complete depletion of 2 % (*w*/*v*) glucose added initially, a concentrated solution containing 800 g/l glucose and 20 g/l $MgSO_4 \cdot 7H_2O$ was fed by the pH-stat feeding mode. When dry cell mass reached approximately 35 g/l, 0.1 mM IPTG was added to induce the expression of the GDP-L-fucose biosynthetic enzymes. Agitation speed was increased to a maximum value of 1,400 rpm in order to prevent the limitation of dissolved oxygen, and airflow rate was maintained at 1 vvm throughout the cultivation. The pH was controlled at 6.8 by 28 % NH_4OH.

For oxidation of cyclohexanone to ε-caprolactone, a fed-batch mode of BV oxidation was done. Culture conditions for the fed-batch BV oxidation were almost the same as the fed-batch fermentation for GDP-L-fucose production (Lee et al. 2011; Lee et al. 2007). When dry cell mass reached approximately 35 g/l, CHMO expression was induced by addition of 0.15 mM IPTG. Subsequently, cyclohexanone was added at a final concentration of 1.5 g/l for adaptation of the *E. coli* cell to cyclohexanone. After the initially added cyclohexanone was exhausted, cyclohexanone was intermittently fed to maintain its concentration at 6 g/l.

Determination of intracellular pyridine nucleotide concentrations

To measure intracellular concentrations of pyridine nucleotides, a modified whole cell assay was used (Lee et al. 2011). Cells were grown to 0.35 g/l in a LB medium at 25 °C and expression of Pos5p was induced by 0.1 mM IPTG. After 4.5 h, the cells were harvested by centrifugation and resuspended at a concentration of 3.5 g/l in the defined medium containing 5 g/l glucose. After 1 h of cultivation at 30 °C, 0.6 g/l of cells were harvested and used for determination of intracellular NADH and NADPH concentrations. All whole cell assays were performed in a 500-ml baffled flask with a 50-ml reaction volume. An agitation speed of 250 rpm was maintained. All experiments were performed in triplicates.

Analytical methods

Dry cell mass was determined by measuring optical density at 600 nm (Ultrospec 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a predetermined calibration curve.

Concentrations of glucose and acetic acid were determined using a M930 high performance liquid chromatography (HPLC) system (Younglin, Seoul, Korea) equipped with an Aminex HPX-87 H cation exchange column (Bio-Rad, Richmond, CA, USA) and a RI detector (Knauer, Germany). The intracellular concentration of GDP-L-fucose was determined using a HPLC system equipped with a CAPCELL PAK C18 MG column (250×4.6 mm, Shiseido, Tokyo, Japan) according to the method described previously (Lee et al. 2011).

The concentrations of cyclohexanone and ε-caprolactone were determined using a gas chromatograph (GC) system (M600D, Younglin, Seoul, Korea) installed with the SPB-5 column (Supelco, Bellefonte, PA, USA) according to the method described previously (Lee et al. 2007).

Intracellular concentrations of NADH and NADPH were determined by EnzyChrom NAD/NADH and NADP/NADPH assay kits (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's protocol.

Overexpression of GDP-L-fucose biosynthetic enzymes, CHMO, and Pos5p inside the cell was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12 % polyacrylamide) (Laemmli 1970). The cells during the fermentations were collected, and its concentration was adjusted around 7.2 g/l by appropriate dilution. They were resuspended in 50 mM potassium phosphate buffer (pH 7.0) and disrupted by an ultrasonic processor. After centrifugation at 15,000×*g* for 20 min, the supernatant (soluble fraction) and debris (insoluble fraction) were separated. Ten microliters of the soluble protein

fraction (approximately 0.04 mg) and the same volume of the total and insoluble protein fractions were subjected to SDS-PAGE.

CHMO activity was measured according to the method reported previously (Lee et al. 2007). The cells were collected and adjusted around 7.2 g/l. After resuspension in reaction buffer (0.1 M glycine-NaOH buffer, pH 9.0), disruption, and centrifugation, the supernatant was collected and used for CHMO enzyme assay. Specific CHMO activity was expressed as units per milligram of cellular protein.

All measurements of fermentations samples were performed in triplicates.

Results

Expression of Pos5p in recombinant *E. coli*

The expression pattern of Pos5p was investigated in a batch culture of recombinant *E. coli* BL21star(DE3) harboring plasmid pHpos5. Expression conditions for Pos5p were the same as the conditions for overexpression of the GDP-L-fucose biosynthetic enzymes. As shown in Fig. 2, heterologous expression of Pos5p was confirmed by SDS-PAGE analysis. A considerable amount of Pos5p was expressed as soluble form.

Determination of intracellular pyridine nucleotides concentrations

To verify whether Pos5p expression increased the NADPH biosynthetic capability, intracellular concentrations of pyridine nucleotides were measured based on the whole cell assay. The *E. coli* strain able to express Pos5p only was used for the whole cell assay to avoid consumption of NADPH by production of GDP-L-fucose and ε-caprolactone. The defined medium was used to validate that the cells were able to increase the intracellular NADPH level in the same condition as the fed-batch fermentation. The results of the control strain, *E. coli* BL21star(DE3) harboring pACYCDuet-1, was described in the previous report (Lee et al. 2011). As shown in Table 1, $NADP^+$ and NADPH levels of the Pos5p expressing strain were elevated by 13 % and 33 %; meanwhile, NAD^+ and NADH levels were reduced by 21 % and 20 %, compared with the control strain. Especially, it was notable that the ratio of NADH/NAD^+ was not changed, whereas the ratio of NADPH/$NADP^+$ increased by 17 % in the Pos5p expressing strain. It is interesting that the sum of NAD^+, NADH, $NADP^+$, and NADPH concentrations in the Pos5p expressing strain was almost the same as the sum in the control strain. These results indicated that Pos5p was functionally expressed and increased the concentration of NADPH inside the cell. These results also suggested that even though the total level of pyridine nucleotides was not changed, effective generation of NADPH was achieved by expression of Pos5p in the recombinant *E. coli* cell.

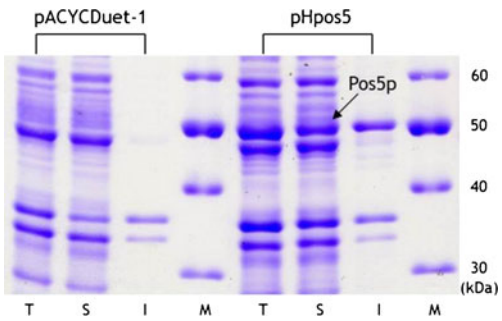

Fig. 2 SDS-PAGE analysis of the cell crude extract of recombinant *E. coli* BL21star(DE3) strains harboring pACYCDuet-1 and pHpos5, respectively. Cells were harvested after 3 h of 0.1 mM IPTG induction. *T*, *S*, and *I* denote total, soluble, and insoluble protein fractions, respectively. The *arrow* indicates the corresponding protein band with the estimated molecular weight of Pos5p. *Lane M* indicates size marker

Effects of Pos5p expression on GDP-L-fucose production

As shown in Fig. 1a, GDP-4-keto-6-deoxy-D-mannose is converted to GDP-L-fucose through epimerization and reduction by WcaG, which requires NADPH as reducing power. In order to investigate the effect of Pos5p expression on GDP-L-fucose production, a fed-batch fermentation of recombinant *E. coli* BL21star(DE3) harboring both plasmid pmBCGW and pHpos5 was conducted. The results of the control strain, *E. coli* BL21star(DE3) overexpressing ManB, ManC, Gmd, and WcaG only, were reported in the previous study (Lee et al. 2011).

After addition of IPTG at 20.75 h of culture, a hyperbolic increase in GDP-L-fucose production was observed (Fig. 3a). Contrary to the control strain, both cell growth and GDP-L-fucose production were maintained till the end of fermentation. After 21 h from induction, 291.5 mg/l of GDP-L-fucose concentration with 3.1 mg/g cell of specific GDP-L-fucose content was obtained, which corresponded to 51 % and 35 % enhancement compared with the control strain, respectively. Along with the enhancement of GDP-L-fucose production, a significant reduction of acetate formation (0.5 g/l) was observed, relative to the control case (5.9 g/l).

To elucidate the expression of GDP-L-fucose biosynthetic enzymes (ManB, ManC, Gmd, and WcaG) and Pos5p in the recombinant *E. coli* cell, SDS-PAGE analysis was done. As shown in Fig. 3b, all enzymes including Pos5p were expressed functionally until the end of fermentation. The results of the fed-batch fermentation for GDP-L-fucose production are summarized in Table 2.

Table 1 Summary of intracellular concentrations of pyridine nucleotides in recombinant *E. coli* BL21star(DE3) overexpressing Pos5p

Plasmids	Specific intracellular concentration (μmol/g cell)				Ratio of NAD(H) NADH/NAD$^+$	Ratio of NADP(H) NADPH/NADP$^+$	Total concentration (μmol/g cell)
	NAD$^+$	NADH	NADP$^+$	NADPH			
pACYCDuet-1[a]	2.91±0.05	0.70±0.16	1.72±0.06	1.41±0.05	0.24	0.82	6.74±0.16
pHpos5	2.30±0.05	0.56±0.13	1.94±0.10	1.87±0.07	0.24	0.96	6.67±0.20

[a] The results of the control strain, *E. coli* BL21star(DE3) overexpressing empty plasmid, were cited in previous study (Lee et al. 2011). Averages and standard errors from three independent measurements are shown

Effects of Pos5p expression on ε-caprolactone production

To confirm that expression of Pos5p can be also effective in oxidation of cyclohexanone, fed-batch BV oxidation of the recombinant *E. coli* BL21(DE3) harboring plasmids pMM4 and pHpos5 was done. The results of the control strain, *E. coli* BL21(DE3) expressing CHMO only, were cited in the previous study (Lee et al. 2007). Cyclohexanone concentration during the fed-batch BV oxidation was maintained less than 6 g/l, which was optimized in the previous study (Lee et al. 2007).

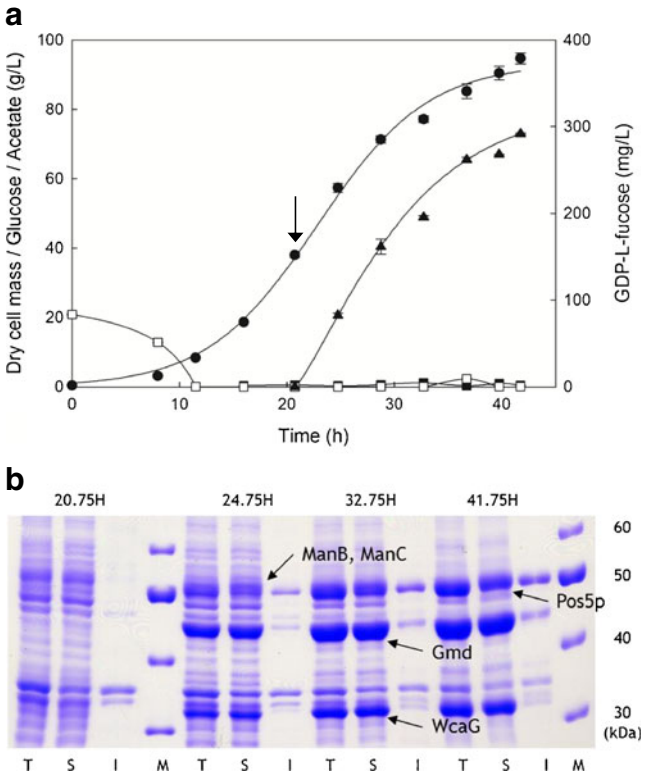

Fig. 3 Profile of GDP-L-fucose production (**a**) and SDS-PAGE analysis of overexpressed enzymes (**b**) in fed-batch fermentation of the recombinant *E. coli* BL21star(DE3) strain harboring pmBCGW and pHpos5. *Arrow* indicates the addition of 0.1 mM IPTG. *Symbols* denote as follows; dry cell mass (*closed circle*); GDP-L-fucose (*closed triangle*)**;** glucose (*open square*); acetate (*closed square*). *M* is a size marker. *T*, *S*, and *I* denote total, soluble, and insoluble protein fractions, respectively. *Numbers* in **b** denote cultivation time. The *arrows* beside protein names indicate the corresponding protein bands with the estimated sizes. All data in the figures are averages from three independent measurements of harvested samples

Figure 4a shows the profiles of ε-caprolactone concentration and CHMO activity in the fed-batch BV oxidation of the recombinant *E. coli* expressing CHMO and Pos5p. After starting BV oxidation at 20.5 h of culture, a drastic increase in ε-caprolactone concentration was observed, twofold faster than the control strain. Reduction of cell growth was not observed until the end of fermentation. Production of ε-caprolactone was still sustained even when ε-caprolactone accumulated up to 20 g/l, which was in contrast with the result of the control strain in the previous study. It is notable that a significant reduction of acetate formation (7.5 g/l) was observed, which was 4-fold lower than the control strain. In the previous study, a maximum ε-caprolactone concentration of 15.3 g/l and a productivity of 0.94 g/l·h were obtained by overexpressing G6PDH (Lee et al. 2007). Meanwhile, with the aid of Pos5p expression, a maximum ε-caprolactone concentration and productivity were further increased to 21.6 g/l and 1.60 g/lh, which corresponded to 96 % and 95 % enhancement compared with the control strain, respectively.

To elucidate such an enhancement of ε-caprolactone production with the soluble expression of enzymes, expression profiles of CHMO and Pos5p were analyzed using SDS-PAGE analysis (Fig. 4b). In the previous study, intracellular stability of CHMO was strongly inhibited by high concentration of ε-caprolactone (more than 11 g/l). Most of intracellular CHMO was even aggregated as inclusion bodies when ε-caprolactone exceeded 15 g/l (Lee et al. 2007). In this study, expression of soluble CHMO was maintained with 193.8 U/g cellular protein of CHMO activity even at 20 g/l of ε-caprolactone (30 h of culture), which was quite contrary to the results observed in the previous study (Lee et al. 2007). The results of fed-batch BV oxidation are summarized in Table 3.

Discussion

Construction of efficient NADPH regeneration systems has been one of the most important issues in the biotransformation processes (Drepper et al. 2006; Duetz et al. 2001; Park

Table 2 Summary of fed-batch fermentations of recombinant *E. coli* BL21star(DE3) strains producing GDP-L-fucose

Plasmids	Dry cell mass (g/l)	GDP-L-fucose concentration (mg/l)	GDP-L-fucose productivity[a] (mg/l·h)	Specific GDP-L-fucose content (mg/g cell)	GDP-L-fucose yield[b] (mg/g consumed glucose)	Acetate concentration (g/l)
pmBCGW[c]	85.0±0.6	193.6±1.9	10.2±0.10	2.3±0.02	1.0±0.01	5.9±0.22
pmBCGW+pMWzwf[d]	84.3±0.8	235.2±3.3	12.7±0.18	2.8±0.04	1.3±0.02	1.4±0.03
pmBCGW+pHpos5	94.7±1.6	291.5±0.8	13.9±0.04	3.1±0.01	1.4±0.01	0.5±0.01

The results of fed-batch fermentation of the Pos5p expressing strain (recombinant *E. coli* BL21star(DE3)/pmBCGW+pHpos5) are averages with standard errors from three independent measurements

[a] Productivity and [b] Yield were estimated during the GDP-L-fucose production period after IPTG induction

[c,d] The results of fed-batch fermentation of the control strain (recombinant *E. coli* BL21star(DE3)/pmBCGW) and the G6PDH overexpressing strain (recombinant *E. coli* BL21star(DE3)/pmBCGW+pMWzwf) were cited in previous study (Lee et al. 2011)

2007; Zhao and van der Donk 2003). A number of metabolic engineering approaches have been made to increase NADPH availability by engineering several metabolic pathways such as PPP, TCA, and transhydrogenation in *E. coli*

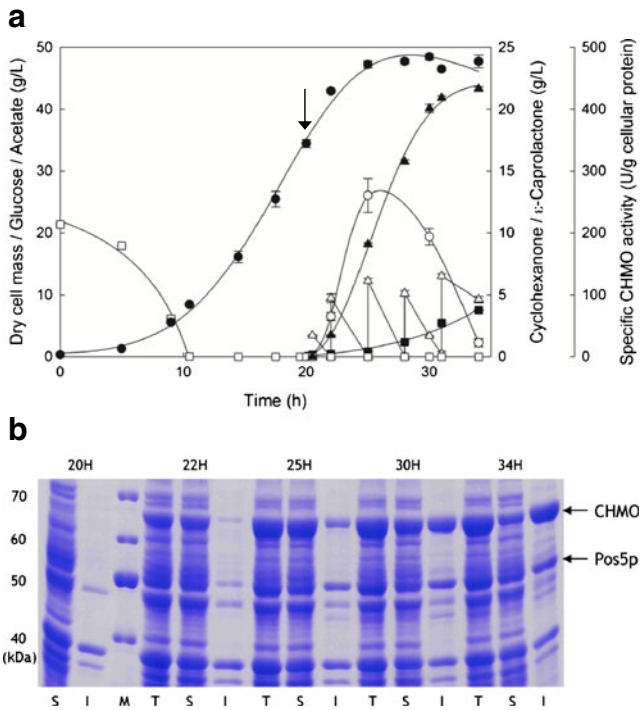

Fig. 4 Profile of ε-caprolactone production (**a**) and CHMO and Pos5p expression pattern (**b**) in fed-batch BV oxidation of the recombinant *E. coli* BL21(DE3) strain harboring pMM4 and pHpos5. *Arrow* indicates the addition of 0.15 mM IPTG. *Symbols* denote as follows; dry cell mass (*closed circle*); specific CHMO activity (*open circle*); glucose (*open square*); acetate (*closed square*); cyclohexanone (*open triangle*); ε-caprolactone (*closed triangle*). *M* is a size marker. *T*, *S*, and *I* denote total, soluble, and insoluble proteins fractions, respectively. *Numbers* in panel **b** denote cultivation time. The *arrows* beside protein names indicate the corresponding protein bands with the estimated sizes. All data in the figures are averages with standard errors from three independent measurements of harvested samples

(Chin et al. 2009; Kabus et al. 2007; Lee et al. 2010; Lim et al. 2002; Sanchez et al. 2006).

Besides the pathways mentioned above, direct phosphorylation of NADH to NADPH can be an alternative way to increase the NADPH level in biotransformation processes because an excess amount of NADH can be generated in glycolysis, indicating that direct phosphorylation of NADH may be easier for efficient NADPH synthesis. However, *E. coli* does not have a native NADH-phosphorylating enzyme. It has NAD kinase that is known to show specificity to NAD^+ only. In addition, NAD kinase in *E. coli* is known to be strongly inhibited by NADH and NADPH (Kawai et al. 2001). Even though overexpression of native NAD kinase was reported to be effective in increasing the productivities of NADPH-dependent PHB production and thymidine production in recombinant *E. coli*, it is hard to draw a clear conclusion because there are a number of unknown factors affecting redox balance between NAD and NADPH (Lee et al. 2009a; Lee et al. 2010; Li et al. 2009).

A number of NAD(H) kinases have been identified from various organisms; however, most of them were reported to show NAD^+-phosphorylating activity with significantly lower NADH-phosphorylating activity (Kawai and Murata 2008). Only a few NADH kinases such as Pos5p from *S. cerevisiae* and NADK3 from *Arabidopsis thaliana* were reported to have higher specificity to NADH than NAD^+ (Kawai and Murata 2008; Outten and Culotta 2003; Turner et al. 2005). Among them, Pos5p from *S. cerevisiae* is one of the well-characterized NADH kinases, and it exhibits more than 50 times higher affinity toward NADH than NAD^+, even though it also phosphorylates NAD^+ weakly (Hou et al. 2009; Outten and Culotta 2003; Strand et al. 2003). Hence, heterologous expression of *S. cerevisiae* NADH kinase was expected to influence the intracellular pyridine nucleotides levels in *E. coli*.

This study was undertaken to investigate the possibility for enhancing the productivity in NADPH-dependent biotransformation processes through direct phosphorylation of NADH by

Table 3 Summary of fed-batch BV oxidations of recombinant *E. coli* BL21(DE3) strains producing ε-caprolactone

Plasmids	Dry cell mass (g/l)	ε-caprolactone concentration (g/l)	ε-caprolactone productivity[a] (g/l h)	Acetate concentration (g/l)
pMM4[b]	38.7	11.0	0.82	29.7
pMM4+pMWzwf[c]	42.0	15.3	0.94	12.4
pMM4+pHpos5	47.7±1.0	21.6±0.2	1.6±0.01	7.5±0.04

The results of fed-batch fermentation of the Pos5p expressing strain (recombinant *E. coli* BL21(DE3)/pMM4+pHpos5) are averages with standard errors from three independent measurements

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

[b,c] The results of fed-batch fermentation of the control strain (recombinant *E. coli* BL21(DE3)/pMM4) and the G6PDH overexpressing strain (recombinant *E. coli* BL21(DE3)/pMM4+pMWzwf were cited in previous study (Lee et al. 2007)

introducing Pos5p into *E. coli*. The recombinant *E. coli* systems producing GDP-L-fucose and ε-caprolactone were used as model systems for NADPH-dependent biotransformation.

The *POS5* gene from *S. cerevisiae* was cloned and expressed in recombinant *E. coli* BL21star(DE3). As shown in Fig. 2, a significant amount of soluble protein (around 50 kDa, truncated protein) was expressed in the recombinant *E. coli*. A previous study revealed that removal of the 17 N-terminal residues led to the soluble expression of Pos5p in recombinant *E. coli* since those residues were used as the mitochondrial targeting sequences in *S. cerevisiae* (Strand et al. 2003). So, the truncated Pos5p was introduced into *E. coli* for soluble expression of Pos5p.

To verify whether this recombinant Pos5p is able to increase the NADPH level, intracellular concentrations of pyridine nucleotides were measured. As shown in Table 1, the Pos5p expressing strain showed elevated levels of intracellular $NADP^+$ and NADPH, which supported Pos5p was able to produce NADPH from NADH in the recombinant *E. coli*. Interestingly, a decrease in the concentrations of both NAD^+ and NADH was observed. Since Pos5p was reported to show much higher NADH specificity than NAD^+ (Outten and Culotta 2003; Strand et al. 2003), it might be due to the consumption of NAD^+ for complementing lack of NADH caused by Pos5p expression.

In addition, expression of NADH kinase may have an advantage in saving carbon molecules in terms of GDP-L-fucose production since this nucleotide sugar can be synthesized from glucose. For comparing direct phosphorylation of NADH with amplification of PPP, the results in the G6PDH overexpressing strain were also summarized in Table 2. The Pos5p expressing strain showed better GDP-L-fucose production and specific GDP-L-fucose content than the control and G6PDH overexpressing strains. Based on the amount of GDP-L-fucose production from glucose consumption, the product yield increased by 40 % and 8 % compared with the control and G6PDH overexpressing strains, respectively (Lee et al. 2011). These results suggest that an efficient NADPH supplementation and a rescue of carbon molecule were possible by expression of Pos5p. Formation of acetate was also significantly reduced in the Pos5p expressing strain compared with other strains. These results also suggest that direct phsophorylation of NADH might lead to faster NADPH regeneration, in turn, minimize acetate formation which is known to be related with the redox balance of pyridine nucleotides (De Mey et al. 2007).

A more dramatic enhancement of product formation was observed in the fed-batch BV oxidation of the recombinant *E. coli* strain expressing Pos5p. As shown in Table 3, ε-caprolactone productivity of the Pos5p expressing strain was 1.6 g/l·h, a significant improvement compared with the control (0.82 g/l·h) and G6PDH overexpressing strain (0.94 g/l·h). Final ε-caprolactone concentration also increased by 96 % and 41 % compared with the control and G6PDH overexpressing strains, respectively (Lee et al. 2007). Similarly to the results from GDP-L-fucose production, the Pos5p expressing strain showed higher final cell mass concentration (23 % increase) and significantly reduced acetate formation (75 % decrease) compared with the control strain, which may be consistent with the previous report where overexpression of an endogenous NADH kinase in *Aspergillus nidulans* led to a 14 % enhancement in cell mass yield (Panagiotou et al. 2009). In addition, soluble expression of CHMO was sustained even at 20 g/l ε-caprolactone (Fig. 4). These results suggested that efficient regeneration of NADPH by functional expression of Pos5p might sustain ε-caprolactone production. Previously, Pos5p has been reported to be involved in the protection against a broad range of oxidative stress, amino acid biosynthesis, and mitochondrial iron homeostasis (Outten and Culotta 2003). It was also reported that Pos5p is required for stabilizing mitochondrial DNA with detoxification of reactive oxygen species (Shianna et al. 2006; Strand et al. 2003). During BV oxidation, *E. coli* cell might suffer from various kinds of oxidative stress caused by cyclohexanone and ε-caprolactone. Probably, Pos5p might play a pivotal role in tolerance of *E. coli* against cyclohexanone and ε-caprolactone stress.

In this study, the effect of Pos5p expression was tested in the fed-batch fermentations with aerobically growing cells in the glucose medium, which were already optimized

in our previous studies (Lee et al. 2007; Lee et al. 2011). These suggest that additional studies may be required to confirm whether Pos5p expression is effective in other fermentation conditions. Also, more researches are in progress to characterize the impact of Pos5p in *E. coli* by combinatorial overexpression (or deletion) of native $NAD(P)^+$-relating enzymes with expression of Pos5p for further applications to NADPH-dependent biotransformation processes.

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Conflict of interest The authors declare no conflict of interest.

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