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Faculty of Textile Engineering

ENZYME IMMOBILIZATION ON MICROFIBROUS OR NANOFIBROUS MATERIALS AND THEIR APPLICATION IN BIOTECHNOLOGY

Diploma thesis

Study programme: N3106 – Textile Engineering
Study branch: 3106T018 – Nonwoven and Nanomaterials

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Supervisor: Ing. Nongnut Sasithorn

Liberec 2015

TECHNICKÁ UNIVERZITA V LIBERCI
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IMOBILIZACE ENZYMŮ V MIKROVLÁKENNÝCH A NANOVLÁKENNÝCH MATERIÁLECH A JEJICH VYUŽITÍ V BIOTECHNOLOGIÍCH

Diplomová práce

Studijní program: N3106 – Textilní inženýrství
Studijní obor: 3106T018 – Netkané a nanovlákenné materiály

Autor práce: **Bc. Milena Maryšková**
Vedoucí práce: Ing. Nongnut Sasithorn

Liberec 2015

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Akademický rok: **2013/2014**

ZADÁNÍ DIPLOMOVÉ PRÁCE

(PROJEKTU, UMĚLECKÉHO DÍLA, UMĚLECKÉHO VÝKONU)

Jméno a příjmení:	**Bc. Milena Maryšková**
Osobní číslo:	**T12000497**
Studijní program:	**N3106 Textilní inženýrství**
Studijní obor:	**Netkané a nanovlákenné materiály**
Název tématu:	**Imobilizace enzymů v mikrovlákenných a nanovlákenných materiálech a jejich využití v biotechnologiích**
Zadávající katedra:	**Katedra netkaných textilií a nanovlákenných materiálů**

Zásady pro vypracování:

1. Vypracujte rešerši týkající se imobilizace enzymů na různých formách přírodních i syntetických polymerních materiálů, zejména ve vlákenné formě.
2. Prostudujte teorii týkající se změny funkčnosti enzymů po jejich imobilizaci.
3. Vytvořte metodiku imobilizace např. lakázy na vlákenném materiálu a vyzkoušejte dostupnou metodiku detekce imobilizovaného enzymu.
4. Ověřte účinnost enzymatické katalýzy na vybraném mikropolulantu.
5. Výsledky shrňte a diskutujte

Rozsah grafických prací:

Rozsah pracovní zprávy:

Forma zpracování diplomové práce: **tištěná/elektronická**

Jazyk zpracování diplomové práce: **Angličtina**

Seznam odborné literatury:

1. Wei, Q , (2007), Surface modification of textiles, Woodhead Publishing in Textiles: Number 97, p. 139-158.
2. Matthews, J.A. et al., (2002), Biomacromolecules, 3, 232-238.
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Datum zadání diplomové práce: **17. června 2014**

Termín odevzdání diplomové práce: **14. května 2015**

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Acknowledgements

I would like to express my gratitude to professor Ivan Stibor and Dr. Michal Řezanka for their expert advice in the chemistry field.

I owe my thanks to my thesis supervisor M.Sc. Nongnut Sasithorn and Assoc. Prof. Lenka Martinová for selecting suitable nanofibrous carriers for my research and to Klára Kučerová and Bc. Monika Řebíčková for fabrication of these nanofibrous layers.

Dr. Alena Ševců and Dr. Jana Rotková deserve their recognition for expert advice in the field of biotechnology and enzyme immobilization.

I am very grateful to Dr. Inés Ardao Palacios and Dr. Carlos García-González for their support to my research made in Université Catolique de Louvain and in Universidad Santiago de Compostela. I would like to thank them for introducing me in the field of enzyme immobilization and its application in biotechnology.

My thanks also go to M.Sc. Kateřina Pilářová for her expert assistance and consultation in the area of electrophoresis that served as an analysis of the applied enzyme. I also need to mention M.Sc Vít Novotný for assisting with the measurement of degradation of EDCs using high performance liquid chromatography.

My research was partly supported by the project Environmental friendly nanotechnologies and biotechnologies in water and soil treatment (NanoBioWat, project TE01010218), project Network for cooperation of academic institution and private sector in the field of environmentally friendly water and soil treatment (WaSot, CZ.1.07/2.4.00/31.0189) and "National Programme for Sustainability I" and the OPR&DI project Centre for Nanomaterials, Advanced Technologies and Innovation CZ.1.05/2.1.00/01.0005.

Abstract

This diploma thesis describes a development of optimal laboratory techniques for enzyme immobilization on nanofibrous matrices and analytical methods for simple and fast determination of their enzymatic activity. The research deals with a screening of different immobilization procedures and adjusting parameters of the process to achieve the best result as a compromise between a high activity and satisfactory stability of the immobilized enzyme. The highest activities over 150 U/g were reached using polyamide 6/chitosan and polycaprolactone/silk fibroin blend nanofibers for covalent attachment while the operational stability showed laccase from *Trametes versicolor* immobilized on polyamide 6 nanofibers by adsorption followed by crosslinking. Selected samples were used for a degradation of two model endocrine disrupting chemicals (BPA and EE2). They showed excellent catalytic efficiency within several degradation cycles. Nanofibers appeared to be an optimal matrix for enzyme immobilization with application for wastewater treatment.

KEY WORDS: laccase, immobilization, nanofibers, chitosan, polyamide 6, silk fibroin, polycaprolactone, wastewater treatment

Abstrakt

Tato diplomová práce popisuje vývoj vhodných laboratorních postupů pro imobilizaci enzymů na nanovlákenné nosiče. Dále popisuje analytické metody pro rychlé a snadné stanovení jejich katalytické aktivity. Výzkum se zabývá různými technikami imobilizace a změnami parametrů procesu za účelem dosažení nejlepšího výsledku, kterým je kompromis mezi aktivitou a stabilitou imobilizovaného enzymu. Nejvyšší aktivity přes 150 U/g bylo dosaženo při kovalentním navázání lakázy z *Trametes versicolor* na nanovlákenné směsi polyamid 6/chitosan a polykaprolakton/silk fibroin. Nejvyšší aktivitu vykazovala lakáza imobilizovaná na polyamid 6 adsorpcí následovanou zesítěním. Vybrané vzorky byly použity pro degradaci dvou modelových endokrinních disruptorů (BPA a EE2). Tyto vzorky prokázaly výbornou katalytickou aktivitu během několika degradačních cyklů. Nanovlákna se osvědčila jako vhodný nosič pro imobilizaci enzymů s aplikací na čistění odpadních vod.

KLÍČOVÁ SLOVA: lakáza, imobilizace, nanovlákna, chitosan, polyamid 6, silk fibroin, polykaprolakton, čištění odpadních vod

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Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AY	activity yield
BCA	bicinchoninic acid
BPA	bisphenol A
BSA	bovine serum albumin
CHIT	chitosan
DF	diclofenac
DIW	distilled water
EDAC	1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride
EDCs	endocrine disrupting chemicals
EE2	17α-ethinylestradiol
GA	glutaraldehyde
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
IY	immobilization yield
Load.	loading
milli-Q	ultrapure water of "Type 1”, 18 MΩ
NFs	nanofibers
PA	polyamide
PEG	polyethylene glycol
PP	polypropylene
RT	room temperature
PCL	polycaprolactone
SEM	scanning electron microscopy
SF	silk fibroin
SDS	sodium dodecyl sulphate
SN	supernatant
S-NHS	sulfo-N-hydroxysuccinimide ester
TEMED	tetramethylenediamine

Introduction

Wastewater management has to deal with increasing concentrations of hazardous compounds including endocrine disrupting chemicals (EDCs). Very low concentrations of EDCs such as pharmaceuticals, polychlorinated biphenyls, dioxins, pesticides or plasticizers may interfere with the endocrine system of humans and other animal species mimicking the effect of hormones (Diamanti-Kandarakis et al. 2009). The main problem with EDCs is in their persistence in water system and difficult break down to harmless compounds. Therefore, there is an enormous worldwide effort to improve the wastewater treatment in order to clean such polluted water.

Recently, a promising approach to remove the EDCs from wastewaters was proposed to be the use of specific enzymes capable of catalyzing oxidations of these chemicals. The most studied enzyme has been laccase which is a multi copper oxidase produced by fungi such as white rot fungus *Trametes versicolor, Pleurotus or Pycnoporus sanguineus* (Ramírez-Cavazos et al., 2014). Laccase belongs to the group of enzymes catalyzing the oxidation of organic and inorganic substrates including EDCs (Madhavi and Lele, 2009).

The efficiency of enzyme catalysis is directly depending on enzyme activity and stability. Especially the enzyme stability and repeated usage are necessary precursors for successful industrial applications in wastewater treatment (Cipolatti et al., 2014).

However, free enzyme is very sensitive to pH, temperature changes and presence of inhibitors in the water environment. These factors may cause conformational changes in enzymes molecules or their direct inhibitions. Immobilization of active enzymes onto various materials might overcome these problems. Enzyme immobilization is a method that specifically fixes the structure of attached molecules which increases their stability and resistivity in time for easier and repeated applications compared to that of the soluble enzyme. The immobilization methods include enzyme entrapment or covalent binding and reversible binding focusing on specific functional groups on the side chains of the biocatalyst (Tisher and Wedekind, 1999).

Materials in form of nanoparticles such as modified silica, carbon, chitosan and other biopolymers or metal oxides commonly used for laccase immobilization with very good results in residual activity and stability in time and repeated catalysing cycles of the immobilized enzyme (Hudson et al. 2008; Xiao et al., 2006; Jiang et al., 2005). However, there are several disadvantages of nanoparticles that complicate their

application in the wastewater treatment. In some cases these materials might represent a certain health and environmental risk during the immobilization process as well as their final applications because of their size and high reactivity allowing them to interact with living systems (Alenius et al. 2014). Reasonable alternative to nanoparticles could be involvement of nanofibers. They are mostly safe and stabile materials providing high specific surface area and numerous reactive sites (Jirsák and Dao, 2009). Furthermore there are elegant ways of using enzyme-modified nanofiber sheets in the final step of waste water treatment. They can simply be arranged into filters or other structures.

This diploma thesis disserts on the immobilization of laccase from a fungus *Trametes versicolor* on specially designed and modified nanofibers formed by synthetic polymers and biopolymers. Activity and stability of the immobilized enzyme is studied upon different operational conditions and various parameters of the immobilization process. The last part focuses on a verification of enzymatic degradation of selected EDCs by materials with the best achieved results.

1. Enzymes

Enzymes are proteins with a catalytic activity. Their primary structure is formed by sequences of 100–1000 amine acids specified by corresponding gene. These polypeptide chains spontaneously fold to form one of three main secondary conformations; α-helix, β-sheet or β-turn. The three-dimensional form of the tertiary structure, essential for the catalytic activity, is given by elements of secondary structure connected together. Proteins consist of several domains which are regions of the secondary structure. Some of them have specific functions such as binding a substrate or a cofactor. Tertiary structures can be also connected together to form the quaternary structure (Bugg, 2004).

Enzymes are highly selective catalysts and they are extensively increasing the rate of a reaction by lowering its activation energy. As a result substrates are converted into products much faster. Enzyme work like other catalyst but they are different for their high specificity for substrates. The part of the enzyme responsible for the catalytic activity is called the "active site". Usually it is a hydrophilic cleft or cavity which makes up 10–20 % of total volume of the enzyme. This place contains 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 (Bugg, 2004).

Their classification comes from the type of the performed catalytic reaction. There are six groups of enzymes:

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 (Murray et al., 2009).

1. 1 Laccase

Laccases, from the group of oxidoreductases, 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 in the way of degradation chemicals produced mainly by paper, textile and petrochemical industry. They effectively replace chlorine-based chemicals used to degrade lignin from wood pulp.

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. These enzymes can also appear as special ingredients in cosmetics Goshev and Krastanov, 2007).

Laccases are produced by higher plants and fungi and they were also observed in some insects and bacteria. These enzymes are commercially extracted from culture medium of different fungi due to their extracellular laccase production as the result of reaction to specific stressful conditions. Extracted enzyme is subsequently purified by centrifugation and lyophilisation (Madhavi and Lele, 2009).

The molecule of laccase is usually dimer or tetramer glycoprotein with molecular mass between 50 and 100 kDa. Glycosides form up to 50% of the molecule which increases the final stability of the enzyme. The isoelectric point is at pH between 3 and 7 depending on the particular type of laccase.

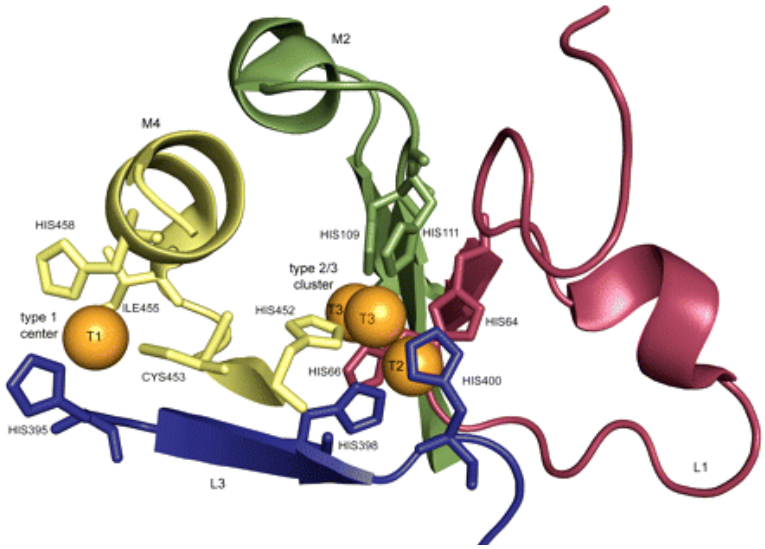


Figure 1 Structure of laccase (Sirim, 2011)

The molecule contains 4 copper atoms in three redox sites (T1, T2, T3). The atom in T1 reduces the substrate while the other atoms bind oxygen and reduce it into water (Thurstor, 1994). Four electrons coming from 4 molecules of the substrate are necessary for the reduction of one molecule of oxygen while only one electron is produced by this reduction. Laccase stores gained electrons and uses them to form water molecules (Claus, 2004). 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 (Kunamneni, 2007). Figures 2 and 3 show examples of the reactions described above.

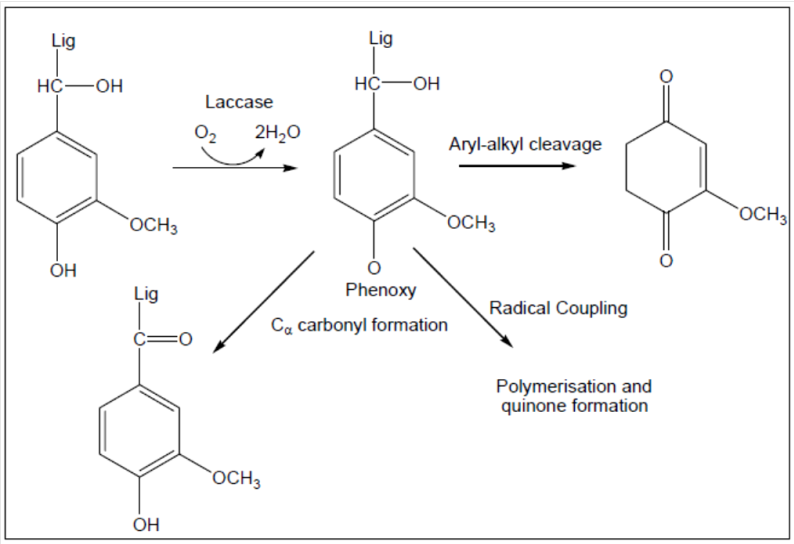


Figure 2 Oxidation of phenolic subunits of lignin by laccase (Kunamneni, 2007)

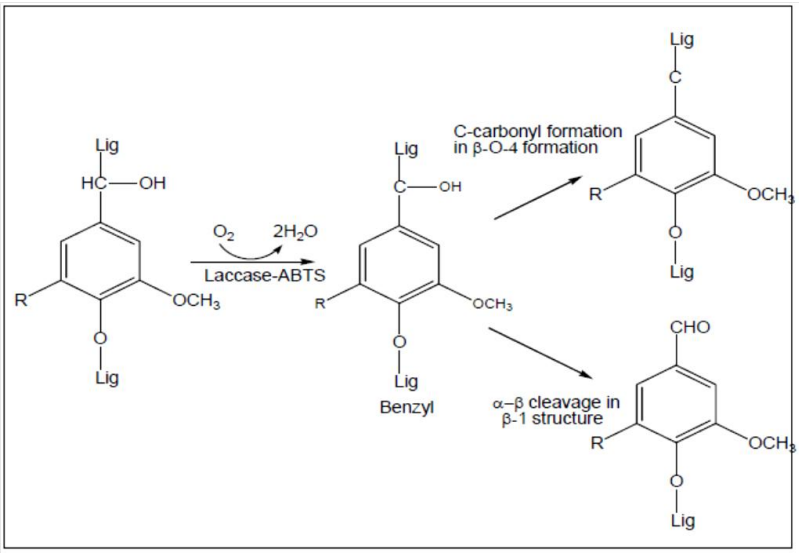


Figure 3 Degradation of non-phenolic part of lignin using laccase mediator system (Kunamneni, 2007)

2. Enzyme immobilization

Although enzymes are excellent biocatalysts with very high efficiency and specificity there are several features that make their applications in industry very complicated. First of all they are soluble which practically disables their removal from the solution. Enzymes have been optimized to be active under specific conditions of biological systems hence they are usually very unstable and strongly inhibited when working in an industrial environment. It is mainly the possibility of re-using the biocatalyst for several catalysing cycles that determines their future industrial applications (Guisan, 2006).

For this reason the methods to maintain the enzymatic activity for a longer time and for number of cycles have been explored. From this point of view the enzyme immobilization may be understood as any method that allows a repeated usage of the enzyme in its solid, insoluble form. There are several ways to immobilize the enzyme. Most of them use a solid matrix (or carrier) that supports and protects molecules of the biocatalyst and in some ways stabilize their structure by protecting them from mechanical damage.

The basic classification of the supports is into organic and inorganic that can be further divided into natural and synthetic. Typical natural materials are polysaccharides (cellulose, chitin,...), proteins (collagen, albumin,...) and carbon. Silica and some pore metal oxides are the most suitable representatives of inorganic carriers. Basically support can be any material with sufficient mechanical robustness, hydrophilicity, biocompatibility, resistance to microbial attack and low cost (Trevan, 1980). There are other features determining the efficiency of the carrier such as size and porosity. These two parameters represent the main influence on enzyme loading (capacity of the support) but they also affect diffusional limitations for the catalytic reactions.

There are two main categories of enzyme immobilization; reversible and irreversible (Cabral and Kennedy, 1991).

2. 1 Irreversible immobilization

The biocatalyst might be connected to the support so strongly that it cannot be detached without a negative impact on the enzymatic activity or solidity of the support (Guisan, 2006; Svec and Gemeiner, 1996).

Covalent coupling

The biggest advantage of the covalent attachment is a multiple re-use with zero leakage of the enzyme into the solution which is an advantage especially when there's a requirement for no release of the enzyme into the product. Most coupling reactions involve side chains of the available amino acids lysine (amine group), cysteine (thiol group) and asparic or glutamic acids (carboxylic group).

There are several ways to connect these side chains to the activated 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 diffusional limitations. Another disadvantage is that the matrix must be disposed together with the enzyme after its activity expiration. In the opposite of these limitations this is mostly quite simple and effective method that can harness common synthetic polymers or biomaterials via their chemical modifications (Guisan, 2006; Svec and Gemeiner, 1996).

Entrapment

Enzyme entrapment or encapsulation is an immobilization process that allows a free flow of a low-molecular weight substrate and leads to products with no protein leaking from the matrix. The enzyme is not held inside the matrix by strong chemical bonds but mainly 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 matrix degradation followed by enzyme release (Guisan, 2006; Fonseca and Meesters, 2013; Svec and Gemeiner, 1996).

Crosslinking

Carrier-free enzyme immobilization has many advantages. The catalyst remains a high specific activity (units per gram) with enhanced stability compared to the free enzyme. The production cost is also lower without preparation and production of a solid carrier. However; solution with the cross-linked enzyme is usually very viscous and uneasy to work with. Cross-linked enzyme aggregates (CLEAs) are usually molecules of soluble catalyst attached to each other via a bifunctional agent such as glutaraldehyde. These CLEAs are easily recovered from the reaction mixture by centrifugation (Cao et al., 2000; Fernandes et al., 2005; Svec and Gemeiner, 1996).

2. 2 Reversible immobilization

Reversibly immobilized enzymes can be detached from the matrix under specific conditions. This method is very attractive for economic reasons because the support can be re-loaded with another enzyme after the previous one is detached. Figure 4 shows schema of possible reversible methods to immobilize enzymes (Guisan, 2006).

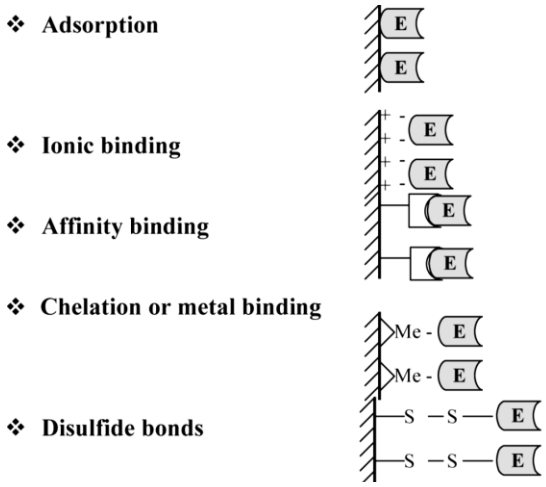


Figure 4 Approaches to reversibly immobilize enzyme

Adsorption

Adsorption is the simplest method based on physical adsorption of an enzyme using hydrogen binding, van der Waals forces or hydrophobic interactions influenced mainly by pH, ionic strength, temperature and polarity of the solvent. Although this method usually preserves the catalytic activity the enzyme leakage might be a very serious disadvantage (Woodward, 1985).

The protein can be attached to the matrix via ionic binding but it is usually very difficult to set optimal conditions to preserve the enzyme strongly bound and yet fully active. The principle of affinity binding often requires covalent attaching of a costly affinity ligand (for example antibody) on the matrix (Solomon et al., 1987).

Chelation or metal binding

Metal salts (titanium or zirconium salts) are first precipitated onto the support (cellulose, chitin, silica,...) by heating or neutralization. Matrix cannot occupy all coordination positions of the metal, therefore there are some positions free for groups of enzymes. However; some metal leakage can occur together with non-uniform adsorption onto the matrix. This problem can be solved by covalent binding instead of adsorption. These metal chelated supports were named "immobilized metal-ion affinity" adsorbents (IMA) (Cabral et al., 1986; Porath, 1992).

2.3 Properties of immobilized enzymes

Some properties of the enzyme molecule might be altered as a consequence of enzyme immobilization. There are great advantages of the immobilization such as an improved operational stability of the biocatalyst which is mostly caused by stabilization of the molecule through multiple covalent binding and established diffusion-controlled catalysis (Hartmeier, 1988).

Enzyme immobilization has also a positive influence on other enzyme stabilities such as thermal stability and durability in a wider range of pH because the interaction between the biocatalyst and the substrate takes place in a different protective environment compared to the soluble enzyme (Trevan, 1980).

On the other hand the immobilization, especially via covalent binding, might have a negative impact on a catalytic activity of the enzyme. First of all the enzyme might be damaged and lose its activity because of its conformational changes caused by the creation of strong linkages with the matrix. Furthermore; the catalytic activity may be suppressed by diffusional limitations determined by the matrix structure which prevents the access of the substrate to the attached biocatalyst (Trevan, 1980).

2. 4 Nanofibers – support for enzyme immobilization

Nanofibers offer many features determining their application for enzyme immobilization. They can be processed into various structures with high surface area depending on fiber diameter and porosity. Probably the simplest method to generate nanofibrous layers is electrospinning with potential 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 immobilization process and possible applications in the industrial field (Tran and Balkus, 2012).

2. 4. 1 Enzyme adsorption and covalent attachment

Both enzyme adsorption and covalent attachment are based on specific interactions between the enzyme and the polymer. 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 leads to shortening of polymeric chains and obtaining higher amount of functional groups required for covalent attachment of an enzyme (Wei Q., 2007).

Several papers report enzyme immobilization on nylon fibers (Da Silva et al., 1991). One of the oldest papers within this topic describes an immobilization of glucose oxidase on a hydrolyzed nylon membrane. 3 M HCl was used to hydrolyze PA-6,6 membrane in order to increase the number of amine groups used to attach the enzyme via glutaraldehyde activation and additional application of different spacers. The best results were obtained with bovine serum albumin (BSA) used as a spacer between two glutaraldehyde molecules. Activity of the immobilized enzyme was close to that of a free enzyme, and after 2 months of storage the immobilized glucose oxidase lost about 50% of its activity.

Similar approach to bind an enzyme on the hydrolyzed nylon was used by Isgrove et al. (2001) to immobilize β-glucosidase and trypsin. In this case the spacer was represented by PEI (polyethyleneimine) or chitosan. Before the enzyme was

covalently attached the nylon film was modified via 2,9 M HCl hydrolyzation followed by GA activation.

Fatarella et al. (2014) continued in this research using partially hydrolyzed nylon films and nanofibers. Laccase *Trametes versicolor* was covalently attached to free aldehyde groups provided by glutaraldehyde. The optimal pH for the immobilization process was 4,5. The laccase immobilized on the nanofibers resulted in the K_m measuring 1,07 mM and V_{max} measuring $1{,}00 \times 10^{-3}$ mM/s (the values of the free laccase were K_m=0,051 mM and V_{max}=$2{,}27 \times 10^{-2}$ mM/s).

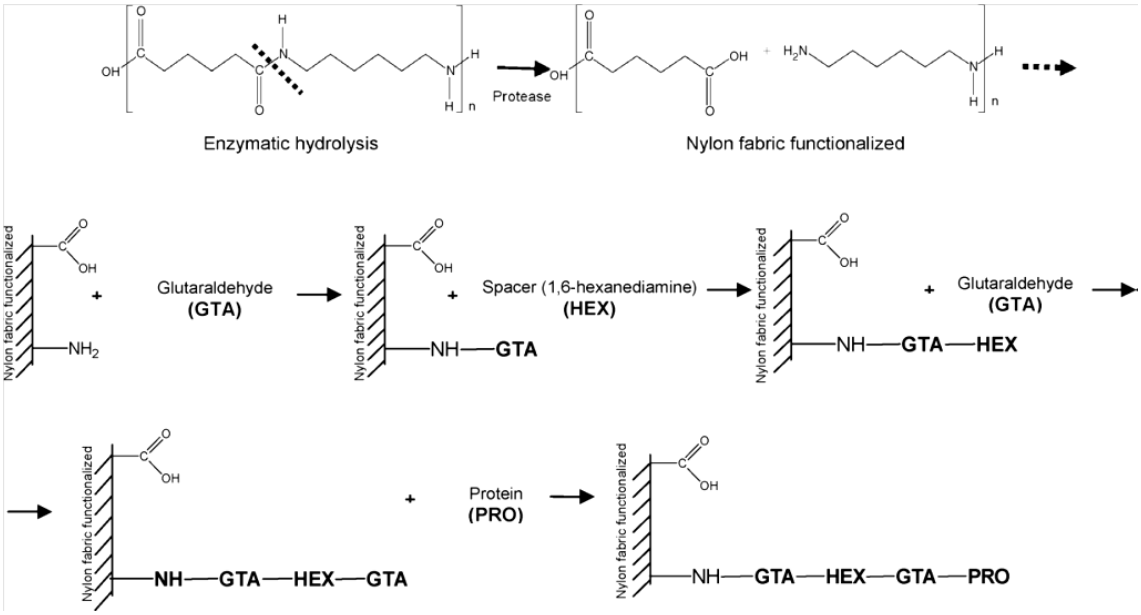


Figure 5 Immobilization procedures via glutaraldehyde and 1,6-hexamethylenediamine and their combinations (Silva et al., 2007)

Silva et al. (2007) applied enzymatically functionalized nylon to immobilize laccase *T. hirsuta* (Figure 5). Enzyme protease cleaved the peptide bonds and increased the quantity of free groups capable of attaching the enzyme. These groups were activated via glutaraldehyde activation with presence of a spacer 1,6-hexandiamine. The immobilization process is schematically described in figure 5. Under optimal conditions the highest achieved immobilization yield was only 2%. The activity was measured by oxidation of 1 ml 0,5 mM ABTS by 1 ml of 0,1 M enzyme at pH 5. However; the actual activity of the immobilized enzyme was not successfully measured because the support was breaking into small filaments during the reaction and these pieces caused serious interference.

Enzymatic surface modification of polyamide 6,6 was also described in another study of Silva et al. (2007) and Araújo et al. (2007) where different enzymes were used to study the effects of hydrolyzation on properties of nylon or polyethylene terephthalate substrates.

Also other synthetic materials were modified in order to obtain a suitable surface for enzyme attachment. Jia et al. (2002) used hydroxyl-modified polystyrene nanofibers as a support for covalently attached α-chymotrypsin. Immobilized enzyme achieved 65 % of activity of the soluble enzyme and storage and chemical stabilities were increased. A similar method is described in a study by Jia et al. (2011).

Li et al. (2007) used PAN nanofibers with fiber diameter in the range of 150–300 nm to immobilize lipase from *Candida rugosa*. The nanofiber membrane was first activated by absolute ethanol and hydrogen chloride to form imidoester derivates enabling the lipase attachment. Activity measurement was performed using p-nitrophenyl palmitate (p-NPP) at pH 7. Activity of the immobilized enzyme retained 95 % of its initial activity after 20 days of storage in 30 °C and after 10 batches of reaction only 30 % of its activity was lost. Another study (Li et al., 2011) describes an immobilization of lipase *Pseudomonas cepacia* on electrospun PAN nanofibers. In this study the activity of the attached enzyme retained 79 % of the activity of the free enzyme and only 2 % of its activity was lost after 10 batch cycles using triolein in n-hexane as the reaction substrate.

Copolymer of PAN and maleic acid (PANCMA) was formed into nanofibers with fiber diameter of 100–180 nm to immobilize lipase (Ye et al., 2006). The nanofiber membrane was subsequently submerged into a low molecular weight chitosan or gelatin solution in the presence of EDAC/NHS (1:1). Lipase was immobilized on these dual-layer membranes using GA modification and enzymatic covalent attachment on the free endings of a crosslinker. The same enzyme was also immobilized on a nascent PANCMA membrane using modification by EDAC/NHS only. The activity retention of the immobilized enzyme was higher on both dual-layer membranes (around 50 %) compared to mono-layer membrane (around 37 %). After ten uses the residual activities of dual-layer supports were 55 % and 60 %.

Another copolymer with PAN was described in a paper by M. R. El-Aassar (2013). β-Galactosidase was immobilized on amine functionalized poly(acrylonitrile-co-methyl methacrylate) nanofibers. The membrane was submerged in PEI solution at 70 °C and then modified via GA. The immobilized enzyme retained 35 % of its initial

activity after 70 days of storage at 4 °C. After 10 batch cycles 36 % of its activity was lost.

Sakai et al. (2010, 7344–7349) used PAN nanofibers with fiber diameter of 400 nm to immobilize lipase via physical adsorption. A highly concentrated enzyme solution was used and only 3x3 mm large membrane samples were immerged into phosphate buffer with pH 7 containing 8 mg/ml of lipase. After an 11h reaction the samples were removed from the supernatant and lyophilized for 24 h. No crosslinker was added to stabilize the immobilized enzyme. Finally 94 % of rapeseed oil was converted into butyl-biodiesel after 24 h.

Gupta et al (2012) covalently immobilized lipase on a modified PAN nanofibrous membrane. The result was; 82 % of the initial enzyme being immobilized via covalent attachment while physical adsorption allowed the entrapment of only 73 % of the enzyme. However; authors did not measure the actual activity of the immobilized enzyme by standardized activity assay using typical substrate. Therefore; this fact enables any comparison with results of other papers.

In some papers authors used a combination of adsorption or covalent attachment on the polymer surface followed by adding a suitable crosslinker and another soluble enzyme. This method enhances the concentration of immobilized enzyme by building more protein layers covering the supporting material. Polystyrene-poly(styrene-co-maleic anhydride) (PS-PSMA) was used as a trypsin carrying material (Lee et al., 2010). The two-step immobilization process consisted of a covalent attachment of highly a concentrated enzyme directly onto the maleic anhydride groups of the support at the pH 7,9 and the temperature of 4°C followed by glutaraldehyde crosslinking at the same temperature. This step resulted in a high concentration of enzyme aggregates. Activity of the system was determined by hydrolysis of BAPNA. Immobilized enzymes retained 90 % of the initial activity after 30 days.

Zhao et al. (2013) used PSMA with grafted branches of polyethyleneimine to covalently attach alcohol oxidase molecules through glutaraldehyde activation. The immobilization yield was over 40 % and the fibers were used for 9 cycles of saliva alcohol concentration without any significant activity decrease.

Laccase *Pleurotus florida* was immobilized on oxidized cellulose nanofibers (Sathishkumar et al., 2014). 500 U of laccase was used for 1 g of nanofiber sample. The adsorption was carried out mainly at 4 °C and was followed by GA crosslinking subsequently. Activity measurement performed by oxidation of ABTS showed that the

immobilized enzyme achieved 88 % of activity of the free enzyme which corresponds to 400 U/g of the carrier and this activity retained for 8 cycles. Huang et al. (2011) also reported a covalent immobilization on cellulose nanofibers. Lipase was attached to aldehyde groups of a modified electrospun cellulose acetate. Authors measured an enzymatic activity of 29,6 U/g of the biocatalyst under optimal conditions using p-NPP as a substrate.

Xu et al. (2013) used electrospun chitosan/poly(vinyl alcohol) to covalently attach laccase *Trametes versicolor*. A precursor for electrospinning consisted of TEOS, 10 wt% PVA and 3 wt% chitosan dissolved in acetic acid. The fiber diameter was in the range of 50–200 nm. After modification by GA the enzyme was immobilized covalently via its amine groups. This reaction was performed at room temperature. Protein content was determined by the Bradford method using BSA protein while the enzymatic activity was detected by oxidation of ABTS at the pH 4. However; the actual activity of the enzyme immobilized on nanofibers is not clearly defined in this study. The removal efficiency of 2,4-dichlorophenol was 87,6 % after 6 h which was almost comparable to the free laccase that removed 82,7 %. Park et al. (2013) also worked with chitosan/PVA but PVA was removed by NaOH in aqueous conditions. Subsequently the cross-linked enzyme aggregates (CLEAs) of lysozyme were immobilized on the nanofibers modified via GA solution. The activity was measured by the lysis of bacterial cells.

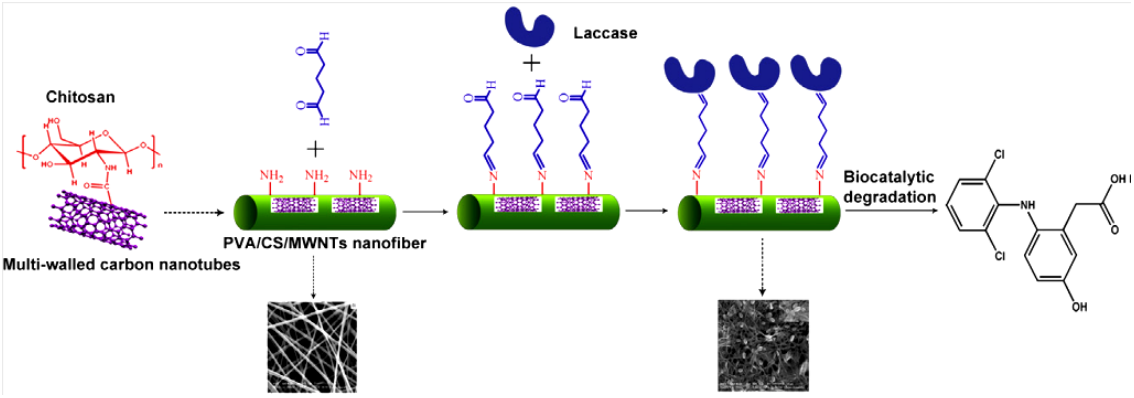


Figure 6 Laccase immobilization on PVA/CS/MWNTs nanofibers (Xu et al., 2015)

Xu et al. (2015) describes a method to immobilize laccase from *Trametes versicolor* onto an electrospun nanofibrous membrane consisting of multi-walled carbon nanotubes (MWNTs), chitosan (CS) and polyvinyl alcohol (PVA). The enzyme was covalently attached via glutaraldehyde which is shown in Figure 6. The final loading was 907 mg of protein per 1 g of membrane which was higher compared to the same

membrane without MWNTs (862 mg/g). With the lowest claimed activity of the used laccase (0,5 U/mg) the maximal potential loading on this membrane is around 450 U/g. However; this number probably was not achieved because of diffusional limitations and protein conformational changes caused by the immobilization process.

Palvannan et al (2014) covalently immobilized laccase *T. versicolor* on electrospun zein-polyurethane nanofibers. DMF:THF (1:1) was a solvent for the polymer solution. Afterwards the nanofibers were activated by water solution of glutaraldehyde and incubated with the laccase at 4°C overnight. The relative activity of the immobilized laccase reached 85 % compared to that of free laccase which corresponded to 0,25 mg of protein immobilized on 5 mg of the nanofibers. The activity of the immobilized enzyme was 1,9 U/mg of the protein therefore; the final enzyme loading was 95 U/g of the carrier. The system was able to degrade phenyl urea herbicide chloroxuron up to 25 reuse cycles.

Another interesting material used as a support for lipase immobilization was polyethersulfone (PES) and its aminated form (Handayani et al., 2012). Interaction with PES was based on weak physical bonds while aminated PES produced a covalent enzyme attachment. More than 95 % of initial activity retained after 4 cycles of p-NP hydrolysis. PES used as a support material for the similar purpose was also reported in a study by Nady et al., 2012. The author used laccase to modify the surface of the membrane by covalently attached (poly)phenolic acids providing interactions with proteins or microorganisms. Other polysulfone electrospun nanofibers were prepared to immobilize lipase by physical adsorption (Wang et al., 2006). Results of this study showed that any used biocompatible surface modification via poly(N-vinyl-2-pyrrolidone) or poly(ethylene glycol) did not improve the enzyme adsorption because it primarily increased the fiber diameter and decreased the surface area.

2. 4. 2 Enzyme entrapment

Other methods of enzyme immobilization using nanofibers as a carrier are encapsulation or entrapment. These methods use a polymer structure of the support as a cage that protects the enzyme and holds it between its molecular chains or integrated microcapsules without a participation of strong chemical bonds (Guisan, 2006). Enzyme encapsulation has many advantages. Protein retains most of its catalytic activity because

its conformation usually stays intact and the matrix is designed to simulate a protective environment that allows the permeability of low-molecular weight compounds without enzyme leaking. In special applications, such as drug delivery, the enzyme is held in the structure until the matrix breaks and releases all enzyme molecules near the target. However; it is very complicated to design an optimal matrix suitable for the enzyme immobilization and also to foresee properties of the surrounding environment where the enzyme operates (Fonseca and Meesters, 2013).

Most of the studies describing encapsulation methods develop a matrix formed by nanoparticles or microparticles. There are several papers that involve nanofibers as well. One of them is a study by Yang et al. (2008). Lysozyme was encapsulated within the core-shell structure of poly(DL-lactide) ultrafine fibers prepared by emulsion electrospinning. The enzyme lost only around 16% of its specific activity during the emulsification procedure.

Dai et al. (2010) encapsulated laccase from *Trametes versicolor* into poly(D,L-lactide)(PDLLA)/PEO-PPO-PEO microfibers by emulsion electrospinning. The activity of the immobilized enzyme retained 67% compared to that of the free laccase. The same laccase was immobilized on four different types of nanofibrous membranes consisting mainly of PLA or PGA copolymers (Dai et al., 2013). The immobilized protein was cross-linked by glutaraldehyde after it was electrospun with the supporting polymer. The immobilized laccase retained more than 70 % of the activity compared to that of the free laccase.

Lipase from *Rhizopus oryzae* immobilized in polystyrene electrospun fibers using emulsion electrospinning retained 77% of the residual activity after 10 catalytic cycles (Sakai et al., 2010, 576–580). In another study the coaxial electrospinning technique was used to immobilize lactate dehydrogenase in poly(vinyl alcohol) nanofibers. The enzyme was released from the structure after 1 month (Moreno et al., 2011).

2. 5 Other materials for laccase immobilization

There are several shapes of materials suitable for enzyme immobilization other than nanofibers, such as particles, polymer membranes or various porous structures. The largest and very fast evolving groups are nano- or microparticles. They can be formed into various shapes with very large specific surface area. There is also quite a large variability of materials suitable for enzyme immobilization. While nanofibers are restricted by spinability of the used material (ability to be formed into fibers) there are other natural or synthetic materials easily formed into effective matrices.

A very popular material for biotechnological applications is carbon. It has several modifications but nanotubes are used most often for their large specific surface area and reasonable manipulation during processing. They have other excellent properties which include superb electrical conductivity, tensile strength and thermal conductivity. Their remarkable electrocatalytic properties make them a promising support for enzyme immobilization because they can enhance direct electron transfers needed for catalytic activity of the attached protein (Gooding et al., 2003).

Liu et al. (2012) used carbon-based mesoporous magnetic composites to immobilize laccase from *Trametes versicolor* via adsorption. The capacity of this matrix was more than 490 mg of protein per 1 g of the support and the immobilized laccase retained 70% of its initial activity after 5 cycles oxidizing ABTS.

Another suitable material is silica formed usually into porous beads. Laccase from *Trametes versicolor* was covalently immobilized on pre-silanized silica beads via glutaraldehyde. The immobilized enzyme showed better stability at higher temperatures and a wider range of pH compared to the free enzyme (Rahmani et al., 2015). A similar immobilization method was used by Areskogh and Henriksson (2011).

Magnetic particles offer a great potential because they can be easily removed from the reaction mixture. Xiao et al. (2006) report the activity 460 U/g of the support (copper tetraaminophthalocyanine (CuTAPc)-Fe304 magnetic nano-composite) by adsorption of non-defined laccase followed by its crosslinking via 10% glutaraldehyde. The immobilized laccase retained 80% of its initial activity after 5 cycles.

Table 1 shows results in different studies on covalent attachment of laccase on magnetic particles.

Table 1 Immobilization of laccase by covalent binding on magnetic particles

Matrix	Laccase	Immobilization yield [%]	Loading [mg/g carrier]	Loading [U/g carrier]	Activity retention [%]	References
GAMNs*	E. taxodi**	60,7	18,2	462	82,4	Shi et al. (2014)
Fe_3O_4/ SiO_2 particles	T. versicolor***	31,3	62,6	224	93,8	Zheng et al. (2012)
Magnetic chitosan	T. versicolor	-	16,3	260	79,6	Bayramoglu et al. (2010)

* glutaraldehyde-activated Fe_3O_4 nanoparticles
** *Echinodontium taxodi*
*** *Trametes versicolor*

Sadighi and Faramarzi (2013) immobilized laccase onto functionalized glass beads through chitosan nanoparticles. First the laccase was attached on the chitosan nanoparticles and afterwards these particles were covalently connected to the glass beads via glutaraldehyde. This two-step lengthy process increased the thermal stability of immobilized laccase from *Paraconiothyrium variabile* up to near boiling temperature.

Chitosan magnetic particles using Fe_2O_3 particles covered by a shell formed by crosslinked chitosan molecules were developed for laccase immobilization in a study of Jiang et al. (2005). The thermal and storage stabilities of the enzyme were improved after the immobilization.

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 on carriers containing TiO_2 in studies of Hou et al. (2014a,b) and Ardao et al. (2015. Other immobilization approaches include various porous structures such as Amberlite IR-120 H beads (Spinelli et al., 2013) and zeolite (Celikbicak et al., 2014), natural materials such as green coconut fibers (Cristóvão et al., 2011) or cellulose (Rekuć et al., 2008) and more complicated structures.

3. Applications of immobilized enzymes

Nowadays immobilized enzymes can find their application in many fields. One of them is medicine where the enzymes are used for diagnostics and treatment. Thanks to their high specificity and reactivity these biocatalysts can be used for very sensitive, accurate and cheap biosensors that could selectively detect biological substances. Other applications of biosensors asides from medicine can be pathogen or toxin detection in food or water (Khan and Alzohairy, 2010). Immobilized enzymes are used for an ecological synthesis of antibiotics, such as β-laktam produced by a reaction catalyzed by Penicillin G Acylase in water at room temperature (Giordano et al., 2006).

Beyond detection applications immobilized enzymes can degrade toxins in food or wastewater. For example; endocrine disrupting chemicals (EDCs) end up in water as waste products from industry, pharmaceutical facilities or from agriculture in the form of pesticides. Therefore immobilized enzymes able to degrade phenolic or other hardly degradable compounds could be used for wastewater treatment (Damstra et al., 2002).

Application of enzymes during a washing process also falls within the same category as water treatment. Especially the washing of extremely dirty textiles containing blood, grass, sweat, oil and different food stains, which require either some specialized condition of washing that could damage the fibers, or there is a chance to clean the textile by an enzyme at mild condition. Some enzymes might improve the quality of the fibers which can be applied into several textile-treating processes that are normally very costly and non-ecological. Using an immobilized enzyme could result in higher savings and higher effectiveness of the ongoing process. Last but not least immobilized enzymes can be used for a production of biodiesel catalyzed by some lipase species (Nisha et al., 2012).

4. Materials and methods

4. 1 Materials and reagents

4. 1. 1 Enzymes

Laccase from *Trametes versicolor* (Sigma–Aldrich): This laccase was purchased in the form of brown powder soluble in water. The 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. The producer did not provide any information about the purity and enzyme extracting method.

Laccase from *Agaricus bisporus* (Sigma–Aldrich): This laccase was purchased in the same form as the previous with a difference in its solubility and activity. One unit corresponds to the amount of enzyme which converts 1 μmol of catechol per one minute at pH 6 and 25°C. The activity of this enzyme was claimed to be ≥4 U/mg. The purity and extraction method are also unknown.

4. 1. 2 Crosslinkers

Glutaraldehyde (GA) (Sigma–Aldrich; 25% in H_2O; grade II): GA is a commonly used crosslinker for molecules containing amine groups, especially proteins. It has several possible forms in aqueous solution depending on its concentration and pH. Glutaraldehyde is used for its reaction with amine groups forming a Schiff base linkage or Michael-type and leaving the other terminal aldehyde free to conjugate with another molecule. The efficiency of the Schiff base increases with higher pH. However; these interactions might not be stable enough to form irreversible linkages (Migneault et al. 2004; Hermanson G.T., 2013).

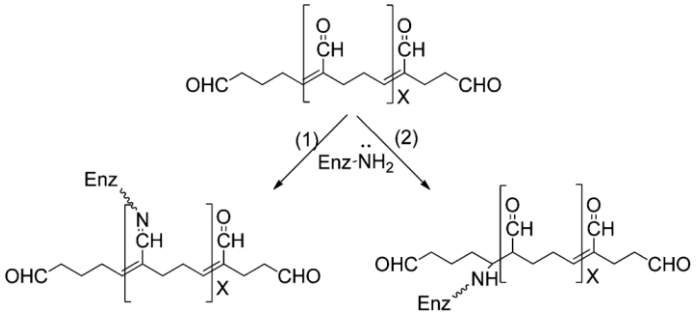


Figure 7 Reaction between an enzyme and glutaraldehyde (1 Schiff base; 2 Michael-type) (Barbosa et al., 2014)

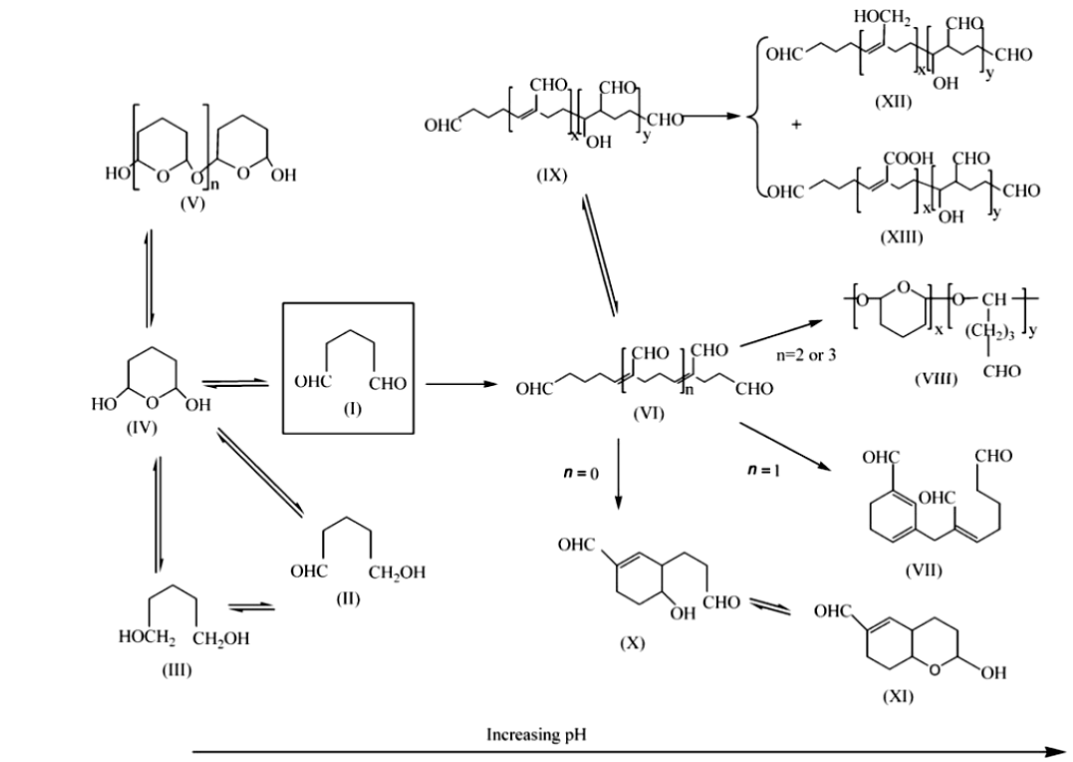


Figure 8 Possible forms of glutaraldehyde in aqueous solution (Migneault et al., 2004)

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and **N-hydroxysulfosuccinimide (S-NHS)** (Sigma–Aldrich): EDAC with S-NHS are used for enzyme immobilization in a two-step reaction with EDAC binding on carboxylates and amine groups on the other end. S-NHS enhances the stability of such linkage (Hrabarek and Gergely, 1990).

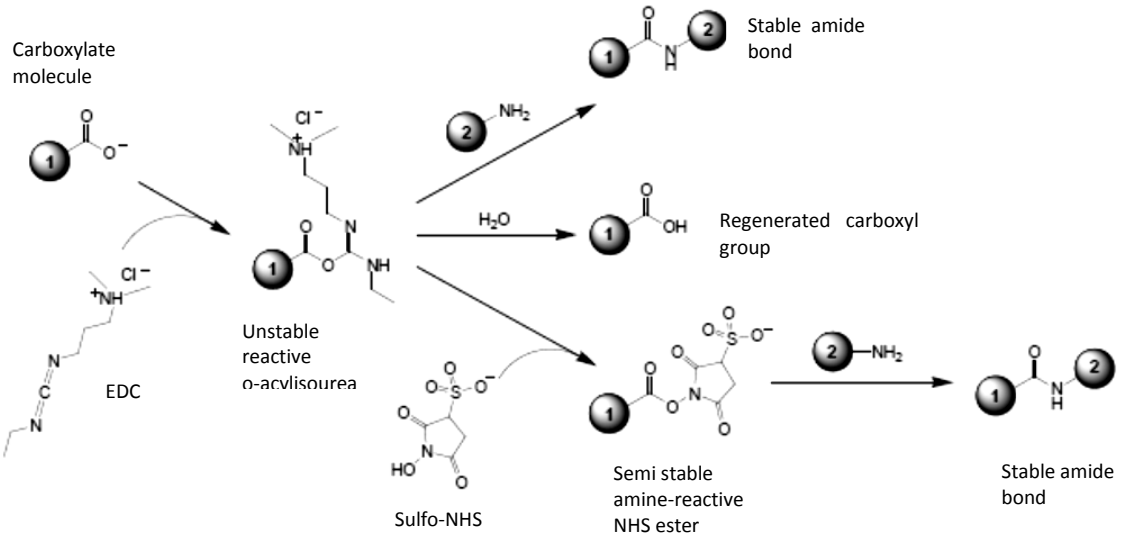


Figure 9 Reaction schema with EDC (Thermo Scientific, 2015)

4. 1. 3 Buffers

McIlvaine's buffer: This buffer system allows to make solutions in pH range from 2,2 to 8 by mixing 0,1M citric acid and 0.2 M Na_2HPO_4. It is commonly used for enzyme kinetics studies (Sigma-Aldrich, 2014).

pH	x ml 0.1M citric acid	y ml 0.2 M Na_2HPO_4	pH	x ml 0.1M citric acid	y ml 0.2 M Na_2HPO_4
2,2	89.10	0.90	5,2	46.40	53.60
2,8	84.15	15.85	5,4	44.25	55.75
3.0	79.45	20.55	5,6	42.00	58.00
3,2	75.30	24.70	5,8	39.55	60.45
3,4	71.50	28.50	6.0	36.85	63.15
3,6	67.80	32.20	6,2	33.90	66.10
3,8	64.50	35.50	6,4	30.75	69.25
4.0	61.45	38.55	6,6	27.25	72.75
4,2	58.60	41.40	6,8	22.75	77.25
4,4	55.90	44.10	7.0	17.65	82.35
4,6	53.25	46.75	7,2	13.5	86.95
4,8	50.70	49.30	7,4	9.15	90.85
5,0	48.50	51.50	7,6	6.35	93.65

Sodium-acetate buffer: Na-acetate buffer system provides solutions with pH in the range 3 to 6. The mixture consists of certain amount of 0,1M acetic acid and 0.1M sodium acetate according to the table 3 (Lambert and Muir, 1986).

Table 3 Sodium-acetate buffer system

pH	x ml 0,1M acetic acid	y ml 0.1M sodium acetate
3	982,3	17,7
4	847,0	153,0
5	357,0	643,0
6	52,2	947,8

4. 1. 4 Polymers

Bovine serum albumin (BSA) (Sigma–Aldrich): lyophilized powder ≥96% (agarose gel electrophoresis), M_w 66 000 Da

Bovine serum albumin (BSA) is a globular protein with a sufficient stability and lack of interference within biological reactions and therefore it is used in numerous biochemical applications (e.g. as a standard for BCA protein determination).

Polyethylene glycol (PEG) (Sigma–Aldrich): M_w 1400–1600 g/mol

PEG is a nontoxic water-soluble polymer with the ability to influence a protein precipitation. It attracts water molecules from the solvation layer around the protein and thereby increases interactions protein-protein (Atha and Ingham, 1981).

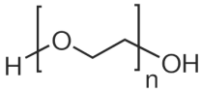


Figure 10 Structure of polyethylene glycol

Polycaprolactone (PCL) (Sigma–Aldrich): M_w 80 000 g/mol

PCL is a synthetic polymer used for manufacturing wrappings or special agricultural plastic films thanks its ability to be easily degraded into harmless low-molecular products. Nowadays; it has been used as a matrix for cell-growth because of its biodegradation and biocompatibility (Hermanová, 2012).

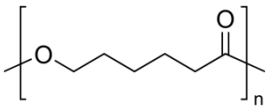


Figure 11 Structure of polycaprolactone

Polyamide 6 (PA 6) Ultramid B24 and B27 (BASF): M_w 37000 g/mol and 45000g/mol

PA 6 is an aliphatic synthetic polymer with good chemical and abrasion resistance. The fibers can absorb up to 2,4% of water and such hydrophilicity determines them to become a possible material used for enzyme immobilization with applications in a water environment (Galanty, 1999).

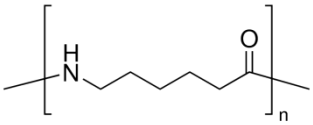


Figure 12 Structure of polyamide 6

Chitosan 5 (CHIT) (Wako): viscosity 0~10 mPa·s, deacetylation rate 80 mol/mol%

Chitosan is a linear polysaccharide made from crustaceans by deacetylation of chitin. This biopolymer is used in agriculture and bioengineering for its biocompatibility and biodegradation (Yogeshkumar et al., 2013).

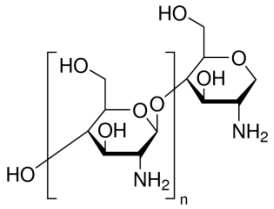


Figure 13 Structure of chitosan

Silk fibroin (SF) (Thai silk of *Bombyx mori* Linn. silkworms: Nang-Noi Srisakate 1)

SF is a biopolymer composed by amino acids and its exact composition is variable. It can be extracted from degummed silk fibres by removing the sericine protein. SF is an excellent material for biomedical applications and bioengineering due to its good biological compatibility (Sah and Pramanik, 2010; Sasithorn and Martinová, 2014). The content of amino acids in the SF extracted from purchased silk is described in Figure 14 and Table 4.

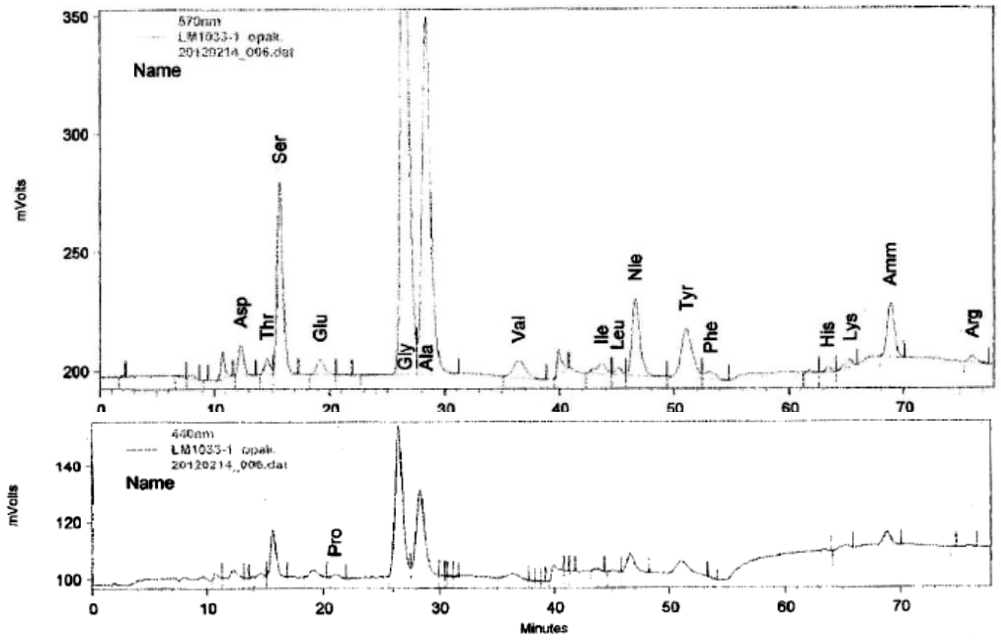


Figure 14 Chemical composition of purchased silk fibroin (Institute of Organic Chemistry and Biochemistry AS CR, v.v.i.; Martin Šafařík)

Table 4 Chemical composition of purchased silk fibroin (Institute of Organic Chemistry and Biochemistry AS CR, v.v.i.; Martin Šafařík)

Peak #	Name	RT (min)	Area	Amount (nmol)
570 nm				
6	Asparagin	12,27	390313	0,712
7	Threonin	14,57	213669	0,387
8	Serin	15,67	2767840	4,712
9	Glutamic acid	19,17	315127	0,56
11	Glycin	26,5	11902384	20,506
12	Alanin	28,33	7917475	13,466
13	Valin	36,4	552803	1,003
15	Isoleucin	43,63	271790	0,48
16	Leucin	45,06	124665	0,215
17	Methionine	46,63	1570880	2,632
18	Tyrosin	51,06	1453145	2,508
19	Phenylalanin	53,16	236683	0,419
21	Histidin	63,33	68220	0,11
22	Lysin	65,3	142977	0,222
23	Amm	68,83	990283	2,139
24	Arginin	76,03	133201	0,217
440 nm				
7	Prolin	21	47892	0,2263

4. 1. 5 Endocrine disrupting chemicals

Bisphenol A (BPA) (Sigma–Aldrich): BPA 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 and kidney diseases and breast cancer (Rezg et al., 2014).

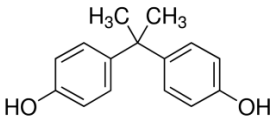


Figure 15 Structure of bisphenol A

17α-ethinylestradiol (EE2) (Sigma–Aldrich): EE2 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. An 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. (Wise et al., 2011).

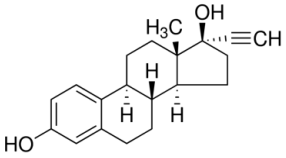


Figure 16 Structure of 17α-ethinylestradiol

4. 1. 6 Other chemicals

Acetic acid (99,8%, Penta)

Acrylamide/Bis-acrylamide (29:1,ultra pure grade, Amresco)

Ammonium persulphate (Lach-Ner)

Calcium chloride (Fluka AG, Switzerland)

Coomassie Brilliant Blue R-250 Dye (Carl-Roth)

Formic acid (98%, Penta)

Glycerol (≥98%, Carl-Roth)

Hexamethylenediamine (HMD) (Sigma–Aldrich)

Hydrochloric acid (HCl) (35%, Penta)

Methanol (G.R., Lach-Ner)

Pierce™ BCA Protein Assay Kit (Thermo Scientific)

Protein MW marker K494, wide range (Amresco)

Sodium azide (Sigma–Aldrich)

Sodium dodecyl sulphate (SDS) (≥98,5%, Sigma–Aldrich)

Tetramethylenediamine (TEMED) (Carl-Roth)

Tris base (ultra pure grade, Amresco)

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (≥98%, Sigma–Aldrich)

2-mercaptoethanol (≥99%, p.a., Carl-Roth)

4. 2 Analytical methods

4. 2. 1 Electrophoresis

Protein electrophoresis SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) was used to analyze a protein distribution of purchased laccase from *Trametes versicolor*. The upper stacking gel (pH 6,8) consisted of:

- 2,1 ml of DIW,
- 0,5 ml of 30% acryl/bis-acrylamide,
- 0,38 ml of 1,5M Tris-HCl buffer (pH 6,8),
- 30 µl of 10% SDS,
- 30 µl of 10% amonium persulphate,
- 3 µl of TEMED,

and the bottom running gel contained:

- 3,3 ml of DIW,
- 4 ml 30% acryl/bis-acrylamide,
- 2,5 ml of 1,5M Tris-HCl buffer (pH 8,8),
- 0,1 ml of 10% SDS,
- 0,1 ml of 10% amonium persulphate,
- 4 µl of TEMED.

The gel was placed in the 10 times diluted running buffer and attached to Enduro 300 V power supply with the voltage of 90 V. The stock laccase with the concentration of 40 mg/ml of DIW was dissolved in 2xSDS-PAGE sample buffer consisting of Tris (pH 6,8), 20% SDS glycerol, 2-mercaptoethanol and Coomassie R250. Then it was denatured by heat and centrifuged. Subsequently, three different concentrations of the stock laccase were sampled on the top of the gel. The gel had following lanes;

- lane 1.....5 µl of the protein marker K494,
- lane 2.....5 µl of the stock laccase (0,2 mg),
- lane 3.....10 µl of the stock laccase (0,4 mg),
- lane 4.....20 µl of the stock laccase (0,8 mg).

The gel was removed after certain time, transferred to a dye solution, decoloured by methanol and acetic acid and finally it was analyzed by software Elfoman version 2.0.

Figure 17 shows SDS-PAGE of laccase from *Trametes versicolor* described in a study by Carabajal et al. (2013). The lane 1 represents culture liquid, lane 2 is an ultra-filtrate (10kDA) and the lane number 3 is the laccase. It is obvious that the laccase has molecular weight of approximately 66 kDa.

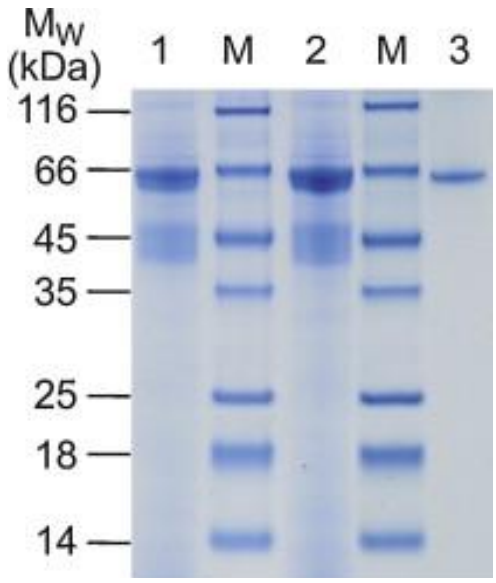


Figure 17 SDS-PAGE of the laccase from *Trametes versicolor* (Carabajal et al., 2013)

Sigma–Aldrich does not provide detailed specifications of the laccase from *Trametes versicolor* but it is supposed that the crude biocatalyst should have similar molecular weight to the laccase described by Carabajal et al (2013).

4. 2. 2 Scanning electron microscopy

Prepared nanofibrous layers were studied by the scanning electron microscopy using Vega 3 Tescan and Vega 3 SB. Captured images were further analyzed by NIS-Elements AR software or VEGA TC software used primarily for the measurement of the average fiber diameter obtained as an average value out of 100 gathered values.

4. 2. 2 Enzyme activity assay

The catalytic activity of the laccase from *Trametes versicolor* was measured according to Arnold and Georgiou (2003) and Hassani et al. (2013) at 25°C using a microplate reader BioTech Synergy HTX (Figure 18). The substrate for the catalytic reaction was 0,5 mM ABTS and the buffer was 100 mM McIlvaine's with pH 3. 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 0,5mM ABTS

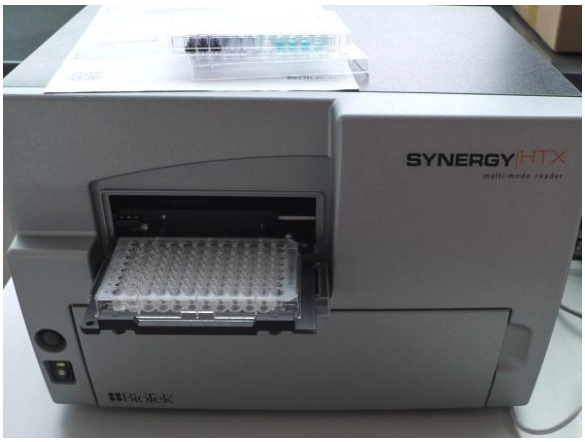


Figure 18 Synergy HTX microplate reader

From the moment ABTS was added in the reaction mixture it began to be oxidized by the laccase producing the stable cation radical $ABTS^{+}$ with green color which was measured by absorbance at 420 nm. The activity was expressed as 1 U which corresponds to the amount of laccase that converts 1 µmole of ABTS per 1 minute. The formula for the expression of 1 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).

The molar extinction coefficient for ABTS cation at 420 nm is 0,036 $\mu mol^{-1}cm^{-1}$ (Zapata-Castillo, 2012), the layer thickness of 200 µl of the reaction solution using 96-well plate was measured to be 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:

$$1\ \mathrm{U} = \frac{\text{slope}\ [\frac{Abs}{min}]}{\varepsilon\ [\mu mol^{-1} \cdot cm^{-1}]\ \cdot\ \mathrm{d}\ [cm]} \cdot \mathrm{V}\ [L] \cdot \mathrm{df}\ [-]$$

The activity of the immobilized laccase was measured likewise using either DR 6000 UV-Vis spectrophotometer (Hach) with cuvette or the microplate reader with 6-well plate (Figure 19). The cuvette had a volume of 3 ml. The volume of the sample was neglected so the reaction mixture consisted of 2,7 ml of buffer and 300 µl of ABTS. Nanofibers with the immobilized laccase were placed on the bottom of the cuvette and weighed down by a stainless steel wire so that the sample did not float and deflect the beam during measurement.

On the other hand; the 6-well plate had 6 beakers with the volume of 10 ml and the beam coming top down through the plate. The reaction mixture consisted of 7,2 ml of the buffer and 800 µl of ABTS. Nanofibers ware attached to the wall of the beaker by a stainless steel wire and the reaction was attended by linear shaking which insured sufficient distribution of the oxidation product. The thickness of the layer *d* was 1 cm in both methods.

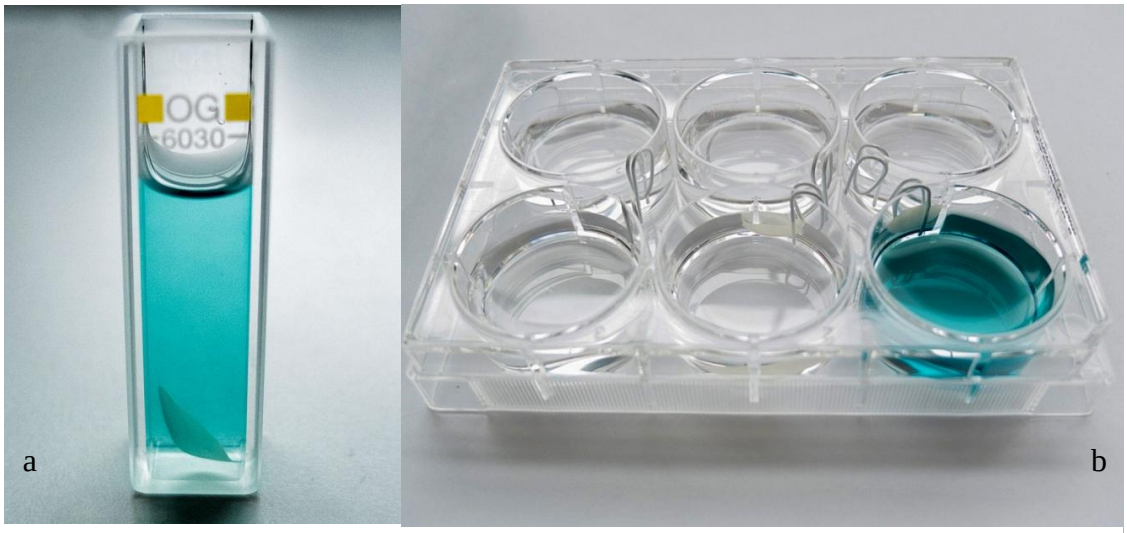


Figure 19 Measurement of the catalytic activity of the immobilized laccase using a cuvette (a) and a 6-well plate (b)

The efficiency of the immobilization procedure was expressed by three values (IY, AY and loading). The first one was the *immobilization yield* (IY). This value is given be following formula:

$$\text{IY}\ [\%] = \frac{\text{initial}\ [U] - \ \text{SN}\ [U]}{\text{initial}\ [U]} \cdot 100$$

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.

The second value is the *activity yield* (AY). It represents the actual activity of the laccase immobilized on the matrix. This value predicates the catalytic activity of the final product. It is given by following formula:

$$\text{AY}\ [\%] = \frac{\text{activity on the NFs}\ [U]}{\text{initial}\ [U]} \cdot 100$$

The last value is the *loading* and it stand for the amount of laccase immobilized on 1 gram of the matrix. Usually the activity is measured just in milligrams of the samples so the final loading is given by the activity $[U]$ on the matrix divided by the mass of the sample $[g]$. In other studies all these values can vary in their symbols as well as in their meanings.

4. 2. 3 Protein quantification assay

BCA protein assay is a method of quantification the protein amount in a testing solution. It is a colorimetric method based on the detection of cuprous cation of bicinchoninic acid (BCA) that comes from the reduction of Cu^{2+} to Cu^{1+} by proteins. In an alkaline environment the copper chelates with peptides containing three or more amino acid residues to form a light blue complex (G-Biosciences, 2015).

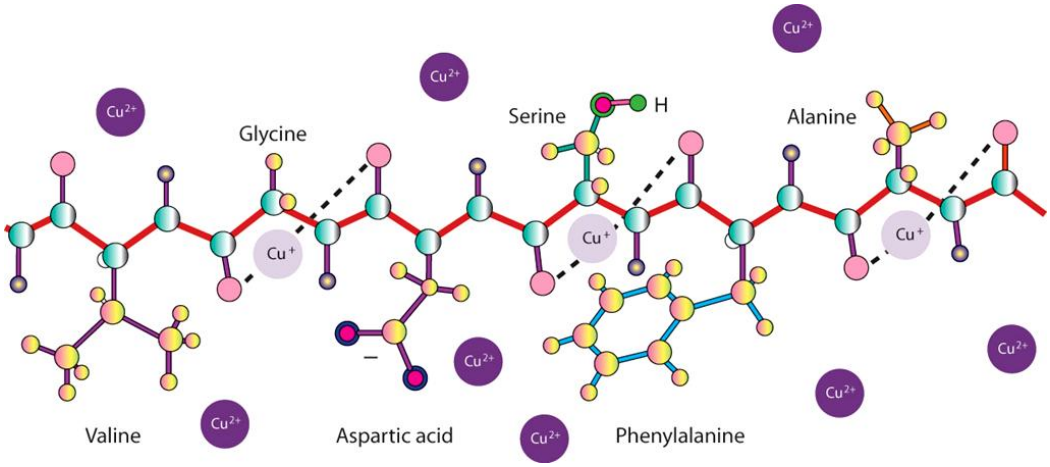


Figure 20 Chelation of copper with peptides (G-Biosciences, 2015)

In the second step BCA reacts with the reduced cuprous cation developing a purple-colored reaction product of two molecules of BCA chelated with one cuprous ion (Figure 21). The color intense can be measured by an absorbance at 562 nm (Figure 22). The absorbance grows linearly with the increasing protein concentration indicated by the amount of BCA/copper complex (A Thermo Fisher Scientific brand, 2015). The assay was carried out according to the manufacturer´s instructions using the microplate reader to measure the absorbance (Figure 23).

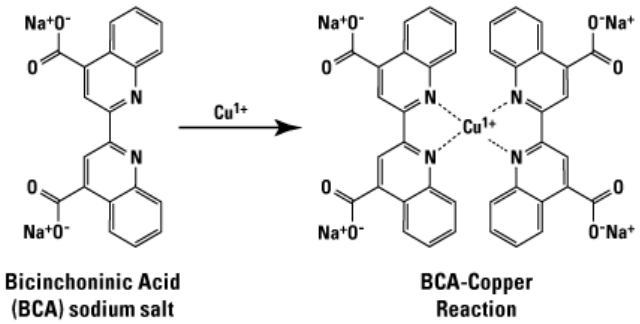


Figure 21 BCA/copper complex (A Thermo Fisher Scientific brand, 2015)

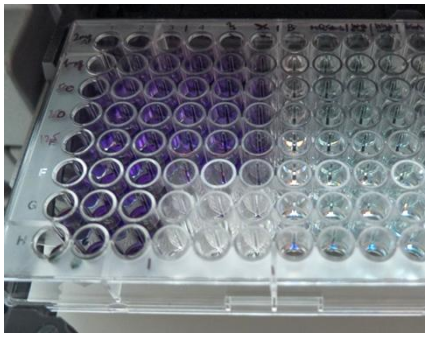


Figure 22 Color development of the reaction of BCA with cuprous cations

4. 2 .4 High-performance liquid chromatography

The degradation of endocrine disrupting chemicals (bisphenol A and 17α-ethinylestradiol) by PA6/CHIT samples was measured by HPCL Dionex Ultimate 3000 with a detector Ultimate 3000 Diode Array Detector. The column Phenomenex was 15 cm long with the diameter of 4,6 mm and pentafluorophenyl stationary phase containing core-shell particles of 2,7 µm. The temperature in the column was set on 40°C. The mobile phase consisted of ultrapure water as an aqueous phase and a mixture of methanol and acetonitrile 10:90 as an organic phase. The flow rate was 1,4 ml/min and the gradient mode of the aqueous and organic phase ratio changing in time is described by Figure 23.

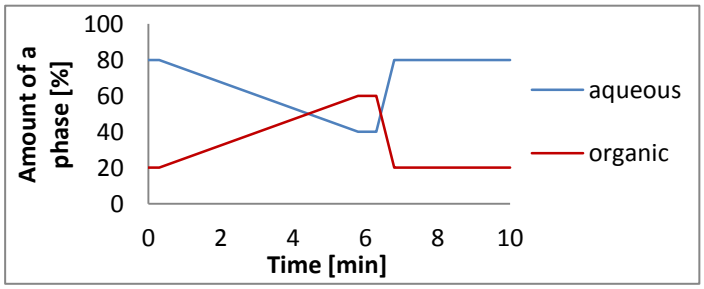


Figure 23 Gradient mode of HPLC phases

The optimal wavelength for the detection of both chemical compounds was 227 nm. Under these conditions the retention time for BPA was 5,6 minutes and 6 minutes for EE2. Figure 24 shows the calibration spectrograms for different concentrations of BPA and EE2 mixture starting with the concentration 50 mM.

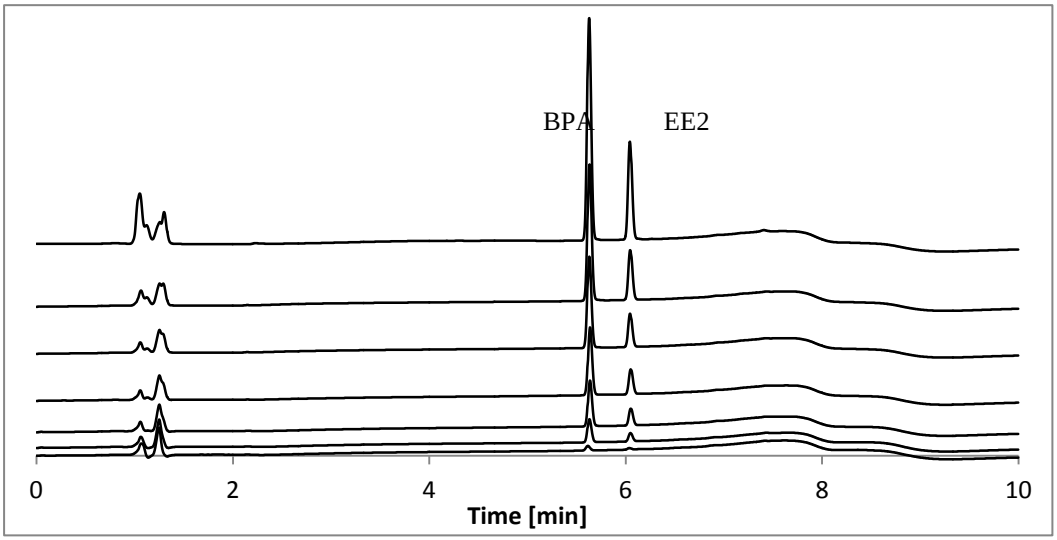


Figure 24 Calibration of BPA and EE2 mixture

Only PCL/SF samples were measured at the University of Santiago de Compostela using Waters HPLC and the column C18. The solvents were methanol and a mixture of methanol and ultrapure water 10:90. The flow rate was 0,5 ml/min and the optimal wavelength for both BPA and EE2 appeared to be 278 nm.

4. 3 Preparation of the nanofibrous matrices

This chapter describes fabrication of four nanofibrous layers used as the matrices for the laccase immobilization. Only PA6/CHIT (20wt%) was prepared by rod electrospinning. The other materials were fabricated by Nanospider technology using NS 1WS500U.

4. 3. 1 Polyamide 6 nanofibers

Spinning solution

Polyamide 6 (B27) pellets were dissolved in a mixture of formic acid and acetic acid (2/1 v/v) at 80°C to make a 14% solution.

Electrospinning

Nanospider™ NS 1WS500U

Scanning electron microscope (SEM, Vega 3 Tescan)

NIS-Elements AR software

- Applied Voltage..... -18,5/61,5 kV
- Wire speed..... 0,2 mm/s
- Distance between electrodes..... 150 mm
- Air flow..... 90 m^3/h
- The size of the girder, ø... 0,6
- Substrate take up speed... 360 mm/min
- Carriage speed..... 350 mm/s on 500 mm distance
- Substrate..... PP spunbond
- Temperature..... 22,4°C
- Humidity..... 35%
- Average fiber diameter... 236 nm
- Surface density.....4 g/m^2

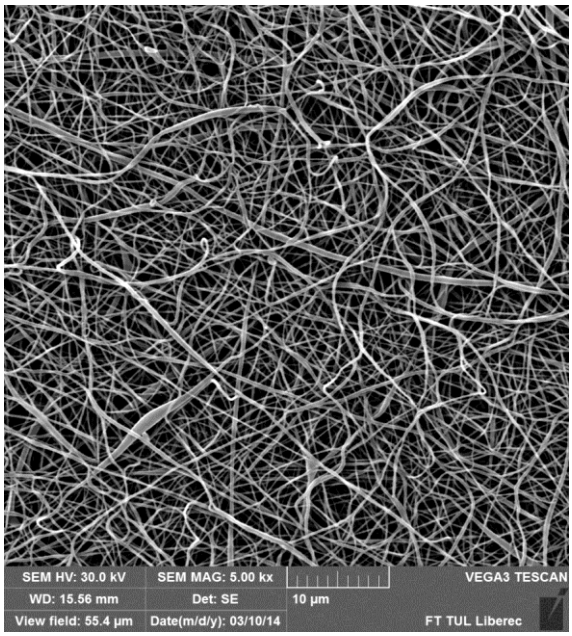


Figure 25 PA 6 nanofibers, SEM image, scale bar 10 µm

After-treatment

The fiber sheets were immersed in ethanol for 30 minutes, then washed with distilled water and eventually dried at room temperature. The final material is shown in Figure 25.

4. 3 2 Polyamide 6/chitosan (20wt%) nanofibers

Spinning solution

Polyamide 6 pellets were dissolved in a mixture of formic acid and acetic acid (2/1 v/v) at 80°C. Meanwhile chitosan 5 was dissolved in the same mixture of formic and acetic acid with the fixed concentration at 8wt% at room temperature overnight. Final spinning solution consisted of PA6/CHIT blend in the ratio 1/1 (w/w).

Electrospinning

Rod electrospinning

Scanning electron microscope (SEM, Vega 3 SB)

VEGA TC software

−Applied Voltage..... 50 kV

−Distance between electrodes..... 150 mm

−Rod diameter..... 20 mm

−Substrate..... PP spunbond

−Temperature..... 22°C

−Humidity..... 35%

−Average fiber diameter..... 240 nm

−Surface density..... 65 g/m^2

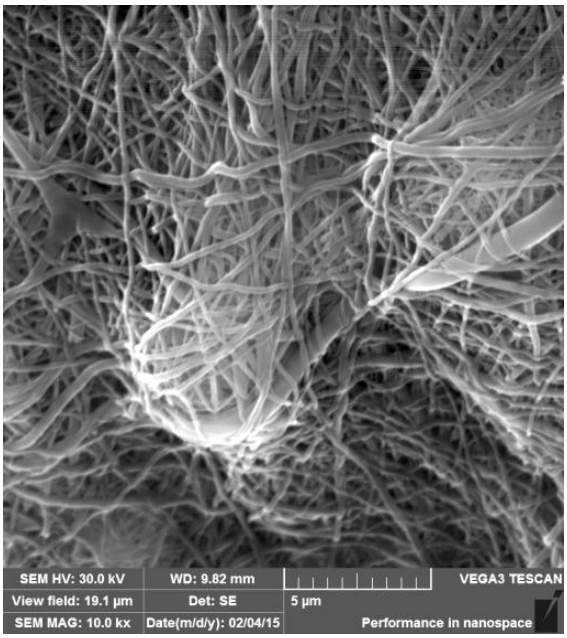


Figure 26 PA6/CHIT (20wt%) nanofibers, SEM image, scale bar 5µm

After-treatment

Fiber sheets were stabilized at 105°C for 15 minutes. Then they were immersed in 0,1M NaOH for 30 minutes to remove the acidic residues. Eventually they were washed with distilled water two times and dried at room temperature. The final material is shown in Figure 26.

4. 3. 3 Polycaprolactone/silk fibroin nanofibers

Spinning solution

The degummed silk fibres were dissolved in a mixture of formic acid and calcium chloride with the fixed concentration of SF at 12wt%. Polycaprolactone pellets were also dissolved in formic acid to prepare 20wt% polymer solution. Final spinning solution consisted of PCL/SF blend in the ratio 80/20 (w/w).

Electrospinning

Nanospider™ NS 1WS500U

Scanning electron microscope (SEM, Vega 3 Tescan)

NIS-Elements AR software

–Applied Voltage..... -10/50 kV

–Wire speed..... 3 mm/s

–Distance between electrodes..... 140 mm

–Air flow..... 90/100 m^3/h

–The size of the girder, ø..... 1.0

–Substrate take up speed.... 15 mm/min

–Carriage speed..... 450-500 mm/s on 500 mm distance

–Substrate..... PP spunbond

–Temperature..... 21,7 °C

–Humidity..... 23,5%

–Average fiber diameter.... 350 nm

–Surface density..... 6,2 g/m^2

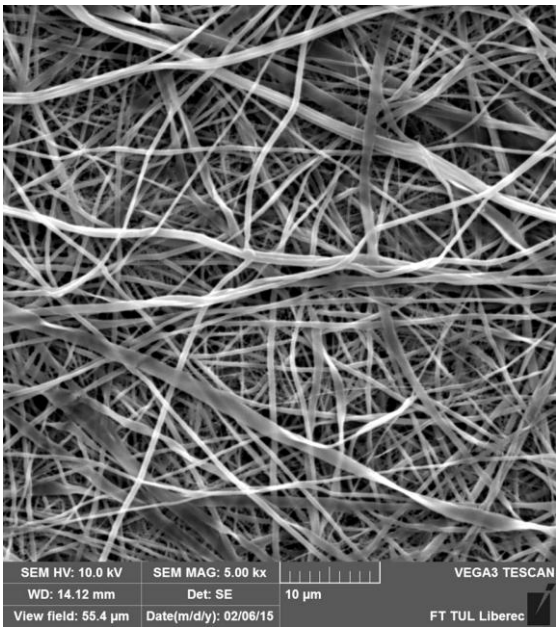


Figure 27 PCL/SF nanofibers, SEM image, scale bar 10 µm

After-treatment

The fiber sheets were immersed in ethanol for 30 minutes to induce crystallization of silk fibroin and to reduce their water solubility. After drying the fiber sheets were washed with distilled water overnight and then rinsed with DIW to get rid of residual salt and eventually dried at room temperature. The final material is shown in Figure 27.

4. 3. 4 Polyamide 6/chitosan nanofibers

Spinning solution

Polyamide 6 (B24) pellets were dissolved in a mixture of formic acid and acetic acid (2/1 v/v) at 80°C to make a 12,5% solution. Meanwhile chitosan 5 was dissolved in the same mixture of formic and acetic acid with the fixed concentration at 8wt% at room temperature overnight. Final spinning solution consisted of PA6/CHIT blend in the ratio 4/1 (w/w).

Electrospinning

Nanospider™ NS 1WS500U

Scanning electron microscope (SEM, Vega 3 SB)

VEGA TC software

- Applied Voltage..... -30/60 kV
- Wire speed..... 0,2 mm/s
- Distance between electrodes..... 175 mm
- Air flow..... 90/100 m^3/h
- The size of the girder, ø.... 0,7
- Substrate take up speed.... 15 mm/min
- Carriage speed..... 480-530 mm/s on 500 mm distance
- Substrate..... paper with silicon
- Temperature..... 23 °C
- Humidity..... 40%
- Average fiber diameter.... 185 nm
- Surface density..... 3 g/m^2

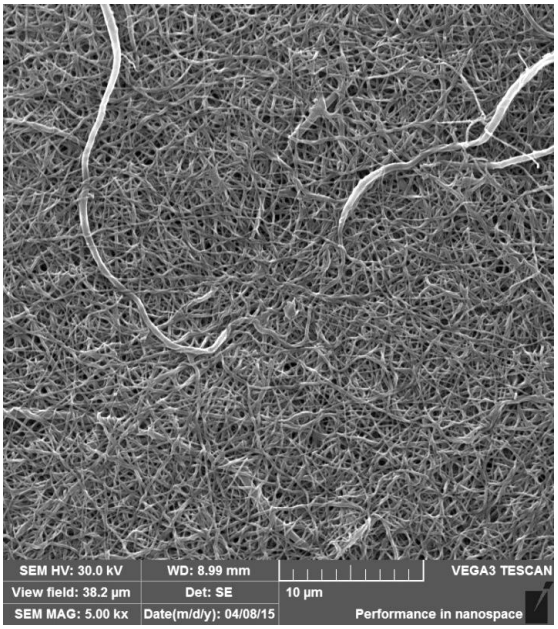


Figure 28 PA/CHIT (10wt%) nanofibers, SEM image, scale bar 10 µm

After-treatment

Fiber sheets were stabilized at 105°C for 15 minutes. Then they were immersed in 0,1M NaOH for 30 minutes to remove the acidic residues. Eventually they were washed with distilled water two times and dried at room temperature. The final material is shown in Figure 28.

4. 4 Immobilization methods

Selected enzymes were immobilized on different nanofibrous materials using different immobilization techniques according to the chemical morphology of chosen matrix (Figure 26). There were two main strategies – *enzyme adsorption on the nanofibrous layer followed by crosslinking* and *covalent attachment on a modified nanofibrous layer*. Adsorption technique required mainly optimal mechanical properties, hydrophilicity, chemical resistance and certain biocompatibility while materials for covalent binding were chosen according to the presence of specific reactive groups within the chemical structure.

Enzyme is a protein sensitive to chemical compounds therefore only nanofibrous materials with sufficient biocompatibility and chemical stability (polyamide 6, polycaprolactone and polyvinylpyrrolidone/polyvinylbutyral) were selected for the adsorption method. However only polyamide 6 appeared to be suitable enough and so the other materials were soon denied.

Polymers for covalent attachment were selected mainly for their free amine groups (chitosan, silk fibroin), enabling a modification via glutaraldehyde, or for the presence of carboxylic groups (polyacrylic acid) modified by EDAC and S-NHS. Amine groups showed to be more promising because of an effective modification via glutaraldehyde. Nanofibers from polyacrylic acid tended to swell in the aqueous environment which was not acceptable for enzyme attachment.

Picture 29 shows a schema of different applied immobilization techniques with described chemicals used for the matrix activation and enzyme attachment.

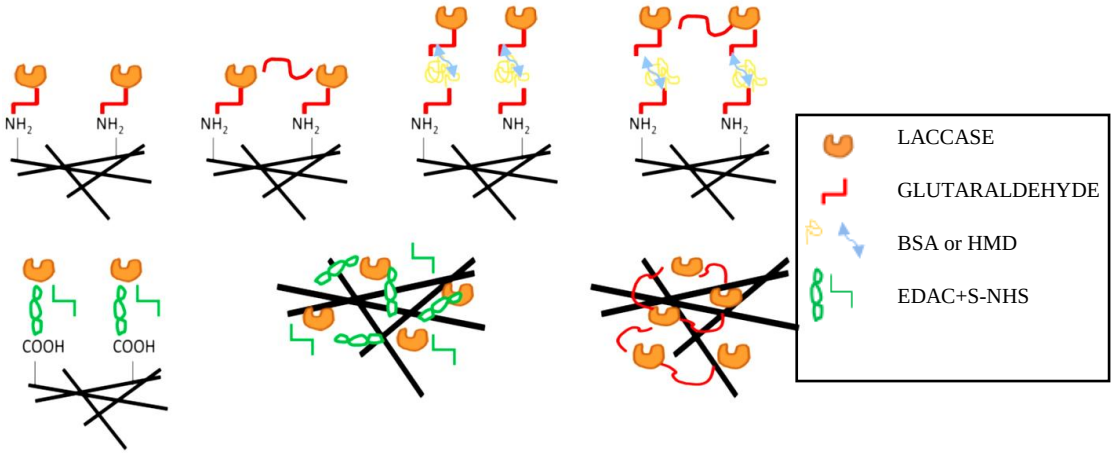


Figure 29 Schema of different applied immobilization techniques

Bovine serum albumin worked as a biocompatible layer that separated the immobilized enzyme from a direct contact with the nanofibrous matrix and in substance it extended the linkage together with two molecules of glutaraldehyde. The main reason for making a longer linkage from the matrix was to provide a larger space for the biocatalyst molecule with a more flexible attachment. Before the BSA was applied it was denatured by heat (100°C, 1 hour) in order to fix its structure and prevent it from its conformational changes that could damage the attached enzyme molecule (Da Silva, 1991).

Hexamethylenediamine had a similar role to extend the linkage with the enzyme. This reactive molecule did not provide a biocompatible environment however it did not require any treatment before it was applied (Da Silva, 1991).

4. 5 Degradation of EDCs

Selected samples with immobilized laccase from *Trametes versicolor* were tested for the degradation of a micropollutant mixture. The mixture consisted of 50μM bisphenol A and 50μM 17α-ethinylestradiol. 500μM stock solutions of these two chemicals were prepared separately by dissolving in methanol. These two solutions were mixed together and diluted with ultrapure water.

All prepared samples with immobilized laccase (1 mg of each, 2 replicates of each) were placed into glass vials with 3 ml of micropollutant mixture and these vials were constantly shaken in a water bath at 37°C. Blank samples that were prepared alike to the actual "samples" but in their case the immobilized laccase was inhibited by 10% sodium azide. The blanks were tested for possible absorption of the endocrine disruptors into the nanofibers. Additional vials contained certain amount of free laccase that approximately corresponded to the units of laccase immobilized on the samples.

In selected time intervals the supernatant from all vials was collected and measured by HPLC. The sampling consisted of 70 μl of the supernatant diluted by 140 μl of methanol and 1,5 μl of 2,8% sodium azide which was added in order to stop the degradation in case some of the laccase was collected within the supernatant.

After the first use all samples were removed from the reaction mixture, washed and stored in the ultrapure water at 4°C until they were used for the next trial.

The degradation of bisphenol A by laccase is well described in a study by Hou et al. (2014b). The final product of the multi step oxidation pathway is quinone (Figure 30).

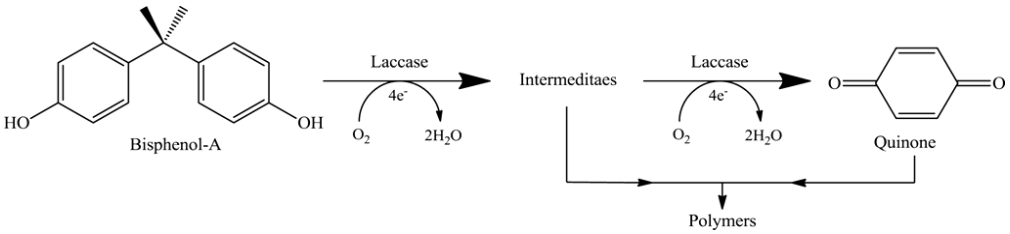
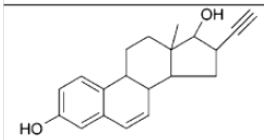
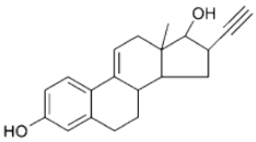
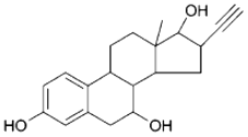
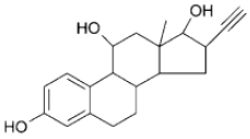


Figure 30 Degradation pathway of BPA (Hou et al., 2014b)

The oxidative degradation of EE2 by the laccase is not as well described in the literature as the degradation of BPA. There are few authors determining possible products of EE2 oxidation in their studies. A study of Kresinova et al. (2012) used the white rot fungus *Plerotus ostreatus* to remove the synthetic hormone 17α-ethinylestradiol. Metabolites were analyzed using gas chromatography and mass spectroscopy system. Table 5 shows the product of the laccase catalysis.

Table 5 Selected EE2 metabolites detected from a study by Kresinova et al. (2012)

Formula	Suggested metabolite structure	Metabolite related structure	Origin	Mw (CI)
	**19-nor-17α-pregna-1,3,5 (10), 6-tetraen-20-yne-3,17β-diol**	**dehydrogenated EE2**	MEG cultivation	294
	**19-nor-17α-pregna-1,3,5(10), 9 (11)-tetraen-20-yne-3,17 β-diol**	**dehydrogenated EE2**	MEG cultivation	294
	**19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,7 (or 6)-17β-triol**	**hydroxylated EE2**	MEG cultivation; mycelial degradation	312
	**19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,1 (or 2)-17β-triol**	**hydroxylated EE2**	MEG cultivation; mycelial degradation	312

5. Results and discussion

5. 1 Characterization of the soluble laccase from *Trametes versicolor*

Several properties of the purchased laccase from *Trametes versicolor* were analyzed. First of all the electrophoresis SDS-PAGE was applied to uncover the distribution of proteins with different molecular weight. This analysis was supported by BCA protein assay to determine the amount of protein in the stock laccase powder and eventually uncover a possible content of impurities. Then the catalytic activity was measured as well as the catalytic stability after two weeks of storage.

5. 1. 1 Electrophoresis

Figure 31 shows SDS-PAGE of the stock laccase. The lane 1 is the marker of standard proteins, lanes 2-4 are different dilutions of the laccase solution.

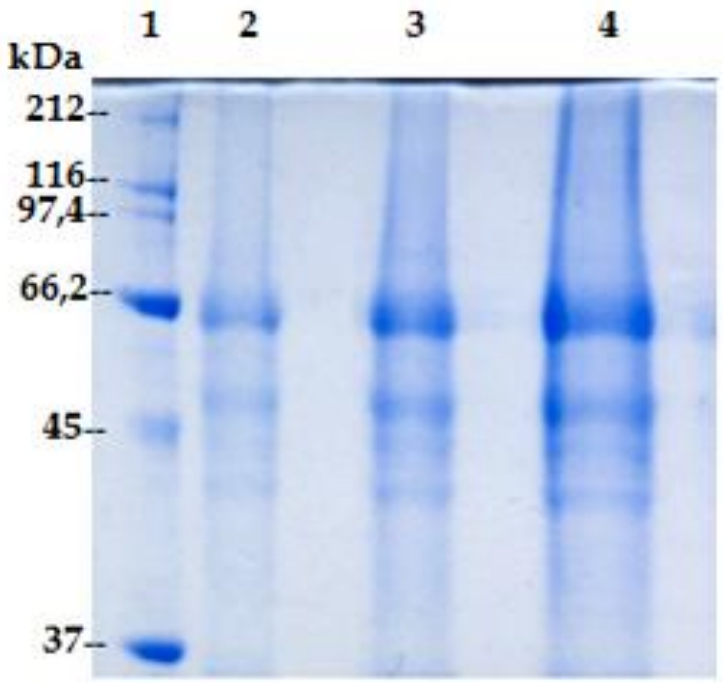


Figure 31 SDS-PAGE of laccase from *Trametes versicolor*, 12% stacking gel, dyed by Coomassie R250

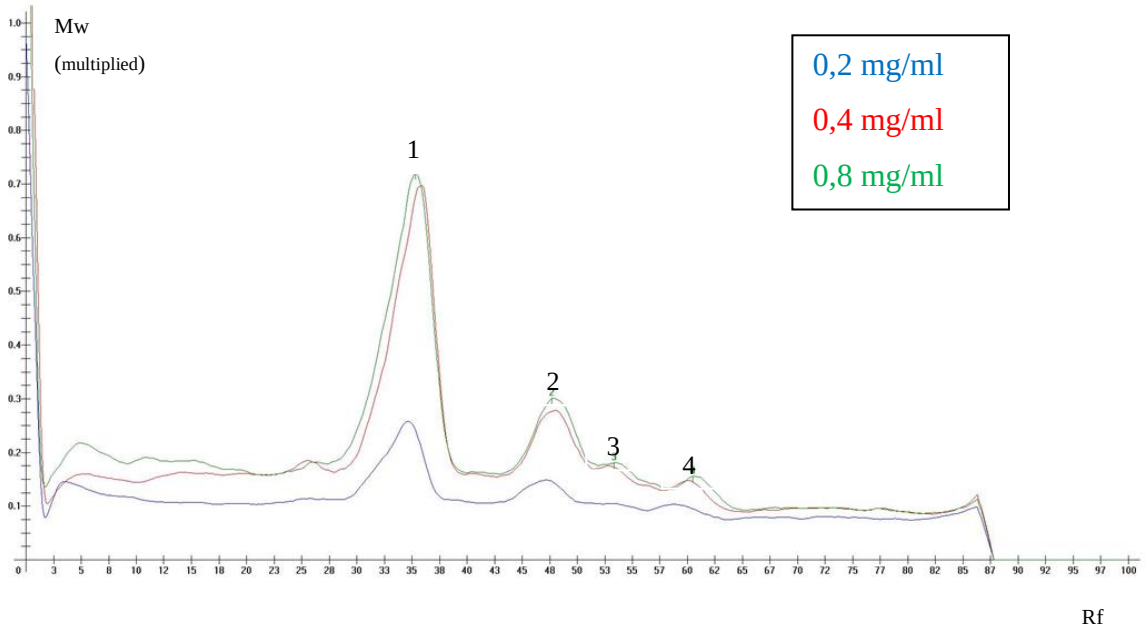


Figure 32 Analysis of SDS-PAGE (densitometric evaluation by the software Elfoman 2.0)

The molecular weight profile of the laccase from *Trametes versicolor* (Figure 31) corresponds to the profile of the cultivation medium described by Carabajal et al. (2013) and Brown et al. (2002). Based on the literature the laccase content is most likely represented by the bend near the area of the protein marker with 66,28 kDa. The other noticeable bends with 47, 41 and 34 kDa represent other proteins contained in the cultivation medium in which the laccase production took place.

Figure 32 and Table 6 show results of the SDS-PAGE analysis. There were four obvious bends on the gel represented by four peaks in the Figure 32. The average molecular weight of supposed laccase was 66,28 kDa.

Table 6 Molecular weight of the laccase from *Trametes versicolor* (calculated by Elfoman 2.0)

Peak	Molecular weight (Da)		
	Lane 2	Lane 3	Lane 4
1	67 360	65 304	66 177
2	48 538	47 265	47 686
3	41 569	41 569	41 020
4	35 600	34 361	33 908

5. 1. 2 Catalytic activity

Laccase powder was dissolved in ultrapure water at the concentration of 2 mg/ml. This stock solution was diluted to obtain required amount of laccase and the catalytic activity of each dilution was measured in 100 mM McIlvaine's buffer at pH 3.

Amount of laccase [µg]	Catalytic activity [U]
50	0,142639 ± 0,01561
25	0,09444 ± 0,02256
10	0,024074 ± 0,005266
4	0,010278 ± 0,000657
3	0,008264 ± 0,002165
1	0,003056± 0,000357

Table 7 Catalytic activity of laccase *Trametes versicolor* at pH 3

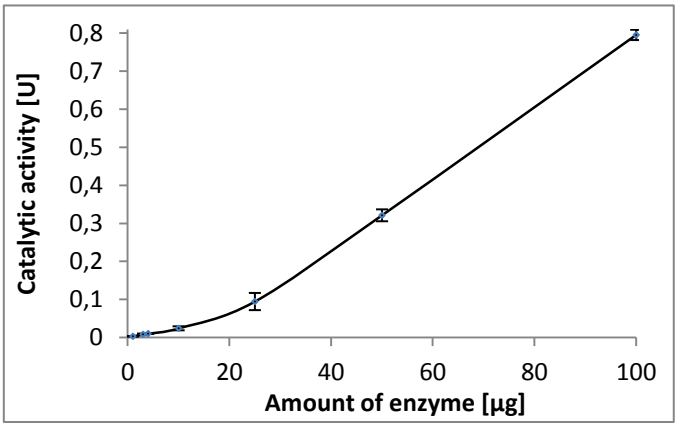


Figure 33 Catalytic activity of laccase from *Trametes versicolor*

Table 7 and Figure 33 show the catalytic activity of a certain amount of the crude laccase. Relation between the amount of the laccase and its catalytic activity is not linear probably because of considerable content of insoluble aggregates and impurities that disabled an equal dilution.

The highest activity of the laccase was achieved when using 100mM McIlvaine's buffer with pH 3. Lower molar concentrations and higher pH decrease the catalytic activity.

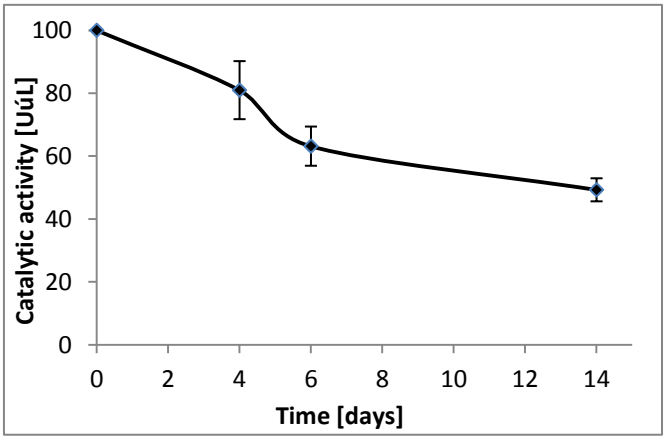


Figure 34 Storage stability of laccase solution

Laccase solution containing 2 mg of the powder enzyme per milliliter of ultrapure water lost around 51% of its initial activity after 14 days of storage at 4°C (Figure 34).

5. 1. 3 Protein determination

Bovine serum albumin and laccase were diluted in ultrapure water and the protein content was determined using Bicinchoninic Acid Kit. Samples of BSA and laccase were incubated at 37°C together with bicinchoninic acid solution and 4% (w/v) $CuSO_4 \cdot 5H_2O$ solution for 30 minutes and the wavelength for absorbance measurement was 562 nm.

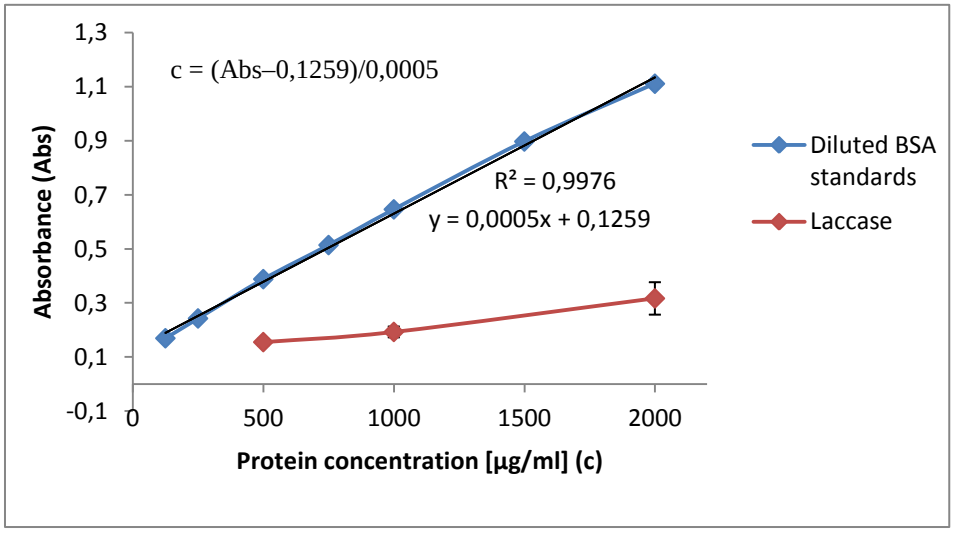


Figure 35 Comparison of BSA calibration curve and different dilutions of laccase from *Trametes versicolor*

BCA protein determination showed that the protein content of the laccase does not correspond to the prepared concentration of the laccase powder (Figure 35). In fact; 2 mg of the stock laccase powder contained only around 382 µg of protein which suggested that more than ¾ of the enzyme powder consisted of non-protein impurities, inactive protein content or insoluble aggregates of the enzyme. Such amount of impurities might have a negative impact on the immobilization process because these inactive molecules can attach to the matrix together with the active enzyme and decrease the diffusion of the substrate and its catalytic products.

5. 2 Immobilization on polyamide 6 nanofibers

Polyamide 6 nanofibrous layer was cut into small pieces. Each of them weighed 5 mg. These samples were washed with ethanol and distilled water and then placed in the solution with dissolved laccase for several hours. Consequently a crosslinking agent was added into the solution to stabilize molecules of the adsorbed enzyme and covalently attach some of the free enzyme in the reaction mixture to form a crosslinked catalytic layer surrounding the nanofibrous matrix.

After certain time these modified nanofibrous pieces were removed and washed with a buffer several times until no enzyme activity was detected in the washing. Immobilization procedure was optimized by changing following parameters;

Table 8 Variable parameters for enzyme adsorption followed by crosslinking

Buffer	McIlvaine	molar concentration	20 mM – 100 mM
		pH	3 – 7,8
	acetate buffer	molar concentration	10 mM – 100 mM
		pH	3 – 6
Enzyme	Tram. versicolor*	concentration in the solution	1 – 10 mg/ml
		solution volume	0,25 – 5 ml
Time for adsorption	1 – 24 hours		
Crosslinker	glutaraldehyde	concentration in the reaction mixture	0,5 – 20 % v/v
	EDAC**	molar ratio of enzyme: EDAC	12:1; 10:1; 6:1
	EDAC+S-NHS***	molar ratio of enzyme: EDAC: S-NHS	60:5:1; 30:10:1
Time for crosslinking	2 – 24 hours		
Temperature	4°C and 20°C		

* Trametes versicolor
** 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride
*** Sulfo-N-hydroxysuccinimide ester

Washed samples were placed in 100 mM McIlvaine buffer with pH3 to measure the activity of the laccase immobilized on the nanofibers. Supernatant (reaction mixture) and the washings were kept for the calculation of the immobilization yield.

5. 2. 2 Results

Table 9 shows the most relevant results comparing immobilization yields, activity yields and the final loadings of samples prepared by different methods. The results are organized into several sections of partially similar parameters of the immobilization process. This arrangement allowed a specification and a comparison of the parameters influencing the activity of prepared samples.

Table 9 Selected results – adsorption on polyamide 6 followed by crosslinking

Enzyme solution	Buffer	Time	Crosslinking	Time	IY [%]	AY [%]	Loading [U/g]
1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	5 h	10 % v/v GA	2 h	**60** ± 4	**0,01** ± 0,008	**15,1** ± 1,15
1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	5 h	5 % v/v GA	2 h	**56** ± 4	**0,06** ± 0,02	**11,6** ± 1,55
1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	5 h	1 % v/v GA	2 h	**85** ± 11	**0,09** ± 0,027	**18,2** ± 2,62
1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	5 h	0,5 % v/v GA	2 h	**70** ± 2,3	**0,09** ± 0,006	**17,7** ± 0,96
1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	24 h	1 % v/v GA	2 h	**76** ± 6	**0,05** ± 0,006	**11,2** ± 0,12
1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	5 h	1 % v/v GA	24 h	**59** ± 4,5	**0,06** ± 0,009	**10,9** ± 0,89
1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	5 h	1 % v/v GA	12 h	**55** ± 7,5	**0,05** ± 0,012	**10,3** ± 0,63
1 ml Trametes versicolor, 2 mg/ml	McIlvaine, pH 7.8, 100 mM, 4°C	24 h	1 % v/v GA	2 h	**68** ± 2	**0,07** ± 0,006	**13,9** ± 1,9
500 µl *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	5 h	1 % v/v GA	2 h	**82** ± 8,2	**0,8** ± 0,12	**18,6** ± 0,65
250 µl Trametes versicolor, 2 mg/ml	McIlvaine, pH 7.8, 100 mM	5 h	1 % v/v GA	2 h	**75** ± 3,5	**1** ± 0,3	**15,8** ± 2,8
500 µl *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 50 mM	5 h	1 % v/v GA	2 h	**69** ± 6,6	**0,8** ± 0,09	**16,5** ± 3,1
500 µl *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 7.8, 20 mM	5 h	1 % v/v GA	2 h	**65** ± 0,8	**0,8** ± 0,06	**18,2** ± 1,3
500 µl *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 3, 50 mM	5 h	1 % v/v GA	2 h	**65** ± 1,6	**0,1** ± 0,03	**22,7** ± 5
500 µl *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 4.5, 50 mM	5 h	1 % v/v GA	2 h	**67** ± 6,9	**0,9** ± 0,11	**20,3** ± 2,2
500 µl *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 6, 50 mM	5 h	1 % v/v GA	2 h	**79** ± 5	**0,7** ± 0,06	**11,8** ± 0,8

1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 4.5, 50 mM	5 h	10 % v/v GA	2 h	**65** ± 8	**0,1** ± 0,02	**20,8** ± 1,6
1 ml *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 4.5, 50 mM	5 h	5 % v/v GA	2 h	**58** ± 3,1	**0,1** ± 0,012	**26,8** ± 2,6
500 µl *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 4.5, 50 mM	5 h	10 % v/v GA	2 h	**67** ± 2	**0,9** ± 0,06	**26,3** ± 1,3
500 µl *Trametes versicolor*, 2 mg/ml	McIlvaine, pH 4.5, 50 mM	5 h	1 % v/v GA	2 h	**66** ± 4,2	**0,8** ± 0,13	**24** ± 2,5
500 µl *Trametes versicolor*, 2 mg/ml	Acetate, pH 4.5, 50 mM	5 h	1 % v/v GA	2 h	**42** ± 16	**0,1** ± 0,06	**8,6** ± 4,1

Time and temperature influence

Optimal time for enzyme adsorption was 5 hours and 2 hours for crosslinking via glutaraldehyde. Increasing time with lower temperature decreased the immobilization yield and the activity of the immobilized enzyme. Longer crosslinking period probably caused a loss of the catalytic activity due to an excessive number of bonds among the enzyme molecules.

Influence of the laccase concentration and volume of the reaction mixture

Laccase concentration was highly depending on its solubility and tendency to form aggregates. The optimal concentration with sufficient activity per millilitre was 2 mg of laccase. However; this amount of enzyme never fully dissolved and after some time it tended to form aggregates on the bottom of a vessel.

Volume of the reaction mixture was set mainly according to the size of the nanofiber samples so that they were fully submerged. The lowest possible volume for the 5 mg samples was 250 µl but 500 µl appeared to be optimal for enzyme loading.

Buffer influence

Type of buffer, molar concentration and pH appeared to be critical for enzyme immobilization. These parameters had a significant influence on the conformation of the protein, interactions with the matrix and the crosslinker and catalytic properties of the enzyme. Although the alkaline pH is recommended for irreversible crosslinking via glutaraldehyde, laccase showed the best results at acidic pH, possibly because the alkaline pH did not provide an optimal charge of the enzyme and the matrix creating strong interactions.

Influence of the crosslinker

Glutaraldehyde is a very effective crosslinker mainly regulated by its concentration in the solution, time, temperature and pH. It binds molecules of enzyme together via their amine groups that form a stable protein layer surrounding the support. Almost all samples crosslinked by GA were very stable during more than five catalytic cycles (stability around 90%) but the activity yield and the enzyme loading were very low.

EDAC or combination of EDAC and S-SHS connects itself to a carboxylic group on one side and amine group on the other side. However; these crosslinkers were not optimal for presented immobilization process because the immobilization yield was zero in all cases.

5. 2. 3 Summary

PA 6 nanofibers showed very good mechanical properties in a dry form as well as in a water environment and they had a sufficient structural stability in time with no marks of degradation. Therefore they were very easy to handle. However; they were not suitable for covalent attachment because of lack of amine groups. The adsorption-crosslinking method was ineffective in terms of the activity yield but very effective in terms of the operational stability when using 10% v/v GA for enzyme crosslinking.

Sathishkumar et al. (2014) reports an optimal time for the adsorption of laccase from *Pleurotus florida* as 30 minutes followed by crosslinking via 0,5% GA at 4°C overnight. In this case, the concentration of the enzyme was 500 U/50 ml of the laccase solution used for an incubation of 1 g of the matrix.

5. 3 Immobilization on polyamide 6/chitosan (20wt%) nanofibers

Nanofiber samples (each of 5 mg) were washed with ethanol and distilled water and modified via glutaraldehyde or combinations of glutaraldehyde, hexamethylenediamine and bovine serum albumin. After each modification step samples were thoroughly washed with distilled water. Bovine serum albumin was denaturized by heat (80°C for an hour) before using.

Samples modified according to techniques described by Silva et al. (1991) were placed into the enzyme solution for certain time. Than they were washed with a buffer several times until there was no activity detected in the washing. The immobilization procedure was optimized by changing following parameters;

Table 10 Variable parameters for covalent attachment on PA6/chitosan (20%) nanofibers

Buffer	McIlvaine	molar concentration	20 mM – 100 mM
		pH	3 – 7,8
	acetate buffer	molar concentration	10 mM – 100 mM
		pH	3 – 6
Enzyme	Tram. versicolor	concentration in the solution	0,5 – 2 mg/ml
		solution volume	0,25 – 1,5 ml
	Agar. bisporus*	concentration in the solution	2 mg/ml
		solution volume	0,25 – 1 ml
Modification of NFs	GA**	concentration	10% – 25% v/v
		reaction time	2 – 4 h
	BSA***	concentration	5 and 10 mg/ml
		reaction time	3 – 5 h
	HMD****	concentration	0,1 M
		reaction time	2 – 4 h
	Combinations	GA; GA-BSA-GA; GA-HMD-GA; GA-BSA-GA-HMD-GA; GA-HMD-GA-BSA-GA	
Time of the enzyme attachment	5 – 24 h		
Additional crosslinkers	glutaraldehyde	concentration in the reaction mixture	1 – 10 % v/v
	EDAC+S-NHS	molar ratio of enzyme:EDAC:S-NHS	60:5:1
Time of crosslinking	10 min – 2 h		
Additional chemicals	PEG*****	concentration in the reaction mixture	0,5 – 1 g/ml
	BSA	concentration in the reaction mixture	1 – 5 mg/ml
Temperature	4°C and 20°C		

* laccase from *Agaricus bisporus*
** glutaraldehyde
*** bovine serum albumin
**** hexamethylenediamine
***** polyethylene glycol

5. 3. 2 Results

Table 11 contains results of selected samples arranged into sections of similar immobilization techniques using different parameters. It appeared that the nanofibrous matrix prepared by rod electrospinning did not have adequately homogenous surface density which caused remarkable standard deviations of the activity measurements. Despite this fact; these samples helped to find an optimal direction of the immobilization process for the further experiments using more developed nanofibrous matrices (chapters 5.4 and 5.5).

Table 11 Selected results – covalent attachment on PA6/chitosan (20%) nanofibers

Modification	Enzyme solution	Time/ °C	Cross-linking	Time/ °C	IY [%]	AY [%]	Load. [U/g]
GA(2h, 20°C, pH 7.8, 10% v/v)	1 ml *T. versicolor*, 2 mg/ml, 10mM McIlvaine, pH 7.8	5 h, 20°C	-	-	**65** ± 8,2	**0,05** ± 0,01	**8,3** ± 1,6
GA(2h, 20°C, pH 7.8, 10% v/v)	1 ml *T. versicolor*, 2 mg/ml, 100mM McIlvaine, pH 7.8	5 h, 20°C	GA(10% v/v)	2h, 20°C	**63** ± 6,3	**0,02**± 0,004	**5,06** ± 0,9
GA(2h, 20°C, pH 7.8, 20% v/v)	1 ml *T. versicolor*, 2 mg/ml, 100mM McIlvaine, pH 7.8	5 h, 20°C	-	-	**77** ± 12,5	**0,02** ± 0,012	**11,3** ± 2,7
GA(2h, 20°C, pH 7.8, 25% v/v)	500 µl *T. versicolor*, 2 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**46** ± 6,2	**3,7** ± 0,64	**19,4** ± 2,5
GA(2h, 20°C, pH 7.8, 20% v/v)	250 µl *T. versicolor*, 2 mg/ml, 100mM McIlvaine, pH 7.8	5 h, 20°C	-	-	**21** ± 3,8	**0,2** ± 0,06	**13,8** ± 1,3
GA(2h, 20°C, pH 7.8, 20% v/v)	250 µl *T. versicolor*, 2 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**44** ± 10,1	**0,4** ± 0,12	**17,8** ± 6,3
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	500 µl *T. versicolor*, 2 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**7** ± 1,2	**0,1** ± 0,06	**13,3** ± 0,76
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	500 µl *T. versicolor*, 1 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**7,3** ± 2,1	**0,5** ± 0,08	**6,7** ± 0,77
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	250 µl *T. versicolor*, 2 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**38** ± 3,9	**0,6** ± 0,2	**29,3** ± 6,3
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	250 µl *T. versicolor*, 1 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**61** ± 4	**1,2** ± 0,65	**37,1** ± 2,5

GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	250 µl *T. versicolor*, 0.5 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**78** ± 14,3	**3,2** ± 1,2	**16,4** ± 3,4
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	500 µl *A. bisporus*, 2 mg/ml, 100mM McIlvaine, pH 6	5 h, 20°C	-	-	**82** ± 13	**7,6** ± 1,9	**27,5** ± 2
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 0.5 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	GA(0,5 % v/v)	1h, 20°C	**36** ± 6,7	**0,2** ± 0,1	**7,3** ± 1,3
GA-HMD-GA-BSA(3h, 30°C , pH 7.8, 1 mg/ml)-GA	500 µl *T. versicolor*, 1 mg/ml, 100mM McIlvaine, pH 7.8	5 h, 20°C	-	-	**5,8** ± 0,6	**0,4** ± 0,21	**9,2** ± 1,3
GA-HMD-GA-BSA(3h, 30°C , pH 7.8, 1 mg/ml)-GA	500 µl *T. versicolor*, 1 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**23** ± 8	**0,7** ± 0,21	**11,4** ± 2
GA(2h, 20°C, pH 7.8, 12.5%v/v)-BSA(3h, 20°C, pH 7.8,1 mg/ml)-GA(2h, 20°C, pH 7.8, 12.5%v/v)	500 µl *T. versicolor*, 1 mg/ml, 100mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**4** ± 1,6	**0,2** ± 0,05	**5** ± 2,74
GA(2h, 20°C, pH 7.8, 12.5%v/v)-BSA(3h, 20°C, pH 7.8,1 mg/ml)-GA(2h, 20°C, pH 7.8, 12.5%v/v)	500 µl *A. bisporus*, 2 mg/ml, 100mM McIlvaine, pH 6	5 h, 20°C	-	-	**13** ± 8	**0**	**0**
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 1 mg/ml, 100mM acetate, pH 4.5	5 h, 20°C	-	-	**41** ± 5,3	**1,3** ± 0,6	**18,8** ± 4,2
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	500 µl *T. versicolor*, 1 mg/ml, 50mM acetate, pH 4.5	5 h, 20°C	-	-	**32** ± 3	**0,6** ± 0,09	**7,3** ± 0,7
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	500 µl *T. versicolor*, 1 mg/ml, 20mM acetate, pH 4.5	5 h, 20°C	-	-	**32** ± 4,7	**1,6** ± 0,8	**24,4** ± 8
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 1 mg/ml, 50mM McIlvaine, pH 3	5 h, 20°C	-	-	**26** ± 1,7	**2** ± 0,9	**22** ± 7,5
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 1 mg/ml, 50mM McIlvaine, pH 4.5	5 h, 20°C	-	-	**27** ± 8	**0,2** ± 0,04	**6,8** ± 3,8
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 1 mg/ml, 50mM McIlvaine, pH 7.8	5 h, 20°C	-	-	**16** ± 1,7	**0,1** ± 0,03	**4,3** ± 1

Time and temperature influence

Obtained results were not reliable because of an uneven structure and a heterogeneous surface density of the nanofiber layer. Longer immobilization process at lower temperature had no positive influence on the activity of the immobilized enzyme. In addition, some of the nanofiber samples were damaged by the acidic buffer after 20 hours which had a negative impact on the activity of the captured catalyst.

Influence of the modification

Experiments showed that more concentrated glutaraldehyde used for the modification of PA6/chitosan nanofibers did not necessarily lead to an enhancement of the immobilization yield but it led to a higher activity of the samples. This phenomenon could have been caused by the presence of a higher number of reactive groups providing multiple covalent bonds with the enzyme that were able to stabilize its native structure. However; there was a very significant irregularity of the results.

Hexamethylenediamine and bovine serum albumin had a positive influence on the immobilization process because they extended the link between nanofibers and the enzyme. However; results did not show which of these chemicals was more beneficial. Creation of new chemical bonds between glutaraldehyde and amine groups of the nanofibers, BSA and HMD were adherent to a significant change of color of the samples changing from white into light brown.

Influence of the laccase, its concentration and volume of the reaction mixture

Laccase from *Agaricus bisporus* was not a better choice for the immobilization process because it had very low activity compared to *Trametes versicolor*. The lower was the volume of the solution the more effective was the immobilization procedure. The minimal volume that kept the samples submerged was 250 μl.

Buffer influence

Experiments showed that the laccase preferred an acidic buffer (pH 3 or 4,5) for the immobilization possibly because the transfer from alkaline environment, used for the immobilization, to the acidic buffer, required for the activity measurement, was too harmful for the protein structure. The laccase showed minimum activity at the alkaline buffer and the same problem appeared when using the acetate buffer because even in this case the matrix with laccase needed to be transferred into the McIlvaine buffer for the activity measurement. The acidic citrate-phosphate buffer with pH 4was also the

optimal environment for the covalent attachment of laccase in studies by Xu et al. (2013, 2015).

Influence of supporting chemicals

Polyethylene glycol increased viscosity of the enzyme solution and its volume however it did not have neither negative nor positive impact on the immobilization efficiency. Results were in the same range as the similar samples without PEG (Cabana et al., 2009).

Bovine serum albumin was added into the catalyst mixture to improve the enzyme attachment on the modified nanofibers (Nair et al., 2013). However; the competition of these two proteins led to a minimal immobilization yield.

Influence of the crosslinker

Additional crosslinkers with different concentrations did not have a positive influence on the immobilization process.

5. 3. 3 Summary

Polyamide 6/chitosan (20wt%) nanofiber samples were not suitable for enzyme immobilization. The reason does not consist in the nature of the polymers but in the structure of the nanofibrous sheet and its macroscopic properties. The rod electrospinning technology is usually applied for testing new polymer materials because of a low consumption of the spinning solution and easy parameter adjustment. However; this technology is a discontinuous process producing uneven nanofibrous layers with a wide range of surface densities and fiber diameters.

The average surface density of described layers was 65 g/m^2 which might be very profitable for manipulation with the material and stability of the mechanical properties. However; the thickness of the matrix might have caused some serious diffusional limitations for the enzyme immobilized under the surface of the textile. Laccase molecules were small enough to fit in the pores between nanofibers and make a linkage with glutaraldehyde,, however the substrate diffusion might have been strongly limited.

The best achieved activity of the laccase immobilized on polyamide/chitosan nanofibers manufactured by rod electrospinning was only around 37 U/g of the NFs. This sample did not excel by its operational stability because there was a significant loss

of its activity after the first catalytic cycle. This low stability could have been caused by inherent enzyme stability, enzyme leakage or increasing diffusional limitation caused by an adsorption of the ABTS cation into the polymer structure.

5. 4 Immobilization on polycaprolactone/silk fibroin nanofibers

5 mg samples were washed with ethanol and distilled water and modified analogous to PA6/chitosan (20%) nanofibers although in case of silk fibroin there was expected presence of markedly lower number of amine groups per milligram of nanofibers. Modified nanofibers were inserted into the enzyme solution for certain time as previous described.

Table 12 Variable parameters for covalent attachment on silk fibroin/PCL nanofibers

Buffer	McIlvaine	molar concentration	20 mM – 100 mM
		pH	3 – 7,8
	acetate buffer	molar concentration	20 mM – 100 mM
		pH	3 – 6
Enzyme	Tram. versicolor	concentration in the solution	0,5 – 2 mg/ml
		solution volume	0,25 – 2 ml
Modification of NFs	GA	concentration	10% – 25% v/v
		reaction time	2 – 4 h
	BSA	concentration	5 and 10 mg/ml
		reaction time	3 – 5 h
	HMD	concentration	0,1 M
		reaction time	2 – 4 h
	Combinations	GA; GA-BSA-GA; GA-HMD-GA;	
Time of the enzyme attachment	5 – 20 h		
Additional crosslinkers	glutaraldehyde	concentration in the reaction mixture	0,5 – 1 % v/v
		time	1– 2 h
Additional chemicals	PEG	concentration in the reaction mixture	0,5 – 1 g/ml
Temperature	4°C and 20°C		

5. 4. 2 Results

The immobilization methods using PCL/SF samples were analogous to the PA6/CHIT (20wt%) samples but the results were more promising in case of the PCL/SF. These nanofibrous layers had required homogenous surface density which ensured lower standard deviation in activities of measured duplicates. In case of several samples the loading exceeded 100U/g of the support which was a very promising result.

Table 13 Selected results – covalent attachment on SF/PCL nanofibers

Modification	Enzyme solution	Time/ °C	Cross-linking	Time/ °C	IY [%]	AY [%]	Loading [U/g]
GA(2h, 20°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 1 mg/ml, 100mM acetate, pH 4.5	5h, 20°C	-	-	**21** ± 2,7	**2,1**± 0,6	**7,7** ± 0,3
GA(2h, 20°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 1 mg/ml, 100mM McIlvaine, pH 4.5	5h, 20°C	-	-	**3** ± 1,8	**0,6** ± 0,2	**9,8** ± 1
GA(2h, 20°C, pH 7.8, 12.5% v/v)	2 ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	5h, 20°C	-	-	**6,7** ± 1,1	**0,6** ± 0,16	**14,4** ± 2,8
GA(2h, 20°C, pH 7.8, 12.5% v/v)	2 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	5h, 20°C	-	-	**3,6** ± 1,5	**0,1** ± 0,06	**6,2** ± 0,6
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 1 mg/ml, 20mM acetate, pH 4.5	5h, 20°C	-	-	**7,5** ± 0,9	**5,3** ± 1,4	**35,6** ± 6,4
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 1 mg/ml, 50mM acetate, pH 6	5h, 20°C	-	-	**9,1** ± 1,2	**0,7** ± 0,06	**12,2** ± 2,5
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	2 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	5h, 20°C	-	-	**9,3** ± 1,4	**1,1** ± 0,2	**13,2** ± 6,1
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	5h, 20°C	-	-	**16,9** ± 2,5	**4,7** ± 1,3	**80,2** ± 5,4
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 4,5	5h, 20°C	-	-	**10,5** ± 1,6	**0,8** ± 0,12	**19,8** ± 2,2
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 6	5h, 20°C	-	-	**2,8** ± 0,8	**0,1** ± 0,01	**12,5** ± 4,7
GA(2h, 30°C, pH 7.8, 12.5% v/v)-HMD(3h, 30°C, DIW, 0.1M)-GA(2h, 30°C, pH 7.8, 12.5% v/v)	1 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 7,8	5h, 20°C	-	-	**0**	**0**	**0**
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	5 h, 20°C	-	-	**15,2** ± 2	**4,4** ± 1,3	**65,8** ± 10,9
GA(2h, 20°C, DIW, 12.5%v/v) -BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 4,5	5 h, 20°C	-	-	**14,8** ± 1,5	**6,5** ± 2,3	**21,6** ± 1,1
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 6	5 h, 20°C	-	-	**15,6** ± 6,2	**5,2** ± 2,8	**16,4** ± 4,4

GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 7,8	5 h, 20°C	-	-	**19,8** ± 2,5	**3,9** ± 0,9	**13,8** ± 1,6
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	5 h, 20°C	GA(0,5% v/v)	2h, 20°C	**26,8** ± 3,6	**5,3** ± 1,3	**55,2** ± 4,8
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	5 h, 20°C	-	-	**18,5** ± 2,6	**6,2** ± 1,9	**75,5** ± 5,9
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	20 h, 4°C	-	-	**23,6** ± 4,8	**10,2** ± 2,1	**156,6** ± 12,4
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 4,4	20 h, 4°C	-	-	**26,2** ± 1,9	**3,9** ± 0,4	**38** ± 5,7
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,5 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	20 h, 4°C	-	-	**21,7** ± 5,1	**9,4** ± 1,6	**121,2** ± 13,6
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,5 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 4,4	20 h, 4°C	-	-	**0**	**0**	**0**
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,5 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1 ml *Tram. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3 + 500 µg PEG	20 h, 4°C	-	-	**36,4** ± 4,8	**2,6** ± 1,2	**166** ± 10,4
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,5 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3 + 500 µg PEG	20 h, 4°C	GA(0,5% v/v)	2h, 20°C	**46,8** ± 10,3	**4,9** ± 1	**94,8** ± 8,7
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,5 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	20 h, 4°C	-	-	**18,9** ± 1,6	**3,3** ± 0,06	**104,8** ± 12,9
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,5 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	20 h, 4°C	GA(0,5% v/v)	2h, 20°C	**21,6** ± 5,3	**1,2** ± 0,07	**70,6** ± 8
GA(2h, 20°C, pH 7.8, 12.5%v/v)-BSA(3h, 20°C, pH 7.8,5 mg/ml)-GA(2h, 20°C, pH 7.8, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	20 h, 4°C	-	-	**19,4** ± 4,2	**1,9** ± 0,06	**43,9** ± 6,1
GA(2h, 20°C, pH 7.8, 12.5%v/v)-BSA(3h, 20°C, pH 7.8,5 mg/ml)-GA(2h, 20°C, pH 7.8, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	20 h, 4°C	GA(0,5% v/v)	2h, 20°C	**23,9** ± 1,9	**0,98** ± 0,0,7	**22,8** ± 2,6

GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,5 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 100mM McIlvaine, pH 3	20 h, 4°C	-	-	**38,4** ± 3,7	**0,23** ± 0,09	**16,4** ± 4,8
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW 1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	-	-	**4,3** ± 1,1	**91** ± 3,6	**244,8** ± 20,5
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	GA(1% v/v)	2h, 20°C	**76,2** ± 6,2	**0,1** ± 0,03	**38,8** ± 6,4
GA(2h, 20°C, DIW, 12.5% v/v)-HMD(3h, 20°C, DIW, 0.1M)-GA(2h, 20°C, DIW, 12.5% v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	GA(5% v/v)	2h, 20°C	**68,2** ± 5,3	**0,14** ± 0,05	**18,5** ± 2,6
GA(2h, 20°C, DIW, 12.5% v/v)-HMD(3h, 20°C, DIW, 0.1M)-GA(2h, 20°C, DIW, 12.5% v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	GA(10% v/v)	2h, 20°C	**74,4** ± 8,1	**0,11** ± 0,02	**25** ± 1,3
GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	GA(10% v/v)	2h, 20°C	**44,6** ± 3,1	**0,13** ± 0,04	**21,4** ± 4,3
GA(2h, 20°C,milli-Q, 12.5%v/v)-BSA(14h,20°C, milli-Q,1mg/ml)-GA(2h, 20°C,milli-Q, 12.5%v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20 mM McIlvaine, pH 3	20 h, 4°C	-	-	**25,1** ± 6,7	**4,3** ± 1,2	**95** ± 8,4

Time and temperature influence

The best results were achieved when the laccase was immobilized for 20 hours at 4°C. The enzyme probably needed a slower reaction with the modified matrix to form an active conformation obtaining an accessible active site. This might be the reason why an additional crosslinker inactivated most of the enzyme immobilized for 20 hours by binding to amine groups in the active sites. Samples where the laccase was immobilized only for 5 hours at 20°C did not show such remarkable inactivation compared to the similar samples without the additional crosslinker.

Influence of the modification

Three hours for modification via GA and four hours for BSA or HMD spacing under alkaline pH around 8 and 40°C were used in a study by Da Silva et al. (1991). Silva et al. (2007) also used alkaline pH (9,5) for introducing of GA and HMD during 2-hour and 4-hour reactions. However; longer time for each modification step other than described in Table 13did not show any improvement in activity of the immobilized

laccase. Using distilled water for glutaraldehyde and BSA or HMD solutions was eventually more efficient than a citrate-phosphate buffer with pH 7.8 that was supposed to ensure more stable Schiff base. Glutaraldehyde aqueous solution was also used in studies by Xu et al. (2013, 2015).

During the first trials bovine serum albumin appeared to be a better modification compound compared to hexamethylendiamine therefore HMD was excluded from subsequent experiments with SF/PCL nanofibers.

Influence of the laccase concentration and volume of the reaction mixture

The best results were achieved when using laccase solution with the concentration of 2 mg/ml in 20mM McIlvaine's buffer with pH 3. The lowest volume acceptable for the nanofibrous samples was 500 µl which appeared to be the most efficient amount of the enzyme solution with the highest achieved activity after immobilization.

Buffer influence

As mentioned, the molar concentration and pH played a very important role in enzyme immobilization. The optimal pH was 3 and the most preferable concentration was 20mM. Xu et al. (2013) and Liu et al. (2012) used 0,1M citrate-phosphate and 0,1M citrate buffers both with pH 4 for the immobilization of the laccase from *Trametes versicolor*.

Influence of supporting chemicals

The influence of polyethylene glycol on the immobilization efficiency is unclear. However; it was observed that PEG increased viscosity of the laccase solution and positively influenced its solubility with lower marks of the enzyme sedimentation on a bottom of a vial.

Influence of the crosslinker

In case of all samples the additional crosslinker had a negative influence on the immobilization efficiency.

5. 4. 3 Summary

PCL/SF showed to be a promising material for enzyme immobilization for its sufficient mechanical stability and affinity to the enzyme. After long immobilization periods it tended to degrade and partially break down into small filaments, especially

when stored at low pH. This fact might have affected the storage stability of the immobilized enzyme. The highest achieved loading of the immobilized laccase was more than 240 U/g which is a reasonable result with regard to the considerable amount of impurities or inactive protein in the stock laccase. The best immobilization procedure included a modification via GA-BSA-GA and 20 hours for the laccase attachment at pH 3 and 4°C.

The highest achieved activity is comparable to the loading of 224 U of the immobilized laccase from *Trametes versicolor* per gram of Fe_2O_3/SiO_2 nanoparticles described in the study by Zheng et al. (2012).

5. 5 Immobilization on polyamide/chitosan (10wt%) nanofibers

Nanofiber sheets were cut into circles with the diameter of 120 mm. Each sample weighed 1± 0,2 mg. Samples were washed with ethanol and distilled water and modified analogous to PA/chitosan (20%) nanofibers. Modified nanofibers were inserted into the enzyme solution for certain time.

Table 14 Variable parameters for covalent attachment on silk fibroin/PCL nanofibers

Buffer	McIlvaine	molar concentration	20 mM – 100 mM
		pH	3 – 7,8
Enzyme	Tram. versicolor	concentration in the solution	2 mg/ml
		solution volume	0,5 – 1 ml
Modification of NFs	GA	concentration	12.5% – 25% v/v
		reaction time	2 – 4 h
	BSA	concentration	1 mg/ml
		reaction time	3 – 5 h
	HMD	concentration	0,1 M
		reaction time	2 – 4 h
	Combinations	GA-BSA-GA; GA-HMD-GA;	
Time of the enzyme attachment	20 h		
Temperature	4°C and 20°C		

5. 5. 2 Results

The immobilization procedures using PA6/CHIT (10wt%) as a matrix were based on the best results collected from the previous chapters. The nanofibrous layers were fabricated specially for the purposes of the enzyme immobilization. The developed material combined mechanical properties of PA 6 and PA6/CHIT (20wt%) nanofibers with an enhanced specific surface area compared to the PA6/CHIT matrix prepared by rod electrospinning.

Table 15 Selected results – covalent attachment on PA6/CHIT nanofibers

Modification	Enzyme solution	Time/ °C	Cross-linking	Time/ °C	IY [%]	AY [%]	Loading [U/g]
GA(2h, 20°C, pH 7.8, 12.5%v/v)-BSA(3h, 20°C, pH 7.8,5 mg/ml)-GA(2h, 20°C, pH 7.8, 12.5%v/v)	1 ml *T. versicolor*, 2 mg/ml, 50mM McIlvaine, pH 3	20h, 20°C	-	-	**30,1** ± 8,3	**0,35** ± 0,03	**60,8** ± 9,3
GA(2h, 20°C, milli-Q, 12.5%v/v)-BSA(3h, 20°C, milli-Q,1 mg/ml)-GA(2h, 20°C, milli-Q, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20h, 4°C	-	-	**3,43** ± 1,7	**16,2** ± 3,2	**150,8** ± 21,6
GA(2h, 20°C, milli-Q, 12.5%v/v)-BSA(3h, 20°C, milli-Q,1 mg/ml)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5 ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20h, 4°C	-	-	**50,9** ± 6,9	**6,4** ± 1,3	**150** ± 13,1
GA(2h, 20°C, milli-Q, 12.5%v/v)-BSA(3h, 20°C, milli-Q,1 mg/ml)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5 ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20h, 4°C	GA(10% v/v)	2h, 20°C	**63,9** ± 8,5	**1,8** ± 0,9	**18,5** ± 2,1
GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	1ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20h, 20°C	-	-	**61,3** ± 12,9	**0,6** ± 0,03	**117,2** ± 12,7
GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	-	-	**46,3** ± 3,9	**7,3** ± 2,1	**148** ± 21,3
GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	GA(5% v/v)	2h, 20°C	**50** ± 8,3	**4,3** ± 1,7	**42,7** ± 11,1
GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml *T. versicolor*, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	GA(10% v/v)	2h, 20°C	**61,6** ± 3,5	**0,9** ± 0,11	**8,9** ± 2,1
GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml *T. versicolor*, 2 mg/ml, milli-Q	20 h, 4°C	-	-	**31,4** ± 3,4	**11,5** ± 4,2	**220,5** ± 5,9

Influence of the modification

Both types of modifications (via bovine serum albumin and hexamethylene-diamine as a middle step) had similar results over 100 U/g. Using HMD instead of BSA was more advantageous because it did not require the denaturation step.

Buffer influence

McIlvaine's buffer with pH 7.8 was not an efficient solvent for the modification procedures. Using distilled water or ultrapure water for this purpose was more convenient. On the other hand, the 20mM McIlvaine's buffer pH 3 was an optimal solvent for the laccase immobilization although the last sample using ultrapure water had a higher activity of the immobilized laccase. In the chapter "5.6 Stability of the immobilized enzyme" it is proved that the non-buffer environment did not provide a stable enzyme attachment to the matrix.

Influence of the crosslinker

In case of all samples the additional crosslinker had a negative influence on the immobilization efficiency.

5. 5. 3 Summary

PA6/CHIT (10wt%) nanofibers were the best matrix for laccase immobilization. They had sufficient mechanical properties over a long period and the best results excelled the PCL/SF samples in their immobilization efficiency. The highest loading reached 220,5 U/g of the matrix using non-buffer solution for the enzyme attachment. This result can be compared with the study by Bayramoglu et al. (2010) where the maximum loading reached 260 U/g using magnetic chitosan particles as a matrix for immobilization of the laccase from *Trametes versicolor*.

5. 6 Stability of the immobilized enzyme

Stability of immobilized enzyme can be expressed different ways. First of all it is the operational stability which is a residual enzyme activity after a number of catalytic cycles. Each cycle represent the measurement of the catalytic activity of the laccase immobilized on NFs using the described protocol and ABTS as a substrate.

Another type is the storage stability. Several replicates of prepared samples were stored in ultrapure water at 4°C and they were taken out one by one to measure their activity in selected time periods.

There are also pH and temperature stabilities that might be enhanced by enzyme immobilization. However; the behaviour of the immobilized laccase was not studied in this diploma thesis. Six samples described in the table below were selected for the measurement of the operational and storage stabilities.

Table 16 Samples selected for the operational and/or storage stabilities

#	NFs	Modification	Enzyme	Time, °C	IY [%]	AY [%]	Loading [U/g]
1	PA6/ CHIT (20%)	GA(2h, 20°C, milli-Q, 12.5%v/v)-BSA(3h, 20°C, milli-Q,1 mg/ml)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml, 2 mg/ml, 20 mM McIlvaine, pH 3	20 h, 4°C	**50,9** ± 6,9	**6,4** ± 1,3	**150** ± 13,1
2	PA6/ CHIT (20%)	GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml, 2 mg/ml, 20 mM McIlvaine, pH 3	20 h, 4°C	**46,3** ± 3,9	**7,3** ± 1	**148** ± 21,3
3	PA6/ CHIT (20%)	GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml, 2 mg/ml, milli-Q	20 h, 4°C	**31,4** ± 3,4	**11,5** ± 4,2	**220,5** ± 5,9
4	PCL/ SF	GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5 ml, 2 mg/ml, 50mM McIlvaine, pH 3	20 h, 4°C	**23,6** ± 4,8	**10,2** ± 2,1	**156,6** ± 12,4
5	PCL/ SF	GA(2h, 20°C, DIW, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5 ml, 2 mg/ml, 20mM McIlvaine, pH 3	20 h, 4°C	**4,3** ± 1,1	**91** ± 3,6	**244,8** ± 20,5

#	NFs	Modification	Enzyme	Time, **°C**	Cross-linking	IY [%]	AY [%]	Load. [U/g]
6	PCL/ SF	GA(2h, 20°C, DIW, 12.5%v/v)-BSA(3h, 20°C, DIW,1 mg/ml)-GA(2h, 20°C, DIW, 12.5%v/v)	0,5ml, 2mg/ml,20mM McIlvaine, pH 3	20 h, 4°C	GA(10% v/v), 2h, 20°C	**44,6** ± 3,1	**0,13** ± 0,04	**21,4** ± 4,3

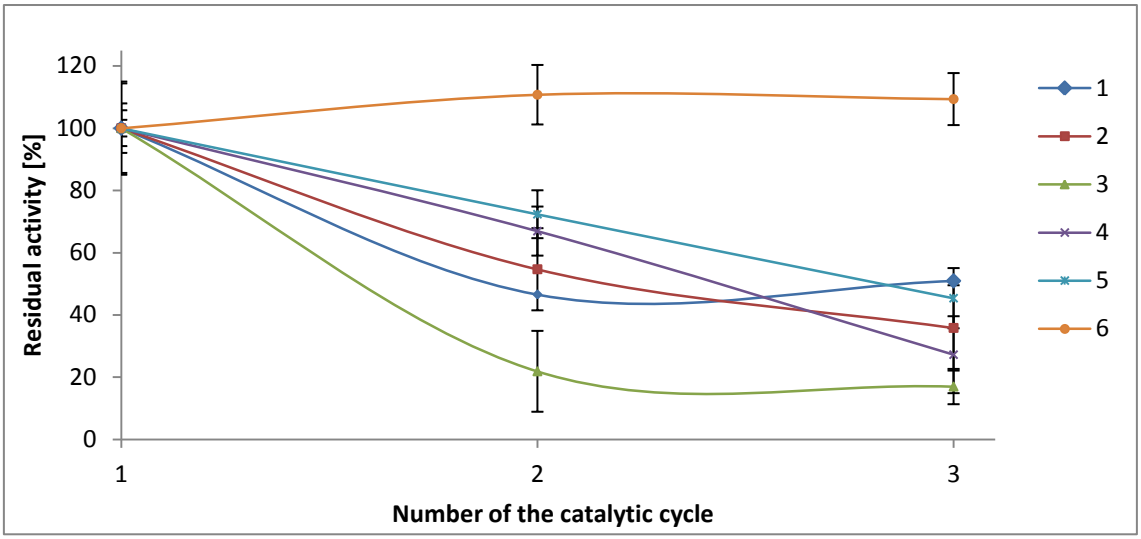


Figure 36 Operational stabilities of selected samples

The operational stability is one of the most important indicators of the linkage strength between the enzyme and the matrix. Generally it is reasonable if the immobilized enzyme retains at least 50% of its initial activity after 5 catalytic cycles. For instance; laccase covalently immobilized on carbon nanotubes lost only around 20% of its initial activity after 5 cycles of ABTS transformation (Xu et al., 2015). The same laccase from *Trametes versicolor* immobilized on magnetic bimodal mesoporous carbon lost 30% after 5 catalytic cycles in a study by Liu et al. (2012).

The remarkable loss indicates either a weakness of the linkage or a significant deactivation of the catalyst after the reaction. However; the activity is usually measured at special conditions containing pH, temperature, molar concentration and substrate. These conditions might differ from the conditions of the environment of the final applications, such as wastewater.

Figure 36 shows operational stabilities of six selected samples. Most of them lost more than 50% of their initial activity within the third catalytic cycle. This might point out that the enzyme was not attached covalently. However; there are other options explaining this remarkable activity loss. First of all there were conditions of the measurement such as multiple washing and manipulation with the samples, that could have damaged their surface, such as transfer to five times concentrated buffer system (from 20mM to 100mM) and very fast agitation providing a homogenous distribution of colorful product of ABTS in the whole volume. These factors might have led to a fast inactivation of the immobilized enzyme. The other option is that the catalytic product did not flow through the nanofibrous structure and cumulated in the pores restricting the

diffusion of a fresh substrate. This theory is motivated by a color change of most samples turning into bluish after the measurement.

The worst result had the sample number 3 where the laccase was dissolved in ultrapure water instead of a buffer. This might suggest that non-buffer system supported adsorption rather than the covalent binding.

The best operational stability showed sample number 6 that differed from the other samples by the immobilization procedure including adsorption on polyamide 6 nanofibers followed by crosslinking via glutaraldehyde. This procedure was not effective regarding the activity during the first catalytic cycle, which was almost ten times lower compared to some of the best samples, but the protein was immobilized very firmly with no drop in activity after the third catalytic cycle.

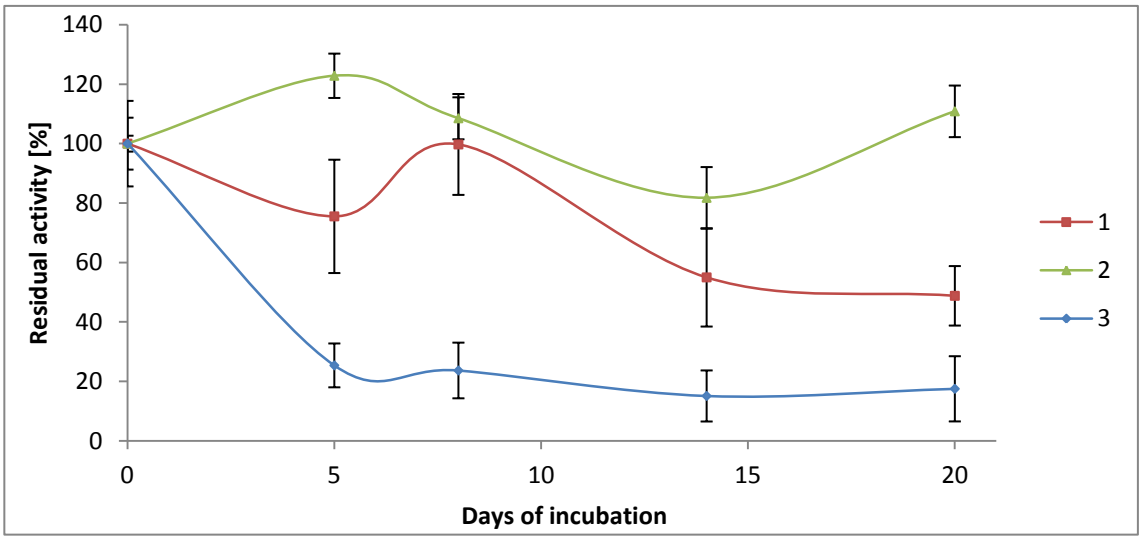


Figure 37 Storage stabilities of selected samples

The storage stability is an important indicator of the enzyme ability to stay active and attached to the matrix during certain time of incubation. During this time of storage in ultrapure water at 4°C there was no mechanical or chemical treatment so there was no other factor but the time influencing their activity.

Three samples were tested (Figure 37) by measuring their activity after certain time of storage. Sample number 3 showed the most significant activity drop after 20 days of storage which again suggests that the laccase was immobilized rather via some weak intermolecular interactions or that it was inactivated during the storage. On the

other hand, the sample number 2 did not show decreasing trend of the residual activity after 20 days although there was about 20% of activity drop after 14 days which was probably caused by the dispersion of the data. Therefore; this sample is considered to be stable for 20-day period.

These results can be compared to the storage stability of the laccase solution (Figure 30). The laccase stock solution (10 mg of laccase in 5 ml of ultrapure water) lost about 51% of its initial activity after 14 days of incubation. The immobilization procedure successfully enhanced the storage stability of the laccase.

In the study by Xu et al. (2015) the immobilized laccase retained 70% of its initial activity after 20 days of storage at 4°C, while the free laccase lost around 60%. Huang et al. (2006) used copper-tetraaminophtalocyanine-Fe_3O_4 nanoparticles for the covalent attachment of the laccase from *P. sanguinens* and after 1 month of storage the immobilized enzyme retained 85 % of its activity in contrast with the free laccase which lost more than 30%.

5. 7 Degradation of bisphenol A and 17α-ethinylestradiol

Four selected samples (Table 15) and five solutions with different concentrations of the free laccase were tested for the degradation of micropollutant mix containing 50µM BPA and 50µM EE2.

Table 17 Selected samples for degradation of endocrine disrupting chemicals

#	NFs	Modification	Enzyme	Time, °C	IY [%]	AY [%]	Load. [U/g]
1	PA6/ CHIT (20%)	GA(2h, 20°C, milli-Q, 12.5%v/v)-BSA(3h, 20°C, milli-Q,1 mg/ml)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml, 2 mg/ml, 20 mM McIlvaine, pH 3	20 h, 4°C	**50,9** ± 6,9	**6,4** ± 1,3	**150** ± 13,1
2	PA6/ CHIT (20%)	GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml, 2 mg/ml, 20 mM McIlvaine, pH 3	20 h, 4°C	**46,3** ± 3,9	**7,3** ± 1	**148** ± 21,3
3	PA6/ CHIT (20%)	GA(2h, 20°C, milli-Q, 12.5%v/v)-HMD(3h, 20°C, milli-Q,0,1M)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml, 2 mg/ml, milli-Q	20 h, 4°C	**31,4** ± 3,4	**11,5** ± 4,2	**220,5** ± 5,9
4	PCL/ SF	GA(2h, 20°C, milli-Q, 12.5%v/v)-BSA(14h, 20°C, milli-Q,1 mg/ml)-GA(2h, 20°C, milli-Q, 12.5%v/v)	0,5ml, 2 mg/ml, 20 mM McIlvaine, pH 3	20 h, 4°C	**25,1** ± 6,7	**4,3** ± 1,2	**65** ± 8,4

Figure 38 shows the decrease in concentration of BPA and figure 39 shows the degradation of EE2 in the micropollutant mixture after incubation with different amount of the laccase from *Trametes versicolor*. The degradation was more rapid with growing amount of laccase expressed by its activity in units.

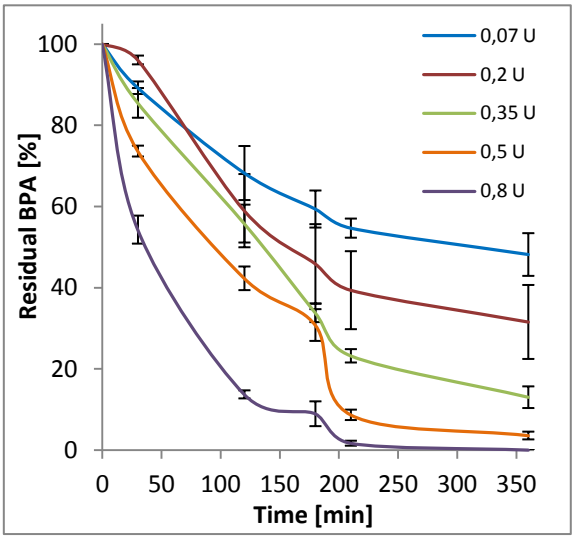


Figure 38 Degradation of BPA by different amounts of laccase from *Trametes versicolor*

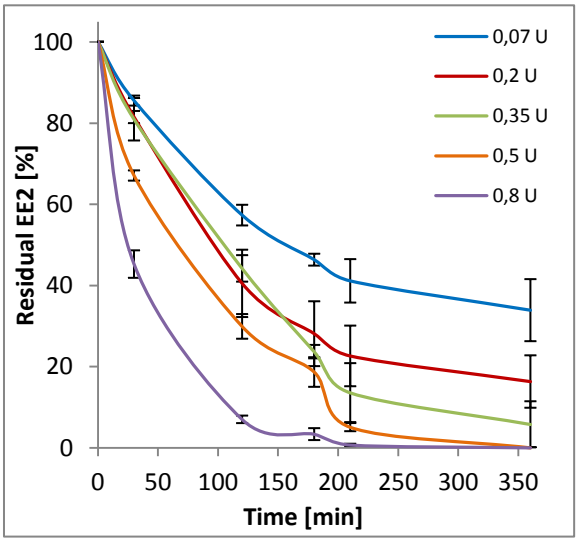


Figure 39 Degradation of EE2 by different amounts of laccase from *Trametes versicolor*

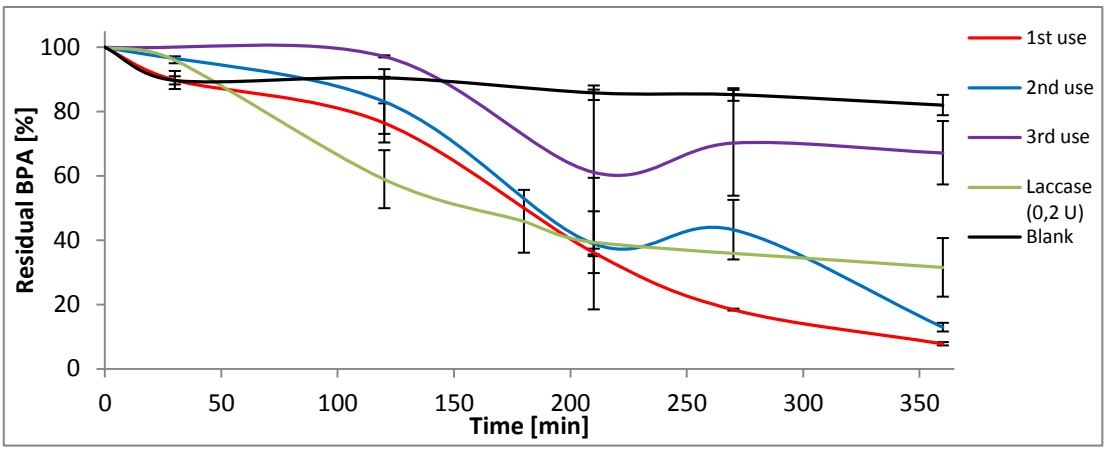


Figure 40 Degradation of BPA by the sample number 1 (PA/CHIT; 150 U/g)

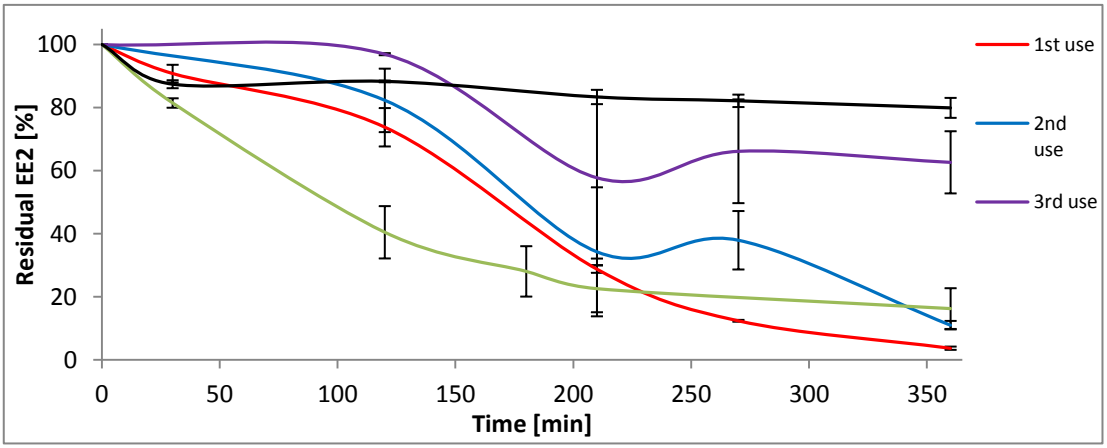


Figure 41 Degradation of EE2 by the sample number 1 (PA/CHIT; 150 U/g)

The first sample showed very good results in the degradation of both BPA (Figure 40) and EE2 (Figure 41). There was a very low concentration of the micropollutants remaining after six hours of incubation of the first use (only 8% of BPA and 4% of EE2). The experiment was repeated the next day and although the degradation curve had a different shape almost 87% of BPA and 89% of EE2 was degraded. The catalysis of the EDCs became slower at the third use one week after the first day of degradation with the concentration of bisphenol A decreasing to 67,1% and EE2 to 62,7% after 360 minutes. The blank samples proved that the decrease in the concentrations of BPA and EE2 was not caused only by their absorption into the structure of nanofibers.

When we compare the degradation curves of the first sample usage with the immobilized laccase of the activity of 0,15 U and the degradation progress of the free laccase with 0,2 U we can observe that the immobilized laccase was more efficient. In six hours the free laccase degraded 23% less of BPA and 12% less of EE2 than the immobilized enzyme.

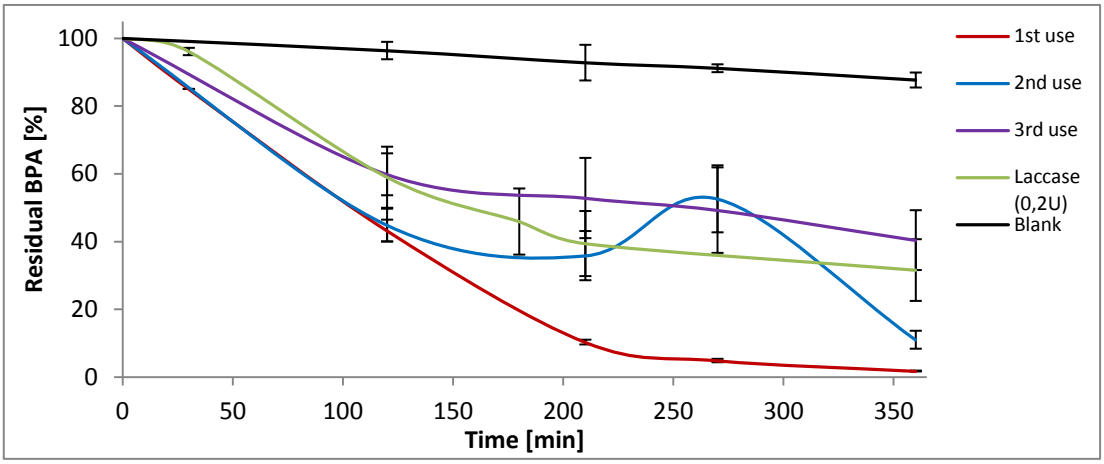


Figure 42 Degradation of BPA by the sample number 2 (148 U/g)

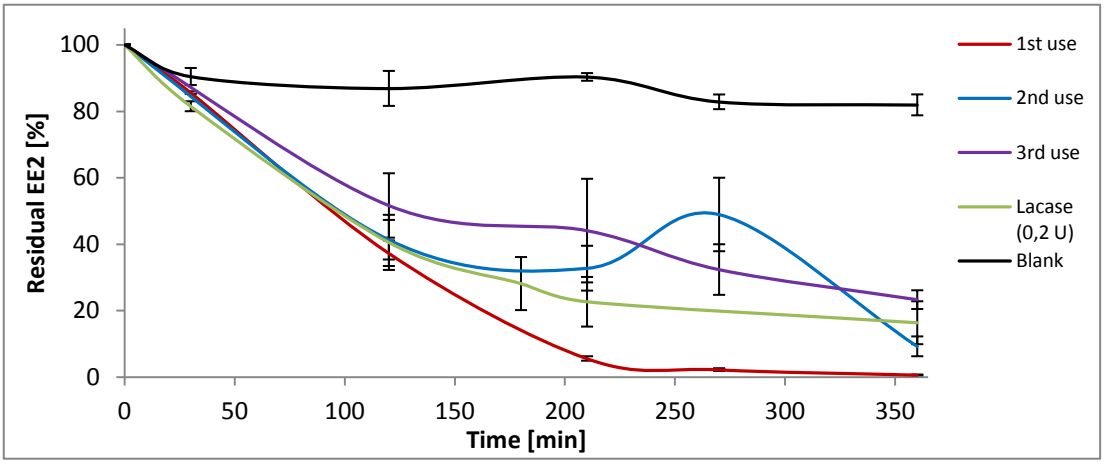


Figure 43 Degradation of EE2 by the sample number 2 (148 U/g)

Figures 42 and 43 show the degradation of BPA and EE2 by the sample number 2. Almost all of the BPA and EE2 was degraded after six hours of the first measurement which is a better result compared to the free laccase with the activity of 0,2 U. Blank samples suggest no significant absorption of the micropollutants. The degradation curve of the second measurement has a similar shape to the second measurement of the sample number 1 but after 360 minutes the concentration of BPA decreased by 89% and EE2 by 91%. The third use after one week of storage in ultrapure water and 4°C the samples still showed some degradation. Only 40,4% of BPA and 23,3% of EE2 remained in the micropollutant mixture after another 6 hours.

When we compare the sample with the immobilized laccase of 0,15 U (red line) and the free laccase of 0,2 U (green line) in the first degradation cycle we can observe that the better result was achieved using the attached enzyme.

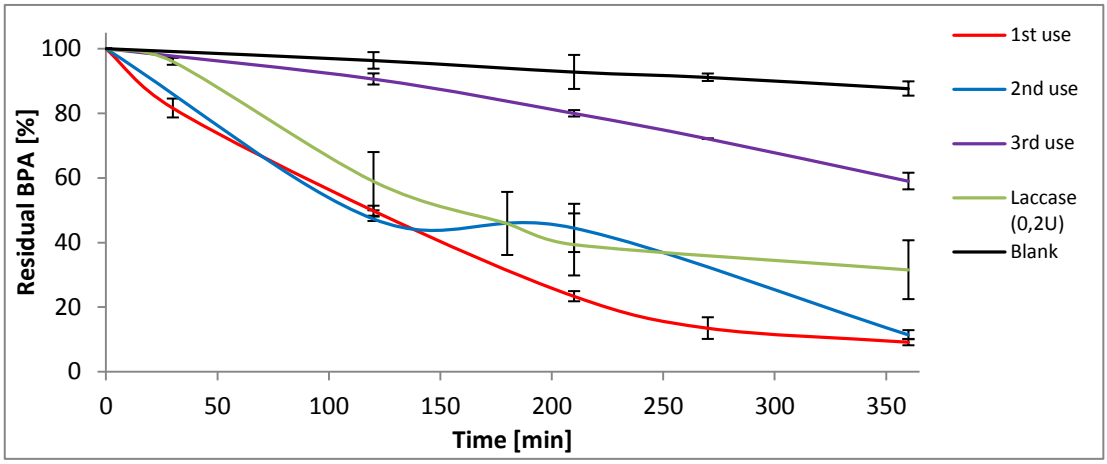


Figure 44 Degradation of BPA by the sample number 3 (PA/CHIT; 220,5 U/g)

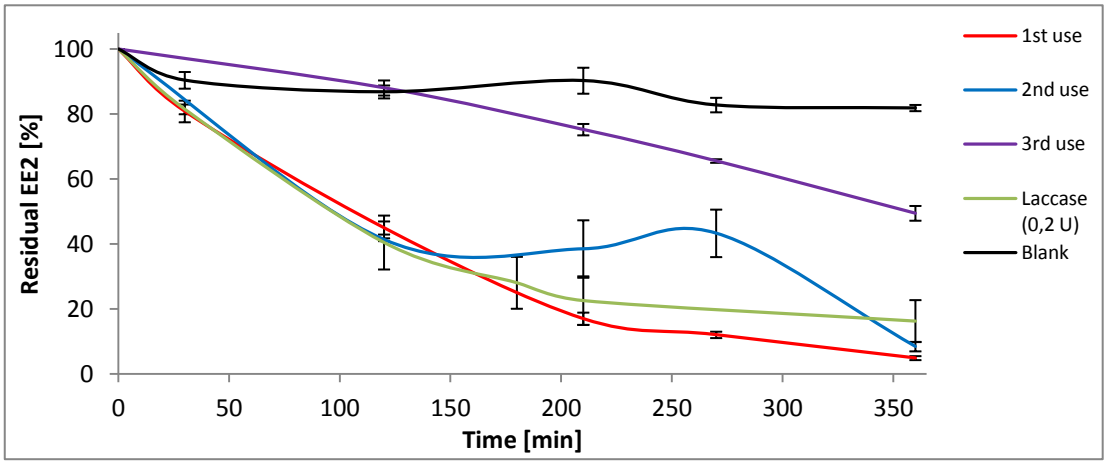


Figure 45 Degradation of EE2 by the sample number 3 (PA/CHIT; 220,5 U/g)

The degradation of BPA (Figure 44) and EE2 (Figure 45) by the sample 3 with the 0,22 U of the immobilized laccase had a very similar course as degradations by previous samples. However; the sample number 3 achieved almost the same decrease in the concentrations (by more than 90%) of both micropollutants in the first and second cycle. The third degradation lowered the concentration of BPA to 59% and EE2 to 49,5%. The free laccase of 0,2 U consumed less BPA and EE2 in six hours compared to the sample with the immobilized laccase.

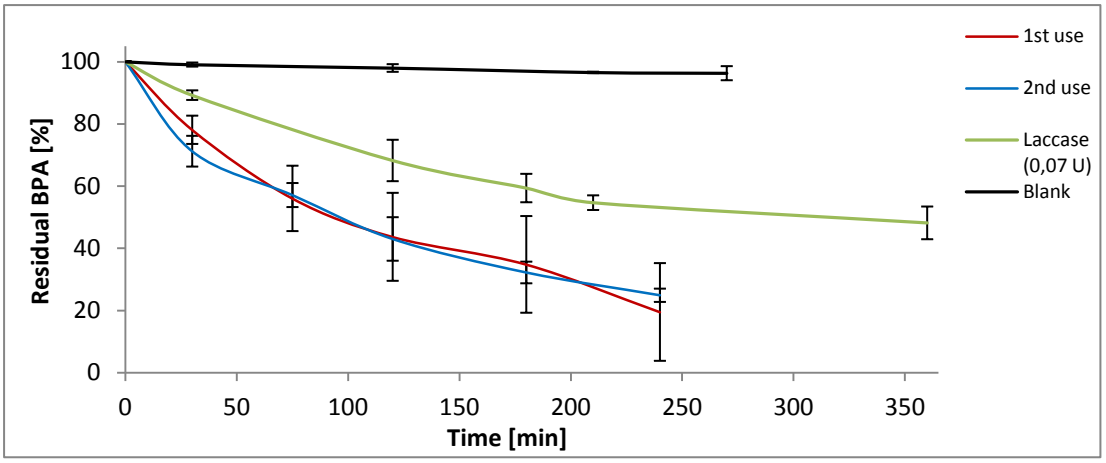


Figure 46 Degradation of BPA by the sample number 4 (PCL/SF; 65 U/g)

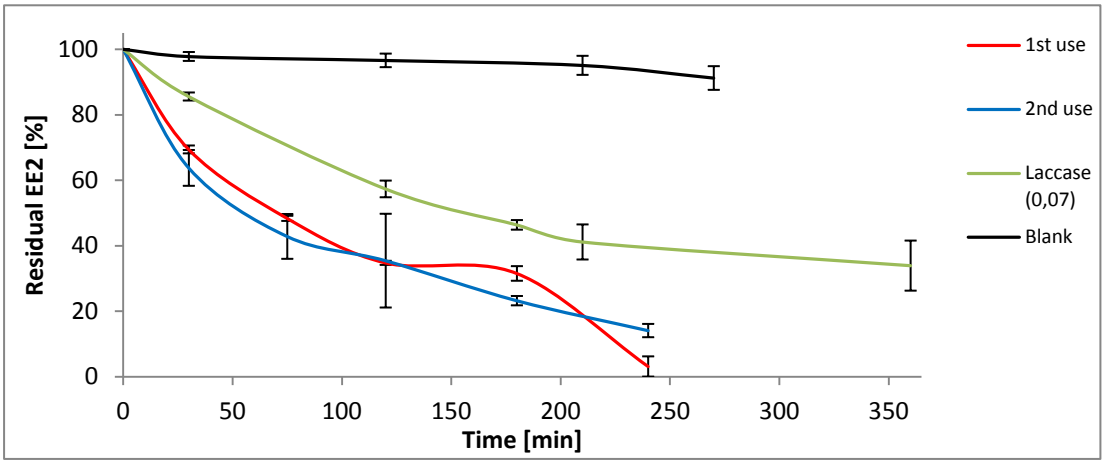


Figure 47 Degradation of EE2 by the sample number 4 (PCL/SF; 65 U/g)

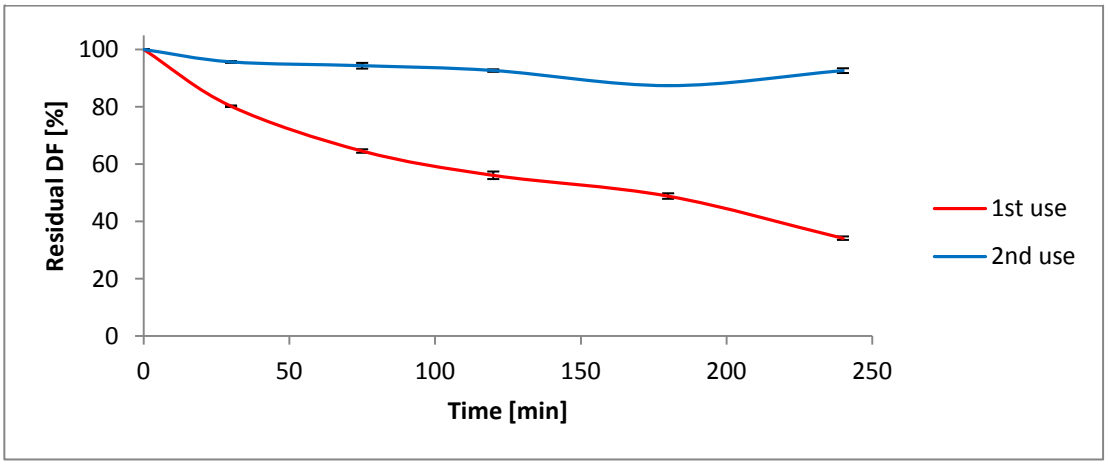


Figure 48 Degradation of DF by the sample number 4 (PCL/SF; 65 U/g)

The sample number 4 is representing PCL/SF that were measured as the first trials using Waters HPLC. Although they were less active compared to the samples 1, 2 and 3 they were able to degrade around 80% of BPA (Figure 46) and 97% of EE2 (Figure 47) after four hours which was a better result compared to the free laccase with

0,7 U. Samples were used the next day after an incubation in ultrapure water at 4°C overnight. Even during the second usage they consumed 75% of BPA and 86% of EE2. However; these samples cannot be compared to the previous samples used for the degradation because the presence of 50μM diclofenac in the micropollutant mixture influenced elimination of BPA and EE2 (Nair et al., 2013).

PCL/SF nanofibers showed marks of mechanical damage during the long time process. Their edges were gradually losing small filaments so it was impossible to recover the original mass of the samples for the second measurement.

All tested samples showed more rapid degradation than the free laccase. This finding was not expected because the free laccase had an advantage against the immobilized enzyme. This advantage was its solubility in the micropollutant mixture offering the laccase to constantly catalyze the degradation in the whole volume of the liquid. On the other hand; the immobilized laccase was attached to the square matrix with the diameter of only 1 cm and this piece of textile was haphazardly floating in the 3 ml of the liquid. The higher catalytic effectiveness of the modified nanofibers might be explained by an increased stability of the immobilized laccase at 40°C and constant shaking of 120 rpm compared to the free enzyme which could have lost its activity at these conditions.

In some cases, the degradation curve had different shape because of a point where the concentration of the micropollutants increased although it generally showed decreasing tendency. This phenomenon was probably caused by a sampling error.

It is very difficult to compare obtained results with the literature. Most of the studies focused on the degradation of EDCs deal with biocatalysts immobilized on nanoparticles using different substrates for the degradation and different conditions of the reaction. Songulashvilli et al. (2012) covalently attached laccase from *C. unicolor* on porous silica beads and used it for the elimination of 50μM BPA. In this study the immobilized laccase of 120 U per 50 ml (7,2 U/3 ml) of the micropollutant mixture eliminated about 90% of BPA after 60 minutes. Although their samples were 36 times more active the degradation rate was only 3 times more efficient compared to the PA6/CHIT samples with only 0,2 U.

Conclusions

This diploma thesis disserts on an investigation of methods for enzyme immobilization on selected nanofibrous matrices and a development of fast and accurate analytical methods for the measurement of their catalytic properties. It describes a screening of different methods by adjusting parameters of the process in order to achieve an optimal proportion between activity and stability of the developed samples. It further deals with an application of selected samples for the degradation of model endocrine disrupting chemicals for the purposes of wastewater treatment.

The first part of the research was focused on developing the most efficient method for the immobilization of an enzyme onto microfibrous or nanofibrous matrices. The enzyme was laccase from *Trametes versicolor* which was purchased for its ability to oxidize several types of endocrine disrupting chemicals.

There were two main approaches for the enzyme immobilization and the materials for the matrices were chosen according to the selected methods. The first immobilization technique consisted of enzyme adsorption on polyamide 6 nanofibers followed by crosslinking of the adsorbed enzyme molecules.

The second approach was a covalent attachment onto specially modified nanofibers. There are several types of modification using different reactive groups binding the enzyme but after a screening of different methods using various matrices and crosslinking agents the most profiting method showed to be the attachment via glutaraldehyde. Two blend nanofibrous layers were chosen for the purposes of the covalent attachment; polycaprolactone/silk fibroin and polyamide 6/chitosan.

Nanofibrous matrices have not been used as widely as other nanostructures. The process of enzyme immobilization on such layers is different and requires specialized techniques. Nanoparticles are usually used in form of a suspension hence the whole immobilization process proceeds within small volumes of liquid and the matrix separation mainly consists of centrifuging and removing the supernatant. On the other hand, nanofibers are a solid material that can be easily removed from the liquid but even very small samples with only few milligrams require higher volumes and different handling.

The optimal immobilization method was an incubation of circle samples, weighing approximately 1 mg, in small-volume inert vessels such as multiwell plates

designed for cell cultivation or spectrophotometric measurement in a microplate reader. This technique allowed a modification of several samples at the same time with the same conditions. Samples were completely submerged and floating in the liquid and yet not adherent on a side of the vessel which could make part of the support inaccessible for the enzyme.

It was also necessary to develop optimal methods for the analysis of the immobilized enzyme. In some studies the value of the actual activity of the captured enzyme has been neglected (Silva et al., 2007), probably because it was technically difficult to measure the kinetic activity of the nanofibers. In this research the enzyme activity was measured spectrophotometrically by an absorbance growth of an increasing product of the catalysis formed by a catalytic reaction of the immobilized enzyme.

The first type of the measurement consisted of the catalytic reaction proceeded in a test-tube and the absorbance growth analysed by measuring an absorbance of 100 μl supernatant samples extracted from the test-tube in a certain time interval. This separated system, consisting of the reaction tube and measurement vessels (94-well plates), allowed constant agitation of the reaction tube providing a homogenous distribution of the substrate and the products within the whole volume. However; the sampling was too slow so it was very difficult to measure the linear growth in absorbance because within two or three samplings the absorbance values were already in the non-linear part of the curve.

Another measurement approach dealt with a cuvette spectrophotometer when the sample was attached to the bottom of the cuvette and after the substrate was added the absorbance was measured directly in the reaction vessel. Unfortunately; this measurement was inaccurate when the surface of the sample was partially restricted by a weight holding the nanofibers on the bottom and the concentration of colorful ABTS cation did not grow linearly because this measurement did not provide the agitation of the liquid.

An optimal vessel for the activity measurement appeared to be a 6-well plate where the samples were attached to a side of the well by a thin wire and after the substrate was added the absorbance was measured in the microplate reader providing a shaking step before and during the reading. This method appeared to be fast and accurate although the surface of the samples was also slightly covered by the adjusting wire. This problem could be solved by developing an inert cage that would separate a certain section of the vessel apart from the middle area where the absorbance reading

proceeds. The sample could be placed in the separated section surrounded by the cage providing sufficient diffusion of substrate and product within the whole volume of the vessel.

After the immobilization and measurement techniques had been developed the screening of parameters of the immobilization process began in order to find an optimal sample with high activity and satisfactory stability. The variable parameters included type and modification of nanofibers and parameters of the immobilization (time, temperature, concentration of the enzyme, type of buffer, molar concentration and pH).

The adsorption method followed by crosslinking via glutaraldehyde was the fastest procedure. It took only seven hours to prepare a sample and it did not require any modification steps of the nanofibers, changing liquid or washing the matrix between each step of a modification. This method provided a very good operational stability of the immobilized laccase with almost no activity loss after 3 catalytic cycles. However; the highest activity reached only around 26 U/g of the support. This low activity yield was probably caused by multiple covalent bindings causing conformational changes of the enzyme molecules.

Many studies refer about activity of the immobilized laccase of hundreds of units (Bayramoglu et al., 2010; El-Aassar, 2013; Shi et al., 2014; Xiao et al., 2006; Zheng et al., 2012 etc), nevertheless each strain of laccase is different and there are various methods to express their activities. There are also various matrices for enzyme immobilization providing different surface densities. Therefore the immobilization efficiency expressed in units per gram of nanoparticles cannot be equal to the same U/g of nanofibers with perhaps ten times lower surface density and hence much lower immobilization capacity.

Satisfactory activity of the immobilized laccase was achieved when using PCL/SF and PA6/CHIT nanofibers for the covalent attachment. Both materials showed very similar results over 150 U/g although their chemical structures and mechanical properties were different. The highest achieved activity was around 220 U per gram of the PA6/CHIT nanofibers when using a modification consisting of glutaraldehyde-hexamethylenediamine-glutaraldehyde and 20-hour immobilization step. Although this procedure was longer than the adsorption technique, the result was almost ten times better.

Unfortunately; the operational stability of these samples was not satisfactory. Most of them lost more than 50% of their activity within three catalytic cycles of ABTS oxidation. Such activity loss would suggest a weakness of the linkage between the enzyme and modified nanofibers or the enzyme preferring rather an adsorption than a covalent attachment. However; there is also a possibility of the enzyme inactivation after a rapid agitation during the measurement, multiple transfers and changes of conditions, such as a significant concentration gradient from 20mM buffer used for the immobilization to 100mM required for the measurement. Another explanation might be related to diffusional limitations of the nanofibrous structures of the samples caused by the product of catalysis cumulating in their pores. This theory can be supported by a conversely satisfactory storage stability of the best sample which retained its initial activity after 20 days of storage although the stock enzyme lost around 20% after 14 days at the same conditions.

In the future it would be interesting to investigate stabilities of the immobilized enzyme and the soluble laccase over a wide range of temperature and pH. It is known that the immobilization process highly increases the enzyme robustness in conditions that normally cause denaturation of the biocatalyst.

Selected samples were used for the elimination of micropollutants in a solution containing 50μM BPA and 50μM EE2 in ultrapure water. The best PA6/CHIT sample weighing only 1 mg with approximately 50 μg of immobilized laccase (0,15 U) removed 92% of BPA 96% of EE2 in 3 ml of the solution after six hours at 40°C. A very similar result was achieved in the second catalytic cycle using the identical sample after 24 hours of storage in ultrapure water. The immobilized laccase still showed some catalytic activity in the third catalytic cycle one week after the first degradation usage by removing more than 30% of both micropollutants after six hours.

All selected samples with the immobilized laccase were more efficient in the degradation of these two EDCs than approximately same amount of the free laccase and all of them remained exceptionally highly active in additional catalytic cycles. The principle of a repeated usage and application of a solid matrix providing a stable protective support for the immobilized enzyme are the main motivations for the enzyme immobilization. The soluble laccase appeared to be less active in the micropollutant

mixture and once it was used it could not be recovered from the solution and used again in comparison with the laccase attached to the nanofibrous matrix.

Only two types of micropollutants were tested however there is a number of endocrine disrupting chemicals in the water environment influencing aquatic animal species as well as animals and humans consuming thus polluted water. It might be profitable to investigate immobilization of an enzyme mixture (e.g. laccase, peroxidase, tyrosinase) in order to enhance the catalytic efficiency of the final product over wide ranges of temperature and pH. It has been described that there is a synergic effect between two immobilized enzymes affording opportunity to catalyse degradation of a larger spectrum of micropollutants (Hirsh et al., 2010; L. Björck, 1976).

Nanofibers used for this research appeared to be valuable carriers for the immobilization of laccase because they had sufficient mechanical properties, hydrophilicity and chemical stability. Their major advantage against matrices in form of particles in a suspension is the feasibility to handle them as a textile. They can be produced continuously by the electrospinning technique which allows fabrication into various structures with adjustable shapes and parameters.

Whereas there must be developed unique reactor systems for the particle-matrices allowing their safe use in a water environment, nanofibers could be applied as a component of established water filtering systems. These filters might be in future dedicated for a treatment of effluents coming from facilities known for their high production of EDCs that end up in the wastewater (e.g. hospitals, industrial facilities).

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