UNIVERSITY OF NIŠ
Faculty of Technology, Leskovac

PROCEEDINGS

13th SYMPOSIUM „NOVEL TECHNOLOGIES AND ECONOMIC DEVELOPMENT“

Leskovac, October, 18-19, 2019.

Faculty of Technology, Leskovac

Publisher and Printing: Faculty of Technology, Leskovac

For the Publisher: Prof. Ljubiša Nikolić

Editor: Prof. Miodrag Lazić

CIP- Каталогизација у публикацији
Народна библиотека Србије

6(082)(0.034.2)

SYMPOSIUM with international participation "Novel Technologies and Economic Development" (13 ; 2019 ; Leskovac)
Proceedings [Elektronski izvor] / 13th Symposium [with international participation] "Novel Technologies and Economic Development" Leskovac, October, 18-19, 2019. ; [organized by] Faculty of Technology, Leskovac [and] Serbian Academy of Sciences and Arts (SANU), Branch in Niš] ; [editor Miodrag Lazić]. - Leskovac : Faculty of Technology, 2019 (Leskovac : Faculty of Technology). - 1 elektronski optički disk (CD-ROM) ; 12 cm

Sistemski zahtevi: Nisu navedeni. - Nasl. sa naslovne strane dokumenta. - Tiraž 100. - Bibliografija uz svaki rad. - Registar.

ISBN 978-86-89429-36-7

а) Технологија -- Зборници б) Технолошки прогрес -- Привредни развој -- Зборници

COBISS.SR-ID 280089356

Printing: 100

Proofreader: Biljana Mitić Stanković
Paging and graphical design: Vesna Marinković

ISBN 978-86-89429-36-7

13th Symposium with international participation "Novel Technologies and Economic Development" was also supported by Ministry of Education, Science and Technological Development Republic of Serbia.

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CONTENS

Section: ENVIRONMENTAL ENGINEERING

Original scientific paper
UDC 579.84:504.5

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *Klebsiella* sp. ISOLATED FROM ENVIRONMENT POLLUTED WITH PERFLUOROALKYL SUBSTANCES

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The development of chemical industry in the last century has contributed to an increase in food production, more effective disease control and improved living standards. Nevertheless, it left huge quantities of toxic substances in the environment. Persistent organic pollutants are chemicals that persist, accumulate throughout the food chain and have harmful effects on human health and the environment. Perfluoroalkyl substances such as perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride, are on the list of persistent organic pollutants. Several recent publications have shown that microorganisms isolated from environments polluted by perfluoroalkyl substances can reduce the level of these compounds. In this paper, bacterial strain isolated from such an environment, which demonstrated capability to reduce perfluoroalkyl substances in a preliminary study, was physiologically, biochemically and molecularly characterized. The microorganism is Gram-negative, nonmotile, oxidase negative and catalase positive. The microorganism can produce a variety of hydrolase enzymes. Fatty acid methyl ester profile was also determined. Molecular characterization confirmed that the bacterium belongs to *Klebsiella* sp. Microorganism was successfully characterized using different methods and in the future this strain will be used in a laboratory study to analyze the mechanisms of reduction of perfluoroalkyl substance concentrations.

Keywords: *Klebsiella sp.*, PFOS, POPs

INTRODUCTION

Development of industry has contributed a lot to the quality of human life. However, uncontrolled discharge during production, use and disposal of various chemicals led to

polluted environment. In trace concentrations (ng / L), organic micropollutants can affect aquatic ecosystems and human health [1].

Perfluoroalkyl substances (PFASs) are synthetic organic chemicals and they consist of hydrophobic alkyl chain in which hydrogen atoms are replaced by fluorine. Usually, hydrophobic chain is attached to sulfonate or carboxyl hydrophilic groups. Due to their properties, water and oil repellence, thermal and chemical resistance, these compounds have been used for a large variety of applications in various industrial and commercial products. They have been used for production of personal care products and surfactant agents, water-resistant textiles, fire-fighting foams, grease-proof coating for food packaging, as well as paint formulations [2-6]. On the other hand, in recent years PFASs have come to public and scientific attention and they have been recognized as environmental contaminants [3]. They are persistent in the environment, resistant to thermal, photolytic, biological degradation, and also bioaccumulative [2, 4, 6]. PFASs are ubiquitous in diverse environmental matrices, in aquatic environments (groundwater, surface water, tap water), soil and sediments.

Two long-chain fully fluorinated compounds, perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), are the dominant PFASs in the environment. PFOS and PFOA can affect the immune system, act like endocrine disruptors, cause hepatotoxicity and carcinogenesis in animals and humans. Since 2009, PFOS and its salts have been added to the list of persistent organic pollutants (POPs) under the Stockholm Convention by the United Nations Environmental Programme (UNEP) [2-5, 7].

Polluted environment is a source of microorganisms which can reduce the amount of PFASs [8]; some strains are highly efficient in production of extracellular polymeric substances [9] which can act as bioemulsifiers [10]; or produce electricity [11, 12]. Research on the possible biochemical pathways and use of microorganisms in bioremediation processes is in progress. It is assumed that microorganisms can biotransform or biodegrade PFASs [8, 13, 14]. Also, they can be adsorbed on the surface or absorbed inside the microbial cell [8]. The aim of this paper was to conduct physiological, biochemical and molecular characterization of microorganism isolated from Ajifu Waterway, Osaka, Japan, which can reduce concentration of PFASs based on preliminary laboratory tests.

EXPERIMENTAL PART

Selection of PFASs degrading microorganism AW-03

Sampling and basic characteristics of the sediment from Ajifu Waterway, Osaka, Japan were previously described [7]. This place is known for long-term PFOA pollution and microbial consortia, when incubated with PFOS and PFOA, were capable of decreasing concentrations of these compounds [8]. During analysis of bacterial strains selected from a consortium of microorganisms isolated from Ajifu Waterway, some of the strains grown as a pure culture were also found to reduce concentration of PFASs (data not yet published). One of these isolated and selected bacterial strains (AW-03) was further physiologically, biochemically and molecularly characterized.

The growth of bacterial populations
The generation time of isolated AW-03 strain was determined. Nutrient broth (100 mL) was inoculated with culture of isolated strain in physiological solution (0.1 mL). After one night at rotary shaker (150 rpm / min), 28°C, culture of AW-03 was transferred from overnight nutrient broth to a fresh broth. Fresh broth was shaken on a rotary shaker (same conditions) and absorbance was measured during 300 min at 540 nm (0-70 min every 10 min; 90-300 min every 30 min) [15]. The nutient broth consisted of peptone 1, 15.0 g; meat extract, 3.0 g; sodium chloride, 5.0 g; potassium hydrogen phosphate, 0.3 g; distilled water, 1 L.

Physiological and biochemical characterization of AW-03
Gram-staining, catalase and oxidase tests
Standard methods were used for Gram-staining, catalase and oxidase tests of the isolated strain [16, 17].

Analytical Profile Index (API)
API (BioMérieux, France) kits were used: API ZYM for semiquantitation of enzymatic activities; API 20 NE – 24 – 48 h identification of Gram-negative non-*Enterobacteriaceae* (qualitative); API CORYNE – 24 h identification of *Corynebacteria* and corynebacteria-like organisms (also qualitative) [18].

Fatty acid methyl ester profile (FAME)
The FAME content was determined as described in Djurić et al. [10]. Wet biomass was refluxed with toluene : methanol : sulfuric acid mixture (5 : 5 : 0.2) for 3 hours. Solution was extracted with equal volumes of chloroform and hexane. Extract was evaporated to dryness. Comprehensive two-dimensional gas chromatography–mass spectrometry (2D GC×GC-MS) was used for analysis (GCMS -QP2010 Ultra, Shimadzu, Kyoto, Japan; 2D GC×GC thermal modulator – Zoex Corp). Data were analyzed by GCMS Solution software ChromSquare Ver.2 (Shimadzu), using NIST11 and Wiley8 database libraries.

Molecular characterization
The following reagents were used in the experiment: DNeasy Blood & Tissue Kit (Germany) – for DNA extraction; 27F and 1492R primers – for 16S rRNA gene amplification; QIAquick PCR Purification Kit – for purification; and Macrogen (Netherlands) – for sequencing process.

RESULTS AND DISCCUSION

The growth of bacterial populations. In the first 70 min, based on the absorbance as consequence of bacterial growth (Fig. 1), it has been noticed that there was no increase in the number of cells. It could be because the microorganism AW-03 was adapting to the fresh medium (lag phase). After 70 min, AW-03 entered the exponential phase with generation time of 51 minutes.

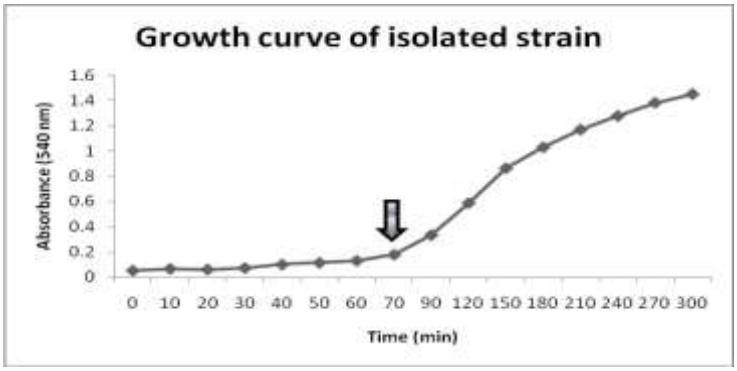

Figure 1. Growth curve of the isolated bacterial strain AW-03. Nutrient broth was used as medium. Broth was shaken on a rotary shaker (150 rpm / min), at 28°C.

Gram-straining, catalase and oxidase tests. Colonies of isolated strain AW-03 were pale yellow, mucoid. Cells were single or in pair, small, oval, nonmotile. Isolated strain is a Gram-negative, aerobic or facultative anaerobic, catalase positive and oxidase negative bacterium. The characteristics of AW-03 strain corresponded to the description from literature for genus *Klebsiella* [9, 19, 20].

API results. There is no high percentage of agreement with the API database (API test software). However, broad biochemical characterization was obtained. API test results are shown in the Fig. 2. Isolated strain AW-03 produced a wide range of different enzymes: alkaline phosphatase, acid phosphatase, esterase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, pyrazinamidase, pyrrolidonyl arylamidase, β-galactosidase, α- and β-glucosidase. The microorganism can reduce nitrates to nitrite and it also performs fermentation and assimilation of a large number of mono- and disaccharides. Small molecules, malic acid and trisodium citrate can also be assimilated. This microorganism does not produce indole. There are some differences, but a large part of the results is in accordance with the literature for genus *Klebsiella* [9, 19, 20, 21].

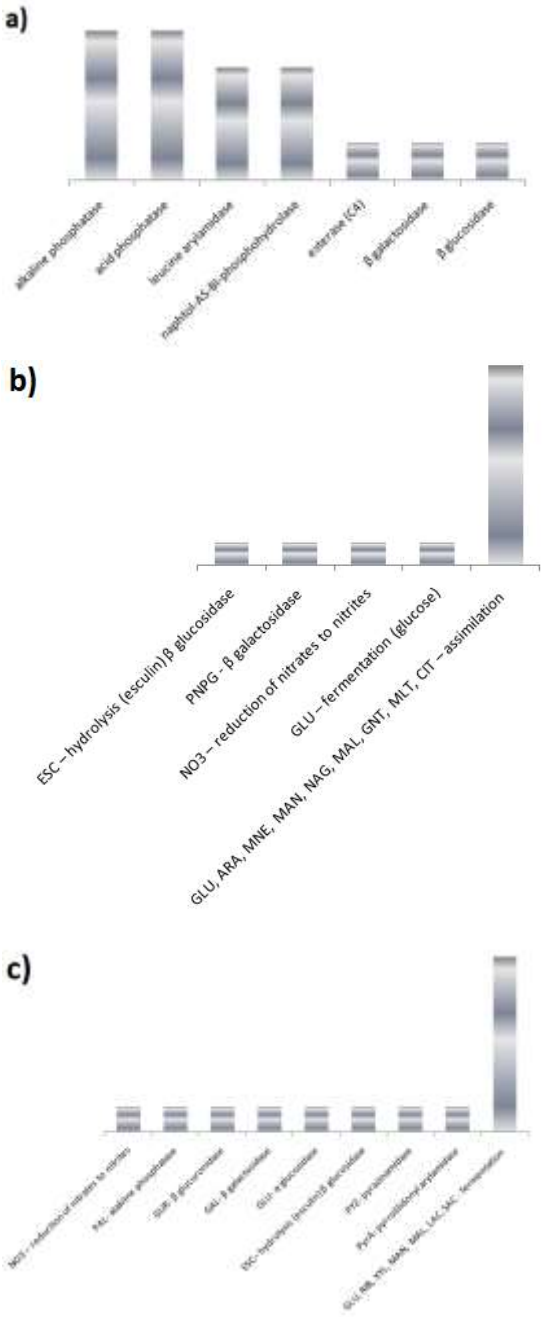

Figure 2. Results of API test of the isolated strain AW-03: a) API ZYM - semiquantitation of activities, b) API 20 NE (qualitative), and c) API CORYNE (qualitative test).

Fatty acid methyl ester. The FAME profile (Fig. 3) consists of normal, cyclo, hydroxy fatty acids methyl esters, and iso methyl-branched fatty acids: *i*C14:0; C14-3OH; C15:0; C16:0; C16:1; C17:0*cy*; C17:0 and C17:0. FAME profile was relatively different compared to previous study of genus *Klebsiella* [22], which could indicate an adaptation to the life in a polluted habitat.

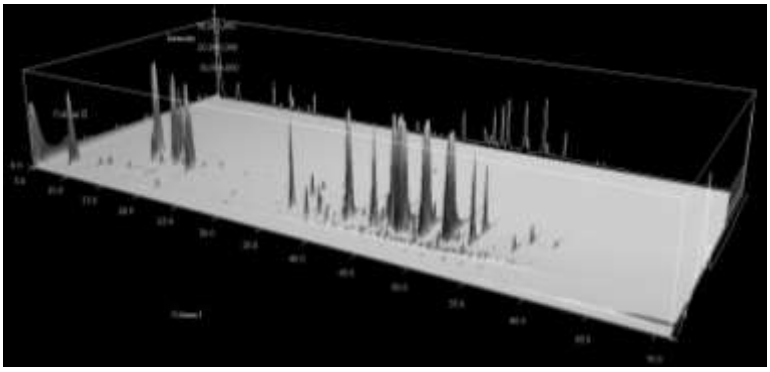

Figure 3. 2D GCxGC–MS spectrum: the fatty acid methyl ester profile of the isolated AW-03 strain.

Molecular characterization. The gene for 16S rRNA was compared to the NCBI sequence database. The sequence has shown the highest homology with *Klebsiella variicola* (99.12% similarity with *K. variicola* CP017289.1). *Klebsiella variicola* belongs to genus *Klebsiella*, family Enterobacteriaceae, order Enterobacteriales, class Gammaproteobacteria, phylum Proteobacteria, kingdom Bacteria [19].

CONCLUSION

The microorganism AW-03 isolated from PFASs-polluted environment was successfully characterized by biochemical and molecular methods. A detailed physiological and biochemical characterization confirmed that isolated bacterial strain AW-03 belongs to the genus *Klebsiella*. Molecular characterization confirmed that this bacterium has the highest homology with *Klebsiella variicola*. Characterized microorganism will be further used for testing of reduction of PFASs concentration.

ACKNOWLEDGMENT: This work has been supported by the Ministry of Education, Science and Technological Development, Republic of Serbia, Project No. III 43004. and JICA grassroot project "Capacity Building for Analysis and Reduction Measures of Persistent Organic Pollutants in Serbia"

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IZVOD

BIOHEMIJSKA I MOLEKULARNA KARAKTERIZACIJA *Klebsiella* sp. IZOLOVANE IZ ŽIVOTNE SREDINE ZAGAĐENE PERFLUOROVANIM JEDINJENJIMA

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Razvoj hemijske industrije u prošlom veku doprineo je povećanoj proizvodnji hrane, efikasnijoj kontroli bolesti i poboljšanju životnog standarda. Ipak, usled industrijskog razvoja došlo je do nagomilavanja velikih količina toksičnih supstanci u životnoj sredini. Dugotrajni organski zagađivači su hemikalije koje se zadržavaju, akumuliraju u čitavom lancu ishrane i imaju štetan uticaj na ljudsko zdravlje i životnu sredinu. Perfluorovana jedinjenja, kao što su perfluoroktan sulfonska kiselina, njene soli i perfluoroktan sulfonil fluorid, nalaze se na listi dugotrajnih organskih zagađivača. Nekoliko skorašnjih publikacija je pokazalo da mikroorganizmi izolovani iz životne sredine zagađene perfluorovanim jedinjenjima mogu smanjiti nivo istih. U ovom radu je bakterijski soj izolovan iz takve životne sredine fiziološki, biohemijski i molekularno okarakterisan. U preliminarnim laboratorijskim testovima soj je pokazao sposobnost smanjenja količine perfluorovanih jedinjenja. Mikroorganizam je Gram-negativan, nepokretan, oksidaza negativan i pozitivan na katalazu, proizvodi različite hidrolazne enzime. Mikroorganizmu je određen i masno-kiselinski profil. Molekularna karakterizacija je potvrdila da izolovani soj pripada rodu *Klebsiella*. Mikroorganizam je uspešno okarakterisan različitim metodama i ubuduće će biti korišćen u detaljnoj laboratorijskoj studiji analize mehanizama smanjenja koncentracije perfluorovanih jedinjenja.

Ključne reči: *Klebsiella sp.*, PFOS, POPs