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Biodegradation of Crude Oil by *Bacillus* species isolated from Different Sources

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ABSTRACT

The consequences of oil pollution to the environment and human health as a result of exploration activities, transportation and usage of petroleum compounds, has become a serious challenge to environmental scientists. This study evaluates the potential of using *Bacillus subtilis* in biodegradation of petroleum compounds. Ten *Bacillus* strains from non-petroleum contaminated sites were identified on the basis of microscopy and biochemical tests. The ability of *B. subtilis* S4 to degrade crude oil was tested by growing in crude oil contaminated soil. The biodegradation products were analysed by gas chromatography-mass spectroscopy (GCMS). The GCMS indicated that some high molecular mass components of the crude oil were broken down to lower molecular mass compounds. Octacosane (MM = 394) disappeared after biodegradation with *Bacillus* S4. It is suggested that *B. subtilis* has the potential to degrade crude oil.

Keywords: Biodegradation, *Bacillus,* Crude oil, Gas chromatography-Mass spectroscopy.

1. INTRODUCTION

Environmental pollution caused by crude oil has become a common and daily phenomenon and has caused a global ecological and social catastrophe [1]. Crude oil continues to be used as the principal source of energy and plays an important role in the global environmental pollution considerations. Oil will remain as a major source of energy in the next several decades, because reliable alternative energy sources have not yet been found [2]. A major environmental impact of petroleum exploration in Nigeria and other oil producing countries is crude oil spillage. The oil spill causes destruction both on cultivated and virgin lands. Oil spills on land and sea has increased with explorative activities [3]. These oil spills devastate the soil, surface and ground water and alter the microbial population at the polluted site [4].

Oil spills affect both vegetation and animals in the soil by contact toxicity and also by reducing oxygen concentration and thus increasing anaerobiosis in soil, which is harmful to plant roots [5]. The most noticeable sources of contamination are releases from manufacturing and refining installations, oil-tanker spills and accidents during transportation of the oil. A great part of the oil pollution problem results from the fact that the oil refineries are located far away from the drilling sites. This requires pumping the crude oil in pipelines with its associated risks.

Remediation of the contaminated soil can be done in many ways which include both physico-chemical and biological methods. Biological methods are usually preferred over physico-chemical treatment in removing oil pollution because of the cost effective *in situ* degradation of hydrocarbons by microorganisms. Bioremediation methods are favoured as environmentally friendly treatment technologies for the removal of hydrocarbons. But the low solubility and adsorption of high molecular mass hydrocarbons limits their availability to microorganisms [6].

Degradation of oil pollutants is carried out *in situ* by a consortium of microorganisms. Many species of bacteria, fungi and algae are capable of degrading

hydrocarbons. Bacteria of various genera have been reported to contain oil degrading species, including *Pseudomonas, Corynebacterium, Brevibacterium, Bacillus* and *Arthrobacter* [7]. Several *Bacillus* spp. have been identified as efficient hydrocarbon degraders by growing on a large number of hydrocarbon compounds as a source of carbon and energy [8, 9, 10,11]. They have the ability to degrade different hydrocarbons by the production of biosurfactants and also of competent hydrocarbon degrading enzymes [12]. The present study was designed to determine the oil degradation potential of *Bacillus* sp. isolated from different environmental sources.

2. MATERIALS AND METHODS

2.1 Isolation of organisms

Bacillus were isolated from contaminated soils, wastewater and cow dung samples. The samples were subjected to thermal shock before pour plating onto nutrient agar medium. The isolates were identified as *Bacillus* biochemically by reference to Barrow GI et al (1993) [13]. The following biochemical tests were carried out for identification: Gram Stain, Citrate test, Catalase test, Starch hydrolysis, Motility test, Nitrate reduction test, Indole test, Voges Proskauer (VP) test, and Urease test.

2.2 Screening of *Bacillus* isolates for crude oil degradation ability

The experiments were set up using 1% v/v crude oil as carbon source in basal mineral salt medium as described by Akhavan SA, et al (2008) [2]. The composition of the mineral salt medium used in this study was as follows (g/L): $NaNO_3$ (2.0), NaCl (0.8), KCl (0.8), $CaCl_2.2H_2O$ (0.1), KH_2PO_4 (2.0), $Na_2HPO_4.12\ H_2O$ (2.0), $MgSO_4$ (0.2), $FeSO_4.7H_2O$ (0.001); 2 ml trace element stock solution composed of (g/L): $FeCl_3.6H_2O$ (0.08), $ZnSO_4.H_2O$ (0.75), $CoCl_2.6H_2O$ (0.08), $CuSO_4.5H_2O$ (0.075), $MgSO_4.\ H_2O$ (0.75), H_3BO_3 (0.15), $Na_2MoO_4.2H_2O$ (0.05).

The pH of the medium was adjusted to 6.9. The isolates were inoculated into 50 ml conical flasks containing the sterilized medium and incubated in rotary shaker programmed at 200 rpm and 30ºC for 14 days. The optical density (OD 600 nm) and pH of the culture medium were monitored at 48-hours' time intervals as biodegradation indices.

2.3 Microbial biodegradation test

Soil samples were air dried, sieved and sterilized fractionally in hot air at 160ºC for 24 - 48 hours to ensure elimination of indigenous soil microorganisms. One hundred grams of the sterilized soil sample was introduced into sterilized flasks and then 20 ml of sterilized Bonny light crude oil was poured into all the flasks along with 10 ml of 48 hours old broth culture of *Bacillus subtilis* S4 [14]. Control flasks contained sterilized Bonny light crude oil with sterilized soil only. The flasks were thoroughly mixed, moistened with sterile water to homogenize the mixture and incubated for 28 days at ambient temperature (28 ±2°C).

2.4 Extraction of residual crude oil

One hundred ml of carbon tetrachloride was poured into each of the test and control flasks. The flasks were shaken vigorously and decanted into sterile test tube. The extraction was repeated twice with 100 ml each of the carbon tetrachloride. The total extract which contains the residual crude oil was then heated over a hot water bath for the evaporation of the carbon tetrachloride [15].

2.5 Gas chromatographic and mass spectrometer (GC-MS) analysis

Gas chromatography and mass spectrometer was used to determine the residual hydrocarbon present in the soil after 28 days of incubation and extraction as used by Bijay T, et al (2012) [16]. The GC-MS used has the following profile: (GCMS-QP2010 Plus model), Column Oven Temp.: 45.0 °C, Injection Temp.: 250.00 °C, Injection Mode: Split, Flow Control Mode: Linear Velocity, Pressure: 94.3 kPa, Total Flow: 21.0 mL/min, Column Flow: 1.64 mL/min, Linear Velocity: 46.3 cm/sec, Purge Flow: 3.0 mL/min, Split Ratio: 10.0, Solvent Cut Time: 3.00 min

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of isolates

Ten (10) *Bacillus species* were isolated, characterized and identified based on their morphological and biochemical properties. Five (5) of the isolates were *Bacillus subtilis,* four (4) were identified as *Bacillus cereus* and one (1) as *Bacillus coagulans. B. subtilis* was isolated from all samples except in cow dung and domestic wastewater. *B. cereus* was not isolated from waste dump and herbicide treated soil, while *B. coagulans* was isolated from cow dung only.

3.2 Crude oil degradation screening test

Table 1 shows the degradative abilities of *Bacillus* in a medium containing crude oil. After 14 days of incubation, the microorganism grew well in 1% crude oil mineral salt medium. The highest optical density (OD_{600}) of 0.66 was seen in cultures of *Bacillus subtilis* S2a, *B. subtilis* S3a and *B. subtilis* S4. The increase in OD_{600} with time is in agreement with the report of [2]. The increase in the OD could be as a result of the incorporation of simple carbon compounds produced during crude oil degradation into new cellular constituents to produce additional biomass [11]. The lowest pH values were obtained in cultures of *B. subtilis* S4 (pH 6.20), *B. cereus* S3b (pH 6.25) and *B. subtilis* S6 (pH 6.25). The pH of the medium was another biodegradation index that was used to determine the degradation potential of the isolates. The decrease in the pH of the medium containing the test isolates with time agrees with the works of [2] and of [11]. The decrease in pH might result from the production of acidic metabolic products during the utilization of crude oil by these bacteria. From the reduction in pH level as well as the increase in OD_{600}, *B. subtilis* S4 was selected for biodegradation of crude oil on crude oil polluted soil.

Table 1: Screening of *Bacillus* isolates for degradation potential

SAMPLE	Day 2		Day 4		Day 6		Day 8		Day 10		Day 12		Day 14	
	pH	OD	pH	OD	pH	OD	pH	OD	pH	OD	pH	OD	pH	OD
Bacillus subtilis S1	6.90	0.15	6.70	0.16	6.65	0.19	6.65	0.25	6.60	0.38	6.45	0.51	6.45	0.54
Bacillus subtilis S2a	6.85	0.18	6.65	0.22	6.55	0.30	6.45	0.37	6.40	0.41	6.40	0.53	6.35	0.66
Bacillus cereus S2b	6.80	0.19	6.65	0.20	6.55	0.27	6.50	0.38	6.40	0.46	6.30	0.58	6.30	0.61
Bacillus subtilis S3a	6.80	0.16	6.60	0.26	6.50	0.34	6.40	0.39	6.35	0.48	6.30	0.54	6.30	0.66
Bacillus cereus S3b	6.90	0.18	6.60	0.21	6.50	0.30	6.40	0.40	6.30	0.46	6.25	0.57	6.25	0.54
Bacillus subtilis S4	6.75	0.19	6.60	0.22	6.50	0.36	6.40	0.42	6.25	0.53	6.25	0.60	6.20	0.66
Bacillus coagulans S5a	6.85	0.16	6.65	0.18	6.60	0.26	6.50	0.35	6.50	0.48	6.45	0.55	6.40	0.56
Bacillus cereus S5b	6.90	0.15	6.75	0.22	6.70	0.28	6.65	0.40	6.60	0.50	6.60	0.53	6.60	0.59
Bacillus subtilis S6	6.90	0.18	6.70	0.23	6.65	0.36	6.35	0.47	6.30	0.53	6.25	0.62	6.25	0.60
Bacillus cereus S7	6.85	0.18	6.80	0.22	6.80	0.33	6.75	0.36	6.75	0.44	6.60	0.47	6.55	0.59
Control	6.90	0.14	6.90	0.14	6.90	0.14	6.90	0.14	6.90	0.15	6.90	0.15	6.85	0.16

Initial pH at day 0: 6.90, Initial Optical Density (OD) at day 0: 0.14 at 600nm, Control = crude oil + mineral salt medium only

3.3 Biodegradation of crude oil on crude oil polluted soil

Result of the GC-MS shows a breakdown of large molecules of hydrocarbon present into shorter chains. The profile of the crude oil before subjecting it to biodegradation test as shown in Figure 1 and Table 2 indicates that there were twenty three (23) compounds with 3,3-dimethyl-2-hexanone and 3-methyl-2-heptanone having the least molecular mass of 128 MM and octacosane with the highest molecular mass of 394 MM. The profile of the crude oil degraded by the *Bacillus subtilis* S4 indicated that the crude oil was broken down to molecules with lower molecular weight and some molecules were removed from the recovered crude oil sample after degradation. Octacosane which happens to have a long chain and higher molecular weight of 394 MW in the crude oil disappeared in the test samples indicating that this compound was broken down into shorter chain hydrocarbons and more peaks were formed indicating increase in number of compounds with shorter chain hydrocarbon and lower molecular mass (Fig. 2; Table 3). Even if obtained with bacteria, these results did agree with those reported by [16] working with fungi species isolated from Red Sea coast of Saudi Arabia, able to degrade tetrapentacontane present in diesel fuel to short chain hydrocarbons. This phenomenon demonstrates the ability of *Bacillus* to biodegrade crude oil by breaking the long chain hydrocarbon to short chain hydrocarbons. [11] also reported the increase in the number of peaks observed after biodegradation showing compounds with lesser molecular mass compared to the molecules observed before biodegradation. Peaks with different retention times disappeared and several new peaks were observed just as in this study, indicating the biodegradation of the crude oil compounds.

There are many different varieties of hydrocarbons and over millions of years bacteria have evolved catalytic enzymes that are specific for particular degradation reactions. Some of the simpler compounds can be degraded by a very wide variety of bacteria but the ability to degrade hydrocarbons is found in fewer species [11]. No single bacterium can make all the different enzymes, instead; each kind of bacterium specializes in a few hydrocarbons as preferred food sources [17].

From the study it was concluded that *Bacillus subtilis* isolated from non-oil contaminated sites has the potential to degrade crude oil as seen by changes in optical density, pH, and GC-MS analysis.

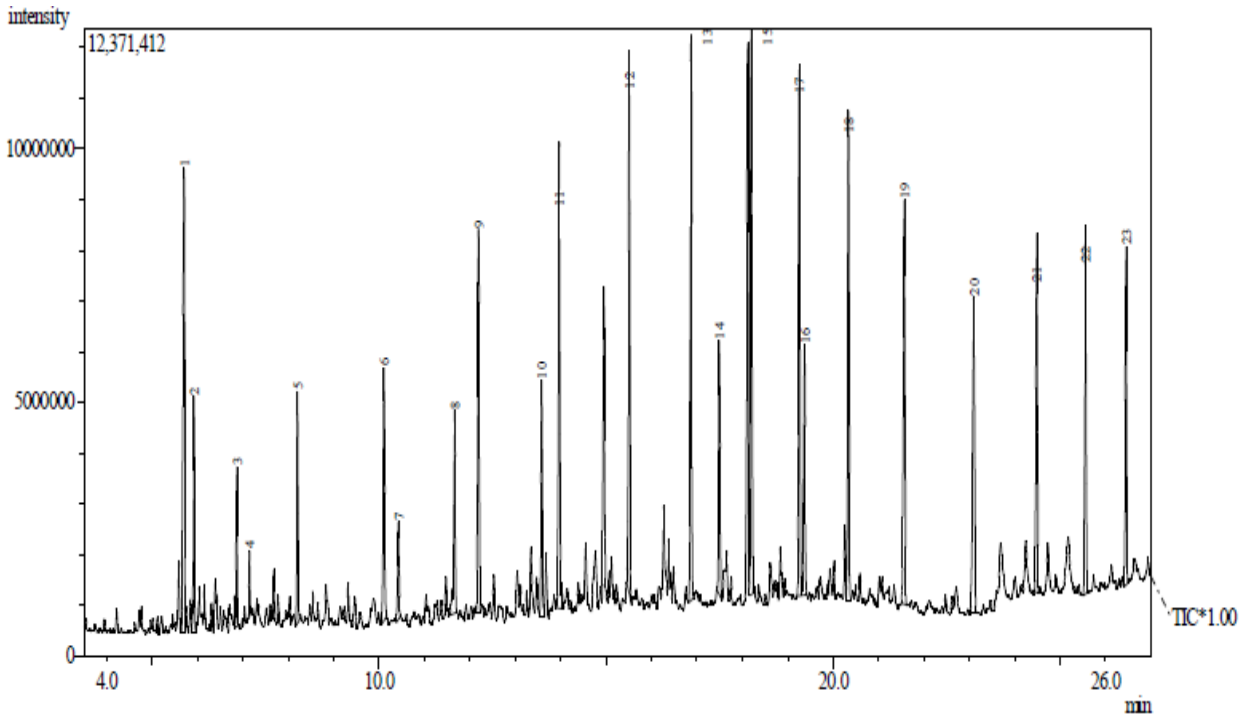

Figure 1: Chromatogram of Bonny light crude oil before subjecting it to biodegradation.

Table 2: Profile of Bonny light Crude oil before biodegradation

Peak Number	Retention Time (Min)	Area %	Name of compound	Chemical formula	Molecular mass
1	5.705	7.03	3,3-dimethyl-2-Hexanone	$C_8H_{16}O$	128
2	5.925	2.32	3, methyl-2- heptanone	$C_8H_{16}O$	128
3	6.865	1.46	2,7-dimethyloctane	$C_{10}H_{22}$	142
4	7.146	0.58	4-methyldecane	$C_{11}H_{24}$	156
5	8.202	2.50	n-Dodecane	$C_{12}H_{26}$	170
6	10.108	3.56	n-Tridecane	$C_{13}H_{28}$	184
7	10.425	1.30	2,6-dimethylundecane	$C_{13}H_{28}$	184
8	11.665	2.91	2-Bromooctane	$C_8H_{17}Br$	192
9	12.191	4.86	4,8-dimethyltridecane	$C_{15}H_{32}$	212
10	13.582	2.91	Hexadecane	$C_{16}H_{34}$	226
11	13.966	5.87	Nonadecane	$C_{19}H_{40}$	268
12	15.506	6.66	Hexadecane	$C_{16}H_{34}$	226
13	16.872	6.99	Hexadecane	$C_{16}H_{34}$	226
14	17.493	3.28	2,6,10 trimethylpentadecane	$C_{18}H_{38}$	254
15	18.197	7.55	2,6,10,14–Tetramethylpentadecane	$C_{19}H_{40}$	268
16	19.255	6.33	2,6,10,14–Tetramethylhexadecane	$C_{20}H_{42}$	282
17	19.371	3.31	Octadecane	$C_{18}H_{34}$	254
18	20.339	6.13	Eicosane	$C_{20}H_{42}$	282
19	21.565	5.80	Eicosane	$C_{20}H_{42}$	282
20	23.096	5.80	2-methyleicosane	$C_{21}H_{44}$	296
21	24.483	4.59	Tetrapentacontane	$C_{54}H_{110}$	758
22	25.556	4.43	Tetracosane	$C_{24}H_{50}$	338
23	26.448	3.85	Octacosane	$C_{28}H_{58}$	394

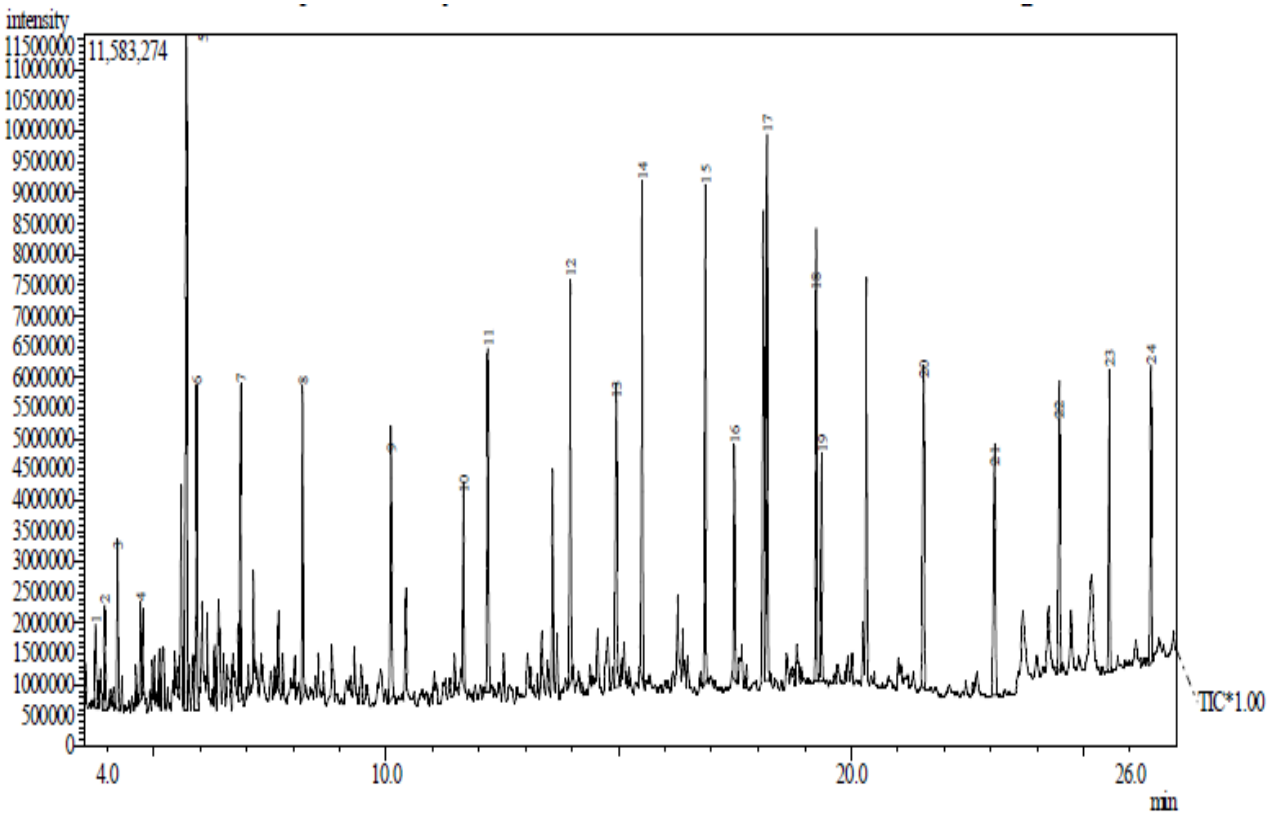

Figure 2: Chromatogram of Bonny light crude oil degraded by *Bacillus subtilis* S4 after 28days of incubation.

Table 3: Profile of degraded Bonny light crude oil by *Bacillus subtilis* S4

Peak number	Retention time (min)	Area %	Name of compound	Chemical formula	Molecular mass
1	3.751	1.00	n-Hexane	C_6H_{14}	86
2	3.945	1.37	1,4-dimethylcyclohexane	C_8H_{16}	112
3	4.220	1.81	n-Octane	C_8H_{18}	114
4	4.716	1.00	1,ethyl – 3, methylcyclohexane	C_8H_{16}	112
5	5.709	11.08	4-Methyl, 1-Octene	C_9H_{18}	126
6	5.929	4.04	3 methyl, 2-pentanone	$C_6H_{12}O$	100
7	6.866	3.04	n-Nonane	C_9H_{20}	128
8	8.204	3.75	3,6-dimethyldecane	$C_{12}H_{26}$	170
9	10.107	4.09	n-decane	$C_{10}H_{22}$	142
10	11.664	2.54	3-methyl nonane	$C_{10}H_{22}$	142
11	12.188	4.75	n-dodecane	$C_{12}H_{26}$	170
12	13.961	5.31	2-methyl dodecane	$C_{13}H_{28}$	184
13	14.947	4.35	n-Dodecane	$C_{12}H_{26}$	170
14	15.500	6.10	n-Tetradecane	$C_{14}H_{30}$	198
15	16.866	6.00	n-Tridecane	$C_{13}H_{28}$	184
16	17.490	3.16	n-Dodecane	$C_{12}H_{26}$	170
17	18.190	7.47	2,7,10-trimethyldodecane	$C_{15}H_{32}$	212
18	19.249	5.40	n-tridecane	$C_{13}H_{28}$	184
19	19.365	3.11	n-tetradecane	$C_{14}H_{30}$	198
20	21.556	4.83	n-Tetradecane	$C_{14}H_{30}$	198
21	23.084	4.68	n-Hexadecane	$C_{16}H_{34}$	226
22	24.474	3.84	n-Eicosane	$C_{20}H_{42}$	282
23	25.550	3.82	n-Heptadecane	$C_{17}H_{36}$	240
24	26.443	3.45	n-Octadecane	$C_{18}H_{38}$	254

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