Isolation and Characterization of a Nitrile-Hydrolysing Bacterium *Isoptericola variabilis* RGT01

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Abstract A nitrile-hydrolysing bacterium, identified as *Isoptericola variabilis* RGT01, was isolated from industrial effluent through enrichment culture technique using acrylonitrile as the carbon source. Whole cells of this microorganism exhibited a broad range of nitrile-hydrolysing activity as they hydrolysed five aliphatic nitriles (acetonitrile, acrylonitrile, propionitrile, butyronitrile and valeronitrile), two aromatic nitriles (benzonitrile and *m*-Tolunitrile) and two arylacetonitriles (4-Methoxyphenyl acetonitrile and phenoxyacetonitrile). The nitrile-hydrolysing activity was inducible in nature and acetonitrile proved to be the most efficient inducer. Minimal salt medium supplemented with 50 mM acetonitrile, an incubation temperature of 30 °C with 2 % v/v inoculum, at 200 rpm and incubation of 48 h were found to be the optimal conditions for maximum production (2.64 ± 0.12 U/mg) of nitrile-hydrolysing activity. This activity was stable at 30 °C as it retained around 86 % activity after 4 h at this temperature, but was thermolabile with a half-life of 120 min and 45 min at 40 °C and 50 °C respectively.

Keywords Acrylic acid · Biotransformation · *Isoptericola variabilis* · Nitriles

Introduction

Nitriles (general formula, $RC \equiv N$) are widespread in the environment due to their excessive use in industries. Nitriles are toxic in nature, but can be hydrolysed to corresponding less-toxic and valuable carboxylic acids either by nitrilase (EC 3.5.5.1), or by nitrile-hydratase (EC 4.2.1.84) and amidase (EC 3.5.1.4). Nitrilases have been reported in a variety of microbes including *Alcaligenes* [1], *Bacillus* [2], *Burkholderia* [3], *Labrenzia* [4], *Pseudomonas* [5], *Pyrococcus* [6] and *Rhodococcus* [7]. In spite of many nitrilases reported in literature, the quest for their new sources has never ended. Owing to their capability of environment friendly transformation of nitriles, nitrilases are employed for the synthesis of pharmaceuticals and commodity chemicals [8]. These versatile biocatalysts have potential biotechnological applications like carboxylic acid production, wastewater treatment, herbicide degradation and polymer surface modification [9]. Bioconversion of acrylonitrile to acrylic acid is a better option than its chemical synthesis, which has drawbacks of high temperature conditions, side reactions, environmental pollution and high cost [7]. Acrylic acid is used in the manufacture of paint formulations, paper coatings, polishes, adhesives, acrylic esters, flocculants, etc. [7, 10].

In this paper, we report on the isolation, identification and media optimization for nitrile-hydrolysing activity of an isolate identified as *I. variabilis* RGT01.

Materials and Methods

Chemicals and Media

All nitriles, acrylamide and acrylic acid were obtained from Sigma-Aldrich, USA. Butyronitrile and propionitrile

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were purchased from Merck (Germany) and Himedia (India) respectively. All reagents were of analytical grade. The Minimal Salt Medium (MSM) contained following in g/l of deionised water: $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2; KH_2PO_4 , 2; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1; NH_4Cl , 0.1. The final pH of the medium was 7.0. Autoclaving was done at 121 °C for 15 min. Nitriles were added aseptically to the medium after autoclaving.

Screening and Cultivation of Nitrile-Hydrolysing Bacteria

Industrial effluent from Samana, Punjab, India; and soil samples from Churu, Rajasthan, India; Solan, Himachal Pradesh, India were collected. Soil sample (1 g) or effluent (1 ml) was added to 50 ml MSM supplemented with 25 mM acrylonitrile and incubated at 30 °C for 72 h under shaking conditions (orbital, 200 rpm). One ml of the culture was transferred to 50 ml of fresh medium and incubated under similar conditions. This step was repeated once again and an aliquot of the culture was spread on MSM agar plates supplemented with 25 mM acrylonitrile and incubated at 30 °C to obtain morphologically distinct colonies, which were streaked onto the fresh medium to obtain a pure culture.

The isolates obtained were streaked on MSM plates containing one of the four nitriles (acrylonitrile, propionitrile, butyronitrile and benzonitrile) at a final concentration of 25 mM. Concentration of the nitriles was increased serially up to 500 mM. The bacterial colonies growing on MSM plates supplemented with nitriles (500 mM, final concentration) were further selected for shake flask studies.

A seed culture was prepared by inoculating a single colony of the bacterial isolate into 100 ml Erlenmeyer flask containing 20 ml nutrient broth (NB), and incubated under shaking conditions (orbital, 200 rpm) at 30 °C, till $\text{OD}_{600\text{nm}}$ reached 0.4–0.6 ($\approx 3 \times 10^8$ cells per ml). One ml of seed culture was transferred to a 250 ml flask containing 49 ml of MSM supplemented with required nitrile concentration (50–200 mM). The flasks were incubated at 30 °C under shaking conditions (orbital, 200 rpm) for 120 h. Cells were harvested by centrifugation at $10,000 \times g$ for 10 min at 4 °C.

Conventional physiological and biochemical characterization tests were carried out as described in Bergey's manual of systematic bacteriology [11]. RGT01 was identified by 16S rRNA gene sequencing technique at IGIB, New Delhi, India.

Enzyme Assay Using Resting Cells

The cell pellet was washed with 0.1 M potassium phosphate buffer (pH 7.0) and suspended in the same buffer [30 mg wet pellet (≈ 4.8 mg dry weight) in 1 ml of buffer, having

$\text{OD}_{600\text{nm}} \approx 9.0$]. Nitrile-hydrolysing activity was measured by phenol-hypochlorite method [12]. One ml reaction mixture contained 945/940 μl potassium phosphate buffer (0.1 M, pH 7.0), 50 μl cell suspension (1×10^7 cells) and 5/10 μl nitrile (10 M prepared in methanol) (final conc. 50 or 100 mM). Enzyme control and substrate control were also taken into consideration. The reaction was carried out at 30 °C in a water bath for 15 min. One unit of the enzyme activity was defined as the amount of cells that hydrolysed the nitrile to release one micromole of ammonia per minute under standard assay conditions. Weight of wet cells in milligrams was used for the assay.

Optimization of Culture Conditions for Maximal Production of Nitrile-Hydrolysing Activity by *I. variabilis* RGT01

Nutrient rich media were tested for growth and activity production. The effect of various carbon sources/metal ions/nitriles/ ϵ -caprolactam on growth and nitrile-hydrolysing activity by *I. variabilis* RGT01 was studied. All factors were studied by changing one factor at a time.

Substrate Specificity of Nitrile-Hydrolysing Activity of *I. variabilis* RGT01

To study substrate specificity of nitrile-hydrolysing activity of *I. variabilis* RGT01, a wide range of nitriles (listed in Table 4) at a concentration of 50 mM was used.

Thermostability and pH Stability of Nitrile-Hydrolysing Activity of *I. variabilis* RGT01

Resting cells were suspended (30 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.0) and were incubated at different temperatures (30 °C, 40 °C and 50 °C) and at different pH values (6.5, 7.0, 7.5 and 8.0). Samples (200 μl) were withdrawn after every half an hour. To the cell suspension, 50 mM acrylonitrile (from 1 M stock prepared in methanol) was added and incubated at 30 °C. The reaction was stopped after 15 min with the addition of 200 μl of 0.1 M HCl and the amount of acrylic acid formed was determined by phenol-hypochlorite method [12]. Residual activity (RA) was calculated in accordance with the activity at 0 h.

Biotransformation of Acrylonitrile Using Resting Cells

Resting cell suspensions were prepared by suspending the cell pellet in 0.1 M potassium phosphate buffer (pH 7.0) to get a concentration of 300 mg/ml. The reaction mixture (10 ml) consisted of 0.1 M potassium phosphate buffer (pH 7.0), 2 mM acrylonitrile and a final resting cell

concentration of 30 mg/ml. The reaction was performed at 30 °C for 2 h under shaking conditions (orbital, 200 rpm); cells were removed from the reaction mixture by centrifugation at 12000×g for 10 min at 4 °C, and the supernatant obtained was then analyzed by HPLC.

Analytical Methods

HPLC (LC-20AD; Shimadzu, Kyoto, Japan) equipped with a C18 column (4.6 × 250 mm, 5 µm particle size; Spinco-tech, India) at a flow rate of 1 ml/minute with a solvent system of HPLC-grade water and acetonitrile (70:30, v/v) in combination with 0.05 % (v/v) formic acid was employed. The UV detector set at a wavelength of 210 nm was used to detect the presence of acrylic acid and acrylamide.

Results and Discussion

Selection and Identification of a Nitrile-Utilizing Strain

Using enrichment culture technique, 30 bacterial isolates having nitrile-hydrolysing activity were isolated (data not shown). After primary screening on MSM agar plates supplemented with different nitriles (500 mM, final concentration), 24 bacterial cultures were selected for shake flask studies. One isolate, RGT01 exhibited good growth on various nitrile substrates like acrylo-, propio-, butyro- and benzo- nitrile and was used for further studies. Cells of RGT01 were small rods, non-motile, positive for Gram-staining and catalase; and negative for urease, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, phenylalanine deaminase, indole production and Voges-Proskauer test. According to its 16S rDNA sequence (940 bp), isolate RGT01 was found to be 99 % identical with the sequence of *Isoptericola variabilis* (GenBank accession no. AJ298873) by examining the phylogenetic tree (Fig. 1). The sequence was deposited in the GenBank database with accession no. KF135213. The result of the phylogenetic analysis was consistent with that of the phenotypic tests. Therefore, isolate RGT01 was identified as a strain of *I. variabilis* and named *I. variabilis* RGT01. To the best of our knowledge, this is the first report on the presence of nitrile-hydrolysing activity in the genus *Isoptericola*. Till date, members of this genus are reported to have only cellulolytic [13] and chitin-degrading activities [14].

Effect of Different Media/Inducers on Nitrile-Hydrolysing Activity

Though, all nutrients rich media like nutrient broth, brain-heart infusion broth, terrific broth etc. supported the growth

of *I. variabilis* RGT01, but significant nitrile-hydrolysing activity was observed only in MSM supplemented with 200 mM acrylonitrile (Table 1). Very little or no nitrile-hydrolysing activity by *I. variabilis* RGT01 in the nutrient-rich environment indicated the strong possibility of production of the enzyme responsible for nitrile hydrolysis under stress conditions.

Most nitrilases are induced by nitriles, amides and carboxylic acids [9]. In *Rhodococcus rhodochrous* J1, *nitR* was found to be required for isovaleronitrile-dependent induction of nitrilase synthesis [15]. Our experiments also indicated inducible nature of nitrile-hydrolysing activity like many nitrilases reported in literature [9]. However, there are also reports on the constitutive nature of nitrilases as observed in *Bacillus subtilis* [2] and in *Rhodococcus ruber* [7]. Choice of inducer may affect the substrate specificity of nitrilase [16, 17]. After confirming that the nitrile-hydrolysing activity was produced only in the presence of the inducer and ruling out any constitutive expression of the enzyme, the effect of different inducers on substrate specificity was studied. The host range of nitrile-hydrolysing activity in the presence of a single inducer is listed in Table 2. Using different inducers, there was a change in the substrate specificity. Presence of more than one nitrilase might be responsible for this trend. It is possible that different substrates led to the induction of different nitrilases. There are reports in literature mentioning the occurrence of two types of nitrilases in the same organism [18–20]. Acetonitrile was finally chosen as the inducer for the cultivation, due to its cheaper price and good effectivity than other inducers. It was found to act as an efficient inducer for nitrilases of *Pseudomonas putida* [5] and *Rhodobacter sphaeroides* [21]. MSM supplemented with 50 mM acetonitrile, an incubation temperature of 30 °C with 2 % v/v inoculum, at 200 rpm and incubation of 48 h was found to be the optimal condition for maximum production (2.64 ± 0.12 U/mg) of nitrile-hydrolysing activity by *I. variabilis* RGT01 (Fig. 2). The logarithmic phase of growth was concomitant with the production of nitrile-hydrolysing enzyme. In literature, acrylonitrile-hydrolysing activities of various strains were: *Arthrobacter nitroguajacolicus*—2.02 U/mg dcw [10], *Rhodococcus* sp.—1.22 U/mg dcm [16] and *Alcaligenes faecalis*—0.04 U/mg wet cell weight [17].

ε-Caprolactam (0.1 %, w/v) reported to be a hyper-inducer of nitrilase in *Rhodococcus rhodochrous* J1 [22], did not cause any enhancement in the enzyme production. The addition of Fe²⁺ and Co²⁺ ions (required for the active expression of nitrile-hydratase enzyme) in minimal medium showed no significant effect on the nitrile-hydrolysing activity. It might be due to the presence of nitrilase enzyme in the *I. variabilis* RGT01 which is not a metallo-protein [6].

Fig. 1 Phylogenetic tree for *I. variabilis* RGT01 and related organisms based on the 16S rDNA sequences. Evolutionary history was inferred using the Neighbour-Joining method. Numbers in parentheses are accession numbers of published sequences. The evolutionary distances were computed using Kimura 2-parameter method and were conducted in MEGA5. Bootstrap values were based on 1,000 replicates

Effect of Various Carbon Sources on Nitrile-Hydrolysing Activity

Carbon sources may affect growth and nitrile-hydrolysing activity. So their effect was tested for growth as well as

Table 1 Growth and nitrile-hydrolysing activity of *I. variabilis* RGT01 in different media

Medium + acrylonitrile (200 mM)	Biomass (mg dcw/ml culture)	Acrylonitrile (100 mM) hydrolysing activity (U/mg)
Nutrient broth	1.78 ± 0.19	ND
Brain–heart infusion broth	1.34 ± 0.16	0.75 ± 0.12
Terrific broth	1.93 ± 0.032	ND
MSM	0.118 ± 0.011	1.81 ± 0.15
MSM + FeSO ₄ ·7H ₂ O (0.001 %, w/v)	0.101 ± 0.012	0.68 ± 0.009
MSM + CoCl ₂ ·6H ₂ O (0.001 %, w/v)	0.098 ± 0.023	1.02 ± 0.034
MSM + ε-Caprolactam (0.1 %, w/v)	0.120 ± 0.008	1.67 ± 0.02

Nitrile-hydrolysing activity was absent in medium devoid of inducers ε-Caprolactam (0.1 %) was used as an inducer while acrylonitrile (final conc. 100 mM) was used as substrate
dcw dry cell weight, ND not detected

Table 2 Effect of different inducers on growth and nitrile-hydrolysing activity of *I. variabilis* RGT01

Inducer (50 mM)	Biomass (mg dcw/ml culture)	Nitrile-hydrolysing activity (U/mg) with substrates (100 mM)				
		Acetonitrile	Acrylonitrile	Propionitrile	Butyronitrile	Benzonitrile
Acetonitrile	0.46 ± 0.03	4.19 ± 0.14	2.64 ± 0.12	3.14 ± 0.18	2.56 ± 0.01	1.31 ± 0.02
Acrylonitrile	0.14 ± 0.04	2.38 ± 0.12	2.05 ± 0.014	1.86 ± 0.13	1.58 ± 0.007	0.58 ± 0.03
Propionitrile	0.54 ± 0.05	0.89 ± 0.06	ND	4.80 ± 0.12	1.62 ± 0.006	ND
Butyronitrile	0.56 ± 0.023	ND	1.18 ± 0.02	1.55 ± 0.15	2.45 ± 0.04	ND
Benzonitrile	0.13 ± 0.012	ND	0.89 ± 0.14	0.58 ± 0.007	0.95 ± 0.12	1.97 ± 0.15

dcw dry cell weight, ND not detected

enzyme production (Table 3). Presence of simple sugars such as glucose, sucrose, fructose and maltose (final concentration—1.0 %) in MSM increased the growth of *I. variabilis*, but resulted in diminished nitrile-hydrolysing activity. Glucose and other hexoses are well known to repress microbial enzymes which are responsible for the catabolism of carbon compounds [23]. Whereas, use of carbon sources like lactose, starch, glycerol and sodium acetate lead to no nitrile-hydrolysing enzyme production.

Fig. 2 Cell growth and nitrile-hydrolysing activity of *I. variabilis* RGT01 in MSM supplemented with acetonitrile (50 mM)

Table 3 Effect of various carbon sources on growth and nitrile-hydrolysing activity by *I. variabilis* RGT01

C source (1.0 %, w/v)	Biomass (mg dcw/ml)	Acrylonitrile (50 mM) hydrolysing activity (U/mg)
None	0.46 ± 0.03	2.05 ± 0.19
Sucrose	0.89 ± 0.012	0.16 ± 0.03
Maltose	0.78 ± 0.016	0.89 ± 0.13
Lactose	0.73 ± 0.01	ND
Fructose	0.81 ± 0.013	0.21 ± 0.02
Glucose	0.76 ± 0.018	0.26 ± 0.03
Mannitol	0.82 ± 0.017	0.7 ± 0.12
Starch	0.81 ± 0.015	ND
Glycerol	0.88 ± 0.019	ND
Sodium acetate	0.29 ± 0.005	ND

I. variabilis RGT01 was cultivated in MSM supplemented with 50 mM acetonitrile and a single carbon source (1.0 %, w/v)

dcw dry cell weight, ND not detected

Substrate Specificity of Nitrile-Hydrolysing Activity

I. variabilis RGT01 exhibited broad substrate specificity, as it could hydrolyse five aliphatic nitriles, two aromatic nitriles and two arylacetoneitriles (Table 4). With saturated aliphatic mononitriles, the shorter the chain length, the greater the rate of hydrolysis was observed. The enzyme activity toward nitriles of shorter chain length suggested that a more compact structure may have a better access or affinity for the active site of the enzyme. The highest activity was observed with acetonitrile, followed by propionitrile, acrylonitrile, butyronitrile and valeronitrile. However, it showed no activity with branched substrates like isobutyronitrile and isovaleronitrile. In case of benzonitrile, substitution at the *meta* position by a methyl group decreased the enzyme activity; whereas at the *para* position by the amino group, lead to complete loss of the enzyme activity. Substitution at the *para* position by methoxy group on the phenyl ring increased the enzyme activity, whereas by most strong electron donating amino group lead to no activity. In case of arylacetoneitriles, 4-Methoxyphenyl acetoneitrile proved to be the best substrate.

Kinetic Parameters of Nitrile-Hydrolysing Activity

The kinetic parameters of nitrile-hydrolysing activity were estimated over a range of acrylonitrile concentration (1–10 mM) at 30 °C in potassium phosphate buffer (0.1 M, pH 7.0). The apparent K_m and V_{max} values calculated for acrylonitrile were found to be 0.909 mM and 0.862 $\mu\text{mol min}^{-1}\text{mg}^{-1}$ respectively as calculated from Lineweaver–Burk plot (Fig. 3).

Table 4 Substrate specificity of nitrile-hydrolysing activity of *I. variabilis* RGT01

Nitrile (50 mM)	Nitrile-hydrolysing activity (U/mg)
Aliphatic nitriles	
Saturated nitriles	
Acetonitrile	6.93 ± 0.011
Propionitrile	4.86 ± 0.16
Butyronitrile	1.81 ± 0.12
Valeronitrile	1.63 ± 0.012
Isobutyronitrile, Isovaleronitrile, Allyl cyanide	ND
Unsaturated nitrile	
Acrylonitrile	2.04 ± 0.017
Dinitriles	
Iminodiacetonitrile, Adiponitrile, Glutaronitrile, Succinonitrile, Malononitrile	ND
Aromatic nitriles	
Benzonitrile	1.02 ± 0.010
4-Aminobenzonitrile	ND
m-Tolunitrile	0.62 ± 0.011
Arylacetoneitriles	
Mandelonitrile, 4-Aminobenzyl cyanide	ND
4-Methoxyphenyl acetoneitrile	5.19 ± 0.009
Phenoxyacetoneitrile	1.52 ± 0.008
Heterocyclic nitrile	
Indole 3-acetonitrile	ND

ND not detected

Fig. 3 Lineweaver-Burk plot for nitrile-hydrolysing activity

Thermostability and pH stability of the nitrile-hydrolysing activity

High thermal and pH stability of a biocatalyst is always a desired trait. Nitrile-hydrolysing activity was stable as it

Fig. 4 Thermostability of nitrile-hydrolysing activity of *I. variabilis* RGT01

Fig. 5 pH stability of nitrile-hydrolysing activity of *I. variabilis* RGT01

retained around 86 % and 80 % activity after 4 h (Fig. 4) and 8 h (data not shown) at 30 °C respectively, but was thermolabile with a half life of 120 min and 45 min at 40 °C and 50 °C respectively. Nitrile-hydrolysing activity showed higher stability at pH 7.0 (Fig. 5). The stability decreased as the pH was varied, both above and below pH 7.0.

Involvement of Possible Nitrile-Hydrolysing Route

HPLC analysis of the reaction product(s) showed that acrylonitrile was converted to only one product i.e. acrylic acid (Fig. 6). No detection of acrylamide indicated no nitrile-hydratase activity in the cells. To confirm that acrylamide was not removed by the action of an amidase, the cells were examined for this activity with acetamide and acrylamide as substrates. These amides were not

Fig. 6 HPLC analysis. **a** 0.5 mM acrylamide (RT-3.0), **b** 1 mM acrylic acid (RT-3.9), **c** biotransformed product (RT-3.9) from the reaction mixture. RT: retention time

hydrolyzed by the resting cells (data not shown). Thus, indicating absence of amidase production under the experimental conditions used. Therefore, acrylamide, if formed by nitrile-hydratase, would mostly remain unreacted in the reaction mixtures. This suggested that hydrolysis of acrylonitrile by *I. variabilis* RGT01 was catalyzed by the nitrilase in the present study.

Conclusions

We have successfully isolated a novel nitrile-hydrolysing strain, *I. variabilis* RGT01. The substrate range of the microbe included aliphatic, aromatic and arylaceto-nitriles.

The microorganism produced nitrile-hydrolysing enzyme in MSM supplemented with a cheaper inducer acetonitrile. Thus, reducing overall enzyme production cost. It was able to biotransform acrylonitrile to acrylic acid. It could be utilized as a biocatalyst for the hydrolysis of low molecular weight nitriles.

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