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

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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.

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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].

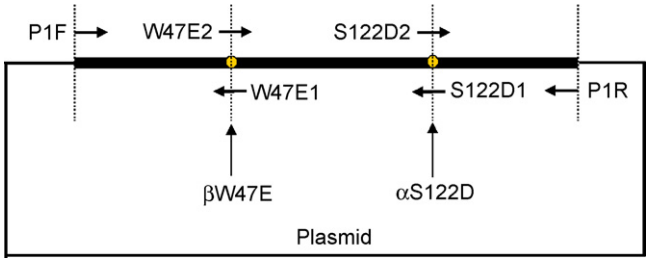


Fig. 1. The sketch map of the primer design for βW47E and αS122D mutations.

### 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.