Growth Patterns of Mixed Microbial Culture in the Biodegradation of Phenol in Petroleum Refinery Effluent

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ABSTRACT

Background/Aim: *Effective description of microbial growth patterns in the presence of organic contaminants is imperative in understanding the dynamics involved in the biodegradation of toxic materials from a microbiological standpoint. This study investigated the growth patterns of microbial culture involved in the biodegradation of phenol in refinery effluents.*

Materials and Methods: *Physicochemical characterization of the refinery effluent was performed using standard methods. The biodegradation experiment was carried out in a 3L Erlenmeyer's flask incubated in a rotary shaker under experimentally determined optimum cultural conditions, previously reported. Phenol concentration (mg/ml) was monitored daily throughout the experiment using spectrophotometric method. Phenol-utilizing bacterial and fungal counts were monitored using plate count method.*

Results: *Results obtained from the study revealed maximum phenol-utilizing bacterial (PUB) counts of $2.35\pm0.35x10^6$ and $9.5\pm0.42x10^6$ for T1 (control treatment) and T3 (experimental treatment), respectively. The respective maximum phenol-utilizing fungal (PUF) counts for T1 and T3 were $3.5\pm0.54x10^4$ and $3.05\pm0.07x10^3$. Growth patterns of PUB and PUF counts for the treatments were irregular throughout the monitoring period. However, a more consistent trend was observed for T3 than for T1 in PUB count while a contrary trend was observed in PUF counts. During the period under study, over 99% biodegradation of phenol was achieved after the first three days in the experimental treatment (T3). Cultural, biochemical, and microscopic characteristics of the microbial culture involved in the biodegradation revealed the presence of bacteria belonging to nine genera and fungi belonging to five genera. The dominant fungal genus was Aspergillus while Acinetobacter was the dominant bacterial genus.*

Discussion: *This study has demonstrated that the growth patterns of a mixed microbial culture involved in the biodegradation of organic pollutant did not follow a well-defined trend but however, complied with standard microbiological growth curve. This finding is important in designing bioremediation of refinery effluent using mixed microbial culture.*

Keywords: *Petroleum refinery effluent (PRE); biodegradation; phenol; microbial growth; mixed microbial culture.*

1.0 Introduction

Microorganisms respond differently to various ranges of organic substrates. The capacity of microorganisms to grow on a given substrate indicates their ability to utilize such substrates as source of food for their metabolic processes. As microorganisms breakdown organic pollutants, they tend to derive nutrients from the metabolic process. Organic and inorganic compounds vary in their susceptibility to microbial degradation as well as their support for microbial growth [1, 2].

Commonly found pollutants in refinery effluents include: phenol, polycyclic aromatic hydrocarbons, ammonia, chlorides, sulphides etc. There are several studies on the ability of microorganisms to degrade organic pollutants. According to World Bank Group [3] "refineries generate polluted wastewaters containing biological oxygen demand (BOD) and chemical oxygen demand (COD) levels of approximately 150 mg/L – 250 mg/L and 300 mg/L respectively; phenol levels of 20 – 200 mg/L; oil level of 100 – 300 mg/L in desalter water and up to 5,000 mg/L in tank bottoms; benzene levels of 1 – 100 mg/L; heavy metals of 0.1 – 100 mg/L for chromium and 0.2 – 10 mg/L for lead; as well as other pollutants." The different kinds of pollutants that exist in refinery wastewater can greatly affect amenability to microbial degradation.

The existence of different categories of pollutants in refinery effluents means that microorganisms that are able to attack the pollutants must be as well diverse in their metabolic characteristics. Different microorganisms have been reported to have different growth requirements. These differences in growth requirements mean that in a complex system like a petroleum refinery effluent, the microorganisms will grow at different rates and densities. This will be different from a single pure culture in which the microorganism has defined characteristics that ensure a more regular growth pattern. Lacasta *et al*. [4], reported a spatio-temporal pattern in bacterial colonies growing on a medium amended with an organic substance. Because of this observed difference in organic substance susceptibility to microbial biotransformation, studying the growth patterns of mixed microbial cultures in biodegradation of organic pollutants have become even more challenging. This study was aimed at investigating growth patterns of mixed microbial culture in the biodegradation of phenol in petroleum refinery effluent (PRE).

2. MATERIALS AND METHODS

2.1 Sample collection and physicochemical analyses

Samples were collected using the method described by APHA, AWWA, and WPCF [5]. Samples for microbiological examination were collected in a non-reactive borosilicate glass bottles that have been cleansed and sterilized (in a thermostatically regulated oven at 160 ^{o}C for 1hr). Representative portions of effluent samples were collected from the biodisks with the sterile containers. In collecting the effluent samples, air space was left in the bottle to facilitate mixing by shaking and aseptic techniques were adopted to avoid sample contamination. The samples were labelled for proper identification and transported to the laboratory in an ice pack for further analyses. Physicochemical characteristics of the effluent sample were analysed using standard methods.

2.2 Isolation and enumeration of phenol-utilizing microorganisms in the biodegradation of phenol in PRE

Mineral salt medium reported by Hill and Robinson [6] was prepared for the isolation and enumeration of phenol-degrading bacteria and fungi with the following components in mg/L of deionized water: Phenol, 235; KH_2PO_4, 420; K_2HPO_4, 375; $(NH_4)_2SO_4$, 244; NaCl, 30; $CaCl_2$, 30; $MgSO_4$, 30; and $FeCl_2$, 3. For the isolation and enumeration of bacteria, 0.01% w/v nystatin was added while the medium for fungi was amended with 0.1% w/v

chloramphenicol. The number of phenol-utilizing bacteria and fungi was determined by counting the colonies on the respective bacterial and fungal agar plates bearing colonies between 30 and 300.

2.3 Phenol Determination

Phenol concentration in the biodegradation set-up was monitored daily using the direct photometric method described by American Society for Testing and Materials (ASTM D1783-01). Phenol standard curve was prepared and the phenol concentration in the sample determined by comparing the absorbance reading with the standard curve.

2.4 Statistical Analysis and Modelling of Phenol Degradation in the Refinery Effluent

The data obtained from this study were compared using one-way analysis of variance (one-way ANOVA) and multiple range tests to find the differences between the measurement means at 5% ($p<0.05$) significance level. The analyses were performed using IBM® SPSS® Statistics Version 20.0 (Gailly and Adler, US). Mathematical modelling of phenol degradation was performed through a non-linear regression method using Microsoft Excel 2010. The equation for the graph as well as coefficient of determination (R^2) (also known as the Goodness of Fit) were derived. R^2 is a measure of how well the derived model fits the experimental data.

3. RESULTS

3.1 Physicochemical characteristics of refinery effluents

The physicochemical characteristics of the refinery effluent used in this study have been previously reported by Agu *et al*. [7]. The result revealed that the refinery effluent was contaminated with hydrocarbons and phenol.

3.2 Identification of phenol-degrading bacteria and fungi involved in the bioremediation of the PRE using cultural, biochemical characteristics

Twelve (12) bacteria belonging to nine different genera were identified based on their phenotypic and biochemical characteristics. The phenol-degrading bacteria identified included: *Acinetobacter* sp., *Pseudomonas putida, Pseudomonas* sp., *Burkholderia* sp., *Xanthomonas* sp., *Azotobacter* sp., *Enterobacter cloacae*, *Streptomyces* sp., *Serratia* sp., *Xanthomonas* sp., *Campylobacter jejuni*, and *C. lari*. Almost all the bacterial isolates identified through biochemical characteristics were Gram negative organisms with the exception of the Gram positive bacterium, *Azotobacter*.

The following phenol-degrading fungi namely: *Aspergillus flavus, Aspergillus sydowii, Cladosporium tenuissimum, Aspergillus japonicas, Trichosporon montevideense, Phanerochaete sordida,* and *Monacrosporium eudermatum* were isolated and identified from the refinery wastewater. The fungi belonged to five (5) different genera with the genus *Aspergillus*, dominating.

3.3 Phenol-utilizing bacterial (PUB) and phenol-utilizing fungal (PUF) counts

Figures 1 and 2 show the results obtained from monitoring PUB and PUF counts during the biodegradation study. The results revealed that TI, and T3 recorded their highest PUB count as $2.35\pm0.35\text{x}10^6$, and $1.61\pm0.75\text{x}10^7$on day 1, respectively. In terms of trend, the PUB counts for both treatments fluctuated throughout the monitoring period. T1 recorded its peak on day 1 after which the value declined although not in a sequential order. The same trend observed for T1 was recorded for T3.

For PUF, T1 and T3 recorded highest values of $3.5\pm3.54x10^4$, and $1.0\pm0.00x10^4$, respectively at day 3 for T1 and day 1 for T3. The PUF for the treatments fluctuated throughout the monitoring period. However, T1 followed a more consistent trend throughout the period under investigation.

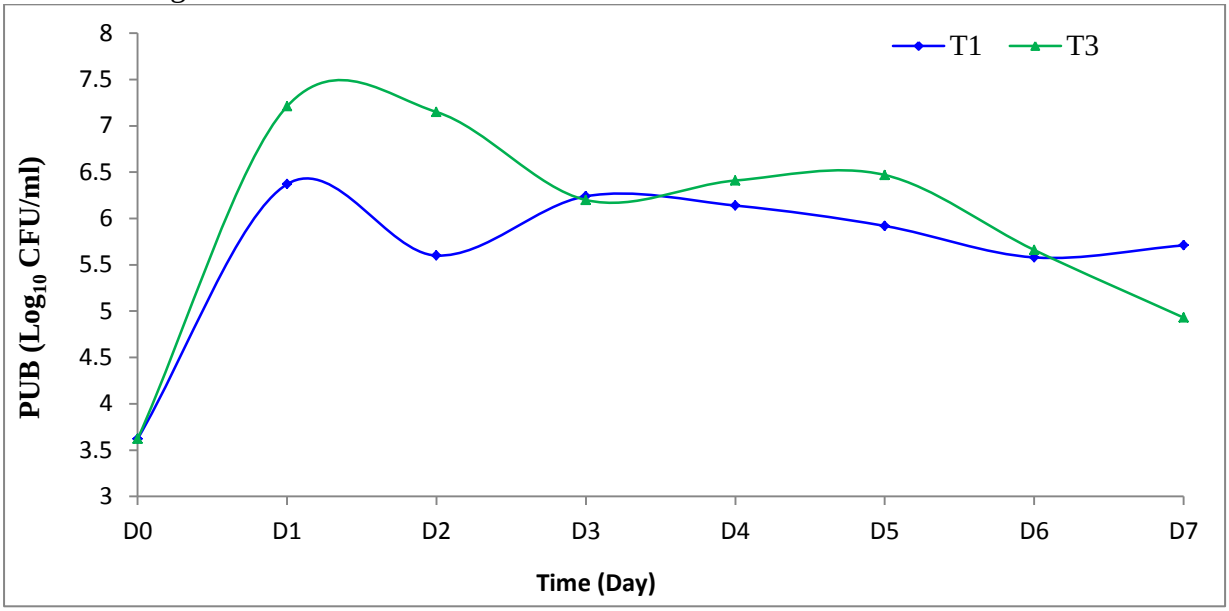

Figure 1: PUB count during the biodegradation study. (T1- control (i.e.PRE only); T3: PRE, optimized nutrients and inoculum; PUB: Phenol-Utilizing Bacteria).

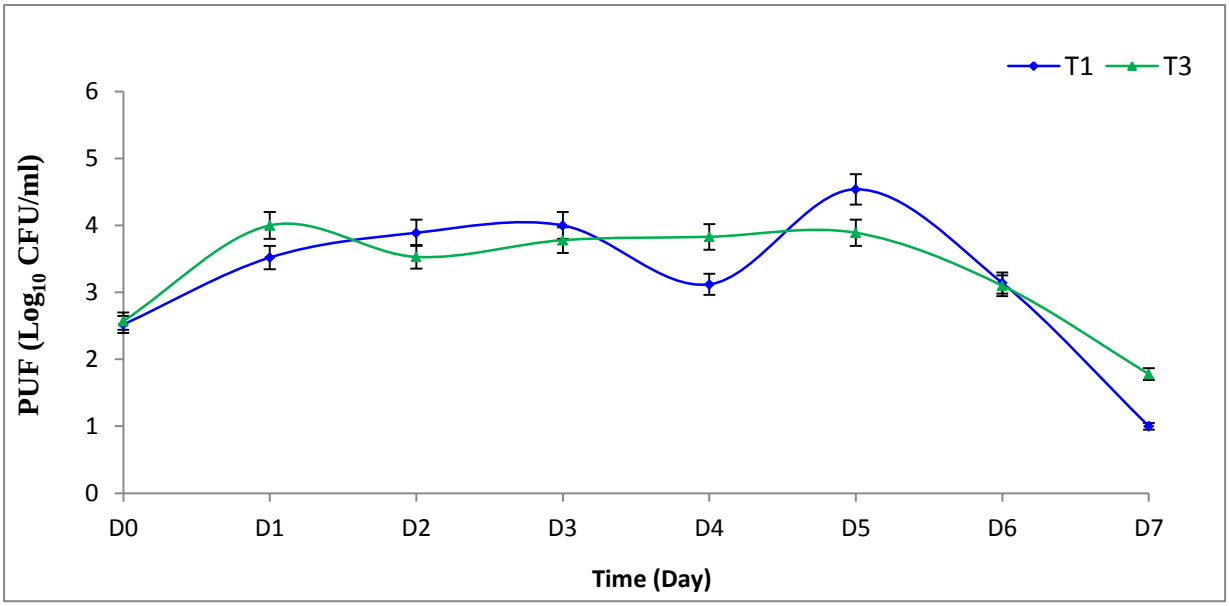

Figure 2: PUF count during the biodegradation study. (T1- control (i.e. PRE only) T3: PRE, optimized nutrients and microbial inoculum; PUF: Phenol-Utilizing Fungi).

3.4 Modelling of phenol degradations in PRE using the optimized medium

Phenol degradation in PRE was monitored for a period of seven (7) days using the optimized medium. Mathematical modelling of phenol degradation through non-linear regression method revealed that phenol content degradation for the experimental treatment (T3) followed an exponential pattern (Figure 3). The derived phenol degradation model is given by the equation $y = 84.998e^{-2.302x}$. The coefficient of determination R^2 (also known as the Goodness of Fit), a measure of how well the derived model fits the experimental data, had a

value of 0.961 (96 %). The R^2 value can be interpreted as the proportion of the variance in phenol content attributable to the variance in time.

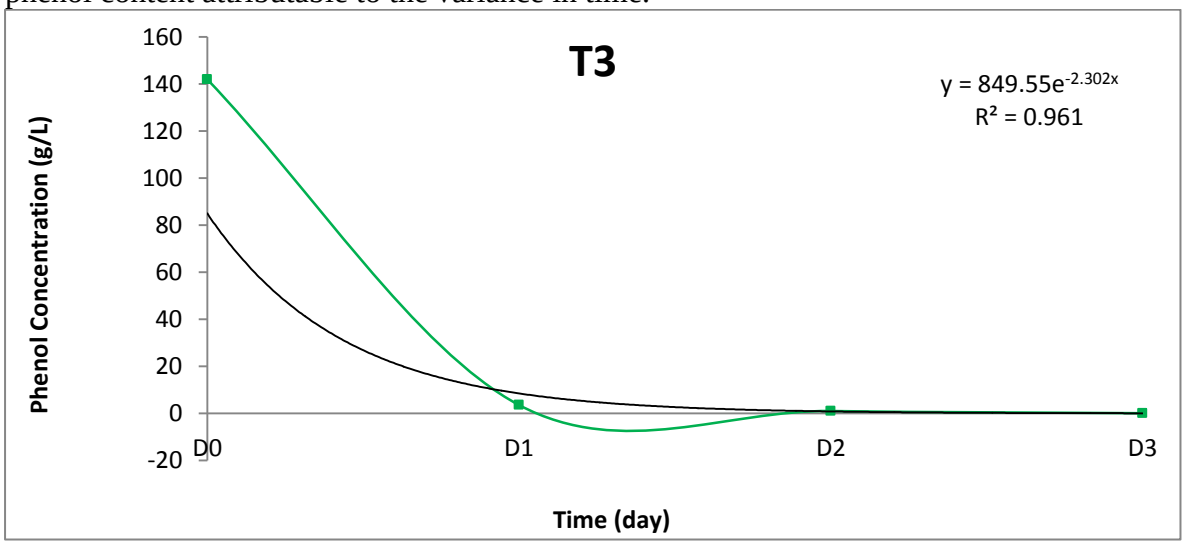

Figure 3: Model of phenol degradation over time. (T3: PRE, optimized nutrients and inoculum; PUB: Phenol-Utilizing Bacteria).

4. Discussion

Analyses of physicochemical parameters in the biodegradation experiment revealed that the PRE was contaminated with phenol and other organic pollutants. Agu *et* al. [7] reported that, "regulatory discharge limit for PRE as stipulated by EGASPIN is 0.5 mg/L". According to Hou *et al*. [8] and Poulopoulos *et al*. [9], discharge of wastewater from refinery into the environment without compliance with regulatory limits can: affect drinking water and groundwater resources, endanger aquatic lives and the health of human beings; cause pollution of the atmosphere; limit optimal crop production; and lead to general land degradation.

Biodegradation of organic pollutants in the PRE was carried out in this study using optimized parameters (pH, 8; temperature, 35; 5% inoculum from the PRE; micronutrient and macronutrient combination ($CoSO_4$+$MnSO_4$+NPK); and incubation, performed at 100 rpm in a rotary shaker incubator).

The biodegradation set-up was monitored for changes in microbial counts. The monitored microbial counts included: phenol utilizing bacteria (PUB) and phenol-utilizing fungi (PUF). The results showed fluctuation in the different counts throughout the biodegradation period monitored. The fluctuation observed in the biodegradation set-up could be as a result of the mixed microbial population involved in the bioremediation process. The existence of diverse microbial population in the system can explain the observed fluctuation in the system. This is because the microorganisms have different growth rates, nutritional and cultural requirements. The degradation of phenol by mixed culture of bacteria has been reported. Jame *et al*. [10] reported the degradation of phenol by mixed cultre of locally isolated *Pseudomonas* spp. Sethilvelan *et al*. [11] also reported that the mixed microbial culture employed in the biodegradation of phenol was better compared to when the individual culture was used for phenol degradation. Farrell and Quilty [12] examined the biodegradation of mono-chlorophenols in aerobic batch cultures using mixed microbial community, specially designed to degrade a wide range of substituted aromatic compounds. Biodegradation of phenol by mixed microbial culture has also been reported by Prpich and Daugulis [13].

The irregular growth pattern observed in the biodegradation experiment could be attributed to the complex nature of the organic contaminants and the diverse nature of the microbial

population. These claims are buttressed by the resluts of the physicochemical characteristics of the effluent sample and the diversity of microbial species recovered from the effluent.

Twelve phenol-degrading bacteria and seven phenol-degrading fungi were identified based on phenotypic, biochemical and microscopic characteristics [7]. Other studies [11,14-15,] have reported the biodegradation of phenol by bacterial strains isolated from wastewater. These bacteria: *Acinetobacter junii*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Burkholderia* sp., *Xanthomonas* sp., *Azotobacter chroococcum*, *Enterobacter cloacae*, *Streptomyces* sp. *Serratia marcescens*, *Xanthomonas sacchari*, *Campylobacter jejuni*, and *Campylobacter lari* have been implicated in phenol degradation. Liu *et al*. [16] reported the biodegradation of phenol by *Acinetobacter calcoaceticus* PA isolated from phenolic wastewater. The genus *Acinetobacter* was dominant in the PRE treatment system. This bacterial genus has been widely implicated in phenol degradation. Therefore their involvement in the biodegradation of organic pollutants (phenol, TPHs, PAHs etc.,) is not surprising. Similarly, Zhang *et al*. [17], identified amongst cultured bacteria, *Pseudomonas* sp., in addition to *Bacillus subtilis* and *Nitrospira* sp.. Gu *et al*. [18] identified the phenol-degrading bacterium *Campylobacter* sp. as well as other bacterial strains different from the ones identified in this study such as *Niastella* sp., *Deinococcus* sp., *Delftia* sp., *Achromobacter* sp., and *Agrobacterium* sp., from drinking water biofilters. Krastanov *et al*. [19] reported that *Pseudomonas* spp. and *Acinetobacter* spp. are some of the most widely implicated phenol-degrading bacteria; a result which is consistent with the findings of this present study.

Juárez *et al*. [20] reported that *Azotobacter chroococcum* can grow up using polyphenolic compounds as an individual source of carbon and energy supply. They studied the degradation of simple phenolic compounds by this strain using a gas chromatography coupled mass spectrometry method. Other studies have reported the ability of *Serratia marcescens* [21], *Streptomyces* sp. [22], *Burkholderia* sp. [23], *Xanthomonas* sp. [24], and *Enterobacter cloacae* [25] to bio-remediate phenolic wastewater.

This study through microscopic and cultural characteristics of the fungal isolates revealed the presence of *Aspergillus flavus*, *Aspergillus sydowii*, *Cladosporium tenuissimum*, *Aspergillus japonicas*, *Trichosporon montevideense*, *Phanerochaete sordida*, and *Monacrosporium eudermatum* in the refinery wastewater. The capacity of these fungal isolates to grow in the presence of phenol is supported by other studies. *Trichosporon* spp. are one the most widely reported phenol-degrading fungi [19]. The identification of *Aspergillus* spp. in the PRE is consistent with another study Supriya and Neehar [26]; which implicated *Aspergillus* sp. in the degradation of phenol in wastewater.

Conclusion

This study has demonstrated that the growth pattern of mixed microbial culture involved in biodegradation of organic pollutants did not follow a well-defined trend. The study revealed that the mixed microbial culture however, efficiently degraded phenol content in the refinery wastewater; an indication of the ability to grow in the presence of the organic substrates.

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Author's Contribution

This work is the collaborative effort of all the authors. Author IVA designed the study under the guidance of author GCO, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches. Authors IVA, AAI and GCO managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Competing Interests

Authors have declared that no competing interests exist.

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