UDC 577.112.083/577.112.34 **https://doi.org/10.15407/biotech16.03.051**

L-DOPA BIOSYNTHESIS WITH *Agaricus bisporus* TYROSINASES ASSISTANCE

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Received 2022/04/20
Revised 2023/05/31
Accepted 2023/06/30

L-DOPA (3,4-dihydroxyphenyl-L-alanine) is the drug of choice in treatment of Parkinson's disease, however, the chemical method of its synthesis has a number of significant drawbacks, so biotechnological approaches are being explored as an alternative.

The aim is to develop a new affordable and effective method for L-DOPA biosynthesis by mushroom tyrosinase immobilized on a cost-effective carrier, which ensures the stability and multiple use of the enzyme.

Methods. Tyrosinase isolated from *Agaricus bisporus* was used in the work. The biosynthesis of L-DOPA was carried out in aqueous and organic media. The resulting product was analyzed by mass spectrometry, specific rotation and melting point. Tyrosinase was immobilized in poly-N-vinylpyrrolidone (PVP), the interaction with the carrier, pH-optimum, and frequency of application were determined.

Results. A partially purified tyrosinase was isolated from *Agaricus bisporus*. In an aqueous solution in the presence of the enzyme, only 5.1 % L-DOPA was obtained due to the further formation of complex polycyclic compounds. L-DOPA derivative biosynthesis in methylene chloride containing buffer solution made it possible to obtain the product in 55 % yield. Tyrosinase immobilized in PVP exhibited 30% higher activity than free tyrosinase in CH_2Cl_2 and carried out biocatalysis for 7 cycles.

Conclusions. A method has been developed for the synthesis of L-DOPA using an effective cost-effective biocatalyst based on immobilized tyrosinase, which in an aqueous-organic medium made it possible to obtain L-DOPA within 7 cycles of use.

Key words: L-DOPA synthesis; biocatalyst; tyrosinase4 immobilization; poly-N-vinylpyrrolidone.

Parkinson's disease (PD) is a slowly progressive, chronic neurological disease characterized by neurons death in the brain basal ganglia and causes a range of effects, including rigidity, akinesia, bradykinesia, and resting tremor. Worldwide, about 5 million people suffer from this disease [1].

3,4-dihydroxyphenyl-L-alanine (L-DOPA, levodopa) is the drug of choice in the treatment of CP, synthetic levorotatory dihydroxyphenylalanine is used as a drug, which is much more active than the dextrorotatory isomer [2]. Currently, the main way to obtain L-DOPA is the chemical method of asymmetric synthesis, which is characterized by a low degree of conversion, low enantioselectivity and requires an expensive metal catalyst. Therefore, as an alternative, various biotechnological approaches using enzymes, microorganisms and drug isolation biological objects are being investigated. In comparison with chemical synthesis, microbial transformation usually exhibits a sufficiently high enantioselectivity, but requires long-term cultivation and complex methods of isolation and purification of the target product from the culture medium [3].

Enzymatic synthesis of L-DOPA, catalyzed by tyrosinase, is characterized by high enantioselectivity, proceeds under mild conditions of pH and temperature. However, its use on an industrial scale is restrained by denaturation and the one-time use of the biocatalyst. To eliminate the

described shortcomings, immobilization of the enzyme is used, which facilitates the separation of tyrosinase from the reaction medium, increases stability, preventing its denaturation, and makes multiple use possible. Thus, for the development of biocatalysts for the enzymatic synthesis of L-DOPA, various carriers (multilayer coating of epoxy resin with electrolytes, poly(ethylene oxide)/polypyrrole, polyhydroxyalkanoate nanogranules, agar-agar granules crosslinked with glutaraldehyde, etc.) and immobilization methods (inclusion in a gel) were used , cross-linking, covalent bonding). However, in many cases, after immobilization, the activity of tyrosinase decreased significantly, the frequency of use was small, and the price of the carrier was high [4–8]. Therefore, the purpose of this study is to develop a new, affordable and effective method of biosynthesis of L-DOPA using partially purified tyrosinase from *Agaricus bisporus* mushrooms, immobilized with an economical carrier using, which ensures stability and multiple uses of the enzyme.

However, in some cases, after immobilization, the activity of tyrosinase significantly decreased, as shown by Yildiz S. et al., who developed two methods of immobilizing tyrosinase on polypyrrole derivatives [5]. In addition, the frequency of biocatalysts use was not high in some places, for example, Botta G. and co-authors used tyrosinase immobilized in Eupergit®C250L resin with additional coating by layer method. Enzyme activity retention after immobilization was 16–38%, the multiple use was only 5 cycles [6]. Works using tyrosinase immobilized on polyhydroxyalkanoate nanoparticles are promising; the obtained drug had high activity during 8 cycles of use, however, the isolation of the enzyme and the method of its immobilization are quite complex and expensive [7, 8].

Therefore, the purpose of this study was to develop a new, affordable and effective method of biosynthesis of L-DOPA in an aqueous-organic medium using partially purified tyrosinase isolated from *Agaricus bisporus* mushrooms, immobilized using an economical non-toxic carrier, which ensures the stability and reusability of the enzyme.

Materials and Methods

In the work, a tyrosinase preparation from *Agaricus bisporus* mushrooms was used, and a partially purified preparation was isolated according to [8]. In the selected tyrosinase preparation, the protein content was determined according to the Lowry method in Hartree's modification [9], the content of copper ions [10], the activity according to L-DOPA [11] and L-tyrosine according to [12]. The L-DOPA content was determined as modified by the 4-aminoantipyrine method [13].

L-DOPA synthesis using tyrosinase in an aqueous solution was carried out as follows. 0.5–2.5 mol/dm^3 L-tyrosine in a buffer solution (0.05 mmol/dm^3 Na-phosphate buffer solution pH 6.5) was added to 100 cm^3 1–5 mol/dm^3 of ascorbic acid and 5 cm^3 of tyrosinase solution (activity of 750 units/mg of protein per minute). After 60 minutes, 10 cm^3 of 10% benzoic acid solution (pH 7.0) was added. To obtain N-acetyl derivative of L-tyrosine ethyl ester, 10.5 g of free base of L-tyrosine ethyl ester, 5 cm^3 of acetic acid and 8 cm^3 of acetic anhydride were added to a three-necked flask with a capacity of 100 cm^3 with stirring, brought to a boil, kept for 1 min and allowed to cool to room temperature. The cooled solution was evaporated, treated twice with ethyl acetate. The obtained oil was dissolved in 50 cm^3 of methyl alcohol, water was added until cloudy and placed in the refrigerator. The precipitate was filtered and dried.

L-DOPA biosynthesis in an organic solvent was carried out by adding to 10.8 mg of N-acetyl derivative of L-tyrosine ethyl ester in 5 cm^3 of methylene chloride, 0.4 cm^3 of 0.05 mol/dm^3 Na-phosphate buffer solution (pH 6.5) and 400 units/cm^3 of tyrosinase preparation. The synthesis was carried out with intensive stirring for 1 h at room temperature. The reaction was monitored by thin layer chromatography (TLC). After the substrate disappearance, the biocatalyst was removed and the organic layer was treated with an equal volume of sodium dithionite solution (1%) to reduce DOPA-quinone with protected functional groups to the corresponding catechin. The mixture was stirred for 5 min and the phases were separated. The aqueous phase was acidified with HCl (1.0 M) and extracted twice with ethyl acetate. The organic extracts were treated with saturated NaCl solution and dried over anhydrous Na_2SO_4, then filtered and concentrated to give a colored crude product. The residue was purified by flash chromatography [7]. The functional groups of the L-DOPA derivative were deprotected by boiling in 2 M hydrochloric acid solution, the reaction was monitored by TLC. The obtained product was analyzed by mass spectrometry,

TLC, specific rotation and melting point (yield 55%; M = 197 g/mol, melting point — 276–277 °C; FAB mass spectrum, m/z (I, %):198 (100%)$[M + H]^+$; specific rotation: — 13.0° (c = 5.12 in 1 N HCl), which corresponds to the literature data [14].

Immobilization of the isolated tyrosinase in poly-N-vinylpyrrolidone (PVP, M.m. 20000) was carried out as follows: 2000 units of the tyrosinase preparation were added to 3.5 cm^3 of 7.7% PVP solution and the solution was poured into a glass container. The form was kept open until drying. The enzyme activity in the organic medium was determined by increase in optical density of the solution at 389 nm. The enzyme amount that caused an increase in optical density by 0.001 per minute at 25 °C was taken as a unit of activity [7].

The pH-Optimum of free and immobilized enzyme preparations was determined by adding a solution of the substrate in methylene chloride and 0.4 cm^3 of the appropriate buffer solution with different pH values (4.0–8.0) to samples of equal activity. The effect of tyrosinase on the viscometric characteristics of PVP solutions was determined by measuring the viscosity of their aqueous solutions and the viscosity of polymer solutions when the appropriate amount of enzyme was added using an Ostwald viscometer (capillary diameter 0.73 mm). Characteristic viscosity was calculated according to [15].

The immobilized tyrosinase use multiplicity was determined by transforming the N-acetyl derivative of ethyl ester L-tyrosine according to the method described above. Multiple application of the biocatalyst was studied up to 50% preservation of enzyme activity. Statistical processing of the results was carried out in the Statistica program using the Student's t-test, the results were considered reliable at ($P < 0.05$).

Results and Discussion

Since commercial drugs have an extremely high cost, we isolated a partially purified preparation of tyrosinase with a protein yield of 0.82 mg/g of mushrooms, a copper ion content of 0.19%, and a specific activity of 750 and 4300 units/mg of protein per minute for L-tyrosine and L-DOPA, respectively, was isolated from *Agaricus bisporus* mushrooms.

Under the developed conditions (0.05 mol/dm^3 Na-phosphate buffer solution (pH 6.5), temperature 25 °C), L-DOPA biosynthesis from L-tyrosine was carried out. Tyrosinase catalyzes the tyrosine o-hydroxylation to L-DOPA, but the enzyme also catalyzes the subsequent oxidation reaction of L-DOPA to DOPA-quinone, which turns into DOPA-chromium, which further forms complex polycyclic compounds. At the same time, L-tyrosine oxidation is a rate-limiting stage in tyrosinase catalysis, i.e. the production of L-DOPA-quinone proceeds much faster, as a result of which it is impossible to isolate the L-DOPA intermediate product. Therefore, to obtain L-DOPA in the process of biocatalysis, ascorbic acid was added to the reaction mixture, which reduced DOPA-quinone to L-DOPA (Fig. 1).

However, most reducing agents, including ascorbic acid, are tyrosinase inhibitors [16]. Therefore, it was necessary to select such a concentration of ascorbic acid, which would restore the DOPA-quinone that was formed, and at the same time, to a small extent, reduce the activity of the enzyme (Table 1).

It was shown that the best concentration of ascorbic acid was 2.5 mmol/dm^3, a decrease in the concentration of the reducing agent led to almost complete conversion of L-DOPA into DOPA-quinone, and an increase in the concentration of ascorbic acid contributed to an even stronger decrease in product yield due to a significant inhibition of tyrosinase activity.

Table 1

Effect of ascorbic acid concentration on the formation of L-DOPA catalyzed by tyrosinase

Ascorbic acid concentration, mmol/dm3	L-DOPA formation *	
	mmol/dm^3	Yield, %
1	0.040±0.001	1.6±0.1
2	0.093±0.003	3.7±0.1
2.5	0.128±0.006	5.1±0.2
3	0.088±0.003	3.5±0.1
5	0.031±0.001	1.2±0.1

* $P < 0,05$; $n = 5$.

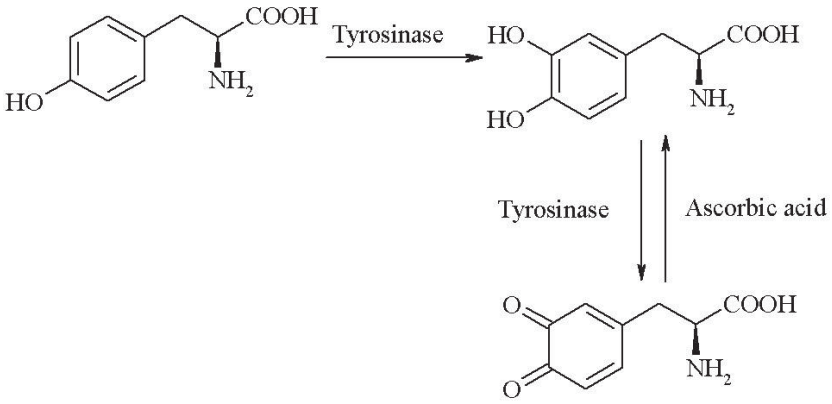

Fig. 1. **L-DOPA synthesis catalyzed by *Agaricus bisporus* tyrosinase**

The effect of tyrosine concentration on the L-DOPA formation in the presence of tyrosinase was investigated. It was found that the product concentration increases non-linearly, which is probably due to the unsaturation of the enzyme with the substrate at low concentrations of tyrosine (Fig. 2).

The highest L-DOPA formation is observed at a substrate concentration of 2.5 mmol/dm^3, further increase in concentration is impossible due to poor solubility of tyrosine in water. Thus, with the help of isolated tyrosinase in an aqueous solution, the L-DOPA formation with a yield of only 5.1% was shown, due to the further oxidation of the product by an enzyme, with the production of reactive *o*-quinone, which is transformed into DOPA-chromium with subsequent formation of complex polycyclic compounds.

Therefore, in order to prevent the intramolecular cyclization of DOPA-quinone, it was necessary to protect the functional groups of L-tyrosine. For this, the ethyl ester of L-tyrosine was subjected to the action of acetic acid and acetic anhydride (5:8 v/v) (Fig. 3).

The resulting N-acetyl derivative of the ethyl ester of L-tyrosine is almost insoluble in water. Therefore, its oxidation to the L-DOPA derivative with protected carboxyl and amino groups, catalyzed by tyrosinase (Fig. 4), was carried out in an organic solvent (methylene chloride). The resulting L-DOPA derivative is also oxidized by tyrosinase to the *o*-quinone derivative, but due to the protection of the functional groups, intramolecular cyclization does not occur.

In the course of biosynthesis, a low degree of substrate conversion is shown. It is known that adding small amounts of water to enzymes that catalyze reactions in organic solvents significantly increases speed of the process. It was established that adding 0.4 cm^3 of 0.05 mol/dm^3 Na-phosphate buffer solution pH 6.5 to the studied reaction mixture led to complete bioconversion of the tyrosine derivative. Yield of the L-DOPA derivative with protected functional groups was 83%, which agrees with the literature data [7]. After deprotection from the carboxyl and amino groups of L-DOPA, the synthesis

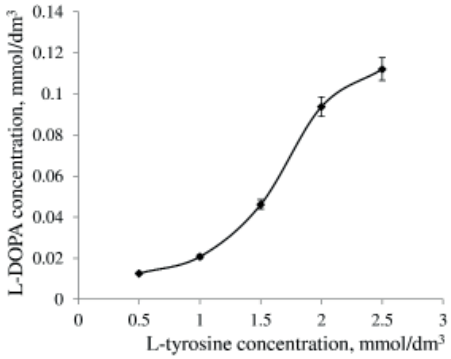

Fig. 2. **The dependence of L-DOPA concentration formed with the help of tyrosinase on the tyrosine concentration ($P < 0{,}05$; $n = 5$)**

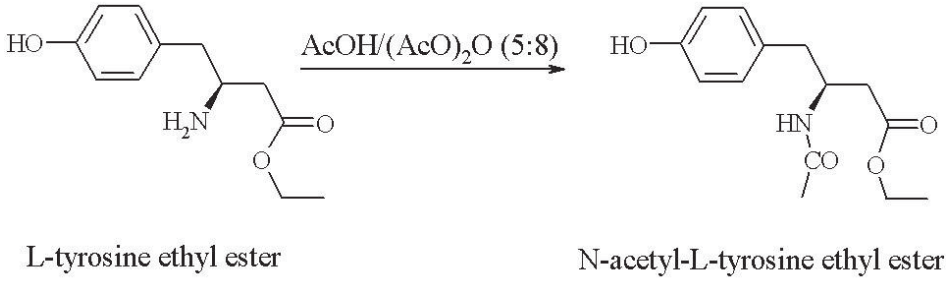

Fig. 3. **Obtaining of N-acetyl derivative of L-tyrosine ethyl ester**

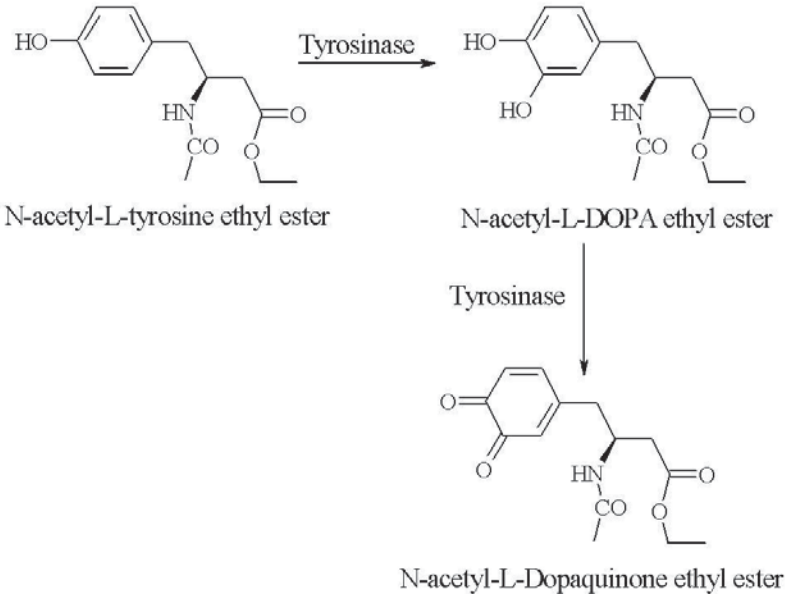

Fig. 4. **Synthesis of L-DOPA derivative with protected functional groups catalyzed by *Agaricus bisporus* tyrosinase**

product yield was 55%. The obtained product was analyzed using mass spectrometry, TLC,measurement of melting point and specific rotation. To create an effective, economical, stable, reusable biocatalyst, the selected tyrosinase preparation was immobilized in an available synthetic polymer — poly-N-vinylpyrrolidone.

Important properties of PVP, which are of great practical importance, are its high adsorption capacity and tendency to complex formation. PVP is a non-toxic promising carrier for inclusion of cells of microorganisms and enzymes, BAR. Thus, immobilized forms of enzymes with a prolonged effect were obtained on the basis of high molecular weight PVP [17, 18].

As a result of immobilization of tyrosinase in PVP, polymer films were obtained with 80% preservation of the enzyme original activity for tyrosine, when used in a buffer solution, and the characteristics presented in the Table. 2.

The interaction of tyrosinase with the carrier was studied by the viscometry method, measuring the viscosity of PVP solutions of various concentrations and the viscosity of these solutions when the appropriate amount of tyrosinase was added (Fig. 5).

When comparing the obtained viscosity values, it is shown that the addition of the enzyme to PVP solutions increases their indicated viscosity (Fig. 5).

By extrapolating the graph of the dependence of the indicated viscosity of polymer solutions on their concentration to the intersection with the ordinate axis, the values of the characteristic viscosity were obtained ($2.31 \cdot 10^2$ and $2.53 \cdot 10^2$ cm^3/g for PVP and PVP with tyrosinase, respectively), which can testify to the presence of interaction between enzyme and polymer. That is, immobilization of tyrosinase is probably carried out due to physical inclusion in the PVP structure and non-valent interactions of enzyme with the matrix.

Table 2

Characteristics of the tyrosinase preparation immobilized in PVP

Characteristics	Results *
Tyrosinase activity by tyrosine, U/g immobilized enzyme	2000 ± 22
Enzyme content, mg/g of film	3.3 ± 0.7
Organoleptic characteristics	Homogeneous transparent films
Diameter, cm	1.5 ± 0.1
Area of a film, cm^2	5.55 ± 0.3
Thickness, mm	0.20 ± 0.01
Mass, g	0.3 ± 0.07

*$P < 0,05$; $n = 5$.

Table 3

Influence of the pH of the buffer solution added to the incubation medium on the activity of free and immobilized tyrosinase

pH	Tyrosinase activity, % *	
	Free enzyme	Immobilized enzyme
4	28.3 ± 0.8	47.1 ± 1.4
5	53.3 ± 1.6	58.8 ± 1.8
6	95.8 ± 3.0	96.1 ± 2.8
6,5	100.0 ±3.1	100 ± 2.8
7	97.2 ± 2.8	97.5 ± 2.9
8	43,7 ± 1.4	50.5 ± 1.4

*$P < 0,05$; $n = 5$.

In the work, the possibility of using the obtained biocatalyst in the environment of methylene chloride (with the addition of 7.5% buffer solution) was investigated. It was established that the immobilized drug showed 30% higher activity than free tyrosinase, which is explained by stabilization of the enzyme activity — immobilized tyrosinase, under adverse conditions, which allows using a smaller amount of biocatalyst.

In the process of studying the properties of the obtained drug, the pH effect of the buffer solution added to the methylene chloride medium during the synthesis process on the activity of free and immobilized tyrosinase was determined.

It was established that as a result of immobilization, the pH-optimum of tyrosinase activity (6.5) does not change. An expansion of the tyrosinase activity pH profile in the area of acidic values was revealed (Table 3).

The inclusion of an enzyme in PVP allows the obtained biocatalyst to be used multiple times. The study of the possibility of repeated use of immobilized tyrosinase in the environment of methylene chloride with the addition of a buffer solution (7.5% v/v) showed that the obtained preparation retains high phenoloxidase activity during 6 cycles of use (Fig. 6). While G. Botta and co-authors [7] found that the biocatalyst developed by them on the basis of tyrosinase immobilized using Eupergit C250L epoxy resin covered with electrolytes, under similar conditions, retained activity only for 3 cycles of use.

Thus, the L-DOPA biosynthesis in an aqueous-organic environment, catalyzed by isolated tyrosinase from *Agaricus bisporus* mushrooms, with 55% product yield was developed. A highly efficient, stable, affordable, reusable, non-toxic biocatalyst based on tyrosinase immobilized in poly-N-vinylpyrrolidone was created to obtain L-DOPA during six cycles of use in a batch reactor.

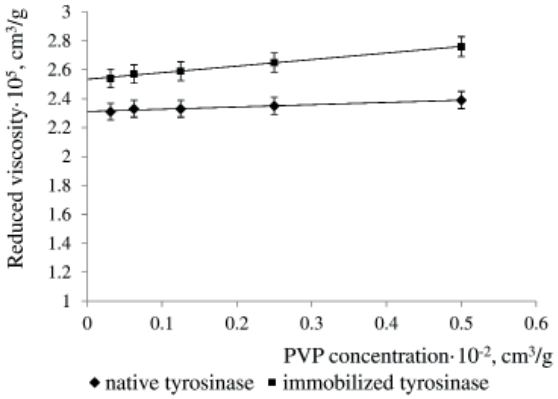

Fig. 5. **Dependence of viscosity on the concentration of free and immobilized tyrosinase ($P < 0,05$; $n = 5$)**

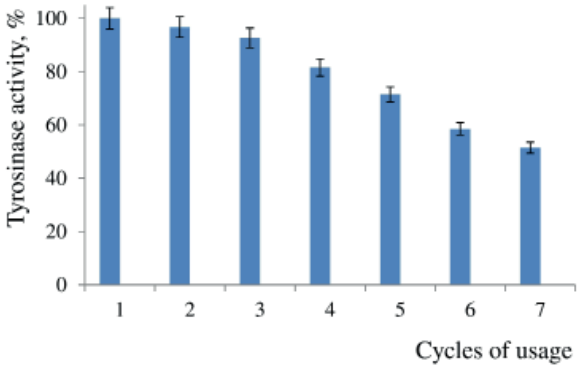

Fig. 6. **Dependence of tyrosinase activity on the frequency of use of the biocatalyst ($P < 0,05$; $n = 5$)**

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БІОСИНТЕЗ L-ДОФА ЗА ДОПОМОГОЮ ТИРОЗИНАЗИ *Agaricus bisporus*

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L-ДОФА (3,4-дигідроксифеніл-L-аланін) є препаратом вибору при лікуванні хвороби Паркінсона, однак хімічний метод його синтезу має ряд недоліків, тому як альтернатива досліджуються біотехнологічні підходи.

Мета — розробити новий доступний і ефективний спосіб біосинтезу L-ДОФА за допомогою тирозинази грибів, іммобілізованої з використанням економічного носія, що забезпечує стабільність і багаторазовість використання ензиму.

Методи. У роботі використовували виділену тирозиназу *Agaricus bisporus*. Біосинтез L-ДОФА проводили у водному і органічному середовищі. Отриманий продукт аналізували за допомогою масспектрометрії, питомого обертання і температури плавлення. Іммобілізацію ензиму проводили в полі-N-вінілпіролідон (ПВП), визначали взаємодію з носієм, pH-оптимум і кратність застосування.

Результати. З *Agaricus bisporus* виділений частково очищений препарат тирозинази. У водному розчині в присутності ензиму було показано отримання лише 5,1 % L-ДОФА, через подальше утворення складних поліциклічних сполук. Біосинтез похідного L-ДОФА у хлористому метилені з додаванням буферного розчину дозволив отримати продукт з виходом 55 %. Тирозиназа, іммобілізована в ПВП, проявляла активність на 30 % вище за вільну у середовищі CH_2Cl_2 і здійснювала біокаталіз протягом 7 циклів.

Висновки. Розроблено спосіб синтезу L-ДОФА за допомогою доступного біокаталізатора на основі іммобілізованої тирозинази, що у середовищі хлористого метилену дозволила отримувати L-ДОФА протягом 7 циклів використання.

Ключові слова: синтез L-ДОФА; біокаталізатор; тирозиназа; іммобілізація; полі-N-вінілпіролідон.