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Laccase Immobilization on Biopolymer Carriers – Preliminary Studies

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ABSTRACT

This preliminary studies concerns preparation of biopolymer carriers for immobilization of laccase from *Trametes versicolor,* based on sodium alginate, chitosan and on a combined alginate-chitosan biopolymers as well as the evaluation of their potential use in the decolourization process. The study is related to the assessment the using of various carriers in the immobilization methods of laccase. The dropping method using sodium alginate (2%) proved to be the most effective technique of enzyme immobilization. The study showed an improvement in the stability of immobilized laccases under the conditions of variable pH, relative to a free laccase. A loss in the stability of enzymes in alginate beads occurs at high temperatures, together with enzyme leaching and degradation. Enzyme leaching from the beads inhibits their preliminary low-temperature drying. Immobilization and drying of obtained capsules constitutes a promising method for improving enzyme stability. The results obtained as part of this study offer a valuable contribution to the future research on the possibility of using the prepared alginate beads to remove colour contamination from wastewater.

Keywords: laccase, immobilization, biopolymer, biocatalysis.

INTRODUCTION

There has been a great focus in recent years on conducting technical processes in the ways that are both effective and environmentally friendly. Enzymes deserve particular attention, as they constitute natural biocatalysts in the processes occurring in living organisms, and they make it possible to increase the reaction rate by several factors. Consequently, broad-scale research is being conducted to discover stable and efficient catalysts serving as "green tools" in industrial processes, which would lower the costs of conducting chemical reactions by accelerating them (Bassanini et al., 2021). The use of enzymes is an increasingly common technological solution in industry. They have found application in the removal of many and diverse contaminants, including dyes (Ramirez-Montoya et al., 2015; Verma et al., 2019; Lopes et al., 2020). The widespread use of dyes and the issues related to their removal from wastewater motivate researchers to seek out effective, inexpensive and environmentally safe methods of their elimination. To date, these methods were based on the adsorption technology, e.g. on active carbon (disadvantages: high costs; technology used primarily for single dyes, rarely for their mixtures generated by industry) (Wong et al., 2018; Okoniewska, 2021) and using activated sludge (disadvantages: high dye toxicity results in sludge bulking, impoverishment of biodiversity, microbe cell deformations) (Didier de Vasconcelos et al., 2021). Algae, bacteria and fungi found application in the biological methods for removing contaminants, including dyes (Mishra & Maiti, 2019). White rot fungi have particular significance, considering their ability to produce exoenzymes (laccase, lignin peroxidase, Mn-dependent peroxidase) with various properties and great redox potential (Shi et al., 2021). Given the low specificity of the abovementioned enzymes, the fungi that generate them are capable of degrading numerous compounds, including synthetic dyes.

In the context of the industrial use of enzymes, the primary factor limiting their effective application in technical processes is the reduction of their activity as a result of the sensitivity to the reaction environment conditions. This led to a dynamic development of the research focused on improving enzyme resistance to variable reaction conditions. Immobilization appears to be a promising method in this regard (Deska & Kończak, 2020). Its application results in an increased thermal, chemical and operational stability of the biocatalyst. Immobilization is a process based on binding the enzyme to a polymer carrier that is stable and insoluble in the reaction environment, with the simultaneous retention of catalytic properties by the biocatalyst. A broad literature review concerning immobilization as a promising technique for improving the operational stability of laccase was carried out by Deska and Kończak (2020). In the light of current literature reports, innovative enzymatic preparations consisting of immobilized laccases, given their high operational stability, can find application in various branches of industry: in water treatment (Zhou et al., 2021), in bioremediation processes (Viswantath et al., 2014), particularly for degrading pharmaceutical agents (Yang et al., 2017), and as biocatalysts (Shokri et al., 2021), particularly for treating sewage containing hard to degrade contaminants, including coloured sewage (Deska & Kończak, 2019). Apart from the appropriate enzyme immobilization method selection, the key factor is to choose the proper carrier for immobilization in order to ensure the stable binding of the biocatalyst and to limit enzyme leaching from the carrier, while retaining the appropriate enzymatic activity of the immobilized biocatalyst. A review of the most recent achievements in laccase immobilization on various carriers was presented in the publication by Jafri et al. (Jafri et al., 2021). The purpose of the preliminary studies presented therein was to prepare enzymatic beads, including the selection of the carrier type and their concentration, the immobilization method and the enzyme concentration, in a way that would yield stable enzymatic beads for potential use in dye removal from wastewater. An important parameter of the enzymatic beads is their stability under storage conditions. Therefore, the presented tests also determined such storage conditions of the immobilized beads that would enable the retention of the high catalytic activity of the biocatalysts even after several or more than a dozen days of storage, which is of great significance for their practical application.

MATERIALS AND TEST METHODOLOGY

Materials

Enzyme

Laccase from *Trametes versicolor* (powder, light brown, ≥0.5 U/mg) was purchased from Sigma Aldrich (Sigma Aldrich, Poznań, Poland).

Biopolymers for immobilization

Alginic acid sodium salt from brown algae (low viscosity) and chitosan (low molecular weight) were purchased from Sigma Aldrich (Sigma Aldrich, Poznań, Poland). The characteristics of the biopolymers used for the laccase immobilization are presented in the table below (Table 1).

Other chemicals

Calcium chloride (anhydrous, granular, ≤ 93%), acetic acid and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma Aldrich, Poznań, Poland), potassium hydroxide, sodium hydroxide, sodium acetate buffer (pH5) and aminoacetic acid (glycine) (Chempur, Piekary Śląskie, Poland), methylene blue (Eurochem BGD Sp. z o.o., Tarnów, Poland).

Biopolymer bead formation

The ability of the polymer solution to produce the appropriate beads (alginate beads (AL), chitosan beads (Ch), alginate-chitosan beads (AL-Ch)) was assessed visually. The correct bead formation

Table 1. Biopolymer characteristics

Biopolymer	Quality level	Physical form	Absolute viscosity
Alginic acid sodium salt from brown algae	200	Powder	4-12 cP, 1% in H_2O (25°C)
Chitosan	100	powder; 75-85% deacetylated	20-300 cP, 1 wt. % in 1% acetic acid (25°C)

Source: Data sheets for chemicals from www.sigmaaldrich.com

process occurred when the biopolymer drops falling into the solution underwent immediate gelling in a water bath, obtaining a spherical and regular shape and sinking to the bottom of the vessel. When the drops disintegrated in contact with the cross-linking solution, or if the beads obtained irregular shapes or floated on the surface of the cross-linking solution, the bead formation conditions were deemed incorrect. Empty beads – not containing an enzyme – were formed using the same methodology, without adding an enzyme to the biopolymer solution.

Alginate-Laccase beads (AL-L)

The slightly modified methodology of Niladevi and Prema (Niladevi & Prema, 2007) was used to prepare alginate beads with immobilized laccase. Various sodium alginate concentrations were used to prepare the alginate beads, at 1%, 2%, 3% (w/v). The appropriate amount of sodium alginate (in the form of powder) was mixed with distilled water, mixing constantly at a temperature of 21°C for 15 minutes until the complete dissolution of the sodium alginate. The obtained solution was left at room temperature for 15 minutes to eliminate the air vesicles generated as a result of mixing. The most appropriate concentration of sodium alginate was then used for further experiments. In the study, 70 μL of the laccase solution at a concentration of 0.6% w/v were mixed with 500 μL of the sodium alginate solution (2.0% w/v). The enzymatic beads were obtained by introducing the enzyme-containing sodium alginate solution into the cross-linking solution containing $CaCl_2$ at a concentration of 2% (w/v) through a dosing needle with a diameter of 0.45 mm (Figure 1d). During the dropwise addition, the cross-linking solution was constantly mixed on a magnetic stirrer with a rate of 70 rpm. The dosing needle was placed at a height of about 2 cm from the surface of the cross-linking solution. The formation and behaviour of the beads in the cross-linking water bath was assessed visually. The produced enzymatic beads were left to harden in the $CaCl_2$ cross-linking solution for 15 minutes. After the encapsulation process was finished, the obtained gel beads with the immobilized enzyme were recovered via decantation and subsequently rinsed thrice in distilled water as well as filtered through a sieve to remove excess water. Afterwards, they were immediately used for further testing.

The microbeads formed using atomizers were produced according to the methodology described above, differing in that the enzyme-containing biopolymer was sprayed directly into the cross-linking solution using 3 kinds of different atomizers instead of a dosing needle (Figure 1a-c).

Chitosan-Laccase beads (Ch-L)

The slightly modified methodology of Jaiswal et al. (Jaiswal et al., 2016) was used to prepare chitosan beads with immobilized laccase. The appropriate amount of chitosan (with low molecular weight) in the form of powder was mixed with acetic acid at a concentration of 5% (w/v), mixing constantly at a temperature of 60°C for 15 minutes until the complete dissolution of the chitosan. The obtained solution was left at room temperature for 20 minutes to eliminate the air vesicles generated as a result of mixing. Various chitosan concentrations were used to prepare the chitosan beads with immobilized laccase, at 1.5–3.0% (w/v). The most appropriate concentration of chitosan was then used for further experiments. In the study, 70 μL of the laccase solution (0.6% w/v) were mixed with 500 μL of the chitosan solution (2.5% w/v) and added dropwise to a KOH solution (1M), which was constantly mixed on a magnetic stirrer with a rate of about 70 rpm. The chitosan beads were left to harden in the solution (60 min). Subsequently, the beads were recovered from the cross-linking KOH solution via decantation. Afterwards they were rinsed twice with a glycine-NaOH buffer (Gly-NaOH buffer) (100 mM, pH 10) and filtered through a sieve to remove the excess Gly-NaOH buffer. The produced beads were immediately used for further testing.

The chitosan microbeads formed using atomizers were produced according to the methodology described above, differing in that the enzyme-containing biopolymer was sprayed directly into the cross-linking solution using 3 kinds of different atomizers, instead of a dosing needle.

Alginate-Chitosan-Laccase beads (AL-Ch-L)

The alginate-chitosan beads with immobilized laccase (AL-Ch-L) were prepared according to the methodology of Segale et al. (2016). The procedure was identical as in the case of the alginate beads, except for the cross-linking solution composition, which contained 0.2% (w/v) of chitosan in acetic acid (1%) containing $CaCl_2$ at

a concentration of 100 mM. The sodium alginate concentration was 2%. The alginate-chitosan microbeads formed using atomizers were produced according to the methodology described above, differing in that the enzyme-containing biopolymer was sprayed directly into the cross-linking solution using 3 kinds of different atomizers instead of a dosing needle (Figure 1a–c).

Immobilization yield

Immobilization yield (*IY*) was calculated according to formula (Eq. 1):

$$IY\ (\%) = \frac{Total\ activity\ of\ immobilized\ enzyme}{Total\ activity\ of\ free\ enzyme} \cdot 100\% \quad (1)$$

Instruments and analytical methods

Enzyme assay

Laccase activities were measured spectrophotometrically by spectrophotometer UV-VIS Jasco V-730 (Kraków, Poland) using a dedicated substrate 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) by monitoring the oxidation of ABTS to its cation radical ($ABTS^{+}$). Activity of laccase was measured according to a modified method of Bourbonnais & Paice (1990) (Bourbonnais and Paice, 1990) and Olajuyigbe (2019) (Olajuyigbe et al., 2019). The reaction mixture for immobilized enzyme consisted of beads with immobilized laccase (AL-L) and 2.0 ml substrate solution (1 mM ABTS in 50 mM sodium acetate buffer pH 5.0). The reaction mixture for free enzyme consisted 2.0 ml substrate solution and proper amount of free laccase (FL) solution (0.06% w/v). Blank contained only 2.0 ml substrate solution (1 mM ABTS in 50 mM buffer pH 5.0). During the process, the increase of absorbance was measured. The measurements were carried out at the wavelength 420 nm (A420), at 1 min intervals, with the absorbance coefficient value of (ε_{max} = 36 000 M^{-1} cm^{-1}). The reaction time was 5 min. One unit of laccase activity was defined as the amount of enzyme that is needed to catalyze 1 µmol of substrate (ABTS) per 1 minute. The tests of the effect of pH on laccase activity were conducted over a pH range of 3.0–7.0 (pH 3 – in citrate-phosphate buffer; (pH5 – in sodium acetate buffer, pH 7 – in citrate phosphate buffer) at 21°C to determine the pH profiles for FL and AL-L. The obtained results were converted to relative activities as percentage of the maximum activity obtained in that series. Each measurement was carried out in triplicate and the mean was calculated.

Influence of bead storage on enzymatic activity

The influence of storage temperature and time on the enzymatic activity of the stored enzymes immobilized in sodium alginate was inspected. The tests were conducted at temperatures of -10°C, 4°C and 21°C over a period of 21 days. The tests were conducted both in a water medium – beads stored in distilled water, as well as with no water medium – in a glass vessel secured from evaporation. Before determining the activity (as per the methodology described in section 2.3.1), the beads were incubated at room temperature for 5 minutes.

Tests of dye (methylene blue) absorption on beads

The tests of dye (methylene blue) absorption on empty (enzyme-free) polymer beads (AL, Ch, AL-Ch) were carried out at temperatures of 30°C and 40°C. The tests were conducted in a glass vessel secured from evaporation. For this purpose, 2 g of wet beads were placed in 20 ml of a methylene blue dye solution in distilled water at a concentration of 25 mg/L and pH 6. The process was conducted over 28 h.

RESULTS AND DISCUSSION

Immobilization method effects

To obtain beads with immobilized laccase, characterized by a spherical shape and a desired diameter as well as appropriate mechanical properties, two immobilization methods were tested as part of these preliminary studies – using an atomizer, and per dropwise addition using a dosing needle. The first method involved the use of 3 kinds of atomizers with different technical parameters (stream width, pressure, capacity) (Table 2, Figure 1 a–c).

The atomizer method did not make it possible to obtain uniform polymer microbeads with the desired and repeatable geometric parameters. The microbeads would merge and their geometric parameters were varied. The second method – encapsulation via dropwise addition – involved the use of two kinds of encapsulation instruments, namely syringes with a capacity of 5 ml, differing in the diameter of the polymer-dosing needle

Table 2. Technical parameters of atomizers used to obtain enzymatic microbeads

Atomizer	Tank capacity [ml]	Nozzle diameter [mm]	Operating pressure [bar]
1a	15	0.5	1–3.5
1b	500	1.4*	3
1c	6000	2.0	3–4

* With smooth regulation from a round, point-wide stream to a broad, surface-wide stream.

(0.45 mm, 0.85 mm). The beads with the desired shape and size (wet bead diameter of about 2–3 mm), repeatable geometric parameters and the appropriate strength were obtained via dropwise addition using a syringe (with a capacity of 5 ml) with a dosing needle having a diameter of 0.45 mm (Figure 1d). Therefore, the dropwise addition method using a feeding module in the form of a syringe with a capacity of 5 ml and a dosing needle with a diameter of 0.45 mm was selected to prepare enzymatic beads for further analyses.

Three types of polymer carriers were tested to prepare the enzymatic beads – sodium alginate (AL), chitosan (Ch) and a combined alginate-chitosan carrier (AL-Ch). After optimising the bead formation process, in the case of the alginate beads, the appropriate biopolymer solution concentration used for encapsulation was 2%, and the appropriate cross-linking solution concentration ($CaCl_2$ solution) was 2%. Convergent results were obtained by Kończak (2017) in her study concerning the optimization of the sewage sludge encapsulation process. As part of that study, the author attempted to convert sewage sludge into an industrially valuable product through its immobilization in a hydrogel carrier formed from cross-linked sodium alginate. It was determined that the sodium alginate solution should have a concentration of at least 2%, which makes it possible to obtain the sewage sludge beads with

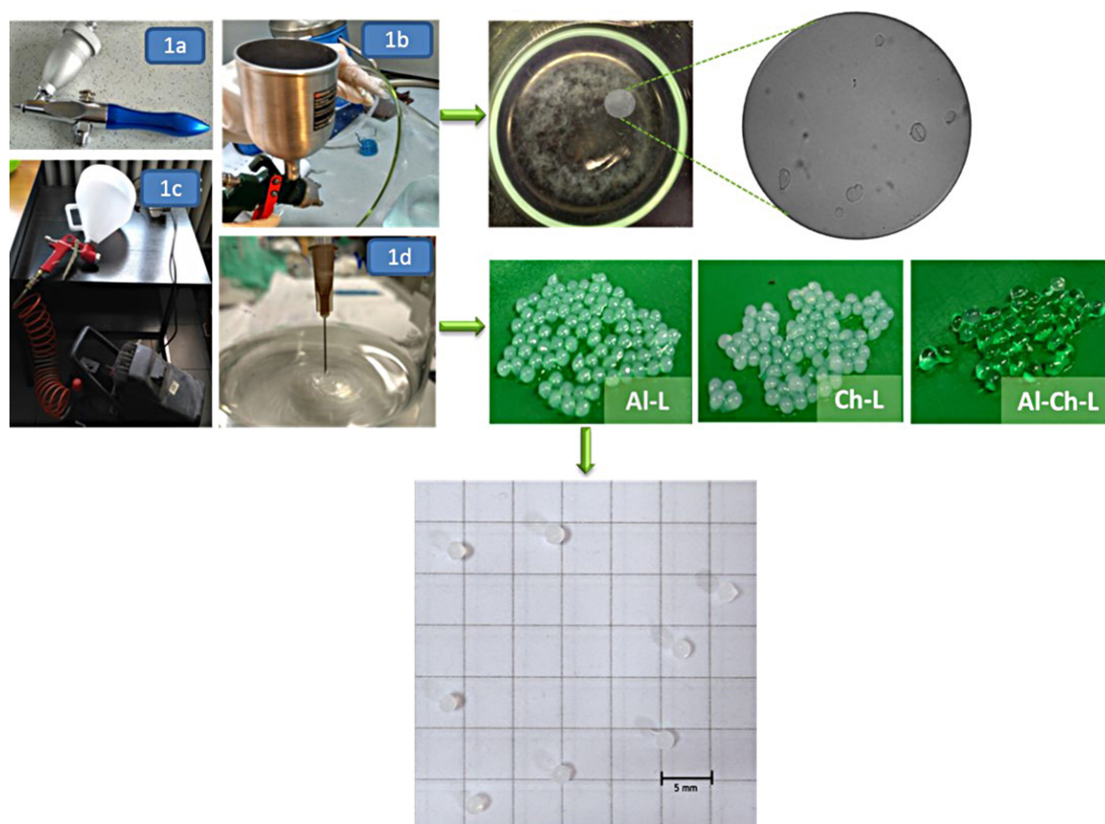

Figure 1. Laccase immobilization methods: a) atomizer φ 0.5; b) atomizer φ 1.4; c) atomizer φ 2.0, d) dropper φ 0.45; AL-L – alginate-laccase beads; Ch-L – chitosan-laccase beads; AL-Ch-L – alginate-chitosan-laccase beads

a satisfactory mechanical stability (Kończak, 2017). Similar test results were obtained by Daâssi et al. (2014), who demonstrated that the optimal concentrations for immobilizing the laccase obtained from *Coriolopsis gallica* are: 2% (w/v) sodium alginate, 2% (w/v) $CaCl_2$ and 1:4 E/A (v/v). On the other hand, Noreen et al. (2016) demonstrated that the maximum enzyme immobilization efficiency (89%) can be obtained when the sodium alginate concentration is 4% (w/v) together with a 2% (w/v) cross-linking calcium chloride solution (Noreen et al., 2016). Ratanapongleka and Punbut (2018) indicate that the optimal conditions for immobilization involve a solution of 5% (w/v) sodium alginate, 5% (w/v) barium chloride and a gelation time of 60 min.

Chitosan is a polyelectrolyte characterized by biocompatibility, antibacterial properties and environmental safety. It finds numerous diverse applications e.g. in water treatment processes, as well as decolourization processes as a carrier for immobilizing (encapsulating) enzymes (Ali & Ahmed, 2018). The study presented herein was based on the chitosan bead formation methodology per Jaiswal et al., differing in that the formation process involved chitosan with low molecular weight (low viscosity). This made it possible to obtain smaller beads of a repeatable shape. The obtained beads were characterized by a milky-white colour and high mechanical strength. Stable beads with the desired geometric parameters and strength were obtained at a chitosan concentration of 2.5%. Jaiswal et al. also indicated 2.5% as the optimal chitosan concentration (chitosan of medium molecular weight, 75% degree of deacetylation, >80% purity) in their study concerning the immobilization of laccase (isolated from *Carica papaya*) in chitosan for the decolourization processes of indigo carmine (at a concentration of 20–150 μg/ml). At a chitosan concentration of 2%, they observed the formation of the beads that proved too brittle as well as vulnerable to damage during processing. On the other hand, using a biopolymer at a concentration of 2.5% yielded stable and uniform beads. Furthermore, in the context of using chitosan as a matrix for immobilizing laccase, the method presented therein was characterized by an additional advantage, in that it involved no toxic chemical substances such as glutaraldehyde or polyepichlorohydrin as cross-linking agents for immobilization. According to the authors' best knowledge, it was the first reported case of plant laccase immobilization on chitosan via entrapment with no use of additional toxic components, simultaneously providing a high immobilization yield (98%) (Jaiswal et al., 2016).

Alginate and chitosan cross-linking through the formation of combined alginate-chitosan carriers found application in the form of materials useful in medicine and the pharmaceutical industry, particularly as modern hybrid materials used for the prolonged release of active substances (Berger et al., 2004). The combined networks thus developed are characterized by increased stability, compared to the networks obtained using one type of biopolymer (Segale et al., 2016). For example, Segale et al. (2016) developed the alginate and alginate-chitosan beads containing celecoxib dissolved in a self-emulsifying phase in order to obtain a drug delivery system for oral administration, capable of delaying the drug release in an acidic environment (Segale et al., 2016).

The alginate-chitosan beads developed by the authors of this study were characterized by slightly greater sizes than the alginate or chitosan beads – 4–5 mm; they were also transparent, and their structure had lower mechanical strength – the bead structure resembled the consistence of very thick gel. Lu et al. (2007) carried out a multiple-criteria analysis of the factors influencing the laccase immobilization yield in an alginate-chitosan carrier. They demonstrated that the factors in the following sequence had key significance for the immobilization: sodium alginate concentration > the volume ratio of the enzyme to the sodium alginate > the $CaCl_2$ concentration > the chitosan concentration. Optimal immobilization conditions were obtained when the sodium alginate concentration was 2%, $CaCl_2$ was 2%, and chitosan was 0.3%, at a 1:8 volume ratio of the enzyme to the sodium alginate (Lu et al., 2007). Another example of a combined carrier for laccase immobilization is a carrier based on chitosan/halloysite. Immobilizing laccases from *Trametes versicolor* on this carrier resulted in improved enzyme catalytic properties and operational stability, and also enabled the repeated use of the enzymatic preparations in decolourization processes (Hürmüzlü et al., 2021).

Enzymatic activity of the developed beads

Enzymes may constitute an incredibly valuable tool as "green catalysts" in many technical processes, including coloured wastewater treatment in particular. Using free laccase in decolourization processes involves disadvantages, such as e.g. the loss of enzyme activity, too high sensitivity to environmental conditions as well as low stability during storage, which considerably limit efficiency and the practical ability to reuse the laccase (Zheng et al., 2016). In this context, particular focus is devoted to the research on increasing the process efficiency and to the investigations into environmentally friendly technologies. Immobilized enzymes are a subject of great interest in industry, pharmacy, chemistry and biochemistry given their various advantages, such as their separability, the potential for repeated use, nontoxicity, biocompatibility, high activity and resistance to changing process conditions (Rafiee & Rezaee, 2021). Thus far, the results of the broad-scale research on increasing the process stability of enzymes through their immobilization on various types of carriers appear promising. Immobilization is a tool for improving such enzyme properties as operational stability, but it also enables repeated enzyme use in successive process cycles. The type of the employed carrier is of key significance in the immobilization process. The reason for this is that the carrier material has a significant influence on the properties of the produced enzymatic preparation. Stable and efficient carriers for enzyme immobilization encompass various materials. Carriers can be classified based on two main groups: organic and inorganic carriers. Natural and synthetic carriers can be found in both the aforementioned categories (Jafri et al., 2021). A broad review of immobilization carriers from the perspective of their characteristics, properties and practical application for the production of high-efficiency biocatalytic systems for use in various processes was prepared by Zdarta et al. (Zdarta et al., 2018). Among the presented carriers, the classic carrier group included biopolymers – the polymers of natural origin, such as alginate and chitosan. Biopolymers – sodium alginate and chitosan – were used as carriers for immobilization as part of the studies present herein as well. A combined carrier was also prepared based on the above – alginate-chitosan. The next step was to determine the enzymatic activity of the prepared alginate, chitosan and alginate-chitosan beads, as well as of the free enzyme.

Immobilization results in a loss of enzymatic activity relative to the activity of native enzymes. Enzyme activity after immobilization in the case of alginate beads was about 3 times lower relative to the activity of the enzyme in the native form (IY = 33.6%). The alginate-chitosan beads were characterized by the highest activity, with an obtained immobilization yield of about 77% (Figure 2). However, during the conducted tests, they sustained fractures and damage too easily. It is

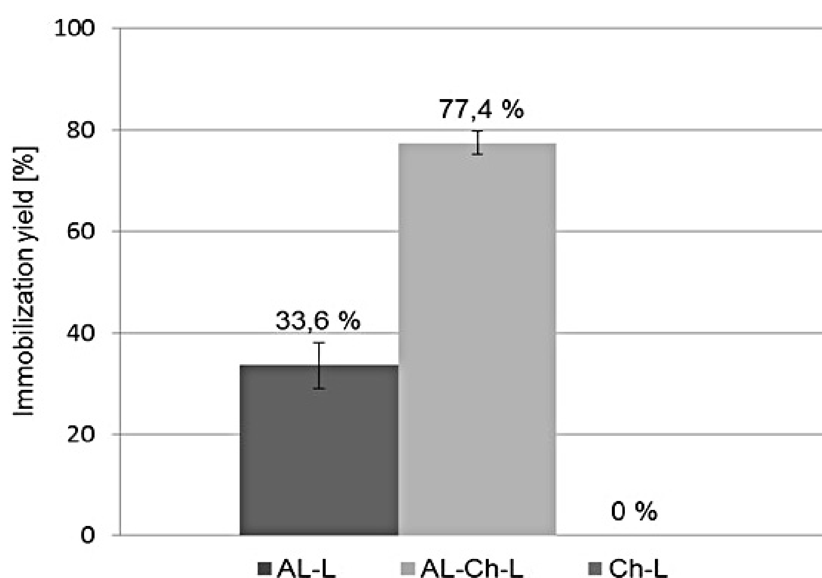

Figure 2. Immobilization yield of alginate-laccase (AL-L), chitosan-laccase (Ch-L) and alginate-chitosan-laccase (AL-Ch-L) beads

therefore recommended to conduct further testing to optimize the bead production process using a combined carrier based on sodium alginate and chitosan, in order to ensure the appropriate mechanical properties of the enzymatic preparations, while simultaneously retaining their desired activity. Testing revealed no activity of enzymes immobilized in a chitosan carrier. This may be the consequence of the spatial limitation of substrate availability, as a result of the stable, dense cross-linking of chitosan.

Enzyme immobilization leads to increased operational stability by securing the enzymatic proteins from the influence of unfavourable process conditions (e.g. pH, temperature). It also enables the repeated use of the biocatalyst, facilitating its recovery from the process mixture. The potential use of immobilized enzymes (including laccases) in industrial processes in various branches of industry has become the subject of particular interest in recent time (Bilal et al., 2019). Enzyme immobilization results in lower affinity for the substrate, as a consequence of the changing conformation of the enzymatic protein, and because of the lower availability of its active centre and the functional group modification in the catalytic centre, but may also be the result of the internal resistance of the diffuse mass transfer towards/from the active centre. The reasoning above has been confirmed by numerous findings in the literature regarding the analysis of the kinetics of enzymatic conversion by free and immobilized laccase, where it was demonstrated that immobilized enzymes have a greater K_m value (Brugnari et al., 2020; Ratanapongleka & Punbut, 2018; Mohammadi et al., 2018). On the other hand, lower affinity is associated with longer action, which in turn may result in more stable operational conditions of reactors with immobilized enzymes and a greater resistance to substrate concentration variations compared to free laccases. The tests presented herein have demonstrated that immobilized enzymes remain active for longer than free enzymes. This is important information in the context of the possibility of the practical application of immobilized enzymes in removing hard to degrade contaminants, including dyes. The rapid degradation of such compounds may result in the generation of metabolites characterized by greater toxicity compared to the degraded compounds themselves. Therefore, an extension of the reaction time can potentially make it possible to also degrade intermediate metabolites into simple and harmless end products. This was confirmed in the studies by Gahlout et al. (2017), who demonstrated that 3 intermediate metabolites with low molecular weight were generated during the biodegradation of azo dyes using immobilized laccases, such as 3-chloro-4, 5-dihydroxybenzene sulphonate, sodium-4-amino-5-hydroxyl-naphthalene-2, 7-disulphonate and sodium-3-(4-chloro-1, 3, 5-triazine-2-ylamine) benzene sulphonate. Further degradation of sodium-3-(4-chloro-1, 3, 5 triazine-2-ylamine) benzene sulphonate led to the generation of further reaction products, such as sodium 3-amino-4-hydroxyl benzene sulphonate and 4-chloro-1, 3, 5-triazine-2-ol. The authors conducted the toxicity assessment of dyes and intermediate metabolites by inspecting the zones of bacterial growth inhibition of *Rhizobium radiobacter, Azotobacter sp., Bacillus subtilis, Streptococcus aureus, Streptococcus typhi* and *Escherichia coli*. The dye degradation reaction metabolites demonstrated no growth inhibition of *R. radiobacter* and *Azotobacter sp.* They also exhibited a lower growth inhibition of *B. subtilis, E. coli* and *S. typhi*. However, in the case of the *S. aureus* bacterium, the inhibition of pure dyes and degraded metabolites was similar (Gahlout et al., 2017).

Dye absorption on enzyme-free polymer beads

The absorption level of a dye (methylene blue at a concentration of 25 mg/L) on empty (enzyme-free) polymer beads was assessed at temperatures of 30 and 40°C for 28 h. The pictures of polymer beads with no immobilized enzyme after a 28-h dye absorption test at temperatures of 30 and 40°C are presented in Table 3. After 28 h, the chitosan beads exhibited the lowest dye absorption level – 29.7% at a temperature of 30°C and 41.7% at a temperature of 40°C. Such a situation is most likely the result of the dense structure of

Table 3. Polymer beads with no immobilized enzyme after a 28-h dye absorption test at temperatures of 30 and 40°C

Temperature [°C]	AL	AL-Ch	Ch
30			
40			

the crosslinked polymer, which would simultaneously confirm that the chitosan beads were characterized by the greatest hardness and no enzymatic activity (Figure 3).

An increase in temperature by 10°C resulted in over 10% greater dye absorption by the chitosan beads. At a temperature of 30°C, the absorption was comparable among the alginate and alginate-chitosan beads (about 55%). However, at a higher temperature, the absorption level of the combined carrier-based beads was slightly lower (by 4.7%) relative to the AL beads. Increased temperature resulted in lower dye absorption for both the AL and the AL-Ch beads, which may be related to the influence of temperature on the chemical structure of the beads, which becomes less dense as a result. On the other hand, the situation was opposite in the case of the chitosan beads, and a temperature increase of 10°C resulted in an absorption increase of about 10%. An explanation for such a situation is that at a temperature of 30°C, the chitosan bead structure is dense enough to absorb a minor amount of the dye. An increase in temperature, however, results in a loosening of the structure and enables the absorption of a greater amount of the dye by the beads. The obtained results presented above constitute important knowledge with regard to the potential use of enzymatic beads in decolourization processes, as it potentially enables two ways of dye decolourization: sorption on the carrier material and through the enzymatic method, using immobilized enzymes. To date, the decolourization research devoted the most attention specifically to adsorption processes by seeking inexpensive and efficient adsorbents (Singh et. al., 2017; Wong et al., 2020).

Influence of environmental parameters (pH and temperature) on the activity of immobilized and free enzymes

The next stage of the studies involved determining the influence of environmental parameters (pH and temperature) on the activity of immobilized laccase (AL-L) and the enzyme in the native form (FL). Enzyme activity, as a function of pH, was determined using phosphate-citrate (pH 3), acetate (pH 5) and phosphate-citrate (pH 7) buffers at a temperature of 21°C for 15 min. Testing demonstrated that an enzyme exhibits the greatest activity at pH 3 in both the native and the immobilized form (Figure 4). An increase in pH influences the decrease in enzyme activity. In the case of the native form, increasing pH to 5 results in an activity loss as high as about 55%. The enzyme in the native form exhibits only slight activity at pH 7. Testing shows that immobilization has an influence on the increase in enzyme resistance to external environment variations. Increasing pH to 5 (Figure 5 a)) results in a significantly lower reduction in enzymatic activity (by 18%) in the case of an immobilized enzyme, relative to free laccase. At pH 7, the enzymes in both the native and the immobilized form exhibit very low enzymatic activity. Furthermore, testing shows that at pH 7, the ABTS oxidation occurs only inside the enzymatic bead (bead interior coloured green) (Figure 5 b)).Laccase is characterized by a variable charge, depending on pH. At low pH, the enzyme molecules typically have a positive charge, and the distribution of the charge on the enzyme molecule surface is uniform. At higher pH, the enzyme molecules have a negative charge (Drozd et al., 2018). On the other hand, alginate

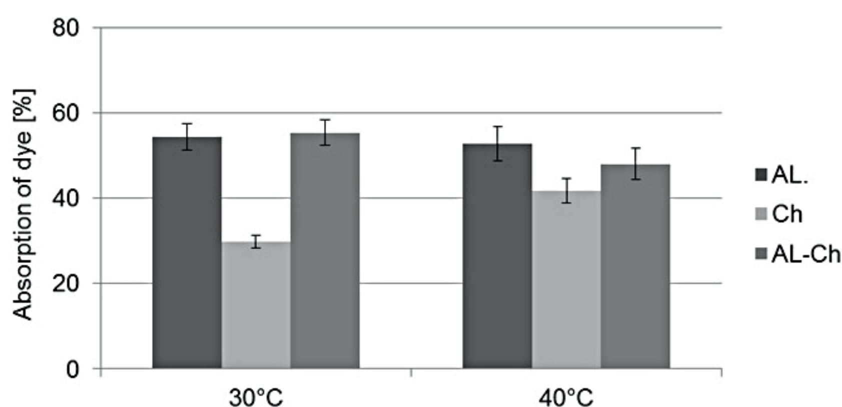

Figure 3. Dye absorption by polymer beads (AL – alginate beads; Ch – chitosan beads; AL-Ch – alginate-chitosan beads)

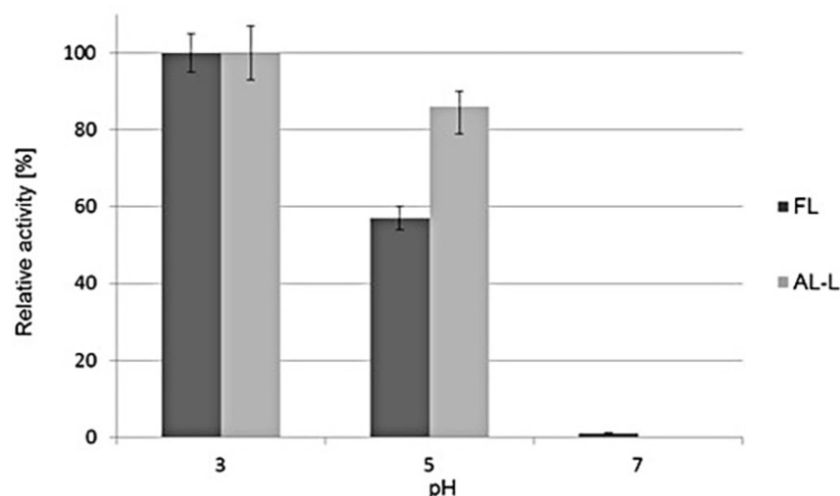

Figure 4. Effect of pH on the activity of FL and AL-L (FL – free laccase, AL–L alginate laccase bead)

fibres in aqueous solutions are characterized by a negative charge, resulting from the interaction of the microfibres with water molecules and the presence of a group of single OH bonds on the fibre surface. Therefore, a lower pH improves the immobilization yield of laccase in an alginate carrier due to the mutual interactions of the electrostatic charges of the laccase and alginate fibres. The studies by Sampaio et al. (2016) demonstrated that polymer microfibres exhibit greater negative zeta potential together with the increase in pH. This may result in gradient variations of the OH- and H+ ions present in the vicinity of the enzyme adsorbed on the carrier. High pH accelerates the bonding process of the OH- ions with the trinuclear copper cluster (T2/T3) located in the laccase active centre, which inhibits the enzyme activity due to the interruptions in electron transfer (Sampaio et al., 2016; Saoudi & Ghaouar, 2019). The effect of temperature on the activity of free and immobilized laccase was examined by measuring the activity of a free enzyme dissolved in distilled water after prior incubation (15 min) at temperatures of 21, 25, 30, 40, 50 and 60°C. The enzyme in the native form exhibited the highest activity at a temperature of 30°C. At room temperature, the enzymatic activity was at 71% of the maximum activity, whereas incubating the free enzyme for 15 min at a temperature of 60°C resulted in a loss of activity by about 40%, relative to the maximum activity (Figure 6).

Interesting test results were also obtained for the immobilized laccase activity measurements under variable temperature conditions. Hydrated beads were incubated in distilled water for 15 minutes at temperatures of 21, 25, 30, 40, 50 and 60°C. A decrease in the enzymatic activity of immobilized

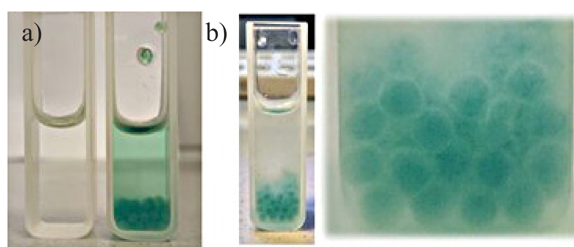

Figure 5. ABTS oxidation by enzyme-containing alginate beads at pH 5 a) and at pH 7 b)

laccase was observed together with the increase in temperature. Bead pre-incubation in distilled water most likely resulted in a change to the bead structure and a release of the enzyme to the liquid, which underwent degradation under increased temperature conditions (Figure 7). The studies by Taqieddin and Amiji (2004) demonstrated that storing the beads in citrate and phosphate buffers accelerates the process of enzyme leaching from the beads (Taqieddin & Amiji, 2004). Furthermore, the studies by Lu et al. (2007) revealed that the process of enzyme leaching from the beads varies under different pH conditions. The greater the pH, the more rapid the leaching process becomes due to the structural changes and increased diffusivity of sodium alginate.

Influence of bead storage conditions on enzymatic activity

The industrial use of enzymatic beads will require their periodic storage. The tests of the influence of storage conditions on variations in bead enzymatic activity were carried out to determine

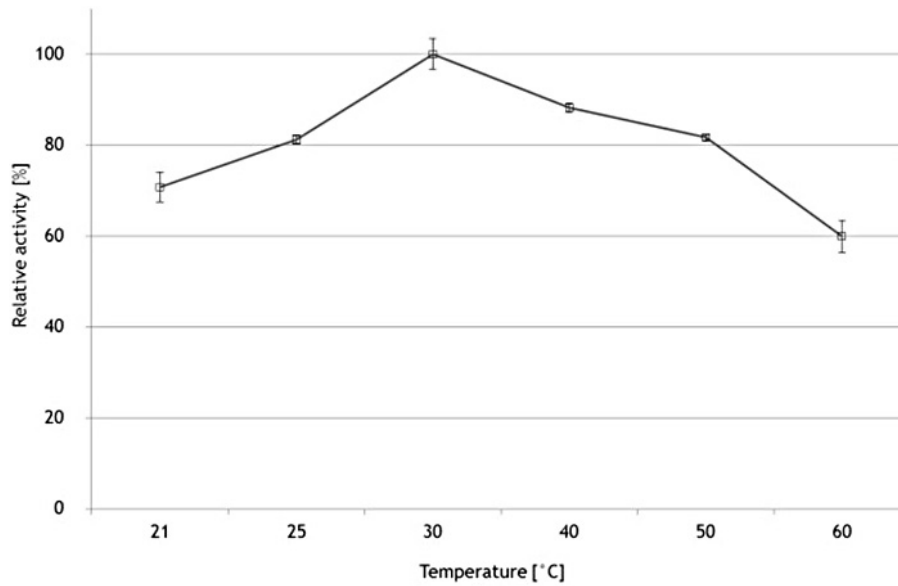

Figure 6. Effect of temperature on the activity of free laccase (FL)

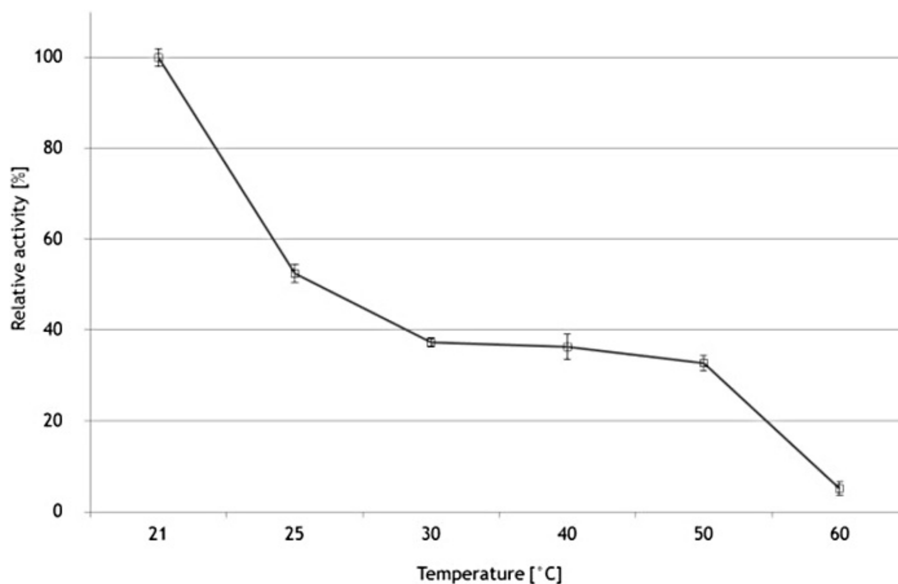

Figure 7. Decrease in immobilized laccase (AL-L) activity under increased temperature conditions

the stability during storage. The enzymatic beads were stored at temperatures of 21°C, 4°C and -10°C. Testing demonstrated that the best bead storage method is to store them with no water carrier and no air access (Figure 8–9). Regardless of the storage temperature, a decrease in the laccase activity was observed in the first 3–6 days of storage. In the case of the beads stored with no water carrier, the greatest loss in activity, as high as by 75%, was observed in the beads stored at a temperature of -10°C, and the highest activity loss occurred on the first day (66%), whereas on the 21st day of the experiment, they retained only 15% of their original activity. Storing the beads at a temperature of 21°C resulted in a loss in activity by about 38%, whereas a storage temperature of 4°C resulted in a loss by about 16% (Figure 8).

A rapid loss in the enzymatic activity of laccases immobilized in alginate beads and stored in a water carrier was observed on the first day of the process (Figure 9). In the case of a storage temperature of -10°C, the loss in activity on the 1st day was as high as 80%, whereas at temperatures of 4°C and 21°C, the loss in activity was about

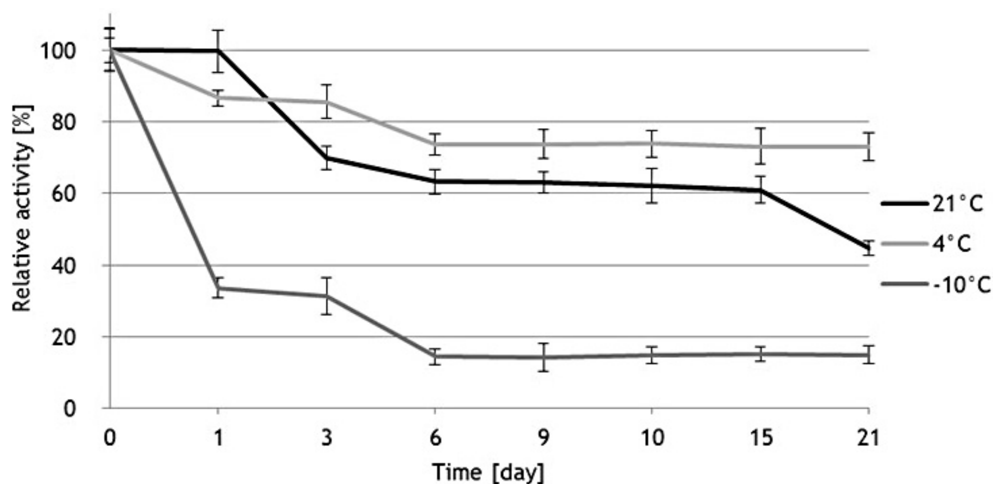

Figure 8. Alginate bead enzymatic activity during storage in parafilm-sealed Petri dishes for 21 days

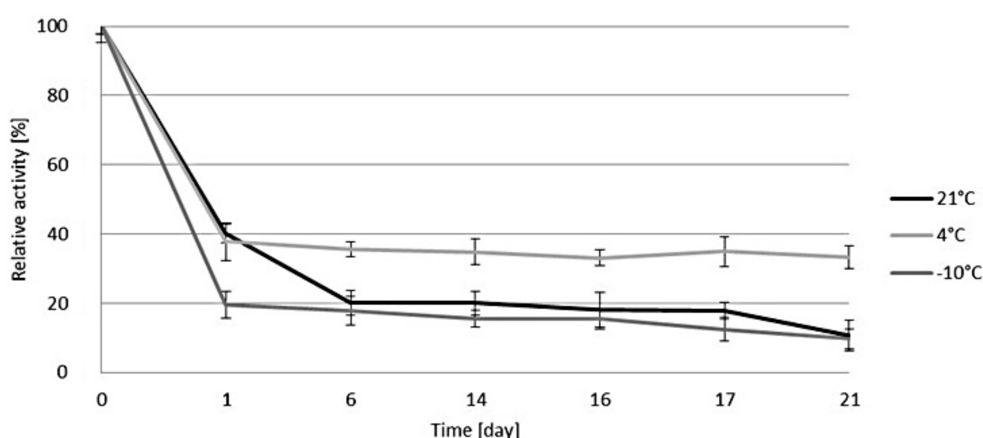

Figure 9. Alginate bead enzymatic activity during storage in water for 21 days

60%. A further loss in the enzymatic activity of beads stored at a temperature of 21°C was observed over the next days, as a result of enzymes leaching from the beads and being released to the water medium. In the case of a temperature of 4°C, no significant extended loss of laccase enzymatic activity was observed – the activity loss was about 4% over the next days.

Alginate bead activity after low-temperature drying

An assessment of the influence of slow low-temperature drying on the catalytic activity of immobilized enzymatic preparations was carried out as well. For this purpose, the enzymatic activity of wet beads was inspected immediately after their preparation. Afterwards, the beads were dried for 5 h at a temperature of 30°C. After drying, the beads exhibited a less regular, oblate shape, and were characterized by a beige-yellow colour as well as very high hardness (Figure 10).

After 5 h of drying at a temperature of 30°C, the bead activity nearly doubled, relative to the wet beads. This demonstrates that low temperature drying may be a promising method for preparing the enzymatic beads for further storage as well as for maximizing their efficiency.

CONCLUSIONS

As part of the investigated immobilization methods using various carriers, the dropwise method using sodium alginate (2%) proved to be the most effective technique of laccase immobilization. The tests of the influence of environmental parameters (pH and temperature) made it

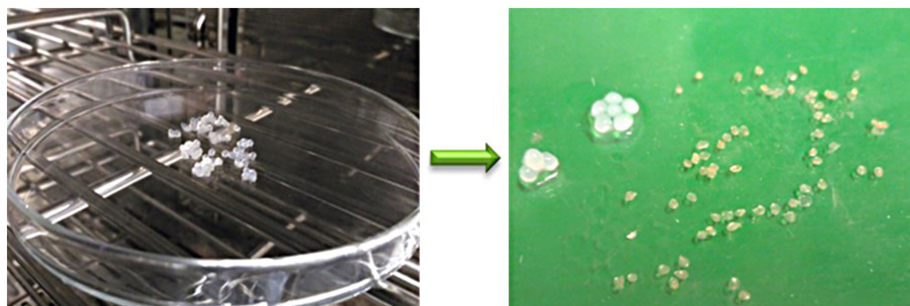

Figure 10. Alginate beads after low-temperature drying

possible to optimize the conditions of enzymatic bead action to achieve optimal activity. Despite the fact that the laccase after immobilization exhibited lower activity than free laccase, it was demonstrated that the immobilization process has an influence on the increase in enzyme stability under the conditions of variable pH. The studies showed that enzymatic bead damage or alginate cross-linking structure variation occurs under increased temperature conditions, which may result in the release of the enzyme to the liquid and consequently the rapid loss of its activity. The beneficial influence of immobilized biocatalyst drying was demonstrated. Preparation immobilization and drying is a promising method for improving enzyme stability in dye degradation processes utilizing biocatalysts, and it also enables the repeated use of the immobilized preparations, which is extremely significant with regard to the possibility of the continued practical application of immobilized laccases. The information obtained as part of this study offers a valuable contribution to the future research on the possibility of using the prepared alginate beads to remove colour contamination from wastewater.

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