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ENHANCED PRODUCTION OF NHASE OF ALKALI STABLE *RHODOCOCCUS PYRIDINIVORANS* NIT 36 AND ITS APPLICATION IN ACRYLAMIDE PRODUCTION

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ABSTRACT

The evaluation of medium components for enhancement of NHase activity by the *Rhodococcus pyridinivorans* NIT-36 bacterial isolate was optimized using Central Composite Design of Response Surface Methodology. Maximum activity was observed in medium having alkaline pH i.e. 9 supplemented with acrylamide as inducer at 37°C for 24 h. Further, the individual and interactive effects of five independent variables i.e. $MgSO_4$, KH_2PO_4, K_2HPO_4, glycerol and yeast extract were studied using Central Composite Design for their enhanced enzyme activity. The model was significant having *p* value less than 0.05. A two fold increase in enzyme activity (6 U/mgdcw) was observed in comparison to the enzyme activity obtained by one factor at a time approach (3 U/mg dcw). Multiple feedings of inducer (acrylamide) at different time intervals appreciably improved growth and activity. The process parameters like buffer pH, temperature and enzyme-substrate ratio further enhanced the enzyme activity to 7.4 U/mg dcw. Various buffer systems of different pH were tested and nitrile hydratase was active over a wide pH range from pH 6.0 to 10.0. Finally bench scale acrylamide production (1L) was carried out by both batch and fed batch methods. The final yield of acrylamide was 50g/L and 60 g/L for batch and fed batch, respectively. The unique feature of this *Rhodococcus* strain is that it gives maximum NHase activity at optimum pH of 9. Another important factor which facilitated high acrylamide production was that amidase

was not activated when acrylamide was used as inducer. This is the first report on acrylamide production at 40°C.

Keywords: Nitrile hydratase (NHase) • Acrylamide • Central composite design • Response surface methodology • Reaction optimization

INTRODUCTION

Compounds containing cyano (C≡N) group belong to the family of nitriles that occur naturally in plants and as intermediates in microbial metabolism [1-4]. They are also used as starting materials for the synthesis of wide range of commercially important amides and acids. The chemical process of nitrile conversion have certain drawbacks such as multistep reaction production of toxic byproducts, very low substrate specificity, require harsh condition (very low or high pH and high temperature) which requires high energy inputs and increase the cost of synthesis [5]. These limitations can be overcome by their enzymatic synthesis.

Nitrile hydratase (E.C.4.2.1.84) is a potential biocatalyst which perform bioconversion of nitriles into amides. Bacteria such as *Arthrobacter* [6], *Bacillus* [7,8], *Corynebacterium* [9,10], *Pseudonocardia* [11], *Rhodococcus* [12–14], and *Pseudomonas* [15] contain NHase activity, which can be utilized for the synthesis of various amides [4].

Acrylamide used as monomer for the preparation of synthetic polymers, which are employed as flocculants in sewage treatment, textile sizers, additives for enhanced oil recovery and many other uses on industrial scale. The chemical synthesis requires hydration of acrylonitrile with sulfuric acid. This process leads to the formation of by products, difficult recovery of product [16]. This problem overcomes by the microbial bioconversion of Acrylonitrile to acrylamide.

Several microbes can convert acrylonitrile to acrylamide involving NHase viz., *Rhodococcus* sp. N-774 *Pseudomonas chlororaphis* B23, *Bervibacterium* R312, *Rhodococcus rhodochrous* J1 [17-19], *R. rhodochrous* M33 [20] and *R. rhodochrous* PA-34 [21]. Recently the acrylamide is produced through the hydrolysis of acrylonitrile using immobilized or free resting cells of *R. rhodochrous* J1 or M33 at an industrial level [19,22].

In microorganisms that catabolyze nitriles by NHase, this enzyme, if inducible, is generally induced by amides (reaction products), not by nitriles (reaction substrates) [23].

In the present study, nitrile degrading organism was identified as *R.*

pyridinivorans NIT 36. The NHase activity of this organism has not been utilized for the production of acrylamide earlier. The culture conditions and the process parameters for the production of acrylamide were optimized using CCD of RSM which involves a minimum number of experiments for large number of factors resulting in improved enzyme production. The unique feature of the NHase of this *Rhodococcus* strain is that it stable over a wide pH range making it highly suitable for production of industrially important amides. High acrylamide production was also attained because of high NHase and low amidase activity in presence of acrylamide as inducer. This facilitated the reaction even at higher temperatures.

MATERIALS AND METHODS

Microorganism and growth medium

A microbial isolate *R. pyridinivorans* NIT-36 was isolated from the hot water spring of Tattapani (HP). It was identified according to morphological and biochemical characteristics followed by 16S rDNA gene sequencing. The bacterial culture was maintained on nutrient agar plates for further experimental work.

The growth medium (g/l): Yeast Extract: 3.0; K_2HPO_4:0.5; KH_2PO_4: 0.5; $MgSO_4$.$7H_2O$: 0.5; $CoCl_2$: 0.01 and 0.1% acrylamide used as inducer. Seed culture was prepared by inoculating nutrient broth (without inducer) with microbial isolate and incubated in an incubator shaker at 160 rpm for 24 hours at 37°C and 4% of seed culture (1 O.D) was further transferred to the above mentioned production medium (with 0.1% inducer) and incubated for 36 hour under same conditions. After cultivation, the cells were harvested and washed twice with 0.1M potassium phosphate buffer and resuspended in same buffer for further use.

Characterization of the microbial isolate

Molecular characterization

16S rDNA amplification and sequencing

The chromosomal DNA was isolated by alkali lysis method and subsequently amplified by PCR using the universal forward and reverse primers 5' AGAGTTTGATCCTGGCTCAG3' and 5'ACGGCTACCTTGTTACGACT3', respectively. The strain was identified by 16S rDNA sequence comparison. The PCR reactions were performed at 25µl scale under following conditions: DNA-1µl, 16s forward primer and reverse primer-1µl (20pmol) 1µl (20pmol), 10× Taq DNA polymerase assay buffer, Taq DNA polymerase- 1µl. A total of 25 cycles was performed with initial denaturation of 96°C for 5 min., denaturation of 96°C for 30 sec, hybridization of 50°C for 30 sec and final elongation of 60°C for 1.30 min. The amplified product was analyzed on 0.8%

agarose gel. The product was eluted from gel using a gel extraction kit and then sequenced.

Enzyme assay

NHase activity was assayed at 40°C in a reaction mixture (2ml) consist of 335mM acrylonitrile, 0.1M phosphate buffer at 7 pH and an 200µl of resting cells mixture was added. The reaction was stopped by adding 1ml of 0.1 N HCl after 15 minutes. Acrylamide formation was estimated with UV – VIS spectrophotometer at 224 nm and confirmed by HPLC [24, 25].

Analytical method

The amount of acrylamide formed was assayed by HPLC with Agilent Technologies reverse – phase column INNOVAL, C18, 5µm, 100 A°, 4.6 × 250mm at a flow rate of 0.6 ml/min at 30°C. The solvent system used consisted of 20% methanol and 80% HPLC grade water and 5µl amount of sample injected. The conversion was detected with PDA detector at λ=240nm. The Retention time 5.247 minutes was observed for the production of acrylamide

Response surface methodology

Culture conditions

Using the 'one variable at a time approach', experiment was conducted to study the effect of media types, carbon source, nitrogen source, temperature, incubation time, inoculum size, pH and inducer concentration. Based on these experimental data, five independent variables were selected for further optimization by using RSM. Individual variables were studied at three levels (-1, 0, +1). The experimental design consists of 32 runs. A total of three replicate runs with their centrally coded values were performed. The statistical analysis of the present model was analyzed using analysis of variance technique (ANOVA).

Reaction conditions

On the basis of preliminary experiments, four different parameters were chosen (pH, temperature, substrate concentration, cell volume) which were expected to have significant effect on acrylamide production. Five levels of these individuals were studied using RSM. The experiments were designed using EXPERT DX8 in order to study the effect of different temperature, pH, substrate concentration and cell volume on the bioconversion of acrylonitrile to acrylamide. The statistical design was analyzed using ANOVA which include F-test, its probability p(F), correlation coefficient which measures the goodness of fit for regression model.

Table-1 Actual and Predicted responses of RSM experiments of culture conditions for NHase production

Run	Y.E.	K_2HPO_4	KH_2PO_4	$MgSO_4$	Glycerol	NHase activity (actual)	NHase activity (predicted)
1	0.35	0.06	0.06	0.06	1.0	1.718	2.241
2	0.60	0.10	0.01	0.01	3.00	0.747	0.744
3	0.35	0.06	0.06	0.06	3.00	1.972	1.391
4	0.60	0.01	0.01	0.10	3.00	0.006	0.197
5	0.60	0.01	0.10	0.10	1.00	2.144	2.280
6	0.60	0.01	0.01	0.01	1.00	1.511	1.261
7	0.60	0.06	0.06	0.06	2.00	0.883	0.354
8	0.35	0.06	0.06	0.06	2.00	1.21	2.147
9	0.35	0.06	0.01	0.06	2.00	3	3.372
10	0.10	0.06	0.06	0.06	2.00	0.452	0.923
11	0.35	0.06	0.06	0.10	2.00	3.42	2.247
12	0.35	0.10	0.06	0.06	2.00	1.9	1.434
13	0.60	0.01	0.10	0.01	3.00	1.2	1.188
14	0.60	0.10	0.01	0.10	1.00	0.595	0.740
15	0.10	0.10	0.10	0.10	1.00	0.01	0.130
16	0.35	0.06	0.06	0.06	2.00	2.191	2.147
17	0.10	0.01	0.01	0.10	1.00	6.4	6.311
18	0.10	0.10	0.01	0.01	1.00	0.342	0.076
19	0.35	0.01	0.06	0.06	2.00	2.342	2.749
20	0.10	0.10	0.01	0.10	3.00	0.283	0.441
21	0.35	0.06	0.06	0.06	2.00	3.196	2.147
22	0.60	0.10	0.10	0.01	1.00	1.335	1.294
23	0.35	0.06	0.06	0.06	2.00	2.3	2.147
24	0.35	0.06	0.10	0.06	2.00	4	3.570
25	0.10	0.01	0.10	0.10	3.00	0.45	0.599
26	0.60	0.10	0.10	0.10	3.00	2.34	2.723
27	0.35	0.06	0.06	0.01	2.00	0.952	2.067
28	0.35	0.06	0.06	0.06	2.00	2.37	2.147
29	0.10	0.01	0.01	0.01	3.00	2.373	2.136
30	0.10	0.01	0.10	0.01	1.00	4.263	3.988
31	0.35	0.06	0.06	0.06	2.00	1.384	2.147
32	0.10	0.10	0.10	0.01	3.00	1.303	1.275

Scale up

The biotransformation of acrylonitrile to acrylamide with resting cells of *Rhodococcus pyridinivorans* NIT 36 was carried out at 1L scale by fed batch and batch mode of reaction. The substrate and cell concentrations were determined based on previous optimization experiments. The reaction mixture containing 730 ml phosphate buffer, 200 ml cells (1.7mg/ml), 7% substrate (v/v) was incubated at 40°C for 6 hours in batch mode. For fed batch mode, the substrate (1575 mM) was added after every 30 minutes to the reaction mixture and continued till 6 hours. The reaction mixture was centrifuged to remove the cells. The supernatant was air dried and acrylamide formation was confirmed by HPLC.

Table-2 Actual and Predicted responses of RSM experiments of reaction conditions for NHase production

Run	pH	Temperature	Substrate Conc.	Cell vol.	Enzyme activity	Enzyme activity
(Actual)		(Predicted)				
1	7.00	20.00	275.00	150.00	3.72	3.745
2	7.00	20.00	475.00	150.00	5.8	5.802
3	7.00	40.00	475.00	150.00	5.1	5.036
4	8.00	30.00	575.00	200.00	6.89	6.929
5	7.00	20.00	275.00	250.00	3.92	3.840
6	8.00	30.00	375.00	300.00	6.42	6.492
7	8.00	30.00	375.00	100.00	4.73	4.823
8	9.00	40.00	475.00	250.00	4.6	4.592
9	10.00	30.00	375.00	200.00	4.326	4.477
10	9.00	20.00	275.00	150.00	5.12	4.970
11	8.00	30.00	375.00	200.00	6	5.988
12	8.00	30.00	175.00	200.00	3.37	3.496
13	7.00	40.00	275.00	250.00	5.49	5.475
14	9.00	20.00	475.00	150.00	6.231	6.263
15	8.00	30.00	375.00	200.00	5.9	5.988
16	9.00	40.00	475.00	150.00	3.12	3.018
17	9.00	40.00	275.00	250.00	3.4	3.216
18	7.00	20.00	475.00	250.00	6.99	7.031
19	7.00	40.00	475.00	250.00	7.648	7.615
20	8.00	30.00	375.00	200.00	5.925	5.988
21	8.00	30.00	375.00	200.00	6.1	5.988
22	9.00	40.00	275.00	150.00	2.8	2.766
23	6.00	30.00	375.00	200.00	6.26	6.275
24	9.00	20.00	275.00	250.00	3.98	4.061
25	8.00	10.00	375.00	200.00	4.05	4.048
26	8.00	30.00	375.00	200.00	6	5.988
27	9.00	20.00	475.00	250.00	6.599	6.487
28	8.00	50.00	375.00	200.00	2.27	2.438
29	8.00	30.00	375.00	200.00	6	5.988
30	7.00	40.00	275.00	150.00	4.1	4.030

RESULTS AND DISCUSSION

Identification of microbial isolate

Biochemical characterization: The microbial strain Nit 36 was initially characterized according to the physiological, morphological and biochemical analysis. The isolated colonies on nutrient agar medium after 24 h of incubation were yellow in color, convex margin, and circular in form with smooth surface. The isolate was gram positive and cells are coccus in shape.

The physiological and biochemical characteristics of the isolate NIT-36 is as follows- positive for catalase, MR, oxidase and citrate and found negative for indole test, starch and VP test.

Phylogenetic study of *R. pyridinivorans* NIT-36 according to 16Sr DNA sequence

16S rDNA sequence from NIT-36 was amplified using universal primers, yielding an amplicon of 1,400 bp. The sequence was analyzed using BLAST and was found to share 99 % homology with *Rhodococcus*

pyridinivorans 16S ribosomal DNA (NR_121768). The next closest homologue was found to be *Rhodococcus pyridinivorans* SB3094 (CP006996.1). The 16s rDNA sequence of *R. pyridinivorans* NIT-36 has been deposited with NCBI having accession number KP055054.

Culture condition optimization

Medium composition is a crucial factor for a microorganism to attain optimum enzymatic activity, therefore by applying 'one factor at a time approach' different media were checked M-I, M-II, M-III, M-IV, M-V [19,26]. The maximum enzyme production (0.88 U/mg dcw) was observed in medium II (Fig-1) supplemented with (g/l) Yeast Extract-3.0, K_2HPO_4-0.5, KH_2PO_4- 0.5, $MgSO_4$.$7H_2O$ -0.5, $CoCl_2$-0.01 which clearly demonstrate the impact of media component on enzyme activity as well as on the ability of strain *R. pyridinivorans* to synthesize the enzyme. However, Pratush *et al.* (26) observed similar growth patterns of mutants of *R. rhodochrous* PA-34 in medium supplemented with glycerol 10.0, peptone 5.0, malt extract 3.0, yeast extract 3.0, $CoCl_2 \cdot 6\ H_2O$ 0.01 and propionitrile 0.2% (v/v).

pH and temperature are crucial factors influencing enzyme activity. Maximum nitrile hydratase activity (2.92 U/mg dcw) was observed at pH 9.0 which indicates that *Rhodococcus pyridinivorans* NIT-36 is highly alkalophilic in nature (Fig 2). The effect of different temperatures ranging from 20°C to 60°C on the production of NHase was studied at pH 9. The enzyme activity (3.1 U/mg dcw) increased till 37°C and then declined sharply as shown in Fig. 3. These readings shows that *R. pyridinivorans* NIT-36 is mesophilic in nature. Most of nitrile degrading microorganisms specially the ones belonging to *Rhodococcus* group are mesophilic in nature and work in the temperature range of 30°C to 35°C. The unique feature of *R. pyridinivorans* NIT-36 which makes it of particular interest is that it is strongly alkalophilic in nature. The other *Rhodococcus* species exhibit growth and activity in the pH range of 7-8. They tend to show reduced growth under alkaline conditions along with negligible nitrile hydratase activity. Glycerol is taken as best carbon source as is reported earlier in literature for production of NHase (Fig 5) [16, 27].

Effect of inoculum size ranging from 4% to 20% on NHase activity was studied. The maximum number of cells and activity (3.01 U/mg dcw) was observed when 10% of seed culture (1 O.D) was used as inoculum in the production medium. However, a decline in enzyme activity was observed when inoculum of

less than or more than 10% was used. The incubation time was observed during 0-72 hour incubation. Maximum enzyme activity (2.9 U/mg dcw) was observed after 24 hours incubation and subsequently declined thereafter.

Acrylamide is observed as best enhancer of NHase which was also shown by [19], where they have used methacrylamide as NHase inducer. However, in contrast to this Bhalla et al [21] found propionitrile as best inducer for acrylamide production.

Table 3: Inducer feeding for hyperinduction of Nitrile hydratase

Experiment	Acrylamide feeding (v/v)	Growth (mg dcw/ml)	NHase Activity U/mg dcw
Control 1	-	3.0	0.03
Control 2	0 h : 0.2%	3.2	5.2
Set 1	0 h : 0.3% 12 h : 0.6% 24 h : 1.2% 36h: 1.5%	3.204	3.4
Set 2	0 h : 0.4% 12 h : 0.6% 24 h : 1.2% 36h: 1.8%	3.876	4.2
Set 3	0 h : 0.3% 12 h : 0.4% 24 h : 0.6% 36h : 0.8%	3.304	3.9
Set 4	0 h : 0.1% 12 h : 0.2% 24 h : 0.3% 36h : 0.4%	2.496	3.6
Set 5	0 h : 0.3% 12 h : 0.6% 24 h : 1.0% 36h : 1.2%	3.006	5.7
Set 6	0 h : 0.3% 12 h : 0.6% 24 h : 1.4% 36h : 1.8%	3.284	5.8
Set 7	0 h : 0.2% 12 h : 00 24 h : 0.2% 36h: 0.4%	3.5	6.5

Hyperinduction of NHase of *Rhodococcus pyridinivorans* NIT-36

To enhance the NHase activity various sets of experiments were performed for *Rhodococcus pyridinivorans* NIT-36. Growth and enzyme activity were monitored till 48 h. Total seven sets of different reactions were performed and two controls were used. In control 1 no inducer was added and in control 2, inducer (0.1%) was added only once. In these experiments different permutations and combinations were analyzed as shown in the Table - 3. The highest activity (6.5 U/mgdcw) was recorded in set 7 where, the first inducer feeding (0.2%) was given at 24 h and was

followed by second feeding (0.4%) at 36 h. The culture was incubated up to 48 h. In the set 7 experiment the highest activity was recorded at 36 h and further incubation led to reduced activity. The prolonged incubation may have resulted in less enzyme production and more cell production, hence an overall decrease in specific activity was observed beyond 36 h. Therefore for the subsequent experiments the cells were allowed to grow for 36 h only. These hyperinduced cells were eventually used for further scale up studies.

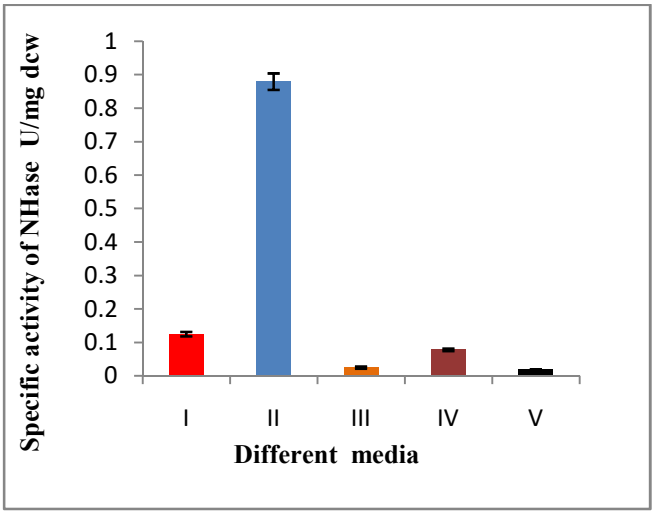


Fig. 1: Effect of media for the production of Nitrile hydratase by *Rhodococcus pyridinivorans* NIT-36. Each value shows the mean of three replicates error bars standard deviation.

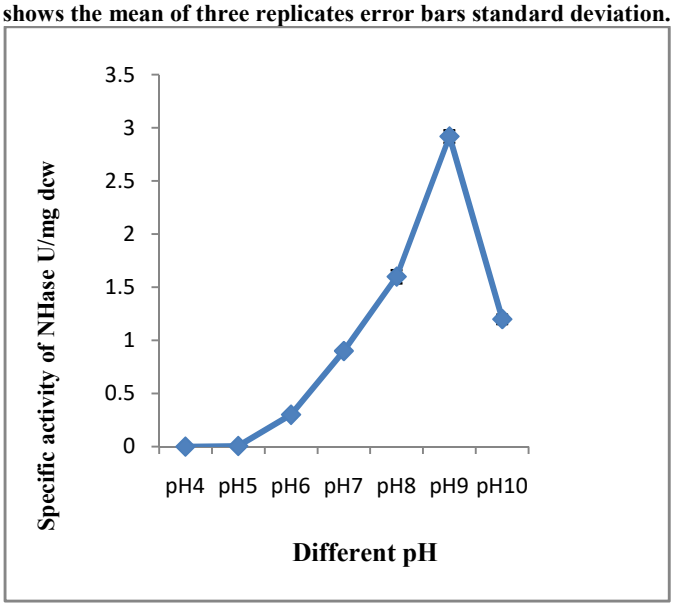


Fig. 2: Effect of pH on the production of Nitrile Hydratase by *R. pyridinivorans* NIT-36. Each values shows the mean of three replicates error bars standard deviation

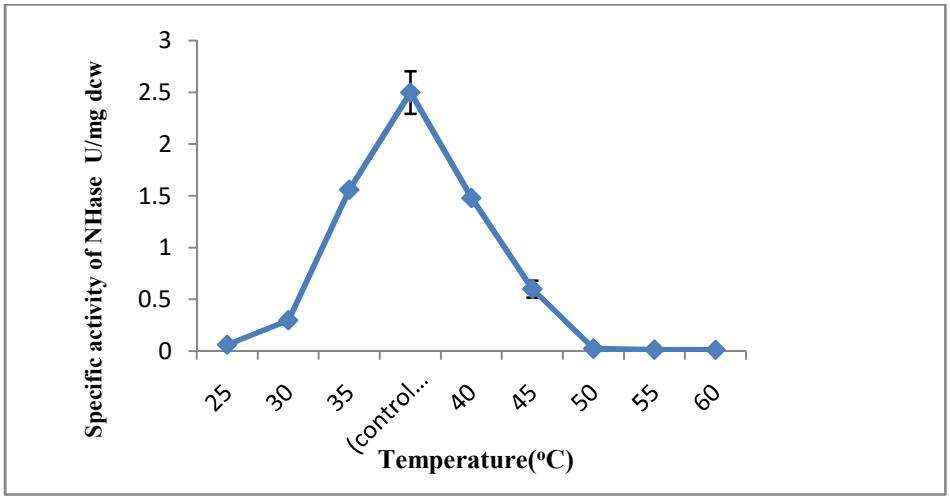


Fig: 3- The effect of different temperature on the production of nitrile hydratase by *R. pyridinivorans* NIT-36. Each value shows the mean of three replicates with error bars standard deviation.

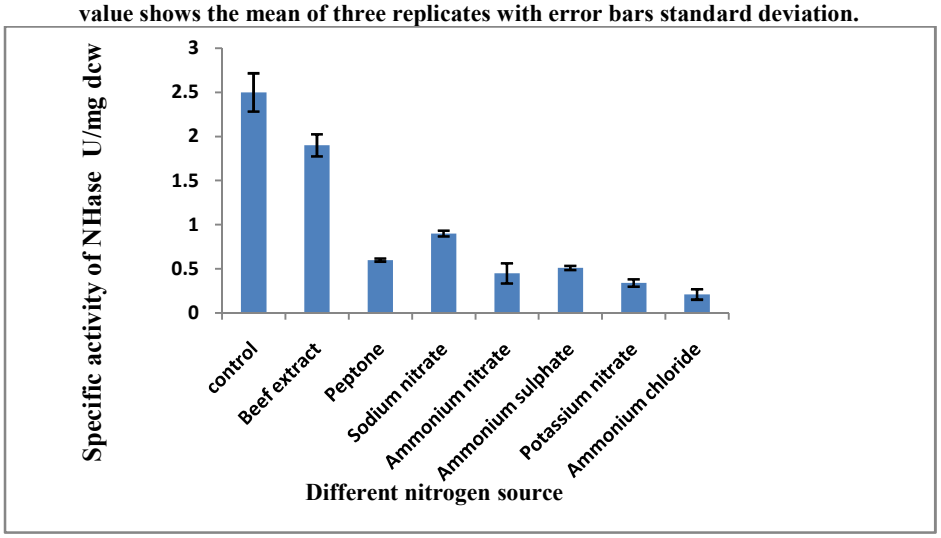


Fig. 4: Effect of nitrogen source on the production of Nitrile Hydratase by *R. pyridinivorans* NIT-36. Each value shows the mean of three replicates. Error bars standard deviation

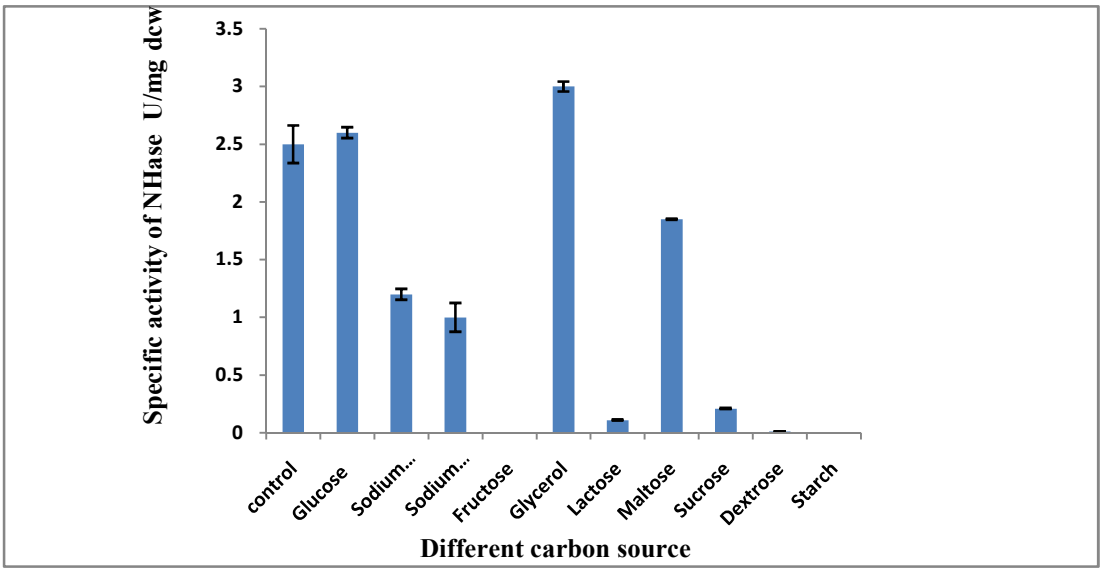


Fig. 5 Effect of different carbon sources on the production of Nitrile hydratase by *R.pyridinivorans* NIT -36. Each value shows the mean of three replicates. Error bars standard deviation.

Nitrile Hydratase optimization using Response Surface Methodology

In order to perform RSM, prior knowledge of various factors like cultivation conditions and other parameters is necessary. A total of five parameters were selected as independent variables for RSM. The interaction of five independent variables (Yeast extract, $MgSO_4$, KH_2PO_4, K_2HPO_4 and glycerol concentration) was studied using RSM for further optimization of NHase production statistically. The results of 32 experiments with 6 replicates of their center coded values, mean of actual and predicted response shown in Table 1 and Fig 7. To fit the response results to the experimental data, regression analysis was done. The regression coefficient of determination (R^2) for NHase production as a function of the independent variables was found to be 87 %, which showed that the model correlated well with measured data and was statistically significant ($P \leq 0.05$). Multiple regression analysis was applied to experimental data to obtain following polynomial equation.

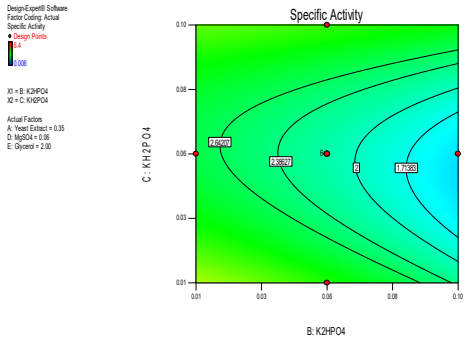
Response (Nitrile Hydratase activity):

6.44301+6.79087* A-78.24387* B-92.25449* C+20.02131 * D-0.43644 * E+65.02222 * A * B+41.84444 * A * C+7.94444 * A * D +1.32850 * A* E+162.16049 * B* C -4.69136 * B* D+17.61111 * B* E-168.76543 * C* D+4.13889 * C* E-5.89167 * D* E -24.14164 * A^2-27.33461 * B^2+653.65304 * C^2 +4.76415 * D^2-0.33135 * E^2

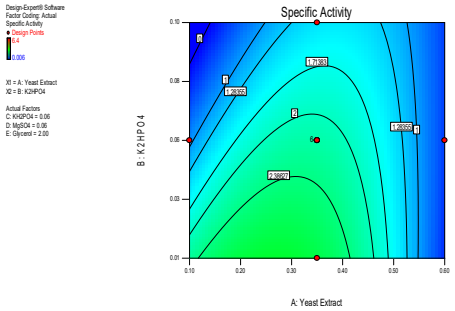
The individual effect of B, A^2, C^2 and the interactive effect of AB, AC, BE found to be the significant terms of present model. Where, A correspond to Yeast Extract concentration, B is level of K_2HPO_4, C is concentration of KH_2PO_4, D is $MgSO_4$, and E is the concentration of glycerol. After eliminating the non significant values from polynomial equation, the final equation as follows:

Response=

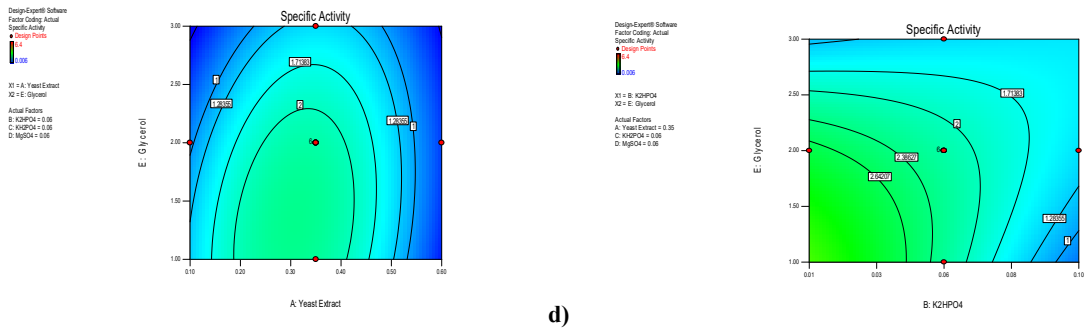
6.44301+6.79087-78.24387* B-92.25449+20.02131 -0.43644 +65.02222 * A * B+41.84444 * A * C+7.94444 +1.32850 +162.16049 -4.69136 +17.61111 * B* E-168.76543 +4.13889 -5.89167 -24.14164 * A^2-27.33461 * +653.65304 * C^2 +4.76415 -0.33135



a)



b)



c) d)

Fig- 6-a) to d) Contour plots showing the effect of the CCD (central composite design) experiments on the production of alkali stable NHase by *Rhodococcus pyridinivorans* NIT-36. a)the interaction between K_2HPo_4 and KH_2Po_4, b) yeast extract and K_2HPO_4, c) Yeast extract and Glycerol and d) K_2HPO_4 and glycerol. The values indicated are the activity of NHase.

The statistical significance of the polynomial equation was studied by applying F-test for Analysis of variance (ANOVA) along with the values of regression coefficient (R^2) and the adjusted coefficient of determination (adj. R^2), which are shown in Table - 4. In all terms the lack of fit analysis gave non significant P- values(>0.05) and F- values was lower then the tabulated value, hence proving the model to be highly significant. The interaction among the each independent variables was investigated according to the regression equation and the contour plots and 3D plot response were represented accordingly.

The effects of various concentration of five independent variables plotted in contour and 3D graphs shown in (Fig-6 and Fig-8).The NHase production in the non optimised medium was 3.0 U/mg dcw which was very low than the maximum actual value obtained by CCD experiments (6.4U/mg dcw) in optimised medium i.e. (Yeast extract- 0.10 %, K_2HPo_4- 0.01 %, KH_2Po4- 0.01 %, $MgSo_4$- 0.10 % and Glycerol- 10%) in Run 17, which is very close to the predicted enzyme activity (6.311 U/mg dcw). A two fold increase was observed with Central composite design incomparison to the enzyme activity obtained by one factor at a time approach.

Statistical experimental designs are gaining great attention of researchers for the optimization of various parameters in order to study their individual and interactive effects [28-31]. Most of the optimization work previously attempted did not include a comprehensive statistical approach. The culture optimization resulted in two fold increase in the enzyme activity. The application of RSM for the production of NHase enzyme gives the rapid approach to identify the important factors, their individual and interactive effects [28].

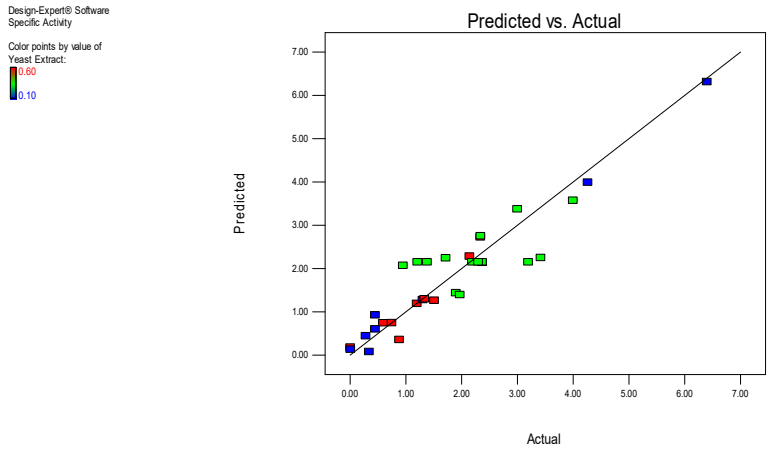


Fig- 7-Graph shows the predicted vs. actual values.

Table- 4 ANOVA (analysis of variance) for Response Surface Quadratic Model for NHase production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	52.27	20	2.61	3.77	0.0139	significant
A-Yeast Extract	1.45	1	1.45	2.10	0.1755	
B-K2HPO4	7.78	1	7.78	11.22	0.0065	
C-KH2PO4	0.18	1	0.18	0.26	0.6227	
D-MgSO4	0.15	1	0.15	0.21	0.6550	
E-Glycerol	3.25	1	3.25	4.68	0.0533	
AB	8.56	1	8.56	12.35	0.0048	
AC	3.55	1	3.55	5.12	0.0450	
AD	0.13	1	0.13	0.18	0.6759	
AE	1.76	1	1.76	2.55	0.1389	
BC	1.73	1	1.73	2.49	0.1429	
BD	1.444E-003	1	1.444E-003	2.083E-003	0.9644	
BE	10.05	1	10.05	14.50	0.0029	
CD	1.87	1	1.87	2.70	0.1289	
CE	0.56	1	0.56	0.80	0.3900	
DE	1.12	1	1.12	1.62	0.2290	
A2	5.60	1	5.60	8.08	0.0160	
B2	7.540E-003	1	7.540E-003	0.011	0.9188	
C2	4.31	1	4.31	6.22	0.0298	
D2	2.290E-004	1	2.290E-004	3.304E-004	0.9858	
E2	0.27	1	0.27	0.39	0.5451	
Residual	7.62	11	0.69			
Lack of Fit	5.00	6	0.83	1.59	0.3148	nonsignificant
Pure Error	2.63	5	0.53			
Cor Total	59.89	31				
R^2	0.8727					
Adj R^2	0.6412					
Pred R^2	-6.7048					
CV	4.54					

The Model F-value of 3.77 implies the model is significant. There is only a 1.39% chance that a "Model F-Value" this large could occur due to noise.Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, AB, AC, BE, A^2, C^2 are significant model terms.Values greater than 0.1000 indicate the model terms are not significant. A negative "Pred R-Squared" implies that the overall mean is a better predictor of your response than the current model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 9.244 indicates an adequate signal. This model can be used to navigate the design space.

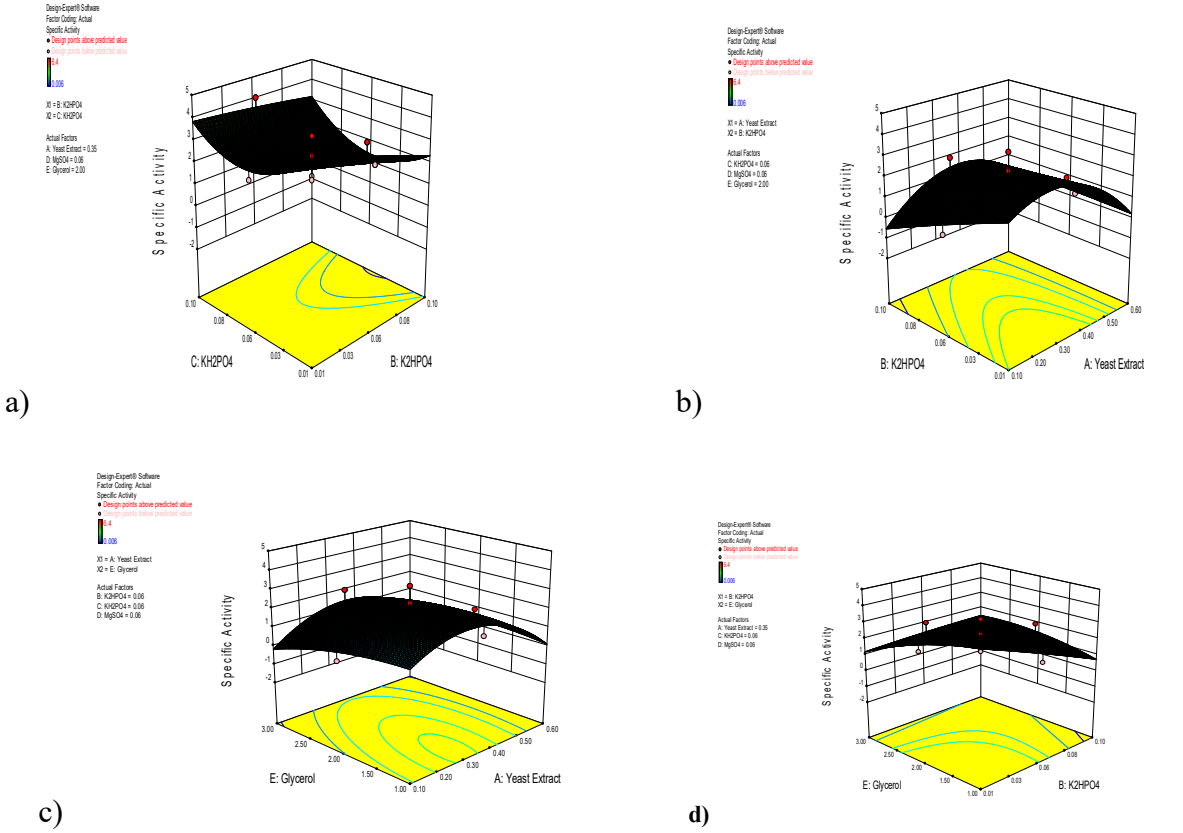


Fig:8- a) to d) 3D response plots of the RSM experiments for highly alkaline NHase by *Rhodococcus pyridinivorans* NIT-36. a) Interaction between K_2HPO_4 and KH_2PO_4, b) Yeast extract and K_2HPO_4, c) Yeast extract and glycerol, and d) K_2HPO_4 and Glycerol

Reaction conditions optimization using RSM

Based upon the preliminary experimental results, the four independent variables were selected (pH, substrate concentration, temperature, cell volume) for CCD. A total of 30 reactions were performed with 6 replicates. To fit the response results of experimental data, regression analysis was performed. After analysis, the polynomial equation generated was given below:

Specific activity = -37.40639 +5.57893 * A +0.83084 * B +0.034938 * C +0.020224 * D -0.061988 *A * B-1.91125E-003 * A * C -5.02500E-003 * A * D -2.62625E-004 * B * C +6.75000E-004 * B * D +5.67000E-005 * C * D - 0.15294 * A^2 -6.86188E-003 * B^2-1.93688E-005 * C^2 -3.29750E-005* D^2

The individual effect of all terms A, B, C, D, A^2, B^2, C^2, D^2 and interactive effect of AB, AC, AD, BC, BD, CD found to be the significant terms. Where A, corresponds to the varying pH, B is the temperature, C is the substrate concentration and D is the cell volume. The second order response surface model was checked using F- test, its associated probability and R-square, which

measures the effectiveness of fit for regression model. The model F-value of 279.97 and the model P- value < 0.0001 shows the model was significant. The lack of fit F-value was 3.83 and the corresponding P- value 0.0758 represent the non-significant Lack of fit. Lack of fit is the variation of the data of fitted model. If the model does not fit the data well, the Lack of fit will be significant.

The corresponding analysis of variance (ANOVA) of the quadratic model along with the regression coefficient (R^2), adjusted R^2 and the predicted R^2 are given in Table 5. The F values which was below the tabulated F-values indicate that the model is highly significant. The significantly high value of R^2 (0.99%) shows the fitness of the model, which explain around 99% of the total variation in the responses. These results indicate that the quadratic model was appropriate to fit the experimental data satisfactorily for acrylamide production. The effect of varying the values of different variables in different combinations are shown in Fig. 9 and 10, which represents the response surfaces of four different combinations, and which are similar to each other. According to the quadratic model, the optimum levels of four different variables are pH-7, temperature - 40°C, substrate concentration 475mM, Cell volume-250µl (1.7mg/ml). The results was confirmed by HPLC with 97% purity.

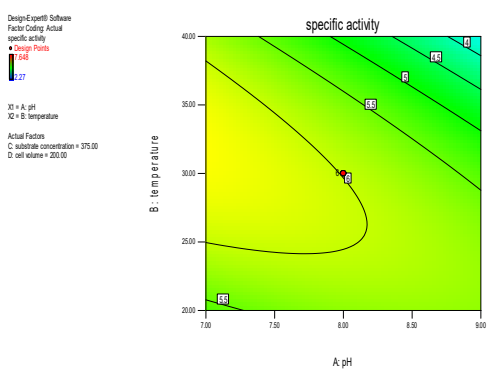
NHase producing microbes mostly show optimum activity at neutral pH. Some of these *Rhodococcus* species which are mesophiles and work in neutral pH range include *Rhodococcus sp.* N-774 (32), *R. rhodochrous* PA-34 (21) and *R. rhodochrous* J1(33). Whereas *Rhodococcus pyridinivorans* NIT-36 was active over a wide pH range from pH 6.0 to 10.0, which shows its enhanced suitability for scale up operations.

In reaction conditions, the optimum temperature observed was 40°C whereas, in similar studies conducted by Keiichi et al. 1987 [17], the NHase activity from *Rhodococcus* sp. N-774 was inactivated at or above 40°C.

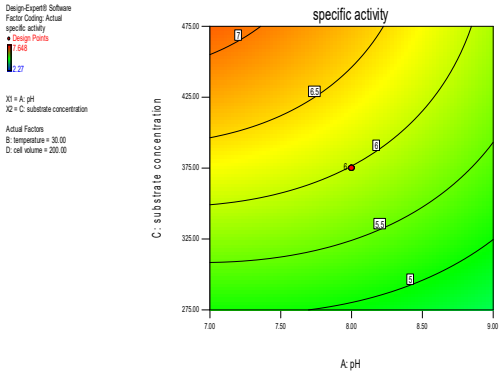
Table- 5 Analysis of variance(ANOVA) for quadratic model of reactions conditions.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	55.80	14	3.99	279.97	< 0.0001significant
A-pH	4.85	1	4.85	340.48	<0.0001
B-temperature	3.89	1	3.89	273.21	<0.0001
C-substrate concen.	17.68	1	17.68	1241.69	<0.0001
D-cell volume	4.18	1	4.18	293.60	<0.0001
AB	6.15	1	6.15	431.82	<0.0001
AC	0.58	1	0.58	41.05	<0.0001
AD	1.01	1	1.01	70.94	<0.0001
BC	1.10	1	1.10	77.51	<0.0001
BD	1.82	1	1.82	128.01	<0.0001
CD	1.29	1	1.29	90.32	<0.0001
A^2	0.64	1	0.64	45.06	<0.0001
B^2	12.91	1	12.91	907.12	<0.0001
C^2	1.03	1	1.03	72.27	<0.0001
D^2	0.19	1	0.19	13.09	0.0025
Residual	0.21	15	0.014		
Lack of Fit	0.19	10	0.019	3.83	0.0758 notsignificant
Pure Error	0.025	5	4.937E-003		
Cor Total	56.02	29			
R^2	0.9962				
Adj. R^2	0.9926				
Pred R^2	0.9799				

The Model F-value of 279.97 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 3.83 implies there is a 7.58% chance that a "Lack of Fit F-value" this large could occur due to noise. The "Pred R-Squared" of 0.9799 is in reasonable agreement with the "Adj R-Squared" of 0.9926. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 61.368 indicates an adequate signal. This model can be used to navigate the design space.



a)



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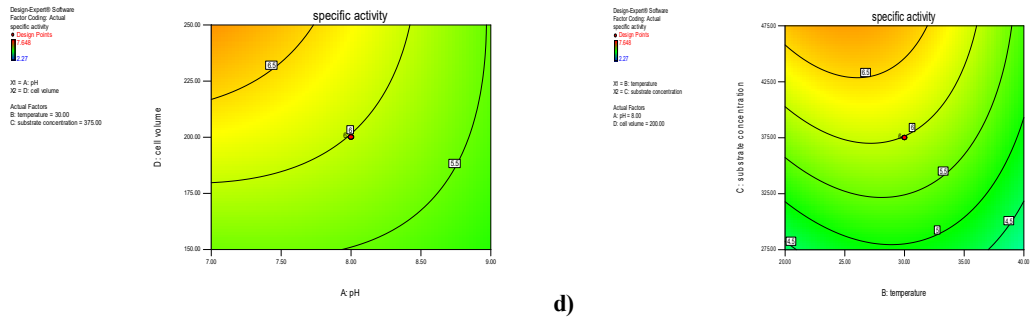
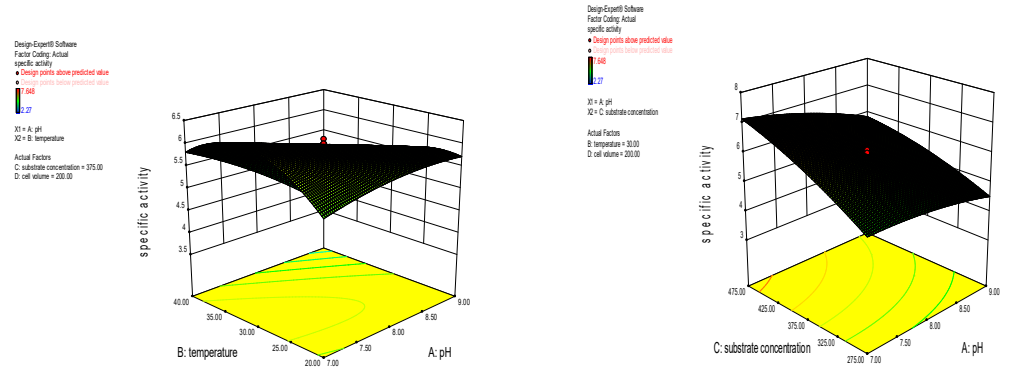
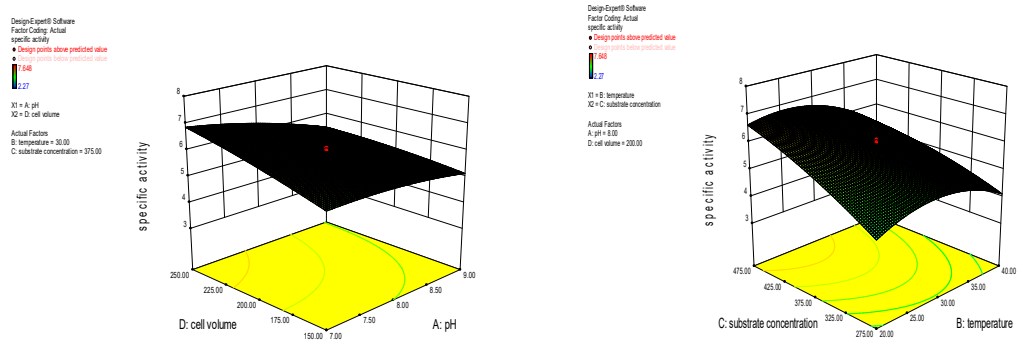


c) d)

Fig- 9 a)-d) Contour plots showing the response for Quadratic model for Acrylamide production. a)interaction between pH and temperature, b)pH and substrate concentration, c)pH and cell volume and d) shows the interaction between temperature and substrate concentration.



a) b)



c) d)

Fig- 10 a)- d) three dimensional(3D) plots shows the response for RSM experiments of acrylamide production. . a) Interaction between pH and temperature, b) pH and substrate concentration, c) pH and cell volume and d) shows the interaction between temperature and substrate concentration

Production of acrylamide at bench scale

According to the previous experimental data obtained for culture and reaction conditions optimization by RSM, the production of acrylamide was carried out at bench scale (1L) by using resting cells of *Rhodococcus pyridinivorans* NIT-36 in fed batch and batch mode for the high concentration of product.

For batch mode reaction, scale up was done by using 730 ml of 100 mM potassium phosphate buffer, 7% substrate (acrylonitrile) and 200 ml cells (1.7 mg/ml). The total of 50 g of acrylamide

was obtained in 1L of reaction mixture at 40°C.

In fed batch reaction, initial reaction mixture consisted of 500 ml of buffer of 100mM, 7% of substrate and 40 ml of cells (1.7 mg/ml). The substrate (1575mM) was added into the reaction mixture at an interval of 30 min. The total of 60 g of acrylamide was obtained after 6 hours of incubation. The results were confirmed by HPLC.

No acrylic acid formation observed due to the negligible activity of amidase. High NHase and low amidase activity can be attributed to the fact that acrylonitrile acts as a powerful nucleophilic reagent which inactivates the thiol residue of the amidase, whereas the NHase is less susceptible to acrylonitrile [33].

CONCLUSION

Statistical analysis of culture conditions and process parameters eventually led to a better understanding of various factors affecting NHase activity of *Rhodococcus pyridinivorans* NIT-36. The validation of model was confirmed by close agreement between predicted and actual values. This newly isolated bacterium under un-optimized conditions demonstrated 0.2 U/mg dcw NHase activity. A multifold increase (7.4 U/mg dcw) in activity was attained due to the statistical approach. The highest growth and activity was attained in an alkaline medium thereby illustrating the alkalophilic nature of this microbe. In addition the NHase was active over a broad pH range (6-10). High yield (60g/L) and high purity (97%) of final product (acrylamide) in the fed batch operation further indicated the suitability of this microbe for industrial applications. Since the feedings were carried out only for 6 h, there is scope for further increase in yield if the fed-batch is carried out for longer duration.

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