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ORIGINAL PAPER

An improved nitrilase-mediated bioprocess for synthesis of nicotinic acid from 3-cyanopyridine with hyperinduced *Nocardia globerula* NHB-2

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Abstract Nitrilase of *Nocardia globerula* NHB-2 was induced by short-chain aliphatic nitriles (valeronitrile > isobutyronitrile > butyronitrile > propionitrile) and exhibited activity towards aromatic nitriles (benzonitrile > 3-cyanopyridine > 4-cyanopyridine > *m*-tolunitrile > *p*-tolunitrile). Hyperinduction of nitrilase (6.67 U mg_{DCW}^{-1}, 18.7 U mL^{-1}) was achieved in short incubation time (30 h, 30°C) through multiple feeding of isobutyronitrile in the growth medium. The nitrilase of this organism exhibits both substrate and product inhibition effects. In a fed batch reaction at 1 L scale using hyperinduced resting cells corresponding to 10 U mL^{-1} nitrilase activity (1.5 mg_{DCW} mL^{-1}), a total of 123.11 g nicotinic acid was produced at a rate of 24 g h^{-1} g_{DCW}^{-1}.

Keywords Nitrilase · *Nocardia globerula* NHB-2 · Isobutyronitrile · Multiple feeding · Hyperinduction · Nicotinic acid · 3-Cyanopyridine

Introduction

Nitrilases (nitrile aminohydrolases, EC 3.5.5.1) catalyse hydrolysis of the triple bond of the cyano group of nitriles to form corresponding carboxylic acids with removal of nitrogen as ammonia (Fig. 1). Nitrilases have been grouped together to form a branch under the nitrilase superfamily and include the characteristic catalytic triad of glutamate, lysine and cysteine at their active site, operating through a thiol acylenzyme intermediate [20]. Nitrilases are able to hydrolyse the nitrile group of aliphatic/aromatic nitriles, arylacetonitriles and hydroxynitriles to a carboxylic group with release of ammonia at physiological pH and temperature [19]. In recent years, some fine chemicals of pharmaceutical importance have been commercially produced from their corresponding nitriles by industry using nitrilase-catalysed processes, e.g. (*R*)-mandelic acid, (*R*)-4-cyano-3-hydroxybutyric acid and (*S*)-ibuprofen [3, 7, 24, 27].

Nicotinic acid (vitamin B_3) is a pellagra-preventing factor, commercially synthesised through liquid-phase oxidation of either 2-methyl-5-ethyl pyridine with nitric acid under high temperature and pressure [28], or 3-methylpyridine in combination with cobalt acetate, manganese acetate and aqueous hydrobromic acid at 100 atm pressure and 210°C to produce 32% picoline and 19% nicotinic acid [9]. Electrochemical methods for oxidation of 3-picoline to nicotinic acid with 70% yield have also been described [25]. Chuck [5], in his review of developments in nicotinate production process technology, revealed that only nicotinamide production through biological process (nitrile hydratase based) has been commercialized, and that nicotinic acid is still manufactured by chemical routes.

In 1988, the first nitrilase-mediated bioprocess for conversion of 3-cyanopyridine (3-CP) to nicotinic acid, using *Rhodococcus rhodochrous* J1, was described [13]. Thereafter, *Nocardia rhodococcus* LL100-21 and *Bacillus pallidus* Dac21 were also explored to develop similar processes [1, 26]. The nitrilase-mediated processes developed to date for conversion of *N*-substituted aromatic nitriles have not been commercialized, due to low substrate tolerance, low product yield and slower conversion rates. These limitations are the main bottlenecks for any commercial process. Nicotinamide (BASF AG, Germany and Lonza AG, Switzerland) and acrylamide (Nitto Chemicals, Japan) are currently synthesized commercially from the

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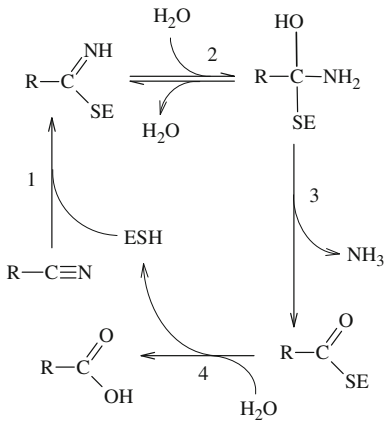

Fig. 1 Mechanism of nitrilase-catalysed hydrolysis of nitrile to corresponding acid and ammonia (*R* aliphatic, aromatic or aryl group)

corresponding nitrile using nitrile-hydratase-based processes. The high product accumulation in nitrile-hydratase-based processes (7–12 M nicotinamide, 600–656 g L^{-1} acrylamide) is the main advantage for its commercialization [4, 15, 18, 21, 22, 31]. Thus, there is a need to search for hyperactive nitrilases capable of tolerating high substrate and product concentrations to replace the existing chemical technology of nicotinic acid production. In this regard, a wild nitrilase from eDNA library was mutated to increase substrate tolerance (2.25 M 3-hydroxyglutaronitrile), and finally 619 g L^{-1} (*R*)-4-cyano-3-hydroxybutyric acid was synthesised [7].

In our laboratory, two strains of nitrilase-producing organism—*Nocardia globerula* NHB-2 and *Rhodococcus rhodochrous* NDB1165—have been used previously for production of nicotinic acid [21, 23]. In the present work, isobutyronitrile feeding in the growth medium for nitrilase hyperinduction in *N. globerula* NHB-2 in short incubation time was optimized. Biotransformation of 3-CP to nicotinic acid was performed using hyperinduced cells of *N. globerula* NHB-2 to develop a bioprocess for conversion of 3-CP to nicotinic acid at a faster rate than in earlier reports. All previously reported nitrilase-based processes have used biocatalyst in terms of dry cell weight to perform biotransformation of nitrile to acid. This is the first report of using biocatalyst (resting cells) in terms of U mL^{-1} nitrilase activity in the reaction for optimization of bioprocess parameters.

Materials and methods

Materials

3-CP (99% pure) was a gift from Jubilant Organosis (India). All the nitriles and amides were from Alfa Aesar, a Johnson Matthey Company (previously Lanchaster Synthesis). The media components were procured from HiMedia, Mumbai (India). All other reagents were of analytical or high-performance liquid chromatography (HPLC) grade as per the requirement.

Microorganism and culture conditions

Nocardia globerula NHB-2 was deposited and identified at the Microbial Type Culture and Gene Collection (MTCC), Institute of Microbial Technology, Chandigarh (INDIA), with accession no. MTCC 6278 [2]. Preculture was prepared by transferring a single colony of *N. globerula* NHB-2 grown over nutrient agar for 48 h at 30°C to 50 mL medium (1.0% glucose, 0.5% peptone, 0.3% beef extract, 0.1% yeast extract, pH 7.5) in a 250-mL Erlenmeyer flask and incubated at 30°C, 160 rpm in an incubator shaker to $OD_{660} \approx 10$. The preculture (2 mL) was inoculated into 50 mL of above medium containing 40 mM isobutyronitrile in a 250-mL Erlenmeyer flask and incubated under similar conditions. After specified incubation times, cells from the culture were harvested by centrifugation at 8,000 × *g* (4°C, 5 min) and washed twice with 0.1 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.5), then finally suspended in the same buffer (referred to as resting cells).

Nitrilase assay

The nitrilase assay was performed in a reaction mixture (1.0 mL) containing 0.1 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.5), 50 mM nitrile and resting cells at 30°C in a water bath shaker. After 15 min of incubation, reaction was stopped with equal volume of 0.1 N HCl.

Analytical methods

The amount of ammonia released in the reaction mixture was estimated colorimetrically using the phenate-hypochlorite method [8], and the amount of nicotinic acid formed due to hydrolysis of 3-CP by nitrilase was estimated using HPLC, as described previously [23]. One unit of nitrilase activity was defined as that amount of resting cells (mg dry cell = mg_{DCW}) which catalyzed the increase of one micromole of ammonia/acid per min by hydrolysis of nitrile under assay conditions. The unutilized glucose of the spent medium was estimated by the 3,5-dinitrosalicylic acid (DNSA) method [14]. Growth of the organism was monitored turbidometrically at 660 nm (1 OD_{660} = 0.2 mg_{DCW}).

Hyperinduction of nitrilase

Effect of various inducers on nitrilase induction

Various inducers (nitriles or amides) were added at concentration of 40 mM in the production medium to select an

appropriate inducer for hyperinduction of nitrilase in *N. globerula* NHB-2.

Multiple feeding of different concentrations of isobutyronitrile in growth medium

Culture of *N. globerula* NHB-2 was fed with varied amount of isobutyronitrile: set 1 (constant level)—10 mM was fed every 6 h interval up to 24 h; set 2 (high exponential level)—10, 20, 40, 80, 160 mM; set 3 (low exponential level)—2.5, 5, 10, 20, 40 mM isobutyronitrile was fed at 0, 6, 12, 18, 24 h of incubation, respectively. In control 1, no nitrile was added to the medium, whereas in control 2, isobutyronitrile (40 mM) was added.

Furthermore, as maximum nitrilase induction was obtained in set 1 and nitrilase activity declined after 18 h in control 2, simplified feeding in terms of percentage (v/v) of isobutyronitrile was performed: set 1*: 0.3%, 0.6%, 1.2%; set 2*: 0.3%, 1.2%, 2.4%; set 3*: 0.3%, 1.0% and 1.0% isobutyronitrile (v/v) fed at 0, 18 and 24 h during cultivation. The cells were harvested after 30 h of incubation. Nitrilase activity and growth were determined.

Bioprocess development for production of nicotinic acid

Effect of 3-cyanopyridine concentration on nitrilase activity

Different concentrations of 3-CP ranging from 10 to 400 mM were added in the reaction mixture to study the effect of substrate concentration on enzyme activity. The reaction was performed at 35°C for 15 min. The nitrilase units were determined by estimating the micromoles of nicotinic acid by HPLC. Nitrilase activity in the presence of 50 mM 3-CP at 35°C was used to calculate the amount of biocatalyst to be added in the reaction in terms of U mL^{-1}.

Time course of 3-cyanopyridine conversion

The nitrilase of *N. globerula* NHB-2 encountered substrate inhibition. This experiment was carried out to study the effect of increasing enzyme concentration on the formation of nicotinic acid at different 3-CP concentrations in the reaction. The reaction was performed in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.5) containing 100, 200, 300, 400 or 500 mM 3-CP and resting cells required to convert the whole substrate to product in 2 h, i.e. resting cells corresponding to 0.83, 1.67, 2.5, 3.33 or 4.17 U nitrilase activity per mL reaction mixture, respectively. The reaction was performed in 2.0 mL reaction mixture at 35°C, and nicotinic acid formed was estimated at an interval of 15 min.

Effect of resting cells concentration on conversion of 300 mM 3-cyanopyridine

To determine the optimum biocatalyst concentration in reaction required for complete conversion of substrate to product, the reaction was performed using 2.5, 5, 7.5 and 10 U nitrilases per mL reaction mixture containing 300 mM 3-CP at 35°C, and after every 15 min, samples were withdrawn for estimation of nicotinic acid and 3-CP.

Fed batch reaction at 40 mL scale (300 mM 3-cyanopyridine)

In the presence of 10 U mL^{-1} nitrilase in the reaction mixture, complete conversion of 300 mM 3-CP to nicotinic acid in 1 h was achieved. Thus, feeding of 300 mM substrate was performed after every 1 h interval in a 250-mL conical flask containing 40 mL reaction mixture with 10 U mL^{-1} nitrilase. The reaction was performed in a water bath shaker with reciprocal shaking at 35°C. Finely powdered 3-CP (1.25 g) was added directly to the reaction mixture. A total of eight feedings (10 g 3-CP) were added, and a sample was withdrawn before every feed for nicotinic acid estimation by HPLC.

Fed batch reaction at 40 mL scale (100 mM 3-cyanopyridine)

In the above experiment, substrate and product inhibitions were encountered after the second feed, decreasing the product formation rate sharply. Thus, a lower level of substrate feeding (100 mM) was applied at an interval of 20 min. Again the reaction volume was 40 mL, which contained resting cells corresponding to 10 U mL^{-1} nitrilase. In total, 8.34 g 3-CP was added in the reaction mixture in 400 min.

Fed batch reaction at 1 L scale (100 mM 3-cyanopyridine)

Based on the above experiment, the reaction volume for conversion of 3-CP was scaled up to 1 L. This reaction was performed in a 1.5-L fermenter (BioFlow C-32; New Brunswick Scientific, USA). Substrate corresponding to 100 mM (10.41 g) was fed every 20 min. A total of 1 mol (104.1 g) substrate was added to the reaction in 200 min. The temperature of the reaction mixture was maintained at 35°C, and impeller speed of 200 rpm was set for proper mixing of substrate and resting cells.

Results

Effect of nitriles/amides on induction of nitrilase

Growth of *N. globerula* NHB-2 was inhibited in presence of benzonitrile, *p*-tolunitrile, *m*-tolunitrile, phenylacetonitrile, 4-CP, acrylonitrile and mandelonitrile. Most of the investigated aliphatic nitriles induced nitrilase expression in *N. globerula* NHB-2 and at the same time inhibited the growth of the organism (Table 1). In the screening of inducers, isobutyronitrile emerged as the best inducer for nitrilase expression in *N. globerula* NHB-2 (1.62 U mg_{DCW}^{-1}), with ~50% growth inhibition.

Nitrilase-induced cells of *N. globerula* NHB-2 were used to hydrolyse various nitriles to determine substrate specificity (Table 1). Aromatic nitriles were efficiently hydrolysed, and highest activity was recorded against benzonitrile, followed by 3-CP, 4-cyanopyridine, *m*-tolunitrile and *p*-tolunitrile. Aliphatic nitriles were hydrolysed at a lower rate, though they acted as better inducers. This enzyme showed 78% reactivity towards acrylonitrile with respect to 3-CP, but for its saturated counterpart, i.e. propionitrile, very low reactivity (9%) was observed.

Hyperinduction of nitrilase

N. globerula NHB-2 was found to be a fast-growing bacterium, attained stationary phase in 30 h and efficiently utilized glucose as carbon source (Fig. 2, control 1). Presence of 40 mM isobutyronitrile (Fig. 2, control 2) decelerated growth and glucose utilization rates, and it took *N. globerula* NHB-2 48 h to reach stationary phase. Growth and glucose utilization rates in the exponential phase were 0.45 mg_{DCW} h^{-1}, 0.63 mg h^{-1} and 0.15 mg_{DCW} h^{-1}, 0.17 mg h^{-1} in control 1 and control 2, respectively. After 18 h of incubation, maximum nitrilase

Table 1 Effect of nitriles/amides on growth and nitrilase induction in *N. globerula* NHB-2 and its substrate specificity

	Growth[a] (mg_{DCW} mL^{-1})		Nitrilase activity[b]		Relative substrate reactivity[c] (%)
			U mg_{DCW}^{-1}	U $mL_{culture}^{-1}$	
Aromatic nitriles	Benzonitrile	0	0	0	111 ± 2.22
	p-Tolunitrile	0	0	0	72 ± 3.16
	m-Tolunitrile	0	0	0	76 ± 2.28
	Phenylacetonitrile	0	0	0	0
Heterocyclic nitriles	2-Cyanopyridine	0.52 ± 0.03	1.57 ± 0.08	0.82 ± 0.04	1 ± 0.02
	3-Cyanopyridine	1.11 ± 0.06	0	0	100 ± 2.34
	4-Cyanopyridine	0	0	0	87 ± 2.17
Aliphatic nitriles	Acetonitrile	2.92 ± 0.18	0	0	5 ± 0.03
	Propionitrile	2.66 ± 0.13	0 ± 0.03	1.74 ± 0.06	9 ± 0.45
	Butyronitrile	2.52 ± 0.15	0.82 ± 0.05	2.08 ± 0.12	6 ± 0.36
	Isobutyronitrile	2.34 ± 0.16	1.62 ± 0.11	3.80 ± 0.04	12 ± 0.84
	Valeronitrile	0.35 ± 0.03	1.98 ± 0.16	0.70 ± 0.01	3 ± 0.24
	Isovaleronitrile	0.26 ± 0.02	1.81 ± 0.09	0.47 ± 0.03	1 ± 0.05
	Acrylonitrile	0	0	0	78 ± 3.9
	Adiponitrile	2.14 ± 0.09	0.22 ± 0.02	0.46 ± 0.02	3 ± 0.21
Hydroxynitriles	Acetone cyanohydrin	0.09 ± 0.01	0	0	0
	Mandelonitrile	0	0	0	0
Amides	Acetamide	2.00 ± 0.14	0	0	–
	Methacrylamide	3.66 ± 0.29	0	0	–
	Butyramide	4.69 ± 0.42	0	0	–
	Cyanoacetamide	3.67 ± 0.15	0	0	–
	ε-Caprolactam	1.78 ± 0.04	0	0	–
	Urea	2.65 ± 0.13	0	0	–
Control (without inducer)		4.59 ± 0.14	0	0	0

[a] 40 mM inducer was supplemented in the growth medium, and nitrilase assay was performed using 50 mM 3-cyanopyridine as substrate

[b] One unit (U) of nitrilase activity was defined as one micromole of ammonia released per minute under assay conditions

[c] Substrate specificity of nitrilase was tested against various nitriles added at 50 mM concentration in 1.0 mL reaction mixture containing cells of *N. globerula* NHB-2 grown in medium supplemented with 40 mM isobutyronitrile. Release of ammonia by hydrolysis of 3-cyanopyridine at a rate of 1.62 μmoles NH_3 min^{-1} $mg_{DCW^{-1}}$ was taken as 100%

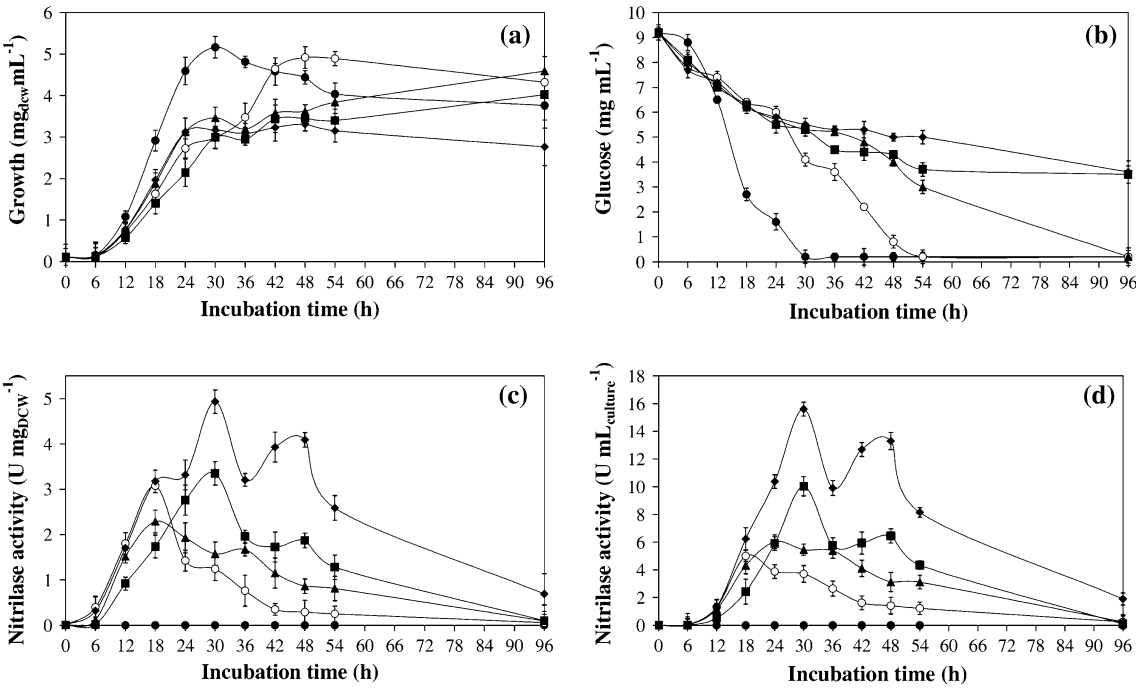

Fig. 2 Effect of isobutyronitrile on growth (**a**), glucose utilization (**b**) and nitrilase induction (**c**,**d**) in *N. globerula* NHB-2: control 1 (*filled circles*), without isobutyronitrile; control 2 (*open circles*), 40 mM isobutyronitrile added at 0 h; set 1 (*filled triangles*) (constant level), 10 mM fed every 6 h interval up to 24 h; set 2 (*filled diamonds*) (high exponential level), 10, 20, 40, 80, 160 mM; set 3 (*filled squares*) (low exponential level), 2.5, 5, 10, 20, 40 mM isobutyronitrile fed at 0, 6, 12, 18, 24 h of incubation, respectively. Growth was determined turbidometrically at 660 nm (1 OD_{660} = 0.2 mg_{DCW} mL^{-1}). Nitrilase activity was determined using 50 mM 3-cyanopyridine as substrate in the reaction at 30°C

activity (3.07 U mg_{DCW}^{-1}, 5.01 U mL^{-1}) in control 2 was observed, which sharply declined on further incubation. High-level (Fig. 2, set 2) exponential feeding of isobutyronitrile in the medium resulted in hyperinduction of nitrilase (4.93 U mg_{DCW}^{-1}, 15.6 U mL^{-1}) after 30 h of incubation, however glucose utilization rate almost ceased during 24–54 h, leaving 36% of added glucose unutilized even after 96 h of incubation. Although feeding of 160 mM isobutyronitrile in the growth medium (set 2) almost ceased the growth of the organism, expression of nitrilase was immensely increased. At 36 h of incubation, nitrilase expression was lowered and once again increase in activity was observed after 48 h of incubation. Nitrilase activity declined on further incubation. A similar effect with lower concentration of inducer was observed for set 1 (constant level) and set 3 (low exponential level). It was also observed that the glucose utilization rate in the initial 6 h of incubation was higher in the medium supplemented with nitrile.

The results of feeding of isobutyronitrile in production medium [set 1*: 0.3%, 0.6%, 1.2%; set 2*: 0.3%, 1.2%, 2.4%; set 3*: 0.3%, 1.0%, 1.0% isobutyronitrile (v/v) at 0, 18, 24 h during cultivation] are summarized in Table 2. In set 1*, highest nitrilase induction (6.67 U mg_{DCW}^{-1}) as well as growth (2.84 mg_{DCW} mL^{-1}) were achieved.

Bioprocess development for production of nicotinic acid

Highest 3-CP hydrolysing activity was recorded at 55°C in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.5), with a rapid decline as incubation time progressed (compete loss in 3 h) (data not shown). The enzyme showed 50% lower activity at 35°C and better stability (16% loss in 24 h) (data not shown). Considering these results, bioprocess parameters for conversion of 3-CP to nicotinic acid were optimized at 35°C.

Effect of 3-cyanopyridine concentration on nitrilase activity

In the presence of high 3-CP concentration, a typical substrate inhibition effect was observed (Fig. 3). The highest nitrilase activity was recorded at 50 mM 3-CP (6.67 U mg_{DCW}^{-1}). Substrate inhibition resulted in lowering of the 3-CP hydrolysis rate to 87% and 64% at 100 and 300 mM substrate concentration, respectively. Non-linear regression fit analysis of the reaction velocity (V_0) at different substrate concentrations (S_0) using GraphPad Prism software resulted in $V_{max} = 7.984 \pm 0.335$ U mg_{DCW}^{-1}, $K_m = 4.968 \pm 1.102$ mM and $K_{si} = 325.7 \pm 42.73$ mM

Table 2 Effect of isobutyronitrile feeding on growth and nitrilase expression of *N. globerula* NHB-2

Run	Isobutyronitrile feedings (v/v)#	Growth (mg_{DCW} mL^{-1})	Nitrilase activity	
			U mg^{-1}_{DCW}	U $mL^{-1}_{culture}$
Set 1*	0 h: 0.3% (40 mM) 18 h: 0.6% (80 mM) 24 h: 1.2% (120 mM)	2.84 ± 0.09	6.67 ± 0.27	18.7 ± 0.75
Set 2*	0 h: 0.3% (40 mM) 18 h: 1.2% (120 mM) 24 h: 2.4% (240 mM)	2.10 ± 0.10	4.76 ± 0.38	10.0 ± 0.80
Set 3*	0 h: 0.3% (40 mM) 18 h: 1.0% (133 mM) 24 h: 1.0% (133 mM)	2.64 ± 0.05	4.81 ± 0.24	12.7 ± 0.63

mM of isobutyronitrile in the medium

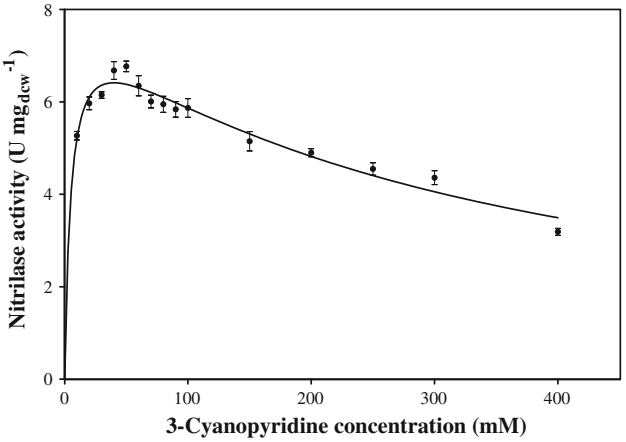

Fig. 3 Substrate inhibition effect on nitrilase activity of resting cells of *N. globerula* NHB-2 at higher concentrations of 3-cyanopyridine (V_{max} = 7.984 ± 0.335 U mg^{-1}_{DCW}, K_m = 4.968 ± 1.102 mM and K_{si} = 325.7 ± 42.73 mM)

(Eq. 1) [6]. The low K_{si} value means that this enzyme is unable to tolerate substantially high concentrations of substrate in the reaction.

$$V_0 = \frac{V_{max}[S]}{K'_m + [S] + \frac{[S]^2}{K_{si}}}. \quad (1)$$

Time course of 3-cyanopyridine conversion

The effect of increasing enzyme concentration with respect to substrate concentration on formation of nicotinic acid is shown in Fig. 4. Deviation from normal enzymatic reaction curves was observed due to substrate/product inhibition, which resulted in linear curves of nicotinic acid formation. Reaction mixture containing 2.5 U mL^{-1} nitrilase and 300 mM 3-CP showed the highest conversion rate (11 g h^{-1}) and also the highest conversion of nicotinic acid (180 mM) in 2 h. The rate of formation of nicotinic acid was similar in the presence of 200 and 400 mM 3-CP or 100 and 500 mM 3-CP. High substrate concentration (500 mM) had an inhibitory effect on the formation of nicotinic acid,

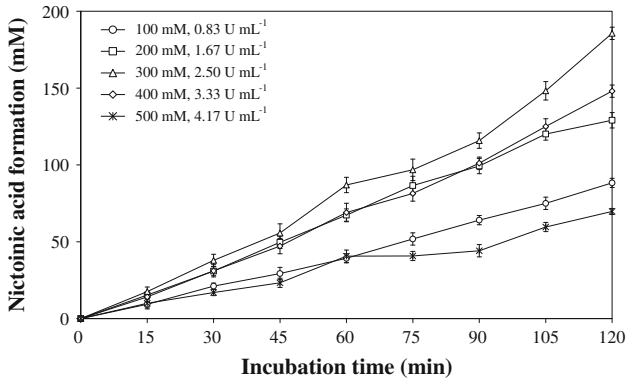

Fig. 4 Nicotinic acid formation in the reaction mixture containing different 3-CP concentrations. Resting cells were added in the reaction mixture as per the legend

although the enzyme concentration in the reaction mixture was increased accordingly (4.17 U mL^{-1}).

Effect of resting cells concentration on conversion of 300 mM 3-cyanopyridine

Biotransformation of 300 mM 3-cyanopyridine was performed in the presence of 2.5, 5, 7.5 and 10 U mL^{-1} nitrilase (Fig. 5). As the biocatalyst concentration in the reaction was raised, the rate of formation of nicotinic acid increased, and a conversion rate of 37 g h^{-1} was achieved at 10 U mL^{-1} nitrilase concentration. This 336% increase in the rate of nicotinic acid formation as compared with the reaction containing 2.5 U mL^{-1} nitrilase activity resulted in 100% conversion of the added substrate in 60 min.

Fed batch reaction at 40 mL scale (300 mM 3-cyanopyridine)

The results of feeding of 300 mM substrate in the reaction mixture (40 mL) containing 10 U mL^{-1} nitrilase are shown in Fig. 6. In the first feed, 100% substrate was converted to

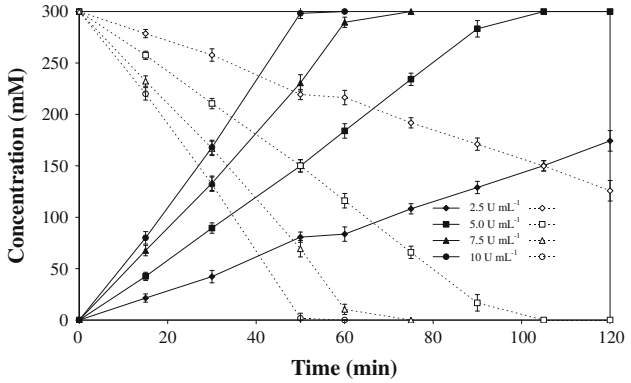

Fig. 5 Effect of resting cells concentration on the formation of nicotinic acid in the reaction containing 300 mM 3-cyanopyridine. (*solid symbols* nicotinic acid, *empty symbols* 3-CP)

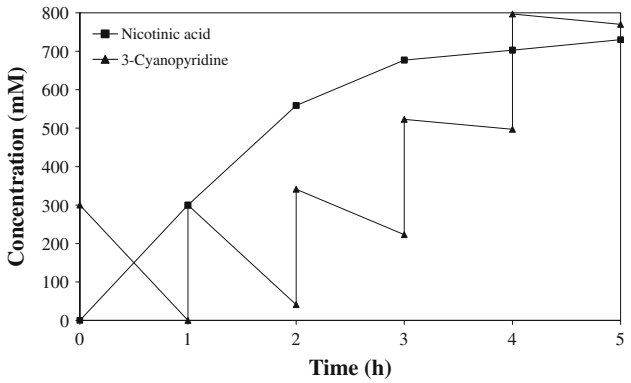

Fig. 6 Accumulation of nicotinic acid and 3-cyanopyridine in 40 mL reaction mixture containing resting cells corresponding to 10 U mL^{-1} nitrilase activity. The reaction was carried out at 35°C, and 300 mM 3-cyanopyridine was fed after every 1 h

product in 1 h. The rate of nicotinic acid formation declined sharply on further feeding of 3-CP. This resulted in formation of only 730 mM nicotinic acid in 5 h, and a total of 770 mM 3-CP accumulated after five feedings.

Fed batch reaction at 40 mL scale (100 mM 3-cyanopyridine)

Substrate feeding at lower concentration (100 mM) at an interval of 20 min showed no inhibitory effect on the rate of formation of nicotinic acid until 10 feeds. After the 10th feed, the rate of nicotinic acid formation declined sharply, and a total of 864 mM 3-CP accumulated in the reaction mixture after 20 feeds. Finally, 1,136 mM nicotinic acid was produced in the reaction mixture (Fig. 7).

Fed batch reaction at 1 L scale (100 mM 3-cyanopyridine)

One litre reaction mixture contained resting cells (1.5 g_{DCW}) corresponding to 10 U mL^{-1} nitrilase. A total

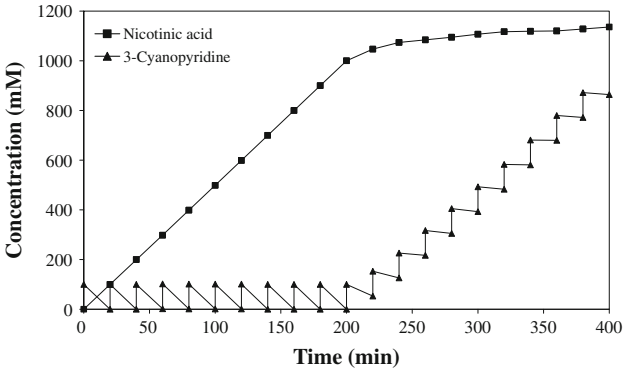

Fig. 7 Accumulation of nicotinic acid and 3-cyanopyridine in 40 mL reaction mixture containing resting cells corresponding to 10 U mL^{-1} nitrilase activity. The reaction was carried out at 35°C, and 100 mM 3-cyanopyridine was fed after every 20 min

of one mole of 3-CP fed in 10 feedings of 0.1 mol (every 20 min) was converted to nicotinic acid in 200 min (3 h 20 min). The rate of nicotinic acid formation was 24.6 g h^{-1} g_{DCW}^{-1}.

Recycling of recovered cells

Cells recovered from the reaction showed 85% residual (5.75 U mg_{DCW}^{-1}) nitrilase activity. There was 50% loss of resting cells during reaction. The recovered cells, when used at concentration of 10 U mL^{-1} in 40 mL reaction mixture for the next fed batch experiment, resulted in 100% conversion of 1 M 3-CP to nicotinic acid. The residual activity of cells recovered from the reaction mixture was 71% (4.08 U mg_{DCW}^{-1}).

Discussion

Growth, glucose utilization and nitrilase expression kinetics of *N. globerula* NHB-2 were investigated in detail. The results showed that this organism grew faster in medium devoid of nitrile without expression of nitrilase. The presence of aliphatic nitriles in the medium retarded growth of the bacterium, and higher nitrilase expression was observed in the presence of isobutyronitrile. Isobutyronitrile has been reported to induce nitrilase in *R. rhodochrous* J1, *R. rhodochrous* PA-34 and *R. rhodochrous* K22. The nitrilase of *N. globerula* NHB-2 was not induced by ε-caprolactam, which has been reported as a powerful inducer of nitrilase in *R. rhodochrous* J1 [17]. Furthermore, this nitrilase had higher reactivity activity towards aromatic nitriles, being maximum for benzonitrile, followed by 3-CP, 4-cyanopyridine, *m*-tolunitrile and *p*-tolunitrile. The cyano group of 2-cyanopyridine was not efficiently hydrolysed by the nitrilase, which might be due

to steric hindrance. Acrylonitrile was hydrolysed to acrylic acid at a higher rate (78%), while much less activity against propionitrile (saturated from of acrylonitrile) and other aliphatic nitriles was observed. Similar activity for the nitrilase of *R. rhodochrous* K22 was observed against propionitrile and acrylonitrile [11]. The π-electrons of C=C in acrylonitrile and aromatic nitriles might play some role in the positioning of the –CN group for interaction with the cystine residue of the catalytic site. Recently, mutational analysis of *R. rhodochrous* ATCC nitrilase showed that Tyr-142 is essential for catalysis of aliphatic nitriles and that conjugated π-electron either in the substrate or in the amino acid at 142 is required for enzyme activity [32]. Thus, the nitrilase of *N. globerula* NHB-2 might contain non-polar aliphatic amino acid at position 142, thus reducing its specificity towards aliphatic nitriles. Interestingly, the nitrilase of *N. globerula* NHB-2 was also unable to hydrolyse phenylacetonitrile, suggesting that CN group directly bound to the C=C bond was necessary for efficient hydrolysis.

Growth kinetic studies of *N. globerula* NHB-2 revealed that isobutyronitrile had an inhibitory effect on both glucose utilization and growth rate. Furthermore, with progression of incubation time, nitrilase activity declined due to depletion of inducer in the medium. Multiple feeding of isobutyronitrile to increase its concentration exponentially resulted in high-level expression of nitrilase in short incubation time (30 h) as compared with earlier reports: *R. rhodochrous* J1 (120 h, ε-caprolactam), *R. rhodochrous* K22 (120 h, isovaleronitrile) and *Aspergillus niger* K10 (48 h, 2-cyanopyridine and valeronitrile) [10, 12, 16]. The high concentration of isobutyronitrile in the production medium almost stopped glucose utilization by *N. globerula* NHB-2 from 24 to 54 h, and even after 96 h of incubation, 3.6 mg mL^{-1} glucose remained unutilized. The effect of nitrile on the metabolism of carbon source (glucose) by a nitrile-metabolizing microorganism has been studied for the first time.

The nitrilase of the organism was active at high concentration of substrate, with a typical substrate inhibition effect. The low substrate inhibition constant (325.7 mM) suggests that the nitrilase of *N. globerula* NHB-2 does not have high substrate tolerance. The addition of resting cells in terms of U mL^{-1} nitrilase activity in the reaction showed reproducibility in the results of the bioprocess development. Though the substrate and enzyme ratio were constant, the rate of nicotinic acid formation varied with different initial substrate concentrations. The substrate concentration affected the rate of nicotinic acid formation. Linear increment of nicotinic acid in the reaction mixture with increasing biocatalyst concentration at 300 mM 3-CP might be due to the substrate inhibition effect. This is a deviation from normal enzymatic reaction, which follows the Michaelis–Menten equation. Fed batch operation with high substrate feeding (300 mM) at a longer time interval (1 h) had an inhibitory effect on the reaction rate. This effect was overcome by low substrate feeding (100 mM) in shorter time interval (20 min). One litre reaction mixture containing resting cells (1.5 g_{DCW}) corresponding to 10 U mL^{-1} nitrilase resulted in conversion of one mole of 3-CP fed in 10 feedings of 0.1 mol (every 20 min) to nicotinic acid in 200 min (3 h 20 min). The rate of nicotinic acid formation was 24.6 g h^{-1} g_{DCW}^{-1}. Practically, only 57% product (123.11 g) was produced by the bioprocess with respect to the theoretical yield of 214.2 g nicotinic acid using 10 U mL^{-1} nitrilase in 200 min, due to substrate inhibition (87% nitrilase activity at 100 mM 3-CP). After one mole accumulation of nicotinic acid in the fed batch reaction, product inhibition was the main factor which almost ceased nitrilase activity. The product inhibition effect could be solved through in situ product removal, which would require the design of a special bioreactor for removal of nicotinic acid formed in the reaction. Protein engineering is another approach that could be employed to increase the substrate and product tolerance of the native enzyme.

Hyperinduction of nitrilase in *N. globerula* NHB-2 led to a 275% increase in the rate of nicotinic acid formation per hour as compared with previously reported work; the amount of biocatalyst in the reaction was reduced to 36% and conversion time to 37% [23]. Use of resting cells of *N. globerula* NHB-2 (hyperinduced with nitrilase) for biotransformation of 3-CP to nicotinic acid at a faster rate is a first step towards commercialization of this bioprocess. Molecular studies of the nitrilase of *N. globerula* NHB-2 would clarify the hyperactive behaviour of the enzyme. Protein engineering of this nitrilase could improve further its specific activity and substrate/product tolerance, similar to *Acidovorax facilis* 72 W (T210A) and uncultured nitrilase (A190H) [7, 29, 30].

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