Plovdiv University "Paisii Hilendarski"

Faculty of Biology
Department of Biochemistry and Microbiology

ANGEL ILIEV PESHKOV

INVESTIGATION OF THE PROPERTIES OF OXIDOREDUCTASES IMMOBILIZED IN BIOCOMPATIBLE MATRICES

ABSTRACT

of a dissertation
for the acquisition of the educational and scientific degree **"Doctor"**
Field of higher education: **4.** Natural sciences, mathematics and informatics
Professional direction: **4.3.** Biological sciences;
Doctoral program: **Biochemistry**

Supervisors:
Prof. Dr. Iliya Nikolov Iliev PhD
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The experimental studies were carried out at the Department of Biochemistry and Microbiology at the Faculty of Biology of the University of Plovdiv "Paisii Hilendarski" and in the laboratories of the Technology Center at the University of Plovdiv "Paisii Hilendarski".

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The open closing meeting of the scientific jury will take place on .....07.2025. from … hours in the "Compass" hall of the University of Plovdiv "P. Hilendarski".

The materials on the protection are provided for free access to those interested in the library of the University of Plovdiv "Paisii Hilendarski".

Author: Angel Iliev Peshkov

Title: Investigation of the properties of oxidoreductases immobilized in biocompatible matrices

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INTRODUCTION

The growing population and rapid industrial development of the world is directly related to the pollution of the environment with various pollutants, both emerging ones and micro- and nano plastics, pharmaceuticals, dangerous dyes, hormones and organic pollutants, well known from various industrial processes. They have been found to cause various side effects and diseases such as cancer, neurotoxic and mutagenic effects, gastrointestinal disorders, loss of peripheral vision, impaired coordination of movements and weakened muscles, as well as a number of other degenerative diseases.

The presence of these pollutants in the environment and water sources affects all living organisms and disturbs the ecological balance, so their removal from the environment is aimed at smarter, greener and more environmentally friendly technology, which has become the main goal in the fight against pollutants of all sources.

A huge part of the pollutants are extremely stable and their removal is a serious challenge. Therefore, the use of enzymes in the processes of detection of toxic substances is an important part of modern biotechnological processes for environmental purification.

Oxidoreductases are placed in Class I in the nomenclature of enzymes, whose oxidative properties show an enviable positive effect on the degradation of various pollutants. The unique properties of this class of enzymes in terms of selectivity and catalytic characteristics make them stable candidates for biocatalytic treatment. Since many chemical and biochemical transformations involve these processes, the development of practical biocatalytic applications of oxidoreductases is an important goal in biotechnology.

In detoxification processes, oxidoreductases laccase (EC 1.10.3.2) and catalase (EC 1.11.1.6) are widely used. Catalase has one of the highest values of the catalytic rate constant (Km), with one enzyme molecule catalyzing the decomposition of over a million molecules of hydrogen peroxide per second. The main mechanism of action of this enzyme involves the disproportionation of two molecules of hydrogen peroxide (H_2O_2) to oxygen and water, without forming reactive oxygen particles. Laccase is most often produced by filamentous fungi, as well as by some bacteria and higher plants. The enzyme has the potential for numerous biotechnological applications, such as the degradation of lignin, as well as many environmental applications for the biodegradation of organic pollutants, with laccase enzymes being able to transform various types of hard-to-destroy pollutants, including pharmaceutical contaminants such as antibiotics, chlorophenols and dyes.

Laccase and catalase are enzymes that play a significant role in biosensor technology due to their catalytic properties and potential for specific interactions with substrates. Lacasse-based biosensors can detect phenolic contaminants, such as those found in water or industrial waste, by oxidation of phenolic groups,

providing a simple and cost-effective method of monitoring the environment. In bioelectrochemical sensors, laccase can be integrated into biofuel cells or environmental sensors, where it serves as a biocatalyst for electron transfer, aiding in the conversion of chemical energy into electrical signals.

Catalase, together with glucose oxidase and peroxidase, are the biological part of biosensors for quantifying blood glucose in humans. A cholesterol-based oxidase and catalase-based biosensor was developed jointly immobilized on a graphene/ionic liquid-modified fiberglass electrode to develop a highly sensitive amperometric cholesterol biosensor. Catalase is often used as a component of food and cosmetic emulsions to prevent the oxidation of the oil fraction and extend the shelf life.

The prospect of using catalase and laccase in the construction of biosensors with multiple applications presupposes scientific research for the development of new methods for immobilization of enzymes and their inclusion in new biosensors.

AIM AND TASKS

The aim of the present thesis is to investigate the capacity of the enzymes catalase and laccase obtained from various sources for inclusion in biosensors for conducting catalytic reactions under conditions of heterogeneous biocatalysis and in the presence of organic solvents.

To achieve the goal, the following tasks have been set:

1. To investigate the kinetic parameters of native and immobilized form catalase of different origins (mold, bacterial and bovine) in a heterogeneous medium in the presence of an organic solvents.
2. To investigate the kinetic parameters of native and immobilized forms of laccase from *Trametes versicolor* in a heterogeneous medium in the presence of an organic solvents.
3. To optimize the electrochemical methods for determining the enzymatic activity of the native and immobilized form of the studied enzymes catalase and laccase in a heterogeneous medium in the presence of an organic solvents.
4. To investigate the parameters of biosensors made with the participation of immobilized forms of the studied enzymes catalase and laccase.
5. To investigate the applicability of the constructed laccase biosensor for quantitative analysis of phenolic acids and polyphenols in herbal extracts.

MATERIALS AND METHODS

1. Biochemical methods

1.1. Extraction, concentration, purification and drying of the enzyme catalase *Pen. chrysogenum*

The mold catalase is produced by a mutant strain *Pen.crysogenum* and is isolated and purified using a patented technology. The purified form of the enzyme preparation was generously provided for the purposes of the present thesis by Prof. Dr. Iliya Iliev.

1.2. Determination of catalase enzyme activity from different sources in homogeneous and heterogeneous environments

Catalase activity in a homogeneous and heterogeneous environment of enzymes from *Pen.crysogenum, Micrococcus lysodeictikus* and catalase from bovine liver was determined according to the Worthington analysis procedure, briefly: 100 µl of enzyme solution was added to 2.9 ml of solution of its substrate (50 mM hydrogen peroxide in 20 mM buffer, pH 7.00) at a constant temperature of 20 °C in a 4.5 mL quartz cuvette. Enzymatic kinetics were traced at 240 nm, at which wavelength the change in light absorption is recorded. The change in the concentration of the substrate – hydrogen peroxide, is calculated on the basis of the reported light absorption according to the Bouguet-Lambert-Beer law, assuming an extinction coefficient ε of 240 = 43.6 $mol.l^{-1}$ cm^{-1}. To determine the catalytic activity of catalases, 3 to 5 measurements were carried out with each enzyme. A thermostatic spectrophotometer Shimadzu UV-VIS 2600 was used.

1.3. Titrimetric method for determining the enzymatic activity of catalase from different sources in a homogeneous medium (Chaga, G., *et al.,* 1992)

1.4. Determination of the enzymatic activity of laccase from different sources by spectrophotometric method

The absorption increase in 3 min was measured spectrophotometrically (Shimadzu UV-VIS 2600) at 420 nm wavelength. The reaction mixture contains: 100 µl 50 mM ABTS, 800 µl 20 mM Na-acetate buffer (pH 4.5) and 100 µl suitably diluted enzyme (Patel, H., *et al.,* 2014). Measurement is carried out under standard conditions versus control sample. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 µM substrate per minute at room temperature.

1.5. Determination of the enzymatic activity of laccase in the presence of organic solvents

Initially, enzyme activity was determined in the absence of organic solvents, and the results obtained were counted as the control. In this case, the laccase from *Trametes versicolor* dissolved in 20 mM Na-acetate buffer with a pH of 4.5 and the enzymatic reaction with 50 mM ABTS was observed for 3 minutes. The analysis was then repeated following the same procedure, adding 10% and 20% to the reaction mixture in successive measurements of each organic solvent – acetone, dimethyl sulfoxide (DMSO), methanol and ethanol. The measurement is carried

out minutes after the addition of each solvent. In order to track the influence of each of the solvent on the enzyme molecule and how the enzyme activity changes, based on the initial results, the incubation of the enzyme takes place for one and two hours in the presence of organic solvents with a concentration of 10% and 20%, where the activity of the enzyme is observed immediately after incubation.

1.6. Quantification of protein according to Bradford, M.M. (1976)

1.7. Tris-Glycine SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1.8. Tris-Glycine Native Polyacrylamide Gel Electrophoresis (Native-PAGE)

2. Electrochemical methods

2.1. Obtaining Modified Electrode Materials

Before being modified, all glassy carbon surfaces were polished with γ-Al_2O_3 suspensions with particle sizes of 0.3 and 0.05 μm on polishing pads (LECO, Plzeň, Czech Republic and Hereus Kreuz, Germany) and rinsed with ultrapure water. Each polishing step was followed by two sonication in ultrapure water for 1–2 minutes.

The enzymatic immobilization of the enzyme laccase is carried out as follows: 2 μl of enzyme solution is drop cast on the surface of the electrode. Then a 4-μl drop of the binder (Nafion 117 diluted with ultrapure water to 0.2%) is added. The surface is air-dried at room temperature (RT). The two types of laccase are immobilized on the electrode surface and the amount of the enzyme in terms of enzyme units is identical. Enzyme electrodes are rinsed with ultrapure water and stored at 4°C when not in use. The regeneration of the working enzyme electrodes can be carried out after mechanical removal of the enzyme-polymer layer after a polishing procedure and following the above steps.

2.2. Immobilization of the enzyme catalase on glass pads

For this purpose, glass pads for microscopic preparations are used, which, before enzymatic immobilization, are cleaned by washing with ethanol, washed with ultrapure water and subjected to ultrasonic treatment for 3 minutes, after which they are air-dried. A freshly prepared solution of catalase with a concentration of 5 $mg.ml^{-1}$ is applied on the prepared pads by dropping in phosphate buffer with pH 7.0 and stay in the refrigerator at 4◦C for 2 hours until the volume of the liquid is reduced by three times. Then 0.5 mM glutaraldehyde is added to the catalase-treated substrate, observing a ratio of 2:1 with the enzyme solution (for every 10 μl of enzyme solution, a portion of 5 μl of crosslinking agent is added). The resulting reaction mixture is allowed to react in the refrigerator at 4˚C for 24 hours.

2.3. Electrografting of the enzyme catalase on a gold electrode

A gold electrode shall be used pre-cleaned in accordance with paragraph **2.1**, after which its surface shall be modified with nitrophenyl groups, in accordance with the following steps.

For the attachment of nitrophenyl groups to the gold electrode, cyclic voltammetry from 0.6V to -0.6V against Ag/AgCl (3 M KCl) and a rate of 0.2 V/s is performed in a solution of 0.1 M BF4NBu4 and 2 mM diazonium salt dissolved in anhydrous acetonitrile. The electrochemical modification is repeated twice so that the nitrophenyl groups can be deposited on the surface of the gold electrode, after which the electrode is washed with water and a new cyclic wattle-amperometric reduction takes place with a 0.1M solution of KCl in ethanol/water (1:9) from 0 to -1.4V and a rate of 0.1 V/s.

It is necessary to immerse the modified electrode in a 1 mM solution of mercaptohexanol (MN) in ethanol overnight in order to modify the places that remain unmodified on the surface of the electrode with a so-called "spacer" and generate an aminophenyl-MH layer.

2.4. Covalent Immobilization of Catalase on the Electrochemically Modified Gold Electrode

Covalent immobilization of catalase on the electrochemically modified gold electrode is achieved by forming amide bonds between the carboxylic acid groups of the enzyme and the amino groups of the functionalized surface of the gold electrode. In this case, a 10 μL solution of the enzyme (8.9 μg/ml in 0.1 M phosphate buffer, pH=6.0) is added to a 400 μl 10 mM solution of 2-(N-morpholino)ethanesulfonic acid (MES) (pH=6.0) containing 36 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 17 mM N-hydroxysuccinimide (NHS). The electrode is immersed in this solution for 2 hours, then washed with a 10 mM solution of 2-(N-morpholino) ethanesulfonic acid and water.

3. Electrochemical characterization of modified glassy carbon or gold electrodes

For this purpose, a three-electrode electrochemical cell consisting of: 1) a working (*unmodified or modified glassy carbon; or gold electrode with electrografted catalase);* 2) a comparative electrode (*Ag/AgCl, sat. KCl)*; and 3) and counter electrode (*Pt plate*) containing 10 ml of background electrolyte (0.1 M *phosphate buffer*, *pH = 7.0, or 0.05 M citrate buffer containing 0.1 M NaClO4*, *pH = 4.0*) is connected to the Autolab 302N electrochemical station. Through the specialized software of the device, the cyclic voltammetry method is set and specific operating values of the parameters are set: initial and final potential, number of scans, scanning speed and operating current range. The cyclic volt-ampere curves are registered – the dependence of the current on the potential of the working electrode, as the latter changes according to a linear law over time in the forward and reverse direction (cyclically). After the background dependencies are established, a certain volume *(μl*) of the solution of the test electrochemical active substance (*0.12 M H_2O_2, or 10 mM solutions of catechol, pyrogallol, resorcinol, caffeic or gallic acid) is added to the cell* and the volt-ampere curves of the electrode under these conditions are recorded again in the manner described above. If

necessary, the electrolyte is purged with chemically pure argon (99.99%) before measurements.

The experimental setup for performing the studies with differential pulse voltammetry is analogous, with the difference that the scanning speed (v = 0.07 V.s-1), amplitude (E = 25 mV) and pulse duration (t = 50 ms) and operating current range are additionally set. A voltampere curve is recorded – the dependence of ΔI (the difference between the current registered before the imposition of the pulse and the current registered at the end of the imposed pulse) on the potential of the working electrode.

A constant operating potential, E (V) is set and a steady-state background current, I_0 (μA) is awaited. After its stationing, a certain volume (μl) of the solution of the test substances (*0.12 M H_2O_2, or 10 mM solutions of catechol, pyrogallol, resorcinol, caffeic or gallic acid) is added to the cell* and the new value of the steady-state current I_s is reported (μA) as a result of the addition of the analyte. Chronoamperometric measurements are performed by constant agitation with a rotating disc electrode (500 rpm for laccase biosensor studies or 2000 rpm for immobilized catalase catalytic activity) at room temperature of 20 ± 1°C. When necessary, buffers are purged either with chemically pure argon (99.99%) or with air during measurements.

4. Scanning electron microscopy (SEM)

The surface morphology of the polymer-binding catalase Nafion immobilized on glass substrates was investigated by scanning electron microscopy. Two types of samples were characterized, on which microscopic photographs were made and elemental analysis was performed

- a sample of immobilized catalase that has not been used for catalytic experiments;
- a sample of immobilized catalase, which was used for the catalytic decomposition of H_2O_2.

The samples were deposited by dripping onto glass pads with approximate dimensions of 2.5x1.2 cm. Before microscopic observation, they were coated with a layer of cathodically evaporated gold to ensure high conductivity on the surface.

RESULTS AND DISCUSSION

1. Study of the kinetic parameters of catalase of different origin

1.1. The enzymatic activity of catalases from different sources in a homogeneous and heterogeneous environment

In order to construct the biosensor, it is necessary to determine the appropriate type of catalase based on its catalytic characteristics. In this regard, the catalytic activity of catalases isolated from various sources – from fungi (*Pen. chrysogenum*), bacteria (*Micrococcus lysodeictikus*) and one from bovine liver is investigated The *Pen. chrysogenum* strain is provided by prof. Iliya Iliev. The other two enzymes, catalase from bovine liver and catalase from *Micrococcus lysodeictikus,* are commercial products purchased from Sigma-Aldrich.

A comparative analysis of the activities of native enzymes was carried out, which were subsequently immobilized in order to obtain a heterogeneous biocatalyst for multiple use. Table 1 shows the results of determining the enzyme activity of all studied enzymes in a homogeneous and heterogeneous environment.

Table 1. Initial enzymatic activity of catalase enzyme preparations from three different sources (U/mg product).

Catalase	Activity in the enzyme preparation	Specific enzymatic activity (U/mg protein)
Fungal *(Penicillium chrysogenum)*	258 550 ± 50 U/mg	5910(U/mg protein)
From bovine liver	1 146 427 ± 30 U/mg	18650(U/mg protein)
Bacterial *(Micrococcus lysodeictikus)*	130 700 ± 50 U/ml	191(U/mg protein)

After the screening of the catalytic activity of the enzymes, the two most active enzymes – catalase of animal origin and catalase of mold source, were subjected to electrophoresis in order to determine their molecular weights, similarities and differences in their subunits. The third enzyme studied, catalase of bacterial origin (*Micrococcus lysodeikticus*) in the form of an enzyme solution (Sigma-Aldrich), was not further subjected to electrophoretic studies and was used in the form in which it was purchased.

Both enzymes analyzed by SDS-PAGE electrophoresis were obtained in a crystalline state. In this way, the purity of the protein samples can be evaluated and the progress of the fractionation or purification procedure can be traced.

From the presented results (Fig. 1) it is evident that the two enzyme preparations differ in their homogeneity: fungal catalase has a homogeneous composition, in which two high-molecular fractions differ – with molecular masses respectively of about 60 kDa and about 50 kDa, corresponding approximately to the mass of one catalase subunit (catalase exists in the form of a tetramer). Bovine liver catalase consists of at least 4 different fractions. The predominant presence is the fraction with a molecular mass of approx. 55 kDa, i.e. the “decay” of the catalase tetramer into separate subunits is again observed.

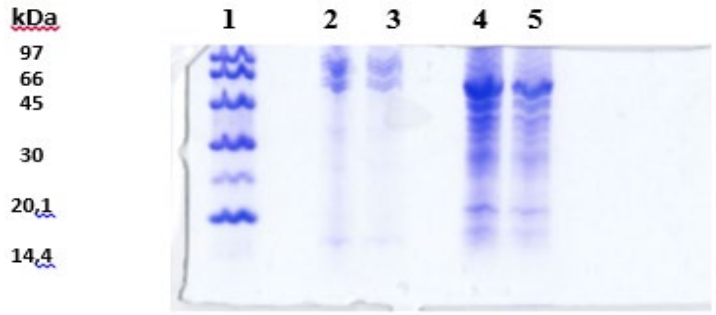

Figure 1. Tris-tricine SDS-PAGE of catalases of *Penicillium chrysogenum* and bovine liver at 4 % concentration gel and 12 % separation gel.
Legend: **1** – marker (5 µl); **2**– 50x dilution *Pen. chrysogenum*; **3** – 100x dilution *Pen. chrysogenum*; **4**- 50-fold dilution of catalase from bovine liver; **5** – 100-fold dilution of catalase from bovine liver.
Marker (Amersham – LMW): phosphorylase b – 97 kDa; bovine serum albumin – 66 kDa; egg albumin – 45 kDa; anhydrase – 30 kDa; trypsin inhibitor – 20.1 kDa; α-lactalbumin – 14.4 kDa.

The remaining lower molecular weight fractions are probably due to the presence of other proteins in the enzyme preparation (e.g. isoenzymes or other proteins). The electrophoretic treatment of the enzyme preparation from *Penicillium chrysogenum* was repeated with lower dilutions and a lower concentration of the separation gel so that all the streaks could be seen, but the result remained unchanged.

2. Comparative analysis of the kinetic parameters of three types of catalase – fungal, bacterial and catalase from bovine liver

For the determination of the kinetic constants of the three studied enzymes (V_{max} and K_m), a more in-depth analysis of kinetic curves was performed. The resulting dependencies of reaction rate on substrate concentration follow a hyperbolic trend typical of Michaelis-Menten kinetics. The constants were calculated by regression analysis of the dependencies plotted in double-reciprocal coordinates (Lineweaver-Burk graph), i.e. the reciprocal rates of the enzyme-catalytic process relative to the reciprocal concentration of the substrate and the determined values of the kinetic constants are presented in Table 2.

Table 2. Comparison of kinetic parameters of catalases of different origins.

Catalase (homogeneous conditions)	**K_M (mM)**	**V_{max} (mol l^{-1} s^{-1})**	**Specific enzymatic activity, $\times 10^3$ Mmol l^{-1}min $^{-1}$ mg^{-1}**
Fungal (*Penicilium chrysogenum)*	112	9.90×10^{-4}	5.91
Bovine liver	121.32	9.02×10^{-3}	18.65
Bacterial *(Micrococcus lysodeikticus)*	77.39	2.77×10^{-4}	0.191

The spectrophotometric method is applicable to H_2O_2 concentrations over the micromolar range, while titrimetric methods can be applied at higher concentrations of peroxide. In this regard, the enzymatic activity of catalase in the absence and in the presence of aliphatic alcohols was determined. The results of this study are presented in Table 3. The catalytic activities of the three selected catalases are accepted as control. Low molecular weight alcohols – methanol or ethanol – are added to the reaction mixture and their effect on enzymatic activity is monitored. The tests were carried out in the presence of 1, 3, 5 or 10% alcohol. It

has been found that catalase from *Micrococcus lysodeikticus* loses its activity with the addition of any alcohol, and the catalases from *Penicillium chrysogenum* and bovine liver show that both catalases – fungal and bovine liver, retain their enzymatic activity to a significant extent in the presence of low concentrations of alcohols (1% ethanol or methanol), however, with increasing concentrations, both alcohols have a denaturing effect on enzymes and they lose their activity.

Similarly, the dependence of the homogeneous catalase activity of catalase on bovine liver was investigated as a function of the concentration of ethanol and methanol in the reaction medium. Experimental results showed lower stability of bovine catalase compared to mold.

Table 3. Enzymatic activity of catalase of fungal origin (*Pen. chrysogenum*) in the presence of ethanol and methanol in the reaction medium.

Catalase Pen. chryso-genum	**A_{sp} U/mg protein, Control**	**Control, %**	**A_{sp} U/mg protein**	**Enzyme activity at 1% alcohol**	**A_{sp} U/mg protein**	**Enzyme activity at 3% alcohol**	**A_{sp} U/mg protein**	**Enzyme activity at 5% alcohol**	**A_{sp} U/mg protein**	**Enzyme activity at 10% alcohol**
Ethanol	8.57	100	7.41	86.50	7.07	82.46	5.43	63.44	0	0
Methanol	8.57	100	8.40	98.03	6.08	70.93	4.94	57.66	4.94	57.66

It is known from the literature that some catalase enzymes possess in addition to catalase activity (Equation 2):

$$2H_2O_2 \rightarrow 2H_2O+O_2 \qquad (1)$$

and peroxidase-like activity (Equation 2), which is manifested by the ability of catalase to oxidize low concentrations of hydrogen donors in the presence of hydrogen peroxide:

$$2H_2O_2 + R\text{-}C(H)OH \rightarrow 2H_2O + R\text{-}CHO \qquad (2)$$

An increase in the catalytic activity of fungal catalase in solution to 83% in the presence of 1% methanol is a manifestation of peroxidase activity of this enzyme, which explains the noticeable increase in catalytic activity. On the other hand, the data obtained on bovine liver catalase suggest that it does not exhibit peroxidase activity under equivalent experimental conditions. The activities of both catalases decrease with increasing percentage of different alcohols, but catalase from *Pen.chrysogenum* is more stable than bovine liver catalase. The residual activity of fungal catalase, a native enzyme, was found to be approximately 83%, while for bovine liver catalase 58% in the presence of methanol relative to baseline activity. On the opposite, when ethanol is added to the reaction mixture – the activity of catalase from *Pen. chrysogenum* is 64% compared to baseline, and that of bovine liver catalase is 60%, suggesting that fungal catalase exhibits greater stability in the presence of alcohols than bovine liver catalase.

In our subsequent studies of the heterogeneous-catalytic activity of the enzyme, mold catalase was selected and immobilized on a polysaccharide biopolymer – water-insoluble glucan URE 13-300, and the results are presented in Table 4. The residual enzyme activity of mold catalase immobilized on water-insoluble glucan after exposure of the enzyme for 30 minutes under the influence of 1 and 5 %, respectively methanol and ethanol, is presented.

Table 4. Enzymatic activity of fungal catalase (*Pen. chrysogenum)* immobilized on glucan URE13-300 in the presence of methanol or ethanol.

Alcohol	Alcohol concentration, %		
	0	1	5
	Residual enzyme activity, %		
Ethanol	100	100	76.1
Methanol	100	98	63.5

In other studies, significant inhibition has also been observed when treating catalase with organic solvents. In their study, Aggez and Karakus (2022) report that the catalase isolated and purified by them from Aspergillus fumigatus It retains its catalytic activity up to about 80% compared to the baseline of a non-immobilized enzyme, at 5% ethanol, as in our study. As the concentration of organic solvent increases, significant differences are observed. In our study, the results show that at a concentration above 5% ethanol, fungal catalase completely loses its activity, and at exposure to 10% methanol, it retains it to about 60%. In the experimental work of Aggez and Karakus (2022), the catalase from Aspergillus fumigatus It retains catalytic activity of up to about 70% for both organic solvents. The situation with bovine liver catalase is different. The results clearly show that the enzyme has difficulty retaining activity even at organic solvent concentrations of up to 3%, resulting in a loss of activity of about 50%. About The catalase of *Aspergillus fumigatus* is reported to retain between 40 and 80% activity of its catalytic activity even in the presence of 20% organic solvent.

There is a significant difference in kinetic parameters. In catalase from *Aspergillus fumigatus*, the authors report Km and Vmax values defined as 7.4 mM and 1250 μmol.min-1 L respectively, while the data from our study shows higher K_m and V_{max} values for catalase from Pen.chrysogenum 112 mM and 9.90x10-4 mol l-1 s-1, respectively.

3. Immobilization of catalase on polysaccharide URE 13-300

The polysaccharide URE 13-300 is a glucan-type homopolysaccharide, water-insoluble. It was obtained as a result of an enzymatic reaction using glycosyltransferase URE13 300 with a molecular weight of 300 kDa. As a result of which, water-insoluble glucan is obtained, which can be used as a carrier of biologically active substances with prolonged action. The method is described in patent No. 67404B1.

The studied fungal catalase, bovine liver catalase and bacterial catalase were immobilized on the water-insoluble glucan URE13-300 according to the following procedure: prepare a solution of each of the studied enzymes with a baseline activity between 100 and 200 U/mg dry enzyme preparation in 50 mM sodium phosphate buffer with a pH of 7.5. To various amounts of purified and lyophilized water-insoluble glucan (from 0.5% to 5%), the catalase solution is added at a total enzyme activity of 50-100 U/ml. The composition is homogenized and incubated for 24 hours at 4°C. In parallel, a control is prepared that does not contain the studied polysaccharide. The glucan with the bound catalase is separated from the sam-

ples by centrifugation at 3000 rpm. for 10 minutes. The amount of immobilized catalase is determined by the residual enzyme activity in the spurious fluid at substrate H_{2O2} according to the titrimetric method described in the thesis.

The best results for immobilized catalase were recorded for fungal catalase. Physical immobilization of catalase was achieved at a 3% concentration of the water-insoluble polysaccharide of 35%. When testing the activity of immobilized catalase, it was found that more than 95% of the activity was retained after storage at 4°C for 30 days and over 70% of the activity was retained for 60 days under the same storage conditions.

Additional studies conducted with immobilized mold catalase revealed that due to the reversible nature of immobilization, the peroxidase-like activity of fungal catalase was observed only in the presence of 1% methanol, while the same concentration of ethanol caused a significant loss of catalytic activity of the enzyme (Table 5).

Table 5. Kinetic constants of immobilized on URE 13,300 catalases from fungal, bovine liver and bacterial origin.

Catalase (immobilized on URE 13 300)	K_M **(mM)**	V_{max} **(µmol l^{-1} s^{-1})**
Fungal (*Penicillium chrysogenum)*	109.4	14.42
Bovine liver	16.4	454.00
Bacterial *(Micrococcus lysodeikticus)*	24.4	75.73

The obtained results show that the enzyme isolated from fungal origin is more stable in an environment with alcohol than enzymes derived from eukaryotic organisms. Thus, immobilized catalase shows remarkable denaturation stability in the presence of aggressive chemical agents such as ethanol and methanol.

The biochemical characteristics of the studied catalases showed that:

- Bacterial catalase does not tolerate the presence of both alcohols, while the enzyme of fungal origin shows an activity similar to peroxidase, most noticeable in the presence of up to 1% of added methanol. Under the same conditions, catalase from bovine liver retains some activity.
- Immobilized fungal catalase is more stable in the presence of alcohols than the dissolved enzyme, and retains about 76% in the presence of 5% methanol, while in the presence of the same concentration of ethanol, the residual heterogeneous activity of the enzyme is 63.5%.

4. Electrochemical study of the catalytic activity of immobilized catalase from different origin

Electrochemical methods are suitable for the study of oxidation-reduction processes, as the measured current strength is proportional to the speed of the process under study. Moreover, unlike spectrophotometric and titrimetric methods, electrochemical methods provide a sufficiently high sensitivity to the determination of analytes with concentrations in the micro- and sub-micromolar range.

The experiments were carried out in an electrochemical cell with a working volume of 20 ml in a three-electrode configuration with Ag|AgCl, KCl sat. as a com-

parative and Pt-foil as an anti-electrode. The concentration of H_2O_2 was tracked in the course of the experiments using a hydrogen peroxide-sensitive electrode, which will be hereinafter referred to as a catalytic peroxide electrode used as an indicator (operational) electrode. The measurements were made at a constant potential of -0.2 V (relative to Ag|AgCl), in which the electroreduction of hydrogen peroxide proceeds at a significant rate and without significant influence of the competitive reaction of electrochemical oxygen reduction and with continuous agitation at 2000 rpm.

The electrochemical determination of the activity of immobilized catalase is carried out in two stages: abiotic and biotic. In the first step, the signal on the working electrode was registered as a function of the concentration of hydrogen peroxide, which is gradually increased by adding aliquots from a starting 0.1 M solution of H_2O_2. The results of the measurements were used to construct a calibration graph of the peroxide catalytic electrode. The calibration stage is designated in the text as the abiotic stage of the experiment.

In the second stage of the study, called biotic, the experiment was carried out in an identical way, but in the presence of a glass substrate with immobilized catalase, placed at the bottom of the electrochemical cell of approx. 0.8 cm distance from the indicator electrode. The biotic step can be repeated as many times as necessary within the same day.

Catalase is known to be one of the most efficient biocatalysts with a number of revolutions (catalytic efficiency) of up to 40 million per second (Smejkal, G. B., & Kakumanu, S. 2019). A widely accepted method for analyzing its enzymatic activity in both dissolved and immobilized states is to track a decrease in the concentration of H_2O_2 in stationary solutions, where the peroxide decomposes catalytically under diffusion control, which can potentially lead to underestimated levels of enzymatic activity. Taking this into account, to determine the activity of immobilized catalase, electrochemical studies were carried out under hydrodynamic conditions (stirring of the solution with a rotating disc electrode, RDE).

Figure 2 presents the hydrodynamic linear voltamograms recorded in the presence of 0.46 mM hydrogen peroxide. It can be clearly seen that in the region of potentials from -0.3 V to + 0.2 V, the electrochemical reduction of H_2O_2 passes under diffusion control. Obviously, the reduction current increases with an increase in the rotational speed of the RDE from 500 to 3000 rpm. It was found that when the catalytic electrode rotated at 2000 rpm, the sensitivity of hydrogen peroxide determination increased by about 25% compared to that determined under static conditions. In addition, the increase in sensitivity does not lead to a significant increase in the noise level. Further increasing the rotation speed to 3000 rpm did not result in significant sensitivity improvements, but the noise levels were much higher compared to the results obtained at 2000 rpm.

During chronoamperometric experiments aimed at determining the catalytic activity of immobilized catalase performed in stationary solutions, no visible differences were noted between the current values recorded in the absence and presence of catalase up to a concentration of 4 mM H_2O_2. It should be mentioned that the differences in electrode signals obtained in the abiotic and biotic steps only become

apparent if the working electrode rotates at a significant speed, thus reducing the effect of the diffusion of H_2O_2 through the electrolyte. This fact undoubtedly indicates that the diffusion of the enzyme substrate is a speed-determining stage of decomposition on H_2O_2 catalyzed by immobilized catalase. All the results discussed further were performed at a 2000 rpm rotational speed of the working electrode.

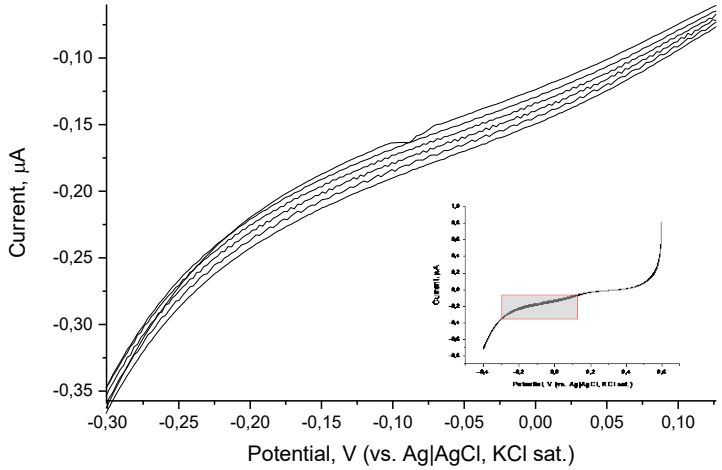

Figure 2. Hydrodynamic linear voltamograms recorded in the presence of 0.46 mM hydrogen peroxide on the catalytic peroxide electrode; rotational speed, rpm: 500; 832; 1248; 1748; 2332 and 3000; electrolyte: 0.1 M phosphate buffer, pH = 7.0; Temperature: 21±1 degrees.

The dependence of the reaction of the catalytic peroxide electrode on the concentration of peroxide is presented in Fig. 2 C. It can be seen that the signal at the same peroxide concentration is lower when immobilized catalase is present during the measurements (Fig. 3 B, blue and red series).

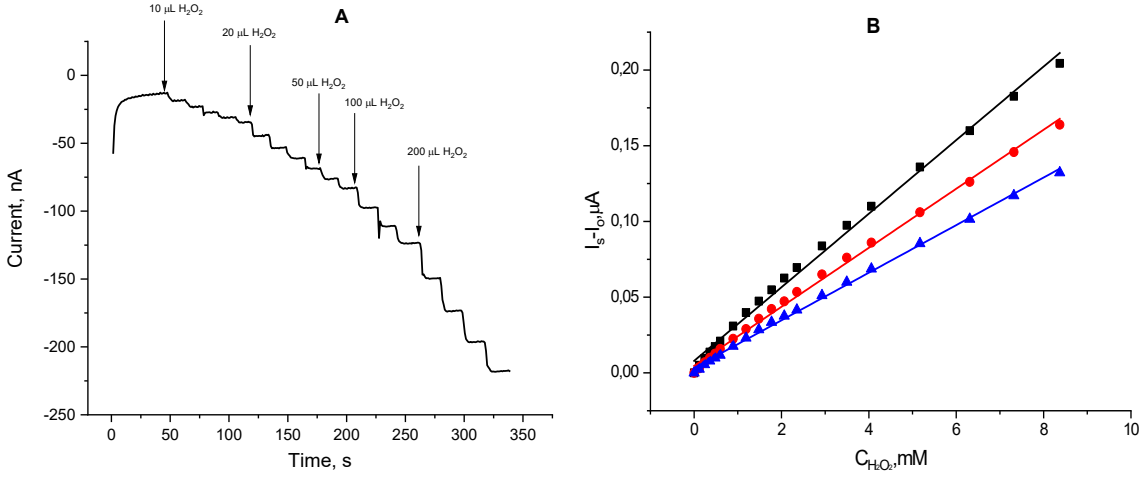

Figure 3. A) Authentic record of the current change over time when aliquots of hydrogen peroxide are added; operating potential: -0,20 V (relative to Ag|AgCl, saturated KCl), PB, pH = 7.0;
B) Dependence of the reaction of a catalytic peroxide electrode on the concentration of hydrogen peroxide in the electrochemical cell in the absence (black series) and in the presence of 0,25 mg catalase immobilized in a film by Nafion (red series) and crosslinked with glutaraldehyde (blue series).

The observed effect is the result of the reduced concentration of H_{2O2} in close proximity to the surface of the electrode due to the catalytic disproportion of the latter. The difference between the current recorded in the abiotic and biotic phases under equivalent other conditions is directly proportional to the rate of disproportionation of H_2O_2 catalyzed by immobilized catalase.

The immobilized enzyme cannot be reused due to a significant loss of enzyme activity during a repeated cycle of biotic experiments. Studies with scanning electron microscopy combined with energy dispersive X-ray spectroscopy (SEM-EDX) confirmed that after one experiment, the amount of enzyme retained in the polymer film was negligible. Figure 4 shows SEM micrographs of immobilized catalase with a producer *Penicillium chrysogenum* in a polymer layer of Nafion on a conductive substrate and the corresponding EDX spectrum.

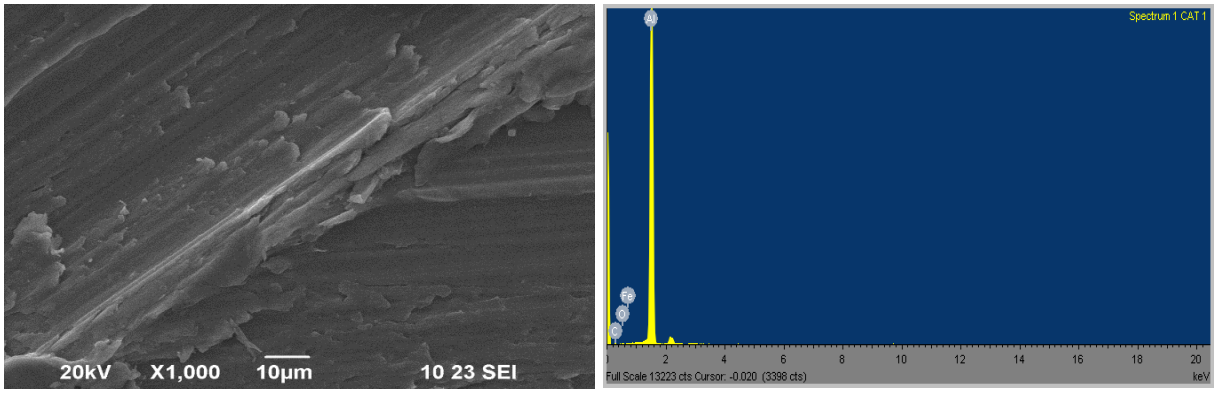

Figure 4. SEM image of immobilized catalase (left) before use in catalytic disproportionation of H_2O_2 and the corresponding EDX spectrum (right) showing the presence of iron from the active site of the enzyme.

By comparing Fig. 4 and Fig. 5 it becomes apparent that the polymer layer with the immobilized catalase is destroyed during the catalytic action and turns into a cracked and dendritic layer, as can be seen from Fig. 4. Analysis of the EDX spectra reveals that the presence of catalase, a Fe-containing enzyme, can be visualized in the spectrum by the Fe-peak representing 0.7 atomic % in the case of unused immobilized catalase (Fig. 4), and only traces of Fe without the possibility of its quantification can be observed in the case of an already used heterogeneous biocatalyst (Fig. 5). Most likely, the enzyme passes into the solution during the catalytic experiment. Given this, it can be assumed that the higher catalytic activity observed when using crosslink-immobilized catalase is a cumulative result of homogeneous (in solution) and heterogeneous decomposition of H_2O_2, whereas in the case of catalase immobilized in a polymer film, the observed catalytic activity is mostly due to the heterogeneous disproportionation of peroxide.

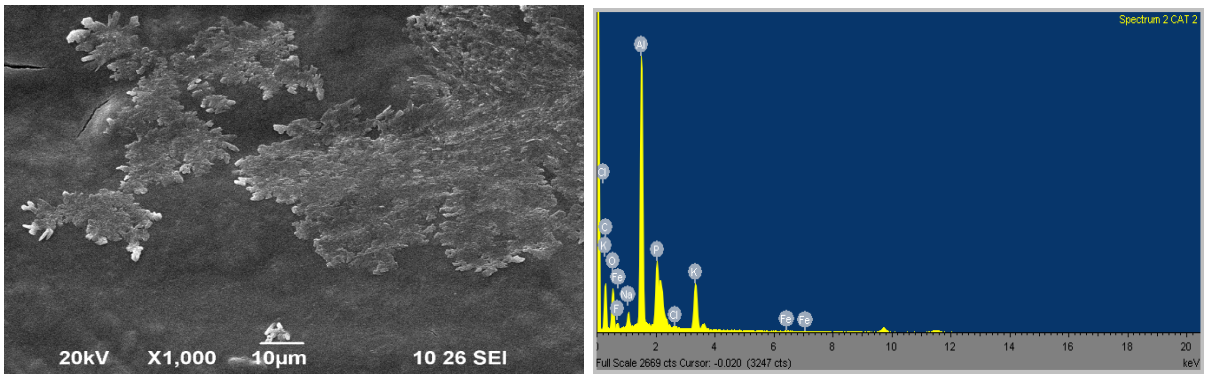

Figure 5. SEM image of immobilized catalase (left) after one cycle of catalytic disproportionation of H2O2 and the corresponding EDX spectrum (right) indicating the absence of an enzyme.

All these results raise the question of how reproducible the measurements are if each biotic experiment needs a freshly prepared heterogeneous biocatalyst. In Figure 6 depicts the experimental results obtained from three consecutive measurements with 3 equally prepared glass pads with immobilized catalase on them. It is obvious that the experimental points are practically superimposed on each other, and the deviation from the average does not exceed 2%.

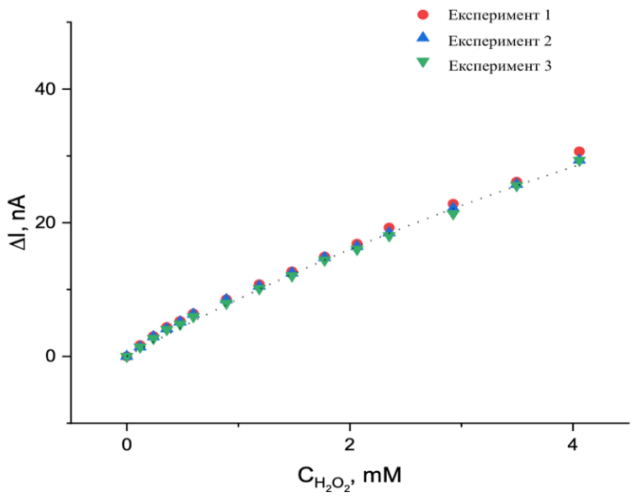

Figure 6. Difference between the electrode signal obtained in the abiotic stage and in the biotic phase as a function of the hydrogen peroxide concentration at which it was recorded for 3 independent measurements. RSD = 1,96 %.

5. Electrochemical study of the catalytic activity of immobilized catalase in the presence of aliphatic alcohols

In the late 1990s, Magner and Klibanov (1995) reported the unusual catalytic activity of dissolved catalase, which mimicked that of the enzyme peroxidase, finding that the enzyme was capable of oxidizing alcohols in the presence of hydrogen peroxide.

Using the same electrochemical approach, we were able to track the catalytic behavior of immobilized catalase in the presence of up to 1% aliphatic alcohols with low molecular weight – ethanol and methanol, due to their denaturing effect on the enzyme at higher concentrations.

Figure 7 shows the dependence of the rate of decomposition of hydrogen peroxide (in nanoamperes) as a function of the concentration at which it was measured in the presence of 0.5% and 1.0% ethanol. Obviously, the typical Michaelis hyperbole becomes a sigmoid curve when small amounts of alcohols are added to the electrolyte. The sigmoid form of dependence is characteristic of allosteric enzymes capable of binding two different substrates in different active centers, whose kinetics obey the Hill equation:

$$V = \frac{V_{max}.[S]^n}{[K]_{0.5}^n + [S]^n} \qquad \textbf{(3)}\ ,$$

Where:

– **V** is the rate of the enzyme-catalyzed reaction;
– **Vmax** is the maximum rate achievable when saturating the enzyme with the substrate,
– $\mathbf{K_{0.5}}$ is the Hill constant, which represents the concentration of the substrate at which 1/2 of the maximum velocity is reached,
– **n** is the Hill coefficient, indicative of the cooperativeness of substrate bonding.

For n>1, cooperativeness is positive, i.e., each binding substrate molecule facilitates the binding of the next substrate molecule; for n=1 there is no cooperative binding of the substrate and the Hill equation becomes the Michaelis-Menten equation, while for n<1 there is negative cooperativeness, where each molecule of the substrate of the bond prevents the next one from binding.

The apparent kinetic constants calculated from the nonlinear regression of the experimental data depicted in Fig. 6 are presented in Table 6. It is obvious that the apparent kinetic constants are highly dependent on the type of enzymatic immobilization – the determined apparent V_{max} in the absence of alcohol, it is approximately twice as high for cross-linked catalase compared to that embedded in the polymer membrane. However, it can be seen how detrimental to enzymatic catalytic activity is the addition of alcohol to the working environment – cross-linked catalase exhibits more than 40 times lower apparent V_{max} in the presence of 1% ethanol, which is a drastic loss of activity, especially compared to catalase immobilized by trapping in a polymer membrane under equivalent experimental conditions. This finding supports the earlier hypothesis that the cross-linked enzyme hardly lingers on the glass substrate and most likely leaks into the solution, thus showing mixed activity – heterogeneous and homogeneous.

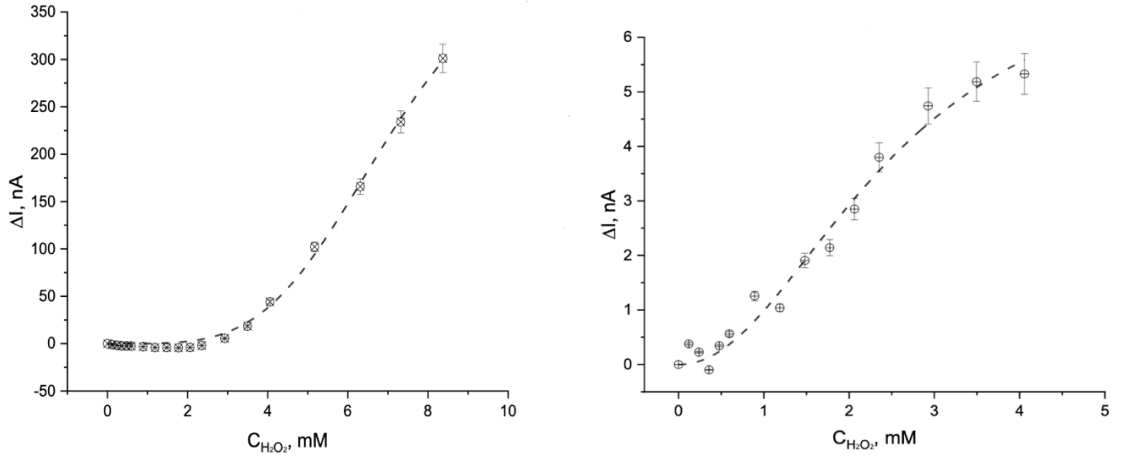

Figure 7. Difference between the electrode signal obtained in the abiotic stage and in the biotic phase in the presence of immobilized catalase from *Penicillium chrysogenum,* as a function of the concentration of hydrogen peroxide at which it is recorded in the presence of 0.5% (left) and 1.0% (right) ethanol.

Interestingly, the presence of 0.5% ethanol in the solution causes an approximately 5-fold increase in the apparent maximum rate, while the apparent Hill constant remains sufficiently close to the Michaelis constant determined in the absence of alcohol. The calculated Hill coefficient drops to 2, the apparent V_{max} decreases dramatically, and the apparent $K_{0.5}$ decreases significantly, which could potentially indicate protein damage.

Table 6. Comparison of the apparent kinetic constants of immobilized catalase from *Penicillium chrysogenum* immobilized in a Nafion membrane or crosslinked with glutaraldehyde determined electrochemically in the decomposition of hydrogen peroxide in the absence and in the presence of 0.5% and 1% ethanol.

Catalase *Penicillium chrysogenum* immobilized in a film by Nafion®			
Kinetic constants	***Water buffer, pH = 7.00***	***0.5 % ethanol***	***1% ethanol***
K^{app}, mmol L^{-1}	10.1 ± 0.9	7.2 ± 3.5	2.5 ±0.5
V_{max}^{app} nA μmol.s^{-1}	87.9 ± 5.4 3.62x10-3 ± 2.2 x10-4	456.6 ± 41.4 18.8x10-3 ± 1.7x10-3	7.7 ±1.5 3.17x10-5 ± 0.7x10-5
Hill coefficient, n	1.0	4.11 ± 0.27	2.06±0.36
Catalase *Penicillium chrysogenum* cross-linked with glutaraldehyde			
K^{app}, mmol L^{-1}	13.1 ± 0.99	n.a.	1.85 ±0.7
V_{max}^{app} nA μmol.s^{-1}	181.2 ± 9.7 7.46x10-3 ± 4.0 x10-4	n.a.	4.10 ±0.7 1.70x10-4 ± 2.9 x10-5
Hill coefficient, n	1.0	n.a.	3.36±1.25

By applying regression analysis of the obtained experimental data from electrochemical measurements, it was found that the enzymatic activity of immobilized catalase obeys the equation:

$$A_{sp} = \frac{(\Delta I_{abiot} - \Delta I_{bio}) \cdot 10^6}{s \cdot t} \frac{V}{m}, \quad \textbf{(4)}$$

Where:

– **Asp** is the specific enzyme activity, U/mg;
– ***ΔIabiot*** **and** ***ΔIbiot*****,** in [A], are the differences between the current recorded at a given concentration with the background signal removed for the abiotic and biotic experiments, respectively;
– **s** is the sensitivity of the catalytic peroxide electrode (slope of the calibration graph, in [A L/mol]);
– **t** is the time, [min]; V is the volume of the bioreactor, mL;
– **m** is the amount of immobilized enzyme [mg].

6. Comparison of the activity of immobilized catalase determined by electrochemical measurements and by spectrophotometry

The specific enzymatic activity of immobilized fungal catalase, determined by electrochemical and spectrophotometric methods, presented in Table 8, is compared. The electrochemically determined activity (under hydrodynamic conditions) is approximately 200 times higher than that determined under static conditions, probably as a result of increased mass transport during measurements. The presence of alcohols complicates the measurement process, as ethanol and methanol are surfactants that affect both the sensitivity and precision of measurements. It can be seen that the higher the percentage of alcohol in the work environment, the greater the variance of the data. Data analysis shows that the presence of 0.5% methanol increases the specific enzymatic activity by approximately 20%, which can be read as a manifestation of catalase peroxidase function. Such a trend is not observed for the same percentage of ethanol, which can be attributed to its more denaturing effect on protein.

Table 7. Specific enzymatic activity of immobilized catalase from *Penicillium chrysogenum*, determined electrochemically and spectrophotometrically, (n≥3).

	Electrochemical determination	**Spectrophotometric determination**	**Electrochemical determination**	**Spectrophotometric determination**
	Methanol		**Ethanol**	
Alcohol, %	**Asp., μmol. $min^{-1}mg^{-1}$**	**Asp., μmol. $min^{-1}mg^{-1}$**	**Asp., μmol. $min^{-1}mg^{-1}$**	**Asp., μmol. $min^{-1}mg^{-1}$**
0	1672.6 ±32.7	8.57	1672.6 ±32.7	8.57
0.5	2033.8 ± 113.8	n.a.	1235.5 ± 143.0	n.a.
1.0	1076.3 ± 269.1	8.40	537.1 ± 127.5	7.41

This also raises the question of whether the activity of immobilized catalase can be determined if the enzyme is immobilized directly on the surface of an electrode. Given that catalase, unlike other metalloproteins, is considered electrochemically inactive, a covalent immobilization methodology was chosen on a gold electrode, which is known to be electrocatalytically active in the electrochemical reduction of oxygen released during the decomposition process of hydrogen peroxide.

The differential pulse voltamograms of a gold electrode with catalase immobilized on its surface by electrografting, recorded in the absence and presence of H_2O_2, are shown in Figure 8.

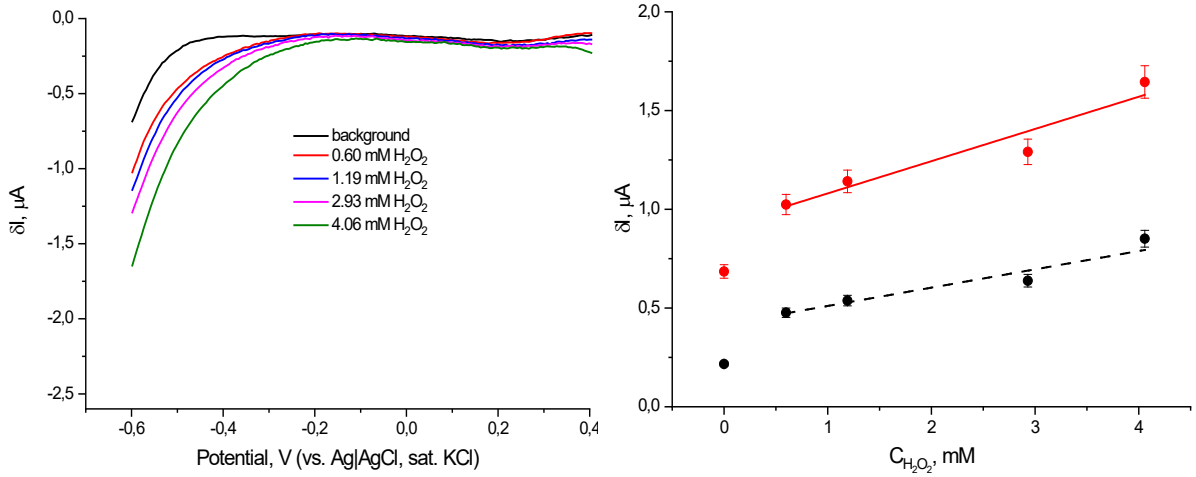

Figure 8. ***(Left)*** Differential pulse voltamogram of a gold electrode with covalently immobilized catalase (by electrografting) in the presence of hydrogen peroxide at different concentrations; ***(Right)*** dependence of the electrode response on the concentration of H2O2 at a potential of -0.5 V (black series) and -0.6 V (red series).

The catalytic effect of gold in the electrochemical reduction of oxygen is clearly expressed at potentials less than -0.2 V, and the increase in the reduction current is evident with an increase in the concentration of H_2O_2 in the working medium, which obviously indicates that the immobilized catalase is catalytically active.

The operational stability of a catalase bioelectrode is determined, presented in Fig. 9. It is observed that the resulting biosensor retains its activity up to 76% after 15 days of use and storage.

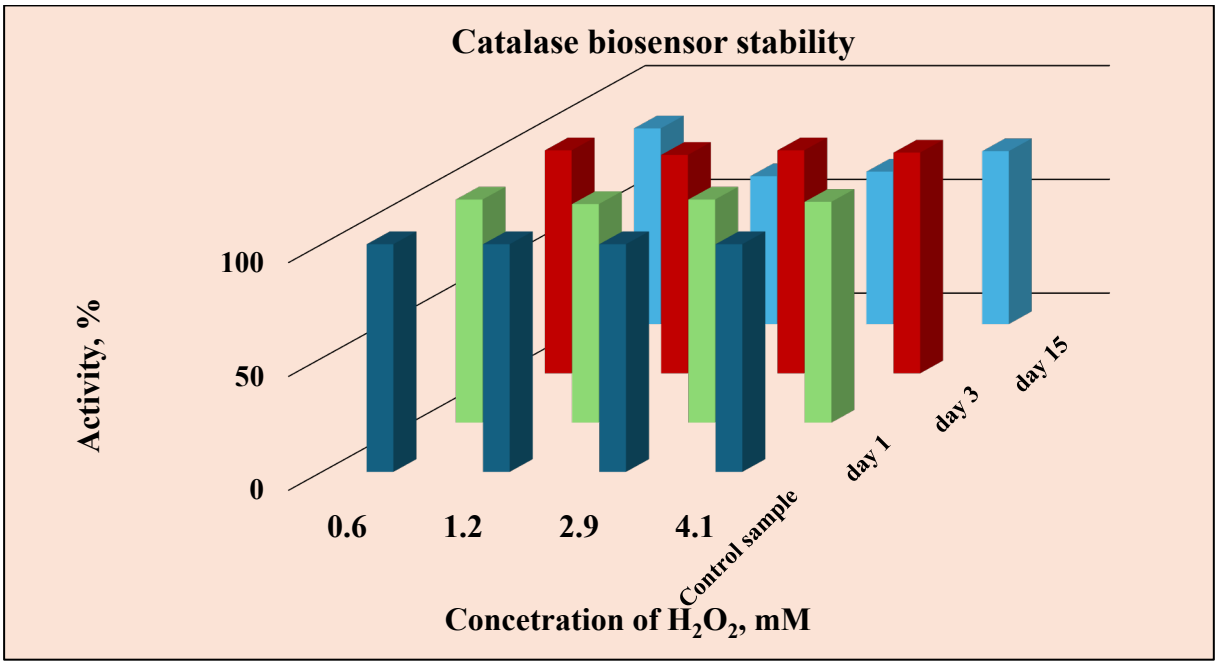

Figure 9. Residual enzymatic activity of catalase immobilized by electrografting on a gold electrode as a function of the hydrogen peroxide concentration measured on the day of immobilization, on the 3rd and 15th days.

7. Laccase

In the current study, two enzymatic preparations of laccase were used, from *Aspergillus oryzae* and *Trametes versicolor*. Preliminary data on the enzymatic properties of the laccases used are necessary as a starting point for immobilizing the enzyme and incorporating it into a biosensor.

The *A. oryzae* enzyme is a technical preparation with enzymatic activity according to the specification of the manufacturer Shannxi Honghao bio-tech Co., Ltd China not less than 11,000 U/g. For the purposes of our research, we need a purified form of the enzyme with high specific enzymatic activity. In this regard, we have applied various methods for its purification and production.

7.1 Purification of technical laccase

7.1.1. Fractional precipitation with ammonium sulfate

Fractional precipitation of laccase from *Aspergillus oryzae* with saturation of the protein solution with ammonium sulphate, corresponding to a percentage ratio of 50, 60, 70, 80 and 100 %, respectively. The enzymatic activity of the purified enzyme from each fraction in the presence of ABTS as an enzyme substrate was determined. The activity was studied in an aqueous medium (0.02 M Na-acetate buffer, pH = 4.6) at room temperature using spectrophotometric observation and the results obtained are presented in Table 8. The best results are shown by the purification of laccase from *Aspergillus oryzae* at saturation with ammonium sulfate 60%, under the same conditions. The enzyme was purified to a specific enzymatic activity of 315 U/mg protein, achieving a total of 10.3-fold purification, which is almost 2.5 times more than the results obtained by Kumar, R., et al. (2016). Kumar, R., and collegium (2016), propose an optimized method for the production of laccase from *Aspergillus flavus,* purified using ammonium sulphate at saturation of 50% and 60%. Under these conditions, the authors report that the enzymatic activity of 30 ml of precipitate buffer solution is 25.66 IU/ml with a protein concentration of 0.048 mg/ml. The enzyme was purified to a protein concentration of 0.036 mg/ml with an enzymatic activity of 30.57 IU/ml, achieving a total of 4.24-fold purification of the crude enzyme extract.

Table 8. Enzymatic activity of laccase *Aspergillus oryzae* after purification with ammonium sulfate in the presence of ABTS as an enzyme substrate.

Ammonium sulfate, %	V (ml)	A total, U	Yield, %	Asp., U/mg Protein	Yield relative to Asp., %	Protein, mg/ml	Residual activity, %	Purification level, U/mg
Control	33	2766	100	30.6	100	2.74	-	-
Starting solution	10	2847	100	1409.6	100	0.202	-	-
50% saturation	16.5	660	24.0	84	6.0	0.44	23.0	2.75
60% saturation	37	933	34.0	315	22.0	0.08	33.0	10.3
70% saturation	7	129	5.0	194	14.0	0.09	4.5	6.3
80% saturation	6	76	3.0	125	9.0	0.1	2.7	4.1
100% saturation	28	484	18.0	35	2.5	0.5	17.0	1.1

7.1.2. Fractional precipitation with polyethylene glycol

Fractional precipitation with polyethylene glycol was carried out in order to improve the yield of purified enzyme with high specific activity. Table 9 presents the experimental data from the analysis. We achieved a yield of 60% by applying PEG 6000 for enzyme precipitation. Although the yield was satisfactory, phase separation of the enzyme was observed at different degrees of PEG saturation. A greater amount of the enzyme is observed in the PEG 6000 phase, while in PEG 1500 and 4000 the enzyme is in the buffer phase. As a comparison, laccase from *Cerena unicolor* was purified with polyethylene glycol of different molecular weights, and the recovery yield achieved in the study was reported to be 97.4% after purification with PEG 6000 and phosphate buffer (Antecka, *A., et., al* 2019).

Table 9. Enzymatic activity of laccase from *Aspergillus oryzae* after purification with polyethylene glycol.

PEG, %	***Solution***	***V, (ml)***	***A total, U***	***Yield, %***	***A_{sp}, U/mg Protein***	***Yield relative to A_{sp}, %***	***Purification level***
Control	Initial laccase sol.	60	12 153 424	100	2026	100	-
30 % PEG 1500	Supernatant	108	899 424	7.4	833	41.12	0.4
	Precipitate	10	17 640	0.14	51	2.52	0.03
30 % PEG 4000	Supernatant	105	1 665 720	13.7	793	39.41	0.4
	Precipitate	15	59 328	4.9	161	7.95	0.1
30 % PEG 6000	Supernatant	60	166 935	7.35	159	7.85	0.1
	Precipitate	18	1 360 710	6.00	189	9.33	0.1
50 % PEG 1500	Supernatant	125	4 275 000	35	488	24.08	0.24
	Precipitate	12.5	601 800	5	112	5.53	0.1
50 % PEG 4000	Supernatant	122	792 146	6.5	130	6.42	0.1
	Precipitate	12	2 299 393	19	456	22.51	0.23
50 % PEG 6000	Supernatant	65	136 500	1.12	151	7.45	0.1
	Precipitate	25	4 824 000	40	1206	59.53	0.6

7.1.3. Determination of molecular weight with SDS-PAGE and in *situ* analysis

The level of purification of laccase from *Aspergillus oryzae* is confirmed by in situ electrophoretic analysis. The molecular weight of purified laccase with both detergents is about 50 kDa.

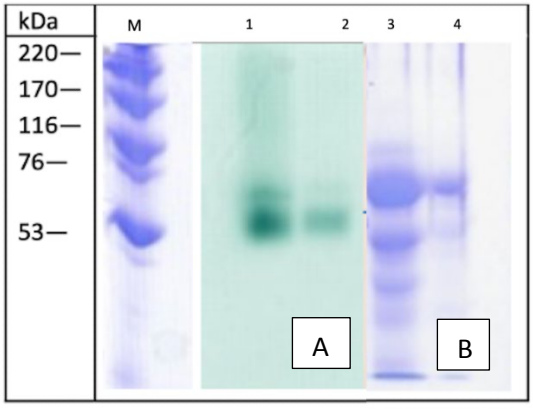

Figure 10. Determination of the molecular weight of laccase from *Aspergillus oryzae* where: **A**) *in situ* NATIVE-PAGE of purified lacse from *Aspergillus oryzae* with polyethylene glycol manifested with ABTS; **(B**) SDS-PAGE of a commercial laccase preparation developed with Comassie Brilliant Blue R 250.
Legend: – **1** – fraction of *Aspergillus oryzae* after purification with 50% polyethylene glycol 6000 and yield 60%; **2** – fraction of *Aspergillus oryzae* after purification with 30% polyethylene glycol 1500 and yield 40%; **3** – Control sample of a commercial preparation of Trametes versicolor (Fluka); **4** – Control sample of a commercial preparation of *Trametes versicolor* (Sigma); **M** Amersham High Molecular Weight Marker Standard.

8. Influence of organic solvents on laccase activity

The enzymatic activity of laccase by *Aspergillus oryzae* and *Trametes versicolor* is tested in the presence of an organic solution such as methanol, ethanol, acetone and DMSO to select an enzyme with high activity and stability for application in biosensor construction. It was found that the laccase from *Aspergillus oryzae* is too labile in the presence of the organic solvents used, and even in the presence of 1% ethanol, methanol and acetone and DMSO, it is completely inactivated. In the analyses carried out with the same organic solvents for the laccase of *Trametes versicolor* found that she retains its catalytic function. An experiment was also carried out in presence of 10% and 20% of polar organic solvents, and the duration of incubation of the enzyme in the respective medium was also varied.

Figure 11 compares the influence of each solvent on enzymatic activity, and the activity of laccase determined in the absence of an organic solvent is accepted as a reference.

The data showed preservation of enzymatic activity when adding 10% or 20% of any organic solvent in the first 5 minutes of the reaction. This can be assumed that the addition of an organic solvent leads to a partial further loosening of the structure of the enzyme, which increases the access of the substrate to the active site. The enzyme activity is reduced to about 60% after one hour of exposure in a solution with 10% ethanol and methanol, and in DMSO and acetone it is reduced below 50%.

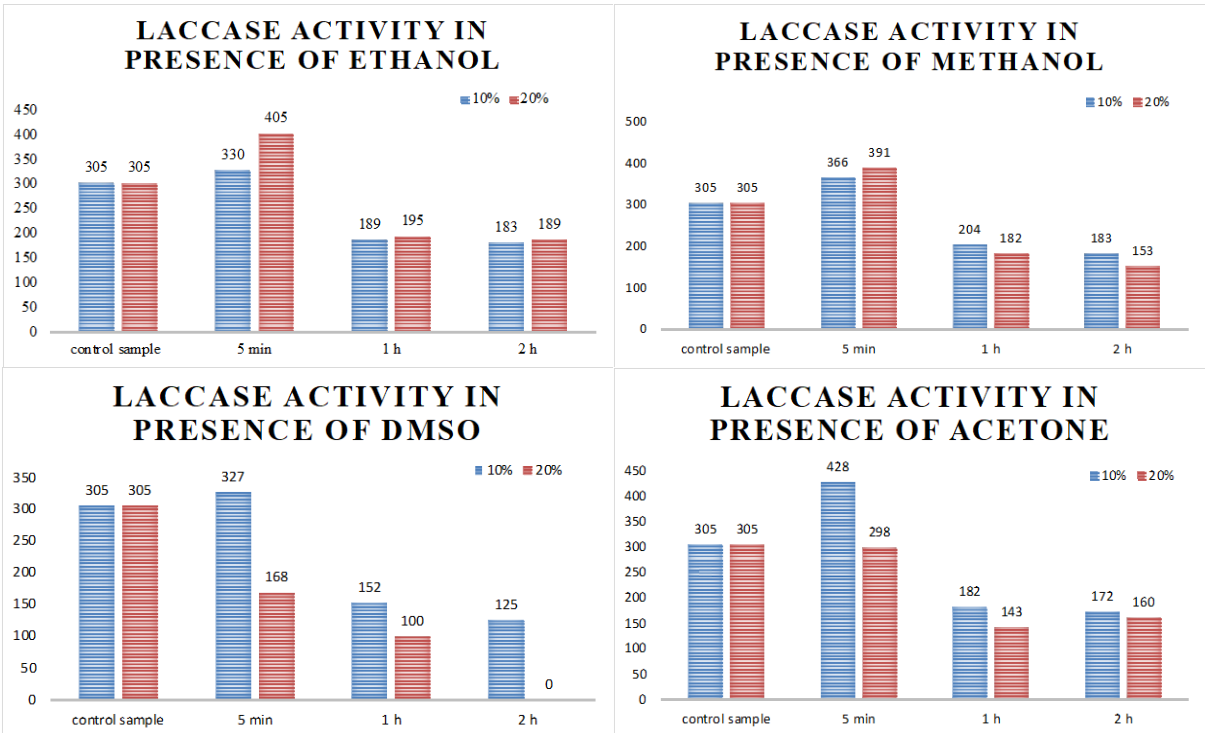

Figure 11. Influence of organic solvents on the activity of laccase from *Trametes versicolor* at the beginning of the reaction, first and second hours of enzyme incubation.

The results presented in Table 10 show that the enzymatic activity of laccase remains stable even after two hours of incubation in the presence of methanol and ethanol, compared to DMSO and acetone.

Table 10. Influence of exposure time in organic solvents on laccase activity by *Trametes versicolor.*

Solvent %	Incubation time	Enzyme activity with Ehanol, %	Enzyme activity with Methanol, %	Enzyme activity with DMSO, %	Enzyme activity with Acetone, %
10%	5 min.	108	120	107	140
	1 hour	62	67	50	60
	2 hours	60	60	41	56
20%	5 min.	123	128	55	98
	1 hour	64	60	33	47
	2 hours	62	50	0	52

Similar results were found in 20% of the organic solvents studied, confirming that DMSO was the most unacceptable solvent for lackase. The addition of acetone and DMSO to the medium at a concentration of 20% causes a sharp decrease in enzymatic activity. The most obvious example is the incubation of laccase in a buffer containing DMSO at a concentration of either 10% or 20%, where the solvent has a degrading effect on it: the enzyme loses between 50% and 100% of its activity when incubated for 1 or 2 hours.

There are a limited number of studies (Milstein *et al.,* 1989), which demonstrate that immobilized laccase can primarily tolerate the presence of water-saturated hydrophobic organic solvents. For practical purposes, however, it is often necessary to carry out studies in mixed water-non-aquatic environments (e.g. removal of polyphenols from beer, wine, etc.).

9. Monitoring of the enzymatic activity of immobilized laccase using electrochemical methods

9.1. Immobilization of laccase from *Trametes versicolor* in a polymer layer of Nafion and determination of catalytic enzymatic activity in the presence of oxygen and phenolic compounds

Subsequent studies were carried out with *Trametes versicolor* because it showed more stable activity in the presence of organic solvents needed in the process of immobilization.

For this purpose, the enzyme laccase from *Trametes versicolor* was immobilized on the surface of a glassy carbon electrode under a semi-permeable membrane of the Nafion polymer and its behavior was compared to an identically prepared laccase by *Trametes pubescens* boelectrode. The electrochemical behavior of the two laccase-based bioelectrodes was investigated by cyclic voltammetry (CV) in the absence and presence of the two laccase substrates – oxygen and phenolic compounds. Voltammetry studies show that for both types of laccase biosensors, a reduction wave is observed in aerated buffer solutions (i.e., in the presence of oxygen) starting at potentials less than -0.25 V, which is not observed in deaerated solutions (Fig. 12, black lines). These observations indicate that immobilized laccase catalyzes the electrocatalytic reduction of O_2 to water, thus proving that the enzymes are electrochemically active.

Comparison of the CV of the enzyme electrode recorded in aerated solutions in the absence and presence of pyrogallol and catechol as substrates (Fig. 13) reveals lacase-catalyzed oxidation of the two phenols to semiquinones, followed by electrochemical regeneration of the oxidized products.

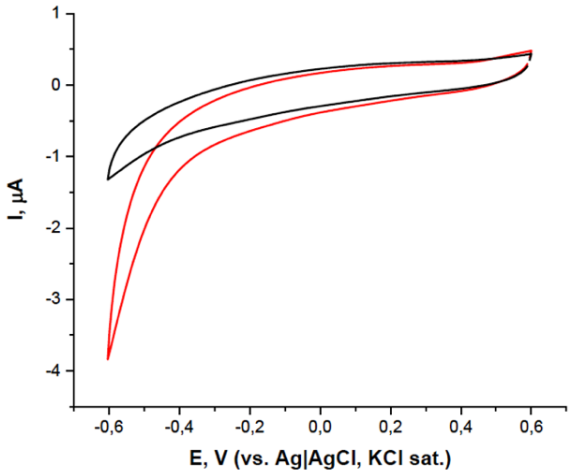

Figure 12. Cyclic voltamograms of a laccase electrode recorded in the presence (red) and absence (black) of dissolved O_2, in citrate buffer, pH = 4.

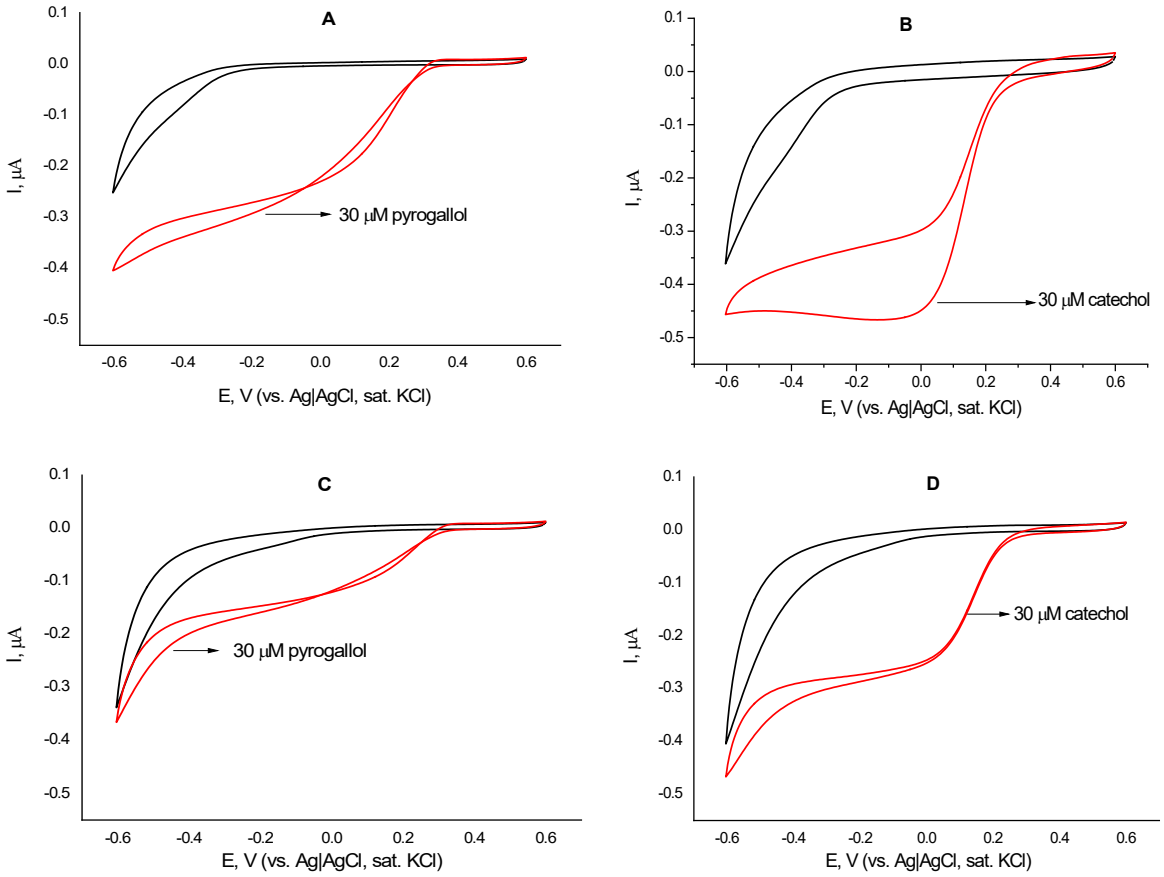

Figure 13. Cyclic voltamograms of enzyme electrodes modified with laccases of: *Trametes pubescens* (A, B) and *Trametes versicolor* (C, D) in background electrolyte (black solid lines) and in the presence (red solid lines) of 30 µM substrates of laccase: pyrogallol (A, C) and catechol (B, D).

9.2. Catalytic enzymatic activity of immobilized laccase in the presence of phenolic acids

Caffeic acid and gallic acid can be considered as derivatives of catechol and pyrogallol phenolic compounds. Their structural similarity to the above di-hydroxyl and tri-hydroxyl aromatic compounds, as well as the fact that they are the usual constituents of the polyphenolic complex in various natural products, deter-

mined our interest in studying the voltametric behavior of the obtained biosensors in the presence of both phenolic acids. It was recorded that in the absence of phenolic compounds (Fig. 11, black lines), a reduction wave occurs at potentials less negative than -0.3 V, which is the result of the electrochemical reduction of dissolved molecular oxygen catalyzed by the immobilized enzyme, which undoubtedly proves that both laccases are not only electrochemically but also catalytically active. Cyclic voltamograms recorded in the presence of either gallic or caffeic acid (Fig. 12, red lines) show a pronounced reduction wave starting much earlier – below +0.1 V, which is due to the fact that the two phenolic acids mediate the electrochemical reduction of dissolved oxygen and therefore significantly reduce the oxygen reduction superpotential on electrodes with immobilized laccase. As shown in the graphs presented, the interactions of the two laccases with the two phenolic acids resemble the forms of the voltamograms recorded in the presence of catechol and pyrogallol. It is evident that the efficiency of the enzymatic interaction with the two phenolic acids is different and is much higher in the presence of caffeic acid, as can be inferred from the pronounced reduction waves (Fig. 13 C, D). The interaction between the two laccases (*Trametes versicolor and Trametes pubescens*) with gallic acid is of lower intensity, probably as a result of electrostatic repulsion of its anionic form generated at operating pH = 4.0 and the electrode surface, which also carries negative charges due to the fine membrane coating of Nafion.

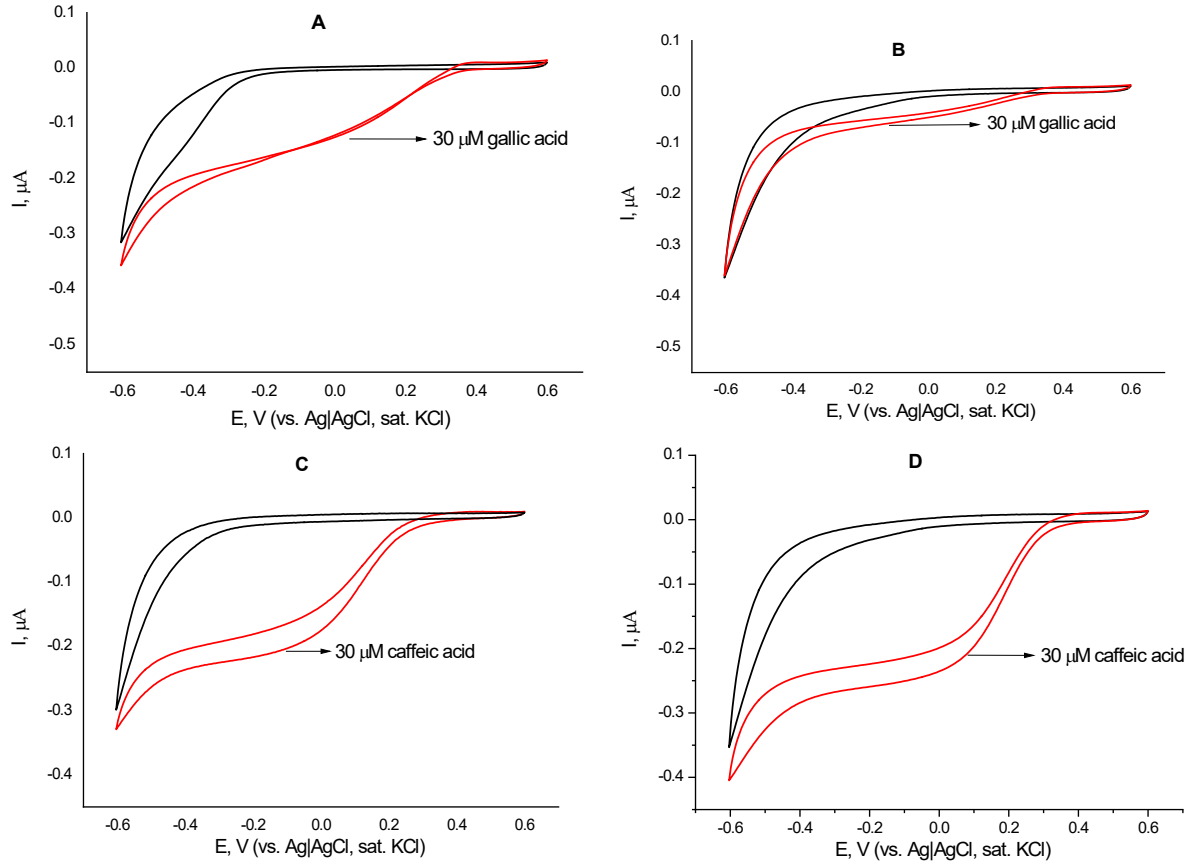

Figure 13. Cyclic voltamograms of enzyme electrodes modified with lacazes by: *Trametes pubescens* (A, C) and *Trametes versicolor* (B, D) in a background electrolyte (black solid lines) and in the presence of 30 μM (red solid lines) gallic acid (A, B) and caffeic acid (C, D).

9.3. Study of laccase-based biosensors from *Trametes pubescens* and *Trametes versicolor* using the differential pulse voltametry (DPV) method

Differential pulse voltamograms (DPVs) of the same laccase electrodes are depicted in Figure 14. No peaks are observed on the background curves (Fig. 14, A, B, dotted curves), and a sharp increase in the reduction current at potentials less than -0.4 V indicates that oxygen reduction occurs on the surface of the bioelectrode.

Subsequent recording of the DPV curves in the presence of increasing concentrations of gallic acid (Fig. 14, A, solid lines) shows a noticeable increase in current with a peak with increasing height at -0.2 V. Similarly, in the presence of caffeic acid (Fig. 14 B, solid lines), a pronounced peak was found at a potential more positive than that observed for gallic acid. Similarly, the height of the peak increases in proportion to the concentration of the substrate. The difference in the behavior of the laccase biosensor in the presence of caffeic acid as an enzyme substrate is that the reduction peak occurs at a potential of + 0.2 V, and the peak potential slightly shifts in a positive direction with increasing substrate concentration. It is likely that the penetration of gallic acid is inhibited by the deprotonation of its carboxyl group at the working pH due to the electrostatic repulsion between the negatively charged membrane of Nafion and the anionic form of gallic acid, resulting in a significant shift in the reduction potential to more negative values than those observed for caffeic acid. The latter does not deprotonate at the working pH of the medium, and therefore its molecules penetrate the membrane more easily. A similar behavior of the second laccase, from *Trametes pubescens*, is observed under equivalent experimental conditions.

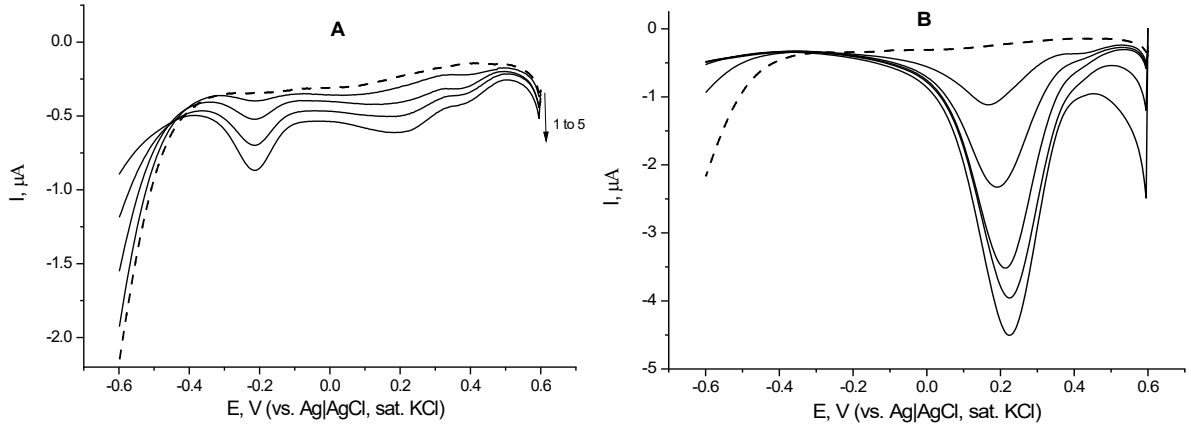

Figure 14. Differential pulse voltamograms of enzyme electrodes modified with laccase from: *Trametes versicolor* in a background electrolyte (black dotted lines) and in the presence of different concentrations of (solid lines) gallic acid (A) and caffeic acid (B).

9.4. Analysis of gallic and caffeic acids with biosensors based on laccase from *Trametes versicolor* and *Trametes pubescens* by constant potential amperometry

The information obtained from the DPV studies clearly shows that this electrochemical technique can hardly be used for the analysis of mixtures of the target di- and tri-phenolic compounds.

Figure 15 shows the dependencies of the electrode signal on the concentration of gallic and caffeic acids, determined by the two types of laccase-based electrodes under operating conditions selected as optimal: operating potential -0.2 V and pH = 4.0. The similarity in the shape of the curves is obvious – for both types of electrodes, the dependence of the electrode signal on the concentration of the substrate has a hyperbolic stroke. However, the apparent kinetic constants for the two immobilized lacases, determined by nonlinear regression analysis of experimental data, show noticeable differences. Michaelis apparent constants for immobilized laccase *Trametes versicolor* (Fig. 15, A) in terms of caffeic and gallic acid are very similar (Table 11), while the corresponding apparent maximum rates of the enzyme-catalyzed reaction differ significantly with for caffeic acid, which is almost 5 times higher than that for gallic acid. In contrast, for the immobilized lacaza of $V_{max}^{app} K_M^{app}$ *Trametes pubescens* (Figure 15, B) for caffeic acid and gallic acid, they were found to differ by more than 3 times (0.19±0.03 mM and 0.06±0.04 mM, respectively, Table 11), while the apparent maximum reaction rates differed even more than those calculated for *Trametes versicolor*. These differences in the apparent kinetic constants of the two identically immobilized enzymes suggest that, despite the biochemical similarity of the two laccases, there are noticeable differences in enzymatic affinity for di-hydroxy and tri-hydroxy aromatic compounds.

Table 11. Apparent kinetic constants of the two types of laccase biosensors when interacting with gallic and caffeic acid and regression coefficient (R^2). The kinetic constants were determined by nonlinear regression analysis of the experimental data.

	Trametes pubescens		***Trametes versicolor***	
Kinetic constants	**Caffeic acid**	**Gallic acid**	**Caffeic acid**	**Gallic acid**
V_{max}^{app}, **μA**	21.90± 2.70	3.11 ± 0.12	3.77 ± 0.17	0.79 ± 0.02
K_M^{app}, **mM**	0.191 ± 0.030	0.061 ± 0.004	0.075 ± 0.056	0.077 ± 0.006
K_I^{app}, **mM**	0.319 ± 0.083	0.621 ± 0.088	0.464 ± 0.055	–
R2	0.999	0.999	0.999	0.993

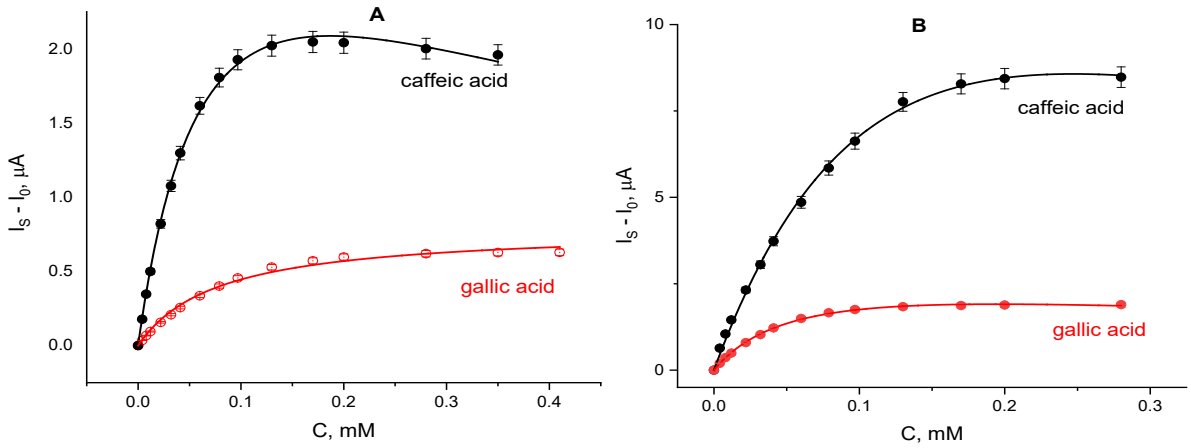

Figure 15. Dependence of the biosensor signal on the concentration of the substrate for laccase-based electrodes from: **A**) *Trametes versicolor* and **B**) *Trametes pubescens* in the presence of gallic acid (red series) and caffeic acid (black series). The graphs were constructed based on chronoamperometric records at -0.2 V relative to Ag|AgCl (saturated KCl), in citrate buffer containing 0.1 M $NaClO_4$, pH = 4.0; room temperature.

10. Operational stability

The operational stability of the laccase electrodes was tested for a period of 5 days and the resulting residual activity as a function of the number of measurements made with the same electrode is depicted in Fig. 16. It can be seen that in the first four measurements, the electrode gradually loses about 7% of its activity, but retains about 30% of its initial activity after 10 measurements. This is explained by a loss of catalytic activity of the enzyme due to both enzyme leakage into the buffer and inactivation of the enzyme due to its prolonged exposure to room temperature.

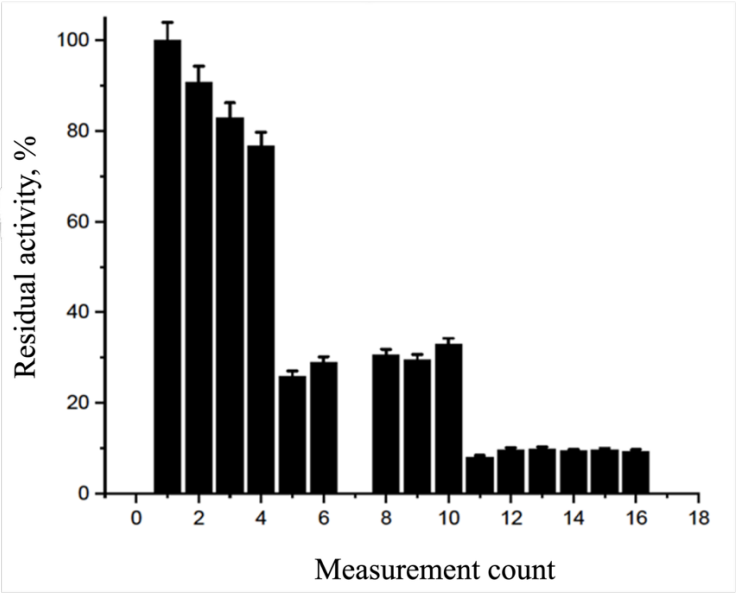

Figure 16. Residual electrode activity as a function of the number of measurements.

10.1 Application of laccase biosensors

The developed laccase biosensors were applied in the analysis of the total phenolic content of three different herbal extracts. It was found that the laccase-based biosensor from *T. pubescens* reacted approximately three times more intensely than the analogue based on laccase from *T. versicolor*. Quantitative analysis of herbal extracts was performed by means of constant potential amperometry, applying the Method of Standard Addition (MSA) as the main analytical approach. The standard addition method is usually performed by adding small volumes of concentrated analyte solution to the sample. Its main advantage is the ability to virtually eliminate the effects of the complex matrix in real samples. 10 mM gallic acid solutions were used as the external standard.

The results of the analysis with the laccase biosensor were compared with those from the HPLC analyses of the three types of extracts (Table 12). As established by the HPLC analysis, the studied herbal extracts included gallic, caffeic, chlorogenic, p-coumaric and trans-ferulic acids. The amounts of phenolic compounds determined by MSA were further multiplied by dilution factor and the resulting values were recalculated in gallic acid equivalents per gram of dry weight of the herbs. The calculated recovery (in %) is within the acceptable limits only for herbal extract 1, while for the other two extracts the biosensor method shows significant deviations from acceptable values (from 95 to 105%). This may be due to the presence of significant amounts of *m*-benzenediols *(e.g. resorcinol and its derivatives)* and/or other types of polyphenols that do not react with laccase.

Table 12. Comparison of phenolic content expressed in milligrams gallic acid equivalents per gram of dry herbs (GAE. DW^{-1}), analyzed with a laccase biosensor from *Trametes pubescens* and analysis with high-performance liquid chromatography of the phenolic acid content.

Herbal extract	Determination with a biosensor *(GAE. DW^{-1})*	Determination of the total amount of polyphenols* *(GAE. DW^{-1})*	Analytical yield, %
1	214.2	216.0	99.0
2	131.0	189.0	69.3
3	48.3	56.5	85.4

* *It is determined by HPLC as the sum of all phenolic acids.*

However, the biosensor method provides two important advantages over chromatographic analysis – it can be carried out quickly with minimal sample pretreatment and with equipment that is very susceptible to miniaturization, allowing on-site analysis.

CONCLUSIONS:

Based on the results obtained, the following conclusions can be drawn:

1. When studying the enzymatic activity of catalases of various origins both in the absence and in the presence of low molecular weight aliphatic alcohols, it was found that the catalase from *Penicilium chrysogenum* is the most resistant to the effects of the organic solvent. It retains 83% and 98% of its activity respectively in the presence of 1% ethanol and 1% methanol, respectively, and when their content is increased to 5%, 63% and 57% of the activity of the native enzyme is preserved, respectively.
2. In a similar study of catalases of different origins in a heterogeneous environment, it was found that catalase from *Penicilium chrysogenum* immobilized on glucan type URE13-300 retains up to 76% of its initial activity in the presence of 5% ethanol and 63.5% of the initial activity in the presence of 5% methanol.
3. An electrochemical method has been developed to determine the enzymatic activity of immobilized catalase from *Penicilium chrysogenum* in the absence and in the presence of methanol and ethanol, and a formula for determining enzymatic activity has been derived. It has been found that the kinetics of the enzymatic reaction in the absence of alcohols is described by the Michaelis–Menten equation, while in the presence of ethanol or methanol it obeys the Hill equation for allosteric enzymes.
4. Two types of electrochemical biosensors have been obtained by immobilizing the enzyme laccase from *Trametes versicolor* and *Trametes pubescens* in a polymer film and have been characterized using electrochemical techniques such as cyclic and pulsed voltammetry, as well as chronoamperometry at constant potential. The electrochemical behavior of biosensors in the presence of di- and trihydroxy phenols has been investigated, and it has been found that:
   – as the most appropriate electrochemical method for studying enzyme activity, chronoamperometry is determined at a constant potential of -0.2 V. Operating conditions of the biosensor guarantee low energy consumption of the biosensor and minimal interference in the analysis of complex matrices;
   – the two laccase enzymes show a significant difference in their heterogeneous biocatalytic activity, with the enzyme from *Trametes pubescens* characterized by many times higher specific activity;
   – the apparent kinetic constants of the two immobilized enzymes, determined by nonlinear regression analysis in the presence of gallic and caffeic acids, differ significantly;
5. The biosensor method is applied to determine the total phenolic content in herbal extracts. When compared with chromatographic analysis of the phenolic content of the samples, a good correlation of the results of the chromatographic and biosensory analyses was established.

CONTRIBUTIONS:

1. An electrochemical method has been developed to monitor the catalytic action of immobilized catalase from *Penicilium chrysogenum* and an equation for determining its enzymatic activity based on electrochemical data has been derived.
2. A methodology for immobilization of laccase enzyme from *Trametes versicolor* and *Trametes pubescens* has been developed and has been applied in the creation of electrochemical biosensors for the analysis of di- and trihydroxy phenolic compounds. It has been shown that the biosensors obtained in this way are stable for 5 days.

PUBLICATIONS RELATED TO THE TOPIC OF THE SCIENTIFIC WORK:

1. **Peshkov, A.,** Angelova, S., Avesque, C., Iliev, I., Dimcheva, N. „Determination of kinetic parameters of catalase of different origin immobilized on water-insoluble glucan synthesized by recombinant glycosyltransferase URE13-300". Ecol. Balk., 15(2) for 2023. ,**(Q4)**
2. Shukri, M., Cherneva, T., **Peshkov, A.,** Nikolova, M., Iliev, I., & Dimcheva, N. (2025). „Comparison of Two Laccase Enzymes from Trametes versicolor and Trametes pubescens for the Assessment of Phenolic Acids Content Using Laccase-Based Biosensor". Applied Food Biotechnology, 12(1), 1–11. https://doi.org/10.22037/afb.v12i1.46955 , **(Q3)**
3. **Peshkov, A.,** Shukri, M., Pimpilova, M., Iliev, I. & Dimcheva, N. (2025). „Electrochemical approach for monitoring the catalytic action of immobilized catalase". Open Chemistry, 23(1), 20250143. https://doi.org/10.1515/chem-2025-0143 , **(Q3)**

PARTICIPATION IN SCIENTIFIC FORUMS:

1. Angelova, S., **Peshkov, A.,** Vasileva, T., Iliev, I., Bivolarski, V. Comparison study on the effect of organic solvents on different types of glucansucrases in heterogenic catalysis. 13th International Conference Protein Stabilization (ProtStab 2021), online**, 07 – 09 October** 2021. Poster and abstract, p. 61.
2. **Peshkov, A.,** Angelova, S., Avesque, C., Iliev, I., Dimcheva, N. Determination of kinetic parameters of catalase of different origin immobilized on water-insoluble glucan synthesized by recombinant glycosyltransferase URE13-300. 13th International Conference Protein Stabilization (ProtStab 2021), online**, 07 – 09 October** 2021. Poster and abstract, p. 67.
3. Determination of kinetic parameters of catalase of different origin immobilized on water-insoluble glucan synthesized by recombinant glucosyltransferase URE13-300. Student Scientific Session in Organic Chemistry. Plovdiv, **15-16 April** 2022. Oral report and abstract, p. 12
4. **Peshkov, A.,** Dimcheva, N., Iliev, I. Study of the influence of organic solvents on the activity of the enzyme laccase. THIRD NATIONAL YOUNG SCIENTISTS CONFERENCE ON BIOLOGY. Plovdiv, **November 1,** 2022. Poster and abstract p. 47
5. **Peshkov, A.,** Pimpilova, M., Iliev, I., Dimcheva, N. ELECTROCHEMICAL APPROACH FOR THE MONITORING OF IMMOBILIZED CATALASE ACTIVITY. 7th International Conference on Novel Enzymes '23, Gracewald, Germany, **28-31 March** 2023. Poster and abstract p.100. *ISBN 978-3-9504809-4-8*
6. **Peshkov, A.,** Iliev, I., Dimcheva, N. Electrochemical method for assaying immobilized catalase activity in the presence of alcohol. BelChem'24, Haskovo, Bulgaria, **25-28 September** 2024. Poster and abstract p. 49

7. **Peshkov, A.,** Dimcheva, N., Iliev, I. OXIDOREDUCTASES – PROPERTIES, FUNCTIONS AND POTENTIAL APPLICATION IN BIOTECHNOLOGY, BIOREMEDIATION AND BIOSENSORS. 2nd National Scientific Conference Physics-Engineering-Technologies, Plovdiv, Bulgaria, **27-28 November** 2024. Oral report and abstract, p. 57
8. **Peshkov, A.,** Dimcheva, N., Shukri, M., Stoynova, T., Nikolova, M., Iliev, I. DETERMINATION OF GALLIC ACID IN DIFFERENT HERBAL EXTRACTS VIA ELECTROCHEMICALLACCASE-BASED BIOSENSOR. 12th International Conference on Nutrition and Development, Athens, Greece, **20-22 February** 2025. Poster and abstract p. 18
9. Dimcheva, N., Shukri, M., Stoynova, T., **Peshkov, A.,** Nikolova, M., Iliev, I. Evaluation of phenolic acids in herbal extracts with laccase-based biosensors. 8th International Symposium on New Enzymes Novel Enzaims'25, Budapest, Hungary, **25-28 March** 2025. Poster and abstract p. 80.

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