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# Hydrophobins from *Trichoderma* for immobilization of industrially relevant enzymes

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der Technischen Universität Wien

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“When every purpose has an action,

every action has a result!”

Greg Plitt

„Our greatest weakness lies

in giving up. The most certain way

to succeed is always to try just one more time“

Thomas A. Edison

# DANKSAGUNG

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ABSTRACT

Hydrophobins are common for filamentous fungi. Since now, their role in surface hydrophobicity, fruit body formation and sporulation has been reported. Due to their ability to attach both to hydrophilic and to hydrophobic surfaces, we hypothesized that they could enhance the enzymatic degradation of polymers by creating a monolayer, which aids the enzyme to bind the polymeric substrate or they can increase the efficiency of enzyme binding in the non-covalent enzyme immobilization.

In this work, we present the effects of GST-HFB4 fusion protein from *Trichoderma virens* on the enzyme activity both in solution and when immobilized, as a proof of concept. Our initial hypothesis was that hydrophobins from *T. virens* could enhance activity of enzymes used for polymer degradation. However, due to the fact that GST-HFB4 has not affected cellulases' activity in their free form, we focused on the hydrophobin-mediated immobilization of enzymes to test the hypothesis, if hydrophobins are able to bind more enzyme activity to the surface of porous glass beads.

The data obtained in this thesis depicts a positive effect on the immobilizing of glucose oxidase activity on the surface of kapton membrane with HFB7-$(His)_6$ layer. However, in order to fully understand the differences between activities of different enzymes both in solution and when immobilized, further examinations would be needed and this data could be useful for that purpose.

# KURZFASSUNG

Hydrophobine sind in Pilzen weitverbreitet. Bekannt ist ihre Rolle in der Herstellung hydrophober Oberflächen, Fruchtkörperbildung, und Sporulation. Dank deren Fähigkeit sowohl an hydrophile- als auch hydrophobe Oberflächen zu binden, habe ich angenommen dass Hydrophobine auch den enzymatischen Abbau von Polymeren oder die Immobilisierung von Enzymen positiv beeinflussen könnten, indem sie ein Monolayer bilden der die Bindung der Enzyme an polymere Substrate begünstigt.

In dieser Masterarbeit zeige ich den Effekt der Anwesenheit von *Trichoderma virens* GST-HFB4 Fusions-Proteinen auf die enzymatische Aktivität von gelösten und immobilisierten Enzymen als Proof of Concept. Die ursprüngliche Hypothese war, dass *T. virens* Hydrophobine die Aktivität von Polymer-abbauenden Enzymen erhöhen könnten. Die Aktivität von freien Cellulasen im Vorhandensein von GST-HFB4 zeigte sich hingegen als nicht beeinträchtigt und aufgrund dieses Ergebnisses fokussierten wir auf der Enzymimmobilisierung mittels Hydrophobinen.

Die in dieser Arbeit beschriebenen Ergebnisse zeigen die Steigerung der Aktivität von Glucose Oxidase durch Immobilisierung an Kapton, mittels HFB7-$(His)_6$.

ABBREVIATIONS

| | |
|---|---|
| APS | Ammonium persulfate |
| Asp | Aspartic acid |
| CMC | Carboxymethylcellulose |
| $ddH_2O$ | Double destilled water |
| $dH_2O$ | Destilled water |
| FPA | Filter paper |
| GOx | Glucose oxidase |
| GST | Gluthathione-S-transferase |
| GST-HFB4 | Fusion protein of gluthathione-S-transferase to hydrophobin 4 |
| HCl | Hydrochloric acid |
| HFB4 | Hydrophobin 4 |
| HFB4-$(His)_6$ | Fusion protein of hydrophobin 4 to six histidine rests |
| HFB7 | Hydrophobin 7 |
| HFB7-$(His)_6$ | Fusion protein of hydrophobin 7 to six histidine rests |
| His | Histidine |
| LB | Lysogeny Broth |
| $Na_2CO_3$ | Sodium carbonate |
| o-DND | o-dianisidine |
| PBS | Phosphate buffered saline |
| PBS Triton X-100 | Phosphate buffered saline polyethylene glycol p-(1,1,3,3-tetra-methylbuthyl)-phenyl ether |

| | |
|---|---|
| PNPL | 4-nitrophenyl-β-D-lactopyranoside |
| POD | Horseradish peroxidase |
| RT PCR | Real Time Polymerase Chain Reaction |
| SDS | Sodium Dodecylsufate |
| SDS PAGE | Sodium dodecyl sulfate polyacrylamid gel electrophorese |
| Ser | Serine |
| TEMED | Tetramethylethylenediamine |
| TRIS – HCl | Tris(hydroxymethyl)aminomethane hydrochloride |

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# 1. INTRODUCTION

## 1.1. ENZYMES

Enzymes are complex globular proteins and catalyze both many metabolic (anabolic or catabolic) reactions and substrate conversions in many chemical reactions. Enzymes possess a unique ability to perform their specific chemical transformation in isolation. Their another remarkable attribute is a high specificity to designated types of compounds. The activity of an enzyme depends not only on the primary structure but also on intricate folding configuration of a whole molecule. The destruction of this configuration via for example a change of pH or temperature, may led to the loss of the activity (Smith, 2009).

The major application fields are production of biodetergents, bulk products in the backing industry, brewing, dairy industry, starch industry, textile industry, leather industry, bioraffinery, medicine and pharmacy (Smith, 2009).

The development of the enzyme technology caused that approximately 90% of bulk enzyme production is derived from filamentous fungi, bacteria and yeast. The remaining 10% come from animal (6%) and plant (4%) sources. Owing to the genetic engineering, the production of enzymes from extremophyles that have higher temperature resistance, can now be grown in a mesophyles (Smith, 2009).

As an example for numerous advantages of using enzymes in industrial processes can be the biocatalytical production of the widely applied emollient mirystyl myristate in cosmetics, which shows attractive results in form of a 60% lower energy consumption and greenhouse gas emission and up to 90% lower emission of pollutants emission when compared to chemocatalysis, as shown in table 1 (Sheldon, 2011).

**Tab. 1:** Environmentally key parameters of chemo- and biocatalytic estrification (Sheldon, 2011).

| Parameter | Units | Chemocatalytic | Biocatalytic | Savings |
|---|---|---|---|---|
| **Energy** | GJ | 22.5 | 8.63 | 62% |
| **GHG emissions**[a] | kg $CO_{2\ eq.}$ | 1518 | 582 | 62% |
| **Acidification**[b] | kg $SO_{2\ eq.}$ | 10.58 | 1.31 | 88% |
| **Eutrophication** | kg $PO_{4\ eq.}$ | 0.86 | 2.24 | 74% |
| **VOC** | kg $C_2H_{4\ eq.}$ | 0.49 | 0.12 | 76% |

[a] Greenhouse gas emissions.

[b] Volatile organics (smog formation)

Due to their stereochemical specificity for substrates and their specificity to produce only one enantiomer, enzymes found application in the production of chemicals or pharmaceuticals, in which stereochemistry plays a key role. Such an example for the process is the production of enantiomerically pure amino acids by an improved racemase/acylase system from N-acetylated amino acids, where the limiting step for this process is racemisation of D – amino acids into L – amino acids through N – amino acid racemase. However, thanks to protein engineering the achieved yield reached up to 98% (Baxter et al., 2012).

Moreover, biotransformation of toxic compounds by naturally occurring or genetically modified enzymes from microorganisms is used for the utilization of soil contaminations (e.g. polycyclic compounds, halogenated polycyclic hydrocarbons). Such an example is aerobic soil biotransformation of fluorotelomer iodide (Ruan et al., 2013).

In the last few decades, more attention has been put on the problem of decreasing natural resources of petroleum. Since then, scientists have been looking for renewable sources of energy, like wind energy, water energy, but only renewable carbon sources such as plants containing biopolymers, such as starch or vegetable oils can be used for bioethanol or biodiesel production. This aspect became a driving force for an enzyme usage, where especially enzymes, which could increase highly the efficiency of a process, are a focus of attention.

The growing prices of enzymes caused a searching in the XX century for methods prolonging their shelf life and enabling their reusability, as mentioned in advantages of this process. Those criteria are fulfilled by enzyme immobilization.

The major advantage of enzyme immobilization are:

- a possibility of reusage of a single batch enzyme.
- a higher stability of an immobilized enzyme in most cases
- purity of obtained product after a reaction
- a possibility of a development of a multienzyme reaction system
- reduction of disposal problems.

On the other hands, the process of immobilization is not free from disadvantages, to which belongs:

- effects on the stability and activity of enzymes
- problems, when the substrate of the reaction is found to be insoluble
- enzyme inactivation by a heat generated in the system
- higher costs bounded to the purification and recovery of active enzyme.

Although we can distinguish a few simple methods of immobilization, such as immobilization by adsorption, covalent binding, encapsulation and cross – linking, which are described below, since then many combined methods have been developed.

Immobilization by adsorption results either from hydrophobic or ionic interactions between a support matrix and an enzyme (Spahn and Minteer, 2008; Mateo et al., 2000). As a support for this method the following materials can be used e.g. coconut fibers, microcrystalline cellulose, porous glass. In case of immobilization by adsorption with porous silica as a support, it is often needed to functionalize the groups on the surface of a support to increase the efficiency of the process (Datta et al., 2012; Hanefeld et al., 2008).

Covalent binding of enzymes is a more stable form of immobilization and results in the maintenance of most activity in comparison to the adsorption method (Schnapp et al., 1976). One important advantage of using covalently immobilized enzymes is the purity of a reaction product from an enzyme. The figure 1 shows possible and commonly used functional groups for covalent binding, which involves the amino groups of the enzyme. The

nucleophilic character of the amino group causes, that this group can attack for instance an epoxide or an aldehyde, what can cause irreversible immobilization of enzymes or the protection of immobilized enzyme against inactivation by chemicals compared to free enzyme. The biggest disadvantage of covalent binding is that enzymes are chemically modified, but on the other side it offers the possibility for better orientation of the enzyme (Hanefeld et al., 2008).

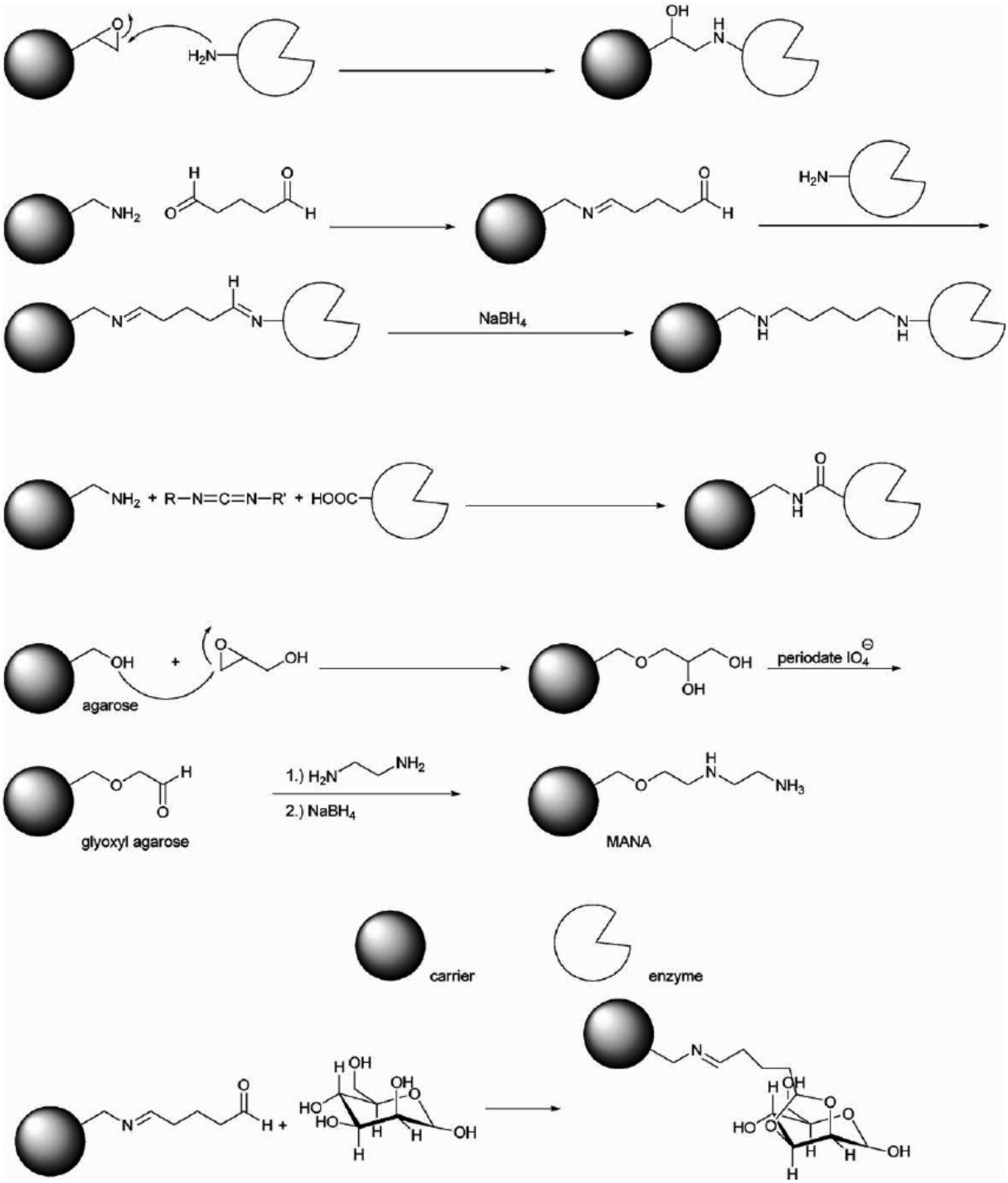



**Fig. 1.**Funtional groups (mostly amino groups) on the surface of the enzyme can easily react with the carrier creating covalent bonds (Hanefeld et al., 2008).

Another method of enzyme immobilization called crosslinking was developed from the covalent binding method. In comparison to covalent binding a carrier is not needed,

because the enzymes themselves play the role of a carrier. The first step of Cross Linked Enzyme Aggregates preparation, the aggregation of enzymes using of precipitants such as acetone, ethanol or 1,2-dimethoxyethane is followed by crosslinking by commonly used glutaraldehyde (see figure 2.).

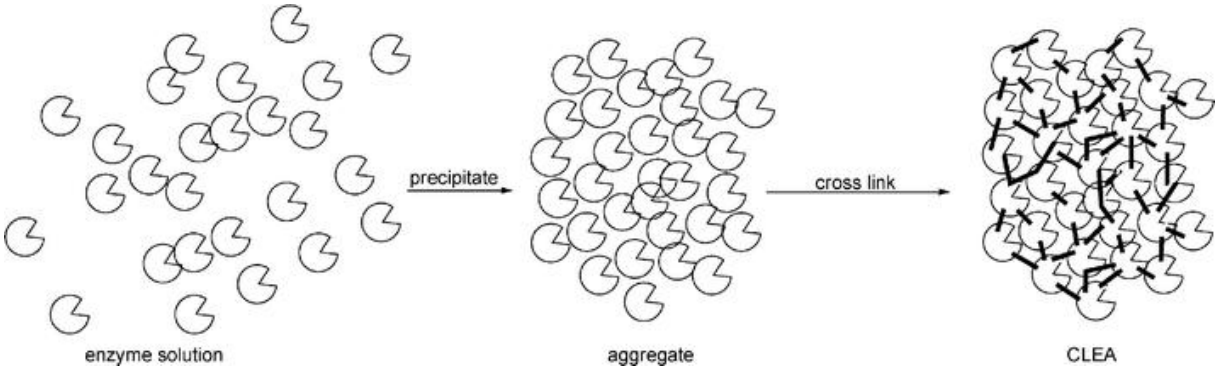



**Fig. 2.** Aggregation and crosslinking of an enzyme to form CLEA (Hanefeld et al., 2008).

The best method to avoid any negative influence on the enzyme structure is encapsulation of an enzyme. The most prominent and widely used technique is the sol – gel encapsulation. Sol – gel is a chemical inert and highly porous silica glass, that can be shaped in any desired form. Despite their porous structure, the diffusion of substrate to the enzyme can be restricted (Hanefeld et al., 2008).

## 1.2. EXAMPLES OF INDUSTRIAL IMPORTANT ENZYMES

In order to reduce energy and waste costs, many chemical catalyzed reactions have been replaced by a biocatalytical reactions with enzymes as catalysators. The three following important industrial enzymes were selected for further investigation in this work.

## Cellulases, their properties and applications

The most abundant organic compound on Earth is cellulose, which builds the main structure of plant cell walls. Cellulose contains linear chains of about 8000 to 12000 residues of D – glucose linked by β - 1,4 bonds (Timell and Syracuse, 1967; Aro et al. 2005). Therefore, from the industrial for example petrochemical or food industry point of view, cellulases are important enzymes.

The enzymatic degradation of cellulose ensues thanks to synergistically acting enzymes, produced mostly by aerobic filamentous fungi. This cellulolytic system (see fig. 3) comprises three types of cellulases:

- ✓ Endoglucanases (EC 3.2.1.4) attack randomly and hydrolyze β - 1,4 bonds of amorphous chains of cellulose to produce cello – oligosaccharides.
- ✓ Exoglucanases (EC 3.2.1.91) act on non reducing (CBH II) or on reducing (CBH I) ends of cellulose chains
- ✓ β-glucosidases (EC 3.2.1.21) hydrolyze soluble cellodextrins and cellobiose to glucose. (Alonso Bocchini Martins et al., 2011)

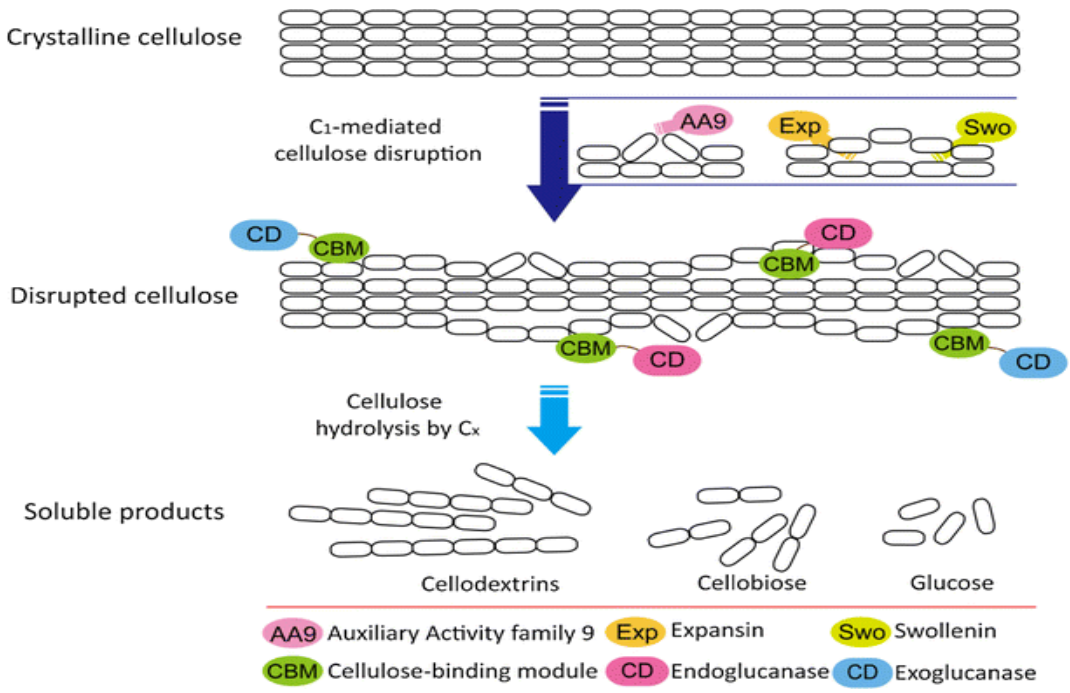



**Fig. 3.** Schematic representation of the recent cellulolytic system (Kim et al., 2014).

The former cellulose degradation model assumed that only carbohydrate–binding modules (CMB) expansins and expansin-like proteins take part in the disruption of high-ordered cellulose matrix. However, the most recent model takes into consideration the activity of synergistically acting proteins called Auxiliary Activity family 9 (AA9, formerly known as glycoside hydrolase or lytic copper-dependent polysaccharide monooxygenases) which enhances cellulose saccharification after being added to the mixture of cellulases. It is supposed that the oxidative activity of AA9 occurs via copper-bound active site through chemical reactions involving hydrogen abstraction and subsequent hydroxylation which require molecular oxygen and electron donors. This process appears to be cost-effective and could be useful to reduce the expenditures of the biofuel production (Phillips et al., 2011).

Swollenin, a small fungal protein which is homologous to plant β- Expansin protein family, also plays a role in the mechanism of cellulose biodegradation. It is thought to enhance the hydrolysis of cellulose by weakening and disrupting of the substrate by promoting amorphogenesis (Gourlay et al., 2013).

Initially, cellulases were investigated only for biodegradation of biomass for bulk production but nowadays they also found applications in animal foods, textiles, biorafinery and detergents industry (Sukumaran et al., 2005).

## Trypsin

Trypsin is an enzyme from a group of serine proteases, possessing a catalytic triad, composed of a serine, histidine and an aspartate residue (see Figure 4.). It is a so – called oxyanion hole formed by backbone NH groups.

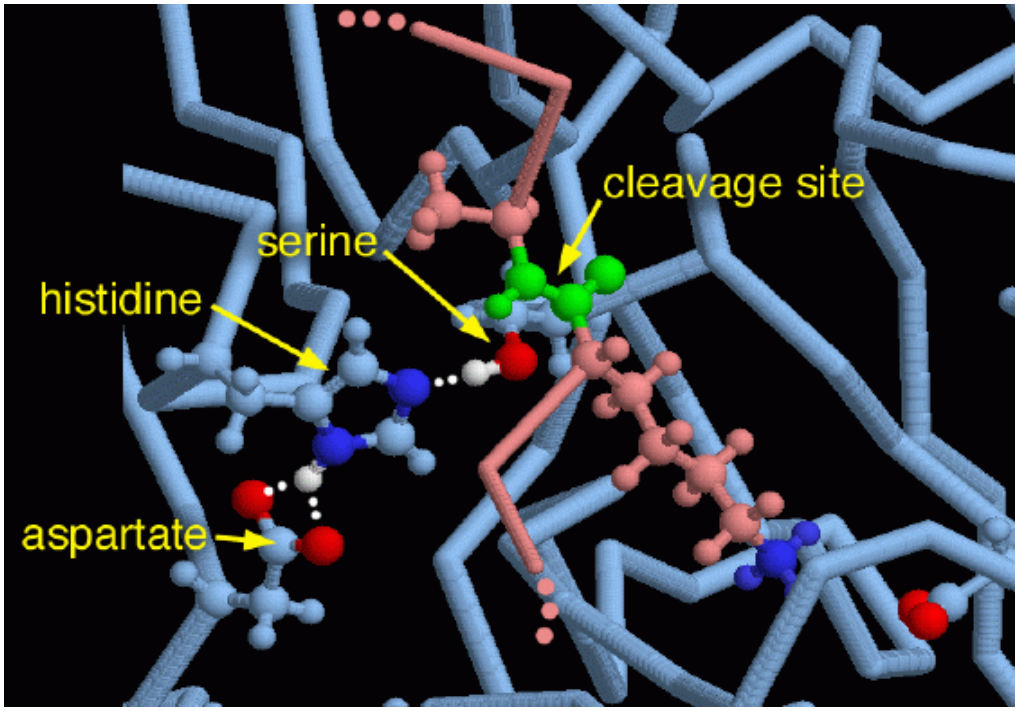



**Fig. 4.** Active site of trypsin from PDB.

The mechanism of proteolytic hydrolysis begins with the formation of a non-covalent Michaelis complex between enzyme and substrate and proceed a nucleofilic attack of the substrate C – atom by the hydroxyl group of $Ser^{195}$. That leads to the formation of a covalent tetrahedral intermediate And the next essential step is the transfer of a proton from the OH group of $Ser^{195}$ to an imidazole N – atom of $His^{57}$. The following stage involves $Asp^{102}$ which stabilizes the positively charged form of $His^{57}$ in the transition state. The imidazolium proton is transferred onto the N-atom of the cleaved amide bond, leaving an acyl – enzyme intermediate. Finally, there is the deacylation where $H_2O$ substitutes the amine component in the trypsin cleaving. And because of activation of $H_2O$ by imidazole, another tetrahedral intermediate is formed which decomposes in turn and liberates the carboxylic acid and the free enzyme (Testa and Mayer, 2003).

Trypsins has already found applications in medicine, food industry and in proteomics. In medicine, it is used in a pure or crystalline form for a treatment of wounds. Food industry uses trypsins for improvement of food quality and functional properties like solubility, emulsification, improvement of digestibility and reduction of allergens content in some foods.

Trypsins found also usage in the field of proteomics for fingerprinting (Yu and Ahmedna, 2012).

## Glucose oxidase

Glucose oxidase is a FAD-containing glycoprotein and was obtained in the process of purification of *Aspergillus niger*, what was reported for the first time by Müller in 1928. The enzyme is made up of two identical subunits with a molecular weight about 80000 Da each, which binds one molecule of FAD with a high binding constant (Gibson et al., 1964). The characteristics glucose oxidase is the catalysis of the oxidation of β-D-glucose using a molecular oxygen to glucono-δ-lactone which hydrolyzes spontaneously to gluconic acid (Whitaker, 1985). The figure 5 shows the principle of glucose oxidase activity test coupled with horseradish peroxidase. The latter catalyzes the oxidation of o-dianisidine which is used for spectroscopy detection. However, due to the light sensibility of the oxidized form of o-dianisidine, it is needed to stabilize it by adding sulfuric acid.

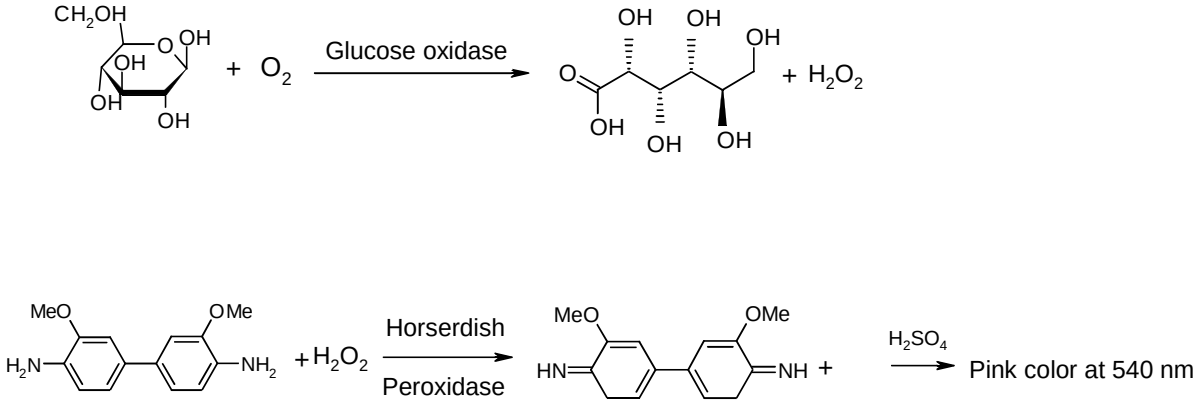



**Fig. 5.** Activity assay of glucose oxidase with coupled horseradish peroxidase for spectroscopy detection.

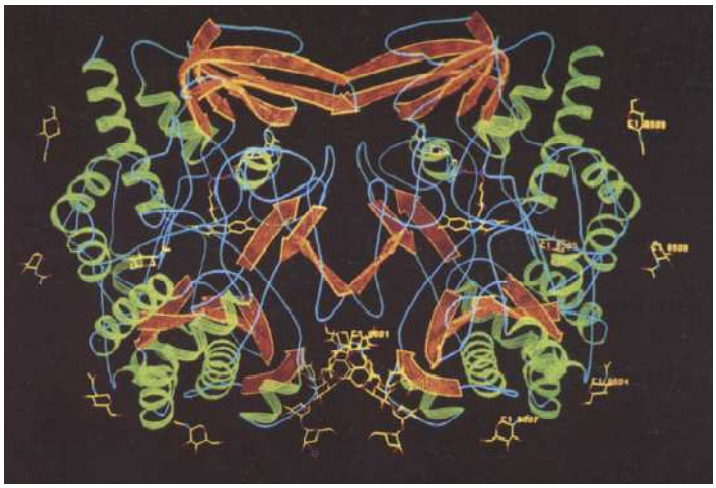


**Fig. 6.** The secondary structure of GOx from *A. niger* (Hecht et al., 1993).

The overall 3D topology depicted in Figure 6 presents the secondary structure of glucose oxidase from *Aspergillus niger*. The dimeric protein displays a rather ellipsoidal shape with high content of secondary structure with 28% of helices and 18% of β-sheets. The latter are located near the interface between monomers with the partly amphiphilic α-helices. At the bottom of a deep cavity, there is a flavin ring system is located with one part of the active site formed by a six-stranded β-helix supported by four α-helices. The bottom of the active site, there is partly formed by mostly buried Asp 548 (Hecht et al., 1993).

Because of its high specificity, glucose oxidase found applications in food industry for example in the glucose oxidase/catalase system for removal of glucose from egg-white before drying or for color stabilization.

In wine production, GOX showed its potential to lower the alcohol content. A various number of technologies was established for this purpose but the easiest way is to add glucose oxidase before the fermentation with *Saccharomyces cerevisiae* (Pickering et al., 1998).

The growing number of people suffering from diabetes caused the need for measurement of glucose in the blood at regular intervals and therefore, glucose oxidase immobilized on the electrodes found application in medicine.

Those are only some applications of this enzyme, however there seems to be plenty of potential possible applications which have to be studied or are being studied.

## 1.3. HYDROPHOBINS

Hydrophobins are a large family of low – molecular – mass proteins secreted at different life stages of filamentous fungi which contain a characteristic pattern of eight cystein residues responsible for creation of stabilizing disulfide bonds. They assemble themselves into amphipathic polymeric films at the interface between hydrophilic and hydrophobic surfaces, thus facilitating the creation of hyphae, spores and fruiting bodies (Kwan et al., 2007).

Until now, there have been two classes of hydrophobins identified based on their physical properties, such as hydrophobicity. Hydrophobins from both classes are typified by the presence of 8 cysteine residues at conserved places along the amino acid sequence. The cysteine residues are separated by spacing sequences which length is different in both classes. However, the number of amino acid residues between cystein residues is more variable among the pseudo-class I hydrophobins (Seidl-Seiboth et al., 2011).

Class I hydrophobins, produced by Ascomycetes und Basidiomycetes, create very robust films which are resistant to boiling in detergents and strong alkalis. Class II hydrophobins (only Ascomycetes) show ready solubility in water solutions (up to 100 mg/ml) (Cox et al., 2007).

Due to the discovery of pseudo class I hydrophobins in *Trichoderma atroviride* and *Trichoderma virens* reported by Seidl-Seiboth 2011, the concept of their classification, based on the amino acid sequence and their properties, is already questioned. Seidl-Seiboth showed that hydropathy plots and cysteine spacing are clearly different from well known class I and class II hydrophobins. Therefore, the expandation of the former classification into two groups is needed (Seidl-Seiboth et al., 2011).

The first hydrophobin genes were found in *Schizophyllum commune* but until now, they have been identified in many different Ascomycetes, such as *Trichoderma* species. Hydrophobins play several different roles in fungi, such as helping the fungi to survive in and adapt to the environment through controlling of the surface tension (Linder et al., 2005).

## Properties of hydrophobins

Hydrophobins owe their name to the high content of hydrophobic amino acids in their sequences. It has been estimated that about 10% of total proteins expressed by *Schizophyllum commune* at the time of emergent growth were hydrophobins (de Vries et al., 1993). To the remarkable properties of class I hydrophobins lie in their ability to assemble into an SDS – insoluble amphipathic membranes, when confronted with the interface between a hydrophilic and a hydrophobic phase. The class II hydrophobins exist as monomers at a lower concentration and an increase of the protein concentration leads to the formation of oligomers. Monomers of class II hydrophobins seem to have a higher affinity for surfaces to create monolayer films at hydrophobic/hydrophilic interfaces than for oligomer formation. In contrast to monolayers formed by Class I hydrophobins, which can be only be solved in 100% trifluoroacetic acid or formic acid (Wessels, 2000), monolayer formed from class II hydrophobins can be dissociated already with 60% ethanol or pressure (Zampieri et al., 2010).

The first well characterized *Trichoderma* spp. hydrophobin was class II HFBII which three dimensional structure was determinated at 1.0 Å resolution. The results showed that HFB II is a single domain protein consisting two β-hairpin motives, created via disulfide bonds between cysteine residues, linked by an α-helix. The first β-hairpin is created by a disulfide bond between C14 and C26 and the second β-hairpin is formed by connecting of two strands by cystein residues at positions 53 and 64. The bridge 3-52 connects the N-terminal loop to β-strand S3 and the bridge 13-43 connects β-strandS1 and the α-helix together. A hydrophobic surface patch is formed from two β-hairpins containing aliphatic hydrophobic amino acid residues. The first β-hairpin contains Val-18, Leu-19, Leu-21, Ile-22 and Val-24. Residues Val-54, Val-57, Ala-58, Ala-61 and Leu-63 are located in the second hairpin. The completed hydrophobic patch contains also Leu-7 located in the terminal N-loop (Hakanpää et al., 2004).

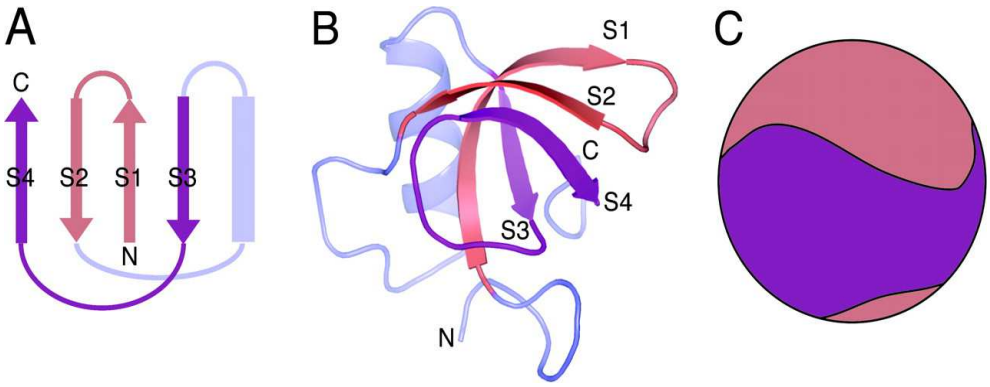



**Fig. 7. Topology and structure of the HFB2.A.** Topology. Arrows mark β-strands and the rectangle α-helix. B. Three-dimensional structure of HFB2. The first hairpin is in red and the second in purple. The central barrel consist of two β-hairpins that interlock in the same way as the leather pieces of the baseball (Hakanpää et al., 2004).

So far, beside of the mentioned above hydrophobin, HFBI, HFB3, HFB4, HFB5, HFB6 and HFB7 belonging to the class II have been identified. However, until now only HFBI, HFBII, HFB4 and HFB7 have been well characterized (Espino-Rammer et al., 2013; Neuhof et al., 2007; Hakanpää et al., 2006).

Since 2011, the group of pseudo-class I hydrophobins from *Trichoderma* genus, has been spread by the new members such as HFB9a and HFB9b from *T. virens* (Seidl-Seiboth et al., 2011; Neuhof et al., 2007).

In a fungal life, HFBs show an influence on the growth of hyphae and on the sporulation. The deletion of *hfb 1* gene from *T. reesei* strain QM9414 caused an easily wettable phenotype when compared to wild-type strain QM 9414 and there was no difference in sporulation for both strains on glucose (see Figure 8). Furthermore, on the third day of cultivation, the colony of Δ*hfb1* were more fluffier than colonies of QM 9414. However after 7 days, the differences were no more detectable. On the cellulose, differences in sporulation between strains were not observed. As well as for HFB I, the *hfb 2* gene was also deleted. The transformant strain was cultivated as in the first case. However, based on the observation, it is concluded that HFB II renders aerial hyphae and spores hydrophobic and is involved in aerial sporulation (Askolin et al., 2005).

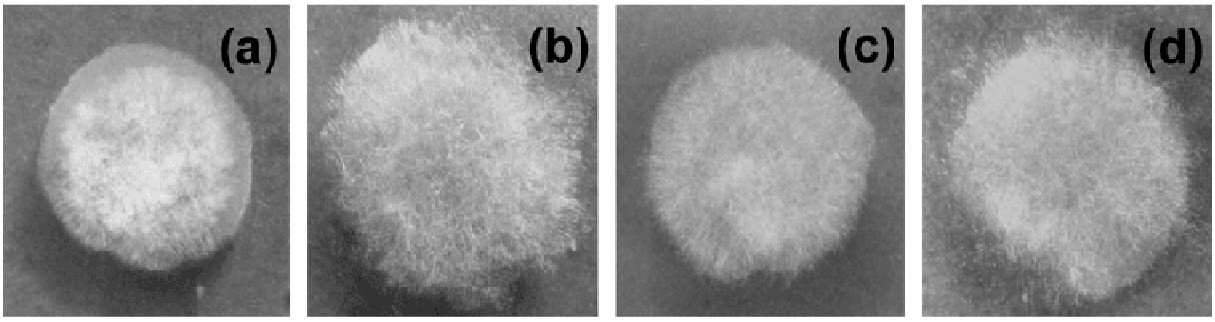



**Fig. 8.** Morphology of *T. reesei* parent strain (a), the Δ*hfb1*(b), the Δ*hfb2* (c) and Δ*hfb1*Δ*hfb2* (d) growing for 3 days on the glucose-containing medium (Askolin et al., 2005).

Another study showed that class II hydrophobins from *T. reesei* show differences in the degree of surface activity and the wettability of the hydrophilic side of a membrane, when compared to the class I hydrophobins SC3 from *S. commune*. Nevertheless, class II hydrophobins from *T. reesei* seem not to co-assemble with the class I hydrophobin SC3 but assemblies seem to interact with each other which results in a mixed membrane consisting of patches of assembled class I and class II hydrophobins. Moreover, the self –assembling of class II hydrophobins at the air–water interface does not influence the secondary structure and ultra structure, what can explain the more rapid reduction of surface tension caused by class II hydrophobins (Askolin et al., 2006).

This surface activity of HFB4 from *T. atroviride, T. virens and T. reesei* and the novel HFB7 from *T. virens* were tested by Espino-Rammer (see fig. 9). A strong binding of the first protein to the surface of polyethylene terephtalate (PET), a non-biodegradable polymer, lead to 30% loss of hydrophobicity which was reflected in a reduced water contact angle (WCA). HFB7 showed a different picture, whereas WCA is reduced for PET and the value on glass was not affected (Espino-Rammer et al., 2013).

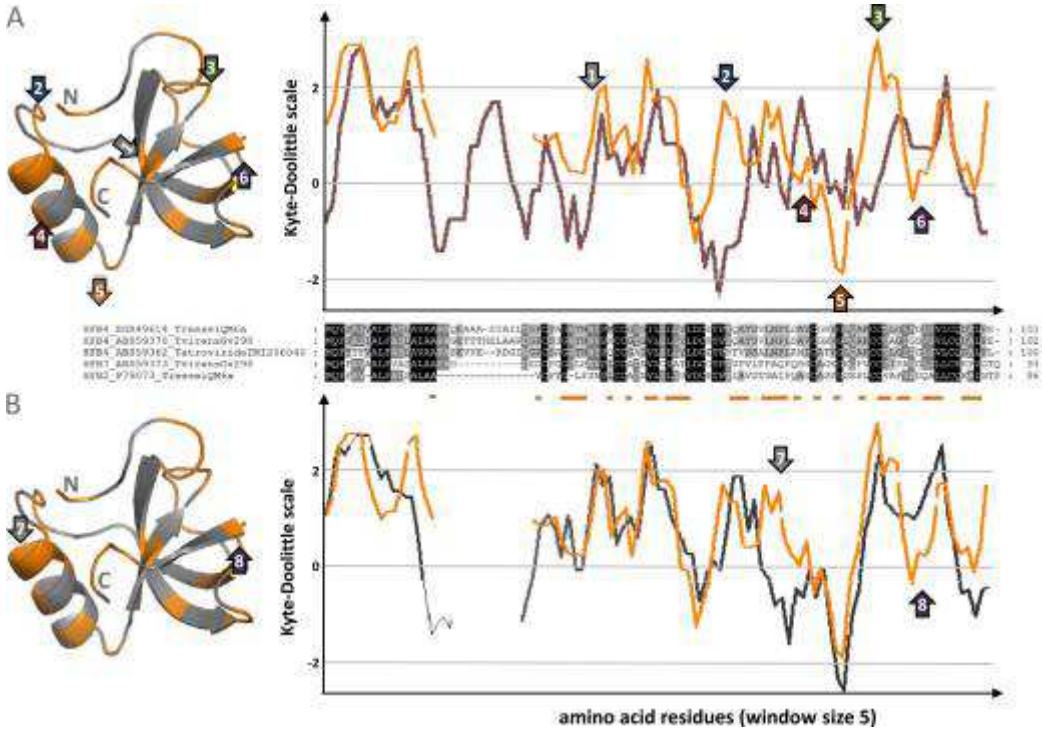



**Fig. 9.**Hydropathy profiles of HFB4 (A) and HFB7(B) compared to HFBII. Numbered arrows indicate increased or decreased hydropohobicity of HFB4 and HFB7 (Espino-Rammer., et al., 2013).

The behavior of *T. reesei* HFBI and HFBII in a bulk solution was studied by Szilvay et al. 2006 using Fluorescence Resonance Energy Transfer (FRET) imaging microscopy and size exclusion chromatography (SEC). Those studies have shown that the water surface tension can be reduced with increasing concentration of hydrophobins up to about 7 µM, while above no significant change in the water surface tension is observed, what indicates that in this point hydrophobins start to form multimers as shown at figure 10. The size of formed multimers could not be measured by FRET and therefore, SEC was used to obtain the size of hydrophobins complexes. Szilvay et al. showed that tetramers are spontaneously formed in solution but it is still unclear, how the tetramer formation in solution is connected to the tetramerized units in surface films (Szilvay et al., 2006).

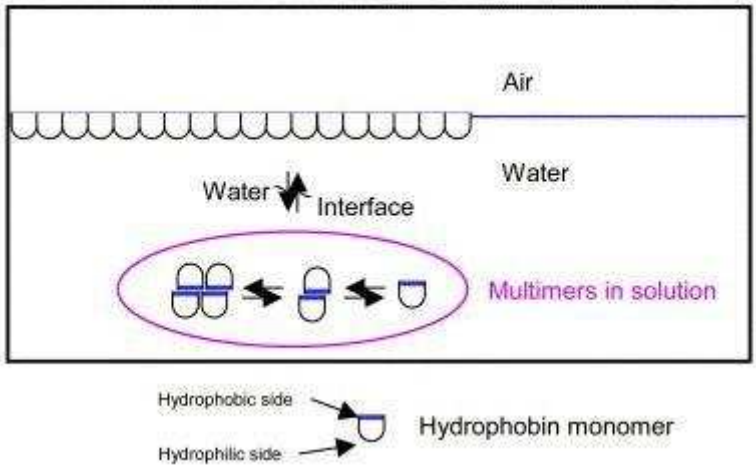



**Fig. 10.** Possible model for the formation of different complexes of hydrophobins in aqueous solution (Linder et al., 2005).

## *Trichoderma* hydrophobins

The ascomycetous genus *Trichoderma* contains many mycoparasitic species. Some of them e.g. *T. harzianum, T. virens, T. atroviride, T. asperellum* are widely used as biological active fungicides. Kubicek et al. reported that only one class I hydrophobin in the genus of *Trichoderma* in *T. asperellum*. is expressed and this protein is involved in root colonization (Kubicek et al., 2008). However, Seidl-Seiboth showed that there are also the presence of hydrophobins that are not classified into class II hydrophobins but they are more likely class I hydrophobins (Seidl-Seiboth et al., 2011).

The class II of hydrophobins genes of *Trichoderma* are highly duplicated and in two cases Kubicek et al. reported the presence of pseudogenes, what suggest that this class of hydrophobins which evolves by a death – and – birth mechanism (Kubicek et al., 2008). The phylogenic analysis of *Trichoderma* hydrophobins depicts that *Trichoderma* has many HFBs genes (see Fig.11) and most of them form 3 significantly supported clades, where the most probability was obtained for HFB4 clade (Kubicek et al., 2008).

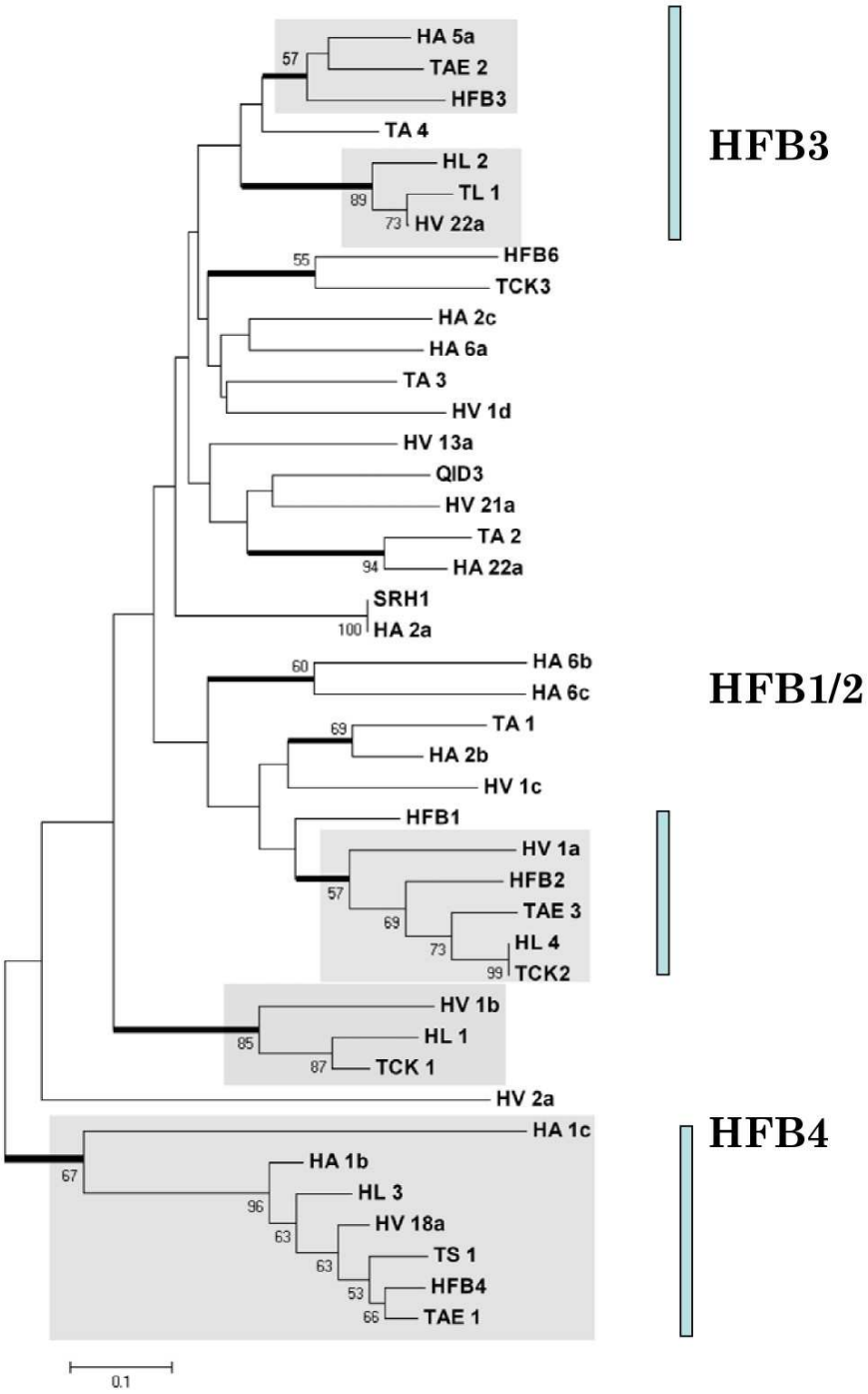



**Fig. 11.** Neighborhood joining analysis of 26 HFBs sequences from *Trichoderma* and other ascomycetes species. *Trichoderma* clades are highlighted in grey (Kubicek et al., 2008).

Askolin et al. reported for the first time a homologus production of HFB I in a large scale in 2001. High production of HFB I from *T. reesei* was obtained by a construction of overproducing *T. reesei* strain containing multiple copies of *hfb 1* gene in its genome. The overproducing strain was able to produce 0.60 g/l of HFB I of which 81 % was cell bound. In the comparison to the constructed strain, wild–type strain production level was much lower and came to 0.17 g/l (Askolin et al., 2001).

## Applications of hydrophobins

Hydrophobins can be used as coatings, emulsion stabilizers or can be applied in separation technology. Some of those proteins have an ability to disperse hydrophobic particles in water or stabilize oil – water emulsions. Despite the fact that studies on HFBI and HFB II from *Trichoderma* have shown their powerful reduction of surface tension, it does not indicate the ability to provide a long-term stability of a film or monolayer to the surface or interface. Owing to their properties, they can find an application as effective aerating agents or as linkers for enzyme immobilization (Cox et al., 2007).

Due to their versatile properties they can be applied in drug delivery as a stabilizer of oil vesicles or as intermediates for attaching molecules to a surface in the immobilization of antibodies or enzymes in a biosensor (Scholtmeijer et al., 2001).

Based on their ability to coat surfaces and the fact that hydrophobins do not seem to be toxic, cytotoxic or immunogenic, they can find many applications also in biomedical engineering (Scholtmeijer et al., 2001).

Bilewicz et al. showed that hydrophobin HYDPt-1 from *Pisolithus tinctorius*, which was used to modify of hydrophobic surfaces of a glassy carbon electrode, a thin mercury film electrode or hydrophilic surfaces such as a gold electrode before they were functionalized with ubiquinone, quinine and azobenzene, can stable attach the electroactive reagents to electrodes (Bilewicz et al., 2001).

The ability of hydrophobin SC3 from *S. commune* to bind GOx and HRP to glossy carbon electrode was tested by Corvis et al. Transmission electron microscopy showed that enzyme film on the adsorbed SC3 was stable and its stability was higher, when compared to

the enzymes adsorbed directly on the electrode, however the specific activity of both enzymes was lower in comparison to dissolved enzymes (Corvis et al., 2005).

Hou S. et al showed that HFBI/collagen modified poly(dimethylsiloxane) (PMDS) surface could support the adhesion and the growth of cells, whereas the native PMDS surface could not (Hou et al., 2008). Similar results, but obtained with neural stem cells were described by Li et. al, whereas poly(lactic-co-glycolic acid) film was modified only by HFBI (Li et al., 2009).

# 2. AIM OF THE THESIS

The aim of the thesis was the investigation of the influence of two hydrophobins from *T. virens* on the activity of industrial important enzymes in solution and the ability of their usage as linkers for enzyme immobilization.

In order to realize this aim the following points were established:

- The proof of concept was conducted using the fusion protein HFB4 linked with glutathione-S-transferase expressed in a bacterial system that was tested with cellulases with soluble and insoluble substrates, glucose oxidase and trypsin in solution.
- Use of other hydrophobins from *T. virens* expressed in *P. pastoris* expression system for the same enzymes.
- Carrying out the enzyme immobilization with hydrophobins as linkers to various polymeric matrices including borosilicate, PET, biaxially-oriented polyethylene terephtalate and polyimide.

We were interested to test if *T. virens* hydrophobins can improve the enzyme activity or increase the binding efficiency of the enzymes activity on the different carriers and their activity varies when produced in different hosts.

# 3. MATERIALS AND METHODS

The *Trichoderma* hydrophobins used for the experiments presented in this work, were heterologously overexpressed as fusion proteins in *E. coli* and *P. pastoris.*

## 3.1. HYDROPHOBINS

The HFB4 N-terminally linked to Glutathione-S-*transfera*se was overexpressed in *E. coli* BL21 DE3 transformed with plasmid pGEX-4T-2 provided by GE Healthcare (Amersham, England).

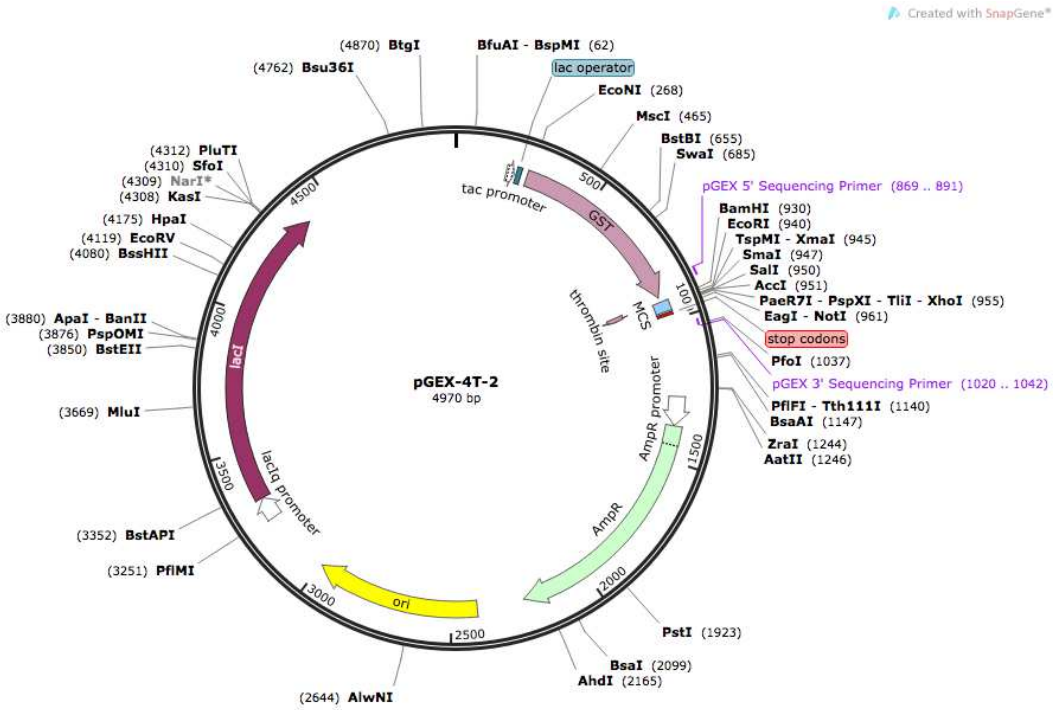



**Fig.11.** The features of pGEX-4T-2 vector used for the transformation.

The pGEX-4T-2 vector is used for the bacterial expression and contains a chemically inducible tac promoter activated by IPTG. It contains also the fusion partner for the gene of interest, what leads to the facilitation of the purification process. The protein of interest can be cleaved from the fusion partner using thrombin.

For the preparation of GST-HFB fusion protein, the pGEX-4T-2 vector was linearizated using restriction enzymes Bam HI (Fermentas) and Xho I (Fermentas), which

generate sticky ends. For this purpose, the mixture containing 8 µl pGEX-4T-2, 1µl enzyme Bam HI, 1 µl enzyme Xho I, 5µl Buffer G and 35 µl water was prepared. The mixture was incubated overnight at 37ºC. Afterwards the agarose gel electrophoresis was carried out to verify the completion of the digestion and the band containing the linearized vector was purified using QIAquick Gel Extraction Kit following the manufacturer's protocol. In the next step, the gene of interest was ligated with the linearized vector by mixing of 50-200 ng of the vector with 1-2 µl of purified cDNA and the volume of mixture was filled up to 10 µl with deionized water. The mixture was incubated for 15 minutes at 50ºC, and afterwards was put on ice. (Przylucka, A., 2012)

The transformation of *Escherichia coli* competent cells was done by adding 5 µl of ligation product to 100 µl of *E. coli* competent cells. The mix was gently mixed with a pipette tip. The tubes containing a mix were incubated on ice for 30 minutes and then placed into thermocycler for 90 seconds at 42ºC. Afterwards, the tubes were put on ice for 2 minutes. In the next step, 400 µl of LB medium was added and the mixture was shaken at 37ºC for 1 hour. After this time, 50, 100 an 200 µl of the mixture were transferred onto LB plates containing ampicillin. The plates were incubated overnight at 37ºC. From each transformation were picked approximately 10 colonies. (Przylucka, A., 2012)

Overexpression of the protein of interest was carried out following a protocol and purified on Gluthatione Sepharose 4B provided by GE Healthcare as decribed in 3.1.2. The protein of interest was not cleaved from Glutathione tag. The protein concentration after purification was measured via Bradford method (3.5) and the purity was checked via SDS – PAGE (3.3).

The HFB4and HFB7linked c-terminally to a 6xHis tag overexpressed in *P. pastoris* were received from Agnieszka Przylucka. (Przylucka A. 2016)

### 3.1.1. OVEREXPRESSION OF GLUTHATHIONE-S-TRANSFERASE FUSION PROTEIN

Transformed *E. coli* with pGEX – 4T – 2 Vector containing *hfb4* gene linked N-terminally to Glutathione-S-transferase was plated on LB amp plates (section 8.) and grown overnight at 37 ºC.

Single, well isolated colonies were picked from the plate and used to inoculate 50 ml LB amp medium (section 8.) in 0.5 L Erlenmeyer flasks. All flasks containing either GST-HFB4 or GST only were incubated overnight for 12 – 15 hours at 37 ºC, 170 rpm. After 12 – 15 hours, the overnight preculture was added to 250 ml of fresh LB amp in 1 L Erlenmeyer flask to give an $A_{600}$= 0.2 – 0.3. After inoculation, all flasks were grown at 37 ºC, 170 rpm to an $A_{600}$= 0.6 – 0.7. Then the expression of the fusion protein was induced by the addition of 250 µl of IPTG stock solution (section 8.) to the final concentration of 1 mM and cells were grown for 6 hours, shaking 170 rpm at 37 ºC.

After the protein of interest was expressed, the liquid cultures were transferred into centrifuge bottles and then centrifuged at 4 ºC, 4000 rpm for 15 minutes. The supernatant was discarded. Pellets were frozen and stored for next steps at -20 ºC.

The frozen pellets were thawed on ice. Then they were resuspended in10 ml of ice – cold PBS – Triton X-100 (section 8.) in 50 ml Falcon tubes. Cell suspension was incubated on ice for 30 minutes with occasionally vortexing.

The cell suspension was sonificated 10 times for 20 seconds with 1 minute interval. During the sonification cells were kept on ice. Cell debris was centrifugated for 10000 rpm at 4 ºC for 15 minutes. The supernatant was recovered in a 50 ml tube.

### 3.1.2. PURIFICATION OF GLUTHATHIONE-S-TRANSFERASE FUSION PROTEIN

#### Preparation of Gluthathione Sepharose 4b

The bottle containing Gluthathione Sepharose 4b was gently shaken to resuspend the slurry. Using $dH_2O$, 8 ml 50% slurry was prepared.

#### Column preparation

As columns were used 10 ml plastic syringes, which were placed upright in an appropriate rack. Glass wool was placed in the bottom of each syringe. The glass wool was wet with a little amount of PBS (section 8.). 4 ml of 50% slurry was transferred to the column. All air bubbles in the chromatography medium bed were removed using needles. The bed was settled for at least 20 minutes. After settling of bed medium, the column was drained slowly.

## Equilibration of the column

The Gluthathione Sepharose 4B was washed with 10 ml of PBS-Triton X-100 (section 8.). Then 10 ml of PBS was added to the column which was then incubated for 15 minutes and drained with a flow rate of 0.5ml/min. This step was repeated at least three times.

## Binding of the fusion protein

After equilibration of the column, filtered bacterial cell free extract was applied to the medium in the equilibrated Gluthathione Sepharose 4B column. The filtrate was kept for 15 minutes at the column and then drained. After draining of the first volume of the filtrate, the remaining sample was added and let to flow through. The column was subsequently washed 5 times with 10 ml of PBS Triton X – 100 (section 8.).

## Elution of the fusion protein

2 ml of elution buffer(section 8.) was added to the column. The elution buffer was incubated on the column at room temperature for 30 minutes, then the GST – tagged protein was eluted. The protein sample was stored at -20 ºC.

### 3.1.3. SODIUM DODECYL SULFATE POLYACRYLAMIDE ELEKTROPHORESIS

## Gel preparation

The 15% (v/v) monomer solution was prepared by mixing dd$H_2O$ and 30% (v/v) acrylamide/bisacrylamide solution (1,125 ml for stacking gel and 7,5 ml for separating gel), gel buffer ( 3,75 ml 1.5M TRIS-HCl pH 8.8 for separating gel and 1,875 ml 0.5 M TRIS-HCl pH6.8 for stacking gel). The mixture was degassed for 15 minutes. To the monomer solution, 10% (w/v) APS (50 µl for stacking gel and 150 ml for separating gel) and (15 µl for stacking gel and 9 µl for separating gel) TEMED were added immediately prior to pouring the gel.

## Sample preparation

A 10 µl protein sample was diluted by an addition of 9,5µl of 2x SDS – PAGE sample buffer. As a reducing agent, 0,5 µl of β-mercaptoethanol was added. Afterwards, the diluted sample was heated at 90 – 95ºC for 5 minutes in a Thermomixer and centrifuged at 14000 rpm for 1 minute.

## Electrophoretic run

After pipetting, the adjusted volume of the sample into the gel (20µl), the electrophoresis was run under denaturating conditions and constant voltage of 200V as long as the blue dye front reached the bottom.

## Visualisation of SDS PAGEreults

The gel was removed from plates and placed in the colloidal Coomassie G250 staining solution (section 8.) The staining was performed overnight, and after this step the gel was washed several times with $dH_2O$ until the background was transparent.

### 3.1.4. SPECIFIC DETECTION OF GLUTATHIONE-S-TRANSFERASE FUSION PROTEIN VIA WESTERN BLOT

The specific detection of Gluthathione-S-transferase was done by Western Blotting. For this purpose, we separated proteins via SDS-PAGE as described in 3.1.3. The separated proteins were transferred overnight from the gel by electroblotting using a constant current of 10 mA onto a nitrocellulose membrane which was then blocked using 20 ml of 3% bovine serum albumin in 1x PBS buffer to prevent the interactions between the membrane and antibodies. After this step, the membrane with transferred proteins was incubated with 15 ml of the diluted 1:5000 primary antibodies Anti GST under gentle agitation for 1 hour. Then the membrane was rinsed three times for 5minutes with 1x PBS buffer to remove unbound primary antibody, and then we incubated the membrane with 15 ml of the secondary antibody IgG against Anti GST antibodies diluted1:10000 which is linked to a alkaline phosphatase. This step was also performed for 1 hour. The membrane was washed three times using 20 ml of solution containing 1x PBS and 0.1% Tween (v/v) after the incubation with the secondary antibody and the enzymatic reaction using Biorad alkaline phosphatase kit (containing 25x AP color development buffer, AP color reagent A, AP color reagent B) (section 8.) was done to visualize the overexpressed fusion protein. For this purpose, 12,5 ml of 1x AP were prepared from 25x color development buffer (section 8.). Then immediately before the use, 125 µl of AP color reagent A (section 8.) and 125 µl of AP color reagent B (section 8.) were added to the diluted color development buffer. Then the nitrocellulose membrane was incubated at RT with gentle agitation until color development was completed. The color

development was stopped via washing the membrane with dd$H_2O$ for 10 minutes with gentle agitation. The membrane was dried.

### 3.1.5. PROTEIN CONCENTRATION DETERMINATION VIA BRADFORD ASSAY

In order to determine the protein concentration, the dye binding assay (Bradford, 1976) was used. For this purpose, firstly the dye reagent was diluted by mixing 1:4 with d$H_2O$, then 20 µl of the protein sample was diluted with 1 ml dye reagent containing methanol and phosphoric acid (Biorad). The mixture was incubated with 1x dye for 5 minutes at room temperature to allow the dye to bind to basic and aromatic amino acid protein residues. At the same time, different concentrations of bovine serum albumin (BSA) were prepared in the same way as the samples to generate a standard curve. The mixture of water with the dye was used as the blank. The absorbance was measured in a Microreader at 595 nm.

## 3.2. CELLULASES ACTIVITY ASSAYS

To test the influence of *Trichoderma* hydrophobins on the activity of the three industrial relevant enzymes, we investigated activity tests in the presence of the protein. The cellulase activity assay was performed using insoluble and soluble substrates. As insoluble substrate we used cellulose filter paper from Whatman (1 cm x 0.5 cm). 4-nitrophenyl-β-D-lacto-pyranoside and 4-nitrophenyl-β-D-cellobioside were used as soluble substrates for cellulases.

### 3.2.1. CELLULASE ACTIVITY ASSAY WITH PNPL AS SOLUBLE SUBSTRATE

For the assay for free enzyme incubated with GST-HFB4 we used 20 µl of enzyme diluted 1:200 in 50 mM citrate buffer pH 4.8 40 µl of 3mM PNPL in the same buffer. HFB4 in 50 mM phosphate buffer pH 7.6 (to the end concentration of HFB4 in the reaction of: 5, 10, 20, 35 and 50 µg/ml) was added and the reaction mixture was filled up with 50 mM citrate buffer pH 4.8 to a total volume of 250 µl. The reaction was performed at 50°C for 30 minutes and stopped with 250 µl of 1M $Na_2CO_3$. The absorbance of released p-nitrophenol was measured in Microreader against the reaction mixture without enzyme at 410nm.

### 3.2.2. CELLULASE ACTIVITY ASSAY WITH NON-SOLUBLE SUBSTRATE

Filter papers were cut into small pieces (1 cm x 0.5 cm) and then incubated with 160 µl of different concentrations of GST-HFB4 (5, 10, 20, 35 and 50 µg/ml) for 16 hours. After incubation the activity assay was carried out as described below.

Filter papers preincubated with different GST-HFB4 or GST concentrations were put to the deep well plate. Then 88,3µl of 50 mM citrate buffer pH 4.8 and 41,7 µl of enzyme in 2 different concentrations (13,5 µg/ml and 5,4 µg/ml) were added. The reaction was performed for 30 minutes at 50°C. All was done in triplicates. Afterwards the reaction was stopped by using 250 µl of 40 mM DNS solution and heating at 100ºC for 5 minutes. Then the deep well plate was put on ice to cool down. The absorbance was measured at 540 nm in Microreader. For the measurement each sample was diluted in proportion 1:16 with $dH_2O$.

### 3.2.3. IMMOBILIZED CELLULASES ACTIVITY ASSAYS

PRETREATMENT OF POROUS GLASS BEADS

Glass beads were put into a 250 ml beaker and covered with 25 ml of 10 M $HNO_3$. The glass beads were put under the hood for 24 hours and from time to time mixed using a glass rod. After incubation, glass beads were decanted and nitric acid was poured out. Afterwards glass beads were washed with 100 ml of $dH_2O$ three times and put into a graduated cylinder and washed with 100 ml of $ddH_2O$ until pH value about 5.4 was reached.

IMMOBILIZATION OF ENZYMES ONTO NON-ACTIVATED GLASS BEADS

100 mg of pretreated porous glass beads were weighted on the analytical balance and put into an eppi. Glass beads were incubated with 750 µl of 0.02 mg/ml or 0.2 mg/ml GST or GST-HFB4 solution in 100 mM TRIS – HCl pH 7.6 for 18 hours. Afterwards the supernatant was removed and the glass beads were washed three times with $dH_2O$. Then glass beads were incubated with 1 ml of enzyme solution (0.5 mg/ml) for 18 hours with shaking. After incubation with the enzyme the glass beads were washed with $dH_2O$ and centrifuged at 18000 rpm as long as the absorbance was near 0.02 at 280 nm.

In order to test if hydrophobins can bind enzymes more efficiently onto a matrix of non-activated glass beads the enzyme solution was added . The reaction was performed in 800µl of 50 mM citrate buffer pH 4.8 and 200 µl of 3 mM PNPL dissolved in the same buffer were added. The reaction was stopped after 30 minutes using 1 ml of 1 M $Na_2CO_3$.The absorbance of released p-nitrophenol was measured at 405nm.

### 3.2.4. CELLULASES ACTIVITY ON DIFFERENT POLYMERS AS SUPPORTS

Borosilicate, PET, biaxially-oriented polyethylene terephtalate and polyimide (0,57 $cm^2$) were prepared in triplicates and coated with 0.1M $KH_2PO_4$pH 6.6 buffer containing 5 µM of HFBs overnight at 50 ºC. All membranes were washed three times with the same buffer using gentle shaking. 1 ml of Tex Cellulases were incubated for 1 hour at 1000 rpm. Afterwards, membranes were washed again, since the supernatant shows no enzymatic activity. Cellulases were tested using 200µl of 2 mM 4-nitrophenyl β-D-cellobioside dissolved in 50 mM citrate buffer pH 4.8 at 45ºC for 30 minutes. The reaction was stopped using 200 µl of 100 mM $Na_2CO_3$. The absorbance was measured at 410 nm.

## 3.3. GLUCOSE OXIDASE ACTIVITY ASSAYS

The free GOx Assay was performed according to the manufacturer's protocol, however the used volume of each component was scaled down to the end volume of 250 µl.

### 3.3.1. FREE ENZYME ACTIVITY TEST

The glucose oxidase assay was performed in discountinous system. For the reaction we used 178.4 µl of reaction cocktail containing 0.17 mM o – DND, 1.72% (w/v) of glucose and 50 mM sodium acetate buffer pH 5.1 and 6 µl of 60 Purpurogallin U/ml of POD and 6µl of 0.8 U/ml GOx. In the assay for glucose oxidase activity in the presence of GST-HFB4 we used 0, 5, 10, 15, 35 and 50 µg/ml concentrations of fusion protein to test the differences. Additionally, we stopped the reaction after 30 minutes using 250 µl of 6 M sulfuric acid, what stabilized the product of the reaction. The reaction was performed at 37 °C in the darkness due to the sensibility of the substrates and products of the complex reaction. The absorbance of oxidized and stabilized by sulfuric acid o- dianisidine was measured in Microreader at 540 nm.

### 3.3.2. IMMOBILIZED GOX ACTIVITY ASSAY

For the immobilized GOx Activity Assay we prepared glass beads and immobilized enzyme as described in 3.2..For the assay we filled up the eppi with 50 mM sodium acetate buffer pH 5.1 and added the reaction cocktail and performed as described in 3.2.2.1. After 30 minutes the reaction was stopped with 6 M sulfuric acid and the absorbance was measured as described above.

### 3.3.3. GOX ACTIVITY ON DIFFERENT MEMBRANES AS CARRIERS

The membranes were prepared as described in 3.2. The membranes were incubated for 20 minutes at 35ºC with the substrate solution pH 7 containing p-hydroxybenzoic acid, peroxidase and D-glucose. The formation of the reaction product quinoneimine was measured spectrophotometrically at 510 nm.

## 3.4. TRYPSIN ACTIVITY ASSAYS

### 3.4.1. FREE ENZYME ACTIVITY ASSAY

The enzyme was dissolved in 1 mM HCl and stored at -20°C. Then, the enzyme solution containing 500 U/ ml was prepared in cold 1 mM HCl. 30 µl of prepared enzyme solution was preincubated with different concentrations (0, 5, 10, 20, 35, 50 µg/ml – end concentrations) of GST-HFB4 or GST for 30 minutes, afterwards 60 µl of cold 1 mM HCl was added, filled up with the 67mM sodium phosphate buffer pH 7.6 buffer up to 300 µl. Finally 900 µl of 0.31 mM BAEE was added. The absorbance of free $N_{\alpha}$- Benzoyl – L-arginine was measured every 30 seconds at 253 nm.

### 3.4.2. TRYPSIN ACTIVITY ASSAY ON POROUS GLASS BEADS

To the test of the immobilized trypsin, we used 100 mg of glass bead with immobilized enzyme as described in 3.2.4. We filled up an eppi with 28,33 µl of 1mM HCl and 850 µl of 0.31 mM BAEE. The reaction was performed for 10 minutes in an eppi and after 10 minutes, the solution was transferred into a cuvette. The changes of absorbance were monitored at 253 nm every 1 minute.

### 3.4.3. Trypsin Activity Assay On Different Supports

All support membranes were prepared as described in 3.6.2.3 and immobilized trypsin was tested using 200 µl of azocasein (4% (w/v)). The reaction was performed at 40 ºC for 20 minutes and then stopped using 1,2 ml of 5% TCA. The mixture was centrifugated for 5 min at 3000 rpm. The determination of released azo-dye was measured at 440 nm.

# 4. RESULTS

## 4.1. HYDROPHOBINS AND THEIR SUCCESSFUL OVEREXPRESSION

### NON-SPECIFIC DETECTION OF HYDROPHOBINS

Prior to the use of the HFBs for the intended experiments, their successful overexpression and purification was confirmed by SDS-PAGE (Fig. 12 and Fig. 13).

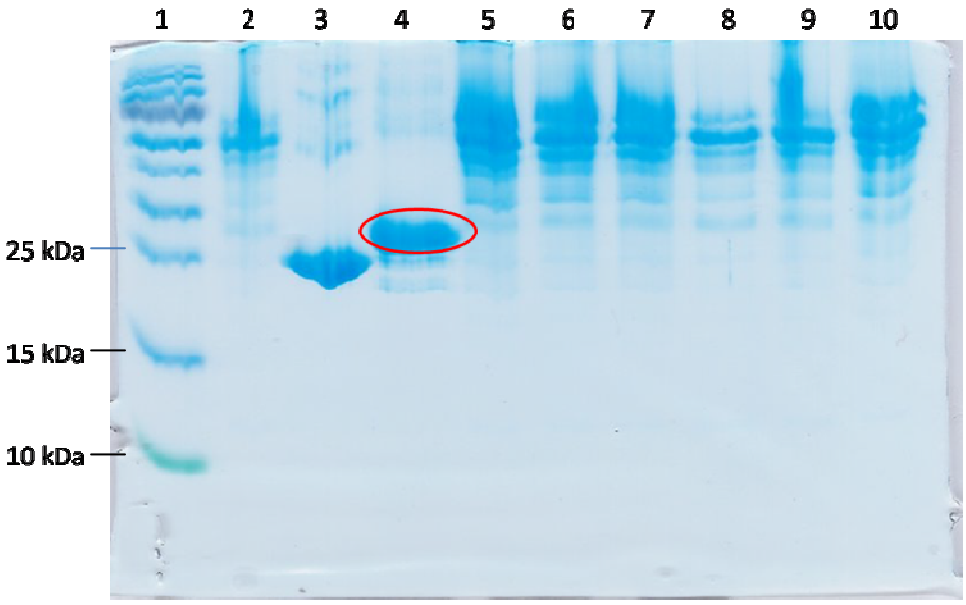



**Fig. 12.** Gel after SDS-PAGE separation for Gluthathione-S-transferase. 1- Protein Ladder, 2- bacterial crude (0.013 mg) , 3- flow through (0.06 mg) , 4- purified GST (0.038 mg) , 5 - 10- washing repetition steps (0.065 mg, 0.052 mg, 0.04mg, 0.023 mg, 0.26 mg and 0.31 mg respectively.

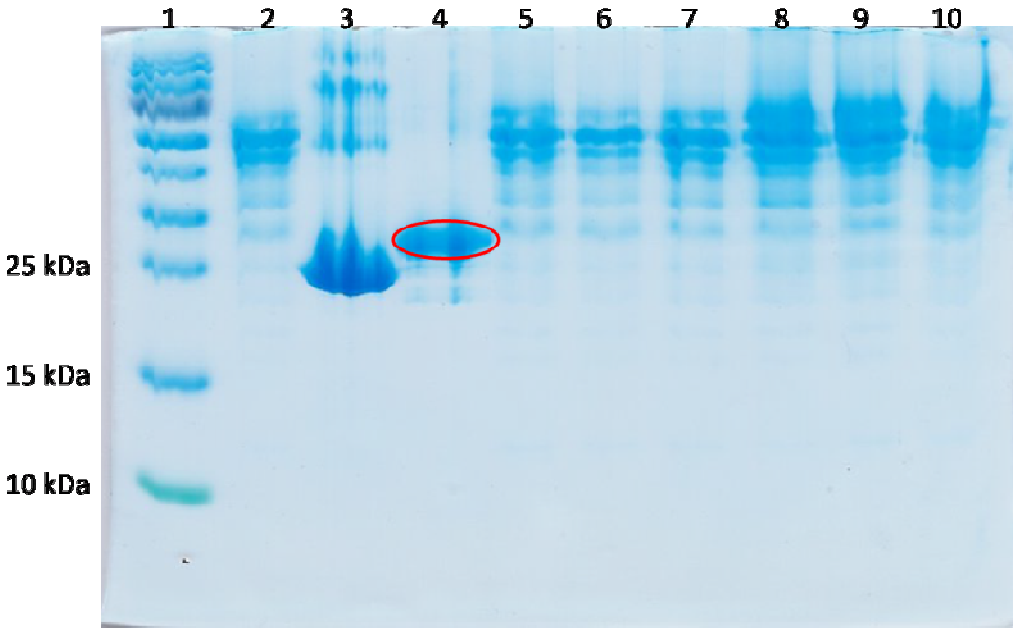



**Fig. 13.** Stained Gel after SDS PAGE separation for HFB4-Glutathione-S-transferase.1- Protein Ladder, 2- bacterial crude (0.008 mg), 3- flow through (0.08 mg), 4- purified HFB4-Gluthathione-S-transferase fusion protein (0.034 mg) , 5- 10- washing repetition steps (0.01 mg, 0.006 mg, 0.009 mg, 0.018 mg, 0.015 mg and 0.011 mg respectively).

Although, the SDS PAGE results showed that the overexpression and purification step were successful, there are still some degradation products visible.

SPECIFIC PROTEIN DETECTION BY WESTERN BLOT VISUALISATION

To prove specific that the band seen in SDS-PAGE was indeed the overexpressed GST-HFB4, Western Blotting was performed. The results shown in Fig. 13 and 14. present the obtained gel visualization.

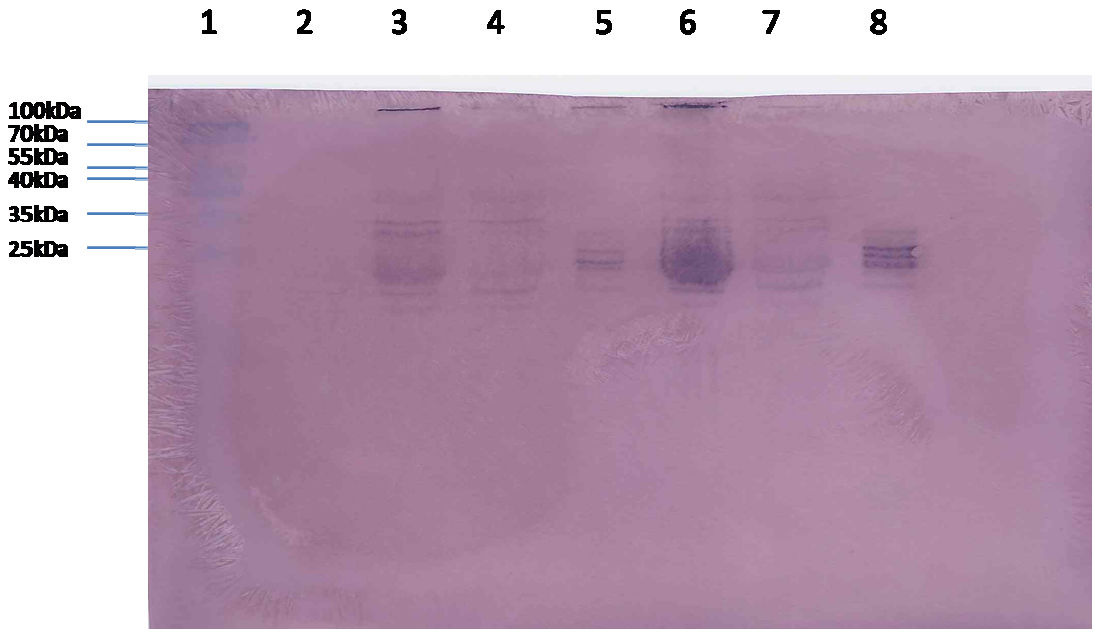



**Fig. 13.** Western bloting for overexpressed Gluthathione-S-transferase. . 1- Protein Ladder, 2- crude extract (0.013), 3- collected supernatant (0.09 mg), 4- flow through (0.06), 5- purified HFB4-Gluthathione-S-transferase (0.05 mg), 6-9- washing steps (0.1 mg, 0.62 mg, 0.08 mg, 0.009 mg respectively).

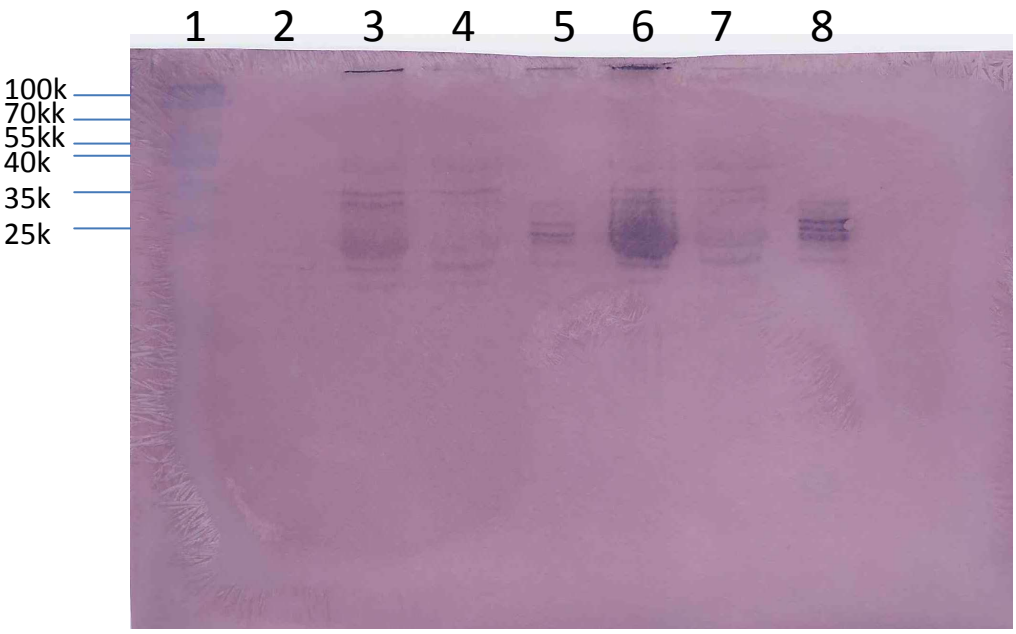


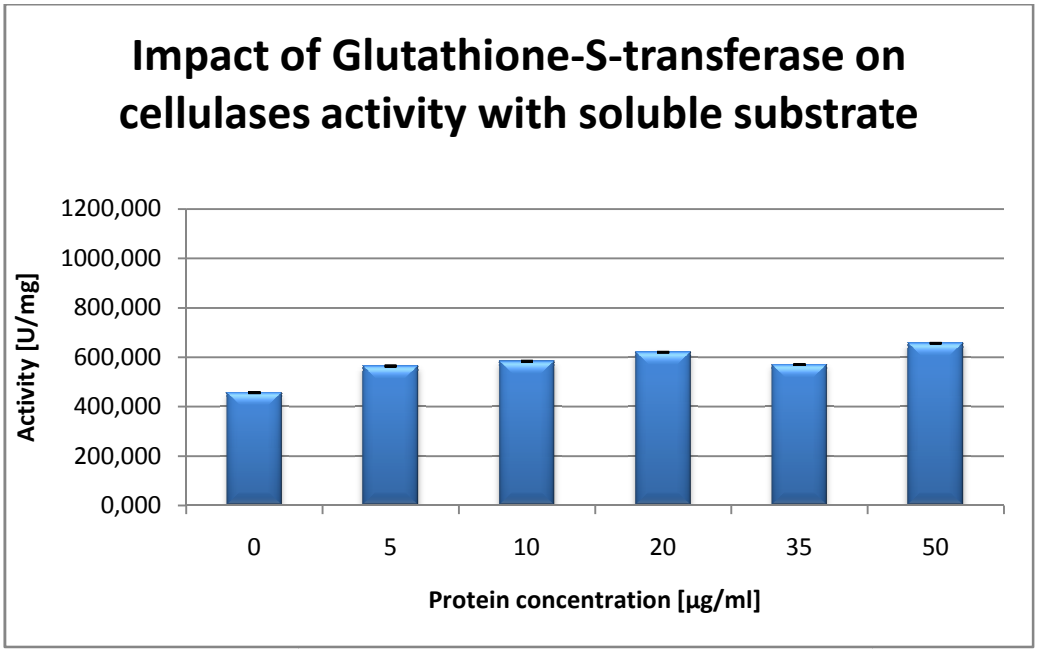

**Fig. 14.** Results from Western blotting for overexpressed HFB4-Gluthathione-S-transferase fusion protein. 1- Protein Ladder, 2- crude extract (0.013 mg), 3- collected supernatant (0.07 mg) 4- flow through (0.055), 5- purified HFB4-Gluthathione-S-transferase fusion protein (0.005 mg), 6-8- washing steps (0.081 mg, 0.049 mg, 0.009 mg respectively).

The specific detection of heterological overexpressed fusion protein proves the presence of this protein.

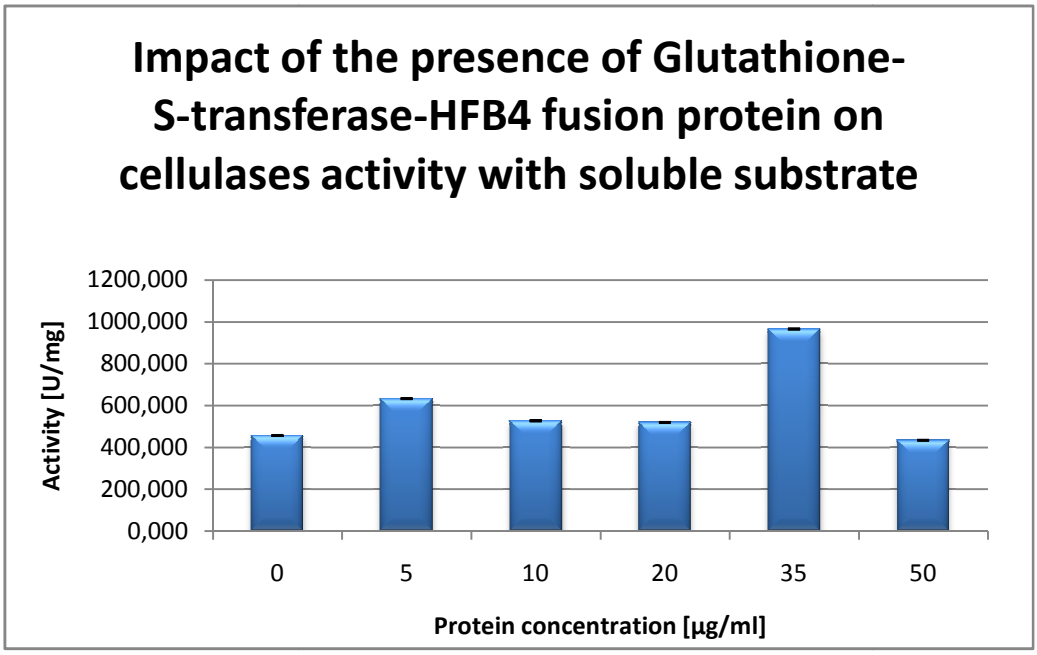
## 4.2. IMPACT OF HYDROPHOBINS ON CELLULASES

### 4.2.1. CELLULASES ACTIVITY WITH PNPL IN THE PRESENCE OF GLUTATHIONE-S-TRANSFERASE OR FUSION PROTEIN

The plots 1 and 2 show the results of the activity test for cellulases with 3mM PNPL in the presence of GST-HFB4 and GST. For the testing of a hypothesis, that hydrophobin can enhance the enzyme activity, we investigated the cellulases activity tests without any other protein, with GST as a control and a fusion protein.




**Plot 1**. Impact of the presence of the Glutathione - S- transferase on the cellulases activity

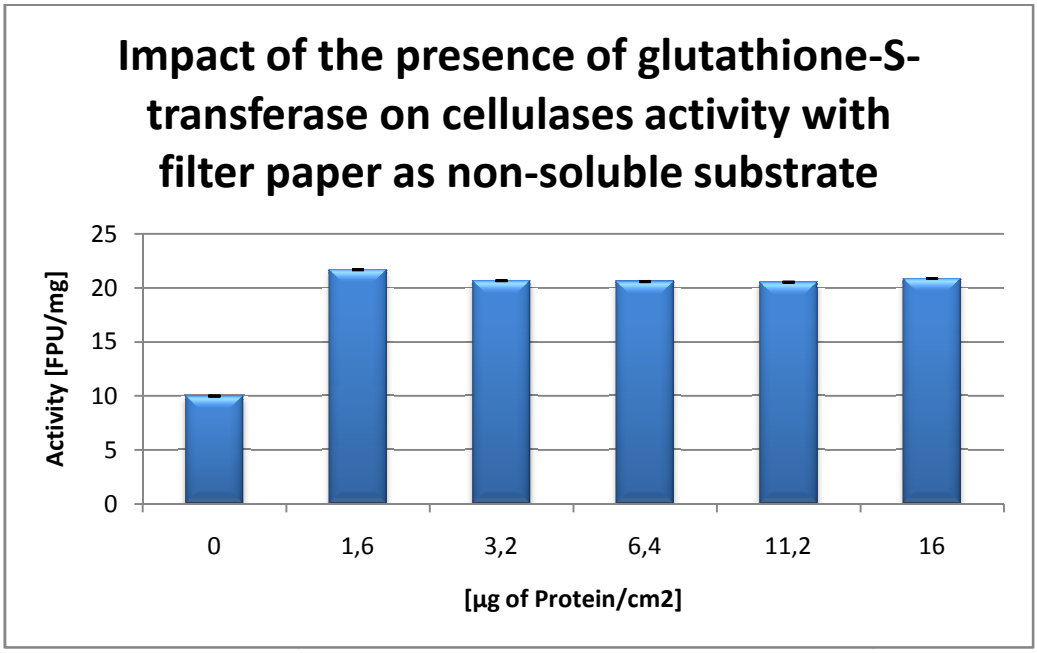



**Plot 2.** Influence on the Cellulase activity by HFB4 - Glutathione-S-transferase.

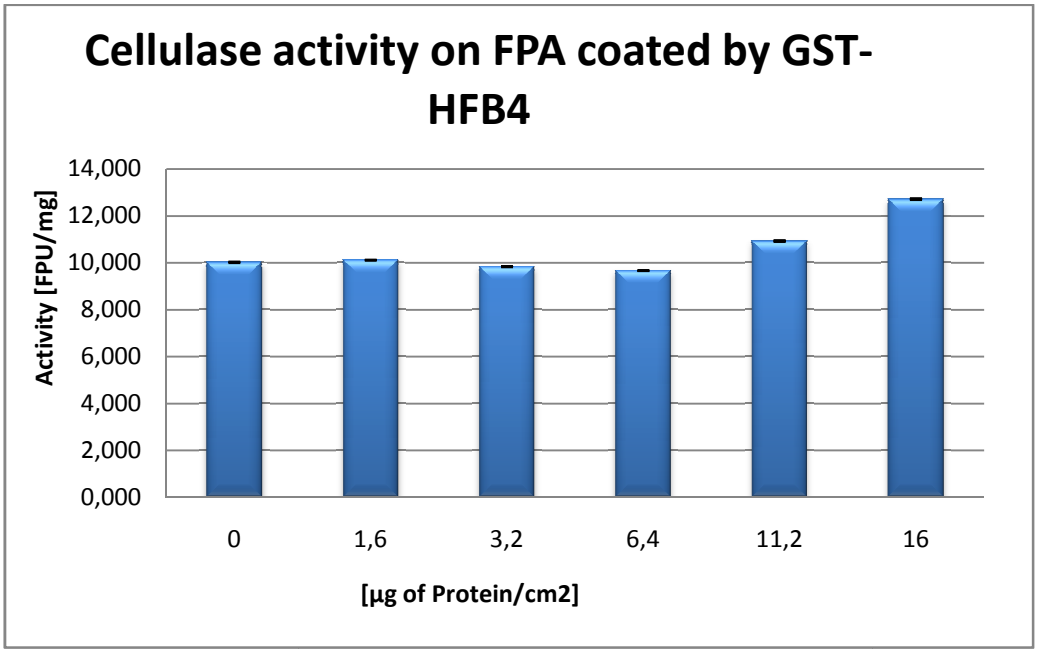
In case of GST-HFB4, we observed a little increase of the activity at the lowest concentration of proteins, however then the decrease of the activity occurred at higher protein concentrations. Interestingly, for GST, we observed a little increase and the highest activity was observed at the highest concentration of GST, whereas at this point we obtained the lowest activity for GST-HFB4.

### 4.2.2. CELLULASES ACTIVITY WITH NON-SOLUBLE SUBSTRATE COATED BY PROTEINS

Because of intra-molecular and intra-strand hydrogen bond formed between hydroxyl groups and oxygen in a D-glucopyranose units, cellulose shows a hydrophobic surface. However, enzymes may need a hydrophilic surface for the surface binding. Therefore, we tested the hypothesis, if due to an amphiphilic character of the hydrophobins, they can also enhance the efficiency of cellulose degradation, by creating of a hydrophobic surface by the coating, enabling a better binding of an enzyme to the cellulose, on the example of a filter paper as the solid surface.




**Plot 3.** Impact of the filter paper coating with Glutathione-S-transferase on the cellulases activity

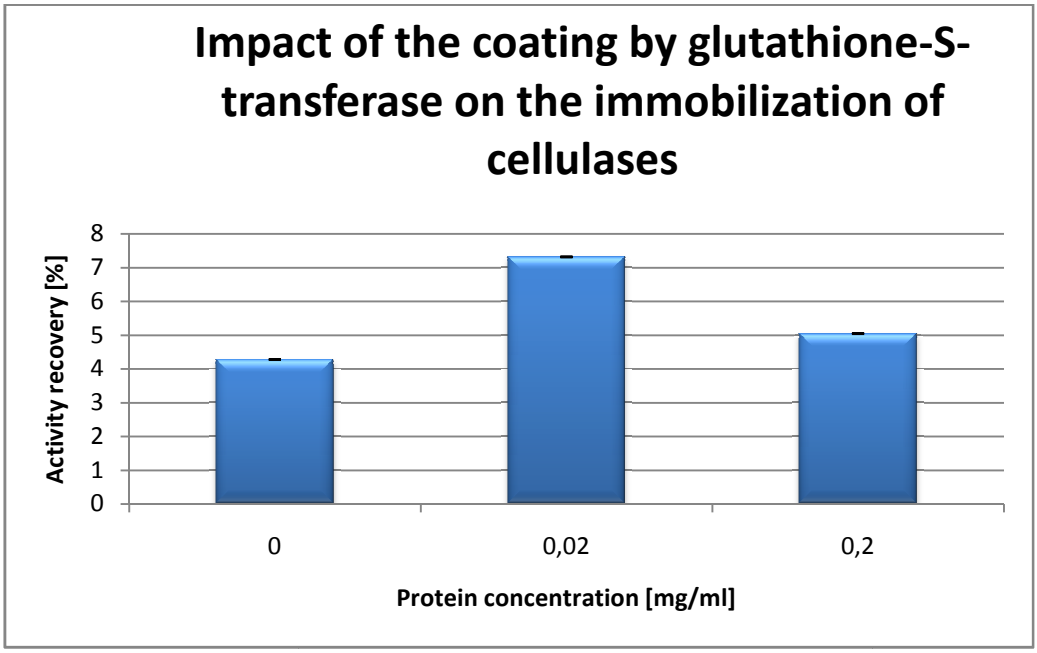



**Plot 4**. Impact of the filter paper coating with HFB4-Glutathione-S-transferase

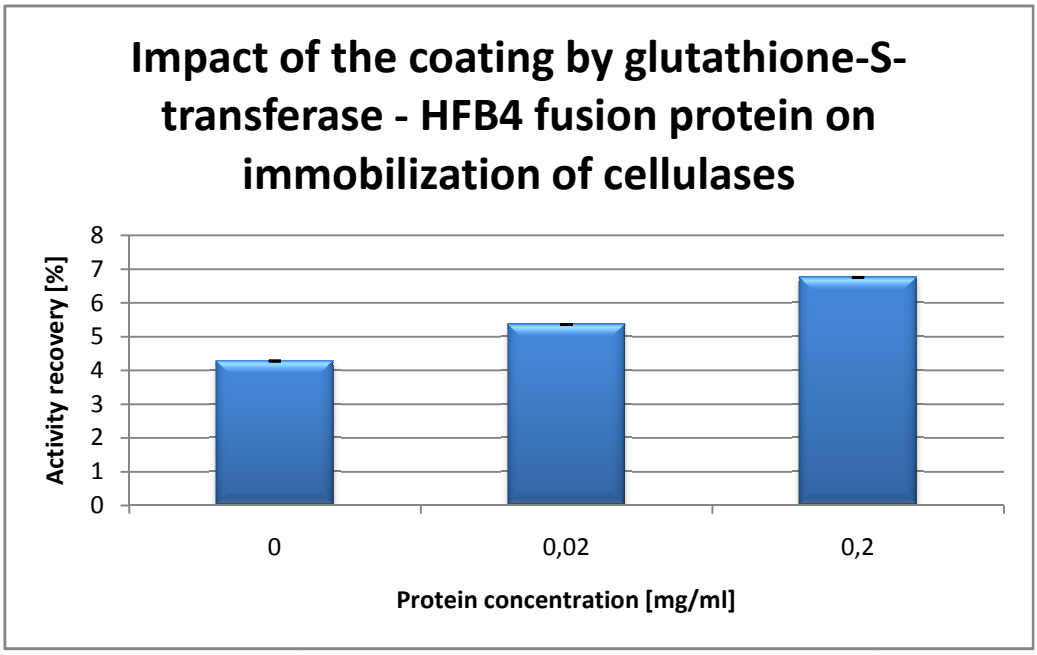
In this case we observed higher activity of the cellulase at higher concentrations of Glutathione-S-transferase, whereas in case of FPA coated by Glutathione-S-transferase-HFB4 fusion protein there is almost no improvement of the enzymatic activity. We observed only higher activity at the highest concentration of GST-HFB4, however it is much lower as for GST.

### 4.2.3. IMMOBILIZED CELLULASES ACTIVITY ON POROUS GLASS BEADS

Based on the results from tests with a solid substrate coated by hydrophobins before a reaction, we tested, if this fungal proteins can bind more enzyme, what should result in a higher activity in comparison to a carrier non-coated by hydrophobins. In this case we tested the activity of the cellulases with 3 mM PNPL on glass beads without the fusion protein with only GST or with the fusion protein using PNPL as a substrate. The plots7 and 8, shown below, represent all data obtained in this experiments.




**Plot 5.** Impact of the glass beads coating by Gluthathione-S-transferase on the activity binding




**Plot 6.** Impact of the glass beads coating by HFB4-Gluthathione-S-transferase on the enzyme binding.

We observed the different activity on the glass bead of cellulases. For only GST the highest activity is obtained, when we added 0.02 µg/ml of GST to the glass bead. At the higher concentration, the reduction of cellulase activity is observed. In comparison only to GST, the fusion protein shows an increase of activity at higher concentration of added fusion protein. However, in both cases we observed increasing activity at the lowest concentration of GST or GST-HFB4, when compared to cellulases immobilized only on porous glass beads.

### 4.2.4. BINDING CELLULASES ONTO DIFFERENT CARRIERS USING HFB4-($His_6$) AND HFB7-($His_6$) AS LINKERS

**Tab.2.** Cellulases activity recovery [%] after immobilization onto different polymers using HFB4-$His_6$ and HFB7-$His_6$ from *P. pastoris*. A-PET, B - kapton, C - mylar.

| Polymer | HFB4-$His_6$ | HFB7-$His_6$ |
|---|---|---|
| **PET** | 0.33±0.003 | 0.32±0.005 |
| **kapton** | 0.31±0.003 | 0.32±0.004 |
| **mylar** | 0.32±0.011 | 0.35±0.006 |
| **Quarzglass** | 0.33±0.020 | 0.30±0.001 |

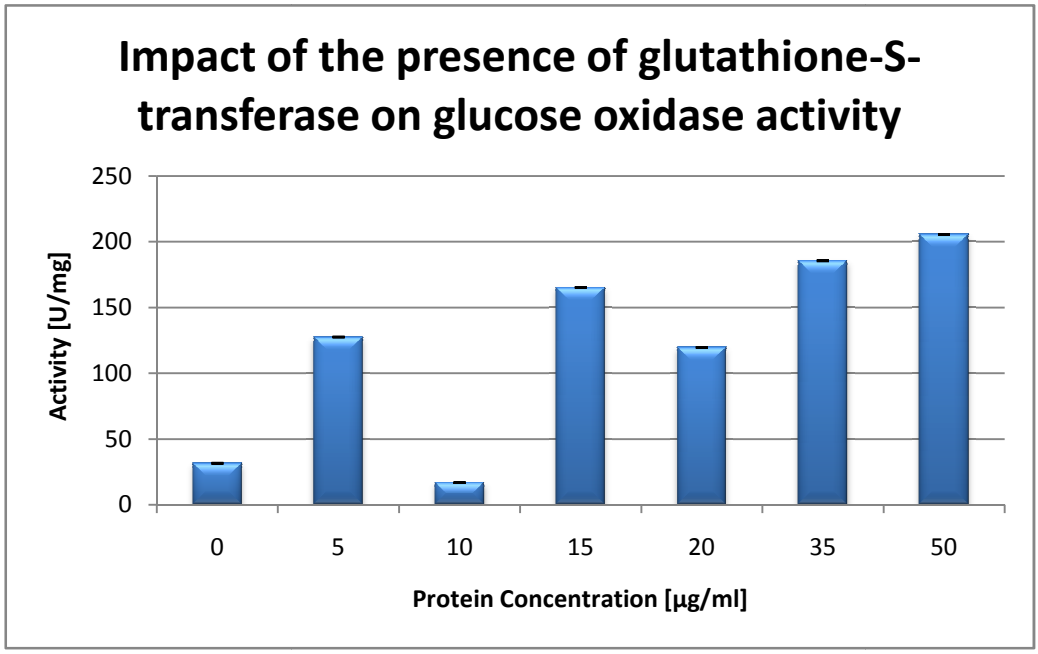
The activity recovery obtained for cellulases immobilized onto different polymers has shown, that cellulases activity was not efficiently bound onto any polymer.

## 4.3. IMPACT OF HYDROPHOBINS ON GLUCOSE OXIDASE

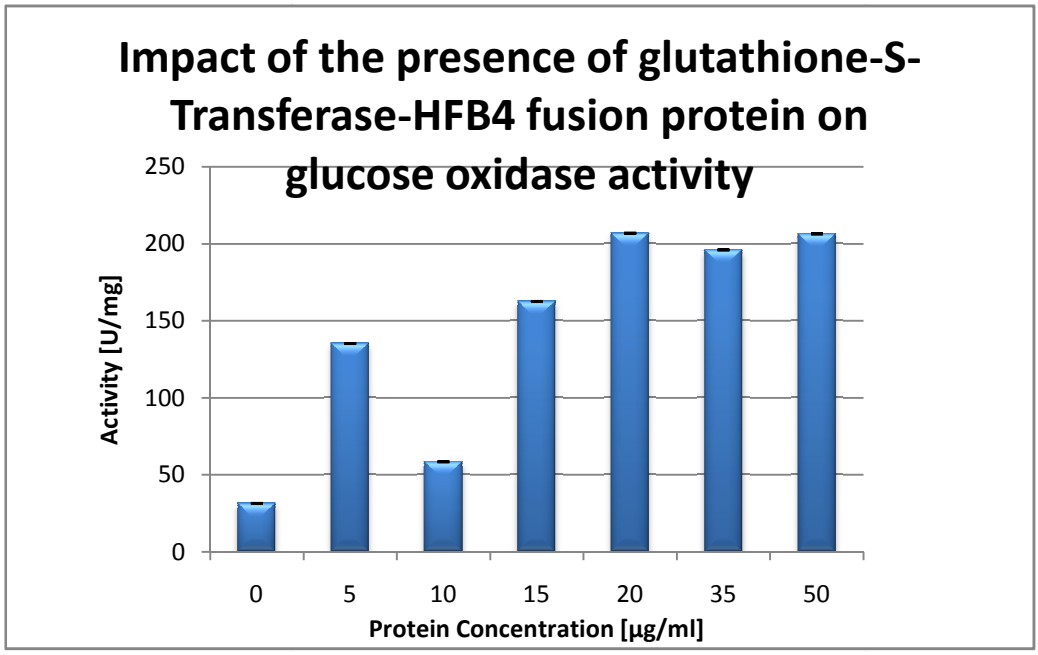
Because of the growing interest in glucose oxidase and its possible applications in industry, we decided to test this enzyme and its activity in the presence of the *Trichoderma* hydrophobins.

### 4.3.1. GLUCOSE OXIDASE ACTIVITY IN THE PRESENCE OF GST-HFB4

Firstly, as in the case of cellulases, we tested a hypothesis concerning that hydrophobin can enhance the activity of the glucose oxidase. For this purpose, we investigated the activity tests, where the glucose oxidase was preincubated for 30 minutes with the hydrophobin solution and afterwards we performed the activity test with β-D-glucose as a substrate. The plot shown below represents the data received from this experiments.




**Plot 8.** Impact of the enzyme preincubation with Glutathione-S-transferase on the activity of 0.8 µg/ml GOx.

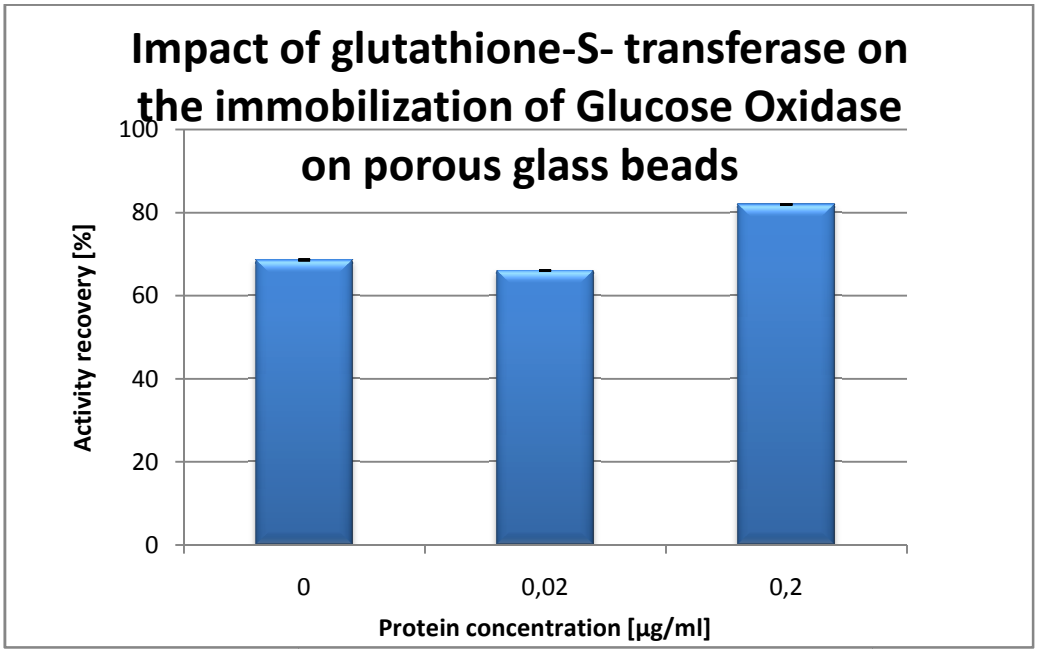



**Plot 9.** Impact of the enzyme preincubation with HFB4-Glutathione-S-transferase on the activity of 0.8 µg/ml GOx

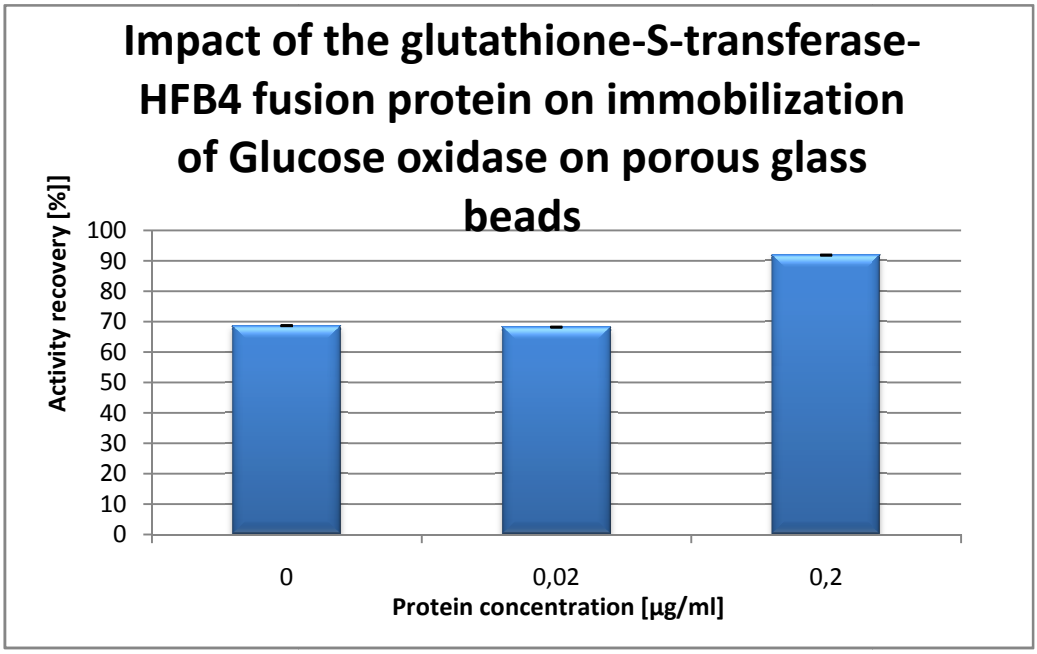
Plots 8 and 9 show the activity of GOx after 30 minutes of preincubation with different concentrations of GST and GST-HFB4 fusion protein. In both cases, we observed a strong decrease of the activity at two lowest concentrations of proteins and an increase of activity at higher concentrations. Moreover, at concentration of 20 µg/ml of protein we observed, that the activity of GOx is significantly higher for GST-HFB4 fusion protein as for GST alone.

### 4.3.2. Immobilized Glucose Oxidase Activity

In order to tests with immobilized cellulases, we tested also the activity of GOx when immobilized on glass bead with GST and HFB4- GST fusion protein as linkers. In the plots below we can see the influence of the presence of GST and HFB4- GST fusion protein on the efficiency of glucose oxidase activity binding.




**Plot 10.** Impact of the coating of porous glass beads by Glutathione-S-transferase on the binding of GOx.

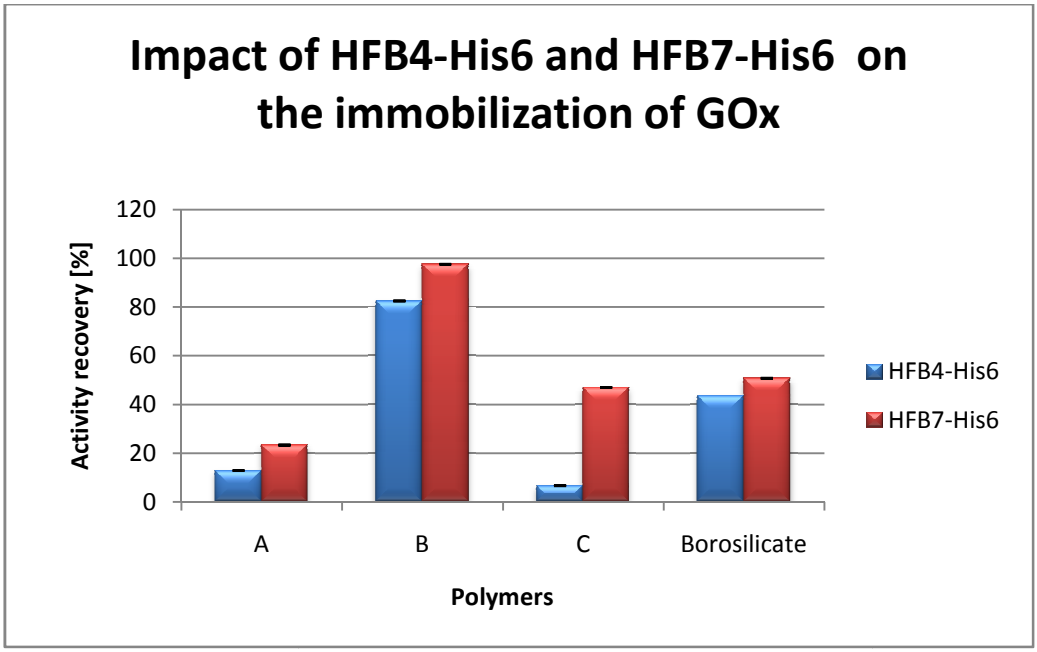



**Plot 11.** Impact of the coating of porous glass beads by HFB4-Gluthathione -S- transferase fusion protein on the binding of GOx

We observed a little increase of the activity of the enzyme at a higher concentration of both proteins as well as in comparison to the activity of the GOx immobilized on the glass

beads without any protein. At lower concentration of added proteins, the activity recovery is decreased than for glucose oxidase immobilized on glass beads without any linker.

### 4.3.3. IMMOBILIZED GOX ACTIVITY ASSAY ON DIFFERENT CARRIERS USING HYDROPHOBINS AS LINKERS



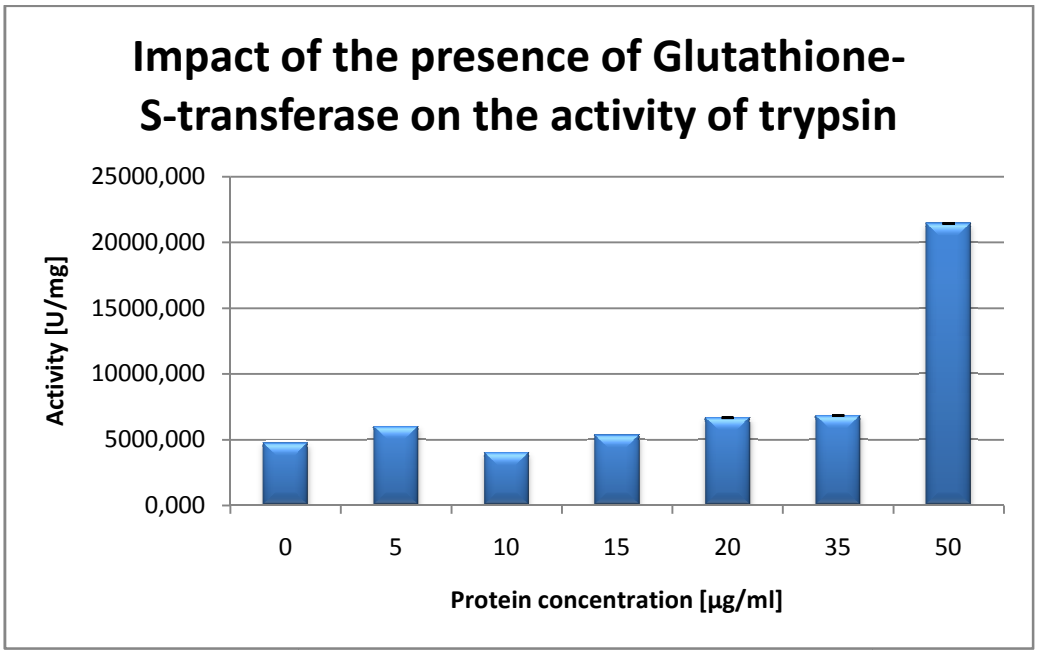

**Plot 12.** Activity recovery after immobilization of GOx on different polymers using HFB4-$His_6$ and HFB7-$His_6$ from *P. pastoris* as linkers. A - PET, B - kapton, C - mylar.

The plot 12. shown above depicts the activity of GOx immobilized on four different non-porous carriers. The activity of the enzyme is retained but it is visible that there are differences both in the case of used carrier and used hydrophobin. In case of kapton (Polymer B) we observed a significant binding of glucose oxidase in both cases in comparison to other carriers. For all cariers, the HFB7-$His_6$ shows higher binding of enzyme, when compared to HFB4-($His_6$). Inspite of the differences in binding affinities of HFB4-$His_6$ and HFB7-$His_6$, the results have shown that the type of the carriers plays a vital role in the binding process. Borosilicate and kapton have achieved better results when compared to PET and mylar.

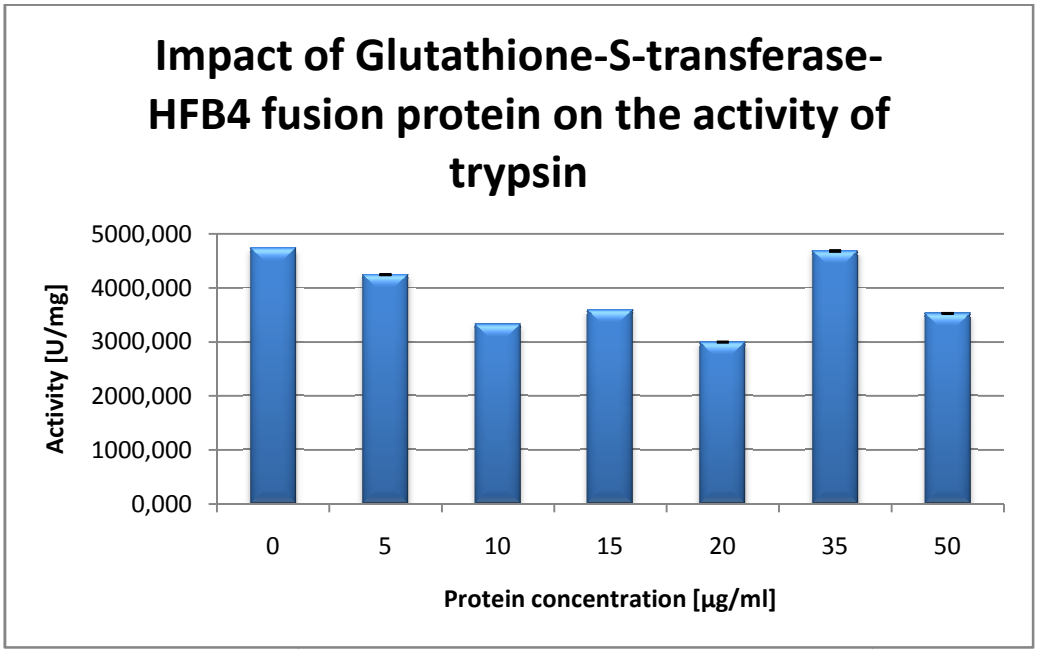
## 4.4. IMPACT OF HYDROPHOBINS ON TRYPSIN

### 4.4.1. TRYPSIN ACTIVITY IN THE PRESENCE OF HFB4

As in the earlier tests with cellulases and GOx, we tested our hypothesis using the next enzyme – trypsin.




**Plot 13.** Impact of the preincubation of 0.05 mg/ml trypsin with Glutathione-S-transferase on its activity.

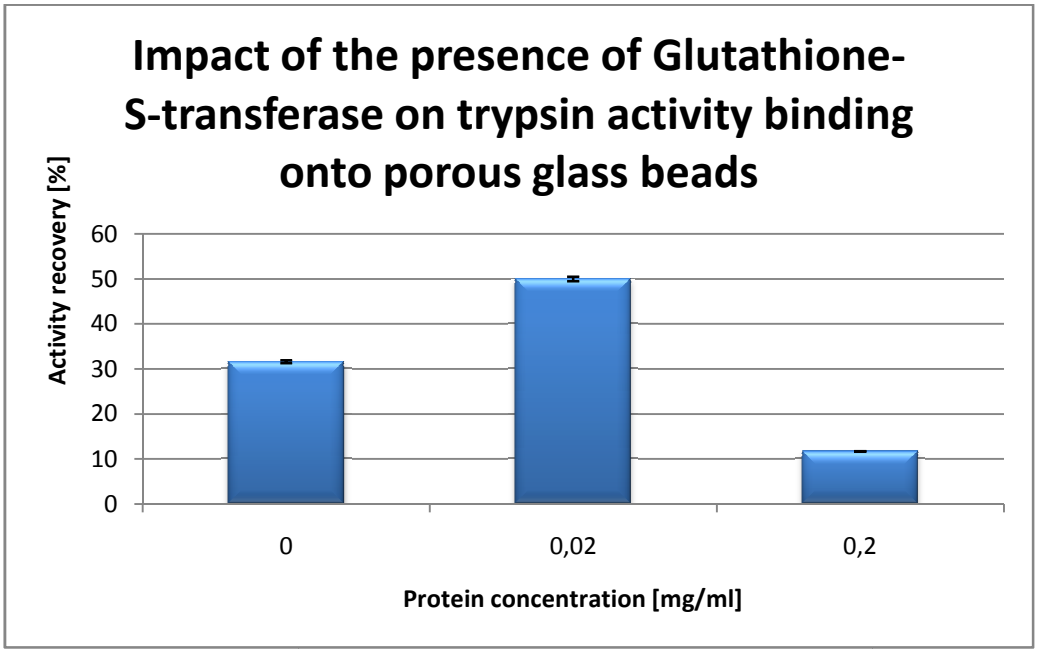



**Plot 14.** Impact of the preincubation of 0.05mg/ml trypsin with Glutathione-S-transferase-HFB4 fusion protein on its activity

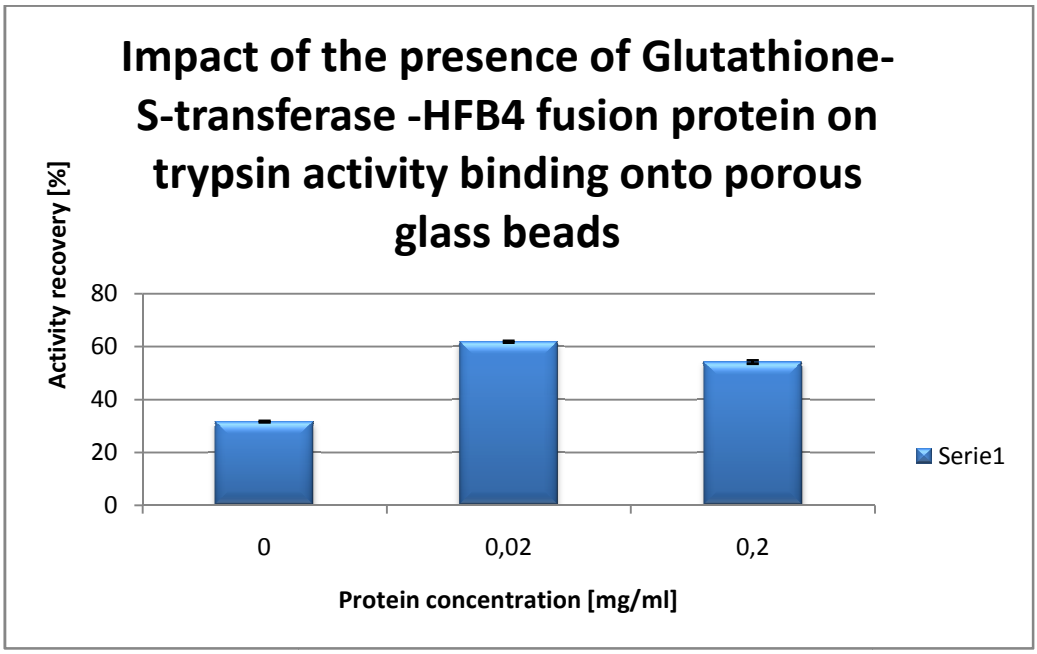
Plots 13 and 14 show the activity of trypsin after 30 minutes of preincubation with GST and GST-HFB4. Here, we did not observe any enhancement of the trypsin activity in the presence of the fusion protein. For GST the activity seems to be concentration-depending.

### 4.4.2. ACTIVITY OF TRYPSIN IMMOBILIZED ON POROUS GLASS BEADS

To test the hypothesis, whether trypsin can better bind to the glass bead when it is coated by GST-HFB4, what we could observed based on the activity.




**Plot 15.** Impact of the coating by Glutathione-S-transferase on the binding of trypsin onto porous glass beads

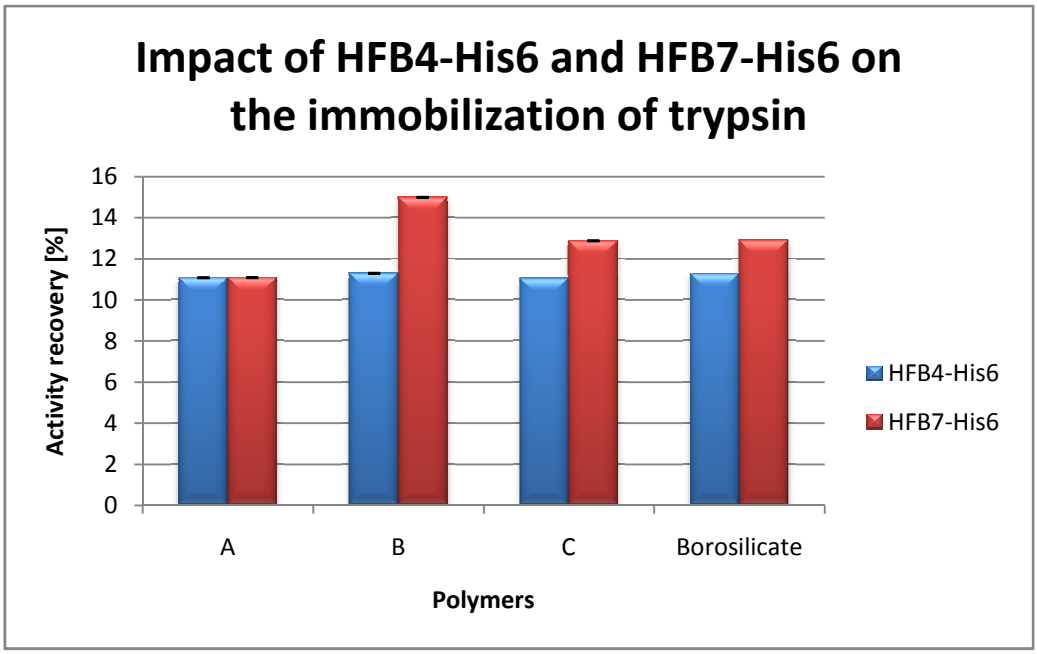



**Plot 16.** Impact of the coating by HFB4-Gluthathione-S-transferase fusion protein on the binding of trypsin onto porous glass beads

For both GST and GST-HFB4, we observed an increase activity recovery at lower protein concentration and its decrease at higher concentration of added proteins in comparison to the activity of trypsin immobilized on the only pretreated glass bead for GST and GST-HFB4, whereas at a higher concentration of GST, the decrease is significantly higher.

However, Glutathione-S-transferase fusion protein has shown better enzyme activity binding, when compared to the Glutathione-S-transferase.

### 4.4.3. IMMOBILIZED TRYPSIN ACTIVITY ON DIFFERENT POLYMERS WITH HFB4-($His_6$) AND HFB7-($His_6$) AS LINKERS




**Plot 17.**Trypsin activity recovery on different polymers using HFB4-$His_6$ and HFB7-$His_6$ from *P. pastoris* as linkers. A - PET, B - kapton, C - mylar.

On the plot 17. is shown the activity recovery of the trypsin immobilized on different membranes. In all cases, the enzyme activity was retained, however in this case HFB4-$His_6$ coated polymers have shown lower enzyme activity binding, when compared to carriers coated by HFB7. There are no significant differences between used polymers for HFB4-$His_6$ in the activity recovery, however, in the case of HFB7-$His_6$, these differences are observed.

# 5. DISCUSSION

The results of this master thesis present experimental observations of the influence of the heterologously overexpressed HFB4 and HFB7 as fusion proteins from *T. virens* on the activity of some important industrial enzymes such as cellulases, glucose oxidase and trypsin, which were used for the experiments. The method of immobilization of enzymes using hydrophobins as a linker for non-covalent binding has been proposed.

The outcome of activity test of cellulases preincubated with GST-HFB4 have demonstrated that hydrophobins have a concentration depending impact on cellulases activity. Only at two concentrations (5 and 35 µg/ml) is the activity higher, when compared to the activity of cellulases in the presence of the GST. In this case, we can suppose that the fusion protein, despite the higher solubility due to the presence of the fusion partner, can create multimers (Szilvay et al., 2006), which can block the enzyme active site due to their size. We cannot also reject the possibility of a negative interaction between monomers and the enzyme active site.

Although, HFB2 and HFB1 from *T. reesei* are identified as some of substrate binding proteins acting via cell adhesion (Adav et al., 2012), in case of cellulases activity with filter paper as non-soluble substrate, the results disputed the hypothesis that hydrophobin GST-HFB4 can enable a better cellulases binding to hydrophobic cellulose surfaces. A reason for this observation could lie in the presence of the fusion partner, however, the cleavage of the tag was not done, because of the loss of the protein of the interest. (Espino-Rammer et al., 2013)

The immobilization of cellulases on porous glass beads using GST-HFB4 as linker showed a slightly enhancement of enzyme binding, however, the activity recovery is too low, when compared to the recovered activity of cellulases bounded on porous glass beads via covalent binding (Rogalski et al., 1985). As the reason for so slight activity recovery could be a influence of the enzyme orientation in the space or mass transport limitations due to the size of the fusion protein. If enzyme is bounded in that way, that active site is not available to the substrate, or enzyme unfolding upon immobilization is occurred then the activity would be lower. (Liu et al., 2013).

In case of immobilization of cellulases on different membranes using HFB4-$(His)_6$ and HFB7-$(His)_6$, the obtained results show that neither type of membrane nor type of used

hydrophobin has any impact on the immobilization of the cellulases and therefore we assume that the immobilization of cellulases using this both $His_6$-tagged HFBins seems to be not possible. As for immobilization of cellulases on glass beads, the reason can lie in the enzyme inactivation upon immobilization process or in the enzyme orientation on the membrane.

Activity test of glucose oxidase in the presence of GST-HFB4 has shown no significant enhancement of the enzyme activity. As in the case of free cellulases, we suppose that monomers or created multimers (Szilvay et al., 2006) can block the enzyme active site and thus make the enzymatic catalysis not possible.

Although, hydrophobin SC3 from *Schizophyllum commune* belonging to class I hydrophobins has shown its possibility to successful binding of GOx on the glassy carbon electrode (Corvis et al., 2005), the immobilization of GOx on the non-activated porous glass beads has shown that the usage of GST-HFB4 fusion protein as a linker between a carrier and enzyme is insufficient. In this instance, it is supposed that the size of fusion partner can play a crucial role causing increase of mass transport limitations. We also cannot exclude the impact of the enzyme's space orientation after immobilization on the layer created by GST-HFB4. (Liu et al., 2013).

Different enzyme activity binding is presented in the experiments using hydrophobins c-terminally linked to $His_6$ overexpressed in *P. pastoris.* Here, the kind of hydrophobin and membrane has an impact of the glucose oxidase activity recovery, however, HFB7-$(His)_6$ shows better possibility to bind enzyme activity on all non porous carriers, when compared to HFB4-$(His)_6$. Thus, we can conclude that enzyme can be immobilized. However, the type of membrane and used hydrophobin plays a role in this process.

As for cellulases and glucose oxidase, the results obtained for the third enzyme, namely trypsin, has shown loss of enzyme activity in the presence of GST-HFB4 fusion protein. Also in this case, we cannot exclude the impact of monomers or multimers, formed by hydrophobins in a free solution, which can block the enzyme active site.

Despite the fact that trypsin undergoes an inactivation upon adsorption on hydrophobic surface, it has been recently shown that trypsin can be successfully immobilized in active form on Teflon coated by hydrophobin SC3 from *S. commune* (Harm et al., 2002). It approved the ability of hydrophobins to modulate the surface hydrophobicity. Moreover, the immobilized enzyme activity and its stability were maintained for 1 month at 4°C. (Harm, 2002) Hydrophobin-mediated immobilization of trypsin on porous glass beads has shown

successful enzyme binding compared to GST. Our results indicates, that HFB4 is able to modulate a hydrophobic surface into hydrophilic and thus enable enzyme biding.

The findings of immobilization of trypsin via hydrophobins HFB4 and HFB7 c-terminally linked to $His_6$ and overexpressed in *P. pastoris* has shown that membranes bind more enzyme when layers of HFB7-$(His)_6$ are used. However the activity recovery is too low. As the reason could be a small surface of the used by us membranes, because non-porous carriers have small specific area compared to porous carriers, what can reflect in smaller specific activity. Thus, it could be a good reason for a usage of porous, made of e.g. kapton beads, to test if the activity recovery would be higher. For membranes with layer of HFB4-$(His)_6$, we could not see any significant differences between used for immobilization membranes.

# 6. CONCLUSION

Enzymes are widely-used proteins catalyzing many chemical reactions. Since, they have found applications in medicine, pharmacy and industry, it is needed to search for methods of their activity improvement or lifetime prolonging. So far, there have been reported many methods of enzyme immobilization, which caused their reusability and prolonged action.

In this thesis, we tested the impact of hydrophobins from *T. virens* on the enzyme activity and enzyme immobilization for cellulases, glucose oxidase and trypsin. The obtained results have shown, that there is no enzyme activity enhancement of all used free enzymes. However, the hydrophobin-mediated immobilization of glucose oxidase has shown significant differences between used supports and used hydrophobin, when we used non-porous membranes.

Nevertheless, we do not deny the application of hydrophobins as linkers for immobilization, however, these results can be used for further investigations, because of a need for research, how they interact with enzymes, how alter the conformation of the enzyme after the immobilization an how exactly the presence of the fusion partner influences the interaction between hydrophobin and enzyme and if the homologously overexpressed hydrophobins could have better properties for this purpose.

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# 8. CHEMICALS

## CHEMICAL LIST

The table below shows all chemicals in an alphabetical order, we used during the procedures under section 3.

| **Name** | **Formula** | **MW [g/mol]** | **Purity/Concentration** | **Supplier** | **Comments** |
|---|---|---|---|---|---|
| acetic acid (glacial) | $C_2H_4O_2$ | 66.05 | 100% p.a. | Roth | dangerous |
| Acrylamide* | $C_3H_5NO$ | 71.08 | | Roth | highly toxic |
| agar agar | n/a | n/a | n/a | n/a | n/a |
| Bis acrylamide* | $C_7H_{10}O_2N_2$ | 154.10 | n/a | Roth | highly toxic |
| Bovine serum albumine | n/a | 69,000 | 98% | Roth | n/a |
| Colloidal staining solution | n/a | n/a | n/a | n/a | n/a |
| D-glucose | $C_6H_{12}O_6$ | 180.16 | n/a | Roth | n/a |
| dipotassium hydrogen phosphate | $K_2HPO_4$ | 174.20 | n/a | Roth | n/a |
| disodium hydrogen phosphate | $Na_2HPO_4$ | 141.96 | | | |
| Distilled water | $dH_2O$ | 18.01 | n/a | n/a | n/a |

| | | | | | |
|---|---|---|---|---|---|
| Double destilled water | $ddH_2O$ | 18.01 | n/a | n/a | n/a |
| Gluthathione Sepharose 4B | n/a | n/a | n/a | GELifesciences | n/a |
| Hydrochloric acid | HCl | 36.46 | 37% | | |
| IPTG | $C_9H_{18}O_5S$ | 238.3 | 99.0% | Fermentas | Storage at 4°C |
| Isopropanol | $CH_3CH(OH)CH_3$ | 60.10 | 99.0% | | |
| L-Gluthathione reduced | n/a | 307.32 | 98% | Sigma Aldrich | n/a |
| Nitric acid | $HNO_3$ | 63.0 | 67% | Roth | dangerous |
| o-dianisidine | $C_{14}H_{16}N_2O_2$ | 244.29 | | Sigma Aldrich | Soluble in acid |
| peptone from casein | n/a | n/a | n/a | Roth | n/a |
| Phenol | $C_6H_6O$ | 94.11 | n/a | n/a | toxic |
| Potassium chloride | KCl | 74.56 | 99.995% | Roth | n/a |
| Potassium dihydrogen phosphate | $KH_2PO_4$ | 136.09 | 99.9% | | |

| | | | | | |
|---|---|---|---|---|---|
| Protein ladder prestained PAGE Ruler | n/a | n/a | n/a | Thermo Scientific | Storage at -20°C |
| sodium acetate | $CH_3COONa$ | 82.03 | 99.0% | Sigma Aldrich | |
| Sodium carbonate | $Na_2CO_3$ | 105.99 | 99.5% | Roth | |
| Sodium dodecylsulfate (SDS) | $C_{12}H_{25}NaO_4S$ | 288.38 | 99.0% | Roth | |
| Sodium chloride | NaCl | 58.44. | 99.0% | Roth | |
| Sodium hydroxide | NaOH | 40.0 | 99.0% | Roth | |
| sodium potassium tartarate tetrahydrate | $KOCOCH(OH)CH(OH)COONa \cdot 4H_2O$ | 282.22 | | | |
| sodium sulfate | $Na_2SO_4$ | 142.04 | | | |
| Sulfuric acid | $H_2SO_4$ | 98.08 | 99.999% | Sigma Aldrich | danger-ous |
| Tetramethylethylenediamine | $C_4H_{11}NO_3$ | 121.14 | 99.9% | Roth | n/a |
| Triton X-100 | $C_{14}H_{22}O(C_2H_4O)_n$ | n/a | | | |
| yeast extract | n/a | n/a | n/a | Roth | |
| β-mercaptoethanol | $C_2H_6OS$ | 78.13 | 99.0% | Merck | Toxic |

| | | | | | |
|---|---|---|---|---|---|
| 1x dye reagent for Bradford | n/a | n/a | n/a | Biorad | Contains methanol and phosphoric acid |
| 4-nitrophenyl-β-D-lactopyranoside | $C_{18}H_{25}NO_{13}$ | 463.39 | 99.0% | Sigma aldrich | White powder storage at - |
| Ammonium persulfate | $(NH_4)_2S_2O_8$ | 228.20 | 98.0% | Sigma Aldrich | n/a |
| ampicillin | $C_{16}H_{18}N_3NaO_4S$ | 371.39 | 99.0% | Roth | n/a |
| Dinitrosalicylic acid | $(O_2N)_2C_6H_2$-2-$(OH)CO_2H$ | 228.12 | 98% | Sigma Aldrich | toxic |
| N-Benzoyl-L-arginine hydrochloride | $C_{15}H_{22}N_4O_3 \cdot HCl$ | 342.82 | n/a | Sigma Aldrich | Storage at 4°C |

## BUFFERS

**Phosphate buffered saline (PBS)**

For 1 liter of buffer weigh 8.0 g of NaCl, 0.2 g of KCl, 1.44 g $Na_2HPO_4$ and 0.24 g of $KH_2PO_4$. Dissolve all compounds in 800 ml of $dH_2O$, adjust to pH 7.4 with HCl or NaOH. Bring then the volume with deionized water until 1L. Sterilize the buffer by autoclaving at 15 psi, at 121°C for 15 minutes. Store the sterile buffer at 4°C.

**PBS – Triton X – 100**

For PBS – Triton X – 100 buffer mix 200 ml of PBS buffer with 10 ml of 20% (v/v) Triton X – 100 in deionized water.

**100 mM TRIS – HCl pH 7.6**

Prepare 12.11 g of TRIS and dissolve it with 800 ml of $dH_2O$. Adjust using HCl to pH 7.6. Complete with $dH_2O$ until 1L.

**1.5 M TRIS – HCl pH 8.8**

Dissolve 90.75g of TRIS and 2g of SDS in 350 ml of $dH_2O$. Adjust pH to 8.8 with 6N HCl and Bring the volume to 500 ml.

**0.5 M TRIS – HCl pH 6.8**

For 0.5 liter of the buffer dissolve 30.25 g of TRIS and 2 g of SDS in 350 ml of $dH_2O$. Adjust pH to 6.8 and bring the volume to 0.5 L.

**50 mM TRIS – HCl pH 8.0**

Weigh 6.055 g of TRIS and dissolve it with 800 ml of $dH_2O$, Adjust with HCl to pH 8.0. Bring the volume with $dH_2O$ up to 1 L.

**Elution buffer**

For preparing the elution buffer weigh 30 mg of reduced Glutathione and dissolve it in 10 ml of 50 mM TRIS – HCl pH 8.

**50 mM citrate buffer pH 4.8**

Dissolve 19.21 g of citric acid in 800 ml of distilled water. Dissolve NaOH pellets in citric acid solution until you get pH 4.5. Complete the volume until 1L. Take 500 ml of 100 mM sodium citrate buffer pH 4.5 and bring the volume until 800 ml. Adjust pH with 1M NaOH to 4.8. Complete the volume until 1L.

**67 mM potassium phosphate buffer pH 7.6**

Prepare 0.5 liter of 67mM potassium phosphate buffer pH 7.6 by dissolving 4.02 g of anhydrous potassium diphosphate and 4.76 g of dipotassium phosphate in 400 ml of distilled water. Adjust pH with NaOH up to pH 7.6 and fill the solution up to 500 ml.

**50 mM sodium citrate buffer pH 5.1**

Weigh out 3.4 g of sodium acetate (trihydrate) and dissolve it in 400 ml of $dH_2O$. Bring the pH down using glacial acetic acid to 5.1 and finally adjust the volume to 500 ml.

MEDIA FOR FERMENTATIONS

**LB Plates with Amp**

For 1 liter of LB Agar prepare 10 g of peptone from casein, 5 g of yeast extract, 10 g of NaCl and 15 g of Agar – Agar. To dissolve agar, heat the mixture until it boils and then sterilize by autoclaving at 15 psi at 121°C for 15 minutes. Afterwards allow the medium tocool down to about 60°C and then add 1ml of the Ampicillin Stock solution (100 mg/ml). Then pour the medium into petri dishes under sterile conditions.

**LB Medium with Amp**

For 1 liter of liquid LB Medium the same amount of peptone, yeast extract and sodium chloride are needed as described above except Agar – Agar.

OTHER SOLUTIONS

**DNS**

Weigh out 182 g of NaKtartarate, 10 g NaOH, 10 g DNS, 2 g Phenol and 0.5 g $Na_2SO_4$. Dissolve all ingredients in 600 ml of distilled water. Bring the volume up to 1 L. Store under the hood in a brown coloured bottle to avoid light exposure.

**COOMASSIE BRILLIANT BLUE G250 STAINING SOLLUTION**

Mix 16 ml of $H_3PO_4$ with 768 ml of distilled water. To this solution add 80 mg of ammonium sulfate. Prepare 50 ml of 5% CBB G250 by dissolving 2,5 g of CBB G250 in distilled water. Mix 16 ml of of 5% CBB G250 solution with solution of ortho-phosphoric acid containing ammonium sulphate. Immadiately before the use, slowly add 200 ml methanol to the solution.

ANTIBIOTICS

**Ampicillin Stock Solution**

Dissolve 5 g of Ampicillin in 30 ml of 50% Ethanol and then fill it up to 50 ml. Sterilize the solution using 0.22 µm filter under sterile conditions. Aliquot and store at -20°C.

ENZYMES

| Enzyme | Supplier | Comments |
|---|---|---|
| **Cellulases** | Tex Biosciences Ltd. | Store at 4°C |
| **Glucose Oxidase from *Aspergillus niger*** | Sigma Aldrich | Store at -20°C |
| **Horseradish Peroxidase** | Sigma Aldrich | Store at -20°C |
| **Trypsin from bovine pancreas** | Sigma Aldrich | Store at -20°C |

EQUIPMENT AND KITS

| Name | Supplier |
|---|---|
| Alkaline phosphate conjugate kit | Biorad |
| Analytical balance | Sysmatec |
| Centrifuge | Eppendorf |
| Deep well plates | |
| Glass beads | Sigma aldrich |
| Incubator | Heraus |
| Microreader | |
| Mini-PROTEAN Tetra Cell | BIORAD |
| Pipette tips (0.1-10µl) | Eppendorf |
| Pipette tips (50-1000µl) | |
| Power supply for Mini-PROTEAN Tetra Cell | BIORAD |

| | |
|---|---|
| QIAquick Gel Extraction Kit | Qiagen |
| Shaker Type multitron | Infors HT |
| Thermomixer compact | Biometra |

# 9. CURRICULUM VITAE

**Persönliche Daten:**


**Name:** Małgorzata Boraca
**Geburtsdatum:** 9. Juli 1988
**Staatsangehörigkeit:** polnisch
**Anschrift:** RudolfZeller Gasse 51/7/5, 1230 Wien
**Telefon:** 0 681 209168 94
**E-Mail:** malgorzata@boraca.pl


**Ausbildung/Praktikum**

| | |
|---|---|
| 2004-2007 | IV Lyzeum in Tarnów |
| 2007-2011 | Technische Universität Rzeszów, Bachelorstudium in Biotechnologie |
| September 2009 | Berufspraktikum bei der Sanitär - epidemiologischen Anstalt in Brzesko |
| 2011-2011 | Technische Universität Rzeszów, Masterstudium in Biotechnologie |
| 2011-2012 | Erasmus Stypendium an der Technischen Universität Wien, Masterstudium Biotechnologie und Bioanalytik |
| Oktober 2012 | Technische Universität Wien, Masterstudium Biotechnologie und Bioanalytik |

**Sprachkenntnisse**

| | |
|---|---|
| Polnisch | Muttersprache |
| Deutsch | B2 Niveau |
| English | B1 Niveau |

**Methodenkenntnisse:**

SDS-PAGE, Nativ Electrophorese, 2D Electrophorese, IEC, HIC, SEC, GPC, PRC, DSC, Western Blot, Northern Blot, Southern Blot, ELISA.

**EDV-Kenntnisse:**

Microsoft Office (Word, Excel, One Note, PowerPoint), Origin Pro, Matlab, CaChe.

**Führerschein:**

Vorhanden (Klasse B)

# 10. APPENDIX

## CALCULATIONS

Calculation of Cellulases Activity with PNPL as soluble substrate

Given: Molar extinction coefficient of , ε= 0,078 μmole$^{-1}$cm$^{-1}$

Calculate: Using the Lambert-Beer's law

$$A=\varepsilon cl$$

And the definition of Unit

$$U = \frac{\mu mole}{min}$$

Substitute Lambert-Beer's equation into equation for Unit definition and divide it by mass of enzyme

$$\frac{U}{mg} = \frac{Av}{l\,t\,w}$$

A- Absorbance

v – total volume of reaction mixture

l – length (1 cm)

t – time

w - weight of used enzyme

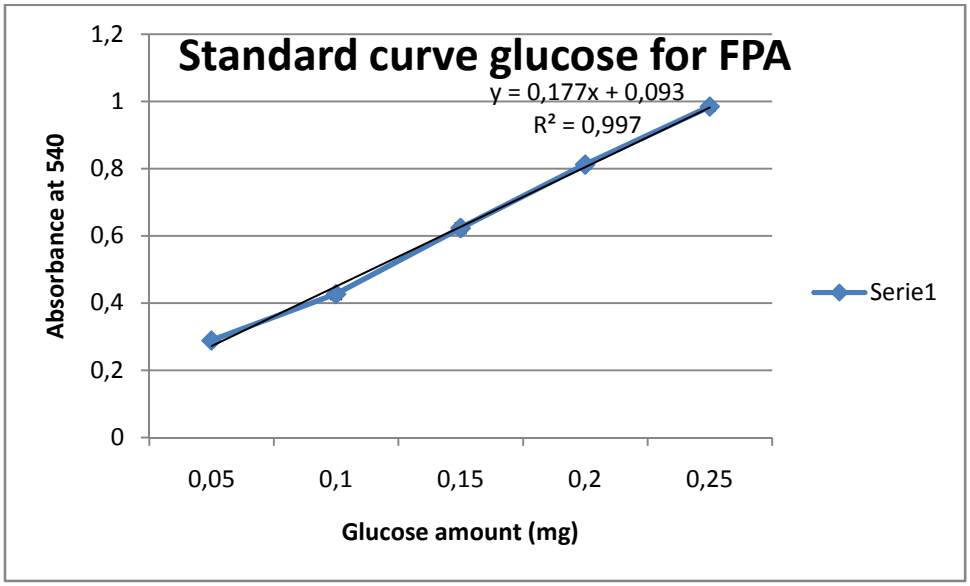
| Conc. Of GST | Average | SD | Activity [U/mg] |
|---|---|---|---|
| 0 | 0,707 | 0,06 | 457,06 |
| 5 | 0,862 | 0,014 | 564,57 |
| 10 | 0,889 | 0,014 | 583,99 |
| 20 | 0,945 | 0,016 | 621,44 |
| 35 | 0,876 | 0,039 | 570,12 |
| 50 | 1 | 0,025 | 657,51 |

| Conc. Of GST-HFB4 | Average | SD | Activity [U/mg] |
|---|---|---|---|
| 0 | 0,707 | 0,060 | 457,53 |
| 5 | 0,963 | 0,055 | 634,16 |
| 10 | 0,809 | 0,036 | 528,51 |
| 20 | 0,800 | 0,057 | 521,11 |
| 35 | 1,444 | 0,109 | 967,08 |
| 50 | 0,679 | 0,013 | 435,57 |

Calculation of Cellulases activity with Whatman 1 filter paper as non-soluble substrate

Given: Standard curve for glucose

Measured enzyme amount: 0,0135 mg




$$\text{FPU/mg} = \frac{\text{A540 sample}}{A540/mg\ standard}\ (5.55\ \mu\text{mole/mg})\ (1/t)\ (1/x\ \text{mg})$$

t – time

| µg/cm$^2$ | Average | SD | Activity [FPU/mg] |
|---|---|---|---|
| 0 | 0,238 | 0,036 | 4,12 |
| 1,6 | 0,516 | 0,030 | 8,94 |
| 3,2 | 0,492 | 0,017 | 8,52 |
| 11,2 | 0,489 | 0,022 | 8,46 |
| 16 | 0,496 | 0,013 | 8,60 |

| Conc. Of GST-HFB4 | Average | SD | Activity [FPU/mg] |
|---|---|---|---|
| 0 | 0,707 | 0,060 | 12,130 |
| 1,6 | 0,963 | 0,055 | 12,249 |
| 3,2 | 0,809 | 0,036 | 11,910 |
| 11,2 | 1,444 | 0,109 | 13,230 |
| 16 | 0,679 | 0,013 | 15,396 |

Activity determination of immobilized cellulases

for glass beads

Given:

Molar extinction coefficient: ε=0.078 µmole$^{-1}$cm$^{-1}$

The activity was calculated using formula below:

$$U = \frac{Av}{\varepsilon t l}$$

The activity of the total enzyme solution used for the reaction was determined like above.

The activity recovery [%] was calculated using the formula

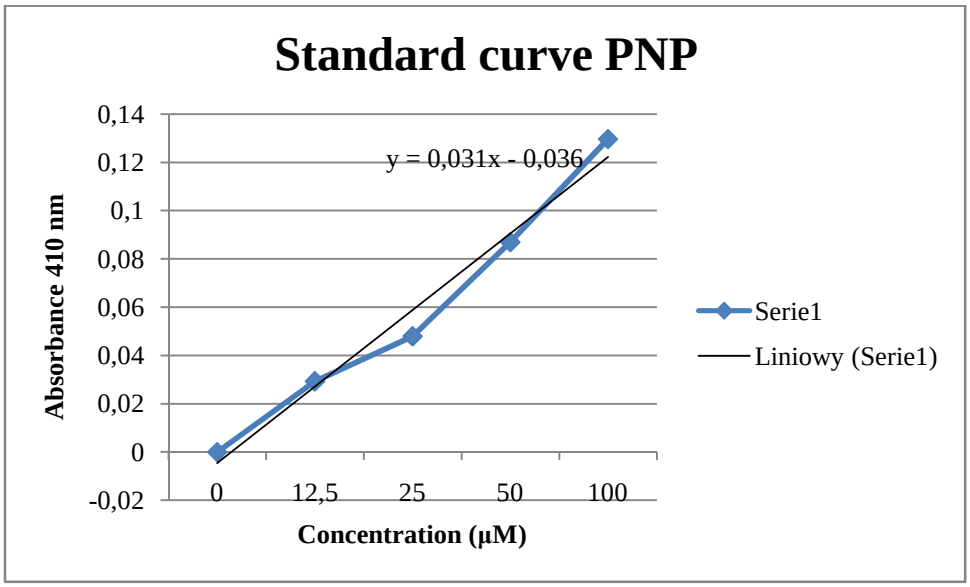
$$\text{activity recovery} = \frac{U\ sample}{U\ total}\ 100\%$$

| GST conc. [µg/ml] | Activity sample [U] | Activity Total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| 0 | 0,479 | 11,17 | 0,002 | 4,28 |
| 0,02 | 0,819 | | 0,002 | 7,32 |
| 0,2 | 0,564 | | 0,004 | 5,04 |

| Conc. Of GST-HFB 4 | Activity sample | Activity total | SD | Activity recovery [%] |
|---|---|---|---|---|
| 0 | 0,479 | 11,17 | 0,002 | 4,28 |
| 0,02 | 0,599 | | 0,001 | 5,36 |
| 0,2 | 0,756 | | 0,001 | 6,76 |

For membranes

Given: standard curve




HFB4-($His_6$)

| Membrane | Activity sample [U] | Activity total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| PET | 0,018638 | 5,597133 | 0,002517 | 0,332992 |
| Kapton | 0,017204 | | 0,002517 | 0,307377 |
| Mylar | 0,017634 | | 0,011269 | 0,315061 |
| borosilicate | 0,018065 | | 0 | 0,322746 |

HFB7-($His_6$)

| Membrane | Activity sample [U] | Activity total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| PET | 0,017778 | 5,597133 | 0,005774 | 0,317623 |
| Kapton | 0,017778 | | 0 | 0,317623 |
| Mylar | 0,019785 | | 0,002828 | 0,353484 |
| borosilicate | 0,016774 | | 0 | 0,299693 |

GLUCOSE OXIDASE

GOx activity determination

Given:

Molar extinction coefficient: 9,6 $mol^{-1}cm^{-1}$ of oxidized o-dianisidine stabilized by sulfuric acid

Weight of used enzyme in solution: 0.000048 mg

The activity of the enzyme was calculated using a formula:

$$U/mg = (A*1,2)/ (9,6*1*0,000048)$$

| Conc. Of GST | Absorbance Average | SD | Activity [U/mg] |
|---|---|---|---|
| 0 | 0,049 | 0,006 | 31,6 |
| 5 | 0,0065 | 7E-04 | 25,5208 |
| 10 | 0,0635 | 7E-04 | 3,3854 |
| 20 | 0,0460 | 0,01 | 33,0729 |
| 35 | 0,0713 | 0,006 | 23,9583 |
| 50 | 0,0790 | 0,009 | 37,1528 |

| Conc. Of GST-HFB4 | Absorbance Average | SD | Activity [U/mg] |
|---|---|---|---|
| 0 | 0,049 | 0,006 | 31,6 |
| 5 | 0,052 | 0,006 | 27,0833 |
| 10 | 0,023 | 0,005 | 11,7188 |
| 20 | 0,063 | 0,006 | 32,5521 |
| 35 | 0,08 | 0,004 | 41,4063 |
| 50 | 0,075 | 0,006 | 39,2361 |

Immobilized GOx activity test

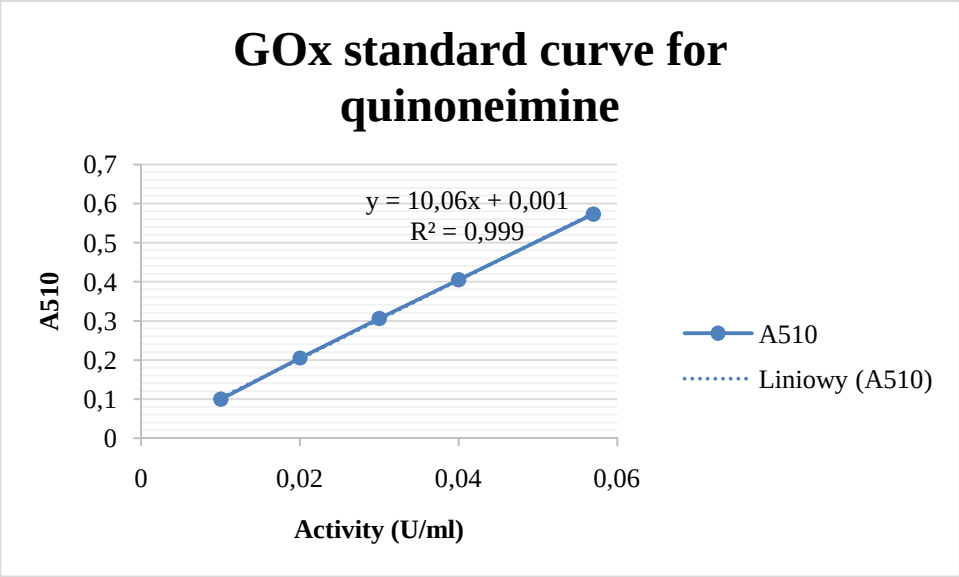
The activity [U] and then the activity recovery [%] was calculated as for cellulases on glass beads and on membranes

| GST conc. [µg/ml] | Activity sample [U] | Activity Total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| 0 | 2,250E-03 | 3,275E-03 | 0,008 | 68,70 |
| 0,02 | 2,167E-03 | | 0,0014 | 66,15 |
| 0,2 | 2,688E-03 | | 0,002 | 82,06 |

| Conc. Of GST-HFB 4 | Activity sample | Activity total | SD | Activity recovery [%] |
|---|---|---|---|---|
| 0 | 2,250E-03 | 3,275E-03 | 0,008 | 68,70 |
| 0,02 | 0,599 | | 0,001 | 68,32 |
| 0,2 | 0,756 | | 0,001 | 91,98 |

GOx on membranes

In case of membranes the activity was calculated using a standard curve for quinoneimine.




HFB4-($His_6$)

| Membrane | Activity sample [U] | Activity total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| PET | 0,00159 | 0,012326 | 0,007572 | 12,90323 |
| Kapton | 0,010172 | | 0,041477 | 82,52688 |
| Mylar | 0,000828 | | 0,000577 | 6,72043 |
| borosilicate | 0,005368 | | 0,046669 | 43,54839 |

HFB7-($His_6$)

| Membrane | Activity sample [U] | Activity total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| PET | 0,002883 | 0,012326 | 0,009539 | 23,3871 |
| Kapton | 0,012028 | | 0,052144 | 97,58065 |
| Mylar | 0,005799 | | 0,015535 | 47,04301 |
| borosilicate | 0,006262 | | 0,004 | 50,80645 |

TRYPSIN

The activity of the trypsin was calculated using formula below:

$$U/mg = (A-(-0.003)/(0.001*0.03*0.05)$$

| Conc. Of GST | Average | SD | Activity [U/mg] | | Conc. Of GST-HFB4 | Average | SD | Activity [U/mg] |
|---|---|---|---|---|---|---|---|---|
| 0 | 0,004 | 0,001 | 4740,741 | | 0 | 0,004 | 0,001 | 4740,741 |
| 5 | 0,006 | 0,001414 | 6000,000 | | 5 | 0,0034 | 0,000 | 4250,000 |
| 10 | 0,006 | 0,000707 | 4000,000 | | 10 | 0,0050 | 0,003 | 3333,333 |
| 20 | 0,008 | 0,000707 | 5333,333 | | 20 | 0,0054 | 0,003 | 3600,000 |
| 35 | 0,01 | 0,001414 | 6666,667 | | 35 | 0,0045 | 0,001 | 3000,000 |
| 50 | 0,01025 | 0,003182 | 6833,333 | | 50 | 0,0070 | 0,002 | 4688,889 |

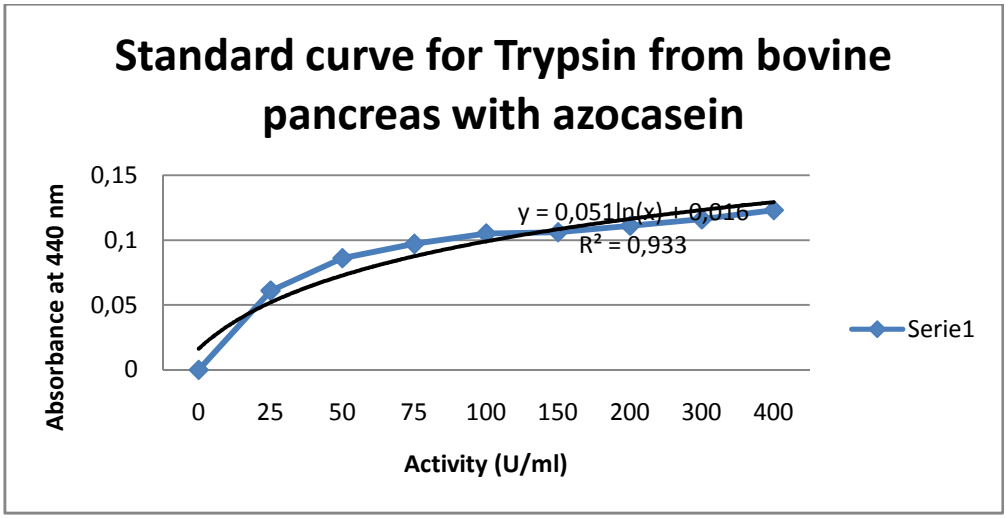
Immobilized trypsin activity test

The activity [U] and then the activity recovery [%] was calculated as for cellulases on glass beads and on membranes

| GST conc. [µg/ml] | Activity sample [U] | Activity Total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| 0 | 49683,33 | 1157000 | 0,30052 | 31,64544 |
| 0,02 | 97166,67 | | 0,346482 | 61,8896 |
| 0,2 | 85111,11 | | 0,636894 | 54,2109 |

| Conc. Of GST-HFB4 | Activity sample | Activity total | SD | Activity recovery [%] |
|---|---|---|---|---|
| 0 | 49683,33 | 157000 | 0,30052 | 31,64544 |
| 0,02 | 78555,56 | | 0,485833 | 50,03539 |
| 0,2 | 18416,67 | | 0,03182 | 11,73036 |

In case of trypsin on membranes with his-tagged HFB4 and HFB7, the activity [U] was calculated from a standard curve for asocasein depicted below by multiplication by the volume of the reaction mixture.




Afterwards, the activity recovery was calculated as for other immobilized enzymes.

**HFB4-($His_6$)**

| Membrane | Activity sample [U] | Activity total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| PET | 0,988665 | 8,919459 | 0,003606 | 11,08436 |
| Kapton | 1,008301 | | 0,008622 | 11,30451 |
| Mylar | 0,986689 | | 0 | 11,06221 |
| borosilicate | 1,005817 | | 0 | 11,27666 |

**HFB7-($His_6$)**

| Membrane | Activity sample [U] | Activity total [U] | SD | Activity recovery [%] |
|---|---|---|---|---|
| PET | 0,989597 | 8,919459 | 0,009238 | 11,09481 |
| Kapton | 1,338438 | | 0,005132 | 15,00582 |
| Mylar | 1,149372 | | 0,007778 | 12,88612 |
| borosilicate | 1,151628 | | 0 | 12,91141 |