BIODEGIUDATION OF REFINERY EFFLUENT BY *Glcophylum sepiratus* AND *Pleurotus ostreatus.*

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BEING A THESIS SUBMITTED TO THE POST-GRADUATE SCHOOL OF FEDERAL UNIVERSITY OF TECHNOLOGY. MINNA. IN PARTIAL FULFILMENT OF THE ~QUIREMENT FOR THE AWARD OF DOCTOR OF PHYLOSOPHY (Ph. D) DEGREE IN APPLIED (ENVIRONMENTAL) MICROBIOLOGY.

CERTIFICATION

This thesis titled "Biodegradation of Refinery Effluent by Gleophylwn sepiratus and Pleurotus ostreatus" was examined and found to meet the regulations governing the award of the Doctor of Philosophy of the Federal University of Technology; Minna. and is approved for its contribution to knowledge and literary presentation.

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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, and the information derived from all literatures have been acknowledged in the references.

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(Solomon B.O. Oyeleke)

DEDICATION.

This study is dedicated to the almighty God who alone has the power and key to success.

[have also dedicate it to the blessed memory of my late wife (Mrs. Dorcas Adeyiola Oyeleke) who laboured with me but could not wait to eat of the fruit of her labour. May her soul rest in the bossom of om Saviour.

Also dedicated to my wife (Mrs. Victoria Bola Oyeleke) and children (Oluwabunmi, Oluwaseyi, Oluwasegun and Oluwakemi) who supported me financially and prayerfully.

I finally dedicate it to my father (Mr. James B. Oyeleke) who had always been my source of inspiration and encouragement.

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ABSTRACT.

The biodegrading capability of Gleophylum separaWs and Pleurotus ostreatus isolated [rom decayed wood and sawdust on refinery effluent was determined in effluent incorporated minimal salt medium by using the mycelial extension rate measurement method and compared with that of other known degraders, namely Aspergillus niger, Penicillium and Fusarium species. The mean mycelial growth rate/day for Gleophylum sepiratus and Pleurotus ostreatus over 18 days' of fermentation was comparable to the values obtained for A. niger, Penicillin and Fusarium species over 12 days of fermentation. The optimum concentration of efffhent (as carbon source), NH_4N0_3 (as nitrogen source) and KH2P04K2HP04 (as phosphate source) for Gleophylum sepiratus and Plellrotus ostreatus were 10%(ml), 0.098%(g) and 0.1 0/0. 12%(g) respectively in the minimal salt medium. The growth of the isolates was significantly higher in sawdust and effluent incorporated minimal salt medium (43.5 *gll* and 36.6 g/l) for Gleophylum sepiratus and Pleurotus ostreatlls compared to that obtained in only effluent incorporated minimal salt medium (33.5 g/I and 19.8 !.!,/l). The total bacterial count for the soil samples, namely loamy, clay and sandy soils isolated from the discharged sit were 2.5xlO!1, 2.3xl07 and 4.8x10s cfu/g . Whereas the respective values or the fungal count were 3.3xl06, 5.1xl05 and 4.0x105 cfu/g. The mean percentage values of mission of carbon dioxide in three effluent contaminated samples were higher than those of ffluent contaminated nonsterile soil samples for Gleophylum sepiratus and PlelU'otus ostreatus. he growth of Gleophylum sepiratus in crude oil and other petroleum products was insignificant en compared to the growth obtained in case of effluent. G. sepiratlls utilised phenol and cyanide minimal salt medium whereas .e. ostreatus could not degrade them even after a long period of entation. Recycling experiments showed that spent effluent could be used as substrate for

fermentation by G. sepiratus in minimal salt medium. Because of high protein cont nt (24%), G. sepiratus was quantitatively incorporated into animal feed. In the test animals, gained weight after a period of 12 weeks (42% weight increment for tested animals and 17% weight increase for the control animals). The survival rate was 100%. Hence these slow degraders, also reported to be lignocellulose degraders, may be u ed to degrade recalcitrant substances in the refinery effluent. Biodegradation using G. sepiratus and ;e. ostreatus with other flora may become an integral part of the total strategy of treating oil-polluted aTeas. It is also worthy to do further study on possible inclusion ofGleophylum sepiratus as a source of protein in animal feed.

TABLE OF CONTENT

LIST OF TABLES

LIST OF PLATES.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW.

Starting from crude oil brought by pipeline or crude oil tankers, an oil refinery manufactures gas, gasoline, kerosene gas oil and fuel oil in the proportions and the quantities required by industrialised society.

The more complex refineries can also produce lubricating oil, wax and asphaltic bitumen. It is a known phenomenon that the refinery industries in oil producing countries such as Kuwait, Nigeria, Libya and the United Kingdom are undergoing a period of considerable growth in the twentieth century. But at the same time it is also taking into account that a side effect of such a growth could increase pollution of the environment and more particularly of surface waters. A refinery unit can cause pollution by discharging refinery effluent, cooling water, ballast water and rainwater to the soil and aquatic environment. Generally a typical refinery effluent contains low levels of a very large number of contaminants. Oil is of course the main contaminant and not only represents the complex mixture of hydrocarbons and other organic compounds present in the crude oil, but also includes those substances produced by the refinery process.

In addition to oil, other substances frequently present are sulfides, mercaptans, cyanides, ammonia, phenols and inorganic salts. Traces of certain heavy metals may also be present. Product tankers arriving at the refinery in ballast usually discharge their ballast water containing dispensed oil, which can be skimmed off in reception tanks. The rain water runoff from paved areas in the product loading stations generally contain some oil due to small oil spillages. During refinery, the wastewater [rom a cooling tower is not normally contaminated with oil but it may contain low concentrations of

conditioning chemicals. Trace quantities of some compounds including oil and metal are known to disrupt metabolic processes, development, morphology and growth of aquatic organisms (Cote, 1976; Sprague *et al.,* 1978; Rowe et aI., 1983a, 1983b: Westlake *et al.,* 1983a, 1983b; Onwumere and Oladimeji, 1990). A hundred percent mortality was recorded in rainbow trout exposed to three Canadian refinery effluents (Pessah *et al.,* 1973). The water soluble fraction of crude oil has an anaesthetic effect on the cilia of gill epithelium of shellfish and cause a decrease in the pumping rate of the gills (Galsof *et al.,* 1935). Sprague *et al.,* (1978) reported an increase in the respiratory rate and coughing in rainbow trout exposed to treated refinery effluent. Cote, (1976) and Rowe *et al.,* (1983) reported that source component of refinery effluent may accumulate in fish posing a serious health hazard to human. Thus an oil refinery can be a great source (If health hazard if we do not take proper care of waste disposal associated with oil refinery.

A modern industrialised society should concentrate on improvement of old refineries as well as establishment of new ones to fulfil the ongoing demand but at the same time it should also develop different suitable disposal methods of refinery wastes for a healthy environment.

1.1 Characteristics of refinery effluent

A typical refinery effluent may consist of petroleum hydrocarbons (PHC)-designated as oils in general, phenols, nitrogen compounds, organic and inorganic sulphur compounds, cyanides and heavy metals.

The hydrocarbons, which mainly occur as mixtures, represent such a large number of chemically defined individual substances that it has still scarcely been possible to isolate or determine all of them, despite enormous expenditure on analysis.

Therefore in practice PHC in refinery effluent generally of the following chemical classes: alkanes, cycloalkanes, aromatics and polycyclic aromatics. The phenols are hydroxyl compounds in which one or more OH groups are bound directly to the benzene ring and they are designated as 1110110-, di- and trihydric phenols. The monohydric phenols are volatile with steam but the polyhydric phenols are not. It has been shown that the phenols that are volatile with steam predominate in refinery effluent (Sprague *et al.,* 1978). From the various refinery processes, ammonia is obtained exclusively, generally in a mixture with hydrogen sulphide. Free ammonia (NH_3) and undissociated ammonium hydroxide (NH_4OH) are relatively toxic whereas the dissociated NH_4^+ has relatively low toxicity. This property explains why refineries employ stripping of acid waters with the addition of acid, resulting in fixation of the ammonia to ammonium. After the hydrocarbons, the sulfur compounds are the second most abundant constituent of petroleum. In refinery waste waters, predominately only sulfides (mercaptans, thioethers and disulfides) and hydrogen sulfides may be present. The major proportion of the cyanide arises in refinery effluent during the pyrolysis of longer chain hydrocarbons.

In cracking processes, the weakness of hydrogen cyanide as an acid is of importance to the extent that in the pH ranges occurring in natural waters, it is largely present in the molecular and therefore more toxic form. For example, 93% is present as HCN at a pH value of 8.1 at 25°C, whereas the figure is only 50% at pH 9.5. If heavy metals (Vanadium, nickel) occur in refinery wastewaters they are for the most part boun I (o sulfides of low solubility, or other solids. and can be readily separated. This is confirmed by their content in refinery effluents, with concentrations (excluding iron) between 0.5 and 1000ppb, so that heavy metals should not be described as contributing to the specific environmental impact of refineries.

The oxygen demand (00) of an effluent gives an idea of the oxygen depleting stress impose on a receiving water. In refinery effluent the main pollutants exerting an 00 are dissolved organics, ammonium salts and sulphides. Submitting the wastewater streams to various treatments can reduce the 00.

1.2 Fate of refinery eftluent discharged in the sea

Physical and chemical changes in oil or waste discharged into the sea have been reported (Lee,1977). Physical processes of evaporation, dispersion, solution adsorption and sinking redistribute the oil relatively quickly. Lee (1977) studied the adsorption of HCs onto particles. Radio labelled compound and estuaries detritus particles composed of a mixture of clay, organic matter, plankton remains and living microbes was used. These particles are likely to sink sooner or later to the bottom of the sea, where the rate of He degradation will depend not only on the hydrocarbon type but also on the local physical and chemical conditions.

Degradation of oils occur by chemical oxidation (especially under the influence of ultra violet light) and by biological processes. This processes may affect both petroleum and oils of natural origin. Photochemical degradation products include toxic compounds, which are relatively water soluble and will loam mainly at or near the surface of the sea while undergoing degradation (Larson *et al., 1976).*

1.3 Fate of refinery effluent discharged in the soil

Ilydrocarbons are naturally occurring organic biological compounds hence the ability to utilize them by microbes is a surprise as nature has a way of making the environment safe. The relative abundance of organic substrates in the soil environment is a crucial factor that favours microbial abundance and diversity (Alexander, 1977; Atlas and Bartha, 1981). The high microbial biomass, the great microbial diversity and the abundant representation of bacterial and fungal genera capable of degrading or metabolising hydrocarbons render the soil a relatively favourable environment for petroleum or refinery effluent biodegradation (Bosssert and Bartha, 1984).

The heterogeneous microbiota of most contaminated soils include naturally occurring hydrocarbon degrading population (Perry and ScheId 1968; Odu, 1978; Pinholt *et al.,* 1979) and this inherent characteristic imparts a large hydrocarbon assimilatory potential to most soils. The addition of petroleum effluent to soils therefore selectively enriches that sector of the microbial community able to adapt and utilise the new substrate.]t increases the organic matter total carbon and nitrogen compared to uncontaminated soil (Elhs and Adam 1961; Adams, 196]; Odu, 1972; Jobson *et al., 1979).*

Bacteria and fungi are the principal agents of petroleum degradation in soi l. Though the relative contribution of each group is not clear (Anderson and Domsch, 1975), but bacteria tends to respond more rapidly to oil contamination of soil, whereas fungi may be inhibited initially (Pinholt *el al.,* 1979). Conversely, the activity of fungi tend to persist long after bacterial activity has declined (Jensen, 1975); Obire (1988) reported the degradation of three different crude oils in Nigeria by species of *Pseudomonas fluorescens* and *Bacillus subtilis* ; the three rude oils used were Bony light, Cados blend and Bony medium. The degradation of crude oil by microorganisms depend on the

chemical composition of the oil, nature of the habitat and other environmental factors (Jobson *et al.,* 1972; Atlas, 1981).

1.4 Impact of refinery effluent on the Aquatic Environment

Refinery effluent may conceivably cause changes in discharge areas through:

(i) Direct toxic effects on flora and fauna leading to reduction in numbers, lower growth rates or changes in other variables.

(ii) Avoidance behaviour of some species which may include fish of commercial importance.

(iii) Tainting of some species, which may include fish and shellfish of commercial importance.

(iv) Other factors such as reduction in dissolved oxygen or eutrophication (Murphy *et (ll.,* 1975; Ramband *et at.,* 1975; Dicks, 1976; Cote, 1976; Hiscock, 1976)

The discharge of unchanged petroleum hydrocarbon (PHC) in faeces have being recorded for several planktonic and shore organisms. Hydrocarbon may also be stored in the liver of marine fish and in the hepatopancreas of several invertebrates (Lee and Benson, 1973). This results in the bioaccumulation and bio-rnagnification of these substances.

Bioaccumulation IS the ability of organisms to concentrate substances from the environment. Examples of organisms that accumulate petroleum hydrocarbon are many especially in the estuarine or marine environment. The release time are different for different hydrocarbons and organisms even though it appears that polynuclear components are released relatively slowly. Anderson and Neil, (1974) worked on discharges of oil hydrocarbons from oysters, which had been exposed to 400 ppm,

dispersed No.2 fuel oil (light gas oil) [or eight hours. At the end or the exposure, the oysters had accumulated 312 ppm oil hydrocarbons in their tissues. When the oysters were returned to clean sea water; more than 90% of the Nsalkanes were discharged in 24 hrs; although the aromatic components were released much more slowly. After 28 days, only small amounts of mono-di- and trimethylnaphthalenes remained in the oyster tissues (Baker, 1979)

Biomagnification is the increase in concentration of a given material at successive states in a food chain i.e. the predatory organism will have a higher concentration in the tissues of the material in question than those organisms upon which it feeds.

This has been demonstrated in terrestrial environment for organochlorine pesticide such as dieldrin and DDT. To date, there is no convincing evidence for the food chain biomagnification of petroleum hydrocarbon (GESAMP, 1977) though there may in some cases, be concentration from sea water to organisms by direct uptake.

Varanasi and Matins (1977) reviewed the metabolism of ingested hydrocarbons by rnanuc organisms. The aromatic hydrocarbon hydroxylase (AHH) enzyme system is known to be induced in fish by environmental sources or polycyclic aromatic hydrocarbons but AHH enzyme activities are generally low and/or uninducible in marine invertebrates (Payne *et (11.,* 1979). Hence, relatively long period or time, may be required for complete removal of such hydrocarbon from many marine invertebrate species. Sabon and Maline (1977), provided evidence that marine crustaceans do not readily degrade some metabolites of aromatic hydrocarbons. The carcinogenicity of several polycyclic aromatic hydrocarbons is thought to be due to metabolic activation by the target organism. Thus, mammals incorporated one atom of molecular oxygen into these compounds to form arene oxides, and this process also occurs in fish and large

crustaceans such as crabs (Varanasi and Matins, 1977). Arene oxides are electrophiles that can react with cellular constituents (Baker, 1979). Phenolic compounds are produced through thermal and catalytic cracking of crude oil. Biodegradation of phenol appears to occur easily. Lee (1977) released radio-labelled phenol into an estuary in the so Litheast United States and found that it was rapidly degraded to carbon dioxide. Degradation may be by a range of bacteria and fungi. Portman (1975), states that phenol is degradable in sewage Works but other phenolic compounds such as trihydric phenols are not necessarily d graded easily. Ristanovic *et of* (1975) isolated marine degrading fungi from the Adriatic sea.

Toxicity of phenol to aquatic life have been reported within the range of I-*100mg/l* depending on the species, temperature and salinity. Petpiroon (1976), tested the effect or concentrations of phenol in sea water solution and found that higher dose (i.e. 75, 50, 30 ppm) has an obvious effect on the wrinkle *Littoria seratiliss* (using 2 hours static test). With lower concentration the effect did not differ significantly [rom the control. The U.S. environmental protection agency (USEPA, 1976), has a standard for water quality criterion I)lg/I phenol for domestic water supplies and to protect against fish tainting. No criterion is given for sea water. The toxic effect of sulphide is influenced by pl I (Cote, 1976). In cases of low pH effluent, the sulphide may become undissociated hydrogen sulphide and the toxicity increases. The main effect of hydrogen sulphide at low concentration is believed to be inhibition of the iron containing oxidative enzymes (Smith and Gosselin, 1966; Coldwell, 1975). Petpiroon (1976), examined six species of marine invertebrates for the effect of dissolved hydrogen sulphide in sea water for four days, they found that the activity of the invertebrates was lowered than the control. EPA water quality standard for hydrogen sulphide is 2).1g/1for fresh water and sea water.

Cyanides hydrolyse in water, forming toxic hydrocyanic acid. Hydrolysis and therefore toxicity increases with a decrease of pH (Cairns *et al.* 1975). Cyanide level of 0.06 mg/l is reported to be lethal to trout in five days in fresh water environment (Mckee and Wolf, 1963). Free ammonia (NH_3) and undissociated ammonium hydroxide (NH_4OH) are relatively toxic. The dissociation ammonium ion NH_4 has relatively low toxicity. High temperatures lead to greater proportions of un-ionised ammonia, and therefore an increase of toxicity (Cairns *et al.,* 1975). The lowest lethal concentration of un-ionised ammonia is 0.2 ppm for young fish - *Salmo gairdneri* (Cote 1976; Wickins, 1976; Hampson 1977). The EPA water quality criterion is 0.02 mg/l of undissociated ammonia for fresh water. Huber (1977) has pointed out that dissolved heavy metals do not exist in water in purely ionic form but as hydroxy, oxyhalogen or other complexes with strong pH dependence. The toxicity of heavy metals is affected by temperature though not consistently - according to Cairns *et al. ()* 975), mercury compounds are more acutely toxic to fresh water fish at high temperature but low temperature accentuated the toxicity of mercury chloride to fiddler crabs *(Uca pugilatori.* Heavy metals are of concern because some, notably mercury, are toxic at very low concentration and bioaccumulate along the food chain (Davies *et al.1976).* Concentration of 4ppb of inorganic mercury in water are lethal to some fish and 0.4 has been reported to kill 60% of Daphnia in 64 hours. Photosynthesis in marine diatoms has been inhibited by as little as 0.1 ppb of some organic mercury compounds (Wat r Pollution Research Laboratory, 197]) . Jernelov (1975) has reported acute toxicity in methyl mercury concentrations in the tissues of 20 pg/g. I-Ie further pointed out that methyl mercury in fish is bound to protein and is not dissolved in the fat as are chlorinated hydrocarbons. Therefore, the mercury content of fish is not very fat dependent but is more related to trophic level, size and age of fish.

Lower form of aquatic life are sensitive 10 chromium, and it can accumulate at all trophic levels (EPA 1972). Oshida (1977) reported tl at trivalent chromium was not toxic to the marine polychaete worm Neanthes arenaceodentata, but that at concentration of 12.5-50~Lg/1 hexavalent chromium began to cause detrimental effects on reproduction. Vanadium is known to have very high accumulation factors from sea water to marine organisms. The extent of man's impact on vanadium concentration in marine organisms is unknown an I so are the biological and ecological effects of vanadium contamination (Jernelov, 1975). The following are EPA standards for other heavy metals (Gregory, 1973).

- Chromium *100llg/i* in fresh and sea water;
- Iron 1.0 *mg/l* for fresh water;
- Lead 0.01 LC 50 for sensitive fresh water,
- Mercury *0.051lg/l* for fresh water 0.1 *Oflg/l* for sea water,
- Nickel 0.01 LC 50 for appropriate fresh and sea water organisms.

1.5 Impact of refinery effluent on soils

Petroleum contaminants or pollution of terrestrial ecosystems affects not only the microbial flora of the soil, but also the resident macro community. The deleterious effects of oil are best seen on the dominating flora of terrestrial environments though a less conspicuous effect is also exerted on the animal community (Woodwell, 1970). Petroleum pollution of soil has strong negative effects on plant community in a complex way involving both contact toxicity and indirect deleterious effects mediated by interactions of the petroleum with the abiotic and microbial component of soil. The low boiling components of petroleum exhibit a high degree of contact toxicity to tender

portions of plant shoot and roots, but little effect on woody parts 01' tree and shrubs. Contact toxicity occurs primarily by the solvent effect of low boiling hydrocarbons on the lipid membrane structures of cell. Toxicity is positively correlated to increasing polarity and inversely correlated to increasing molecular weight. (Haider, 1965; Holcombe *et al.,* 1976; Davies *et al.,* 1976; McGill *et al.,)*98); Orwumere and Oladimeji, 1990). Toxicity of hydrocarbon has also been detected in fish (Onwumere and Oladimeji 1990).

The low boiling petroleum components are readily removed from the biologically active surface layer in moist, well- drained soils through evaporation and leaching (Hunt *et al.,)*973), therefore the effects are of short duration. Indirect effects include oxygen deprivation of plant roots, due to the exhaustion of soil oxygen by hydrocarbon degrading microbes; such anaerobic conditions resulting may bring about microbial generation of phytotoxic compounds such as hydrogen sulphide. Oil degrading microbes compete with plants for mineral nutrient and the oil also affects the physical structure of the soil, decreasing its capacity to store moisture and air (De long,' 1980). All these negative effects manifest themselves either immediately or during the biodegradation of the pollution oil. However, once the biodegradation process of a moderate-size pill is complete, the negative effects tend to disappear and the soil may actually show improvement in its ability to support plant growth. When compared with its pre-spill status, such improvements have been due to increased organic matter and combines nitrogen in soil after the biodegradation of the spill. However, the severity and duration of the effects of petroleum spill on a plant community are highly dependent on the quantity and quality of the petroleum on the post-spill treatment and type of the contaminated soil (Mcgill *et al.,]*981; Bossert and Bartha, 1984).

1.6 Treatment and disposal of petroleum waste

Petroleum is a major source of energy, and remains the principal source of lubricants, solvents and a variety of chemical feedstock for synthesis of plastics, fibres, detergents, pharmaceutical and cosmetics. The vast scale of the operations necessitates by the above demand renders the petroleum refining industry a potentially severe source of air, water and soil pollution. Though the refining industries undertook an effective and largely voluntary programme to reduce environmental pollution by effective waste treatment and disposal facilities, including those for biological treatment, the state of refinery waste treatment has remained a major concern to the environmentalist (Burrough, 1963; Mckinney, 1963).

Treatment of refinery effluent is similar to the treatment of liquid waste from other industrial and domestic sources. A unique ingredient of refinery affluent is floating or emulsi lied oil. that is a liquid lighter than water; some of the oil can be physically separated and reclaimed but hydrocarbons associated with inorganic and organic particulates becomes sludges that ultimately need to be disposed of as solid wastes. Adding to the disposal problems are tank bottoms, accumulated sediments from oxidation ponds, oil-contaminated soil and some spent lubricants that cannot be economically reprocessed. Most of these materials have been classified as hazardous waste and their ultimate disposal is becoming tightly regulated. Many techniques have been developed for the treatment of petroleum waste, which include the following physicochemical and biological methods. Incineration, one of the physicochemical methods, is sometimes used but is not economically attractive, though it decreases the bulk of the wastes (Welmantl]98]; Remrez]982). The residue, especially the heavy metals will still have to be

disposed off. The escape of gaseous emissions would have to be controlled so as to reduce the threat to global warming (the green house effect). Other physicochemical methods include gravity separation and floating recovery. This technique recovers floating oil by skimming and emulsifying for re-use. A technique for removing the chlorinated hydrocarbons by adsorption on activated carbon has b en developed (Robertson *et Cll.,* 1980) but it is co tly to operate. The use of anaerobic land fills and injection wells pose hazards to the ground water.

Biological treatments are similar to those used for sewage treatment. Oxidation ponds arc sometimes used, but its limitations are due to the necessity for large land areas because of long retention times. The use of biofillers suffers from the problems of biomass clogging which requires expensive replacement of filter beds. With activated sludge, the problem of variation in effluent quality and bulking arises. These three methods generate sludges which includes recalcitrants like the chlorinated hydrocarbons. These sludges would still have to be properly disposed of.

Another biological method that make use of the soil rnicroflora is the "Land farming" or land treatment; it is less costly and requires simple technology which could be suitable for developing countries especially those with warm climates. The micro-organisms reported to be responsible for the transformation of the hazardous wastes into harmless ones in the soil are the bacteria and lower fungi.

1.7 Land treatment Method

Soil or land treatment otherwise called "Land farming" is a relatively new technique in the disposal of refinery waste. It relies on soil micro-organisms to render this toxic sludge innocuous by complete mineralization. Even the heavy metals may be immobilised by

ph. .ical and chemical forces such as complexing, cation exchange capacities and adsorption to soil organic matter (Mckinney, 1963; Davies, 1967; Schroeder, 1977; Phung and Ross, 1978).

Reports have shown that bacteria and fungi are involved in the degradation of petroleum hydrocarbons in the soil (0 Ill, 1978; Atlas *et 01.,* 1981). Bossert and Bartha (1984) gave a comprehensive list of the bacteria and fungi in the review. A diverse range of bacteria are involved in the degradation of the petroleum, whereas for the fungi the Moniales and Mucorales are the dominant types. Although the degradation of petroleum in the soil is a direct result of co-operative action of bacteria and fungi it has been reported that bacteria tend to respond more rapidly to oil contamination in the soil while fungi may be inhibited initially (Pinholt *et al.,* 1979). The activities of the fungi seem to persist long after that of bacteria have diminished. The role of algae and protozoa has not been elucidated.

Biodegrading bacteria and lower fungi have evolved special traits to overcome the problem of pollution by hydrocarbon in the environment. Three of such distinguished traits or specialities are:

I. Flora possessing efficient hydrocarbon uptake system by having special receptor sites for binding hydrocarbon and/or production of hydrocarbon in the cell.

2. A group of microorganisms that possess specific oxygenases to introduce molecular oxygen into the hydrocarbon and, with relatively few reactions, generate intermediates that subsequently enter common energy yielding pathways.

3. For the enzyme oxygenase to function in some microorganisms in the presence of petroleum and its components, inducer specificity and substrate specificity may not need to coincide (Gutnick and Rosenberg, 1977).

The most dominant bacterial genera in soils are *Corynebacterium. Micrococcus, Mycobacterium, Arthrobacter, Pseudomonas, Bacillus, Agrobacterlum. Alcaligenes* and *Flavobacterium* (Alexandar, 1977). These genera have been found to have the ability LO degrade at least some hydrocarbon components (Bossert and Batha 1984). The filamentous fungi of the genera *Aspergillus, Pencillum* and *Cladosporium* and some strains of *Candida* and *Rhodotorula* have been implicated in hydrocarbon biodegradation (Atlas, 1981).

A unique group of hydrocarbon degrading bacteria however, not included in the genera, is the Methanotrophs, which possess a highly specialised Carbon 1 metabolism (Higgins *et al.1981).* It was observed that methane escaping from underground gas pipes was rapidly utilised by Methanotrophs in soil (Adams *e11l1.1971* and 1Ioeka, 1(72).

Microbial seeding of petroleum and its waste as opposed to mechanical removal (Sabba 1971) had been proposed by a number of investigators over the years. Seeding has been examined in the laboratories (Liu and Dika, 1972; Maget, 1973). Both laboratory and field studies demonstrated that bacteria enhanced oil degradation, 5% degradation after 42 days, especially in the presence of sufficient nitrogen and phosphorous and 70% degradation after 42 days, (Atlas and Bartha 1973). A single microorganism will not possess the enzymatic capacity to metabolize all of the many compounds present in a spilled oil or waste. A mixture of microorganisms including fungi, yeasts and bacteria would be required. Either pure, mixed or enriched cultures may serve as inoculaum. Oil

contaminated soil was seeded with bacteria by Johnson *et al,* (1974). They found that when compared with addition of fertilizer i.e. nitrogen and phosphate; bacteria alone had little effect on the removal of the oil.

].8 Effect of Environmental parameters on Land Treatment

The degradation of organic constituents in refinery effluents depend not only upon the nature of the compound but also upon temperature, oxygen, nutrients and microbial species present. Giving optimum conditions, many petroleum hydrocarbons and phenols will degrade easily but higher molecular weight polynuclear aromatic and trithydric phenols degrade slowly and it is not clear how long they are likely to remain in different types of marine environments.

Incorporation into anaerobic bottom sediment may greatly increase degradation times of all organic compounds, except those occurring naturally as well as those derived from effluents. Sulphides and ammonia may be oxidised chemically or by bacteria and are not likely to accumulate in well-aerated discharge areas. High sulphide levels may be found in anaerobic mud. both in effluent discharge areas and in naturally anaerobic areas distant from industry (Baker 1971; Bakel', 1973; Vos *et (.[1.* 1977). The fate of petroleum hydrocarbon in the environment is largely determined by abiot ic factors, which influences the rate of microbial growth and enzymatic activities and degradation. The persistence of petroleum pollutants depends on the quality and quantity of the hydrocarbon mixture and on the properties of the affected ecosystem. Petroleum hydrocarbon may persist indefinitely in an environment under certain conditions, whereas under another set of conditions the same hydrocarbon can be completely biodegraded within a few hours or days (Atlas, 1981).

Biodegradation of petroleum products occur at a wide range of soil temperature. Freezing of the soil solution, of course, interrupts microbial activity, but it was reported that petroleum biodegradation occurs at a temperature as low as 1.1 "C, provided the soil solution remained liquid; the highest degradation rate occurs generally between 30°C-40°C (Huddleston and Cresswel, 1976). Dibble and Bartha (1979) however, observed that the highest hydrocarbon degradation rate occms above 20°C, with no further increase in rate at *3rc.* Hydrocarbon degrading microbes isolated from oil-contaminated soil in Alaska have however been able to grow at 50°C (Atlas *et al.1978;* Sexstene *et al.i* 978a, b).

The initial steps of hydrocarbon biodegradation are oxygen dependent, since sporadic reports on anaerobic hydrocarbon biodegradation in vitro remains controversial (Senez and Azoulay 1961; Chouteau *et al.)*962; Traizulea *et al.,)*969; Trexler and Bernard, 1969; Perkh *et al.* 1977). Intermediates of aerobic hydrocarbon biodegradation can however, be metabolised further under anaerobic conditions using sulphate as electron sink (Shelton and Hunter, 1975; Jobson *et al.,* 1979). Reports of oxygen requirement for hydrocarbon biodegradation in soils are scanty, but the few available support of an absolute oxygen requirement significant biodegradation activity since oxygen depletion leads to sharply reduced hydrocarbon utilization in the soil (Huddleston and Cresswell, 1976; Atlas *et al.* 1978). Studies on soil derived enrichments and on oil-impregnated soil consistently showed the highest rate of oil degradation when aeration was maximised (Jobson *et al.1972;* Lehtomaki and Niemela 1975).

The moisture content of the soil is essential to the biodegrading organisms. As a result a reduction in moisture content, it could lead to reducing the water holding capacity of the soil (Dibble and Bartha) 979) and hydrocarbon contamination, generally reducing the

bulk density of soil while it increases the porosity of such soils. In such cases soil aggregates are broken down and dispersion results (Ellis and Adam, 1961; Buckman and Brady, 1969).

The pH of various soils have a wide range, but most are acidic and most bacteria have a limited tolerance far acidic condition, so the pH of the soil often determines the types of microbes participating in hydrocarbon degradation. It was reported that, the rate of biodegradation is higher under slightly alkaline (pH 7-9) condition for bacteria than under acidic conditions (Vanloocke *et al.197S).* It was also observed that degradation was minimal in a naturally acidic soil (PH 3.7), but stimulation of hydrocarbon biodegradation increases with rising soil pH up to a level of pH 7-8 (Dibble ancI Bartha, 1979). 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 the presence of inorganic and organic nitrogenous substances.

1.9 Biodegradation of Petroleum Hydrocarbons (J>HC).

The biodegradation normally proceeds by a monoterminal attack of alkanes by oxidation on predominantly the carbon 1. A primary alcohol is formed. This is followed by an aldehyde on further oxidation and formation of a monocarboxylic acid. This proceeds the incorporation of molecular oxygen into the oxidation product by hydrogen molecule oxidation with subsequent formation of a 2 carbon unit, shorter fatty acid and acetyl co-enzyme A with eventual liberation of carbondioxide (Atlas, 1981).

It has been shown that classical Beta-oxidation may not be the only long chain fatty acid degradation pathway occurring by microbes. As it has been suggested that Alpha-oxidation may occur to some extent in certain microbes at the expenses of hexadecane,

producing not only acetyl palmitate but also palmitic, palrnitolcic, stearic and oleic acid as well. Branched MId highly branched alkanes undergo omega oxidation with the formation of dicarboxylic acids. Even though methyl branching has been found to increase the resistance of hydrocarbons to microbial attack, terminal branching inhibits degradation of the hydrocarbon completely since the Carbon 1 is not free for oxidation (Atlas 1981).

Bacterial degradation of aromatic compound involves the initial oxidation by incorporating two atoms of molecular oxygen into the substrate to form a dihydrodiol with cis-configuration (Gibson 1977). The reaction being catalysed by a oxygenase. a multi-component enzyme system consisting of a flavoprotein, an iron-sulphur protein and a ferredoxin (Yeh *et al.* 1977, Crutcher and Geary 1979). Further oxidation of the cis-dihydrodiols leads to the formation of catecols that are substrates for another dioxygenase that brings about enzymatic fission of the aromatic ring (Dagley, 197 I). Lighter aromatic hydrocarbons are more readily subject to evaporation and microbial attack in a dissolved state. The initial aromatic compound oxidation can be inhibited by extensive methyl substitution but initial enzymatic attack may be on the alkyl substituent or alternatively, directly on the ring (Atlas, 1981)(Fig. 1.1 - 1.3). The biodegradation of petroleum and its subsrituents ultimately results in carbondioxide, water and a microbial biomass partially oxygenated biodegradation intermediates of hydrocarbons and fatty acids and phenolic substances. Some petroleum carbon may become part of the soil humus via microbial biomass or directly.

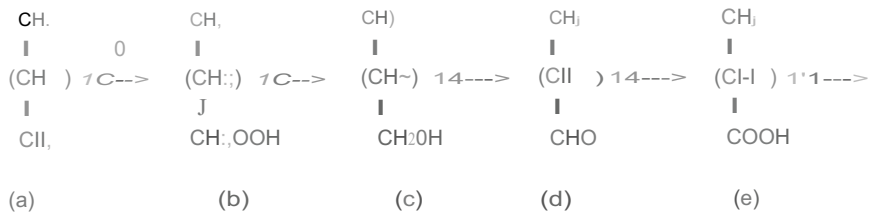


fig. 1.1 Proposed pathway of hexadecane metabolism in Acetob~cter sp. (Singer 1984).

Key:
(a) n-hexadecane, (b) n-hexadecyl hydroperoxide,
(c) n-hexadecanol, (d) n-hexadecyl aldehyde,
(0) f1-h,'xdck·canoicacid.

E'ig.1.2 Mechanism of oxidation where alkane is converted to primary alcohol and water (Singer, 1984)

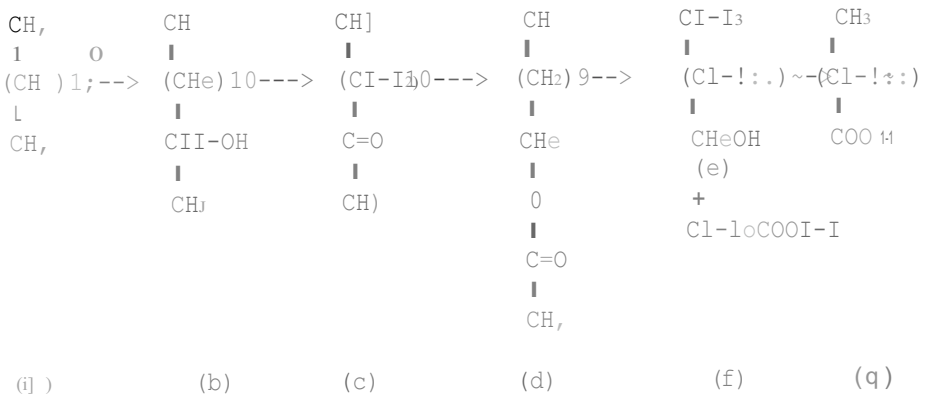
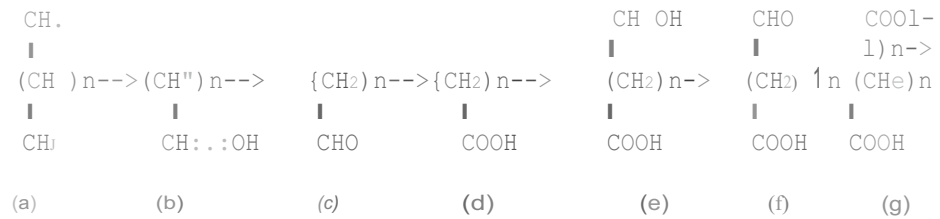


Fig. 1.3 Pathway of subterminal alkane oxidation in Psuedomonas (Singer, 1984)

Key;

a = n-tridieane; b n-tridicane-2-01;
c = tridican-2-01; d Undecy lacetate.
e = Undecan-l-al. f acetate
g Undecanoic acids.

Higtll'y branched isoprenoids like pristance undergo omeqa (diterminal) oxi ation (Fi 1.4) to form dicarboxylic acids as th major degraddtive parhway.



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
9 dicarboxylic acids

Fig. 1.4 Pathway of diterminal alkane oxidation (Sil1g1e]984).

This results from the production of extracellular products e.g alkane hydroxylase. However, methyl branching increases resistance of PHC to microbial attaches

(Andrernoni *et al.*. 1993). Cycloalkanes are resistant to microbial attack, but arc substrates of co-oxidation (Monna *et al.,* 1983; Sikkena and De Bout, 1993) forming ketone and aldehydes. Once oxygenated, degradation of cycloalkane can proceed with ring cleavage. Substituted cycloalkanes are liable to degradation more readily than unsubtituted ones, especially 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 1.5).

Fig. 1.5 Proposed pathway for catabolism of cyclohexane by *Norcardia* sp.{Perry 1984).

Aromatic hydrocarbon have gained notoriety as environment pollutants that are resistant to biodegradation (Bossert and Bartha, 1984; Miller *et al.,* 1988). Microbial degradation involves the formation of a diol followed by cleavage and formation of a diacid such as cis, cis-muconic acid (Fig 1.6).

Fig. 1.6 Pathway of aromatic hydrocarbon oxidation by Prokaryote Singer (1984)

Light aromatics are subject to evaporation and 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).

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

1.10 Immobilisation technique in PHC treatment

Immobilisation technique has been used for detecting the rate of utilization of xenobiotic compounds by microorganisms. Michael *et (II.,* (1988) used five selected enzymes of *Geotrichum candidum* to demonstrate immobilization and subsequent fate of Cvlabelled 4 methylphenol and 2-4-dichlorophenol in soil columns. They concluded that enzymatic immobilization of phenolic contaminant in soil appears to be a promising technique for the reduction of ground water pollution. Also Sahasrabudhe and Modi (1987) used immobilized *Aspergillus niger* mycelia pellet in calcium alginate to study the

dechlorination of 2-Chlorobenzoate, 3-Chlorobenzoate and 4-Chll rophenoxyacetate at a 0.5mM concentration of the substrate. They used *Pseudomonas* sp. B 13 cells and by electron microscopic studies, they revealed that the immobilized cells are protected from chlorobenzoate toxicity and therefore can be used for a longer time compared to the free cells. Calcium alginate entrapped Psuedornonas sp. 813 cells were used in a fluidized bed column reactor to study the dechlorination or 3-chlorobenzoate. 2.5nM f 500mls 3-Chlorobcnzoate was completely dechlorinated within 5-6 hours, when air fluidization was provided and substrate was continuously recycled through the column. 4-chlorophenol degradation by *Alcaligenes* sp. A7-2 entrapped in calcium alginate showed that increasing concentrations of 4-chlorophenol are better tolerated and degraded at a faster rate (Westmeier and Rehm 1985). Vinylchloricle, I,2-clichloroethane and dichloromethane CDCM) are said to make up over 50% of all the volatile chlorinated hydrocarbons emitted from gaseous industrial sources. The removal of these compounds from waste gas streams is possible using microbial processes since all of these compounds have been found to be aerobically degradable In the reactors, the bacteria grew on a solid support which was tludidized by uptlowing medium. DCM as the sole carbon source was supplied dissolved in the feed, oxygen was absorbed externally by sparging air in a well-mixed tank (Niemann and Dunn, 1992). The biodegradation of three chlorophenols, 2,4,6-trichlorophenol, 2,4-dichlorophenol and 4-chlorophenol was studied under aerobic andlor anaerobic conditions using adapted biofilm in fluidized sand bed reactors. Two reactor configurations were compared with a single stage aerobic digester. In one configuration the effluents were first treated anaerobically and then aerobically, in a single pass. In a second configuration the effluents were first treated anaerobically, after that, aerobically and then the effluents of the aerobic reactor were

partially recirculated to the anaerobic reactor. The three chlorophenols were almost completely removed in all 3 systems (Fehmy *et al.,* 1991).

1.11 Importance of Genetic manipulation technique in Land treatment

M icro-organisms are known to degrade both aliphatic and aromatic hydrocarbons, as well as synthetic hydrocarbon derivatives such as halogenated as well as aromatic organic compounds. According to Chakraborty (1985), the genes for the degradation of such hydrocarbons can be borne on either chromosome or plasm ids. He has demonstrated that a set of genetic loci, on both the OCT plasmid and the chromosome enable *P. putida* to grow on short-chain alkanes. Some organisms, namely, *Acinetobacter* contain the genetic information for long chain alkane metabolism that is localized solely on the chromosome. Dunn and Gunsulus, (1973) have suggested that a plasmid, designated NAH, carried all the genetic information necessary for the dissimilation of naphthalene ancl salicylate via the inducible metabolic pathway. Olukoya (1992), has surveyed the Nigerian environment for indigenous hydrocarbon utilizing bacteria and genetic studies into the degradative capacity of these isolates has implicated the activity 0f plasmids. These plasrnids can now be genetically engineered and may be used in oil industries in Nigeria. This may lead to the development of efficient hydrocarbon degrading microorganisms for use in clearing oil spills, safe disposal of refinery effluents and production of microbial surfactants (Olukoya, 1992). Finally, it may be suggested that success in the isolation and cloning of specific aromatic degrading genes and of entire regulated gene sequences gives considerable promise for the application of constructed strains of microorganisms serving useful and functional purposes in environmental pollution problems.

1.12 Advantages and Disadvantages of Land treatment

llydrocarbons may be present in the following physical phases: gas, liquid, solid, "accommodated phase". It is assumed that the microorganisms absorb hydrocarbons by direct contact with the oil. As the cells need to be in contact with water for other vital needs such as oxygen, minerals and carbon dioxide disposal, the active micro flora is found at oil-water interfaces. Normal soil always contains small quantities of biogenic hydrocarbons and hence hydrocarbon-oxidising microorganisms are present, albeit in small numbers. When the supply of hydrocarbons is increased, the number of hydrocarbon-decomposing microbes also increases. Microbial adaptation to hydrocarbons is a rapid phenomenon. a lag-phase may be caused by the presence of toxic low-boiling hydrocarbons but these disappear rapidly as a result of the frequent mixing and aeration of the soil.

It has been found that accumulation of metallic elements in the crops is curtailed by maintaining the soil pH between 6.5-7.5, by the addition of lime. But the main concern is to avoid build-up to phytotoxic levels of elements such as nickel, copper and zinc in the soil since these can have a detrimental effect on vegetation and on animals which may eat the plants. Certain other metals like cadmium have no phytotoxic effects but can accumulate in vegetation to reach a level which is toxic for animals (Wainwright *et al., 1986).*

In determining admissible limits for metals considered as toxic elements, the soil nitrogen content must be taken into account since a high level can inhibit uptake of metals. Excess nitrogen can both reduce output and make plants more susceptible to disease or pests. But it should also be noted that metals such as iron and other elements

such as phosphorous and magnesium which may be present in refinery effluent may have a beneficial effect on the soil flora and fauna (Wainwright, 1992).

1.13 Role of Basidiomycetes in treatment of wastes

Considerable research effort is being devoted world- wide to the use of Basidiomycetes to decompose environmental pollutants both in soil and in liquid effluents. Most of the research have involved the use of a single species *Phenerechaete chrysosporium.* This basidiomycete is especially versatile at degrading xenobiotics because it produces a variety of non-specific ligninases, which acts upon organic molecules other than those present in lignin. Although these enzymes are used in natural environments and in ecosystem to degrade lignin, they can be employed in biotechnology to degrade a variety of complex phenol containing compounds such as pesticides. Similarly, wastewater discharges containing trinitrotoluene effluent and chlorinated lignin wastes produced by the pulp industry (Martonet *et al., 1969).*

Wood decomposing fungi are also used to discolour and defoarn pulping waste liquors. Lignin and their derivatives are common in these liquors causing them to be blackish brown in colour and presenting an unsightly pollution problem. Decolourization of Kraft block liquor is achieved by a number of fungi including *Trametes versicolor* (Bergbauer *et (.l/.,* 1991) and non wood rotting fungi such as *Aspergillus* sp. Decolorization is often associated with adsorption of the colour on the surface of the mycelium.

Tinctoporia borbonica, for example, can reduce waste pigment colour by 70% or when sugar is added by up 10 99% in this case decolouration does not rely solely upon surface adsorption, but result from partial reduction and delignification of o tylignin. Chlorinated lignin derivatives present in bleach plant effluents can also be degraded by the white rot

fungus *Trametes versicolor,* when glucose is provided as co-substrate, close to a 90% reduction in colour can be achieved in 3 days. Simultaneously, the concentration of chloro-organic compounds decreases by about 45%. Efficient colour removal from such effluents can also be achieved by immobilising these in calcium alginater beads (Bergbauer *et (II.,* 1991).

Phenerochaete chrysosporium produces several peroxidases or ligninases involved in the degradation of lignin, including Manganese peroxidases. These enzymes catalyse a one electron oxidation of lignin to an aryl cation radical; which then undergoes cleavage and further oxidation to benzylic alcohols and aldehydes. Isolated peroxidases from this fungus also oxidises polycyclic aromatic compounds to quinones, for example anthracene is converted to anthroquinone, while pyrene is converted to a mixture of pyrene-, 6-quinone and pyrene-18-quinone. Ligninases will oxidise aromatic substrate with ionization potentials as high as 7.6eV, which make them substantially more oxidising than classical horseradish peroxidase. Lignin peroxidases can attack 1110staromatic structures, while other peroxidases are limited in their action to phenolic moieties (Weinwright, 10(2).

The lignin degrading system of white rot fungi can be used to break down a wiele variety of environmental pollutants (Zurer, 1987); a usage based on the ability of these fungi to produce peroxidases, enzymes intimately involved in the. minerilization of chemical pollutants. White rot peroxidases are produced in response to nutrient limitation rather than repression of enzyme synthesis, as a result, white rot fungi do not need a period of adaptation before they can degrade chemical pollutants. The fungus can be grown on lignocellulose wastes, which are then added to the soil. Wastes such as wood chips act a all ideal nutrients source since they contain only small amounts of nitrogen

but all other necessary nutrients (Bumpus *et al.,* 1985; Anst, 1990). Culture of *P. chrysosporium* can degrade environmental pollutants such as chlorinated biphenols, aromatic hydrocarbons, and chlorinated dibenzoquinones; It has also been used to degrade so-called "pink water" produced during the manufacture of 2,4,-6 trinitrotoluene (TNT). In this case the concentration of TNT is reduced by *P. chrysosporium* from 90~lg per ml to zero in about 24 hours. White rot fungi are potentially of great importance in treating effluent from paper mills. Some 3 Kg of lignin bound chlorine is discharged into the environment for each ton of bleached paper produced in U.S.A. These high molecular weight compounds are not degraded by conventional treatment systems and their ultimate fate in the environment is largely unknown but the use of *P. chrysosporium* on a rotating contactor some 70% of the organic chlorine in bleach plant effluent can be removed in less than 48 hours. *P. chrysosporium* has also been used to degrade polycyclic aromatic hydrocarbons (PAl-I) and pentachlorophenol. These pollutants are found in anthracene oil, a product of coal tar distillation (Bumpus, 1989). Also, *P. chrysosporium* when grown on nitrogen free media containing a suitable carbon source degrade persistent pesticide such as DDT and Lindane. The use of *P. chrysosporium* to degrade hazardous wastes has rec ntly been described (Lewandowski *et al.,* 1990). The biodegradation rate was found to be improved by a factor of 40% when the fungus was immobilized; this was achieved using a parked bed reactor employing a silica-based porous support for the fungus and a well mixed reactor with alginate beads as the immobilizing medium. Both proved effective in degrading 2- chlorophenol at concentration of Lipto *520llg* per ml. The abilities of the white rot fungi *Chrysosporium lignorum, Trametes versicolor, Phanerochaete chrysosporium* and *Stereum hirsutum* to mineralize 3,4- dichloroaniline, dieldrin and phenanthrene was investigated by Morgan *et al.,* (1991). They observed that

with the exception of *S. hirsutum* all of these species mineralized this potential pollutant, with *r. versicolor* showing the greatest degradative ability and *P. chrysosporium* the least of the three. Highest rate of mineralization were achieved when the medium was supplemented with minerals, vitamins, and veratryl alcohol. But the mineralization of these compounds did not involve the production of detectable levels of extracellular ligninase activity.

The LIseof white rot fungi in soils to break down xenobiotic substances was carried out with *P. chrysosporium.* This was done by growing the organism on a suitable substrate such as wood chips, which are then ploughed into the contaminated soil. The fungus grows from this inoculum into the surrounding soil where it degrades the pollutant, thereby leading to soil reclamation (Lamar *et al.,* 1990). The rate of pollutant degradation achieved by *P. chrysosporium* in soil never approaches those produced in liquid culture. This is because environmental factors such as unfavourable soil type, pH, temperature and the presence of microbial antagonists detrimentally influenced the growth and activity of *P. chrysosporium* in the soil. Lamar and co-workers (1990) recently showed that pentachlorophenol can be rapidly degraded in the soil by *P. chrysosporium.* However they autoclaved their soils prior to inoculation. This process removes competing organisms and also releases large amounts of available carbon. As a result, fungal growth and metabolism in autoclaved soil differs from that found in non- sterile soil, which contain only a small amount of available carbon and are highly competitive. Also George and Neufed (1989) used autoclaved soil when they showed that *P. chrysosporium* could be used to enhance the degradation of fluorene in soil.

1.1'" Role (If Basidiomycetes in disposal of' refinery effluent

Some of the basidiomycetes especially the white rot fungi, are capable of degrading lignin found on wood (Silverbory, 1953; Kalpoor, 1978; Michra *et al.,* 1979; Hatakka, 1985). The structure of the lignin is diverse but aromatic and seems to be made up of phenyl propane units (Boidin, 1951; Kahlon *et al.,)*983; Chebci *et (/l.,)*985; Hatakka 1985; Chen and Chong 19851). These are similar to those found in petroleum hydrocarbons. The wood contains some chemical substances extractable by water and chemical solvents which are mainly phenolic (Silverbory, 1953: Kapoor *et (ll., 1978;* Airkan *et al.,* 1989). These extractives which enhance the resistance of wood to decay are removed or detoxified by some wood decaying fungi (Upcher, 1971; Taylor, 1974; Mishra *et al.,* 1979). The study of two brown rot fungi *Gleophyllum sepiratus* and *Gleophyllum* sp. and a white rot fungus *Pleura/us ostreatus* have been found to removed some of the extractive of opepe (Maulea diderrichii) and mahogany (Khaya ivo-rensis) wood (Fjechi, 1991; Ejechi & Obuekwe 1993).

Some researchers have found that *Phenerochaete chrysosporium* and *P. sordida* clecr'(ISI.! the level of pentachlnroph '1)01. by *9M/o* and 81% after 6~1 lays. Also the lise of' *OSYO* (v/v) pentachlorophenol fa)' preservation *01'* obeche anti Mahogany timber only reduced the loss in weight and mechanical properties caused by Pleurotus, ostreatus and Glecophyllum sp. (Ejechi, 1991). This ligninolytic activity can be linked with degradation of hydrocarbon and its derivatives in the soil (Merrit and Franch 1966; Kaarik 1974; Kirk *et al.* 1976; Ryan and Bumpus 1989). So these Basidiomycetes may be efficient petroleum effluent degraders in the soil, more so when chlorinated phenols constitute parts of the refinery wastes added to soil.

I.J 5 Advantage and disadvantages of Basidiornyeetes used in Land treatment

The nutritional imbalance in land treatment techniques (C:N: P ratio) created by large amount of hydrocarbons, is evedent though phosphate may come from those employed for softening water used for boilers and heat exchangers, but those of nitrogen may have to be added to the oil in the form of fertilizers. However, such fertilizers application have been reported to enhance the degradation of oil sludges in the soil (Dibble and Bartha, 1979). But the danger of ground water pollution by nitrites cannot be ignored and loss of nitrogen by denitrification may add to the cost of the treatment. The wood decaying Basidiomycities are known to have adapted to the low nitrogen content of woods which is about 0.03 - 0.1% (Kaarik,)974). This indicates that they would survive in the soil treatment of these wastes that will minimise the need for fertilizer application.

Dibble and Bartha (1979) reported a decline in soil pH during degradation owing to the appearance of fatty acid intermediates. This fall in pH was corrected by addition of lime and degradation was enhanced, but with Basidiomycetes, the addition of lime may not be necessary since they proliferate within an acid pH range. In warm climates where evaporation is high, the soil moisture can be lowered to 50% to limit inhibition to the bacteria and fungi, the need for addition of water may not be as high for the Basidiomycetes which tolerate close to 30% of moisture. Application of orgamc supplement and sewage was found to interfere with oil degradation (Dibble and Bartha. 1979). The study attributed this to diauxic phenomenon on the part of the degraders undoubtedly the lower fungi and bacteria. Some ligninolytic Basidiomycetes degrade lignin only in the presence of easily metabolizable substrate such as cellulose and glucose (Kirk *et al.,)*976). For instance the 2,4,S-trichloro-phenoxy-acetic acid was mineralized by *Phanerochaete chrysosporium* in soil amended with ground corn cobs (Ryan and

Blimps, 1989). The addition of complex nutrient such as sawdust, maize cobs, rice straw and yam peds to soil containing hydrocarbons will probably enhance their degradation by white rot fungi.

II' white rot fungi can degrade the aromatics of lignin, it is possible for these organisms to equally degrade the sludge from oil refineries, being aromatics. Groups of non structural substances extracted by water and natural solvents which contribute to plant resistance and known as extractives are also generally phenolic, these phenols are also removed by these wood decay fungi (Ejechi, 1991). Lamar *et al.,* (1990) reported that a number of xenobiotics are mineralized by ligninolytic cultures of Phanerochaete sp., white rot fungi in aqueous media and in soil.

One major reason why the basidiomycetes are unpopular in microbiological techniques, even though there are over 1000 species (Lamar *et al.* 1990), is because of their slow growth habit in vitro and difficulties and long period required for fruitification which aid their identification. In degradation of woody tissues and liters in the soil bacteria and the lower fungi are the initial colonizers (Dibbles and Bartha. 1979). These are later replaced by the traditional wood rot higher fungi- the Basidiomycetes. The bacteria and microfungi consume cytoplasmic constituent as well as other nitrogenous materials. They are unable to degrade the cell wall further due to its low nitrogen content of 0.3% (Kaarik, 1971). The Basidiomycetes then become established since it appears they are adapted to the level of nitrogen of woody substrate, while the bacteria and mierofungi are active, they inhibit the activities of the Basidiomycetes (Ejechi and Obuekwe 1993).

1.16 Biomass Production using crude oil and refinery wastes

Hydrocarbon fermentations, for the most part, involve either an oxidative transformation of the hydrocarbon molecule, or a total degradation of the hydrocarbon to the acetate level followed by resynthesis of a fermentation product. However in certain instances, the microbial cells, which grow at the expense or the hydrocarbon, become the fermentation products. Considerable publicity has been accorded a hydrocarbon fermentation process that produces the edible yeast *Candida llpolytica* (Champagant, 1963). This yeast is grown on an aqueous salt medium with various oil fractions, including furnace oil, as the hydrocarbon substrate. lnorganic N-P-K fertilizers provide the salts and nitrogen for the aqueous medium, and the fermentation is continuous in that the hydrocarbon is recycled. During its growth, the yeast removes paraffins from the oil to bring about dcwaxing, thus yielding as a by-product oil improved as to its pour-point characteristics. The yeast cells re overed from this fermentation contain a nutritionally balun .ed protein that should find lise as a food or food supplement in the undernourished areas of the world. Imperial Chemical Industries have heen successful in developing a COll111~ri·1Iprocess for production of bacterial biomass from methanol at an annual rate of 54,000 to 70,000 tonnes. The process utilizes a novel air-lift Iermentor, of 3000 m3 capacity, and is the first commercial process to introduce single cell protein (Sep) from methanol (King, 1982). Candida yeast was cultivated from alkane with a growth rate comparable to that obtained from glucose and with a conversion factor of 100 g. dry yeast per I00g of paraffin consumed (Dasilva *et al., 1987)*

Single cell protein from petroleum hydrocarbon (Engel, 1972; Shennan, 1983) and from methanol (Stringer,1983; Lloyd, 1983) have been submitted for nutritional and

toxicological testing on laboratory animals and the result has certified the high nutritional value and the complete safety of the SCP tested.

It is an established fact that some Basidiomycetes are capable of degrading the lignin component of wood (Hataka, 1985; Ejechi, 1990). The structure of lignin is diverse but aromatic and seems to be made up of phenyl propane units. These are similar to those found in hydrocarbons. But it is a very surprising fact that they have not been reported as petroleum degraders in the soil, more so when chlorinate phenols constitute part of the refinery wastes added to the soil. So, the objectives of this study are:

1. To screen some Basidiomycetes isolated from decayed wood and sawdust collected from Timber shed, Maitumbi on their ability to break down recalcitrant components of refinery effluent.
2. To evaluate the rate of biodegradation of refinery effluent by the isolated organisms by gravimetric method using the emission of carbon dioxide.
3. To generate biomass of *Gleophylum seplratus* using refi nery ern uent as substrate.
4. To determine the nutritive value of *Gleophylum sepiratus.*
5. To establish a recycling method for the biodegradation of refinery effluent for its safe disposal and to mitigate the environmental pollution problem.

CHAPTER 2

MATERIALS AND METHODS

2.1 Collection of materials:

(i) Escravos (Petroleum crude):

The escravos crude oil was collected from NNPC, Kaduna in a sterile 2.5 litre plastic bottle and stored in the laboratory cabinet for further use.

(ii) Refinery effluent:

The effluent during refining of Escravos crude was collected in a sterile 2.5 litre plastic container NNPC, Kaduna and stored in the laboratory cabinet for the purpose of analysis.

(iii) Engine and diesel oil:

Samples were collected in sterile containers from Total filling station located in Minna.

(iv) Decayed wood and sawdust as source of microorganisms:

Samples were collected from Maitumbi timber shed located in Minna in sterile MacCartney bottles and kept in the refrigerator at 4°C for isolation purpose.

(v) Soil samples:

These were collected from the discharged site of refinery effluent at NNPC, Kaduna in sterile containers and kept in the refrigerator at 4°C for further analysis.

2.2 Isolation and identification of fungi from decayed wood and sawdust.

Exactly one gram of each sample was transferred into 9 011 of distilled water and then using this as a stock solution. Serial dilution up to !0-9 were made following the method of lawole and Oso (1988).1.0 ml of each dilution was plated in Potato dextrose agar

(rDA) and Malt extract agar (MSA) (Answorrth,1995; Smith, 1977). Sterilised glass spreader was used aseptically to spread the suspension on the surface of the agar medium. The plates were incubated at 28°C for 48 hours. Distinct colonies were selected to re-inoculate into PDA slants for further Lise. The morphological characteristics of the isolates were studied by growing the cultures in potato dextrose medium (Smith, 1977) and the cultures of different age were observed under the microscope (x I0 objectives). The cultures of different age were also studied under the microscope (x40) using Indophenol cotton blue as mountant.

2.3 Utilisation of refinery effluent by isolated fungi

For this purpose, minimal salt medium (MSA) (Ejechi, 1990) was used as basal medium (composition: 2.78 NH4N03; 0.98g KH2P04; 0.7g K2HPO..j; 0.00 I ZnS04,7H20; 0.005g MnS04,4H20, 0.05g CaCI2,2H20; 0.001 g CaCb, 61-hO; 0.00 Ig Thiaminehydrochloride, water-I litre; pH 6). One hundred ml of refinery effluent was added as sole carbon source to the basal medium. About 18-20 1111 of the solid MSA basal medium was poured onto sterile agar plates, and dried at room temperature for 3-4 hours before the plates were coated with 10% refinery effluent. Then the isolates were streaked onto the refinery effluent-coated agar plates and the plates were incubated at 28° for 28 days. Growth on the plates indicated the refinery effluent degrading capability of the isolates.

The fungi, which are capable of utilizing refinery effluent, were subcultured on MSA medium where 10% refinery effluent was used as carbon source.

2.4 Experiment to identify the best degraders.

In this experiment, about 18-20 ml of the solid MSA basal medium was poured onto sterile agar plates, and dried at room temperature for 3-4 hours before the plates were coated with 10% refinery effluent. Then the isolates were streaked onto the refinery effluent-coated agar plates and the plates were incubated at 28°e for 28 days. The rate of utilization was determined by measurement of mycelia growth (Van Etten, 1973; Smith, 1977) at di fferent time intervals over 28 days of incubation.

The isolates *Gleophylum sepiratus* and *Pleura/lis as/rea/us* selected lor further research work (Plate 1).

2.5 Effect of various factors on biodegrading capability of *Gleophylum sepiratus* and *Pleurotus ostreatus*.

The optimum conditions for utilisation of refinery effluent were determined by keeping all the factors constant except one, which was varied within reasonable limits. I Icre the factors studied were:

(i) Inoculum volume

(ii) Initial pl J of the medium

(iii) Temperature of incubation

(iv) Time period of fermentation

(v) Volume of the fermentation medium.

Plate 2.1: G. *sepiratus* and *P. ostrcatus* on minimal salt liquid medium (MSLM) and Minimal salt agar (MSA) after two weeks of culti vation.

(i) Inoculum volume:

In this experiment, each of *Gleophylum sepiratus* and *Pleurotus ostreatus* was grown far 7 days in 50 ml Potato dextrose agar medium. After 7 days or fermentation, the growth was filtered through Whalman No. I filter paper. The growth on the paper was washed twice thoroughly with distilled water and then the growth was transferred into 30 llll sterile water in 50 ml Erlenmeyer flask and was shaken vigorously with few glass beads for 20-30 minutes. This uniform cell suspension was used as inoculum for this purpose. The optimum volume of the inoculum was determined by using different volumes of this uniform cell suspensions used as the inoculum. Different volumes of the cell suspensions namely 5, 10, 15 and 20 ml respectively were used to inoculate 125 ml of fermentation medium (MS medium with refinery effluent at 10% as carbon source) taken in each 250 ml Erlenmeyer flask. Fermentation experiments were carried out at 28°e and cell growth was determined on 5th, 9th, 12th, 15th, ISth and 21st day of fermentation. Cell growth was determined by taking the dry weight of cells. 1'01' this purpose, the cells were filtered and washed twice thoroughly with distilled water and then transferred to a pre-weigh ed (W I) aluminium Clip, dried at 60-70oe for 24 hours. A ncr 24 hours, the weight of the cells in aluminium CLIp (W2) was measured and the di tference in weight (W2- WI) was taken as the dry wt of the cells.

(ii) Determination of optimum pH of the medium:

The optimum pH of the fermentation medium was determined by carryrng out the fermentation at different pH values (initial) of the medium. 125 ml of each of media with

pl-l's of 4, 6 and 8 in 250 ml Erlenmeyer flask was inoculated with 10 ml of each of the isolates and then incubated at

noe tar 21 days. The dry cell weight was measured on the 5th, 9th, 12th, 15th, 18th and 21 sr day of fermentation.

(iii) Temperature of incubation:

Fermentation was carried out at different temperature between 28°e to 40°C namely 20, 30 and 40°C.Cell growth was determined on 5th, 9th, 1Zth, 15th, 18th and 21 st day of fermentation.

(iv) Time period of fermentation:

The opti mum period of fermentation was determined by carrying out the fermentation for 28 days, keeping the initial pH of the medium at 6.0. The dry cell weight. was measured on the Sth, 9th, 1Zth, 15th, 1Sth, 21 st and 25th day of incubation.

(v) Volume of the fermentation medium:

In all the preceding experiments, the volume of the medium was 125 1111n a 250 llll Erlenmeyer flask. The effect of the volume of the medium was therefore studied by taking diff rent volumes of fermentation medium (50 ml, 75 ml, 100 ml, 125 ml and 150 llll) in 250 ml Erlenmeyer flasks and the fermentation period was 21 days. The dry cell weight. was measured on 5th, 9th, 12th, 15th, 18th and 21 st day of fermentation.

2.6 Effect of carbon, nitrogen and potassium on the biodegrading capability of *Gleophylum sepiratus* and *Pleurotus ostreatus.*

Since refinery effluent was selected as sole carbon source, different concentrations of refinery effluent (1.0, 5.0, 7.5, 10, 15 and 20 %) were tested to

determine the optimum concentration of refinery eftluent utilised by *Gleophylum sepiratus* ancl *Pleurotus ostreatus.*

For studying the effect of nitrogen sources, the MS medium omitting NR1NO] but containing 10% refinery effluent was used. Each of the nitrogen sources (urea, NH.jNO], NII.jCI, NaNO] and (NH4)2S04 was employed at a concentration equivalent to 98mg of nitrogen per 100mi of the medium. The optimal concentration of NH.jNO] (the best nitrogen source) utilized by *Gleophylum sepiratus* and *Pleurotus ostreatus* was next determined using different concentrations of NH.jNO]. The concentrations of NaNO] used were 24, 48, 72, 98, 120 mg/l 00 ml respectively.

A combination of different concentrations of Kl-hPO.j and $K_2HPO.j$ (0.25:0.20, 0.5:0.4, 0.98:0.7, 1.25: 1.00, 1.50: 1.25 *gil)* were employed for this purpose using MS medium containing 10% refinery effluent and 0.15% NH_4N0_3.The dry cell weight. was determined on 21 st day of fermentation.

2.7 Effect of complex nutrients on utilisation of refinery effluent by *G. sepiratus* and *P. ostreatus.*

Complex nutrients (Maize husk, Beans husk, Rice husk, sawdust and Guinea corn husk) containing materials of natural origin were examined to know their effect on the biodegradation of recalcitrant chemicals of refinery effluent.

The method of preparation of the complex nutrients in order to use them as substrates for G.sepiratlls and P.ostreatus indicated below:

25g of each waste were suspended in 200 rnl of hot water in a 500 1111 beaker. The suspension was kept at 900C for 18 hours. The hot extract was filtered through Whatman

no. I filter paper. The extract thus obtained was concentrated to 100 ml. The solid contents of rice bran extract, Maize husk extract, beans husk extract, saw dust and guinea corn extract were 4%,3.5%,2.9%,3.0 and 3.4% respectively. These materials were then used as carbon source, either alone or in combination with refinery effluent in MS medium. Each of the complex nutrients was employed at 0.1 % concentration (according to solid content) either alone or in combination with 10% refinery effluent in MS medium and the cell growth was determined on 21 st day of fermentation.

2.8 Determination of the rate of biodegradation using gravimetric method.

(i) Structural characteristics of soil pH

Two grams of each soil sample was mixed thoroughly in 10 rnl of sterile water and the pl l or each of soil samples was determined using a pl I meter (Croon Micro pl-l 200).

(ii) Moisture: The moisture content was determined by the method or Concawe (1980). Ten grams of each soil sample was placed in a pre-weighed petridish and the soil in the petridish was dried in the oven (Gallenharnp oven size, England) at 105°C tor 24 hours. Weighing of the samples was done at regular time intervals (after every 12 hrs.) until a constant weight was obtained.

(iii) Determination of total microbial counts:

Two grams of each soil sample was dissolved in 10 ml of sterile water to prepare a stock solution .Serial dilutions up to 10-7 were then prepared out using the method described by Fawole and Oso (1988). 1 ml of each dilution was plated onto dry agar medium (for bacteria, NA plates and for fungi, PDA plates were used). In either case, the pour plate

technique was used. The plates were incubated at 370C [or bacteria and 280C for fungi. The total microbial count was then determined after 24 hours and 48 hours respectively.

Topography of the different soil samples:

Fifty grams of each of 5 air-dried samples collected from the discharged site of refinery effluent at NNPC, Kaduna were used for this purpose. Each of air-dried soil samples, which have been passed through 2 mm sieve, was transferred into a milk shake cup. 50 ml. or 5% sodium xametaphosphate and 100 ml. of water were added to the soil and the solution was mixed thoroughly with a stirring rod. Then the suspension was stirred for 15 minutes with a multi-mix machine. The suspension was transferred from the cup into a sedimentation cylinder. Distilled water was added up to the one litre mark of the cylinder. The cylinder was inverted several times covering the top of the cylinder with the hand. The cylinder. was placed on a flat surface and the soil hydrometer was immediately placed into the suspension until the hydrometer was floating. The first reading on the hydrometer was taken at 40 seconds after the placement of the hydrometer in the suspension. Then the hydrometer was removed and the temperature or the suspension was recorded with a thermometer.

After the first hydrometer reading, the suspension was allowed to stand for 3 hours before the second hydrometer reading was taken. The first hydrometer reading indicates the percentage of silt and clay in the suspension, while the second reading represent the total clay in the suspension. A similar experiment was done for a blank (control) which had no sample.

(ii) Microbiological and nutritional analysis of soil and oil contaminated soil incubated with G. *sepiratus* and P.ostreatus.

(a) Determination of soil nitrogen content

The nitrogen (N) content of the soil was determined by Kjeldahl method as described below:

One gram of each ground soil sample was weighed into Kjeldahl tube. 30mls of concentrated. sulphuric acid was added and 2 Kjeldahl catalyst tablets was added.

The digestion is then carried out in Kjeldathem equipment (Gerhardt Germany). Digestion continued until a clear digest was obtained. The digest was then transferred quantitatively into 100 ml volumetric flask and was adjusted to the mark of the flask with distilled water. Ten ml of digest was pipetted into the *Markham setninitro* nitrogen still.l 0 ml of 40% of NaOH was then added to the digest and steam distilled. The liberated ammonia was collected into 5 ml boric acid solution containing 4 drops of mixed indicator taken in a conical flask. The distillation was continued for 2 more minutes after the indicator colour had changed from pink to green. The titration was carried out with standard hydrochloric acid (1N) in a burette this was added drop wise until the colour turned just pinkish. A blank titration was also carried out.

(b) Determination of Phosphorous available in the soil.

Bray P-I method (1945) was used for the determination of available phosphorous in the soil samples. One gram of air-dried soil, which has been passed through 2 mm

sieve, was transferred into a 50 ml Erlenmeyer flask. Seven ml. of extracting solution (0.03 N NH~F in 0.02 N HCl) was added to the soil sample and was vigorously shaken for one m inute using a mechanical shaker. The suspension was centri fuged to obtain a clear filtrate. A two ml. aliquot of the clear filtrate was pipcrted into a clean test tube and 5 mls. of water and 2 ml of ammonium molybdate solution were added and the contents were mixed thoroughly. To this mixture 1 ml of dilute $SnC1_2.21hO$ solution was added and the mixture was shaken for proper mixing. Then colour was allowed to develop for the next 20 minutes. After development of colour the % transmission and absorbance (Optical density) was determined using a colorimeter, at a wave length of 660nm. A standard curve was also prepared with a range of 0.1 ug/ml. This was used in calibrating the colorimeter before the sample readings were taken. A plot of optical density of the standard against the concentration of standard was drawn. The available P in soil samples was calculated from the plot.

(c) Microbial and nutritional analysis of refinery effluent-incorporated soil and also of refinery effluent- contaminated soil incubated with *G. sepiratus* and *P. ostreatus*.

The total microbial count and the nitrogen and phosphate COntent of each soil sample was determined following the methods described in section 2.7(i) and 2.7(ii).

2.9. Determination of the rate of biodegradation of refinery effluent by *G. sepiratus* and *P. ostreatus*.

Five hundred grams of each of the soil samples was steilised and then refinery effluent was incorporated at]0% level. Each of the sterilised soil samples was distributed into five 250 ml Erlenmeyer flasks so that each flask contained 100g refinery effluent incorporated soil. The flasks were marked as A,B,c'D and E. The soil sample in Band C were inoculated with a cell suspension of G. *sepiratus* and the flasks D and E were inoculated with a cell suspension of *P. ostreatus*. The same thing was done with each non-sterilised soil samples. A control (no refinery effluent and no organism) was also used for comparison purpose. One hundred grams of each type of soil taken in 250 ml -rlenmeyer flask was then mixed with 20 ml of sterile water. 0.5 g of Barium peroxide was taken in a vial and to this, 5 llll of distilled water was added. Each of the vials was then lowered into each of the Erlenmeyer llasks containing soil and each flask was covered with sterile cotton wool. The vial was placed at 20° angle so that there is an increased surface area for the alkaline solution in the vials and it can promote CO_2 absorption. The flask was agitated gently two to three times daily to break lip the scum of BaCO], which had been formed, on the surface of the liquid vials. At 5 days intervals the vial was removed and a new one was placed inside the Erlenmeyer flask. This was done for a peri?d of 30 days. The amount of CO_2 ahsorbed by Ba(OH)2 was determined by titration of BaCO] with lN HCI at room temperature, with phenophralin as indicator. The amount of CO_2 produced was calculated using Stotky's formula (1965).

2.10 Utilization of other petroleum products by G. *sepiratus* and *P. ostreatus*.

The ability of G. *sepiratus* and *P. ostrcatus* to utilize Kerosene, Engine oil and cruclc petroleum as sole carbon source in MS medium was examined and compared with that of refinery effluent. Minimal salt liquid medium (MSLM) was incorporated with kerosene as sole carbon source at 10% concentration The two organisms (G.sepiratus and P.ostrealus) were then inoculated into the medium and incubated for 18 days at room temperature. Cell growth was determined on 3rd, 5th, 7th, 9th, 12th, 15th and 18th day of incubation. Same experiment was repeated for engine oil (as sole carbon source at 10% concentration) and crude oil (as sole carbon source at 1.0% concentration).

2.11 Utilization of the Phenol and cyanide content of refinery effluent by G. *sepiratus* and *P. ostreatus*.

(a) Phenol:

Minimal sail liquid medium (MSLM) was incorporated with phenol as sole carbon source at eliIlerent oncentrations (I %, 2% and 3%). The two organisms (G.sepiralus and P.OSlreatus) were then inoculated into the medium and incubated for two months at room temperature. Cell growth was determined on 3rd, 7th, 9th and 12th weeks (Plate 2 and Plate 3).

Plate 2:2 Utilisation of Phenol and Cyanide as sole carbon source by *Gleophylum sepiratus* during three months cultivation.

Plate 2.3: Utilisation of Phenol and Cyanide as sole carbon source by *Pleurotus ostreatus* during three months cultivation.

(b) Cyanide:

Cyanide in different ratio was incorporated into MSLM as sole carbon source (0.3%, 0.5% and O.R%). The medium was inoculated with the test organisms G.sepiratus and P.ostrcatus incubated at room temperature for two months. Growth was determined on 3rd, 7th, 9th and 12th weeks. (Plate 2.2 and Plate 2.3).

2.12 Immobilization of cells of *G. sepiratus* for repeated utilisation of refinery effluent:

The organism was grown in Potato dextrose agar medium for 15 days and the cells were harvested by filtration through No I Whatrnan filter paper and washed twice with sterile water. The cell (2gm/litre dry weight) were then placed in a sterile mortar in an iced box and ground with a pestle with 5-10 ml of sterile water. The ground cells were then mixed with 20ml molten agar (I % agar-agar). This was sucked into hypodermic syringe (20 1111) and by depressing the plunger drops of the mixture were dropped into chilled sterile water kept in a beaker dropwisc to form little beads. The beads were then used as the source of C. *sepiratus* in MS medium with phenol as the sole carbon source and incubated at room temperature [or 28 days.

2.B Production of Biomass of G scpiratus and P .ostreatus by the recycling method:

(a) Microferrnentation:

Each of the organisms was maintained on MSA medium with 10% refinery effluent as sole carbon source and was subcultured at monthly intervals. For the development of

flask A were oven dried at 60° C for 18 hrs in order to gel the dry weight(W') of the cells. The washed growth from flask B was later reinoculated into 5 litre MSLM in a 10 L glass fermentor incorporated with 100% refinery effluent as carbon source (first fermentation). some glass beads were kept in the fermentor to break the clump of the cells and after 15 days of fermentation a pedrollo pump (Model no. PKM 60, 0.5 HP, Germany) was used to pump the cell and fermentation broth to a filtration unit and the cells were collected and dry weight of the cells were taken (W''). The filtered broth marked as M' was transferred to another sterile 10-litre glass fermentor for the second fermentation (Plate 4).It was then incubated at room temperature for 15 days. After 15 days of incubation, the cells and the broth were transferred to the filtration unit using the pump and the dry weight of the filtered cells (W''') was taken. The spent medium from the liltration marked as Mil was kept in the 10-litre ferrnentor for inoculation with fresh inoculum (third fermentation). After the third fermentation. The dry weight of the cells (Willi)was taken.

2.14 Determination of Biomass protein content:

The crude protein content of dry cells of G. *sepiratus* and *P. as/rea/us* was determined by the MicroKjeldahl method described earlier in section 2.7(ii).

Plate 2.4: Recycling for Biomass production of G. *sepiratus* and *P. as/rea/us.*

2.15 Determination of Biomass fat content:

One gram of dried cells was taken in a 10 ml test tube and l ml of concentrated. Hel was added 10 it and the mixture wa heated until it became dark brown according to the methods or lumbe (1963). The mixture was allowed to 001 and then t the contents of the test tube 5 ml Of 1(N) Hel and 3 ml of ether was added. The test tube was stoppered and the contents of the test tube was shaken vigorously and allowed for phase separation. Then the aqueous part was transferred into a centrifuge tube and 2ml of ether were added. The ether extract was transferred in a pre-weighed distillation flask and was measured using a balance. Then the ether was distilled off in a water bath and the flask re-weighed. The difference in weight was used to calculate the percentage lat content of the dried cells (Appendix).

2.16 Determination of Biomass total carbohydrate:

Total carbohydrates was determined using the following calculation:

Carbohydrate content *CY0)* = 100 - (%moisture + %ash + % Protein + %[at)

(Since the fibre content of fungal growth is very insignificant).

(ii) Preliminary assessment of G. *sepiratus* as animal feed.

Two sets of African hamster of 4 weeks old (each set contains 2 hamsters) and a control set contains two African hamster were used for this purpose. The first two sets were fed with O.5g of dried G. *sepiratus* cells mixed with 30g of normal feed (a mixture of corn, rice, and guinea corn grains). The control set was on 30g of normal diet (a

mixture of corn, rice and guinea corn grains) 4 weeks and the effect of the meals were observed both on the control and G. *sepiratus* fed African hamster.

CHAPTER THREE

RESULT.

3.1 Isolation of fungi from decayed wood and saw dust:

Thirty-two distinct colonies were isolated from samples of decayed wood. The colonies were divided into four groups: (i) A 1- A 10 (ii) B 1- B II (iii) SP 1- SP7 and (iv) WI- W-L

Twenty-two distinct colonies were isolated from sawdust. These were divided into three groups:

(i) AI3I-ABII

(ii) BW 1- IlW9 and

(iii)S.'\ 1- SA~.

3.2 Identification of isolates from decayed wood and sawdust:

From the microscopic an I morphological observations, the microorganisms Id\.~1 ti li ed nre shown iii Tabl ' I und Table 2. SP5, A6, A 7.13 I0, B2 and 83 were round to be unidentified species in case of isolation of fungi from decayed wood. AI33. AB7, B WS. 13;V9 and AI3 10 were found to be unidentified species in case of isolation of fungi I'rlliil -:,\'ll usl (Table 2). Table 3 shows the distribution of microbial non. in samples 0f dccavcd \\ ood and sawdust.

Table 1

Identi lication of isolates from decayed wood

Organism	Microscopic description	Morphology on PDA	Fungus identi tied
SPI 131 139 W2 AS 136 B4	Septate conidio-sphores that form foot cell spores Rough, in black color and spherical.	White colony that becomes rough, black in color and spherical.	*Aspergillus niger*
SP2 SP4 A6 A-+ SP6 AIO	Septate conidio-sphore, aggregate into clusters of sterigmata,	Aerial hyphae in broom like fashion blue-green ill colour.	*Penicillium sp*
SP3 1\1 *t\2* B7 A3	Non-septate.short hyphal.sphcrical HnJ smooth.	Alternate ring or pink and white fuzzy colonies changing colour to pink, purple or yellow.	*Fusari 1/111 sp*
SP7 138	Broken hyphae arthrospores are Oval.	Initially white but later becomes yellowish-brown and powdery.	*Gleophylum sepiratus*
Bil AS WI A9	Broken hyphae, rooted in medium, spores are attached to mycelia.	deeply Initially-white and later becomes concentric with browning and later a hard crust.	*P/eurot us ost reo/ us*
W3 W4 85	Broken hyphae and arthrospores, wh ite, later dark brown.	Initially white cottony, colonies that later becomes concentric dark brown.	*(;feo/)llY/1I1J1 sp*

Table 2

Identification of isolates from sawdust

Organism	Microscopic description	Morphology on PDA	rungi identified
BW3,AB9 BWI,SAI AB6,BW6 ABS,BW5	Same as srI	Same as SPI	*Aspergillus myer*
BW2,ABI AB2,SAI	Same as SP2	Same as SP2	*Penicillium* sp
AB4,AB5 AB9,ABII	Same as SPJ	Same as SP3	*Fusuri IIII/* sp
SA2,BW4	Ascospores with lhi k stroma, aerial hyphae with conidia cl ustered at the tip.	Cushioned shaped and bright-green in colour.	*Tri chode rn m sp*
BW7,BW9	Same as WJ	Same as WJ	*Gleophylum Sp.*

Tahle J

Relative distribution of isolates in decayed wood and sawdust.

Isolate	Distribution		% distribution	
	Wood	Sawdust	Wood	Sawdust
A. niger	7	8	21.9	36.4
Penicillium sp	5	3	15.6	13.6
Fusarium Sp.	5	3	15.6	13.6
Gleophylum Sp.3	2	3	9.4	9.1
G *sepiratus*	2	0	6.3	0
P. ostreatus	4	0	13.0	0
Trichoderma spO	2	3	9.1	9.1
Unidentified	6	4	18.8	18.2

3.3 Utilisation of refinery effluent by isolated fungi

Cl. *sepirutus, P. ostreatus, Gleophylum Sp., A. niger, Penicillium Sp.* and *lusuriutn Sp.* showed significant growth on MSA medium incorporated with refinery effluent as sole carbon source. These isolates were then used to determine the best degrader by measuring mycelial extension rate in MSA medium.

3.... Selection of best degraders:

The results are shown in Tables 4a, 4b and 4c. The mean mycelial length of the isolates (Table 4b) *A. niger, Penicillium Sp.* and *Fusarium* SjJ. are 7.20, 7.08 and 6.83 IIIIII \\ hercas for G. *sepiratus, P. ostreatus* and *Gleophylum Sp.* the values arc 6.00,5.3 J and -l.7lllllll respectively. The mean growth rate/day (Table -lb) for the isolates also indicate that the lower fungi can utilise refinery effluent at a higher rate within a short period compare to the higher fungi. The results in Table 4c show that statistically there is no significant statistical difference between growth of the organisms CP>0.05) over 25 d<1Y-or incubruion, Though the low r fungi can efficiently use refinery effluent in a short period oltimc. the slow degraders namely, *Cl.seplratus* and *Piostreatus* arc selected to LIS' lor further research work.

Table 4a

Utilisation of Refinery Effluent by isolates.

	Length ofmycelia (111m) at different day							
Organisms"	3	6	9	12	15	18	21	25
Gs	-	3.0	5.0	6.2	8.0	8.6	8.6	8.6
Po	-	2.5	4.0	5.6	6.8	7.6	8.0	8.0
G	-	2.0	3.5	5.3	6.2	6.6	7.0	7.0
An	3.5	4.3	6.8	8.6	8.6	8.6	8.6	8.6
P	2.8	4.6	6.2	8.6	8.6	8.6	8.6	8.6
F	2.0	3.8	5.8	8.6	8.6	H.6	8.6	H.6

* (is- (; *scpiratus;* Po- P. *us/rea/us,* U- *Gleophylum Sp.* ,An-A. *niger,* P- *Penicillium Sp., 1:-Fusarium Sp.*

Table 4b

Determination of means growth ancl mean growth rate/clay of the isolated species

Source	Mean growth (mm)	* Mean growth rate (nun/day)
C. sepiratus	6.00	0.41
P. ostreatus	5.31	0.33
Gleophylum Sp.	4.78	0.29
A. niger	7.20	0.57
Penicillium Sp.	7.00	0.57
Fusarium Sp.	6.83	0.57

* calculated over the number or days until the day of observation of a zero or negative gro\\ til rutc.

Table 4c

Analysis of variance of growth of the isolated species.

Source	Of	SS	Ms	F	p
Organisms	5	41.86	8.37	1.19	0.33
Error	42	295.86	7.04		
Total	47	337.72			

Key:

01'= Degree of freedom (n-I) ss = Sum of square (x2)

Ms = Mean slim F = Fisher distribution P = probability

N = Number of variables.

3.4 Effect of various factors on biodegrading capability of *Gisepiratus* and *Piostreatus*.

(i) Inoculum volume

The results are shown in Table Sa, 5b and 5c. Table 5b shows the mean growth of G. *.\(')iru/lls* in MS medium using different inoculum volumes. It also shows that the Jl1e~111 groxvtl: rate/day is higher in MS medium (1.888 g/I) using 10m!. of inoculum compared to thc mean growth rate/day in MS medium using 5,15 and 20 ml. of inoculum. Table 5c reveals that there is a significant statistical difference ($P<0.05$) in the growth of G. *sepirutus* in MS medium using different inoculum volumes. For *P. ostreat us,* the mean gro« th rate /day (1.443 g/I) is also higher in case or 10 ml inoculum. There is also a signi Iicnnt statistical difference ($P<0.05$) in the growth of *P. ostreatus* in MS medium using different inoculum volumes. There is no significant statistical difference in the gro\\th *\)U; scpiratus* and *P. ostreatus* ($P>0.05$) over the fermentation period 01'21 days. So the optimum volume of inoculum is 10 ml per 125 ml of Icrmcnunion medium i.c. about 8 percent of the volume of fermentation medium.

Table Sa

Effect of inoculum volume on biodegrading capability of G. *sepiratus* and *P. ostreatus*.

"Organisrn	Inoculum volume (rnl)	Dry cell wt. (g/lit.) at cliff 'rent day 5	9	12	15	18	21
	5	8.0	8.4	9.6	12.0	15.6	15.6
	10	10.8	18.0	21.0	30.0	34.5	34.0
C.S	15	11.0	19.8	20.8	32.2	36.5	32.0
	20	10.7	20.0	22.0	28.0	28.8	28.3
P.o	5	2.4	4.0	8.0	12.0	15.0	13.0
	10	3.2	4.8	18.0	22.0	26.0	~
	15	4.0	5.7	19.~	23.0	25.6	23.4
	20	4.2	6.0	16.8	22.0	23.2	21.8

*G.s- *U.sepirutus*, *P.o*- *P.ostre(/fus*

Table 5b

Effect or inoculum volume on the mean growth and mean growth rate/day of *U. sepiratus* and *P. ostreatus.*

Species	Inoculum vol.(ml)	Mean growth(g/I)	Mean growth rate/day(g/I/day)
G. *sepiratus*	5	11.43	0.683
	10	24.72	1.888
	15	25.38	1.777
	20	23.13	1.601
P. as/rea/us	5	9.07	0.720
	10	16.92	1.-+43
	15	16.92	1.30 I
	20	15.67	1.211

Statistical analysis of G. *seplratus* find *P. ostreatus* using elifferent ina .ulum volume.

Factors	OF	SS	MS	F	p
Organisms"		510.91	510.91	7.42	0.009
Concentration#	3	958.1	39.37	4.64	0.007
Concentration@	3	70.72	23.57	0.34	0.795
Error	40	2752.69	68.82		
Total	47	4292.42	91.33		

* Probability in case of G. *sepiratus* and *P. ostreut us.*

#" "" " C. *sepiratus.*

@" "" " *P. ostreatus.*

(ii) Initial pH of the medium:

The results are in Table 6a, 6b and 6c. The mean growth of G. *Sepiratus* (Table 6b) at pH 6 is higher *(25.05g/l)* than at pH 4 and pH 8 (11.53 *gil* and 10.87 *gil).* The table indicates that the mean growth rate/day for C. *sepiratus* at pH 6 is 1.550 *gil* whereas the respective values at pi [4 and pll 6 are 0.663 *gil* and 0.495 *gil.* The results in Table 6<.show that there is a significant statistical difference (P<O.05) in the growth of CJ. *sepiratus* at pll 4, 6 and 8. For *P. ostreatus,* the mean growth at pII 6 is 0.765 whereas the mean growth at pH 4 and pH 8 are 0.645 *gil* and 0.637 *gil.* There is no significant statistical difference (P> 0.05) in the growth of *P. ostreatus* at pH 4, 6 and 8. There is also ıı ı) signilicant statistical difference (P...U.U5) ill growth ol *G. sepiratus* and I'. *ostreatus* over the said period of incubation. I knee an initial pH of 6.0 appears to be the optimum tor both G. *sepiratus* and *P. ostreatus*.

Table 6a

Effect of pl I of the medium on utilization of refinery effluent by *G.sepirotus* and *P. estreat us.*

"Organism	Initial pH	Dry cell wt.(g/I) at different day 5	9	12	15	18	21
	4	8.0	*8A*	9.6	12.0	15.6	15.6
G.s	6	10.8	18.0	21.0	30.0	36.5	34.0
	8	3.2	4.8	10.0	15.0	16.6	15.6
	4	2.4	4.0	S.0	12.0	15.0	1:"LO
P.o	()	4.8	10.0	12.0	16.0	22.0	20.0
	8	2.0	5.0	7.0	11.0	13.0	13.0

*G.s =: G. *sepiratus, P.o = P. ostreatus*

Table 6b

l.Ilcct 01' initial pIlon the mean growth and mean growth rate/day 0f G. *sepiratus* and *l),* *()S/I'eU/IIS,*

Species	pH	Mean growth	Mean growth rate/day (g/I)
G. *sepiratus*	4	11.53	O.6lD
	6	25.0S	I.SSO
	8	10.87	0.495
P. ostreatus	4	9,07	0,645
	6	14,13	0,765
	8	10.87	0,637

Table 6c

Analysis or variance of growth of G. *sepiratus* and *P.* ostriatus at different pi r.

Source	DF	SS	MS	F	p
Organisms		162.6	162.6	2.69	0.1 I
Error	34	2057.1			
Total	35	2219.7			

Source@	OF	SS	MS		p
pH	2	768.6	384.3	7.87	0.005
Error	15	732.9	48.9		
Towl	17	1501.5			

Source#	OF	SS	MS	F	P
pH	2	77.1	38.5	1.21	0.326
Error	15	478.6	31.9		
Total	17	555.6			

* Probability in case of b th of G. *sepiratus* and *P. ostreatus.*
(ci - Probability in case of G. *sepiratus.*
\# – Probability in case of *P. as/rea/us.*

(iii) Temperature of incubation:

Fermentation was carried out at different temperatures between nOC to 40°l' for 21 days using the initial pH of 6.0. The results are given in Table *Te.* 7b ancl 7c. The mean growth of *U sepiratus* in refinery effluent incorporated MS medium at 20, 30 and 40°C are 10.23 *gil,* 24.33 *gil* and 10.63 *gil* where as the respective growth ratelday are *0.793 gil,* 1.928 *gil* and 0.678 *gil* (Table 7b). Table 7c reveals that there is a significant statistical difference *(P<O.S)* in growth of G. *sepiratus* at different incubation temperatures namely 20, 30 and *40DC.* the mean growth of *P. as/rea/us* at different temperatures is g.07 *gil,* 13.43 *gil* ancl 9.20 *gil.* The mean growth rate /day at *30DC* was higher (1.166 gil) than those at 20 and *40DC* respectively. There is no significant statistical difference (P>O.OS) in growth of *P. as/rea/us* at different temperatures. There is also no statistical difference in growth of *U sepiratus* and *f. ostreatus* at different temperatures over the period of fermentation. So it may be concluded that optimum utilisation of effluent takes place in the temperature range of *28-30DC.* for subsequent studies, a temperature of 30oC was therefore selected.

Table 7a

Effect or temperature of incubation on effluent utilization by *CJ.sepira/us* and *P. ost real us.*

Organism	Tempt. (oc.)	Dry cell wt. *(gil)* at different day or incubation 5	9	12	15	18	21
	20	4.0	4.4	9.0	12.0	16.0	16.0
G.s.	30	12.0	14.0	18.0	32.0	40.0	40.0
	40	5.3	6.0	10.0	13.0	15.0	14.5
	20	2.0	3.2	6.0	11.0	13.0	13.0
P.o.	30	3.7	5.9	10.0	15.0	23.0	13.0
	40	3.2	5.U	8.0	11.0	15.0	13.0

*Gs- *G.septmtus,* Po- *P. ostreatus*

Table 7b

Eflect of temperature on mean growth and mean growth rate/day of G. *sepiratus* and *P. ostreatus*.

Species		Mean growth (g/I)	Mean growth rate/ day(g/I)
G. *sepiratus*	20	10.23	0.793
	30	24.33	1.928
	40	10.63	0.678
P. ostreatus	20	8.07	0.661
	30	13.43	1.166
	40	9.::W	0.595

Table 7e

Analysis of variance of growth of G. *sepiratus* and F. *ostreatus* at different temperatures.

Source	Dr	SS	MS	F	P
Ore.*	I	210.2	210.1	2.89	0.10
Error	34	2475.1	72.8		
Total	35	2685.3			

Source	OF	SS	MS	F	P
Temp/G. *sepiratus*	2	773.3	386.7	2.89	0.10
Error	15	1029.3	68.6		
	17	1R02.6			

Source	DF	SS	MS	F	P
Temp/P. *ostreatus*	2	96	415	1.25	0.351
Error	15	576.4	JR.4		
Total	17	672.4			

(iv) Time period of fermentation:

The optimum period of incubation was determined by carrying out the fermentation for 21 days, keeping the initial pH of the medium at 6.0. The results are given in Tables Sa, 8b and 8c.The mean growth rate *lday* (Table Sb) of G. *sepiratus* on 1Sth (by 0f fermentation is higher (2.003 *gil)* compared to the mean growth rate 01 *P. as/rca/lis* (1.428 *gil).* There is no statistical difference in growth of G. *sepiratus* and *P. ostreatus* (P 0.05) over 25 days of fermentation (Table 8c). So the optimum period of fermentation for subsequent experiments is] 8 days of fermentation.

Effect of incubation period on utilisation of effluent by *G.sepira/lis* and *Piostreatus.*

"Organism	Growth *(gil)* at di ITerent period of incubation (Days)						
	5	9	12	15	18	21	25
G.s	12.8	20.0	26.0	30.0	46.0	43.0	43.0
P.o	6.6	8.0	12.6	19.0	36.0	33.0	33.0

**U s-Gisepirutus, P. o-P. ostreatus*

Table Rb

Determination of mean growth and mean growth rate of G. *sepiratus* and *P. as/rea/lis* over a period of 21 days.

Species	Mean growth *(gil)*	Mean growth ratelday *(gil)*
G. *sepiratus*	31.54	2.003
P. ostreatus	21.17	1.428

Table 8c

Analysis of variance of growth or G. *sepiratus* and *P. ostreatus.*

Source	DF	SS	MS	F	p
organisms	1	376	376	2.31	0.154
Frror	12	1953	163		
Total	13	2329			

v) Volume! of the fermentation medium.

In all the previous experiments, the volume of the medium was 125 ml in a 250 llll Erlenmeyer flask. The effect of the volume of the medium was therefore studied by taking different volumes of fermentation medium in 250 rnl. Erlenmeyer flasks which w crc then iucub.ucd lor 21 days at JO"c. Tables I)a, ()b and IJc show the results o I the experiments, The results in Table 9b show that the mean growth of G. *sepiratus* in 125 llll MS medium (fermentation medium) is higher *(27.5 gil)* when compared to the mean growth obtained in other volumes of fermentation medium. From Table 9c it has been shown that there is a significant statistical difference in the growth of G. *sepiratus* ($P<0.05$) using different volumes of fermentation medium. The mean growth rate/day *(2.428. gil)* is also higher compared to the values obtained for 50, 75, 100 and 150 rnl fermentation medium. For *P. ostreatus,* the mean growth rate/day (1.342 *gil)* obtained using 125 llll Iennentation medium is higher than the values obtained for other volumes (Ii' II:I'IIIcnt<IIioII medium. There is a significant statistical difference in the growth o I J'. *ox/real us* (1.342 *gil).* When we consider the growth 0r both G. *sepiratus* and *P ostreatus,* there is a signi ficant statistical difference ($P<O.04$) over 21 days of fermentation. So the optimum volume of the fermentation medium is 125 ml in 250 ml Erlenmeyer flask.

Table 9a

1.Ilcct of volume of the medium on utilisation of effluent by G. *sepiratus* and *P. ostrea/lis.*

*Organisl11s	vol. of medium	Dry cell wt. *(gil)* at different day 5	y	12	15	I~	21
C.S	50	2.0	4.0	4.4	5.3	9.0	6.0
	75	1.8	5.9	8.0	9.0	16.0	12.0
	100	10.5	12.0	18.0	23.0	34.0	34.0
	125	12.0	14.0	23.0	38.0	40.0	38.0
	150	12.5	13.0	24.5	33.0	36.0	33.8
	50	1.0	2.0	2.5	3.2	6.7	5.5
P.o	75	2.5	3.7	5.9	8.0	10.0	11.0
	100	כ·י־	6.4	10.0	12.0	20.0	16.0
	125	4.6	12.6	19.0	23.0	29.8	28.0
	150	4.9	13.4	18.5	22.1	25.0	25.2

**O.s-o.sepiralus, .P.o-P. ostreutus.*

Table 9b

Determination of mean growth and mean growth rate/day of G. *sepiratus* and *P. as/rea/us* using different volumes of fermentation medium.

Species	Volume	Mean growth *(gil)*	Mean growth rate/dayrg/l)
G. *sepiratus*	50	5.12	0.422
	75	8.73	0.75&
	100	21.58	1.746
	125	27.50	2.428
P. as/rea/us	50	3.48	0.412
	75	6.85	0.593
	IU0	I I .27	U.995
	125	19.50	1.220

Table 9c

Analysis of variance of growth of *U sepiratus* and *J'. ostreatus.*

Source	OF	SS	MS	r	P
VoUG.sepiratus	4	2445.6	611.4	7.46	0.000
Error	25	2048.4	81.9		
Total	29	4494.1			

Source	OF	SS	MS	F	P
Vol.! *P. ostreatus*	4	177.9	294.5	6.95	0.00
Error	25	1058.7	42.3		
Total	29	2236.7			

Source	OF	SS	MS	F	P
Vol.IBoth	1	497	497	4.28	0.04
Error	58	6731			
Total	59	*7127*			

3.5 Effect of carbon, nitrogen and potassium on the hiodegruding capabiliry of G. *sepiratus* and *P. ostreatus.*

(i) Effect of different concentrations of refinery effluent:

Refinery effluent was used here as sole carbon source, different concentrations of eftluent were tested to determine the optimal concentration degradable by *Gisepiratus* and *P. ostreatus.* The Dry cell wt. *(gil)* was measured on 18th day of fermentation. The results are shown in Table IDa and 1Db. The results (Table IDa) show that refinery effluent at 10% concentration gives maximum cell growth *(gil),* a higher concentration does not increase the cell yield significantly. From Table lOb we can see that there is no significant statistical difference (P>0.05) in the growth of both *U. sepiratus* and P. *as/rea/us.*

Table lOa

Effect of refinery effluent concentration on the growth ofG. *sepiratus* and *Piostreatus*

Isolate	Dry cell wt.(g/I) at different conc.(%)					
	1.0	5.0	7.5	10	15	20
C. *sepiratus*	7.5	16.0	28.0	38.0	38.4	39.0
P. ostreatus	3.5	12.0	18.0	24.6	32.5	32.0

Table lOb

Analysis of variance of growth of G. *sepiratus* and *P. as/rea/us.*

Source	DF	SS	MS	F	p
Species	I	164	164	1.05	0.33
Error	10	1554	155		
Total	11	1717			

(ii) Effect of different nitrogen sources.

The data are presented in Table))a and 11b. The result shows that NH4N03 is the best nitrogen source for the two organisms, closely followed by NaN03 and urea. The results in Table 11b reveals that there is no significant statistical difference (P>O.05) between the growth of G. *sepiratus* and *P. as/rea/us* under these conditions.

Table lla

Effect of urea and inorganic nitrogen sources on the growth of G. *sepiratus* and *P. ostreatus*.

Organism	Growth *(gil)* using nitrogen sources				
Gisepiratus	*38.0*	23.5	34.5	28.0	30.3
Piostreatus	*34.6*	19.2	27.6	21.0	29.9

Table 11b

Analysis of variance of growth of G. *sepiratus* and *P. as/rea/us.*

Source	OF	SS	MS	F	p
N.sources	I	48.4	48.4	1.34	0.28
Error	8	288.8	36.1		
Total	9	337.2			

(iii) Effect ofNH_4NO) concentration on growth of G. *sepiratus* and *P. ostreatus.*

The optimal concentration of NH_4N03 was next determined using different concentrations of NH_4N03. The concentration of refinery effluent was 10% of the fermentation medium and fermentation period was 21 days. Table 12a shows that NI-J.jN03 at 98 mg/l OOml is the optimum for both G. *sepiratus* and *P. ostreatus.* Table 12h shows that there is no significant statistical difference (P>O.05) in the growth of G. *sepiratus* and *P. us/rea/us* over the chosen period of fermentation.

Table 12a

Effect ofNH_4NO_3 concentration on growth of *Gisepiratus* and *P. ostreatus.*

Organism	Dry cell *wt.(g/l)* at different NH_4NO_3 conc.(mg ofNH_4NO_3 per 1001111)				
	24	48	72	98	120
G. *sepiratus*	15.0	23.0	28.9	42.5	43.6
P. ostreatus	6.0	16.2	22.0	35.6	37.0

Table 12b

Analysis of variance of growth of G. *sepiratus* and *P. as/remus*

Source	OF	SS	MS	F	p
Substrate	1	117	117	0.76	0.41
Error	8	1239	155		
Total	9	1356			

(iv) Effect of different concentrations of $Kf\text{-}hP0_4$ and K_2HP0_4.

The data are shown in Table 13a and 13b. The results in Table 13a reveals that the optimum ratio of" $Kl\cdot hP0_4$ and $K21IPO\text{-}l$ is 1.50: 1.25 for both G. *sepiratus* and *P. ostreatus*. However, from Table 13b we can see that there is no significant statistical difference (P>O.05) in the growth of G. *sepiratus* and *P. ostreatus* over the said concentration range.

Table 13a

Effect of combination of KI-I_2P0_4 and K_2HP0_4 on G *sepiratus* and *P. as/rea/us.*

Organism	Dry cell wt. *(gil)* at different ratio of KH_2P0_4 and *K2I-1P0₄(g/l)*				
	0.25 :0.20	0.50:0.40	0.98 :0.7	1.25: 1.00	1.50: 1.25
Gsepim/us	)9	23	28	36	38
Piostreatus	)0	13	20	25	25

Table 13b

Analysis of variance of growth of C. *sepiratus* and *P. as/rea/us.*

Source	DF	SS	MS	I,	P
Substrate	1	260.1	260.1	4.56	0.065
Error	8	456	57		

Total	9	716.1			

(v) Effect of complex nutrients:

The effect of complex nutrients on the utilisation of effluent by *Gisepiratus* and *P.oslreulus* are shown in Table 14a and 14b. The result in Table 14a shows a remarkable growth on sawdust and RE + Sawdust for both organisms. Other wastes did not have any significant effect on the growth of G. *sepiratus* and *P. ostreatus*. However, results in Table 14b reveals that there is no significant statistical difference (P>O.05) in the growth of G. *sepiratus* and *P. as/rea/us* for the said complex nutrients.

Table 14a

Effect of different complex nutrients on growth of G. *sepiratus* and *P. as/real us.*

dry cell wr.(g/I) using diff.cornplex nutrients

#)solate *	RE	RE+B	B	M	M+RE	R	R+RE	GC	*U*	C+RE	S	S+RE
C.s	33.5	29.2	13.0	12.2	24.0	5.0	15.3	4.2	25.3	38.0	43.5	
P.o	19.8	23.0	6.0	9.2	18.0	2.0	8.5	1.6	17.0	30.0	36.6	

* RE- refinery effluent, B-beans extract, M- maize extract, R- rice extract, G.C- guinea corn, S-saw dust.

G.s- G. *sepiratus,* *P.o*- *P. as/rea/lis*

Table 14b

Analysis of variance of growth of G. *sepiratus* and *P. ostreatus*.

Source	OF	SS	MS	F	p
Substrate		192	192	1.28	0.27
Error	20	2991.5	149.6		
Total	21	3183.6	151.6		

3.6 Determination of rate of biodegradation using the gravimetric method.

(i) Characteristics of collected soil samples.

The soil samples have the following characteristics listed below: The pH of the loamy and sandy soil is slightly alkaline but that of the clay soil is slightly acidic. The moisture content of the sandy soil is very high (94%) but that of the loamy soil is the least. The microbial count of the loamy soil is the highest *2.S* x 10^8 and 3.3 x 10^6 cfu for both bacteria and fungi. and that of sandy is the least, 4.8x 10^5 and 4.0 x 10^5 cfu (Table ISa). Table 1Sb shows that there is a significant statistical difference ($P<0.05$) in the bacterial load of all the soil samples tested. However, there is no significant statistical difference ($P>O.OS$) in the fungal count on all three samples.

Table 15a

Structural Characteristics of soil

Sample	pH	Moisture (%)	TBC	TFC	Soil identified
A	7.9	*n.7*	2.5xI0^{8}	3.3xI0^{6}	loamy
B	6.7	81.6	2.3 xl07	5.1 xl0^{5}	sandy loam(clay)
C	7.4	94.0	4.8 xl 0^{5}	4.0 xl 0^{5}	sandy learn (sandy)

TBe = Total bacterial count. TFC ;::;Total fungal count.

Table 15b

Analysis of variance of microbial load count of different soil samples.

Source	DF	SS	MS	F	p
Bacteria	2	9.023E+14	4.511E+14	156.3	0.00
Error	6	1.732E+13	2.886E+12		
Total	8	9.196E+ 14			

Source	DF	SS	MS	F	p
Fungi	2	2.706E+I~	1.353E+14	1.33	0.33
Error	6	6.166E+14	1.01912		
Total	8	8.822E+14			

(ii) Nutritional analysis of soil and refinery eftluent contaminated soil incubated with G. *sepiratus* and *P. ostreatus.*

The nitrogen and phosphate content of soil samples collected from the discharged site were analysed. Then the soil samples were contaminated with refinery effluent and were seeded with *G.sepiralus* and *Piostreatus* and incubated at *28-30*^{D}C for 25 days. At the end of the incubation period the parameters were analysed again.

The results are tabulated in Table 16. The result in table 16 indicated a slight increase in the nitrogen and phosphate content of the soil when the effluent was added to the three samples.

Table 16

Determination of total Nitrogen and Phosphate in three types of soil before and after incorporation with R.E. and Inoculation with G. *sepiratus* and *P. as/rea/us.*

Sample	N content(%)	P content (%)
L	0.5	0.2
L*	0.2	0.2
L**	0.2	0.2
C	0.3	0.2
C*	0.03	0.3
*C***	0.03	0.2
S	0.03	0.05
S*	0.07	0.08
S**	0.03	0.01

L-loamy,L *- loamy+RE with G. *sepiratus,* L**-loamy+RE with *P. ()Slreallls,C-clay, C*- clay +" ,C**-clay+ "
Svsandy, S*-salldy +" ,S**- sandy+ "

3.7 Determination of the rate of biodegradation of refinery effluent by *Gisepiratus* and *Piostreatus.*

The data are shown in Tables 17a, 17b and 17c. Table 17b shows that the mean CO_2 (%) emission for loamy soil (in case of both sterile and nonsterile soil) inoculated with *G.sepiratus* is high compared to those of clay and sandy soil. But for *Piostreatus* there is no significant statistical difference in the mean CO_2 emission in all three types of soil. The mean rate of CO_2 emission per day (%) for *G.sepiratus* in sterile loamy, clay and sandy soil are 3.08,2.00 and 2.16 whereas the mean CO_2 emission per day for *Piostreatus* in sterile loamy, clay and sandy soil are 1.92,2.00 and 2.12. From Table 17c we can see that there is a significant statiscal difference in the CO_2 emission ($P<0.05$) in loamy, calay and sandy sterile soil by G. *sepiratus.* There is also a significant statistical difference in CO_2 emission in the three sterile samples ($P<;0.05$) by *P. ostreatus.* The statistical di fference is significant in CO_2 emission by G. *sepiratus* and *P. ostreatus* in three sterile soil samples. The statistical difference is insignificant ($P>0.05$) in CO_2 emission by G. *sepiratus* in sterile and nonsterile samples.

Table 17A.

Determination of rate of biodegradation by G. *sepiratus* and *P. as/rea/us* (% CO_2 emission).

Samples		% CO_2 emission at different day					
		5	10	15	20	25	30
Sterile soil + R.E+G.seP.	L	65	76	83	88	76	60
	C	32	42	52	56	50	50
	S	30	36	56	60	54	48
Sterile soil +R.E + *P.ost.*	L	22	49	50	58	48	40
	C	30	40	53	58	42	36
	S	24	36	50	58	55	52
Nonsterile soil + R.E. +G.seP.	L	20	36	66	70	66	60
	C	25	46	55	65	52	50
	S	20	36	50	53	48	40
Nonsterile soil + R.E + *P.ost.*	L	30	49	59	68	54	47
	C	28	45	52	66	50	42
	S	20	36	50	53	48	40

G.s- G. *sepiratus, P.o-P. as/rea/lis*
L- Loamy; C- Clay; S- Sandy.

Table 17b

Determination of mean CO_2 and mean rate/day (%) $C02$ emission.

"Samples		Mean C02 (%)	Mean rate/day CO_2 ermssion
SS+RE+G.sepira/us	L	74.66	3.08
	C	47.00	2.00
	S	47.33	2.16
SS+RE+P.os/rea/us	L	44.50	1.92
	C	43.16	2.00
	S	45.83	2.12
NS+RE+G.sepiralus	L	53.00	2.64
	C	48.83	2.08
	S	41.17	1.92
NS+RE+P.oslreatu.s	L	51.17	2.16
	C	47.16	2.00
	S	41.17	1.92

*SS-sterile soil, RE-refinery effluent, L-loamy,C-clay,S-sandy and NS-nonsterile soil.

Table 17c

Analysis of variance of CO_2 (%) emission by G. *sepiratus* and *P. as/rea/us.*

Source	DF	SS	MS	F	P
SS+RE+GS	2	3088	1544	7.2	0.003
Error	33	7075			
Total	35	10163			

Source	DF	SS	MS	F	P
SS+RE+PO	2	115	57	0.4	0.676
Error	JJ	4780			
Total	35	4895			

Source	DF	SS	MS	F	P
GS+PO	1	903	903	4.2	0.04
Error	70	15058	215		
Total	71	15961			

Source	DF	SS	MS	F	P
Strains	4	12821	3206	19.04	0.00
Error	85	14306	168		
Total	89	27126			

SOLU'ce	DF	SS	MS	F	P
Factor	1	870	870	3.18	0.083
EITor	34	9293			
Total	35	10163			

G. *s-G.sepira/us,P,o-P. as/rea/us.*

3.8 Utilisation of other petroleum products by *G. sepiratus* and *P. ostreatus.*

For this purpose, kerosene, engine oil and crude oil were selected. The data are presented in Table 18a and 18b. From the results in Table 18a it has been shown that both G. *sepiratus* and *P. as/rea/us* can utilize crude oil as sole carbon source in MS medium. Both can also utilize engine oil but at a different rate from that of crude oil. The growth of both G. *sepiratus* and *P. as/rea/us* on kerosene incorporated MS medium is negligible. The mean growth of *Gisepiratus* in crude oil incorporated MS medium (7.27 *gil)* is higher compare to the mean growth obtained with kerosene and engine oil utilised as carbon source. The mean growth of P.ostriatus is also greater (2.10 *gil)* than the values obtained with other PHC products used as carbon source in MS medium. Table 18b shows that there is a significant statistical difference (P<O.05) in response of G. *sepiratus* to the above mentioned petroleum products. For *P. ostreatus* also there is a significant statistical difference (P<O.05) in response to these PHC components. In case of both G. *sepiratus* and *P. ostreatus* there is a significant statistical difference (P<O.05) in their growth in these PHC products incorporated medium.

Table 18a

Utilisation of other petroleum products by G. *sepiratus* and *P. ostreatus*.

"Organism	Petroleum products	Dry cell *wt.(g/l)* at diff.day						mean growth *(g/l)*
		5	7	9	12	15	18	
GS	Kerosene	0.3	0.3	0.7	1.2	1.6	1.8	0.98
	Engine oil	1.2	2.2	3.4	5.7	6.8	7.2	4.42
	Crude oil	2.4	4.8	6.8	8.6	9.8	11.2	7.27
PO	Kerosene	0.4	0.4	0.35	0.3	0.4	0.4	0.31
	Engine oil	0.4	0.3	0.5	0.6	1.2	1.2	0.70
	Crude oil	0.7	1.3	1.8	2.0	2.8	4.0	2.10
@Control	-	+	+	+	+	+	+	+

**G. s-Gisepiratus, P. o-P. oSlrealus,@* +v-negligible.

Table 18b

Analysis of variance of growth of G. *sepiratus* and *P. as/rea/us.*

Source	OF	SS	MS	F	p
Substrate+GS	2	66.85	33.42	5.3	0.02
Error	18	113.44	6.3		
Total	20	180.29			

Source	Dr	SS	MS	I'	P
Substrate+PO	2	8.941	4.47	7.85	0.004
Error	18	10.249	0.57		
Total	20	19.190			

Source	OF	SS	MS	r	P
Organism+Substr.	1	57.75	57.75	11.58	0.002
Error	40	199.48	4.99		
Total	41	257.23			

Source	OF	SS	MS	F	P
Control/orG.	6	158.91	26.48	8.99	0.00
Error	42	123.72	2.95		
Total	48	282.63			

3.9 Utilization of phenol and cyanide by *G. sepiratus* and *P. ostreatus.*

The results shown in Table 19a and 19b that G. *sepiratus* can utilize phenol and cyanide as carbon source in MS medium whereas *P. as/rea/us* is incapable of using phenol and cyanide even after three months. From Table 19b it has been shown that there is a significant statistical difference (P 0.05 for phenol) in growth of G. *sepiratus* for different concentrati n of phenol. The same thing is applicable for cyanide. There is also a significant statistical difference ($P<0.05$)in the growth of G. *sepiratus* at different concentrations of phenol and cyanide.

Table 19a

Utilization of phenol and cyanide by G. *sepiratus* and *P. as/rea/us.*

Component	Cone. (%)	Dry cell wt.(g/l) at different week interval				Mean growth
		J	7	9	12	
Control		0.05	0.1	0.15	0.20	0.12
Phenol	0.0001	1.6	2.6	3.30	6.70	3.55
	0.0002	2.4	3.2	3.80	6.8	4.05
	0.0003	3.0	4.2	5.2	5.6	4.50
Cyanide	0.0003	0.1	0.6	0.8	1.4	0.75
	0.0005	0.3	0.8	1.2	1.4	0.93
	0.0008	0.02	OJ	0.7	0.9	0.48
Control		0.05	0.05	0.1	0.1	0.08

Table 19b

Analysis of variance of growth of G. *scpiratus* and *P. ostreatus*.

Source	DF	SS	MS	F	P
Phenol cone.	3	48.16	16.05	6.5	0.01
Error	12	29.82			
Total	15	77.98			

Source	OF	SS	MS	F	P
Cyanide conc.	3	1.59	0.53	3.33	0.06
Error	12	1.91	0.16		
Total	15	3.49			

Source	Of	SS	MS	F	P
Factor	1	49.53	49.53	18.2<1-	0.000
Error	30	8l.48	2.72		
Total	31	131.00			

3.10 Immobilization of *G.* sepiraus.

The beads were used to inoculate refinery effluent incorporated MS medium and then incubated at JOoe for 21 days. The dry wt. *(gil)* of the beads before and after incubation almost remained more or less the same throughout

3.11 Production of Biomass of *G. sepiratus* by the recycling method.

The results are shown in Table 20. Up to second recycling there was good growth or G *sepiratus* but after that there was a decline in the weight or the cell mass. In)0 litre ferrnentor the cell mass and broth after first fermentation was filtered and transferred to another Ierrnentor using a pumP. Because of the thick cell mass, it was not possible to transfer all the cciI growth from the first fermentor to the second fermentor. Also, the cell growth after 10-12 days of fermentation in the first cycle; the rate of growth was insignificant. [t may be due to inadequate oxygen availability in the fermentor. However, the approximate cell growth after the first fermentation, second fermentation and third fermentation in 10 litre fermentor were 11.5 *gil,S gil* and 2.0 *gil* respectively.

Table 20

Production of Biomass by recycling method.

Period of recycle	Initial pH	Adjusted pH	Dry weight of cell *(gil)*
	6.02		19.2g/l
2	5.38	6.03	9.6 "
3	5.03	6.00	2.0 "

3.12 Proximate analysis of *G. sepiratus* and *Piostreatus.*

Table 21 shows the protein, fat, ash and carbohydrate content of *G.sepiratlls* and *Piostreatus.* The crude protein, fat, ash and carbohydrate content of the fermentation broth after 18 days of fermentation was very low.

TabJe 21

Crude nutritional composition or C. *sepiratus* and *P. ostreutus.*

	Crude Protein (%)	Fat (%)	Ash (%)	Carbohydrates
Giseptratus	24.0	1.5	0.8	72.0
G.sepira/us	4.0	1.5	0.6	93.0
Ui ltrate)				
P.os/rea/us	22.0	2.5	I.J	74.0
P. ostreatus	2.5	1.4	0.6	94.5
(filtrate)				

3.13 Quantitative assessment of Biomass for Toxicity.

The two marked *African hamster* used for this experiment fed very well without any recognisable abnormality or death, they weighed 23g, 25g and 24g,26g before experimental feeding; at the expiration of four weeks there was an increase in body weight to 28g, 32g and 29g, 32g respectively but the control *African hamster* weighted 27g, 30g as compared to their original weight of 24g, 260. This means that the feed is well tolerated in the body of the African hamster, and it may be used to increa e the weight of animals.

Table 22

Quantitative assessment of Biomass for toxicity

A/dean hamster	Body WI.(o.) Initial	Final	Survival(%)
Control	24,27	27,30	100
Set A	23,25	28,32	"
Set B	24,26	29.32	"

CHAPTER FOUR

DISCUSSION

Microbes play an important role in the degradation of PHC in contaminated ecosystems. Land treatment using the biodegrading capability of naturally occurring microorganisms for mitigating the environmental polution is a new and exciting area i Biotechnology. However, not all microorganisms in the environment are able to degrad petrochemical hydrocarbon (PCHC) and its products in case of spillage or discharge. Th rate of degradation and the amount of oil removed depend on the type and quantity of o and the environmental conditions and also the soil microbial community.

The use of lower fungi *(Aspergillus niger* and *Fusarium* species) for degradation of chlorinated aromatic compound has been reported (Sahasrabudhe and Modi 1987). I this study, some lower and higher fungi namely *A. niger, Penicillin sp., Fusarium* sp *Gleophyliun sepiratus* an I *Pleurotus ostreatus,* isolnted from de .ayed wood an sawdust were identified and later tested for their biodegrading capability of refinery effluent by using the mycelial extension measurement method (Smith 1977; Van Ette 1973). The mycelial growth extension rate/day for G. *sepirutus* and *P. ostreatus* over 1 days of fermentation was almost the same in effluent incorporated MS medium as that o lower fungi namely *A. niger,* Penicillin and *Fusarium* species over 12 days o fermentation under laboratory conditions in situ. *A. niger,* Penicillin and *Fusarium* specks are nonseptate fungi whereas G. *sepiratus* and *P. ostreatus* are septate ones. Though G. *sepiratus* and *P. ostreatus* are slow degraders compared to lower fungi, these ,..ere chosen because 0f the fact that they have been reported as lignocellulose degraders

(Ejechi 1993), capable of degrading the phenolic substances from the wood surface. However there has been no report of using these in reducing the toxic pollutants from the envi ronment.

Ramadan *et, of.* (1990) observed that the size of inoculum aids in stimulating rapid growth of cells because of the fact that the more the inoculum the greater the rate of metabolism. The optimum inoculum size for G. *sepiratus* and *P. ostreatus* was 8% of the fermentation medium. The optimum pH, for the two organisms was found to be 6.0 in agreement with the previous study (Ejechi, 1991) However like *Fusarium aquaeductum,* these two organisms, G. sepiratus and *P. ostreatus* especially *P. as/rea/us* are tolerant to the fluctuations in the pH of effluent (Rees and Dickinson,1977). These organisms were later tested for their optimum growth in MSA incorporated with effluent as sole carbon source at different incubating temperatures of 20°C, 28°C and 40°C for 21 days; Growth was observed at all temperatures. But the growth at 28°C was more luxuriant than at 20°C and 40°C. This is in agreement with the findings by Ejechi (1993). The optimum concentration of effluent for best utilisation is 10% though at higher concentration there is no inhibitory action. This may be due to the insignificant amount of PHC present in effluent compare to the crude oil which has an inhibitory action above 5% un some lower fungi (Bello, 1995). NH_4NO) at 0.098% was found to be the optimum followed by $NaNO_J$ and urea at 0.098% *(gil* 00 rnl) concentration. The optimum concentration of KH_2P0_4/K_2HP0_4 was *0.15/0.12 (gil* 00 ml). This agrees with the findings of Bumpus *et. al.* (1985) and Aust (1990) that white rot fungi only need a small amount of nitrogen and phosphorous. These organisms (G. *sepiratus* and *p. ostreatusy* were cultivated on complex nutrients, namely sawdust, rice beans and maize husk etc.

Generally complex media containing natural origin precursors, vitamins, inorganic materials and organic nitrogenous substances which can promote microbial growth when used as carbon and energy sources or when used with C and N source in a particular medium as a source of growth stimulant (Bello, 1995). Eftluent contaminated MS medium forti tied with sawdust showed tremendous growth or G. *sepiratus* and *P. ostreatus* compared to the growth of G. *sepiratus* and *P. ostreatus* in only effluent contaminated MS medium. This may be due to presence of lignolytic materials in the sawdust (Ejechi 1991). Soil samples that were taken at the dumping site of the refinery effluent were analysed for their topography, pH, moisture content, total bacterial count, total fungal count and nitrogen and phosphorous content (Black 1965). The soil samples were classified as loamy, clay and sandy. Their total microbial count for loamy soil is highest at 2.5 X 10^8 and 3.3 X 10^6 for bacteria and fungi respectively with the sandy soil having the lowest count of 4.8 X 10^5 and 4.0 X 10^5 for bacteria and fungi, respectively. The nitrogen and phosphorous content of the loamy and clay were slightly higher having a phosphorous level of 0.2% and 0.3% whereas sandy soil has 0.05% which is the lowest; The nitrogen content was comparably high for loamy (0.5%) followed by clay (0.3%) and sandy (0.05%). Though this is very low [or degradation by bacteria in the soil (Atlas and Bartha, 1973), some higher fungi can proliferate at very low level of phosphorous and nitrogen (Bumper 1985,and Aust 1990). There was an increase in the nitrogen and phosphorous content of soils contaminated with refinery effluent; This could be attributed to the activities of the nitrogen fixing bacteria whose presence have been reported by other workers (Odu, 1972; 1978; Gudin and Syratt, 1975). In situ nitrogen fixing

capabilities of heterotrophic hydrocarbon degrading bacteria have also been reported (Coty, 1967).

The rate of biodegradation of the refinery effluent by G. *sepiratus* and *P. as/rea/us* was assessed by gravimetric method using emission of C02 as a yard stick for the three soil samples. It was observed that the mean *COl* (%) emission for loamy soil (in case of both steri Ie and nonsterile) contaminated with refinery effluent and inoculated with G. *sepiratus* has the greatest emission compared to other soils. But for *P. ostreatus,* sterile sandy soil has the least emission compared to other sterile soils. Both isolates in nonsterile soil were found to compete favourably with other soil microbes. Decrease in the emission of C02 (%) immediately after contamination with refinery effluent could be due to the toxic effect or other unfavourable conditions which may occur as a result of the introduction of refinery effluent (Jenser 1975). The gradual increase in the emission of CO_2 (%) after the initial repression could indicate the adaptation of these organisms to the new environment, also the pollutant refinery effluent could stimulate the growth of the resistant strain of these fungi though the rate of biodegradation of PCH are dependent on temperature, concentration of inorganic nutrients, extents of dispersion of refinery effluent, the abundance and kind of microbes, and the chemical composition of refinery effluent (Zobell, 1969).

Other petrolewum products (Kerosene, Engine oil, and crude oil) were examined for their ability to support the growth of both G. *sepiratus* and *P. ostreatus,* The result shows that G. *sepiratus* was able to metabolize all the products efficiently while *P. as/rea/us* was not able to utilize Kerosene and had poor utilization of crude and engine oil. This result compared favourably with the finding of Walker *et. al.(* 1976), where it

was recorded that crude oil are most susceptible to degradation than the refine products of crude oil due to increased aromatic content of the refined products. For safe disposal of refinery effluent, spent refinery effluent obtained from the first fermentation was used as substrate for the second fermentation and the spent refi nery effluent from the second fermentation was used as substrate for the third fermentation so that the discharged effluent does not contain the toxic pollutants at high concentration. Then the liquid waste after the third fermentation may be applied directly to land as irrigation water and fertilizer when they are claimed to have a number of beneficial effects on the soil and plants (Stanbury and Whitaker, 1984). Growth of these organisms (O. *sepiratus* and *P. ostreatusy* in the two most toxic components of refinery effluent (phenol and cyanide) were examined by using them as sole carbon source in MS medium and growth was recorded for G. *sepiratus* which shows the ability of the fungus to use these as carbon source. Recent work shows that organisms can detoxify cyanide by converting it to non-toxic form of amide (Haris *et. af. 1987).*

HC'N + H20 -------) HCONH2

Though the utilization of both compounds takes a very long time, it agrees with the findings of Haris *et. al.* (1987) that fungal system works well with cyanide containing wastes (Wainwright 1992). Sahasrabudhe and Modi (1987) used immobilized cells of Aspergillus niger in calcium alginiate to study the dechlorination of 2.CB 3-CB, 4-chlorophenoxyacetate and 2,4-0, at a O.5mM concentration, This enabled them to use the immobilized cells for five successive cycles whereas the free mycelia cells lysed at the end or the second cycle. Immobilization or *Glcophylum scpiratus* may be improved by using other techniques and then can be used as substrate for few successive cycles of

fermentation for production of this organism at less cost to lise as animal feeds as well as its use for biodegradation and biorernediation.

In view of the ever-increasing population of the world effort are made to source for food proteins that are of direct microbial source both for human and animal consumption. Human consumption of yeast are in small quantities to supply protein, vitamins, and minerals; their antitoxicant properties stabilize food products and they are therefore incorporated into the body flours, cereals, soups, and sauces, as texture and flavour-enhancing components. They are also used as diet food because of their low-calorie content (lnchauspe, 1986). In our preliminary analysis of *Gleophylum sepiratus* cells, we found that it has a total protein of 24%, which is adequate for normal growth and production of both layers and broilers chicken (Horn, 1978). But the fat content is low compared to other animal feed (Oyenuga, I968).Even though some nutririonists stated thai yeast consumption could lead to an over production of uric acid in the hlooe.!stream which may cause gout and urinary lithiasis (Sasson 1988). In 1984 Vrignand fed undernourished children with dried lactic yeast that have been deprived of 50% of its nucleic acid content: There was aJ1 increase in the weight of the children and adult for a minimum amount of 1Og/day per child and 20g/day per adult. Also the unextracted yeast nucleic acid nitrogen improves the storage of vitamin A in the liver. As such, French nutritionist were of the opinion that nucleic acid should not be extracted from yeast cells which are to be consumed by human and that the eventual kidney disorders due to uric acid could be prevented through the consumption of a daily amount of yeast similar to that recommended for animal proteins (Vrignand 1984).

The toxicity of the cells were tested quantitatively by feeding some African hamster on feeds incorporated with *Gleophylum sepiratus* cells for four weeks. At the end of the four weeks, the two animals were alive and well. This could be due to the absence of toxic components in the Basidiomycetes and the level of tolerance of the animals.

CONCLUSION AND RECOMMENDATION

4.2 CONCLUSION.

Gleophylum sepiratus and *Pleurotus ostreatus* may be used to degrade some toxic components of refinery effluent. The emission of $C02$ by these organisms using gravimetric method also lend credence to their ability to degrade refinery effluent even though G. *sepiratus* tends to be the better degrader of the two organisms studied.

The synthetic medium for optimum utilisation of refinery effluent by these organisms is as follows: Refinery effluent 10 ml, NI-L,$N0_3$ 0.098 g, K2HP04 1.25g, KI-h$P0_4$ 1.5 g and other elements used in ML medium (as indicated in Appendix A), water-IOOml.pJI 6.0. The optimum temperature for fermentation is 28 - 30° C. they have the ability to grow at a very low nitrogen and phosphate content. The complex medium for optimum utilisation of refinery effluent is: Refinery effluent 10 rnl, saw dust 0.1 g, $NH.jN0_3$ 0.098 g, KH2PO., 1.5 g, K2HPO.j 1.25 g and other trace elements used in MS

medium, water 100ml, pH 6.0. The optimum temperature is also 28 - 30° C. The growth of G. *sepiratus* on some of the most toxic components of refinery effluent (phenol and cyanide) shows that G. *sepiratus* may be used to detoxify these toxic components prior to its disposal to aquatic environment or soil. The refinery effluent may be recycled for mass production of microbial biomass before its safe disposal to environment and this treated refinery effluent may be used for irrigation and other purposes. The analysis of nutritional content of the organisms (protein content 24 and 22 % for G. *sepiratus* and *P. ostreatus* respectively) and 100 % survival rate of all the experimental organisms reveals that these organisms may be used in animal feed as protein source although this requires further investigations.

4.3 RECOMMENDATIONS.

There should be further intensive studies on:

1. The use of these organi ms with other known hiodegraders for biodegradation of crude oil, refinery effluent and other recalcitrant.
2. The usc of these organisms in degrading other toxic industrial waste e. g. lignocellulose wastes from paper industry and wastes from bleaching industries.
3. The immobilization of G. *sepiratus* and *P. ostreatus* should further be carried out using other technique e. g cross-linking or entrapment using other gel matrix and encapsulation.
4. To determine whether these immobilized cells can be used for recycling of effluent purpose.
5. Establishing a suitable method for recycling of refinery effluent as a substrate for mass production of G. *sepiratus.*
6. Genetic manipulation of these organisms IJ1 order to increase their biodegrading capability of toxic pollutants in the environment.

REFERENCES

Ainsworrth, A.M. (1995) Technical information sheets No II. Isolation Techniques for Basidiomycetes. World Journal of Microbiology and Bacteriology 11: 364-366.

Airken, M.D.; R. Yenkatadin and R. L Trusine (1989) Oxidation of phenolic pollutant by lignin degrading enzymes from the white rot fungus Phanerochaete chrysosporium. Water Resource 23: 443-450.

Alexander, M. (1977) Introduction to soil Microbiology Wiley, New York pp126-180.

Amund,O.O.; C.A. Omole; N. Esiobu; and E.O. Ugoji (1993) Effects of waste Engine oil spillage on soil, physicochemical and microbiological properties.

Anderson, lO.E and K.H. Domseh (1975) Measurement of bacteria and fungal contributions to respiration of selected agricultural and Forest soil. Canadian 1. of Microbiol. 21: 3I4-322.

Anderson, J.W. and J. M. Neff. (1974) Accumulation and release of petroleum hydrocarbons by edible marine animals. In: Proceedings of the international symposium. Report advance in the assessment of health effects of environmental pollution. June] 974.

Andreoni, Y.; C. Baggi; C. Guita and P. Manfun (1993) Bacteria degradation of 6-Amino caproic acid polyamides (Nylon-6) of low molecular weight. .International Biodeterioration and Biodegradation 31 (I): 41

Anselmo, A. M.; J. M. S. Cabral and J. M. Novanic (1989) The absorption of *Fusarium* flocciferum species on white particles and their us~ in the degradation of phenol. Aim. Micro. Bioch.ll: 200-203.

Anst, S. O. (1990) Degradation of environmental pollutants by Phaenerochaetes chrysosporium. Microb. Eco!. 20: 197-209.

Atlas, R. M. and R. Bartha (1981) Microbial ecology fundamentals and applications. Wesley; New York. pp 87-150.

Atlas, R.M. (1981) Microbial degradation of petroleum hydrocarbon: An enveronmental perspective. Microbial Review 45: 180-209.

Atlas, R.M.; A. Sexstone; P. Gustin ; O. Miller; P. Linkins and K. Evereh (1978) Biodegradation of crude oil by tundra soils microbes In: Biodeterioration proceedings of the 4th International symposium.Barlin (T. A. Oxley. N. Alsop and G. Beckerieda) pp22-28. Pitman London.

Atlas, R.M.; R. Bartha (1973) Stimulated hiodegradation of oil slick using Oleophilic fertilizers. Environ. Sci. Techno!. *1:* 538.

Bakers,.J. M. (1973b) Effects of refinery effluents on the plants of the Crymlyn Bog field studies Council pollution Research Unit. Anmnual Report 1973. Penbrinke, U.K, pp.~9-35.

Bakers, 1M. (1971) Refinery effluents: In the ecological effects of oil pollution on Litteral communities by Cowell, E. S. ed. Applied Sci. Publication; Barking pp.33- 43.

Bartha, R. (1986) Biotechnology of petroleum pollutants biodegradation. Microbial Ecology. 11:155-172.

Bello, Y.M. (1995) Oil utilization capabilities of Microorganisms isolated from waste contaminated soil. Msc. Thesis (1995) Federal University of Technology, Minna.

Bergbuver, M.; C.Eggert and G. Kraeplin (1991) Degradation of chlorinated lignin compounds in a bleach plant effluent by white rot fungus Trametes versicolor. 6m2. Micro. Biotech. 35: 105-109.

Black, C. A. (1965) Method of soil analysis Il Chemical and Microbiological properties. Arne. Soc. Agron. Madison; Wisconsin 157 (2).

Boldin, 1. (1951) Research de la tyrosinase et. de la laccuse ch los B, sidiomy ete en culture pure. Revue. Mycologia *1.Q: 173-197.*

Bossert, T. and R. Bartha (1984) Fate of petroleum in soil ecosystem. Petroleum Microbiology. (Atlas R. M. ed.) McMillan, London pp 4335-473.

Bray. R.II.; E. T. Kuriz (1945) Determination of the total organic and available form of phosphorous in soil. Soil Sci. 59: 39-45.

Buckman, H. O. and N. C. Brandy (1969) The nature and properties of soils. 7th ed oil Microbiology .. McMillan New York. pp.217-238.

Bumpus, 1. A. (J 989) Biodegradation of polycyclic aromatic hydrocarbon by Phanerochaetes chrysosporium . .6Jml. Environ. Microbiol. 53: 154-158.

Bumpus, 1. A.; M. Tien; D. Wright and S. D. Aust. (1985) Oxidation of persistent environmental pollutants by white rot fungus. Science 228: 1432- J 436.

Buonicore, A. J. (J 980) Ail' pollution control. Chem. En C. 87(13): 81 - J 0 I.

Burroughs.I. C. (1963) Recent developments in control of air and water pollution in U.S refineries. In proc. 6th World Petroleum congress. Frankfurt Sect. 325. 77-86.

Cairns, J.(Jr.);]Ieath, A. G. and Parker. B.C. (1975). Temp rature influnce on chemical toxicity to aquatic organisms. I. Wat. Poll. Control. Fed. 47.(2), 267-280.

Champagnat, A.. Vernet *C.;* Laine, B. and Filosa, J. (1963) Biosynthesis of protein-vitamin concentrates. Nature 197: pp 13-14.

Chen, C. 1. and H.M. Chang (1985) Chemistry lignin biodegradation. In T. Higuchi (Editer) Biosynthesis and Biodegradation of wood components. Academic press Inc. New York pp 35.

Chouteau, .1;E. azoulay and J. . Senez (1962) Anaerobic formation of n-hept-l-ene from , n-heptane by resting cells of P. aeruginosa. Nature 194: 576-578.

Confield, A.H. (1961) A sample technique for determining mineralization of carbon during incubation of soil treated with organic materials. Plant and Soil. .2(1):90-93.

COle, R. H,(1976) The effect of petroleum liquid wastes on aquatic Jives with special emphasis on Canadian environment. Natl.Res. Coun. Canad. NRC. Assoc. Comm. Sci. Criter. Environ. Qual. Rep. NNPC No 15021 pp77

Coldwell, R.R; .1:F. Carney; T.Kaneko; J.D. Nelson and J.D.

Crafts, A.S. and H. G. Reiber (1948) Herbicidal properties of oils. Hilgardia~: 77-156.

Crutcher, S.E. and P. J. Geary(1979) Properties of the iron-sulphur protein of benzen dioxygenase system from P. putida. Biochem.1. 177: 393-400.

Dagley. S.(1971) Catabolism of aromatic compounds by microbes. Adv. Microbial Physiol. 2: 1-46.

Dailva, E.1.; Y.R. Dommergue; EJ. Nyns; and C. Ratledge (1987) Microbial Technology in the developing world. Oxford University press. New York. pp 240-250.

Davies, P.I!.; J.P. Goettl;J.R. Sinley and N.F. Smith(1976) Acute and chronic toxicity of lead to rainbow trout. Salmo gairdneri in hard and sort water. Water Res. l.Q: 199-206.

Dejong, E.(I980) The effect of a crude oil spill on cereals. Environ. Pollution Service 22:187-196.

Dibble, J.1. and RBartha (1979) The effect of environmental parameters on the biodegradation of oil sludge. AP.nJ.. Environmental Microbiology.37:729-739.

Dicks, B.(1976a) The importance of behavioural patterns in toxicity testing and ecological prediction. In. Marine ecology and oil pollution by Baker I.M ed. Applied Science publisher, Barking pp. 303-319.

Dietrich, D.M. and R.T. Lamar (1990) Selective medium for isolating Phanerochaeto chrysosporium from soil. Env. Microbiol. 56:3088-3092.

Ejechi, B.O. (1991) Biodeterioration of some Nigerian Timber by fungi. Ph.D Thesis University of Benin. Benin city.

Ejechi, B.O. and e.0. Obuekwe (1993) Prelimnary studies on the effects of crude Opepe *(Nauclea diderrichi)* Timber extractive on Timber biodetrioration. International Biodeterioration and Biodegradation. 11(1) 71-75.

Ellis. R.S.; R.S. Adams (1961) Contamination of oil by petroleum hydrocarbons. Adv. Agron.ll: 197-216.

Engel, C. (1972) Safety evaluation of yeast growing on hydrocarbons in protein hydrocarbons (ed. H. G. de Pontanel) Academic press, New York, pp 53-81.

Environmental Protection Agency (1972). Water Quality Criteria, EPA R3,73033, Washington. D.C., USA, P 594.

Fahmy, M., Heinzle,O.M. and Kut, O.M. (1991) Comparison of 3 anaerobic/aerob fluidized bed biofilm systems to degrade chlorophenolic compounds in p bleaching effluents In Recent advances in Biotehnology (ed. F. Vardar-Sukan a S.S. Sukan) Vol. 210,pp 543.

Fawole, M.O. and B.A. Oso(1988) Laboratory manual of Microbiology. Spectrum Boo Limited lbadan Nig.pp 24-56.

Galsof, P.S.; H.F. Prutherah; R.O. Smith; and V.Keohring (1935) Effect of crude pollution on Oyster in Louisiana water. Bull. Bur. Fish Wash. _lli: 143- 210.

George. E.J.and R.D. Neufeld(1989) Degradation of fluorene in soil by fungu Phanerochaete chrisosporium. Biotech. Bioen G. 33: 1306-1310.

GESAMP:IMCO/F *AO/UNESCO/WIIO/WHD/IAEA/UN(* 1977) Joint group of expe on the scientific aspects of marine pollution(GESAMP)(1977). Impact of oil the marine environment. Rep. Stud. GESAMP, 6,2,SOp.

Gibson, O.T.; (1976) Microbial degradation of carcinogenic hydrocarbons and relat compounds. Proceedings of symposium on sources,effects,and sinks hydrocarbons in the aquatic environ.America Instit, of Biological Scienc Washington D.C.Au G. pp. 224-238.

Gregory, J.B. (1973). An investigation of trace heavy metal concentrations on shellfis from the sdouth Walescoastline. B.Sc. Applied Chemistry fourth year projec Kingston Polytechnic. Ms., pp 33.

Gutrick, D.C. and Rosenberg (1977) Oil tankers and pollution: a microbiologica approach. A1U1Rev. Microbial. 11: 379-396.

lIaider, G (1964) Studies on heavy metals poisoning of fishes. 1. (ead poisoning of rainbow traut *(Salmo gairdneri)*. Z. Angero Zoo. it: 347-368.

Halcornbe, G. W.; D.A. Benoit; E.N. Leonard; and J.M. Makim (1976) Long term effect of lead exposure onthree generation of brook trout (Salvalinns fontinalis) J. Fish Res. Board Can. 33: 1713-1741.

Hampson, B.L. (1977). Relationship between total ammonia and free ammonia III terrestrial and ocean waters. Journal du Conseil, 37: 117-122.

Ilarris, R.E.; A.W. Bunch; C.T. Knowles (1987) Microbial cyanides and nitrite metabolism. Sci. Pro G. Oxford 11: 293-304.

Hat-akka, A.T. (1985) Degradation of veratic acid and other lignin related compounds by white rot fungus. Pycneporus cinnabarinus. Arch. Microbiol. 141: 22-28.

Havis, 1.R.(J950) Herbicidal properties of petroleum hydrocarbons. Cornel Univ. Agr. expo Sta. Memar Ithaca, New York 298:20p.

Higgins, U; DJ. Best; R.C.Hammand and D.Scott (1981) Methane oxidising microbes. Microbiolo 'ical Review 45: 556~590.

Hiscock, C. (1976) Investigation into the toxicity of refinery effluents-Field studies council oil pollution Research unit. Annual Report 1976 pembroke U.K. pp24-25.

JluddJeston, R.L. and L.W. CrosswelJ (1976) Environmental and nutritional constrains of microbial hydrocarbon utilization in soil. In proceeding of 1975 Engineering foundation conference. The role of microbes in recovery of oil. pp71-72 NSF/RANN, Washington.

Hunt, P. C.; W.E. Rickard; F.1. Deneke; F.R.Kontz; and R. Murman (1973) Terrestrial oil spill in Alaska; Environmental effect and recovery, Prevention and control of oil spills. pp 733-746.

Jensen, V. (1975) Bacteria nora of soil after application of oily wastes. Oikas 26:152-158.

Jernelov, A. (1975). Heavy metals in the marine environment. In: Discharge of sewage from sea outfalls by Gerneson. A.L.H. ed. In: Proccdings of an international symposium, held at hurch I louse, London, 27th Au G. -2nd Sep. 1974. Pergamon Press. pp.115-122 .

Jobson, A.; r.D. Cook; D.W.S. Westlake (1979) Interaction of aerobic and anaerobic bacteria in petroleum degradation. Chern. Oeo!. 244:355-356.

Jobson, A.; M. Mclaughlin; F.C. Cook and D.W.S. Wesstlake (1974) Effects of arnmendments on the microbial utilization of oil applied to soil. AQQl.Microbiol.27: 166-171.

Jobson, A; F.D.Cook; and D.W.S. Westlake (1972) Microbial utilization of crude oil. AQQl. Microbiology 23: 1082-1089.

Johnson, A.M.; M. Mclaughlin; F.D.Cook; and D.W.S. Westlake (1974) Effects of ammendments on the microbial utilization of oil applied to soil. AQQl. Microbiol. 27: 166-) 69.

Kaarik, A.A.(1974) Decomposition of wood In Dickinson CH. Pugh; G. *T.F* (Fd) Biology of plant liter decomposition. Academic press. pp.129-174.

Kapoor, K.K. M.K Jain and C.P. Singh (1978) Cellulose activity degradation of cellulose and lignin and humus formation by cellulolytic fungi. Annal. of Microbiol. 129: 613-620.

King, P.P. (1982) Biotechnology:an international view.J.Chem. Tech. Biotechnol. 32: 2-8.

Kirk, K.T..J.; W.J. Coners and .l. G. Zeikus (1976) Requirement for growth substrate during lignin decomposition by two wood rotting fungi. Alm. and Environ Microbio1.32: 192-194.

Lamar, R.T.; .LA. Glases; and T.K. Kirk (1990) Fate of pentachlorophenol (PCP) in sterile soil inoculated with white rot Basidiomycetes, *Phanerochaete chrysosporium;* Minerilization, volatilization and depletion of PCP. Soil BioI. Biochem. 22:433-440.

Lamar. R.T.; M.J. Larson and K.T. Kirt (1990) Sensitivity to degradation of pentachlorophenol by Phanerochaete spp. LUm. and Environ. Microbiol. 56: 3519-3526.

Larson, R.A.; D.W. Blankenship and L.1. Hunt (1976) Toxic hydroperoxides, photochemical f rmation from petroleum constituents. In: Sources, effects and sinks of hydrocarbons in the aquatic environment. American Institute of Biological Sciences pp. 298-308.

Lee. R.F. (1977) Fate of petroleum components in estuarine waters od South eastern United State:In proceeding of 1977 oil spill conference New Orleans EPA/API/USC G. pp. 611-616.

Lee, R.F.; A.A. Benson (1973) Fate of petroleum in the sea: Biological aspect. In proceedings of workshop on inputs, fates, and effects of petroleum in marine environment. 21-25 May 1973. Airline Virginia, National Academy of Sciences. Washington D.C. pp.541-551.

Lehtomaki, M.; and S. Niemela (1975) Improving microbial degradation of oil in soil. Ambio. :!: 126-129.

Lewandowski, G. A.; P.M. Arrnenantc and D. Pak (1990) Reactor design for harzadouss waste treatment using a white rot fungi. Water Res. 24:75-82.

Liu, D.T.S. and B..I. Durkan (1972) Bacteria seeding techniques: novel approach to oil spill problems. Can. Res. Dev. 2,:1.

Lloyd, D.R. (1983) The nutritional evaluation of Pruteen. In Proc. Int. Symp. on from hydrocarbons for animal feeding (ed. I.Y. Hamdans) Fed. Arab Scientist Res. Comn, pp 159-175.

Loddcr, J (1971) The yeast a taxonomy study. North Iioliand pub. cornp. Amsterdam. pp.1385.

Magel, R. (1973) Bacteria seeding to enhance biodegradation of oil slick, in the microbial degradation of oil pollutants. Ahearn, D. G. and Meyers. S.P. Eds; Lousiana State University Baton Rouge 291.

Matron, .l; A.M. Stern and J. Matron (1969) Decolourization of krott bin k liquor with *Polyperus versicolor,* a white rot fungus. Tappi 1. 52: 1975-1981.

McGill, W.B.; MJ. Rowell and D.W. Westlake (1981) Biochemistry, Ecology and Microbiology of petroleum components in soil. Soil Biochem. (Paul, E.A. and I.add IN. eds) Vol.2,:229-296.

advances in Biotehnology (cd.F. Vardar-Sukan and S.S. Sukan) Vol. 210,pp 541-542.

Oberie, O (1988) Studies on the biodegradation of ome microorganisms isolated from water system of two petroleum producing area in Nigeria. Nig. Journal of Botany 1:88-90.

Odu, C.T. (1972) Microbiology of soil contaminated with petroleum hydrocarbon I. Extent of contamination and some soil and microbial properties after contamination. 1- Institute of Petroleum 58:201-208.

Odu. C.T. (1978) Effects of nutrient application and aeration on oil degradation in soil. Environ. Pollution 11: 235-240.

Ogoke, .1.;1. Ngozi (1992) Rehabilitation ofcrucle oil pollutecllancl in Oml.: 61. Seminar on the petroleum industry and the Nigerian environment menton Ni G. Ltd. Ibadan. pp 1-14.

Olukoya. D.K (1992) Genetical-engineering microorganisms 111 the oil industry-Paper presented at International Seminar on Petroleum lnclustry and Nigerian Environment (10-14 Feb., 1992).

Onwumere, B. *C.;* and A.A. Oladimeji (1990) Accumulation of metals and histopathology in Oreochronis niloticus exposed to treated NNPC Kaduna (Nigeria) Petroleum refinery effluent. 1. Ecotoxicology and Environmental Scofety 19: 123-134.

Olumbe Bassil' (1963) Determination of fat by Werner Schmid method: Handbook of Practical Biochemistry. Ibadan University Press. Ibadan.Nigeria, pp. 87.

Oshida, P.S. (1977). A safe level of hexavalent chromium for a marine polychaete. Coastal Water Research Project Annual Report for the year ended 30th June, 1977. Southern California Water Research Project, pp 169-180.

Oyenuga, V.A. (1968) Nigerian food and feeding stuff. Ibadan University Press. pp. 19-23.

Payne, .I.F.; R. Maloney and Rahimtula (1979) Are petroleum hydrocarbon an important source of mutagens in the marine environment? .In: Proceedings of 1979 oil spills conference, Los Angelis. EPA/API/USCO. Pp 533-536.

Perkh, V.R.; R.W. Traxler and J.M. Subek (1977) N-alkane oxidation enzymess of Pseudornonads, £\QQ. Environ. Microbiol. 33:381-384.

Perry, 1..1.and H.W. Shield (1968) Oxidation of hydrocarbon by microbes isolated from soil. Canads. *L* of MicrobioI.11:403-407.

Pessah, E.; 1.S.Loch; and J.C. Mclead (1973) Prelimnary report on the acute toxicity of Canadian petroleum refinery effluent to fish. Fish Res. Board Canad. Tech. Report No. 48.

Petpiroon, S. (1976). Effects of ammonia, sulfide and phenol on winkle behaviour. Field Studies Council Cil production Research Unit, Annual Report 1976. Pembroke, U.K. pp 26..

Pinholt, Y; S. Struwe; A. Kjoller (1979) Microbial changes during oil decomposition in soil. Holartic Ecology~: 195-200.

Porttman, .I.E. (1995) Persistent organic residue In Discharge of sewage from sea out falls by Gemeson, A.L.H.; ed. In proceedings of an international symposium, held at

church house London. 27th Au G. -2nd Sept. 1994. Pergamon Press. pp.123-130.Puticla. L Bacteriol. ill: 974-979.

Ramadan, M.A.; M.E. Ossama; A. Martins (1990) Inoculum size as a factor limiting success of inoculation for biodegradation . .tUm. Environ. Microbiol. 56(5) 1392-1396.

Rarnband, A.; K.S. Chan; B. Reboul and J. Bentoux (1975) Toxicate des effluents de la refinerie Mobil-oil a Frontignan. Universite de Montpellier Laboratoire d'hydrologie et d'hygiene. Manuscrip 4p.

Rernirez, R. (1982) Inciniration at sea - Are the odds now in favour? Chem. Eng. 86(6): 49-53

Ress, R.C. and A.M. Dickinson (1977) Mycology in Pharmaceutical Microbiology. ed. Hugo, W.B. and A.D.Russel. Blackwell Scientific Publication pp 50.

Ristanovic, S.D., Muntajola-Cvetkovic, M. and Munjko, I. (1975). Phenol degrading fungi from South Adriatic Sea and Lake Skader. European 1. Alml· Microbiol. 1, p 313-322.

Robertson, J.H.; W.F. Cowen; and 1.y. Longfield (1980) Water pollution control. Chem. Eng.87 (13): 102-119.

Rowe. D.W.; J.B. Sprague; T.A. Hening (19R3a) Sublethal effects of treated liquid effluent from a petroleum refinery 1 chronic toxicity of flag fish. Aquat. Toxicol *1: 1-+9-159.*

Rowe, D.W.; J.B. Spraque; T.A. Haning (1983b) Sublethal effects of treated liquid effluent from a petroleum refinery II Growth of a rainbow traut. Aquat Toxicoll: 161-169.

Smith, D.A. (1977) Enumerating fungi. Phytopatholog *B,:81.*

Smith, R.P. and Gosselin, R.E. (1966). On the mechanism of sulfide inactivation by methemoglobin. Toxicity and Applied Pharmacology.,~: 159-172.

Spraque, J.B.; D.W. Rowe, G. E. Westlake; T.A. Herring and T.T. Browns (1978) Sublethal effects of treated liquid effluent from a petroleum refinery on fresh water organisms Canada Dept. of Fisheries and Environmental protection services Canadian Environ. Ottawa.

Stanbury, P.F. and A. Whitaker (1974) Spray irrigation in Principle of fermentation Technology. A Wheaton and Co. Ltd. Great Britain pp 224.

Stringer, D.A. (1983) Toxicological evaluation of Pruteen.]n Proc. Int. Symp. on from hydrocarbons for animal feeding (ed. I.Y. Harridans) Fed. Arab Scientist Res. Cornn, pp 147-158.

Taylor. J.B. (1974) Biochemical tests for identification of mycelial culture of Basidiomycetes. Annal. of Applied Biol.78: 113-123.

Traxler. R.W. and .I.M. Bernard (1969) The utilization of n-alkanes by P. aeruginosa under condition of anaerobicsis.jjjj. Biodclcrioration Bulletin 2:21-25.

Upsher, F.J.(1971) Methods for Isolation of Biodeterioration fungi. In A.H. Walter (editor) Biodeterioration investigation Techniques. Applied Science Publisher.

Van Etten; H.D.(1973) Enumerating fungi. Phytopathology 63: 1477.

Van loocke. R.; R. DeBorger; J.P. Voets and W. Verstraete (1975) Soil and ground water contamination by oil spill problems and remedies. Int'l 1. of Environ. Studies.~: 99-111.

Zurer, R. S. (1987) Fungus shows promise in hazardous waste treatment. Chem. Industry 11:17-19.