Antimicrobial packagings based on enzyme-functionalized fibrillated celluloses

COST action AktinPak

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1. Presentation of partners

Partner 1: University of Natural Resources and Life Sciences (BOKU) Vienna, Inst. of Environmental Biotechnology:

The institute is part of the Department of Agrobiotechnology (IFA Tulln) of BOKU, and focuses on the exploitation of the microbial metabolism to safeguard the quality of life and preserve natural resources. Therein, the research group (enzyme and biomaterial technology) of Prof. Georg Guebitz is dedicated to biomaterial processing and biomaterial modifying enzymes. The group of Prof. Guebitz currently counts around 30 people including 10 PhD students and 7 PostDocs.

Persons involved: Georg M Guebitz, Gregor Tegl, Verena Ambros (master students doing the STSM)

Contact Person:

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Partner 2: Grenoble INP LGP2:

The Laboratory of Pulp & Paper Science (LGP2) is a joined research unit (UMR 5518) with National Center for Scientific Research (CNRS). Its activity starts from wood science to converting & printing of packaging. Laboratory is then considered as expert in: Biorefinery, Multiscale Biobased Materials, Functionalization of surface & printing. About 100 people are working in thus laboratory including 36 PhD students & 5 post-docs.

Persons involved: Julien Bras

Contact Person:

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2. Background

The inability of chronic wounds to heal, especially in combination with multidrug resistance, is severe health issue today. Long-term treatments and special wound management strategies prove to be expensive. By introducing antimicrobial agents into wound dressing the healing process can be influenced on-site. A prominent example is silver, which has been used in wound treatments for centuries. The cytotoxic activity of silver on intact skin tissue within the scope of long-term treatment is a major disadvantage. Topical wound dressings release the dose of antimicrobial agents at once, whereas a slow and steady release would be preferable.

Glucose oxidase (GOx) is a Flavin-enzyme, which catalyses the oxidation of glucose to gluconic acid, whereby hydrogen peroxide (H_2O_2) is produced via a two-electron transfer to molecular oxygen.

Cellulose is one of the most abundant biomaterials worldwide. This highly versatile polymer shows properties, like high biodegradability, biocompatibility, hydrophilic character, mechanical strength and possibility of functionalization. Microfibrillated cellulose (MFC) and nanofibrillated cellulose (CNF) are produced from wood pulp by mechanical disintegration. Those materials are able to form tight networks in the dry state and therefore show great mechanical properties, such as high strength, high surface area and flexibility. Furthermore, MFCs and CNFs can be chemically modified resulting in higher compatibility to other biomaterials. Functionalised MFCs and CNFs find application a variety of different fields and processes, like composites, antimicrobial surfaces, biomedical field, and controlled drug release.

3. Objective

Grafting CNF with glucose oxidase for the use of food packaging is a promising approach to ensure non-contaminated food or beverages.

Since H_2O_2 is a strong oxidative agent it would be of interest to incorporate GOx into packing materials to enhance their antimicrobial effect. GOx will be entrapped in Nanocellulose films (CNF), immobilized in CNF-films and its activity ($\mu M*min^{-1}$ H_2O_2) measured. Furthermore, a surface coating of the CNF-GOx-mix will be performed.

Those experiments will give insight whether enzyme/CNF composites are suitable for packaging materials.

4. Material and Methods

For all experiments TEMPO and enzyme treated nanocellulose fibres (CNF-T/CNF-E) were used, which were kindly provided by LGP2, INP Pagora, Grenoble. Glucose oxidase from *Aspergillus niger* was purchased from Sigma Aldrich.

For the entrapment, the CNFs were first diluted in double distilled water (ddH_2O) to reach concentrations of 0.1% and 0.5% before GOx (1mg/ml) was added. 40 ml of suspension, containing 0.3 mg/ml GOx, were plated into Petri dishes and incubated at 30°C until dry.

For the immobilization *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) and *N*-Hydroxysuccinimide (NHS) were purchased from Sigma Aldrich. Just like for the entrapment the CNFs were diluted in ddH_2O to reach concentrations of 0.5% and 0.1%. Then EDC and after 10 minutes NHS was added, both in 2 mol, 5 mol and 10 mol equivalents. Afterwards, GOx (10 mg/ml) was added and

the first sample (5ml; t0 h.) drawn. The suspension was stirred for three hours, taking two more samples after 1.5 and 3 hours. The supernatants were centrifuged at 10.000 rpm for 15 minutes at 6°C. After taking the last sample the remaining suspension was plated á 40 ml (5mg/ml GOx) and incubated at 30°C.

For the activity measurement two assays were used. Horse radish peroxidase type VI and glucose were purchased from Sigma Aldrich.

For the Amplex Red assay, Amplex Red reagent was purchased from Molecular Probes, life technologies. 1x1 cm^2 of entrapped film (0.5% +/- GOx; 0.1% +/- GOx) were placed in an Eppendorf tube and 2 µl Amplex red (10 mM), 4 µl HPO (0.1 U/ml) and 335 µl PBS (1x; pH 7.4) were added. The reaction was started by adding 60 µl of glucose. As blanks the same reaction was performed using GOx (1 mg/ml) instead of the piece of film or leaving it out altogether. The tubes were examined only visually.

For the ABTS assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS was purchased from Sigma Aldrich. 1.5 cm^2 pieces of film were placed into cuvettes before premixed 656 µl PBS (1x; pH 7.4), 120 µl glucose (200 mM), 8 µl HPO (0.1 U/ml), and 16 µl ABTS (100 mM) were added. The samples were measured at 420 nm in 10 second intervals for 1 minute each using a UV-spectrophotometer (UV – 1800; SHIMADZU).

For the surface coating an Endupap Universal coating machine was used. CNF-E was diluted to reach a 2% concentration. Samples of just enzyme treated CNF (2%), just GOx (5 mg/ml), CNF-E with 5 mg/ml GOX and 1 mg/ml GOX were prepared. A 2% suspension of enzyme treated CNF was made by weighing in 6.7 g of CNF-E and filling up to 10 g with ddH_2O. A 10 mg/ml stock of Glucose oxidase was prepared. 1667 µl or 333 µl of it were added to 6.7 g of CNF-E apiece and filled up to 10 g. Also 0.5 ml GOx were added to 3 g of ddH_2O. Depending on the viscosity of the sample an appropriate bar for coating was chosen. For viscose samples (CNF-E, CNF-E + 5 mg/ml GOx; CNF-E + 1 mg/ml GOx) a grooved bar was used. For the GOx (5 mg/ml – no CNF-E) a smooth bar was chosen. The sample was applied with a Plastic Pasteur pipette in a line along the bar. The coated cardboards were dried in a constant temperature and humidity room.

5. Results and Discussion

By entrapping the GOx in CNF-films the encapsulation efficiency of the enzyme in nanocellulose fibres could be tested. This method is fairly simple and quick, however, those films have the major disadvantage of leaching enzymes, since the entrapment is only based on absorption.

To check the enzyme activity in the films a preliminary examination was performed using the Amplex Red assay. By means of this assay a simple yes or no determination concerning the GOx activity could be made.

Figure 1: Amplex Red assay: (A): top row: blank without CNF-T - without GOx, with GOx, bottom row: CNF-T (0.1%) without GOx, with GOx; CNF-T (0.5%) without GOx, with GOx; (B): top row: blank without CNF-E – without GOx; with GOx; bottom row: CNF-E (0.1%) without GOx, with GOx; CNF-T (0.5%) without GOx, with GOx

In figure 5 the enzyme activity can be observed. Glucose oxidase is active in both CNF types and in both concentrations (0.5% and 0.1%) as well as in the positive control.

Table 1: Activity of GOx entrapped in CNF-E and CNF-T

CNF-E			CNF-T		
	Av. µM*min^{-1} H_2O_2 produced	stdev		Av. µM*min^{-1} H_2O_2 produced	stdev
0.5% + GOx	91.3310	4.4307	0.5% + GOx	68.2703	1.8402
0.5% -GOx	-0.5082	0.2824	0.5% - GOx	-0.2190	0.1015
0.1% + GOx	15.2724	0.6234	0.1% + GOx	77.6072	2.3965
0.1% - GOx	0.9750	2.0107	0.1% - GOx	0.1714	0.5686

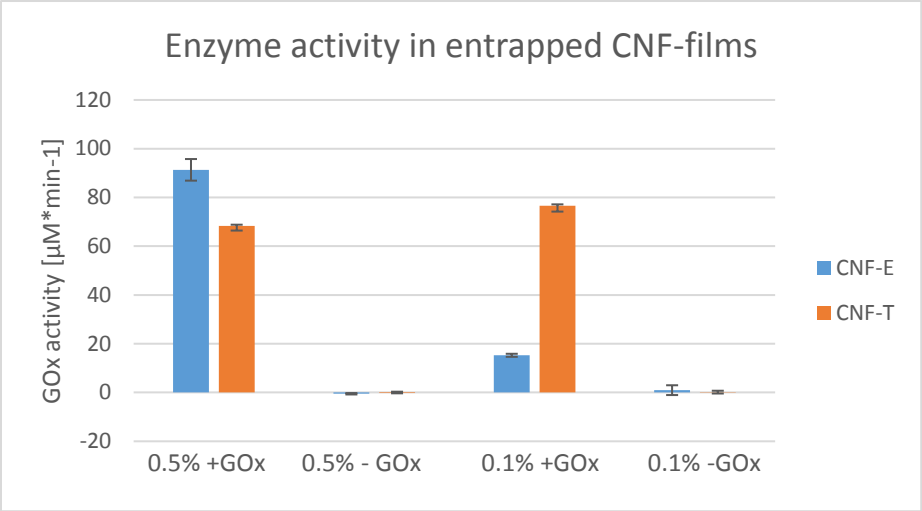

Figure 2: Enzyme activity in entrapped films

In figure 2 the enzyme activity in the entrapped films can be observed. The blanks (0.1% - GOx and 0.5% - GOx) show practically no activity as it should be. The activity of GOx in CNF-E (0.5%) is considerably higher than in CNF-E (0.1%), whereas the activity in CNF-T is almost equal in both concentrations.

Covalent immobilization is performed to avoid leaching of the enzyme but often goes hand in hand with a decrease in activity.

The immobilization of GOx in the CNFs over a period of three hours seems to be too short. Supernatant samples taken at timepoints 0, 1.5 and 3 hours did not much differ from one another in terms of produced H_2O_2 and were still rather high and therefore not sufficiently immobilized. The films only showed very low activities.

Furthermore, enzyme activity seems to be influenced in the presence of cellulose fibres (figure 3). In fact, the activity decreased about 3-fold. It is important to say, that shearing forces of the magnetic stirrer, three hours at room temperature and maybe the centrifugation might also influence the GOx activity.

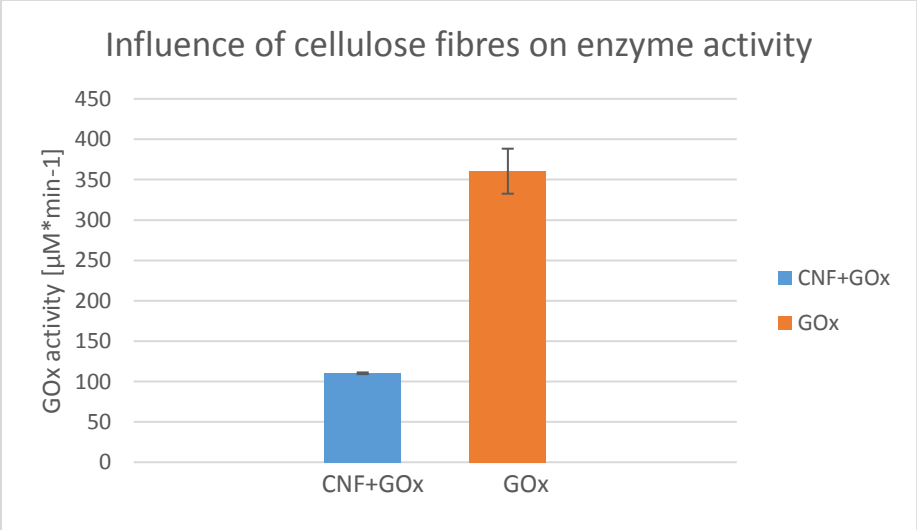

Figure 3: Influence of cellulose fibres on enzyme activity

For the analysis of the surface coating 10x10 cm^2 pieces of coated cardboard and uncoated reference were cut and weighed.

Table 2: Analysis of coated cardboards

	g/100 cm^2	g/100 cm^2	g/m^2	Average g/m^2	Stdev	% coated	Av. % coated	Stdev
Reference	2.4923	average: 2.4925	-	-	-	-	-	-
	2.4928							
	2.4924							
		Corr.						
CNF+1mg GOx	2.5244	0.0319	3.19	3.2133	0.0252	1.2798	1.2892	0.0101
	2.5246	0.0321	3.21			1.2879		
	2.5249	0.0324	3.24			1.2999		
CNF+5mg GOx	2.5170	0.0245	2.45	2.4567	0.0115	0.9829	0.9829	0.0046
	2.5170	0.0245	2.45			0.9829		
	2.5172	0.0247	2.47			0.9910		
CNF	2.5180	0.0255	2.55	2.5800	0.0265	1.0231	1.0231	0.0106
	2.5184	0.0259	2.59			1.0391		
	2.5185	0.0260	2.60			1.0431		
GOx 5mg	2.5313	0.0388	3.88	3.8933	0.0153	1.5567	1.5620	0.0061

Table 2 shows that there is not much weight difference between CNF-E, CNF-E + 1 mg/ml GOx and CNF-E 5 mg/ml GOx, but it is clearly visible, that GOx 5 mg/ml coated without the CNF has the highest g/m^2 value. This might be due to the fact, that this suspension was much more fluid than the viscous CNF suspensions and therefore more liquid might penetrate the cardboard, whereas excess viscous material simply will not be coated. As shown in the table above, roughly 1% of the viscous suspensions and 1.6% of a 5 mg/ml GOx solution were coated.

Antimicrobial tests to prove that the surface coating shows an antimicrobial affect will be performed by Dr. Seema Saini of LGP2.

6. Further plans

The covalent immobilization of GOx in CNF-films will be repeated in Tulln over the course of three days, as well testing the antimicrobial properties and pH-dependent swelling. Hippolyte Durand, PhD student at LGP2, will perform dynamic mechanical analysis and take SEM images of the entrapped and immobilized films.

The work with the staff of LGP2 was very insightful and the results seem very promising indeed. Analogue experiments in collaboration with the MatBio team of LGP2 might be carried out by entrapping or immobilizing other enzymes, like lactate oxidase or laccase into CNF-films, which would also be interesting to apply to packaging materials. Future projects will surely include similar research on the nanocrystals and nanochitosan, which were kindly provided by LGP2.