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An over expression and high efficient mutation system of a cobalt-containing nitrile hydratase

Hui-Min Yu [a,*], Yue Shi [a], Hui Luo [b], Zhuo-Ling Tian [a], Yan-Qin Zhu [a], Zhong-Yao Shen [a,*]

[a] *Institute of Biochemical Engineering, Department of Chemical Engineering, Tsinghua University, Beijing 100084, China*
[b] *Department of Environmental Engineering, University of Science and Technology Beijing, Beijing 100083, China*

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Abstract

A superior novel recombinant strain, *E. coli* BL21(DE3)/pETNH^{M}, containing the start codon mutation of the α subunit, was constructed and selected as an overexpression and high efficient mutation platform for the genetic manipulation of the nitrile hydratase (NHase). Under optimal conditions, the specific activity of the recombinant strain reached as high as 452 U/mg dry cell. Enzymatic characteristics studies showed that the reaction activation energy of the recombinant NHase^{M} was 24.4 ± 0.5 kJ/mol, the suited pH range for catalysis was 5.5–7.5, and the K_m value was 4.34 g/L (82 mM). To assess the feasibility of the NHase improvement by protein rational design using this *E. coli*, site-directed mutagenesis of αS122A, αS122C, αS122D and βW47E of the NHase^{M} were carried out. The NHase^{M} (αS122A) and NHase^{M} (αS122D) mutants were entirely inactive due to the charge change of the side-chain group. The product tolerance of the NHase^{M} (αS122C) mutant was enhanced while its activity decreased by 30%. The thermo-stability of the NHase^{M} (βW47E) mutant was significantly strengthened, while its activity reduced by nearly 50%. These results confirmed that the specific activity of the mutant NHase expressed by the recombinant *E. coli* BL21(DE3)/pETNH^{M} can reasonably change with and without mutations. Therefore, this recombinant *E. coli* can be efficiently and confidently used for the further rational/random evolution of the NHase to simultaneously improve the activity, thermo-stability and product tolerance of the target NHase.

Keywords: Nitrile hydratase; Recombinant *E. coli*; Site-directed mutagenesis; Thermo-stability; Product tolerance

1. Introduction

Nitrile hydratase (NHase) [EC 4.2.1.84], catalyzing the hydration of nitriles to the corresponding amides [1], is a heterodimeric enzyme from microorganism and contains either a non-heme iron or a non-corrin cobalt at the respective catalytic center. NHase is extensively applied as a novel biocatalyst for acrylamide (AM) production from acrylonitrile (AN).

In recent years, great progresses have been achieved after many years of efforts in the screening and optimization of wild strains, such as *Rhodococcus*, *Pseudomonas* and *Bacillus* [2,3]. Furthermore, some species of the wild *Rhodococcus* and *Nocardia* had already been successfully applied in the industrial production of acrylamide from acrylonitrile, in which the activity of NHase could reach as high as 248 U/mg dry cell [4]. However, the wild strains still have some disadvantages, such as the poor thermo-stability and AM inactivation of the NHase, and the accumulation of the by-product, acrylic acid. In order to overcome these obstacles, the heterogenous cloning and expression of NHase in different recombinant strains had already been carried out by some researchers, but the results were not as good as expected [5–7], except a recombinant *Rhodococcus* harboring a shuttle-plasmid showing the NHase activity of 518 U/mg dry cell [8].

In this study, a novel recombinant strain, *E. coli* BL21(DE3)/pETNH^{M}, was successfully constructed for overexpression of the NHase. Subsequently, site-directed mutagenesis was further run for examining the feasibility to improve the thermo-stability and AM tolerance of the recombinant NHase. Finally, an active and confident expression and mutation system was constructed as the technical platform for genetic manipulations of the recombinant NHase.

* Corresponding authors. Tel.: +86 10 62788568; fax: +86 10 62770304.
E-mail addresses: yuhm@tsinghua.edu.cn (H.-M. Yu), szy-dce@tsinghua.edu.cn (Z.-Y. Shen).

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Table 1
Plasmids and bacterial strains used in this study

	Features and genotypes	Sources
Plasmids		
pUC18	2.7 kb, *Amp*^{r}	Dingguo
pET-28a	5.4 kb, *Kan*^{r}	Novagen
pUC18-NHase	4.4 kb, NHase, *Amp*^{r}	This study
pUC18-NHase^M	4.4 kb, NHase, αMet(*gtg*)1Met (*atg*), *Amp*^{r}	This study
pETNH^M (pET28-NHase^M)	6.6 kb, NHase, αMet(*gtg*)1Met (*atg*), *Kan*^{r}	This study
pETNH^M (αS122A)	6.6 kb, NHase, αMet(*gtg*)1Met (*atg*), αSer122Ala, *Kan*^{r}	This study
pETNH^M (αS122C)	6.6 kb, αMet(*gtg*)1Met (*atg*), αSer122Cys, *Kan*^{r}	This study
pETNH^M (αS122D)	6.6 kb, αMet(*gtg*)1Met (*atg*), αSer122Asp, *Kan*^{r}	This study
pETNH^M (βW47E)	6.6 kb, αMet(*gtg*)1Met (*atg*), βTrp47Glu, *Kan*^{r}	This study
Host strains		
E. coli JM105	*sup*E *end*A *sbc*B15 *hsd*R4 *rps*L *thi* Δ(*lac* proAB)	This laboratory
E. coli XL1-Blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F^- *Pro*AB *lac*I^{q}ZΔM15 Tn10 (*Tet*^{R})]	Stratagene
E. coli BL21(DE3) pLysS	F^- *omp*T *hsd*S_B($r_B{}^-m_B{}^-$) *dcm gal*(DE3)pLysS *Cm*^{r}	Promega

2. Experimental

2.1. Plasmids and strains

Nocardia YS-2002, kindly endowed by Shengli Administration Bureau of Oil Field, Shandong Province, China, with the NHase activity of over 290 U/mg, was used for genomic DNA extraction to amplify the NHase gene. The recombinant plasmids and bacterial strains used in this study, containing different antibiotic resistances, are listed in Table 1.

2.2. Enzymes, chemicals and culture conditions

Restriction endonucleases, T4 DNA ligase, Pfu DNA polymerase and isopropyl-1-thio-beta-D-galactopyranoside (IPTG) were obtained from Takara Biotechnology Dalian Co. Ltd. The plasmid pUC18 (Dingguo Biotechnology Co. Ltd., China) and pET28a (Novagen, USA) was used for cloning and expression of the NHase in *E. coli*. All other chemicals were of reagent grade and obtained from the local commercial sources.

The composition of the culture medium and growth condition of the *Nocardia* strain was previously described [9]. Cultivation of the recombinant *E. coli* was carried out in flask as described previously [10].

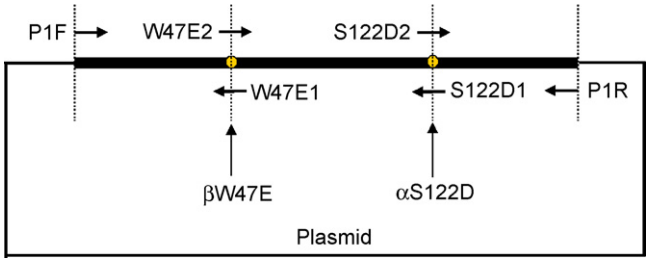

Fig. 1. The sketch map of the primer design for βW47E and αS122D mutations.

2.3. Site-directed mutagenesis and primer design

The ExSite^{TM} PCR-Based Site-Directed Mutagenesis Kit (Stratagene, USA) was used for the manipulation of site-directed mutagenesis of the start codon of α subunit, αS122A and α S122C mutation. The overlap PCR method was used for the mutations of αS122D and βW47E, as designed in Fig. 1. The primers for NHase gene and all of the mutations were listed in Table 2.

2.4. Gas chromatography assay of the enzyme activity

The concentration of acrylamide and acrylonitrile in the reaction mixture and NHase activity were measured by gas chro-

Table 2
PCR primers used in this study

Primer name	Primer sequence (5′ → 3′)	Digestion site	T_m (°C)
S122A1	GCCATGGAGTACCGGTCCCGAGTGG	*Nco*I	52
S122A2	CTTGTACCAGGCGGGCGGGA		52
S122C1	ATGCATGGAGTACCGGTCCCGAGTG	*Eco*T22I	54
S122C2	TTGTACCAGGCGGGCGGGAG		54
P1F	TTTAAGAAGGAGATATACCATGGATGGAT	*Nco*I	56
P1R	CCGCAAGCTTTCATACGATCACTTC	*Hin*dIII	56
W47E1	CGCGACTTGTCCCACTCCGACATGCCCTTGAG		56
W47E2	CTCAAGGGCATGTCGGAGTGGGACAAGTCGCG		56
S122D1	GACCGGTACTCCATGTCCTTGTACCAGGCGGG		56
S122D2	CCCGCCTGGTACAAGGACATGGAGTACCGGTC		56

Note: the underlined sequences are the restriction sites in the primers.

matography (GC-10Avp, Shimadzu, Japan), as described by Shi et al. [10]. One unit of NHase activity corresponds to the amount of enzyme producing 1 μmol AM/min. Specific activity (U/mg) is denoted as μmol (AM)/mg (dry cell) min. SDS-PAGE analyses on whole cell proteins were performed with 4% stacking gel and 12.5% running gel as described [11].

3. Results and discussion

3.1. Construction and cultivation of the superior E. coli BL21(DE3)/pETNH^M

Generally, the NHase is a kind of in-cell enzyme and the intact cells of *Nocardia* or *Rhodococcus* have been broadly applied as the biocatalysts to produce acrylamide in industry. The cell wall is of great importance for maintaining the operational stability of the NHase to resist the denaturing caused by the product. Therefore, the in-cell expression is more preferred to the secretion expression for the NHase in the recombinant *E. coli*. As described in our previous studies [10], the NHase gene from *Nocardia* sp. YS-2002 (GenBank AY168347) contained two subunits, the β subunit with regular start codon, *atg*, and the α subunit with the rare start codon, *gtg*. When this NHase gene was cloned by PCR and expressed in *E. coli* JM105 (pUC18-NHase), no activity of NHase could be detected, which was because that the translation of the α subunit of NHase could not be properly started up by the rare start codon. The site-directed mutagenesis of *gtg* into *atg* enables the NHase^M activity increase to 51 U/mg dry cell, when expressed in *E. coli* XL1-Blue (pUC18-NHase^M).

For overexpression of the NHase, the mutated NHase^M gene was amplified from plasmid pUC18-NHase^M and inserted into pET28a, an inducible plasmid with T7 promoter. A novel plasmid, pETNH^M, was constructed and transformed into *E. coli* BL21(DE3), as shown in Fig. 2. The new recombinant strain, *E. coli* BL21(DE3)/pETNH^M, was subsequently selected and used for the studies of NHase expression and mutation.

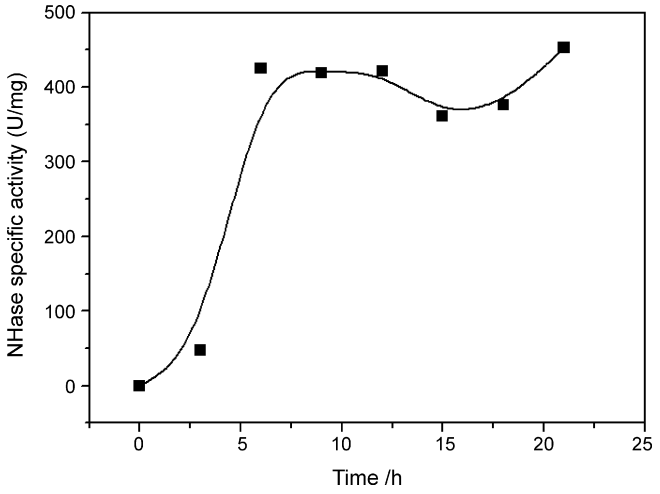

Fig. 3. The optimal expression of the NHase^M in *E. coli* BL21(DE3)/pETNH^M at 28 °C in the 300 mL flask with 0.1 mM IPTG or 3.75 M lactose, 0.4 mM Co^{2+}, LB plus corn steep medium, 1 g/L glucose. The initial inducing OD_{600} was 1.8–2.0.

The cultivation and inducing conditions of *E. coli* BL21(DE3)/pETNH^M were optimized in the shaking flasks to realize the active expression of the NHase^M. All of the important factors affecting cell growth and NHase^M expression were evaluated, i.e. the inoculation volume, the initial inducing OD_{600} and thereafter incubation time, the inducing temperature and dissolved oxygen, the Co^{2+}, IPTG or lactose concentration, the fed-batch approaches of the inducers (Co^{2+}, IPTG or lactose), the composition of the culture medium, the addition of other possible inducers of NHase^M, such as urea, acrylonitrile, acylamide, metal ions and amino acids. The results showed that NHase could be actively expressed in *E. coli* BL21(DE3)/pETNH^M, its final

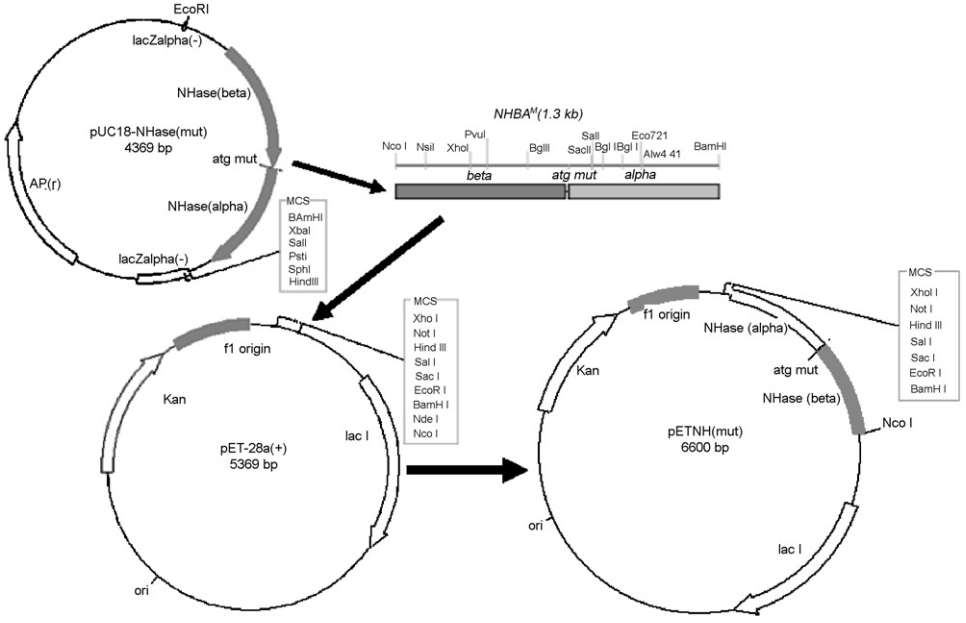

Fig. 2. The sketch map of the construction of plasmid pETNH^M.

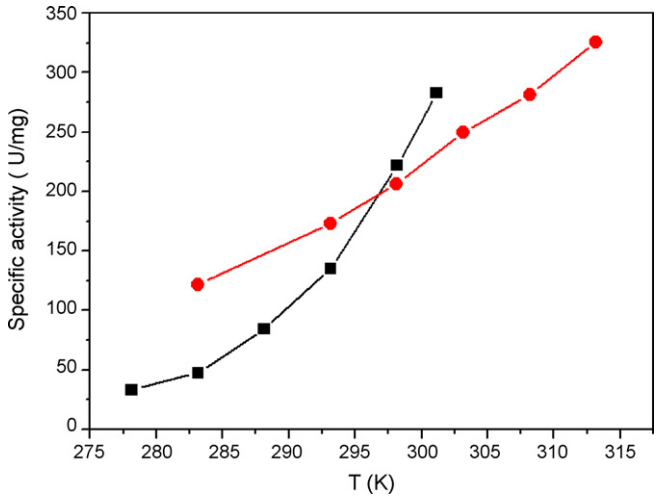

Fig. 4. Effect of temperature to the catalysis rate of the recombinant NHase^{M} in *E. coli* BL21(DE3)/pETNH^{M} (●) and the natural NHase in *Nocardia* (■).

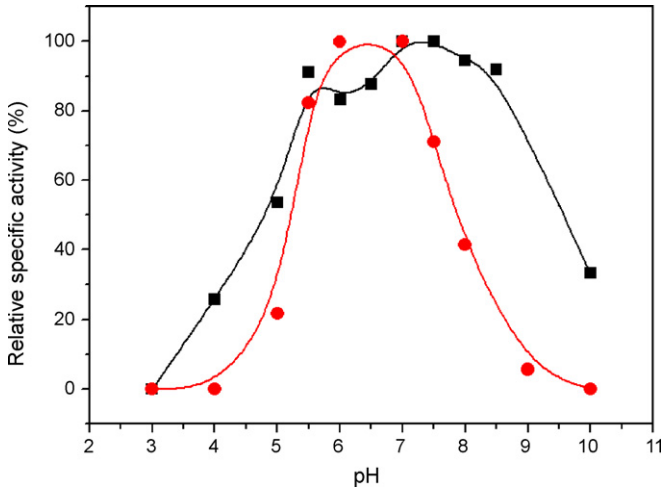

Fig. 5. Comparison of the optimal pH range of the recombinant NHase^{M} in *E. coli* BL21(DE3)/pETNH^{M} (●) and the natural NHase in *Nocardia* (■). Reaction temperature was controlled at 28 °C.

activity reached 452 U/mg, as shown in Fig. 3, which is the highest value of the NHase expressed in the recombinant *E. coli*.

3.2. Enzymatic characteristics of the recombinant NHase^{M} expressed in E. coli BL21(DE3)/pETNH^{M}

Based on the active expression of NHase^{M} in *E. coli* BL21(DE3)/pETNH^{M}, the enzymatic characteristics of the recombinant NHase^{M} in cell was focused on subsequently. At first, the effect of temperature on the catalysis reaction rate (v, U/mg, μmol/(min mg), the same as the specific activity) was assessed by detecting the specific NHase^{M} activity of the recombinant *E. coli* cells incubated at constant concentration of the reaction substrate, acrylonitrile, and different temperatures for 20 min. The results showed that the catalysis rate of NHase^{M} would increase with the elevated temperatures, as plotted in Fig. 4. That is, the enzymatic catalysis reaction of the NHase was apt to generate at higher temperatures. Because the substrate concentration maintained constant under different temperatures, the natural log of v ($\ln v$) would be in positive proportional to $1/T$ according to the Arrhenius equation. Thus, the reaction activation energy (E_a, kJ/mol) of the recombinant NHase^{M} were calculated from the liner fit of $\ln v$ and $1/T$ as 24.4 ± 0.5 kJ/mol, which was 37.2% of the NHase in the wild *Nocardia* YS-2002.

Next, the impact of the hydration pH on the activity of the recombinant NHase^{M} was further reviewed. Carrying out the catalysis reaction under different pH conditions, it could be found that the optimal pH range is 5.5–7.5 for the NHase^{M} in *E. coli* BL21(DE3)/pETNH^{M}, which is a little different from the natural NHase in *Nocardia* having a wider optimal pH range, 5.5–8.5, for the hydration catalysis, as shown in Fig. 5.

Followed studies focused on the K_m value of both the recombinant and the natural NHase. Changing the concentration of substrate, acrylonitrile, in the reaction system, and detected the specific activity of the NHases. The results showed that when the substrate concentration was higher than 15 g/L, the reaction rate of the recombinant NHase^{M} stopped the linear increase, but the natural NHase did not, indicating that the natural NHase in the Gram-positive *Nocardia* behaves better tolerability to acrylonitrile than the recombinant NHase in *E. coli*, as shown in Fig. 6. Regarding the both NHases as the enzymes obeying the Michaelis–Menton law, the Michaelis–Menton constant (K_m) values were calculated out by the Hanes equation. The K_m of the recombinant NHase^{M} was 4.34 g/L (82 mM), slightly lower than that in wild *Nocardia*, 4.56 g/L (86 mM). This infers that the recombinant NHase^{M} in *E. coli* behaves the similar substrate affinity to the natural NHase in *Nocardia* during the catalysis.

3.3. Site-directed mutagenesis of the recombinant NHase in E. coli BL21(DE3)/pETNH^{M}

Site-directed mutagenesis was further performed to evaluate the feasibility of using this recombinant *E. coli* to accomplish genetic manipulations for improving the acrylamide tolerability and thermo-stability of the recombinant NHase^{M}. Two amino acid residues, α122Ser and β47Trp, were selected as the muta-

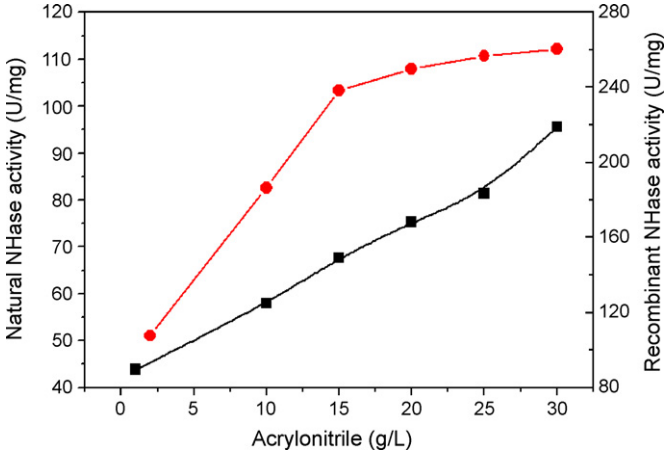

Fig. 6. Effect of the substrate concentration on the catalysis rate of the recombinant NHase^{M} in *E. coli* BL21(DE3)/pETNH^{M} (●) and the natural NHase in *Nocardia* (■). Reaction temperature was controlled at 28 °C.

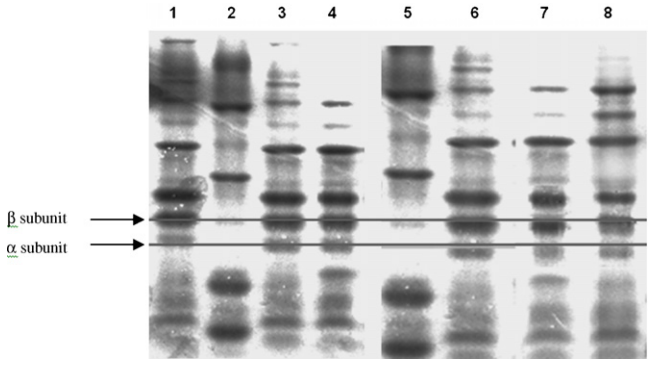

Fig. 7. SDS-PAGE analyses of the whole cells of *E. coli* BL21(DE3) harboring a series of plasmids with or without point mutation. Lane 1: αS122C mutant; lane 2: protein size marker with molecular weight 97, 66, 45, 31, 20.1 and 14.4 kD, respectively; lane 3: original NHase^{M}; lane 4: αS122A mutant; lane 5: protein size marker; lane 6: original NHase^{M}; lane 7: αS122D mutant; lane 8: βW47E mutant. The arrows indicated the positions of β and α subunits of the NHase^{M}, respectively. All recombinant strains grew at optimal conditions as described in Section 3.1.

tion targets, because their positions are located in the N-cap structure of the corresponding α helixes. Moreover, the α helix flanking α122Ser is just adjacent to the active center of NHase, and the α helix flanking β47Trp is the one most close to the active center in the β subunit, according to the secondary and tertiary structure prediction of the NHase (AY168347) by SOPMA [12] and Geno3D [13]. The α122Ser residue was designed to be mutated into three different residues, i.e. Ala, without nucleophilic group, Cys, with weak nucleophilic group, and Asp, with strong nucleophilic group. The β47Trp residue was mutated directly into Glu with strong nucleophilic group. Because the negative charge in the carboxyl group of Asp or Glu could stabilize the dipole of the α helix, one or two of the above mutations are expected to improve the stability of the recombinant NHase^{M}.

By running the site-directed mutagenesis as described in 'experimental', four recombinant strains, *E. coli* BL21(DE3)/pETNH^{M} (αS122A), BL21(DE3)/pETNH^{M} (αS122C), BL21(DE3)/pETNH^{M} (αS122D), and BL21(DE3)/pETNH^{M} (βW47E) were successfully constructed then cultured in the flasks. After 8 h of induction, the whole-cell SDS-PAGE analyses of all of the recombinants were performed, as shown in Fig. 7. It was found that the recombinant NHase^{M}s before and after point mutations displayed the same two clear bands of both α (27.5 kD) and β (31.6 kD) subunits, indicating that the point mutations did not affect the translation of the recombinant NHases.

GC analyses on the specific activity of each point mutation were performed and the results were listed in Table 3, in which the original NHase^{M} mutant was used as the control. Two single-colonies of each strain were simultaneously cultured for ensuring the reproducibility. It was found that both the αS122A and αS122D mutations lost the NHase activity completely, while the αS122C and βW47E mutations still remained 70 and 50% activity of the control, respectively. The inactivation of the αS122A mutation might be caused by the excision of the nucleophilic group for catalysis. On the contrary, the inactivation

Table 3
Specific activities of the NHase^{M} mutants before and after the site-directed mutation

Control and mutation	Dry cell concentration (mg/mL)	Specific activity (U/mg)
Control 1#	1.15	189.2
Control 2#	1.21	195.1
αS122A 1#	1.40	0
αS122A 2#	1.27	0
αS122C 1#	1.32	133.2
αS122C 2#	1.38	128.7
αS122D 1#	1.17	0
αS122D 2#	1.27	0
βW47E 1#	1.32	94.3
βW47E 2#	1.24	96.8

of αS122D mutation was probably caused by the introduction of the strong negative carboxyl group into the hydrophobic center of the NHase. These results also indicated that αS122 is an important residue for the catalysis mechanism of NHase.

Further evaluation of the acrylamide tolerance ability and thermo-stability of the recombinant strains containing the αS122C and βW47E mutation, respectively, were carried out by measuring their inactivation behavior before and after mutation. When the whole cells of *E. coli* BL21(DE3)/pETNH^{M} (αS122C) and the control strain, *E. coli* BL21(DE3)/pETNH^{M} were immersed into different concentrations of acrylamide (AM) to denature the NHase for 570 min, the residual specific activity was analyzed and plotted in Fig. 8. It was obvious that the αS122C mutant always exhibited higher activity than the control during the inactivation process, especially at elevated concentrations of AM. The residual specific activity of the NHase^{M} (αS122C) was 2.8-folds higher than that of the original one. That is, the AM tolerance of the NHase^{M} (αS122C) was significantly improved. However, its thermo-stability was almost the same with the original NHase^{M}, when immersing the both in-

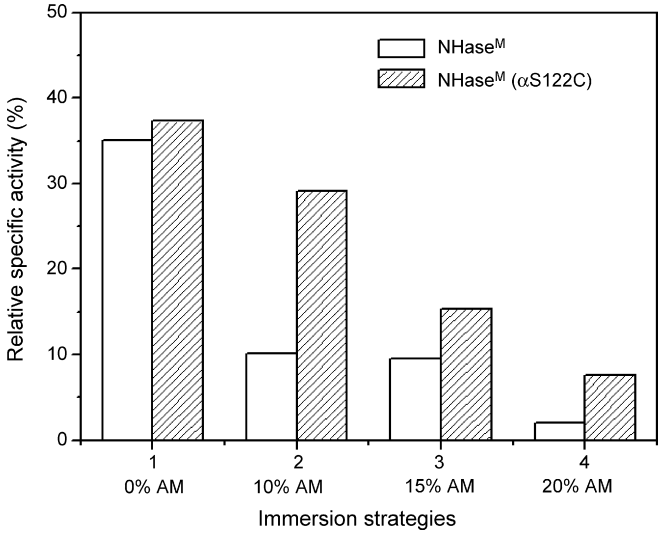

Fig. 8. Comparison of the acrylamide (AM) tolerance of the NHase^{M} (αS122C) and the original NHase^{M} in *E. coli* BL21(DE3)/pETNH^{M} (αS122C) and BL21(DE3)/pETNH^{M}, respectively. The 0, 10, 15 and 20% AM solution were used for the cell-immersion.

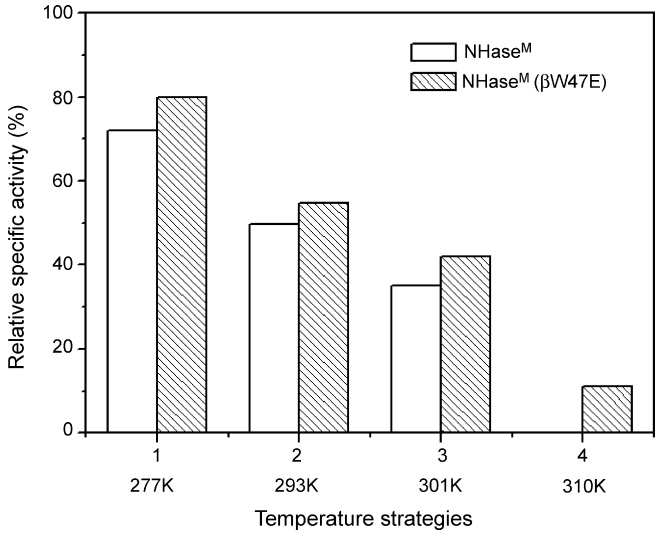

Fig. 9. Comparison of the thermo-stability of the βW47E mutant and the original NHase^M in the corresponding *E. coli* BL21(DE3)/pETNH^M (βW47E) and BL21(DE3)/pETNH^M. The two bacteria were incubated in the PBS buffer (50 mM, pH7.0) under 5, 20, 28 and 37 °C, respectively.

cell enzymes into the PBS buffer (50 mM, pH 7.0) for 570 min under different temperatures.

Reproducing the experiments above using *E. coli* BL21(DE3)/pETNH^M (βW47E) as the target strain, it was found that the AM tolerance of the βW47E mutant was similar to the original NHase^M. However, its thermo-stability was markedly increased when immersed both of the recombinant cells into the PBS buffer at enhanced temperatures, 277 K (5 °C), 293 K (20 °C), 301 K (28 °C) and 310 K (37 °C), respectively. As shown in Fig. 9, the 570 min incubation of the NHase^M under 28 °C resulted in more than 50% loss of the specific activity for both enzymes, implying that the thermo-stability of the NHase^M was really not good. Relatively, the βW47E mutant could always remain higher activity than the control under different temperatures, especially at 37 °C that when the original NHase^M had been completely inactivated, 11% of the activity still remained for the βW47E mutant.

4. Conclusions

A superior recombinant *E. coli* BL21(DE3)/pETNH^M, containing the start codon mutation of the α subunit, was constructed as a high efficient expression and mutation platform for genetic manipulation of the NHase^M. After the optimizations of the cell culture and NHase inducing conditions, the specific activity of the NHase^M reached as high as 452 U/mg dry cell. Enzymatic characteristics studies showed that the recombinant NHase^M in *E. coli* has lower reaction activation energy and K_m value than the natural NHase in the wild *Nocardia* YS-2002, but its pH-stable range was a little limited. Point mutations of αS122 and βW47 of the NHase^M were performed to evaluate the feasibility of protein rational design using this recombinant *E. coli*. The results confirmed that the specific activity of the recombinant strain can reproducibly and reasonably response before and after mutation. The AM tolerance of the αS122C mutant and the thermo-stability of the βW47E mutant were, respectively, improved, although paying for the expense of the activity loss.

In short, the novel strain, *E. coli* BL21(DE3)/pETNH^M, exhibiting high efficiency and high confidence for the expression and mutation studies of the NHase, can be used as a technical platform for further modification of the NHase to achieve a superior mutant with not only high activity, but also high thermo-stability and AM tolerance ability. These experiments are in progress in our lab under the guide of bioinformatics [14] and protein rational design.

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