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In Vitro Biodegradation of Palm Oil Mill Effluent (POME) by *Bacillus subtilis, Pseudomonas aeruginosa* and *Aspergillus niger*

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Abstract

In vitro Comparison of Palm Oil Mill Effluent (POME) degradation was carried out using *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Aspergillus niger* over a period of 16 days using POME as carbon source. pH, nitrate, phosphate, turbidity, and bacterial counts were carried out at intervals of 0, 4, 8, 12, and 16 days to assess the progress of degradation. There was appreciable increase in pH and turbidity in medium containing the organisms while phosphate and nitrate decreased with time. Colony counts showed a decline after day 8 in all organisms. The counts in *P. aeruginosa* and *B. subtilis* were higher than that of *A. niger* throughout the period of study. There were no significant differences ($p>0.05$) in the pH and nitrate while significant differences were observed in the turbidity, phosphate and colony counts at 5% probability level. Gas Chromatography Mass Spectrophotometric (GCMS) analysis carried out on days 0, 7, and 14 for *Pseudomonas aeruginosa* and *Aspergillus niger* showed decrease in peaks after day 7 while samples containing *B. subtilis* showed no reduction in peak numbers rather new compounds were formed in the process. At the end of the study, it was deduced that *P. aeruginosa* was able to efficiently utilize POME as a carbon source for growth better than *Aspergillus niger* and *Bacillus subtilis* and could play a great role in large scale treatment of palm oil effluent.

Keywords: POME; *In vitro*; *Aspergillus niger*; *Bacillus subtilis*; *Pseudomonas aeruginosa*

Introduction

Biodegradation is the use of microorganisms to clear up pollutions or contaminations [1]. These organisms are able to do this due to their ability to utilize certain compounds of the contaminant as nutrient source resulting in by-products of microbial metabolism e.g., carbon dioxide as well as an increase in cell mass [1]. Microorganisms gain energy by catalysing energy producing chemical reactions that involves breaking bonds and transferring electron away from the contaminants. In this type of reaction, the organic contaminant is oxidized while the chemical that gains the electron is reduced. The energy gained from this transfer is then invested in growth and metabolism of the biodegrading microbe. Aerobic organisms use oxygen as their electron acceptor while anaerobes use nitrates, sulphates, or even carbon dioxide as their own electron acceptor and in addition to new cell mass, by products formed here may include; nitrogen gas, hydrogen sulphide, reduced forms of metals, and methane depending on the electron acceptor [2]. Petroleum hydrocarbon, gasoline, fuel, oil, alcohol, ketones, and esters have been successfully biodegraded at contaminated sites [1]. Organisms that have been successfully reported to be capable of degrading these hydrocarbons include *Pseudomonas aeruginosa, Bacillus subtilis*, *Alcaligenes, Acinetobacter iwoffi*, *Flavobacterium* sp., *Aspergillus* sp., and *Rhizopus* [3,4].

Palm oil mill effluent (POME) is a waste generated during the process of palm oil production. During the production process of palm oil, about 50% of the water results in palm oil mill effluent and it is estimated that for every tonne of crude oil produced, 5.0 to 7.5 tons of water ends up as POME [5-7]. The effluent is an acidic, brownish, and colloidal suspension with 95-96% of water, 0.6-0.7% of oil and 2-4% of total suspended solid (TSS). This can cause considerable environmental problems if discharged without effective treatment by polluting water and destroying aquatic biota [8]. In addition, untreated POME contains high amount of fatty acid, proteins, carbohydrates, and other plant materials which have the tendency of altering environmental parameters, particularly, BOD, dissolved oxygen, carbon/nitrogen ratio, as well as chemical oxygen demand (COD). Okwute and Isu, Sridhar and Adeoluw, and Awotoye et al. [6,9,10] are all of the opinion that POME can cause pollution of water ways due to oxygen depletion, land use and other related effects.

Over the past decades, several economically viable technological solutions have been utilized for the treatment of palm oil mill effluent including biological processes and other specialized treatments [11] Owing to the increasing amount of POME generated, disposal remains a challenge and as such bioconversion has been considered as an alternative for pollution control [12-18]. Bioconversion process is achieved by using this rich organic residue as a medium where some microbial species grow, consume the organic components, and at the same time, produce biomass and other valuable products. The industrial treatment of POME is aimed at reducing the amount of potentially toxic compounds and environmental contaminants in POME to their acceptable threshold limit value (TLV), according to some standards of the Federal Environmental Protection Agency, FEPA [19] by introducing microorganisms that are capable of using the effluent as a nutrients source, and in the process, degrading the palm oil mill effluent into less toxic forms.

This study is aimed at comparing the biodegradative ability of *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Aspergillus niger* on the effluent and to ascertain which one of these three organisms is most efficient for the purpose and can be adapted for large scale /industrial biodegradation of POME at a lesser cost. This is due to the fact that the three organisms do not require complex growth media, and are able to grow on oily waste.

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Materials and Methods

Study area

This study was carried out at the Microbiology laboratory of the National Institute of Pharmaceutical Research Development (NIPRD), Idu, FCT-Abuja, Nigeria.

Sample collection

The POME sample was obtained from a local processing plant in Ankpa Local Government Area of Kogi State into sterile containers with sterile spoons using sterile hand gloves and then transported to the Microbiology laboratory of the National Institute of Pharmaceutical Research Development (NIPRD), Idu, FCT-Abuja, Nigeria.

Collection and identification of pure isolates

Pure isolates of *Aspergillus niger* was obtained from stock culture in University of Abuja Microbiology Laboratory while *Pseudomonas aeruginosa* and *Bacillus subtilis* were collected from the Microbiology laboratory of the National Institute of Pharmaceutical Research Development (NIPRD), Idu, FCT-Abuja, Nigeria and were subjected to further identifications.

Determination of physicochemical characteristics of POME

Growth pattern of the organisms were determined by measuring the turbidity using turbidity meter (WGZ-113 Shanghai, China) at 520 nm. pH was determined at ambient temperature using glass electrode pH and conductivity meter (Hannia, Italy). Phosphorus was determined using the method described by Lasslo [14]. Nitrate was determined using the modification adapted by APHA [15].

Inoculation of POME with microbial isolates

A modified Zajic and Suplisson medium [16] consisting of palm oil mill effluent and various inorganic salts were dissolved in 1000 mL of distilled water (Mineral Salt Medium containing 2.0 g of Na_2HPO_4, 0.17 g of K_2SO_4, 4.0 g of NH_4NO_3, 0.53 g of KH_2PO_4, 0.10 g of $MgSO_4$. $7H_2O$ was prepared in 1000 mL of distilled water. 10 mL of Mineral Salt Medium was dispensed into forty test tubes each. 2 mL of POME was added into each test tubes and the solution sterilized by autoclaving. Two millimetres (2 mL) of 3 hours broth culture (peptone broth) of *Bacillus subtilis, Pseudomonas aeruginosa* and *Aspergillus niger* were seeded into ten test tubes each while the last ten test tubes containing the medium without the bacteria and fungus served as the control. *Bacillus subtilis* and *Pseudomonas aeruginosa* were incubated at 37 ± 2°C for 16 days while the test tubes containing *Aspergillus niger* together with controls were incubated at ambient temperature, All the experiments were carried out in duplicates.

Determination of total viable count

Total viable count of the inoculated organisms was carried out using nutrient agar at 4 days interval for the bacteria while Sabouraud Glucose agar (SGA) with chloramphenicol was used for *Aspergillus niger*. The POME+medium+organisms samples were serially diluted in 10 folds (that is for the two organisms). The plates were incubated at 37°C for 18-24 hours. After incubation, the total viable counts were determined.

Gas Chromatographic-Mass Spectrophotometric analysis

The Gas Chromatographic-Mass Spectrophotometric analysis was carried out at day 0 (control), day 7 and day 14 for POME inoculated separately with *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Aspergillus niger* as described by Stephen et al. [17].

Statistical analysis

The degradation ability was tabulated and the statistical analysis was determined using Analysis of Variance (ANOVA) using SPSS Software version 19.0.

Results

Figure 1 shows the turbidity during biodegradation of POME. There was a gradual increase in the turbidity after four days in *A. niger* and *B. subtilis* compared to *P. aeruginosa* medium. However, the turbidity in the *B. subtilis* medium decline after eight days till day sixteen compared to *A. niger* and *P. aeruginosa* media. The highest turbidity was observed in the *A. niger* medium at day 16. There was significant difference ($p>0.05$) in the turbidity produced by the three organisms.

Figure 2 shows the level of phosphate utilized during biodegradation of POME. The phosphate concentration declined with time in the *A. niger* and *P. aeruginosa* media compared to *B. subtilis* medium. The highest phosphate utilization was observed in the *A. niger* followed by *P. aeruginosa* medium. There were significant differences ($p>0.05$) in the phosphate level of the three medium and the control.

Figure 3 shows the changes in nitrate concentration during biodegradation of POME by the three organisms. The nitrate concentration was fairly stable within the first four days. Notable nitrate utilization was observed after eight days which continued till the end of the study. The highest nitrate utilization was observed in *A. niger* followed by *P. aeruginosa* medium compared to *B. subtilis* medium. The highest level of nitrate utilization was observed in *A. niger* medium. There was no significant difference ($p>0.05$) in nitrate concentration of the three medium.

Figure 4 shows the result of the total viable counts of the microbial isolates during biodegradation. The *A. niger* population increased gradually from day zero reaching its peak after twelve days and then declined after sixteen days. *B. subtilis* counts were higher than that of *P. aeruginosa* throughout the period of study. There were significant differences (<0.05) in the colony counts of the three organisms.

Figure 5 shows the Gas Chromatography Mass Spectrophotometric analysis of uninoculated POME (Control) at day 0. The chromatogram showed that 3,3-Dimethyl-2-hexanone (5.58), pentadecanoic acid

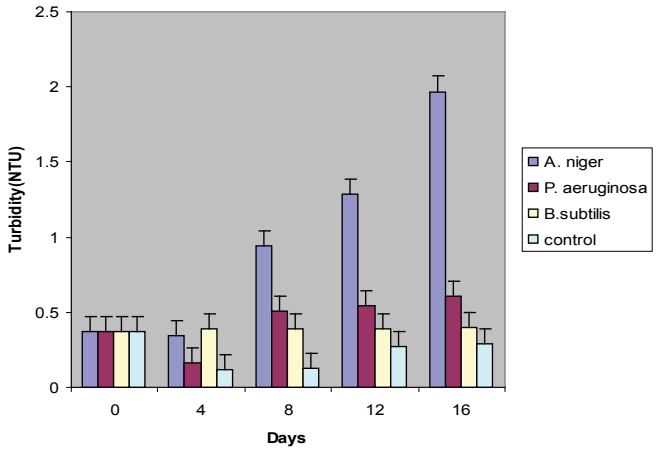

Figure 1: Turbidity of POME during biodegradation.

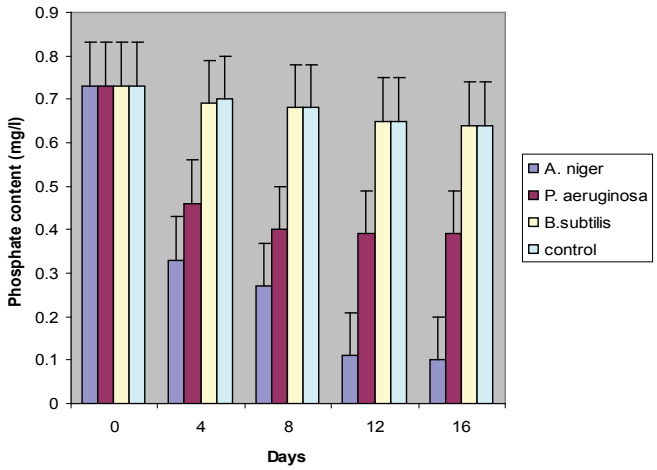

Figure 2: Phosphate concentration during biodegradation of POME.

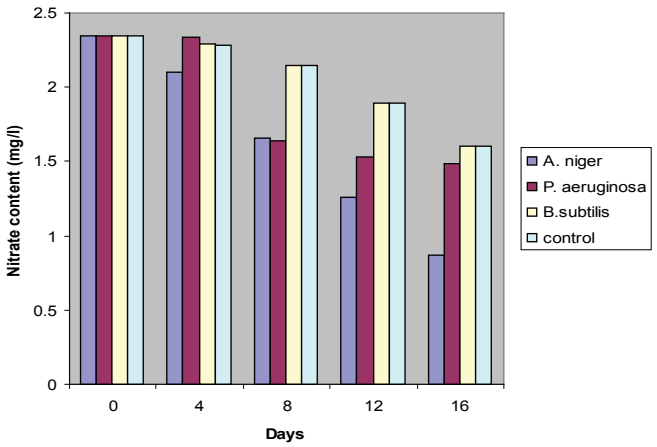

Figure 3: Levels of Nitrate utilization during biodegradation of POME.

(2.20), hexadecanoic acid (2.29), oleic acid (4.65) and 9-octadecanol (2.29) compounds were present in the raw POME.

Figures 6 and 7 show the GCMS analysis of POME degraded by *Pseudomonas aeruginosa* at days 7 and 14 respectively. The compounds present at days 7 and 14 were hexadecanoic acids (3.97 and 4.23) and oleic acid (4.82 and 4.54). 3,3- Dimethyl-2- hexanone was degraded to hexanoic acid by *Pseudomonas aeruginosa* after 7 days.

Figures 8 and 9 are chromatograms of POME degraded by *B. subtilis* at days 7 and 14. The compounds present at day 7 were hexadecanoic acid (2.38), 9-octadecanoic acid (2.68), ethyldocosonate (2.72) and Nonadecanoic acid (4.55) while at day 14, in addition to hexadecanoic acid (2.32) and oleic acid (3.96), pentadecanoic acid (2.76), methyl-11-octadecanoate (2.62), methylheptacosonate (2.26) were also present.

Figures 10 and 11 shows the chromatograms of POME degraded by *A. niger* at days 7 and 14. There were more compounds in the POME after seven days of degradation. Tridecane (2.07) and 2-methylnonadecane (2.58) were observed in the chromatogram after seven days of degradation. However, after 14 days, the peaks were lower in numbers compared to the raw POME at day zero. The compounds 3,3-dimethyl-2-hexanone (5.60), oleic acid (3.57) and hexadecanoic acid (2.20) were not degraded after fourteen days.

Table 1 depicts the change in pH in the POME during the study. The pH in each sample increased with time. The pH ranged from 3.9 ± 0.20 to 7.53 ± 0.20. The highest pH value for each organism was observed at day 16. There was no significant difference ($p>0.05$) in the pH of the control, *A. niger, P. aeruginosa* medium and *B. subtilis* medium.

Discussion

The turbidity increased with time. Turbidity was higher in the samples inoculated with *P. aeruginosa* than *B. subtilis* and the control. This indicates that the inoculated samples were able to grow and utilize the POME as carbon source. The reason for the increase in turbidity after 4 days till the end of the study may have been due to the presence of nitrogen and phosphorus in the Mineral Salt media which are necessary for biodegradative activity [18] and also played a role in overcoming nutrient limitation during the biodegradative process.

The phosphate decreased from day 0 to day 16. The phosphate concentration was lower in the samples inoculated with the microbial isolates than control. Phosphate content was lower in *A. niger* and *P. aeruginosa* than *B. subtilis*. This implied that both *A. niger* and *P. aeruginosa* utilized phosphate more than *B. subtilis* during the biodegradation process. This may be as a result of their ability to solubilise phosphorus [19]. This result is in agreement with the observation of Panapanaan et al. [20]. The phosphate concentration of 0.91 – 0.725 was within the FEPA [13] effluent limitation guideline of 5 mg/L.

Nitrate content also decreased during biodegradation from day 0 to day 16 in all samples. The decline in the concentration of nitrate between days 0 to 16 could be attributed to high metabolic activity and utilization of nitrate during the biodegradation process by the microbial isolates [21]. Nitrate content was lower in *A. niger* than *P. aeruginosa* and *B. subtilis* and the control. This observation may be due

Days	*P. aeruginosa*	*B. subtilis*	*A. niger*	Control
0	3.9 ± 0.20[a]	3.9 ± 0.20[a]	3.9 ± 0.20[a]	3.9 ± 0.20[a]
4	5.8 ± 0.02[a]	6.0 ± 0.01[a]	5.73.9 ± 0.0[a]	4.8 ± 0.05[a]
8	6.1 ± 0.06[a]	6.1 ± 0.05[a]	6.33.9 ± 0.0[a]	6.1 ± 0.12[a]
12	6.6 ± 0.04[a]	6.6 ± 0.02[a]	6.73.9 ± 0.20[a]	6.6 ± 0.00[a]
16	6.7 ± 0.07[a]	6.9 ± 0.03[a]	7.53 ± 0.20[a]	6.4 ± 0.04[a]

[a]Superscript with same alphabets on each column are not significantly different

Table 1: Changes in pH during biodegradation of POME.

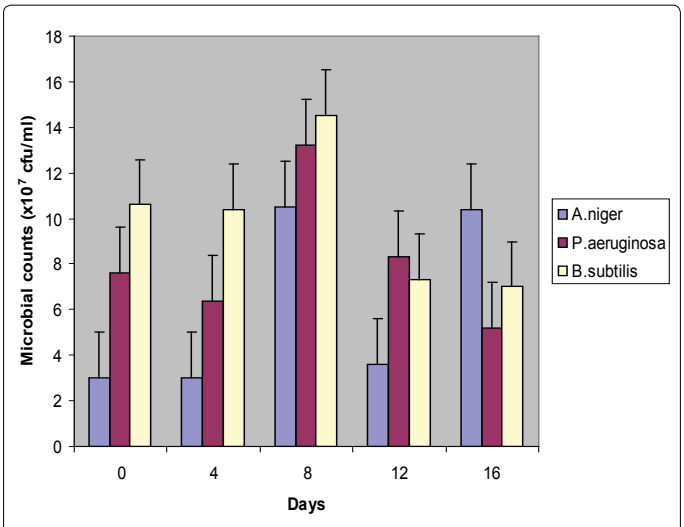

Figure 4: Microbial population during biodegradation of POME.

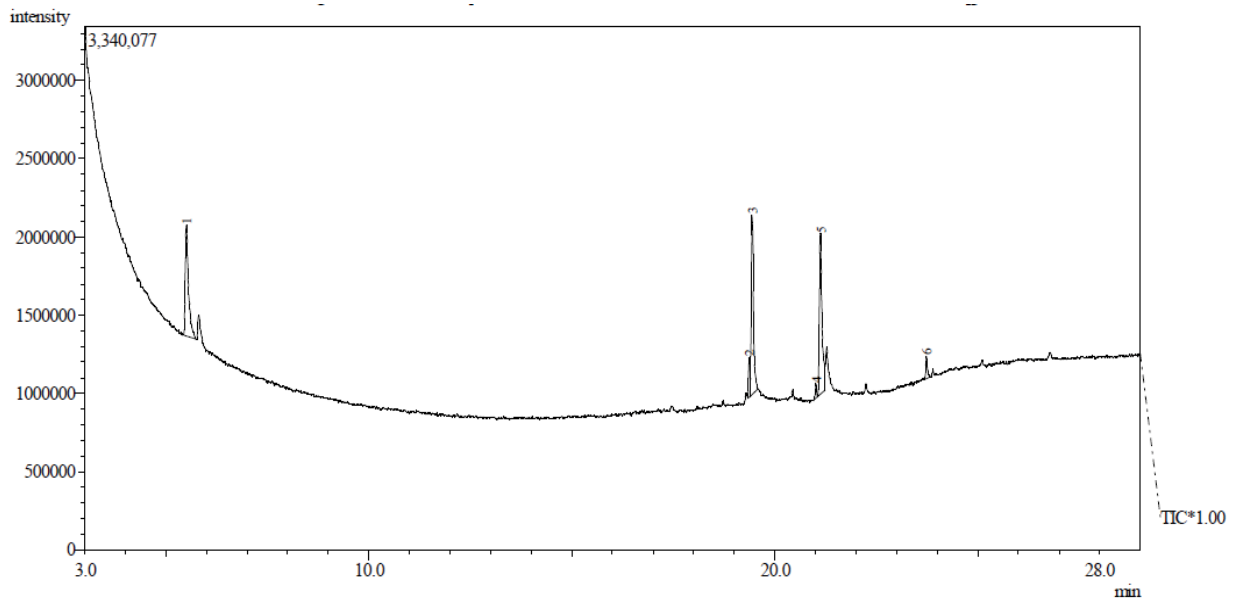

Figure 5: Gas chromatogram of POME at day 0 (control).

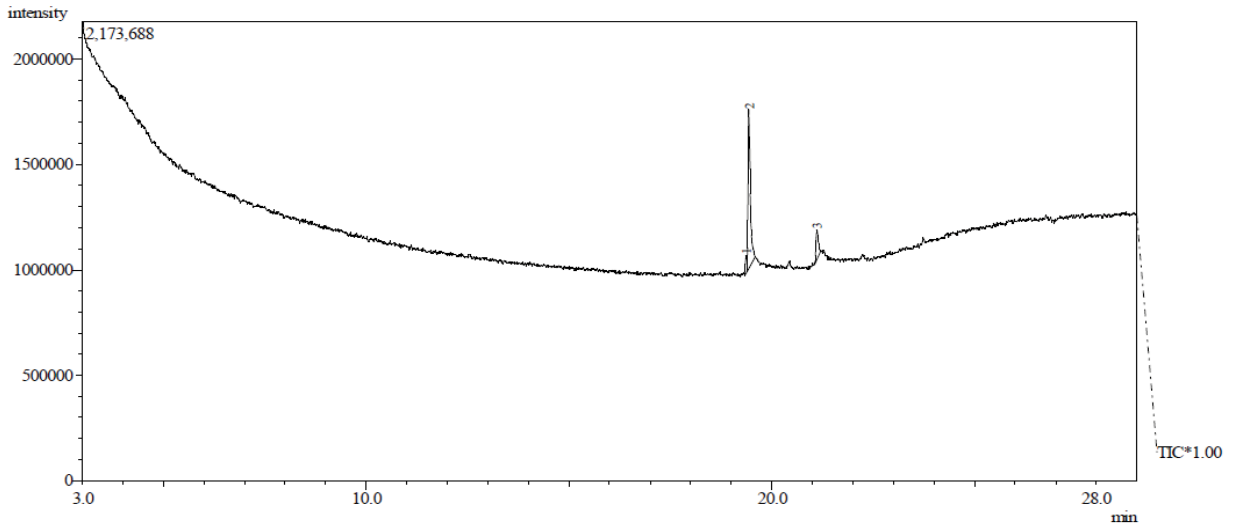

Figure 6: Gas Chromatogram of POME degraded by *Pseudomonas aeruginosa* at day 7.

to ease of utilization of nitrate by *A. niger* and *P. aeruginosa* than *B. subtilis*. The nitrate concentration of 0.086-2.356 at the end of the study was within the Federal Environmental Protection Agency, FEPA [13] effluent limitation guideline of 20 mg/L.

The pH increased from day 0 to day 16 in all samples. The pH of the inoculated samples was higher than the control. *A. niger* had the highest pH followed by *B. subtilis, P. aeruginosa* and the control. The pH range of 5.8-7.53 in this study fell within the range reported by Ma [22]. This researcher reported that the pH of POME ranged from 4.0-9.0. The higher pH observed in this study maybe due to metabolism of POME by the various organisms. Shamshuddin et al. [23] reported that when raw POME is discharged, the pH is acidic but seems to gradually increase to alkaline as biodegradation takes place. This progression to alkaline state is in line with reports of other researchers [24,25]. The pH recorded in this study however, was within FEPA [13] effluent limitation guideline of 6-9.

The viable count showed gradual increase from day 0 to 8 and then decreased as the day progressed by. The increase in the first 8 days showed that the organisms were able to utilize the substances present in the POME but the reduction as the days progressed was a result of the complete degradation of POME. This is in agreement with earlier work by Okwute et al. [26]. They reported that the reduction with time may be due to nutrient limitation with time.

The GCMS analysis of raw POME showed that oleic acid which are monosaturated omega-9 fatty acid, pentadecanoic acid, hexadecanoic acid (palmitic acid) and octadecanoic acid (stearic acid) were the main

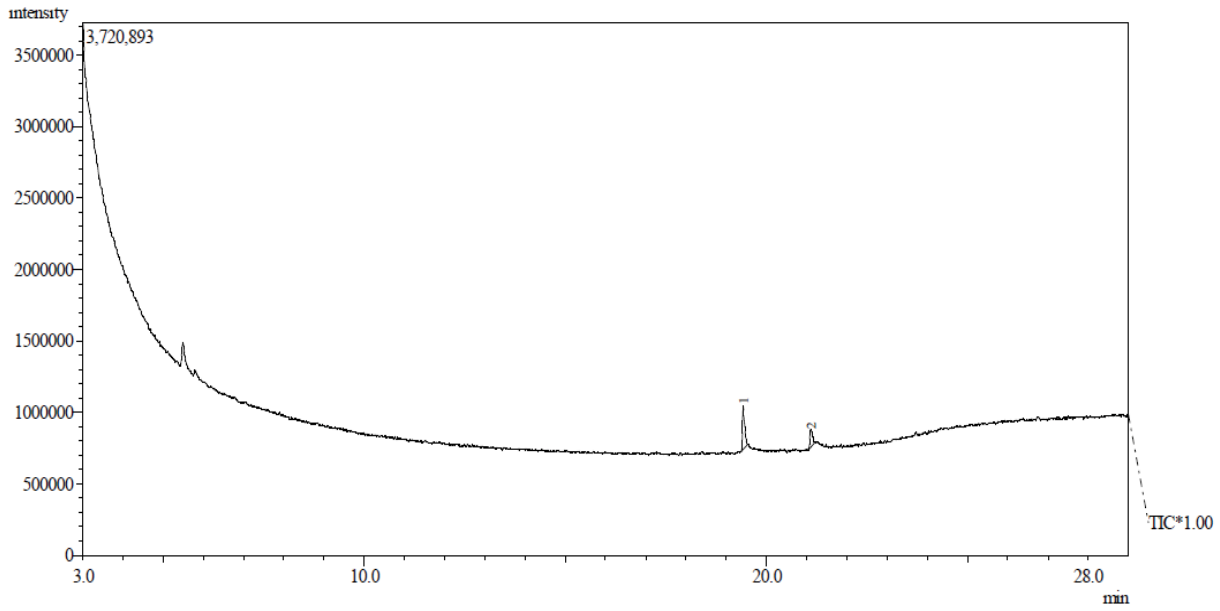

Figure 7: Gas Chromatogram of POME degraded by *Pseudomonas aeruginosa* at day 14.

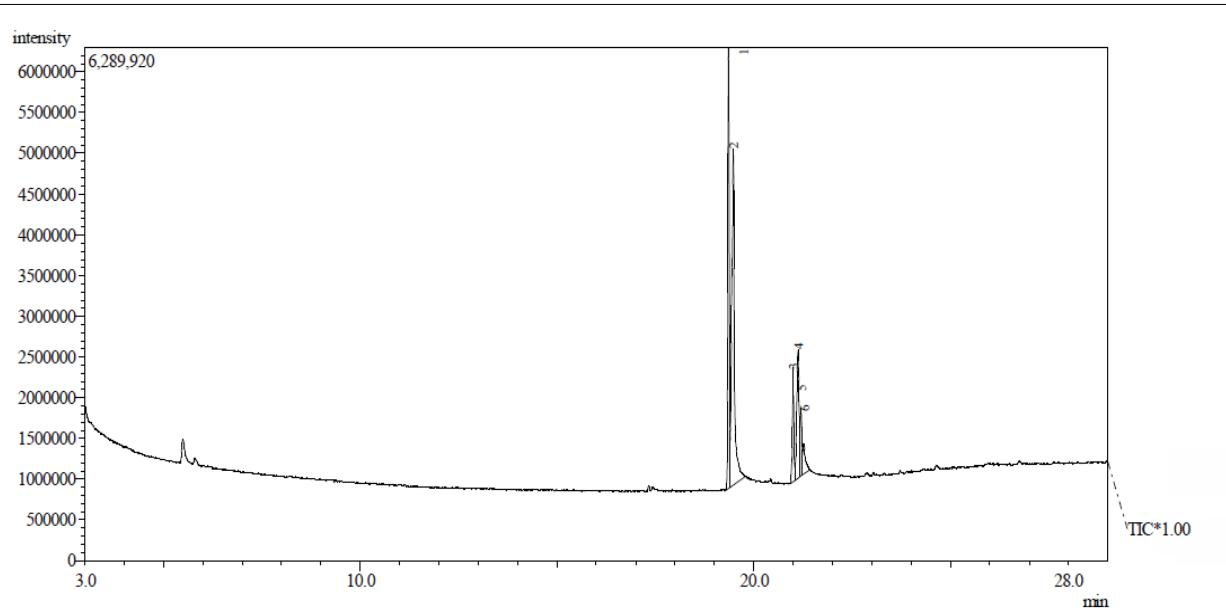

Figure 8: Gas Chromatogram of POME degraded by *Bacillus subtilis* at day 7.

active compounds present in the palm mill effluent at day zero (0). This is in agreement with the findings of Nwigbo et al. [27]. They reported that oleic acid, hexadecanoic acid (palmitic acid) and octadecanoic acid (stearic acid) are the main active compounds present in the palm oil mill effluent. The number of peaks / compounds present in the raw effluent is quite low compared to works of other researchers such as Stephen et al. [28] who had 16 peaks and Okwute and Ijah [29] whose chromatogram had 17 peaks. An explanation for this could be because freshly prepared POME was used in this study as opposed to Stephen et al. [28] and Okwute and Ijah [29] who used spent lubricating oil and aged POME samples.

Gas chromatography-mass Spectrophotometric analysis of POME degraded by *Pseudomonas aeruginosa, Bacillus subtilis* and *A. niger* at days 7 and 14 showed that hexadecenoic acid (palmitic acid) octadecanoic acid and oleic acid were not degraded by the three organisms. Their inability to degrade these compounds may be due to the absence of suitable enzymes for the mineralization of oleic acid, hexadecanoic acid (palmitic acid) and octadecanoic acid [29,30]. 3,3-Dimethyl-2- hexanone was degraded to hexanoic acid by *Pseudomonas aeruginosa* and *Bacillus subtilis* after 7 days. This suggests the presence of suitable degradative enzymes in the bacteria to break down 3,3-Dimethyl-2- hexanone. However, *A. niger* could not degrade 3,3-Dimethyl-2- hexanone after 14 days.

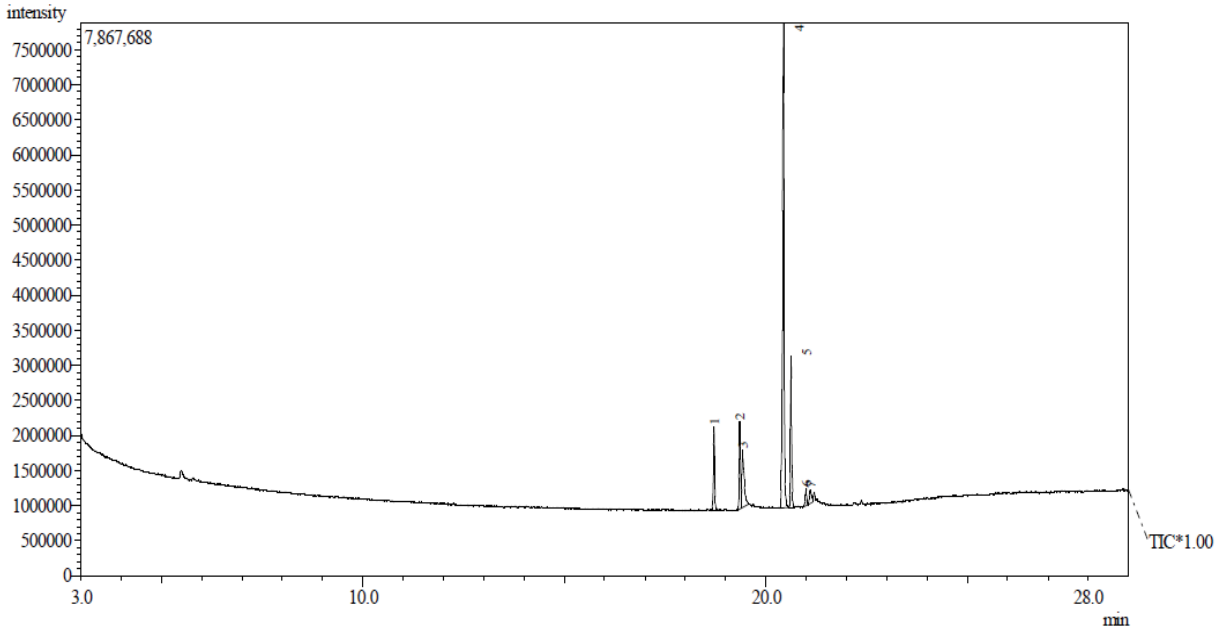

Figure 9: Gas Chromatogram of POME degraded by *Bacillus subtilis* at day 14.

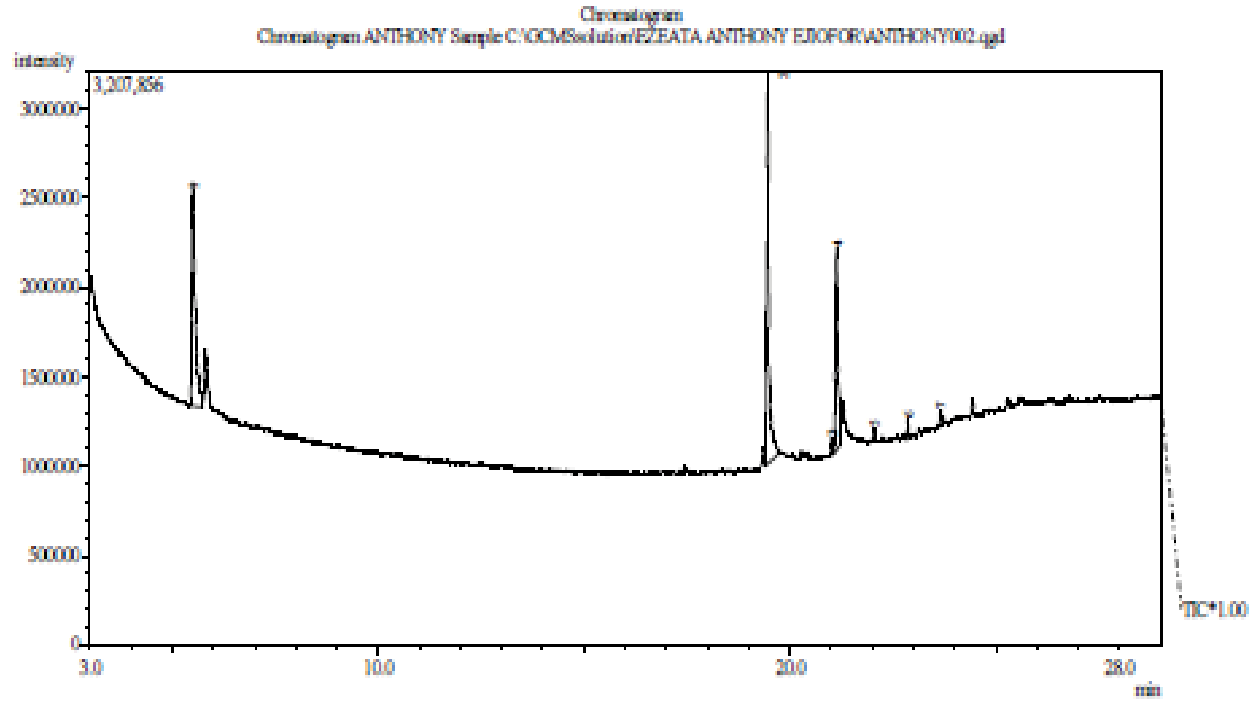

Figure 10: Gas Chromatogram of POME inoculated with *Aspergillus niger* after 7 days.

Conclusion

The present study revealed that the three organisms could not degrade the main active components of POME (oleic acid, hexadecanoic acid and octadecanoic acid) but *Pseudomonas aeruginosa* and *A. niger* degraded other components of POME better than *Bacillus subtilis*. The degradative enzyme produced by *Pseudomonas aeruginosa, A. niger* and *Bacillus subtilis* were capable of breaking down complex substrates in nature, and thus may be responsible for the biodegradation of POME in polluted habitats. However, from this study, *Pseudomonas aeruginosa* which had a better degradative ability compared to *B. subtilis* and *A. niger* should be employed in large scale clean-up of POME contaminant in the environment.

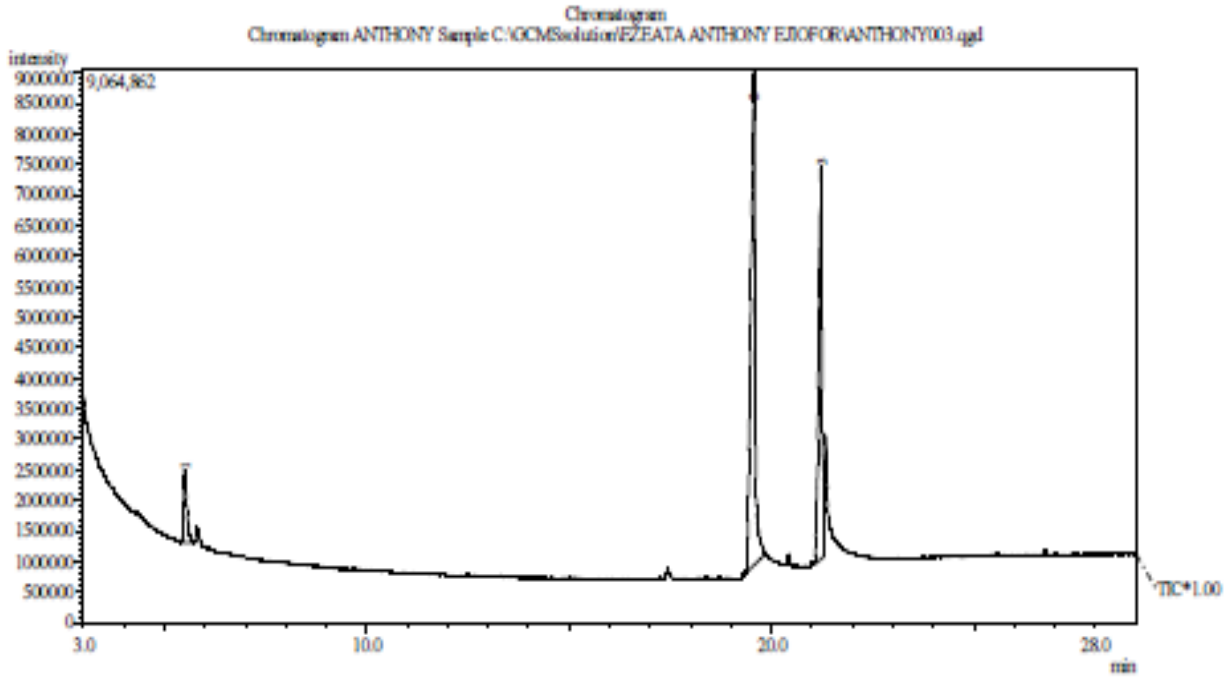

Figure 11: Gas Chromatogram of POME inoculated with *Aspergillus niger* after 14 days.

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