Biotransformation of 3-cyanopyridine by utilizing free and immobilized whole cell nitrilase enzyme from *Nocardia globerula* NHB-2.

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Abstract

Nitrile hydrolysing enzymes are potential biocatalyst for direct conversion of toxic nitriles into non-toxic and high value amides and acids. The microorganism *Nocardia globerula* NHB-2 was grown and induced with three different short chain aliphatic nitriles and exhibited activity towards aromatic nitriles observed. Propionitrile was found to be the optimal inducer for this microorganism at a concentration of 0.3% v/v. Three factor Box Behnken design was used to optimize the reaction parameters for the biotransformation of 3-cyanopyridine to nicotinic acid. The optimized conditions include substrate concentration of 210 mM, resting cell concentration of 30 U/mg DCW and time of conversion of 70 min. Then, immobilization of the resting cells of *Nocardia globerula* NHB-2 with agar was performed and the nitrilase activity was compared with that of the free resting cells in terms of thermal stability. Packed bed reaction system of the immobilized cells was utilised for the production of nicotinic acid in a continuous operation wherein the substrate is recycled. The immobilized resting cells were reused four times and the residual nitrilase activity observed was 40% up to the third recycle.

Keywords: Nitrilase, Response surface methodology, 3-cyanopyridine, Nicotinic acid, Immobilization, Agar.

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Introduction

Nitrile is an organic compound that has a C≡N functional group [1,2]. These compounds are found in agriculture, chemical, and pharmaceutical industries as intermediates, products, by-products, and waste products [3]. Many nitriles are toxic, carcinogenic, and mutagenic. Nitriles can be degraded either using chemicals or microbial systems. The chemical conversion of toxic nitriles to acids requires expensive environmental conditions, which may result in the production of unwanted by-products [4,5] The degradation of the variety of nitriles including aliphatic, aromatic, and heterocyclic nitriles is thus considered for bioremediation of toxic nitriles from environmental contaminants [6,7].

Microorganisms through green catalysis can degrade nitrile compounds. Microbial biotransformation is used in the transformation of various pollutants compounds including hydrocarbons, pharmaceutical substances and metals [8,9]. Microbes use different enzymatic pathways to catabolize nitrile. The enzymatic biotransformation is two-step conversion of nitrile to carboxylic acid using enzymes, nitrile hydratase and amidase with amide as intermediate or a single step conversion of nitriles into corresponding acids by nitrilase without formation of amide [10,11].

Nitrilases catalyse the hydrolysis of natural and xenobiotic nitriles, under mild conditions, and with high enantioselectivity, to their corresponding carboxylic acid [12]. The nitrilases (EC 3.5.5.1) are an important class of industrial enzymes belonging to the nitrilase superfamily and are widely expressed in both prokaryotes and eukaryotes [13].

The superfamily members include microbial nitrilases (nitrilases, cyanide dihydratases and cyanide hydratases), aliphatic amidases, amidohydrolases and acyl transferases [14]. The nitrilases are potentially important finding major application in biotransformations, particularly for the production of pharmaceutical intermediates and fine chemicals [15]. Based on the substrate specificity, nitrilase is distinguished into three subclasses: aliphatic nitrilase, aromatic nitrilase and heterocyclic nitriles and arylacetonitrilase [16,17]. Nitrilases are often inducible in nature. Most nitrilases are usually inducible by products, their analogs or substrates. Generally certain nitriles, amides, and carboxylic acids function as nitrilase inducers [18].

Nitrilase enzymes have a range of applications including food industries, chemical manufacturing, pharmaceutical processes, wastewater treatment and textile industries [3,18]. Some of, the microorganisms that have gained a versatile application in the nitrile biotransformation include the genus *Rhodococcus*, *Pseudomonas*, *Arthrobacter*, *Alcaligenes*, *Bacillus* [19].

All the nitrile-metabolizing enzymes hydrolyse several structurally diverse nitriles. Numerous commercially important organic compounds, such as p-aminobenzoic acid, benzamide, acrylamide, nicotinic acid, pyrazinoic acid, thiophenamide, have been prepared from the corresponding nitriles using microbial whole cells.

An alternative for synthesizing acrylic acid is currently known to be the enzyme-based biotransformation of acrylonitrile using nitrilase [20]. Another important aspect of nitrile biocatalysis is the ability of these enzymes to perform stereoselective

transformations [21]. Nitrilases have applications in surface modification of polymers as well [22]. Nitrile hydrolysing enzymes are also used for bioremediation and have biodegradation potential [23]. Immobilization of the resting cells enables repeated and continuous use of the cells with retention of some or all of the catalytic activity [24,25].

In this study, we have investigated the effect of inducer type and inducer concentration for inducing nitrilase enzyme from *Nocardia globerula* NHB-2 for the biotransformation of 3-cyanopyridine to nicotinic acid. Further, to optimize the induction, the reaction parameters such as substrate concentration, resting cell concentration and time of conversion were analysed using response surface methodology.

The reusability of the resting cells was determined by immobilizing the cells in agar and used in a packed bed reactor system for the production of nicotinic acid.

Materials and Methods

Microorganism

The microorganism for the nitrilase production *Nocardia* globerula NHB-2 obtained from microbial type culture and gene collection, institute of microbial technology, Chandigarh with accession number MTCC 6278. The culture was maintained in agar slants stored at 4°C. The microorganism was revived using nutrient broth media and allowed to grow for 3 days in 35°C at 160 rpm.

The media containing glucose (1%), peptone (0.5%), beef extract (0.3%), yeast extract (0.1%) (pH 7.5) was used for the production of nitrilase. The nitrilase enzyme production confirmed by the pH indicator plates using 0.02% phenol red indicator with 0.3% acetonitrile as carbon source described [26,27].

Preparation of nitrilase producing cells with various inducers

Preculture was prepared by transferring a single colony of *Nocardia globerula* NHB-2 grown over nutrient agar for 48 hrs at 30°C to 5 mL of the nutrient broth medium and incubated at 30°C, 160 rpm for 24 hrs. This seed culture transferred into 100 ml fresh media and incubated at 30°C, 160 rpm for 24 hrs in 500 ml Erlenmeyer flasks.

After 24 hour incubation, freshly prepared 100 ml media with 0.3% of inducer added and incubated at 30°C, 160 rpm for 24 hrs. Then the inducer added again in the medium after 24 hrs and further incubated for 6 hrs at similar conditions.

For the induction of nitrilase acetonitrile, propionitrile and isobutyronitrile were supplemented in the media with the concentrations of 0.3%, 0.6% and 1%. Each experiment carried out in triplicates with control study.

For the control study, same inoculation procedure followed without adding any inducers In another study, the above-mentioned induction procedure was followed with combined inducers of 0.3% (0.1% acetonitrile, 0.1% propionitrile and 0.1% isobutyronitrile).

Preparation of resting cells

After completion of growth and induction, the cells were harvested using centrifugation at 7000 rpm, 4°C for 15 min. The supernatant was discarded and the cell pellet was washed twice with 0.1 M NaH2PO4/Na2HPO4 buffer (pH 7.5). The cell pellet was then suspended in the same buffer (referred to as resting cells) [28].

Nitrilase assay

2 ml reaction mixture containing resting cells and 3-cyanopyridine was prepared in phosphate buffer (pH 7.5) and incubated at 35°C in a water bath for 20 min. The reaction was quenched by the addition of 2 ml of 0.1N HCl. The reaction mixture was centrifuged at 7000 rpm for 10 min.

The resting cells were used in the control reaction without the presence of substrate. To 1 ml of the clear supernatant, 5.5 ml of buffer solution, 4 ml of salicylate/nitroprusside solution and 2ml of hypochlorite solution is added and incubated for 20 min at 37°C [29].

The absorbance of the blue green coloured solution was measured at 654nm and the nitrilase activity was estimated. One unit of nitrilase activity was defined as that amount of resting cells (mg dry cell=mg DCW) which catalysed the increase of one micromole of ammonia/acid per min by hydrolysis of nitrile under assay conditions.

Optimization of reaction parameters for biotransformation

The optimization of the reaction parameters was performed using response surface methodology software, design expert (version 11.0, Stat-Ease Inc., Minneapolis, USA). Box Behnken experimental design used to provide data modelling for the optimization of variables such as substrate concentration, resting cell concentration and time of conversion. The experimental data were analysed by the response surface regression procedure to fit the following quadratic model.

$$Y=\beta_0+\sum_{i=1}^{N}\beta_i X_i+\sum_{i=1}^{N}\beta_{ii}X_i^2+\sum_{i=1}^{N-1}\sum_{j=i+1}^{N}\beta_{ij}X_{ij}$$

Where Y is the predicted response (nitrilase activity), N is number of variables, Xi is the independent variables, β0 is the intercept term, βi, βii, and βij, are the coefficients of the independent variables. The parameters varied according to the RSM design table with low, high and centre values. The reaction conditions varied and the corresponding nitrilase activity measured as the response shown in Table 1.

Factor	Name	Units	Minimum	Maximum	Coded low	Coded High	Mean	Std. Dev.
X1	Substrate concentration	mM	20	400	-1 ↔ 20.00	+1 ↔ 400.00	210.00	134.35
X2	Resting cell concentration	U/mg DCW	10	50	-1 ↔ 10.00	+1 ↔ 50.00	30.00	14.14
X3	Time of conversion	min	20	120	-1 ↔ 20.00	+1 ↔ 120.00	70.00	35.36

Table 1. *Factors involved in RSM for optimization of reaction of 3-cyanopyridine to nicotinic acid using free resting cells.*

Immobilization of resting cells

The whole resting cells were immobilized using agar entrapment method for enhancing the reusability of the cells. 3% of agar was prepared in normal saline and heated to 110°C and then cooled to 50°C in a water bath. 1.5 mg DCW/mL and 2.5 mg DCW/mL of resting cells were added to the agar and was immediately poured into a petri dish and kept at 5°C for gelling. The agar gel cut into discs of 1 cm diameter and stored in phosphate buffer for further use. Three different substrate concentrations 100 mM, 200 mM, 300 mM of 3-cyanopyridine was studied in the immobilized cell system.

The agar entrapped cells were packed in a column sealed using glass wool and the substrate was recycled with the help of a peristaltic pump (RH-P100S). The rpm was maintained at 12 with elapse time of 0.5 sec. The reaction was continued for 5 hrs and the nitrilase activity was measured for the samples taken after each hour. The storage temperature was varied from 30°C-70°C for 1 hr for both the free and immobilized cells for comparison of the stability of the resting cells.

The reusability of the immobilized cells was evaluated with the substrate concentration of 200 mM 3-cyanopyridine while varying the resting cell concentrations of 1.5 and 2.5 U/mg. The process was repeated 4 times and the nitrilase activity was determined using sodium hypochlorite method and quantified using HPLC.

Analytical methods

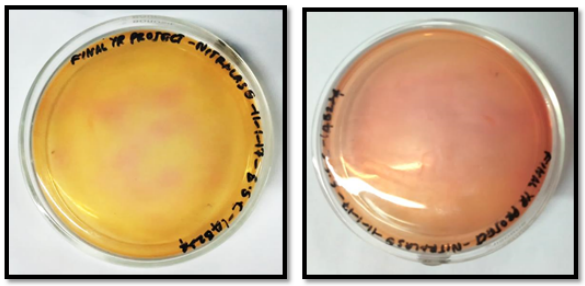
3-Cyanopyridine and nicotinic acid in the reaction mixture were quantitatively analysed by HPLC system (Agilent 1200) equipped with C18 reverse phase column (Waters Reliant, 4.6 mm × 250 mm) at a flow rate of 1 ml min-1 at ambient temperature (23°C) with the elution buffer of composition methanol: acetonitrile: water (20:20:60, v/v). The detection wavelength was set at 216 nm and the injection volume of samples was 20 µl.

Results and Discussion

Growth on phenol red indicator plates

Phenol red acts as an indicator of change in pH, often in cell culture. Its colour shows a gradual change from yellow to red over the 6.8 to 8.2 pH range. Above pH 8.2, phenol red turns into bright pink. The initial pH of the medium was neutral and hence the plates appear yellow in colour (Figure 1a). After 24 h of incubation, the colour of the plates changes from yellow to pink due to the increase in pH (Figure 1b).

Bacterial cultures capable of utilizing acetonitrile as source of carbon and nitrogen result in the release of ammonia. This released ammonia causes increase in the pH of the indicator plate, resulting in colour change of the indicator dye from red to pink (pH 7.0–9.2). The increase in pH is due to the utilization of nitrile compound during bacterial growth which leads to the release of ammonia in the medium. Other indicators such as bromothymol blue, phenolphthalein can also be used for other pH ranges.



a. Before incubation b. After incubation

Figure 1. *Growth of Nocardia globerula NHB-2 on phenol red indicator plates.*

Similar plating method was used by Kumar et al. for screening bacteria capable of degrading aliphatic nitriles. The phenol red indicator plates were used to identify the bacteria Paracoccus species, which is capable of degrading acetonitrile with the release of ammonia. Phenol red shows good colour change from red to pink in the pH range from 7 to 7.8; other dyes take longer for the colour change to be discerned.

Effects of inducer type on nitrilase production

Nitrilases are induced in the presence of a variety of nitrile compounds based on substrate specificity. A large number of nitriles including acetonitrile, benzonitrile, propionitrile, valeronitrile, isobutyronitrileetc are known to induce nitrilase production. In this study, acetonitrile, propionitrile, isobutyronitrile and the three combined were used in 0.3% v/v for inducing nitrilase enzyme production during growth. Among the nitriles used, propionitrile was found to have maximum induction of nitrilase activity (Figure 2).

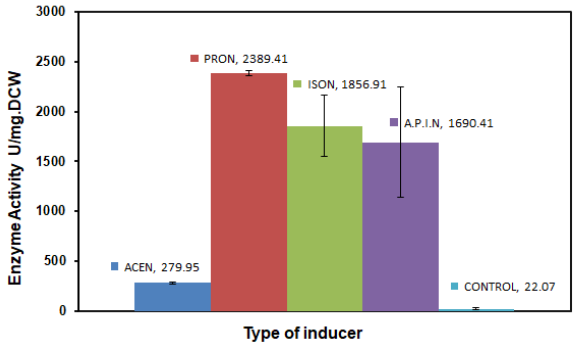


Figure 2. *Effect of various inducers on the production of nitrilase in terms of nitrilase activity in U/mg DCW: ACEN-Acetonitrile (0.3%), PRON: Propionitrile (0.3%), ISON: Isobutyronitrile (0.3%), APIN–Acetonitrile+ Propionitrile +Isobutyronitrile (0.1%+0.1%+0.1%), Control: 0% inducer.*

Bhalla et al. used *N. globerula* NHB-2 for production of nitrilase using several inducers such as acetonitrile, propionitrile, benzonitrile and acrylonitrile. When acetonitrile was added in the nutrient broth as an inducer, the organism exhibited the production of nitrile hydratase amidase activity, which could also hydrolyse aromatic amide (benzamide) in addition to the hydrolysis of saturated nitriles and amide.

While propionitrile was used as an inducer, these cells showed higher affinity for aromatic and unsaturated aliphatic nitrile, although they also hydrolysed saturated nitriles representing broad substrate affinity. Nitrilases of microbial systems vary as far as their affinity towards nitrile substrates is concerned. Some of the nitrilases are specific for aromatic nitriles, while others have affinity for aliphatic nitriles.

Effect of inducer concentration on nitrilase production

The optimal inducer for nitrilase production was found to be propionitrile among other nitriles. The presence of nitrile induces nitrilase production, however high concentration of nitrile may be toxic to the bacterial growth. Therefore, it is necessary to optimize the concentration of nitrile used for induction of nitrilase.

0%, 0.3%, 0.6% and 1% v/v of propionitrile was supplemented in the growth medium for inducing nitrilase production and incubated for 30 hour. The nitrilase activity was determined using sodium hypochlorite method for these inducer concentrations. Propionitrile concentration of 0.3% was found to have maximum induction of nitrilase in *Nocardia globerula* NHB-2 cells.

Varying concentration of isobutyronitrile was used for induction of nitrilase using *Nocardia globerula* NHB-2 by Sharma et al. Three different sets of concentration variation (constant level, exponential level and low exponential level) approach were used to induce nitrilase production.

The highest nitrilase production as well as growth was observed in the set having constant level of propionitrile. Since most of the nitriles are toxic, their presence in high concentration in the growth medium may be inhibitory for nitrilase induction. As the concentration of nitrile increases, the nitrilase activity decreases representing the inhibition of nitrilase induction (Figure 3).

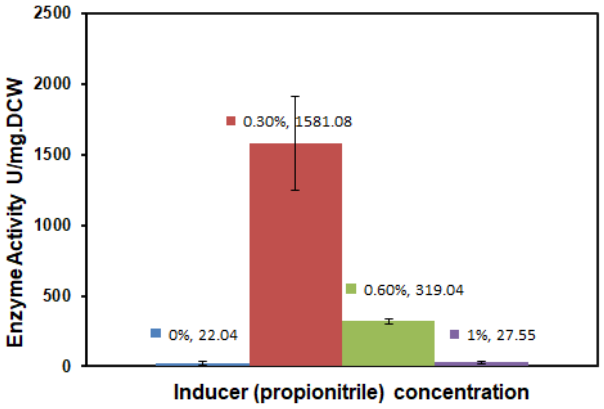


Figure 3. *Effect of inducer (propionitrile) concentration on the production of nitrilase in terms of nitrilase activity in U/mg DCW.*

Optimization of reaction parameters for biotransformation

Three factor Box Behnken design is used to optimize substrate concentration, resting cell concentration and time of conversion. The independent variables and their levels used in the study are shown in Table 1. The design table consisting 17 runs with 5 central points is given in Table 2.

STD	Run	Substrate Conc (mM)	Resting cell conc (U/mg DCW)	Time of conversion (min)	Actual activity (U/mg DCW)	Predicted activity (U/mg DCW)
6	1	400	30	20	12.64	7.18
7	2	20	30	120	15.09	20.55
10	3	210	50	20	17.93	26.1
3	4	20	50	70	23.25	21.75
12	5	210	50	120	6.88	2.92
4	6	400	50	70	7	4.28
13	7	210	30	70	69.96	64.34
14	8	210	30	70	64.19	64.34
9	9	210	10	20	0.3692	4.33

8	10	400	30	120	48.66	55.33
11	11	210	10	120	37.05	28.88
17	12	210	30	70	61.96	64.34
2	13	400	10	70	9.66	11.16
15	14	210	30	70	71.69	64.34
1	15	20	10	70	16.35	19.06
5	16	20	30	20	74	67.33
16	17	210	30	70	53.88	64.34

Table 2. *Box Behnken design matrix for the experimental and predicted results in the production of nitrilase.*

The highest nitrilase activity was measured as 71.68 U/mg DCW for run 14. The obtained experimental data were analyzed by multiple regression analysis by using design-expert software to determine the regression coefficient of quadratic model given below:

Y=+64.34-6.34 X1-1.05 X2+0.3437 X3-2.39 X1X2+23.73 X1X3-11.93 X2X3-14.12 X12-36.16 X22-12.62 X32

Where Y is the nitrilase activity (U/mg DCW), X1 is substrate concentration (mM), X2 is resting cell concentration (U/mg DCW) and X3 is time of conversion (min). The Analysis of Variance (ANOVA) results are summarized in Table 3.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	10771.2	9	1196.8	15.7	0.0007
X1-Substrate conc	321.71	1	321.71	4.22	0.079
X2-Resting cell conc	8.78	1	8.78	0.1152	0.7443
X3-Time of conversion	0.9452	1	0.9452	0.0124	0.9145
X1 X2	22.88	1	22.88	0.3002	0.6008
X1 X3	2253.28	1	2253.28	29.57	0.001
X2 X3	569.4	1	569.4	7.47	0.0292
$X1^2$	838.95	1	838.95	11.01	0.0128
$X2^2$	5504.25	1	5504.25	72.23	< 0.0001
$X3^2$	670.93	1	670.93	8.8	0.0209
Residual	533.46	7	76.21		
Lack of Fit	332.74	3	110.91	2.21	0.2294
Pure Error	200.72	4	50.18		
Cor Total	11304.6	16			

Table 3*. Results of ANOVA for the proposed quadratic model.*

The model F-value of 15.70 implies the model is significant. There is only a 0.07% chance that an F-value this large could occur due to noise. P-values of less than 0.0500 shows that model terms are significant.

In this case X1 X3, X2 X3, $X1^2$, $X2^2$, $X3^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The lack of fit F-value of 2.21 implies the lack of fit is not significant relative to the pure error.

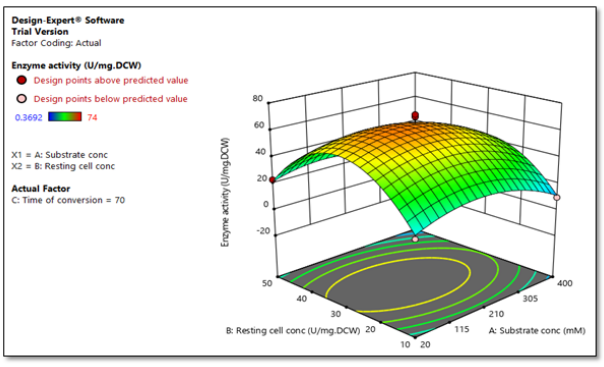


Figure 4. *Response surface plot for the relative activity of nitrilase as a function of substrate concentration and resting cell concentration.*

Figure 4 shows the response surface plot of relative activity change of nitrilase production depending on substrate concentration and resting cell concentration at fixed time of conversion of 70 min. Similarly, the response surface plot for

the relative activity of nitrilase as a function of substrate concentration and time of conversion with constant resting cell concentration of 30 U/mg DCW is shown in Figure 5.

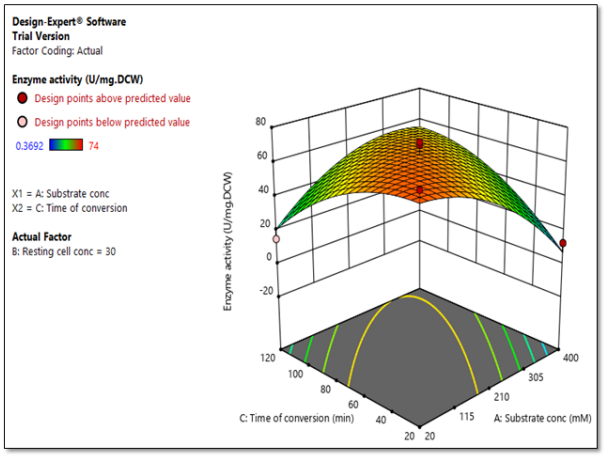


Figure 5. *Response surface plot for the relative activity of nitrilase as a function of substrate concentration and time of conversion.*

The relative activity of nitrilase as a function of resting cell concentration and time of conversion at a constant substrate concentration of 210 mM is shown in Figure 6.

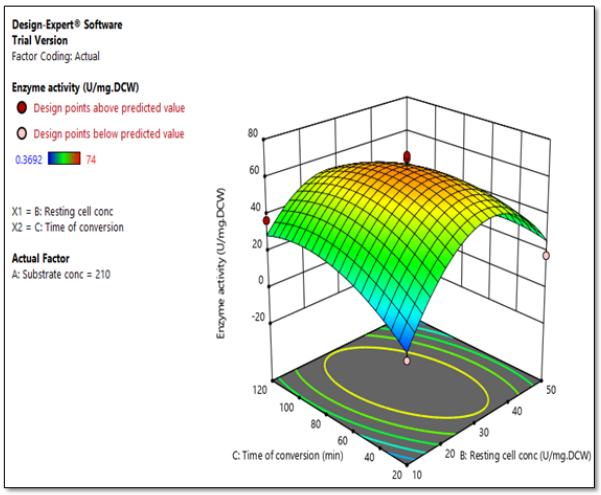


Figure 6. *Response surface plot for the relative activity of nitrilase as a function of resting cell concentration and time of conversion.*

The increase in substrate concentration leads to increase in nitrilase activity however higher concentration of nitrile may lead to substrate inhibition or may be toxic to the bacteria. The substrate concentration and the resting cell concentration vary with respect to each other. The resting cell concentration influences the nitrilase production as it increases due to the availability of the resting cells. Time of conversion varies because of the variation in the initial substrate concentration and resting cell concentration.

As the reaction progresses, the substrate concentration declines gradually and thereby there is a reduction in the nitrilase activity. The optimal nitrilase activity was observed for the substrat concentration of 210 mM, resting cell concentration of 30 U/mg DCW and a time of conversion of 70 min (Runs 7, 8, 12, 14 and 17). Under these conditions, the nitrilase activity ranges from 53-71 U/mg DCW. The optimized parameters from the method were validated using HPLC analysis for the conversion of 3-cyanopyridine to nicotinic acid.

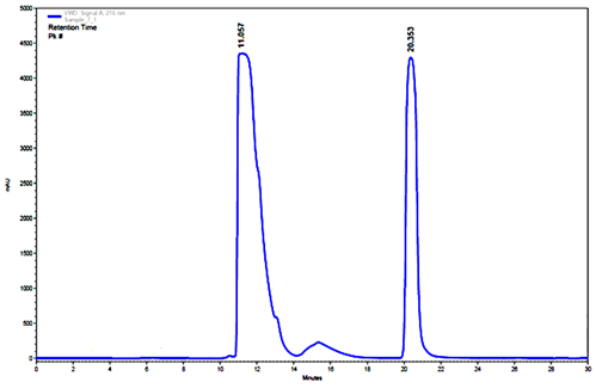


Figure 7. *HPLC analysis profile showing conversion of 3-cyanopyridine to nicotinic acid by the free resting cells.*

77% conversion of 3-cyanopyridine into nicotinic acid was observed for the reaction parameters of 210 mM substrate concentration, 30 U/mg DCW resting cell concentration for a time period of 70 min (Figure 7).

The retention time of nicotinic acid and 3-cyanopyridine were 11.057 min (peak 1) and 20.353 min (peak 2) respectively. The results obtained by the HPLC analysis were in accordance to the results obtained from the statistical tool. The conversion is affected by substrate concentration above 210 mM which leads to the decreasing nitrilase activity.

Immobilization of resting cells

Immobilization of whole cells and purified enzymes can make the biocatalytic process more economical. Compared with the free catalysts, separating the immobilized biocatalysts from the bioconversion reaction mixture is easier, making the repeated batch operations possible and simple. The immobilized and free cells were stored at temperatures ranging from 30°C-70°C for 1 hr. The cells were then assayed for nitrilase activity and are summarized in Table 4.

Temperature (0C)	Free resting cells		Immobilized resting cells	
	OD654 nm	Nitrilase activity (U/mg DCW)	OD654 nm	Nitrilase activity (U/mg DCW)
30	0.0762	75.99	0.0017	1.695
40	0.069	68.8083	0.004	3.9883
50	0.1709	170.416	0.0074	7.3791

60	0.0402	40.083	0.0024	2.3933
70	0.0387	38.5916	0.0015	1.4958

Table 4. *Nitrilase activity of free and immobilized resting cells – thermal stability at various temperatures.*

Both the free and immobilized cells were able to withstand temperature up to 50°C with minimal activity. On further increase in temperature, the nitrilase activity decreased abruptly. This may be due to denaturation of enzyme at temperatures greater than 50°C and *Nocardia globerulais* a mesophilic organism.

The material used for immobilization plays an important role in the thermal stability of the cells. Agar is known to undergo melting in high temperatures which might lead to the loss of activity of the immobilized cells. Immobilization improved the operational stability, and immobilized cells were found to hydrolyse a wider range of nitriles than free cells.

The immobilized cells were used in a packed bed reactor to convert 3-cyanopyridine to nicotinic acid with the release of ammonia. Different concentrations of substrate and resting cells were utilised for the conversion of 3-cyanopyridine and the nitrilase activity was determined.

For each substrate concentration, resting cell concentration of 1.5 U/mg DCW and 2.5U/mg DCW were utilised for a time of conversion of 5 hrs. The nitrilase activity is given as a function of time in Figure 8 for the substrate concentration of 100 mM of 3-cyanopyridine.

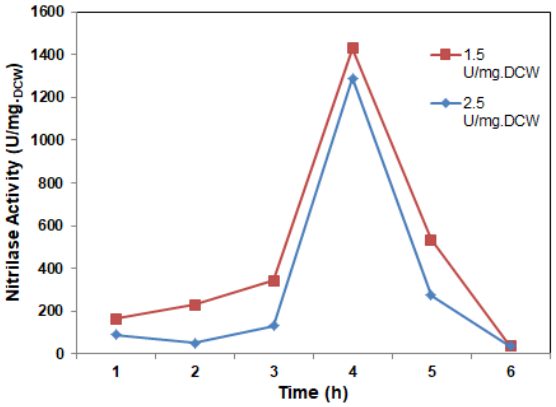


Figure 8. *Immobilized nitrilase activity profile for the concentration of 100 mM 3-cyanopyridine.*

As the time progresses, the nitrilase activity in both 1.5 U/mg DCW and 2.5 U/mg DCW agar entrapped cells were found to increase up to 4th hr. After 4th hr, the nitrilase activity was found to decline gradually which is due to the increase in the concentration of the product leading to the inhibition of nitrilase induction.

Substrate concentration of 200 mM of 3-cyanopyridine was used for the evaluation of nitrilase activity of the immobilized cell system. The nitrilase activity was found to increase with time reaching its maximum at 3rd hr for both cell concentrations (Figure 9).

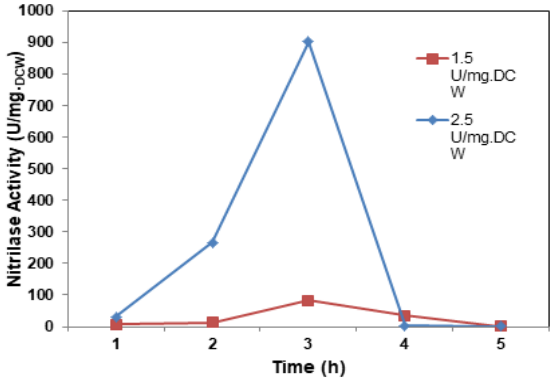


Figure 9. *Immobilized nitrilase activity profile for the concentration of 200 mM 3-cyanopyridine.*

However, compared to the 1.5 U/mg DCW resting cell concentration, the 2.5 U/mg DCW cell concentration was observed to have higher nitrilase activity at 3rd h (901.83 U/mg DCW). The presence of increased amount of resting cell provides enhanced nitrilase activity. For the substrate concentration of 300 mM 3-cyanopyridine, the nitrilase activity profile represents maximal conversion within 2-3 hrs for both of the cell concentrations (Figure 10).

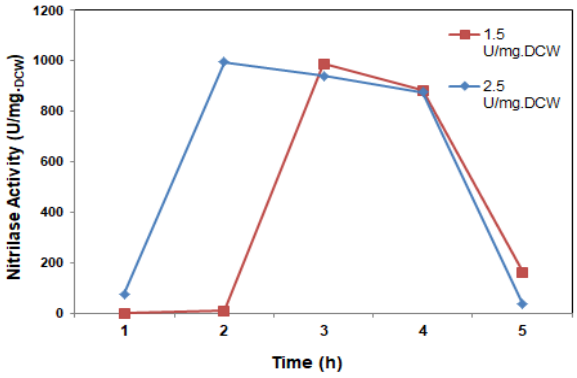


Figure 10. *Immobilized nitrilase activity profile for the concentration of 300 mM 3-cyanopyridine.*

The higher substrate concentration was utilised efficiently by 2.5 U/mg DCW resting cells due to increased availability of nitrilase enzyme for conversion. Immobilization of the resting cells enables to operate at higher substrate concentrations for better conversion efficiency and enhanced reusability.

The reusability was studied to determine the maximum number of times the immobilized cells can be used for the efficient conversion of the substrate. The immobilized resting cells were

reused 4 times for the biotransformation over a period of 3 hrs and the nitrilase activity was evaluated each time. The cells retained the nitrilase activity upto third time of usage. After 3 cycles, the nitrilase activity was found to be reducing indicating that the efficiency of the conversion is affected after continuous usage (Figure 11).

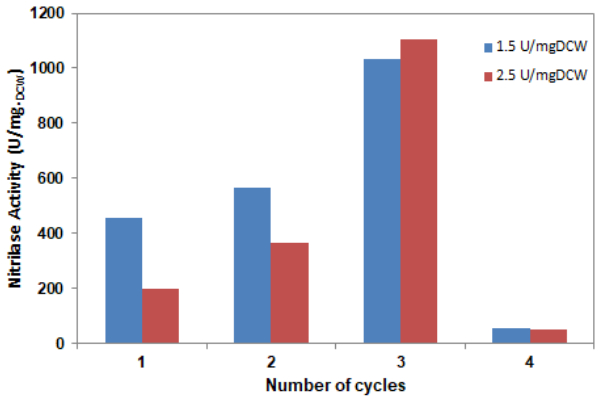


Figure 11. *Nitrilase activity profile showing the reusability of the immobilized resting cell system.*

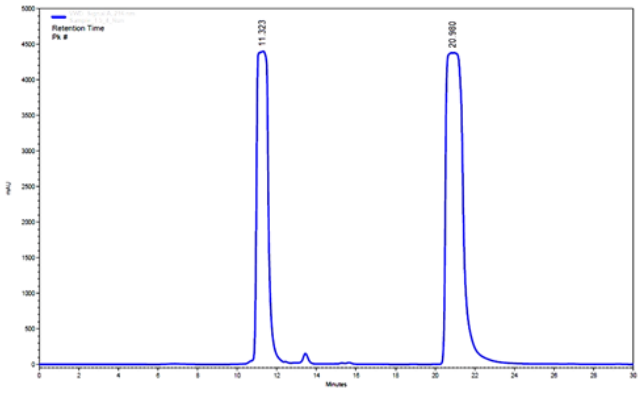


Figure 12. *HPLC profile showing production of nicotinic acid by the immobilized resting cells (1.5 U/mg DCW) after 3rd recycle.*

The residual nitrilase activity considerably decreased after the third recycle which is also validated using HPLC analysis. Figure 12 represents the HPLC profile of the third recycle of 1.5 U/mg DCW resting cells with 200 mM substrate concentration.

40% of conversion of 3-cyanopyridine into nicotinic acid was observed after third recycle. For the resting cell concentration of 2.5 U/mg DCW, 39% of residual activity
was observed after the third recycle (Figure 13).

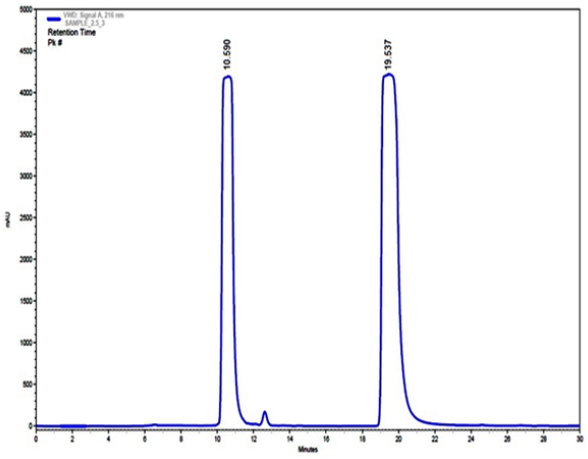


Figure 13. *HPLC profile showing production of nicotinic acid by the immobilized resting cells (2.5 U/mg DCW) after 3rd recycle.*

The residual activity after fourth recycle was found to be 24% which is in order with the results obtained by Raj et al. They reported the reusability of the *N. globerula* NHB-2 cells for the conversion of benzonitrile to benzoic acid. The residual enzyme activity after second, third and fourth recycle was 70%, 45% and 12% respectively. Thus immobilization of the resting cells aid in providing a sustainable technique for enhanced production of nicotinic acid through reusing the cells.

Conclusion

The use of biocatalyst is a solution to green chemistry because of its excellent catalytic efficiency and environmentally benign characteristics. In order to establish such readily available biocatalysts, the microorganism *Nocardia globerula* NHB-2 was grown in specified media. Propionitrile was found to have higher nitrilase activity compared to the other nitriles employed in the growth media. Among the propionitrile concentrations used, 0.3% v/v was found to be optimal for the induction of nitrilase in *Nocardia globerula* NHB-2. The reaction parameters such as substrate concentration, resting cell concentration and time of conversion were optimized using response surface methodology and were found to be 210 mM, 30 U/mg DCW and 70 min respectively. The immobilization of the resting cells was done to overcome the issues of thermal stability, reusability and conversion efficiency of 3-cyanopyridine to nicotinic acid. Immobilized and free resting cells were pre incubated at different temperatures and found to be stable at 50°C. The immobilized resting cells were reused 4 times and were found to be efficient until third recycle with 40% residual activity. The retention time of nicotinic acid and 3-cyanopyridine was found to be 6.233 min and 16.150 min respectively. The nicotinic acid used as additive in pharmaceuticals and food industry can thus be produced using biocatalysts without the harmful effects of chemical method.

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