J. Korean Soc. Appl. Biol. Chem. **54**(6), 986-992 (2011) **Article**

Improving the Catalytic Activity of Cyclohexanone Monooxygenase-based Whole-cell Biocatalysts under Substrate Toxic Conditions

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Received August 23, 2011; Accepted September 23, 2011

The catalytic activity of oxygenase-based whole-cell biocatalysts is heavily influenced by substrate and product toxicities due to cell membrane permeabilization and protein denaturation effects of the organic substrates and products. Therefore, stability of oxygenase-based whole-cell biocatalysts against solvent stress was investigated with recombinant *Escherichia coli* BL21 and *Corynebacterium glutamicum* ATCC13032 expressing the *chnB* gene of cyclohexanone monooxygenase of *Acinetobacter calcoaceticus* NCIMB 9871. The cyclohexanone oxygenation activity of the recombinant biocatalysts rapidly decreased as cyclohexanone concentration increased from 2.4 to 26 g/L. However, treatment of the recombinant cells with non-lethal doses of cyclohexanone or preadaptation to the toxic substrate led to the oxygenation activity being relatively maintained. For instance, the oxygenation activity of cyclohexanone-treated *E. coli* cells was ca. 13 U per g dry cells at the substrate concentration of 26 g/L, which was almost 5-fold higher than that of the cyclohexanone-nontreated cells. In addition, biocatalytic activity was better maintained when the genes encoding chaperones (i.e., GroEL-ES and DnaKJ-GrpE) were coexpressed with the *chnB* gene. The positive effects of chaperones on the catalytic activity of the recombinant *E. coli*-based biocatalyst appeared to be related with expression level of biotransformation enzymes rather than with solvent stress-response metabolism. Overall, molecular chaperones, of which expression can be induced by solvent treatment, were involved in catalytic stability of whole-cell biocatalysts during biotransformations involving toxic compounds as the reactants.

Key words: *Corynebacterium glutamicum*, cyclohexanone monooxygenase, *Escherichia coli*, molecular chaperones, whole-cell biocatalysis

The efficiency of oxygenase-based whole-cell biocatalysts, in terms of specific product formation rates, is influenced by many factors including the activity of catalytic enzymes (e.g., k_{cat}), efficiency of substrate transport, the cofactor (NAD(P)H) regeneration activity of the host cells, and biocatalyst stability against lipophilic substrates and products [Park *et al.*, 2006a; 2006b; Bae *et al.*, 2008; Blank *et al*., 2008b; Buhler *et al*., 2008; Julsing *et al*., 2008; Yim *et al*., 2010; Lee *et al*., 2011]. Among these, substrate and product toxicities are especially critical for the activity of whole-cell biotransformation, where hydrophobic molecules are involved as the reaction substrates and/or products [Park *et al*., 2006a; Park, 2007]. Lipophilic compounds may cause cellular membranes to be permeabilized and cellular enzymes to be denaturated [Sikkema *et al*., 1994; Ramos *et al*., 2002]. For instance, toluene was shown to accumulate in the cellular membrane bilayer and to result in permeabilization of the cell membrane [Isken and de Bont, 1998]. Cyclohexanone monooxygenase (CHMO) was denatured and aggregated in the presence of a high concentration of cyclohexanone [Lee *et al*., 2007].

There could be a number of ways to improve the stability of whole-cell biocatalysts against solvent stresses. One approach would be to use chaperoning proteins and foldases to increase the functional expression and stability

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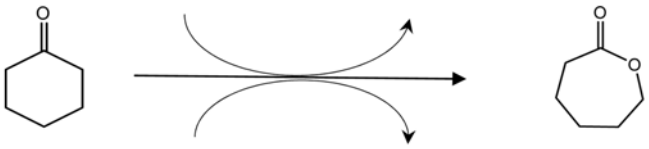
http://dx.doi.org/10.3839/jksabc.2011.148



Scheme 1. Oxygenation of cyclohexanone into ε-caprolactone by cyclohexanone

of catalytic enzymes inside cells [Bae *et al*., 2009; Kolaj *et al*., 2009; Ratajczak *et al*., 2009]. GroEL-ES and DnaK chaperone systems were reported to prevent the aggregation of proteins and support refolding to their native conformation. DnaK cooperates with its cofactor DnaJ and nucleotide exchange factor GrpE to promote folding to the native or partially folded conformation. The partially folded proteins may complete folding to their native state with assistance from the GroEL-ES complex. The DnaK system was also shown to disaggregate small aggregates of denatured proteins. In addition, caseinolytic peptidase B (ClpB) and small heat-shock proteins (e.g., IbpA, IbpB) were reported to be involved in the disaggregation of aggregates of misfolded proteins [Kolaj *et al*., 2009]. ClpB, a member of the AAA+ superfamily of ATPases, was postulated to unfold insoluble protein aggregates. IbpA and IbpB act to partially hold unfolded proteins to proceed into further folding processes.

In the present study, we investigated the effects of chaperoning proteins on the activity and stability of *Escherichia coli*- and *Corynebacterium glutamicum*-based biocatalysts expressing the *chnB* gene encoding CHMO of *Acinetobacter calcoaceticus* NCIMB 9871. Biotransformation of cyclohexanone into ε-caprolactone was used as a model reaction (Scheme 1). The expression of genes encoding chaperoning proteins (e.g., DnaKJ-GrpE, GroEL-ES, IbpAB, and ClpB) was induced by solvent stress or facilitated by the introduction of recombinant plasmids.

Materials and Methods

Bacterial strains and cultivation. Recombinant *E. coli* BL21 pMM4 expressing the *chnB* gene [Lee *et al*., 2007] was cultivated at 25°C in Luria-Bertani (LB) medium containing 50 mg/L ampicillin. Plasmid pMM4 (7.0 kb, pBR322 replicon, Amp^r), a kind gift from Prof. J. Stewart (University of Florida, Gainesville, FL), harbors the *chnB* gene under the control of the T_7 promoter. The expression of the *chnB* gene was induced by adding 0.01 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture broth as reported by Lee *et al.* [2007]. Depending on the chaperone plasmids introduced, chloramphenicol and/or kanamycin and L-arabinose and/or tetracycline were added to the cultures at the beginning of cultivation to induce the expression of the chaperone genes. Recombinant *C. glutamicum* pEKEx2-*chnB* [Doo *et al*., 2009] was cultivated at 25°C in brain-heart infusion (BHI) medium containing 25 mg/L kanamycin. Plasmid pEKEx2-*chnB* harbors the *chnB* gene under the control of the P_{tac} promoter. The expression of *chnB* was induced by 0.1 mM IPTG as reported in our earlier study [Doo *et al*., 2009]. Cyclohexanone was added to the cultures at a concentration of 5 g/L at $OD_{600\ nm}$ of 1.0 to cause solvent stress to the recombinant cells or for preadaptation of the cells to cyclohexanone.

Plasmid construction. The plasmid pG-KJE8, containing *dnaKJ-grpE* and *groEL-ES* genes, was purchased from Takara Bio Inc (Otsu, Japan). The pBBR2ABC plasmid, containing *ibpAB* and *clpB* genes of *E. coli*, was constructed as follows; the genes of *ibpA*, *ibpB*, and *clpB* encoding inclusion body-associated proteins and protein aggregation chaperone were amplified from *E. coli* MG1655 genomic DNA by using Phusion DNA polymerase (Finnzymes, Vantaa, Finland). Polymerase chain reaction (PCR) was performed according to the protocol of the supplier. Following sets of forward and reverse PCR primers were used for the PCR reaction: 5'-CGGTACCT**ATG**CGTAACTTTGATTTATCCCCG-3' (forward)/5'-CCTCGAG**TTA**GTTGATTTCGATACGG CGC-3' (reverse) for *ibpA*, 5'-CCTCGAGAGGAGGTAA TAAAT**ATG**CGTAACTTCGATTTATCCCC-3' (forward) /5'-GAAGC**TTA**GCTATTTAACGCGGGACGTTC-3' (reverse) for *ibpB*, and 5'-TTCTAGAAGGAGGTAATA AAT**ATG**CGTCTGGATCGTCTTACTAATAAATTC-3' (forward)/5'-CCCGCGG**TTA**GCGATGAGACAACGT CGC-3' (reverse) for *clpB*.

Restriction enzyme sites, *Kpn*I/*Xho*I for *ibpA*, *Xho*I/*Hin*dIII for *ibpB* and *Xba*I/*Sac*II for *clpB*, are underlined in the PCR primer sequences. The start and stop codon sites are presented in bold letters. The PCR products with expected sizes (*ibpA*, 429 bp; *ibpB*, 455 bp; *clpB*, 2062 bp) were obtained and subsequently confirmed by DNA sequencing. The PCR products were digested with indicated restriction enzymes and then ligated into multi-cloning sites of a broad-host-range pBBRMCS2 vector by using T4 ligase (NEB, Ipswich, MA). The plasmid containing *ibpA, ibpB* and *clpB* genes was designated as pBBRABC.

Protein electrophoresis. After disruption of the recombinant *E. coli* cells with an ultrasonic processor (Cole-Parmer, Vernon Hills, IL), the crude protein solutions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide) according to the protocol previously reported by Sambrook and Russell [2001].

Determination of whole-cell biotransformation rates. The biotransformation rates of the recombinant cells were measured based on our previous reports [Lee *et al.*, 2007; Doo *et al.*, 2009]; the cells were grown in LB or BHI medium containing appropriate antibiotics and/or 5 g/L cyclohexanone. Four hours after induction of the target gene expression, the recombinant cells were harvested by centrifugation and resuspended to a concentration of 1.0 or 3.5 g CDW (cell dry weight)/L in sodium-potassium phosphate buffer (pH 7.0) containing 5 g/L glucose and various concentrations of cyclohexanone. One unit of the whole cell reaction rate is defined as the amount of dry cells able to produce 1 μmol of oxidation products per min.

Analysis of reactants and metabolites. The concentrations of cyclohexanone and ε-caprolactone were measured according to the method described in our previous report [Doo *et al.*, 2009]. After removing the cells, the culture broth was mixed with an equal volume of ethyl acetate containing 1.0 g/L methyl benzoate as an internal standard. The organic phase was harvested after vigorous vortexing and centrifugation and then injected into a gas chromatograph (GC) (7890A, Agilent Technologies, Santa Clara, CA) installed with a SPB-5 column (Supelco, Bellefonte, PA). The column temperature was increased from 50 to 215°C at a rate of 15°C/min with 2 and 5 min of holding times for the initial and final temperatures, respectively.

Concentrations of glucose and organic acids (e.g., acetic acid, lactic acid) were measured by high performance liquid chromatography (HPLC) (Waters, Milford, MA) equipped with an HPX-87H column (Bio-rad Aminex, Hercules, CA). The column was eluted with 5 mM H_2SO_4 at a constant rate of 0.6 mL/min at 45°C. A 410 RI refractive index detector and UV dual λ absorbance detector (Waters) were used.

For measurement of CO_2 content, the gas phase of the side-arm flasks was taken up with a syringe and analyzed via injection into a GC (6890N, Agilent Technologies) installed with an Agilent 19095P-MS6 column (30 m × 530 μm × 50 μm) and thermal conductivity detector. Helium was used as a carrier gas at a flow rate of 9 mL/min. The oven, injector, and detector temperatures were kept at 60, 100, and 250°C, respectively. The CO_2 content per liter of reaction medium was calculated based on the CO_2 concentration in the gas phase.

Results and Discussion

Effect of cyclohexanone treatment on the biotransformation activity of *E. coli*. Molecular chaperones (e.g., DnaKJ, GrpE, GroEL-ES) were reported to be induced via heat-shock and/or treatment with organic solvents (e.g., benzyl alcohol) [de Marco *et al.*, 2005]. Therefore, the effect of treatment with cyclohexanone on the biocatalytic activity of recombinant *E. coli* BL21 pMM4 expressing the *chnB* gene was investigated.

The oxygenation activity of *E. coli*-based biocatalysts, which was calculated based on the biotransformation rate of cyclohexanone into ε-caprolactone, was reduced significantly as the concentration of the reaction substrate cyclohexanone was increased in the reaction medium (Fig. 1). On the other hand, the oxygenation activity of recombinant *E. coli* cells, which had been harvested after growth in the presence of 5 g/L cyclohexanone, remained unchanged up to the cyclohexanone concentration of 10.5 g/L. Moreover, the specific oxygenation activity of the cyclohexanone-treated cells was ca. 5-folds higher at the substrate concentration of 26 g/L. These results indicate that the stability of recombinant *E. coli* BL21 pMM4 against substrate toxicity was greatly increased via solvent treatment before biotransformation or preadaptation toward the toxic substrate.

Effect of cyclohexanone treatment on the oxygenation activity of *C. glutamicum*. To examine the effects of solvent treatment or preadaptation to toxic substrates on the biocatalytic activity of another class of microorganisms, biotransformation of cyclohexanone into ε-caprolactone by recombinant *C. glutamicum* ATCC13032 pEKEx2-*chnB* expressing the CHMO gene was investigated at reaction conditions comparable to those used in the experiments shown in Fig. 1. The effect of solvent treatment on the oxygenation of cyclohexanone was similar to that of the *E. coli*-driven biotransformation (Fig. 2). Specific oxygenation rates were better maintained over increasing cyclohexanone concentrations in the solvent-treated whole-cell biocatalysts. This result indicates that the stabilizing effect of solvent treatment, or preadaptation to the toxic substrate, was also valid with the Gram-positive bacterium, *C. glutamicum*-based biocatalyst.

Another interesting point was that the biotransformation rate of recombinant *C. glutamicum* was greater than that of recombinant *E. coli* and comparable to that of cyclohexanone-treated *E. coli* cells at substrate concentrations of over 5.8 g/L (Figs. 1 and 2). The expression level of *chnB* was higher in the *E. coli* cells; thus, the greater activity of the *C. glutamicum* cells than the non-treated *E. coli* cells was not related to the biotransformation enzymes. Because the solvent tolerance (supplemental material) and NADPH regeneration capacity of *C. glutamicum* ATCC13032 [Doo *et al.*, 2009], which are critical for the cyclohexanone oxygenation activity of CHMO (Scheme 1), were reported to be greater than those of *E. coli* cells, the higher

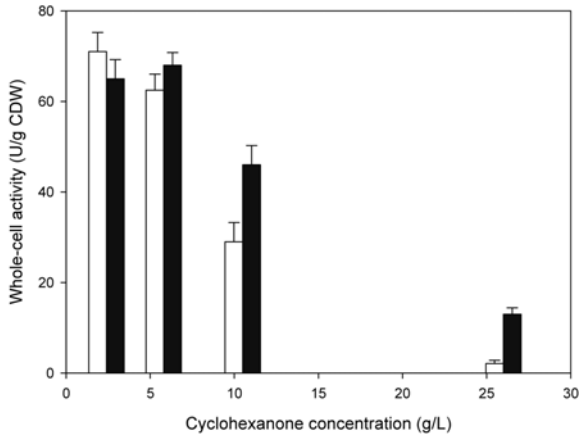


Fig. 1. Effects of cyclohexanone treatment or preadaptation to cyclohaxanone on the biotransformation activity of recombinant *E. coli* BL21 pMM4. The oxygenation activities of cyclohexanone-treated cells and non-treated cells were measured in sodium-potassium phosphate buffer (pH 7.0) containing 5 g/L glucose and varying amounts of the reaction substrate cyclohexanone. Symbols indicate specific ε-caprolactone production rates of cyclohexanone-treated cells (■) and non-treated cells (□).

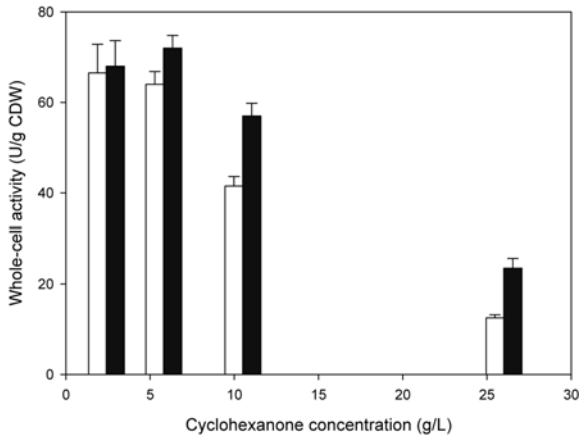


Fig. 2. Effects of cyclohexanone treatment on the biotransformation activity of recombinant *C. glutamicum* pEKEx2-*chnB*. The oxygenation activities of cyclohexanone-treated cells and non-treated cells were measured in the sodium-potassium phosphate buffer (pH 7.0) containing 5 g/L glucose and varying amounts of cyclohexanone. Symbols indicate specific ε-caprolactone production rates of cyclohexanone-treated cells (■) and non-treated cells (□).

oxygenation activity at high substrate concentrations could be ascribe to the higher metabolic and cofactor (NAPDH) regeneration activity of recombinant *C. glutamicum* during biotransformation.

Effect of chaperoning protein coexpression on biotransformation activity. In order to understand the stabilizing mechanisms of solvent treatment on the whole-cell biocatalysts, we overexpressed some specific chaperoning proteins in recombinant *E. coli* pMM4 and determined their effects on oxygenation activity. The specific oxygenation rate of cyclohexanone into ε-caprolactone by *E. coli* BL21 pMM4 expressing the *chnB* gene decreased linearly over the reaction time under the whole-cell biotransformation conditions used for the experiments shown in Fig. 1 (Fig. 3). However, the specific ε-caprolactone production rates of recombinant *E. coli* pMM4/pBBR2ABC expressing *chnB* and molecular chaperones (IbpAB and ClpB), and of *E. coli* pMM4/pG-KJE8 expressing *chnB* and other molecular chaperones (DnaKJ-GrpE, and GroEL-ES), were better maintained during the biotransformation. Notably, the specific product formation rate of *E. coli* pMM4/pG-KJE8 was almost the same as that of *E. coli* pMM4, which had been cultivated in the presence of cyclohexanone at the cyclohexanone concentration of 5.8 g/L (Fig. 1). These results indicate that the chaperoning proteins such as DnaKJ-GrpE and GroEL-ES were involved in the stabilizing effect of cyclohexanone treatment as shown in Fig. 1.

To investigate the influence of molecular chaperones on the expression level and conformation of CHMO, SDS-PAGE analysis of proteins from recombinant *E. coli* cells prepared during biotransformation shown in Fig. 3 was carried out (Fig. 4). The density of the CHMO bands from soluble and insoluble fractions of *E. coli* pMM4, *E. coli* pMM4/pG-KJE8, and *E. coli* pMM4/pBBR2ABC cells appeared to remain unchanged over the reaction times (Fig. 4). This indicates that the molecular chaperones did not have a significant impact on the conformation of CHMO during the biotransformation. On the other hand, the CHMO bands from soluble fractions of *E. coli* pMM4/pG-KJE8 appear to be thicker than those from *E. coli* pMM4 or *E. coli* pMM4/pBBR2ABC. This result suggests that the content of the soluble form of CHMO would be one of the factors influencing the activity of the *E. coli*-based biocatalysts.

Effect of chaperoning protein coexpression on carbon metabolism of *E. coli*. Molecular chaperones were reported to be involved not only in soluble expression and (re)folding of proteins, but also in adaptation and/or tolerance of microbial cells (e.g., *E. coli*) to environmental stresses [Techtmann and Robb, 2010]. Therefore, effects of molecular chaperones (i.g., DnaKJ-GrpE and GroEL-ES) on the carbon and cofactor metabolism of *E. coli* BL21 in the presence of cyclohexanone were evaluated. The specific glucose uptake rate as well as the specific production rate of the major metabolic products (i.e., CO_2 and acetic acid) during incubation with cyclohexanone was monitored (Fig. 5 and Table 1). The specific CO_2 evolution rate indicates cofactor regeneration rates in *E. coli* cells; during complete oxidation of 1 mol glucose

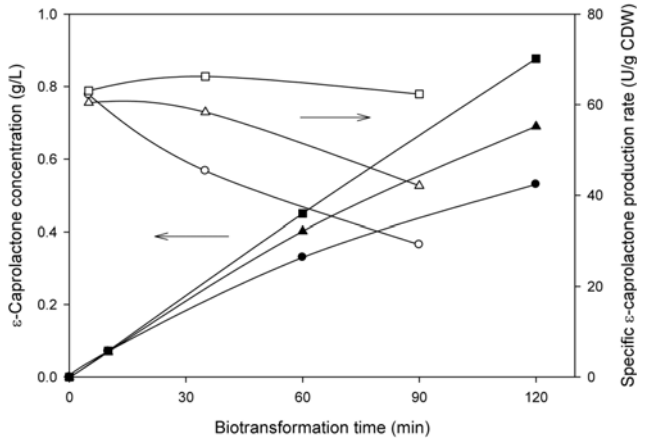


Fig. 3. Effects of chaperoning protein coexpression on the biotransformation rates of recombinant *E. coli*-based biocatalysts. The biotransformation was carried out in the sodium-potassium phosphate buffer (pH 7.0) containing 5 g/L glucose and 5.8 g/L cyclohexanone. Symbols indicate ε-caprolactone concentration and specific ε-caprolactone production rates of *E. coli* pMM4 (●, ○), *E. coli* pMM4/pG-KJE8 (■, □), and *E. coli* pMM4/pBBR2ABC (▲, △).

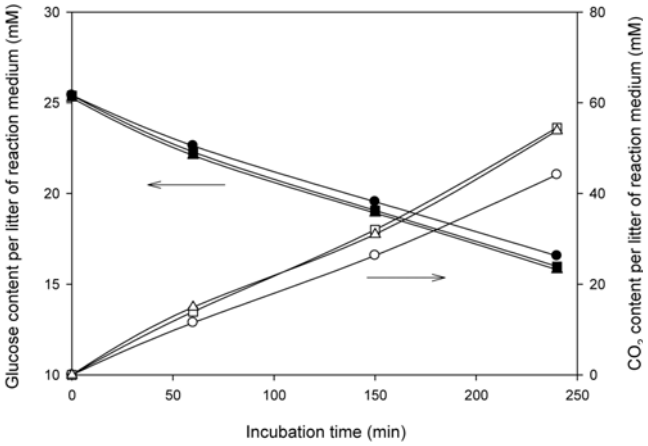


Fig. 5. Effects of chaperoning protein expression on carbon metabolism of *E. coli*. The glucose and CO_2 contents per liter of reaction medium were measured by HPLC and GC, respectively (see Materials and Methods for details). The *E. coli* BL21 culture was incubated in sodium-potassium phosphate buffer containing 5.0 g/L glucose (●, ○). The *E. coli* BL21 (■, □) or *E. coli* BL21 pG-KJE8 culture (▲, △) was incubated in the sodium-potassium phosphate buffer containing 5.0 g/L glucose and 5.8 g/L cyclohexanone. The closed and open symbols indicate the glucose and CO_2 contents per liter of reaction medium, respectively.

into 6 mol CO_2, approximately 10.6 mol NAD(P)H are regenerated, thus ca. 1.8 mol NAD(P)H are regenerated during formation of 1 mol CO_2 [Blank *et al*., 2008a; 2010]. Acetic acid was reported to be formed as a result of solvent stress on the *E. coli* cells [Park *et al*., 2006a; Buhler *et al*., 2008].

The specific CO_2 evolution rate of *E. coli* BL21 was substantially higher in the presence of 5.8 g/L cyclohexanone (Fig. 5 and Table 1), indicating that the NAD(P)H regeneration rate increased with addition of cyclohexanone. Cyclohexanone could accumulate in the cytoplasmic membrane, resulting in disintegration of the lipid bilayer and thereby dissipation of proton gradients across the cell membrane, as shown with toluene [Isken and de Bont, 1998]. This assumption was also supported by increase of acetic acid formation rate (Table 1) and reduction of the specific growth rate of *E. coli* BL21 by over 30% in the LB medium containing 5.8 g/L cyclohexanone (supplemental material). As a result of the toxic effects of cyclohexanone on the cells, the cellular requirement of nicotine amide cofactors would be increased in the presence of cyclohexanone. On the other hand, there was little difference in the specific CO_2 evolution rates between *E. coli* BL21 and *E. coli* BL21 pG-KJE8 during incubation with cyclohexanone. This result suggested that the molecular chaperones did not have a marked impact on the integrity of the cytoplasmic membrane and/or stress-response metabolism of *E. coli*

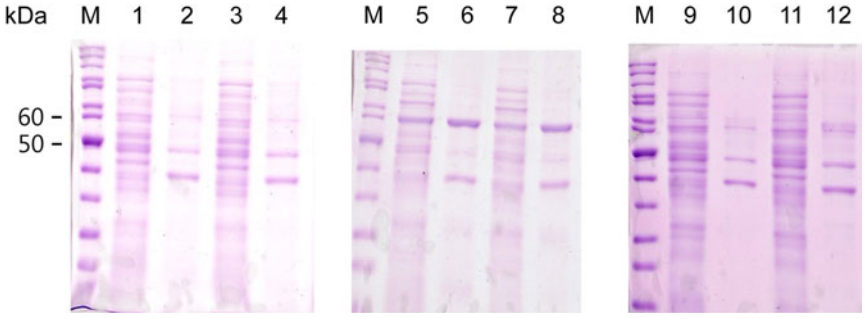


Fig. 4. SDS-PAGE analysis of CHMO (M.W. 61.5 kDa) prepared from recombinant *E. coli* cells. *E. coli* pMM4 expressing *chnB* (lane 1-4), *E. coli* pMM4/pG-KJE8 expressing *chnB*, *dnaKJ-grpE*, and *groEL-ES* (lane 5-8), and *E. coli* pMM4/pBBR2ABC cells expressing *chnB*, *ibpAB*, and *clpB* (lane 9-12) were taken at t = 0 and 120 min in the biotransformation experiments (Fig. 3). The CHMO band is indicated by an arrow. Lane M: size marker; Lanes 1, 3, 5, 7, 9, 11: soluble fractions; Lanes 2, 4, 6, 8, 10, 12: insoluble fractions.

Table 1. Effects of chaperoning protein expression on carbon metabolism of *E. coli*

	Specific glucose uptake rate (mmol/g CDW/h)	Specific CO_2 evolution rate (mmol/g CDW/h)	Sp. acetic acid production rate (mmol/g CDW/h)
[a]*E. coli* BL21	2.21	11.0	<0.10
[b]*E. coli* BL21 at 5.8 g/L cyclohexanone	2.39	13.6	0.30
[b]*E. coli* BL21 pG-KJE8 at 5.8 g/L cyclohexanone	2.37	13.5	0.41

[a]Incubated in sodium-potassium phosphate buffer containing 5.0 g/L glucose
[b]Incubated in sodium-potassium phosphate buffer containing 5.0 g/L glucose and 5.8 g/L cyclohexanone

under the experimental conditions used in the present study.

In summary, preadaptation to toxic substrates and/or expression of molecular chaperones (i.e., DnaKJ-GrpE and GroEL-ES) allowed the oxygenation activity of the recombinant *E. coli* and *C. glutamicum*-based biocatalysts to be better maintained under high substrate concentrations. The positive effects of chaperones on the catalytic activity of the recombinant *E. coli*-based biocatalyst appeared to be related with expression level of biotransformation enzymes rather than with structural integrity of cellular membranes and/or solvent stress-response metabolism. These results may contribute to engineering a solid whole-cell biocatalyst for biotransformations, in which toxic compounds are involved as the reactants.

Acknowledgment. This work was supported by a Korea Research Foundation Grant funded by the Korean Government (KRF- 2010-0009169).

References

Bae JW, Doo EH, Shin SH, Lee SG, Jeong YJ, Park JB, and Park S (2009) Development of a recombinant *Escherichia coli*-based biocatalyst for styrene epoxidation with high product yield on energy source and with low oxygen requirement. *Proc Biochem* **45**, 147–52.

Bae JW, Shin S, Raj SM, Lee SE, Lee SG, Jeong YJ, and Park S (2008) Construction and characterization of a recombinant whole-cell biocatalyst of *Escherichia coli* expressing styrene monooxygenase under the control of arabinose. *Biotechnol Bioproc Eng* **13**, 69–76.

Blank LM, Ebert BE, Buehler K, and Buhler B (2010) Redox biocatalysis and metabolism: molecular mechanisms and metabolic network analysis. *Antioxidants Red Signal* **13**, 349–394.

Blank LM, Ebert BE, Buhler B, and Schmid A (2008a) Metabolic capacity estimation of *Escherichia coli* as a platform for redox biocatalysis: Constraint-based modeling and experimental verification. *Biotechnol Bioeng* **100**, 1050–1065.

Blank LM, Ionidis G, Ebert BE, Buhler B, and Schmid A (2008b) Metabolic response of *Pseudomonas putida* during redox biocatalysis in the presence of a second octanol phase. *FEBS J* **275**, 5173–5190.

Buhler B, Park JB, Blank LM, and Schmid A (2008) NADH availability limits asymmetric biocatalytic epoxidation in a growing recombinant *Escherichia coli* strain. *Appl Environ Microbiol* **74**, 1436–1446.

de Marco A, Vigh L, Diamant S, and Goloubinoff P (2005) Native folding of aggregation-prone recombinant proteins in *Escherichia coli* by osmolytes, plasmid- or benzyl alcohol-overexpressed molecular chaperones. *Cell Stress Chap* **10**, 329–339.

Doo EH, Lee WH, Seo HS, Seo JH, and Park JB (2009) Productivity of cyclohexanone oxidation of the recombinant *Corynebacterium glutamicum* expressing *chnB* of *Acinetobacter calcoaceticus*. *J Biotechnol* **142**, 164–169.

Isken S and de Bont JAM (1998) Bacteria tolerant to organic solvents. *Extremophiles* **2**, 229–238.

Julsing MK, Cornelissen S, Buhler B, and Schmid A (2008) Heme-iron oxygenases: Powerful industrial biocatalysts? *Curr Opin Chem Biol* **12**, 177–186.

Kolaj O, Spada S, Robin S, and Wall J (2009) Use of folding modulators to improve heterologous protein production in *Escherichia coli*. *Microb Cell Fact* **8**, 1–17.

Lee NR, Yoon JW, and Park JB (2011) Effect of lipopolysaccharide mutation on oxygenation of linoleic acid by recombinant *Escherichia coli* expressing CYP102A2 of *Bacillus subtilis*. *Biotechnol Bioproc Eng* **16**, 7–12.

Lee WH, Park JB, Park K, Kim MD, and Seo JH (2007) Enhanced production of epsilon-caprolactone by overexpression of NADPH-regenerating glucose 6-phosphate dehydrogenase in recombinant *Escherichia coli* harboring cyclohexanone monooxygenase gene. *Appl Microbiol Biotechnol* **76**, 329–338.

Park JB (2007) Oxygenase-based whole-cell biocatalysis in organic synthesis. *J Microbiol Biotechnol* **17**, 379–392.

Park JB, Buehler B, Habicher T, Hauer B, Panke S, Witholt B, and Schmid A (2006a) The efficiency of recombinant *Escherichia coli* as biocatalyst for stereospecific epoxidation. *Biotechnol Bioeng* **95**, 501–512.

Park MS, Bae JW, Han JH, Lee EY, Lee SG, and Park S (2006b) Characterization of styrene catabolic genes of *Pseudomonas putida* SN1 and construction of a recombinant *Escherichia coli* containing styrene monooxygenase gene for the production of (S)-styrene oxide. *J Microbiol Biotechnol* **16**, 1032–1040.

Ramos JL, Duque E, Callegos M-T, Codoy P, Ramos-Gonzlez MI, Rojas A, Teran W, and Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. *Annu Rev Microbiol* **56**, 743–768.

Ratajczak E, Zietkiewicz S, and Liberek K (2009) Distinct activities of *Escherichia coli* small heat shock proteins IbpA and IbpB promote efficient protein disaggregation. *J Mol Biol* **386**, 178–189.

Sambrook J and Russell RW (2001) In *Molecular Cloning: A Laboratory Manual,* (3rd ed). Cold Spring Harbor Laboratory Press, Cold Spring, NY.

Sikkema J, de Bont JAM, and Poolman B (1994) Interactions of cyclic hydrocarbons with biological membranes. *J Biol Chem* **269**, 8022–8028.

Techtmann SM and Robb FT (2010) Archaeal-like chaperonins in bacteria. *Proc Natl Acad Sci USA* **107**, 20269–20274.

Yim SK, Kim DH, Jung HC, Pan JG, Kang HS, Ahn T, and Yun CH (2010) Surface display of heme- and diflavin-containing cytochrome P450 BM3 in *Escherichia coli*: A whole-cell biocatalyst for oxidation. *J Microbiol Biotechnol* **20**, 712–717.