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ORIGINAL ARTICLE

Potential of pure and mixed cultures of *Cladosporium cladosporioides* and *Geotrichum candidum* for application in bioremediation and detergent industry

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Abstract The effect of ethoxylated oleyl–cetyl alcohol (Henkel, "Merima", Serbia) on the growth and metabolic activity of *Cladosporium cladosporioides*, *Geotrichum candidum* and their mixed culture was in the focus of this paper. The cultures were grown in Czapek-Dox liquid nutrient medium with the addition of 0.5% pollutant and without it. The physico-chemical and biochemical changes of pH, the total biomass dry weight, the quantity of free and total organic acids, proteolytic activity and the quality of carbohydrates were evaluated from 4th to 19th day of fungal growth. The pollutant caused an inhibitory effect on biomass dry weight of *C. cladosporioides* and *G. candidum* for 10.36% and 4.65% respectively, and stimulatory effect on biomass of mixed culture for 3.80%. The pollutant had influence on the decrease in pH value of the media in the phase of culture growth, and pH changes were correlated with the amount of excreted total organic acids. The highest quantity of free and total organic acids was noted in media with pollutant of mixed culture and *C. cladosporioides*, respectively. The alkaline protease activities of *C. cladosporioides*, *G. candidum* and mixed culture were enhanced by addition of pollutant for 56.88%, 55.84% and 30.94% respectively. The obtained results indicate the potential of both pure and mixed cultures in mycoremediation environment contaminated by alcohol ethoxylated and detergent industry.

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1. Introduction

Fatty alcohol ethoxylates (FAEs) are the leading group of non-ionic ethoxylated surfactants (Szymanski et al., 2000) and make up the second highest volume group of surfactant after linear alkylbenzene sulfonate. By far, the greatest use of FAEs is in domestic detergents, household cleaners and

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personal care products such as shampoos, but they are also used as penetration promoters in insecticides. After use, surfactants as well as their products are mainly discharged into sewage treatment plant and then dispersed into environment by releasing effluents into surface waters and by sludge disposal on land (Olkowska et al., 2014). FAEs are easily biodegraded in aerobic and anaerobic conditions, although the massive dumping of these surfactants into sewage waters requires strict control to prevent the pollution of the aquatic environment. In order to perform the purification of wastewaters from different xenobiotics, combinations of physical, chemical and biological methods have been developed (Araujo et al., 2008; Mo et al., 2008). Due to the side effects that accompany the use of the physical and chemical methods, the application of new technologies such as bioremediation is necessary (Haritash and Kaushik, 2009).

Bioremediation is a process in which indigenous or inoculated microorganisms (e.g., fungi, bacteria, and other microbes) degrade (metabolize) organic contaminants found in soil and water, converting them to innocuous end products (Bavarva, 2015). Thus, the identification of microorganisms indigenous to high-contaminated environment ought to be the first step of bioremediation process. A large number of fungal enzymes (e.g., peroxidases, oxidoreductases, cellulolytic enzymes, proteases, amylases, etc.) have been reported to play an important role in an array of waste treatment application, because there is growing interest in application of fungi in bioremediation. Published papers (Fakhru'l-Razi and Molla, 2007) report the use of a wide variety of pure and mixed cultures of fungi, including micromycetes of genus *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, *Cladosporium*, etc. for biopurification of soils and waters contaminated by different hydrocarbons.

The occurrence of *Geotrichum candidum* and *Cladosporium cladosporioides* in sewage and industrial wastewater and sewage sludge is reported in studies of Cooke and Pipes (1970), Diener et al. (1976) and Fluery (2007). The ability of *G. candidum* to degrade various organic wastes such as phenolics (Garcia Garcia et al., 1997, Landeka Dragičević et al., 2010) and glycerol trinitrate (Singh and Ward, 2004) or its ability to decolorize different azo and anthraquinone dyes (Kim et al., 1995) is well documented. On the other hand, *C. cladosporioides* is effective in degradation of [*o*,*o*-diethyl-*o*-(3,5,6-trichloro-2-pyridyl)phosphorothioate] and its hydrolysis product (Chen et al., 2012), of concrete (Wei et al., 2013) and different azo and triphenylmethane dyes (Vijaykumar et al., 2006). However, no literature data exist about the role of these fungi in biodegradation ethoxylated alcohol or other non-ionic surfactants (NSs). Our previous studies (Jakovljević et al., 2014; Stojanović et al., 2011a,b) confirmed that several species of fungi (*Fusarium oxysporum*, *Aspergillus niger*, *Trichothecium roseum*, etc.) originated from municipal wastewaters, able to grow and metabolize EOCA at a wide concentration range 0.01–1%. Taking into consideration the fact that literature provides the evidence of the powerful biodegradation potential of *G. candidum* and *C. cladosporioides* and due to the fact that they are abundant in man-made contaminated wastewater, these particular, abovementioned fungal species are chosen as test organisms in this study.

The current study was conceived in order to investigate the effect of EOCA on the growth and changes of metabolic activity of pure cultures *G. candidum* and *C. cladosporioides* and their mixed culture isolated from wastewater, since these parameters are crucial for the application of fungi in mycoremediation. Additionally, stability and high activity of fungal alkaline proteases in the presence of tested pollutant can have a practical application in the detergent industry.

2. Experimental

2.1. Isolation and identification of fungi from wastewater

Pure cultures of micromycetes *C. cladosporioides* (Fresen) G. A. de Vries (1952) and *G. candidum* link (1809) were isolated from samples of wastewater river basin of Lepenica (Kragujevac, Serbia) (the place of wastewater flood, sewage) according to the standard procedure. The isolation and identification of pure cultures from a sample of wastewater were carried out based on the macroscopic and microscopic morphology at the Faculty of Biology, University of Belgrade, Serbia. The fungi were raised on PDA plates at (28 ± 2 °C) from 3- to 5-days until sporulation. After having sufficient population of spores, the plates were stored at (4 ± 0.5 °C) and subcultured monthly in sterile conditions.

2.2. Spore inocula preparation

Inoculum suspensions were prepared from fresh, mature cultures as described above. The colonies were covered with 5 mL of distilled sterile water. The inoculums were achieved by carefully rubbing the colonies with a sterile loop; the isolates were shaken vigorously for 15 s with a Vortex mixer and then transferred to a sterile tube. The inoculum sizes were adjusted to 1.0×10^6 spores/mL by microscopic enumeration with a cell-counting hematocytometer (Neubauer chamber).

2.3. Experimental procedure and culture condition

Three sets of triplicate 250 mL Erlenmeyer flasks were prepared for screening of *C. cladosporioides* and *G. candidum* and their mixed culture. Each flask contained 100 mL of Czapek Dox liquid nutrient media, which was prepared according to the procedure presented in Table 1. The pH value of nutrient media was adjusted at 4.8 with 0.1 mol L^{-1} HCl before sterilization. All flasks were sterilized at 121 °C in an autoclave for 15 min. After cooling the liquid media to room temperature, 1 mL spore suspension of individual and mixed cultures of fungi was inoculated in liquid media in aseptic condition. Inoculated flasks were incubated on an electric shaker (Kinetor-*m*, Ljubljana) at 150 rpm and ambient temperature for 19 days. The flasks were harvested at 4th, 7th, 10th, 14th and 19th day after inoculation. To observe the fungal growth, mycelium was removed by filtration of fermentation broth, according to the procedure described below. Fermentation broth was used for determination of pH, organic acids, carbohydrates and protease activity.

2.4. Determination of dry weight biomass

After the separation of the mycelium from the fermentation broth by filtration through a filter paper of a known weight, the mycelium was washed with deionized water several times.

Table 1 Composition of growth media in 1000 mL distillated water.

Growth medium	Denote	C (g L^{-1})					
		$NaNO_3$	K_2HPO_4	$MgSO_4 \times 7H_2O$	$FeSO_4 \times 7H_2O$	Sucrose	EOCA[a]
Control	C	3	1	0.5	0.01	30	–
C + 0.5% EOCA	EOCA	3	1	0.5	0.01	30	5

[a] Ethoxylated oleyl–cetyl alcohol (Henkel, "Merima", Serbia).

Thereafter, the filter paper with the mycelium was dried in an oven at 80 °C to a constant weight and re-weighed. Mycelia dry weight was calculated by subtracting the initial weight of the filter paper from the weight of dried mycelia and filter paper. Results are expressed in grams per liter (g L^{-1}) of submerged culture.

2.5. The measurement of pH values

A pH value of the fermentation broth in the experiment was measured by a pH meter (type MA-5705, the product "Iskra", Kranj) with calomel electrode that was initially standardized with appropriate buffer solution of pH 4, 7 and 10.

2.6. Determination of concentrations free (FOA) and total (TOA) organic acids

Concentrations of FOA and TOA were determined by ion exchange chromatography method according to the method by Bulen et al. (1952). The quantity of 50 mL of ethanol (70%) was added to 10 mL of fermentation broth and the reaction mixture was incubated at 70 °C in water bath for 1 h. The mixture was filtered through Whatman filter paper No. 1 and filtrate was concentrated at 50–60 °C under reduced pressure to final extract volume of 40 mL. Active charcoal was added to the extract followed by incubation (30–45 min) in the water bath at 70 °C. After incubation, the extract was filtrated to remove active charcoal; the residue was made up to a volume of 100 mL with distilled water. Ten milliliters of aliquots of filtrate was sampled for the determination of concentration of the FOA by titration 0.1 mol L^{-1} NaOH. Phenolphthalein (0.1%) was used as indicator. The residual of sampling (90 mL) was passed through a cationic column (Amberlite IR-120) previously activated, to the volumetric flask of 250 mL. By washing the column with distilled water, volumetric flask was supplemented to 250 mL. To determine the concentration of TOA, 25 mL aliquots were sampled and titration was carried out as previously described. The results were presented in grams per liter (g L^{-1}).

2.7. The qualitative analysis of carbohydrates by paper chromatography

The rest of fermentation broth (225 mL) passed through the activated anionic column (Amberlite IR-400) followed by evaporation to a volume of 5 mL. Ascending paper chromatography was employed for separation of carbohydrate by Williams's method with slight modifications. Whatman No. 1 filter paper strips (10.5 cm by 35 cm) were used for the separations of carbohydrate. A solution of *n*-butanol: acetic acid: water in volume proportions 4:1:5, respectively was used as developing solvent for separation of the sugar. Triplicate samples of fermentation broth in the volume of 4 μL were placed on the Whatman No. 1 filter paper strips, dried, and developed for 8 h in the irrigating solvent. Upon the completion of development, the strips were dried rapidly in a hot air current. After drying, the chromatograms are treated by silver nitrate solution in acetone, and again dried in an oven at 100 °C for 5–10 min. The dried chromatograms were treated by 0.5 mol L^{-1} potassium hydroxide solutions in ethanol. After drying, the strips appear at points where there are carbohydrates. Qualitative identification was made by comparing the R_f values of the samples used with those of known sugars. The R_f values were determined by dividing the distance of the unknown spot moved by the distance of the solvent front moved.

2.8. Assay of alkaline protease activity (EC 3.4.21–24)

The assay of alkaline protease was carried out according to Anson's method (1938). The reaction mixture, which contained 5 mL of casein and 1 mL fermentation broth, was incubated at 37°C for 30 min. The reaction was stopped by adding 1 mL of 5% trichloroacetic acid (TCA). The mixture was centrifuged at 4000 rpm/min and then 5 mL of 6% Na_2CO_3 and 1 mL diluted Folin–Ciocalteu's phenol reagent were added to supernatant. The solution was kept at room temperature for 30 min and absorbance was read at 660 nm using tyrosine standard. One unit of alkaline protease activity was defined as the amount of enzyme capable of producing 1 μg of tyrosine from casein in a minute under assay condition.

2.9. The statistical analysis

All experiments were performed in triplicate and the results were expressed as means ± standard deviation. For the statistical analysis, the following tests were used: Mann–Whitney, Kruskal–Wallis, Spearman's and Pearson tests for correlation coefficient by SPSS (Chicago, IL) statistical software package (SPSS for Windows, v. 13, 2004). Coefficient of correlation at the 0.05 and 0.01 levels of significance was tested.

3. Results and discussion

3.1. Effect of pollutant on fungal biomass

The production of biomass dry weight of pure and mixed cultures measured in C and EOCA media after 19 days' cultivation is shown in Table 2. In C medium, the highest biomass dry weight was produced by *C. cladosporioides* (1.93 g L^{-1}), followed by mixed culture (1.84 g L^{-1}) and *G. candidum*

Table 2 Effect of ethoxylated oleyl–cetyl alcohol on biomass dry weight production.

Biomass (dry weight) comparison after 19-day cultivation				
Fungal isolate	C (g L^{-1})	EOCA (g L^{-1})	Inhibition or stimulation via treatment (EOCA)	
			Weight (g L^{-1})	(%)
Cladosporium cladosporioides	1.93 ± 0.24	1.73 ± 0.23	−0.20[a]	10.36[a]
Geotrichum candidum	1.72 ± 0.20	1.64 ± 0.22	−0.08[a]	4.65[a]
Mixed culture	1.84 ± 0.16	1.91 ± 0.30	0.07[b]	3.80[b]

[a] Inhibition.
[b] Stimulation.

(1.72 g L^{-1}). Different growth rates between pure cultures can be explained by their morpho-physiological differences that affect different responses for the adoption and nutrient transport. On the other hand, biomass dry weight of mixed culture produced in C medium was between the biomass values of respective pure cultures. This result can be interpreted by synergistic effect between pure cultures due to mutual interaction (Oliveira et al., 2011). Apparently, the interactions between the members of community are important in a mixed culture. According to More et al. (2010), the growth of interaction fungi is influenced by the time interval, which is higher for fast growing fungi than slow growing fungi. In addition, one member of community usually synthesizes some strong fungal metabolites, which restricted the growth of the other. As a result of mutual interaction in current mixed culture, the biomass of *G. candidum* was enhanced for 6.52% whereas the biomass of *C. cladosporioides* was suppressed for 4.89%.

In a medium with EOCA, biomass of *C. cladosporioides* and *G. candidum* was inhibited for 10.36% and 4.65%, respectively. Obviously, *G. candidum* had better response to the presence of this pollutant and lower extent of biomass inhibition than *C. cladosporioides*. This finding could be explained of both morpho-physiological characteristics of fungi and the influence of the surfactant on active centers of the key enzymes in fungal metabolism. The obtained results are consistent with our previous studies, which revealed a mild stimulatory effect of EOCA on the biomass dry weight of *T. roseum* (Stojanović et al., 2011b) and *F. oxysporum* (Jakovljević et al., 2014) and very strong inhibitory effect on the biomass of *A. niger* (Stojanović et al., 2011a). Recently, Hasan (2014) has revealed a significant stimulation in fresh weight of *A. niger* in 10% kerosene broth. Unlike pure cultures, biomass dry weight of mixed culture in medium with EOCA was stimulated for 3.80%. In this case, interaction between pure cultures caused stimulation of biomass production of both *C. cladosporioides* and *G. candidum* for 9.42% and 14.14%, respectively. This result indicates the ability of mixed culture to degrade EOCA efficiently than pure cultures. The results of other authors (Asgher et al., 2012; Cetin and Donmez, 2006) also provided the evidence that mixed cultures are more efficient in biodegradation processes than single cultures. Ammen et al. (2016) reported that among five pure cultures and their consortium, consortium achieved the highest amount of biomass in a medium with 20% diesel fuel.

3.2. Effect of EOCA on pH media

Since the pH value has a significant impact on fungal physiology and biochemistry and efficiency of biodegradation, the changes in pH value of growth media of pure and mixed cultures were examined. The obtained results are summarized in Table 3. Fungi generally alter the pH of the medium in which they grow due to uptake of the anions and cations in the medium. The pH values of C media of *C. cladosporioides*, *G. candidum* and mixed culture measured at 4th day after inoculation were 8.31, 7.02 and 6.08 units, respectively. During the growth of pure cultures from 4th to 10th day, the pH value of C media was decreasing. The highest decrease in pH value was observed in medium of *C. cladosporioides* (1.22 units), followed by in medium of *G. candidum* (0.67 units). From that point on, the pH value of media of both pure cultures was increasing until the end of experiment. This result could be an indicator of autolysis. The pH value of C medium of mixed culture was increasing during whole cultivation period. The considerable increase in pH value (0.77 units) was noted from 4th to 10th day. The addition of EOCA in medium caused an increase in initial pH value toward alkaline condition compared to control. From the fermentation beginning until 10th (*G. candidum* and mixed culture) and 14th day (*C. cladosporioides*), the pH value of EOCA media was continuously decreasing. The most significant decrease in pH value was measured in medium of *C. cladosporioides* (2.26 units) followed by in mixed culture (0.60 units) and *G. candidum* (0.27 units). According to statistics, negative correlation was found between the pH value and the amount of FOA ($r = -0.528$, $p < 0.05$) as well as between the pH value and the amount of TOA ($r = -0.514$, $p < 0.05$) in EOCA media. The decrease in pH value during treatment of sludge by fungi was observed and well discussed by several authors (Fakhru'l-Razi and Molla, 2007). Hong et al. (2015) revealed decreasing the pH values of the four fun-

Table 3 Changes in pH value of growth media of pure and mixed cultures.

Changes in pH value during 19-day cultivation			
Fungal isolate	Days	C (Units)	EOCA (Units)
Cladosporium cladosporioides	4	8.31 ± 0.15	8.56 ± 0.26
	7	8.23 ± 0.20	8.36 ± 0.24
	10	7.01 ± 0.10	6.10 ± 0.15
	14	7.21 ± 0.18	5.98 ± 0.15
	19	7.22 ± 0.12	6.84 ± 0.28
Geotrichum candidum	4	7.02 ± 0.10	7.22 ± 0.25
	7	6.98 ± 0.15	7.05 ± 0.20
	10	6.19 ± 0.23	6.78 ± 0.29
	14	6.66 ± 0.19	7.09 ± 0.25
	19	6.54 ± 0.15	7.35 ± 0.16
Mixed culture	4	6.08 ± 0.14	7.89 ± 0.23
	7	6.85 ± 0.18	7.89 ± 0.25
	10	7.32 ± 0.10	7.29 ± 0.19
	14	7.33 ± 0.15	7.37 ± 0.15
	19	7.46 ± 0.23	7.68 ± 0.20

gal cultures in submerged fermentation process using paper mill sludge as the substrate.

3.3. The effect of the pollutant on the quantity of FOA and TOA

The exudation of organic acids by fungus mycelium plays an important role in the effectiveness of bioremediation and uptake of contaminants (Magdziak and Drzewiecka, 2011). Due to, the production of organic acids in growth media of pure and mixed cultures was examined in this study. The cultures excreted variable amounts of organic acids in C and EOCA media, depending on medium composition and a period of cultivation, as Table 4 shows. The quantity of FOA measured in C medium of *C. cladosporioides*, *G. candidum* and mixed culture was in the following range: 0.36–0.96 g L^{-1}, 0.12–1.0 g L^{-1} and 0.20–0.36 g L^{-1}, respectively. Evidently, the mixed culture excreted about 2-fold lower amount of FOA compared to pure cultures. A high correlation was found between the quantity of FOA and biomass dry weight of cultures in C media ($r = 1.000$, $p < 0.01$). The quantity of FOA achieved in EOCA media of *C. cladosporioides*, *G. candidum* and their mixed culture was in the range: 0.16–0.68 g L^{-1}, 0.20–0.64 g L^{-1} and 0.20–1.40 g L^{-1}, respectively. In this medium, the mixed culture excreted about 2-fold higher quantity of FOA than pure cultures. According to statistics, a moderate correlation was found between the quantities of FOA and TOA in EOCA media ($r = 0.577$, $p < 0.05$) and between the quantity of FOA and length of cultivation ($r = 0.564$, $p < 0.05$), which can be explained by the fact that FOA are secondary metabolites which need time to accumulate (Cvetković and Markov, 2002).

The amount of TOA excreted in C media of pure and mixed cultures was also variable (Table 4). *C. cladosporioides* excreted from 1.16 to 3.36 g L^{-1}, whereas *G. candidum* and mixed culture excreted from 2.24 to 4.64 g L^{-1} and 1.60 to 3.36 g L^{-1} of TOA respectively. A moderate negative correlation was found between the amount of TOA excreted in C media and the length of the cultivation ($r = -0.631$, $p < 0.05$). In EOCA media, the highest amount of TOA was produced by *C. cladosporioides* (5.76 g L^{-1}) followed by mixed culture (3.68 g L^{-1}) and *G. candidum* (3.36 g L^{-1}). The presented results show that *C. cladosporioides* excreted about 1.5-fold higher amount of TOA in relation to *G. candidum* and mixed culture. A high positive correlation was found between the quantity of TOA and biomass dry weight of cultures ($r = 0.999$, $p < 0.01$). To data, numerous studies have confirmed that organic acids produced by fungi could be applied as leaching agent for toxic metals. Sukla et al. (1992) found that culture filtrates obtained from *A. niger* have been used to leach Cu, Ni and Co from copper converter slag. Bosshard et al. (1996) revealed that some organic acids such as citric acid produced by *A. niger* may cause the leaching of Cu, Cd, Zn, Mn, Pb, Cr and Al from red mud and ashes derived from the incineration of municipal waste. The current study has provided the evidence that tested fungi, particularly *C. cladosporioides* produce a considerable amount of organic acids in applied experimental conditions, which is a good base for further investigation in terms of their practical application in bioremediation processes.

3.4. Effect of pollutant on alkaline protease activity (EC 3.4.21–24)

The examination of hundreds of fungal and bacterial genomes shows that most encode proteases. For practical application of protease in detergent formulation, an important characteristic is their stability in the presence of surfactants (Niyonzima and More, 2015). For this purpose, the alkaline protease activity of *C. cladosporioides* and *G. candidum* and their mixed culture in the presence of EOCA was evaluated in this study.

Table 5 shows the results of alkaline protease activities of pure and mixed cultures in C and EOCA media. As the table shows, the enzyme activity of cultures in C medium was variable extent, from the total inhibited to pronounce. The minimum protease activity was generally observed in first half of cultivation, from 4th to 10th day. The maximum protease

Table 4 Quantities of organic acids measured in growth media of pure and mixed cultures.

Quantities of organic acids during 19-day cultivation					
Fungal isolate	Days	Free organic acids		Total organic acids	
		C (g L^{-1})	EOCA (g L^{-1})	C (g L^{-1})	EOCA (g L^{-1})
Cladosporium cladosporioides	4	0.60 ± 0.18	0.16 ± 0.03	2.56 ± 0.52	2.72 ± 0.70
	7	0.64 ± 0.15	0.16 ± 0.05	3.36 ± 0.74	3.04 ± 0.84
	10	0.96 ± 0.23	0.36 ± 0.10	1.16 ± 0.25	3.36 ± 0.80
	14	0.76 ± 0.16	0.68 ± 0.16	2.36 ± 0.43	5.76 ± 1.16
	19	0.36 ± 0.09	0.24 ± 0.06	2.08 ± 0.36	1.60 ± 0.35
Geotrichum candidum	4	0.12 ± 0.03	0.20 ± 0.05	3.04 ± 0.56	1.44 ± 0.28
	7	0.48 ± 0.16	0.28 ± 0.08	3.36 ± 0.83	3.20 ± 0.65
	10	1.00 ± 0.25	0.64 ± 0.20	4.64 ± 0.82	3.36 ± 0.63
	14	0.56 ± 0.15	0.28 ± 0.08	2.72 ± 0.60	3.04 ± 0.24
	19	0.16 ± 0.10	0.24 ± 0.05	2.24 ± 0.54	1.28 ± 0.15
Mixed culture	4	0.32 ± 0.06	0.20 ± 0.04	3.36 ± 0.62	1.12 ± 0.26
	7	0.36 ± 0.09	0.25 ± 0.05	2.72 ± 0.50	2.24 ± 0.57
	10	0.36 ± 0.09	0.64 ± 0.12	2.24 ± 0.27	3.68 ± 0.72
	14	0.24 ± 0.05	0.48 ± 0.19	2.08 ± 0.34	2.72 ± 0.35
	19	0.20 ± 0.02	1.40 ± 0.35	1.60 ± 0.40	2.40 ± 0.20

Table 5 Activities of alkaline protease measured in growth media of pure and mixed cultures.

Alkaline protease activities during 19-day cultivation				
Fungal isolate	Days	C (IU mL^{-1})	EOCA (IU mL^{-1})	Enhance (%)
Cladosporium cladosporioides	4	0.212 ± 0.024	0.088 ± 0.015	56.88
	7	0.159 ± 0.036	0.513[a] ± 0.125	
	10	0.097 ± 0.025	0.198 ± 0.052	
	14	0.327[a] ± 0.078	0.299 ± 0.073	
	19	0.176 ± 0.046	0.106 ± 0.038	
Geotrichum candidum	4	0.087 ± 0.015	1.041[a] ± 0.234	55.84
	7	0.668[a] ± 0.174	0.353 ± 0.092	
	10	0.056 ± 0.010	0.120 ± 0.025	
	14	0.449 ± 0.055	0.101 ± 0.010	
	19	0.141 ± 0.023	0.124 ± 0.015	
Mixed culture	4	0.047 ± 0.015	0.016 ± 0.008	30.94
	7	0.040 ± 0.012	0.146 ± 0.024	
	10	0.131 ± 0.025	0.592 ± 0.132	
	14	0.398 ± 0.088	0.601[a] ± 0.175	
	19	0.459[a] ± 0.119	0.512 ± 0.125	

[a] Enzyme maximum.

activity of cultures measured in C media was in the following order: 0.668 IU mL^{-1} (*G. candidum*), 0.459 IU mL^{-1} (mixed culture) and 0.327 IU mL^{-1} (*C. cladosporioides*). These maximal values were expressed in different phases of culture growth and can be connected to autolysis (*C. cladosporioides* and mixed culture) and mycelial growth (*G. candidum*). The statistical analysis showed that significant differences ($p < 0.05$) in alkaline protease activity were found between pure cultures as well as between the pure and mixed cultures. According to our opinion, the differences in protease activity between pure cultures could be influenced by the metabolism of carbohydrates (inhibition of some key enzymes or different flux of glucose and fructose). The synergism of pure cultures in growth medium was influenced balance in protein degradation. Therefore, protease activity of mixed culture was between the enzyme activity values of pure cultures.

In EOCA media of *C. cladosporioides* and mixed culture, minimum of alkaline protease activity was measured at the beginning of fermentation (4th day). The maximum protease activity of cultures achieved in EOCA media was in the following order: 1.041 IU mL^{-1} (*G. candidum*), 0.601 IU mL^{-1} (mixed culture) and 0.513 IU mL^{-1} (*C. cladosporioides*). Apparently, the mentioned pollutant had the strongest stimulatory effect on protease activity of *C. cladosporioides* (56.88%) followed by *G. candidum* (55.84%) and mixed culture (30.94%). An overview of literature provides the contradictory data related to the impact of NSs on proteolytic activity of microorganisms. Evans and Abdullahi (2012) underlined that surfactants affect the permeability of the cell membrane through disruption of lipid bilayer thereby increasing the uptake of nutrient into the organism and the secretion of enzyme into the culture medium. Grbavčić et al. (2009) emphasized that NSs type of ethylene oxides, binds to active site of enzymes through hydrogen bonds in order to enhance conformation flexibility. Stability of fungal proteases in the presence of NSs is well studied among *Aspergillus* sp. According to Choudhary (2012), alkaline protease from *Aspergillus versicolor* PF/F/107 retained about 84%, 60% and 70% activity in the presence of 1% Tween-20, Tween-80 and Triton X-100 respectively. Mulimani et al. (2002) reported that the stimulation in protease activity was obtained from *Aspergillus flavus* in the presence of Tween-20. Verma et al. (2001) revealed that protease activity from *Aspergillus fumigatus* was stable in the presence of Tween-80 and Triton X-100. The obtained results report for the first time that alkaline protease activities of pure and mixed cultures were enhanced in the presence of 0.5% EOCA, at ambient temperature. In the light of obtained results, it is clear that tested fungal proteases could have practical application as an additive in laundry detergent formulations, and beyond, in bioremediation processes.

3.5. The effect of the pollutant on the qualitative composition of carbohydrates

Chromatographic analysis of C and EOCA media of pure and mixed cultures during 19-day cultivation confirmed the presence of different sugars, as Table 6 shows. In the early phase of growth (at 4th day) in C media, the pure cultures produced identical carbohydrate content with exception of sucrose, which was not detected in the medium of *G. candidum*. This finding clearly indicates that differences in the rate of sucrose transformation between pure cultures exist. It may be caused by the concentration of sucrose in the medium or by various mechanisms of transformation of sucrose, which is species specific. The decrease in sucrose level in the medium of pure cultures correlated with liberation of glucose and fructose. As the result of synergism, in C medium of mixed culture was detected monosaccharide xylose and disaccharide maltose. The presence of ribose, which occurs from glyceraldehyde (an intermediate in the metabolism of carbohydrate) was confirmed in both pure and mixed cultures. This finding clearly indicates enzyme activity of hexose monophosphate shunt of cultures. In the period from 4th to 7th day, carbohydrate metabolism flowed in the direction of synthesis di- and trisaccharide. Thus, at 7th day after inoculation, trisaccharide raffinose wad detected in C media of both pure cultures, and disaccharide lactose in mixed culture. Synthesis of polysaccharide is important because they can be subsequently consumed during stationary and death growth phase (Bahat-Samet et al., 2004).

Table 6 Qualitative composition of carbohydrates determined in growth media of pure and mixed cultures.

Qualitative composition of carbohydrates during 19-day cultivation			
Fungal isolate	Days	C	EOCA
Cladosporium cladosporioides	4	Sucrose, glucose, fructose, ribose	Lactose, ribose
	7	Glucose, raffinose	Fructose, ribose, raffinose
	10	Ribose	Ribose
	14	Arabinose, ribose	Glucose, ribose
	19	Raffinose, ribose	Galactose, ribose
Geotrichum candidum	4	Glucose, fructose, ribose	Mannose
	7	Raffinose, xylose	Glucose, ribose
	10	Ribose, lactose	Sucrose
	14	Glucose	Fructose, ribose
	19	Glucose, ribose, xylose	Glucose, ribose
Mixed culture	4	Maltose, ribose, xylose	Fructose, ribose
	7	Lactose	Ribose
	10	Galactose	Maltose
	14	Ribose, arabinose	Maltose, ribose
	19	Raffinose, galactose	Xylose

At 10th day, both pure cultures produced ribose whereas *G. candidum* produced lactose. Galactose, created by decomposition of lactose with activity of *β*-galactosidase was detected only in medium of mixed culture. As galactose causes toxicity and cell lysis in some microorganisms (Chai et al., 2012) it could be an indicator of autolysis in mixed culture. At 14th day, ribose and arabinose were detected in media of *C. cladosporioides* and mixed culture, whereas galactose was detected in medium of *G. candidum*. Production of arabinose usually occurs during stationary phase and dead growth phase, so it could be an indicator of these phases of *C. cladosporioides*. At the end of fermentation (at 19th day), cultures produced sugars as follows: raffinose and ribose (*C. cladosporioides*), ribose and xylose (*G. candidum*), and raffinose and galactose (mixed culture). The obtained results showed that ribose was the most dominant carbohydrate in C media of pure cultures throughout the whole period of cultivation. Moreover, some of carbohydrates were species specific: arabinose for *C. cladosporioides*, lactose and xylose for *G. candidum*, maltose and galactose for mixed culture.

The EOCA added into the growth medium of pure and mixed cultures influenced different carbohydrate compositions compared to the control one. The differences in carbohydrate composition between pure cultures were best expressed in the early fermentation phase. At 4th day of cultivation, *C. cladosporioides* produced ribose and lactose; *G. candidum* produced mannose, whereas mixed culture produced fructose and ribose. In contrast to C media, in EOCA media of pure cultures were no identified neither sucrose nor those of its products, glucose and fructose. Thus, the presence of EOCA in the growth medium was affected by the rapid decomposition of sucrose and transport of fructose and glucose. Glucose is essential for degradation of surfactants, so its absence in the medium can be brought into connection with the degradation of ethoxylated alcohol. A surplus of glucose is obviously incorporated in the form of lactose in *C. cladosporioides*. Mannose in the form of mannoproteins is a constituent of fungal cell wall and is liberated in the medium during growth and remodeling of the cell wall (Křen et al., 1984). Therefore, their presence in the medium of *G. candidum* can be considered as a physiological indicator of growth intensity. The carbohydrate composition of mixed culture in EOCA medium is also reported in Table 6. Apparently, the presence of the EOCA in medium of pure and mixed cultures influenced the absence of some specific sugars compared to the control. In this medium, *C. cladosporioides* did not produce sucrose and arabinose whereas *G. candidum* did not produce raffinose, lactose and xylose. On the other hand, the mixed culture did not produce galactose, lactose, raffinose and arabinose. Furthermore, some of carbohydrates were species specific: lactose and galactose for C. *cladosporioides*; mannose and sucrose for *G. candidum*, and maltose and xylose for mixed culture.

According to our best knowledge, this is the first study which identified the qualitative carbohydrate composition in the growth media of tested fungi. Bearing in mind that the carbohydrate industry is of central importance for industrial biotechnology, this study provides a good basis for further research of both pure and mixed cultures toward the optimization of cultivation conditions and the quantification of carbohydrates.

4. Conclusions

The current results have shown that the pure and mixed cultures can grow and develop in the presence of EOCA at a concentration of 0.5%. Unlike biomass of pure cultures which were inhibited, the biomass of mixed cultures was stimulated by the presence of EOCA. The pollutant had influence on the decrease in the pH value of the media in the phase of culture growth, and pH changes were correlated with the amount of excreted TOA. The alkaline protease activities of *C. cladosporioides*, *G. candidum* and mixed culture were enhanced for 56.88%, 55.84% and 30.94% respectively, in the presence of EOCA. These results clearly indicate that the tested fungi can have practical application in the detergent industry and beyond, in bioremediation processes. Finally, the presence of pollutant in growth media of tested fungi caused different compositions of carbohydrates compared to the control one, so additional research is needed in terms of the confirmation of their practical application in biotechnology.

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