International Journal of Trend in Scientific Research and Development (IJTSRD)
Volume 5 Issue 5, July-August 2021 Available Online: www.ijtsrd.com e-ISSN: 2456 – 6470

Biodegradation Potentials of *Aspergillus Flavipes* Isolated from Uburu and Okposi Salt Lakes

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ABSTRACT

Saline lakes are water bodies with salinity greater than 3 g/l (0.3%), while hypersaline lakes are water bodies that surpass the moderate 35 g/l (3.5%) salt of oceans. **Hypersaline lakes, could either be thalassohaline (which are creations of evaporation of seawater and as such contain sodium chloride as the major salt,** with a salinity that surpasses that of seawater by a factor of 5–10, with a neutral or slightly alkaline pH**). Whereas, athalassohaline lakes stem from non-seawater sources and are made up of** high concentrations of ions such as **magnesium and calcium and sundry other ions such as potassium, or sodium** in smaller amounts. This work has revealed the biodegradation potentials of some halophiles isolated from Uburu and Okposi salt lakes. The isolates recovered in descending order of salt tolerance were *Aspergillus flavipes* (13mm at 40%), *Penicillium citrinum* (10mm at 40%), *Aspergillus ochraceus* (9mm at 40%), *Aspergillus nomius* (15mm at 35%), *Microsphaeropsis arundinis* (12mm at 35%), *Aspergillus sydowi* (28mm at 30%), *Penicillium janthinellum* (26mm at 30%), *Mucor* sp (13mm at 30%), *Aureobasidium* sp (12mm at 30%), *Trichoderma* sp (9mm at 30%), *Alternaria* sp. (22mm at 25%), *Aspergillus* sp (18mm at 25%), *Penicillium* sp (20mm at 20%), *Cladosporium* sp. (7mm at 15%) and identified using ITS rDNA Sequencing (Macrogen, South Korea). They belonged to the borderline extreme halophiles and moderate halophiles respectively. The biodegradative potential of *Aspergillus flavipes* was ascertained by testing it against 2%, 4% and 6% crude oil and it grew only on 2 % crude oil-*Bushnell-Haas* broth with a fungal count of 2.56×10^5 cfu/ml. Crude oil degradation rate was evaluated biweekly gravimetrically with 22% degradation in 2 weeks, 36% in 4 weeks, 67% in 6 weeks and 89% in 8 weeks; as well as by way of gas chromatography (GC-FID), which showed that fractions C_{10}-C_{11} were significantly degraded, C_{12}-C_{20}, moderately degraded and C_{26}-C_{34}, insignificantly degraded.

KEYWORDS: *Biodegradation, Potentials, Aspergillus flavipes, Uburu, Okposi, Salt Lakes*

How to cite this paper: Kingsley C. Agu | Frederick J. C. Odibo "Biodegradation Potentials of *Aspergillus Flavipes* Isolated from Uburu and Okposi Salt Lakes" Published in International Journal of Trend in Scientific Research and Development (ijtsrd), ISSN: 2456-6470, Volume-5 | Issue-5, August 2021, pp.1160-1170, URL: www.ijtsrd.com/papers/ijtsrd44949.pdf

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INTRODUCTION

The existence of petroleum predates man, but the present day petroleum industry was established in 1859 by Colonel E.A. Drake in Romania [1]. The early usage of crude oil was primarily for lighting as it conveniently replaced whale oil which was expensive; nonetheless, today, it is used as fuel and it is at present Nigeria's and indeed, the world's leading energy source [2]. Petroleum is the name given to a mix of condensate, natural gas and crude oil. Crude oil is a complex combination of varying molecular weight hydrocarbons comprising hydrogen and carbon in the ratio of 2:1. It also consists of about 3% (v/v) oxygen, nitrogen and sulphur; trace amounts of phosphorus and heavy metals namely nickel and vanadium [1,3-6]. There are 4 groups of hydrocarbons in crude oil *viz.* the saturated hydrocarbons and the aromatics; the more polar, non hydrocarbon components; the resins and the asphaltenes. Moreover, crude oil can be categorized according to individual distillation residues as naphthenes, paraffins and aromatics; and on the basis of heavy molecular weight components as heavy,

medium or light; and also based on the age, location and depth of the oil well as paraffin-based, asphalt-based or mixed based [7,8]. In Nigeria, oil fields litter the Niger-Delta region, having a large network of pipelines which convey crude oil to various refineries including the Kaduna refinery up north. These pipes get vandalized occasionally by hoodlums thus resulting in oil spillage and environmental pollution. Oil spillage is therefore, the discharge of oil into the natural environment with its consequent perils. Bioremediation is the application of micro organisms, plants, or microbial or plant enzymes to decontaminate and reclaim an environment [9]. The concept embodies a host of other processes including biodegradation. Biodegradation is the biologically catalyzed break down in the structural and molecular complexity of compounds into smaller components such as carbondioxide and minerals by enzymatic or metabolic processes in the environment [10]. Nonetheless, in marine, saline and hypersaline environments, biodegradation is an uphill task owing to the harmful impact of salt on micro organisms and as such in order to successfully bioremediate such environments, dilution is done which is costly; therefore, to clean up this type of environment, a distinct bioremediation approach employing halophiles and halotolerant micro organisms capable of biodegrading crude oil in saline or hypersaline conditions becomes necessary [11]. Much of the earth's surface is occupied by water and a greater percentage of the earth's hydrosphere is salt water (Oceans and seas), in spite of this, literature on crude oil degradation by halophilic and halotolerant micro organisms especially molds remains meager [12]. Of this paltry amount of literature on crude oil degradation by halophilic micro organisms, those associated with molds are relatively insufficient, regardless of the fact that they are decomposers.

MATERIALS AND METHODS

Isolation, Characterization and Identification of Halophilic Fungal Species

Fungal Isolation and Characterization

The water samples obtained from the lakes quarterly from January to December were stored in ice chests and transported to the laboratory before being transferred to refrigerators. Exactly 0.1ml of the water samples were transferred into the centre of already prepared agar plates using sterile pipettes. With the aid of a sterile glass spreader, the aliquot was spread evenly on the surface of the agar plate. All fungal media were amended with 0.5mg/ml of Chloramphenicol to inhibit bacterial growth. Plates were incubated at room temperature for 10 days each. Developing fungal isolates were purified by repeated subculture technique and transferred to Bijou bottles with agar slopes for identification and storage. Czapex-Dox Agar (CzA) and SDA prepared with the lake water were used for the isolation of halophilic fungal species.

Identification of Fungal Isolates

Preliminary fungal characterization were done by studying the cultural characteristics and employing the slide culture wet mount technique for evaluating the fungal microscopic features with reference to the Manual of Fungal Atlases according to [13-16]. The identities of 7 most halophilic isolates were confirmed at Macrogen Inc., 10F, 254 Beotkkot-ro, Geumcheon-gu, Seoul, Republic of Korea, using the ITS rDNA Sequence Analyses. Molecular assays were carried out on each sample using nucleic acid as a standard. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, Polymerase Chain Reaction (PCR) was employed to amplify copies of the rDNA *in vitro*. The quality of the PCR product was assessed by undertaking gel electrophoresis. PCR purification step was carried out to remove unutilized dNTPs, primers, polymerase and other PCR mixture compounds and obtain a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK). Modules containing pre-hydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing Big Dye® terminators. Dye removal was followed by suspension of the purified products in highly deionized formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. Sample was loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide. Following sequencing, identifications were undertaken by comparing the sequence obtained with those available from the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI). The strains were

identified using Inter specific region sequencing analyses [17].

Halotolerance Test of the Isolates

Salt tolerance of the isolates was checked by inoculating the developing cultures in triplicates on CzA amended with salt up to concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 % w/v. Growth was recorded after 7 days incubation in terms of colony diameter. Plates that did not show growth up to 7 days were further incubated till the fifteenth day to check for delayed growth. Thereafter, the colony diameter of the molds in millimetres plotted against percentage salt concentration in the medium was used to determine their salt tolerance [18]. The salt tolerance study was used to classify the isolates as slight halophiles (0.2-0.5 or even 0.85 M salt equivalent to 1-5%), moderate halophiles (0.5-2.5 M or 0.85-3.4 M salt equivalent to 5-20%), borderline extreme halophiles (1.5-4.0 M salt equivalent to 9-23%) and extreme halophile (2.5-5.2 M salt equivalent to 15-30%) according to various classification schemes proposed by [19-23]. The most halophilic isolate was then used for the biodegradation studies.

Growth Tolerance of *Aspergillus flavipes* to different Percentage of Crude Oil.

The Bonny light crude oil used for this study was obtained from from Warri Refinery and Petrochemicals. Three set of 100ml conical flasks were prepared containing 49 ml of 2%, 4% and 6% crude oil in Bushnell-Haas broth. Exactly 1 ml of the 10^{-2} dilution tube of the 24 hours Sabourand dextrose broth culture of *Aspergillus flavipes* was used to seed each flask, incubated at room temperature and the developing colonies counted after 48 hours. Upon completion of the 48 hours of incubation period, 0.1 ml aliquots of the 2%, 4%, 6% of crude oil amended *Bushnell-Haas* broth culture were plated out on *Bushnell-Haas* agar and incubated at room temperature for 48 hours and the total fungal count obtained.

Preliminary Hydrocarbon Degradation Studies

Modified method of [12] was employed for this study. The concentration of crude oil that gave the best growth rate on agar plate (Bushnell-Haas amended with 2% crude oil) was used for this study. Four Erlen-meyer flasks (100ml) containing 50 ml Bushnell-Haas broth amended with 2% crude oil, were inoculated with 1ml of 24 hours broth culture of *Aspergillus flavipes* and incubated in a rotary shaker for two months. Biweekly, one flask was withdrawn and the crude oil degradation rates evaluated gravimetrically using a separating funnel and also chromatograpically by GC-FID technique.

Evaluation of Hydrocarbon Degradation Rate by Gravimetry

The hydrocarbon degradation potential of the mold was evaluated using the modified gravimetric analysis method according to [25]. Exactly 5 ml of n-hexane was added to the fermentation flask containing the crude oil degradation set up and the contents transferred to a separating funnel. Extraction was done thrice to ensure thorough oil recovery. The extract was however mixed with 0.4g of anhydrous sodium sulphate to remove moisture and carefully transferred into a beaker leaving the sodium sulphate behind. This was evaporated to dryness by heating in a rotary evaporator. The amount of residual oil was measured after extraction of oil from the medium and evaporating it to dryness.

The crude oil degradation equation was derived thus:

$$W_4 = W_3 - W_1$$

$$W_5 = W_2 - W_1$$

$$\%\ \text{Oil Degradation Rate} = \frac{W_5 - W_4}{W_5} \times 100$$

$$\textbf{Or}\ \frac{(W_2 - W_1) - (W_3 - W_1)}{(W_2 - W_1)} \times 100$$

Where:
W1: Weight of empty beaker
W2: Weight of beaker + crude oil before degradation
W3: weight of beaker + recovered crude oil after degradation
W4: Residual crude oil
W5: Original weight of crude oil before degradation

Preparation of Samples for GC Analysis TPH Florisil Clean Up

Standard methods of [27] was used for this study. Florisil was heated in an oven at 130 ^{0}C overnight (ca.15h) and transferred to a 250ml size beaker and placed in a desicator. Then, 0.5g anhydrous $NaSO_4$ was added to 1.0g of activated flosiril (florisil charged with magnesium silicate) with mesh size of 60–100nm on an 8ml column plugged with glass wool. Packed column was filled with 5ml n–hexane for conditioning and the stopcock opened to allow n–hexane run out until it just reaches the top of the sodium sulphate into a receiving vessel whilst tapping gently the top of the column till the florisil settled well in the column. Extract was transferred into the column with disposable Pasteur pipette from an evaporating flask. Each evaporating flask was rinsed twice with 1ml portions of n–hexane to dislodge any residual sample and added to column. Eluent was collected into an evaporating flask and evaporated to dryness in a rotary evaporator. Dry eluent was

dissolved in 1ml acetone for Chromatographic analysis using Buck Gas Chromatograph Model No: 910 equipped with an HP 88 capillary on-column (100ml x 0.25µm film thickness,) CA, USA, automatic injector, Flame Ionisation Detector (Detector Temperature A: 250 ^{0}C, Injector temperature: 22 ^{0}C), Integrator chart speed: 2cm/min, Oven temperature: 180 ^{0}C.

RESULTS

Halotolerance Test of the Isolates

The isolates recovered in descending order of salt tolerance were *Aspergillus flavipes* (13mm at 40%), *Penicillium citrinum* (10mm at 40%), *Aspergillus ochraceus* (9mm at 40%), *Aspergillus nomius* (15mm at 35%), *Microsphaeropsis arundinis* (12mm at 35%), *Aspergillus sydowi* (28mm at 30%), *Penicillium janthinellum* (26mm at 30%), *Mucor* sp (13mm at 30%), *Aureobasidium* sp (12mm at 30%), *Trichoderma* sp (9mm at 30%), *Alternaria* sp. (22mm at 25%), *Aspergillus* sp (18mm at 25%), *Penicillium* sp (20mm at 20%), *Cladosporium* sp. (7mm at 15%) as seen in figures 1 to 14. From the above conclusions, it can be inferred that *Aspergillus flavipes*, *Penicillium citrinum*, *Aspergillus ochraceus*, *Aspergillus nomius*, *Microsphaeropsis arundinis*, *Aspergillus sydowi*, *Penicillium janthinellum*, *Mucor* sp, *Aureobasidium* sp, *Trichoderma* sp, *Alternaria* sp, and *Aspergillus* sp belonged to the extreme halophiles class, whereas, *Penicillium* sp and *Cladosporium* sp belonged to the borderline extreme halophiles and moderate halophiles respectively. No slight halophile was isolated in this work as seen in Table 1.

Table 1: Phenotypic and Genotypic Characteristics of Fungal Isolates from Uburu and Okposi Salt Lakes

Code	Colony morphology	Microscopy	Identity
UBA	Colonies on SDAwere dark green with yellowish tint, with cushion-shaped structures distributed. Needle-shaped crystals were characteristically formed in cultures. Reverse side was tan	Conidiophores were hyaline, erect, branched, bearingspore masses apically at verticillate phialides: phialides were short andthick. Conidia werephialosporous, hyaline, globose, subglobose, or ovate, one-celled. Chlamydospores were brown and subglobose.	*Trichoderma sp*
UBD	On SDA, colonies were floccose (cottony in texture), pale greyish-brown. Growth rate was rapid, thus, colonies filled the entire petri-dish in 3 days. Colour on the reverse side was yellow. Colonies were incubated at 30 ^{o}C for 5 days.	Sporangiophores were hyaline, erect, non-septate and branched sympodially and circinate. Sporangia were terminal,dark-brown, finely echinulate to smooth and spherical (20- 80 µm in diameter). Sporangiospores were hyaline or pale-brown. Collumellae were ellipsoidal and 4.5-7 x3.5-5 µm in size. Chlamydospores were absent.	*Mucor sp*
UBE	On SDA colonies displayed moderately slow growth on at 30°C. texture is velutinous (soft, velvety surface) to floccose (woolly tufts of soft "hairs"). The colonial growth appeared radially sulcate (narrow, deep furrows or radial grooves –like spokes on a wheel). The mature colony had a central greyish-turquoise to greyish-orange colour with a white periphery (outer edge). Exudates (extrolites) were frequently produced which appeared as drops of liquid upon the surface of the colony, which appeared to be clear, to pale yellow, to reddish-brown in colour. The reverse was a pale yellow to a light yellow-brown.	*Organism* produced septate, hyaline (clear, not pigmented) hyphae. Smooth-walled conidiophores stipes were rather long (100 – 300 µm) and is biverticillate. Metulae were 12 – 15 µm in length which were found in whorls of 3 – 5 divergent structures. Phialides were ampuliform (flask-shaped) and about 7 – 12 µm in length. Conidia (2.2 – 3.0 µm diameter) were globose to sub-globose (round to off-round) and were smooth or had a finely roughened surface. Conidia resist disruption and formed rather long chains. These characteristics: the metulae longer than the phialides and the conidia being both spherical and produced in well-defined chains, were distinguishing features of *Penicilliumcitrinum.*	*Penicillium citrinum*

OKA	On SDA organisms were dull white to yellow to light yellow. Dark fluorescent yellow colour was observed on the reverse side. No sclerotia were observed and growth rate was good.	Conidiophore length was 403-521 µm, breadth 7.3-8.7 µm. conidiophores were yellowish to pale brown and coarsely roughened. Conidiospore size was 2.5-2.7 µm, ellipsoid, smooth to finely roughened. Vesicle diameter is 20-24 µm. phialides are biseriate, 7-8 µm in length and and 2.2-2.4 µm in diameter.	*Aspergillus Ochraceus*
OKC	On SDA, colonies were green with with white margins. The reverse side was cream to light cream-brown. Good growth rate observed.Coonial texture was velvety to floccose	Conidiophores are echinulate/colourless. Vesicle is globose and covered by biseriate phialides with globose echinulate conidia.sclerotia were dark and bullet shaped, conidia were subglobose/ellipsoidalwith fine rough walls.	*Aspergillus nomius*
UA	On SDA, colonies were slow growing, with dense aerial mycelium, initially greenish-grey, later becoming dark brown to grey-brown	Hyphae were septate, pigmented, and irregularly shaped, with swollen segments up to 4 µm in diameter. Pycnidia were sub-spherical, 250-350 µm in diameter; with a pseudoparenchymatous wall composed of very densely packed cells that appear angular in cross section (*textura angularis*). conidiogenous cells ampulliform, up to 5 µm long. conidia brown, thick- and smooth-walled, cylindrical, 3.5-4.5 × 1.0-1.5 µm.	*Microsphaeropsisarundinis*
UB	Colonies on SDA agar are velvety, pale grayish green on the surface. The colour on the reverse side is pale yellowish brown. Growth rate: 2–3 cm in diameter in 10 days after incubation at room temperature	Conidiophores hyaline, erect, branched penicillately at the apexes with verticillate metula, terminal phialides and catenulate conidia on each phialide, forming rather divergent conidial heads: phialides pen-pointed with abruptly tapered tips. Conidia phialosporous, pale green, dark in mass, ellipsoidal or subglobose, 1-celled, smooth, apiculate at one end.	*Penicillium janthinellum*
UC	On SDA,growth was moderately rapid and matured within 7 days of incubation. The colony diameter was 1-3 cm following incubation at 25°C for 7 days. Colonies were flat, smooth, resupinate, moist, yeast-like, mucoid to pasty, shiny and leathery in appearance. The surface was white, pale pink or yellow at the beginning and became brown to black and velvety with a grayish fringe by aging. Reverse was pale or black.	Blastoconidia were pale in color. Synchronous development of blastoconidia in tufts was observed. Hyphae were septate appearing hyaline at the beginning and getting dark brown by aging. The width of the hyphae was 2-10 µm but may be as thick as 15-20 µm. Conidiogenous cells, which were not much differentiated, were either intercalary or located terminally in the hyphae. The conidia (4-6 x 2-3 µm in size) were one-celled, hyaline and oval to cylindrical in shape. They formed clusters or were located along the hyphae. Blastoconidia, chlamydoconidia and arthroconidia may also be observed. Thick-walled, one- to two-celled, phaeoid arthroconidia were produced in old, mature cultures.	*Aureobasidiumsp*

OA	On SDA, green mycelium with yellowish, dull buff, aerial brownish with age. Slow growing colonies were observed. Reverse was orange-brown to red	Largely tightly packed conidia chains were observed Vesicles were subglobose to elliptical, up to 30 by 40 µm in the largest forms, usually with diameter twice that of the conidiophore in smaller forms. Sterigmata were in two series, colourless or nearly so, closely packed over the apex of the vesicle in smallheads, and covering the vesicle in large heads, primary sterigmata about6 or 8 µm by 2 to 3 µm, secondary sterigmata 5 to 8 µm by 1.5 to 2 µm. Conidia were 2to 3 µm, smooth, subglobose, colorless or nearly so under high magnification, with chains aggregated to form columns as seen with a handlens in oldcultures.	*Aspergillus flavipes*
OD	On SDA, Growth rate was moderate. Colour was blue-green to dark green to greyish-turquoise. Reverse was maroon to reddish-brown. Texture was as lanose *(woolly) and* velutinous *(dense, silky hairy*). Colonies produced rather long conidiophore stipes *approximately* up to 200 µm which gave the colony that woolly or hairy appearance.	The long, smooth-walled stipes which bore the conidiophores were hyaline generally (translucent/colourless) or slightly brownish. The vesicles (7.0 µm - 17 µm wide) were sub-spherical, pyriform (pear or teardrop shaped) to somewhat clavate (club shaped). Conidiogenous structures were biserate with metulae (2 µm -3.5 µm by 4 µm -6 µm) and phialides (2 µm – 3 µm by 5 µm – 7 µm) in size. Diminutive conidial structures were produced by many isolates which may resemble penicillate (like*Penicillium*) heads. Conidia were spherical, echinulate or spinose (rough, jagged texture), and were about 2.5 µm to 4.0 µm in diameter. Hülle cells were also present.	*Aspergillus sydowi*
UE	Cultures on SDA were fluffy, bright yellowish green with bluish green tint, funiculose with bundles of hyphae, reverse yellowish pink with reddish purple tint. Rather good in growth.	Conidiophores were hyaline, erect, developed from aerial hyphae, branched penicillately at the apexes with primary and secondary metula. verticillate phialides and catenulate conidia in each phialide, were seenwith open-spaced yellowish green conidial heads: phialides lanceolate or abruptly sharpened. Conidia werephialosporous, pale green, dark in mass, globose to subglobose, one-celled, minutely echinulate on the surface.	*Penicillium sp*
OKB	On SDA, colonies are fast growing, black to olivaceous-black or greyish, and are suede-like to floccose. Reverse was brown-black on reverse due to pigment production	Microscopically, branched acropetal chains (blastocatenate) of multicellular conidia (dictyoconidia) were produced sympodially fromsimple, sometimes branched, short or elongate conidiophores. conidia are obclavate, obpyriform, ovoid to ellipsoidal, often with a short conical or cylindrical beak, pale brown, smooth-walled and/or verrucose. *Alternaria* produces the pigment melanin therefore structures appeared brown to black in	*Alternaria sp*

		colour with dark septate hyphae	
OB	On SDA, colonies were powdery, flat, white at first, then turned yellowish-brown with age. Colour on the reverse side was yellow. Colonies were incubated at 30 ^{o}C for 5 days	Conidiophores appeared blue and terminated in a vesicle of uniseriate phialides. Conidia were one-celled, rough-walled and produced in long chains which were divergent.	*Aspergillus sp*
OKPb	On SDA, the growth rate *was* moderately good at room temperature and the texture was velvety to powdery. The colour was olivaceous green to black from the front and black from the reverse.	Conidia were elliptical to cylindrical in shape, pale to dark brown in colour and have dark hila. They occurred in branching chains that readily disarticulate. Conidial wall was smooth or occasionally echinulate. *They* produced unicellular conidia.	*Cladosporium sp*

Table 2: Halophilic Classification of Isolates

Slight Halophiles (1-5% Salt)	Moderate Halophiles (5-20% Salt)	Borderline Extreme Halophiles (9-23% Salt)	Extreme Halophiles (15-30% Salt)
	Cladosporium sp. (7mm at 15%)	*Penicillium* sp (20mm at 20%),	*Aspergillus flavipes* (13mm at 40%)
			Penicilliumcitrinum (10mm at 40%)
			Aspergillus ochraceus (9mm at 40%)
			Aspergillus nomius (15mm at 35%)
			Microsphaeropsisarundinis (12mm at 35%)
			Aspergillus sydowi (28mm at 30%)
			Penicillium janthinellum (26mm at 30%)
			Mucor sp (13mm at 30%)
			Aureobasidiumsp (12mm at 30%)
			Trichoderma sp (9mm at 30%)
			Alternaria sp. (22mm at 25%)
			Aspergillus sp (18mm at 25%)

Growth Tolerance of*Aspergillus flavipes* to different Percentages of Crude Oil

The growth tolerance of *Aspergillus flavipes* on different percentage concentrations of crude oil (2%, 4% and 6%) in *Bushnell-Haas* broth was studied. There was insignificant growth on 4% and 6% crude oil broth as shown by total fungal count. Only 2% crude oil broth had significant confluent growth with a fungal count of $2.56x10^{5}$ CFU/ml. thus the biodegradation study was performed using 2% crude oil in broth, as shown in Table 3.

Bi-weekly Degradation Rate of of crude oil by *Aspergillus flavipes* as shown by Gravimetry

The crude oil degradation rate of *Aspergillus flavipes* was evaluated by gravimetric method bi-weekly. After the first two weeks, it was observed that the crude oil had been degraded by 36%, by 4 weeks, it was 67%; by 6 weeks, it was 89%; and upon completion of the 8 weeks, the percentage degradation had reached 91% as shown in Figure 1.

Bi-weekly Total Petroleum Hydrocarbon Degradation of crude oil by *Aspergillus flavipes* as shown by GC-FID Analysis

The bi-weekly total petroleum hydrocarbon (TPH) degradation was investigated for a period of 8 weeks and the following inferences were drawn. Fractions C10 and C11 were significantly degraded. Whereas, fractions C12-C20 were moderately degraded; however, fractions C26-C34 were insignificantly degraded as shown in Figure 2.

Table 3: Growth Tolerance of *Aspergillusflavipes* on different Percentages of Crude Oil

Samples	No. of colonies on plate	Total fungal count (CFU/ml) 10^{-2}
2% crude oil + Bushnell-Haas broth	256	2.56×10^5
4% crude oil + Bushnell-Haas broth	TFTC	TFTC
6% crude oil + Bushnell-Haas broth	TFTC	TFTC

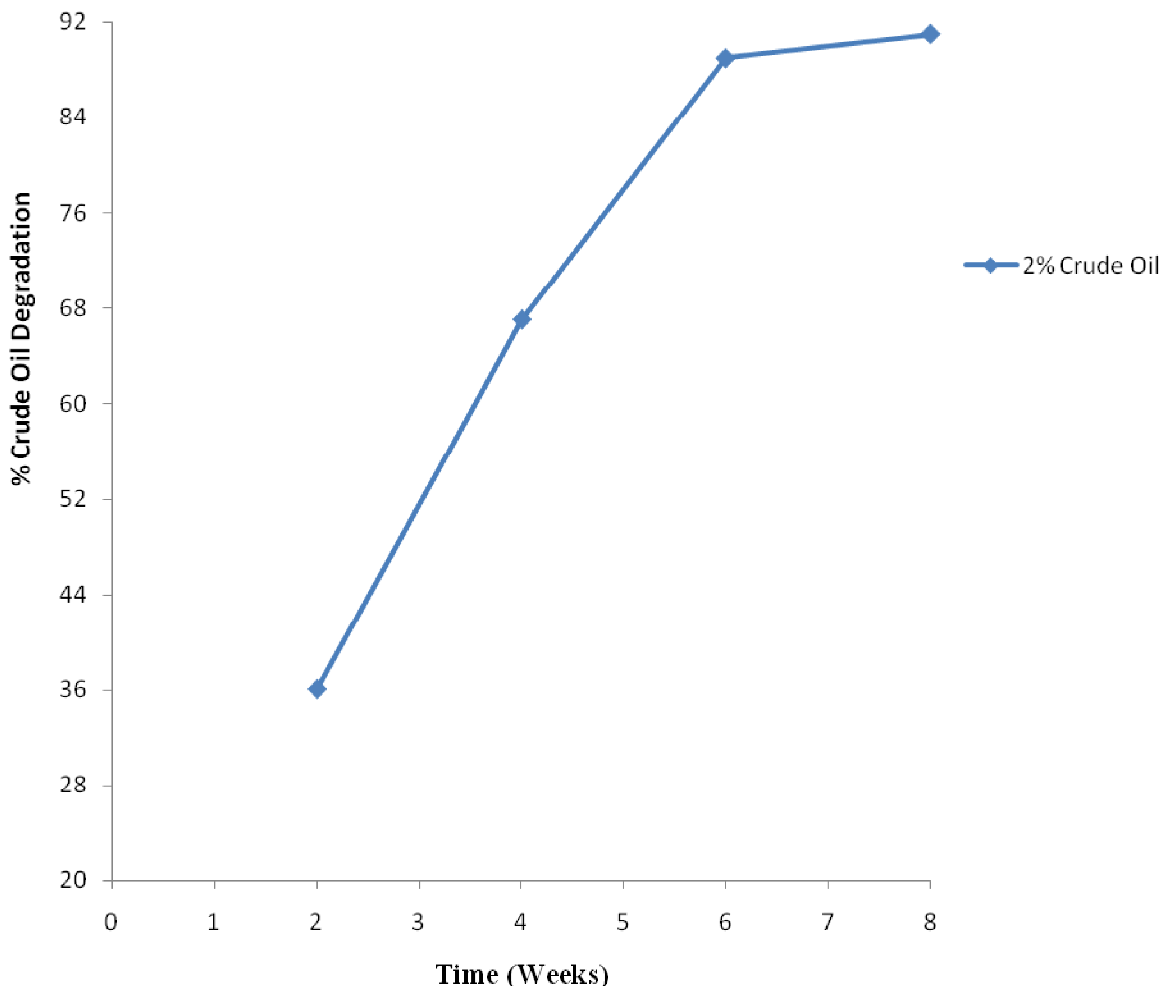

Figure 1: Bi-weekly Degradation Rate of crude oil by *Aspergillus flavipes* as shown by Gravimetry

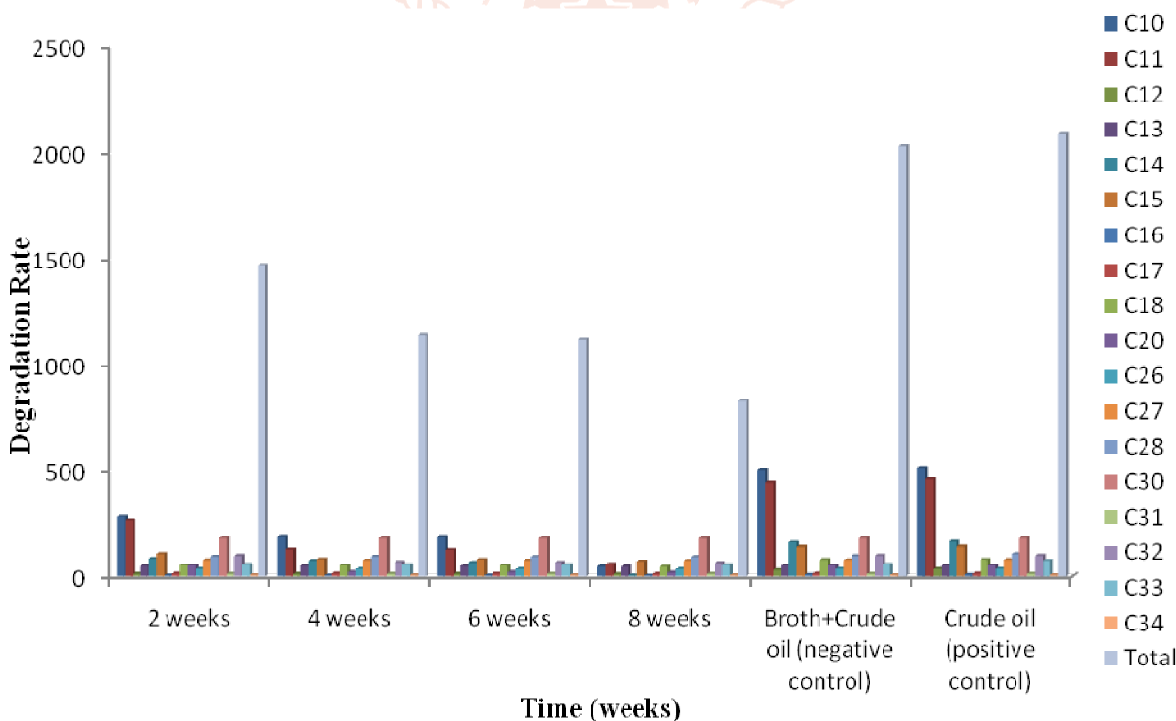

Figure 2: Bi-weekly Total Petroleum Hydrocarbon Degradation of Crude Oil by *Aspergillus flavipes* as shown by GC-FID Analysis

DISCUSSION

A little above two decades ago, scientists believed that hypersaline environments were occupied by archaea, bacteria and a eukaryote, the alga named *Dunaliella salina.* But today, several researchers such as [28-33] have isolated a plethora of eukaryotic fungal species predominantly *Cladosporium*, different species within the anamorphic *Aspergillus* and *Penicillium*, the teleomorphic *Emericella* and *Eurotium*, certain species of non-melanized yeasts represented by black, yeast-like hyphomycetes: *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum*, and *Aureobasidium pullulans*, together with phylogenetically closely related *Cladosporium* species, all belonging to the order Dothideales, and *Wallemia* sp from various hypersaline waters of the world and this work has corroborated that fact by isolating numerous halophilic fungi from uburu and Okposi salt lakes (Tables 1 and 2). A total of 14 different fungal isolates namely *Aspergillus flavipes* (13mm at 40%), *Penicillium citrinum* (10mm at 40%), *Aspergillus ochraceus* (9mm at 40%), *Aspergillus nomius* (15mm at 35%), *Microsphaeropsis arundinis* (12mm at 35%), *Aspergillus sydowi* (28mm at 30%), *Penicillium janthinellum* (26mm at 30%), *Mucor* sp (13mm at 30%), *Aureobasidium sp* (12mm at 30%), *Trichoderma* sp (9mm at 30%), *Alternaria* sp. (22mm at 25%), *Aspergillus* sp (18mm at 25%), *Penicillium* sp (20mm at 20%) and *Cladosporium* sp. (7mm at 15%) were isolated from Uburu and Okposi salt lakes throughout the 4 quarters and two seasons of the the year (Table 2). The seasons were divided in to rainy (April-September) and dry (October-March). *Aspergillus* and *Penicillium* species occurred throughout the study period in both lakes. However, the top 7 extreme halophilic fungi *viz. Aspergillus flavipes*, *Penicillium citrinum, Aspergillus ochraceus, Aspergillus nomius*, *Microsphaeropsis arundinis*, *Aspergillus sydowi and Penicillium janthinellum* were characterized and identified phenotypically and genotypically, while, the rest were characterized only phenotypically (Tables 1 and 2). Of the 14 isolates, 12 were extremely halophilic, one was a borderline extreme halophile and another was a moderate halophile; moreover, Gunde-Cimerman *et al*. (2009) stated that fungi isolated from hypersaline habitats of 1.7 M (~10%) salt concentration and can grow *invitro* at or above 3 M (~18%) salt concentration should be considered as a halophilic fungus (Table 2). All the organisms isolated in this study tolerated this salinity range, and some of them exceeded the salinity range as well. No slight halophile was isolated in this work as seen in table 2. These findings agrees to some extent to the work of Mansour (2017) who collected sandstone samples from the Medamoud, Egypt, and halophilic fungi (*Aspergillus nidulans, Aureobasidium pullulans* and *Cladosporium sphaerospermum*) from a local culture bank. Mansour estimated salinity tolerance by growing these fungi at 0% to 25% of NaCl concentration supplemented on potato dextrose agar and broth and posited that 5% of NaCl was the best suited growth supplement for halophilic fungi and that halophiles showed better tolerance to salt as compared to the solid media; and that, 25% of NaCl concentration was found to inhibit any fungal growth. The fact that no slight halophilic fungus was isolate in this work is also in concordance with the reports of [12,19,20,23], who stated that micro organisms belonging to the slight halophiles are mostly marine bacteria such as *Vibrio*. The most halophilic fumgi that was isolated in this study was *Aspergillus flavipes* which had a colony diameter of 13mm at 40% salt concentration; hence it was used for biodegradation studies.

The growth tolerance of *Aspergillus flavipes* on different percentage concentrations of crude oil (2%, 4% and 6%) in *Bushnell-Haas* broth was studied. There was insignificant growth on 4% and 6% crude oil broth as shown by total fungal count. Only 2% crude oil broth had significant confluent growth with a fungal count of $2.56x10^5$ CFU/ml. thus the biodegradation study was performed using 2% crude oil in broth, as shown in Table 3. Obuewe *et al.* (2005) have demonstrated that two halophilic fungi namely *Fusarium lateritium and Drechslera* sp. were able metabolize crude oil, with degradation efficiency improved more than thrice if petroleumwas an additional source of carbon and energy instead of being the exclusive one [36]. However, in this work crude oil was an exclusive source of carbon and energy and hence reason why *Aspergillus flavipes* tolerated 2% crude oil concentration out of the 3 concentrations assayed (Table 3).

The crude oil degradation rate of *Aspergillus flavipes* was evaluated gravimetrically and chromatographically bi-weekly. The gravimetric analysis showed that after the first two weeks, the crude oil had been degraded by 36%, by 4 weeks, it was 67%; by 6 weeks, it was 89%; and upon completion of the 8 weeks, the percentage degradation had reached 91% as shown in Figure 1. However, the bi-weekly total petroleum hydrocarbon (TPH) degradation was investigated for a period of 8 weeks also by way of Gas chromatograph coupled with flame ionization detection, and the following inferences were drawn. Fractions C10 and C11 were significantly degraded. Whereas, fractions C12-C20 were moderately degraded; and fractions C26-C34

were insignificantly degraded as shown in Figure 2. This agrees with the findings of [37, 38] who stated that hydrocarbons differ in their susceptibility to microbial attack and generally degrade in the following order of decreasing susceptibility: n-alkanes >branched alkanes>low molecular weight aromatics >cyclic alkanes, >polyaromatic hydrocarbons.

Conclusion

Crude oil contamination affects the environment and in return, the environmental conditions impact its bioremediation processes. As a complex mixture, different hydrocarbon groups have varying bioavailability and biodegradability leading to different intrinsic bioremediation applicability. *Aspergillus flavipes* tested in the present study can be used in oil bioremediation programmes as it has the activity to grow in crude oil amended *Bushnell-Haas* broth as evidenced by the decline in the total petroleum hydrocarbon content of bonny light crude oil over an eight week period with 89 % TPH degradation rate, as shown by gravimetry and gas chromatography.

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