Evaluation of Fungicidal Effects of Post-culture Medium of Selected Mold Fungi and Bacteria in Relation to *Basidiomycetes* Fungi, Causing Wood Destruction

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The results of the post-culture fungicidal medium from *Trichoderma viride* Pers. and *Alternaria alternata* (Fr.) Keissl. mold fungi and *Acetobacter xylinum* bacteria were studied relative to selected fungi belonging to *Basidiomycetes*, which cause wood decay. The obtained results confirmed that post-culture liquids derived from the cultivation of various microorganisms might have a differentiated fungicidal effect on wood-decaying fungi. The lowest concentration of fluid from *A. xylinum* culture added to the growth medium of the studied fungi that completely inhibited the growth was 5mL/100mL.The fungicidal effect of the liquid from the mold fungus culture on the tested wood-decaying fungi turned out to be definitely low. *Trametes versicolor* (L.) Lloyd proved to be the most sensitive species. *Pleurotus cornucopiae* (Paulet) Rolland showed complete resistance to the liquid added to the growth medium, derived from mold fungi. The *A. xylinum* bacterial culture-fluid may be subject to further analysis as a natural biocide in protecting wood against wood-decaying fungi.

Keywords: Fungicides; Post-culture liquid medium; Basidiomycetes

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INTRODUCTION

The durability and preservation of wood intended for specific use can be guaranteed by appropriate selection of protection means and impregnation methods. Wood preservatives are chemical compounds, whose market value and usage are subject to the restrictive provisions of Regulation EU 528/2012 on the supply and use of biocidal products on the market. The restrictive biocidal law does not allow the market placement of compounds with proven harmfulness to human, animal, and the environment. Changes in the biocidal law have resulted in the withdrawal of many effective impregnants formerly used in the protection of wood. Notably, products containing chromium and arsenic compounds, phenols, petroleum compounds, and fluorine compounds were permanently withdrawn from the market. The current market situation of wood preservatives includes a limited group of active substances, mainly copper compounds, boron, quaternary ammonium salts, HDO complexes, propiconazole, tebuconazole *etc*. Based on the data provided by the European Chemicals Agency (ECHA 2019), it is allowed to use biocidal products for wood impregnation containing 39 types of substances. The list of active substances is not closed, because Regulation EU 528/2012 provides the opportunity to conduct scientific research in the selection of new active substances with high fungicidal effectiveness and a guaranteed level of toxicological and

eco-toxicological safety. Bearing in mind the safety in the use of biocides, research is being carried out to assess the fungicidal activity of substances of natural origin, such as plant extracts, alkaloids, essential oils, but also substances produced by microorganisms (Kundzewicz and Ważny 1994; Cofta *et al.* 2014; Adedeji *et al.* 2017). Many substances of natural origin in fact have fungicidal activity, which was confirmed from *in vitro* tests (Barbero-López *et al.* 2018).

Studies on the effect of catechins, in particular 1,2-benzenediol (catechol), 1,3,5-benzenetriol, and dimethyl terephthalate on the growth of the *Schizophyllum commune* Fr. were conducted by Nandika *et al.* (2019). These researchers confirmed that a 12% concentration of compounds effectively inhibits the development of wood-decaying fungus. The fungicidal properties of teak extracts against fungi causing white and brown wood decay were the subject of research by Brocco *et al.* (2017). The authors of the study observed that wood samples impregnated in a vacuum method with extracts from 20-year-old teak were resistant to biodegradation processes caused by test fungi. Silveira *et al.* (2017) obtained similar positive results from the use of tannins to protect acacia wood against white wood rotting fungi.Substances derived from wood as fungicide were the subject of research by Salido *et al.* (2015), Alejo-Armijo *et al.* (2017), and Kadir and Hale (2019).

Fungicidal or fungistatic properties are also attributed to certain groups of microorganisms and their metabolites. Fidanza and Caneva (2019) reported that selected species of *Bacillus* sp. and numerous mold fungi belonging to the genera: *Aspergillus*, *Penicillium*, *Candida*, *Fusarium* or *Trichoderma* exhibited fungicidal and bactericidal activities against a wide group of microorganisms. At the same time, the data provided by ECHA that microorganisms such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus thuringiensis* subsp. *israelensis*, Strain SA3A and Serotype H14, Strain AM65-52 are listed as active substances and authorized for use in biocidal products intended for disinfection in the veterinary area and for insect control (ECHA 2019).

The analysis of the possibility of using metabolites from the *Trichoderma viride* culture for biological protection of wood against decomposition was already carried out in early 90s by Kundzewicz and Ważny (1994).

In this study, an attempt was made to assess the action of fungicide post-breeding medium from the culture of selected mold fungi: *Trichoderma viride* Pers., *Alternaria alternata* (Fr.) Keissl. and bacteria: *Acetobacter xylinum* against fungi causing decay of wood. The selection of the indicated microorganisms was dictated by the following criteria: selected mold fungi exhibit high growth expansion and are relatively resistant to wood preservatives, suggesting that they can produce metabolites that protect fungal cells from biocide toxicity. *Acetobacter xylinum* is a component of Kombucha biofilm, which produces many chemical substances with antimicrobial or antioxidant activities that could also be tested as biocides acting against wood-decaying microorganisms. The presented results have the nature of screening research, the purpose of which is preliminary and approximate determination of fungicidal activity as potential factors supporting the resistance of biomaterials, such as wood and wood-based materials against fungi. From the point of view of assessing the effectiveness of biocides, this type of research is important, and in addition it allows for a relatively quick assessment of the impact of the test substances on the development of microorganisms. The research was carried out on media having an increasing proportion of influence with respect to potential bioprotective properties. Preliminary assessment of the fungicidal activity of the culture media derived from the cultivation of mold fungi and bacteria made it possible to determine the lowest

concentration (MIC), completely inhibiting the growth of the tested fungi able to decay wood. The results obtained are the basis for planning further research work aimed at improving the bioprotective properties of various materials (wood, wood-based materials) based on the use of natural resistance factors obtained from the cultivation of microorganisms.

EXPERIMENTAL

Materials

The material that was used to assess fungicidal properties was post-culture liquid medium, derived from a 14-day-old culture of mold fungi *Trichoderma viride* Pers., strain A-102, *Alternaria alternata* (Fr.) Keissl., strain A-166, and bacteria *Acetobacter xylinum*, strain ATCC 23767. Mold fungi were grown on a liquid maltose medium containing 2.5% maltose extract (Biomaxima, Lublin, Poland). Bacteria were grown on liquid medium containing 2.5% mannitol (Pol-Aura, Zabrze, Poland), 0.3% peptone (Biomaxima, Lublin, Poland), and 0.5% yeast extract (Biomaxima, Lublin, Poland). Cultivation of microorganisms was carried out for 14 days in a heat-incubator under the following conditions: mold fungi at 22 °C, and bacteria at 26 °C. The relative humidity at the test site was 66% ± 2%. Only liquid post-culture medium was used to evaluate the fungicidal effect, and it was not modified. For this purpose, the liquid was separated from the microorganisms with a paper filter. Then, in order to eliminate the presence of microorganisms in the prepared post-culture liquid medium, it was sterilized using the syringe filter method. To eliminate all microbiological structures, 0.25 µm pore diameter filters (Nalge Company, Rochester, New York, USA) were used. The fluid prepared in this way was used as research material to assess the biocidal effect. The evaluation of fungicidal properties of the post-culture liquid from mold fungi and bacteria was carried out against strains of *Basidiomycetes* fungi causing deep wood decomposition: *Trametes versicolor* (L.) Lloyd strain 30, *Coniophora puteana* (Schumach.) P. Karst. strain EB 97, and *Pleurotus cornucopiae* (Paulet) Rolland (n.d.). Basal fungal cultures were grown on a maltose-agar medium containing 2.5% maltose extract (Biomaxima, Lublin, Poland) and 2.5% agar (Difco, New Jersey, 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.

Methods

The fungicidal properties of post-culture liquid medium from the cultivation of mold fungi and bacteria against *Basidiomycetes* were carried out by determining the percentage of growth inhibition in relation to the growth of reference fungi, *i.e.*, in control cultures not containing fungicides in the medium. The sterilized post-culture medium was added to a sterile medium containing 2.5% maltose and 2.5% agar. The temperature of the medium was kept at 42±2 °C. The fluid medium from the microbial culture was added to the maltose-agar medium in an amount of 1, 5, 10, 15, 20, and 30 mL in such a way that the final volume of medium was 100 mL. After mixing the contents thoroughly, about 10 mL of the medium was added onto 90 mm diameter Petri dishes, where the test fungi were centrally inoculated. The inoculum size of each fungi was 5 mm. Cultivation was carried out under temperature and humidity conditions of 25°C and 66%±2%, respectively. The evaluation of the effect of post-culture liquid

medium on the growth of test fungi was carried out by measuring the diameter of the growth of *T. versicolor*, *C. puteana*, and *P. cornucopiae* mycelium in two perpendicular directions. Height measurement was taken at 48 h intervals. Tests were completed on the day of complete growth of control medium. Each study was performed in triplicate.

Test Standards

To verify statistical hypotheses, analysis of variance was used for single classification using Snedecor statistics. Statistical inference was carried out for the significance level $\alpha = 0.05$. In case of rejection of the null hypothesis, the next step was to compare the mean multiple comparison test - Tukey's. The statistical hypothesis was as follows,

H0: Ø 0.5= Ø 1= Ø 5= Ø 10= Ø 15= Ø 20= Ø 30= Ø K

H1: There are at least two means that differ significantly.

RESULTS AND DISCUSSION

Analysis of the test results showed that the post-culture liquid medium, derived from the cultivation of *T. viride* and *A. alternata* mold fungi, inhibited the growth of *T. versicolor*, which causes white wood decay, but only at a specific concentration level. In the case of low share of post-culture fluid medium (1mL/100mL and 5mL/100mL) in the growth medium *T. versicolor*, no differences in growth diameter were observed compared to the control conditions, on the sixth day of observation. A clearly visible zone of inhibition of fungal growth appeared in the test in which the density of the post-culture medium in the growth medium was 10mL/100mL (Table 1). In contrast to *T. versicolor*, the fungus causing brown wood decay - *C. puteana* was more resistant to the fungicidal effect of the post-culture liquid medium, than *T. versicolor*, as indicated in the diameter of the fungus growth on the medium (Table2). Nevertheless, it was noted that fungicidal effects already appeared at the lowest concentrations of post-culture fluid in the growth medium. The results obtained during the last day of measurement, in which the complete mycelium growth of the medium was achieved in the control sample was analyzed. It was found that the inhibitory effect of the growth of *C. puteana* fungus by the ingredients contained in the post-culture liquid medium from mold fungi occurs in the test in whose share of post-culture medium was the smallest. A complete lack of growth inhibitory activity was found relative to the *P. cornucopiae* test fungus (Table 3). In the last day of observation, no significant differences were observed in the diameter of the mycelium growth of the medium under control conditions and in all research variants containing various shares of the media from *T. viride* and *A. alternata* cultures. Bruce and Highley (1991) observed a very similar effect of the effect of *T. viride* on the growth of *T. versicolor*, which may suggest that the indicated fungus causing white wood decay is relatively resistant to the potential fungicidal action of metabolites synthesized by *T. viride*. The low biocidal activity of the *T. viride* post-culture medium on the growth of *C. puteana* does not correlate with the results of other authors who at the cellular level obtained strong lysis of *C. puteana* cells due to the action of metabolites derived from mold fungal culture (de Vries and Wessels 1973). A much stronger action of the fungicide was obtained by examining the effect of the medium derived from *A. xylinum* bacterial culture on the growth activity of the studied fungi. The addition of 10%

bacterial culture medium to the fungal growth medium practically completely inhibited the growth of *T. versicolor*. On the last day of breeding, a slight growth of the fungus was observed. In the case of the effect of *A. xylinum* post-culture liquid medium on the growth of *P. cornucopiae* and *C. puteana*, effective fungicidal activity was demonstrated for concentrations of 5% and higher. The fungicidal properties of *A. xylinum* cultures against fungi causing wood decay have not been the subject of analysis, unlike other organisms belonging to *Actinobacteria* such as *Streptomyces exfoliatus* or bacteria *Pseudomonas* spp., the use of which in the protection of wood has been evaluated by other researchers (Susi *et al*. 2011; Sharma *et al*. 2016).

Table 1. Growth Diameter of *T. versicolor* on Media Containing Post-culture Liquid Medium from a *T. viride, A. alternata*, and *A. xylinum* Culture

Concentration of post-culture liquid from *T. viride* in growth medium (mL/100 mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	113.17	2.37
	Growth diameter of *T. versicolor* mycelium (mm)					Tukey's test	
0	8.2	51.0	90.0	-	-	a	
1	7.2	44.5	90.0	-	-	a	
5	7.3	45.0	90.0	-	-	a	
10	8.8	47.0	71.7	-	-	b	
15	5.5	28.0	64.2	-	-	c	
20	5.2	24.5	56.2	-	-	d	
30	(nd)	(nd)	(nd)	-	-	(nd)	
Concentration of post-culture liquid from *A. alternata* in growth medium (mL/100mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	81.03	2.56
	Growth diameter of *T. versicolor* mycelium (mm)					Tukey's test	
0	10.5	52.5	90.0	-	-	a	
1	(nd)	(nd)	(nd)	-	-	(nd)	
5	14.3	58.5	90.0	-	-	a	
10	10.3	46.0	72.3	-	-	b	
15	9.8	48.0	77.5	-	-	bc	
20	10.5	44.7	72.3	-	-	bc	
30	8.5	39.7	65.5	-	-	d	
Concentration of post-culture liquid from *A. xylinum* in growth medium (mL/100mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	5434.86	2.53
	Growth diameter of *T. versicolor* mycelium (mm)					Tukey's test	
0	16.5	49.2	81.8	-	-	a	
1	(nd)	(nd)	(nd)	-	-	(nd)	
5	0.0	19.2	44.8	-	-	b	
10	0.0	0.0	0.0	-	-	c	
15	0.0	0.0	0.0	-	-	d	
20	0.0	0.0	0.0	-	-	d	
30	0.0	0.0	0.0	-	-	d	
(nd) – no data							

Table 2. Growth Diameter of *C. puteana* on Media Containing Post-culture Liquid Medium from a *T. viride, A. alternata*, and *A. xylinum* Culture

Concentration of post-culture liquid from *T. viride* in growth medium (mL/100mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	81.03	2.39
	Growth diameter of *C. puteana* mycelium (mm)					Tukey's test	
0	16.2	47.8	73.8	90.0	-	a	
1	15.7	47.2	73.5	88.5	-	ab	
5	14.8	46.8	72.8	90.0	-	a	
10	12.8	41.7	66.8	83.7	-	bc	
15	11.7	40.0	62.5	79.7	-	cd	
20	13.8	41.2	65.8	78.0	-	cde	
30	(nd)	(nd)	(nd)	(nd)	-	(nd)	
Concentration of post-culture liquid from *A. alternata* in growth medium (mL/100mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	9.81	2.50
	Growth diameter of *C. puteana* mycelium (mm)					Tukey's test	
0	13.5	55.5	90.0	-	-	a	
1	(nd)	(nd)	(nd)	-	-	(nd)	
5	14.8	52.3	87.8	-	-	ab	
10	14.5	53.2	80.5	-	-	abc	
15	11.8	50.5	79.2	-	-	bc	
20	11.5	48.0	78.8	-	-	bc	
30	12.2	48.7	75.5	-	-	c	
Concentration of post-culture liquid from *A. xylinum* in growth medium (mL/100mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	4.7×10^{13}	2.53
	Growth diameter of *C. puteana* mycelium (mm)					Tukey's test	
0	11.5	36.0	75.0	90.0	-	a	
0,5	11.8	39.5	79.3	90.0	-	a	
1	11.5	42.7	83.3	90.0	-	a	
2	12.0	39.3	78.8	90.0	-	a	
5	0.0	0.0	0.0	0.0	-	b	
10	0.0	0.0	0.0	0.0	-	b	
(nd) – no data							

Table 3. Growth Diameter of *P. cornucopiae* on Media Containing Post-culture Liquid Medium from a *T. viride, A. alternata*, and *A. xylinum* Culture

Concentration of post-culture liquid from *T. viride* in growth medium (mL/100mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	2.44	2.39
	Growth diameter of *P. ostreatus* mycelium (mm)					Tukey's test	
0	9.5	36.8	83.8	90.0	-	a	
1	10.3	36.2	78.8	87.7	-	a	
5	11.8	39.8	77.0	87.2	-	a	
10	10.0	38.2	78.0	87.8	-	a	
15	11.2	34.8	51.5	85.8	-	a	
20	11.0	33.7	44.5	85.3	-	a	
30	(nd)	(nd)	(nd)	(nd)	-	(nd)	
Concentration of post-culture liquid from *A. alternata* in growth medium (mL/100mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	1.00	2.53
	Growth diameter of *P. ostreatus* mycelium (mm)					Tukey's test	
0	11.7	47.8	74.8	90.0	-	a	
1	(nd)	(nd)	(nd)	(nd)	-	(nd)	
5	8.3	42.5	69.7	90.0	-	a	
10	8.2	38.0	63.2	90.0	-	a	
15	8.8	45.5	68.7	90.0	-	a	
20	10.2	45.8	73.2	90.0	-	a	
30	10.3	44.3	69.8	90.0	-	a	
Concentration of post-culture liquid from *A. xylinum* in growth medium (mL/100mL)	Day of observation					F_{emp}	$F_{0,05}$
	2	4	6	8	10	23.98	2.53
	Growth diameter of *P. ostreatus* mycelium (mm)					Tukey's test	
0	10.5	27.8	71.7	90.0	-	a	
0,5	9.5	28.8	70.5	90.0	-	a	
1	9.8	28.8	68.5	85.3	-	ab	
2	9.3	28.7	63.7	80.3	-	abc	
5	0.0	0.0	0.0	0.0	-	d	
10	0.0	0.0	0.0	0.0	-	d	
(nd) – no data							

The percentage growth rate of fungi on the media containing various proportions of post-culture liquid medium, derived from the cultivation of mold fungi and bacteria is presented in Table 4 and Figs. 1, 2, and 3. In studies involving the fungicidal action of the post-culture liquid medium from mold fungi on the *T. versicolor* fungus, statistically significant differences were found between the control and the medium containing 5% and a higher concentration of post-culture liquid medium. In the case of the *C. puteana* fungus, statistically significant differences in growth compared to the control sample were found on a medium containing 15% and a higher concentration of post-culture medium. Similar analyzes of the effect of fungal extracts on the growth of *C. puteana*

were conducted by Yildiz *et al*. (2019). The fungicidal effects of fungal extracts on many saprophytic and parasitic fungi have been studied by Okeke *et al*. (1992), Sudirman *et al*. (1992), and Highley (1997). This activity was attributed to the production of metabolites with antibiotic functions by many fungi, which inhibit the development of other organisms (Yang *et al*. 1993).

Table 4. Percentage Growth of Fungi on Medium, Containing a Post-culture Liquid Medium from Mold Fungi and Bacteria

The amount of post-culture liquid medium (mL/100mL)	Percentage of overgrowth on the last day of breeding (%)		
T. viride	*T. versicolor*	*C. puteana*	*P. cornucopiae*
0	100	100	100
1	100	98.3	97.4
5	100	100	96.9
10	79.0	93.0	97.6
15	71.3	88.5	95.3
20	62.4	86.7	94.8
30	(nd)	(nd)	(nd)
A. alternata			
0	100	100	100
1	(nd)	(nd)	(nd)
5	100	97.6	100
10	86.1	89.4	100
15	80.3	88.0	100
20	80.3	87.5	100
30	72.8	83.9	100
A. xylinum			
0	100	100	100
0,5	(nd)	100	100
1	(nd)	100	94.8
2	(nd)	100	89.3
5	83.1	0.0	0.0
10	8.3	0.0	0.0
15	0.0	(nd)	(nd)
20	0.0	(nd)	(nd)
30	0.0	(nd)	(nd)
(nd) – no data			

None of the applied post-culture liquid medium concentrations from the mold fungi culture achieved complete inhibition of the growth of the test fungi. It is likely that, despite the use of high concentrations of post-culture medium, the proportion of active substances with fungicidal activity did not have to be high. It has been demonstrated that many factors can affect biocidal effectiveness. Both the bioavailability of given substances, and the extraction method and conditions may affect the results of toxicity obtained (Jo *et al*. 2014).

In the case of evaluation of the fungicidal effect of the post-culture liquid medium, derived from the culture of *A. xylinum* bacteria in relation to the tested fungi, statistically significant differences between the control and the tested samples were found at much lower concentrations than it was the case when assessing the effectiveness of the culture medium from mold fungi.

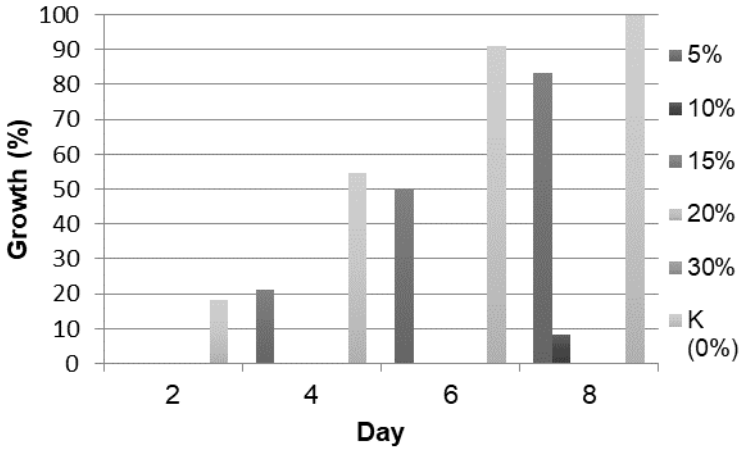

Fig. 1. Relative percentage of growth of *T. versicolor* on medium containing post-culture liquid medium from *A. xylinum* culture

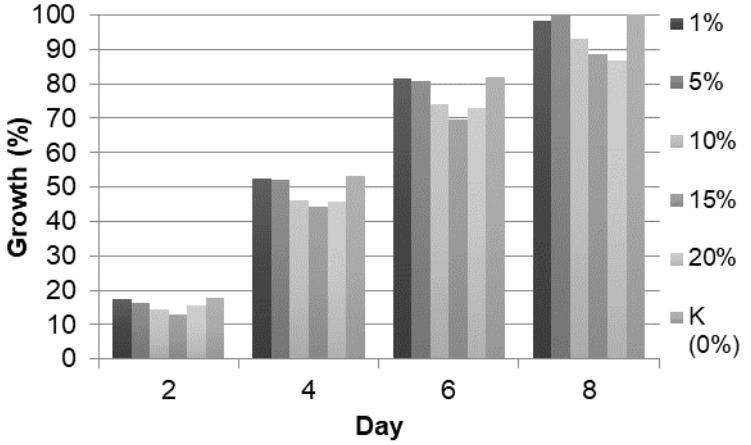

Fig. 2. Relative percentage of growth of *C. puteana* on medium containing post-culture liquid medium from *T. viride* culture

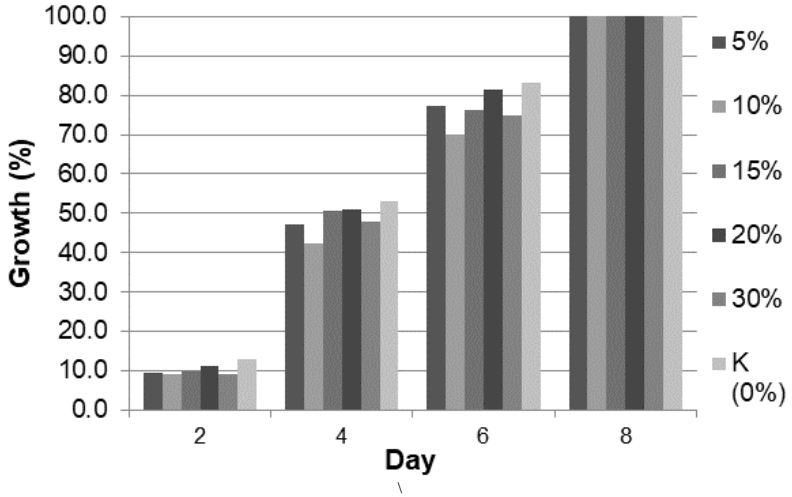

Fig. 3. Relative percentage of growth of *P. cornucopiae* on medium containing post-culture liquid medium from *A. alternata* culture

CONCLUSIONS

1. Results of this study showed that the tested fungi exhibited various levels of sensitivity to the toxic effects of medium from microbial culture.
2. Post-cultural medium derived from the cultivation of molds *A. alternata* and *T. viride* did not cause as pronounced inhibition of fungal growth as it was in the experiment using liquid medium from bacterial culture.
3. *P. cornucopiae* turned out to be a species insensitive to the toxic effects of post-cultural medium from mold fungi.
4. The post-culture medium from *A. alternata* and *T. viride* fungi cultures did not show a clear fungicidal activity against the tested fungi causing deep wood decay.
5. The post culture medium from *A. xylinum* bacteria showed strong fungicidal activity against tested wood-decaying fungi, which gives the basis for its further use in tests for determining bio-protective values under practical conditions of use in wood and wood-based materials.

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