Chapter 8

The Potential Application of Selected Fungi Strains in Removal of Commercial Detergents and Biotechnology

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Additional information is available at the end of the chapter

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Abstract

The consumption of synthetic detergents is increasing year by year due to increasing urbanization, which reflects on higher concentration of this pollutant in the environment. In order to purify wastewaters from different pollutants, the application of new technologies such as bioremediation is necessary. From the environmental point of view, it is important to identify microorganisms that are tolerant to the presence of this pollutant. This chapter presents the experimental evaluation of ability of several fungal species, isolated from municipal sewage and industrial wastewater, in removing a high detergent concentration (anionic surfactants) from the environment as well as their potential application in biotechnology. The selected fungi were cultivated in Czapek-Dox liquid medium supplemented with commercial detergent "Merix" (Henkel, Kruševac, Serbia). Changes of physicochemical and biochemical parameters such as pH, redox potential, dry weight biomass, and enzymes activities such as alkaline protease and phosphatase were evaluated during 16 days of cultivation. The obtained results could be useful in the implementation of tested fungi in bioremediation processes and in biotechnology.

Keywords: alkaline protease, alkaline phosphatase, biodegradation, commercial detergent, fungi

1. Introduction

The detergents that we use for our daily laundry have been recognized as one of the major pollutants responsible for water pollution [1]. Detergents are synthetic organic compounds, which contain three main ingredients (%): phosphate builders (50% by weight, approximately), surface-active substances (surfactants) (between 10 and 20%), and bleaches (7%), as well as very small percentages of additives (wetting agents, optical brighteners, softeners, and enzymes).

Surfactants are the components mainly responsible for the cleaning action of detergents, whereas the additives are designated to enhance the cleaning action of surfactants [2]. Surfactants commonly used as main ingredients in commercial detergents are linear alkyl-benzene sulfonates (LASs) (anionic) and ethoxylated alcohols (AEs) (nonionic) [3]. Commercial LAS also contain coproducts called dialkyltetralinsulphonates (DATS) and iso-LAS. Furthermore, over 70 major isomers of DATS have been detected in commercial LAS [4]. The molecular structure of a synthetic detergent influences its biodegradation potential, which is the principal criterion for their ecological behavior. The biodegradation rate and acute toxicity of LAS on aquatic life are very much related to both the chain length and phenyl position of the alkyl chain [5, 6].

The main environmental impact of detergents is related to their post use effect when the wash water is discharged into sewage treatment plants (STPs) or discharged directly to the aquatic environment in areas where there is no sewage treatment [7]. These compounds can act on biological wastewater treatment processes and cause problems in sewage aeration and treatment facilities due to their high foaming, lower oxygenation potentials, and the ability to kill waterborne organisms [8]. Biodegradation processes and adsorption on active sludge remove these chemicals from wastewater to a greater or lesser extent, depending on the chemical structure of the surfactant molecule and operating conditions of the STP. Under aerobic conditions, LAS is degraded through ω-oxidation of terminal carbon in the alkyl chain followed by *ß*-oxidation. In this process, which is known as the primary biodegradation, sulfophenyl carboxylic acids (SPACs) are forming [9]. In the next step, known as ultimate biodegradation, SPACs are transforming ultimately into CO_2, H_2O, inorganic salts, and biomass. After treatment, residual surfactants, refractory coproducts, and biodegradation products dissolved in STPs effluents or adsorbed on sludge are discharged into the environment. These chemicals through several transport mechanisms enter the hydrogeological cycle. However, even biodegradable detergents can have a toxic effect upon the living beings if they are present in quantities above the permitted level [10]. In line with their high-environmental relevance, surfactants have to meet certain requirements issued in the European detergent regulation 684/2004 [11]. According to Legislative acts, the maximal amount of detergent allowable in wastewater which effluent in public sewage is 4 mg/L, and 0.5 mg/L in natural recipient.

Traditional methods for the reduction of pollutants and environmental cleanup contain combination of physical, chemical, and biological methods [12, 13]. Bioremediation, as less expensive and eco-friendly alternative to conventional technology for decontaminating environment from wide range of pollutants by microorganisms, has been extensively studied during past two decades [14]. Mycoremediation is a type of bioremediation, which uses fungal mycelium to decontaminate or filter the toxic waste from contaminated area. Filamentous fungi have the ability to grow and transform or degrade hazardous compounds in polluted environment, as response to severe environmental conditions [15]. Because of that, they represent power useful potential in bioremediation processes. The numerous examples of fungi used in biodegradation of certain toxic pollutants (petroleum hydrocarbons, chlorofenols, pesticides, nitroaromatic explosives, etc.) have been observed [16–20]. The potential of filamentous fungi in removing of commercial detergent has been continuously studied over the past three decades by Stojanović et al. [21–23]. These studies identified a total of 15 strains of *Actinomycetes*, which have the ability to grow and metabolize synthetic detergent "Merix" (Henkel, Serbia) and its particular components: ethoxylated oleyl-cetyl alcohol and sodium

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tripolyphosphate at wide concentrations range 0.01–1.0%. Since the application of new technology in detergent industry leads to the development of new detergent performance, the identification and investigation of new microbial strains are necessary and justified.

The ability of filamentous fungi to produce and excrete a variety of extracellular hydrolytic enzymes is significant not only for bioremediation processes but also for fermentation industries and biotechnology. This study was focussed on two types of hydrolases: alkaline protease and phosphatase, due to their importance in different industrial areas. Proteases (EC 3.4.21-24 and 99) are one of the key enzymatic constituents in detergent formulation in which they act as protein stain removers. They represent about 60% of total worldwide sale of enzymes. The most significant commercial detergent protease additives (Savinase®, Esperase®, Alcalase®, etc.) are produced by *Bacillus* spp. [24]. In addition, *Pseudomonas* species are also recognized as sources of alkaline proteases with advantageous properties for industrial applications [25, 26]. In recent years, a great number of fungi from genus *Aspergillus, Penicillium, Rhizopus, Mucor, Humicola, Thermoascus, Thermomyces* have been identified as producers of different types of protease of biotechnological relevance. The increased demand for proteases with specific properties has led researchers to explore new sources of proteases.

***Alkaline phosphatase (ALP: EC 3.1.3.1) catalyzes the hydrolytic cleavage of phosphate monoesters under alkaline conditions and plays important roles in microbial ecology through its involvement in phosphate metabolism [27] and molecular biology [28] applications. There has been considerable effort in recent years toward the application of ALPs for bioremediation of heavy metals and radionuclides from nuclear wastes [29]. ALP can be isolated from variety of microorganisms including *Escherichia coli* [30], *Pseudomonas* [31], *Aerobactor* [32], and *Bacillus* species [33]. Usually the ALP is produced at commercial level from *E. coli* or calf intestine. However, there is no literature data about production of ALP among fungi strains so far. This study investigated potential of several filamentous fungi to produce the enzyme for the first time.

The aim of the study was isolation of fungi (micromycetes) from municipal sewage wastewater originating from households and industrial wastewater from Henkel factory (Kruševac, Serbia); selection of fungi strains that are tolerant to a high detergent concentration; cultivation of selected fungi in Czapek-Dox liquid medium supplemented with commercial detergent mark "Merix" (Henkel, Serbia), and *in vitro* investigation of their growth and metabolic activity. The investigation of these parameters is crucial for the practical application of fungi in bioremediation processes. The obtained results could be beneficiary in clarifying the potential role of fungi in bioremediation of environment contaminated with a high concentration of tested pollutant as well as in biotechnology.

2. Materials and methods

2.1. Isolation and identification of fungi

The fungi used in this work were isolated from wastewater samples, which contain commercial detergents. The wastewater samples were collected from rivers basins of Lepenica (Kragujevac, Serbia) and West Morava (Čačak, Serbia), at places where municipal wastewater

discharges into the rivers. In addition, samples of wastewater were collected from river basin of Rasina (Kruševac, Serbia), downstream where the industrial wastewater of factory Henkel discharges into the river. Samples of wastewaters were taken in sterile glass bottles, transferred to the microbiology laboratory and disposed to refrigerate at 4°C. Within 24 hours, different dilutions of samples were transferred on Petri plates with malt agar and streptomycin to prevent the bacterial growth. The Petri plates were maintained at room temperature for 5 days. Positive cultures of fungi were subcultured on malt agar (MA) and potato dextrose agar (PDA) for the isolation of a pure, single colony for identification.

The identification of pure fungal cultures was carried out according to Systematic key at the Faculty of Science, University of Kragujevac, Serbia, by Prof. Branislav Ranković. For spores production, pure cultures were aseptically maintained at (28 ± 2)°C from 3 to 5 days on PDA, composed of (g/L): peeled potatoes 200, dextrose 20, and agar 15. After having sufficient population of spores, the plate were stored at (4 ± 0.5)°C with periodical subculturing in sterile conditions.

2.2. Inoculum preparation

An inoculum suspension was prepared from fresh, mature cultures of selected fungal species. The colonies were covered with 5 mL of distilled sterile water. The inoculum was achieved by carefully rubbing the colony with a sterile loop; the tube with isolate was shaken vigorously for 15 seconds with a Vortex mixer and then transferred to a sterile tube. The inoculum size was adjusted to 1.0×10^6 spores/mL by microscopic enumeration with a cell-counting hematocytometer (Neubauer chamber).

2.3. Experimental procedure and culture conditions

The fungi were grown in 250 mL Erlenmeyer flasks with 200 mL of modified Czapek Dox liquid nutrient medium the following composition (g/L): $NaNO_3$ is 3.0; K_2HPO_4 is 1.0; $MgSO_4 \times 7H_2O$ is 0.5, and sucrose is 30.0 and distilled water up to 1000 mL (control-C). The pH value of this liquid media was about 4.80 (adjusted with 0.1 M HCl). The media with addition of detergent "Merix" (Henkel, Serbia) at concentrations of 0.3% (D3) and 0.5% (D5) were prepared according to same procedure. The pH values of these media were measured and recorded as 9.35 and 9.80, respectively. All flasks were sterilized at 121°C in an autoclave for 15 minutes. After cooling the liquid media to room temperature, 1 mL spore suspension of each fungus was inoculated in liquid media in aseptic condition. Inoculated flasks were incubated on an electric shaker (Kinetor-*m*, Ljubljana) at 150 rpm and room temperature for 16 days. Summary, one positive control without detergent with spores (C), two test flask with detergent and with spores (D3 and D5), and two negative controls with detergent but without spores (ncD3 and ncD5) were used for each fungal species. Three flasks of each fungus were used for collecting samples at 3rd, 6th, 9th, 12th, and 16th day. To determine that each fungal biomass dry weight, mycelia were removed by filtration of fermentation broths, according to procedure described below. The filtrates of fermentation broths were collected by centrifugation at 12,000 g for 2 minutes. The supernatants were used as the source for determination of pH, redox potential, concentration of ASs and enzymes activities.

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2.4. The measurement of dry weight (DW) biomass

At the time intervals above-mentioned, the mycelium of each fungus was filtered through filter paper (Whatman No. 1) of a known weight, washed with distilled water and dried at constant weight at 80°C. The filter paper with the mycelium was placed in the desiccator and then reweighed. Mycelium DW was calculated using the Eq. (1), and results are expressed in grams per liter (g/L) of submerged culture.

$$\mathrm{DW}(\mathrm{g/L}) = (\mathrm{Wcf} - \mathrm{Wif}) \times 5 \quad (1)$$

where DW is total biomass dry weight, Wcf is weight of culture with filter paper, and Wif is initial weight of filter paper.

2.5. The measurement of pH and redox potential values

The pH and redox potential values of the culture filtrates were measured by digital PHS-3BW microprocessor pH/mV/temperature meter model 65-1 (Bante Instruments Ltd., China) with reference electrode Ag/AgCl/3 mol/kg KCl that was initially standardized with appropriate buffer solution of pH 4.0, 7.0, and 10.0. The redox potential values are expressed in mV, and are calculated using the following Eq. (2):

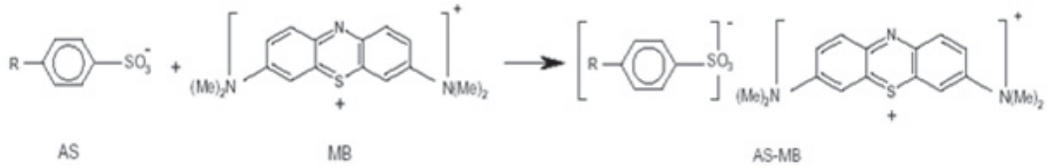
$$\mathrm{E(mV)} = E_{\mathrm{ems}} + E_{\mathrm{ref}} \quad (2)$$

where E_{ref} is the potential of the reference electrode (+210 mV at 25°C), E_{ems} is measured potential.

2.6. Determination of concentration of anionic surfactants (ASs) and calculation of biodegradation rate

The concentration of ASs in the detergent and fermentation broth was determined by spectrophotometrically using methylene blue (MB). The method for determining the concentration of methylene blue-active substance (MBAS) in the detergents was adapted from *Standard Methods for the Examination of water and wastewater* [34]. The method is based on the reaction presented in **Scheme 1**.

The solutions of detergent were transferred into the separatory funnels. One drop of 1% phenolphthalein solution as indicator was added to the detergents solutions, afterward 1 M NaOH was adding until obtained change in color from colorless to pink. Then, 1 M H_2SO_4 was added carefully until the solution in the funnel had become acidic, which is reflected in



Scheme 1. Mechanism of formations an ionic pair methylene blue-active substance (AS-MB) between the anionic surfactants (AS) and the methylene blue (MB).

appearance of colorless. The procedure of extraction was continued by adding 5 mL chloroform and 13 mL methylene blue reagent in each funnel. The funnels were shaken about 30 seconds. In order for phase's separation the funnels were stored at ambient temperature for at least 30 minutes. Thereafter, the chloroform layer was decanted into a clear 100 mL Erlenmeyer bottle. The same procedure was repeated three times employing 5 mL of chloroform for each time. The chloroform layers were collected in a 100-mL Erlenmeyer bottle and then reversible transferred to the separatory funnel. To each funnel, 25 mL of wash solution (6.7×10^{-3} 1 M phosphate buffer, pH 7.1) was added; then they were shaken once more for 30 seconds and stored at ambient temperature for 30 minutes. Finally, the chloroform layer was drawn off through glass wool into a 50-mL volumetric flask. Absorbance of chloroform layer was measured using Perkin-Elmer Lambda 25 UV-Vis spectrophotometer at 652 nm against blank chloroform. The concentration of the residual surfactant present in test detergent in terms of MBAS were then plotted against the time (days) for the 16-day experimental period. The result obtained with the SDS (Fluka, Switzerland), an alkylsulfate anionic surfactant, as the referent anionic surfactant compound served as the standard. The percentage of surfactants degradation was calculated using Eq. (3):

$$\% \text{ Degradation} = 100 - [(A_{625} \text{ exp} - A_{625} \text{ blank}) / A_{625} \text{ std}] \times 100, \quad (3)$$

where A_{625} *exp* is absorbance of test sample, A_{625} *blank* is absorbance of blank sample, and A_{625} *std* is absorbance of standard sample at 625 nm.

2.7. Assay of alkaline protease activity (EC 3.4.21-24)

The protease activity was assayed by the Anson method [35]. The fermentation broth (1 mL) was mixed with 5.0 mL of substrate (0.65% casein in 25 mM tris-HCl buffer, pH 8.0), was incubated at 37°C for 30 minutes. After incubation, 1 mL of 5% trichloroacetic acid (TCA) was added to attenuate the reaction. The mixture was allowed to incubate for 30 minutes at room temperature and filtered to remove the precipitate. 5 mL of 6% Na_2CO_3 and 1 mL of diluted Folin-Ciocalteu's phenol reagent were then added to the filtrate. The solution was kept at room temperature for 30 minutes and absorbance was read at 660 nm. A standard curve was generated using tyrosine standard. One unit enzyme activity was defined as the amount of enzyme capable of producing 1 μg of tyrosine from casein in a minute under assay condition.

2.8. Assay of alkaline phosphatase activity (EC 3.1.3.1)

The activity of alkaline phosphatase (ALP) was assayed by using *ß*-glycerophosphate as substrate. The substrate solution was prepared by mixing 0.5 mL of 0.05 mol/L glycol buffer with 1.0 mM Magnesium chloride, pH 9 and 0.5 mL of substrate to both test and blank tubes. Then, 0.1 mL of enzyme solution (fermentation broth) and deionized water were added to test and blank tubes, respectively. The tubes were mixed and incubated at 37°C for 30 minutes. After incubation, 10% TCA was added to each tube in order to stop the enzyme reaction. The solution of NH_4-molybdate was added as color reagent. The amount of liberated inorganic phosphate (Pi) was quantitatively determined spectrophotometrically (Perkin-Elmer Lambda 25) at 660 nm [36]. One unit of enzyme activity (IU) was defined as the amount of enzyme that released 1 μg of inorganic phosphate per minute under the assay conditions.

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2.9. Statistical analysis

Statistical analysis was performed using SPSS statistical software package (SPSS for Windows, ver. 13.0, Chicago, IL, USA). For testing the normality of distribution, means and standard deviation, student *t*-test was used. To compare the differences between growth media, Mann-Whitney and Kruskal-Wallis tests were used. Pearson's correlation coefficient was used for the measurement of the strength of the association between variables. All significance tests were two-tailed (0.05 and 0.01) and $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Identification and selection of fungi species used in this study

From collected samples of wastewaters the following fungi were identified: *Aspergillus niger, Penicillium chrysogenum* (both isolated from sewage wastewater of Lepenica River, Kragujevac, Serbia), *Penicillium cyclopium* (from sewage wastewater of West Morava River, Čačak, Serbia), and *Trichoderma harzianum* and *Mucor racemosus* (both from wastewater plant of detergent industry (Henkel, Kruševac, Serbia)). The identification of fungi strains were performed using systematic keys at Faculty of Science, University of Kragujevac.

Systematic and morphological (macro- and microscopic) characterizations of isolated species are reported in **Table 1**.

Aspergillus niger (**Table 1**)—the colony color and texture varies with age. Initially, the colony is white but changes color to dark brown or black with age and conidial production. Hyphae are septate and hyaline. Conidiophores are hyaline, upright, simple, smooth-walled, length between 400 and 3000 μm, terminating in spherical to globose vesicles, 30–75 μm in diameter. *A. niger* has both metulae and phialides (biseriate), which cover the entire surface of the vesicle. The stipe measured 440–680 × 6–12 μm, smooth-walls, slightly brown in color. Conidia are 1-celled, size from 4–5 μm, very rough structure, globose, and variously in mass (brown to black color) in dry basipetal chains [37].

Penicillium chrysogenum (**Table 1**)—colony broadly spreading, blue-green to bright green, with broad white margin during the growing period, smooth velvety, usually becoming grayish or purplish brown in age with overgrowth of white or rosy hyphae; reverse yellow, with color diffusing somewhat; drop usually branches with all parts smooth; stipes 2.5–4 μm diameter; phialides ampuliform with a reduced neck, 7–10 × 2–2.5 μm; conidia elliptical to subglobose, 3–4 in μm long axis, smooth [38].

Penicillium cyclopium (**Table 1**)—colonies rather dull blue-green, with brighter zone inside the white margin, almost velvety but showing distinct fascilutation in the younger areas; reverse usually pale peach but occasionally fairly bright yellow or purplish brown; the penicillin with normally two to three stages of branching, often with branches and metulae born at the same level, stipes rough, long, 100–400 × 2.5–4 μm; phialides flask-shaped, 7–10 × 2–3 μm; conidia globose to subglobose, sometimes elliptical when first formed, smooth to very finely roughened, 3–4 μm diameter [39].

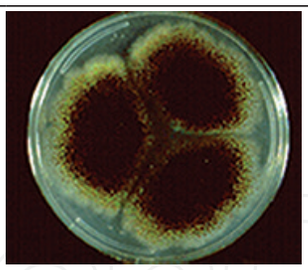
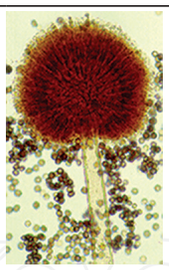
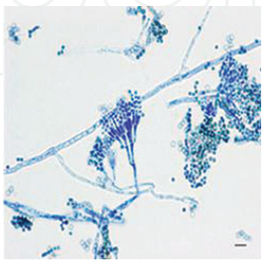
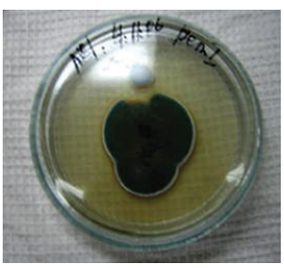
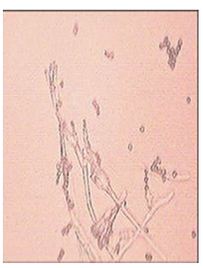
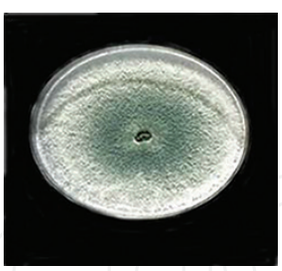
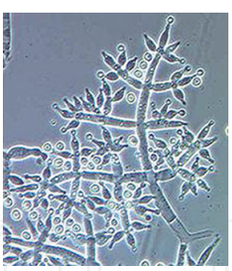
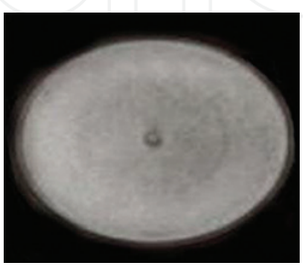
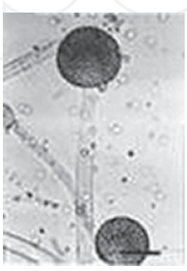
Fungi species	Macroscopic	Microscopic
Phylum: Ascomycota Class: Ascomicetes Subclass: Euromycetide Order: Eurotiales Family: Trichocomaceae Genus: *Aspergillus* *Aspergillus niger* Van Tieghem (1867)		
Phylum: Ascomycota Class: Ascomicetes Subclass: Euromycetidae Order: Eurotiales Family:Trichocomaceae Genus: *Penicillium* *Penicillium chrysogenum* Thom (1910)		
Phylum: Ascomycota Class: Ascomicetes Subclass: Euromycetidae Order: Eurotiales Family: Trichocomaceae Genus: *Penicillium* *Penicillium cyclopium* Westling (1911)		
Phylum: Ascomycota Class: Sordariomycetes Order: Hypocreales Family: Hypocreaceae Genus: *Trichoderma* *Trichoderma harzianum* Rifai (1969)		
Phylum: Zygomycota Order: Mucorales Family: Mucoraceae Genus: *Mucor* *Mucor racemosus* Fresenius (1976)		

Table 1. Systematic and morphological appearance of fungi [41–45].

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Trichoderma harzianum (**Table 1**)—the surface of colony is initially white or yellow, then becoming yellow-green with age. Colony texture is wooly. Hyphae are septate and hyaline. Conidiophores are hyaline, much branched, not verticillate, and may sporadically demonstrate a pyramidal arrangement. Phialides are divergent, typically flask shaped, enlarged in the middle, sharply constricted below the tip to form a narrow neck and slightly constricted at the base. Conidia are hyaline, 1-celled, smooth or roughened, range in shape from globose to ellipsoidal, born in a small terminal clusters at the tips of phialides, diameter of 3 μm [40].

Mucor racemosus (**Table 1**) is a dimorphic, facultative anaerobic zygomycete, capable of vegetative growth in either a filamentous phase or as spherical yeasts. Colonies grows rapidly at 25–30°C and quickly cover the surface of the agar. Its fluffy appearance with a height of several cm resembles cotton candy. From the front, the color is white initially and becomes dark grayish-brown or light olive-grey in time when grown on typical laboratory media. It is easily recognizable microscopically by its tall (up to 2 cm) needle-like sporangiophores and large sporangium. Sporangiophore born from aerial hyphae; stipes simpodially branched; sporangia spherical, approximately 50–300 μm in diameter; columella have ellipsoidal to pyriformal shape. Sporangiospores are hyaline, ellipsoidal, mostly diameter of 4–8 μm, with smooth wall [46].

3.2. Effect of commercial detergent on fungal biomass and growth curves

Biomass is an indicator of fungal metabolic activity and their bioremediation potential. Very important factor for biodegradation processes is physicochemical interaction between surfactants and fungal membranes and cell wall [47]. Further, the surfactants can cause inhibitory or stimulatory effect on enzymes involved in key metabolic pathways and change their metabolic activity in these two ways. Overview of the literature provides the evidences that growth of fungi depends on the type of surfactant in such a way that nonionic surfactants, Triton X-100 and Tween 80 supported, whereas anionic-type surfactant, SDS, inhibited their growth [48]. An investigation of the impact of surfactants on the growth and development of fungi is not simple process due to numerous factors such as applied concentration of surfactants, type of fungus and its genetic properties, experimental conditions, and so on influence these processes.

The current study investigated the effect of commercial-powdered detergent "Merix" (Henkel, Kruševac, Serbia) on the growth and development of five fungi species, which were quantitatively dominated in wastewaters. Previously, the maximal concentration of detergent on which fungi can grow was determined and defined as 3 mg/mL or 0.3% for all tested fungi with exception of *M. racemosus*. This fungus was able to grown at higher concentration of detergent, 5 mg/mL or 0.5%. In this case, the detergent at both concentrations was used for investigation of the growth and metabolic activity of the fungus compared to control (Czapek-Dox liquid medium). The obtained results were presented in **Figure 1**. As **Figure 1** shows, all fungi had monophasic exponential growth in C medium. The growth curves of fungi in this medium have following phases: exponential growth, stationary phase, and autolysis. A little deviation from this growth profile was observed in the C medium of *A. niger* [**Figure 1 (1)**]. This fungus had very pronounced the exponential growth phase until the 9th day, followed by the stationary phase until 16th day, without autolysis [49]. The other fungi, *P. chrysogenum*

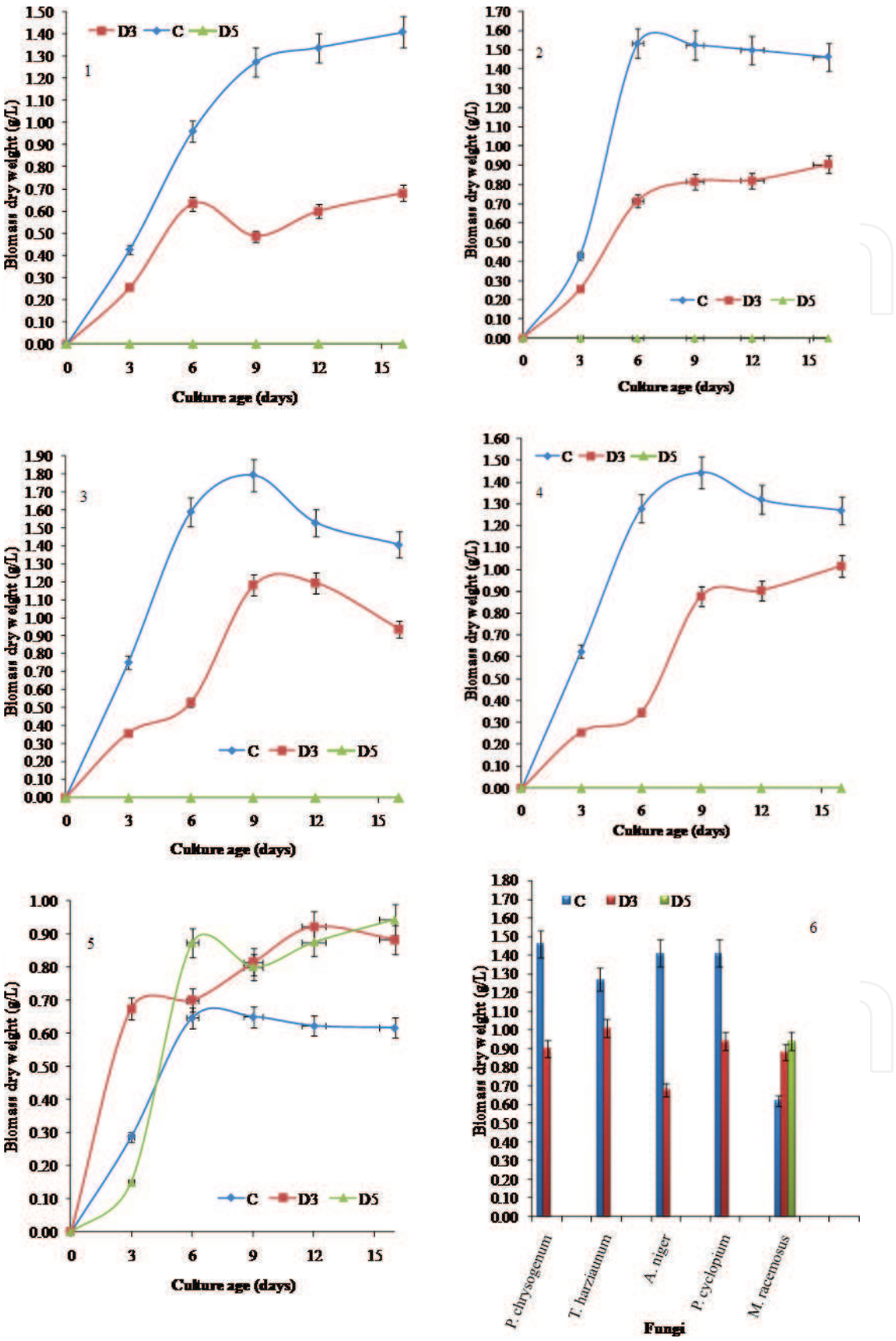
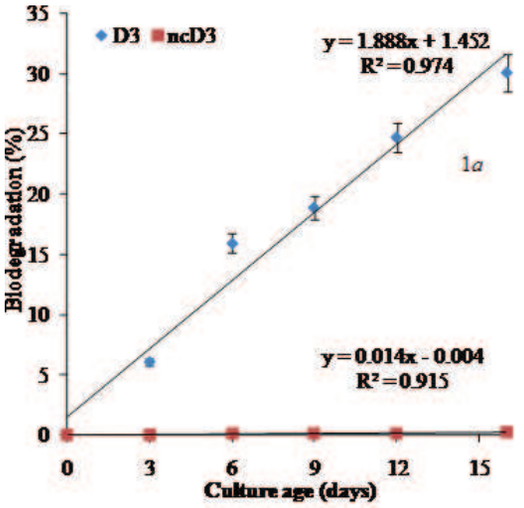


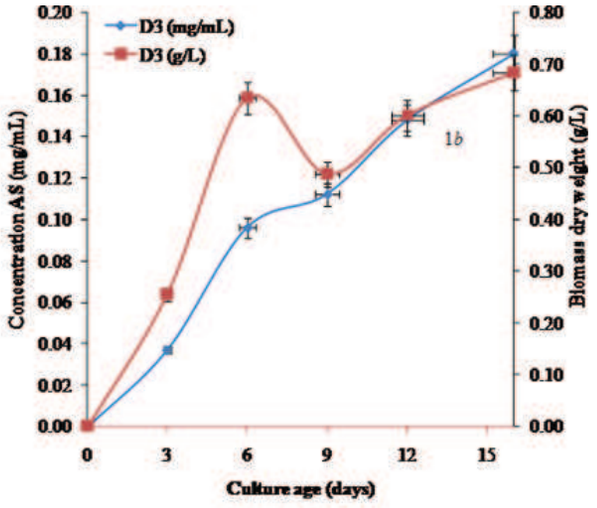
Figure 1. Fungal biomass dry weight and growth curves: *A. niger* (1), *P. chrysogenum* (2), *P. cyclopium* (3), *T. harzianum* (4), *M. racemosus* (5), and comparison of total biomass of fungi after 16-day cultivation (6).

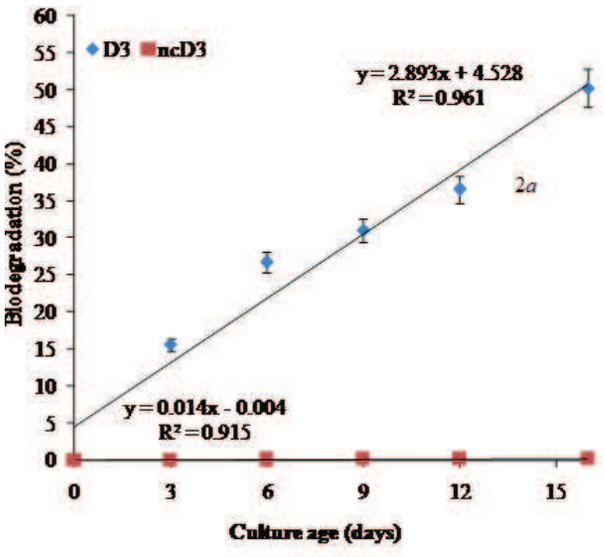
[**Figure 1(2)**], *P. cyclopium* [**Figure 1(3)**], *T. harzianum* [**Figure 1(4)**], and *M. racemosus* [**Figure 1(5)**] had the exponential growth phase from inoculation until the 6th day and stationary phase from 6th to 9th day, when the maximal growth and biomass dry weight were observed. After stationary phase, autolysis was noted, which was reflected on the total biomass reduction. In contrast to C medium, the fungi cultivated in D3 medium had biphasic exponential growth [49–52]. The growth curve of *P. cyclopium* [**Figure 1(3)**] had cascade shape with five distinct phases: the early exponential growth (until 3rd day), the first stationary phase (from 3rd to 6th day), the second exponential phase (from 6th to 9th day), the second stationary phase (from 9th to 12th day) and autolysis (from 12th to 16th day). In the profile of *A. niger* [**Figure 1(1)**], the autolysis was observed between 6th and 9th day; afterward the fungus has slow growth until the end of experiment. The growth curves of *P. chrysogenum* [**Figure 1(2)**] and *M. racemosus* [**Figure 1(5)**] revealed the autolysis between 12th and 16th day. On the other hand, autolysis was not observed in the profile of *T. harzianum* [**Figure 1(4)**]. As we mentioned above, *M. racemosus* had the ability to grow in D5 medium and their growth curve in this medium was slightly modified in respect to D3 medium. The growth curve of the fungus in D5 medium [**Figure 1(5)**] revealed the early growth phase during the first 3 days, followed by the first exponential growth (from 3rd to 6th day), autolysis (from 6th to 9th day), and second exponential growth phase (until the 16th day) [53]. In order for better comprehension of detergent impact on the fungi growth, biomass dry weight was measured after 16 days of cultivation and was compared with the control. The results were presented in **Figure 1(6)**. The amount of biomass produced by fungi in C medium ranged in the following direction: *P. chrysogenum* > *A. niger* > *P. cyclopium* > *T. harzianum* > *M. racemosus*. This observation showed that chemical composition of Czapek-Dox liquid medium was optimal for the growth of tested fungi, except for *M. racemosus*. This finding is consistent with results of other research studies [21–23]. As the figure shows, the tested detergent at a concentration of 0.3% influenced the inhibition of biomass in the following direction: *A. niger* is 51.42%, *P. chrysogenum* is 50.0%, *P. cyclopium* is 33.38%, and *T. harzianum* is 20.0%. The inhibition of fungi growth by detergent could be explained by toxic effect of some detergent ingredients or degradation products and by autolysis. From these results, it is evident that *A. niger* and *P. chrysogenum* are the most sensitive species according to tested detergent. However, the detergent at both concentrations had stimulatory effect on the biomass production of *M. racemosus*; even the higher concentration had stronger stimulatory effect. The obtained results indicate the possible application of fungi, first of all *M. racemosus*, in bioremediation process.

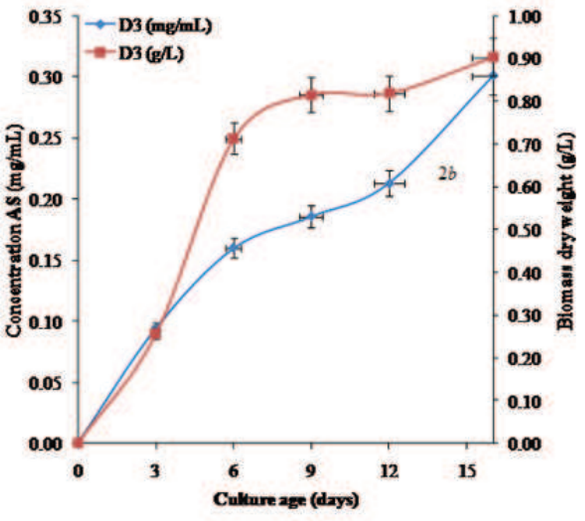
3.3. Biodegradation rate of anionic surfactants incorporated in detergent and its relationship according to fungal biomass dry weight

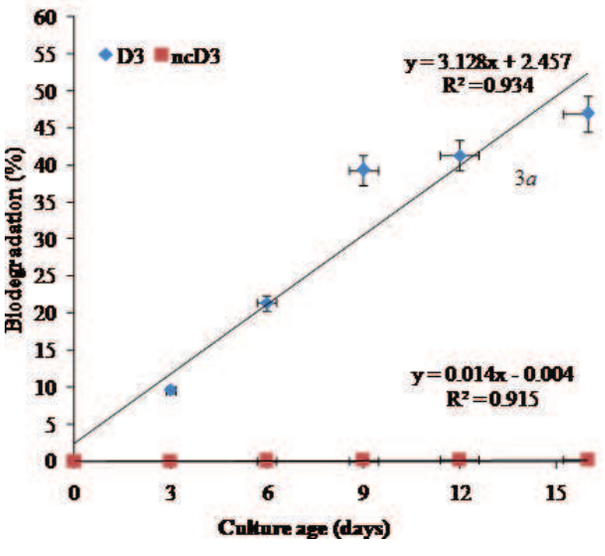
The next step in this study was confirmation and comparison ability of the fungi to degrade anionic surfactants (ASs) of detergent in terms of their potentially application in bioremediation processes. First, it is defined percentual share of ASs in the tested detergent (about 20%) by MBAS assay. By conversion of percentage, it was obtained 600 and 1000 μg/mL of ASs in D3 and D5 media. The concentration of ASs during 16-day cultivation of fungi in liquid medium was monitored and compared with negative controls (abiotic). They were used in order to monitor the stability of detergent during the cultivation time and a process of its adsorption on the walls of glass (flasks). The obtained results were presented in **Figure 2**.

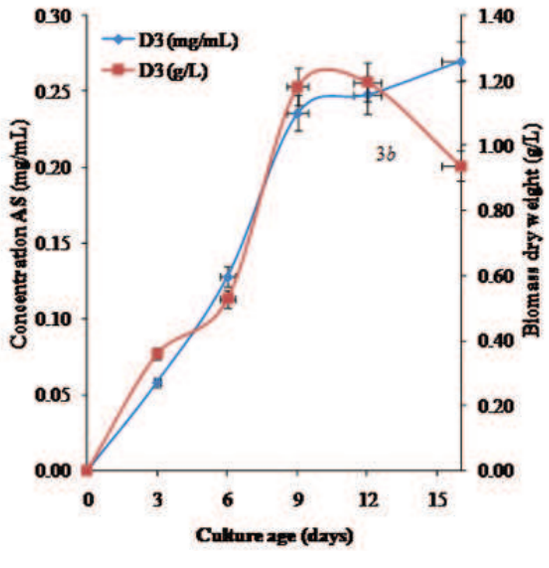

D3
ncD3
y = 1.888x + 1.452
R² = 0.974
y = 0.014x - 0.004
R² = 0.915
1a
Biodegradation (%)
Culture age (days)


D3 (mg/mL)
D3 (g/L)
1b
Concentration AS (mg/mL)
Biomass dry weight (g/L)
Culture age (days)


D3
ncD3
y = 2.893x + 4.528
R² = 0.961
y = 0.014x - 0.004
R² = 0.915
2a
Biodegradation (%)
Culture age (days)


D3 (mg/mL)
D3 (g/L)
2b
Concentration AS (mg/mL)
Biomass dry weight (g/L)
Culture age (days)


D3
ncD3
y = 3.128x + 2.457
R² = 0.934
y = 0.014x - 0.004
R² = 0.915
3a
Biodegradation (%)
Culture age (days)


D3 (mg/mL)
D3 (g/L)
3b
Concentration AS (mg/mL)
Biomass dry weight (g/L)
Culture age (days)

http://dx.doi.org/10.5772/intechopen.68184

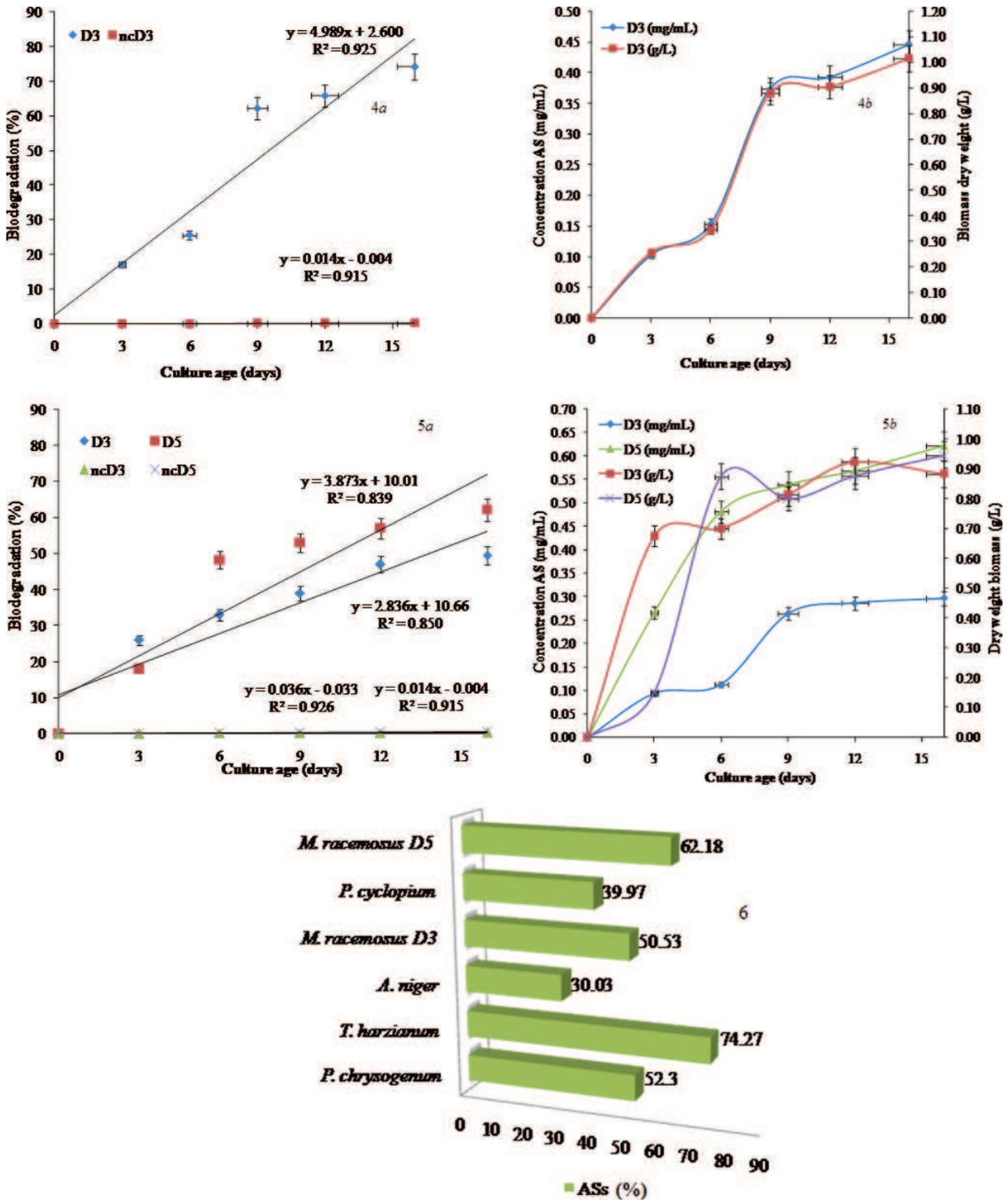


Figure 2. (a) Biodegradation rate of ASs, (b) relationship between concentration of ASs and fungal biomass dry weight: *A. niger* (1), *P. chrysogenum* (2), *P. cyclopium* (3), *T. harzianum* (4), *M. racemosus* (5), and comparison of biodegradation ability among fungi (6).

Over a period of 16 days, the tested fungi were degraded different amount of ASs, depending on fungi species and their locality (wastewater sample) [49–53]. The initial concentration

of ASs in D3 medium decreased continuously with the growth and development of mycelia. The highest biodegradation rate was observed during the first exponential growth phase of *A. niger* [**Figure 2(1a)**], *P. chrysogenum* [**Figure 2(2a)**], *T. harzianum* [**Figure 2(4a)**], and *M. racemosus* [**Figure 2(5a)**] or second exponential growth phase of *P. cyclopium* [**Figure 2(3a)**]. The current results are in line with the results of other authors who found that biodegradation rate of surfactants changes concomitant with cellular growth [54, 55]. **Figure 2(6)** shows a comparison of biodegradation capabilities of the fungi. Over a period of 16 days, *A. niger, P. chrysogenum, P. cyclopium, and T. harzianum* decomposed a total of 30.0, 50.2, 46.97, and 74.27% of ASs, which is equivalent to 180.20 μg/mL [**Figure 2(1b)**], 301.0 μg/mL [**Figure 2(2b)**] and 281.80 μg/mL [**Figure 2(3b)**], and 445.0 μg/mL [**Figure 2(4b)**] of ASs. At the same time, *M. racemosus* removed about 49.50 and 62.2% or 300.0 and 620.0 μg/mL of ASs [**Figure 2(5b)**] from D3 and D5 media, respectively.

From the equation of regression curves for each fungus (**Figure 2**) it was calculated the time needs for biodegradation at 80% of ASs (detergent). By this statistical test, the time predicted for the removing 80% of ASs, using *A. niger* [**Figure 2(1a)**], *P. chrysogenum* [**Figure 2(2a)**], *P. cyclopium* [**Figure 2(2a)**], *T. harzianum* [**Figure 2(4a)**], in applied experimental conditions, was calculated: 41.6, 26.1, 24.8, and 16.8 days, respectively. The *M. racemosus* [**Figure 2(5a)**] will be able to remove 80% of ASs for 24.3–18.3 days, depending on applied concentration. These statistical data indicate that ASs of detergent satisfying required the limit for 80% biodegradability in applied experimental conditions when *T. harzianum* and *M. racemosus* were used. Bearing this in mind their resentence to high detergent's concentrations, they could be used as test organisms in laboratory biodegradation studies. The main reason of their strong resistance to tested detergent could be their origin (industrial wastewater) and adaptation to a high concentration of detergents. However, despite the fact that they isolated from same wastewater sample, they had different response to different concentrations of detergent. Obviously, morpho-physiological characteristics of fungi have significant influence on its biodegradation capacity.

As the literature review does not provide quantitative information about the capacity of fungi to degrade ASs, the current results can only be compared with results obtained on bacterial species. The biodegradation capacity of *T. harzianum* and *M. racemosus* is very similar to the capacity of *Pseudomonas* spp., which can reduce the level of ASs up to 70% after 20 days [56]. The results of degradation of surfactants using a few bacteria strains that are available in the literature are far better compared to the current results. For example, according to Schleheck et al. [57], *Citrobacter* spp. can decompose about 90% of ASs after 35 hours of growth. Hosseini et al. [58] revealed that *Acinetobacter johnson* can use about 94% of the initial concentration of the SDS in the medium during 5-day growth. Ojo and Oso [59] investigated the capacity of two types of bacteria MH1 and MH2 to degrade LAS on alkaline pH and ambient temperature and found high biodegradation rate (93.6 and 84.6%, respectively) of LAS after 5 days. However, in above-mentioned studies with bacteria, the pure ASs were tested and their concentrations were far less compared to the current study. In our study, a commercial-type anionic detergent was used, whose composition is very complex and contains a variety of toxic substances in addition to ASs. Because of, the current results are very important from the aspect of purification of wastewaters which contain commercial detergents.

3.4. Changes of physicochemical parameters (pH and redox potential)

The normal functioning of basic cell processes and biodegradation reactions are closely related with acid-base and oxidation-reduction reactions. The acidic pH value (range 4.5–5.0) is necessary for the optimal growth of most fungi. On the other hand, the pH values between 6.5 and 8.5 are optimal for biodegradation processes in most aquatic and terrestrial systems, and pH values between 5.0 and 9.0 are considered acceptable. Bearing in mind their significant impact on mentioned processes, this study considered the changes of the pH values of media in all phases of fungal growth. The results are presented in **Table 2**.

The initial pH values recorded in C, D3, and D5 media were 4.80, 9.35, and 9.85 units, respectively. During cultivation of fungi, the pH values of C, D3, and D5 media were changing in relation to their composition, type of fungi, and the growth phases. These changes are influenced by the uptake of anions or cations from the medium by the fungal cells [60, 61] and excretion of organic acids in medium [62]. Over a period of 16 days, the pH values of the C media of *A. niger, P. chrysogenum*, and *T. harzianum* decreased toward strong acidic range. Contrarily, it was noted that the pH values of *M. racemosus* and *P. cyclopium* increased toward slightly acidic (neutral) range. The most significant changes in the pH value of C media were recorded during the exponential growth phase of fungi. From 3rd to 9th day, the pH values of *A. niger, P. chrysogenum*, and *T. harzianum* were dropped from 4.80 to 2.53, 3.42 and 4.56 units, respectively. The pH values of *P. cyclopium* and *M. racemosus* were increased from 4.80 to 6.92 and 6.13 units, respectively. These significant changes recorded in the pH values can be interpreted by intensive metabolism of fungi. Thereafter, during the stationary phase and autolysis, the changes in the pH value of the C media were less pronounced [40–53].

The pH values of the D3 media decreased throughout the cultivation time, except in phase of autolysis. The most significant changes in the pH values were observed during the first exponential growth phase of *P. chrysogenum* (from 9.12 to 6.07 units) followed by *M. racemosus* (from 9.40 to 6.24 units), *A. niger* (from 9.13 to 6.49 units), *T. harzianum* (from 9.05 to 6.07 units) or second exponential growth phase of *P. cyclopium* (from 8.75 to 6.33 units). Similar to the C medium, the changes of pH values were less pronounced during the stationary phase and autolysis [49–53]. The obtained results are consistent with results of other authors who confirmed decreasing of pH media during extensive mycelium development of *Glomus intraradices* [63], *Fusarium oxysporum* [64], etc. The obtained results suggest that fungi have different mechanisms for regulation of environmental pH, which depend on the initial pH. It could be speculated that the presence of detergent in the medium, which can be considered as strong alkali agent, probably induces the expression of different sets of genes, compared to C medium, as reflected in the regulation of external pH. Based on literature data, fungi can response to alkaline pH by two possible mechanisms: proteolytic activation of PacC transcription factors (*A. niger, C. albicans, S. cerevisiae*, etc.) or calcium-mediated pathway.

The *Eh* clearly influence the development of microorganisms. Each microorganism type is adapted to specific *Eh* conditions and is characterized by its ability to develop within a wide of narrow *Eh* range. The concentration levels of oxidants or reductants have an impact on the enzymatic activity via effects on three-dimensional conformation. Many investigations demonstrate the influence of the *Eh* value on the activity of some enzymes such as ADP-glucose pyrophos-

pH (Units)					Redox potential (mV)		
Fungi	**Day**	**C**	**D3**	**D5**	**C**	**D3**	**D5**
A. niger	3	3.92 ± 0.10	9.13 ± 0.26	-	390 ± 4	91 ± 2	-
	6	3.12 ± 0.15	6.49 ± 0.20	-	434 ± 2	238 ± 0.4	-
	9	2.53 ± 0.10	7.06 ± 0.10	-	457 ± 2	207 ± 5	-
	12	2.73 ± 0.18	5.63 ± 0.15	-	441 ± 5	309 ± 3	-
	16	2.52 ± 0.12	5.59 ± 0.28	-	435 ± 5	392 ± 5	-
P. chrysogenum	3	5.26 ± 0.10	9.12 ± 0.25	-	312 ± 0.1	91 ± 0.4	-
	6	3.77 ± 0.15	6.07 ± 0.20	-	403 ± 5	261 ± 2.5	-
	9	3.42 ± 0.23	6.36 ± 0.29	-	397 ± 3	246 ± 2	-
	12	3.44 ± 0.19	6.05 ± 0.25	-	401 ± 10	271 ± 5	-
	16	3.61 ± 0.15	7.02 ± 0.16	-	388 ± 8	210 ± 1	-
P. cyclopium	3	5.78 ± 0.14	7.89 ± 0.23	-	281 ± 0.1	101 ± 0.5	-
	6	6.68 ± 0.18	7.89 ± 0.25	-	229 ± 0.5	111 ± 0.5	-
	9	6.92 ± 0.10	7.29 ± 0.19	-	213 ± 2	247 ± 1	-
	12	5.63 ± 0.15	7.37 ± 0.15	-	283 ± 2.5	279 ± 1	-
	16	6.21 ± 0.20	6.48 ± 0.25	-	272 ± 2	243 ± 2	-
T. harzianum	3	5.40 ± 0.15	8.95 ± 0.26	-	299 ± 1	90 ± 2	-
	6	4.82 ± 0.20	8.75 ± 0.24	-	336 ± 0.5	97 ± 0.5	-
	9	4.56 ± 0.10	6.33 ± 0.15	-	341±2.5	261 ± 0.1	-
	12	4.67 ± 0.18	5.90 ± 0.15	-	335 ± 0.5	290 ± 2	-
	16	4.44 ± 0.12	6.48 ± 0.28	-	344 ± 2	301 ± 2	-
M. racemosus	3	5.24 ± 0.10	6.24 ± 0.25	9.36 ± 0.14	313 ± 1.6	250 ± 2	79 ± 2
	6	6.13 ± 0.15	6.37 ± 0.20	6.46 ± 0.10	261 ± 1.8	245 ± 2.5	240 ± 1.4
	9	6.01 ± 0.23	6.14 ± 0.29	6.89 ± 0.05	262 ± 2.5	257 ± 1	217 ± 2
	12	5.87 ± 0.19	6.36 ± 0.25	6.31 ± 0.12	270 ± 0.2	294 ± 2	257 ± 2.2
	16	5.80 ± 0.15	5.50 ± 0.16	5.62 ± 0.08	272 ± 2	297 ± 2	290 ± 2.5

Table 2. Changes in the pH and redox potential values of media.

phorylase [65], the activity and binding of α-glucan, water dikinase (SEX1) to starch granules [66], the activity of β-amylase [67]. The *Eh* values within a range from +100 to +350 mV indicate well-aerated conditions that are optimal for biodegradation processes. However, the addition of detergent in medium caused a significant shift in *Eh*, as **Table 2** shows. The initial *Eh* values of C, D3, and D5 media (before inoculation) were 340, 80, and 60 mV, respectively. From inoculation until the 16th day, the changes in *Eh* values of nutrient media of fungi were expressed with different intensity respect to the type of media, fungi species, and growth phase. During the growth of *A. niger, P. cyclopium, T. harzianum*, and *M. racamosus*, the *Eh* value decreased in C medium.

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In contrast, the *Eh* value of *P. chysogenum* increased during most time of cultivation, with exception in first 3 days. In D3 medium, the *Eh* value increased during the growth of all fungi species, without exception. Statistically significant changes were observed during the exponential growth phase whereas during stationary and autolysis phase these changes were insignificant [51, 52].

3.5. Fungal alkaline protease (EC 3.4.21-24) activity

Alkaline proteases have been maximally exploited in food, leather, silk, detergent industries, and waste management. The use of alkaline protease as active ingredient in laundry detergent is the single largest application of this enzyme [68]. From this aspect, isolation and characterization of new promising microbial strains is a continuous process [69]. For the practical application of alkaline proteases in detergents industry, the following conditions are important: their compatibility with the detergent, efficiency at lower temperatures, and stability.

The current study investigated the effect of tested commercial detergent on alkaline protease activity of selected fungi species, and results are presented in **Table 3**. In C medium, the maximum enzyme activity was produced by *P. cyclopium* (0.73 IU/mL) followed by *P. chrysogenum* (0.31 IU/mL), *T. harzianum* (0.27 IU/mL), *A. niger* (0.18 IU/mL), and at least *M. racemosus* (0.15 IU/mL). The addition of detergent in the C medium influenced the changes of enzyme activity depending on the fungi species. The detergent at a concentration of 0.3% showed slight inhibitory effect on alkaline protease activity of *P. cyclopium* (for 12.3%) and strong inhibitory effect of enzyme activity of *P. chrysogenum* (for 89.87%). The detergent at concentrations of 0.3 and 0.5% inhibited enzyme activity of *M. racemosus* for 81.7 and 21.57%, respectively. On the other hand, the activity of alkaline protease of *A. niger* and *T. harzianum* was enhanced in presence of tested detergent for 372 and 128.0%, respectively [49, 51].

Overview of the literature provides contradictory results about the impact of pure surfactants or commercial detergents on activity and stability of alkaline proteases of microbial origin. Choudhary and Jain [70] have reported the detergent compatibility of the alkaline protease of *Aspergillus* sp., but enzyme was not able to retain maximum activity more than 1 hour of incubation. Rani et al. [71] found that alkaline protease from *Aspergillus flavus* AS2 retained 56–92% activity in presence of commercial detergents in following range: Rin (India) < Surf Excel (India) < Tween 80 < Ariel (India) <Tween 20. According to Sankeerthana et al. [72], alkaline proteases from *A. niger* and *A. flavus* retained about 75–70% activity in the most of tested commercial detergents with maximum activity (65–85%) obtained in Surf Excel (India). Niyonzima and More [73] observed that protease of *Aspergillus terreus* gr. was 100% stable and compatible for 2 hours at 60°C with all the detergents except for Super wheel (India), and retained of 84–89% activity after 24 hours at 60°C in following order: Super wheel < More choice < Ariel < Henko < Surf excel (all from India). The alkaline protease was also active and retained 79.1–83.2% and 55.8–75.1% of activity in the presence of tested detergents at 28 and 90°C, respectively, after 24 hours. The different proteolytic activities of fungi in the presence of detergents could be the consequence of experimental conditions (medium composition, aeration, temperature, etc.) as well as morphological characteristics and genetic basis of fungi. Therefore, examination of the above mentioned effects on each individual type of fungus is very important and justified. Finally, this study clearly indicates that alkaline protease of *A. niger, T. harzianum*, and *P. cyclopium* could be used as an additive in formulation of tested detergent.

Alkaline protease activity					Alkaline phosphatase activity		
Fungi	**Day**	**C**	**D3**	**D5**	**C**	**D3**	**D5**
A. niger	3	0.18 ± 0.15	0.09 ± 0.26	-	12.60 ± 0.15	1.65 ± 0.26	-
	6	0.001 ± 0.20	0.67 ± 0.24	-	0.07 ± 0.20	17.74 ± 0.24	-
	9	0.002 ± 0.10	0.001 ± 0.15	-	21.57 ± 0.10	24.14 ± 0.15	-
	12	0	0.001 ± 0.15	-	0	24.31 ± 0.15	-
	16	0.13 ± 0.12	0 ± 0.28	-	2.57 ± 0.12	11.19 ± 0.28	-
P. chrysogenum	3	0.004 ± 0.10	0.005 ± 0.25	-	5.10 ± 0.10	9.79 ± 0.25	-
	6	0.03 ± 0.15	0.001 ± 0.20	-	0.21 ± 0.15	8.40 ± 0.20	-
	9	0.15 ± 0.23	0.03 ± 0.29	-	25.00 ± 0.23	1.17 ± 0.29	-
	12	0.31 ± 0.19	0.01 ± 0.25	-	3.45 ± 0.19	8.23 ± 0.25	-
	16	0.29 ± 0.15	0.01 ± 0.16	-	0.10 ± 0.15	18.29 ± 0.16	-
P. cyclopium	3	0.26 ± 0.14	0.03 ± 0.23	-	31.71 ± 0.14	18.36 ± 0.23	-
	6	0.01 ± 0.18	0.64 ± 0.25	-	96.58 ± 0.18	9.76 ± 0.25	-
	9	0.73 ± 0.10	0.03 ± 0.19	-	22.26 ± 0.10	19.14 ± 0.19	-
	12	0.22 ± 0.15	0.09 ± 0.15	-	12.57 ± 0.15	4.79 ± 0.15	-
	16	0.003 ± 0.23	0.01 ± 0.20	-	0.35 ± 0.23	15.21 ± 0.20	-
T. harzianum	3	0.23 ± 0.15	0.05 ± 0.26	-	0.55 ± 0.15	9.57 ± 0.26	-
	6	0.27 ± 0.20	0.59 ± 0.24	-	0.07 ± 0.20	0.02 ± 0.24	-
	9	0.01 ± 0.10	0.03 ± 0.15	-	0.55 ± 0.10	21.37 ± 0.15	-
	12	0 ± 0	0.63 ± 0.15	-	10.21 ± 0.18	4.87 ± 0.15	-
	16	0.03 ± 0.12	0.01 ± 0.28	-	4.21 ± 0.12	26.24 ± 0.28	-
M. racemosus	3	0.001 ± 0.10	0.03 ± 0.25	0.10 ± 0.01	2.43 ± 0.10	0.07 ± 0.25	2.41 ± 0.21
	6	0.15 ± 0.15	0 ± 0	0.03 ± 0.01	73.34 ± 0.15	4.86 ± 0.20	8.98 ± 1.45
	9	0.14 ± 0.23	0.001 ± 0.29	0.001 ± 0	26.64 ± 0.23	28.64 ± 0.29	24.48 ± 1.92
	12	0.15 ± 0.19	0.002 ± 0.25	0.07 ± 0.02	9.00 ± 0.19	28.56 ± 0.25	0.43 ± 0.02
	16	0.002 ± 0.15	0.002 ± 0.16	0.013 ± 0.01	6.54 ± 0.15	2.43 ± 0.16	16.26 ± 0.16

Table 3. Activity of alkaline protease and phosphatase of fungi.

3.6. Activity of alkaline phosphatase (EC 3.1.3.1) of fungi

Alkaline phosphatase (ALP) enzyme hydrolyzes the phosphomonoesters from number of organic molecules like ribonucleotides, deoxyribonucleotides, proteins, alkaloids, phosphate esters, and anhydrides of phosphoric acid [74] ALP enzymes are involved in various biological processes (cell cycle, differentiation, etc.) and industries; therefore have a wide range

http://dx.doi.org/10.5772/intechopen.68184

of applications [75]. Since the relevant literature provide the information about production of the enzyme only using bacterial strains, the current study investigated the potential of selected fungi to produce ALP. The obtained results are presented in **Table 3**.

In C medium, the maximum enzyme activity was produced by *P. cyclopium* (96.58 IU/mL) followed by *M. racemosus* (73.84 IU/mL), *P. chrysogenum* (25.0 IU/mL), *A. niger* (21.57 IU/mL), and *T. harzianum* (10.21 IU/mL). The addition of detergent in growth medium influenced the inhibition of enzyme activity of *P. cyclopium* (81.18%), *P. chrysogenum* (for 26.86%), and *M. racemosus* (for 61.33 to 66.85%; at applied concentration of 0.3 and 0.5%, respectively). The inhibition of ALP activity by detergent is understood, considering the specific action of the enzyme on *ß*-glycerophosphate and the composition of the growth media [52, 53]. According to Aseri et al. [76], ALP hydrolyses more easily monoesters originating from the carbohydrate metabolism than ester bonds in the alkyl chain of surfactants. The study of Koffiet al. [77] found that SDS has a strong inhibitory effect (about 98%) on phosphatase activity. The current results are in agreement with observations of mentioned authors with exception of *A. niger and T. harzianum*. As **Table 3** shows, the ALP activity of *A. niger and T. harzianum* was slightly (for 12.70%) or significantly (for 156.86%) enhanced by detergent. Finally, this study showed for the first time that fungi grown in Czapek-Dox liquid medium, in applied experimental conditions, can be considered as significant source of ALP. Moreover, the addition of a commercial detergent in liquid medium with *A. niger and T. harzianum* can be used as a strategy for improving the enzyme activity. The knowledge obtained in this study on ALP can have considerable effort in application of tested fungi in biotechnology and waste management, and provides a good base for further investigation in this manner.

4. Conclusions

The main conclusion of this study is that all fungi showed the ability to degrade a high concentration of tested detergent during experimental period of 16 days. The fungi *T. harzianum* and *M. racemosus* had the best biodegradation ability, which is expected since they were isolated from industrial wastewater of Henkel Factory (Kruševac, Serbia). Second conclusion, the alkaline protease and phosphatase activities of *A. niger* and *T. harzianum* were significantly enhanced by detergent. On the other hand, the alkaline protease of *P. cyclopium* and alkaline phosphatase of *P. chrysogenum* retained a high percentage of activities in the presence of detergent. The obtained results could have practical application of tested fungi in bioremediation processes and in biotechnology.

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