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Optimization of Crucial Reaction Conditions for the Production of Nicotinamide by Nitrile Hydratase Using Response Surface Methodology

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Abstract The reaction conditions for the nicotinamide production by *Rhodococcus erythropolis* MTCC 1526 have been optimized by statistical experimental design. Application of this approach in the bioprocess can result in rallied product yield, reduced development time, and process variability. In this investigation, response surface methodology and central composite design were employed to predict the levels of variables such as reaction pH (6.5, 7, and 7.5), temperature (15, 20, and 25 °C), cell concentration (190, 200, and 210 mg/ml), and substrate concentration (18, 20, and 22 mM) on the production of nicotinamide. A total of 22 experiments were carried out in shake flasks, and a three-dimensional response surface was generated to determine the effect of crucial reaction parameters for the maximum conversion of 3-cynopyridine to nicotinamide. Using this methodology, the optimal values for the reaction conditions were reaction pH 6.85, temperature of 24.8 °C, cell concentration of 190.98 mg/ml, and substrate concentration of 21.98 mM. This statistical approach led to the increase of conversion of 3-cynopyridine (93%) as compared to the conversion obtained by one-factor-at-a-time approach (84%).

Keywords Nitrile hydratase · Nicotinamide · 3-cyanopyridine · Response surface methodology · Reaction optimization · Reaction temperature

Introduction

Nicotinamide is one of the important forms of vitamin B_3, which is mainly used in pellagra and niacin deficiency [1]. It has also an antioxidant and cytoprotective effect. Another form of vitamin B_3 is nicotinic acid, which is equally important as nicotinamide. Most vitamins and related compounds are now industrially produced by chemical and biocatalytic ways.

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There are many chemical processes for the production of nicotinamide and nicotinic acid. One of the chemical process for nicotinamide production involves the hydration of 3-cyanpopyridine, which is synthesized by ammoxidation of picoline with alkali. The disadvantages of this method are low yield and the production of significant amount of by-product, i.e., nicotinic acid, which complicates the downstream processing [2]. On the contrary, production of nicotinamide by nitrile hydratase (NHase) is the most convenient method, as the enzyme-mediated conversion of 3-cyanopyridine to nicotinamide leads to 100% conversion and nicotinamide is the sole product, with simplified isolation procedure under ambient conditions [3, 4]. Nitrile biotransformation has important applications in the industry for the production of pharmaceuticals, drug intermediates, and pesticides, etc. [5]. NHase from *Rhodococcus rhodochrous* has been used for the industrial production of tuberculostatic intermediates such as isonicotinamide, picolinamide, and pyrazinamide [6], while plant hormones like indole-3-acetic acid from indole-3-acetonitrile has been synthesized by NHase of *Agrobacterium tumefaciens* [7]. To date, *Rhodococcus* R312 [8, 9], *R. rhodochrous* J1, and *Nocardia rhodochrous* strains have been used for nicotinamide production with significant yield [10–12]. In above mentioned cases, optimization of nicotinamide production was done using conventional approach, which involved varying one parameter at a time and keeping the other constant. This approach is easy to apply without any use of statistical analysis. This method is time consuming and requires a relatively large number of experiments, and it does not bring about the effect of interaction of various parameters; thus, it frequently fails to anticipate the optimal condition [13, 14]. In comparison, response surface methodology (RSM) is a powerful technique for testing multiple process variables with fewer experimental trials. Interaction between variables can be identified and quantified by using the statistical approach [15, 16]. Central composite design (CCD) is a well-established and widely used statistical technique for determining the crucial factors from a large number of variables by a small number of experiments. Experimental designs have been used for the optimization of multiple variables with minimum number of experiments [17–19].

The reaction optimization is a relevant aspect to be considered in the biotransformation, which has an influence on product yield. Optimization of reaction parameters for conversion of acrylonitrile to acrylamide by *R. rhodochrous* PA-34 showed rallied effect on acrylamide yield [20]. Successful experimental design and RSM have been applied for the reaction optimization of pectin hydrolysis and for the synthesis of sorbitan methacrylate [21, 22]. To the best of our knowledge, there are few reports that describe the use of RSM for the optimization of reaction conditions in the production of nicotinamide by NHase. The aim of the present investigation is to study the effect of reaction conditions (pH, temperature, substrate, and cell concentration), which have been predicted to play a very significant role on the biotransformation of 3-cynopyridine to nicotinamide using statistical approach.

Materials and Methods

Microorganism

Different strains from natural and culture collection sources were screened. Among the screened strains, *Rhodococcus erythropolis* MTCC 1526 (procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh) had shown remarkable NHase activity and was used for further optimization studies.

Table 1 Levels of factor chosen for experimental design.

Factors	Symbols	Code levels		
		−1	0	+1
pH	A	6.5	7	7.5
Temperature	B	15	20	25
Substrate concentration	C	18	20	22
Cell concentration	D	190	200	210

Culture Conditions

Rhodococcus erythropolis strain was maintained on a nutrient agar medium (pH 7.0). Seed culture was developed by inoculating a single colony of *R. erythropolis* into 20 ml nutrient medium (pH 7.0) containing (*w*/*v*) peptone 0.5%, beef extract 0.15%, yeast extract 0.15%, and NaCl 0.5% and incubated at 25 °C for 20 h in a rotary shaker (160 rpm). This inoculum (4%, *v*/*v*) was transferred to the production medium (pH 8.0) and grown for 48–60 h at 25 °C in a rotary shaker (160 rpm). The composition of production medium was (*w*/*v*) peptone 0.5%, beef extract 0.15%, yeast extract 0.15%, and NaCl 0.5%, KH_2PO_4 0.5%, K_2HPO_4 0.5%, and $MgSO_4$ 0.5%. Culture medium was supplemented with $CoCl_2$ (0.01%), which was required for the catalytic activity of NHase.

Table 2 Central composite design matrix for the experimental and predicted results in the production of nicotinamide.

Run	Block	Factors				Actual value (%)	Predicted value (%)
		A	B	C	D		
1	1	7.00	20.00	83.24	200.00	85.24	92.82
2	1	7.00	20.00	83.24	200.00	82.50	93.71
3	1	7.50	25.00	93.73	190.00	90.20	88.42
4	1	7.50	25.00	93.86	190.00	80.24	90.93
5	1	7.00	20.00	83.24	200.00	83.24	91.36
6	1	6.50	15.00	85.19	190.00	70.10	84.28
7	1	6.50	25.00	91.55	210.00	69.50	90.64
8	1	6.50	15.00	79.88	190.00	78.40	79.73
9	1	7.50	15.00	18.00	210.00	75.24	84.30
10	1	6.50	15.00	18.00	190.00	77.12	84.30
11	1	7.00	20.00	20.00	200.00	90.80	84.30
12	1	7.50	15.00	22.00	210.00	78.40	84.30
13	2	7.00	20.00	20.00	200.00	79.90	87.25
14	2	7.00	20.00	20.00	216.82	65.90	85.64
15	2	7.00	11.59	20.00	200.00	81.80	73.29
16	2	7.00	11.59	20.00	200.00	55.76	90.91
17	2	7.84	20.00	20.00	200.00	75.20	92.36
18	2	7.00	20.00	20.00	200.00	88.70	92.54
19	2	7.00	20.00	20.00	183.18	80.20	83.06
20	2	7.00	28.41	20.00	200.00	60.10	89.53
21	2	6.16	20.00	20.00	200.00	82.10	83.51
22	2	7.00	20.00	23.36	200.00	85.24	83.51

Table 3 Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental design.

Source	Sum of squares	*df*	*P>F*
Model	1,378.19	14	0.0447
A	114.00	1	0.0708
B	215.07	1	0.0236
C	17.60	1	0.4219
D	102.24	1	0.0831
A^2	264.29	1	0.0156
B^2	337.32	1	0.0093
C^2	12.88	1	0.4889
D^2	76.79	1	0.1220
AB	16.57	1	0.4351
AC	15.79	1	0.4455
AD	52.52	1	0.1872
BC	64.52	1	0.1500
BD	2.50	1	0.7562
CD	10.40	1	0.5324
Residual	142.21	6	
Lack of fit	61.24	2	0.3242

Preparation of Resting Cell Suspension

Resting cell suspension was prepared by centrifugation of the cell-slurry at 7,000×*g* for 20 min at 4 °C. Pellet was washed with phosphate buffer (10 mM, pH 7.0) at 10,000×*g* for 10 min and resuspended in the same phosphate buffer.

Biocatalytic Reaction Setup

Biotransformation of 3-cyanopyridine to nicotinamide was performed by subjecting the resting cells of *R. erythropolis* (200 mg/ml) to 20 mM 3-cyanopyridine and incubated at 20 °C. Samples were taken at regular time interval and analyzed for the percentage conversion.

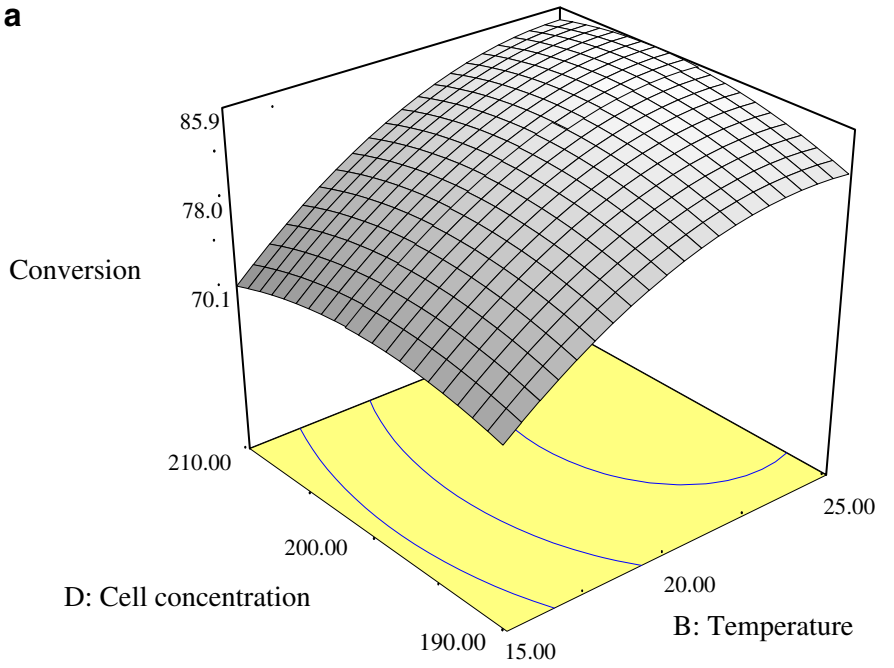
Analytical Method

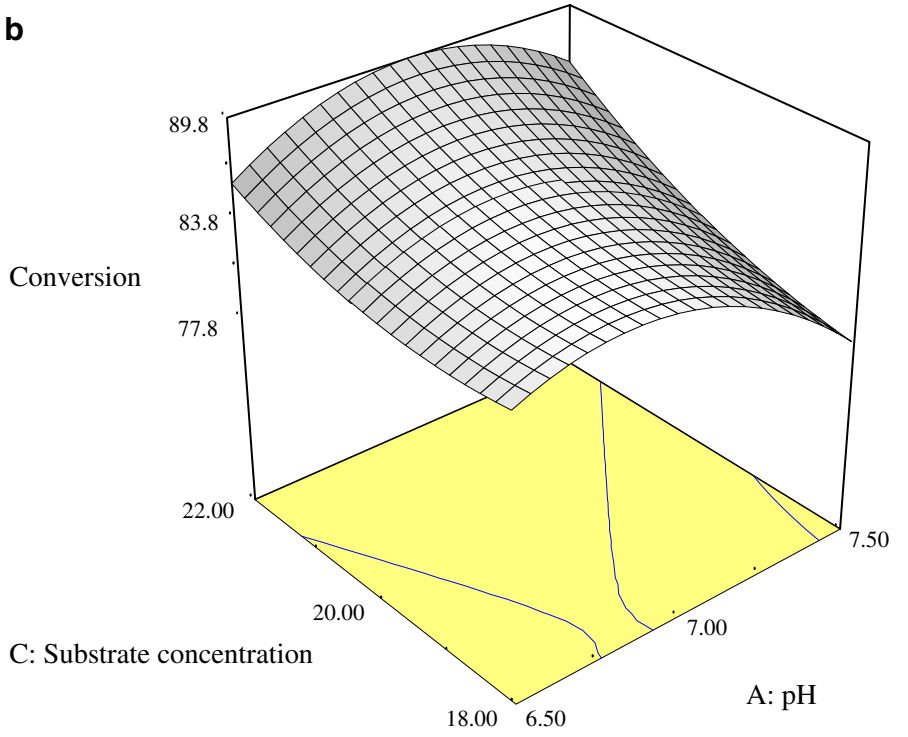
The conversion of 3-cyanopyridine to nicotinamide in the reaction mixture was measured by high performance liquid chromatography (Shimadzu 10AD VP, Kyoto, Japan) using a packed C_{18} column (reverse-phase column, 4.6×250 mm; Waters, Milford, MA, USA and Ireland). The mobile phase consisted of phosphate buffer (50 mM, pH 7.0) and acetonitrile (95:5) at a flow rate of 1 ml/min and was detected at 230 nm.

Experimental Design

Four experimental factors (pH, temperature, substrate, and cell concentration), which were expected to have an effect on nicotinamide synthesis, were identified by preliminary

Fig. 1 Three-dimensional contour plots of the nicotinamide production by *Rhodococcus erythropolis* as a function of **a** cell concentration and temperature and **b** substrate concentration and pH ▶


a
85.9
78.0
70.1
Conversion
210.00
200.00
190.00
D: Cell concentration
15.00
20.00
25.00
B: Temperature


b
89.8
83.8
77.8
Conversion
22.00
20.00
18.00
C: Substrate concentration
6.50
7.00
7.50
A: pH

experiment. These variables were found to have significant effect on the conversion. Therefore, these four variables were chosen for further optimization through RSM. Three level CCD was used to optimize the response of four variables. A 2^4 factorial design was generated to study the effect of reaction pH, temperature, and substrate and cell concentration on the conversion of 3-cyanopyridine. The statistical analysis of the results was performed with the aid of Design Expert ver. 6.0.9 statistical software (Stat-Ease, Minneapolis, MN, USA). The conversion was analyzed using the analysis of variance (ANOVA) combined with the Fisher test to evaluate if a given term has a significant effect ($p \leq 0.05$). The optimum levels of the variables were obtained by graphical and numerical analysis using Design Expert program.

Results and Discussion

Central Composite Design

Based on the results obtained in preliminary experiments, reaction pH, temperature, substrate, and cell concentration were chosen as important process parameters for the nicotinamide synthesis and were selected to find out the optimized conditions for higher nicotinamide production using CCD and RSM. A CCD with three-coded levels for all the four factors, pH (A), temperature (B), substrate (C), and cell concentration (D), were used for this purpose. The levels of variables for the CCD were based on the preliminary results. The range of the variables is given in Table 1. The experimental design and the results of the CCD obtained for nicotinamide production are presented in Table 2. The coefficients of regression were calculated, and the experimental results of the CCD were fitted with a second-order polynomial equation. The fitted equation (in terms of coded values) for predicting conversion (Y) was:

$$Y = +83.60 + 4.49A + 6.17B + 1.14C - 4.25D - 4.14A^2 - 4.68B^2 + 0.91C^2 - 2.23D^2 - 2.24AB + 1.41AC + 3.98AD + 2.84BC + 0.87BD + 1.14CD$$

Analysis of Variance

The data of ANOVA proved that the applied model fits the experimental values (Table 3). The Model F value of 4.15 implies the model is significant. The regression equation obtained from ANOVA indicated that the R^2 value (multiple correlation coefficient) is 0.9065 (a value >0.75 indicates aptness of the model), indicating that 90.65% of the variability in the response could be explained by the model. Furthermore, the model has an "adequate precision value" of 7.711. According to the fitted model, the optimum levels for four variables were A=6.85, B=24.8, C=21.98, D=190.98. The predicted 3-cyanopyridine conversion corresponding to these values is 90.1%. The conversion of 3-cyanopyridine to nicotinamide at a reaction temperature of 25 °C and pH 8.0 were reported for NHase by *Rhodococcus* R312 [8].

Verification of the Experimental Model

Verification of the predicted results was accomplished by carrying out three sets of experiments using these optimized reaction parameters. These triplicate experiments yielded an average maximum conversion of 93%. The experimentally determined

conversion value was in close agreement with the statistically predicted value, thus confirming the model's authenticity. The response surface shown in Fig. 1a describes the quadratic effect of cell concentration and temperature on nicotinamide production by *R. erythropolis*. Here, the substrate concentration and pH were kept constant at the centre point values. The increase of the conversion (%) occurs when cell concentration is at its lowest level, and the production increased considerably due to the increase of reaction temperature. The response also varied noticeably at different levels of cell concentration along the axis suggesting that there is a considerable interaction between these two factors. Figure 1b showed the effect of substrate concentration and pH on nicotinamide production, where quadratic effect of pH is significant in comparison to the effect of substrate concentration.

Conclusion

This study demonstrated that the use of statistically based designs can serve as efficient tools in process development, where an analysis of the effect and interaction of many experimental factors are required. CCD allowed smaller and less time-consuming experimental designs with identification of the important factors and interaction between them. In this study, CCD and RSM were useful to determine the optimum levels of the components that significantly influence the nicotinamide production. The optimum reaction conditions for the production of nicotinamide by *R. erythropolis* was established as follows: reaction pH 6.85, temperature 24.8 °C, cell concentration 190.98 mg/ml, and substrate concentration 21.98 mM.

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