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Assessment of Microorganisms Isolated from Steeping Maize (*Zea mays* L.) and Sorghum (*Sorghum bicolour* L.) on the Hydrolysis of some Hydrocarbon Products

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Abstract

In this work, the degradative ability of microorganisms isolated from steeping maize and sorghum on some hydrocarbon products namely petrol, diesel, engine oil, and kerosene is investigated. The steeping microorganisms were isolated and identified by standard microbiological methods, while its physicochemical parameters were determined. In addition, all the isolates were screened for their ability to utilize some hydrocarbon products. The microbial utilization was monitored by measuring the absorbance of each of the isolates in culture media in which the hydrocarbon products served as carbon sources. The associated bacterial isolates included *Bacillus subtilis*, *Erwinia herbicola* and *Lactobacillus plantarum*, while the fungal isolates were *Aspergillus flavus*, *A. niger*, *A. repens*, *Neurospora crassa*, *Penicillium italicum,* and *Candida krusei*-yeast. The pH values ranged from 4.07 to 5.09 (maize), 4.18 to 5.66 (sorghum); titratable acidity (TTA) ranged from 0.06 to 2.44% (maize), 0.19 to 2.32% (sorghum). Whilst the steeping water turned turbid, the colour of the grains changed from yellow to pale, and from red to brown. All the microbial isolates utilized the hydrocarbon products with the exception of *Erwinia herbicola* on diesel. Therefore, cereals steeping water could be a reservoir of microorganisms with the potential to bioremediate environments polluted with hydrocarbon products.

Keywords: Hydrocarbons, Steeping, Bioremediate, Maize, Sorghum

1. Introduction

Microbial degradation is important in the elimination of spilled petroleum products from the environment. Many bacteria and fungi have been demonstrated to have the ability to degrade specific fractions of petroleum compounds. Mixed cultures of bacteria and fungi have been used to degrade petroleum-derived hydrocarbon mixtures (Diaz et al., 2002), but single cultures of fungi have been found more effective than mixed cultures of bacteria and fungi or using traditional techniques involving bacteria (Okerentugba and Ezeronye, 2003; Ojumu et al., 2004). Fungi digest hydrocarbons through the secretion of extracellular enzymes, and they have the ability to grow in environments with low pH, nutrients and water activity. These group of fungi include *Articulospora, Aspergillus*, *Candida*, *Cladosporium*, *Fusarium*, *Helminthosporium*, *Mucor*, *Penicillium*, *Rhodosporium*, *Saccharomyces*, *Trichoderma*, *Umbelopsis*, *Varicosporium* (Ojumu et al., 2004; Ezeji et al., 2005; Akinyosoye et al., 2011). Bacteria capable of utilizing hydrocarbon compounds as a source of nutrients include *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Corynbacterium*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* (Ijah and Abioye, 2003; Ojumu et al., 2004; Ojo, 2006; Olalemi and Arotupin, 2012).

Maize (*Zea mays* L.) constitutes a staple food in many parts of the world. In Africa, it has become the most important staple food crop. Maize can be cooked or roasted to become palatable during consumption. Also, it is cooked to be served as a vegetable in side dishes, salads, garnishes as well as cornbread, cornflakes, and other baked products (Nuss and Tanumihardjo, 2010). The flour of maize is common in home cooking and industrialized food products. Maize starch can be hydrolysed and treated enzymatically to produce syrups used as sweeteners, and it may also be fermented and distilled to produce alcohol (Nuss and Tanumihardjo, 2010). The corn steep liquor is the watery by-product of maize wet-milling process and is widely used in the biochemical industry, and in research as a medium for culturing microorganisms. It has been demonstrated to be a potential resource for the bioremediation of soils polluted with hydrocarbon (Salam and Ishaq, 2019).

Sweet sorghum may be used to produce syrups, bread, beverages and ethanol (Nuss and Tanumihardjo, 2010; Maryke, 2010). Fermentation refers to the processes involving the production of ethanol by yeasts or organic acids by lactic acid bacteria. The process is a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate, is incompletely oxidised and acts as the electron acceptor (Adams, 1990). The fermentation of maize and sorghum grains contribute significantly to food technological processes in most low- and middle-income countries including Nigeria (Inyang and Idoko, 2006; Omemu et al., 2007).

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This study is aimed at determining the degradative ability of microorganisms isolated from steeping maize and sorghum on some hydrocarbon products such as petrol, diesel, engine oil and kerosene. It also aims at isolating and identifying microorganisms associated with fermenting maize and sorghum grains; and determining the ability of the isolates to utilize various hydrocarbon products.

2. Materials and Methods

2.1 Collection of Samples and Fermentation

Maize and sorghum samples were obtained from Oja-Oba in Akure, Ondo State, Nigeria. The samples were transported immediately in sterile black polythene bags to the Microbiology Research Laboratory. Impure grains were sorted and discarded, while the clean and apparently healthy grains were stored at ambient temperature. Fifty grams of each of maize and sorghum were weighed using a weighing balance (Triple beam 700/800 series, 2610g-5lb 2oz capacity) and were poured into a separate clean, sterile fermenter. Approximately, 150 ml of sterile distilled water was poured into each fermenter until the grains were fully submerged. Fermentation of the grains was allowed to take place for a period of seven days under appropriate conditions.

2.2 Determination of pH and Total Titratable Acidity

The pH of the steeping water was determined using Hanna multi-meter instrument (HI 98107). The total titratable acidity (TTA) was determined by titrating 0.1 N sodium hydroxide (NaOH) against 10 ml of steeping water containing three drops of phenolphthalein. The total titratable acidity was calculated and expressed as percentage by multiplying the molarity and volume of NaOH used and dividing it by the volume of the sample. The pH and %TTA were determined daily over the period of seven days

2.3 Enumeration and Identification of Bacterial and Fungal Population

One millilitre each of the steeping water was diluted in a ten-fold serial dilution. An aliquot of 1 ml from the third, fourth, and fifth dilutions was pour-plated with freshly prepared media: Nutrient agar (NA) and Potato Dextrose agar (PDA). The agar plates were incubated at 37°C for twenty-four hours (NA); 25°C for seventy-two hours (PDA) and were observed for growth. The discrete colonies of bacteria and fungi were counted, calculated and expressed as colony-forming units per millilitre (CFU/ml) and spore-forming units per millilitre (SFU/ml) respectively. The isolates were sub-cultured repeatedly to obtain pure isolates, and were characterized using standard microbiological techniques.

2.4 Determination of Rates of Utilization of Refined Petroleum Products by Isolated Bacteria and Fungi

Refined petroleum products including petrol, kerosene, diesel, and engine oil were obtained from Total Petrol Station along Oba Adesida road, Akure, Ondo State, Nigeria. Czapek broth was prepared containing; 3 g $NaNO_3$, 1 g K_4HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7H_2O$, 1 g peptone and 1000 ml distilled water with 1% refined petroleum product (petrol, diesel, kerosene, engine oil) as the only source of carbon. A positive control was also prepared with 30 g sucrose as the carbon source. The prepared media were inoculated with 0.1 ml of a nutrient broth of twenty-four-hour old cultures of isolated bacteria. The setup was incubated at 30°C for five days. Turbidity produced as a result of bacterial growth was monitored visually on a daily basis and the absorbance reading at 650 nm on UNICO 1100RS spectrophotometer was determined. Similarly, the Czapek agar (20 g agar added to the prepared broth) was inoculated with a 9 mm diameter culture disc of a forty-eight-hour old culture of isolated fungi and was incubated at 30°C for five days. Nutrient utilization was measured by determining the radial diameter of the fungal growth.

2.5 Statistical Analysis

The data obtained were subjected to general descriptive statistics and the single factor analysis of variance (ANOVA), while the significant means were separated with the new Duncan's multiple range test (DMRT) at 5% confidence level ($p = 0.05$) using Statistical Package for Social Sciences (SPSS) Version 20.

3. Results

3.1 The pH and Total Titratable Acidity of the Steeping Water of Maize and Sorghum

The epicarp of the yellow maize and red sorghum grains was hard before fermentation and the steeping water was clear. As fermentation progressed, the maize turned pale and sorghum turned brown with the epicarp of both grains becoming soft, and the colour of the steeping water turning turbid. The values of pH of the steeping water ranged from 4.07 to 5.09 (maize) and 4.18 to 5.66 (sorghum) (Figure 1). Also, the values of pH decreased steadily as the fermentation progressed, the total titratable acidity (%) increased steadily. TTA ranged from 0.06 to 2.44% (maize) and 0.19 to 2.32% (sorghum) (Figure 2).

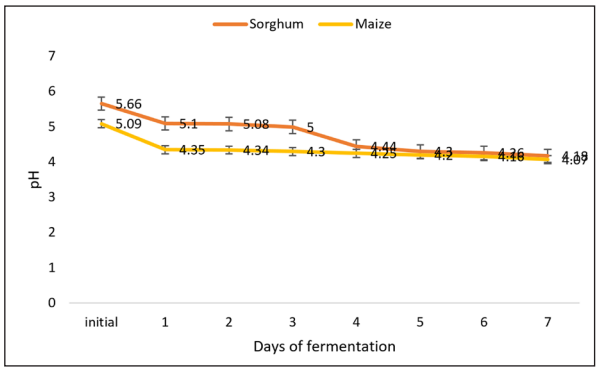

Figure 1. pH of the steeping water of maize and sorghum grains.

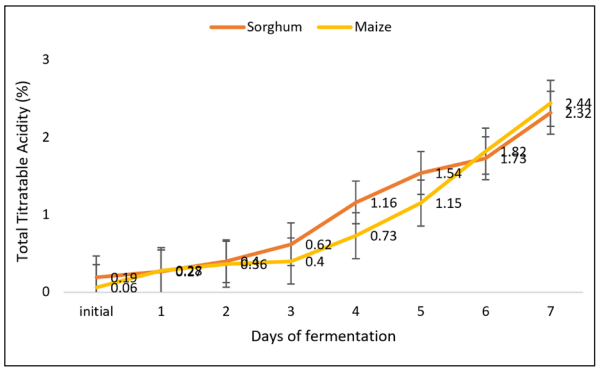

Figure 2. Total titratable acidity (%) of the steeping water of maize and sorghum grains.

3.2 Detection of Bacteria and Fungi in the Steeping Water of Maize and Sorghum Grains

Three bacterial isolates were detected in the steeping water of maize and sorghum. Isolate A was observed to be Gram-positive, motile, spore former, catalase-positive

and hydrolysed starch. Isolate B was observed to be Gram-positive, motile, spore former, catalase-positive and does not utilize citrate. Isolate C was observed to be Gram-negative, motile, spore former, catalase-positive and does not produce indole (Table 1). On the basis of these Gharacteristics, isolates A, B, and C were tentatively identified as *Bacillus subtilis*, *Lactobacillus plantarum* and *Erwinia herbicola* respectively. *Bacillus subtilis* (47%) had the highest percentage of occurrence in the steeping water of maize and sorghum, followed by *Lactobacillus plantarum* (41%) and *Erwinia herbicola* (12%) was the least (Table 2). Furthermore, six fungal isolates were detected in the steeping water of maize and sorghum. Based on their morphological characteristics, the isolates were tentatively identified as *Aspergillus flavus*, *A. niger*, *A. repens*, *Candida krusei*, *Neurospora crassa,* and *Penicillium italicum*. Interestingly, *Neurospora crassa* (33%) had the highest percentage of occurrence in the steeping water of maize and sorghum, while *Candida krusei* (5%) had the least (Table 3).

Table 1. Morphological and biochemical characteristics of bacterial isolates from the steeping water of maize and sorghum grains.

Characteristics	Isolates		
	A	B	C
Morphological			
Colour	Creamy	Creamy	Creamy
Surface	Rough	Rough	Rough
Cell shape	Rod	Rod	Rod
Cell arrangement	Pairs	Singly	Singly
Colony morphology	Opaque	Opaque	Opaque
Biochemical			
Gram reaction	+	+	-
Catalase	+	+	+
Motility	+	+	+
Spore	+	+	-
Urease	-	+	-
Oxidase	-	+	-
Indole	+	+	-
Citrate	+	-	+
Hydrogen sulphide	+	+	+
Carbohydrate utilization			
Starch hydrolysis	+	+	-
Glucose	+	+	+
Galactose	+	+	+
Fructose	+	+	+
Maltose	+	+	+
Lactose	-	+	+
Sucrose	+	+	+
Arabinose	+	-	+
Mannitol	+	+	+
Sorbitol	-	-	-

Key: + = Positive; - = Negative; Probable organisms: A – Bacillus subtilis; B – Lactobacillus plantarum; C – Erwinia herbicola

Table 2. Occurrence of bacterial isolates in the steeping water of maize and sorghum.

Isolated bacteria	Maize (n=10)	Sorghum (n=10)	Occurrence (%)
Bacillus subtilis	8	7	47
Erwinia herbicola	3	1	12
Lactobacillus plantarum	5	8	41

Key: Values represent the number of times the bacterial isolates were detected in the samples (n) of the steeping water of maize and sorghum.

Table 2. Occurrence of fungal isolates in the steeping water of maize and sorghum grains.

Isolated fungi	Maize (n=10)	Sorghum (n=10)	Occurrence (%)
Aspergillus flavus	3	5	14
Aspergillus niger	5	4	16
Aspergillus repens	6	7	22
Candida krusei	3	0	5
Neurospora crassa	9	10	33
Penicillium italicum	4	2	10

Key: Values represent the number of times the fungal isolates were detected in the samples (n) of the steeping water of maize and sorghum.

3.3 Utilization of Refined Petroleum Products by Bacterial and Fungal Isolates

The growth of bacterial isolates in media containing refined petroleum products measured at 650 nm absorbance revealed that *Bacillus subtilis*, *Lactobacillus plantarum*, and *Erwinia herbicola* grew heavily in petrol-based media. *Bacillus subtilis* exhibited the highest ability to utilize the refined petroleum products, whereas *Erwinia herbicola* demonstrated the least ability to utilize the products and exhibited no growth in diesel-based media. In addition, all the bacterial isolates exhibited moderate growth in kerosene- and engine oil-based media and minimal growth in diesel-based media. After forty-eight hours, bacterial growth in the control medium containing sucrose declined, whereas growth in media containing refined petroleum products continued even after ninety-six hours (Figure 3).

The growth of fungal isolates measured as radial diameter (cm) in media containing refined petroleum products showed that *Aspergillus flavus*, *A. niger*, *A. repens*, *Candida krusei*, *Neurospora crassa* and *Penicillium italicum* had the ability to utilize the refined petroleum products. Although, at forty-eight hours, the fungal growth in the control medium containing sucrose was greater than that in the media containing refined petroleum products. Generally, *A. flavus*, *A. repens*, *Neurospora crassa* and *Penicillium italicum* exhibited heavy growth in media containing refined petroleum products. Interestingly, *A. flavus* exhibited the highest ability to utilize petrol- and kerosene-based media, while *Neurospora crassa* showed the highest ability to utilize engine oil- and diesel-based media. On the other hand, *A. niger* and *Candida krusei* had a minimal growth in media containing refined petroleum products (Figure 4).

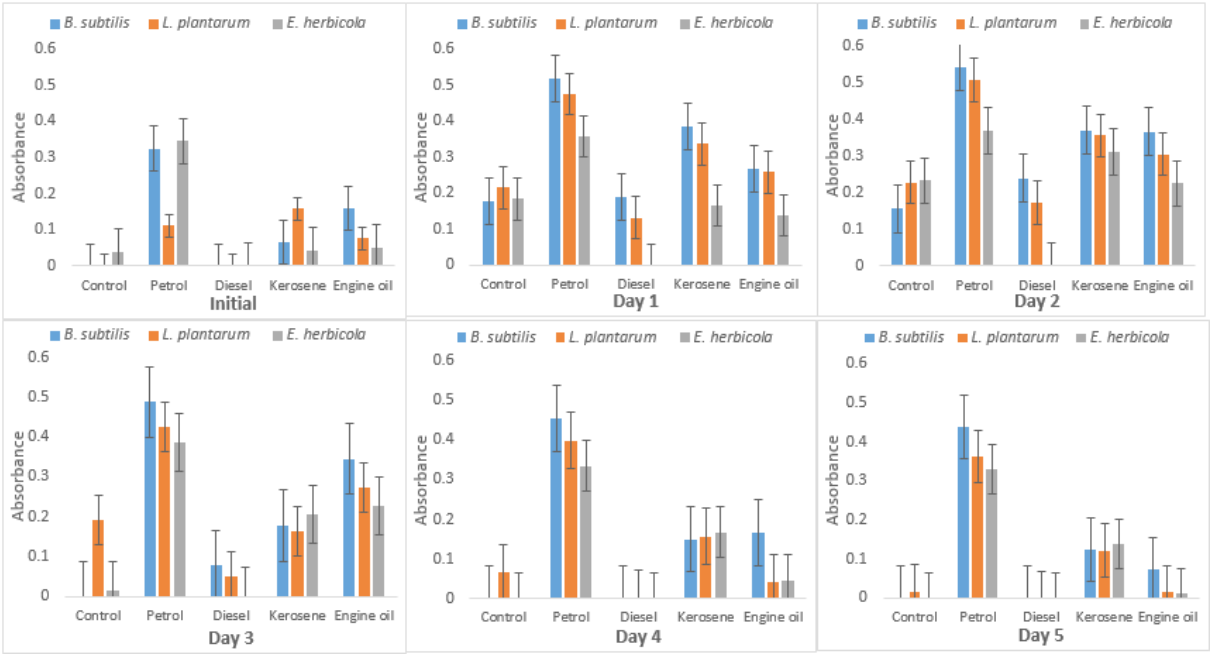

Figure 3. Growth of bacteria in media containing refined petroleum products measured at 650 nm absorbance.

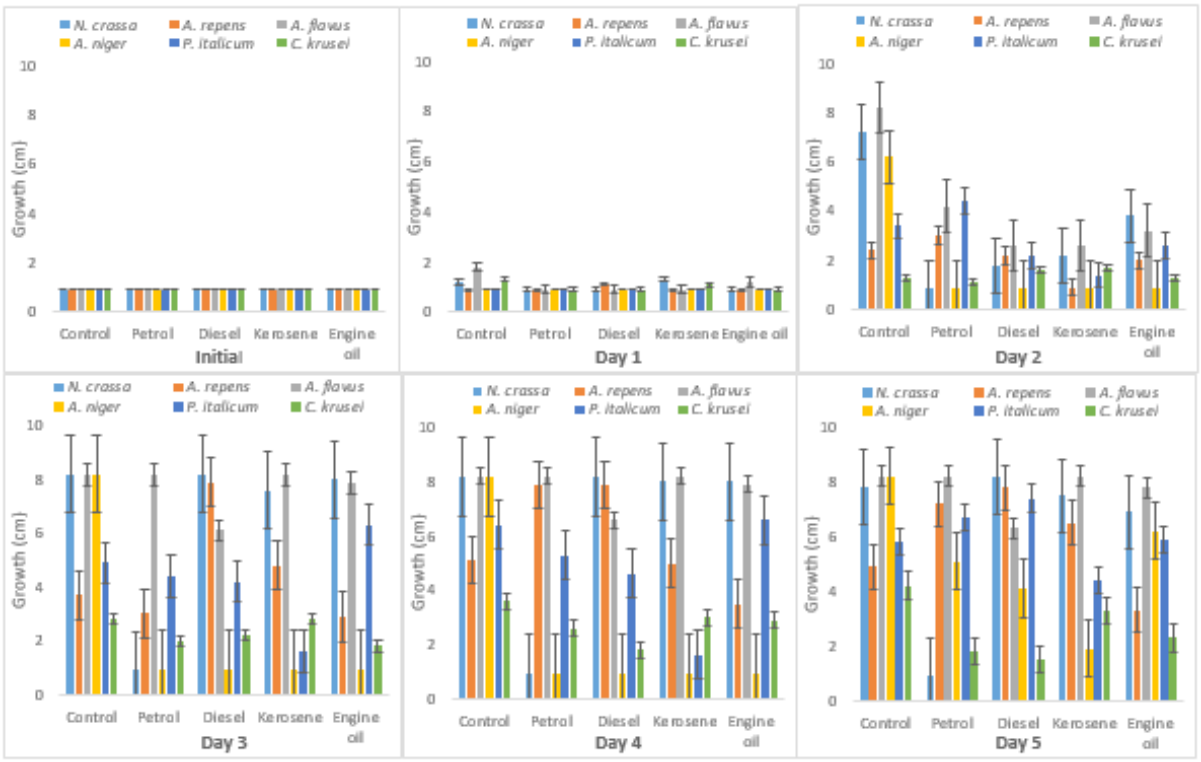

Figure 4. Growth of fungi measured as radial diameter (cm) in media containing refined petroleum products.

4. Discussion

This study investigates microorganisms associated with fermenting maize and sorghum grains. It examines whether their ability to utilize the carbon in some hydrocarbon products (such as petrol, diesel, engine oil and kerosene) as a basic source of carbon for growth may be useful especially for the remediation of environments polluted with refined petroleum products. The pH of the steeping water of maize and sorghum grains decreased steadily, while the total titratable acidity (TTA) increased as the fermentation progressed over the period of study. The decreasing pH and the increasing TTA may be a result of the accelerated growth of lactic acid bacteria in the steeping water. Studies have demonstrated that a decrease in pH and accumulation of lactic acid bacteria are common during the fermentation of foods (Choi et al., 1994; Inyang and Idoko, 2006; Akpinar-Bayizit et al., 2007). The lactic-acid bacteria hydrolyses the starch in the grains to produce acid which eventually reduces the pH and increases the TTA of the steeping water. The decrease in pH gives rise to conditions favourable for the souring of the fermented grains (Akpinar-Bayizit et al., 2007).

In this study, the bacteria detected in the steeping water of maize and sorghum grains were *Bacillus subtilis*, *Lactobacillus plantarum* and *Erwinia herbicola.* Counts of *Lactobacillus plantarum* were moderate at the onset of the fermentation of the grains, but increased as the fermentation progressed. This may likely be a result of acidification in the steeping water. This observation is in agreement with Abegaz (2007) who observed increasing counts of lactic-acid producing bacteria attributed to the acidification of the fermentation medium. However, the decrease in the counts of lactic-acid producing bacteria at the later stages of fermentation may be attributed to the depletion of available nutrients in the cereal slurry (Muyanja et al., 2003). Fungi detected in the steeping water of maize and sorghum were *Aspergillus flavus*, *A. niger*, *A. repens*, *Candida krusei*, *Neurospora crassa* and *Penicillium italicum.* Species of *Aspergillus* and *Penicillium* were eliminated during the early steeping period and after twenty-four hours of fermentation of the grains. This observation may likely be due to the reduction in pH and the acidification of the fermentation medium (Abegaz, 2007).

The rates of utilization of refined petroleum products by *Bacillus subtilis*, *Lactobacillus plantarum* and *Erwinia herbicola* varied. All the bacterial isolates utilized petrol, diesel, engine oil, and kerosene suggesting that the organisms can adapt, survive, and grow easily in the refined petroleum-based medium, except for *Erwinia herbicola* which could not utilize diesel at all. The decline in bacterial growth in the control medium containing sucrose after forty-eight hours may likely be attributed to the exhaustion of carbon and other nutrients necessary for growth. On the other hand, the massive bacterial growth after ninety-six hours in the media containing refined petroleum products may not be unconnected with the high number of carbon atoms per molecule of hydrocarbons in the refined petroleum products (Collins, 2007). In this study, *Bacillus subtilis* exhibited the highest ability to utilize the refined petroleum products and this is in agreement with many studies that have demonstrated the high ability of *Bacillus subtilis* in the degradation of petroleum hydrocarbon (Ijah and Abioye, 2003; Ijah and Antai, 2003; Olalemi and Arotupin, 2012). The findings of this investigation points to *Bacillus subtilis*, *Lactobacillus plantarum* and *Erwinia herbicola* as promising isolates in the clean-up of petroleum hydrocarbon pollutants. The rates of utilization of refined petroleum products by *Aspergillus flavus*, *A. niger*, *A. repens*, *Candida krusei*, *Neurospora crassa* and *Penicillium italicum* were generally high suggesting that hydrocarbons in petroleum products do not resist attack by fungi.

Aspergillus flavus, *A. repens*, *Neurospora crassa* and *Penicillium italicum* exhibited the highest degradative ability as a result of their high cell densities with a concomitant visual increase in the radial diameter of their spread. This observation is in agreement with Ezeji et al. (2005) who reported that the species of *Aspergillus* and *Penicillium* are often implicated in the degradation of petroleum hydrocarbons. These findings suggested that *Aspergillus flavus*, *A. niger*, *A. repens*, *Candida krusei*, *Neurospora crassa*, and *Penicillium italicum* are potential fungi for the degradation of petroleum hydrocarbon products.

5. Conclusions

The findings of this study demonstrate the degradative ability of bacteria (*Bacillus subtilis*, *Lactobacillus plantarum* and *Erwinia herbicola*) and fungi (*Aspergillus flavus*, *A. niger*, *A. repens*, *Candida krusei*, *Neurospora crassa,* and *Penicillium italicum*) isolated from steeping maize and sorghum grains on hydrocarbon products such as petrol, diesel, engine oil and kerosene. Further understanding of this essential low-cost approach and metabolic processes of these organisms on the hydrocarbons would increase the possibilities of developing techniques that may be useful for the remediation of an environment polluted with refined petroleum products.

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