

UNIVERSITY OF BELGRADE
TECHNICAL FACULTY BOR



XXV International Conference
"ECOLOGICAL TRUTH"

Eco-Ist'17

PROCEEDINGS

Edited by
Radoje V. PANTOVIC
and
Zoran S. MARKOVIC

12 – 15 June 2017
Hotel "Breza", Vrnjačka Banja, SERBIA

FOR THE PUBLISHER:
DEAN: Prof. dr Nada STRBAC

EDITORS:
Prof. dr Radoje V. PANTOVIC and
Prof. dr Zoran S. MARKOVIC

TECHNICAL EDITOR
Zeljko PAJKIC, MSc.

PRINTED BY:
»HAPPY TREND« Zajecar
Printed: 150 copies

CIP – Каталогизacija y публикацији
Народна библиотека Србије, Београд

502/504(082)
613(082)

**INTERNATIONAL Conference "Ecological Truth"
(25 ; 2017 ; Vrnjačka Banja)**

Proceedings / XXV International Conference
"Ecological Truth", Eco-Ist '17, 12-15 June 2017,
Vrnjačka Banja, Serbia ;
[organizer] University of Belgrade, Technical Faculty, Bor ;
edited by Radoje V. Pantovic and Zoran S. Markovic. - Bor :
University of Belgrade, Technical Faculty, 2017
(Zaječar : Happy Trend). - XVII, 697 str. : ilustr. ; 26 cm

Tiraž 150. - Bibliografija uz svaki rad. - Registar.

ISBN 978-86-6305-062-4

1. Tehnički fakultet (Bor)

a) Животна средина - Заштита - Зборници

b) Здравље - Заштита – Зборници

COBISS.SR-ID 236276748



THE POTENTIAL OF *Aspergillus niger* IN
BIODEGRADATION/BIOREMEDIATION OF ETHOXYLATED
OLEYL-CETYL ALCOHOL

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ABSTRACT

This study was investigated the effect of 0.5% EOCA (Henkel, Serbia) on the growth and metabolic activity of *Aspergillus niger*. Changes in chemical and biochemical parameters: pH value, organic acids and biomass dry weight were observed during fungal growth for 19 days. The capacity of fungus to decrease concentration of pollutant in medium was determined at 19th day. The pollutant was influence on increase in pH value of medium but decrease in organic acids excretion and biomass production of *A. niger*. The biodegradation capacity of fungus was about 83%. The obtained results indicate on potential application of fungus in bioremediation.

Key words: Biomass, biodegradation, ethoxylated oleyl-cetyl alcohol, organic acids, pH.

INTRODUCTION

Ethoxylated oleyl-cetyl alcohol (EOCA) is non-ionic surfactant from the group of fatty alcohol ethoxylates (FAEs), which has application in various industries (detergents, household cleaners, shampoos, paper, oil, agriculture, pharmaceuticals) (1). The increase in industrial production and consumption of these active substances makes them one of the leading pollutants of the environment. After use, the residues of surfactant and their degradation products falling due to the wastewater treatment plants (WWTP) or directly on the surface of the water and sediments (2). Numerous studies have confirmed a high level of primary and total biodegradability of these surfactants in the environment. Alcohol ethoxylates (AEs) are broken down by biological treatment in WWTP, in a high percentage (95-99%) (3,4). The concentration of total AEs in effluents varied in interval 1-23 µg/L in Europe, Canada and the USA. Also, the study estimates the risk AEs for the environment is carried out continuously since the 70s until today (5,6). Toxicity for aquatic organisms, expressed as the EC₅₀ varies from very toxic (<1 mg/L) to very dangerous (10-100 mg/L). Simultaneously with aforementioned studies,

the studies concerning understanding of the mechanism for biodegradation of AEs in the presence of complex microbial communities have been carried out using various methods.

Bioremediation is an attractive technology that utilizes the metabolic potential of microorganisms in order to clean up the environmental pollutants to the less hazardous or non-hazardous forms with less input of chemicals, energy and time (7,8). During last two decades, many mycologists have tried the use of various fungal species in the degradation of organic compounds. Some of the best known fungi species used as mycoremediators are: *Pleurotus ostreatus*, *Rhizopus arrhizus*, *Phanerochaete chrysosporium*, etc., (9).

The key to mycoremediation is determining the right fungal species to target a specific pollutant. Thus, isolation and identification of fungi from environment that are resistant to the presence of high concentrations of pollutants on the one hand, and the effect of pollutants on their metabolism on the other hand, are crucial parameters for the application of fungi in mycoremediation. The occurrence of different fungal genera in sewage and industrial wastewater and sewage sludge is well documented. However, despite of that fungi have not yet been significantly exploited for mycoremediation of such polluted environments. More extensive research needs to be carried out on the use of fungi in mycoremediation.

The current study was designated with aim to investigate and promote the potential of *A. niger* in bioremediation of wastewater from AEs.

MATERIALS AND METHODS

Isolation and identification of the fungus from wastewater. Pure culture of *A. niger* van Tieghem (1867) was isolated from wastewater samples of Lepenica river basin (Kragujevac, Serbia) at a place where municipal wastewater discharge into the river. Identification of the fungus was based on the macroscopic and microscopic morphology by using Systematic key (10) and carried out at the Faculty of Biology, University of Belgrade, Serbia. The fungus was cultivated on potato dextrose agar (PDA, Sigma-Aldrich, Germani) plates at 28°C for 5-7 days until sporulation. After having sufficient population of spores, the PDA plates were stored at 4°C and sub-cultured monthly under sterile conditions.

Preparation of spore inoculum. Spore suspensions were prepared according to procedure described in our previous work (11). The concentration of spores in the suspension was evaluated by microscopic enumeration with a cell-counting hemocytometer, Neubauer chamber (Lonza Cologne AG, Germany) and adjusted to $5.0 \cdot 10^6$ spores/mL.

Culture conditions. Shake flask cultivation was carried out in 250 mL Erlenmeyer flasks containing 200 mL of Czapek-Dox liquid medium (g/L): NaNO₃ – 3.0, K₂HPO₄ – 1.0, MgSO₄·7H₂O – 0.25, FeSO₄·7H₂O – 0.01, sucrose – 30.0 (control – C). The same medium was supplemented with 0.5% EOCA (Henkel, Serbia) (medium EOCA). The pH value of C medium was about 6.8±0.2 (without adjustment) before

sterilization. Thereafter, 1 mL of spore suspension (5.0×10^6 spores) was inoculated in liquid media. Inoculated flasks were incubated at 150 rpm on a shaker (Kinetor-m, Slovenia) and ambient temperature ($28^\circ\text{C} \pm 3^\circ\text{C}$) for 19 days in triplicate. The mycelium was collected by filtration of fermentation broth using Whatman No.1 (Germany) filter paper at 19th day.

Determination of dry weight biomass (DW). The fungal biomass DW produced in C and EOCA media was determined gravimetrically according to standard procedure (11). The obtained results were expressed in g/L of submerged culture.

Determination of concentration of non-ionic surfactant (NS) in medium. To investigate the biodegradation capacity of the tested fungi, NS concentration in fermentation broth of fungi was measured by cobalt thiocyanate active substances (CTAS) method. Fermentation broth of fungi was extracted sequentially with ethyl acetate, according to procedure described by Traczyk *et al.*, (12). The reaction of NS with cobalt thiocyanate reagent forming Co-complex in which cobalt concentration is determined at OD₆₂₀. Triton X-100 (Sigma-Aldrich, USA) was used as a standard for preparation of calibration curve. The percentage of the NS degradation was calculated using Eq. (1):

$$\% \text{ Degradation} = 100 - [(OD_{620 \text{ exp}} - OD_{620 \text{ blank}}) / OD_{620 \text{ std}}] \times 100 \quad (1)$$

where OD_{620 exp} is optical density of test sample, OD_{620 blank} is optical density of blank sample and OD_{620 std} is optical density of standard sample at 620 nm.

Measurement of pH values. A pH value of the fermentation broth was measured by pH meter (type MA-5705, Iskra, Slovenia) at 4th, 7th, 10th, 14th and 19th day of fungal growth.

Determination of free (FOA) and total (TOA) organic acids. The quantities of FOA and TOA were determined by ion exchange chromatography method according to Bullen *et al.*, (13) as described in detail in our previous paper (11). To determinate concentrations of FOA and TOA, 10 or 25 mL aliquots of ethanolic extracts of fermentation broth were sampled and titration was carried out with 0.1 M NaOH. Phenolphthalein (0.1%) was used as indicator. The obtained results were presented in %.

Statistical analysis. Statistical analysis was performed using SPSS statistical software package (SPSS for Windows, ver. 13.0, Chicago, IL, USA). For tested the normality of distribution, means and standard deviation, Student *t*-test was used. Pearson's and Spearman's correlation coefficients were 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.

RESULTS AND DISCUSSION

Effect of EOCA on fungal biomass DW. The biomass DW of *A. niger* measured in C and EOCA media at 19th day of cultivation was presented in Fig. 1(a). The slightly higher biomass was produced by *A. niger* in C (2.17 g/L) than in EOCA medium (1.98 g/L). On the other words, the addition of EOCA in growth medium caused slight inhibitory effect (8.5%) on the fungal growth compared to control. Overview of literature provides evidence that NS can both stimulate and inhibit the fungal growth, depending on fungal species and concentration of pollutant. The study of Zeng *et al.*, (14) found that Tween 80 has a mild stimulating effect on biomass of *Penicillium simplicissimum*. Stojanović *et al.*, (15,16) revealed a mild stimulatory and very strong inhibitory effect of 1% EOCA on biomasses of *Trichothecium roseum* and *A. niger*, respectively. Our previous work (17) showed a mild stimulatory effect of aforementioned types of surfactant at a concentration of 0.1% on the production of *Fusarium oxysporum*. The results aforementioned confirmed that the effect of surfactants on the growth and metabolism of fungi is very complex. This phenomenon could be explained by the interaction of the surfactant with functional groups in fungal membranes, influence on rheological properties and pH value of medium, oxygen uptake, and on active centers of the key enzymes of fungal metabolism.

Biodegradation assay of EOCA. The result of the biodegradation ability of *A. niger* grown in liquid medium with EOCA are presented in Fig. 1(b). CTAS assay used for determination of EOCA concentration in inoculated medium confirmed the significant biodegradation potential of the fungus. After 19 days of cultivation, the concentration of EOCA in medium decreased from initial value of 5.0 to 0.85 mg/mL. Expressed in percentages, about 83% of EOCA was removed from medium as result of metabolic activity of *A. niger*. Only a few data about biodegradation of NS by single microorganisms, especially by fungi can be found in the literature. The study of Mohamed *et al.*, (2014) confirmed the degradation of naphthalene by *A. niger* isolated from soil of petroleum refinery. According to this study, under the optimal conditions (pH 7.0, concentration of naphthalene 100 ppm and temperature 30°C), *A. niger* degraded 87.90% of naphthalene after 8 days. Recently, Jakovljević and Vrvic (19) revealed that *A. niger* decomposed about 30% of AS (components of commercial detergent Merix, Henkel, Serbia) after 16 days of incubation. The current result confirmed that *A. niger* is about 2.7-fold more efficient in biodegradation of NS than AS. These result is in accordance with conclusion of Jurado *et al.*, (20) that biodegradation of NS by *Pseudomonas putida* is much more efficient compared to AS. Jun *et al.*, (21) found that *Trichoderma viride* can ability to degrade Tween 80 up to more than 8 g/L at pH 7.0 and 20°C. The obtained results clearly show that *A. niger* could be applied in the purification of wastewater which contains detergents.

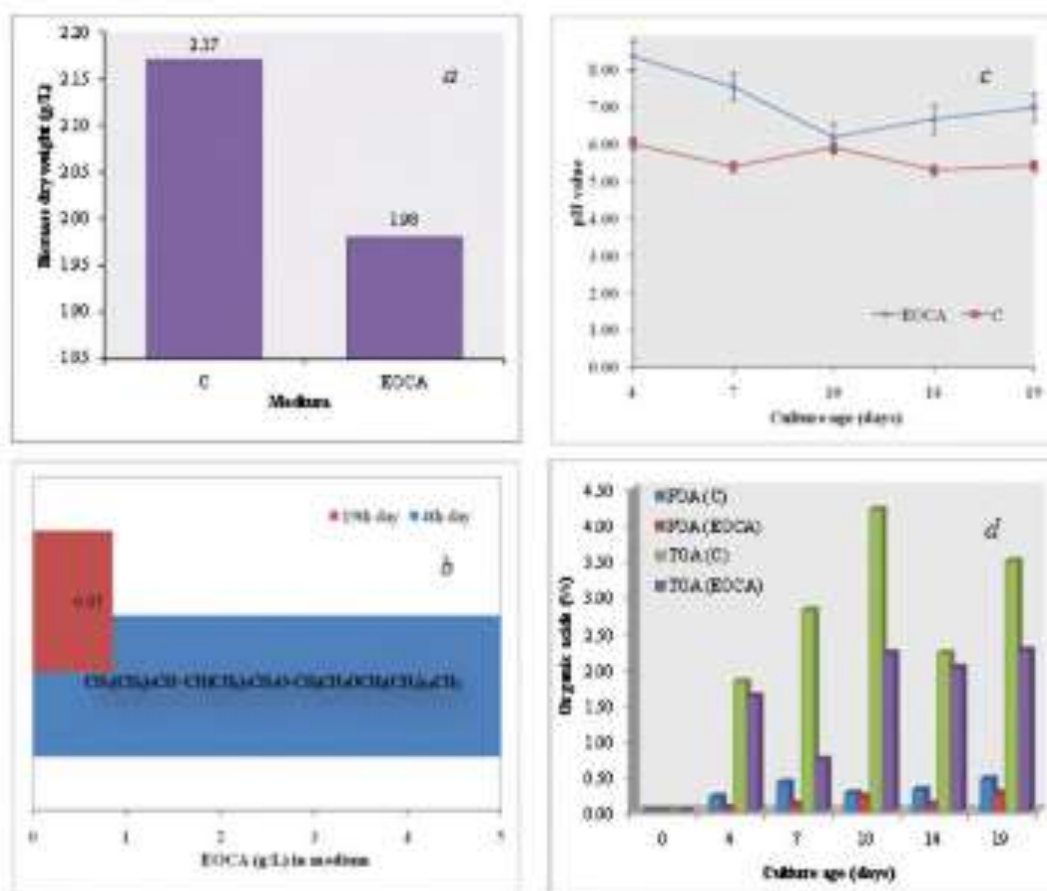


Figure 1. Display of the biochemical parameters of *Aspergillus niger* in control (C) and medium with ethoxylated oleyl-cetyl alcohol (EOCA): production of biomass dry weight (a); biodegradation ability (b); pH value (c); quantities of organic acids (free (FOA) and total (TOA) (d).

Effect of EOCA on pH of medium. The changes in pH values of C and EOCA media of *A. niger* are presented in Fig. 1(c). Altering pH medium is result of utilization nutrients from growth medium and excretion of some acid and alkaline metabolites by fungi (22). Thus, the intensity of pH changes is in correlation with intensity of metabolic activities of the fungus. The pH values of C medium of *A. niger* decreased continually from inoculation until 10th day and moved toward acidic condition. The most significant changes in pH were noted in first 4 days after inoculation, which means that the most significant metabolic changes are associated with spores germination and mycelial growth. Afterwards, the only increase in pH values was noted at 14th day, probably as a result of autolysis and excretion of alkaline metabolites. Addition of EOCA in medium caused a significant increase in initial pH value (8.50 units) compared to C, and, thus, this compound can be considered as factor of alkaline stress. As respond to alkaline stress the fungus excreted organic acids, due to the pH value of medium was

considerable decreasing until 10th day. From this point, the pH value of medium was increasing until 19th day. Statistical analysis revealed a negative correlation between changes in pH values and quantities of FOA ($r = -0.830, p < 0.01$) and TOA ($r = -0.707, p < 0.05$). The decrease of media pH values by fungi during treatment of sludge was observed and well discussed by several authors (27, 28).

Effect of pollutant on quantity of organic acids (FOA and TOA). The fungus excreted variable amounts of organic acids in the liquid media, depending on medium composition and a period of cultivation (Fig. 1(d)). The quantity of FOA produced in C and EOCA media by *A. niger* ranged from 0.20% to 0.45% and 0.05% to 0.25% respectively. The quantity of TOA measured in C and EOCA media was 1.80% to 4.20% and 0.70% to 2.20% respectively. Based on presented results, the EOCA caused an inhibitory effect on quantity of FOA (44.4%) and TOA (47.6%) compared to control. Statistical data revealed strong correlation between quantities of FOA and TOA ($r = 0.873, p < 0.01$) in EOCA medium. The quantities of FOA and TOA excreted in medium were correlated with duration of experiment ($r = 0.705, p < 0.05$ and $r = 0.664, p < 0.05$, respectively). These results are in agreement with literature data about capability of *Aspergillus* genus to produce high amounts of useful organic acids (citric, gluconic, malic, itaconic acids) (23). The growth of fungus expressed as biomass DW was correlated with quantity of TOA ($r = 0.973, p < 0.05$). The correlation between biomass and quantity of TOA was also found in our previous biodegradation study with *Penicillium chrysogenum* (11). Briefly, the quantity of excreted TOA depends on the fungal growth and intensity of metabolic activity.

CONCLUSIONS

During fungal growth in Czapek-Dox liquid media supplemented with EOCA at a concentration of 0.5%, the total 83% of pollutant was removed by *A. niger* for 19 days. The obtained results indicate the potential application of tested fungus in wastewater treatment, detergent industry and biotechnology.

Acknowledgment

This research was financially supported by Serbian Ministry of Education, Science and Technology Development (Grant number III 43004).

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ISBN 978-86-6305-062-4

2017