Determination of kinetic parameters of catalase of different origin immobilized on water-insoluble glucan synthesized by recombinant glycosyltransferase URE13-300

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Abstract. The kinetic constants determined for three enzymes of different origins – fungal, bacterial, and mammal, have been compared in the process of hydrogen peroxide dispropor-tionation as a model reaction. In order to stabilize the enzyme, it has been immobilized on water-insoluble glucan whilst the studied process has been carried out in both the absence and the presence of low molecular weight aliphatic alcohols over a wide range of concentrations. In this study, purified catalases originating from the fungus *Penicilium chrysogenum*, bacteria *Micrococcus lysodeikticus*, and mammalian from the bovine liver, have been used. Their activities were determined by means of a spectrophotometric method, following the decay of the absorbance at 240 nm at a constant temperature, over the temperature range from 0 to 25°C. The catalytic processes were performed in the presence of methyl and ethyl alcohols in concentrations from 1% to 10%. For catalase immobilization, the water-insoluble glucan URE13-300 was used as a carrier when determining heterogeneous catalytic enzyme activity. The best and most stable was found to be the enzyme from *P. chrysogenum*, both immobilized and in the native state. It was found that the immobilized enzyme retains its activity over the temperature range – from 0 to 25°C. Its apparent kinetic constants were calculated to be: Km =109.4 mM and Vmax =14.42 μmol l^{-1} s^{-1}. Preservation of the catalytic activity above 90% of the initial one was found in the presence of ethanol and methanol at concentrations not exceeding 3%.

Key words: α-glucan synthesis, immobilized catalase, enzyme kinetics, apparent kinetic constants, peroxidase-like activity, low-molecular weight aliphatic alcohols.

Introduction

Catalase (hydrogen peroxide oxidoreductase; EC 1.11.1.6) is an enzyme found in all aerobic living organisms. Catalases are abundant in all aerobic organisms being a part of the antioxidant system of the living cells with the main function to eliminate hydrogen peroxide formed during metabolic processes via a radical-free mechanism (Karakus, 2020). Due to their diversity, it is expected that catalases isolated from different organisms will show noticeable differences in their catalytic activity and mechanism of action. Dissimilar to the non-catalyzed hydrogen peroxide disproportionation, its cata-

lase catalyzed decomposition to water and molecular oxygen goes without the formation of free radicals (Alfonso-Prieto et al., 2009). This ability of catalase together with its high catalytic efficiency is the main reason for the application of enzyme as an agent for the removal of excess H_2O_2 in numerous industrial and technological processes such as cotton bleaching (Arabaci & Usluoglu, 2013; Purich, 2010), milk processing (Singh, et al., 2019) and wine ageing (Inanan, 2019).

Recently, various biomedical applications of catalase have been reported, such as treatment of osteoarthritis (Chen et al., 2023a), alcohol-associated liver disease (Chen et al., 2023b), oxidative stress (Kehrer et al., 2010), age-associated degenerative diseases (Nandi et al., 2019), discharge of cytokine storm during Covid-19 disease (Qin et al., 2020), as well as treatment of some cancer types (Glorieux & Calderon, 2017; Najafi et al., 2022).

Typically, catalases include in their structure a protein and a prosthetic group, the functional part of which is made up of an iron porphyrin. The four polypeptide chains that make up catalase contain over 500 amino acids (Karakus, 2020), and the heme functional groups included in the protein molecule allow the enzyme to easily react with hydrogen peroxide. There is a variety of pH optimum, especially the human catalase, around 7 (Purich, 2010), but depends on the species the pH could vary between 4 and 11 (Singh & Kumar, 2019). Temperature is also a varying factor (Aebi, 1984).

Enzyme immobilization is of crucial importance especially when aiming at its industrial applications. Immobilization process may dramatically lower the costs of biocatalyst used due to the option for running multiple catalytic cycles. With increasing public concern for food and agricultural sustainability, food safety and environment care, food industry is seeking for greener strategy for the production of immobilized biocatalysts. A critical point in implementing this strategy is the enzyme immobilization on appropriate carriers. The carriers shall comply with several specific characteristics, e.g.: large specific surface area, stability during immobilization and catalytic process, good mechanical strength to enable multiple cycles of usage, inertness towards the active site of enzyme, abundance, sustainability, and non-toxicity (Grigoras, 2017).

In this study, insoluble glucan produced by recombinant glycosyltransferase URE13-300 is used to immobilize *P. chrysogenum* catalase, *Micrococcus lysodeikticus* catalase and bovine liver catalase as a sustainable and eco-friendly alternative of petroleum-based polymers commonly employed in catalase immobilization. Public concerns for the environment and food safety, pushed researches to seek for renewable and recyclable bio-derived structures such as α-glucans. These glucans are produced by lactic acid bacteria from different genera (mainly *Streptococcus, Leuconostoc, Lactobacillus, Weissela*). The physiological functions of the polymers are to create a protective biofilm which shields bacteria from environmental stress, and mediates the adhesion to surfaces (Molina et al., 2021).

The enzymes synthesizing α-glucans from the glucose residues of sucrose are called glucansucrases and belong to the glycoside hydrolase family 70 (GH70), the number of its biochemically characterized representatives has increased significantly in the recent years (Li et al., 2020; Moulis et al., 2016). Thanks to advances in the rational design of glucansucrases to obtain polymers with desirable properties, they have the potential for multiple applications in the food, medical, and cosmetic industries as value-added products (Claverie et al., 2019; Meng et al., 2017). Subfamily of GH70 enzymes, called branching sucrases (BRS) are able to add single α-(1→2) or α-(1→3) branched glucose residues to linear dextran with mainly α-(1→6) linkages as an acceptor, resulting in highly branched polysaccharides (Moulis et al., 2016).

Strain *Leuconostoc mesenteroides* URE 13, isolated from Bulgarian fermented vegetables, produces high molecular weight glucansucrase with molecular mass about 300 kDa. The gene encoding glucansucrase URE 13-300 was successfully cloned and expressed in *E. coli* BL21. The synthesized glucan and oligosaccharides have branched structure and possible prebiotic potential (Bivolarski et al., 2018). Structure of the insoluble glucan, synthesized by the glucansucrase harbors significant amount of α-(1→3) linkages.

Keeping in mind the public concerns about food safety and care for the environment, in this study we focused on the applicability of

bio-derived polymer α-glucan as a substrate for catalase immobilization in view of the prospective application of thus immobilized enzyme as a heterogeneous biocatalyst in beverage processing. With this respect, the activity of so immobilized catalases has been studied in hydrogen peroxide decomposition in both the absence and the presence of low – to moderate concentrations of methanol and ethanol, commonly present in alcoholic beverages. The catalytic activities of three catalases differing by their origin (mammal, bacterial and fungal), have been examined in both native and immobilized states. The resulting kinetic constants have been determined as well.

Materials and Methods
Materials

In this study, the following catalases and chemicals were used - *Penicillium chrysogenum* with specific activity ≥ 200 kU/mg, isolated and purified by prof. Iliya Iliev in the form of powder; catalase from bovine liver (deep brown powder, specific activity ≥ 1000 U/mg, Sigma-Aldrich); catalase from *Micrococcus lysodeikticus* (dissolved, specific activity ≥ 100 kU/mL, Merk, Sigma-Aldrich), Hydrogen peroxide, p.a., 34.5-36.5% solution (Sigma-Aldrich), Potassium Phosphate (purity > 99.00%, Sigma-Aldrich), Potassium Phosphate dibasic (purity > 99.00%, Sigma-Aldrich), Sodium phosphate monobasic (purity > 99.00%, Sigma-Aldrich), Ammonium molybdate (purity > 99.98%, Sigma-Aldrich), ethanol 95% (Labscan) and methanol (HPLC grade, Labscan). All chemicals were of analytical grade and used without additional purifycation.

The salts were used for preparation of buffer solutions with concentration of 0.1 M and pH = 7.0. All the solutions were prepared with ultra-pure water (18.2 MΩ resistance, total carbon ≤ 2 ppb, Adrona Bio, Vilnius, Lithuania).

Characterization of catalase activity by means of spectrophotometry

For this criterion, Worthington assay procedure was used as a template: 20 mg of catalase is dissolved in 20 mM phosphate buffer with pH 7.00 ± 0.05 and different concentrations of hydrogen peroxide. The measurement is made at 240 nm wavelength on spectrophotometer Shimadzu UV-VIS 2600.

The homogeneous enzymatic activity of catalases was determined by the Worthington method as follows: 100μl enzyme solution is added to 2.9 ml of substrate solution (15, 30, and 50 mM hydrogen peroxide in 20 mM phosphate buffer, pH 7.0) at a temperature of 20°C. The enzyme kinetics was monitored at a wavelength of 240 nm at which the variation in absorbance was registered, with an extinction coefficient used ε_{240} = 240 = 43.6 mol l^{-1} cm^{-1}. For the determination of the catalytic activity of the three catalases 3 to 5 measurements with each enzyme were carried out.

Characterization of catalase activity using internal titrimetric method

All the crystalline catalases as *P. chrysogenum* (0.0501 g) and bovine liver (0.0501 g) were dissolved in 10 ml 20 mM phosphate buffer with pH 6.8 (K$_2$HPO$_4$ – 0.4084 g, NaH$_2$PO$_4$ – 0.5340 g), while for the dissolved catalase from *Micrococcus lysodeikticus* – 250 μl were taken and added to 10 ml 20 mM of phosphate buffer.

All the samples (1 ml) were incubated for 10 min at 0°C in the presence of 0.01 M hydrogen peroxide solution (5 ml). After the incubation, the reaction was stopped by the addition of 20% sulfuric acid (2 ml) and 2-3 drops of 1% ammonium molybdate solution for 3 minutes, followed by the addition of 5% KI (1 ml). Determination of the residual H$_2$O$_2$ was performed by titration with 0.01 N Na$_2$S$_2$O$_3$ in the presence of 2-3 drops 1% solution of starch as indicator.

Same procedure was repeated in the presence of either methanol or ethanol added to the reaction mixture until 1, 3, 5 or 10% final concentration.

Catalase immobilization

All types of catalases were immobilized on water-insoluble glucan synthesized by recombinant glycosyltransferase URE13-300. For this purpose, lyophilized catalases (0.050 g) were dissolved in 20 ml phosphate buffer (20 mM) with pH 7.0, and the water-insoluble glucan was soaked in the enzyme solution. Same procedure was used for the dissolved *Micrococcus lysodeikticus* catalase, 250 μl of which were added to

the same amount of phosphate buffer, and then the insoluble glucan was soaked in the enzyme solution. Further, the three samples were frozen at -20ºC and then lyophilized.

In vitro glucan synthesis

Glucan synthesis by glucansucrase URE 13-300 was carried out at 30°C with 0.5 U/mL enzyme (unless otherwise stated), in 0.02 M sodium acetate buffer with pH 5.3 and 100 g/L sucrose as a substrate (Iliev I., December 15, 2021, Bulgarian Patent No. 67404 B1). All of the performed reactions were supplemented with 0.1% (w/v) sodium azide for prevention of bacterial growth. Synthesis reactions were stopped by heating at 100°C for 10 min. The α-glucan fractions were separated by precipitation with two volumes of 96% ethanol. They were purified by triple washing with ultrapure water, followed by centrifugation at 8000 x g for 15 min. Glucan preparations were dissolved in distilled H_2O, frozen at -20°C and then lyophilized (Labconco FreeZone 4.5, USA) for the immobilization of catalase.

Statistical analysis

The statistical software package SigmaPlot v12.0 (Systat Software, Inc., Chicago, USA) and Microsoft Excel were used for data analysis and graphical representation. All reactions of enzyme activity were performed in triplicate from different experiments with standard deviation (±SD).

Results and Discussion
Optimization of glucan synthesis by glycosyltransferase URE 13-300

Series of reactions were performed in order to explore the dynamics of the insoluble glucan synthesis. Low-volume – synthetic reactions were performed in volumetric flasks in a rotary shaker for 24 to 48 hours. The influence of glucansucrase URE 13-300 activity on glucan synthesis was investigated by using enzyme solutions with gradually increasing activity – 0.1 U mL-1; 0.2 U mL-1; 0.5 U mL-1 and 0.7 U mL-1. The obtained glucan fractions, derived from each reaction mixture indicate that there is no significant change in glucan yield. Therefore, glucansucrase concentration was kept at the level of about 0.1 U mL-1 for the subsequent analyses.

Both small scale (performed in flasks) and large scale (performed in a bioreactor) reactions were monitored dynamically over a time interval of 24 to 48 hours. In both cases the reactions were carried out at a 30°C temperature and 100 rpm agitation. The obtained results manifest rapid accumulation of α-glucan between reaction initiation and the 8th hour (Fig. 1). The rate of glucan synthesis notably decayed from the 8th hour onwards. Finally, between the 24th and 48th hour from the start of the reactions, only a low amount of glucan was synthesized. Taking into account the achieved results, it was concluded that efficient glucan synthesis was performed both in flasks and in a bioreactor (Fig. 2). The lyophilized glucan represent a white crystalline-like mass with cotton-like appearance (Fig. 3).

Catalase homogeneous and heterogeneous activity

The kinetics of enzyme-catalyzed decomposition of hydrogen peroxide was investigated. For this purpose, the catalytic activity of 3 catalases isolated from different sources - from fungi, from bacteria, and one of animal origin - was screened. The purpose of this screening was to select the most active of the studied enzymes, which will subsequently be immobilized with a view to obtaining a heterogeneous biocatalyst for repeated use. In Table 1 are shown the results from the determination of the enzymatic activity of all studied enzymes. Notably, the homogeneous activity of immobilized catalase *from Penicillium chrysogenum* did not change substantially over the temperature range of 0-25°C.

In order to determine the kinetic constants of the three investigated enzymes (Vmax and Km), a more thorough analysis of the kinetic curves was performed. The obtained dependencies of the reaction rate on substrate concentration followed a hyperbolic trend typical for Michaelis-Menten kinetics. The constants were calculated by regression analysis of the dependencies plotted in double-reciprocal coordinates (Lineweaver-Burk plot), i.e. the reciprocal rates of the enzyme-catalytic process versus the reciprocal substrate concentration, and the determined values of the kinetic constants are presented in Table 2.
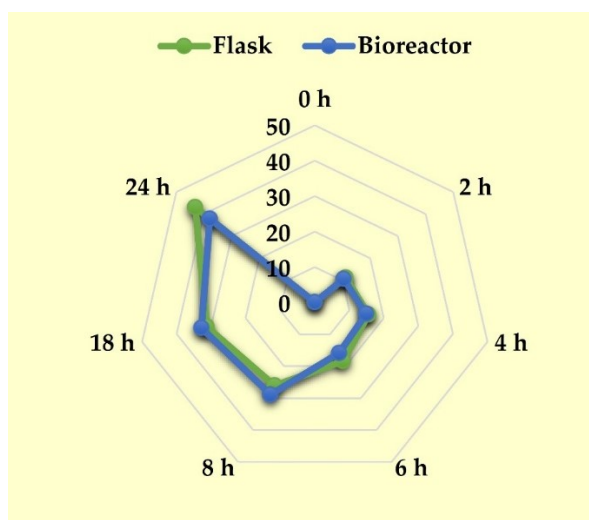
Fig. 1. Synthesis of insoluble α-glucan at different hours of the polymerization reaction, performed in flasks (green) and bioreactor (blue). Glucan yield is presented in mg/mL concentration.
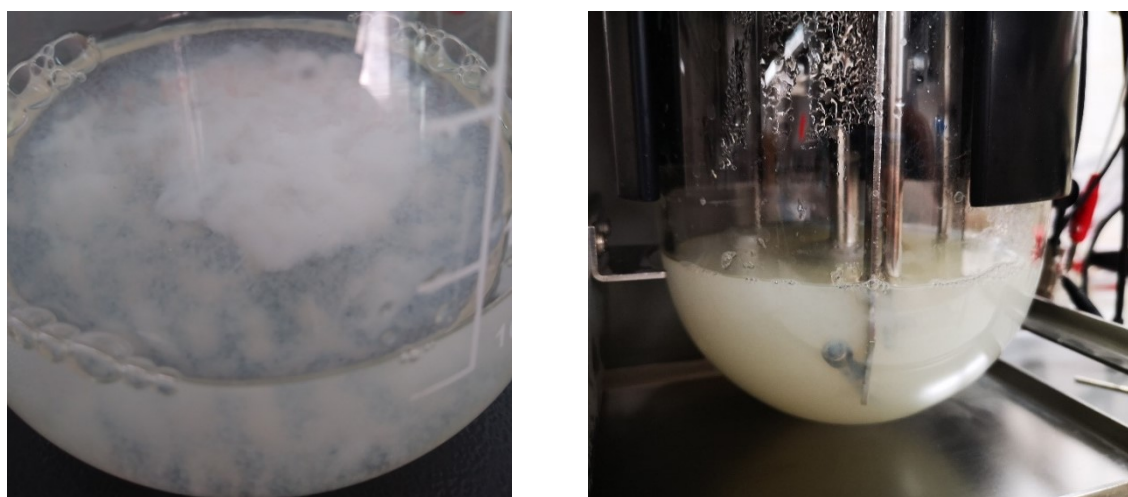

Fig. 2. Glucan synthesis in flask (24th hour, left) and in bioreactor (18th hour, right).

Fig. 3. Appearance of lyophilized glucan.

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Table 1. Homogeneous catalytic activity of catalases isolated from fungal, mammalian, and bacterial sources.

Catalase	*Activity*
Fungal *(Penicilium chrysogenum)*	258 550 U/mg
Mammalian *(Bovine liver)*	1 146 427 U/mg
Bacterial *(Micrococcus lysodeictikus)*	130 700 U/ml

Table 2. Kinetic constants and specific enzyme activity of catalases from fungal, mammalian, and bacterial origin.

Catalase (homogeneous experiment)	K_M (mM)	V_{max} (mol l^{-1} s^{-1})	Specific enzyme activity, $\times 10^3$ µmol l^{-1} min^{-1} mg^{-1}
Penicilium chrysogenum	112	9.90x10^{-4}	5.91
Bovine liver	121.32	9.02x10^{-3}	18.65
Micrococcus lysodeikticus	77.39	2.77x10^{-4}	0.191

When immobilized, the apparent kinetic constants of catalase determined in the reaction of hydrogen peroxide radical-free decomposition change to a large extent, mostly due to strong interferences from the diffusion of the substrate to the active site of the immobilized enzyme. Another possible explanation of this finding is the effect of substrate distribution between the bulk solution and the enzyme carrier.

It might be hypothesized that hydrogen peroxide adsorbs strongly on the surface of the glucan, thus creating substrate saturated environment for the immobilized enzyme, which in turn affects both apparent kinetic constants: the K_M^{app} value tends to lower drastically due to the concomitant effect of diffusion and substrate distribution between the surface of the carrier (Table 3). Similarly, the V_{max}^{app} value decreases approximately 1.5 orders of magnitude for all studied catalases – most probably due to the change of the rate-limiting stage of the heterogeneous multistep reaction (Table 3).

Table 3. Kinetic constants of immobilized on URE 13 300 catalases from fungal, mammalian, and bacterial origin.

Catalase (Immobilized on URE 13 300)	K_M^{app} (mM)	V_{max}^{app} (mol l^{-1} s^{-1})
Penicilium chrysogenum	109.4	0.00001442
Bovine liver	16.4	0.000454
Micrococcus lysodeikticus	24.4	0.00007573

### Comparative studies of catalase activity in the presence of alcohols

As determined in the previous subsection, catalytic activities of the three selected catalases – both homogeneous and heterogeneous ones, have been taken as control. Afterwards, low molecular weight alcohols - methanol or ethanol, were added to the reaction mixture, and their effect on the enzyme activity was monitored. Studies were carried out in the presence of 1, 3, 5, or 10% of either alcohol.

Catalase from *Micrococcus lysodeikticus* was found to lose its activity upon the addition of each alcohol. The results obtained with the catalases from the fungus *Penicillium chrysogenum* and from mammalian origin are presented in Table 4 and Table 5.

These results show that both catalases (fungal -P.c. and mammal - B.l.) retain their enzyme activity to a significant extent in the presence of low concentrations of alcohols (1% ethanol or methanol), but with increasing concentrations, the two alcohols have a

denaturing effect on the enzymes, and they lose their activity.

It is known from the literature that some catalase enzymes possess, in addition to catalase activity (eq. 1):

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (1)$$
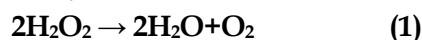
and peroxidase-like activity (eq. 2), which is manifested by the ability of catalase to oxidize low concentrations of hydrogen donors in the presence of hydrogen peroxide:

$$2H_2O_2 + R\text{-}C(H)OH \rightarrow 2H_2O + R\text{-}CHO \quad (2)$$
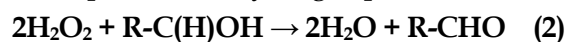
The increase in the native activity of fungal catalase in solution up to 83% in the presence of 1% methanol is the manifestation of peroxidase-like activity of this enzyme that explains the noticeable catalytic activity increase. On the other hand, the data obtained for mammalian catalase imply that it does not exhibit peroxidase-like activity under the equivalent experimental conditions.

Comparison of the activities of both catalases keep decreasing activity with increasing percentage of different alcohol, but *P.*

chrysogenum is more stable and active than bovine liver catalase when in dissolved state: the remaining activity of *P. chrysogenum* – native enzyme has been found to be approximately 83%, whilst the one of bovine liver catalase is 58% in the presence of methanol. The situation is different when ethanol is added to the reaction mixture – the average activity of *P. chrysogenum* is 64% and the one of bovine liver catalase is 60%, which suggests that the *P. chrysogenum* catalase has greater stability in the presence of alcohols than bovine liver catalase. Therefore, the fungal enzyme was immobilized on a polysaccharide biopolymer for further studies.

Analogous studies, carried out with immobilized fungal catalase (Table 6), revealed that due to the reversible nature of the immobilization, the peroxidase-like activity of fungal catalase was observed only in the presence of 1% methanol, while the same concentration of ethanol caused some loss of catalytic activity of the enzyme.

Table 4. Dependence of the native catalytic activity of catalase of fungal origin (*P.c.*) on the concentration of ethanol and methanol in the reaction medium.

P.c. CAT	A_{sp} U/mg Protein (control sample)	Native enzyme activity (control sample), %	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

Table 5. Dependence of the native catalytic activity of mammal origin (B.l) on the concentration of ethanol and methanol in the reaction medium.

B.l. CAT	A_{sp} U/mg protein (control sample)	Native enzyme activity (control sample), %	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	9.83	100	8.12	82.65	6.08	61.85	5.95	60.50	0	0
Methanol	9.83	100	6.66	67.80	5.68	57.80	0	0	0	0

Table 6. Heterogeneous enzyme activity (determined as a percentage from the catalase activity in the absence of alcohols) of immobilized on URE13-300 fungal catalase (*Penicilium chrysogenum*) in the presence of methanol or ethanol.

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

Conclusions

The outcome of the present study can be summarized as follows:

- The reaction conditions for the enzyme-catalyzed synthesis of α-glucan were optimized with respect to the enzymatic activity of the bio-catalyst, reaction temperature, duration of the process, and volume of the reactor;

- The enzymatic activities of catalases isolated from fungal, bacterial, and mammalian sources have been determined in dissolved and immobilized state at the model reaction decomposition of hydrogen peroxide.

- Similarly, the activities of the same catalases (dissolved) have been determined in the presence of methanol and ethanol. The results demonstrate that bacterial catalase does not tolerate the presence of either alcohol, whilst the enzyme from fungal origin shows peroxidase-like activity, most noticeable in the presence of up to 1% added methanol. Under the same conditions, mammalian catalase retains some activity.

- Immobilized fungal catalase is more stable in the presence of alcohols than the dissolved enzyme, and retains ca. 76% in the presence of 5% methanol, while in the presence of the same ethanol concentration, the residual heterogeneous activity of the enzyme is 63.5%.

The obtained results demonstrate that the enzyme isolated from fungi is more durable and alcohol-tolerant than the enzymes derived from eukaryotic organisms. The support for catalase immobilization - α-glucan, obtained by enzymatic synthesis catalyzed by glucansucrase is a promising and eco-friendly alternative to the traditionally used polymers synthesized from fossil fuels. The thus immobilized catalase shows remarkable stability and resistance to denaturation in the presence of aggressive chemical agents – e.g. ethanol and methanol.

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