

Fungicidal Properties of the Medium from SCOBY Microorganism Cultivation in Saturated Wood against *Coniophora puteana* Fungus

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Effects were assessed for the post-culture liquid medium originating from the cultivation of microorganisms that are present in the ecosystem called symbiotic consortium bacteria and yeast (SCOBY). The effectiveness of protecting Scotch pine wood samples against decomposition caused by the fungus *Coniophora puteana* was evaluated. The obtained results confirmed that impregnation of wood with post-culture medium reduces the possibility of decomposition of this wood by the test fungus. The potential biocidal effect of the post-culture medium from SCOBY was attributed to the presence of substances of a potential fungicidal nature, which were synthesized in the culture medium during metabolic processes occurring in the culture. The obtained results encourage further studies on the potential use of metabolites obtained from SCOBY breeding to protect wood against biodegradation.

Keywords: Fungicides; Post-culture liquid medium; SCOBY microorganisms

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INTRODUCTION

The search for new biocides for effectively protecting wood, on the one hand, and on the other hand, safety for human health and the environment, are the consequences of legal regulations introduced in European Union countries. The implementation of the provisions of Directive 98/8/EC of the European parliament resulted in the withdrawal of many effective biocides from the market (Directive EC, 1998). Because of their high toxicity, these biocides did not meet the standards for acceptable risk and exposure assessment. The overriding legal document regulating the trade in biocidal products currently in force on the European Union market is the regulation of the European parliament and of the Council No. 528/2012 (EC EU Regulation 2012) concerning the availability on the market and use of biocidal products. Despite the restrictive regulations on the marketing of biocides, the possibility for the research and development work on new biocidal substances and products is still on the rise. Bearing in mind the applicable law, it is worth undertaking research on new ways to protect wood using substances that do not carry any toxicological and ecotoxicological risks. Examples of this type of substance include essential oils, alkaloids, or natural resins obtained from plants or substances produced by microorganisms.

Attempts to use microorganisms or their metabolites to protect wood against decay caused by fungi have been the subject of research by many authors (Highley and Ricard

1988; Kreber and Morrell 1992; Moita *et al.* 2005; Susi *et al.* 2011; Sajitha *et al.* 2018). Sajitha *et al.* (2016) isolated and characterized lyclopeptides with fungicidal properties, synthesized by the bacterium *Bacillus subtilis* B1, protecting rubber wood against colonization of the fungus *Lasiodiplodia theobromae*. Successful attempts to use *Bacillus subtilis* B1 as a biocontrol agent before fungal growth *Ceratocystis* sp., *Fusarium* sp., *Aspergillus* sp., and *Penicillium* sp. were conducted by Sajitha *et al.* (2018). Vicente *et al.* (2013) found that the active substances produced by *Bacillus amyloliquefaciens* inhibited the growth of 15 species of fungi. Attempts have been made to use the *Pseudomonas putida* bacteria, actinomycetes *Streptomyces* sp., *Saccharomyces cerevisiae* yeast, and mold fungi to protect ponderosa pine against color changes caused by phytopathogens (Kreber and Morrell 1992). The fungicidal properties of metabolites produced by *Streptomyces exfoliatus* MT9 were also evaluated (Sharma *et al.* 2016). The authors of the study showed that the ability of actinomycetes to inhibit the growth of fungi is associated with the synthesis of enzymes such as chitinase and β -1,3 glucanase, which cause cell wall lysis.

An interesting solution to protect oriented strand boards (OSB) against the development of mold and basidiomycetes was proposed by Yang *et al.* (2007). The authors of the study used extracts from *Phaeotheca dimorphospora* fungus cultures for saturating the OSB samples. After 8 weeks of incubation with mold fungi and fungi causing deep decomposition of wood on OSB boards, it was found that *Phaeotheca dimorphospora* extracts protect samples of wood-based materials against fungal colonization. The studies of the antagonistic ability of *Trichoderma viride* and *Gliocladium virens* in relation to fungi that cause deep wood decay were the subject of research by Highley and Ricard (1988). Benitez *et al.* (2004) showed that the biocontrol properties of *Trichoderma* species before the development of phytopathogens in wood resulted not only from the ability of fungi to produce metabolites of antibiotic importance, but also from purely environmental mechanisms, such as competition for nutrients and space. Similar observations were made by Smolińska *et al.* (2014).

An interesting study in the protection of wood is an attempt to assess the fungicidal properties of metabolites produced by microorganisms belonging to the kombucha ecosystem. The kombucha ecosystem consists of bacteria, mainly belonging to *Komagataeibacter*, with the dominant species of *Acetobacter xylinum*, *Aerobacter*, *Achromobacter*, *Agrobacterium*, *Pseudomonas*, and *Sarcina*, *Zooglea* i *Rhizobium*. Mayser *et al.* (1995) identified yeast-like fungi belonging to *Brettanomyces*, *Zygosaccharomyces*, *Saccharomyces*, and *Candida* in fermented kombucha drinks. During the fermentation process, these microorganisms produce a number of compounds with antimicrobial properties (Ivanišová *et al.* 2020). Sreeramulu *et al.* (2001) identified numerous substances in fermented kombucha drink that exhibit potential bactericidal activity. The killing properties of fermented kombucha drinks against dangerous pathogens, such as *Staphylococcus aureus*, *Shigella sonnei*, *Escherichia coli*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Staphylococcus epidermis*, *Campylobacter jejuni*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Bacillus cereus*, *Helicobacter pylori*, and *Listeria monocytogenes* have been studied by Sreeramulu *et al.* (2000). The fungicidal properties of the post-culture medium from *A. xylinum* bacteria were observed by Betlej *et al.* (2020). Researchers found that small amounts of bacterial post-culture medium are sufficient to completely inhibit the growth of fungi that cause deep wood decay. The ability of kombucha to inhibit the growth of *Aspergillus flavus* and *Candida albicans* was also observed by Yuniarto *et al.* (2016). The biocidal properties of the kombucha culture fluid are related to the presence of numerous

metabolites produced during the culture. Identification and characterization of substances with potential biocidal activity is crucial in the context of planning the possibility of using kombucha in the formulation of wood preservatives.

This study attempts to assess the effectiveness of wood protection with post-culture liquid medium, derived from the cultivation of SCOBY microorganisms, rich in numerous substances with a potential fungicidal effect, against the *Coniophora puteana* fungus causing brown decomposition of wood. Identification of substances with a potential fungicidal effect was carried out using the GCMS method. The use of gas chromatography allows the detection of volatile substances with low molecular weight. According to numerous scientific studies conducted at the symbiotic consortium bacterial and yeast SCOBY, it is precisely the numerous compounds and low-molecular substances produced in fermentation processes that often exhibit strong antimicrobial properties (Tu *et al.* 2019).

EXPERIMENTAL

Materials

The post-culture liquid derived from the cultivation of SCOBY (Organic farm Wolanin, Szczawnik, Poland) microorganisms was used as the testing sample. The microorganisms were homogenized, and then 1 mL of the homogenate was added to 100 mL of the medium containing sucrose, peptone, and yeast extract to produce starter cultures. 10 days after inoculation, the entire starter culture (cellulose with post-culture fluid) was added to 1000 mL of substrate, which was used to impregnate the wood. The weight of cellulose produced in the starter culture was 21.058 g. SCOBY microorganisms were grown on liquid medium containing 10% sucrose (Polski cukier, Toruń, Poland), 0.3% peptone (Biomaxima, Lublin, Poland), and 0.5% yeast extract (Biomaxima, Lublin, Poland). The microorganisms were cultured for 30 days in the liquid medium in an incubator (Selecta, Barcelona, Spain) in the dark. The temperature and humidity conditions of the culture were: 26 ± 2 °C and $66 \pm 2\%$. After the assumed cultivation time, the cellulose produced on its surface was removed from the medium, and the post-culture liquid medium was sterilized by passing it through syringe filters with a pore diameter of 0.22 µm to eliminate the microorganisms contained in it. The post-culture medium, prepared in this way was used for saturating the wood samples. Pine samples (*Pinus sylvestris* L.) with dimensions according to GOST 16712-71 (1971) standard were used. Wood samples containing only sapwood, without knots or other defects, with an average density of 0.50 g/cm³, were taken randomly from a previously prepared batch of samples. The average absolute humidity of sample was 7%.

The evaluation of the fungicidal effect of the post-culture medium from SCOBY microorganisms was conducted against the fungus *Coniophora puteana* (Schumach.) P. Karst. strain EB 97. Basal fungal cultures were grown on a maltose-agar medium containing 2.5% maltose extract (Biomaxima, Lublin, Poland) and 2.5% agar (Difco, Franklin Lakes, NJ, USA). The strains of fungi used in the study were obtained from the collection of the Department of Wood Science and Wood Protection, Warsaw University of Life Science - SGGW in Warsaw, Poland.

Methods

Assessment of fungicidal properties

The assessment of the fungicidal properties of the post-culture medium from the cultivation of SCOPY microorganisms was performed based on the requirements of Polish Standard, introducing European Standard PN-EN 113 (2000).

Before impregnation, the wood samples were dried at a temperature of 103 ± 2 °C for 24 h. After this time, they were weighed to determine the initial dry weight. Saturation of the samples was performed in a vacuum oven (ShelLab, Cornelius, OR, USA). The saturation parameters used were as follows: pressure 150 mbar, saturation time 45 min. After saturation, the wood samples were dried, weighed with an accuracy of 0.01 g, and then dried under the same conditions as before saturation. Wood samples were sterilized in a steam autoclave (Spółdzielnia Mechaników SMS, Warsaw, Poland). The wood sample retention (kg/m^3) was calculated by following Eq. 1,

$$R_c = m_c/V \quad (1)$$

where R_c denotes the liquid retention (kg/m^3), m_c represents liquid mass (kg), and V is sample volume (m^3)

The evaluation of the fungicidal properties of the culture medium in saturated wood was performed in Petri dishes onto which the *C. puteana* mycelium had previously been inoculated. The wood samples were placed on sterile glass pads. Fifty wood samples were used for the tests: 30 test samples were saturated with post-culture medium, 10 control samples were used for contact with the mycelium, and 10 control samples as the so-called control without fungus. This term applies to samples saturated with biocide, the so-called control samples, used to calculate the correction factor (C), i.e. changes in the weight of test samples resulting from reasons other than wood decomposition by test fungi. The fungus was cultivated in a heat incubator (Selecta, Barcelona, Spain) for 8 weeks. The temperature and humidity conditions of the cultivation were 22 ± 2 °C and $70 \pm 5\%$.

Identification of volatile substances of low molecular weight in the post-culture medium by gas chromatography coupled with a mass spectrometer

The analysis of components was performed using a Shimadzu (Kyoto, Japan) gas chromatograph GC-2010 connected to the GCMS-QP2010 mass spectrometer. A ZB-5MS capillary column with a length of 30 m, a diameter of 0.25 mm, and a deposit of 0.25 μm was used. Samples were introduced to the column with the AOC-20i autosampler. The samples were analyzed via a dedicated program – GCMS solution Version 2.72. The peaks were identified with NIST11 and NIST11b libraries.

The gas chromatograph-mass spectrometry (GC-MS) analysis program was as follows: Temperature, 50 °C maintained for 5 min, increased to 200 °C at 13 °C/min rate, increased later to 300 °C and maintained for 10 min; a flow rate of 0.8 mL/min; injection temperature of 250 °C; the detector voltage of 1.1 kV; and carrier gas of helium - "Gas helium 5.0" (PGNiG, Warsaw, Poland).

Polarimetric determination of sucrose in the culture medium

The concentration of sucrose in the post-culture medium was determined by the polarimetric method, using a model P3000 polarimeter (Krüss, Hamburg, Germany). Prior to measurement, the culture medium was purified using syringe filters. Measurement of the specific rotation of the sugar solution was performed in a glass tube with a length of

1.0 dm. The sucrose content of the SCOBY culture medium was determined using Eq. 2 (Szczeniowski 1983),

$$S = s \times (l_1/l_2) \quad (2)$$

where S represents tested concentration (%), s denotes concentration of sucrose in medium at the beginning of the cultivation (%), l_1 is the specific rotation capacity of the nutrient solution before cultivation of SCOBY microorganisms ($^{\circ}$), and l_2 is the specific rotation capacity of the nutrient solution after the cultivation ($^{\circ}$).

Statistical analysis

The results were statistically analyzed by performing an analysis of variance for a single classification using the Snedecor statistic (F statistic). Statistical inference was performed for the significance level $\alpha = 0.05$. Then, the means of the individual sample groups were compared using the multiple comparison test (*post hoc* test). For this purpose, the Tukey test was used. Statistical analyzes were developed using Statistica version 13.3 software (StatSoft Polska, Cracov, Poland).

RESULTS AND DISCUSSION

The fungicidal effectiveness of the post-culture medium from the cultivation of SCOBY against *C. puteana* was assessed from the average weight loss of wood samples, as presented in Table 1.

Table 1. The Effectiveness of Wood Protection with Post-Culture Medium from SCOBY Cultivation against *C. puteana*

Samples	Average Wood Mass at MC = 0%			Loss of Wood Mass			Average Corrected Loss of Mass (%)	SD (g)	CV (%)
	Initial	After Saturation	Final	Min.	Average	Max.			
	(g)	(g)	(g)	(g)	(g)	(g)			
Test specimen	4.42	4.66	4.41	0.16	0.26	0.42	4.34	0.045	17.49
Control	4.43	4.43	3.98	0.34	0.49	0.65	9.40	0.08	16.33
Control without fungus	4.41	4.41	4.34	0.06	0.07	0.08			

CV- Coefficient of variation, SD – Standard deviation

The obtained results indicated that fungal activity on test samples caused an average of 4.34% weight loss. At the same time, it was found that the weight loss in the control sample was less than 10%. Therefore, it should be stated that impregnation of wood with SCOBY post-culture medium has a protective effect against the destructive influence of the fungus, which causes brown decomposition of wood. The analysis of variance made it possible to determine the significant differences between the weight loss of the test and control samples caused by the action of *C. puteana* (factor A) (Table 2). The observed high coefficient of variation indicated a strong differentiation of the feature, which in the case of biological tests involved the use of a biocide of natural origin, could be considered as an acceptable result. The high coefficient of variation may result from the different retentions

of bioactive substances in the impregnated wood. The sucrose content in the post-culture liquid medium, which was used to saturate the wood, was 2.17%. Therefore, it can be concluded that such a concentration of sugar in the post-culture medium may stimulate the growth of the fungus. However, it should be noted that despite the remains of sucrose in the post-culture medium, the inhibitory effect of the fungus growth on the test samples was visible, compared to the results obtained for the control wood.

Table 2. Analysis of Variance and Division of Sample Groups in Tukey Test

Variability	F _{emp.}	p-value	F _{0.05}
Factor A	109.83685	4.936 × 10 ⁻¹²	4.13925
Samples	Average	Sample Groups According to Tukey Test	
Control	-0.257	a	
Test	-0.486	b	

Thus far, there have been no available data in the literature on the effectiveness of protecting softwood or hardwood with SCOBY extracts or microorganisms. Research by Betlej *et al.* (2020), carried out as a nutrient test, confirm the ability of the liquid from *A. xylinum* culture to inhibit the growth of wood-decaying fungi. The ability of kombucha microorganisms (SCOBY) to synthesize antimicrobial substances that are natural preservatives is established and analyzed in detail in the context of food quality and safety. Battikh *et al.* (2013) demonstrated the killing properties of fermented kombucha against various *Candida* species. The bactericidal properties of kombucha tea against numerous groups of bacteria were confirmed by Al-Kalifawi (2014) and Ebrahimi Pure and Ebrahimi Pure (2016). On the basis of the conducted analyzes, nine substances have been identified with a biocidal effect, and three substances that may indirectly affect the growth activity of *C. puteana*. The fungicidal properties of hexadecanoic acid were demonstrated by Bukhari *et al.* (2020). The fungicidal effectiveness of 5-hydroxymethylfurfural was positively assessed by Elsherbiny *et al.* (2015). Yuniarto *et al.* (2016) reports that the factor inhibiting the growth activity of microorganisms is, among others, acetic acid, produced during the fermentation process conducted by kombucha microorganisms.

Table 3. List of Substances Identified in Liquid Kombucha Culture Medium with Potential Importance in Inhibiting the Growth of *C. puteana*

Substances with a Fungicidal Effect	Concentration (%)
Hexadecanoic acid, ethyl ester	1.03
n-Hexadecanoic acid	0.59
2H-Pyran-2,6(3H)-dione	26.63
Acetic acid, hydroxy-, ethyl ester	12.76
Benzoic acid	1.57
D-Gluconic acid, δ-lactone	0.60
5-Hydroxymethylfurfural	25.83
D-Galactonic acid, γ-lactone	0.60
2-Pentenoic acid, 4-methyl-	2.86
Substances with an Indirect Fungicidal Effect	Concentration (%)
5-Acetoxyethyl-2-furaldehyde	0.77
Butanedioic acid, diethyl ester	1.77
Manganese, pentacarbonyl-, (oc-6-21)-	3.58

Biocidal properties can also be attributed to substances such as lactic acid, glucuronic acid, ethanol, and usnic acid (Blanc 1996; Cardoso *et al.* 2020), produced as products of SCOBY metabolism. Polyphenol fractions, such as catechin and isorhamnetin, produced by SCOBY influence on the physiological activity of microorganisms (Bhattacharya *et al.* 2020) by neutralizing bacterial toxins or inhibiting biofilm formation (Cardoso *et al.* 2018).

The method used allowed the identification of volatile, low-molecular compounds of potential fungicidal importance. However, it is not excluded that the composition of the medium derived from the cultivation of SCOBY microorganisms may contain many other substances that have not been identified due to the limitations of this method. There is a probability that the composition of the post-culture medium may contain numerous enzymes and bacteriocins that exhibit fungicidal properties (Bhattacharya *et al.* 2020, Tu *et al.* 2019).

CONCLUSIONS

1. Post-culture liquid medium, obtained from the cultivation of kombucha microorganisms, inhibits the decomposition of wood caused by *C. puteana*.
2. Nine substances with potential antimicrobial properties were found in the kombucha culture medium.

ACKNOWLEDGMENTS

The financial support from Warsaw University of Life Sciences – SGGW is thankfully acknowledged.

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Article submitted: October 22, 2020; Peer review completed: October 25, 2020; Revised version received and accepted: December 14, 2020; Published: December 23, 2020.
DOI: 10.15376/biores.16.1.1287-1295