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The Biotechnological Potential of Mushroom Tyrosinases

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Summary

Over the last decade there has been a significant interest in developing biotechnological applications of tyrosinases. These applications include the production of L-DOPA (3,4-dihydroxyphenyl-L-alanine) from L-tyrosine, the production of cross-linked protein networks for use as novel food additives and the detection of phenolic compounds in wastewater or their removal from it. Much of the research into these applications has involved mushroom tyrosinases. We review the potential biotechnological applications of mushroom tyrosinases and evaluate the state of knowledge about their production, recovery and immobilization. We conclude that much more research is necessary in these areas if mushroom tyrosinases are to fulfill their biotechnological potential.

Key words: mushroom tyrosinase, *Agaricus bisporus*, biosensors, cross-linked biopolymers, phenol removal, submerged culture

Introduction

Tyrosinase (EC 1.14.18.1; tyrosine, L-DOPA:oxygen oxidoreductase; catecholase; diphenol oxidase; polyphenol oxidase – PPO) is a copper-containing enzyme that catalyzes sequential oxidation steps with various phenolic substrates. In the first reaction, often referred to as the »monooxygenase« or »cresolase« activity, a hydroxyl group is introduced into the *ortho* position of the aromatic ring while in the second reaction, often referred to as the »diphenolase« or »catecholase« activity, the *o*-dihydroxy compound produced in the first reaction is oxidized to an *o*-quinone. Both reactions involve molecular oxygen (*1*). These activities can be used as the basis for several biotechnological applications (*2*).

Although tyrosinases are widely distributed in microorganisms, plants and animals, much of the current interest in the development of biotechnological applications has focused on the use of mushroom tyrosinases. Several aspects of mushroom tyrosinases have been extensively reviewed, such as their biochemical characteristics, their roles in the metabolism of the producing organism and some of their potential biotechnological applications (*3*). However, relatively little attention has been given to the production and purification of mushroom tyrosinases and a realistic appraisal of their biotechnological potential. This review addresses these issues.

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Biochemical Characteristics of Mushroom Tyrosinases

In this section we give a brief overview of the biochemical characteristics of the most studied macrofungal tyrosinase, namely that produced by *Agaricus bisporus*, although characteristics of other fungal tyrosinases are mentioned as appropriate.

As with other macrofungal tyrosinases, the tyrosinase of *A. bisporus* is intracellular (*4,5*). It is a heterotetramer with an apparent molecular mass of 120 kDa with two heavy and two light polypeptide chains (*3,6*). Note that tyrosinases from other fungi have different quaternary structures: the tyrosinase from the macrofungus *Amanita muscaria* is a heterodimer with a molecular mass of about 50 kDa (*7*), while that of the filamentous fungus *Neurospora crassa* is a 46-kDa monomer (*8*).

The active site of the tyrosinase of *Agaricus bisporus* contains two Cu binding sites, Cu_A and Cu_B, which interact with molecular oxygen and histidine residues (*3*). Depending on the copper-ion valence and the linking with molecular oxygen, this active site exists in three intermediate states: deoxy (CuI–CuI), oxy (CuII–O_2–CuII) and met (CuII–CuII) (*9*). These three states determine the ability of tyrosinase to bind to its substrates and therefore determine the reaction kinetics. The met form of the enzyme is predominant *in vivo* (*10*). This form can bind diphenols and, during the subsequent reaction in which it oxidizes the diphenol and releases the *o*-quinone, is converted into the deoxy-form. The deoxy-form is able to bind reversibly with molecular oxygen, producing the oxy-form, which can act on both monophenols and diphenols (*9,11*).

With respect to the reaction kinetics, the second reaction typically follows Michaelis-Menten kinetics, while analysis of the first reaction is complicated by the lag period during which the enzyme is converted from the met to the oxy form (*9*). The first reaction step may suffer substrate inhibition by L-tyrosine, the monophenol most commonly used to characterize its kinetics (*12*), while substrate inhibition of the second reaction by L-DOPA (3,4-dihydroxyphenyl-L-alanine) has also been reported (*13*).

Applications of Mushroom Tyrosinases

Initial studies of tyrosinases were motivated by a desire to understand and to prevent the enzymatic browning that occurs in the presence of air when mushrooms, fruits or vegetables are cut or bruised. This phenomenon is intimately linked to the action of tyrosinase and causes severe economic losses in the food industry. Recently the focus has moved to the biotechnological and environmental applications of tyrosinases, and macrofungal tyrosinases have commonly been chosen for these studies. The most important of the applications being investigated are the biosynthesis of L-DOPA, the detection and quantification of phenolic compounds in water samples, the removal of phenolic compounds from wastewaters and the production of cross-linked protein networks.

Use of mushroom tyrosinases for L-DOPA production

Since 1967, L-DOPA has been the preferred drug for treatment of Parkinson's disease (*14*). It is also used for controlling the myocardium following neurogenic injury (*15*). The world market for L-DOPA is about 250 tons per year (*16*). Most of the L-DOPA sold commercially is produced from vanillin and hydantoin by a chemical process that involves eight reaction steps, one of which is an optical resolution (*17*).

Several investigators have studied biological routes for production of L-DOPA. For example, systems involving whole cells of *Escherichia coli* (*18*), *Erwinia herbicola* (*16*) or *Aspergillus oryzae* (*5,15*) have been used to transform L-tyrosine into L-DOPA in the laboratory. However, the specific activities in these processes are low and both the initial substrate (L-tyrosine) and the desired product (L-DOPA) suffer side reactions that produce other metabolites (*15*).

The poor performance of whole-cell systems stimulated researchers to use tyrosinases in cell-free systems. So far all studies have involved mushroom tyrosinase obtained from Sigma. The productivities of L-DOPA that have been obtained in batch reactors using immobilized tyrosinase range from 1.44 to 54 mg/(L·h) (Table 1) (*19–23*). Unfortunately, these productivities are still relatively low, which is due to two factors. Firstly, the conversion of the added L-tyrosine is incomplete, with less than 30 % of it being consumed during the process. Secondly, although side reactions with L-tyrosine are avoided in these enzymatic systems, some of the L-DOPA that is produced is lost in a second tyrosinase-catalyzed reaction that produces dopaquinone (Fig. 1). This dopaquinone isomerizes spontaneously to leukodopachrome, which in turn reacts with a second dopaquinone mole-

Table 1. Productivities obtained for L-DOPA production using immobilized mushroom tyrosinase

Immobilization method	Productivity/(mg/(L·h))	Source
Adsorption on nylon 6,6	33 (using a single 7-h batch experiment)	(*19*)
Entrapment in copper-alginate gels	4.5 (in batch reactor) 110 (in packed bed reactor when air was used)	(*20*)
Adsorption on sodium aluminosilicate and calcium aluminosilicate (two separate forms of zeolite)	34 (using a single 7-h batch)	(*21*)
Adsorption on polystyrene-polyamino styrene (PSNH) and polymethylchloride styrene (PSCL)	1.4 for the PSNH and 2.33 for the PSCL at 30 °C 3.9 for the PSNH at 60 °C 5.5 for the PSCL at 70 °C (using a batch reactor)	(*22*)
Adsorption on chitosan-based support	54 (on chitosan flakes using glutaraldehyde as crosslinking agent and using a batch reactor)	(*23*)

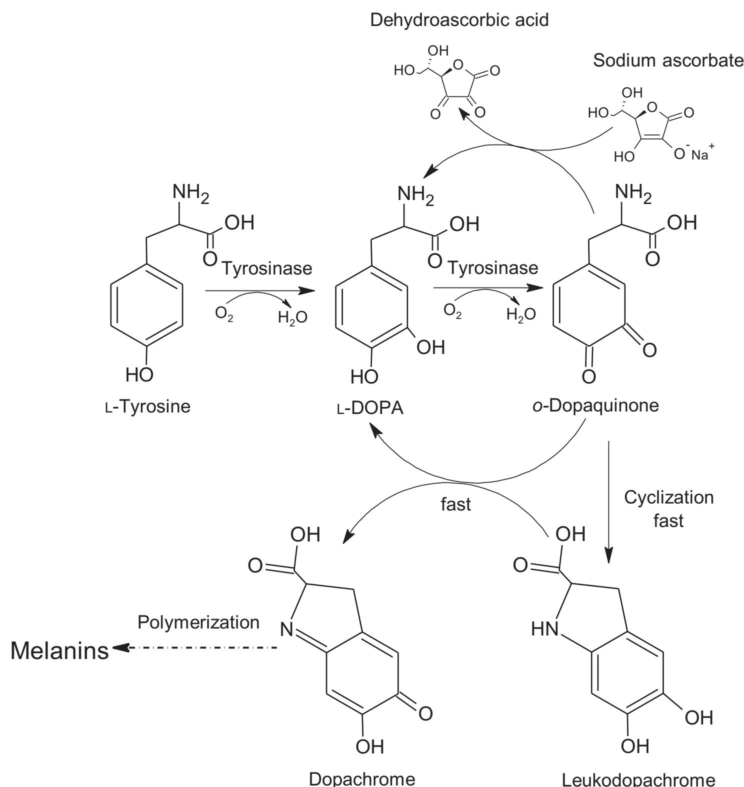

Fig. 1. Mechanism by which tyrosinase converts L-tyrosine firstly to L-DOPA and then to *o*-dopaquinone. Also shown are the spontaneous chemical reactions that lead to conversion of the *o*-dopaquinone into dopachrome and melanins and to the regeneration of L-DOPA from *o*-dopaquinone if L-ascorbate is added to the reaction mixture

cule, regenerating one molecule of L-DOPA but also producing a dopachrome molecule, which then polymerizes spontaneously to produce melanin. L-ascorbate is added to the reaction medium in an attempt to minimize melanin production. It acts by reducing dopaquinone back to L-DOPA, in a non-enzymatic reaction (Fig. 1). However, since high concentrations of L-ascorbate not only inhibit the cresolase activity responsible for L-DOPA synthesis but also irreversibly inactivate the tyrosinase, the L-ascorbate is added in the same concentration as L-tyrosine (*19*). The problem is that the L-ascorbate is consumed continuously during the process. Although it could be continuously fed into the medium in order to maintain its concentration constant, the problem would not be solved because the continued accumulation of diphenols would activate the melanin formation pathway anyway (*24*).

Amongst the results shown in Table 1, those of Ates *et al.* (*20*) deserve special mention. They used tyrosinase immobilized in Cu-alginate gels in continuous and batch systems. In a continuous packed-bed, they obtained a productivity of 110 mg/(L·h) when air was bubbled through the bed, this productivity being 22-fold greater than that in a batch reactor through which air was not bubbled (*20*). The product of two effects explains the much greater productivity in the aerated packed-bed. Firstly, the ratio of immobilized enzyme preparation to reaction medium was 9.7 g to 2.4 mL in the packed bed and 2.4 g to 50 mL in the batch reactor (*i.e.* it was about 80 times higher in the packed bed), while the residence time was approximately 15 times shorter in the packed bed. This combination of higher enzyme concentration but shorter residence time would be expected to give an approximately 5-fold higher productivity in the packed bed. Secondly, the bubbling of air was important, since the packed-bed had a 5-fold greater productivity when it was aerated compared to when it was not. The increase in reaction rate with aeration occurs because molecular oxygen is a substrate in the reaction.

Despite the progress in tyrosinase-based production of L-DOPA, this process will probably never be commercialized, since an enzymatic process involving another enzyme, tyrosine phenol-lyase (TPL), appears to have better potential. TPL transforms a mixture of pyruvate, ammonia and catechol into L-DOPA in a different reaction. It has the advantage that it does not have a diphenolase activity and so does not react with the L-DOPA that it produces. Ajinomoto Co. recently implanted a cell-free process that uses the TPL from *Erwinia herbicola* and results in an L-DOPA concentration of 110 g/L after 36 h (*25*).

Detection and quantification of phenolic compounds

Wastewaters containing phenols and phenolic derivatives are generated by the textile, coal, chemical, petrochemical, mining and paper industries, amongst others (*18,26,27*). Increasingly strict environmental laws are providing an impetus for the development of analytical techniques for fast monitoring of these compounds. Traditionally, analysis has been based on spectrophotometric or chromatographic methods. New techniques that are currently being developed include capillary electrophoresis, immunoassays, and biosensors. They potentially pro-

vide better specificity, lower costs, and faster and simpler sample processing (*26*).

Biosensors are currently being developed for the detection of phenolic compounds, based on the reaction of these compounds with an immobilized mushroom tyrosinase. Immobilization is essential because it ensures intimate contact between the enzyme and the underlying signal detector and also prevents the enzyme from being washed off the electrode when readings are made in aqueous samples (*28*). The detection and quantification of the reaction within the biosensor can be based on various different principles, such as detection of oxygen consumption (*29*), direct reduction of liberated *o*-quinone (*30–34*) or reduction of the *o*-quinone using a redox mediator such as hexacyanoferrate (*35*).

Despite the intensive research into biosensors based on mushroom tyrosinase, a major obstacle to commercialization of these biosensors is the relatively poor stability that the immobilized tyrosinase has under operational and storage conditions. The instability of tyrosinase biosensors is mainly due to the fact that quinones can form intermediate radicals in both the enzymatic and electrochemical reactions and these radicals readily react and polymerize to produce aromatic compounds that can inactivate the enzyme and foul the electrode (*36*).

Another challenge is to ensure that the analyses are cost-competitive and suitable for processing large numbers of samples. Probably the main advantage of tyrosinase-based biosensors would be the possibility to determine the levels of phenolic compounds *in situ*, allowing on-the-spot checks.

Removal of phenolic compounds and bioremediation

The presence of phenolic compounds in drinking and irrigation water or in cultivated land represents a significant health and environmental hazard. In recent years, methods for the removal and transformation of phenolic compounds have received attention. Numerous conventional methods have been used to remove phenols from industrial wastewaters, these methods being based on chemical or physical principles such as solvent extraction, chemical oxidation and adsorption onto activated carbon (*37*). However, these methods typically involve high capital and operating costs and do not remove the phenol completely (*38*). Further, they can generate secondary effluent problems. For example, the chlorinated phenols that are produced by chemical processes can be more toxic than the original compounds (*39*). Some chemical and physical methods, such as oxidation and adsorption, work cost-effectively only in waters with high phenol concentrations (*40,41*).

Due to the disadvantages of physical and chemical methods, microbial and enzymatic methods have been investigated (*27*). Enzymatic processes have several potential advantages over conventional biological treatments. Firstly, they do not need an acclimatization period. Secondly, they suffer less from charge shocks and toxic compounds than microorganisms do. Finally, they are highly specific and do not generate undesired side products (*42*).

Enzymatic methods are based on oxidative enzymes such as peroxidases and phenoloxidases (*43*). Peroxidases require hydrogen peroxide as a co-substrate, which complicates the process and increases costs (*44*). They are used commercially, but the costs of these processes are high (*44,45*). Phenoloxidases include laccases and tyrosinases. These have the advantage that they can react with molecular oxygen without the need for externally-supplied co-substrates, which leads to lower costs. Further, when tyrosinases oxidize phenols and other aromatic compounds in wastewaters, typically the oxidized product will polymerize to insoluble compounds that can be removed by filtration or precipitation (*27*).

Due to these features, tyrosinases have been studied for phenol removal from wastewaters. Several studies have involved mushroom tyrosinases. For example, Edwards *et al.* (*46*) immobilized commercial mushroom tyrosinase on chitosan-coated polysulphone capillary membranes within a capillary bioreactor and used this system to oxidize a range of phenols present in synthetic and industrial effluents. The removal was efficient even at low phenol concentrations, which typically cause problems for physical and chemical methods. Further, the chitosan adsorbed *o*-quinones generated during the reaction, thereby helping to reduce enzyme inactivation. In another example, Ensuncho *et al.* (*44*) used *Agaricus bisporus* tyrosinase, immobilized on a chitosan-alginate support, in both bench-scale and pilot-scale bioreactors in which air was bubbled through the system. More than 92 % of the phenol was removed in the bench-scale reactor and 60 % in the pilot-scale reactor.

Production of cross-linked biopolymers

The food industry has an interest in developing new biopolymers with special properties, for use not only as emulsifying and thickening agents, but also in low-calorie and low-fat foods. The cross-linking of natural polymers by mushroom tyrosinase has the potential to produce such new biopolymers.

Tyrosinase forms *o*-quinones from tyrosine and these *o*-quinones cross-link proteins by reacting with their amino and sulfydryl groups (*47*). Halaouli *et at.* (*48*) used this capability to form a cross-linked protein network from casein using the tyrosinase from *Pycnoporus sanguineus*. Caffeic acid was supplied as an external phenolic source that was oxidized by the tyrosinase, with the *o*-quinone product then acting as the cross-linker in the production of protein-protein conjugates made with lysozyme, α-lactalbumin and β-lactoglobulin (*49*). Anghileri *et al.* (*50*) used tyrosinase to produce conjugates from sericin, a peptide found in the wastewater of silk textile industries.

In addition, a polysaccharide-protein biopolymer was obtained from gelatin and chitosan by tyrosinase cross-linking (*51*). In this process, the tyrosinase oxidized tyrosine residues of the gelatin, which then reacted with the available amino groups of the chitosan. The gelatin-chitosan gels formed had chemical and physical properties that were different from those of gelatin gels.

Production, Purification and Immobilization of Mushroom Tyrosinases

Although it is unlikely that a commercial process will be established for the tyrosinase-based production of L-DOPA, other applications of tyrosinase that are currently under study do have the potential to create a large demand for the enzyme. Mushroom tyrosinases would seem to be natural candidates for the establishment of commercial processes, based on the fact that many of the laboratory studies have involved enzymes from this group of organisms. The current section briefly outlines the current state of the art in relation to production and purification of mushroom tyrosinases.

Traditional methods for obtaining mushroom tyrosinase

To date many researchers have either bought tyrosinase from commercial sources or extracted the enzyme themselves from fruiting bodies. The enzyme sold by Sigma Co. is mainly extracted from *Agaricus bisporus* and purified as described by Kertesz and Zito (*52*). Those researchers who extract tyrosinase from the fruiting body typically buy mushrooms at local markets. In this case it is important to select fruiting bodies that are young, white and fresh (*52*).

Production of tyrosinase in culture

If the applications of mushroom tyrosinases currently being studied become so successful that large amounts of enzyme are required, then it will be necessary to optimize tyrosinase production in mycelial culture, either by submerged culture or by solid-state fermentation. Such studies have not yet been done, although several tyrosinase-producing basidiomycetes have been cultured in bioreactors for the production of other products, providing experience that will be useful if a decision is made to develop a process using one of these macrofungi.

Qualitative results from agar plate cultures of basidiomycetes may give insights into the conditions necessary to stimulate tyrosinase production in submerged culture. For example, tyrosinase is involved in the production of DOPA-melanins, and the production of these melanins in agar plate cultures may be stimulated by incubation above the optimal growth temperature or by the presence of competing microorganisms (*53,54*). These results suggest that, depending on the producing organism, it might be appropriate either to start the culture at the optimal temperature for growth and later increase the incubation temperature to stimulate tyrosinase production or to add extracts of antagonistic organisms to the culture broth. However, such strategies have not yet been tested in liquid cultures.

The only reported study of tyrosinase production by submerged culture of a basidiomycete was undertaken by Halaouli *et al.* (*48*), who produced intracellular tyrosinases from various strains of *Pycnoporus cinnabarinus* and *Pycnoporus sanguineus*, grown in Erlenmeyer flasks in a liquid medium containing maltose, yeast extract, salts and vitamins. *P. sanguineus* CBS 614.73 was identified as the best tyrosinase producer, reaching monophenolase and diphenolase productivities, based on the protein in the crude extract, of 45 and 164 U/(g·day), respectively. The highest tyrosinase activity was reached after one week, when maltose was totally consumed.

Although there have been no studies aiming to optimize the production of tyrosinase in mycelial cultures of basidiomycetes, a heterologous expression strategy has been tried. Halalouli *et al.* (*55*) noted that their strain of *P. sanguineus* BRFM49 produced an intracellular tyrosinase at relatively low levels. They cloned the gene for this tyrosinase into *Aspergillus niger*, with the preprosequence of the glucoamylase from *A. niger* being used to target secretion. Their recombinant *A. niger* was able to produce monophenolase and diphenolase activities in the culture broth of 534 and 1.67 U/L, respectively. This strategy of heterologous expression offers promise for obtaining high levels of mushroom tyrosinases and the secretion of the enzyme into the culture medium facilitates recovery and purification. Work with a microfungus shows the potential of this approach: Selinheimo *et al.* (*56*) used the *cbh1* promoter for the homologous superexpression of the *Trichoderma reesei tyr2* gene, obtaining tyrosinase concentrations of up to 1.0 g/L in the culture medium.

Extraction and purification

When tyrosinase is produced in the fruiting body or by fermentation, it is necessary to recuperate and purify it. In the case of the fruiting body, frozen mushrooms are homogenized in a blender and then passed through a French press (*57,58*). Acetone or ammonium sulfate is added to precipitate the enzyme and other proteins (*7, 52,57,58*). The resuspended precipitate is passed through one or more chromatography columns for further purification. The most commonly used columns contain hydroxylapatite (*57*), DEAE-cellulose (*59,60*) or DEAE Sepharose (*48*), although various other packings have been used, including an immunoaffinity resin (*61*) and a Sephadex size exclusion gel (*7,62*). SDS-PAGE is used to monitor efficiency during the purification steps (*7,63*). A comparative study has not been undertaken, so it is not possible to define an efficient standardized purification protocol.

Immobilization

Most applications of tyrosinase typically require immobilization, to enable the enzyme to be used repeatedly. It is desirable for the immobilized preparation to have a high stability against chemical denaturants, pH change and high temperature.

The highest productivities in batch processes for L-DOPA production were obtained when the tyrosinase was immobilized by adsorption on various forms of zeolite. Not only do these supports possess a high surface area, but also they are excellent free radical scavengers that enhance tyrosinase stability (*21*). In the case of phenol biosensors and wastewater treatment processes, the enzyme has been immobilized on various supports: an Al_2O_3 sol-gel membrane overlaid on a glassy carbon electrode (*64*), a glassy carbon electrode modified with electrodeposited gold nanoparticles (*65*), a carbon-paste electrode (*30,36,66*), a graphite electrode (*31,67*), chitosan (*68*), a glassy carbon electrode with its surface modified by

electropolymerization of a mixture of enzyme and amphiphilic pyrrolylalkylammonium tetrafluoroborate monomers (*69*), carbon-paste composites (*70*), and a carbon paste electrode with a poly(vinyl alcohol) film on its surface (*71*). However, some of these immobilization methods are rather complicated and do not give good enzyme stability or retention (*72*). Immobilization of tyrosinase on solid graphite electrodes leads to the most sensitive electrodes, capable of detecting micromolar concentrations of phenol and catechol (*3*). These electrodes combine high versatility with low cost, the surfaces are easily renewed and the noise and background currents are low (*32*,*71*).

In order to be able to select an adequate immobilization method for a particular application, it would be necessary to have a broad database about the performance of different immobilized tyrosinase preparations in different applications. Although the work cited above does represent the initial steps in this direction, our current knowledge is not sufficient to allow us to reach this ideal.

Conclusions

Although the use of tyrosinases to produce L-DOPA will probably not compete with the enzymatic process based on tyrosine phenol-lyase, other applications, such as the detection and removal of phenols and the production of cross-linked protein networks, will probably lead to increased demands for tyrosinase. In order to satisfy these demands, it will be necessary to develop processes for the production of tyrosinases by submerged culture. Given that many of the studies that have aimed to develop applications have been undertaken with mushroom tyrosinases, it would not be surprising if mushrooms were chosen for such production processes. Unfortunately, our current state of knowledge about the production by submerged culture and the recuperation, purification and immobilization of mushroom tyrosinases is limited. Also, although the heterologous expression approach could work well, it has not been widely investigated for tyrosinase production. Much more research and development work will be required before successful large-scale production processes can be established.

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