The influence of detergent and its components on metabolism of *Fusarium oxysporum* in submerged fermentation

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Abstract

The influence of detergent and its components (sodium tripolyphosphate and ethoxylated cetyloleyl alcohol) at 0.1% concentration on the enzymatic and metabolic activity of *Fusarium oxysporum* during exponential growth was investigated in this paper. The fungus *Fusarium oxysporum* was isolated from wastewater originating from households which contain detergent. The following biochemical parameters were analyzed: pH, redox potential, proteolytic activity, production of carbohydrates, free and total organic acids, proteins and total dry weight biomass. The detergent had influence on the significant decrease of redox potential, slight increase of pH and quantity of glucose and total organic acids, while the proteolytic activity was triple insensitive in relation to control. The sodium tripolyphosphate had influence on the slight decrease of pH, significant increase of redox potential and quantity of glucose and free and total organic acids, whereas the proteolytic activity was intensive only 5th and 6th day. The total dry weight biomass of the fungus *F. oxysporum* was slightly inhibited by ethoxylated alcohol, but significantly inhibited by detergent and sodium tripolyphosphate.

Keywords: biomass, carbohydrates, ethoxylated cetyloleyl alcohol, organic acids, proteins, proteolytic activity, sodium tripolyphosphate.

Available online at the Journal website: http://www.ache.org.rs/HI/

SCIENTIFIC PAPER

UDC 661.187:582.28:66

Hem. Ind. **68** (4) 465–473 (2014)

doi: 10.2298/HEMIND130620071J

Over 2000 years, mankind has used surface-active components or their ingredients in the various aspects of daily life, personal care products, laundry washing, chemical cleaning and cosmetics. Chemical surfactants are amphiphilic compounds, which can reduce surface and interfacial tension by accumulating at the interface of immiscible fluids, and increase the solubility and mobility of hydrophobic or insoluble organic compounds [1,2]. Their main application is used by the manufacturers of detergents. A modern detergent powder is complicated multicomponent mixture consisting of active substances (surfactants), builders (sodium phosphates, sodium carbonate, sodium silicate and zeolite), bleach (perborate and percarbonate), wetting agents, optical brighteners, softeners, enzymes [3]. Based on ionization of polar head the surfactants are divided into four groups: anionic, cationic, nonionic and amfotheric. The most widely used in all regions of the world are linear alkyl benzene sulfonates (LASs), alcohol ether sulfates (AESs), aliphatic alcohols (AEs), alcohol sulfates (ASs) and alkaline salts of fatty acids (soap). The linear chain alkylbenzene sulfonate types (LAS) are the most popularly used synthetic anionic surfactant as major ingredient in domestic and industrial detergents more than 30 years [4]. Alcohol ethoxylated are presently the largest volume nonionic surfactants. Growth in use of linear primary AE has been rapid over the past 20 years because of their many desirable qualities such as rapid biodegradation, low to moderate foaming ability, superior cleaning of manmade fibers, tolerance of water hardness and ability to perform in cold water [5]. Builders boost the efficiency of surfactants by counteracting hard water, emulsifying oil and grease, and preventing soil from redeposition. Phosphates are an environmentally delicate chemical and a commonly used detergent builder. Phosphorus is in the form of sodium tripolyphosphate ($Na_5P_3O_{10}$) and is used to soften hard water and suspend dirt in the water. But, by dissolving in water phosphates create orthophosphates which are more toxic and because of that phosphates can be substituted with a phosphate free zeolite ($Na_2Al_2Si_3O_{10}\cdot 2H_2O$ – aluminum silicate) [6]. Increased use of household's detergents leads to their accumulation in the wastewater and natural aquatic ecosystems, causing a number of harmful effects on the microbial community and hydrobionts [7]. Numerous investigations showed that detergent causes anaerobic condition in aquatic ecosystems, disrupts cells structure of live beings, changes activities of their enzymes, *etc.* [8]. Various reports are available on the simulative effect of nonionic and ionic surfactants in fermentation

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Paper received: 20 June, 2013
Paper accepted: 6 September, 2013

broth of microorganism, this resulted in many fold increases in the production and secretion of enzyme such as cellulase [9], phytase [10] and amylase [11]. Inclusion of nonionic surfactants, Tween 80 into culture medium increases extracellular protease activity without altering of enzyme properties [12]. Also, it was found that addition of detergent have enhancing effects on extracellular production of some metabolites with microorganism [13] and may be useful method for over-production of hydrophobic compounds by means of biological process. The *Fusarium oxysporum* is mesophilic fungus ubiquitous in soils worldwide. It commonly inhabits aquatic ecosystems and wastewater with high rate of organic matter. The fungus *F. oxysporum* produces severe hydrolytic enzymes which were found to be active on a wide range of natural substrates of either vegetable or animal origin, *e.g.*, lipases, cellulases, hemicellulases, pectinases, xylanases, *etc*. Lignocellulolytic enzyme produced by *F. oxysporum* has been used for conversion of lignocellulosic material to ethanol [14].

The aim of this study was to investigate how commercial detergent (Merix, Henkel, Serbia) and its mainly components at concentration of 0.1% influence on metabolic process of fungus *Fusarium oxysporum* under submerged fermentation during exponential growth phase. The submerged culture technique is widely used for biotechnological application as it is intrinsically less problematic, making it more reliable and reproducible easier to monitor and to control key operational parameters and it is more flexible.

EXPERIMENTAL

Isolation of *Fusarium oxysporum* and cultivation

The fungus species *F. oxysporum* was isolated from the river basin of Lepenica (the place of wastewater flood, sewage). Identification of fungus from a sample of wastewater was carried out at the Institute for Biology and Ecology, University of Kragujevac. The fungus was maintained in a chamber with a constant temperature at 4±0.5 °C, on potato-dextrose agar slant (PDA), in sterile conditions. A monosporial culture was developed by the method of exhaustion on a poor agar, in Petri dishes, in sterile conditions. Meat peptone agar was used for sterility control. During the experiment, the fungus was cultivated in the sterile Czapek Dox liquid medium of the following composition (Table 1).

Erlenmeyer flasks with growth mediums were sterilized at 120 °C for 20 min (autoclave pressure, 0.14 MPa). The pH control was adjusted about 4.70 before sterilization with 0.1 mol/dm^3 HCl.

Inoculation and sampling

The liquid growth mediums were stored in Erlenmeyer flask (100 ml of medium in 250 ml flask). Each flask was inoculated with one ml spore suspension of (1×10^4 CFU ml^{-1}) in three replicates. Erlenmeyer flasks were placed on an electric shaker (Kinetor-m, Ljubljana) thus enabling uniform and constant mixing. All experiments were carried out at room temperature, under alternate light and dark for 8 days. Sampling was started at 4th day after inoculation and repeated every day until the 8th day of the experiment which corresponding exponential growth phase of fungus. The exponential growth phase was confirmed by screening test on solid state fermentation (results of this test were no shown in this paper).

Measurement of pH and redox potential

pH-meter (type MA-5705, the product "Iskra", Kranj) was used for measuring value of pH and electrochemical potential. Redox potential was determined by Petersen's method [15].

Assays of proteolytic activity

Activity for the alkaline protease was determined spectrophotometrically by Anson's method, with casein as substrate [16]. Reaction mixture incubated at 37 °C for 10 min and arrested by addition of 1 ml 5% trichloroacetic acid (TCA). The mixture was centrifuged at 4000 rpm and the supernatant 5 ml of 6 % Na_2CO_3 and 1ml diluted Folin–Ciocalteu phenol reagent were added. The resulting solution was incubated at room temperature for 30 min and absorbance of the blue color developed was read at 660 nm using tyrosine standard. One unite enzyme activity (IU) was defined as the amount of enzyme that liberated 1μg of tyrosine from casein per minute under assay condition.

Table 1. Composition of growth media in 1000 ml distilled water

Growth medium	Mark	c / g dm^{-3}				Sucrose	Detergent[a]	TTP[b]	AOC[c]
		$NaNO_3$	K_2HPO_4	$MgSO_4\cdot7H_2O$	$FeSO_4\cdot7H_2O$				
Control	K	3	1	0.5	0.01	30	–	–	–
K + 0.1 % D	D	3	1	0.5	0.01	30	1		–
K + 0.1% TTP	TTP	3	1	0.5	0.01	30	–	1	–
K + 0.1 % AOC	AOC	3	1	0.5	0.01	30	–	–	1

[a]Detergent "Merix", Henkel, Kruševac; [b]sodium tripolyphosphate; [c]ethoxylated cetyloleyl alcohol

Protein assay

Protein was determined according to Kjeldahl, on the basis of the nitrogen amount present in the fungus tissue [17], according to the Eq. (1):

The quantity of proteins (mg) =
= 6.25×Quantity of nitrogen (1)

Determination of concentrations total and free organic acids

Concentrations of free and total organic acids were determinate by ion exchange chromatography method. To 10 ml of fermentation broth was added 50 ml of ethanol (70 %) and reaction mix 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 extract following by incubation (30 to 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 aliquots of filtrate were sampling for determination of concentration the free organic acids by titration 0.1 mol/dm^3 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 determination of concentration the total organic acids, 25 ml aliquots were sampling and titration was carried out as previously described [18]. The results were presented as a percentage.

Determination of monosaccharides quantity

Monosaccharides, glucose and fructose were determined after passing 225 ml of filtrate through a previous activated anion column of type Amberlite IR-120 and after evaporation of filtrate to volume 5 to 10 ml. A small volume of sample was inflicted on paper chromatography Whatman No.1 and descending chromatography was performed. The quantity of glucose and fructose was measured by the spectrophotometer at 600 nm, followed by reaction with suitable reagent to produce a blue-green complex [19].

Determination of dry weight biomass

The mycelia previously removed from fermentation broth were washed with sterile distilled deionized water several times. Both filter paper and mycelia were then dried in an oven at 80 °C to a constant weight. The dry weight of the mycelia was determined by subtracting the initial weight of the filter paper from the weight of mycelia and filter paper.

RESULTS

The effects of detergent and its main components at a concentration of 0.1% on the metabolism of fungus *Fusarium oxysporum* are presented on Figs. 1–7. These effects were observed by the following biochemical parameters: pH, redox potential, proteolytic activity, quantity of proteins, organic acids and carbohydrates from 4th to 8th day, whereas total dry weight biomass was measured on 8th day.

The fungus *F. oxysporum* showed the express or slight decrease of pH value of fermentation broth. The maximum values of pH measured on 4th day in all variation of liquid growth medium except in control. The maximum deviation of pH value was observed in the medium with D and TTP at 0.1% concentration compared to control. The detergent at 0.1% concentration influenced on the significant increase of pH value whereas the TTP at 0.1% concentration influenced on the significant decrease of pH value, as shown in Fig. 1.

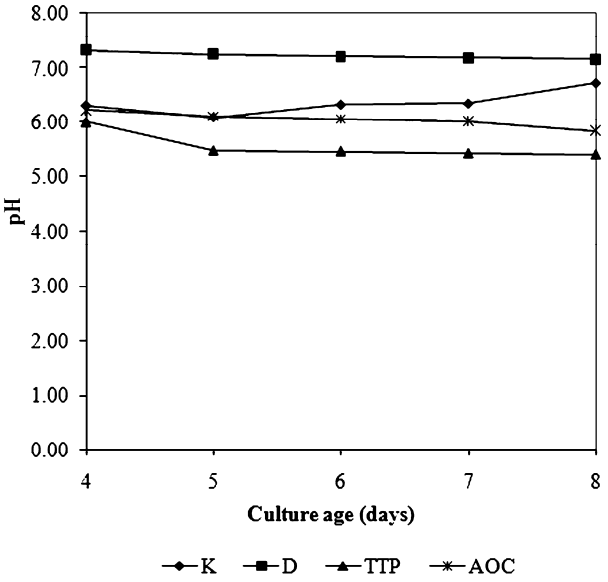

Fig. 1. pH value of different media during growth of fungus F. oxysporum.

Redox potential of the fungus *F. oxysporum* grown in all variants of liquid nutrient medium had a positive value throughout the experimental period, with exception in medium with detergent (negative value). Increasing values of redox potential proceeded in parallel with the development of mycelia, the maximum values were measured on 7th day (control medium) and on 8th day (TTP and AOC media). The results are shown in Fig. 2.

The proteolytic activities of the fungus *F. oxysporum* in the liquid growth medium according to Czapek (the control) and the variants of liquid medium with D, TTP and AOC were particularly expressed on 6th or on 7th day. Presence of AOC in medium had stimulating effect

while the presence of detergent had inhibitory effect on proteolytic activity during the experimental period. The results are shown in Fig. 3.

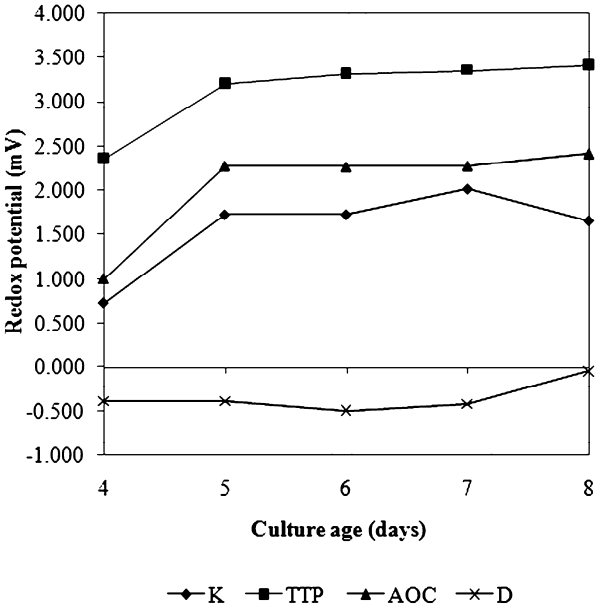

Fig. 2. Redox potential of different media during growth of fungus F. oxysporum.

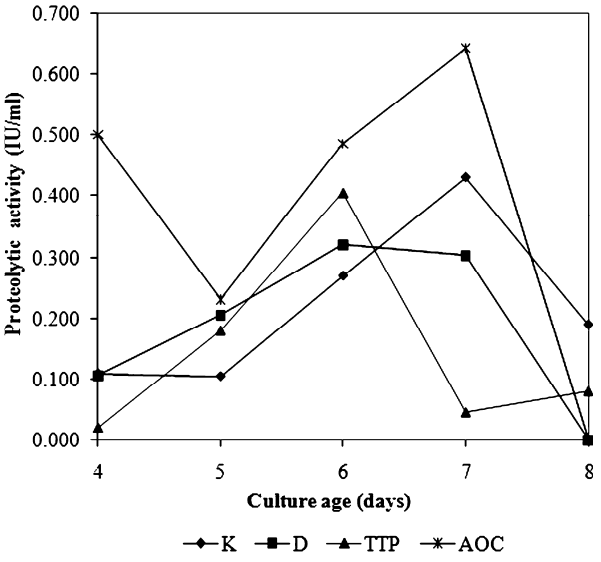

Fig. 3. Profile of proteolityc activity of F. oxysporum in different media during exponential growth.

Protein production increased in parallel with exponential mycelium growth, but concentrations of total protein was different in relation with type of medium. The highest amount of protein was produced by fungus in medium with TTP on the 7th day whereas the least amount of protein measured in medium with detergent at the same time of experiment. The results are shown in Fig. 4.

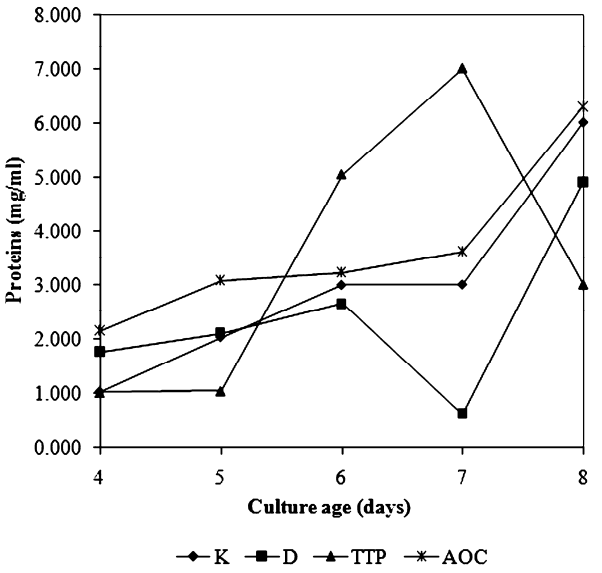

Figure 4. Concentrations of total proteins measured in different media during exponential fungal growth.

The concentration of total organic acids in the control medium and in the medium with AOC was considerably or significantly smaller on the 8th day compared to the 4th day. Contrary, the fungus *F. oxysporum* produced higher percent of total organic acids in the medium with D and TTP, at the same time. The fungus produced different concentration of free organic acids depending on the type of medium. The percentage of free organic acids was lesser in medium with detergent, but it was higher in the medium with TTP and AOC. The TTP showed strong inhibitory effect on the production of free organic acids in the early exponential growth phase, but a strong stimulatory effect in the late exponential growth phase. The results are shown in Fig. 5.

The concentration of glucose, produced by the fungus *F. oxysporum,* was significantly higher in the growth medium with D and AOC at concentration of 0.1% on the 8th day in relation to the control. The presence of AOC in growth medium reduced the concentration of fructose to half while the presence of D influenced on increase of concentration of fructose. The detergent showed the strongest stimulating effect on the bioproduction of glucose whereas ethoxylated cetyl-oleyl alcohol was the strongest stimulator of the fructose bioproduction compared to control, as shown in Fig. 6.

The amount of the total dry weight biomass of the fungus *F. oxysporum* grown in the control nutritious medium was significantly or slightly higher compared to the biomass of the same fungus grown in the variants of liquid media with D, TTP and AOC. Detergent and the sodium tripolyphosphate of 0.1% concentration showed the most significant inhibitory effect on the total dry weight biomass bioproduction in relation to the control (about 50%). The results are shown in Fig. 7.

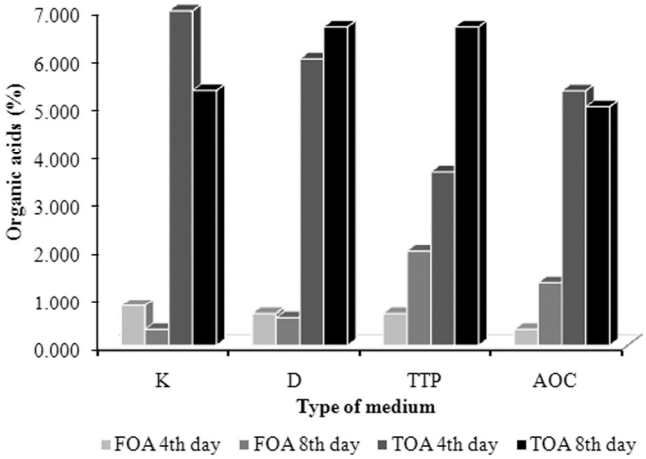

Fig. 5. Concentrations of free (mark-FOA) and total (mark-TOA) organic acids in different media in early and late exponential growth phases.

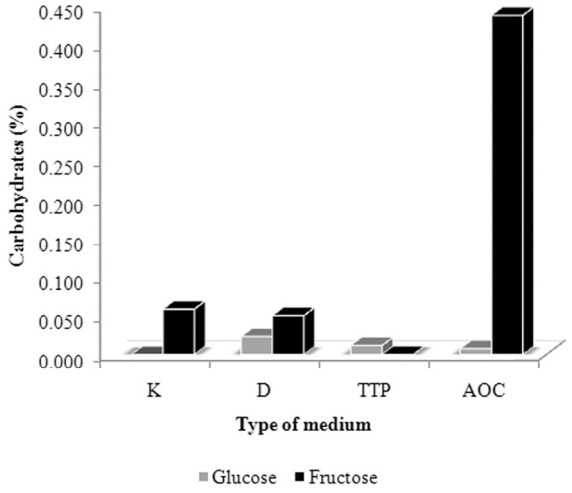

Fig. 6. Concentrations of glucose and fructose measured in different media.

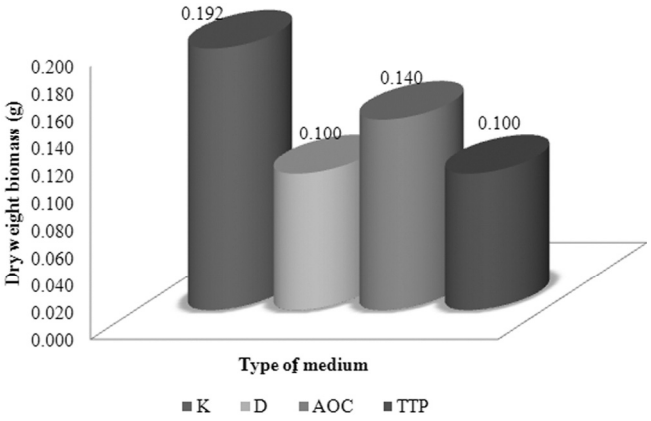

Fig. 7. The total dry weight biomass of fungus F. oxysporum produced in different media at 8th day.

DISCUSSION

The optimal pH environment is necessary for the regular growth and development of microorganisms. It affects the morph-physiological characteristics and biochemical properties of microorganisms. The different initial pH value occurs with addition of detergent and its components in growth media, but these differences aren't influence on the spores germinating and development of mycelia. St Leger *et al.* [20]

observed that *F. oxysporum* tolerated a wide range of pH values. However, these pollutants change metabolic activity of fungus on different ways, which reflects on fungal growth and total dry weight biomass. The utilization of nutrients from growth media changes the pH values. Simultaneously, with changes in pH values and redox potential is changing too. This implies the role of organic acids in pH homeostasis and growth of fungus in a flexible media with detergent and its components. Increase of glucose and fructose concentration in the medium leads to acidification or pH reduction indicating that the acidity of medium originates from organic acids which are released by glycolysis and Krebs cycle. Production of organic acids depends both on the microorganism and source of P in which the microorganism is grown. Many researchers observed that the accumulation of oxalic acid in the cytoplasm can help regulate cytoplasmic pH. Strasser *et al.* [21] reported that oxalic acid is produced in citoplasm of *Aspergillus niger* with little or no participation of the tricarboxylic acids cycle. The chemical composition of commercial detergent is very complex and consists of a mixture of active components, builders, fillers, phosphates, bleach, *etc*. Each of these components can react with active center of the enzyme, leading to their inhibition or activation, which reflects on growth and development of fungi. On the other hand, the individual components of detergent, as substrates for nutrition, are far more acceptable for fungi. As we predicted, the treatment with detergent, AOC and TTP, led to the production of different amounts of free and total organic acids. The quantity of free organic acids was insignificantly lesser in the medium with detergent, but it was significantly higher in the medium with TTP and AOC at the end of experimental period. Many researchers observed that many fungi species produce different organic acids in medium with polyphosphates because of solubilization of P. In the case of the total organic acids bioproduction, the TTP is behaving as a significant stimulator while the detergent is insignificant stimulator of bioproduction. At the same time, the AOC is behaving as an inhibitor of the total organic acids bioproduction.

The presence of detergent, AOC and TTP, in the liquid growth medium affects the production of different protein concentration during the fungal exponential growth. With culture aging, fungus produced higher amount of proteins in control and AOC media probably due to increasing of N consumption from these media. On the 7^{th} day of the experiment, significant changes of proteins bioproduction were observed in medium with D and TTP. The production of protein was rapidly decreased in D medium whereas the opposite effect was observed in TTP medium. These results are in accordance with the results of other authors which observed that. According to Scervino *et al.* [22] consumption of N increases with decreasing of phosphates concentration, which is in agreement with results of this study. Also, our previous studies on other filamentous fungi [23,24] confirmed that pollutants such as LAS detergent type have inhibitory effect on protein bioproduction.

The proteolytic activity of the fungus *F. oxysporum* was expressed in late exponential phase of fungal growth (6^{th} or 7^{th} day). Inclusion of AOC in growth medium showed significant stimulatory effect on proteolityc activity whereas the treatment with the detergent and TTP had inhibitory effect on proteolytic activity. As well known, numerous microorganisms have ability to synthesized different proteases depending on type of medium. The protease of *Bacillus* species showed excellent stability and compatibility with some commercial detergents [25]. However, anionic surfactants, such as SDBS and SDS, express strong inhibitory effect on enzymes activity in the most of microorganisms [26,27]. Polar heads of SDS or SDBS bind to the active site of the enzyme through ionic interaction which causes conformational changes of enzymes. Nonionic surfactants type of ethylene oxides bind to the active site of enzymes through hydrogen bonds in order to enhance the conformation flexibility. In agreement with observations of Pradhan and Sukla [28] and Scervino *et al.* [29] our experiment confirms the hypothesis that solubilization of P is related with releasing of organic acids in medium. Many researchers observed relation between the beginning of autolysis after depletion of the exogenous carbon source and proteolytic activity to a greater or lesser degree in various species of filamentous fungi [30–32]. However, it is not uncommon for the maximum of proteolytic activity to coincide with the growth phase [33], which it was confirmed by our investigation. Similar observations with *A. fumigatus* and *N. crassa* suggest that this phenomenon can be widespread among fungi.

The concentration of carbohydrates (glucose and fructose) was measured in fermentation broth of growth medium on the 8^{th} day. Fungal metabolism of carbohydrates is influenced by the composition of growth medium. When sucrose is only carbon source, the fungus utilizes preferably glucose than fructose for its metabolism which is consistent with the results of Peynaud and Domercq [34]. Because of that, the concentration of glucose is very low compare to fructose in liquid medium by Czapek. As well known, under aerobic condition the fungus *F. oxysporum* metabolizes glucose *via* Embden–Meyerhof–Parnas (EMP) pathway, resulting in high acetic acids production, while under anaerobic condition of the glucose is metabolized via pentose phosphate pathway (PPP) resulting in high ethanol production [35]. The inclusion of D, AOC and TTP leads to decreasing of glucose incorporation in biomass and

its higher concentration in medium. The detergent shows the highest inhibitory effect on glucose metabolism. Interesting, TTP and detergent added in medium showed the stimulatory effect on fructose metabolism and its incorporation in fungal biomass. On the contrary, AOC shows the highest inhibitory effect on metabolism fructose and its incorporationin biomass. This striking difference in the utilization of sugars could be caused either by differences in the transport systems or by the subsequent intracellular metabolism of the sugars [36]. Probably, detergent and TTP influence on synthesis of inducible enzymes involving in regulation of carbohydrates metabolism or some degradation products have a role of competitive inhibitor of these enzymes. These results are in accordance with the results of other authors and our previous studies on other filamentous fungi [37,38].

The total dry weight biomass which the fungus *F. oxysporum* produced during the exponential growth was measured on 8th day. The total amount of biomass of the fungus grown in control is higher compare to media with the detergent, AOC and TTP. These results confirm that these compounds behavior as inhibitors on the bioproduction of biomass. Detergent and the sodium tripolyphosphate at 0.1% concentration show the significant inhibitory effect on the total biomass (about 50%), while the AOC showed little inhibitory effect compared to control.

CONCLUSION

Based on the obtained results it can be concluded that detergent and its components influence on fungal metabolism in different way. These pollutants didn't express fungicide effect, but they have inhibitory influence on total dry weight biomass production at different rates. Inclusion of pollutants in growth medium causes production of different metabolites which could have practical application in biotechnology. The alkaline protease from fungus is stimulated by the presence of AOC, so it could have potential utilization for catalysis and application in detergent formulation. High solubilization of P in medium with TTP indicated the potential role of *F. oxysporum* in bioremediation of environment. The production of higher amount of organic acids is stimulated by detergent and its components, so the fungus could be applied in different industrial area. The fungal ability to produce different amount of glucose and fructose could be practically applied in biotechnology.

Acknowledgments

This research was financially supported by Ministry Education, Science and Technological Development of the Republic of Serbia (Grant No. III 43004).

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IZVOD

UTICAJ DETERGENTA I NJEGOVIH KOMPONENTI NA METABOLIZAM *Fusarium oxysporum* U SUBMERZNOJ FERMENTACIJI

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(Naučni rad)

U ovom radu ispitivan je uticaj detergenta (“Merix”, Henkel, Kruševac) i njegovih komponenti (natrijum-tripolifosfata i etoksilovanog cetil-oleil alkohola) 0,1% koncentracije na enzimsku i metaboličku aktivnost gljive *Fusarium oxysporum* tokom eksponencijalne faze rasta. Ova vrsta gljive je izolovana iz rečnog korita reke Lepenice na mestu ulivanja otpadnih voda u reku. Praćene su promene sledećih biohemijskih parametara: pH, redoks potencijala, proteolitičke aktivnosti, produkcije ugljenih hidrata, slobodnih i ukupnih organskih kiselina, proteina i ukupne suve biomase. Dodatak detergenta u hranljivu podlogu je izazvao značajno smanjenje redoks potencijala, blago povećanje pH, koncentracije glukoze i ukupnih organskih kiselina, dok je proteolitička aktivnost bila trostruko intenzivnija u odnosu na kontrolu. Dodatak natrijum-tripolifosfata u hranljivu podlogu uticao je na blago smanjenje pH, značajno povećanje redoks potencijala i količine glukoze, slobodnih i ukupnih organskih kiselina, dok je proteolitička aktivnost bila izražena samo 5. i 6. dana. Ukupna suva biomasa gljive *Fusarium oxysporum* bila je blago inhibirana prisustvom etoksilovanog cetil-oleil alkohola ali značajno inhibirana prisustvom detergenta i natrijum-tripolifosfata u podlozi.

Ključne reči: Biomasa • Etoksilovani cetil-oleil alkohol • Natrijum-tripolifosfat • Organske kiseline • Proteini • Proteolitička aktivnost