

Production of isonicotinic acid using agar entrapped whole cells of *Nocardia globerula* NHB-2

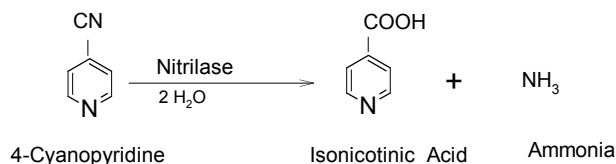
Bhalla T. C., Mehta P.K., Sharma N.N. and Bhatia S.K.
 Department of Biotechnology, Himachal Pradesh University, Summer Hill,
 Shimla-171005, Himachal Pradesh, INDIA bhallatek29@gmail.com



INTRODUCTION

Isonicotinic acid or pyridine-4-carboxylic acid is an important pyridine derivative which is mainly used for the synthesis of isoniazid (an antitubercular drug), inabenfide (a plant growth regulator), terefenadine (an antihistamine) and nialamide (an antidepressant) (Yadav G.D. *et al.* 2005, Scriven E.F.V. *et al.* 1998). Isonicotinic acid also finds application as an anticorrosion reagent, plating additive, and photosensitive resin stabilizer (Wu F. *et al.* 1991). Isonicotinic acid is chemically synthesized through several chemical methods including potassium permanganate oxidation, air oxidation, and ozone oxidation of alkylpyridines (Qiao Q.D. *et al.* 2000). The chemical processes that are used for the manufacturing of isonicotinic acid are hazardous, energy demanding and expensive (Yadav G.D. *et al.* 2005).

Nitrilases are finding applications in organic synthesis and a range of nitriles are being converted into corresponding acids (Banerjee A. *et al.* 2002, Martinkova L. and Kren V., 2002). The 4-cyanopyridine can be converted to isonicotinic acid by nitrilase enzyme as per reaction scheme given below:



An enzyme mediated process that involved the co-immobilization of nitrilase (5.5 U) from *Aspergillus niger* and an amidase (5 U) from *Rhodococcus erythropolis* on Butyl Sepharose column for the conversion of 4-cyanopyridine to isonicotinic acid has been reported (Vejvoda V. *et al.* 2006). The time and space productivity of this process was 88 mg isonicotinic acid ml⁻¹ h⁻¹. In total, 3.102 g isonicotinic was produced through this reactor in 35 h. A more productive process for the synthesis of isonicotinic acid that uses whole cells of *Nocardia globerula* NHB-2 (hyper induced for nitrilase) entrapped in agar for catalyzing hydrolysis of 4-cyanopyridine to isonicotinic acid is being reported here.

MATERIALS AND METHODS

N. globerula NHB-2 previously isolated in our laboratory as an efficient nitrile degrading organism as it harbors three enzymes i.e. nitrilase, nitrile hydratase and amidase that are involved in nitrile degradation (Bhalla T.C. and Kumar H., 2005). The nitrilase of *N. globerula* NHB-2 was hyper induced (5.71 U mg dwt⁻¹, 16.2 U ml⁻¹) in short incubation time (30 h) through multiple feeding of

isobutyronitrile in the growth medium containing 1% glucose, 0.5% peptone, 0.3% beef extract and 0.1% yeast extract, pH 7.5 (Sharma N.N., 2009). The organism was cultured at 30°C in an incubator shaker at 160 rpm. The cells were sedimented at 10000 'g' and suspended in 0.1M sodium phosphate buffer, pH 7.5 and were termed as resting whole cells. The nitrilase activity of the resting cells was assayed as reported earlier using 4-cyanopyridine as substrate (Sharma N.N. *et al.* 2006). One unit of nitrilase activity was defined as that amount of enzyme (whole cells) which catalyzed the conversion of one μ mole of 4-cyanopyridine to isonicotinic acid per min under the assay conditions.

The whole cells of *N. globerula* NHB-2 were immobilized in agar (1%, w/v) and small beads (4 mm x 2.5 mm) were prepared following the methods described previously (Raj J. *et al.* 2007). The parameters optimized for immobilization of cells and transformation 4-cyanopyridine to isonicotinic acid included: agar concentration, cell concentration and substrate concentration. A fed batch reaction (100 ml scale) with a substrate feed of 50 mM 4-cyanopyridine every 20 min using immobilized cells equivalent to 30 Uml⁻¹ nitrilase activity was designed.

RESULTS AND DISCUSSION

Immobilization of whole cells of *Nocardia globerula* NHB-2

A number of concentration of agar (1-5% in 0.1M Sodium phosphate buffer, pH 7.5) as matrix for entrapment of cells were tested and maximum nitrilase activity of the immobilized cells was observed in beads prepared with 1% agar (Fig. 1 and 2). The concentration of cells of *Nocardia globerula* NHB-2 were varied (10-50 Uml⁻¹) in 1% agar and cells equivalent to 30 Uml⁻¹ in matrix exhibited optimum conversion of 4-cyanopyridine to isonicotinic acid (Fig. 3). The various concentrations (50mM-250mM) of substrate (4-cyanopyridine) tested for finding out optimum substrate concentration for reaction with the immobilized cells, at 100 mM substrate concentration the immobilized cells gave highest activity (Fig. 4). The substrate concentration higher than this inhibited the nitrilase activity of the immobilized cells. It seems that at higher concentration than the optimized concentration of matrix, cells and substrate, limitation due to diffusion of substrate/product across beads might have become more operational leading to decrease in nitrilase activity of immobilized cells.



Fig. 1. Cells of *N. globerula* NHB-2 immobilized in agar gel beads

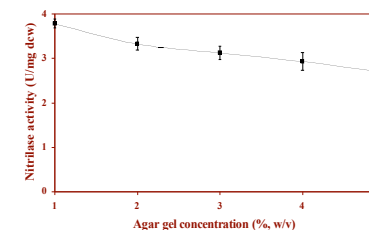


Fig. 2. Effect of agar gel concentration on nitrilase activity

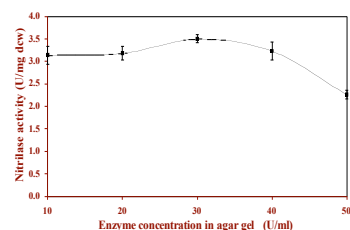


Fig. 3. Effect of enzyme loading in agar gel on nitrilase activity.

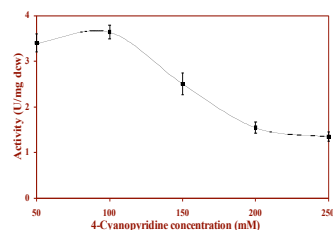


Fig. 4. Effect of substrate concentration on nitrilase activity of cells immobilized in agar gel (1% w/v).

Fed batch reaction for production of isonicotinic acid

The experiment involving optimization of substrate concentration revealed that the nitrilase of immobilized cells experience substrate inhibition beyond 100mM concentration of 4-cyanopyridine and therefore at a given time substrate higher than this concentration can not be added for efficient conversion of 4-cyanopyridine to isonicotinic acid. However, higher concentration of product (upto 400 mM) did not affect the enzyme catalysis. Therefore, a fed fed batch reaction for addition of substrate was designed to overcome the problem of inhibition of nitrilase activity of immobilized cells at higher concentration of 4-cyanopyridine. Most of the nitrilases are inhibited at higher concentration of substrates (Sharma N.N. et al. 2006).

In a fed batch reaction at 100 ml scale using agar immobilized resting cells of *N. globerula* NHB-2 corresponding to 30 U ml⁻¹ nitrilase activity (1.75 mg dew ml⁻¹) in 0.1 M NaH₂PO₄/ Na₂HPO₄ (pH 7.5). The reaction was performed at 35°C and eight feeds of 50 mM 4-cyanopyridine (solid) were added in the reaction each at an interval of 20 min. A total of 5 g isonicotinic acid was produced at a rate of 12.2 g h⁻¹ g⁻¹ dew in 160 min (Fig 1). No residual substrate was detected in the medium after the reaction and this showed that there was a 100% conversion of the substrate to product. The absence of isonicotinamide in the reaction mixture further revealed that the nitrilase of this organism has an edge over the fungal nitrilases since latter also convert substrate to amide. The present process developed for the production of isonicotinic acid is simple as it employs whole cell. This time and space productivity of isonicotinic acid using immobilized biocatalyst is much higher than what has been reported earlier (Vejvoda V. et al. 2006).

CONCLUSION

The parameters for immobilization of *N. globerula* NHB-2 cells in agar were optimized. The cells of this bacterium can be best immobilized 1% agar for nitrilase activity. The immobilized cells efficiently catalysed the conversion of 4-cyanopyridine to isonicotinic acid. The process reported here is simple and less expensive as compared to previously reported process as it uses only whole cells and not the purified enzymes. The time and space productivity of isonicotinic acid observed in the present studies is higher than so for reported by other investigators.

REFERENCES

- Banerjee A. et al. (2002) *The nitrile degrading enzymes: current status and future prospects*. Appl Microbiol Biotechnol **60**, 33-44.
- Bhalla T.C. and Kumar H. (2005) *Nocardia globerula* NHB-2: a versatile nitrile degrading organism. Can J Microbiol **51**:705-708.
- Matinkova L. and Kren V. (2002) *Nitrile- and amide-converting enzymes: Sterio-rgio- and chemolelectivity*. Biocat Biotransform **20**, 73-93.
- Qiao Q.D. et al. (2000) *Electrochemical synthesis of 4-picolinic acid from 4-methylpyridine*. Fine Chem., 17.
- Raj J. et al. (2007) *Bioconversion of benzonitrile to benzoic acid using free and agar entrapped cells of Nocardia globerula* NHB-2. Acta Microbiologica et Immunologica Hungarica, **54**, 79–88.
- Scriven E.F.B. et al. (1998) *Pyridine and pyridine derivatives*. In: Krik-Othmer *Encyclopedia of Chemical Technology*, 4th edition, Vol 20 JI Kroschwitz and M Howe-Grant(Eds.), John Wiley & Sons Inc. pp 641-679.
- Sharma N.N. (2009) *Hyperinduction of nitrilase in Nocardia globerula* NHB-2 and its application in synthesis of *N*-heterocyclic carboxylic acids. Ph.D. Thesis, Himachal Pradesh University, Shimla, India.
- Sharma N.N. et al. (2006) *Nocardia globerula* NHB-2: Bench scale production of nicotinic acid. Process Biochemistry, **41**, 2078-2081.
- Vejvoda V. et al. (2006) *Immobilization of fungal nitrilase and bacterial amidase-two enzymes working in accord*. Biocatalysis and Biotransformation, **24**, 414-418.
- Yadav G.D. et al. (2005) *Enzymatic synthesis of isoniazid in non-aqueous medium*. Enzyme and Microbial Technology, **36**, 217-222.
- Wu F. et al. (1991) *Electrochemical synthesis of 4-picolinic acid*. J Guang Dong Chem Eng, **4**, 21–24.