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EXPLORING THE CATALYTIC POTENTIAL OF NITRILASE FROM ALKALOPHILIC *Rhodococcus pyridinivorans* NIT-36 FOR NITRILE DEGRADATION

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ABSTRACT

An alkalophilic nitrilase-producing strain capable of transforming both aliphatic and aromatic nitriles was isolated from hot-water springs of Tattapani, Himachal Pradesh. On the basis of 16S rDNA gene sequencing this isolate was designated as *Rhodococcus pyridinivorans* NIT-36. The optimization of reaction parameters was performed by using benzonitrile and acrylonitrile as substrates and maximum conversion to respective acids was observed in 0.1M sodium bicarbonate buffer having alkaline pH 10. Whole cell immobilization of *R. pyridinivorans* NIT-36 was carried out in polyacrylamide, sodium alginate and agar as matrix. Highest activity and thermostability was observed for agar immobilized cells and they retained 70% activity even after 5 h.Optimization of reaction conditionsby using response surface methodology led to 3.8 and 1.5 fold increase in nitrilase activity of free and immobilized cells respectively. Further, scale-up studiesresulted in production of 53 g/L of benzoic acid for free cells and and 10g/L for agar immobilized cells. Since *R. pyridinivorans* is capable of degrading nitriles under wide pH range especially in highly alkaline conditions, it is a suitable candidate for industrial applications.

KEYWORDS: ***Acrylonitrile, alkalophilic, benzonitrile, Response Surface Methodology, Rhodococcus pyridinivorans***

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INTRODUCTION

Enzymes from microorganisms thriving in thermophilic sites have received special attention since these enzymes are resistant to high temperatures, extreme pH and chemical reagents in comparison to their mesophilic counterparts.[1] Several industrially important enzymes have been isolated from thermophilic sites like Manikaran and Tattapani Himachal Pradesh, India.[2-5]Nitriles are organic cyanides which are widely distributed in nature and many of them are synthesized chemically.[6]Various chemical and pharmaceutical industries require these nitriles as feedstock for synthesis of a wide range of compounds, drug intermediates, pesticides, polymers and solvents. These nitriles are poisonous and occur as pollutants except the ones which protect against enemies. These compounds can be degraded either chemically or by microbial systems. The nitrile degradation by chemical procedure requires harsh environmentalconditions and it often results in production of many unwanted by-products along with inorganic wastes, hence biotransformation has become a viable alternative route which involves conversion with the help of microorganisms. This not only has greater specificitybut they produce less secondary byproducts along with easy purification of product.[7]It provides environment friendly approach for the synthesis of various carboxylic acids and their corresponding nitrile educts. Biotransformation of nitriles has attracted considerable attention in recent years owing to great potential in organic synthesis. The use of microbial processes in industrial production of chemicals has increased rapidly, due to the high purity of the products, environmental acceptability, and energy savings.[8] Further, it offers a valuable alternative to traditional chemical reactions due to their mild conditions (physiological pH and ambient temperature), environmentally attractive catalysts, high activities and inherent excellent selectivity including chemo, regio and enantioselectivities.[9] Alkalophilic micro organisms offer an opportunity to appreciate the array of possible applications in the field of biotechnology. They not only produce compounds of industrial interest, but they also possess useful physiological properties which can facilitate their exploitation for commercial purposes. However, a major disadvantage of using nitrilases in industrial application is their relative poor stability as the temperature requirement for product synthesis is comparatively higher as compared biotransformation at a small scale. In order to meet the current requirement there is a need to find and develop an ideal catalyst which fits into category constrained by a set of parameters. Thus, enzymatic hydrolysis of nitriles to carboxylic acids or amides play an increasingly important role in organic synthesis and environment remediation, due to the mild reaction conditions, high activities and selectivities of the enzymes.[10] Benzoic acid is an important chemical used as an antimicrobial additive to a wide variety of foods, fruit products, baked goods, drinks, condiments, cheeses and frozen dairy products. The compound is not only recognized for its active role in the food industry, but also has a significant contribution to industrial and pharmaceutical spectrum. It is also converted to caprolactam, a monomer used in the industrial production of nylon fibers.[11]Acrylic acid and its derivatives (esters, salts and amides) are the primary building blocks or monomers in the manufacture of acrylate polymers andco-polymers with numerous applications such as surface coatings, adhesives, artificial teeth, orthopedic cement, sealants, textiles, adsorbents and dispersing agents.[12-14]The present study was carried out to isolate and identify a novel alkalophilic nitrile degrading microbe. The effects of various physico-chemical parameters for the production of acrylic acid and benzoic acid have been analysed. The parameters affecting nitrilase activity have been statistically optimized.

MATERIALS AND METHODS

Soil samples were collected from hot water springs of Tattapani which is situated at an altitude of 1403m in Western Himalayas of Himachal Pradesh, India where maximum temperature was 55°C. The soil samples were brought to laboratory in sterile screw cap bottles and were kept at 4ºC in the research laboratory of Shoolini University for further screening.

Isolation of nitrile hydrolyzing bacteria

Isolation of nitrile degrading bacteria from soil sample was performed by enrichment culture technique where 1gm of soil sample was suspended in sterilized minimal salt medium[15] supplemented with filter sterilized benzonitrile (0.1%) as sole source of carbon and nitrogen. Microbes were isolated by spreading enriched medium on the surface of nutrient agar petriplates and incubating at 37ºC for 24 h. Circular colonies were picked,re-streaked to ensure purity and were subjected to nitrilase assay. Plates of pure isolates having ability to degrade nitrile were stored at 4ºC for further use. The selected isolatewas subjected to molecular characterization for identification.

Molecular characterization based on 16S rRNA gene

The genomic DNA was isolated by alkali lysis method and subsequently amplified by PCR using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTTACGACTT-3'). The product was eluted from gel using a gel extraction kit and identified by 16S rRNA gene sequencing.

Growth conditions

Seed culture of the strain was prepared by transferring a loopful of microorganism from the stock culture to 2ml nutrient broth which was further transferred to 50ml of production medium supplemented with 0.4% (v/v) of propionitrile/acetonitrile as inducer and was incubated at 37°C in an incubator shaker (150 rpm) for 24 h. Cells were harvested by centrifugation and washed twice with 0.1M potassium phosphate buffer (pH 7.0). The resting cells were suspended in the same buffer and further used for hydrolysis of benzonitrile and acrylonitrile to benzoic and acrylic acid respectively.

Enzyme Assay

The amount of ammonia released during the hydrolysis of nitriles to respective acids by nitrilase was estimated according to Fawcett and Scott method.[16] Benzonitrile and acrylonitrile degradation was determined after 24 h of growth using variable amount of resting cells (0.5 to 2.5 mg dcw ml^{-1} reaction mixture) and substrate concentration (90 mM-300 mM). The nitrilase activity

was estimated in various buffers with pH ranging from 3 to 10, viz., 0.1M solutions of sodium acetate (pH 3 to 5), sodium phosphate (pH 6 to 8), sodium borate (pH 8 to 9) and sodium bicarbonate buffer (pH 9 to 10) in the presence of substrates. The assay for the determination thermostability of nitrilase (1h to 6h) was carried out at different temperature ranging from 20°C to 60°C. One unit of nitrilase enzyme activity is defined as the amount of enzyme that catalyzes release of one micromole ammonia in one minute from the prospective substrate under the standard assay conditions.

Immobilization of cells

The immobilization of cells was carried out in three different matrices namely agar-agar, calcium alginate and polyacrylamide.

Immobilization with agar-agar[17]

Cell suspension was mixed aseptically with sterilized agar 1% (w/v) and 0.1 M potassium phosphate buffer (pH 7.0) at 40-50°C. The agar plates were kept at room temperature for solidification. The agar blocks after solidification were washed and stored in buffer at 4°C for further use.

Immobilization with calcium alginate[18]

Cells were mixed with 3% (w/v) aqueous sodium alginate solution was slowly extruded as droplets through syringe, with attached needle no. 20 into beaker with 0.2 M calcium chloride solution which results in instant formation of gel beads. The beads were washed and stored in buffer at 4°C until use.

Immobilization with polyacrylamide[19]

The resting cell suspension (2 ml) was mixed with 3 ml of chilled potassium phosphate buffer. In another tube 1.425g of acryl amide, 0.075g of bis-acrylamide and 5mg ammonium persulphate were added to 5 ml of chilled potassium phosphate buffer (pH 8.5). After mixing the contents properly 50µl of TEMED was added to the second test tube. The contents of two tubes were mixed properly and poured in a glass cavity and were left undisturbed for 1 h so as to facilitate the polymerization process. Subsequently the gel contents were washed and beads of uniform size having 1 cm diameter were cut and stored in buffer at 4°C for further use.

Optimization of reaction parameters and scale up for conversion of benzonitrile to benzoic acid using free cells and agar entrapped resting cells

The reaction conditions were initially optimized for free and immobilized cells by using one variable at a time approach where benzonitrile was used as a substrate. Different variables which were optimized included buffer systems with pH ranging from 3.0-11.0, reaction temperature (30-50°C)and thermostability which was determined by pre-incubating reaction mixture at temperature for 6 h. The time course of conversion of benzonitrile to benzoic acid with free and agar immobilized cells was carried out for 30 minutes and samples were withdrawn after every 5 minutes for enzyme assay. Three different parameters viz. temperature, pH and substrate concentration were expected to have an interactive effect on nitrilase activity. These independent variables were chosen for optimization by RSM using CCD experiments. The experimental design included 20 reactions with three replicates at their central coded value.[20] The statistical software package Design-Expert 8.0.4 (StatEase, Minneapolis, MN) was used for regression analysis of experimental data to obtain working parameters and to generate response surface graphs. The statistical design was evaluated using ANOVA which include F-test, its probability p(F), correlation coefficient which measures the goodness of fit of regression model.

Scale up production of benzoic acid

The conversion of benzonitrile to benzoic acid using free and immobilized cells of *R. pyridinivorans* NIT-36was carried out to check the validity of the method. The reaction was scaled up 0.1 L in a fed batch mode of reaction. The reaction mixture was incubated at 40ºC on incubator shaker. Benzonitrile (200 mM) was added at a regular interval of 15 minutes and the reaction was continued for 6 h. The reaction mixture was centrifuged to separate the free cells and the supernatant was dried at 100ºC.

RESULTS AND DISCUSSION

Nitriles are xenobiotics responsible for pollution of environment and the major role is being played by herbicides like Dichlobenil, Bromoxynil, Ioxynil etc. Nitrilases are capable of degrading nitriles and find a principal application in biodegradation of hazardous aromatic nitriles compounds and results in the production of acids. Nitrilases are highly specific in nature and hence they have been explored for effective degradation of a number of aliphatic and aromatic nitriles. The present study was carried out with the aim of the isolation, identification of nitrile degrading microbe from Tattapani, a thermophilic site in Himachal Pradesh and its utilization in the production of benzoic acid.

Isolation and Identification of microorganism

Out of 110 microbial isolates, 24 isolates exhibiting nitrilase activity were subjected to secondary screening (Table 1) and the most stable isolate was selected for further optimization of process parameters.The colony morphology of the selected isolate showed small raised circular pink colonies on nutrient agar plate which changed to dark pink on storage. The cells were coccus and Gram positive. The 16S rRNA gene sequence of the isolate exhibited 98% similarity with *Rhodococcus pyridinivorans* SB3094 and was designated as *Rhodococcus pyridinivorans* NIT-36. The microorganism was deposited at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh and assigned an accession number MTCC 12617. The cells of *R. pyridinivorans* NIT-36 were inducible in nature as presence of nitrile as an inducer enhanced nitrilase activity.However, nitriles showed inhibitory effect on NHase production and no amidase activity was observed.[21]

Effect of pH, temperature and substrate on nitrile hydrolysis

The free cells of *R.pyridinivorans*NIT-36 were used to study the effect of different buffers namely sodium citrate, sodium phosphate, potassium phosphate, sodium borate and sodium bicarbonate with varying pH

(3-11) on hydrolysis of benzonitrile and acrylonitrile. The isolate showed nitrilase activity over a broad range of pH (6-11) and optimum pH was 10.0 for nitrile degradation. The nitrilase activity increased sharply with the increase of pH from 6.0-9.0 and maximum activity was observed at pH 10.0 (Fig 1a)indicating that the enzyme was active and more stable in alkaline conditions. It can be conferred that it is highly alkalophilic. Similarly, in *R. rhodochrous*and *R. fascians,* maximum enzyme activity was obtained at pH 8.5 and 8.0 respectively[22] and both *Rhodococcus* species were stable over a wide range of pH retaining about 72% and 68% activities at pH 9.5 respectively. However, a narrow pH range was observed for *Rhodococcus rhodochrous* J1[23] in comparison to broad pH ranges exhibited by the nitrilase of *Nocardia* NCIB 11215.[24] Maximum nitrilase activity of free cells of *Fusarium solani* IMI196840[25] and *Klebsiella pneumoniae* sp.[26] was observed at pH 9.0 which indicated that these isolates were alkalophilic in nature. Nitrilases of *Arthobacter* sp strain J1[27] and *Pseudomonas* species S1[28] have shown maximum activity in the pH range of 7-8.Likewise, the bioconversion of benzonitrile and aryrlonitrile was carried out at temperatures ranging from 20-60ºC.An increase in nitrilase activity was observed from 20-40°C (Fig 1b).Maximum enzyme activity was recorded at 40°C which decreased gradually with further increase in temperature. Majority of microorganisms have shown maximum nitrilase activity at temperature ranging from 30ºC-40ºC. An increase in nitrilase activity of *R. rhodochrous* upto 45°C was observed which decreased drastically at 50°C.[22] Similarly, maximum nitrilase activity of *Arthrobacter nitroguajacolicus*[29],*Arthrobacter* sp. strain J1 and *Fusarium oxysporium* sp. was recorded at 40ºC.[30] However, nitrilase of *Acidovorax facilis* 72W[31-32], *Geobacillus pallidus*[33]showed optimum activity at 65ºC which indicates that these are partially thermostable.

Optimization of reaction parameters using free and immobilized cells

Cell immobilization offers numerous technical and economical advantages such as feasibility of continuous processing, lower costs of recovery, recycling and downstreaming process. As a result biotransformation reactions with immobilized microbial cells have become a subject of increasing interest and are used in production of valuable chemicals. The free cells of *R. pyridinivorans* NIT-36 showed greater nitrile degrading potential for aromatic nitriles in comparison to aliphatic nitriles so benzonitrile was used as substrate for immobilization and scale up studies. Further, free and immobilized cells of *R. pyridinivorans* NIT-36 were used to study the effects of various parameters like temperature, substrate concentration, incubation time, pH and thermostability on the nitrilase activity for conversion of benzonitrile to benzoic acid.The free cells were immobilized by using agar, calcium alginate and polyacrylamide as gelling agents. Maximum activity was observed in agar entrapped cells (0.9 U/mgdcw) followed by polyacrylamide entrapped cells (0.07 U/mgdcw) and sodium aliginate entrapped cells (0.03 U/mgdcw). Therefore, agar entrapped cells were used in subsequent experiments. It has been observed earlier that whole cell immobilization of nitrilase of *R. pyridinivorans* NIT-36with chitosan as matrix not only enhanced stability but also reusability of nitrilase[34].

Effect of pH on benzoic acid production

Different buffers sodium citrate, sodium phosphate, potassium phosphate, sodium borate and sodium bicarbonate with pH ranging from (3.0 to 11.0) were used for optimization. Maximum nitrilase activity in free cells (0.6 U/mgdcw) and immobilized cells (1.2 U/mgdcw) was recorded in sodium bicarbonate buffer with pH 10. The enzyme exhibited low enzyme activity in sodium citrate and potassium phosphate buffers. The nitrilase activity increased sharply from 0.3 U/mgdcw to 0.5 U/mgdcw with the increase of pH i.e. 6 to 9 in sodium phosphate and sodium borate. Similar pattern of increasing nitrilase activity with the increase of pH from 6 to 9 was observed in case of immobilized cells which was retained up to pH 11.0 (Fig 2a,b) indicating that the enzyme was more stable and active in alkaline pH. Most of the reported species of *Rhodococcus* harbouring nitrilase enzyme are active at neutral pH.[35-36]

***Thermostability of free and immobilized cells of R. pyridinivorans* NIT-36**

The thermal stability of both free and immobilized cells of *R. pyridinivorans* NIT-36 was determined by pre-incubating cells or beads at varying temperatures ranging from 30-50°C for 1h to 5h. The residual activity was assayed at different time intervals. The nitrilase activity was almost stable at 30°C and at 40°C up to 5 h (Fig 3 a,b). However, a sharp decrease in activity was observed at 50°C which may be attributed to thermal inactivation or denaturation of the enzyme. Agar entrapped cells exhibited better thermostability as compared to free cells and showed higher residual nitrilase activity even after 5 h of incubation. Bioconversion of benzonitrile to benzoic acid using immobilized cells of *Nocardia globerula* NHB-2 which was evaluated by researchers and they observed 100% enzymatic activity at 30°C and a loss of 50% activity at 40°C.[37]

Effect of time course for hydrolysis of benzonitrile

The enzymatic hydrolysis of benzonitrile to benzoic acid using free and agar immobilized cells of *R. pyridinivorans* NIT-36started after 10 min and continued upto 30 min. The samples were assayed after every 5 minute Maximum nitrilase activity of free cells and agar immobilized cells was observed when the cells were incubated for 15 minute followed by a sharp decreased after 25 minutes in both free and immobilized cells (Fig 4 a, b).

Statistical optimization of process parameters for biotransformation of benzonitrile using free cells and immobilized cells of R. pyridinivorans* NIT-36 *using RSM

Optimization by one variable at a time approach results in increase of nitrilase activity. However, it fails to assess the cumulative effect of various physicochemical parameters which lead to the production of enzyme by the microorganism. An experimental design should be used to study the interaction and influence of various operational.[38] RSM is a good statistical tool which uses factorial designs and regression analyses for generating empirical models.[39] RSM has been used for optimization

of process parameters to enhance nitrilase production from *Arthrobacter nitroguajacolicus*[40], *Rhodococcus erythropolis* ZJB-0910[41], *Fusarium proliferatum*[42],*Rhodococcus pyridinivorans* SN2.[43] In order to enhance the growth and enzyme production by aCentral Composite Design of Response Surface Methodology, using different optimization parameters, the growth and enzyme activity was predicted by ANOVA.

Regression model of Response

The interaction of three independent variables (pH, substrate concentration and temperature) was studied for optimization of nitrilase production by free and agar immobilized cell of *R. pyridinivorans* NIT-36 using CCD experiments. In order to determine the optimum concentration of each factor for maximum nitrilase production, the contour plot and three-dimensional response surfaces were plotted. Tables 2 and 6show maximum and minimum levels of variables chosen for trials in the Central Composite design for free and immobilized cells respectively. The optimization of independent variables by RSM based on Central Composite Design consisted of 20 experimental runs for free and immobilized cells respectively, with different combinations of three factors: pH (A), substrate (benzonitrile) concentration (B) and temperature (C) as shown in Tables 3 and 7 along with the results of the effect of three variables along with the mean actual and predicted response from RSM experiments.Regression analysis was performed to fit the response function to the experimental data. The corresponding analysis of variance (ANOVA) of the empirical model obtained along with the values of the coefficient of determination (R^2) and the adjusted determination coefficient (adj.R^2) for free and immobilized cells are represented in Table 4 and 8 respectively, where, the lack-of-fit analysis gave non-significant P-values (>0.05) and F-values lower than the corresponding tabulated F-values, thus proving that the model obtained was highly significant. Tables 5 and 9 showed that the coefficient of determination (R^2) for nitrilase production as a function of the independent variables was 73% for free cells and 75% for immobilized cells which indicate that the model correlated well with measured data and was statistically significant at $P \leq 0.05$.

Table 1
Secondary screening of soil isolates possessing nitrilase activity along with biomass

S. No	Sample number	Dry cell weight (mgdcw/ml)	Specific nitrilase activity (U/mgdcw)
1	12	9.0	0.01
2	16	18.0	0.01
3	22	7.0	0.16
4	32	4.0	0.20
5	**36**	**6.0**	**0.34**
6	46	10.0	0.01
7	53	7.0	0.04
8	58	9.0	0.17
9	64	8.0	0.02
10	65	3.0	0.01
11	68	7.0	0.31
12	70	10.0	0.02
13	73	7.0	0.01
14	86	9.0	0.12
15	87	10.0	0.01
16	88	9.0	0.01
17	90	4.0	0.01
18	91	1.0	0.01
19	92	11.0	0.13
20	93	7.0	0.02
21	94	6.0	0.01
22	95	7.0	0.03
23	98	5.0	0.16
24	102	4.0	0.01

Table 2
Coded values of independent variables at different levels used in Central Composite Design (CCD) for free cells

Independent variables	Symbol	Levels		
		-1	**0**	**+1**
pH	A	7	8.5	10
Substrate concentration (mM)	B	150	200	250
Temperature (°C)	C	30	40	50

Table 3
Actual and predicted values of nitrilase recorded in experimental setup of Response Surface Methodology for free cells

Std	Run	pH	Temperature	Substrate concentration	Actual Value	Predicted Value	Specific Activity (U/mgdcw)
2	1	1	-1	-1	0.050	0.057	0.03
14	2	0	0	1	0.030	0.083	0.01
18	3	0	0	0	0.40	0.28	0.2
11	4	0	0	0	0.40	0.48	0.08
1	5	-1	-1	-1	0.060	0.019	0.05
19	6	0	0	0	0.100	0.20	0.05
17	7	0	0	0	0.060	-0.021	0.05
6	8	1	-1	1	0.30	0.27	0.1
13	9	0	0	-1	6.000E-003	0.11	0.2
12	10	0	1	0	0.50	0.42	0.2
4	11	1	1	-1	0.080	9.136E-003	0.4
3	12	-1	1	-1	0.20	0.30	0.4
20	13	0	0	0	0.20	0.21	0.05
10	14	1	0	0	1.000E-002	0.027	0.5
8	15	1	1	1	0.040	0.10	0.3
7	16	-1	1	1	0.20	0.10	0.06
15	17	0	0	0	0.050	0.10	0.04
9	18	-1	0	0	0.20	0.10	0.006
16	19	0	0	0	0.050	0.10	0.2
5	20	-1	-1	1	0.050	0.10	0.06

Table 4
ANOVA for Response Surface Quadratic Model (Nitrilase Activity) for free cells

Source	Sum of squares	df	Mean square	F-Value	P-value	Prob>F
Model	0.31	9	0.034	3.44	0.0336	Significant
A	0.097	1	0.097	9.72	0.0109	
B	0.084	1	0.084	8.40	0.0159	
C	0.034	1	0.034	3.42	0.0942	
A^2	0.042	1	0.042	4.23	0.0669	
B^2	4.102E-003	1	4.102E-003	0.41	0.5365	
C^2	4.069E-004	1	4.069E-004	0.041	0.8443	
AB	6.050E-003	1	6.050E-003	0.60	0.4549	
AC	0.011	1	0.011	1.12	0.3140	
BC	0.034	1	0.034	3.38	0.0960	
Residual	0.10	10	0.010			
Lack of Fit	0.069	5	0.014	2.22	0.2009	not significant
Pure Error	0.031	5	6.217E-003			
Cor Total	0.41	19				

Table 5
Model fitting values for free cells of R. pyridinivorans NIT-36

Std. Dev.	0.10	R-Squared	0.7561
Mean	0.15	Adj R-Squared	0.5365
Coefficient of variance(%)	67.02	Pred R-Square	-0.4996
PRESS	0.62	Adeq Precision	6.276

Table 6
Coded values of independent variables at different levels used in Central Composite Design (CCD) for agar immobilized cells of of R. pyridinivorans NIT-36

Independent variables	Symbol	Levels		
		-1	0	+1
pH	A	7	8.5	10
Substrate concentration (mM)	B	15	20	25
Temperature (°C)	C	30	40	50

Table 7
Actual and predicted values of nitrilase recorded in experimental setup of Response Surface Methodology for agar immobilized cells and actual values of other responses observed along with nitrilase activity of R. pyridinivorans NIT-36

Std	Run	pH	Temperature	Substrate concentration	Actual Value	Predicted Value	Specific Activity (U/mgdcw)
2	1	1	-1	-1	0.40	0.53	0.4
14	2	0	0	-1	0.60	0.40	0.6
18	3	-1	1	-1	0.30	0.42	0.3
11	4	0	0	0	0.40	0.92	0.4
1	5	-1	1	1	0.26	0.31	0.26
19	6	0	0	0	1.10	0.92	1.1
17	7	0	0	0	0.060	6.080E-003	0.06
6	8	1	1	-1	0.090	0.29	0.09
13	9	0	0	0	1.30	0.92	1.3
12	10	1	-1	1	0.30	0.36	0.3
4	11	1	0	0	0.20	0.068	0.2
3	12	-1	1	1	0.100	0.12	0.1
20	13	1	1	1	7.000E-003	0.058	0.07
10	14	0	0	0	1.40	0.92	1.4
8	15	-1	0	0	0.050	8.580E-003	0.05
7	16	0	0	0	0.90	0.92	0.9
15	17	0	1	0	0.020	- 0.100	0.02
9	18	-1	-1	-1	0.100	0.22	0.1
16	19	0	0	0	0.60	0.92	0.6
5	20	-1	-1	1	0.20	0.17	0.2

Table 8
ANOVA for Response Surface Quadratic Model (Nitrilase Activity) for agar immobilized cells of R. pyridinivorans NIT-36

Source	Sum of Squares	DF	MeanSquare	FValue	P-value Prob > F
Model	2.61	9	0.29	3.05	0.0486 Significant
A	0.011	1	0.011	0.12	0.7385
B	3.511E-003	1	3.511E-003	0.037	0.8513
C	0.079	1	0.079	0.83	0.3836
A^2	1.47	1	1.47	15.51	0.0028
B^2	1.22	1	1.22	12.90	0.0049
C^2	0.68	1	0.68	7.16	0.0232
AB	0.093	1	0.093	0.98	0.3454
AC	1.891E-003	1	1.891E-003	0.020	0.8906
BC	7.381E-003	1	7.381E-003	0.078	0.7860
Residual	0.95	10	0.95		
Lack of Fit	0.17	5	0.035	0.22	0.9365 not significant
Pure Error	0.78	5	0.16		
Cor Total	3.56	19			

Table 9
Model fitting values for immobilized cells of R. pyridinivorans NIT-36

Std. Dev.	0.31	R-Squared	0.7330
Mean	0.42	Adj RSquared	0.4927
C.V.	73.47	Pred R-Square	0.2981
PRESS	2.50	Adeq Precision	4.685

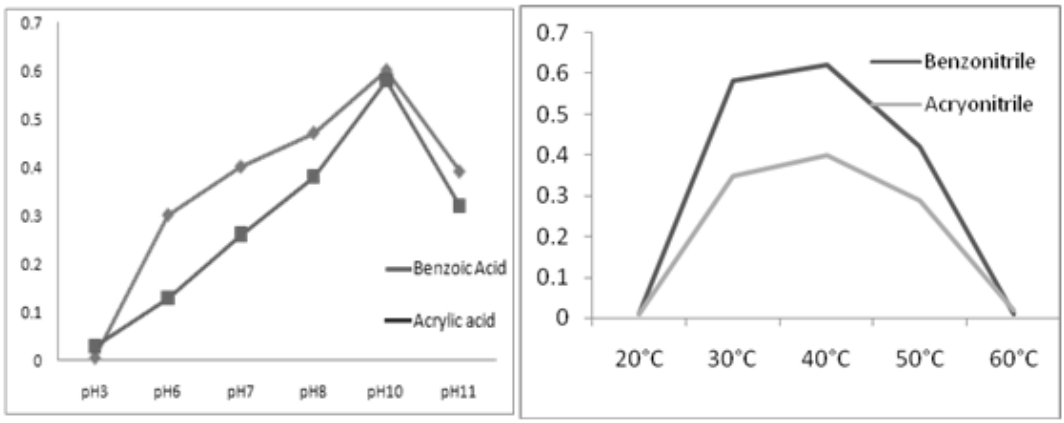
Determination of optimum conditions

The 3D response surface plots were drawn to illustrate the effects of the independent variables on the response variables (Fig 5 and 6). An elliptical nature of the contour plot indicates that the interactions between the independent variables are significant. The optimal values of the independent variables and the interaction between each independent variable's pair could be observed from the 3D response surface plots and the corresponding contour plots.The highest nitrilase activity for free cells was obtained with pH 10 at 40°C and 200 mM substrate concentration (Table 3) and for immobilized cells maximum nitrilase activity was obtained with pH 8.5, substrate concentration 20 mM

and temperature 40°C (Table 7).The optimization of reaction parameters resulted in 3.8 and 1.5 fold increase of enzyme activity free cells and immobilized cells respectively. The results of CCD indicate the significance of pH, temperature and substrate concentration on enzyme activity for free and immobilized cells.

Scale up of benzoic acid

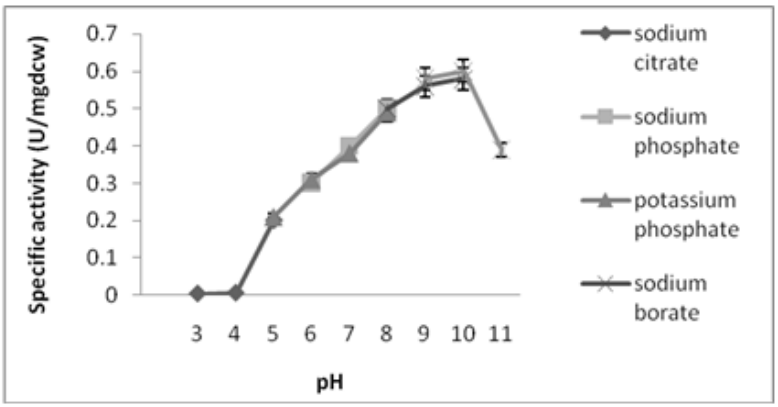
R. pyridinivorans NIT-36 has more affinity for aromatic nitriles in comparison to aliphatic nitriles. Benzonitrile was used as substrate for scale up of benzoic acid to 0.1L at 40°C. Both free and immobilized cells of microbial isolate were used for the production of benzoic acid in fed batch mode of reaction and the reaction was performed at 40°C for 6 h which produced 5.4g and 1g of benzoic acid respectively.



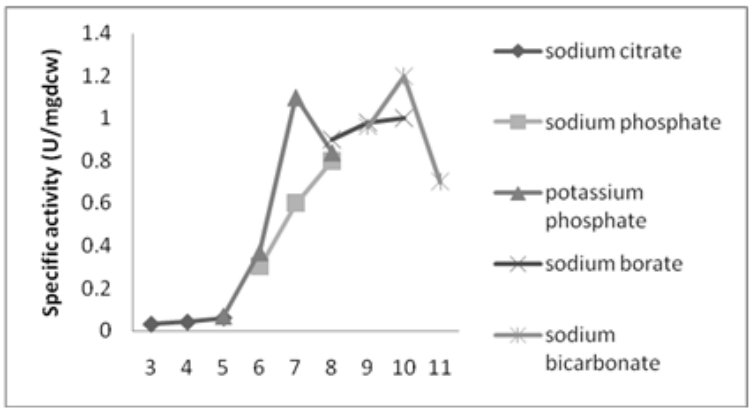
(a) ***pH*** **(b)** ***Temperature***

Figure 1

Effect of a) pH and b) temperature on the conversion of benzonitrile and acrylonitrile using free cells.



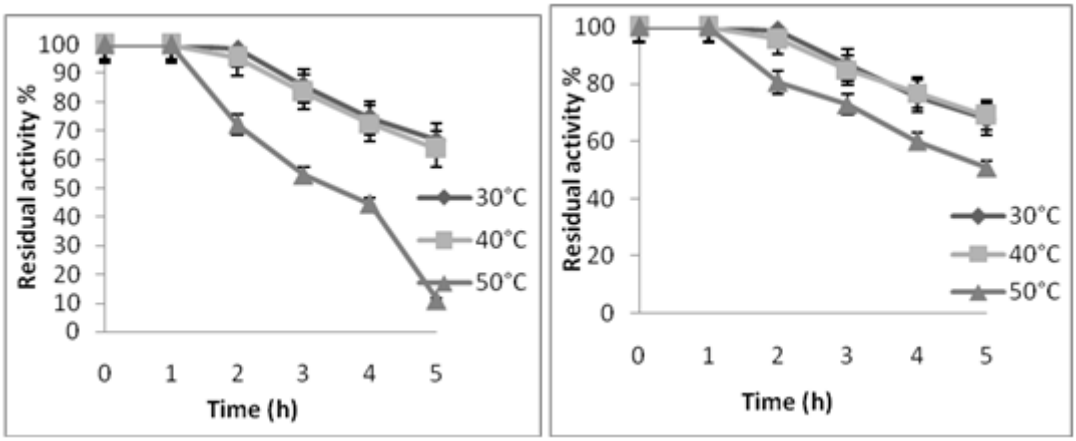
(a)
Free cells



(b)
Immobilized cells

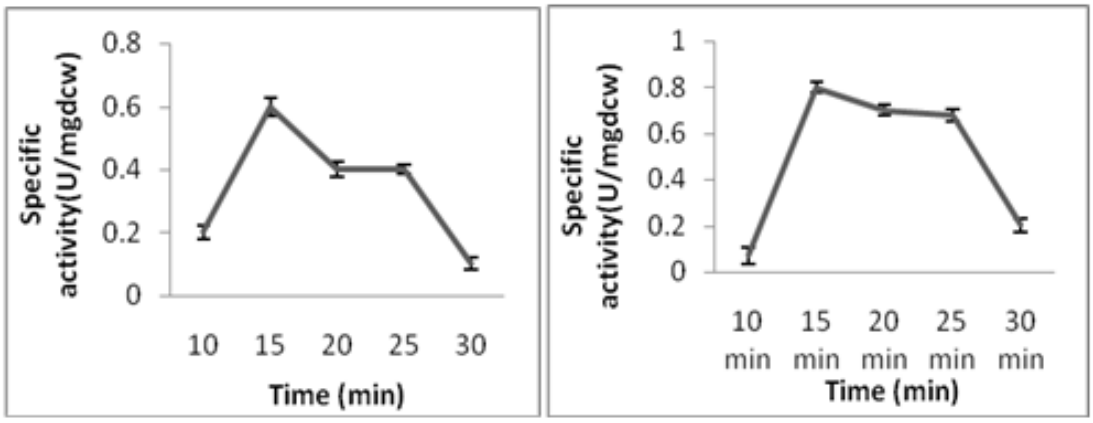
Figure 2

Effect of buffer on benzoic acid production showing optimum production at pH 10. (a) Free cells (b) Immobilized cells.



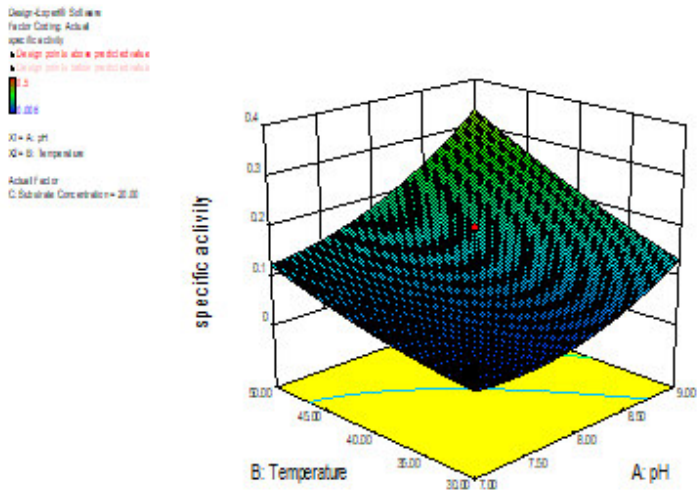
(a) *(b)*
Free cells *Immobilized cells*

Figure 3
Effect of thermostability on the benzoic acid production showing nitrilase stability at 30°C-40°C. a) Free cells b) Immobilized cells.



(a) **(b)**
Free cells ***Immobilized cells***

Figure 4
Effect of time of hydrolysis of benzonitrile a) Free cells b) Immobilized cells.



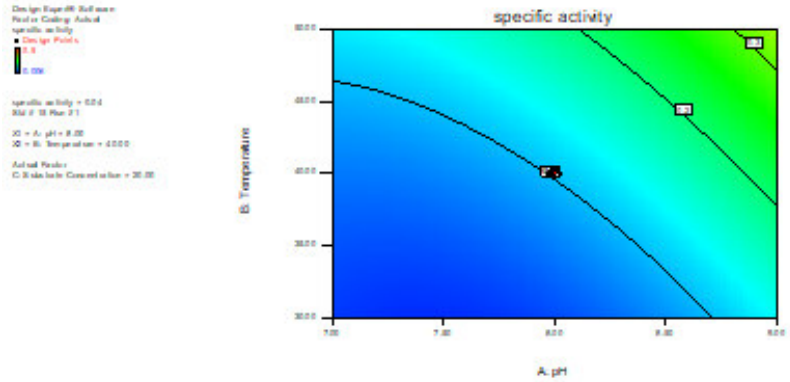


Figure 5
Response surface plot and contour plot of the combined effects of pH, temperature on the nitrilase activity of free cells of R. pyridinivorans NIT-36.

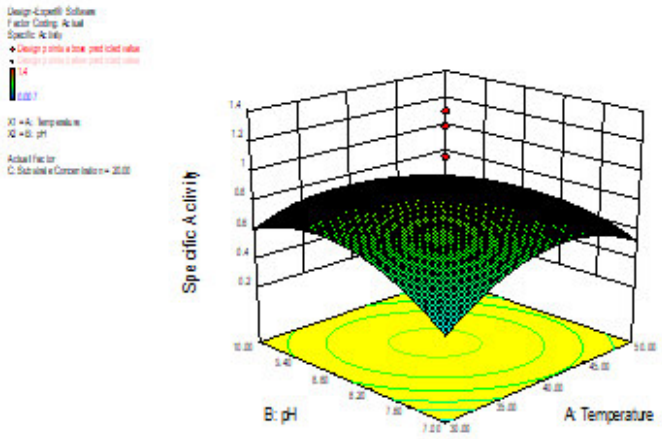


Figure 6
Response surface plot of the combined effects of pH, temperature on the nitrilase activity by agar immobilized cells of R. pyridinivorans NIT-36.

CONCLUSION

A microbial isolate *Rhodococcus pyridinivorans* NIT-36 has been isolated and characterized which exhibited appreciable nitrilase activity over a wide range of pH making it a suitable candidate for industrial applications. The use of agar entrapped cells for the production of benzoic acid can be beneficial as immobilization ofcells enhanced the stability and reusability of nitrilase enzyme making it suitable for biotransformation applications.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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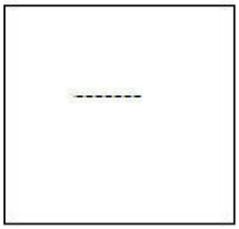

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