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Saprophytic and crude oil degrading fungi from cow dung and poultry droppings as bioremediating agents

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The population and types of saprophytic and crude oil degrading fungal genera from cow dung and poultry droppings was investigated monthly for a period of four months using standard methods. The total counts of saprophytic fungi ranged from 28.33 x 10^2 to 34.69 x 10^2 cfug^{-1} for the cow dung while that of poultry droppings ranged from 44.67 x 10^2 to 48.33 x 10^2 cfug^{-1}.The total counts of petroleum-utilizing fungi ranged from 4.67 x 10^1 to 6.67 x 10^1cfug^{-1} for cow dung and ranged from 9.67 x 10^1 to 14.33 x 10^1 cfug^{-1} for poultry droppings. The average counts of the total petroleum-utilizing fungi for cow dung and poultry droppings was 5.67 x 10^1 cfug^{-1} and 11.17 x 10^1 cfug^{-1} respectively. Statistical analysis using analysis of variance (ANOVA) and paired (t - test) comparison on the data obtained showed that there is no significant difference between cow dung and poultry droppings in both total saprophytic and petroleum-utilizing fungi. However, there was a significant difference between cow dung and poultry droppings in the counts of petroleum-utilizing fungi expressed as a percentage (%) of total saprophytic fungi at $P \leq 0.05$. Calculated t-value was 6.325 while tabular t-value is 3.182. The result suggests that the addition of cow dung or poultry droppings to polluted soils is beneficial because they can enhance the proliferation of mycoflora that may be suppressed by the addition of crude oil to the soil.

Key words: petroleum-utilizing fungi, polluted soils, mycoflora

Introduction

Different types of methods of restoration of oil-polluted sites exist. These vary from complete removal of the affected soil to doing nothing at all and "letting nature take its course" (McGill and Nyborg, 1975). According to Baker (1970); Odu (1972); Stebbings (1970), natural revegetations of the area affected by light spillages of crude oil have occurred without any special treatment. At low levels of contamination of crude, cultivation of soil without

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nutrient amendment is possible since reclamation of the minerals in the soil can take place in a very short time (Plice, 1948; Toogood, 1974). Naturally-occurring microbial communities that respond to the presence of contaminating hydrocarbons normally have more than one type of hydrocarbon utilizing microorganisms. For seeding oil slicks therefore, mixture of hydrocarbon utilizing microorganisms or a genetically engineered microorganism have been suggested (Horowitz and Atlas, 1978).

Bioremediation is the use of naturally-occurring microorganisms or genetically-engineered microorganisms (bacteria and fungi) by man, to detoxify man-made pollutants (Odgen and Adams, 1989). Since bioremediation is a microbial process, it requires the provision of nutrients among other factors or requirements.

Nutrient is one factor that can hinder biodegradation if not handled properly and could limit the rate of hydrocarbon degradation in the terrestrial environment (McGill and Nyborg, 1975).

According to OTA (1990), the addition of nutrients that can limit biodegradation to the spill site is necessary and those nutrients are not different from fertilizer. There are enough populations of hydrocarbon-utilizing organisms in the soil environment (Stone *et al*., 1942; Davis, 1967; Parkinson, 1973). In any case when oil is spilled in large quantities, the microorganisms in that environment will limite in their ability to degrade the petroleum due to lack of nutrients, but when nutrients are added to the environment, the organisms will regain enough ability to overcome the limitations and biodegradation will then take place unhindered.

The relatives contributions of both inorganic and organic nutrient supplements for the development of a cheaper and more effective rapid biodegradation (with less toxic by-product) supplementary substrates to augment the native soil fertility status, improve rate of oil recovery and crop yield as to sustain agricultural development has been investigated (Amadi *et al*., 1993; Obire and Akinde, 2006). They noted that nutrient supplementation of oil-polluted soil with poultry droppings as organic nutrient source in particular is beneficial for maize growth and it also enhances both biodegradation of oil and soil recovery.

Although there has been reports of laboratory investigations on the use of organic nutrients such as cow dung and poultry droppings in bioremediation of oil polluted sites (Amadi and Ue-Bari, 1992; Johnson *et al*., 1994; Obire and Akinde, 2006), there has been no investigation on the population and types of fungi (mycoflora) of these organic nutrients. Organic nutrients such as cow dung and poultry droppings when added to polluted sites act both as a source of nutrients and of microorganisms. It is therefore necessary to carry out

studies on the population and types of saprophytic and petroleum degrading mycoflora of cow dung and poultry droppings.

Materials and methods

Source of materials

Cow dung and poultry droppings used for the study were aseptically collected from abattoir and poultry farm respectively, situated within Nkpolu - Rumuigbo area of Rivers State. Collection of cow dung and poultry droppings was carried out monthly for a period of four months. All microbiological analyses were carried out within 24 hours after sample collection.

Media for isolation of fungi

The following media were used for isolation and enumeration of fungi. Potato dextrose agar (PDA) was used for isolation and enumeration of total heterotrophic fungi. The composition of the medium was potato, 200g; distilled water, 500ml; glucose – D, 15g; and agar No. 1, 20g. Tetracycline was added to prevent bacterial growth and permitted selective isolation of yeasts and moulds (Walker and Colwell, 1976; Paul and Clark, 1988; Harrigan and McCance, 1990). The medium was allowed to cool to 45^{o}C under aseptic condition, mixed thoroughly and then dispensed into sterile Petri dishes to set.

Oil agar medium was prepared according to the mineral salts medium (MSM) composition of Mills *et al*. (1978) as modified by Okpokwasili and Okorie (1988). The composition of the medium was NaCl, 10.0g; $MgSO_4.7H_2O$, 0.42g; KCl, 0.29g; KH_2PO_4, 0.83g; Na_2HPO_4, 1.25g; $NaNO_3$, 0.42g; agar, 20g; distilled water, 1 litre and pH of 7.2. The medium was used for isolation, enumeration and preliminary identification of petroleum-utilizing fungi (oil-degraders). The medium was prepared by the addition of 1% (v/v) crude oil sterilized with 0.22µm pore size Millipore filter paper Moslein France (Obire, 1988) to sterile MSM, which has been cooled to 45^{o}C under aseptic condition. Tetracycline was added to prevent bacterial growth. The MSM and crude oil were then mixed thoroughly and dispensed into sterile Petri dishes to set. Saprophytic and oil-utilizing fungi in cow dung and poultry droppings were isolated.

Saprophytic fungi in cow dung and poultry droppings were estimated by dilution plate count method (IPS, 1990). Sterile physiological saline i.e. 0.85% (w/v) sodium chloride was used as diluent for inoculum preparation. One gram (1.0gm) of homogenized cow dung or poultry droppings was aseptically

transferred, using a flame-sterilized steel spatula, into a sterile test tube containing 9.0ml of the diluent. This gave 10^{-1} dilution. Subsequently, a two-fold (10^2) serial solutions were prepared from the 10^{-1} dilution.

A zero point one millitre (0.1ml) aliquot of 10^{-2} dilution of each sample was aseptically removed with a sterile pipette and separately spread plated with flame-sterilized glass spreader on well-dried PDA plates and onto oil-agar plates in triplicates. The cultured plates were incubated at 28 ± 2°C for 5 to 7 days. After incubation, the colonies that developed on the PDA plates were counting and recorded as counts of total viable saprophytic fungi. For the estimation and preliminary identification of petroleum-utilizing fungi, oil agar plates were inoculated with 0.1ml aliquots of 10^{-1} dilutions of the soil samples incubated at 28 ± 2°C for 7 days. Colonies which developed and showed growth of colonies and zones of clearance of oil on the oil-agar plates were counted as petroleum-utilizing moulds. The colonies counted were computed and expressed as colony forming unit (cfu) per gram of cow dung or of poultry dropping. Discrete colonies were subcultured onto fresh medium for the development of pure isolates, which were stored on potato dextrose agar slants for subsequent characterization and identification tests.

Confirmatory identification of true petroleum-utilizing fungi

Crude oil utilization test was carried out for the confirmatory identification of actual petroleum-utilizing moulds using isolates obtained from the oil agar preliminary isolation medium. The composition and preparation of the crude oil utilization test medium was the same as that of oil agar medium except that oil was made available via vapour phase transfer (Thijsse and van der Linden, 1961).

Putative petroleum-utilizing mould isolates were streaked on plates of agar medium (one isolate per plate). In the inside of the Petri dish cover was placed a sterile filter paper (Whatman No. 1) saturated with filter-sterilized crude oil used in the study. This was aimed at supplying hydrocarbons as sole sources of carbon and energy for the growth of the microorganisms on the mineral salts agar medium surface through vapour phase transfer. All the plates were inverted and incubated at room temperature for 7 – 14 days (Okpokwasili and Amanchukwu, 1988). Uninoculated plates served as control. Colonies which appeared on the mineral salts agar medium plates were picked and purified on plates of potato dextrose agar. They were finally transferred onto petroleum dextrose agar slants. These were then considered confirmed petroleum-utilizing fungi.

Presumptive identification of fungal isolates

Pure Fungal cultures were observed while still on plates and after wet mount in lacto-phenol on slides under the compound microscope. Observed characteristics were recorded and compared with the established identification key of (Malloch, 1997).

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) on the data obtained for the fungal counts, carbon (IV) oxide evolution, and on the quantity of crude oil utilized. ANOVA was performed on all the treatment, while least significant difference test (LSD) was performed between each treatment and control with reference to Gomez and Gomez (1984).

Results and discussion

The present investigation has revealed the population and types of fungi (mycoflora) present in organic nutrients such as cow dung and poultry droppings which could be used in bioremediation of polluted environments. The results of the counts of total saprophytic fungi (x 10^2 $cfug^{-1}$), petroleum-utilizing Fungi (x 10 $cfug^{-1}$), and counts of petroleum-utilizing fungi expressed as a percentage (%) of total saprophytic fungi in cow dung and poultry droppings are as shown in Table 1.

The total counts of saprophytic fungi ranged from 28.33 x 10^2 to 34.69 x 10^2 $cfug^{-1}$ for the cow dung while that of poultry droppings ranged from 44.67 x 10^2 to 48.33 x 10^2 $cfug^{-1}$. The average counts of total saprophytic fungi for cow dung and poultry droppings were 32.25 x $10^2 cfug^{-1}$ and 46.84 x $10^2 cfug^{-1}$ respectively.

The total counts of petroleum-utilizing fungi ranged from 4.67 x 10^1 to 6.67 x 10^1 $cfug^{-1}$ for cow dung while it ranged from 9.67 x 10^1 to 14.33 x 10^1 $cfug^{-1}$ for poultry droppings. The average counts of the total petroleum-utilizing fungi for cow dung and poultry droppings were 5.67 x 10^1 and 11.17 x 10^1 $cfug^{-1}$ respectively.

Table 1. Counts of Total Saprophytic Fungi (x 10^2 $cfug^{-1}$), Petroleum-utilizing Fungi (x 10 $cfug^{-1}$), and of Petroleum-utilizing Fungi Expressed as a percentage (%) of Total Saprophytic Fungi in Cow Dung and Poultry Droppings.

Sampling	Cow dung			Poultry droppings		
	Saprophytic fungi (SPF) (x 10^2cfu)	Petroleum-utilizing fungi(PUF) (x 10)	PUF/SPT (%)	Saprophytic fungi (SPF) (x 10^2 cfu)	Petroleum-utilizing fungi(PUF) (x 10)	PUF/SPF (%)
1	34.67	6.67	1.92	44.67	9.67	2.16
2	28.33	6.00	2.11	46.67	14.33	3.07
3	33.67	4.67	1.39	47.67	10.33	2.17
4	32.33	5.33	1.65	48.33	10.33	2.14
Total	129	22.67	7.07	187.34	44.66	9.54
Average	32.25	5.67	1.77	46.84	11.17	2.39

The counts of petroleum-utilizing fungi expressed as a percentage (%) of total saprophytic fungi in cow dung and poultry droppings ranged from 1.39% to 1.92% for cow dung while for poultry droppings it ranged from 2.14% to 3.07%. Statistical analysis using analysis of variance (ANOVA) and paired (t - test) comparison on the data obtained showed that there is no significant difference between cow dung and poultry droppings in both total saprophytic and petroleum-utilizing fungi. However, there was a significant difference between cow dung and poultry droppings in the counts of petroleum-utilizing fungi expressed as a percentage (%) of total saprophytic fungi at $P \leq 0.05$. Calculated t-value was 6.325 while tabular t-value is 3.182 with the percentage of petroleum-utilizing fungi expressed as a percentage (%) of total saprophytic fungi being higher in the poultry droppings than in the cow dung. A range of 0.7% to 2.68% was report by Obire (1988) for water systems of petroleum producing areas.

The saprophytic fungi (yeasts and moulds) isolated from cow dung used for the investigation were *Alternaria* sp*., Aspergillus* sp*., Cephalosporium* sp*., Cladosporium* sp*., Geotrichum* sp*., Monilia* sp*., Mucor* sp*., Penicillium* sp*., Rhizopus* sp*., Sporotrichum* sp*., Thamnidum* sp*., Candida* sp*., Rhodotorula* sp*. and Torulopsis* sp. The saprophytic fungi isolated from poultry droppings were *Alternaria* sp*., Aspergillus* sp*., Cladosporium* sp*., Fusarium* sp*., Geotrichum* sp*., Mucor* sp*., Penicillium* sp. and *Trichoderma* sp*., Candida* sp*., Rhodotorula* sp*., Torulopsis* sp. and *Trichosporon* sp. The petroleum-utilizing fungi isolated from cow dung were *Aspergillus* sp*., Cephalosporium* sp*., Cladosporium* sp*., Geotrichum* sp*., Mucor* sp. *Penicillium* sp*., and Candida* sp. While the petroleum-utilizing fungi isolated from poultry droppings were *Aspergillus sp., Cladosporium* sp*., Fusarium* sp*., Geotrichum* sp*., Mucor* sp*., Penicillium* sp*., Trichoderma* sp*., Candida* sp. and *Rhodotorula* sp. The result suggests that the addition of cow dung or poultry manure to polluted soils can enhance the

proliferation of mycoflora that may be suppressed by the addition of crude oil to the soil.

This shows that apart from improved soil fertility brought about by the addition of these organic nutrients (i.e. cow dung and poultry droppings) to soil, the addition of cow dung and poultry dropping to oil-polluted soils will result in an increase in the population of total saprophytic fungi and an increase in the population of petroleum-utilizing in soil. The fungi reported to have been isolated from cow dung by Gadre *et al.*, (1986) include *Aspergillus* spp*., Piromonas commonis, Sphaeromonas communis, Rhizopus* spp*., Mucor* spp*.,* and *Penicillium* spp. Ahearn *et al*. (1971) isolated strains of *Candida, Rhodosporidium, Rhodotorula, Saccharomyces Sporobolomyces,and Trichosporon*, which are capable of oil degradation. Cladosporium resinae has been isolated from soil (Cooney and Walker, 1973; Walker *et al*., 1973). Westlake *et al*., (1974) reported that the most important fungal genera (based on the frequency of isolation) were *Candida, Rhodotorula, and Sporobolomyces.* However, Bartha and Atlas (1977) listed 14 genera of fungi which had been demonstrated to contain members which utilize petroleum hydrocarbons. The genera were *Aspergillus, Aureobasidium, Candida, Cephalosporium, Cladosporium, Cunningamella, Hansenula, Penicillium, Phodosporidium, Rhodotorula, Saccharomyces, Sporobolomyces, Torulopsis, Trichosporon.* In Nigeria, the fungi reported as oil-degraders in aquatic environments of petroleum producing areas by Obire (1988) were *Candida, Rhodotorula, Saccharomyces* and *Sporobolomyces* species and the moulds were *Aspergillus niger, Aspergillus terreus, Blastomyces* sp*., Botryodiplodia theobromae, Fusarium* sp*., Nigrospora* sp*., Penicillium chrysogenum, Penicillium glabrum, Pleurofragmium* sp., *and Trichoderma harzianum.*

The result showed that the mycoflora of cow dung and poultry droppings possess the ability to utilize crude oil and that nutrient supplementation of oil-polluted soils, especially with organic nutrient sources is beneficial. However, poultry droppings supported the growth of a greater variety of fungi than cow dung which suggests that poultry droppings may therefore have more utilizable nutrients than the cow dung.

Complex mixtures of components are contained in the petroleum hydrocarbon contaminants and microbial degradation differs in the susceptibility of each component. Miget (1973) reported that naturally mixed populations degrade crude oils and hydrocarbons better than single isolates from the mixed populations. The present investigation has shown that cow dung and poultry droppings possess a mixed culture of petroleum degrading fungi. The addition of organic nutrients such as cow dung and poultry droppings as bioremediating agents to polluted environments will increase both

the population and diversity of the mycoflora (fungi) of such polluted environments to enhance bioremediation. Moreover, the mixed culture of petroleum degrading fungi present in cow dung and poultry droppings can be harnessed by researchers in the search for mixed culture of microorganisms with naturally enhanced oil degrading capabilities or which could be genetically engineered for enhanced bioremediation of polluted sites.

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