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Enzyme profile in samples of bacterial cellulose film treated with *Trametes versicolor* and *Coniophora puteana*

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Abstract: *Profile of enzymes in samples of bacterial cellulose film treated with Trametes versicolor and Coniophora puteana*. The paper presents the results of research showing the profile of hydrolytic enzymes synthesized by fungi that cause decomposition of wood during growth on film samples made of bacterial cellulose. The performed analyzes allowed for the conclusion that the presence of cellulose in the culture medium stimulates the fungal cells to synthesize full groups of enzymes that are not produced on the agar-maltose medium. Among the synthesized enzymes, both species of fungi produced two enzymes: β - glucosidase and N-acetyl-β - glucuronidase with the highest observed activity.

Keywords: apiZYM test kit, bacterial cellulose, *Basidiomycetes*, enzymes

INTRODUCTION

Cellulosic materials, such as wood, paper, and all building and finishing materials based on cellulose polymer can be colonized by biodegradable fungi. The decomposition of cellulose materials by fungi belonging to *Basidiomycetes* results from their ability to synthesize various types of metabolites, mainly enzymes, but also other initiators of the decomposition process, such as organic acids (Betlej and Grąz, 2006). The activity of enzymes synthesized by fungi, and thus the intensity of the biodegradation processes of cellulose and other foreign polymers in wood, may be modified by various factors, both environmental (Rębkowski *et al*. 2016), and factors related to the properties of wood (Bellon *et al*. 2020). Alfaro *et al*. (2020) showed that *Pleurotus ostreatus*, the mkPC9 strain and the mkPC15 strain produced glycosidic hydrolases, carbohydrate esterases and polysaccharide lyases in the presence of wood as a carbon source, while in the assessment of glucose as a carbon source, the set of enzymes synthesized by the studied fungi was slightly different. The activity of extracellular enzymes synthesized by *Coniophora puteana* and *Trametes versicolor* in immersion culture and on solid medium with the addition of portable bran was studied by Irbe *et al.* (2014). The authors of the study showed that the fungus causing white decay of wood in both types of culture produced hydrolytic enzymes with relatively low activity. An interesting observation of the authors of the research was the identification of enzymes produced by fungi on samples of birch, aspen and alder wood, both thermally modified and unmodified. The authors of the study indicated that wood modification increases its resistance to biodegradation, but does not disturb the secretion of enzymes involved in the decomposition of wood. Identification of enzymes involved in the decomposition of cellulose materials is a valuable source of information about the nature of the decomposition process taking place. As reported by the studies by Dhull *et al*. (2020) the recently discovered ability of *C. puteana* to synthesize laccase changes the interpretation of the wood decomposition process caused by the indicated brown decay fungus.

Cellulose produced by a consortium of bacteria and fungi called SCOBY, in contrast to plant cellulose, is characterized by a greater degree of polymerization, crystallinity, the size of the fibers and their spatial arrangement. Numerous studies show that a chemically modified biopolymer, on the one hand, may be resistant to colonization by *Escherichia coli* (Pasaribu *et*

al. 2020) and *Staphylococcus aureus* (Żywicka *et al*. 2018), but it is rapidly biodegradable in an environment with high cellulolytic activity. Camargo *et al*. (2020) showed that bacterial cellulose decomposes rapidly in the soil environment, which is associated with the presence of a large and diverse group of microorganisms capable of polymer decomposition. The fact that bacterial cellulose, unlike petrochemical polymers, degrades quickly and safely in the natural environment, leaving no hazardous pollutants in it, makes this polymer a possibility of wide application in various sectors of industry.

The presented work presents the results of tests of semi-quality activity of hydrolytic enzymes produced by two species of fungi, causing decomposition of wood, synthesized in the presence of cellulose produced by SCOBY microorganisms. The aim of the presented research was to determine what types of extracellular enzymes are activated by fungi in contact with bacterial cellulose.

MATERIALS

Bacterial cellulose was obtained as a result of the synthesis carried out by the consortium of bacteria and SCOBY fungi on a culture medium containing 10% sucrose, 0.03% peptone and 0.05% yeast extract. The cultivation of cellulose-synthesizing microorganisms was carried out for 14 days in a thermal incubator at the temperature of 26±2˚C and relative air humidity of 66±2%. After the assumed cultivation time, the produced polymer was removed from the culture, which was then purified according to the procedure described by Betlej *et al*. (2020), using 0.1% NaOH and 0.1% citric acid for this purpose. After the purification process was completed, the cellulose was dried at the temperature of 26 ± 2˚C until the weight of the polymer did not change. The cellulose film obtained in this way was cut into 25 x 25 mm squares, and then stored in the dark until the beginning of the tests. The thickness of the film samples, measured with a thickness gauge, ranged from 0.15 to 0.17 mm.

The produced cellulose films were treated with wood decomposing fungi - *Trametes versicolor* (L.) Lloyd strain 30 and *Coniophora puteana* (Schumach.) P. Karst. strain EB 97. Wood-decaying fungi came from the collection of the Department of Wood Science and Wood Protection. Fungi were grown on a maltose-agar medium in Petri dishes with a diameter of 90 mm. Cellulose film samples were placed on a 25x25mm glass plate embedded in the medium, but in such a way that the upper surface of the plate was not covered with the substrate. An inoculum of test fungi was placed at four locations on the substrate 1 cm from each side of the square plate. The fungi were grown on the substrate and the bacterial cellulose therein was carried out for 20 days. After the assumed cultivation time, the cellulose was removed from the medium and gently cleared of mycelial hyphae, and then placed in a 100ml phosphate buffer at pH=7 in order to extract the enzymes produced by the fungi during polymer fouling. Extractions were performed by shaking the samples on a rotary shaker for 15 min. For control purposes, a qualitative analysis of enzymes was performed in maltose-agar media, covered with tested fungi, but not containing cellulose film additive. The method of extracting enzymes from the medium consisted in taking the medium without mycelium and suspending it in 100 ml of phosphate buffer. The medium suspended in the buffer was shaken for 15 min on a rotary shaker. After the specified extraction time, 65 µl of fluid was withdrawn and added to the appropriate wells for apiZYM assays. The enzyme identification process was performed according to the apiZYM test manual. The color reaction was induced by adding the ZYM A and ZYM B reagents, respectively, included in the test kit to the enzyme wells. The types of identifiable enzymes in the apiZYM tests are presented in Table 1.

Table 1. List of enzymes identified in the apiZYM method (source: instruction on the use of apiZYM tests by BioMerieux)

No.	Enzymes	Reaction result	
		positive	negative
1	Control	colorless	colorless or pale yellow
2	Alkaline phosphatase	purple	
3	Esterase	purple	
4	Lipase esterase	purple	
5	Lipase	purple	
6	Leucine arrylamidase	orange	
7	Valine arrylamidase	orange	
8	Cystine arrylamidase	orange	
9	Trypsin	orange	
10	α-chymotrypsin	orange	
11	Acid phosphatase	purple	
12	Naphthyl-AS-BI phosphohydrolase	blue	
13	α-galactosidase	purple	
14	β-galaktozydaza	purple	
15	β-glucuronidase	blue	
16	α-glucosidase	purple	
17	β-glucosidase	purple	
18	N-acetyl-β-glucuronidase	brown	
19	α-mannosidase	purple	
20	α-fucosidase	purple	

The results of the determinations are presented visually, by indicating the enzyme numbers, identified in the individual test wells and on a point scale (0-5), indicating the intensity of the enzymatic reaction.

RESULTS

ApiZYM screening tests are a simple and quick method of identifying the metabolic activity of fungi, including their biodegradation processes. The tests allow the detection of 19 types of hydrolytic enzymes. The presented studies show that the profile of enzymes synthesized by fungi, which cause the decomposition of cellulose materials, largely depends on the environment in which a given organism develops (Table 2). Therefore, it is the substrate on which fungi grow and develop that determines the targeted metabolism of fungi in the production of specific groups of enzymes. Similar observations were made by Gutarowska (2010). The author of the research pointed out that the fungi that cause molding of technical materials, such as wallpapers or drywall, synthesize different spectra of enzymes depending on the types of materials on which they grow. In the experiment, it was shown that during the decomposition of the cellulose film, the tested test fungi synthesized additional enzymes that were not found in the control medium, without cellulose.

Table 2. Identification of enzymes produced by test fungi in bacterial cellulose film samples and maltose-agar medium

Test fungi	Maltose-agar medium (control)	Bacterial cellulose
T. versicolor	3, 4, 12,16, 17, 18	3, 4, **6**, **11**, 12, **13**, 16, 17, 18
C. puteana	2, 3, 6, 7, 12, 15, 16, 17	2, 3, **4**, 6, 12, **14**, 16, 17, **18**

bold – enzyme appears only in bacterial cellulose film

T. versicolor fungus growing on a biopolymer sample - cellulose produced by the SCOBY microorganisms consortium synthesizes two enzymes - naphthyl-AS-BI phosphatohydrolase and α-galactosidase, which were not found on maltose-agar medium. In the case of *C. puteana*, a slightly different set of enzymes synthesized during the fouling of the cellulose film was observed than in T. versicolor. In addition, it was found that the *C. puteana* fungus growing on the maltose-agar medium synthesizes the same amount of enzymes as on the cellulose medium, but in terms of quality, these are mostly different enzymes. On a medium with bacterial cellulose, *C. puteana* synthesizes lipase esterase, β-galactosidase and α-fucosidase, which were not found on the control medium. At the same time, on the maltose-agar medium, the presence of leucine arylamidase, valine arylamidase and glucuronidase were found, which were not detected in bacterial cellulose films. When assessing the activity of enzymes identified in the polymer samples grown by the tested fungi, it should be stated that the activity of β-glucosidase and N-acetyl-β-glucuronidase was at the same high level in the samples grown by both test fungi species (Table 4). The influence of the fungal growth substrate on the result of enzymatic activity was the subject of research by Nabrdalik (2008). The author of the research, using the apiZYM tests to identify the enzymes of mold fungi growing on materials such as cellulose, starch, gypsum, and mortar, confirmed that the enzymatic activity of the tested mold fungi is related to the food substrate on which they grow. On cellulose samples, the author of the study identified, inter alia, β-glucurunidase, α and β-glucosidase, esterase and lipase esterase, which was also confirmed in the presented studies.

Table 3. Assessment of the level of enzyme activity in bacterial cellulose in relation to the level of enzyme activity in the control

Test fungi	Enzyme	The level of enzyme activity in bacterial cellulose compared to that in control		
Trametes versicolor	Esterase	Increase (↑)	Decrease (↓)	No difference (0)
	Lipase esterase			(0)
	Naphthyl-AS-BI phosphohydrolase			(0)
	β-glucosidase	(↑)		
	α-glucosidase			(0)
	N-acetyl-β-glucuronidase	(↑)		
Coniophora puteana	Alkaline phosphatase			(0)
	Esterase			(0)
	Leucine arrylamidase			(0)
	Naphthyl-AS-BI phosphohydrolase		(↓)	
	α-glucosidase	(↑)		
	β-glucosidase	(↑)		

Comparing the level of activity of identical enzymes, which were identified in cellulose samples and under control conditions, it was found that some enzymes, such as N-acetyl-β-glucuronidase and β-glucosidase, synthesized by *T. versicolor* on cellulose medium, are characterized by higher activity (more intense color reaction in the test), than in the control. The activity level of the remaining enzymes, both in cellulose and in maltose food, was determined to be unchanged. Only naphthyl-AS-BI phosphohydrolase, synthesized by *C. puteana*, was characterized by a higher level of activity in the control samples.

Table 4. Assessment of enzymatic activity in samples of bacterial cellulose exposed to fungi

Test fungi	Enzyme	Semi-quantitative evaluation of enzyme activity in samples of bacterial cellulose film				
		1	2	3	4	5
Trametes versicolor	Esterase					+
	Lipase esterase			+		
	Leucine arrylamidase	+				
	Acid phosphatase	+				
	Naphthyl-AS-BI phosphohydrolase		+			
	α-galactosidase		+			
	α-glucosidase			+		
	β-glucosidase					+
	N-acetyl-β-glucuronidase					+
Coniophora puteana	Alkaline phosphatase	+				
	Esterase				+	
	Lipase esterase			+		
	Naphthyl-AS-BI phosphohydrolase			+		
	β-galactosidase		+			
	α-glucosidase			+		
	β-glucosidase					+
	N-acetyl-β-glucuronidase					+
	Leucine arrylamidase	+				

1, 2, 3, 4, 5 - visual ocean of enzymatic activity, 1 - minimum activity, 5 - maximum activity

The enzymatic activity of fungi that decompose cellulose materials is an important factor determining their pathogenicity. The ability to synthesize many groups of enzymes typically leads to the rapid degradation of material that grows over the fungus. The presented research with the use of apiZYM tests confirmed the ability of wood-decomposing fungi to synthesize a diverse group of hydrolytic enzymes. The variety of enzymes and the degree of their activity can be a valuable indicator of the intensity of the degradation process. Interesting ideas on the influence of conditions in which wood decay fungi develop are presented by Tekere *et al*. (2001). The authors of the study, using the apiZYM tests, also indicated how diverse the enzymatic activity is within the same species of fungi.

The high activity of selected hydrolytic enzymes, identified in the conducted study, allows the conclusion that bacterial cellulose is a polymer susceptible to decomposition. On

the other hand, the easy biodegradability of bacterial cellulose gives a chance for its future use as an environmentally friendly polymer (Betlej et al. 2021).

CONCLUSION

- The presence of cellulose in the growth substrate of wood-decomposing fungi induces the synthesis of extracellular hydrolytic enzymes, which in the absence of polymer are not synthesized.
- In the presence of bacterial cellulose, an increase in the activity of extracellular enzymes: α-glucosidase, β-glucosidase and N-acetyl-β-glucuronidase, synthesized by fungi, is visible in relation to the enzymatic activity measured in the control samples.
- Wood-decaying fungi *Trametes versicolor* and *Coniophora puteana* synthesize a slightly different set of hydrolytic enzymes in the presence of bacterial cellulose.
- High activity of enzymes such as β-glucosidase or N-acetyl-β-glucuronidase testify to the high metabolic activity of the tested fungi on the cellulose substrate.

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Streszczenie: *Profil enzymów w próbkach folii celulozy bakteryjnej, poddanych działaniu grzybów Trametes versicolor oraz Coniophora puteana*. W pracy przedstawiono wyniki badań obrazujące profil enzymów hydrolitycznych, syntetyzowanych przez grzyby powodujące rozkład drewna w trakcie wzrostu na próbkach folii wytworzonych z celulozy bakteryjnej. Wykonane analizy pozwoliły na stwierdzenie, że obecność celulozy w podłożu hodowlanym stymuluje komórki grzybów do syntezy pewnych grup enzymów, które nie są wytwarzane na podłożu agarowo-maltozowym. Spośród syntetyzowanych enzymów obydwa gatunki grzybów wytwarzały enzymy: β – glukozydazę i N-acetylo- β – glukuronidazę o najwyższej, stwierdzonej aktywności.

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