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Short communication

Isolation and characterization of a novel *Arthrobacter nitroguajacolicus* ZJUTB06-99, capable of converting acrylonitrile to acrylic acid

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ABSTRACT

A nitrilase-producing strain ZJUTB06-99, capable of biotransforming acrylonitrile into acrylic acid, was newly isolated from soil samples. Based on the morphology, physiological tests, ATB system and its 16S rDNA sequence, strain ZJUTB06-99 was identified as *Arthrobacter nitroguajacolicus*. Optimal reaction conditions were investigated by the manipulation and measurement of various parameters including pH, temperature and certain cationic metals. The highest nitrilase activity was obtained when reaction was carried out at a pH of 6.5 phosphate buffer and in temperature of 40 °C water bath. The nitrilase of *A. nitroguajacolicus* ZJUTB06-99 exhibited excellent thermostability. Nitrilase activity was strongly inhibited by Hg^{2+}, Ag^{+} and Cu^{2+}, but Ni^{2+} and Ca^{2+} increased enzyme activity to 163% and 158%, respectively. The investigation of substrates spectrum showed that *A. nitroguajacolicus* ZJUTB06-99 exhibited the highest nitrilase activity towards aromatic nitriles such as phenylacetonitrile. However, no detectable activity was recorded when any of the tested amides were used as substrates.

1. Introduction

Acrylic acid is a commodity chemical with an estimated annual production capacity of 4.2 million metric tons [1]. It is widely used in the production of many commercial products including paints, coatings, polymeric flocculants and paper. Currently, most commercial acrylic acid is produced through the oxidation of propene, a process which generates several unwanted by-products such as acrolein and a large amount of inorganic waste. Developments in biotechnology, however, offer the possibility of alternative methods for industrial-scale production of acrylic acid using biocatalyst.

There is considerable interest in using nitrile-converting enzymes as biocatalysts in preparative organic chemistry because of their ability to readily convert available nitrile into the corresponding acid and amides [2–5]. This environmental-friendly bioconversion is not only a much cleaner process than traditional chemical methods, but also a relatively mild synthesis with high selectivity and yield. Early studies demonstrated that some organisms possessed versatile nitrile-converting characteristics [3,6–10]. Nitrile-metabolizing enzymes have been used as powerful tools for industrial production of some valuable amides and acids [11–14]. To date, however, there are scanty reports of acrylonitrile hydrolysis to produce acrylic acid by *Arthrobacter nitroguajacolicus*.

The objective of this work was to isolate a novel bacterial strain capable of converting acrylonitrile to acrylic acid. The strain was identified according to physiological and biochemical characteristics, ATB system and its 16S rDNA gene sequence. Furthermore, the biotransformation conditions from acrylonitrile to acrylic acid by resting cells of ZJUTB06-99 were optimized.

2. Materials and methods

2.1. Materials and medium

Acrylonitrile, acrylic acid and other chemicals were obtained from commercial sources and were of analytical grade.

The selective enrichment medium contained glucose (10 g), K_2HPO_4 (0.5 g), KH_2PO_4 (0.5 g) and $MgSO_4{\cdot}7H_2O$ (0.5 g) in 1 L of tap water. The enzyme production medium contained glucose (20 g), yeast extract (5 g), K_2HPO_4 (0.5 g), KH_2PO_4 (0.5 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), ε-caprolactam (4 g) and monosodium glutamate (0.75 g) in 1 L of distilled water. The media were adjusted to pH 7.0 with NaOH and then autoclaved at 121 °C for 20 min. The strain was incubated aerobically at 150 rpm, 30 °C for 72 h. Cells were collected by centrifugation at 4 °C, 10,000 rpm for 10 min and were washed with 0.9% sodium chloride solution. Then cells were harvested by centrifugation and stored at 4 °C for further use.

2.2. Isolation of strains producing nitrilase

Soil samples were collected from different locations in Zhejiang Province, China. Each soil sample (2 g) was added to 98 mL of distilled water and stirred for 10 min with glass beads to help keep particles in suspension. 1 mL of the soil–water suspension was transferred to 40 mL selective enrichment medium supplemented with 0.1% (v/v) acrylonitrile as the sole nitrogen source after being autoclaved [2,15]. The culture was incubated at 30 °C on a rotary shaker (150 rpm) for 72 h, after which 1.0 mL bacteria solution was transferred to the same medium and held under the same incubation conditions. After repeating this procedure four times,

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the enriched cultures were diluted and plated onto an enrichment medium in order to isolate pure colonies. The isolate exhibited high conversion rate from acrylonitrile to acrylic acid and was selected for further studies.

2.3. Identification and characterization

2.3.1. Phenotypic and biochemical characterization

Cell morphology of strain was observed using a transmission electron microscope (TEM) (JEM-1200EX, Japan). Conventional physiological and biochemical characteristics were identified by the procedures described in Bergey's Manual of Systematic Bacteriology [16,17]. The oxidase activity was assayed using an oxidase reagent (bioMerieux, Marcy-l'Etoile, France). Carbon source utilization was examined by a standardized micromethod, with ID32 GN (bioMerieux). The results were obtained using an automated reader (bioMerieux, ATB Expression System) and analyzed with the database V 3.0.

2.3.2. 16S rDNA sequence determination and phylogenetic analysis

Chromosomal DNA of the isolate was extracted using a TAKARA MiniBEST Bacterial Genomic DNA Extraction Kit ver2.0 (TAKARA Biotechnology Co. Ltd Dalian, China). The 16S rDNA genes were amplified using polymerase chain reaction (PCR) with the universal primers p16s8 (5′-AGAGTTTGATCCTGGCTCAG-3′) and p16s1492 (5′-GGCTACCTTGTTACGACTT-3′), and subsequently sequenced by TAKARA Biotechnology (Dalian) Co. Ltd. Related sequences were obtained from the GenBank database (National Center for Biotechnology Information [NCBI], Bethesda, MD, USA) using the BLAST search program. The sequences were aligned using multiple sequence alignment software, CLUSTAL W ver. 1.81. A phylogenetic tree was constructed with MegAlign software (DNASTAR, Inc., Madison, WI, USA) based on the partial 16S rDNA sequences of 19 strains similar to strain ZJUTB06-99.

2.4. Biotransformations

The conversion of acrylonitrile to acrylic acid was performed at 30 °C on a rotary shaker at 150 rpm. The reaction system consisted of 0.2 g wet cells, 10 mL of 50 mM pH 6.5 phosphate buffer and 30 μl acrylonitrile as the substrate. Samples were withdrawn at regular intervals, and the reaction was terminated through removal of cells by centrifugation.

To determine the optimal reaction conditions for conversion of acrylonitrile to acrylic acid using resting cells of ZJUTB06-99, we studied the effects of pH, temperature and metal ions on the reactions as well as the substrate specificity of strain ZJUTB06-99. The optimal pH for reaction by the cells was determined with 30 μl acrylonitrile in 10 mL reaction system, in a pH range of 3.6–10.4 using NaAC–HAC (pH 3.6–5.8), phosphate (pH 5.8–8.0), Tris–HCl (pH 7.1–8.9) or glycine (pH 8.6–10.4).

The optimum reaction temperature was determined from 25 to 50 °C with two different cell masses (0.2 and 0.4 g wet wt.) at each temperature. The thermostability of the nitrilase was assessed at 4, 30 and 40 °C. The cells were firstly incubated at 4, 30 and 40 °C in 10 mL 50 mM phosphate buffer (pH 6.5). At a regular interval, cell suspension added with 0.3% acrylonitrile was reacted at 40 °C water bath for 2 h. Residual activity was calculated by comparing final values to the initial activity of nitrilase in cells which had not been incubated. The half-life of nitrilase at each temperature was calculated by extrapolating the line which was plotted by the natural logarithm of residual activity (ln RA) at each temperature against time.

The effects of different metal ions on biotransformation were assessed by suspending 0.2 g of cells (wet wt.) in 9 mL of 50 mM phosphate buffer, then added 1 mL of the appropriate metal ion solution (to achieve a final ion concentration of 1 mM).

The substrate specificity of nitrilase from ZJUTB06-99 was evaluated using different nitriles and amides. The concentration of each substrate was 10 mM. The reaction system was composed as mentioned above. And the reaction was carried out at the optimal conditions.

2.5. Analytical methods

Reaction samples of biotransformation were withdrawn, centrifugated and microfiltrated by 0.45 μm membrane. The resultant 0.2 μl filtrate was directly quantified by Agilent 6890N gas chromatograph (Agilent, Santa Clara, CA, USA), equipped with a flame ionization detector (FID) and a FFAP column (30 m × 0.25 mm × 0.33 μm). The operating conditions were as follows: temperature of oven 150 °C, the injection and detector temperature were kept at 220 °C. The flow rates of N_2, H_2 and air gas were 64, 40 and 400 mL/min, respectively. One unit of nitrilase activity (1 U) was defined as the amount of enzyme that catalyzed the formation of 1 μmol acrylic acid per min under standard conditions. All assays were performed in triplicate. The values were the average of the triplicate groups.

3. Results and discussion

3.1. Screening of microorganisms

Approximately 50 soil samples were collected. Isolates were obtained by enrichment using 0.1% (v/v) acrylonitrile as the sole nitrogen source. After initial screening, pure colonies were incubated and cells were collected to convert the substrate. Three strains exhibited nitrilase activity. Among them, strain ZJUTB06-99 was found to exhibit highest activity with the conversion rate of 10.71 μmol/(min g DCW) before optimization of culture conditions. It was chosen for additional evaluation. In recent years, the formation of organic acids or amides from nitrile precursors by enzyme hydrolysis is likely to become increasingly important [18]. For this purpose, isolation of strains harboring nitrilase activity was becoming more attractive. The strains were usually isolated with the target substrate or structural analogues as the sole source of nitrogen. In this study, we used acrylonitrile as the sole nitrogen source in the enrichment steps, and a bacterium ZJUTB06-99 with the ability of converting nitrile to acid was obtained.

3.2. Identification of strain ZJUTB06-99

3.2.1. Morphological and physiological characteristics

The morphology of the ZJUTB06-99 colonies on a plate were light yellow, convex, smooth, wet and with a single edge. The morphological characteristics observation of the strain by TEM was in Fig. 1a. It is found that cells were short, rod-like, 0.4–0.8 μm × 1.5–2.5 μm in size, with polar tufts of one to four flagella. The detailed conventional biochemical tests and physiological characteristics resulting from ATB system analysis are summarized in Table 1. The strain was Gram-staining negative, as well as oxidase reaction, nitrate reduction and indole production. However, catalase reaction, starch hydrolysis, H_2S production and citrate utilization were positive. The ATB system analyzed the carbon sources utilization of the strain. According to these data mentioned above, the strain was preliminarily confirmed as *Arthrobacter* genus.

3.2.2. 16S rDNA gene sequencing and phylogenetic analysis

The partial 16S rDNA sequence of ZJUTB06-99 was determined and a phylogenetic tree was constructed based on the 16S rDNA sequence (Fig. 1b). The sequence was deposited in the GenBank database with accession No. EF206344. Comparative ribosomal DNA gene sequence analysis supported a strong relationship between strain ZJUTB06-99 and members of the genus *Arthrobacter*. Particularly, strain ZJUTB06-99 had 100% sequence similarity with *A. nitroguajacolicus* (GenBank accession No. AJ512504). By examining physiological and biochemical characteristics and comparing its 16S rDNA gene sequence, the strain was identified as a strain of *A. nitroguajacolicus* and named *A. nitroguajacolicus* ZJUTB06-99.

3.3. Effects of transformation conditions on nitrilase activity of ZJUTB06-99

3.3.1. Effect of pH

The optimum enzyme activity was observed in the NaAC–HAC buffer at a pH of 5.8 (Fig. 2a). The enzyme exhibited a broad range of high activity in the phosphate region. Given the stability, we selected phosphate pH 6.5 as the optimum buffer for use in the later experiments. Usually, cellular activity of nitrilase was stable at the near-neutral range. Nitrilase from *Bacillus pallidus* strain Dac 521 was found to have a consistent pH of between 6 and 9, with an optimum pH of 7.6 [19]. The resting cells of *Rhodococcus rhodochrous* J1, using acrylonitrile as the substrate, reacted suitably at a pH of approximately 7.8 [20]. In addition, at pH of 3.6 and 9.4, enzyme activity was reduced to 36% and 27% of the maximum activity, respectively.

3.3.2. Effect of temperature

Reaction temperature is another important parameter for nitrilase activity. The optimum temperature for maximum