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Full Length Research Paper

Lipase activities of microbial isolates from soil contaminated with crude oil after bioremediation

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This study involving assessment of the enzyme lipase as indicator of microbial degradation of crude oil was carried out using indigenous and exogenous soil microorganism. Standard microbiological, physiological and biochemical methods were adopted. Result obtained revealed *Edwardsiella tarda*, *Bacterium aliphaticum*, *Bacillus* megaterium, *Bacillus cereus*, *Pseudomonas maltiphilia*, *Fusarium vertiaculloide*, *Botryodiphodia thiobroma*, *Fusiarum oxysporum*, *Cryptococcus neofomas*, *Aspergillus niger* and *Candida tropicalis* as organisms indigenous to soil that have the potential to degrade crude oil. While biochemical analysis revealed that except *B. aliphaticum* which had lipase activity of 3.99 µ/ml, fungal isolates generally recorded higher lipase activities than bacterial isolates. *A. niger* showed the highest lipase activity of 4.00 µ/ml while *P. maltophilia* gave the least activity of 0.45 µ/ml after 6 weeks of remediation. The findings have possible industrial and environmental implications especially towards developing a bioremediation protocol at the enzymatic level.

Key words: Bioremediation, crude oil, enzymes.

INTRODUCTION

Remediation of contaminated aquifers continues to be a major focus of regulatory activity and an area of scientific and technical challenge for environmental professionals. Improper storage and waste management practices have left a legacy of contaminated soil and aquifers, threatening drinking water supplies and many other areas (Fetter, 1993). Ecosystems, including human communities, are faced with release of quantities of pollutants each year as a result of human activities. In some cases, these releases are deliberate and well regulated (e.g. Industrial emissions), while in other cases, they are accidental and largely unavoidable (e.g. Chemical spills) (Akpofure et al., 2000). Environmental pollution is one of the major problems facing industrialized world today. It is estimated that most underground water tables in the United States are leaking (Kovalick, 1991). While regulatory steps have been implemented to reduce or remove or eliminate production and release of these chemicals into the environment, significant environmental contamination has occurred in the past and will probably continue to occur in the future (Baker and Diana, 1994). The need to remediate these sites has led to the development of new technologies that emphasize on the detoxification and destruction of the contaminants rather than the conventional approach of disposal. Bioremediation, the use of microorganism or microbial process to detoxify and degrade environmental contaminants is among these new technologies. Regardless of the exact nature of the treatment technology, all bioremediation techniques depend on having the right microbes in the right place with the right environmental conditions for degradation to occur (Atlas and Bartha, 1987).

The right microbes are those bacteria or fungi, which have the physiological and metabolic capabilities to degrade the contaminants. In many instances these organisms will already be present at the site (indigenous microbes). In other circumstances, such as bioreactors treating wastes with high concentration of toxic materials, there may be a need to add exogenous microbes to the material. In order for the microbes to degrade the contaminants they must be in close proximity to the contaminants (Baker and Diana, 1994). Once the right microorganisms are present in the right place, the environmental conditions must be controlled or altered to

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optimize the growth and metabolic activity of the microbes. Such environmental factors as temperature, inorganic nutrients (primarily nitrogen and phosphorous), electron acceptors (oxygen, nitrate, sulfate and pH) can be modified to optimize the environment for bioremediation. The ultimate objective of bioremediation is to gain control over the biological activity in a given system on the premise that such control is best achieved through knowledge (Subhas and Robert, 1998). The aim of this research is to assess the relative abilities of microbes isolated from crude oil polluted soil to degrade crude oil hydrocarbons, to assay and possibly determine some kinetic parameters of crude lipase extract from the different microbial isolates.

MATERIALS AND METHODS

Materials

Bonny light (B –iii Nigeria) crude oil from Technology Partners Institute (Port Harcourt). Mac conkey Agar (Antec Diagnostic laboratories), Nutreint Agar (Biotec laboratories) and Sabouraud Agar medium (Biotec laboratories) were used for isolation. Peptone water sugar medium (V-Patricks laboratories), Motility test medium (V–Patricks laboratories), Simmions citrate agar (Biotech laboratories), Urea agar (christensens urea agar) (V- Patricks laboratories), Glucose (dextrose) phosphate peptone water (Biotech laboratories) were used for identification. Fresh palm oil was obtained from Ekenuwa Market, Owerri, Nigeria.

Study area description

The study area for the experiment is located at Federal University of Technology, Owerri Nigeria. The area has a lowland forest, which is relatively dense with a luxuriant woody plant structure, arranged in strata. The tallest trees are about 50 m in height while the middle tree layers have trees ranging in height between 20 and 30 m and the lowest tree layer has their tree also ranging in height between 10 and 20 m.

The soil is moist all the year round due to the extensive rainfall of about 2700 m. This volume of rainfall provides great amount of surface run–offs rivulets and occasional streams which may carry substance like crude oil, granulated fertilizers and other pollutants to nearby lands and rivers.

Collection of soil sample

A soil auger was used in collecting soil sample for analysis. The auger was used to make a depth of 30 cm using a grid or Zig zag sampling system as reported by the National Association of testing Authorities (NATA, 2005). The soil sample for physiological analysis was collected with unused plastic bag sealed with heavy-duty rubber bounds. All samples were labeled with a permanent waterproof maker, while the microbiological analyses were collected using 200 ml capacity sterile glass sampling container. The soil sample was taken to the Laboratory within 1 h of collection for analysis.

Isolation, characterization and identification of soil microorganisms

The soil samples were aseptically subjected to serial dilutions and plated on oxoid Nutrient Agar (NA), MacConkey agar (MCA) and Dextrose Agar (SDA) using spread plate technology and incubated at 30°C for 24, 48 and 72 h as described by Cruckshank et al. (1982). Acceptable plate counts for bacteria were between 30 – 300 cfu/ml per plate. After incubation, bacterial species were isolated and subjected to morphological, cultural and biochemical examination as reported by cheesbrough (1985) and Cruchshank et al. (1982) to identify them, while fungal isolates were subjected to culture and staining to identify them.

Experimental design

Soil microcosm were used in the experiment .The soil microcosm were prepared in glass- Stoppard 50 ml flask each containing soil equivalent to 20 g (dry weight), excluding sterilized soils. The soil microcosms were divided into the following:

Microcosm A = soil + *Edwardsiella tarda* isolate + crude oil + N.P.K. fertilizer
Microcosm B = Soil + *Bacillus aliphaticum* isolate + crude oil + N.P.K. fertilizer
Microcosm C = Soil + *Bacillus megaterium* isolate + crude oil + N.P.K. fertilizer
Microcosm D = Soil + *Bacillus cereus* isolate + crude oil + N.P.K. fertilizer
Microcosm E = Soil + *Pseudomonas maltophilia* isolate + crude oil + N.P.K. fertilizer
Microcosm F = Soil + *Fusarium vertiaculloide* isolate + crude oil + N.P.K. fertilizer
Microcosm G = Soil + *Botryodiphodia thiobroma* isolate + crude oil + N.P.K. fertilizer
Microcosm H = Soil + *Fusiarum oxysporum isolate* + crude oil + N.P.K. fertilizer.
Microcosm I = Soil + *Cryptococcus neofomas* isolate + crude oil + N.P.K. fertilizer.
Microcosm J = Soil + *Aspergillus niger* isolate + crude oil + N.P.K. fertilizer.
Microcosm K = Soil + *Candida tropicalis* isolate + crude oil + N.P.K. fertilizer.
Microcosm L' (control) = Soil + All microorganism (Consortia), no crude oil.

Soil treatment, crude oil, fertilizer and microbes for challenge test

Soil samples were collected as described by NATA (2005) from a depth of 0 – 30 cm. The soil was dried under the sun to a constant weight. Stones and debris were removed manually. Simulated Crude oil spills were achieved by adding 20 ml of the crude oil to 100 g fractions of the dried composite soil. This is referred to as simulated crude oil polluted soil (S.C.O.P.S). There were three replicates of each and controls. Mixing was carried out with a horizontal arm shaker at 120 rpm for about 30 min. The soil used had no history of crude oil pollution or any other known pollution and was characterized prior to crude oil application at the Department of Crop Science Laboratory, Federal University of Technology, Owerri. The soil used for control experiment was sterilized in a hot air oven at a temperature of 120^{o}C for 30 min before spiking with crude oil (Onwura, 2004).

Fertilizer application

A 15:15:15 NPK fertilizer was used for the soil microcosm. A 100 g of the fertilizer was dissolved in 20 ml of water and 20 ml of the solution was applied into soil microcosm treatment types that required fertilizer application, the set up was mixed thoroughly

using a glass rod (Odokuma and Dickson, 2002).

Inoculation of microorganism

The process of scaling as described by Odokuma and Dickson (2002) was used. Nutrient agar slants of all the microorganisms isolated from the site were prepared. 9 ml of sterile normal saline (0.85% NaCl) was transferred into a 20 ml sterile test tube. A sterile inoculating wire loop was used to scrape organism from these slants into the normal saline. The suspension was then transferred into a conical flask containing 50 ml of sterile mineral salt medium. The mixture was placed on a shaker and incubated at room temperature of 30°C for 7 days. This was done for each of the microorganism isolated from the soil.

The pH of each of the culture at all stages was monitored daily and maintained at 7.2 by adjusting with standard phosphate buffer (APHA, 1985). Aliquots of the final culture were weighed. The process (centrifugation) was repeated until the weight (wet) desired for deposition on treatment tests were achieved. These deposits served as inoculation for each soil microcosm treatment type. The microbial load applied per each treatment type was 20 ml. The above was used to produce the set of treatment shown below.

Total petroleum content of crude oil polluted soil

The method used was as described by Onwurah and Nwoke (2004). At time zero, before inoculation of SCOPS samples with bacteria, extractable crude oil as petroleum hydrocarbon (PHC) or total hydrocarbon content (THC), was carried out on portions of the soil, using an ethanol–chloroform mixture (1:1). Double step–wise extractions was performed through agitation of 1.0 g fractions of SCOPS and uncontaminated soil samples with 2 cm^3 of the extraction mixture, for 30 min, in a horizontal arm shaker, at 120 rpm. The optical density of each filtrate was read at 520 nm against a blank of the extraction mixture that had been run through uncontaminated soil samples. A calibrated standard curve was developed from known concentrations of crude oil in the extraction mixture, plotted against the corresponding absorbances at 520 nm. Subsequent THC was analyzed after 2 weeks, 4 weeks and 6 weeks using the standard curve.

Measurement of lipase enzyme activity

The method used was as described by Doye et al. (1984). A quantity of 20 ml of each soil microcosm treatment cells of soil sample was introduced into a mortar and pistil and ground gently for 5 min, to release the extracellular microbial enzyme in a liquid medium. The whole solution was then transferred into a glass-separating funnel and allowed to stand for 30 min at room temperature. A quantity of 3 ml each of the supernatant was used as source of crude enzyme solution.

The titration method of Doye et al. (1984) for lipase determination was used. Lipase activity was measured by titrating the fatty acid released with 0.1 M NaOH using 0.1% alcoholic phenolphthalein as indicator. Fresh palm oil was used as the source of glyceride. The oil (0.5 ml) was taken in a glass stopper Erlenmeger flask and 10 ml of acetate buffer (0.107 M) and 1 ml of hexane were added. The contents were stirred for 5 min (for thorough mixing). Then 1 ml of the enzyme solution was added with vigorous shaking. The set – up was allowed to stand for 20 min for hydrolysis to take place with continuous shaking on a rotary shaker at 30 ± 1°C. At the end of the hydrolysis, 20 ml of ethyl alcohol was added. The liberated fatty acid was titrated against 0.1 M NaOH. The determination of the blank was also carried out with the only difference that the enzyme solution was added after 20 min. The activity was expressed in arbitrary units–one arbitrary unit of enzyme activity corresponds to 1 ml of 0.1 M NaOH required to neutralize the fatty acid liberated during the incubation period of 20 min at 30°C. Appropriate controls were always run to compensate for spontaneous hydrolysis and the inherent acidity of the enzyme preparation. Zero time control titres were subtracted from each of the determinations and the averages of duplicate analyses were reported.

Table 1. Lipase activities of microbial isolates from soil two to six weeks after remediation.

Microbial isolate	Enzyme activity (units/ml)		
	2 weeks	4 weeks	6 weeks
E. tarda	0.08	0.85	0.90
B. ailiphaticum	1.80	2.80	3.99
B. megaterium	0.79	0.80	0.82
B. cereus	0.50	0.65	0.75
P. maltophilia	0.20	0.30	0.45
F. vertiaculloide	2.40	3.50	3.98
B. thiobroma	2.30	3.40	3.72
F. oxysporum	1.20	2.00	2.50
C. neoformans	2.10	2.50	2.90
A. niger	2.40	3.80	4.00
C. tropicalis	2.20	3.00	3.20

Results represent mean differences between the blank and sample text. One unit of enzyme activity corresponds to 1 ml of 0.1 M Na0H required to neutralize the fatty acid liberated during the incubation period of 20 min at 30°C.

RESULTS AND DISCUSSION

The results of changes in lipase activity during the 6 weeks of bioremediation for the 11 isolates as shown in Table 1. Results obtained showed that there is a great parity in the abilities of these microbes to degrade crude oil hydrocarbons. Chemical analyses have shown that crude oil is a complex mixture of polar and non polar compounds (Dragun, 1988). It has also shown that, crude oil contains more of non polar compounds than polar (Subhas and Robert, 1998). Carbon atoms of these polar and non polar constituents of crude oil serve as growth substrates and non growth substrates for microbial enzymes during degradation of this biogenic (hydrocarbons) compound (Polleroni, 1994). Since the same enzyme catalyze the initial degradation of both the growth and non growth substrates, competition for the enzyme can occur, reducing the rate of growth substrate degradation (Subhas and Robert, 1998). Results have also shown that the reduction in the rate of non growth substrate degradation can occur in the presence of growth substrates (Gill, 1989). This shows that the decrease in their action will depend on the relative affinity of each of the substrate for the microbial enzymes. This shows that a non growth substrate with high enzyme affinity will significantly decrease the rate at which the growth substrate is degraded, while a non growth sub-

strate with low enzyme affinity will have much less effects, thus explaining the great parity in the abilities of these organisms to degrade crude oil hydrocarbons as obtained in this study which corroborates the findings of Subhas et al. (1989) and Dixon and Webb (1979). More research is encouraged in this area especially to determine whether there is the possibility of the non growth substrate-enzyme interaction leading to the formation of suicide-enzyme intermediate after a longer time thereby leading to dead-end since some of the treatment samples showed very low production of lipases which have been used as indicators of bioremediation.

In addition to competitive inhibition, unproductive binding of a non growth substrate conceivably could cause uncompetitive inhibition or noncompetitive inhibition of growth substrate degradation as shown in the findings of Dixon and Webb (1979), thus explaining the very low production of lipase obtained in this study.

All the organisms in simple forms showed moderate utilization of crude oil hydrocarbons in all treatment samples, this may result from the reaction of intermediates and products that are highly reactive and that inactivate the microbial enzymes by covalently binding to it and changing its structure (Rasche et al., 1991). More research is encouraged in this area especially in using pure enzyme cultures.

This study generally has used lipase as a biochemical indicator of bioremediation and other studies in this direction will help to unravel the missing link in the role biochemists and biochemistry can play in the remediation of biogenic pollutants in our environments. Looking also at the performance of the individual isolates from the soil (both fungal and bacterial); result obtained revealed that fungal isolates showed higher lipase activities than bacterial isolates generally. However, *A. aliphaticum* performed beyond expectation among other bacterial isolates showing that this organism can be optimized for bioremediation protocols; hence it can be used as initial consortium before fungal consortium. This could be because bacteria have shorter generation time compared to fungi.

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