

# Biosurfactant-Assisted Biodegradation of Fluoranthene in a Two-Stage Continuous Stirred Tank Bio-Reactor System using Microorganism

Tshilidzi B. Lutsinge\*, Evans M. N. Chirwa

Water Utilisation and Environmental Engineering Division, Department of Chemical Engineering, University of Pretoria, Pretoria 0002

[blutsinge@gmail.com](mailto:blutsinge@gmail.com)

Polycyclic aromatic hydrocarbons (PAHs) are known to be resistant to environmental degradation due to their hydrophobicity which results in low solubility in water. PAHs are therefore typically less bioavailable than other non-aromatic compounds in the same range of molecular weight. One of the PAHs, fluoranthene, is a four-ring PAH rated among the top 16 PAHs which are included in the list of priority pollutants by the U.S.EPA. Like other PAHs, fluoranthene is a mutagenic, carcinogenic and toxic compound with known potential risks to human health and the environment. In this study, cultures of biosurfactant producing bacteria were isolated from engine oil contaminated soil at a car servicing facility in Pretoria (South Africa). Biodegradation of fluoranthene was conducted using the isolated culture in two Continuous Stirred Tank Reactors (CSTRs) in series followed by polishing treatment in a packed-bed biofilm reactor. Optimum operation times for the CSTR and biofilm system was based on the optimum incubation time and experimental results from batch systems. Results showed that up 93 % fluoranthene was degraded during runs that utilised the enriched inoculum from engine oil contaminated soil. The open system was easily optimised based on target feed rate, hydraulic retention time (HRT) and biomass yield. Analysis of the 16S rRNA gene of the biosurfactant producing inoculum showed that the strains isolated were 100 % homologs of *Pseudomonas aeruginosa*.

## 1. Introduction

PAHs refer to compounds composed of two or more fused benzene rings. They originate from both natural and anthropogenic sources (Haritash and Kaushik, 2009) and are known to be carcinogenic and mutagenic to living organisms (Gan et al. 2009). Compounds consisting of two or three benzene rings are classified as low molecular weights (LMW) PAHs and those with four or more benzene rings as high molecular weights (HMW) PAHs. HMW PAHs are more likely to adsorb to soil particles or solid surfaces (Ukiwe et al., 2013). Due to the carcinogenicity, mutagenicity and/or toxicity of PAHs in the environment, it has become increasingly important to properly manage all PAH pollution sources in the environment.

Remediation strategies for PAHs contaminated sites using physical, chemical and biological technology have been studied and developed in many countries. Methods such as incineration, excavation, landfilling, and storage are still used (Chirwa et al., 2017). But these methods have been shown to be difficult to execute. Alternatively, biological methods offer several advantages over conventional technologies because they are cheaper and environmentally compatible (Bustamante et al., 2012). Most biological processes employ enzymatic processes within microorganisms to facilitate the processes of biodegradation and complete mineralisation of the offending contaminants to more benign products such as low molecular weight carboxylic acids, CO<sub>2</sub> and H<sub>2</sub>O. To achieve higher degradation rates for hydrophobic compounds, biosurfactants are used to enhance the bioavailability of the hydrophobic substrates by increasing solubility in water (Benincasa, 2007). Sarubbo et al. (2015) reported the favourable properties of biosurfactant produced by *Pseudomonas sp.* in the bioremediation of hydrophobic compounds in soil and water.

Degradation of HMW PAHs is mostly achieved in batch experiments (Patowary et al., 2015). Saiu et al. (2016) efficiently removed greater than 90 % of pyrene in a batch system using *Pleurotus sajor-caju*. The present

study is aimed at degrading fluoranthene as a representative of a HMW PAHs, by means of cultures grown from pure colonies of *Pseudomonas aeruginosa* bred in a stirred tank reactor.

## **2. Materials and methods**

### **2.1 Microorganisms enrichment**

Cultures containing biosurfactant-producing bacteria were isolated from engine oil contaminated soil from a car service yard in Pretoria, South Africa. The microorganisms were rapidly grown in broth by adding 5 g of contaminated sample soil to 100 mL nutrient broth in a 250 mL Erlenmeyer flask. The flask was incubated in a rotary shaker with temperature 37 °C, Rotary speed 150 rpm, pH = 7 and for a period of 42 h. After 24 h, 1 mL of grown microorganisms was added to a fresh sterile mineral salt medium (MSM) supplemented with 25 mg of PAH as carbon and energy source in a 250 mL Erlenmeyer flask and incubated for 21 d in the same conditions. After 21 d, 10 mL from the sample was inoculated into a new sterile MSM solution supplemented with PAH as sole carbon and energy source and incubated for 7 d.

### **2.2 Cell Isolation and purification**

After microbial consortia enrichment, 1 mL sample was extracted and serially diluted in sterile mineral salt medium using glass test tubes. 100 µL from the fourth test tube was transferred onto a prepared nutrient agar plate and incubated at 30 °C for 48 h for bacterial colonies to grow (APHA, 2005). Colonies were streaked on new nutrient agar plates two times to further purify bacterial isolates. Colonies with different morphological characteristics were evaluated for their potential to produce biosurfactants. Cell overproduction, was conducted in nutrient agar followed by growth in mineral medium with composition of (g/L): 0.1 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 4.5 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 0.68 g KH<sub>2</sub>PO<sub>4</sub>, 4.5 g NaNO<sub>3</sub> and 0.5 yeast (Roslev et al., 1998).

### **2.3 Screening for potential biosurfactant-producing isolates – Drop-collapse Test**

First method used was drop-collapse method which was performed using a procedure adopted from Bodour and Miller-Maier (1998). A quantitative method was performed on a 96-micro well plate which were coated with mineral oil and incubated for 24 h at a room temperature prior to the test. 5 µL aliquot of a sample was delivered into the centre of the well using a pipette. The drop will bead up, spread slightly or collapse depending on the amount of surfactant in the sample. If the drop collapses and spreads out it indicates the presence of biosurfactant and if the drop remains bead up, it indicates the absence of the surfactants.

### **2.4 Emulsification index**

E<sub>24</sub>, defined as the percentage of the height of emulsified layer divided by the total height of the liquid column (Bento et al., 2005) was used to examine the quality of biosurfactant producing culture using their ability to form and stabilize emulsions. 2 mL of cell free supernatant of the cultured sample was added to 2 mL of hexane. The mixture was then mixed for 2 min with a vortex mixer and allowed to stand for 24 h.

### **2.5 Culture Identification**

Cultures were sent to be analyzed using a sequence of gene encoding 16S rDNA. 16S rRNA genomic material was extracted and prepared following the method outlined by Chirwa et al. (2017). Cultures identified as biosurfactant producing *Pseudomonas aeruginosa* were stored in 30 % glycerol solution at -80 °C for further use. To prepare microbial inoculum, pre-culture was done by adding a strain of stored culture into 50 mL of broth solution and kept in an orbital shaker at 150 rpm, 37 °C and pH 7 for 24 h.

### **2.6 Biosurfactant production**

Biosurfactant production was achieved after growth on engine oil or cranola oil followed by starvation using the method derived by Bezza and Chirwa (2016). After biosurfactant production, cell biomass was removed by centrifugation at 6000 rpm, 4 °C for 20 min. The cell free supernatant was pH adjusted to a value of 2 using 2M HCl. Equal amount of Chloroform:methanol (2:1) was added to the solution and left overnight in a refrigerator at 4 °C.

### **2.7 Continuously Stirred Tank Reactor (CSTR) – Biofilm Reactor setup**

The CSTR has three stages aimed at executing different objectives. First stage involves production of biosurfactants in reactor one using the methodology described in 2.3 with the aim of using these biosurfactants to dissolve HMW PAHs. Second stage involves growth of microbial consortia capable of degrading dissolved PAHs from the first stage. Finally, the third stage involves growth of biofilm on stone as media aimed at further degradation of PAHs and aeration which is injected through recycled flow. The reactor

was operated in a warm room with temperature between 27 °C and 35 °C dependent on the time of the day and the weather.

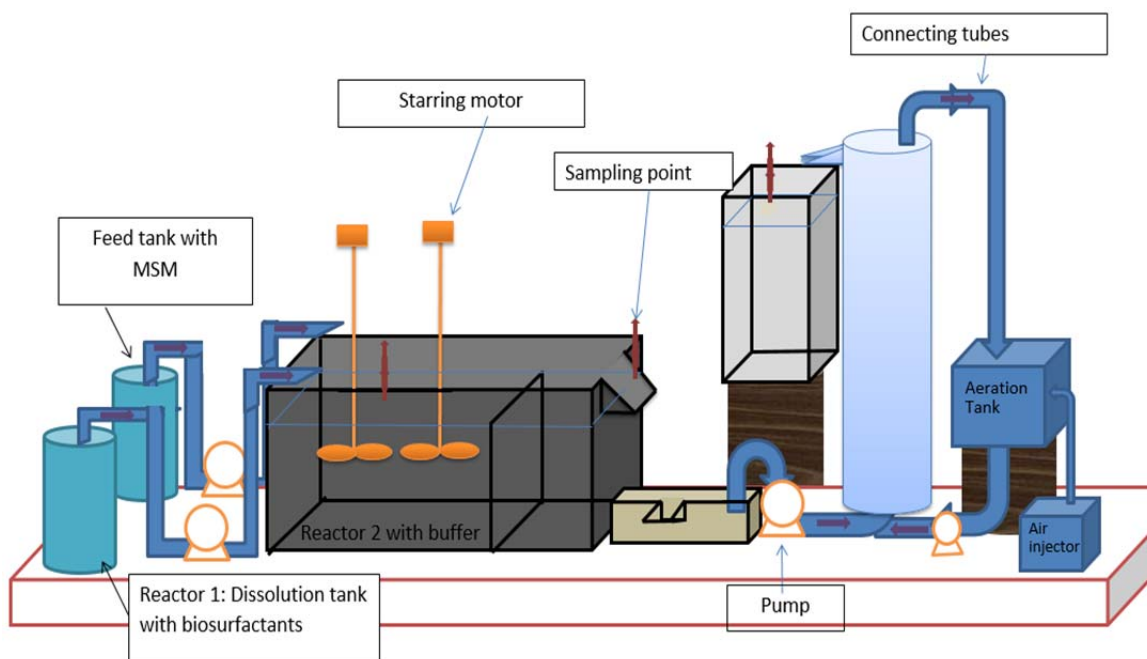


Figure 1: Diagram showing the layout of the CSTR-Biofilm reactor connected in series with dual feed for growth medium and PAH dissolution chamber

The reactor was run for a period of 114 h while sampling after 4 h for the first 64 h and after 6 h for the remaining running period. 2 mL samples were drawn from the reactor using a pipette and vortexed vigorously with hexane to extract the PAHs in the sample. Using a separation funnel, hexane was extracted and evaporated to leave PAHs coating at the bottom of test tubes.

## 2.8 Analytical method

PAHs were re-dissolved using acetonitrile and filtered into vials for analyzing using a high performance liquid chromatograph (HPLC). HPLC, Waters 2695, separation module equipped with waters Photodiode Array Detector Model 2998 was used. The PAHs were separated using a reverse phase mode, a Waters PAH C<sub>18</sub> column (250 mm × 4.6 mm, 5 µm particle size), temperature of 25 °C and pressure of 4000 psi. The detection wavelength operated at 254 nm. The mobile phase (Acetonitrile:Water) was programmed with a reciprocating pump.

Table 1: Reactor configuration

	Feed tank with MSM	Reactor 1	Reactor 2	Biofilm tube
Tank size	30 L	1 L	63 L	3.82 L
Water volume	16 L	400 mL	47.7 L	1.001 L
Surface area	N/A	N/A	N/A	0.676 m <sup>3</sup>
Influent flow	N/A	N/A	0.20 L/h MSM feed 0.55 L/h PAH feed	0.531 L/h
Effluent flows	0.2 L/h	0.55 L/h	0.531 L/h	0.531 L/h
Hydraulic retention time	N/A	N/A	63.6 h	1.88 h
Recycle flow	N/A	N/A	N/A	6.529 L/h

### 3. Results and Interpretation

#### 3.1 Screening and identification of biosurfactant producing bacteria

After enrichment of the contaminated soil, five different colonies were isolated. The colonies were tested for their biosurfactant production capability using the drop-collapse test and emulsification index ( $E_{24}$ ) method. Three strains were identified that produced positive results in the Drop-collapse test high  $E_{24}$  values (52 %, 68 %, 56 %) (Figure 2a). A batch culture of strain B with  $E_{24} = 68 %$  was confirmed to produce a large amount of biosurfactant during a batch test under low carbon source (Figure 2b).

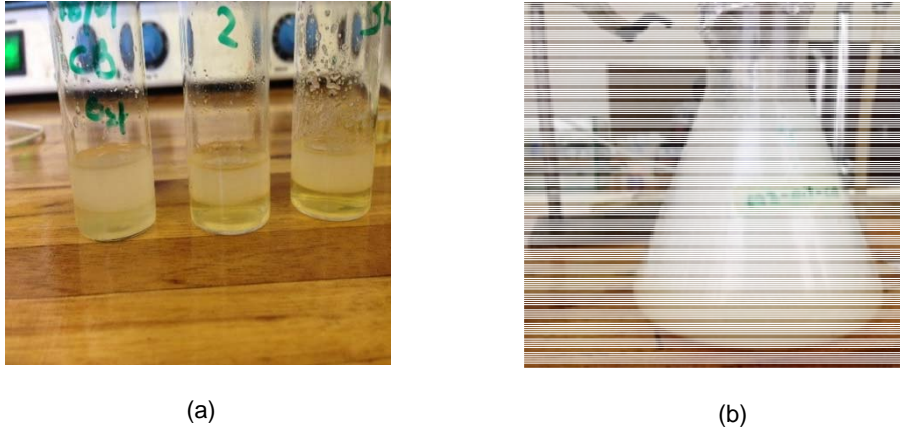


Figure 2: (a) Test-tubes showing the visual results for  $E_{24}$  index results and (b) the forming caused by biosurfactant under low carbon source conditions

The DNA sequence from each culture was then uploaded to the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed from the identified 16S rRNA sequences using the neighbor-joining method in the MEGA Version 6 software (Tamura et al., 2013). The results showed that the strain A, B, and D belong to members of genus *Pseudomonas*, with a 100% sequence similarity to *Pseudomonas aeruginosa* (Figure 3).

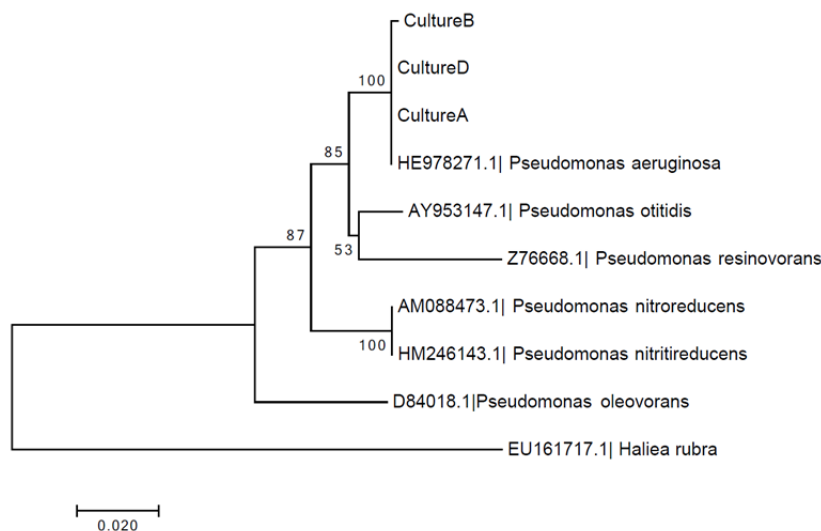


Figure 3: Phylogenetic tree of 16S rRNA identification showing *Pseudomonas aeruginosa* as the most predominant homolog of the biosurfactant producing isolates - Culture A, Culture B, and Culture C

A total of 20.75 g/L of crude biosurfactant was produced from the purified colonies for further characterisation of the physical attributes of the biosurfactant. The biosurfactant produced had a yellow-brownish colour. 8.3 g

of crude biosurfactants suspended in 400 mL, of produced biosurfactant supernatant after cell removal by centrifugation, was used to dissolve 600 mg of fluoranthene. After 24 h in a shaker a total of 456.94 mg/L of fluoranthene was dissolved.

### 3.2 Fluoranthene degradation in CSTR

The results in **Errore. L'origine riferimento non è stata trovata.**4 shows that there is degradation of fluoranthene in the system when comparing the tracer studies run results and the degradation run results. The graphs do not show smooth curves due to factors such as human errors and instrumental errors.

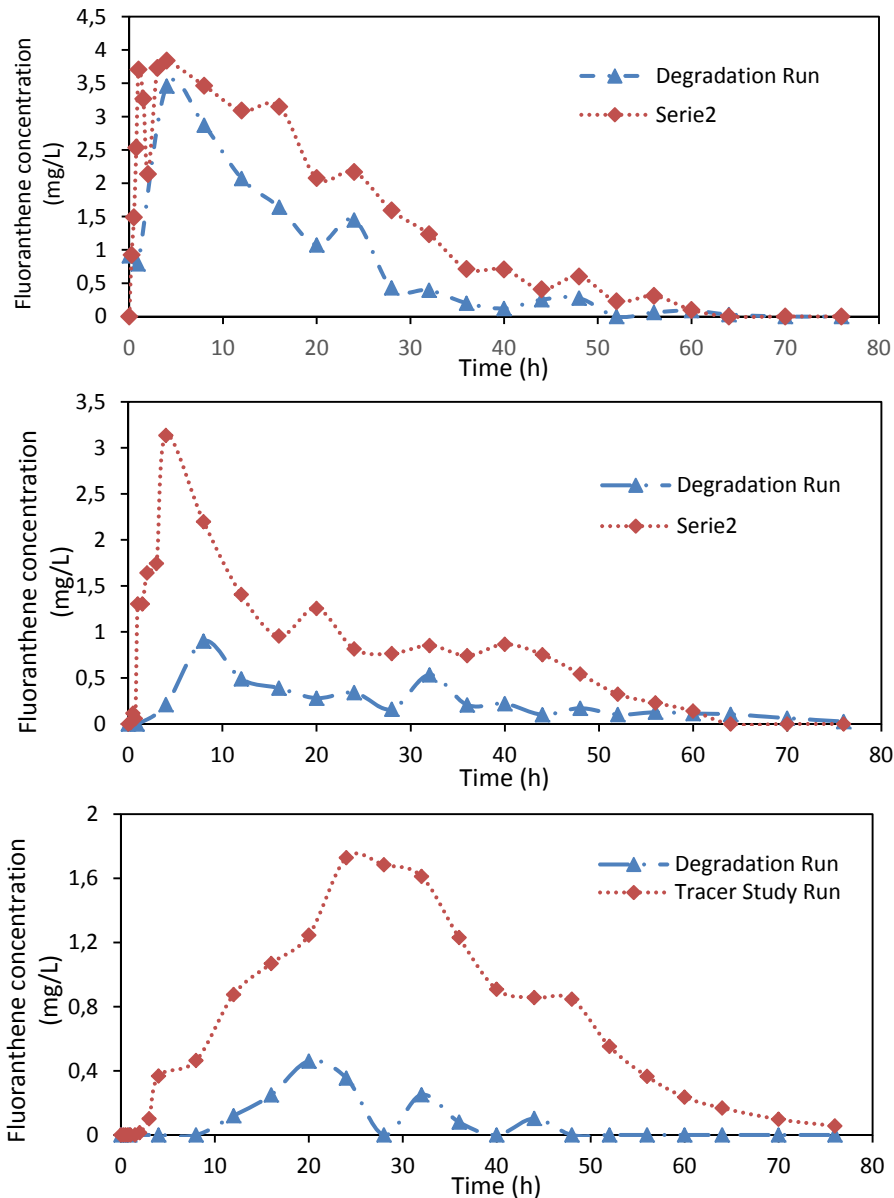


Figure 4: Effluent fluoranthene concentration 'blue' from (a) PAH biodegradation zone 2A, (b) biodegradation zone 2B, and (c) final removal of PAH residual in the biofilm reactor with tracer concentration (red) respectively

Using the area under the tracer concentration curve (red line) and fluoranthene concentration curve, total fluoranthene removed from reactor 2A, reactor 2B and the biofilm tank compartments was calculated. Removal percentages from reactor compartment 2A, 2B and biofilm were 39.18 %, 70.75 % and 97.87 %

respectively. An overall removal percentage of fluoranthene considering initial concentration of 3.79 mg/L in the reactor 2 and an average of 0.28 mg/L as an effluent from the biofilm tank 93 %.

#### 4. Conclusion

The isolated strain from oil spill contaminated soil in an automobile and car service yard was identified as *Pseudomonas aeruginosa*. The strain had the capacity to produce biosurfactants utilising olive oil and glycerol as carbon and energy source. Approximately 20.75 g/L of crude biosurfactant was produced with 8.3 g of biosurfactant in a liquid form dissolving 68 % of added solid fluoranthene. In the CSTR system, approximately 93 % of fluoranthene was degraded, which shows that it is possible to dissolve high molecular weight polycyclic aromatic hydrocarbons with biosurfactants produced by *Pseudomonas aeruginosa* strain and use the same strain to degrade the dissolved PAHs. From the results from the experiments, the open system can be introduced to the water treatments with effluents containing PAHs to efficiently remove them.

#### Acknowledgments

The authors thank the National Research Foundation (NRF) of South Africa who funded the research through the NRF Competitive Support for Unrated Researchers Grant No. CSUR160506164236 and the NRF Incentive Finding for Rated Researchers Grant No. IFR170214222643 awarded to Prof Evans M.N. Chirwa of the Department of Chemical Engineering, University of Pretoria.

#### References

- APHA, 2005, Standard Methods for the Examination of Water and Wastewater - Centennial Edition. American Public Health Association, American Water Works Association, Water Environment Federation, USA.
- Benincasa M., 2007, Rhamnolipid Produced from Agroindustrial Wastes Enhances Hydrocarbon Biodegradation In Contaminated Soil, *Current Microbiology*, 54, 445–449.
- Bento F.M., De Oliveira Camargo F.A., Okeke B.C., Frankenberger W.T., 2005, Diversity of Biosurfactant Producing Microorganisms Isolated from Soils Contaminated with Diesel Oil, *Microbiological Research*, 160, 249–255.
- Bezza F.A., Chirwa, E.M.N., 2016, Biosurfactant-Enhanced Bioremediation of Aged Polycyclic Aromatic Hydrocarbons (PAHs) in Creosote Contaminated Soil, *Chemosphere*, 144, 635–644.
- Bodour A.A., Miller-Maier R.M., 1998, Application of a Modified Drop-Collapse Technique For Surfactant Quantitation and Screening Of Biosurfactant-Producing Microorganisms, *Journal of Microbiological Methods*, 32, 273–280.
- Bustamante M., Durán N., Diez M., 2012, Biosurfactants are Useful Tools for the Bioremediation of Contaminated Soil: A Review. *Journal of Soil Science and Plant Nutrition*, 12, 667–687.
- Chirwa E.M.N., Mampholo C.T., Fayemiwo O.M., Bezza F.A., 2017, Biosurfactant Assisted Recovery of Oil And C<sub>11</sub>-C<sub>15</sub> Hydrocarbon Preservation in a Biological Oil Recovery Process from Oily Sludge, *Journal of Environmental Management*, 196, 261–269.
- Gan S., Lau E.V., Ng H.K., 2009, Remediation of Soils Contaminated with Polycyclic Aromatic Hydrocarbons (PAHs), *Journal Of Hazardous Materials*, 172, 532–549.
- Haritash A.K., Kaushik C.P., 2009. Biodegradation Aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A Review, *Journal of Hazardous Materials*, 169, 1–15.
- Maliszewska-Kordybach B., 1999, Sources, Concentrations, Fate and Effects of Polycyclic Aromatic Hydrocarbons (PAHs) in the Environment, Part A: PAHs in Air, *Polish Journal of Environmental Studies*, 8, 131–136.
- Patowary K., Kalita M.C., Deka S., 2015, Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) Employing Biosurfactant Producing *Pseudomonas aeruginosa* KS3, *Indian Journal of Biotechnology*, 14, 208–215.
- Roslev P., Madsen P.L., Thyme J.B., Henriksen K., 1998, Degradation of Phthalate and Di-(2-Ethylhexyl) Phthalate by Indigenous and Inoculated Microorganisms in Sludge-Amended Soil, *Applied and Environmental Microbiology*, 64, 4711–4719.
- Saiua, G., Tronci S., Grosso M., Cadonib E., and Currelic N., 2016, Biodegradation of Polycyclic Aromatic Hydrocarbons by *Pleurotus sajor-caju*, *Chemical Engineering Transactions*, 49, 487–492.
- Sarubbo L.A., Luna J.M. & Rufino R.D., 2015, Application of a Biosurfactant Produced in Low-cost Substrates in the Removal of Hydrophobic Contaminants, *Chemical Engineering Transactions*, 43, 295–300.
- Tamura K., Stecher G., Peterson D., Filipski A., Kumar S., 2013, MEGA6: Molecular Evolutionary Genetics Analysis version 6.0, *Molecular Biology and Evolution*, 30, 2725–2729.
- Ukiwe L.N., Egereonu U.U., Njoku P.C., Nwoko C.I.A., Allinor J.I., 2013, Polycyclic Aromatic Hydrocarbons Degradation Techniques: A Review. *International Journal of Chemistry*, 5, 43–55.