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Analysis of the feasibility of using biopolymers of different viscosities as immobilization carriers for laccase in synthetic dye removal

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Abstract: The main aim of the study was to assess the feasibility of using biopolymers of different viscosities (high, medium and low viscosity) as immobilization carriers for laccase in synthetic dye removal. The following dye solutions were decolorized: indigo carmine (IC, anionic dye), methylene blue (MB, cationic dye), and their mixture in a molar mass ratio MB/IC=0.69, using biopolymers of different viscosities as laccase immobilization carriers. Toxicity tests were also carried out to assess the toxicity of the post-decolorization samples.
Decolorization tests showed that the main decolorization mechanism depends on the dye class. The removal of IC (max. total removal efficiency 72.15%) was mainly by biocatalysis. The mechanism of the MB decolorization process was mainly by sorption on alginate beads, and the efficiency of enzymatic removal was low. However, the highest efficiency of MB decolorization (45.80%) was obtained for beads prepared using the high viscosity alginate when decolorization occurred by both sorption and biocatalysis. The results of mixture decolorization tests differ from the results obtained for single dyes.
The results showed differences in the efficiency of the dye sorption process depending on the alginate used for immobilization. Moreover, the varying mechanisms of dye removal from the dye mixture were confirmed by toxicity tests. The occurrence of both biocatalysis and sorption promotes reduced toxicity.

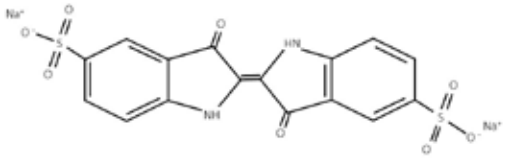
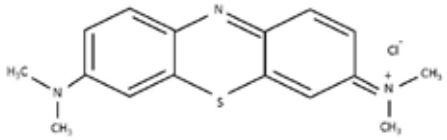
Introduction

The rapid growth of the textile industry has led to the widespread use of dyes in industrial processes. With over 100,000 commercial dyes available, global production exceeds $7*10^5$ tonnes annually, of which approximately 10-15% is released into the environment during the technological processes (Moon et al. 2023). Dyes are difficult to biodegrade and are considered extremely toxic and potentially carcinogenic due to the various chromophores (e.g. -N = N-, = C = O, C-NH, CH = N-, C-S) in their structures (Islam et al. 2021). Most dyes are toxic, mutagenic and carcinogenic to living organisms (Fernandes et al. 2021), highlighting the pressing need for sustainable water management practices, including water reuse and the adoption of circular economy principles to eliminate of toxic compounds, including dyes (Al-Tohamy et al. 2022). Notably, dyes commonly used in industrial applications, such as indigo carmine (IC) and methylene blue (MB), among others, are of particular concern.

IC, a synthetic acid dye, finds extensive applications across various industries including pharmaceuticals, textiles, tanning and food (Tabti et al. 2022). Additionally, it serves as a redox indicator in analytical chemistry (Edwin et al. 2021) and as a photometric detector (Eswaran et al. 2022). Particularly in the textile sector, IC is widely used in denim clothing dyeing process (Choi 2021). However, due to its frequent use and toxic effects on aquatic ecosystems, IC is classified as a contaminant that must be treated and/or disposed of before discharge into the environment (Behera et al. 2021). Recognized as a highly toxic indigo dye. IC exposure can lead to skin irritation, permanent corneal damage, gastrointestinal discomfort, and symptoms like nausea, diarrhea and vomiting (Ahlawat et al. 2022, Leonties et al. 2022, Mohan et al. 2022).

MB is an aromatic dye characterized by the molecular formula $C_{16}H_{18}CIN_3S$ and containing a benzene ring with redox properties (Li et al. 2023). It is a commonly used synthetic dye in fabric dyeing for clothing and textile industries, as well as in paper and leather dyeing processes (Oladoye et al. 2022). Therapeutic applications of MB are also known. However, its therapeutic applications are restricted to strictly recommended uses (Kofidis et al. 2001). The discharge of dye into the environment via wastewater poses various environmental hazards, including potential risks to human health (Oladoye et al.

Table 1. Characteristics of the tested dyes.

Name	Indigo carmine	Methylene blue
Abbreviation used in the study	IC	MB
Name according to International Union of Pure and Applied Chemistry (IUPAC)	3,3'-dioxo-2,2'-bis-indolyden-5,5-disulfonic acid disodium salt	3,7-bis (dimethylamino) phenothiazine chloride tetra methylthionine chloride
Chemical formula	$C_{16}H_8N_2Na_2O_8S_2$	$C_{16}H_{18}ClN_3S$
C.I.	73015	52015
CAS number	860-22-0	61-73-4
Molecular mass	466.36 g/mol	319.85 g/mol
Chemical structure	The chemical structure of IC presents two essential groups, $NaSO_3$ and a chromophore group. The chromophore group is a conjugate system of a C=C bond replaced by two C=O groups and two NH groups. 	The chromophore group of MB is the N–S conjugated system on the central aromatic heterocycle, while the auxochrome group is N-containing groups with lone pair electrons on the benzene ring. 
λ max	610 nm	664 nm

Source: own elaboration based on Khan et al. (2022), Ristea and Zarnescu (2023)

2022). Human exposure to this dye may lead to symptoms such as cyanosis, tissue necrosis, Heinz body formation, vomiting, jaundice, shock and tachycardia (Ahmad and Kumar 2010). In addition, the presence of MB can induce detrimental effects in plants, including inhibited growth and reduced pigment and protein components in microalgae such as *Chlorella vulgaris* and *Spirulina platensis* (Moorthy et al. 2021). Therefore, effective treatment of wastewater containing MB is crucial to mitigate its adverse environmental impacts. The characteristics of the tested dyes are summarized in Table 1.

Due to the multifaceted problem of environmental contamination by dyes, extensive research is ongoing to improve current methodologies and techniques and develop new approaches for treating wastewater containing dyes. Researchers are exploring a range of methods, including physico-chemical, biological, advanced, and combined approaches (Zaied et al. 2011, Veeranna et al. 2014, Kalyana et al. 2017, Kishor et al. 2021, Kuśmierek et al. 2023). Physicochemical methods include adsorption and coagulation/flocculation. Adsorption methods based on the transfer of contaminants from one phase to another (Saha et al. 2013, Ahmed et al. 2017, Arenas et al. 2017, Pavithra and Jaikumar 2019, Siyal et al. 2020, Micheletti et al. 2023). Coagulation/flocculation methods effectively decolorize dyes, such as sulfur and disperse dyes, but are less effective for acidic, reactive, direct and vat dyes (Kumar et al. 2020). However, these treatment methods have limitations: they are expensive, time-consuming, have limited application, and generate large amounts of highly toxic sludge as secondary pollution, contributing to environmental pollution.

The main advantages of adsorption and coagulation/flocculation include their ease of use, established effectiveness, utilization of readily available chemicals, simple working conditions, and high color removal efficiency (Kishor et al. 2021). Advanced oxidation processes (AOPs) are recognized as innovative and rapid methods for removing persistent contaminants, including dyes. AOPs use various oxidizing agents such as O_3, H_2O_2 and catalysts like Fe_2O_3, ZnO, CdS, TiO_2, and ZnS, along with UV radiation (Oriol et al. 2019, Ramos et al., 2020, Deska and Zawadzki, 2021, Kishor et al., 2021, Zawadzki and Deska, 2021). However, it is important to note that during photocatalysis and other advanced oxidation processes may produce by-products that could be more toxic than the original dye (Genázio Pereira et al., 2017). In addition to the chemical methods, biological approaches have been shown to be effective in decolorizing dyes. Bacteria such as Bacillus sp. MZS10, *Bacillus subtilis*, as well as fungi like *Phanerochaete chrysosporium* and *Trametes versicolor*, have demonstrated efficacy in removing dyes like IC through enzymatic activity (Diorio et al. 2021, Ahlawat et al. 2022).

Biological processes for dye removal are lauded as green and environmentally friendly technologies. These methods harness bacteria, fungi, yeast, and algae capable of decolorizing, degrading, detoxifying, and mineralizing a variety of contaminants, including dyes, through diverse metabolic pathways and biosorption mechanisms. The primary benefits of biological approaches are their eco-friendly nature, low cost, absence of sludge generation, complete mineralization, and global acceptance. However, they are challenged by long treatment time and limited efficacy against highly toxic compounds (Deska and Zawadzki 2021, Kishor et al. 2021). Research is also exploring combined dye-removal methods that integrate physical, chemical, and biological processes. For instance, the combination of an AOP process and biological

treatment holds promise. During AOP, complex contaminant structures are broken down through free radical attack, yielding more biodegradable compounds. Subsequently, biological processes facilitate further degradation and mineralization into smaller, simple and non-toxic metabolites through microbial consortium involvement (Waghmode et al. 2019). Furthermore, the utilization of enzymes in various technological processes is a focal point of research worldwide (Gonçalves et al. 2019). Enzymes like azoreductase, laccase, peroxidases, and polyphenol oxidases exhibit significant potential for degrading pollutants present in industrial wastewater (Kishor et al. 2018, Al-Tohamy et al. 2022).

Conventional physico-chemical processes utilized for dye removal often exhibit certain drawbacks, such as the generation of toxic intermediates and the accumulation of large amounts of sludge or sediment. Moreover, these processes frequently necessitate large quantities of chemicals to be effective. Current research trends are oriented toward identifying new, more environmentally friendly methods for removing hard-to-degrade organic compounds, including textile dyes (Al-Tohamy et al. 2022). In the contemporary context, where significant emphasis is placed on ensuring that technological processes are not only effective but also environmentally friendly, enzymes, known as "green catalysts", emerge as invaluable tools (Deska and Kończak 2019). Therefore, the importance of these biocatalysts is growing in white biotechnology.

Laccases, also known as benzenediol oxidoreductase (EC 1.10.3.2), are enzymes capable of oxidizing a wide range of substrates, including ortho- and para-diphenols, phenolic acids, aromatic amines and other electron-rich substrates, while concurrently reducing molecular oxygen to water. These enzymes are present in higher plants, most fungi, certain bacteria, and insects. However, commercially viable laccase is predominantly derived from white-rot fungi such as *Trametes versicolor*. Notably, white-rot fungi, including *Trametes versicolor* and *Pleurotus ostreatus*, have been extensively studied in recent laccase research and are regarded as model organisms in both basic and applied research for various environmental applications (George et al. 2023, Kumar et al. 2022, Tišma et al. 2021). Due to its low substrate specificity, laccase is considered a "green tool" with a wide range of potential applications (Deska and Kończak 2019, Deska and Zawadzki 2021).

The extensive body of scientific literature on laccase reflects considerable interest in both its production and its diverse application, including its use in immobilized forms for various industrial applications as biocatalysts (Alvarado-Ramírez et al. 2021). Laccase fins wide application in biotechnological processes involving the degradation of dyes and other contaminants, including those characterized by high persistence and resistance to degradation, such as phenolic compounds, pesticides, pharmaceuticals, personal care product (PCP) ingredients, and other organic chemicals. Furthermore, laccase plays a pivotal role in several industries including textile industry, biosensors development, food production, pulp and paper manufacturing, and polymer synthesis processes (Bilal et al. 2019, Zhou et al. 2021, Neha et al. 2022). For instance, Malinowski et al. (2020) developed a laccase-based biosensor, GCE/Lac, for the detection of dihydroxybenzene isomers in real water samples. In other studies, laccase-based biosensors were successfully prepared using one-step Soft Plasma Polymerization technique for dopamine determination (Wardak et al. 2020), and a biosensor based on immobilized laccase was developed for the determination of catechol (Palanisamy et al. 2017).

The BRENDA database – The Comprehensive Enzyme Information System (https://www.brenda-enzymes.org/index.php), catalogs over 300 laccases, predominantly produced by fungi. In scientific literature, laccases are hailed as promising alternative "green tools" for conventional chemical processes, mainly owing to the reduction or absence of side reactions in the processes (Deska and Kończak 2019). A report on the global market for industrial enzyme applications estimated a billion-dollar growth in this sector, projecting a value of USD 8.7 billion in 2026, with a compound annual growth rate (CAGR) of 6.3% from 2021 to 2026 (BBC Research 2021). Laccase is a potential target for the global enzyme market, with its size estimated at approximately USD 3 million in 2020, poised to reach USD 4 million by the end of 2027, maintaining a CAGR of 4.3% from 2021 to 2027. The surge in demand for laccase is reflected in the number of patents filed by both industry and research centers. A patent search using terms like "laccase immobilization" or "immobilized laccase" over the past five years at the World Intellectual Property Organization (WIPO; https://www.wipo.int) yielded 5,966 documents, with higher numbers in 2019. Over the last five years, the United States has seen the highest number of patent applications related to "laccase" (Gonçalves et al. 2019, Brugnari 2021).

Enzymes in their native form often exhibit limited potential for industrial applications. Consequently, significant research efforts have been directed towards enhancing enzyme stability under adverse process conditions through enzyme immobilization on various carriers (Daâssi et al. 2014; Zhou et al. 2021). Among these carriers, biopolymers, particularly alginates, have emerged as promising materials for immobilization (Deska and Kończak 2020, Hurtado et al. 2022, Dalginli and Atakisi 2023, Thirumavalavan 2023). Alginates possess a range of desirable properties, including non-toxicity, biodegradability, biocompatibility, and ease of accessibility, rendering them valuable across various industries such as biomedicine, bioengineering, biotechnology, pharmaceuticals, paper and packaging (Hurtado et al. 2022, Marszałek 2023). Purified alginates find widespread industrial use primarily due to their ability to form hydrogels, beads, fibers and films. The main structure of alginates is composed of two monomeric units: β- (1,4) linked d-mannuronic acid (M) and α- (1,4)-linked l-guluronic acid (G). Gelation process occurs through the introduction of crosslinkers, usually divalent ions such as calcium ions, which interact with regions rich in GG blocks. The monomer molecules are epimers, displaying an inverted spatial arrangement at the C5 carbon site. While the M regions show an elongated ribbon shape, the G regions are regularly curved. The bent arrangement of the G-blocks relative to the linear M-blocks creates hollow spaces that readily accommodate multivalent ions, particularly calcium ions. When calcium ions are introduced into the alginate solution, they bind the two alginate molecules, forming a three-dimensional gel network through a process known as ionotropic gelation.

The three-dimensional, congealed structure of the gel matrix envelops the active substance, forming a protective

barrier that restricts the diffusion of molecules based on their size and charges, thereby minimizing the harmful effects of external environmental factors that could degrade the enzyme activity of the catalytic protein (Ching et al. 2017). The M-blocks in alginate lack the ability to gel in the presence of calcium cations due to their low affinity for these cations. Sodium alginate also forms gels in the presence of ions of other multivalent metals, such as barium, cobalt, zinc, copper, iron, aluminum, but the lack of biocompatibility of gels produced in this manner precludes their use. Consequently, these areas of the gel remain in liquid form, providing a platform for immobilizing particles or microbial cells. Crosslinking follows the so-called "egg-box model", wherein the composition of the individual monomers and the length of the regions depend on the species and type of tissue from which the alginate is derived. The chemical composition of the various types of alginates affects their properties as well as the gels they form. The rheological properties of these gels are determined by the proportion of individual blocks and their distribution in the alginate chains, with the order of chain stiffness being MG < MM < GG. Gels obtained from alginates with high G-block content are stiff, brittle and prone to syneresis, whereas alginates with high M-block content yield weak, flexible, deformable gels that retain water (Abka-Khajouei et al. 2022, Hurtado et al. 2022).

Low molecular weight alginates with a high number of G units have been identified as producing the strongest and most robust gels suitable for encapsulation purposes, particularly for protecting probiotics (Bennacef et al. 2021). Similar to other polysaccharides, the properties of alginates vary based on their molecular weight and polydispersity. The physical and chemical attributes of alginates are contingent upon the arrangement of individual monomers within the chain, including their molecular weight, uronic acid chain length, and the composition percentage of each monomer (guluronic acid and mannuronic acid), leading to distinct structural differences and specific physicochemical properties. The content, composition, and M/G ratio of alginate may vary depending on the plant species, algae age, natural alginate source, geographic location, and seasonal variations, which collectively influence alginate's functional properties, including viscosity, solubility, reactivity with metal ions, and gel-forming capabilities. The linear and flexible structure of alginate features a steric barrier surrounding the carboxyl groups, with G-blocks assuming folded and rigid structures. Commercially, alginates are available as sodium, potassium, or ammonium salts, with molecular weights ranging from 60 000 to 700 000 Daltons, depending on the application (Abka-Khajouei et al. 2022). Regarding enzyme immobilization on alginate matrices, the porosity and permeability of the alginate are crucial.

Studies using electron microscopy and gel permeation chromatography have shown that the pore size of alginate gels ranges from 5 to 200 nm. The pore size of alginate gels is influenced by the gelation mechanism (external or internal), and the composition of the alginate monomers. Alignate gel rich in G monomers shows an open pore structure that is less susceptible to shrinkage (Abka Khajouei et al. 2022). Our recent study suggests that biopolymers, particularly alginates, are promising compounds for laccase immobilization (Deska and Kończak 2022). The results of this study are also a significant contribution to research into the use of biopolymers, particularly alginates, as carriers for enzyme immobilization to remove synthetic dyes from wastewater.

In the present study, laccase was immobilized on alginic acid with varying viscosities. The efficacy of laccase immobilized through this method in decolorization processes was assessed by conducting tests on the removal of textile dyes. Luminescence inhibition test was used to determine dye toxicity both before and

Table 2. Properties of the alginic acids used in the study

Name	Alginic acid sodium salt from brown algae (Alginic acid low viscosity)	Alginic acid sodium salt (Alginic acid medium viscosity)	Alginic acid sodium salt from brown algae (Alginic acid high viscosity)
Abbreviation used in the studies	AL-LV	AL-MV	AL-HV
Number	A1112	180947	71238
Initial appearance (form)	Powder		
Lot number	SLBT1081	MKCG6779	BCCB8704
Appearance (color)	Faint Yellow	Light beige	Faint beige
Solution (color)	Slightly hazy	Light beige	Almost colorless
Viscosity c = 1%, H_2O	11 cps	21 cps	Data n.a
Guluronic acid content (%)	39	Data n.a.	65 – 70
Molecular weight [g/m]	80 000-120 000	120 000-190 000	100 000-200 000
pH c=1%, H_20	Data n.a	7.2	6.1
Quality level	200	200	100
Origin	algae (brown)	algae (marine)	algae (brown)
Additional information	-	Kinematic viscosity: 15 – 25 mm^2/s	Suitable for immobilization of microorganisms

Source: Product specification and certificate of analysis available at https://www.sigmaaldrich.com and other properties obtained from Sigma Aldrich.

Table 3. Types of alginates used in research along with their corresponding acronym names

Alginate type	Enzyme solution	
	in buffer pH 5.0	in water
AL low viscosity (AL-LV)	ALe_b-LV	ALe_w-LV
AL medium viscosity (AL-MV)	ALe_b-MV	ALe_w-MV
AL high viscosity (AL-HV)	ALe_b-HV	ALe_w-HV

after decolorization. The primary objective of this study aims to assess the potential of different alginates with varying viscosities, as carriers for immobilizing laccase for synthetic dye removal. Moreover, most previous research in decolorization, particularly involving enzymes or microorganisms, has mainly focused on single dyes. Considering that industrial wastewater often contains dye mixtures, this study aims to evaluate the effectiveness of alginates with different viscosities as carriers for laccase immobilization. This evaluation involves assessing immobilized laccase capability to decolorize both single dyes and dye mixture.

Materials and methods

Materials

Biopolymers

In the study, 3 types of sodium alginate with different properties were used (Table 2). The sodium alginates were purchased from Sigma Aldrich (Poznań, Poland).

Chemicals and enzymes

Calcium chloride (anhydrous, granular, ≤ 93%) and laccase (EC 1.10.3.2) from *Trametes versicolor* were purchased from Sigma Aldrich (Poznań, Poland). Sodium acetate buffer solution pH5 and indigo carmine were purchased from Chempur (Bytom, Poland). Methylene blue was sourced from Eurochem BGD Sp. z o.o. (Tarnów, Poland).

Methods

Formation of Alginate-Laccase beads (ALe) with different types of alginic acid

For the preparation of alginate beads with immobilized enzyme (ALe), the methodology of Niladevi and Prema (2007) and the methodology used by Deska and Kończak (2022) were used with minor modifications. To form ALe, different types of alginic acid were used:

- low viscosity alginic acid (AL-LV);
- medium viscosity alginic acid (AL-MV), and
- high viscosity alginic acid (AL-HV).

For the preparation of alginate beads, appropriate concentrations of sodium alginate, 2% (w/v), were used. The appropriate concentration of sodium alginate was determined in preliminary studies (Deska and Kończak, 2022). Sodium alginate was dissolved in distilled water with continuous stirring for 10-20 minutes (for low viscosity alginic acid - 10 minutes, for medium viscosity alginic acid - 15 minutes, and for high viscosity alginic acid - 20 minutes) at 21°C until the sodium alginate was completely dispersed. The solutions prepared in this way were left at room temperature for 15 minutes to remove air bubbles formed during mixing. To prepare the biopolymer laccase beads, 400 µL of 200 mg/ml laccase enzyme solution (in water or sodium acetate buffer solution, pH 5.0) was mixed with 3000 µL of sodium alginate solution (2.0% w/v). The enzyme doses used in the decolorization process resulted from previous studies on the optimization of the MB and IC decolorization process conducted by the authors. Abbreviations for each type of enzyme beads are summarized in Table 3.

The alginate-laccase beads were prepared by dropping a solution of sodium alginate and laccase into a crosslinking solution of 2% $CaCl_2$ (w/v) using a 0.45 mm diameter dispensing needle. During the dropping, the crosslinking solution was continuously stirred. The dispensing needle was positioned 2 cm above the crosslinking solution. The formed alginate-laccase beads were kept in CaCl2 crosslinking solution for 60 minutes to harden. Then, the beads were recovered by decanting and rinsed three times with distilled water and were filtered through a sieve. Immobilization of laccase using $CaCl_2$ as hardeners gave spherical and regular shaped alginate-laccase beads. The diameter of the wet beads was between 2-3 mm. Wet beads were then immediately used for further research. The scheme of the enzyme immobilization process is shown in Figure 1.

Biopolymer bead formation – alginate beads

Based on the methodology used by Daasi et al. (2014) to assess the respective contributions of sorption and biochemical processes to the decolorization process, the experiments were conducted simultaneously using alginate beads both with and without enzymes. The alginate beads (AL) were developed using similar methodology as the alginate-laccase beads (ALe)

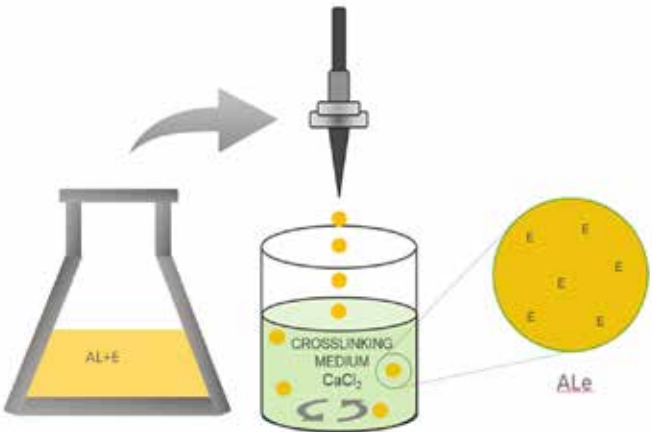


Figure 1. Scheme of enzyme immobilization process
Symbols: AL – alginic acid, E – laccase enzyme, ALe – alginate-laccase beads

described above, except that the dropped solution did not contain the laccase. The diameter of the wet alginate beads was between 2-3 mm.

Viscosity tests of biopolymers
A method based on measuring the flow time of the test material using a 100 ml Ford viscosity cup with an orifice diameter of 4 mm - was used. The flow time is the time that elapses from the moment the test product starts to flow out of the orifice of the full cup until the flow stops at the orifice. The test was conducted for 3 types of sodium alginate with a concentration of 2% at 21ºC (+/- 0.5ºC) and 30ºC (+/- 0.5ºC). Before testing, the cup was thoroughly cleaned with a suitable solvent and dried. The Ford viscosity cup was calibrated according to viscosity standards, and measurements were then carried out for the tested alginates. The cup was filled with the alginate solution, the orifice was covered, and after 5 seconds, the orifice was uncovered, and the time taken for the liquid to flow out was measured until flow of liquid broke for the first time. The test was repeated three times. Consequently, the arithmetic mean of the two selected determinations, not differing by more than 5% from their mean value, was taken.

Decolorization tests
The dye solutions for the decolorization tests were prepared in distilled water. The tests were conducted in 50 mL glass bottles at temperature 30°C within 10 days. The concentrations of the dye solutions were as follows: methylene blue (MB) 5 mg/l, indigo carmine (IC) 5 mg/l and a 1:1 mixture (M) of MB and IC (MB 2.5 mg/l and IC 2.5 mg/l), respectively, in molar mass ratio MB/IC=0.69. The initial dye concentrations resulted from previous studies on optimizing the decolorization process carried out by the authors. To assess the respective contributions of sorption and biocatalysis to the decolorization process, the experiments were conducted simultaneously using alginate beads without enzymes.

The total removal efficiency ($T_{eff,}$ sorption + biocatalysis) was measured for the beads with the immobilized enzyme and was calculated by monitoring absorbance changes at the maximum absorbance wavelength of IC (610 nm) and MB (664 nm), for the dye mixture at λ_{max} for IC and MB, calculated using the formula (eq. 1).

$$T_{eff} = A_0\text{-}A_t/A_0*100\ [\%] \qquad \text{(eq. 1)},$$

where
A_0 is the initial absorbance of the reaction mixture, A_t is the absorbance after decolorization.

The sorption efficiency (S_{eff}) was measured by using beads without enzyme.

The biocatalysis efficiency (B_{eff}) was measured as the difference between the total removal efficiency (T_{eff}) and sorption efficiency (S_{eff}).

Sorption coefficient (SC) was calculated according to the formula below (eq. 2).

$$SC = S_{eff}[\%]/T_{eff}[\%] \qquad \text{(eq. 2)},$$

where
S_{eff} – sorption efficiency [%], T_{eff}– the total removal efficiency of alginate beads with enzyme [%]

The study assumed an coefficient value in the range of 0-1. If sorption on alginate beads (S_{eff}) was higher than 1, then the SC value was taken as 1.

Toxicity tests: Luminescence inhibition test - Microtox system
Toxicity tests were carried out in accordance with Microtox technology, on a Microtox® model 500 instrument (Modern Water, USA) with a built-in photometer, temperature control and auto-calibration functions, and using the US EPA›s recommended procedure for toxicity testing of environmental samples - the Whole Effluent Toxicity Test (WET).

The Microtox system works with a selected strain of the marine luminescent bacteria *Aliivibrio fischeri*, which is sensitive to a broad spectrum of toxic substances. The luminescent bacteria produce light in the visible range as a result of normal metabolic processes. The change in metabolism following exposure to the test sample triggers a response of changing the intensity of the light produced.

As the test samples were colored, their absorbance (also for dilutions, if made) at 490 nm was determined before the test. The absorbance values were used to correct the luminescence measurement during the actual toxicity test. This correction was performed using MicrotoxOmni software provided by the manufacturer of the Microtox system.

Initial solutions of M, MB and IC dyes were tested using a concentration of 5 mg/L as the starting concentration. Subsequently, a series of dilutions of the tested dyes were prepared in the range of 5.0-0.125 mg/L with a dilution factor of 2. 2% NaCl in distilled water was used as the dilution solution. Each dilution was tested 3 times.

Post-process samples were tested without dilution. Only the salinity of the sample was corrected by adding Osmotic Adjusting Solution (Modern Water, USA), as required by the used procedure. The post-process samples were tested 4 times.

Toxicity tests were conducted in glass cuvettes by introducing 1 mL each of the properly prepared sample. The bacterial suspension obtained from lyophilized bacteria was then added according to the manufacturer's instructions (10 µL), the samples were mixed thoroughly and then incubated for 15 minutes at 15ºC. After this time, the intensity of bioluminescence was measured against a control sample containing only a 2% NaCl solution.

All results obtained from the conducted experiments were expressed as mean values and standard deviation using Microsoft Excel software.

Results

Viscosity of sodium alginate solutions tested at 21ºC and 30ºC

The present study was designed to determine the viscosity of alginate solutions at a concentration of 2% (w/v), at 21ºC and 30ºC (the selected temperature values were determined by the formation of alginate beads at 21ºC, while the decolorization process was conducted at 30ºC). Among the tested alginates, AL-HV has the highest viscosity, as determined by kinematic viscosity coefficient (ν), at 176.93 mm^2/s and 153.01 mm^2/s, at 21ºC and 30ºC respectively. In contrast, the lowest viscosity (almost two times lower than that of AL-MV alginate and 17 times lower than that of AL-HV) was that of AL-LV alginate,

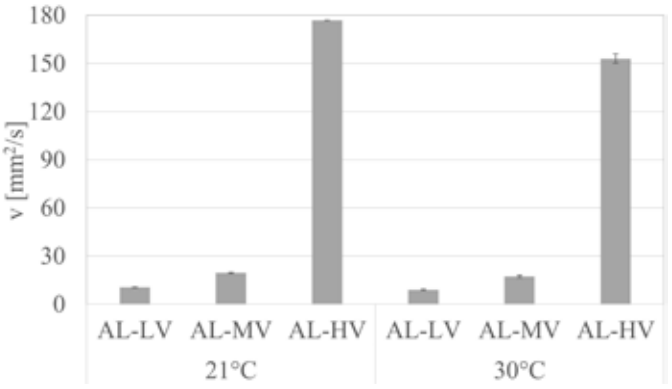


Figure 2. Viscosity of sodium alginate solutions (2%) tested at 21ºC and 30ºC

for which *v* was 10.43 mm²/s at 21ºC and 8.97 mm²/s at 30 ºC. Among the alginates tested, AL-HV had a significantly higher viscosity compared to the other alginates tested. Compared to AL-HV, AL-MV had a viscosity that was approximately 89% lower, and AL-LV had a viscosity that was approximately 94% lower (at 21ºC). The tested alginate solutions showed a higher viscosity (by about 13%) at 21ºC than at 30ºC (Figure 2).

Decolorization experiment

To assess the respective contributions of sorption and biochemical processes to the decolorization process, the experiments were conducted simultaneously using alginate beads based on different alginates as supports both with enzyme (enzyme alginate beads based on alginate of high viscosity ALe-HV, medium viscosity ALe-MV and low viscosity ALe-LV) and without enzyme (AL-HV, AL-MV, AL-LV, respectively). In addition, the enzyme beads tested contained enzyme dissolved in water (w) or buffer (b).

It was found that using the enzyme beads prepared based on the three analyzed alginates, the primary mechanism for indigo carmine dye removal was biocatalysis (Figure 3a). Among the beads prepared on AL-HV and AL-MV alginate carriers, the sorption of the dye occurred at approximately 9%, while for the beads prepared on AL-LV carrier, it occurred at nearly 5% (Figure 3b).

Table 4 summarizes the sorption coefficients for IC decolorization, using different enzyme beads.

For IC decolorization, a dependency became apparent - the lower the viscosity of the alginate used, the lower the sorption coefficient. Overall, the use of enzyme beads with immobilized laccase on alginates of different viscosities allowed for the removal of the dye in a range from 55.31% (ALe_w-HV) to 72.15% (ALe_b-MV) within 10 days. In the IC decolorization process, the removal of the IC dye by biocatalysis (B_{eff}) was 46.64% for ALe_w-HV, and 65.15% for ALe_w-LV. No differences were identified in the enzymatic degradation efficiency of IC due to the different physicochemical parameters of the tested alginates. No differences were identified in the

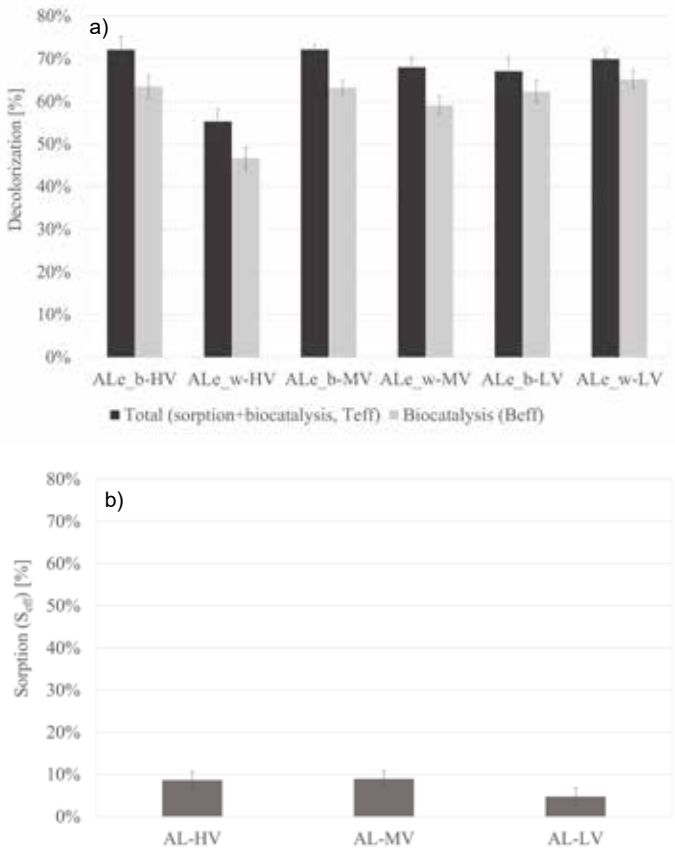


Figure 3. Indigo carmine removal, a) decolorization by enzyme-alginate beads and b) sorption of alginate beads

efficiency of the decolorization process using enzyme beads when laccase was dissolved in water and buffer solution and when laccase was immobilized on different alginates. The lowest IC total removal efficiency was observed for enzyme beads ALe_w-HV (T_{eff} = 55.31%).

In the case of MB decolorization overall, the use of enzyme beads with immobilized laccase on alginates of different viscosities resulted in total removal efficiency (T_{eff}) ranging from 30.68% (ALe_b-MV) to 45.80% (ALe_w-HV) (Figure 4a). Regardless of the alginate used, the sorption process prevailed over biocatalysis (Figure 4b). For the high viscosity enzyme-alginate beads (ALe-HV), it could be observed that the removal process of MB occurred due to the process of sorption and biocatalysts. The total removal efficiency (T_{eff}) ranged from 41.14% to 45.8%, depending of the type of enzyme solution (water (w) or buffer (b)).

Thus, the highest degree of MB removal occurred where both processes, biocatalysis and sorption, occurred. The sorption removal efficiency (S_{eff}) was about 29.60%, and biocatalysis (B_{eff}) about 11.5 to 16.2%. The less viscous the alginate, the sorption process dominated in the MB removal process. For the alginates with the lowest viscosity, the dye removal process occurred only by sorption. For the AL-LV, the

Table 4. Sorption coefficients for different enzyme beads used during IC decolorization

Alginate beads	ALe_b-HV	ALe_w-HV	ALe_b-MV	ALe_w-MV	ALe_b-LV	ALe_w-LV
Sorption coefficient SC	0.12	0.16	0.12	0.13	0.07	0.07

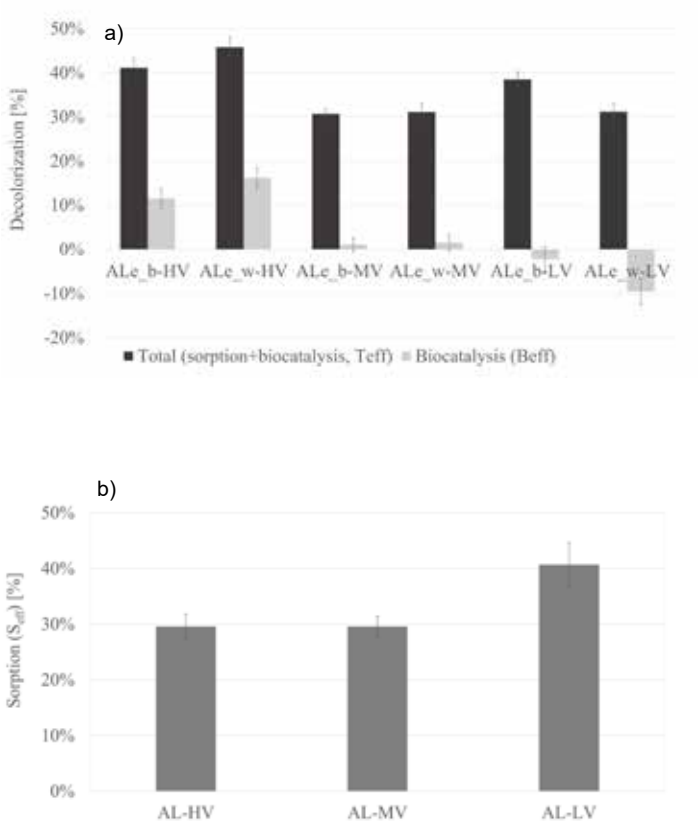


Figure 4. Methylene blue removal: a) decolorization by enzyme-alginate beads and b) sorption of alginate beads

sorption efficiency on beads without enzyme (S_{eff}) was higher than the dye removal efficiency of beads with immobilized enzyme (T_{eff}). This was most probably due to the lower sorption surface area of the enzyme-filled beads. The empty beads removed more than 40% of the dye by sorption, while the enzyme beads only removed 31.18% to 38.48% of the MB.

In the case of MB decolorization, the value of the sorption coefficient increases with decreasing alginate viscosity, with a value of 1 for the alginate with the lowest viscosity, which means that the decolorization process occurred exclusively by sorption (Table 5).

The removal of the dye using enzyme-alginate beads with the lowest viscosity occurred only by sorption (SC=1). In this case, approximately 31 to 38% of MB total removal efficiency (T_{eff}) from solution was achieved.

The degree of removal of the individual dyes IC and MB from a mixture of dyes M was also investigated. The results of this test differ from those obtained for the individual dyes, particularly evident in the case of IC in a mixture, where removal by biocatalysis (B_{eff}) accounted only for nearly 7% for ALe_w-HV and 1.44% for ALe_b-LV. The IC total removal efficiency (T_{eff}) of ALe_b-HV, ALe_b-MV, ALe_w-MV, ALe_w-LV was lower than sorption (S_{eff}) on beads formed from

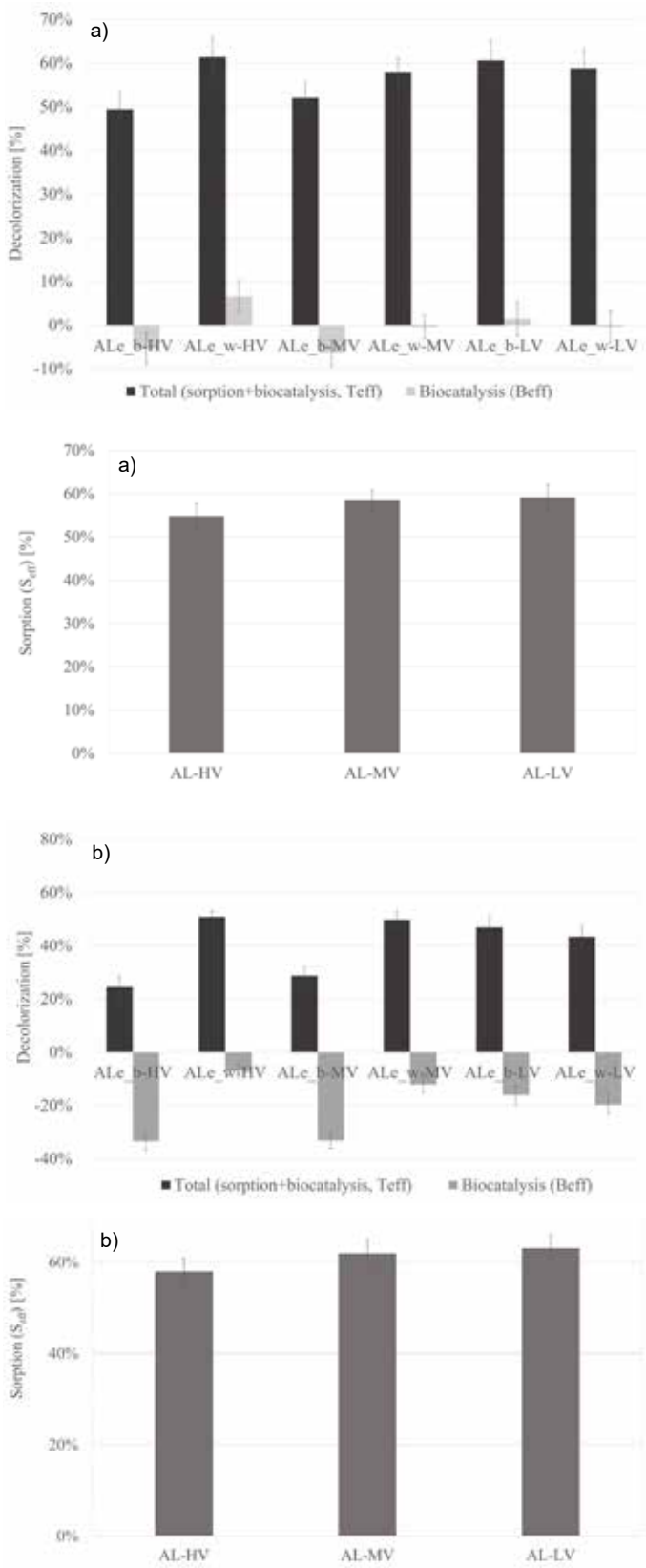


Figure 5. Decolorization of a) IC in a mixture and b) MB in a mixture

Table 5. Sorption coefficients for different enzyme beads used during MB decolorization

Alginate beads	ALe_b-HV	ALe_w-HV	ALe_b-MV	ALe_w-MV	ALe_b-LV	ALe_w-LV
Sorption coefficient SC	0.72	0.65	0.96	0.95	1.00	1.00

the corresponding alginates (Figure 5a). Also, the total removal efficiency of IC from a mixture was lower, about 50 to 61%, while decolorization of the dye alone achieved decolorization of about 70%. Decolorization of IC in the mixture was mainly by sorption on the beads.

Only in the case of ALe_w-HV and ALe_b-LV beads, occurred the removal of the dye by biocatalysis, respectively 6.56% with a total decolorization of 61.38% and 1.44% with a total decolorization of 60.61%, respectively.

Decolorization of MB in the mixture occurred only by sorption on the beads regardless of the type of alginate used for immobilization (Figure 5b). Interestingly, the use of the enzyme-alginate beads with the lowest viscosity, ALe-LV, in both cases, whether the enzyme was dissolved in water or in a buffer solution, resulted in a decolorization of approximately 45%. It should be noted, however, that in the case of MB removal from the mixture, dye removal by sorption (S_{eff}) by enzyme-free beads was higher than dye removal by enzyme beads. This is most probably due to the lower sorption surface area of the enzyme-filled beads. Moreover, the obtained results indicate that the mechanism of removal of dyes in a mixture is different from that during decolorization of single dyes.

Toxicity testing

Dye solutions

The results of the luminescence inhibition test for the initial M, MB and IC dye solutions are shown in Figure 6. The results are presented as the relationship between dose (concentration) of dyes and corresponding average effect (inhibition of luminescence).

The toxicity results obtained for the standard solutions indicated an increase in toxicity with increasing concentration for the MB and the M. MB showed the highest toxicity, as measured by inhibition of *Aliivibrio fischeri* bioluminescence. As the concentration of the MB solution tested increased (0.3125 mg/L to 5 mg/L), an increase in bioluminescence inhibition was observed. In contrast, the results for the IC were different; in the range of concentrations tested, no increase in toxicity was observed with increasing dye concentration.

Toxicity test results for post-decolorization samples

Toxicity tests were carried out to assess whether the toxicity of the post-decolorization samples were increased compared to the standard solutions as a result of the presence of possible minor metabolites during decolorization using immobilized enzymes. The results of the post-decolorization samples are shown in Table 6.

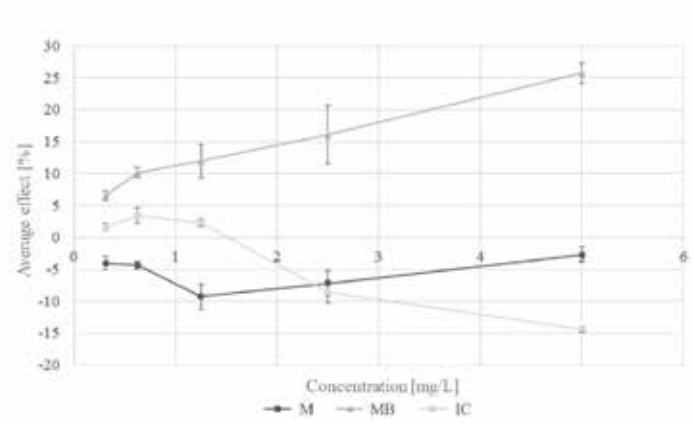


Figure 6. Toxicity testing: dose-effect relationship of M, MB and IC in the Aliivibrio fischeri luminescence inhibition test

The toxicity of the post-decolorization samples containing IC did not increase compared to that of the initial dye, with total removal efficiency between 55% and 72%, over 10 days. The study showed a decrease in toxicity of the samples after the decolorization process. At the same time, the results of toxicity tests confirm different mechanisms involved in the removal of cationic and anionic dyes using alginate beads and immobilized laccase. The results for IC indicate that both before and after decolorization, the samples showed a stimulating effect on bioluminescence. At the same time, an increase in bioluminescence is evident for the samples after decolorization using both empty beads and enzymatic beads.

The highest efficiency of MB decolorization was obtained for beads prepared based on the high viscosity alginate (ALe-HV). The results indicate that decolorization occurred by both sorption and biocatalysis (41.14% for the enzyme in buffer solution; 45.80% for the enzyme in water, of which biocatalysis occurred in approximately 12% and 16%, respectively), promoting reduced toxicity. At the same time, it should be noted that in this case of AL-HV alginate, a reduction in luminescence inhibition was observed in relation to the original dye solution, which may indicate a reduction in its toxicity. Based on the data obtained, it can be concluded that the simultaneous occurrence of sorption and biocatalysis processes is beneficial in decolorization processes when using high viscosity alginate.

Total removal efficiency of MB using enzyme-alginate beads, which had the lowest viscosity among those tested, was about 31 to 38% and occurred mainly by sorption. In this case, the toxicity of the post-decolorization samples remained at a similar level or decreased slightly compared to the initial dye solution. In the case of beads without enzyme, a decrease in the toxicity of the samples is evident. MB has a high affinity for alginate, especially for AL-LV. The removal of MB by sorption reduces the toxicity of the whole solution. An increase in toxicity was only shown for beads with AL-MV, which is related to the low overall efficiency of these beads in dye decolorization processes.

In the case of decolorization of the dye mixture, the highest luminescence inhibition (80%) occurred in the post-decolorization sample using laccase (enzyme in buffer solution) immobilized on AL-HV alginate (the high viscosity alginate). Interestingly, also the use of beads ALe_b-HV resulted in the lowest removal rates of IC and MB from the mixture of these dyes (49.43% and 24.49%, respectively).

Discussion

In order to assess the feasibility of removing dyes from wastewater through the use of laccase immobilization technology on biopolymer carriers, a part of the study was to determine the viscosity of the alginates tested for immobilization. Viscosity tests on the alginates used in the experiment showed that at both 20°C and 30°C, AL-LV had the lowest viscosity (10.43 mm^2/s and 17.38 mm^2/s, respectively), almost twice as low as AL-MV alginate and 17

Table 6. Post-decolorization samples results

Dye	Alginate used	Beads without enzyme (after 10 days)		Beads with enzyme (after 10 days)			
		Average effect [%]	SD	Average effect [%] (enzyme in water)	SD	Average effect [%] (enzyme in buffer)	SD
MB	AL-HV	50	4	-10	1	-3	6
	AL-MV	-27	5	73	6	15	8
	AL-LV	-46	6	22	4	-20	19
M	AL-HV	-32	8	-6	4	80	1
	AL-MV	27	4	9	0	3	5
	AL-LV	-47	6	-26	1	14	4
IC	AL-HV	-60	3	-51	4	-64	11
	AL-MV	-74	6	-47	37	-64	13
	AL-LV	-78	6	-59	26	-43	10

times lower than AL-HV (176.93 mm^2/s and 153.01 mm^2/s, respectively).

In alginates, the arrangement of the individual monomers in the chain, as well as their molecular weight, the chain length of the uronic acid, and the percentage of each monomer are factors that cause significant structural differences and confer specific physicochemical properties. Results obtained in this study showed that AL-LV also had a lower guluronic acid content (39%), with AL-HV contained approximately 65-70% of this acid. The content, composition and M/G ratio of alginate can vary not only according to the species and age of the algae from which the alginate is extracted but also according to the location, geographical location and season. This variablility translates into the functional properties of a given alginate, including viscosity, solubility, reaction with metal ions, and gel-forming properties (Ma et al. 2014, Abka-Khajouei et al. 2022).

Alginates are composed of two hexuronic acids: β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked by 1-4 bonds. These are randomly distributed in a linear chain and can also be arranged as homogeneous MM or GG blocks, as well as heterogeneous or alternating MG blocks. The physical and chemical properties of the alginate determine the characteristics of the product made from them and the application possibilities for the specific industry sector. The proportions of MM, GG and MG blocks affect the properties of the material. The guluronic acid content of alginate gels determines their brittleness and strength, while the mannuronic acid content determines the elasticity and lower strength of the gel formed. The high G content in alginates contributes to their high gelling capacity (Fertah et al. 2014). Conversely, alginates with a high M/G ratio produce flexible gels, while those with a low M/G ratio result in more brittle gels (Łabowska et al. 2019, Peteiro 2018). The chemical and physical properties of alginates are significantly influenced by their molecular characteristics, in particular the M/G block ratio, the concentration of the crosslinking solution (e.g., calcium ion concentration), the molecular weight, the degree of polymerization and the block structure of the alginate framework. The sequence of M and G blocks can vary not only among different algal species from which alginate is extracted but also within different tissues of the same species (Silva et al. 2012, Ferath et al. 2017, Rhein-Knudsen et al. 2017). The viscosity of the solution before gelation, as well as the stiffness and strength after gelation, can be individually controlled by the molecular weight and its distribution. Indeed, the molecular weight depends on the extraction method, e.g., it is possible to increase it by cold extraction. Higher temperatures during extraction can lead to the breakdown of the uronic acid chains and consequently lower the viscosity of the extracted alginate. Controlling the viscosity of alginate is an important factor for the industry, as the application of alginate depends significantly on its viscosity. Low-viscosity alginates are desirable for applications in the paper and fruit industries, while high-viscosity alginate is typically used in the food and cosmetic industries. The ability to form viscous solutions and gels in aqueous media is also important in the pharmaceutical industry (Chee et al. 2011, Lee and Mooney 2012). Increasing the molecular weight of alginates can improve the physical properties of gels, although too high a molecular weight of alginate can lead to extreme viscosity, which may be undesirable when used in specific processes. The beads formed on AL-LV were slightly softer and more flexible compared to AL-HV-based beads, but despite of this, they showed adequate strength and were not damaged or cracked during the decolorization process. AL-HV alginate with a high guluronic acid content of 65-70% also had the highest viscosity. It should be noted that the range of molecular weight values of AL-HV (100,000-200,000 g/m) is similar to that of AL-MV (120,000-190,000 g/m). However, due to the rather wide range of values, the actual molecular weight may adopt extreme values from the indicated range for the two alginates analyzed, which may significantly impact such differences in viscosity. The literature also explores the possibility of using a fusion of high and low-molecular-weight alginate polymers, potentially increasing the elastic modulus and slightly raising the viscosity of the solution (Lee and Mooney 2012). In summary, the ratio

of M/G blocks in a given alginate is important to obtain the appropriate rheological properties of the developed gel. These issues are of particular interest to researchers in the biomedical field and the agricultural industry (Łabowska et al. 2019, Martínez-Cano et al. 2022). It is equally intriguing to explore these matters in the context of using alginates as matrices for enzyme immobilization, as undertaken in the present work.

The viscosity of alginate solutions is highly dependent on temperature, the molecular conformation of the polymer, and the ionic strength of the solvent. Alginate solutions exhibit higher viscosity at low temperatures and low ionic strengths. Conversely, alginate solutions demonstrate lower viscosity at high temperatures and higher ionic strengths, attributed to increased intermolecular distances due to thermal expansion and a more compacted conformation, respectively (Ma et al. 2014, Abka-Khajouei et al. 2022), consistent with the results obtained in the present study.

The mechanism of dye removal using immobilized enzymes can occur either by enzymatic biodegradation (via biocatalysis) or by sorption of the dye onto alginate beads. Decolorization tests were carried out for dyes from two different groups, MB (cationic dye) and IC (anionic dye), as well as their mixtures in molar mass ratio MB/IC=0.69. Decolorization was conducted using immobilized laccase from *Trametes versicolor* on biopolymer supports and using alginate beads without enzyme. The results showed that different decolorization mechanisms prevailed depending on the dye class. In the case of IC, its removal was mainly attributed to enzymatic activity, and the viscosity of alginate did not affect the efficiency of enzymatic degradation of the indigo carmine dye. This preference for enzymatic removal of IC is likely due to its classification as a negatively charged anionic dyes. The negatively charged surface of alginate (Tyagi et al. 2021) reduces IC's affinity for alginate, thereby increasing the likelihood of enzymatic removal of the dye, as confirmed by the results obtained in this study. The effectiveness of biological methods in removing IC was further supported by results obtained by Ahlawat et al. (2022), demonstrating degradation to anthranilic acid by oxidoreductases, particularly laccase, present in the culture medium of a fungus grown on wheat bran and orange peels. This was further confirmed by treating IC with purified laccase (Ahlawat et al. 2022).

In the case of MB (cationic dye), its removal was primarily through sorption. The higher sorption of MB on the alginate beads compared to IC may be influenced by MB's affinity to alginate resulting from the ionization of this dye, as it is a cationic dye, while IC is an anionic dye. The results obtained suggest that the physicochemical parameters of the alginates studied, in particular their viscosity, affect the efficiency of enzymatic degradation of the MB. This efficiency is mainly associated with the sorption process of this dye.

Furthermore, from the results obtained, it can be concluded that lower alginate viscosity promotes the MB sorption process on the beads, which is confirmed by the calculated sorption coefficient, the value of SC increases with decreasing alginate viscosity. However, it should be noted that the greatest removal of MB occurred where both processes, biocatalysis and sorption, occurred. In most types of enzyme beads tested, the sorption efficiency (S_{eff}) was higher than the removal by enzyme beads. This was most probably due to the lower sorption surface area of the enzyme-filled beads. The results of a study by Daâssi et al. (2014) also indicate a high percentage of sorption of the cationic dye Bismark Brown R (BBR) on Ca-alginate beads with immobilized laccase extracted from *Coriolopsis gallica* (34.6%) within 24 h compared to the other dyes tested: Remazol Brilliant Blue R, Reactive Black 5, Bismark Brown R and Lanaset Grey G. In the decolorization process using immobilized and free enzymes, the dye was removed by nearly 53% and 47%, respectively, within 24 h. It can, therefore, be concluded that the immobilized laccase was not able to degrade the dye adsorbed to such a high degree on the beads, and that the decolorization of this dye occurred mainly by sorption. It is also interesting to note that even the addition of the redox mediator, 1-hydroxybenzotriazole (HBT), did not significantly enhance the decolorization of this dye (Daâssi et al. 2014). Also, a study by Enayatzamir et al. (2010) showed that the cationic dye BBR was resistant to biodegradation by the fungus *Phanerochaete chrysosporium* immobilized in alginate beads, and that dye removal was mainly due to adsorption of the dye on the alginate beads (Enayatzamir et al. 2010). Dye removal by sorption is considered to be a promising method for relatively easy removal of dyes or dye mixtures. Similar to the enzymatic degradation method, the adsorption method can also be repeated over several cycles until the adsorbent is used up or saturated with the dye. The disadvantage of this method is that these adsorbents can be relatively expensive. However, a solution to this problem may be the use of alternative sorbents of low-cost waste materials, often modified in various ways (Katheresan et al. 2018). Also, other researchers have shown that the decolorization of MB occurs mainly by sorption, indicating that for such dyes the sorption method may be the best solution for its removal efficiency (Hamad and Idrus 2022, Radoor et al. 2022, Shah et al. 2022). The present study indicates that it is possible to remove methylene blue by biocatalysis, provided a higher viscosity alginate is used. In addition, recent literature has shown that both enzymatic degradation and decolorization by adsorption are effective in removing a variety of dyes, hence it may be promising to combine these methods into a single hybrid dye removal method and should therefore be considered for future technologies with industrial applications (Katheresan et al. 2018).

To date, research in the area of decolorization, particularly using enzymes or microorganisms, has mainly focused on single dyes. Thus, one element of the present study is to determine the decolorization efficiency of a dye mixture (M), using immobilized laccase on alginates of different viscosities. The removal mechanisms of the dyes tested in pure solutions and in their mixture differ. This different mechanism is particularly evident in the case of IC removal, where decolorization of the pure dye occurred mainly by biocatalysis, while the removal from the mixture occurred mainly by sorption. Only when the alginate with the highest viscosity was used, there was a slight removal by biocatalysis (6.56%). Furthermore, the degree of IC decolorization in the mixture was lower than in the single dye solution. The decolorization of the MB in the mixture occurred only by sorption. Difficulties in removing dyes from their mixtures may be influenced by the diverse reactions that can occur between dyes in solution. Variations in spatial structure or redox potential can have a significant impact on decolorization efficiency (Park et al. 2007).

One of the biggest challenges facing industry today is the move towards greener, more environmentally friendly processes. All innovations are directed at minimizing or completely eliminating the pollutants generated and abandoning the use of toxic and hazardous raw materials. Hence, a crucial aspect is to minimize the formation of compounds during the process that could be more toxic than the pollutants themselves that were removed. Therefore, the toxicity of the original dye solutions, control samples and post-decolorization sample solutions were assessed in the present study.

The toxicity test results obtained for the standard solutions showed an increase in toxicity with increasing concentration for the MB dye and mixture M. In contrast, luminescence was stimulated for the IC. This can occur when testing environmental samples such as wastewater or, as in this case, samples that contain conversion products of enzyme catalysis. The presence of substances other than those in the control sample, such as ions themselves (e.g., Cl^-), can stimulate the enzymatic activity of *Vibrio fischeri*, resulting in an observed increase in luminescence. In the case of wastewater or post-process samples, the presence of organic compounds as a carbon source is also significant. The literature also points to the possibility of a stimulation phenomenon resulting from the hormesis effect. Hormesis is an ecotoxicological phenomenon defined as a biphasic dose response. At low concentrations of toxic substances there is a stimulatory effect of hormesis, while at higher concentrations there is an inhibitory effect. The hormesis phenomenon can hinder the interpretation of toxicity test results and reduce the actual toxicity of the samples tested (Drzymała and Kalka 2020). Ahlawat et al (2022) in their studies on biological methods of IC removal showed that IC was degraded to anthranilic acid by laccase. Interestingly, the addition of IC to the culture medium of the white-rot fungus *Cyathus bulleri* resulted in stimulated growth of the fungus. The toxicity of IC before and after treatment with culture filtrates was measured by the Ames test using *Salmonella typhimurium* TA98. Toxicity analysis indicated that the dye was highly mutagenic. Plates containing untreated dye (50 µl of 100 ppm aqueous solution) yielded 500 to 550 revertants. When IC was treated with fungal culture filtrate (0.5 U/mL of laccase equivalent), a reduction in mutagenicity of 87% (65-70 revertants) was obtained. When treated using ABTS, almost complete removal of toxicity was achieved (Ahlawat et al. 2022). Results obtained in this study show that the toxicity of the post-decolorization samples containing IC, did not increase, compared to the toxicity of the initial dye, while the color was removed, so the results obtained in the present work are in line with those quoted above. Furthermore, it should be noted that both before and after decolorization, the samples showed a stimulating effect on bioluminescence.

During the decolorization of MB using high-viscosity alginate, simultaneous sorption and biocatalysis processes occurred, which proved beneficial, resulting in the highest degree of decolorization as well as a reduction in luminescence inhibition relative to the original dye solution. A study by Ezike et al. (2020) on the decolorization of dyes including methylene blue using laccase from *Trametes polyzona* WRF03 indicated that this enzyme was not effective in decolorizing methylene blue and thiazine dye (Azure B) (Ezike et al. 2020). Similarly, no decolorization methylene blue by laccase from *Perenniporia tephropora* was observed even in the presence or absence of HBT (Younes et al. 2007). In contrast, a study by Wu et al. (2022) shows that an isolated strain of *Bacillus thuringiensis* was effective in decolorizing methylene blue. The ability of the strain to efficiently degrade this dye was due to the synergistic action of laccase, manganese peroxidase, lignin peroxidase and NADH-DCIP reductase. Furthermore, based on the intermediates identified by GC-MS, a new mode of degradation of methylene blue by *B. thuringiensis* was proposed. Also, research on phytotoxicity showed that MB was degraded to metabolites with lower toxicity (Wu et al. 2022). The toxicity test results obtained in the present study indicate varying mechanisms of dye removal from the mixture. In an experiment where laccase beads ALe-HV were used (laccase in a buffer solution), an increase in toxicity of the post-decolorization solutions was observed. It should be noted that the research on decolorization included solutions of two different types of dyes (cationic and anionic) in a 1:1 ratio. The laccase solution in a buffer solution offers greater stability and resistance to external influences, ensuring a better reaction environment for the laccase. It should be noted that the biodegradation of IC could result in an increase in the toxic effect of MB, especially in the case of AL-HV beads with a lower affinity for dyes, as confirmed by the test results obtained. Since the toxicity tests were carried out on samples after 10 days of the decolorization process, given the complexity of the decolorization process of the dye mixtures, it is recommended to continue the tests with extended analyses, including measurements at day 20 and day 40 of the process. This will help determine the mechanism of toxicity changes during decolorization process and after complete removal of the dye.

Conclusions

Recent research by many scientists has focused on the immobilization of biocatalysts for use in biotechnological processes. One of the most promising applications of immobilized biocatalysts, especially laccase, is their use in processes for removing dyes from wastewater. Biological decolorization and detoxification of synthetic dyes by immobilized laccase from white-rot fungi could potentially serve as a “green tool”. Some of the most promising carriers for immobilized laccase are biopolymers, including sodium alginate. Therefore, the aim of the study was to assess the feasibility of using biopolymers with different viscosities (sodium alginate: high, medium and low viscosity) as carriers for immobilizing laccase in the decolorization of synthetic dyes. Based on the results obtained in the present study, it can be concluded that:

1. The laccase from *Trametes versicolor* immobilized on sodium alginate, during 10 days, was able to decolorize:
   - indigo carmine in a range from 55.31% by enzyme beads based on high viscosity alginate to 72.15% by enzyme beads based on medium viscosity alginate.
   - methylene blue in a range from 30.68% by enzyme beads based on medium viscosity alginate to 45.80% by enzyme beads based on high viscosity alginate.
2. The dye removal efficiency by enzyme beads is independent of the type of enzyme solution used (water or buffer solution).

3. Decolorization of the tested dyes and their mixture using alginate-laccase beads is based on various mechanisms – sorption and/or biocatalysis.
4. The results showed differences in the efficiency of the dye sorption process depending on the alginate used for immobilization and the class of tested dye.
5. Decolorization of indigo carmine occurred mainly by biocatalysis, the removal by biocatalysis was 46.64% for enzyme beads based on high viscosity alginate to 65.15% for enzyme beads based on medium viscosity alginate, when enzyme was dissolved in water. In decolorization of indigo carmine, sorption on beads made using high and medium viscosity sodium alginate was about 9%, whereas on beads formulated on low viscosity alginate it was about 5%.
6. The decolorization of methylene blue occurred mainly by sorption. The removal of the methylene blue was 30.68% by enzyme beads based on medium viscosity alginate to 45.80% by enzyme beads based on high viscosity alginate during 10 days. For alginates beads based on lowest viscosity alginate, the dye removal process occurred only by sorption. The sorption removal efficiency on empty beads was higher than the total removal efficiency by beads with immobilized enzyme. The highest total removal efficiency of methylene blue removal (45.80%) was obtained using enzyme beads based on high viscosity alginate, where both processes, biocatalysis and sorption, occurred.
7. The results of mixture decolorization tests differ from the results obtained with single dyes. The total removal efficiency of indigo carmine from a mixture was lower than that from single dye solution, about 52.00 to 61.38%. The removal process due to biocatalysis was low and occurred only for enzyme beads based on high viscosity alginate when enzyme was dissolved in water (B_{eff} 6.56%) and for enzyme beads based on low viscosity alginate when enzyme was dissolved in buffer (B_{eff} 1.44%). For the medium viscosity alginates the sorption removal efficiency on beads without enzyme was higher than the total dye removal efficiency T_{eff} of beads with laccase. The total removal efficiency (T_{eff}) of methylene blue decolorization in the mixture was 24.49% to 50.85% for enzyme beads based on high viscosity alginate. The methylene blue removal by sorption was higher than dye removal by enzyme beads in all types of alginate used.
8. The varying mechanisms of dye removal from the dye mixture were confirmed by toxicity tests. The occurrence of both biocatalysis and sorption promotes reduced toxicity.
9. The toxicity of post-decolorization samples containing indigo carmine did not increase compared to that of the initial dye, with color removal between 55% and 72% over 10 days.
10. Simultaneous occurrence of sorption and biocatalysis processes is beneficial in methylene blue decolorization processes when using high viscosity alginate.

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https://www.wipo.int

Analiza możliwości zastosowania biopolimerów o różnej lepkości jako nośników do immobilizacji lakazy w usuwaniu barwników syntetycznych

Streszczenie. Głównym celem badań była ocena możliwości zastosowania biopolimerów o różnej lepkości (wysoka, średnia i niska lepkość) jako nośników do immobilizacji lakazy w celu usuwania barwników syntetycznych. Dekoloryzacji poddano następujące barwniki: indygo karmin (IC, barwnik anionowy), błękit metylenowy (MB, barwnik kationowy) i ich mieszaninę w stosunku molowym MB/IC=0.69, przy użyciu biopolimerów o różnej lepkości jako nośników do immobilizacji lakazy. W celu oceny toksyczności próbek poprocesowych przeprowadzono również testy toksyczności. Wyniki testów wykazały, że główny mechanizm dekoloryzacji zależy od klasy barwnika. Usunięcie IC (max. całkowita efektywność 72.15%) nastąpiło głównie na drodze biokatalizy. Dekoloryzacja MB następowała głównie poprzez sorpcję na kapsułkach alginianowych, a efektywność usuwania enzymatycznego była niska. Jednak najwyższą efektywność dekoloryzacji MB (45.80%) uzyskano przy użyciu alginianu o wysokiej lepkości, gdzie dekoloryzacja zachodziła zarówno na drodze biokatalizy jak i sorpcji. Wyniki testów odbarwiania mieszaniny różnią się od wyników uzyskanych dla pojedynczych barwników. Uzyskane wyniki wykazały różnice w efektywności procesu sorpcji barwnika w zależności od użytego do immobilizacji alginianu. Ponadto odmienne mechanizmy usuwania barwnika z ich mieszaniny zostały potwierdzone testami toksyczności. Występowanie zarówno biokatalizy jak i sorpcji sprzyja redukcji toksyczności próbek poprocesowych.