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Abilities of *Achyla orion* and *Allomyces anomalus* to degrade petroleum and petroleum products as sole carbon sources

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Abilities of *Achyla orion* and *Allomyces anomalus* isolated from some crude oil polluted aquatic environments in Nigeria to biodegrade petroleum and petroleum products were determined. Baiting method using hemp and sesame seeds was used to isolate the two species of aquatic phycomycetes. The species were grown in liquid broth culture made of minimal mineral salts supplemented separately with petrol, diesel and kerosene and incubated at room temperature with agitation for two weeks. Biodegradation was monitored using spectrophotometer at 600 nm wavelength. Fat/lipid was extracted from pellets resulting from centrifugation of the final broth culture using selected fat extractor and quantified. *A. anomalus* gave highest mean growth values in broth medium supplemented with diesel (0.970) and kerosene (1.302) while that supplemented with petrol recorded the least mean growth value of 0.663. The mean growth values for *A. orion* showed a similar trend. Crude fat/lipid production was highest for both *isolates* grown in diesel supplemented broth culture medium and least for both isolates grown in petrol supplemented broth culture medium. These results imply that these two species of aquatic phycomycetes were able to degrade diesel and kerosene better than petrol with corresponding production/accumulation of fat/lipid as biodegradation product.

Key words: Aquatic phycomycetes, petroleum, fractions, mineral salt and supplement.

INTRODUCTION

Petroleum and petroleum products form one of the major pollutants of the aquatic environment especially in the

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Niger-Delta region of Nigeria and in freshwater bodies used for industrial and domestic purposes like washing of petrol and diesel engine automobiles. Microorganisms especially fungi have a higher tolerance to the toxicity of hydrocarbons due to their physiology and adaptation to such variations in the environment and have mechanisms for the elimination of spilled oil from the environment (Abatenh et al., 2017; Yuniati, 2018). The effect of oil on microbial populations depends upon the chemical composition of the oil and on the species of microorganisms present. Populations of some microbes increase; typically, such microbes use the petroleum hydrocarbons as nutrients according to Abatenh et al. (2017) and Mahjoubi et al. (2017). Okerentugba and Ezeronye (2003) showed that bacteria and fungi spp. (*Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp.) isolated from rivers and refinery effluents in the Niger-Delta region of Nigeria could degrade crude oil. They reported increase in biomass for fungal isolates after a 35-day growth period. Their result also showed changes in pH, optical density and total viable count for bacterial isolates after a 17-day period. Many researchers have studied the role of fungi in biodegradation process of petroleum products. The most common fungi, which have been recorded as biodegraders, belong to many genera. These genera include Alternaria, Aspergillus, *Candida*, *Cephalosporium*, *Fusarium*, *Gliocladium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Pleurotus*, *Polyporus*, *Rhizopus*, *Rhodotolura*, *Talaromyces* and *Torulopsis* (Gesinde et al., 2008; Obire and Anyanwu, 2009; Hadibarata and Tachibana, 2009; Romero et al., 2010). Furthermore, reviews on fungi in bioremediation and biodegradation of crude oil across the world (Das and Chandran, 2011; Jahangeer and Kumar, 2013) and in Nigeria, Obire and Putheti (2009) recorded yeasts and filamentous fungi as fungi biodegraders of crude oil. None of the reviewers encountered in this study reported aquatic phycomycetes as biodegraders of crude oil. Aquatic phycomycetes are the group of primitive fungi that have adapted to the aquatic environment by the possession of flagella for motility. Two groups based on possession of one or two flagella as *Chytridiomycetes* and *Oomycetes*, respectively are associated with aquatic phycomycetes (Khulbe, 2001). These fungi contribute to the energy flow and productivity of aquatic and semi aquatic ecosystem by their active role in the utilization and bio deterioration of a variety of organic and inorganic materials (Khulbe, 2001). Most research carried out on biodegradation of crude oil and its fractions have centered mainly on yeasts and filamentous fungi. There is a dearth of information on the ability of aquatic phycomycetes to degrade crude oil and its fractions especially in some water bodies in Jos, Plateau State and the oil rich Bayelsa State of Nigeria. Therefore, this study seeks to evaluate the biodegradation potentials of two species of aquatic phycomycetes by growing them in suitable broth culture medium that mimics their natural aquatic environment, using spectrophotometer to measure degradation of the substrates and finally confirming biodegradation by extraction of fatty acid/lipid as a metabolite produced from biodegradation.

MATERIALS AND METHODS

Sampling sites and sampling procedures

The sampling sites for the study were two sampling points on River Nuns at Nembe seaport (N4° 32' 32'', E6° 24' 02'') (Marine) and Ogbia waterside (Brackish water) in Bayelsa State and one freshwater body, Dorowa pond by College of Health and Technology, Zawan, in Jos, Plateau State of Nigeria (N9°46' 20'', E8° 52' 17''). Water samples collection for Plateau State were done in February 2013 while those from Bayelsa State were collected in the first week of March 2013. Water samples collection were done with the aid of sterile 500 ml bottles aseptically and transported to the Dermatophilosis laboratory of National Veterinary Research Institute (NVRI), Vom, Jos, Plateau State in coolers designed as hand refrigerator with icepacks maintained at 5°C. Water samples analyses were carried out as soon as practicable on the day of collection but not more than 24 h after collection especially water samples from Bayelsa State (Rankovic, 2005; Marano et al., 2008).

Isolation of aquatic phycomycetes for culture based studies

Aquatic phycomycetes were isolated using hemp seed (*Canabis sativum*) and sesame seed (*Sesamum indicum*) as baits according to the methods of de Almeida Nascimento et al. (2011) and Trifa and Adiba (2011). Direct observation and identification of isolated aquatic phycomycetes were made using the compound light microscope according to de Almeida Nascimento et al. (2011) and Trifa and Adiba (2011). Results as seen from the microscope under appropriate magnifications were then compared for similarity with pictures and descriptions found in manual for identification of aquatic fungi (Khulbe 2001) with the support of other standard references, which included Fuller and Jaworsky (1987) and Dick (1990). Two species of aquatic phycomycetes showing consistency in occurrence and presenting with clear identification properties both morphologically and microscopically were selected for the biodegradation experiment. The said species of aquatic phycomycetes isolates used for the study were *Achyla orion and Allomyces anomalus. A. orion* was isolated from Dorowa pond by College of Health and Technology, Zawan and Nembe seaport in Bayelsa State while *A. anomalus* was isolated from Ogbia waterside in Bayelsa State and Dorowa pond by College of Health and Technology, Zawan, Plateau State, Nigeria.

Abilities of test fungi (*A. orion and A. anomalus)* to degrade petroleum and petroleum products

Overnight cultures of the two test species (*A. orion* and *A. anomalus*) grown in Malt extract broth were introduced into aseptically prepared mineral salt medium supplemented with 1% v/v petroleum, diesel and kerosene separately in well corked 250 ml conical flasks. The biodegradation of petroleum and petroleum products was observed via optical density as measured

spectrophotometrically at 600 nm wavelength using broth culture of aquatic fungi isolated from experimental sites for a period of two weeks on minimal salt broth as used by Sebiomo et al. (2011) and Ekundayo et al. (2012).

Petroleum and petroleum products namely diesel and kerosene were purchased from NNPC mega filling station at Secretariat junction in Jos, Plateau State and were dispensed into 200 ml sterile bottles and transported to the laboratory. A sterile inoculating needle was used to pick aquatic phycomycetes mycelium or pinhead of hyphae and inoculated into sterile malt extract broth (35 ml quantity prepared with drops of antibiotics, streptomycin sulphate, to suppress bacteria growth). The broth cultures of the two test isolates were then incubated at room temperature (25°C) and left to stand for 24 h. These served as the overnight broth cultures used in the biodegradation experiment. The broth culture medium used was prepared according to the pioneering method of preparing mineral salt medium by Mills et al. (1978) as modified by Okpokwasili and Okorie (1988) and further used by Sebiomo et al. (2011). The composition of the medium was Nacl=10.0 g, $MgSO_4.H_2O$ =0.42 g, Kcl=0.29 g, KH_2PO_4=0.83 g, Na_2HPO_4=1.25 g, $NaNO_3$=0.42 g, distilled water=1000 ml.The mineral salt medium prepared as above was sterilized by autoclaving at 121°C for 15 min and allowed to cool to 45°C. Simultaneously petroleum, diesel and kerosene were filtered using Millipore filter core paper (AP2029300) made in Bedford, Massachusette, USA.

A known volume (150 ml) of the sterilized and cooled mineral salt medium was dispensed into seven 250 ml conical flasks. Two three sets of the conical flasks were separated for the introduction of overnight broth culture of each of the test isolates of aquatic phycomycetes after supplementing with petroleum and petroleum products. The seventh flask that served as control was not supplemented but had one of the isolates inoculated. 1% v/v of each of the filtered petroleum and petroleum products were introduced into each of the flasks containing 150 ml MSM except the seventh flask. 35 ml of overnight broth cultures (using malt extract broth) of each isolate of aquatic phycomycetes were then separately seeded into different flasks supplemented with petrol, diesel and kerosene, resulting into six flasks, three per test organism.

A. anomalus was seeded into the seventh flask without supplement and used as control. The resulting flasks with test organisms were incubated at room temperature with constant agitation by clipping flasks to a laboratory gyratory shaker to mix the contents in order to enhance biodegradation. Biodegradation of petrol, diesel and kerosene by *A. orion and A. anomalus* were then monitored at two days interval for 14 days by measuring the optical density of the content of each of the appropriately labeled flasks using Jenway-6405 uv/vis spectrophotometer at 600 nm wavelength (Sebiomo et al., 2011).

Changes in pH were also determined in the course of the experiment using a portable pH meter. At the end of the 14 days period, the aquatic phycomycetes isolates in each of the flask that have visibly increased in biomass as a result of growth were harvested by centrifugation in a rotary centrifuge at standard setting of the centrifuge. The solid residues (increased biomass of aquatic phycomycetes isolates) were each dried on sterile whatman No1 filter paper as pellets and the weights were taken with a digital weighing balance. The supernatant was decanted. The dried residues were then wrapped in separate filter papers and used for the extraction of accumulated fat/lipid.

Fat/lipid extraction from biodegradation experiment

The seven resulting pellets from the biodegradation experiment above were subjected to fat/lipid extraction using the Soxhlet method of AOAC (1980). Weights of the resulting pellets were less than 1 g and the extraction of fat/lipid was carried out using selecta fat extractor at the Biochemistry laboratory of National Veterinary Research Institute, Vom, Plateau State, Nigeria.

RESULTS

The results of growth measurement using spectrophotometer readings for *Achyla orion* in Figure 1 were high on the first day (start day, 0) for growth on all the media supplemented with petrol, diesel and kerosene with 0.632, 0.450 and 0.451 readings respectively. These readings lowered for day 2 and day 4, after which there were progressive, increases on day 6 and day 8. This continued on day 12 for only the medium supplemented with kerosene. The media supplemented with petrol and diesel showed a decline on the day 12 of measurement. It was also observed as shown in Figure 1, that *A. orion* recorded the highest reading on medium supplemented with diesel which peaked at 1.306 on day 10. The medium supplemented with petrol conversely showed the lowest peaks.

The result of growth measurement with *A. anomalus* in Figure 2 shows that the readings on the 1st days (0 day) were slightly higher than that of day 2. Growth continued to increase from day 2 to day 10 and slightly decline on day 12 for growth on media supplemented with diesel and kerosene but increased on day 12 for media supplemented with petrol. *A. anomalus* recorded the highest growth readings on media supplemented with kerosene, which peaked also on day 10 at 2.150. Another observation from the result in Figure 2 shows growth readings on media without any supplement presented with lower values throughout the duration of the study when compared with growth on media supplemented with diesel, kerosene and even petrol. The growth on medium without supplement showed a progressive increase in growth from the start day 0 to the last day, day 12 representing a somehow normal pattern.

The mean values of the spectrophotometer readings with respect to *A. anomalus* in Figure 3 shows lowest mean value of 0.622 for medium without supplement followed by that supplemented with petrol (0.663) and diesel (0.970).

In comparison, the growth of *A. orion* and *A. anomalus* on media supplemented with petrol showed the least readings for both test isolates with respect to Mean measurements as can be seen in Figure 3. This tends to suggest that petrol is the least utilized supplement as compared with diesel and kerosene.

The mean pH of experimental culture media for the two test isolates fell within the acidic range as shown in Figure 4.

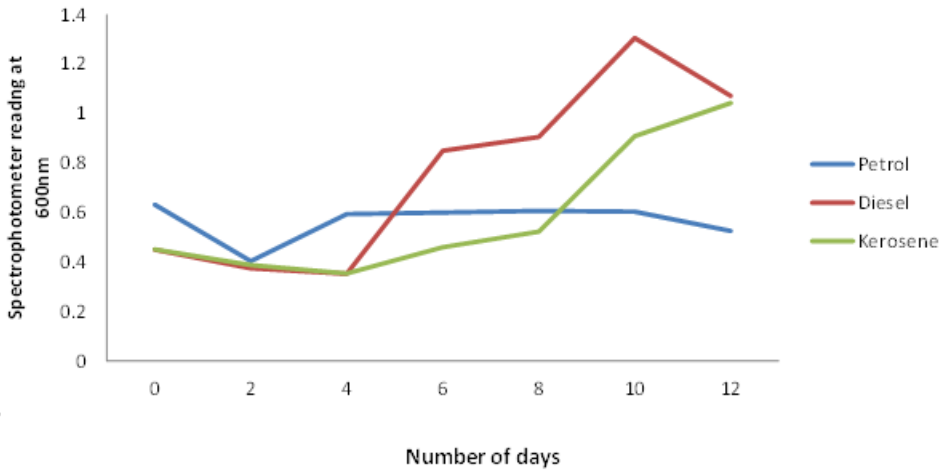

Figure 1. Biodegradation/Utilisation of Petroleum and Petroleum products by *Achyla orion.*

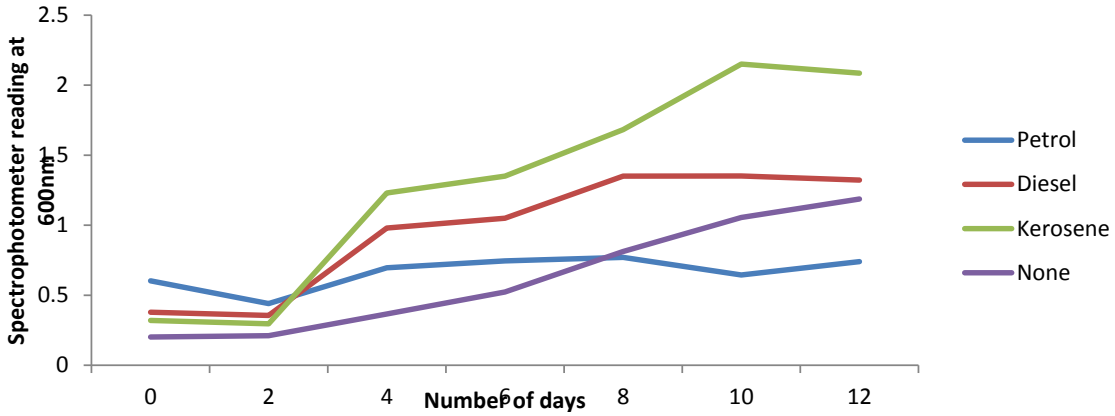

Figure 2. Biodegradation/Utilisation of Petroleum and Petroleum products by *Allomyces anomalus.*

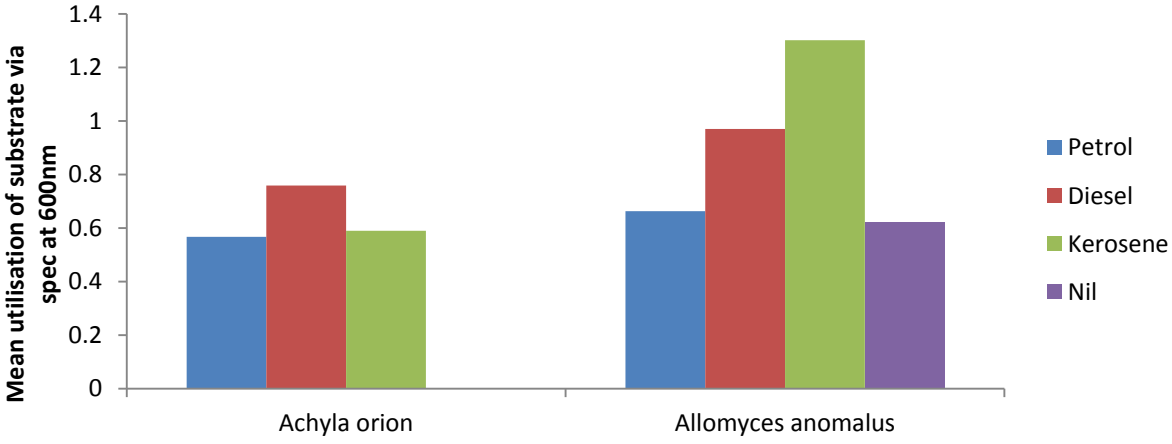

Figure 3. Mean utilization of Petroleum and Petroleum products by test isolates.

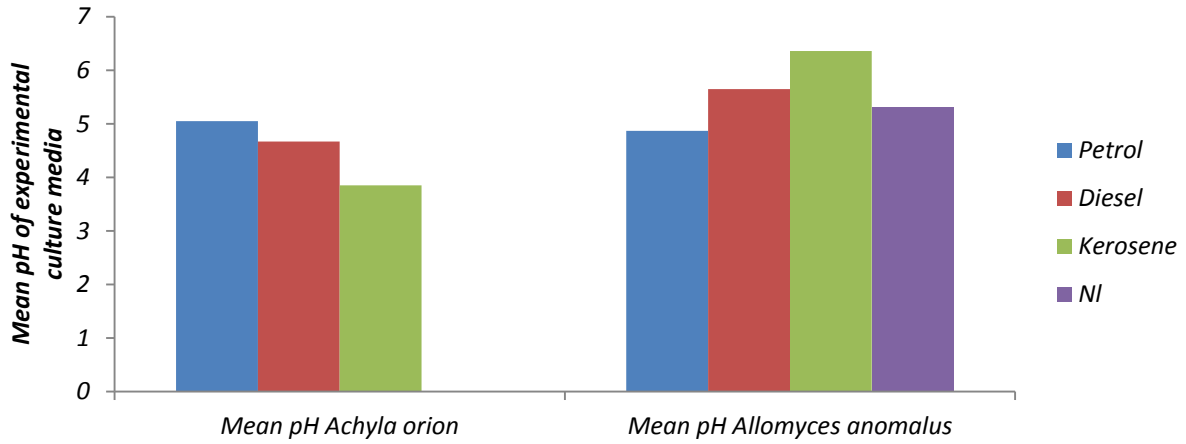

Figure 4. Mean pH of experimental culture media.

Table 1. Crude fat/lipid from biodegradation experiment.

Sample name	pH of final broth culture	Weight of pellet from fbc (g)	Weight of extracted fat/lipid	% crude fat /lipid g/100 g
Achyla +petrol	5.08	0.028	0.00	0.00
Achyla+kerosene	4.26	0.0140	0.00	0.00
Achyla+diesel	5.89	0.1260	0.0390	30.95
Allomyces+petrol	4.92	0.0120g	0.00	0.00
Allomyces+kerosene	7.16	0.0800	0.0050	6.25
Allomyces+diesiel	6.38	0.1880	0.0240	12.77
Allomyces+nil	6.15	0.0890	0.0060	6.74

Determination of by-product of petroleum and petroleum products degradation by test fungi (crude fat/lipid)

The by-product of the petroleum and petroleum products determined was crude fat/lipid produced at the end of the experiment as an accumulated product. The result of crude fat/lipid extraction from aquatic phycomycetes pellets from the biodegradation experiment in Table 1 shows the highest crude fat/lipid extract from pellets of *A. orion* and *A. anomalus* grown in mineral salt broth culture medium supplemented with diesel producing 30.95 and 12.77 g/100 g crude fat respectively. *A. anomalus* grown on kerosene and without supplement also produced 6.25 and 6.74 g/100 g crude fat respectively

DISCUSSION

The pattern of rise in the spectrophotometer reading at 600 nm for *A. orion* from day 0 to day 10 for petrol and diesel and decline in day 12 and rise from day 0 to day 12 for kerosene enriched broth culture media shows that the inoculated aquatic phycomycetes species was actively growing and metabolizing the substrate. With respect to *A. orion* the growth on diesel and kerosene enriched broth culture media gave the highest mean spectrophotometer readings of 0.759 and 0.590 respectively. Petrol enriched broth culture medium recorded the least mean of 0.567. This seems to suggest that the isolate, *A. orion* utilized or degraded diesel and kerosene better than petrol as supplements. For *A .anomalus*, the growth on kerosene and diesel enriched broth culture media gave the highest mean spectrophotometer readings of 1.302 and 0.970 respectively. Petrol enriched broth culture medium again recorded the least mean of 0.663. The pattern of rise and fall in spectrophotometer readings for *A. anomalus* signifying growth was similar to that of *A. orion*. The pH values of the growth media of both test aquatic phycomycetes isolates fell within the acidic range. This seems to be in line with Davis and Westlake (1979) who stated that fungi could grow in environmentally stressed condition like low pH and poor nutrient status while

bacteria cannot. Sebiomo et al. (2011) work on utilization of crude oil and gasoline by ten bacteria and five fungi isolates also reported reduction in pH of the culture fluid in flasks within their 14 days of incubation with pH readings also falling within the acidic range. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products (Nwachukwu and Ugoji, 1995; Okpokwasili and James, 1995). Thus organic acids probably produced account for the reduction in pH levels (Oboh et al., 2006).

The aspect of the experiment that may further buttress the utilization of petroleum and petroleum products is probably the result gotten when *A. anomalus* was grown in MSM broth culture medium without supplements of petrol or petroleum products. The growth on these MSM broth medium without supplement was a normal growth of consistent increase in the spectrophotometer reading from day 0 to day 12 while the spectrophotometer reading for MSM broth culture medium supplemented with petrol and petroleum products declined on the day 12 after an initial increase from day 0 to day 10.

Another good evidence of utilization of petroleum and petroleum products was that *A. anomalus* grown on MSM broth culture without any supplement recorded the least mean as signified by the spectrophotometer reading when compared with its growth on the MSM broth medium supplemented with petrol, kerosene and diesel. The higher spectrophotometer reading signifying growth activity for the MSM broth culture supplemented with petrol and petroleum products must have arisen from the metabolism of these added supplements by the aquatic phycomycetes species.

Comparing the growth patterns of *A. orion* and *A. anomalus* on MSM broth culture supplemented with petrol and petroleum products, one finds that both species shows the least growth values on broth culture supplemented with petrol and the highest spectrophotometer readings from the broth cultures supplemented with diesel and kerosene for *A. orion* and *A. anomalus* respectively. This somehow presupposes that these two aquatic phycomycetes species have abilities of utilizing these supplements as carbon sources, most importantly that they also have the least preference for utilization of petrol than diesel and kerosene. Therefore, there must be a structural reason that facilitates better growth values on MSM broth culture supplemented with diesel and kerosene than that supplemented with petrol. It is therefore important to note that diesel fuel is commonly heavier and more powerful than gasoline (petrol) engines. Martinez (2014) gave the average densities of gasoline (petrol), kerosene and diesel as 750, 780 and 830 kg/m^3, respectively. Diesel and kerosene were utilized better than petrol by the two aquatic phycomycetes species in this research work. Most of the earlier reported works on biodegradation of petrol and petroleum products by aquatic fungi have not involved aquatic phycomyctes, this seems to be one of such reports of biodegradation of petrol and petroleum products by species of aquatic phycomycetes.

Looking at the crude fat/lipid extraction from the final broth culture (FBC) at the end of the biodegradation experiment, one finds interesting results that supports the growth rate experiments. For instance, looking at the weight of the pellets from the final broth culture, one finds the least final weight values for the two species on broth culture supplemented with petrol. This may imply that the two species did not metabolize or accumulate the supplemented petrol to the extent of causing any appreciable increase in the final weight of the species as shown from the weight of the pellets from the final broth culture. In the same vein, since the final weight of aquatic phycomycetes grown in broth medium supplemented with petrol was the least, crude fat/lipid was not extracted from the pellets gotten from the final broth culture supplemented with petrol. The support of the fact that the aquatic phycomycetes species may have better abilities of utilizing diesel and kerosene more than petrol may also be buttressed from the final weight of the pellets resulting from the species grown on broth culture supplemented with diesel and kerosene. For instance, the weight of the pellets of Achyla *orion and Allomyces anomalus* grown on diesel supplemented broth culture were the highest at 0.1260 and 0.1880 g/100 kg respectively and equally produced the highest quantities of crudefat/lipid of 30.95 and 12.77 g/100 g respectively. For the kerosene supplemented final broth culture, *A. orion* did not yield any crude fat/lipid but *A. anomalus* yielded some 6.25 g/100 g of crude fat/lipid. *A. anomalus* grown on broth culture without supplement also yielded some 6.74 g/100 g of crude fat/lipid, which possibly shows that under normal circumstances, these species of aquatic phycomycetes are characterized by the possession of oil globules in their life cycle, which may be equivalent to the crude fat/lipid that was extracted. Hence, the highest quantity of crude fat/lipid produced by both species of aquatic phycomycetes grown on broth culture supplemented with diesel may therefore suggest better utilization and accumulation of diesel than both kerosene and petrol. In line with the results of this work where population of the species increased as evidenced by the increase in biomass as reflected by the weight of the pellets from the final broth culture, Abatenh et al. (2017) and Mahjoubi et al. (2017) also stated that population of some microbes increases and that such microbes use petroleum hydrocarbon as nutrients.

Conclusion

The study shows that both *A. orion* and *A. anomalus*

metabolized or utilized diesel and kerosene better than petrol as supplements in mineral salt broth culture medium. The study further concludes that the aquatic phycomycetes isolates after degrading the substrates probably also accumulated crude fat/lipid as a bye product of the degradation experiment since crude fat/lipid was extracted from the pellets resulting from the final broth culture.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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