Constitutive acetonitrile hydrolysing activity of *Nocardia globerula* NHB-2: Optimization of production and reaction conditions

H Kumar, S Prasad, J Raj & T C Bhalla*
Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla 171 005, India

Received 24 June 2005; revised 7 December 2005

*Nocardia globerula* NHB-2 exhibited an intracellular acetonitrile hydrolysing activity (AHA) when cultivated in nutrient broth supplemented with glucose (10.0 g/l) and yeast extract (1.0 g/l), at pH 8.0, 30 °C for 21 hr. Maximum AHA was recorded in the culture containing 0.1 M of sodium phosphate buffer, (pH 8.8) at 45 °C for 15 min with 600 μmol of acetonitrile and resting cells of *N. globerula* NHB-2 equivalent to 1.0 ml culture broth. This activity was stable up to 40°C and was completely inactivated at or above 60°C. About five-fold increase in AHA was observed after optimization of culture and reaction conditions. Under the optimized conditions, this organism hydrolyzed various nitriles and amides such as propionitrile, benzonitrile, acetamide, and acrylamide to corresponding acids. This nitrile/amide hydrolysing activity of *N. globerula* NHB-2 has potential applications in enzymatic synthesis of organic acids and bioremediation of nitriles and amides contaminated soil and water system.

**Keywords:** Acetonitrilase, Amidase, Nitrile hydratase, *Nocardia globerula* NHB-2

The nitrile and amide degrading enzymes (nitrilase, EC 3.5.5.1; nitrile hydratase, EC 4.2.1.84 and amidase, EC 3.5.1.4) have emerged as important biocatalysts for conversion of nitriles and amides to corresponding amides and acids of industrial and pharmaceutical value. The sources of these enzymes are plants and microbes. However, the microbes are most common source of nitrile and amide converting enzymes. A number of microorganisms such as *Rhodococcus rhodochrous* J1, *Pseudomonas chlororaphis* B 23, *Rhodococcus* sp. Strain YH3-3, *R. rhodochrous* PA-34, *R. rhodochrous* NHB-2, *Rhodococcus equi* A4 and *Brevibacterium* R 312 have been isolated and explored for their application in the transformation of nitriles/amides to corresponding acids. Acetonitrile hydrolysing activity (AHA) has been reported in *Agobacterium* sp., *Arthrobacter* sp. J-1, *Candida famata*, *Nocardia rhodococcus* LL 100-21, *R. erythropolis* A10, *R. erythropolis* BL1, *Rhodococcus* sp. as these organisms efficiently utilised acetonitrile as carbon and nitrogen source. In the present communication, optimisation of culture conditions for production of acetonitrile hydrolysing activity (AHA) of *Nocardia globerula* NHB-2 has been reported.

**Materials and methods**

**Chemicals**

Nitriles, amides and acids used in the present studies were purchased from Lancaster synthesis (England). All other chemicals and media ingredients were of AR grade from Merck (Germany) and HiMedia (Mumbai), respectively.

**Bacteria and culture conditions**

*Nocardia globerula* NHB-2 is a versatile nitrile-degrading organism, was isolated from the soil of a forest near Manali, India and maintained on nutrient agar slopes. A pre-culture of *N. globerula* NHB-2 was prepared by transferring a loopful of culture from agar slope to 50 ml of modified nutrient broth. Two ml of pre-culture was transferred to 50 ml of production medium supplemented with 0.4% (v/v) of acetonitrile (filter sterile) for induction of AHA in *N. globerula* NHB-2. It was incubated at 30°C in an incubator shaker (160 rpm) for 24 hr. Cells of *N. globerula* NHB-2 were harvested by centrifugation at 5,000 g for 15 min at 4°C. The cell pellet was washed twice with 0.1 M potassium phosphate buffer (pH 7.2) containing 5 mM of 2-mercaptoethanol and finally suspended in the same buffer. These cells were termed as resting cells and used as biocatalyst for hydrolysis of acetonitrile to acetic acid.
Enzyme assay

AHA was assayed in 2 ml reaction mixture containing resting cells of *N. globerula* NHB-2 (i.e. equivalent to 1 ml of culture broth), 400 μmol acetonitrile and 0.1 M potassium phosphate buffer, (pH 7.2). It was incubated at 30°C for 15 min and the reaction was ceased by addition of 2 ml of 0.1 N HCl. In the control, acetonitrile was omitted during incubation and added after the reaction was stopped. The reaction mixture was centrifuged at 5,000 g for 10 min and ammonia released during enzymatic hydrolysis of acetonitrile was estimated in the supernatant following the method of Fawcett and Scott (1960)\(^{18}\). One unit of acetonitrile hydrolyzing activity (AHA) was defined as that amount of enzyme, which released 1 μmol of NH\(_3\)/min under assay conditions. In case of hydrolysis of acrylonitrile to acrylamide, the amount of acrylamide formed was estimated in the supernatant of reaction mixture by high performance liquid chromatography, HPLC\(^{19}\).

All experiments were carried out in triplicate and mean value was taken under consideration.

Optimisation of culture conditions for production of AHA in *N. globerula* NHB-2

Six different media (M1-M6 supplemented with 0.4% v/v acetonitrile) were tested for the production of AHA by *N. globerula* NHB-2 (Table 1). Effect of temperature (20°C-45°C), incubation time (0-63 h), inoculum size (1-10%, v/v) and pH (5.5-10.0) were also studied on this activity. Effect of acetonitrile concentration (0-4% v/v) was studied on AHA, and growth of *N. globerula* NHB-2 in the production medium M2.

Optimization of reaction conditions using AHA of *N. globerula* NHB-2

Effect of reaction buffer (pH 6.0-9.0), various buffer systems (i.e. sodium phosphate, potassium phosphate, borate, bicarbonate and Tris-HCl buffers of 0.1 M and pH 8.8), incubation temperature (20°C-60°C), amount of resting cell (i.e. equivalent to 1-5ml culture broth), incubation time (7-77 min), substrate (acetonitrile) concentration (200-900 μmol) on AHA of *N. globerula* NHB-2 were studied. Thermostability of AHA was studied after 1h incubation at 30°C-80°C.

Substrate affinity of AHA of *N. globerula* NHB-2

*N. globerula* NHB-2 was grown at optimized production conditions. The substrate affinity of AHA of this organism was studied at optimized reaction conditions against 600 μmol of various nitriles/amides (e.g. acetonitrile, acrylonitrile, benzonitrile, acrylamide, acrylamide and benzamide) to produce corresponding acids and ammonia.

Effect of metal ions, carbonyl reagent and chelators on AHA of *N. globerula* NHB-2

Effect of metal ions (e.g. Zn\(^{++}\), Fe\(^{++}\), Ca\(^{++}\), Pb\(^{++}\), Cu\(^{++}\), Co\(^{++}\), Mn\(^{++}\), Al\(^{+++}\), Cd\(^{++}\), Hg\(^{++}\) and Ag\(^{+}\)), carbonyl reagent (phenyl hydrazine hydrochloride), chelators (EDTA and sodium azide) and urea on AHA of *N. globerula* NHB-2 was also studied.

Results and Discussion

Optimization of culture conditions for production of AHA in *N. globerula* NHB-2

Medium — Among the six medium [M1-M6 supplemented with acetonitrile (0.4% v/v)] tested, the maximum AHA (0.06 U/ml) was observed in the modified nutrient broth medium (M2) supplemented with glucose (10 g/l) and yeast extract (1 g/l), whereas slightly low enzyme activity (0.05 U/ml) was also recorded in *N. globerula* NHB-2 cells grown in nutrient broth medium (M1; Table 1). Aliphatic nitrile/amide hydrolysing activity of *Bacillus pallidus* Dac 521 was also noted in nutrient broth. \(^{20}\) In media M3 and M6, 50 and 66% activity, respectively was observed in comparison to medium M2. No activity could be detected in cells cultured in mineral salt

<table>
<thead>
<tr>
<th>Medium*</th>
<th>Composition (g/l, pH 7.2)</th>
<th>AHA (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Nutrient broth Peptone 5.0 g and beef extract 3.0 g</td>
<td>0.05</td>
</tr>
<tr>
<td>M2</td>
<td>Modified nutrient broth Glucose 10.0 g, yeast extracts 1.0 g, peptone 5.0 g and beef extract 3.0 g</td>
<td>0.06</td>
</tr>
<tr>
<td>M3</td>
<td>MY medium Glucose 15.0 g, peptone 5.0 g, yeast extract 3.0 g and malt extract 3.0 g</td>
<td>0.03</td>
</tr>
<tr>
<td>M4</td>
<td>Modified MY medium Glycerol 10.0 g, peptone 5.0 g, yeast extract 3.0 g and malt extract 3.0 g</td>
<td>0.03</td>
</tr>
<tr>
<td>M5</td>
<td>Mineral salt medium Na(_2)HPO(_4).12H(_2)O 2.5 g, KH(_2)PO(_4) 2.0 g, MgSO(_4).7H(_2)O 0.5 g, FeSO(_4).7H(_2)O 0.03 g, CaCl(_2).2H(_2)O 0.06 g and yeast extract 0.1 g</td>
<td>ND</td>
</tr>
<tr>
<td>M6</td>
<td>APY medium (NH(_4))(_2)PO(_4) 5.0 g, peptone 5.0 g, yeast extract 3.0 g, K(_2)HPO(_4) 5.0 g, MgSO(_4) 7H(_2)O 0.2 g and FeSO(_4).7 H(_2)O 0.02 g</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*0.4% Acetonitrile was added in each medium
ND: not detected
medium (M5) due to poor growth of the organism, while the optimum production of nitrilase has been reported in *R. rhodochrous* PA-34 cells grown in M5 by Bhalla *et al.* (1992)4. A comparison of the composition of media used in the present investigation showed that glucose and beef extract together in the medium M2 might have enhanced AHA in *N. globerula* NHB-2.

**Temperature** — Optimum temperature for the production of AHA (0.06 U/ml) in *N. globerula* NHB-2 was found to be 30°C. It showed that AHA of *N. globerula* NHB-2 was mesophilic in nature. Most of the nitrile degrading organisms grew and nitrile-hydrolysing enzymes operated around 30°C4. There was 67 and 50% decline in activity at 20° and 45°C, respectively.

**Time course of enzyme production** — AHA in *N. globerula* NHB-2 was low during lag phase of growth (0-7 h), it increased rapidly in early exponential phase (7-14 h) and maximum activity (0.08 U/ml) was observed in mid exponential phase at 21 h (Fig. 1). Thereafter, a rapid fall in activity was recorded in stationary phase (28 h onward) and it was almost constant, 0.01 U/ml beyond 42 h incubation. Reduction in activity on prolonged incubation may be due to production of intracellular proteases and AHA may be prone to action of proteolytic enzymes. A similar trend has been reported in *Arthrobacter* sp. strain C-38 which exhibits maximum growth at 60 h, but increase in nitrile hydratase activity has been recorded only up to 30 h and a loss of enzyme activity has been observed on further incubation31.

**Inoculum size** — At 2 and 4% (v/v) of inoculum, optimum production of AHA was recorded i.e. 0.08 and 0.083 U/ml, respectively. Above 4% (v/v) inoculum, gradual decline in enzyme activity was observed and about 54% of enzyme activity was recorded at 8-10% (v/v) of inocula in comparison to control (0.4 % inoculum). Decrease in enzyme production at higher level of inocula may be due to relatively more abundance of cells which had achieved late log phase or stationary phase of growth because of early depletion of nutrients. Earlier investigators have also used 1-4% (v/v) pre-culture for optimum production of nitrile/amide hydrolysing activity5,22.

**pH** — *N. globerula* NHB-2 was grown in the medium (M2) at pH 5.5 to 11, but AHA was only detected at pH range 6 to 9.5. Maximum activity (0.10 U/ml) was found at pH 8. At pH range 8.5-9.5, about 40% enzyme activity was observed as compared to optimum. AHA was not produced when pH of the medium was below 5.5 and above 10. It indicated that optimum pH for production of AHA was near neutral/slightly alkaline pH. Acetonitrilase (nitrile hydratase-amidase system) production in *Brevibacterium* R312 has been reported to be maximum at pH 7 (Ref.8). However, *N. globerula* NHB-2 expressed two significant activities, one at acidic (pH 6) and other at alkaline (pH 8) condition. It seemed that this organism produced two types of AHA (one at pH 6 and other at pH 8) for catalyzing the hydrolysis of acetonitrile to acetic acid.

**Acetonitrile concentration** — In most of the nitrile-degrading enzyme systems reported hitherto, nitriles have been added in the culture medium as inducer of nitrile hydrolysing enzymes10,12. However, in the present studies, it was observed that maximum production of AHA in *N. globerula* NHB-2 (0.11 U/ml) was in the absence of acetonitrile in the medium M2. It seemed that this activity was constitutive. With increasing the concentration of acetonitrile (0-4% v/v) in medium M2, gradual decrease in AHA was recorded. It appeared that acetonitrile partially suppressed the genes involved in the expression of AHA in this organism. At 4% of acetonitrile concentration in the medium, the organism could exhibit only 17.6% activity. However, *N. globerula* NHB-2 could tolerate 4.5% (v/v) of acetonitrile concentration in the medium M2. Such a high tolerance of acetonitrile by *N. globerula* NHB-2 may be of potential application for removal of acetonitrile from industrial wastes, bioremediation of nitrile contaminated soil or water systems.

---

Fig. 1 — Time course of AHA production in *N. globerula* NHB-2 (–○– indicates the growth of *N. globerula* NHB-2 and –●– shows status of AHA production)
Optimization of reaction conditions for AHA of *N. globerula* NHB-2

**Buffer pH** — The resting cells of *N. globerula* NHB-2 exhibited AHA in the pH range of 6-9. However, maximum activity (0.12 U/ml) was recorded at pH 8.8. As observed during production of AHA, two significant enzyme activities (0.09 and 0.12 U/ml) were also observed at pH 6.5 and 8.8, respectively. It further supported the apparent role of two different enzymes in the hydrolysis of acetonitrile. However, the optimum pH for hydrolysis of acetonitrile in *Brevibacterium* R 312 was 7.

**Buffer system** — Four buffers [sodium phosphate, potassium phosphate, borate, bicarbonate and tris-HCl (0.1M) and pH 8.8] were tested, and recorded maximum AHA (0.13 U/ml) in sodium phosphate buffer. However, AHA, 0.12, 0.05 and 0.09 U/ml, were reported in potassium phosphate, bicarbonate and borate buffer, respectively, while no AHA was detected in Tris-HCl buffer possibly due to inhibition of enzyme system. It seemed that the potentially reactive primary amine of Tris (hydroxymethyl)aminomethane (Tris) might be interfering with the enzyme protein leading to its inhibition.

**Incubation temperature and thermal stability** — AHA increased gradually from 0.05 U/ml at 20°C to 0.15 U/ml at 45°C and optimum temperature was found to be 45°C. However, insignificant decrease in activity (0.14 U/ml) was observed at 40°C and 50°C. Above 50°C, a rapid decline in activity was recorded. It might be due to thermal inactivation of enzyme. Majority of the nitrile degrading enzymes optimally catalyse nitrile hydrolysis in the mesophilic range (23°C-37°C), but the nitrile hydrolysing activity of *Bacillus* strain is thermophilic in nature.

Thermostability of AHA was also investigated by preincubating the resting cells of *N. globerula* NHB-2 at various temperatures (30°C-80°C) for 1 hr. This activity was stable upto 40°C and thereafter, enzyme activity decreased with the rise in temperature. The residual AHA after 1 hr preincubation at 50°C was 67% and above 60°C it was completely inactivated.

**Biocatalyst and substrate concentration** — There was linear increase in total ammonia production with the increase in resting cell (biocatalyst containing AHA) in reaction mixture from 10-50% v/v (i.e. equivalent to 1-5 ml culture broth), however, AHA (0.15 U/ml) remained almost unchanged.

AHA gradually increased from 0.04 U/ml at 200 μmol to 0.16 U/ml at 600 μmol of acetonitrile as substrate, then it declined with increasing concentration of acetonitrile. It might be due to substrate inhibition of enzyme. About 50% AHA was observed at substrate concentration of 900 μmol.

Substrate affinity for AHA of *N. globerula* NHB-2 Among six substrate, maximum AHA was recorded with acetamide as substrate followed by acetonitrile (Table 2). The higher relative activity against acetamide proved that this reaction was catalysed by two enzymes (i.e. nitrile hydratase and amidase) and amidase, which catalyse acetamides hydrolysis, had more activity in comparison to nitrile hydratase of acetonitrile hydrolysis pathway. Therefore, nitrile hydratase was a step regulating enzyme. Similar result has been observed in *Kluyveromyces thermotolerans* MGBY 37. AHA of *N. globerula* NHB-2 could not hydrolyse acrylonitrile, while acrylamide was hydrolysed into acrylic acid. However, acrylamide peak was detected (Fig. 2), when reaction mixture of acrylonitrile hydrolysis was analysed using high performance liquid chromatography (HPLC), this indicated that acrylonitrile somehow inhibited amidase activity of the strain, NHB-2.

In case of benzonitrile and benzamide hydrolysis, AHA activity could not be detected using benzamide as substrate, while benzonitrile was hydrolysed to small extent. This could be explained on the basis of presence of a third enzyme i.e. nitrilase, which directly converted benzonitrile to benzoic acid.

**Effect of metal ions on AHA of N. globerula NHB-2** — Among the metal ions studied, Fe++, Ca++, Cu++, Co++, Cd++, Al+++ and Mn++ enhanced AHA ranging from 4 to 71%. These might have played an important role in the assembly of subunits of the enzyme and subsequently stabilization of the native enzyme in active form. However, Hg++, Pb++, Zn++ and Ag++ inhibited the activity up to 60, 35, 24 and 7%, respectively (Table 3).

<table>
<thead>
<tr>
<th>Table 2 — Substrate affinity of AHA of <em>N. globerula</em> NHB-2</th>
<th>Relative nitriles/amides hydrolysing activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrile/Amide</td>
<td></td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>100.0</td>
</tr>
<tr>
<td>Acrylonitrile</td>
<td>0.0</td>
</tr>
<tr>
<td>Benzonitrile</td>
<td>19.0</td>
</tr>
<tr>
<td>Acetamide</td>
<td>107.0</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>45.0</td>
</tr>
<tr>
<td>Benzamide</td>
<td>0.0</td>
</tr>
</tbody>
</table>
AHA of *N. globerula* NHB-2 had some interesting features, e.g. its production was constitutive, it optimally catalysed the hydrolysis of nitrile/amides at 45°C, and the organism could tolerate up to 4.5% acetonitrile during growth that made it a potential biocatalyst for application in synthesis of organic amides and acids or bioremediation.

**Acknowledgement**

The author (HK) gratefully acknowledges CSIR, New Delhi, for financial support.

**References**

10. Asano Y, Fujishiro K, Tani Y & Yamada H, Aliphatic nitrile hydratase from *Arthrobacter* sp. J-1: Purification and


