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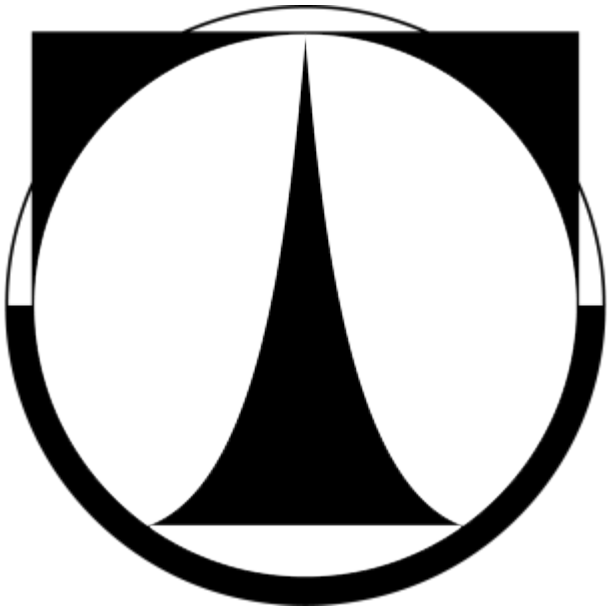

Self-report

Enzymatically activated filters for water treatment

Liberec 2020 Ing. Milena Maryšková

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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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.

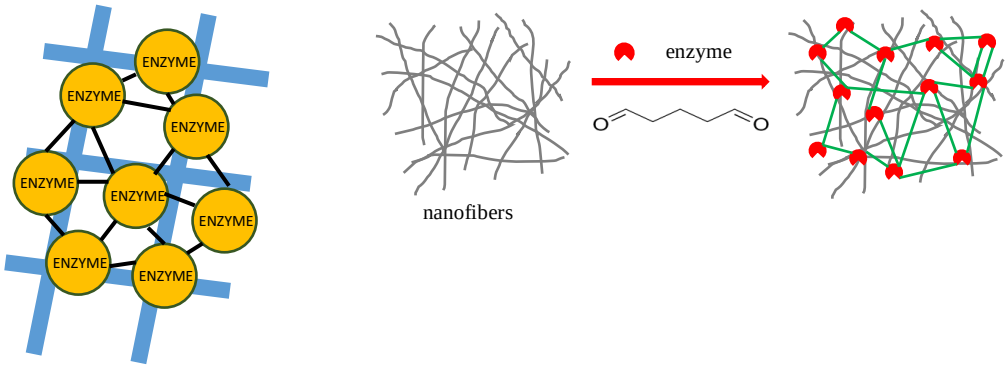
Conventional wastewater treatment methods are insufficient for complete reduction of some pollutants, and especially endocrine disrupting chemicals (EDCs). Wastewater treatment plants are only capable of removing or transforming a limited amount of such compounds, either through sorption onto activated 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.

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.

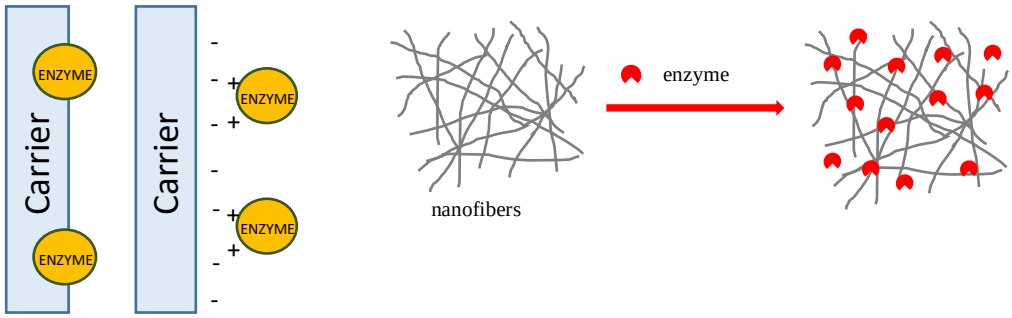
+ suitable for most types of nanofibers

+ 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

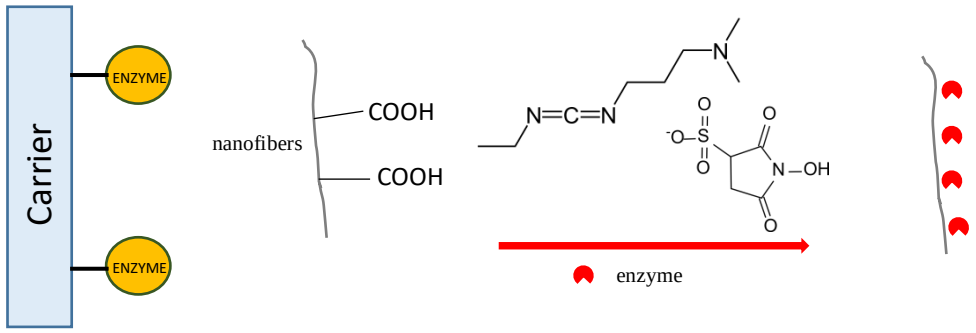
+ low cost

+ one-step method (short time immobilization)

+ low enzyme damage due to the absence of strong bonding

- enzyme leakage [174]–[176]

c) 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

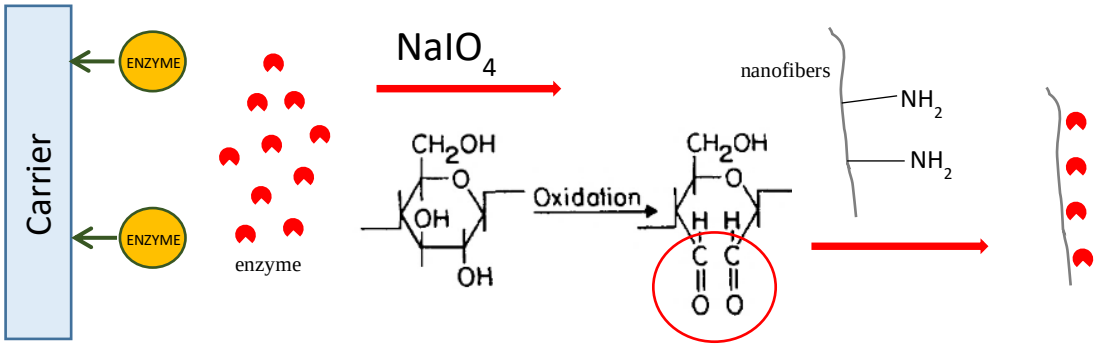
- suitable for nanofibers with free primary carboxylic groups (e.g. PAA)

- 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]

d) 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]

2. 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.

2. 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 1a). 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. 1b). 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.

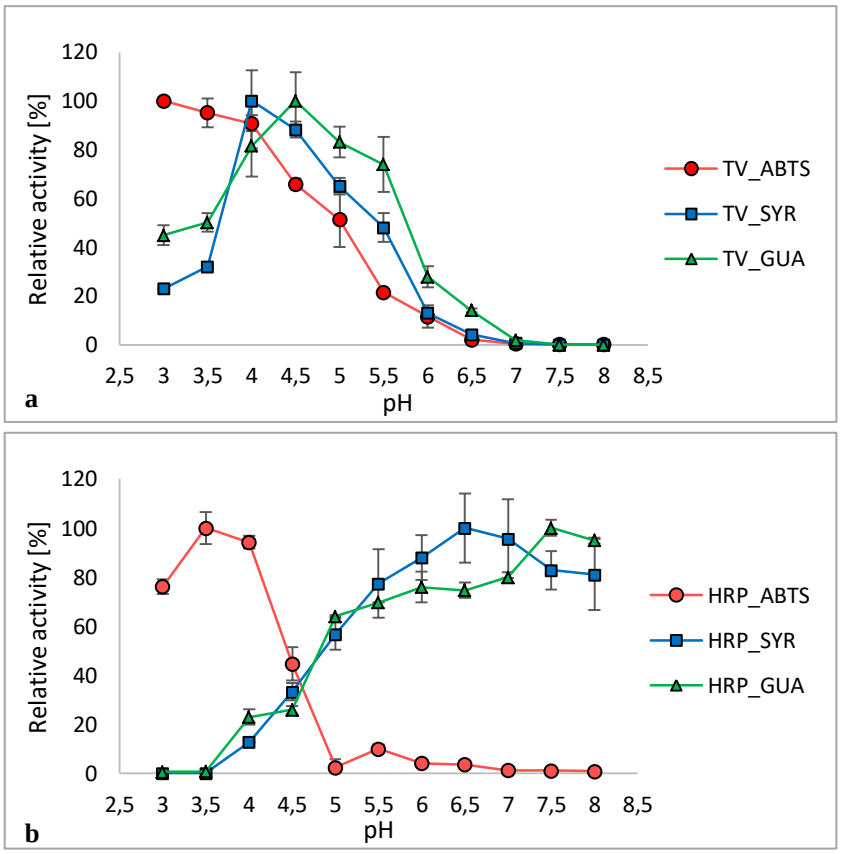


Fig. 1 Activity of (a) laccase from Trametes versicolor (TV) and (b) horseradish peroxidase (HRP) at different pH

2. 2. 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 1) 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 1 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 2 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 2). 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.

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 (McIlvaine's with pH 3) content in the case of laccase (Fig. 2a) and 2.5–5% (v/v) content in the case of peroxidase (Fig. 2b). 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. 1. 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].

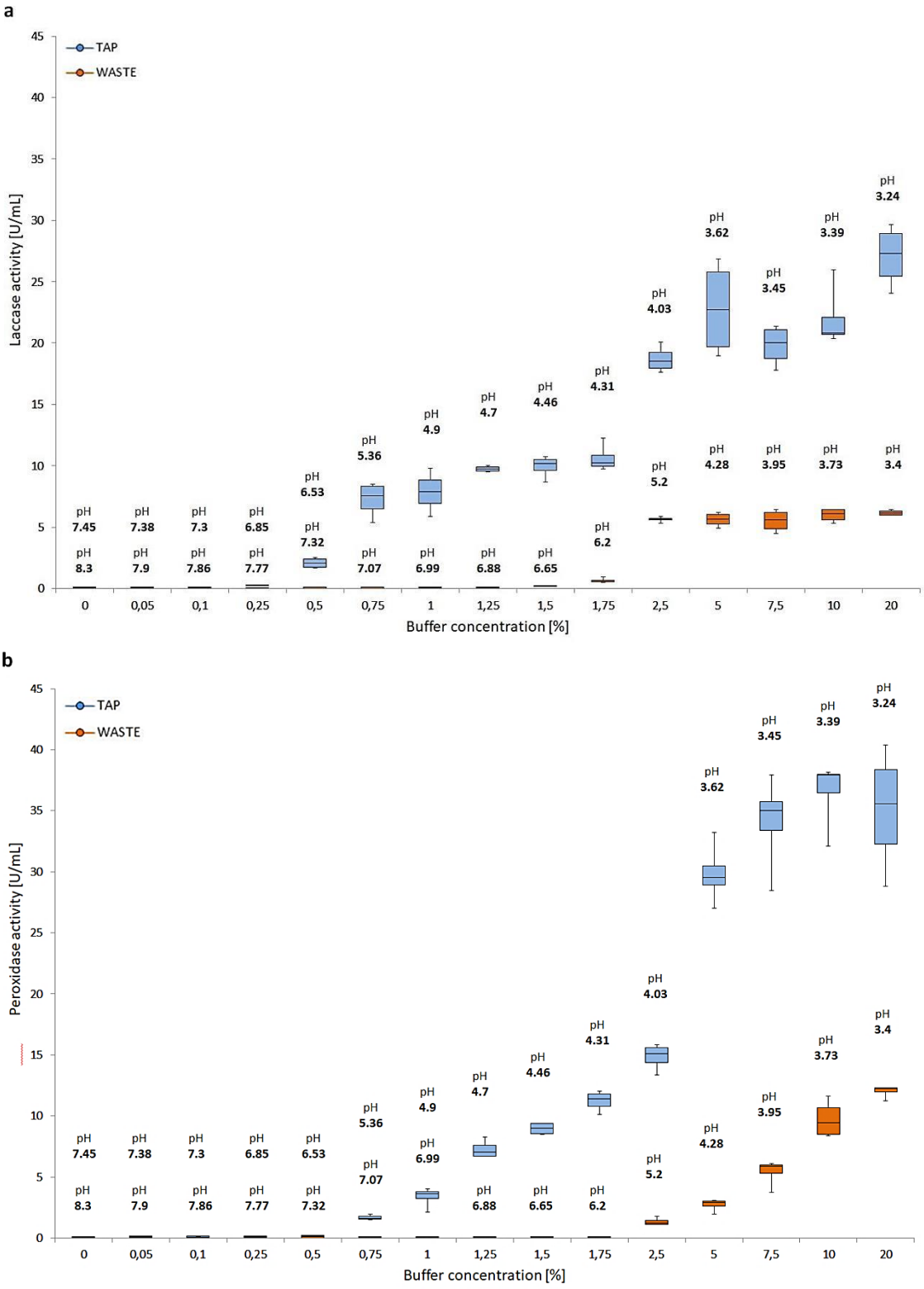


Fig. 2 Catalytic activity of laccase (a) and peroxidase (b) in tap water and wastewater infused with McIlvaine's buffer with pH 3

2. 3. 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. 3). 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. 3d), while peroxidase retained 53% of its initial activity at pH 7 for 30 days (Fig. 3e), 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. 1a), practical use of laccase at this pH is highly restricted due to the poor storage stability.

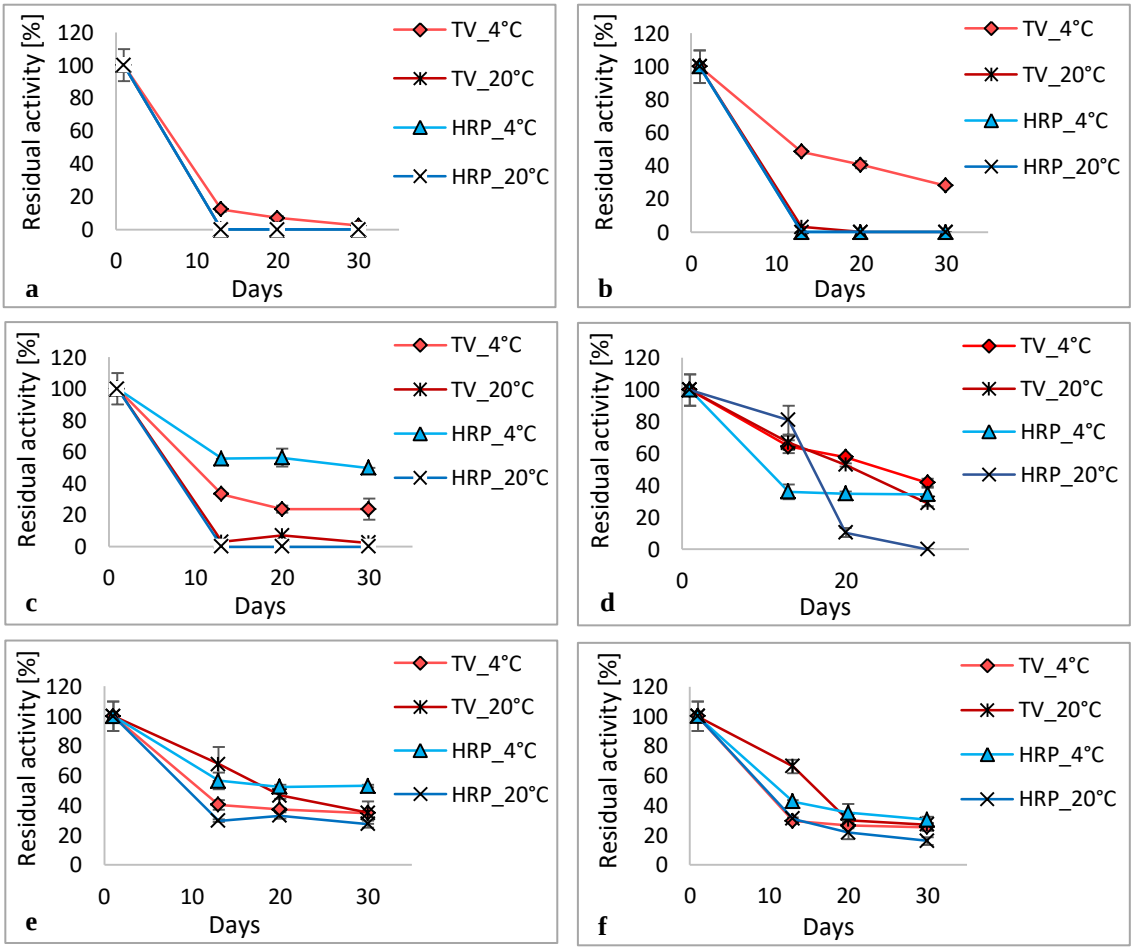


Fig. 3 Storage stability of laccase and peroxidase in McIlvaine's buffer at pH 3 (a), 4 (b), 5 (c), 6 (d), 7 (e), and 8 (f)

2. 4. 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.

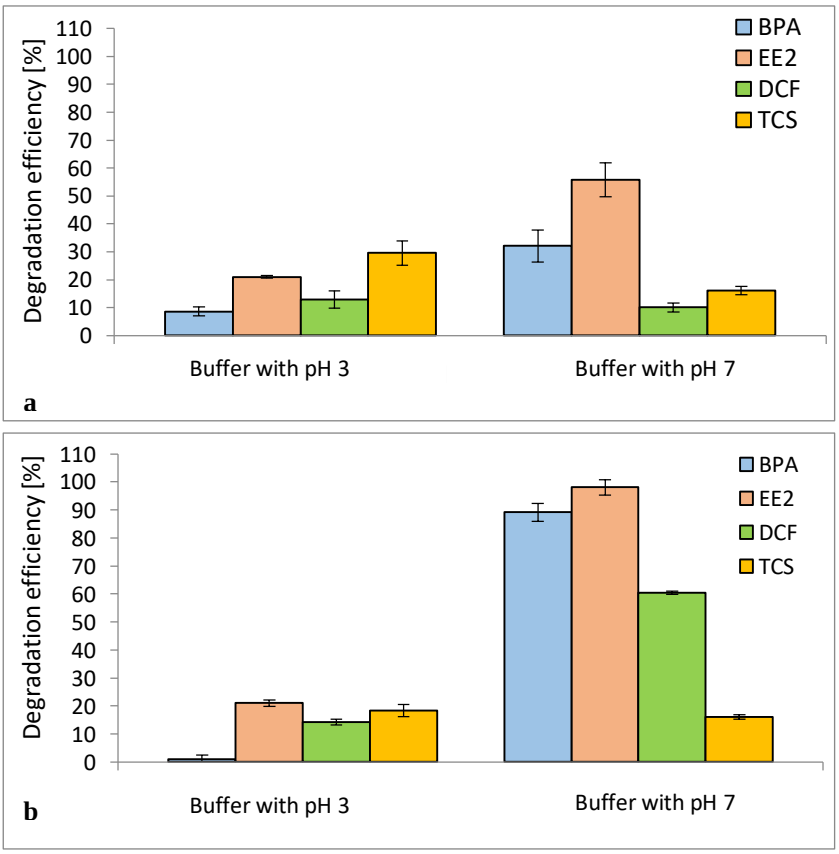


Fig. 4 Elimination of a mixture of BPA, EE2, TCS and DCF in pure McIlvaine's buffer with pH 3 and 7 using laccase (a) and peroxidase (b)

In general, peroxidase was more efficient in degradation of all four contaminants. Based on the previous results from ABTS oxidation (Fig. 1a), 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. 4a). 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. 4b).

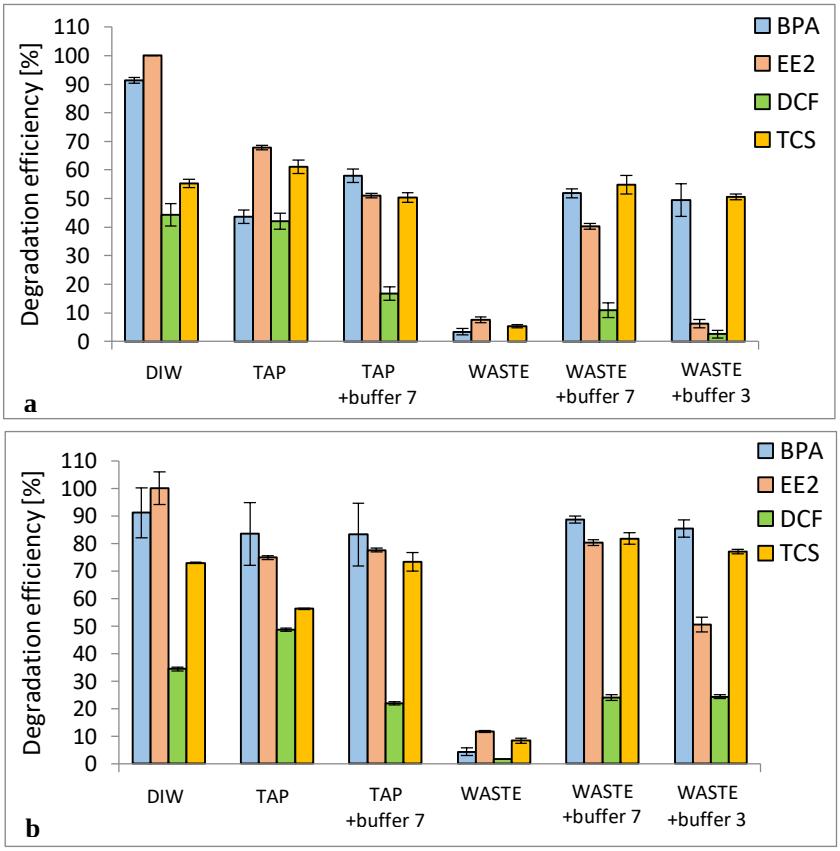


Fig. 5 Elimination of BPA, EE2, TCS and DCF in different water samples using laccase (a) and peroxidase (b)

When testing degradation efficiency in DIW and real waters (Fig. 5), 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. 5a), although our previous results using ABTS, GUA and SYR at neutral pH buffer showed almost zero catalytic activity (Fig. 1a). 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. 5b).

3. 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 (Fig. 6). 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.

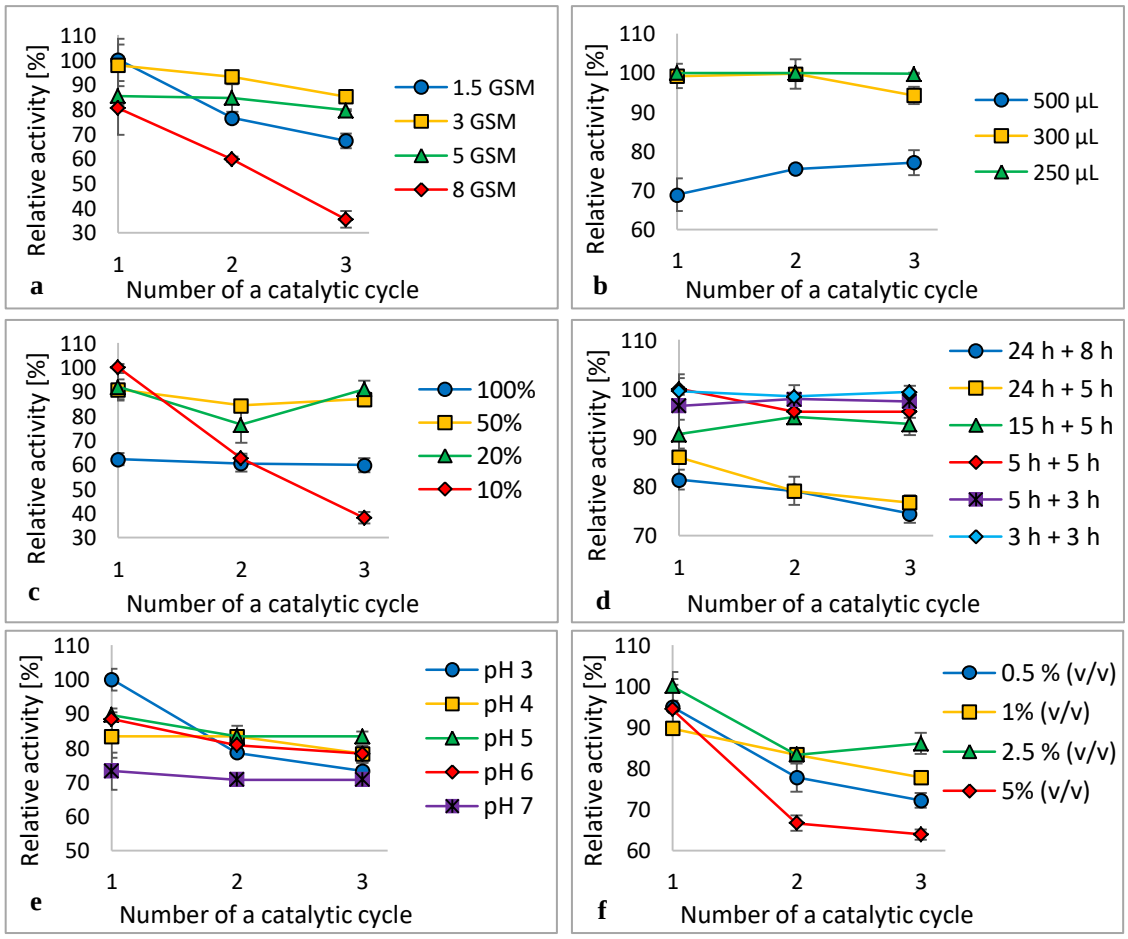


Fig. 6 Effect of (a) nanofibers' surface density, (b) solution volume, (c) McIlvaine's buffer concentration, (d) adsorption and crosslinking time, (e) pH, and (f) glutaraldehyde concentration on catalytic activity of PA6-laccase

3. 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 **20%** buffer at **pH 5**, 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 McIlvaine's buffer at pH 3. Pristine PA6 nanofibers display an homogenous structure and smooth surface (Fig. 7a), while PA6-laccase displays a grainy surface formed by crosslinked laccase clusters strongly attached to the nanofibers, even after thorough washing (Fig. 7b).

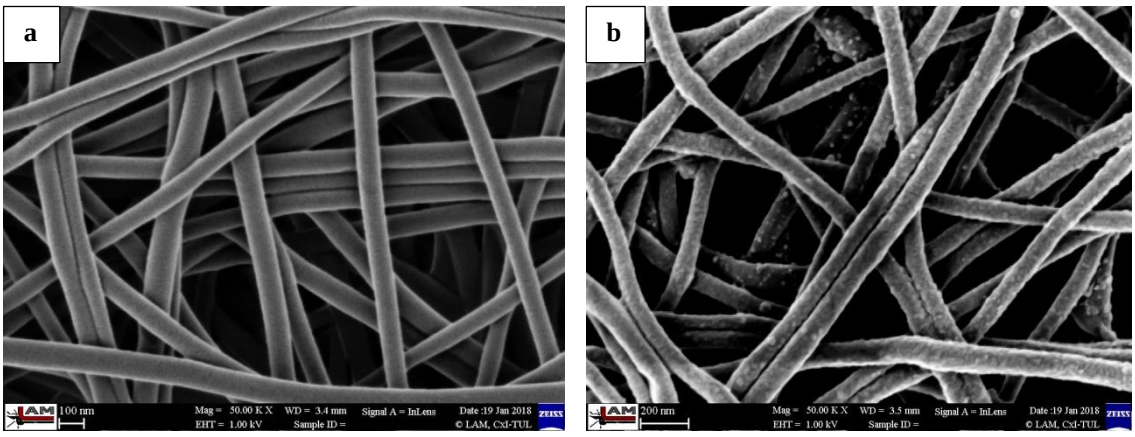


Fig. 7 Comparison of SEM images of (a) pristine polyamide 6 nanofibers, and (b) nanofibers with immobilized laccase. Magnitude 50 kx.

3. 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.

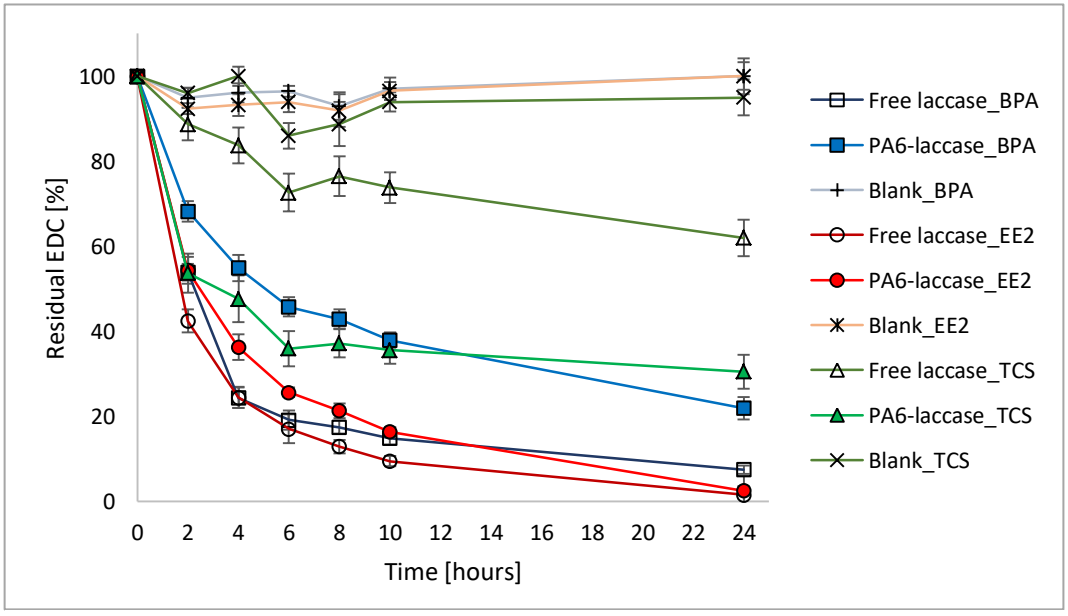


Fig. 8 Degradation efficiency of free and immobilized laccase (PA6-laccase) over the elimination of bisphenol A (BPA), 17α-ethinyl estradiol (EE2), and triclosan (TCS)

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. 8).

4. 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 (Fig. 9). 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 McIlvaine's buffer at pH 3 until no laccase activity was detected in the washings, following which the activity of the immobilized laccase was determined.

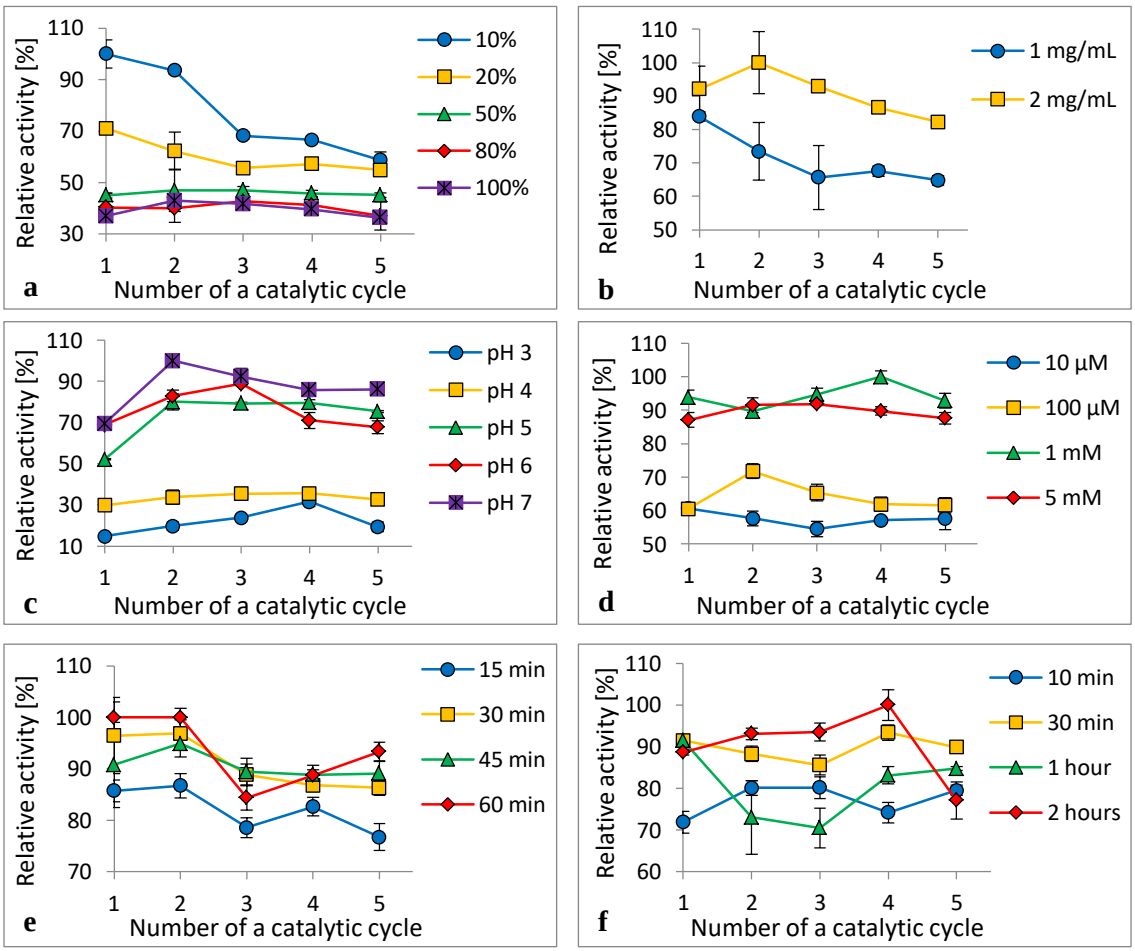


Fig. 9 Effect of (a) ˇMcIlvaine's buffer concentration, (b) laccase concentration, (c) pH, (d) concentration of $NaIO_4$, (e) oxidation time, and (f) immobilization time on catalytic activity of PA/PEI-laccase

4. 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 20% McIlvaine's buffer **pH 7** to produce a solution with a concentration of **2 mg/mL** (Fig. 9b). 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.

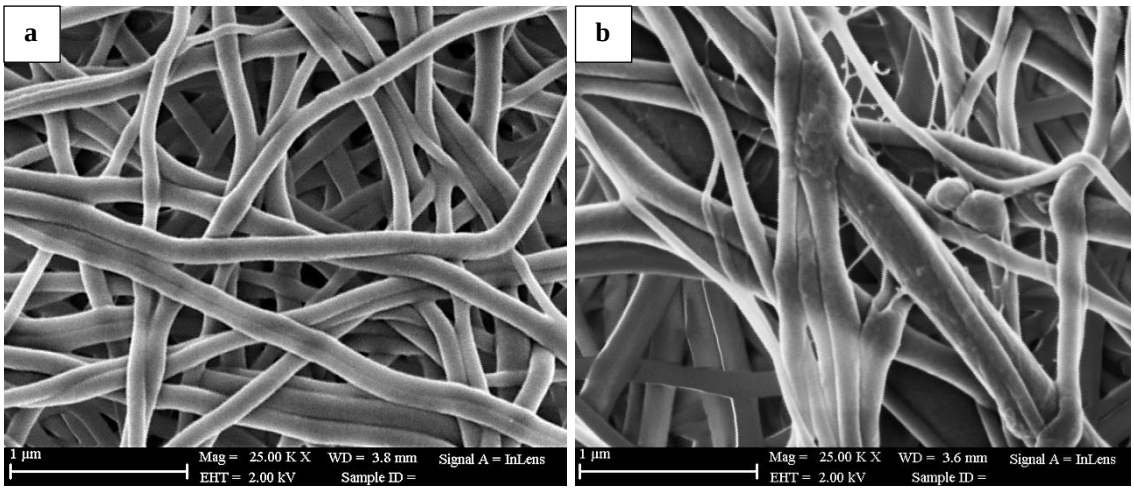


Fig. 10 Comparison of SEM images of pristine PA/PEI nanofibers (a) and PA/PEI with immobilized T. versicolor laccase (b); Magnitude 25 kx

The PA/PEI-laccase sample (Fig. 10b) had a noticeably different surface morphology to the smooth pristine PA/PEI nanofibers (Fig. 10a), being somewhat grainy and containing thinner fibrous structures.

4. 2. 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.

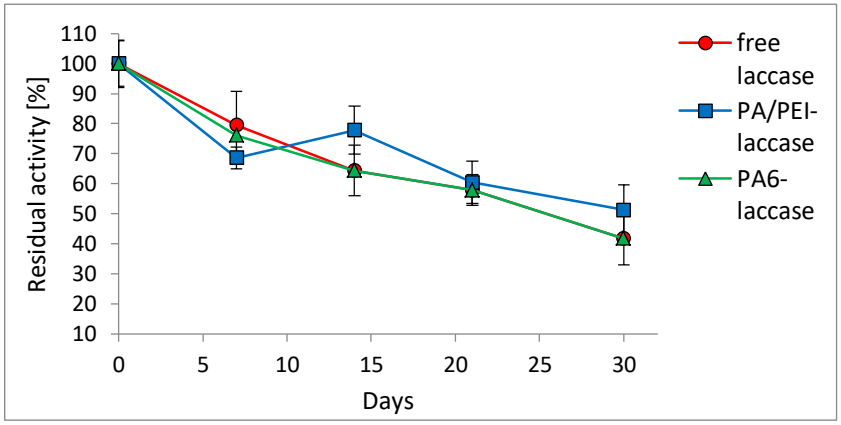


Fig. 11 Storage stability of PA/PEI-laccase compared to free laccase and PA6-laccase

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. 11). 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].

4. 3. 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.

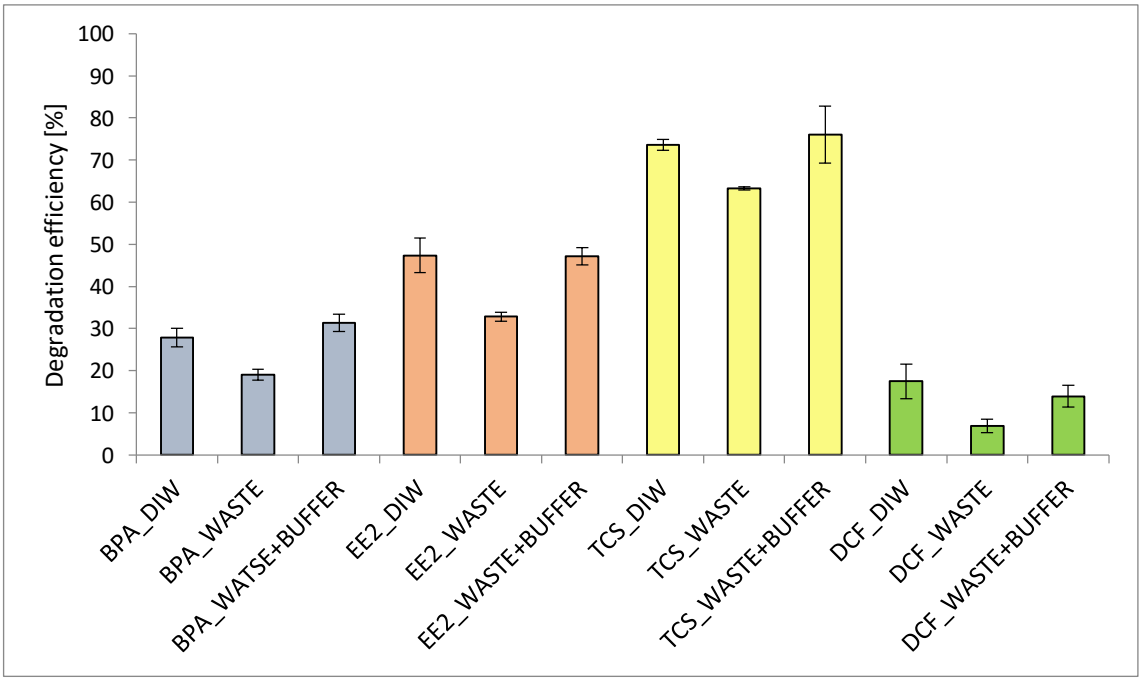


Fig. 12 Degradation efficiency of PA/PEI-laccase towards a mixture of 10 mg/mL of BPA, EE2, TCS and DCF in deionized water (DIW), wastewater effluent (WASTE) and wastewater infused with 2.5% (v/v) of McIlvaine's buffer of pH 7 (WASTE+BUFFER)

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 1) [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. 12).

5. *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 (Fig. 13), 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 McIlvaine's buffer at pH 4 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.

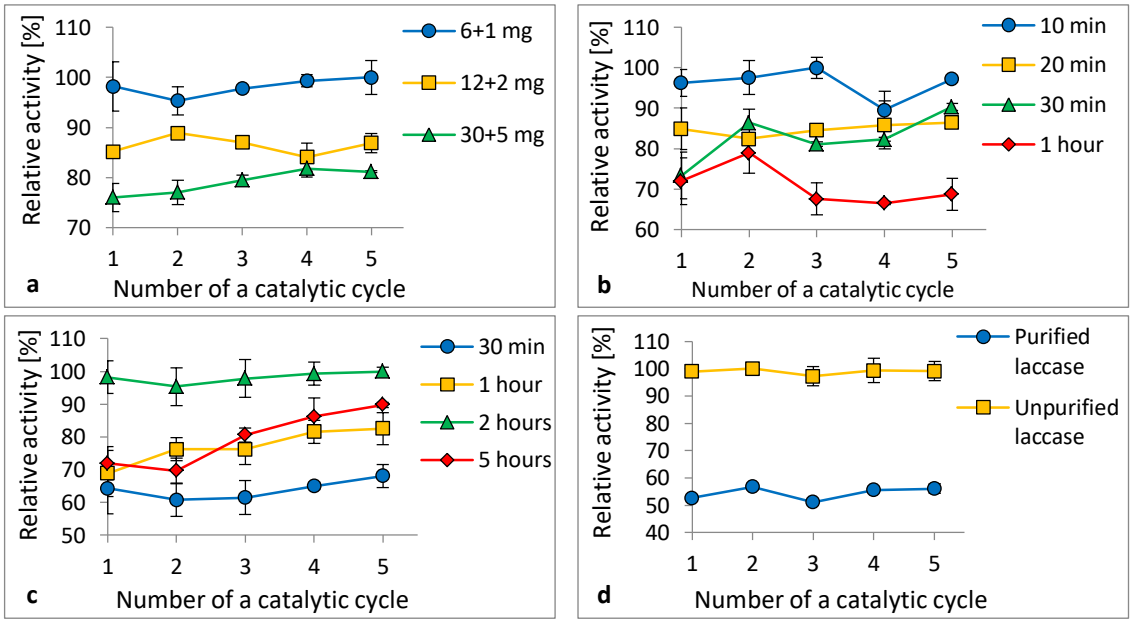


Fig. 13 Effect of (a) EDAC+S-NHS quantity, (b) activation time, (c) immobilization time, (d) type of enzyme on catalytic activity of laccase immobilized onto PAA nanofibers

5. 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% McIlvaine's buffer at **pH 4.0** (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.

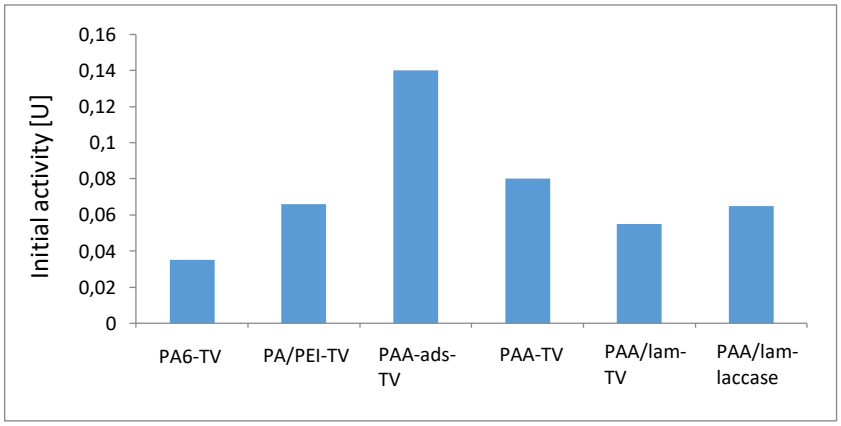


Fig. 14 Comparison of initial activity levels of commercial and crude laccase immobilized onto different types of nanofiber support

Fig. 14 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. 15a, c), laminated nanofibers (Fig. 15e), PAA-TV (Fig. 15b), PAA-laccase (Fig. 15d), and PAA/lam-laccase (Fig. 15f) 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 non-woven 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.

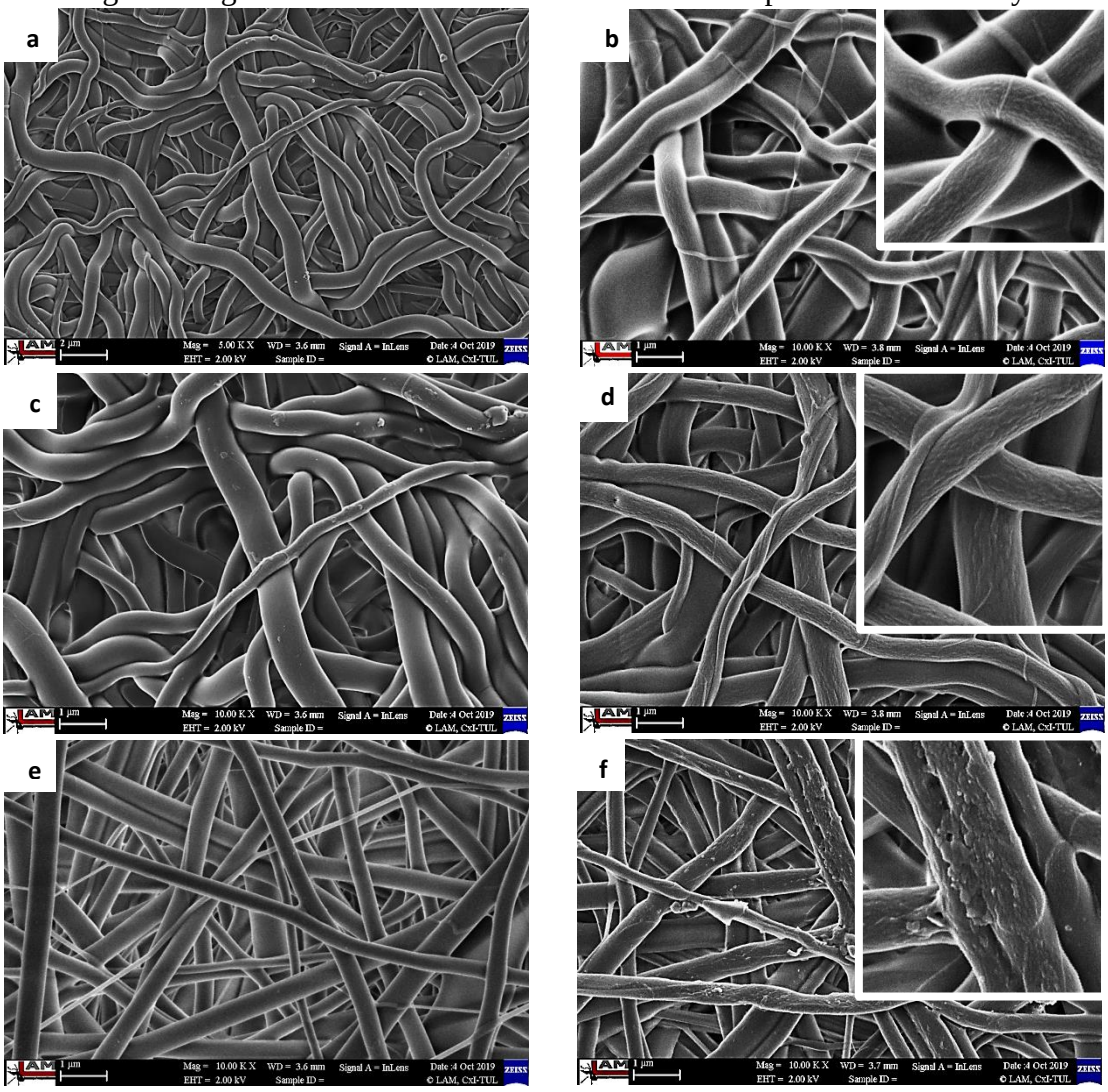


Fig. 15 Comparison of SEM images for pristine PAA nanofibers (a,c), laminated PAA nanofibers (e), PAA-TV (b), PAA-laccase (d) and PAA/lam-laccase (f). Magnitude 5 kx and 10 kx

5. 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. 16). 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].

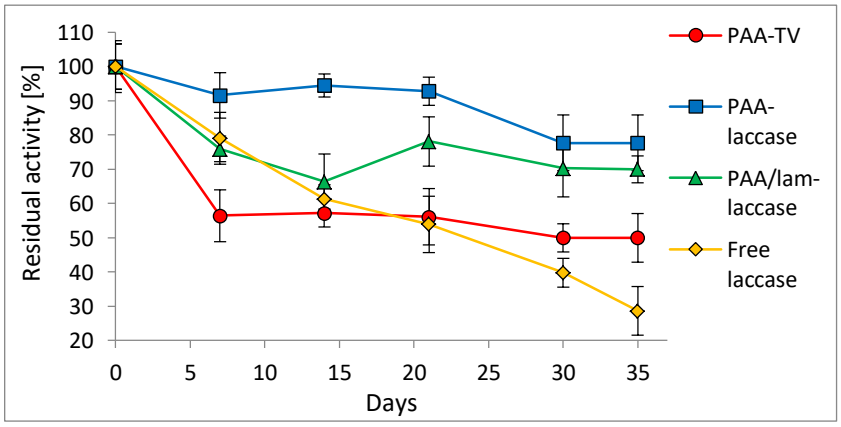


Fig. 16 Storage stability of PAA-TV, PAA-laccase, and PAA/lam-laccase samples compared to free TV laccase

5. 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. 17). PAA/lam-laccase achieved highest and most even degradation efficiency (Fig. 17c), indicating that support stability and mechanical durability had a major impact on stability of the immobilized enzyme. Both PAA-TV (Fig. 17a) and PAA-laccase (Fig. 17b) 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. 17), though DCF elimination did not follow this trend exactly.

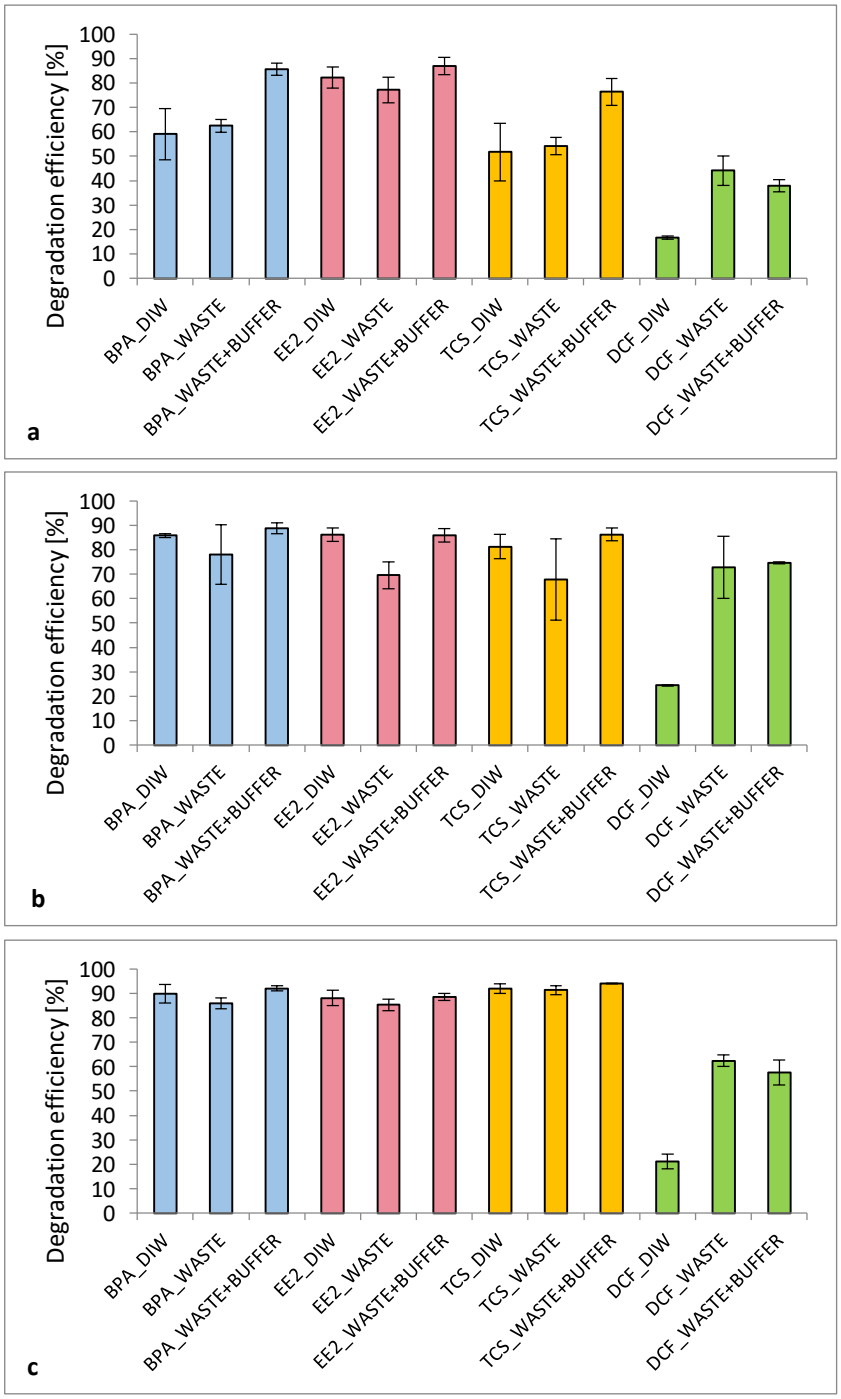


Fig. 17 Degradation of a mixture of BPA, EE2, TCS and DCF in deionized water (DIW), wastewater (WASTE) and wastewater with pH 7 buffer (WASTE+BUFFER) using PAA-TV (a), PAA-laccase (b), and PAA/lam-laccase (c)

Fig. 18 shows reuse of PAA-laccase (Fig. 18a) and PAA/lam-laccase (Fig. 18b) 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.

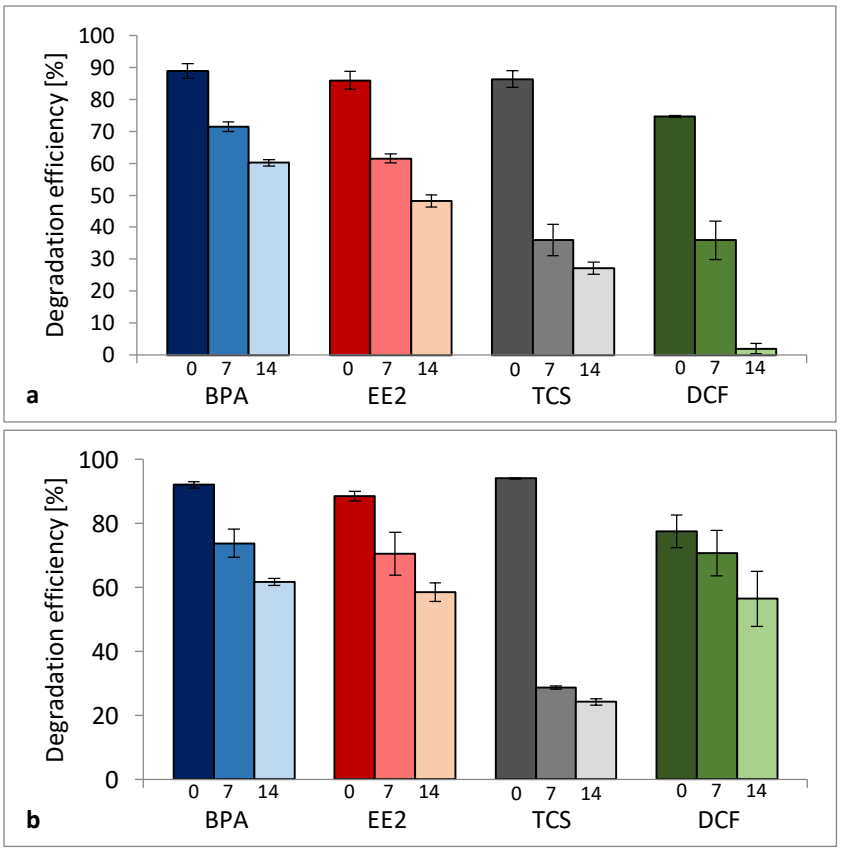


Fig. 18 Reuse in the degradation of a mixture of BPA, EE2, TCS, and DCF using PAA-laccase (a) and PAA/lam-laccase (b) samples after 7 and 14 days storage in wastewater effluent at 4°C

5. 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.

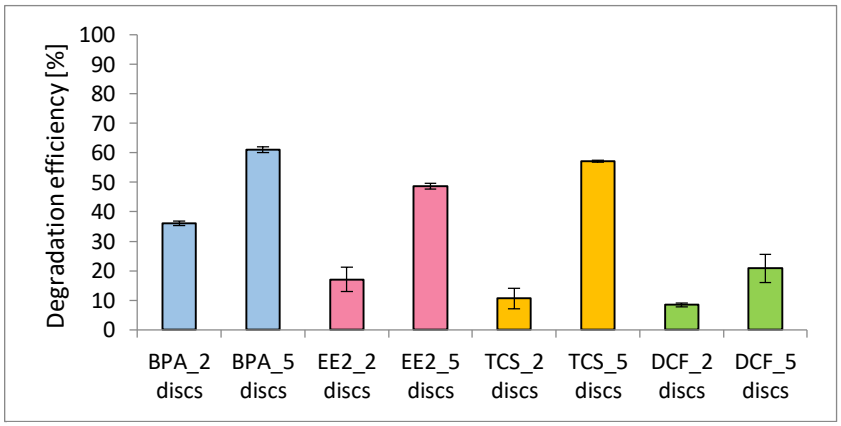


Fig. 19 Degradation of a mixture of BPA, EE2, TCS, and DCF (100 µg/L) in 200 mL of wastewater with 2.5% (v/v) pH 7 McIlvaine's buffer content using two and five PAA/lam-laccase discs

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. 19). 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].

6. 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.

6. 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.

6. 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. 20).

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.

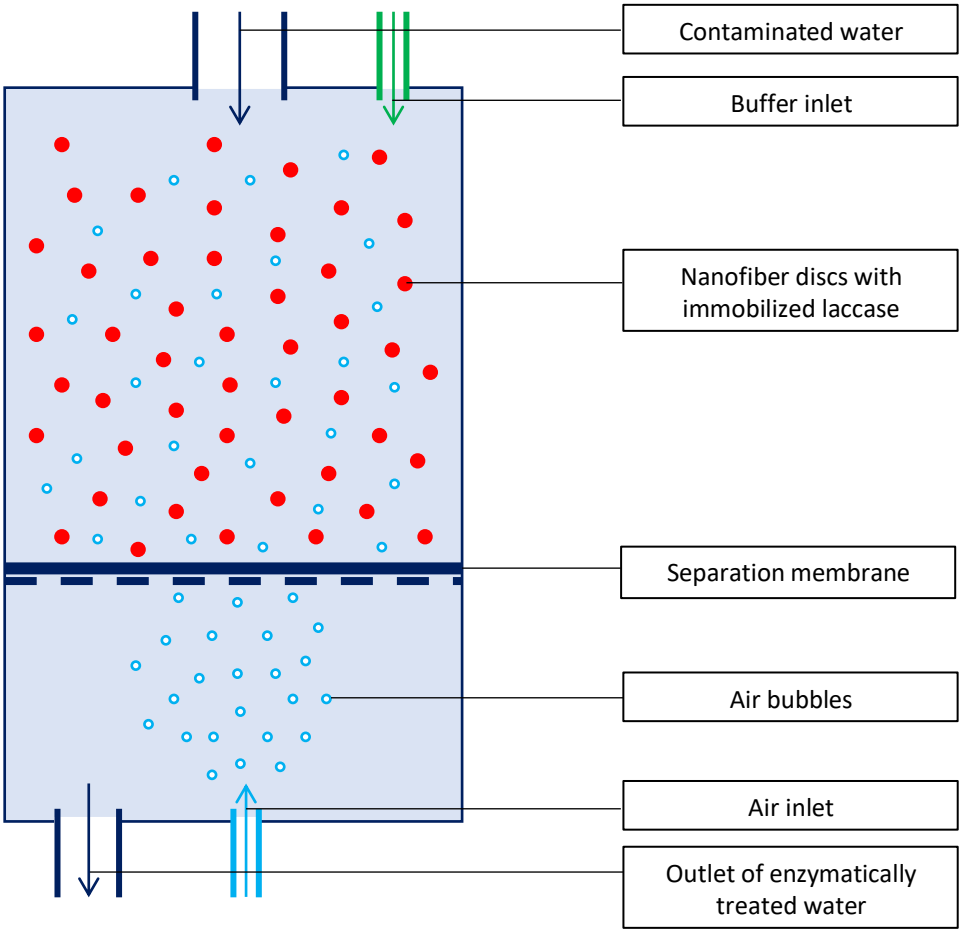


Fig. 20 Design for a reactor-based filtration system using laminated nanofiber discs with immobilized laccase

A potential filtration system allowing semi-continuous water treatment (see Fig. 20) 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.

6. 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. 22).
- 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. 21).
- 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.

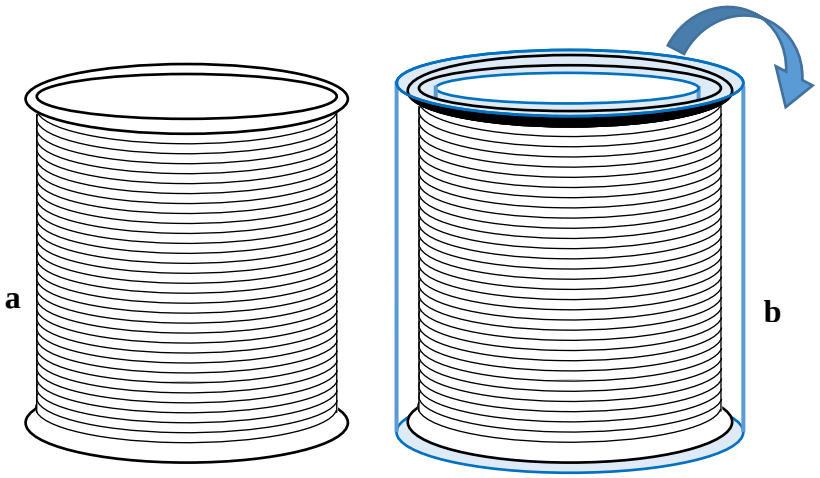


Fig. 21 Nanoyarn on a bobbin (a) and a special rotating vessel for enzyme immobilization onto coiled nanoyarn (b)

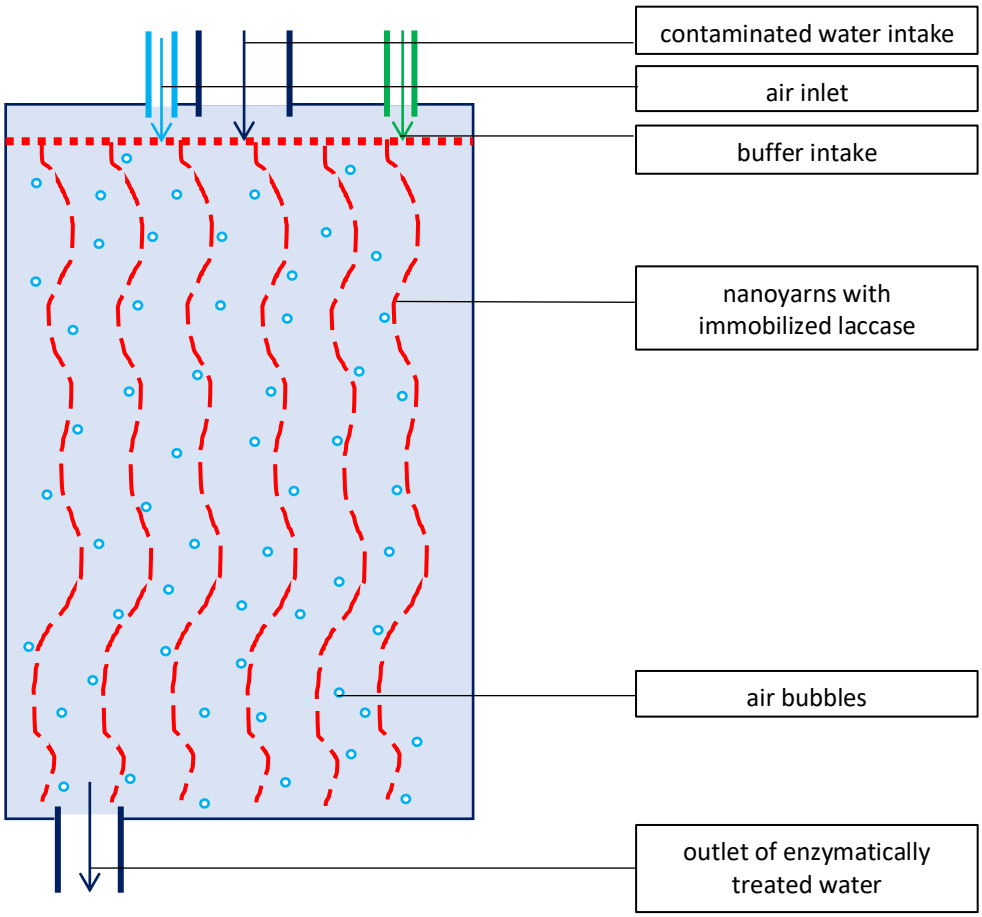


Fig. 22 Design of a reactor-based filtration system using nanoyarns with immobilized laccase

A reactor-based filtration system using uncoiled nanoyarns is illustrated in Fig. 22. 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.

List of publications

1. Maryšková, M.; Ardao, I.; García-González, C.A.; Martinová, L.; Rotková, J.; Ševců, A. Polyamide 6/chitosan nanofibers as support for the immobilization of Trametes versicolor laccase for the elimination of endocrine disrupting chemicals. *Enzyme Microb. Technol.* **2016**, *89*, 31–38.

50% contribution; impacted journal

2. Maryšková, M.; Vaňátková, P.; Schaabová, M.; Maryška, J. Immobilization of Laccase from *T. versicolor* on Nanofiber Matrix. *Scientific.net*., Material Science Forum, **2018**, *937*, 123–128.

70% contribution; conference paper

3. Maryskova, M.; Rysova, M.; Novotny, V.; Sevcu, A. Polyamide-Laccase Nanofiber Membrane for Degradation of Endocrine-Disrupting Bisphenol A, 17α-ethinylestradiol, and Triclosan. *Polymers* **2019**, *11*, 1560.

60% contribution; impacted journal

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