Archives of Environmental Protection
Vol. 50 no. 1 pp. 45–55

PL ISSN 2083-4772
DOI:10.24425/aep.2024.149431

Alginate-laccase beads in the decolourization of indigo carmine

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Keywords: immobilization, sodium alginate, indigo carmine, decolourization, laccase

Abstract: The aim of the study was to assess the feasibility of utilizing sodium alginate biopolymer as an immobilization carrier for laccase in the removal of indigo carmine (IC), an anionic dye. The main goal of this work was to optimize the decolourization process by selecting the appropriate immobilized enzyme dose per 1 mg of dye, as well as the process temperature. The effective immobilization of laccase using sodium alginate as a carrier was confirmed by Raman spectroscopy. An analysis of the size and geometric parameters of the alginate beads was also carried out. Tests of IC decolourization using alginate-laccase beads were conducted. Applying the most effective dose of the enzyme (320 mg of enzyme/1 mg of IC) made it possible to remove 92.5% of the dye over 40 days. The optimal temperature for the IC decolourization process, using laccase immobilized on sodium alginate, was established at 30-40ºC. The obtained results indicate that laccase from *Trametes versicolor* immobilized on sodium alginate was capable of decolourizing the tested dye primarily based on mechanism of biocatalysis.

Introduction

Indigo carmine (IC) is a synthetic blue dye belonging to the group of acidic dyes. Of the total global production of dyes, 20,000 tonnes/year are indigo derivatives. IC is widely used in the textile industry for dying clothing, primarily jeans and jackets, as well as other products made from blue denim. It is considered a highly toxic dye from the indigo class that provokes skin irritation, permanent corneal damage, and digestive tract irritation by nausea, diarrhea, and vomiting. IC is considered as pollutant that must be treated and/or removed before its discharge into the environment together with wastewater (Ahlawat et al. 2022, Leonties et al. 2022, Mohan et al. 2022).

A variety of physical, chemical and biological methods are employed to eliminate synthetic dyes from wastewater. Coagulation, adsorption, chemical oxidation, membrane processes, ion exchange and advanced oxidation processes (AOPs) are among the physical and chemical techniques most commonly referenced in the literature (Kishor et al. 2021, Kuśmierek et al. 2023). However, these methods can be quite expensive and may result in the accumulation of substantial amounts of sludge or sediment as well as additional pollution caused by chemical agents. Within the field of colored wastewater treatment, the commonly used biological methods include activated sludge processes and biological filters. However, the presence of dyes in wastewater leads to significant issues, such as activated sludge swelling, microorganism cell deformation and reduced biodiversity. Despite these drawbacks, biological methods that use microorganisms for dye removal offer a comprehensive approach to the problem (Rane and Joshi 2021). While these methods have some limitations compared to physicochemical approaches, they are cost-effective, exhibit low energy consumption, produce minimal waste and require less water than other procedures. Moreover, they are environmentally friendly, especially when dealing with non-toxic by-products generated by biodegradation processes (Deska and Zawadzki 2021).

Bioremediation, specifically through biocatalysis, has recently emerged as a promising research area, and an increasing trend in the use of biocatalysts in various scientific and technological fields is now becoming apparent. Due to their unique catalytic and physicochemical properties, biocatalysts can be utilized as "green catalysts" in intricate technological processes across diverse industrial sectors (Deska and Kończak 2019). Nevertheless, the relatively low stability of free enzyme proteins and their sensitivity to varying reaction and environmental conditions highlight the need for developing new techniques to enhance their properties.

Recent research has been focused on immobilizing biocatalysts for use in biotechnological processes, and one promising application of immobilized biocatalysts is to employ them in the processes of dye removal from wastewater. The most commonly used enzyme for colored pollutant removal is laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2), which belongs to the large family of multicopper oxidases (MCOs). The extensive number of scientific publications regarding it not only indicates the interest in the production of laccase, but also in the development of immobilization methods for the industrial applications of these biocatalysts (Alvarado-Ramírez et al. 2021). The most frequently cited applications of laccase in biotechnological processes include its use in the degradation of dyes and other environmental pollutants (phenolic compounds, pesticides, pharmaceuticals, personal care product (PCP) ingredients), in the textile industry, in biosensors, food production, the pulp and paper industry, and in polymer synthesis processes (Bilal et al. 2019, Neha et al. 2022, Zhou et al. 2021). The application of laccase in industrial processes is considered environmentally feasible and aligns with the concept of circular economy, following the principles of "reduce-reuse-recycle".

Enzymes in their native form face limitations in industrial applications due to sensitivity to variable environmental conditions of process, such as pH and temperature. It can lead to a reduction or complete loss of catalytic activity. Moreover, the use of laccase in its native form makes it unable to be recovered and reused. To overcome these limitations, there has been a surge in research to enhance the enzymes' resilience to variable process conditions. One promising technique that has emerged is the immobilization of enzymes on diverse carriers (Daâssi et al. 2014, Zhou et al. 2021). Among the carriers of interest, biopolymers, specifically alginates, have shown promise (Deska and Kończak 2020).

Sodium alginate, a salt of alginic acid, is naturally found in the cell walls of marine brown algae (Phaeophyceae) and some species of bacteria. It is commercially extracted from various algae species. Sodium alginate was first discovered in bladderwrack (kelp) in 1883 and has been extensively studied by researchers since then (Hurtado et al. 2022, Marszałek 2022). Alginate is a linear polysaccharide composed of two types of monomers – beta-D mannuronic acid (ManAp or M block) and alpha-L-guluronic acid (GulAp or G block) – linked by glycosidic bonds that can be arranged in different patterns along the chain. The quantity of each monomer in the molecule, as well as the length of each G G M M G block, depends on the type of algae and the tissues from which the polymer was extracted (Ching et al. 2017).

Alginate is considered one of the best carriers for biocatalyst immobilization, which is due to such factors as the presence of carboxyl groups, its hydrophilicity, natural origin, relatively good mechanical strength as well as the inexpensive and simple procedure for obtaining a gel that is non-toxic and gentle to the immobilized enzyme. Typically, after biomolecules are immobilized in the alginate, there are no covalent bonds between the enzyme and the substrate, and therefore the functional groups of the matrix and the enzyme interact based on relatively weak ionic bonds or adsorptive forces. Since interference with the enzyme structures is strongly limited, immobilized enzymes usually retain most of their catalytic properties. On the other hand, the formation of relatively weak interactions can lead to the enzyme leaching from the matrix, resulting in a decrease in the biocatalytic activity of the generated system. Additionally, poorer catalytic properties may be related to diffusion limitations in the transport of substrates and products through the alginate layer (Zdarta et al. 2018). When selecting a method for immobilizing enzymatic proteins, it is important to consider various factors such as the enzyme-substrate/product interaction, enzyme-carrier interaction, substrate/product-carrier interaction, and the intended technological process for the immobilized enzymes (Deska and Kończak 2022a).

Recent research by Deska and Kończak (2022b) suggests that biopolymers, specifically alginates, are promising compounds in the context of laccase immobilization for the removal of dyes from wastewater. Therefore, this study aims to evaluate the potential of applying sodium alginate as a support in laccase immobilization for decolourization processes, using indigo carmine as the tested dye. Literature reports reference two possible ways of removing the dye by means of enzymes immobilized on biopolymers – sorption and/or biotransformation/biocatalysis (Daâssi et al. 2013, Rodriguez-Couto and Herrera 2006). The novelty of the present study is to identify the mechanism of indigo carmine (anionic dye) decolourization by immobilized laccase and to optimize process conditions as well as the determination of the geometrical parameters of the beads and the confirmation of laccase immobilization by Raman spectroscopy. The acquired knowledge will be useful for further research on the application of immobilized laccase in color wastewater decolourization process.

Materials and methods

Materials

Enzyme

Laccase (EC 1.10.3.2) from *Trametes versicolor* (CAS Number: 80498-15-3, appearance - color: light brown; form: powder; solubility - color: very light brown, turbidity: clear; batch number: BCCJ3036, BCBX0087) was purchased from Sigma Aldrich (Sigma Aldrich, Poznań, Poland). *Trametes versicolor* and *Pleurotus ostreatus* are frequently referenced in recent research on laccases and can be considered model organisms in basic and applied research for different purposes concerning the use of laccase.

Chemicals

Sodium alginate was used as a carrier in the study. The alginic acid sodium salt from brown algae was purchased from Sigma Aldrich (Sigma Aldrich, Poznań, Poland).

Calcium chloride (anhydrous, granular, ≤ 93%) was obtained from Sigma Aldrich, Poznań, Poland. "Indigo carmine was sourced from Chempur, Bytom, Poland."

Methods

Biopolymer bead formation – Alginate-Laccase beads (ALe)

The methodology used for preparing alginate beads with immobilized laccase from *Trametes versicolor* (ALe) was based on a slightly modified approach, combining elements from the methods of Niladevi and Prema (2007) and Deska and Kończak (2022b). Sodium alginate concentrations of 2%

(w/v) were employed, a determination made in preliminary studies (Deska and Kończak 2022b). The preparation of beads with appropriate permeability and rigidity for enzyme gel entrapment was based on the concentration of the sodium alginate and the ability of the calcium ions to cross-link with sodium alginate as well as the amount of the enzyme. Sodium alginate was mixed with distilled water and stirred continuously for 20 minutes at 21°C until complete dispersion. The resulting solution was set aside for 20 minutes at room temperature to eliminate any air bubbles generated by mixing. In this study, 400 µl of the laccase enzyme solution, with a concentration of 200 mg/ml, was mixed with 3000 µl of the sodium alginate solution (2.0% w/v). The enzyme doses used in the decolourization process were determined based on prior studies optimizing the indigo carmine decolourization process (unpublished data). The enzyme was dissolved in distilled water. Alginate-laccase beads were prepared by introducing the enzyme-containing sodium alginate solution into the cross-linking solution of 2% $CaCl_2$ (w/v) through a 0.45 mm diameter dispensing needle. Drops of the sodium alginate solution with the enzyme were added to the continuously stirred cross-linking solution at a rate of 70 rpm, maintaining a distance of 2 cm from the dispensing needle to the cross-linking solution surface. The produced alginate-laccase beads were left to harden in the $CaCl_2$ cross-linking solution for 60 minutes. Subsequently, they were recovered by decantation, rinsed three times with distilled water, and filtered through a sieve to remove excess water. The beads were then immediately used for further research. The alginate-laccase beads (ALe) are presented in the figure below (Figure 1).

The alginate beads without the enzyme (AL) were produced using the same methodology as described for alginate-laccase bead (ALe) preparation above, with the only difference being that the introduced solution did not contain laccase.

Polymer bead size, shape and chemical composition analysis

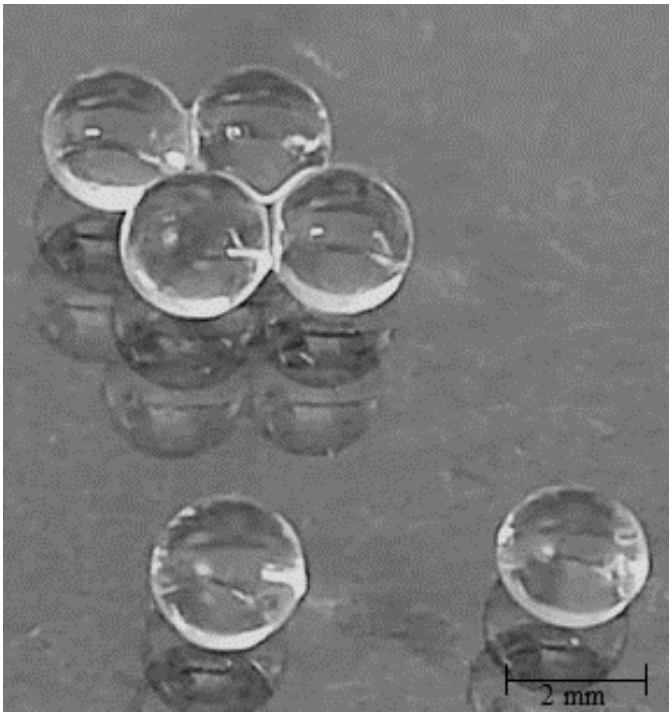

Figure 1. Alginate-laccase beads (ALe)

Polymer bead size, shape, and chemical composition analysis were performed using an optical particle size and shape analyzer coupled with a Raman spectrometer (Morphologi G3S-ID, Malvern, UK). Raman spectra were recorded with a Kaiser Optical System at a wavelength of 785 nm (Rockwell Collins Company, USA) and the spectral range 150 cm^{-1} to 1850 cm^{-1} with a 4 cm^{-1} spectral resolution. Raman spectra were registered for the AL and ALe alginate beads to confirm the enzyme presence in the beads, both on their surface and in their cross-section. The registered spectra were compared to the spectrum of the reference substance, i.e., the enzyme – the laccase (E) from *Trametes versicolor.*

The bead size and shape were immediately inspected after preparation to assess the influence of laccase presence and drying time on the beads' geometric parameters. After removal from the distilled water, the beads were immediately placed on a glass plate (ambient temperature of 22°C±1°C, humidity of 51.0%). The analysis was performed for 5 randomly selected beads, with measurement repeated at intervals of 10, 20, 30, 40, and 60 min., as well as after 5 days (when the beads were dry). Both beads with the immobilized enzyme (ALe) as well as empty beads (AL) were included in the analyses.

The determined parameters characterizing size and shape included: *i*) length (L), defined as the distance between two points on the circumference with the greatest length among all the possible projections on the major axis; *ii*) width (W), defined as the distance between two points on the circumference with the lowest length among all possible projections on the major axis; *iii*) aspect ratio (AR), defined as the proportion of length to width; *iv*) circularity (C), defined as the proportion of the circumference of a circle with an area identical to a given particle to the particle's actual circumference. The circularity parameter ranges from 0 to 1, where 1 denotes a perfect circle.

Decolourization tests

The dye solutions for the decolourization process were prepared using distilled water. The decolourization tests were conducted in 50 ml glass bottles at a temperature of 21°C (main decolourization experiment). Additional experiments were conducted at temperatures of 21°C, 30°C and 40°C to evaluate the influence of temperature on the decolourization efficiency. For the decolourization experiment to determine the effective dose of the enzyme, tests were carried out for 40 days with the following enzyme (E) doses applied in the IC decolourization tests: 20 mg E/mg IC, 40 mg E/mg IC and 320 mg E/mg IC. The experiment to assess the effect of temperature on decolourization efficiency was conducted for 20 days and the following enzyme (E) dose was applied: 320 mg E/mg IC. The concentration of the IC solution was 5 mg/l, determined based on prior studies on decolourization process optimization by the authors – unpublished data. Dye decolourization was calculated by monitoring absorbance changes at the maximum absorbance wavelengths for indigo carmine (610 nm). The decolourization values were expressed as decolourization % = $(A_0-A_t)/ (A_0 \times 100)$, where A_0 is the initial absorbance of the reaction mixture, and A_t is the absorbance after an incubation time. All the results obtained in the conducted experiments were expressed as mean values of the three replicates with standard deviation.

Statistical analysis

Statistical analysis of the experimental data was performed using Statistica 13.3 (StatSoft, Kraków, Poland) and Jamovi 2.3.28 (open-source software available under the AGPL3 license). Group comparisons were made using analysis of variance (ANOVA) and the post-hoc Bonferroni test (parametric test) or the Kruskal-Wallis test and the DSCF method (non-parametric one-way ANOVA). A probability level of p under 0.05 was considered statistically significant.

Results and discussion

Biopolymer bead analysis by Raman spectroscopy

Previous studies conducted by the authors focused on assessing the use of various carriers, including sodium alginate, chitosan, and combined alginate-chitosan biopolymers for laccase immobilization. The results showed that the dropping method using sodium alginate (2%) proved to be the most effective technique for enzyme immobilization (Deska and Kończak 2022b).). In this study, to confirm the effective immobilization of laccase using alginic acid as a carrier, biopolymer bead analysis by Raman spectroscopy was carried out.

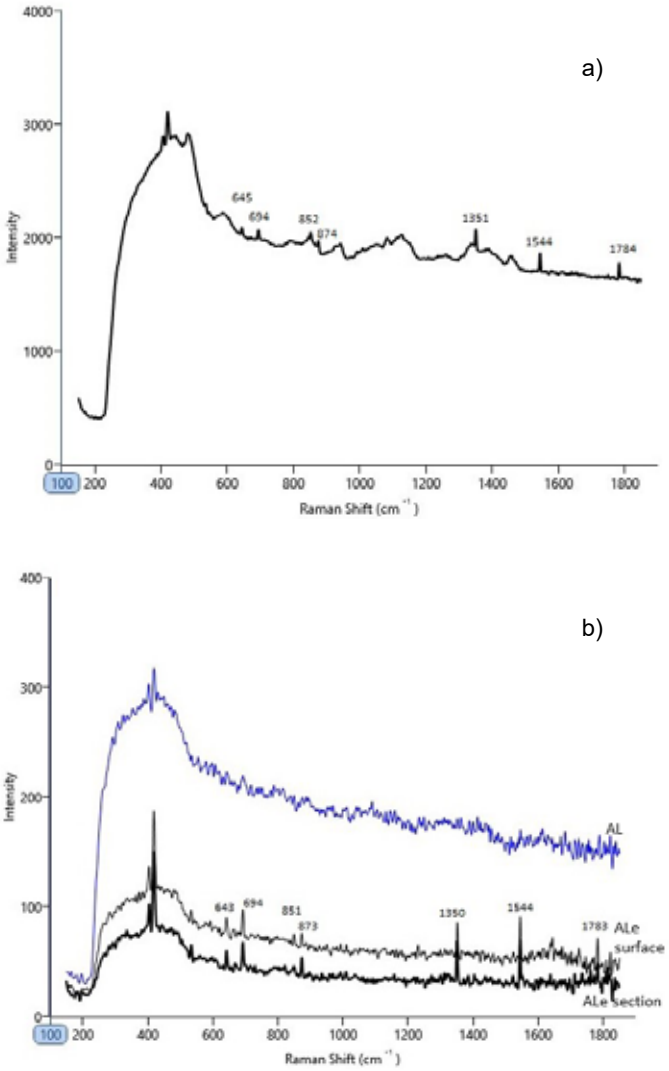

Figure 2. Raman spectra registered for:
a) the laccase enzyme (powder) (E), exposure time 10 s,
b) alginate beads with no enzyme (AL) and the alginate-laccase bead surface (ALe surface) and cross-section (ALe section), exposure time 5 s.

Denotations: bead with no enzyme (AL), bead with an immobilized enzyme – measurement on the bead surface (ALe surface), bead with an immobilized enzyme – measurement over the bead cross-section (ALe section)

Figure 2 presents example Raman spectra registered for the tested samples. Characteristic peaks were identified in the spectrum of the reference substance E at 645 cm^{-1}, 694 cm^{-1}, 852 cm^{-1}, 874 cm^{-1}, 1351 cm^{-1}, 1544 cm^{-1} and 1784 cm^{-1}. These peaks also occurred in spectra registered for ALe beads, but they were absent in the spectrum registered for the AL bead, confirming the presence of the laccase enzyme in the alginate-laccase beads prepared for further studies, both on the surface and in the cross-section of the bead.

Klis et al. (2005) conducted research on laccase immobilized on a thiol-modified Au surface using Raman spectroscopy. The surface-enhanced resonance Raman scattering (SERRS) spectrum of the surface-bound laccase exhibited a general similarity to the resonance Raman (RR) spectrum of the enzyme sample, suggesting the retention of the structure of the "blue" active sites of copper in the immobilized enzyme. Almulaiky and Al-Harbi (2022) analyzed the immobilization of the polygalacturonase (PG) enzyme through a calcium alginate-coated polypyrrole/silver nanocomposite. The immobilized PG was assessed by FTIR, TGA, SEM, EDX as well as Raman spectroscopy. Krzyczmonik et al. (2023) applied Raman spectroscopy in their studies on developing an electrochemical biosensor using the laccase enzyme for polyphenol detection. They confirmed the presence of laccase on an electrode by identifying Raman bands attributed to pure laccase at 482 cm^{-1}, 580 cm^{-1}, 853 cm^{-1}, 935 cm^{-1}, 1121 cm^{-1}, 1350 cm^{-1}, 1337 cm^{-1}, 1386 cm^{-1}, 1456 cm^{-1} and 2906 cm^{-1} in the Raman spectrum of the electrode with this enzyme (Krzyczmonik et al. 2023).

Analysis of the alginate bead size and shape variation as a function of enzyme presence and drying time

An analysis of the size and geometric parameters of the alginate beads was also carried out. The figures below present the beads before and after drying (Figure 3) as well as the variations in parameters describing the studied bead size and shape as a function of drying time (Figure 4).

Immediately after preparation, the AL and ALe beads had an average length of 2400 μm and 2183 μm respectively. Consequently, the AL beads were therefore 9.9% longer on average compared to the ALe beads. Both types of beads, AL and ALe, exhibited a nearly spherical shape, indicated by the circularity parameter, averaging 0.99 and the aspect ratio (AR) parameter averaging 0.98. Upon drying, the AL and ALe beads exhibited an average length of 798 μm and 811 μm respectively. In this case, the ALe beads were approximately 2% longer on average than the AL beads. Additionally, the bead size changed after drying, reflected in the average AR parameter values of 0.86 for AL beads and 0.82 for Ale beads. After 5 days of natural drying, both types of beads underwent a considerable reduction in length, decreasing by over 60% (62.84% for the enzymatic beads and 66,75% for the non-enzymatic beads).

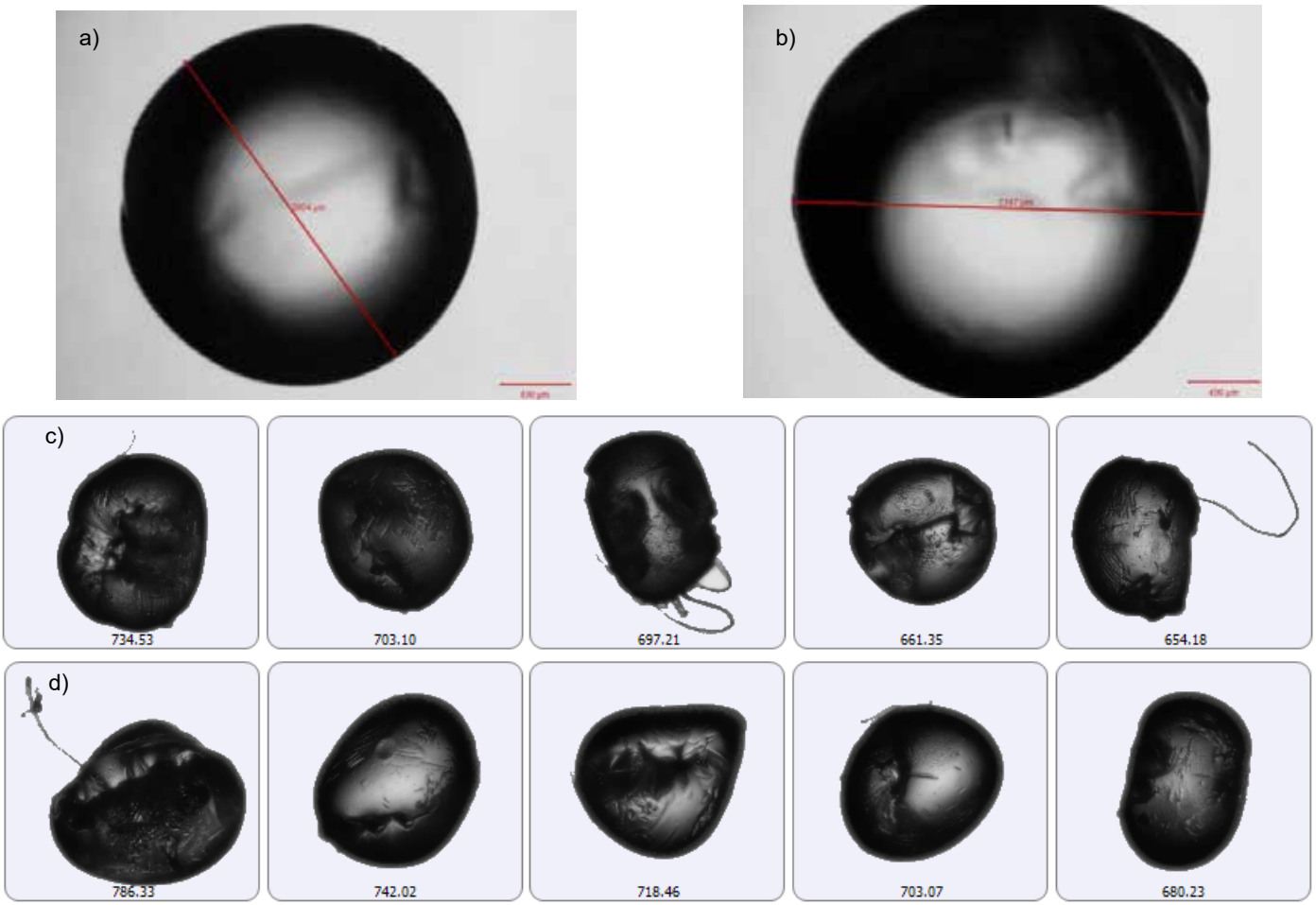

Figure 3. Bead pictures: a) ALe bead immediately after preparation, b) AL bead immediately after preparation, c) ALe beads after natural drying, d) AL beads after natural drying

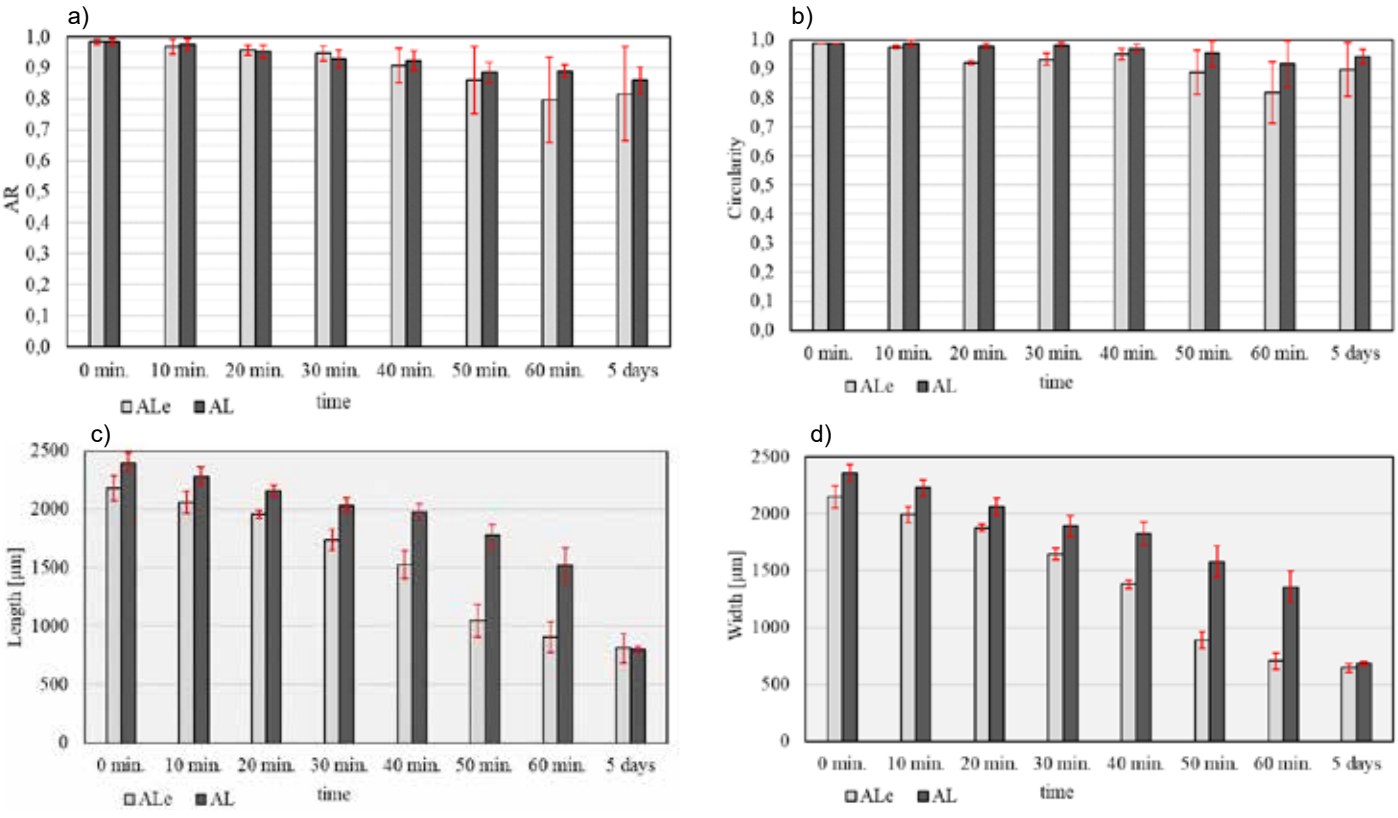

Figure 4. Geometric parameter variations of ALe and AL beads as a function of time: a) aspect ratio (AR), b) circularity, c) length, d) width (mean value with standard deviation)

Table 1. ANOVA results for hypothesis H1.

Variable	df	F	p	η^2
Presence of laccase (E)	1	77.42	<0,001	0.108
Drying time (t)	7	128.88	<0,001	0.811
E*t	7	138.54	<0,001	0.045

df – degrees of freedom, F – test statistic (variance of the group means/mean of the within group variances), p – probability level, η^2 – effect size

A hypothesis (H1) was formulated that the enzyme presence in the bead and the drying time have a significant influence on the bead size. The bead size was defined by the length parameter. The parametric test assumptions concerning homogeneity and normality were fulfilled. A two-way analysis of variance was used to verify the hypothesis, using a dataset of 2 (enzyme absence, enzyme presence) x 8 (drying time: 10 min, 20 min, 30 min, 40 min, 60 min, 5 days). The analysis of variance revealed that the evaluated variables, i.e., the presence of the enzyme and the drying time, differentiate the beads in terms of the dependent variable, i.e., the bead length (Table 1).

The next stage involved a post-hoc test with Bonferroni correction. Table 2 presents comparisons between selected groups. This served as the basis to conclude that the AL bead immediately after preparation (AL-0min) exhibits significant statistical differences in terms of size compared to the bead after a drying time of 40 min or more (Ale-40min). In the case of ALe beads, this dependence occurs already after 30 min of drying (Ale-30min). The AL bead exhibits statistical differences in terms of size relative to the ALe bead after 40 min, 50 min and 60 min of drying. After complete drying (5 days), the difference in size between the AL (AL-5day) and ALe (ALe-5day) beads is not statistically significant.

A hypothesis (H2) was formulated that the enzyme presence in the bead and the drying time have a significant influence on the bead shape. The bead shape was defined by the AR parameter. The parametric test assumptions concerning homogeneity and normality were not fulfilled, therefore the Kruskal-Wallis test was applied (Table 3). The analysis results indicated no statistically significant influence of the laccase presence ($p = 0.750$) as well as a statistically significant influence of the drying time ($p < 0.001$) on the bead shape. Further pairwise comparisons using the DSCF method demonstrated statistically significant differences in beads shape between the immediate post-preparation (0 min) and after a drying time of 30 min or more.

Influence of the enzyme dose on indigo carmine decolourization

Alginate gelling can be initiated by mixing sodium alginate and a calcium chloride solution. However, the preparation of beads with appropriate geometric parameters and strength depends on the sodium alginate concentration, the capability of the calcium ions to cross-link with the sodium alginate as well as the ratio of the enzyme to the alginate (E/A). In the paper by Deska and Kończak (2022b) beads with laccase immobilized on biopolymers, including sodium alginate, characterized by a spherical shape and the desired diameter as well as the appropriate mechanical properties were produced. The first stage of the studies concerning the possibility of using the enzymatic preparations thus developed in decolourization processes was to determine the effective enzyme dose required to remove the tested dye solution of IC, at room temperature (21°C). The following enzyme (E) doses were applied in the IC decolourization tests: 20 mg E/mg IC, 40 mg E/mg IC, and 320 mg E/mg IC. Such a selection of the enzyme doses was determined based on prior studies on decolourization process optimization as conducted by the authors (unpublished data). Applying the individual enzyme doses: 20 mg E/mg IC, 40 mg E/mg IC, and 320 mg E/mg IC made it possible to achieve dye removal at a level of 19.3%, 35.0% and 92.5% respectively, over 40 days at room temperature (21°C) (Figure 5a).

Table 2. Results of the post-hoc test with Bonferroni correction.

Group 1	Group 2	pBonferroni	Group 1	Group 2	pBonferroni
AL-0min	ALe-40min	<0.001	AL-0min	ALe-0min	1.000
	ALe-50min	<0.001	AL-10min	ALe-10min	0.912
	ALe-60min	<0.001	AL-20min	ALe-20min	1.000
	ALe-5day	<0.001	AL-30min	ALe-30min	0.079
ALe-0min	ALe-30min	<0.001	AL-40min	ALe-40min	0.001
	ALe-40min	<0.001	AL-50min	ALe-50min	0.001
	ALe-50min	<0.001	AL-60min	ALe-60min	0.001
	ALe-60min	<0.001	AL-5day	ALe-5day	1.000
	ALe-5day	<0.001			

Table 3. Kruskal-Wallis test results for hypothesis H2.

Variable	χ^2	df	p	ε^2
Presence of laccase (E)	0.101	1	0.750	0.002
Time (t)	37.4	7	<0.001	0.645

$\chi 2$ – distribution (approximates the distribution of test statistic H), df – degrees of freedom, p – probability level, ε^2 – effect size

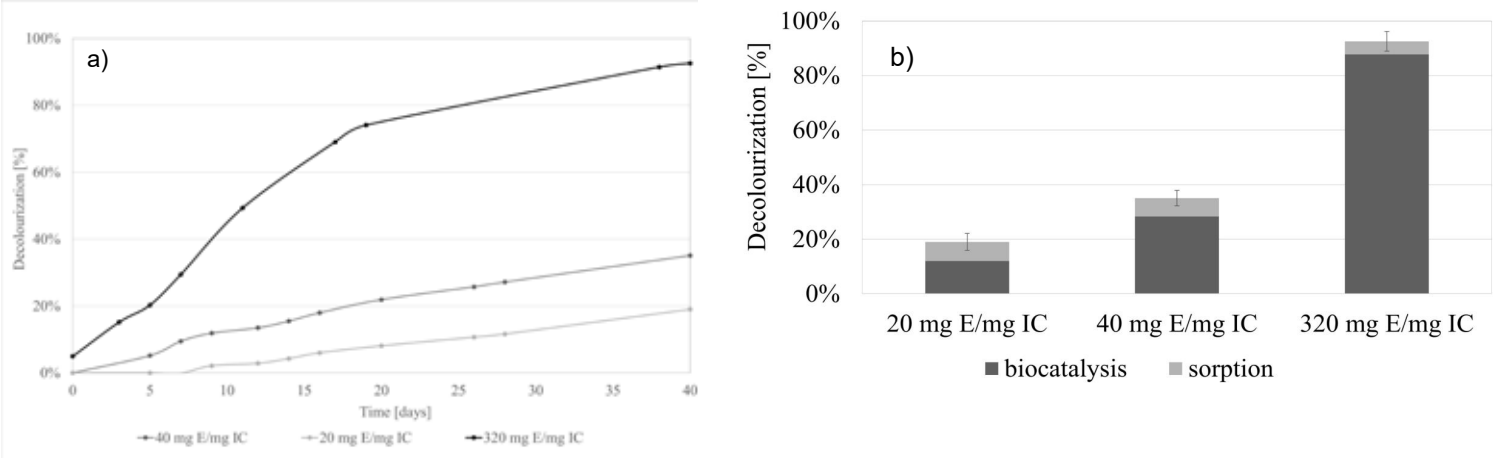

Figure 5. Decolourization efficiency by alginate-enzyme beads of a) different enzyme doses per 1 mg of indigo carmine (IC) dye over 40 days at 21ºC, b) with an identification of the dominant mechanism.

The results obtained reveal that, at the lowest tested enzyme dose, the maximum decolourization efficiency was approximately 19% This outcome could be attributed to an insufficient enzyme dose relative to the substrate, coupled with rapid enzyme saturation. Conversely, employing the highest enzyme dose led to decolourization efficiency of up to 92.5% over 40 days. To discern the primary decolourization mechanism for the IC dye (anionic dye), the decolourization process was conducted concurrently for beads with the immobilized enzyme as well as using beads without the enzyme. The findings indicate that biocatalysis is the predominant process in IC removal (Figure 5b). Notably, the higher the enzyme dose per 1 mg of IC, the more dominant biocatalysis became over sorption. At the lowest tested dose (20 mg E/mg IC), biocatalysis constituted 63.16% of the process, while for a dose of 320 mg E/mg IC, it approached 95%. Additionally, sorption coefficients were also calculated for the individual enzyme doses (Table 4).

The sorption coefficient decreases with an increase in the enzyme dose per 1 mg of IC. Rapid saturation of the enzyme with the dye occurs at low enzyme doses, leading to a higher degree of dye sorption on the alginate bead (sorption coefficient of 0.36 for 20 mg E/mg IC). At higher enzyme dose, the sorption coefficient is lower, equaling 0.05 for 320 mg E/mg IC, as the saturation of the enzyme with the dye is slower and the biocatalysis is the dominant mechanism during the indigo carmine decolourization. Studies by Daâssi et al. (2014) on anionic and cationic dye decolourization by means of *Coriolopsis gallica* laccase immobilized on alginates also confirmed that biocatalysis is the dominant mechanism for removing dyes of the anionic group (RBBR and RB5).

Wang et al. (2017) discovered that the efficiency of indigo carmine biocatalysis by laccase is primarily influenced by the conformational flexibility in the laccase active site. Adding reaction mediators affects the redox potential of the laccase and leads to a higher catalytic decolourization efficiency of indigo carmine. The IC decolourization efficiency can be increased up to even 85% by adding syringaldehyde as a reaction mediator. However, it should be noted that the addition of a mediator significantly increases the process cost, due to the price of the mediator and the impossibility of its co-immobilization. In the tests performed as part of this work, the appropriate selection of process conditions resulted in an equally high efficiency (up to 87.77% by biocatalysis) of decolourization using laccase as a biocatalyst. Furthermore, mediators very often constitute

Table 4. Decolourization efficiency and sorption coefficients for the individual enzyme doses.

Amount of enzyme		[mg E/ mg IC]	20	40	320
Decolourization (whole process)		[%]	19.00	35.08	92.55
Specification	biocatalysis		12.00	28.32	87.77
	sorption		7.00	6.76	4.78
Sorption coefficient			0.36	0.19	0.05

highly toxic substances, which makes their application in processes based on safe and green biocatalysis impossible.

An analysis of the pure IC spectrum as conducted by Wang et al. (2017) revealed characteristic peaks for wavelengths of 610 nm, 287 nm and 250 nm in the UV adsorption spectrum. Figure 6 below presents the results of this work's indigo carmine spectrum analysis before and after the biocatalysis process. An inspection of samples before the treatment process revealed the presence of a peak at 610 nm, which corresponds to the blue color of IC. The peak disappeared after applying treatment by means of immobilized laccase. Hence, the test results indicate that the chromophore of indigo carmine underwent cleavage.

As reported by Holkar et al. (2016), chromophore cleavage is the key stage of dye degradation, rendering the dye fragments more susceptible to further biodegradation (Holkar et al. 2016). The indigo carmine chromophore center comprises a double C=C bond substituted by two NH donor groups and two CO acceptor groups (Vautier et al. 2001). The results obtained, presented in Figure 6, indicate the disappearance of the peak at 287 nm, while the absorption band at 250 nm increased and shifted towards blue at 235 nm. The results suggest the cleavage of the dye chromophore structure and the generation of a new compound. The spectrum of the solution subjected to the influence of laccase closely resembles the spectrum of isatin sulphonic acid (ISA) (Kandelbauer et al. 2008), which may be formed from indigo carmine by cleaving the double bond in the H-chromophore. The results obtained in this work align with those by Wang et al. (2017). Peaks at wavelengths of 610 nm, 288 nm and 253 nm are visible in the pure dye spectrum. After the IC decolourization process involving 320 mg of laccase/1 mg of IC was performed, the peaks at 610 nm and 288 nm in the spectrum disappeared. Meanwhile, the absorption spectrum at 250 nm increased and shifted towards blue at 235 nm (Figure 6).

Based on the obtained results, it is recommended to continue research involving the toxicity testing of samples after the decolourization process by means of laccase immobilized in sodium alginate.

Furthermore, the test results obtained indicate that the efficiency of IC removal by sorption is only 5-7% Indigo carmine is an anionic dye, and the structure of IC reveals two points of $–SO^-$ on the compound, which are electrostatically

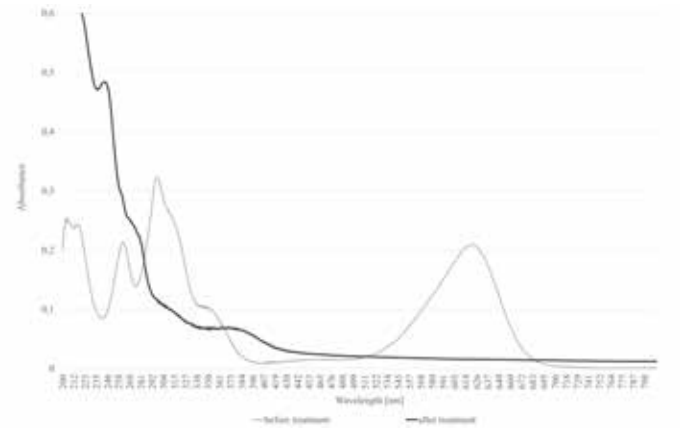

Figure 6. UV-vis absorption spectra of indigo carmine (IC) before and after immobilized laccase treatment (40 days, temperature: 21ºC, enzyme dose: 320 mg E/mg IC).

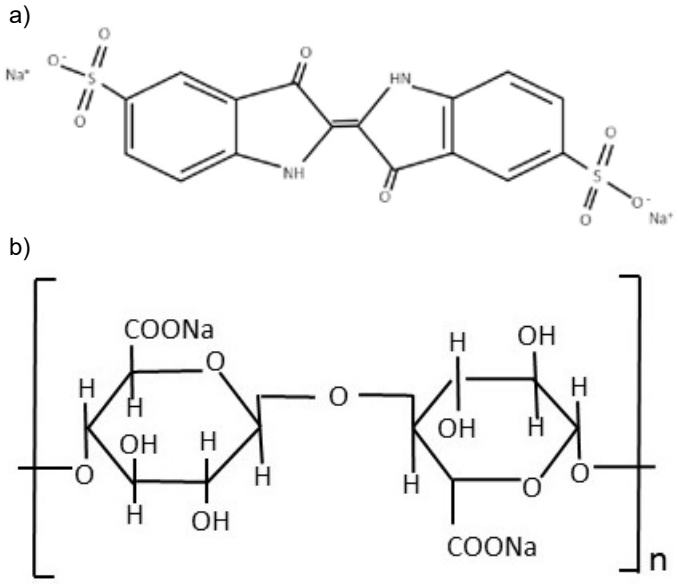

Figure 7. Chemical structure of a) indigo carmine and b) sodium alginate.

negative (Hevira et al. 2020) (Figure 7a). This situation can be explained by the presence of deprotonated carboxylic groups that impart a negative charge on alginate, hindering its interaction with other negatively charged molecules (Tyagi et al. 2021) (Figure 7b). Consequently, it can be concluded that IC, as anionic dye, has a lower affinity for alginate beads, and as a result, the possibility of enzymatic degradation of the dye increases. Furthermore, in studies on the decolourization of cationic and anionic dyes, such as anthraquinone dye (RBBR), diazo dyes (RB-5 and BBR) and a complex metal dye (LG), using immobilized laccase on an alginate carrier, it was found that during decolourization of the anionic RBBR dye, biocatalysis was the primary decolourization mechanism, while sorption on alginate beads without enzyme was only about 12% (Daâssi et al. 2013).

However, the IC sorption process may be of key significance for optimizing the costs of wastewater treatment involving this dye, and it can also constitute a secondary wastewater treatment procedure performed after biocatalysis. Research is already underway to develop adsorptive materials using waste as sorbents for dye removal. An example of this is the use of *Moringa oleifera* seeds from oil extraction processes, ground to obtain an adsorptive material at nanoscale. Adsorption tests conducted with this material demonstrated the removal of 85% of IC from textile wastewater, while the removal efficiency of the pure solution was 91%. Research focused on finding effective sorbents for removing indigo carmine has also explored *Terminalia catappa* shells modified with the egg-white of broiler chickens (Zein et al. 2022), activated carbon prepared from sawdust (Bhowmik et al. 2021), and fish scale biochar (Achieng et al. 2019). According to various analyses conducted by Hevira et al. (2020), the possible biosorption mechanism of IC onto *Terminalia catappa* shells includes electrostatic interactions between the $–SO^-$ on the compound and the biosorbent surface, physical adsorption and anion exchange. Continued research in this scope is recommended, seeking methods that can combine the biocatalysis process

with sorption processes for the most efficient wastewater decolourization.

Influence of process temperature on decolourization efficiency

One of the most significant physicochemical parameters influencing the enzymatic activity, and, consequently, the decolourization efficiency is the process temperature. Numerous literature reports confirm that the laccase immobilization process leads to lower enzyme sensitivity to temperature. Increased thermal stability is a crucial parameter that broadens possibilities of applying immobilized enzymes in catalytic reactions (Olajuyigbe et al. 2018, Saoudi and Ghaouar 2019, Shokri et al. 2021). The influence of temperature on the decolourization process was tested at 21ºC, 30ºC, and 40ºC, using indigo carmine over 20 days. The tests indicated that the optimal temperature for conducting the indigo carmine decolourization process falls within the range of 30-40ºC (Figure 8).

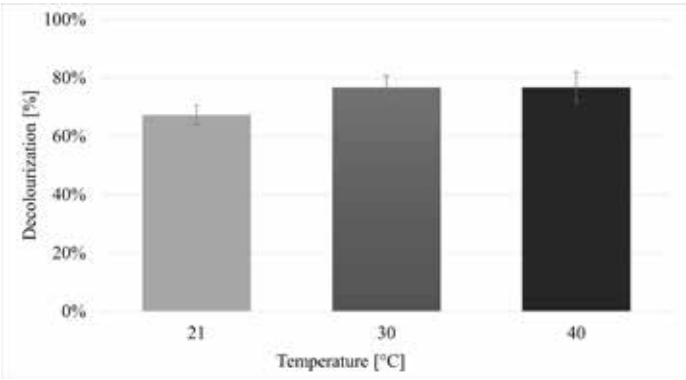

Figure 8. Influence of temperature (21, 30 and 40ºC) on the decolourization efficiency of indigo carmine over 20 days, enzyme dose: 320 mg/mg IC.

The decolourization of indigo carmine at temperatures of 30 and 40ºC resulted in the removal of nearly 77% of the dye. These test results align with findings by Lassouane et al. (Lassouane et al. 2019), who demonstrated that free and immobilized laccase exhibited the greatest thermal stability at 30ºC. However, at temperatures of 40ºC and over 50ºC, immobilized laccase retained over 90% and 50% of its initial activity, respectively. In contrast, the stability of free laccase considerably decreased at temperatures exceeding 30ºC. The enhanced thermal stability of immobilized laccase, compared to the native form of the enzyme, may be attributed to the enzymatic protein protection provided by the alginate sheath, shielding it from the influence of the process temperature (Lassouane et al. 2019). Teerapatsakul et al. (2017) successfully demonstrated the multiple decolourization of synthetic dyes in a 5-litre airlift bioreactor. The process involved *Ganoderma* sp. KU-Alk4 laccase immobilized on copper alginate. The immobilized enzyme exhibited high degradation efficiency for various synthetic dyes under unbuffered conditions, particularly for indigo carmine. Additionally, the immobilized laccase showed a substantial increase in stability concerning temperature and pH compared to the free enzyme. The optimal temperature for laccase activity remained 37°C, and it did not change after immobilization by means of copper alginate. Immobilization led to an increase in the thermal stability of the enzymatic beads; the initial enzymatic activity of the preparations was retained at temperatures up to even 55°C for 1 hour (ten degrees higher than for the free enzyme), which is beneficial for the practical application of the immobilized enzyme. Furthermore, the immobilized enzyme retained 98% of its initial activity after 1 h of incubation at a temperature of up to 65°C (Teerapatsakul et al. 2017).

Conclusions

Recent research conducted by numerous scientists has been focused on immobilizing biocatalysts for use in biotechnological processes. The most promising application of the immobilized biocatalysts, particularly laccase, is their use in processes involving dye removal from wastewater. The biological decolourization and detoxification of synthetic dyes by means of immobilized laccase obtained from white-rot fungi can constitute a potentially useful “green tool”. Some of the most promising carriers are biopolymers, including sodium alginate. Therefore, the aim of this study was to assess the feasibility of utilizing sodium alginate biopolymers as immobilization carriers for laccase in indigo carmine decolourization processes. Based on the results obtained in this study, it can be concluded that

1. The laccase obtained from *Trametes versicolor* immobilized on a sodium alginate carrier was capable of decolourizing indigo carmine. The most effective enzyme dose per 1 mg of the tested dye was determined at 320 mg E/mg of IC, which yielded a decolourization efficiency of 92.5% over 40 days.
2. The optimal temperature for the indigo carmine decolourization process by means of laccase immobilized on alginic acid was established at 30-40ºC.
3. Removing the dye in a process involving enzymes immobilized on biopolymers can be based on sorption and/or biotransformation/biocatalysis. The results demonstrated that indigo carmine removal by means of the immobilized enzyme occurred primarily by biocatalysis.
4. The effective immobilization of laccase by using alginic acid as a carrier was confirmed by Raman spectroscopy.

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Immobilizowana lakaza na nośniku biopolimerowym w dekoloryzacji indygo karminu

Streszczenie. Celem pracy była ocena możliwości wykorzystania biopolimeru alginianu sodu jako nośnika do immobilizacji lakazy w procesie usuwania indygo karminu (IC) (barwnik anionowy). Głównym celem pracy była optymalizacja procesu dekoloryzacji poprzez dobór odpowiedniej dawki immobilizowanego enzymu na 1 mg barwnika oraz temperatury procesu. Skuteczną immobilizację lakazy przy użyciu alginianu sodu jako nośnika potwierdzono za pomocą spektroskopii Ramana. Przeprowadzono także analizę wielkości i parametrów geometrycznych kapsułek polimerowych. Przeprowadzono również testy dekoloryzacji IC przy użyciu kapsułek alginianowych z lakazą. Zastosowanie najbardziej efektywnej dawki enzymu (320 mg enzymu/1 mg IC) umożliwiło usunięcie 92,5% barwnika w ciągu 40 dni. Optymalna temperatura dla procesu dekoloryzacji IC za pomocą lakazy immobilizowanej na alginianie sodu mieści się w zakresie 30-40ºC. Uzyskane wyniki wskazują, że lakaza z *Trametes versicolor* immobilizowana na alginianie sodu umożliwiła dekoloryzację badanego barwnika głównie w oparciu o mechanizm biokatalizy.