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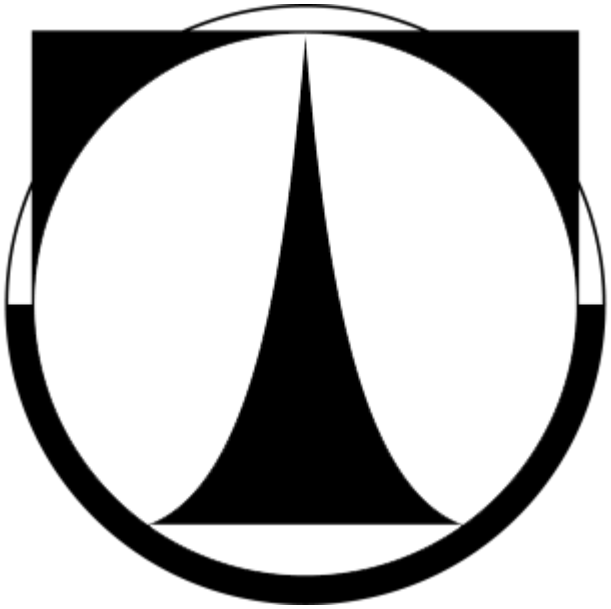

DISSERTATION THESIS

Enzymatically activated filters for water treatment

Liberec 2020 Ing. Milena Maryšková

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Dissertation thesis

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Abstract

Over recent decades, emerging pollutants have come to represent an increasing threat to aquatic organisms due to their high persistence and tendency to accumulate in living organisms, even at low concentrations. Amongst these, several enzymes of the oxidoreductase group have shown an ability to oxidize phenolic, polyphenolic, aniline and even some inorganic compounds.

This dissertation thesis comprises an outline of a water treatment method using an enzymatically activated filtration system. The thesis starts by comparing suitable enzyme candidates and methods of enzyme production and isolation and continues with methods of enzyme immobilization onto selected nanofiber supports, testing of degradation efficiency toward the most common endocrine disrupting chemicals in real water, and ends with a discussion around possible variants of a feasible model filtration system.

Of two potential enzyme candidates (laccase, peroxidase), laccase was selected as the most suitable candidate for immobilization onto a nanofiber support. Subsequently, the optimal immobilization method was determined using polyamide 6, polyamide/polyethylenimine and poly(acrylic acid) nanofibers as enzyme carriers. The most effective immobilization process involved bonding laccase with poly(acrylic acid) via EDAC and S-NHS activation, which provided both high activity and stability of the attached enzyme.

Finally, the best samples (with immobilized crude laccase) were tested for degradation efficiency on a mixture of micropollutants (bisphenol A, 17α-ethinyletsradiol, triclosan and diclofenac) in real wastewater effluent. The samples proved both robust and highly active, and thus represent an efficient candidate for final wastewater treatment technology.

KEYWORDS: laccase, peroxidase, enzyme immobilization, nanofibers, wastewater treatment, endocrine disrupting chemicals

Abstrakt

V posledních desetiletích se ve vodních zdrojích začaly akumulovat polutanty, které negativně ovlivňují zdraví organizmů i při nízkých koncentracích. Některé enzymy z třídy oxidoreduktáz však mají schopnost oxidovat fenolické, polyfenolické, anilinové a dokonce určité anorganické sloučeniny.

Tato dizertační práce pojednává o možnosti využití enzymaticky aktivovaných filtračních systémů, počínaje porovnáním vhodných enzymů, jejich produkcí a izolací, následující imobilizací na vhodný nanovlákenný nosič, testováním efektivity při degradaci nejběžněji se vyskytujících endokrinních disruptorů v reálné vodě a nakonec nastíněním možností vývoje vhodných filtračních systémů.

Ze dvou potenciálních enzymatických kandidátů (lakáza, peroxidáza) byla vybrána lakáza jako nejvhodnější pro imobilizaci na nanovlákenný nosič. Následně byla vyvinuta metoda pro imobilizaci na nanovlákna z polyamidu 6, směsi polyamid/polyetyleniminu a z kyseliny polyakrylové (PAA). Právě imobilizace na PAA prostřednictvím aktivačních činidel EDAC a S-NHS byla nejefektivnější a bylo při ní dosaženo vysoké aktivity a stability imobilizovaného enzymu.

Následně byly testovány nejlepší vzorky s imobilizovanou nepřečištěnou lakázou při degradaci směsi mikropolutantů (bisfenol A, 17α-ethinyletsradiol, triklosan, diklofenak) v reálné odpadní vodě. Vzorky byly velmi odolné a vysoce aktivní, a proto se ukázaly jako vhodný kandidát v technologii dočištění odpadních vod.

KLÍČOVÁ SLOVA: imobilizace lakázy, nanovlákna, peroxidáza, čištění odpadních vod, endokrinní disrutproy

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Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AY	activity yield
BPA	bisphenol A
CLEAs	crosslinked enzyme aggregates
DCF	diclofenac
DIW	deionised water
EDAC	1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride
EDCs	endocrine disrupting chemicals
EE2	17α-ethinylestradiol
GA	glutaraldehyde
GUA	guaiacol (2-Methoxyphenol)
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
IY	immobilization yield
MEG	malt-extract glucose
Na_2EDTA	ethylenediaminetetraacetic acid disodium salt
NFs	nanofibers
PA	polyamide
PAA	poly(acrylic acid)
PAA-laccase	PAA nanofibers with immobilized crude laccase
PAA/lam	laminated PAA nanofibers
PAA/lam-lacccase	laminated PAA nanofibers with immobilized crude laccase
PAA-TV	PAA nanofibers with immobilized commercial laccase
PA6	polyamide 6
PA/PEI	polyamide 6/polyethylenimine
PEI	polyethylenimine
PET/CV	Poly(ethylene terephatalate)/cushion vinyl
PO	*Pleurotus ostreatus*
SN	supernatant
S-NHS	sulfo-N-hydroxysuccinimide ester
SPE	solid-phase extraction
SPME	solid-phase microextraction
STAB	triacetoxyborohydride
SYR	syringaldazine (4-Hydroxy-3,5-dimethoxybenzaldehyde azine)
TCS	triclosan
THF	tetrahydrofuran
TV	*Trametes versicolor*
WWTP	wastewater treatment plant

Introduction

Wastewater treatment plants have to deal with increasing amounts of micropollutants that have a negative effect on both environmental and human health. These tend to occur at very low concentrations (μg/L to < ng/L) and most have only become detectable following significant advancements in available analytical methods. These 'emerging' micropollutants represent a new and, as yet, insufficiently explored form of toxicity, not least due to their remarkable persistence in the aquatic environment and their ability to bioaccumulate in living organisms. A number of chemical compounds, mostly originating from pesticides, pharmaceuticals, cosmetics, flame retardants, perfumes, waterproofing agents, plasticizers and insulting foams [1], [2], interfere with human and other vertebrate endocrine systems by mimicking the effect of hormones.

Conventional wastewater treatment methods are insufficient for complete reduction of some pollutants, and especially endocrine disrupting chemicals (EDCs). Wastewater treatment plants are only capable of removing or transforming a limited amount of such compounds, either through sorption onto activated sludge or through common degradation processes [3]. While progressive technologies, such as photocatalysis, UV oxidation, ozonation, super-critical water oxidation and ultrasound and ionizing radiation, appear to be more effective in removing some EDCs [4], [5], most of these approaches require high energy and reagent input. The future strategy of EDC treatment in the EU, according to Directive 2013/39/EU of the European Parliament, is based on just two alternative processes: ozonation and treatment with powdered activated carbon [6]. Ozonation is potentially hazardous due to toxicity associated with the formation of possible harmful by-products (e.g., the suspected human carcinogen bromate, when bromine appears in water) [7]. While activated carbon possesses a high adsorption capacity for organic matter (requires a small particle size and prolonged contact time), at the end of the process the carbon needs to be separated and sent for destruction/re-activation through incineration [8]. Alternative technologies now under consideration involve nanofiltration, reverse osmosis, and enzymatic treatment and, from this perspective, nanofibers appear to represent the most promising material [9], [10].

Numerous previous studies have addressed enzyme immobilization, including immobilization of oxidoreductases, for wastewater treatment. Most of these focus on laccase as the optimal candidate [11]–[15], with peroxidase [16]–[19] and fungal tyrosinase [20]–[22] less often chosen. More recent studies have also described immobilization of two enzymes synergistically, thereby combining their efficiencies [23]–[26]. Of the available immobilization techniques tested using different forms of matrix (e.g., nanoparticles, beads, foams, nanofibers, mats), nanofibers appear to be the most promising for wastewater treatment as they can be used to form safe and easily handled macroscopic mats with a high specific surface area. However, in order to be applicable in water treatment technology, the final nanofiber-laccase membrane needs to be cost-effective and safe.

This dissertation thesis is based on the immobilization of laccase onto specifically designed and modified nanofibers formed by synthetic polymers. The activity and stability of the immobilized enzyme was determined under different operational conditions and immobilization process parameters. Samples with immobilized commercial and crude laccase were then tested to verify enzymatic degradation of selected EDCs (bisphenol A, 17α-ethinylestradiol, triclosan and diclofenac) in real wastewater effluent. The final section focuses on the design of feasible filtration options.

Theoretical background

1. Enzymes

Enzymes are biocatalytically active proteins with the primary structure formed by sequences of 100–1000 amine acids. These polypeptide chains spontaneously fold into secondary conformations (α-helix, β-sheet or β-turn) that connect together to form the three-dimensional tertiary structure, essential for the catalytic activity. Proteins consist of several domains, regions of the secondary structure with specific functions such as binding a substrate or a cofactor [27].

Enzymes are highly selective biocatalysts as they are considerably increasing the rate of a reaction by lowering its activation energy and converting substrates into products much faster. The part of the enzyme mainly responsible for the catalysis is the active site, which is usually a hydrophilic cleft or a cavity containing amino acid side chains able to bind substrate by one of four types of interactions (electrostatic interactions, hydrogen bonding, Van der Waals and hydrophobic interactions). In some cases the catalytic reaction might be supported by cofactors attached to the active site of the enzyme [27].

There are six classes of enzymes according to the type of the performed catalytic reaction:

1. *Oxidoreductases*... catalyze oxidations and reductions,
2. *Transferases*..... catalyze transfer of glycosyl, methyl, phosphoryl groups etc.,
3. *Isomerases* ..... catalyze geometric or structural changes inside of the molecule,
4. *Hydrolases* ..... catalyze hydrolytic cleavage of chemical bonds,
5. *Lyases* ..... catalyze cleavage of chemical bonds by means other than hydrolysis leaving double bonds or a new ring structure,
6. *Ligases*..... catalyze the joining of two large molecules producing a new chemical bond [28].

1. 1. Oxidoreductases

Oxidoreductases are enzymes widely occurring among microbial, plant and animal organisms. They catalyze the transfer of electrons from one molecule to another which results in oxidation or reduction, whether the enzyme represents electron donor or electron acceptor [29].

1. 1. 1. Laccase

Laccases are interesting enzymes able to degrade phenolic, polyphenolic, aniline and even some inorganic compounds. This ability determines them to be used in biotechnological processes that include wastewater treatment due to their ability to degrade chemicals produced mainly by paper, textile, pharmaceutical, agricultural or petrochemical industry.

Additional usage of laccases represents polymer synthesis, bioremediation of contaminated soil, stabilization of wine and other beverages. Currently, laccase immobilization has been studied for potential applications in ecological field including degradation of endocrine disrupting chemicals as well as medical applications such as cancer treatment [30].

Laccases are produced by higher plants and fungi but they were also observed in some insects and bacteria. They can be commercially extracted from a culture medium of different fungi because they are produced extracellularly as the result of a reaction to specific stressful conditions. Extracted enzyme solution is subsequently purified by multiple procedures consisting of centrifugation and lyophilisation [31].

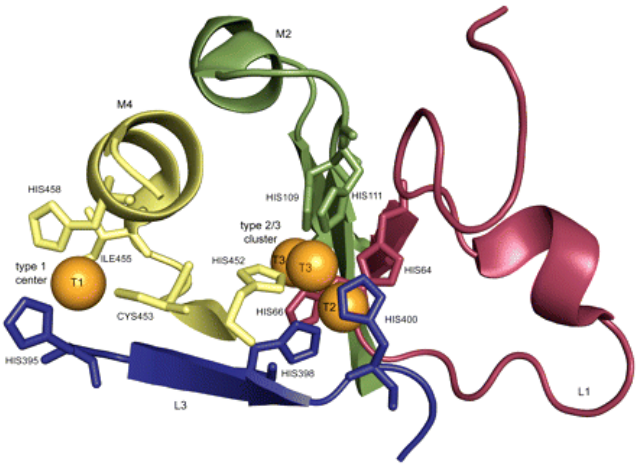


Fig. 1 Structure of laccase [40]

The molecule of laccase (Fig. 1) is usually dimer or tetramer glycoprotein with molecular mass between 50 and 100 kDa. Up to 50% of the molecule is formed with glycosides which increase the final stability of the enzyme. The isoelectric point is at pH between 3 and 7 depending on the particular type of laccase [32].

The molecule (Fig. 2) contains four copper atoms in three redox domains (T1, T2, T3). The atom in T1 reduces the substrate while the other atoms bind oxygen and reduce it into water [33]. Four electrons coming from four molecules of the substrate are necessary for the reduction of one molecule of oxygen while only one electron is produced by this reduction. The enzyme stores produced electrons and uses them to form water molecules [34], [35]. The first step of the substrate oxidation is usually formation of a radical followed by oxidation or non-enzymatic reaction such as hydration or polymerization. Substrate degradation can also be realized via a mediating molecule (for example 2,2′-azino-bis(3-ethybenzthizoline-6-sulfonic acid)) that transports electrons donated by enzyme to attack other molecules (Fig. 3) [36], [37].

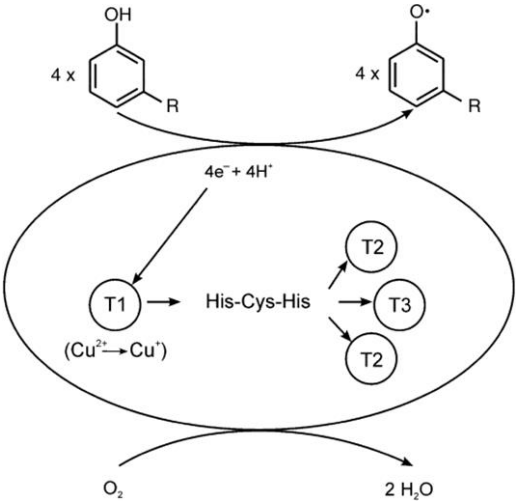


Fig. 2 Catalytic cycle of laccase [43]

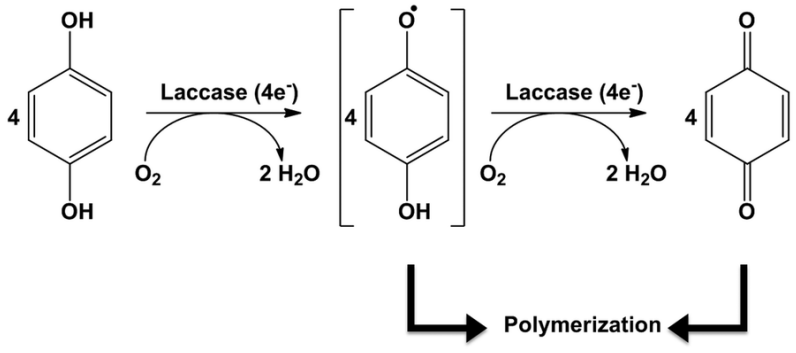


Fig. 3 Oxidation of a phenolic compound by laccase [45]

1. 1. 2 Peroxidase

Peroxidases are iron (III) containing enzymes with a molecular weight ranging from 30 to 150 kDa (approximately 44 kDa in case of horseradish peroxidase). They catalyse the reduction of peroxides and the oxidation of a various organic and inorganic compounds, mostly aromatic phenols, phenolic acids, indoles, amines and sulfonates [38]. The typical catalytic reaction consists of following reactions (Eq 1, 2, 3) [39]:

$$HRP + H_2O_2 \longrightarrow HRP^{-I} + H_2O \quad (1)$$

$$HRP^{-I} + AH_2 \longrightarrow HRP^{-II} + AH^{\cdot} \quad (2)$$

$$HRP^{-II} + AH_2 \longrightarrow HRP + AH^{\cdot} + H_2O \quad (3)$$

The structure (Fig. 4) consists of two centers, one containing iron heme group and two calcium atoms. The planar heme group is considered the active site of the enzyme because it is open for the peroxide to attach [40].

Peroxidases are widely used in biochemistry for enzyme immunoassays but they find their applications in novel fields such as wastewater treatment or synthesis of organic and polymer chemicals. Several studies focus on degradation of phenolic contaminants from the aqueous environment in the presence of hydrogen peroxide [41]–[43]. Another peroxidase application is decolorization of hardly degradable synthetic dyes, such as azo dyes [44]–[47].

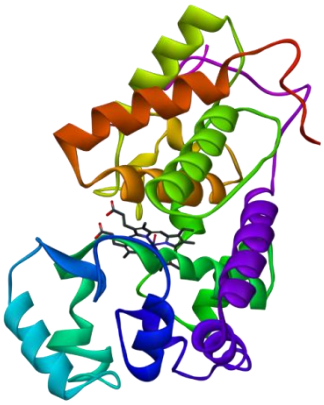


Fig. 4 Structure of horseradish peroxidase [51]

2. Enzyme isolation and purification

Most enzymes are present inside cells in the cytoplasm or in organelles, therefore the first isolation step is tissue homogenization in a selected medium at cold condition. Homogenizing techniques involve blending, ultra-sonic disruption, enzymatic lysis, freeze-thaw method, drying with acetone powder etc. In case of extracellular enzymes, such as fungal laccase, these techniques are skipped and the extracellular medium is used directly as the enzyme source (Figs. 5 and 6).

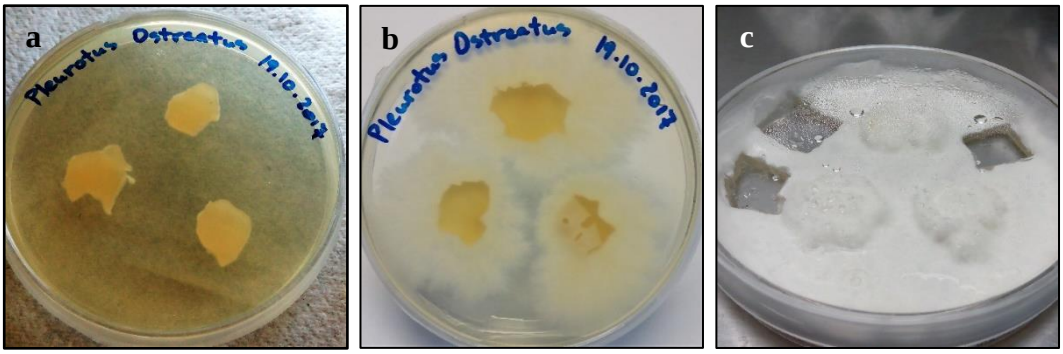


Fig. 5 (a) Plugged malt-extract glucose agar plate with Pleurotus ostreatus; (b) Pleurotus ostreatus after 7 days of incubation; (c) fully cultivated PO with removed plugs for further cultivation (internship at the University in Maribor, Slovenia, 2017)

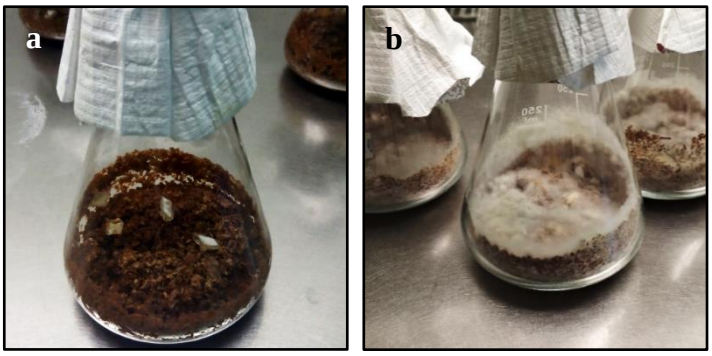


Fig. 6 (a) Fungi filtration through cotton cloth; (b) extracted fungi medium (internship at the University in Maribor, Slovenia, 2017)

The homogenized tissue or the extracellular medium consists of many other protein and non-protein substances and the multi-step enzyme purification is based on enzyme biochemical and biophysical characteristics (solubility, size, charge, hydrophilicity, pH and temperature stability). The techniques selected for enzyme purification should be moderate in order to achieve highly purified enzyme with preserved native conformation [48].

2. 1. Purification methods

Commonly, the first step is based on *fractionation of proteins on the basis of solubility* using ammonium sulfate, organic solvents (e.g. chilled acetone), nonionic polymers (polyethylene glycol) or by heat treatment in case of enzymes stable at temperatures over 55°C.

The next step is *chromatographic separation of the enzyme proteins* involving ion exchange, adsorption, gel filtration or affinity chromatography. In principle, the enzyme sample is applied onto the pre-equilibrated column and afterwards, the sample is eluted with elution buffer. The effluent is collected as a series of fractions and tested for enzyme activity and protein. Other techniques, such as electrophoresis or isoelectrofocusing, might be used. The fractions containing enzyme dissolved in buffer are commonly diluted, therefore concentrating methods should be used (Fig. 7). These techniques involve ultrafiltration, dialysis and crystallization using mostly ammonium sulfate [48]–[50].

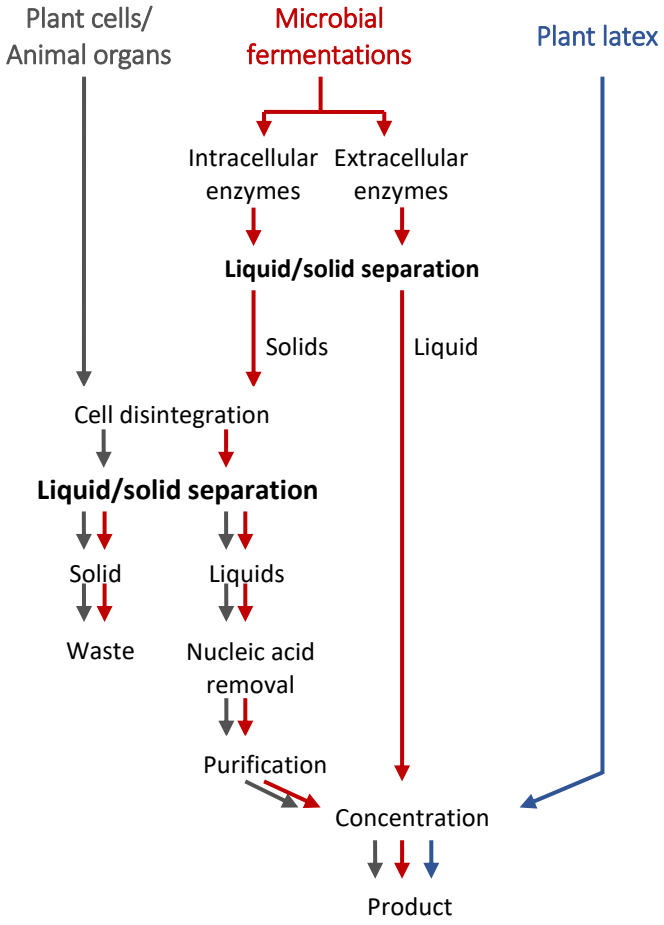


Fig. 7 Scheme of laccase isolation

3. Enzyme immobilization

Although enzymes are excellent biocatalysts with very high efficiency and specificity, there are several complications for their applications in industry. First of all, they are water soluble which practically disables their removal from the solution and repeated usage. Also they might be very unstable and strongly inhibited when working in an industrial environment [51].

The use of enzymes capable of catalyzing the oxidation of these chemicals is regarded as a promising approach to remove EDCs from wastewater. The efficiency of enzyme catalysis is directly dependent on enzyme activity, and stability as repeat usage is a necessary feature for its successful industrial application in wastewater treatment [52], [53]. However, free enzyme is very sensitive to pH, temperature changes, and the presence of inhibitors in the wastewater environment as such factors can cause conformational changes in enzyme molecules, leading to inactivation or direct inhibition.

For this reason the methods to maintain the enzymatic activity for a longer time and for a number of cycles have been explored. Enzyme immobilization increases the rigidity of the attached molecule's structure, thereby enhancing its stability and resistance and allowing repeated application [54]–[59]. Immobilization methods rely on different forms of interaction between biocatalyst's side-chain functional groups and the immobilization support [60]. The type of interaction and the number and strength of the enzyme-support bonds influence the final activity and stability of the immobilized enzyme. The development of immobilized biocatalysts is especially important in a low added value sector of bioeconomy such as environmental services [61]. Enzymes are the major cost-determining factor of enzyme-assisted bioremediation/biodegradation treatments. Therefore, the increase in enzyme stability and reuse possibility provided by immobilization methods contribute to reduce the overall process cost and to make biocatalysis a feasible and attractive alternative to conventional environmental processes.

There are several approaches basically divided into reversible and irreversible methods. To the reversible immobilization belong: *adsorption*, *ionic binding*, *affinity binding*, *chelation* or *metal binding*. The irreversible methods are *covalent coupling*, *entrapment* and *crosslinking* (Fig. 8).

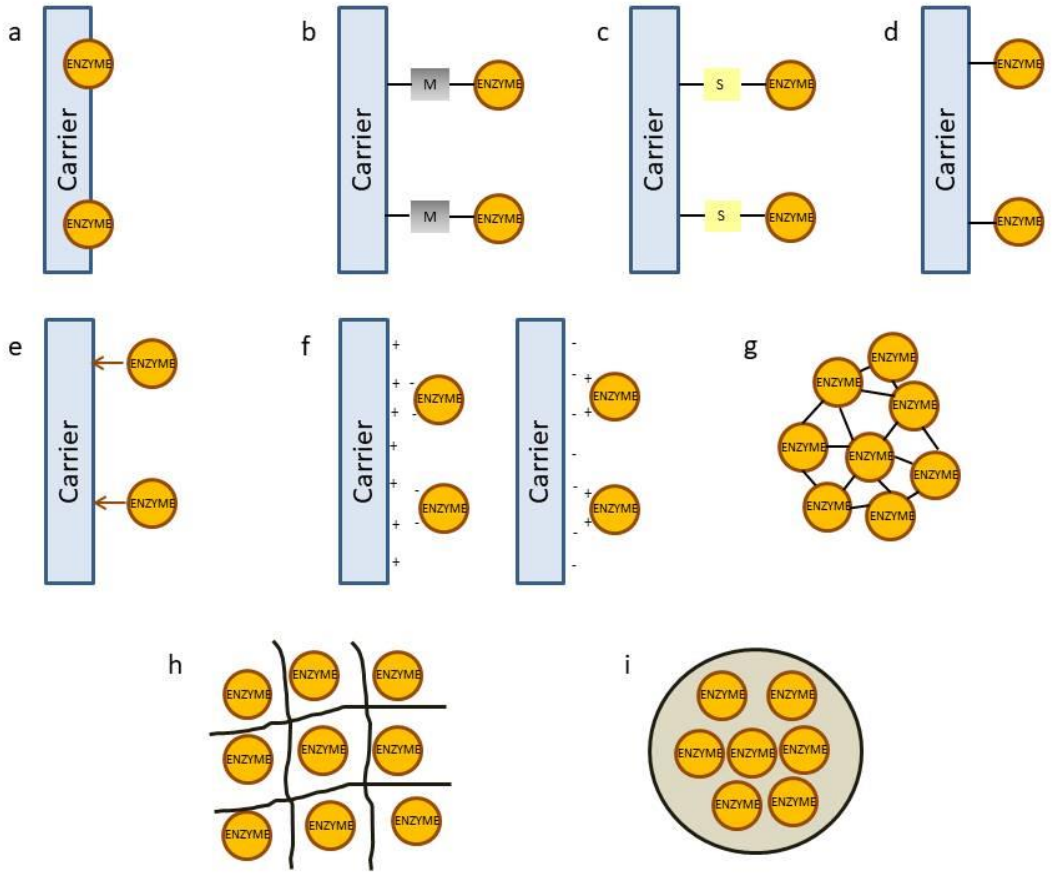


Fig. 8 Most common immobilization techniques: (a) adsorption, (b) chelation, (c) disulfide bonding, (d) covalent binding, (e) affinity binding, (f) ionic binding, (g) crosslinking, (h) entrapment, and (i) encapsulation

3. 1. Irreversible immobilization

In principle, after the irreversible immobilization the enzyme becomes a part of a structure of a carrier. This method evolves chemical binding which is usually very harmful for the enzyme activity and might cause some conformational changes, on the other hand this method usually hughly increases enzyme's stability [51], [62], [63].

Covalent attachment

The biggest advantage of the covalent attachment is a multiple re-use with minimal leakage of the enzyme into the solution. Most coupling reactions involve free functional groups of the available amino acids lysine (amine group), cysteine (thiol group) and asparic, acrylic or glutamic acids (carboxylic group).

There are multiple procedures to connect these side chains to the matrix based on the chosen coupling agent and types of groups of the protein and the chemistry of the support. However, there is always a significant probability of activity loss after the

attachment caused by conformational changes within the protein structure or by diffusional limitations [51], [62].

Entrapment

Enzyme entrapment or encapsulation is an immobilization process that allows a free flow of a low-molecular weight substrate. The enzyme is not attached via strong chemical bonds but is mainly held by surrounding molecular chains representing a cage. This cage can be formed by gels, fibers or microencapsules. The biggest disadvantage of this method is usually mass transfer limitations that occur in most cases. However, this method can be optimal for specific applications, such as drug delivery, that enable the gradual matrix degradation followed by enzyme release [51], [62], [64].

Crosslinking

Crosslinked enzyme aggregates (CLEAs) are usually molecules of soluble enzyme attached to each other via a bifunctional agent such as glutaraldehyde. These CLEAs are easily recovered from the reaction mixture by centrifugation or filtration. Carrier-free enzyme immobilization has many advantages. The enzyme remains a high specific activity with enhanced stability compared to the free enzyme. The production cost is also lower without a solid carrier. However, solution with the cross-linked enzyme is usually very viscous and uneasy to work with [62], [65], [66].

3. 2. Reversible immobilization

Reversibly immobilized enzymes can be detached from the matrix under specific conditions. These methods are very attractive for economic reasons because the support can be re-loaded with another enzyme and the method might not requires special modification of the support [51].

Adsorption

Adsorption is the simplest method based on physical attachment of an enzyme using hydrogen binding, van der Waals forces or hydrophobic interactions. The process is influenced mainly by pH, ionic strength, temperature and polarity of the solvent. Although this method usually preserves the catalytic activity of the enzyme, leakage might be a very serious disadvantage [51].

The protein can be attached to the matrix via ionic bonding but this principle of affinity bonding might require a covalent support modification using costly affinity ligand [67].

Chelation or metal binding

Metal salts (titanium or zirconium salts) are first precipitated or covalently bound onto the support by heating or neutralization. There are some coordination positions of the metal remaining free for enzymes attachment. These metal chelated supports were named "immobilized metal-ion affinity" adsorbents. Problem might occur when some metal leaks from the matrix cause the leakage of the immobilized enzyme as well [68], [69].

3. 3. Applications of immobilized enzymes

Nowadays, immobilized enzymes can be utilized in various applications. Specifically, they are widely used in medicine due to their high specificity and reactivity providing an element for very sensitive, accurate and cheap biosensors that could selectively detect biological substances. Enzymes can be applied either in diagnostics or treatment. Other biosensoric applications asides from medicine are pathogen or toxin detection in liquid or solid state (water, food or soil) [70]. Furtermore, immobilized enzymes catalyze an ecological synthesis of antibiotics, such as β-laktam [71]. Beyond detection applications immobilized enzymes can degrade toxins, such as phenolic or other hardly degradable compounds, in food or wastewater [72].

Application of enzymes during a washing process, as an ecological substitution for normally used detergents, also falls within the similar category as water treatment. Using immobilized enzyme could result in higher savings and higher effectiveness of the process of washing extremely dirty textiles causing no damage both to the textile and to water environment [73].

4. Enzyme immobilization for wastewater treatment

Nanofibers offer many features determining their application for enzyme immobilization. They can be processed into various structures with high surface area depending on nanofiber morphology (average fiber diameter, surface density and porosity). The most common method to generate nanofiber layers is electrospinning with potentially high productivity, sufficient mechanical properties and chemical stability of the samples. These properties are essential for materials carrying immobilized enzymes. Chemical stability and nanoscaled fibers formed into a macroscaled membrane or a layer, guarantee a safe material for the immobilization process and possible applications in the industrial field [74].

4. 1. Carriers for enzyme immobilization

Numerous previous studies have addressed enzyme immobilization, including immobilization of oxidoreductases for wastewater treatment. Most of these focus on laccase as an optimal candidate [12]–[15], with peroxidase [16]–[19], and fungal tyrosinase [20]–[22] less often chosen. More recent studies have also described immobilization of two enzymes synergistically, thereby combining their efficiencies [23]–[26]. Of the available immobilization techniques using different forms of matrix (e.g. nanoparticles, beads, foams, nanofibers, mats), nanofibers appear to be the most promising for wastewater treatment as they can be used to form safe and easily-handled macroscopic mats with a high specific surface area.

Laccase from *Pleurotus florida* was immobilized onto oxidized **cellulose nanofibers** [75] via adsorption and glutaraldehyde crosslinking, while a reverse process was described by Xu et al. (2015) as a method to immobilize laccase from *T. versicolor* onto an electrospun nanofibrous membrane consisting of multi-walled **carbon nanotubes** (MWNTs), **chitosan** (CS) and **polyvinyl alcohol** (PVA) [76] modified via glutaraldehyde prior the enzyme attachment.

Dai et al. (2010) proposed encapsulation of laccase from *T. versicolor* into **poly(D,L-lactide)/poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) microfibers** by emulsion electrospinning [77].

Both enzyme adsorption and covalent attachment are based on specific interactions between the enzyme and the carrier. In most cases it is necessary to modify the surface in order to increase hydrophilicity, remove components or introduce functional groups on the surface. For example; polyamide materials can be enzymatically modified by cutinase, amidase or protease. This cleavage, also obtainable using a strong acid, leads to shortening of polymeric chains and obtaining higher amount of functional groups required for covalent attachment of an enzyme [78].

Several studies report enzyme immobilization on **nylon fibers** [79]–[82] and laccase was no exception. Laccase from *T. versicolor* was covalently attached to partially hydrolyzed nylon films and nanofibers using 3 M HCl, further modified by glutaraldehyde.

Enzymatically functionalized nylon was selected to immobilize laccase from *T. hirsuta*. Protease cleaved the peptide bonds and increased the quantity of free groups capable of attaching the enzyme. These groups were then activated via glutaraldehyde with presence of a spacer 1,6-hexandiamine [83].

A very popular material for biotechnological applications is carbon. There are several forms of **carbon** (nanotube, nanosphere, fulleren, nanosheet etc.) but nanotubes are very favourite for their large specific surface area and reasonable manipulation during processing [84]. These structures are no self-supporting, therefore they must be used in a combination with another robust material.

For example Liu et al. (2012) used **carbon-based mesoporous magnetic composites** to immobilize laccase from *T. versicolor* via adsorption [85]. Another study describes immobilization of laccase from *Aspergillus oryzae* onto chemically functionalized **multi-walled carbon nanotubes** supported by **polysulfone membranes**.

Another suitable material is silica formed usually into porous beads. Laccase from *T. versicolor* was covalently immobilized on **pre-silanized silica beads** via glutaraldehyde [86] and silica based carriers were used in other multiple studies [86]–[93]. **Chitosan** proved to be an optimal candidate for immobilization of various biomolecules including enzymes. It is biocompatible, hydrophilic and offers high amount of free primary amino groups available for a covalent modification. Chitosan has been previously formed into enzyme-loaded carriers in form of particles, beads, hydrogels or other sructures [94]–[102] including nanofibers or membranes [12], [103], [104].

Magnetic particles offer a great potential because they can be easily removed from the reaction mixture. However, these materials are rarely used without a suitable organic (e.g. cellulose, chitosan, dopamine) or innorganic (e.g. copper sulphate, titanium oxide, silica) modification [105]–[111]. Another interesting group of matrices are **titanium nanoparticles** or various polymer membranes functionalized by TiO_2 because this material may be modified via 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde as well as often used silica. Laccase was immobilized onto carriers containing TiO_2 in studies of [112] and [113]. Other immobilization approaches include various porous structures such as **Amberlite IR-120 H beads** [114] and **zeolite** [115], natural materials such as **green coconut fibers** [116], **alginate** or **cellulose** [117], [118]. Of the available immobilization techniques using different forms of matrix (e.g. nanoparticles, beads, foams, nanofibers, mats), nanofibers appear to be most promising for wastewater treatment as they can be used to form safe and easily-handled macroscopic mats with a high specific surface area.

However, enzyme immobilization is only the first part of the research focused on the degradation of xenobiotics. In order to develop biocatalytic systems with a potential for further use in water treatment, the immobilization procedure must be reasonable in the sense of economy of the used chemicals and enzyme, duration of the immobilization process and sufficient stability of the immobilized enzyme in water environment.

4. 2. Immobilization of laccase and peroxidase for degradation of micropollutants

Laccase from *Trametes pubescens* was immobilized into Ca-alginate beads via glutaraldehyde crosslinking following by entrapment. Furthermore, the immobilized laccase (1500 U/L using 1 μM ABTS as a substrate) was tested against the removal of bisphenol A at 30°C using 100 mL of 20 mg/mL solution in succinic buffer with pH 5. As the result, more than 99% of **BPA** was removed after 2 hous of incubation, and immobilized laccase showed higher than 70% efficiency within 10 catalytic batches [119].

Different type of laccase (*Cyberlindnera fabianii*) was entrapped using Na-alginate as the carrier. The laccase-alginate beads (activity unknown) were then tested against the degradation of 12 mL of approximately 20 mg/mL (100 μM) solution of **BPA** in distilled water. After 24-hour incubation the immobilized laccase degraded

around 40% of the micropollutant and continued with the degradation with resulting 70% of eliminated BPA after 14 days of incubation [120].

Monoaminoethyl-N-aminoethyl agarose (MANAE–agarose) was used for encapsulation of laccase form *Pleurotus ostreatus*. The immobilized laccase (5000 U/L) was further tested against the degradation of 100 mg/L **BPA** solution in acetate buffer with pH 5. All of the BPA was eliminated after one hour of incubation and the immobilized laccase remained effective after 15 degradation cycles [121].

Barrios-Estrada et al. (2018) described immobilization of laccase from *Pycnoporus sanguineus* and *Trametes versicolor* onto multichannel ceramic membrane deposited with gelatin and modified via glutaraldehyde. Subsequently, the biocatalytically active membrane was tested towards the degradation of 20 mg/L solution of BPA in McIlvaine buffer with pH 5. All **BPA** was eliminated within 24 hours [122].

Degradation of 100 mg/L solution of **BPA** was also explored using laccase from *Trametes versicolor* immobilized onto copper phosphate hybrid nanoflowers (laccase precipitated covering amino-functionalized magnetic nanoparticles). All BPA was eliminated in 5 minutes using highly concentrated laccase system (14.4 mg of enzyme protein per 1 L of BPA solution) [106].

Zdarta et al. (2018) reported immobilization of laccase from *Trametes versicolor* onto *Hippospongia communis* sponges with application for the degradation of **BPA** (bisphenol A), **BPF** (bisphenol F) and **BPS** (bisphenol S) at a concentration of 2 g/L at pH 5. Approximately 50 mg of the biocatalytic system containing 5 mg of laccase were used for the experiment performed at 30°C and 40°C in a total volume of 30 mL. Almost all BPA, BPF and BPS were eliminated within 24 hours of incubation [123].

Laccase from *T. versicolor* was immobilized onto magnetic Fe_3O_4/chitosan microspheres via Cu(II) and Mn(II) reversible chelation. Approximately 85% of **BPA** (the initial concentration 20 mg/L in 50 mL of buffer with pH 5) was removed by 100 mg of immobilized laccase after 12 hours of incubation [124].

Maryšková et al. (2016) used laccase from *Trametes versicolor* immobilized onto polyamide 6/chitosan nanofibers for the degradation of a mixture of approximately 10 mg/mL solution of **BPA** and **EE2** (17α-ethinylestradiol) in ultrapure water. Immobilized laccase showed complete elimination of both chemicals within initial 6 hours of incubation, and remained highly effective when reused the next day and seven days after the first degradation test [125].

Apart from BPA, which is probably the most often selected compound for water treatment modeling, there are other emerging pollutants hazardous for the environment. Several studies focus on enzymatic degradation of triclosan. For instance, Xu et al. (2014) chose laccase from non-specified source immobilized onto PAA/SiO_2 nanofiber membrane via EDAC/NHS chemistry for the degradation of 10 mg/L solution of **TCS** at pH 4. Almost 100% was eliminated within 24 hours of incubation in 50 mL of buffer using 5 mg of the membrane with immobilized laccase [126].

Cabana et al. (2011) immobilized laccase from *T. versicolor* onto EDAC-crosslinked chitosan in order to eliminate triclosan. 250 U/L of immobilized laccase was tested towards the degradation of 5 mL of **TCS** solution (5 mg/L) in McIlvaine's buffer at pH 5. All triclosan was eliminated within 6 hours of incubation [127].

Degradation of triclosan solution (5 mg/L) was studied by Bokare et al. (2010). Initially, they used Pd/nanoFe particles for the reduction of **TCS** into 2-phenoxyphenol, which was subsequently polymerized by soluble laccase from *T. versicolor* [128].

Immobilized laccase from *T. versicolor* was tested towards the efficiency in degradation of **DCF** (diclofenac) in a study of Lonappan et al. (2018). Laccase was immobilized onto pinewood, pig manure and almond shell biochar modified via citric acid and using glutaraldehyde as crosslinking agent. Furthermore, 0.5 g of immobilized laccase was added into 25 mL of effluent wastewater with pH 6.35 which was spiked to the final concentration of 500 μg/L. All DCF was eliminated by a combination of adsorption and enzymatic degradation within less than 6 hours of incubation [129]. Similar study was performed using crude laccase adsorbed into biochars [130].

Laccase from *T. versicolor* was immobilized onto polyvinyl alcohol/chitosan/multi-walled carbon nanotubes composite nanofibers via glutaraldehyde activation. Carbon nanotubes improved electron transfer between the enzyme and **DCF** molecules resulting in full degradation of 12.5 mg/L solution within 6 hours of incubation [76].

Potential use of laccase from *Pleurotus florida* immobilized onto poly(lactic-*co*-glycolic acid) nanofibers for **DCF** degradation was investigated in a study of Sathishkumar et al. (2012). After 5-hour incubation of 4 U of immobilized laccase in 1 mL of DCF solution (50ppm) at pH 4 there was full elimination of the contaminant [131].

Fumed silica nanoparticles amino-modified via APTES ((3-Aminopropyl) triethoxysilane) and activated via glutaraldehyde were used for immobilization laccase

from *C. polyzona, Phoma* sp. [132], [133], and from *T. versicolor* and *Myceliophthora themophila* [134]. The immobilized laccase was used for the degradation of **BPA** (78 μg/L) and **DCF** (93 μg/L) from secondary effluent from a municipal wastewater treatment plant. After 24-hour incubation of highly active immobilized laccase (224 U in 28 mL) all BPA was removed, however DCF elimination was not observed at all.

Similar approach used Arca-Ramos et al. (2016) when harnessing magnetically-separable crosslinked laccase aggregates [135] for the elimination of a mixture of pharmaceuticals from biologically treated wastewater effluent. The removal efficiency of approximately 900 U/L of immobilized laccase after six hours of incubation is displayed in Fig. 9.

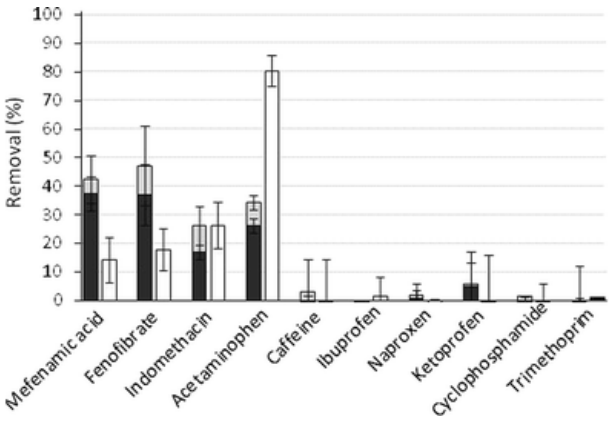


Fig. 9 Removal efficiency of magnetic laccase CLEAs in wastewater effluent [135]

Laccase-based catalytic system has been known for the efficiency to degrade a wide spectrum of pollutants, including the above outlined endocrine disruptors (BPA, EE2, TCS, DCF). The use of immobilized laccase has been reported in several works, however, in most of these studies the EDCs removal was conducted in a buffer under favorable pH or in distilled water and at high concentrations of target micropollutants.

Moreover, most studies focus on a very low number of targeted molecules, although biocatalytic activity is highly dependent on a presence of other potential substrates. This fact was often embraced using synthetic laccase mediators, such as 1-hydroxybenzotriazole or ABTS, to enhance the catalytic potential of laccase. However, further use of these mediators for water treatment was refused, because they are expensive, can generate toxic derivatives, and their presence could affect the quality of the treated water [136].

Equally, the cost-effectivness of immobilized laccase should be considered. We can observe high EDCs conversions within several hours of incubation. But what price of such biocatalytic system? Many authors still work with highly pure and active laccase, which is remarkably costly and its availability is very limited. Their immobilization process also often requires long and costly techniques, and in order to ensure sufficient efficiency in EDCs removal, they use an extreme amount of the

catalyst for relatively low volume of the solution. In addition, tests on repeated or long-term degradation are often skipped in the research.

Peroxidases catalyze the oxidation of a wide spectrum of substrates using H_2O_2 or other peroxides that serve as a hydrogen donor [38]. Sakuyama et al. (2003) reported an oxidative degradation of alkylphenols (e.g. **BPA**) by horseradish peroxidase using 3mM H_2O_2. Furthermore, HRP was immobilized on aluminum-pillared clay and used for **phenol** oxidation [137]. Removal of **chlorophenols** using immobilized peroxidase was reported in [138] and [139]. Krim et al. (2010) immobilized peroxidase on alginate-starch beads and the system was used for oxidation of **anthracene** in presence of 0.7 mM H_2O_2 [140].

Other studies focus on the degradation of industrial dyes. Kim et al. (2005) combined enzymatic catalysis of HRP immobilized onto graphite felt with electrochemical generation of hydrogen peroxide in order to degrade **orange II** azo dye [42]. HRP has been also studied for the degradation of **Remazol blue** [44], [46] or **anthraquinone dyes** [45].

5. Emerging micropollutants

Over recent decades, the list of known environmental pollutants has been widened by chemical compounds occurring at very low concentrations. Most of these only became detectable following significant progress in available analytical methods. These emerging micropollutants represent a new and, as yet, insufficiently explored form of toxicity, not least due to their remarkable persistence in the aquatic environment and their ability to bioaccumulate. Many of these compounds are capable of short- and long-term toxicity, disruption to the endocrine system, or contribute to the antibiotic resistance of microorganisms [141].

Emerging pollutants are chemicals that are not monitored by default but have a potential to cause ecological and health effects by entering the aquatic environment. These pollutants are a large group of different kinds of chemicals, including medicines, personal care products, household cleaners or agricultural products [142]. Fig. 10 outlines the most occurring sources and paths of contamination. One group of these micropollutants, the endocrine disrupting chemicals (EDCs), interfere with the vertebrate (including human) endocrine system by imitating or blocking the effect of natural hormones.

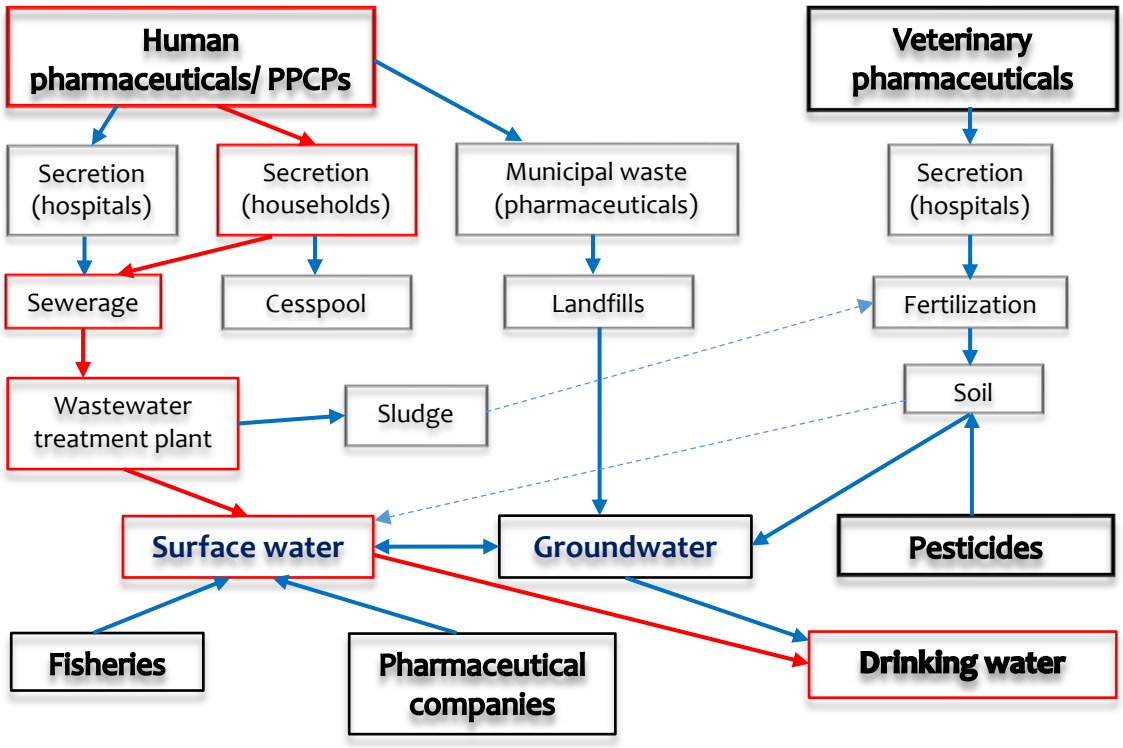


Fig. 10 A scheme of emerging pollutants entering the water environment

6. Water treatment processes for organic micropollutants' removal

Conventional wastewater treatment methods are not sufficient for the reduction of organic micropollutants. Only limited amount of these compounds is removed in the sewer and about half of the micropollutants is removed or transformed in wastewater treatment plants (WWTPs), either by sorption to sludge or by degradation [3]. In March 2014 the Swiss government decided to implement technical measures on WWTPs in densely populated regions in Switzerland in order to optimize the wastewater infrastructure regarding micropollutant elimination. The current strategy, that will involve 100 WWTPs over the next 20 years, is based on two alternative processes: ozonation and treatment with powdered activated carbon [143].

5. 1. Wastewater treatment with ozone

Ozone is a powerful disinfectant and oxidizing agent. A typical ozonation system consists of an ozone generator and a reactor where ozone is bubbled into the water. Conventional ways to produce ozone are UV-light and corona-discharge, which is more efficient and produces longer lifespan of the unit compared to UV-light. The generated ozone directly attacks the organic compounds, and therefore might induce their degradation. Low pH of the wastewater favors direct elimination of the organic compound containing phenols, tertiary amines or double bonds, whereas high pH results in a production of OH radicals that effectively eliminate a wider range of micropollutants.

This water treatment process has several disadvantages. Ozone production requires large amount of energy, it involves potential fire hazard and toxicity associated with ozone generation, and formation of potentially harmful by-products (e.g. created suspected human carcinogen -bromate- in case of bromine existence in water) [7]. On the other hand, ozone is a strong disinfectant that can replace chlorination [144].

5. 2. Wastewater treatment with activated carbon

Activated carbon is an effective adsorbent, which has a very high adsorption capacity of organic matter in a combination of small particle size and long contact time. The high amount of dissolved organics decrease the adsorption efficiency and

hydrophilic or polar compounds are adsorbed at very low level. Moreover, used carbon needs to be separated and sent for destruction and re-activation through incineration [8].

5. 3. Alternative technologies for the wastewater treatment

Nanofiltration is a pressure-driven separation process using membrane filters with pore size of 1–5 nm. These membranes has been used for desalination of surface and ground water, however, they are efficient also in removal of organic micropollutants including pesticides, hormones, and pharmaceuticals [145], [146].

Reverse osmosis uses semipermeable membrane and high pressure, that ideally allows only water molecules to pass through the membrane. However, this method has a very high energy requirement [147].

Ultraviolet light has been used for disinfection of drinking water because it can degrade organic compounds by direct photolysis or in combination with a chemical oxidant (H_2O_2 or TiO_2), which can increase the water treatment efficiency by production of hydroxyl radicals [148].

Biological treatment is not able to completely remove micropollutants from wastewater, however, some microorganisms such as anaerobic bacteria and activated sludge are able to degrade some pharmaceuticals (e.g. caffeine, carbamazepine, chlortetracycline, 17β-estradiol or 17α-ethinylestradiol) [149]–[151]. In order to include microorganisms into the water treatment plant, for example activated sludge can be placed in a bioreactor in the middle of two separation membranes [152], [153].

Last but not least, **enzymatic degradation** has been widely studied. Enzymes dispose high catalytic activity and selectivity within mild conditions. Recent studies regarding harnessing enzymatic activity report filtration systems in form of enzymatic reactors containing the catalyst immobilized onto various supports or in the form of crosslinked enzyme aggregates [154].

Materials and methods

7. Materials and reagents

7. 1. Enzymes

Laccase from *Trametes versicolor* (Sigma–Aldrich, EC number 1.10.3.2): Laccase was purchased in the form of brown powder soluble in water. Claimed activity of the enzyme was ≥10 U/mg. One unit (1 U) corresponds to the amount of enzyme which converts 1 μmol of catechol per minute at pH 4.4 and 25°C when the enzyme powder (2 mg/mL) is dissolved in 50mM citrate buffer.

Horseradish peroxidase (Sigma–Aldrich, EC number 1.11.1.7): Peroxidase was purchased in the form of beige lyophilized powder with the claimed activity of ~150 U/mg. One unit corresponds to the amount of enzyme which oxidizes 1μmol ABTS per minute at pH 6.0 and 25°C.

Unpurified precipitated broth (crude laccase): Fungal broth containing mainly enzymes from *T. versicolor*, precipitated via ammonium sulfate, was kindly provided by the Department of Chemistry and Biochemistry of the Mendel University in Brno. The native fungal strain *Trametes versicolor* was isolated from a dead tree trunk of *Alnus glutinosa* in a Czech forest. Then the strain was grown on agar for 10 days at 22°C and transferred into Erlenmeyer flasks containing 100 mL of potato dextrose broth [155]. The samples were cultivated in a shaker (1 week, 150 rpm, 28°C) and finally, ammonium sulphate (75 wt.% saturation) was added for 30 minutes to the crude culture supernatant and pH of the sample was adjusted to the desired pH 6.0 according to Solcany et al. (2016) [156].

7. 2. Endocrine disrupting chemicals

Bisphenol A (BPA), ≥99% (Sigma–Aldrich): BPA (Fig. 11) is used in the manufacturing of polymers, polyvinyl chloride plastics and flame retardants. There is a concern about its implications in the etiology of some human chronic diseases such as diabetes, obesity, reproductive disorders, cardiovascular diseases, birth defects, chronic respiratory, kidney diseases, and breast cancer [157].

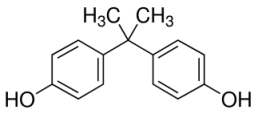


Fig. 11 Structure of bisphenol A

17α-ethinylestradiol (EE2), ≥98% (Sigma–Aldrich): EE2 (Fig. 12) is a synthetic estrogen used in contraceptive pills. Women generally metabolize only 20–48% of the daily dose of EE2 and the rest of it is excreted and enters the wastewater. Exposure to estrogenic compounds has an influence to several aqueous species and it is a risk factor for human health outcomes including testicular dysgenesis syndrome, testicular cancer, breast cancer etc. [158].

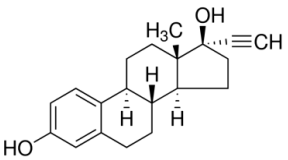


Fig. 12 Structure of 17α-ethinylestradiol

Triclosan (TCS), pharmaceutical secondary standard (Sigma–Aldrich): TCS (Fig. 13) is an antibacterial and antifungal agent. It is used in personal care products (soaps, toothpaste, detergents etc.) and can be found in toys, bedding, socks, trash bags, or as a part of some surgical equipment. This compound binds to both androgen and estrogen receptors, therefore its endocrine disruptive activity has been proved [159].

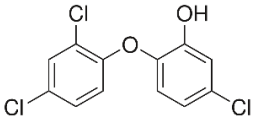


Fig. 13 Structure of triclosan

Diclofenac sodium salt (DCF), (Sigma–Aldrich): DCF (Fig. 14) is a nonsteroidal anti-inflammatory drug that reduces inflammation and pain. It was first introduced under trademark Voltaren. Diclofenac belongs to the group of emerging pollutants mainly in a context of a disruptive influence on water species, however, its impact on human health has also been discussed [160].

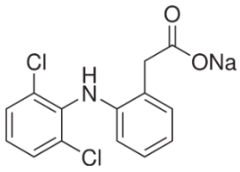


Fig. 14 Structure of diclofenac

7. 3. Other chemicals

- **Acetic acid** (99,8%, Penta)
- **Formic acid** (98%, Penta)
- **Glutaraldehyde** (Sigma–Aldrich, Grade I, 25% in H_2O), specially purified for use as an electron microscopy fixative
- **Guaiacol** (Sigma–Aldrich, 98%, M_w 124.14 g/mol)
- **Methanol** (Lach-Ner, G.R.)
- **N-hydroxysulfosuccinimide sodium salt** (Sigma–Aldrich, ≥99% HPLC)
- **Poly(ethylenimine)** (Sigma–Aldrich, branched, Mw 25 000 g/mol)
- **Poly(acrylic acid) Sokalan® PA 110 S** (BASF, M_w 250 000 g/mol)
- **Polyamide 6 Ultramid B24** (BASF, M_w 37000 g/mol)
- **Polyethylenimine, branched** (Sigma–Aldrich, M_w 25 000 g/mol)
- **Poly(ethylene terephatalate)/cushion vinyl**, nonvowen textile with polyethylene dotcoating (Hoftex GmbH)
- **Quebec Ministry of Environmental Phenol Mix** (Sigma–Aldrich), 2000 μg/mL in methanol, 27 components including 19 chlorophenols, cresols, nitrophenols and dimethylphenol
- **Sodium azide** (Sigma–Aldrich, ReagentPlus®, ≥99.5%)
- **Sodium periodate** (Sigma–Aldrich, ≥99.0%)
- **Syringaldazine** (Sigma–Aldrich)**,** indicator for laccase and peroxidase activity: M_w 360.36 g/mol
- **1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride** (Sigma–Aldrich, ≥99.0%)
- **2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (**Sigma–Aldrich, ≥98%,)

8. Analytical methods

8. 1. Enzyme activity assays

Enzyme activity assay using ABTS

The catalytic activity of laccase from *Trametes versicolor* was measured according to [161], [162] at 25°C using a microplate reader BioTech Synergy HTX. The substrate for the catalytic reaction is 0.5 mM ABTS and the buffer is pH 3 McIlvaine's buffer. The activity of the soluble laccase was measured in 96-well plates where the contents were following:

- 160 μL of buffer
- 20 μL of the laccase solution
- 20 μL of 0.5 mM ABTS

From the moment ABTS iss added into the reaction mixture it is being oxidized by laccase producing stable cation radical $ABTS^{+}$ (Fig. 15). Typical green color of the product can be measured by absorbance at 420 nm [163]. The activity is expressed as 1 U which corresponds to the amount of laccase that converts one μmol of ABTS per one minute. The formula for the expression of one U is derived from the Lambert-Beer law; **Abs = c · ε · d** (*Abs* stands for absorbance, *c* is concentration, *ε* is a molar extinction coefficient and *d* is a path length of the beam passing through the testing material, or the thickness of the layer).

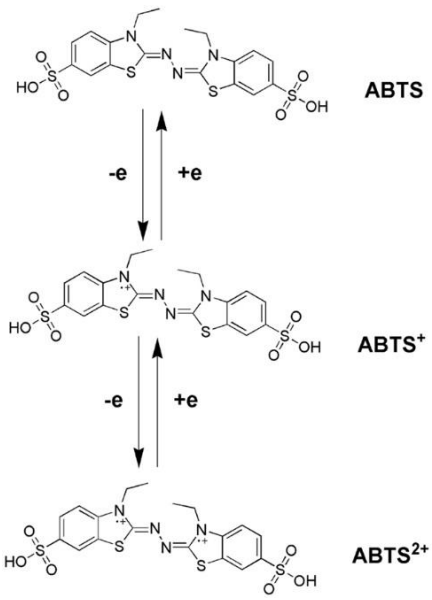


Fig. 15 ABTS oxidation by laccase-catalysis [163]

The molar extinction coefficient for $ABTS^{+}$ at 420 nm is 0.036 $\mu mol^{-1}cm^{-1}$ [164], the layer thickness of 200 μL of the reaction solution using 96-well plate was measured as 0.6 cm and *df* represents the dilution factor. With the slope deducted from the linear part of the absorbance growth in time the final formula for the activity measurement was following Eq. 4:

$$1\ \mathrm{U} = \frac{\mathrm{slope}\ [\frac{Abs}{min}]}{\varepsilon\ [\mu mol^{-1}\cdot cm^{-1}]\cdot \mathrm{d}\ [cm]\cdot\ \mathrm{V_{sample}}\ [L]}\ \cdot \mathrm{V}\ [L] \cdot \mathrm{df}\ [-] \qquad (4)$$

An optimal method for precise detection of activity of the immobilized enzyme consists of a method where the reaction takes place in a reaction tube separated from the spectrophotometer containing 4 mL of the buffer, nanofiber sample with the immobilized enzyme, and 0.4 mL of 0.5 mM ABTS. This reaction tube must be constantly shaken in order to avoid adsorption of the ABTS product into the nanofibers, and to insure sufficient distribution of the oxidation product within the whole volume. In selected time intervals, 0.1 mL of the liquid is sampled into the 96-well plate and the absorbance is measured. After each measurement the liquid is returned from the plate back to the reaction tube.

The efficiency of the immobilization procedure can be expressed by three values (IY, AY and loading). *Immobilization yield* (IY) is given by following formula (Eq. 5):

$$\text{IY}\,[\%] = \frac{\text{initial}\,[U] - \text{SN}\,[U]}{\text{initial}\,[U]} \cdot 100 \qquad (5)$$

The "initial" represents the activity of the laccase initially added to the reaction and the "SN" is the activity of the laccase remaining in the supernatant after the nanofibers are removed from the immobilization bath. IY represents the amount of laccase immobilized on the nanofibers.

Activity yield (AY) represents the active part of laccase immobilized on the matrix. This value predicates the catalytic activity of the final product. It is given by following formula (Eq. 6):

$$\text{AY}\,[\%] = \frac{\text{activity on the NFs}\,[U]}{\text{initial}\,[U]} \cdot 100 \qquad (6)$$

Loading [U] stands for the amount of laccase immobilized on the nanofiber sample.

Enzyme activity assay using syringaldazine

The catalytic activities of laccase and peroxidase using syringaldazine were measured similarly to the reaction using ABTS (concentration of SYR = 0.5 mM, ε = 65 $mM^{-1} \cdot cm^{-1}$), only the absorbance peak of the product (Fig. 16) was at 526 nm [165].

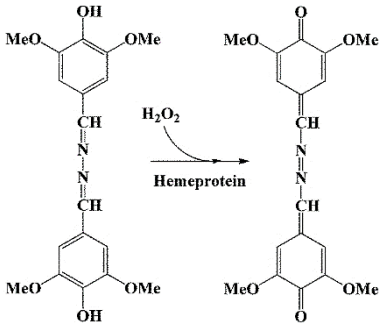


Fig. 16 Oxidation of syringaldazine by peroxidase-catalysis [165]

Enzyme activity assay using guaiacol

The product of the catalytic activity of laccase and peroxidase was measured at 470 nm (ε = 26.6 $mM^{-1} \cdot cm^{-1}$) at the same conditions as above. The initial concentration of guaiacol (2-methoxyphenol) (Fig. 17) was also 0.5 mM [166].

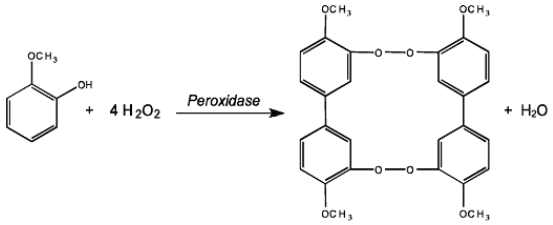


Fig. 17 Oxidation of guaiacol by peroxidase-catalysis [166]

8. 2. Determination of kinetic constants

The kinetic parameters (K_m, V_{max}), describing how efficiently the enzyme converts a substrate into a product, were determined for both enzymes at their optimal pH at 25°C. The parameters were calculated from the Lineweaver–Burk plot according to Michaelis-Menten Eqs. (4) and (5):

$$V = V_{max} \frac{[S]}{K_m + [S]} \quad (7)$$

$$\frac{1}{V} = \frac{K_m}{V_{max}\ [S]} + \frac{1}{V_{max}} \quad (8)$$

where [S] is the concentration of substrate, V is the reaction rate, Vmax represents the maximum rate, Km is the Michaelis constant which is the indicator for the enzyme's affinity to the substrate.

8. 3. Scanning electron microscopy

Images of pristine nanofibers and nanofibers with immobilized laccase were obtained using SC7620 sputter coater (Quorum Technologies, Lewes, UK) with 10 nm gold layers, Carl Zeiss ULTRA Plus (Zeiss, Oberkochen, Germany) and VEGA3 Tescan (Tescan, Czech Republic) scanning electron microscopes (SEM). Subsequently, the SEM images were analyzed using VEGA TC software for assessing average fiber diameter obtained by averaging the values of 100 individual measurements.

8. 4. Characterization of amino groups

The amount of available primary amino groups of a nanofiber support was estimated using a method described in [168]. In short, methyl orange is an acid dye that binds with amino groups and can subsequently be removed by bases (Fig. 18). Firstly, the nanofiber samples were incubated in 0.05% (w/v) methyl orange solution in 0.01 M phosphate buffer for one hour. Next, the unbound methyl orange was thoroughly washed with deionized water and 0.1 M sodium carbonate solution was added to the samples in order to release all bound methyl orange. The amount of amino groups was estimated spectrophotometrically by reading the absorbance at 465 nm and using equation from the study of Hartwig et al. (1994).

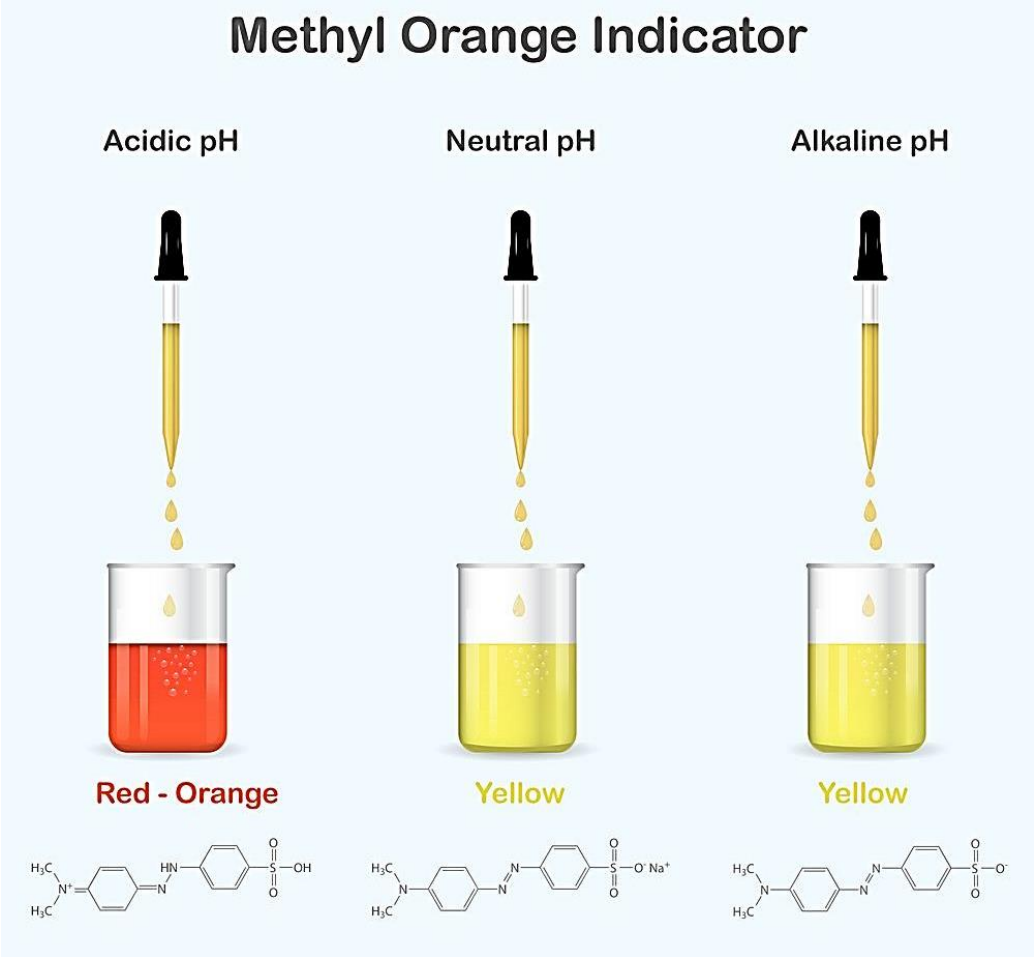


Fig. 18 Colorful changes in the structure of methyl orange in acidic and alkaline pH [176]

8. 5. Solid-phase extraction

Solid-phase extraction (SPE) was used as a sample enrichment method in degradation experiments with very low concentration of analyzed micropollutants. Firstly, the samples (200 mL) were acidified by adding 10 mL of a solution containing 20 % methanol, 1 M HCl and 10 mM Na_2EDTA. Subsequently, Phenomenex Strata RP 18-E SPE cartridges containing 200 mg of sorbent and a volume of 6 mL were conditioned by passing 5 mL of 10% methanol and then 5 mL of 100% methanol. After passing the sample, cartriges were washed with 5 mL of 10% methanol and eluted via 4 mL of THF. Finally, the sample extracts were dried under nitrogen at 40°C and redissolved in 1 mL of a solution identical to the innitial mobile phase composition.

8. 6. Solid-phase microextraction

Degradation of chlorophenols was measured via solid-phase microextraction method (SPME) [167]. The apparatus consisted of tandem gas chromatograph (Thermo trace 1310) equipped with a mass spectrometer triple quadrupole detector (Thermo TSQ 8000 EVO) and a programmed temperature vaporizing injector (PTV). A gas chromatography column DB-5MS (30 m long, 0.25 μm thick and with 0.25 μm film thickness of stationary phase) was installed into GC oven. Temperature program of the chromatographic oven started at 60°C held for 2 min, followed graduating by 8°C/min up to 100°C, which was held for 4 min, followed by second temperature gradient 15°C/ min to final temperature 300°C, which was held for 1 min. The carrier gas (helium 5.0) flow was adjusted to 1.0 mL/min. For automatic handling of prepared samples GC/MSMS was equipped by an autosampler (CTC Analytics AG, PAL RTC), which was set to SPME headspace operation mode (samples were agitated during enrichment time). During injection step, PTV injector was held at 250°C in splitless mode for 1 min, for cleaning phase the temperature was set to 250°C and the flow of carrier gas to 30 mL/min. SPME fiber (100 μm polydimethylsiloxane) was used during all measuring. The temperature of ion source was set to 250°C and transfer line to 200°C.

8. 7. High-performance liquid chromatography

The degradation of selected EDCs (BPA, EE2, TCS, DCF, NF) by soluble oxidoreductases and immobilized enzyme samples was measured using HPLC Dionex Ultimate 3000 with LPG-3400SD quaternary gradient pump, SR-3000 solvent rack, WPS-3000TSC autosampler, TCC-3000SD column compartment, and DAD-3000 detector. A Phenomenex Kinetex F5 core-shell column with a length of 150 mm and internal diameter of 4.6 mm were used. The aqueous component (A) of the mobile phase consisted of 10 mM phosphoric acid in 5% acetonitrile. The organic component (B) of the mobile phase consisted of 10mM phosphoric acid in a mixture of 90% acetonitrile and 10% methanol.

A set of linear gradients started with the proportion of B in the mobile phase of 10 %. At 0.7 min it was 15%, at 1.7 min it was 25%, at 3 min 35%, at 4.2 min 40%, at 5.9 min 50%, and at 7.6 min 70%. The proportion of the B component reached 80% at 8.8 min and from this point till 9.3 min the composition of the mobile phase returned to the starting conditions. The chromatogram for each sample was recorded for 12.65 min. The flow rate was 1.5 mL/min and the column was kept at 40°C. The injection volume used was 20 μL. The chromatograms were recorded at wavelengths of 200, 227, 278 and 285 nm with a sampling rate of 2 Hz.

9. Bioconjugate techniques for enzyme immobilization

Among the scale of various nanofibers we chose polyamide 6 (PA 6), blend of polyamide 6 and branched polyethylenimine (PA/PEI), and poly(acrylic acid) (PAA). While PA 6 has a very limited number of free primary amino groups and large number of secondary groups, therefore it enables adsorption with enhanced affinity to an enzyme, PEI has numerous free amino groups on each polymer unit which is necessary for a successful covalent bonding. Partially crosslinked PAA offers available carboxylic groups (Fig. 19) suitable for covalent bonding, ionic bonding, and adsorption.

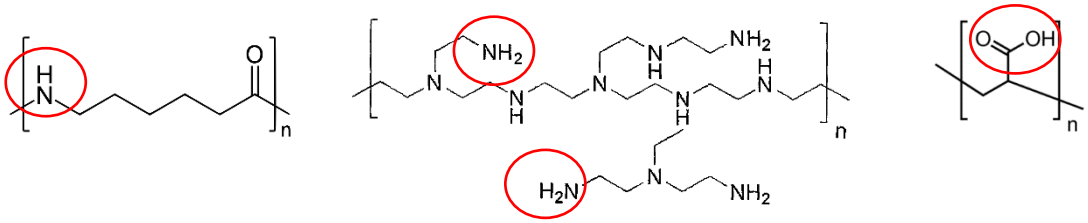


Fig. 19 From the left: polyamide 6; branched polyethylenimine; poly(acrylic acid)

It has been reported that laccase has several amino acid chains containing lysine with primary amino groups available for a bond formation [169]. Therefore, chemical linkers connecting amino-amino or amino-carboxyl are in consideration. Apart from well-known glutaraldehyde crosslinking producing relatively stable Schiff's base (Fig. 21) [170], there are other coupling agents such as NHS-esters or imidoesters with a longer chain of the linker providing larger space for the enzyme, therefore higher enzyme recovery after the bonding. However, these chemicals are rather costly. One of the most suitable linker is BS3 (bis(sulfosuccinimidyl) suberate) which is soluble in water and highly reactive towards free primary amino groups. There is a cheaper version of the biochemically popular BS3 which is water insoluble DSS (disuccinimidyl suberate) (Fig. 20). This compound, however, must be dissolved in dimethylformamid prior the application, which is a major complication when working with proteins.

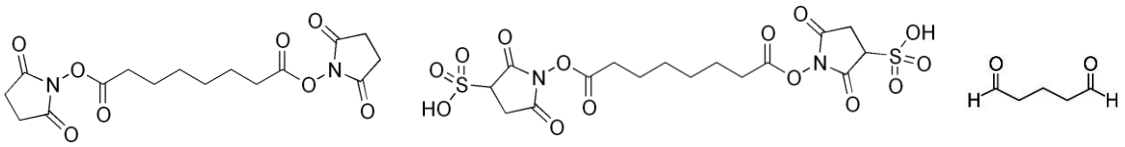


Fig. 20 From the left: DSS (disuccinimidyl suberate); BS3 (bis(sulfosuccinimidyl) suberate); glutaraldehyde

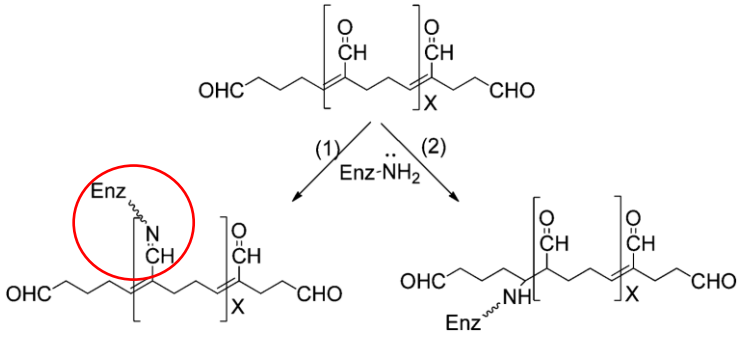


Fig. 21 Formation of Schiff base produced by the reaction between glutaraldehyde and enzyme [3]

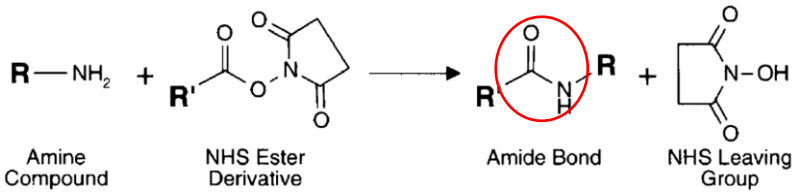


Fig. 22 Covalent binding of free amino group and NHS-ester derivate forming amide bond [171]

Carboxylic group, on the other hand, does not offer so many possibilities for coupling via linkers, because most of them target on hydroxyl only. However, there is commonly used zero-length reagent EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) usually used in a combination with S-NHS (N-hydroxysulfo-succinimide) in order to enhance durability in water and to prolong its reactivity (Figs. 22 and 23) [171].

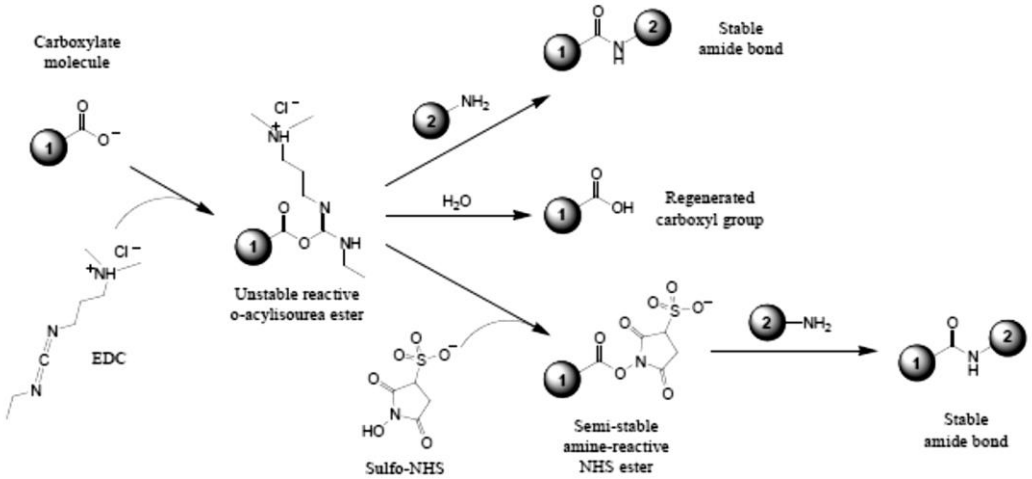
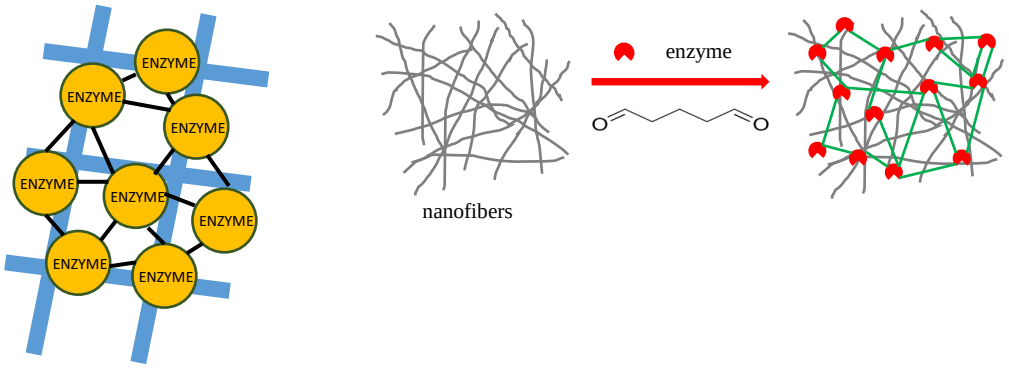


Fig. 23 Formation of amide after zero-length reaction between carboxyl and amine using EDC and/or S-NHS [171]

9. 1. Selected immobilization techniques

a) Enzyme adsorption → glutaraldehyde crosslinking



Variable parameters: i) Time for adsorption and crosslinking, *ii)* concentration of enzyme solution, buffer and glutaraldehyde, *iii)* pH, *iv)* volume of the enzyme solution.

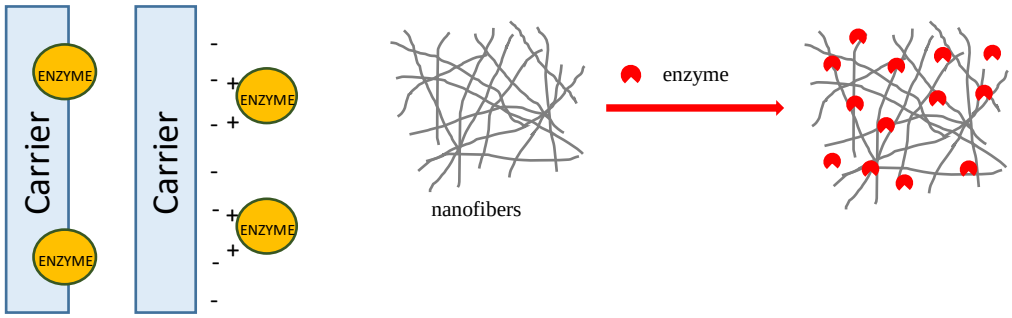
+ suitable for most types of nanofibers

+ low cost

- effectivity dependent on a sorption capacity of the carrier [172], [173]

- enzyme activity loss due to glutaraldehyde crosslinking

b) Enzyme adsorption or ionic binding



Variable parameters: *i)* Time for adsorption, *ii)* concentration of enzyme solution and buffer, *iii)* pH, *iv)* volume of the enzyme solution.

+ suitable for many types of nanofibers

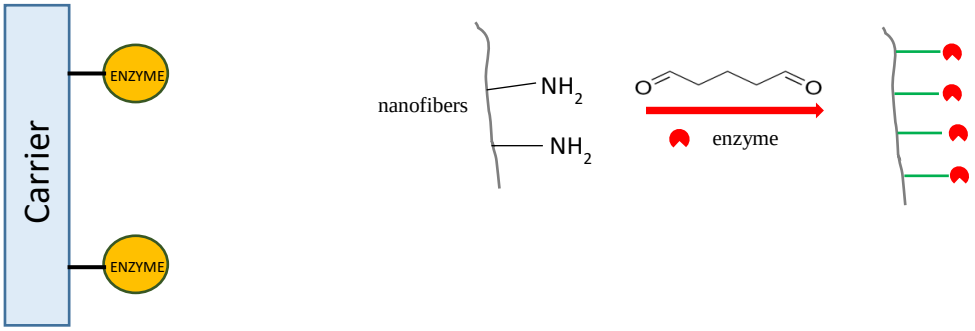
+ low cost

+ one-step method (short time immobilization)

+ low enzyme damage due to the absence of strong bonding

- enzyme leakage [174]–[176]

c) Glutaraldehyde activation → enzyme attachment



Variable parameters: *i)* Time for activation and enzyme bonding, *ii)* concentration of glutaraldehyde, enzyme solution and buffer, *iii)* pH, *iv)* volume of the enzyme solution.

+ higher stability

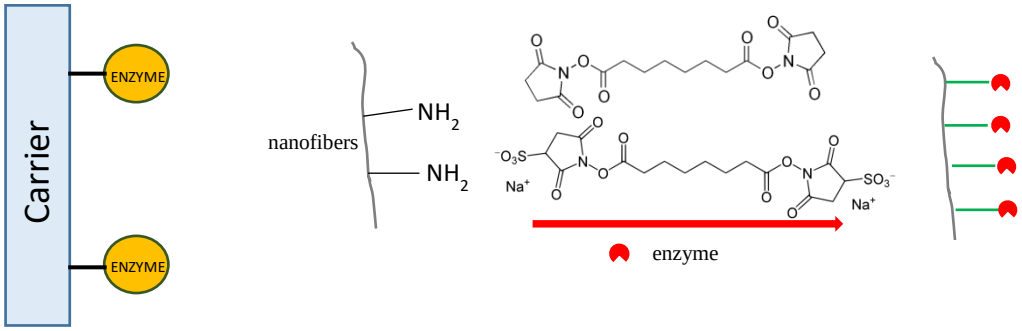
- suitable for nanofibers with free primary amino groups (e.g. PA/PEI)

- potential enzyme damage due to strong bonding

- effectivity depending on a number of functional groups of the support

- difficult setting of suitable parameters (possible GA crosslinking of two NH_2 groups of the carrier) [173], [177]

d) Activation via DSS or BS3 → enzyme attachment



Variable parameters: *i)* Time for activation and enzyme bonding, *ii)* concentration of DSS/BS3, enzyme solution and buffer, *iii)* pH, *iv)* volume of the enzyme solution.

+ higher stability

- suitable for nanofibers with free primary amino groups (e.g. PA/PEI)

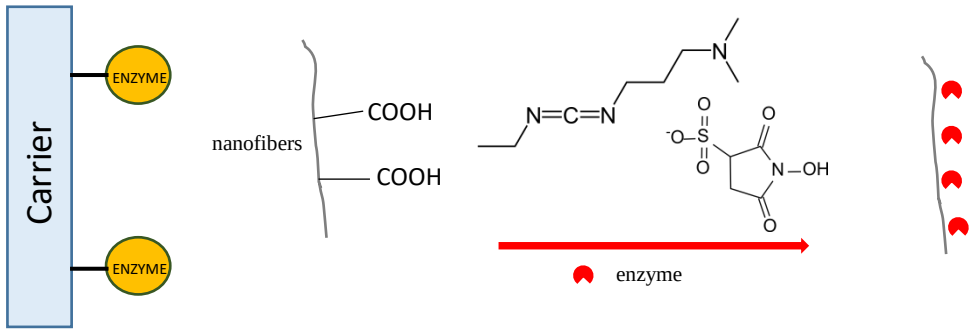
- costly DSS/BS3

- effectivity depending on a number of functional groups of the support

- potential enzyme damage due to strong bonding

- difficult setting of suitable parameters (possible crosslinking of two NH_2 groups of the carrier) [178], [179]

e) Activation via EDAC and S-NHS → enzyme attachment



Variable parameters: *i)* Time for activation and enzyme bonding, *ii)* concentrations of EDAC and S-NHS, enzyme solution and buffer, *iii)* pH, *iv)* volume of the enzyme solution.

+ higher stability

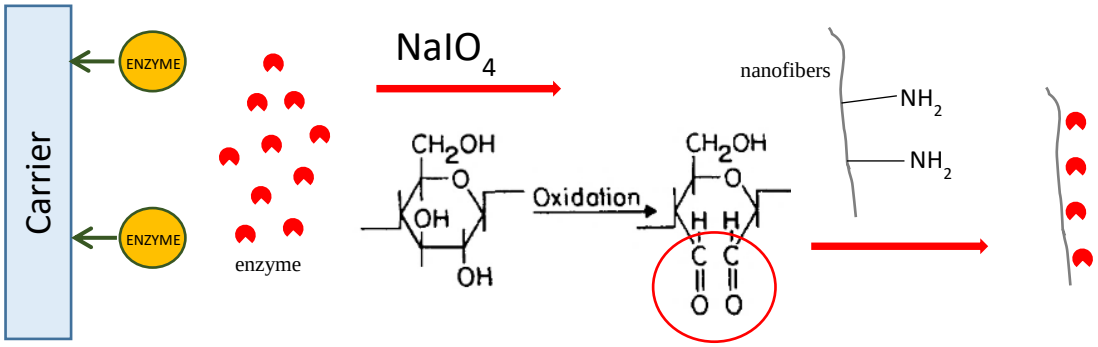
- suitable for nanofibers with free primary carboxylic groups (e.g. PAA)

- costly EDAC and S-NHS

- effectivity depending on a number of functional groups of the support

- potential enzyme damage due to strong bonding [180], [181]

f) Laccase oxidation via sodium periodate → attachment



Variable parameters: *i)* Time for oxidation and enzyme bonding, *ii)* concentration of $NaIO_4$, enzyme solution and buffer, *iii)* pH, *iv)* volume of the enzyme solution.

+ higher stability

- suitable for nanofibers with free primary amino groups (e.g. PA/PEI)

- effectivity depending on a number of functional groups of the support

- potential enzyme damage due to oxidation or strong bonding [182]–[184]

10. Nanofiber carriers

10. 1. Polyamide 6 nanofibers

PA 6 (B24, Mw 37000 g/mol) pellets were dissolved in a mixture of formic acid and acetic acid (2:1; v/v) to prepare a 12% wt. solution. The nanofibers were then prepared by electrospinning, using Nanospider™ NS 1WS500U equipment (Elmarco, Czech Republic) with a voltage of -20/60 kV and distance between electrodes set at 180 mm. Images of PA6 nanofibers were obtained using VEGA3 Tescan (Tescan, Czech Republic) scanning electron microscope.

Four types of PA6 nanofiber sheets with different surface densities were prepared by adjusting the speed of the electrospinning process. The finest sheet had a surface density of 1.5 g/m^2 (Fig. 24a), and an average fiber diameter of 79.3 ± 19.8 nm. Subsequent sheets had a surface density of ca. 3 g/m^2 and a fiber diameter of 87.9 ± 14.7 nm, ca. 5 g/m^2 and 109.4 ± 19.1 nm, and 8 g/m^2 (Fig. 24b) and 100.4 ± 23.8 nm.

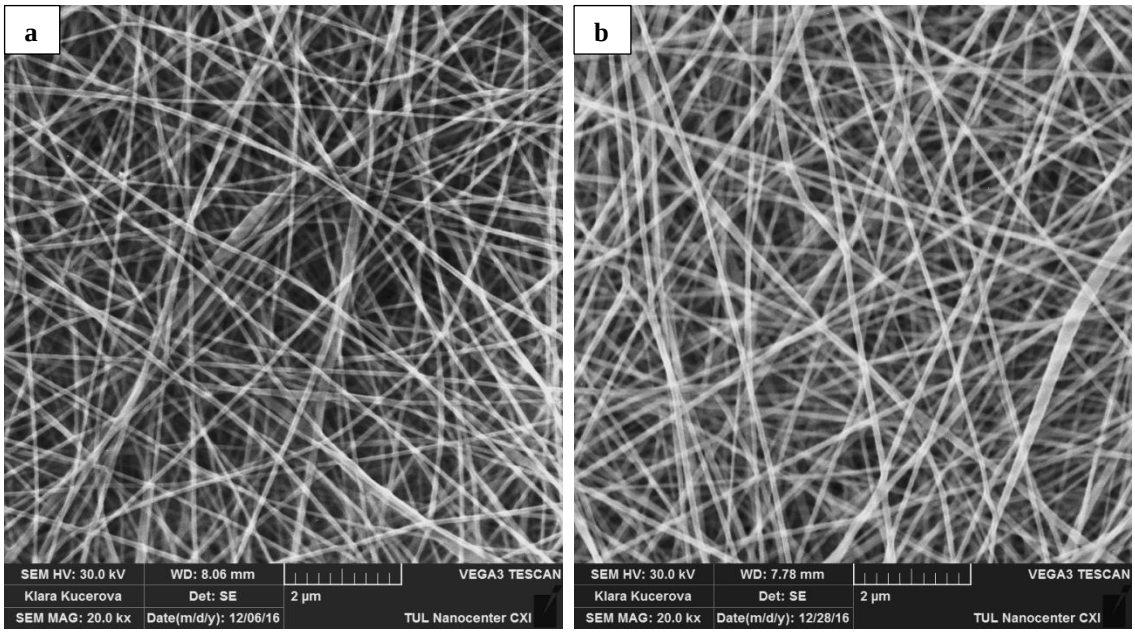


Fig. 24 SEM images of a) PA6 nanofibers with 1.5 g/m^2, and b) 8 g/m^2. Magnitude 20 kx.

10. 2. Nanofibers from poly(acrylic acid)

PAA (Sokalan 110S, Mw 250000 g/mol) was dissolved in a mixture of ethanol and isopropanol (1:1; w/w). Subsequently, the crosslinking agent ethylene glycol (ca. 5% wt.) was added into the solution, and finally, 1 M sulfuric acid (0.4% wt.) was added in order to improve spinnability of the polymer solution. The nanofibers were then prepared by electrospinning, using Nanospider™ NS 1WS500U equipment (Elmarco, Czech Republic) with a voltage of -10/45 kV and distance between electrodes set at 150 mm. Prepared nanofiber sheets were stabilized at 130°C for 30 minutes thus they became water insoluble.

Images of PAA nanofibers before (Fig. 25a) and after (Fig. 26b) stabilization were obtained using VEGA3 Tescan (Tescan, Czech Republic) scanning electron microscope. The average fiber diameter of non-stabilized and stabilized nanofibers was very similar; 374 ± 133.8 nm and 385.3 ± 128.6 nm, correspondingly.

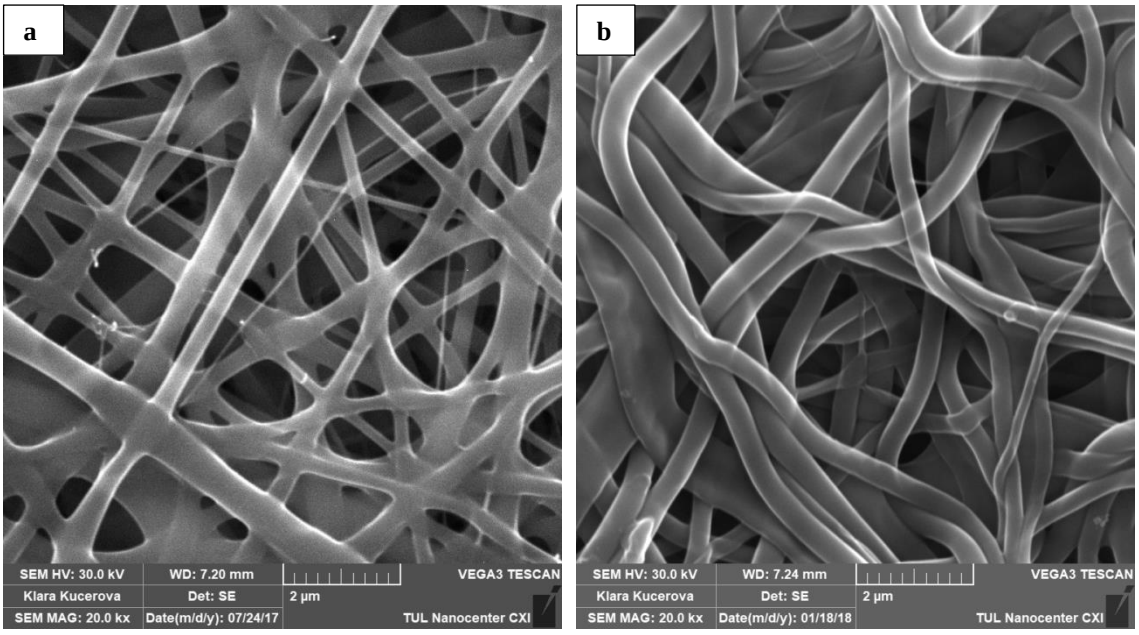


Fig. 25 SEM images of a) PAA nanofibers before crosslinking, and b) stabilized PAA nanofibers. Magnitude 20 kx.

10. 3. Nanofibers from polyamide 6/branched polyethylenimine

The polymer blend electrospinning solution was prepared by a two-step method. In the first step, PA6 pellets were dissolved in a mixture of acetic acid and formic acid (65/35, v/v) to the final concentration of 12 wt%. Subsequently, polyethylenimine (PEI; branched, Mw 25 000) solution was added and the blend was gently stirred overnight. The final PEI concentration in the polymer blend solution was 2.15 wt%. The polymer solution was electrospun using Nanospider^{TM} NS 1 WS500U (Elmarco Ltd., Liberec, Czech Republic) equipment. The distance between electrodes was firmly set to 175 mm and the voltage applied was -15/60 kV. The electrospun nanofiber sheets containing approximately 15% of the dry weight of PEI required no further stabilization or modification.

Images of PA/PEI nanofibers (surface density 7.5 g/m^2) were obtained using Carl Zeiss ULTRA Plus (Zeiss, Oberkochen, Germany) scanning electron microscope (Fig. 26). The average fiber diameter was 108.7 ± 24.9 nm.

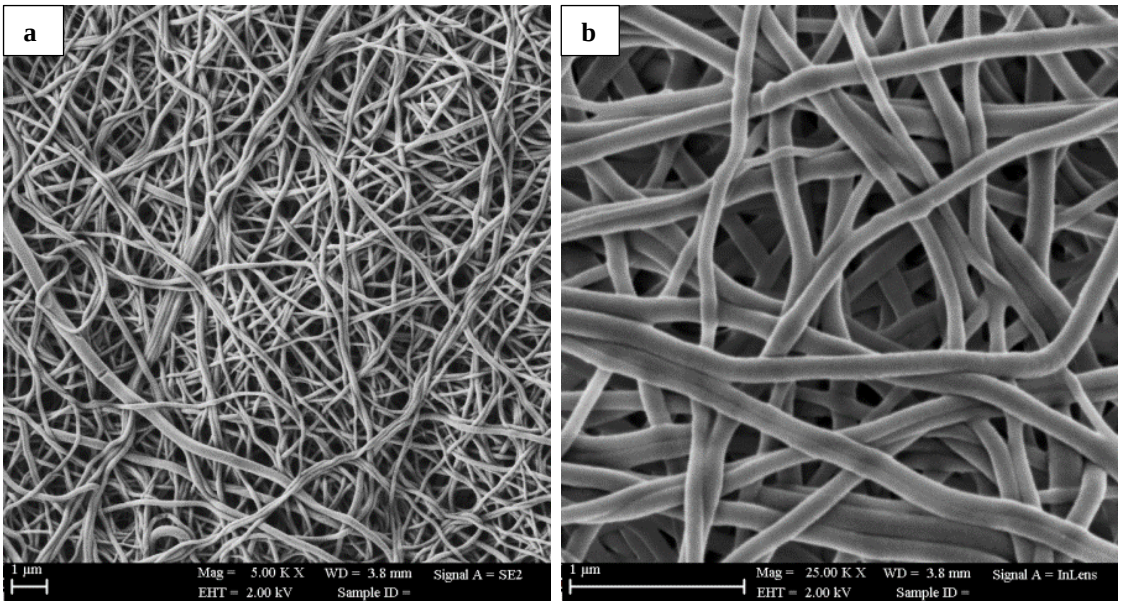


Fig. 26 SEM images of PA/PEI nanofibers. Magnitude 5 kx (a) and 25 kx (b).

Results and discussion

11. Comparison of laccase from *T. versicolor* and horseradish peroxidase

In this part the catalytic activity of two commercially available enzymes from the group of oxidoreductases is compared by unified methods across a range of substrates (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), syringaldazine, guaiacol). The activity of laccase from *T. versicolor* (TV) and horseradish peroxidase (HRP) was evaluated at different conditions (pH, temperature, type of a substrate) in real water samples exploring optimal parameters for their potential application in immobilization on a nanofiber matrix and subsequent degradation of a mixture of EDCs.

11. 1. Effect of pH on enzymatic activity

The influence of pH on enzyme catalytic activity was determined in McIlvaine's buffer at pH 3, 4, 5, 6, 7 and 8 at 25°C. The pH range used was the maximum available by mixing 0.1 M citric acid and 0.2 M disodium phosphate. Prior to determination, the soluble enzyme was pre-incubated in buffer at the required pH for 24 h.

Commercial *T. versicolor* laccase showed the highest catalytic activity toward ABTS and SYR at pH 3 and 4, respectively; with pH 4.5 proving optimal for GUA. For all substrates, therefore, optimal pH ranged between 3 and 5.5, indicating that laccase shows highest activity at more acidic pH levels (Fig 27a). According to the literature, laccases isolated from different strains have an optimal pH of between 1.8 and 4.4 when using ABTS as a substrate, between 4.8 and 8.2 when using SYR [185], and between 4.0 and 6.0 when using GUA [186], [187]. Several studies have suggested that a pH 3 buffer is the most efficient for ABTS oxidation [188]–[190].

In comparison, horseradish peroxidase was most active at more neutral pH of between 6.5 and 8 for all substrates except ABTS, where the optimal pH was 3.5 (Fig. 27b). In other studies, buffers at pH 5 [191] and 6 have been used for ABTS oxidation [192], [193], with a similar pH being used for SYR [46] and GUA [194] for peroxidase-catalysis.

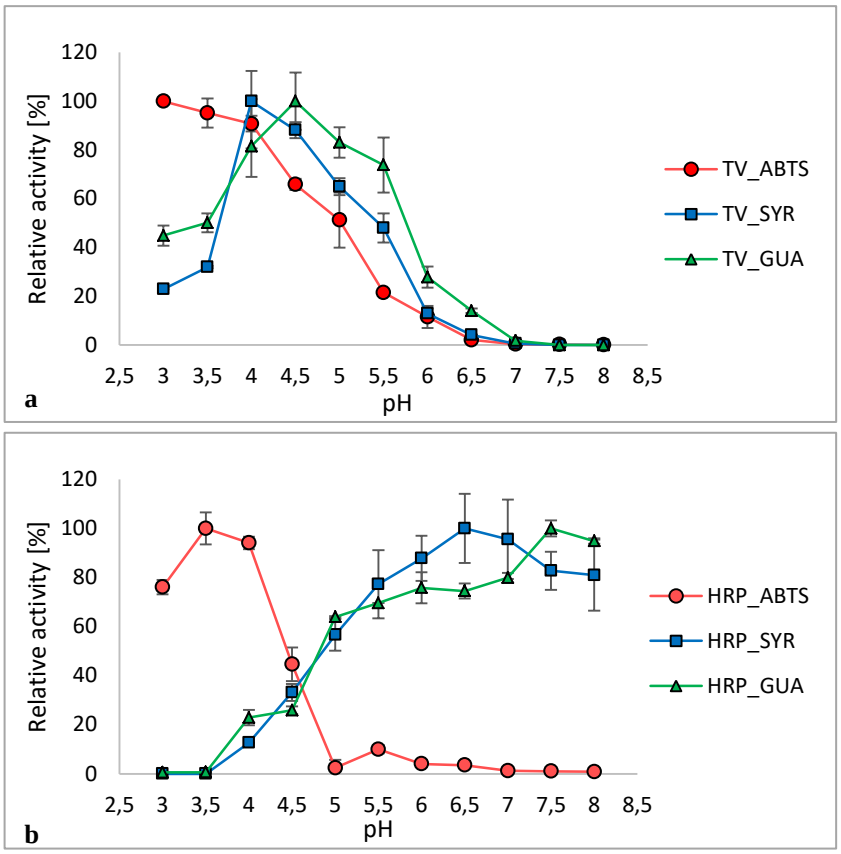


Fig. 27 Activity of (a) laccase from Trametes versicolor (TV) and (b) horseradish peroxidase (HRP) at different pH

Wastewater treatment plants often adjust pH according the chemical composition of the influent water. While an acidic pH leads to concentration of positive hydrogen ions, which can help eliminate pathogenic bacteria, a basic pH is more suitable for the removal of heavy metals. As the pH of the effluent water should always be set close to neutral, use of enzymes with a catalytic optimum at neutral pH would confer a major advantage. Though catalysis of each substrate may require different pH levels, as shown in Fig. 27 [195], laccase had almost no activity at neutral pH using all three tested substrates.

11. 2. Effect of H_2O_2 concentration on activity of HRP

Six H_2O_2 solutions with different concentrations (0.5, 1, 2.5, 5, 7.5 and 10%) were prepared and used for the assessment of catalytic activity of peroxidase using ABTS as a substrate. Effect of the concentration was evaluated from four replicate activity measurements using one-factor analysis of variance (ANOVA).

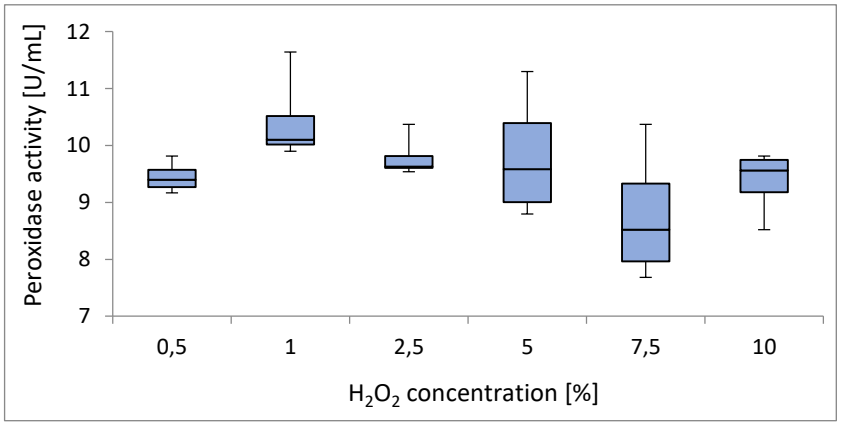


Fig. 28 Effect of H_2O_2 concentration on catalytic activity of peroxidase

There were no statistically significant differences between group means as determined by one-way ANOVA ($F(5,18) = 1.87$, $p = 0.1496$). The boxplot (Fig. 28) supports results of the analysis of variance with a conclusion that there was no optimal H_2O_2 concentration between 0.5 and 10%. Amount and H_2O_2 concentration used in the literature significantly diverge as well as amount, type and concentration of a substrate. In principal, very low concentrations are required for the assays (0.0085–3% [196], [197]), which corresponds to 1% concentration used in this study.

11. 3. Catalytic activities of enzymes

Activity (U/mg) and kinetic parameters (K_m, V_{max}) were determined for each enzyme at 25°C and its optimal pH (according to Fig. 27).

Table 1 Kinetic values and activities of laccase and peroxidase using ABTS, syringaldazine and guaiacol as substrates

Enzyme	Substrate	K_m [mM]	V_{max} [mM/min]	Activity [U/mg]	pH
TV (10 U/mg)	ABTS	0.4248 ± 0.0165	2.0207 ± 0.0805	2.1735 ± 0.1727	3
	SYR	0.1969 ± 0.0284	1.5573 ± 0.1412	4.6509 ± 0.2483	4
	GUA	0.7469 ± 0.0248	0.0384 ± 0.0006	0.0305 ± 0.0007	4.5
HRP (150 U/mg)	ABTS	0.5443 ± 0.0902	1.8123 ± 0.1586	2.3012 ± 0.1390	3.5
	SYR	1.0609 ± 0.2478	1.9946 ± 0.3978	1.6751 ± 0.0059	6.5
	GUA	0.9523 ± 0.0679	0.0424 ± 0.0017	0.0288 ± 0.0007	7.5

Laccase proved to be the most active and widely applicable enzyme, with the lowest K_m values and the highest activity (Table 1). Other studies have reported *T. versicolor* laccase K_m values of 0.43 mM for ABTS [198], 0.5 mM for SYR [199] and 0.128 mM for GUA [200], which corresponds when using ABTS as a substrate.

ABTS is the most frequently used substrate as it is commonly available, safe to handle, easy to store and highly soluble in water. On the other hand, both laccase and peroxidase show high affinity toward ABTS, increasing the speed of catalysis and making it difficult to measure kinetics over the linear part of catalytic oxidation. Such a problem may also occur when using SYR, which has a similar affinity to laccase and peroxidase. Moreover, SYR has to be dissolved in alcohol, a considerable disadvantage compared to ABTS. Enzymes' affinity towards GUA is lower, which can be utilized as an advantage for more precise detection of highly active enzymes. Problems related to rapid catalytic reactions are usually solved using higher volumes of a reaction mixture or lower concentration of the substrate, though this could lead to inaccurate measurements. To conclude, while all substrates have constraints that should be carefully considered, ABTS appears to be the most suitable for both enzymes.

11. 4. Catalytic activity of laccase and peroxidase in real water samples

Enzymatic activity of laccase and peroxidase was measured using ABTS as a substrate, only real water samples (Table 2) replaced McIlvaine's buffer. The experiment was performed in four replicates and detected enzymatic activities were compared to activities in buffer of pH 3 and 7, and deionized water (DIW). Similar approach was used in order to study influence of buffer infusion. McIlvaine's buffer with pH 3 was added in concentrations 0–20% (v/v) into tap and wastewater. Water samples consisted of following water sources, all collected in February 2019 Czech Republic;

Tap water (TAP)…….....tap water in Technical University of Liberec

Snow (W1)……..... snow from Janov nad Nisou

Well (W2)…….....water from a private well in Janov nad Nisou

Pond (W3)……..... water from a pond in a center of Liberec

Lake 1 (W4)…….....water from the lake Matylda in Most

Lake 2 (W5)…….....water from the lake Milada in Ústí nad Labem

Wastewater (WASTE).....ultrafiltered wastewater from the company Amazon near Prague

Table 2 Chemical analysis of water samples

mg/L	Tap water	Melted snow	Well	Pond	Lake 1	Lake 2	Waste-water
fluoride	<0.05	<0.05	0.25	0.19	0.36	0.77	0.16
chloride	1.6	0.56	3.0	133	24.9	62.7	213
nitrate	0.66	0.28	0.96	15.1	0.13	0.33	9.5
nitrite	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
sulfate	0.66	0.28	0.96	15.1	0.13	0.33	9.5
TOC	1.6	1.5	<1	2.1	2.3	2.3	6.2
Ag	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.0019
Al	0.033	0.008	0.012	0.029	0.027	0.02	0.035
Be	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Ca	37.6	0.439	9.9	57.4	41.2	39.6	127
Co	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002	< 0.002
Cr	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Cu	0.01	0.029	< 0.001	< 0.001	< 0.001	0.015	< 0.001
Fe	0.02	0.004	< 0.002	< 0.002	0.039	0.002	0.003
K	0.35	0.08	0.91	3.79	18.6	26.1	74.8
Mg	0.897	0.067	1.53	8.44	37.1	51.5	21.3
Mn	0.003	0.008	0.001	0.001	0.006	0.001	< 0.001
Na	2.59	0.44	5.37	69	58.2	174	119
Ni	0.004	< 0.002	< 0.002	< 0.002	0.002	0.009	0.002
Pb	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
V	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Zn	0.094	0.013	0.014	0.029	0.015	0.177	0.014
pH	7.5	6	6.8	6.8	7.9	8.1	8

Table 3 Relative activity of laccase (TV) and peroxidase (HRP) in real water samples

TV	Relative activity [%]	HRP	Relative activity [%]
pH 3	100 ± 3.879	**pH 3**	100 ± 10.340
DIW	29.894 ± 2.470	**DIW**	17.420 ± 3.500
Melted snow	12.937 ± 1.771	**pH 7**	2.105 ± 0.193
Well	3.069 ± 0.786	**Melted snow**	2.049 ± 0.782
pH 7	2.565 ± 0.362	**Lake 1**	0.476 ± 0.219
Pond	0.388 ± 0.010	**Pond**	0.373 ± 0.124
Tap water	0.246 ± 0.066	**Well**	0.349 ± 0.112
Lake 2	0.067 ± 0.005	**Lake 2**	0.234 ± 0.094
Lake 1	0.066 ± 0.005	**Wastewater**	0.204 ± 0.138
Wastewater	0.038 ± 0.010	**Tap water**	0.175 ± 0.118

Both laccase and peroxidase reached their highest activities in a pH 3 buffer (Table 3, Fig. 29). In DIW the activities dropped to approximately 20% most probably due to higher pH (pH 7.5) and absence of beneficial ions. Although all real water samples had neutral pH similar to DIW (6–8.1), the main factor affecting catalytic activity was the presence of inhibiting ions. It has been previously reported that water content, especially presence of inorganic salts (namely those containing divalent and trivalent cations or halides), has a negative impact on catalytic activity of laccase [201], [202]. The most unfavourable type of water for laccase activity was, as expected, the wastewater having the highest ionic concentration. Measurement of peroxidase activity was more complicated compared to laccase. H_2O_2 consumption significantly fastened with increasing water pollution and ion concentration, which resulted in shortening of the linear part of the kinetic activity measurement, therefore, the data might be burdened with error.

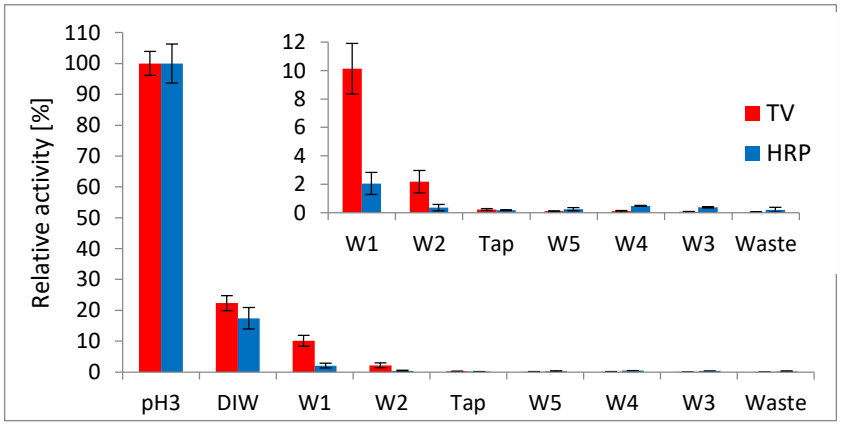


Fig. 29 Comparison of relative activities of laccase and peroxidase in different water sources

Specific effect of each salt was not explored in this study; however, Fig. 30 demonstrates the impact of tap water content in deionized water on the activity of laccase using ABTS as a substrate. With only 10% (v/v) of the tap water diluted with DIW the activity of laccase decreased by 90%.

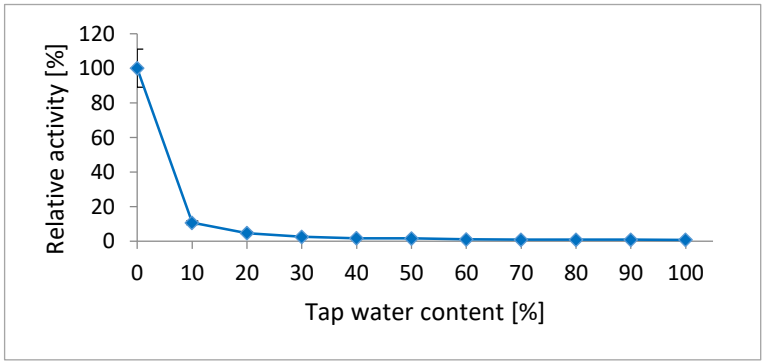


Fig. 30 Catalytic activity of laccase in mixtures of tap and deionized water

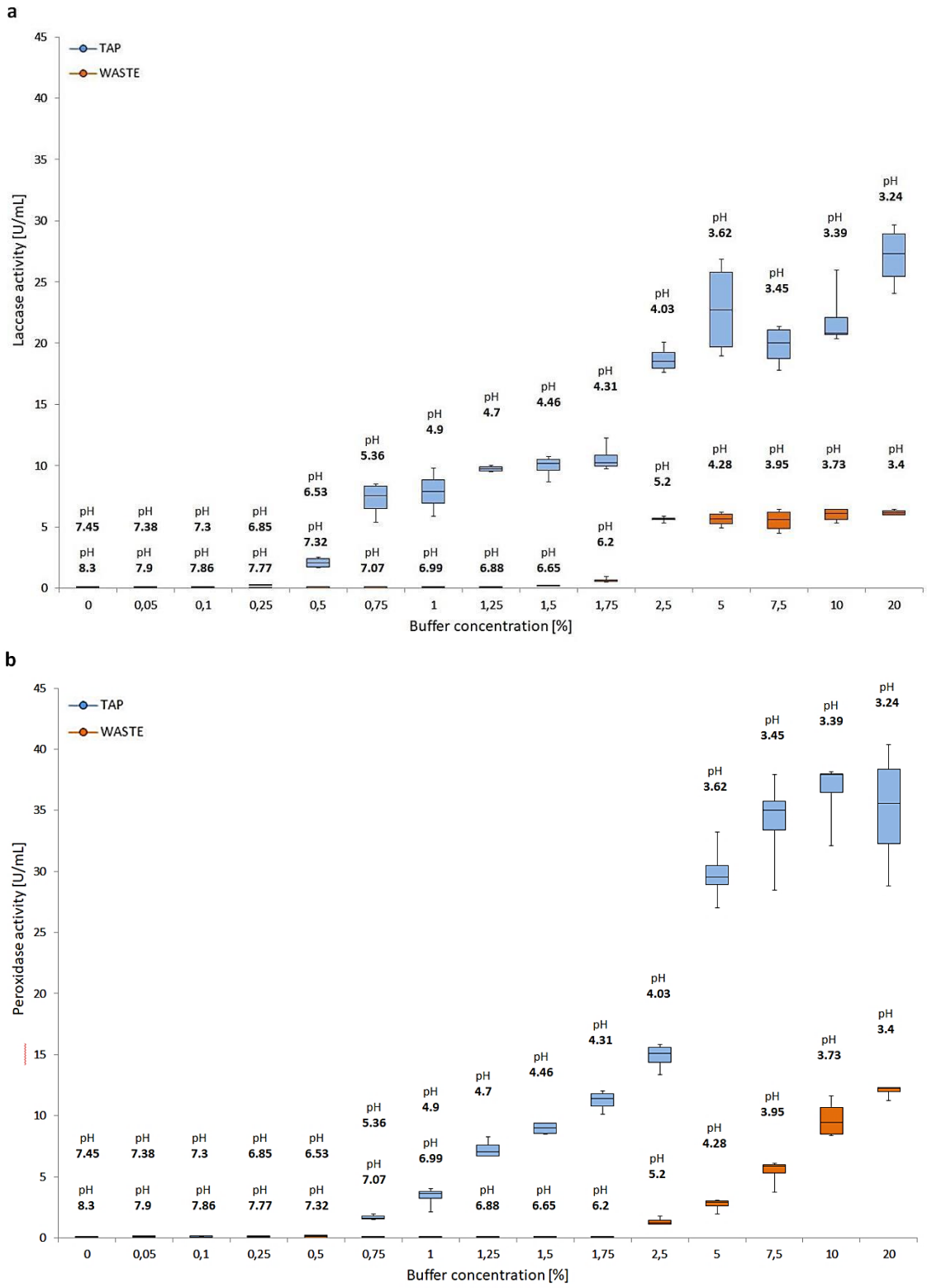


Fig. 31 Catalytic activity of laccase (a) and peroxidase (b) in tap water and wastewater infused with McIlvaine's buffer with pH 3

Similar approach was used in order to study influence of buffer infusion. McIlvaine's buffer with pH 3 was added in concentrations 0–20% (v/v) into tap and wastewater. Significant increase in catalytic activity was observed at 2.5% (v/v) of the

buffer (pH 3 McIlvaine's) content in the case of laccase (Fig. 31a) and 2.5–5% (v/v) content in the case of peroxidase (Fig. 31b). Both enzymes reached higher activities in tap water where the buffer caused larger pH decrease (pH 4 at 2.5% buffer content in tap water and pH 5.2 in wastewater). This finding corresponds with the results of pH optima from Fig. 27. The highest peak of catalytic activity of both laccase and peroxidase using ABTS as a substrate at pH lower than 4. Adjustment of pH has been discussed as a necessary step for wastewater treatment in many studies. An optimal pH for the removal of phenolic compounds was reported between pH 4.5 and 6 [203]–[205].

11. 5. Storage stability

Storage stability of the two enzymes was studied upon two temperatures (4°C and 20°C) in McIlvaine's buffer with 6 different pH using ABTS as a substrate (Fig. 33). Optimal storage conditions for preservation of enzyme activity were recorded at 4°C, with laccase retaining 42% of its initial activity after 30 days at pH 6 (Fig. 32d), while peroxidase retained 53% of its initial activity at pH 7 for 30 days (Fig. 32e), which is comparable to previously reported levels of >50% of initial activity retained after 30 days for another horseradish peroxidase under similar conditions [206]. Although the optimal condition for laccase activity is presumably at pH 3 (Fig. 27a), practical use of laccase at this pH is highly restricted due to the poor storage stability. Better results could conceivably be achieved by using different types of buffer. For example, over 70% of the initial activity of *T. versicolor* laccase was preserved after 28 days storage in 100 mM sodium acetate buffer at pH 4.5 at 4°C [207], however pH plays the main role in storage stability together with chemical composition.

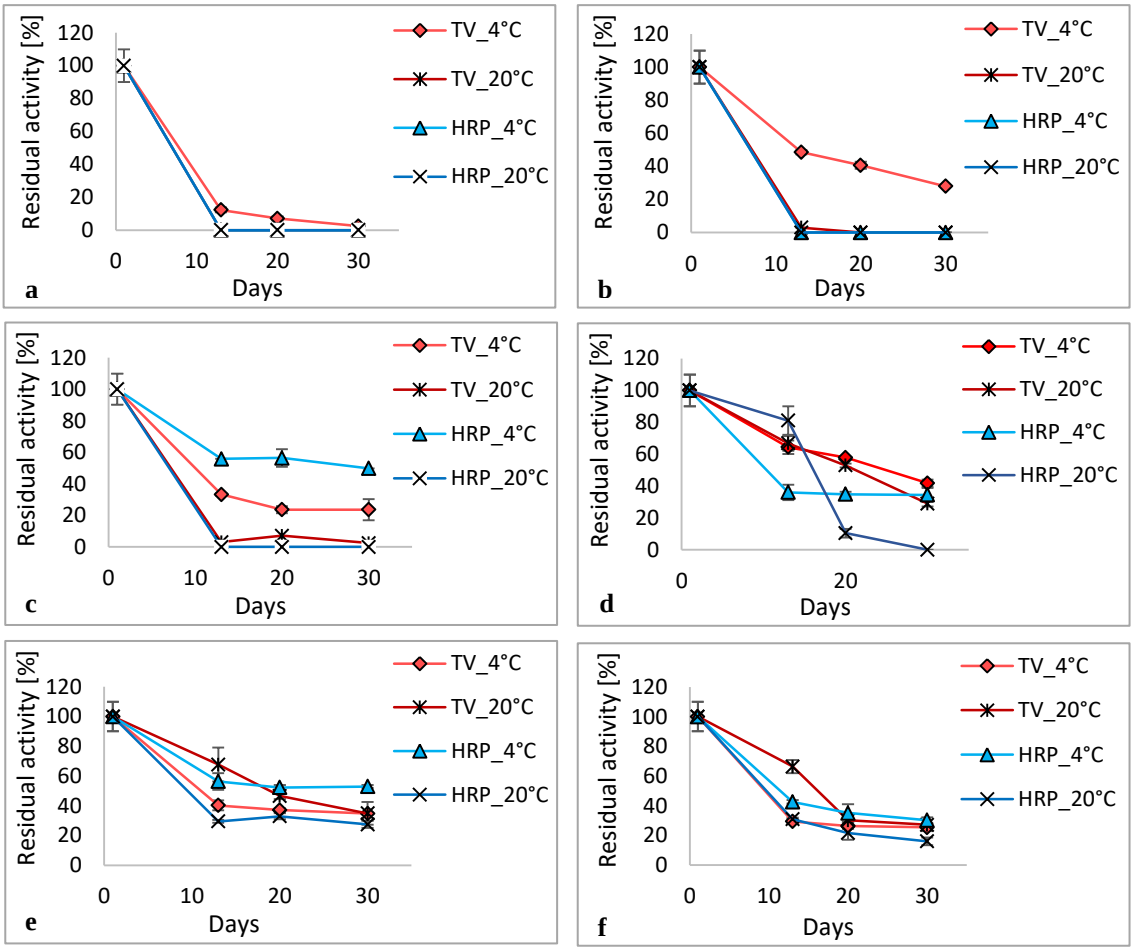


Fig. 32 Storage stability of laccase and peroxidase in McIlvaine's buffer at pH 3 (a), 4 (b), 5 (c), 6 (d), 7 (e), and 8 (f)

11. 6. Degradation of a mixture of bisphenol A (BPA), 17α-ethinylestradiol (EE2), triclosan (TCS) and diclofenac (DCF)

Degradation efficiency of laccase and peroxidase was determined by decreasing the concentration of micropollutants over 20 hours of incubation at 25°C. 25 µL of each enzyme stock solution (2 mg of enzyme per 1 mL of ultrapure water, approximately 0.07 U) were added to glass vials containing 5 mL of a mixture of BPA, EE2, TCS and DCF (10 mg/L) in deionized water, McIlvaine's buffer with pH 3 and pH 7, tap water injected with 2.5% (v/v) of buffer with pH 7, and wastewater with 2.5% (v/v) of buffer with pH 3 and 7. In the case of samples with peroxidase, 700 µL of the EDCs mixture was replaced with the same volume of 1% hydrogen peroxide. Amount and concentration of H_2O_2 resulted from our preliminary measurements. After 20-hour incubation, 100 µL of 10% sodium azide was added, thereby preventing further

degradation of the EDCs [208]. Each experiment was performed in duplicate and the results presented as the mean value ± standard deviation.

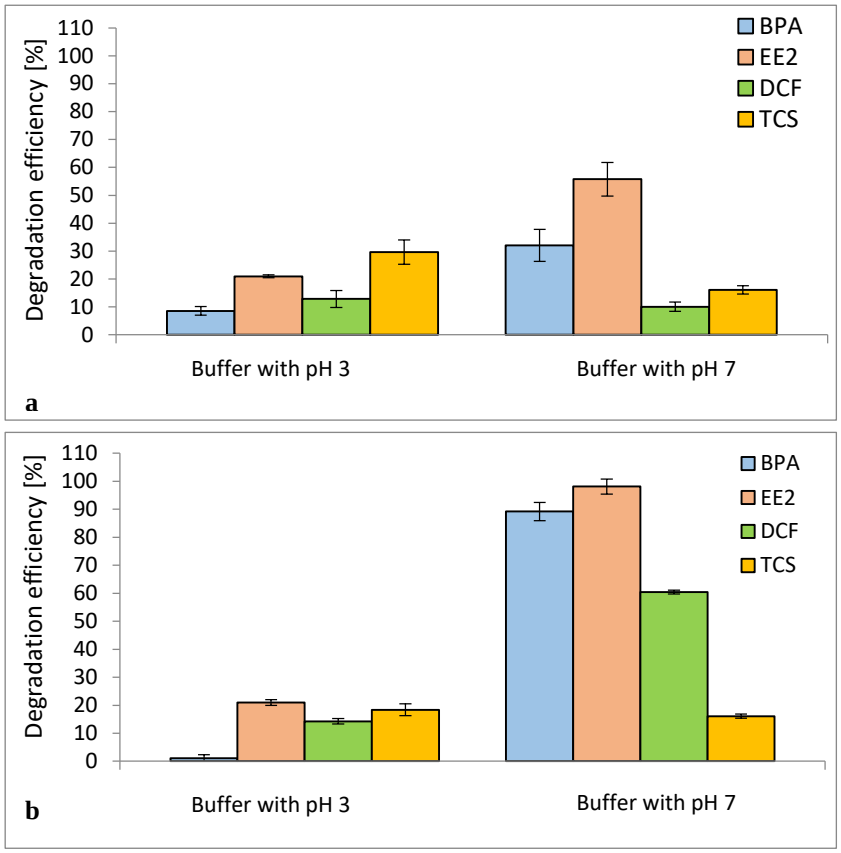


Fig. 33 Elimination of a mixture of BPA, EE2, TCS and DCF in pure McIlvaine's buffer with pH 3 and 7 using laccase (a) and peroxidase (b)

In general, peroxidase was more efficient in degradation of all four contaminants. Based on the previous results from ABTS oxidation (Fig. 27a), we had presumed the highest effectivity using laccase in deionized water and buffer of pH 3. Compared to that, buffer of pH 3 was not an optimal long-term storage medium for both enzymes and rather neutral pH was preferable for peroxidase when using GUA and SYR as substrates. Contrary previous results, pure buffer with pH 3 was not significantly beneficial for the degradation BPA, EE2 and DCF in case of laccase, and only TCS was eliminated by 30% at pH 3 compared to the 16% removal in buffer with pH 7 (Fig. 33a). Peroxidase confirmed its preference of neutral pH by significantly higher oxidation of BPA, EE2 and DCF, while elimination of TCS was similar at both pH 3 and 7 (Fig. 33b).

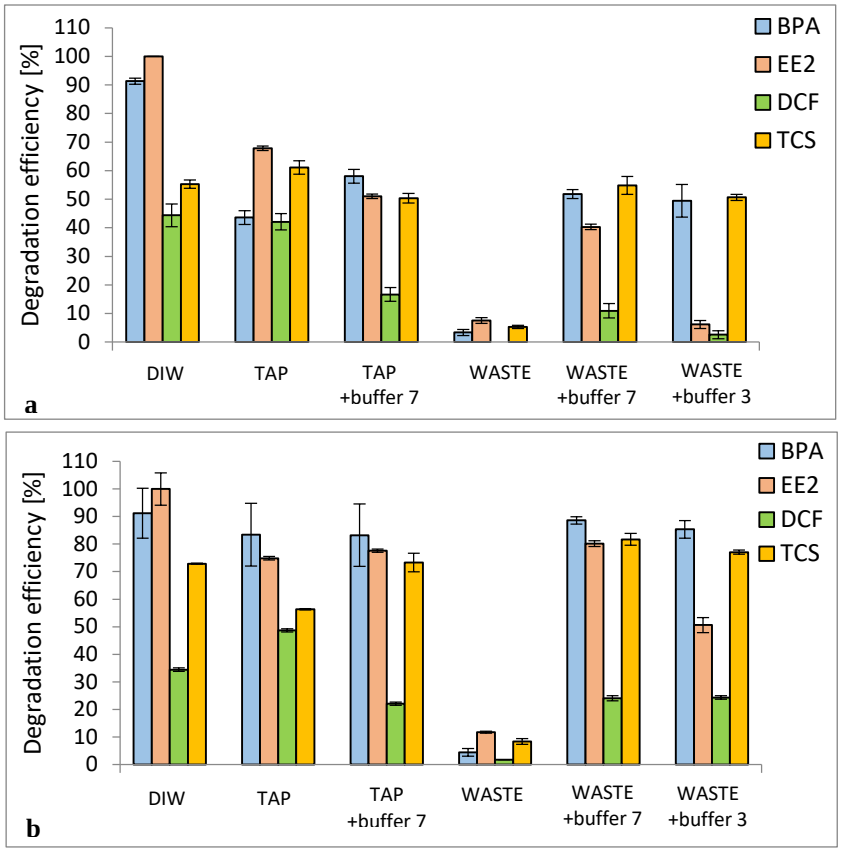


Fig. 34 Elimination of BPA, EE2, TCS and DCF in different water samples using laccase (a) and peroxidase (b)

When testing degradation efficiency in DIW and real waters (Fig. 34), DIW was optimal for laccase and most favorable for the degradation of BPA and EE2 when using peroxidase as well. There was no significant improvement in degradation when doping the tap water with the buffer of pH 7. However, there was a definite progress in the degradation of all EDCs in wastewater injected with the buffer with pH 3 and 7. This fact implies that the benefit of buffer infusion is not simply pH adjustment but rather presence of beneficial ions of the McIlvaine's buffer improving conditions for enzymatic catalysis, especially in such unfavorable environment as wastewater. Surprisingly, after 20-hour incubation in wastewater injected with pH 7 buffer, laccase degraded over 51% of BPA, 40% of EE2, 10% of DCF and 54% of TCS (Fig. 34a), although our previous results using ABTS, GUA and SYR at neutral pH buffer showed almost zero catalytic activity (Fig. 27a). Peroxidase was even more successful when oxidizing over 88% of BPA, 80% of EE2, 24% of DCF and 81% of TCS at the similar conditions (Fig. 34b).

A comparison of these results with those of other studies is difficult as the authors used different activity assays, chemical concentrations or performed the tests

under different pH levels or temperatures. For example, Hongyan et al. (2019) reported fast degradation of BPA in optimal buffer system [209], as well as Olajuyigbe et al. (2019) who used compared free and immobilized laccase [120] at conditions incomparable with real waters. Garcia-Morales et al. (2015) used a 100 U/L laccase cocktail to eliminate EDCs (10 mg/L) in a solution containing BPA, EE2, TCS and nonylphenol at buffer with pH 5, with over 90 % of the micropollutants degraded within five hours [210]. However, in this study oxidoreductases were tested of much lower activity (ca. 35 U/L) to eliminate a similar concentration of EDCs, which explains the lower degradation efficiencies observed. More importantly, our experiments were performed using real waters, which is great progress in the study of water treatment using oxidoreductases.

11. 7. Degradation of a mixture of chlorophenols

Degradation efficiency of laccase and peroxidase was determined by decreasing the concentration of 19 isomers of chlorophenols over 1 hour at 25°C. 25 μL of each enzyme stock solution (1 mg of enzyme per 1 mL of ultrapure water, approximately 0.07 U) were added to glass vials containing 7 mL of a mixture of 19 standard chlorophenols (5 μg/L) in deionized water. In the case of samples with peroxidase, only 25 μL of the EDC mixture was replaced with the same volume of 1% hydrogen peroxide. After the selected time interval, 10 μL of 10% sodium azide was added to stop the catalysis, thereby preventing further degradation of the EDCs. Each experiment was performed in duplicate and the results are presented as the mean value ± standard deviation.

Although the incubation time was only 1 hour, peroxidase was able to degrade all molecules reaching around 30–50% of turnover when degrading 3-chlorophenol (3-CLF), 2,6-dichlorophenol (2.6-diCLF), 3,4,5-trichlorophenol (3,4,5-triCLF), 2,3,5,6-tetrachlorophenol (2,3,5,6-tetraCLF), and pentachlorophenol (PentaCLF). Degradation of remaining contaminants was even more successful, when HRP reached up to 92% of turnover. On the other hand, laccase reached its maximum (over 50%) at the elimination of 2,3,6-triclorophenol (2,3,6-triCLF), however, there was no elimination of five chlorophenols and the overall degradation was much lower compared to HRP (Fig. 35). The catalytic mechanism of HRP seems to be more universal compared to TV.

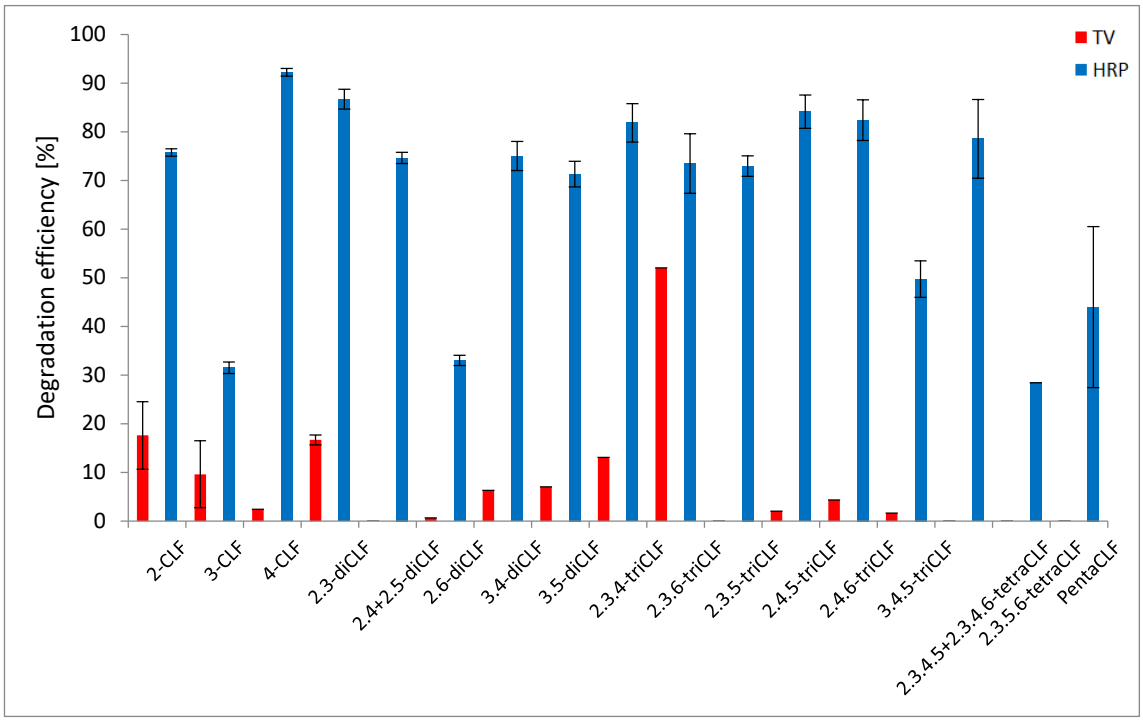


Figure 35 Degradation of a mixture of chlorophenols using laccase and peroxidase

Degradation of chlorophenols using both laccase and peroxidase was previously reported, however, only mixtures containing limited number of compounds were used in most studies. For instance, Tandjaoui et al. (2016) used crosslinked peroxidase aggregates to degrade 2-chlorophenol in silicone oil [211], and Lai and Lin (2005) immobilized peroxidase in order to degrade p-chlorophenol [212]. A mixture of three chlorophenols was successfully eliminated using HRP immobilized onto magnetic particles [213]. Immobilized laccase was also tested against single or multiple chlorophenols mixtures [108], [214], [215], however, obtained results cannot be compared to those achieved in this study, since all selected parameters, including number of observed contaminants, degradation time or enzyme concentration, were different in each study.

12. Laccase immobilized on polyamide 6 (PA6) nanofibers via adsorption and crosslinking

T. versicolor laccase was immobilized onto the PA6 nanofibers via adsorption followed by glutaraldehyde (GA) crosslinking. A range of parameters, including nanofiber matrix surface density, enzyme solution volume, buffer concentration and pH, adsorption and crosslinking time, and GA concentration, were examined in order to establish the most effective immobilization method. Preliminary experiments identified the optimal immobilization process temperature as 4°C and the most convenient mode of agitation providing uniform enzyme molecules distribution as orbital shaking at 150 rpm. After each immobilization process, the samples were washed with pH 3 McIlvaine's buffer until no laccase activity was detected in the washings, following which the activity of the immobilized laccase was determined in order to compare the effectivity of each immobilization process.

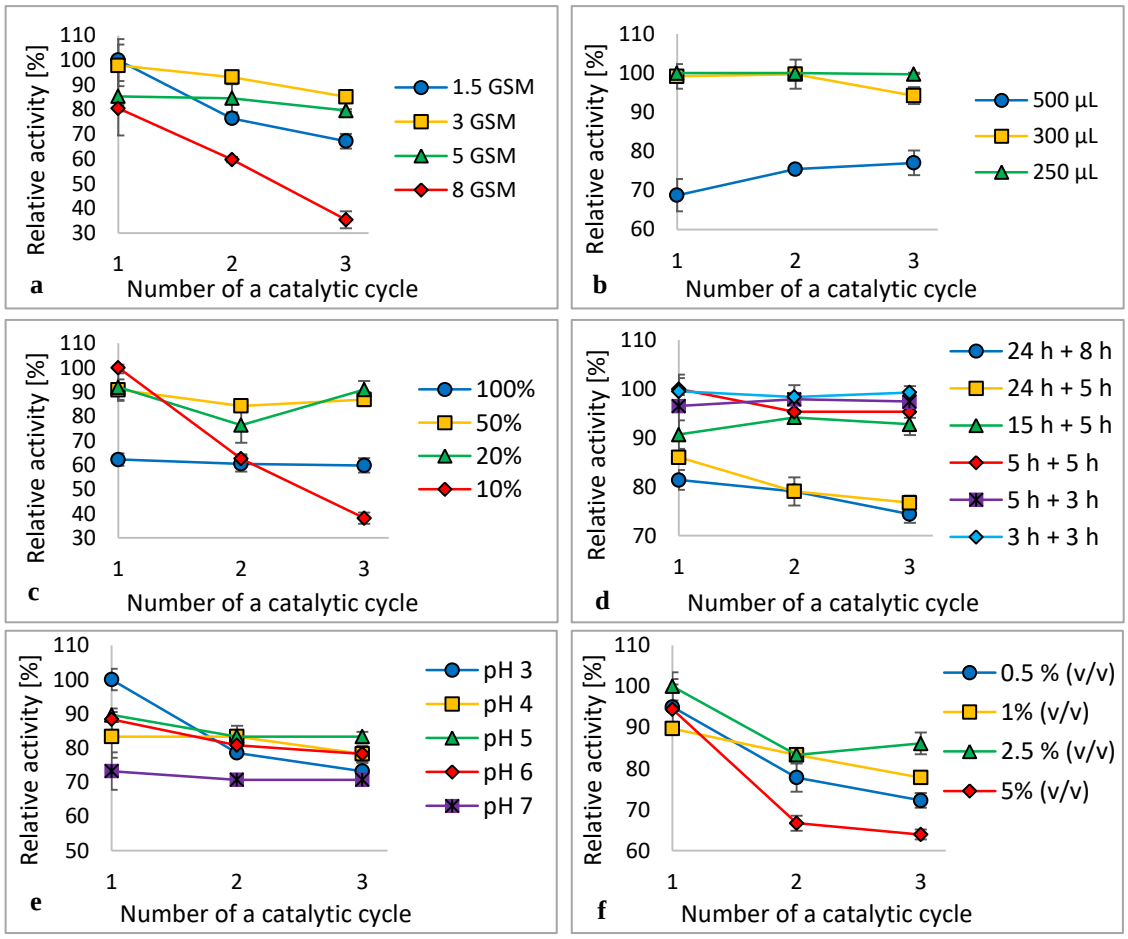


Fig. 36 Effect of (a) nanofibers' surface density, (b) solution volume, (c) McIlvaine's buffer concentration, (d) adsorption and crosslinking time, (e) pH, and (f) glutaraldehyde concentration on catalytic activity of PA6-laccase

a) Effect of nanofiber surface density on immobilization of laccase onto PA6 nanofibers

Support material morphology is an important parameter affecting the enzyme immobilization process. Nanofiber materials are mainly characterized by average fiber diameter and surface density, the combination of these two parameters influencing specific surface area and the immobilization capacity of the matrix [216]. As all PA6 nanofiber sheets were produced under similar electrospinning conditions, average fiber diameter was similar for all the nanofiber samples at 79.3 nm up to 109.4 nm. In comparison, the nanofiber materials used in previous studies were all thicker, with typical diameters ranging from 150 to 500 nm [75], [217], [218]. As such, PA6 nanofiber sheets had a greater specific surface area for laccase immobilization.

Despite surface density being an important parameter influencing enzyme loading, substrate/product diffusion, and mechanical properties of the nanofiber sheet, most studies focus solely on the physico-chemical nature of the immobilization process and tend to ignore the 'usability' of the product under actual water treatment conditions [75], [219]–[221].

Four types of PA6 nanofiber sheets with different surface densities were prepared by adjusting the speed of the electrospinning process. The finest sheet had a surface density of 1.5 g/m^2 and an average fiber diameter of 79.3 ± 19.8 nm. Subsequent sheets had a surface density of ca. 3 g/m^2 and a fiber diameter of 87.9 ± 14.7 nm, ca. 5 g/m^2 and 109.4 ± 19.1 nm, and 8 g/m^2 and 100.4 ± 23.8 nm. To evaluate the most suitable nanofiber sheet, circular PA6 samples (diameter 1.5 cm) were immersed into 0.5 mL of 2 mg/mL laccase solution in 50% McIlvaine's buffer at pH 3 and shaken for 15 hours at 4°C and 150 rpm. GA was then added to achieve a final concentration of 2.5% (v/v), and the mixture was shaken for a further five hours under the same conditions.

The highest activity levels recorded for immobilized laccase, and the most effective reuse within three ABTS oxidation catalytic cycles, was achieved by PA6-laccase nanofibers with a density of 3 g/m^2 (Fig. 36a). Unfortunately, these nanofibers were not easy to handle due to their poor mechanical properties, a tendency to tear, and deteriorative wettability compared to nanofibers of higher surface density. As such, PA6 nanofibers with a surface density of 5 g/m^2 were chosen for enzyme immobilization, despite displaying 13 % less enzymatic activity than 3 g/m^2 PA6 nanofibers.

b) Effect of laccase solution volume on immobilization of laccase onto PA6 nanofibers

Were pristine PA6 nanofibers to be used for enzyme adsorption, it can be assumed that there would be insufficient functional groups to selectively bind the enzyme during the adsorption. Thus, the volume of enzyme solution will have a strong effect on adsorption efficiency by forcing the liquid to fully adsorb into the nanofiber structure and, as such, the lowest volume ensuring full wetting of the carrier would be most desirable. With insufficient agitation, however, enzyme molecules tend to agglomerate and form sediment. Thus, the volume selected must enable satisfactory enzyme flux in the solution. The lowest volume of laccase solution tested required a very small vessel (500 µL vial), compared to more often used 24-well microplate, which required a higher agitation speed (around 220 rpm) to ensure sufficient motion in the solution. In comparison, a 24-well non-treated polystyrene microplate was able to accommodate PA6 nanofiber samples with a diameter of 1.5 cm, meaning that the samples remained flat throughout the immobilization process. Further, though a slightly higher liquid volume was required (>300 µL), such a vessel allowed easy monitoring of nanofiber immersion level and required a lower agitation speed (<150 rpm).

PA6 samples were immersed into different volumes of 2 mg/mL laccase solution (250 µL, 300 µL, and 500 µL) and incubated according to the procedure described above. While the best results were obtained using 250 µL of enzyme solution (Fig. 37b), such a low volume required a special vessel in ensure complete immersion of the nanofiber sample. Hence, using 300 µL is recommended when using a 24-well plate for immobilization of 1.5 cm nanofiber samples as this encourages full adsorption of the enzyme solution into the nanofibers and ensures that most of the enzyme molecules are in contact with the matrix.

c) Effect of buffer concentration on immobilization of laccase onto PA6 nanofibers

McIlvaine's buffer has been used for laccase immobilization and activity assays in numerous previous studies [15], [189], [222], [223]; however, none of these have addressed optimization of the buffer concentration. Ionic strength is an important parameter influencing both enzyme solubility and the charge of the free functional groups on the enzyme molecules and nanofiber matrix, which in turn enables the

development of electrostatic protein-protein and protein-matrix interactions. Generally speaking, lyophilized enzyme powder requires a buffer of sufficient ionic strength to fully solubilize and separate clusters formed by enzyme oligomers [224].

Undiluted (100%) McIlvaine's buffer was prepared from a mixture of 0.2 M disodium hydrogen phosphate and 0.1 M citric acid at appropriate ratios that depended on the required pH [225]. Subsequently, the buffer was diluted with ultrapure water to produce a 10%, 20% and 50% solution (no pH change detected after dilution). All four concentrations (10, 20, 50, and 100%) were then used for laccase immobilization. All other parameters of the immobilization process were identical to those described in previous sections (i.e. 300 μL of 2 mg/mL laccase solution, pH 3).

The clearest influence of ionic strength on the immobilization process was observed between 10% and 100% buffer (Fig 36c). While low ionic strength buffer increased the amount of immobilized laccase, it did not allow for a sufficiently strong interaction between carrier, enzyme, and GA molecules formed during adsorption and crosslinking, resulting in immobilized laccase of poor stability. In comparison, the highest buffer concentration promoted formation of strong interactions, resulting in increased enzyme stability; however, possibly due to conformational changes caused by the formation of strong bonds, this concentration yielded the lowest activity of all the samples tested. As a compromise, both reasonable activity and stability was achieved when using 20% and 50% McIlvaine's buffer, though, from an economic point of view, the less concentrated buffer would be preferable.

d) Effect of adsorption and crosslinking time and pH on immobilization of laccase onto PA6 nanofibers

Six different combinations of adsorption time (3, 5, 15, and 24 hours) and crosslinking time (3, 5, and 8 hours) were used to determine the optimal immobilization duration. The optimal pH for immobilization was evaluated using 20% McIlvaine's buffer with a pH of 3, 4, 5, 6, and 7.

Time taken for laccase adsorption and GA crosslinking has a clear influence on the activity and stability of immobilized laccase. Surprisingly, the longest time (24 + 8 h) for both adsorption and crosslinking gave the worst results, with the lowest immobilized enzyme activity and stability (Fig. 36d), probably as the catalyst was damaged through long agitation or due to extreme crosslinking rate causing

conformational changes of immobilized laccase. Enzyme activity and reuse increased, however, at shorter times. Optimal results were obtained after three hours adsorption and three hours GA crosslinking. This total of six hours compares well with previous studies, where the most common time for GA crosslinking was up to two hours, though adsorption usually took place overnight [75], [226].

The most suitable pH for immobilization of laccase onto PA6 nanofibers was pH 5, which resulted in the second highest enzyme activity level, while the highest stability over three ABTS oxidation catalytic cycles was recorded over pH 3–7 (Fig. 36e). In previous studies [75], [103], [217]–[219], [226], a similar pH (4–5.5) was also considered as optimal, probably as laccase is best able to maintain its functional conformation when attached to a matrix.

f) Effect of GA concentration on immobilization of laccase onto polyamide 6 nanofibers

Of the four GA concentrations tested (0.5, 1, 2.5, and 5% v/v), the highest operational stability was observed at the highest GA concentration (5% v/v). Surprisingly, the optimum concentration turned out to be 2.5% v/v, providing both the highest activity and the best reuse of immobilized laccase. Most probably, larger enzyme clusters were formed outside the matrix area at this concentration (2.5% v/v), which decreased the number of bonds between the enzyme and the support (Fig. 36f).

GA has long been favored as a crosslinking agent for enzyme immobilization. In a number of cases it has been used for matrix stabilization [94], [227] or for functionalization of the supporting material [105], [174], [217], [228], the optimal GA concentration in such cases varying between 1–4 % v/v. In most cases, however, GA has been used as a bifunctional agent for introduction of aldehyde groups to the matrix surface prior to enzyme attachment [119], [129], [172], [229]. However, this type of functionalization requires the presence of free primary amino groups on the matrix. In comparison, our method does not require the matrix to have such a chemical composition and, as such, it provides much greater freedom in the selection of materials for enzyme immobilization.

12. 1. Summary of the optimal immobilization process

Overall, the results suggest an optimal average PA6 nanofiber diameter of 105 ± 19.1 nm, with a surface density of **5 g/m^2**. Under these conditions, the nanofibers display excellent mechanical properties, ease of handling, repeatability, low cost, and homogenous surface density; thereby providing perfect conditions for laccase immobilization [230]. *T. versicolor* laccase was immobilized onto PA6 nanofibers via adsorption followed by GA crosslinking. The optimal immobilization process required the PA6 nanofiber samples (1.5 cm in diameter) to be submerged separately into **300 µL** of laccase stock solution (**2 mg/mL**) in **pH 5 20%** buffer, shaken at **4°C** in an orbital shaker at 150 rpm for **3 hours**, following which GA was added in order to achieve a final concentration of **2.5 % v/v** and the samples shaken in the immobilization solution for a further **3 hours**. Finally, the samples were washed with pH 3 McIlvaine's buffer. Pristine PA6 nanofibers display an homogenous structure and smooth surface (Fig. 37a), while PA6-laccase displays a grainy surface formed by crosslinked laccase clusters strongly attached to the nanofibers, even after thorough washing (Fig. 37b).

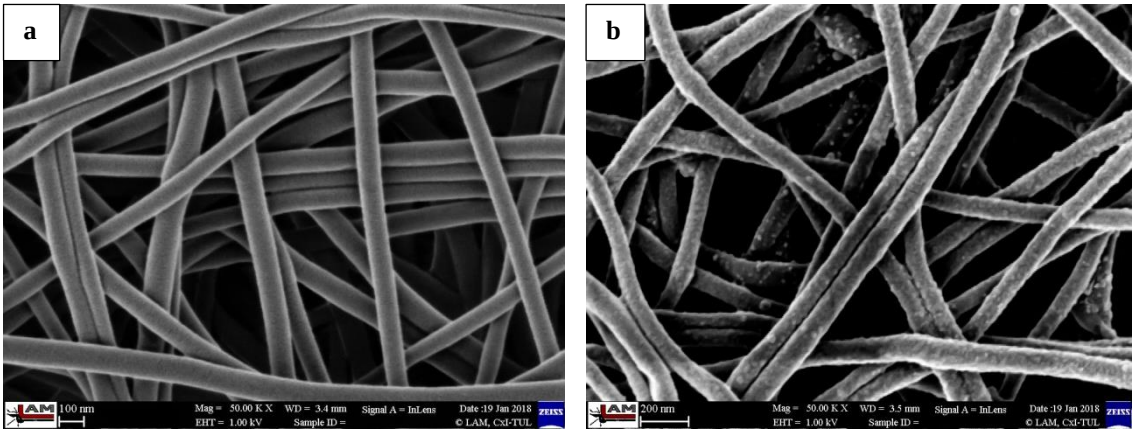


Fig. 37 Comparison of SEM images of (a) pristine polyamide 6 nanofibers, and (b) nanofibers with immobilized laccase. Magnitude 50 kx.

12. 2. Storage stability and reuse

Immobilized laccase (PA6-laccase) was incubated in pH 6 McIlvaine's buffer (20%) at 4°C in order to assess storage stability. Two replicate samples were taken at selected time points (13, 20, and 30 days) and their enzyme activity was measured. Free laccase solution was stored under the same conditions for comparison with the immobilized laccase.

Reuse of the PA6-laccase nanofibers was determined by measuring enzyme activity over several catalytic cycles using ABTS as a substrate. The samples were removed from the ABTS mixture and thoroughly washed with fresh buffer at pH 3 after each catalytic cycle.

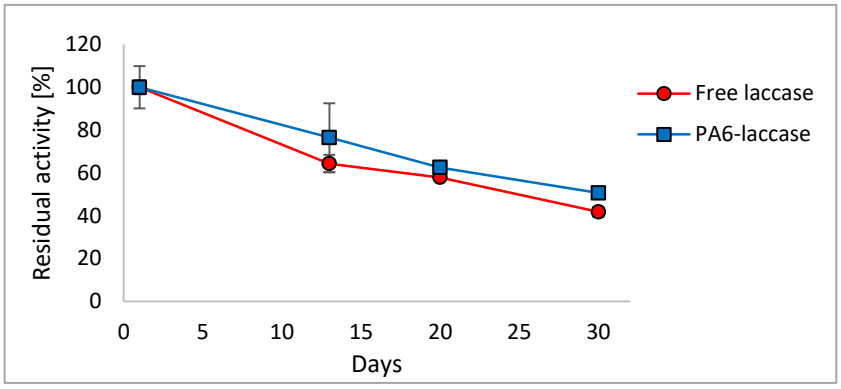


Fig. 38 Comparison of storage activity of free laccase and immobilized laccase onto polyamide 6 nanofibers

The storage stability of free and immobilized laccase was tested by assessing activity after 13, 20, and 30 days of storage at 4°C. Use of pH 6 McIlvaine's buffer and a temperature of 4°C provided optimal conditions for preservation of enzyme activity, with free laccase retaining 42% of its initial activity. PA6-laccase displayed better storage stability than the soluble enzyme, with 50 % of activity retained after 30 days (Fig. 38).

In previous studies, laccase immobilized onto fibrous polymer-grafted polypropylene chloride film preserved around 57% of initial activity after 30 days storage in a pH 5.5 buffer at 4°C [231], while laccase immobilized onto a PVDF membrane retained 43% of initial activity after 36 days storage in pH 4 McIlvaine's buffer at 4°C [15]. In an exceptional case, laccase immobilized onto carbon nanotubes retained 80% of initial activity under similar storage conditions, while free laccase retained a similar activity level as that in this study [232]. Thus, it appears that particle or nanoparticle carriers are better able to preserve enzyme activity, probably due to their

favorable pore size distribution compared to fibrous or nanofibrous structures. However, these nanomaterials cannot compete with the PA6 nanofiber matrix regarding safety, ease of handling, and applicability under actual wastewater treatment conditions. PA6-laccase retained 88% of its initial activity after five ABTS oxidation catalytic cycles; a very promising result compared with the study of Xu et al. (2015), where laccase covalently immobilized onto carbon nanotubes retained ca. 80% activity after five ABTS transformation cycles, and the study of Liu et al. (2012) [233], where *T. versicolor* laccase immobilized onto magnetic bimodal mesoporous carbon retained 70% activity after five cycles.

12. 3. Degradation of bispehnol A (BPA), 17α-ethinylestradiol (EE2), and triclosan (TCS)

Degradation efficiency of free and immobilized laccase was determined by decreasing the concentration of micropollutants over time. One sample of PA6-laccase, PA6 (blank) or 25 µL of the enzyme stock solution (2 mg of enzyme per 1 mL of ultrapure water) were added to glass vials containing 5 mL of a mixture of BPA, EE2, and TCS (50 µM) in ultrapure water containing 30 % methanol. Over the selected time intervals, 70 µL of the mixture supernatant was collected into vials containing 65 µL of deionized water and 5 µL of 10% sodium azide, thereby preventing further EDC degradation if some of the enzyme had been collected with the supernatant [208]. Each experiment was performed in duplicate and the results presented as the mean value ± standard deviation.

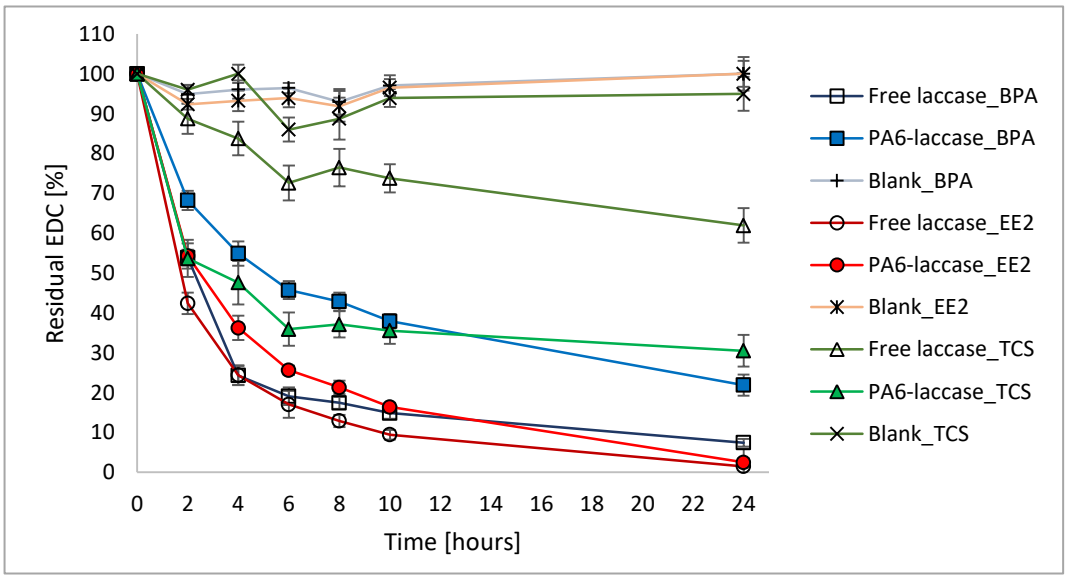


Fig. 39 Degradation efficiency of free and immobilized laccase (PA6-laccase) over the elimination of bisphenol A (BPA), 17α-ethinyl estradiol (EE2), and triclosan (TCS)

The degradation activity of free laccase and PA6-laccase was tested against a 50 μM BPA, EE2, and TCS micropollutant mixture. PA6-laccase displayed activity of 0.03 U, similar to that of free laccase in 25 μL of the stock enzyme solution (2 mg/mL). PA6-laccase removal efficiency of BPA was lower than that of free laccase (22% remaining after 24 hours), though the removal profile was similar to that of free laccase with EE2. PA6-laccase was notably more efficient in TCS reduction within 24 hours of incubation (ca. 70% decrease compared with 38% for free laccase; Fig. 39). In comparison, a blank sample containing only PA6 nanofibers did not adsorb any EDCs over the 24 hours.

Degradation of EDCs using immobilized laccase has been the subject of a number of studies. *Cerrena unicolor* laccase (12 U/5 mL) captured on porous silica beads, for example, has been shown to eliminate around 90% of BPA (50 μM) after 60 minutes of incubation [235]. Application of nanoparticles for enzyme immobilization has recently been criticized due to issues connected with their commercial application, such as their tendency to agglomerate and the need to separate them from treated water [234], [236], [237]. The mechanical properties of PA6, on the other hand, showed no signs of damage over 30 days of storage. Hence, PA6 has a major advantage over particle- or nanoparticle-matrices in that their macroscopic and compact form allows them to be handled as textiles, providing great potential for their use as stable filters in water treatment processes.

13. Laccase immobilized onto polyamide 6/ polyethylenimine (PA/PEI) nanofibers via Schiff's base formation

T. versicolor laccase was immobilized onto the PA/PEI nanofibers via oxidation of the enzyme and it's covalent attachment (as described in section 9.1.). A range of parameters, including buffer concentration and pH, concentration of laccase and oxidation agent ($NaIO_4$), oxidation and immobilization time, were examined in order to establish the most effective immobilization method. Preliminary experiments identified the optimal immobilization process temperature as 4°C and the most convenient mode of agitation providing uniform enzyme molecules distribution as orbital shaking at 150 rpm and the optimal volume of laccase solution as 300 µL. After each immobilization process, the samples were washed with pH 3 McIlvaine's buffer until no laccase activity was detected in the washings, following which the activity of the immobilized laccase was determined.

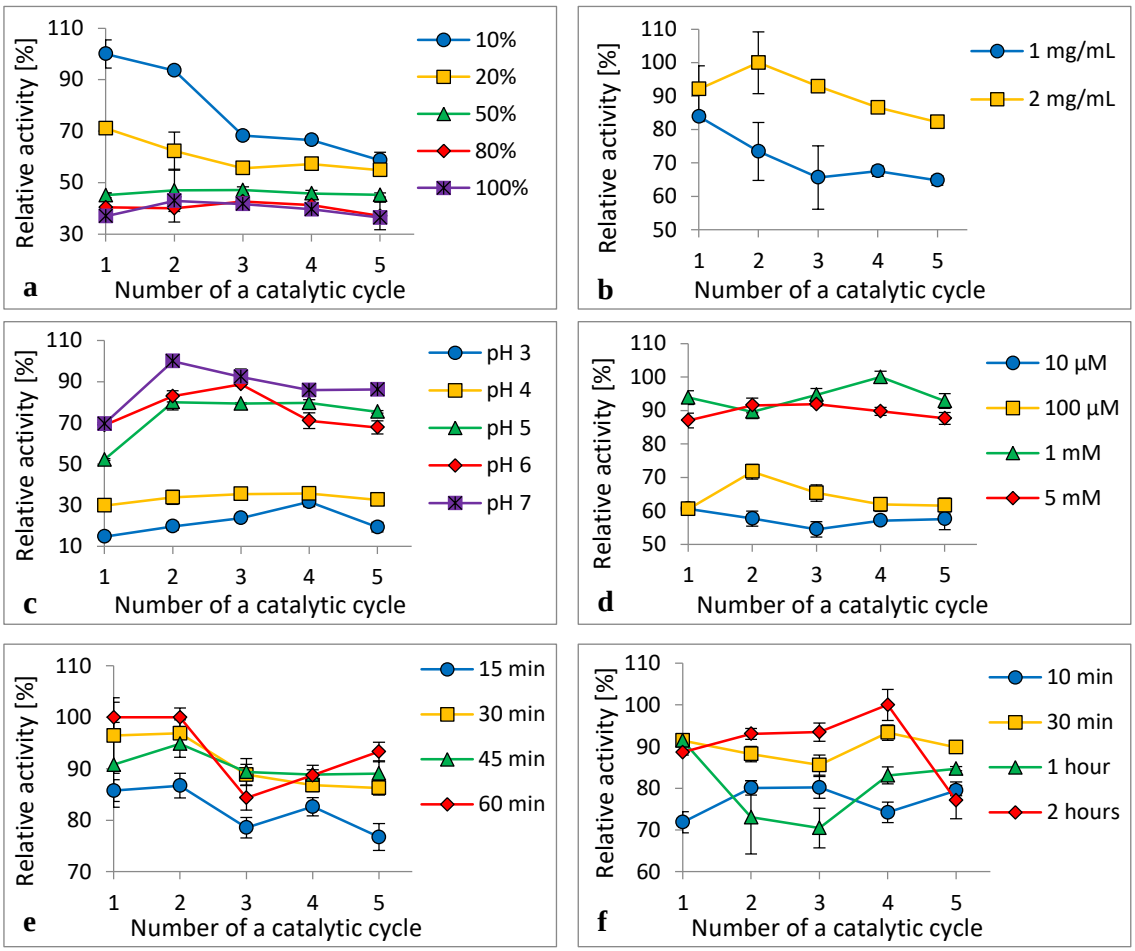


Fig. 40 Effect of (a) McIlvaine's buffer concentration, (b) laccase concentration, (c) pH, (d) concentration of $NaIO_4$, (e) oxidation time, and (f) immobilization time on catalytic activity of PA/PEI-laccase

a) Effect of buffer concentration and pH on immobilization of laccase onto PA/PEI nanofibers

Buffer concentration and pH mainly influence the total charge of enzyme molecules and the supporting material, thereby affecting emerging bonds between laccase and PA/PEI nanofibers. In general, each type of binding requires different conditions as the optimal pH for laccase adsorption may be different from those for creation of a covalent bond. In this study, enzyme activity increased with lower buffer concentration (Fig. 40a). Considering both catalytic activity and reusability of the immobilized laccase, 20% McIlvaine's buffer proved to be the best at providing an optimal combination of both parameters. In terms of optimal pH, a neutral pH was preferable for immobilization of the oxidized laccase (Fig. 40c). This corresponds with previous studies, where neutral or somewhat alkaline pH (range 7–10) proved optimum for formation of Schiff's bases [184], [238].

b) Effect of concentration of $NaIO_4$ and oxidation time on immobilization of laccase onto PA/PEI nanofibers

In this study, we tested four different concentrations of the $NaIO_4$ oxidizing agent and four reaction times in order to assess the optimal combination giving highest activity and allowing reuse of the immobilized laccase. As in the study of Jolivalt et al. (2000), the most appropriate concentration proved to be 1 mM (Fig. 40d) as it provides a sufficient oxidation time of ca. 30 minutes (Fig. 40e) [182].

c) Effect of immobilization time on immobilization of laccase onto PA/PEI nanofibers

There was no significant difference in immobilized laccase activity in samples taken between 10 minutes and 2 hours of immobilization time (Fig. 40f). Overall, however, the best combination of activity and stability was achieved at 30 minutes.

13. 1. Summary of the optimal immobilization process

Overall, the optimal immobilization process required a **10 mg/mL** laccase stock solution to be oxidized via **1 mM $NaIO_4$** for **30 minutes** at **4°C**. Subsequently, any residual oxidizing agent was removed using gel filtration (PD MiniTrap G-25, centrifugation for **2 minutes at 2400 rpm**). The oxidized laccase solution was diluted with **pH 7** 20% McIlvaine's buffer to produce a solution with a concentration of **2 mg/mL** (Fig. 40b). Next, the PA/PEI nanofiber samples (1.5 cm diameter) were submerged separately into 300 µL of laccase solution and shaken at 4°C in an orbital shaker at 150 rpm for **30 minutes**. Finally, the samples were washed with McIlvaine's buffer in order to remove all unattached enzyme molecules.

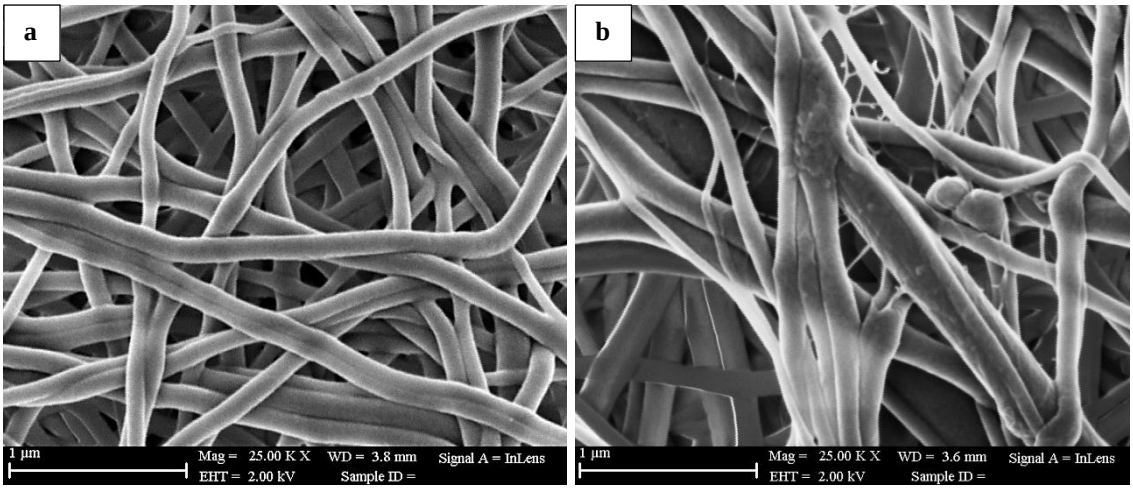


Fig. 41 Comparison of SEM images of pristine PA/PEI nanofibers (a) and PA/PEI with immobilized T. versicolor laccase (b); Magnitude 25 kx

The PA/PEI-laccase sample (Fig. 41b) had a noticeably different surface morphology to the smooth pristine PA/PEI nanofibers (Fig. 41a), being somewhat grainy and containing thinner fibrous structures.

13. 2. Quantification of amino groups

The quantification method was performed as described in chapter 8.6. Both the pristine PA/PEI nanofibers and the PA/PEI-laccase samples had comparable specific surface areas, both having a surface density of ca. 7–7.5 g/m^2 and an average fiber diameter of ca. 100 nm. While PA6 nanofibers possessed only 16.96 ± 0.61 nmol/mg of free amino groups, branched PEI increased the concentration in PA/PEI nanofibers almost 40-fold to 670 ± 57.2 nmol/mg (Fig. 42a). The difference was further displayed by the amount of attached methyl orange molecules, which gave the samples a distinct orange hue (Fig. 42b).

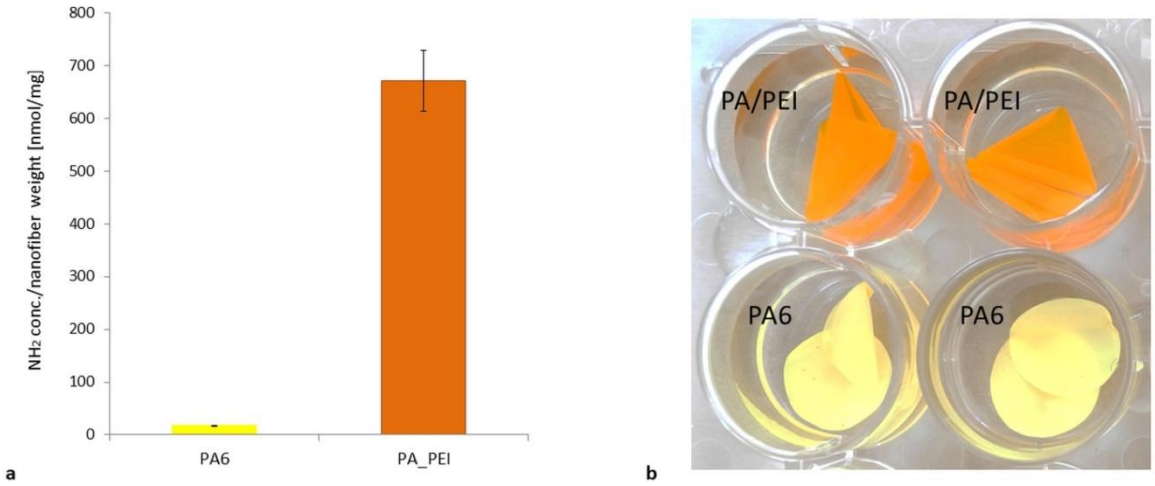


Fig. 42 Quantification of available amino groups of polyamide 6 (PA6) and polyamide 6/polyethylenimine (PA/PEI) nanofibers using methyl orange

The novel PA/PEI nanofibers prepared for this study excelled in several parameters. First, the nanofiber sheets were easy to handle due to their mechanical properties, provided by presence of PA6, and thickness, while PEI ensured presence of multiple primary amino groups together with excellent nanofiber wettability.

13. 3. Storage stability and reuse

Immobilized laccase (PA/PEI-laccase) was incubated in DIW at 4°C in order to assess storage stability. Two replicate samples were taken at selected time points (7, 14, 21 and 30 days) and their enzyme activity measured.

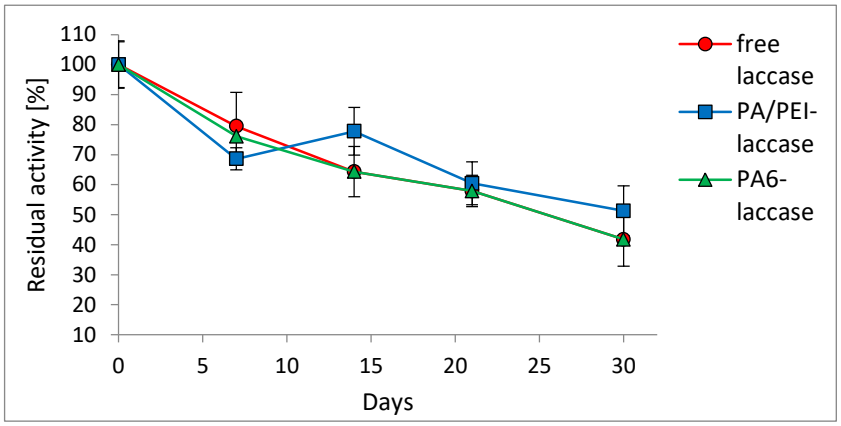


Fig. 43 Storage stability of PA/PEI-laccase compared to free laccase and PA6-laccase

While reaching 47% higher initial activity compared to PA6-laccase samples the PA/PEI-laccase samples retained more than 52 % of initial activity after 30 days of storage (Fig. 43). This is comparable to the previous results with polyamide/chitosan and pristine PA6 as a nanofiber support [125], [239], though the optimal buffer in the current study was exchanged for the less profitable DIW. Reuse of PA/PEI-laccase samples was excellent, reaching up to 100% of initial activity after five rounds of ABTS oxidation. This indicates a very high level of stability compared to the literature [240], [241].

13. 4. Degradation of bisphenol A (BPA), 17α-ethinylestradiol (EE2), triclosan (TCS), and diclofenac (DCF)

Degradation of a mixture of BPA, EE2, TCS and DCF with an initial concentration of 10 mg/L of each contaminant was performed in three different water systems: *i)* DIW, *ii)* wastewater, and *iii)* wastewater infused with 2.5% (v/v) of undiluted McIlvaine's buffer at pH 7. One PA/PEI-laccase sample was immersed into 5 mL of EDCs mixture and incubated for 20 hours at room temperature under mild agitation (80 rpm). Subsequently, samples were removed and 10 µL of 10% sodium azide was added in order to inhibit possible leakage of laccase molecules from the nanofiber carrier.

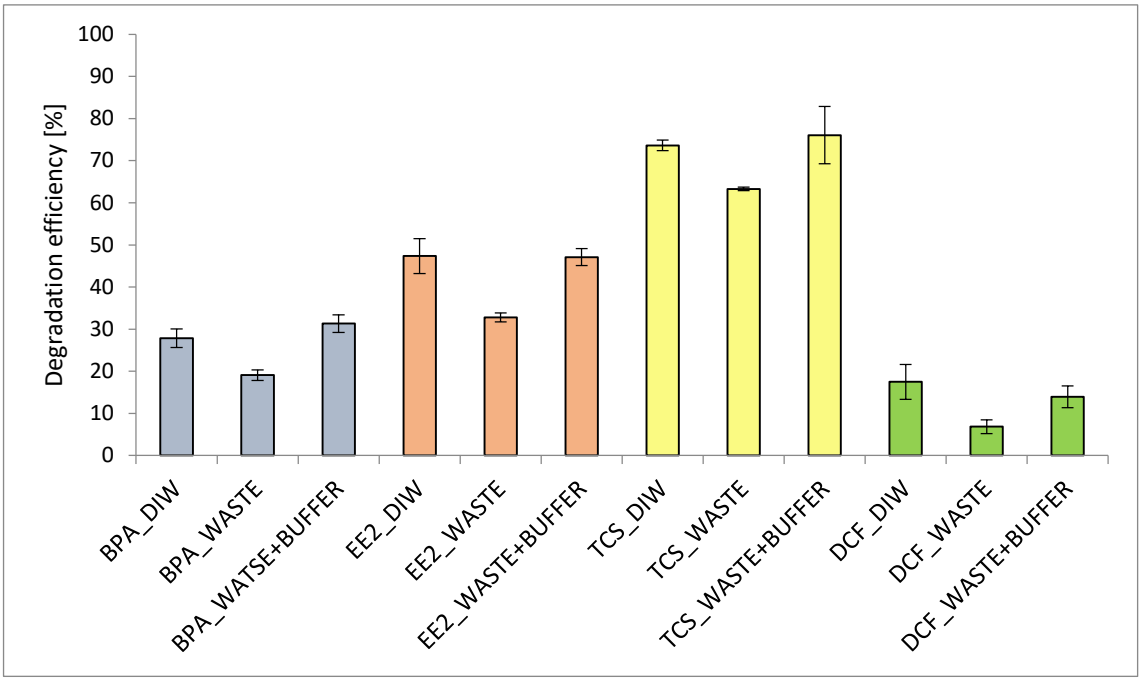


Fig. 44 Degradation efficiency of PA/PEI-laccase towards a mixture of 10 mg/mL of BPA, EE2, TCS and DCF in deionized water (DIW), wastewater effluent (WASTE) and wastewater infused with 2.5% (v/v) of McIlvaine's buffer of pH 7 (WASTE+BUFFER)

In general, PA/PEI-laccase removal efficiency of a mixture of BPA, EE2, TCS and DCF was higher in DIW than wastewater effluent, reaching highest efficiency in elimination of TCS (73.6 %), followed by EE2 (47.3 %), BPA (27.9 %), and DCF (17.5 %). Although EDC removal in real wastewater effluent proved somewhat less efficient, the PA/PEI-laccase samples were still successful, considering the degree of pollution and the presence of highly concentrated ions that could negatively affect enzyme activity (Table 2) [201], [202]. When using wastewater infused with McIlvaine's buffer, however, PA/PEI-laccase degradation efficiency achieved a similar level of efficiency to those in DIW (Fig. 44).

14. Laccase immobilized onto poly(acrylic acid) (PAA) nanofibers via adsorption

Trametes versicolor laccase was immobilized onto PAA nanofibers via simple adsorption. In the first trials, the procedure included GA crosslinking, as described in chapter 12; however, use of GA provided no improvement in immobilized laccase (PAA-laccase) activity or stability (Fig. 45f). Consequently, the range of parameters used to determine the most effective immobilization method included only adsorption time, buffer concentration, pH, and concentration and volume of laccase solution. Preliminary experiments identified the optimal temperature for the immobilization process as 4°C, and the most convenient mode of agitation providing uniform enzyme molecules distribution as orbital shaking at 150 rpm. The optimal volume of laccase solution was determined as 300 µL using a 24-well non-treated polystyrene microplate as the optimal vessel. After each immobilization process, the samples were washed with pH 3 McIlvaine's buffer, following which the activity of the immobilized laccase was determined.

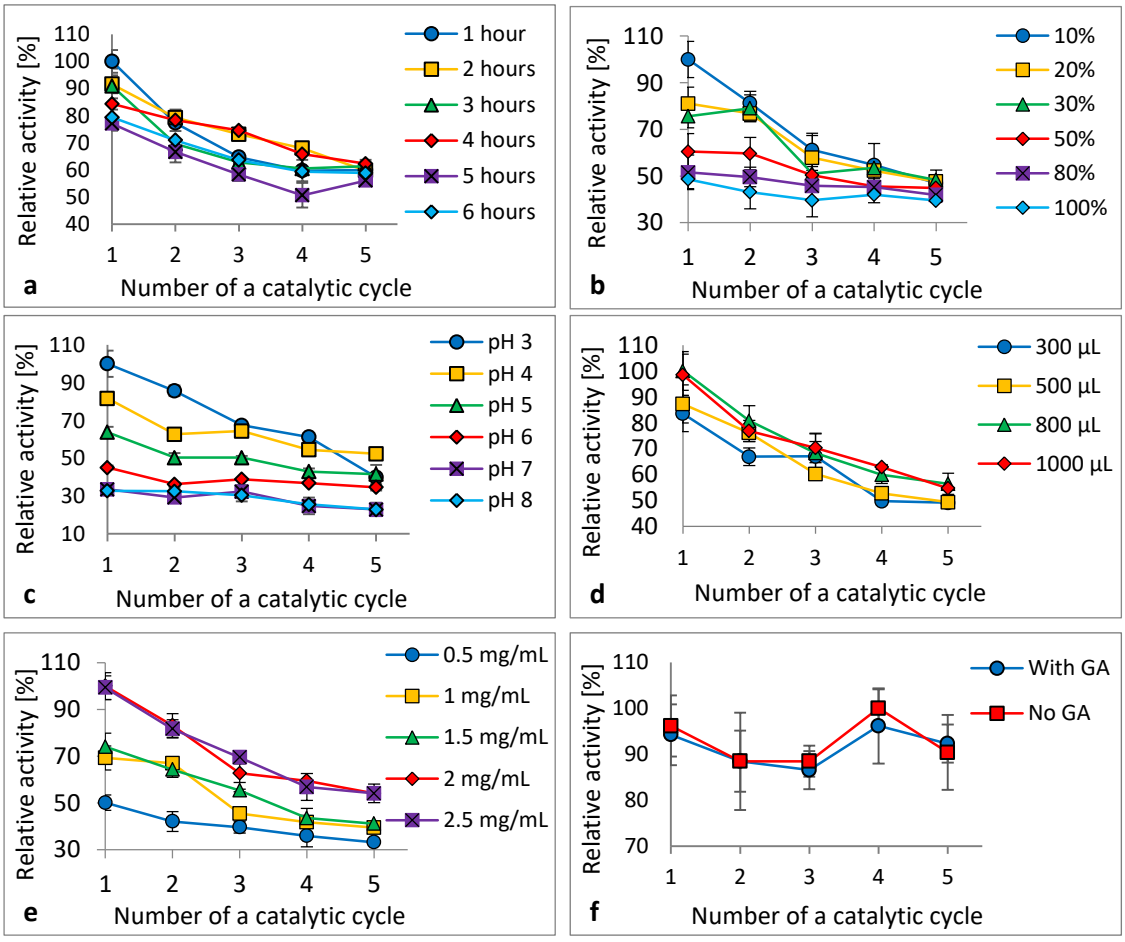


Fig. 45 Effect of (a) immobilization time, (b) McIlvaine's buffer concentration, (c) pH, (d) volume of laccase solution, (e) laccase concentration, (f)presence of glutaraldehyde on catalytic activity of immobilized laccase onto PAA

a) Effect of adsorption time on immobilization of laccase onto PAA nanofibers

Physical adsorption requires that the support be soaked for a sufficient period in the enzyme solution and adequate incubation in order to obtain optimal conformation once the enzyme has been adsorbed into the nanofiber structure [242]. Tests showed that an adsorption period of two hours to be sufficient (Fig. 45a). Prolonged soaking did not improve reuse; rather, it negatively affected the activity of PAA-laccase, with increased contact time causing either enzyme damage due to excessive agitation or increased diffusion limitation due to adsorbed enzyme blocking the interfiber pores. Measurements of the amount of laccase adsorbed (immobilization yield) indicated that there was no significant difference between one-hour (IY=58.5%) and six-hour adsorption (IY=53.8%).

b) Effect of buffer concentration on immobilization of laccase onto PAA nanofibers

As mentioned previously, the most distinctive difference in the effect of ionic strength on the immobilization process was observed between 10% and 100% buffer (Fig 45b). While low ionic strength increased the amount of laccase immobilized, the stability of these samples was very low. In comparison, the highest buffer concentration promoted formation of strong interactions, resulting in increased enzyme stability. However, the highest buffer concentration yielded the lowest activity of all samples tested, possibly due to conformational changes caused by formation of strong bonds. As a compromise, we achieved reasonable activity and stability when using 20% and 30% McIlvaine's buffer; though, from an economic point of view, the less concentrated buffer would be preferable.

c) Effect of pH on immobilization of laccase onto PAA nanofibers

The optimal pH for immobilization of laccase onto PAA nanofibers was evaluated using 20% McIlvaine's buffer at pH 3, 4, 5, 6, 7, and 8. The tests indicated pH 4 to be the most suitable, with the second highest enzyme activity level and reasonable stability over five ABTS oxidation catalytic cycles (Fig. 45c). This slightly acidic pH proved favorable for adsorption, probably because the isoelectric point of most fungal laccases is usually between 2.6 and 6.9, and most often around pH 4 [35],

[243], [244]. Moreover, PAA is well-known for its pH responsive behavior [245], [246]; with ionization of carboxylic groups occurring ($COOH \rightarrow COO^-$) at slightly acidic or neutral pH.

d) Effect of laccase solution volume on immobilization of laccase onto PAA nanofibers

PAA samples were immersed into different volumes of 1 mg/mL laccase solution (300 μL, 500 μL, 800 μL, and 1000 μL) and incubated for three hours in pH 4 McIlvaine's buffer (20%) at 4°C under constant shaking. A 24-well non-treated polystyrene microplate was previously identified as the optimal vessel for immobilization onto nanofiber samples (diameter 1.5 cm). While the best results were obtained using 1000 μL of enzyme solution (Fig. 45d), where initial activity was highest, operational stability was poor compared to lower volumes. In fact, all samples acheived approximately the same activity after five catalytic cycles. Thus, the lowest volume (300 μL) proved sufficient, this also being the least costly variant.

e) Effect of laccase concentration on immobilization of laccase onto PAA nanofibers

PAA nanofiber samples (diameter 1.5 cm) were immersed into 300 μL of different concentrations of laccase solution (0.5, 1, 1.5, 2, and 2.5 mg/mL) and shaken for three hours at 4°C and pH 4, as described above. Fig. 45e demonstrates the most common issue encountered when trying to achieve maximal initial activity versus maximal reuse of immobilized enzyme. High initial enzyme activity usually results from the preserved native conformation of enzyme molecules due to formation of weak non-specific (van der Waals, hydrophobic interactions, hydrogen bonds) or ionic bonding [247]. In the case of a highly concentrated laccase solution, enzyme molecules tend to agglomerate (i.e. form clusters) that are immobilized via the aforementioned weak bonding. Although this type of immobilization preserves the enzyme, it usually provides insufficient stability in enzyme-enzyme and enzyme-carrier complexes during pH or temperature changes and rapid agitation, causing cluster disintegration and significant leakage.

While the most concentrated enzyme solution (2.5 or 2 mg/mL) is recommended in order to achieve the highest possible activity, the almost 50% loss of activity that

results within five ABTS oxidation cycles indicates that a lower enzyme concentration would be more reasonable economically. According to the data obtained, therefore, the most suitable laccase concentration for adsorption onto PAA nanofibers is 1 mg/mL, which provides sufficient initial activity and only 30% activity loss after five catalytic cycles.

14. 1. Summary of the optimal immobilization process

Utilization of PAA for enzyme adsorption has previously been described in studies by Chen et al. (2005) [248], Ahmed et al. (2017) [249] and Levin et al. (2016) [250]. In each of these studies, however, PAA was used as a monolayer or surface brush coated onto different supporting materials, unlike our self-supporting, partially crosslinked nanofiber layer, which required no further surface modification. Overall, the data indicates an optimal immobilization process requiring PAA nanofiber samples (diameter 1.5 cm) to be submerged into **300 µL** of **1 mg/mL** laccase stock solution in **20% pH 4** McIlvaine's buffer and shaken at **4°C** in an orbital shaker at 150 rpm for **two hours** with no GA crosslinking.

14. 2. Storage stability and reuse

PAA-adsorbed laccase was incubated in deionized water at 4°C in order to assess storage stability. Two replicate samples were taken at selected time points (7, 15, 22, and 30 days) and their enzyme activity measured. Reuse of immobilized laccase was determined by measuring enzyme activity over five catalytic cycles using ABTS as a substrate, as described in previous chapters.

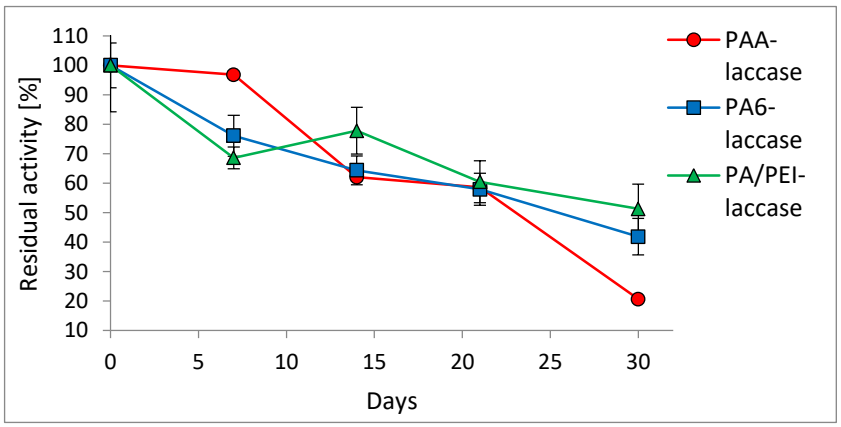


Fig. 46 Storage stability of PAA-adsorbed laccase (PAA-laccase) samples compared with PA/PEI-laccase and PA6-laccase

The PAA-adsorbed laccase samples only retained around 20 % of initial activity after 30 days storage (Fig. 46), which could constitute an obstacle for long-term use in real water treatment application. However, initial activity was 53% higher than that of PA/PEI-laccase samples. Reuse of laccase immobilized onto PAA nanofibers using the optimal immobilization process outlined above achieved more than 90% of initial activity after five rounds of ABTS oxidation (Fig. 45f). Though reuse of PA/PEI-laccase samples has reached 100%, this is still a very high level of stability compared to the literature [240], [241].

14. 3. Degradation of bisphenol A (BPA), 17α-ethinylestradiol (EE2), triclosan (TCS) and diclofenac (DCF)

Degradation efficiency of free laccase and PAA-adsorbed laccase was determined by decreasing the concentration of micropollutants over 24 hours. Samples of either immobilized laccase or free enzyme with a corresponding catalytic activity of 0.09 U were added to glass vials containing 5 mL of a mixture of BPA, EE2, TCS, and DCF (50 µM) in ultrapure water. Over selected time intervals (0, 2, 4, 6, 8, 10, 22, and 24 hours), 70 µL of the supernatant was collected into vials containing 65 µL of deionized water and 5 µL of 10% sodium azide, thereby preventing further EDC degradation if some of the enzyme had been collected with the supernatant [208]. Each experiment was performed in duplicate and the results presented as the mean value ± standard deviation.

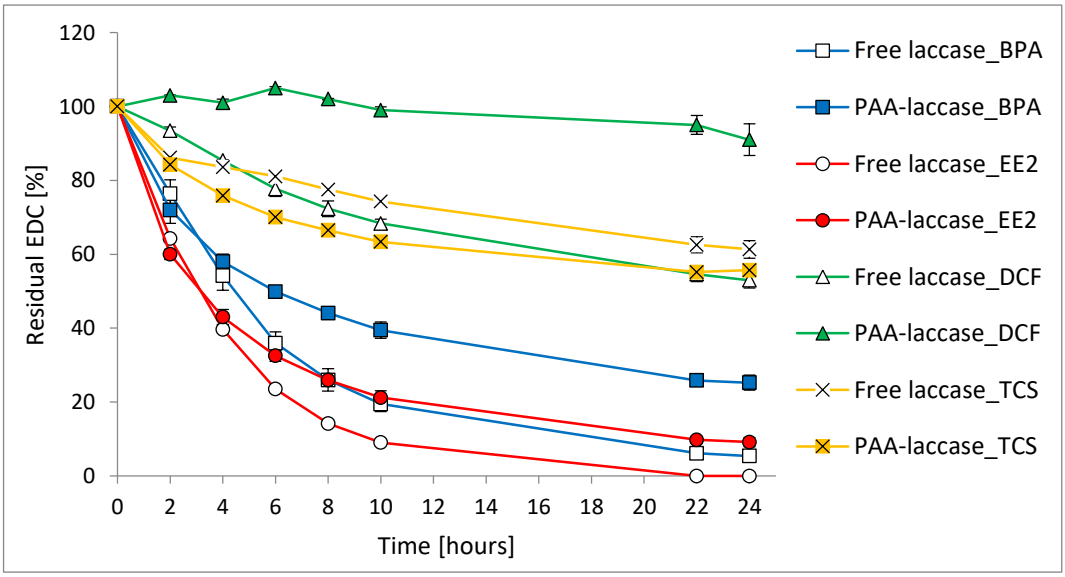


Fig. 47 Degradation efficiency of free and PAA-adsorbed laccase over the elimination of bisphenol A, 17α-ethinylestradiol, triclosan and diclofenac

PAA-adsorbed laccase removal efficiency of BPA, EE2, and DCF was lower than that of free laccase, with 24%, 9%, and 91% remaining after 24-hours incubation, respectively. On the other hand, the PAA-laccase removal profile was slightly better than that of free laccase with TCS (Fig. 47). While the results of the previous degradation experiment using PA6-laccase appear more successful in terms of BPA, EE2, and TCS degradation (22%, 2.5%, and 30% remaining EDC, respectively), the two experiments cannot be fully compared due to the presence of highly resistant DCF in measurements using PAA-adsorbed laccase.

15. *T. versicolor* laccase from and crude laccase immobilized onto poly(acrylic acid) nanofibers (PAA)

Commercial laccase from *Trametes versicolor* (TV) and crude laccase (laccase) were immobilized onto PAA and PAA/lam nanofiber supports via EDAC and S-NHS activation, followed by covalent attachment of the enzyme. EDAC and S-NHS concentration, activation time, and enzyme immobilization time, were examined using commercial laccase as the model enzyme, while optimal laccase and crude laccase concentration, volume of activation mixture and enzyme solution, nanofiber sample size, temperature, and agitation, were all based on previous experiments. Deionized water and pH 4 McIlvaine's buffer were identified as the optimal EDAC and S-NHS solvent for laccase immobilization, based on preliminary experiments and previously published data [251], [252].

Several nanofiber sheets (PAA/lam) were laminated to poly(ethylene terephatalate)/cushion vinyl (PET/CV; 80/20; surface density 35 g/m^2; with polyethylene dotcoating; Hoftex GmbH, Selbitz, Germany) nonwoven textile in order to enhance mechanical stability of the nanofiber layer. Lamination was undertaken using a discontinuous laminator preheated to 105°C and set to a pressure of 1 kN for 45 seconds.

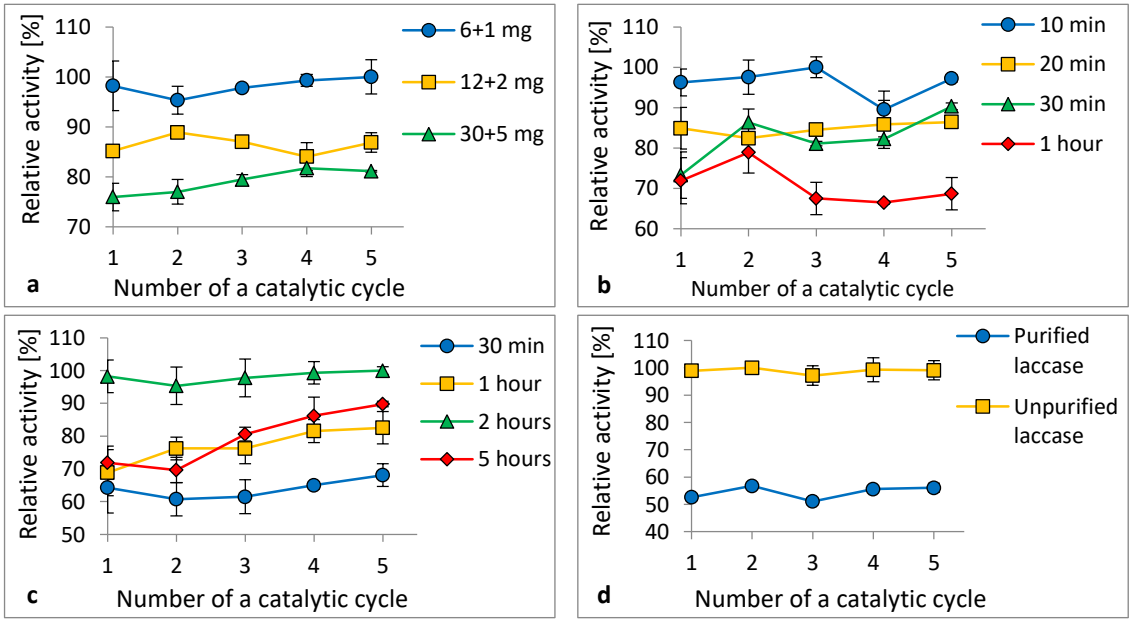


Fig. 48 Effect of (a) EDAC+S-NHS quantity, (b) activation time, (c) immobilization time, (d) type of enzyme on catalytic activity of laccase immobilized onto PAA nanofibers

a) Effect of EDAC and S-NHS concentration and activation time on immobilization of laccase onto PAA nanofibers

Three different concentrations of EDAC and S-NHS were tested, with the ratio of the two molecules preserved, i.e. 6/1 mg, 12/2 mg, and 30/5 mg of EDAC/S-NHS in 500 μL of the final solution volume. Fig. 48a identifies the least concentrated solution as the most suitable activation agent. As has previously been shown, EDAC is highly labile in the presence of water [252]; thus, the shortest activation time (10 minutes) will be the most suitable (Fig. 48b). A similar approach was reported in the papers of Tsai et al. (2019), Raghav and Srivastava (2016), and Li et al. (2007), where EDAC/NHS was used for activation of gold nanoparticles [253]–[255], while Zdarta et al. (2019) used EDAC/NHS to immobilize laccase onto PMMA/Fe_3O_4 nanofibers [205]. In principal, the strategies described vary in activation time, concentration of reagents used, and the order of usage, with the possibility to use EDAC and S-NHS (or NHS) in either one-step or two-step reactions.

b) Effect of immobilization time and type of laccase on immobilization of laccase onto PAA nanofibers

Of the four immobilization times tested, the optimal results regarding activity and stability of PAA-TV were obtained after two hours, though outstanding stability was also observed over five catalytic cycles of ABTS oxidation after 30 minutes of TV attachment (Fig. 48c). Regarding activity and reuse of PAA-TV and PAA-laccase, the TV and crude laccase stock solutions both showed similar catalytic activity (approximately 6.5 U/mL using ABTS as a substrate), though PAA-laccase achieved an almost two times higher activity rate than PAA-TV (Fig. 48d). This can be explained by the presence of multiple isozymes and other biomolecules originating from the cultivation medium, which became attached or adsorbed into the nanofiber structure together with the targeted laccase. As a result, these biomolecules provided an environment enhancing both activity and stability of the immobilized enzyme molecules [130], [256]–[258].

15. 1. Summary of the optimal immobilization process

First, **50 mg** of precipitated broth (corresponding to a catalytic activity of 2 mg of commercial TV laccase) was dissolved in 1 mL of deionized water. The supernatant was then diluted with 20% **pH 4.0** McIlvaine's buffer (1:1) in order to achieve an approximate activity of **1 mg/mL** of TV. At the same time, nanofiber samples (diameter 1.5 cm) were activated with a mixture of **6 mg EDAC** and **1 mg of S-NHS** in **500 µL of deionized water** for **10 minutes** at room temperature, after which the samples were thoroughly washed to remove unreacted coupling agents. Finally, **300 µL** of crude laccase solution was added to the nanofiber samples then shaken at **150 rpm** for **two hours** at **4°C**. The samples were then washed with the buffer until no laccase activity was detected in the washings, following which activity of the PAA-laccase was determined.

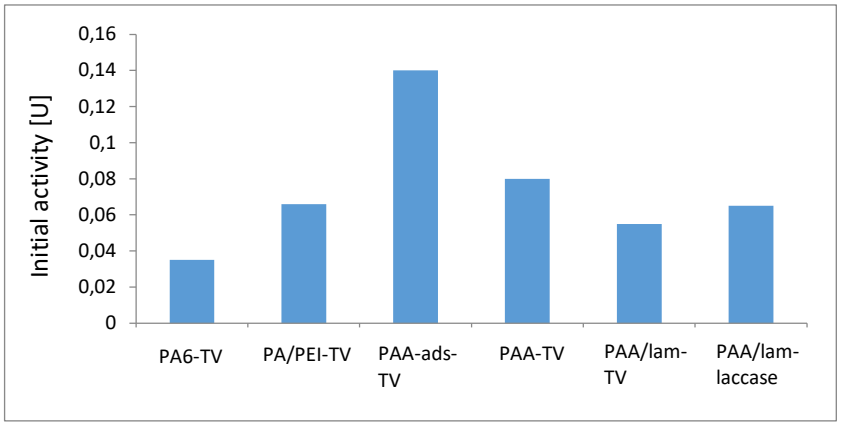


Fig. 49 Comparison of initial activity levels of commercial and crude laccase immobilized onto different types of nanofiber support

Fig. 49 provides a comparison of the initial activity levels of commercial laccase immobilized onto polyamide 6 (PA6-TV) and polyamide/polyethylenimine (PA/PEI-TV) nanofibers, poly(acrylic acid) nanofibers immobilized via adsorption (PAA-ads-TV) and covalent bonding (PAA-TV) with commercial laccase immobilized onto laminated PAA nanofibers (PAA/lam-TV), and crude laccase immobilized onto laminated PAA nanofibers (PAA/lam-laccase). The results indicate highest initial activity after adsorption onto PAA nanofibers, while the least effective method proved to be immobilization via adsorption and GA crosslinking onto PA6 nanofibers.

PAA nanofibers displayed exceptional wettability, with increased surface density and partial crosslinking of NFs with ethylene glycol guaranteeing both ease of handling, mechanical stability and sufficient reactivity of the remaining free carboxylic

groups. SEM images of pristine PAA nanofibers (Fig. 50a, c), laminated nanofibers (Fig. 50e), PAA-TV (Fig. 50b), PAA-laccase (Fig. 50d), and PAA/lam-laccase (Fig. 50f) show that the lamination process caused no significant damage to nanofiber structure. PAA/lam nanofibers appear to be straighter than those of pristine PAA, probably because the supporting nonwoven textile fixed the nanofiber structure and restricted natural flexibility of the polymer. More importantly, compared to the smooth surface of pristine nanofibers, PAA-TV, PAA-laccase, and PAA/lam-laccase nanofibers were rougher and grainier due to chemical modification and presence of the enzyme.

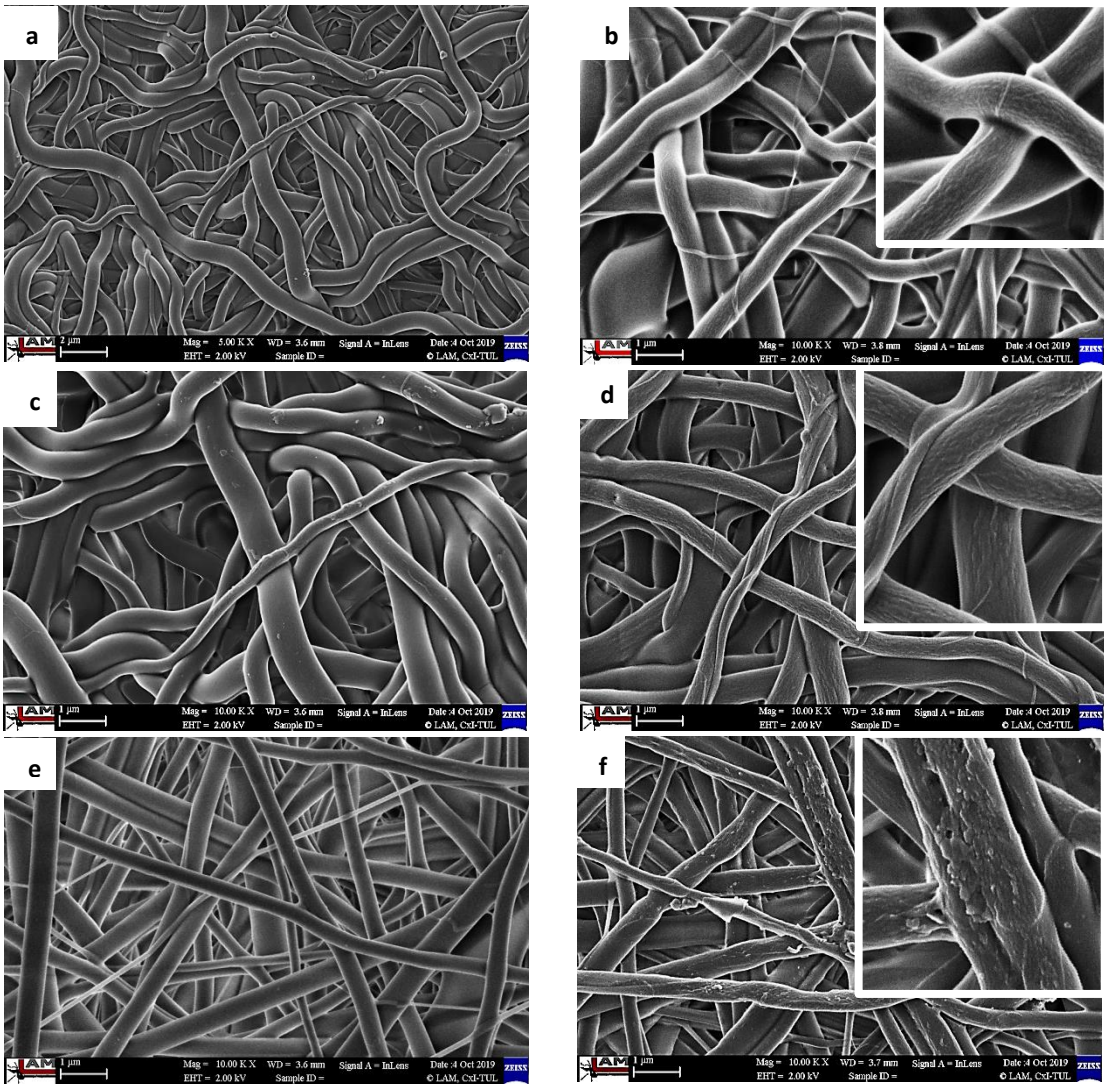


Fig. 50 Comparison of SEM images for pristine PAA nanofibers (a,c), laminated PAA nanofibers (e), PAA-TV (b), PAA-laccase (d) and PAA/lam-laccase (f). Magnitude 5 kx and 10 kx

15. 2. Storage stability and reuse

The storage stability of free laccase, PAA-TV, PAA-laccase, and PAA/lam-laccase samples was tested by assessing activity after 7, 14, 21, 30, and 35 days of storage at 4°C in wastewater infused with 2.5% (v/v) of undiluted McIlvaine's buffer at pH 7.0 (Fig. 51). As expected, free laccase showed lowest stability, with initial activity decreasing by more than 70% after 35 days of storage. PAA-TV showed better stability, retaining about 50% activity, while immobilized crude laccase (PAA-laccase) provided best stability, retaining almost 80% of initial activity. The storage stability of PAA/lam-laccase was lower compared to PAA-laccase as some of the enzyme was adsorbed onto the supporting non-woven textile during the immobilization procedure and, as such, was not strongly attached to the nanofiber matrix. Zdarta et al. (2019), for example, reported around 10% loss of initial activity for immobilized laccase and Xu et al. (2014) reported 40% loss after 30 days storage at 4°C. In contrast to these previous studies, our samples were stored in real wastewater effluent [218], [259].

All three PAA-immobilized samples displayed excellent reusability by retaining 100% of initial activity after five ABTS oxidation cycles, which was higher than previous experiments and similar to those from previous studies [125], [239], [241].

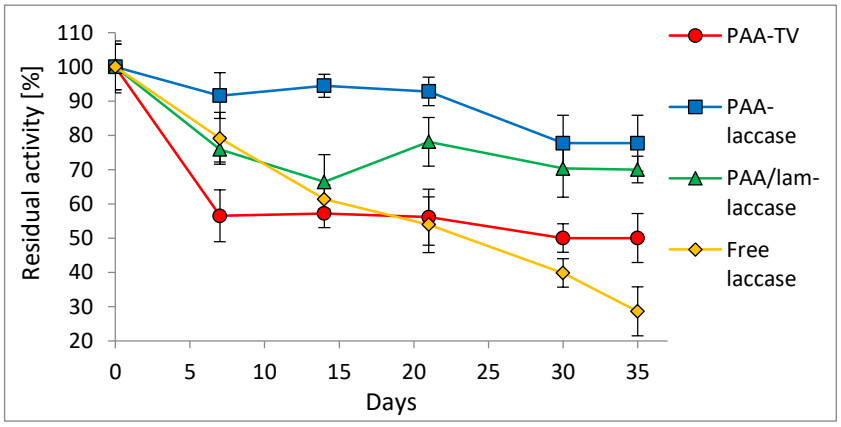


Fig. 51 Storage stability of PAA-TV, PAA-laccase, and PAA/lam-laccase samples compared to free TV laccase

15. 3. Degradation of bisphenol A (BPA), 17α-ethinylestradiol (EE2), triclosan (TCS), and diclofenac (DCF)

Samples of immobilized laccase were compared in order to assess their efficiency in degrading a mixture of four EDCs. Samples of PAA-TV, PAA-laccase, PAA/lam-laccase, and blank samples PAA and PAA/lam were added to glass vials containing 5 mL of a mixture of BPA, EE2, TCS, and DCF (10 mg/L) in either deionized water, wastewater, or wastewater containing 2.5 % (v/v) of undiluted McIlvaine's buffer at pH 7.0. Each experiment was performed in duplicate and the results presented as the mean value ± standard deviation. After 20 hours incubation under constant shaking (200 rpm) at room temperature, samples were removed and stored in fresh wastewater with buffer at 4°C. Furthermore, 10 μL of 10% sodium azide added to the vials with the residual EDCs to prevent further degradation in the case that some enzyme had been collected along with the supernatant. After 7 and 14 days, the samples were tested under the same conditions previous described.

In general, samples with immobilized crude laccase were more successful at degradation than PAA-TV due to their higher stability and presumed presence of multiple isozymes extending substrate specificity [260]. Highest efficiency was recorded towards elimination of BPA, EE2, and TCS (up to 94% removal), with DCF being the most durable micropollutant (Fig. 52). PAA/lam-laccase achieved highest and most even degradation efficiency (Fig. 52c), indicating that support stability and mechanical durability had a major impact on stability of the immobilized enzyme. Both PAA-TV (Fig. 52a) and PAA-laccase (Fig. 52b) samples exhibited higher standard deviations between duplicates, caused by potential damage of the nanofibers and enzyme leakage over the 20-hour agitation. The lamination step, therefore, was a necessary precaution preventing nanofiber and enzyme damage.

All samples were tested in deionized water, wastewater, and wastewater with 2.5% (v/v) buffer content. There was a general trend indicating that buffer-enriched wastewater was the most suitable environment for degradation of all four contaminants, with the presence of McIlvaine's buffer helping to improve final degradation efficiency (Fig. 52), though DCF elimination did not follow this trend exactly.

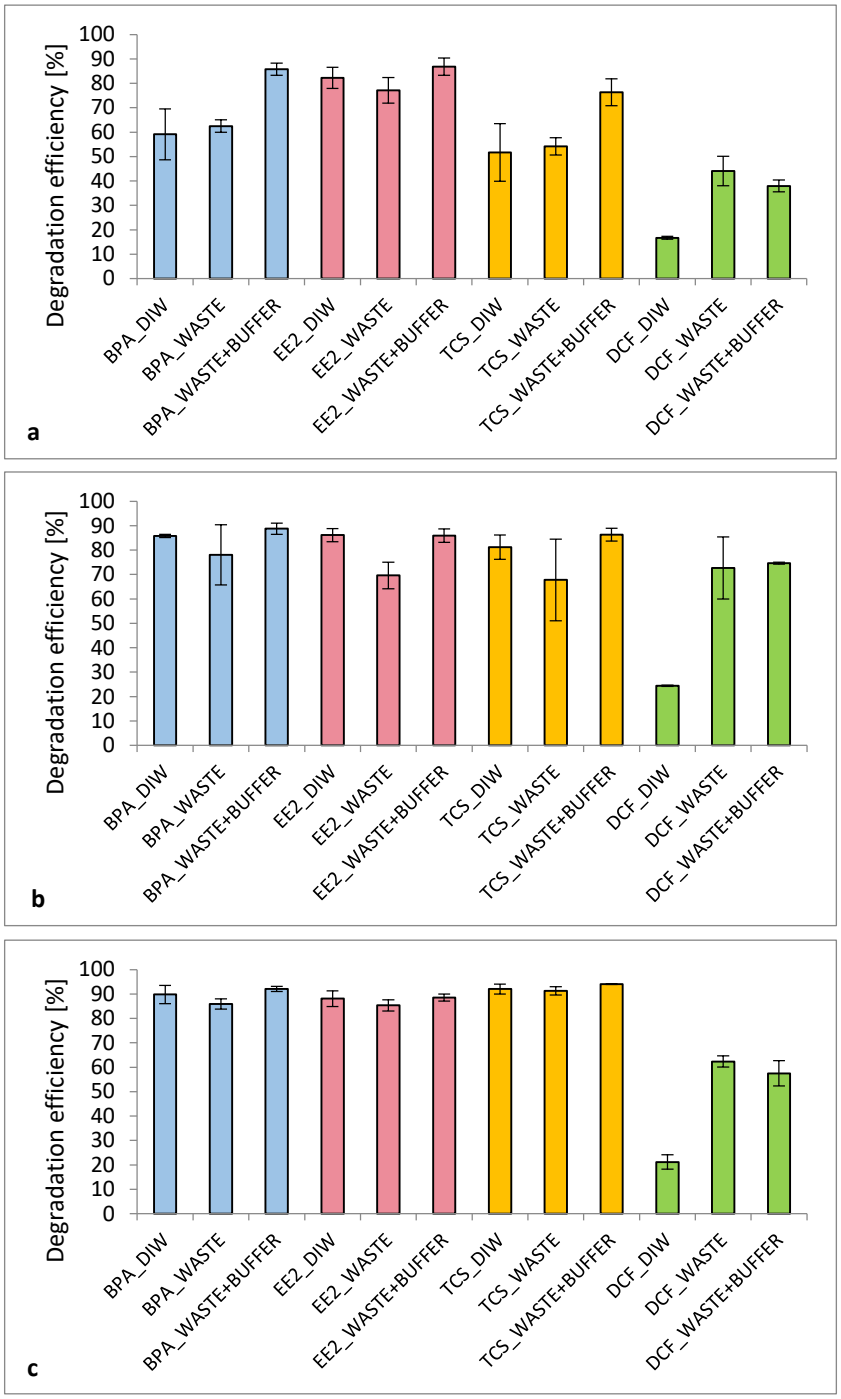


Fig. 52 Degradation of a mixture of BPA, EE2, TCS and DCF in deionized water (DIW), wastewater (WASTE) and wastewater with pH 7 buffer (WASTE+BUFFER) using PAA-TV (a), PAA-laccase (b), and PAA/lam-laccase (c)

Fig. 53 shows reuse of PAA-laccase (Fig. 53a) and PAA/lam-laccase (Fig. 53b) samplesin buffer-infused wastewater over three degradation cycles, with seven-day intervals between testing. Both samples gave comparable results, though laminated nanofibers were a mechanically more durable support for the immobilized laccase, slightly increasing degradation efficiency compared to unreinforced PAA-laccase. The samples remained highly active even during the third degradation cycle, at which point

efficiency had dropped by approximately 33% for BPA, 34% for EE2, 74% for TCS, and just 27% for DCF when using PAA/lam-laccase.

These tests provided novel results compared with previous studies due to testing sample reuse for degradation of a mixture of four EDCs and use of crude enzyme in real wastewater. The data obtained suggests that immobilization of laccase onto laminated PAA nanofibers (PAA/lam) has high potential for wastewater treatment application due to its enhanced micropollutant elimination activity and favorable reuse properties. Though elimination of BPA, EE2, TCS, and DCF using immobilized laccase has been widely described in the literature over recent decades [131], [205], [218], this study cannot be compared with these due to differing experimental conditions.

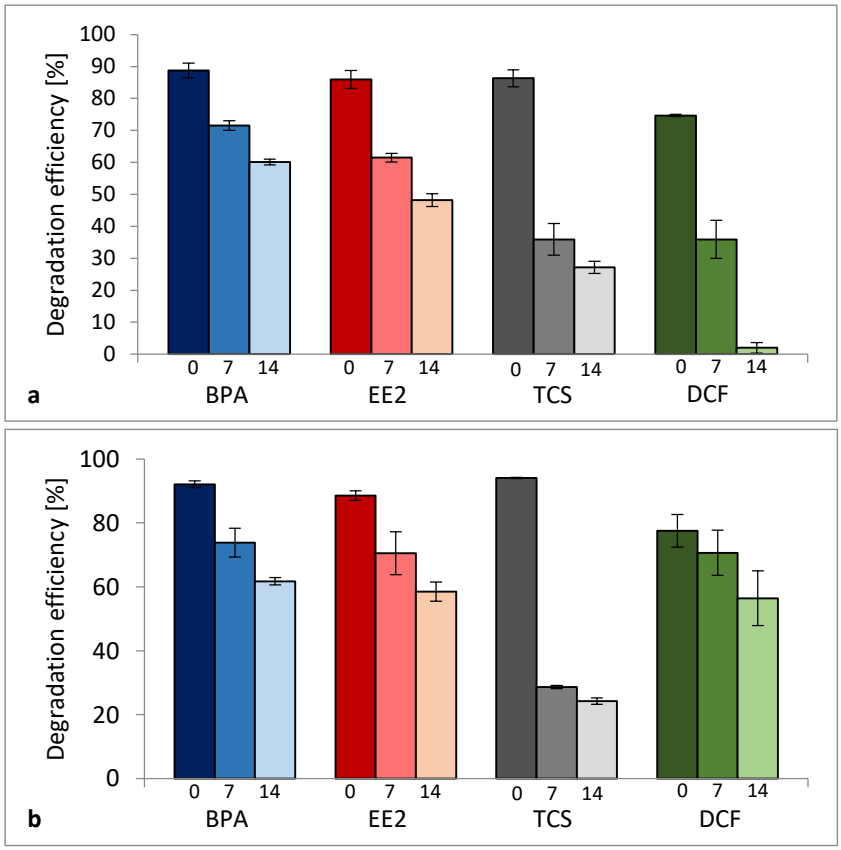


Fig. 53 Reuse in the degradation of a mixture of BPA, EE2, TCS, and DCF using PAA-laccase (a) and PAA/lam-laccase (b) samples after 7 and 14 days storage in wastewater effluent at 4°C

15. 4. Degradation of bisphenol A (BPA), 17α-ethinylestradiol (EE2), triclosan (TCS) and diclofenac (DCF) in decreased concentration and increased volume

PAA/lam-laccase samples (two and five discs of 1.5 cm diameter) were tested for degradation against a mixture of BPA, EE2, TCS, and DCF (100 µg/L) in 200 mL of wastewater infused with 2.5% (v/v) undiluted pH 7 McIlvaine's buffer content. After 20 hours incubation under constant shaking (80 rpm) at room temperature, the samples were removed and 100 µL of 10% sodium azide added to the reagent bottles to prevent further EDC degradation. The EDC solutions were then analyzed using SPE and HPLC.

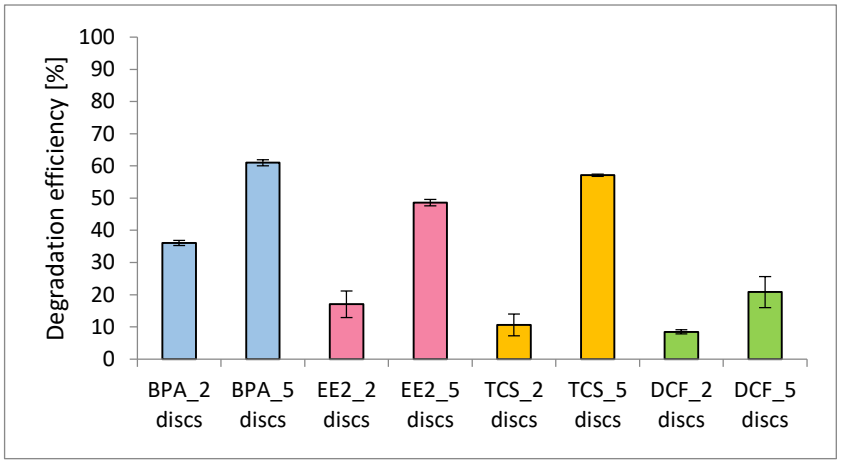


Fig. 54 Degradation of a mixture of BPA, EE2, TCS, and DCF (100 µg/L) in 200 mL of wastewater with 2.5% (v/v) pH 7 McIlvaine's buffer content using two and five PAA/lam-laccase discs

Though the EDCs mixture was 100-times less concentrated than in previous experiments (chapter 15. 3.), and the reaction volume was only 8-times higher for five discs and 20-times higher for two discs, we did not record as higher degradation efficiency as predicted. Maximum elimination was achieved using five discs with approximately 60% BPA, 48% EE2, 57% TCS, and 20% DCF degradation (Fig. 54). The removal rate using two discs was adequate in the case of BPA, EE2, and DCF; however, TCS removal was lower than expected.

The higher volume negatively influenced removal speed, and showed a more significant effect than EDC concentration. The probability of enzyme-substrate encounter was distinctly lower compared to the highly concentrated mixture. Furthermore, laccase catalysis usually involves formation of radicals followed by oxidation combined with non-enzymatic reactions such as hydration and polymerization, achieved via mediating molecules and nascent metabolites [36], [37].

As such, transformation speed grew with the concentration of radicals and reactive metabolites present. An absence of supportive nascent radicals could be replaced by adding a suitable co-substrate such as ABTS. This laccase/mediator system has previously been proposed in several studies [261]–[264].

16. Filtration systems based on laccase immobilized onto a nanofiber carrier

One of the great advantages of using nanofibrous materials is their structural variability. While 2-D mats are the most common form, single fibers, yarns, or nanofibrous layers covering different supporting materials are also produced [265], [266]. Out of the wide range of structures possible, laminated discs, membranes, and nano-yarns are discussed further in this thesis.

16. 1. Laminated nanofiber membranes

Nanofiber membranes are homogenous mats designed for flow filtration. The membranes require mechanical stabilization via lamination to a more robust supporting textile.

Advantages:

- All the nanofibers studied (PA6, PA/PEI, PAA) are capable of producing membranes.
- Flow filtration is a common mechanism used in wastewater treatment plants; hence, enzymatically activated membranes could be easily applied in current systems.

Disadvantages:

- In addition to an increased demand for mechanical stability and the possibility of enzyme damage from water flow, this system requires exceptional homogeneity and immobilized laccase of a sufficient surface density.

16. 2. Laminated nanofiber discs

Circular nanofiber discs with a diameter of 1.5 cm were used throughout the immobilization experiments. The circular shape of these discs provided increased mechanical durability than concurrently tested square filters in previous experiments, which showed a greater tendency to tear. Lamination onto a mechanically stable nonwoven textile would increase the service life of enzymatically activated membranes, especially in an aquatic environment.

Advantages:

- Nanofiber discs possess a shape suitable for all types of nanofibers studied (PA6, PA/PEI, PAA).
- Nanofiber discs with immobilized laccase, floating in a container of water, can spread easily throughout the tank, allowing micropollutant molecules to be attacked efficiently and homogeneously (Fig. 55).

Disadvantages:

- In order to ensure the discs circulate, the container must be equipped with an air pump or other type of stirring or whirling mechanism, which brings additional financial costs. However, presence of oxygen in the water may also positively influence catalytic activity of the immobilized laccase.

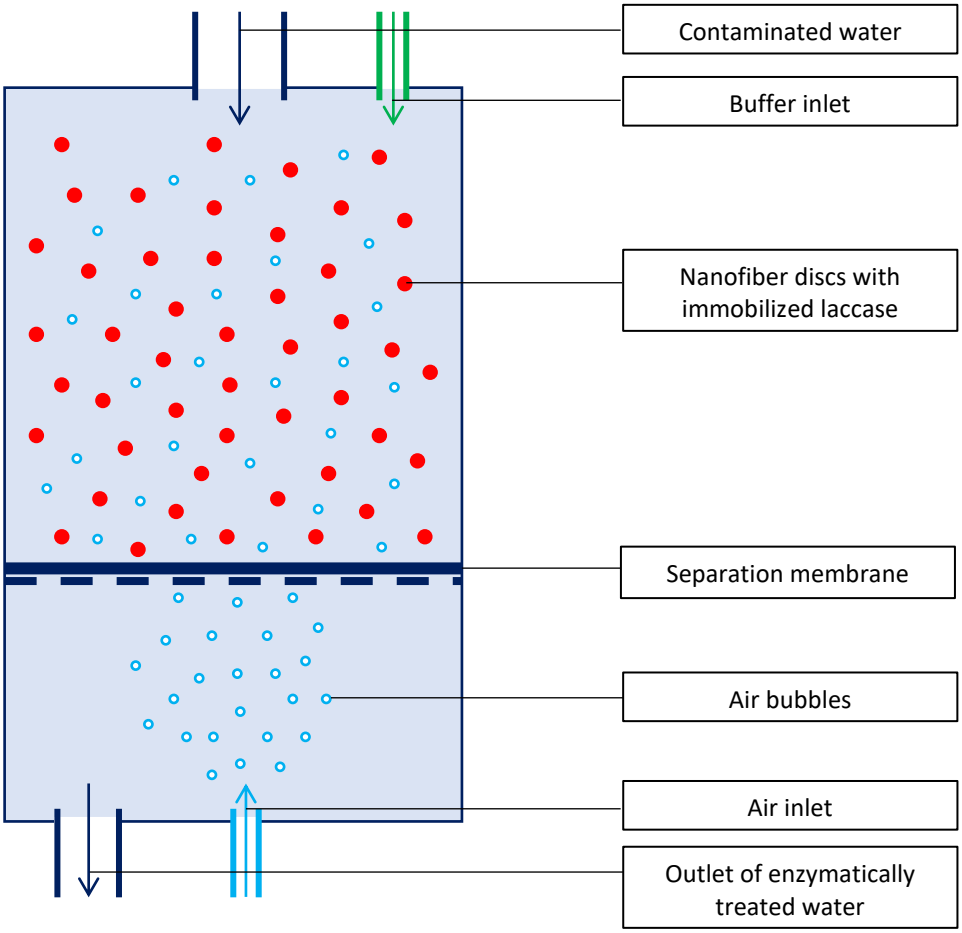


Fig. 55 Design for a reactor-based filtration system using laminated nanofiber discs with immobilized laccase

A potential filtration system allowing semi-continuous water treatment (see Fig. 55) might comprise a reactor with numerous laminated PAA nanofiber discs coated with immobilized crude laccase floating within the reaction chamber. These would be separated from the outlet section of the reactor by a membrane or screen, preventing the discs from passing out through the outlet tube. An air inlet produces bubbles that keep the nanofiber discs floating, provide oxygen for laccase catalysis, and help mix the wastewater and buffer. Wastewater and buffer inlet tubes are positioned at the top of the reaction chamber.

16. 3. Nanoyarns

Nanoyarns are 3-D nanofibrous structures prepared from either a stream of electrospun nanofibers twisted together or comprise a core made of a yarn or cable coated with a nanofibrous layer. While both systems require special collector adjustment, the core-shell method provides higher nano-yarn mechanical stability due to the strong core [267], [268].

Advantages:

- Nanoyarns with immobilized laccase can be distributed throughout a tank of water, allowing micropollutant molecules to be attacked efficiently and homogeneously (Fig. 57).
- Nanoyarns coiled onto a bobbin can be used in present-day coil-type flow filtration systems.

Disadvantages:

- Nanoyarns can only be prepared from a limited range of polymers. PAA is unsuitable as the nanofiber layer becomes water insoluble following heat stabilization, resulting in significant technical challenges as regards core/shell systems and prospective winding onto a bobbin.
- Immobilization of laccase onto a coated yarn coiled onto a bobbin would require a specially designed vessel for enzyme immobilization, utilizing the optimal volume of reaction mixture and sufficient agitation (Fig. 56).
- Immobilization yield (amount of laccase immobilized) would be negatively affected by the number of ‘circuits’ around the bobbin, each ‘circuit’ decreasing enzyme molecule permeability within the whole nanoyarn structure.

- The winding process that follows the immobilization process could cause serious mechanical damage to the immobilized enzyme.

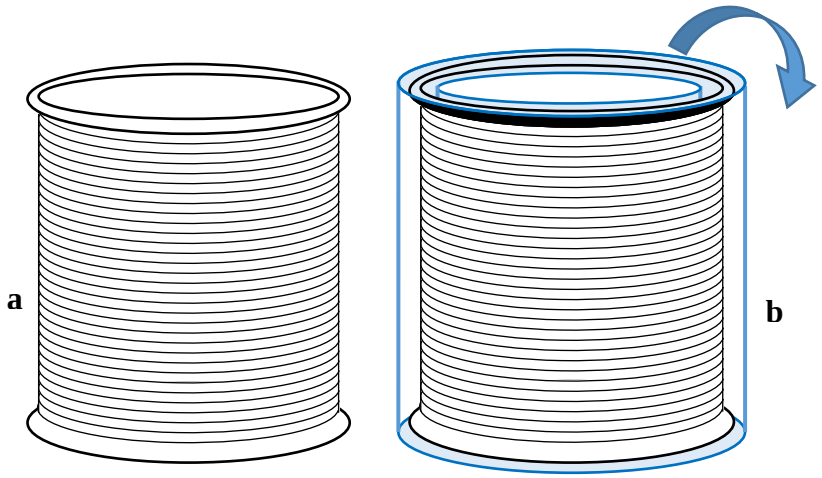


Fig. 56 Nanoyarn on a bobbin (a) and a special rotating vessel for enzyme immobilization onto coiled nanoyarn (b)

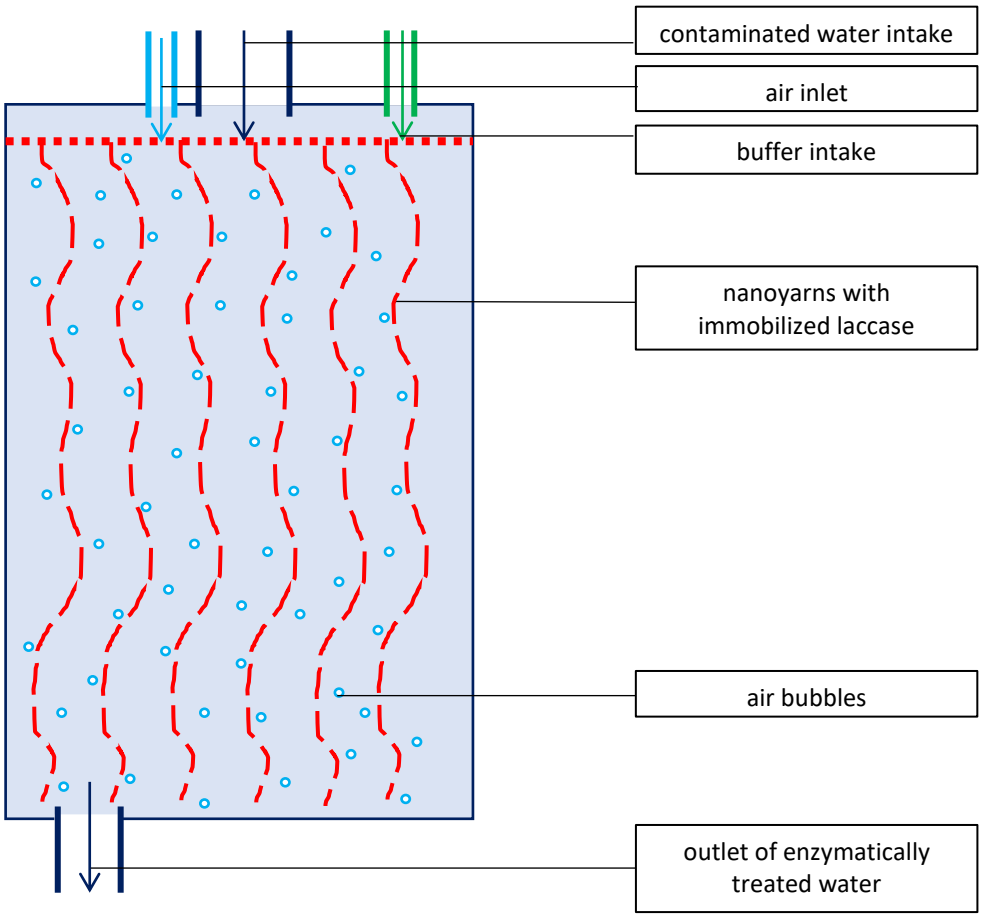


Fig. 57 Design of a reactor-based filtration system using nanoyarns with immobilized laccase

A reactor-based filtration system using uncoiled nanoyarns is illustrated in Fig. 57. Unlike the previous design using laminated nanofiber discs, the nanoyarns are fixed to the upper part of the reactor, along with an air inlet that produces bubbles, which ensure sufficient whirling while preventing the yarns from tangling together. The main difference consists in the construction of the enzyme carrier. First, nanoyarns with a suitable nanofibrous layer (e.g. PA6, PA/PEI) are cut into an equal number of long sections. These are then attached to a textile or screen that also acts as a pre-filter. This unit (nanoyarns attached to a pre-filter) must enable successful enzyme immobilization and subsequent placement into a reactor without significant damage to the immobilized enzyme. This design is based on future tests for immobilization of laccase onto core-shell nanoyarns, though the stability of the unit under semi-continuous water filtration has yet to be tested.

Conclusion

There is now an urgent need for efficient solutions addressing the increasing presence of emerging micropollutants in open waters. Most of these micropollutants enter waterbodies as byproducts of personal care products, household or hospital pharmaceuticals, and agricultural pesticides. The amount of hazardous or potentially harmful chemicals that people use and dispose of every day is surprisingly high; hence, it is highly unlikely that their use could be restricted, replaced, or stopped in the short-term. As such, novel mitigation and cleansing technologies are urgently required. The solution presented in this thesis was specifically developed for effectively eliminating such contaminants from water.

The majority of emerging micropollutants are organic compounds with a wide range of structures and properties. Some resemble naturally occurring biomolecules and, as such, their chemical structures can mask the threats they pose. On the other hand, this resemblance to naturally occurring biomolecules could be successfully used to promote their degradation during water treatment, using biological or biochemical systems with the ability to distinguish such compounds and catalyze their destruction or transformation. A number of ligninolitic fungi produce extracellular digestive enzymes that catalyze oxidation of lignin. These 'oxidoreductases' have the potential to catalyze a much wider variety of organic molecules, making them a potential candidate for water treatment.

In this study, the catalytic activity of two commercially available oxidoreductases (laccase from *T. versicolor* and horseradish peroxidase) were compared in order to evaluate their potential use for wastewater treatment, particularly as regards removal of widely occurring endocrine disrupting chemicals (EDCs). Enzyme activity was measured under a wide range of conditions using three substrates (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt [ABTS], guaiacol [GUA] and syringaldazine [SYR]) in McIlvaine's buffer, deionized water, and real water samples (tap water, several types of natural water, and wastewater effluent).

Though pH optima and catalytic activity measurements, using ABTS, GUA, and SYR as substrates, implied that real wastewater effluent was unfavorable for enzyme application, degradation efficiency of a highly concentrated mixture of bisphenol A

(BPA), 17α-thinylestradiol (EE2), triclosan (TCS) and diclofenac (DCF) proved satisfactory after addition of McIlvaine's buffer. These findings may be considered as a guideline for utilizing these two enzymes in real wastewater treatment conditions.

Both enzymes were also tested for degradation of a mixture of 19 chlorophenols in deionized water, the results demonstrating their low substrate specificity and economic potential, thereby extending their potential for wastewater treatment.

Laccase and peroxidase proved both robust and universal in application. Although peroxidase was more efficient in eliminating all contaminants, especially the mixture of chlorophenols, laccase proved to be a more favorable choice as, unlike peroxidase, it did not require hydrogen peroxide as a co-substrate. In conclusion, laccase appeared to be the most suitable enzyme for wastewater treatment.

Based on this knowledge, further research focused on finding an optimal laccase immobilization method using specially designed nanofiber carriers. The criteria for successful immobilization included sufficient stability over time and in real water environments, low cost, safety when handling, and zero environmental risk. Based on these requirements, polyamide 6 (PA6), polyamide 6/polyethylenimine (PA/PEI), and poly(acrylic acid) (PAA) were chosen as suitable nanofiber carries.

While PA6 represented a robust material with low cost and remarkable mechanical stability, it had the disadvantages of hydrophobicity and a lack of suitable chemical groups for modification or covalent immobilization. In comparison, PA/PEI possessed multiple primary amino groups providing both nanofiber hydrophilicity and opportunities for a variety of chemical modifications. On the other hand, this material was more costly than PA6 due to the addition of PEI. Partially crosslinked PAA was both stable in water and hydrophilic, with high sorption capacity and a sufficient number of available carboxylic groups, allowing immobilization via adsorption or covalent bonding.

Commercial laccase *T. versicolor* was immobilized onto PA6 nanofibers via adsorption and glutaraldehyde crosslinking. While this rapid and low-cost method ensured reasonable stability, activity of the attached enzyme was lowest compared to methods using PA/PEI or PAA. As such, PA6 nanofibers were considered the least convenient carrier of the three tested.

PA/PEI nanofibers were designed for a more targeted and considerate form of immobilization, based on oxidation of the glycoside elements of laccase followed by formation of Schiff's base with the primary amine groups of PEI. As a result, immobilized laccase achieved 47% higher initial activity, 12% higher reuse and 9% higher storage stability in deionized water compared to laccase immobilized onto PA6.

A further improvement in initial activity was achieved using PAA as the support for adsorption of immobilized laccase. This method was the most considerate in terms of preserving laccase's native structure, as manifested by the highest initial activity (53% higher than PA/PEI-laccase). However, adsorption failed to provide long-term strong attachment of the enzyme, resulting in unsatisfactory reuse and storage stability.

The implementation of covalent bonding of laccase was tested as a means of improving the stability of immobilized laccase when using PAA as a carrier. In this case, selected conjugation using carbodiimides resulted in the creation of an amide bond between PAA carboxylic groups and laccase primary amine groups. Note that attachment using a zero-length crosslinker is usually extremely inconsiderate as regards the preservation of native enzymatic structure. Once the optimal activation and immobilization process parameters had been identified, the final method resulted in an approximately 17% increase in initial activity compared with PA/PEI-laccase; highest reuse (100% after five catalytic cycles) and storage stability in real wastewater effluent (70% activity preservation after 35 days storage); and showed exceptional degradation efficiency against a highly concentrated mixture of BPA, EE2, TCS, and DCF in real wastewater effluent.

The cost-effectiveness of the final filtration system presented here is crucial for its future application in water treatment technology. The most expensive item in filtration systems based on PAA-laccase samples is the high-quality commercial laccase. As such, the most logical step for increasing the cost-effectiveness of our system is to find a less costly source of laccase and, at the same time, to increase the durability of the supporting nanofibers.

The simplest method for increasing PAA nanofiber sheet durability is to laminate them with a mechanically stable nonwoven textile. This process makes nanofiber manipulation easier and increases their durability and long-term stability in aquatic environments, while having no significant negative effect on immobilization capacity.

The initial experiments of this thesis were based on the use of unpurified (crude) laccase and took place during an internship at the University in Maribor (Slovenia) when cultivating *Pleurotus ostreatus* fungi in order to achieve a laccase-rich broth. After the internship, we established a cooperative agreement with Mendel University in Brno (Czech Republic), where we focused on isolation of crude laccase from *T. versicolor*. Subsequently, we successfully immobilized this "low-cost" crude laccase onto specially prepared laminated PAA nanofibers. The resulting nanofiber sheets with immobilized laccase provided sufficient catalytic activity while also increasing laccase stability, the laccase remaining effective after 14-days storage in wastewater effluetn at 4°C. Furthermore, degradation efficiency was also increased, with exceptional 80–95% elimination of a highly concentrated mixture of BPA, EE2, TCS, and DCF (10 mg/L) in wastewater after 20 hours incubation.

In order to better address conditions similar to those found during real wastewater treatment, the concentration of micropollutants was reduced to 0.1 mg/L and the volume of the reaction mixture increased 40-times per one PAA-crude laccase sample. Against our expectations, however, maximum elimination was only 20–60%. This was primarily due to the increased liquid volume and decreased concentration of micropollutants, which lowered the probability of enzyme-substrate encounters and formation of reactive by-products that usually participate in the degradation process. These unique findings may prove crucial for future utilization of immobilized laccase in real water treatment processes.

In this study, several filtration models were designed in order to fully exploit the enzymatically activated nanofiber structures developed. Nanofiber carriers can be formed into a range of structures, including laminated discs, laminated membranes or core-shell nanoyarns, and each of these variants are discussed in this study, along with their advantages and disadvantages for use in semi-continuous filtration systems. The most suitable technological solution in this case proved to be a reactor containing laminated PAA discs with immobilized crude laccase. The discs are homogenously distributed within the reaction chamber via air bubbles fed into the reactor, while the wastewater is doped with McIlvaine's buffer to improve enzymatic catalysis.

While we were unable to fully test this design due to the limited amount of crude laccase available for larger experiments and analytical limitations (e.g. we were unable to achieve detection limits under realistic micropollutant concentrations [1 ng/L–

1 μg/L]), we believe that this unique system offers an effective and economically feasible solution for the elimination of multiple persistent micropollutants.

Conventional wastewater treatment methods have proved insufficient for complete reduction of some pollutants, and especially EDCs. The current strategy of generalized wastewater treatment is mainly based on two alternative processes, i.e. ozonation and treatment with powdered activated carbon. Ozone production requires high energy input, represents a potential fire hazard, has known toxic associated with ozone generation, and forms potentially harmful by-products, while activated carbon needs to be separated from the waste and sent for destruction or re-activation through incineration.

Alternative technologies include nanofiltration, reverse osmosis, and biological or enzymatic treatment. This dissertation thesis shows that nanofibers represent a promising material for wastewater treatment as they are safe and easy-to-prepare. Their major advantage over particle- or nanoparticle-matrices is their ability to be handled as textiles while still possessing sufficient immobilization capacity for strong attachment of laccase, which gives them great potential for use as enzymatically activated filters in water treatment technologies.

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