The effect of ethoxylated oleyl-cetyl alcohol on metabolism of some fungi and their potential application in mycoremediation

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Abstract

The effect of ethoxylated oleyl-cetyl alcohol at a concentration of 1% on the growth and metabolism of *A. tenuis* Nees and *P. verrucosum* Dierckx was examined in this paper. The fungal growth was investigated by monitoring the diameter of colonies on solid media and dry weight biomass in liquid media. *A. tenuis* had better response to applied pollutant in solid medium, whereas *P. verrucosum* had better response in liquid medium. During exponential fungal growth in liquid media with and without pollutant (control), the following physico-chemical and biochemical parameters were carried out: pH, quantity of free and total organic acids, proteins, carbohydrates, proteolytic activity. The ethoxylated oleyl--cetyl alcohol had influence on decrease in pH value and increase in free organic acids of both fungi. Furthermore, it has influenced production in way that lower amount of total organic acids, proteins, glucose and fructose were gained in fermentation broth of *P. verrucosum* compared to *A. tenuis*. The proteolityc activity of fungi was partially (*A. tenuis*) or fully inhibited (*P. verrucosum*) by presence of pollutant in liquid medium. Based on the obtained results, these fungal species act as potential candidates for mycoremediation of alcohol ethoxylated contaminated environments and biotechnology.

Keywords: colony diameter, dry weight biomass, monosaccharides, organic acids, proteins, proteolytic activity.

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Ethoxylated oleyl-cetyl alcohol (Fig. 1) is surfactant from the group of fatty alcohol ethoxylates (FAEs) that are representing the most important group of non-ionic surfactants from economical point of view. FAEs are widely used in domestic and commercial detergents, household cleaners and personal care products. They are employed as wetting and washing agents in the cosmetics, agriculture, paper, oil and other sectors of processing industry. Constant increase in production volume of these non-ionic surfactants in the world over the past 20 years, especially in Europe, is conditioned by many FAEs desired characteristics, such as rapid biodegradation, low-to-moderate foaming ability, superior cleaning of man-made fibers, tolerance of water hardness and ability to perform in cold water [1].

On the other hand, rapid growth of production alcohol ethoxylates (AEs) in the world points to the possibility of an increased quantity of this pollutant in aquatic ecosystems at concentrations above expectations. After usage, residual surfactants and their deg-

Name	Structure	Formula
		$R\text{-}O(CH_2CH_2O)n\text{-}H$
Ethoxylated oleyl-	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CH_2O\text{-}$	R-blend of oleyl and cetyl
cetyl alcohol (AOC)	$CH_2CH_2OCH_2(CH_2)_{14}CH_3$	alcohol
		n-number of ethylene oxide

Figure 1. Chemical structure surfactant.

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radation products are discharged to sewage treatment plants or directly to surface water sand sediments [2]. Experimental results from many biodegradation studies in laboratory and field conditions suggest that there is a high quantity of primary and ultimate biodegradation

of these surfactants in the environment [3]. AEs are degraded biologically by wastewater treatment plant (WWTP) in excess 95–99% [4,5]. Concentration of total AEs in WWTP effluents is in the range 1.0–23 μg/l in Europe, Canada and USA [6,7]. From the middle of '70s until today, several environmental risk assessments were carried out on AEs [8,9]. These surfactants have a strong affinity for sorption to solids such as activated sludge, river water solids and, ultimately, sediments [10–12]. Level of toxicity to aquatic organisms, measured by EC_{50}, ranges from very toxic (< 1 mg/l) to harmful (between 10–100 mg/l). Also, during the mention time period, studies related to understanding the biodegradation mechanisms of AEs in the presence of complex microbial communities were carried out using different methods. Several AEs degrading bacteria were isolated under aerobic [13] and anaerobic conditions [14,15]. In the last two decades, bacteria were in the focus of bioremediation studies, unlike fungi which were studied much less. Mycoremediation is an innovative biotechnology that uses living fungus for *in situ* and *ex situ* cleanup and management of contaminated sites [16]. Filamentous fungi have ability to grow on wide spectrum of substrates by secreting extracellular hydrolytic enzymes, even capable of growing under ambient environment. Moreover, due to the low substrate specificity of their degradative enzyme machinery, fungi are able to perform the breakdown of a wide range of organic and xenobiotic pollutants: petroleum hydrocarbons, chlorophenols, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and furans, pesticides, herbicides and nitroaromatic explosives [17,18]. These fungal properties are utilized in a variety of processes (biological control agent, biobleaching, bioremediation, waste treatment).

Based on our previous researches [19–22], it was identified that several species of fungi such as *A. niger*, *T. roseum*, *F. oxysporum, etc.*, can grow and metabolize ethoxylated oleyl-cetyl alcohol at a wide concentration range 0.01–1%. For this reason, current study was conceptualized in order to investigate the effect of ethoxylated oleyl-cetyl alcohol at a concentration of 1% on the growth of selected fungi and changes of their metabolic activity. Isolation and identification of fungi from aquatic ecosystems that are resistant to the presence of high concentrations of this pollutant on the one hand, and the effect of a pollutant on their metabolism on the other hand, are crucial parameters for application of fungi in mycoremediation.

EXPERIMENTAL

Isolation and identification of fungi from wastewater

The fungi applied in this study were isolated from the sample of wastewater river basin of Lepenica, Kragujevac (the place of wastewater flood, sewage). The sample of water was taken in a sterile container. The sample was transferred to the microbiology laboratory and was afterwards inoculated onto Petri plate's nutrient malt agar with streptomycin (in duplicates). The Petri plates were then incubated for 5–7 days at standard temperature 28±2 °C. Pure cultures were obtained by the method of exhausting on poor malt agar plates and potato dextrose agar (PDA) plates. Identification of the fungi was based primarily on the macroscopic and microscopic morphology and was carried out by Systematic keys at the Faculty of Biology, University of Belgrade, Serbia. The fungi selected as test organisms in this study were: *Alternaria tenuis* Nees (1817) and *Penicillium verrucosum* Dierckx (1913). The fungi were maintained on PDA plates, stored at 4±0.5 °C and subcultured monthly in sterile conditions.

Inoculums preparation

Inoculums suspensions were prepared from fresh, mature (from 3- to 5-day-old) cultures grown on PDA plates. 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 inoculums sizes were adjusted to 1.0×10^6 spores/ml by microscopic enumeration with a cell-counting hematocytometer (Neubauer chamber).

Cultivation of fungi on solid media and culture condition

The Czapek Dox's solid media was prepared according to the formulation shown in Figure 2, with addition of 20 g agar-agar and autoclaved at 121 °C for 20 min (autoclave pressure, 0.14 MPa). After cooling to 45 °C, culture media were dispensed into sterile Petri dishes

Growth medium	Mark	*c* (g/l)					
		$NaNO_3$	K_2HPO_4	$MgSO_4 x7H_2O$	$FeSO_4 x7H_2O$	Sucrose	AOC[a]
Control	C	3	1	0.5	0.01	30	
C + 1 % AOC	AOC	3	1	0.5	0.01	30	10

[a]AOC - Ethoxylated oleyl-cetyl alcohol (Henkel, Krusevac)

Figure 2. Composition of growth media in 1000 ml distillated water.

for solidification. The tested fungi were inoculated at the center of the agar plates. The plates were incubated at room temperature over 8 days, in order to examine the exponential growth of fungi.

Cultivation of fungi in liquid media and culture condition

The Czapek Dox's liquid growth media (100 ml) was prepared in 250 ml Erlenmeyer flask, according to procedure mentioned above, but without addition of agar. One ml spore suspension of both fungi was inoculated in liquid media. Following inoculation, Erlenmeyer flasks were placed on an orbital shaker (Kinetor-m, Ljubljana) thus enabling uniform and constant mixing. All Erlenmeyer flasks were incubated at room temperature, under alternate light and dark for 8 days. Sampling has begun on day 4 and repeated daily until the end of experiment. All experiments were conducted in triplicate.

Measurement of pH values

A pH value of the fermentation broth (initial pH value about 5.0) in the experiment was measured by a pH meter (type MA-5705, the product "Iskra", Kranj) during fungal growth from day 4 to day 8.

Determination of colony diameters

Colony diameters (*CD*) were measured with a ruler at intervals of 24 h from inoculation until day 8. The growth curves were constructed from the diameter of the colonies (cm) *versus* incubation time (day). From the growth curves, the exponential growth phases of fungi were determined in the period of cultivation from day 4 to day 8. This period was selected for further study of physicochemical and biochemical parameters of fungi in liquid growth media.

Determination of dry weight biomass

The 8-day-old mycelia was separated from the fermentation broth by filtration through pre-weighed filter paper. The mycelia was washed with distilled water several times. Filter papers with mycelia were dried in an oven at 80 °C to constant weight. The dry weight of the mycelia was calculated by subtracting the initial weight of the filter paper from the weight of mycelia and filter paper. The results are presented as g/l.

Determination of concentrations total and free organic acids

The concentration of free and total organic acids (FOA and TOA) was determined by ion exchange chromatography according to method by Bullen *et al.* [23], as described in greater detail in our previous work [20]. The results are presented as percentages (%).

Determination of monosaccharides quantity

The quantity of monosaccharides, glucose and fructose, were also determined by ion exchange chromatography according to procedure which is discribed in our previous work [21]. The results are presented as percentages (%).

Determination of protein concentration

Protein concentration in fermentation broth of fungi was determined according to the method by Kjeldahl [24]. A sample was digested with a strong acid so that it releases nitrogen, which was determined by a suitable titration technique. A conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein) was used for calculating the quantity of protein, according to the Eq. (1):

$$\text{The quantity of proteins (mg)} = 6.25 \times \text{quantity of nitrogen} \quad (1)$$

Assays of alkaline protease activity (EC 3.4.21-24)

The assay of alkaline protease was carried out by Anson's method [25]. 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 4.000 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 unite of alkaline protease activity was defined as the amount of enzyme capable of producing 1μg of tyrosine from casein in a minute under assay condition.

Statistical analysis

The results were expressed as mean ± standard deviation of data obtained from three independent measurements. The database was analyzed using the Software Package for Social Science for Windows 14.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISSCUSION

Our previous studies emphasize that some fungi species which originated from wastewater can metabolize the detergent components (*e.g.*, ethoxyled oleyl-cetyl alcohol and sodium tripolyphosphate) for growth and biomass accumulation [19–22]. Having this in mind, this study was designated in order to investigate the influence of ethoxyled oleyl-cetyl alcohol, which is added to nutrient medium in a high concentration (1%) on the growth, development and metabolic activity of *A. tenuis* Nees and *P. verrucosum* Dierckx. The obtained results should serve as a theoretical basis for practical

application of tested fungi in mycoremediation of environment. Research results of this study are presented in Figures 3–5.

The effect of surfactant on fungal growth on solid medium

Chemical composition of Czapek Dox's nutrient medium has optimal properties for growth and high biomass production of numerous fungi [19–22,26]. In addition, the presence of various pollutants (*e.g.* dye, heavy metals, pesticides, surfactants) in the culture medium may have an inhibitory or stimulatory effect on the fungal growth depending on type and applied concentration of pollutant, and the fungal species. For testing of fungal growth, the fungi were grown previously on solid media with (AOC) and without pollutant (C) over a period of 8 days. The colonies diameters were measured daily and growth curves were constructed. For both fungi, the exponential growth phase occurred in the period of cultivation from day 4 to day 8 (Figure 3). As Figure 3 shows, *CD* of *A. tenuis* had gradually increased on C medium, from day 4 to day 8, whereas *P. verrucosum* had a lower growth rate compared to *A. tenuis.* Different growth rates between the tested fungi in C medium can be explained by their morphophysiological differences that affect different response for the adoption and nutrient transport.

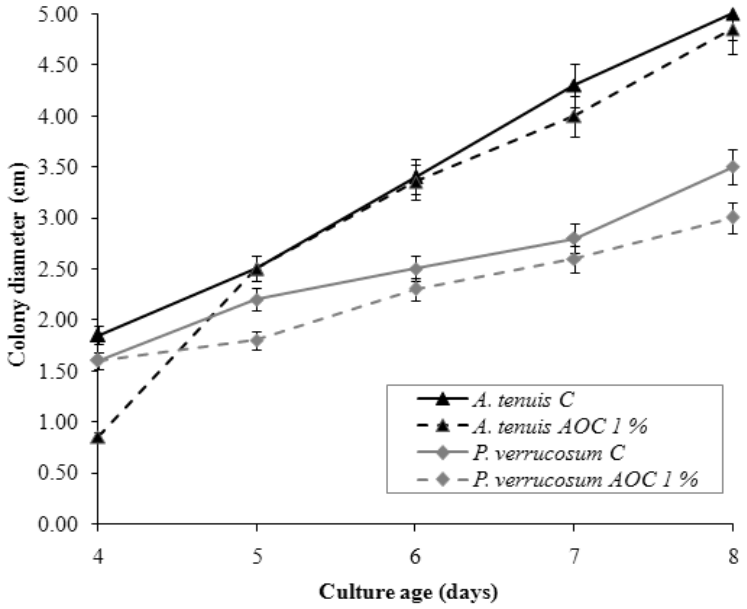

Figure 3. The colonies diameter of A. tenuis and P. verrucosum during exponential growth on solid media (C-control, AOC-medium with 1% ethoxylated oleyl-cetyl alcohol).

Fungi	Medium / Days	Dry weight biomass (g/l) C	Dry weight biomass (g/l) AOC	pH C	pH AOC	Proteolytic activity (μg/ml) C	Proteolytic activity (μg/ml) AOC
Alternaria tenuis	4	0	0	7.14±0.01	7.03±0.06	0	0.19±0.07
	5	nd	nd	7.16±0.02	7.04±0.12	0.28±0.04	0
	6	nd	nd	7.18±0.02	7.05±0.05	0.63±0.10	0.02±0.01
	7	nd	nd	7.16±0.04	7.06±0.05	0.31±0.08	0.32±0.14
	8	0.19±0.09	0.55±0.12	7.15±0.01	7.05±0.01	0.19±0.05	0.40±0.08
Penicillium verrucosum	4	0	0	7.12±0.05	7.25±0.02	0.18±0.09	0
	5	nd	nd	7.05±0.04	7.08±0.05	0.20±0.10	0
	6	nd	nd	6.88±0.02	6.84±0.09	0.35±0.12	0
	7	nd	nd	6.73±0.07	6.82±0.06	0.18±0.05	0
	8	0.14±0.05	0.17±0.06	6.75±0.01	6.66±0.01	0.15±0.05	0.05±0.01

Each value is expressed as mean ± standard deviation ($n = 3$), nd-not determined

Figure 4. Total dry weight of biomass, pH value and proteolitic activity of fungi in liquid media (C – control, AOC – medium with 1% ethoxylated oleyl-cetyl alcohol).

		Proteins (mg/ml)		Free org. acids (%)		Total org. acids (%)		Glucose (%)		Fructose (%)	
Fungi	Medium / Days	C	AOC	C	AOC	C	AOC	C	AOC	C	AOC
Alternaria tenuis	4	0.61±0.03	0.76±0.21	0.67±0.04	0.17±0.05	7.07±1.02	1.67±0.35	0.98±0.16	0.03±0.01	0.49±0.12	0.03±0.01
	5	0.68±0.09	0.62±0.26	nd	nd	nd	nd	nd	nd	nd	nd
	6	0.72±0.10	0.63±0.42	nd	nd	nd	nd	nd	nd	nd	nd
	7	0.68±0.12	0.68±0.32	nd	nd	nd	nd	nd	nd	nd	nd
	8	0.70±0.05	0.76±0.25	0.33±0.06	2.33±0.55	5.67±1.26	7.67±1.48	0.32±0.24	0.73±0.15	0.32±0.09	0.44±0.10
Penicillium verrucosum	4	0.10±0.02	0.08±0.04	0.28±0.02	0.28±0.08	0.56±0.10	2.22±0.65	0.10±0.06	0.07±0.05	0.05±0.02	0.07±0.02
	5	0.20±0.07	0.10±0.05	nd	nd	nd	nd	nd	nd	nd	nd
	6	0.24±0.04	0.11±0.05	nd	nd	nd	nd	nd	nd	nd	nd
	7	0.97±0.19	0.21±0.07	nd	nd	nd	nd	nd	nd	nd	nd
	8	1.80±0.62	0.05±0.01	1.94±0.09	3.33±0.05	8.89±1.32	7.78±1.28	0.78±0.09	0.21±0.04	0.71±0.09	0.37±0.16

Each value is expressed as mean ± standard deviation ($n = 3$), nd-not determined

Figure 5. Quantity of proteins, free and total organic acids and monosaccharides (glucose and fructose) in liquid media (C – control, AOC – medium with 1% ethoxylated oleyl-cetyl alcohol).

When fungi were grown on solid medium with addition of AOC, they had lower CD compared to the control. The growth of *A. tenuis* on AOC medium was significantly ($p < 0.01$) or slightly inhibited on day 4 and from day 7 to day 8, respectively. These results can be explained by a period of fungal adaptation to the presence of AOC in medium, during the initial stage of growth, as well as the creation of some degradation products that slow down fungal growth. Ethoxylated alcohol showed an inhibitory effect on *CD* of *P. verrucosum* over a period of three days (from day 5 to day 8), but had no influence on fungal growth on day 4. Obviously, the fungus *P. verrucosum* is more tolerant to a high concentration of the AOC in medium than *A. tenuis*, considering its CD was two times higher that CD of *A. tenuis* on day 4. However, the AOC degradation products had a higher intoxicating effect on the growth of *P. verrucosum* than parent molecule. Consequently, CD of *P. verrucosum* was significantly lower (14.29%) on day 8, whereas CD of *A. tenuis* was lower only by 3% compared to the control.

The effect of surfactant on fungal growth in liquid medium

The DW biomass of tested fungi grown in the liquid Czapek Dox's media was measured on day 8 (at the end of exponential growth) and results are presented in Figure 4. The fungus *A. tenuis* had higher DW biomass (0.55 g/l) than fungus *P. verrucosum* (0.17 g/l) in C medium. On the other hand, AOC added in liquid medium showed strong inhibitory effect (67%) on DW biomass of *A. tenuis* and mildly stimulating effect (13.5%) on DW biomass of *P. verrucosum,* compared to control. The finding that fungi could survive and grow in solid and liquid Czapek Dox's media with AOC at a high concentration (1%) provided evidence for the fungal resistance to this pollutant. The different response of fungi to growth on solid and liquid media with AOC was also confirmed in this study. Therefore, *A. tenuis* had better response to presence of AOC in solid medium, whereas *P. verrucosum* had better response to presence of AOC in liquid medium. These characteristics of fungi make them utilizable in bioremediation of solid and liquid environments. The obtained results are consistent with the results of our previous studies, which revealed the influence of AOC on the DW biomass of fungi *T. roseum*, *F. oxysporum* and *A. niger*. Therefore, this pollutant has a mild stimulating effect on the biomass of *T. roseum* and *F. oxysporum* but has a very strong inhibitory effect on the biomass of *A. niger*, under the same experimental conditions [21,22].

The influence of surfactant on pH media

Transport of nutrients through the cell membranes and growth of microorganisms are closely related with ambient pHs. Although, most fungal species live in a wide range of external pH, they proliferate more rapidly at acidic pH. When grown in an unbuffered medium, filamentous fungi often rapidly acidify their environment to very low and even sometimes detrimental pH values [27]. The major mechanism behind this acidification is controversially discussed, although it is most often attributed to either organic acid excretion [27,28] or proton release by the plasma membrane H-ATPase [29]. The addition of some organic molecules (*e.g.*, AOC, sodium tripolyphosphate or commercial detergent) in nutrient medium influences the change of pH values towards an alkaline environment that can be considered as a stress condition. According to litera-

ture, fungal response to alkaline pH is based on two possible mechanisms. First is the proteolytic activation of PacC transcription factors (*A. nidulans, C. albicans, S. cerevisiae,* Y. *lipolytica*) [30] and second mechanism is existence of the calcium-mediated pathway [31].

This study has evaluated the changes of pH value of fermentation broth during fungal growth from day 4 to day 8, as Table 3 shows. The pH values of liquid control media (C) were closely related and in neutral range (*P. verrucosum* 7.12 units, *A. tenuis* 7.14 units) on day 4. During the growth of fungi, the pH values of C media were changed to different intensity, depending on the fungal species. Therefore, during those four days, the pH value of C medium of *P. verrucosum* was gradually decreasing and the largest decrease in pH value (0.17 units) was measured from day 5 to day 6. On the contrary, the changes of pH value of C medium of *A. tenuis* were slightly increasing from day 4 to day 6. From that point on, the pH value was then decreasing with the almost same intensity until day 8. The presence of AOC at a concentration of 1% in a liquid media resulted in pH changes of media. The pH value of AOC medium of *A. tenuis* was insignificantly lower on day 4, whereas the pH value of *P. verrucosum* was significantly higher as compared to control. During the growth of *P. verrucosum*, pH value of AOC medium was decreasing, and the largest decrease in pH value (0.24 units) was observed from day 5 to day 6. On the contrary, the pH values of AOC medium of *A. tenuis* changed in the opposite manner, but the changes were very small. Decreasing of media pH is widespread phenomenon observed during extensive mycelium development of many fungal species such as *A. niger*, *F. oxysporum*, *P. chrysogenum*, *etc*. The results obtained in this study evidently suggest that fungi probably have different mechanism regulation of external pH, which depends on numerous factors (*e.g.*, pH value, chemical composition of medium, fungal morphology, *etc.*).

Activity of alkaline protease (EC 3.4.21-24) in liquid media

Proteases are degradative enzymes, which catalyze the cleavage of peptide bonds in proteins. They have wide-ranging applications in industrial products and processes such as detergent, food, pharmaceuticals, tannery, waste treatments, *etc*. In literature, several microbial strains including fungi (*Aspergillus flavus, Fusarium graminarum, Penicillium griseofulvin, etc.)* and bacteria *(Bacillus licheniformis, B. firmus, B. subtilis, etc.*), are reported to produce protease. Due to these facts, the alkaline protease activity of *A. tenuis* and *P. verrucosum* was evaluated in this paper.

Data presented in Figure 4 has shown that tested fungi produced extracellular protease when grown in C medium more effectively than in AOC medium. The proteolytic activity of fungi was increased parallel with the fungal growth (*A. tenuis* and *P. verrucosum*) in C medium, from day 4 to day 6. The maximum proteolytic activity was measured in the fermentation broth of *A. tenuis* (0.63 mg/ml) on day 6. Fungus *P. verrucosum* had two times lower proteolytic activity in same medium, with its maximum (0.35 mg/ml) achieved on day 6. Addition of ethoxylated oleyl-cetyl alcohol, in liquid nutrient medium at a concentration of 1%, contributed to a partial (*A. tenuis*) or complete inhibition (*P. verrucosum*) of proteolytic activity, during fungal growth. Proteolytic activity of *A. tenuis* in AOC medium was expressed on day 4 and from day 6 to day 8, with its maximum (0.400 mg/ml) achieved on day 8. In the presence of AOC, fungal proteolytic activity was inhibited about 37% (or remained about 63% activity) in relation to control. Obviously, various morpho-physiological characteristics of the fungi and some degradation products of AOC in medium have caused differences in effects of this pollutant on proteolytic activity. These results are also supported by the findings of Stojanović *et al.* [22] who reported that AOC at concentration of 1% has a strong inhibitory effect on the activity of proteolytic enzymes the fungus *T. roseum*. However, the same authors also reported that the AOC at concentration of 1% has a strong stimulating effect on the proteolytic activity of *A. niger*, under the same experimental conditions [19]. According to Evans and Abdullahi [32], surfactants may have improved 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. Non-ionic surfactants type of ethylene oxides, bind to active site of enzymes through hydrogen bonds in order to enhance conformation flexibility [33]. Zeng *et al.* [34] revealed that incorporation of Tween-80 into fermentation medium have shown to enhance production and secretion of protease. Li *et al.* [35] demonstrated that Tween-80 and acetonitril increased the yield of protease activity of *Serratia sp.* SYBC H by 5.0 and 4.3 folds, respectively. Maruthiah *et al.* [36] has reported enhanced protease activity of *Bacillus flexus* by non-ionic surfactant Tween-20, Tween-40, Tween-60 and Triton X-100. According to Barberis *et al.* [37], proteolytic activity of *araujiain* increased or remained constant while non-ionic surfactant concentration was being increased (0.1, 0.4 and 1%). Enzyme stability in the presence of detergent ingredients, such as surfactants, builders and activated bleach, *etc.*; optimum activity at alkaline pH; effectiveness at low wash temperatures of 20–40 °C; are very important properties for its use in detergent formulations [38]. Based on presented results and facts mentioned above, performances of *A. tenuis* alkaline protease are suitable for its potential application as an additive in laundry detergent formulations ethoxylated oleyl-cetyl alcohol type.

Production of protein in liquid media

Numerous studies of protein secretion has been made with filamentous fungi, but the molecular basis for the protein secretion in fungi is still lacking. Bearing that in mind, the fungi were referred as "a highly productive black box" by Peberdy [39]. Therefore, the examination of protein secretion of each fungal species in various media is very important. The tested fungi had produced a different amount of protein in C medium on day 4, and it was ranged from 0.10 (*A. tenuis*) to 0.67 mg/ml (*P. verrucosum*), Figure 5. The amount of proteins secreted in this medium was increased parallel with the fungal growth. Fungus *A. tenuis* has secreted the highest amount of protein (0.72 mg/ml) on day 6, and *P. verrucosum* (1.80 mg/ml) on day 8. Ethoxylated oleyl-cetyl alcohol added in the culture medium seems to have slightly stimulated the proteins secretion of *A. tenuis* (0.76 mg/ml), whereas it strongly inhibited the proteins secretion of *P. verrucosum* (0.21 mg/ml). The least deviation in the secretion of proteins between the media was found by *A. tenuis*. Data that was found in the literature confirmed inhibitory/stimulatory effect of AOC at a concentration of 1% on protein production of *T. roseum* [22], and *A. niger* and *F. oxysporum* [19,21]. The results of this study as well as results of mentioned authors are evidently indicating that fungal morphology is also directly correlated with protein production.

The influence of surfactant on organic acids excretion

The tested fungi excreted different amount of FOAs and TOAs depending on the type of medium and culture age (Figure 5). The amount of FOAs measured in C medium was significantly higher (*P. verrucosum*) or low (*A. tenuis*) on day 8, compared to day 4. During the same cultivation period, AOC at a concentration of 1% showed strong stimulatory effect on FOAs excretion of both fungi, in relation to control. To summarize, the fungus *P. verrucosum* excreted about 1.5-fold larger amount of FOAs and *A. tenuis* excreted about 7-fold larger amount of FOAs in AOC medium in relation to control. The amount of TOAs measured in fermentation broth of C medium of *A. tenuis* was significantly lower or, as in the case of *P. verrucosum*, significantly higher on day 8 than on day 4. The AOC added in medium with a concentration of 1% has influenced TOAs amount in both fungi by increasing it significantly on day 8 compared to day 4. Generally, *A. tenuis* excreted higher amount of TOAs in both culture media than *P. verrucosum*. These results provide evidence that significant differences exist between the tested fungi in organic acids excretion in both media. Decreasing of pH media of *P. verrucosum* is in positive correlation with increasing of organic acids excretion. In contrast, increasing of pH control medium of *A. tenuis* is in negative correlation with amount of organic acids excreted, which could be explained with a reuptake of organic acids. Therefore, organic acids excreted in media are serving another purpose (charge balance, energy spilling or chelation of trace elements) besides acidification of external medium.

The influence of surfactant on amount of monosaccharides

Monosaccharides, glucose and fructose, are the reducing sugars produced by the action of invertase on sucrose. Generally, when sucrose is used as an only carbon source, the fungus utilizes rather glucose than fructose for its metabolism [40]. Sucrose is necessary in medium with AOC for fungal biodegradation of pollutant. Taking this into consideration, the effect of AOC on amount of monosaccharides was investigated in this study. The concentration of glucose and fructose in the fermentation broth of fungi was determined at the beginning (day 4) and at the end of exponential growth phase (day 8), as Figure 5 shows. On day 4, very small amount of glucose and fructose was measured in C medium of *P. verrucosum* but significant amount of monosaccharides was measured in C medium of *A. tenuis*. Regardless of these differences, both fungi produced lower amount of fructose than glucose, which means that fungi metabolized fructose rather than glucose in medium with sucrose as only carbon source. These results are opposite from results of above-mentioned authors. Obviously, parameters such as fungal morphology and experimental conditions (pH medium, chemical composition of medium, aeration, *etc.*), have influenced monosaccharides uptake rates. At the same time, very low amount of glucose and fructose was also measured in both AOC media. In this medium, fungi metabolized equal amount of monosaccharides. On day 8, the amount of glucose and fructose measured in C medium of *P. verrucosum* was significantly higher than on day 4. Nevertheless, the amount of glucose and fructose in same medium of *A. tenuis* was lower, especially glucose. In both AOC media, amount of monosaccharides was higher on day 8 compared to day 4. Accordingly, *A. tenuis* produced about 0.71% glucose and 0.44% fructose, whereas *P. verrucosum* produced about 0.18% glucose and 0.35% fructose. These results indicate that tested fungi have different flux for monosaccharides in presence of AOC. Therefore, *A. tenuis* utilizes fructose rather than glucose, whereas *P. verrucosum* rather utilizes glucose in medium with AOC. Results obtained in this study are in accordance with report by Stojanović *et al.* [22] who found that ethoxylated alcohol stimulates the production of glucose of *F. oxysporum* and fructose of *T. roseum* and *F. oxysporum*. However, the same authors confirmed the inhibitory effect of this pollutant on production of monosaccharides in experiment with *A. niger* [21]. The differences in the fungal utilization of

glucose and fructose observed in this study could be caused either by differences in the transport systems or by the subsequent intracellular metabolism of the sugars. Also, AOC could influence synthesis of inducible enzymes involved in regulation of carbohydrates metabolism or some of its degradation products have a role of competitive inhibitor of these enzymes.

CONCLUSIONS

Based on obtained results, ethoxylated oleyl-cetyl alcohol at a concentration of 1% had different effect on the growth, development and metabolic activity of the tested fungi, depending on the species of fungi. Both fungi species could survive and grow in solid and liquid Capek Dox's medium with addition of AOC at a high concentration (1%). The *A. tenuis* has better response to AOC on solid medium whereas *P. verrucosum* has better response to AOC in liquid medium. Ethoxylated oleyl-cetyl alcohol has influenced metabolic activity of fungi in direction of production of significant amount of organic acids, proteins, fructose (*P. verrucosum*) and glucose (*A. tenuis*). The alkaline protease activity of fungus *A. tenuis* had retained about 63.50% activity in the presence of AOC, so it could have a potential application in detergent formulation. The results presented in this study undoubtedly indicate the possible application of the tested fungi in both mycoremediation of contaminated solid and liquid environments and in different areas of industries.

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REFERENCES

[1] HERA, Human & Environmental Risk Assessment on ingredients of European household cleaning products, Alcohol Ethoxylates, 2009. http://www.heraproject.com

[2] G.G. Ying, Fate, behavior and effects of surfactants and their degradation products in environment, Environ. Int. **32** (2006) 417–431.

[3] Danish EPA, Environmental and Health Assessment of Substances in Household Detergents and Cosmetic Detergent Products. Environmental Project No. 615, 2001. http://www.mst.dk/udgiv/ Publications/2001/87-7944--596-9 /html/default_eng.htm

[4] T. Wind, R.J. Stephenson, C.V. Eadsforth, A. Sherren, R. Toy, Determination of the fate of alcohol ethoxylate homologues in a laboratory continuous activated-sludge unit study, Ecotoxicol. Environ. Saf. **64** (2006) 42–60.

[5] T.W. Federle, N.R. Itrich, Fate of free and linear alcohol-ethoxylate-derived fatty alcohols in activated sludge, Ecotoxicol. Environ. Saf. **64** (2006) 30–41.

[6] S.W. Morrall, W.S. Eckhoff, A. Evans, M.L. Cano, J.C. Dunphy, D.C. McAvoy, Removal and environmental exposure of alcohol ethoxylates in US sewage treatment, Ecotoxicol. Environ. Saf. **64** (2006) 3–13.

[7] C.V. Eadsforth, A.J. Sherren, M.A. Selby, R. Toy, W.S. Eckhoff, D.C. McAvoy, E. Matthijs, Monitoring of environmental fingerprints of alcohol ethoxylates in Europe and Canada, Ecotoxicol. Environ. Saf. **64** (2006) 14–29.

[8] S.E. Belanger, P.B. Dorn, R. Toy, G. Boeije, S.J. Marshall, T. Wind, R.Van Compernolle, D. Zeller, Aquatic risk assessment of alcohol ethoxylates in North America and Europe, Ecotoxicol. Environ. Saf. **64** (2006) 85–99.

[9] L. Bin-Le, Y. Meng, Y. Ishikawa, A. Tokai, A new approach to estimate concentrations of alcohol ethoxylate in rivers in Japan for screening-level risk assessment, J. Risk Res. **14** (2011) 1109–1126.

[10] D.C. McAvoy, K.M. Kerr, In: C.E. Clapp, M.H.B. Hayes, N. Senesi, P.R. Bloom, P.M. Jardine (Eds.), Association of alcohol ethoxylates with a dissolved humic substance. Humic substances and chemical contaminants, Madison, WI: Soil Science Society of America, 2001, pp. 177–186.

[11] L. Zhu, K. Yang, B. Lou, B. Yuan, A multi-component statistic analysis for the influence of sediment/soil composition on the sorption of a nonionic surfactant (Triton X-100) onto sediments/soils, Water Res. **37** (2003) 4792–4800.

[12] S.M. Mudge, P.C. De Leo, S.D. Dyer, Quantifying the anthropogenic fraction of fatty alcohols in a terrestrial environment, Environ. Toxicol. Chem. **31** (2012) 1209––1222.

[13] E.C. Tidswell, N.J. Russell, G.F. White, Ether-bond scission in the biodegradation of alcohol ethoxylate surfactants by *Pseudomonas* sp. strain SC25A, Microbiology **142** (1996) 1123–1131.

[14] M.A. Grant, W.J. Payne, Anaerobic growth of *Alcaligenes faecalis* var. *denitrificans* at the expense of ether glycols and nonionic detergents, Biotechnol. Bioeng.**25** (1983) 627–630.

[15] S. Wagener, B. Schink, Fermentative degradation of non-ionic surfactants and polyethylene glycol by enrichment cultures and by pure cultures of homoacetogenic and propionate-forming bacteria, Appl. Environ. Microbiol. **54** (1988) 561–565.

[16] R. Ramachandran, J.J. Gnanadoss, Mycoremediation for the treatment of dye containing effluents, IJCOA **2** (2013) 286–293.

[17] A.L. Juhasz, R. Naidu, Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[*a*]pyrene, Int. Biodeter. Biodegr. **45** (2000) 57–88.

[18] M.L. Rabinovich, A.V. Bolobova, L.G. Vasilchenko, Decomposition of natural aromatic structures and xenobiotics by fungi, Appl. Biochem. Microbiol. **40** (2004) 5––23.

[19] J. Stojanović, M. Grbavcić, A. Cosović, M. Stojanović, The influence of detergent, the active component of detergent and sodium tripolyphosphate on the biochemical process of some fungi, Riv. Biol. **97** (2004) 329–339.

[20] J. Stojanović, V. Jakovljević, I. Matović, Z. Mijušković, T. Nedeljković, The influence of detergent, sodium tripolyphosphates and ethoxyled oleyl-cetyl alcohol on metabolism of the fungi *Penicillium verrucosum* Peyronel, Acta Vet-Beograd **60** (2010) 67–77.

[21] J. Stojanović, V. Jakovljević, I. Matović, O. Gajović, Z. Mijušković, T. Nedeljković, Influence of detergent on metabolic activity of fungi *Aspergillus niger*, Natural Science **3** (2011) 466–470.

[22] J. Stojanović, J. Milićević, O. Gajović, V. Jakovljević, I. Matović, Z. Mijušković, T. Nedeljković, The effects of detergent, sodium tripoly-phosphate and ethoxyled oleyl-cetyl alcohol on metabolic parameters of the fungus *Trichothecium roseum* Link, Arch. Biol. Sci. **63** (2011) 1001–1006.

[23] W.A. Bulen, J.E. Varner, R.C. Burrell, Separation of Organic Acids from Plant Tissues, Anal. Chem. **24** (1952) 187–190.

[24] J.T. Kjeldahl, Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern, Z. Anal. Chem. **22** (1883) 366–382.

[25] M.L. Anson, The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin, J. Gen. Physiol. **22** (1938) 79–89.

[26] V.D. Jakovljević, J.M. Milićević, J.D. Stojanović, S.R. Solujić, M.M. Vrvić, Influence of detergent and its components on metabolism of *Fusarium oxysporum* in submerged fermentation. Hem. Ind. **68** (2014) 465–473.

[27] J.K. Magnuson, L.L. Lasure, In: J.S. Tkacz, L. Lange (Eds.), Organic acid production by filamentous fungi. Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine, Kluwer Academic/Plenum Publishers, New York, 2004, pp. 307–340.

[28] M.R. Andersen, L. Lehmann, J. Nielsen, Systemic analysis of the response of *Aspergillus niger* to ambient pH, Genome Biol. **10** (2009) R47.

[29] K. Jernejc, M. Legisa, Activation of plasma membrane H^+-ATPase by ammonium ions in *Aspergillus niger*, Appl. Microbiol. Biotechnol. **57** (2001) 368–373.

[30] W. Li, A.P. Mitchell, Proteolytic activation of Rim1p; a positive regulator of yeast sporulation and invasive growth, Genetics **145** (1997) 63–73.

[31] R. Serrano, A. Ruiz, D. Bernal, J.R. Chambers, J. Ariño, The transcriptional response to alkaline pH in Saccharomyces cerevisiae: evidence for calcium-mediated signaling, Mol. Microbiol. **46** (2002) 1319–1333.

[32] E.C. Evans, A. Abdullahi, Effect of surfactant inclusions on the yield and characteristics of protease from *Bacillus subtilis*, Proc. Rom. Acad. Ser. B **2** (2012) 108––112.

[33] S.Ž. Grbavčić, D.I. Bezbradica, I.M. Karadžic, Z.D. Knežević-Jugovic, Lipases and proteases produced by indigenous *Pseudomonas aeruginosa* strain as potential detergent additives, Hem. Ind. **63** (2009) 331–335.

[34] G.-M. Zeng, J.-G. Shi, X.-Z. Yuan, J. Liu, Z.-B. Zhang, G.-H. Huang, J.-B. Li, B.-D. Xi, H.-L. Liu, Effects of Tween 80 and rhamnolipid on the extracellular enzymes of Penicillium simplicissimum isolated from compost, Enzyme Microb. Technol. **39** (2006) 1451–1456.

[35] G.Y. Li, Y.J. Cai, X.R. Liao, A novel nonionic surfactant- and solvent-stable alkaline serine protease from *Serratia sp.* SYBC H with duckweed as nitrogen source: production, purification, characteristics and application, J. Ind. Microbiol. Biotechnol. **38** (2011) 845–853.

[36] T. Maruthiah, P. Esakkiraj, G. Immanuel, A. Palavesam, Alkaline serine protease from marine *Bacillus flexus* APCMST-RS2P: purification and characterization, Curr. Biotechnol. **3** (2014) 238–243.

[37] S. Barberis, E. Quiroga, C. Barcia, C. Liggieri, Effect of laundry detergent formulation on the performance of alkaline phytoproteases, Electron. J. Biotechn. **16** (2013) 1-8. http://dx.doi.org/10.2225/vol16-issue3-fulltext-1

[38] E. Smulders, W. Rähse, W. Von Rybinski, J. Steber, E. Sung, F. Wiebel, In: E. Smulders (Ed.), Detergent ingredient. Laundry detergent, Wiley-VCH, Verlag GmbH & CoKGaA, Germani, 2002, pp. 38–98.

[39] J.F. Peberdy, Protein secretion in filamentous fungi – trying to understand a highly productive black box, Trends Biotechnol. **12** (1994) 50–57.

[40] E. Peynaud, S. Domercq, Sur les espbces de levures fermentant selectivement le fructose, Annales de l'Institut Pasteur, **89** (1955) 346–351.

IZVOD

UTICAJ ETOKSILOVANOG OLEIL-CETIL ALKOHOLA NA METABOLIZAM NEKIH GLJIVA I NJIHOVA POTENCIJALNA PRIMENA U MIKOREMEDIJACIJI

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(Naučni rad)

Uticaj etoksilovanog oleil-cetil alkohola 1% koncentracije na metabolizam gljiva *A. tenuis* Nees and *P. verrucosum* Dierckx, koje su izolovane iz otpadnih kanalizacionih voda, bio je predmet istraživanja ove studije. Dejstvo 1% etoksilovanog oleil-cetil alkohola na rast gljiva ispitivano je praćenjem prečnika kolonija na čvrstoj i merenjem suve biomase micelija u tečnoj Čapekovoj podlozi. Gljiva *A. tenuis* imala je bolji odgovor na prisustvo polutanta u čvrstoj podlozi dok je *P. verrucosum* ispoljila bolji odgovor na polutant u tečnoj podlozi. Tokom eksponencijalnog rasta gljiva u tečnoj podlozi sa i bez navedenog polutanta (kontrola), praćene su promene sledećih fizičko-hemijskih i biohemijskih parametara: pH, količina: slobodnih i ukupnih organskih kiselina, proteina i ugljenih hidrata, proteolitička aktivnost. Etoksilovani oleil-cetil alkohol uticao je na smanjenje pH vrednosti podloge i povećanje količine slobodnih organskih kiselina obe gljive. Pomenuti polutant uticao je na produkciju manje količine ukupnih organskih kiseline, proteina, glukoze i fruktoze, u fermentacionoj tečnosti gljive *P. verrucosum* u odnosu na *A. tenuis.* Proteolitička aktivnost gljiva bila je delimično (*A. tenuis*) ili potpuno inhibirana (*P. verrucosum*) prisustvom polutanta u tečnoj hranljivoj podlozi. U prisustvu 1% etoksilovanog oleilcetil alkohola alkalna proteaza *A. tenuis* zadržala je oko 67% aktivnosti tako da bi se mogla koristiti kao aditiv u formulaciji deterdženta. Na osnovu dobijenih rezultata može se zaključiti da se testirane gljive mogu smatrati potencijalnim kandidatima za mikoremedijaciju životne sredine (zemljišta, voda) kontaminirane alkoholnim etoksilatima.

Ključne reči: Monosaharidi • Organske kiseline • Prečnik kolonije • Proteini • Proteolitička aktivnost • Suva biomasa