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Nitrile-metabolizing potential of *Amycolatopsis* sp. IITR215

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ABSTRACT

Strain *Amycolatopsis* sp. IITR215 was isolated from a sewage sample using polyacrylonitrile powder as the sole nitrogen source. Identification was performed by 16S rDNA analysis. The isolated strain harbored multiple nitrile-metabolizing enzymes having a wide range of substrate specificities. It metabolized nitrile and amide compounds with constitutive enzymes. Studies using an amidase inhibitor showed that hydrolysis of acrylonitrile and acrylamide occurred due to nitrile hydratase and amidase, respectively, while hydrolysis of hexanenitrile was due to the action of either nitrilase or a second nitrile hydratase/amidase system. The inhibitory effects of N-bromosuccinimide and N-ethylmaleimide on enzymes of this culture were also studied and this further indicated the involvement of either a nitrilase or a second nitrile hydratase/amidase system for hydrolysis of hexanenitrile. Interestingly, hexanenitrile hydrolysis exhibited an optimum temperature of 55 °C, whereas acrylonitrile and acrylamide hydrolysis showed an optimum temperature of 45 °C. The optimum pH was 5.8 for hexanenitrile hydrolysis and 7.0 for acrylonitrile and acrylamide hydrolysis. Hexanenitrile hydrolysis by enzymes of this strain showed better organic solvent tolerance in the presence of alcohols. The maximum enzyme activity of nitrile-metabolizing enzymes was found using media containing isobutyramide as the nitrogen source. This is the first report on constitutive multiple enzymes from the *Amycolatopsis* genus.

1. Introduction

Nitrile compounds are widely used by chemical industries for the synthesis of various chemicals. These compounds are highly neurotoxic and carcinogenic in nature and considered as environmental pollutants [1]. Therefore, remediation of such toxic compounds is necessary for a pollution-free environment. Nitrile compounds can be detoxified by transformation of the –CN group to $-CONH_2$ or –COOH. These types of transformations can be achieved chemically as well as enzymatically. Enzymatic biotransformation has many advantages over chemical transformation, namely: (1) it is effective at very low concentrations; (2) it acts under mild conditions; (3) it displays chemo-, regio- and enantioselectivity; and (4) it is not restricted to a natural substrate and can work outside an aqueous environment.

Nitrile metabolism is common in bacteria and plants but has also been reported in fungi. Nitrile-metabolizing enzymes are generally inducible in nature. Nitrile-hydrolyzing biocatalysis is due to the activity of two enzyme systems: nitrilase (EC 3.5.5.1) and nitrile hydratase (EC 4.2.1.84) plus amidase (EC 3.5.1.4). Multiple nitrile-metabolizing enzyme systems have been reported in some strains. *Rhodococcus rhodochrous* J1 was found to contain two nitrile hydratases, one of which was induced by urea and the other by cyclohexane–caboxamide–crotonamide [2,3]. A constitutive nitrilase was found in *Bacillus subtilis* ZJB-063 whereas nitrile hydratase and amidase were induced by the addition of ε-caprolactam [4]. Zhu et al. (2008) reported two nitrilase genes (blr 3397 and bll 6402) in *Bradyrhizobium japonicum* USDA 110. Both nitrilases were active against phenylacetonitrile. Nitrilase blr 3397 also showed highest activity with hydrocinnamonitrile while nitrilase bll 6402 showed comparable activity towards aliphatic nitriles [5]. Multiple nitrile-metabolizing enzymes have also been reported in *Nocardia rhodochrous* LL100–21 [6], *R. rhodochrous* NCIMB11216 [7,8], *Bacillus pallidus* DAC521 [9,10] and *Nocardia globerula* NHB-2 [11].

In the present study, we isolate a novel microbial strain from sewage that is capable of growth on agar plates containing polyacrylonitrile as the sole nitrogen source. The strain was identified by 16S rDNA sequence homology. This strain produced multiple nitrile-metabolizing enzymes having wide range of substrate specificities. To our knowledge this is the first report showing constitutive multiple nitrile-metabolizing enzymes from the *Amycolatopsis* genus.

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Table 1
Activities of different isolates with different inducers.

Culture no.	Site of sample collection	Inducer/nitrogen sources	Activity with 10 mM acrylonitrile (IU/l)
4/a	Soil near the ETP of pharmaceutical industry	Phenylacetonitrile	10.43
		Isovaleronitrile	15.73
		Benzonitrile	1.722
		Acrylonitrile	N.D.
6/a	Sewage of Mohali	Phenylacetonitrile	12.16
		Isovaleronitrile	15.05
		Benzonitrile	9.58
		Acrylonitrile	N.D.
6/b	Sewage of Mohali	Phenylacetonitrile	13.01
		Isovaleronitrile	23.32
		Benzonitrile	11.74
		Acrylonitrile	N.D.

2. Materials and methods

2.1. Materials

Soil and water samples were collected from various chemical and pharmaceutical manufacturing industries as well as sewage and organic manure of Chandigarh, India (Table 1). Polyacrylonitrile and other nitrile substrates were obtained from Sigma–Aldrich (USA). Media components were obtained from S.D. Fine Chem. (India) and were of analytical grade.

2.2. Isolation and screening of nitrile-metabolizing microorganisms

Isolation of nitrile-metabolizing organisms was carried out from various soil (2 g/50 ml) and water samples (2 ml/50 ml) using mineral base (MB) media. The composition of mineral base medium was as described by Khandelwal et al. [12]. In this work, polyacrylonitrile powder was used as the sole nitrogen source. Two grams of each sample from different locations were added to 50 ml sterilized MB medium in a 250 ml flask and incubated at 45 °C and 160 rpm. After 2 weeks of incubation, 1 ml of broth was transferred to 50 ml of the same medium and incubated for 1 week at 45 °C and 160 rpm. After 1 week, 50 μl of the sample was spread onto mineral base agar plates containing 20 mM acrylonitrile as the sole nitrogen source. Individual colonies growing on these plates were further purified on 20 mM acrylonitrile plates and stored at 4 °C. The purity of isolates was further confirmed by microscopy.

One loopful of culture from an agar plate was transferred to 50 ml MB media containing various nitriles as the sole nitrogen source, and incubated at 45 °C and 160 rpm for 36 h. Harvested cells were used for checking the biotransformation ability of whole cell enzymes.

2.3. Identification and characterization

2.3.1. Isolation of chromosomal DNA

Cells were harvested from media containing 10 mM adiponitrile as the sole source of nitrogen after 30 h of growth, and centrifuged at 20,000 × *g* followed by two washes with TE buffer. The pellet was suspended in 500 μl solution containing 1 g/l lysozyme and incubated for 1 h at 37 °C. Total genomic DNA was isolated by a modified CTAB-NaCl protocol [13]. Finally, the isolated DNA was suspended in 100 μl of nuclease-free water and stored at −20 °C.

2.3.2. 16S rRNA gene amplification and DNA sequencing

PCR amplification of 16S ribosomal DNA was carried out with universal primers p16S-8 (5′-AGAGTTTGATCCTGGCTCAG-3′) and p16S-1541 (5′-AAGGAGGTGAT CCAGCCGCA-3′) and the following conditions were used: 5 min at 95 °C, 30 cycles of 40 s at 95 °C, 60 s at 53 °C and 2 min at 72 °C and a final step of 10 min at 72 °C. The amplified PCR product was analyzed in 0.8% agarose.

2.3.3. Sequencing and molecular phylogenetic analysis

The amplified PCR product was sequenced by Ocimum Biosolutions, Hyderabad (India). Forward and reverse 16S rRNA gene sequences were used to search the Genebank database using the BlastN algorithm to determine phylogenetic position.

2.3.4. Nucleotide sequence accession number

The 16S rRNA gene sequence of strain *Amycolatopsis* sp. IITR215 determined in this study has been deposited in GenBank under the accession number FJ744759.

2.4. Substrate specificity of whole cells and cell-free extracts

The substrate specificity of whole cell enzymes was investigated using *Amycolatopsis* sp. IITR215 grown in MB medium for 36 h containing 10 mM adiponitrile as the sole nitrogen source. Cells were harvested by centrifugation at 20,000 × *g* for 10 min at 4 °C followed by washing with 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA. Finally, the cell pellet was suspended in the same buffer and the suspension used to check the biotransformation ability of enzymes with 10 mM of various substrates (benzonitrile, phenylacetonitrile, valeronitrile, propionitrile, isobutyronitrile, adiponitrile, acrylonitrile, acetonitrile, butyronitrile, isovaleronitrile, glutaronitrile, 3-hydroxypropionitrile, methacrylonitrile, hexanenitrile, cyclohexanecarbonitrile, 2-cyanopyridine, 4-hydroxybenzonitrile, 4-aminobenzylcyanide, 4-phenylbutyronitrile, 3-cyanopyridine, 4-cyanopyridine, 3-hydroxyglutaronitrile and acrylamide). Phenoxyacetonitrile, phenylthioacetonitrile, hydrocinnamonitrile, mandelonitrile, malononitrile and indole-3-acetonitrile were dissolved in 5% (v/v) methanol in 0.1 M potassium phosphate buffer at pH 7 containing 1 mM EDTA due to their low solubility in water. The substrate specificity of cell-free extracts was also determined using lysate obtained from cells, using 2 g/l lysozyme in the cell suspension and incubating at 30 °C for 1 h. Cell debris after lysis was separated by centrifugation at 20,000 × *g* for 10 min at 4 °C and the supernatant was designated as the cell-free extract for determining substrate specificity. Protein in the cell-free extract was estimated by the Lowry method with bovine serum albumin (Sigma fraction V) as a protein standard. The optical density of the cell suspension was determined at 600 nm and the dry weight of cells was determined from a standard curve.

2.5. Characterization of enzymes

From the substrate specificity data, it was postulated that *Amycolatopsis* sp. IITR215 cells might contain more than one nitrile-metabolizing enzyme. Therefore, some reported inhibitors for nitrilase, nitrile hydratase and amidase were used to determine the precise nature of the enzymes. Experiments with inhibitors were carried out by preparing 10 mM substrates in presence and absence of 10 mM DEPA (diethylphosphoramidate), an amidase inhibitor [15]. Similarly, separate experiments were performed with different concentrations of N-bromosuccimide and N-ethylmaleimide as inhibitors in acrylonitrile, acrylamide and hexanenitrile as substrates. *Amycolatopsis* sp. IITR215 cells were harvested from MB media containing adiponitrile as the sole nitrogen source, and harvested cells were then incubated at 45 °C with these substrates in a shaking water bath.

2.6. Enzyme assays

Nitrile hydratase and amidase activities were determined using 10 mM acrylonitrile along with 10 mM DEPA and 10 mM acrylamide, respectively. Assays for these enzymes were carried out in 0.1 M phosphate buffer pH 7 containing 1 mM EDTA. In the assay mixture, 400 μl of substrate in phosphate buffer was incubated with 100 μl of cell suspension for 30 min at 45 °C in a shaking water bath. The reaction was terminated by adding 10 μl 1N HCl. Cells were removed by centrifugation at 20,000 × *g* for 10 min at 4 °C. Ammonia released in the reaction was determined by the Bertholet method [14]. One unit of enzyme was defined as the amount that catalyzed the hydrolysis of amide to release 1 μmol of ammonia per min under the assay conditions. In the case of nitrile hydratase, the presence of acrylamide in the context of DEPA was confirmed by GC analysis. The amount of amide formed in the mixture was determined by gas chromatography with a sol–gel wax capillary column (0.32 mm i.d., 30 m length and 0.5 μm film thickness) equipped with a flame ionization detector. The operational conditions were: column temperature, 100–200 °C at 15 °C/min with 2 min hold at 200 °C; injector and detector temperature, 260 °C; and carrier gas N_2 at a flow rate of 2 ml/min. One unit of nitrile hydratase was defined as the amount of enzyme that catalyzed formation of 1 μmol of acrylamide per min. The activity of enzyme towards hexanenitrile was determined using 10 mM hexanenitrile in 0.1 M phosphate buffer (pH 7) containing 1 mM EDTA, and the methods followed for the assay were similar to those with acrylamide. This activity was referred as hexanenitrile activity in our study. Thus, in this work, three enzyme activities were monitored, namely nitrile hydratase, amidase and hexanenitrile activity.

2.7. Effect of temperature

The optimum temperature for the nitrile-metabolizing enzymes was determined by measuring activities at various temperatures, ranging from 30 °C to 60 °C in 0.1 M phosphate buffer (pH 7) containing 1 mM EDTA with a whole cell suspension. The optimum temperature was determined by comparing enzyme activities at various temperatures.

2.8. Effect of pH

The effect of pH on nitrile-metabolizing enzymes was studied in 50 mM buffers of varying pH {acetate buffer (pH 4.0–5.8), phosphate buffer (pH 5.8–8.0), borate buffer (pH 8.0–9.2), carbonate buffer (pH 9.2–10.0)} containing 1 mM EDTA with 10 mM hexanenitrile, 10 mM acrylonitrile with DEPA and 10 mM acrylamide as