ASSESSMENT OF MICROBIAL DEGRADATION OF BONNY LIGHT CRUDE OIL BY SOIL ISOLATES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF POST GRADUATE DIPLOMA (P.GD) INDUSTRIAL MICROBIOLOGY

BY

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DEDICATION

This work is dedicated to my parents.

Mr and Mrs Patrick Mbah

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My profound gratitude goes to my Supervisor Dr. N. A. Oranusi for his able supervision.

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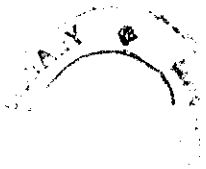
May I also thank the rest of my family for their prayers

Above all, I thank the Lord Jesus Christ for his love, grace and mercy that saw me through.



ABSTRACT

The role of microbial degradation of Bonny Light Crude Oil (BLCO) was assessed by laboratory determinations of counts of hydrocarbon utilizing bacteria, optical density and measurement of microbial hydrocarbon biodegradation potentials by gravimetric analyses of samples incubated for a two weeks period under room temperature. The bacteria isolated were members of the genera *Pseudomonas, Alcaligenes,, Corynebacteria, Bacillus and Arthrobacter.* All the isolates caused the degradation of Bonny Light Crude Oil. (BLCO). Counts of hydrocarbon - utilizing population increased from 10^4/ml to 10^{15}/ml with concomitant increase in optical density. *Bacillus sp* showed the highest degradation with a percent degradation of 34.2%. Increase in counts of hydrocarbon utilizers and optical density in some cases did not reflect corresponding increase in rates of biodegradation. It therefore becomes necessary to utilize total viable count (TVC), optical density (O.D) and residual oil determinations in assessing the role of microbial degradation.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION:

The high demand for crude oil, as a source of revenue, energy and as a principle raw material for petrochemical industry has generated in recent times, increase in production (Udeme and Antai, 1988).

Until 1937, oil exploration remained an uncharted terrain in Nigeria, in fact all across West Africa, it was like a fresh venture. The search continued till 1957 when the first oil was struck in Oloibiri Rivers State by Shell D'arcy Exploration Company Limited now Shell Development Company Limited.

Without doubt, Petroleum is at present Nigeria's and indeed, the world's most important derived energy supplier (NEST, 1991). Other sources of energy such as natural gas, coal, tar, sands and oil shale do exist but cannot be compared to oil and coal in terms of exploitation and utilization in Nigeria. Nigeria's proven oil reserves are close to three billion tones of flowing oil. There is also the need to notice "trapped " or non -flowing oil. The latter comprises vast quantities of petroleum left over after the more accessible oil has been pumped out. The amount of oil trapped in rock pores and cracks may range up to 50 or 60 percent of the original gross oil deposit estimates. Intimately associated with oil in Nigeria is natural gas. The nations natural gas reserves are estimated to be some 2,600 billion cubic meters, of this tremendous quantity, only 20 billion cubic meters is produced annually; Again, it is only a mere ten percent of this yearly production figure that is utilized, while the rest is deliberately flared (NEST, 1991).

The Niger Delta is the hub of oil exploration, exploitation and transportation in Nigeria. The increased activity in the petroleum industry has made accidental spillage inevitable in this area. (Okpokwasili and Nnorom, 1990).

Presently, oil is the most significant pollutant in the environment due to the fact that it is capable of causing serious damage to life and vast destruction of the environment.

In this regard "Pollution" is defined as the addition to any segment of the environment, any material which has detrimental effect on the ecosystem.

The greatest single environmental problem connected with petroleum exploitation in Nigeria is oil spillage both on-shore and off-shore. The rate of spill has been rising with increasing tempo of petroleum production. Only a single spill was reported in the country in 1970 whereas in 1971, the number shot up to 14. In 1974, 105 oil spills were reported in Nigeria, 154 in 1978 and 216 in 1982 (NEST, 1991). Although there has been no clear estimates of the total input of petroleum into the soil environment, Morgan and Watkinson (1989) identified three main sources from which hydrocarbon enter the soil:

i. Continuos low level inputs from road surface, domestic waste and slow seepage from old underground tanks.

ii Major spillage from tankers, pipelines and storage tanks.

iii Slow natural seepages from natural oil reservoirs.

Major spills, despite their news worthy nature, are only a minor overall source while the main source of introduction of hydrocarbons into the soil environment is continuos low-level inputs. (Morgan and Watkinson, 1989; Shaheen, 1992).

Regardless of the source of contamination, some oil or decomposition products may reach ground water reserves, lakes or water courses providing water for domestic and industrial use; posing serious threat to public health and socio-economic hazards to the inhabitants especially in areas which rely on groundwater and rivers as major sources of drinking water and productive fishing.

The 1980's were a period of rapid advances in knowledge of the genetics and molecular biology of bacteria degradation of different hydrocarbons and of renewed interest in the microbial ecology of pollution - stressed environments. Much progress has been made in clearing up terrestrial and enclosed sights polluted with oil. When an accidental oil spill occurs, The first priority is mechanical recovery which helps to decontaminate locations that have high cumulative or shock loading of petroleum (Morgan and Watkinson, 1989) Upon spilling into the environment, oil begins to

undergo a series of physical and chemical changes. The processes leading to these changes include spreading, emulsification, photochemical oxidation, sinking, dissolution/ leaching, evaporation, sedimentation and adsorption. These collectively make up weathering processes. From the moment oil is spilled and weathering begins, microbial infection takes place. This is followed by microbial growth and the consequent biodegradation (Okpokwasili and Nnorom, 1990). The weathering process can either be biological as regards ingestion or abiological for example evaporation, leaching, photochemical oxidation etc. where as some of these processes such as evaporation and dissolution are rapid, others such as oxidation and biological degradation are long term processes on which depend ultimate evolution of the hydrocarbon. (Ladousse and Tramier, 1991).

The impact of an oil spill depends on the magnitude of the spill, the chemical composition of the oil and the nature of the contaminated ecosystem. The initial impact can vary from minimal (e.g following some open ocean spills) to the death of everything in a particular biological community. A mangrove swamp which has trapped crude oil, leading to death of the mangrove trees and associated fauna, can present a particularly bleak future.

These impact include

a) Devegetation and other forms of ecological damage;
b) Fall in productivity of both animals and plants;
c) Loss of fish, crustaceans and other aquatic life; loss of drinking and industrial water and eutrophication of water bodies

Biodegradation refers to the natural process whereby bacteria or other microorganisms alter and break down organic molecules into other substances such as fatty acids and carbon dioxide. Bioremediation is the act of adding materials to contaminated environments, such as oil spill sites to cause an acceleration of the natural degradation process. Compared with other processes such as incineration, bioremediation is - relatively inexpensive and it is not a new technology. The

biological agents used, which often are commonly occurring bacteria and fungi that degrade pollutants are normally indigenous to the contaminated area or may be isolated elsewhere or produced genetically to degrade a specific pollutant in a particular locale (Atlas and Prammer, 1990).

The mode of hydrocarbon alteration by bacteria that consume petroleum known as "hydrocarbon oxidizers" is the addition of oxygen to the petroleum molecules, the newly formed oxygenated molecules are further consumed until only the final products, biomass and carbondioxide remain. The process of converting the oil to carbondioxide and biomass is called mineralisation. (Chianelli et al 1991). The wide array of microbial metabolic capabilities enables microorganisms to act as "natures" incinerator to remove or reduce pollutant concentrations to levels that no longer present a risk to human health and to the environment (Atlas and Pramer, 1990). The microbial degradation of complex inimical pollutants on a large scale makes bioremediation possible and attractive.

It is known that greater degradation of oil pollutants is carried out in situ by a consortia of microorganisms (Okpokwasili and Nnorom, 1990). According to C.E. Zobell (1969), more than 200 species of bacteria, yeast and even algae can biograde hydrocarbon. These microorganisms can be found in a variety of different environments such as oceans, lakes, estuaries and sediments and particularly environments chronically exposed to hydrocarbons. The presence of microorganisms especially adapted to the degradation of hydrocarbons was shown by Atlas and Bartha in the early 1970s. The various microbial genera that are reported to contain hydrocarbon degrading species include *pseudomonas, Achromobacter, vibrio, Arthrobacter, Micrococcus, Nocardia, Acinetobacter, Flavobacterium, Corynebacterium, Candida, Rhodotarula and mycobacterium.* These organism have been isolated abundantly in many oil polluted waters and soils but are found in less number in unpolluted environments (Floodgate 1984).

The principle by which mixture of microorganisms act together to bring about the oxidation of a complex compound is known as cometabolism. In this

process, compounds which otherwise would not be degraded can be enzymatically attacked within the crude oil mixture due to the abilities of the individual microorganisms to grow on other hydrocarbons within the oil. Crude oil provides an excellent chemical environment in which cometabolism can occur because it contains multitudes of potential primary substrate (NEST, 1990; Chianelli et al, 1991).

The mere disappearance of oil (e.g. through emulsification by living cells) technically is not biodegradation if the oil has not actually been chemically transformed by microbes. (Cooney, 1990). The ideal may be difficult in reasonably short time, given the recalcitrance of some petroleum fractions to biodegradation and the many variables that affect its rate and extent. Man-made bioremediation technologies are intended to improve the effectiveness of natural biogradation. In general, the natural biodegradation process requires the following factors.

- Adequate macro and micro nutrients
- Source of oxygen
- Presence of optimal temperature ranges and pH levels
- Freedom from toxic or inhibiting substances
- Presence of acclimated microbial population. (Safferman, 1991).

Having known that natural biodegradation of hydrocarbons occurs at a slow rate due to certain environmental limitations, the above factors can be modified to support or accelerate microbial growth and consequently biodegradation.

Much attention is being given to bioremediation which reflects a growing public concern with environment quality and an increase interest in natural biological processes that prevent or minimise pollution. When pollution prevention fails, bioremediation can help improve environmental quality as part of a balanced program that include measures to enhance natural cleaning process. This study is focused generally on natural biodegradation of crude oil by soil isolates.

1.2 LITERATURE REVIEW

1.2.1 THE SOIL ENVIRONMENT AND ITS MICROFLORA

The transmission of oil from the point of contamination either to the ground water or a nearby stream depends on the nature of the oil and soil. The soil is a relatively favourable environment for petroleum biodegradation because of its great microbial diversity, less scarcity of mineral nutrients (although sometimes, limiting) and the high absorption capacity of clay and soil organic matter respectively (Bossert and Bartha, 1984).

The soil is composed of organisms belonging to these classes: bacteria, Actinomycetes, Fungi, Algae, protozoa and nematodes. The microorganisms together represents a minute proportion of the soil mass but accounts for most of soil biochemistry and are numerous in numbers (Blakebrough, 1978). The biological characterisation of natural soil is centered on the soil microorganisms since they exert a profound impact on waste constituent mobility. Because their activity is not readily discernable, they are often overlooked.

It is an established fact that in the presence of high amounts of toxic substances complete destruction of the microflora is rarely possible. Soil "sterilisation" is at best, temporary for most of the indigenous soil microorganisms. In other words, the soil microflora in the natural habitat is highly versatile and resistant to destruction even by the most drastic treatment and will recover fully in time (Fuller and Warrick, 1985). Another important characteristic of the soil microflora is that they are well established ecological unit resistant to invasion or settlement of new microorganisms not indigenous to the habitat. Introduction of a specific culture into an environment have been observed by Lee and Levy (1989) to cause competition between them and the indigenous microflora; leading to the rapid disappearance of the new culture, success can only be achieved by manipulation of environmental condition to reduce competition, or availability of a host plant e.g. legume or infecting plant.

The single most important means of altering the natural soil microflora is to alter the energy or food source. The microflora adjusts to the kind of material and circumstance with which it is confronted, whether gas, liquid, solid, organic or inorganic substance. Microorganisms will attack almost any material at a given concentration. They synthesize as well as biodegrade. The transformation may be beneficial or detrimental to good waste disposal operations and management. The soil microorganisms in waste management processes either perform entirely or mediate:

a) Degradation of carbonaceous waste

b) Transformation of cyanide to mineral nitrogen compounds and denitrification to inert N_2 Gas.

c) Initiation of metal ion oxidation-reduction

d) Production of CO_2 with subsequent formation of weakly ionized carbonic acid.

e) Production of simple organic acids, methane, hydrogen, and carbon monoxide.

f) Production of large or small molecular species upon which trace contaminants may be absorbed

g) Production of complex organic compounds which may react with waste contaminants.

h) Production of small-sized organic debris which can infiltrate small pore spaces and move downwards in the soil profile. (Fuller and Warick, 1985).

1.2.2 THE CHEMICAL NATURE OF PETROLEUM AND ITS SUSCEPTIBILITY.

Crude oil is a complex mixture of different chemical compounds. In addition, the composition of each accumulation of oil is unique, varying in different producing regions and even in different unconnected zones of same formation (NRC, 1985). The composition of oil also varies with the amount of refining. Significantly, the many compounds in oil differ markedly in volatility, solubility and susceptibility to biodegradation.

Some compounds are readily degraded, others stubbornly resist degradation, still others are virtually non-biodegradable. The biodegradation of different petroleum compounds occurs simultaneously but at very different rates (Atlas, 1988).

Petroleum hydrocarbons in general can be divided into four broad categories namely, saturates, aromatics asphaltenes and resins (Leahy and Colwell, 1990).

Saturated hydrocarbons constitute the largest group of hydrocarbons. They are made up of compounds with only single carbon-carbon bonds, commonest of which are the normal or straight chain alkanes (alkanes). These n-alkanes are the most susceptible to biodegradation. Therefore the higher the content of n-alkanes the more biodegradable the oil.

Atlas (1988) reported that compounds with chains of up to 44 carbon atoms can be metabolized by microorganisms but those having 10 to 24 carbon atoms (C_{10} to C_{24}) are usually the easiest to metabolize. Shorter chains (up to about C_{12}) also evaporate relatively easily. Only a few species can use C_1 to C_4 alkanes, C_5 to C_9 alkanes are degraded by some microorganisms but toxic to others. The saturated fraction also contains branched chain alkanes and cycloalkanes and these are less vulnerable to microbial attack. The cycloalkanes (napthenes) are those alkanes having carbon atoms in ring-like central structures. They are more resistant to biodegradation than branched alkanes.

Branched alkanes are increasingly resistant to microbial attack as the number of branches increases. Similarly as the number of rings in a cycloalkane increases, the susceptibility to biodegradation decreases. Fingas, (1990) reported that light oils contain 10-40 per cent of normal alkanes, but weathered and heavier oils may have only a fraction of a per cent. Heavier alkanes constitute 5 to 20 per cent of light oils and up to 60 percent of heavier oils.

Aromatic hydrocarbon are defined as those having at least one benzene (or substituted benzene) ring. Low-molecular-weight aromatic hydrocarbons are subject to evaporation and although toxic to microorganisms are also relatively easily degraded. Light oils contain between 2 and 20 percent light aromatic compounds

whereas heavy oils contain 2 percent or less. Susceptibility to biodegradation of these fraction decreases as molecular weight and complexity increases. Thus the degradation rate of polyaromatics is slower than that of monoaromatics. Aromatics with five or more rings are not easily attacked and may persist in the environment for long periods (NCR, 1985).

High molecular-weight-aromatics comprise 2 to 10 percent of light oils and up to 35 per cent of heavy oils (Fingas, 1990). Sulfur containing aromatics are roughly twice as recalcitrant as their non-sulfur analogues. The asphaltic fraction contains compound that either are not biodegradable or are degraded but very slowly (Cooney, 1990).

Resins include petroleum compounds containing nitrogen, sulfur, and/or oxygen as constituents. If not highly condensed, they may be subject to limited microbial degradation. Extensive losses of asphaltenes and resins have been observed in some cases. The microbial degradation of these relatively recalcitrant fraction have been ascribed to co-oxidation. (Leahy and Colwell, 1990).

Light oils may contain about 1 to 5 percent of both asphaltenes and resins, heavy or weathered oils may have up to 25 percent asphaltenes and 20 percent resins (Fingas, 1990). It was concluded by Leahy and Colwell, (1990) that biodegradation rates are typically highest for the saturates, followed by the light aromatics with high-molecular-weight aromatics asphaltenes and resins exhibiting extremely low rates of degradation.

1.2.3 UPTAKE OF HYDROCARBON BY MICROORGANISM

Microorganisms have evolved their capacity to degrade hydrocarbons over millions of years. These compounds are a rich source of carbon and energy that microbes require for growth. Before that carbon is available to microorganisms, however, large hydrocarbon molecules must be metabolized or broken down into simpler molecules suitable for use as precursors of cell constituents. Ultimately, hydrocarbon molecules have to enter cytoplasm of microorganisms. Two main

theories have been proposed about the mechanism of uptake of hydrocarbon (Higgins and Gilbert, 1978). The first involves uptake of hydrocarbon that has become solubilized in the aqueous phase while the second involves physical adhesion between the microorganisms and oil droplet with the hydrocarbon taken up without solubilization in the aqueous phase; (Moo-Young and Shimizu, 1971). The first theory supports the uptake of gaseous hydrocarbon whilst the latter favours that of liquids, although there remains some uncertainty about the size of the droplets involved. An important requirement for uptake and subsequent biodegradation of hydrocarbons is close association between microorganisms and droplet. In some cases direct entry by solubilisation in the membrane probably occurs, whilst in others the hydrocarbon may be solubilized by a surfactant produced at the interface between organism and droplet. This suggest that some form of facilitated diffusion mechanism is involved since there is no evidence for active transport process requiring direct utilization of metabolic energy. Whatever the mechanism, hydrocarbon uptake is a very rapid process (Higgins and Gilbert, 1978).

1.2.4 IMPACT OF HYDROCARBON ON FUNCTIONING OF MICROBIAL COMMUNITY

The environmental impacts of oil spills are highly diverse. Some spills, for example those that result from break up of super-tankers in high seas or blow-out of off-shore wells, often have immediate and catastrophic impact on the neighbouring ecosystems. They also attract maximum publicity and public attention. However, the vast majority of oil spills are of moderate scale (e.g. 100-100m^3), and they occur mainly on land. Such spills attract little public concern although they can cause subtle but long lived ecological disturbances of various kinds. (Duffy et al, 1979). The effect of petroleum hydrocarbon on microbial metabolic activities, will depend on the characteristics of the indigenous microbial community, environmental factors composition and concentration of the oil and whether the microorganisms were previously exposed to oil (Pfaender and Buckley, 1984).

Vestal and colleagues reported that hydrocarbon specific microflora exist naturally and comprise approximately 1 percent of the total heterotrophic populations in unpolluted environment. The oil degrader population increases to as much as 10 percent in response to a spill. At many spill sites, a very low level of oil is often present as "chronic" input, inducing oil degradability in naturally occurring microorganisms, hence microorganisms previously exposed to crude oil have a higher capacity for its degradation due to adaptation and adverse effect on such community is low.

Oil degrading microorganisms are ubiquitous and can metabolize a wide range of oil components under diverse environmental conditions (Atlas, 1981). The introduction of hydrocarbon to the soil represents basically an increase in carbon content. To enhance natural biodegradation, enough nitrogen, phosphorous and oxygen are required to balance the available hydrocarbon contamination in order for microbial growth and hydrocarbon reduction to occur.

There is a potential inhibiting effect of crude oil constituents on both the indigenous biomass and their biodegradative activity on readily biodegradable or biogenic substrates at certain levels. (Tabak et al 1991). These effects include damage to biological organisms by direct toxicity, sublethal effects, inducement of behavioral changes, tissue damage, tainting etc.

Generally, microbial numbers and activity are enhanced in contaminated soil.

1.2.5 OIL SPILL ENTRY AND MIGRATION

Crude oil contamination of soil could either be from a point source such as leakage or failure of a tank/pipe or dispersion over the land surface due to routine low level input by rainwash off and inland farming process (Morgan and Watkinson 1989). Bossert and Bartha (1984) observed that the physical distribution of oil spilled onto soil affects its ecological impact as well as its clean up.

Once spilled into the soil, oil is distributed by lateral spreading and vertical penetration. Lateral spreading facilitates the removal of the low molecular weight components but increases the contaminated area. Vertical penetration is the downward movement of oil until it exhaust itself to immobilization and remains in the soil at a residual saturation level or it reaches the water table where it forms a "pancake" or layer. It results in deep seated pollution of subsoil or groundwater contamination, decreased evaporation and reduced available oxygen but reduces the area of contamination. Most oil spill undergo vertical penetration due to the influence of gravitational and capillary forces.

Factors affecting the rate of movement of oil include the spilled volume, contour of land, soil structure, viscosity of oil, vegetation cover, temperature and rainfall (Bossert and Bartha 1974).

If oil loading is high, penetration into some sediments may be enhanced, and there is greater likelihood of oil masses incorporating stones and gravel and hardening to relatively persistent asphalt pavements. Sheltered sand and shores tend to be sedimentary with mud flats, Marshes and (in the tropics) mangroves, such vegetated areas have a high biological productivity but are also the worst oil traps. Oil pathways are provided by burrows of worms, mollusc and crustaceans and the stems and root systems of marsh plants. Oiling of such area results in sub-surface penetration. Oil penetrates relatively easily on sand, gravel or stones because they are porous. In contrast, oil does not readily penetrate into firm water-logged, fine sand or mud. Temperature affects the viscosity of the oil (and so the ease with which it can be dispersed, and with which it can penetrate into sediments),

The downward movement of oil in freshly contaminated soil generally occurs as a multiple phase flow (Somers, 1974). With the more hydrophillic hydrocarbons migrating in the aqueous phase (Shallubhai, 1986). As they become emulsified and solubilized by weathering and biodegradative processes, the system approaches the state of single phase flow. The leaching hydrocarbon is dependent on soil texture, amount and intensity of rainfall, water solubility of individual hydrocarbons (Bossert

and Bartha, 1984). Mobility is also enhanced by microbial activity such as hydrocarbon oxygenation and biosurfactant production (Somers, 1974; Price, 1980; Zajic et al, 1974; Bossert and Bartha, 1984). As soon as the hydrocarbon reaches its destination which is the water table, its hydrophillic components may dissolve in the water to cause tainting of water sources (aquifers) (Somers, 1974) or its hydrophobic components tend to spread along the water table forming a "pancake".

1.2.6 ELIMINATION OF HYDROCARBON FROM SOIL

Crude oil and petroleum distillate products introduced to the soil environment can be eliminated either through abiological weathering process or biological processes. In most cases, both mechanisms are at play.

1.2.6.1 ABIOLOGICAL PROCESSES

The abiological processes that determine the fate of hydrocarbon in the natural environment are volatilization, leaching and chemical or photochemical oxidation. Abiotic removal of hydrocarbon from a contaminated site is relatively unimportant unless enhanced by specific environmental conditions (Morgan and Watkinson, 1989).

1.2.6.1.1 VOLATILIZATION

Evaporation is a process by which low to medium weight crude oil components with low boiling points volatilize into the atmosphere. It is the most important weathering process during the first 48 hours of a spill. Evaporation can be responsible for the loss of one to two thirds of an oil spill mass during this period (NCR, 1985). With the loss rate decreasing rapidly over time (Floodgate, 1984). Evaporation loss is controlled by the composition of the oil, its surface area and physical properties, wind velocity, air and soil temperatures and intensity of solar radiation. (Payne and Mcnabb, 1984). It was observed by McGill et al (1981) that up to 20-40% of crude oil may volatilize from soil in high temperature regions such as

the tropics, also an aid is extensive lateral spreading over impermeable surfaces and adsorption onto surface vegetation. Franckie and Clark (1974) reported that less than 0.1% of crude oil evaporated from soils in a temperate arable soil. This may be due to the fact that the oil is absorbed into the surface strata and migrates into the sub-surface layers.

Floodgate (1984) concluded that the material left behind after evaporation is richer in metals (mainly nickel and vanadium), asphaltenes and waxes than the original oil. With evaporation, the specific gravity and viscosity of the original oil also increases since the volatile components tend to be membrane disruptive, their removal may therefore enhance subsequent biodegradation and increased amount of recalcitrant components.

1.2.6.1.2 LEACHING

The leaching process does not contribute much to hydrocarbon elimination from polluted soil. For hydrocarbon to be leached into surface and ground water, their solubility in water is of great importance, also the soil texture, amount and intensity of rainfall. Duffy et al (1980) reported that more than the equivalent of 140 times an average yearly rainfall of 40cm/yr was required to reduce the oil concentration in the leachate to below 0.4mg/l.

There is a decrease in water solubility of liquid hydrocarbons as molecular weight increases within each hydrocarbon class. Lighter and more soluble hydrocarbon are leached more rapidly (e.g. the light aromatic compounds which are toxic to organisms). Microbial activities like oxygenation and surfactant production also promotes solubilization and leaching (Bossert and Bartha, 1984).

The water soluble components of crude oil spilled on land are very persistant and represents long term environmental threats to groundwater.

1.2.6.1.3 CHEMICAL AND PHOTOCHEMICAL DEGRADATION

Karrick (1977) stated that chemical degradation of petroleum involves primarily, photocatalytic oxidation and polymerizations. He succinctly reviewed these process and reported that 1-napthol acted as a photosensitizer for crude oil. The mechanism involves the formation of free radicals and polymerization process. Circumstantial evidence for such a mechanism comes from a number of studies on the weathering of oil. In most studies mono-and di-aromatic compounds disappear during weathering with an apparent increase in high molecular weight poly-aromatic hydrocarbon. Riley (1970) noted that small naturally occurring particles formed larger organic aggregates which settled out. It is unknown whether petroleum will enhance or inhibit this reaction but similar aggregation of hydrocarbons have been noted.

1.2.6.2 BIOLOGICAL PROCESSES:

MICROBIAL METABOLISM OF HYDROCARBON

Biodegradation of petroleum hydrocarbons by microorganisms play a major role in the natural decontamination process. The activity of microorganism at a spill sight is governed by the organisms ability to produce enzymes to catalyze metabolic reactions. This ability is in turn governed by their genetic composition. The availability of carbon sources induces enzyme production by microorganisms which attack the hydrocarbon molecules. Other enzymes are utilized to breakdown hydrocarbons further. (Applied Biotreatment Association, 1990).

An inhibition to complete hydrocarbon degradation or prevention of attack is lack of appropriate enzyme.

Atlas (1988), stated that the complex series of steps by which biodegradation occurs constitutes a metabolic pathway. Many different enzymes and metabolic pathways, not all of which can be found in any single species, are required to degrade a significant portion of the hydrocarbons contained in petroleum (He thus advocates of using specially selected mixtures of microorganisms to bioremidiate oil spills or of

creating, through recombinant DNA technology, genetically engineered organism that are motivated in part by the desire to combine all the requisite enzymes and pathways).

Knowledge of the numerous metabolic pathways involved in the breakdown of hydrocarbons is far from complete. Studies carried out by various researchers, (Watkinson, 1978; Gibson, 1984; Atlas, 1984; Connan, 1986) have revealed that hydrocarbon degradation is an oxidative process. The saturated and aromatic hydrocarbons are transformed into oxygenated products by several metabolic processes. Such products include fatty acids, alcohol, ketones, phenols etc. These products are further broken down by alpha and beta oxidation, ring cleavage, etc. (Connan, 1986).

The metabolic steps involved in biodegradation entails formation of increasing smaller molecules resulting ultimately to carbon-dioxide, water and biomass (Ladousse and Tramer, 1991; Jones and Greenfield, 1991).

Bartha and Atlas (1987) reported that the most common type of primary metabolic attack by microorganisms on n-alkanes is mediated by mixed function oxidases (Mono-oxygenases) which act on the terminal carbon converting the hydrocarbon molecule to a primary alcohol. This oxidation is mediated by both cytochrome P-450 and rubredoxin systems that act in an aerobic condition. Some microorganisms have been noted by Markovetz (1971) to attack hydrocarbons subterminally converting them to secondary alcohol.

The primary alcohol whether derived from terminal or subterminal oxidation are further oxidized to aldchydes and fatty acids. Such fatty acids are subsequently shortened to C-2 units by beta oxidation. If beta oxidation is hindered by branching, the fatty acid can be attacked at the other terminal carbon by omega oxidation. This terminus is progressively oxidized to an alcohol aldehyde and carboxyl group (Higgins and Gilbert, 1978; Connan, 1986).

Cycloalkanes are reportedly metabolized in nature primarily through cometabolizm followed by commensal utilization of the products by other microbial

strains (Perry, 1977). Co-oxidation degradation of cycloalkanes involves hydroxylation to a corresponding cyclic alcohol. This product is inturn degraded to the corresponding cyclic ketone. The next step is the conversion of the cyclic ketone to a lactone by the monoxygenase systems. (Blakebrough, 1978, Higgins and Gilbert, 1978).

Ring opening is catalysed by a lactatone hydrolase or may occur spontaneously resulting in an omega hydroxylated carboxylic acid. The hydroxyl group is successively dehydrogenated resulting in a dicarboxylic acid which is further metabolized by beta oxidation. Degradation of hydrocarbons in a microbial community occurs simultaneously but at widely differing rates. A condition explained by the presence of large spectrum of potential hydrocarbon substrates and differing susceptibility of the hydrocarbon fractions to degradation. This leads to the sequential disappearance of individual components of petroleum over time and to successional changes in the degrading microbial community (Atlas, 1988). Presence of one hydrocarbon substrate may influence the biodegradation of another in either a positive or negative way by several possible mechanisms.

'Sparing' is the term used to describe a situation in which the presence of a readily degradable substrate will influence a microorganism to ignore other available substrates. Conversely, certain compounds, may only be metabolized in the presence of others - a process known as "cometabolism". It has been well characterized in the degradation of cyclo-alkane (Perry, 1984) and also proposed for the partial oxidation of polynuclear aromatics in soil (Morgan and Watkinson, 1989).

1.2.7 ENVIRONMENTAL INFLUENCES ON BIODEGRADATION

Biodegradation rates and extent are greatly influenced by a number of environmental variables. This variables include oxygen, temperature, water, soil pH and mineral nutrients. Microbial removal of hydrocarbon contaminant occurs in nature but is a slow process, since most contaminated soils lack the balance of elements necessary for bacterial growth.

1.2.7.1 TEMPERATURE

Petroleum biodegradation is temperature dependent occurring over a wide range of temperature. It has been reported by various workers (Klug and Markovetz, 1967) to occur in the thermophillic range - up to 70°C and also by Zobell and Agosti (1972) to occur at 0°C. As much as this range exist, hydrocarbon degradation is limited by temperature. The importance of this limiting effect has been shown in different ecosystems in both Artic by Atlas and Bartha (1972) and tropical zones (Oudot and Dutriex, 1989; Scherer and Mile, 1989). The rates of biodegradation are fastest at the range of 30-40°C and usually decreases - sometimes dramatically in very cold climates with decreasing temperatures. At low temperature; the rate of hydrocarbon metabolism by microorganisms decreases (Leahy and Colwell, 1990). Apart from this slowing of degradation kinetics, there is inhibition of the microorganisms due to the presence of certain volatile compounds in the petroleum. The microorganisms inhibition causes latency periods which delay biodegradation. (Atlas and Bartha 1972).

Petroleum also becomes more viscous at low temperature, hence less spreading occurs and less surface area is available for colonization by microorganisms.

1.2.7.2 MINERAL NUTRIENTS

The driving force behind nearly all of the nutrient cycling reactions involving organic compounds in soils and sediments is the never-ending search by micro-organisms for the energy tied up in the C-H bond. Microorganisms convert the carbon in organic materials to CO_2 and thereby complete the biological carbon cycle that was initiated during photosynthesis. The carbon transfers and transformation at the soil or even the cell level also delineate the other nutrients such as sulphur, nitrogen and phosphorous, that become available to plants and microorganisms (Paul and Clark, 1989).

Petroleum as a microbial substrate supplies carbon and energy but is extremely deficient in nitrogen and phosphorus. The nitrogen and sulphur of the NSO fraction are mostly heterocyclic rings and generally insufficient and unavailable (Atlas, 1984; Bartha and Atlas, 1987; Morgan and Watkinson, 1989). Consequently, petroleum degrading microorganisms must obtain their essential mineral requirements from the soil. Most ecosystems are often deficient in these substances because non-oil degrading microorganisms consume them in competition with the oil degrading species. Also the input of a large quantity of petroleum tends to result in a rapid depletion of the available pools of the major inorganic nutrients.

Studies variously carried out by Atlas and Bartha (1972); Frankenberger (1988); Lee and Levy (1989); have shown that the availability of the nutrients, nitrogen and phosphorus are the main limiting factors to hydrocarbon degradation. Scientist have attempted to adjust nutrient levels (e.g. by adding nitrogen - and phosphorus - rich fertilizers) to stimulate biodegradation of petroleum hydrocarbons. This is the approach employed by some boremediation techniques.

1.2.7.3 SOIL pH

Biodegradation of hydrocarbon can occur over a wide range of soil pH values. As with the other factors, decomposition at the extreme ranges is slower than at the mean. The optimum range for most soil microorganisms have been reported by Morgan and Watkinson (1989) to be between PH values 6.0 and 8.0. Many organisms are not greatly affected by a unit change from their specific optimum.

1.2.7.4 OTHER ORGANIC COMPOUNDS

Microorganisms when faced with an array of substrate will preferentially utilize the more readily assimilable carbon substrate. Dibble and Bartha (1979) confirmed that hydrocarbon utilization rates was greatly reduced in the presence of easily assimilable source of carbon for microorganisms.

1.2.7.5 WATER

Soil water affects not only the moisture available to organisms but also the soil aeration status, the nature and amount of soluble materials, the osmotic pressure and the PH of the soil solution.

Microbial activity are generally optimal in soils with a water content between 50% and 80% water holding capacity. (Atlas, 1984; Lynch, 1983). The moisture status of the soil is best expressed in terms of a percentage of its moisture-holding capacity (Buckman and Brady, 1969). Moisture is essential to active life processes but too much moisture in soils interferes with the availability of oxygen (Bossert and Bartha, 1984). At 100% saturation, all available pores are filled with water creating anaerobic condition while at 10% water holding capacity, osmotic and matric forces reduce the availability of water to a degree that metabolic activities remain marginal (Bossert and Bartha, 1984).

1.2.7.6 OXYGEN

Oxygen is one of the most important requirements for microbial degradation of hydrocarbons. Oxygen - incorporating enzymes are employed by microorganisms to initiate attack on hydrocarbons. Blumer and Sass (1972) reported that anaerobic degradation does occur but at a very slow rate.

The use of hydrocarbon as the only source of carbon and energy has not been demonstrated in strictly anaerobic condition (Bartha and Atlas, 1987). However, intermediates of aerobic hydrocarbon biodegradation can be metabolized further under anaerobic condition. (Shelton and Hunter, 1975; Jobson et al, 1979).

Oxygen is generally necessary for the initial breakdown of hydrocarbons and subsequent reactions may also require direct incorporation of oxygen. Requirements can be substantial, 3 to 4 parts of dissolved oxygen are necessary to completely oxidize 1 part of hydrocarbon into carbon dioxide and water. (Lee and Levy, 1989).

Oxygen is usually not a factor limiting the rate of degradation on or near the soil surface where there is adequate aeration and oil spread out to provide a large

exposed surface area. When oxygen is less available, however, the rates of biodegradation decrease. Oxygen availability in these case is determined by the oil concentration, its degradation rate, depth in the sediment and aerobic condition of the soil with respect to aeration. Soil aeration depends on the total amount of air filled pore spaces, soil water, soil texture, pore sizes and layer.

High oxygen reserves are ensured by particle size of the soil grain with large pore sizes and rapid replacement of oxygen by diffusion (Atlas, 1984).

1.2.8 BIODEGRADATION POTENTIALS

Biodegradation is the most effective and economical means of eliminating the capacity of most organic materials and compounds to pollute the environment. As a tool in population control, we have just begun to understand how to use biodegradation processes efficiently. Yet it has been available for time immortal. Microorganisms demonstrate great capacities to produce unusual products and perform unusual functions in commercial industry for mankind, yet in the field of pollution abatement, biodegradation is a waste control management practice that has been developed little beyond its original crude state.

It is the purpose of this research, therefore to assess the role of microbial degradation in removing contaminating hydrocarbons by determination of viable counts of hydrocarbon utilizing microorganism (TVC) optional density (O.D) and residual crude oil in the medium.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 SOURCE OF SAMPLES

The polluted soil samples used for the study were collected from Mbiama. The samples were collected using containers which were sterilized with ethanol (70% w/v)

Crude oil (Bonny light crude oil-BLCO) was obtained from the Nigerian National Petroleum Corporation (NNPC) Refinery, Eleme, Rivers State.

2.2 All sterilization, unless otherwise stated were done by autoclaving at 121°C for 15 minutes at 15psi.

2.3 MEDIA

Two types of media were used for these study: nutrient agar (oxoid) and nutrient broth (oxoid) and the modified mineral salts medium of Mills *et al* (1978). The nutrient broth and nutrient agar were prepared in accordance with the manufacturer's procedure.

Nutrient agar = 28g per litre

Nutrient broth = 8g per litre

Both were sterilized. The nutrient broth was used for the preparation of active innoculum for use in the various tests; while the nutrient agar was used for isolation and characterisation of crude oil utilizing bacteria, stock preservation and enumeration of hydrocarbon utilizing bacteria.

The mineral salt medium had the following composition 10.0g sodium, chloride (NaCl), 0.42 magnesium sulphate ($MgSO_4$, $7H_2O$), 0.29g potassium chloride (KCl), 1.25g disodium hydrogen phosphate (Na_2HPO_4), 0.42 g Ammonium nitrite ($NH_4 NO_3$), 0.83g. Potassium dihydrogen phosphate (KH_2PO_4) and 1000ml deionized water. The agar medium was prepared by adding 15.0g of agar (Oxoid) to the mineral

salt medium. The medium was sterilized and used for isolation of Hydrocarbon utilizing microorganisms and Biodegradation tests.

2.4 DILUENT USED

Physiological saline, sodium chloride, (0.85% w/v) was used for isolation and total viable count. The diluent was dispensed in 9ml amounts into clean test-tubes which were plugged with non-absorbent cotton wool, wrapped with aluminium foil and sterilized.

2.5 ISOLATION OF CRUDE OIL UTILIZING BACTERIA

One gram of the polluted soil was weighed into 9ml of sterile diluent and shaken manually. This gave 10^{-1} dilution. From this diluent, subsequent ten-fold serial dilutions were carried out up to 10^{-5} dilutions.

Using pour plate technique, 1ml of each dilution was aseptically withdrawn and added into sterile petridishes. Into each of these plates was added 15ml of modified molten mineral salt agar medium. The plates were rotated to ensure uniform distribution of the organisms. The medium were allowed to set after which sterile filter papers (Whatman No.1), saturated with crude oil (BLCO) were aseptically placed on the inside of the lid and the cultures incubated at 37°C for five days. After the incubation period, colonies were picked based on cultural and morphological characteristics. The isolates were further purified as outlined below.

2.6 PURIFICATION OF THE ISOLATES

The colonies that were picked based on cultural and morphological characteristics were further purified by inoculating into sterile nutrient broth contained in test-tube incubated for 18-24 hours period at 37°C. The innoculum was streaked on to a sterile nutrient agar plates using an inoculating loop. The culture was incubated for 18-24hours. Distinct colonies were picked from the agar plates and tested for gram reaction as outlined below.

2.7 STOCK CULTURE

Based on their reaction to gram staining, pure isolates were carefully picked and inoculated onto sterile nutrient agar slants in Bijou bottle; incubated at 37°C for 18-24hours; then stocked for further use after observing growth.

2.8 GRAM STAINING

The method used was that described by Gerhadt *et al* (1981). A drop of water was placed on a clean grease free slide using a sterile wire loop and emulsified. The smear was air dried and fixed by passing the under side of the slide thrice over blue flame of a burnsen burner.

Each smear was stained with crystal violet for 60 seconds, excess stain was dripped off and then flooded with Lugol's iodine solution. The stain was allowed to act for 60 seconds and then rinsed off with 85% (v/v) ethanol until the violet colour disappeared. The ethanol was immediately washed off with slow running tap water before finally staining with safranin for 30 seconds.

Finally the slides were washed with slow running tap water, blotted dry and viewed under oil immersion objective of the microscope.

Gram positive organisms retained the purple colour of the crystal violet while gram negative organisms absorbed the pink colour of the safranin.

2.9 BIOCHEMICAL CHARACTERISATION AND IDENTIFICATION OF THE ISOLATES

2.9.1a The innoculum for the various test was prepared by innoculating from the stock culture of the appropriate isolate(s) into sterile nutrient broth contained in test-tubes. The cultures were incubated for 18-24hours at 37°C and were used to carry out the following tests:

2.9.1b For each test, controls were set up which consisted of uninoculated medium.

2.9.2 CATALASE TEST

Certain microorganisms growing aerobically posses the ability to produce the enzyme catalase which oxidizes hydrogen peroxide to water and oxygen. The method outlined by Cruishank (1981) was used.

Into a series of labelled sterile tubes were added 3mls of hydrogen peroxide (3% v/v). A loopful of each of the isolate picked from culture plates (prepared by plating out 0.1ml of the inoculum in section 2.9.1. onto sterile nutrient agar plates and incubating for 18-24 hours at 37^{o}C) was inoculated into these tubes. Effervescence, caused by the liberation of oxygen as gas bubbles was used as an indication of the presence of catalase in the culture under test.

2.9.3 OXIDASE TEST

This test is used to demonstrate the presence of oxidase in the bacteria that catalyzes the transfer of electron from the bacteria donor to a redox dye; tetra-methyl-p-phenyl diamine hydrocholoride.

One percent aqueous solution of tetra-methyl-p-phenylenediamine hydrochloride was prepared by dissolving 1g of the reagent in 10mls of distilled water. Strips of Whatmans No.1 Filter paper were soaked in these freshly prepared solution. A sterile loop was used to pick isolates from culture plates that were prepared as in section 2.9.2 and inoculated onto the filter paper by streaking. Development of purple colour within 5-10 seconds indicated oxidase positive cultures while no coloration indicated negative reactions.

2.9.4 INDOLE PRODUCTION TEST

This test demonstrates the ability of certain bacteria to decompose amino acid, tryptophan with the release of indole which accumulates in the medium. Peptone water which has the following composition 10.0g peptone, 5g sodium chloride (Nacl) and one litre of deionized water, was the medium used.

The pH of the medium was adjusted from 7.2 to 7.4 using O.IN-sodium-Hydroxide. The medium was dispensed in 5ml amounts into test tubes and sterilized

by autoclaving. The peptone water broth were each inoculated with the appropriate isolate from the stock culture and incubated at 37°C for 48 hours. After incubation, 0.5ml of kovac's reagent was added to each of the test-tubed and shaken gently. A deep red colour at the surface of the tube was indicative of indole production while the yellow colour at the surface of the test-tube indicated a negative result.

2.9.5 CITRATE UTILIZATION

The medium used for this test is the Simmon citrate agar (SCA). It is a differential medium used to test for the ability of organisms especially members of the Enterobacteriaceae to utilize citrate as the sole carbon and energy source for metabolism, the medium was prepared and sterilized according to the manufacturer's instruction.

After preparation, 10ml amounts were dispensed into each of the test-tubes and sterilized by autoclaving. The medium was inoculated by streaking and incubated at 37°C. The cultures were observed for change of colour from light green to blue which indicated positive result.

2.9.6 STARCH HYDROLYSIS

The medium used was nutrient agar to which was added 4% (w/v) soluble starch. The medium was sterilized by autoclaving, allowed to cool and dispensed in 15ml amounts into sterile petridishes. The plates were inoculated with each isolate by streaking and incubated at 37°C for 24 hours. The plates were then flooded with Gram's iodine solution, plates which showed halo (zone of hydrolysis) were recorded as positive while plates which retained the blue-black colour were recorded as negative.

2.9.7 SUGAR FERMENTATION

This test was carried out to ascertain the ability of the isolates to metabolize some sugars (glucose, sucrose, lactose and mannitol) with the resultant production of acid and gas or either.

Into one-litre of 1% (w/v) peptone water was added 3ml of 0.2% (w/v) bromocresol purple and dispensed in 9ml amounts, into test-tube which contained inverted Durham tubes. The medium was then sterilized by autoclaving.

The sugar solutions (glucose, sucrose, mannitol and lactose) were each prepared at 10% (w/v) and sterilized at 105°C for 5 minutes. One millilitre amount of each sugar was dispensed aseptically into the test-tubes. The medium was then inoculated with the appropriate isolate and the cultures incubated at 37°C for 48 hours and examined for acid and gas production.

A change in a colour from purple to yellow indicated acid formation while gas production was assessed by the presence of gas bubbles in the inverted Durham tubes.

2.9.8 METHYL RED-VOGES PROSKAUER (MRVP) TEST

The method used was adapted from Cruishank et al (1980).

The medium is commercially prepared (DIFCO) and its composition is an follows: Peptone (oxoid L49) 5.0g, phosphate buffer 5.0g and one litre of deionized water.

Into one litre of deionized water was added 15g of the medium. The broth was dispensed in 10ml amounts into test-tubes and sterilized. On cooling to room temperature, the medium was inoculated in duplicate with each isolate and incubated at 37°C for 48 hours. One of the tubes was labelled M.R while the other was V.P.

M.R test was carried out by adding 5 drops of methyl red indicator to M.R broth test-tubes and shaken gently. Cherry pink (red) colour formation indicated M.R positive while a yellow coloration within seconds of adding methyl red was M.R negative.

For the V.P, six drops of Baritt reagent (α - naphthol) was added to the broth culture so labelled; followed by addition of 0.5ml of 40% potassium hydroxide. This was shaken gently and left to stand for 10 minutes. A pink red colour indicated V.P positive organism (Gerhadt *et al*, 1981). V.P negative organism did not show any colour change.

2.9.9. SPORE STAINING

Members of the genera *Bacillus* and *Clostridium* produce endospores that form inside the vegetative cells when conditions are unfavourable for continued growth of the cell.

The test was performed as described by Carpenter (1977). A smear of each isolate was made on a clean, dry, grease-free slide and allowed to air dry. The slide was then placed on the rim of a beaker of water heated over a burnsen flame so that the water vapour would heat the underside of the slide. The smear was then flooded with 5% (v/v) aqueous malachite green solution. Further heating was carried out for one minute, then the slide was washed in gentle running tap water and then counterstained with safranin for 30 seconds . This was washed, blotted dry and viewed under oil immersion objective of the microscope. Spores stained green while the vegetative cells stained red.

2.10 GROWTH OF PURE BACTERIAL ISOLATES ON CRUDE OIL AS SOLE CARBON SOURCE

The isolates used were *Alcaligenes sp, Pseudomonas sp, Corynebacterium. sp; Bacillus sp and Arthrobacter. sp.* 100 ml of the mineral salts medium was dispensed into each of seven 250ml Erlenmeyer flasks. One millilitre of the crude oil (BLCO) was then added to each flask and the medium sterilized by autoclaving. After cooling the flasks were inoculated with 1ml of the appropriate isolate(s) as follows:

Flask A was inoculated with *Alcaligenes sp*

Flask B was inoculated with *Pseudomonas sp*

Flask C was inoculated with *Corynebacterium sp*

Flask D was inoculated with *Arthrobacter sp*

Flask E was inoculated with *Bacillus sp*

Flask F was inoculated with *Alcaligenes sp, Pseudomonas sp, Corynebacterium sp, Arthrobacter sp and Bacillus sp.*

Flask G was not inoculated

Flask F was inoculated with all five isolates using 0.2ml of each isolate as the inoculum. The flasks were shaken manually and 7ml aseptically withdrawn for the analysis (section 2.10.1).

The cultures were then incubated at room temperature for 14 days. At two-day intervals, 7.0ml samples were aseptically withdrawn for the analysis described in section 2.10.1 below.

2.10.1a MEASUREMENT OF pH CHANGES USING A pH METER

The pH of the samples were measured using a pH meter (Mettler Delta 340 pH meter). The meter was standardized using freshly prepared pH buffers at pH 4 and 7 3mls of the previously filtered sample (filtration was done to remove the oil) was transferred into a test-tube and the electrode arm of the meter inserted. The pH of the sample was read off the display panel.

2.10.1b OPTICAL DENSITY MEASUREMENT

The optical density of each of the samples was measured using a spectrophotometer (Jenway 6100 spectrophotometer). The spectrophotometer was standardized with distilled water. After standardization, 3ml of the appropriate sample was added into the cuvette and the absorbance read.

2.10.1c TOTAL VIABLE COUNT

Growth of the isolates on mineral salt medium was monitored every two days by using the plate count technique.

One millilitre of the sample was aseptically withdrawn and added to 9mls of sterile physiological saline. The test-tube was thoroughly shaken this gave 10^{-1} dilution. Subsequent ten-fold serial dilutions were carried out up to 10^{-6} dilutions. The various dilutions were plated out onto nutrient agar plates and the plates were then incubated at 37°C. The number of colonies that developed were counted after 24 hours and 48hours.

2.11 DETFRMINATION OF RESIDUAL CRUDE OIL IN THE MEDIUM

The method used was as outlined in section 2.10 above except that each isolate was inoculated into triplicate set of 250ml of flask. The cultures were not sampled during incubation and were analyzed for residual crude oil after 14 days of incubation.

2.11.1 STANDARDIZATION OF THE INSTRUMENT

The spectrophotometer (Cornin 253 Spectrophotometer) was set at wavelength 425nm, switched on and allowed to stabilize for 5 minutes 2 ml of chloroform was then added to the cuvette and used to zero the instrument. The standardize control was adjusted until the meter was exactly at the centre zero mark.

2.11.2 CALIBERATION CURVE

The standard stock solution of crude oil (BLCO) was prepared by weighing 1.0g of the crude oil into 1,000ml of chloroform. This gave 1,000 part per millilitre (ppm). The flask was shaken manually to effect complete solubility of the crude oil. From this stock solution, various concentrations were obtained as shown:

Stock Solution	Chloroform added	Concentration of Standard Solution (ppm)
0.1ml	0.9ml	100
0.3ml	0.7ml	300
0.5ml	0.5ml	500
0.7ml	0.3ml	700
1.0ml	0.0ml	1,000

The absorbance of the above standard concentrations were read by filling each to within 1cm of the top of the cuvette. The blank cuvette was removed from the sample well and placed with that of the standard. The control knob under the scale window was adjusted until the meter needle returned to center zero position.

The absorbance was read in the scale and used to plot the standard curve.

2.11.3 EXTRACTION PROCEDURE

After incubation for fourteen days, the contents of each flask was transferred into a 1-litre separatory funnel.

15ml of chloroform was added to each of the flasks which were then shaken to dissolve the remaining crude oil. The resulting solution was then mixed with the contents in the separatory funnel. The contents were shaken for 5 minutes and allowed to stand for 10 minutes to allow for the separation of the mixture into two phases. The Chloroform phase was carefully withdrawn into a 50ml volumetric flask.

Another 10ml aliquot of solvent was added to the content of the separatory funnel and shaken for five minutes, allowed to separate for ten minutes. The chloroform layer was withdrawn into the 50ml volumetric flask which contained the first 15ml extract.

To bring the absorbance of the extract within the linear range of the curve, 0.1ml of the extract was diluted with 4ml of chloroform; the diluted extract was used to fill the cuvette to within 1cm of top, inserted in the well and absorbance was read.

The concentration of residual oil was determined by reading off this absorbance from the standard curve.

The concentration of crude in the extract was given by:

$$\frac{\text{Total volume of Extract x Dilution Factor x Concentration from graph in ppm}}{\text{Total volume of Test sample}}$$

CHAPTER THREE

3.0 RESULTS

3.1 GRAM REACTION

Twenty hydrocarbon utilizing bacteria were isolated. Nine out of the twenty isolates were gram-negative rods. While eleven were gram -positive rods (Table 1)

3.2 BIOCHEMICAL TESTS

The results obtained for the various biochemical tests are as shown in Table 1. The uninoculated controls gave negative results.

3.2.1 CATALASE TEST

All the twenty isolates gave positive results for the catalase test.

3.2.2 OXIDASE TEST

Nine of the isolates were oxidase positive while the remaining eleven were negative.

3.2.3 INDOLE PRODUCTION TEST

The degradation of Tryptophan to release indole could not be effected by any of the isolates.

3.2.4 METHYL RED AND VOGES-PROSKAUER TEST

Three of the isolates were methyl red positive, seventeen gave a negative result.

Nine of the isolates were positive for Voges-Proskaur test while the remaining eleven were negative.

3.2.5 CITRATE UTILISATION TEST

Seventeen of the isolates had the ability to utilize citrate as shown by change in colour of the medium from light green to deep blue. There was no colour change in the medium inoculated by the other isolates.

3.2.6 STARCH HYDROLYSIS

Nine of the isolates hydrolyzed starch, eleven isolates gave negative results.

3.2.7 SPORE STAINING

Six of the isolates were spore formers, the remaining fourteen did not indicate presence of spore.

3.2.8 SUGAR FERMENTATION TEST

Glucose: Eighteen of the isolates fermented glucose, thirteen with the production of acid and gas. Five with the production of acid only. Two of the isolates did not ferment glucose

Mannitol: Sixteen isolates utilized mannitol - eight with the production of acid only, two produced gas only, six with the production of acid and gas. Four of the isolates did not utilize mannitol.

Sucrose: Sucrose was fermented by Fourteen of the isolates (nine with acid production and five, acid and gas). Six of the isolates did not ferment sucrose.

Lactose: Lactose was not fermented by any of the isolates

TABLE 1: CHARACTERISATION AND IDENTIFICATION OF BACTERIAL ISOLATE FROM CRUDE OIL POLLUTED SOIL

										Sugar Fermentation				
Isolate No.	*Gram Reaction*	*Catalase*	*Oxidase*	*Indole*	*M.R*	*V.P*	*Citrate*	*Starch Hydrolysis*	*Spore Stoining*	*Glocose*	*Mannitol*	*Sucrose*	*Lactose*	*Probable Identity*
1.	-ve Rods	+	+	-	-	+	-	-	-	A	A	A	-	*Alcaligenes sp*
2.	-ve Rods	+	+	-	-	-	+	-	-	A/G	A/G	-	-	*Pseudomonas sp*
3.	+Rods	+	-	-	-	+	+	+	-	A	A	-	-	*Corynebacterium sp*
4.	+Rods	+	-	-	+	-	+	+	+	A/G	-	A	-	*Bacillus sp*
5.	+Rods	+	-	-	-	-	+	+	+	A/G	A/G	A	-	*Bacillus sp*
6.	+Rods	+	-	-	+	-	+	+	+	A	A	A	-	*Bacillus sp*
7.	-Rods	+	+	-	-	+	+	+	-	A/G	A	A	-	*Alcaligenes sp*
8.	+Rods	+	-	-	-	-	-	+	+	A/G	A	A	-	*Bacillus sp*
9.	-Rods	+	+	-	+	-	+	-	-	A/G	A/G	A/G	-	*Pseudomonas sp*
10	-Rods	+	+	-	-	-	+	-	-	A	A	A/G	-	*Pseudomonas sp*
11.	-Rods	+	+	-	-	+	-	-	-	A/G	A	-	-	*Pseudomonas sp*
12.	+Rods	+	-	-	-	+	+	-	-	-	-	A	-	*Arthrobacter sp*
13.	-Rods	+	+	-	-	-	+	-	-	-	A/G	A/G	-	*Pseudomonas sp*
14.	+Rods	+	-	-	-	+	+	-	-	A/G	-	A	-	*Arthrobacter sp*
15.	+Rods	+	-	-	-	+	+	-	-	A/G	-	-	-	*Arthrobacter sp*
16.	-Rods	+	+	-	-	+	+	-	-	A/C	A/G	A/G	-	*Pseudomonas sp*
17.	+Rods	+	-	-	-	-	+	+	+	A	A	A	-	*Bacillus sp*
18.	+Rods	+	-	-	-	+	+	+	-	A/G	G	A/G	-	*Corynebacterium sp*
19.	+Rods	+	-	-	-	-	+	+	+	A/G	G	-	-	*Bacillus sp*
20.	-Rods	+	+	-	-	-	+	-	-	A/G	A/G	-	-	*Pseudomonas sp*

A – ACID G – GAS + – POSITIVE - – NEGATIVE

Based on the above results (Table 1), and with reference to Bergey's manual of Determinative Bacteriology, the isolates were identified as the following:

Seven of the isolates were of the genus *Pseudomonas.* Two were *Alcaligenes.* Six belonged to the genus *Bacillus.* Two were *Corynebacterium* and three were of the genus *Arthobacter.*

3.3 GROWTH OF PURE BACTERIAL ISOLATES ON CRUDE OIL AS SOLE CARBON SOURCE

The indices used to monitor growth were pH, optical density (O.D) and total viable count (TVC). The results obtained for the changes in pH, optical density and TVC are as shown in fig. 1-6 and Appendices 1 and 2 for the various isolates.

Fig. 1 shows a steady increase in the number of viable cells of *Alcaligenes sp* from an initial count of $7.8x10^6$ to $1.4x10^{15}$ c.f.u. ml^{-1} by the sixth day. TVC decreased from the eight day to the end of the incubation period.

O.D profile increased from .009 to a final value of .560 while the pH decreased from an initial value of 6.35 to 5.70, increased again to 6.07 and remained relatively constant.

The growth profile of *Pseudomonas sp* (Fig 2) shows that TVC increased from an initial count of $3.7x10^6$ to $1.0x10^{15}$ c.f.u. ml^{-1} by the sixth day and decreased afterwards. O.D. increased from .003 to .510 at the end of the incubation period. A decrease was observed for the pH from 6.37 to 6.13 by the 2^{nd} day after which it remained relatively constant.

Fig. 3 shows the growth profile of *Corynebacterium sp.* Its TVC increased from an initial count of $2.9x10^7$ x $2.0x10^{15}$ c.f.u.ml^{-1} by the sixth day and decreased from the eight day. The O.D. increased from an absorbance of .036 to .380 with a concormitant decrease in pH from a value of 6.39 to 5.71. The pH increased again to 6.01 and remained relatively constant till the end of incubation.

The growth profiles of *Arthrobacter sp* (Fig. 4) revealed increase in TVC from $3.2x10^6$ to $2,0x10^{15}$ c.f.u.ml^{-1} by the sixth day with counts decreasing from the eight day. O.D. increased steadily from 0.04 to .557 at the end of incubation. pH decreased from 6.46 to 6.03 and remained relatively constant.

Fig. 5 shows the profile obtained for *Bacillus sp.* TVC increased from an initial count of 3.0 x 10^6 to a maximum of $7.6x10^{14}$ c.f.u.ml^{-1} by the sixth day counts decreased

afterwards. O.D increased gradually from .004 to a value of .343 at the end of incubation. The pH decreased from 6.32 to 5.77, at the end of incubation.

Fig. 6 shows the growth profile obtained for the consortium of the isolates. Counts increased from $2.1x10^7$ to $1.14x10^{15}$ by the 4^{th} day and remained static up to the sixth day after which it began to decrease.

3.4 DETERMINATION OF RESIDUAL CRUDE OIL

The residual concentration of the crude oil measured as the disappearance of chloroform-soluble components using gravimetric determinations is given in Table 2.

The percent degradation which is represented as percent loss in concentration of oil was obtained after substraction of the loss due to evaporation (i.e. the uninoculated control).

Bacillus sp caused the highest degradation of BLCO (34.2%) with the consortium causing 20% degradation.

The data for the other isolates were as follows:

Pseudomonas sp (29.8%), *Alcaligenes sp* (28.2%), *Corynebacterium sp* (24.6%) and *Arthrobacter sp* (23.5%).

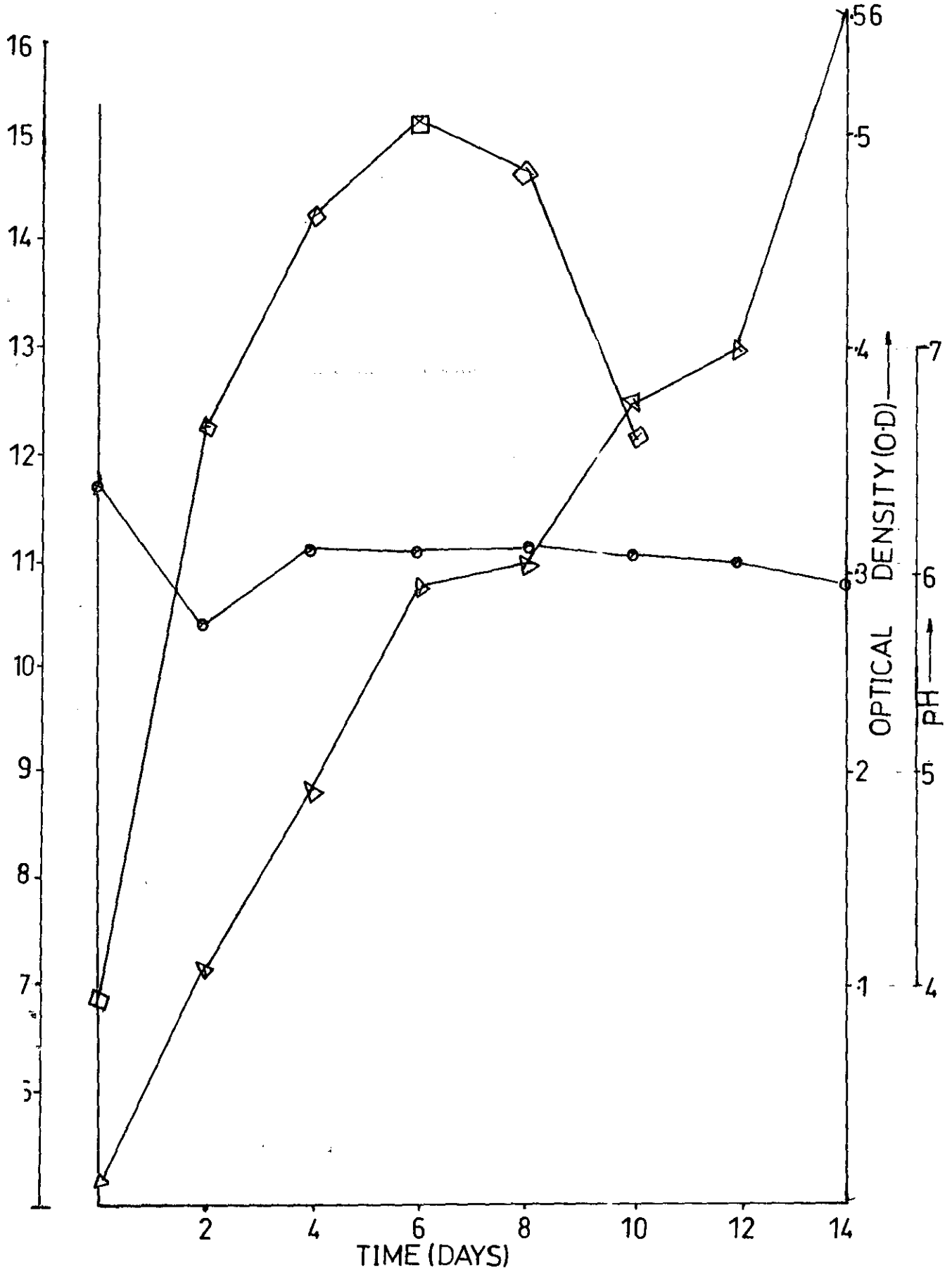

Fig 1; Changes in pH (o), optical density (Δ) and Total viable count (□) of Alcaligenes Sp when grown on mineral salts medium containing crude oil as sole carbon source.

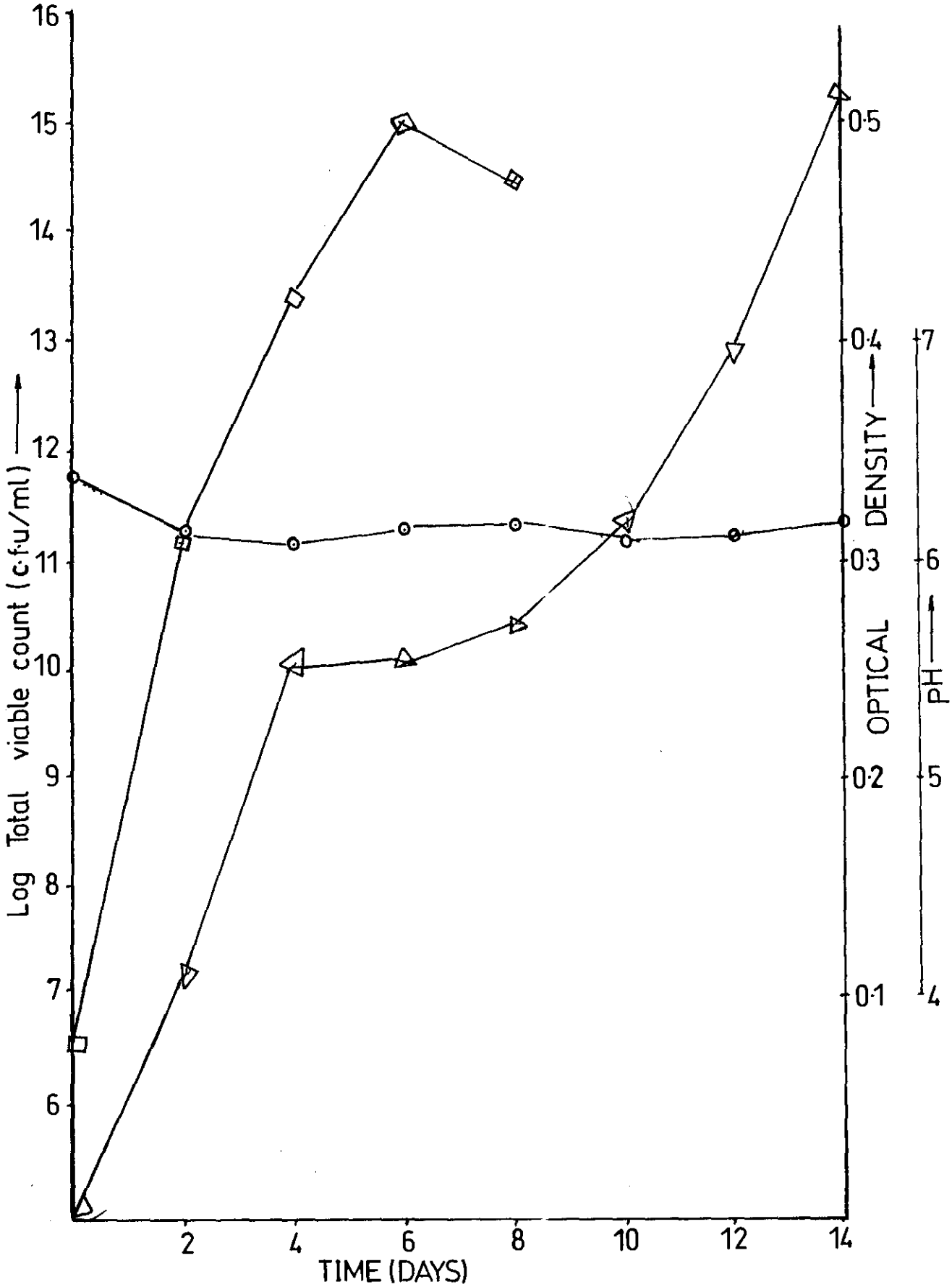

Fig 2: Changes in pH (o), optical density (Δ) and Total viable count (□) of Pseudornonas Sp when grown on mineral salt medium containing crude oil as sole carbon source.

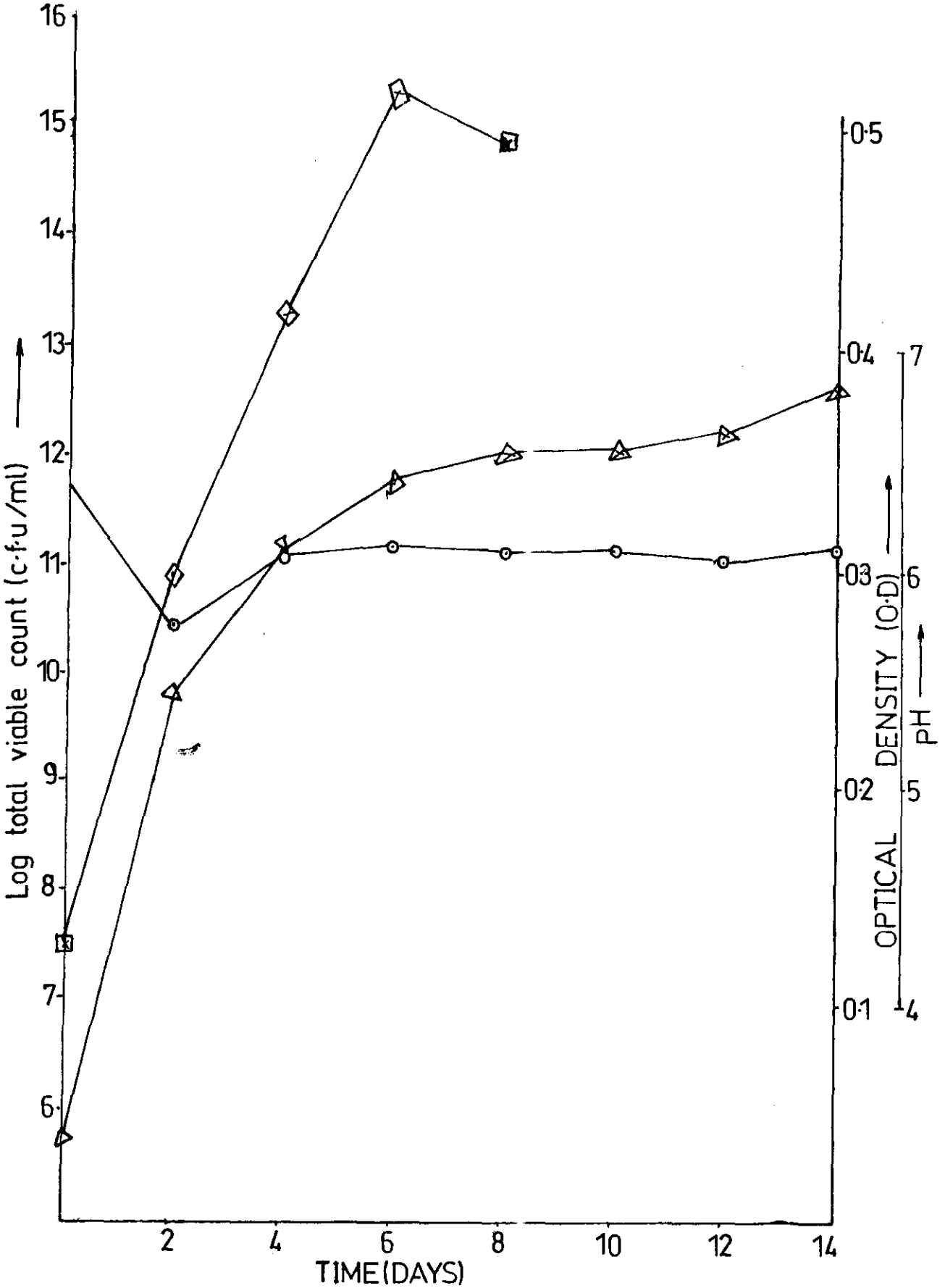

Fig 3: Changes in pH (o), optical density (Δ) and total viable count (□) of Corynebacterium Sp when grown on mineral salt medium containing crude oil as sole carbon source.

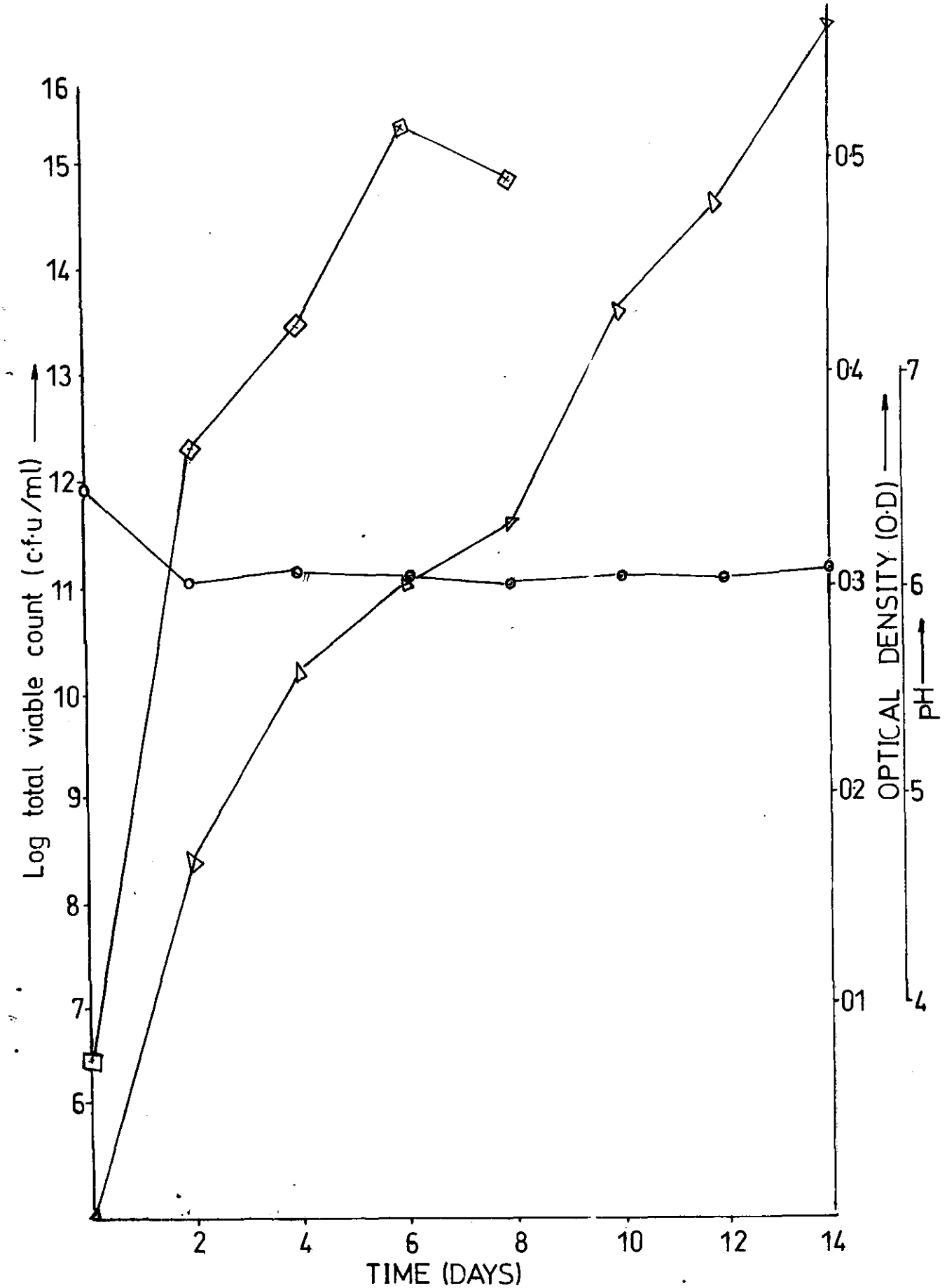

Fig 4: Changes in pH (o), optical density (Δ) and Total viable count (□) of Arthrobacter Sp when grown on mineral salt medium containing crude oil as sole carbon source.

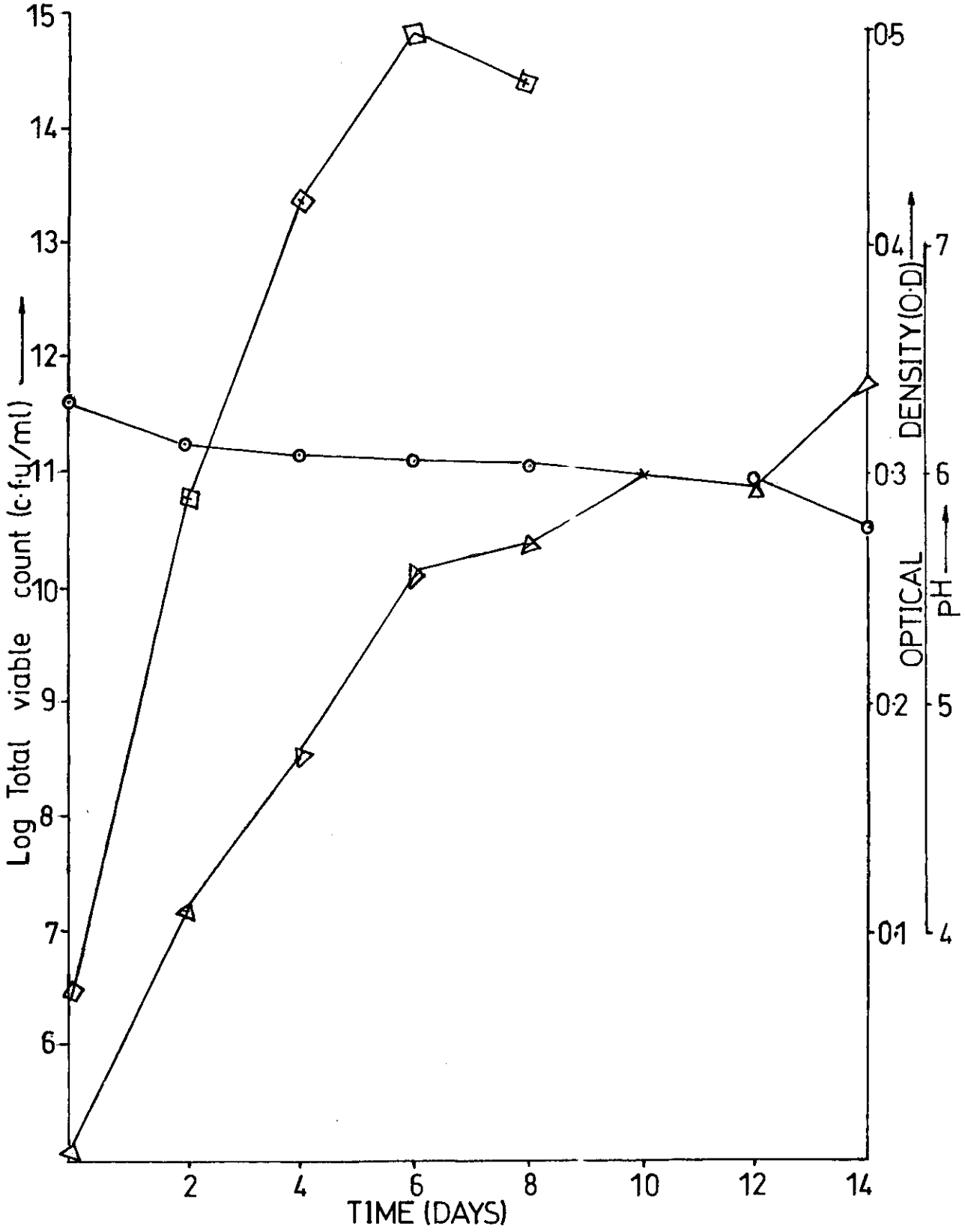

Fig 5: Changes in pH(o), optical density (Δ) and Total viable count(□) of Bacillus Sp when grown on mineral salt medium containing crude oil as sole carbon source.

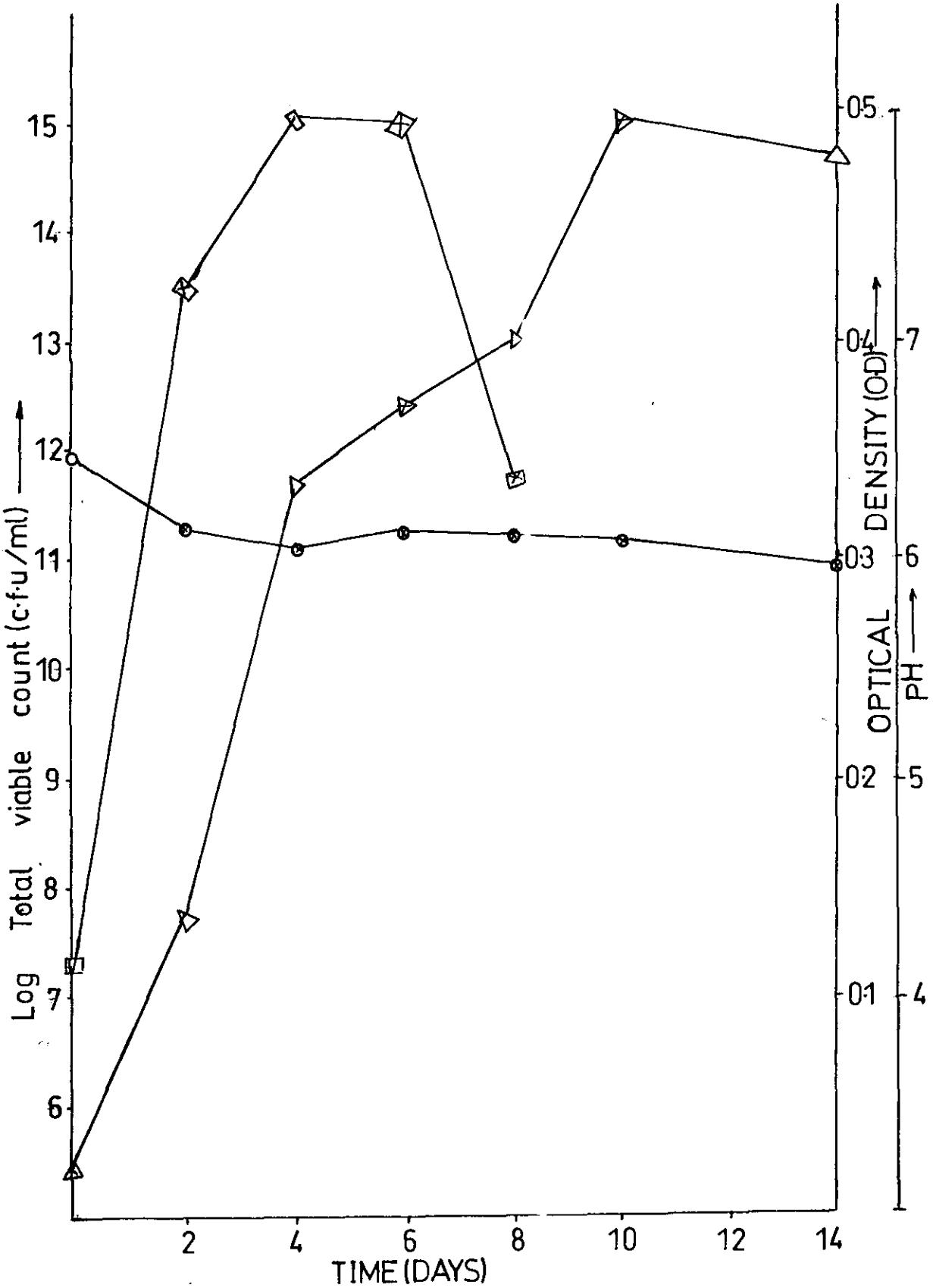

Fig 6: Changes in pH (o), optical density (Δ) and Total viable count (□) consortium of the isolates when grown on mineral salt medium containing crude oil as sole carbon source.

STANDARD CURVE FOR RESIDUAL OIL

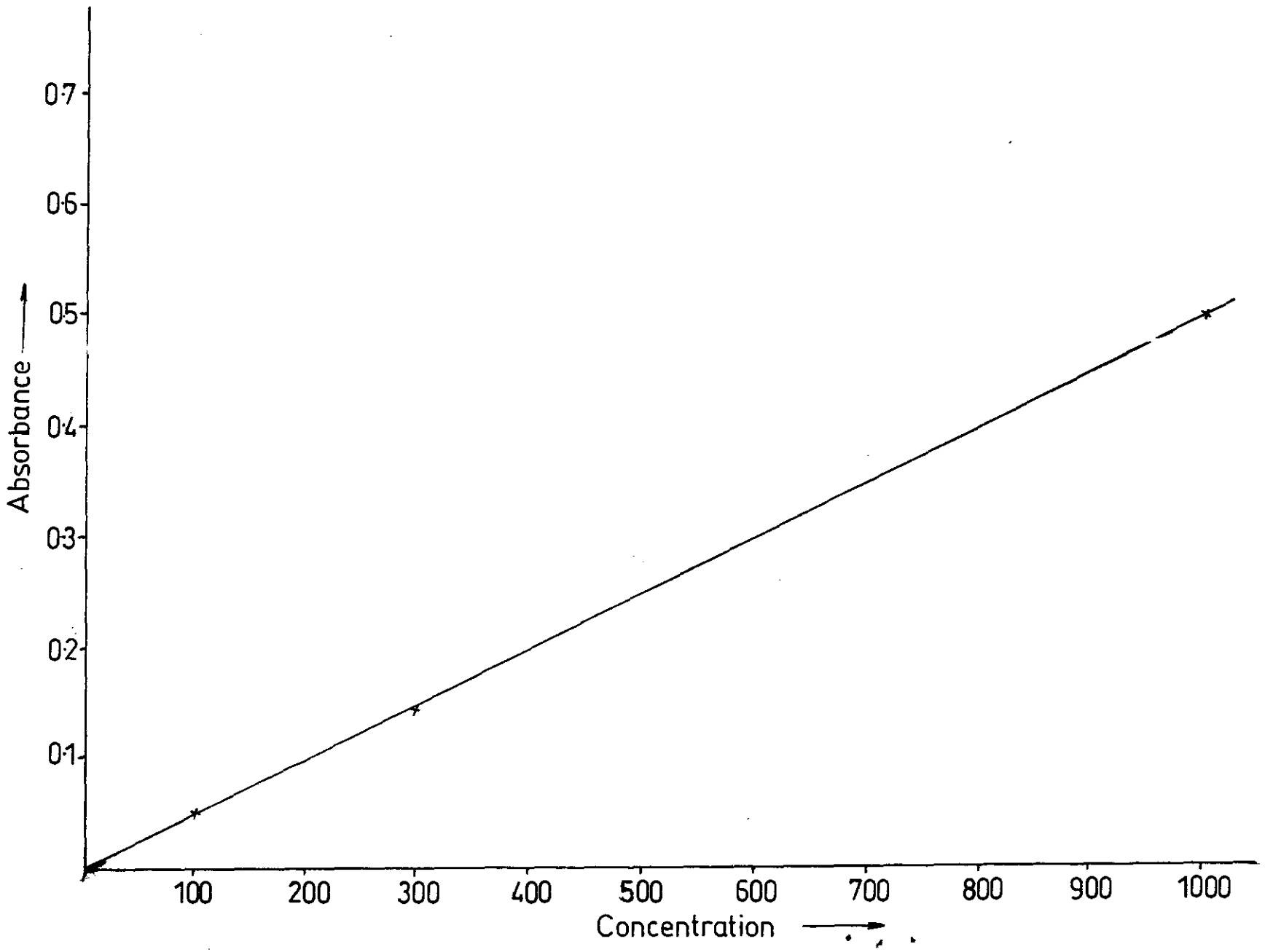

TABLE 2:

ISOLATES	DAY O			DAY 14				VALUES FOR STANDARD CURVE (CONCENTRATION IN PPM)	
	Absorbance (425nm)	Concentration of crude oil from graph	Actual Crude Concentration	Absorbance at 425nm	Concentration from Graph	Actual Residual oil Concentration	% loss in concentration of crude oil	Concentration	absorbance
Control	0.569	1160	11600	0.523	1074	10740	7.4	100	0.05
Alcaligenes sp	0.553	1126	11260	0.353	725	7250	35.6	300	0.143
Pseudomonas sp	0.553	1126	11260	0.346	707	7070	37.2	500	0.277
Corynebacterium sp	0.538	1096	10960	0.361	745	7450	32.0	700	0.370
Arthrobacter sp	0.569	1160	11600	0.390	801	8010	30.9	1000	0.488
Bacillus sp	0.569	1160	11600	0.331	677	6770	41.6	% loss in concentration of oil is given by	
Consortium of the isolates	0.553	1126	11260	0.398	818	8180	27.4	$\frac{\text{conc. of Crude oil (day 0) - Conc. Of crude oil (Day 14) x 100}}{\text{Concentration of Crude oil (Day 0)}}$	

CHAPTER FOUR

4.0 DISCUSSION AND CONCLUSION

The isolates that were used for this study were tentatively identified as *Bacillus sp., Pseudonomas sp, Corynebacterium sp, Arthrobacter sp* and *Alcaligenes sp* (Table 1) . Members of these genera have been reported by various workers (Floodgate, 1984; Austin et al, 1977; Odu, 1978; Bartha and Atlas, 1977; West et al 1984;. Okpokwasili *et al*, 1986) as petroleum hydrocarbon degrader. The predominance of *Pseudomonas sp* (Seven) and *Bacillus sp* (six) maybe explained on the basis that *Pseudomonas sp* is one of the most common, occurring aerobic soil bacteria and *Bacillus sp* have been shown by Udeme and Antai, (1988) to have relatively high capability in degrading crude oil.

The growth profile of the axenic and mixed bacterial cultures obtained by measuring the total viable count (TVC), Optical Density (O.D)and pH of the cultures as crude oil was being utilized presented in figures 1-6 indicates an increase in counts for all the bacteria with the mixed bacteria culture giving the highest total viable count of $1.4x10^{15}$ c.f.u. ml^{-1} by the 4th day. Optimal growth of 10^{15} ml^{-1} was observed for all the isolates except *Bacillus sp* with 10^{14} ml^{-1} by the 6th day followed by a decline from the 8th day. Just as the consortium of the isolates (mixed bacterial culture) grew the fastest, its rate of decline was the highest. Increase in microbial numbers is one of the indicators of crude oil utilization.

Generally O.D of each of the cultures increased with increasing viable counts. This increase in biomass correlated strongly with TVC for all the isolates. As observed from the growth profile of the isolates, decrease in counts did not lead to any decrease in O.D. Rather O.D continued to increase until the end of the incubation. The only exception was the consortium of the isolates, its O.D. continued to increase up to the 10th day and became relatively constant till the end of incubation. Jawetz *et al* (1989) observed that frequently, after the majority of cells have died, the death rate decreases drastically, so that a small number of survivors persist for some time. This persistance may in some cases reflect cell turnover (i.e. increase in O.D.). also O.D.

can be increased by cellular components released into the medium by some of the cells that die and Lyse.

The pH of the samples were relatively constant, fluctuating between pH 5.7 and 6.9 (Appendix 1). A slight decrease was observed for all the isolates. This may be as a result of metabolic activities of the organisms. That means that utilization of crude oil has resulted in production and accumulation of acidic metabolic products which accounted for the decrease in pH (Okpokwasili and Nnorom 1990). The observed pH values throughout the study is of special consideration since microbial populations are highly dependent on this parameters (Manual Pradojater et al, 1993) The results are in agreement with observations made by other workers that a pH range of 6-8 provides better conditions for mineralization of hydrocarbons since most bacteria capable of metabolizing hydrocarbon develop best at pH conditions close to neutral (Phung, 1988; Morgan and Watkinson, 1989; Manual Pradojatar *et al* 1993).

All the isolates utilized crude oil as carbon and energy source (Table 2) but the rate of biodegradation varied: *Bacillus sp* (34.2%), *Pseudomonas sp* (29.8%) *Alcaligenes sp* (28.2%), *Corynebacterium sp* (24.6%), and *Arthrobacter sp* (23.5%). *Baccillus sp* and *Pseudomonas sp* caused the highest degradation. This work is in agreement with the report of Udeme and Antai (1988) that *Bacillus sp* degraded medium and light crude oil at a faster rate than the other bacteria they worked with. The least amount of degradation was by the consortium (20.0%). Similar finding was reported by Udeme and Antai, (1988). Soli and Bens (1973) proposed that no particular benefit was derived by the mixing of different bacterial strains, that inhibitory effect might occur which would result in a reduced degradation of hydrocarbons. However, in accordance with the proposal of Cerniglia and Perry (1973) that crude oil degradation was enhanced when there was "associative" growth of microorganisms, Ekundayo and Obire (1988) reported that the rate of biodegradation was higher with the consortium of organism, *(Pseudomonas flourescens* and *Bacillus subtilis)* compared with the axenic culture. This was similarly observed by Zobell, 1969; Davies, 1967, and Horowitz, 1975. The

performance of mixed cultures could be attributed to synergistic activities of the organisms.

It is possible that the low value obtained with the mixed culture (20%) was due to either nutrient inadequacy as well as inhibitory metabolites resulting from the biotransformation of the crude oil or antagonism displayed by one of the bacterial species.

A comparison of TVC, O.D. (Fig. 1-6) and the rate of biodegradation (Table 2) of each of the isolates shows that in some cases, high viable count and O.D obtained for an isolate did not reflect high rate of degradation of BLCO *Arthrobacter sp (* O.D, .557) and *corgnebacterium sp* (O.D, .380) had the highest TVC ($2.0x10^{15}$ c.f.u ml^{-1}), their percent degradation were 23.5% and 24.6% respectively. *Alcaligenes sp* (TVC, $1.4x10^{15}$ c.f.u. ml^{-1}) gave the highest O.D (.560), its percent degradation was 28.2%. The consortium of the isolates (TVC, $1.0x10^{15}$ c.f.u. ml^{-1}; O.D. .485) caused the lowest degradation (20%). *Bacillus sp* had the lowest TVC ($7.6x10^{14}$) and O.D. (.343) but its rate of crude oil degradation was the highest (34.2%). Crude oil is a complex mixture of different chemical compounds that differ markedly in volatility. solubility and susceptibility to biodegradation. Different species of microbes preferentially attack different compounds in the mixture. Gunkel and Gassman (1980) contended that some hydrocarbons could be oxidized or utilized by micro-organisms without such hydrocarbons supporting their growth and some other hydrocarbon could act as a co-substrate. This agrees with some of the following general observation made by Zobell (1964):

i) aliphatic hydrocarbons are degraded and assimilated by a wide range of microorganisms, other classes including aromatics may be partly oxidized but are assimilated by only a few bacteria.

ii) n-alkanes of chain length shorter than n-nonane are not usually assimilated but may be oxidized. Relatively few bacteria have the ability to grow on alkanes

shorter than n-octane. As the chain length of the alkanes increases beyond C_9, the yield factor increases but the rate of oxidation decreases.

iii) Saturated compounds are degraded more readily than unsaturated ones.

iv) Straight chain compounds are degraded more easily than branched ones.

4.2 CONCLUSION:

The results of this study has shown that BLCO is a source of carbon and energy required for growth by the isolates used. To assess the role of microbial degradation in removing contaminating hydrocarbon, high viable counts and optical density may not reflect a corresponding increase in percentage degradation. Measurement of microbial hydrocarbon biodegradation potentials, by chemical analyses (i.e. gravimetric analysis) of the sample should be included for satisfactory results.

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APPENDIX 1:

The pH and optical density (O.D) for the axenic and mixed bacterial cultures in mineral salt medium containing Bonny Light crude oil as sole carbon and energy source is given below.

Alcaligenes sp

Day	*pH*	*O.D. (420nm)*
0	6.35	0.009
2	5.70	0.109
4	6.07	0.192
6	6.06	0.290
8	6.07	0.300
10	6.04	0.375
12	6.01	0.400
14	5.91	0.560

Pseudomonas sp

Day	*pH*	*O.D. (420nm)*
0	6.37	0.003
2	6.13	0.109
4	6.06	0.250
6	6.14	0.253
8	6.16	0.270
10	6.08	0.317
12	6.10	0.395
14	6.16	0.510

Corynebacterium sp

Day	*pH*	*O.D. (420nm)*
0	6.39	0.0036
2	5.71	0.240
4	6.01	0.308
6	6.08	0.339
8	6.05	0.350
10	6.07	0.352
12	6.02	0.360
14	6.07	0.380

Arthrobacter sp

Day	*pH*	*O.D.*
0	6.46	0.004
2	6.03	0.170
4	6.08	0.260
6	6.05	0.300
8	6.02	0.327
10	6.05	0.428
12	6.02	0.478
14	6.08	0.557

Bacillus sp

Day	*pH*	*O.D.*
0	6.32	0.04
2	6.13	0.111
4	6.08	0.179
6	6.06	0.260
8	6.05	0.272
10	6.02	0.300
12	5.99	0.295
14	5.77	0.343

Consortium of the Isolates

Day	*pH*	*O.D. (420nm)*
0	6.46	0.24
2	6.14	0.138
4	6.05	0.330
6	6.12	0.370
8	6.10	0.400
10	6.07	0.503
12		
14	5.96	0.485

APPENDIX 2:

HYDROCARBON UTILIZING BACTERIAL COUNTS (C.F.U Ml^{-1}) OF BROTH CULTURE

ORGANISM	*DAY 0*	*DAY 2*	*DAY 4*	*DAY 6*	*DAY 8*	*DAY 10*
Alcaligenes sp	$7.8x10^{6}$	$1.9x10^{12}$	$1.7x10^{14}$	$1.4x10^{15}$	$4.8x10^{14}$	$1.5x10^{12}$
Pseudomonas sp	$3.7x10^{6}$	$1.3x10^{11}$	$2.4x10^{13}$	$1.0x10^{15}$	$3.0x10^{14}$	
Corynebacteriam sp	$2.9x10^{7}$	$7.7x10^{10}$	$1.9x10^{13}$	$2.0x10^{15}$	$3.2x10^{14}$	
Arthrobacter sp	$3.2x10^{6}$	$2.0x10^{12}$	$2.8x10^{13}$	$2.0x10^{15}$	$6.5x10^{14}$	
Bacillus sp	$3.0x10^{6}$	$5.8x10^{10}$	$2.4x10^{13}$	$7.6x10^{14}$	$2.7x10^{14}$	
Consortium	$2.1x10^{7}$	$2.8x10^{13}$	$1.14x10^{15}$	$1.0x10^{15}$	$5.0x10^{11}$	