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Research article

Response surface methodology: An effective optimization strategy for enhanced production of nitrile hydratase (NHase) by *Rhodococcus rhodochrous* (RS-6)

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ABSTRACT

Nitrile hydratase is an enzyme which catalyze the hydration of nitriles into amide and their role as catalysts for acrylamide production in industries are well known. The present study aims at statistically optimizing physiological and nutritional parameters for NHase production from *Rhodococcus rhodochrous* (RS-6). The effect of incubation period, temperature, pH, carbon and nitrogen sources on the production of NHase was investigated by one factor at a time strategy. Further optimization process was carried out by response surface methodology for studying the interactive effect of these variables using central composite design. The optimized levels of variables obtained by statistical analysis were: incubation period 48 h, temperature 33 °C, pH 7.0, glycerol 1% and urea 0.75%, which resulted in maximum NHase production. The results of ANOVA were significant with the *F*-value of the model being 296.78, value of R^2 is 0.9983 and the lack of fit test was not significant. The contour and response surface plots showed significant interaction between the variables. The NHase yield was enhanced up to 6.22 fold by statistical optimization using RSM. Thus, the developed experimental design is effective towards process optimization for NHase production from *R. rhodochrous* (RS-6).

1. Introduction

Enzymes are an important group of biomolecules, which play key roles in the production of numerous biotechnology products. Microorganisms are the largest and useful source of many industrially important enzymes. Enzymes from microorganisms are gaining remarkable interest in developing industrial bioprocesses. In nature, nitrile compounds are widespread and extensively used for the production of an array of compounds such as amides, acids, and polymers [1, 2]. Nitrile degrading enzymes have gained much importance as biocatalyst in industrial bioconversion, as substitute to chemical methods to convert nitriles into most valuable compounds such as amides and acids under moderate conditions. Nitrile hydratases are class of nitrile degrading enzymes belonging to hydrolases which catalyzes the breakdown of nitriles into acids and amides [3]. In recent years, this enzyme has gained increased attention because of its role in the conversion of nitriles into useful products and for the production of acids and amides [4, 5, 6]. The potential of these enzymes has been exploited in industries for commercially producing acrylamide [7]. The role of these enzymes in the treatment of nitrile contaminated soil and water is also reported [8]. Microbes have proved to be better source of NHase enzyme as they can be easily cultured and the enzymes purified, aiding their production in large scale. The NHase production has been reported from many microorganisms which includes both bacteria and fungi. The bacterial strains like *Pseudomonas chlororaphis* B23 [9], *Arthrobacter* sp. [10], *Bacillus smithii* [11], *Rhodococcus rhodochrous* [12] and *Nocardia* sp [13]. and the fungal strains like *Candida* sp. [14], *Cryptococcus* sp., *Henseniaspora* sp. and *Rhodotorula glutinis* [15], *Kluyveromyces thermotolerans* [16] and *Geotricum* sp [17]. have been reported to exhibit NHase activity. Currently, industrial production of acrylamide was carried out using NHase from a potential strain *Rhodococcus rhodochrous* J1 [18].

Owing to the importance of NHase for producing amides from nitriles in industries and also wild strain always secrets low levels of enzymes it is necessary to optimize the cultural conditions for NHase production. Therefore, designing a suitable culture medium and determining the ideal conditions for improving NHase production are of foremost importance. In microbial system, the one factor at a time (OFAT) optimization approach was commonly used for improving the cultural

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Table 1. Composition of different medium used for NHase production by *Rhodococcus rhodochrous* (RS-6).

Medium	Composition	Quantity g/L
M1	Glucose	3.0
	K_2HPO_4	0.5
	KH_2PO_4	0.5
	$MgSO_4$	0.5
	$CaCl_2$	0.01
M2	Glycerol	10.0
	Peptone	5.0
	Yeast extract	3.0
	Malt extract	3.0
M3	Starch	5.0
	Na_2HPO_4	0.6
	KH_2PO_4	0.3
	$MgSO_4$	0.1
	NaCl	5.0
M4	Glucose	10.0
	Peptone	5.0
	Yeast extract	3.0
	Malt extract	3.0
M5	Sucrose	10.0
	Peptone	5.0
	Yeast extract	3.0
	Malt extract	3.0
M6	Beef extract	3.0
	Peptone	5.0

conditions. This simple method consumes much time and does not consider the interactive effects between multiple variables. The above problem can be overcome by statistical based experimental designs which involve designing the experiments specifically that reduces the errors in analyzing the effects of variables and the results are obtained in most economical ways. These optimization techniques allow rapid screening of large experimental data and consider the role of each variable and also their interactions. The problems associated with classical optimization technique can be overcome by the application of response surface methodology which is an effective statistical technique widely used to optimize process parameters. RSM technique assists in building models and designing experiments by using multiple regression and factorial design analysis. These designs further help in studying the interactive effects among the variables by reducing the number of experiments in a short span of time and for finding the optimal levels of the variables for obtaining accurate response [19, 20]. This method also analyzes the data obtained from the experiments and predicts their relationship [21]. Previously, we have reported that a strain of *Rhodococcus rhodochrous* (RS-6) isolated by us produces appreciable amount of NHase [22]. The present study was taken up for optimizing the cultural conditions and nutritional factors for enhancing NHase production from *Rhodococcus rhodochrous* (RS-6) using RSM with a central composite design.

2. Materials and methods

2.1. Chemicals

The components of the medium used for present investigation are standard HPLC and analytical grade. The acrylamide standard and substrate acrylonitrile are of HPLC standards and purchased from Merk (Germany) and Sigma-Aldrich (St Louis, USA) respectively.

2.2. The culture conditions for microorganisms

The strain *Rhodococcus rhodochrous* (RS-6) used for present investigation was isolated in our laboratory from soil [22]. The bacterial strain was streaked onto nutrient agar (NA) slants and sub-cultured at regular intervals on the same medium and stored under cold conditions (4 °C). Six different liquid nutrient medium (Table 1) were tested of NHase production in *R. rhodochrous* (RS-6). Fifty millilitre (50 ml) of each production medium was prepared in 100 ml Erlenmeyer flask with 1% of substrate (acrylonitrile). A loop full of culture was inoculated and the flasks were incubated in temperature controlled shaking conditions (160 rpm) for 24 h at 30 °C. The culture was centrifuged in 50 ml eppendorff tubes under cooling conditions for 8 min at 8000 rpm. The cell pellets were transferred into fresh tubes and washed two times with K_2PO_4 buffer 0.1 M and pH 7.2. The pellets were again suspended in the same buffer and stored under cold conditions until future use [22].

The M2 culture medium used for optimization was used for optimization studies. The pre culture was prepared with 50 ml of M2 medium, a loop full of *R. rhodochrous* (RS-6) culture was inoculated and incubated in temperature controlled shaking conditions (160 rpm) at 30 °C for 24 h. To 50 ml of production medium, $CoCl_2$ (0.01 g/l) and 1 % acrylonitrile as substrate (inducer) was supplemented and the pre culture (2 ml) was inoculated and incubated in temperature controlled shaking conditions (160 rpm) at 30 °C for 36 h. The cell pellets were obtained by the above mentioned methods [22].

2.3. Optimization studies for NHase production

Enzyme production by microorganisms mainly depends on the important parameters like nutrients (carbon and nitrogen source), pH, temperature and salt concentration which are comparatively linked with the metabolic activities of the strain and has significant effect on enzyme biosynthesis [23]. OFAT strategy was initially applied for optimizing physiological and nutritional parameters for NHase production. In this approach one factor is varied at once while the remaining factors are maintained at fixed level. The effects of different cultural parameters (both physiological and nutritional) like time of incubation (12–72 h), incubation temperature (27, 30, 33, 37 and 40 °C), initial pH (4.0–9.0) on the production of NHase has been investigated. To study the impact of carbon source on NHase production, glycerol (1%) was substituted in the production medium at same concentration with other carbon sources like lactose, glucose, sucrose, fructose, maltose and starch. The impact of supplementation of additional nitrogen sources like tryptone, beef extract, corn steep liquor, urea, potassium nitrate, ammonium sulfate,

Table 2. Experimental range and levels of variables used for NHase production by *Rhodococcus rhodochrous* (RS-6).

Independent variables	Symbols	Coded levels of variables				
		–2	–1	0	1	2
Incubation period (hours)	A	24	36	48	60	72
Temperature (°Celsius)	B	27	30	33	37	40
pH	C	5	6	7	8	9
Glycerol (%)	D	0.5	0.75	0	1.25	1.5
Urea (%)	E	0.25	0.5	0.75	1	1.25

Table 3. NHase production in different culture media by *Rhodococcus rhodochrous* (RS-6) under submerged fermentation.

Medium	Growth (mg/dcw/ml)	NHase activity (U/ml/min)	Specific activity (U/mg dcw)
M1	4.46	0.24 ± 0.02	0.05 ± 0.02
M2	4.12	3.16 ± 0.04	0.76 ± 0.04
M3	3.98	0.35 ± 0.03	0.08 ± 0.01
M4	5.17	1.19 ± 0.06	0.23 ± 0.04
M5	3.60	1.02 ± 0.04	0.28 ± 0.02
M6	4.88	0.74 ± 0.03	0.15 ± 0.02

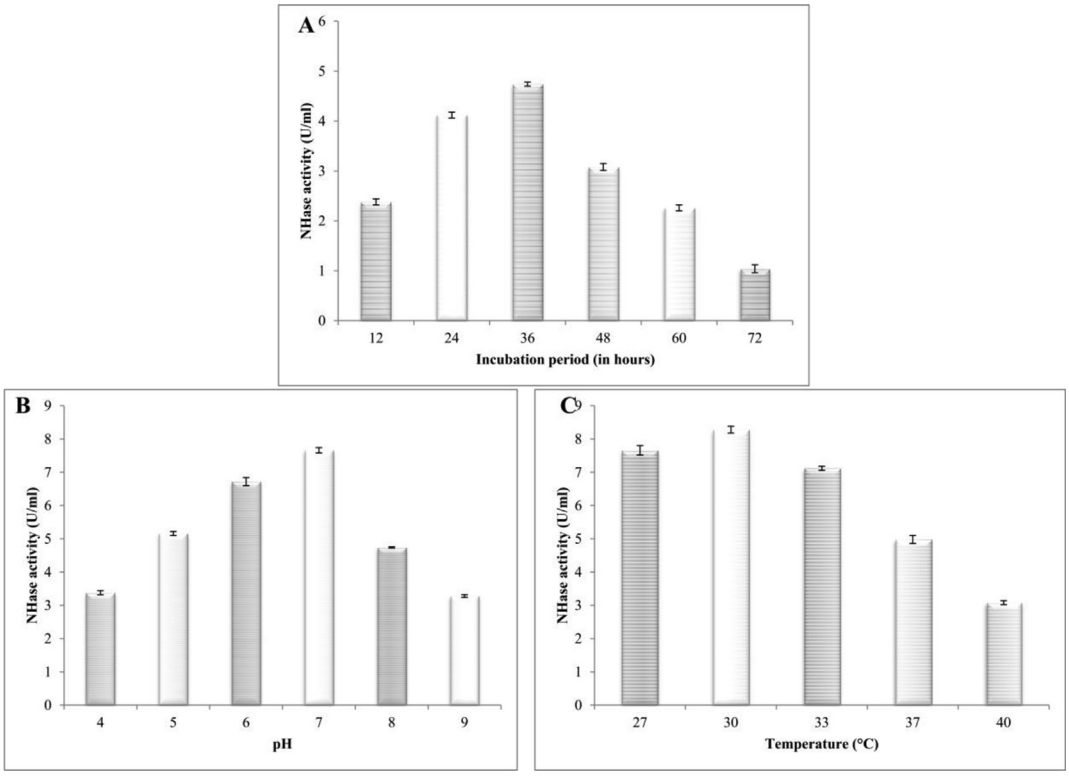

Figure 1. Effects of incubation period (A), pH (B) and temperature (C) on NHase production in *Rhodococcus rhodochrous* (RS-6) under submerged fermentation (SmF).

ammonium nitrate and ammonium chloride at 0.5 % on NHase production has been studied. The experimental procedures were done in triplicates. The conditions which exhibited maximum NHase production by *R. rhodochrous* (RS-6) were recognized and each factor levels which showed maximum yield were identified and fixed as center point in the central composite design [24].

2.4. Estimation of nitrile hydratase activity

The activity of NHase was estimated by spectrophotometric method [22]. The nitrile hydratase activity one unit is defined as the quantity of the enzyme required to produce one μ mole of acrylamide from acrylonitrile per min under standard assay condition. HPLC analysis was also performed to detect the amount of acrylamide produced [22, 25].

2.5. Experimental design for optimization of NHase production using RSM

Initially the physiological and nutritional parameters were optimized by (OFAT) approach. Further, optimization for NHase production by *R. rhodochrous* (RS-6) was performed by RSM using CCD. In the initial screening procedure, the physiological conditions like incubation period, temperature and pH and the nutritional parameters like glycerol and urea which exhibited increased levels of NHase were further optimized by RSM using M2 medium. The interactive effects of physiological and nutritional parameters on NHase production was studied by full factorial (2^k) analysis by changing variables which resulted in a combination of 30 runs (k: independent variable) [26].

The experiments with the five parameters were carried out in duplicates at the five levels coded as –2, –1, 0, +1 and +2 to analyze 2^k factorial designs. The experimental errors of the design were analyzed by carrying out the experiments with central point in triplicates [27]. Table 2 gives the coded values of the design. The factor incubation period was coded as A, temperature as B, pH as C, glycerol as D and urea was coded as E.

To depict the relationship between independent and dependent variables in second order quadratic equation, a mathematical model was developed. The data obtained from the experimental model were similar to polynomial Eq. (1).

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ii} \chi_i^2 + \sum \beta_{ij} \chi_i \chi_j \quad (1)$$

where the predicted response is represented as Y, the input variables were represented as χ_i χ_j, the intercept term is represented as β_0 and the terms β_i, β_{ii}, β_{ij} represents linear, squared and interactive effect of the variables respectively. Design Expert software, version 12 (Stat Ease Inc) was used for obtaining the polynomial equation and thereby accessing their response on NHase production. Further iterative method was used to optimize the equation and to obtain the optimal values [28].

To analyze the significance of regression coefficients we performed Student's *t*-test. To access the statistical significance of the designed model we performed Fisher's F-test. To demonstrate the interactive and main effects of independent factors on NHase production 3D response surface plots were drawn and optimal levels of each factors we analyzed the RSM contour plots.

3. Results and discussion

In order to choose an appropriate medium for the production NHase the strain *R. rhodochrous* (RS-6) was grown in six liquid nutrient medium (M1–M6) of different composition and acrylonitrile (1%) as substrate. Although all medium supported the growth and production of NHase, the maximum production of the enzyme was observed in M2 medium rich in nutrients (Table 3). The other three medium (M4, M5 and M6) with defined carbon source exhibited minimal NHase production. However, the mineral medium (M1 and M3) without a defined carbon source, supported the growth of bacteria but not the production of NHase. Raj et al. [24] also reported earlier that for NHase production in *R. rhodochrous* PA-34, nutrient rich medium was most suitable. Therefore, nutrient rich medium M2 has been selected for optimization process.

3.1. Effect of incubation period

In submerged fermentation, incubation period is one of the most crucial factor. The effect of incubation time on NHase production was tested from 12 to 72 h with periodic estimation of enzyme activity. The production of NHase was exponentially increased with increasing time of incubation and the maximum production was observed at 36 h of incubation time (Figure 1A). But, the production of NHase was moderately reduced with increase in incubation period. This reduction in NHase production may be due to the reduction of nutrients in the fermentation medium [29, 30].

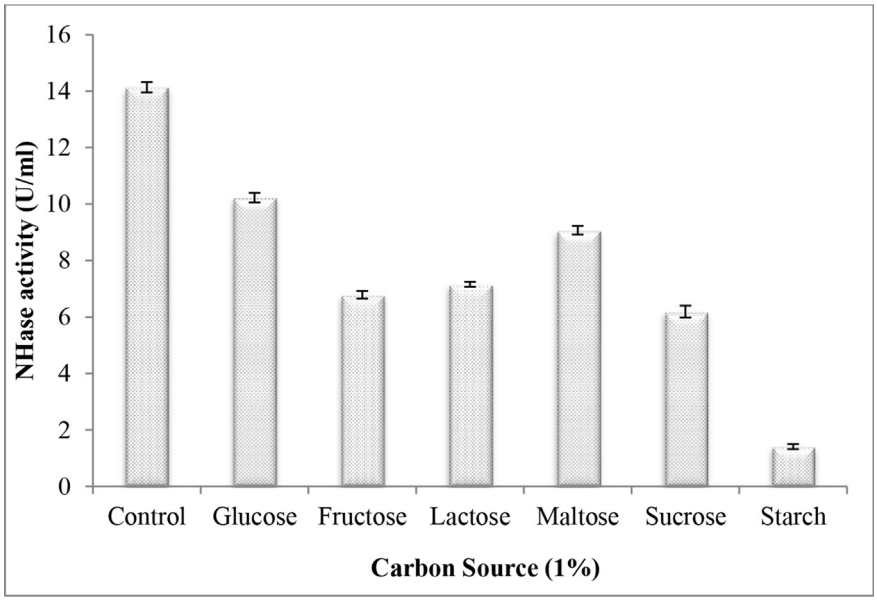

Figure 2. Effect of carbon sources on NHase production in *Rhodococcus rhodochrous* (RS-6) under submerged fermentation (SmF).

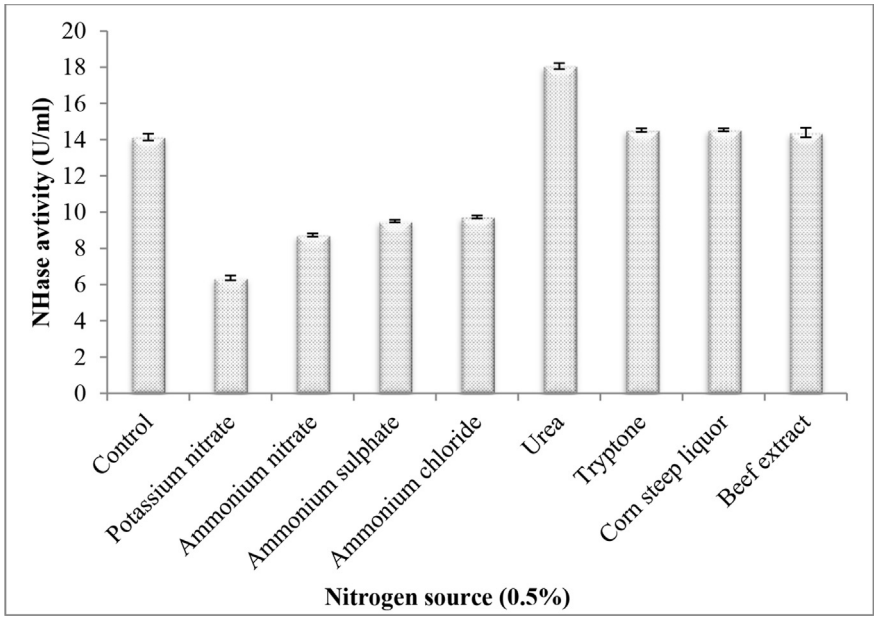

Figure 3. Effect of nitrogen sources on NHase production in *Rhodococcus rhodochrous* (RS-6) under submerged fermentation (SmF).

3.2. Effect of medium pH

Enzyme production always relies upon pH of the production medium. The effects of medium pH on NHase production were investigated by altering the pH of the production medium (pH 4 to pH 9). We observed that the production of NHase substantially increased from pH 5 to pH 8 and the maximum production was observed at pH 7 (Figure 1B). Similar pH optimum was reported for NHase production in *R. rhodochrous* J1 and *R. rhodochrous* PA-34 [24,31]. However, the production of NHase by *R. pyridinivorans* NIT-36 and *Bacillus* sp. APB-6 was highest at pH 9.0 and pH 7.5 respectively [29, 32]. NHase production decreased below pH 5 and above pH 8 (Figure 1B). This may be because of modification of enzymes by alteration of side chains of amino acids at lower pH or at higher pH the peptide bonds may break down.

3.3. Effect of temperature

Temperature is an important physical parameter which has major role in enzyme production. In order to select an optimal temperature for NHase production, we varied the incubation temperature (27–40 °C) of the growth medium. We observed maximum NHase production at 30 °C (Figure 1C). Sankhian et al. [33] also reported a similar such temperature optima for NHase production in *R. rhodochrous* NHB-2. However, the production of NHase drastically reduced with increase in temperature above 30 °C. This may be because, at high and low temperature the microorganisms slow down their metabolic activities due to denaturation of proteins which results in less enzyme production [34].

3.4. Effect of carbon source

Among the different nutrients in the production medium, the source of carbon plays an important role in growth, metabolism and enzyme production. Among the various sources of carbon examined, none of the carbon source influenced the production of NHase compared to the control medium containing glycerol as carbon source. Instead, all the other carbon sources tested decreased NHase production (Figure 2). Glycerol was found to be the most suitable carbon source which recorded maximum NHase production. Similar findings were reported by Seth et al. [29] in *R. pyridinivorans* NIT-36. Previously in many studies, glucose was reported as the most suitable carbon source for the production of NHase [35, 36]. However, Singh et al. [32] reported that lactose is most suitable source of carbon for the production of NHase in *Bacillus* sp. APB-6. They also reported that different concentration of glycerol has profound effect on NHase production. In our study, maximum production was observed at 1% glycerol and further increase in concentration however, decreased NHase production. Possibly, this may be either due to catabolite repression or due to increase in the concentration of the osmolytes in the medium compared to cell [32].

3.5. Effect of additional nitrogen source

The sources of nitrogen play a major role on microbial growth and enzyme production. The influence of additional organic and inorganic nitrogen source on the production NHase by *R. rhodochrous* (RS-6) was examined. Among the different sources of nitrogen tested, we observed

Table 4. Experimental and predicted values in RSM for NHase production by *Rhodococcus rhodochrous* (RS-6).

Standard order	Incubation (A)	Temperature (B)	pH (C)	Glycerol (D)	Urea (E)	NHase activity (U/ml)	
						Experimental Value	Predicted Value
1	36	40	5	1.5	1.25	3.08	3.10
2	24	27	9	1.25	0.5	14.56	14.59
3	48	27	9	0.75	1.25	8.88	8.96
4	72	33	7	0.5	1.25	7.66	7.68
5	48	33	8	1.5	0.75	12.74	12.58
6	24	40	6	0.5	0.25	13.74	13.77
7	60	40	5	0.75	1	13.84	13.78
8	48	33	8	1	0.25	16.98	17.51
9	24	37	8	0.75	1.25	11.32	11.29
10	48	33	8	1.5	0.75	12.32	12.58
11	72	40	6	1.5	0.25	11.84	11.90
12	60	27	7	1	0.75	17.70	17.42
13	60	27	7	1	0.75	17.20	17.42
14	36	27	5	1.5	0.25	11.22	11.26
15	24	33	5	1	0.75	17.46	17.50
16	24	33	7	1.5	0.25	12.72	12.76
17	72	27	9	1.5	0.25	10.78	10.81
18	72	30	5	1.5	1.25	4.74	4.77
19	36	27	5	0.5	1.25	10.94	10.91
20	60	40	9	0.5	0.5	13.76	13.74
21	72	40	9	1.25	1.25	1.04	1.02
22	72	30	5	0.5	0.25	12.72	12.75
23	24	40	5	1.25	0.25	14.14	14.02
24	48	33	8	1	0.25	18.06	17.51
25	72	30	6	1.5	0.25	13.16	13.08
26	24	27	9	0.5	0.5	10.52	10.48
27	48	33	8	1.5	0.75	12.74	12.58
28	24	27	7	1.5	1.25	8.28	8.24
29	24	40	9	1.5	0.25	11.74	11.74
30	36	40	7	1	0.75	17.44	17.56

the NHase production was maximum when urea was supplemented in the production medium (Figure 3). Similar results were reported by Leonova et al. [37]. There was no significant difference in NHase production when organic nitrogen source such as beef extract, tryptone and corn steep liquor was used. However, the inorganic nitrogen such as ammonium chloride and potassium nitrate showed repressive effect by decreasing the production of NHase (Figure 3). The decrease in the production of NHase in *R. rhodochrous* NHB 2 and *Serratia marcescens* ZJB-09104 was also observed with the addition of ammonium ions [33, 36].

3.6. Response surface methodology optimization of NHase production

Among different statistical tools used in scientific research response surface methodology (RSM) is one of the popular statistical tools extensively used for optimizing the cultural conditions during fermentation process of various enzymes in recent years. This statistical approach has been used by many researchers for optimizing the production of enzymes from microorganisms [38, 39, 40, 41]. The economics of the fermentation process for the production of enzymes in industries is one of the most crucial factors. The process optimization by RSM will improve the process efficiency, reduce the time and the cost of labor, thereby contributing towards the complete economy of the process. The initial optimization by OFAT approach produced maximum NHase production when glycerol and urea was used as carbon and nitrogen source with incubation time 36 h, temperature 30 °C and pH 7. On the basis of initial optimization results the physiological conditions incubation period 36 h, temperature 30 °C, pH 7 and the nutritional parameters glycerol and urea are carbon and nitrogen source were predicted for optimizing the production of NHase by RSM. Out of five factors incubation period represented variable 1 (A), temperature represented variable 2 (B), pH represented variable 3 (C), glycerol as carbon source, represented variable 4 (D) and urea as nitrogen source, represented variable 5 (E). A total of 30 experiments with different combinations of 5 factors were performed using central composite experimental design (CCD). All the thirty responses are entered into the design and responses that were predicted by the design is presented (Table 4). The quadratic model was selected from the above obtained responses, while the other regression models were aliased (Table S1 and S2). The responses were analyzed using analysis of variance (ANOVA) and a polynomial equation was derived which explains how NHase production was depended on the composition of the medium and the responses obtained from the model were fit into the Eq. (2). The complete equation for the production of NHase:

$$Y\ (\text{NHase production}) = 18.34 - 0.6915A - 0.2754B - 0.9441C - 1.18D - 3.07E - 0.3138AB - 0.4185AC - 0.2134AD - 1.12AE + 0.2245BC - 0.9309BD - 1.24BE + 0.2702CD - 0.6925CE - 0.9839DE - 0.8210A^2 - 1.25B^2 - 4.02C^2 - 3.38D^2 \quad (2)$$

Table 5. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for the production of NHase by *Rhodococcus rhodochrous* (RS-6).

Source	Sum of squares	*df*	Mean square	*F* value	*P*-value *Prob* > *F*	
Model	531.88	20	26.59	296.78	< 0.0001	significant
A-Incubation period	6.63	1	6.63	74.02	<0.0001	
B-Temperature	1.19	1	1.19	13.29	0.0045	
C-pH	13.11	1	13.11	146.28	<0.0001	
D-Glycerol	21.51	1	21.51	240.07	<0.0001	
E-Urea	147.28	1	147.28	1643.53	<0.0001	
AB	0.9475	1	0.9475	10.57	0.0087	
AC	1.60	1	1.60	17.83	0.0018	
AD	0.4861	1	0.4861	5.42	0.0421	
AE	14.83	1	14.83	165.47	<0.0001	
BC	0.5628	1	0.5628	6.28	0.0311	
BD	8.29	1	8.29	92.50	<0.0001	
BE	15.63	1	15.63	174.44	<0.0001	
CD	0.7096	1	0.7096	7.92	0.0183	
CE	4.73	1	4.73	52.75	<0.0001	
DE	11.11	1	11.11	123.93	<0.0001	
A^2	2.83	1	2.83	31.62	0.0002	
B^2	3.25	1	3.25	36.25	0.0001	
C^2	6.12	1	6.12	68.35	<0.0001	
D^2	70.90	1	70.90	791.21	<0.0001	
E^2	49.54	1	49.54	552.84	<0.0001	
Residual	0.8961	10	0.0896			
Lack of Fit	0.0703	5	0.0141	0.0851	0.9914	not significant
Pure Error	0.8258	5	0.1652			
Corrected Total	532.78	30				

Table 6. Fit statistics of response surface quadratic model.

Term	Value	Term	Value
Standard deviation	0.299	R^2	0.9983
Mean	12.28	Adjusted R^2	0.9950
Coefficient of variation (%)	2.44	Predicted R^2	0.9886
PRESS	6.05	Adequate precision	67.148

PRESS: Predicted residual sum of squares.

Where Y is NHase production; A (incubation period); B (temperature); C (pH); D (glycerol) and E (urea).

The model's significance we evaluated using Fisher's *F*-test. The *F* and *p*-value of the model were used to determine the significant differences between the model terms of each coefficient. The ANOVA results of the regression equation showed a confidence level of 99.9% which revealed the significance of the model (Table 5). The *F*-value of model is 296.78 which indicate the significance of model. The $P > F < 0.05$ value is in the acceptable range for a model to be significant. The value of $P > F$ is 0.0001 indicating that the individual terms of the model was highly significant. The term values of the model $\leq$0.1 were significant and the term values of the model $\geq$0.1 were not significant. If the model should be significant than the model *F*-value should be higher and *p*-value should be lower [42]. Therefore, from the results obtained the designed model was statistically significant for the production of NHase.

To study the good fit of the model, we used determination coefficient (R^2). The designed model R^2 value is 0.9983 which implies that the model was fit for NHase production. The value of R^2 was in considerable concurrence with the value of adjusted R^2 i.e. 0.9950 which described the model's efficacy to predict response. The value of adequate precision was used to study the ratio of signal to noise. The adequate precision value $\geq$ four is preferable. The designed model showed the signal to noise ratio was 67.148 which signifies the signals were adequate (Table 6) and the current model could be used to operate the model space. The value of lack of fit is 0.085 which indicates it is not significant, and also implies the designed model was appropriate for illustrating the impact of the variables on NHase production. For a model to be an appropriate, the lack of fit test values should not be significant Bas and Boyaci, [43]. The value of coefficient of variation is lower i.e. 2.44 % which indicates the experiments conducted are accurate and good.

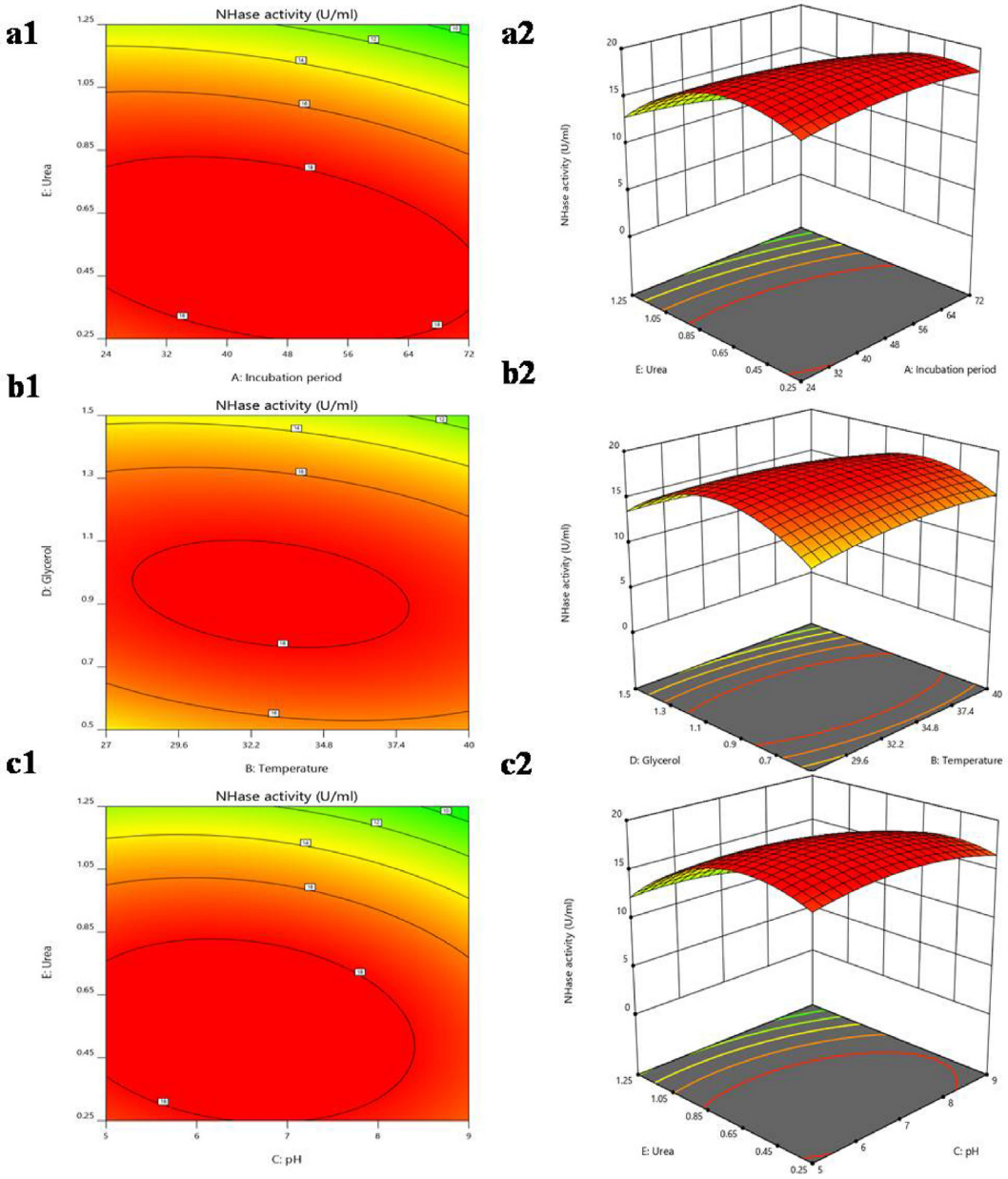

Figure 4. The contour and response surface plots displaying their effects of interactions of (a1 and a2) represents interaction between incubation period and urea, (b1 and b2) represents interaction between temperature and glycerol and (c1 and c2) represents interaction between pH and urea on NHase production by *Rhodococcus rhodochrous* (RS-6).

The model *P* value analyzes the importance of every coefficient. The *P* value for all the coefficient were shown in Table 5. The results revealed that higher quadratic effect ($P < 0.0001$) was shown by pH, glycerol and urea. The mutual effects of incubation period versus urea, temperature versus glycerol, temperature versus urea, pH versus urea and glycerol versus urea also showed higher significance. However, the mutual effects of incubation period versus glycerol, pH versus temperature and pH versus glycerol was found to be 0.042, 0.031 and 0.018 respectively, which indicates that these interactions showed lesser significance in the model.

3.7. Contour and response surface plots displaying interactive effect of variables on the production of NHase

The 2D contour and 3D response surface plots of the regression equation provide a visual interpretation of the interactions between the experimental values and the responses obtained from the model for individual variables. These plots also aid in finding their optimal levels for maximum production of NHase [44]. The contour plot shape indicates the robustness of the interaction between corresponding variables [45]. The elliptical contour plots indicate significant interaction among the variables, whereas the circular contour plots represents non-significant interactions among the variable [46, 47]. The three-dimensional (3D) graphs were generated for the pair wise combination of the two interacting variables while the remaining variables were kept at zero.

The interactions among the two variables, incubation period and urea are shown in Figure 4a1 and a2. The 2D contour and 3D response surface plot indicated that the interactions among these two variables showed elliptical shape of contour plots indicating their significant influence on NHase production. As incubation time and concentration of urea increases, the enzyme yield was also increased but further increase in incubation time and urea concentration resulted in decreased enzyme production. The interaction between other variables such as temperature and glycerol (Figure 4b1 and b2) and pH and urea (Figure 4c1 and c2) also showed elliptical contour plots indicating their significant influence on NHase production. The contour and response surface plots for the interaction between incubation period and pH (Fig. S1 a1 and a2) and incubation period and temperature (Fig. S1 b1 and b2) showed nearly elliptical shape of contour plots indicating these interactions had moderately significant influence on NHase production. The contour and response surface plots of glycerol and urea (Figure 5a1 and a2) and temperature and pH (Figure 5b1 and b2) displayed circular shape of contour plots indicating these interactions had less significant influence on NHase production.

3.8. Model validation

The experiments were conducted for validating the model using the optimal conditions which the model predicted. The response surface plots predicted the following optimal levels: incubation period 48 h, temperature 33 °C, pH 7.0, glycerol 1% and urea 0.75%. Under these optimal conditions, the yield of NHase was 18.06 U/ml which found to be in close agreement with response predicted by the model 17.56 U/ml, which proves that the model is valid. There was significant increase in NHase production by *R. rhodochrous* (RS-6) when the validation experiments were performed using the predicted levels based on RSM.

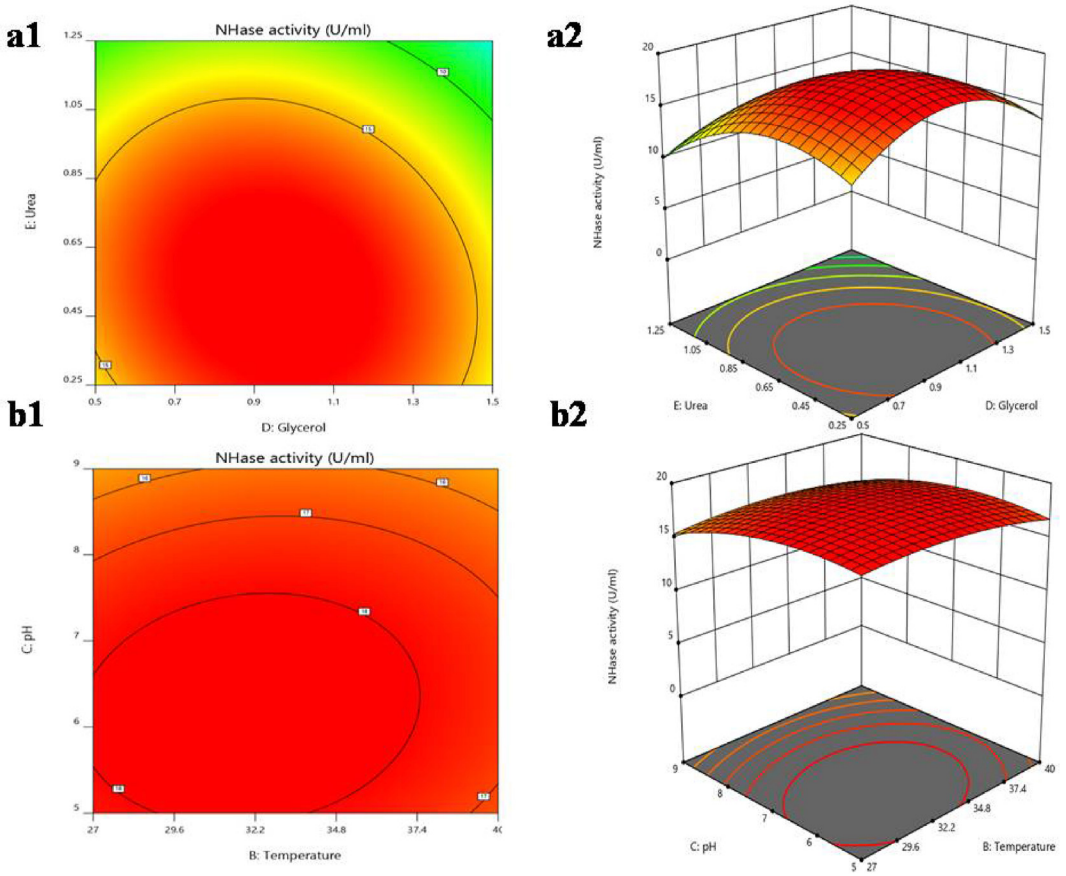

Figure 5. The contour and response surface plots displaying their effects of interactions of (a1 and a2) represents interaction between glycerol and urea and (b1 and b2) represents interaction between temperature and pH on NHase production by *Rhodococcus rhodochrous* (RS-6).

4. Conclusion

Acrylamide, a commercially important product, is produced mainly by the enzyme nitrile hydratase. Therefore, it is significant for optimizing the nutritional and physiological parameters for enhancing NHase production. In this study we explored the use of statistical approach i.e. OFAT and RSM for optimizing the growth conditions for increasing NHase production by *R. rhodochrous* (RS-6). The main factors influencing the process of fermentation for the production of NHase were investigated and the optimum levels were recognized. It is significantly noted that optimization by statistical methods enhanced the yield of the enzyme from 3.16 U/ml to 18.06 U/ml. Thus, the sequential optimization of culture conditions and nutritional factors by statistical methods OFAT followed by RSM was most effective and flexible which enhanced NHase production by 6.22 fold in the soil isolate *R. rhodochrous* (RS-6). Our findings reports the statistical optimization approach in laboratory conditions, which in turn, would shed light for future optimization efforts for large scale bioreactors and bioprocess development for commercial production of NHase.

Declarations

Author contribution statement

Ruchi Sahu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Anil Kumar Meghavarnam: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Savitha Janakiraman: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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References

[1] M.X. Wang, Enantioselective biotransformations of nitriles in organic synthesis, Top. Catal. 35 (2005) 117–130.
[2] A. Banerjee, R. Sharma, U.C. Banerjee, The nitrile-degrading enzymes: current status and future prospects, Appl. Microbiol. Biotechnol. 60 (2002) 33–44.
[3] T. Nagasawa, H. Yamada, Application of nitrile converting enzymes for the production of useful compounds, Pure Appl. Chem. 62 (1990) 1441–1444.
[4] Z. Wang, Z. Liu, W. Cui, Z. Zhou, Establishment of bioprocess for synthesis of nicotinamide by recombinant *Escherichia coli* expressing high molecular mass nitrile hydratase, Appl. Biochem. Biotechnol. 182 (2017) 1458–1466.
[5] R. Egelkamp, D. Schneider, R. Hertel, R. Daniel, Nitrile-Degrading bacteria isolated from compost, Front. Environ. Sci. 5 (2017) 1–8.
[6] A. Pratush, A. Seth, T.C. Bhalla, Expression of nitrile hydratase gene of mutant 4D strain of *Rhodococcus rhodochrous* PA 34 in *Pichia pastoris*, Biocatal. Biotransform. 35 (2016) 19–26.
[7] H. Yamada, M. Kobayashi, Nitrile hydratase and its application to industrial production of acrylamide, Biosci. Biotechnol. Biochem. 60 (1996) 1391–1400.
[8] A.B. Vesela, M. Franc, H. Pelantova, D. Kubac, V. Vejvoda, S. Sulc, T.C. Bhalla, M. Mackova, P. Lovecka, P. Janu, K. Demnerova, L. Martinkova, Hydrolysis of benzonitrile herbicides by soil actinobacteria and metabolite toxicity, Biodegradation 21 (2010) 761–770.
[9] H. Yamada, K. Ryuno, T. Nagasawa, K. Enomoto, I. Watanabe, Optimum culture conditions for production by *Pseudomonas chlororaphis* B23 of nitrile hydratase, Agric. Biol. Chem. 50 (1986) 2859–2865.
[10] I. Watanabe, S. Yoshiaki, K. Enomoto, Screening, isolation and taxonomical properties of microorganisms having acrylonitrile-hydrating activity, Agric. Biol. Chem. 51 (1987) 3193–3199.
[11] Y. Takashima, T. Kawabe, S. Mitsuda, Factors affecting the production of nitrile hydratase by thermophilic *Bacillus smithii* SC-J05-1, J. Biosci. Bioeng. 89 (2000) 282–284.
[12] S. Prasad, J. Raj, T.C. Bhalla, Optimization of culture conditions for hyper production of nitrile hydratase of *Rhodococcus rhodochrous* PA-34, Indian J. Microbiol. 44 (2004) 251–256.
[13] Y.J. Wang, Y.G. Zheng, J.P. Xue, Y.C. Shen, Characterization of nitrile hydration catalysed by *Nocardia* sp. 108, World, J. Microbiol. Biotechnol. 23 (2007) 355–362.
[14] J.C.T. Dias, R.P. Rezende, C.A. Rosa, M.A. Lachance, V.R. Linardi, Enzymatic degradation of nitrile by a *Candida guilliermondii* UFMG-Y65, Can. J. Microbiol. 46 (2000) 525–531.
[15] R.P. Rezende, J.C.T. Dias, C.A. Rosa, F. Carazza, V.R. Linardi, Utilization of nitriles by yeast isolated from a Brazilian gold mine, J. Gen. Appl. Microbiol. 45 (1999) 185–192.
[16] S. Prasad, D.R. Sharma, T.C. Bhalla, Nitriles and amides hydrolysing activity in *Kluyveromyces thermotolerans* MGBY 37, World, J. Microbiol. Biotechnol. 21 (2005) 1447–1450.
[17] R.P. Rezende, J.C.T. Dias, A.S. Monteiro, F. Carazza, V.R. Linardi, The use of acetonitrile as the sole nitrogen and carbon source by *Geotrichum* sp. JR1, Braz, J. Microbiol. 34 (2003) 117–120.
[18] Y. Lan, X. Zhang, Z. Liu, R. Shen, X. Zhong, W. Cui, Z. Zhou, Over expression and characterization of two types of nitrile hydratase from *Rhodococcus rhodochrous* J1, PloS One 12 (2017), e0179833.
[19] K. Shivam, P. Chandra, M. Tripathi, S.K. Mishra, Culture conditions for the production of a-galactosidase by *Aspergillus parasiticus* MTCC-2796: a novel source, Electronic, J. Biotechnol. 12 (2009) 1–9.
[20] D. Gangadharan, S. Sivaramakrishnan, K.M. Nampoothiri, R.K. Sukumaran, A. Pandey, Response Surface Methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*, Bioresour. Technol. 99 (2008) 4597–4602.
[21] A. Rajendran, V. Thangavelu, Optimization of medium composition for lipase production by *Candida rugosa* NCIM 3462 using response surface methodology, Can. J. Microbiol. 53 (2007) 643–655.
[22] R. Sahu, A.K. Meghavarnam, S. Janakiraman, A simple, efficient and rapid screening technique for differentiating nitrile hydratase and nitrilase producing bacteria, Biotechnol. Rep. 24 (2019) 1–7.
[23] M. Hymavathi, T. Sathish, C.H. Subba Rao, R.S. Prakasham, Enhancement of L-asparaginase production by isolated *Bacillus circulans* (MTCC 8574) using response surface methodology, Appl. Biochem. Biotechnol. 159 (2009) 191–198.
[24] J. Raj, S. Prasad, T.C. Bhalla, *Rhodococcus rhodochrous* PA-34: a potential biocatalyst for acrylamide synthesis, Process Biochem. 41 (2006) 1359–1363.
[25] J. Raj, A. Seth, S. Prasad, T.C. Bhalla, Bioconversion of butyronitrile to butyramide using whole cells of *Rhodococcus rhodochrous* PA-34, Appl. Microbiol. Biotechnol. 74 (2007) 535–539.
[26] P.W. Araujo, R.G. Brereton, Experimental design II. Optimization, Trends Anal. Chem. 15 (1996) 63–70.
[27] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for Experiments, John Wiley and Sons, NY, USA, 1978, pp. 291–334.
[28] K. Theodore, T. Panda, Application of response surface methodology to evaluate the influence of temperature and initial pH on the production of b-1,3-glucanase and carboxymethyl cellulose from *Trichoderma harzianum*, Enzym. Microb. Technol. 17 (1995) 1043–1049.
[29] A. Seth, P. Singh, A. Kumari, C. Attri, Enhanced production of NHase of alkali stable *Rhodococcus pyridinivorans* NIT 36 and its application in acrylamide production, Int. J. Biol. Pharm. Allied Sci. 6 (2017) 278–299.
[30] F.N. Niyonzima, S.S. More, Concomitant production of detergent ompatible enzymes by *Bacillus flexux* XJU-1, Braz. J. Microbiol. 54 (2014) 903–910.
[31] T. Nagasawa, K. Takeuchi, V. Nardi-Dei, Y. Mihara, H. Yamada, Optimum culture conditions for the production of cobalt-containing nitrile hydratase by *Rhodococcus rhodochrous* J1, Appl. Microbiol. Biotechnol. 34 (1991) 783–788.
[32] R. Singh, D. Pandey, D. Dhariwal, P. Sood, D. Chand, Bioconversion of acrylonitrile using nitrile hydratase activity of *Bacillus* sp, APB-6. 3 Biotech. 2 (225) (2018) 1–8.
[33] U.D. Sankhian, H. Kumar, D. Chand, D. Kumar, T.C. Bhalla, Nitrile hydratase of *Rhodococcus rhodochrous* NHB-2: optimization of conditions for production of enzyme and conversion of acrylonitrile to acrylamide, Asian J. Microbiol. Biotechnol. Environ. Sci. 5 (2003) 217–223.

[34] R. Tunga, R. Banerjee, B.C. Bhattacharya, Some studies on optimization of extraction process for protease for production in SSF, Bioprocess Eng. 20 (1999) 485–489.
[35] Y.G. Zheng, J. Chen, Z.Q. Liu, M.H. Wu, L.Y. Xing, Y.C. Shen, Isolation, identification and characterization of *Bacillus subtilis* ZJB-063, a versatile nitrile converting bacterium, Appl. Microbiol. Biotechnol. 77 (2008) 985–993.
[36] L.Q. Jin, Z.Q. Liu, Y.G. Zheng, Y.C. Shen, Identification and characterisation of *Serratia marcescens* ZJB-09104, a nitrile-converting bacteria, World, J. Microbiol. Biotechnol. 26 (2010) 817–823.
[37] T.E. Leonova, O.B. Astaurova, L.E. Ryabchenko, A.S. Yanenko, Nitrile hydratase of *Rhodococcus*, Appl. Biochem. Biotechnol. 88 (2000) 231–241.
[38] C.D. Tang, H.L. Shi, Y.Y. Jia, X. Li, L.F. Wang, J.H. Xu, L.G. Yao, Y.C. Kan, High level and enantioselective production of L-phenlyglycine from racemic mandelic acid by engineered *Escherichia coli* using response surface methodology, Enzym. Microb. Technol. 136 (2020) 109513.
[39] Y. Liu, H. Guo, J. Gu, W. Qin, Optimize purification of a cellulase from *Bacillus velezensis* A4 by aqueous two phase system (ATPS) using response surface methodology, Process Biochem. 87 (2019) 196–203.
[40] E. Omidinia, H. Shahbazmohammadi, Z. Mohsenipour, R. Mahdizadeh, Partitioning of recombinant *Pseudomonas putida* POS-F84 proline dehydrogenase in aqueous two phase systems: optimization using response surface methodology, Appl. Biochem. Biotechnol. 189 (2019) 498–510.
[41] M.C. Da-Cunha, L.C. Silva, H.H. Sato, R.J.S. De-Castro, Using response surface methodology to improve the L-asparaginase production by *Aspergillus niger* under solid state fermentation, Biocatal. Agric. Biotechnol. 16 (2018) 31–36.
[42] M. Yolmeh, N.M. Habibi, R. Farhoosh, Optimization of ultrasound assisted extraction of natural pigment from annatto seeds by response surface methodology (RSM), Food Chem. 155 (2014) 319–324.
[43] D. Bas, I.H. Boyaci, Modeling and optimization I: usability of response surface methodology, J. Food Eng. 78 (2007) 836–845.
[44] S.N. Surwase, S.A. Patil, S.B. Jadhav, J.P. Jadhav, Optimization of L-Dopa production by *Brevundimonas* sp. SGJ using response surface methodology, Microbial. Biotechnol. 5 (2012) 731–737.
[45] R.V. Muralidhar, R.R. Chirumamila, R. Marchant, P. Nigam, A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources, Biochem. Eng. J. 9 (2001) 17–23.
[46] P.K. Bhagwat, S.B. Jhample, P.B. Dandge, Statistical medium optimization for the production of collagenolytic protease by *Pseudomonas* sp. SUK using response surface methodology, Microbiology 84 (2015) 520–530.
[47] S. Wu, G. Gong, Y. Wang, F. Li, S. Jia, F. Qin, H. Ren, Y. Liu, Response surface optimization of enzyme assisted extraction polysaccharides from *Dictyophora indusiata*, Int. J. Biol. Macromol. 61 (2013) 63–68.