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Kinetic Characteristics of Immobilized Tyrosinase of Edible Mushroom in Synthesizing L-3, 4-Dihydroxyphenylalanine (L-Dopa)

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ABSTRACT

Tyrosinase (EC 1.14.18.1) from edible mushroom was immobilized onto agar particles activated by various concentrations of sodium periodate solution. It was found that agar activated with 200 and 300 mM periodate solution could adsorb the highest amount of tyrosinase. The progress of the reactions of two forms of tyrosinases (soluble tyrosinase and tyrosinase covalently bound to activated agar particles) were linear up to 50 and 120 min, respectively. The Michaelis-Menten constant (K_m) values of free and immobilized tyrosinases were determined as 0.8 and 0.9 mM, respectively by Lineweaver-Burk plots. The optimum temperature of the immobilized tyrosinase increased from 25 to 35°C while the optimum pH of the immobilized form remained unchanged relative to free tyrosinase. The immobilized tyrosinase on agar was recycled 6 times maintaining 50% of its original activity at the end of the last cycle.

Keywords: activated agar, effectiveness factor, immobilization, tyrosinase

Abbreviations: EC, Enzyme Commission number (1.14.18.1); $\mathbf{K_m}$, Michaelis-Menten constant; $\boldsymbol{\eta}$, effectiveness factor; $\mathbf{V}_{imm}$, rate of reaction catalyzed by immobilized tyrosinase; V_{free}, rate of reaction catalyzed by free tyrosinase

INTRODUCTION

At present, biotechnology is considered to be an alternative and useful technology to the conventional chemical technology. This could be due to the biological systems which can bring about the conversion of complex chemicals into more specific compounds with high efficiency and less undesirable product(s) under mild environmental conditions. In biotechnological processes, the chemical transformations are catalyzed by enzymes but their applications in industry have been restricted for many reasons such as: high cost, instability, availability in small amount and solubility in water which makes the enzyme difficult to be recovered from the reaction mixture at the end of enzymatic process hence limiting the application of soluble enzyme in biotechnological processes. To circumvent such obstacles, enzymes could be stabilized, converted into their insoluble counterpart, recovered from the reaction mixture and reused by different immobilization techniques but there are still some other obstacles such as effects of mass transfer limitation, rate limitation and leakage of enzymes from the supports in employing immobilized enzyme in biotechnological processes. There are many reports indicating the use of stabilized/immobilized enzymatic transformation of substrates to products (Norouzian 2003; Norouzian *et al.* 2003; Kuroiwa *et al.* 2005). Tyrosinase (EC 1.14.18.1) is a copper containing enzyme with mono oxygenase activity which is responsible for the biosynthesis of melanin and other polyphenolic compounds, acting at mild temperature and neutral pH (Fenoll *et al.* 2002). Tyrosinase shows cresolase and catecholase activities via separate active sites, oxidation of L-tyrosine is brought about via chemical reducing agents such as ascorbic acid, NADH, NH_2OH. Henceforth, L-Dopa is synthesized in the presence of ascorbic acid, tyrosinase and L-tyrosine, in such reaction ascorbic acid gets converted into ascorbate and this ascorbate inhibits further conversion of L-Dopa to melanine leaving L-Dopa as a final product and un-reacted L-tyrosine (Seetharam and Saville 2002). Tyrosinase has attracted the attention and interest of scientists in the synthesis or modification of valuable compounds such as coumestrol, known for its estrogenic activity and L-3,4 dihydroxyphenylalanine (L-Dopa) used for the treatment of Parkinson's disease (Kong *et al.* 2000; Sikandar and Ikram-ul-H 2002; reviewed by Pugalenthi and Vadivel 2007). Immobilized tyrosinase of mushroom has been employed to synthesize L-Dopa in the presence of ascorbate which is a route yielding the desired product while leaving out some un-reacted L-tyrosine (Jimenez-Hamann and Saville 1996; Carvalho *et al.* 2000; Seetharam and Saville 2002; Neeru *et al.* 2003; Norouzian *et al.* 2007a). In this paper we attempted to immobilize commercially obtained tyrosinase (Sigma) covalently onto activated agar particles in order to improve the efficiency and stability of tyrosinase.

MATERIALS AND METHODS

Materials

Tyrosinase and glutaraldehyde were obtained from Sigma Chemical Company. L-Tyrosine, ascorbic acid and L-Dopa were purchased from Merck. Agar was procured from High Media, India. Other reagents used were of analytical grade.

Methods

Agar (10 g) was mixed in 1 L of a solution containing sodium periodate at various concentrations (50, 100, 200 and 300 mM) at 4°C in the dark for activation as reported by Tien *et al.* (2004). The degree of oxidation was determined by 3,5-dinitrosalysilic acid reagent using maltose as a standard (Miller 1959).

Enzyme immobilization

Tyrosinase solution with the specific activity of 7.07 (units/mg of

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protein) was used to couple onto agar (1 g) activated with 50, 100, 200 and 300 mM periodate solutions at 4°C using a magnetic stirrer overnight. Uncoupled tyrosinase solution was recovered by filtration. Tyrosinase coupled to modified agar was cross-linked with 2% (v/v) glutaraldehyde solution prepared in phosphate buffer pH 7.0, at 4°C for 4 hrs. The cross-linked tyrosinase with agar was again recovered from the glutaraldehyde solution by filtration and washed with cold de-ionized water several times in order to wash out glutaraldehyde solution.

Assay of tyrosinase activity

Tyrosinase activity was determined colorimetrically from the amount of L-Dopa produced by tyrosinase action on tyrosine in the presence of ascorbic acid and molecular oxygen as reported by Munjal and Sawhney (2002). 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) 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 were added: 2 M HCl, 2 M NaOH, 15% (w/v) sodium nitrite and finally 15% (w/v) sodium molybdate. Absorbance was recorded at $\lambda_{460\ nm}$ after 1 h against a blank which contained inactivated tyrosinase (tyrosinase solution was inactivated by keeping the screw capped tube containing appropriately diluted enzyme solution in boiling water bath for 5 min). Units of enzyme activity were defined as the amount of enzyme that produces 1 µM of L-Dopa in one minute under the above assay conditions, employing L-tyrosine as a substrate. The protein content of tyrosinase was measured by Lowry's method (Lowry et al 1951), using bovine serum albumin as standard. Desorption of tyrosinase from the activated agar particles was measured as follows: after an appropriate time of reaction catalyzed by tyrosinase immobilized on activated agar particles, the immobilized tyrosinase was separated from the reaction mixture. A fraction of the reaction mixture was immediately assayed as mentioned above and the remaining solutions were further incubated at 25°C for 30, 60, 90, 120 min and 24 hrs. Then aliquots were taken to assay the variations in the content of L-Dopa produced by immobilized tyrosinase. Each assay was conducted in duplicate.

Statistics

Data obtained for each set of experiments were computed using Microsoft Excel. The analyses of R^2, variance, co-variance, sum square, t-test, f-test and standard deviation were performed by the same program. Each set of experiment was performed in duplicate and subsequently repeated.

RESULTS AND DISCUSSION

Different concentrations of periodate solutions at the range of 50, 100, 200 and 300 mM were employed to activate agar. A known amount of the enzyme was used to be immobilized onto agar activated with different concentrations of periodate solutions. It was found that maximum amount of tyrosinase was bound to agar activated by 200 mM periodate solution. Tien *et al.* (2004) activated alginate to immobilize catalase, by increasing the degree of oxidation of alginate, the concentration of catalase used to be immobilized onto alginate was also increased, reaching a plateau. We observed a somewhat similar trend in immobilizing tyrosinase onto activated agar (**Fig. 1**) The reactions that progressed the catalysis of L-tyrosine in the presence of ascorbic acid leading to the formation of L-Dopa by free and immobilized tyrosinase over time were also studied. It was found that the formation of L-Dopa by free tyrosinase and tyrosinase coupled to activated agar particles was linear up to 50 and 120 min (**Figs. 2, 3**). The K_m values of free and immobilized tyrosinase on agar particles were also studied by the Lineweaver-Burk plot (**Fig. 4**). The K_m values from these plots were determined to be 0.8 mM and 0.9 mM for free tyrosinase and tyrosinase immobilized onto agar particles, respectively (**Table 1**). Hülya and Ayten (2002) found the K_m value of tyrosinase of quince immobilized onto alumina was lower than that of free enzyme; but our finding shows that there is not much difference between the K_m values of the both forms of tyrosinase. Enzyme immobilization means restriction of the mobility of the enzyme which can affect the mobility of the solutes. These phenomena can be referred to as mass-transfer effect and can be led to a re-

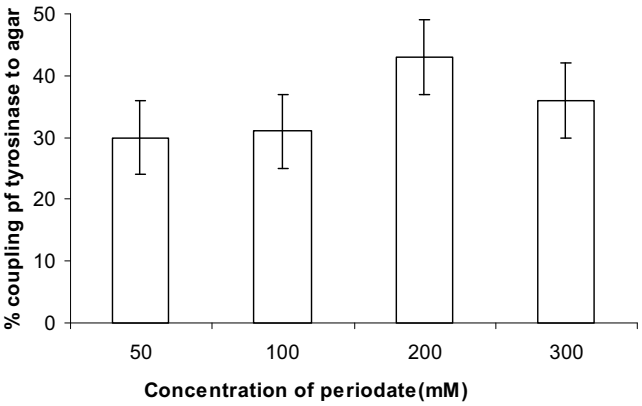

Fig. 1 Covalent binding of tyrosinase to activated agar with different concentrations of periodate solution.

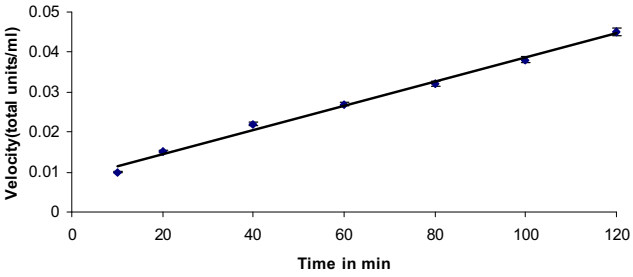

Fig. 2 Linearity of immobilized tyrosinase onto activated agar particles catalyzed L-tyrosine to L-Dopa.

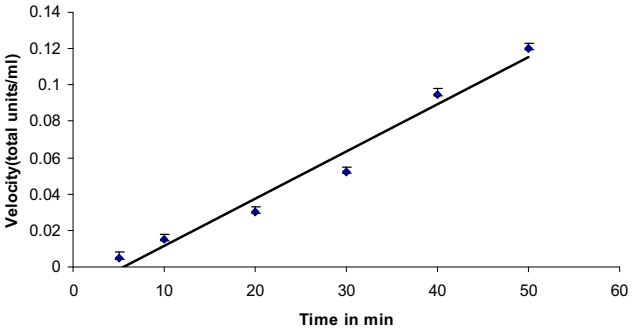

Fig. 3 Linearity of free tyrosinase catalyzed L-tyrosine to L-Dopa.

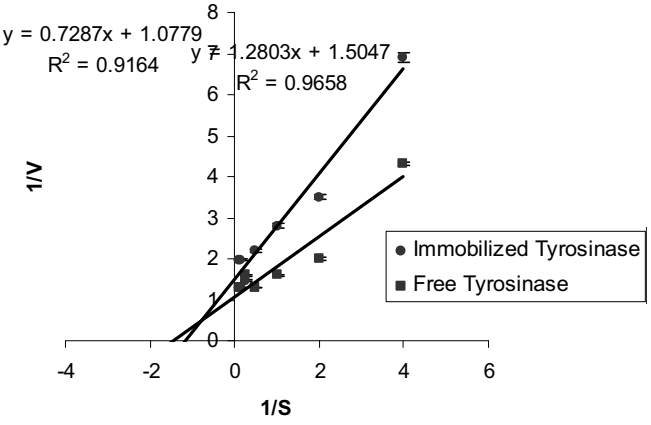

Fig. 4 Line-weaver Burk plot of tyrosinase in free, immobilized onto activated agar.

Table 1 Kinetic parameters of tyrosinase.

Forms of tyrosinase	K_m	V_m	η
Soluble	0.8 mM ± 0.07	1(U/ml/min) ± 0.14	-
Immobilized onto agar particles	0.9 mM ± 0.12	0.6(U/ml/min) ± 0.17	0.6

duced reaction rate and consequently to decrease efficiency as compared with soluble enzyme. For Michaelis-Menten enzyme kinetics, the extent of mass transfer control is usually expressed by the efficiency coefficient or effectiveness factor (η) (Tischer and Kassche 1999).

$$\eta = \frac{V_{imm}}{V_{free}}$$

where V_{imm} and V_{free} are the rates of the reaction catalyzed by the same enzyme concentration with immobilized and free tyrosinase under identical conditions. The effectiveness factors for tyrosinase immobilized onto activated agar particles was determined to be 0.6 ($\eta<1$) indicating diffusional limitation imposed onto the reaction process leading to the formation of L-Dopa by tyrosinase immobilized onto activated agar particles. However, the enzymatic synthesis of L-Dopa by immobilized tyrosinase is controlled by mass transfer limitation i.e. the availability of the substrate to the active site of the enzyme is somewhat affected by immobilized tyrosinase onto agar particles. Desorption of tyrosinase from the activated agar matrix was also studied. Desorption of tyrosinase from activated agar could not be detected by further incubating the reaction mixtures at room temperature. Therefore, desorption of tyrosinase immobilized onto activated agar particles could not contribute to the overall reactions catalyzed by immobilized tyrosinase as there are strong evidence in the literature (Bieniarz *et al.* 1998; Norouzian *et al.* 1999; Yakup 2000; Torres-Bacete 2000; Wang *et al.* 2001) indicating that the covalent attachment of the enzyme to the activated supports would be strong and the desorption of the enzyme from the activated supports might be negligible although inactivation of the enzyme during the immobilization process was observed. Furthermore, the optimum conditions like temperature and pH for the immobilized tyrosinase onto activated agar catalyzed L-tyrosine reaction leading to the synthesis of L-Dopa in the presence of ascorbate were studied. It was found that the optimum pH of the immobilized tyrosinase onto agar remained the same as the free form i.e. 6.8 while that of temperature of the immobilized form increased from 25 to 35°C indicating that the immobilized tyrosinase needs more activation energy to catalyze the targeted reaction (**Figs. 5, 6**). Finally the operational stability of the developed biocatalyst was studied in terms of the number of recycles performed. **Fig. 7** shows the recycling of the immobilized tyrosinase where at the end of the 6th cycle 50% of the original enzyme activity was retained (duration of each cycle was 5 hours). Enzyme immobilization/stabilization is the state of art in biotechnological process development, since the soluble enzymes behave differently from their immobilized/stabilized counterparts, therefore choices of supports and methods are important which should not much affect the kinetic properties of the immobilized/stabilized enzymes. In this regard, tyrosinase has been immobilized onto different supports/matrices according to its application. Its application ranges from the synthesis of drugs to the monitoring of the environment contaminated with phenolic compound by constructing biosensors (Pialis *et al.* 1996; Espin *et al.* 2001; Carvalho *et al.* 2000; Krastanov 2000; Ho *et al.* 2003; Ensuncho *et al.* 2005). Several workers have endeavored to immobilize tyrosinase through entrapments in alginate, polyacryalamide and gelatin gels/beads. Although enzymes entrapped in either natural or synthetic polymers providing friendly environment to the biocatalyst but the yields of entrapment varied for different matrixes (Munjal and Sawhney 2002). Seetharam and Saville (2002) modified sodium and calcium aluminosilicates by 1% glutaraldehyde solution prepared in phosphate buffer pH 7.0 to immobilize tyrosinase. The uptake of the enzyme was comparable by the two supports. However, the operational stability of the enzyme on both sodium and calcium aluminosilicates were determined to be 48 and 40 hours respectively. Tyrosinase has been conjugated to Fuller's earth and then entrapped in gelatin gels. This technique has also improved the stability of the enzyme on reusing such biocatalyst (Neeru *et al.* 2003). To improve the operational stability and maintain somehow the catalytic properties of the immobilized/stabilized enzymes as compared to their soluble counterparts, enzymes can be crystallized and then cross linked in order to prepare cross linked enzyme crystals. Recently Norouzian *et al.*

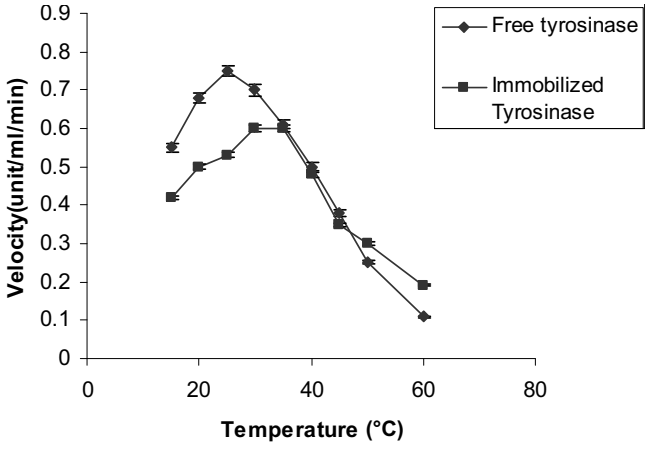

Fig. 5 Optima temperatures of free and immobilized tyrosinase onto activated agar.

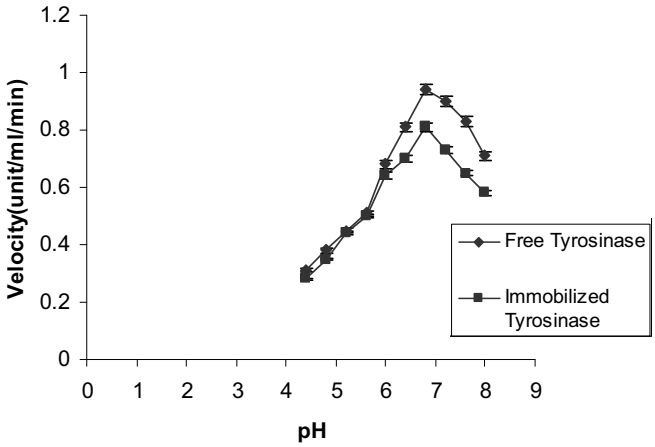

Fig. 6 Optima pH of free and immobilized tyrosinase onto activated agar.

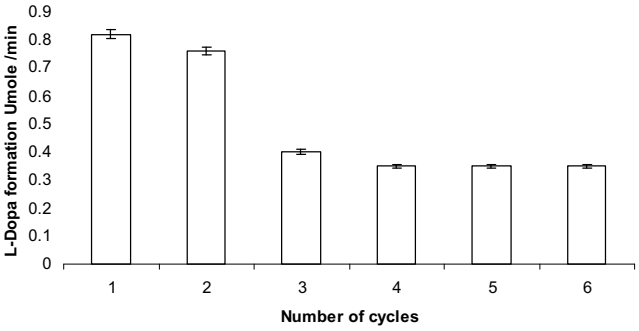

Fig. 7 Recycling of tyrosinase immobilized onto activated agar.

(2007b) crystallized tyrosinase along with bovine serum albumin and then cross-linked such crystal by glutaraldehyde solution. The developed biocatalyst was stable over the consequent uses and upon the storage. Most of the reports are concerned with the improvement of the tyrosinase stability. During the process of immobilization particularly through covalent attachment of the enzyme to the supports, the loss of enzyme sometimes reaches to above 50% of its original activity but the bond formed between the enzyme-supports or enzyme-arm-support is strong and the leaching of enzyme can occur during the subsequent operations. In this way we have developed a biocatalyst that kinetically behaves fairly similar to its soluble counterpart with the afore-mentioned kinetic characteristics.

REFERENCES

Bieniarz C, Cornwell MJ, Young DF (1998) Alkaline phosphatase activatable polymeric cross-linkers and their use in the stabilization of proteins. *Bioconjugate Chemistry* **9**, 390-398

Carvalho GM, Alves TL, Freire DM (2000) L-Dopa production by immobilized tyrosinase. *Applied Biochemistry and Biotechnology* **84-86**, 791-800

Ensuncho L, Alvarez-Cuenca M, Legg RL (2005) Removal of aqueous phenol using immobilized enzymes in a bench scale and pilot scale three phase fluidized bed reactor. *Bioprocess and Biosystems Engineering* **27**, 185-191

Espin JC, Soler-Rivas C, Cantos E, Tomas-Barberan FA, Wichers HJ (2001) Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst. *Journal of Agricultural and Food Chemistry* **49**, 1187-1193

Fenoll LG, Rodriguez-Lopez JN, Garcia-Molina F, Garcia-Canovas F, Tudela J (2002) Michaelis constants of mushroom tyrosinase with respect to oxygen in the presence of monophenols and diphenols. *The International Journal of Biochemistry and Cell Biology* **34**, 332-336

Ho PY, Chiou MS, Chao AC (2003) Production of L-Dopa by tyrosinase modified polystyrene. *Applied Biochemistry and Biotechnology* **111**, 139-152

Hülya Y, Ayten S (2002) Non-covalent immobilization of quince poly phenol oxidase. *Acta Chemica Solvvenia* **49**, 893-902

Jimenez-Hermann MC, Saville BA (1996) Enhancement of tyrosinase stability by immobilization on Nylon. *Food and Bioproduct Processing* **74 (C)**, 47-52

Kong HK, Hong MP, Choi SS, Kim YT, Cho SH (2000) Purification and characterization of a highly stable tyrosinase from *Thermomicrobium roseum*. *Biotechnology and Applied Biochemistry* **31**, 113-118

Krastanov A (2000) Removal of phenol from mixtures by co-immobilized laccase/tyrosinase and polyclar adsorption. *Journal of Industrial Microbiology and Biotechnology* **24**, 383, 388

Kuroiwa T, Shoda H, Ichikawa S, Sato S, Mukataka S (2005) Immobilization and stabilization of pullulanase from *Klebsiella pneumoniae* by multiple attachment method using activated agar gel support. *Process Biochemistry* **40**, 2637-2642

Lowry OH, Rosebrough NJ, Al Far, Randol RJ (1951) Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275

Miller GL (1959) Use of dinitrosalysilic acid reagent for determination of reducing sugar. *Analytical Chemistry* **31**, 426-28

Munjal N, Sawhney SK (2002) Stability and properties of mushroom tyrosinase entrapped in alginate, polyacrylamide and gelatin gels. *Enzyme and Microbial Technology* **30**, 613-619

Neeru MS, Sushil K, Surrender SK (2003) A novel method for the immobilization of tyrosinase to enhance stability. *Biotechnology and Applied Biochemistry* **38**, 137-141

Norouzian D, Akbarzadeh A, Mirdamadi S, Khetami Sh, Farhangi A (2007a) Immobilization of mushroom tyrosinase by different methods in order to transform L-tyrosine to L-3,4 dihydroxyphenylalanine (L-Dopa). *Biotechnology* **6**, 436-439

Norouzian D, Akbarzadeh A, Abnosi MH, Atyabi SM, Farhangi A, Mehrabi MR (2007b) Stabilization of tyrosinase-bovine serum albumin crystals by glutaraldehyde. *American Journal of Biochemistry and Biotechnology* **3**, 110-113

Norouzian D (2003) Enzyme immobilization: The state of art in biotechnology. *Iranian Journal of Biotechnology* **1 (4)**, 197-206

Norouzian D, Akbarzadeh A, Nouri Inanlou D, Farahmand B, Saleh M, Sheikh-ul-Eslam F, Vaez J (2003) Biotransformation of alcohols to aldehydes by immobilized cells of *Saccharomyces cerevisiae* PTCC 5080. *Enzyme and Microbial Technology* **33**, 150-153

Norouzian D, Hosseinzadeh A, Inanlou D, Moazami N (1999) Various techniques used to immobilize naringinase produced by *Penicillium decombens* PTCC 5248. *World Journal of Microbiology and Biotechnology* **15**, 501-502

Pialis P, Jimenez H, Maria C, Bradley AS (1996) L-Dopa production from tyrosinase immobilized on nylon 6.6. *Biotechnology and Bioengineering* **51**, 141-147

Pugalenthi M, Vadivel V (2007) L-Dopa (L-3,4-Dihydroxyphenylalanine): A non-protein toxic amino acid in *Mucuna pruriens* seeds. *Food* **1**, 322-343

Seetharam G, Saville BA (2002) L-dopa production from tyrosinase immobilized on zeolite. *Enzyme and Microbial Technology* **31**, 747-753

Sikander A, Ikram-ul-H, Qadeer MA (2002) Novel technique for microbial production of 3, 4-dihydroxyphenylalanine by a mutant strain of *Aspergillus Niger*. *Electronic Journal of Biotechnology* **5 (2)**, 118-123

Tien CL, Millette M, Lacroix M, Mateescu MA (2004) Modified alginate matrices for the immobilization of bioactive agents. *Biotechnology and Applied Biochemistry* **39**, 189-198

Tischer W, Kasche V (1999) Immobilized enzyme: crystals or carriers. *Trends in Biotechnology* **17**, 326-335

Torres- Bacete J, Arroyo M, Torres-Guzmán R, de la Mata I, Pilar Castillon M, Acebal C (2000) Optimization of 6-aminopenicillanic acid (6-APA) production by using a new immobilized penicillin acylase. *Biotechnology and Applied Biochemistry* **32**, 173-177

Yakup MA (2000) Immobilization of polyphenol oxidase on carboxymethylcellulose hydrogel beads. *Polymer International* **49**, 775-781

Wang P, Dai S, Waezsada SD, Tsao AY, Davison BH (2001) Enzyme stabilization by covalent binding in nonporous sol-gel glass for nonaqueous biocatalysts. *Biotechnology and Bioengineering* **74**, 249-255