Comparative study of *in vitro* biodegradation of Spent Lubricating Oil by *Aspergillus niger* and *Bacillus subtilis*

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Abstract: Comparative study of biodegradation of spent lubricating oil by *Aspergillus niger* and *Bacillus subtilis* was studied for 16 days. pH, turbidity, nitrate, phosphorus and Gas Chromatographic analysis (GC-MS) of the medium was carried out. The result showed lower pH, nitrate and phosphorus level in *B. subtilis* medium compared to *A. niger* medium and the control. The GC-MS revealed that more compounds were degraded by *B. subtilis* than *A. niger*. Methylbenzene, ethlybenzene, hexadecane were degraded into benzene and hexadecanoic acid while Azulene and benzene were not degraded further by both organisms. This study suggests that *B subtilis* grows and metabolize compounds in spent lubricating oil better than *A. niger*.
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Key words: azulene, biodegradation, benzene, spent lubricating oil, turbidity

1. Introduction

Biodegradation refers to biological activities resulting in the breakdown of a compound (Ismail, 2008, Stephen *et al.*, 2013a), and is a sustainable and inexpensive remediation method for contaminated soil (Aislabie *et al.*, 1998, Stephen *et al.*, 2013b). Biological degradation of hydrocarbons in the environment is linked to a number of physical and chemical factors including the concentration and chemical structure of contaminant, moisture, oxygen, temperature and pH (Stephen *et al.*, 2013a).

According to Hawrot and Nowak (2006) and Anene and Chika (2011), the rate and efficiency of biodegradation depends on the occurrence of adequately numerous and active microflora in the contaminated environment. Organisms that have been reported with potential to degrade hydrocarbons include *Alcaligenes*, *Acinetobacter* spp, *Flavobacterium* spp, *Micrococcus* spp, *Bacillus* spp, *Pseudomonas* spp, *Trichoderma* spp, *Candida* spp, *Aspergillus* spp, *Rhizopus* spp (Anene and Chika, 2011, Stephen and Egene, 2012, Stephen *et al.*, 2013a). According to Okereke *et al.* (2007) and Chikere *et al.* (2009), *Aspergillus* species especially *A,niger, A. flavus* and *A. fumigatus* have been found in oil spilled site. Watkinson and Morgan (1990) reported that *Aspergillus* spp are capable of initiating the degradation of n-alkanes by sub-terminal oxidation, hence their relative abundance in hydrocarbon polluted soil. Ijah and Abioye (2003) reported the presence of *Bacillus* spp on kerosene polluted soil while Stephen *et al.* (2013b) reported the presence of *Bacillus* spp on diesel polluted soil undergoing biodegradation. Okwute and Ijah (2014) also reported the presence of *Aspergillus niger* and *Bacillus* spp in palm oil mill effluent (POME) polluted soil undergoing biodegradation.

Abdulhadi and Kawo (2006) reported that spent lubricating oil refers to any lubricating oil that has served its service properties and considered not fit for its initial purpose. Huge amount of spent lubricating oil are produced world wide (Khaled *et al.*, 2012). All types of lubricants become contaminated and lose their performance due to changes in their properties (Shakirullah *et al.*, 2006).

Some compounds in hydrocarbons may not be degraded by organisms (Atlas and brag, 2009) while others may be degraded and broken down into carbon dioxide, water and cell mass (Anene and Chika, 2011). *Aspergillus niger* and *Bacillus subtilis* have always been implicated in biodegradation studies. Hence, this study was undertaken to compare the ability of *A. niger* and *B. subtilis* to degrade spent lubricating oil separately and the products of their degradation.

2. Materials and methods

Collection of isolate and lubricating oil:

Aspergillus niger and *Bacillus subtilis* were collected from stock cultures from the Department of Microbiology, Kogi State University Anyigba while spent lubricating oil was collected from the mechanic workshop opposite First City Monument Bank, Anyigba, Nigeria. Both organisms were inoculated into peptone broth for 24 hours. Mineral Salt Medium containing 2.0g of Na_2HPO_4, 0.17g of K_2SO_4, 4.0g of NH_4NO_3, 0.53g of KH_2PO_4, 0.10g of $MgSO_4.7H_2O$ was prepared in 1000 ml of distilled water. 10 ml of

Mineral Salt Medium was dispensed into twenty test tubes each. 2ml of spent lubricating oil was added into each test tubes and the solution sterilized by autoclaving. 2ml of overnight broth culture (peptone broth) of *Aspergillus niger* and *Bacillus subtilis* were seeded into seven test tubes separately while the remaining six without *Aspergillus niger* and *Bacillus subtilis* served as the control. The test tubes were incubated at ambient temperature for 16 days without shaking. Degradation of the spent lubricating oil was monitored at 4 days interval for 16 days. Growth pattern of the organisms were determined by measuring the turbidity using turbidity meter (WGZ-113 Shanghi, China). pH was determined at ambient temperature using glass electrode pH and conductivity meter (Hannia, Italy). Phosphorus was determined using the method of Murphy and Riley (1962). Nitrogen was determined by the micro Kjedahl method as described by Ibitoye (2006).

2.1GasChromatography-Mass Spectrophometry

The Gas Chromatographic –Mass Spectrophotometric analysis was carried out at day 0 (for control) and days 7 and 14 (for spent lubricating oil inoculated with *Aspergillus niger* and *Bacillus subtilis*). The mineral salt medium containing spent lubricating oil and the organisms were decanted into a 50 ml beaker using Whatmann filter paper. The oil on the filter papers was recovered by rinsing with 25ml of carbon trichloromethane (chloroform) in another 50ml beaker. The oil was placed in a water bath for 20 minutes to evaporate the solvent. The oil was then analysed using gas-liquid chromatography-mass spectrophotometer (GCMS Qp 2010 plus, Shimadzu, Japan). The oil was diluted with n-hexane to a volume of 10ml from which one micro litre was injected into the GC-MS. Injector and detector chamber temperatures were set at 250^0C and 380^0C respectively. The oven temperature was initially set at 60^0C for 4 minutes, ramped at 10^0C per minutes to 210^0C for 3 minutes. It was further held for 2 minutes and ramped at 20^0C per minutes to 280^0C.

3. Result

Figure 1 shows the change in pH of the spent lubricating oil undergoing biodegradation. The temperature ranged from 4.4 to 7.6. The highest pH was observed in *Aspergillus niger* inoculated medium followed by *Bacillus subtilis* medium and the control. The pH in all samples decreased from day 14 till the last day of the experiment.

Figure 2 shows the turbidity produced by the organisms in the spent lubricating oil medium during the course of the experiment. Turbidity decreased in all treatments from day 0. This decreased continued till day 14 in *Bacillus subtilis* medium while increased in turbidity was observed in *Aspergillus niger* medium after day 4 till day 12. In the control, slight increase in turbidity was observed at day 12 which later decline at day 16.

Figure 3 shows the change in nitrate concentration of the spent lubricating oil undergoing biodegradation. The nitrate content was high in all treatments after 4 days and then decline till day 12. An appreciable increase was observed in *Aspergillus niger* medium and the control at day 16.

Figure 4 shows the phosphorus content of the spent lubricating oil undergoing biodegradation. The phosphorus contents were higher in samples inoculated with *Aspergillus niger* and *Bacillus subtilis* than the control. However, there was a gradual decline until day 16 of the experiment in both *Aspergillus niger* and *Bacillus subtilis* samples. The phosphorus content was higher in the control at the end of the experiment followed by *Aspergillus niger* and *Bacillus subtilis.*

Figure 5 shows the gas chromatographic tracing of the uninoculated spent lubricating oil (control). The compounds present were methylbenzene, ethylbenzene, o-xylene, propylbenzene, octane, pentadecane and hexane. The chromatogram showed that spent lubricating oil had more aromatic and cycloalkanes than straight chain alkanes.

Figure 6 shows the chromatogram of the spent lubricating oil inoculated with *Aspergillus niger* after one week (7 days). The branched aromatic compounds were degraded after one week. There was a reduction in the peak heights after one week. New compounds such as azulene (bicyclo (5,3,0) decapentene) was introduced as a result of the metabolism of the spent oil by *Aspergillus niger*.

Figure 7 shows the gas chromatograms of spent lubricating oil degraded by B.*subtilis* after one week. The results revealed the presence of benzene, azulene, alkanes (tetradecane, nonane, heptadecane) and carboxylic acids. More carboxylic acids were found in figure 7 compared to figure 8.

The chromatogram of the inoculated spent oil by *Aspergillus niger* after 14 days is shown in figure 8. The peak heights and compounds were further reduced compared to figures 5 and 6. Organic acids were more in figure 8 than 7. The compounds remaining in the spent lubricating oil after 14 days were benzene, azulene, dodecane, tetradecane, heptadecane, hexadecanoic acid, 9-octadecanoic acid and octadecanoic acid.

Figure 9 shows the chromatograms of the degraded spent lubricating oil after two weeks by *B.subtilis*. The peaks were less than those of *A. niger* after two weeks (figure 8). More carboxylic compounds were also observed in the chromatograms of *B. subtilis* after two weeks compared to *A. niger*.

Azulene, benzene and naphthalene were not degraded by both organisms after two weeks.

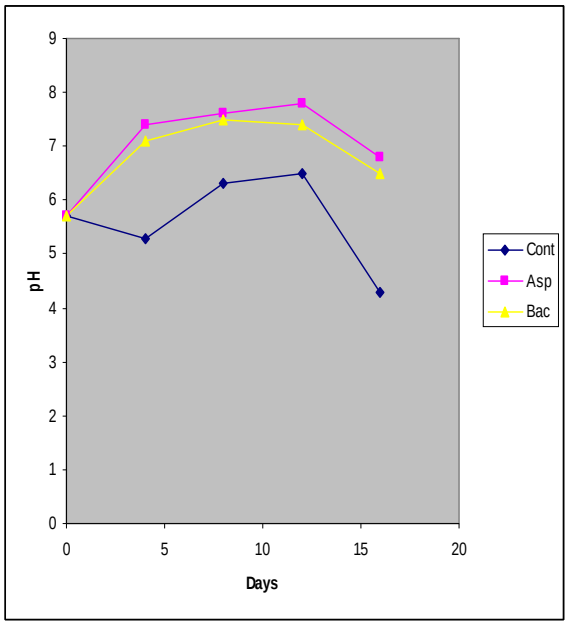

Figure 1: pH of spent lubricating oil undergoing biodegradation
Cont: control, Asp: *Aspergillus niger*, Bac: *Bacillus subtilis*

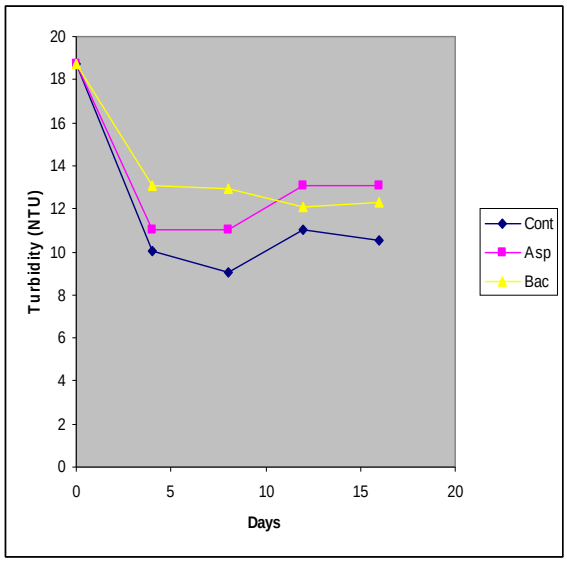

Figure 2: Turbidity of spent lubricating oil undergoing biodegradation
Cont: control, Asp: *Aspergillus niger*, Bac: *Bacillus subtilis*

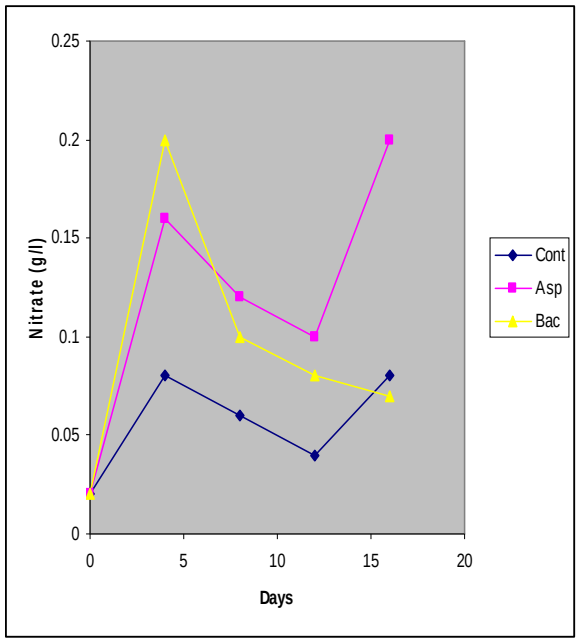

Figure 3: Nitrate of spent lubricating oil undergoing biodegradation
Cont: control, Asp: *Aspergillus niger*, Bac: *Bacillus subtilis*

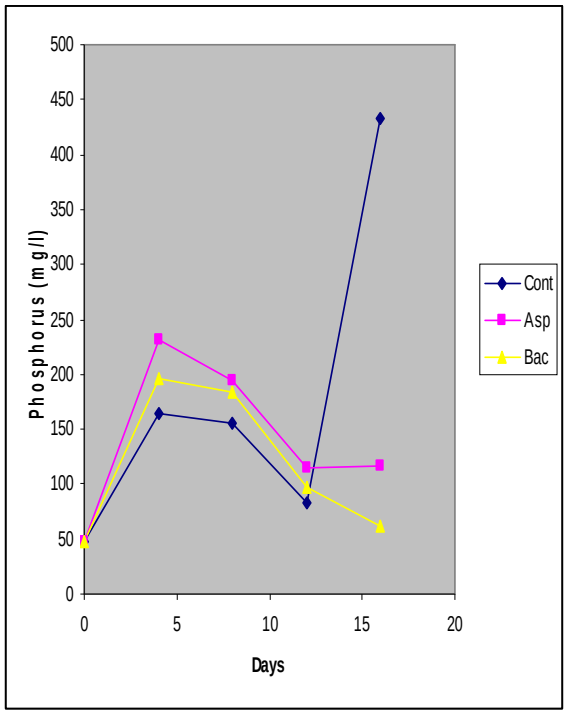

Figure 4: Phosphorus content of spent lubricating oil undergoing biodegradation
Cont: control, Asp: *Aspergillus niger*, Bac: *Bacillus subtilis*

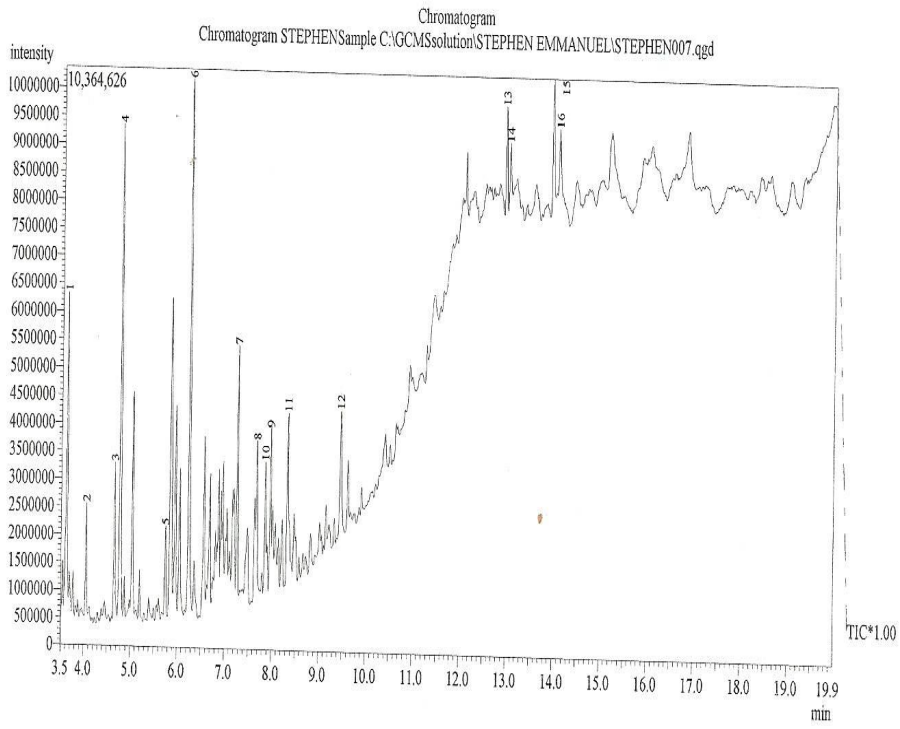

Figure 5: Gas Chromatographic analysis of Spent Lubricating Oil, SLO.
Peak sequences: 1: methylbenzene 2: octane 3: ethylbenzene 4: o-xylene 5: propylbenzene 6: ethylbenzene 7: benzene 8: 1-bromomethyl-4-isopropylbenzene 9: methyl-p-ethyltoluene 10: 1-phenyl-1-butene 11: cyclopentacycloheptene 12: 1,6-methanol 13: pentadecane 14 : pentadecane 15: Hexadecane 16: hexadecane

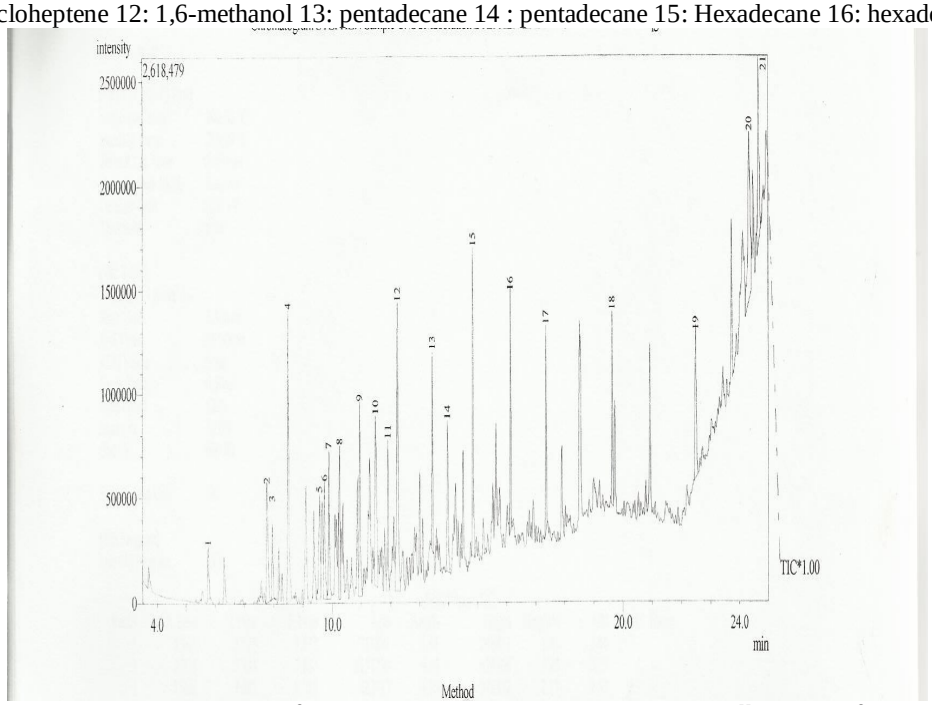

Figure 6: Gas chromatographic analysis of spent lubricating oil degraded by *Aspergillus niger* after one week.
Peaks : 1:benzene 2: benzene 3: benzene 4: benzene 5: benzene 6: benzene 7: 4,7- methanoindene 8: 1,3-cyclohexadiene 9: benzene 10: 3-phenyl-1-butene 11: undecane 12: azulene 13: undecane 14: napthalene 15: tetradecane 16: pentadecane 17:hexadecane 18:heptadecane 19: heptadecane 20: tetracosanoic acid 21: tricontane

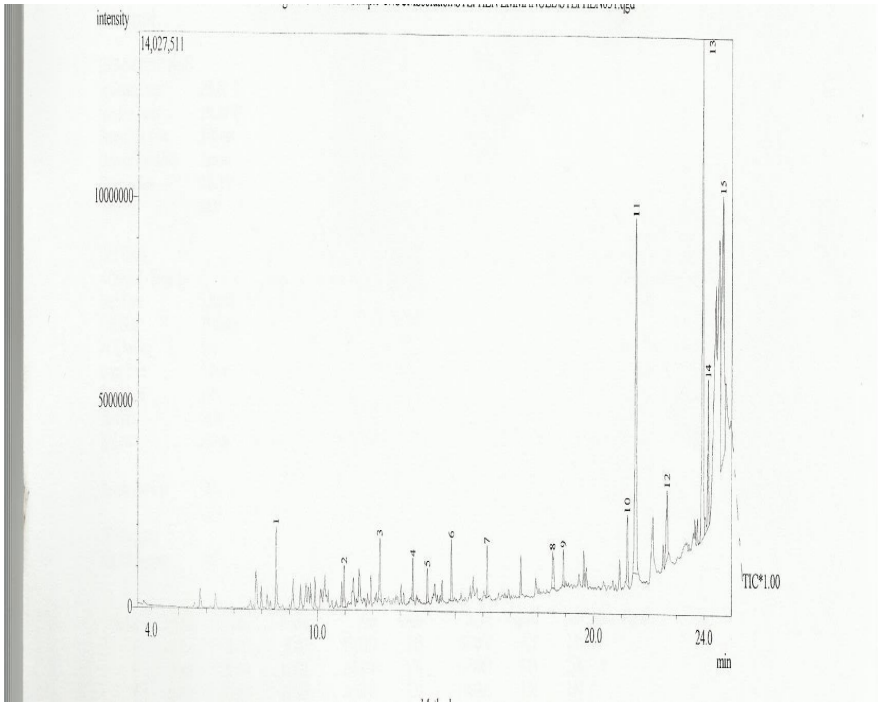

Figure 7: Gas chromatographic analysis of spent lubricating oil degraded by *Bacillus cereus* after one week.
Peaks : 1: benzene 2: benzene 3: azulene 4: pentadecane 5: naphthalene 6: tetradecane 7: nonane 8: heptadecane 9:methyltetrradecane 10: 9-hexadecanoic acid 11: hexadecanoic acid 12: hexadecanoic acid 13: 9-octadecanoic acid 14: octadecanoic acid 15: dodocanoic acid

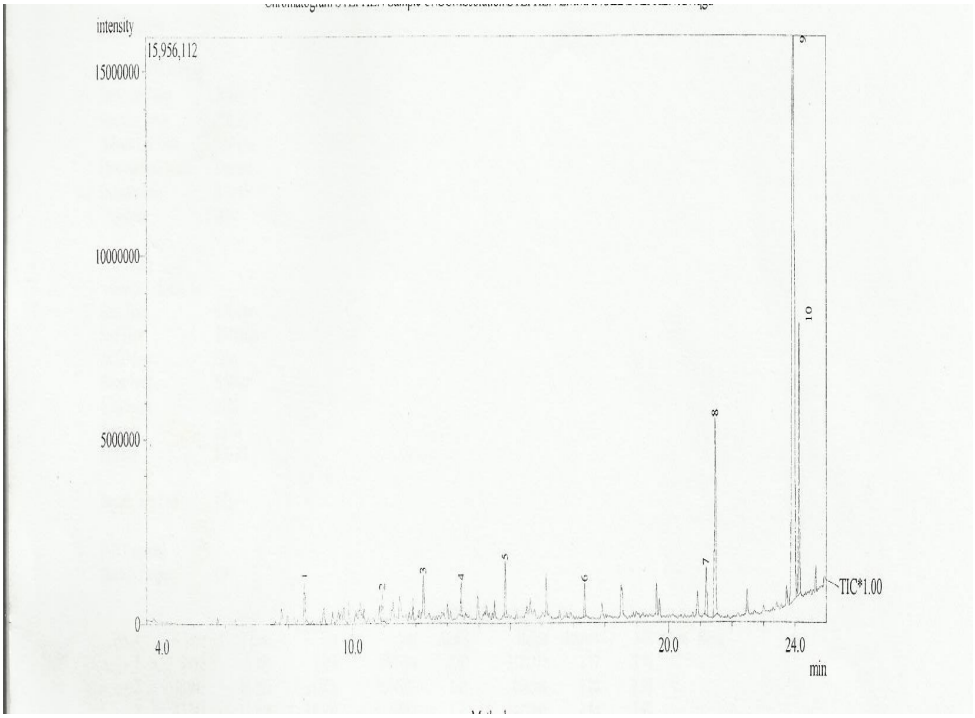

Figure 8: Gas chromatographic analysis of spent lubricating oil degraded by *Aspergillus niger* after two weeks.
Peaks: 1: benzene 2: benzene 3: azulene 4: dodecane 5: tetradecane 6: heptadecane 7: hexadecanoic acid 8: hexadecanoic acid 9: 9- octadecanoic acid 10: octadecanoic acid

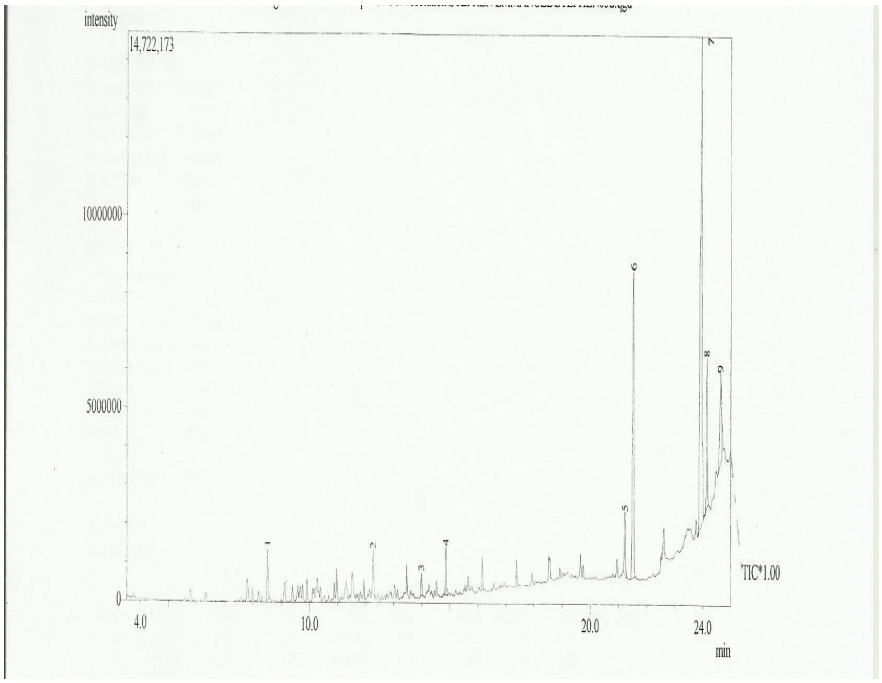

Figure 9: Gas chromatographic analysis of spent lubricating oil degraded by *Bacillus cereus* after two weeks.
Peaks: 1: benzene 2: azulene 3: napthalene 4: hexadecane 5: 9-hexadecanoic acid 6: hexadecanoic acid 7: 9-octadecanoic acid 8: octadecanoic acid 9: 9, 12- octadecanoic acid

4. Discussion

The pH tends towards neutrality. The range of pH observed in both *A.niger* and *B. subtilis* media compared to the control favours biodegradation of hydrocarbon. The range in pH observed in this study were similar to those reported earlier by Stephen *et al.* (2013a, b). The highest pH observed in *A.niger* medium is an indication of higher metabolic activity by *A.niger* compared to *B. subtilis* (Stephen *et al*., 2013c).

The turbidity produced during biodegradation was higher in *B. subtilis* medium up till the 12th day compared to the control and *A. niger* medium. This implied that *B. subtilis* was able to utilize the spent lubricating oil better than *A. niger*. This result is in line with earlier report by Stephen *et al.* (2013a) who observed higher metabolic activity in an *A. niger* medium compared to the control. The higher turbidity produced at the end of the experiment indicates that metabolic activity increases with time in the fungus after it might have adapted to the spent lubricating oil medium.

The nitrate concentration was initially high in *B. subtilis* medium compared to *A.niger* and the control. The steady reduction in *B. subtilis* compared to *A.niger* implies high nitrate utilization by the bacterium in its metabolic activity compared to *A.niger* (Stephen *et al*., 2013b).

The phosphorus content was lower in *B. subtilis* medium than *A.niger* and control all through the study. Phosphorus like nitrate is required by organisms during biodegradation.This result is an indication that phosphorus was more utilized by *B.subtilis* than *A.niger*.

Some of the compounds present in the spent lubricating oil (control) may be due to prolonged usage. The branch aromatic compounds may result from chemical contamination during usage (Domingueq-Rosado and Pichtel, 2004).

The GC-MS of the spent lubricating oil degraded by *A.niger* after 7 days revealed the presence of more benzene than *B. subtilis* medium and the control. This may be due to the cleaving of the methyl, ethyl, propyl branches present in the control by the organisms. However, by *B.subtilis* medium had fewer benzene compounds and carboxylic acids compared to *A. niger* medium. This is an indication that the rate of degradation and disappearance of some of the compounds were higher in *B.subtilis* than *A. niger* medium. The presence of benzene in both medium shows that benzene cannot be degraded by *A.niger* and *B. subtilis*. This is in agreement with Atlas and Brag (2009). These

researchers reported that some aromatic hydrocarbons cannot be degraded by some organisms.

The chromatographic analysis of the samples after 14 days revealed peaks and compounds in both *A. niger* and *B. subtilis* media compared to the chromatograms of both organisms at day 7. This may be attributed to increased biodegradation (Susarla *et al*., 2002). Benzene and azulene could not be degraded after 14 days by both organisms. The increased number of carboxylic acids in both medium (*A. niger* and *B. subtilis*) is an indication of biodegradation of the alkanes found in the spent lubricating oil (control) and after 7 days of biodegradation (Meredith *et al*., 2000). The higher number of carboxylic acids in *B. subtilis* medium compared to *A. niger* also indicates that *B.subtilis* degrades spent lubricating oil better than *A.niger*.

5. Conclusion

This study revealed that *B. subtilis* grew and utilize spent lubricating oil better than *A. niger*. pH, nitrate and phosphorus concentration were lower in *B. subtilis* medium than *A. niger*. Some of the branch aromatic compounds such as ethylbenzene, methylbenzene, propylbenzene and straight chain alkanes such as hexadecane, heptadecane and octane were degraded into benzene, hexadecanoic acid and octadecanoic acid. This study also revealed that benzene and azulene present in the spent lubricating oil could not be degraded further by *A. niger* and *B. subtils* after two weeks.

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