

Bioremediation of soil contaminated with crude oil and amended with *Moringa oleifera*

ABSTRACT

The potential of *Moringa Oleifera* in increasing the rate of the microbial degradation of crude oil-spilled soil was investigated. The Proximate analysis of *Moringa oleifera* seed cake was found to contain nitrogen (10.55%), phosphorus (18.33ppm)...more? and hydrocarbon degrading bacteria. Likewise, hydrocarbon-degrading bacteria were isolated using standard procedures. The results revealed that the counts of crude oil degrading bacteria in crude oil polluted soil amended with *Moringa oleifera* increased to 3.6×10^6 CFU/g on the 21st day as compared with while that in unamended soil could only get to having 1.5×10^6 CFU/g. *Moringa oleifera* seed cake was found to enhance the degradation in crude oil polluted soil by up to 64.3% compared to the crude oil polluted and unamended soil (%). pH of crude oil polluted soil sample amended with *Moringa oleifera* ranged between 7.24 and 7.70 while that of crude oil polluted soil ranged between 7.24 and 7.44. This implies that *Moringa oleifera* has a buffering effect which provides an enabling environment for the micro-organisms to proliferate. The results of this study shows that *Moringa oleifera* seed cake is efficient and can be used in bioremediation of reclaiming crude oil polluted soils.

Keywords: Microbial degradation, spilled oil, degrading bacteria, polluted soil, buffering effect, *Moringa oleifera*.

1. Introduction

Oil and gas has become a major source of energy, the world over. This is deduced from application of its various products in our lives and how much revenue is being generated from it. With the immense benefits accrued generated from the oil and gas industry notwithstanding, through its exploration and exploitation, also brings along with it significant negative effect impact to the ecological and human environments (Oyegun, 1998), subsequently impacting on the socio economy of the nation. Oil spillage is one of the problems that could result from oil drilling. In Nigeria, oil pollution problems have been prevalent since the commencement of oil exploration and development of the petroleum industry (Okoh *et al.*, 2001). However, different methods have been used in restoring petroleum polluted soil, some of these include the use of oil degrading microorganisms, inorganic fertilizers, chicken droppings, periwinkle shell, liming and tilling (Leahy and Colwell 1990; Ijah *et al.* 2003a; and Ijah *et al.* 2003b). Bio-stimulation using inorganic fertilizer has been extensively employed worldwide in reclaiming oil polluted soil (Jobson *et al.* 1974; Dibble and Bartha 1979; and Song *et al.* 1990 and USEPA 1990). However *Moringa Oleifera* due to its high nitrogen content can be used to enhance biodegradation of crude oil in soil and at the same time solving the problem of wastes from the environment. Treatment of oil contaminated soil is necessary to protect

UNDER PEER REVIEW

water supplies, human health and environmental quality (Chang *et al.* 1996), owing to the fact that plants derive nutrients for their living from soil, animals derive theirs from plants and human derive theirs from plants and animals living on soil. Bioremediation is a method whereby the natural abilities of micro-organisms are employed for the reduction of concentration or /and toxicity of various chemical substances, such as, petroleum derivatives, aliphatic and aromatic hydrocarbons, industrial solvents, pesticides and metals (Korda *et al.*, 1997).

41 The speed and efficiency of bioremediation of a soil contaminated with petroleum and
42 petroleum products depends on the number of hydrocarbon-degrading microorganisms in
43 the soil. The most important factors for population growth are temperature, oxygen, pH,
44 content of nitrogen and phosphorus, hydrocarbon class and their effective concentration. A
45 single microorganism can degrade only certain types of petroleum compounds, but a mixed
46 population – microbial community enables a higher level of degradation. The crude oil
47 degrading bacteria and fungi identified in soil amended with *Moringa oleifera* seeds and
48 pods are species of *Bacillus*, *Acinetobacter*, *Micrococcus*, *Pseudomonas*, *Rhizopus* and
49 *Aspergillus* species respectively.

50 The objectives of this work were to determine the potential of *Moringa oleifera* seed in
51 enhancing crude oil degradation in soil and to determine some changes in physicochemical
52 properties of soil during bioremediation of oil polluted soil [A.F9].

53 2. Materials and Methods

54 2.1 Collection and Processing of Samples

55 Nigerian light crude oil was obtained collected from Niger Delta University, Bayelsa State, Nigeria.
56 The *Moringa Oleifera* [A.F10] seeds were collected from Zaria town, Kaduna State, Nigeria. The
57 *Moringa Oleifera* seeds were grinded into fine powder to pass through [A.F11] a Tyler sieve of 2mm
58 mesh-size. The soil sample [A.F12] used was collected from the farm of National Research Institute
59 for Chemical Technology (NARICT) Zaria, Kaduna State, Nigeria. The soil was sieved with
60 2mm mesh size before use.

61

62 2.2 Bioremediation Studies

63 Two kilograms [A.F13] of soil samples each was introduced into four [A.F14] different plastic buckets
(PB)

64 labelled A to C. The PB B and C were treated with 200ml of crude oil each, while A had no
65 crude oil and served as control. PB C was treated with 400g of grinded seeds of *Moringa*
66 *oleifera*, 250ml of distilled water was introduced into each of the PB and the contents were
67 thoroughly mixed and incubated at room temperature. Periodic sampling from each PB was
68 carried out at seven days intervals for 28 days [A.F15]. The samples were analyzed for changes in
69 pH, moisture, biodegradation of crude oil as well as microbial counts. [A.F16]

70

71 2.3 Biodegradation of Crude Oil

72 The amount of crude oil degraded in each soil sample was determined by the weight loss
73 method of Bossert and Bartha (1984). This was done by suspending 3g of soil in 10ml of
74 diethyl ether in a universal bottle and shaken vigorously to extract the oil. The solvent-oil
75 mixture was transferred slowly into a beaker of known weight. This was repeated until all the
76 oil was extracted from the soil. The solvent-oil mixture was kept open exposed at room
77 temperature
78 overnight to allow the solvent to evaporate completely. The new weight of the beaker
79 containing the residual oil was taken and the percentage of oil degraded was calculated [A.F17] (Ijah
80 and Ukpe 1992).

80

81 2.4 pH Determination

82 pH of the sample was determined using pH-meter (Crison pH 210 micro-processor) on 1:2.5
83 (w/v) soil/water mixture (ref.).

UNDER PEER REVIEW

133

84

85 2.5 Microbial analysis Counts and Type

86 Bacteria and fungi were enumerated by spread plate [A.F18] technique by inoculating 0.1ml
[A.F19] serially

87 diluted sample onto nutrient agar (NA) plates and Potato dextrose agar (PDA) plates
88 respectively. The NA plates were incubated at 30°C [A.F20] for 48 hours while PDA plates were
89 incubated at room temperature for 72 hours. The crude oil utilizing bacteria were
90 enumerated on oil agar OA (1.8g K₂HPO₄, 4.0g NH₄Cl, 0.2g MgSO₄·7H₂O, 1.2g KH₂PO₄,
91 0.01g FeSO₄·7H₂O, 0.1g NaCl, 20g agar, 1% crude oil in 1000ml distilled water, pH 7.4).

92 The oil agar was incubated at 30°C for seven days. The colonies which developed were
93 counted and expressed as colony forming units per gram (CFUg⁻¹) of sample. Isolated

94 bacterial colonies were identified characterized based on their gram stain reaction and biochemical

95 tests. The organisms were identified to the genus level using the scheme of Cowan (1974).
96 The fungi isolates were characterized based on macroscopic and microscopic examinations
97 (Ijah et al. 2003a) and were identified using the scheme of Alexopoulos and Mims (1979).
98

99 2.6 Determination of Utilization of Crude Oil by Bacterial Isolates

100 The method of Kokub et al. (1989) was used, 5ml of mineral salts medium of Zajic and
101 Supplisson (1972) was dispensed into each universal bottles containing 0.05ml of crude oil
102 and sterilized for 15 minutes at 121°C, and the bottles were allowed to cool and inoculated
103 with 0.1ml of nutrient broth-grown culture of crude oil degrading bacterial isolates. The
104 bottles were incubated at room temperature (28±2°C) for 21 days. The turbidity produced as
105 a result of bacterial growth was monitored visually at the end of incubation period and
106 assigned (+) to (+++) depending on the intensity of the growth.
107

108 3. Results and Discussion

109 Table 1: Proximate analysis Biological and Physiochemical Properties of *Moringa Oleifera* 110 Seeds used 111 for Bioremediation Studies

Biological/Physiochemical
Properties parameters

Moringa

Oleifera seeds

pH 7.06

Moisture (%) 1.95

Nitrogen (%) 10.55

Phosphorus (ppm) 18.33

Organic carbon (%) 1.06

Total aerobic bacteria 6.0×10^6 CFU/g

Crude oil utilizing bacteria 3.0×10^2 CFU/g

Total fungi 6.0×10^1 CFU/g

Introduce.....properly

112 Table 1 shows the proximate composition biological and physiochemical properties of *Moringa*
113 *Oleifera* seed cake

113 used for bioremediation studies. The results from Table 1 above revealed that the *Moringa*
114 *oleifera* seed cake used had appreciable quantities of nitrogen and phosphorus 10.55 % and
115 18.33 ppm, which are major nutrients required by bacteria to degrade organic substances
116 from the environment (Dibble and Bartha 1979). The results of bioremediation studies with
117 crude oil polluted soil amended with *Moringa oleifera* shown in Figure 1, shows that the
118 counts of aerobic heterotrophic bacteria in unpolluted soil ranged from 2.0×10^6 CFU/g to
119 3.5×10^6 CFU /g of soil that of polluted but unamended soil ranged from 1.0×10^6 CFU/g to
120 1.5×10^6 CFU /g of soil (Lüring and Beekman, 2010). The counts in oil- polluted soil
121 amended with *Moringa oleifera* seed cake ranged from 1.2×10^6 CFU /g to 3.6×10^6 CFU/g

UNDER PEER REVIEW

134

of soil. The results indicate an increase in the counts of 122 total aerobic heterotrophic bacteria
123 in soil amended with *Moringa oleifera*.

124

125

126 Fig. 1. Microbial Growth Pattern in the Soil Samples

127 KEY:

128 Sample A = Unpolluted control soil;

129 Sample B = Untreated polluted soil;

130 Sample C = Polluted soil treated with *Moringa oleifera* seed cake;

131

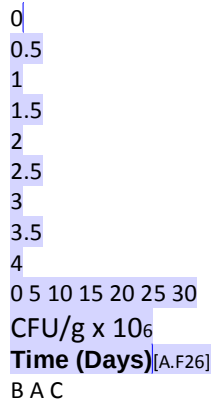
132 The crude oil utilizing bacteria isolates were identified as species of *Bacillus*, *Micrococcus*,

133 *Pseudomonas* and *Acinetobacter* -with Bacillus sp.(how?)

134

135 The microbial growth pattern in the untreated polluted soil sample (B) begins at the initial
136 point when the crude oil was first introduced into the soil some of microorganisms that were
137 in the oil were unable to survive, while some microorganisms were trying to gather enough

enzymes, adapting to the new environment and was able to feed on the substrate (crude oil) to some extent and started dying due to high concentration of the substrate and insufficient microorganisms in the soil. [arbitrary statements?](#) In samples C, [as shown in](#) Figure 1, the lag phase is very much reduced compared to the growth phase. The growth phase is elongated which indicate that there is adequate proliferation of the microbes in sample C and subsequently higher rate of bio-degradation of crude oil in the samples, compared to sample B. Sample A is regarded as the control in this case. The reason for the proliferation of the microbes in the samples can be attributed to the variation of pH in the samples, as shown in Figure 3. The microbial growth pattern begins with the lag phase, as shown in Figure 2, at which phase the microorganism keeps adapting to the new environment and trying to gather enough enzyme to survive. Beyond that lag phase begins the experimental growth phase meaning that the microorganisms are now used to the environment and can effectively make use of the substrate (feeding on crude oil). At the end of the experiment, growth sets in the stationary phase where there is possible depletion of the substrate, there is possible inhibition, there is possible [contamination](#)^[A.F25] and change in temperature. This is the period of dynamic equilibrium where the death phase is equal to the growth phase. In the death phase, the activities of the enzymes reduce because the substrate and nutrients are used up and the microorganisms begin to die gradually.



UNDER PEER REVIEW

Fig. 2. Analysis of the Growth Pattern in the Soil Sample (C) Treated with *Moringa Oleifera* Seed Cake

Fig.3: pH of the Soil Samples

KEY:

- Sample A = Unpolluted control soil;
- Sample B = Untreated polluted soil;
- Sample C = Polluted soil treated with *Moringa oleifera* seed cake;

The pH of the polluted soil amended with *Moringa oleifera* seed cake was raised from 7.24 to 7.70, as shown in Figure 3. This is one of the conditions that increased the rate biodegradation of crude oil in amended soil since crude oil degrading bacteria grow and utilize hydrocarbons better at slightly alkaline pH (Dibble and Bartha 1979).

3.5
4
4.5
0 5 10 15 20 25 30

CFU/g x 10⁶

Time (days)

C

Stationary phase

6.8

7

7.2

7.4

7.6

7.8

0 7 14 21 28

pH

Time (Days)

A B C

Lag phase

UNDER PEER REVIEW

136

174

175

Fig.4: Rate of Biodegradation of Crude Oil in Soil [176](#) Amended with *Moringa Oleifera* Seed Cake.

178 KEY:

179 Sample B = Untreated polluted soil;

180 Sample C = Polluted soil treated with *Moringa Oleifera* seed cake;

181 The results of bioremediation study also show that the rates of oil breakdown in oil-polluted
182 soil with time. In untreated polluted soil, sample B, 10% of the oil was biodegraded after 28

183 days whereas in polluted soil treated with *Moringa oleifera* seed cake, sample C, 74.3%[2](#) of

184 oil was degraded within the same period of time, as shown in Figure 4. This is most likely

185 due to the high nitrogen and phosphorous contents in the *Moringa oleifera* seed cake which

186 favour the growth of crude oil utilizing bacteria in the treated soil. [Ref?](#)

187

188 4. CONCLUSION AND RECOMMENDATION

189 From the results of this study, it can be concluded that *Moringa Oleifera* seed cake

190 possesses the ability to enhance the biodegradation of crude oil in soil by up to 64.3%.

191 *Moringa Oleifera* seed cake can serve as good materials for reclaiming/rehabilitating crude

192 oil polluted soil. Their use in reclaiming oil polluted soil could also solve the problem of solid
193 waste disposal in the environment.

194 [There should be collaboration between Federal Environmental Petroleum Agency \[FEPA\]](#)

195 [and various Research bodies on the use of bioremediation in soil reclamation.](#)

196 [There should be an oil pollution Act on the development of a National \[A.F27\]contingency plan on](#)

197 the use of bioremediation as an oil spill response tool.

198

199

200 REFERENCES

201 Alexopoulos, C.J.; and Mims, C.W. 1979. Introductory mycology, 3rd ed., Wiley, New York,

202 NY, USA. [pp](#)

203

204 Bossert, I.; and Bartha, R. 1984. The fate of petroleum in soil ecosystem. *In*: R.M. Atlas (Ed.)

205 Petroleum microbiology, Macmillan, New York, NY, USA, pp. 435-73.

206

207 Chang, Z.; Weaver, R.W.; and Rhykerd, R.L. 199). Oil bioremediation in high and low

208 phosphorus soil. *J. Soil Contam.* 5: 215-24.

209

210 Cowan, S.T. 1974. Manual for identification of medical bacteria. 2nd ed., Cambridge

211 University Press, Cambridge, England, pp. 56-82.

212

0

20

40

60

80

0 7 14 21 28

Wt. loss of Crude Oil

(%)

B Time(Days)C

UNDER PEER REVIEW

137

Dibble, J.T.; and Bartha, R. 1979. Effects of environmental 213 parameters on biodegradation of 214 oil sludge. Appl. Environ. Microbiol. 37: 729-739.

215

216 Ijah, U.J.J.; and Ukpe, L.I. 1992. Biodegradation of crude Oil by *Bacillus* strains 28A and 217 61B isolated from oil spilled soil. Waste Management 123: 55-60.

218

219 Ijah, U.J.J. 1998. Studies on relatives capabilities of bacterial and yeast isolates: Technical 220 Report 37AU J.T. 13(1): 34-38 (Jul. 2009). from tropical soil in degrading crude oil.

221 Waste Management 18: 293-9.

222

223 Ijah, U.J.J.; and Antai, S.P. 2003. The potential use of chicken-drop micro-organisms for oil 224 spill remediation. The Environmentalist 23: 89-95.

225

226 Ijah, U.J.J.; and Ndana, M. 2003. Stimulated biodegradation of crude oil in soil amended with 227 periwinkle shells. The Environmentalist 23: 249-54.

228

229 Jobson, A.; McLaughlin, M.; Cool, F.D.; and Westlake, D.W.S. 1974. Effects of amendments 230 on the microbial utilization of oil applied to soil. Appl. Environ. Microbiol. 27: 166-71.

231

232

233 Kokub, O.; Shafeeq, M.; Kahlid, Z.M.; and Malik, K.A. 1989. Isolation, screening and 234 characterization of biosurfactant producing bacteria. In: K.A. Malik; S.H.M. Maqui; and 235 M.I.H. Aleem (Eds). Proc. Int. Symp. Biotech. for Energy, 12-16 Dec. 1989, 236 NIAB/NIBGE, Faisalabad, Pakistan, pp. 221-32.

237

238 Korda A., Santas P., Tenente A., Santas R. (1997), *Appl. Microbial. Biotechnol.* **48** 77

239

240 Leahy, J.C.; and Colwell, R.R. 1990. Microbial degradation of hydrocarbons in the 241 environment. Microbiol. Rev. 54: 305-15.

242

243 Lüring, M and Beekman, W. (2010). Anti-cyanobacterial activity of *Moringa oleifera* seeds. 244 Journal of Applied Phycology, 22 (4): 503-510

245

246 Okoh, I.A.; Ajisebutu, S.; Babalola, G.O.; and Trejo-Hernandez. M.R. 2001. A Study of the 247 potentials of *Burkholderia cepacill* strain (RQ1) in the biodegradations of heavy crude oil 248 (Maya). Int. Microbiol. 4: 83-7.

249

250 Oyegun C.U. (1998). "Land Degradation and Coastal Environment of Nigeria" CATENA 251 vol. 20 pp 215-225

252

253 Song, H.; Wang, X.; and Bartha, R. 1990. Bioremediation potential of terrestrial fuel spills. 254 Appl. Enviro. Microbiol. 56: 652-6.

255

256 USEPA². 1990. Alaskan Bioremediation Project. Publication EPA/600/889/073, 1-21.

257

258 Zajic, E.; and Supplisson, B. 1972. Emulsification and degradation of "Banker C" fuel oil by

259 microorganisms. Biotech. and Bioengin.14: 331-43.
260
261

UNDER PEER REVIEW