Optimization of Process Parameters for Conversion of 3-cyanopyridine to Nicotinamide using Resting Cells of Mutant 4D Strain of *Rhodococcus rhodochrous* PA-34

Amit Pratush, Amit Seth, Tek Chand Bhalla*

Department of Biotechnology
Himachal Pradesh University
Summer Hill, Shimla – 171005, India
E-mails: amit.pratush@gmail.com, amitsethshimla@gmail.com, bhallatc@rediffmail.com

**Correspondent author*

Received: May 22, 2011 **Accepted: October 10, 2011**

Published: November 30, 2011

Abstract: *Mutant of Rhodococcus rhodochrous PA-34, named as 'mutant 4D' has been reported for the hyperconversion of 3-cyanopyridine to nicotinamide. This mutant 4D generated through chemical mutagenesis has much more hydration potential than its wild strain. The reaction conditions for prolonged reaction and process parameters for the conversion of 3-cyanopyridine to nicotinamide were optimized. Under the optimized reaction conditions the mutant 4D is stable at higher temperature (55°C), high ionic strength (0.3 M) and at acidic pH conditions (5.5) and exhibited 8.0, 7.9 and 7.0 U/mg dcw NHase activity, respectively. In a batch reaction of (One litre), 7M 3-cyanopyridine was completely converted to nicotinamide in 3h at 55°C using 7g resting cells (dry cell mass) of mutant 4D of R. rhodochrous PA-34.*

Keywords: *Rhodococcus rhodochrous PA-34, 3-cyanopyridine, Nicotinamide, Conversion.*

Introduction

Nitrile hydratases (EC 4.2.1.84) are unusual metalloenzyme that catalyze the hydration of nitriles to their corresponding amides. They are used as biocatalysts in nicotinamide production, one of the few commercial scale bioprocesses, as well as in environmental remediation for the removal of nitriles from waste streams. Nicotinamide (niacin) is vitamin B3 and is also known as the pellagra-preventing (P-P) factor as its deficiency in human beings causes pellagra. Nicotinamide is also used for animal feed supplementation. The world nicotinamide production is 22 000 tons per annum [13]. The chemical synthesis of this vitamin is well defined but it gives a large number of byproducts, which are harmful to our ecosystem [3], whereas the enzymatic synthesis of nicotinamide is ecofriendly and byproduct free [6]. There are a large number of nitrile hydratase producing organisms, which are able to produce nicotinamide by hydration of 3-cyanopyridine [6, 14]. Among these organisms *R. rhodochrous* PA-34 [8, 9] and *R. rhodochrous* J1 [6] exhibited high NHase activity for the transformation of 3-cyanopyridine to nicotinamide.

Keeping the biotransformation capabilities of *R. rhodochrous* PA-34 [9] in mind we generated a mutant strain i.e. mutant 4D, having hyper NHase activity by using chemical mutagen. This mutant strain (mutant 4D) of *R. rhodochrous* PA-34 produces hyper active nitrile hydratase having two time high NHase activity than its wild strain [10]. The nitrile hydratase of mutant strain is thermostable. It gives NHase activity at 55ºC. The NHase of this

strain is also stable at low buffer pH and high potassium ion concentration (0.3 M) [10]. The productivity of mutant resting cells is much higher than the wild strain. Hence this mutant can act as an efficient biocatalyst for the biotransformation of 3-cyanopyridine to nicotinamide at industrial scale. In this communication we report the optimization of parameter for the conversion of 3-cyanopyridine to nicotinamide at one litre scale.

Materials and methods

Chemical materials

3-cyanopyridine and nicotinamide used in this study were purchased from Sigma-Aldrich, while other media components and chemicals of analytical grade were purchased form Hi-media and Merck India Ltd.

Preparation of mutant 4D resting cells

Mutant 4D was cultured in A5 medium as described in Pratush *et al*. [10]. These resting cells having NHase activity were used as biocatalyst for the biotransformation of 3-cyanopyridine to nicotinamide.

Nitrile hydratase assay

The nitrile hydratase activity of resting cells of mutant 4D was assayed at 55ºC for 30 min by following the procedure reported earlier [5, 8]. The conversion of 3-cyanopyridine to nicotinamide in the reaction was determined by HPLC [11]. One unit of nitrile hydratase activity is defined as the amount of enzyme which catalyzes the formation of 1 µmol nicotinamide min^{-1} from 3-cyanopyridine under the assay conditions.

Optimization of reaction conditions for assay of nitrile hydratase activity in resting cells of mutant 4D

The various reaction parameters such as buffer system (potassium phosphate, sodium phosphate and Tris buffer pH 7.0), buffer pH (4-8), temperature (5-80°C), and substrate concentration (0.05-0.25 M) were studied for NHase activity by using 0.50 mg (dcw) resting cells of mutant 4D. The Lineweaver Burk plot was plotted by varying the substrate (3-cyanopyridine) concentration (0.05-0.25 M) to determine K_m and V_{max} value of mutant cells.

Optimization of process parameters for the production of nicotinamide from 3-cyanopyridine by the mutant 4D cells

Temperature for prolonged reaction at higher concentration of substrate (3-cyanopyridine)

Under optimized reaction conditions the nitrile hydratase assay of mutant resting cells were carried out at different temperatures 50, 55, 60 and 65°C separately for 12 h in 25 ml reaction mixture containing high substrate concentration i.e. 3 M and 3 mg dcw/ml resting cells in reaction mixture.

Effect of substrate (3-cyanopyridine) concentration and resting cell concentration on the nicotinamide production

The reaction was performed at 55°C in 25 ml reaction mixture to determine complete conversion of 4-8 M 3-cyanopyridine to nicotinamide using 4-8 mg dcw/ml of resting cells in the reaction mixture Table 1.

Table 1. Optimization of substrate (3-cyanopyridine) and resting cells concentration of mutant of *R. rhodochrous* PA-34 for nicotinamide synthesis at 55ºC

Substrate 3-CP* (M)	Mutant resting cells (mg/ml dcw)	Time and % age Conversion of 3-cyanopyridine of nicotinamide at 55ºC	
		Time (h)	% Conversion rate
4	4	3	100
5	5	3	100
6	6	3	100
7	7	3	100
8	8	3 6 9	75 94 87
9	9	No reaction carried out as the reaction mixture become viscous due to large amount of substrate 3-cyanopyridine	
10	10		

*3-cyanopyridine

Production of nicotinamide by mutant 4D resting cells at one litre scale

On the basis of the results of previous experiments performed for the optimization of process parameters, the conversion of 3-cyanopyridine to nicotinamide was scaled up to one litre. This one litre biotransformation reaction was carried out in a BioFlow C-32 fermenter (New Brunswick scientific, USA). The substrate conversion status was analyzed by HPLC at the interval of 3 h each. After the completion of biotransformation reaction nicotinamide was recovered by the procedure of Nagasawa *et al*. [6].

HPLC analysis of product

The product formed as a result of one litre reaction was analyzed by HPLC (Perkin Elmer Series 200 LC pump and Applied Biosystems 785A programmable absorbance detector) using a C18 reverse phase chromatography column (Intersil ODS, 4.6×250 mm, Macherey Nagel, Germany). The flow rate of following solvent system: acetonitrile: 0.01 M potassium phosphate, orthophosphoric buffer (pH 2.8) in 1:4 (v/v) ratio was 1 ml/min at 25ºC. Absorbance was measured at 230 nm for nicotinamide (product).

Result and discussion

Optimization of reaction condition for assay of nitrile hydratase activity of resting cells of mutant 4D strain

Buffer system, buffer pH and ionic strength of buffer

Among three buffer systems tested for NHase activity. The resting cells of mutant 4D strain exhibited maximum NHase activity (6.5 U/mg dcw) in potassium phosphate buffer (0.1 M, pH 7.0) and followed by 3.9 U/mg dcw and 2.9 U/mg dcw in sodium phosphate and Tris buffer of similar ionic strength and pH, respectively. Therefore potassium phosphate buffer was selected for further experiments. Most of the earlier reported NHase including the wild strain i.e. *R. rhodochrous* PA-34 had exhibited maximum activity in this buffer [8, 12]. The mutant resting cells exhibited maximum NHase activity (7.4 U/mg dcw) in the above optimized buffer at a pH range of 5.5 (Fig. 1a). However most of NHase producing organisms exhibited their maximum NHase activity at optimum pH around 7.0 [1]. The mutant resting cells showed 17.6% decrease at pH 7.0 in comparison to activity recorded at the optimum pH 5.5. This concluded that the mutant NHase was stable in acidic conditions (pH 5.5).

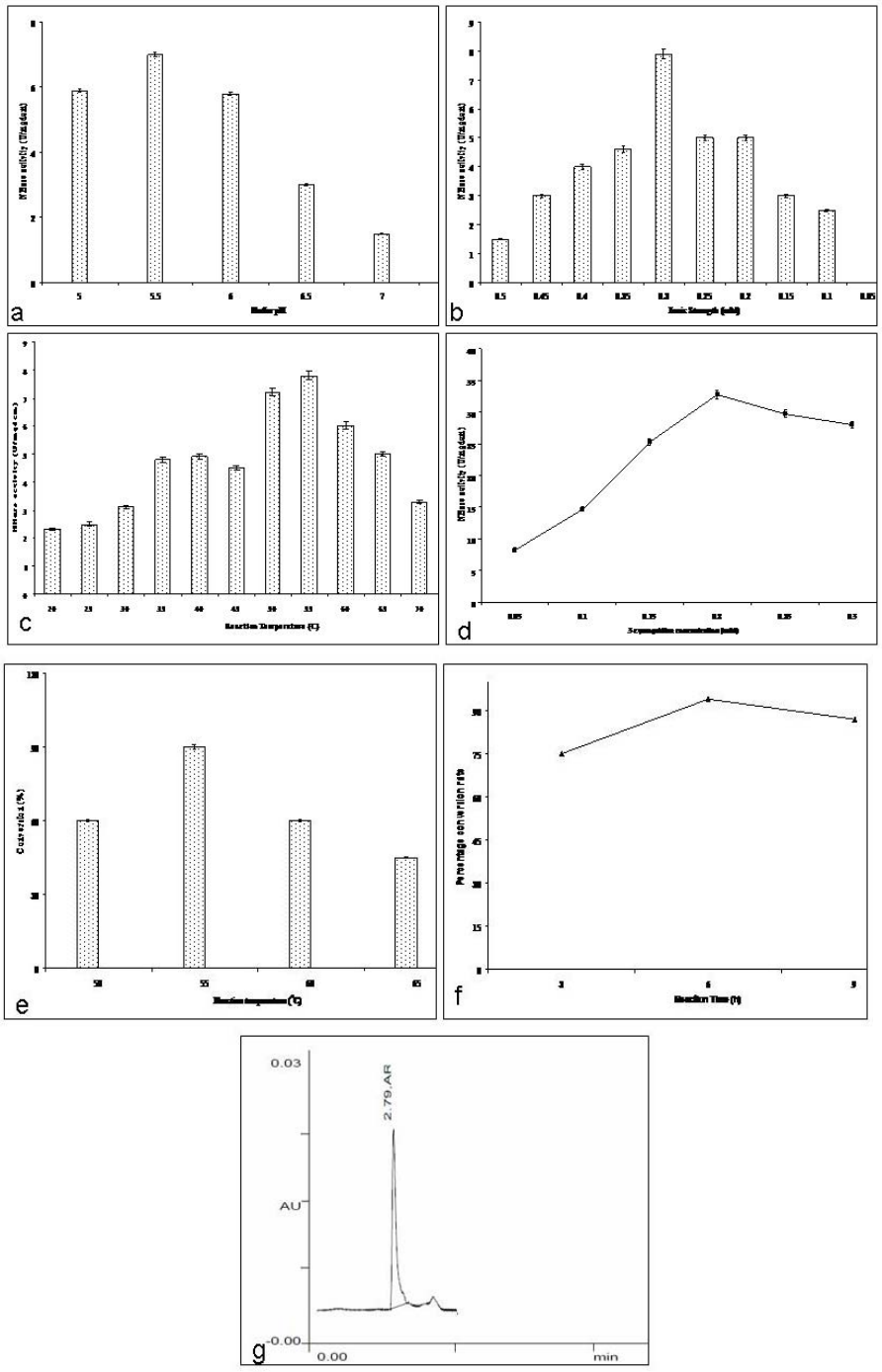

Fig. 1 Effect of different reaction conditions on the activity of nitrile hydratase of the mutant 4D. (a) buffer pH; (b) ionic strength; (c) reaction temperature; (d) substrate concentration; (e) reaction temperature optimization for selection of substrate concentration; (f) substrate hydration with time; (g) HPLC chromatogram of product i.e. nicotinamide.

The ionic strength of buffer also showed its impact on the NHase activity of mutant resting cells. The mutant 4D resting cells exhibited maximum NHase activity (7.9 U/mg dcw) in 0.3 M potassium phosphate buffer and with the rise (0.35 M) and decrease (0.25 M) in ionic strength, 64% and 71% decrease in NHase activity was recorded with comparison to the NHase activity at 0.3 M (Fig. 1b).Whereas the earlier reported NHase were stable at low concentration (0.1 M) of same buffer [4, 7, 8]. These all results confirmed that the mutant

cells were much more stable at low buffer pH (pH 5.5) and high concentration (0.3 M) potassium ions.

Temperature

The nitrile hydratase of mutant resting cells was stable at higher temperature. These cells exhibited maximum NHase activity (8.0 U/mg dcw) at 55ºC. The every 5ºC rise or fall from this optimum temperature i.e. 60ºC and 50ºC resulted in the 76.9 and 92.3% decrease in NHase activity when compared to the NHase activity at 55ºC, respectively (Fig. 1c). At higher temperature i.e. 70 and 75ºC resting cells retains only 42.3 and 21.8% of NHase activity. The NHase of wild strain *R. rhodochrous* PA-34 and other reported strains showed their best results at temperature 20-40ºC [2, 8, 11].

Substrate (3-cyanopyridine concentration)

The NHase activity of mutant 4D strain of *R. rhodochrous* PA-34 was assayed at 55ºC using various concentration of 3-cyanopyridine (0.05-0.25 M). The NHase activity increased from 8.4 to 32 U/mg dcw when substrate (3-cyanopyridine) concentration varied from 0.05-0.25 M, respectively. Mutant resting cells exhibited maximum NHase activity (32 U/mg dcw) at concentration of 0.19 M. As the substrate concentration increased or decreased up to 0.2 M from optimum concentration (0.19 M) there was a decrease of 9% and 4.3% in NHase activity was observed, when compared to the optimum NHase activity (32 U/mg dcw) (Fig. 1d). The K_m (0.350 M) and V_{max} (63.5 µmol/m/min) value of whole cells of mutant 4D strain was calculated from Lineweaver-Burk plot.

Optimization of process parameters for synthesis of nicotinamide at one litre scale

Temperature selection for prolonged reaction at high substrate concentration

In this experiment the suitable temperature for the maximum production of nicotinamide was selected. The prolonged reaction (12 h) was carried out for the conversion of 3 M substrate (3-cyanopyridine) to nicotinamide by using resting cells (3 mg dcw/ml) of mutant 4D. 80-90% of 3 M, 3-cyanopyridine was converted in to nicotinamide at 55ºC in 3 h, however at lower temperature (50ºC) or higher (60ºC) only 60% conversion achieved. Therefore 55ºC is selected for prolonged reaction (Fig. 1e).

Substrate (3-cyanopyridine) and resting cell concentration for prolonged reaction

The concentration of 4-8 M substrate (3-cyanopyridne) using 4-8 mg dcw/ml resting cells at 55ºC was carried (Table 1). The reaction was performed till 9 h and conversion rate was determined after each 3 h at this optimum temperature (55ºC), the 4, 5, 6 and 7 M substrate (3-cyanopyridne) was completely converted to nicotinamide in 3 h. However, only 75, 94 and 87% of 8 M substrate was converted into product in 3, 6 and 9 h, respectively. The reaction having 9 and 10 M substrate was not carried as the reaction mixture become viscous because large amount of substrate (Table 1). The wild strain showed 100 and 85 % conversion of 7 M substrate (3-cyanopyridne) into nicotinamide by using 9 mg dcw/ml of resting cells at 25 and 30ºC, respectively [8].

Production of nicotinamide at one litre scale

In one litre batch reaction 100% of the 7 M substrate (3-cyanopyridne) has been converted into nicotinamide by using 7mg dcw/ml resting cells of mutant strain in 3 h at 55ºC (Table 1). This one litre reaction produce 7 M product (nicotinamide) from 7 M substrate i.e. 855 g nicotinamide and productivity of 40.7 g nicotinamide/g resting cells (dcw)/h. The wild strain (*R. rhodochrous* PA-34) had 100% conversion producing 855 g nicotinamide with a

productivity of 7.92 g nicotinamide/g resting cells (dcw)/h [8]. The above conversion results showed that the resting cells of mutant strain were much more efficient than the wild strain. The mutant resting cells hydrolyse about 94% of substrate into product in 6 h (8 mg dcw/ml for 8 M substrate) (Fig. 1f) whereas the wild strain showed the 100% conversion in 12 h by using more amount of resting cells (9 mg dcw/ml for 7 M substrate). The results pertaining to productivity of NHase resting cells of mutant 4D revealed that mutant 4D was much more efficient than the wild strain. The productivity of mutant cells was higher as compared to mutant strain; it was about 40.7 g and 7.92 g nicotinamide/g resting cells (dcw)/h, respectively [9]. Therefore, the mutant 4D has shown very good potential for the production of nicotinamide from 3-cyanopyridine. The comparison of catalytic productivity of various NHase reveals that NHase of mutant 4D is quite efficient in conversion of 3-cyanopyridine to nicotinamide.

The product recovery was carried out following the procedure of Nagasawa [5]. About 87% of the product was recovered from reaction mixture. The product purity was analyzed by HPLC (Fig. 1g).

Conclusion

It is concluded that the mutant 4D has much more hydration potential than its wild strain. The mutant 4D is stable at high temperature (55ºC), high ionic strength (0.3 M) of buffer and at acidic pH (pH 5.5). The mutant 4D is able to biotransform higher concentration (7 M) of substrate (3-cyanopyridine) than its wild strain (7 M) in less time period i.e. 3 h. So the mutant strain can be the better option for the production of nicotinamide at industrial scale.

Acknowledgement

We acknowledge the financial support in term of Junior Research Fellowship from University Grants Commission, Government of India to Mr. Pratush and also gratefully acknowledge the internet facilities provided by Sub- Distributed Information centre, Department of Biotechnology, Himachal Pradesh University, Shimla-5, India.

References

1. Almatawah Q. A., D. A. Cowan (1999). Thermostable Nitrilase Catalysed Production of Nicotinic Acid from 3-cyanopyridine, Enzyme Microbial Technology, 25, 718-724.
2. Cramp R. A., D. A. Cowan (1999). Molecular Characterisation of a Novel Thermophilic Nitrile Hydratase, Biochemistry Biophysics Acta (BBA) – Protein Structure and Molecular Enzymology, 1431, 249-260.
3. Furniss B. S., A. J. Hannaford,, P. W. G. Smith, A. R. Tatchell (1996). Vitamins. In: Vogel's Text Book of Practical Organic Chemistry, ELPB, British Government.
4. Kobayashi M, S. Shimizu (1998). Metalloenzyme Nitrile Hydratase: Structure, Regulation and Application to Biotechnology, Nature Biotechnology, 16, 733-736.
5. Kobayashi M., M. Nishiyama, T. Nagasawa, S. Horinouchi, T. Beppu, H. Yamada (1991). Cloning, Nucleotide Sequence and Expression in *Escherichia coli* of Two Cobalt-containing Nitrile Hydratase Genes from *Rhodococcus rhodochrous* J1, Biochemistry Biophysics Acta, 1129, 23-33.
6. Nagasawa T., C. D. Mathew, J. Mauger, H. Yamada (1988). Nitrile Hydratase-catalyzed Production of Nicotinamide from 3-cyanopyridine in *Rhodococcus rhodochrous* J1, Applied Environmental Microbiology, 54, 1766-1769.
7. Pereira R. A., D. Graham, F. A. Rainey, D. A. Cowan (1998). A Novel Thermostable Nitrile Hydratase, Extremophiles, 2, 347-357.

8. Prasad S., J. Raj, T. C. Bhalla (2004). Optimization of Culture Conditions for Hyper Production of Nitrile Hydratase of *Rhodococcus rhodochrous* PA-34, Indian Journal of Microbiology, 44, 251-256.
9. Prasad S., J. Raj, T. C. Bhalla (2007). Bench Scale Conversion of 3-cyanopyridine to Nicotinamide using Resting Cells of *Rhodococcus rhodochrous* PA-34, Indian Journal of Microbiology, 47, 34-41.
10. Pratush A., A. Seth, T. C. Bhalla (2010). Generation of Mutant of *Rhodococcus rhodochrous* PA-34 through Chemical Mutagenesis for Hyperproduction of Nitrile Hydratase, Acta Microbiologica Immunologica Hungarica, 57(2), 135-146.
11. Raj J., S. Prasad, T. C. Bhalla (2006). *Rhodococcus rhodochrous* PA-34: A Potential Biocatalyst for Acrylamide Synthesis, Process Biochemistry, 41(6), 1359-1363.
12. Sankhian U. D., H. Kumar, D. Chand, D. Kumar, T. C. Bhalla (2003). Nitrile Hydratase of *Rhodococcus rhodochrous* NHB-2: Optimisation of Conditions for Production of Enzyme and Conversion of Acrylonitrile to Acrylamide, Asian Journal of Microbiology Biotechnology and Environmental Sciences, 5, 79-85.
13. Shimizu S. (2001) Vitamins and Related Compounds: Microbial Production. In: Biotechnology: Special Processes, Vol. 10, (Eds. Rehm H.-J. and G. Reed), Wiley-VCH, Weinheim, Germany.
14. Yamada H., K. Ryuno, T. Nagasawa, K. Enomoto, I. Watanabe (1986). Optimum Culture Conditions for Production by *Pseudomonas chlororaphis* B23 of Nitrile Hydratase, Agricultural Biological Chemistry, 50, 2859-2865.

Mr. Amit Pratush
E-mail: amit.pratush@gmail.com

Mr. Amit Pratush, Diligent and Proactive Research Fellow, pursuing Ph.D. in Enzyme Technology and Molecular Biology at Research Lab II at Department of Biotechnology, Himachal Pradesh University, Shimla, India. Research Interests: nitrile degrading enzymes (nitrile hydratase, nitrilase and amidase) & chemical mutagenesis of microorganisms for hyper induction of enzymes.

Mr. Amit Seth
E-mail: amitsethshimla@gmail.com

Mr. Amit Seth is a Ph.D. Research Fellow at Research Lab II at Department of Biotechnology, Himachal Pradesh University, Shimla, India. His major research areas are isolation, characterization and purification of nitrile degrading enzymes. He also has expertise in molecular biology techniques.

Prof. Tek Chand Bhalla
E-mail: bhallatc@rediffmail.com

Prof. Tek Chand Bhalla, Corrdinator Department of Biotechnology, Himachal Pradesh University, Shimla India.

Professional teaching experience: about 30 year teaching and research experience. Research areas: (i) nitrile metabolizing enzyme, isolation, purification, cloning and expression in suitable host; (ii) biotransformations with immobilized or free biocatalysts for industrial and environmental applications. Published more than 80 research article in peer reviewed journals and supervised 20 Ph.D. and 25 M.Phil. students for their thesis.