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 R² 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 bioreconversion, 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 *Nocardiopsis* sp. [13], and the fungal strains like *Candida* sp. [14], *Cryptococcus sp.* [15], *Hanseniaspora sp.* and *Rhodotorula glutinis* [16], *Kluyveromyces thermodotolerans* [17] and *Geotricum* sp [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
Table 1. Composition of different medium used for NHase production by Rhodococcus rhodochrous (RS-6).

| Medium | Composition | Quantity g/L |
|--------|-------------|--------------|
| M1     | Glucose     | 3.0          |
|        | K2HPO4      | 0.5          |
|        | KH2PO4      | 0.5          |
|        | MgSO4       | 0.5          |
|        | CaCl2       | 0.01         |
| M2     | Glycerol    | 10.0         |
|        | Peptone     | 5.0          |
|        | Yeast extract | 3.0       |
|        | Malt extract | 3.0        |
| M3     | Starch      | 5.0          |
|        | Na2HPO4     | 0.6          |
|        | KH2PO4      | 0.3          |
|        | MgSO4       | 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          |

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

| Independent variables | Symbols | Coded levels of variables |
|-----------------------|---------|---------------------------|
| Incubation period (hours) | A       | -2  | -1  | 0   | 1   | 2   | 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|
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 μmole 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 triplicates 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).

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i^2 + \sum \beta_{ij} X_i X_j \]  

(1)

where the predicted response is represented as Y, the input variables were represented as \(X_i\), \(X_j\), the intercept term is represented as \(\beta_0\) and the terms \(\beta_i, \beta_{ij}, \beta_{ij}\) 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].

| 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).
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].
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

| Standard order | Incubation (A) | Temperature (B) | pH (C) | Glycerol (D) | Urea (E) | NHase activity (U/ml) |
|---------------|---------------|----------------|--------|-------------|---------|---------------------|
| 1             | 36            | 40             | 5      | 1.5         | 1.25    | 3.08                |
| 2             | 24            | 27             | 9      | 1.25        | 0.5     | 14.56               |
| 3             | 48            | 27             | 9      | 0.75        | 1.25    | 8.88                |
| 4             | 72            | 33             | 7      | 0.5         | 1.25    | 7.66                |
| 5             | 48            | 33             | 8      | 1.5         | 0.75    | 12.74               |
| 6             | 24            | 40             | 6      | 0.5         | 0.25    | 13.74               |
| 7             | 60            | 40             | 5      | 0.75        | 1       | 13.84               |
| 8             | 48            | 33             | 8      | 1           | 0.25    | 16.98               |
| 9             | 24            | 37             | 8      | 0.75        | 1.25    | 11.32               |
| 10            | 48            | 33             | 8      | 1.5         | 0.75    | 12.32               |
| 11            | 72            | 40             | 6      | 1.5         | 0.25    | 11.84               |
| 12            | 60            | 27             | 7      | 1           | 0.75    | 17.70               |
| 13            | 60            | 27             | 7      | 1           | 0.75    | 17.20               |
| 14            | 36            | 27             | 5      | 1.5         | 0.25    | 11.22               |
| 15            | 24            | 33             | 5      | 1           | 0.75    | 17.46               |
| 16            | 24            | 33             | 7      | 1.5         | 0.25    | 12.72               |
| 17            | 72            | 27             | 9      | 1.5         | 0.25    | 10.78               |
| 18            | 72            | 30             | 5      | 1.5         | 1.25    | 4.74                |
| 19            | 36            | 27             | 5      | 0.5         | 1.25    | 10.94               |
| 20            | 60            | 40             | 9      | 0.5         | 0.5     | 13.76               |
| 21            | 72            | 40             | 9      | 1.25        | 1.25    | 1.04                |
| 22            | 72            | 30             | 5      | 0.5         | 0.25    | 12.72               |
| 23            | 24            | 40             | 5      | 1.25        | 0.25    | 14.14               |
| 24            | 48            | 33             | 8      | 1           | 0.25    | 18.06               |
| 25            | 72            | 30             | 6      | 1.5         | 0.25    | 13.16               |
| 26            | 24            | 27             | 9      | 0.5         | 0.5     | 10.52               |
| 27            | 48            | 33             | 8      | 1.5         | 0.75    | 12.74               |
| 28            | 24            | 27             | 7      | 1.5         | 1.25    | 8.28                |
| 29            | 24            | 40             | 9      | 1.5         | 0.25    | 11.74               |
| 30            | 36            | 40             | 7      | 1           | 0.75    | 17.44               |
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 physiological conditions (Figure 3). Similar results were reported by Leonova et al. [38]. There was no significant difference in NHase production when incubation time 36 h, temperature 30 °C and pH 7.

\[
Y = 18.34 - 0.6915A - 0.2754B - 0.9441C - 1.18D - 0.307E - 0.3138AB - 0.4185AC - 1.24BE - 0.2702CD - 1.12AE + 0.2245BC - 0.9309BD - 1.24BE + 0.2702CD - 0.6925CE - 0.8210A^2 - 1.25B^2 - 4.02C^2 - 3.38D^2
\] (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 ≤0.1 were significant and the term values of the model >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 ≥ 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](image-url)
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](image-url)
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 *Rhodococcus* (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 *Rhodococcus* (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; 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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