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L-DOPA Production by Immobilized Tyrosinase

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Abstract

The production of L-DOPA using L-tyrosine as substrate, the enzyme tyrosinase (EC 1.14.18.1) as biocatalyst, and L-ascorbate as reducing agent for the *o*-quinones produced by the enzymatic oxidation of the substrates was studied. Tyrosinase immobilization was investigated on different supports and chemical agents: chitin flakes activated with hexamethylenediamine and glutaraldehyde as crosslinking agent, chitosan gel beads, chitosan gel beads in the presence of glutaraldehyde, chitosan gel beads in the presence of polyvinylpyrrolidone, and chitosan flakes using glutaraldehyde as crosslinking agent. The last support was considered the best using as performance indexes the following set of immobilization parameters: efficiency (90.52%), yield (11.65%), retention (12.87%), and instability factor (0.00). The conditions of immobilization on chitosan flakes were optimized using a two-level full factorial experimental design. The independent variables were enzyme-support contact time (t), glutaraldehyde concentration (G), and the amount of enzyme units initially offered (U_C). The response variable was the total units of enzymatic activity shown by the immobilized enzyme (U_{IMO}). The optimal conditions were t = 24 h, G = 2% (v/v), and U_C = 163.7 U. Under these conditions the total units of enzymatic activity shown by the immobilized enzyme (U_{IMO}) was 23.3 U and the rate of L-DOPA production rate was 53.97 mg/(L·h).

Index Entries: Tyrosinase; enzymatic production; L-DOPA; immobilized enzyme.

Introduction

L-DOPA is the most commonly used drug for the treatment of Parkinson disease and is currently chemically produced on a commercial scale by Monsanto. The high production cost and its high commercial value

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have motivated many researchers to study alternative routes of synthesis of this drug. Recent investigations report the microbiological production of L-DOPA by microorganisms such as *Escherichia coli* and *Erwinia herbicola*. However, the need for removal of other components and the low concentrations of L-DOPA in the product stream increase the cost of the microbial synthesis, making it economically unfeasible. Another alternative that has been showing promising results is the synthesis catalyzed by the enzyme tyrosinase with L-tyrosine as substrate *(1–7)*. Because tyrosine is an expensive enzyme, its immobilization is worthwhile in order to make reutilization possible.

Tyrosinase is a copper-containing oxidase that has two enzymatic activities: monophenol monooxygenase and orthodiphenol oxidoreductase. Both L-tyrosine and L-DOPA, the respective natural substrates for each of these activities, give the final product dopachrome. To produce L-DOPA from L-tyrosine, it is necessary to inhibit further oxidation to dopachrome with simultaneous ascorbate oxidation *(1,8–10)*.

The aim of the present study was to evaluate several immobilization procedures in order to obtain an immobilized system suitable for L-DOPA synthesis. The best system was then optimized through a factorial design to find the immobilization conditions that give optimal conversion rate.

Materials and Methods

Enzyme

Mushroom tyrosinase (T7755) was obtained from Sigma (St. Louis, MO).

Protein Determination

The protein content of the enzymatic preparation was determined using the Lowry et al. *(11)* method.

Immobilization Procedure

Chitin Flakes

Chitin flakes were purchased from Sigma. Tyrosinase was immobilized on chitin by modifications of a previous work *(12)*. Chitin (1 g) was incubated with 12.8 mL of a 2% aqueous hexamethylenediamine (HEMDA) solution for 2 h at room temperature. After the reaction, the supernatant was removed, and the activated chitin was treated with 12.8 mL of a 5% aqueous glutaraldehyde solution for 1 h at room temperature with occasional stirring. The material was then washed with water and sodium phosphate buffer (0.1 *M*, pH 7.0). Activated chitin was incubated with 7.5 mL of enzyme solution with an activity of 13.0 U/mL for 24 h at 4°C. Next, the flakes were washed twice (15 min each wash) with 100 mL of sodium phosphate buffer (0.05 and 0.1 *M*, pH 7.0). The activity of the immobilized enzyme system was determined on the same day and the enzyme was stored at 4°C in sodium phosphate buffer (0.1 *M*, pH 7.0) for further experiments.

Chitosan Flakes

Chitosan flakes were purchased from Aldrich. The immobilization procedure was similar to that just described, excluding treatment with HEMDA.

Chitosan Gel Beads

The procedure employed by Patel et al. *(13)* was slightly modified. Chitosan flakes (1 g) were dissolved into 50 mL of acetate buffer (0.3 *M*, pH 4.0) and mixed with 7.5 mL of enzyme solution with an activity of 13.0 U/mL. After binding the enzyme to the support, the beads were generated by dropwise addition into 150 mL of an NaOH solution (2% [w/v]) with continuous stirring. The beads were removed from the NaOH solution after 30 min, and washed with distilled water and sodium phosphate buffer (0.1 *M*, pH 7.0). The enzymatic activity of the beads was determined on the same day, and the enzyme was stored at 4°C in sodium phosphate buffer (0.1 *M*, pH 7.0) for further experiments.

Chitosan Gel Beads in Presence of Glutaraldehyde

Tyrosinase was immobilized on chitosan as described for chitosan gel beads. After beads were formed, 20 mL of a 5% glutaraldehyde solution was added to the NaOH solution for 30 min with continuous stirring. Then, the beads were removed, washed with distilled water, and put into contact with 100 mL of a 5% aqueous glutaraldehyde solution for 1 h with continuous stirring. Finally, the beads were washed with distilled water and sodium phosphate buffer (0.1 *M*, pH 7.0).

Chitosan Gel Beads in Presence of Polyvinylpyrrolidone

The procedure was similar to that for chitosan gel beads, differing only in the preparation of the chitosan solution. Polyvinylpyrrolidone (PVP) (0.05 g) was added to the chitosan flakes (1 g) prior to its dissolution in 50 mL of acetate buffer (0.3 *M*, pH 4.0).

Reaction Conditions

Twenty milliliters of substrate solution (2.50 m*M* L-tyrosine and 2.50 m*M* ascorbate in 0.1 *M* sodium phosphate buffer, pH 7.0) were incubated in a 50-mL reactor at 25°C with continuous stirring. The reaction was initiated by adding the immobilized enzyme to the substrate solution. One-milliliter aliquots were taken every 10 min for the next hour.

Cresolase Activity

Cresolase activity of the mushroom tyrosinase was assessed by L-DOPA production following a modified procedure reported by Arnow *(14)*. The L-DOPA content in the samples was determined as follows: one milliliter of HCl (2 *M*), 1 mL of sodium hydroxide (2 *M*), and 1 mL of a solution containing sodium molybdate (15% [w/v]) and sodium nitrite (15% [w/v]) were added, in this order, to each sample. The absorbance was read at 460 nm

precisely 1 h after the addition of the last reagent. One unit of tyrosinase activity was defined as the amount of enzyme that produced 1 μmol of L-DOPA/min at 25°C, pH 7.0, measured at a wavelength of 460 nm.

Immobilization Parameters

Different parameters were employed to evaluate the performance of the immobilized biocatalyst. Because enzyme activities are expressed as units per milligram for immobilized biocatalysts and units per milliliter for soluble biocatalysts, in all subsequent definitions enzyme units (U) were used.

Efficiency (*E*)

Efficiency (E) is defined as the ratio of theoretically immobilized enzyme units (U_T) to total soluble enzyme units initially offered (U_C). U_T is calculated by mass balance as the difference between enzyme units initially offered (U_C) and enzyme units remaining in the supernatant after immobilization (U_S):

$$E = U_T/U_C \times 100 = (U_C - U_S)/U_C \times 100 \quad (1)$$

Yield (η)

Yield (η) is defined as the ratio of really immobilized enzyme units (U_{IMO}, measured experimentally) to total enzyme units initially offered (U_C):

$$\eta = U_{IMO}/U_C \times 100 \quad (2)$$

Retention (*R*)

Retention is defined as the ratio of yield to efficiency. The retention gives an idea of steric hindrance, denaturation, partition, and diffusion limitations caused by the immobilization procedure:

$$R = \eta/E = (U_{IMO}/U_T) \times 100 \quad (3)$$

Instability Factor (*IF*)

The instability factor (IF) indicates enzyme leakage from the support during the reaction. It is quantified by removing two equal aliquots from the supernatant in the reactor after the reaction begins, at a time t_i. The amount of activity units of one of the samples is immediately measured (U_i), and the other sample is stored at reaction temperature for a time interval t_f = 10 min, after which its amount of activity units is determined (U_f). An eventual increase in the amount of activity units of the supernatant is caused by desorption of tyrosinase from the support:

$$IF = (U_i - U_f)/U_{IMO} \quad (4)$$

Results and Discussion

Table 1 gives the performance parameters of the different supports. In spite of high values of E, η, and R, immobilization of tyrosinase on chitin flakes was unfeasible owing to the high value of IF, indicating that the enzyme was released from the support. Additionally, the use of HEMDA

Table 1
Performance Parameters Obtained
in Different Tyrosinase Immobilization Procedures[a]

Support	Method	*E* (%)	η (%)	*R* (%)	*IF*
Chitin flakes	Covalent bonding by enzyme crosslinking	86.88	19.10	21.98	0.1600
Chitosan gel beads	Occlusion	97.68	15.26	15.57	0.0122
Chitosan gel beads in the presence of PVP	Occlusion	97.05	12.16	12.56	0.0016
Chitosan gel beads in the presence of glutaraldehyde	Occlusion	—	—	—	—
Chitosan flakes (nonoptimized)	Covalent bonding by enzyme crosslinking	90.52	11.65	12.87	0.0000

[a]*E*, efficiency; η, yield; *R*, retention; *IF*, immobilization factor.

as support activator, even though ensuring that this reagent was used in low concentrations and that it had been removed from the support by repeated washes, would negatively affect possible pharmaceutical use of the L-DOPA produced.

The immobilization of tyrosinase on chitosan gel beads, in the presence or absence of PVP, resulted in high values of the immobilization parameters, when compared with the ones reported in the literature *(1,2,4,15)* and with those obtained in other supports tested in this present work (Table 1). However, the particles presented serious diffusion problems, because a long lag phase was observed during enzyme activity measurements. PVP was added to the beads to increase their rigidity and to improve their mechanical properties. There was reduction in *IF* of 87%, indicating that PVP greatly increased the rigidity of beads, preventing the release of enzyme to the substrate solution. However, this increase in rigidity reduced the activity of the immobilized enzyme, generating a slight decrease in the immobilization parameters and worsening the diffusion problems. The beads prepared using glutaraldehyde as a crosslinking agent were unstable under reaction conditions, being completely destroyed during the reaction.

Immobilization of tyrosinase on chitosan flakes was efficient in terms of its immobilization parameters (90.52% efficiency, 11.65% yield, 12.87% retention, and 0.00 instability factor), when compared with those reported in the literature and those obtained in other supports tested in the present work (Table 1). Vilanova et al. *(10)*, when immobilizing mushroom tyrosinase on CPG-AA support, obtained the following immobilization parameter values: $E = 8\%$, $R = 60\%$, and $\eta = 5\%$. Note that the high retention value obtained by these investigators could be owing to the low efficiency

Table 2
Comparison of L-DOPA Production Rates Found in This Study, Using an Enzymatic Method, and in the Literature, Employing Different Microbial and Enzymatic Methods

Method	Production rate (mg/[L·h])	Scale (mL)	Reference
Mucuna pruriens, single-stage culture	0.025	100	*14*
M. pruriens, two-stage culture	0.39	100	*14*
E. coli–cloned *E. herbicola* culture	0.39	25	*4*
Immobilized tyrosinase, batch reactor	27.6	20	*10*
Immobilized tyrosinase, plug flow reactor	53.1	15	*10*
E. herbicola culture, serine substrate	670.0	100	*3*
E. herbicola culture, pyruvate substrate	760.0	100	*3*
Immobilized tyrosinase, batch reactor	1.70	500	*1*
Immobilized tyrosinase, batch reactor (nonoptimized)	44.86	20	This study
Immobilized tyrosinase, batch reactor (optimized)	54.00	20	This study

value. Pialis et al. *(1)*, who immobilized mushroom tyrosinase on nylon support, obtained lower efficiency values (in the range of 50–70%). *IF* was equal to zero in all experiments employed using this support, demonstrating that the enzyme was strongly bound to the support, eliminating the possible existence of mixed kinetics of immobilized and soluble enzyme. The rate of L-DOPA production obtained with this support (under nonoptimized conditions) was 44.86 mg/(L·h). This rate was almost equal *(10)* or higher than the literature values shown in Table 2, being just lower than the results reported by Enei and Yamada *(3)*, who, however, had difficulties in separating the products. Because of the good results obtained when compared with the literature, this support was chosen to be used in the next stage of our study, which is the optimization of the immobilization procedure, having the amount of immobilized enzyme units (U_{IMO}) as the response variable.

The immobilization conditions of tyrosinase on chitosan flakes were optimized to increase the L-DOPA production. The two-level full factorial experimental design was employed with three independent variables: enzyme-support contact time (t), glutaraldehyde concentration (G), and the amount of enzyme units initially offered (U_c). The dependent variable chosen was the amount of activity units shown by the immobilized enzyme (U_{IMO}). Four replicates of the central point were used as a measure of the experimental deviation.

Table 3 gives the experimental results obtained in the two-level full factorial experimental design. The empiric model that best described the experimental data is shown in Eq. 5:

Table 3
Experimental Conditions Employed
for Immobilization of Tyrosinase on Chitosan Flakes
and Immobilized Enzyme Units (U_{IMO}) Obtained,
According to a Two-Level Full Factorial Experimental Design[a]

Experiment	t (h)	U_C (U)	G (% [v/v])	U_{IMO} (U)
01	4.0 (–)	27.8 (–)	2.0 (–)	3.13
02	24.0 (+)	27.8 (–)	2.0 (–)	5.77
03	4.0 (–)	212.0 (+)	2.0 (–)	16.31
04	24.0 (+)	212.0 (+)	2.0 (–)	21.19
05	4.0 (–)	27.8 (–)	10.0 (+)	10.14
06	24.0 (+)	27.8 (–)	10.0 (+)	9.52
07	4.0 (–)	212.0 (+)	10.0 (+)	6.41
08	24.0 (+)	212.0 (+)	10.0 (+)	15.71
09[b]	14.0 (0)	113.3 (0)	6.0 (0)	18.76

[a]t, support-enzyme contact time; U_C, units of soluble enzyme initially offered; G, glutaraldehyde concentration.

[b]This result represents the arithmetic average of the results of four experiments done in the central point.

Table 4
Parameter Values with Their Corresponding Independent Variables
and SDs According to Model Proposed
for Description of Immobilized Tyrosinase Units on Chitosan Flakes

Parameter	Variable	Parameter value	SD
A0	—	0.01876	0.00041
A1	t	0.00202	0.00015
A2	U_C	0.00388	0.00015
A3	G	–0.00058	0.00015
A4	t, U_C	0.00152	0.00015
A5	U_C, G	–0.0033	0.00010
A6	t, U_C, G	0.000958	0.000146
A7	U_C^2	–0.0077	0.0004

$$U_{IMO} = A_0 + A_1 \cdot t + A_2 \cdot U_C + A_3 \cdot G + A_4 \cdot t \cdot U_C + A_5 \cdot U_C \cdot G + A_6 \cdot t \cdot U_C \cdot G + A_7 \cdot U_C^2 \quad (5)$$

Table 4 gives the values of the parameters in Eq. 5 and their standard deviations (SDs). The experimental and model variances were 5.12×10^{-5} and 1.69×10^{-7}, respectively. The F-test, at a confidence level of 95%, showed that the proposed model describes well the experimental results. As shown in Figs. 1 and 2, there is good agreement between the observed and predicted values.

Analyzing the proposed model and the values of its parameters, it is observed that U_C and t had a positive primary effect, indicating that higher values of these variables led to higher values of U_{IMO}. The glutaraldehyde

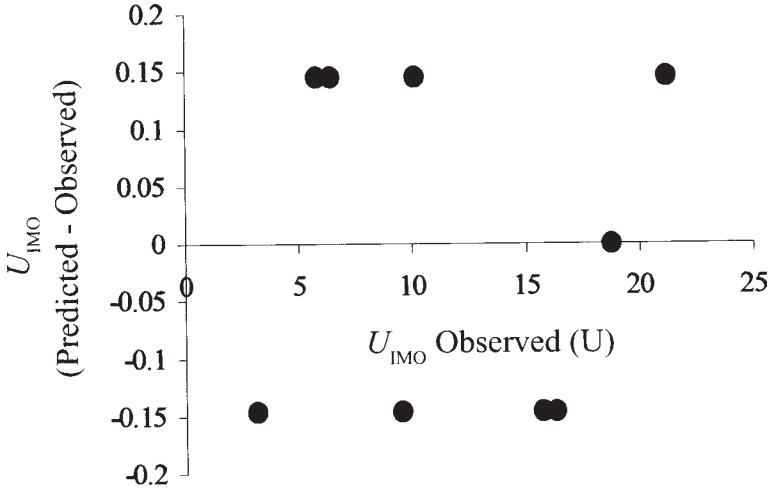

Fig. 1. Deviation between U_{IMO} predicted by the empiric model and U_{IMO} experimentally observed.

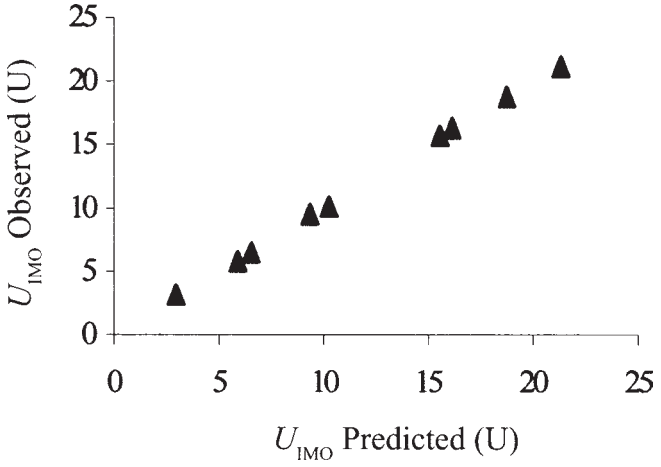

Fig. 2. Comparison between the U_{IMO} values observed experimentally and those predicted by the empiric model.

concentration (G) had a small and negative primary effect, indicating that its increase causes a small decrease in U_{IMO}. This occurs because glutaraldehyde, when used in high concentrations, is able to deactivate enzymes. There was a strong interaction among the independent variables. The presence of a quadratic term different from zero and negative indicated that the process is not linear and has a maximum point.

The dependent variable U_{IMO} was optimized by calculating the values of t, G, and U_C in the maximum point of the empiric model (Eq. 5). The calculated values were t = 24 h, G = 2% (v/v), and U_C = 163.7 U. Under these conditions, the total number of units of enzymatic activity shown by the immobilized enzyme (U_{IMO}) was 23.3 U.

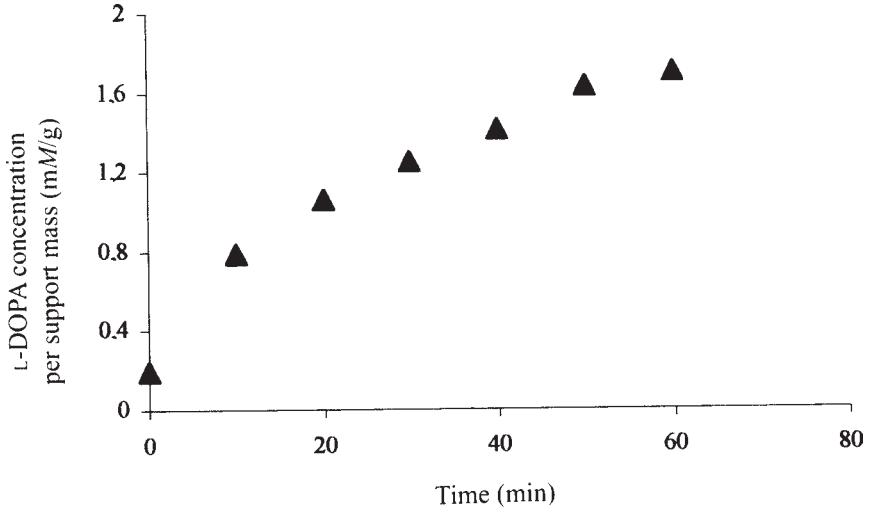

Fig. 3. Time course of L-DOPA production using tyrosinase immobilized on chitosan flakes under optimized conditions (t = 24 h, U_C = 163.7 U, and G = 2% [v/v]).

Figure 3 shows the time course of L-DOPA production using tyrosinase immobilized under optimal conditions on chitosan flakes. The support mass used in this experiment was 0.5 g. The production rate of L-DOPA (53.97 mg/[L·h]) was 20% higher than the rate obtained with enzyme immobilized under nonoptimized conditions. The immobilization parameters of the enzyme immobilized under optimal conditions were E = 68.84%, η = 14.20%, R = 18.01%, and IF = 0.00. As desired, optimization enabled an increase in U_{IMO}, beyond improving activity retention (R) and immobilization yield (η) as well. The efficiency was drastically reduced owing to an increase in U_C from 97.5 to 163.7 U. However, this reduction did not prevent the desired increase in U_{IMO}. This indicates that the most important parameters for evaluating immobilization performance are η, R, and IF.

Conclusion

The present results showed that the immobilization of tyrosinase on chitin flakes, in spite of the high values of E, η, and R, was unfeasible because of enzyme leakage from support. Chitosan gel beads, in the presence or absence of PVP, showed long lag phases during activity measurement, indicating serious diffusion problems. The chitosan gel beads treated with glutaraldehyde were destroyed when they came in contact with the substrate solution, probably owing to the ascorbate present in this solution.

Tyrosinase immobilized on chitosan flakes showed the best combination of performance parameters and lack of enzyme leakage and diffusional limitations. Therefore, this support was chosen to be used in the optimization of the amount of immobilized enzyme units (U_{IMO}).

The empiric model proposed to represent the relation between U_{IMO} and the variables U_C, t, and G described the experimental results well, showing a variance of 1.69×10^{-7}. U_C and t presented a positive primary effect, whereas G presented a small negative primary effect. There was interaction among the dependent variables, and the process was not linear. The L-DOPA production rate using tyrosinase immobilized on chitosan flakes under optimal conditions was comparable with the rates found in the literature, and was 20% higher than the rate presented by the enzyme immobilized under nonoptimized experimental conditions.

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