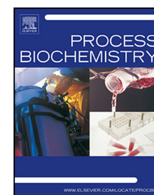
Process Biochemistry 48 (2013) 1921–1927

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Efficient cloning and expression of a thermostable nitrile hydratase in *Escherichia coli* using an auto-induction fed-batch strategy

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ARTICLE INFO

Article history:
Received 10 June 2013
Received in revised form 1 September 2013
Accepted 4 September 2013
Available online 12 September 2013

Keywords:
Nitrile hydratase
Thermostable
Recombinant expression
Escherichia coli
Auto-induction
Fed-batch cultivation

ABSTRACT

A nitrile hydratase (NHase) gene from *Aurantimonas manganoxydans* was cloned and expressed in *Escherichia coli* BL21 (DE3). A downstream gene adjacent to the β-subunit was necessary for the functional expression of the recombinant NHase. The structural gene order of the Co-type NHase was α-subunit beyond β-subunit, different from the order typically reported for Co-type NHase genes. The NHase exhibited adequate thermal stability, with a half-life of 1.5 h at 50 °C. The NHase efficiently hydrated 3-cyanopyridine to produce nicotinamide. In a 1-L reaction mixture, 3.6 mol of 3-cyanopyridine was completely converted to nicotinamide in four feedings, exhibiting a productivity of 187 g nicotinamide/g dry cell weight/h. An industrial auto-induction medium was applied to produce the recombinant NHase in 10-L fermenter. A glycerol-limited feeding method was performed, and a final activity of 2170 U/mL culture was achieved. These results suggested that the recombinant NHase was efficiently cloned and produced in *E. coli*.

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1. Introduction

Nitrile hydratase (NHase, EC 4.2.1.84) catalyzes the hydration of diverse nitriles to their corresponding amides [1]. Several microbes with NHase activity have been successfully used for the production of acrylamide, nicotinamide, and 5-cyanovaleramide [2]. However, these original strains also transform amides into carboxylic acids using amidase, which is expressed along with NHase in the same operon [3]. Consequently, the purity and yield of the target amide is reduced. To solve this problem, the NHase gene is typically cloned into heterologous cells. Cloning facilitates over-expression, which is an alternative method for the production of amide as the only product. Moreover, the recombinant NHase can be conveniently tailored and improved through protein engineering. *E. coli* is the most commonly used prokaryotic host for the expression of heterologous proteins and biocatalysts [4,5]. However, previous attempts to express NHase in *E. coli* were not successful because of the formation of inactive and insoluble protein [6,7]. Although recent progress has been made in this field, only a dozen NHases have been cloned and functionally expressed in *E. coli* cells [8,9]. Therefore, exploring new NHases is necessary to expand the range of feasible reactions for the production of valuable chemicals. With the rapid increase in gene data in this post-genomic era, mining gene data could be more convenient than traditional cultivation techniques for identifying novel biocatalysts.

Auto-induction is a simple method for recombinant protein expression, requiring little user intervention from inoculation until cell harvest. This strategy omits biomass monitoring for the correct time of inducer addition and automatically performs a shift from growth to recombinant protein expression under the metabolic control of *E. coli* [10]. Auto-induction has been used to efficiently produce recombinant proteins in *E. coli*, particularly in high-throughput experiments using 96-well microtiter plates. This method is based on the function of *lac* operon regulatory elements in mixtures of glucose, lactose and glycerol under diauxic growth conditions. During the initial growth period, glucose is preferentially used as the carbon source, and protein expression is prevented through catabolic repression. When glucose is depleted, catabolic repression is relieved, thereby shifting cellular metabolism toward the import and consumption of lactose and glycerol [11]. Lactose is further generated into allolactose through β-galactosidase, which acts as a physiological inducer of the *lac* operon for the expression of recombinant proteins. The original auto-induction media has been previously described [12]. Subsequently, several auto-induction systems have been marketed, such as Overnight Express™, MagicMedia™ and Staby™ [13]. However, these media are complex and expensive and only used for small-scale screening studies. Thus, a simple and economical auto-induction medium (AIM) is required for the large-scale preparation of some industrial recombinant enzymes.

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1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.09.004

In this study, a putative NHase gene from *Aurantimonas manganoxydans* (*A. manganoxydans*) ATCC BAA-1229 was cloned and functionally expressed in *E. coli* BL21 (DE3). A downstream region next to the β-subunit gene was necessary for the functional expression of recombinant NHase *in E. coli*. The enzyme is a member of the Co-type NHase family, and exhibits higher thermal stability than most mesophilic NHases. Moreover, the NHase efficiently catalyzes 3-cyanopyridine to produce nicotinamide. Thereafter, a fed-batch strategy was developed to efficiently produce recombinant NHase using a simple AIM in a 10-L stainless steel fermenter.

2. Materials and methods

2.1. Strains, plasmids and chemicals

The NHase gene was cloned from *A. manganoxydans* ATCC BAA-1229. The *E. coli* strains DH5α and BL21 (DE3) were used for gene cloning and protein expression, respectively. Plasmid pET28a (+) (Novagen™, Merck KGaA, Germany) was employed to construct a recombinant vector for the expression of NHase, defined as pNh1229. The chemicals used in this work were either purchased from Aladdin Chemistry (China) or Sinopharm Chemical Reagent (China), unless otherwise specified. Yeast extract and tryptone were obtained from OXOID (England).

2.2. Media and cultivation conditions

2.2.1. Media

Luria–Bertani (LB) medium comprised 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl. The pH was adjusted to 7.0 using 3 M NaOH. For solid media, a total of 15 g/L of agar was added to the LB medium before sterilization.

An AIM with locally available cheap yeast extract as the nitrogen source was used in all fermenter cultivations. The AIM contained 20.0 g/L glycerol; 5.0 g/L glucose; 3.0 lactose; 23.0 g/L yeast extract (Xiwang, China); 17.1 g/L $Na_2HPO_4 \cdot 12H_2O$; 3.0 g/L KH_2PO_4; 1.0 g/L NaCl; 1.0 g/L NH_4Cl; 1.3 g/L $MgSO_4 \cdot 7H_2O$; 0.05 g/L Kanamycin sulfate; and 0.02 g/L $CoCl_2$. Glucose, lactose, $CoCl_2$, and $MgSO_4$ were sterilized separately. The feeding medium used in the fed-batch cultivations comprised 200 g/L of glycerol, 50 g/L of lactose, and 0.2 of g/L $CoCl_2$.

2.2.2. General culture

A single clone was transferred to 5 mL of LB medium and shaken overnight at 37 °C. This preinoculum culture was inoculated into 100 mL LB media in 500-mL shaker flasks. After 3 h of culture at 37 °C to a density of OD_{600} = 0.6, 20 μL of a 0.5 M IPTG stock solution and 200 μL of a 10 g/L $CoCl_2$ stock solution were added to induce the expression of the recombinant NHase at 18 °C for 14 h.

2.2.3. Fed-bath cultivations in 10 L fermenter using AIM

Fed-batch cultivations were performed in a 10-L stainless steel bioreactor (Shanghai Baoxing, China) with an initial 6 L of AIM. The preinoculum culture was transferred to a 1-L Erlenmeyer flask containing 300 mL LB liquid medium and shaken at 220 rpm at 37 °C. When the OD_{600} was higher than 1.5, the culture was inoculated into the fermenter under sterile conditions. The inoculation volume was 5%. The culture was performed at 37 °C for 6 h until an OD_{600} of approximately 16 was obtained. Subsequently, the temperature was gradually reduced to 18 °C and maintained until the end of the fermentation. During the entire process, the pH was maintained at 7.0 through the automatic addition of ammonium solution (25%, w/v). Antifoam was added manually when necessary. The dissolved oxygen level (DO) was maintained at approximately 30% air saturation throughout fermentation. The initial airflow rate was 0.45 m^3/h, and increased with the need for DO. The end of the cultivation was determined by a reduction in the oxygen consumption rate and an increase in the pH value. To avoid the accumulation of glycerol in the broth, the feeding solution was added using a continuous model by monitoring the changes in the DO level, pH value and glycerol concentration during fermentation.

2.3. Construction of the NHase expression plasmid

The following oligonucleotides were designed and synthesized as primers to amplify the NHase gene from the chromosomal DNA of *A. manganoxydans* (ATCC BAA-1229): PAur_UP (5′-CGGGATCCATGACGGGATCGCACGGCAG-3′), PAur_DOWN1 (5′-CCCAAGCTTTCAGGCGTGCGCAAGATAGG-3′) and PAur_DOWN2 (5′-CCCAAGCTTTCAGTCGTGTGGGTTCGGCAGG-3′). *Bam*HI and *Hind*III restriction enzyme sites (underlined) were introduced into these primers. PCR was conducted using TransStar™ *FastPfu* DNA polymerase (TransGen Biotech, China). The PCR reaction included preliminary denaturation at 95 °C for 2 min, followed by 30 cycles of 20 s denaturation at 95 °C, 20 s annealing at 58 °C, and 1 min extension at 72 °C. A final extension was performed at 72 °C for 5 min. The PCR product was digested using *Bam*HI/*Hind*III and ligated into the expression vector pET28a (+) digested with the same restriction enzymes. The successful clones were subsequently transformed into *E. coli* DH5α. The resulting recombinant plasmids were further confirmed through gene sequencing (Shanghai Sangon Biological Engineering, China) and transformed into the expression host *E. coli* BL21 (DE3) for the production of the recombinant NHase.

2.4. Purification of the recombinant NHase

The collected cells were washed twice and resuspended in 50 mM phosphate buffer (pH = 8.0) at room temperature and subsequently disrupted through sonication (Sonicator 400, Misonix, USA) in an ice bath. The crude cell extract was centrifuged at 12,000 × *g* for 30 min to remove the insoluble debris. The obtained supernatant was loaded onto a nickel chelate affinity column (Ni-NTA Resin, Bio Basic) to purify the recombinant NHase [14]. The purified enzyme was subsequently lyophilized.

2.5. Enzyme assay and kinetics

NHase activity was determined using reversed-phase high-performance liquid chromatography (HPLC, Agilent 1100, USA), equipped with a C_{18} reverse-phase column (Varian pursuit C_{18}, 5 μm, 4.6 mm × 250 mm) [14]. One unit (U) of NHase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol nicotinamide per minute. The kinetic analyses were completed at pH 8.0 and 25 °C in 50 mM phosphate buffer containing 3-cyanopyridine at different concentrations ranging from 0.5 to 20 mM. The K_m and V_{max} values were determined using the Hanes–Woof plot.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration assays

The expression of the recombinant NHase was analyzed using SDS-PAGE (15%) with a 4% stacking gel. The insoluble debris from the cell lysate was resuspended in 50 mM phosphate buffer (pH = 8.0), and mixed in proportion with SDS-PAGE loading buffer (Takara, Dalian). All samples were denatured in a boiling water bath for 10 min. The molecular weight protein markers (ProteinRuler ™ I, 12–80 kDa; ProteinRuler™ II, 12–100 kDa, TransGen Biotech, China) were used as references. The SDS-PAGE Gels were stained with Coomassie Brilliant Blue G-250. The protein concentrations were determined using the Bradford protein assay kit (Quick Start™, Bio-Rad, USA).

2.7. Optimum temperature and pH

The activities of the recombinant NHase were measured at 6–70 °C to determine the optimum reaction temperature. The optimum pH of the NHase was determined after measuring the activity at different pH values between 4.8 and 10.4. The following buffers were used: 50 mM citric acid-Na_2HPO_4 buffer (pH 4.8–6.8), 50 mM sodium phosphate buffer (pH 6.8–8.0), 50 mM Tris–HCl buffer (pH 8.0–8.8), and 50 mM glycine–NaOH buffer (pH 8.8–10.4). These experiments were repeated three times.

2.8. Thermal stability

The thermal stability of the enzyme was measured after incubating 0.1 mL of the purified enzyme in 0.1 mL of 100 mM sodium phosphate buffer (pH 8.0) at 30, 40, 50, and 60 °C for different times. The residual activity was measured using the standard assay method previously mentioned. All experiments were performed in triplicate. The exponential decay process can be described using the following equivalent formula: $N(t) = N_0 e^{-\lambda t}$, where λ is the decay constant of the decaying quantity. The activity half-life ($t_{1/2}$) was calculated using the following equation: $t_{1/2} = \ln 2/\lambda$.

2.9. Substrate specificity

To determine the substrate specificity, 100 mM of different substrates and an appropriate amount of purified lyophilized enzyme was mixed with 50 mM sodium phosphate buffer (pH = 8.0) in a 0.5-mL reaction mixture. Dimethyl sulfoxide (20%, v/v) was added to the mixture to dissolve hydrophobic substrates [9]. The reaction was cultured at 25 °C for 1 min and terminated upon the addition of 0.1 mL of 2 M HCl. The aliphatic nitriles were determined through gas chromatography (FULI™ GC9790 II, China) using an SE-54 capillary column (60 m × 0.54 mm × 1.0 μm). Dodecane was used as the internal standard. The temperatures of flame ionization detector and injector were 280 °C, and the temperature program of the column oven was 100 °C for 5 min, and was raised to 260 °C for 10 min at a rate 20 °C/min. The presence of aromatic compounds was determined through HPLC.

2.10. Biotransformation of 3-cyanopyridine to nicotinamide

To examine the effect of substrate concentration on the production of nicotinamide, resting cells (2.28 mg dry weight) from 2 mL of culture broth were added. The concentration of 3-cyanopyridine (i.e., 0.2 mol/L to 1.5 mol/L) varied in the 5-mL reaction mixture. The reaction was performed in a 1.0-L continuous fed-batch model to obtain a high amount of nicotinamide. Four batches of 0.9 mol 3-cyanopyridine were added to the reaction mixture. These reactions were performed at 20 °C at 150 rpm. The process was sampled periodically and analyzed through HPLC.

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