

Biosurfactant Assisted Degradation of High Ring Number PAHs Using a Two-Stage CSTR/Fully Submerged Fixed-Film Bioreactors in Series

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Polycyclic aromatic hydrocarbons (PAHs) are released into the environment through natural activities such as forest fires and incomplete combustion of wood and decay of vegetable matter. Anthropogenic activities that release PAHs into the environment include activities such as combustion of coal and petroleum product processing. PAHs are not easily accessible to bacteria for degradation due to their low solubility in water. Among the notorious PAH are the high ring number PAHs (ring-number 4 - 7) that are extremely difficult to degrade due to their hydrophobicity which renders them insoluble in water. One such compound, fluoranthene (Flu), is a four ring PAH classified as a High Molecular Weight (HMW) compound. Due to the difficulty to degrade these compounds, it is necessary to find novel and environmentally compatible methods for treating them. In this study, PAH degrading organisms isolated from engine oil contaminated soil achieved 92 % removal of Flu in a fixed-film bioreactor operated at retention time of 1.19 h at 0.86 L.h⁻¹ under fully submerged conditions. The predominant species of biosurfactant producing bacteria in the reactor were determined to be dominated by *Pseudomonas aeruginosa* using 16S rRNA genotype fingerprinting analysis.

1. Introduction

PAHs are discharged from both industrial human activities and natural sources. Combustion of vegetable materials at high temperatures in the range of 400-5,000 °C, produces thermodynamically un- or metastable organic compounds such as carbon monoxide (CO) which in turn recombine to form larger and more complex PAHs (Kabziński, 2002). Natural production of PAHs in the environment occurs through many pathways including: vegetation burning in forest and bush fires, thermal geological reactions associated with fossil-fuel and mineral production, volcanic eruptions and biosynthesis. Natural sources of PAHs such as volcanic activities and forest fire do not significantly contribute to the overall PAH emission (Maliszewska-Kordybach, 1999).

PAHs together with other chlorinated and non-chlorinated aromatic organics are generally resistant to environmental degradation due to their greater hydrophobicity (Gan et al., 2009). Compounds consisting of two or three benzene rings are classified as low molecular weight LMW-PAHs and those with four or more benzene rings are classified as high molecular weight HMW-PAHs. HMW-PAHs are more likely to adsorb to soil particles or solid surfaces due to their hydrophobicity which results in higher surface affinity and are less biodegradable than the LMW compounds (Ukiwe et al., 2013).

Remediation strategies for PAH contaminated sites include physical, chemical and biological methods. Methods such as incineration (Chen et al., 2013), excavation and landfilling (Das and Chandran, 2011), and land-farming (Bezza and Chirwa, 2016), have been used in a variety of contexts. These methods have been found to be expensive, difficult to execute, and inefficient when applied for treatment of large flows of contaminated wastewater or decontamination of large land sites (Das and Chandran, 2011). The process of biodegradation where compounds are completely mineralised to CO₂ and H₂O is considered more effective and environmentally compatible. In this study, Flu was chosen as the surrogate compound representing a class of high-ring number PAHs. Bacteria from PAH contaminated soil was considered the most likely candidates to achieve both biosurfactant production to facilitate the dissolution of the compounds and degraders of the aromatic ring

compounds (Lutsinge and Chirwa, 2018).

2. Materials and methods

2.1 Culture isolation and media

A mixed culture of biosurfactant-producing/PAH-degrading bacteria was obtained from oil contaminated from a motor service yard in Pretoria West, South Africa. The culture was first grown in nutrient broth (NB) from commercial powder by dissolving 16 g of nutrient broth solid in 1 L distilled water. A nitrogen limited growth medium with composition of (g/L): 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 7.8 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 4.5 g KH_2PO_4 and 2 mL of trace element solution was used for the purpose of biosurfactant production. All media were sterilised by autoclaving the solution at 121 °C for 15 min.

2.2 Biosurfactant production

Screening for potential biosurfactant producing cultures was done using a Drop-collapse and emulsification index techniques (Bodour and Miller-Maier, 1998). Isolates with positive results were used to produce biosurfactants using different kinds of mineral salts medium to evaluate the optimum conditions. The biosurfactant was characterised chromatographically by the Thin Layer Chromatography (TLC) and spectrophotometrically by the Fourier Transform Infra-Red spectroscopy (FTIR) to determine its molecular structure and chemical group classification.

2.3 Continuous flow reactor

The reactor consisted of a 14 L mixing chamber where biosurfactants were produced followed by a Pyrex glass column (0.90 m long x 0.10 m diameter) (Figure 1) filled with 8 mm diameter ceramic beads with a liquid volume of 1.001 L and attachment surface area of 0.676 m². The reactor was operated in an up-flow mode to ensure operation under fully submerged conditions.

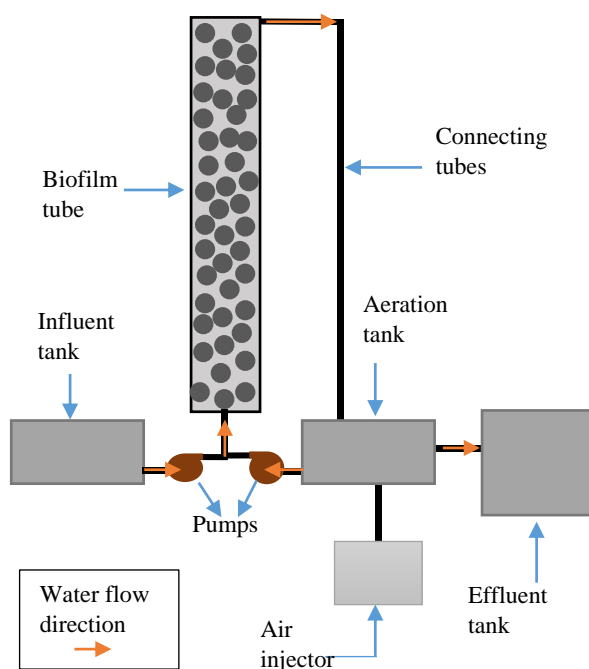


Figure 1: The configuration of the biofilm phase showing the recirculation and external aeration.

2.4 Species Identification

Genomic DNA extracted from purified colonies was amplified by a reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8-27; Primer pH to position 1,541-1,522 following methods specified earlier. Species identification was conducted by BLAST search of the NCBI database. Phylogenetic tree diagrams were then constructed using the neighbour-joining method (Tamura et al., 2013). Confidence in the tree topology was determined the bootstrap analysis based on 100 re-sampling (Felsenstein, 1985).

2.5 Analytical Methods

PAHs were measured using a Waters 2,695 high performance liquid chromatograph (HPLC) (Waters Corporation, Massachusetts, USA) separation module equipped with waters Photodiode Array Detector Model 2,998. The PAHs were separated using a reverse phase mode and a Waters PAH C18 column (250 mm × 4.6 mm, 5 µm particle size) at a temperature of 25 °C and pressure of 275.8 bar at a detection wavelength of 254 nm.

3. Results and discussion

3.1 Microbial culture composition

From the 16S rRNA gene analysis, it was shown that the predominant species of biosurfactant producing bacteria in the reactor, samples submitted as *3na10*, *5pca9*, and *9na8* were most closely related to *Pseudomonas aeruginosa* HE978271.1 (Figure 2). *Pseudomonas aeruginosa* is a well-known aromatic compound degrading organism. Its ability to produce biosurfactant and degrade benzene ring compounds (Meliani and Bensoltane, 2014) and lower ring number PAHs was demonstrated earlier in batch studies by Bezza and Chirwa (2016).

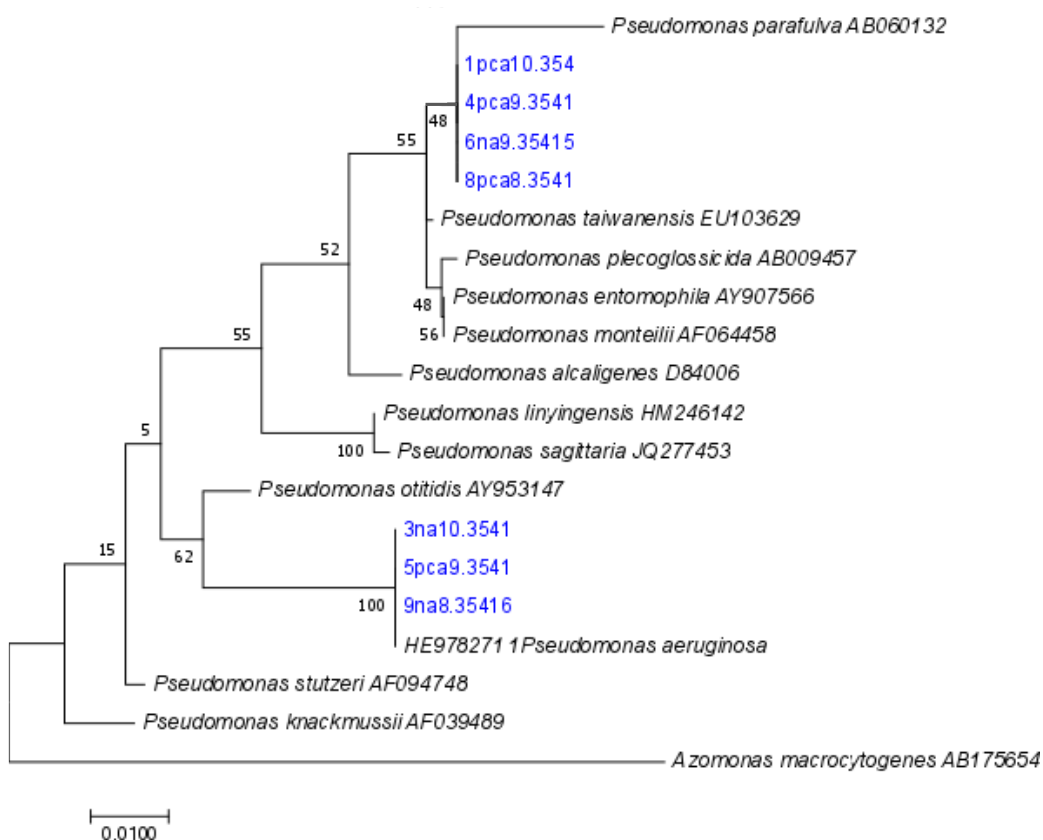


Figure 2: Phylogenetic tree showing the biosurfactant producing cultures *3na10*, *5pca9*, and *9na8* as 100 % homologs of *Pseudomonas aeruginosa* HE978271.1.

3.2 Biosurfactant chemical structure characterisation

FTIR spectrum of purified biosurfactant showed peaks correlated to -NH_2 (peptide group) at $3,264\text{ cm}^{-1}$, C-H (alkane group) at $2,925\text{ cm}^{-1}$, -C=O-N (amide group) at $1,713\text{ cm}^{-1}$, $\text{-CH}_2\text{-CH}_2\text{-}$ (aliphatic chains) at $1,457$ to $1,418\text{ cm}^{-1}$ and $\text{-CH-NH}_2\text{-}$ (amine group) indicated by a strong stretching mode peak around $1,084\text{ cm}^{-1}$ (Figure 3). Many literature sources present similar FTIR results for biosurfactants produced by *Pseudomonas* sp. with characteristics indicating a lipopeptide chemical structure (Mandal et al., 2013).

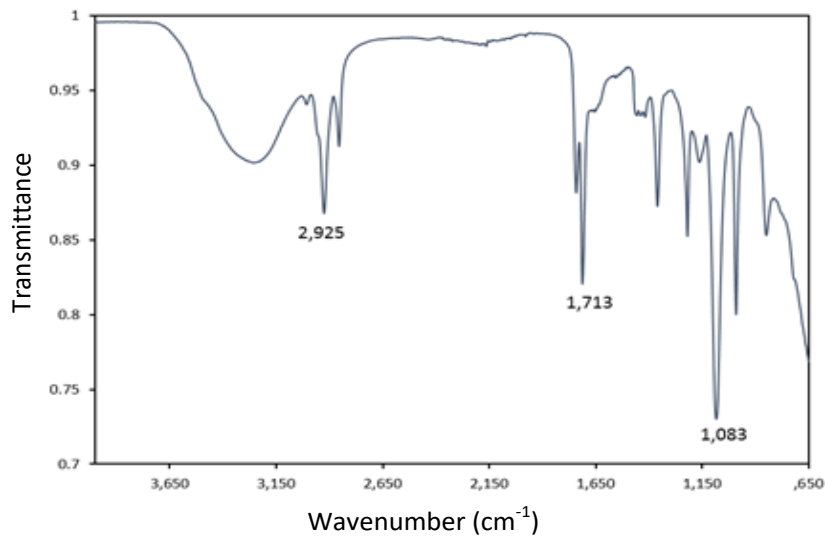


Figure 3: FTIR absorption spectrum of biosurfactant produced by *Pseudomonas aeruginosa* strain.

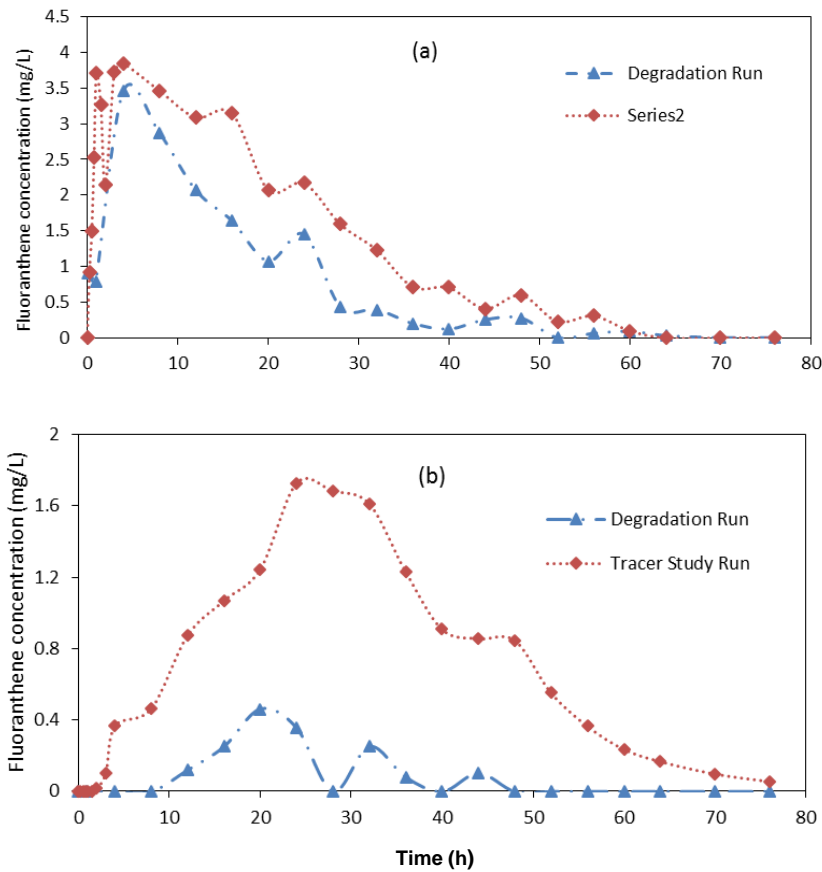


Figure 4: Influent and effluent fluoranthene concentration in (a) the CSTR stage and (b) the biofilm reactor based on results reported earlier by Lutsinge and Chirwa (2018).

3.3 Fluoranthene degradation in the CSTR and Biofilm Stages

Dissolution of Flu was facilitated by mixing the PAH with a harvested biosurfactant in the CSTR (stage 1) of the reactor. Effluent from stage 1 containing the dissolved surfactant passed through a second CSTR (stage 2) in

which it was partially degraded and proceeded to the biofilm reactor (stage 3) where it was almost completely degraded. The results from the first bioreactor (dissolution phase) and the large removal in the biofilm reactor is shown in Figure 4a and 4b. The plots show the input to the given phase (red line) and the effluent concentration for the corresponding loading (blue line). The plot takes into consideration that there is time difference between corresponding points on the influent and effluent data points due to the long retention time across each stage as indicated. The retention time across the biofilm reactor (1.19 h) was much shorter in comparison to the retention time across the two CSTRs which was 43.83 h.

3.4 Mass balance analysis

The cumulative removal efficiency was plotted against cumulative loading for the biofilm reactor based on the data from Figure 4b. Complete conversion of Flu was represented by a match of measured total Flu removed to theoretical Flu removed (Figure 5). Based on the trend of the cumulative removal line, four modes of operation were identified, these being Moderate Loading (I), Transitional Inhibited (II), Severely Inhibited (III) and Recovery (IV). In Mode I, the very close relationship between the two plots indicates that almost all the Flu was degraded during this phase. In Mode II the degradation efficiency progressively decreased indicating a shift away from 100% removal. In Mode III, the system failed. In Mode IV the parallel line of the measured data to the theoretical curve reflects that the system recovered after lowering the influent Fluoranthene concentration to safe levels. The demonstrated self-recovery of the system shows that the system can be operated continuously without need for re-inoculation after failure operation. This is not normally the case with suspended growth CSTR systems.

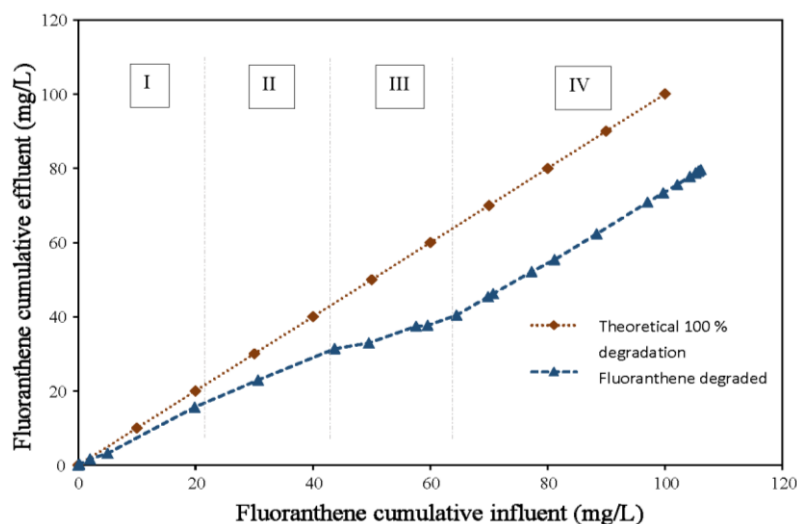


Figure 5: Cumulative fluoranthene influent to the biofilm tank and cumulative effluent from the biofilm tank. Moderate Loading (I), Transitional Inhibited (II), Severely Inhibited (III) and Recovery (IV).

3.5 Fluoranthene biodegradation kinetics

Ordinarily, the degradation of PAHs and phenolic compounds follows substrate inhibited Haldane substrate inhibition kinetics Eq(1) (Andrews, 1968):

$$-\frac{dC}{dt} = \frac{k_c \cdot C \cdot X}{K_c + C + C^2/K_i} \quad (1)$$

where C = fluoranthene concentration (mgL^{-1}), k_c = maximum specific fluoranthene degradation rate coefficient ($\text{mgL}^{-1}\text{h}^{-1}$), K_c = half velocity concentration (mgL^{-1}), K_i = substrate inhibition concentration coefficient (mg^2L^{-2}), and X = viable biomass concentration (mgL^{-1}). Under low concentrations, Haldane equation approaches simple first order kinetics Eq(2) when the Michaelis-Menten constant is higher, then C is not enough in this case; it has to be $K_c > C^2/K_i$.

$$-\frac{dC}{dt} = k_c \cdot C \cdot X \quad (2)$$

In this study, it was observed that the reaction rate approached first order with the Flu concentration, C , as the main variable over time. The degradation rate coefficient k_c was determined to be constant at $0.0054 \text{ mgL}^{-1}\text{h}^{-1}$ through the four modes of operation so far tested as indicated in Figure 5.

4. Conclusions

The biofilm system achieved 92 % removal of a high ring number PAH, Fluoranthene, and the worst loading conditions (Mode III). Removal of Fluoranthene was 100 % during low to moderate loading conditions (Modes I-II, and VI). The results demonstrated that a self-optimizing biological process for degradation of all PAHs in including the HMW-PAHs is possible by inoculating a biofilm reactor with PAH degrading-biosurfactant producing cultures of bacteria.

Acknowledgments

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