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ORIGINAL ARTICLE

Bench scale conversion of 3-cyanopyidine to nicotinamide using resting cells of *Rhodococcus rhodochrous* PA-34

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Abstract The nitrile hydratase (NHase, EC 3.5.5.1) activity of *Rhodococcus rhodochrous* PA-34 was explored for the conversion of 3-cyanopyridine to nicotinamide. The NHase activity (~18 U/mg dry cell weight, dcw) was observed in 0.1 M phosphate buffer, pH 8.0 containing 1M 3-cyanopyridine as substrate, and 0.75 mg of resting cells (dry cell weight) per ml reaction mixture at 40°C. However, 25°C was more suitable for prolonged batch reaction at high substrate (3-cyanopyridine) concentration. In a batch reaction (1 liter), 7M 3-cyanopyridine (729 g) was completely converted to nicotinamide (855 g) in 12h at 25°C using 9.0 g resting cells (dry cell weight) of *R. rhodochrous* PA-34.

Key words: *Rhodococcus rhodochrous* PA-34 · bioconversion · nitrile hydratase (NHase) · 3-cyanopyridine · nicotinamide.

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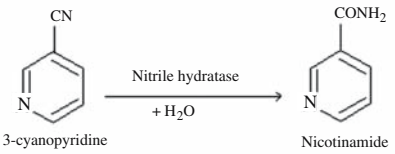
Introduction

The nicotinic acid (niacin) and nicotinamide (niacinamide) comprise Vitamin B3. This vitamin is also popularly known as pellagra-preventing (P-P) factor as its deficiency in human being causes 'pellagra'. Nicotinamide is the physiologically active form of Vitamin B3 in animals and it is a component of coenzymes NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate)[1].

The world production of nicotinamide and nicotinic acid is estimated to be 22,000 tons per annum. Out of the total market demand for nicotinamide and nicotinic acid, 45%, 30% and 10% is in USA, Europe and Japan respectively [2].

Nicotinamide is chemically synthesized either by the action of ammonia on nicotinyl chloride, or heating nicotinic acid with urea in the presence of molybdenum or reacting 3-cyanopyridine with hydrogen peroxide in alkaline solution[3]. It can also be synthesized by refluxing nicotinic acid with absolute ethanol, sulphuric acid and ammonia[4]. However, these processes require high temperature and pressure, harsh acidic and basic conditions, and yield undesired byproducts such as nicotinic acid and HCN. In order to overcome these limitation, nitrile hydratase (EC 3.5.5.1) mediated conversion of 3-cyanopyridine to nicotinamide has been proposed[5] and the reaction is outlined below:



Among the nitrile hydratases so far reported[5–11] the nitrile hydratase of *R. rhodochrous* J1[5] and *R. rhodochrous* PA-34[6] have exhibited high activity for transformation of 3-cyanopyridine to nicotinamide. In this paper, the bench scale conversion of 3-cyanopyridine to nicotinamide using resting cells of *R. rhodochrous* PA-34 is being reported.

Materials and Methods

Chemicals: 3-Cyanopyridine used in the present studies was a gift from Jubliant Organosys Ltd., Jyotiba Phoolay Nagar, India. Nicotinamide and nicotinic acid were from Sigma Chemical Company, St. Louis, USA. While other chemicals were of analytical grade and purchased from the standard commercial supplier. Media ingredients were procured from HiMedia, Mumbai, India.

Preparation of resting cells of Rhodococcus rhodochrous PA-34: R. rhodochrous PA-34 was cultured and resting cell of this organism was prepared according to procedure reported earlier[6]. These cells were used as biocatalyst having nitrile hydratase activity for conversion of 3-cyanopyridine to nicotinamide.

Assay of nitrile hydratase activity: The nitrile hydratase activity of resting cells was assayed at 20°C for 30 min according to the method of *Prasad et al*[6]. The reaction mixture (1 ml) contained 0.1M 3-cyanopyridine, resting cell of *R. rhodochrous* PA-34 (i.e. equivalent to 0.75 mg dry cell weight, dcw) and 0.1M potassium phosphate buffer (pH 7.0). One unit of nitrile hydratase (NHase) activity was defined as that amount of enzyme which catalysed the hydrolysis of one µmol of 3-cyanopyridine to nicotinamide per min under the assay conditions.

Optimization of reaction conditions for assay of nitrile hydratase activity in resting cells of R. rhodochrous PA-34: The following parameters such as buffer system (potassium phosphate, sodium phosphate and Tris-HCl buffer of 0.1M concentration and pH 7) and buffer concentrations (0.025 to 0.2M), pH (5-8.5 pH using 0.1M KH_2PO_4/K_2HPO_4 buffer and 9-11 pH employed using 0.1M K_2HPO_4/KOH buffer), temperature (15° to 70°C) and substrate (3-cyanopyridine) concentration (0.1 to 1.5M) were studied for nitrile hydratase activity using 0.75 mg (dcw) resting cells of *R. rhodochrous* PA-34. The Km and Vmax values of NHase of *R. rhodochrous* PA-34 was calculated from a Lineweaver-Burk plot (1/v versus 1/[S]). The initial velocities of the NHase-catalyzed reaction (v) recorded using 0.75 mg (dcw) of resting cells of *R. rhodochrous* PA-34 at 40°C using various substrate concentration [S] i.e. 0.1-1.0M 3-cyanopyridine were used for plotting Lineweaver-Burk plot.

Optimization of process parameters for nicotinamide synthesis at 1 litre scale

Temperature for prolonged reaction at higher concentration of 3-cyanopyridine: Under optimized assay conditions, the reactions were carried out at 20°, 25°, 30°, 35°C and 40°C separately for 24 hr in 25 ml reaction mixture containing 3M 3-cyanopyridine and 1.5 mg resting cells (dcw)/ml reaction mixture.

Effect of substrate and resting cell concentration on production of nicotinamide: The batch reactions were carried out at 25°C and 30°C in 25 ml reaction mixture for complete conversion of 3–7 M 3-cyanopyridine to nicotinamide using 3-9 mg resting cells of *R. rhodochrous* PA-34 (dcw)/ml reaction mixture) (Table 1).

Production of nicotinamide at one litre scale: On the basis of preceding experiments, the conversion of 3-cyanopyridine to nicotinamide was scaled up to one litre and carried out in a BioFlow C-32 fermenter (New Brunswick Scientific, U.S.A.). The status of the conversion of substrate to product was analyzed by HPLC in reaction sample at an interval of 3h. Nicotinamide was recovered from reaction mixture according to procedure of Nagasawa *et al*[5].

Gas chromatographic analysis: The nicotinamide and 3-cyanopyridine were estimated in the reaction mixture by gas chromatography (GC) system (Netel India Limited, New Delhi, India) equipped with 2 m Chromosorb WHP 15 % SE-30 column (methyl silicone 80/100, 50/300°C). The oven temperature was 250°C while detector and injector temperature was 260°C. Flow rates of nitrogen (carrier) gas, hydrogen gas and oxygen gas were 4, 3 and 2 kg cm^{-2}.

Results and discussion

Optimization of reaction conditions for assay of nitrile hydratase activity of resting cells of *R. rhodochrous* PA-34

Buffer system: Among the three buffer systems tested, the resting cells of *R. rhodochrous* PA-34 exhibited maximum nitrile hydratase activity (2.27 U/mg dcw) in potassium phosphate buffer (0.1 M, pH 7.0) and followed by 2 U/mg dcw in sodium phosphate buffer of similar ionic strength and pH. Therefore, potassium phosphate was selected for further experiments. Most of the earlier reported nitrile hydratases had maximum activity in this buffer[7]. However, the cell could not exhibit NHase activity in the Tris-HCl buffer possibly due to the potentially reactive primary amine of Tris(hydroxymethyl)aminomethane which might have interfered with the enzyme protein leading to its inhibition[12].

Concentration of potassium phosphate buffer: Maximum nitrile hydratase activity (2.31 U/mg dcw) was recorded in 0.1 M potassium phosphate buffer. Below and above 0.1 M, nitrile hydratase activity of the resting cells of *R. rhodochrous* PA-34 declined (Fig. 1). At 0.025 M and 0.2 M, the cells exhibited about 54 and 87 % nitrile hydratase activity respectively in comparison to activity observed in 0.1 M buffer.

Buffer pH: Maximum nitrile hydratase activity (2.31 U/mg dcw) was observed in the pH range of 6.5 to 9.5 and pH 8.0

Table 1. Optimization of concentration of substrate (3-cyanopyridine) and resting cells of *R. rhodochrous* PA-34 for nicotinamide synthesis at 25°C and 30°C.

		Time and % conversion of 3-cyanopyridien to nicotinamide at			
		25°C		30°C	
3-cyanopyridine (M)	PA-34 (mg dcw mL^{-1} reaction)	Time (h) %	Conversion	Time (h)	% Conversion
3	3.0	6	100.0	6	100.0
4	4.0	12	100.0	9	100.0
5	5.0	12	100.0	12	100.0
6	7.0	12	100.0	12	92.0
7	9.0	12	100.0	12	85.0

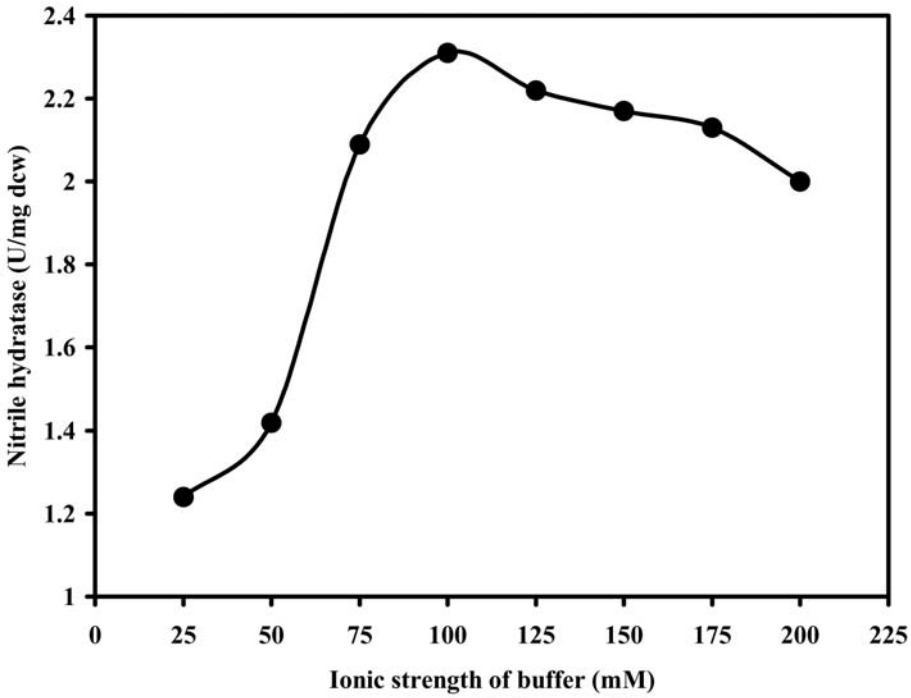


Fig. 1 Effect of concentration of potassium phosphate buffer (pH 7) on nitrile hydratase activity of *R. rhodochrous* PA-34 at 20°C.

being in the mid of this range was selected for subsequent experiment (Fig. 2). However, most of the nitrile hydratases have optimum pH around 7.0[7]. A slow decrease in activity of the enzyme was observed above pH 9.5 and about 71 % activity was recorded at pH 11 in comparison to activity in the optimum pH range. However, there was a rapid loss of enzyme activity below pH 6.0 and a complete loss of this activity was recorded at pH 5.5 which showed that the nitrile hydratase of *R. rhodochrous* PA-34 was more vulnerable to acidic pH.

Temperature: Nitrile hydratase of *R. rhodochrous* PA-34 exhibited broader temperature range i.e. 40–60°C in which the activity was 4.44 U/mg dcw. Nagasawa *et al*[5] had also reported 40°C as optimum temperature for the nitrile hydratase of *R. rhodochrous* J1. Temperatures < 40°C and >60°C decreased the activity of NHase (Fig. 3). At 65°C and 70°C this enzyme could only retain 39 % and 19 % activity as compared to its maximum activity. At these temperatures, conversion of nicotinamide to nicotinic acid was not observed. While, amidase of *R. rhodochrous* PA-34 become active at > 20°C and converted the acrylamide to acrylic acid and butyramide to butyric acid with equimolar ammonia[13]. It meant that the amidase of this organism did not convert nicotinamide to nicotinic acid. Therefore, the conversion of 3-cyanopyridine to nicotinamide was feasible at higher temperature (>20°C) without production of nicotinic acid.

Substrate (3-cyanopyridine) concentration: The NHase activity of *R. rhodochrous* PA-34 was assayed at 40°C using various concentration of 3-cyanopyridine (0.1–1.5

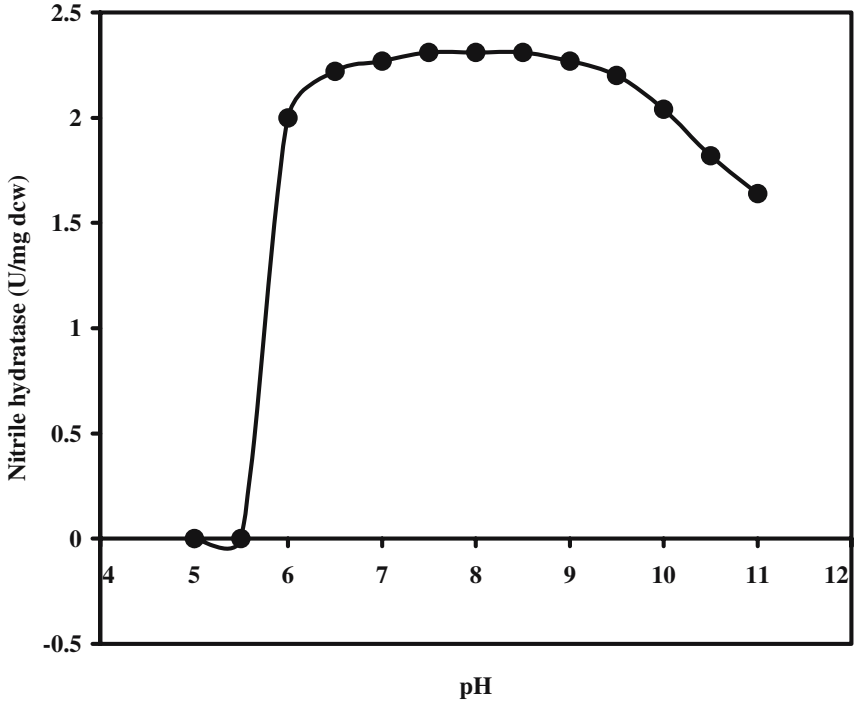


Fig. 2 Effect of pH of 1.0 M potassium phosphate buffer on nitrile hydratase activity of *R. rhodochrous* PA-34 at 20°C.

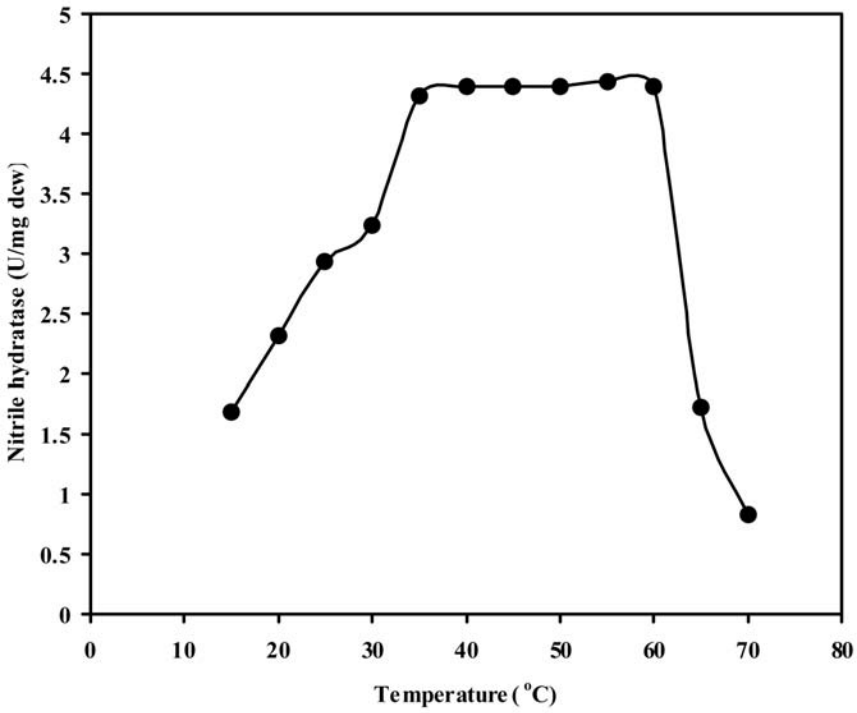


Fig. 3 Effect of temperature on nitrile hydratase activity of *R. rhodochrous* PA-34 in 1.0M potassium phosphate buffer, pH 8.0 at 20°C.

M). This activity increased from 4.40 to 17.78 U/mg dcw when 3-cyanopyridine concentration was varied from 0.1 to 1.0 M, respectively. While it slightly decreased beyond 1.0 M 3-cyanopyridine and enzyme activity of 15.66 U/mg dcw was recorded at 1.5 M 3-cyanopyridine (Fig. 4). The Km (0.625 M) and Vmax (22.22 µmole/ml/min) values of

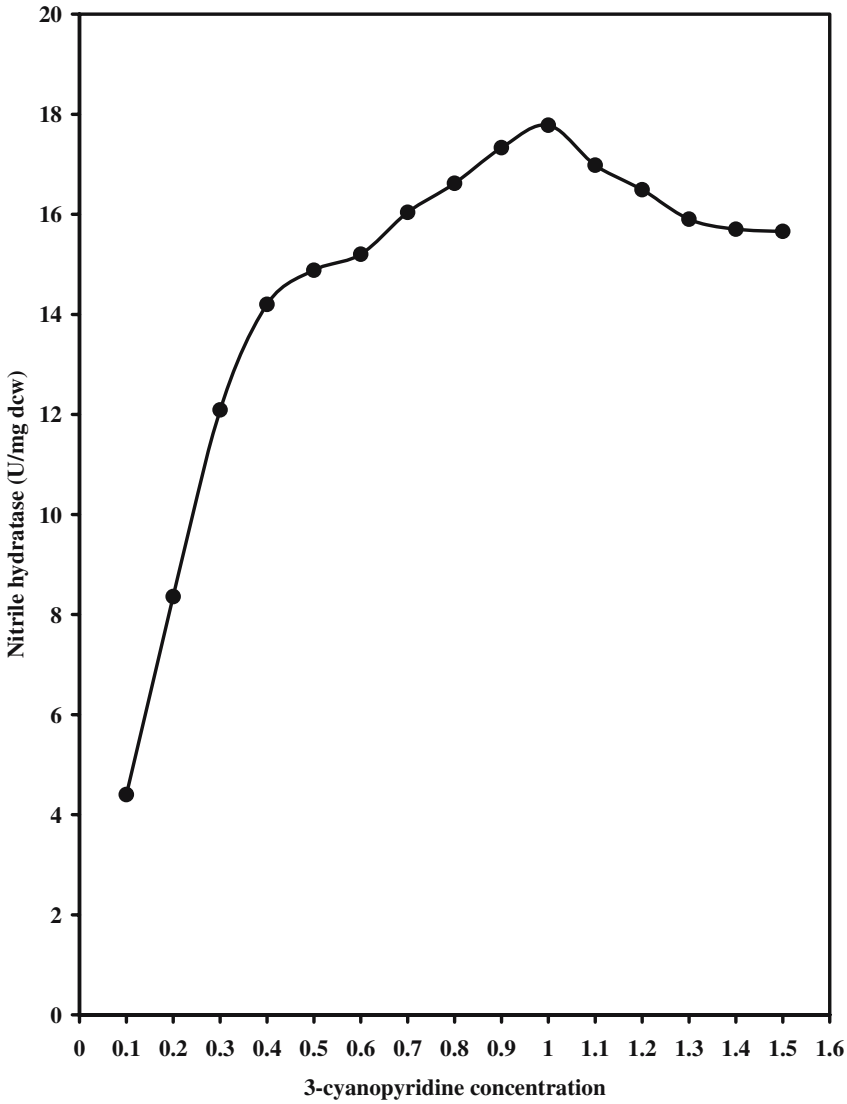


Fig. 4 Effect of 3-cyanopyridine concentrations on nitrile hydratase activity of *R. rhodochrous* PA-34 at 40°C in 1.0M potassium phosphate buffer, pH 8.

R. rhodochrous PA-34 NHase were calculated from Lineweaver-Burk plot (Fig. 5).

Optimization of process parameter for synthesis of nicotinamide at 1 litre scale

Selection of temperature for prolonged reaction at higher substrate concentration: The objective of this experiment was to study the effect of various temperatures on the prolonged reaction (upto 24 h) for conversion of higher concentration of 3-cyanopyridine (3 M) to nicotinamide using resting cells (1.5 mg dcw/ml) of *R. rhodochrous* PA-34 and to select a suitable temperature for the prolonged reaction. Maximum conversion (i.e. 77 and 80 %) of 3 M 3-cyanopyridine to nicotinamide was detected at 25° and 30°C, respectively. However, 70 and 60% conversion of substrate to product was observed at 20° and 35°C, respectively in 24 h. Therefore, 25° and 30°C were observed as suitable reaction temperature for prolonged reaction (Fig. 6). Although at 40°C, the resting cells of *R. rhodochrous* PA-34 had shown the maximum nitrile hydratase activity in short time reaction with low substrate concentration i.e., 0.1 M 3-cyanopyridine (Fig. 3) but at high substrate concentration of 3-cyanopyridine at temperature 40°C and above decreased the activity of NHase (Fig. 6). It means that 3M 3-cyanopyridine (at 40°C) might have inactivated NHase leading to loss of its activity.

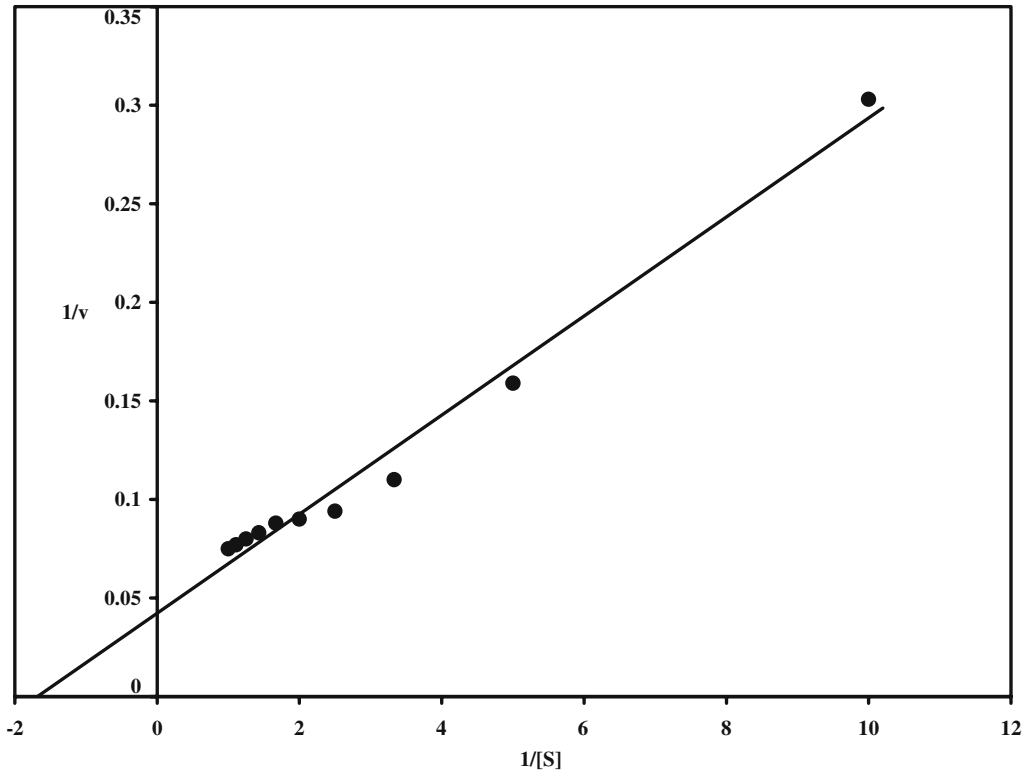


Fig. 5 A Lineweaver-Burk plot (1/v versus 1/[S]) of the data from velocity of the NHase-catalyzed reaction using 0.75 mg (dcw) of resting cells of *R. rhodochrous* PA-34 at 40°C using various substrate concentration [S] i.e. 0.1-1.0M 3-cyanopyridine.

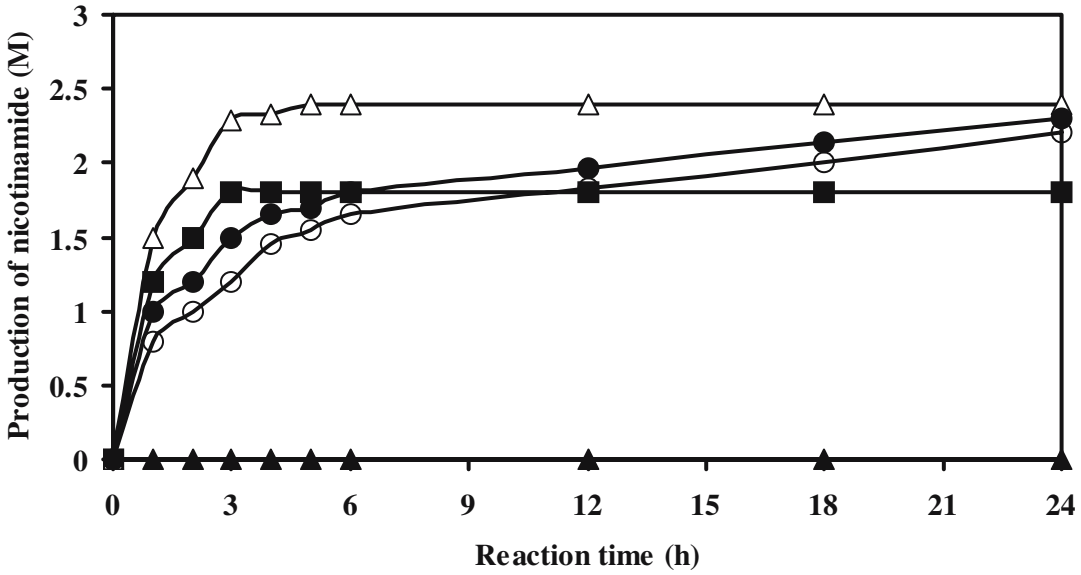


Fig. 6 Effect of various temperatures on prolonged reaction for conversion of 3 M 3-cyanopyridine to nicotinamide using resting cells (1.5 mg dcw/ml) of *R. rhodochrous* PA-34. (-○-, -●-, -Δ-, -■- and -▲- indicate the reaction at 20, 25, 30, 35 and 40°C respectively)

Optimization of substrate (3-cyanopyridine) and resting cell concentration for maximum production of nicotinamide at 25° and 30°C: A complete conversion of 3, 4, 5, 6 and 7 M 3-cyanopyridine was achieved using 3, 4, 5, 7 and 9 mg resting cells (dcw)/ml of reaction mixture at 25°C (Table 1). Time taken for these conversions was 6 hr for 3 M substrate

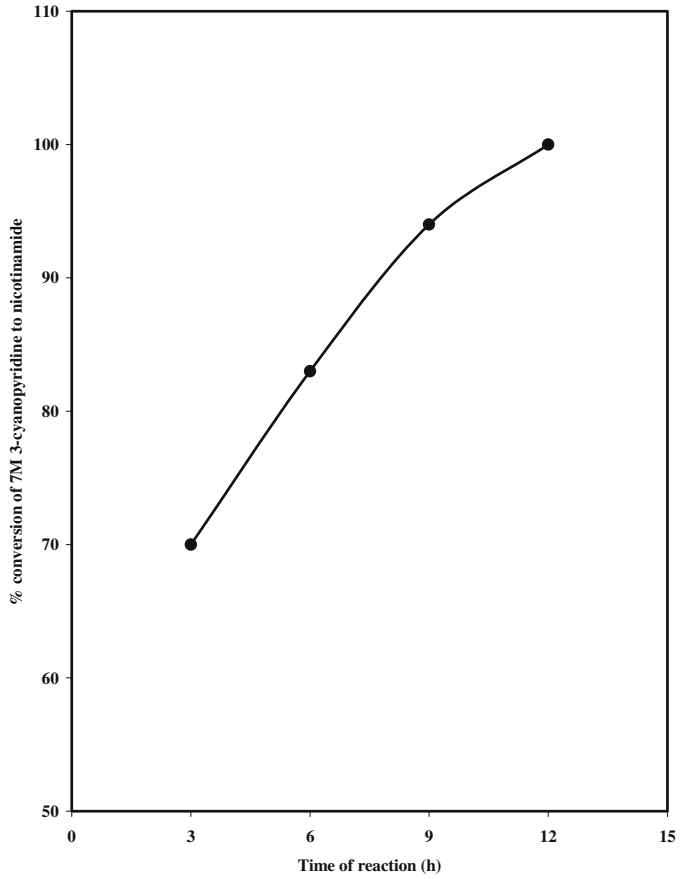


Fig. 7 Gas chromatographic analysis report on the recovered nicotinamide from reaction mixture. (Peaks at retention time 0.24 and 0.91 min, are of potassium phosphate buffer and nicotinamide, respectively. However, the retention times of 3-cyanopyridine and nicotinic acid standard were 0.45 and 1.45 min, respectively).

and 12 hr for 4, 5, 6 and 7 M of 3-cyanopyridine. At 30°C, 3, 4 and 5 M 3-cyanopyridine was also completely converted to nicotinamide in 6, 9 and 12 hr, respectively. However, only 92 and 85 % of 6 and 7 M 3-cyanopyridine respectively was transformed to nicotinamide. Nagasawa et al.[5] have reported the complete conversion of 12 M 3-cyanopyridine to nicotinamide in 25 ml reaction mixture using resting cells (1.48 g dcw/l) of *R. rhodochrous* J1 in 9 hr and they have also reported an yield of 1465 g nicotinamide/l reaction mixture. It is quite surprising to achieve such a high yield of nicotinamide (1465 g) in one litre reaction mixture. In the present studies, it was difficult to keep reaction mixture containing >7M 3-cyanopyridine in liquid/semi liquid state to allow proper mixing of substrate, biocatalyst and product during the reaction at 25°C. Since this enzymatic reaction requires equimolar content of water, therefore, it became difficult to carry out reaction at water content <12.5 % in the reaction mixture.

Production of nicotinamide at 1 litre scale: In the one litre batch reaction, 7 M 3-cyanopyridine has been completely hydrated to nicotinamide using resting cells of *R. rhodochrous* PA-34 (9.0 g dcw) in 12 h at 25°C. This reaction produced 855 g nicotinamide with a productivity of 7.92 g nicotinamide/g resting cells (dcw)/h. However, the yield 82 g nicotinamide/g resting cells (dcw) of *R. rhodochrous* J1/h have been reported. The cells of R. rhodochrous PA-34 are pinkish orange due to pigment and the nicotinamide formed was crystalline white in appearance but did not exhibit any trace of contamination by the pigment which indicated that there was no lysis of cells. Had there been any lysis of the cells during the reaction that would have shown orange tinge either in the reaction mixture or in the product recovered from the reaction mixture after removal of cells. About 85% (w/w) nicotinamide produced was recovered from reaction mixture. The HPLC analysis of the final product (nicotinamide) exhibited very high purity (Fig. 7). *R. rhodochrous* PA-34 has shown very good potential for the production of nicotinamide from 3-cyanopyridine and therefore, it can be of industrial importance. The production of acrylamide and

butyramide has also been carried out using whole cell of this organism[13,14].

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