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Biodegradation of Pyrene Using Corynebacteria SP and Pseudomonas Putida in Contaminated Water

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ABSTRACT: The hazardous nature of pyrene have been posed serious threat to humans on exposure through industrial effluent discharged and other combustion activities of petroleum products. The biodegradation of pyrene as a bioremediation method by the activity of *Corynebacteria sp and Pseudomonas putida* with effectiveness of these microbes was aim to be investigated. The enriched pure culture of *Corynebacteria sp and Pseudomonas putida* was inoculated into pyrene contaminated water at room temperature of 28°C with mineral salt medium under an optimum pH of 7.2 for 96 hours, thereby decreased the pyrene concentration in the contaminated water and biodegradation kinetics parameters were evaluated from the experimental results. The result favourable to both *Corynebacteria sp and Pseudomonas putida* as pyrene degraders in the contaminated water but Pseudomonas putida was preferred due to higher content of pyrene degraded.

Keywords: Biodegradation, pyrene, corynebacteria sp, pseudomonas putida and Monod's kinetics

1. INTRODUCTION

Petroleum fuel spills are major hazards that introduced polycyclic aromatic compounds (PAHs) to the environment as they damage the surrounding ecosystems through pipeline ruptures, tank failure, combustion, accidental spills and transportation accidents.[19, 20] PAHs such as anthracene, pyrene, benzo (a) pyrene etc. have been considered as the persistent organic pollutants of soil, aquatic environment and hazardous wastes due to their cytotoxic, mutagenic, and their carcinogenic effects on human.[14, 21, 23, 24] Researchers have reported many clean up techniques such as by thermal desorption, soil washing, incineration, some landfilling,[18] phytoremediation which employed living higher organisms like green vegetation, plants, aquatic plants, trees and grasses, to remove toxic compounds with advantage of in situ treatment of contaminated soils, sediments, groundwater, surface water and external atmosphere and biodegradation using microbes.[2, 3, 6, 18, 27] The search for effective and efficient methods of contaminants removal from contaminated soils has indicated that biological degradation of contaminant by microbes is a promising bioremediation technique.[7] The favourable factors such as availability of microbes, accessibility of contaminants and environment conditions of the contaminant to the microbes such as pH had been employed to liberate environment from danger posed by contaminants due to its ecofriendly, bio-based treatment, cost-effective and ecologically adaptable biodegradation method.[2, 24, 30,] The interest in discovering how bacteria are dealing with hazardous environmental pollutants has driven a large research community and it has resulted in important biochemical, genetic and physiological knowledge about applications in bioremediation.[6, 13] Microorganisms are equipped with metabolic machinery bacteria known for their potential degradation of pyrene as a carbon and energy sources and metabolic pathways have been proposed by many researchers based on mineralization of pyrene and degraded to carbon (IV) oxide and water by the different microorganisms.[3, 6] Pathway for the metabolism of pyrene by *Corynebacterium variabilis sp. Sh42 and Pseudomonas sp* was proposed by El-Gendy et al.[15] This is similar to metabolic pathway proposed by Liang et al (2006) for biodegradation of Pyrene using *Mycobacterium sp*.[32] More so, metabolic pathway for *biodegradation* of 2, 2'-bihydroxybiphenyl (2,2'-BHBP) by *Pseudomonas sp* was proposed by Sondossi et al.[14] Many researchers have also work on clean up of pyrene by employed the activity of different microbes such as *pseudomonas putida, Bacillus subtilis, Corynebacterium variabilis sp.Sh42, Pseudomonas sp, Leclercia adecarboxylata, Saccharothrix sp* and Mycobacterium sp without study the kinetics parameters of microbes on pyrene.[1, 8, 9, 11, 15, 17, 20, 21, 26, 28, 29, 31] The motivation of this research was to have quantitative insight on kinetics parameters of biodegradation of pyrene contaminated wastewater using *Corynebacteria sp and Pseudomonas putida* under an optimum pH to liberate environments from danger posed by pyrene to humans as a major constituent of crude oil at room temperature and effectiveness of these microbes.

2. MATERIAL AND METHODS

Pyrene, dichloromethane and hexane (Analytical grade Chemicals) were purchased from Patanne Chemicals, a renowned laboratory chemicals and equipments dealer in Benin City.

Collection of Soil Samples

Corynebacterium sp and *Pseudomonas putida* for the experiment were isolated from the subsurface soil of about 0-15cm depth obtained from an uncultivated land in the Nigerian Institute for oil palm research (NIFOR), Benin City in Nigeria. The soil material used has been described by Salami (2006).[4] The soil was sieved using 2mm mesh screen for uniform particle size and stored in sterilized polyethylene bag at room temperature covered with aluminium foil for further use.

Preparation of Mineral Salt Medium (MSM)

MSM was used to avoid drastic fluctuation of pH which may be detrimental to the viability of the microbes in the batch medium and it was carbon free before anthracene was added after autoclaved at 121°C for 15 minutes. The MSM was prepared with Analytical grade chemicals composition: KH_2PO_4 (9.0g/l), K_2HPO_4 (1.5g/l), NH_4Cl (1.5g/l), $CaCl_2$ (20mg/l), and $MgSO_4$ (0.2g/l). The pH of the medium was standardized to 7.2 using 0.1N NaOH. The MSM was sterilized in an autoclaved at 121°C for 15 minutes and then stored in a secured corner in the laboratory until the experiment was set up.

Microbial Isolation

0.5 g of soil samples were added into 100 ml MSM. The medium containing the soil and 0.1% w/v anthracene was incubated at 29 ± 2°C on a rotary incubator shaker at 150 revolutions per minute for 24 h. The pure culture of colonies of *Corynebacterium sp* and *Pseudomonas putida* were maintained on nutrient agar plates for 72 hours at 29 ± 2°C temperature for production of the microbes' enmasse, reduction of the lag phase and suitability of the inoculums in anthracene contaminated environment before the biodegradation.

Biodegradation of Anthracene

0.6 g of pyrene was dissolved in 10% dichloromethane solution and make up to 4 liters of water. The applied pyrene concentration was 75 mg/l of water. The solvent was volatized from pyrene solution under fume-hood. 250 ml of each of the pyrene solution measured as *A, B* and *C* in 500 ml cotton-plugged Erlenmeyer flasks as reactors wrapped with aluminium foil to prevent contamination. 5 ml of inoculums was transferred from each agar plate of *Corynebacterium sp* and Pseudomonas putida into pyrene contaminated water A and B respectively in 500 ml cotton-plugged Erlenmeyer flasks wrapped with aluminium foil to prevent contamination incubated at 29 ± 2°C on a rotary incubator shaker at 150 revolutions per minute for 96 h except only when a flask was withdrawn for the aliquots to be taken for analysis of the sediment. Non-inoculated *C* sample was taken as the control of the experiment.

Biodegradation Analysis

10 ml of aliquots were taken from *A* and *B* at every 12 hours for the analysis of microbial mass concentration of *Corynebacterium sp* and *Pseudomonas putida* respectively, and utilized pyrene concentration. The biomass concentration was determined using dry weight analysis procedure described by Azeez.[27] The supernatant was centrifuged, decanted and *Corynebacterium sp* and *Pseudomonas putida* cells that settled down at the bottom of the centrifuge tube were scooped and dried in an oven at a temperature of 60°C for 8 hours to a constant weight and recorded. The weight obtained was taken as the dry weight of *Corynebacterium sp* and *Pseudomonas putida* respectively in the analyzed samples. The method of Kumar was employed using UV visible spectrophotometer to measure absorbance of the pyrene in aliquot.[5] The absorbance of the pyrene was recorded at a wavelength of 286 nm in the UV region after isolation of the microbes by centrifuge 10 ml aliquots of rotating at 10,000 revolutions per minute for 20 minutes and allowed to settle for 30 minutes to get a clear supernatant. 5 ml of the clear supernatant was extracted with 5ml of hexane for 10 minutes in a separating funnel. The top solution in a separating funnel at the end of the extraction was a solution of the pyrene in hexane and poured into the corvettes of the spectrophotometer and absorbance readings at a wavelengths of 286 nm was recorded. The procedure was repeated in twelve hourly intervals immediately after inoculation with *Corynebacteria sp* and *Pseudomonas putida* for 96 hours of incubation and the kinetics parameters were determined.

Preparation of the Standard Plots

Solutions of pyrene in the hexane were prepared to give a concentration of 0.3mg/ml. The absorbance of the solutions was read at the appropriate wavelengths 286 nm for the pyrene solution. Calculated quantities of the solution of pyrene were taken and calculated quantities of hexane were added to give lower concentration of the pyrene in hexane 0.27, 0.24, 0.21, 0.18mg/ml etc. The concentration of anthracene (mg/ml) against the absorbance of the pyrene solutions were determined, recorded and then plotted as shown in the Figure 1. A line of best fit plotted for the points obtained. The model of the standard plots was determined and used to convert the pyrene concentration readings from values in absorbance to µg/ml.

Mathematical Model for the Evaluation of Biodegradation of kinetics Parameters

Biodegradation of anthracene was based on the growth of the microbial mass of Malthus correlation concept of first order reaction rate in pyrene contaminated area. The rate of formation of microbial mass on consumption of pyrene by Malthus correlation is given as:

$$\frac{\partial X}{\partial t} = \mu X \tag{1}$$

$\frac{\partial X}{\partial t}$ is the rate of formation of biomass, X is the biomass concentration at any time t, μ is the specific growth rate of biomass and t is the degradation time. Integration of equation (1) at the boundary conditions: $X = X_0 = 0$ at $t = 0$ because there is no inoculation of the microbes in the anthracene broth medium and $X = X$ at $t = t$ gave

$$\frac{\ln(X)}{t} = \mu \tag{2}$$

But Monod's model expressed the growth rate of the microbial cells as function of concentration of the pyrene and it is given as;

$$\mu = \frac{\mu_m X}{k_s + C} \tag{3}$$

C is the mass concentration of pyrene at any time t, k_s Monod's kinetics constant which indicates the affinity of the microbes and μ_m is the maximum specific growth rate of the microbes.

Linearization of equation (3) gave

$$\frac{1}{\mu} = \frac{k_s}{\mu_m}\frac{1}{C} + \frac{1}{\mu_m} \tag{4}$$

Combined equation (2) and (4) obtained,

$$\frac{t}{\ln(X)} = \frac{k_s}{\mu_m}\frac{1}{C} + \frac{1}{\mu_m} \tag{5}$$

Equation 4 is a linear model in which $\frac{k_s}{\mu_m}$ is the gradient of the curve of $\frac{1}{\ln(X)}$ against $\frac{1}{C}$ and $\frac{1}{\mu_m}$ is an intercept of the curve.

More so, the production rate of microbial mass is a function of the rate of consumption of pyrene. [16]

$$\frac{\partial X}{\partial t} = -Y_{x/s}\frac{\partial C}{\partial t} \tag{6}$$

Integration of equation 6 with the boundary conditions:

$C = C_0$, $X = X_0$ at $t = 0$ and

$C = C$, $X = X$ at $t = t$

$$X - X_0 = Y_{x/s}(C_0 - C) \tag{7}$$

C_0 and X_0 is the mass concentration of pyrene and biomass concentration respectively prior to commencement of degradation at time zero.

3. RESULTS AND DISCUSSION

Figure 1 depicts the standard plot of the mass concentration of pyrene against absorbance at 286nm and the standard model for the conversion of absorbance to mass concentration of pyrene obtained was given as equation 8. The correlation coefficients obtained using linear regression method was 0.999 and indicating the reliability of the model from experimental data.

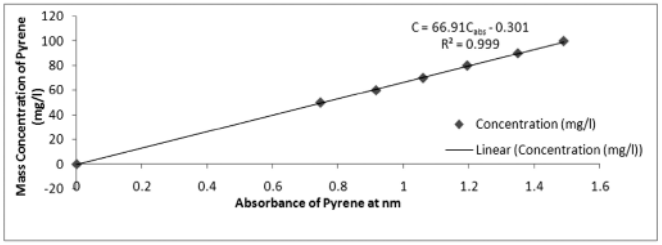

Figure 1: Mass Concentration of Pyrene (mg/1) Against Absorbance at 286nm

$$C = 66.91\text{Cabs} - 0.301 \tag{8}$$

C_{abs} is the absorbance of pyrene at any time.

Figure 2 depicts the degradation rate of pyrene using *Corynebacteria sp* and *Pseudomonas putida* while Figure 3 depicts the growth rate of Corynebacteria sp and Pseudomonas putida. The pyrene depleted to concentration of about 4.52mg/L and 9.40mg/L after 72hours of biodegradation time by the activity of *Pseudomonas putida* and *Corynebacteria sp* respectively. The concentration of *Corynebacteria sp* and *Pseudomonas putida* increased to 0.087mg/l and 0.094mg/L respectively after 72hours of incubation. This indicates that the rate formation of microbes increased with increased rate of degradation of the pyrene. Moreover, there was no significant change in the formation of microbes after 84hours of the biodegradation time which might be attributed to optimum condition of biodegradation of pyrene, formation of the toxic substance or death phase of the microbes as a result of shortage of pyrene as a food. This indicates that the rate formation of microbes increased with increased rate of degradation of the pyrene until when lowest concentration of the pyrene was reached and

biodegradation can no longer proceed. It can be deduced from Figure 2 and 3 that the increased rate of formation of microbes used resulted in the depletion of the pyrene. This indicates that the microbes used were able to utilized and degraded pyrene as a source of carbon and energy as reported by many researchers and stop when pyrene no longer sufficient for the microbes as a substrate. [15, 17]

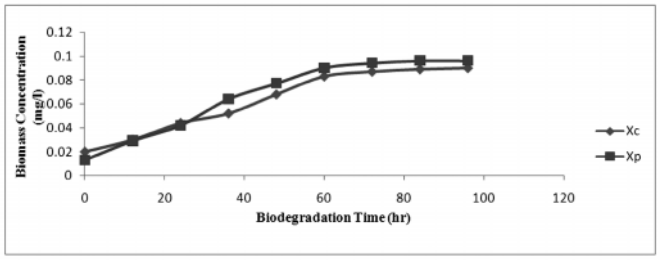

Figure 2: Biomass Concentration Against Biodegradation Time

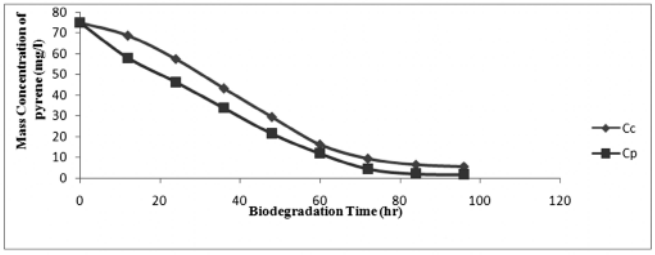

Figure 3: Mass Concentration of Pyrene Against Biodegradation Time

Figure 4 and 5 depicts the yield coefficient of *Corynebacteria sp* and *Pseudomonas putida* respectively and gave the same magnitude of 0.001 which indicated that the yield of microbes was independent of concentration of the substrate and the nature of the microbes but the fitness of the microbes for the bioremediation was evaluated using correlation coefficient (R^2) in which the correlation coefficient of 0.989 for *Corynebacteria sp* was less than that of *Pseudomonas putida* with a value of 0.991. This implies that both *Pseudomonas putida* and *Corynebacteria sp* were fits for biodegradation as pyrene in contaminated water due to high degree of correlation coefficient.

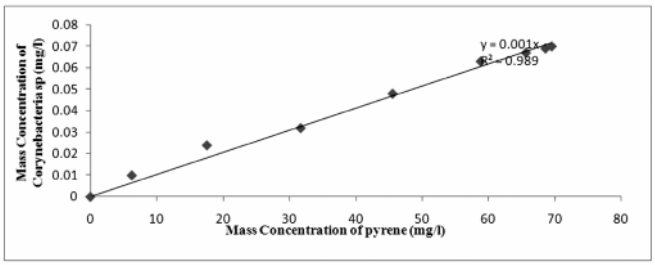

Figure 4: Evaluated Yield of Corynebacteria sp on Pyrene

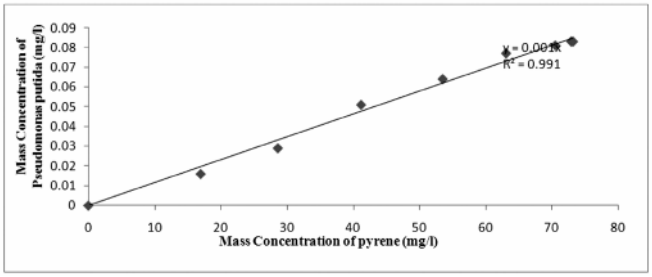

Figure 5: Evaluated Yield Pseudomonas Putida on Pyrene

The magnitude of μm and k_s obtained for *Corynebacteria sp* was 0.0441hr^{-1} and 0.1621mg/L and for Pseudomonas putida was 0.0512 hr^{-1} and 2.7320mg/L from Figure 6 and 7 respectively as shown in the Table 1. Because of higher value of μm of *Pseudomonas putida* was favourable for the condition of pyrene in the water than that of *Corynebacteria sp* while higher value of k_s depicts that the *Pseudomonas putida* has lower affinity compared with *Corynebacteria sp* which cannot be only parameter to determine effectiveness and efficient of the microbes.

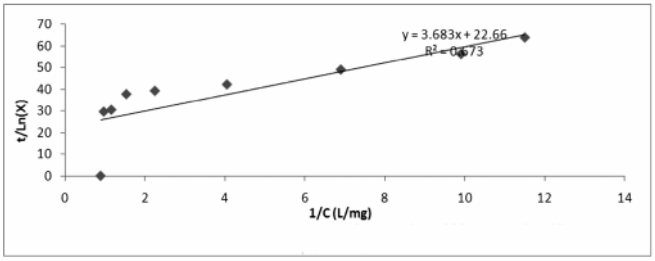

Figure 6: Simulation of Monod's Kinetics Constant k_s and Maximum Specific Growth Rate μ_m of Corynebacteria sp

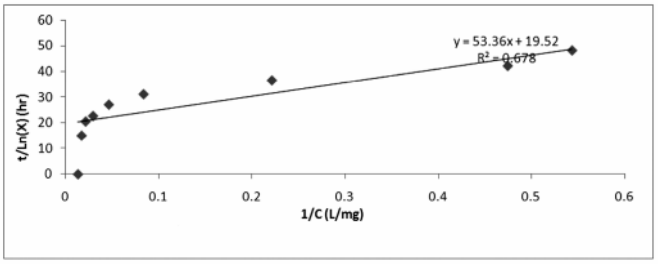

Figure 7: Simulation of Monod's Kinetics Constant k_s and Maximum Specific Growth Rate μ_m of Pseudomonas Putida

Table 1
Biodegradation Kinetics and Degradation Percentage of Pyrene

Microorganism	$Y_{x/s}$	μ_{max}	k_s (mg/L)	*Degradation Percentage (%)*
Corynebacteria sp	0.001	0.0441	0.1621	92.6
Pseudomonas putida	0.001	0.0512	2.7320	97.6

The value of R^2 obtained when employed the activity of *pseudomonas putida* was 0.678 and higher than when *corynebacteria sp* was used with a value of 0.673 but

statistically, there is no significant difference in the correlation coefficients and data obtained for both microbes were fit for bioremediation of pyrene in contaminated water.

Moreover, it was deduced that about 92.6 percent of pyrene was degraded by activity of *Corynebacteria sp* and 97.6 percent of pyrene was degraded by activity of *Pseudomonas putida* as shown in the Table 1. The experiment result shows that the two microbes used have higher degree of effectiveness of degradation of pyrene compared with other microbes as researchers reported *Bacillus subtilis* degraded about 40 percent of pyrene,[22] *Pseudomonas sp* degraded about 47-68 percent of pyrene,[15] degraded 70-79 percent of pyrene to CO_2 by the activity of *Mycobacterium spp*,[28] about 92.8 percent of pyrene was utilized by Mycobacterium spp with Fenton's solution,[12] and *Corynebacterium variabilis sp. Sh42* was mineralized about 70 percent of pyrene to carbon (VI) oxide and water.[15] This might be attributed to the susceptibility of the microbes to pyrene, kinetics and viability of the microbes, accessibility of contaminants and favourable environment for the microbes such as pH and light effect.

4. CONCLUSION

The experimental results shows that about 92.6w/w% of pyrene was utilized and degraded by the activity of *Corynebacteria sp* and *Pseudomonas putida* utilized and degraded about 97.6w/w of pyrene in water. The evaluated results of biodegradation kinetics parameters of pyrene using Pseudomonas putida gave higher degree of effectiveness compared with many microorganisms such *Corynebacteria sp* used in this research. This indicates that *Pseudomonas putida* may be preffered for biodegradation of pyrene as a bioremediation technique to liberate pyrene contaminated environment from danger that might be posed by pyrene.

Nomenclature

C	Mass concentration of pyrene (mg/l) at any time t
C_{abs}	Absorbance of pyrene at any time t
C	Mass concentration of pyrene (mg/l) at time $t = 0$
k_s	Monod's kinetics constant (mg/l)
t	Biodegradation time (hr)
$\frac{\partial X}{\partial t}$	Rate of formation of microbial mass ($mgL^{-1}hr^{-1}$)
X	Biomass concentration (mg/l) at any time t
X_0	Biomass concentration (mg/l) at time $t = 0$
μ	Specific growth rate of biomass (hr^{-1})
μ_m	Maximum specific growth rate of biomass (hr^{-1})

Subscript

c refers to corynebacteria *sp*

p refers to pseudomonas putida

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