

Effect of the essential oils addition on the rate of bacterial cellulose surface overgrowth by mold fungi

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Abstract: *Effect of the essential oils addition on the rate of bacterial cellulose surface overgrowth by mold fungi.* The aim of this study was to determine the effectiveness of protecting films made of bacterial cellulose with essential oils against overgrowth by mold fungi. The cellulose film produced by microorganisms forming a pellicle called SCOBY was modified by introducing into the cellulose pulp essential oils: cinnamon and manuka. Samples of the protected film were treated with mold fungi: *Chaetomium globosum*, *Aspergillus niger* and *Trichoderma viride*. On the basis of the tests conducted, the rate of film overgrowth by mold fungi and the effectiveness criteria of cellulose film protection with essential oils were determined. The addition of cinnamon oil protected the film against the growth of *Aspergillus niger* and *Chaetomium globosum* fungi. Manuka oil slowed down the growth of *Chaetomium globosum* microorganisms on the surface of the bacterial cellulose film sample, but did not protect the samples from overgrowth. The essential oils tested were ineffective against the fungus *Trichoderma viride*.

Keywords: bacterial cellulose, mold fungi, essential oils

INTRODUCTION

Cellulose is one of the most common natural polymers found in nature. It is used in various industries, such as woodworking, papermaking, but also in cosmetology or textile industry [Choi et al. 2020, Dehui et al. 2020, El-Saided et al. 2004, Khan et al. 2007]. Cellulose is composed of glucopyranose units linked together by β - 1,4 - glycosidic bonds. In plant cells, cellulose has a structural role. Outside the plant world, cellulose is also produced by microorganisms, especially bacteria belonging to *Gluconacetobacter*, *Acetobacter*, *Agrobacterium*, and *Rhizobium* [Jianbin et al. 2019]. Bacterial cellulose, unlike its plant counterpart, is chemically pure due to the absence of additional polymers such as lignin and hemicellulose. Bacterial cellulose is considered a natural nanocellulose due to the size and diameter of the individual fibers that make up the polymer. The very thin nanofibrils (3.0-8.0 nm) that form its structure and are arranged in a network to form a spatial structure make the biopolymer more porous than plant cellulose [Omoto et al. 2009]. Furthermore, the presence of numerous hydroxyl groups, results in strong hydrophilic properties of the polymer. Bacterial cellulose, unlike its plant counterpart, is also characterized by a higher crystallinity and polymerization level [Choi et al. 2020]. Its higher polymerization and crystallinity are beneficial for tensile strength and Young's Modulus [Tabuchi 2007]. Scientific studies have been conducted to conclude the usage of bacterial cellulose in the packaging and paper industry as a pulp additive [El-Saided et al. 2004]. Such an application of biopolymer allows reducing the consumption of valuable wood raw material. Examples of the application of bacterial cellulose as a packaging material were presented by Olędzki and Walaszczyk [2020]. Biocellulose as a material is characterized by biocompatibility. In the scope of packaging industry, it seems to be a relatively important feature. The production of

packaging from bacterial cellulose is a viable option due to the high strength of this material and the use of a natural raw material that is fully ecological and non-toxic [Mu et al. 2019]. For decades, packaging has been playing a key role in the economy and economics, and undoubtedly, its production and processing affect the state of the environment. Increasing deterioration of the environment is forcing the production of fully bio-degradable materials. Therefore, the use of cellulose as a completely natural and non-toxic material for the production of packaging is not only a modern solution, but first and foremost is in line with the principles of sustainable production

Bacterial cellulose is susceptible to microbial decomposition and fungal overgrowth. One of the factors which enable prolonging its durability, especially in conditions that favor the growth of the microorganisms, is to modify it by using substances inhibiting the growth of microorganisms. Modification of cellulose by the use of various substances with bactericidal or fungicidal properties protects the polymer from the harmful effects of biotic factors. In the scientific literature, there are many reports on the use of substances of plant origin in protecting bacterial cellulose from microbial decomposition [Saravanakumar et al. 2020, Shao et al. 2017, Walsh et al. 2003] One of the substances of natural origin with biocidal properties can be essential oils. They show the ability to inhibit the growth of fungi and other microorganisms [Srikandace et al. 2018]. Essential oils contain mainly terpenes i.e. limonene, sabinene or pinene and some oxidized chemical structures i.e. camphor, camphone or boryl acetate. Research by several authors shows that the use of different types of essential oils has a beneficial effect on the bioresistance of cellulose films [Swistak and Betlej 2020, Noshirvani et al. 2017, Mohamed et al. 2016]. The application of essential oils makes these films acquire resistance to fungal overgrowth [Mohsenabadi et al. 2018, Royo et al. 2010].

MATERIALS AND METHODS

The evaluation of the bioresistance of bacterial cellulose film containing manuka oil and cinnamon oil against overgrowth by mold fungi was carried out based on the guidelines of the method described by Indriyati and Indrarti [2018]. Two species of mold fungi, from the collection of the Institute of Wood Sciences and Furniture, were used as model fungi. Starter cultures of the fungi *Trichoderma viride* Pers., strain A-102, *Chaetomium globosum* Kunze 1817, strain 16 and *Aspergillus niger* van Tieghem, strain 287 were grown on maltose-agar medium (2.5 g maltose, 2.5 g agar per 100 ml water). Such prepared medium was then sterilized in an autoclave for 20 minutes at 121°C.

Bacterial cellulose samples were produced by microorganisms forming the SCOBY ecosystem. The cellulose synthesizing microorganisms were cultured for 14 days in a thermal incubator at a temperature of $26\pm 2^\circ\text{C}$ and $66\pm 2\%$ relative humidity. These cultures were conducted on a medium containing 200 g sucrose, 0.5 g peptone, 0.3 g yeast extract per 1500 ml water. At the end of the culture period, the produced cellulose was removed from the surface of the liquid, which was then cleaned of residual microorganisms. The polymer purification procedure consisted of washing with a detergent containing <10% anionic surfactants and <5% non-ionic surfactants, followed by two rinses in distilled water. Cellulose purification further consisted of a 30-minute soak in 0.1% NaOH, another rinse in distilled water, followed by a rinse in 0.1% citric acid and another rinse in water. After the purification process was completed, the polymer was put in a laboratory blender and ground until the pulp was obtained. The pH of the purified pulp was 6.5. Before the incorporation of essential oils, the water content of the cellulose was determined by the weight method. For this purpose, 3 grams of wet pulp was placed in a weighing dish, roasted to a constant weight in a laboratory dryer. The cellulose dish was placed in the dryer at 100°C and dried until the weight of the cellulose dish showed no variation.

Essential oils were introduced into the resulting pulp. The method of introducing the oils is shown in Table 1. The oils used in this study were not diluted. Only one concentration of essential oils was used in this study, which was introduced into the specified pulp. The pulp content used in the study was 140g. The indicated pulp weight was optimal for a full and uniform filling of the die on which the cellulose film was obtained. The oil content of the dried cellulose film was not determined.

Table 1. Amounts of essential oils introduced into the pulp

Sample type	Wet pulp mass [g]	Essential oil [ml]		Tween 80 [ml]
		manuka	cinnamon	
Manuka oil film	140	0.1	-	0.5
Cinnamon oil film	140	-	0.1	0.5
Control film	140	-	-	-

The method of modifying the pulp by introducing oils was carried out following the methodology described by Indriyati and Indrarti [2018]. The combined ingredients were homogenized very well using a blender. The addition of Tween 80, - emulsifier and dispersing agent was aimed to evenly distribute the essential oils in the ground cellulose mass and thus increase the stability of the system. The addition of Tween to the wet and ground cellulose improves the dispensability of the essential oils in it. The control samples did not contain Tween, at the same time, based on the literature data, Tween 80 was found to have no fungal growth inhibitory effect (EN 13697:2019).

The cellulose pulp with essential oils was vented in a vacuum dryer for 1 h using a vacuum of 100 mbar. The corresponding pulp mass was then placed and distributed in a silicon form. The thus prepared cellulose was dried in a laboratory dryer at $24\pm 2^{\circ}\text{C}$ until the polymer mass remained unchanged.

The resulting cellulose film was cut into 25x25 mm samples, which were stored in the dark until testing. The thickness of the film samples after measuring with a thickness gauge was 0.66 mm on average.

Assessment of the extent of cellulose film overgrowth by mold fungi was carried out on maltose agar medium in 90 mm diameter petri dishes. The samples were placed on glass plates of the same dimensions as the cellulose samples. The glass plates were placed in the center of the empty dishes, to which the medium was then added in such a way that the glass plate remained free of the medium. After placing the film on the plates, the inoculum of the tested fungi was inserted on the surface of the medium. The inoculum of the tested fungi with a diameter of 5 mm was introduced with a cork borer into the medium in 4 places, located in the middle of the length of the edge of the glass plate on which the cellulose was lying. Evaluation of the rate of overgrowth of cellulose films was carried out at 48 h intervals. The means of assessing the rate of overgrowth was carried out by determining the percentage overgrowth of cellulose. For this purpose, a grid with a hole dimension of 2.5x2.5mm was applied on the surface of a 25x25mm transparent film template. The number of holes on the surface of the template was 100. The stencil was placed on the surface of the Petri dish lid in such a way that it lay parallel to the cellulose film. Then, it was counted how many transparent holes of the template were occupied by the fungal hyphae. 1 hole of the grid represents 1% of the overgrowth of the film. Based on the data obtained, three stages of overgrowth evaluation were adopted:

- 0 - no fungal growth on the sample,
- 1 - trace fungal growth from 1-9%, limited to edges of the sample
- 2 - less than 25% of the sample surface is covered with mycelium, but more than 9%.
- 3 - more than 25% of the sample area covered is with mycelium.

The following efficacy criteria were used for essential oils:

- 0 - essential oil effectively protects the film against mold fungi overgrowth,
- 1 - the essential oil provides poor protection against mold fungi overgrowth on the film,
- >1 - the essential oil does not protect the film against mold fungi overgrowth.

This criterion could be adopted with the simultaneous fulfillment of the condition that on the control samples 100% overgrowth of the surface was achieved.

Each test was performed in five repetitions. The control samples were subjected to the same test procedure, but they did not contain essential oils. At the completion of the culture, the fungus-treated film samples were photographed to document the rate of overgrowth of the test film samples.

RESULTS AND DISCUSSION

Based on the results obtained, it was found that the samples treated with cinnamon oil effectively protected the cellulose film from overgrowth by the mold fungi *Chaetomium globosum* and *Aspergillus niger*. In the case of *Trichoderma viride*, the film protected with cinnamon oil was completely overgrown on the ninth day of the experiment. The condition of the correctness of the performed tests was to obtain complete overgrowth of control samples infested with mold fungi. The tests carried out show that the control film samples were completely overgrown by fungi on the eleventh day of the test: *Chaetomium globosum* and *Aspergillus niger*, while the fungus *Trichoderma viride* completely overgrew the samples already on the ninth day of testing. Thus, one can observe, the unprotected film is susceptible to decay caused by mold fungi, and this further indicates the validity of the tests performed. Based on the test results obtained, it was found, manuka essential oil does not protect the film samples from infestation by the fungus *Trichoderma viride*, on the ninth day of testing the samples were completely overgrown by the test organism. As a result of the study, the exceptional resistance of the *Trichoderma viride* fungus to the applied essential oils was noted. A study on the effectiveness of protecting southern yellow pine wood samples with essential oils by Yang and Clausen [2007] also shows exceptional resistance of the *Trichoderma viride* fungus. The samples were immersed for 15 seconds in the essential oils of dill herb, rosemary, lemongrass, tea tree, ajowan, thyme and Egyptian geranium. The results of the immersion method of Yang and Clausen [2007] showed that when four essential oils were used: ajowan, lemongrass, rosemary, and tea tree, the samples were 80% covered with mold after 10 weeks, while by week 10, the mold fungus had overgrown the entire surface of the sample. In contrast, manuka oil slowed the growth of *Chaetomium globosum* on the surface of the bacterial cellulose film sample. The averaged results of the percentage mold fungi overgrowth on the film surface are given in Table 2.

Table 2. Measurement of bacterial cellulose film sample overgrowth by mold fungi

Film sample	Sample overgrowth [%]								Microorganism
	After 4 days		After 7 days		After 9 days		After 11 days		
	x	σ	x	σ	x	σ	x	σ	
Control film	7.8	4.1	46.6	9.3	88.8	5.9	100.0	0.0	<i>Chaetomium globosum</i> Kunze 1817, strain 16
Cinnamon oil film	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Manuka oil film	3.6	1.1	9.6	3.5	25.6	5.4	32.2	2.9	
Control film	3.8	1.8	52.6	2.9	69.0	9.4	100.0	0.0	<i>Aspergillus niger</i> van Tieghem, strain 287
Cinnamon oil film	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Manuka oil film	5.0	5.4	50.2	4.5	60.8	4.6	100.0	0.0	
Control film	21.0	10.1	51.2	3.0	100.0	0.0	-	-	<i>Trichoderma viride</i> Pers., strain A-102
Cinnamon oil film	22.4	6.3	62.3	6.1	100.0	0.0	-	-	
Manuka oil film	43.6	2.6	60.8	4.2	100.0	0.0	-	-	

x – arithmetic mean of the measurements, σ – standard deviation of the measurements

Table 3. Evaluation of bacterial cellulose film overgrowth by mold fungi

Film sample	Evaluation of the rate of overgrowth of samples				Microorganism
	After 4 days	After 7 days	After 9 days	After 9 days	
Control film	1	3	3	3	<i>Chaetomium globosum</i> Kunze 1817, strain 16
Cinnamon oil film	0	0	0	0	
Manuka oil film	1	2	3	3	
Control film	1	3	3	3	<i>Aspergillus niger</i> van Tieghem, strain 287
Cinnamon oil film	0	0	0	0	
Manuka oil film	1	3	3	3	
Control film	2	3	3	-	<i>Trichoderma viride</i> Pers., strain A-102
Cinnamon oil film	2	3	3	-	
Manuka oil film	3	3	3	-	

On the basis of the percentage overgrowth measurement of the film samples, it was possible to assess the rate of overgrowth of mold fungi microorganisms on the samples. In the case of the film with cinnamon oil infested with *Chaetomium globosum* and *Aspergillus niger* fungi, no fungal growth was observed on the tested samples. On the control film infested with *Chaetomium globosum* and *Aspergillus niger* fungi, after four days of testing the growth of

fungi was visible, limited only to the edges of the sample, in the next days of testing more than 25% of the sample surface was covered with mycelium. The film protected with cinnamon oil on the fourth day of the test was covered by *Trichoderma viride* in a degree less than 25% of the sample surface. However, in the following days of observation mycelium covered more than 25% of the surface of the sample. The results of the rate of overgrowth of the samples by the tested mold fungi are shown in Table 3.

A comparison of the overgrowth of the bacterial cellulose film surface with the addition of cinnamon oil and the control film by the fungus *Aspergillus niger* is shown in Fig. 1.

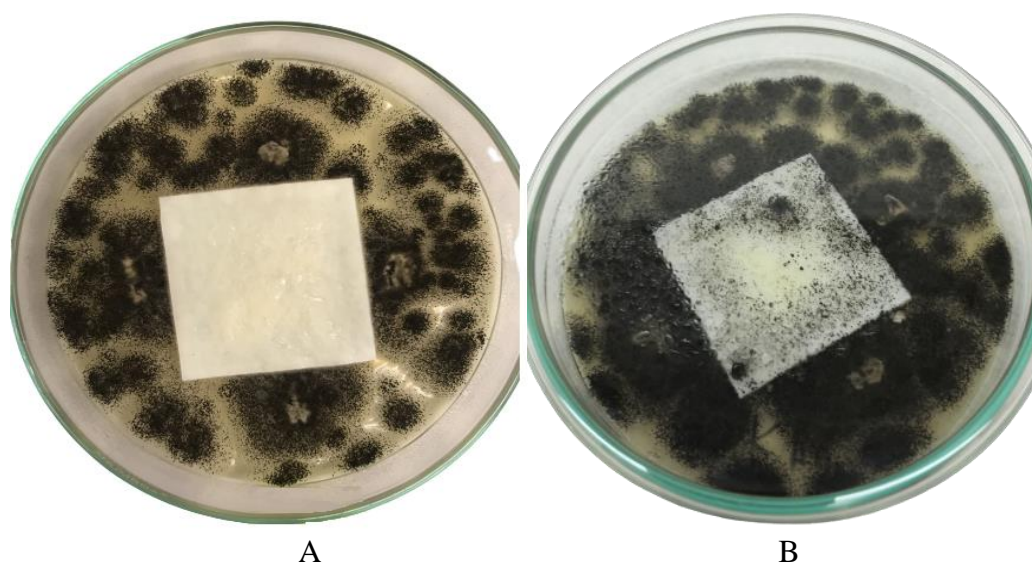


Fig. 1 Growth of *Aspergillus niger* on bacterial cellulose samples A - protected with cinnamon oil, B - control

Based on the data in the available literature, essential oils such as cinnamon, Egyptian geranium, and lemongrass have positive effects on reducing the growth of mold fungi such as *Chaetomium globosum*, and *Aspergillus niger* [Mohamed et al. 2016]. The fungicidal properties of cinnamon oil against the fungi *Chaetomium globosum* and *Aspergillus niger* were confirmed in the presented studies. The resistance of *Trichoderma viride* to the biocidal effects of essential oils is confirmed by the analyses conducted by Yang and Clausen [2007], or Mohamed et. al. [2016]. The addition of manuka oil to the pulp slows the growth of the fungus *Chaetomium globosum* to some extent but ultimately does not protect the film from overgrowth by this mold fungus. A considerable amount of literature data exists on the effectiveness of manuka oil against mold fungi [Chen et al. 2016, Prosser et al. 2014, Tripti and Colleen 2010, Zhang et al. 2017]. However, the biocidal efficacy of manuka oil is much weaker than that of cinnamon oil, as evidenced by studies by Swistak and Betlej [2020], Noshirvani et al. [2017], and Mohamed et al. [2016].

CONCLUSIONS

1. Treating the bacterial cellulose film with cinnamon oil effectively prevents the growth of *Aspergillus niger* and *Chaetomium globosum* mold fungi.
2. Manuka oil has no fungicidal properties against the tested mold fungi.
3. The fungus *Trichoderma viride* is resistant to the biocidal properties of oils added to bacterial cellulose.

Based on the evaluation of the overgrowth of bacterial cellulose film, containing essential oils, by the tested mold fungi, the criteria for the effectiveness of protection of the tested samples were determined:

- cinnamon oil is very effective, showing good properties to protect the cellulose film against the growth of *Chaetomium globosum* and *Aspergillus niger* fungi,
- cinnamon oil is not effective in protecting the film against *Trichoderma viride*,
- manuka oil does not have antifungal properties against the tested mold fungi and does not protect bacterial cellulose film against overgrowth by mold fungi.

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Streszczenie: *Wpływ dodatku olejków eterycznych na stopień porostania powierzchni celulozy bakteryjnej przez grzyby pleśni.* Przedmiotem badań było ustalenie skuteczności zabezpieczenia folii wytworzonej z celulozy bakteryjnej olejkami eterycznymi przed porastaniem grzybami pleśniowymi. Folia celulozowa, wytworzona przez mikroorganizmy tworzące konsorcjum zwane SCOPY została poddana modyfikacji poprzez wprowadzenie do pulpy celulozowej olejków eterycznych: cynamonowego i z manuka. Próbki zabezpieczonej folii zostały poddane działaniu grzybów pleśniowych: *Chaetomium globosum*, *Aspergillus niger* i *Trichoderma viride*. Na podstawie przeprowadzonych badań określono stopień oceny porostania folii przez grzyby pleśniowe i kryteria skuteczności zabezpieczenia folii celulozowej olejkami eterycznymi. Dodatek olejku cynamonowego zabezpieczył folię przed rozwojem grzybów *Aspergillus niger* i *Chaetomium globosum*. Olejek manuka spowolnił rozwój mikroorganizmów *Chaetomium globosum* na powierzchni próbki folii celulozy bakteryjnej, ale nie zabezpieczył próbek przed porastaniem. Badane olejki eteryczne okazały się nieskuteczne wobec grzyba *Trichoderma viride*.

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