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Degradation of 17-alpha-Ethinestradiol by Enzyme immobilization on different hollow Fiber Membranes

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Content

Abstract

The target of this work was the immobilisation of the enzyme Horseradish Peroxidase (HRP) on surfaces. This was successfully achieved by immobilizing HRP on three different kinds of hollow fibre membranes. MFO2 (microfiltration membrane) consisting of Polyethersulfone (PES), HFS (nanofiltration membrane) which consists of PES and Sulfonated-Polyethersulfone (S-PES), and the UFC-membrane (ultrafiltration) where the membranes of choice. By designing functional polysaccharide nanostructures on surfaces, the enzyme HRP got immobilized. On all three membranes three different immobilisation methods have been used. The results show that the enzyme binds best to the HFS-membrane with the chemical linkage method.

Furthermore, this thesis demonstrates the effect of pH, salt concentration, Cd^{2+} and F^{-} on the activity of immobilized Horseradish Peroxidase. It was found out that Cd^{2+} has a crucial impact to immobilized Horseradish Peroxidase.

Also the successful degradation of 17-alpha-ethinylestradiol, a precarious pollutant occurring in diverse water bodies, with free and immobilized HRP has been studied and successfully developed. In addition, a potentially applicable degradation process for the degradation of EE2 has been invented successfully.

Abstract (Deutsch)

Das Ziel dieser Arbeit war die Immobilisierung von Meerrettich Peroxidase(HRP) an verschiedenen Oberflächen. Dies wurde erfolgreich an 3 verschiedenen Membran Oberflächen erreicht. MFO2 (Microfiltrations Membrane) welche aus Polyethersulfone besteht(PES) HFS (Nanofiltrations Membrane) welche aus PES und sulfonierten PES besteht und der UFC-Membrane (Ultrafiltration Membrane) welche auch aus PES bestand wurden hierbei verwendet. HRP wurde durch funktionalisierte Polysaccharide an den Membran Oberflächen immobilisiert. An allen 3 Membranen wurden 3 verschiedene Immobilisierung Methoden getestet. Die Ergebnisse zeigten, dass das Enzyme am besten zu HFS-Membrane gebunden hat durch die „Chemische Bindungs Methode“. Darüber hinaus zeig die Arbeit den Effekt von pH, Salzkonzentration, Cd^{2+} und F^{-} auf die Aktivität von immobilisierten HRP. Ergebnisse zeigen dass Cd^{2+} einen großen Effekt auf HRP hat. Es wurde auch der erfolgreiche Abbau von 17-alpha-Ethinestradiol mit freien und immobilisierten HRP gezeigt und ein potentieller anwendungsorientierter Prozess entwickelt.

Introduction

Through the rising development of chemical industry, chemicals become more and more an issue in every day live. Nowadays chemicals are widely used in nutrients, pharmaceuticals and pesticides. Based on this fact, rising concentrations of harmful chemicals have been found in the environment in recent studies[1]. One harmful pharmaceutical which was found in waste water streams is 17α-Ethinylestradiol (EE2) which is a component in birth control pills. EE2 enters the environment by human urine, because only a very small part of EE2 can be degraded by human body. Pharmaceutical waste constitutes another source of EE2. By consulting the available literature, it is evident that already low concentrations of EE2 in wastewater can have considerable effects on fertility of animals and humans.

In several countries the effect of EE2 on fish has been studied. Researchers suggest that some male fish feminize if they are exposed over a long time to Oestrogen in low concentrations, such as 0,1ng/L. Great Britain is the country where feminisation of fish has been most widely observed. The main indicator of feminisation of male fish is the production of female yolk[2].

A very promising way to deal with endocrine disrupting compounds (EDCs) is the use of enzymes. Enzymes are getting produced via biotechnological processes. The big advantage of these processes is the possibility for specification and affinity to a target substrate and specific environmental conditions. For the practical use of enzymes in industry enzymes have to be immobilized on surface to allow longer use[1].

History of membranes

In the 18th century the first studies considering membranes were made by Jean-Antoine Nollet (1700-1770). Nollet described the first osmosis by permeating water through a semipermeable membrane. Until the 19th century, there was no use of membranes considering commercial or industrial applications. Membrane scientists experimented with various kinds of membranes which were available from animals. This situation changed in 1865, when Thomas Graham (1805-1869) was the first scientist who reported a convenient use of synthetically membranes in dialysis experiments.

In 1930 micro porous membranes consisting of nitrocellulose were for the first time produced in industry. In the next years, industry developed more and more membranes consisting of different polymeric materials. Until 1950 only a few practical applications existed and mostly scientists were interested in membrane development and research. In this year, technical relevant membranes with specific transport characteristics and mechanical and thermal properties became the focus of research. The maybe most important membrane process developed which is still in use is the reversed osmosis process. This process is mainly used for the production of drinking water from sea. The used membrane is consisting of cellulose acetate which provides high salt rejection. With the invention of different polymers such as polyethylene, polysulfones and polyamides and their remarkable disparity in mechanical, thermal and chemical stability, more and more applications were developed from membrane industry[3,4]. Figure 1 provides an illustration of the number of membrane facilities, starting with the year 2000.

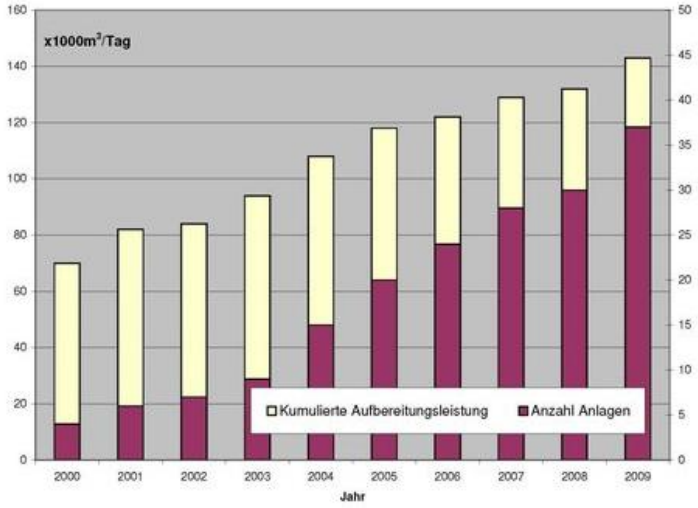


Figure 1: Rising membrane facilities since the year 2000 [3]

Commercially used membrane separation processes

Membranes are widely used in industry for separation processes. According to the driving force of membrane separation processes, these processes can be divided into three different types[5].

- Pressure driven operations
- Permeation operations
- Dialysis operation

Pressure driven operations

Pressure driven membrane separation operations are processes where the driving force is a pressure gradient across the membrane. The subsequent table 1 provides an overview on characteristics, typical pore sizes, materials, and fields of application for micro-, nano-, and ultrafiltration membranes, and membranes used for reversed osmosis. The typical pore diameters are correlated to these membrane types as depicted in Figure 2.

Table 1: Filtration processes and their application

Type of pressure driven Filtration	Characteristics	Cut-Off (Poresize) [nm]	Membrane material	Application
Microfiltration	Cutting off insoluble particles according to their size;	100 – 500 (1000)	Celluloseacetat; Polysulfones, Polyethersulfones; Ceramikmodules	water disinfection; brewing and beverage technology
Ultrafiltration	Cutting off of insoluble particles according to size; Viruses, big proteins and agglomerated biopolymers are retained	10 – 100	Polysulfones, Polyethersulfones; Ceramikmodule	sterilization, removal of globular particles; nutrients technology, antibiotic production; waste water treatment
Nanofiltration	Cutting off all soluble compounds; (dyes, drugs, pesticides and macromolecules; via size exclusion!)	1 – 10	Polysulfones, Polyethersulfones; Ceramic modules	sterilization, ; water treatment
Reversed Osmosis	Separation of small molecules Semipermeable Membrane	0,1 – 1	Polysulfone, Polyethersulfone	desalination of seawater

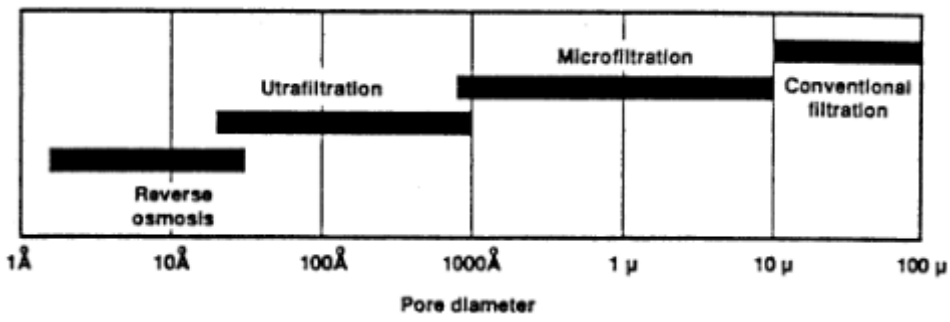


Figure 2: Showing pore diameter related to application (http://ihome.ust.hk/~kexhu/ceng576/576-04.pdf)

Permeation operations

Considering permeation processes, the driving forces are activity difference, for example pressure, across the membrane. When this operation is applied to solution, the permeate is the solvent[5].

Examples of Permeation operation

- **Gas permeation process** is a gas/gas process where gases are separated through a membrane. The activity difference is the difference in pressure across a dense membrane. Examples of applications are for instance the hydrogen recovery in the ammonia synthesis process [5].
- **Pervaporation** is a liquid/vapor separation where liquids are separated through a dense membrane by vaporization. Again, the activity difference is generated by introducing a pressure difference. This is achieved by introducing partial vacuum on the permeate side. This process is mainly used for the dehydration of alcoholic azeotropes and the removal of volatile organic compounds from waste water [5].
- **Membrane distillation** is a temperature driven separation process where a porous hydrophobic membrane is used. The temperature difference generates a vapor pressure difference which acts as the driving force of the process. These kinds of operations are mainly used for desalination [5]. The principle of membrane distillation processes is illustrated in Figure 3.

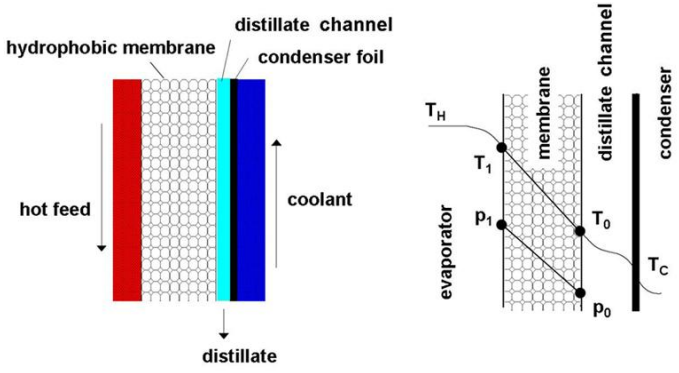


Figure 3: Scheme of membrane distillation [6]

Dialysis operation

The driving force of the dialysis processes is the chemical potential. In general, two phases which differ in the concentration of any component are separated by a membrane. By diffusion of the component through the membrane, the concentration balances [7].

- Electrodialysis is a process where ions are migrating through a membrane by an electrochemical potential.
- Dialysis (Hemodialysis) is an operation where the driving force is a transmembrane concentration difference. This process is mainly used for medical application in purification of blood.

Mass transport through membranes

Since membranes are mainly used for separation processes, the ability to control the rate of permeation of different species is a quite important feature of membranes. To describe the mass transport through a membrane, two different models are distinguished.

- Solution-Diffusion model
- Pore-Flow model

Solution-Diffusion Model

The basic of the Solution-Diffusion model is that permeates dissolve in the membrane and then diffuse through the membrane. The driving force of this process is a concentration gradient. [8] The permeates are separated through their different solubility. This phenomenon was first well described by Fick's law[9].

$$J_i = -D_i \frac{dc_i}{dx}$$

Where J_i is the rate of transfer of component I or flux [g/cm^2*s]. dc_i/dx is the concentration gradient of component i and D_i is called diffusion coefficient [cm^2/s].

Pore-Flow Model

The second model to describe the permeation of permeates through a membrane is the so called Pore-Flow model. This model describes the flow through a membrane by a pressure gradient and is describes by Darcy's law[8].

$$J_i = -K' c_i \frac{dp}{dx}$$

Where dp/dx is the pressure gradient. K' is a coefficient reflecting the nature of the medium. c_i is the concentration of component i and J_i is the rate of transfer of component I or flux [g/cm^2*s].

The separation of different components is achieved through different size of the components and pores.

Implementation of Solution-Diffusion Model and Pore Flow Model

Considering the Solution-Diffusion Model and Pore Flow Model there are applications where one model works superior to the other. In general, non-porous membranes with tiny spaces between the polymer chains which are often caused by thermal motion of polymer molecules are often best described with the Solution-Diffusion Model. This model is most frequently used to describe the diffusion of gases through dense membranes[8]. Membranes with fixed and large pores considering ultrafiltration and microfiltration membranes are best described by the Pore-Flow Model[10].

Membrane Filtration Techniques

Membrane processes separate a volume flow (Feed) through their chemical and physical properties into two different concentration flows, the permeate, the flow which diffuses through the membrane and the retentive, the flow which gets refrained of the membrane (see Figure 4). By this separation, the concentration of the permeate decreases in the feed, and the concentration of the retentive (also known as retentate) increases in the feed.

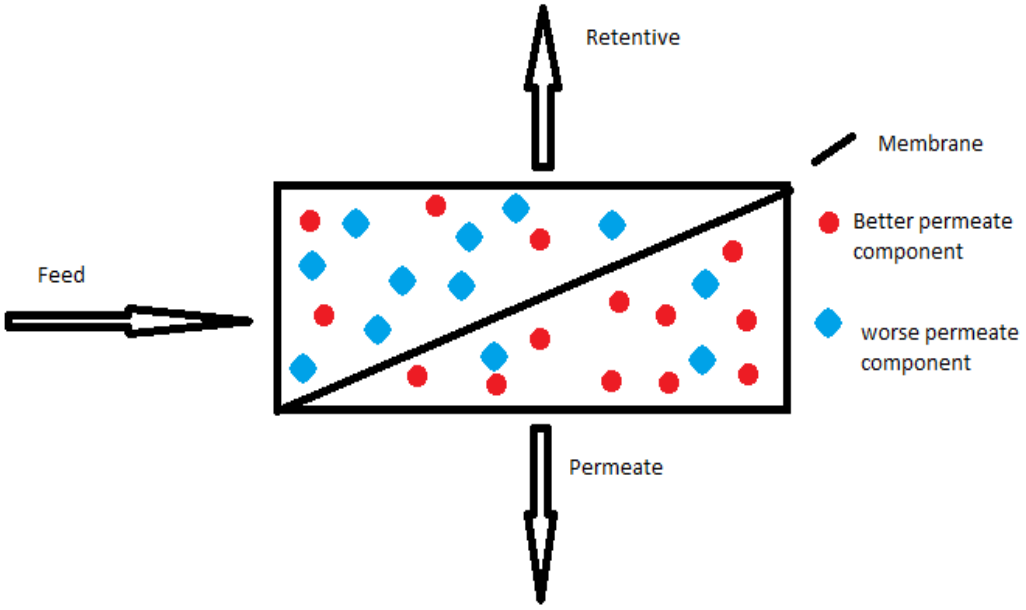


Figure 4: Membrane Seperation Process

In general, there is a distinction between two different separation techniques which are called Cross-Flow Filtration and Dead-End Filtration.

Cross-Flow Filtration Technique

Using the Dead-End Filtration technique, a feed gets circulated over a membrane surface (see Figure 5). Only a small part of the feed permeates through the membrane. The main advantages of this system is that membrane fouling is reduced; thus, membranes which are used in this technique, can be used for a long time without any cleaning required. The main disadvantage is that high shear forces can damage the membrane and more power is required for the filtration process.

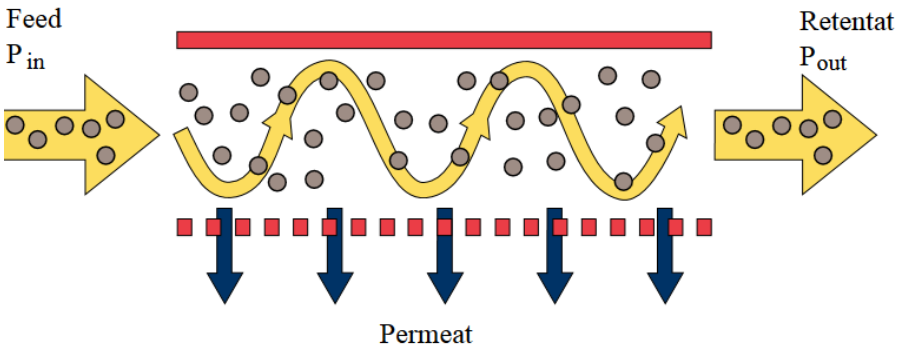


Figure 5: Cross-Flow Filtration Technique[11]

Dead-End Filtration

In the Dead-End Filtration techniques, a pressure drives the feed directly through the membrane and no circulation is needed (see Figure 6). The residue forms a filter cake on the surface if the membrane is operated in Dead-End Filtration mode. Using this process, cleaning is required. In comparison to Cross-Flow Filtration, less energy is needed for the filtration process and, in general, only solutions which contain only a small amount of solids are applied to this process.

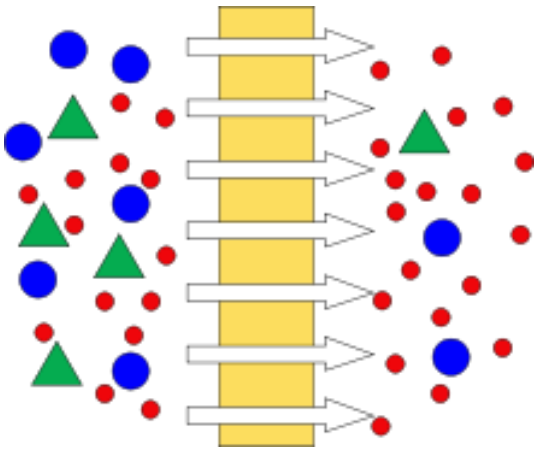


Figure 6: Dead-End Filtration Technique [12]

Production of Hollow Fiber Membranes

For the industrial production of hollow fiber membranes, the so called phase inversion process is applied. In general, three different techniques of this process are used: Melt Spinning, Dry Spinning and Dry/Wet Spinning. The Dry/Wet Spinning process is the most important one for the industrial production of hollow fiber membranes. The pore size and membrane characteristics are determined by several different parameters considering polymer casting concentration, ratio of non-solvent to polymer, average molecular weight of the polymer and temperature [13].

- Melt Spinning Process: The polymer gets heated above its melting point in an inert atmosphere and the liquid polymer gets extruded through an extruder (see Figure 7). After cooling, the polymer solidifies and hollow fiber membranes are obtained[14,15].

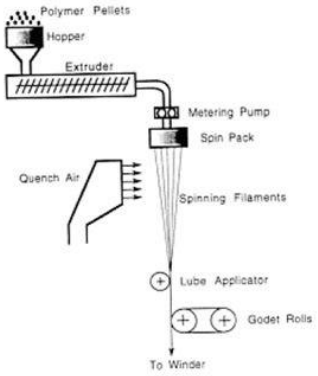


Figure 7: Melt Spinning Process [16]

- Dry Spinning Process: The polymer is dissolved in a solvent with low boiling point and, after extrusion, the polymer solution is heated (see Figure 8). Through evaporation of the solvent the polymer solidifies[14,15].

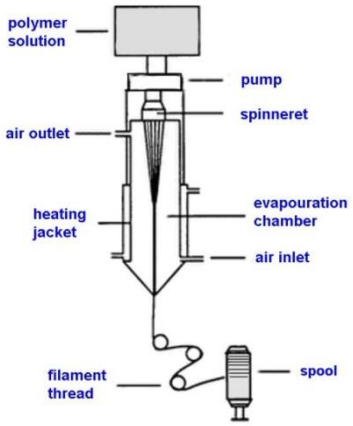


Figure 8: Dry Spinning Process [17]

- Dry/Wet Spinning (see Figure 9): At this method the polymer solution gets extruded to a precipitation bath[14,15].

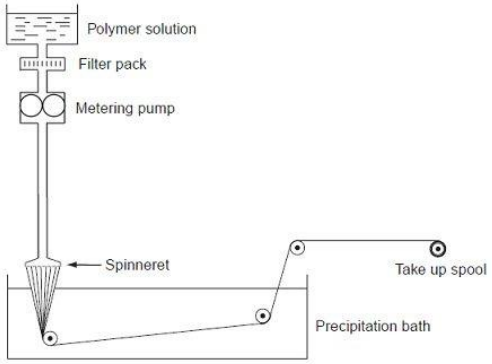


Figure 9: Dry/Wet Spinning Process[18]

Endocrine disrupting compounds

Provoked by the rising concentration of endocrine disrupting compounds (EDCs) in waste water streams, high quality water has become a rare property in our society. US Environmental Protetaction Agency (EPA) has defined EDCs as substances which have an harmful effect on humans and the wild life hormone system[19]. In general it can be said that, from the chemical point of view, these EDCs are mainly steroid hormones, dibenzyldixions and bisphenyls[20].

Scientists in the USA have analyzed waste- and drinking water streams for about 2 years. Lots of different organic compounds like estrone, gemfibrozil, atenolol, atrazine and several more have been identified in drinking water. Most of these compounds are considered to be harmful to humans and the environment. [21].

EDCs can enter the environment by several ways. A major source of these compounds are pharmaceutical and other chemical industries, for example through birth control (contraceptive) pills, soaps, food, pesticides and personal care products[22].

Endocrine disruptors are able to enter human body through different ways. The two most important ways are oral consumption of water or food or entering the living organism through skin or inhalation[20].

In order to remove EDCs from wastewater, different methods have been applied. Conventional treatment processes are not able to remove these kinds of compounds in an efficient way. Today physical, chemical and biological treatments for waste water streams to remove EDCs exist[23]. For the physical reduction of EDCs from aqueous systems, sedimentation is a widely used technique. Through their hydrophobic properties, EDCs tend to enrich on sludge which reduces the amount of these compound significantly[24]. For chemical treatment, activated carbon or coagulants like alumina and iron salts can be used. Chlorination and oxidation processes have been developed as well[23]. The problem accruing from chlorination process is the formation of carcinogenic and mutagenic byproducts of often undefined chemical composition, which turns the chlorination process into a rather inappropriate degradation technique[25].

Estrogen and its derivates

Hormones are biochemically active substances which are produced by certain cells in the body. These cells are arranged in pineal gland, thyroid gland and pancreas. Hormones perform specific effects in the living body. In general, based on their chemical structure, hormones can be classified into three different groups. These groups are called steroid hormones (for example sex hormones like estrogen), the peptide and protein hormones (Somatropin which is a growth hormone) and amino acid-related hormones (Epinephrine). Steroid hormones have the steran (gonan) base body in common (see Figure 10).

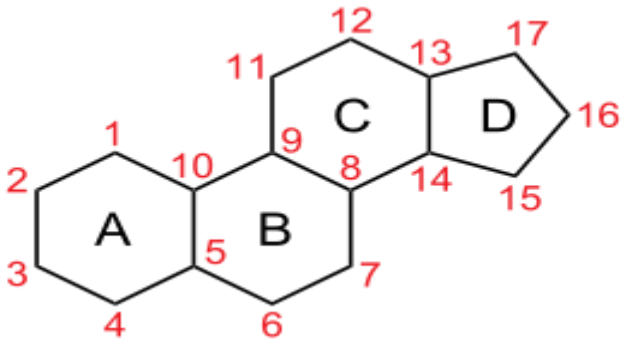


Figure 10: Steran (Gonan) base body

One example of steroid hormones is estrogen. Estrogen is the female sexual hormone and it has several functions in the female human body. In general, these effects can be divided into genital effects, where the main function is the development of sexual attributes of women, and extra genital effects, where it has an important function in growth of bones. The most representative molecule of the estrogen group is 17β-Estradiol (see Figure 11).

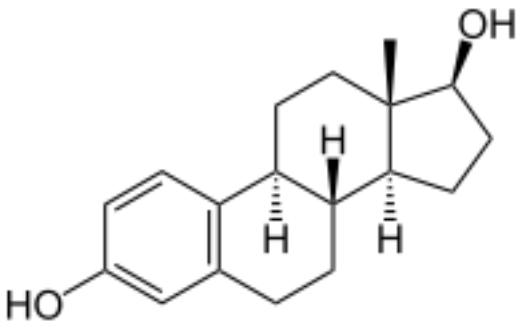


Figure 11: 17β-Estradiol

The target substrate of this work is 17α-Ethinylestradiol (EE2) (structure see Figure 12), which is a derivate of the ethinestradiol. EE2 is a synthetically produced molecule and finds use in pharmaceuticals, like the birth control pill, nutrients and pesticides.

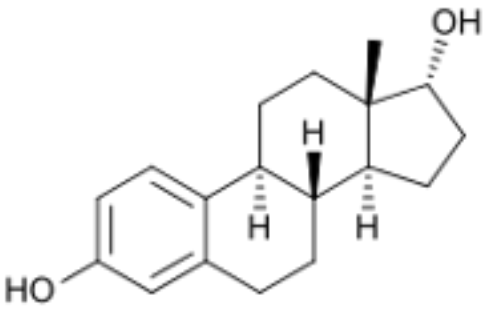


Figure 12: 17α-Ethinylestradiol (EE2)

Since decades, scientists have reported the detection of estrogen in waste water streams. Estrogen enters the environment through agricultural waste, medical waste and human urine[26]. Recent studies report harmful effects on fish and other animals by introducing a feminization of male organisms, thus severely effecting populations of aquatic beeings[27].

Horseradish Peroxidase

Peroxidase enzymes like lignin peroxidase, manganese peroxidase or horseradish peroxidase are suitable enzyme for the degradation of EE2 in waste water streams. The most studied and most promising enzyme among them is the Horseradish Peroxidase (HRP)[28], E.C. 1.11.1.7. HRP uses hydrogen peroxide for the oxidation of organic and inorganic matter. The source of HRP is horseradish which is growing in the moderate regions of the world. HRP is mainly used for diagnostic immunoassays and for cancer therapies. Nowadays HRP is mainly produced through biotechnology processes using recombinant microorganisms as highly efficient production systems. Several types of HRP are known among those the HRP C-enzyme constitutes the most commonly used[29].

HRP contains two metal centers where one is an iron heme group and two calcium metal centers (Figure 13).

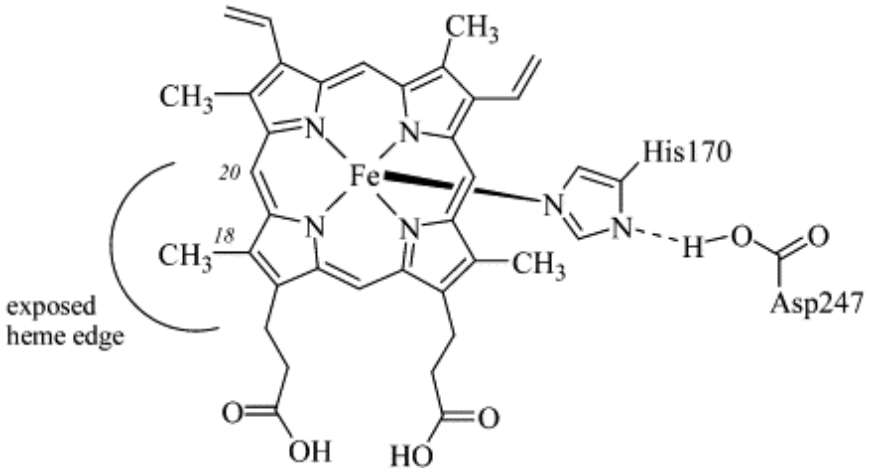


Figure 13: Structure of active center of HRP [29]

In general, both metal centers are essential for the catalytic activity of the enzyme. In addition to the metal centers, HRP consists of 308 amino acids with four disulphide bridges (see Figure 14)[29].

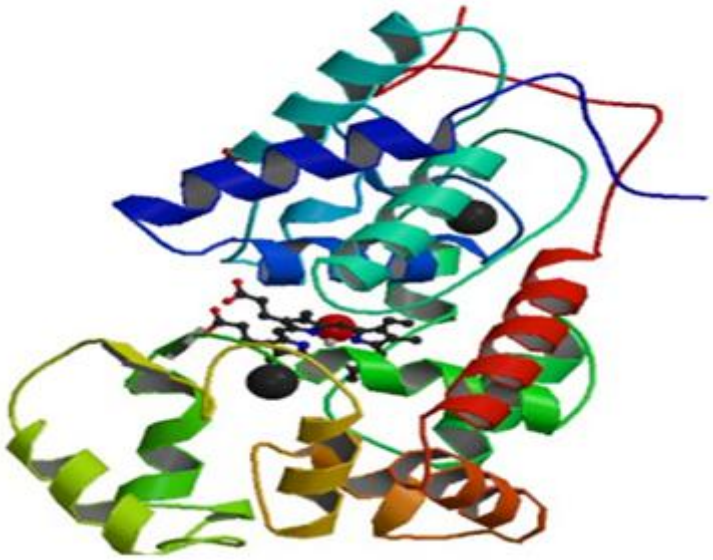


Figure 14: 3-Dimensional-Structure of HRP

The catalytic mechanism is well known as well (see Figure 15).

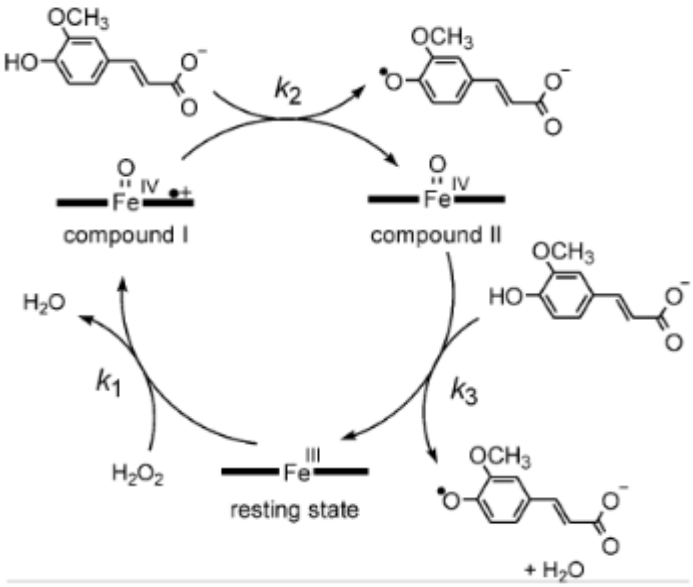


Figure 15: Mechanism of action of HRP[29]

The first step of the catalysis reaction is a reaction between H_2O_2 and Fe(III) which leads to compound I. Compound I merges with one alcohol to compound II and a radical. Compound II reacts to a radical and the resting state Fe(III). Step III is again the reaction with the substrate to radicals. The mechanism shows that one HRP-Protein can convert 2 substrates and H_2O_2 is needed to reactivate the active center of the enzyme (Figure 15). The radicals stemming from these reactions are not stable and decompose to other products[29].

Protein-Carbohydrate Interaction

Important biological phonemes including cell recognition or immune response are based on carbohydrate-protein interaction. To get an understanding of these phenomena it is important to study this kind of interactions[30,31]. Carbohydrate binding proteins are also called lectines. In general, carbohydrate-protein interaction can be studied from two different angles. One is from the view of carbohydrates, the other from the view of the protein.

In general, there are several different binding categories. The most important interactions are:

- Hydrophilic interaction
- Hydrophobic interaction
- Hydrogen bonding
- Van der Waals interaction
- Electrostatic interactions
- Multi side and singe side bonding

Hydrophilic interaction is the interaction of two polar components of the amino acid side chain and sugar residues.

Hydrophobic interaction is the interaction of proteins with C-H or other apolar side chains of amino acids and sugar residues.

Hydrogen bonding is the interaction of hydrogen bonds with any receptor, for example O, N, or F. This kind of interaction is mainly determined by functional groups like –OH and -NH_2 groups. In general the hydroxyl groups of sugars interact with the side chains of the amino acids[32].

Van der Waals interactions are forces which occur through a nonequivalent electron distribution in molecules. This generates temporal dipoles, thus initiating interaction.

Electrostatic interactions are considered to be the strongest interactions. In general, these are Colomb-forces of two charges and can either be attractive or repulsive. These

interactions occur between glycans and charged amino acids in the side chain or metal center of the protein[33].

The difference between multi side and singe side bonding is a quite important feature as well. Multi side bonding is considered to be stronger than singe side bonding because it may prevent the molecule from diffusing away and improves the collision frequency.

Experimental Section

Materials

Redox indicator ABTS

2,2'-Azino-di-(3-ethylbenzthiazolin-6-sulfonsäure) (ABTS), structure see Figure 16, is a special redox indicator. Horseradish peroxidase is an enzyme which is able to metabolise ABTS into radicals which can be detected at a wavelength of λ =405nm *via* photometry.

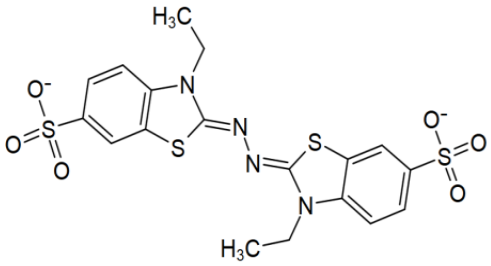


Figure 16: Structure of ABTS

ABTS-Assay Solution

220 mg ABTS was dissolved in in 20 ml 50mM NaOAc buffer with a pH value of 4,5 (11 mg/ml). 1ml (11 mg/ml) was mixed with 19 ml 50mM NaOAc buffer at pH 4,5.

HRP-Solution

HRP was obtained by Sigma Aldrich. The activity of the enzyme was 52 units/mg. The Enzyme concentration in solution was 1 g/L.

EDC

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (structure see Figure 17) is a water soluble carbodiimid. It is generally used as a carboxyl activating agent for the coupling of primary amines to yield amide bonds. In this work it was used to bind the enzyme to carboxy methyl cellulose (CMC) which was immobilised on membranes. The concentration of the EDC solution was 0,1wt%.

Figure 17: Structure of EDC

Carboxymethyl cellulose (CMC)

Carboxymethyl cellulose (CMC) is an ether derivate of cellulose (structure see Figure 18). The hydroxyl groups of natural cellulose are replaced through ether groups, which are connected with a Carboxy-methyl group.

The synthesis of this polymer is accomplished by using NaOH and chloroacetic acid. Applications for CMC are found for example in washing detergents and as adhesive agent.

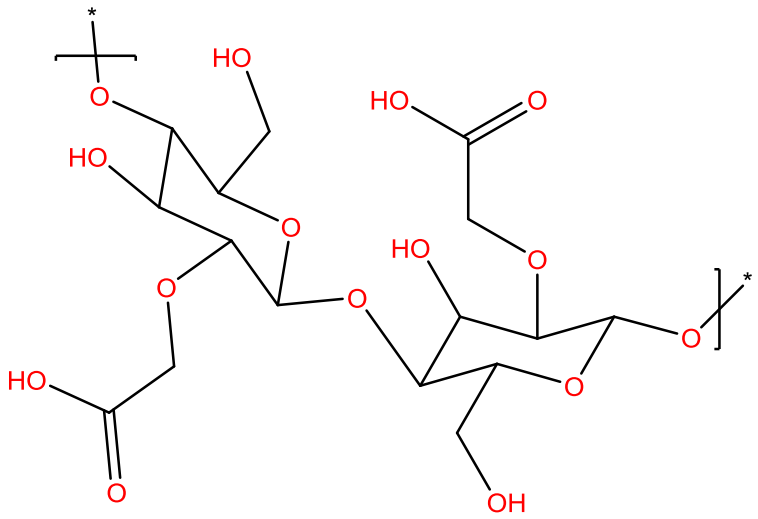


Figure 18: Structure of CMC

Chitosan

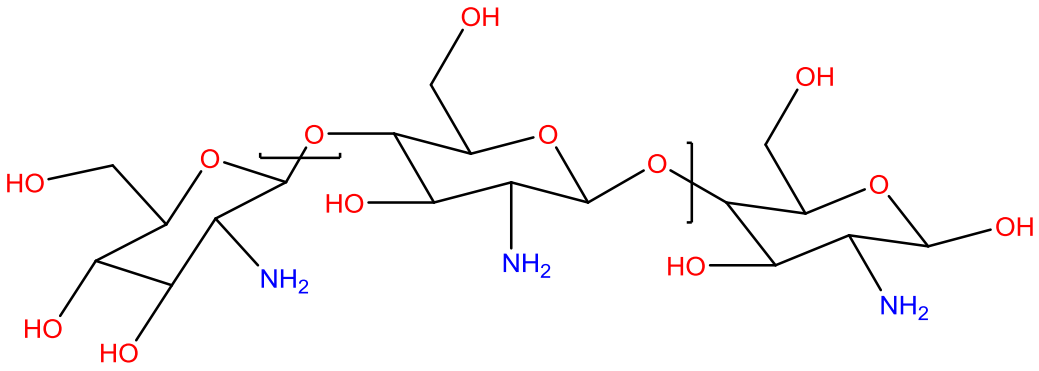


Figure 19: Structure of Chitosan

Chitosan (CHI; structure see Figure 19) is a biopolymer which derives from chitin. In general, chitosan can be produced through deacetylation of chitin using enzymes or NaOH. The unrivaled properties of showing good dissolving properties in acidic medium and bad solubility in neutral or alkaline media are a unique property displayed by no other biopolymer.

Applications for Chitosan are, for example, filter materials, medical products (based on its styptic effects) and as additive in paper industry.

Polymers used in the study

PES

Poly(oxy-1,4-phenylsulfonyl-1,4-phenyl), also known as poly(ethersulfone) (PES), is a polymer with thermoplastic properties, and, chemically seen, constitutes a poly(ether); for the structure see Figure 20. It is a chemically stable material, solid at room temperature and highly resistant towards chemical and enzymatic hydrolysis. PES has an excellent heat resistance and is mainly used by the electrical and medical industry.

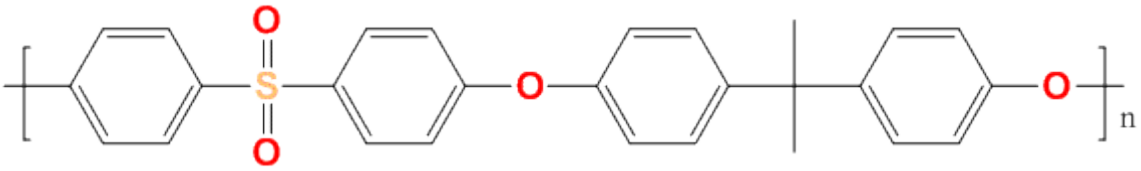
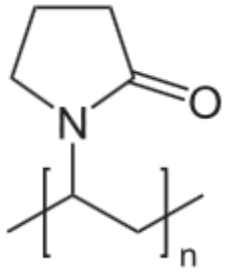


Figure 20: Structure of PES

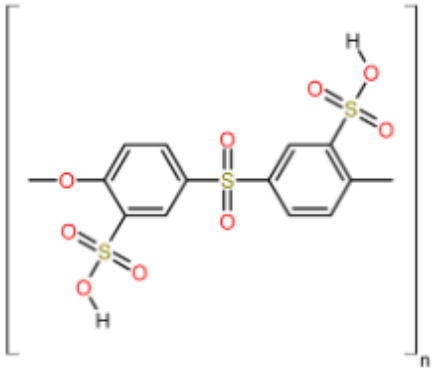
PVP

Poly(vinylpyrrolidon) (PVP) (structure see Figure 21) is a highly polar polymer with hygroscopic and amorphous properties. The polymer has no dripping point but, in contrast to PES, a glass transition temperature.



Picture 21 Structure of PVP

S-PES



Picture 22 Structure of sulfonated polyether sulfone (S-PES)

F

The polymer S-PES (see Figure 21) was prepared by the company Pentair-X-Flow by sulfonization of PES after the polymerisation process. [personal communication Pentair X-Flow]

Measurement of the activity of immobilized HRP with Redox indicator ABTS

All activities of immobilized HRP were determined with an ABTS-assay. For this determination, 220 mg ABTS were dissolved in 20 ml NaOAc buffer at pH 4,5. 1 ml of ABTS solution was mixed with 19 ml NaOAc buffer at pH 4,5. After addition of 1,7 µl H_2O_2, 1ml of the ABTS solution was added to one membrane to be investigated. The increase of the absorption at 420 nm was measured with a Genesys 10 vis spectrophotometer. The activity was calculated with the following equation.

$$ABTS\frac{units}{mL} = \frac{\frac{mAU}{min} * f}{\varepsilon * d} * \frac{V}{v}$$

Calculation of ABTS units/mL: f = dilution factor. Ɛ ABTS = absorption coefficient of oxidized ABTS = 34,700 M^{-1} cm^{-1}. d = path length = 1 cm. V = total reaction volume = 155 µL. v = enzyme solution volume = 15 µL.

Sources

Chemicals used

Commercial Horse Radish Peroxidase (145.7 units/mg solid; Sigma Aldrich)

Commercial Horse Radish Peroxidase (52 units/mg solid; Sigma Aldrich)

Rotiquant protein assay (Carl Roth GmbH)

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma Aldrich)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDC) (Sigma Aldrich)

Sodium acetate (Carl Roth GmbH)

Carboxymethylcellulose (Mw 10,000 Da; Sigma Aldrich)

Chitosan ("Low molecular weight"; Sigma Aldrich)

1,4-Dioxane

N-methyl pyrrolidon (NMP). Sigma Aldric

Poly(ethersulfone) (PES): Obtained from Pentair X-Flow

Sulfonated Poly(ethersulfone): Obtained from Pentair X-Flow

Poly(vinylpyrrolidone) (PVP): Obtained from Pentair X-Flow

Hydrogen peroxide 30% (Carl Roth GmbH)

Cadmium sulfate (Lactan)

Sodium fluoride:

Sodium acetate (Carl Roth GmbH)

Absorption of the Enzyme on hollow Fiber Membranes with prea-treatment of CMC and CHI

Polysaccharides like Carboxymethylcellulose (CMC) and chitosan (CHI) are able to bind to hollow fibre membranes. This binding can make them accessible to enzyme immobilization[34]

The hollow fibre membranes were activated by three different methods (incubation in 0,1M HCl, 0,1M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt.-% CHI, 150 mM KCl at pH 5,5) for 20min.

Methods of immobilization

For the immobilization of HRP on pre-treated Hollow Fibre membranes, three different methods have been used.

- **Adsorption Method**: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated in a HRP solution for 30 min.
- **Chemical Linkage**: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated into an EDC (0,1%) HRP solution in the ratio 1:1 for 3 hours.
- **Combined Method**: The CMC and CHI coated membranes as described before were incubated into a HRP/CMC 0,05%/EDC 0,1% solution in the ratio 1:1:1 for 3 hours.

three different kinds of hollow fibre membranes were studied: MFO2 (microfiltration membrane), HFS (nanofiltration membrane) which consists of PES and S-PES, and the UFC-membrane (ultrafiltration).

After the immobilisation of enzyme all membranes were rinsed with water. An ABTS-Assay was made for the determination of the activity.

Results

Table 2 shows the activity of HRP immobilized by 3 different methods on MFO2 membranes.

Table 3 shows the activity of HRP immobilized by 3 different methods on HFS membranes.

Table 4 shows the activity of HRP immobilized by 3 different methods on UFC-membranes.

Table 2:

MFO2	Absorption Method [Units/min]	Combined Method [Units/min]	Chemical linkage Method [Units/min]
H2O	$35{,}0*10^{-5} \pm 7{,}6*10^{-5}$	$26{,}4*10^{-5} \pm 3{,}9*10^{-5}$	$30{,}0*10^{-5} \pm 5{,}0*10^{-5}$
HCl	$37{,}9*10^{-5} \pm 5{,}7*10^{-5}$	$38{,}7*10^{-5} \pm 7{,}9*10^{-5}$	$34{,}0*10^{-5} \pm 2{,}4*10^{-5}$
KOH	$43{,}6*10^{-5} \pm 3{,}5*10^{-5}$	$44{,}7*10^{-5} \pm 3{,}9*10^{-5}$	27,7*10-5±1,0*10^{-5}

Table 3:

HFS	Absorption Method [Units/min]	Combined Method [Units/min]	Chemical linkage Method [Units/min]
H2O	$26{,}7 *10^{-5} \pm 6{,}9*10^{-5}$	$19{,}3 \pm 1{,}5*10^{-5}$	$41{,}8*10^{-5} \pm 4{,}9*10^{-5}$
HCl	$28{,}8*10^{-5} \pm 3{,}2*10^{-5}$	25,0*10-5±6,0*10^{-5}	32,0*10-5±2,4*10^{-5}
KOH	$33{,}6*10^{-5} \pm 5{,}4*10^{-5}$	$25{,}1*10^{-5} \pm 54{,}6*10^{-5}$	$45{,}4*10^{-5} \pm 6{,}1*10^{-5}$

Table 4:

UFC	Absorption Method [Units/min]	Combined Method [Units/min]	Chemical linkage Method [Units/min]
H2O	$96,4*10^{-4}\pm7,9*10^{-4}$	$37,5*10^{-5}\pm1,3*10^{-5}$	$33,2*10^{-5}\pm3,6*10^{-5}$
HCl	$199*10^{-4}\pm10^{-4}$	$40,5*10^{-5}\pm7,0*10^{-5}$	$50,6*10^{-5}\pm6,7*10^{-5}$
KOH	$56,4*10^{-4}\pm0,1*10^{-4}$	$34,9*10^{-5}\pm2,5*10^{-5}$	$72,7*10^{-5}\pm9,6*10^{-5}$

Conclusion

As suggested by the data, the UFC-membrane appears to be the most promising membrane type for immobilization of HRP. The data show that the absorption method is the best for the purpose however the problem with the absorption method was that the membrane only absorbs the enzyme and doesn't bind it to the membrane. If the membrane gets in contact with the solution, the enzyme diffuses into the solution. Among the other two investigated methods, the combined method with HCl as pre-treatment method performs best on the membrane UFC. It can be conducted that the ideal method of pre-treatment (acidic, alkaline, or neutral, respectively) is very much dependent on the membrane used and on the method of enzyme immobilisation.

Effect of pH and salt concentration to the activity of immobilized enzyme

Enzymes are not the most stable biomolecules and so they have to be treated very cautiously. A change of the environmental condition of the enzyme can have huge effects to the enzyme activity. In the case of waste water treatment, one has to expect fluctuations in environmental conditions regarding various pivotal process parameters such as substrate concentration (expressed as biochemical oxygen demand), pH-value, temperature, or salinity. Therefore, the activity of HRP immobilized on hollow fiber membranes at different pH-values and different salinities is of high interest for practical application. Studies on the effect of pH and ionic strength are very important because changes can have tremendous impact to the activity of the enzyme. Exposure to extreme pH-values may irreversiblly inactivate the enzyme. There are several effects which are dependent on the pH-value which may influence the enzyme activity. This can happen through changes in activity because of dissociable groups of the enzyme, free substrate, enzyme substrate-complex or a combination of these effects[35].

Also the ionic strength can have an effect on the enzyme´s catalytic activity because ionic interactions between surfaces and the enzyme are very dependent to charge density. Another decisive factor to this interaction is the environment of the protein. Solved metal ions like Na^+, K^+, Ca^{2+} are able to shield the protein from the surface. That means that the salt concentration in the solution is very important for the interactions between the surface and the protein.

In the following section the effect of pH and ionic strength on immobilized enzyme on hollow Fiber Membranes have been studied.

Experimental

The hollow fibre membranes were activated by three different methods (incubation in 0,1M HCl, 0,1M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this, they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min.

Methods of immobilization

For the immobilization of HRP on pre-treated Hollow Fibre membranes, three different methods have been used.

- Adsorption Method: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated in a HRP solution for 30 min.
- Chemical Linkage: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated into an EDC (0,1%) HRP solution in the ratio 1:1 for 3 hours.
- Combined Method: The CMC and CHI coated membranes as described before were incubated into a HRP/CMC 0,05%/EDC 0,1% solution in the ratio 1:1:1 for 3 hours

Three different kinds of hollow fibre membranes were studied: MFO2 (microfiltration membrane) consisting of PES, HFS (nanofiltration membrane) which consists of PES and S-PES, and the UFC-membrane (ultrafiltration) consisting of PES.

After the immobilisation of the enzyme, all membranes were rinsed with water and incubated in different solutions with different salt concentrations for 1 week. pH 3 with salt concentration of [0 mol/L, 0,25 mol/L 1 mol/L NaCl], pH 7 with salt concentration of [0 mol/L, 0,25 mol/L 1 mol/L NaCl] and pH 8 with salt concentration of [0 mol/L 0,25 mol/L and 1 mol/L NaCl] were studied. Afterwards the membranes were rinsed again and an ABTS-assay was made to determine the activity.

Results

Table 5:shows the activity of HRP immobilized on MFO2 Membranes at different salinity and with different activation Methods at pH 3. Table 6 shows the activity of HRP immobilized on UFC membranes at different salinity and with different activation Methods at pH 3. Table 7 shows the activity of HRP immobilized on HFS membranes at different salinity and with different activation Methods at pH 3. Table 8 shows the activity of HRP immobilized on MFO2 membranes at different salinity and with different activation Methods at pH 7. Table 9 shows the activity of HRP immobilized on UFC membranes at different salinity and with different activation Methods at pH 7. Table 10: shows the activity of HRP immobilized on f HFS membranes at different salinity and with different activation Methods at pH 7. Table 11 shows the activity of HRP immobilized on f MFO2 membranes at different salinity and with different activation Methods at pH 8Table 12 shows the activity of HRP immobilized on f UFC membranes at different salinity and with different activation Methods at pH 8. Table 13 shows the activity of HRP immobilized on f HFS membranes at different salinity and with different activation Methods at pH 8.

Table 5: Activity of HRP immobilized on MFO2 Membranes at different salinity and with different activation Methods at pH 3

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]	No activity	No activity	No activity	$5*10^{-4}$±0,7*10^{-4}	$1,3*10^{-3}$±0,2*10^{-3}	$1*10^{-4}$±0,7*10^{-4}	$2,5*10^{-4}$±0,3*10^{-4}	$2,5*10^{-4}$±0,2*10^{-4}	$2,4*10^{-4}$±0,3*10^{-4}
0	No activity	No activity	No activity	No activity	$3,5*10^{-4}$±0,6*10^{-4}	$2,5*10^{-4}$±0,2*10^{-4}	No activity	No activity	No activity
0.25	No activity	No activity	No activity	$5,6*10^{-4}$±0,9*10^{-4}	$7,2*10^{-4}$±0,8*10^{-4}	$3,6*10^{-4}$±0,9*10^{-4}	No activity	No activity	No activity
1	No activity	No activity	No activity	$5*10^{-4}$±0,7*10^{-4}	$1,3*10^{-3}$±0,2*10^{-3}	$1*10^{-4}$±0,7*10^{-4}	$2,5*10^{-4}$±0,3*10^{-4}	$2,5*10^{-4}$±0,2*10^{-4}	$2,4*10^{-4}$±0,3*10^{-4}

Table 6: Activity of HRP immobilized on UFC membranes at different salinity and with different activation Methods at pH 3

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]									
0	No activity	No activity	No activity	$4,5*10^{-4}$±$1,5*10^{-4}$	$1,1*10^{-3}$±$0,2*10^{-3}$	$9,7*10^{-4}$±$0,8*10^{-4}$	No activity	No activity	No activity
0.25	No activity	No activity	No activity	4,5*10-4±1,6*10-4	8*10-5±9,4*10-5	4,9*10-4±0,6*10-4	No activity	No activity	No activity
1	No activity	No activity	No activity	8,0*10-4±0,,6*10-4	3,3*10-4±1,1*10-4	3,7*10-4±0,9*10-4	No activity	No activity	No activity

Table 7: Activity of HRP immobilized on HFS membranes at different salinity and with different activation Methods at pH 3

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]									
0	No activity	No activity	No activity	$4,5*10^{-4}\pm1,5*10^{-4}$	$1,2*10^{-3}\pm0,2*10^{-3}$	$2,0*10^{-3}\pm0,8*10^{-3}$	$4,2*10^{-4}\pm0,2*10^{-4}$	$3,3*10^{-4}\pm0,3*10^{-4}$	$4,0*10^{-4}\pm0,5*10^{-4}$
0.25	No activity	No activity	No activity	$4,4*10^{-3}\pm0,6*10^{-3}$	$4,4*10^{-4}\pm0,6*10^{-4}$	6,0*10-$^{4}\pm0,3*10^{-4}$	No activity	No activity	No activity
1	No activity	No activity	No activity	$8,9*10^{-4}\pm0,7*10^{-4}$	$3,4*10^{-4}\pm1,1*10^{-4}$	$0,7*10^{-4}\pm0,5*10^{-4}$	No activity	No activity	No activity

Table 8: Activity of HRP immobilized on MFO2 membranes at different salinity and with different activation Methods at pH 7

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]	$1,0*10^{-3}\pm0,1*10^{-3}$	$6,6*10^{-4}\pm0,7*10^{-4}$	$1,1*10^{-3}\pm0,1*10^{-4}$	$9,6*10^{-4}\pm1,9*10^{-6}$	$8,5*10^{-4}\pm1,9*10^{-4}$	$1,3*10^{-3}\pm0,1*10^{-3}$	$3*10^{-4}\pm0,3*10^{-4}$	No activity	$2,0*10^{-4}\pm1,3*10^{-4}$
0	$1,4*10^{-3}\pm0,5*10^{-3}$	$8,7*10^{-4}\pm0,1*10^{-4}$	$6,5*10-^{4}\pm1,4*10^{*-4}$	$1,2*10^{-3}\pm0,1*10^{-3}$	$1,1*10^{-3}\pm0,2*10^{-3}$	$1,3*10^{-3}\pm0,07*10^{-3}$	No activity	No activity	No activity
0.25	$5,0*10^{-4}\pm1,2*10^{-4}$	$4,2*10^{-4}\pm0,5*10^{-4}$	$4,0*10^{-4}\pm1,6*10^{-4}$	$1,5*10^{-3}\pm0,1*10^{-3}$	$1,3*10^{-3}\pm0,4*10^{-3}$	$1,5*10^{-3}\pm0,3*10^{-3}$	No activity	No activity	No activity
1	$1,0*10^{-3}\pm0,1*10^{-3}$	$6,6*10^{-4}\pm0,7*10^{-4}$	$1,1*10^{-3}\pm0,1*10^{-4}$	$9,6*10^{-4}\pm1,9*10^{-6}$	$8,5*10^{-4}\pm1,9*10^{-4}$	$1,3*10^{-3}\pm0,1*10^{-3}$	$3*10^{-4}\pm0,3*10^{-4}$	No activity	$2,0*10^{-4}\pm1,3*10^{-4}$

Table 9: Activity of HRP immobilized on UFC membranes at different salinity and with different activation Methods at pH 7

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]									
0	$1,1*10^{-3}\pm0,3*10-3$	$1,0*10^{-3}\pm0,1*10^{-3}$	$1,2*10^{-3}$	$4,5*10^{-4}\pm1,5*10^{-4}$	$1,2*10^{-3}\pm0,2*10^{-3}$	$9,7*10^{-4}\pm0,7*10^{-4}$	$1,2*10^{-4}\pm0,3*10^{-4}$	$3,7*10^{-4}\pm0,3*10^{-4}$	$2,4*10^{-4}$
0.25	$1,5*10^{-3}\pm0,*10^{-3}$	$1,2*40^{-3}$	$1,4*10^{-3}$	$4,5*10^{-4}\pm1,6*10^{-3}$	$8,0*10^{-5}\pm9,5*10^{-4}$	$4,9*10^{-4}\pm0,6*10^{-4}$	$1,8*10^{-4}\pm0,1*10^{-4}$	$2,7*10^{-4}\pm1,5*10^{-4}$	$1,5*10^{-4}\pm0,6*10^{-4}$
1	$1,3*10^{-3}\pm0,2*10^{-3}$	$4,3*10^{-4}\pm1,0*10^{-4}$	$5*10^{-4}\pm1,3*10^{-4}$	$4,2*10^{-4}\pm0,1*10^{-4}$	$6,0*10^{-4}\pm0,3*10^{-4}$	$4,2*10^{-4}\pm1,1*10^{-4}$	No activity	No activity	No activity

Table 10: Activity of HRP immobilized on f HFS membranes at different salinity and with different activation Methods at pH 7

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]									
0	$6,7*10^{-4}\pm0,6*10^{-4}$	$9,1*10^{-4}\pm1,1*10^{-4}$	$1,9*10^{-4}\pm0,1*10^{-4}$	$9,0*10^{-4}\pm0,3*10^{-4}$	$1,,1*10-^{3}\pm0,2*10^{-3}$	$8,4*10^{-4}\pm0,5*10^{-4}$	$4,7*10^{-4}\pm1,0*10^{-4}$	No activity	No activity
0.25	$3,3*10^{-4}\pm0,4*10^{-4}$	$3,5*10^{-4}\pm0,6*10^{-4}$	$2,7*10^{-4}\pm0,2*10^{-4}$	$7,9*10^{-4}\pm0,8*10^{-4}$	$9,3*10^{-4}\pm2,5*10^{-4}$	$8,4*10^{-4}\pm1,8*10^{-4}$	$3,7*10^{-4}\pm0,8*10^{-4}$	$1,5*10^{-4}\pm0,6*10^{-4}$	No activity
1	$2,6*10^{-4}\pm0,2*10^{-4}$	$2,9*10^{-4}\pm0,9*10^{-4}$	$8,6*10^{-4}\pm1,5*10^{-4}$	$1,5*10^{-3}\pm2,6*10^{-3}$	$2,0*10^{-3}\pm0,3*10^{-3}$	$1,4*10^{-3}\pm0,1*10^{-3}$	No activity	No activity	No activity

Table 11: Activity of HRP immobilized on f MFO2 membranes at different salinity and with different activation Methods at pH 8

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]									
0	$1,7*10^{-4}\pm0,4*10^{-4}$	$1,9*10^{-4}\pm1*10^{-4}$	$1,7*10^{-4}\pm0,2*10^{-4}$	$3,2*10^{-3}\pm0,4*10^{-3}$	$3,4*10^{-3}\pm0,9*10^{-3}$	$3,6*10^{-3}\pm0,8*10^{-3}$	$1,2*10^{-3}\pm0,2*10^{-3}$	$1,1*10^{-3}\pm0,2*10^{-3}$	$1,1*10^{-3}\pm0,2*10^{-3}$
0.25	$8,2*10^{-5}\pm5,6*10^{-5}$	$5,1*10^{-5}\pm6,9*10^{-5}$	$1,0*10^{-4}\pm0,9*10^{-4}$	$2,9*10^{-3}\pm0,8*10^{-3}$	$2,0*10^{-3}\pm0,5*10^{-3}$	$2,5*10^{-3}\pm1,0*10^{-3}$	$1,2*10^{-3}\pm0,2*10^{-3}$	$1,7*10^{-3}\pm0,3*10^{-4}$	$9,0*10^{-4}\pm3,8*10^{-4}$
1	$2,4*10^{-4}\pm0,6*10^{-4}$	$1,5*10^{-4}\pm0,2*10^{-4}$	$3,0*10^{-4}\pm0,5*10^{-4}$	$3,2*10^{-3}\pm0,3*10^{-3}$	$4,0*10^{-3}\pm0,7*10^{-3}$	$1,8*10^{-3}\pm0,3*10^{-3}$	$1,0*10^{-3}$	$1,4*10^{-3}\pm0,2*10^{-3}$	$7,9*10^{-4}\pm1,1*10^{-4}$

Table 12: Activity of HRP immobilized on f UFC membranes at different salinity and with different activation Methods at pH 8

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]									
0	$2,5*10^{-4}$±$1,1*10^{-4}$	$7,5*10^{-4}$±$3,3*10^{-4}$	$2,9*10^{-4}$±$0,5*10^{-4}$	$3,4*10^{-3}$±$0,2*10^{-4}$	$5,1*10^{-3}$±$2,0*10^{-3}$	$4,0*10^{-3}$±$0,1*10^{-3}$	6,7*10-4±1,6*10-4	9,4*10-4±1,6*10-4	8,5*10-4±0,6*10-4
0.25	$8,2*10^{-4}$±$2,7*10^{-4}$	$8,4*10^{-4}$±$0,3*10^{-4}$	$8,3*10^{-4}$±$0,3*10^{-4}$	$1,1*10^{-3}$±$0,2*10^{-3}$	$3,0*10^{-3}$±$0,6*10^{-3}$	$2,3*10^{-3}$±$0,5*10^{-3}$	$1,4*10^{-3}$±$0,8*10^{-3}$	$2,4*10^{-3}$±$1,0*10^{-3}$	$1,8*10^{-3}$±$0,3*10^{-3}$
1	$2,4*10^{-4}$±$0,6*10^{-4}$	$2,6*10^{-4}$±$0,2*10^{-4}$	$3,6*10^{-4}$±$0,6*10^{-4}$	2,2*10-3±0,3*10-3	$4,5*10^{-3}$±$0,6*10^{-3}$	$2,6*10^{-3}$±$0,2*10^{-3}$	$9,0*10^{-4}$±$3,8*10^{-4}$	$1,8*10^{-3}$±$0,1*10^{-3}$	$1,6*10^{-3}$±$0,3*10^{-3}$

Table 13: Activity of HRP immobilized on f HFS membranes at different salinity and with different activation Methods at pH 8

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
Salinity [Mol NaCl]									
0	$5,3*10^{-4}\pm0,6*10^{-4}$	$5,4*10^{-4}\pm0,4*10^{-4}$	$6,8*10^{-4}\pm1,8*10^{-4}$	$2,5*10^{-3}\pm0,4*10^{-3}$	$2,2*10^{-3}\pm0,2*10^{-3}$	$2,8*10^{-3}\pm0,5*10^{-3}$	$2,4*10^{-3}\pm0,8*10^{-3}$	$2,3*10^{-3}\pm0,9*10^{-3}$	$1,*10^{-3}\pm0,2*10^{-3}$
0.25	$6,0*10^{-4}\pm0,8*10^{-4}$	$5,2*10^{-4}\pm1,4*10^{-4}$	$5,6*10^{-4}\pm1,1*10^{-4}$	$2,0*10^{-3}\pm0,5*10^{-3}$	$2,3*10^{-3}\pm0,4*10^{-3}$	$1,4*10^{-3}$	$1,5*10^{-3}\pm0,1*10^{-3}$	$2,1*10^{-3}\pm0,1*10^{-3}$	$1,8*10^{-3}\pm0,1*10^{-3}$
1	$7,2*10^{-4}\pm1,7*10^{-4}$	$8,2*10^{-4}\pm1,4*10^{-4}$	$1,1*10^{-3}\pm0,2*10^{-3}$	$2,0*10^{-3}\pm0,4*10^{-3}$	$2,5*10^{-3}\pm0,8*10^{-3}$	$1,8*10^{-3}\pm0,1*10^{-3}$	$1,8*10^{-3}\pm0,1*10^{-3}$	$1,8*10^{-3}\pm0,2*10^{-3}$	$1,7*10^{-3}\pm0,2*10^{-3}$

Conclusion

The results of the experiment show clearly that the influence of pH has a much higher effect on the activity of the enzyme than the investigated levels of salt concentration. Considering highly acidic conditions (pH 3,0), the enzyme activity results in nearly a complete loss of activity for the adsorption and chemical linkage method for all membranes. At this low pH, only the combined method shows some activity for MFO2 and HFS membranes. For the storage at slightly alkaline conditions (pH 8), the membranes show higher activity than in the acidic conditions for all immobilization methods.
At pH 7,0, the best results were obtained in the case of UFC and MFO2 membranes. Considering HFS- membranes the activity values are in the same order than for the alkaline storage.
Comparing different salinities, no real effect of the activity of immobilized HRP can be deducted from the obtained results in the salt range investigated.

Stability III: Influence of Inhibitors on the Activity of Enzyme Immobilized on HFs: Example Fluorid

Fluorid (F^-) is a well-known pollutant in diverse waste water streams. Due to the fact that F^- ions are well known to inhibit various catalytic centres of diverse biocatalysts, it was of interest to examine the inhibitory impact of F^- on HRP immobilized on hollow fibre membranes. Literature reports various impacts of F^- on (bio)catalytic systems. Up to now nor reports have been published for the impact of F^- on immobilized HRP in literature. In the following section the impact of different concentration levels of F^- Ions on immobilized HRP on MFO2, UFC and HFS-Membranes have been studied

Experiment

The hollow fibre membranes were activated by three different methods (incubation in 0,1 M HCl, 0,1 M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min.

For the immobilization of HRP on pre-treated hollow fibre membranes, 3 different methods have been used.

After the immobilisation of the enzyme, all membranes were rinsed with water and incubated in different F- containing solutions (0,1 g/L and 1 g/L). Afterwards, the membranes were rinsed again with water and an ABTS-Assay was made to determine the activity.

Results

Table 14 shows the inhibition of F^- ions on the activity of immobilized HRP on UFC-membranes by different methods. Table 15 shows the inhibition of F^- ions on the activity of immobilized HRP on HFS-membranes by different methods. Table 16 shows the inhibition of F^- ions on the activity of immobilized HRP on MFO2-membranes by different methods.

Table 14: Results for the inhibition of immobilized HRP on UFC –Membranes

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
F^- [g/L]									
0.1	$7,6*10^{-3}\pm2,0*10^{-3}$	$3,3*10^{-3}\pm0,1*10^{-3}$	$3,7*10^{-3}\pm0,3*10^{-3}$	$2,9*10^{-3}\pm0,4*10^{-3}$	$3,4*10^{-3}\pm0,2*10^{-3}$	$2,5*10^{-3}\pm0,1*10^{-3}$	$2,1*10^{-3}\pm0,4*10^{-3}$	$2,1*10^{-3}\pm0,4*10^{-3}$	$1,6*10^{-3}\pm0,3*10^{-3}$
1	$2,3*10^{-3}\pm0,3*10^{-3}$	$3,3*10^{-3}\pm0,5*10^{-3}$	$2,2*10^{-3}\pm0,3*10^{-3}$	$3,9*10^{-3}$	$1,5*10^{-3}\pm1,3*10^{-3}$	$1,2*10^{-3}\pm0,2*10^{-3}$	$2,6*10^{-3}\pm0,2*10^{-3}$	$3,0*10^{-3}\pm0,1*10^{-3}$	$3,0*10^{-3}\pm0,4*10^{-3}$

Table 15: Results for the inhibition of immobilized HRP on HFS –Membranes

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
F^- [g/L]									
0.1	$2,5*10^{-3}\pm0,3*10^{-3}$	$8,2*10^{-3}\pm0,5*10^{-3}$	$1,4*10^{-3}\pm0,4*10^{-3}$	$1,5*10^{-3}\pm0,1*10^{-3}$	$1,4*10^{-3}$	$2,0*10^{-3}\pm0,5*10^{-3}$	$2,7*10^{-3}\pm0,2*10^{-3}$	$2,4*10^{-3}\pm0,5*10^{-3}$	$4,6*10^{-3}\pm0,7*10^{-3}$
1	$3,3*10^{-3}\pm0,7*10^{-3}$	$2,3*10^{-3}\pm0,3*10^{-3}$	$1,4*10^{-3}\pm0,2*10^{-3}$	$2,5*10^{-3}\pm0,2*10^{-3}$	$1,5*10^{-3}$	$1,0*10^{-3}\pm0,2*10^{-3}$	$3,2*10^{-3}\pm0,6*10^{-3}$	$1,4*10^{-3}\pm0,1*$ 10-3	$8,8*10^{-4}\pm0,4*10^{-4}$

Table 16: Results for the inhibition of immobilized HRP on MFO2 –Membranes

Immobilization method	Adsorption	Adsorption	Adsorption	Covalent linkage	Covalent linkage	Covalent linkage	Combined method	Combined method	Combined method
Pre-treatment	HCl	KOH	Water	HCl	KOH	Water	HCl	KOH	Water
F^- [g/L]									
0.1	$2,8*10^{-3}\pm0,1*10^{-3}$	$4,0*10^{-3}\pm0,8*10^{-3}$	$1,3*10^{-3}\pm0,3*10^{-3}$	$2,3*10^{-3}$	$2,2*10^{-3}\pm0,5*10^{-3}$	$1,7*10^{-3}\pm1,2*10^{-3}$	$1,8*10^{-3}\pm0,6*10^{-3}$	$1,7*10^{-3}\pm0,3*10^{-3}$	$1,5*10^{-3}$
1	$2,5*10^{-3}\pm0,7*10^{-3}$	$1,7*10^{-3}\pm0,7*10^{-3}$	$3,0*10^{-3}\pm0,3*10^{-3}$	$1,8*10^{-3}\pm1,2*10^{-3}$	$1,6*10^{-3}\pm1,4*10^{-3}$	$3,1*10^{-3}\pm0,3*10^{-3}$	$2,1*10^{-3}\pm0,3*10^{-3}$	$7,0*10^{-4}\pm1,7*10^{-4}$	$0,26*10^{-3}\pm0,2*10^{-3}$

Conclusion

Considering the results of the measurement of the inhibition of F- Ions on immobilized HRP on different hollow fiber membranes it can be deducted that Fluoride Ions have no real effect on the activity of the enzyme.

Comparing the results of 0,1g/L F- and 1g/L Fluoride it can be observed that no significant effect, considering activity of the enzyme can be found through every membrane and adsorption method. UFC membranes with adsorption method shows highest activity considering concentrations of 0,1g/L F^-. Also the other methods show highest activity on the UFC membranes.

Effect of Cadmium Ions on immobilized HRP on UFC hollow fiber membranes

Ultrafiltration is used in many membrane separation processes. Especially in waste water treatment, ultrafiltration is used [36]. Because of its good thermal and chemical stability, UFC membranes often consist of Polysulfone [37]. In ultrafiltration membrane fouling is a serious problem. There are many factors which contribute to membrane fouling. These are, for example, surface properties (chemistry, morphology, etc.), hydrodynamic conditions, ionic strength, and solute concentration. [37]. Rising concentrations of heavy metals in waste water start to become more and more problem. Heavy metals are frequently occurring in contaminated waste water samples. Cadmium, the selected heavy metal used for the subsequently described investigation, enters the environment by several industrial processes like nonferrous metal production and iron and steel production [38]. Environmental problems from cadmium are based on its high toxic effect on humans, animals, microorganisms and plants. Reported toxic effects show that it causes skeletal deformation [39], has carcinogenic effects[40] and decreases in the bone strength [41]. Cadmium is also well known to have an negative impact to the catalytic performance on enzymes [42]. Isoenzymes of HRP play an important role in physiological processes including the metabolism of indole-3-acetic acid, lignification and resistance to infection [43]. However recent studies have shown the inhibition of HRP by metal Ions. In the following study the effect of increasing Cd^{2+} concentrations (between 0 and 10 g/L) on HRP immobilized on hollow fiber membranes have been investigated. The activity was measured with the redox indicator ABTS

Effect of Cadmium^{2+} Ions on HRP in solution (free enzyme)

From a solution containing 0,1 g/L HRP the activity was measured with the redox indicator ABTS. For this 220mg ABTS were solved in 20ml NaOAc buffer pH 4,5. 1 ml of ABTS solution was mixed with 19ml NaOAc buffer pH 4,5. After addition of 1,7 µL H_2O_2 1ml of the ABTS solution was added to one membrane. The increase of the absorption was measured with a Genesys 10 vis spectrophotometer. The activity was calculated with the following equation.

$$ABTS\frac{units}{mL} = \frac{\frac{mAU}{min} * f}{\varepsilon * d} * \frac{V}{v}$$

Calculation of ABTS units/mL: f = dilution factor. Ɛ ABTS = absorption coefficient of oxidized ABTS = 34,700 M^{-1} cm^{-1}. d = path length = 1 cm. V = total reaction volume = 155 µL. v = enzyme solution volume = 15 µL.

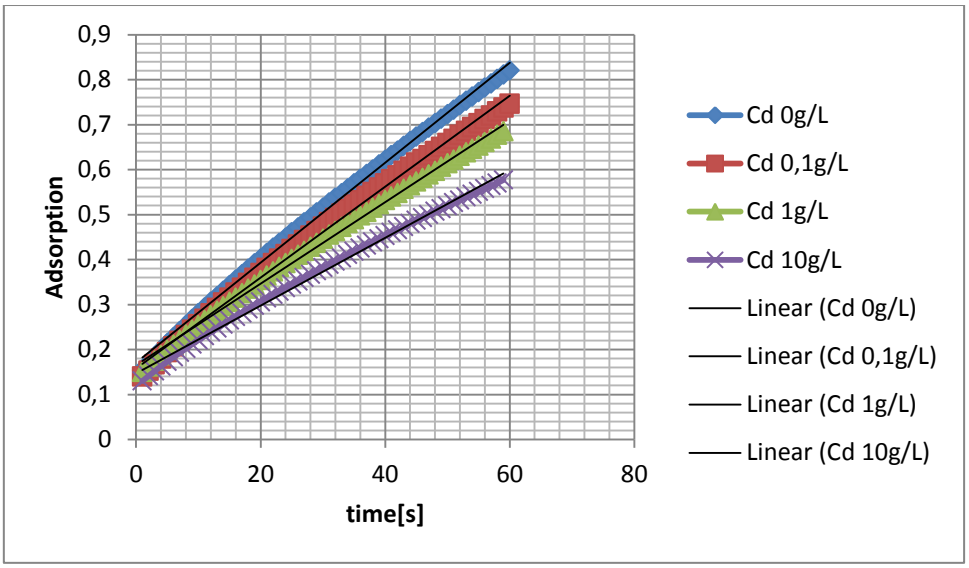


Figure 23 Activity of free Enzyme with different Cadmium concentration

$$Specific\ \ Activity[\ {}^{Units}/_{mg}\ ] = \frac{Activity[Units]}{c[Enzyme]}$$

Equation 1: Calculation of Specific Acitivty

Table 17: Specific activity and activity in Katal

	[Units/mg]	[Katal*10^{-7}]
„Activity calculated according to commercial provider of the enzyme	52	8,6
Measured Activity of HRP in Cd^{2+} 0g/L	57,3±2,6	9,5±0,4
Measured Activity of HRP in Cd^{2+} 0,1g/L	54,6±3,7	9,1±0,6
Measured Activity of HRP in Cd^{2+} 1g/L	47,7±1,3	7,9*10-5±0,2
Measured Activity of HRP in Cd^{2+} 10g/L	35,98±8,0	6,0*10-5±1,3

Effect of Cadmium(2^+) Ions on immobilized HRP

The hollow fibre membranes were activated by three different methods (incubation in 0,1 M HCl, 0,1M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min.

For the immobilization of HRP on pre-treated Hollow Fibre membranes 3 different methods have been used.

After immobilization the membranes got rinsed with water for 20 min and incubated either in 0,1g Cd^{2+}/L, 1g Cd $^{2+}$/L or 10g Cd^{2+}/L for 1 week. After rinsing with water for 20 minutes the HRP activity was measured by an ABTS-Assay.

Results of adsorption methode

Figure 24 shows the Effect of Cadmium Ions on immobilized HRP by absorption method on UFC-Hollow Fiber membranes which are pre-treated with H_2O. Figure 25 Effect of Cadmium Ions on immobilized HRP by absorption method on UFC-Hollow Fiber membranes which are pre-treated with HCl. Figure 26 Effect of Cadmium Ions on immobilized HRP by absorption method on UFC-Hollow Fiber membranes which are pre-treated with KOH

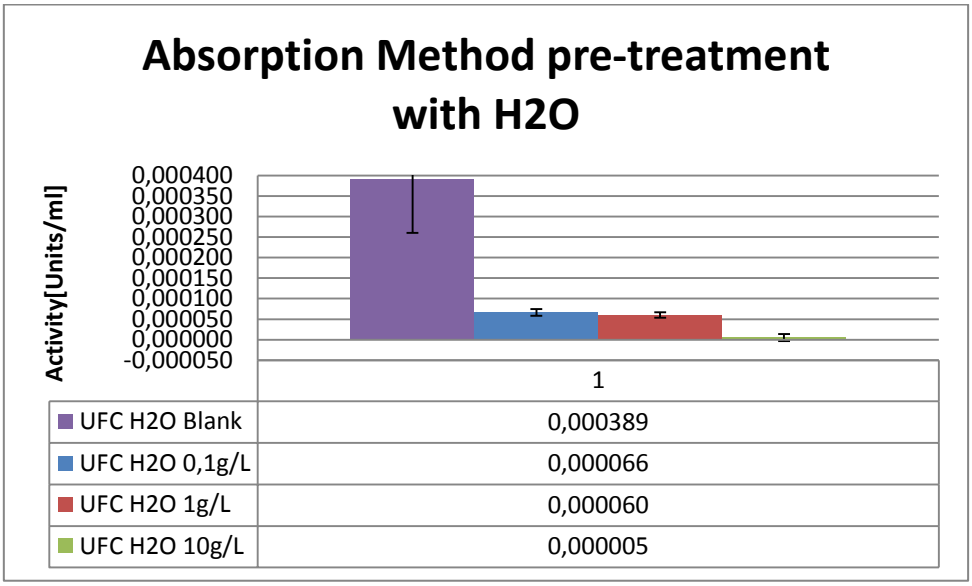


Figure 24 Effect of Cadmium Ions on immobilized HRP by absorption method on UFC-Hollow Fiber membranes which are pre-treated with H_2O

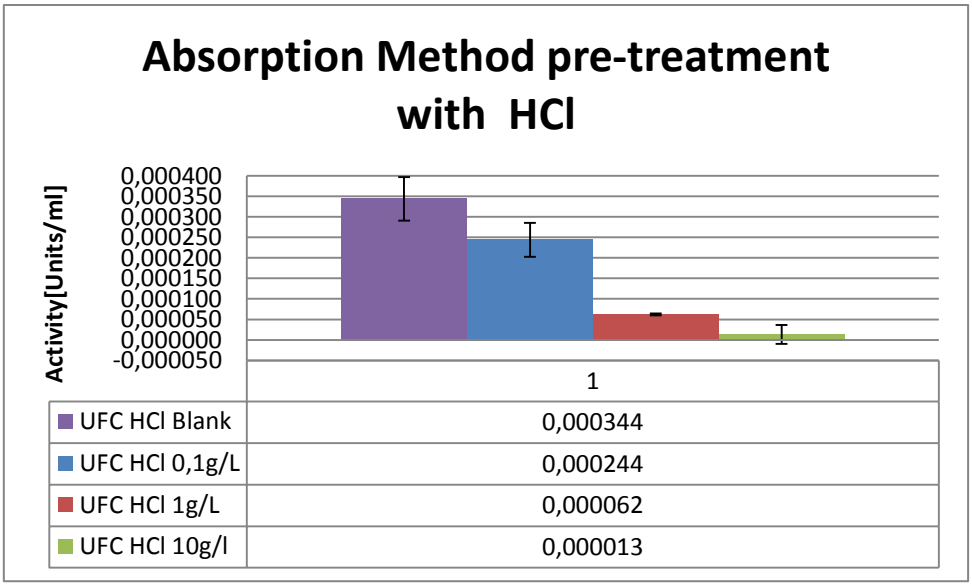


Figure 25 Effect of Cadmium Ions on immobilized HRP by absorption method on UFC-Hollow Fiber membranes which are pre-treated with HCl

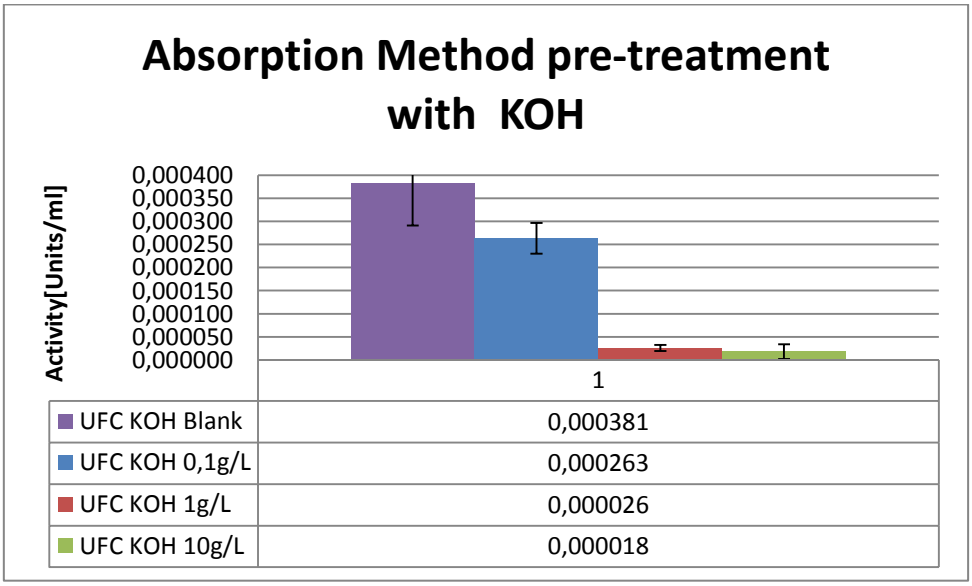


Figure 26 Effect of Cadmium Ions on immobilized HRP by absorption method on UFC-Hollow Fiber membranes which are pre-treated with KOH

Results of chemical linkage

Figure 27 shows the effect of Cadmium Ions on immobilized HRP by chemical linkage on UFC-Hollow Fiber membranes which are pre-treated with H_2O. Figure 28 shows the effect of Cadmium Ions on immobilized HRP by chemical linkage on UFC-Hollow Fiber membranes which are pre-treated with HCl. Figure 29 shows the effect of Cadmium Ions on immobilized HRP by chemical linkage on UFC-Hollow Fiber membranes which are pre-treated with KOH.

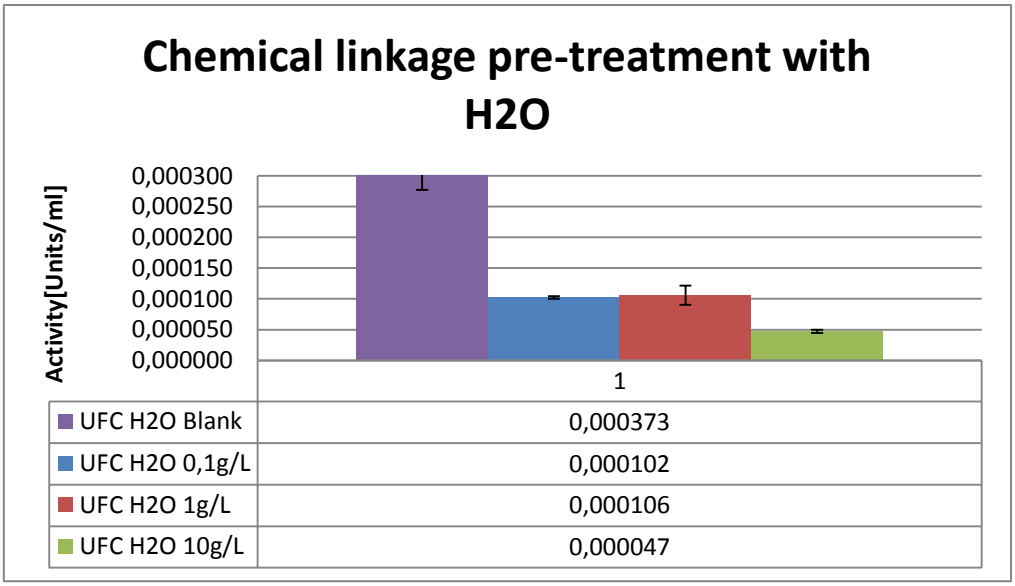


Figure 27 Effect of Cadmium Ions on immobilized HRP by chemical linkage on UFC-Hollow Fiber membranes which are pre-treated with H_2O

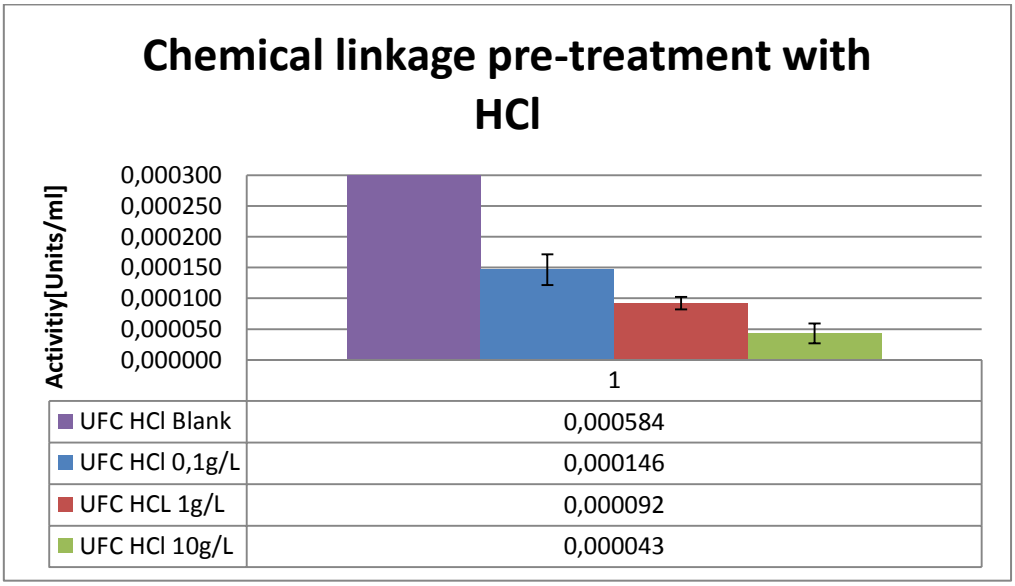


Figure 28 Effect of Cadmium Ions on immobilized HRP by chemical linkage on UFC-Hollow Fiber membranes which are pre-treated with HCl

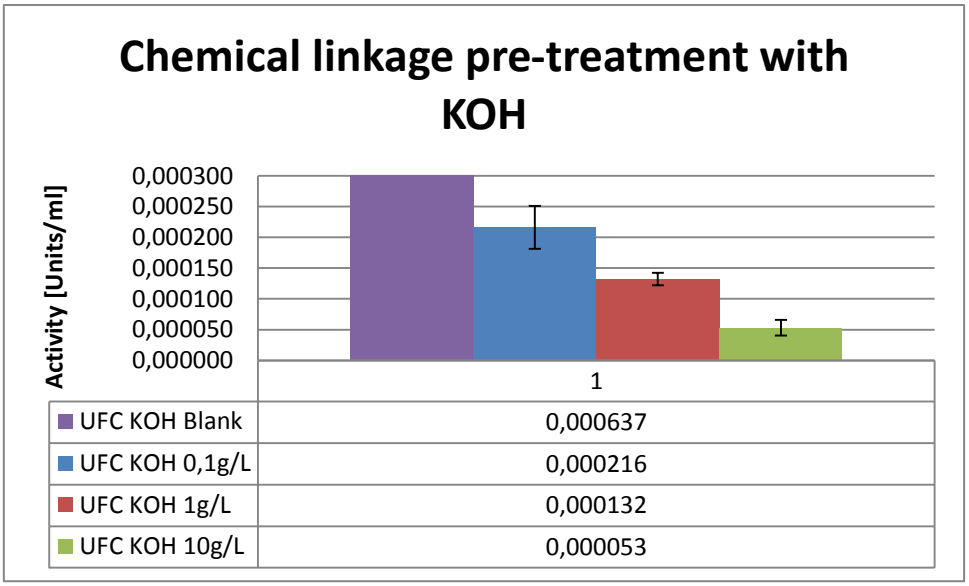


Figure 29 Effect of Cadmium Ions on immobilized HRP by chemical linkage on UFC-Hollow Fiber membranes which are pre-treated with KOH

Results combined Method

Figure 30 shows the effect of Cadmium Ions on immobilized HRP by combined method on UFC-Hollow Fiber membranes which are pre-treated with H_2O. Figure 31 shows the effect of Cadmium Ions on immobilized HRP by combined method on UFC-Hollow Fiber membranes which are pre-treated with HCl. Figure 32 shows the effect of Cadmium Ions on immobilized HRP by combined method on UFC-Hollow Fiber membranes which are pre-treated with KOH

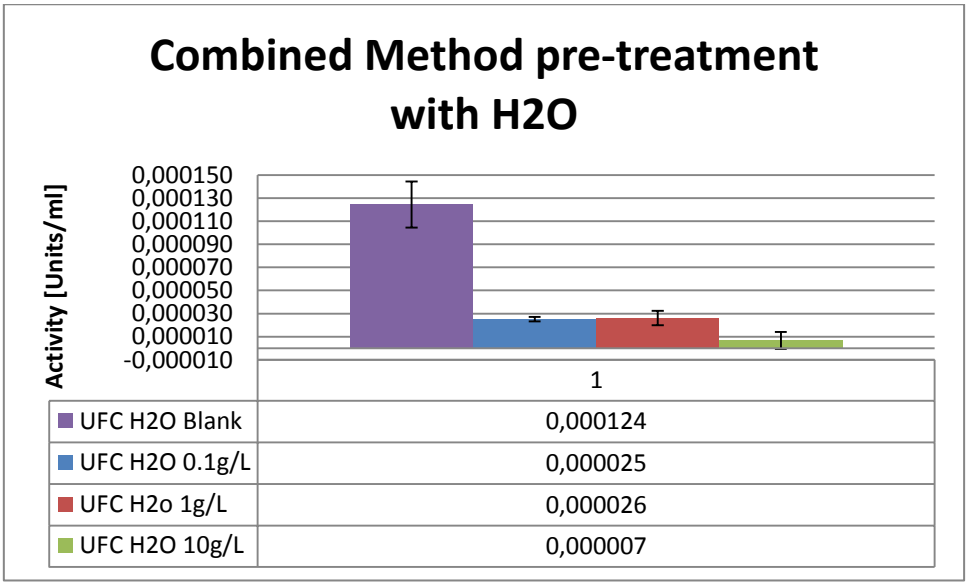


Figure 30 Effect of Cadmium Ions on immobilized HRP by combined method on UFC-Hollow Fiber membranes which are pre-treated with H_2O

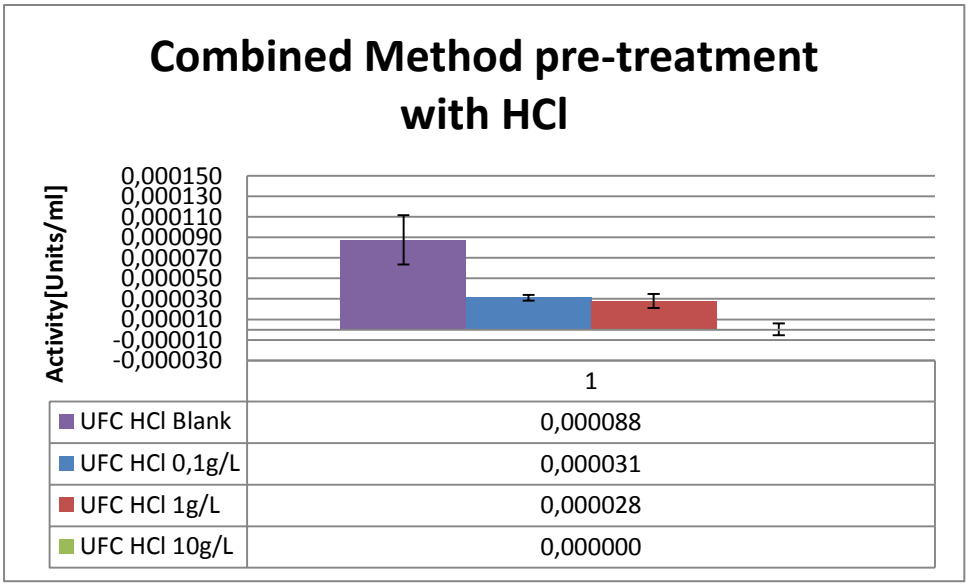


Figure 31 Effect of Cadmium Ions on immobilized HRP by combined method on UFC-Hollow Fiber membranes which are pre-treated with HCl

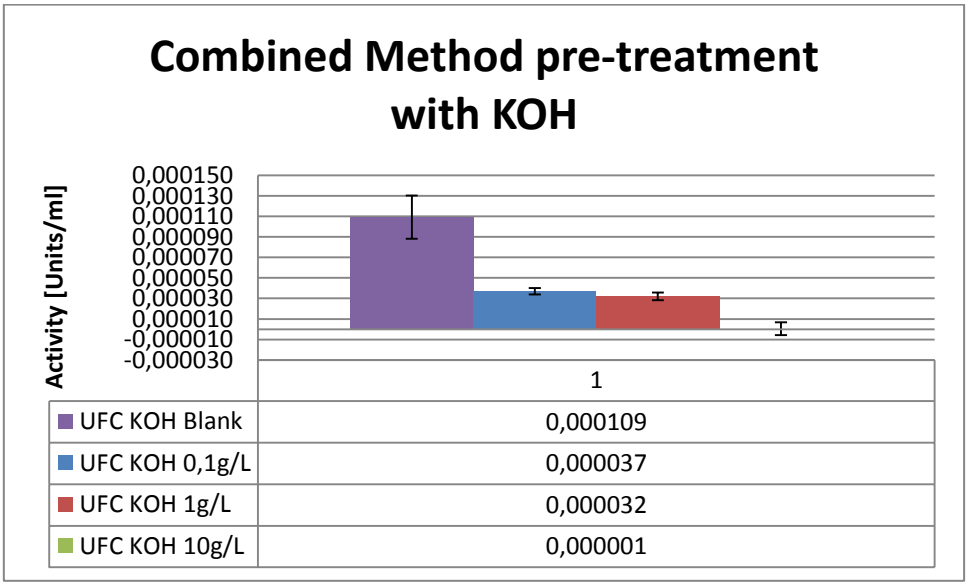


Figure 32 Effect of Cadmium Ions on immobilized HRP by combined method on UFC-Hollow Fiber membranes which are pre-treated with KOH

Conclusion

On all three immobilization methods as well was on free HRP, Cadmium has a high impact to the activity of the membrane already at concentrations not exceeding 0,1 g/L. All three methods show a decrease of the activity by rising Cadmium concentration. Free enzyme incubated in different Cadmium concentration shows quite a high effect to the activity of the enzyme. While incubation over 1 week in Cd^{2+} -free environment shows no loss in activity, all other solutions show a lost in activity. With rising Cadmium concentration

the activity decreases.Comparing the results of the absorption method it can be said that the pre-treatment has an effect on the activity of the hollow fiber membrane. Pre-treated membranes with KOH and HCl are 2 times as active as membranes which are only pre-treated with H_2O.

The Cadmium concentration has a very high effect on immobilized HRP on hollow fiber membranes. With rising Cd^{2+} concentration the activity of the membranes decreases tremendously. If the membranes are incubated for 1 week in 0,1 g/L Cd^{2+} concentration only 70% of the origin activity remain on the membrane. By incubation in 1 g/L Cd^{2+} on 20% of the activity remain. Incubation in 10g/l results to a nearly complete loss of the activity. Comparing the results of the chemical linkage that also there pre-treatment has a huge impact on the activity of the membrane. In this case pre-treated membranes with KOH have the highest activity. Also on the chemical linkage the activity of the membranes decreases with rising Cd^{2+} concentration. By incubation in 0,1 g/L Cd^{2+} the concentration decreases almost about 66%. With rising Cd^{2+} concentration the activity decreases even more. By incubation in 10 g/L Cd the activity of immobilized HRP nearly disappears. Also the combined method shows different activity by different activation. In contrast to both other methods at the combined method H_20 pre-treatment seems to be best for this method. Also on this method the Cadmium concentration has a high impact to the activity of the membrane.

The Cadmium concentration has most impact on the chemical linkage method. The reason for this might be that most enzyme is absorbed by this method.
At concentration of 10gCd^{2+}/L almost all the activity is gone from the membranes. While at concentration of 0,1Cd^{2+}/L and 1gCd^{2+}/L still a remarkable amount of activity is left.

Taking all results into consideration it can be said that the concentration of Cadmium plays a quite big role in the activity of the enzyme. Free HRP as well as immobilized show significant loss of activity with rising Cd^{2+} concentration.

Long Term Storage Experiments.

The storage of membranes with immobilized HRP is of high interest for practical application. To provide a loss of activity the optimal storage conditions have to be found. As prior experiments presented in the thesis at hand showed, activity of immobilized HRP is clearly dependent on the applied pH conditions. Therefore, in the following experiment, HFS-membranes with immobilized HRP were incubated in different solution considering pH for 1 or 2 weeks. The selection of HFS membranes for these experiments was done in accordance with the practical interest of the industrial partner Pentair.

Experimental

The hollow fibre membranes were activated by three different methods (incubation in 0,1 M HCl, 0,1 M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min.

- Chemical Linkage: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated into an EDC (0,1%) HRP solution in the ratio 1:1 for 3 hours.

After the immobilisation of the enzyme all membranes were rinsed with water and incubated in different solutions with different pH-values for a certain time and stored either at room temperature or at 4°C. Afterwards the membranes were rinsed again and an ABTS-Assay was made.

Results

Table 18 shows the storage activity of HFS-Membranes for certain time at pH 4,5. Table 19 shows the storage activity of HFS-Membranes for certain time at 4°C at pH 7. Table 20 shows the storage activity of HFS-Membranes for certain time at 4°C at pH 8. Table 21 shows the storage activity of HFS-Membranes for certain time at 4°C in water. Table 22 shows the storage activity of HFS-Membranes for certain time at room temperature at pH 4,5. Table 23 shows the storage activity of HFS-Membranes for certain time at room temperature at pH 7. Table 24 shows the storage activity of HFS-Membranes for certain time at room temperature at pH 8. Table 25 shows the storage activity of HFS-Membranes for certain time at room temperature in water

Table 18: Storage of HFS-Membranes for certain time at pH 4,5

pH 4,5	Storage Temperature[°C]	H_2O	HCl	KOH
		[Units/min]	[Units/min]	[Units/min]
t=0	4	$5,7*10^{-4}±0,6*10^{-4}$	$8,5*10^{-4}±0,2*10^{-4}$	$8,0*10^{-4}±0,2*10^{-4}$
t=1 week	4	$5,1*10^{-4}±0,2*10^{-4}$	$5,7*10^{-4}±0,2*10^{-4}$	$6,2*10^{-4}±0,1*10^{-4}$
t= 2 weeks	4	$3,7*10^{-4}±0,5*10^{-4}$	$1,7*10^{-4}±0,3*10^{-4}$	$1,1*10^{-4}±0,3*10^{-4}$

Table 19: Storage of HFS-Membranes for certain time at 4°C at pH 7

pH 7	Storage Temperature[°C]	H_2O	HCl	KOH
		[Units/min]	[Units/min]	[Units/min]
t=0	4	$5,7*10^{-4}±0,6*10^{-4}$	$8,5*10^{-4}±0,2*10^{-4}$	$8,0*10^{-4}±0,2*10^{-4}$
t=1 week	4	$3,7*10^{-4}±0,1*10^{-4}$	$2,4*10^{-4}±0,1*10^{-4}$	$4,8*10^{-4}±0,1*10^{-4}$
t= 2 weeks	4	$4,3*10^{-4}±1,1*10^{-4}$	$1,1*10^{-4}±0,3*10^{-4}$	$1,9*10^{-4}±0,3*10^{-4}$

Table 20: Storage of HFS-Membranes for certain time at 4°C at pH 8

pH 8	Storage Temperature[°C]	H_2O	HCl	KOH
		[Units/min]	[Units/min]	[Units/min]
t=0	4	$5,7*10^{-4}±0,6*10^{-4}$	$8,5*10^{-4}±0,2*10^{-4}$	$8,0*10^{-4}±0,2*10^{-4}$
t=1 week	4	$3,8*10^{-4}±0,7*10^{-4}$	$3,7*10^{-4}±0,1*10^{-4}$	$3,6*10^{-4}±0,4*10^{-4}$
t= 2 weeks	4	$2,3*10^{-4}±0,9*10^{-4}$	$2,3*10^{-4}±1,1*10^{-4}$	$1,1*10^{-4}±0,3*10^{-4}$

Table 21: Storage of HFS-Membranes for certain time at 4°C in water

H_2O	Storage Temperature[°C]	H_2O	HCl	KOH
		[Units/min]	[Units/min]	[Units/min]
t=0	4	$5,7*10^{-4}\pm0,6*10^{-4}$	$8,5*10^{-4}\pm0,2*10^{-4}$	$8,0*10^{-4}\pm0,2*10^{-4}$
t=1 week	4	$4,9*10^{-4}\pm1,2*10^{-4}$	$4,3*10^{-4}\pm1,0*10^{-4}$	$1,9*10^{-4}\pm0,3*10^{-4}$
t= 2 weeks	4	$4,7*10^{-4}\pm0,5*10^{-4}$	$3,0*10^{-4}\pm0,3*10^{-4}$	$1,1*10^{-4}\pm0,3*10^{-4}$

Table 22: Storage of HFS-Membranes for certain time at RT at pH 4,5

pH 4,5 RT	Storage Temperature[°C]	H_2O	HCl	KOH
		[Units/min]	[Units/min]	[Units/min]
t=0	RT	$5,7*10^{-4}\pm0,6*10^{-4}$	$8,5*10^{-4}\pm0,2*10^{-4}$	$8,0*10^{-4}\pm0,2*10^{-4}$
t=1 week	RT	$2,0*10^{-4}\pm0,5*10^{-4}$	$1,5*10^{-4}\pm0,5*10^{-4}$	$1,7*10^{-4}\pm0,6*10^{-4}$
t= 2 weeks	RT	$2,1*10^{-4}\pm1,0*10^{-4}$	$1,9*10^{-4}\pm1,6*10^{-4}$	$1,0*10^{-4}\pm0,2*10^{-4}$

Table 23 Storage of HFS-Membranes for certain time at RT at pH 7

pH 7	Storage Temperature[°C]	H_2O	HCl	KOH
		[Units/min]	[Units/min]	[Units/min]
t=0	RT	$5,7*10^{-4}\pm0,6*10^{-4}$	$8,5*10^{-4}\pm0,2*10^{-4}$	$8,0*10^{-4}\pm0,2*10^{-4}$
t=1 week	RT	$2,5*10^{*-4}\pm0,2*10^{-4}$	$2,3*10^{-4}\pm0,5*10^{-4}$	$2,2*10^{-4}\pm0,5*10^{-4}$
t= 2 weeks	RT	$0,9*10^{-4}\pm0,3*10^{-4}$	$0,7*10^{-4}\pm0,3*10^{-4}$	$0,2*10^{-4}\pm0,1*10^{-4}$

Table 24: Storage of HFS-Membranes for certain time at RT at pH 8

pH 8	Storage Temperature[°C]	H_2O	HCl	KOH
		[Units/min]	[Units/min]	[Units/min]
t=0	RT	$5,7*10^{-4}\pm0,6*10^{-4}$	$8,5*10^{-4}\pm0,2*10^{-4}$	$8,0*10^{-4}\pm0,2*10^{-4}$
t=1 week	RT	$1,8*10^{-4}\pm0,2*10^{-4}$	$1,7*10^{-4}\pm0,3*10^{-4}$	$1,6*10^{-4}\pm0,3*10^{-4}$
t= 2 weeks	RT	$1,0\text{-}10^{-4}\pm0,3*10^{-4}$	$1,1*10^{-4}\pm0,6*10^{-4}$	$1,0*10^{-4}\pm0,1*10^{-4}$

Table 25: Storage of HFS-Membranes for certain time at RT in water

H_2O	Storage Temperature[°C]	H_2O	HCl	KOH
		[Units/min]	[Units/min]	[Units/min]
t=0	RT	$5,7*10^{-4}\pm0,6*10^{-4}$	$8,5*10^{-4}\pm0,2*10^{-4}$	$8,0*10^{-4}\pm0,2*10^{-4}$
t=1 week	RT	$1,6*10^{-4}\pm0,3*10^{-4}$	$2,9*10^{-4}\pm0,2*10^{-4}$	$0,4*10^{-4}\pm0,1*10^{-4}$
t= 2 weeks	RT	$1,4*10^{-4}\pm0,5*10^{-4}$	$0,4*10^{-4}\pm0,1*10^{-4}$	$0,5*10^{-4}\pm0,2*10^{-4}$

Discussion

The results show that the incubated membranes lose their activity as longer as they are stored in solution at different pH. At 4 °C it seems that the membranes lose less activity in H_2O activated membranes than in all other solutions. At all other 3 solutions it seems that they lose quite a similar amount of activity. At room temperature the data show that the membranes are best stored at pH 4,5. The reason for this might be because it was investigated that the optimum pH for the activity of HRP is pH=4,5. After two weeks the membranes loose a significant amount of their activity. The reason for this might be activity of microorganisms.

The data show quite convincingly that storage at 4°C is much better than storage at room temperature. The membranes which were stored at room temperature lose after 2 weeks incubation ne nearly all of their activity while membranes stored at 4°C still have some activity left. Hence, storage at pH 8 at 4°C is suggested.

Regeneration after deactivation

The reuse of hollow fiber membranes after loosing their enzyme activity is of high interrest for practical application as well. To study this effect, hollow fiber membranes were reactivated after deactivation.

Experiment

The hollow fibre membranes were activated by three different methods (incubation in 0,1M HCl, 0,1M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min.

For the immobilization of HRP on pre-treated Hollow Fibre membranes 3 different methods have been used.

3 different kinds of hollow fibre membranes were studied: MFO2 (microfiltration membrane), HFS (Nano filtration membrane) which consists of PES and S-PES, and the UFC-membrane (ultrafiltration).

After rinsing with water the membranes were incubated in 10%HCl in order to deactivate the membranes. After the deactivation the membranes got rinsed with water and the enzyme got immobilized by the 3 methods again. After rinsing again with water an ABTS-Assay was made.

Results

Table 26 shows the activity of MFO2-membranes for conventional immobilization. Table 27 shows the activity of UFC-membranes for conventional immobilization. Table 28 shows the activity of HFS-membranes for conventional immobilization. Table 29 shows the activity of MFO2-membranes where HRP got immobilized after deactivation. Table 30 shows the activity of UFC-membranes where HRP got Immobilized after deactivation. Table 31 shows HFS-membranes where HRP got Immobilized after deactivation.

Table 26: MFO2-membranes conventional immobilization

Pre-treatment	H2O	HCl	KOH
Method	[Units/min]	[Units/min]	[Units/min]
Adsorption-Method	$8{,}6*10^{-5}$ $\pm 2{,}1*10^{-5}$	$7{,}8*10^{-5}$ $\pm 0{,}2*10^{-5}$	$7{,}5*10^{-5}$ $\pm 0{,}6*10^{-5}$
Combined Method	$2{,}3*10^{-5}$ $\pm 0{,}3*10^{-5}$	$5{,}3*10^{-5}$ $\pm 4{,}6*10^{-5}$	$2{,}8*10^{-5}$ $\pm 0{,}8*10^{-5}$
Chemical linkage	$8{,}3*10^{-5}$ $\pm 0{,}6*10^{-5}$	$7{,}0*10^{-5}$ $\pm 0{,}9*10^{-5}$	$6{,}5*10^{-5}$ $\pm 0{,}3*10^{-5}$

Table 27: UFC-membranes conventional immobilization

Pre-treatment	H2O	HCl	KOH
Method	[Units/min]	[Units/min]	[Units/min]
Adsorption-Method	$1,4*10^{-4}\pm0,1*10^{-4}$	$1,0*10^{-4}$	$1,9*10^{-4}\pm0,5*10^{-4}$
Combined Method	$5,3*10^{-5}\pm4,6*10^{-5}$	$1,3*10^{-5}\pm0,3*10^{-5}$	$2,9*10^{-5}\pm2,3*10^{-5}$
Chemical linkage	$1,0*10^{-4}$	$8,7*10^{-5}\pm0,2*10^{-5}$	$6,8*10^{-5}\pm0,2*10^{-5}$

Table 28: HFS-membranes conventional immobilization

Pre-treatment	H2O	HCl	KOH
Method	[Units/min]	[Units/min]	[Units/min]
Adsorption-Method	$1,3*10^{-4}\pm0,2*10^{-4}$	$1,9*10^{-4}\pm0,5*10^{-4}$	$2,3*10^{-4}\pm0,4*10^{-4}$
Combined Method	$3,0*10^{-4}\pm0,5*10^{-4}$	$2,3*10^{-4}\pm0,2*10^{-4}$	$2,4*10^{-4}\pm0,3*10^{-4}$
Chemical linkage	5,3*10-4±0,8*10-4	$3,0*10^{-4}\pm0,1*10^{-4}$	$3,3*10^{-4}\pm05*10^{-4}$

Table 29: MFO2-membranes immobilization after deactivation

Pre-treatment	H2O	HCl	KOH
Method	[Units/min]	[Units/min]	[Units/min]
Adsorption-Method	$4,3*10^{-5}\pm0,6*10^{-5}$	$4,1*10^{-5}\pm0,8*10^{-5}$	$4,7*10^{-5}\pm0,2*10^{-5}$
Combined Method	$4,5*10^{-5}\pm1,0*10^{-5}$	$3,1*10^{-5}\pm1,3*10^{-5}$	$3,3*10^{-5}\pm2,4*10^{-5}$
Chemical linkage	$2,4*10^{-5}\pm0,6*10^{-5}$	$1,9*10^{-5}\pm0,4*10^{-5}$	$2,3*10^{-5}\pm0,1*10^{-5}$

Table 30: UFC-membranes Immobilization after deactivation

Pre-treatment	H2O	HCl	KOH
Method	[Units/min]	[Units/min]	[Units/min]
Adsorption-Method	$5{,}1*10^{-5}\pm0{,}3*10^{-5}$	$4{,}8*10^{-5}\pm1{,}4*10^{-5}$	$2{,}9*10^{-5}\pm0{,}4*10^{-5}$
Combined Method	$3{,}0*10^{-5}\pm1{,}3*10^{-5}$	$8{,}3*10^{-5}\pm5{,}9*10^{-5}$	$5{,}3*10^{-5}\pm0{,}8*10^{-5}$
Chemical linkage	$3{,}6*10^{-5}\pm0{,}7*10^{-5}$	$3{,}8*10^{-5}\pm0{,}8*10^{-5}$	$0{,}7*10^{-5}\pm0{,}2*10^{-5}$

Table 31 HFS-membranes Immobilization after deactivation

Pre-treatment	H2O	HCl	KOH
Method	[Units/min]	[Units/min]	[Units/min]
Adsorption-Method	$5{,}2*10^{-5}\pm0{,}2*10^{-5}$	$2{,}7*10^{-5}\pm0{,}1*10^{-5}$	$2{,}0*10^{-5}\pm0{,}6*10^{-5}$
Combined Method	$2{,}0*10^{-4}\pm0{,}2*10^{-4}$	$2{,}2*10^{-4}\pm0{,}6*10^{-4}$	$5{,}3*10^{-4}$
Chemical linkage	$5{,}8*10^{-5}\pm0{,}1*10^{-5}$	$8{,}4*10^{-5}\pm0{,}3*10^{-5}$	$1{,}0*10^{-4}\pm0{,}3*10^{-5}$

Impact of cleaning agents to the activity of HRP immobilized on membranes

Hollow fiber membranes have to be cleaned from time to time. The reason for this is bio-fouling which causes several problems in filtration technology. By blocking the pores of the membranes bio-fouling can decrease the performance of hollow fiber membranes tremendously. In industrial processes (personal communication Dr. Jens Potreck, Pentair), the hollow fiber membranes get cleaned in a 2 step procedure using harsh conditions:

1) A combination of NaOCl and NaOH (200 mg/L of free chlorine and a pH of 12)

2) Followed by H_2SO_4/HCl (equimolar) at pH 2

It is of high interesting for process purposes, if the enzyme stays stable and immobilized on the membranes under these conditions.

Experiment

The hollow fibre membranes were activated by three different methods (incubation in 0,1 M HCl, 0,1 M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min.

Three different methods to bind the commercial enzyme to the polymer were used.

3 different kinds of hollow fibre membranes were studied: MFO2 (microfiltration membrane), HFS (nanofiltration membrane) which consists of PES and S-PES, and the UFC-membrane (ultrafiltration).

After the immobilization the membranes were rinsed with water and incubated in these solutions for 10 min. After rinsing the membranes again with water the activity of the membranes got measured with an ABTS-Assay

Results

All investigated set-ups showed a complete loss of enzymatic activity.

Conclusion

Cleaning with the above mentioned cleaning agents of the hollow fiber membranes immobilized with HRP results in a complete loss of the activity of the enzyme. All 3 methods showed no more activity after the cleaning process.

Testing of alternative polyelectrolytes (PolyDADMAC, PSS) instead of CHI for HRP immobilization

3 different kinds of hollow fiber membranes were studied: MFO2 (microfiltration membrane), HFS (Nano filtration membrane) which consists of PES and S-PES, and the UFC-membrane (ultrafiltration).

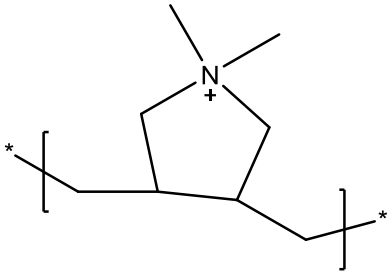


Figure 33 PolyDADMAC

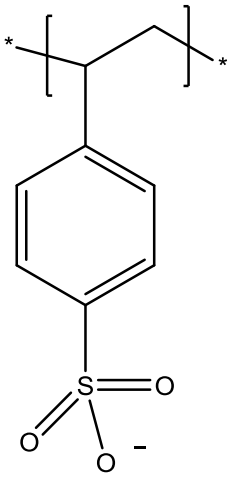


Figure 34 PSS

The hollow fibre membranes were activated by three different methods (incubation in 0,1M HCl, 0,1M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a PolyDADMAC[Figure 33] or either PSS [Figure 34] solution (0,05 wt%) for 20min.

- Chemical Linkage: The CMC and PolyDADMAC or PSS coated membranes as described before were again coated with CMC and afterwards incubated into an EDC (0,1%) HRP solution in the ratio 1:1 for 3 hours.

Results

Table 32 showing different layers with different activity

Table 32: Comparison on the effect of PSS or PolyDADMAC instead of CHI in the bilayer

	CHI-Layer [Units/min]	PSS-Layer [Units/min]	PolyDADMAC-Layer [Units/min]
H_2O	$1{,}0*10^{-3}\pm0{,}04*10^{-3}$	$4{,}7*10^{-4}\pm0{,}3*10^{-4}$	$5{,}7*10^{-4}\pm1{,}3*10^{-4}$
HCl	$9{,}7*10^{-4}\pm0{,}5*10^{-4}$	$4{,}1*10^{-4}\pm0{,}2*10^{-4}$	$6{,}7*10^{-4}\pm0{,}7*10^{-4}$
KOH	$6{,}5*10^{-4}\pm0{,}5*10^{-4}$	$8{,}9*10^{-5}\pm5{,}9*10^{-5}$	$7{,}0*10^{-4}\pm1{,}0*10^{-4}$

Conclusion

The replacement of PSS or PolyDADMAC instead of CHI has huge effect to the activity of immobilized HRP on HFS-Membrane surfaces. The data show that the CHI layer seems to be the best layer for the immobilization except with pretreatment with KOH and PolyDADMAC. In this case KOH increases the affinity of HRP to the surface and shows higher activity. The data show also that PolyDADMAC layer show better performances than PSS-layer.

Additional pre-treatment methods (water buffer HCl, KOH) with EtOH for HFS-Membranes

In order to remove lipophilic residues from the production HFS-membranes got cleaned with 18%-Ethanol for 5 hours. 3 different kinds of hollow fiber membranes were studied: MFO2 (microfiltration membrane), HFS (Nano filtration membrane) which consists of PES and S-PES, and the UFC-membrane (ultrafiltration).

Experiment

Polysaccharides like Carboxymethylcellulose (CMC) and chitosan (CHI) are able to bind to hollow fibre membranes. This binding can make them accessible to enzyme immobilization[34]

The hollow fibre membranes were activated by three different methods (incubation in 0,1M HCl, 0,1M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min.

Three different methods to bind the commercial enzyme to the polymer were used.

- Adsorption Method: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated in a HRP solution for 30 min.
- Chemical Linkage: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated into an EDC (0,1%) HRP solution in the ratio 1:1 for 3 hours.
- Combined Method: The CMC and CHI coated membranes as described before were incubated into a HRP/CMC 0,05%/EDC 0,1% solution in the ratio 1:1:1 for 3 hours

3 different kinds of hollow fibre membranes were studied: MFO2 (microfiltration membrane), HFS (Nano filtration membrane) which consists of PES and S-PES, and the UFC-membrane (ultrafiltration).

After the immobilization the membranes were rinsed with water and incubated in these solutions for 10 min. After rinsing the membranes again with water the activity of the membranes got measured with an ABTS-Assay

Results

Table 33 shows the effect of pretreated HFS membranes with different activation. Figure 35 shows the comparison of Pre-treatment with ethanol with conventional method on HFS-membranes with chemical linkage. Figure 36 shows the comparison of Pre-treatment with ethanol with conventional method on HFS-membranes with adsorption method. Figure 37 shows the comparison of Pre-treatment with ethanol with conventional method on HFS-membranes with combined method

Table 33 Effect of pre-treatment of Ethanol on HFS membranes followed by enzyme immobilization

Pre-treatment	H2O	HCl	KOH
Method	[Units/min]	[Units/min]	[Units/min]
Adsorption-Method w/o EtOH	$6,6*10^{-4}\pm0,8*10^{-4}$	$5,6*10^{-4}\pm0,8*10^{-4}$	$5,4*10^{-4}\pm0,5*10^{-4}$
Adsorption-Method	$5,6*10^{-4}\pm0,8*10^{-4}$	$5,2*10^{-4}\pm0,9*10^{-3}$	$4,6*10^{-4}\pm0,4*10^{-4}$
Combined Method w/o EtOH	$3,8*10^{-4}\pm0,4*10^{-4}$	$2,4*10^{-4}\pm0,4*10^{-4}$	$8,0*10^{-4}\pm1,8*10^{-4}$
Combined Method	$2,6*10^{-4}\pm0,2*10^{-4}$	$3,0*10^{-4}\pm0,3*10^{-4}$	$2,9*10^{-4}\pm0,4*10^{-4}$
Chemical linkage w/o EtOH	$6,4*10^{-4}\pm2,1*10^{-4}$	$7,3*10^{-4}\pm2,0*10^{-4}$	$5,8*10^{-4}\pm1,3*10^{-4}$
Chemical linkage	$4,8*10^{-4}\pm0,5*10^{-4}$	$4,5*10^{-4}\pm0,1*10^{-4}$	$3,1*10^{-4}\pm0,7*10^{-4}$

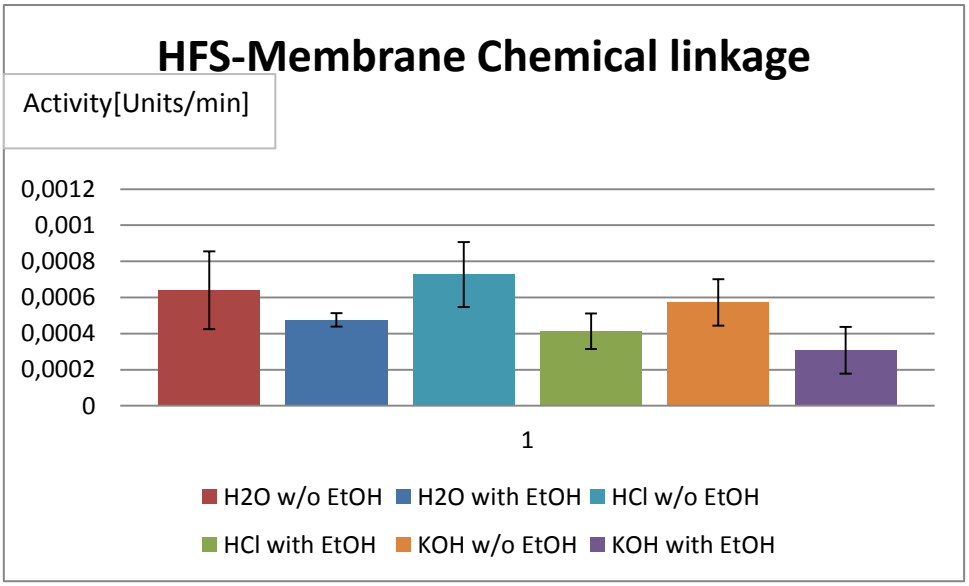


Figure 35 Comparing Pre-treatment with ethanol with conventional method on HFS-membranes with chemical linkage

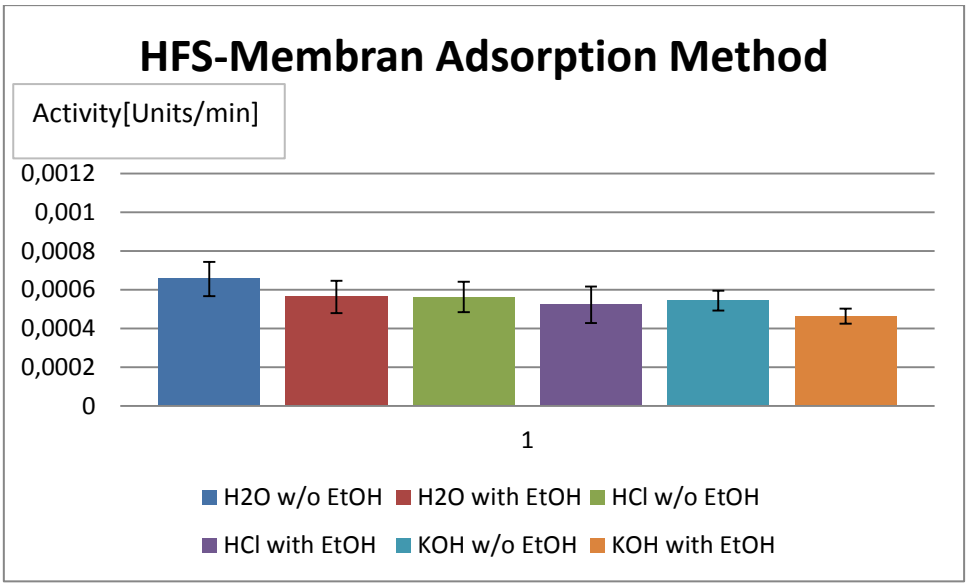


Figure 36 Comparing Pre-treatment with ethanol with conventional method on HFS-membranes with adsorption method

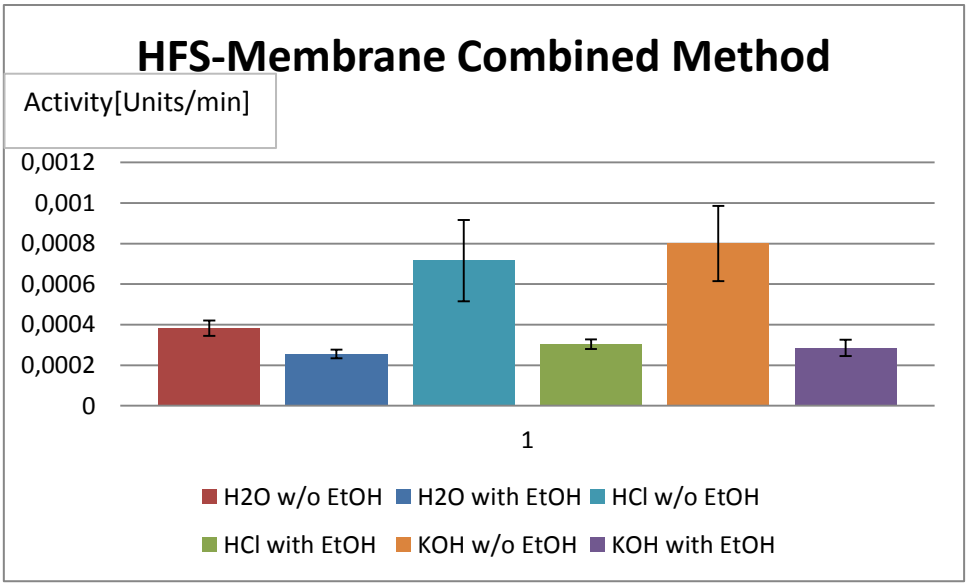


Figure 37 Comparing Pre-treatment with ethanol with conventional method on HFS-membranes with combined method

Conclusion

With 5 hours pre-treatment with Ethanol to remove residues from the production the membrane activity decreases. The absorption Method shows the lowest decrease of activity. The pre-treatment has a very huge effect on the chemical and combined Method. The activity without pre-treatment is much higher than the activity with pre-treatment.

Additional incubation in ethanol in order to remove glycerol residues from the production of UFC-Membranes

Experiment

In order to remove glycerol from the production UFC-membranes got cleaned with ethanol for 5 hours. The hollow fibre membranes were activated by three different methods (incubation in 0,1 M HCl, 0,1 M KOH or H_2O for 20 min). After activation, the membranes were rinsed with water and incubated in an 0,05 % CMC solution at pH 2 for 30 min. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min.

- Adsorption method: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated in a HRP solution for 30 min.

- Chemical Linkage: The CMC and CHI coated membranes as described before were again coated with CMC and afterwards incubated into an EDC (0,1%) HRP solution in the ratio 1:1 for 3 hours.
- Combined method: The CMC and CHI coated membranes as described before were incubated into a HRP/CMC 0,05%/EDC 0,1% solution in the ratio 1:1:1 for 3 hours

Results

Table 34 shows the effect of pre-treatment of water on HFS membranes followed by enzyme immobilization. Figure 38 shows the comparison of Pre-treatment with water with conventional method on UFC-membranes with combined method. Figure 39 shows the comparison of Pre-treatment with water with conventional method on UFC-membranes with adsorption method. Figure 40 shows the comparison Pre-treatment with water with conventional method on UFC-membranes with chemical linkage

Table 34: Effect of pre-treatment of water on HFS membranes followed by enzyme immobilization

Pre-treatment	H_2O	HCl	KOH
Method	Activity[Units/min]	Activity[Units/min]	Activity[Units/min]
Adsorption-Method w/o H_2O	$3,4*10^{-4}\pm1,5*10^{-4}$	$2,8*10^{-4}\pm0,8*10^{-4}$	$2,3*10^{-4}\pm0,9*10^{-4}$
Adsorption-Method	$3,1*10^{-4}\pm0,6*10^{-4}$	$3,0*10^{-4}\pm1,2*10^{-4}$	$3,3*10^{-4}\pm1,2*10^{-4}$
Combined Method w/o H_2O	$2,0*10^{-4}\pm0,6*10^{-4}$	$1,8*10^{-4}\pm0,6*10^{-4}$	$1,7*10^{-4}\pm0,4*10^{-4}$
Combined Method	$2,1*10^{-4}\pm0,3*10^{-4}$	$3,0*10^{-4}\pm0,2*10^{-4}$	$2,9*10^{-4}\pm0,6*10^{-4}$
Chemical linkage w/o H_2O	$3,8*10^{-4}\pm0,4*10^{-4}$	$2,5*10^{-4}\pm0,5*10^{-4}$	$4,3*10^{-4}\pm1,1*10^{-4}$
Chemical linkage	$4,5*10^{-4}\pm0,4*10^{-4}$	$4,5*10^{-4}\pm0,1*10^{-4}$	$5,9*10^{-4}\pm1,2*10^{-4}$

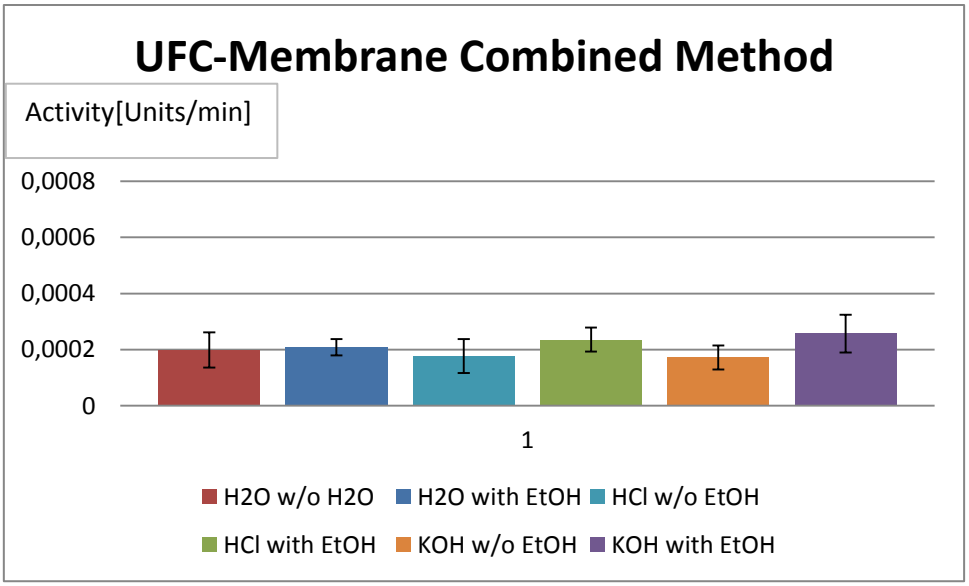


Figure 38 Comparing Pre-treatment with water with conventional method on UFC-membranes with combined method

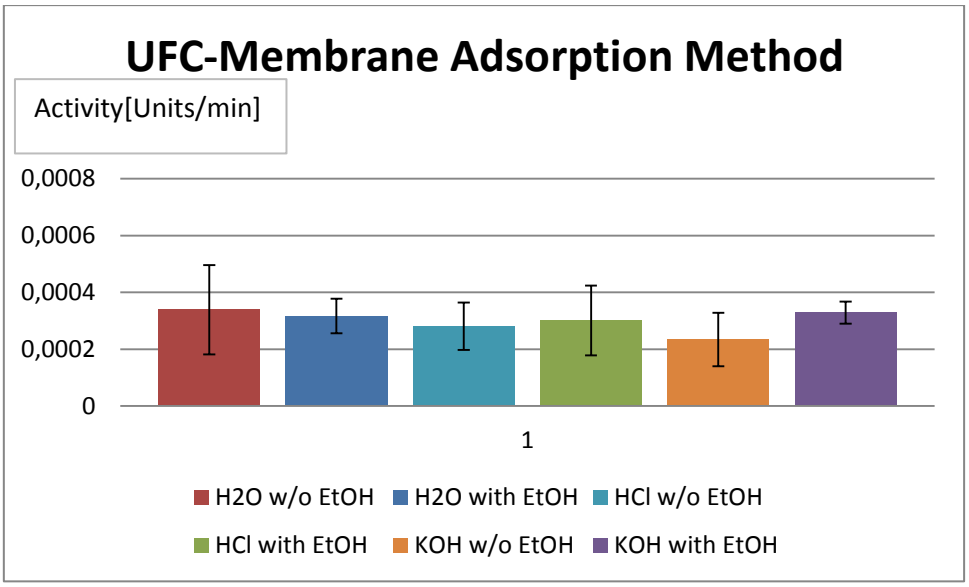


Figure 39 Comparing Pre-treatment with water with conventional method on UFC-membranes with adsorption method

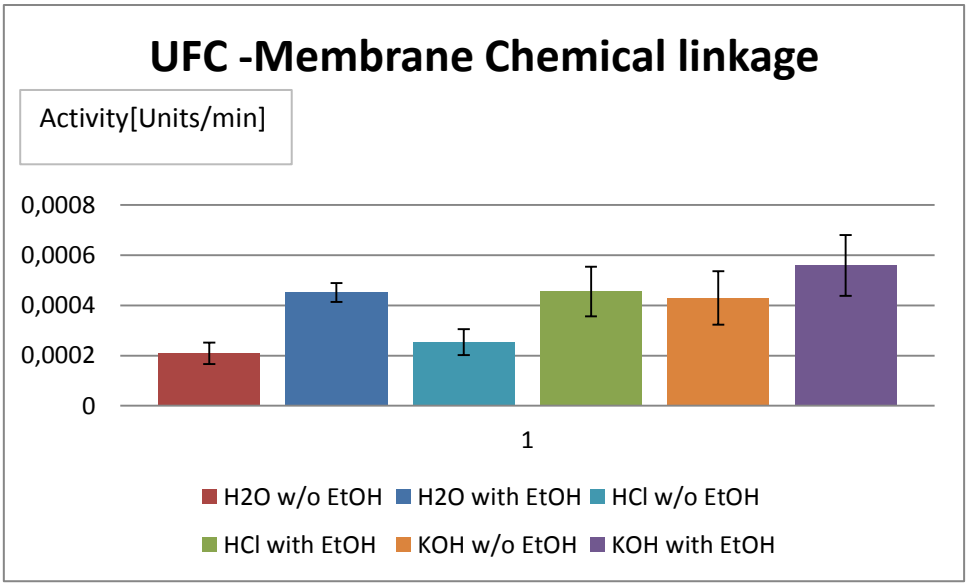


Figure 40 Comparing Pre-treatment with water with conventional method on UFC-membranes with chemical linkage

Conclusion

From the obtained results it can be deducted that no real increase in activity can be observed with addition pre-treatment with ethanol. Comparing different immobilization methods it can be said that the chemical linkage works best.

Dynamic immobilization on MFO2 and HFS membranes

The membranes were **without activation** incubated in an 0,05 % CMC solution at pH 2 for 30 min for creating a negative surface charge. After this they were rinsed with water, and the membranes were incubated in a CHI solution (0,05 wt% CHI, 150 mM KCl at pH 5,5) for 20min. A dynamic immobilization by Watson Marlow pumps has been made. Only the combined Method has been tested.

- Combined method: The CMC and CHI coated membranes were given in a single hollow fibre modul as and a solution containing HRP/CMC 0,05%/EDC 0,1% solution in the ratio 1:1:1 was pumped through for 3 hours

Figure 41 Pump for dynamical immobilization

After the immobilization the membranes were cut into 1cm pieces and the Activity was measured with an ABTS-Assay.

Results

Figure 42 shows the comparison of MFO2 and HFS membranes immobilized dynamical or conventional immobilization.

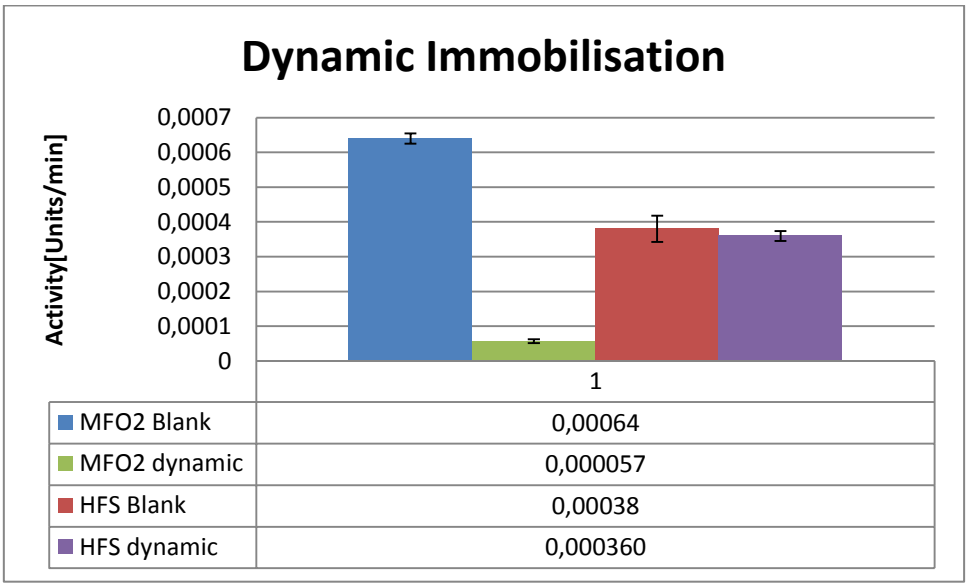


Figure 42 Comparing conventional immobilization(Blank) to dynamical immobilization

Conclusion

The results show that the dynamical immobilization have huge effect on MFO2 membranes. It seems that MFO2 dynamically immobilized membranes loose a big amount of activity. The dynamical immobilization on HFS membranes has no observable effect on the activity of the membranes

Determination of the Biocatalytic Degradation of 17α-Ethinylestradiol by HRP using HPLC-MS

Prior experiments to determine EE2 levels in aqueous systems resort to laborious and time demanding methods of UV-spectroscopy which do not assure a desired degree of reproducibility[1]. Therefore, fast and reliable alternatives had to be developed. The combination of high performance liquid chromatography (HPLC) coupled with a mass spectroscopy (MS) is a technique to separate and detect different substances both qualitatively and quantitatively, and was for the first time used to monitor EE2 degradation.

The HPLC system separates substances based on their chemical properties. The separated fractions of the HPLC-system are transferred to the MS-system. The first step is the ionisation of the analytes. In this case the ionisation was made with electron impact ionization.

$$M + e^- \longrightarrow M^{+\cdot} + 2\,e^-$$

Using an electric field, the ions get to the analyser where they are separated to their mass/charge proportion. From the analyser the ions get to the detector which identifies the mass of the ions.

Experimental

The selected concentration of EE2 in water amounted 11,3 mg/L. Because of the low solubility it was not possible to dissolve the substrate in water and, consequently, it was dissolved in absolute ethanol to create a stock solution (0,5 g/L). 1 mL of the ethanol solution was mixed with 99 mL $CaCl_2$solution (10 mM).

1,4 mL enzyme solution was added to 14 mL EE2 solution and incubated for 2 hours under continuous stirring using a magnetic stirrer. Then the solutions were injected into a HPLC-MS system for measurement.

The subsequent equipment was used for the measurements:

HPLC Agilent Technologies 1260 Infinity coupled to an MS system; the flow rate was set to 1 mL/ min. A gradient – elution was chosen. As eluents performed water, HPLC grade,

obtained from CHEM-LAB and acetonitrile Rotisolv® HPLC from ROTH. The MS device was an Agilent Technologies 6120 Quadropole. Measurments were carried out both in positive and negative mode.

Results of the HPLC-MS system

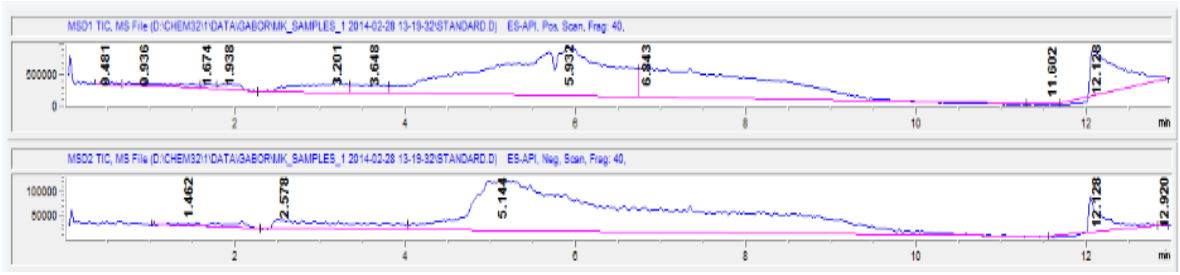


Figure 43 Pos. mode(upper) neg. mode(bellow) of the scan (substrate without enzyme)

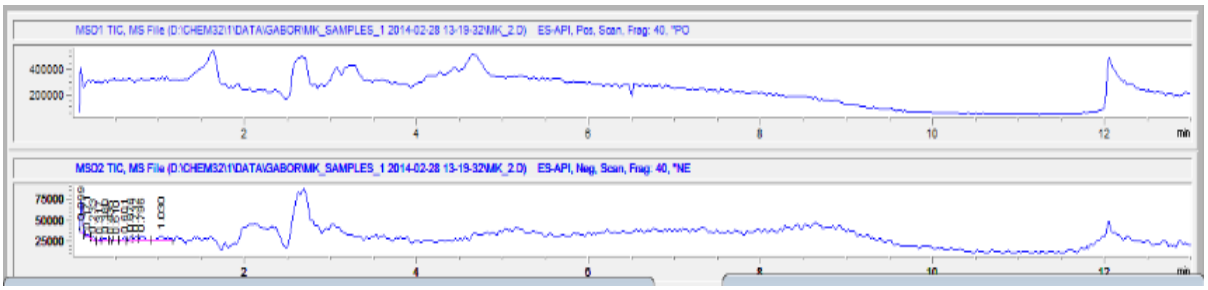


Figure 44 Substrate with enzyme. Positive mode (upper) negative mode(bellow)

CONCLUSION

For the first time, EE2 degradation was shown by means of HPLC-MS. In comparison to the samples standards chromatogram, the chromatograms obtained from measuring the samples after the catalytic action of HRP look quite different. At 2 and 2, 5 min two more peaks appear in the negative mode. In the positive mode, there are peaks at about 1,5 min and 2, 5 min. These new peaks are most likely the degradation products of the substrate with the enzyme. The concentration of the substrate compared to the standard has also decreased which is another evidence for the degradation The detailed identification of the degradation products, together with the expression of the

ecological performance is one task of further activities. Most importantly, the outcomes provide a fast and convenient tool for determination and quantification of EE2 in aqueous systems such as waste water bodies.

Degradation of EE2 in presence of Humic acid

Waste water is containing different organic ingredients which, in addition to the inorganic inhibitors F^- and Cd^{2+}, might severely impact the enzymatic activity of HRP. In order to simulate these conditions, the degradation of EE2 in presence of humic acids [HS] has been studied.

Experiment:

The selected concentration of EE2 in water amounted to 11,3 mg/L. Because of the low solubility of EE2 in water it was not possible to directly dissolve this quantity. First EE2 was dissolved in absolute ethanol to create a 0,5 g/L stock solution.

1 mL of the ethanolic stock solution was mixed with 99 mL 10mM $CaCl_2$ solution with 5mg/L or 50mg/L humic acid filtrated with 0,2 µm filter (10 mM). The enzyme was dissolved in a solution at the optimal pH for their activity. As determined by our experiments (VERWEIS AUF DEINE BAC-ARBEIT!!), the optimal pH for the isoenzyme C1A6 was 4.5, whereas the optimal pH for commercial enzyme is pH 7,0. Both enzyme variants were used for these investigations.

1,5 mL enzyme solution was added to 14 mL EE2 solution under continuous shaking; samples were taken after certain time intervals and H2O2 was added every 12 hours. Then the solutions were sterile filtrated and injected into a HPLC-MS system for measurement. Two different calibration lines have been made to be used external standard for EE2 quantification.

Results

Fig. 45 and 46 show the linear range of the EE2 concentration. Figure 47 shows the degradation of EE2 without HS with enzyme C1A6. Figure 48 shows the degradation of EE2 in presence of HS in the relation 1:1 with enzyme C1A6. Figure 49 shows the degradation of EE2 in presence of HS in the relation 1:10 with enzyme C1A6. Figure 50 shows the

degradation of EE2 without HS with commercial enzyme. Figure 51shows the degradation of EE2 in presence of HS in the relation 1:1 with commerzial enzyme. Figure 52 shows the degradation of EE2 in presence of HS in the relation 1:10 with commerzial enzyme

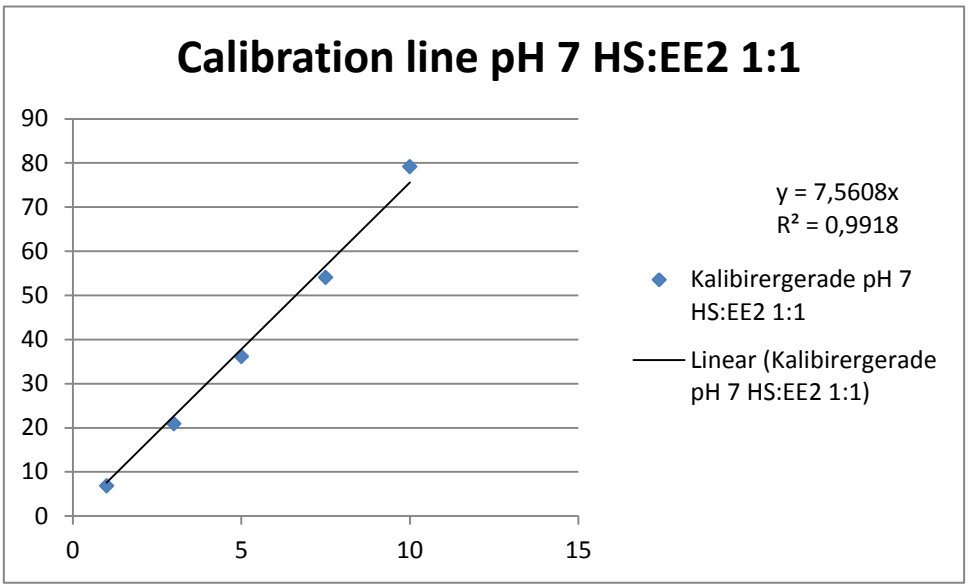


Figure 45 Calibration line for EE2:HS 1:1 at pH 7

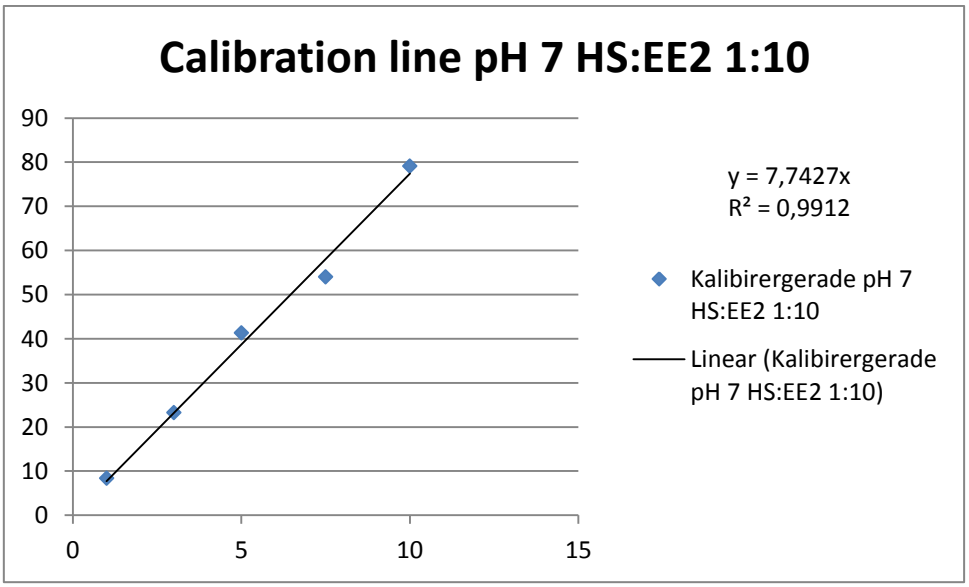


Figure 46 Calibration line for Humic acid : EE2 in the relation 1:10

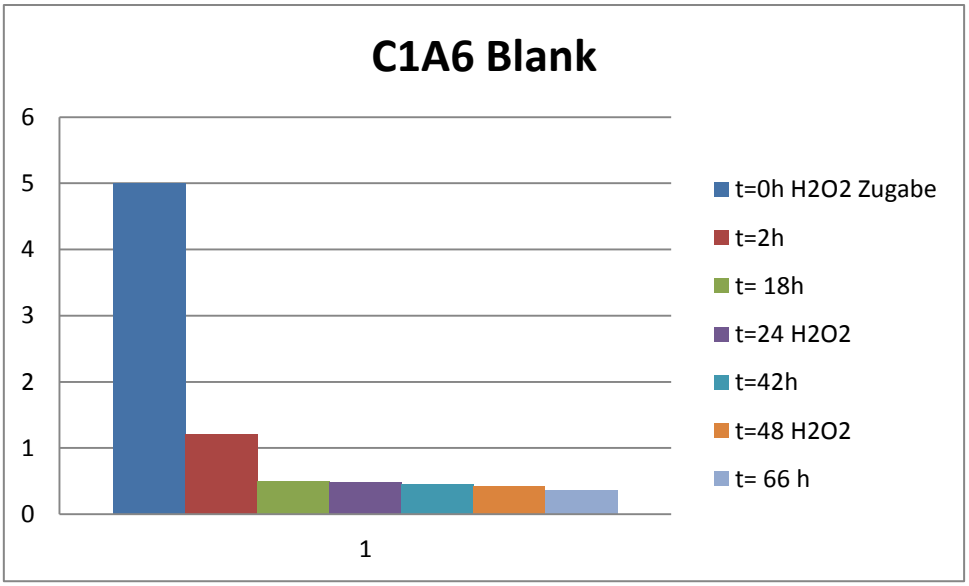


Figure 47 Shows the degradation of EE2 without HS with enzyme C1A6

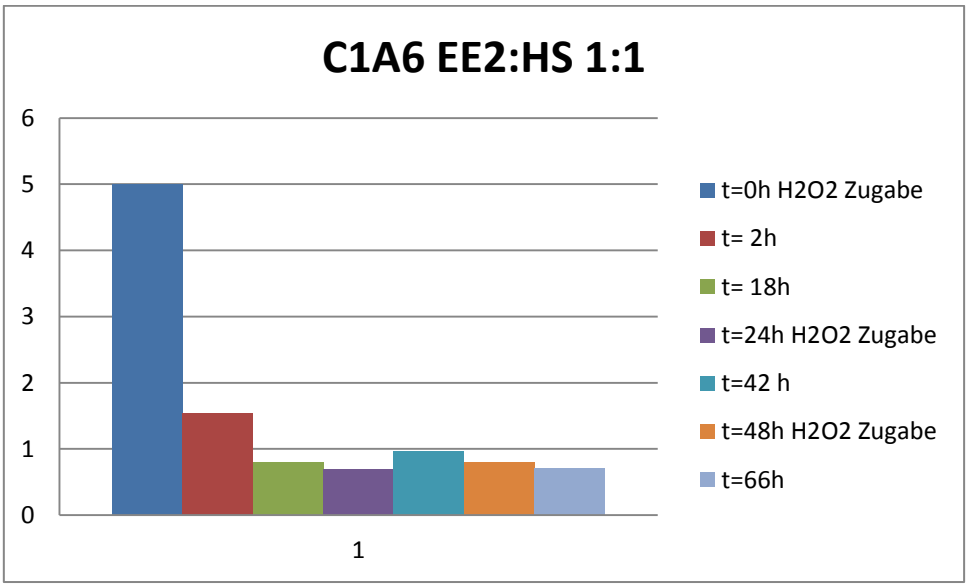


Figure 48 Shows the degradation of EE2 in presence of HS in the relation 1:1 with enzyme C1A6

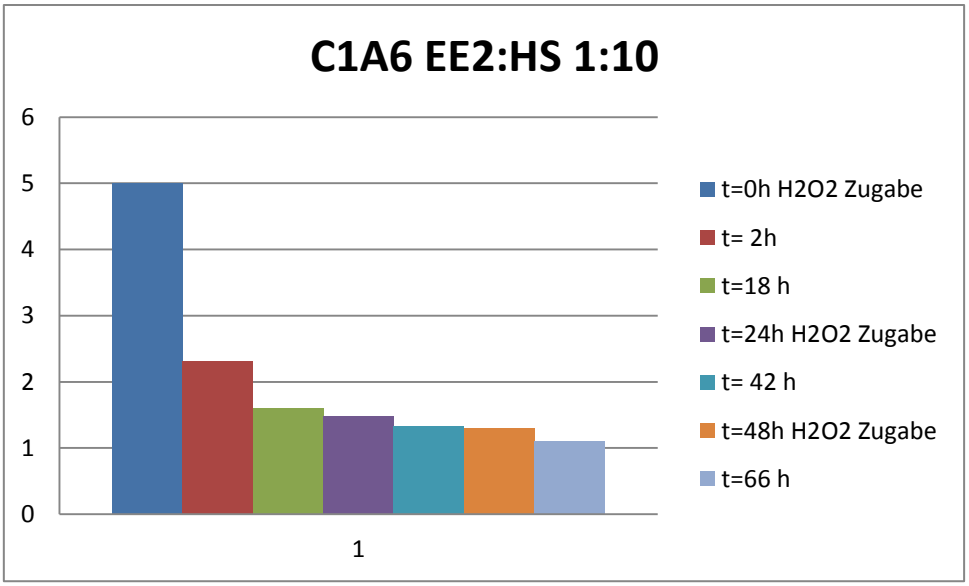


Figure 49 Shows the degradation of EE2 in presence of HS in the relation 1:10 with enzyme C1A6

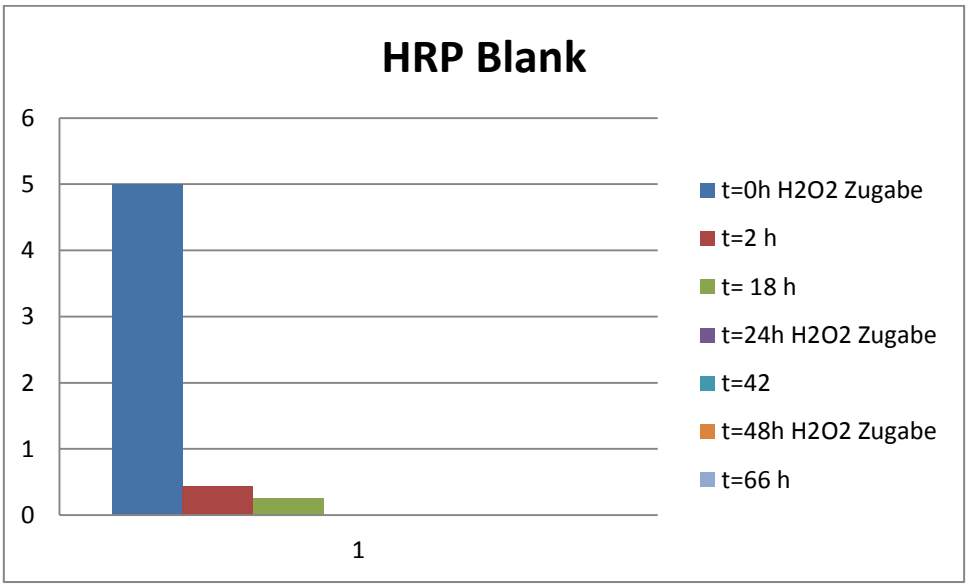


Figure 50 Shows the degradation of EE2 without HS with commercial enzyme

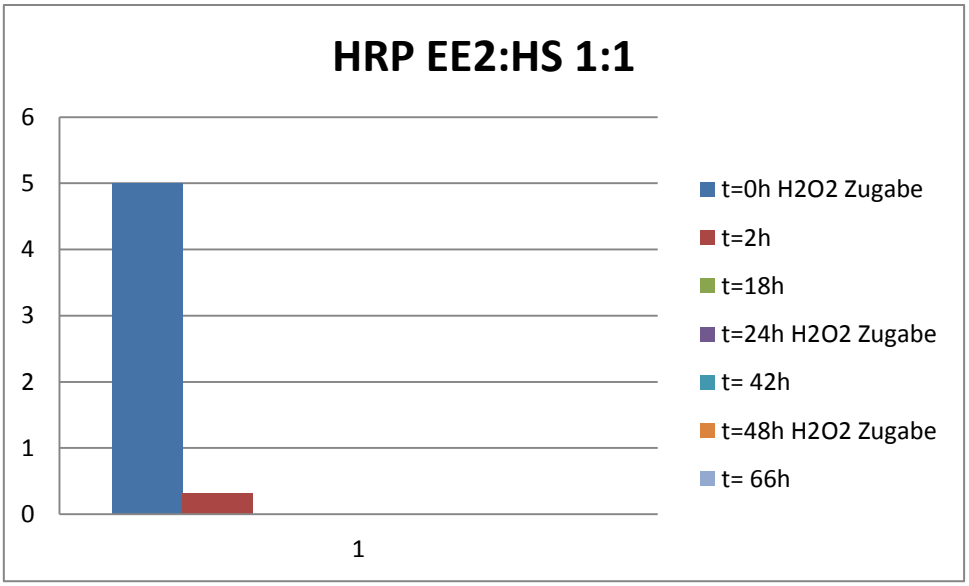


Figure 51 Shows the degradation of EE2 in presence of HS in the relation 1:1 with commerzial enzyme

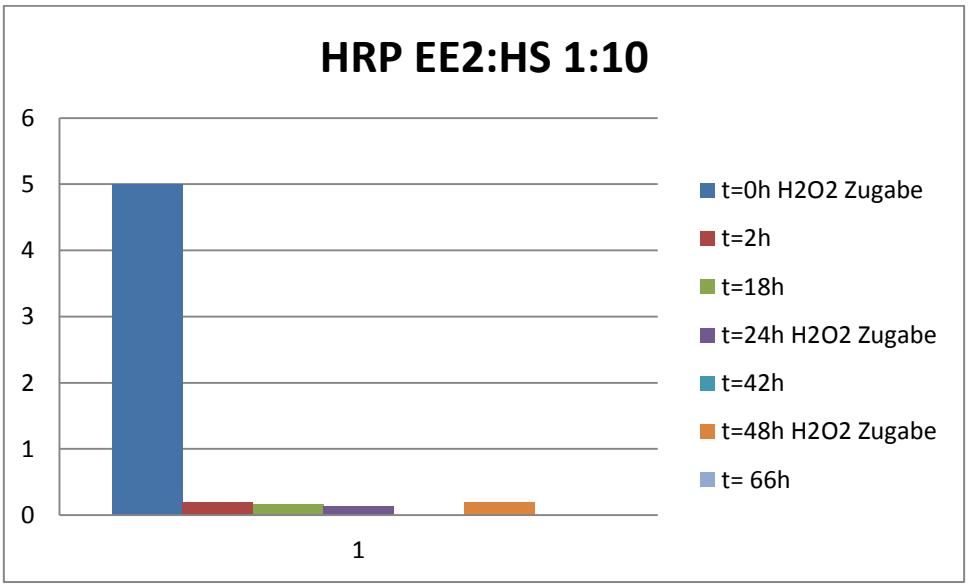


Figure 52 Shows the degradation of EE2 in presence of HS in the relation 1:10 with commerzial enzyme

CONCLUSION

As can be concluded from the results, the degradation of 17α-ethinylestradiol is strongly dependent to the amount humic acid in solution; tis pollutant definitely displays inhibitory effects at all investigated concentration level. It is well visible that this inhibitory effect strongly differns between the two investigated isoenzyme variants. After 66 hours interaction time between the isoenzyme C1A6 and 17α-ethinylestradiol the blank (no humic acids added) shows a remaining amount of only 0,36mg/L EE2, hence, almost 80% of EE2 were already degraded (Figure 47). In presence of humic acid at the same concentration the amount of 17α-ethinylestradiol is nearly twice as much than without the addition of humic acid, hence, degradation rate is almost halfed (Figure 48). If the amount of humic acid is 10 times higher than the amount of 17α-ethinylestradiol the concentration of 17α-ethinylestradiol is 3 times higher than in the blank after 66 hours (Figure 49)

Although the commercial enzyme is much more active than C1A6, it´s enzymatic activity shows a dependency to the concentration of humic acid as well. In the blank the degradation is finished after 18 hours (Figure 50). In the presence of a 10-fold amount of humic acid in comparision to 17α-Ethinestradiol, the degradation was not completed after 66 hours of incubation (Figure 51, Figure 52).

All in all it can be said the sample matrix plays a major role in the degradation performance of the enzyme.

Comparison of Batch and Dynamical Immobilization of Horseradish Peroxidase on Hollow Fiber Membranes

Dynamical Immobilization

CMC/CHI double layer

Single membrane modules were prepared with the equipment provided by Pentair-X-Flow. For removing any residues from the production either an 18% ethanol aqueous solution (For HFS-Membranes) or pure water was pumped through the modules. After this the modules were cleaned with water for 20 min. A solution of 0,05 wt.-% CMC solution at pH 2 was pumped through the Modules for 30 min. After this process, water was pumped through the modules for another 20 min. Subsequently a 150 mM KCl 0,05 wt% CHI solution was pumped through the membranes and cleaning for 20 min with pure water was carried out

PSS/PolyDADMAC double layer

Single membrane modules were prepared with the equipment provided by Pentair-X-Flow. For removing any residues from the production either an 18% Ethanol aqueous solution (For HFS-Membranes and UFC-Membranes). After this the modules were cleaned with water for 20min. A solution of 0,05 wt.-% PSS solution at pH 2 was pumped through the Modules for 30 min. After this process, water was pumped through the modules for another 20 min. Subsequently a 0,05 wt% PolyDADMAC solution was pumped through the membranes and cleaning for 20 min with pure water was carried out.

Batch Immobilization

In comparison to the dynamical immobilization also batch immobilization was accomplished. For this the hollow Fiber-Membranes were cut into 1 cm pieces and immersed in a 0,05 wt% CMC. After rinsing with water for 20 min the membranes were incubated in 150 mM KCl 0,05 wt% CHI solution for another 30min.

Immobilization of Horseradish peroxidase

Because of practical issues (suggestion by the industrial partner Pentair), only the Combined Method of enzyme immobilization has been tested. Details for the Combined HRP immobilization method as used for this experiment: A mixture of EDC, HRP and CMC or PSS solution for 3 hours has been pumped through the membrane for 3h as previously reported.

Results

Picture 53 shows the comparison of Dynamical and Batch immobilisation with CMC/CHI doublelayer. Picture 54 shows the comparison of Dynamical and Batch immobilization with PSS/PolyDADMAC doublelayer.

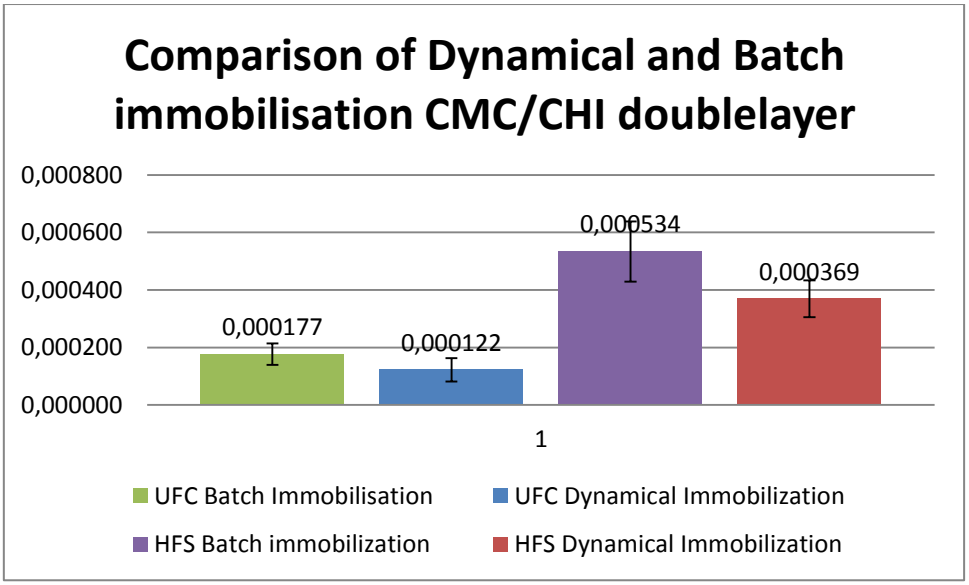


Figure 53 Comparison of Dynamical and Batch immobilization with CMC/CHI double layer

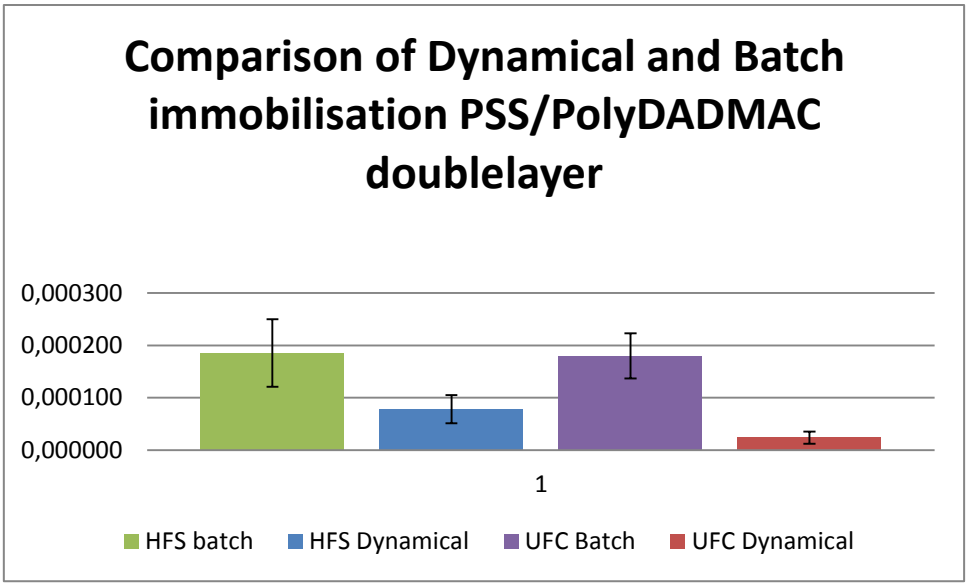


Figure 54 Comparison of Dynamical and Batch immobilization with PSS/PolyDADMAC double layer

Conclusion

The results show that the hollow fiber membranes carry the enzyme HRP both after static or dynamic immobilization respectively. The results show quite big differences between the dynamical and batch immobilization. The dynamical immobilization shows less activity than batch immobilization. HFS-membrane is much more active than the UFC-membrane. Also the replacement of the original layer(CMC/CHI) with PSS and PolyDADMAC has a big impact on the activity of the membranes. HFS-membranes show the highest activity in both cases.

Degradation of EE2 in Fiber modules for 24 hours

Experimental

A membrane (UFC or HFS) in the size of a module was given into a module and both endings were clued with a clue (provided by Pentair X-Flow) in order to prepare single fiber membrane modules. To remove any residues from the production the membranes were treated with 18% EtOH in aqueous solution (for HFS) or in pure water (for UFC) for 3 hours.

Subsequent the membranes got cleaned with water for 20 minutes. Afterwards a 0,05wt% CMC solution got pumped through the modules and cleaning with water for 20 minutes followed. A 0,05 wt% CHI, 150 mM KCl solution was pumped through the membrane and washed again with water for 20 minutes.

Immobilization of Horseradish Peroxidase

Because of practical issues only the Combined Method has been tested. Details for the Combined HRP immobilization method as used for this experiment: A mixture of EDC, HRP and CMC or PSS solution for 3 hours has been pumped through the membrane for 3h as previously reported.

Degradation of EE2

The concentration of EE2 in water amounted to 11,3 mg/L. Because of the low solubility of EE2 in water it was not possible to dissolve. First EE2 was dissolved in absolute ethanol to create (0,5 g/L) solution.

1 mL of the ethanol solution was mixed with 99 mL 10mM $CaCl_2$ solution with. The enzyme was dissolved in a solution at the optimal pH for their activity. The optimal pH for commercial enzyme is pH 7,0.

Afterwards the EE2 solution got pumped in a cycle through the modules and samples were taken in certain time intervals. Samples were taken from the bulk (explain what the bulk is!) and immediately after the release of the membrane module.

Results

Figure 55 shows the EE2 concentration of UFC-Membrane after release of the membrane module in specific time intervals. Figure 56 shows the EE2 concentration of UFC-Membrane Bulk in specific time intervals. Figure 57 shows the EE2 concentration of HFS-Membrane after release of the membrane module in specific time intervals. Figure 58 shows the EE2 concentration of HFS-Membrane bulk in specific time intervals

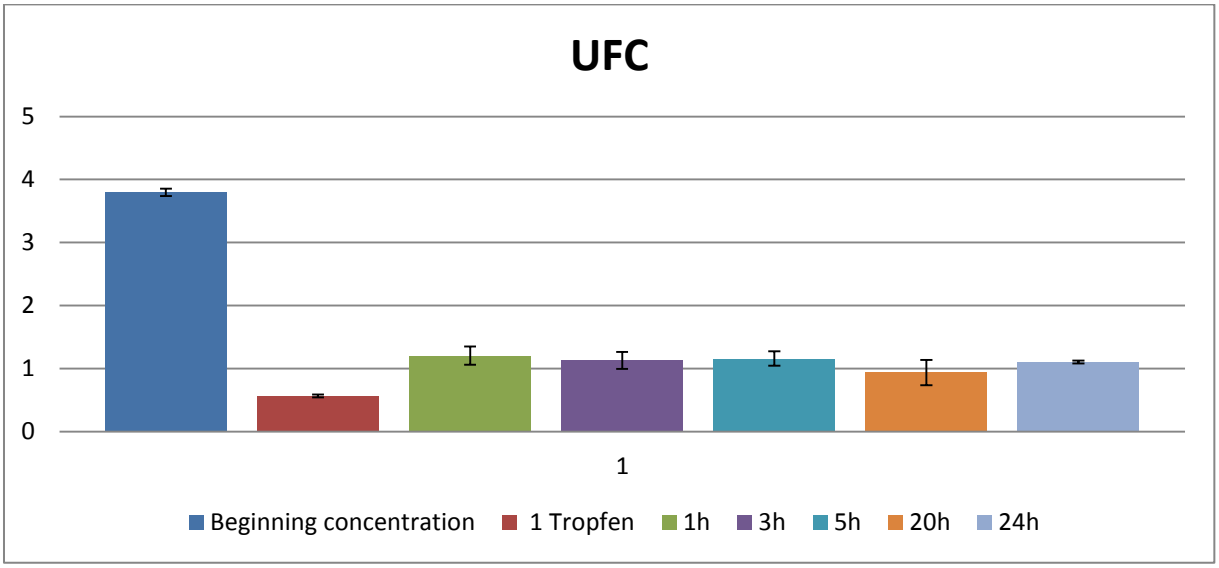


Figure 55 EE2 concentration of UFC-Membrane after release of the membrane module in specific time intervals

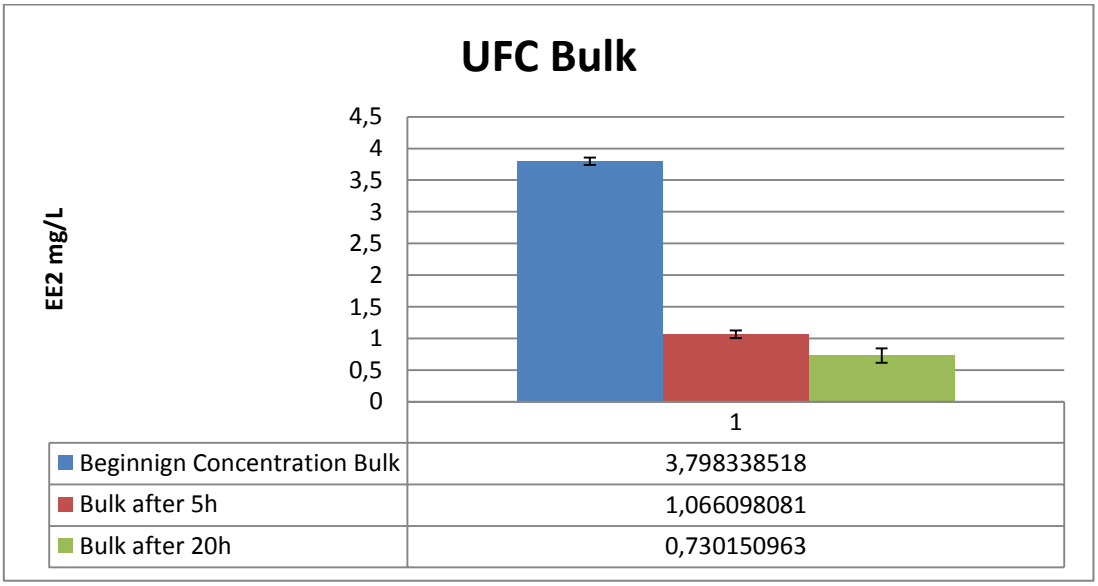


Figure 56 EE2 concentration of UFC-Membrane Bulk in specific time intervals

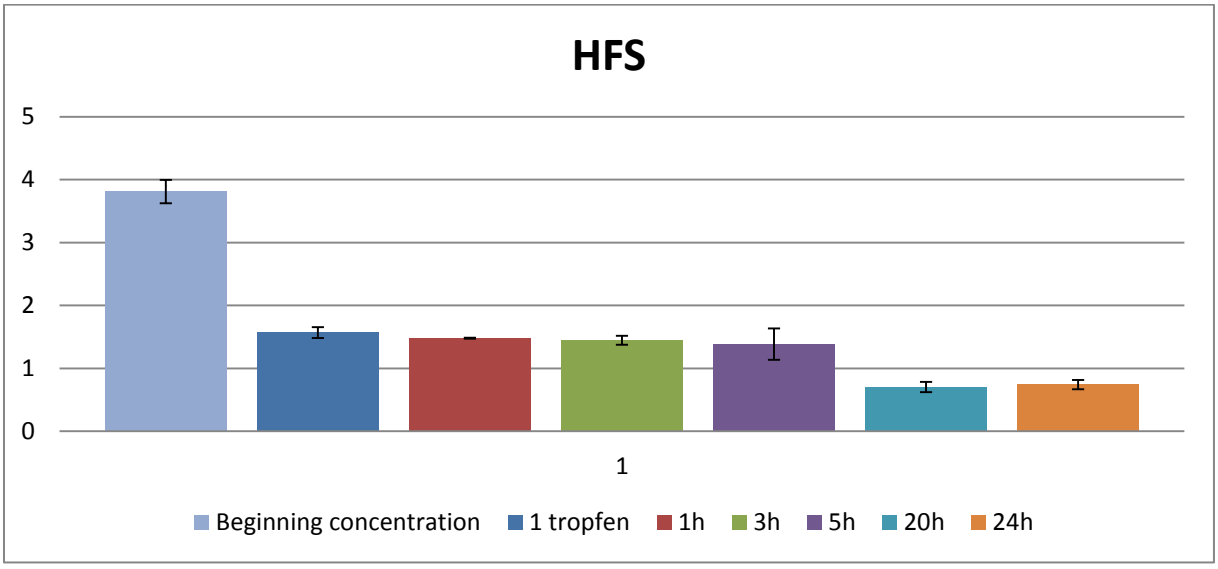


Figure 57 EE2 concentration of HFS-Membrane after release of the membrane module in specific time intervals

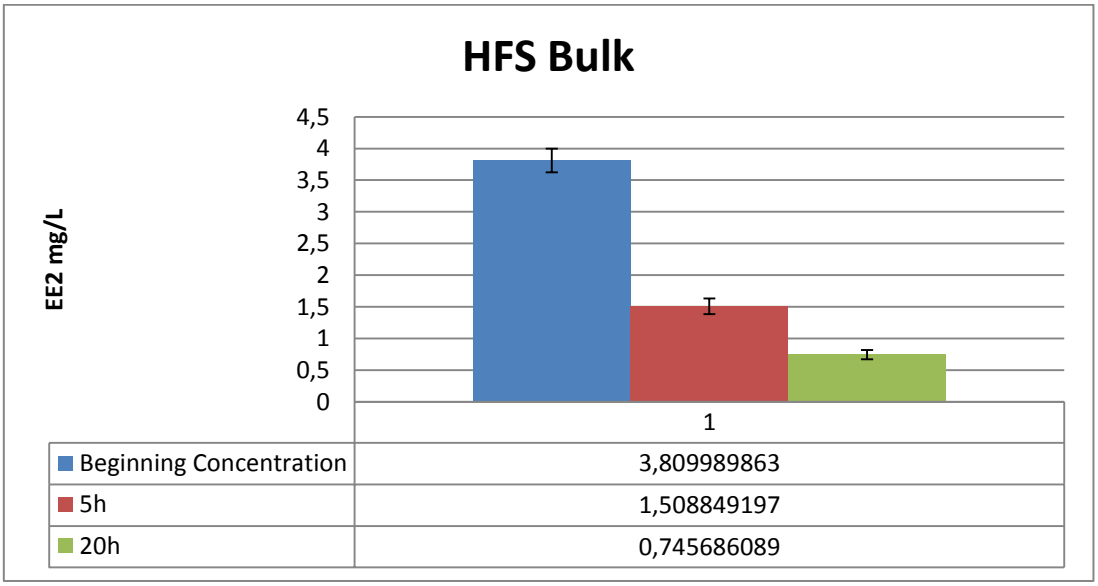


Figure 58 EE2 concentration of HFS-Membrane bulk in specific time intervals

Conclusion

The results show that the concentration of EE2 in water is degraded by immobilized HRP on fibre modules. As expected, the HFS membrane shows higher activity than the UFC membrane the reason for this might be the higher enzyme activity on the membrane. About 80% of the starting concentration in both cases has been degraded by the fiber module. Therefore, the experiments demonstrates the successful application of immobilized HRP on continuously operated single fiber modules for EE2 degradation in aqueous systems.

Degredation of EE2 in Fiber modules with reimmobilization after 24 hours

Experimental

The experiments aimed at elaborating a method to replace the biocatalytic layer on membrane fibers after operation. A membrane (UFC or HFS) in the size of a module was given into a module and both endings were clued with a clue (provided by Pentair X-Flow). To remove any residues from the production the membranes were treated with 18% EtOH in aqueous solution (for HFS) or in pure water (for UFC). Subsequent the membranes got cleaned with water for 20 minutes. Afterwards a 0,05wt% CMC solution got pumped through the modules and cleaning with water for 20 minutes followed. A 0,05 wt% CHI, 150 mM KCl solution was pumped through the membrane and washed again with water for 20 minutes.

Immobilization of Horseradish Peroxidase

Because of practical issues only the Combined Method has been tested. Details for the Combined HRP immobilization method as used for this experiment: A mixture of EDC, HRP and CMC or PSS solution for 3 hours has been pumped through the membrane for 3h as previously reported.

Degradation of EE2

The concentration of EE2 in water amounted 11,3 mg/L. Because of the low solubility of EE2 in water it was not possible to dissolve. First EE2 was dissolved in absolute ethanol to create (0,5 g/L) solution.

2.5 mL of the ethanol solution was mixed with 247,5 mL $CaCl_2$ solution (10 mM).

Afterwards the EE2 solution got pumped in a cycle through the modules and samples were taken in certain time intervals.

After 24 hours of degrading EE2 the membranes get washed with water for about 1 hour. Subsequent the immobilisation of HRP followed. Because of practical issues only the

Combined Method has been tested. Details for the Combined HRP immobilization method as used for this experiment: A mixture of EDC (0,1wt%), HRP (0,1g/l) and CMC (0,05wt%) or PSS solution for 3 hours has been pumped through the membrane for 3h as previously reported. Afterwards a EE2 solution described as before was pumped through the membranes and samples were taken in certain time intervals. Afterwards the concentration was measured by HPLC described as before.

Results of 1st immobilization of HRP HFS

Figure 59 shows the EE2 concentration of HFS-Membrane 1st immobilization after release of the membrane module in specific time intervals. Figure 60 shows the EE2 concentration of HFS-Membrane 1st immobilization bulk in specific time intervals.

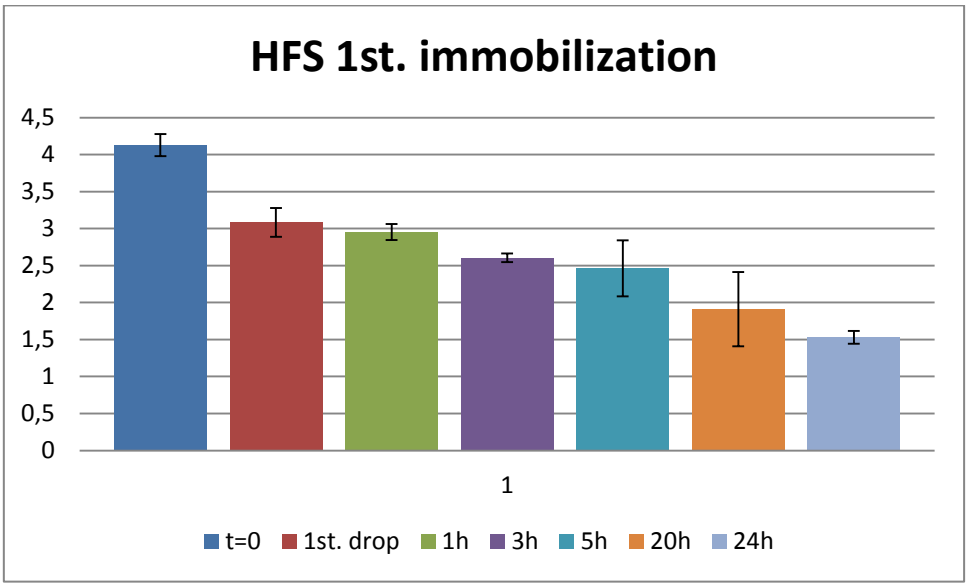


Figure 59 EE2 concentration of HFS-Membrane 1st immobilization after release of the membrane module in specific time intervals

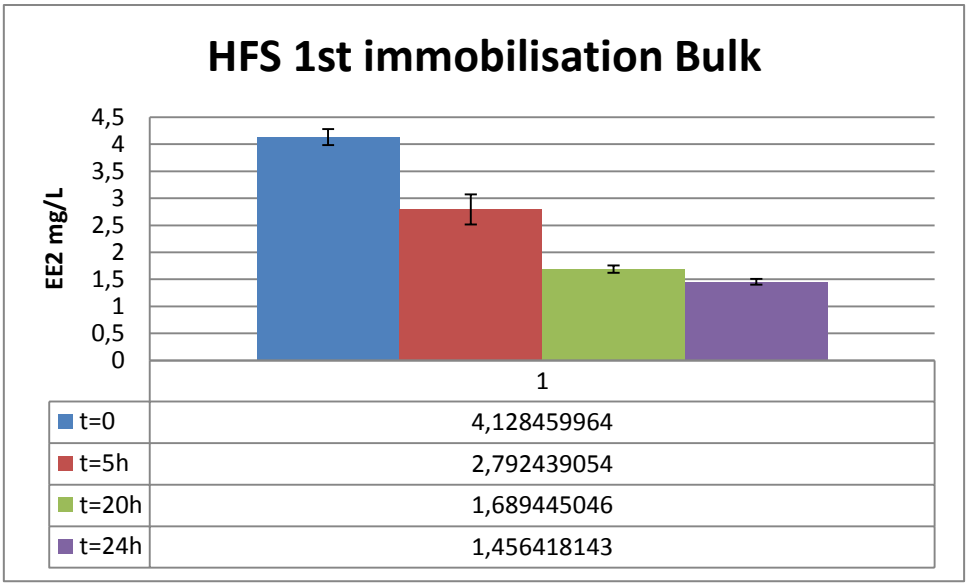


Figure 60 EE2 concentration of HFS-Membrane 1st immobilization bulk in specific time intervals

Results of 1st immobilization of HRP UFC

Figure 61 shows the EE2 concentration of UFC-Membrane 1st immobilization after release of the membrane module in specific time intervals. Figure 62 shows the EE2 concentration of UFC-Membrane 1st immobilization bulk in specific time intervals.

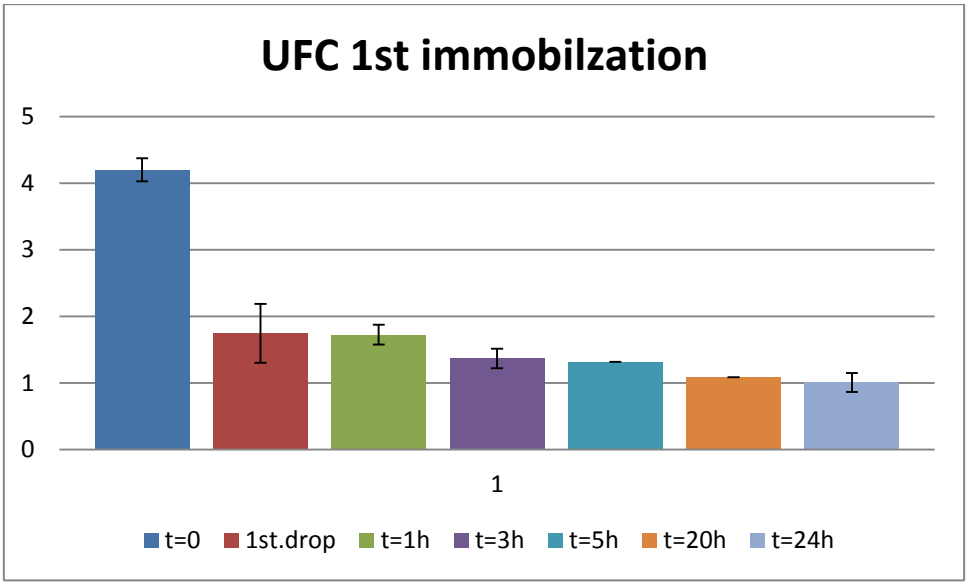


Figure 61 EE2 concentration of UFC-Membrane 1st immobilization after release of the membrane module in specific time intervals

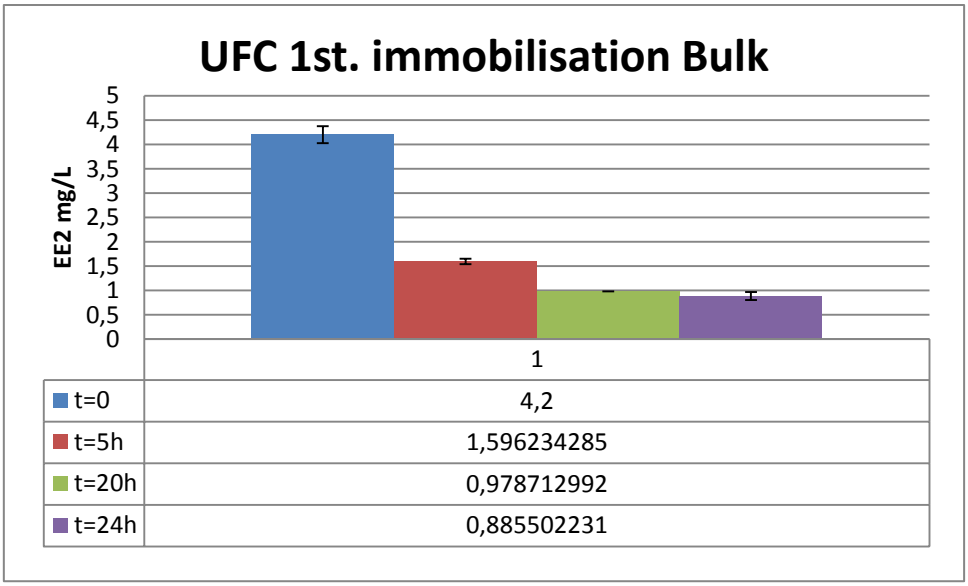


Figure 62 EE2 concentration of UFC-Membrane 1st immobilization bulk in specific time intervals

Results of 2nd immobilization of HRP HFS

Figure 63shows the EE2 concentration of HFS-Membrane 2nd immobilization after release of the membrane module in specific time intervals. Figure 64 shows the EE2 concentration of HFS-Membrane 2nd immobilization bulk in specific time intervals.

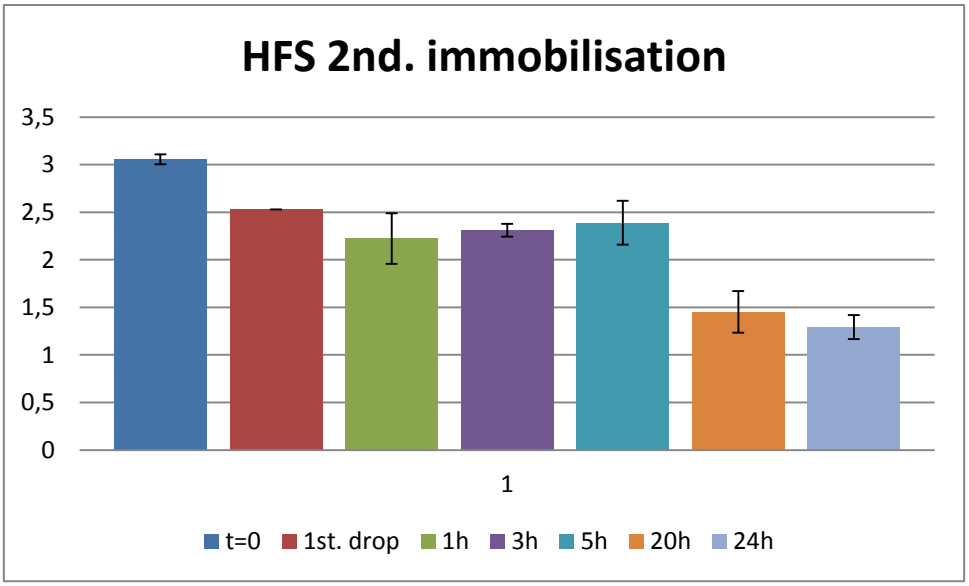


Figure 63 EE2 concentration of HFS-Membrane 2nd immobilization after release of the membrane module in specific time intervals

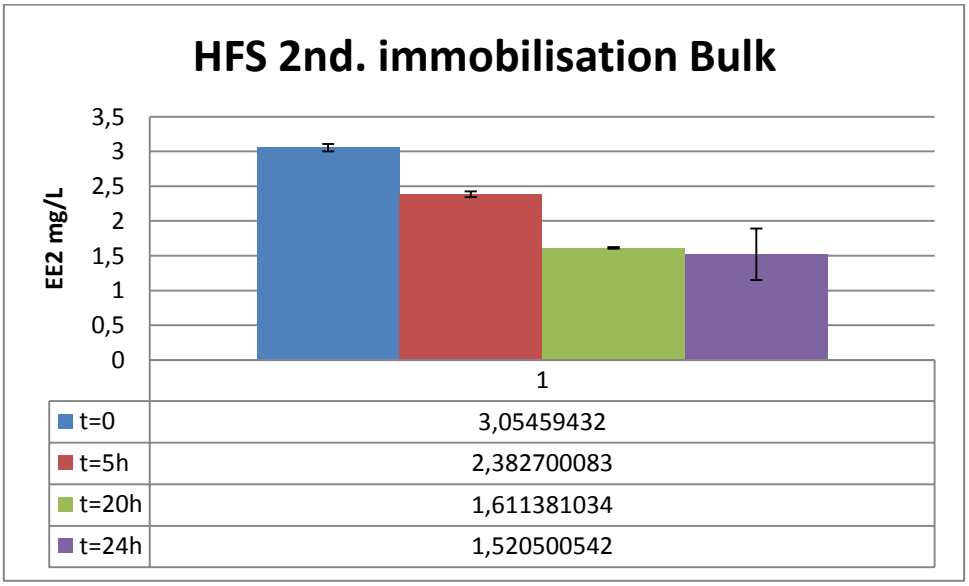


Figure 64 EE2 concentration of HFS-Membrane 2nd immobilization bulk in specific time intervals

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Results of 2nd immobilization of HRP UFC

Figure 65 shows the EE2 concentration of UFC-Membrane 2nd immobilization after release of the membrane module in specific time intervals. Figure 66 shows the EE2 concentration of HFS-Membrane 2nd immobilization bulk in specific time intervals.

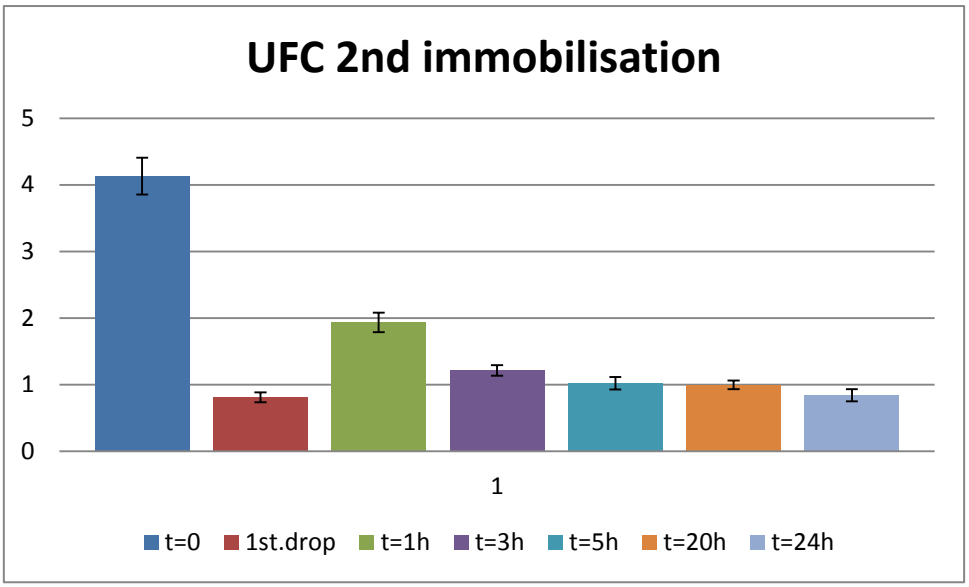

Figure 65EE2 concentration of UFC-Membrane 2nd immobilization after release of the membrane module in specific time intervals

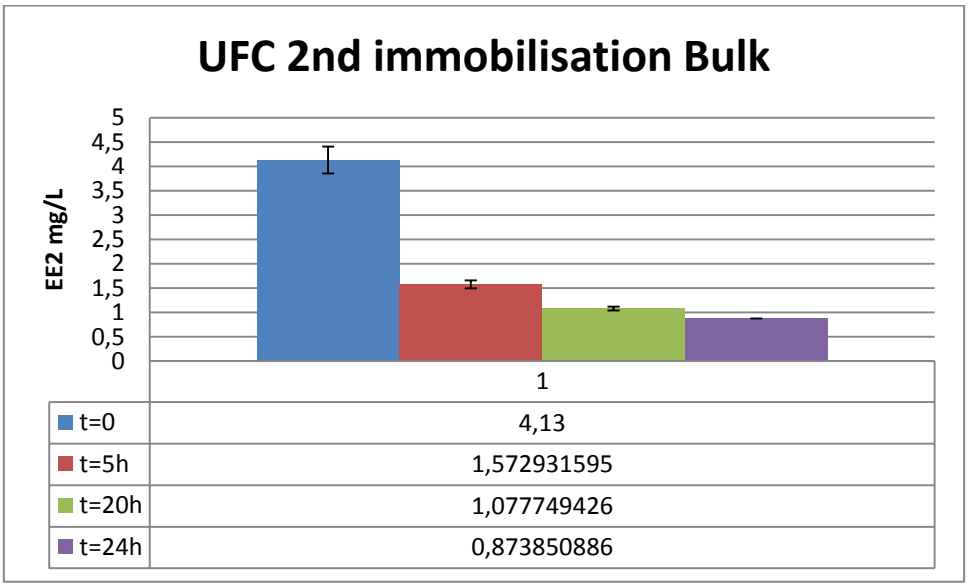

Figure 66 EE2 concentration of HFS-Membrane 2nd immobilization bulk in specific time intervals

Results of 3rd immobilization of HRP HFS

Figure 67 shows the EE2 concentration of HFS-Membrane 3rd immobilization after release of the membrane module in specific time intervals. Figure 68 shows the EE2 concentration of HFS-Membrane 2nd immobilization bulk in specific time intervals.

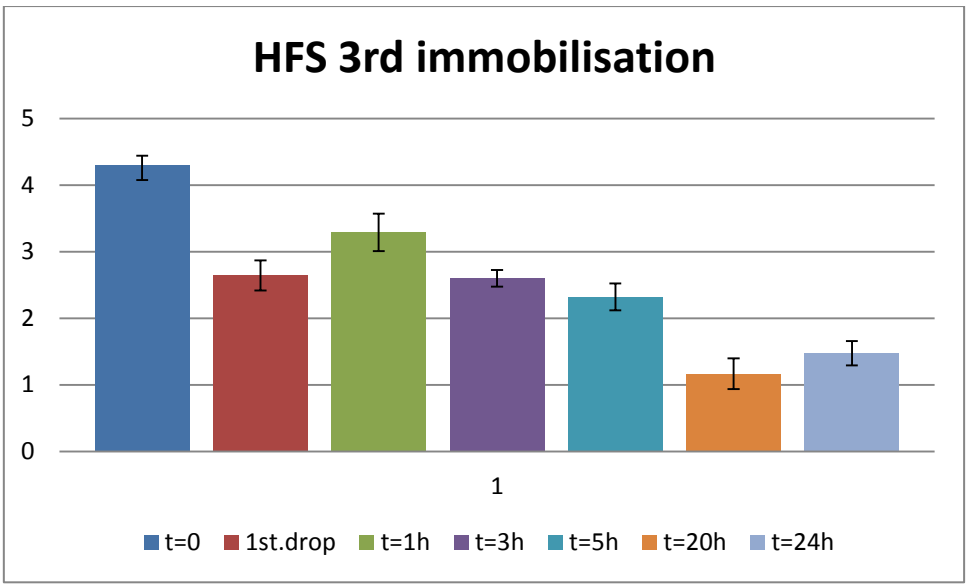

Figure 67 EE2 concentration of HFS-Membrane 3rd immobilization after release of the membrane module in specific time intervals

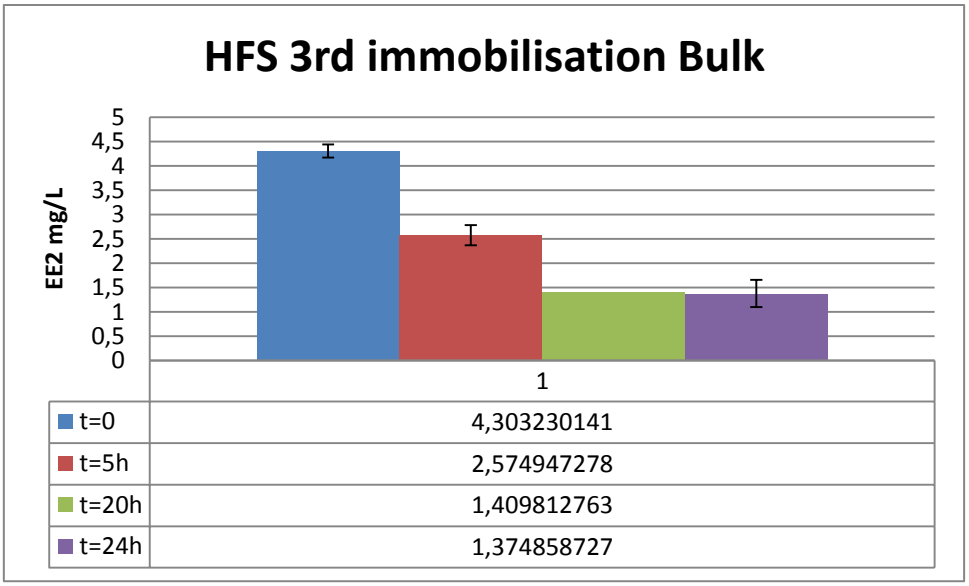

Figure 68 EE2 concentration of HFS-Membrane 2nd immobilization bulk in specific time intervals

Results of 3rd immobilization of HRP UFC

Figure 69 shows the EE2 concentration of UFC-Membrane 3rd immobilization after release of the membrane module in specific time intervals. Figure 70 shows the EE2 concentration of UFC-Membrane 3rd immobilization bulk in specific time intervals.

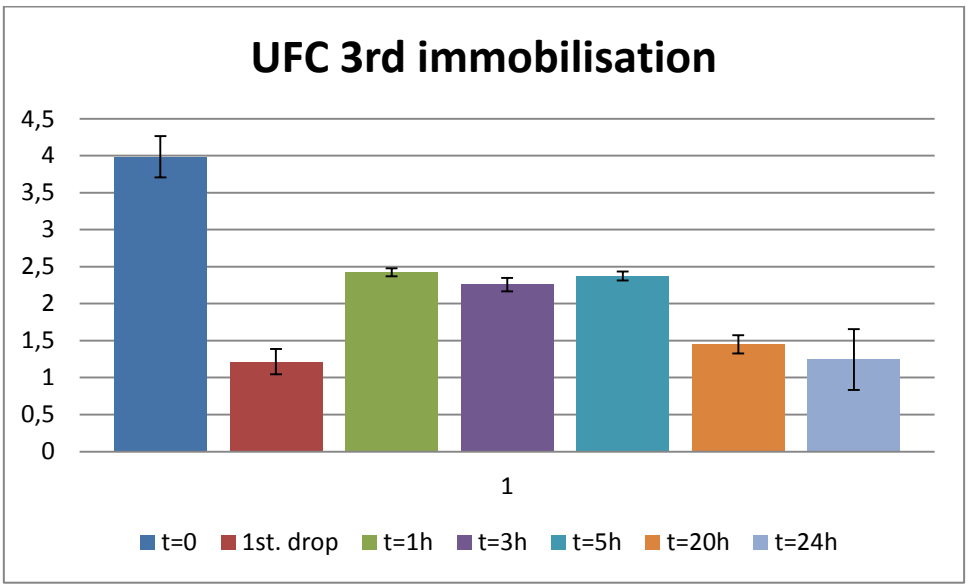

Figure 69 EE2 concentration of UFC-Membrane 3rd immobilization after release of the membrane module in specific time intervals

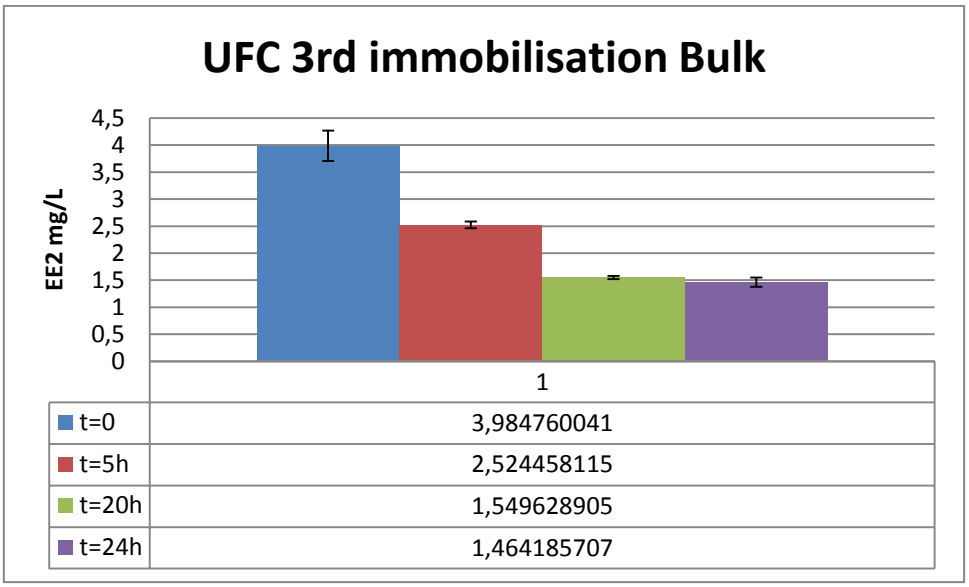

Figure 70 EE2 concentration of UFC-Membrane 3rd immobilization bulk in specific time intervals

Conclusion

The first immobilization of HFS membranes shows significant degradation of EE2 in solution. After 24h only 35% of the starting concentration remains in solution. The 2nd immobilization of HFS membranes shows that the degradation is getting worse with further immobilization. Only 50% of the starting concentration is degraded by the 2nd immobilization of HFS membranes. Also the third immobilization of HFS membranes shows significant degradation of EE2 in solution. Only 30% of the starting concentration remains. The results show that 1,4 EE2 mg/L seems to be the limit of degradation for this method.
The first immobilization of UFC-Membranes shows significant degradation of EE2 in solution. After 24h only 20% of the starting concentration remains in solution in the bulk. The 2nd immobilization of UFC membranes shows that no decrease of the degradation rate is observable in comparison to HFS membranes. Only about 20% of the starting concentration remains in solution. A concentration of 0,87 mg/L EE2 seems to be the limit of degradation for this method. The third immobilization of UFC shows less degradation than the immobilizations before. About 38% of the starting concentration remains in solution.

Taking the results into consideration quite significant degradation of EE2 in solution is observable. It can be said that UFC membranes work better than HFS membranes. The degradation limit for HFS membranes is about 1,4 mg EE2/L while the degradation limit of UFC membranes is about 0,88 mg EE2/L.

Degradation of EE2 on Fiber modules long term study

Experimental

A membrane (UFC or HFS) in the size of a module was given into a module and both endings were clued with a clue (provided by Pentair X-Flow). To remove any residues from the production the membranes were treated with 18% EtOH in aqueous solution (for HFS) or in pure water (for UFC).

Subsequent the membranes got cleaned with water for 20 minutes. Afterwards a 0,05wt% CMC solution got pumped through the modules and cleaning with water for 20 minutes followed. A 0,05 wt% CHI, 150 mM KCl solution was pumped through the membrane and washed again with water for 20 minutes.

Immobilization of Horseradish Peroxidase

Because of practical issues only the Combined Method has been tested. Details for the Combined HRP immobilization method as used for this experiment: A mixture of EDC, HRP and CMC or PSS solution for 3 hours has been pumped through the membrane for 3h as previously reported.

Degredation of EE2

The concentration of EE2 in water amounted 11,3 mg/L. Because of the low solubility of EE2 in water it was not possible to dissolve. First EE2 was dissolved in absolute ethanol to create (0,5 g/L) solution. 10 mL of the ethanol solution was mixed with 990 mL $CaCl_2$ solution (10 mM).

Results of UFC-membrane

Figure 71 shows the EE2 concentration of UFC-Membrane long-term study after release of the membrane module in specific time intervals. Figure 72 shows the EE2 concentration of UFC-Membrane long-term bulk in specific time intervals

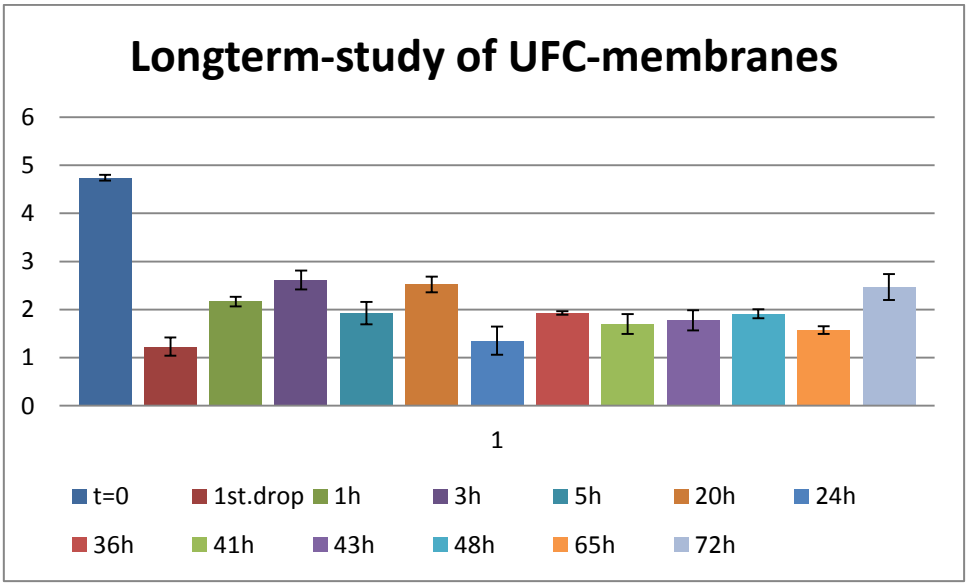

Figure 71 EE2 concentration of UFC-Membrane long-term study after release of the membrane module in specific time intervals

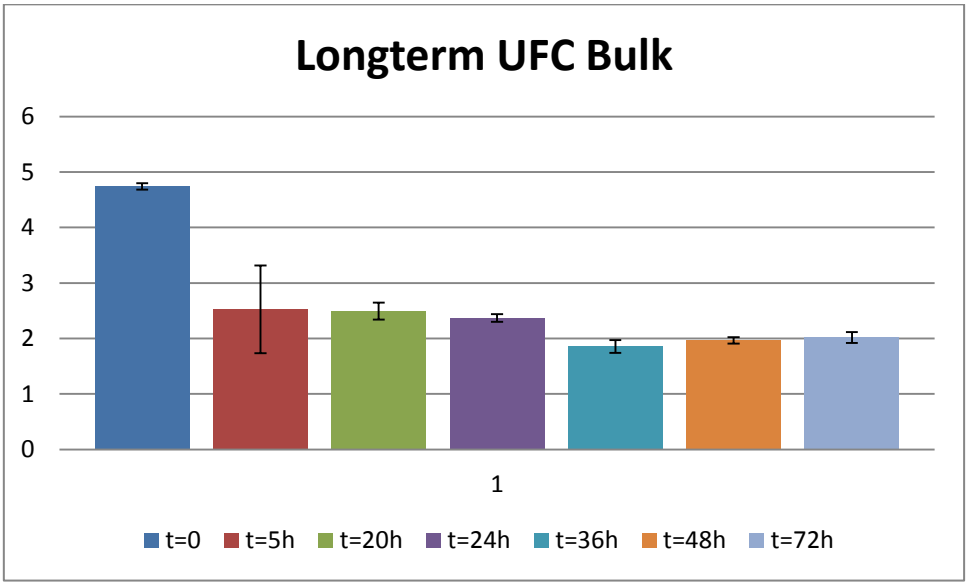

Figure 72 EE2 concentration of UFC-Membrane long-term bulk in specific time intervals

Results of HFS membrane

Figure 73 shows the EE2 concentration of HFS-Membrane long-term study after release of the membrane module in specific time intervals. Figure 71 shows the EE2 concentration of HFS-Membrane long-term study after release of the membrane module in specific time intervals.

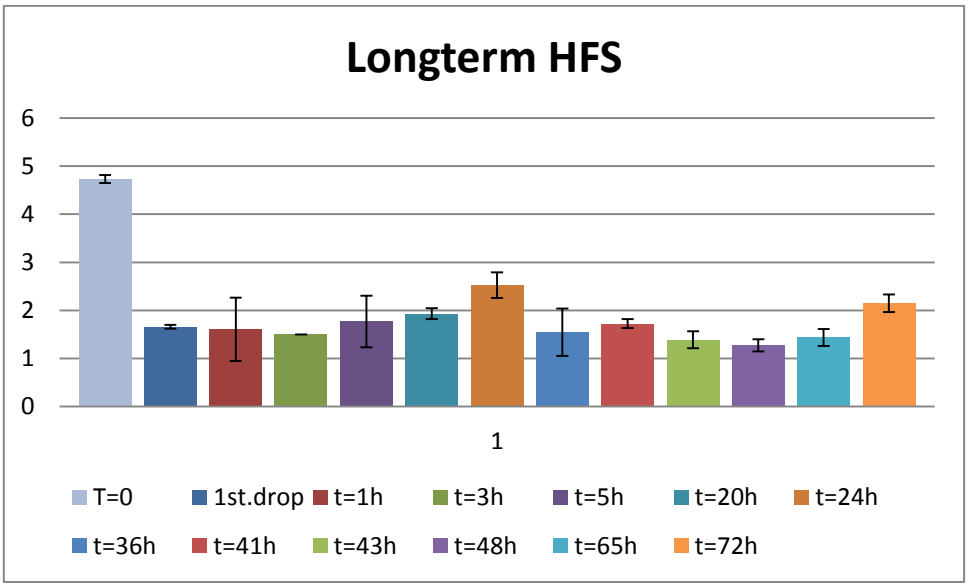

Figure 73 EE2 concentration of HFS-Membrane long-term study after release of the membrane module in specific time intervals

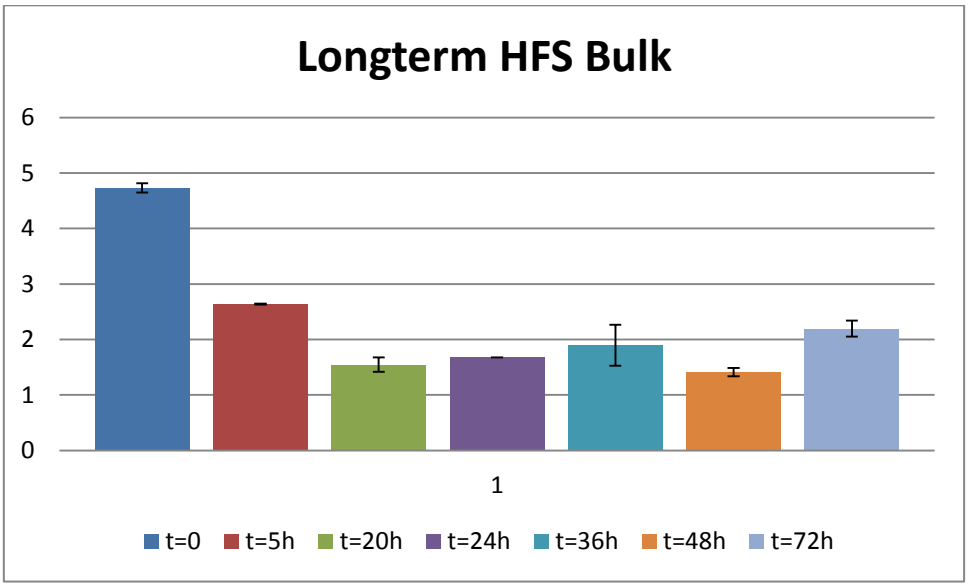

Figure 74 EE2 concentration of HFS-Membrane long-term study after release of the membrane module in specific time intervals

Conclusion of long term study

UFC-membranes show over 3 days significant degradation of EE2 in solution. After 5h nearly 40% of the starting concentration is degraded. After the first 5 hours the degradation rate is decreasing quite fast. After 36h nearly no degradation of EE2 is observable which might be because of a loss of enzyme activity.

The HFS-membranes show similar properties than the UFC-membrane. Also here after 36 hours the enzyme loses its activity.

Overall conclusion and future outlook

The immobilization of HRP on different hollow fibre membranes was presented in this work. For this 3 different immobilization techniques have been used. Considering stability and activity of immobilized HRP, the chemical linkage seems to be the most promising one. The work showed the influence of different conditions (pH and salt concentration) on the activity on the immobilized enzyme. The results show that salt concentration has no influence on the activity of immobilized enzyme while the pH-Value has huge effect on the activity. Strongly acidic conditions results in a complete loss of enzyme activity while at neutral/slightly alkaline conditions no major influence was observed in the experiments. Also the influence of Cd^{2+} and F^- has been studied. The data show that Cd^{2+} ions have huge effect on the activity of free and immobilized enzyme. With rising Cd^{2+} concentration the activity of HRP decrease in case of free and immobilized HRP tremendously.

In the further sections the degradation of EE2 with enzyme in solution and immobilized HRP has been studied. The work shows that free HRP shows significant degradation of EE2 with and without the presence of humic acids. Also immobilized HRP shows significant degradation of EE2 over a certain time. Long term experiments over 72h show event degradation after this time period. Also the remobilization of HRP and another degradation cycles shows significant degradation.

Also a simulation of a potential practical process in waste water treatment has been successful developed. For this hollow fibre modules have been tested. Even here degradation of EE2 was observed.

The work also provides a convenient tool to monitor EE2 degradation by means of HPLC-MS. One step of the future work for this task should be the detailed characterization of the obtained degradation products to make sure that not more toxic compounds than EE2 are getting produced.
Another very important step is to optimize the degradation process. Several parameters like the optimization of desired enzyme concentration, the optimization of the desired degradation time and the time after the enzyme loses its activity should be determined.

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