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Immobilization of Mushroom Tyrosinase by Different Methods in Order to Transform L-Tyrosine to L-3, 4 Dihydroxyphenylalanine (L-dopa)

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Abstract: Tyrosinase of edible mushroom was immobilized on activated agar particles, blocks and egg shell powder coated with polyethyleneimine (PEI) so as to study their efficiency in the transformation of L-tyrosine to L-dopa. The reaction rate for each form (PEI coated egg shell powder, activated agar blocks and activated agar particles) of the immobilized tyrosinase was calculated to be 0.0021, 0.018 and 0.0032 min, respectively. Desorption of tyrosinase from each support was found to be negligible. The production of L-dopa was 25, 73 and 42 mg L^{-1} for tyrosinase immobilized onto egg shell powder coated with PEI, activated agar particles and blocks, respectively.

Key words: L- Tyrosinase, immobilization methods, reaction rate, production rate of L-dopa

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper containing enzyme with mono oxygenase activity which is responsible for the biosynthesis of melanins and other polyphenolic compounds, acting at mild temperature and neutral pH (Fenoll *et al*., 2002). Tyrosinase has attracted the attentions and interests of scientists in the synthesis or modification of valuable compounds such as coumestrol known for oestrogenic activity and L-3, 4dihydroxyphenylalanine (L-dopa) used for the treatment of Parkinson's disease (Kong *et al*., 2000; Sikander *et al*., 2002). Immobilized tyrosinase of mushroom has been employed to produce L-dopa in the presence of ascorbate which is a rout yielding the desired product and leaving out some unreacted L-tyrosine (Seetharam and Saville, 2002; Munjal *et al*., 2003; Jimenez-Hamann and Saville, 1996; Carvalho *et al*., 2000). In this study attempts were made to immobilize commercially obtained tyrosinase of edible mushroom (Sigma) by different techniques such as adsorption on egg shell powder coated with polyethyleneimine, activation of agar by periodate solution and then cross linking the enzyme-supports by glutaraldehyde reagent in order to transform L-tyrosine to L-dopa.

MATERIALS AND METHODS

Materials: Tyrosinase, polyethyleneimine and glutaraldehyde were obtained from Sigma Chemical Company. L- Tyrosine, ascorbic acid and L-3, 4 dihydroxy phenylalanine (L-dopa) were purchased from Merck. Agar was procured from High Media, India. Other reagents used were of analytical grade.

Activation of agar: Agar (10 g) was mixed in 1 L of solution containing 200 mM sodium periodate which was kept at 4°C in the dark for activation as reported by Tien *et al*. (2004). The oxidation of agar was determined by 3,5 dinitrosalysilic acid reagent using maltose as a standard (Miller, 1959).

Immobilization of tyrosinase on activated agar I): In brief, tyrosinase in 0.05 M phosphate buffer pH 7 was used to couple on activated agar particles (1 g) at 4°C using magnetic stirrer for 4 h, uncoupled tyrosinase solution was recovered by filtration. Tyrosinase immobilized on modified agar particles was cross-linked with 2% (v/v) glutaraldehyde solution prepared in phosphate buffer pH 7.0, at 4°C for 4 h. The cross-linked tyrosinase-agar particles was again recovered from the glutaraldehyde solution by filtration. The cross-linked tyrosinase- agar particles were washed with cold deionized water several times in order to remove excess of glutaraldehyde.

Tyrosinase in 0.05 M phosphate buffer pH 7 was attached to agar (2% w/v) blocks of 2.0 mm thickness and 10 mm of length. It was allowed to react with modified agar blocks at 4°C for 4 h and the supernatant containing enzyme solution was drained off. The amount of tyrosinase uncoupled to the agar blocks was determined. Tyrosinase coupled to agar blocks was cross-linked with 2% (v/v) glutaraldehyde solution prepared in phosphate

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buffer pH 7 for 4 h at 4°C. The activity of the immobilized enzyme was then determined. The data are expressed in terms of U g^{-1} of immobilized enzyme.

Immobilization of tyrosinase onto egg shell powder coated with polyethyleneimine (PEI): Egg shell powder was coated by 1% (v/v) polyethyleneimine (PEI) prepared in phosphate buffer pH 7 (the pH was adjusted to 7) for 1 h and then washed with distilled water to remove excess of PEI. After incubation of the enzyme with coated egg shell powder for 4 h at 4°C they were then cross-linked with 2% (v/v) glutaraldehyde solution prepared in phosphate buffer pH 7 for 4 h at 4°C. The enzyme cross-linked egg shell powder was washed with distilled water to remove the excess of glutaraldehyde.

Assay of tyrosinase: Tyrosinase activity was determined colorimetrically from the amount of L-dopa produced by tyrosinase action on L-tyrosine in the presence of ascorbic acid and molecular oxygen as reported by Munjal and Sawhney (2002). In brief, the reaction mixture contained 1 mL of 2.5 mM tyrosine and ascorbic acid in 0.05 M phosphate buffer pH 7.0 to which 0.1 mL [0.1 g of immobilized tyrosinase on agar particles or 0.3 g agar block or 0.1 g tyrosinase immobilized on PEI coated egg shell powder)] of appropriately diluted tyrosinase solution was added. The reaction mixture was incubated at 25°C for 25 min. To the above solution, 1 mL of the following reagents was added: 2 M HCl, 2 M NaOH, 15% (w/v) sodium nitrite and finally 15% (w/v) sodium molybdate. Absorbance was recorded at λ 460 nm after 1 h against blank which contained inactivated tyrosinase (activated agar particles, agar block or egg shell powder containing no enzyme). One unit of enzyme activity can be defined as the amount of enzyme that produced 1 μ mole of L-dopa in 1 min under the above assay conditions, employing L-tyrosine as a substrate. The protein content of tyrosinase was measured by Lowry's method, using bovine serum albumin as a standard.

Study of desorption of tyrosinase from different matrixes: Desorption of tyrosinases from the activated agar particles, blocks or egg shell powder coated with PEI was measured as follows: after an appropriate time of reaction catalysed by tyrosinases immobilized onto activated agar particles, blocks or egg shell powder coated with PEI, the immobilized tyrosinases were separated from the reaction mixtures. A fraction of the reaction mixture was immediately assayed as mentioned above and the remaining of the solutions was further incubated at 25°C, after 30, 60, 90, 120 min and 24 h aliquots were taken to assay the variations in the content of L-dopa produced by different forms of immobilized tyrosinases. Each assay was conducted in duplicate.

RESULTS

Tyrosinase was immobilized on two forms of agar which was activated by 200 mM periodate solution at 4°C, for 24 h in dark. The reaction was terminated by the addition of 10% (w/v) PEG 10000. The oxidation of agar was determined by dinitrosalysilic acid. Egg shell powder was allowed to react with 1% (v/v) PEI glutaraldehyde solution prepared in buffer as mentioned in materials and methods. One gram of agar particles were allowed to react with tyrosinase solution at 4°C with the activity of 14.36 U mL^{-1} min^{-1} and protein content of 2.95 mg mL^{-1}. The uncoupled tyrosinase solution was recovered by filtration. Tyrosinase coupled to activated agar particles was further allowed to react with 2% (v/v) glutaraldehyde solution prepared in buffer at 4°C for 4 h on magnetic stirrer. Therefore, 26% of tyrosinase solution was covalently attached to activated agar particles. When tyrosinase solution was allowed to be coupled to agar blocks. It was observed that 60% of the enzyme was covalently attached to activated agar blocks. After coating egg shell powder by PEI solution, only 39% of tyrosinase solution was found to be adsorbed onto such matrix which was then cross linked by 2% (v/v) glutaraldehyde solution. The desorption of tyrosinases from supports such as activated agar particles, blocks and egg shell powder coated with PEI was studied as mentioned in materials and methods. It was observed that the desorption of tyrosinases from the supports under study was negligible indicating that the attachment of tyrosinase to supports like activated agar and then cross-linking by a bifunctional reagent is strong. PEI imparts negative charge onto egg shell powder as it is a polycationic reagent and tyrosinase coupled to such support was then cross linked by glutaraldehyde solution which makes the immobilization of tyrosinase to be strong. However, immobilized forms of tyrosinases were employed to study the reaction rate of each form catalysed the conversion of tyrosine to L-3, 4 dihydroxyphenylalanine in the presence of ascorbate. Figure 1 shows the reaction rate of each form of the immobilized system. Reaction rates of tyrosinases immobilized onto egg shell powder coated with PEI, activated agar particles and blocks were calculated to be 0.0021, 0.0032 and 0.018 min, respectively. The conditions of the reactions for each system were as follows: reaction temperature 25°C, concentrations of tyrosine and ascorbic acid 2.5 mM (each), reaction time, 10, 20, 40 and 60 min. Reaction rate could be found as follow:

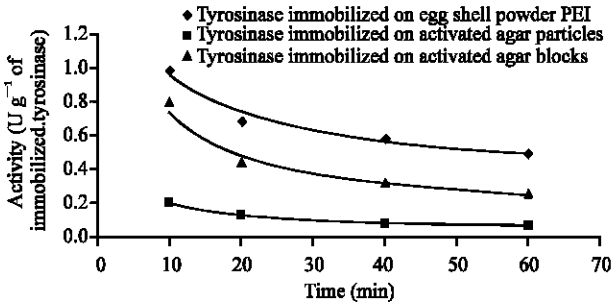

Fig. 1: Reaction rates of immobilized tyrosinases catalysed tyrosine to L-dopa

Reaction rate = 1/2Δ A divided by time that Ai -1/2Δ A took

Where

A_i = Initial activity (in terms of U g^{-1} of immobilized tyrosinase)

A_f = Final activity (in terms of U g^{-1} of immobilized tyrosinase)

$$\Delta A = A_i - A_f$$

A_i-1/2 ΔA after subtraction, the resulting value could be found on the Y-axis

T_{Ai}-1/2 ΔA this is a time that could be found on X-axis

Finally reaction rate = 1/2ΔA divided by T_{Ai}-1/2 Δ

Transformation of L- tyrosine to L-3, 4dihydroxyphenylalanine (L-dopa) by three forms of immobilized tyrosinases was carried out in the presence of ascorbic acid. The production rate of L-dopa reached the maximum of 42, 73 and 25 mg L^{-1} for tyrosinases immobilized on activated agar blocks, agar particles and egg shell powder coated with PEI and activated agar particles following 2 h of batch reaction, respectively.

DISCUSSION

Immobilization of tyrosinase of edible mushroom in order to convert L- tyrosine to L-dopa by entrapment into synthetic polymer, natural polymer, modified polystyrene, adsorption on nylon (Pialis *et al*., 1996), Zeolite (Seetharam and Saville, 2002), glass beads (Warrington and Saville, 1999), Fuller's earth (Munjal *et al*., 2003) and chitin activated with hexamethylenediamine (Carvalho *et al*., 2000) has been studied. However, in this study attempts are made to develop a biocatalyst to transform L-tyrosine to L-dopa by immobilizing tyrosinase of edible mushroom on to different supports such as activated agar in two forms (agar particles and agar blocks) and egg shell powder coated with PEI in order to minimize the leakage of an enzyme from the support, to maximize the access of substrate to the active site of the enzyme. While studying the desorption of tyrosinase immobilized on different supports, no leakages of tyrosinases from any form of the supports could be observed. Furthermore, Table 1 compares the production rates of L-dopa by various techniques as reported by different investigators. Nevertheless, as it can be seen from the table, the methods employed by us are quite efficient and simple in immobilizing tyrosinase of edible mushroom in order to transform L-tyrosine to L-dopa in the presence of ascorbic acid which the later inhibits the further progress of the reaction leading to the synthesis of polyphenolic compounds.

Table 1: Comparison of different techniques used to produce L-dopa

Method	Production rate (mg $L^{-1}h^{-1}$)	Scale (mL)	Total L-dopa produced	Reference
Immobilization of tyrosinase on to PEI coated egg shell	12.5	50	25 mg	Present investigation
Immobilization of tyrosinase onto activated agar blocks	21.0	50	42 mg	Present investigation
Immobilization of tyrosinase onto activated agar particles	36.5	50	73 mg	Present investigation
Immobilized tyrosinase, batch reactor	27.6	25	0.53 mg	Foor *et al*. (1993)
Immobilized tyrosinase, plug flow reactor	53.1	15	10.4 mg	Villanova *et al*. (1984)
Immobilized tyrosinase batch reactors	1.7	500	0.143 g	Pialis *et al*. (1996)
Mucuna pruriens, single-stage culture	0.025	100	0.90 mg	Chattopadyay *et al*. (1994)
Mucuna pruriens, two-stage culture	0.39	100	28.1 mg	Chattopadyay *et al*. (1994)
E. herbicola, culture serine substrate	670.0	100	5.1 g	Enei and Yamada (1986)
E. herbicola culture, *pyruvate substrate*	760.0	100	5.8 g	Enei and Yamada (1986)

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