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# High-level expression in *Corynebacterium glutamicum* of nitrile hydratase from *Rhodococcus rhodochrous* for acrylamide production

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**Abstract** The *nhhBAG* gene of *Rhodococcus rhodochrous* M33 that encodes nitrile hydratase (NHase), converting acrylonitrile into acrylamide, was cloned and expressed in *Corynebacterium glutamicum* under the control of an *ilvC* promoter. The specific enzyme activity in recombinant *C. glutamicum* cells was about 13.6 μmol/min/mg dry cell weight (DCW). To overexpress the NHase, five types of plasmid variants were constructed by introducing mutations into 80 nucleotides near the translational initiation region (TIR) of *nhhB*. Of them, pNBM4 with seven mutations showed the highest NHase activity, exhibiting higher expression levels of NhhB and NhhA than wild-type pNBW33, mainly owing to decreased secondary-structure stability and an introduction of a conserved Shine-Dalgarno sequence in the translational initiation region. In a fed-batch culture of recombinant *Corynebacterium* cells harboring pNBM4, the cell density reached 53.4 g DCW/L within 18 h, and the specific and total enzyme activities were estimated to be 37.3 μmol/min/mg DCW and 1,992 μmol/min/mL, respectively. The use of recombinant *Corynebacterium* cells for the production of acrylamide from acrylonitrile resulted in a conversion yield of 93 % and a final acrylamide concentration of 42.5 % within 6 h when the total amount of fed acrylonitrile was 456 g.

**Keywords** Nitrile hydratase · Acrylamide · *Rhodococcus rhodochrous* · *Corynebacterium glutamicum*

## Introduction

Nitrile hydratase (NHase; EC 4.2.1.84) catalyzes the hydration of nitriles into the corresponding amides. NHase is a multimeric metalloenzyme composed of two subunits, α and β, and requires either non-heme iron (Fe-NHase) or non-corrin cobalt (Co-NHase) at its active site (Brennan et al. 1996; Kobayashi and Shimizu 1998; Kobayashi et al. 1991; Yamada and Kobayashi 1996). Several bacteria including *Pseudomonas chlororaphis* B23, *Brevibacterium* R312, *Rhodococcus* sp. N-774, *Rhodococcus rhodochrous* J1 (Kobayashi and Shimizu 1998; Yamada and Kobayashi 1996), and *R. rhodochrous* M33 (Kim et al. 2001) were shown to catalyze the conversion of acrylonitrile into acrylamide. Acrylamide is an important commodity chemical and is used to manufacture various polymers for petroleum recovery, wastewater treatment, papermaking, pesticide formulations, soil erosion prevention, and gel electrophoresis (Kim et al. 2001; Myagchenkov and Kurenkov 1991; Yamada and Kobayashi 1996). Acrylamide is currently produced through the hydration of acrylonitrile using free or immobilized cells of *R. rhodochrous* J1 or M33 possessing a high molecular mass nitrile hydratase (H-NHase, EC 4.2.1.84) at an industrial scale (Nagasawa et al. 1993; Yamada and Kobayashi 1996).

The genes coding for cobalt-type H-NHase in *R. rhodochrous* J1 are known to be organized as an operon, *nhhBAG*, and regulated by an amide at the transcriptional level (Komeda et al. 1996). Previous studies have revealed

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that the expression level of NHase in *R. rhodochrous* J1 reaches about 50 % of the total soluble proteins when cultured in a medium containing urea and cobalt (Nagasawa et al. 1991; Yamada and Kobayashi 1996). However, a long cultivation time (72–100 h) is generally required to obtain the maximum cell mass of *R. rhodochrous* (Kim et al. 2001; Nagasawa et al. 1991), and cell density is relatively low. In an effort to increase the cell density and shorten the cultivation time, the cloning and overexpression of NHase from various sources in different organisms such as *Escherichia coli*, *Pichia pastoris*, and *Rhodococcus* sp. have been attempted (Kobayashi et al. 1991; Liebeton and Eck 2004; Nojiri et al. 1999; Kim and Oriel 2000; Komeda et al. 1997). Nonetheless, the levels of NHase activity in recombinant cells are much lower than in *R. rhodochrous* J1 (Prasad and Bhalla 2010; Yamada and Kobayashi 1996).

Herein, we report a high-level functional expression of H-NHase from *R. rhodochrous* M33 in *Corynebacterium glutamicum* (ATCC 13032) to enhance the specific activity of NHase in recombinant cells. We chose *Corynebacterium* species as a host organism because *Corynebacterium* cells offer significant advantages over other microorganisms for the production of various organic and biological products (Hermann 2003; Date et al. 2006). In *C. glutamicum*, we first expressed the *nhhBAG* coding for H-NHase of *R. rhodochrous* M33 under the control of an *ilvC* promoter (Patek et al. 1996). To increase the expression level of the enzyme, we modulated the stability of the secondary structure of the translational initiation region and introduced a conserved Shine-Dalgarno (SD) sequence between the 5′-untranslated region of *ilvC* upstream and downstream of the initiation codon in *nhhB* ORF. A fed-batch culture of the constructed strain was carried out to investigate the specific activity and productivity of the enzyme in the recombinant cells. We also conducted a conversion of acrylonitrile into acrylamide using recombinant *Corynebacterium* cells. Details are reported herein.

## Materials and methods

### Bacterial strains, plasmids, and gene manipulation

The bacterial strains and plasmids used in this study are listed in Table 1. *R. rhodochrous* M33 and *C. glutamicum* were used to provide the DNA templates for a cloning of the *nhhBAG* (GenBank accession number: DI030052) and the promoter region of the *ilvC* gene (GenBank accession number: L09232), respectively. An *E. coli*/*C. glutamicum* shuttle plasmid, pCES208, was used for molecular investigation in *C. glutamicum* and *E. coli* (Park et al. 2008). Restriction enzymes were purchased from New England Biolabs, Inc. The DNA was amplified through a polymerase chain reaction (PCR) with *Pfu*-X DNA polymerase (SolGent, Korea). PCR purification and gel extraction kits were purchased from GeneAll Biotechnology (Korea). All of the PCR constructs were verified through DNA sequencing. Plasmid DNA was

**Table 1** The bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristics | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* Top10 | F⁻*mcr*A Δ(*mrr-hsa*RMS-*mcr*BC) φ80*lacZ*ΔM15 ΔlacX74 *recA1 araD139* Δ(*ara-leu*)7679 *gal*U *gal*K *rps* ($Str^R$) endA1 *nup*G | Invitrogen, USA |
| *C. glutamicum* | | |
| ATCC 13032 | Wild-type strain | This lab. |
| AA424 | *C. glutamicum* ATCC 13032/pNBM4 | This work |
| *R. rhodochrous* M33 | | |
| KCCM 10635 | Nitrile hydratase-producing strain | Yanenko et al. (1998) |
| Plasmids | | |
| pKK223-3 | Expression vector with *tac* promoter; $Amp^R$ | This lab. |
| pKBAG26 | pKK223-3 derivative; 1.4 kb of *nhhBAG* ORF | This work |
| pCES208 | *E. coli*/*C. glutamicum* shuttle vector; 5.93 kb, $Kan^R$ | This lab. |
| pNBW33 | pCES208 derivative; 2.38 kb of $P_{ilvC}$-*nhhBAG*-$T_{rm}$ | This work |
| pNBM1 | pCES208 derivative; 2.38 kb of $P_{ilvC}$-M1-*nhhBAG*-$T_{rm}$ | This work |
| pNBM2 | pCES208 derivative; 2.38 kb of $P_{ilvC}$-M2-*nhhBAG*-$T_{rm}$ | This work |
| pNBM3 | pCES208 derivative; 2.38 kb of $P_{ilvC}$-M3-*nhhBAG*-$T_{rm}$ | This work |
| pNBM4 | pCES208 derivative; 2.38 kb of $P_{ilvC}$-M4-*nhhBAG*-$T_{rm}$ | This work |
| pNBM5 | pCES208 derivative; 2.38 kb of $P_{ilvC}$-M5-*nhhBAG*-$T_{rm}$ | This work |

*ATCC* American Type Culture Collection, $Amp^R$ ampicillin resistance, $Kan^R$ kanamycin resistance, *KCCM* Korean Culture Center of Microorganisms

transformed into *C. glutamicum* through electroporation (van der Rest et al. 1999).

## Media and culture conditions

For the production of H-NHase by *C. glutamicum* variants, the cells were cultivated in 20 mL of a Luria-Bertani (LB) medium (tryptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 10 g/L) supplemented with 50 mg/L of kanamycin and 20 mg/L of cobalt chloride in a shaking incubator at 32 °C overnight. For a large-scale production of NHase, the cells were grown in a 5-L jar fermentor containing 2 L of a fermentation medium (50 g of glucose, 10 g of $(NH_4)_2SO_4$, 3 g of $KH_2PO_4$, 3 g of a yeast extract, 5 g of $MgSO_4{\cdot}7H_2O$, 10 mg of $FeSO_4{\cdot}7H_2O$, 10 mg of $MnSO_4{\cdot}4H_2O$, 10 mg of $ZnSO_4{\cdot}7H_2O$, 1 mg of $CuSO_4{\cdot}4H_2O$, 50 mg of $CoCl_2{\cdot}6H_2O$, 10 mg of $CaCl_2{\cdot}2H_2O$, 0.1 mg of biotin, 1 mg of thiamine, and 50 mg of kanamycin per liter of water at a pH of 7.0). A seed culture was grown at 32 °C for 8 h in a 1-L flask containing 200 mL of an LB medium and then inoculated in a 5-L jar fermentor. During the cultivation, a 400mL mixture of glucose, $KH_2PO_4$, and $CoCl_2{\cdot}6H_2O$ at final amounts of 200 g, 2 g, and 20 mg, respectively, was fed twice when the glucose was completely depleted. The pH was controlled at 7.0 with $NH_4OH$, and the temperature was maintained at 32 °C. The aeration rate and agitation speed were fixed at 1 vvm (air volume/culture volume/min) and 800 rpm, respectively.

## Construction of NhhBAG expression vector

The primers used in this study are listed in Table 2. The *nhhBAG* coding for H-NHase from *R. rhodochrous* M33 was cloned and expressed in pKK223-3 and pCES208. Amplification of *nhhBAG* was conducted using primers P1 and P2. An amplified fragment of about 1.63 kb was doubly digested with *Eco*RI and *Hin*dIII followed by ligation with *Eco*RI/*Hin*dIII-cleaved pKK223-3. The resulting plasmid was designated as pKBAG26 and transformed into *E. coli* Top10. A 0.3-kb *ilvC* promoter from *C. glutamicum* and 2.08-kb *nhhBAG* linked with the *rrn* transcriptional terminator in pKBAG26 were amplified through PCR using primer sets P3-P4 and P5-P6, respectively. A second round of PCR was conducted using the combined PCR products with primer set P3-P6. A 2.38-kb fragment was purified and digested by *Xba*I/*Bam*HI, followed by ligation with pCES208/*Xba*I/*Bam*HI, which yielded pNBW33.

## Mutagenesis in translation initiation region and calculation of $\Delta G$

Plasmids harboring five different types of mutations in the translation initiation region (M1 through M5) were constructed through PCR-based mutagenesis as follows. DNA fragments, $P_{ilvC}$-M1, $P_{ilvC}$-M2, $P_{ilvC}$-M3, $P_{ilvC}$-M4, $P_{ilvC}$-M5, M1-*nhhBAG*, M2-*nhhBAG*, M3-*nhhBAG*, M4-*nhhBAG*, and M5-*nhhBAG*, were amplified through PCR using primer sets P3-P7, P3-P8, P3-P9, P3-P10, P3-P11, P12-P6, P13-P6, P14-P6, P15-P6, and P16-P6, respectively. PCR products of 0.3 kb, $P_{ilvC}$-M1, $P_{ilvC}$-M2, $P_{ilvC}$-M3, $P_{ilvC}$-M4, and $P_{ilvC}$-M5, were combined with M1-*nhhBAG*, M2-*nhhBAG*, M3-*nhhBAG*, M4-*nhhBAG*, and M5-*nhhBAG*, respectively, and a second round of PCR was then conducted using primer sets 3–6. Five plasmids, pNBM1, pNBM2, pNBM3, pNBM4, and pNBM5, were constructed by digesting pCES208 with *Xba*I and *Bam*HI, followed by ligation with about 2.38 kb of *Xba*I/*Bam*HI fragments, which contained $P_{ilvC}$-M1-*nhhBAG*, $P_{ilvC}$-M2-*nhhBAG*, $P_{ilvC}$-M3-*nhhBAG*, $P_{ilvC}$-M4-*nhhBAG*, and $P_{ilvC}$-M5-*nhhBAG*, respectively. The minimal folding free energy, $\Delta G$, for the RNA secondary structure(s) of 80 nucleotides within the 5′-untranslated region of *ilvC* upstream and downstream of the initiation codon in *nhhB* ORF (wild-type sequence) was calculated based on the Mfold Web server (Zuker 2003).

**Table 2** Primer sequences used in this study

| Primer | Sequence (5′–3′) | Restriction enzyme site |
|---|---|---|
| P1 | CCCGGAATTCATGGATGGTATCCACGACACAG | *Eco*RI |
| P2 | CCCCAAGCTTTCAGTCGATGATGGCCATCGA | *Hin*dIII |
| P3 | CTAGTCTAGACCAGGCAAGCTCCGCGCACT | *Xba*I |
| P4 | CTGTGTCGTGGATACCATCCATGGGAGAAAATCTCGCCTTTC | |
| P5 | ATGGATGGTATCCACGACACAG | |
| P6 | CCGCGGATCCGCAAAAAGGCCATCCGTCAG | *Bam*HI |
| P7 | TAGAAAATCTCGCCTTTCGTAAAAATTTTGGTTAAAAGATGTCTGCCCCCCACT | |
| P8 | TAGAAAATCTCGCCTGTCGTAAAAATTTTGGTTAAAAGATGTCTGCCCCCCACT | |
| P9 | TAGATAATCTCGCCTTTCGTAAAAATTTTGGTTAAAAGATGTCTGCCCCCCACT | |
| P10 | TAGAAAATCTCTCCTTTCGTAAAAATTTTGGTTAAAAGATGTCTGCCCCCCACT | |
| P11 | TAGATAATCTCTCCTTTCGTAAAAATTTTGGTTAAAAGATGTCTGCCCCCCACT | |
| P12 | TTACGAAAGGCGAGATTTTCTATAATGGACGGAATCCACGACACAGGCGGCA | |
| P13 | TTACGACAGGCGAGATTTTCTATAATGGACGGAATCCACGACACAGGCGGCAT | |
| P14 | TTACGAAAGGCGAGATTATCTATAATGGACGGAATCCACGACACAGGCGGCA | |
| P15 | TTACGAAAGGAGAGATTTTCTATAATGGACGGAATCCACGACACAGGCGGCA | |
| P16 | TTACGAAAGGAGAGATTATCTATAATGGACGGAATCCACGACACAGGCGGCA | |

Restriction enzyme sites are underlined

### Enzyme assay

The nitrile hydratase activity was determined according to the previously reported method with a slight modification (Kim et al. 2001). The 1-mL reaction mixture contained 50 mM of K-Pi buffer (pH of 7.2), 300 mM of acrylonitrile, and 0.03 mg DCW. Following incubation for 10 min at 25 °C, the reaction was stopped by the addition of 80 μL of 1 M HCl. The amount of acrylamide was determined spectrophotometrically at 235 nm. The specific activity of the acrylamide production was defined as the amount of DCW in milligrams that catalyzes the formation of 1 μmol of acrylamide per minute. An enzyme assay was conducted in triplicate.

### Protein expression analysis

Cells were disrupted by a beadbeater (Biospec Product, Inc), and the cell debris was removed through centrifugation (13,000×*g*, 30 min), yielding crude extracts used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined using the Bradford method (Bio-Rad) with bovine serum albumin as the standard. The gel band density was analyzed using TotalLab Quant. Each experiment was performed using two independent applications of SDS-PAGE.

### Bioconversion

The acrylamide production was carried out in a 2-L jacketed stirred reactor containing a 0.5-L reaction mixture of acrylonitrile, recombinant *Corynebacterium* cells, and demineralized water. The temperature was controlled at 18 °C through the circulation of cooling water, and the agitation speed was fixed at 500 rpm. Acrylonitrile was continuously fed into the reactor using a peristaltic pump to maintain the concentration below 4 % during the reaction. The amount of nitrile consumed and amide formed in the reaction mixture were determined using high-pressure liquid chromatography (HPLC, Shimadzu, Japan). The final test solution (20 μL) after the preparation was injected onto a reversed ACE C18 column (4.6×250 mm, 5 μm, 300 Å) maintained at 30 °C. The elution mode was isocratic using a mixture of 10 % acetonitrile and 90 % water containing 0.1 % formic acid as a mobile phase at a flow rate of 1 mL/min.

## Results

### Expression in *C. glutamicum* of NhhBAG from *R. rhodochrous* M33

To express the *nhhBAG* gene coding for H-NHase from *R. rhodochrous* M33 in *C. glutamicum*, the *nhhBAG* with a promoter $P_{ilvC}$ was inserted into pCES208 (Patek et al. 1996), and the resulting plasmid was designated as pNBW33. We tested the expressions of NhhB and NhhA in *C. glutamicum* and observed two thin bands corresponding to molecular masses of about 26 and 23 kDa, respectively, from the crude cell extract (Fig. 2). The NHase activity from the whole cells of *C. glutamicum*/pNBW33 was estimated to be 13.6 μmoL/min/mg DCW (Table 3), whereas *R. rhodochrous* M33 exhibited an enzyme activity of about 61 μmoL/min/mg DCW. The NHase activity in *C. glutamicum* was much lower than in *R. rhodochrous* M33, which seemed to be mainly due to a low expression level of the enzyme.

### Effect of mutations in the translation initiation region of *nhhBAG* genes

It has been suggested that the expression of heterologous proteins in recombinant cells is affected by various factors including the vector design, gene dosage, promoter strength, translational efficiency and stability of the messenger RNA (mRNA), translation termination, and codon usage (Jana and Deb 2005). Of these, the efficiency of the translation initiation is known to be critical for high-level expression of proteins and is strongly influenced by the accessibility of ribosome to mRNA and the secondary structure around the translation initiation region in the 5′-untranslated region of bacterial mRNAs (de Smit and van Duin 1994; Park et al. 2007; Salis et al. 2009; Seo et al. 2009; Tsao et al. 2011). To obtain some insight into the stability of a secondary structure around the translation initiation region, we calculated the $\Delta G$ values for the predicted secondary structures from a wild-type sequence containing 80 nucleotides in the translation initiation region of the *nhhB* genes. The $\Delta G$ values were revealed to range from −12 to −6.59 kcal/mol for four types of RNA secondary structures (Table 3). Previous studies have shown that the change in $\Delta G$ around a hairpin structure harboring the SD sequence by −1.4 kcal/mol results in a 10-fold decrease in the translation initiation rate (de Smit and van Duin 1994). In addition, the introduction of 5′-untranslated region (UTR) with different $\Delta G$ values (−12 to −3 kcal/mol) using a de novo design or directed evolution has been shown to affect the gene expression (Neupert et al. 2008).

To increase the expression level of NHase, we introduced an M1-type mutation into the translation initiation region of the *nhhB* gene through iterative changes within 80 nucleotides to increase $\Delta G$. However, the $\Delta G$ of the mutant sequence decreased to −4.82 kcal/mol. Two mutation types, M2 and M3, were introduced in positions 23 and 34, respectively, for disrupting a hairpin loop structure harboring the SD sequence of M1 (Fig. 1), resulting in an increase in $\Delta G$. The efficiency of the translation initiation was shown to be determined based on the accessibility of the ribosome to mRNA, because the SD sequence facilitates 16S ribosomal RNA (rRNA)-specific

**Table 3** Mutation sequences and predicted $\Delta G$ values of 80 nucleotides in the translational initiation region of *nhhB*

| Mutation | Sequence [a] | Predicted ΔG (kcal/mol) |
|---|---|---|
| Wild-type ($P_{ilvC}$-*nhhB*) | CTTTTCACCAAAATTTTTACGAAAGGCGA GATTTTCTCCC***ATG***GATGGTATCCACGACA CAGGCGGCATGACCGGATACG | −12 – −6.59 |
| M1 ($P_{ilvC}$-M1-*nhhB*) | CTTTTAACCAAAATTTTTACGAAAGGCGA GATTTTCTATA***ATG***GACGGAATCCACGACA CAGGCGGCATGACCGGATACG | −4.82 |
| M2 ($P_{ilvC}$-M2-*nhhB*) | CTTTTAACCAAAATTTTTACGACAGGCGA GATTTTCTATA***ATG***GACGGAATCCACGACA CAGGCGGCATGACCGGATACG | −5.8 – −1.53 |
| M3 ($P_{ilvC}$-M3-*nhhB*) | CTTTTAACCAAAATTTTTACGAAAGGCGA GATTATCTATA***ATG***GACGGAATCCACGACA CAGGCGGCATGACCGGATACG | −3.57 – −2.03 |
| M4 ($P_{ilvC}$-M4-*nhhB*) | CTTTTAACCAAAATTTTTACGAAAGGAGA GATTTTCTATA***ATG***GACGGAATCCACGACA CAGGCGGCATGACCGGATACG | −7.8 – −4.82 |
| M5 ($P_{ilvC}$-M5-*nhhB*) | CTTTTAACCAAAATTTTTACGAAAGGAGA GATTATCTATA***ATG***GACGGAATCCACGACA CAGGCGGCATGACCGGATACG | −6.7 – −3.51 |

[a] Boxes show the putative Shine-Dalgarno sequence. The underlined nucleotides indicate mutated bases in each mutation type compared to the wild-type sequence, and the bold italics indicate a start codon.

ribosome binding during the translation initiation step in prokaryotes (Park et al. 2007; Salis et al. 2009). The putative SD sequence in $P_{ilvC}$ was predicted to be 5′-GAAAGGCGA-3′ that is not fully complementary to the anti-SD sequence of the *C. glutamicum* 16S rRNA, whereas the consensus SD sequence in *C. glutamicum* was proposed as 5′-GAAAGGAG G-3′ (Martín et al. 2003). Based on this finding, the mutation type M4 was designed by changing C into A at 27nt from M1 (Fig. 1). Finally, the mutation type M5 was derived from M1 by introducing the consensus SD sequence and disrupting the hairpin loop structure at positions 27 and 34, respectively.

## Overexpression of nitrile hydratase

We tested the effect of the mutation types in the translation initiation region on the expression and activity of H-NHase in *C. glutamicum* (Fig. 2 and Table 4). The mutation type M1 resulted in a relatively higher expression level and activity compared to the wild type, which seems to be due to an increase in the minimal folding free energy near the translation initiation region. It was reported that a low level of mRNA secondary structure leads to an increase in the expression level of proteins (Kudla et al. 2009). On the other hand, mutation types M2 and M3, with increased $\Delta G$ values owing to a destabilization of the hairpin structure in M1, had a negative effect on the expression level. The M2 type with a mutation in the conserved SD sequence showed the lowest expression level, whereas the mutation type M4 with a change in putative SD sequence of $P_{ilvC}$ revealed the highest expression level and activity of NHase. These results demonstrate that a more conserved SD sequence in the translation initiation region can increase the enzyme translation. Previous studies have also shown a close relationship between the translational efficiency and the ribosome binding affinity (Park et al. 2007; Salis et al. 2009). The mutation type M5 with a more conserved SD sequence and destabilized hairpin structure showed a similar expression level and enzyme activity to those of the M1 type, which seems to be due to the formation of other stable secondary structure(s) and decreased the ribosome binding affinity around the translation initiation region in the M5 type. Collectively, the mutation type M4 showed about a 2.7-fold increase in the NHase activity, which seems to result from a decreased stability of the secondary structure and the conserved SD sequence in the translation initiation region.

## Effect of cobalt ion on the enzyme activity

NHase from *R. rhodochrous* M33 is known to require non-corrin cobalt for the catalytic activity (Kim et al. 2001; Kobayashi and Shimizu 1998). We tested the effect of cobalt

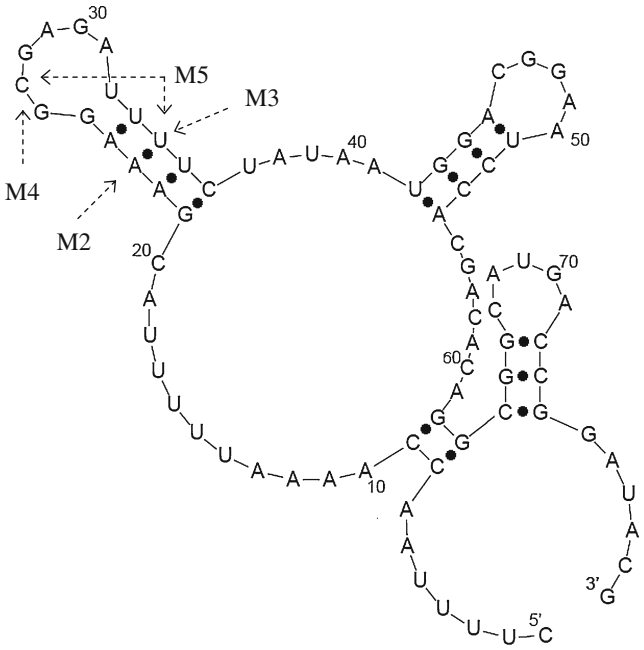


**Fig. 1** The predicted mRNA secondary structure of the M1 sequence. The *dots* indicate a base pairing of A and U or G and C. The *dotted arrows* indicate mutation position(s) of M2, M3, M4, and M5

concentration on the cell growth and NHase activity of recombinant *C. glutamicum* (Fig. 3). The enzyme activity gradually increased with an increase in cobalt concentration and reached a maximum level of 40 to 46 U/mg DCW at the cobalt chloride concentrations of 30 to 70 mg/L, whereas the cell growth remained at similar levels even though the cobalt chloride changed from 10 to 70 mg/L. In contrast, the NHase activity was very low when cobalt was not added, which indicates that cobalt ions are necessary for the expression and activity of NHase in *C. glutamicum*.

## Production of nitrile hydratase by a fed-batch culture

To evaluate the performance of recombinant *Corynebacterium* cells in the expression of nitrile hydratase, we performed a fed-batch culture of the cells and analyzed the specific enzyme activity and cell growth (Fig. 4). The maximum cell mass and cell yield coefficient on glucose were estimated as 53.4 g DCW/L and 44.5 g cell/g glucose, respectively. It was reported that the cultivation of *R. rhodochrous* requires a high concentration of urea for an induction of NHase (Kim et al. 2001; Nagasawa et al. 1991). In the recombinant *Corynebacterium* cells, the enzyme was constitutively expressed without the addition of urea, and the specific enzyme activity remained almost constant after 8 h of cell growth. The total enzyme activity gradually increased with the cell growth until 18 h, and the maximum total activity reached about 1,992 U/mL. The specific activity of the enzyme in *Corynebacterium* cells was lower than that in *Rhodococcus* cells, but the culture time was shortened significantly. Consequently, the production rate of the enzyme by

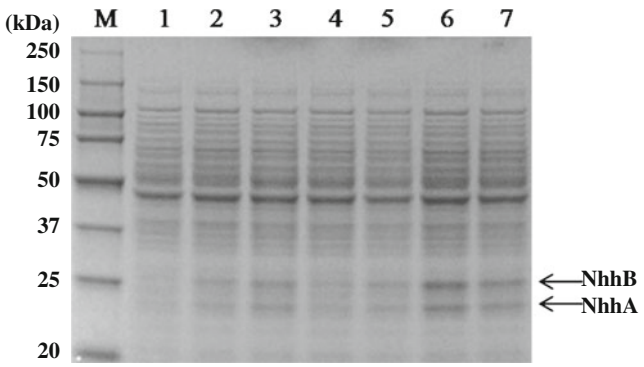


**Fig. 2** SDS-PAGE analysis of NhhB and NhhA expression by *C. glutamicum* harboring different plasmid variants. The proteins were separated by 10 % SDS. Lanes: *M* protein size marker; *1* pCES208, *2* pNBW33, *3* pNBM1, *4* pNBM2, *5* pNBM3, *6* pNBM4, *7* pNBM5

**Table 4** Relative expression ratio of NhhB and NhhA and specific activity of NHase in *C. glutamicum*

| *C.glutamicum*/ plasmid | Relative expression on SDS-PAGE | | Cell $OD_{600nm}$ | Specific activity (U/mg DCW) |
|---|---|---|---|---|
| | NhhB | NhhA | | |
| /pCES208 | – | – | 8.1±0.2 | 0 |
| /pNBW33 | 1.0 | 1.0 | 6.7±1.1 | 13.6±2.4 |
| /pNBM1 | 1.7±0.1 | 1.7±0.1 | 7.2±0.8 | 29.7±3.1 |
| /pNBM2 | 0.8±0.1 | 0.6±0.1 | 7.7±0.9 | 8.8±0.8 |
| /pNBM3 | 1.4±0.1 | 2.2±0.2 | 6.9±0.5 | 22.9±2.7 |
| /pNBM4 | 2.9±0.5 | 3.7±0.6 | 7.4±0.5 | 36.9±2.0 |
| /pNBM5 | 2.1±0.4 | 2.7±0.3 | 7.1±1.6 | 29.9±1.3 |

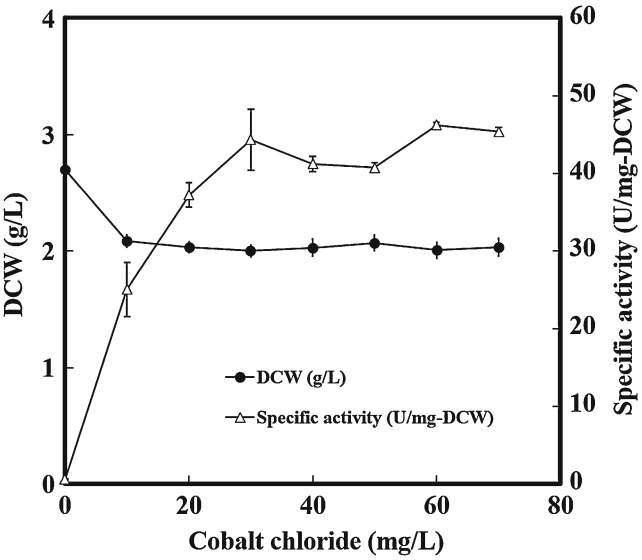


**Fig. 3** Effect of cobalt concentration on the cell growth and production of nitrile hydratase in *C. glutamicum*. The cells were cultivated overnight in 20 mL of an LB medium containing different concentrations of cobalt chloride in a shaking incubator at 32 °C. Experiments were carried out in duplicate, and averages and *error bars* are presented

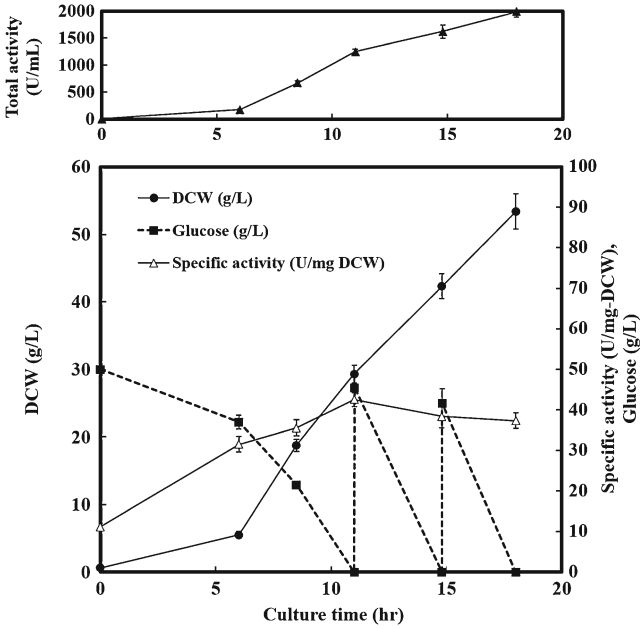


**Fig. 4** Cell growth and the production of nitrile hydratase by a fed-batch culture of recombinant *C. glutamicum* AA424. Experiments were carried out in duplicate, and averages and *error bars* are shown

the recombinant *Corynebacterium* cells was estimated to be 4-fold higher than that by *R. rhodochrous* M33 (Kim et al. 2001).

## Conversion of acrylonitrile into acrylamide by recombinant *Corynebacterium* cells

We conducted a conversion of acrylonitrile into acrylamide using recombinant *Corynebacterium* cells in a 2-L jacketed stirred reactor at 18 °C. The substrate was continuously fed into the reactor as the conversion proceeded, and the substrate concentration was maintained lower than 4.0 %. The production rate and final concentration of acrylamide increased with an increasing amount of cells from 1.4 to 3.7 g DCW and reached 425 g/L in 6 h at 3.7 g DCW (Fig 5). Acrylonitrile was completely consumed until 3 h and accumulated below 40 g/L when 3.7 g DCW was initially added. The conversion yield (mol/mol %) of acrylamide from acrylonitrile was estimated to be 93 % when the total amount of fed acrylonitrile was 456 g. This result indicates that recombinant *Corynebacterium* cells can be effectively used for economic production of acrylamide on an industrial scale.

## Discussion

We have shown that recombinant *Corynebacterium* cells expressing nitrile hydratase from *R. rhodochrous* can be used for the economic production of acrylamide from acrylonitrile. The bacterium *C. glutamicum* is Gram-positive and is widely used for the industrial production of amino acids, offering some advantages over other bacteria in the industrial production of diverse organic chemicals (Toru et al. 2012; Wieschalka et al. 2013). Currently, acrylamide is produced using recombinant *Rhodococcus* cells on an industrial scale (Kobayashi and Shimizu 1998; Ogawa and Shimizu 2002), but the use of *Rhodococcus* cells has some drawbacks such as a long cultivation time and low cell density. The use of recombinant *Corynebacterium* cells expressing nitrile hydratase for the production of acrylamide showed a competitive performance in terms of the final product concentration and conversion yield when compared to *Rhodococcus* cells, mainly owing to the high density of cells in a much shorter cultivation time.

For overexpression of heterologous proteins in *C. glutamicum*, several approaches have been attempted, including the development of promoters and expression modules as well as the changes in the −10 region sequence of promoter, spacer length, and start codon (Nesvera and Patek 2011; Srivastava and Deb 2005; Schneider et al. 2012). We tested the available promoters (tac, ilvc, and sod), but they were not effective for improving the expression level of the enzyme in *C. glutamicum*. Recent studies have demonstrated that the expression level of proteins is strongly influenced by the secondary structure and accessibility of ribosome to the SD sequence around the TIR (Park et al. 2007; Salis et al. 2009; Seo et al. 2009). In our study, we focused on the modification of a secondary structure based on the predicted Gibbs free energies in the 5′-UTR region and introduction of consensus RBS sequence for the overexpression of nitrile hydratase in *C. glutamicum.* The $\Delta G$ values for the wild-

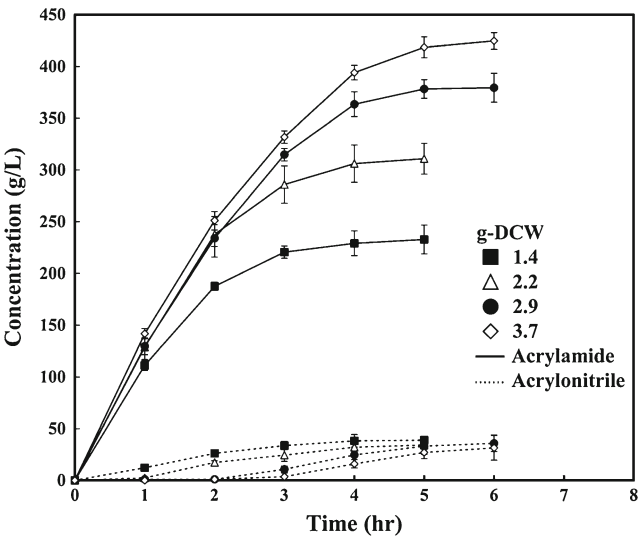


**Fig. 5** Conversion profile of acrylonitrile into acrylamide by recombinant *Corynebacterium* cells. The conversion was carried out in a 2-L jacketed stirred reactor at 18 °C for 6 h. The acrylonitrile solution was continuously fed into the reactor. Experiments were carried out in duplicate, and averages and *error bars* are presented

type sequence were shown to range from −12 to −6.59 kcal/mol, depending on the RNA secondary structures. The M1-type mutation was revealed to contribute to an increase in $\Delta G$ value, leading to a 2.2-fold increase in NHase activity. An analysis of the mRNA structure in the M1 sequence showed that a hairpin loop structure and ribosome binding site overlapped each other (Fig 1). This result suggests that the mRNA secondary structure of TIR in $P_{ilvC}$-M1-*nhhB* hinders the accessibility of the ribosome to the SD sequence and consequently decreases the translation initiation rate. Two mutation types, M2 and M3, which were constructed by disrupting a hairpin loop structure of M1, yielded a slight increase in $\Delta G$ values compared to M1. However, both M2 and M3 types showed a lower expression and NHase activity than the M1 mutant, which implies that an increase in $\Delta G$ value at above −4.83 has no significant effect on the NHase expression (de Smit and van Duin 1994). An introduction of the consensus SD sequence of *C. glutamicum* into the M1 sequence resulted in a 2.7-fold increase in the enzyme activity compared to the wild type. These results clearly indicate that both the secondary-structure instability of mRNA and the introduced SD sequence around the TIR are crucial for the overexpression of NHase in *C. glutamicum*.

The advantage of recombinant *Corynebacterium* cells for the enzyme production was clearly demonstrated through a fed-batch culture. The final cell mass reached 53.4 g DCW in 18 h, and accordingly, the enzyme production rate by the recombinant *Corynebacterium* cells was estimated to be 4-fold higher than that by the *R. rhodochrous* cells. The specific enzyme activity in *Corynebacterium* cells was lower than in *Rhodococcus*, but this low specific activity can be overcome by a much shorter culture time and high cell density. The production of acrylamide from acrylonitrile using recombinant *Corynebacterium* cells showed a competitive performance. The final concentration and maximum conversion yield of acrylamide reached 43 and 93 %, respectively, when acrylonitrile was fed up to 456 g. Based on these results, recombinant *Corynebacterium* cells can be effectively used for the production of acrylamide from acrylonitrile, providing a more economically feasible process compared to the currently used method. Our approach is expected to be applied to other bioconversion processes based on whole cell enzymes.

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