Process Biochemistry 84 (2019) 112–123

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Immobilized fungal laccase as "green catalyst" for the decolourization process – State of the art

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ARTICLE INFO

Keywords:
Laccase
Enzyme
Immobilization
Decolourization
Dye

ABSTRACT

The omnipresence of enzyme decides on their essential role in the infinite number of catalytic transformations taking place in nature as well as on their undisputed physiological significance in a series of metabolic changes. They comprise an extremely valuable tool as “green catalysts” in many technological processes in various industries. Removal of dyes from industrial wastewater constitutes a significant problem for highly developed countries resulting from the continuous economic progress and the increased production of coloured impurities. This generates the need to search for new, effective and ecological technologies for removal of toxic dyes. The enzymes, immobilised on a carrier to increase and facilitate their recovery from the post-process mixture, more and more often find application in various industries. The use of enzymes, including laccase, is a promising technology for the removal of coloured impurities from wastewater. This article reviews the application of laccase as the “green catalyst” in the decolourization process. Research results published so far indicate that the immobilization positively affects the entire protein structure of the enzyme and strengthening, which translates into an increased resistance of the enzyme to unfavourable process conditions. Selected immobilization methods and the carriers most commonly used for this purpose are also presented.

1. Introduction

Nowadays, special attention is paid to the effectiveness and positive environmental impact of technological processes. The specific properties of biocatalysts have quickly become the subject of interest in many diverse fields of science and industry [1]. A clear trend indicating a significant increase in the interest in the use of biocatalysts in many fields of science and technology is visible and confirmed by the ever-increasing value of the enzyme market [2]. Despite the high potential for the use of industrial enzymes, there are still several limitations associated with their wide commercial use. One of the most important limitations is the relatively rapid loss of activity, instability and desired performance only under certain operating conditions, as well as the lack of catalyst recovery and reuse. Achieving the goal of obtaining a specific product is difficult because most of the enzymes in the native form are not adapted to function under the extreme conditions prevailing during the industrial process. In recent years, numerous attempts have been made to improve the properties of enzymes in the context of their industrial use by immobilizing them [3–5]. Laccases, due to their catalytic and physicochemical properties, are used as biocatalysts, in the food, textile, pharmaceutical, medical, pulp and paper industry as well as in bioremediation processes [6–8]. However, the relatively low stability of free enzyme proteins and their sensitivity to changing conditions of the reaction environment supports the development of new methods to improve their properties. Currently, one of the main trends of enzyme research is their modification, improvement of functions and optimization of their performance for commercial use as biocatalysts [1]. The improvement of stability is achieved among others thanks to their immobilization on solid carriers. Immobilization also allows the reuse of immobilized enzymes, which comprises a significant advantage in comparison to free enzymes [9]. The complexity and timeliness of the problem of the presence of synthetic dyes in wastewater makes those cheap, effective and environmentally friendly removal techniques still desirable. Technologies using immobilized laccase seem to be promising in this respect.

2. Characteristics of laccase

For the first time, laccase was isolated from the Japanese *Rhus vernicifera* lacustrine tree [10,11]. Laccases are widely distributed in eukaryotes: fungi and plants as well as in prokaryotes e.g. bacteria and exhibit various functions,depending on their source organism, physiological and pathological conditions. Laccases are dimeric or tetrameric glycoproteins. Due to catalytic function, laccases depend on Cu atoms

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https://doi.org/10.1016/j.procbio.2019.05.024
Received 21 January 2019; Received in revised form 6 May 2019; Accepted 22 May 2019
Available online 23 May 2019
1359-5113/

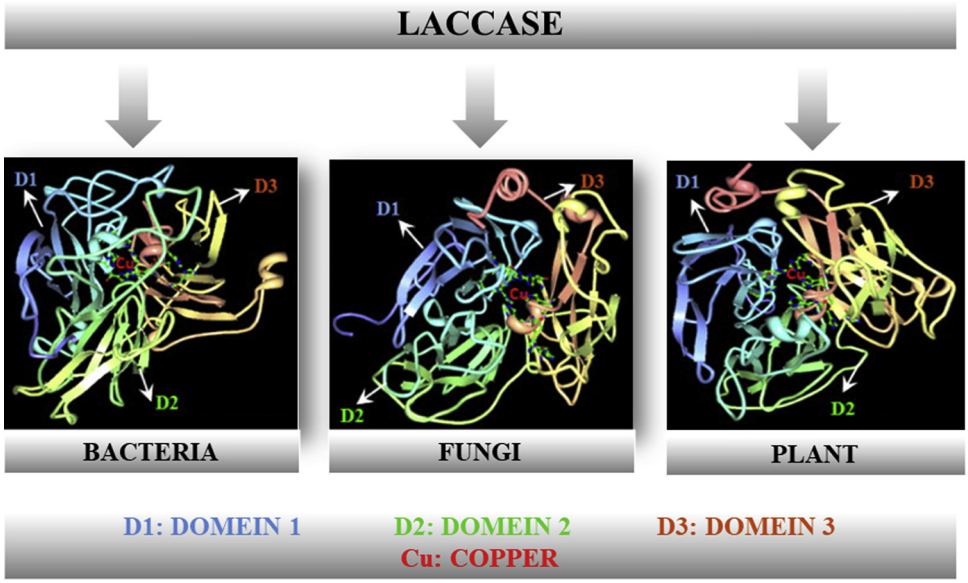

Fig. 1. Dimensional structure of (A) bacterial laccase (*Bacillus subtilis*), (B) fungi laccase (*Tramates versicolor*), and (C) plant laccase (*Populus trichocarpa*) [12, with slight modified].

that are distributed at the three different copper centers: Type-1 or blue copper center, Type-2 or normal copper and Type-3 or coupled binuclear copper centers, that differ in their characteristic electronic paramagnetic resonance (EPR) signals. A comparative analysis of laccases form bacteria, fungi and plant from the point of view of their structure and function was presented by Dwivedi et al. [12]. At three dimensional structure level, laccases (bacterial, fungal and plant) have been suggested to have three sequentially arranged cupredoxin-like domains (Fig.1).

The conducted research showed that this enzyme catalyses a series of reactions, due to which it is possible to use it in various industries [13,14]. Since bacterial laccases have low redox potential [15], fungal laccases are more preferred owing to their high redox potential. So far, the most research and developed directions of applications have been made for fungal laccase, mainly from white rot fungus. Potential for commercial use are mainly laccases obtained from filamentous fungi belonging to Ascomycota and Basidiomycota, decomposing wood such as: *Irpex lacteus, Phanerochaete chrysosporium, Pleurotus ostreatus, Trametes versicolor* [9,16–19]. Attention was also paid to saprophytic Ascomycetes, such as *Myceliophthora thermophila* and *Chaetomium thermophile*, in which laccases are involved in the process of humification during composting [13]. Most of the known fungal laccases are extracellular proteins. Nevertheless, there are also some examples of enzymes found intracellularly, mainly in fungi that cause white rot in wood [20]. Small amounts of intracellular enzyme were identified in *Agaricus bisporus.* In a two-fold way, laccase is also secreted by *Phanerochaete chrysosporium* and *Suillus granulatus* [21]. The fungal laccases are characterized by the presence of a sugar fragment (from 10 to 45% of the enzyme mass), which aims to protect it against the action of proteolytic enzymes [6,16,22]. In contrast, laccases secreted by *Bacillus subtilis, Streptomyces griseus, Azospirillum lipoferum* do not contain sugar fragments, which makes them more sensitive to the external environment and much less likely to find industrial applications [12,23–25].

There are two categories of laccases: white and yellow laccase. The white laccase exhibits neutral pH and due to anomalous metal content has unique characteristic. It exhibits absorption peak at 400 nm but absence of peak at 605 nm. White laccase has been considered under laccase family. This is due to the fact that the original structure of the white laccase is identical to those known laccase and uses oxygen (O_2) as oxidizing substrate. The production of white laccase was identified in few strains, *Pleurotus ostreatus* and *Myrothecium verrucaria* NF-05. Yellow laccase is artificially reduced blue laccase that can occur by the reduction of type I copper site by aromatic product of lignin degradation or binding of specific amino acid of enzyme polypeptide to a molecule of modified product produced by lignin degradation; it can also be due to heterogeneity induced by glycosylation. The production of white laccase was identified among others in *P. ostreatus, Panus tigrinus, Phlebia radiate, Phlebia tremellosa, Sclerotinia sclerotiorum* [14].

There are three types of reactions catalysed by laccase:

- direct oxidation of simple phenolic derivatives without the participation of a mediator (type A reactions),
- indirect oxidation of phenolic and non-phenolic substrates in the presence of a mediator (type B reactions),
- coupling reactions of reactive radicals (type C reactions) [6] (Fig. 2).

A-type reactions include the oxidation of simple organic compounds, such as mono-, di- and polyphenols, and their derivatives that contain amine, carboxyl, methoxy or sulphonic functional groups. Moreover, during the oxidation of phenols by laccases, quinones with cellular protective function are formed [26]. In addition, fungal laccases along with other enzymes are involved in the lignin degradation process. Due to their high oxidation-reduction potential, fungal laccases are capable of direct oxidation even of small phenolics, up to 10% of lignin polymer [14,27–29].

The type B reaction is an oxidation reaction of aromatic derivatives, in which large molecules (e.g. lignin complexes) or molecules with high redox potential (e.g. non-phenolic compounds or aromatic amines), thanks to low molecular weight mediators, first the enzyme oxidizes the mediator and then, the oxidized mediator reacts with the corresponding substrate. [30–39]. The most commonly used synthetic mediators are: hydroxybenzotriazole (HBT), hydroxyanthranilic acid (HAA), syringic acid, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), diammonium salt 2,2`-azyno-bis [3- ethylbenzthiazoline-6-sulphonic acid] (ABTS), hydroxyacetanilide (NHA), hydroxyphthalimide (HPI), violuric acid (VIO) [39–44]. In addition to synthetic mediators, the low molecular weight mediators that are formed during the degradation of lignin polymers also play an important role. Such compounds act as natural mediators, e.g. during the decomposition of lignin by white rot fungi. Examples of such mediators are: acetosyringone, acetovanillon and organic acids: veratric, vanillic, coumaric, ferulic [44–46]. The presence of mediators during the catalytic reaction leads to higher yields and substrate

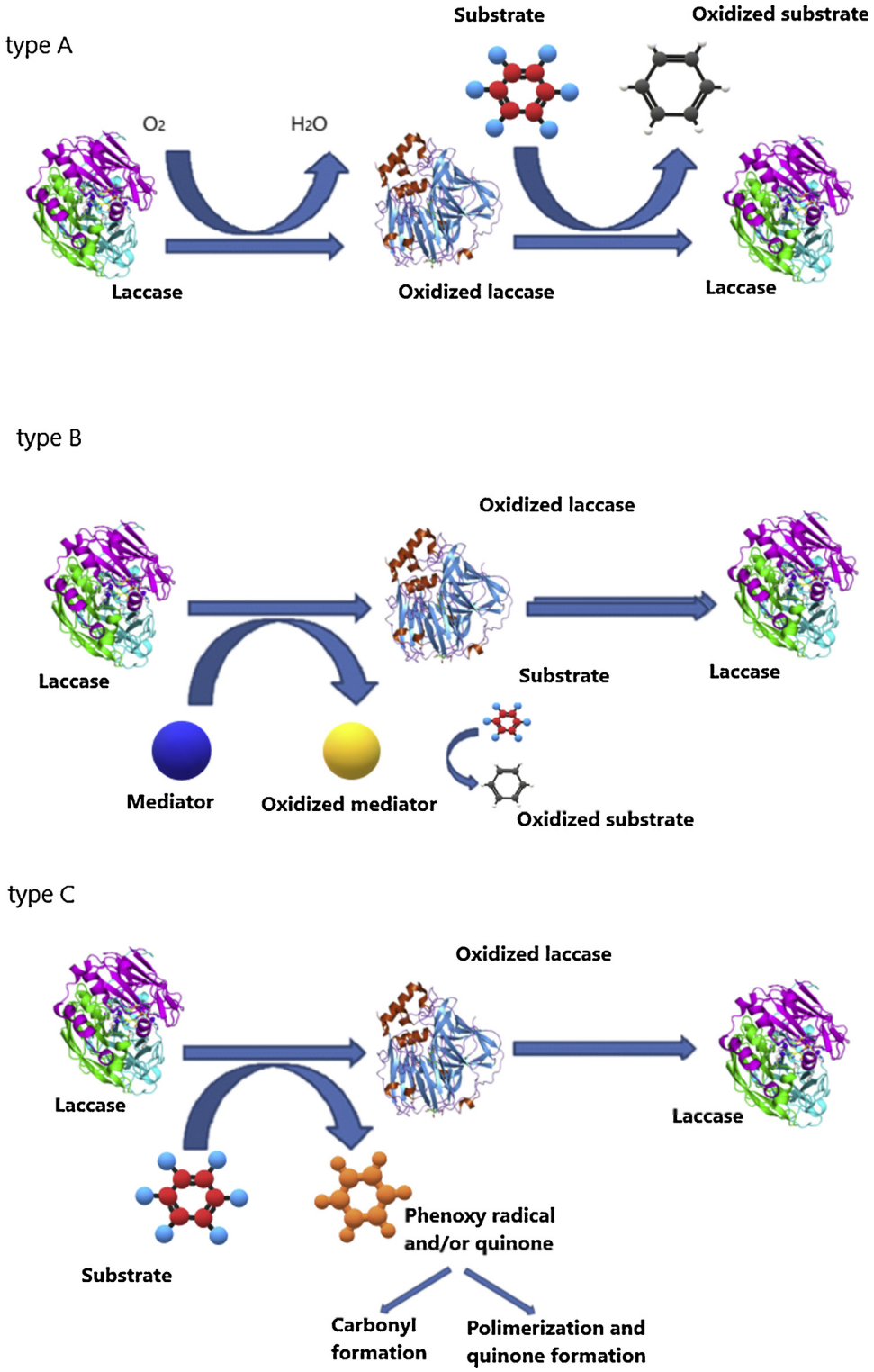

Fig. 2. Three types of reactions catalysed by laccase: type A direct oxidation, type B indirect oxidation, type C coupling reactions.

transformation rates. It also initiates other oxidation reactions of substrates in which the enzyme itself would not show activity or this activity would be negligible. Depending on the type of mediator, various oxidation products of a given substrate are formed [13,18,39,44,47]. In nature, an example of this type of reaction is the degradation of the lignin wood polymer with the presence of mediators with a low redox potential [27,48].

The third type is the C-type coupling reaction. Direct oxidation of phenolic substrates creates intermediates of a reactive nature as well as very unstable radicals. These radicals, in the result of enzymatic oxidation, can be transformed into quinones or also by non-enzymatic (hydration, polymerization, oxidation or reduction) compounds, resulting in new phenolic structures in the form of dimers, as well as oligomers and polymers [29].

3. The use of fungal laccases as green catalysts

In 2017, during the JRC-EC – CEI – ICGEB European Workshop: Smart Specialization Strategy in the Field of Biotechnologies in Europe: A Challenge for CEE Countries, the scientists emphasized that persistent organic pollutants occurring in both soil, surface and underground water are an important environmental problem [49–52]. During the workshop, it was pointed out that it is possible to solve the problem of colour contamination using fungal laccases in the processes of decolourization and detoxification [53,54]. In this context, the possibility of using enzymes as green catalysts, with reference to laccases in a wide range of technological processes, seems promising. The huge number of reports described in the literature concerns the properties, abilities and use of laccases, including fungal laccases to remove organic impurities [55–68]. A comprehensive review on the use of enzymes in many industries was presented in a work developed by Rao et al. (2014). The potential use of laccases includes: water treatment, pulp processing, degradation of polymers and xenobiotics, construction of biosensors, chemoenzymatic synthesis and oxidative polymerization [8,69,70]. As one of the most interesting solutions, they considered the use of enzymes as biosensors, which are an important tool in monitoring the environment in terms of the presence of a specific type of pollution [8]. A model example of a commercially available enzyme biosensor are test strips for the verification of biological samples such as urine or blood [71].

Fungal laccases have relatively low substrate specificity and have a high potential for application to the oxidation of a wide range of pollutants [14]. Isolated fungal oxidases can be successfully used in bioconversion processes. The effectiveness of enzymes from the group of oxidoreductases has been repeatedly confirmed in many studies on the degradation of various groups of pollutants, including phenolic compounds, polycyclic aromatic hydrocarbons (PAHs) or synthetic dyes with various chemical structures [72]. Laccases could be used in processes of detoxification of the environment contaminated with phenol, trichlorophenol, pesticides and polycyclic aromatic compounds, such as benzo (a) pyrene, and thus compounds possessing mutagenic and carcinogenic properties, widely distributed in the environment [73–75]. Fungal laccase has also found application in the bioremediation of soils contaminated as a result of agricultural activity and the use of plant protection products, including herbicides. Compounds, such as diketonitrile, can be oxidized by laccase from *T. versicolor* in the presence of ABTS, used as a reaction mediator [76]. Laccase is also used in the biodegradation of various pharmaceutical substances [77]. The ability of laccases to degrade naproxen, a compound belonging to the group of non-steroidal anti-inflammatory drugs, which pollutes both soil, ground water and water reservoirs, has been demonstrated. Laccase is widely used also in technological processes related to the cellulose-paper and lignine-cellulose industries [78–80]. This enzyme is produced, for example, by *Cerrena unicolor* and used for the delignification of natural linen products [14]. The previous experience in the use of enzymes also indicate their wide application in primary mass bleaching processes, preparation and de-inking of recycled pulp and paper production [81–83]. In the food industry, laccase is used to stabilize and improve the quality of beverages and deoxygenation of products containing vegetable fats that are easily spoiled [76]. This enzyme can remove undesirable phenolic derivatives found in too high concentrations in fruit juices and in wine. Oxidation, polymerization and subsequent precipitation of phenolic substances improve the clarity, taste, aroma and colour of these products [84]. It has also been proved that the oxidation of phenolic components which are present in the lignocellulose extracts a natural inhibitors of alcoholic fermentation, significantly improves the efficiency of ethanol production from lignocellulose by yeast [85].

Laccases also find potential application in the process of removing dyes from industrial wastewater. Traditional physicochemical methods, such as ozonation, electrochemical and ultrasound techniques, membrane filtration, photocatalysis and adsorption, and biological processes – based on the activated sludge method, are not sufficiently effective [86–89]. Combined methods are more effective, such as coagulation and flocculation combined with flotation and filtration, precipitation-flocculation using $Fe(II)/Ca(OH)_2$, electroflotation, electrokinetic coagulation, or conventional oxidation processes using ozone and UV radiation. These techniques are often highly cost-intensive and involve the accumulation of a large amount of slime or sludge and additional contamination of the treated medium, associated with the chemicals used in the process. Biological methods are ineffective due to the problem related to active sludge swelling, deformation of microbial cells, as well as the reduction of their biodiversity. Moreover, the practical application of biological processes is limited mainly by technical problems, i.e. a large area necessary for the process, limitations related to the sensitivity of microorganisms to sewage toxicity and their variable composition, low flexibility in designing and controlling the purification process [90]. Numerous studies indicate that a promising solution in this regard seems to be the use of immobilized enzymes, including laccase, for the purification of coloured wastewater [29].

4. Immobilization as a method for targeted modification of enzyme properties

The catalytic properties of enzymes are determined by many factors, which mainly include the physico-chemical parameters of the reaction environment in which the biocatalyst participates. These include: temperature, pH, ionic strength of the solution, the type of solvent used, the amount and type of ions, inhibitors and cofactors present in the mixture, the concentration of substrates, the number of active enzyme molecules available during the catalytic conversion. Relatively low stability of free enzyme proteins, their sensitivity to changing conditions of the reaction environment, a narrow range of optimal parameters in which they show high activity, supports the development of new methods to improve their properties. Immobilization of enzymes is aimed at obtaining as much of the target product as possible in the shortest possible time, with the maximum limitation of by-product formation and the possibility of reusing the biocatalyst in the process [91–93]. Immobilization of enzymes is a process known since 1916. From that time, the researchers have been continuously conducting work aimed at finding new, better methods and carriers for enzyme immobilization, as well as to optimize efficiency towards the use of commercial biocatalysts [71,94–96]. The immobilization techniques developed to date differ in the type and strength of interactions between the immobilized biocatalyst and the matrix as well as the form and role of the carrier. The type and number of interactions between the enzyme and the carrier have a decisive influence on the coupling strength, as well as on the catalytic capacity of the insoluble biocatalyst. In this context, five basic methods of immobilization can be distinguished: adsorption, covalent binding, entrapment, encapsulation, cross-linking [8,9,96,97]. The biocatalytic systems created in this way, depending on the method used, have different properties, catalytic activity, and also determine the type of process in which they can be used. It is extremely significant to select the proper immobilization method since it affects the catalytic capacity of the biocatalyst and so, the efficiency of the reaction., The carrier allows the development of an insoluble biocatalyst. As a result of this, among other advantages, the enzyme can be reused and it is possible to stop the reaction by removing the biocatalyst by a simple filtration. The wide spectrum of the use of a given carrier is therefore conditioned by such factors as: surface, volume, porosity, shape, form, stability in given reaction conditions and its cost. From the point of view of the economics of the process, the price of the carrier is essential. Therefore, the possibility of its multiple use may generate significant savings [71]. A large diversity of inorganic and organic as well as hybrid or composite materials can be used as carriers for immobilization enzyme. A general overview of the immobilization techniques and matrices are summarized in Table 1. For

Table 1
A general overview of the immobilization techniques and carriers used for the immobilization of enzymes.

Type of carrier	Immobilization technique	Results	Reference
NANOCARRIERS			
Fe_3O_4/SiO_2 nanoparticles	Cross-linking	- almost 100% of color (Procion Red MX-5B) removal was observed in one hour - the half life time of the immobilized laccase towards the decolorization of Procion Red MX-5B is close to 50 cycles - after storage at 4 C for 5 months the activity of the immobilized laccase still remain 96% of colour removal in one hour	[98]
Cellulose nanofiber		- the half life of the immobilized laccase was retained for up to 8 cycles and had greater than 85% SDE decoloration for up to 5 cycles - the thermal and pH stability of the immobilized laccase was significantly improved	[99]
Polyacrylonitrile-biochar composite Nanofibrous membrane	Amidoxime linkage	- storage, temperature and pH stability, - degradation of chlortetracycline (efficiency in the range 226% - 583%), - lost about 50% of initial activity after 7 days of operation, - lost about 662% of initial activity after 7 days of operation.	[100,101]
Cross-linked enzyme aggregates (MAC-CLEAs) on magnetic nanoparticles and chitosan	Cross-linking	- complete removal of acetaminophen, diclofenac, mefenamic acid, atenolol and epoxy carbamazepine within 12 h of incubation, - partial removal of fenofibrate, diazepam, trimethoprim, and ketoprofen, - the catalytic activity was maintained even after a hundred and fifty batch reactions.	[102]
GO nano-sheets	Covalent attachment	- laccase loading of 156.5 mg/g and immobilization yield of 64.6% at laccase concentration of 0.9 mg/ mL - the average decolorization effectiveness of the nanobiocatalyst was more than 75% azo dyes (R23 and AB92) after six cycles, implies its excellent operational stability and good reusability.	[103]
Cu (II)-chelated chitosan-graft-poly (glycidyl methacrylate) nanoparticles	Adsorption on Cu-chitosan	- phenol was removed above 80% in 4 h, - 2,2′-azino-bis(3-ethylbenzthiazoline-6- sulfonic acid) as mediator enhances the phenol degradation efficiency, - reusable up to 8 times.	[104]
Cross-linked protein-metal hybrid nanoflower (CL-NF)	Cross-linking	- the catalytic efficiency of CL-NF laccase was 2.2-fold higher than that of free laccase. - CL-NF laccase showed 2.6-fold higher oxidation potential than free laccase towards phenolic compounds. - up to 10 times of resue in batch conditions.	[105]
Graphene oxide/CuFe2O4/laccase nanocomposite	Covalent bonding	- storage, temperature and pH stability - recoverable laccase (could be reused up to 10 times), - little loss of activity	[106]
Fumed silica nanoparticles/laccase composite	Cross-linking	- more than 81% residual activity in term of micropollutant elimination from wastewater - after 7 days. - with 81.1 ± 0.4% residual activity after 7 days	[107]
MICROCARRIERS			
Ca-alginate beads	Encapsulation	- the immobilized and free laccase maintained 91.2% and 25.6% of their initial activities, respectively, after 90 min of incubation at 55 °C. - at the end of 20 days of storage, the immobilized and free laccase retained about 82.7% and 22.4% of their initial activities, respectively - the immobilized laccase exhibited efficient textile dye decolorization in several successive batches	[108]
Cu-alginate beads	Encapsulation	- triclosan (TCS) removal (89.6%) after 8 h and RBBR decolonization at a range from 54.2% to 75.8% after 4 h.	[109]
Chitosan beads	Cross-linking	- immobilized laccase was less sensitive to changes in pH and temperature, and to storage time, compared with free laccase - the removal effi ciency of metal-complex dye Acid Black 172 by immobilized laccase (68.84%) was 1.22 times higher than that by free laccase.	[110]
Alginate-chitosan microcapsules	Encapsulation	- the immobilized laccase dis-played a lower activity and a lower substrate affinitythan the free enzyme - immobilization improved its stability to other parameters including temperature and pH. - immobilized laccase retained 35.73% activity afterthree reaction cycles	[111]
Biochar	Chemical and physical process of attachment	- high thermal stability of laccase, - degradation of 714% of HO-DiCB	[112]
GA-CBs	Covalent attachment	- crosslinked chitosan beads were used as biosorbents and enzyme immobilization supports, - the removal of SB15 and SBGD reached - 98.0% and 49.5%, respectively, after 12 h of reaction time (an initial dye concentration was 100 mg/L, initial laccase activity was 2.24 U L-1 - the overall removals of SB15 and SBGD from binary mixtures containing 100 mg/l of SB15 and 100 mg/l of SBGD was 78.2% and 52.6%, respectively	[113]

(*continued on next page*)

Table 1 (*continued*)

Type of carrier	Immobilization technique	Results	Reference
PVA-beads	Cross-linking	- very effective (90%) laccase immobilization - stability more than 70% after 5 months of storage at 4 °C - continuous decolorization in a packed bed bioreactor led to nearly 90% decolorization of Acid fiolet 17 for up to 5 days.	[114]
Chitosan/CeO_2microspheres	Adsorption or covalent binding	- the thermal and operational stabilities of the immobilized laccase compare to free laccase were significantly improved - the removal rates of methyl red and orange II by theimmobilized laccase were 83.3% and 92.6%, respectively, which are much higher than that of free laccase(i.e., 49.0% and 67.1%, respectively).	[115]
Sponge-like hydrogel	Encapsulation	- the Lac-PAM-CTS showed enhanced thermal and chemical stability as compared to free laccase - in a continuous six-cycle batch decoloration of MG, immobilized laccase showed much better durability than the free laccase.	[4]
Mesoporous carbon	Adsorption	- immobilization increases the stability of laccase with pH - immobilization of laccase resulted in a decrease in activity enzyme	[116]
Hydrophobic sol-gel	Entrapment	- immobilized laccase was more efficient decolorizer of all reactive dyes as compared to its free counterpart. - immobilization of enzyme led to improve its catalytic and thermostability and reusability.	[117]
Hydrophobic sol-gel	entrapment	- 97.3% decolorization during 24h - immobilized laccase displayed enhanced tolerance against high temperature and inactivating agents	[118]
Polysaccharide-conjugated laccase	Conjugation	- The T1/2 values for conjugated laccase at 50 and 60 °C 3.73 and 2.80-fold, respectively higher than for free one. - Conjugation increase the decolorization rate - up to 36% and 26% of RBBR and Malachite	[119]
Yeast cells	Adsorption on the yeast cells surface	- good storage stability - over 90% of the initial enzyme activity after 25 days storage, - maintaining up to 74% of its initial - activity after 8 repeated batch reactions. - retained 74% of initial activity after eight repeated batch reactions.	[120]
Granular activated carbon (GAC-bound laccase)	Adsorption on GAC	- higher degradation efficiency of pharmaceuticals in comparison to free laccasse (10 - 50% difference) - higher reusability and stability over a broad range of pH and temperature.	[121]
Magnetic mesoporous silica microbeads	Cross-linking	- catalytic potential to transform acetaminophen, mefenamic acid, fenofibrate, and indomethacin, - retained 70% of initial activity after ten repeated batch reactions.	[122]
Porous silica beads	Adsorption	- immobilized laccase removed 71.7% of sulfathiazole (STZ) and 53% of sulfamethoxazole retained 63.3% and 82.6% of the initial activity of the immobilized laccase toward SMZ and STZ after eight successful repeated batch operations.	[123]
Ceramic membrane reactor	Adsorption	- the degradation rate of 0.34 mg of tetracycline per hour was maintained during 10 days	[124]
Nanoporous Zeolite-X (ZX)	Adsorption	- 100% activity after 7 successive decolorization cycles - higher stability over a broad range of pH and temperature.	[125]

the purposes carriers are divided into two main groups, nanocarriers and microcarierrs.

The commonly used group of carriers are natural polymers, such as chitin/chitosan, cellulose, alginates, agar, gelatin and starch [126–131]. Due to their polymeric structure, thanks to which they obtain a significant contact surface with the enzyme, they have high affinity for proteins, and their biocompatibility makes them not toxic to enzymes. The components that build biopolymers are polysaccharides (starch, alginates, pectins), polypeptides, polynucleotides [132,133]. The use of biopolymers reduces the risk of contamination of the product of the catalysis by residues of substances that are used in the synthesis of artificial polymers. Biopolymers also have the ability to create three-dimensional networks. This process consists in the formation of hydrogen, hydrophobic and covalent bonds or ionic interactions between molecules [134]. This mechanism is used in processes of enzyme immobilization. Alginates are important as enzyme carriers [135]. They are linear copolymers composed of two types of monomers: α-L-guluronic acid (G blocks) and β-D-mannuronic acid (M blocks). During the encapsulation process with alginate, a three-dimensional network with liquid regions is formed, within which biocatalyst molecules can be immobilized [136]. Immobilization of particles or microbial cells by entrapping is used on a large scale in the cosmetics [137], pharmaceutical [138–140], chemical industry [141], in medicine in the construction of artificial organs [142–144], and also as an innovative immobilization method of sewage sludge on a hydrogel carrier, made of cross-linked sodium alginate [145].

Another, widely used biopolymer for the immobilization of enzymes is chitosan, obtained in the process of de-*N*-acetylation of chitin, a natural building material found in fungi, molluscs and insects [146]. Depending on the source of chitin (α-, β- and γ-chitin) and the de-*N*-acetylation reaction parameters used (homogeneous or heterogeneous reaction), the commercially available chitosan preparations differ significantly in their physicochemical properties [133,147,148]. Chitosan can be stabilized by chemical cross-linking, cross-linking agents such as dialdehydes (glutaraldehyde (GA), glyoxal), genipin, epichlorohydrin (ECH),ethylene glycol, could be used [149,150]. Another known technology in the biopolymer immobilization is gelatin, which is a protein derived from the denaturation of collagen. It contains a high concentration of hydroxyproline, proline and glycine. Due to the high film-formation capacity, biocompatibility and non-toxicity, it is used for encapsulation in combination with other anionic polysaccharides [151–153].

Systems made of two or more types of carriers are also used for immobilization. These include, for example, silica-magnetite [154], magnetite-nanoparticles of gold [155], chitosan-alginate, silica-chitosan [156] or silica in combination with various polymers [157,158]. The advantage of using such systems is the ability to precisely design their properties in terms of a particular enzyme and the process in which they will be used. In addition, these materials combine the properties of both carriers, which increases functionality and extends the range for potential applications. The immobilization of enzymes enables catalytic transformations to be carried out in continuous and flow bioreactors. An evaluation of the activity and stability of the laccase biocatalyst showed its efficacy in the successful elimination of the pollutants [159]. Moreover, an undoubted advantage of such systems in potential industrial applications is primarily a better control of the processes carried out, mainly due to the possibility of quickly separating the biocatalyst from the reaction mixture and the greater purity of the reaction products. This factor is of particular importance in the food, pharmaceutical and cosmetics industries, where the presence of undesirable compounds prevents its further processing [160].

Drozd et al. (2018) used bacterial cellulose (BC), obtained from the bacterial cultures of *Gluconacetobacter xylinus,* modified and unmodified as a result of the rotating magnetic field, as a carrier for the immobilization of laccase from *Trametes* sp. The influence of temperature and pH on the activity of free and immobilized laccase was investigated. Free laccase had its maximum activity in the pH range from 3.0 to 4.0, while the immobilized enzyme (on both tested carriers) showed a significantly lower activity at pH 3.0 and reached its maximum at pH 4.0. Moreover, free laccase showed much lower activity at pH 5.0, while the activity of the immobilized enzyme was still maximum at this pH. The effect of temperature on the activity of free and immobilized laccase was studied in the range from 30 °C to 80 °C. Laccases immobilized on BC showed an optimum activity at 60 °C, whereas immobilized on a modified carrier showed maximum activity at 70 °C. The observed shift of the optimal temperature may suggest the presence of structural changes in the modified carrier. After 7 catalytic cycles, the laccase immobilized on the modified BC retained 70% of the initial activity, whereas the laccase immobilized on the unmodified BC maintained 50% of the initial activity. The obtained results indicate a high potential of biocellulose as a carrier for enzymes, which can be used in many innovative industrial applications. Modification of bacterial cellulose by a rotating magnetic field leads to favourable changes in the structure of the carrier, resulting in better immobilization of the enzyme, which in turn affects the higher operational stability of the catalyst [161].

Over the last decades the great growth of nanotechnology has allowed the development of new carriers. The carbon nanomaterials that have been obtained so far include such carriers as carbon nanotubes (CNTs), which are the most popular nanomaterial for the immobilization of enzymes, including laccases. CNTs on the background of other nanoparticles are characterized by high environmental stability, ease of preparation and, above all, the possibility of depositing more protein on the surface, which is confirmed by the results obtained by Costa et al. (2019) [162]. Other carbon nanocarriers used for immobilization of laccase are also carbon nanoparticles [163]. The effective elimination of bisphenol A (BPA) was obtained in studies carried out using fungal laccase from *Polysona Coriolopsis*, immobilized on silica nanoparticles [164] and a laccase from *Trametes versicolor*, used in immobilized form on nanoparticles, including carbon nanotubes. In the second case, over 80% of the effectiveness of direct degradation of BPA was observed compared to free laccase, for which a degradation efficiency of 55% was noted [98,165,166]. In comparison to compounds with micro or macro particles, nanoparticles are characterized by high mechanical strength as well as exceptional surface properties that allow more effective interaction with many biological molecules. Graphene oxide (GO) attracts a lot of interest in the scientific community, because it is characterized by high stability in water and a large surface area, on which there are oxygen functional groups. Despite the huge potential associated with the use of GO, literature reports on its impact on the catalytic properties of immobilized molecules are still scarce. Theresearch in this field was carried out by Kashefi et al. (2019). The obtained results prove that graphene oxide is an interesting carrier for immobilizing laccase aimed at removing coloured substances from the aquatic environment [103].

5. Immobilized fungal laccase for removing dyes

Methods using immobilized laccase are an attractive alternative to conventional dye removal technologies used at present, due to their ability to degrade dyes of diverse chemical structure. In comparison with the free, native enzyme, stabilization of the protein structure by immobilization causes its strengthening, which in turn determines the possibility of extending the storage time of the immobilized preparation. Zheng et al (2016) entrapped a laccase from *Trametes pubescens* onto chitosan and evaluated the decolorization capacity of the resulting biocatalyst. Free and immobilized enzyme allowed to achieved almost the same degree of degradation of Acid Black 172 (69%). After 30 days, free enzyme had only 15% of its initial activity while the entrapped enzyme in chitosan and cross-linked with glutaraldehyde kept 40%. Moreover, immobilized laccase had higher pH (range 3.5–12.0) and thermal stabilities than free enzyme. After 2 hs at 60 and 70 °C the

Table 2
Decolourization studies of synthetic dyes by immobilized laccase [96, modified].

Fungal strain	Immobilization matrices	Immobiliza-tion technique	Dyes	Decolourization (%)	Reaction time	Reference
P. ostreatus	Fe_3O_4/SiO_2 nanoparticles	Cross-linking	PR MX-5B	100	20 min	[98]
T. versicolor	Copper alginate beads	Encapsulation	RBBR	75.8	4 h	[109]
T. versicolor	GA-CBs	Covalent attachment	SB15	98	12 h	[121]
			SBGD	49.5		
T. pubescens	Chitosan beads	Cross-linking	AB 172	68.84	48 h	[110]
			CV	54.24		
			DFB FBL	56.28		
			RBB X-BR	52.26		
			RBBR	48.23		
			IB	45.12		
			NR	44.58		
			NG B	37.18		
			MB	25.39		
			CV	20.81		
Cyathus bulleri	PVA-beads	Cross-linking	AR 27	95		[114]
			SE	90		
T. versicolor	Chitosan/CeO_2microspheres	Adsorption or covalent binding	MR	83.3	12 h	[115]
T. versicolor	Sponge-like hydrogel	Encapsulation	AO7	≥ 90	5 h	[4]
			MG	≥ 90		
T. versicolor	Mesoporous carbon	Adsorption	AO7	≥ 90	72 h	[116]
			AB74	≥ 90		
			RR2	≤ 90		
			RB5	≤ 90		
Coriolopsis gallica	Ca-alginate beads	Entrapment	RBBR	90.3	24 h	[108]
			LG	86.5	24h	
			RB5	78.2	8 h	
			BBR	58.2	24h	
P. florida NCIM 1243	Cellulose nanofiber		RBBR	96	12 h	[99]
			RB5	95		
			RR120	89		
			RO16	88		
			RBV5R	79		
			SDE	70		
T. versicolor	Gum Arabic	Entrapment	RBBR	53	2 h	[170]
T. versicolor	Hydrophobic sol-gel	Entrapment	DB K2RL	100	5 h	[117]
			CRT	100	5h	
			DO KGL	≥ 90		
			DBR K4BL	≥ 90		
			RBY 3GL	≥ 90		
			SIT	97		
			NIT	≥ 90		
			KNT	≥ 90		
G. lucidum	Hydrophobic sol-gel	Entrapment	MTE	97.3	24 h	[118]
			CTE	91.5		
			ATE	85		
			CTE	77.8		
Novozyme	Alginate-chitosan microcapsules	Encapsulation	AR	66	6 h	[111]
Trichoderma harzianum	Ca-alginate	Entrapment	MG	61	20 min	[168]
	beads		MB	41	20 min	
	Cu-alginate		CR	33	20 min	
	beads		MG	70	20 min	
	Ca-alginate-chitosan		MB	52	20 min	
	beads		CR	45	20 min	
	Sol-gel matrix		MG	85	16 min	
			MB	66	20 min	
			CR	54	20 min	
			MG	90	16 min	
			MB	87	18 min	
			CR	53	20 min	
Trametes versicolor IBL-04	Chitosan beads	Cross-linking	S.F Violet P4RN	98.7	4h	[171]
			S.F. Golden Yellow CRL	97.6		
			S.F. Black BR	96.9		
			S.F. Turq Blue GWF	89.36		
A. tenuissima KM651985	Polysaccharide-conjugated laccase	Conjugation	RBBR	36	5h	[119]
			MG	26	5h	
Aspergillus	GO nano-sheets	Covalent attachment	DR23	88.7	60 min	[103]
			AB92	48.7	60 min	

entrapped enzyme retained more than 50% of its initial activity, while only 22% was recovered for free enzyme [. Moreover, the free enzyme showed significant loss of activity during storage. After 30 days, the activity decrease to about 15%. In the case of immobilization of this protein by entrapment in chitosan, and then after cross-linking with glutaraldehyde, its activity after the same storage period was about 40%. Moreover, immobilized laccase was less sensitive to changes in pH (range 3.5–12). In addition, after incubation at 60 and 70 °C for 2 h, it showed activity at > 50%, while the activity of free enzyme reached only 22.3%. It was found that the immobilized enzyme showed better

operational stability and the possibility of reuse [110]. In a work published by Daassi et al. (2014) laccase from *Coriolopsis gallica* was entrapped into calcium alginate beads. In order to maintain high catalytic activity of preparations after immobilization (up to 85%), as well as to obtain a high yield immobilization process (96%), a volume ratio of calcium ions to alginate of 1:4 was used. The preparation thus obtained was used in the process of removing aqueous dyes from wastewaters from textile industry. The enclosed of the enzyme in the biopolymer shell allowed to maintain the catalytic activity of the preparation over a longer period of time. The degree of removal of Bismark Brown R dye dissolved in the analysed solution was over 50% [108].

Immobilized enzyme preparations are of great industrial interest due to the fact that they can be used by more than one catalytic cycle, while maintaining satisfactory efficiency. The above observations are confirmed by studies carried out by Chao et al. (2013). In order to immobilize the laccase from *Trametes versicolor*, halloysite nanotubes were used, the surface of which was modified with dopamine to improve enzyme loading on the carrier. About 170 mg of enzyme was immobilized per gram of the carrier. It has been shown that laccase systems - halloysite nanotubes can be used without significant loss of activity even up to ten consecutive catalytic cycles. In addition, after 30 days of storage immobilizad laccase retained over 90%, while the free enzyme retained only 32% of the activity [167]. The possibility of multiple uses of the biocatalyst is one of the important factors determining the profitability of the technology. Bagewadi et al. (2017) studied the effectiveness of decolourization of malachite green, methylene blue and Congo red by laccase from *Trichoderma harzianum* both free and immobilized on various carriers: alginate, chitosan, alginate-chitosan and sol-gel matrix. The highest immobilization efficiency (93%) was obtained on a sol-gel carrier. Moreover, this insoluble biocatalyst kept the catalytic activity over a wide pH range (4.0–7.0) and high temperature (more than 90% at 65 °C). Besides, it could decolorize malachite green (90% after 16 h of incubation), blue methylene (87% after 18 h) and Congo red (53% after 20 h) [168]. Asgher et al (2012) also showed an improvement in thermal stability and decolourization capacity by immobilizing laccase from *Pleurotus ostreatus* IBL-02 on a sol-gel carrier [117].

Analysis of the kinetics of enzymatic transformation by free and immobilized laccase from *Trichoderma harzianum* strain HZN10 showed a greater affinity of the enzyme to the substrate of immobilized laccase. In this case, the K_m was 2.0 mM, while for the free enzyme reached 0.5 mM. The reaction rate (V_{max}) with respect to the immobilized laccase was about twice as high as for the free enzyme and was 500 U/mg for the enzyme immobilized and 285 U/mg for the native enzyme, respectively. Higher values for the immobilized enzyme indicate a lower affinity for the substrate, which is probably related to the change in protein conformation and the difficult access to the active enzyme centre [168]. This is also confirmed by the results obtained by Asgher et al. (2012) [117] and Gahlout et al. (2014) [169]. The use of immobilized fungal laccase is a promising technology for the treatment of wastewater and removing coloured impurities, which is confirmed by many literature reports. The table below presents the most interesting results of the research carried out so far in this respect (Table 2).

6. Conclusion

Dyes are compounds with diverse chemical structure. They are most commonly used in the textile, clothing, paper, cosmetics, dye and plastic industries. Their toxicity results from a complex chemical structure that provides high stability and resistance to chemical, photolytic and biological degradation. New, more effective and environmentally friendly methods of removing coloured substances are searched for. Therefore, the development of processes based on immobilized laccase is of interest due to their potential to degrade different types of dyes. In the light of current literature reports, enzyme immobilization technology offers the possibility of beneficial modification of the catalytic and physico-chemical properties of enzymes in terms of specific applications in continuous industrial scale processes. Modified properties of the enzyme make them more stable and resistant to reaction environment and the possibility of their reuse leads to higher catalytic efficiency and lower process costs.

Acknowledgments

The presented study was performed in the framework of the research work in the Central Mining Institute in Poland [No. 14508017-343], financially supported by the Polish Ministry of Science and Higher Education [No. 3989/E-263/M/2017].

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Operational stability of laccases under immobilization conditions

Stabilność operacyjna lakaz w warunkach immobilizacji

DOI: *10.15199/62.2020.3.22*

A review, with 85 refs., of methods for enzymes immobilization on org. and inorg. carriers as well as of the impact of enzyme immobilization on the kinetics of biocatalyst.

W celu otrzymania komercyjnych biokatalizatorów do zastosowania w procesach technologicznych prowadzone są badania dotyczące białek enzymatycznych w kierunku ich modyfikacji, doskonalenia oraz optymalizacji ich wydajności. W pracy dokonano przeglądu literatury dotyczącej immobilizacji jako obiecującej techniki w zakresie poprawy stabilności operacyjnej lakazy. Innowacyjne preparaty enzymatyczne składające się z immobilizowanych lakaz, z uwagi na wysoką stabilność operacyjną, mogą znaleźć zastosowanie w różnych gałęziach przemysłu (w szczególności do oczyszczania ścieków barwnych).

Wszechobecność białek enzymatycznych stanowi o ich istotnej roli w nieskończonej liczbie przemian katalitycznych zachodzących w naturze, a tym samym niekwestionowanego znaczenia fizjologicznego w wielu przemianach metabolicznych. Obecnie, kiedy szczególną uwagę przywiązuje się do sposobu prowadzenia procesów technologicznych, aby były one nie tylko efektywne, ale również przyjazne środowisku, enzymy stanowią niezwykle cenne narzędzie jako „zielone katalizatory" w wielu procesach technologicznych w obrębie różnych gałęzi przemysłu. Stąd przejście od traktowania białek enzymatycznych jako cząsteczek o właściwościach katalitycznych do ich praktycznego zastosowania w przemyśle jest wyjściem naprzeciw potrzebom współczesnej cywilizacji. Widoczny jest wyraźny trend, który wskazuje na znaczący wzrost zainteresowania wykorzystaniem enzymów jako biokatalizatorów w wielu dziedzinach nauki i technologii, czego potwierdzeniem jest nieustannie rosnąca wartość rynku enzymów[1, 2]. Biotechnologie, w których wykorzystuje się enzymy określane są jako „białe biotechnologie" i stanowią jeden z kluczowych sektorów badań w kierunku innowacji, znacznie przyczyniają się do rozwoju przemysłu wysokich technologii, a tym samym poprawy warunków i jakości życia społeczeństwa. Aktualność i ważność nakreślonej tematyki potwierdzają wnioski z warsztatów JRC-EC przeprowadzonych w Bratysławie (Słowacja) 4–6 września 2017 r.[3–6]. W publikacji[4] poświęconej tym warsztatom poruszono tematykę wybranych obszarów biotechnologii, kierując się najnowszymi trendami w tym zakresie. Wśród nich jako istotny problem wskazano zanieczyszczenie środowiska takimi związkami organicznymi, jak barwniki syntetyczne. Oceniono możliwości zastosowania enzymów, w tym w szczególności lakazy, w procesie dekoloryzacji oraz detoksyfikacji zanieczyszczeń[7, 8].

W celu otrzymania komercyjnych biokatalizatorów do zastosowania w procesach technologicznych trwają badania dotyczące białek enzymatycznych w kierunku ich modyfikacji, doskonalenia oraz optymalizacji ich wydajności[9, 10]. Dotychczasowe doniesienia literaturowe wskazują, że immobilizacja stanowi obiecującą technikę poprawy ich stabilności operacyjnej w trudnych warunkach procesu technologicznego[11–15]. W tym kontekście możliwość zastosowania immobilizowanych enzymów jako „zielonych narzędzi", ze szczególnym uwzględnieniem lakazy wydaje się obiecująca[1, 16–18].

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Lakaza „zielone narzędzie" białej biotechnologii

Lakaza (oksydoreduktaza tlenowa, EC 1.10.3.2), zaliczana do dużej rodziny niebieskich oksydaz wielomiedziowych MCOs (*multicopper oxidases*) zawiera 4 atomy miedzi w centrum aktywnym[19–21]. Masa cząsteczkowa lakaz wynosi 38–150 kDa, chociaż istnieją doniesienia, że niektóre lakazy występujące w grzybach, takich jak *Pleurotus eryngii* i *Podospora anserina* mają masę cząsteczkową odpowiednio 34 i 383 kDa[22]. Cztery atomy miedzi w miejscu aktywnym enzymu reprezentują trzy typy specyficznych właściwości. Podziału tego dokonano na podstawie wyników analizy spektroskopowej elektronowego rezonansu paramagnetycznego (EPR) centrum aktywnego lakazy. Wykazano aktywność paramagnetyczną dla miedzi typu I (T1) i typu II (T2) oraz brak sygnału dla typu III (T3) jako efekt antyferromagnetycznego sprzężenia pary atomów miedzi typu III. Typ T1 miedzi nadaje cząsteczce enzymu niebieską barwę (stąd nazwa niebieska oksydaza), stanowi także miejsce utleniania substratu, jego forma utleniona wykazuje absorbancję przy długości fali 610 nm. Miedź typu T2, a także dwa atomy typu T3 tworzą kompleks, w którym zachodzi wiązanie i redukcja tlenu do wody[21, 23].

Lakazy są szeroko rozpowszechnione w takich roślinach, jak *Rhus vernicifera* i *Rhus succedanea*[24], grzybach zgnilizny drewna (*Trametes versicolor*, *Trametes hirsuta*, *Trametes ochracea*, *Trametes villosa*, *Trametes gallica*, *Ganoderma brownie*, *Ganoderma curtisii*, *Ganoderma lobatum*, *Ganoderma lucidum*, *Cerrena maxima*, *Coriolopsis polyzona*, *Lentinus tigrinus* i *Pleurotus eryngi*[21, 25], *Polyporus versicolor* A, B, *Pleurotus* spp., *Pholiata* spp., *Podospora anserina*, *Neurospora crassa*, *Aspergillus nidulans* i *Pyricularia oryzae*[26, 27]) oraz w grzybach saprofitycznych workowców, takich jak *Myceliophthora thermophila* i *Chaetomium thermophile*[25]. Lakazy znajdują się również w bakteriach, takich jak *Bacillus subtilis*, *Streptomyces griseus* i *Azospirillum lipoferum*[28–31]. Występują także u owadów, gdzie uczestniczą w procesie sklerotyzacji i pigmentacji[32–35].

Grzybowe lakazy najczęściej występują w postaci kilku monomerów (50–110 kDa), które w procesie oligomeryzacji tworzą multimerowe kompleksy. Od innych lakaz odróżnia je obecność w cząsteczce grupy cukrowej, stanowiącej zwykle 10–45% całej cząsteczki enzymu. Reszta cukrowa składa się głównie z mannozy, *N*-acetyloglukozaminy oraz galaktozy[31, 36]. Lakazy katalizują reakcje utleniania aromatycznych i niearomatycznych związków przy równoczesnej redukcji tlenu cząsteczkowego do wody. Właściwości te czynią ten enzym cennym „zielonym narzędziem" wykorzystywanym w wielu gałęziach przemysłu i ochronie środowiska.

Rozróżnia się trzy typy reakcji katalizowanych przez lakazę: (*i*) utlenianie bezpośrednie prostych pochodnych fenolowych bez udziału mediatora (reakcje typu A), (*ii*) utlenianie pośrednie substratów fenolowych i niefenolowych w obecności mediatora (reakcje typu B) oraz (*iii*) reakcje sprzęgania reaktywnych rodników (reakcje typu C)[32].

Sposoby immobilizacji i dobór nośnika

W 1916 r. Nelson i Griffith dokonali pierwszej immobilizacji inwertazy z drożdży. Sam termin „immobilizowany enzym" wprowadzono w 1971 r.[37, 38]. Znaczący wzrost zainteresowania tematem immobilizacji enzymów miał miejsce w latach sześćdziesiątych XX w. Pierwsze badania o tej tematyce obejmowały wytwarzanie L-aminokwasów (L-asparaginian) przy udziale immobilizowanej aspartazy z *E. coli* oraz z oksokwasów w reaktorach membranowych[37, 39]. Stosowanie unieruchomionych enzymów umożliwia znaczne uproszczenie konstrukcji samego reaktora oraz kontrolę procesu np. przerwanie/zatrzymanie procesu poprzez oddzielenie katalizatora od mieszaniny reakcyjnej. Ponadto immobilizacja prowadzi do zwiększenia stabilności enzymu poprzez wielopunktowe oddziaływanie z powierzchnią nośnika, stwarza korzystne mikrośrodowisko, a także chroni przed oddziaływaniami międzycząsteczkowymi[40–43].

Metody immobilizacji obejmują pułapkowanie, kapsułkowanie, sieciowanie oraz immobilizację kowalencyjną i adsorpcyjną[44]. Nie ma jednej, uniwersalnej techniki immobilizacji, a wybierając metodę unieruchamiania, należy rozpatrzyć takie czynniki, jak oddziaływanie enzym-substrat/produkt, enzym-nośnik, substrat/produkt-nośnik, odporność nośnika na warunki reakcji (rozpuszczalnik, temperatura, pH) a także rodzaj procesu technologicznego, w którym zastosowanie znajdą immobilizowane enzymy[40, 43, 45].

Liczne badania wskazujące na immobilizację jako obiecującą technikę poprawy stabilności operacyjnej enzymów spowodowały rozwój badań nad ulepszaniem istniejących lub poszukiwaniem nowych nośników do immobilizacji enzymów. Obszernego przeglądu nośników z podziałem na klasyczne i nowe materiały dokonali Zdarta i współpr.[46]. Wyróżniono nośniki nieorganiczne, organiczne, a także materiały hybrydowe i kompozytowe. Aby materiał mógł zostać wykorzystany jako nośnik, musi posiadać odpowiednie właściwości oraz spełniać wiele kryteriów. Przede wszystkim matryca musi wykazać zdolność do związania enzymu, jednak bez znaczącej utraty jego aktywności katalitycznej. Najważniejsze parametry determinujące wybór nośnika obejmują odporność chemiczną, stabilność w środowisku reakcji, wysokie powinowactwo do enzymu, brak negatywnego wpływu na enzym, obecność reaktywnych grup w strukturze cząsteczki nośnika, dostępność na rynku i stosunkowo niską cenę oraz możliwość ponownego wykorzystania. Ponadto przy wyborze matrycy należy wziąć pod uwagę temperaturę, lepkość, polarność, a także charakter kwasowo-zasadowy mieszaniny reakcyjnej, w której pracować będzie immobilizowany enzym[46].

Do klasycznych nośników nieorganicznych zalicza się szkło porowate, krzemionkę, tlenki metali, hydroksyapatyt, kordieryt, ziemię okrzemkową, bentonit, kaolinit i montmorylonit. Nośniki nieorganiczne dzielą się na syntetyczne i naturalne (biopolimery). Wśród syntetycznych można wyróżnić żywice jonowymienne, żywice epoksydowe, poli(chlorek winylu), polistyren, poliuretany i poliamidy[47–51]. Do najczęściej stosowanych biopolimerów należą chityna/chitozan, celuloza, alginiany, agar, żelatyna oraz skrobia[52–57]. Do nowoczesnych materiałów nośnikowych należą nanomagnetyczne nośniki[58], nanocząstki[59], nanorurki węglowe[60], grafen i tlenek grafenu[61]. Na uwagę zasługują także porowate materiały krzemionkowe. Są one nietoksyczne, mają dużą odporność termiczną i mechaniczną, są odporne na biodegradację oraz wpływ rozpuszczalników organicznych. Ponadto opracowano stosunkowo proste metody modyfikacji ich powierzchni różnymi grupami funkcyjnymi[62–64]. Zainteresowanie budzą także klasyczne żele krzemionkowe oraz mezoporowate materiały o uporządkowanej strukturze, otrzymywane metodą zol-żel za pomocą tzw. szablonów (*templates*), takie jak mezoporowate pianki komórkowe (MCF) i mezoporowate sita typu SBA-15[65].

Parametry katalizy enzymatycznej

Przebieg reakcji enzymatycznej jest warunkowany przez wiele czynników, czego odzwierciedleniem są zmiany w kinetyce przemiany. Parametry kinetyczne charakteryzują enzym, wskazują na jego powinowactwo do substratu, a także opisują powstające opory dyfuzyjne w transporcie substratów do centrum aktywnego enzymu. Procesy katalizy enzymatycznej charakteryzowane są przez dwa główne parametry: maksymalną szybkość reakcji enzymatycznej, V_{maks}, oraz stałą Michaelisa, K_m. Na wartość stałej K_m ma wpływ pH, temperatura, siła jonowa oraz rodzaj i stężenie substratu, a także obecność inhibitorów, jednak jej wartość nie zależy od stężenia enzymu. Wielkość K_m jest wyrazem powinowactwa enzymu do substratu, zatem enzymy o małej wartości K_m działają znacznie sprawniej niż enzymy, które mają duże wartości K_m[66].

Analiza kinetyki przemiany enzymatycznej przez lakazę wolną i immobilizowaną wykazała wartości K_m wynoszące 0,5 mM i 2,0 mM oraz V_{maks} 285 U/mg i 500 U/mg, dla enzymu odpowiednio wolnego i immobilizowanego na nośniku typu zol-żel. Większe wartości dla enzymu immobilizowanego wskazują na mniejsze powinowactwo

do substratu, co prawdopodobnie jest spowodowane zmianą konformacji białka, a także utrudnionym dostępem do centrum aktywnego enzymu[67]. Olajuyigbe i współpr.[17] dokonali pomiarów kinetyki enzymatycznej lakazy wyizolowanej z *C. fabianii* w postaci wolnej (FL), immobilizowanej w kapsułkach Ca-alginianowych (Ca-Al) oraz Cu-alginianowych (Cu-Al). Wartość K_m oraz V_{maks} dla FL, Ca-Al oraz Cu-Al wynosiła odpowiednio 0,032 mM i 15,15 mM/min, 0,078 mM i 6,98 mM/min oraz 0,091 mM i 5,61 mM/min. Wartości te określono przy użyciu soli diamonowej 2,2'-azobis(3-etylobenzotiazolino-6-sulfonianu) (ABTS) jako substratu. Uzyskane wyniki wskazują na wyższe powinowactwo do ABTS wolnej lakazy w stosunku do enzymu immobilizowanego, jednocześnie enzym na nośniku Cu-Al charakteryzował się najniższym powinowactwem do substratu. Większe wartości K_m dla immobilizowanej lakazy wynikają prawdopodobnie z modyfikacji grup funkcyjnych w centrum katalitycznym enzymu[68]. Zwiększenie wartości K_m w przypadku enzymów immobilizowanych mogło wynikać także z wewnętrznego oporu dyfuzyjnego transportu masy do/od jego centrum aktywnego[40, 69]. Mniejsza wartość K_m dla mikrosfer Ca-Al w stosunku do Cu-Al mogła wynikać także z ograniczonej dyfuzji substratu i wzrostu zdolności wychwytywania produktu przez kapsułki Ca-Al[68, 70]. Wcześniejsze badania wykazały, że unieruchomiona lakaza mimo większych wartości K_m i mniejszych wartości V_{maks} w stosunku do formy natywnej, wykazała znaczącą wydajność katalityczną, możliwość ponownego użycia i stabilność termiczną. Ji i współpr.[71] immobilizując lakazę (wyizolowaną z *P. ostreatus*) na nanocząstkach tytanu, zaobserwowali nieznaczne zwiększenie wartości K_m dla enzymu immobilizowanego (42,9 ± 3,3 μM) w stosunku do enzymu wolnego (37,3 ± 2,5 μM), co wskazuje, że taki sposób immobilizacji pozwala na zachowanie dużego powinowactwa enzymu do substratu. Również nieznaczne zwiększenie K_m po kowalencyjnej immobilizacji lakazy z *Myceliophthora thermophila* na modyfikowanej glicydopropylotrimetoksysilanem (związkiem zawierającym grupy epoksydowe) krzemionce typu zol-żel otrzymali Mohammadi i współpr.[64].

Wpływ pH

Obok temperatury, pH środowiska reakcji stanowi jeden z najistotniejszych parametrów fizykochemicznych procesu, mający bezpośredni wpływ na aktywność enzymów. Większość białek katalitycznych wykazuje najwyższą aktywność w bardzo wąskim zakresie pH. Enzymy wykazują także dużą wrażliwość na zmienne warunki pH środowiska reakcji, nawet niewielkie zmiany wartości tego parametru mogą powodować istotną utratę aktywności enzymów. Optymalne pH dla działania białka katalitycznego zależy od rodzaju biokatalizatora, a także procesu, w którym bierze ono udział.

Dotychczasowe wyniki badań w obszarze immobilizacji enzymów jako techniki umożliwiającej swego rodzaju zabezpieczenie białek przed szkodliwym działaniem pH o skrajnych wartościach wydają się być obiecujące. Jaiswal i współpr.[20] badali wpływ pH na aktywność lakazy wolnej i immobilizowanej na nośniku chitozanowym. Zakres optymalnego pH dla unieruchomionego enzymu przesunął się w kierunku zasadowym w porównaniu z pH optymalnym dla enzymu wolnego (z pH 8 dla wolnego enzymu do pH 10 dla enzymu immobilizowanego)[20]. Zbieżne wyniki uzyskali Olajuyigbe i współpr.[17]. Przesunięcie optymalnej wartości pH z 5 do 6 dla odpowiednio wolnej i immobilizowanej lakazy prawdopodobnie wynikało z występowania oddziaływań elektrostatycznych między białkiem a mikrośrodowiskiem matrycy, a także ze zmian enzymu związanych z dysocjacją i jonizacją. Ponadto zmniejszenie aktywności zarówno wolnej, jak i unieruchomionej lakazy w środowisku o wyższym pH mogło być spowodowane inhibicją enzymu, wynikającą z wiązania jonu wodorotlenkowego z miedzią w centrum aktywnym enzymu[72, 73]. Immobilizacja lakazy umożliwiła zachowanie ponad 80% jej aktywności w zakresie pH 7,0–13,0 oraz 100% przy pH 10,0, podczas gdy aktywność wolnej lakazy przy pH powyżej 8 istotnie się zmniejszyła[20]. Zheng i współpr.[74] również wskazali, że unieruchomiona lakaza na nośniku chitozanowym wykazywała mniejszą wrażliwość na zmiany pH (w zakresie pH 3,5–12). Bagewadi i współpr.[67] badali efektywność dekoloryzacji zieleni malachitowej, błękitu metylenowego i czerwieni kongo przez lakazę wyizolowaną z *Trichoderma harzianum* strain HZN10, immobilizowaną na różnych nośnikach: alginianowym, chitozanowym, alginianowo-chitozanowym i matrycy zol-żel oraz wpływ wybranych parametrów środowiska reakcji, w tym pH, na aktywność immobilizowanych i wolnych enzymów. Najwyższą wydajność immobilizacji (93%) uzyskano na nośniku zol-żel, dodatkowo unieruchomienie enzymu tą metodą pozwoliło na utrzymanie aktywności katalitycznej preparatu w szerokim zakresie pH (4–7) oraz zachowanie ponad 90% aktywności[67].

Stabilność termiczna

Mała odporność enzymów na działanie wysokiej temperatury ogranicza ich stosowanie. Proces immobilizacji powoduje, że biokatalizatory stają się mniej wrażliwe na działanie temperatury, a podwyższenie stabilności termicznej jest istotnym parametrem wpływającym na wykorzystanie unieruchomionych enzymów w reakcjach katalitycznych.

Badania prowadzone na lakazach różnego pochodzenia wskazują na ich różną stabilność termiczną[75, 76]. Immobilizowana na chitozanie lakaza z *Trametes pubescens* wykazała najwyższą aktywność w temp. 60°C, a wolny enzym w temp. powyżej 50°C istotnie utracił swoją aktywność. Uzyskane wyniki mogą wskazywać, że immobilizacja enzymu stabilizuje jego strukturę, stwarzając przyjazne mikrośrodowisko i chroniąc białko przed denaturacją[77–79]. Badania przeprowadzone przez Daâssi i współpr.[80] dotyczące dekoloryzacji różnych barwników stosowanych w przemyśle, za pomocą wolnej i immoblizowanej w alginianie lakazy wyizolowanej z *Coriolopsis gallica*, potwierdzają, że enzym immobilizowany charakteryzuje się większą termostabilnością w porównaniu z enzymem wolnym. Stabilność termiczną wolnej i immobilizowanej lakazy badano w temp. 55°C przez 210 min, w interwałach czasowych co 30 min. Po 90 min inkubacji enzym immobilizowany i wolny zachowały odpowiednio 91,2 ± 0,7% i 25,6 ± 1,3% wartości aktywności początkowej. Po 210 min inkubacji aktywność enzymu immobilizowanego wynosiła ok. 67 ± 0,8% wartości początkowej, a enzym wolny zachował tylko 3% aktywności[80].

Również Reyes i współpr.[81] wykazali, że unieruchomiona na agarozie lakaza z *C. gallica* UAMH8260 wykazała wyższą stabilność termiczną w 70°C niż wolny enzym. Irshad i współpr.[82] wykonali profil aktywności lakazy z *Ganoderma leucidum* w formie wolnej i immobilizowanej na matrycy zol-żel, w zakresie temp. 20–80°C przez 4 h. Wolna lakaza charakteryzowała się najwyższą aktywnością w temp. 55°C. Jednak w wyższej temperaturze zachowała tylko 18% aktywności początkowej, podczas gdy enzym immobilizowany nawet po czasie inkubacji 4 h w temp. 80°C zachował prawie 73% aktywności początkowej. Tak wysoka termostabilność enzymu immobilizowanego na matrycy zol-żel stanowi o potencjalnym jego zastosowaniu w różnorodnych procesach technologicznych, podczas których panują niesprzyjające warunki termiczne dla białek enzymatycznych. Badania Saoudi i Ghaouar[66] także potwierdziły wzrost termostabilności lakazy z *Trametes versicolor* po jej immobilizacji na nośniku PEGDA (diakrylan glikolu polietylenowego) o różnych masach cząsteczkowych (6000 i 8000). Uzyskane wyniki wskazują, że temperatura optymalna zarówno dla enzymu wolnego, jak i immobilizowanego (na obu rodzajach PEGDA) wynosi 40°C. Immobilizowana lakaza (na PEGDA o większej masie cząsteczkowej) w skrajnych temp. 4°C i 60°C zachowała odpowiednio ok. 70% i 95% aktywności początkowej, podczas gdy w tych samych warunkach enzym wolny zachował ok. 30% i 75% swojej aktywności początkowej. Uzyskane wyniki wskazują, że termostabilność enzymu może być również zależna od masy cząsteczkowej nośnika. Najwyższą termostabilność wykazał enzym immobilizowany na nośniku PEGDA o większej masie cząsteczkowej[66].

Stabilność operacyjna i wydajność procesowa

Zastosowanie immobilizowanego biokatalizatora warunkuje wiele czynników. Istotnym parametrem technologicznym, który pozwala ocenić celowość zastosowanej metody immobilizacji jest stabilność operacyjna enzymu. Należy zaznaczyć, że stabilizacja struktury białkowej poprzez immobilizację prowadzi do jej wzmocnienia, co warunkuje możliwość wydłużenia czasu magazynowania preparatu po immobilizacji, w porównaniu z przechowywaniem natywnego enzymu. Stabilność preparatów z immobilizowanymi enzymami jest szczególnie istotnym parametrem warunkującym ich praktyczne zastosowanie w skali przemysłowej. Nie zawsze preparat z immobilizowanym enzymem może zostać wykorzystany bezpośrednio po jego wytworzeniu, dlatego też stabilność w trakcie magazynowania jest istotnym i bardzo pożądanym parametrem charakteryzującym jego właściwości[20]. Preparaty immobilizowanych enzymów cieszą się dużym zainteresowaniem przemysłowym również z uwagi na fakt, że mogą być wykorzystywane przez więcej niż jeden cykl katalityczny, przy zachowaniu wymaganej efektywności. Możliwość wielokrotnego wykorzystania to także jeden z istotnych czynników warunkujących opłacalność technologii. Obecnie trwają badania dotyczące wydajności procesowej preparatów z immobilizowanymi enzymami podczas kolejnych cykli operacyjnych, a także warunków ich przechowywania. Uzyskane do tej pory wyniki wydają się obiecujące w kontekście zastosowania w procesach przemysłowych.

Immobilizowana lakaza z *Trametes versicolor* na szkielecie *Hippospongia communis* po 50 dniach przechowywania w temp. 4°C w buforze octanowym (pH 5) zachowała ponad 90% swojej pierwotnej aktywności, podczas gdy wolny enzym zachował tylko 20% pierwotnej aktywności[83]. Według Xu i współpr.[84] większa stabilność podczas przechowywania wynika ze stabilizacji struktury enzymu oraz ochronnej roli nośnika, co ogranicza zmiany konformacji białka katalitycznego. Mostafa i współpr.[85] badając stabilność immobilizowanej lakazy z *Alternaria tenuissima* KM651985 poprzez wiązanie kowalencyjne z utlenionymi polisacharydami wykazali, że podczas przechowywania w temp. 4°C immobilizowane preparaty enzymatyczne utraciły jedynie 0,73% i 13,23% swojej pierwotnej aktywności, po odpowiednio 14 i 41 dniach. Wolny enzym przechowywany w tych samych warunkach utracił odpowiednio 10,7 i 39,26% pierwotnej aktywności[85]. Zbieżne wyniki uzyskał Spinelli ze współpr.[56], określając stabilność preparatów z immobilizowaną lakazą na żywicy jonowymiennej Amberlite IRC 120 H. Zespół Zheng[79] oceniał wydajność katalityczną unieruchomionego enzymu w degradacji różnych syntetycznych barwników. Wolny enzym wykazywał znaczną utratę swojej aktywności podczas przechowywania. Po 30 dniach aktywność wynosiła ok. 15% aktywności początkowej. W przypadku unieruchomienia tego białka przez pułapkowanie w chitozanie, a następnie po usieciowaniu aldehydem glutarowym jego aktywność po takim samym okresie magazynowania wynosiła ok. 40%[79].

Podsumowanie

Wyraźny trend w kierunku możliwości zastosowania enzymów w procesach przemysłowych skłania badaczy do opracowywania innowacyjnych preparatów enzymatycznych charakteryzujących się jak najwyższą stabilnością operacyjną w zadanych warunkach procesu technologicznego. Immobilizacja enzymów stanowi obiecującą technologię w tym zakresie. Spośród dotychczas opracowanych i zaproponowanych technik, immobilizacja enzymów pozwala na istotną poprawę stabilności biokatalizatorów, co powoduje wzrost efektywności przemian przez nie katalizowanych. Trwają badania w kierunku opracowania nowych nośników do immobilizacji. Dotychczasowe badania w tym obszarze wskazują, że immobilizacja stanowi obiecującą technikę zwiększania stabilności operacyjnej (w większym zakresie pH i temperatury), a także umożliwia ponowne wykorzystanie biokatalizatora oraz ułatwia jego odzysk z mieszaniny poprocesowej. Innowacyjne preparaty enzymatyczne składające się z immobilizowanych lakaz, z uwagi na wysoką stabilność operacyjną, mogą znaleźć zastosowanie w różnych gałęziach przemysłu (w tym w szczególności do oczyszczania ścieków barwnych).

Niniejsza publikacja powstała w ramach pracy statutowej (nr 11358019-343) obejmującej badania naukowe i/lub prace rozwojowe, finansowanej przez Ministerstwo Nauki i Szkolnictwa Wyższego, realizowanej w Głównym Instytucie Górnictwa w Katowicach.

Otrzymano: 21-01-2020

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Support materials for laccase immobilization for decolourization processes

Nośniki do immobilizacji lakazy w procesach dekoloryzacji

DOI: *10.15199/62.2022.2.9*

A review, with 82 refs., of modern methods of enzyme immobilization as a technique aimed at increasing the operational stability of biocatalysts in the context of their applicability in industrial processes. The types of inorg. and org. carriers used were presented, with particular emphasis on biopolymers. Attention was paid to the immobilization of enzymes with the use of nanocarriers. The use of innovative carriers for the immobilization of laccase in order to increase its stability in the processes of removing dyes from wastewater was also discussed.

W pracy szczególną uwagę zwrócono na immobilizację jako technikę ukierunkowaną na zwiększenie stabilności operacyjnej biokatalizatorów w kontekście możliwości zastosowania enzymów w procesach technologicznych. Dokonano przeglądu nowoczesnych nośników do immobilizacji lakazy, podkreślając jej szczególne znaczenie aplikacyjne do usuwania barwnych zanieczyszczeń. Wskazano także możliwości i dalsze wyzwania związane z zastosowaniem unieruchomionych biokatalizatorów w procesach dekoloryzacji.

Trendy obecnie prowadzonych badań w obszarze bioremediacji obejmują poszukiwanie nowych technologii, bardziej przyjaznych dla środowiska. Szczególna uwaga zwrócona została na poszukiwanie metod usuwania trudno rozkładalnych związków organicznych, w tym barwników tekstylnych[1]. Barwniki tekstylne mogą być wysoce toksyczne i potencjalnie rakotwórcze, stanowiąc istotne zagrożenie zarówno dla środowiska, jak i ludzi[2, 3]. Charakteryzują się zróżnicowaną budową chemiczną, a co za tym idzie, także właściwościami fizykochemicznymi, co generuje istotne trudności w ich usuwaniu[2, 4]. Obecnie stosowane konwencjonalne procesy chemiczne są skuteczne w degradacji barwników, niemniej jednak nie są pozbawione wad, prowadząc często do wytworzenia toksycznych produktów pośrednich[5]. W tym świetle bioremediacja[6], w szczególności oparta na biokatalizie, postrzegana jest jako obiecujący kierunek badań. Proces ten ma na celu degradację substancji chemicznych za pomocą enzymów w sposób łatwy, szybki, ekologiczny i zrównoważony[7–9]. Stąd też obecnie jednym z kierunków badań jest zastosowanie biokatalizy w usuwaniu barwnych zanieczyszczeń ze ścieków. Szczególnym zainteresowaniem cieczą się enzymy z grupy oksydoreduktaz, zwłaszcza lakazy[10, 11].

Lakaza. „Zielone narzędzie" w usuwaniu barwników

Lakazy należą do wielomiedziowych oksydaz (*multicopper oxidases*, MCOs), które katalizują reakcje utleniania szerokiej gamy substratów z jednoczesną redukcją tlenu cząsteczkowego do wody. Ich miejsca aktywne zawierają cztery atomy miedzi. Miejsce aktywne lakazy zawiera cztery atomy miedzi. W cząsteczce enzymu obecny jest jeden atom miedzi typu I (T1) oraz trójatomowy zespół składający się z jednego atomu miedzi typu II (T2) i dwóch atomów miedzi typu III (T3)[10, 12].

Lakazę po raz pierwszy wyizolowano z japońskiego drzewa lakowego *Rhus vernicifera*[13, 14], jednakże tę o potencjale komercyjnym otrzymuje się przede wszystkim z grzybów zgnilizny drewna[15–17], m.in. z *Trametes versicolor*. Lakaza ze względu na szeroką specyficzność substratową stanowi cenne „zielone narzędzie" w wielu gałęziach przemysłu, wykazując szeroką gamę możliwości aplikacyjnych. Dotychczasowe badania dotyczące możliwości zastosowania lakazy oprócz biodegradacji barwników[18–21] obejmowały m.in. ich wykorzystanie do usuwania farmaceutyków[22–24], pestycydów[25] oraz organicznych związków chemicznych, takich jak bisfenol A[26–28] i 2,4-dichlorofenol[29]. Lakazy są również

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stosowane w procesach technologicznych przemysłu papierniczego[30], a także w przemyśle kosmetycznym[31].

Możliwość przemysłowego wykorzystania wolnych enzymów limitowana jest przez ich wrażliwość na zmienne warunki środowiska reakcji (pH, temperatura), powodującą obniżenie lub całkowitą utratę ich aktywności katalitycznej w warunkach procesowych. Ponadto zastosowanie lakazy w formie natywnej uniemożliwia jej odzysk po procesie oraz ponowne wykorzystanie. Stąd też nastąpił dynamiczny rozwój badań ukierunkowany na poprawę odporności enzymów na zmienne warunki procesowe. Jedną z obiecujących technik okazała się immobilizacja enzymów na różnego rodzaju nośnikach. Immobilizacja lakazy prowadzi do zwiększenia jej stabilności i odporności na niekorzystne warunki środowiskowe oraz na działanie enzymów proteolitycznych, a także umożliwia odzysk biokatalizatora w celu ponownego wykorzystania[32–34]. Obecnie badania skupione są na poszukiwaniu innowacyjnych nośników do immobilizacji lakazy, na modyfikacji i poprawie ich funkcji oraz wydajności w celu ich potencjalnego komercyjnego wykorzystania do usuwania barwnych zanieczyszczeń[1].

Immobilizacja. Metody i nowoczesne nośniki

Pierwszej immobilizacji enzymu, inwertazy z drożdży, dokonali w 1916 r. Nelson i Griffith. Sam termin „immobilizowany enzym" wprowadzono później, bo dopiero w 1971 r.[35, 36]. Znaczący wzrost zainteresowania tematem immobilizacji enzymów nastąpił w latach 60. XX w. Pierwsze badania obejmowały wytwarzanie L-aminokwasów (L-asparaginian) przy udziale immobilizowanej aspartazy z *E. coli* oraz wytwarzanie L-aminokwasów z ketokwasów w reaktorach membranowych[37]. Immobilizacja enzymów jest definiowana jako przyłączenie enzymu do materiału nośnego, co powoduje zmniejszenie lub też całkowitą utratę mobilności przyłączonego biokatalizatora, prowadząc do utworzenia nierozpuszczalnego kompleksu i unieruchomienia enzymu na materiale nośnym[38]. Obok warunków środowiskowych, które wpływają na aktywność immobilizowanych enzymów, istotnym czynnikiem determinującym zachowanie ich właściwości katalitycznych są także warunki procesu immobilizacji. Dobór odpowiedniej metody immobilizacji oraz materiału nośnego stanowi bardzo istotny czynnik z uwagi na silny wpływ zarówno na właściwości katalityczne wytworzonego preparatu enzymatycznego, jak i jego stabilność[39].

W ciągu ostatniej dekady badania wielu naukowców skupiły się na poszukiwaniu nie tylko nowych metod immobilizacji lakazy, ale także na opracowaniu nowych materiałów nośnych, aby otrzymać biokatalizatory bardziej wydajne i odporne na zmienne warunki środowiskowe, jednocześnie umożliwiając ich ponowne wykorzystanie w kolejnych cyklach procesowych. Immobilizacja lakaz na różnych nośnikach ma chronić je przed denaturacją, poprawić ich stabilność, utrzymać dobrą wydajność katalityczną, ułatwić efektywny odzysk i prowadzić do bardziej ekonomicznego procesu. Poprawa ich stabilności poprzez immobilizację wynika z rozszerzenia optymalnego zakresu pH i temperatury (zwiększona stabilność termiczna), co stwarza możliwość pracy unieruchomionych biokatalizatorów w szerszym zakresie warunków środowiskowych[32, 40, 41]. Szeroko zakrojone badania nad immobilizacją enzymów w ostatniej dekadzie świadczą o dużym potencjale tej technologii w kontekście możliwości zastosowania w procesach przemysłowych[34, 41].

Dotychczas opisano wiele metod immobilizacji enzymów, w tym lakazy. Wśród nich wyróżnić można metody fizyczne (adsorpcja, pułapkowanie i enkapsulacja) oraz metody chemiczne (poprzez wiązania kowalencyjne oraz sieciowanie). W tabeli scharakteryzowano główne metody immobilizacji enzymów.

Wymienione techniki różnią się między sobą rodzajem i siłą oddziaływań pomiędzy unieruchamianym białkiem a nośnikiem, jak również formą, a także rolą nośnika. Rodzaj interakcji enzym-nośnik, jak i siła tych interakcji wpływają zarówno na siłę wiązania katalizatora z matrycą, jak i na zachowanie przez unieruchomiony enzym jego właściwości katalitycznych. Opracowane preparaty enzymatyczne w zależności od techniki, która została zastosowana będą charakteryzować się odmiennymi właściwościami i aktywnością katalityczną, przez co będą mogły znaleźć zastosowanie w różnych procesach technologicznych. Szczegółowego

Table. Methods of enzyme immobilization

Tabela. Metody immobilizacji enzymów

Metoda immobilizacji		Charakterystyka	Źródło
Metody fizyczne	adsorpcja	Najstarsza i najpopularniejsza z metod, w której enzym jest przyłączany do zewnętrznej powierzchni nośnika poprzez słabe oddziaływania, w tym wiązania wodorowe, oddziaływania elektrostatyczne, hydrofobowe. Słaba siła interakcji enzym-nośnik nie wywołuje zmian w strukturze białka enzymatycznego, może jednak być przyczyną elucji katalizatora. Powszechnie stosowanymi nośnikami do immobilizacji poprzez adsorpcję są organiczne i nieorganiczne materiały porowate, takie jak kaolinit, diatomit, węgiel aktywny, montmorylonit, szkło porowate, bentonit, żel krzemionkowy i celuloza. Immobilizację wg tej metody można prowadzić poprzez klasyczną adsorpcję, agregację lub depozycję.	20, 42
	pułapkowanie, enkapsulacja	Polega na fizycznym uwięzieniu enzymu w porowatym nośniku stałym poprzez wytworzenie oddziaływań fizycznych. Siła wiązania enzymu z matrycą jest stosunkowo słaba (nie powstają wiązania kowalencyjne), pozwalając z jednej strony na uniknięcie ingerencji w strukturę białka enzymatycznego, z drugiej strony jednak może to stanowić przyczynę wymywania enzymu i w konsekwencji utratę aktywności enzymatycznej preparatu. Ograniczenie w tej metodzie stanowi występowanie oporów dyfuzyjnych w transporcie substratów. Powszechnie stosowanymi nośnikami do immobilizacji poprzez pułapkowanie są m.in. alginiany, karagen, chitozan. Podczas enkapsulacji powstają kapsułki, przez co enzym otoczony zostaje ścianką, która umożliwia transport małocząsteczkowych reagentów, utrzymując jednocześnie białko katalityczne w rdzeniu kapsułki.	18, 41, 43
Metody chemiczne	immobilizacja poprzez wytworzenie wiązań kowalencyjnych	Polega na tworzeniu trwałych oraz stabilnych wiązań kowalencyjnych pomiędzy enzymem a nośnikiem, co ogranicza wymywanie enzymu z matrycy, zwiększa jego stabilność termiczną i możliwość ponownego użycia, jednak może prowadzić do zmian struktury enzymu i znacznej utraty jego aktywności. Istotą doboru nośnika jest obecność w jego strukturze grup funkcyjnych komplementarnych z grupami enzymu, umożliwiając wytworzenie wiązań kowalencyjnych. Metoda ta może być realizowana w procesie jednoetapowym, gdy grupa funkcyjna nośnika nie wymaga modyfikacji. W przeciwnym razie wymaga ona wstępnej modyfikacji nośnika, aby umożliwić jego kowalencyjną koniugację z enzymem.	21, 34, 44
	sieciowanie	Następuje w wyniku międzycząsteczkowego sieciowania enzymów poprzez wiązania chemiczne tworzone przez dwufunkcyjne reagenty, tzw. *cross-linkers*, np. aldehyd glutarowy, diizocyjaniany i sól diazoniową, diiminoestry. W metodzie tej nie jest wykorzystywany nośnik. Biorąc pod uwagę sposób przygotowania enzymu oraz warunki procesu, wyróżnia się preperaty: CLEA (*cross-linked enzyme aggregats*) i CLEC (*cross-linked enzyme crystals*).	20, 45–47

porównania głównych metod immobilizacji pod kątem zalet i wad/ograniczeń dokonali w swojej pracy Bilal i współpr.[18].

Obok ujętych w tabeli głównych metod immobilizacji należy także wskazać nowoczesne techniki wykorzystywane podczas immobilizacji, takie jak druk strumieniowy, elektrospinowanie (*electrospinning*) i druk 3D, które przyczyniają się nie tylko do poprawy wydajności procesu, ale także upraszczają poszczególne etapy immobilizacji[48–51]. Elektrospining jest techniką używaną głównie do wytwarzania włókien i membran w szybki sposób, uzyskując ciągłe włókna o średnicach w skali mikro- i nanometrów. Otrzymane w ten sposób włókna mają wiele zalet, takich jak mała średnica, porowata struktura, duża powierzchnia i doskonałe właściwości mechaniczne[52, 53]. Elektrorozpylanie jest uważane za technologię pokrewną do elektrospiningu, ponieważ w obu technologiach konieczne jest zastosowanie pola elektrycznego o wysokim potencjale. Roztwór polimeru jest rozpylany w celu uzyskania cząstek. Sposób ten polega na delikatnej metodzie jonizacji, która nie powoduje rozbijania ani dezintegracji cząsteczek[48, 54]. Za innowacyjną technikę immobilizacji uważana jest technika impulsowego odparowania laserowego wspomaganego matrycą (*matrix-assisted pulsed laser evaporation*, MAPLE). Uważana jest ona za prostą, tanią i wszechstronną metodę osadzania cienkich warstw[55].

Zespół Liu i współpr.[56] dokonał immobilizacji lakazy metodą druku 3D w celu zastosowania w biodegradacji związków fenolowych. Prace nad immobilizacją enzymów poprzez druk 3D trwają już od 2019 r., gdy zespół Ye i współpr.[51] badali możliwość modyfikacji materiału do druku C-PLA (kwas mlekowy wzmocniony włóknem węglowym) poprzez jego obróbkę chemiczną. W pierwszej kolejności wydrukowany materiał trawiony był za pomocą kwasu nadoctowego oraz mieszaniny stężonych roztworów kwasu siarkowego (H_2SO_4) i nadtlenku wodoru (H_2O_2) (tzw. roztwór pirania), a następnie został pokryty czynnikiem wiążącym na bazie krzemowodorów. W ten sposób badacze zwiększyli powierzchnię właściwą C-PLA i liczbę odsłoniętych grup aktywnych, do których wiążą się cząsteczki enzymu. W swoich badaniach wykazali, że tak unieruchomiona glikozydaza była w stanie syntezować laktosacharozę z sacharozy i laktozy w ilości 20% mas./obj. każdego z substratów, a wydajność procesu utrzymywała się przez 10 cykli. Obiecujące wyniki uzyskali także, wiążąc acylazę penicylinową z modyfikowanym C-PLA i syntezując amoksycylinę z kwasu 6-aminopenicylanowego (6-APA).

Trwają również szeroko zakrojone prace nad projektowaniem nowoczesnych matryc/sieci metaloorganicznych (*metal-organic frameworks*, MOFs) do immobilizacji lakazy[57]. Technologia produkcji MOFs jest wciąż na bardzo wczesnym etapie i polega na mieszaniu enzymów, jonów metali lub ich kompleksów związanych z ligandami substancji organicznych. Pomimo prostoty technologii, wciąż wymaga ona badań z uwagi na dużą utratę aktywności enzymów związanych w taki sposób. Poprawę efektywności wiązania enzymu i ograniczenie spadku jego aktywności uzyskać można dzięki zastosowaniu metody mikroprzepływów z wykorzystaniem reaktora laminarnego, w którym unieruchamianie enzymów przebiega w warunkach kontrolowanego mieszania dyfuzyjnego[58].

Odpowiedni wybór nośnika ma istotne znaczenie w procesie, warunkuje bowiem wydajność reakcji oraz aktywność katalityczną biokatalizatora. Pożądane jest, aby podłoże cechowało się mechaniczną wytrzymałością, biozgodnością, możliwością regeneracji, odpornością na działanie drobnoustrojów oraz dostępnością przy relatywnie niskich kosztach[20, 59]. Nośnik służyć ma stabilizacji enzymu, tak więc powinien cechować się jak największą powierzchnią dostępną dla cząsteczek enzymu. Szerokie spektrum wykorzystania danego nośnika jest zatem warunkowane przez takie czynniki, jak powierzchnia, objętość, porowatość, kształt, forma, stabilność w danych warunkach reakcji oraz jego koszt. Z punktu widzenia ekonomiki procesu szczególnie istotna jest cena nośnika. Dlatego też możliwość wielokrotnego jego użycia może generować znaczne oszczędności. Obecnie głównym nurtem badań dotyczących białek enzymatycznych jest ich modyfikacja, doskonalenie oraz optymalizacja ich wydajności w kierunku zastosowania jako komercyjnych biokatalizatorów, a immobilizacja wydaje się obiecującą metodą w tym zakresie. Dlatego też trwają szeroko zakrojone badania nad poszukiwaniem nowych nośników do immobilizacji, a także badania ukierunkowane na modyfikację dotychczas opracowanych nośników[20, 43]. Poszukiwanie wydajnych nośników w celu poprawy wydajności enzymów jest jednym z najważniejszych kierunków w biotechnologii od momentu dokonania pierwszej immobilizacji enzymów w 1916 r.[60].

Wśród dotychczas najczęściej wykorzystywanych, klasycznych nośników do immobilizacji rozróżnia się nośniki nieorganiczne oraz organiczne. Do nośników nieorganicznych należą takie materiały, jak krzemionka, tlenki metali, hydroksyapatyt, kordieryt, ziemia okrzemkowa, kaolinit, montmorylonit i bentonit. Wśród nośników organicznych należy wymienić nośniki syntetyczne (żywice jonowymienne, żywice epoksydowe, poli(chlorek winylu), polistyren, poliuretany i poliamidy, poliakrylamid, silikony) oraz naturalne, czyli biopolimery, takie jak alginian, chitozan, celuloza, żelatyna, skrobia i kolagen. Wśród biopolimerów stosowanych do immobilizacji dużą popularnością cieszy się chitozan. Ze względu na właściwości chitozanu jego przemysłowe zastosowanie jest szeroko badane. Ponieważ jest on biodegradowalny, biokompatybilny, nietoksyczny, hydrofilowy, tani i charakteryzuje się dobrą stabilnością fizykochemiczną, postrzegany jest jako doskonała alternatywa dla materiałów syntetycznych w poszukiwaniu bardziej zrównoważonych metod produkcji. W pracy Nunes i współpr.[46] dokonano szerokiego przeglądu najnowszej literatury dotyczącej zastosowania chitozanu w procesach immobilizacji enzymów, z nakreśleniem zalet i wad, jak również wskazaniem możliwych modyfikacji chemicznych i kombinacji z innymi związkami w celu opracowania idealnego nośnika do immobilizacji. Immobilizacja lakazy w chitozanie powoduje stabilizację jej struktury, stwarzając przyjazne mikrośrodowisko i chroniąc białko przed denaturacją. Potwierdzają to m.in. badania Zheng i współpr.[61], w których immobilizowana lakaza z *Trametes pubescens* wykazała najwyższą aktywność w temp. 60°C, podczas gdy wolny enzym w temp. powyżej 50°C istotnie utracił swoją aktywność. Także w pracy Jaiswal i współpr.[62] immobilizacja w chitozanie lakazy z papai spowodowała 3-krotny wzrost termostabilności, wzrost optymalnej temperatury o 10°C, a także przesunięcie wartości optymalnego pH z 8,0 do 10,0. Ponadto immobilizacja prowadziła do zwiększenia tolerancji enzymu na wiele jonów metali (w tym metali ciężkich) oraz rozpuszczalników organicznych, takich jak etanol, izopropanol, metanol, benzen i DMF. Immobilizacja lakazy powodowała również zwiększenie efektywności dekoloryzacji barwnika indygokarminu (w stężeniu 50 g/mL), w ciągu 8 h dokonano całkowitej dekoloryzacji, a użycie wolnej lakazy pozwoliło na dekoloryzację tego samego barwnika jedynie w 56%.

Wśród biopolimerów szczególnym zainteresowaniem cieszą się także alginiany. Liczne doniesienia literaturowe świadczą o szerokich możliwościach zastosowania ich jako efektywnych nośników do immobilizacji lakazy w procesach dekoloryzacji[63–66]. W badaniach Gill i współpr.[67] wykazano wzrost aktywności katalitycznej i stabilności lakazy po immobilizacji w alginianie. Ponadto uzyskane wyniki eksperymentalne wskazują, że modyfikacja pH wstępnego roztworu alginianu może zwiększać aktywność lakazy w matrycy alginianowej. Użyty nośnik pozwolił nie tylko na odpowiednią wydajność enkapsulacji i zachowanie aktywności enzymu, ale również odpowiednią stabilność podczas przechowywania. Co więcej, hybrydyzacja alginianu z krzemionką okazała się obiecującym podejściem do enkapsulacji lakazy w polimerach organicznych.

Rehbein i współpr.[68] podają, że hybrydyzacja alginianu z krzemionką wpływa na zwiększenie integralności struktury kapsułek przy jednoczesnym zachowaniu potencjału dyfuzyjnego. Ponadto powłoka nieorganiczna ma mniejsze pory niż powłoka alginianowa, co znacznie utrudnia wymywanie katalizatora z wnętrza kapsułki. Kapsułki alginianowo-krzemionkowe są też mniej podatne na pęcznienie i rozpad osmotyczny.

Mimo że klasyczne nośniki wciąż skupiają znaczną uwagę badaczy na całym świecie, to poszukiwane są także nowoczesne nośniki oraz podejmowanych jest wiele badań w celu modyfikacji już istniejących. Zdarta i współpr.[69] dokonali klasyfikacji nowoczesnych nośników na organiczne, nieorganiczne i hybrydowe. Wśród nośników organicznych dużym zainteresowaniem cieszą się nośniki opracowane metodą elektrospiningu. Ich ogromną zaletą w porównaniu z innymi matrycami jest możliwość zastosowania odpowiednich materiałów, umożliwiających dostosowanie ich do konkretnych enzymów oraz procesu, w którym

ma być zastosowany. Immobilizacja enzymów na tego typu materiałach może być prowadzona poprzez adsorpcję i wiązanie kowalencyjne, jak również przez enkapsulację przeprowadzaną w tym samym czasie, w którym formowany jest nośnik, co dodatkowo obniża koszty procesu[53, 70]. Przykładowo Jankowska i współpr.[53] za pomocą techniki elektrospiningu opracowali (metodą adsorpcji i poprzez wiązania kowalencyjne) nośnik do immobilizacji lakazy w postaci włókien poli(metakrylanu metylu)/polianiliny. Największą aktywność enzymów immobilizowanych metodą adsorpcji i poprzez wiązania kowalencyjne uzyskano po 1 h immobilizacji, stosując roztwór lakazy o stężeniu 1 mg/mL, w pH 5 i temp. 25°C. Stwierdzono, że immobilizowane enzymy, w ilości 110 mg/g i 185 mg/g (odpowiednio dla układów z lakazą adsorbowaną i związaną kowalencyjnie), wykazywały nieco niższe powinowactwo do substratu, a w konsekwencji również mniejszą maksymalną szybkość reakcji niż wolny enzym. Stabilność lakazy uległa znacznej poprawie po jej immobilizacji. Oba układy enzymatyczne zachowały ponad 80% względnej aktywności nawet po 10 cyklach katalitycznych i 30 dniach przechowywania. Otrzymane układy zastosowano do usuwania barwnika Remazol Brilliant Blue R z modelowego roztworu wodnego, uzyskując wydajność usuwania 87% i 58%, odpowiednio za pomocą lakazy adsorbowanej i związanej z nośnikiem poprzez wiązania kowalencyjne.

Wiele prac badawczych koncentruje się także na immobilizacji enzymów z wykorzystaniem nanonośników, do których zaliczane są nanowłókna z mieszanki poli(alkoholu winylowego)-poliakrylamidu (PVA-PAAm NF), bakteryjna membrana nanocelulozowa (BCN), nanożel celulozowy (CN), nanocząstki chitozanu (CS-NPs), nanocząstki magnetyczne pokryte chitozanem (CS-MNPs), nanocząstki Fe_3O_4 pokryte chitozanem (Fe_3O_4-CSNP), nanocząstki magnetyczne pokryte celulozą (CCM-NP), nanocząstki chitozanu o małej masie cząsteczkowej (LMW-CS-NP) i nanocząstki magnetyczne funkcjonalizowane chityną (Ch-MNPs)[71].

Zaletą takich nośników jest duży stosunek powierzchni do objętości, znacznie zwiększona wydajność transferu masy i minimalizacja problemów dyfuzyjnych. Zastosowanie nanotechnologii enzymatycznej w oczyszczaniu ścieków barwnych jest zatem bardzo obiecujące. Przeprowadzone dotychczas badania wskazują na duży potencjał nanotechnologii w tworzeniu nowoczesnych nośników do immobilizacji lakazy w celu usuwania zanieczyszczeń barwnych. Potwierdzają to badania Sadighi i Faramarzi[72], którzy immobilizowali lakazę poprzez nanocząstki chitozanu (CS-NPs) na szklanych kulkach w celu usunięcia czerwieni Kongo. Immobilizacja spowodowała wzrost stabilności termicznej enzymu oraz możliwość jego wielokrotnego użycia. Unieruchomiona lakaza zachowała blisko 100% skuteczności w usunięciu barwnika w temp. 90°C, podczas gdy wolna lakaza straciła całkowicie swoją aktywność. Z kolei Wang i współpr.[73] immobilizowali lakazę na nanocząstkach Fe_3O_4/SiO_2 w celu usunięcia barwnika Procion Red MX-5B. Za pomocą tak immobilizowanego enzymu uzyskano 99% usunięcia barwnika w ciągu 20 min. Ponadto unieruchomiony enzym zachował 79% swojej początkowej aktywności po 10. cyklu katalitycznym. Z kolei Liu i współpr.[74] immobilizowali lakazę na nanokompozycie poli(*p*-fenylenodiaminy)/Fe_3O_4 do usuwania barwnika Reactive Blue, przy czym preparaty enzymatyczne po 10. cyklu zachowały 43% swej początkowej aktywności.

Zahirinejad i współpr.[71] podają, że najnowsze badania skupione są na opracowaniu nośników organicznych umożliwiających ukierunkowaną immobilizację białek, tak aby odsłonić miejsca aktywne enzymów i umożliwić proces rozpoznawania substratu. Zależność pomiędzy aktywnością enzymów a rodzajem i sposobem immobilizacji na nich białek określali na podstawie przeglądu literatury Ashkan i współpr.[75]. Wykazali oni, że w przypadku nanonośników nieorganicznych aktywność enzymów spadła w 42% przypadków, a w 21% przypadków wzrosła. Mniejszy spadek aktywności enzymów obserwowano w procesach immobilizacji na nanonośnikach organicznych, gdzie aktywność spadła tylko w 15,4% przypadków, a wzrosła aż w 54,5% przypadków.

Z kolei zespół Wong i współpr.[20] wśród kluczowych wyzwań stojących przed wykorzystaniem nanotechnologii w immobilizowaniu enzymów wskazał: (*i*) realizację eksperymentów w skali laboratoryjnej do zastosowań przemysłowych, (*ii*) niedostateczne poznanie/zrozumienie nanotechnologii z udziałem enzymów, (*iii*) odzyskiwanie unieruchomionego enzymu, (*iv*) syntezę hybrydowych „nanokwiatów”[76] oraz (*v*) trwałość zastosowanych nanomateriałów.

Jako nośnik do immobilizacji uwagę skupia także tlenek grafenu, z uwagi na doskonałe właściwości sorpcyjne i obecność wielu grup hydroksylowych. Liczne doniesienia literaturowe wskazują na jego duże możliwości aplikacyjne jako nośnika do immobilizacji enzymów do procesów dekoloryzacji[47, 77]. Mulko i współpr.[78] badali immobilizację alfa-amylazy na trzech różnych nośnikach, hydrożelu poliakrylamidowym (PAAm), nanokompozycie poliakrylamid-tlenek grafenu (PAAm-GO) oraz alginianie. Wyniki ich badań pokazały, że zastosowanie nośników na bazie tlenku grafenu znacząco poprawia zdolności retencyjne enzymu wewnątrz kapsułki (97,5%). Ponadto kapsułki te wykazywały większą stabilność operacyjną aż do 5 cykli procesowych, podczas gdy kapsułki alginianowe utrzymywały stabilność operacyjną do 3 cykli. Nowe nośniki wykorzystane mogą być na skalę przemysłową do produkcji bioetanolu dzięki zastosowaniu enzymów i drożdży unieruchomionych w tej samej matrycy PAAm. W ten sposób możliwe jest sekwencyjne lub jednoczesne scukrzanie i fermentacja.

W poszukiwaniu odpowiednich materiałów nośnikowych do immobilizacji coraz więcej badań skupia się na opracowaniu tanich, biodegradowalnych i łatwo dostępnych materiałów, które mogą być poddawane modyfikacjom. Jedną z alternatyw stanowią odpady rolno-przemysłowe[79]. Suman i współpr.[80] w swojej pracy zademonstrowali zastosowanie piór kurzych do immobilizacji lakazy metodą wiązań kowalencyjnych za pomocą aldehydu glutarowego. Otrzymany preparat enzymatyczny (TML@ACFP) wykazywał zwiększoną stabilność w określonym zakresie pH, temperatury, a także podczas przechowywania, jednocześnie z możliwością ponownego wykorzystania. Aktywność enzymatyczną preparatów potwierdzono z wykorzystaniem niefenolowego substratu, alkoholu weratrylowego. Uzyskane wyniki potwierdzają możliwość wykorzystania odpadowych piór kurzych do immobilizacji enzymów i potencjalnego zastosowania w procesach przemysłowych[80]. Zespół Girelli i współpr.[81] badał możliwość zastosowania odpadu w postaci skorupek jaj do immobilizacji lakazy. Pomimo że uzyskane wyniki wskazały na niższą stabilność enzymów immobilizowanych w pH 3 i 4 w porównaniu z wolnym enzymem oraz w zakresie temp. 30–50°C, uzyskane badania wstępne wskazują na możliwość ich wykorzystania, gdyż zapewniają dobrą dostępność do substratu i stabilność operacyjną, a także dają możliwość ponownego wykorzystania enzymu. Ponadto prostota oraz nowatorstwo zaproponowanej procedury immobilizacji z wykorzystaniem nośnika na bazie odpadu otwierają możliwości potencjalnych zastosowań w przemyśle.

Ograniczenia, wyzwania i możliwości

Możliwość zastosowania biokatalizatorów w różnych gałęziach przemysłu stała się w ostatnich latach przedmiotem zainteresowania badaczy na całym świecie. Szczególną uwagę poświęca się technikom immobilizacji enzymów w celu zwiększenia ich wydajności i stabilności operacyjnej. Wśród zalet zastosowania immobilizowanych enzymów niewątpliwie wskazać można: wzrost wydajności procesu biokatalizy, wzrost stabilności enzymu w zmiennych warunkach procesu (np. pH, temperatura), ograniczenie inhibicji enzymu, zwiększenie odporności na działanie czynników denaturujących oraz różnego rodzaju zanieczyszczeń obecnych w mieszaninie reakcyjnej, wydłużenie czasu aktywności preparatów enzymatycznych i możliwość zastosowania ich w procesach ciągłych, możliwość wzrostu koncentracji pożądanych substancji w bioreaktorze oraz zwiększoną kontrolę procesu, możliwość ponownego wykorzystania immobilizowanego biokatalizatora dzięki łatwości jego oddzielenia od mieszaniny procesowej oraz wyższą stabilność podczas przechowywania preparatów immobilizowanych w stosunku do wolnych enzymów. Obok wielu wymienionych zalet immobilizacja enzymów może wiązać się także z występowaniem pewnych ograniczeń, takich jak ograniczenia przenikania substratów i produktów (opory dyfuzyjne), ograniczenia wynikające z zastosowanego nośnika, utrata aktywności enzymu w wyniku immobilizacji i straty ilościowe enzymu podczas procesu immobilizacji. Jednak większość wyżej wskazanych ograniczeń/wad można usunąć lub znacznie ograniczyć, np. poprzez dobór

odpowiedniej metody immobilizacji oraz nośnika do zastosowania w konkretnym procesie technologicznym[18, 82].

Zaleca się, aby przyszłe prace koncentrowały się na opracowaniu nowych, innowacyjnych nośników, a także technologiach modyfikowania istniejących nośników w celu poprawy efektywności immobilizacji enzymów, ze szczególnym uwzględnieniem możliwości zastosowania w skali przemysłowej[65].

Publikacja powstała w ramach pracy statutowej (nr 11331011-343) obejmującej badania naukowe i/lub prace rozwojowe, finansowanej przez Ministerstwo Nauki i Szkolnictwa Wyższego, zrealizowanej w Głównym Instytucie Górnictwa w Katowicach.

Otrzymano: 18-01-2022

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JEE Journal of Ecological Engineering

Journal of Ecological Engineering 2022, 23(4), 235–249
https://doi.org/10.12911/22998993/146611
ISSN 2299–8993, License CC-BY 4.0

Received: 2021.01.20
Accepted: 2022.02.15
Published: 2022.03.01

Laccase Immobilization on Biopolymer Carriers – Preliminary Studies

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ABSTRACT

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

Keywords: laccase, immobilization, biopolymer, biocatalysis.

INTRODUCTION

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

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

MATERIALS AND TEST METHODOLOGY

Materials

Enzyme

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

Biopolymers for immobilization

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

Other chemicals

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

Biopolymer bead formation

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

Table 1. Biopolymer characteristics

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

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

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

Alginate-Laccase beads (AL-L)

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

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

Chitosan-Laccase beads (Ch-L)

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

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

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

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

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

Immobilization yield

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

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

Instruments and analytical methods

Enzyme assay

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

Influence of bead storage on enzymatic activity

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

Tests of dye (methylene blue) absorption on beads

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

RESULTS AND DISCUSSION

Immobilization method effects

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

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

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

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

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

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

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

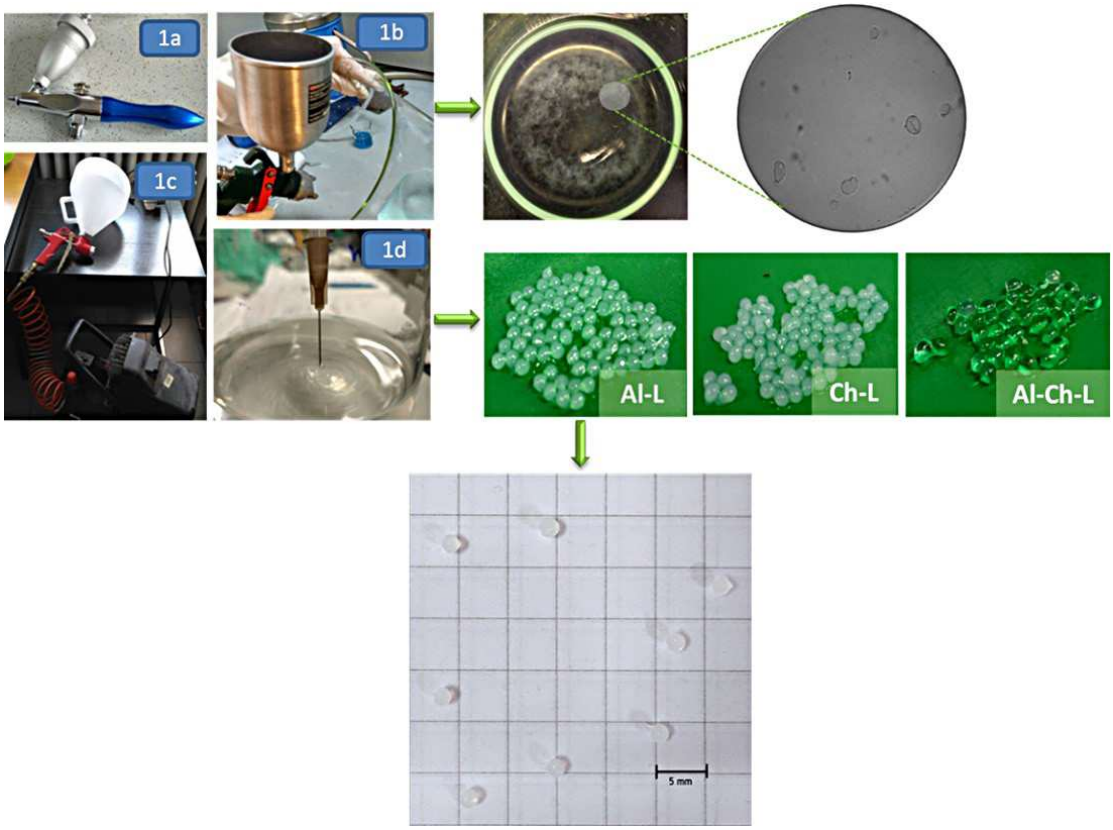


Figure 1. Laccase immobilization methods: a) atomizer φ 0.5; b) atomizer φ 1.4; c) atomizer φ 2.0, d) dropper φ 0.45; AL-L – alginate-laccase beads; Ch-L – chitosan-laccase beads; AL-Ch-L – alginate-chitosan-laccase beads

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

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

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

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

Enzymatic activity of the developed beads

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

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

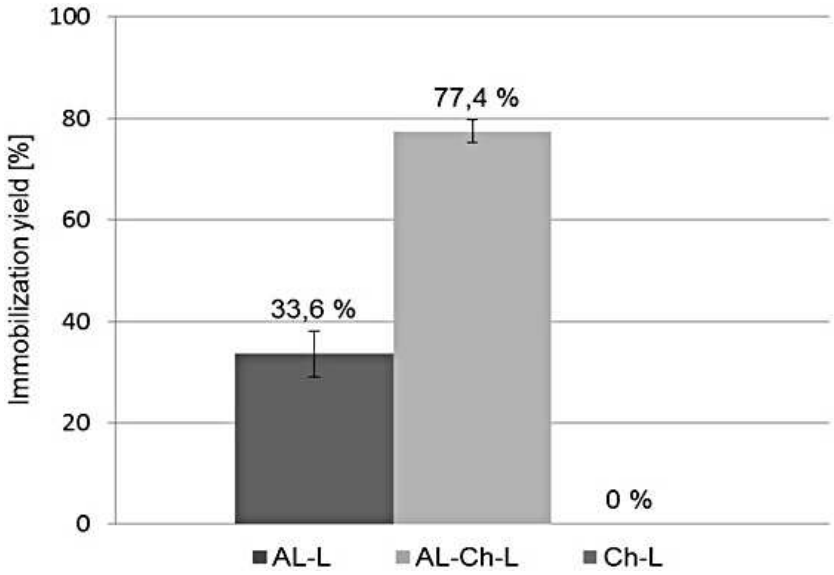


Figure 2. Immobilization yield of alginate-laccase (AL-L), chitosan-laccase (Ch-L) and alginate-chitosan-laccase (AL-Ch-L) beads

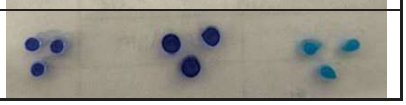
therefore recommended to conduct further testing to optimize the bead production process using a combined carrier based on sodium alginate and chitosan, in order to ensure the appropriate mechanical properties of the enzymatic preparations, while simultaneously retaining their desired activity. Testing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

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

Dye absorption on enzyme-free polymer beads

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

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

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

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

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

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

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

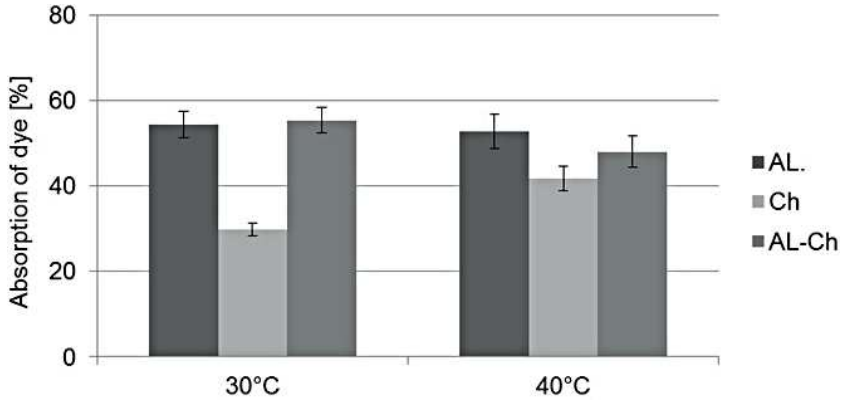


Figure 3. Dye absorption by polymer beads (AL – alginate beads; Ch – chitosan beads; AL-Ch – alginate-chitosan beads)

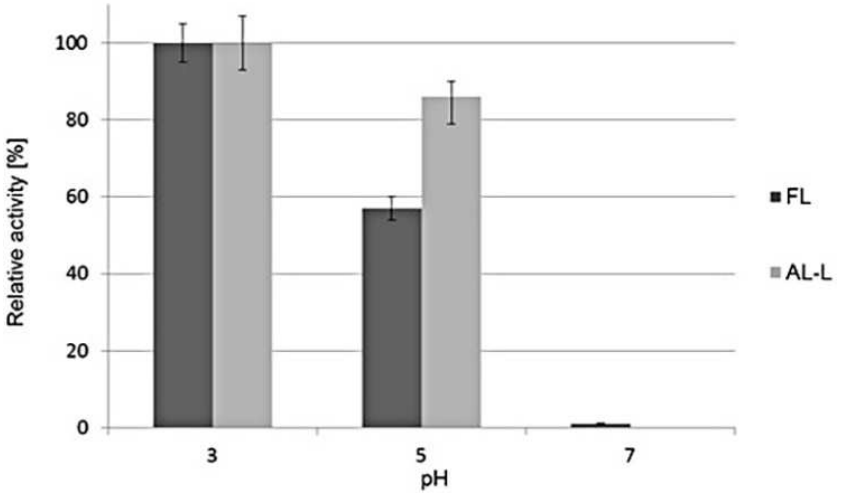


Figure 4. Effect of pH on the activity of FL and AL-L (FL – free laccase, AL–L alginate laccase bead)

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

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

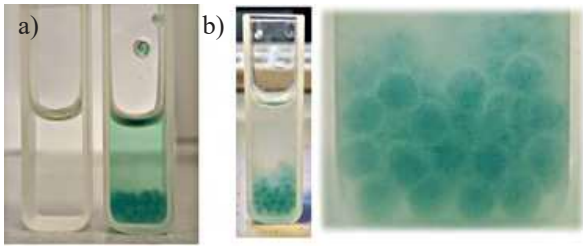


Figure 5. ABTS oxidation by enzyme-containing alginate beads at pH 5 a) and at pH 7 b)

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

Influence of bead storage conditions on enzymatic activity

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

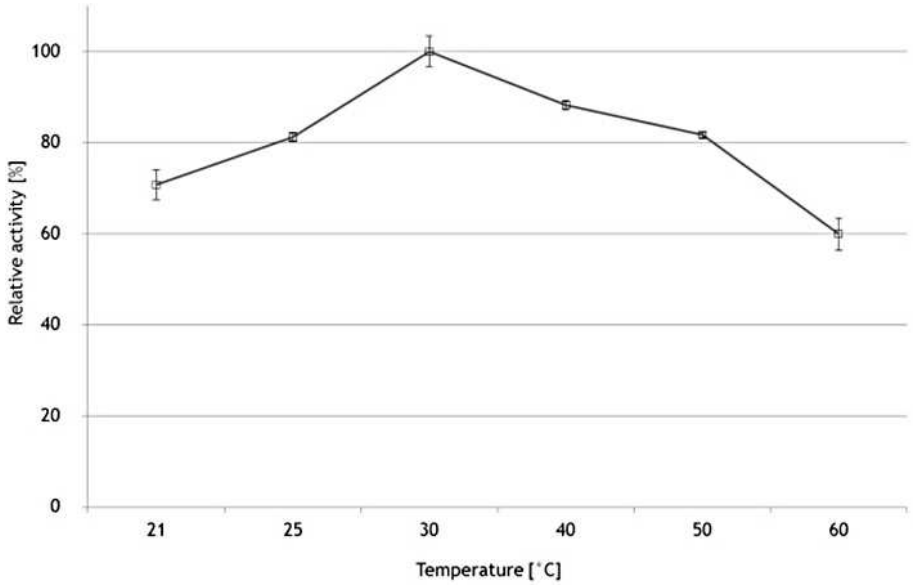


Figure 6. Effect of temperature on the activity of free laccase (FL)

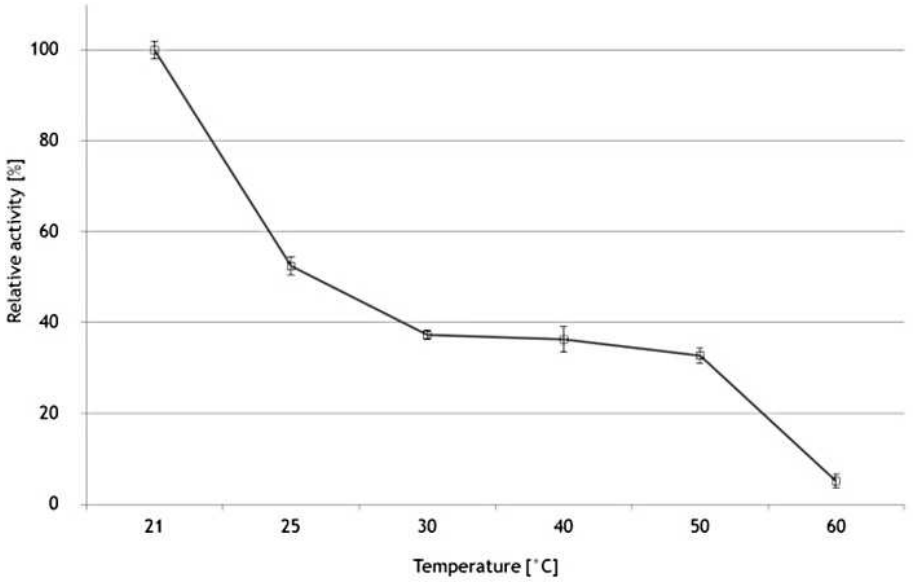


Figure 7. Decrease in immobilized laccase (AL-L) activity under increased temperature conditions

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

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

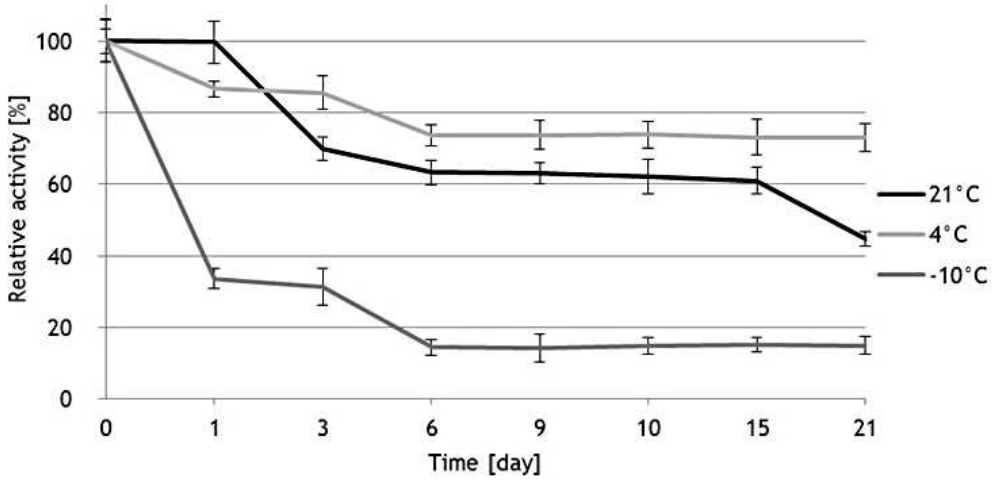


Figure 8. Alginate bead enzymatic activity during storage in parafilm-sealed Petri dishes for 21 days

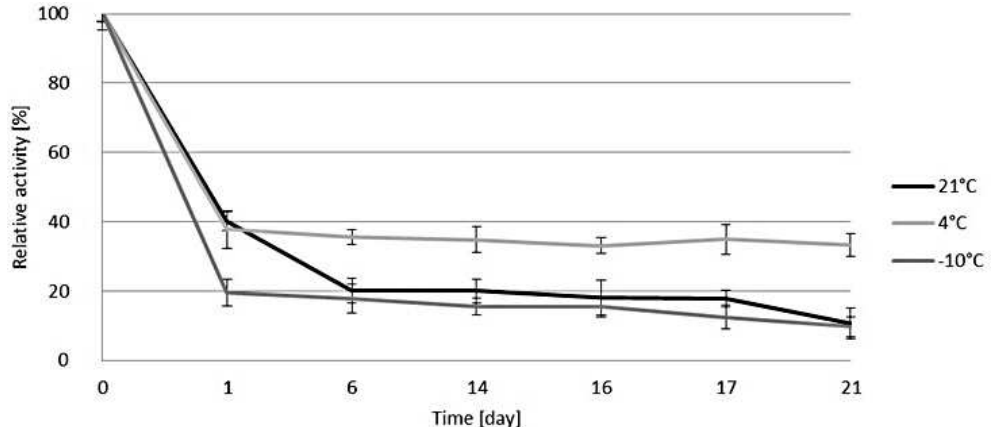


Figure 9. Alginate bead enzymatic activity during storage in water for 21 days

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

Alginate bead activity after low-temperature drying

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

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

CONCLUSIONS

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

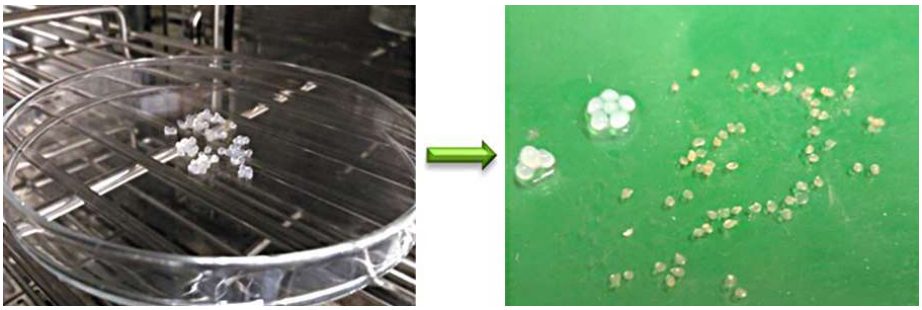


Figure 10. Alginate beads after low-temperature drying

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

Acknowledgements

This work was supported by the Polish Ministry of Science and Higher Education in the framework of the research work [No 11331011-343], in the Central Mining Institute in Poland.

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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

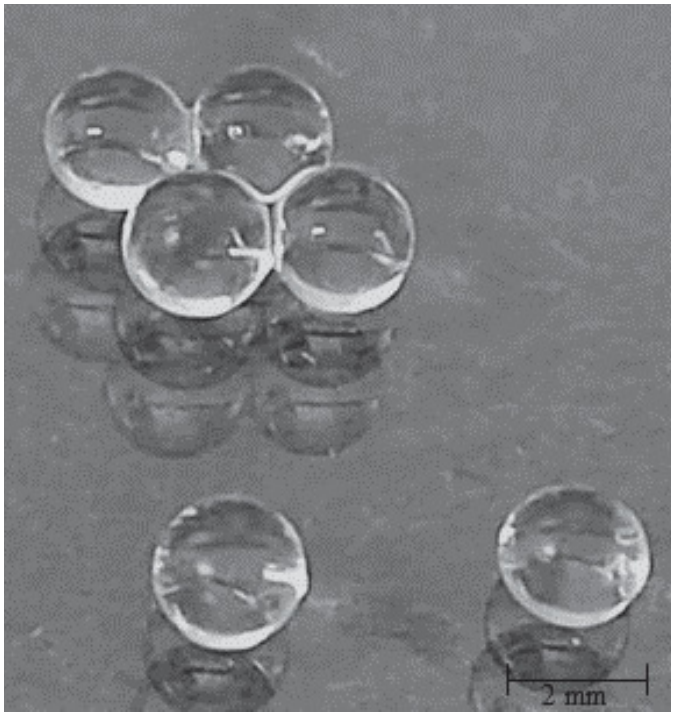

Figure 1. Alginate-laccase beads (ALe)

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 % = $(A_0\text{-}A_t)/ (A_0 \times 100)$, 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.

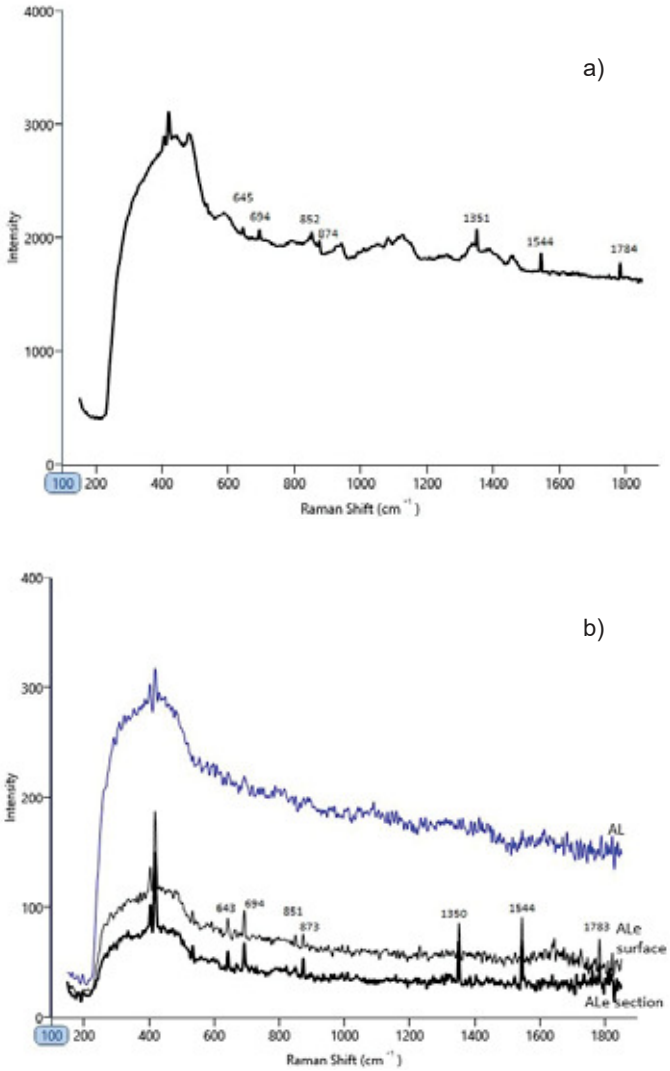

Figure 2. Raman spectra registered for:
a) the laccase enzyme (powder) (E), exposure time 10 s,
b) alginate beads with no enzyme (AL) and the alginate-laccase bead surface (ALe surface) and cross-section (ALe section), exposure time 5 s.

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).

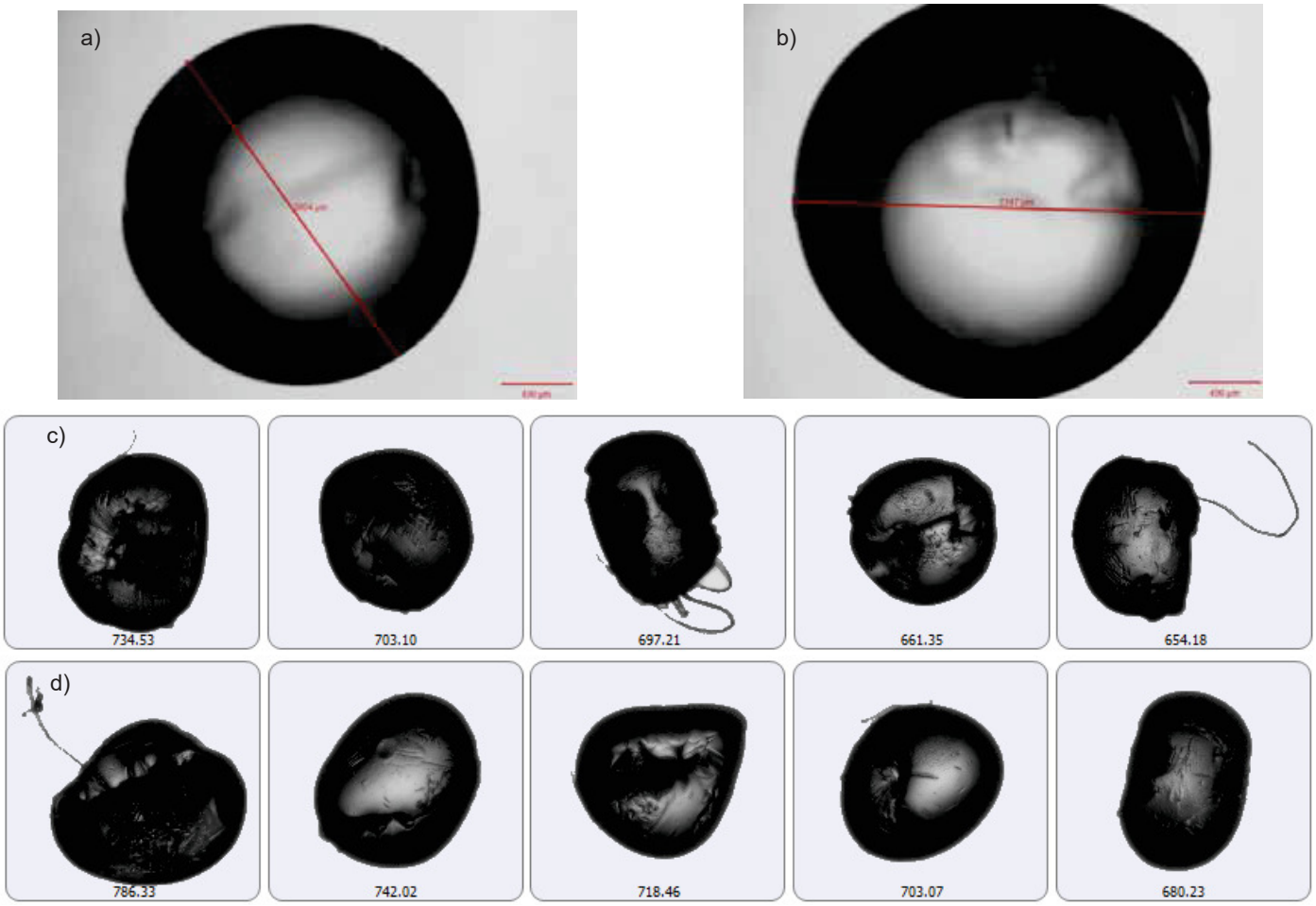

Figure 3. Bead pictures: a) ALe bead immediately after preparation, b) AL bead immediately after preparation, c) ALe beads after natural drying, d) AL beads after natural drying

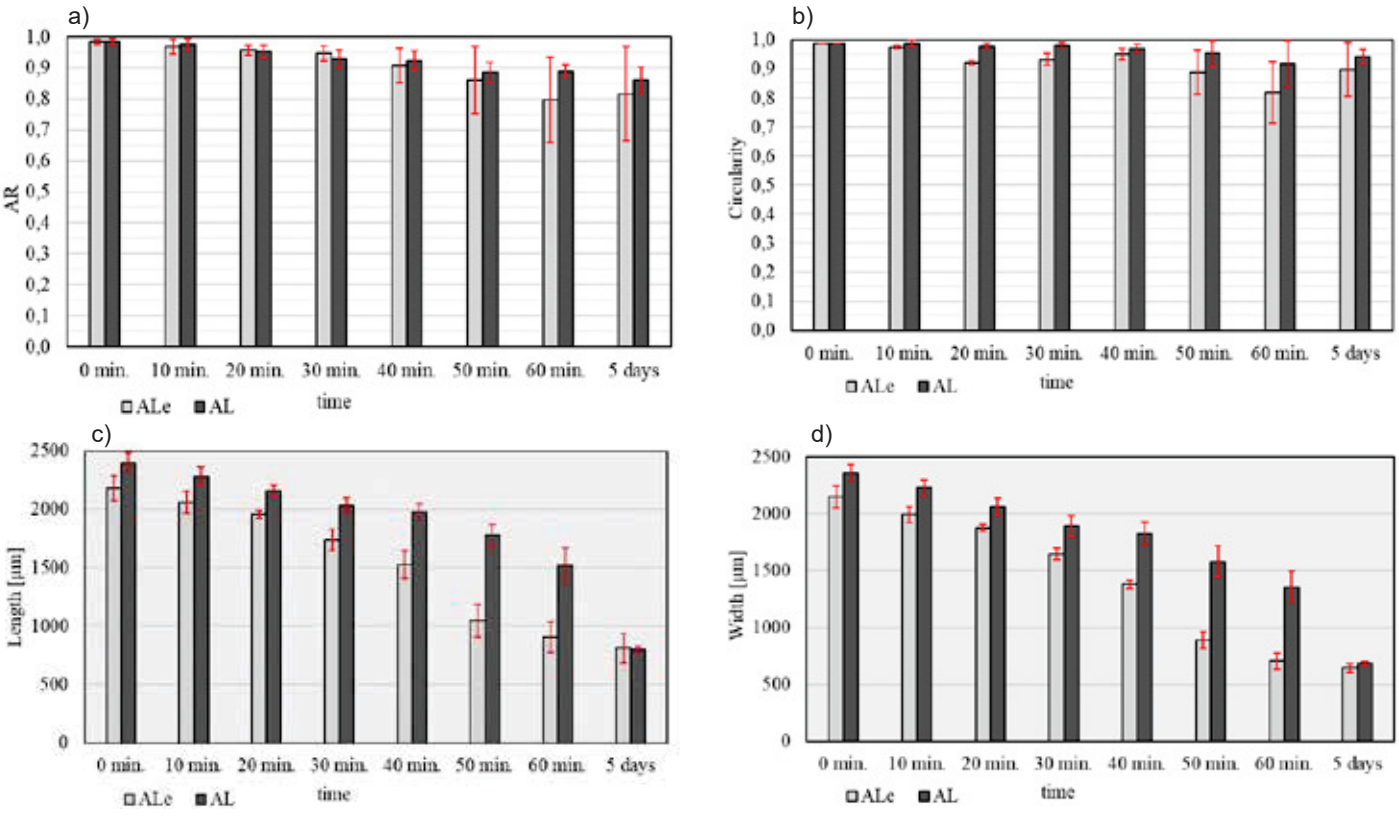

Figure 4. Geometric parameter variations of ALe and AL beads as a function of time: a) aspect ratio (AR), b) circularity, c) length, d) width (mean value with standard deviation)

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

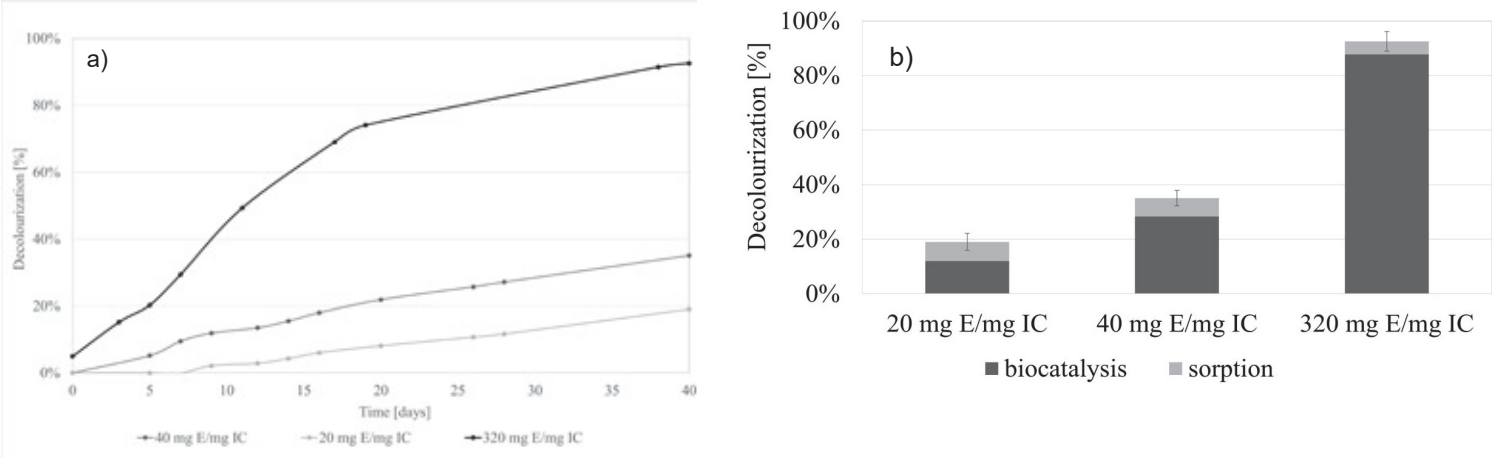

Figure 5. Decolourization efficiency by alginate-enzyme beads of a) different enzyme doses per 1 mg of indigo carmine (IC) dye over 40 days at 21°C, b) with an identification of the dominant mechanism.

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

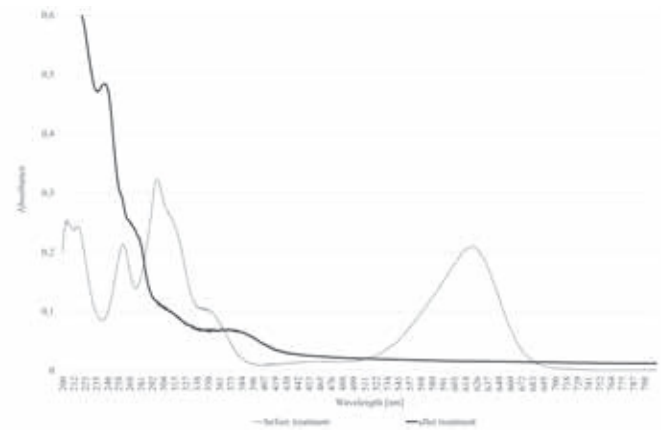

Figure 6. UV-vis absorption spectra of indigo carmine (IC) before and after immobilized laccase treatment (40 days, temperature: 21ºC, enzyme dose: 320 mg E/mg IC).

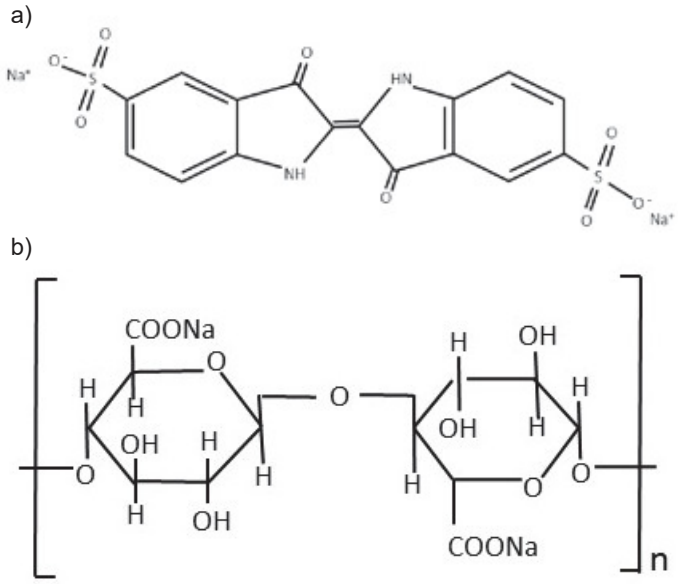

Figure 7. Chemical structure of a) indigo carmine and b) sodium alginate.

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).

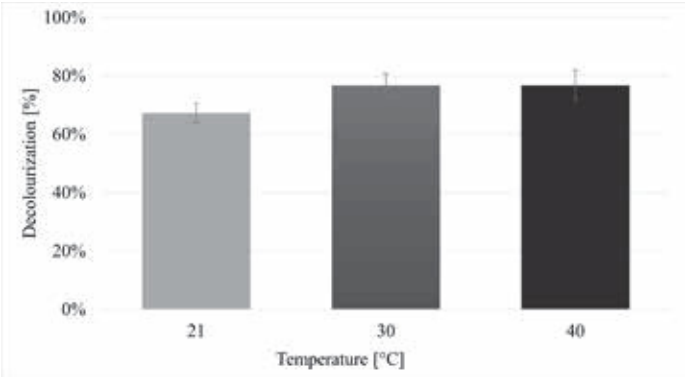

Figure 8. Influence of temperature (21, 30 and 40ºC) on the decolourization efficiency of indigo carmine over 20 days, enzyme dose: 320 mg/mg IC.

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.

Archives of Environmental Protection
Vol. 50 no. 1 pp. 19–34

PL ISSN 2083-4772
DOI:10.24425/aep.2024.149429

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

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Keywords: enzyme, sodium alginate, dye, biocatalysis, immobilization

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.

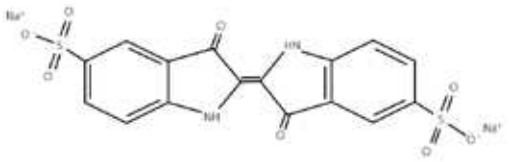
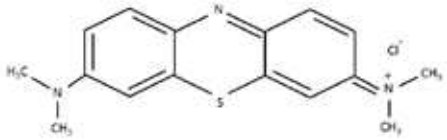
Introduction

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)

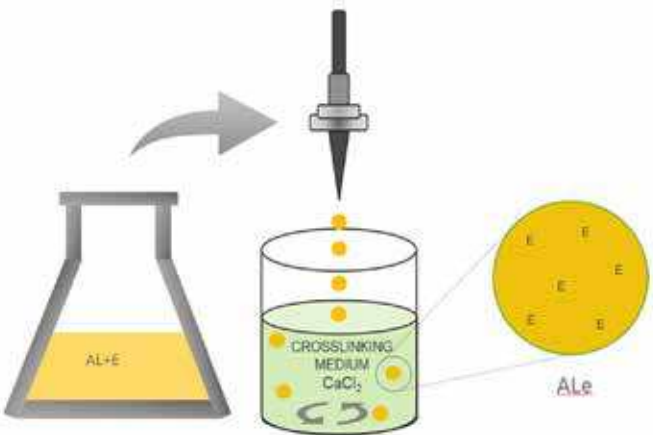


Figure 1. Scheme of enzyme immobilization process
Symbols: AL – alginic acid, E – laccase enzyme, ALe – alginate-laccase beads

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,

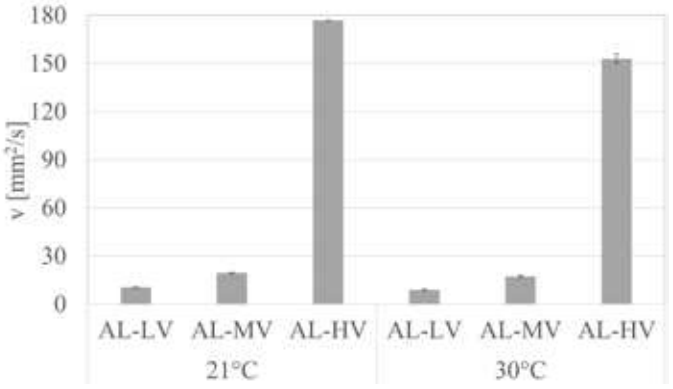


Figure 2. Viscosity of sodium alginate solutions (2%) tested at 21ºC and 30ºC

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

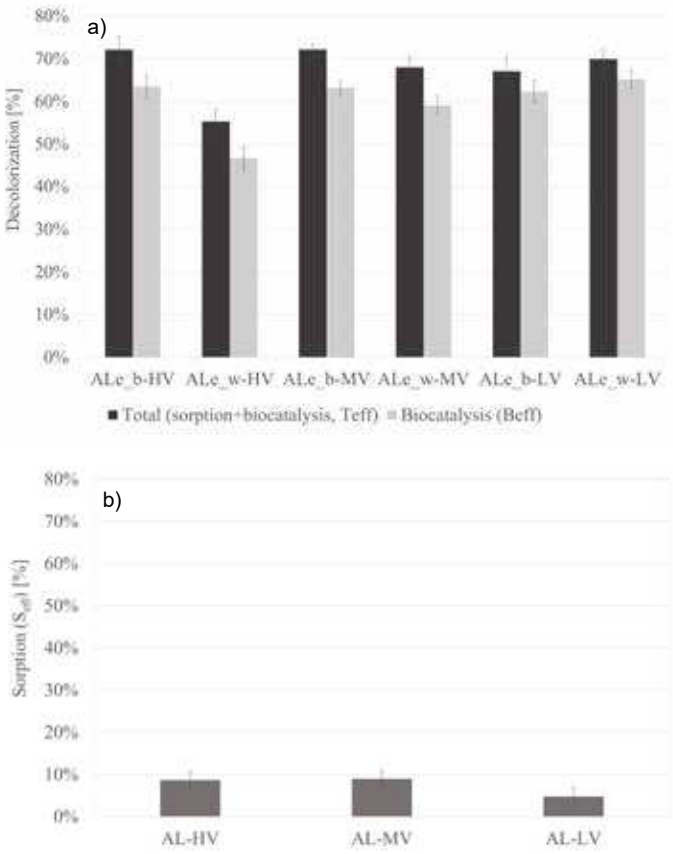


Figure 3. Indigo carmine removal, a) decolorization by enzyme-alginate beads and b) sorption of alginate beads

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

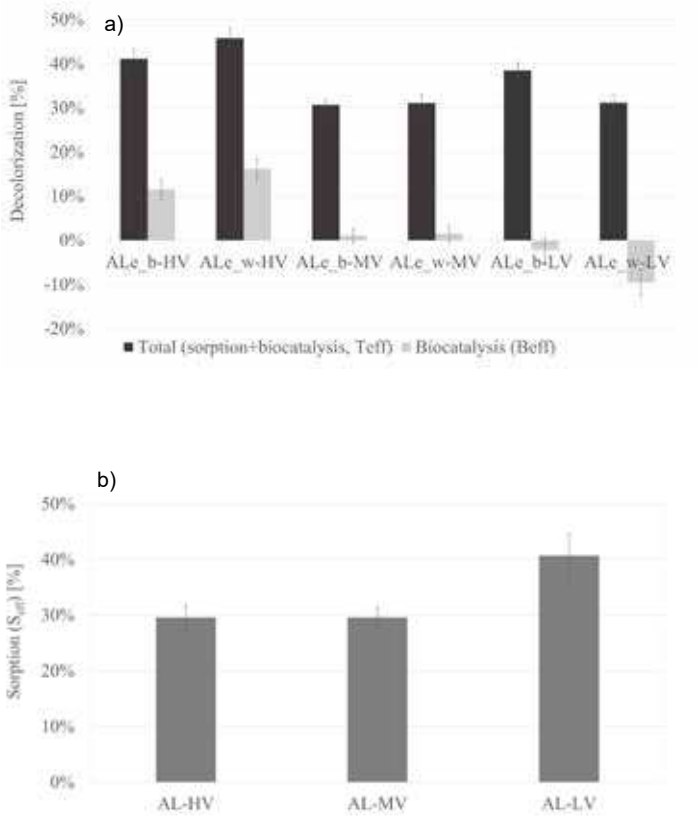


Figure 4. Methylene blue removal: a) decolorization by enzyme-alginate beads and b) sorption of alginate beads

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

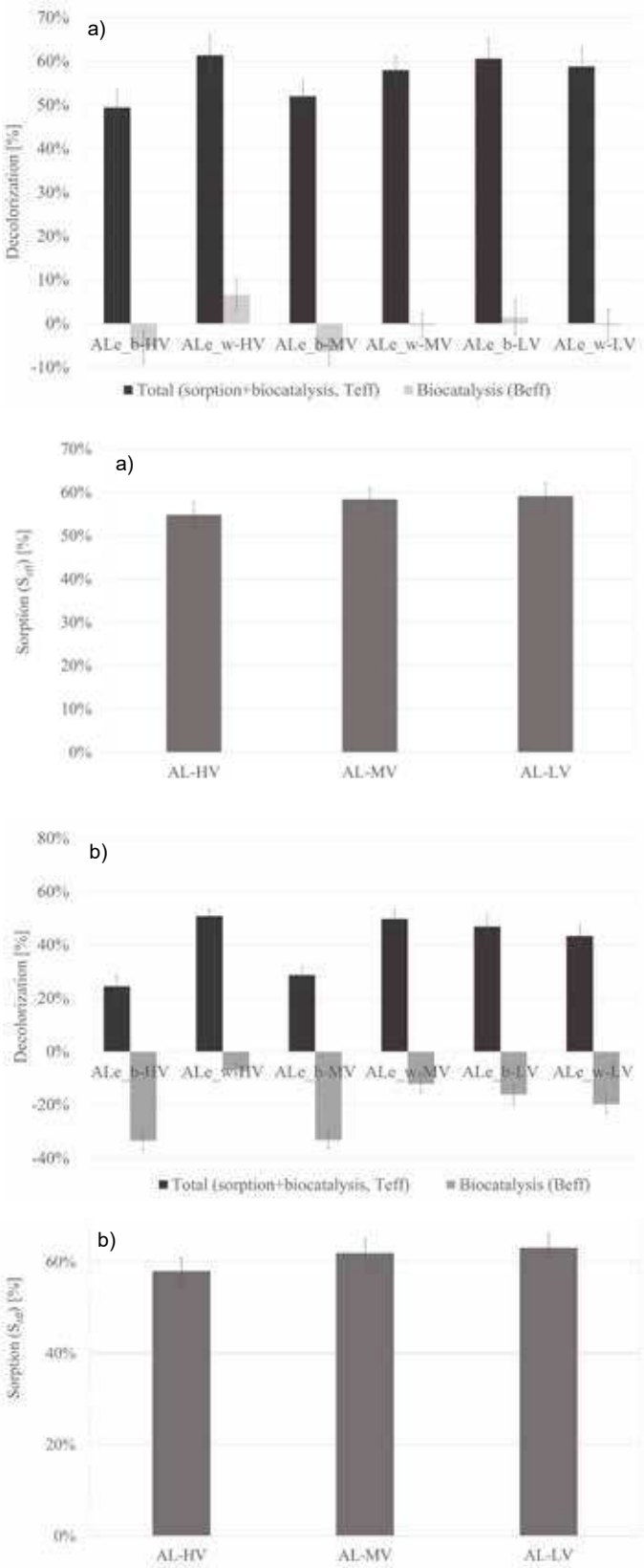


Figure 5. Decolorization of a) IC in a mixture and b) MB in a mixture

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.

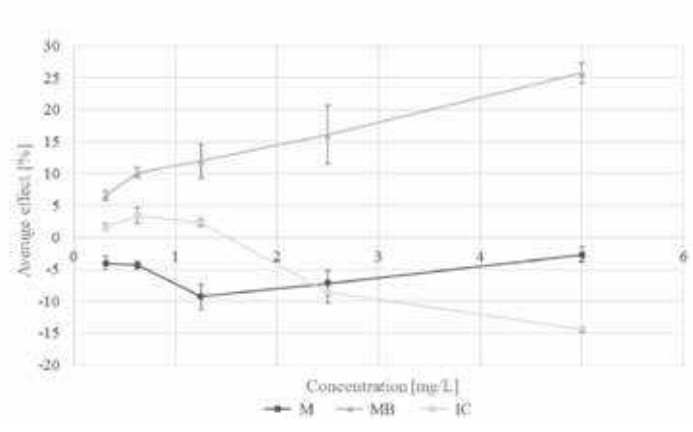


Figure 6. Toxicity testing: dose-effect relationship of M, MB and IC in the Aliivibrio fischeri luminescence inhibition test

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.