OIL UTILIZATION CAPABILITIES OF MICROORGANISMS ISOLATED FRROM WASTE OIL CONTAMINATED SOIL

BY

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B.Sc (ABU)

BEING A THESIS SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL
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DEDICATION

This work is wholly dedicated to my dearest mother

LARAI L. M. BELLO

and my little brothers for their encouragement and prayers to put my dreams to realities.

ACKNOWLEDGEMENT

I am indebted to my supervisor, Dr. (Mrs) N.B. De who is also the Head of Microbiology unit, Department of Biosciences, F.U.T., Minna. She challenged, constructively criticised and painstakingly stamped out errors with progressive suggestions. I say thank you, though it is an understatement of my sincere expression.

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Finally, my gratitude to the creator - ALMIGHTY ALLAH for the strength and guidance. I remain grateful, and may you continue to guide me on to the right path. Al-hamdu - lillah.

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DECLARATION

I hereby declare that this work is original and has not been presented elsewhere for the award of any degree. Information derived from all literatures have been acknowledged in the references.

27/02/95

YAKUBU M. BELLO

'This thesis entitled "oil utilization capabilities of microorganisms isolated from waste oil contaminated soil" was examined and found to meet the regulations governing the award of the degree of Msters of Technology of Federal University of Technology, Minna and is approved for its contribution to knowledge and literacy presentation.'

Naud M. B. De — DATE: 31/3/95

SUPERVISOR

DR. (MS) N.B.DE

H.O.D. — DATE: 31/3/95

DEAN — DATE: 26/07/95

EXTERNAL EXAMINER — DATE: 31/3/95

ABSTRACT

A total of 24 microorganisms were isolated from waste oil contaminated soil of three automechanic workshops in Minna. Out of this number, bacterial isolates constituted 54.16%, fungal isolates was 41.68% and unidentified species was 4.16%. Two of the bacterial isolates (B102B & B104B) could not utilize crude oil (Lagoma light) at 0.1% concentration in MSLM, while the rest utilized the oil poorly at the same concentration. All the isolated fungi utilized the crude oil efficiently, except at higher concentration (5%) which inhibited growth. The fungal isolates had optimum growth at 0.5% concentration of crude oil in MSLM and was also observed that the optimum incubation period was 21 days. Their growth on crude oil, complex nutrients and glucose was similar except for the lag phase which was different for each of the substrates. In MSLM, the fungi were able to use the crude oil as carbon and energy source, their growth being almost same with and without Nitrogen source supplement in crude oil incorporated into MSLM. The fungal isolates preferred acidic pH for Lagoma light crude oil utilization with maximum at pH 5.0. The optimum condition of temperature was at 28 $\pm$ 1 ^{o}C for crude oil utilization by the fungal isolates. Isolated fungi that degraded crude oil also had the capability of utilizing refinery effluent and other petroleum components – gasoline, kerosine, diesel and engine oil. However, crude oil was utilized more

efficiently than effluent and other petroleum components.

In view of their ability to utilize crude oil as carbon and energy source, these microorganisms may be used in mitigating oil spills in the environment.

CHAPTER ONE

INTRODUCTION

The demand for petroleum has been on the increase not only as an energy source, but also as raw material in chemical industries for production of pharmaceuticals, agricultural chemicals and feeds, plastics, cosmetics, detergents, fibres and other polymers like lubricating agents, stabilising agents [Gutnick and Resenberg, 1977]. The proportional increase in the production, refining and distribution of the petroleum in order to fulfil the on-going demand has brought an ever-increasing problem of environmental pollution. According to Bartha [1986], world wide, over 2 billion metric tons per annum (mta) of petroleum are produced, and it has been estimated that 1.7 - 8.8 mta. i.e. 0.08 - 0.4% of the world production end up polluting the oceans. No comparable estimates have been worked out for terrestrial petroleum hydrocarbon (PHC) pollution but considering that the greater part of petroleum is produced, refined and utilized on land, the resulting routine, accidental and illegal discharges are likely to equal if not exceed the figure cited for the marine environment. Both in the marine and the terrestrial environments, the low level routine discharges (effluents, urban run off, cleaning operations, oil treatments of roads for dust control, etc) account for probably over 90% of the

total PHC pollution. Production and transportation accidents such as well blow outs, tanker disasters, and pipeline breaks account for only 5-10% of the total PHC discharge, but because of their drastic and highly visible local effects, press coverage and public attention is focussed disproportionally on these types of incidents.

As pollutants, PHC occupy an intermediate position between highly biodegradable, biogenic and highly recalcitrant, xenobiotic substances. Though, biogenic in origin, PHCs have been altered greatly by geochemical processes and often also by refining. For millions of years, PHCs entered the biosphere only in a localised and rather gradual manner due to natural seeps and erosion. The resulting sporadic exposures allowed for the evolution of some hydrocarbons biodegradation pathways, but globally PHCs exerted little selective pressure until the 20th century when man's activities radically increased the exposure of the biosphere to PHCs. Hence, the removal of the petroleum and its products that are accidentally or deliberately spilled into the environment become of great importance. The developed countries, like USA recognised the dangers and had subsquently taken a number of measures as early as 1920s to reduce the problems [Bartha and Bossert, 1984].

In Nigeria, oil is produced mostly in eastern part, so massive movement of oil occurs for refining and consumption purpose throughout the country. Also another fact is that it is not the major oil consumer, though it is one of the

major oil producing countries. Oil exportation involves heavy transportations of oil from refineries to ports. Hence, there have been frequent reports of oil spillages involving blowouts, and leakages from pipes and oil wells into the environment, since oil production began in 1958 [Obire, 1990 and Ogoke, 1992]. Other spillages come from accidents involving oil tankers, oil prospecting, as well as discharge and wash waters from the tankers. Considering the adverse effect of the pollution on the environment therefore, scientists in different parts of the country are now concentrating on studies of different suitable oil disposal methods.

1.1 NATURE OF CRUDE OIL AND REFINERY EFFLUENT

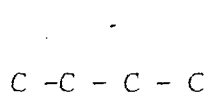
Crude oil is a naturally occuring and extremely complex mixture of hydrocarbons including small quantities of compounds that contain oxygen (Phenol, naphthanic acid), sulfur (alkylthiol, thiophene) and nitrogen (Pyridine, Pyrole, Indole), as well as trace amounts of metallic constituents [Gutnick and Rosenberg, 1977 and Bartha, 1986). Its complete resolution is very unlikely to be ever achieved because of the tremendous number of isomeric structures with similar physical and chemical properties especially in the higher boiling fractions of petroleum [Bartha, 1986]. Gutnick and Rosenberg [1977] reported fractionation of crude oil with respect to molecular size (by distillation) and polarity (by adsorption chromatography).

Distillation gave fractions containing a mixture of compounds of similar molecular weights, but representing all classes of hydrocarbons. They include gasoline, kerosine, gas oil, lubricant and residue (Table 1). Using silica gel chromatography, the crude oil gave asphaltic or polar, saturate or aliphatic and aromatic hydrocarbon fractions. PHC within the saturate fraction have n-alkanes, branched alkanes and cycloalkanes (naphthanes). Alkenes (unsaturated analogs of alkanes) are rare in crude petroleum but occur in many refined petroleum products as a consequence of 'cracking' process. Increasing carbon numbers of alkanes (homology), variations in carbon chain branching (Iso-alkanes), ring condensations, and Interclass combination (e.g. phynylalkanes) account for the enormous numbers of hydrocarbons that occur in crude petroleum [Bartha, 1986].

Table 1- Fractionation of crude oil by distillation.

Fraction	Boiling range (%)	Volume (%)	C-atom
1. Gasoline	40 - 180	30.5	5 - 10
2. Kerosine	180 - 230	9.5	10 - 13
3. Gas oil	230 - 300	14.5	12 - 17
4. Lubricant	300 - 400	17.5	17 - 26
5. Residue	> 400	25	>24

Gutnick and Resenberg, 1977.



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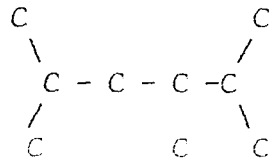

(B)

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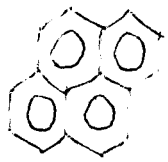

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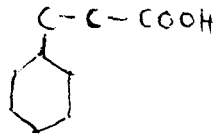

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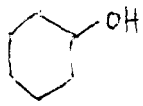


(G)

(H)

(I)

Note: *Only Carbon skeletons are represented!*

Key

A - *Normal alkane* D - *Aromatic hydrocarbon* G - *phenol*

B - *Iso - alkane* E - *Condensed aromatic hydrocarbon* H - *Pyridine*

C - *Cyclo alkane* F - *Naphthenic acid* I - *Thiophene*

Fig. 1 - Examples of Crude oil components.

Bartha, 1986

Refinery effluents vary in composition and concentrations. They contain organic and inorganic matter, heavy metals, and sulphides. Compounds like pyridine, thiols and phenols are also found. Some may contain floating and emulsified hydrocarbons, phosphates used in softening water, and chlorinated hydrocarbon generated in the refining operations, and sludge [Bartha, 1986 (table 2).

Table 2. Composition of refinery effluent.

Particles	Mean concentration (mg/l)
1. Suspended solid	350
2. Sulphides	8.8
3. Phenols	27
4. Oil	57
5. Ammonia nitrogen	87
6. Phosphate	49
7. Chromium	0.5
8. Chlorides	310
9. Calcium carbonate	180

McKinney, 1963.

Table 2. Composition of refinery effluent.

1.2 IMPACTS OF OIL POLLUTION

Oil pollution presents a serious problem, and the toxicity of crude and refined oils on the environment - human, livestock, wildlife, crop, soil and microorganisms have been well documented [Colwell and Walker, 1972; Raymond et al. 1976; Atlas et al. 1978; Amund and Igiri, 1990;

Ogoke, 1992 and Antai and Mbomo, 1993]. The pollutants some of which are toxic components eventually get to the plants and animals via food chain when they enter into sources of water and soil. In soil, an oil spill kills the herbaceous vegetation of the affected area immediately. Trees and woody shrubs are destroyed more slowly via their root system. The mechanism is in part contact toxicity to fine roots and root hairs, and also the effect of the anoxic conditions that develop in oiled subsoil [Bartha, 1986]. Biodegradation activity deprives plant roots of vital oxygen. In aquatic environment, oil spill spreads over the surface of water forming 'oil-slick'. The slick prevents the passage of oxygen as well as reduces the penetration of light (sun), thus depriving aquatic organisms of the above two vital needs. Photosynthetic organisms die off, while anaerobiosis sets in producing toxic products like H_2S that results into foul odour of the environment. The toxic products could accumulate to inhibitory levels killing aquatic animals like fish. Crude oil contains mutagenic, carcinogenic and growth inhibtory chemicals [Gutnick and Rosenberg, 1977 and Bossert and Bartha, 1984]. Directly, these carcinogenic and mutagenic components kill the animals [Cooney, 1984]. Lack of oxygen penetration in part starves the animals to death.

Apart from being directly toxic, some of the products from biodegradation of PHC could be toxic. Bartha and Atlas [1977] reported that $C_5 - C_9$ alkanes were not toxic to a

certain population of bacteria, but the alcohols of these compounds were inhibitory. It is also known that addition of hydrocarbons to an ecosystem may result into selective increase or decrease in the size of microbial populations. This is however dependent on the composition of the contaminating hydrocarbon, and on the species of microorganisms present within that community [Colwel and Walker, 1972; Atlas et al 1978; Federak et al 1984; Bartha, 1986 and Monna et al 1993). The ecosystem is enriched primarily for microoganisms capable of utilizing the hydrocarbon and secondarily for those capable of utilizing metabolites produced by the hydrocarbon-utilizing microorganisms. The enrichment results into increased number of both the primary and secondary biodegraders which gives an overall increase in the total population of ecosystem. On the other hand, some of the hydrocarbons lead to a decrease in microbial population [Monna et al. 1993 and Sikkena and De Bont, 1993). Atlas et al.(1978) found that rate of algal photosynthesis and nitrogen fixation decrease in the presence of crude oil. Compounds like toluene and phenol which are petroleum components have been used as disinfectants.

Toxic volatile components that are bacteriostatic are also found in crude oils. The inhibitory effect is not only characteristic of the chemical nature of the hydrocarbons, but also the kind of microbial population. The effects are in addition influenced by their solubility and

concentration. Some of the microorganisms show neutral response to PHC contamination, thus their size may remain unchanged [Bartha and Atlas, 1977].

1.3 APPROACH TO PETROLEUM AND WASTE DISPOSAL

Presently, a number of approaches to PHC pollution are available, and some of the methods used include incineration, land disposal (e.g. land fills, surface impoundments, use of waste pipes and underground injection wells) and land treatment (or land farming) [Bartha and Bossert, 1984].

Except land farming, all the methods are not associated with microbial metabolism, hence the recycling of petroleum and its waste in the environment is hindered. Mbreover, they are not economically atrractive, and have other side effects. For example, there is gaseous emission into the atmosphere from incineration, and use of land fills and injection wells could be hazardous to ground waters and streams. These effects would need separate attention.

Land farming is a recent and widely used approach to disposal of PHC and its products [Bartha and Bossert, 1984 and Bartha, 1986]. It relies on soil microorganisms to degrade and eventually stabilize the PHC pollutants by mineralization. It is cost-effective and does not involve high-skilled technology, making it acceptable and suitable especially for developing countries. This microbiological

approach to the problem of PHC pollution become possible in the light of emergence of information concerning hydrocarbon-utilizing microorganisms; the specificities of the microbes with their hydrocarbon substrates; and some specific metabolic transformations that result from this interaction [Gutnick and Rosenberg, 1977). Microbial degradation is an 'open' system which plays a major role in the weathering process [Atlas 1981]. The biodegradability depends on the nature and chemical composition of the oil, the microbial community and on environmental factors to degrade, transform or immobilize the hydrocarbons in the soil [Obire 1990; Ogoke, 1992 and Shefer, 1992]. Unlike other methods, land farming has no physical barrier, therefore organic compounds are converted to mineral constituents and humis, heavy metals are bound or precipitated, and hazardous wastes are rendered harmless or are not separated from the environment, instead leaching is minimized by soil process [Bartha, 1986 and Nil-Min Wu and Grogory, 1993].

Apart from the physical, chemical and biological factors, biodegradation of PHC products can only occur if there is a 2-phase oil/water system, but the actual amount of free water can be very small. The degradative process can be classified according to the amount of free water and nutritional status of the petroleum product, but there are many variations, within these classes. The first degradative category is exemplified by those of fuel oils,

particularly light oils. The water phase is small in relation to fuel phase. The fuel is nutritionally sparse, but it turns over rapidly, a new nutrient interface is repeatedly and frequently presented to the microbes in the lower water phase. There is little chemical change in the fuel itself because of the short contact time. The second category is where a small water phase is in contact with or dispersed through the oil phase, the oil is nutritionally adequate and complex, and the contact time is weeks, months or even years. This category is typified by microbial degradation of lubricating oils, hydraulic oils, and straight cutting oils. In the third category is oil in water-emulsions, the nutritionally rich oil formulation is finely dispersed in from 10 - 100 times its volume of water, hence presents an enormous interface that stimulates rapid microbial growth. Oil-in-water emulsions are typically used to cool and lubricate metal-working process.

1.3.1 RECENT STUDIES ON PHC BIODEGRADATION IN SOIL

The biodegradation of PHC in natural ecosystem is complex, however microorganisms have the ability to utilize petroleum as sole source of carbon and energy [Fedorak et al 1984; Ijah and Ukpe, 1992; Ijah and Essien 1993 and Antai and Mgbomo, 1993]. These organisms widely distributed in nature are not restricted to a few genera, because a diverse group of bacteria, yeasts, fungi and algae have been implicated in PHC biodegradation in both aquatic and terrestrial habitats. Bossert and Bartha [1984] have found

22 and 31 genera of bacteria and fungi respectively that are biodegraders in soil ecosystems. Atlas [1981] established that bacteria and yeasts seem to be more dominant degraders in soil ecosystems. Ijah and Okang [1993] determined the rate at which bacterial isolates from tropical rainforest soil could degrade Bonny light crude oil drilled in the area. On same Bonny light crude oil, Antai and Mgbomo [1993] studied the pattern of its degradation by Bacillus sp. and Pseudomonas sp. isolated from oil spilled site. The ability of bacteria isolated from crude oil polluted soil to degrade pesticide (eg Nuvacron 40) was investigated by Ijah and Essien [1993]. A study of the relationship between crude oil quality and its biodegradability by compairing the efffect of growth of pure and mixed cultures of bacteria obtained by soil enrichment on the composition of crude oils of different quality, and the effect of temperature on this relationship was carried out by Jobson et al (1977). Antai (1990) studied the extent to which temperatures, pH, nutrients in the soil during the dry and rainy seasons in Nigeria affect crude oil degrading ability of two crude oil degraders (Bacillus sp. and Pseudomonas sp.) isolated from a crude oil spilled site. Two strains of Bacillus (28A and 61B) were isolated from oil spilled soil by Ijah and Ukpe [1992) and efffects of nitrogen supplements was tested on their biodegration of crude oil. Atlas et al. [1978] examined the ability of Tundra soil microorganisms to

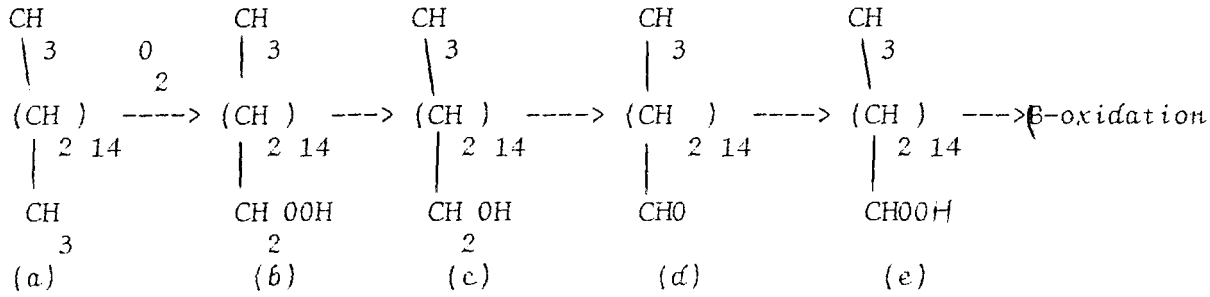
degrade crude oil. Dibble and Bartha [1979] carried out an extensive study on the effects of environmental parameters on the biodegradation of oil sludge. Also, Bossert and Bartha [1984) studied the structure-biodegradability relationships of polycyclic aromatic hydrocarbons in soil. Raymond et al [1976] defined the magnitude of the stimulation of specific hydrocarbon utilization flora in soil after the addition of six different oils to small field plots.

Atlas [1981] showed that several fungi exhibit more efficient degradation of PHC than bacteria. Some of the bacteria encountered include Pseudomnas, Archromobacter, Arthrobacter, Flavobacterium, etc. For fungi, Penicillium, Aspergillus, Verticillium, Fusarium, etc have been denonstrated. Rhodotorula, Candida, Sacharomyces, etc are some of the yeasts involved, while Cerninglia and Perry tested and found some algae (e.g. Anabaena sp, Chlorella sp, Microleus sp. etc) are capable of hydrocarbon degradation.

1.4 MECHANISM OF BIODEGRADATION OF PHCs.

Studies to determine the metabolic pathways for PHC degradation have been undertaken, and a number of reviews are available. The n-alkanes are generally most readily degradable components in a given mixture of PHC [Atlas 1981]. Their degradation is an oxidative process and proceeds with a monoterminal attack on the chain by introduction of molecular oxygen into the molecule by the

action of oxygenases. Hence, the presence of free oxygen is important. A primary alcohol is formed which is followed by aldehyde with subsquent formation of carboxylic acid [Gutnick and Rosenberg, 1977; Singer and Finnerty, 1984 and Bartha, 1986). Further degradation on the carboxylic acid is by β-oxidation which results into a 2-C unit shorter fatty acid and acetyl coenzyme A, with eventual liberation of carbondioxide and water (figs II and III). Extensive methyl branching interferes with the B-oxidation process and necessitates diterminal (Omega) oxidation [Roymond et al. 1976 and Bartha, 1986]. Subterminal oxidation may occur producing secondary alcohols sometimes, and subsquently ketone formation, but it is not the primary pathway for n-alkane degradation. It is rather used by many fungi (molds) and certain yeasts exclusively. (fig IV).



Key

a - n - hexadecane b- n - hexadecylhydroperoxide c-n-hexadecanol d- n-hexadecylaldehyde e- n-hexadecanoic acid

Fig II Proposed pathway of hexadecane metabolism in Acinetobacter sp.
Singer, 1984.

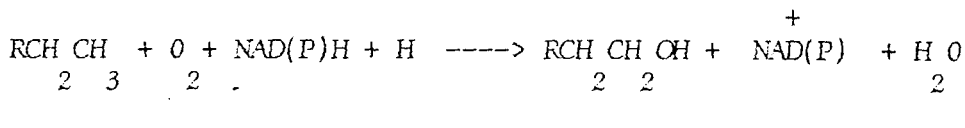
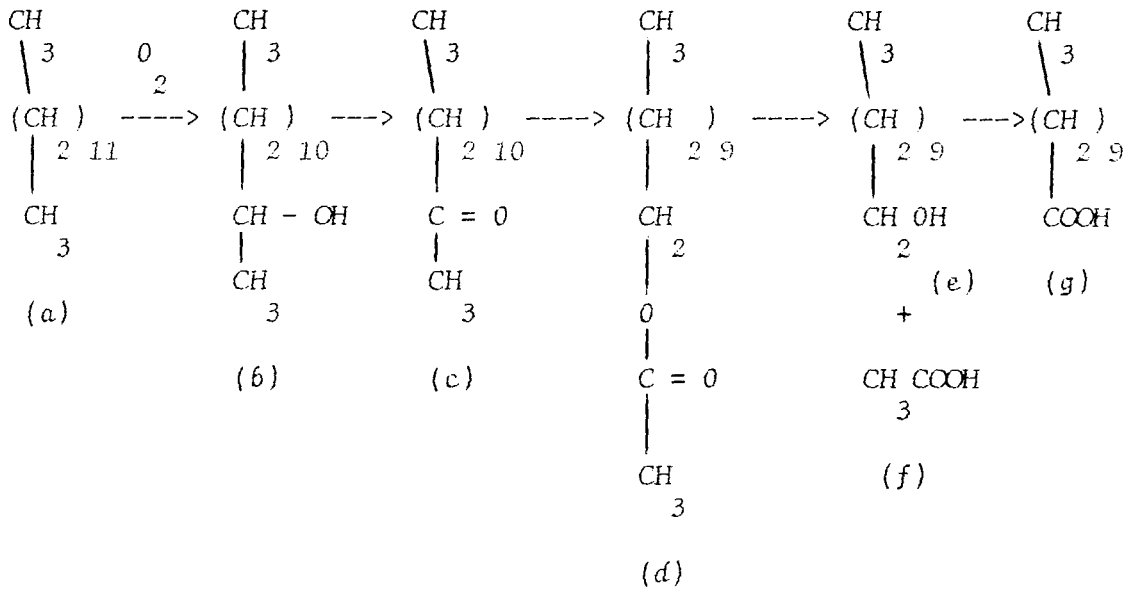


Fig. III. Mechanism of Oxidation:

Singer, 1984.



Key a - n-tridecane b - tridecan-2-ol c- tridecan-2-one

d - undecylacetate e- undecan-1-ol f - acetate

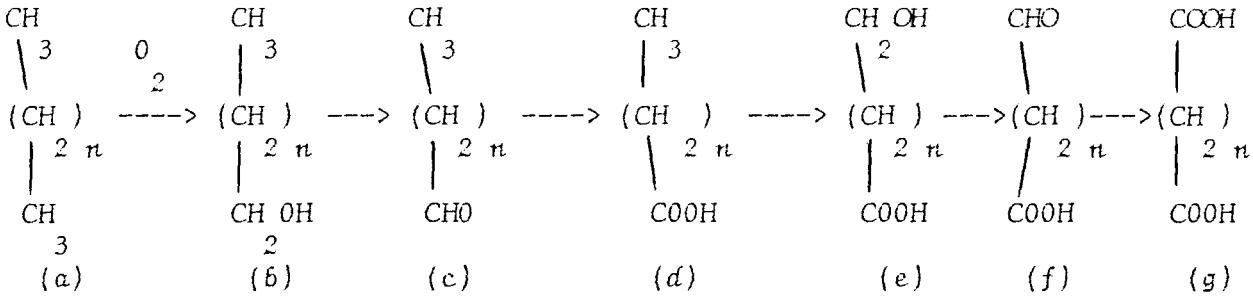
g - undecanoic acid

Fig. IV Pathway of Subterminal attack by Pseudomonas

Singer, 1984

Highly branched isoprenoids like pristane undergo Omega (diterminal) oxidation (Fig. V) to form dicarboxylic acids as the major degradative pathway. This results from the production of extracellular products e.g. alkane hydroxylase. However, methyl branching increases resistance of PHC to microbial attack [Andreoni et al. 1993]. Cycloalkanes are resistant to microbial attack, but are substrates of co-oxidation [Mouna et al. 1993 and Sikkena and De Bont, 1993] forming ketone or aldehyde. Once oxygenated, degradation

can proceed with ring cleavage. Substituted cycloalkanes are liable to degradation more readily than unsubstituted ones, especialy if the substituent is an n-alkane of adequate chain length. Normally, the substituent is attacked first leading to an intermediate product of cyclohexane carboxylic acids or related compounds. (Fig. VI)



Key a - n-alkane b- primary fatty alcohol c - Fatty aldehyde
d - Monocarboxylic fatty acid e - w-hydroxy fatty acid
f - w- aldehyde fatty acid g - dicarboxylic acid.

Fig. V Pathway of diterminal alkane oxidation.
Singer, 1984

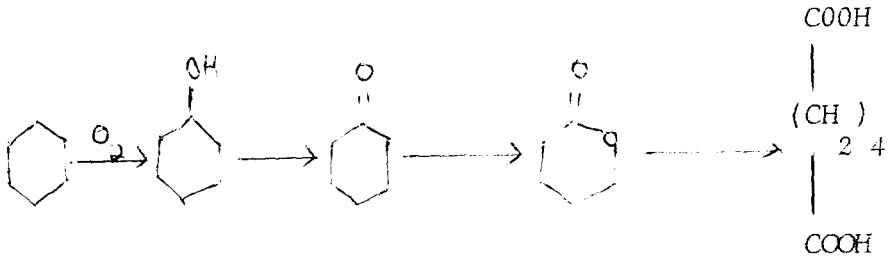


Fig. VI Proposed pathway for the catabolism of cyclohexane by Nocardia sp.

Perry, 1984

Aromatic hydrocarbons have gained notoriety as environmental pollutants that are resistant to biodegradation [Bossert and Bartha, 1984 and Miller et al 1988]. Microbial degradation involves formation of a diol

followed by cleavage and formation of a diacid such as cis,cis-muconic acid (Fig. VII). Light aromatics are subject to evaporation and to microbial degradation in a dissolved state. Extensive methyl substitution inhibits initial oxidation, though the initial enzymatic attack could be on the alkyl substituent or alternatively, directly on the ring [Atlas 1981].

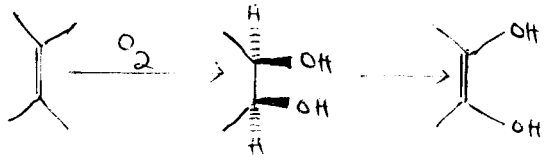


Fig. VII Pathway of aromatic hydrocarbon oxidation by Prokaryotes.

Singer, 1984

Metabolic pathways available for asphaltic components are least well understood. No uniform degradative pathway comparable to those of aliphatic or aromatic hydrocarbons have yet emerged. They are complex structures which are difficult to analyse.

1.5 ENVIRONMENTAL PARAMETERS THAT AFFECT BIODEGRADATION

The degradative potentials of the widely distributed hydrocarbon utilizing microorganisms depend to a large extent on environmental factors, and these include pH, moisture, state of the pollutant, topography, temperature, mineral nutrients, oxygen tension, diauxic effect, product inhibition, etc.

1.5.1 TEMPERATURE

It has a profound influence on rate of all biochemical processes, and so affect the rate of oil-biodegradation. Biodegradation of hydrocarbons occur over a wide range of temperatures, and psychrophilic, mesophilic and thermophilic hydrocarbon-utilizing microorganism have been isolated. At high temperatures, the hydrocarbon tends to be volatile or present in liquid state and could be degraded, but at low temperatures they tend to solid form and cannot be easily or efficiently utilized. The degree of spreading is reduced at low temperatures [Atlas, 1981] and freezing interupts biodegradation [Bossert and Bartha, 1981). Hill (1984) reported PHC biodegradation at below 0 ^{o}C. Dibble and Bartha [1979] found that a rise in metabolic activity of microbial communities is common throughout the mesophlic range. In thermophilic situations, metabolic rates tend to be high but growth rates and cells yield are low since energy is expended for repair of thermal damage to cell constituents [Mulkins-Phillips and Stenwart, 1974).

1.5.2 PHYSICAL STATE OF THE HYDROCARBON

At very low concentrations, some of the compounds may be soluble in water, and at high concentrations, they may exceed solubility limits. The degree of spreading determines in part the surface area of the oil available for microbial colonization. In soils, the pollutants are absorbed by plant matters and soil particles thus limiting

their spreading [Bossert and Bartha, 1984]. Availability of increased surface area accelerates biodegradation because not only are pollutants made available to the microorganisms, but movement of emulsion droplets makes oxygen and nutrients more readily available.

1.5.3. MOISTURE

Aerobic biodegradation of simple or complex organic materials in soil community is greatest at 50-70% soil water holding capacity. Inhibition at lower values is due to inadequate water activity and at higher values interfere with soil aeration. However, Dibble and Bartha [1979] observed that requirements for maximum activity on hydrophobic compounds may be different from the optimal moisture levels for the biodegradation of hydrophilic substrates. This could be due to the fact that the polymers, by rendering some surfaces hydrophobic reduce water holding capacity of the soil and this increase availability of water that is present for use by microorganisms [Bossert and Bartha, 1984].

1.5.4 pH

Extremes of pH are inhibitory to a great majority of microbial degradation processes. Most fungi are less adversely affected by low pH values than bacteria. Dibble and Bartha [1979] observed that liming favoured biodegradation of oil. They also found out an optimal activity of biodegradation at pH 7.4 and a considerable inhibition at pH 8.5 for bacteria.

1.5.5 MINERAL NUTRIENTS

The nutritional imbalance between substrate carbon supplied by petroleum and the nitrogen and phosphorous requirements for microbial growth is a limitation of hydrocarbon biodegradation. This has been extensively studied in the marine environment, and as the nitrogen and phsophorous reserves of most soils are low, a similar situation is expected to exist in the soil. Nutrients availability is limited by organic carbon. Top soils have more organic carbon per unit volume but it is humified and not readily available for mineralization. Stimulation of oil biodegradation is achieved by addition of nitrogen and phosphorous [Bossert and Bartha, 1984].

However, a complete assimilation of petroleum carbon into biomass is not achieved under natural conditions, because some of the compounds are recalcitrant or are metabolized slowly over a long period of time, and for compounds that are readily metabolised, some carbons are mineralised to CO_2. Therefore, efficiency of conversion of oil carbon to cellular materials is not 100% [Dibble and Bartha, 1979].

1.5.6 OXYGEN TENSION

Petroleum and its products are only metabolised oxidatively, because they are completely reduced organic substrates. Though, anaerobic oxidation occurs with nitrate and sulfate serving as electron sinks in laboratory, this

type of biodegradation has a negligible effect on oil pollutants in soils [Bartha and Atlas, 1977 and Bossert and Bartha, 1984]. Therefore, lack of molecular oxygen is considered to be an important limiting parameter of petroleum biodegradation.

1.5.7 DIAUXIC EFFECT

A phenomenon of metabolic regulation first described by Monod (1979) which determines the fate of petroleum products. When an organism with a broad range of substrates is offered more than one type of organic substrates, it may not attack the substrates simultaneously, rather in a definite sequence. Diauxic effect determines which of the petroleum components are degraded and in what order [Bartha and Bossert, 1984]. The presence of non-hydrocarbon substrates, or more utilizable low molecular weight compound may repress inductive synthesis of enzymes required for hydrocarbon utilization.

1.5.8. PRODUCT INHIBITION

The ultimate products of hydrocarbon biodegradation are CO_2 and H_2O, but biodegradation of higher molecular weight compounds involves many intermediates, some of which may accumulate to inhibitory levels. For example, the biodegradation of aromatic hydrocarbons yields phenolics and benzoic acids intermediates. However, the presence of different microbial communities reduce, if not prevent the accumulation of metabolic intermediates to inhibitory levels

[Cerninglia, 1984 and Sikkena and De Bont, 1993]. This is because some intermediates serve as substrates to others.

1.5.9. WASTE MATERIALS

Generally, complex media containing materials of natural origin support better microbial growth compared to synthetic medium. This may be due to various factors, mainly due to presence of inorganic and organic nitrogenous substances. It is interesting to see complex nutrient utilization capability of crude oil biodegraders as nitrogen source, and also as carbon source with or without crude oil.

1.6 JUSTIFICATION

There are volumes of literature on the biodegradation of PHC in developed countries with most attention on aquatic environment. Atlas [1981] attributed this to oceans being the largest and ultimate receivers of hydrocarbon pollutants. In Nigeria, there are few such studies on oil biodegradation for treatment of oil spills in aquatic environment [Obire 1990]. Furthermore, such studies are very scarce on soil ecosystem, and there are very considerable differences in the environmental and nutritional parameters as well as the microbial communities of the two ecosystems. Also, the physical behaviour of oil pollutants in the two habitats is quite distinct.

Therefore, this study is to isolate and identify microorganisms associated with waste oil contaminated soil of mechanic workshop dumps, and to determine their ability

to degrade PHC. Finally, the effect of some environmental parameters were investigated on the biodegradation activity of the microbial isolates.

CHAPTER TWO

MATERIALS AND METHODS

2.1 COLLECTION OF SAMPLES

(a) Soil Samples

The soil samples for this study were collected around January/February, 1994 from three principal mechanic workshop dumps namely - Bosso, Keteren gwari, and Northern bye-pass mechanic lay outs, all in Minna, Niger State, Nigeria. The mechanics in these workshops reported that generally they dump lubricating oil (black oil), gasoline, diesel. After the removal of surface litter, soils were collected at a depth between 15-20cm in sterile screw capped bottles. These were transported to the laboratory and stored at -4^{o} C.

(b) Crude oil, refinery effluent and Petroleum products

The crude oil (lagoma light) used was obtained from the chemical refining laboratory, Kaduna Refining and Petrochemical company, Nigerian National Petroleum Corporation, Kaduna. Though, the intended research was centrally on crude oil, other PHCs were collected for comparative purposes. Refinery effluent was collected at a discharge point where the effluent runs off pavement freely. Gasoline, Kerosine and Diesel were collected from filling

Fig.xviii: Collection of soil sample at Bosso automechanic workshop

stations, while engine oil was obtained from a lubricating shop, all in Minna, Niger State, Nigeria. These were collected in sterile bottles and stored at room temperature for further use.

2.2 EXAMINATION OF SOIL SAMPLES

Properties of the soils were determined on the field and in the laboratory.

2.2.1 Soil examination on the field

Temperature:- Temperature of soil of each mechanic workshop was determined on the spot using soil - thermometer.

Topography:- The type of locality - plain, hilly or mountaineous, low or high land of each site was recorded.

Vegetation:- Whether the sites had vegetation or not was observed.

2.2.2. Soil examination in the laboratory

pH: - A sample of the soil was crushed using a pestle and mortar. 2.0g of the crushed soil was added to a test tube and then filled up with distilled water. It was mixed throughly and pH was taken using pH meter (Croon Micro pH 200). This was done for 3 times and the average was taken.

Mbisture:- The moisture was calculated based on the method described by Akinsanmi [1985]. Empty petridish was weighed and sample of soil was placed on the dish and weighed. The soil in the dish was dried in the oven (FISHER ISOTHEM (R) OVEN MODEL 175) at 105 ^{o}C. Weighing was done at intervals until a constant weight was obtained. The moisture was determined

thus:

$$\text{Percentage of water content} = \frac{\text{loss in weight of Soil}}{\text{Weight of fresh soil}} \times 100$$

Structure:- The structural characteristics of the soils were determined at the Agricultural Science department, Niger State College of Education, Minna.

2.3 ISOLATION AND IDENTIFICATION

2.3.1 ISOLATION

10.0g of soil sample from each of the sites was transfered into 90ml of sterile distilled water, and aseptically, serial dilution as described by Fawole and Oso [1988] was performed to obtain soil suspension up to 10^{-7}. 0.1 ml of each dilution (10^{-7}, 10^{-5} 10^{-3} and 10^{-1}) was introduced onto dry agar medium (For bacterial isolation, NA and for fungal isolation, PDA were used respectively). In either case, sterilized glass spreader was used aseptically to spread the suspension on the surface of agar medium. These were incubated at 37 ^{o}C and room temperature (28 $\pm$ 1 ^{o}C) for bacteria and fungi respectively. Distinct colonies were picked and reinoculated into agar medium to obtain pure isolates. These pure isolates were then kept in the refrigerator for further use.

2.3.2 Identification

A number of microscopy/biochemical tests were done for identification purpose

1. Fungi

a) Cellular and colonial morphology

The isolated pure cultures were visualized by aseptically removing the cover plates to see growth morphology. The plates were placed under the microscope (x 10 objective) and viewed. Wet preparations were done using lactophenol cotton blue as mountant and viewed under the low-power objective and then using x 40 (high-power) objective.

(2) Bacteria (a) Cellular and colonial morphology: Simple staining was employed.

b. Spore staining:-

The isolates were stained for the presence of spores using Wirtz's method [Akinsami 1985]

c. Gram reaction

The bacterial isolates were gram stained following the procedure as described by Fawole and Oso [1988]

d. Motility test:-

The motility medium (Semi-solid nutrient agar) was stabbed with a loop of bacterial isolates to a depth of 1-2cm short of the bottom of the tube. These were incubated at 35 ^{o}C for 48 hours, and the line of inoculation observed at the end of the period.

Line of inoculation was not sharply defined, and the rest of medium was cloudy for motile isolates. While growth was restricted to the line of inoculation which become sharply defined and the rest of medium remained clear

for non-motile isolates.

e. Oxidase test:-

A piece of filter paper was wetted with a few drops of dilute (1%) solution of the oxidase reagent (tetramethy(-p-Phenylene diamine dihydrochloride). A bit of growth of isolate obtained from nutrient agar plate was smeared on the wet piece of paper. The development of an intense purple colour by the bacterial cells in the smears within 30 seconds indicated a positive test and failure within same period was a negative test.

f. Catalase test:-

A drop of 3% hydrogen peroxide was placed on a glass slide. A bit of growth of the isolates was removed from the medium with a wire loop, and the drop was touched with the charged loop. A positive test was indicated by bubbling and frothing. Negative test did not show bubbling or frothing.

g. Triple Sugar Iron (TSI) test:-

Using a sterile wire loop, the surface of the TSI agar slants was streaked and the butt stabbed 2 times with the bacterial isolates. These were capped loosely and incubated at 37 ^{o}C for 24 hours. Several reactions were read after incubation.

Gas formation was determined by the appearance of bubbles in the butt or formation of cracks or pushing the butt from the bottom.

minutes] seperately from the bulk medium, but was added to the bulk medium after cooling. In part B, the salts were dissolved in concentrated HCl (51.3ml/l), and made up to 1 litre with distilled water. pH was adjusted to 7.6 then 1.5% (w/v) agar was added and finally autoclaved separately and combined with part A after sterilization.

About 18-20ml of the solid media was poured onto sterile plates, and dried at room temperture for several hours before coating the agar surface with a mixture of crude oil and carbon tetra chloride 1:1 (v/v). The plates were used when there was no remains of CCl_4 vapour. Initial concentrations of 0.1, 0.5, 1.0, 2.5, and 5% of crude oil were used. Bacterial isolates on NA were streaked onto the "oil agar" aseptically using wire loop. The plates were incubated at 37 ^{o}C for 6 weeks with glucose as control. Growth on the plates indicated oil degrading capabilities of the bacterial isolates (table 6). A set of plates without crude oil also served as controls.

(b) For fungal isolates-

The minimal salt media had the following composition (gram per litre): K_2HPO_4 - 1.0; $NaNO_3$ - 3.0; $MgSO_4.7H_2O$ - 0.5; $FeSO_4.7H_2O$ - 0.01. The medium was adjusted to pH 5.0 after 1.5%(w/v) oxoid No.3 agar had been added before sterilization. The antibiotics - Penicillin G - 0.05; Chloramphenicol - 0.05 in gram per litre were added as filter sterilized solutions to the autoclaved and cooled bulk medium.

To test biodegradability, the same procedure as described above for bacteria was followed except that incubation was at room temperature ($28 \pm 1^{o}C$).

Utilization of crude oil by the isolated bacteria was not pronounced, hence the fungal isolates were selected for further studies (table 6).

2.5 DETERMINATION OF OPTIMUM INCUBATION PERIOD FOR UTILISATION OF CRUDE OIL BY THE FUNGAL ISOLATES

The best five isolated fungal oil degraders B101F, B102F, K602F, B1002F, B1001F (table 7) were selected for this purpose. Each isolate was inoculated into minimal salt liquid media (MSLM) [same as 2.4(6) above]. The medium was stirred using magnetic stirrer (B & T - HOT PLATE Mag stirrer) to ensure uniform mixture of oil throughout the medium before distribution into flasks for inoculation. 50 ml medium was taken into 100 ml Erlemeyer's flask and then inoculated with the degraders. A control without addition of crude oil was used for comparison purpose. The flasks were then incubated at room temperature ($28 \pm 1^{o}C$). Cell mass was measured at 7 days interval up to 28 days to determine the optimum incubation period. (From table 7, it was also shown that 0.5% is the optimum concentration of crude oil for the biodegraders, therefore 0.5% crude oil was incorporated into the sterilized MSLM).

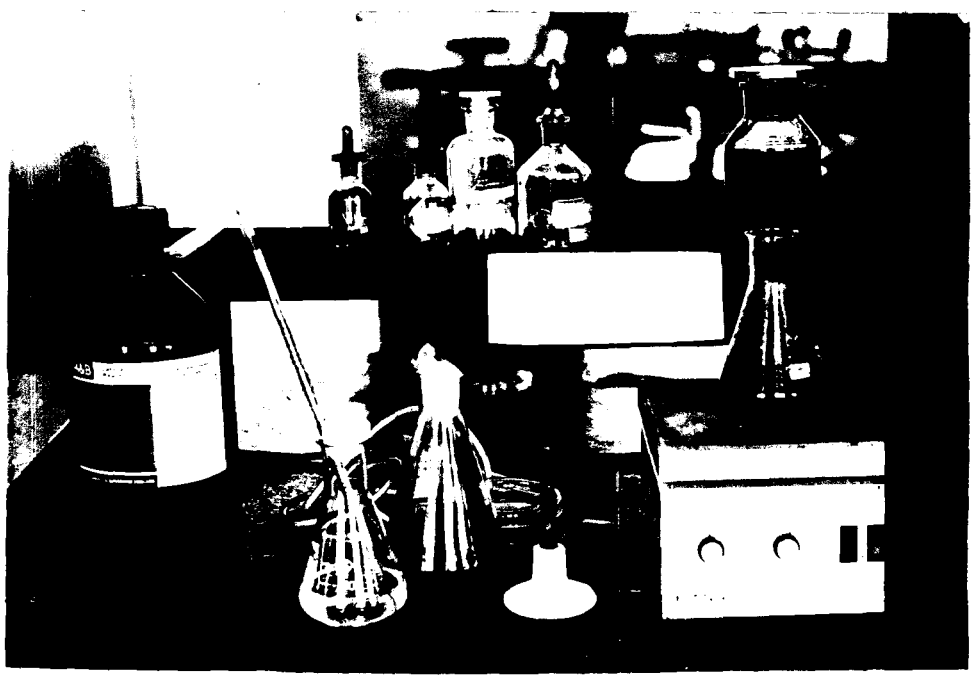


Fig.ix: Mixing of crude oil and MSLM with magnetic stirrer in rediness for dispensing

2.5.1. Measurement of cell mass.

At definite time interval, the broth (growth and medium) was taken and filtered through Whatman No.1 filter paper. The growth on the paper was washed twice thoroughly with distilled water and then transferred to pre weighed aluminium foil and dried at 90 °C for 24 hours.

Weight of empty aluminium foil – x

Weight of aluminium + dry cell – y

Weight of dry cell – $(y - x)$

$$\text{In gram per litre:} \quad \frac{y-x}{\text{Quantity of medium}} \times 1000$$

2.6 COMPARISON OF GROWTH OF FUNGAL ISOLATES ON CRUDE OIL AND GLUCOSE

The five oil degraders were each inoculated into 50 ml MSLM incorporated with 0.5% crude oil in 100 ml flasks. The medium was prepared as described in section 2.4(6). For glucose, 0.5% glucose powder was incorporated into MSLM before sterilization. This was also dispensed (50 ml) into 100 ml flasks and inoculated with the five fungal isolates. These were incubated at 28 ± 1 °C for 21 days. At the end of incubation period, the cell mass was calculated as in section 2.5.1. Flasks having MSLM without crude oil or glucose served as control.

2.7 EFFECT OF pH AND TEMPERATURE ON OIL DEGRADING CAPABILITY OF TWO ISOLATES

The effect of pH and temperature on crude oil biodegradation by two of the isolated fungi was assessed by inoculating the isolates into 50 ml MSLM with 0.5% crude oil (prepared as in section 2.4 (6)) in 100 ml flasks with different pHs and temperatures.

For pH, media with pHs of 3.0, 5.0, 7.0 and 9.0 were used and incubated at 28 ± 1 °C in order to determine the optimum pH for utilization of crude oil by the organisms. The media were adjusted with HCl and NaOH using pH meter (Croon MicropH 2000). (Table 10)

From table 10 it was shown that the optimum pH for crude oil utilization is 5.0. So to determine the effect of temperature on oil degrading capability MSLM media with 0.5% crude oil of pH 5.0 were inoculated with the organisms and incubated at 10 °C, room temperature (28 ± 1 °C) and 37 °C.

The cell mass of the organisms were calculated as in section 2.5.1 after 21 days incubation.

2.8 EFFECT OF COMPLEX NUTRIENTS ON UTILIZATION OF CRUDE OIL

Complex media that contain materials of natural origin were employed to ascertain their effect on hydrocarbon biodegradation. The materials used include beans husks, rice, maize and corn brans. 20g each of the materials was suspended in 200 ml hot water in 500 ml flasks. The suspensions were kept at 90 °C for 24 hours.

The hot extracts were filtered through Whatman No.1 filter paper. The extract thus obtained were each concentrated to 100 ml. The solid content of rice, maize, corn and beans were determined to be 3.43; 4.12; 3.91 and 0.98% respectively.

(a) Complex nutrients as carbon source

To first set, the complex nutrients were incorporated directly into the MSLM (prepared as in section 2.4(b)) prior to sterilization 0.1% each of the nutrients was added to MSLM with 0.5% crude oil. To the second set, 0.5% complex nutrients only was incorporated into the MSLM (no crude oil). To the last set, 0.5% crude oil only was incorporated into MSLM, and glucose (0.5% in MSLM) served as control. All the flasks were incubated under same conditions. The cell mass was calculated as in section 2.5.1.

(b) Complex nutrients as Nitrogen source

To determine the effect of complex nutrients as N-source three sets of media were prepared. One set had crude PHC and MSLM without Nitrogen compound ($NaNO_3$). The second set had crude PHC and MSLM without N-compound but complex nutrients serving as N-source. The last set had crude PHC and MSLM with Nitrogen compound. The results were compared after incubation.

2.9 UTILIZATION OF OTHER PHC; BY SOME ISOLATES

For comparative purposes, the ability of the isolated fungi to utilize refinery effluent and some petroleum

components (Gasoline, Kerosine, Diesel and Engine oil) was determined.

(a) For refinery effluent, 0.5% of the hydrocarbon was incorporated into MSLM (prepared as in section 2.4(b). The medium was dispensed into 100ml flaks of 50 ml each and inoculated with the fungal degraders. Crude oil was used as control. The flasks were incubated at 28 ± 1 °C for 4 weeks.

(b) For gasoline, kerosine, diesel and engine oil, 0.5% each of the hydrocarbons were incorporated into MSLM (propared as in section 2.4(b)). 50ml of the medium was dispensed into 100ml flasks and inoculated with the fungal degraders. All the flasks were incubated at 28 ± 1 °C for 4 weeks.

At the end of incubation period, the cell mass of the fungal degraders were calculated as in section 2.5.1.

2.10 COMPARISON OF RATE OF CELL GROWTH ON CRUDE OIL, CRUDE OIL ± COMPLEX NUTRIENT, AND GLUCOSE

The rate of increase in cell mass of a fungal degrader was monitored over a 28 day period in MSLM (prepared in section 2.4(b) containing separately crude oil, crude oil + complex nutrient and glucose. The flask labelled crude oil was incorporated with 0.5% of crude oil. The one labelled crude oil + complex nutrient was incorporated with 0.1% of maize extract before sterilization and 0.5% crude oil was incorporated after sterilization. While the one

labelled glucose was incorporated with 0.5% glucose before sterilization. The media were each dispensed (50 ml) into 100 ml flasks, inoculated with the fungus and incubated at 28 ± 1 °C. The isolate used was Mortierella sp.

At definite period the cell mass of the fungal degrader was calculated as in section 2.5.1. The measurement was for 28 days, and the results were compared.

CHAPTER THREE

RESULTS

.1 CHARACTERISTICS OF COLLECTED SOIL SAMPLES

The soils of three mechanic layouts examined both in he laboratory and on the field had the following haracteristics listed below.

able 3:

Characteristis of soil samples collected from three mechanics layouts.

ite	Moisture (%)	PH	Temperature (^{o}C)	Particle Size (mm)	Vegetation	Structure	Soil Identified
. Bosso	14	4.8	31	0.003+2	little vegetation	tightly packed	Clay-loam
. Keteren gwari	10	5.2	32	0.009+6	No vegetation	packed	Silt-loam
. Northern bye-pass	8	6.4	35	0.090+5	No vegetation	loose	Sand-loam

.2 ISOLATION AND IDENTIFICATION

From the morphological observation and biochemical tests, the following organisms ere identified.

ible 4

Identification of bacteria isolated from three sites.

TESTS/CHARACTERS

N	OX	VP	UR	GL	SU	LA	HS	GA	SH	MO	CA	CI	GL	PP	SP	GR	SR	BACTERIA IDENTIFIED
101B	−	+	ND	+	−	−	−	−	+	+	+	−	+	−	R	+	+	Bacillus sp
102B	ND	ND	−	−	ND	−	−	−	−	+	ND	ND	+	ND	R	−	−	Erwinia sp
103B	+	ND	−	−	−	−	−	−	ND	+	+	+	+	ND	R	−	ND	Alkaligenes
104B	+	+	−	+	+	+	−	−	ND	+	+	ND	+	ND	R	−	ND	Aeromonas
105B	+	+	+	+	−	−	−	−	−	+	+	+	+	+	R	−	ND	Psendomonas
107B	−	−	−	+	+	+	−	−	ND	+	+	ND	+	ND	R	−	−	Vibrio
108B	ND	−	+	+	+	+	+	−	ND	+	−	+	+	ND	R	−	−	Proteus
501B	−	+	+	+	+	+	−	−	ND	−	+	−	−	ND	R	−	−	Actinobacillu
1001B	±	ND	±	±	±	±	±	±	±	±	±	±	±	ND	R	±	ND	Acinetobacter
1001B	+	ND	−	+	ND	ND	−	−	−	−	+	−	+	+	R	−	ND	Flavobacterium

KEY

N-Isolate number, OX-oxidase, VP-Voges-proskauer, UR-Urease, GL-Glucose, Su-sucrose, LA-lactose, HS- H_2S, GA-Gas, SH-starch hydrolysis, MO-Motility, CA-catalase, CI-citrate, GL-gelatin liquefaction, PP-Pigment production, SP-Shape, GR-Gram, SR-Spore, ND-Not determined, R-Rod + - positive, − - negative tests.

From this table it has been shown that 100% of the isolated bacteira were rod shaped and 90% was gram-ve while 10% was gram +ve.

able 5

Identification of fungi isolated from sites.

SOLATE NO.	MICROSCOPIC EXAMINATION		APPEARANCE ON PDA	FUNGI IDENTIFIED
	Spores	Hyphae		
101F	Smooth and elliptical in shape	Septate branched conidiophores, aggregate into clusters of sterigmata	Serial hyphae in broom-like fashion Blue-green in colour	Penicillium Sp
102F	Smooth and globose in shape	Septate branched conidiophore, aggregate into clusters of sterigmata	Aerial hyphae in broom-like fashion Green in colour	Penicillium Sp
501F	Spherical and Smooth	Non septate short hyphal branches	Alternate rings of Pink and white, like spokes in a wheel.	Fusarium Sp
602F	Too celled ascospores with thick stroma	Aerial hyphae with conidia clustered at the tip	Cushion shaped and bright-green in colour	Trichoderma Sp
1001F	Rough, black in colour and spherical	septate conidiophore that form vesicle at apex. Arise from foot cell.	Dirty bluish appearance which turned black. Rough	Aspergillus niger
.002F	Thick walled chlamydospores	Lack calumella. Large short number of aerial hyphae surround zygosporos.	Yellowish-white cottony appearance with fan-like zones.	Mbtierella Sp

The total number of bacteria isolated was 54.16% and
ngi was 41.68%, which means the bacterial species were
re predominant than that of fungi. The remaining 4.16%
s unidentified species.

3.3 UTILIZATION OF CRUDE OIL BY THE ISOLATES

The crude oil utilization capabilities of all the isolated organisms were tested at five different concentrations of the oil in MSM. The result is shown below.

Table 6 Utilization of Crude oil by the bacterial isolates

ISOLATE	CONCENTRATION OF CRUDE OIL (%)				
	0.1	0.5	1.0	2.5	5.0
B101B	+	–	–	–	–
B102B	–	–	–	–	–
B103B	+	–	–	–	–
B104B	–	–	–	–	–
B105B	+	–	–	–	–
B107B	+	–	–	–	–
B108B	+	–	–	–	–
K601B	+	–	–	–	–
K602B	+	–	–	–	–
B1001B	+	–	–	–	–
Control	–	–	–	–	–

KEY:

+ – Scanty growth; – – no growth.

Two of the bacterial isolates (B102B and B104) could not utilize the crude oil, and the rest of the isolated bacteria grew poorly on the crude oil at 0.1% concentration. As a result of this inefficient utilization of crude oil by the bacterial isolates no further study was performed with

the bacteria.
Table 7

Utilization of Crude oil by fungal isolates.

ISOLATE	CONCENTRATION OF CRUDE OIL (%)				
	0.1	0.5	1.0	2.5	5.0
B101F	++	+++	+	-	-
K101F	++	+++	+	+	-
B102F	++	+++	+	-	-
K602F	+++	+++	++	+	-
B1001F	++	++++	++	+	-
B1002F	+++	++++	++	+	-
B1002F	+++	++++	++	+	-
Control	-	-	-	-	-

KEY:

++++ - abundant growth, +++ heavy growth, ++ - Mederate growth + - scanty growth,- - no growth.

From the table above it is shown that all the isolated fungi grew on crude oil at 1.0% crude oil concentrations and that 0.5% is the optimum concentration of crude oil for the isolates. Also, 0.1% crude oil concentration was utilized more than 1.0% crude oil. Three of the isolates (B101F,B102F and B501F) did not grow on crude oil at 2.5% concentration, while the rest showed scanty growth on same medium. None of the isolates grew on crude oil at 5% concentration. The isolates B1001F and B1002F showed better growth than the rest. These were followed by B101F, K101F, B102 and K602F, while B501F showed least growth of all the

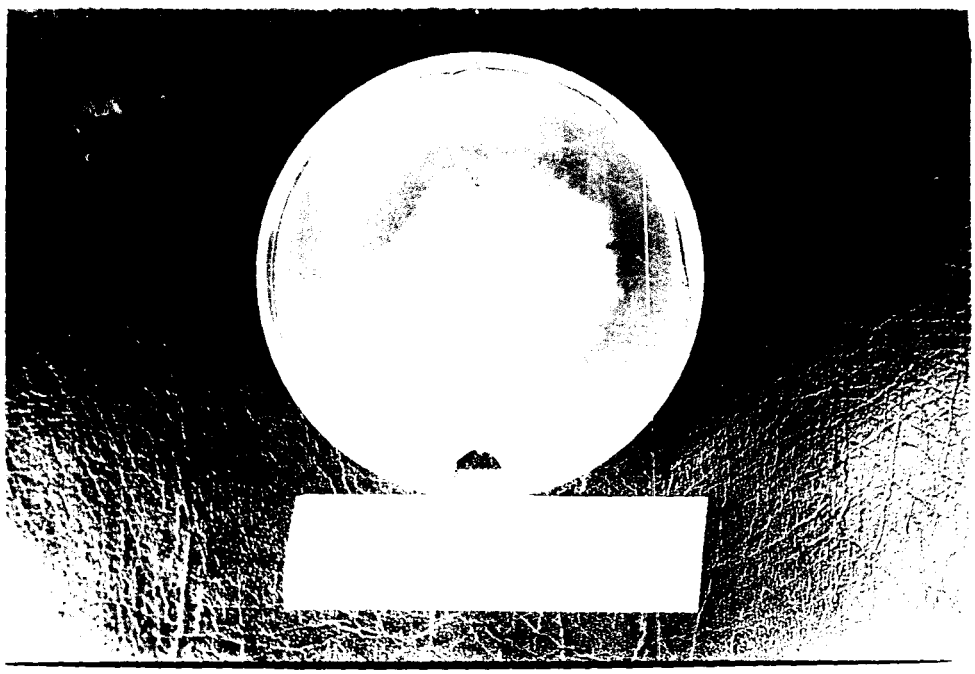


Fig. x: Growth of a fungal isolate (B501F) on minimal salt agar medium coated with crude oil.

isolates.

3.4 COMPARISON OF GROWTH BY THE FUNGAL ISOLATES ON CRUDE OIL

Table 8

Utilization of crude oil by fungal isolates at different time of incubation.

ISOLATES	GROWTH (g/l)			
	7th day	14th day	21st day	28th day
B101F	1.846	3.204	3.528	3.528
B102F	2.033	3.862	4.172	4.178
K602F	1.292	2.208	2.580	2.580
B1001F	0.708	1.889	2.275	2.334
B1002F	1.435	3.271	4.275	4.486
Control	0.194	0.200	0.194	0.198

The rate of increase in cell mass of five best biodegraders is shown above at 7 days' interval for 28 days of incubation. From the table, the optimum incubation period is 21 days. If B1002F had 100% cell growth, then B1001F had 54.33%, K602F had 60.58%, B102F had 95.59% and B101F had 82.75%. The growth of the isolates was very slow in the first 3-5 days, but the growth reached the peak value on 21st day of incubation. At 28th day of incubation, the cell growth was almost same as at 21st day of incubation (fig. xi).

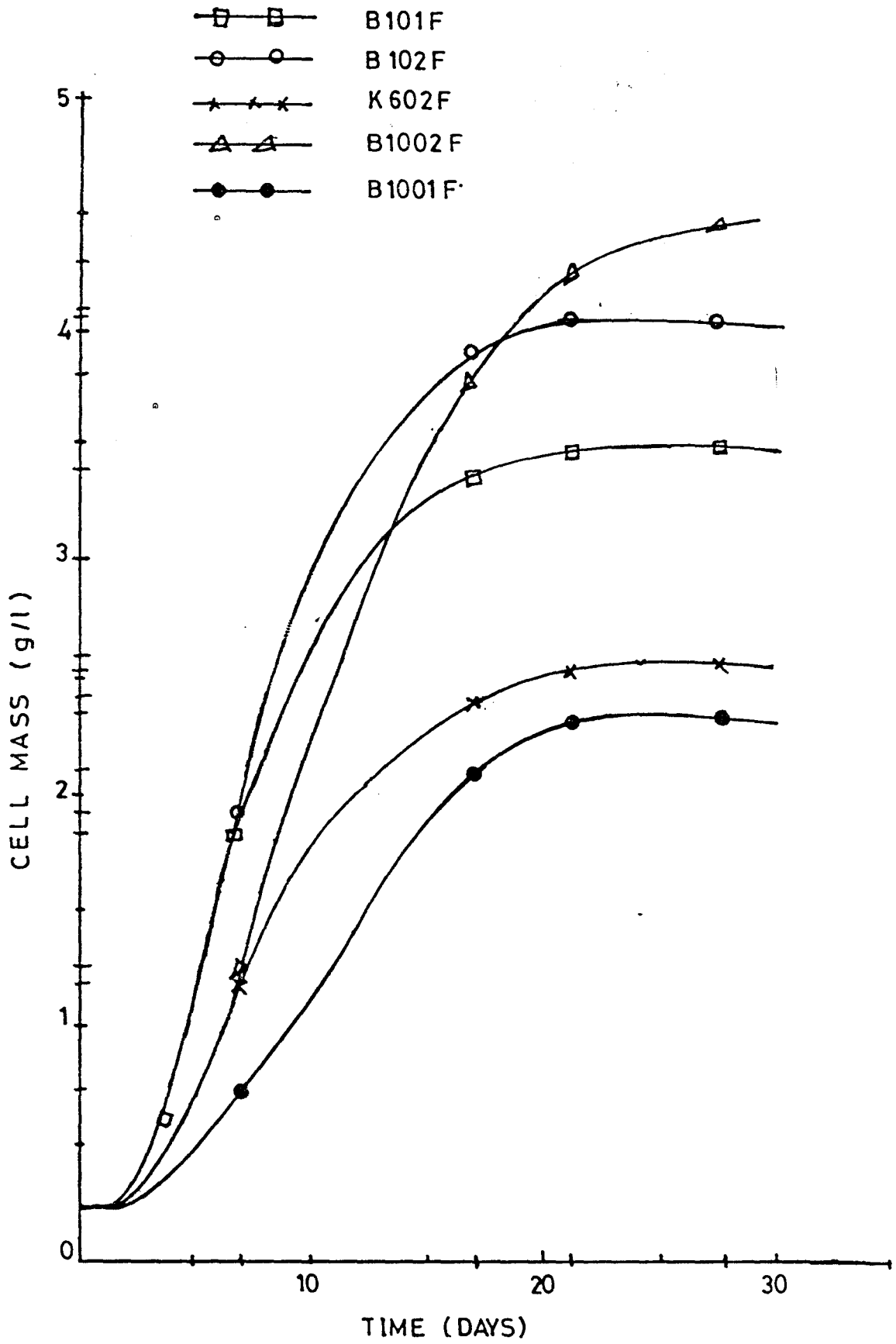


Fig. XI: RATE OF INCREASE IN CELL MASS OF FUNGAL OIL DEGRADERS DURING INCUBATION PERIOD.

3.5 COMPARISON OF GROWTH OF FUNGAL ISOLATES ON CRUDE OIL AND GLUCOSE MEDIA

Five of the fungal oil degraders were cultivated in MSLM incorporated with Lagoma light crude oil in one set, and the other set with glucose only. The cell growth (g/l) after incubation for 4 weeks at room temperature (28 ± 1 °C) is shown below)

Table 9

Comparison of growth of five fungal isolates on crude oil and glucose.

ISOLATES	GROWTH (g/l)	
	CRUDE OIL	GLUCOSE
B101F	3.429	2.575
B102F	3.918	2.945
K602F	2.780	2.400
B1001F	2.681	3.176
B1002F	4.256	3.510
Control	0.234	0.241

There is generally more growth on crude oil compared to glucose after the period of incubation. The percentage of cell growth on glucose ranged between 75 - 92% if those of crude oil were taken as 100%. (Fig. xii)

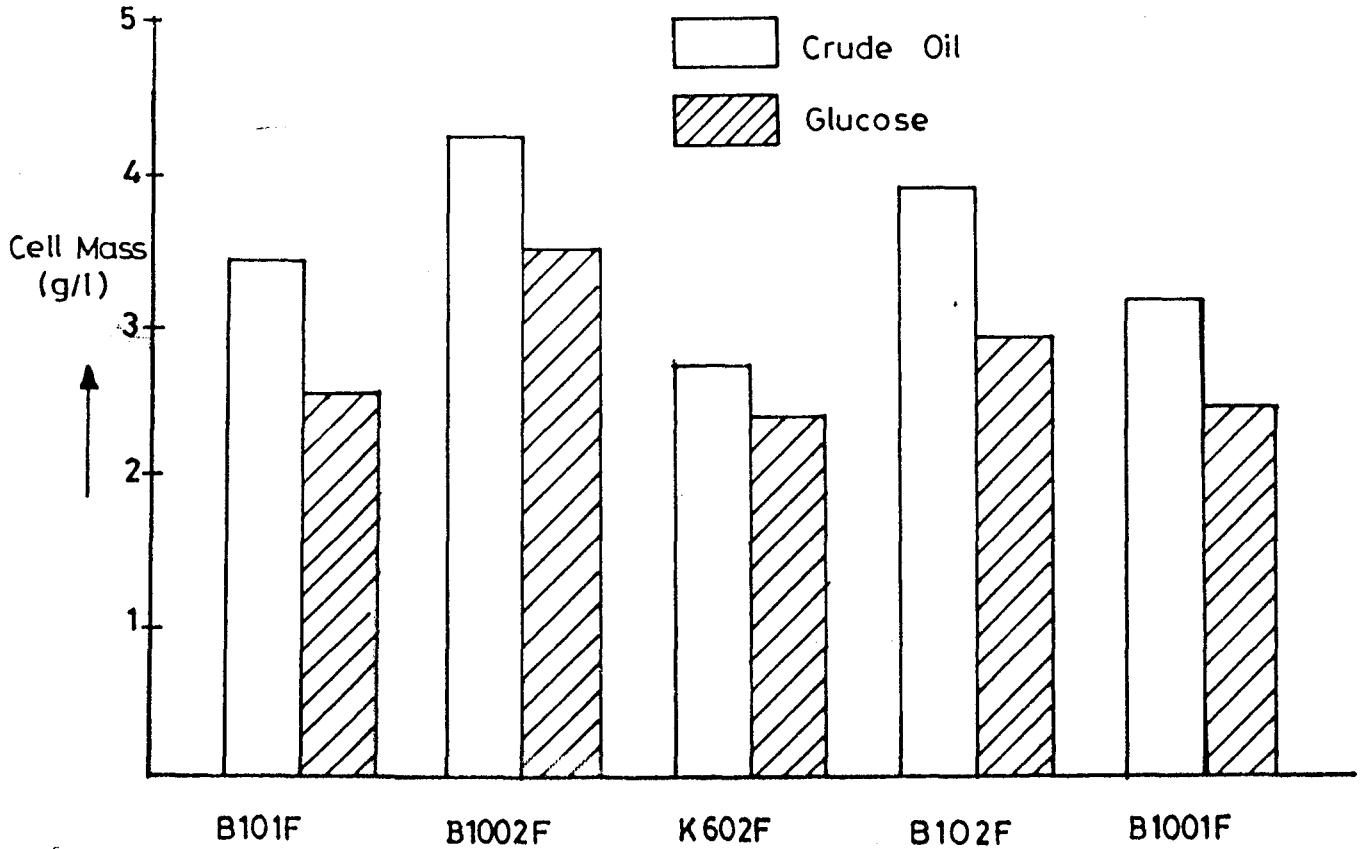


Fig. XII: CELL MASS OF GROWT OF FUNGAL ISOLATES ON CRUDE OIL COMPARED TO GLUCOSE.

3.6 EFFECT OF pH AND TEMPERATURE ON OIL DEGRADING CAPABILITY OF TWO ISOLATES (*Penicillium sp and Fusarium sp.*) (B102F and B501F)

The effect of pH and temperature on the utilization of crude oil was determined by measuring the cell growth at the end of incubation period.

Table 10

Growth of two fungal oil degraders incubated at different pHs and temperatures.

ISOLATES	GROWTH (g/l)						
	PH				TEMPERATURE(°C)		
	3.0	5.0	7.0	9.0	10	28±1	37
B102F	0.195	3.665	2.370	1.490	-	3.440	1.610
B501F	0.125	3.178	1.982	0.994	-	2,853	1.074

It is shown from the table above that the optimum conditions of pH and temperature for the utilization of crude oil by the two fungal isolates are 5.0 and 28 ± 1 °C respectively. (Fig.xii)

For pH, the two isolates utilized crude oil at all the pH media tested, but growth was least at 3.0. If growth at 5.0 is assumed to be 100%, then B102F had 64.66% at pH 7.0; had 40.65% at pH 9.0 and 5.3% at pH 3.0. While B501F sp had 62.57% at pH 7.0; had 31.28% at pH 9.0 and 3.93% at 3.0.

For temperature, none of the isolates grew at 10 °C. If growth at 28 ±1 °C is taken to be 100%, B102F had 46.8% at 37 °C while B501F had 37.64%

Generally, B102F utilized crude oil more than B501F in

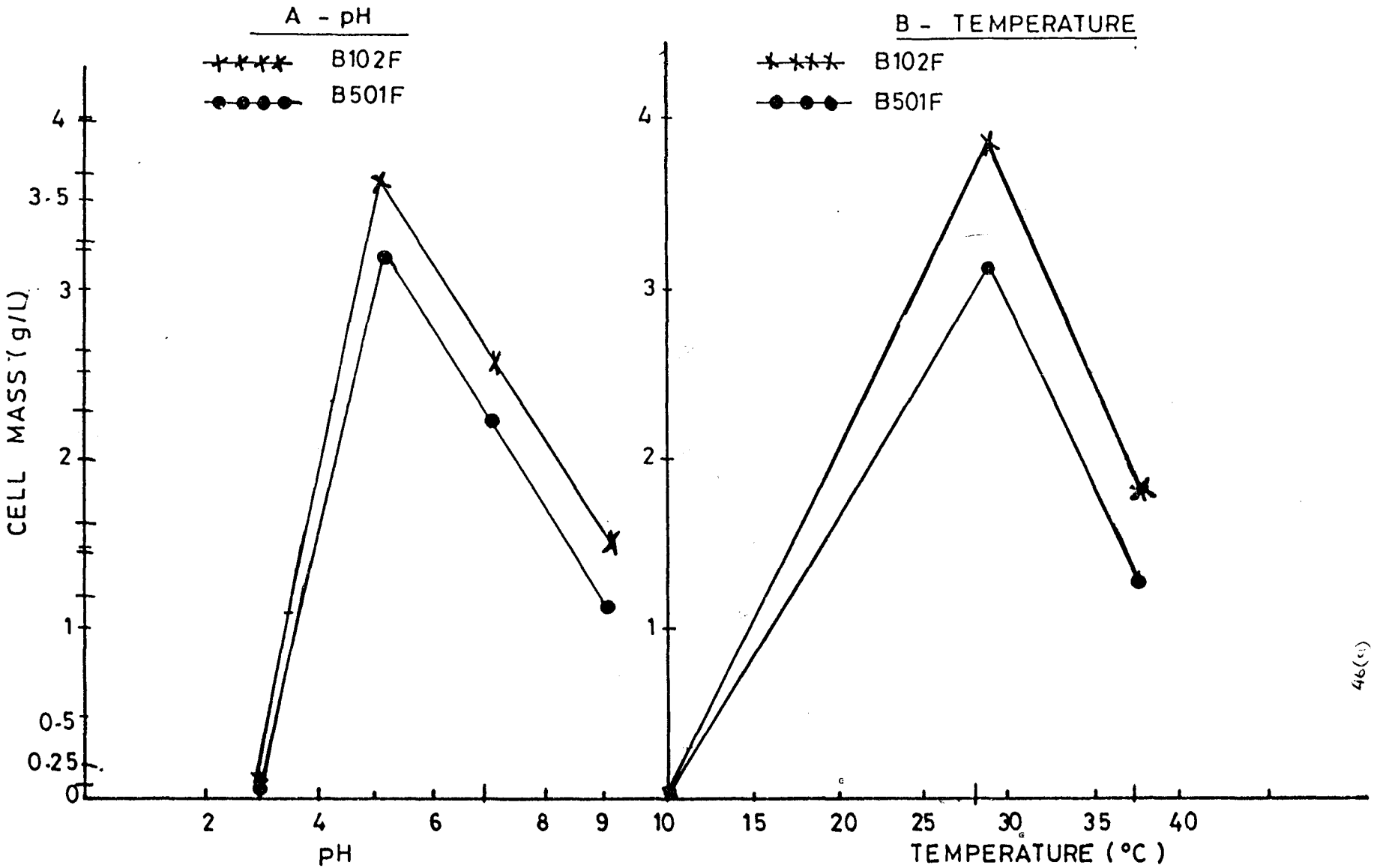


Fig. XIII: EFFECT OF PH AND TEMPERATURE ON OIL DEGRADING CAPABILITY OF TWO FUNGAL ISOLATES.

all the conditions of the experiment (Fig xiii A and B).

3.7 EFFECT OF COMPLEX NUTRIENTS ON CRUDE OIL UTILIZATION

(A) The effect of complex nutrients (serving as carbon source) on crude oil utilization by two of the fungal isolates (Penicillium sp and Mortierella sp (B101F and B1002F] is shown below with glucose and crude oil as controls.

Table 11

Effect of complex nutrients (as C-source) on crude oil utilization by two fungal oil degraders.

ISOLATES	GROWTH(g/l)			
	P	P+BN	BN	G
B101F	3.470	2.905	0.558	1.266
B1002F	3.930	3.580	0.567	1.200
	P	P+MN	MN	G
B101F	2.970	2.255	0.626	1.260
B1002F	3.290	2.690	0.630	1.231
	P	P+RN	RN	G
B101F	3.140	2.605	0.595	1.268
B1002F	3.315	2.804	0.610	1.214
Control	0.189	0.201	0.194	0.198

KEY: P-Petroleum, RN-Rice nutrient, BN-Beans nutrient, MN-Maize nutrient, G-Glucose.

From table 11, it is shown that the 1st set containing crude petroleum only had maximum growth. This is followed by the set that contained crude petroleum and complex

nutrients. The set of glucose followed, while the set containing complex nutrients as carbon source only had least growth of all the sets (Fig xiv). In the set containing glucose B101F grew more than B1002F, but the reverse was the case in the other sets.

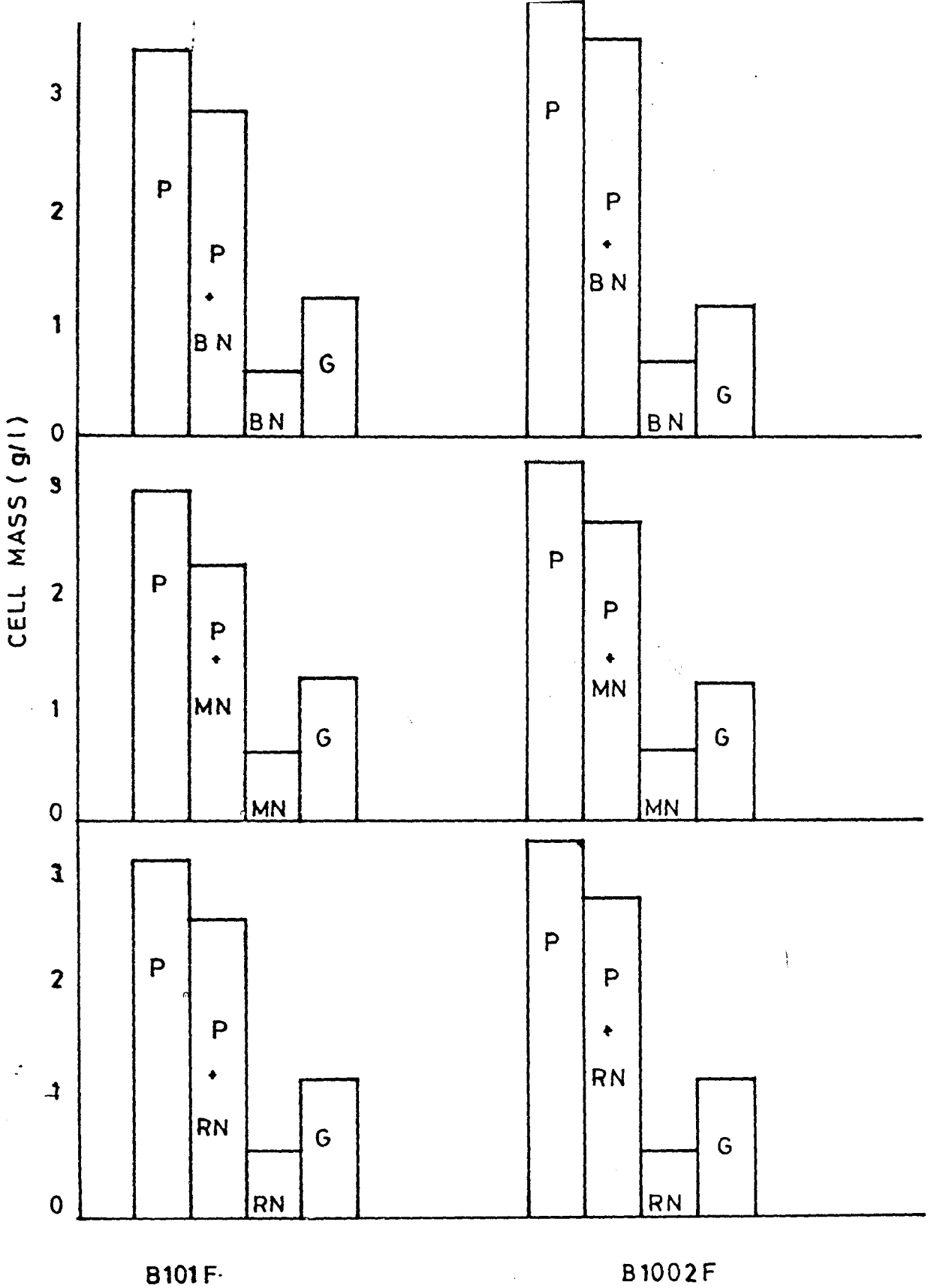


Fig. XIV: EFFECT OF COMPLEX NUTRIENTS (C-SOURCE) ON CRUDE OIL UTILIZATION.

(B) The effeect of complex nutrients (serving as Nitrogen source) on crude oil utilization by two fungal isolates (Penicillium sp (B101F) and Mortierella sp (B1002F)] is shown in the table below with crude petroleum as control.

Table 12

Effect of complex nutrients (as N-source) on crude oil utilization by two fungal isolates with maize brans as N-source.

ISOLATES	GROWTH (g/l)		
	P^-	P+MN	P^+
B101F	2.982	3.045	2.970
B1002F	3.286	3.194	3.290

KEY: P^- - Crude petroleum in MSLM without nitrogen source Compound

P+MN - Crude petroleum in MSLM with maize bran as nitrogen source

P^+ - Crude petroleum in MSLM with N-source compound

From the table, it is shown that B101F grew in all sets, but more in the medium with maize serving as N-source. For B1002F, it grew also in all sets and even more in the medium without N-source.

Generally the fungal isolates did not recognise the absence of N-source in the set up.

3.8 UTILIZATION OF OTHER PHCs BY THE ISOLATED FUNGI

(A) The growth of four fungal oil degraders on refinery effluent and crude oil was compared after the period of incubation.

Table 13

Growth of fungal isolates on refinery effluent and crude oil.

ISOLATES	GROWTH (g/l)	
	CRUDE OIL	REFINERY EFFLUENT
B101F	3.504	2.651
B1001F	2.892	2.284
K602F	2.978	2.305
B1002F	3.901	2.895
Control	0.176	0.169

The fungal isolates had between 74.21 - 79.67% growth on refinery effluent if growth on crude oil were taken to be 100%. From the table above, it is shown that growth of the isolates is proportional on crude oil and refinery effluent, i.e. the isolate that had maximum growth on crude oil also grew most on refinery effluent and the one with least growth on crude oil also had least growth on refinery effluent (Fig. xv).

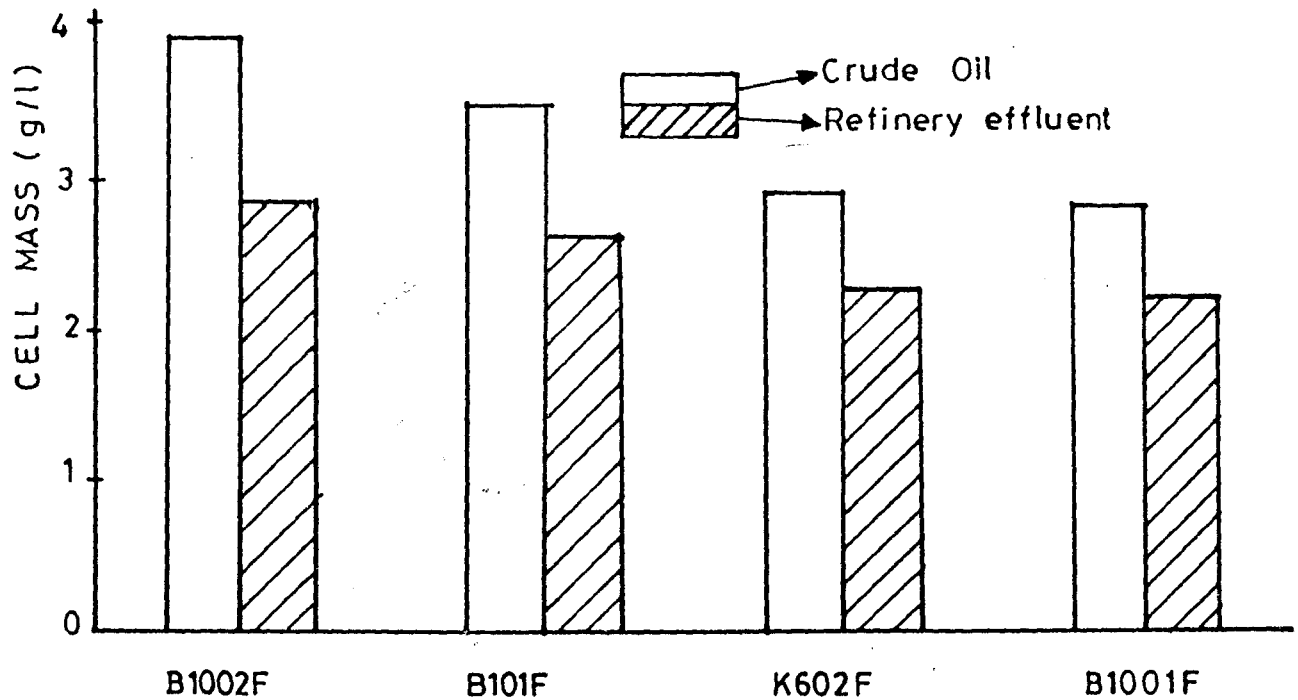


Fig. XV: UTILIZATION OF REFINERY EFFLUENT AND CRUDE OIL.

B. Table 14

Growth of Penicillium sp (B101F) and A.niger (B1001F) on Kerosine, gasoline, diesel and engine oil.

ISOLATES	GROWTH(g/l)				
	CRUDE OIL	KEROSINE	GASOLINE	DIESEL	ENGINE OIL
B101F	2.994	0.80	0.40	1.20	1.60
B1001F	3.192	0.40	0.20	1.00	1.30
Control	0.220	0.05	0.05	0.10	0.12

From the table, it can be seen that the PHCs were utilized in the order Engine oil > Diesel > Kerosine > Gasoline by both the isolates involved (Fig xvi). B101F had more growth in all the petroleum components than B1001F. Also, the growth of the isolates B101 and B1001F on the petroleum components (table 14) were generally lower than their growth on crude oil.

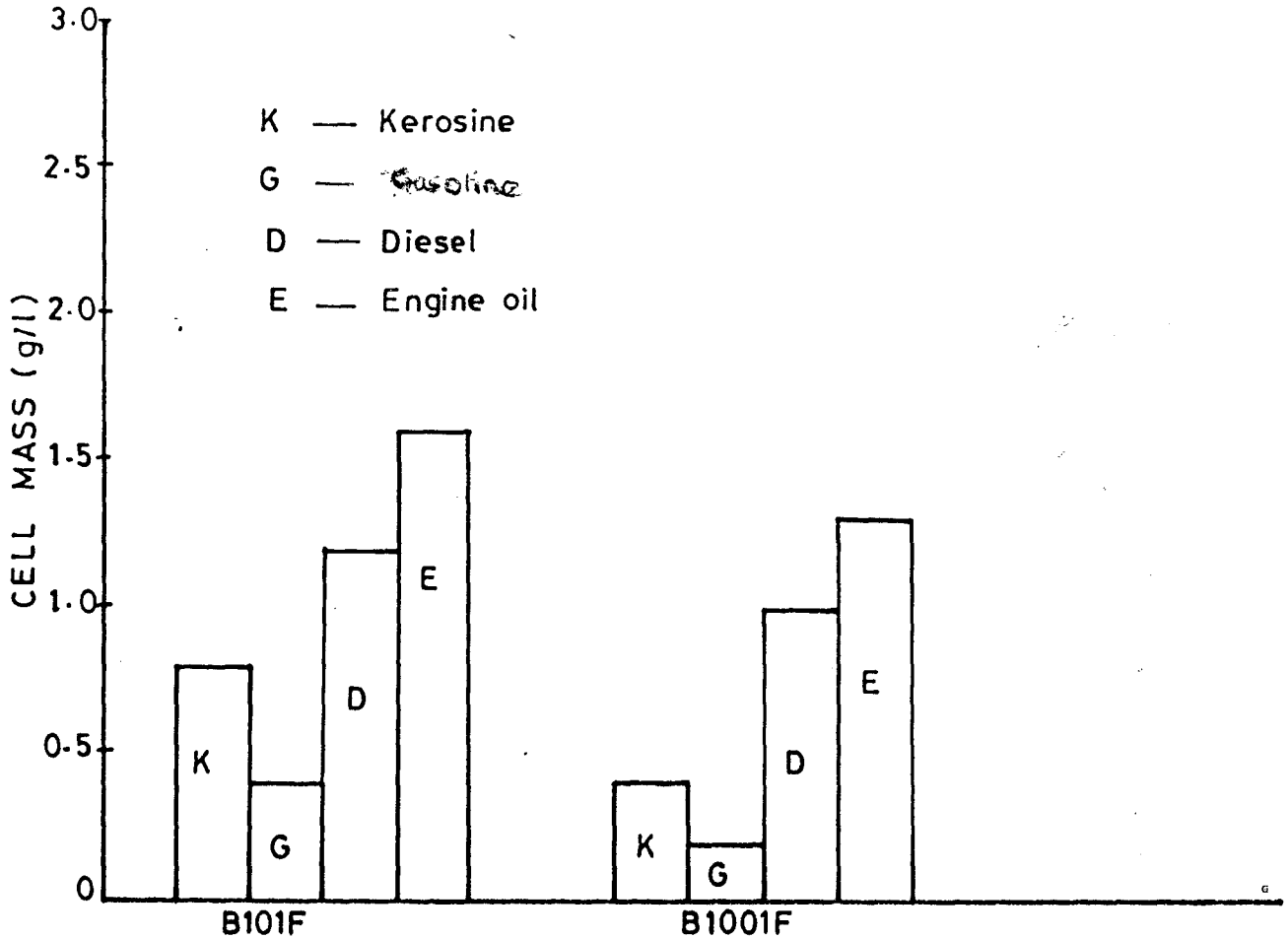


Fig.XVI: UTILIZATION OF SOME PETROLEUM COMPONENTS BY THE ISOLATED FUNGI

3.9 COMPARISON OF RATE OF GROWTH OF MORTIERELLA sp (B1002F) ON CRUDE OIL, CRUDE OIL + COMPLEX NUTRIENT, AND GLUCOSE.

The rate of growth of Mortierella (B1002F) on utilization of Crude oil, crude oil + complex nutrient (maize brans) and glucose was measured at intervals over a 28 day period. The result is shown below.

Table 15

Rate of growth of B1002F on crude oil, crude oil + maize nutrient and glucose.

MEDIA	GROWTH OF MORTIERELLA SP (g/l)					
	3rd day	5th day	7th day	14th day	21st day	28th day
Crude oil	0.80	1.31	1.84	2.64	2.79	2.82
Crude oil + nutrient	1.15	1.60	1.98	2.48	2.73	2.89
Glucose	1.35	1.90	2.26	2.55	2.53	2.31

There was immediate on set of growth in the case of glucose and complex nutrient + crude oil, but more in the case of glucose media (table 15). This gave a sharp rise in the growth curve with a shorter lag phase (fig xvii). Crude oil media has longer lag phase and the rise in growth curve was not as sharp as those of glucose and crude oil + nutrient. Growth on glucose reached the peak in between 7 - 14 days, and there after began to decline. Crude oil + complex nutrient medium's growth reached it peak at about 21st day, though there was slight increase before levelling off. While crude oil reached the peak at 21st day also, because the growth curve levelled as from the 21st day.

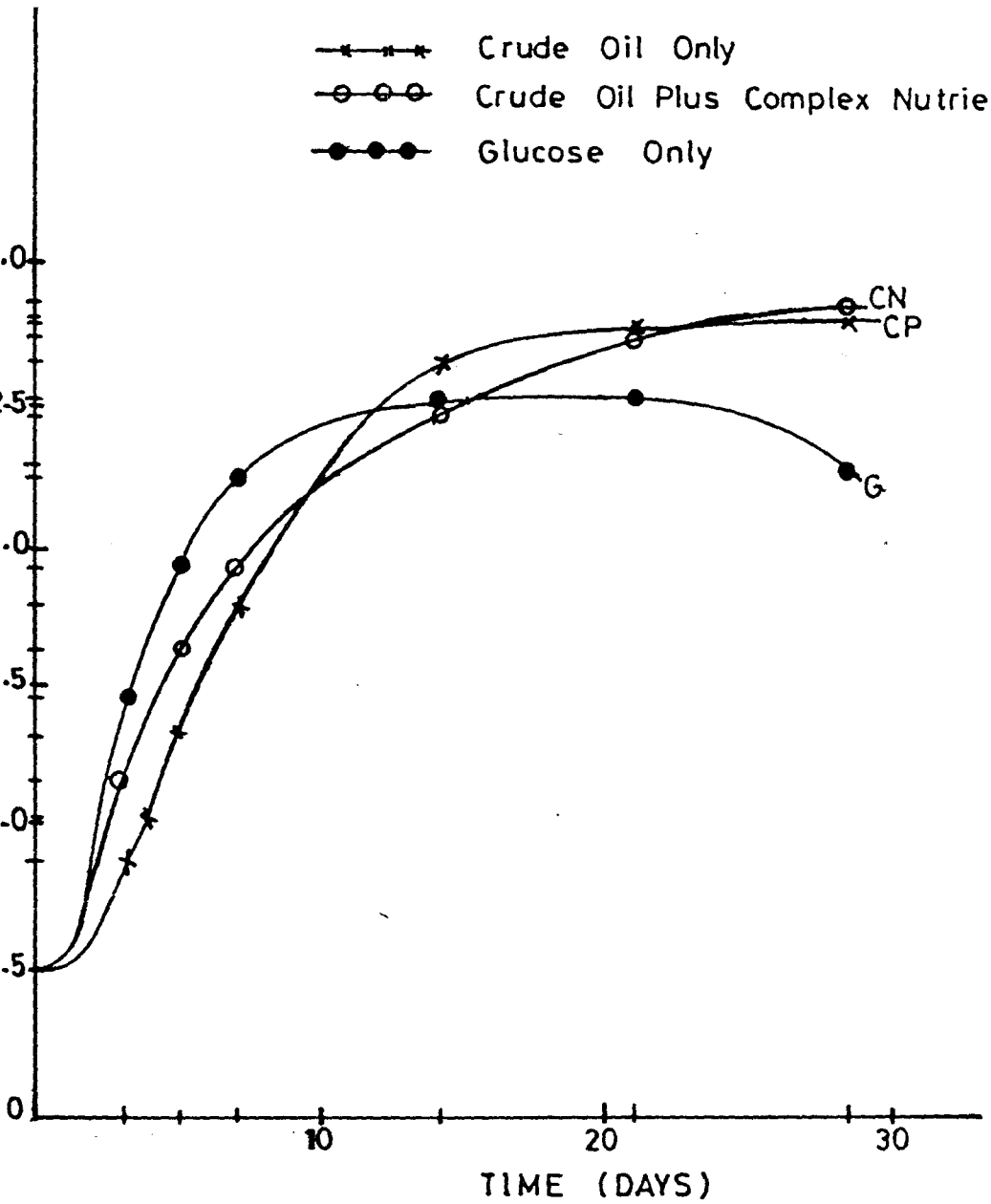


Fig. XVII: THE RATE OF GROWTH OF B1002F ON UTILIZATION OF CRUDE OIL, CRUDE OIL PLUS COMPLEX NUTRIENTS AND GLUCOSE OVER A 28 DAY PERIOD.

CHAPTER FOUR

DISCUSSION

In Nigeria, a major source of oil contamination in soil is an automechanic workshop which is fairly unnoticed. Since there are no rules and regulations concerning their locations or establishments these workshops are seen almost everywhere namely near farmland, source of water e.g. water reservoir, and also in the centre of the city where city dwellers are exposed to this pollution. As a consequence of this, the direct and indirect carcinogenic and mutagenic effects of PHC influence human and microbial populations as well as marine dwellers. In this study, an attempt was made to determine the petroleum degradation capability of those microorganisms that are forcefully or naturally adapted to the PHCs in soil.

The soil samples were collected at a depth between 15-20cm because of the bactericidal effect of sunlight, and inadequate moisture due to evaporation on the surface. Also, if any, some microorganisms might be feeding on some surface litters, those are not really PHC biodegraders. The fact that the percentage of species of bacteria (54.16%) in oil contaminated soil was higher than fungi (41.68%) is not abnormal (tables 4 and 5). Ordinarily, a free non-contaminated soil is expected to have higher population of bacterial species than fungi as reported by Alexander [1977]. Also, in this type of oil-contaminated soil, the concentration of crude oil and other PHC products is not high.

The inability of the bacterial isolates to degrade crude PHC efficiently (table 6) conforms to an earlier study by Atlas [1981] and Bossert and Bartha [1984] that fungi (table 7) are more predominant oil degraders in soil environment than bacteria. The presence of bacteria at these sites could be that they are secondary degraders, and feeding on the products of the activities of primary degraders, which may be the fungi.

Not many studies are avaialble on fungal oil degradation which makes this work very important since current emphasis on integrated oil pollution abatement is microbial seeding, or land farming [Bossert and Bartha, 1984]. In this study, the optimal concentration of Lagoma light crude oil in MSLM for fungal degradtion was 0.5%. This is in contrast to a report by Bossert and Bartha [1984] that stimulation of microbial activity is enhanced up to 5% level of hydrocarbon (though in soil environment). Above 0.5% of crude oil in MSLM there was reduction in growth up to 2.5% of crude oil, and there was no growth for all the isolates at 5% crude PHC concentration (table 7). The increase in concentration of crude PHC likely interfered with medium aeration, hence degradation could not occur because it is an oxidative process. Also at higher concentration the PHC could be toxic to the microorganisms. Below 0.5% concentration of crude PHC, the reduction in cell growth might have been attributed to the amount of substrate available. The two species of Penicillium (B101F and

B102F) exhibited same amount of growth, but in case of Aspergillus strains, B1001F exhibited a superior ability in degrading crude PHC than K101F (table 7).

The five best fungal oil degraders had different rates of crude PHC utilization which yielded different cell mass after optimum incubation period. Out of the five biodegraders, B1002F had best PHC utilization of 100% and B1001F had least of 54.33%. Despite the differences in cell mass, all the fungal isolates had same optimum period of incubation of 21 days. The increase in cell mass after 21st day of incubation was negligible for all the isolates (fig xi). Their growth reached peak value on 21st day of incubation.

When utilization of crude PHC and glucose by the isolated fungi was compared (fig xii), it was found that crude PHC yielded more cell mass than glucose at the end of incubation period. This may be that glucose being a readily utilizable substrate was attacked and used up immeditely, and after sometimes the cells started lysis resulting into lower cell mass at end of incubation period of 21 days. For PHC, utilization was not instant because there was the need for the isolates to adapt, hence it was not exhausted immediately and was available for cell growth till optimum incubation period.

pH was found to have drastic effect on crude oil degradation. Dibble and Bartha [1979] have reported low crude PHC degradation by bacteria in acidic soil. Hambrick

et al. and Antai [1990] also reported low crude PHC degradation by bacterial isolates at acidic pH. Here, all the soils were of acidic region (table 3), so the indigenous bacteria may not be adapted to using crude PHC as carbon and energy source in acidic soil as efficiently as fungi. This may be one of the reasons why the bacteria could not use crude PHC at higher concentration efficiently like fungi.

The fungal isolates utilized crude PHC at all pHs tested with maximum at pH 5.0. There was more growth at pH 7.0 (neutral) compared to that obtained at pH 9.0 and pH 3.0 (fig xiii A) with 3.0 yielding least growth. This supports earlier observations of Bossert and Bartha [1984] and Dibble and Bartha [1979] that fungi degrade oil most efficiently in acidic medium. However, they can also degrade oil in non-acidic medium (up to pH 9.0) but not efficiently. *Fusarium* sp. (B501F) which utilized crude PHC poorly (table 7) was used in this test to see if change in pH could enhance its oil degradation capability. Though, pH 5.0 had remained the optimum condition, hence no effect on change of pH for *Fusarium* sp.

Another parameter tested and found to affect microbial oil degradation is temperature (fig xii B). At low temperatures ($10^{o}C$) fungal degradative capability was arrested. Optimum growth was obtained at room temperature ($28 + 1^{o}C$) and higher above that oil degrading capabilities reduced, e.g. at $37^{o}C$ cell growth was low. This is in line

with acclamed work of Dibble and Bartha [1979] and Bossert and Bartha [1984] that best condition of temperature for maximum fungal activity is the mesophilic range.

Complex nutrients (materials of natural origin) generally contain precursors, vitamins, etc which can promote microbial growth when they are used with other carbon and energy sources. In this study, rice bran, maize bran, bean lusks were used with crude PHC in MSLM. It has been shown (table 15) that when crude oil with waste materials used as carbon source, the growth of isolates were almost the same as of crude petroleum used only as carbon source, and glucose only. From tables 11 & 15 and fig xvii it can be concluded that the growth pattern of the isolates is almost the same, except the difference in lag phases. When complex nutrients was used as nitrogen source replacing $NaNO_3$ (table 12), it was found that the growth was almost same in the three cases where there was no $NaNO_3$ where there was $NaNO_3$; and where complex nutrient replaced $NaNO_3$. Lack of mineral elements eg Nitrogen, sulphur, phosphorous is said to be a limitation in PHC biodegradation [Atlas 1981, Bossert and Bartha, 1984 and Antai, 1990), but crude PHC contains small amount of nitrogen -, oxygen - and sulphur containing compounds [Gutnick and Rosenberg, 1977 and Bartha, 1986]. Therefore, a larger amount of crude PHC may be required to detect the limitation of nitrogen.

From fig xv, refinery effluent, a waste product of petroleum refining yielded less growth of the fungal

isolates compared to crude PHC. This agrees with Bartha and Bossert [1984] that refinery effluent contain chemical compounds which are toxic to microorganims, and therefore, affected their growth. Or the amount of utilizable carbon substrate was not as to support enough growth as crude PHC.

The petroleum components - gasoline, kerosine, diesel and engine oil gave less growth compared to crude PHC (fig xvi). The extreme difference in cell mass might be due to volatility of the components. They are lighter compounds and might have escaped in gaseous form into the atmosphere thereby making them unavailable to the microorganisms. The heavier ones had more growth than the lighter ones. This confirms the work of Bossert and Bartha [1984] and Singer and Finnerty [1984]

CHAPTER FIVE

SUMMARY AND CONCLUSION

For the purpose of identification the isolated microorganisms were coded in the laboratory. Fungal isolates exhibited higher efficient utilization of the Lagoma light crude oil than the bacterial isolates, though the bacterial isolates were more in number (54.16% and 41.68% respectively). The use of bacterial isolates for further studies was discontinued in respect of their poor utilization capabilities of the petroleum hydrocarbon (PHC).

The optimum concentration of the crude oil in Minimal Salt Medium (MSM) for fungal growth was at 0.5%, while the optimum incubation period was 21 days. In terms of pH and temperature, the fungal isolates required pH 5.0 and 28 ± 1^{o} C respectively for optimum growth.

Complex nutrient introduced into the medium as carbon source influenced crude oil biodegradation, but when it was used as nitrogen source there was no any effect. The growth pattern of the isolates on crude oil, crude oil plus complex nutrient, and glucose in MSM was the same, except for lag phases which was different for each medium.

All the isolated fungi that utilized crude oil also had the capability of utilizing other PHCs - refinery effluent, gasoline, kerosine, diesel and engine oil.

In conclusion, the following suggestions are recommended:

- Government should set aside special sites for automechanics and efforts be made to contain the waste oil (used in these places); and arrangements be made for the proper disposal of these wastes.
- Studies should be undertaken to see the effect of these pollutants around the sites, eg Poultry, crops, aquatic inhabitants and inverterbrates near automechanic workshops.
- Fungi isolated from these workshops may be used when dealing with oil pollution on land (soil)
- To arrest oil pollution biologically, a cheap and more readily utilizable substrates may be introduced to increase the cell mass of oil utilizing organisms instantly to in turn attack the PHC.
- A study be undertaken to improve genetically the oil degrading capability of these oil utilizing microorganisms.

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