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BIODEGRADATION OF CRUDE OIL BY *PENICILLIUM sp.* AND *MORTIERELLA sp.* ISOLATED FROM OIL –CONTAMINATED SOIL IN AUTO MECHANIC WORKSHOPS.

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Abstract: Biodegrading capability of fungi isolated from oil-contaminated soil in three auto-mechanic workshops in Minna, Nigeria was determined by mycelial extension rate measurement method. *Penicillium* strain B101F, B201F and B202F and *Mortierella* strain B1002F were identified as best crude oil (lagoma light) degraders compared to the other isolates such *Aspergillus* strain B102F, B104F and B119F, *Fusarium* strain B501F and *Trichoderma* strain K602F and K561F. The optimum pH and temperature for B101F and B1002F to degrade crude oil were 5.0 and 28 °C respectively. When complex nutrient such as maize bran was used as nitrogen source with crude oil in minimal salt liquid medium (MSLM) omitting $NaNO_3$, the optimum growth reached on 14th day of fermentation for both the isolates. B101F and B1002F were capable of using crude oil as both carbon and energy source in minimal salt medium. In view of their ability to utilize crude oil as carbon and nitrogen source, these organisms may be used in mitigating the pollution arising out of oil spills in the environment.

INTRODUCTION

Oil pollution presents a serious problem, and the toxicity of crude and refined oils on the environment – human, livestock, wildlife, crop, soil and microorganisms have been well documented (Amund and Igiri, 1990; Atlas *et al.*, 1978; Bartha, 1986; Bossert and Bartha, 1984; Raymond *et al*, 1976). Land firming technique which relies on soil microorganisms to degrade and eventually stabilize the petroleum hydrocarbon components (PHC) by mineralization is a recent and widely used approach to disposal of petroleum hydrocarbons and its products (Coldwell and Bartha, 1972). Dibbie and Bartha (1979) have isolated 22 and 31 genera of bacteria and fungi that are biodegraders in soil ecosystems. Atlas *et al* (1986) found that a rise ion metabolic activity of microbial communities is common throughout the mesophilic range. In thermophilic situations, metabolic rates tend to be high but growth rate and cell yield are low because of repair of thermal damage to cell constituents. Extremes of pH are inhibitory to a great majority of microbial degradation processes (Amund and Igiri, 1990). It is interesting to see if there is a potential increment in biodegrading capability of microbes in presence of complex materials containing many organic and inorganic nitrogenous substances. In this study, an investigation was made on the biodegrading capability of crude oil by fungal species isolated from oil-contaminated soil of mechanic workshop dumps and the effects of some environmental parameters on the biodegrading capability of these fungal isolates.

MATERIAL AND METHODS

Collection of samples

Soil samples were collected from three auto mechanic workshops located in three areas namely Bosso, Keteran Gwari and Northern bye-pass, Minna, Nigeria where the mechanics generally dump lubricating oil, gasoline and diesel. Three samples were collected at each site for microbiological and biochemical purposes. The crude oil (lagoma light) was collected from Chemical Refining Laboratory, NNPC, Kaduna.

pH and moisture content of samples

The pH of soil samples were measured using a pH meter (Crison Micro pH 2000). The moisture of the soil samples were determined using an electric protimetergrain Master 2000. Ten grams of each of freshly collected soil samples were used for moisture content determination.

Isolation and Identification of Microorganism

One gram of each soil sample was transferred into 9ml of distilled water and then using this as a stock solution, serial dilution up to 10^{-9} were made as described by Fawole and Oso (1988). Then 1.0ml of each dilution was plated in Potato dextrose agar and Malt extract agar. Sterilized glass spreader was used aseptically to spread the suspension on the surface of the agar medium. The plates were incubated at 28°C for 48 hours. Distinct colonies were selected to re inoculate into PDA slants for further use. The morphological characteristics of the isolates were studied by growing the culture in PDA medium and the cultures of different age were observed under the microscope at X10 objectives and also at X40 objectives using lactophenol cotton blue as mountant (Smith, 1977; Pelczar and Chan, 1977).

Selection of crude oil degraders

This was conducted as described by Smith (1977). Sterile PDA plates were inoculated with 2 mm of the mycelium of each of the isolated fungus and cultures were incubated for four days at 28°C. Mycelial plugs measuring 5 mm in diameter were cut with a sterile cork borer from the margin of the fungal colonies on PDA and placed at the center of Modified Czapek (MSA) plates containing the crude oil at different concentrations. To the autoclaved MSA medium developed by Dibbie and Bartha, 1979 (Composition: K_2HPO_4.1.0; $NaNO_3$.30; $MgSo_4$. $7H_2O$-0.5; $FeSO_4$. $7H_2O$- 0.01; agar- 15.0 gram; water- 1000ml; pH 5.0) chloramphenicol at 0.05 gram per liter was added as filtered sterilized solutions. About 18–20ml of the solid medium was poured onto sterile plates and dried at room temperature for 3-4 hours before the plates were coated with crude oil. Crude oil at concentrations of 0.1, 0.5, 1.0, 2.5 and 5.0% were used. Plates with MSA medium without incorporation of crude oil were served as controls. The plates were incubated at 28°C and mycelium extension were recorded for seven days. The isolates capable of degrading crude oil were then inoculated in 0.5% crude oil incorporated MSA slants.

Determination of optimum cultural conditions for the biodegradation of crude oil by strains B101F and B1002F

From the previous experiments it has been shown that B101F and B1002F were best degraders compared to others. So these were selected for further work. For the development of inoculum, each of the isolates was grown for 7 days in 50 ml potato dextrose medium. After 7 days of fermentation, the cells were harvested and washed twice thoroughly with distilled water and then 30 ml sterile water was added to the cells to make a cell suspension. The optimum pH (initial) of fermentation medium was determined by carrying out the fermentation at different pH values (3.0, 5.0, 7.0 and 9.0) of the medium. For this purpose, 5 ml of inoculum was added to 50 ml of Modified Czapek liquid medium (MSLM) at different pH with 0.5% crude oil in each of the 250 ml Erlenmeyer flasks. The flasks were incubated at 28°C for 28 days and the dry cell wt. was determined at definite time intervals. For determination of dry wt., the cells were harvested and washed twice thoroughly with distilled water and then transferred to a constant weight aluminum cup, dried at 60 –70°C for 24 hours. For determination of optimum temperature, fermentation was carried out at different temperatures between 10°C and 40°C. Dry cell wt. was determined on different days of fermentation. Then the optimum incubation period was determined by carrying out the fermentation for 28 days using 0.5% crude oil incorporated 50 ml MSLM in 250 ml flask and the dry wt. was determined at definite time interval.

Effect of complex nutrients on biodegradation of crude oil

The materials used were beans husk extract (BN) and rice (RN) and maize bran (MN) extracts. Twenty grams of each of the materials was suspended in 200 ml hot water in a 500 ml flask. The suspensions were kept at 90°C for 24 hours. The hot extracts were filtered through Whatman no. 1 filter paper. The solid content (%) of rice, maize, corn and beans were determined to be 3.43, 4.12, 3.91 and 0.98 respectively.

To first set, each of the complex nutrients at 0.1% level was added into the crude oil incorporated MSLM (omitting $NaNO_3$) as nitrogen source. To second set, only crude oil at 0.5% level was incorporated into $NaNO_3$ containing MSLM medium. In each case, 5 ml inoculum was used for 50 ml fermentation medium in 250 ml Erlenmeyer flask. Dry cell wt. was calculated as described before.

Crude oil as both carbon and nitrogen source

In the first set, different concentrations of crude oil were added to MSLM omitting $NaNO_3$. In a typical experiment, a definite concentration of crude oil was added to 50 ml MSLM in a 250 ml Erlenmeyer flask and was inoculated with 5 ml inoculum. Crude oil incorporated MSLM with $NaNO_3$ was served as control. Dry cell wt. was determined at definite time intervals.

RESULTS

Characteristics of soil samples

The values of pH of collected soil samples in three sites were in the range of 4.8 – 6.4 and the moisture content (%) of the samples were in the range of 8 – 14. The results are shown in Table 1.

Isolation and identification of microorganisms

Five different types of organisms were identified namely *Penicillium sp.*, *Aspergillus sp.*, *Fusarium sp.*, *Trichoderma sp* and *Mortierella sp.*

Table 1: Characteristics of soil samples collected from auto–mechanic workshops (mean ± SEM; n=3)

Site	Moisture	pH	Isolate	Fungus identified	% of total fungal population
Bosso	14.1± 0.12	4.5 ± 0.08	B101F	*Penicillium sp.*	50.4 ± 0.1
			B1002F	*Moiterella sp.*	14.3 ± 0.1
			K602F	*Trichoderma sp*	19.7 ± 0.2
			B102F	*Aspergillus sp.*	15.4 ± 0.1
Keteran Gwari	10.2 ± 0.16	5.0 ± 0.10	B501F	*Fussarium sp.*	27.1 ± 0.1
			B104F	*Aspergillus sp.*	23.4 ± 0.3
			B202F	*Penicillium sp.*	47.2 ± 0.1
				Unknown	3.1 ± 0.03
Northern Bye-pass	8.1 ± 0.12	6.3 ± 0.08	K561F	*Trichoderma sp*	18.2 ± 0.1
				Penicillium sp	62.1 ± 0.0
				Aspergillus sp.	17.9 ± 0.1
				Unknown	2.1 ± 0.02

Selection of crude oil degraders

All the isolated fungi were capable of utilizing crude oil at 0.5% concentration and B101F and B1002F (Table 2) were capable of utilizing crude oil more efficiently than the other isolates. So these two isolates were selected for further studies.

Determination of optimum cultural conditions

The optimum pH and the optimum temperature for degradation crude oil by B101F and B1002F were 5.0 and 28°C. For B101F, the respective dry cell wt. at temperature 10°C, 28°C and 30°C were 0.16g/l,

3.44g/l and 1.61g/l. For B1002F, the respective values were 0.13g/l, 2.85g/l and 1.07g/l. This shows that the utilization of crude oil by B101F and B1002F was significantly reduced at 10°C and 37°C. The cell wt. (g/l) was maximum (3.67 for B101F and 3.17 for B1002F) at pH 5.0 compare to the pH 3.0, 7.0 and 9. The optimum incubation period was 21st day of fermentation. The results are shown in Tables 3 and 4.

Effect of complex nutrients on biodegradation of crude oil

The results in Table 5 indicate that maize bran extract in crude oil incorporated MSLM gives the maximum growth on 14th day of fermentation whereas in case of crude oil incorporated MSLM medium, the optimum growth reached on 21st day of fermentation.

Table 2: Biodegradation of crude oil by the isolates (mean ±SEM; n=3)

Isolate	Length of mycelia (mm) at diff. Conc.(%)				
	0.1	0.5	1.0	2.5	5.0
B101F	3.4 ± 0.2	9.0 ± 0.1	3.9 ± 0.2	-	-
B1002F	4.2 ± 0.2	9.8 ± 0.1	6.0 ± 0.2	2.2 ± 0.2	-
K602F	1.3 ± 0.2	3.3 ± 0.2	0.8 ± 0.0	0.6 ± 0.0	-
B102F	2.9 ± 0.1	7.3 ± 0.2	3.4 ± 0.2	1.9 ± 0.1	-
B501F	2.0 ± 0.0	2.1 ± 0.1	1.8 ± 0.2	-	-
B104F	2.8 ± 0.2	7.2 ± 0.1	2.9 ± 0.3	-	-
B202F	3.5 ± 0.0	8.8 ± 0.1	3.9 ± 0.2	2.3 ± 0.2	2.0 ± 0.1
K561F	1.6 ± 0.0	3.9 ± 0.3	3.2 ± 0.1	-	-
B201F	3.4 ± 0.2	8.2 ± 0.2	2.2 ± 0.1	-	- B119F
	2.4 ± 0.3	6.9 ± 0.1	3.8 ± 0.1	2.2 ± 0.1	-

Table 3: Effect of pH temperature on utilization of crude oil by B101F and B1002F

Isolate	pH			Growth(g/l)	Temperature(°C)		
	3.0	5.0	7.0	9.0	10	28	37
B101F	0.20	3.67	2.37	1.49	0.16	3.44	1.61
B1002F	0.13	3.17	1.99	0.99	0.13	2.85	1.07
Control	0.11	0.13	0.11	0.12	0.16	0.20	0.17

Table 4: Determination of optimum incubation period

Isolate	Dry cell wt. (g/l) at different days of incubation			
	7th	14th	21st	28th
B101F	2.03	3.87	4.17	4.18
B1002F	0.71	1.89	2.28	2.33
Control	0.19	0.20	0.19	0.19

Table 5: Effect of complex nutrients on biodegrading capability of B101F and B1002F (mean ± SEM; n=3).

Isolate	**CN	Dry cell wt. (g/l) at different days of incubation			
		7th	14th	21st	28th
B101F	RN	1.98±0.08	2.87±0.14	2.40±0.19	2.54±0.14
	BN	2.28±0.14	3.09±0.11	3.19±0.14	2.075±0.01
	MN	3.89±0.18	4.65±0.01	4.31±0.08	4.02±0.18
	*Control	2.48±0.20	3.80±0.08	4.62±0.08	4.32±0.08
B1002F	RN	1.26±0.08	2.43±0.03	2.08±0.01	1.76±0.01
	BN	1.79±0.12	3.11±0.00	2.98±0.02	2.68±0.02
	MN	2.00±0.11	4.35±0.01	4.20±0.02	4.22±0.01
	*Control	1.40±0.13	4.08±0.01	4.75±0.03	4.22±0.02

*0.5% crude oil used as carbon source in MSLM

**Complex nutrients added as nitrogen source in 0.5% crude oil incorporated MSLM (omitting $NaNO_3$).

RN-rice bran extract; BN-beans husk; MN-maize bran extract.

Crude oil as both carbon and nitrogen source

Both the isolates can utilize crude oil at 0.5% concentration both as carbon and energy source in MSLM medium without incorporation of $NaNO_3$, the conventional nitrogen source. The results are shown in Table 6.

Table 6: Utilization of crude oil as both carbon and nitrogen source (means ± SEM; n=3).

Isolate		Dry cell wt. (g/l) at different days of incubation			
		7th	14th	21st	28th
B101F	P–	2.20±0.02	3.81±0.01	4.64±0.00	4.40±0.04
	P+	2.44±0.00	3.61±0.02	4.60±0.01	4.33±0.05
B1002F	P–	1.44±0.02	3.99±0.01	4.25±0.03	4.68±0.04
	P+	1.36±0.13	4.00±0.01	4.45±0.01	4.32±0.02

P– where crude oil only serves as carbon source

P+ where crude oil serves both as carbon and energy source

DISCUSSION

The soil samples were collected at a depth between 15 –20cm because of the bactericidal effect of sunlight, and inadequate moisture due to evaporation on the surface. The fungal isolates were selected for this work because of the fact that there are not many studies available on oil degradation by fungi. In this study, the optimum concentration of lagoma light crude oil in MSLM for fungal degradation was 0.5%. This is in contrast to a report by Bossert and Bartha (1984) that stimulation of microbial activity is enhanced up to 5% level of hydrocarbon. The increase in concentration of crude PHC likely

interfered with medium aeration, hence degradation could not occur because it is an oxidative process. Also at higher concentration the PHC could be toxic to the microorganisms. Despite the differences in cell mass, all the fungal isolates had same optimum period of incubation of 21 days. The fungal isolates utilized crude PHC at all pHs tested with maximum at pH 5.0. This supports earlier observations of Bossert and Bartha (1984) and Dibbie and Bartha (1979) that fungi degrade oil most efficiently in acidic medium. At low temperatures fungal degradative capability was arrested. Optimum growth was obtained at low temperature (28°C) and higher above that oil degrading capabilities reduced. This is in line with acclaimed work of Debbie and Bartha (1979) and Mikkins – Philips and Stewart (1974) that best condition of temperature for maximal fungal activity is the mesophilic range. When complex nutrients were added as nitrogen source with crude oil in MSLM omitting $NaNO_3$, the growth of isolates were almost same as of crude petroleum used as carbon source. However, the optimum growth period was different. The effect of complex nutrients on biodegradation of crude oil may be due to the presence of inorganic materials and organic nitrogenous substances in the complex nutrients. Crude petroleum could serve as both carbon and nitrogen source for these isolates. Lack of mineral elements as nitrogen, phosphorous and sulfur is said to be a limitation in PHC biodegradation (Atlas, 1981; Ogoke, 1992) but crude PHC contains small amount of nitrogen, oxygen and sulfur containing components (Bartha, 1986).

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