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ORIGINAL PAPER

# Enhanced production of amidase from *Rhodococcus erythropolis* MTCC 1526 by medium optimisation using a statistical experimental design

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**Abstract** In the present work, statistical experimental methodology was used to enhance the production of amidase from *Rhodococcus erythropolis* MTCC 1526. *R. erythropolis* MTCC 1526 was selected through screening of seven strains of *Rhodococcus* species. The Placket–Burman screening experiments suggested that sorbitol as carbon source, yeast extract and meat peptone as nitrogen sources, and acetamide as amidase inducer are the most influential media components. The concentrations of these four media components were optimised using a face-centred design of response surface methodology (RSM). The optimum medium composition for amidase production was found to contain sorbitol (5 g/L), yeast extract (4 g/L), meat peptone (2.5 g/L), and acetamide (12.25 mM). Amidase activities before and after optimisation were 157.85 units/g dry cells and 1,086.57 units/g dry cells, respectively. Thus, use of RSM increased production of amidase from *R. erythropolis* MTCC 1526 by 6.88-fold.

**Keywords** Amidase · *Rhodococcus erythropolis* · Plackett–Burman screening · Response surface methodology · Medium optimisation

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## Introduction

Amidases (acylamide amidohydrolases; EC 3.5.1, 3.5.2) catalyse the hydrolysis of carboxylic acid amides to free carboxylic acids and ammonium [1]. These enzymes are distributed widely in almost all kingdoms of the living world [2]. The exact physiological role of amidase is not yet clearly understood; however, in some species amidases (along with nitrile hydratases) are involved in the utilisation of nitrogen from nitriles. In recent years, amidases have gained considerable interest in such diverse research fields as chirotechnology, neurobiochemistry and plant physiology [3].

Microbial amidases have emerged as important industrial biocatalysts. For example, amidase-catalysed synthesis of acrylamide and acrylic acid is one of the largest industrial biotransformations in the world [4]. Moreover, a wide range of commercially useful chemicals can be synthesised using microbial amidases [5–9]. Microbial amidases are abundant in Rhodococci, Nocardia, and Arthrobacteria. Rhodococcal amidases exhibit fairly wide substrate specificity and often demonstrate stereoselective hydrolysis of amides of α-substituted carboxylic acids [1]. Along with nitrile hydratases, amidases can catalyse the unique conversion of nitriles to the corresponding enantiopure carboxylic acids. Rhodococcal amidase (and nitrile hydratase) find applications in the stereoselective synthesis of several drugs/drug intermediates [4, 10–13]. Hence, large scale fermentative production of Rhodococcal amidase is of great commercial interest.

In order to make the fermentative process commercially viable, it is necessary to improve the yield without increasing the cost of production. One method of achieving this objective is selection of appropriate media components and optimal culture conditions. Thus, optimisation of

fermentation conditions is generally regarded as the most crucial primary step in the development of a cost-effective fermentation process [14].

The conventional method of medium optimisation involves changing one parameter at a time and keeping the others at fixed levels. Besides being labourious and time consuming, this method does not account for interactive effects between operational variables during the fermentation process [14]. Furthermore, being linear in nature, the conventional method is incapable of determining the interactive effects of variables and is therefore unable to predict the 'true' optimum [15, 16]. The limitations of one-factor-at-a-time optimisation can be eliminated by employing statistical optimisation methods. Among different statistically based approaches, response surface methodology (RSM) has been used extensively in media optimisation. RSM is a collection of statistical techniques that uses design of experiments (DoE) for building models, evaluating the effects of factors and searching for optimum conditions. It is a statistically designed experimental protocol in which several factors are simultaneously varied [17]. In biotechnology, this technique has been used for a broad range of primary as well as secondary microbial metabolites, viz. enzymes, acids, terpenoids, etc. However, to the best of our knowledge, such a detailed study has not yet been reported for Rhodoccocal amidase.

In present work, production of amidase from seven different *Rhodococcus* strains using different carbon and nitrogen sources was studied and *Rhodococcus erythropolis* MTCC 1526 was selected for further experiments. A systematic and sequential optimisation strategy was adopted to enhance the production of amidase from *R. erythropolis* MTCC 1526. An optimal medium composition for production of amidase was achieved in the following three steps: (1) screening of optimum carbon source, nitrogen source and inducer; (2) use of Placket–Burman experimental design to select the most influential media components; and (3) use of a face-centre central composite design (FCCCD) of RSM for optimisation of critical media constituents.

## Materials and methods

### Strain and cultivation conditions

The seven microbial strains, namely *R. erythropolis* MTCC 1526, 1548 and *Rhodococcus* spp. MTCC 2574, 2678, 2683, 2794 and 3951, were procured from MTCC (Chandigarh, India). All bacterial growth medium components were purchased from Hi-Media (Mumbai, India). All other chemicals were of analytical grade procured from S.D. Fine Chemicals (Mumbai, India).

All *Rhodococcus* strains were maintained on nutrient agar. The liquid fermentation medium used for batch culture experiments contained (g/L): glucose (10), yeast extract (3), meat peptone (7.5), $Na_2HPO_4$ (4.0), $KH_2PO_4$ (2.0), $MgSO_4{\cdot}7H_2O$ (0.2), $CaCl_2{\cdot}2H_2O$ (0.02), ammonium ferric citrate (0.05), acetamide (10 mM) and trace mineral solution (1 ml/l; termed as medium A). The composition of trace mineral medium was (g/L) $H_3BO_3$ (0.1), $MnCl_2{\cdot}4H_2O$ (0.1), $ZnSO_4{\cdot}H_2O$ (0.1), $FeCl_3{\cdot}6H_2O$ (0.1), $CaCl_2{\cdot}2H_2O$ (1), $CuCl_2{\cdot}2H_2O$ (0.05) [12].

### Effect of fermentation time on amidase production

The amidase activities (units/g dry cells) produced by the seven *Rhodococcus* strains were studied at regular time intervals (24, 36 and 48 h) in order to determine the effect of fermentation time on amidase production.

### Selection of optimum nitrogen source, carbon source and inducer

Components containing organic nitrogen (yeast extract and meat peptone) from medium A were replaced with inorganic nitrogen sources (namely urea, ammonium sulfate and ammonium phosphate) at an equivalent nitrogen level. To evaluate the optimum carbon source, glucose was replaced by an equivalent amount of different carbon sources, namely glycerol, sucrose, sorbitol and mannitol. Seven inducers (namely acetamide, acetamidophenol, acetamido acetophenon, 4-acetamido benzaldehyde, acetanilide, acrylamide and urea; 10 mM each) were screened to evaluate the enhancement in amidase production. Among these seven different inducers, acetamide was found to give maximum amidase production and was therefore selected for further experiments.

### Amidase activity profile for *R. erythropolis* MTCC 1526

Cell growth and intracellular amidase activity were studied simultaneously to determine the optimum fermentation time in shake flask fermentation. *R. erythropolis* MTCC 1526 was grown on liquid medium containing (g/L) sorbitol (10.12), yeast extract (3), meat peptone (7.5), $Na_2HPO_4$ (4.0), $KH_2PO_4$ (2.0), $MgSO_4{\cdot}7H_2O$ (0.2), $CaCl_2{\cdot}2H_2O$ (0.02), ammonium ferric citrate (0.05), trace mineral solution (1 ml/L) and acetamide (10 mM; termed as medium B). Samples were removed aseptically at regular intervals from 6 h up to 48 h and analysed for optical density (600 nm), and amidase activity (500 nm).

### Screening of critical media components using a Plackett–Burman design

A total of seven process variables comprising six media components (namely sorbitol, yeast extract, meat peptone, $Na_2HPO_4$, $KH_2PO_4$ and acetamide) and one dummy variable were studied in Plackett–Burman screening experiments. Experiments were performed at various combinations of 'high' (H) and 'low' (L) values of the process variables and analysed for their effects on the response of the process [18]. All trials were performed in duplicate and the average of amidase activity (units/g dry cells) was used as response.

### Experimental design and data analysis

The concentrations of the four media components (sorbitol, yeast extract, meat peptone and acetamide) were optimized by using FCCCD of RSM [17]. The value of the dependent response was the mean of two replications. Second-order polynomial coefficients were calculated and analysed using the trial version of 'Design Expert' software (Version 6.0, Stat-Ease, Minneapolis, MN).

### Amidase activity assay

Amidase activity (acyl transfer activity) was determined spectophotometrically at 500 nm as described previously [5]. *Rhodococcus* cells were isolated from fermentation medium by centrifugation. Cells were washed twice with 0.1 M sodium phosphate buffer (pH 7.2). The washed cells (0.01 g wet weight) were suspended in cold sodium phosphate buffer and subjected to ultrasonic treatment for 5 min. Cell debris was removed by centrifugation (15,000 rpm for 10 min). The supernatant (0.1 ml) was analyzed for amidase activity. The ratio of wet weight to dry weight was determined for each sample and the enzyme activity is expressed in units per gram of dry cells as reported earlier [19]. One unit is defined as amount of enzyme that converts 1.0 μmol acetamide and hydroxylamine to acethydroxamate and ammonia per minute at pH 7.2 at 37°C.

## Results and discussion

### Optimisation of fermentation time for amidase production

Amidase production (amidase activity; units/g dry cells) by seven *Rhodococcus* strains at different time intervals was studied. All *Rhodococcus* strains gave maximum amidase production at 36 h (except MTCC 2794 and MTCC 3951 where maximum amidase production was observed at 24 h). All further fermentations of seven *Rhodococcus* strains were terminated at their respective optimised fermentation time.

### Selection of optimum nitrogen source, carbon source and inducer

Use of inorganic nitrogen source resulted in reduced cell density and amidase activity (results not shown) and therefore organic nitrogen sources (yeast extract and meat peptone) were selected for further studies. *R. erythropolis* MTCC 1526 gave maximum amidase activity when grown on sorbitol as carbon source (Table 1). Hence *R. erythropolis* MTCC 1526 was selected and sorbitol was selected as an optimum carbon source for further studies. The presence of amides in the fermentation medium is known to enhance amidase production [20]. The amidase activities of *R. erythropolis* MTCC 1526 cells grown on different inducers are given in Fig. 1. Among seven inducers tested, acetamide gave maximum amidase activity (464.23 units/g dry cells) and was therefore selected for further experiments.

**Table 1** Effect of carbon source on amidase production by seven *Rhodococcus* strains

| *Rhodococcus* strain | Fermentation time (h) | Amidase activity (units/g dry cells) | | | | |
|---|---|---|---|---|---|---|
| | | Glucose | Glycerol | Mannitol | Sorbitol | Sucrose |
| MTCC 1526 | 36 | 157.85 | 17.54 | 19.66 | 489.14 | 251.43 |
| MTCC 1548 | 36 | 115.40 | Nil | Nil | 11.27 | 136.97 |
| MTCC 2574 | 36 | 160.49 | 0.59 | Nil | 21.83 | 1.09 |
| MTCC 2678 | 36 | 91.38 | 1.96 | 21.40 | 221.38 | 58.53 |
| MTCC 2683 | 36 | 184.82 | Nil | 12.64 | 30.36 | 23.26 |
| MTCC 2794 | 24 | 284.41 | 113.15 | 206.11 | 359.84 | 184.04 |
| MTCC 3951 | 24 | 212.40 | 180.35 | Nil | 359.38 | Nil |

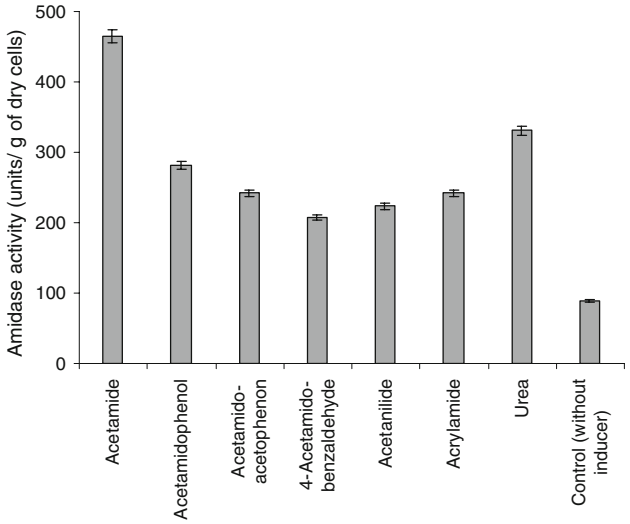


**Fig. 1** Screening for inducers of amidase production by *Rhodococcus erythropolis* MTCC 1526 [medium A + amidase inducer (10 mM); incubated for 36 h at 30°C on rotary shaker at 200 rpm]

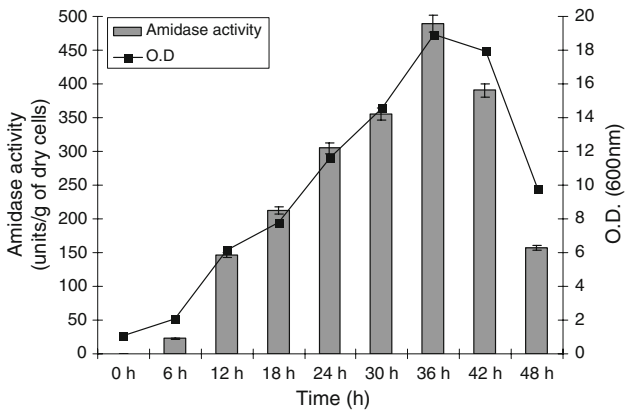


**Fig. 2** Growth profile and amidase production by *Rhodococcus erythropolis* MTCC 1526 (medium B; 30°C at 200 rpm)

## Amidase activity profile for *R. erythropolis* MTCC 1526

Cell growth and amidase activity were simultaneously studied with respect to fermentation time, and the amidase activity profile was established (Fig. 2). During the course of fermentation, maximum amidase activity was found at 36 h and thereafter the activity was found to decline along with cell density from 42 to 48 h. Therefore all further fermentations were terminated at 36 h.

## Screening of significant media components using a Plackett–Burman design

The Plackett–Burman design served the purpose of ascertaining the critical influential process variables. Here, seven independent variables were screened in eight combinations organised according to the Plackett–Burman design as shown in Table 2. The results of the Plackett–Burman design calculations along with the corresponding variable-specific *F*-scores are summarised in Table 3. A high *F*-score value indicates greater significance of the input variable. Thus, depending on the *F*-score, sorbitol, yeast extract, meat peptone and acetamide were found to be the most influential media components. The dummy variable, which is not allocated any value, gives some redundancy required by the statistical procedure. Moreover, incorporation of the dummy variable into an experiment allows an estimation of the variance. The mean square deviation of the dummy variable was found to be very low compared to that of the influential variables.

## Media optimisation by RSM

The DoE and respective experimental and predicted values of amidase activities are given in Table 4. The second-order

**Table 2** Plackett–Burman experimental design

| Trial | Sorbitol | Yeast extract | Meat peptone | $Na_2HPO_4$ | Dummy variable | $KH_2PO_4$ | Acetamide | Amidase activity[a] |
|---|---|---|---|---|---|---|---|---|
| 1 | H | H | H | L | H | L | L | 358.98 |
| 2 | L | H | H | H | L | H | L | 191.50 |
| 3 | L | L | H | H | H | L | H | 266.81 |
| 4 | H | L | L | H | H | H | L | 191.46 |
| 5 | L | H | L | L | H | H | H | 259.93 |
| 6 | H | L | H | L | L | H | H | 417.88 |
| 7 | H | H | L | H | L | L | H | 474.95 |
| 8 | L | L | L | L | L | L | L | 92.57 |

*H* and *L* represent high level and low level of media component, respectively (sorbitol H = 10 g/L and L = 1 g/L, yeast extract H = 5 g/L and L = 1 g/L, meat peptone H = 10 g/L and L = 1 g/L, $Na_2HPO_4$ H = 6 g/L and L = 2 g/L, $KH_2PO_4$ H = 3 g/L and L = 1 g/L, acetamide H = 50 mM and L = 5 mM)

[a] Amidase activity is expressed in units/g of dry cells

**Table 3** Statistical calculations for Plackett–Burman design

| Parameter | Sorbitol | Yeast extract | Meat peptone | $Na_2HPO_4$ | Dummy variable | $KH_2PO_4$ | Acetamide |
|---|---|---|---|---|---|---|---|
| $Y_{i+}$ | 1,443.26 | 1,285.36 | 1,235.17 | 1,124.72 | 1,077.17 | 1,060.77 | 1,419.57 |
| $Y_{i-}$ | 810.81 | 968.72 | 1,018.91 | 1,129.36 | 1,176.91 | 1,193.31 | 834.51 |
| $\Sigma Y_{i+} - \Sigma Y_{i-}$ | 632.45 | 316.64 | 216.25 | −4.642 | −99.73 | −132.54 | 585.06 |
| MSD | 49,999.17 | 12,532.58 | 5,845.96 | 2.694 | 1,243.31 | 2,195.88 | 42,788.04 |
| *F*-score | 40.21 | 10.08 | 4.70 | 0.002 | 1.00 | 1.76 | 34.41 |

**Table 4** Face centre design matrix of independent variables and their corresponding experimental and predicted values of response

| No. | Media components (coded values)[a] | | | | Amidase activity (units/g dry cells) | |
|---|---|---|---|---|---|---|
| | Sorbitol | Yeast extract | Meat peptone | Acetamide | Experimental[b] | Predicted |
| 1 | −1 | −1 | −1 | −1 | 789.05 | 831.48 |
| 2 | 1 | −1 | −1 | −1 | 688.93 | 581.69 |
| 3 | −1 | 1 | −1 | −1 | 679.29 | 702.27 |
| 4 | 1 | 1 | −1 | −1 | 117.58 | 195.02 |
| 5 | −1 | −1 | 1 | −1 | 598.23 | 540.41 |
| 6 | 1 | −1 | 1 | −1 | 432.29 | 592.54 |
| 7 | −1 | 1 | 1 | −1 | 330.63 | 252.40 |
| 8 | 1 | 1 | 1 | −1 | 0.9 | 47.08 |
| 9 | −1 | −1 | −1 | 1 | 534.28 | 566.95 |
| 10 | 1 | −1 | −1 | 1 | 112.07 | 204.05 |
| 11 | −1 | 1 | −1 | 1 | 1,162.09 | 1,015.59 |
| 12 | 1 | 1 | −1 | 1 | 258.58 | 395.24 |
| 13 | −1 | −1 | 1 | 1 | 469.37 | 405.68 |
| 14 | 1 | −1 | 1 | 1 | 288.84 | 344.70 |
| 15 | −1 | 1 | 1 | 1 | 509.44 | 695.53 |
| 16 | 1 | 1 | 1 | 1 | 405.78 | 377.10 |
| 17 | −1 | 0 | 0 | 0 | 819.96 | 897.27 |
| 18 | 1 | 0 | 0 | 0 | 498.98 | 329.04 |
| 19 | 0 | −1 | 0 | 0 | 616.25 | 585.31 |
| 20 | 0 | 1 | 0 | 0 | 550.18 | 488.49 |
| 21 | 0 | 0 | −1 | 0 | 890.21 | 861.28 |
| 22 | 0 | 0 | 1 | 0 | 615.77 | 552.07 |
| 23 | 0 | 0 | 0 | −1 | 54.17 | 47.46 |
| 24 | 0 | 0 | 0 | 1 | 198.86 | 112.95 |
| 25 | 0 | 0 | 0 | 0 | 789.45 | 783.18 |
| 26 | 0 | 0 | 0 | 0 | 780.3 | 783.18 |
| 27 | 0 | 0 | 0 | 0 | 788.22 | 783.18 |
| 28 | 0 | 0 | 0 | 0 | 781.74 | 783.18 |
| 29 | 0 | 0 | 0 | 0 | 778.82 | 783.18 |
| 30 | 0 | 0 | 0 | 0 | 780.56 | 783.18 |

[a] Real values (in sequence of −1, 0, +1) sorbitol 5, 12.5, 20 g/L, yeast extract 1, 2.5, 4.0 g/L, meat peptone 2.5, 6.25, 10 g/L, acetamide 5, 10, 15 mM

[b] Values indicate mean of duplicate observations

polynomial equation was used to correlate the independent process variables with amidase production. The second-order polynomial coefficient for each term of the equation was determined through multiple regression analysis using the Design Expert. After regression analysis, the second-order response model was obtained as follows:

**Table 5** Analysis of variance (ANOVA) of the model

| Source | *df* | *F* value | Probability > *F* (*P* value) |
|---|---|---|---|
| Model significant | 14 | 11.104 | <0.0001 |
| A | 1 | 36.065 | <0.0001 |
| B | 1 | 1.047 | 0.322 |
| C | 1 | 10.679 | 0.005 |
| D | 1 | 0.479 | 0.499 |
| $A^2$ | 1 | 3.690 | 0.074 |
| $B^2$ | 1 | 7.742 | 0.014 |
| $C^2$ | 1 | 0.747 | 0.401 |
| $D^2$ | 1 | 63.084 | <0.0001 |
| AB | 1 | 4.935 | 0.042 |
| AC | 1 | 6.788 | 0.019 |
| AD | 1 | 0.952 | 0.345 |
| BC | 1 | 1.877 | 0.191 |
| BD | 1 | 24.865 | 0.0002 |
| CD | 1 | 1.254 | 0.280 |

$$\begin{aligned}\text{Amidase activity(units/g of dry cells)} &= +783.18167 - 142.05708A - 24.20458B \\ &\quad - 77.30292C + 16.37208D - 42.50510A^2 - 61.57B^2 \\ &\quad - 19.13C^2 - 175.74D^2 - 64.36AB + 75.48AC \\ &\quad - 28.28AD - 39.70BC + 144.46BD + 32.45CD \end{aligned} \quad (1)$$

where A is sorbitol, B yeast extract, C meat peptone, D acetamide, and A, C, $B^2$, $D^2$, AB, AC, BD were identified as significant terms. Thus, the interactive effects between 'sorbitol and yeast extract' (AB); 'sorbitol and meat peptone' (AC) and 'yeast extract and acetamide' (BD) were found to be predominant in the given system. The model significance was analysed using analysis of variance (ANOVA) for the experimental design (Table 5). Model *F*-value was calculated as a ratio of mean square regression and mean square residual. The model *F*-value of 11.104 implied that the model was significant, and that there was only a 0.01% chance that a large 'Model *F*-value' could occur due to noise. The *P*-value was used as a tool to check the significance of each of the coefficients, which, in turn, are necessary to understand the pattern of the mutual interactions between the process variables. The smaller the magnitude of *P*, the more significant is the corresponding coefficient. Values of $P < 0.05$ indicate that model terms are significant.

**Table 6** Model fitting values

| No. | Model terms | Values |
|---|---|---|
| 1 | Coefficient of the variation | 21.301 |
| 2 | $R^2$ | 0.912 |
| 3 | Adjusted $R^2$ | 0.829 |
| 4 | Adequate precision | 11.819 |
| 5 | Standard deviation | 115.883 |

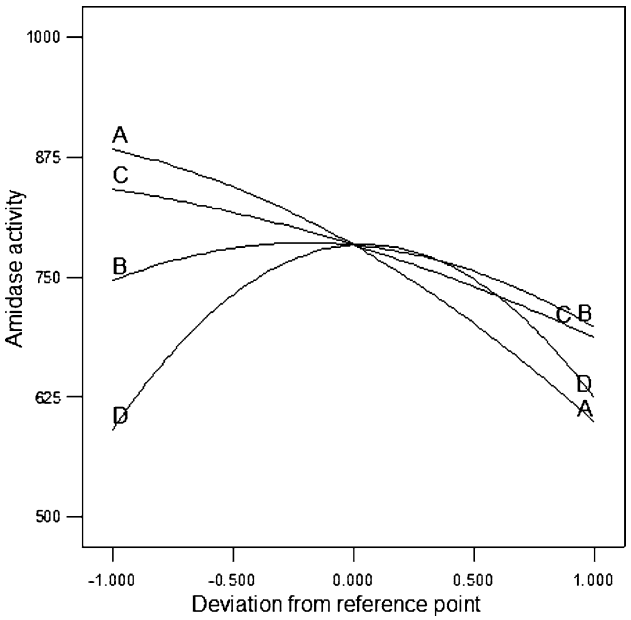


**Fig. 3** Perturbation plot indicating the relative effect of each media component on amidase activity (units per gram of dry cells); *A* sorbitol, *B* yeast extract, *C* meat peptone, *D* acetamide

The model fitting values, which indicate model adequacy, are given in Table 6. A low value of coefficient of variation (21.301%) indicates the very high degree of precision and good reliability of the experimental values. The fit of the model can also be expressed by the coefficient of determination, $R^2$, which was found to be 0.912, indicating that 91.2% of the variability in the response could be explained by the model. The closer the $R^2$ value is to 1, the better is the model fit to experimental data and the less the distance between predicted and observed values. 'Adeq Precision' measures the signal to noise ratio. A ratio >4 is desirable. Here, a ratio of 11.819 indicates an adequate signal.

A perturbation plot was obtained to observe the relative effect of media components on amidase activity (Fig. 3). The perturbation plot indicates that acetamide (D) is the most influential medium component, whereas yeast extract (B) has least influence on amidase activity. The three-dimensional plots of the statistically significant interactions are shown in Fig. 4. These plots were obtained from the pair-wise combination of two independent variables, while keeping the other two variables at their centre point levels. From the bump of three-dimensional plot or the central point of its respective contour plot; the optimal composition of medium components can be identified. The contour

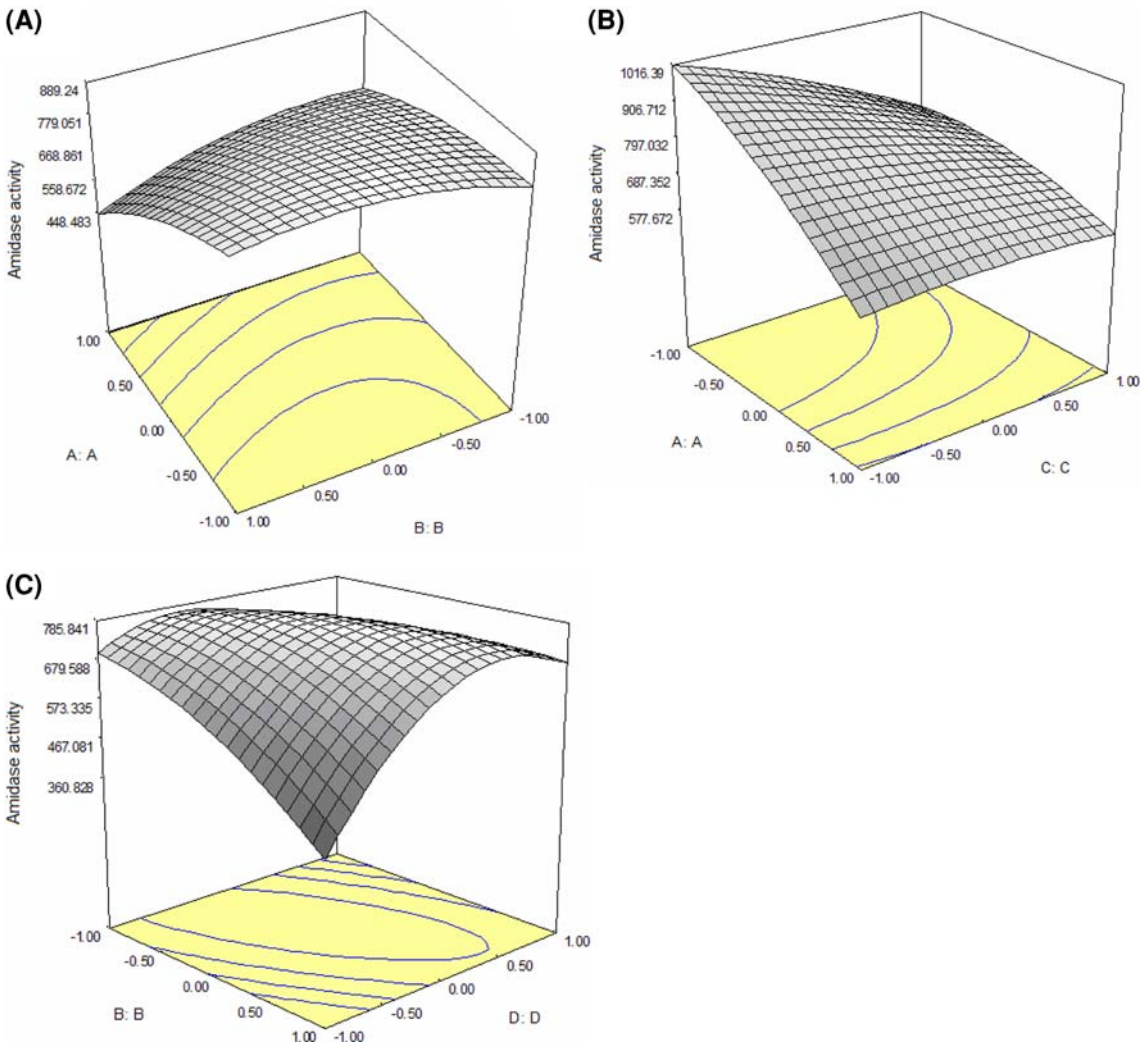


**Fig. 4** Three-dimensional response surface contour plots for amidase activity (units per gram of dry cells). **a** Sorbitol and yeast extract, **b** sorbitol and meat peptone, **c** yeast extract and acetamide

**Table 7** Experimental validation of model predicted values of amidase activity

| Solution | Media composition (g/l) | | | | Amidase activity (units/g dry cells) | | Percentage deviation |
|---|---|---|---|---|---|---|---|
| | Sorbitol | Yeast extract | Meat peptone | Acetamide | Predicted value | Observed value | |
| 1 | 5.00 | 4.00 | 2.50 | 12.25 | 1,069.33 | 1,086.57 | 1.61 |
| 2 | 5.00 | 4.00 | 2.50 | 12.40 | 1,069.24 | 1,084.84 | 1.46 |
| 3 | 5.00 | 3.88 | 2.50 | 11.90 | 1,067.59 | 1,077.73 | 0.95 |

response plots highlight the roles played by process variables and their interactive effects.

The three optimum solutions were obtained by substituting levels of the factors into the regression equation (Eq. 1). The media composition for these optimal solutions and the corresponding predicted responses (amidase activity) are summarised in Table 7. The predicted response of three optimal solutions was verified experimentally by conducting actual fermentation runs. In the first optimal solution, the concentrations of four media components were found as: sorbitol (5 g/L), yeast extract (4 g/L), meat peptone (2.5 g/L), acetamide (12.25 mM; all remaining components are same as mentioned in medium B). The predicted response of first optimal solution (1,069.33 units/g dry cells) was experimentally verified (1,086.57 units/g dry cells). The close agreement between predicted values and experimental values of amidase activities confirms the significance of the model. The amidase activities before and

after optimization were 157.85 units/g dry cells and 1,086.57 units/g dry cells, respectively. Thus, use of RSM has increased the production of amidase from *R. erythropolis* MTCC 1526 by 6.88-fold.

## Conclusions

The production of amidase was found to depend greatly on four media components (namely sorbitol, yeast extract, meat peptone and acetamide). Using RSM, it was possible to model the individual and interactive effects of media components on production of amidase. The validity of the model was confirmed by the close agreement between experimental and predicted values. Medium optimisation by RSM effectively enhanced amidase production by 6.88-fold.

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