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Research Article

ENHANCED NITRILASE PRODUCTION BY *RHODOCOCCUS PYRIDINIVORANS* SN2 USING AN OPTIMIZED BIPHASIC FERMENTATION APPROACH AND ITS USE FOR NICOTINIC ACID SYNTHESIS

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Abstract: In the present study, nitrilase activity of *Rhodococcus pyridinivorans* SN2 was optimized by a combination of response surface methodology and biphasic fed batch strategy. The optimized conditions were 15 g l^{-1} of yeast extract and 6 g l^{-1} of benzonitrile (in feeds of 1 g l^{-1}), followed by a feed of 10 g l^{-1} fructose and 4 g l^{-1} ∈-caprolactam leading to a biomass yield of 10±1 g_{dcw} l^{-1} in 48 h with nitrilase activity of 150±20 U g_{dcw}^{-1} and a nitrilase productivity of around 1500 U l^{-1}. This represented a 16 fold increase in nitrilase productivity from 90 U l^{-1} under unoptimized conditions. Whole cells (2 g_{dcw}) of *Rhodococcus pyridinivorans* SN2 when immobilized in calcium alginate beads completely hydrolysed 10.5 g 3-cyanopyridine (~0.1 mol) to nicotinic acid in 6.5 hours using a fed batch reaction at a scale of 100 ml.

Keywords: Nitrilase; nicotinic acid; *Rhodococcus*; RSM; biphasic

Introduction

The chemical hydrolysis of nitriles using either strong acid or base catalysis is an important reaction in organic synthesis, and a primary method for obtaining various carboxylic acids. The hydrolysis of 3-cyanopyridine to nicotinic acid is an industrially important reaction, part of the commercial process for nicotinic acid production. This process utilizes the gas-phase ammoxidation of picoline to 3-cyanopyridine, followed by its chemical hydrolysis using a strong basic catalyst under elevated temperature and pressure to the final product (Chuck, 2005). Chemical hydrolysis of nitriles carries disadvantages such as non-specificity, low conversion yield and generation of waste byproducts, alongwith the environmental and personnel hazards due to the extreme temperature, pressure and pH employed for the process (Kukushkin and Pombeiro, 2005). Nitrilases (nitrile aminohydrolase, EC 3.5.5.1) hydrolyse nitrile to the corresponding carboxylic acid without the intermediate formation of amide. Nitrilases appear to be a good replacement to chemical catalysis due to their advantages of high conversion yield, specifity of reaction, negligible waste generation, and their ability to be used under physiological conditions (Bhalla *et al.*, 1992; Thuku *et al.*, 2009). Therefore, researchers have attempted nicotinic acid production using nitrile hydrolysing enzymes from a number of organisms, namely *Rhodococcus rhodochrous* J1 (Mathew *et al.*, 1988), *Bacillus pallidus* Dac521 (Almatawah and Cowan, 1999), *Rhodococcus* sp. NDB 1165 (Prasad *et al.*, 2007), *Nocardia globerula* (Sharma *et al.*, 2006; Sharma *et al.*, 2011), *Microbacterium imperiale* (Cantarella *et al.*, 2011), and *Fusarium proliferatum* (Jin *et al.*, 2013; Yusuf *et al.*, 2013).

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In order to make any fermentation process commercially viable, it is important to understand the relations between the different physical and chemical parameters. Response surface methodology (RSM) involves characterization of these relations in a functional form using design of experiments and regression analysis, and predicting values of these parameters for obtaining an optimum response (biomass yield or activity). RSM has been used for optimization of production process parameters for maximizing yield of nitrilases from *Arthrobacter nitroguajacolicus* (Shen *et al*., 2009), *Rhodococcus erythropolis* (Dong *et al*., 2011) and *Fusarium proliferatum* (Yusuf *et al*., 2013).

In the present study, optimization of biomass and nitrilase production by *Rhodococcus pyridinivorans* SN2 was carried out using response surface methodology and biphasic fed batch strategy. This enzyme was then applied for the production of nicotinic acid from 3-cyanopyridine.

Materials and Methods

Chemicals & media components

Nicotinic acid and 3-cyanopyridine were purchased from Sigma, USA. Yeast extract was purchased from Titan Biotech, India, and benzonitrile (puriss) was purchased from Spectrochem, India. ϵ-caprolactam was purchased from Merck. All other chemicals were of analytical grade or above.

Culture conditions

Rhodococcus pyridinivorans SN2 was isolated from farm soil of Allahabad, India. This strain was maintained on solid medium of composition (in g l^{-1}): yeast extract, 8; agar-agar, 15; benzonitrile, 4; pH 7.0, and incubated at 30 °C. An OD@630nm of 1.0 was found to correspond to 0.2±0.01 g_{dcw} l^{-1}.

Experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of production medium. Initial production medium was of composition (g l^{-1}): Na_2HPO_4, 0.5; KH_2PO_4, 0.25; yeast extract, 4; sucrose 4; benzonitrile, 1; and pH 8±0.2. Benzonitrile was sterilized by filtration through 0.2 µm filter and added after autoclaving. The culture was incubated at 30 °C, 200 rpm in a rotary incubator shaker. The cells were harvested by centrifugation at 11000 g at 4 °C in Sigma 3K30 centrifuge, washed twice with distilled water, and the cell pellet was used immediately or stored frozen at –20 °C till further use. To calculate dry weight, the cell pellet was dried in a hot air oven at 80 °C till constant weight. An OD@630nm of 1.0 was found to correspond to 0.2±0.01 g_{dcw} l^{-1}.

Effect of carbon and nitrogen source

Different carbon sources such as benzonitrile, sodium acetate, sodium citrate, ethanol, fructose, glucose, sucrose and glycerol were evaluated at a concentration of 2 g l^{-1} in a medium containing (g l^{-1}): yeast extract, 2; KH_2PO_4, 0.25; Na_2HPO_4, 0.5; pH 8. Benzonitrile was added in two increments of 1 g l^{-1} to prevent its toxic effects. All other cultivation conditions were same as given above.

Different nitrogen sources including yeast extract, malt extract, peptone, urea, and ammonium sulfate were evaluated at 2 g l^{-1} in a medium containing (g l^{-1}): benzonitrile, 2; KH_2PO_4, 0.25; Na_2HPO_4, 0.5; pH 8 under the cultivation conditions mentioned above.

Response surface methodology

Experiments were generated and analysed in R 3.0.1 (R Core Team, 2013), using the ***rsm*** package ver. 2.03 (Lenth, 2009). A 2^2 design with 4 center points was used for the initial model, which was used to generate a path of steepest ascent. A rotatable central composite design (CCD), divided into a cube block and star block ($\alpha = \sqrt{2}$) with 4 centre points each, was used to analyse the region of curvature and determine the stationary point of the response.

Chromatographic analysis of 3-cyanopyridine and nicotinic acid

Nicotinic acid and 3-cyanopyridine were resolved and quantitated using HPLC (Waters 600 pump, 2489 detector) using a C-18 column (Waters Spherisorb ODS2) eluted with 0.01% HCl:acetonitrile::3:1 at a flow rate of 1.0 ml min^{-1} and detection at 230 nm. Thin layer chromatography (TLC) was carried out according to the procedure of Almatawah and Cowan (1999).

Nitrilase assay

Assay was carried out in 1 ml reaction mixture in 2 ml microcentrifuge tubes. The reaction mixture consisted of 0.05 M potassium phosphate buffer pH 7.0 with 100 mM 3-cyanopyridine as substrate, to which cells were added to initiate the reaction. The reaction mix was incubated in a shaking water bath at 30 °C and 200 rpm for 30 minutes. The reaction was terminated by centrifugation at 12000 *g* for 1 minute to remove cells from suspension. The concentration of nicotinic acid and 3-cyanopyridine in the supernatant was assayed by HPLC. One unit of nitrilase is defined as the amount of enzyme required to convert 1 µmol of 3-cyanopyridine to nicotinic acid per minute under the above assay conditions.

Effect of temperature and pH

For studying the effect of temperature, the standard nitrilase assay was carried out at different temperatures of 20, 30, 40, 50 and 60 °C in a shaking water bath. For determining the effect of pH, the standard nitrilase assay was carried out at 30 °C in different buffers: 0.1 M citrate-phosphate (pH 4, 5 and 6), 0.1 M potassium-phosphate (pH 7), 0.1 M Tris-HCl (pH 8) and 0.1 M glycine-NaOH (pH 9 and 10).

Alginate entrapment and 3-cyanopyridine hydrolysis

Sodium alginate solution (3% w/v) was prepared in 0.1 M borate buffer pH 8 with heating. Ten ml of cell suspension (2 g dry weight of cells) was added to 10 ml of alginate solution and mixed well by vortexing. This mixture was extruded using a needle and syringe into ice cold 0.2 M $CaCl_2$ solution to give spherical beads approximately 3 mm in diameter. These beads were washed in borate buffer, and resuspended in 100 ml of fresh buffer in a 250 ml Erlenmeyer flask. The reaction was started by the addition of 2.1 g of finely powdered 3-cyanopyridine (corresponding to a concentration of 200 mM) to the reaction mix, which was then incubated at 30 °C, 200 rpm in a shaker incubator. The reaction was monitored by TLC and 2.1 g 3-cyanopyridine was added on exhaustion of substrate. Addition of substrate was repeated until a total of 10.5 g of substrate had been added. Aliquots were withdrawn every 90 minutes for HPLC analysis.

Results and Discussion

Effect of carbon and nitrogen source

Carbon and nitrogen sources are known to have significant effects on enzyme yield (Gong *et al*. 2012). Different carbon and nitrogen sources were studied for biomass production of *Rhodococcus pyridinivorans* SN2. Among the carbon sources tested, *R. pyridinivorans* SN2 was able to utilize benzonitrile, simple sugars like glucose and fructose, organic acids such as acetate and succinate and ethanol. Benzonitrile was found to support highest biomass yield followed by fructose and glucose (Figure 1). A μ_{max} of ~0.498 and ~0.208 was observed for benzonitrile and fructose respectively. Such rapid and profuse growth on benzonitrile has also been observed by Collins *et al*. (1983) in case of *Rhodococcus rhodochrous* (earlier *Nocardia rhodochrous*). The organism in the present study did not utilize glycerol, unlike *R. rhodochrous* J1 (Mathew *et al.,* 1988). It also did not utilize sucrose, and showed poor growth on citrate and acetate. Citrate has been reported to give poor biomass and nitrilase production in *R. erythropolis* (Dong *et al.,* 2011).

Among the nitrogen sources tested, *R. pyridinivorans* SN2 showed good and comparable growth on the different organic nitrogen sources, but did not utilize urea or ammonia (Figure 2).Yeast extract was chosen for further experiments since it was the cheapest among the organic sources tested. Yeast extract has also been reported to be the best nitrogen source for *R. erythropolis* (Dong *et. al.*, 2011), while Mathew *et al.* (1988) found that a mixture of polypeptone, meat extract and yeast extract was best for nitrilase production by *R. rhodochrous* J1.

Biomass production optimization using RSM

Using a 2^2 full factorial design (Table 1), the growth of *R. pyridinivorans* SN2 was found to depend on concentrations of yeast extract and benzonitrile in the medium, following a first order model with interaction:

$$OD@630nm = 0.2875 + 0.3875 * X1 + 1.075 * X2 + 0.475 * X1 * X2$$

Table 1
2^2 full factorial design

Std order	*Run order*	*Yeast extract (g l^{-1})*	*Benzonitrile (g l^{-1})*	*Response (OD@630nm)*
1	6	1	0.5	1.45
2	4	3	0.5	2.7
3	2	1	1.5	3
4	7	3	1.5	5.2
5	3	2	1	3.1
6	1	2	1	3
7	8	2	1	3.1
8	5	2	1	3.2

where, X1 = Yeast extract; X2 = Benzonitrile

This model had a F-value of 365.09 and p-value of 0.0002, which implied that this model was significant. The coefficient of determination, R^2 was 0.9973, which showed that this model could explain 99.73% of the variability in the data. Adeq precision of 58.095 was obtained for this model, which indicated an adequate signal and suggested that this model could be used to navigate the design space. This model was used to construct a path of steepest ascent, and experiments showed that biomass yield plateaued beyond 12.5 g l^{-1} of yeast extract and 6.4 g l^{-1} of benzonitrile (Table 2). This was used as the new center to design a central composite design (CCD), divided into a cube block and star block ($\alpha = \sqrt{2}$) with 4 centre points each, which was used to analyse the region of curvature and determine the stationary point of the response (Table 3). This design showed the location of stationary point of the response surface (Figure 4) to be at 13.157 g l^{-1} of yeast extract and 6.493 g l^{-1} of benzonitrile which validated the results from the previous experiment. *R. pyridinivorans* SN2 could be finally grown to an OD@630nm of 50±5 in 30 h, corresponding to a dry weight of 10±1 g l^{-1} of cells having a specific activity of 10±5 U g_{dcw}^{-1} (Figure 5). Beyond this point growth was found to plateau, with increase in yeast extract or benzonitrile concentration not yielding any significant increase in growth. Since this culture is highly aerobic, oxygen concentration might be the limiting factor at higher cell concentrations.

Biphasic fed batch strategy

Benzonitrile was unable to act as a good inducer under high cell concentrations, probably due to rapid degradation. Furthermore, due to its toxicity, it cannot be supplemented to the medium in excess. This necessitates the use of a stable inducer of nitrilase activity, such as ϵÀ-caprolactam (Nagasawa *et al.*, 1990b). When ϵÀ-caprolactam was used as an inducer in this study, it was observed to have no effect on cells growing on benzonitrile, giving a nitrilase activity of only 10±2 U g_{dcw}^{-1}. Therefore, a biphasic strategy was employed where biomass was first grown on benzonitrile using RSM optimized conditions, followed by growth on fructose in the presence of ϵÀ-caprolactam for maximum induction of nitrilase activity (Figure 5). This strategy resulted in biomass yield of 10±1 g_{dcw} l^{-1} in 48 h with nitrilase activity of 150±20 U g_{dcw}^{-1}. Using this

Table 2
Path of steepest ascent

Std order	*Run order*	*Distance in steps*	*Yeast extract (g l^{-1})*	*Benzonitrile (g l^{-1})*	*Predicted OD@630nm*	*Actual OD@ 630nm*
1	1	0	2	1	3.094	3.2
2	10	3	4	2.1	8.145	8.7
3	4	6	6.1	3.2	15.331	17.4
4	2	9	8.2	4.2	24.652	27.3
5	8	12	10.4	5.3	36.111	38.2
6	9	15	12.5	6.4	49.718	54
7	7	18	14.6	7.4	65.446	51
8	3	21	16.7	8.5	83.323	53.5
9	6	24	18.8	9.6	103.459	54.2
10	5	27	21	10.6	125.571	55.8

Table 3
Central composite design (CCD)

Std order	*Run order*	*Yeast extract (g l^{-1})*	*Benzonitrile (g l^{-1})*	*Response (OD@630nm)*
		Cube block		
1	4	11.5	6	46
2	7	13.5	6	53.5
3	8	11.5	7	47
4	6	13.5	7	51
5	5	12.5	6.5	54
6	1	12.5	6.5	51.5
7	2	12.5	6.5	53
8	3	12.5	6.5	53.5
		Star block		
1	3	11	6.5	44
2	2	14	6.5	53
3	5	12.5	5.8	47
4	6	22.5	7.2	51
5	4	12.5	6.5	53.5
6	7	12.5	6.5	56
7	1	12.5	6.5	54
8	8	12.5	6.5	54.5

strategy, a 16 fold enhancement in nitrilase yield was attained. RSM has been used by Shen *et al*. (2009) to optimize nitrilase production by *Arthrobacter nitroguajacolicus* resulting in 2.86 fold increase in activity, by Dong *et al*. (2011), achieving 1.77 fold enhancement in nitrilase yield from *Rhodococcus erythropolis* and by Yusuf *et al.* (2013), who reported 2.24 fold increase in nitrilase activity of *Fusarium proliferatum*.

Effect of pH and temperature

The *Rhodococcus pyridinivorans* SN2 whole cell preparation is a mesophilic nitrilase showing good activity at 20 to 40 °C, with an optimum of 30 °C (Figure 6). It displayed high activity in a broad pH range (6-10) with an optimum at pH 8 (Figure 7). Gong *et al*. (2012) have reported in their review that most known bacterial and fungal nitrilases have pH optimum of 7 - 8 and are maximally active at 30 - 55 °C. This implies that the *Rhodococcus pyridinivorans* SN2 nitrilase is comparable to most other known nitrilases in these respects.

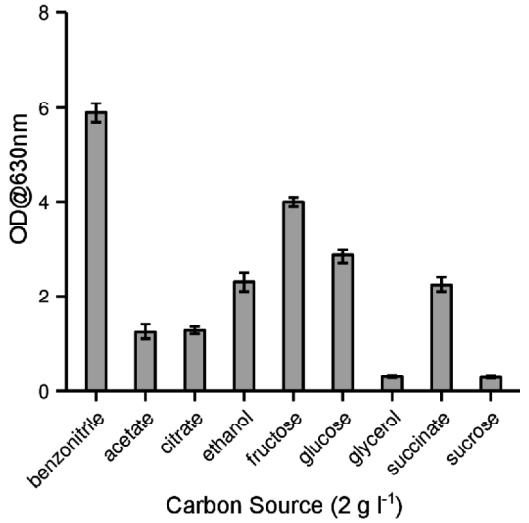

Figure 1: **Effect of carbon source on biomass production**

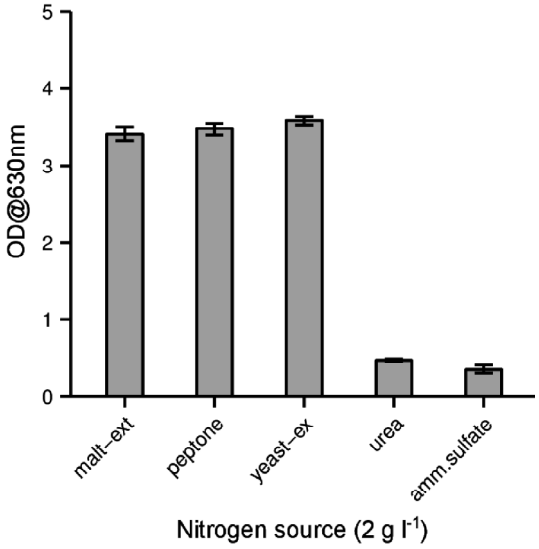

Figure 2: **Effect of nitrogen source on biomass production**

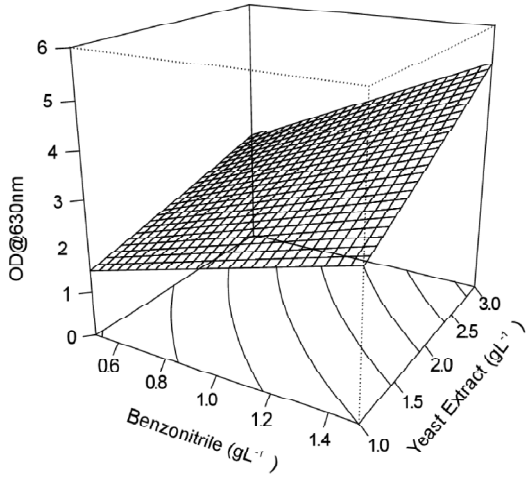

Figure 3: **Response surface of biomass production as function of benzonitrile and yeast extract concentration showing first order model with positive interaction**

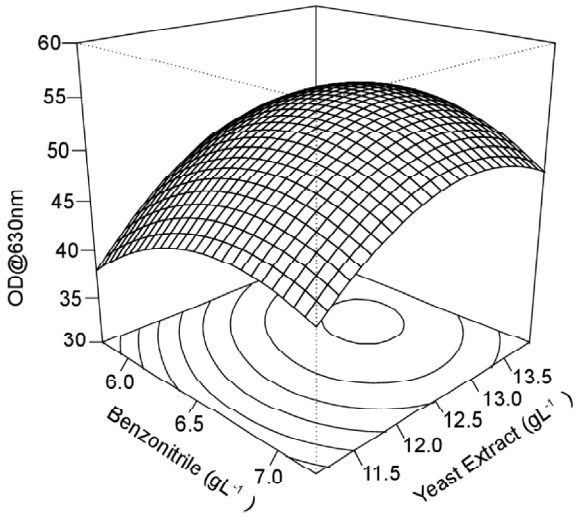

Figure 4: **Response surface of biomass production as function of benzonitrile and yeast extract concentration showing quadratic curvature and stationary point (region of optimum response)**

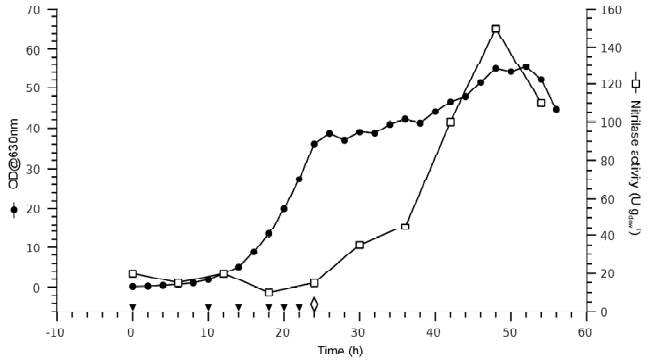

Figure 5: **Biphasic strategy for maximum biomass production (filled circle) and nitrilase activity (open square) in medium containing 15 g l^{-1} yeast extract as nitrogen source. Inverted triangles represent feed of 1 g l^{-1} benzonitrile. Diamond represents feed of 10 g l^{-1} fructose and 4 g l^{-1} ϵÀ-caprolactam**

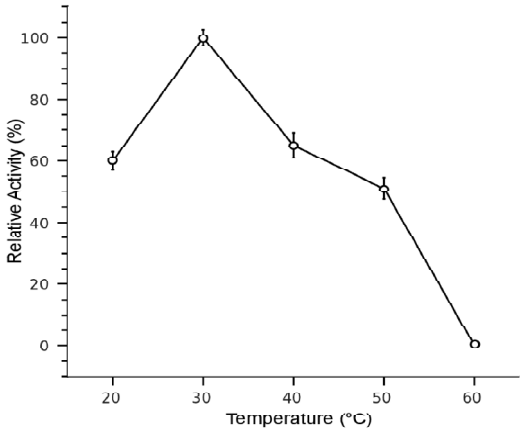

Figure 6: **Effect of temperature on nitrilase activity. 100% relative activity corresponds to 160±5 U g_{dcw}^{-1}**

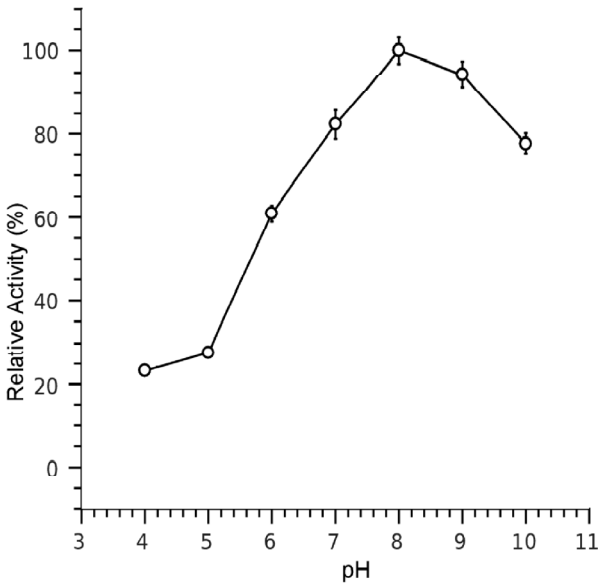

Figure 7: **Effect of pH on nitrilase activity. 100% relative activity corresponds to 140±5 U g_{dcw}^{-1}**

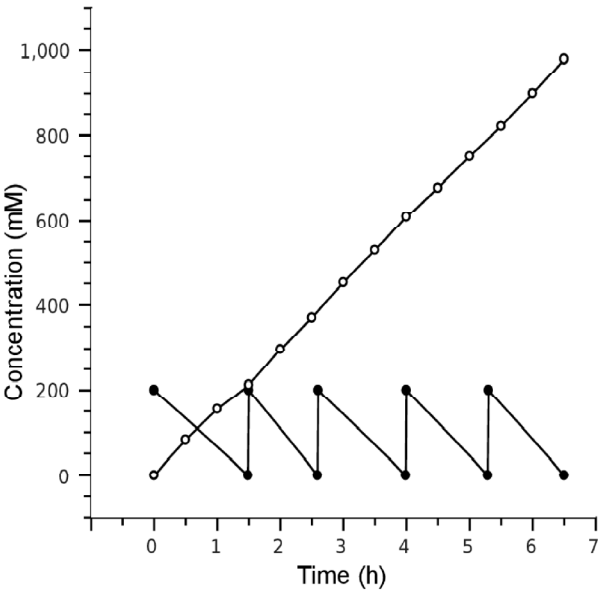

Figure 8: **Hydrolysis of 3-cyanopyridine (closed circle) to nicotinic acid (open circle) by *Rhodococcus pyridinivorans* SN2 immobilized in alginate beads**

Hydrolysis of 3-cyanopyridine to nicotinic acid

Alginate immobilized cells (2 g_{dcw}) completely hydrolysed 10.5 g of 3-cyanopyridine to nicotinic acid in 6.5 hours at a 100 ml scale, giving a substrate conversion rate of 0.81 g h^{-1} g_{dcw}^{-1} (Figure 8). Almatawah and Cowan (1999) reported a conversion efficiency of 0.208 g h^{-1} g_{dcw}^{-1} using alginate entrapped cells of *Bacillus pallidus*

Dac521. Much interest is being shown in biocatalytic routes for the synthesis of nicotinic acid by major companies like Lonza, Switzerland (Shaw *et al*., 2003) due to the advantages of high yield, high specificity, high purity and environmental safety. However, in order for nitrilases to be economical, different strategies, such as that in the present work, have to be employed to reduce the cost as well as to increase the yield of these valuable enzymes.

Summary

In the present work, response surface methodology and a biphasic fed batch strategy was used to optimize nitrilase production by *Rhodococcus pyridinivorans* SN2. On applying Response surface methodology an optimum biomass yield of 10±1 g l^{-1} was obtained using benzonitrile and yeast extract. Subsequently on using the biphasic fed batch strategy, a nitrilase activity of 150±20 U g_{dcw}^{-1} and a nitrilase productivity of around 1500 U l^{-1} were achieved. This represented a 16 fold increase over initial unoptimized nitrilase yield. This nitrilase could be successfully applied for synthesis of nicotinic acid from 3-cyanopyridine.

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Abbreviations

g_{dcw} = gram dry cell weight

OD@630nm = Optical density at 630 nm

RSM = Response surface methodology

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