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Gene Cloning, Overexpression, and Characterization of the Nitrilase from *Rhodococcus rhodochrous* tg1-A6 in *E. coli*

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Abstract A DNA fragment containing the entire coding sequence of nitrilase gene was amplified from *Rhodococcus rhodochrous* tg1-A6 with high nitrilase activity using PCR and sequenced. The open reading frame of the nitrilase gene contains 1,101 base pairs, which encodes a putative polypeptide of 366 amino acid residues. The nitrilase gene was cloned into an expression vector pET-28a and expressed in an *Escherichia coli* strain BL21 (DE3). The enzymatic activity of nitrilase, which converts various nitriles to the corresponding carboxylic acids, was detected to reach 24.5 U/ml at 9 h in the recombinant bacteria.

Keywords Nitrilase · Nitrile · Carboxylic acid · Acrylic acid · Recombinant *E. coli* · Induction · Expression

Introduction

As synthesis intermediates, nitriles are widely used in the chemical industry. For instance, carboxylic acids can be converted from nitriles using nitrilases, which are enzymes hydrolyzing the nitriles to corresponding carboxylic acids and ammonia. Nitrilase catalyzing reactions have several advantages such as milder conditions of pH and temperature and less byproducts than the conventional chemical hydrolysis [1–3]. Nitrilases

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have been found in many plants and microorganisms. A variety of nitriles have been transformed by different kinds of nitrilases [2–6].

To obtain the interested nitrilases for certain nitrile hydrolysis, the bacterial species with nitrilase activity are usually isolated from enriched soil samples using the nitriles as the sole source of nitrogen. From some of these strains, genes coding for nitrilases are cloned and expressed in a recombinant manner [7–9].

In our previous work, a strain of *Rhodococcus rhodochrous* tg1-A6, with high nitrilase activity in converting acrylonitrile to acrylic acid, was isolated and kept in our laboratory. After optimization, the nitrilase activity (acrylonitrile as the substrate) in the culture reached to 22 U/ml, and the concentration of acrylic acid catalyzed by the resting cell of *R. rhodochrous* tg1-A6 reached to 414.2 g/l [10]. Due to its high enzymatic activity and tolerance to the high concentration of the products, this strain seems to possess some potential for the industrial usage in the production of carboxylic acids from nitriles.

In the present work, a gene encoding the nitrilase from *R. rhodochrous* tg1-A6 was isolated and cloned into an expression vector for overexpression in a recombinant strain of *Escherichia coli* BL21(DE3)/pET-Nit. The enzymatic activity of recombinant nitrilase was further evaluated using a few kinds of nitriles as substrates.

Materials and Methods

Strains, Plasmids, Enzymes, and Chemicals

R. rhodochrous tg1-A6, isolated and kept in this lab and having a high nitrilase activity of 22 U/ml, was used for extraction of the genomic DNA by which the nitrilase gene was to isolate. *E. coli* TOP-10F' (Invitrogen, USA) and BL21(DE3) (Promega, USA) were used as host strains for genetic cloning and expression, respectively.

Restriction endonuclease, T4 DNA ligase, Taq DNA polymerase, and isopropyl-1-thio-beta-D- galactopyranoside (IPTG) were obtained from Takara Biotechnology Co. Ltd (Dalian, China). The plasmid pGM-T (Tiangen Biotechnology co. Ltd. China) and pET-28a (Novagen, USA) were used for cloning and expression of the nitrilase in *E. coli*. All other chemicals were of reagent grade and were obtained from the local commercial sources.

Amplification by PCR and Subsequent Cloning of the Gene

Restriction enzymes and T4 DNA ligase were used according to the manufacturer's instructions. Genomic DNA from *R. rhodochrous* tg1-A6 was obtained using the Wizard Genomic DNA purification Kit (Promega, USA) and used as the template for polymerase chain reaction (PCR).

Two oligonucleotide primers, 5-AGGTACG**CATATG**GTCGAATACACAA-3 (P1) and 5-TAC**AAGCTT**CGAGTCAGATGGAGGC-3 (P2), incorporating *Nde*I and *Hin*dIII restriction sites, respectively, were designed according to the nucleotide sequence of nitrilase gene from *R. rhodochrous* J1 [8]. The reaction mixtures for PCR contained 1× PCR buffer, each deoxynucleoside triphosphate (dNTP) at a concentration of 200 μM, each primer at a concentration of 1 μM, DNA templates at a concentration of 1 ng/μl, and 1 U of *Taq* DNA polymerase in a final volume of 25 μl. DNA amplification was performed with a GeneAmp thermocycler (Techne TC312, UK) using the following program: a 5-min hot start at 94 °C; followed by 30 cycles consisting of denaturation (30 s at 94 °C), annealing (60 s at 50 °C), and extending (90 s at 72 °C); and a final extending at 72 °C for 5 min. The

PCR products were verified by the electrophoresis in 0.7% agarose gel and stained with ethidium bromide.

The interested DNA fragment (around 1.1 kb) was purified by the agarose gel electrophoresis and then ligated into pGM-T TA cloning vector, and the recombinant DNA was used to transform *E. coli* TOP-10F'. The clones harboring recombinant plasmid pGM-T-Nit were screened and verified by gene sequencing. Plasmid DNA was isolated using the plasmid isolation kit (Tiangen, China). The nitrilase gene fragment was digested with *Nde*I and *Hin*dIII from pGM-T-Nit and subcloned into the plasmid pET-28a between *Nde*I site and *Hin*dIII sites to yield pET-Nit, in which the expression of nitrilase gene is under the control of T7 promoter. The recombinant *E. coli* BL21(DE3)/pET-Nit was finally constructed by transforming pET-Nit into the host strain BL21(DE3). The correct cloning of the nitrilase gene was confirmed in all cases both by sequencing and by restriction enzyme analysis.

Culture Conditions

The composition of the culture medium and growth condition of the *R. rhodochrous* tg1-A6 strain was previously described [10].

Cultivation of the recombinant *E. coli* BL21(DE3)/pET-Nit was performed at 37 °C in 300-ml conical flasks containing 50 ml of Luria–Bertani (LB) medium at 200 rpm. Then 0.5 mM IPTG (or 20 mM lactose) was added to the medium as an inducer when OD_{600} (optical density at 600 nm) reached 0.6. The cultures were incubated at different temperatures (25–37 °C) for another 24 h, and the cells were harvested by centrifugation at 4 °C and 10,000×*g* for 5 min. The cultivation of *E. coli* BL21(DE3)/pET-Nit was further optimized by using lactose as the inducer in different mediums as listed in Table 1.

Enzyme Assay

Nitrilase activity was assayed by measuring the production of NH_4^+ using a spectrophotometric method [11]. After cultivation and induction, cells were collected by centrifugation and resuspended in 100 mM phosphate buffer (pH 7.0). The reaction mixture contained 10 μl acrylonitrile (or other nitriles) and 1 ml cell suspension. One unit (U) of nitrilase activity corresponds to the amount of enzyme producing 1 μmol NH_4^+ per min at 25 °C. Specific activity (unit per milligram) of the culture is denoted as micromoles (acrylic acid) per milligram (dry cell) minute. SDS-PAGE analysis on whole cell proteins was performed with 4% stacking and 12.5% running gel as described [12].

Table 1 Culture mediums used in this work.

Medium	Components (g/l)
LB	Luria–Bertani medium: tryptone 10.0, yeast extract 5.0, NaCl 10.0, pH 7.0
LBSuc	Luria–Bertani medium, sucrose 5, pH 7.0
LBGly	Luria–Bertani medium, glycerol 5, pH 7.0
LBGlu	Luria–Bertani medium, glucose 5, pH 7.0
CSL	Corn steep liquor 50, KH_2PO_4 2.5, K_2HPO_4 12.5, glucose 4, pH 7.5
M9	$Na_2HPO_4{\cdot}7H_2O$ 12.8, KH_2PO_4 4.8, NaCl 0.5, NH_4Cl 1.0, glucose 4, pH 7.0

Results and Discussion

Cloning of the Nitrilase Gene

In our previous work, the nitrilase of *R. rhodochrous* tg1-A6 was effectively induced with ε-caprolactam, and the optimal catalyzing pH is about 8.0, which shows very similar properties to those of the nitrilase from *R. rhodochrous* J1 [8, 10]. Accordingly, the oligonucleotide primers for PCR were designed after the nucleotide sequence of nitrilase gene from *R. rhodochrous* J1 [8].

Following PCR using the genomic DNA of *R. rhodochrous* tg1-A6 as the template, the amplification product was detected using the agarose gel electrophoresis. As shown in Fig. 1, a specific DNA fragments (about 1.1 kb), which matched the correct size of the nitrilase gene, was successfully amplified. This DNA fragment was ligated into the pGM-T TA cloning vector, and the insert of the recombinant plasmid pGM-T-Nit was then sequenced.

A single open reading frame of 1,101 base pairs, encoding a putative polypeptide of 366 amino acid residues, was identified. The genbank accession number for this gene is EF467367. Sequence comparison of this gene to the nitrilase gene from *R. rhodochrous* J1 resulted in 97% similarities at DNA level and 100% identity at the amino acid level.

To overexpress the new nitrilase in *E. coli*, the nitrilase gene was then cut out from pGM-T-Nit with *Nde*I and *Hin*dIII and cloned into the prokaryotic expression vector pET-28 to obtain a recombinant plasmid pET-Nit, in which the expression of nitrilase gene is under the control of T7 lac promoter (shown in Fig. 2). The recombinant *E. coli* BL21 (DE3)/pET-Nit was finally constructed by transforming pET-Nit into the host strain BL21 (DE3).

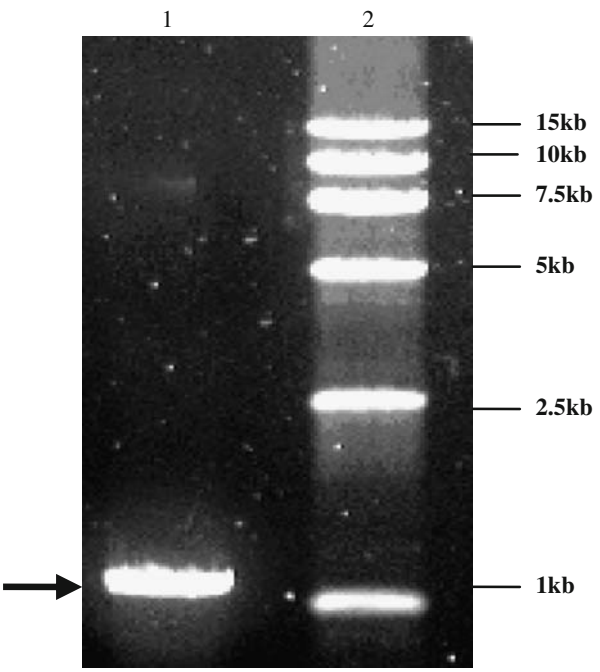

Fig. 1 PCR amplification of the nitrilase-encoding gene. *Lane 1* Nitrilase gene amplified from the genomic DNA of *R. rhodochrous* tg1-A6. *Lane 2* DNA standards. *Arrow* indicates the amplified products (about 1.1 kb) corresponding to the nitrilase gene

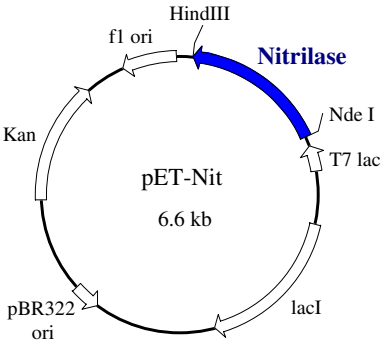

Fig. 2 Physical map of expression plasmid pET-Nit. *T7 lac* T7 lac promoter, *pBR322 ori* origin of replication, *lacI* the mutant repressor gene of lac operon, *kan* kanamycin-resistant marker. The transcriptional direction of the nitrilase gene is indicated with *arrows*

Expression of the Nitrilase

To verify the expression level of recombinant nitrilase, BL21(DE3)/pET-Nit was grown in LB medium at 28 °C followed by induction using 0.5 mM IPTG or 20 mM lactose. SDS-PAGE analysis revealed a recombinant protein (corresponding to the bands of molecular weight 42 kDa), representing about 50% of the total protein (Fig. 3).

Using IPTG as the inducer in LB medium, the temperature for the maximum production of the recombinant protein is 25 °C. When using lactose as the inducer, the optimal temperature is increased to 33 °C, a more suitable temperature for *E. coli* growth. Induced by IPTG and lactose in LB medium, the nitrilase activity of the recombinant strain was 7.9 and 12.8 U/ml, respectively. An interesting observation was that whole cell extract prepared by ultrasonic treatment and cell pellet of intact cells showed no difference in specific

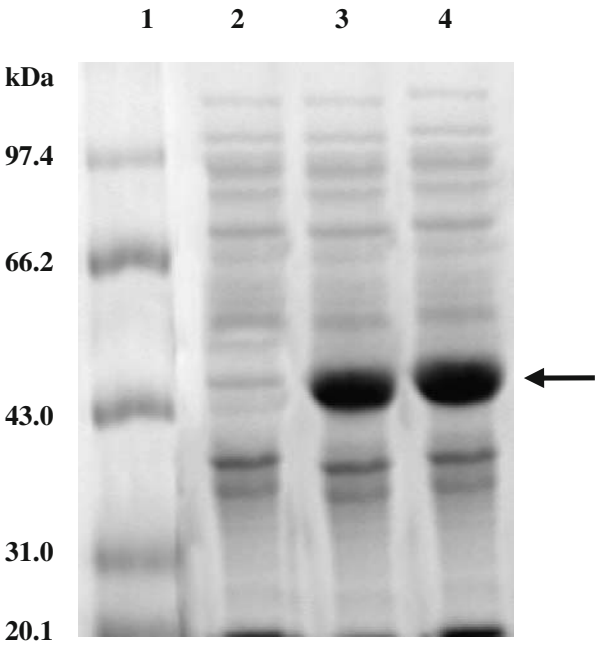

Fig. 3 SDS-PAGE analysis of nitrilase produced by *E. coli* BL21(DE3)/pET-Nit. *Lane 1* Protein size marker, *lane 2* without induction, *lane 3* with 0.5 mM IPTG induction, *lane 4* with 20 mM lactose induction. *Arrow* indicates the bands corresponding to recombinant nitrilase

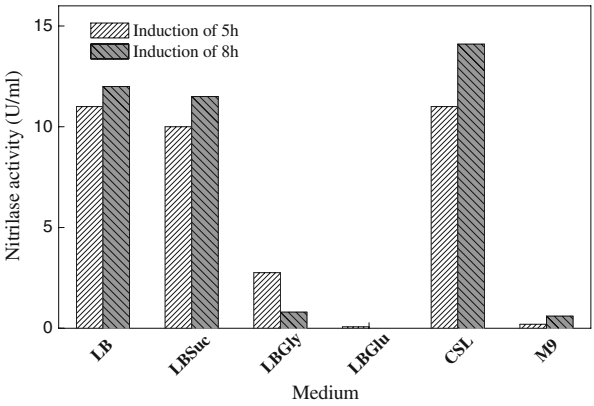

Fig. 4 Nitrilase activity of *E. coli* BL21(DE3)/pET-Nit cultured in different media

activity of nitrilase. This phenomenon facilitated the assaying and catalyzing, which could be rapidly performed with intact cell.

To determine whether any other growth conditions may affect expression of the nitrilase, the recombinant *E. coli* was grown in different media with 20 mM lactose as the inducer (Table 1), and the activity of the different enzyme preparations prepared from these cultures was measured at 5 and 8 h after induction. Under the growth conditions described, the highest nitirilase activity was found in the culture with CSL medium, in which the cost-effective corn steep liquor was used as the nitrogen source (Fig. 4). While cultured in some rich (LBGly and LBGlu) or minimal (M9) media, the recombinant *E. coli* exhibited low nitrilase activity. These data indicate that lactose can serve simultaneously as an inducer and carbon source for the production of recombinant proteins in *E. coli*, acting as a complex role different from IPTG [13].

The induction conditions such as lactose concentration, the time of induction, the induction temperature, and the dissolved oxygen level were also optimized (data not shown). The recombinant strain was cultured and induced in 300-ml flask under the optimal condition. It was shown that the activity of nitrilase reached as high as 24.5 U/ml in shaking flask at 9 h. The nitrilase accumulation stopped when the culture reached stationary phase at approximately 9 h and then decreased (Fig. 5).

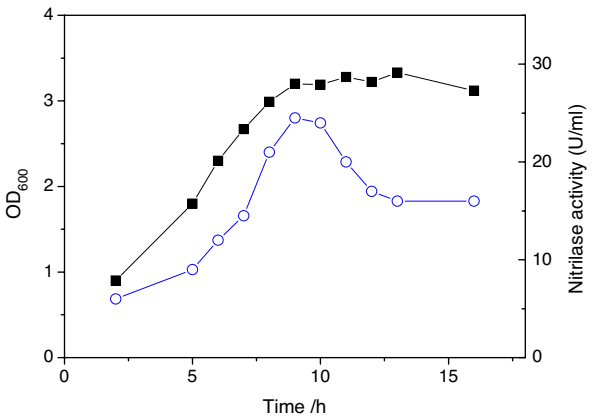

Fig. 5 Cell density (*square*) and nitrilase activity (*circle*) of *E. coli* BL21(DE3)/pET-Nit in a batch culture

The recombinant *E. coli* effectively produced nitrilase with a specific activity of 7.7 U/mg dry cell, a value higher than that of the wild strain *R. rhodochrous* tg1-A6 (5.5 U/mg dry cell). The time to reach the highest activity of culture is much shorter than that of the wild strain (approximately 120 h). In addition, the absence of nitrile hydratase that exists in *R. rhodochrous* tg1-A6, which may cause the production of byproduct acrylamide, demonstrated the superiority of the recombinant *E. coli* over the wild strain in nitrilase production [10].

Conversion of Various Nitriles by the Recombinant Nitrilase

The optimum temperature and pH of the recombinant nitrilase were 45 °C and 8.0, respectively, and the optimum substrate concentration was 225 mM acrylonitrile. These characteristics of nitrilase expressed in *E. coli* were similar to those of native enzyme from *R. rhodochrous* tg1-A6 [10].

The nitrilase of *R. rhodochrous* tg1-A6 exhibited relatively broad substrate specificity, similar to that of *R. rhodochrous* J1 [8, 10]. The ability of recombinant nitrilase of intact cell to catalyze the hydrolysis of various nitriles was examined. The generation of ammonia by nitrilase was assayed in 50 mM phosphate buffer (pH 7.5) at 30 °C, with the following nitriles as the substrate: acrylonitrile, glycolonitrile, aminoacetonitrile, 3-cyanopyridine, and iminodiacetonitrile. Acrylonitrile was found to be the best substrate for the nitrilase (Fig. 6). The enzymatic reactions showed that the nitrilase of *E. coli* BL21(DE3)/pET-Nit may be very promising for the nitrile hydrolysis of various labile nitrile compounds. Under present conditions, the enzyme activity for some nitriles (i.e., aminoacetonitrile and iminodiacetonitrile) are relatively low (about 20~30% of the activity with acrylonitrile). Gene engineering of the nitrilase gene may offer one way to improve its enzymatic activity toward such substrates.

In summary, cloning, expression, and characterization of the nitrilase gene from *R. rhodochrous* tg1-A6 is described in this report. The amino acid sequence of *R. rhodochrous* tg1-A6 nitrilase had a remarkable homology to that of nitrilase from *R. rhodochrous* J1. To overexpress the nitrilase from *R. rhodochrous* tg1-A6, a recombinant *E. coli* BL21(DE3)/pET-Nit was constructed. After the optimizations of the cell culture and inducing

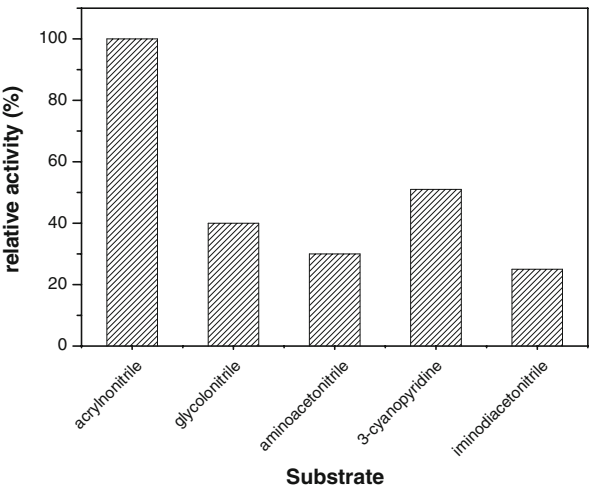

Fig. 6 Catalyzing of various substrates with the recombinant nitrilase

conditions, the nitrilase activity reached as high as 24.5 U/ml in a very short time. Enzymatic characteristics studies showed that the recombinant nitrilase in *E. coli* had similar properties to the native enzyme from *R. rhodochrous* tg1-A6, such as substrate specificity, catalyzing temperature, and pH. The novel strain, *E. coli* BL21(DE3) /pET-Nit, which exhibited high efficiency for the expression of the nitrilase, could be used as a template for further modification of the nitrilase to generate a better mutant with less substrate specificity to various nitriles. These experiments are in progress in our lab with help of bioinformatics [14] and protein rational design.

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