Optimization of Biotransformation of L-Tyrosine to L-DOPA by *Yarrowia lipolytica*-NCIM 3472 Using Response Surface Methodology

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Abstract L-DOPA (3,4-dihydroxyphenyl-L-alanine) is the most widely used drug for treatment of Parkinson's disease. In this study *Yarrowia lipolytica*-NCIM 3472 biomass was used for transformation of L-tyrosine to L-DOPA. The process parameters were optimized using response surface methodology (RSM). The optimum values of the tested variables for the production of L-DOPA were: pH 7.31, temperature 42.9 °C, 2.31 g l⁻¹ cell mass and 1.488 g l⁻¹ L-tyrosine. The highest yield obtained with these optimum parameters along with recycling of the cells was 4.091 g l⁻¹. This optimization of process parameters using RSM resulted in 4.609-fold increase in the L-DOPA production. The statistical analysis showed that the model was significant. Also coefficient of determination (R²) was 0.9758, indicating a good agreement between the experimental and predicted values of L-DOPA production. The highest tyrosinase activity observed was 7,028 U mg⁻¹ tyrosine. L-DOPA production was confirmed by HPTLC and HPLC analysis. Thus, RSM approach effectively enhanced the potential of *Y. lipolytica*-NCIM 3472 as an alternative source to produce L-DOPA.

Keywords L-DOPA · L-Tyrosine · Tyrosinase · *Yarrowia lipolytica* · RSM · Box–Behnken design

Introduction

L-DOPA (3,4-dihydroxyphenyl-L-alanine), is a widely used drug for treatment of Parkinson's disease. The world market for L-DOPA is about 250 tons per year, and the total market value is about \$101 billion per year [1]. The production of L-DOPA has been reported earlier by several biological sources that include *Bacillus* sp. JPJ [1], *Aspergillus oryzae* [2], *Yarrowia lipolytica* NRRL-143 [3] and *Brevundimonas* sp. SGJ [4], *Acremonium rutilum* [5]. In addition, plant sources, such as cell suspension cultures of banana and *Portulaca grandiflora*, seeds of *Mucuna pruriens* [6], *Mucuna monosperma* [7] have also been used for L-DOPA production. Half of the total L-DOPA produced per year involves use of enzymatic method; as, chemical processes use harsh conditions that increases overall cost [5].

Process optimization has long been used to enhance the yield and productivities of many bioprocesses. Optimization of process parameters by classical methods, which involves the change of one variable at a time, is extremely time consuming and expensive when a large number of variables are considered. Also, it fails to determine the combined effect of different factors. In order to overcome this difficulty and determine the interaction between the studied variables, an experimental factorial design and response surface methodology have been employed in optimization [8–10]. As the biotransformation of L-tyrosine to L-DOPA has been reported using different biological sources, it is essential to optimize the process parameters by the use of statistical methods. Hence in this study,

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optimization of critical factors for biotransformation of L-DOPA by *Y. lipolytica*-NCIM 3472 was carried out by using RSM.

Materials and Methods

Chemicals and Microorganism

L-Tyrosine and L-DOPA were purchased from Sigma-Aldrich (St. Louis, MO, USA), cupric sulfate and all other chemicals were procured from Himedia (India). *Y. lipolytica*-NCIM 3472 was purchased from National Collection of Industrial Microorganism (NCIM), NCL, Pune, India. The medium for the cultivation of the *Y. lipolytica* strain composed of (g l^{-1}) yeast extract 1, peptone 0.5 and glucose 1 at pH 7 and 37 °C growth temperature. Two milliliter of cell suspension after 24 h of growth was inoculated in the same fresh medium for growth of the yeast cells. After a growth period of 24 h, cells were harvested by centrifugation at 6,000 rpm.

Biotransformation of L-Tyrosine to L-DOPA

The bioconversion of L-tyrosine to L-DOPA was carried out in a reaction mixture containing 30 ml of potassium phosphate buffer (0.1 M, pH 7) and 0.5 g l^{-1} of L-tyrosine. This mixture was taken in 250 ml of Erlenmeyer flask and autoclaved at 15 psi (121 °C). The cell mass of 0.5 g l^{-1} was re-suspended in the sterilized reaction mixture and incubated at 30 °C for 90 min at 120 rpm in an incubator shaker and the samples were then assayed for tyrosinase activity and L-DOPA produced in the reaction mixture was determined according to Arnow's method [3, 4, 11].

Experimental Design

Based on the results obtained in the previous experiments and literature review [1, 3, 4], pH, temperature, cell mass and L-tyrosine were found to be the determining variables in L-DOPA production. Hence, these variables were evaluated toward achieving enhanced production of L-DOPA. Box–Behnken design was used to study the impact of these parameters on L-DOPA production. As shown in Table S1, factors were arranged into low, middle and high levels, respectively. The full experimental plan with respect to their coded forms obtained from Box–Behnken experiments are listed in Table S2. The variables, each at level with three replicates at the centre points, were used to fit a polynomial model [12]. The Design Expert software (Stat-Ease, USA) was used in the experimental design and data

analysis. The analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model.

Biomass Trend, Tyrosinase Activity and L-DOPA Production

After validation of the experiment using the Design Expert software, the L-DOPA production was observed by comparing the conditions before and after optimization. The biomass trend and tyrosinase activity were observed during growth of *Y. lipolytica*. High-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) analysis of standard L-DOPA (1 g l^{-1}), and reaction was carried out using conditions described earlier [1, 4].

Results and Discussion

RSM and Statistical Analysis

The final empirical model in terms of four factors in coded units was as follows:

$$\begin{aligned} \text{L-DOPA} = & 0.74 - 0.059X_1 + 0.10X_2 + 0.081X_3 \\ & + 0.088X_4 - 0.040X_1X_2 - 3.250E \\ & - 0.03X_1X_3 - 0.074X_1X_4 + 0.028X_2X_3 \\ & + 0.060X_2X_4 + 0.048X_3X_4 - 0.3X_1^2 \\ & - 0.20X_2^2 - 0.15X_3^2 - 0.16X_4^2 \end{aligned}$$

where X_1 is pH, X_2 is temperature, X_3 is cell mass, and X_4 is L-tyrosine.

The results of 29 experiments with predicted values and externally standardised residual response are presented in Table S2. According to the ANOVA analysis in Table S3, the model F value was found to be 40.48013 and <0.0001 , respectively. The parameter estimate and the corresponding p values suggested that all the independent and interactive terms were significant. The lack-of-fit value 0.1822 implied that it is insignificant. Insignificant lack-of-fit is good to fit the model. The coefficient of determination (R^2) was 0.9758, which indicated a good agreement between the experimental and predicted values of L-DOPA production. “Adeq Precision” measures the signal to noise ratio. Adeq Precision ratio >4 is desirable and in this model the ratio value was found to be 14.0093, which indicated a satisfactory outcome. This model can be used to navigate the design space.

Interaction Between Variables

The response surface curve for the interaction between temperature and pH is shown in Fig. 1a, this curve indicated

that lower (below 20 °C) and higher (above 50 °C) levels of both the factors resulted in low yields of L-DOPA. Whereas the temperature towards higher range (40–50 °C) enhanced the L-DOPA production as compared to lower (10–20 °C) temperature, which ultimately resulted into positive interaction between these variables. This might have been possible due to a faster conversion of L-DOPA to other metabolites at alkaline condition, and also tyrosinase might have inhibited [1, 3].

Figure 1b shows 3D response surface curve for the interaction of pH and cell mass. This indicated that yield of L-DOPA was decreased at higher cell mass and pH. L-DOPA production was drastically affected even by a slight change in the values of these two components. The

interaction of pH and cell mass indicated that lower levels of L-DOPA were produced at a lower cell mass possibly due to insufficient cell number that was not enough to convert L-tyrosine to L-DOPA; and a higher cell number might have absorbed all of the L-tyrosine quickly from the buffer and failed to produce L-DOPA in a short time. Another possibility was that at a high cell mass, some of the L-tyrosine might have been used for cell metabolism, which ultimately resulted in lower yields. In addition to this, the decreased yield of L-DOPA with alkaline pH might be due to alkaline lysis of the cells.

Figure 1c represents the interaction of pH and L-tyrosine. This interaction showed that these two factors strongly affected the L-DOPA yield. The pH and L-tyrosine

Fig. 1 Three-dimensional response surface curve showing the effect of interactions of **a** pH and temperature, **b** pH and cell mass, **c** pH and L-tyrosine, **d** temperature and cell mass, **e** temperature and L-tyrosine, and **f** cell mass and L-tyrosine

interaction showed the significant interaction as implied from response surface curve. The lower L-DOPA production was observed at higher L-tyrosine concentrations and neutral pH because solubility of L-tyrosine was less at this pH. Though L-tyrosine was more soluble at acidic conditions, tyrosinase activity of yeast was affected by the pH range that ultimately resulted in less L-DOPA yield [1–3].

Three dimensional response curves from interaction of temperature and cell mass indicated less significant effect on the L-DOPA production. (Fig. 1d). The interaction of temperature and L-tyrosine is illustrated in Fig. 1e, which showed that L-DOPA yield was affected less significantly by these two parameters as compared other parameters. The interaction of cell mass and L-tyrosine is as illustrated in Fig. 1f, which showed that these factors influence L-DOPA production moderately. The maximum L-DOPA production was recorded in the mid-levels of both the factors, whereas further increase in the levels resulted in a gradual decrease in yield.

Validation of the Model

Validation was carried out under conditions predicted by the model. The optimum conditions were pH 7.31, temperature 42.9 °C, 2.31 g l⁻¹ cell mass and 1.488 g l⁻¹ L-tyrosine. The predicted yield of L-DOPA with these conditions was 1.163 g l⁻¹ and the actual yield obtained was 1.158 g l⁻¹. The close correlation was observed between the experimental and predicted values, which validated the model.

L-DOPA Production, Biomass Trend, and Tyrosinase Activity

The L-DOPA production before optimization is illustrated in Fig. S1b, which suggested that using the pre-optimization conditions L-DOPA production resulted in 0.251 g l⁻¹ at 90th min, and then decreased. With the optimized conditions L-DOPA yield resulted in 1.157 g l⁻¹ at 90th min, and finally decreased. Thus, the medium optimization by RSM resulted in a 4.609-fold increase in L-DOPA yield over the yield before optimization. The biomass trend and tyrosinase activity during the growth of *Y. lipolytica*-NCIM 3472 are shown in Fig. S1a, which showed that the dry cell weight increased gradually up to 24th h (0.291 g l⁻¹) and then remained nearly constant. Meanwhile, tyrosinase activity increased slowly up to 7,028 U mg⁻¹ at 24th h and then decreased suddenly to 4,179 U mg⁻¹ at 30th h.

Recycling of the Cells

Recycling of the cell mass in five cycles was carried out at the optimum conditions obtained after validation of the

model. The cumulative L-DOPA yield of 4.091 g l⁻¹ was achieved after 5 reaction cycles using the same cells. The bioconversion of L-tyrosine to L-DOPA reported by using biomass of *A. oryzae* was 1.686 g l⁻¹ with consumption of 1.525 g l⁻¹ L-tyrosine [2] while *Y. lipolytica*-NRRL-143 biomass has been reported to produce 2.960 g l⁻¹ L-DOPA by utilizing 2.680 g l⁻¹ L-tyrosine [3]. Also *Bacillus* sp. JPJ and *Brevundimonas* sp. SGJ have been reported to produce 0.497 and 3.81 g l⁻¹ L-DOPA, respectively [1, 4]. The repeated use of *Y. lipolytica*-NCIM 3472 cells in present study resulted in the highest yield (4.091 g l⁻¹), which is encouraging and proved to be a cost-effective option as compared to present technique. Also the yeast system has several advantages over earlier sources.

Analysis of L-DOPA

The HPTLC peak profile of the reaction mixture showed distinct peaks at 0.24, which was identical to that of standard L-DOPA (0.23) (Fig. S2). The HPTLC profile of the cell free reaction mixture showed distinct peaks with a similar R_f value (0.23), which primarily confirmed the production of L-DOPA in buffer.

The HPLC elution profile of standard L-DOPA peaked at the retention time of 2.723 min., while the HPLC elution profile of the cell free reaction mixture showed a prominent peak at the retention time of 2.722 min (Fig. S3a, b). These results confirmed the L-DOPA production.

Conclusion

This work has demonstrated the use of a multifactor statistical approach for determining the conditions that lead to the optimum bioconversion of L-tyrosine by *Y. lipolytica*-NCIM 3472. This statistical method will be useful in large scale production of L-DOPA using *Y. lipolytica*-NCIM 3472 as a yeast model.

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