# BIOREMEDIATION OF WASTEWATER FROM KADUNA REFINERY AND PETROCHEMICAL COMPANY (KPRC) USING IMMOBILISED BURKHOLDERIA CEPACIA AND CORYNEBACTERIUM KUTSCHERI

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PhD/SCIEN/10107/2007-08

**DEPARTMENT OF MICROBIOLOGY, FACULTY OF SCIENCE,**

**AHMADU BELLO UNIVERSITY, ZARIA.**

**MAY, 2014**

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**A DISSERTATION SUBMITTED TO THE SCHOOL OF POST-GRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILMENT FOR THE AWARD OF Ph.D MICROBIOLOGY**

**DEPARTMENT OF MICROBIOLOGY,**
**FACULTY OF SCIENCE,**
**AHMADU BELLO UNIVERSITY, ZARIA.**

**May, 2014**

## DECLARATION

I declare that the work in the dissertation entitled ‘’**Bioremediation of Wastewater From Kaduna Refinery and Petrochemical Company (KPRC)) Using Immobilised *Burkholderia cepacia* and *Corynebacterium Kutscheri*’’** has been performed by me in the Department of Microbiology under the supervision of

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2. Prof. (Mrs) V. J Umoh Dept. of Microbiology, Faculty of Science, Ahmadu Bello University, Zaria.
3. Prof. J.B Ameh Dept. of Microbiology, Faculty of Science, Ahmadu Bello University, Zaria.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

_____ _____

AJAO, Abdullahi Taiwo Date

## CERTIFICATION

This dissertation titled "**BIOREMEDIATION OF WASTEWATER FROM KADUNA REFINERY AND PETROCHEMICAL COMPANY (KPRC) USING IMMOBILISED BURKHOLDERIA CEPACIA AND CORYNEBACTERIUM KUTSCHERI**" meets the regulations governing the award of Doctorate degree in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge.

_____ _____

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Dean School of Postgraduate Studies (Date)
(Prof. A. A. Joshua)

## DEDICATION

This dissertation is dedicated to my mother Alhaja Ramat Remilekun Ajao

## ACKNOWLEDMENT

All the praises and glory be to Allah (SWT), the Supreme Being and master of the day of resurrection to whom nothing is impossible for making me witnessed this glorious day of my life.

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## ABSTRACT

Refinery sludge and raw wastewater samples were collected from Kaduna Refining and Petrochemical Company, Kaduna, Nigeria were collected and analyzed by selective enrichment technique.This resulted in the isolation of two bacterial species, *Burkholderia cepacia* and *Corynebacterium kutscheri*. Crude oil degradation was performed by the pure and mixed culture of the isolates incubated in the shake flask culture. Dehydrogenase activitie (DHA), Fluorescein diacetate (FDA) Protein Estimation, pH, Emulsification index and Optical density ($OD_{600}$) were used were used to evaluate degradation processes while $CO_2$ production and GC-MS were used to analyze mineralization potential.Growth, $CO_2$ production, FDA and protein reached maximum after 96h-192h of incubation while, DHA followed the same trend up to 162hours and declined.This suggested that the microbial activities and their hydrolytic activities correlated to biodegradation of Bonny light crude oil which is a function of $CO_2$ production. The pH was found to increase between 24h to 48h of incubation which corresponded to the period during which maximum emulsification was recorded and subsequently decreased progressively.The activities of all measured parameters between 24h-48h were not statistically significant ($p< 0.05$). Activities of all the measured parameters in *Burkholderia cepacia* and *Corynebacterium kutsheri* were characterized by flunctuations and also their activities not statistically different from each other in both isolates ($p< 0.05$).This result correlated with GC-fingerprints that showed disappearance of majority of hydrocarbons after 5days of incubation except a peak that represented high molecular weight hydrocarbon that later broken down to low weight hydrocarbons after 10days of incubation. Inspite of low catabolic potential of the axenic culture of *Burkholderia cepacia*, it produced more of Catechol-2,3-dioxygenase that played a major role in biodegradation of Bonny Light crude oil. Specifically, this enzyme cleaved aromatic rings of

several environmental pollutants such as toluene, xylene, naphthalene and biphenyl derivatives. Hence, the importance of Catechol-2, 3-dioxygenase and its role in the degradation of environmental pollutants accounted for the prediction of the three-dimensional structure of Catechol 2, 3-dioxygenase from *Burkholderia cepacia*. The 10ns molecular dynamics simulation was carried out to check the stability of the modeled Catechol 2, 3- dioxygenase. The results showed that the model was energetically stable, and it attained their equilibrium within 2000 ps of production MD run. The docking of various petroleum hydrocarbons into the Catechol 2,3-dioxygenase revealed that the benzene, O-xylene,Toluene, Fluorene, Naphthalene, Carbazol, Pyrene, Dibenzothiophene, Anthracene, Phenanthrene, Biphenyl made strong hydrogen bond and Van der waals interaction with the active site residues of H150, L152, W198, H206, H220, H252, I254, T255, Y261, E271, L276 and F309. Free energy of binding and estimated inhibition constant of these compounds demonstrated that they were energetically stable in their binding cavity. Chrysene shows positive energy of binding in the active site atom of Fe. Except Pyrene all the substrates made close contact with Fe atom by the distance ranging from 1.67 to 2.43 Å. In addition the substrates except pyrene made π-π stacking interaction with H252 by the distance ranging from 3.40 to 3.90 Å. All these docking results revealed that, except Chrysene all other substrate has good free energy of binding to hold enough in the active site and made strong VdW interaction with Catechol-2,3-dioxygenase. These results suggest that, the enzyme is capable of catalyzing the substrates. Standard methods were used to determine the physicochemical parameters of raw refinery wastewater. The results obtained indicated that the mean values of physicochemical parameters of raw refinery wastewater were statically significant ($p< 0.05$).. Parameters such as pH, Total Suspended Solids, Biochemical Oxygen Demand, Chemical Oxygen Demand, phosphate, Nitrate, sulphate, phenol, lead, were all above maximum permissible limit as

specified by Federal Environmental Protection Agency (FEPA) in raw wastewater. Mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri* isolated from refinery sludge immobilized on agar-agar was used for bioremediation of refinery wastewater in a laboratory scale bioreactor. Physicochemical parameters were determined before and after treatment.The results showed that the test organisms significantly reduced all the measured parameters below permissible limit except nitrates and sulphates ($p < 0.05$).. Each of the bacteria was screened for the presence of plasmid DNA and for the involvement or otherwise of plasmid in the bioremediation of wastewater.The gel electrophoresis resultof the isolated plasmid from *Burkholderia cepacia* and *Corynebacterium kutsheri* suggested that the catabolic trait may have some relationship with single plasmid detected in each of them. Further, investigation via curing of plasmids confirmed that the plasmid is the ultimate source of catabolic traits. The plasmid was transferred to soil-derived mixed culture recipients in plate mating experiments and isolated transconjugant colonies were immobilized and used for bioremediation process.The results caused the multiplicative effect by achieving the same percentage achieved by donor cell after 20days of treatment in10days which implied that Plasmid mediated bioaugmentation resulted in the most rapid bioremediation of refinery wastewater. Therefore, bioaugmentation of wastewater using transmissible catabolic plasmid will enhance efficiency of the bioremediation by spreading the plasmid among indigenous microbial community either through horizontal gene transfer or transformation.

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# CHAPTER ONE

## 1.0 INTRODUCTION

The Kaduna Refining and petrochemical company (KPRC) was designed to process two types of crude oils: The imported heavy crude and Nigerian light crude. It supplies the domestic market in the Nothernwest and Northeast regions of the country (Bureau of Public Enterprise, 2003-2007). The high demand for petroleum and associated products during the last ten decades has made petroleum spills inevitable consequences of oil production and refining.Despite fluctuations in its prices, oil will remain a major source of energy in the next several decades because a reliable alternative has not yet been found (Adieze *et al.*, 2003).However, Chang and Lin (2005) reviewed 242 accidents of storage tanks that occurred in industrial facilities over last 40 years and observed that Accidents occurred more frequently at petroleum refineries with 116 cases (47.9%).The second most frequently involved place was terminals and pumping stations (64 cases, 26.4%).Only 25.7% of accidents occurred in petrochemical plants (12.8%), oil fields (2.5%), and other types of industrial facilities (10.3%) such as power plants, gas plants, pipelines, fertilizer plants. Major causes are Operational error, Equipment/Instrument failure, Tank crack/rupture, Piping rupture/leak and Maintenance Error. Therefore, result, the problem of pollution during production and transportation of oil would remain a major issue.

Oil spillage has occurred for sometimes in the Midwestern areas of Southern Nigeria from 1995 to 2000 (Benka-Coker and Ekundayo, 1995; Ekundayo and Obuekwe, 1997; and Ekundayo and Obuekwe, 2000). The spillages were attributed to flow line, pipe line leakages, hose failures, blowouts, sabotage to well heads and tankers loading systems (Okpokwasili *et al*., 1998). Such spillage usually causes pollution of surrounding farmland. This occurrence of

oil spillages on land is of major concern as it could result in a substantial increase in organic load (Adams and Ellis, 1961).

The impacts of crude petroleum, prospecting and production operations on oil producing countries of the world had resulted in enormous pollution of air, water and particularly agricultural land for food production (Atlas, 1981; Amund *et al.*, 1987). Crude oil pollutant is a recalcitrant molecule toxic to biotic factors and persists in the environment for many years depending on the natural and quality of oil spilled. (Ward *et al.*, 1980; Teal *et al.*, 1992; Wang, *et al.*, 1994) Such contamination may result in an extensive damage to the natural environments and these effects may be long term and extended over many seasons.

Crude oil components are of environmental concern due to their toxic, mutagenic, and carcinogenic properties (Nelson-smith, 1973; Freedman, 1995; Rozas *et al.*, 2000). Consequently, crude oil is deleterious to a wide spectrum of marine plants, animals and microbial communities, through oxygen stress (from organic enrichment) and direct toxic effects (mortality) (Carman *et al.*, 2000). Generally speaking, oil spills into salt marsh ecosystems imparts potential damage to their physical and ecological integrity even in minimal spill level let alone catastrophic accidents like the Exxon Valdez in 1989 (36, 000 tons of crude oil covered approximately 500 kilometers of shoreline) (Miller, 1999). The removal of oil that has been accidentally or purposely spilled into the environment is therefore of great concern to the petroleum industry (Sanni and Ajisebutu, 2003).Previous investigations have shown that crude oil and other related organic pollutants can be degraded (Philips and Stewart, 1994; Okpokwasili and Okorie, 1998; Ijah and Ukpe, 1992;Ekpo and Ekpo, 2006). To remediate petroleum contaminants in these environments, bio-stimulation and bio-argumentation are generally considered as environmentally friendly techniques. However, the use of extrinsic micro-organism is unlikely to be acceptable to the public or

introduction of a single species has a tendency to use limited compounds, and had low survival rates due to predation, parasitism and competition with indigenous species in the limited environment (Kim, 2003). Crude oil usually consists of 83 - 87% carbon and 10 - 14% hydrogen, 0.1 - 2% nitrogen, 0.1 - 1.5% oxygen, 0.5 - 6% sulfur, and < 0.1% metals (Speight, 1999). The predominant hydrocarbons are theoretically degradable but the components are rather complicated. It contains aliphatic and polycyclic aromatic hydrocarbons (PAH), for example, crude oil consists of paraffins 15 - 60%, naphthenes 30 - 60%, aromatics 3-30% and asphaltic 6% by weight (Hyne, 2001).

Microbial degradation present one of the major routes through which petroleum hydrocarbon can be removed from contaminated environments (Prince, 1993; Hougton and Shanley, 1994 and Venossa *et al*., 1999). As pointed out by Minas and Gunkel (1995) since hydrocarbons are natural products, it is not surprising to find organisms that are able to degrade these energy rich substrates. The degree of degradability of crude oils also depend on the proportion of low or high molecular weight hydrocarbon components of crude oil.High molecular weight hydrocarbon in proportion to low molecular weight hydrocarbon compound in the crude oil are termed “light oils” and flow easily as opposed to “heavy oils” which are the reserve (Atlas andBartha, 1998).

Biostimulation is a technique that relies on increasing the activity of the resident bacteria by adding the factors that are limiting activities such as nutrient or air. This technique seems to be effective because the indigenous bacteria are likely to be better adapted to the soil environment requiring treatment (Rahman *et al.*, 2003). However, biostimulation sometimes does not work well and may take longer time because bacteria with the ability to degrade xenobiotics may be scarce at contaminated sites or because the high-concentration of xenobiotics reduces the activity of degrading microorganisms.

Bioaugmentation is a promising and low-cost bioremediation method in which an effective bacterium isolates or microbial consortium capable of degrading xenobiotics is administered to contaminated site (Gentry *et al.,* 2004). The number of petroleum degrading microbial isolates available for bio-argumentation is increasing (Van Hamme *et al.,* 2003; Singer *et al.,* 2005). However, the soil environment is very complicated and the degrading ability of exogenously added microorganisms tends to be affected by the physicochemical and biological features of the soil environment. Sometime, the administration of petroleum-degrading microorganisms leads to a failure of bioaugmentation (Vagel 1996; Gentry *et al.,* 2004).

Microbial degradation appears to be the most environmentally friendly method of removal of oil pollutant since other methods such as surfactant, washing and incineration lead to introduction of more toxic compounds to the environment. Hydrocarbon-degrading microorganisms are widely distributed in marine, freshwater, and soil ecosystems (Atlas and Bartha, 1973). The ability to isolate high numbers of certain oil-degrading microorganisms from an environment is commonly taken as evidence that those organisms are active degraders of that environment (Okerentugba and Ezeronye, 2003).

## 1.1 Statement of Research Problem

The process of refining crude oil consumes large amounts of water. Consequently, significant volumes of wastewater are generated (Coelho *et al.*, 2006). Coelho *et al.* (2006) reported that the volume of refinery effluent generated during processing is 0.4–1.6 times the amount of the crude oil processed. Thus, based on the current yield of 84 million barrels per day (mbpd) of crude oil, a total of 33.6mbpd of effluent is generated globally (Doggett and Rascoe, 2009).World oil demand is expected to rise to 107mbpd over the next two decades, and oil will account for 32% of the world's energy supply by 2030. Bio-fuels, including

ethanol and biodiesel, are expected to account for 5.9mbpd by 2030, and the contributions from renewable energy sources like wind and solar power are estimated to be 4–15% (Marcilly, 2003; Doggett and Rascoe, 2009). These data clearly indicate that effluents from the oil industry will continually be produced and discharged into the world's main water bodies. However, these pollutants pose serious toxic hazards to the environment. Petroleum refinery effluent can vary greatly depending on the type of oil being processed, the plant configuration, and operation procedures (Saien and Nejati, 2007). They enter into waterways and adversely affect water quality.

## 1.2 Justification

A number of investigations which focus on the treatment of refinery waste water reported in the scientific literature are purely biotransformation or co-metabolism rather than mineralization and this can result into the formation of intermediate products that are not only toxic but also recalcitrant and persistent in the ecosystem (Atlas, 1995). Also, most previous works centred on the refinery effluent which should have undergone partial or whole treatments in the company before being discharged rather than raw wastewater generated in the company. Ability of any isolate to treat refinery effluent is not an indication that such organism can be used for the treatment of their raw wastewater because of the differences in concentration of pollutants

In KRPC the only waste water purification systems are mechanical cleaning and use of foreign bacteria coated on the film which seem to be ineffective due to poor qualities of the physicochemical properties of their effluent discharged into water ways as reported by Bako *et al*. (2008) and Idise *et al*. (2010).However, It is evident that both the waste water and process water are treated in a water treatment plant, but the level of treatment can only be

ascertained through the analysis of the discharged effluent at different points of their exit into the adjoining environment (Osibanjo *et al.*, 2011).

Improvement in the ability to mineralize crude oil could be achieved by immobilizing indigenous crude oil degrading bacterial cells so as to protect them from the toxic effect of hazardous compound, increasing their survival and metabolic activities so also the purpose of their reusability. There is need for the survival of inoculum and to remain physiologically stable for a very long time in a pollutant, the inoculation of strains with mobile genetic elements that encode the relevant genetic information and their subsequent genetic horizontally transfer to one or various well established and competitive indigenous bacterial population of an ecosystem is an alternative strategy. In this case, the survival of the introduced donor strain is no longer needed once the catabolic genes are transferred and expressed in the autochthonous bacteria within the ecosystem.

### 1.3 Aim

Bioremediation and Mineralization of refinery wastewater using immobilized bacterial consortium of *Burkholderia cepacia* and *Corynebacterium kutscheri*

The specific objectives of this study were:

1. To isolate oil degrading bacteria indigenous to the refinery sludge by selective enrichment technique.
2. To evaluate the mineralization potential of the isolates on Bonny light crude oil.
3. To carry out homology & molecular docking of Catechol-2,3-dioxygenase from *Burkhoderia cepacia.*
4. To determine physicochemicalproperties of Refinery wastewater

5. To carry out Bioremediation of refinery wastewater using immobilized bacterial consortium.
6. To examine plasmid profile and curing of the isolates.
7. To carry out Bioremediation of refinery wastewater using transconjugants.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

Crude oil continues to be used as the principal source of energy and play an important role in the global environmental pollutant consideration. On the other hand, it will remain as a major source of energy in the next several decades, because reliable alternative energy consumption has not yet been substituted (Trinadade *et al.*, 2005; Al-Saleh and Obuekwe, 2005).

In the last years, a large number of ecosystems have been changed by the growing influence of human activity. As a result, many people have become aware of the need to protect ecosystems as well as to evaluate the damage caused by contamination.During the previous years, the frequency and risk of oil pollution has led to extensive research. Most of the petroleum goes in the ecosystem via leak of coastal oil refineries. This fact increased the interest of scientists to investigate the oil distribution and its fate in the environment, especially the marine environment. Approximately five million tons of crude oil and refined oil enter the environment each year as a result of anthropogenic sources such as oil spills (Hinchee and Kitte, 1995). Past analysis of reported oil spills indicated that most of the oil comes from tankers, barges and other vessels as well from land pipeline spills. Extensive changes in marine, as well as terrestrial ecosystems resulting from the grounding of the Exxon Valdez (1989), the Nahodka oil spill, the Erica spill (1999) and the Prestige spill (2002), increased the attention of environmentalists, chemists, biotechnologists and engineers (Braddock *et al.*, 1995; Chaerun *et al*., 2004; Tazaki *et al*., 2004). An oil spill is the release of a liquid petroleum hydrocarbon into the environment due to human activity, and is a form of pollution. The term often refers to marine oil spills, where oil is released into the ocean or coastal waters. The oil may be a variety of materials, including crude oil, refined petroleum

products (such as gasoline or diesel fuel) or by-products, oily refuse or oil mixed in waste (Christopherand Christopher, 2004). Interest in the microbial biodegradation of pollutants has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environments. These bioremediation and biotransformation methods endeavor to harness the astonishing, naturally occurring, ability of microbial xenobiotic metabolism to degrade.

One example of the most recent oil spill accident is the Deep water Horizon oil spill or the BP oil spill in the Gulf of Mexico which happened in 2010. It is the largest accidental marine oil spill in the history of the petroleum industry. About 4.9 million barrels of crude oil was released into the sea (Rick and Alan, 2010). Oil spillage is a major environmental problem in Nigeria. Between 1976 and 1996 Nigeria recorded a total of 4835 oil spill incidents, which resulted in a loss of 1,896,960 barrels of oil to the environment. In 1998, 40,000 barrels of oil from Mobil platform off the Akwa Ibom coast were spilt into the environment causing severe damage to the coastal environment. Oil spillage has led to very serious pollution and destruction of flora, fauna and resort centers, pollution of drinkable water, destruction of properties and lives along the Nigerian coast. Oil spillage has also caused regional crisis in the Niger Delta. Factors responsible for oil spillage in the zone are; corrosion of oil pipes and tanks, sabotage, port operations and inadequate care in oil production operations and engineering drills (Nwilo and Badejo, 2005).

Oil spillage, which often results from accidental or operational discharges of petroleum hydrocarbon into the environment, is a global issue that has been occurring since the discovery of crude oil (Ite *et al*., 2013).

According to estimates, over 2,567,966 barrels of crude oil has been spilled in 5733 incidents in the Niger Delta from 1976-2000 and about 549,060 barrels were recovered while

1,820,411 barrels were lost to the environment (Edoho, 2008) . The cumulative spilt volume within the period is nearly 10 times more than the total quantity that was spilled in the Exxon Valdez disaster. However, multinational oil companies operating in the Nigeria's Niger Delta often underestimate the quantity of oil spilt and a large number of other spills are not frequently reported, the total volume of oil spilt may be 10 times higher than the officially reported figure (Moffat and Olof, 1995). Further, the cases of unreported spills by some oil exploration and production companies could be related to the Nigerian National Petroleum Corporation (NNPC) inspectorate classification guidelines whereby spillage are classify into minor, medium, major and disaster.

In past years, major oil spills has attracted a global attention and created awareness due to the associated ecological, human health and environmental risks and/or damages that result from such spillages (Ite *et al.*, 2013). The main sources of oil spill and pollution in the Niger Delta are equipment failure, oil blowouts from the flow stations, leakages from aged and corroded network of the pipelines, operational mishap, sabotage and vandalization of the oil pipelines by the local militant groups. However, oil spill resulting from the vandalization of pipelines either as a result of civil disaffection with the political process or as a criminal activity causes a serious contamination of the environment (Nwilo and Badejo, 2006).

Shipping accidents and the discharges of petroleum–derived waste streams from oil and gas exploration and productions are toxic to the coastal waters, soils and sediments near the discharge points. For example, disposal of produced water poses several threats to marine ecosystems and local effects have been observed in shallow estuarine or coastal waters (Neff, 2002; Ramirez, 2002; Johnsen *et al*., 2004).

It is very important to characterize oil spills as already known sources. This can help environmentalists to predict the behavior of oil and estimate the long-term impact on the

environment. It is necessary to select an appropriate clean-up method. The recovery of oil was studied extensively since the 1970s (Malatova, 2005). Conventional remediation methods include physical removal of contaminated material. These methods also use chemicals, especially shoreline cleaners, which are often organic solvents with or without surfactants (Riser-Roberts, 1992). The shoreline cleaners with surfactants emulsify the adsorbed oil, which entrain adjacent waters or is transported deeper into the shoreline soil. The oil solvent mixtures are collected using conventional skimming methods. Mechanical recovery of oil includes the use of the oil sorbents. Sorbents help to transform oil to a transportable form for short-term storage. However, most of the used sorbents end up in the landfills (Malatova, 2005). Most of the physicochemical methods use chemical agents and their emulsion with oil cause toxicity, to aquatic organisms. They produce another source of pollution and also increase the oil recovery cost (Khairiali, 2012). Additionally, abiotic losses due to evaporation of low molecular hydrocarbons, dispersion and photooxidation (involves only aromatic compounds) play a major role in decontamination of the oil spill environments (Mills *et al*., 2003).There is an increased interest in promoting environmentally methods in the process of cleaning oil-polluted sites. These methods are less expensive and do not introduce additional chemicals to the environment. Compared to physiochemical methods, bioremediation offers a very feasible alternative for an oil spill response. This technique is considered an effective technology for treatment of oil pollution. One reason is that the majority of the molecules in the crude oil and refined products are biodegradable (Malatova, 2005, Khairiali, 2012).

Oil spills are a common event in Nigeria (Baird, 2010). Half of all spills occur due to pipeline and tanker accidents (50%), other causes include sabotage (28%) and oil production operations (21%), with 1% of the spills being accounted for by inadequate or non-functional

production equipment. Corrosion of pipelines and tankers is the rupturing or leaking of old production infrastructures that often do not receive inspection and maintenance (Nwilo and Badejo, 2001). A reason that corrosion accounts for such a high percentage of all spills is that as a result of the small size of the oilfields in the Niger Delta, there is an extensive network of pipelines between the fields, as well as numerous small networks of flowlines—the narrow diameter pipes that carry oil from wellheads to flowstations—allowing many opportunities for leaks. In onshore areas most pipelines and flowlines are laid above ground. Pipelines, which have an estimate life span of about fifteen years, are old and susceptible to corrosion. Many of the pipelines are as old as twenty to twenty-five years (Bronwen, 2007). Lightning was the most frequent cause of accident and the maintenance error was the second most frequent cause. The rest were operational error, equipment failure, sabotage, crack and rupture, leak and line rupture, static electricity, open flames etc.Causes and effects of oil spillage is illustrated as a fishbone diagram shown in Fig. 2.0

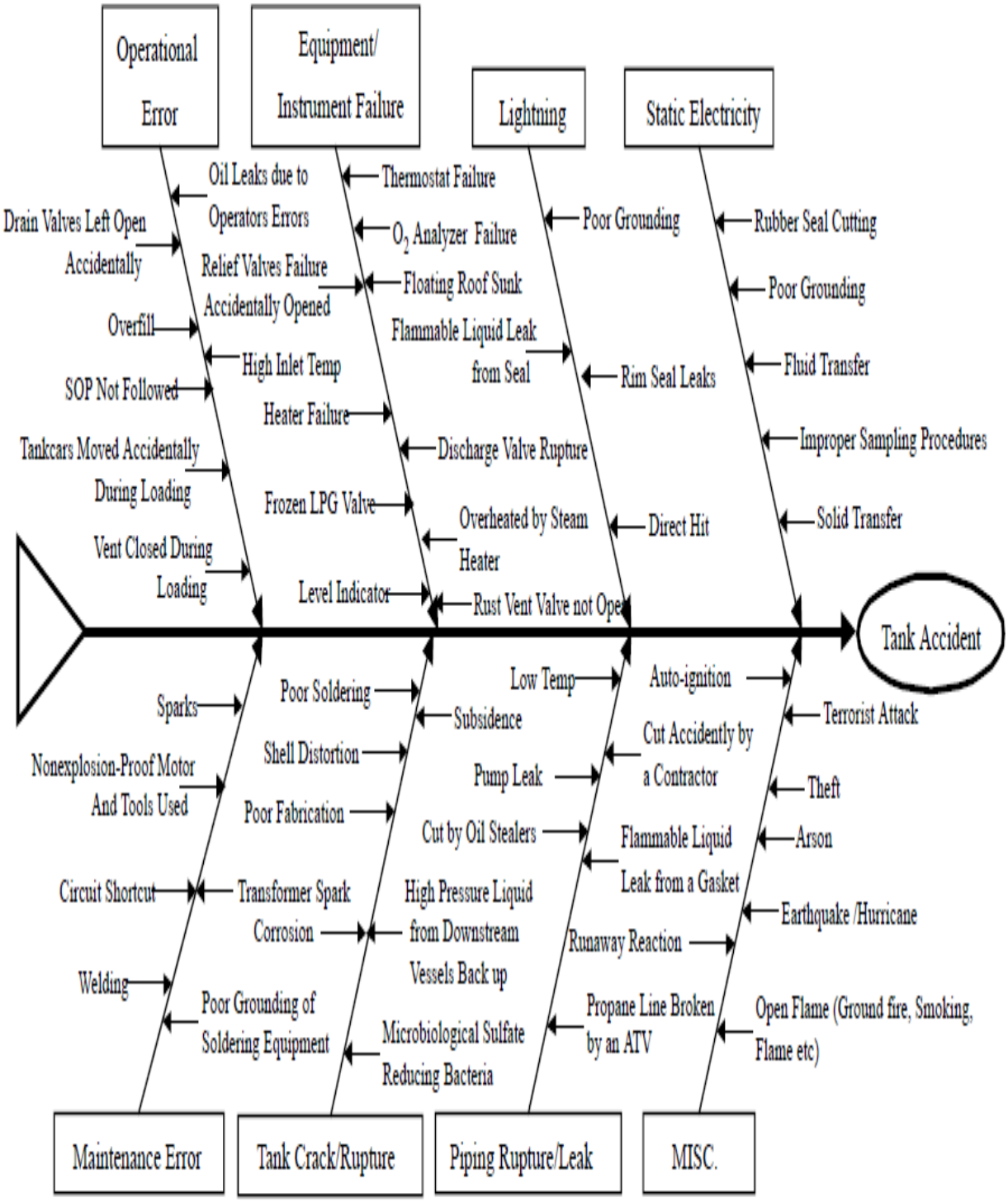



Fig.2.0: Fishbone diagram causes of oil accidents (Chang and Lin, 2006).

## 2.1 Hydrocarbons

Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallo-constituents. Petroleum constituents represent: saturates, aromatics, resins and asphaltenes. Saturates are defined as hydrocarbons containing no double bonds. They are categorized according to their chemical structures into alkanes (paraffins) and cycloalkanes. Saturates represent the highest percentage of crude oil constituents. Aromatic hydrocarbons with one or several aromatic rings are usually substituted with different alkyl groups. In comparison to the saturated and aromatic fractions, the resin and asphaltenes contain non-hydrocarbon polar compounds. Resins and asphaltenes have very complex and mostly unknown carbon structure with addition of many nitrogen, sulfur and oxygen atoms (Harayama, 2004).

Hydrocarbon is an organic compound consisting entirely of hydrogen and carbon. Hydrocarbons are mainly the pollutant from oil refineries and oil spills. Hydrocarbons differ in their susceptibility to microbial attack and, in the past, have been generally ranked in the following order of decreasing susceptibility: n-alkanes [branched alkanes [low molecular weight aromatics [cyclic alkanes [high molecular weight aromatics (Leahy and Colwell, 1990).

Hydrocarbons contain benzene, cyclopentadiene, dicyclo-pentadiene, stryenetoluene, and xylene as major components and many other hydrocarbons as minor components. Many microorganisms have the ability to utilize hydrocarbons as the sole source of carbon and energy. Such microorganisms are widely distributed in naturally occurring crude oil, diesel, and petroleum products (Santhini *et al.,* 2009).

Petroleum recovered from different reservoirs varies widely in compositional and physical properties (Malatova, 2005).The composition of particular petroleum product ranges

from the very low molecular weight hydrocarbons to the very high. A hydrocarbons' chemical structure affects its biodegradation in two ways. First, the molecule may contain groups or substituent that cannot react with available or inducible enzymes. Second, the structure may determine the compound to be in a physical state where microbial degradation does not easily occur. Usually, the larger and more complex the structure of a hydrocarbon, the more slowly it is oxidized (Navarre, 2014) Also the degree of substitution affects the degradation. Compounds that contain amine, methoxy and sulfonate groups, ether linkages, halogens and branched carbon chains are generally persistent. Adding aliphatic side-chains increases the susceptibility of cyclic hydrocarbons to microbial attack (Riser-Roberts, 1992).

Hydrocarbon composition affects their physicochemical properties. Hydrocarbons differ in their solubility, from polar compounds, such as methanol to very low solubility non-polar compounds, such as high molecular weight polynuclear aromatic hydrocarbons. The solubilization is not the only factor determining the degradation of hydrocarbons. Many microorganisms, such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus laterospor* excrete emulsifiers that increase the surface area of the substrate. On the other hand, these microorganisms modify their cell surface to increase its affinity for hydrophobic substrates and, thus facilitate their absorption (Cybulski *et al*., 2003; Carvalho and Fonseca, 2004). Hydrocarbons can be very fluid or very viscous and very volatile or relative nonvolatile. Viscosity of polluting oils is an important property. It determines the spreading and dispersion of the hydrocarbon mixture and also the surface area available for microbial attack (Leahy and Colwell, 1990).The variability in the physicochemical character of hydrocarbons causes changes in the behavior of individual hydrocarbons as well as mixtures (Malatova, 2005)

The concentration of organic compounds in the environment also affects the level of tolerance. At low concentrations, all fractions are likely to be attacked. However, at high

concentrations, only those fractions most susceptible to degradation will be broken down. Also the concentration of contaminants will affect the number of organisms present. It has been shown that the higher concentrations of gasoline in contaminated water were related to higher counts of microorganisms (Doong and Wu, 1995).

## 2.2 Factors Affecting Hydrocarbon Biodegradation

. Hydrocarbon biodegradation can occur over a wide pH and temperature range. The optimum pH for petroleum bioremediation in soil ranges from 6.0 to 8.0. Generally, lime is added to increase the pH of soil if it is in the acidic range (pH < 6), and elemental sulfur or ammonium sulfate is added to lower the pH if the soil is too alkaline (pH > 8). The biodegradation rate generally increases from the psychrophilic to mesophilic temperatures. The optimum temperature for biodegradation has been reported in the range of 25–40°C (Van Hamme *et al.*, 2003). Temperature influences hydrocarbon biodegradation by affecting the physical state and chemical composition of oil as well as the metabolic activities and composition of the microbial community (Crawford and Zhou, 1995; Walworth and Reynolds, 1995). *Bacillus* sp. was reported to be capable of growing on petroleum hydrocarbons in the range of 45–70°C (Klug and Markovetz, 1971; Sorkhoh *et al.*, 1993). A psychrophilic strain of *Rhodococcus* sp. Q15 was found to mineralize short-chain alkanes to a greater extent than long-chain alkanes at low temperatures of 0 and 5°C (Whyte, *et al.*, 2002). Low temperatures also reduce the rates of volatilization of low molecular weight hydrocarbons.

Studies on the feasibility of bioremediation as a treatment option for a chronically diesel-oil-polluted soil in an alpine glacier area at an altitude of 2,875 m above sea level for three summer seasons indicated that at moderate temperatures (20–40°C), there were both greater abiotic losses and higher rates of hydrocarbon metabolism (Margesin and Schinner, 2001).

Nutrient availability plays an important role in adaptation of microbes and their growth on hydrocarbons. Two major nutrients, nitrogen and phosphorus, are considered to be the most important, as they are required for incorporation of carbon into the biomass. Nitrogen and phosphorus often become limiting factors in hydrocarbon biodegradation, as was found to be the case in Prince William Sound after the spill from Exxon Valdez (Pritchard and Costa, 1991), and the high C:N and/or C:P ratio became unfavourable for microbial growth (Toccalino *et al.,* 1993). Oil-sludge biodegradation was found optimal at C: N and C:P ratios of 60:1 and 800:1 respectively (Dibble and Bartha, 1979).

The importance of oxygen for hydrocarbon degradation is realized by the fact that the major degradative pathways in saturated, cyclic and aromatic hydrocarbons in bacteria and fungi involve oxygenases for which molecular oxygen is required (Cerniglia 1984; Atlas, 1995). To maintain metabolic activities of microbial cells, the oxygen supply rate must match the overall oxygen consumption rate under equilibrium conditions (Anderson and Helder, 1987; Huesemann and Truex, 1996). Oxygen diffusion in soil can be severely restricted in wet or flooded soils. Addition of bulking agents and periodic tilling can increase soil porosity, and the rate of oxygen consumption due to bacterial metabolism can be improved (Devinny and Islander, 1989). Appropriate aeration is provided by a network of spargers in biopiles, and optimum mixing and aeration in bioslurry reactors (Ward *et al.*, 2003).

Anaerobic degradation of petroleum hydrocarbons has also been reported, but the degradation rates were found to be very slow for a practical bioremediation system (Ward and Brock, 1978). It has been demonstrated that sulphate-reducing bacteria can utilize aliphatic and aromatic hydrocarbons directly under anoxic/ anaerobic conditions (So and Young, 1999). Bolliger *et al.* (1999) have shown that petroleum hydrocarbon mineralization in anaerobic aquifers was linked to the consumption of oxidants such as $O_2$, $NO_3$ or others, with subsequent production of corresponding reduced species. Denitrifying microorganisms

also carry out degradation of alkane and alkylbenzenes under anaerobic conditions (Rabus *et al.,* 1999; Ehrenreich, *et al.,* 2000).

Reduced bioavailability of hydrocarbons can limit biodegradation, particularly in aged soils that have been contaminated for many years and during the final stages of a soil bioremediation treatment processes (Alexander, 1999). Mass transfer of hydrocarbons into microbial cells is a significant determinant of biodegradation rates and extent. Hydrocarbon bioavailability and subsequent degradation can be improved by addition of chemical surfactants and biosurfactants. However, depending on the physiology of the microbes involved, mass transfer may be affected positively, negatively, or not at all in the presence of a biosurfactant (Das and Mukherjee, 2007). In a study, a biosurfactant was found to greatly increase desorption of four-ring PAHs from soil and enhance biodegradation by *Pseudomonas alcaligenes* PA-10, possibly due to a combination of increased solubility and increased biomass, given that the biosurfactant could also serve as a carbon source (Hickey *et al*., 2007). Thus, microorganisms that overproduce biosurfactants may have an important role in the hydrocarbon degradation process.

## 2.3 Microorganisms

In recent years, many microbial ecologists have identified various microbial species that are effective degraders of hydrocarbons in natural environments. Many of these microbial consortia have been isolated from heavily contaminated coastal areas. They were isolated on their ability to metabolize various carbon sources, such as aliphatic and aromatic compounds and their chlorinated derivates. The microorganisms were obtained originally by enrichment culture procedures, where maximum specific growth rate or maximum final cell concentration was used as the selection criterion (Horowitz *et al.*, 1975; Malatova, 2005).

Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae (Riser-Roberts, 1992; Bundy *et al*., 2004). However, bacteria play the central

role in hydrocarbon degradation. Biodegradation of hydrocarbons by natural populations of microorganisms is the main process acting in the depuration of hydrocarbon-polluted environments. The mechanism has been extensively studied and reviewed (Atlas, 1984; Leahy and Colwell, 1990; Korda *et al.*, 1997; Kanaly and Harayama, 2000; Vam Hamme *et al.*, 2003; Pinzon-Martinez *et al*., 2010). Recognized as one of the most metabolically versatile (Stammer *et al.,* 1966) of bacterial species, *B. Cepacia* can degrade a wide range of compounds as carbon and energy source (Lassie and Gaffney, 1986), including a variety of aromatic compounds (Grenfell *et al.,* 1995), pesticides and herbicides. It has been suggested that the remarkable degradation potential of this bacterium may be due to the presence of large number of insertion elements in the genome of the organism (Waasbergen *et al.*, 1998) and extra chromosomal elements such as plasmids (Gonzalez, 1970; McKevitt, 1984;Lenon *et al.*, 1991).

Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment (Rahman *et al*., 2003; Brooijmans et al., 2009). Several bacteria are even known to feed exclusively on hydrocarbons (Yakimov *et al*., 2007) Floodgate (1984) listed 25 genera of hydrocarbon degrading bacteria and twenty five genera of hydrocarbon degrading fungi which were isolated from marine environment. A similar compilation by Bartha and Bossert (1984) included twenty two genera of bacteria and 31 genera of fungi. In earlier days, the extent to which bacteria, yeast, and filamentous fungi participate in the biodegradation of petroleum hydrocarbons was the subject of limited study, but appeared to be a function of the ecosystem and local environmental conditions (Leahy and Colwell). *Acinetobacter* sp. was found to be capable of utilizing n-alkanes of chain length $C_{10}$–$C_{40}$ as a sole source of carbon (Throne-Holst *et al.*, 2007).

Bacterial genera, namely, *Gordonia*, *Brevibacterium*, *Aeromicrobium*, *Dietzia*, *Burkholderia*, and *Mycobacterium* isolated from petroleum contaminated soil proved to be the potential organisms for hydrocarbon degradation (Chaillan *et al.*, 2004). The degradation of polyaromatic hydrocarbons by *Sphingomonas* was reported by Daugulis and McCracken, (2003). Fungal genera, namely, *Amorphoteca*, *Neosartorya*, *Talaromyces*, and *Graphium* and yeast genera, namely, *Candida*, *Yarrowia*, and *Pichia* were isolated from petroleumcontaminated soil and proved to be the potential organisms for hydrocarbon degradation (Chaillan *et al.*, 2004). Singh (2006) also reported a group of terrestrial fungi, namely, *Aspergillus*, *Cephalosporium*, and *Pencillium* which were also found to be the potential degrader of crude oil hydrocarbons. The yeast species, namely, *Candida lipolytica*, *Rhodotorula mucilaginosa*, *Geotrichum* sp, *and Trichosporon mucoides* isolated from contaminated water were noted to degrade petroleum compounds (Bogusławska-Was and Da¸browski, 2001). Though algae and protozoa are the important members of the microbial community in both aquatic and terrestrial ecosystems, reports are scanty regarding their involvement in hydrocarbon biodegradation. Walker *et al.* (1975) isolated an alga, *Prototheca zopfi* which was capable of utilizing crude oil and a mixed hydrocarbon substrate and exhibited extensive degradation of n-alkanes and isoalkanes as well as aromatic hydrocarbons. Cerniglia *et al.* (1980) observed those nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms could oxidize naphthalene. Protozoa, by contrast, had not been shown to utilize hydrocarbons (Das and Chandran 2010).

*B. cepacia* strains grow on fluorene and degrade this compound by a mechanism analogous to naphthalene catabolism. The system has wide specificity, and the range of substrates includes many other polycyclic aromatic compounds (Grifoll *et al.*, 1995). But in spite of the remarkable catabolic versatility of the species, there are some limitations to the

range of susceptible substrates. Thus, strains isolated on phenanthrene from polyaromatic hydrocarbon (PAH)-contaminated soils had limited capacity to use higher PAHs (Mueller *et al*., 1997).

Genetically Modified Organisms (GMOs) can also be used to improve petroleum degradation efficiency, but other limitation may complicate the procedure, such as problem with international legislations (Alcalde *et al*, 2006; Paul *et al.*, 2009). In both cases (GMO or wild – type strains), the potential impact of introducing degrading microorganisms in the presence of indigenous microbes must be evaluated (Peixoto *et al*., 2004; Alcalde *et al.*, 2006; Santos *et al*., 2011).

## 2.4 Petroleum Degrading Enzymes (PDE)

Oil planet hosts many different environments from the arctic to the Antarctic; there are deserts, rainforests, abyssal regions, and many other places where different forms of life can be found. Not all organisms can adapts and survive in diverse environments, but instead, they inhabit specific environments according to their biotic and abiotic characteristics. However, microorganisms are everywhere, they have colonized diverse environments for thousands of years, including those that for most organisms, are considered "extreme" in addition to colonizing the environment, microorganism colonize other organisms and are essential to life on our planet as we know it. Only a small portion of bacteria are harmful. In fact, microorganisms are key component of food webs and biogeochemical cycles and in the maintenance and survival of plants, animals, and other organisms through symbiotic relationships.

Several microorganisms may be involved in the reactions of biogeochemical cycles, and in some cases they are the only biological agents capable of regenerating forms of elements needed for other organisms (Madigan *et al.,* 2010). Collectively, microorganisms

have great metabolic diversity which allows their ubiquity. Because of their ubiquitous nature, the biotechnological potential of microorganisms is virtually endless, with many possible applications. One of these applications is the utilization of microorganisms or their enzymes in petroleum bioremediation approaches (Madigan *et al.*, 2010).

Biocatalysts can provide alternative ways to improve petroleum bioremediation approaches (Cao *et al.*, 2009), the screening for enzymes for this purpose is necessary. This method presents some enzymatic applications for the degradation of petroleum toxic compounds and a discussion about improvements that could be used in petroleum enzymatic bioremediation.

Numerous microorganisms such as bacteria, cyanobacteria, green algae, and fungi are capable of degrading different components of petroleum under different environmental conditions (e.g. aerobic and anaerobic conditions at varied salinity and pH). The enzymatic apparatus provides the capacities for microorganisms. Petroleum degradation occurred gradually by sequential metabolism of its compounds. The genes involved in degradation of petroleum may be located in chromosomal or plasmid DNA (Broderick, 1999).

Biodegradation of hydrocarbons, both aliphatic and aromatic compounds may occur under anaerobic and aerobic conditions (Van Hamme, 2003). Under aerobic conditions oxygenase enzymes introduce one oxygen atom into hydrocarbons (mono-oxygenases introduce one oxygen atom to a substrate while di-oxygenases introduce two). The anaerobic degradation is catalyzed by anaerobic bacteria, such as sulphate – reducing bacteria, using different terminal electron acceptors (Van Hamme, 2003).

Degradation of hydrocarbons aerobically are often faster, because of the metabolic advantage of getting the supply of O2 as an electron acceptor (Cao *et al*., 2009). Aerobic

catabolism of hydrocarbons is often faster, because of the metabolic advantage of getting the supply of $O_2$ as an electron acceptor (Cao *et al*., 2009). The final product of the oxidation of saturated aliphatic hydrocarbon is acetyl-CoA, which is catabolized in the citric acid cycle, together with the production of electrons in the electron transport chain. This chain is repeated, further degrading the hydrocarbons, which are normally fully oxidized to $CO_2$ (Madigan *et al.,* 2006). Aromatic hydrocarbons, such as benzene, toluene, xylene, and naphthalene, can also be degraded in aerobic conditions. The degradation of the compound usually serves as an initial step in the formation of catechol or a structurally related compound. Once formed, catechol can be degraded, resulting in compounds that can be introduced into the citric acid cycle. Also these compounds can be completely degraded to $CO_2$ (Cao *et al.*, 2009; Madigan, 2010).

Alkane hydroxylases are alkane-degrading enzymes that are distributed among many different species of bacteria, yeast, fungi and algae (vanBeilen and Funhoff, 2007). Furthermore, Van Beden and Funhoff (2007) proposed three categories of alkane-degrading enzyme systems: $C_1$-$C_4$ (methane to butane, oxidized by methane-monooxygenase-like enzymes), $C_5$-$C_{16}$ (pentane to hexadecane, oxidized by integral membrane nonheme iron or cytochrome p450 enxyme), and $C_{17}$ + (longer alkanes, oxidized by essentially un-known enzyme system). They, then reported the composition, cofactors, substrate ranges, and presence of the main groups of alkane hydroxylase (Soluble methane monooxygenase (Smno) < particulate methane monooxygenase (pMNO), Alk B-related-alkane hydroxylase, eukaryotic p450 (CYP52), class I). These authors also noted that microorganisms that are able to degrade alkane can contain multiple alkane hydroxylases and can thus consume different substrate ranges. One among the foremost studied alkanedegradation pathways is that described for *Pseudomonas putida* Gpo1, encoded by the OCT plasmid (Beilen *et al.,*

1994, Beilen *et al*., 2001). During this case, the conversion of an alkane into an alcohol is mediated by a membrane monooxygenase and rubredoxin reductase (Hamme *et al.*, 2003).

The catechol deoxygenase class of bacterial iron containing enzyme is an example of an enzyme class involved in the degradation of aerobic aromatic hydrocarbons. These enzymes are able to catalyse the addition of molecular oxygen atom to 1,2-dihydration xybenzene (catechol) and its derivatives, with subsequent cleavage and are responsible for the wide varieties of micro organisms capable of degrading aromatic compounds (Broderick, 1999).

Despite the fact that petroleum degradation under aerobic conditions occurs faster than under anaerobic conditions, it is important to note that anaerobic degradation is also essential to the bioremediation process because in several cases the environmental conditions can include limitations of the oxygen availability, such as in mangrove, aquifers and sludge digester (Santox *et al*., 2011). In anaerobic metabolism, generally, aromatic compounds are converted into benzoyl-CoA, which is target of the benzole-CoA reducate (BCR) action (Hosoda *et al.*, 2005). Depending on the environmental conditions, different terminals elections acceptors can be used, such as nitrate, sulphate and $Fe^{3+}$ (iii); generally, the degradation pathway converge to benzolyl-CoA (Cao *et al*., 2009).

Biocatalyst is opening new path toward improving the development of products and process to reduce industrial cost and generation of toxic sub products and consequently, the impact on the environment. Both enzymes bioremediation are contributing to minimizing fossil fuel damage (Alcalde *et al.*, 2006); enzymatic remediation can be simpler than working with whole organisms. Some advantages, including the enzymatic potential, can be increased in laboratory conditions (Weir *et al.*, 2006). The use of isolated enzymes does not generate

toxic product (Alcalde *et al.*, 2006) and whole cell competitiveness is not necessary (Alcalde *et al.,* 2006).

Alcalde *et al.* (2006) summarized the main aspects to be considered; from search to production, in enzymatic bioremediation. First, for an enzymes to be selected for a bioremediation application, it needs to have the capacity to degrade the target contaminant into less – toxic products. It is also important to search for enzymes that do not depend on cofactors, which would increase process cost at the commercial level. After screening, the next step is to identify the gene encoding the selected enzymes and if necessary improve enzymatic production. Commercial companies produce their enzymes via large-scale industrial fermentation; unlysed cells are removed during downstream processing. Sutherland *et al.*, (2004) also noted that the purification of enzymes from the soluble material in the fermented liquor is not required for environmental remediation, which can facilitate the production process and reduce costs, but they highlighted that self-life and environmental stability must be evaluated to ensure effectiveness of the enzymes against the contaminants. The steps and considerations outlined by Sutherland *et al.*, (2004) can be extrapolated to the bioremediation of any contaminant, their report describes how to produce an enzymatic bioremediation agent for different applications.

Polycyclic Aromatic Hydrocarbons (PAHs) are mutagenic cytotoxic and carcinogenic organic chemicals. PAHs are widely distributed in the environment as a result of the incomplete combustion of organic matter, emission sources, automobile exhaust, domestic matter, and other factors (Samanta *et al.,* 2002). The enzymatic remediation of PAHs has been proposed by many authors (Samanta *et al.,* 2002; Whitelay and Lee, 2006). PAH degradation under aerobic condition involves the oxidation of aromatic ring by specific deoxygenase, as described above, and a complete biotransformation into $CO_2$ and water

(Whitley and Lee, 2006).The BTEX compounds (benzene, toluene, ethylbenzene, and xylene) can be degraded or both aerobic and anaerobic condition by microorganisms such as sulphate reducers.

As an example of enzymatic bioremediation PAH detoxification can be achieved by the use of lactose (Alcalde *et al.*, 2005) (enzymes capable of catalyzing the oxidation of phenols, polyphones and anilines, coupled to the 4 – electron reduction of molecular oxygen and water) (Smith *et al.*, 1997). A great advantage of the enzymatic bioremediation of xenobiotics that are either hydrophobic or poorly soluble in aqueous solutions, such as PAHs, is that oxidation can occur in the presence of organic solvent (Alcalde *et al.*, 2005). A disadvantage is that the relevant enzyme can be unstable, inhibited, or denatured in organic solvents. In the work of Bulter *et al.*, (2003) laccase was expressed from Mycelrophythora (MtL) in *Saccharomyces cerevisiae*, using directed evolution, and extensively improved laccase expression.

Scott *et al.*, (2010) successfully reported an initial field trial with enzyme-based products, based on the enzyme TrzN, demonstrating that the technology can efficiently remediate water bodies contaminated with herbicides. However, few field studies with enzymatic bioremediation are currently available. Whiteley and Lee (2006) reported that until 2004, there were over 1000 described enzyme involved in the biodegradation of aromatic system (organic pollutants on otherwise).

Despite the advantage of enzyme bioremediation, there are also limitations and factors required for enzymatic remediation which restrict its applicability to a few enzyme classes. Bioremediation enzymes must be adapted to relatively specific environmental condition and must be rather independent of cofactor (Alcalde *et al.*, 2006; Sutherland *et al.*, 2004).

## 2.5 Microbial Diversity and Biodegradation

Today use of microorganisms for removing crude oil pollution from contaminated sites as bioremediation was considered by scientists, because other methods such as use of surfactants, washing and incineration lead to production of more toxic compounds and they are non-economic (Margesin, 2000; Balba *et al.*, 2002; Urum *et al.*, 2003).

A wide range of studies have dealt with biotransformation, biodegradation and bioremediation of petroleum hydrocarbons and interest in exploiting crude oil – degrading organisms for environmental clean-up has become central to petroleum microbiology. There are so many bacteria and fungi with this ability (biodegradation of oil pollutants) and these organisms are widely distributed in marine, fresh water and soil habitat (Head and Swarmell, 1999), but scientists reported that indigenous and adapted microorganisms are more efficient for biodegradation of all pollutants than introduced microorganisms.The adapted organism degrades pollutants normally but rate of this action is critically depend on different factors including microbial composition, contaminants type, geology of polluted site and chemical conditions of the contaminated site. There are different strategies for enhancing the rate of bioremediation of soil contaminated with crude oil, these include: stimulation of the indigenous microorganisms, by introducing nutrients and oxygen into the soil (bioaugmentation), (Barathi and Vasudevan, 2001; Seklemova *et al.*, 2001; Okoh and Trejo – Hernandez, 2006). Microbial degradation of oil has been known to occur by attack on the aliphatic or light aromatic fractions of the oil. A number of well known microorganisms are responsible for the biodegradation of oil hydrocarbon. The rate of microbial degradation of hydrocarbon in soil is affected by several physical, chemical and biological parameters including the number and species of microorganisms present in the conditions for microbial degradation activity (e.g. presence of nutrient, oxygen, pH and temperature). The quality,

quantity and bioavailability of the contaminants and the soil characteristics such as particle size distribution.

The population of microorganisms found in a (pollutant) polluted environment will degrade petroleum differently and at a different rate than micro-organisms in relatively clean environmental (Obire and Okudu, 1997). Petroleum hydrocarbon utilizing bacteria can tolerate oil contaminated environments because they possess the capability to utilize oil and energy source other species are gradually eliminated. Microbial decomposition of petroleum product is of considerable importance (Satloni *et al.,* 2009).

Mechanical method to reduce hydrocarbon pollution is expensive and time consuming. Hydrocarbons include PAHs have been long recognized as substrates supporting microbial growth (Bushnell and Haas, 1941; Speight, 1991; Ehrlich, 1995). Bioremediation makes use of indigenous oil consuming microorganisms, called petro-philes, by enhancing and fertilizing them in their natural habitats. Petrophiles are very unique organisms that can naturally degrade large hydrocarbon and utilize them as a food source (Harder, 2004). Microorganisms degrade these compounds by using enzymes in their metabolism and can be useful in cleaning up contaminated site (Alexander, 1999; Mandri and Lin, 2007).

Microbial remediation of a hydrocarbon contaminated site is accomplished with the help of a diverse group of microorganisms, partially the indigenous bacteria present in soil. This microorganism can degrade a wild range of target constituents present in oily sludge (Eriksson *et al.*, 1999; Barathi and Vasudervan, 2001; Mishra *et al.*, 2001). A large number of *Pseudomonas* strains capable of degrading PAHs have been isolated from soil and aquifers (Fall *et al.,* 1979; Kiyohara *et al.,* 1992; Johnson *et al.,* 1996). Other petroleum hydrocarbon-degraders include: *Yokenella spp., Alcaligenes* spp., *Roseomo-nas* spp., *Stenotrophomonas* spp., *Acinetobacter* spp., *Streptococus* spp., *Providencia* spp., *Sphingobacterium* spp.,

*Cyanocytophaga* spp., *Moraxella* spp., *Bacillus* spp., (Rusan-sky *et al.*, 1987; Antai, 1990; Bhaltacharya *et al.*, 2002). Other organisms such as fungi are also capable of degrading the hydrocarbons. However, they take longer periods of time to grow as compared to their bacterial counterparts (Prenafeta – Boldu *et al.*, 2001).

The fate of petroleum hydrocarbon in the environment is largely controlled by abiotic factors which influence rate of microbial growth and enzyme activities that determine the rate of petroleum hydrocarbon utilization (Leahy and Colwell, 1990; Atlas, 1995). The persistence of petroleum pollutants depends on the quantity and quality of hydrocarbons mixture and on the properties of the affected ecosystem. In one environment, petroleum hydrocarbon persist definitely where as under another set of conditions the same hydrocarbon may be completely biodegraded within a few hours or days (Atlas and Bartha, 1992). Although, hydrocarbon degraders may be expected to be readily isolated from an oil-associated environment, the same degree of expectation may be anticipated by microorganisms isolated from a total unrelated environment such as domestic waste water (Ojo, 2006).

Biodegradation of crude oil is a natural process. The changes in the composition of polluting hydrocarbons, induced by the various physical, chemical and biological factors, are collectively known as weathering. A large number of microorganisms belonging to a variety of genera are able to utilize hydrocarbons as the sole source of carbon and energy, and these microorganisms are widely distributed in nature (Van Hamme *et al.*, 2003). Hydrocarbons in the natural environment are biodegraded by a diverse group of bacteria, yeast and filamentous fungi, algae, cyanobacteria and some protozoan organisms (Paul *et al.*, 2005). Most of the hydrocarbon-degrading bacteria belong to a Gram-negative group. Bartha and Bossert (1984) have listed 22 genera of hydrocarbon-degrading bacteria and 31 genera of fungi isolated from

soil. In a comparative study of hydrocarbon degradation by bacteria and fungi, Song *et al.* (1986) found that 82% of *n*-hexadecane mineralization in a sandy plume was attributed to bacteria, while only 14% was due to fungi.

Based on published reports in the literature, among the most common and efficient hydrocarbon-degrading bacteria in both soil and marine environments are species of *Acinetobacter, Pseudomonas, Alcaligenes, Arthrobacter, Achromobacter, Bacillus, Flavobacterium* and *Nocardia*; among the yeasts are *Aureobasidium, Candida, Rhodotorula and Sporobolomgers.* Among fungi the dominant organisms are species of *Trichoderma, Mortiecerella, Penicillium* and *Aspergillus* (Leahy and Colwell, 1990, Atlas and Bartha 1992; Ubalua, 2011). Species of *Aspergillus, Penicillium* and *Phanerochaete,* capable of degrading hydrocarbons have been frequently isolated fromsoil and marine environment. In a successful biopile remediation test, the soil microbial characterization showed one *Bacillus*, two *Corynebacterium*, and three *Micrococcus* species among the existing Gram-positive bacteria, and *Enterobacter amnigenus*, *Enterobacter cloacae*, and *Pseudomonas* sp. were detected as the main Gram-negative bacteria present in the soils (Iturbe *et al*., 2007).

Biodegradation mediated by indigenous microbial communities is the ultimate fate of the majority of oil hydrocarbons that enter the marine environment (Leahy and Colwell**,** 1990; Atlas, 1991; Prince, 2010). Many interesting marine bacteria have been isolated and characterized to use hydrocarbons as a carbon source (Head *et al*., 2006; Malatova, 2005). Some of the examples include species of *Alcanivorax*, *Cycloclasticus*, *Oleiphilus*, *Oleispira*, *Thalassolituus* and some members of the genus *Planomicrobium* (previously known as *Planococcus*). *Alcanivorax* spp., *Oleiphilus* spp., *Oleispira* spp., *Thalassolituus* spp. and *Planomicrobium* spp. use a variety of branched- and/or straight-chain saturated hydrocarbons, whereas *Cycloclasticus* spp. can use a range of polycyclic aromatic hydrocarbons. The

selection of the microbial population is determined by the characteristics of the pollutant to be treated. The challenge of characterizing the roles of a range of organisms in degrading contaminants present in natural media is clearly substantial.

Most of the marine bacteria are well known for their association with the wide variety of functions like antibiotics and enzyme production (Okami, 1986), heavy metal bioremediation (Rainbow, 1995), biosurfactant production (Maneerat and Phetrong, 2007), biodegradation and bioremediation of hydrocarbons (Margesin and Schinner, 2001), oil biodegradation (Nweke and Okpokwasili, 2003), bioremediation of diesel-contaminated soils (Gallego *et al*., 2001).

It is important to analyze the community structure and its changes during bioremediation processes. The challenge of characterizing the roles of a range of hydrocarbon-metabolizing organisms in degrading the myriad of petroleum substrates present in hydrocarboncontaminated soils is clearly substantial. However, such studies can provide major insights into important biochemical and physiological aspects of bioremediation and microbial catabolism. Culturedependent and -independent methods are being applied to microbial community characterization. The temporal and spatial changes in bacterial populations and the diversity of the microbial community during bioremediation can be determined using sophisticated (Stapleton *et al*., 1998; van Elsas et al. 1998; Widada *et al*., 2002). Recent advances in molecular techniques, combined with genomic information, are greatly assisting microbiologists in unraveling some of the mysteries related to the diverse roles of microbes in these communities. One of the exciting outcomes of these studies is an advance in our understanding of the degree and importance of lateral gene transfer in complex microbial communities. Catabolic genes have the ability to spread through a microbial community at high frequencies (Top and Springael 2003; Van der Meer and Senchilo 2003).

Since the majority of the biological treatment processes rely on the activities of complex microbial communities, there still is much to learn about the interactive and interdependent roles played by individual species in these communities (Ram, 2005). Community dynamics are mainly followed using cultivation-independent, 16S-rRNA-gene-based methods or functional-gene-based methods (Spiegelman *et al.,* 2005). Careful monitoring of microbial community dynamics can lead to the discovery of common biodegradation patterns, which can help in the development of new tools for rapid evaluation of ongoing bioremediation processes (Head *et al*., 2006).

Among saturated hydrocarbons, *n*-alkanes are generally considered to be readily degradable components of crude oil (Setti *et al.,* 1993). Based on the chain length, *n*-alkanes are divided into three degradation groups: lower alkanes ($C_8$– $C_{16}$), medium alkanes ($C_{17}$– $C_{28}$), and higher alkanes, (> $C_{28}$). A number of microbes including bacteria, fungi and yeasts have been reported to degrade alkanes using them as the source of carbon and energy (van Beilen *et al.,* 2003; Wentzel *et al*. 2007). Depending on the solubility, the alkanes may be arranged as follow: linear alkanes > branched alkanes > cyclic alkanes with regard to their susceptibility to microbial degradation (Singh *et al*., 2012).

Biodegradation of n-alkanes with carbon chain length from $C_8$– $C_{16}$ is generally favoured by increased solubility and reduced surface tension. Biodegradation of *n*-alkanes up to $C_{44}$ has been demonstrated (Sakai *et al.,* 1994). In most instances, enhanced solubilization was a result of surface-active biological remediation of petroleum contaminants compounds produced by the hydrocarbon-degrading microorganisms. Emulsification of the substrate enhanced the contaminant surface area, and this facilitated higher dissolution rates and overcame mass-transfer limitations (Herman *et al.,* 1997; Yuste *et al.,* 2000; Koma *et al.,* 2001; Singh *et al.,* 2006).

The metabolism of branched-chain alkanes has received relatively little attention, in contrast to metabolism of *n*-alkanes. Most hydrocarbon-degrading microorganisms cannot effect the complete degradation of branched-chain alkanes. Transformation of cyclohexane by mixed or pure culture has been reported (de Klerk and van der Linden, 1974; Uribe *et al.,* 1990). Earlier transformation of cyclohexanes was demonstrated using pure cultures (Ooyama and Foster, 1965) in the presence of co-substrates only. The degradation of aromatic hydrocarbon has been extensively reviewed (Gibson and Parales, 2000; Spormann and Widdel, 2000; Foght, 2008; Phelps and Young, 2001; Gibson and Harwood, 2002; Chakraborty and Coates, 2004; Andreoni and Gianfreda, 2007; Farhadian *et al.*, 2008; Cao *et al.*, 2009; Das and Chandran, 2010; Martins and Peixoto, 2012) and examination of these reviews reveals that our understanding of the biodegradation pathways is constantly changing.

Light aromatic hydrocarbons are subjected to evaporation and to microbial degradation in a dissolved state, but extensive methyl substitution does inhibit initial oxidation. A general comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that the initial conversion steps are carried out by different enzymes, but those compounds are transformed to a limited number of common metabolites such as protocatechuate and catechols (Reineke and Knackmuss, 1988; Commandeur and Parsons, 1990; Chaudhary and Chapalamadugu, 1991).

These key intermediates are subjected to one of the two ring-cleavage pathways, either to an extradiol (meta) or to an intra-diol (ortho) cleavage-type pathway (Harayama *et al.,* 1999). In the majority of the cases, ortho-fission of catechol by catechol-1,2-dioxygenase and of prototechuate by protocatechuate 3,4 dioxygenase has been demonstrated (Fewson, 1991).

The resins and asphaltenes are generally considered to be recalcitrant to biodegradation, and the metabolic pathways for the degradation of these fractions are less understood (Van Hamme *et al.,* 2003). Bacterial strains capable of utilizing resins as the sole source of carbon and energy have also been isolated (Venkateswaran *et al.*, 1995). These strains, isolated from mixed populations, were clustered into five phenotypic groups: *Pseudomonas, Vibrionaceae, Enterobacteriaceae, Moraxella* and *Neisseria*-like rods. Among them, one *Pseudomonas* strain, when grown on resins as the sole source of carbon, showed significant adaptation towards these high molecular weight classes of crude oil. Microorganisms that biodegrade resins and asphaltenes by cometabolism have been identified (Raymond *et al.*, 1975). Biological degradation of asphaltenes is a process that indeed occurs in nature, but in very low percentages, from 5 to 35%. It is mediated by microbial consortia, isolated from sediments and other sources polluted with hydrocarbons (Venkateswaran *et al*., 1995; Thouand *et al*., 1999). These complex molecular structures of asphaltenes make them resistant to biodegradation and cause their accumulation in ecosystems where petroleum and its refining byproducts are spilled either in accidental or purposeful ways (Atlas, 1981; Guiliano *et al.*, 2000 ).

Mixed populations of marine microorganisms *Pseudomonas, Brevibacterium, Alcaligenes, Arthrobacter and Bacillus* were found to cometabolize resins and asphaltenes. The presence of saturated hydrocarbons ($C_{12}$–$C_{18}$) appeared to be a pre-requisite for the degradation of resins and asphaltene. The *n*- alkanes probably served as carbon and energy source for the bacteria, or might be useful in solubilization and emulsification of resins and asphaltene by their fatty acid degradation products. Characterization studies of crude oil resins by Fourier Transform Infrared Spectroscopy (FTIR) have shown that resins are more oxidized, more aliphatic and less aromatic than asphaltenes (Boukir *et al.*, 2001).

In many ecosystems there is already an adequate indigenous microbial community capable of extensive oil biodegradation, provided that environmental conditions are favorable for oil-degrading metabolic activity (Richard and Vogel, 1999; Capelli, *et al*., 2001). There are several advantages relying on indigenous microorganisms rather than adding microorganisms to degrade hydrocarbons. First, natural populations have developed through many years. These microorganisms are adapted for survival and proliferation in that environment. Secondly, the ability to utilize hydrocarbons is distributed among a diverse microbial population. This population occurs in natural ecosystems and either independently or in combination metabolizes various hydrocarbons. Many times, when the amount of microorganisms is sufficient in the contaminated environment, microbial seeding is not required. Nutrient availability, especially of nitrogen and phosphorus, seems to be the most limiting factor. It was confirmed that these nutrients enhance growth of microorganisms, which leads to more rapid decomposition of contaminants (Coulon *et al.*, 2004; Chaineau *et al.*, 2005). Accepted values for a mixed microbial population in the soil are C: N, 10:1; and C: P, 100:1. Nitrogen and phosphorus can be supplied with common inorganic fertilizers with the N:P ratio at 16:1 when the optimum nitrogen fertilization for a sandy matrix is lower than 100 mg N kg dry soil mates (Ferguson *et al*., 2003; Kim, *et al*., 2004). Adding yeast extract or domestic sewage as source of nitrogen did not prove beneficial. Urea formaldehyde was found to be the most satisfactory nitrogen source (Riser-Roberts, 1992).

Microorganisms are equipped with metabolic machinery to use petroleum products as a carbon and energy source. The metabolic pathways that hydrocarbon-degrading heterotrophs use can be either aerobic (i.e. they utilize oxygen as the primary electron acceptor) or anaerobic (i.e. they utilize an alternative electron acceptor such as nitrate or sulfate). Aerobic degradation usually proceeds more rapidly and is considered to be more

effective than anaerobic degradation. One reason is that aerobic reactions require less free energy for initiation and yield more energy per reaction (Malatova, 2005).

## 2.6 Petroleum Biodegradation by Microbial Consortia

Researches indicate that use of mixed bacterial consortium is more efficient in biodegradation of crude oil than individual bacterial strains. For example, according to Sathishkumar *et al.* (2008), the mixed consortium of four bacterial strains degraded a maximum of 77% crude oil, followed by 69% by *Pseudomonas* sp. BPS1-8, 64% by *Bacillus* sp. ISS1-7, 45% by *Pseudomonas* sp. HPS2-5, and 41% by *Corynebacterium* sp. BPS2-6 at 1% crude oil concentration. Increasing crude oil concentration from 1 to 12%, the degradation rate by the same consortium was decreased still it reached up to 45%. Many biodegradation processes require more than a single species. Individual organism can metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with overall broad enzymatic capacities are required to bring the and extent of petroleum biodegradation further. Microbial strain belonging to various genera have been detected in petroleum contaminated soil or water (Sorkhoh *et al.*, 1995; Dagher *et al*., 1997; Radwan *et al.*, 1999). This strongly suggests that each strain or genera have their roles in the hydrocarbon transformation processes.

Further evidence for the cooperation of mixed cultures in biodegradation is apparent when Sorkhoh *et al.* (1995) observed a sequential change of the composition of oil degrading bacteria over a period of time in oil-contaminate soil was reported in sequential enrichments in medium containing residual crude oil (Venkateswaran and Harayama, 1995). After exhaustive growth of one strain in crude oil, the residual oil supported the growth of a second and third strain of bacteria (Horowitz *et al.,* 1975). When grown together in a mixed culture or sequentially, there was over 75% oil transformation.

To maximize petroleum biodegradation, many studies employed mixed bacterial and bacterial fungal cultures (Jørgensen *et al*., 2000; Li *et al.*, 2000; Del' Arco and França, 2001; Vasudevan and Rajaram, 2001; Mishra *et al.*, 2001; Shelton *et al.*, 2002; Mrayyan and Battikhi, 2005; Morais and Tauk-Tornisielo, 2009; Varjani and Upasani, 2013). Rambeloarisoa *et al.* (1984) demonstrated consortia of eight strains that were able to grow in pure cultures using a variety of hydrocarbons. However, when the other 3 strains were removed from the consortium, the effectiveness of the mixed culture was remarkably reduced. Marquez – Rocha *et al.* (2001) demonstrated diesel – biodegradation effectiveness of 85, 91 and 96% using bacterial consortium of white rot fungus, *Pleurotus ostreatus*, and a combination of the bacterial consortium and *P. ostreatus* respectively. The biodegradation rate of diesel was also highest in the bacteria and fungus mixture. Thus, each member in a microbial community has significant role, and may need to depend on the presence of other species or strain to be able to survive.

The degradative capacity of any microbial consortium is not necessarily that the result of mere solding together of capacities of the individual strains forming the association. Surotseva *et al.* (1997), when removing three strains from an association, observed a decrease in efficiency of the consortium's degradation of aromatic fractions.

Komukai – Nakamura *et al*. (1996) reported the sequential degradation of Arabian light crude oil by two different genera. *Acinetobacter* sp. T4 biodegraded alkanes and other hydrocarbons producing the accumulation of metabolites following that, *Pseudomonaes putida* PB4 began to grow on the metabolites and finally degraded aromatic compounds in the crude oil. Further, Nadarajah *et al.* (2002) suggested that single pure bacterial culture would require the use of sterile fermenters and culture media, which have high capital and operating costs, hence using a mixed bacterial culture offers an economically feasible

bioremediation process. The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been attributed to the effects of synergistic interactions among members of the association. It is possible that one species removes the toxic metabolites (that otherwise may hinder microbial activities) of the species preceding it. It is also possible that the second species is able to degrade only partially (Alexander, 2000).

## 2.7 Metabolic Machinery

Aerobic biodegradation of hydrocarbons and crude oil is a long known and well-studied process. However, the ability of anaerobic microorganisms to oxidize and utilize crude oil as a complex organic substrate under aerobic conditions was discovered just recently (Malatova, 2005). These microorganisms decompose most organic compounds into carbon dioxide, water and mineral matter, such as sulfate, nitrate and other inorganic compounds. They do not produce hydrogen sulfide or methane as reaction products. The aerobic pathway proceeds most rapidly and most efficiently, because aerobic reactions require less free energy for initiation and yield more energy per reaction (Malatova, 2005).

Aromatic hydrocarbons, e.g benzene, toluene, ethylbenzene and xylenes (BTEX compounds), and naphthalene belong to the large volume of petrochemicals, widely used as fuels and industrial solvents. Phenols and chlorophenols are released into the environment as products and waste materials from industry. Aromatic compounds are formed in large amounts by all organisms, e.g., as aromatic amino acids, phenols, or quinones. Thus, it is not surprising that many microorganisms have evolved catabolic pathways to degrade aromatic compounds. In general, man-made organic chemicals (xenobiotics) can be degraded by microorganisms, when the respective molecules are similar to natural compounds. The diversity of man-made aromatics can be converted enzymatically to natural intermediates of the degradation: catechol and protecatechuate. In general, benzene and related compounds are

characterized by a higher thermodynamic stability than aliphatics are. Only few reports on bacteria capable of attacking benzene have been published (Smith, 1990; Zylstra, 1994; Gülensoy and Alvarez, 1999). Monooxygenases incorporate one atom of oxygen into the substrate; the second atom is reduced to $H_2O$. Dioxygenases incorporate both atoms into the substrate.

The first step of benzene oxidation is a hydroxylation catalyzed by a dioxygenase (Fig. 2.1).The product, a diol, is then converted to catechol by a dehydrogenase. These initial reactions, hydroxylation and dehydrogenation, are also common to pathways of degradation of other aromatic hydrocarbons. The introduction of a substituent group onto the benzene ring renders alternative mechanisms possible to attack side chains or to oxidize the aromatic ring (Das and Chandran, 2011). The versatility and adaptability of bacteria is based on the existence of catabolic plasmids. Catabolic plasmids have been found to encode enzymes degrading.

The metabolism of a wide spectrum of aromatic compounds by one species requires the metabolic isolation of intermediates into distinct pathways. This kind of metabolic compartmentation seems to be realized by metabolic regulation. The key enzymes of the degradation of aromatic substrates are induced and synthesized in appreciable amounts only when the substrate or structurally related compounds are present. Enzyme induction depends on the concentration of the inducing molecules. The substrate specific concentrations represent the threshold of utilization and growth and are in the magnitude of μM.A recent report on the regulation of TOL catabolic pathways has been published by Ramos *et al.* (1997).

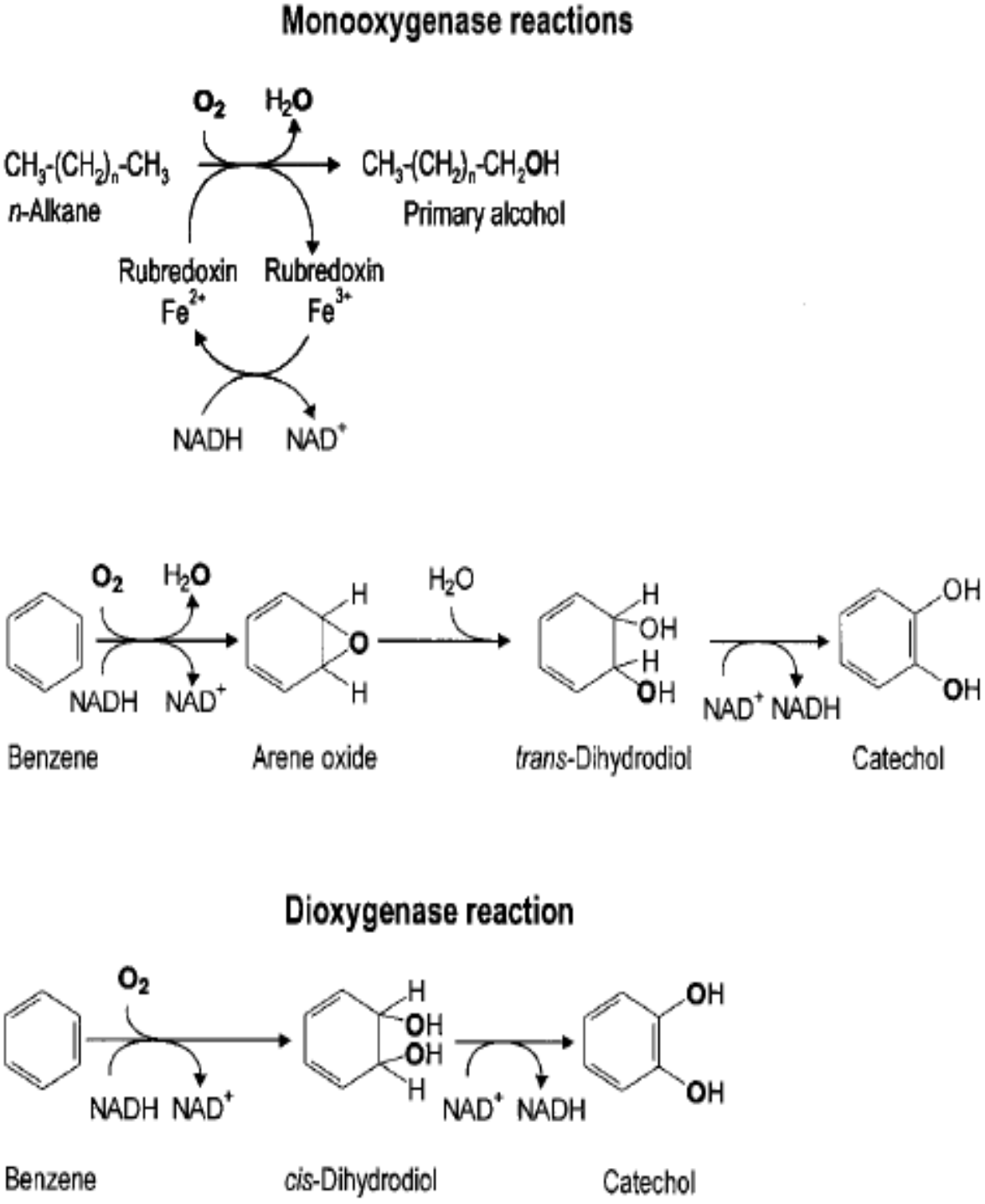



**Fig.2.1:** Initial attack on xenobiotics by oxygenases (Das and Chandran, 2011).

Fig 2.2 shows the pathways of the oxygenolytic ring cleavage to intermediates of the central metabolism. At the branchpoint catechol either is oxidized by the intradiol *o*-cleavage, or the extradiol *m*-cleavage. Both ring cleavage reactions are catalyzed by specific dioxygenases. The product of the *o*-cleavage – *cis, cis* muconate – is transferred to the instable enollactone, which is in turn hydrolyzed to oxoadipate. This dicarboxylic acid is activated by transfer to CoA, followed by the thiolytic cleavage to acetyl-CoA and succinate. Protocatechuate is metabolized by a homologous set of enzymes.The additional carboxylic group is decarboxylated and, simultaneously, the double bond is shifted to form oxoadipate enollactone. The oxygenolytic *m*-cleavage yields 2- hydroxymuconic semialdehyde, which is metabolized by the hydrolytic enzymes to formate, acetaldehyde, and pyruvate. These are then utilized in the central metabolism (Fritsche and Hofrichter, 2000). In general, a wealth of aromatic substrates is degraded by a limited number of reactions: hydroxylation, oxygenolytic ring cleavage, isomerization, and hydrolysis. The inducible nature of the enzymes and their substrate specificity enable bacteria with a high degradation potential, e.g., *Pseudomonads* and *Rhodococci*, to adapt their metabolism to the effective utilization of substrate mixtures in polluted soils and to grow at a high rate (Jördening and Winter, 2005). The hydrocarbons are broken down by a series of enzyme-mediated reactions. Oxygen serves as an external electron acceptor, while an organic component of the contaminating substance functions as the electron donor or energy source. The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde and a fatty acid. The fatty acid is cleaved, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule in a process known as beta-oxidation.

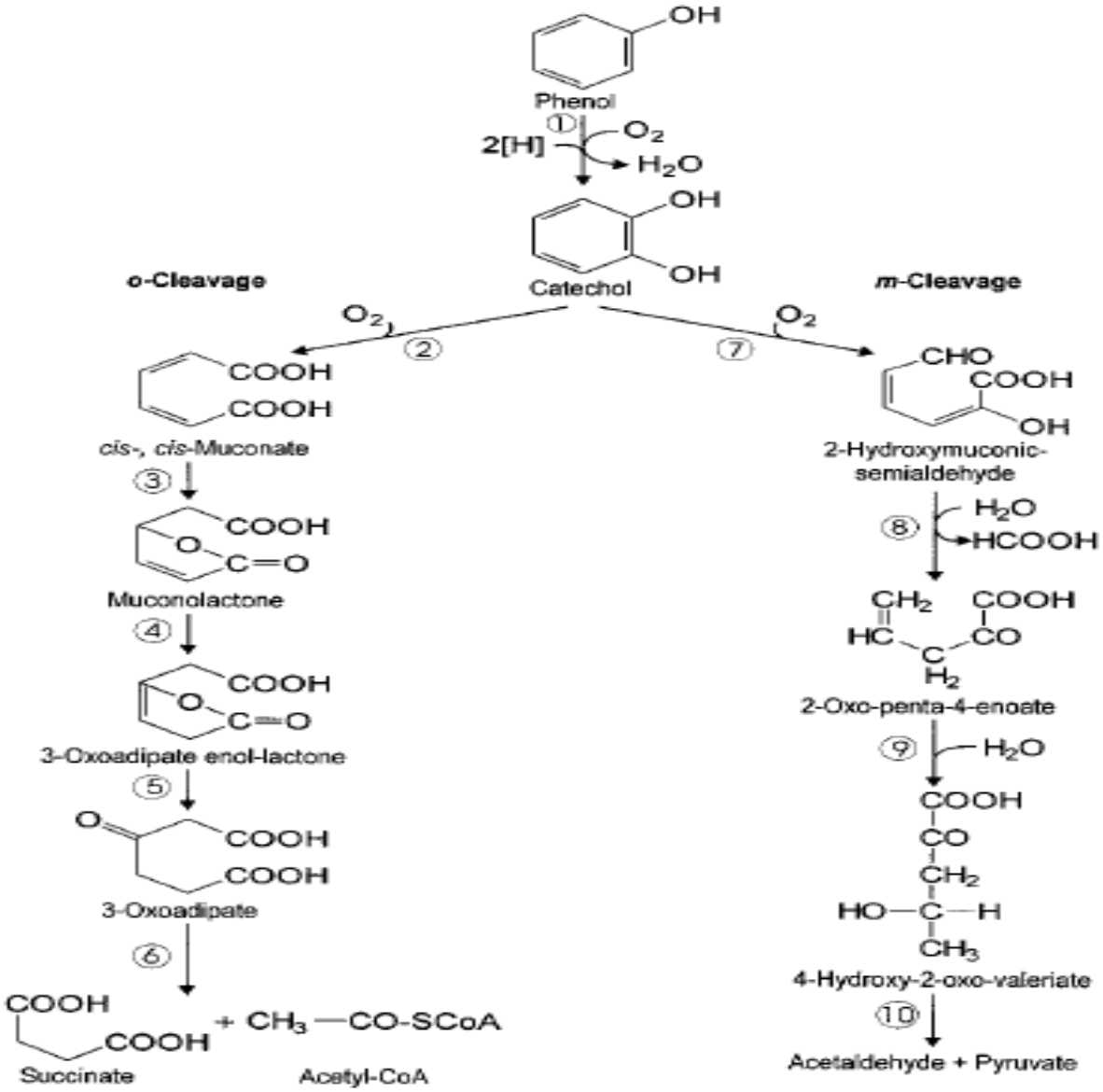



**Fig.2.2:** The two alternative pathways of aerobic degradation of aromatic compounds: *o*- and *m*-cleavage (Fritsche and Hofrichter, 2000)

(1) phenol monoxygenase,(2) catechol 1,2-dioxygenase,(3) muconate lactonizing enzyme, (4) muconolactone isomerase, (5) oxoadipate enol-lactone hydrolase,(6) oxoadipate succinyl-CoA transferase,(7) catechol 2,3 dioxygenase, (8) hydroxymuconic semialdehyde hydrolase,(9) 2-oxopent-4- enoic acid hydrolase,(10) 4-hydroxy-2- oxovalerate aldolase

The initial enzymatic attack involves a group of monooxygenases. The general pathway for aromatic hydrocarbons involves cis-hydroxylation of the ring structure forming a diol (e.g. catechol) using dioxygenase. The ring is oxidatively cleaved by dioxygenases, forming a dicarboxylic acid (e.g. muconic acid). Oxidation of substituted aromatics generally proceeds by initial beta-oxidation of the sidechain, followed by cleavage of the ring structure. The degradative pathway for a highly branched compound, such as pristane or phytane, may proceed by omega oxidation forming a dicarboxylic acid, instead of only monocarboxylic acid (Hamme *et al*., 2003).

Aerobic degradation in soil is associated with a variety of microorganisms, including bacteria, actinomycetes and fungi. *Acinetobacter* and *Rhodococcus* are two bacterial strains often associated with petroleum contaminated habitats. Intense interest has arisen on the study of alkane oxidation by these bacteria in the last decade. *Acinetobacter* utilizes an alkane monooxygenase (terminal oxidation) to convert the hydrocarbon to a primary alcohol to allow for the subsequent breakdown and utilization of the hydrocarbon.The degradation of alkylcyclohexane by *Acinetobacter* sp. ODDK71 was investigated by Koma *et al*. (2003). Strain ODDK71 degraded alkylcyclohexanes (alkyl sidechain length of >12) by co-metabolism when hexadecane was used as a growth substrate. GC/MS analysis of co-metabolized products from dodecylcyclohexane suggests that strain ODDK71 degraded dodecylcyclohexane via a ring oxidation and an alkyl sidechain oxidation pathways. The ring oxidation pathway of dodecylcyclohexane is a novel pathway of microbial degradation of dodecylcyclohexane (Koma *et al*., 2003). Significant correlation between aliphatic, aromatic and asphaltic fractions of the crude oil and the *Rhodococcus* genera able to utilize such hydrocarbons was observed in several studies (Finnerty,1992; Warhurst and Fewson, 1994; Ruberto *et al*., 2005; Malatova, 2005; Jördening and Winter, 2005). After oil pollution of soil, representative strains of the *Rhodococcus* showed the ability to metabolize a broad spectrum

of hydrocarbons (Peressutti *et al*., 2003). *Rhodococcus* possess an alkane monooxygenase as *Acinetobacter*, but in which the second carbon atom is oxidized (subterminal oxidation) leading to the production of a secondary alcohol and the subsequent ketone is further metabolized to a primary alcohol for further breakdown (Figure 2.3). Both microorganisms, *Acinetobacter* as well *Rhodococcus*, utilize respiration to generate ATP where oxygen serves as the terminal electron acceptor in electron transport. *Pseudomonas* appears to be the most ubiquitous bacteria found in oil contaminated soils and soil in general. This bacterium is able to adapt to many different hydrocarbons. They are responsible for degrading most of the aromatics in gasoline, although the efficiency in degrading aromatic hydrocarbons can vary among strains.The most extensively characterized polycyclic aromatic hydrocarbons degradation pathway is encoded by the NAH7 plasmids from *Pseudomonas putida (*Yen and Gunsalus, 1985; Sanseverino *et al.*, 1993; Eaton, 1994; Grimm and Harwood, 1999; Samanta and Jain, 2000; Obayori and Salam, 2010). The first operon encodes the pathway for naphthalene conversion to salicylate. The second codes for the conversion of salicylate via catechol meta-cleavage to acetaldehyde and pyruvate. Molecular oxygen is introduced into the aromatic nucleus via naphthalene dioxygenase (Hamme *et al.*, 2003).

The catabolic pathways for three- and four-ring PAHs in *Pseudomonas putida* were examined in several studies (Prabhu and Phale, 2003; Parales and Haddock, 2004; Malatova, 2005). For example, phenanthrene was degraded by *Pseudomonas* sp. strain PP2 via a dioxygenase-initiated pathway that converged with the naphthalene degradation pathway. Secretions of a surfactant into the medium and increased cell-surface hydrophobicity during growth were postulated to increase uptake of poorly soluble phenanthrene (Parales and Haddock, 2004).

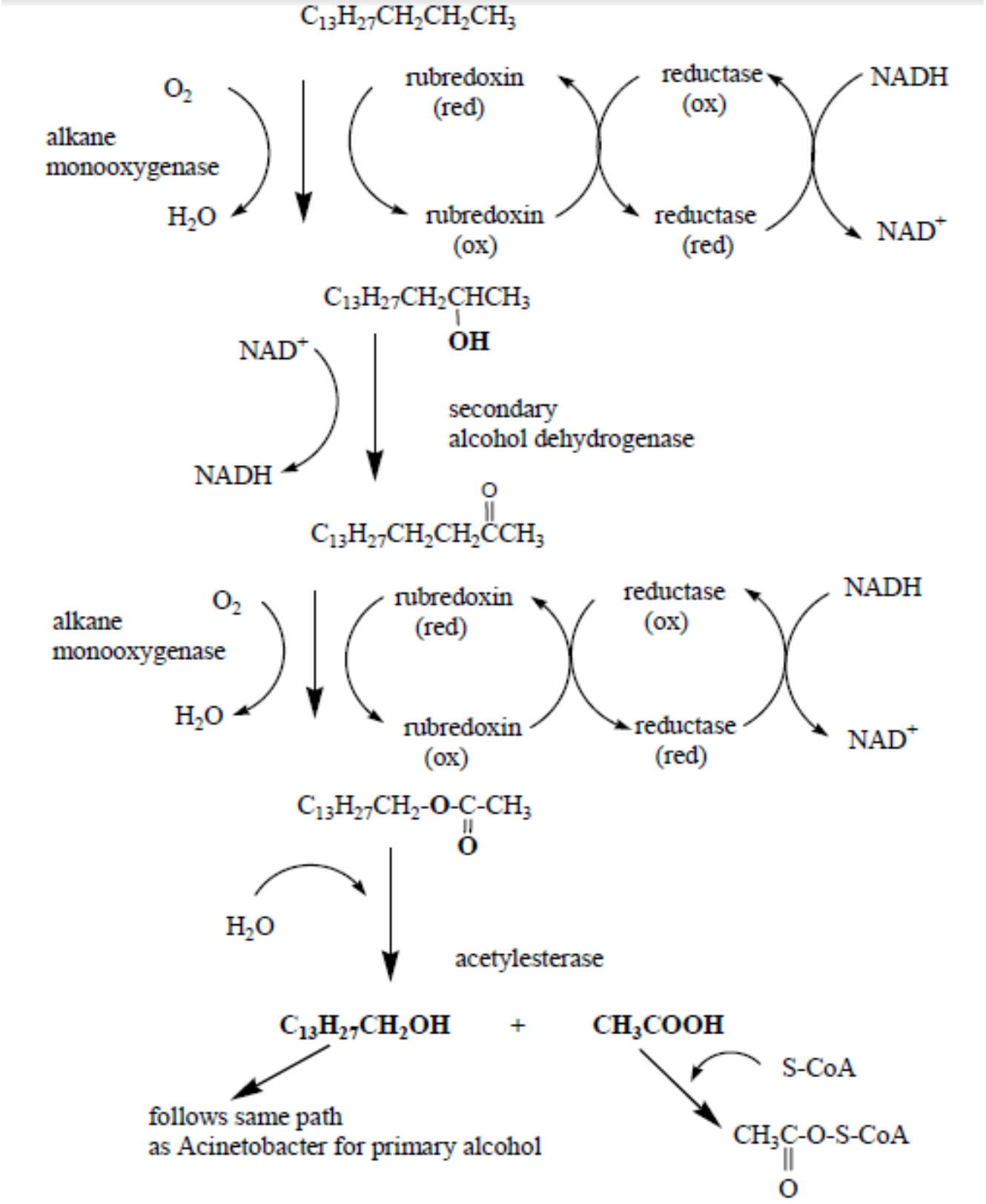



Fig. 2.3**:** Degradation of alkanes by *Rhodococcus sp*. (Hamme *et al*., 2003)
NAD/NADH= nicotine amide adenine dinucleotide; S-CoA= acetyl coenzyme

Specific attention has also been given to other dioxygenases associated with the biodegradation of polycyclic aromatic hydrocarbons (PAHs). These enzymes represent multicomponent systems that catalyze a specific regioselective dioxygenation. These reactions are required for degradation of dibenzofurans, dibenzo-*p*-dioxin and carbazole. Carbazole dioxygenase activity had been observed only in *Pseudomonas* strains (Ouchiyama *et al*.,1993) The archetype carbazole 1,9-dioxygenase, isolated from *Pseudomonas resinovorans* CA10, has been characterized in detail (Pieper *et al*., 2004).

Aromatic compounds, including chlorinated ones, are frequently a major fraction of petroleum hydrocarbons. Further, most of the aromatic compounds, including polyaromatic hydrocarbons, are known to be metabolized to a common intermediate, catechol, which is then oxidized through the two ring-cleavage pathways, *ortho* and *meta* cleavage pathways, catalyzed by $C_{12}O$ and $C_{23}O$, respectively (Uchiyama and Miyazaki, 2013).

The substrates of the ring-cleavage dioxygenases usually contain two hydroxyl groups on two adjacent aromatic carbons and ring-cleavage dioxygenases can be classified into two groups according to the mode of scission of the aromatic ring. Intradiol enzymes cleave the aromatic ring between two hydroxyl groups, whereas extradiol enzymes, such as catechol-2, 3-dioxygenase, cleave the aromatic ring between one hydroxylated carbon and another adjacent non-hydroxylated carbon. These enzymes play a key role in the metabolism of aromatic compounds. In bacteria, they are responsible for the cleavage of aromatic rings during aerobic catabolism of compounds such as toluene and xylene, naphthalene or biphenyl derivatives, some of which are environmental pollutants of serious concern (Harayama and Rekik, 1989). Consequently, these enzymes were the subject of different studies and have been suitable targets for site directed mutagenesis to improve their activities by selecting the

best mutant which had the best activity in oil degradation and use them in future for cleaning oil spills in the environment (Andujart and Santero, 2003).

Aerobic and anaerobic biodegradation of aromatic compounds have several common features. Structurally diverse compounds are degraded through many different peripheral pathways to a few intermediates that are further channeled via a few central pathways to the central metabolism of the cell (Diaz, 2004). In the aerobic catabolic funnel, most peripheral pathways involve oxygenation reactions carried out by monooxygenases and/or hydroxylating dioxygenases that generate dihydroxy aromatic compounds (catechol, protocatechuate, gentisate, homoprotocatechuate, homogentisate, hydroquinone, hydroxyquinol). These intermediate compounds are the substrates of ring-cleavage enzymes that use molecular oxygen to open the aromatic ring between the two hydroxyl groups (ortho cleavage, catalyzed by intradiol dioxygenases) or proximal to one of the two hydroxyl groups (meta cleavage, catalyzed by extradiol dioxygenases) (Fig. 2.4) (Harayama and Timmis, 1992).

Central pathways involve a series of reactions leading to the formation of Krebs cycle intermediates (central metabolism). In the anaerobic catabolism of aromatic compounds, the peripheral pathways converge to benzoyl-CoA (occasionally to resorcinol and phloroglucinol), which becomes dearomatized by a specific multicomponent reductase that requires confronted with alternative carbon sources and they must “decide” which of them will be preferentially consumed before metabolizing less preferred substrates, such as pollutants, to guarantee, therefore, a satisfactory metabolic return. In order to achieve this goal, bacteria have developed a physiological response (superimposed regulation) that controls and adjusts the specific regulation of catabolic operons to the physiological and metabolic state of the cells (Cases and de Lorenzo, 2001). The catabolic clusters are usually present in mobile genetic elements, such as transposons and plasmids, which facilitate their

horizontal transfer of the respective genes and, therefore, rapid adaptation of microorganisms to the presence of new pollutants in a particular ecosystem (Van der Meer *et al*., 1992; Tan, 1999). A PAH - and phenol-degrading microorganism, *Pseudomonas putida* (ATCC 17484), was also used to study the substrate interactions during cell growth on carbazole-containing mixtures with *p*-cresol and sodium salicylate. Both *p*-cresol and sodium salicylate could be utilized by the bacteria as the sole carbon and energy sources (Gen-Yu and Loh, 2002). Some other *Pseudomonas* sp. was found to grow on aromatic constituents of gasoline as a sole source of carbon. Strains of the *Bijerinckia* genus are also very active in aerobic hydrocarbon degradation. The presence of biphenyl dioxygenase enable these microorganisms to oxidize benzo(a) pyrene, benzo(a)anthracene and the aromatic N-heterocycle carbazole (Resnick *et al*., 1993).

## 2.8 Anaerobic Degradation

In contrast to the fact that aerobic microbial hydrocarbon metabolism has been extensively investigated, the same is not true about anaerobic hydrocarbon metabolism, the roles of bacteria that participate in these processes under anoxic/anaerobic conditions during biodegradation are not fully understood. Oxygen is not available in all environments where hydrocarbons occur (e.g. in deep sediments, flooded soils, eutrophic lagoons, stagnant fresh and ocean waters and in oil reservoirs). Several studies have investigated the question, whether or not the biodegradation of hydrocarbons is possible under anoxic conditions. It was not until the late 1980s that new groups of microorganisms were found to degrade hydrocarbons under strictly anoxic conditions. Studies have confirmed that these microorganisms activate organic compounds by special biochemical mechanisms that differ completely from those employed in aerobic hydrocarbon metabolism (Riser-Roberts, 1992; Malatova, 2005; Saxena e*t al.*, 2013).

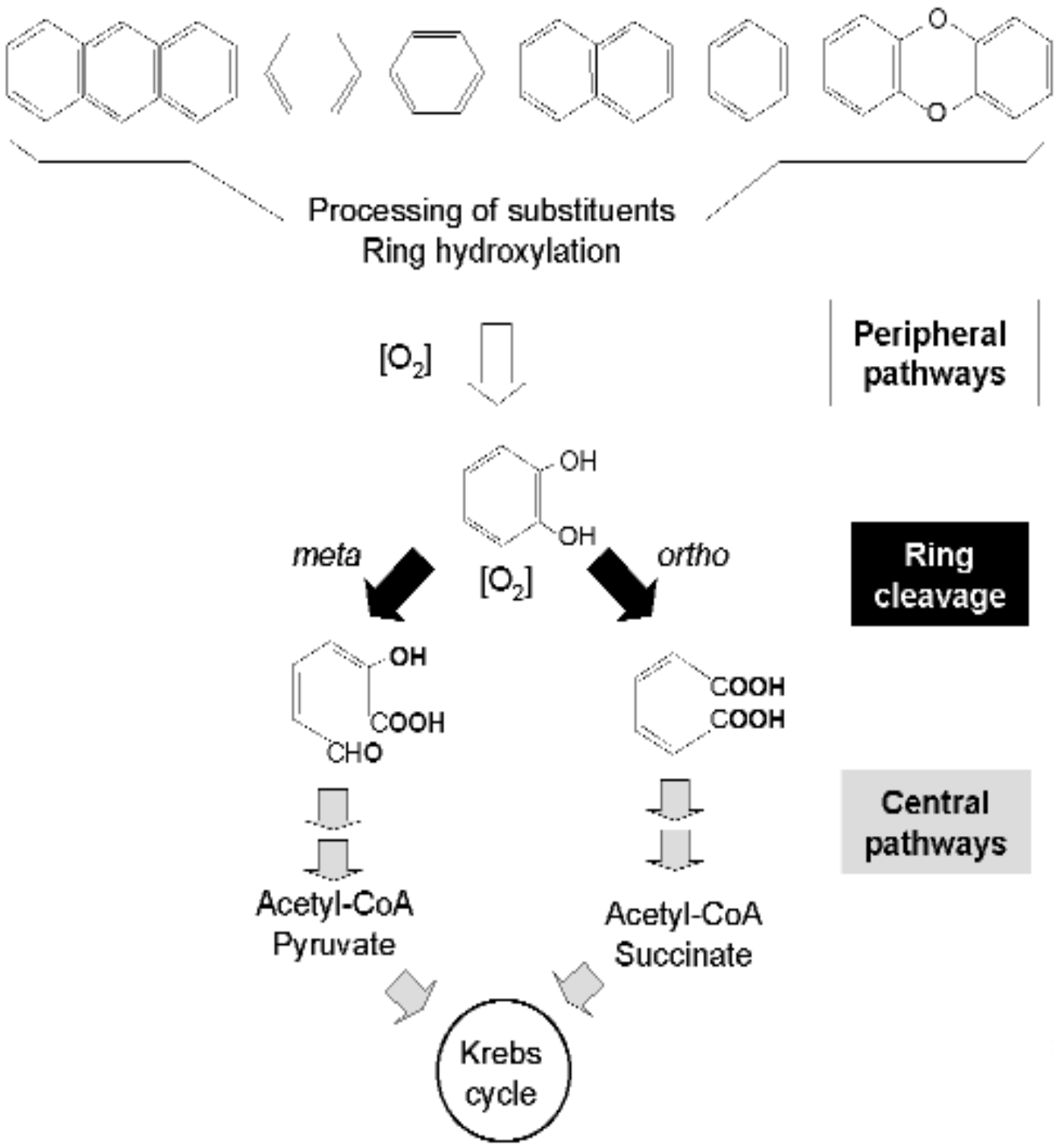



Fig. 2.4: The catabolic funnel for the aerobic degradation of aromatics.
**White arrows**, peripheral pathways, **black arrows**, cleavage of the ring; **gray arrows**, central pathways (Harayama and Timmis, 1992)

N-alkanes, branched alkanes, cycloalkanes, and some alkenes have been shown to be degraded under anaerobic conditions. For example, unsubstituted, methyl-substituted, and ethyl-substituted cyclopentenes, cyclopentanes and cyclohexanes were consumed without a substantial lag in the presence of sulfate but rather less effectively under methanogenic conditions. Dimethyl-substituted cyclopentanes and cyclohexanes were biodegraded only in the presence of sulfate (Widdel and Rabus, 2001). Several laboratory and field studies conducted on biodegradation had clearly demonstrated biodegradation of BTEX (benzene, toluene, ethylbenzene and xylene isomers) compounds. The biodegradation of these individual compounds depends on the terminal electron acceptor. Usually, toluene and *m*, *p*-xylenes degrade under anaerobic conditions. One typical example is benzene degradation. Benzene is cleaved aerobically. On the other hand, it is usually recalcitrant under nitrate-reducing conditions. Benzene cleavage is highly site-specific in the presence of Fe (III) and sulfate terminal electron acceptor. Ethylbenzene degradation was described under aerobic as well as nitrate-reducing conditions. Nevertheless, degradation of this compound is site-specific under Fe (III) and sulfate reducing as well as methanogenic conditions. Utilization of *o*-xylene is enhanced by aerobic conditions, and is site-specific under anaerobic conditions (Schreiber *et al*., 2004).

Hydrocarbons such as alkylbenzenes including m-, o-, and p-xylene, trimethylbenzenes, naphthalene and phenanthrene as well as n-alkanes and branched alkanes can also be metabolized under anaerobic conditions (Figure 2.5A) (Thakur, 2006). These reactions may take place under Fe (III)-reducing, denitrifying and sulfate reducing conditions, by anoxygenic photosynthetic bacteria, or in syntrophic consortia of proton-reducing and methanogenic bacteria. Other terminal electron acceptors than $O_2$ shown to be used during this metabolism include manganese oxides, soil humic acids and fumarate in a

fermentative oxidation. These microorganisms that use nitrate, ferric iron or sulphate as electron acceptors grow in syntrophic cocultures with other anaerobes (Boopathy, 2004).
Most recent studies showed that *n*-hexane was activated under anaerobic conditions at carbon-2 in connection with an addition to fumarate, yielding (1-methylpentyl) succinate (Figure 2.5B) (Malatova, 2005).

The biochemistry of the degradation of alkylsuccinates has not been elucidated (Aeckersberg *et al*., 1998; So and Young, 1999). However, it is expected that these reactions lead to fatty acid metabolism. Toluene has been the most studied hydrocarbon with respect to enzymatic and genetic characterization in the denitrifying bacteria *Azoarcus* sp. In the proposed pathway, fumarate addition to toluene is mediated by benzylsuccinate synthase to form benzylsuccinate. Series of β-oxidation reactions convert benzylsuccinate to acetyl CoA and benzoyl-CoA which is a central intermediate in the anaerobic degradation of aromatic compounds. Benzoyl-CoA undergoes reductive dearomatization and ring cleavage followed by reactions that again resemble those in the β-oxidation reactions of fatty acids (Widdel and Rabus, 2001). For ethylbenzene, oxidation under denitrifying conditions starts with dehydrogenation by ethylbenzene dehydrogenase to produce 1-phenylethanol (Figure 2.5C). This reaction is followed by oxidation to acetophenone. Acetophenone is carboxylated and after activation yields 3-oxo-3phenylpropionyl-CoA and thiolytic cleavage to acetyl-CoA and benzoyl-CoA. The metabolic pathway for sulfate-reducing bacteria is similar to that of toluene metabolism. In this case (1-phenylethyl) succinate was detected in enrichment cultures of *Azoarcus* sp. indicating ethylbenzene addition to fumarate (Figure 2.5C). There is evidence that anaerobic degradation of *m*-xylene proceeds, in analogy to that of toluene, via *m*-methylbenzylsuccinate to *m*-methylbenzoyl-CoA (Riser-Roberts, 1992; Widdel and Rabus, 2001).

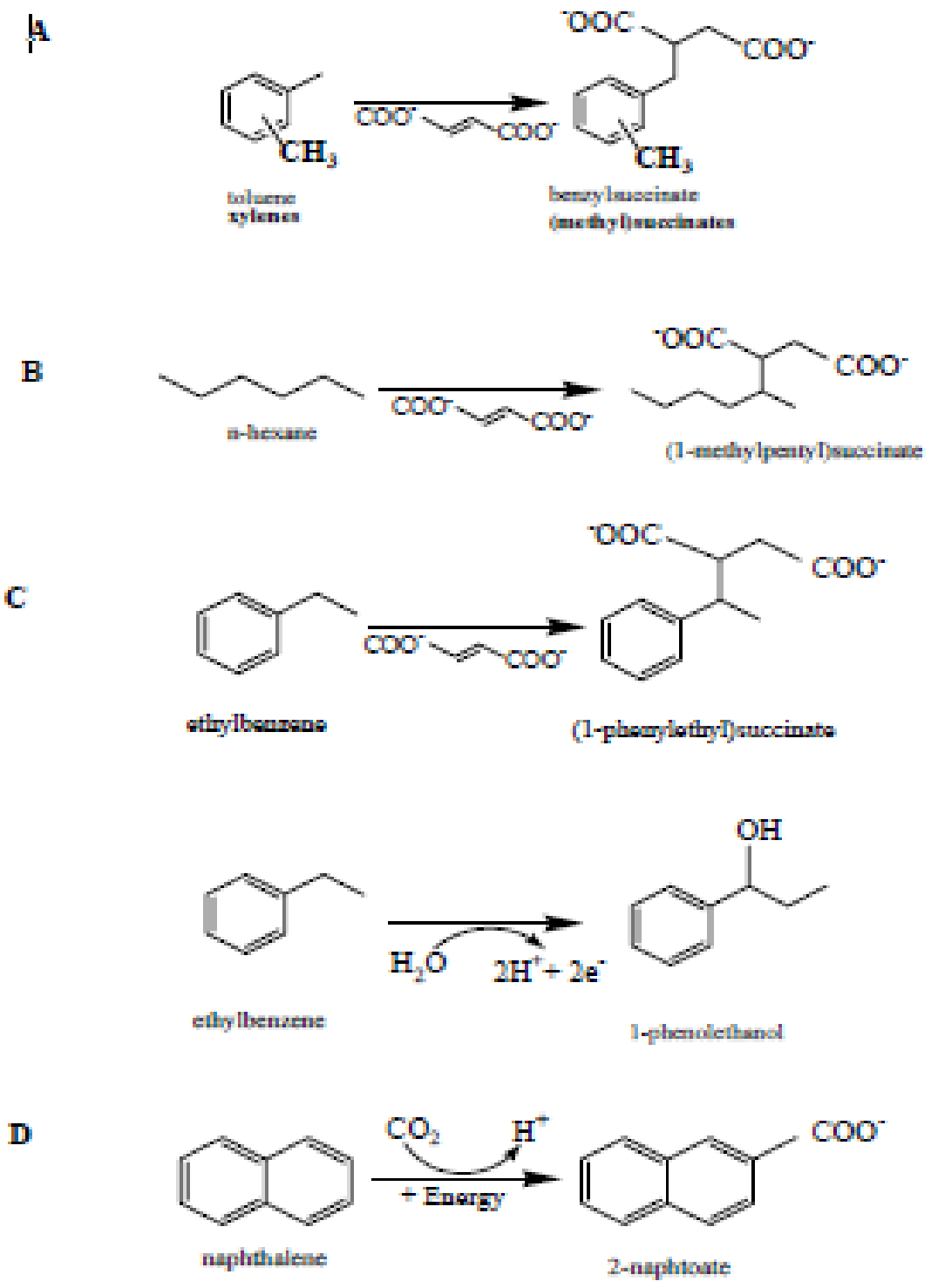



**Fig. 2.5** The initial reactions during anaerobic degradation of saturated and aromatic hydrocarbons (Townsend *et al*., 2004).

Succinate was detected in enrichment cultures of *Azoarcus* sp. indicating ethylbenzene addition to fumarate. There is evidence that anaerobic degradation of *m*-xylene proceeds, in analogy to that of toluene, via *m*-methylbenzylsuccinate to *m*-methylbenzoyl-CoA (Widdel and Rabus, 2001; Riser-Roberts, 1992).

PAHs are also metabolized under anaerobic conditions. Naphtalene degradation proceeds via carboxylation to form 2-naphtoate (the central intermediate in a pathway analogous to the benzoyl-CoA pathway for monoaromatic compounds) in sulfate-reducing as well as denitrifying bacteria (Figure 2.5D) The identification of other metabolites in a sulphate-reducing enrichment culture indicated the further metabolism of 2-naphthoate (presumably as activated acid) via subsequent reduction of the two rings to yield decalin-2-carboxylate. Alkylnaphthalenes appear to be activated by a mechanism similar to that of toluene (Hamme *et al.*, 2003).

## 2.9 Approaches to Bioremediation

Petroleum refinery effluents contain floating or emulsified oil, which can be physically separated and recovered, but some of this oil associate with inorganic and organic particulates to form sludge which need appropriate disposal as solid wastes. Oily sludge from the separators in the wastewater treatment plants and ‘tank bottoms’; where sediments accumulate in the storage tanks can not be economically reprocessed. Most of these materials are classified as hazardous wastes, and therefore disposal of this sludge is tightly regulated and monitored. Bioremediation technology is considered an effective and economically viable method for cleanup of hydrocarbon-contaminated soils and sludge, either in-situ or ex situ after excavation or transportation (Singh *et al*., 2009; Bhatnagar and Kumari, 2013)

Bioremediation has long been applied as a treatment technology that is cost-effective, ecologically friendly and efficient for the decontamination of hydrocarbon polluted soils (Leahy and Colwell, 1990; Rosenberg and Ron, 1996; Balba *et al*., 1998; Bouchez-Naitali *et*

*al*., 1999; Margesin *et al*., 2003; Nweke and Okpokwasili, 2004; Kaplan and Kitts, 2004; Quatrini *et al*., 2008). Bioremediation techniques for removing petroleum hydrocarbons in the soil are developed around strategies for delivering moisture, aeration and nutrients in order to optimize microbial activity and degradation of the pollutants (Macnaughton *et al*., 1999; Kaplan and Kitts, 2004; Ayotamuno *et al*., 2006; Stroud *et al*., 2007). Although experimental and climatic conditions differed considerably in each study, some general trends have indicated that Gram negative *Proteobacteria* and *Cytophaga-Flavobacterium-Bacteroides* group dominates during bioremediation and the diversity shift with time to this group (Macnaughton *et al*., 1999; Kaplan and Kitts, 2004). These groups were usually associated with the fast degradation phase and their abundance was positively correlated to hydrocarbon attenuation. Gram positive bacteria if detected are never diverse and dominant during bioremediation (Kaplan and Kitts, 2004). However, recent reports have shown that Gram positive bacteria mainly the *Actinobacteria* can actually dominate during bioremediation of petroleum hydrocarbons owing to their metabolic versatility and their widespread occurrences both in pristine and hydrocarbon polluted soil (Bell *et al*., 1998; Bouchez-Naitali *et al*., 1999; Bogan *et al*., 2003; Margesin *et al*., 2003; Larkin *et al*., 2005; Hamamura *et al*., 2006; Quatrini *et al*., 2008).

Bioremediation techniques can be carried out in two ways. First, bioremediation by autochthonous microorganisms can be activated by nutrient additions (N and P) which are often limiting factors in the environment (Thouand *et al*., 1999). The success of this method has been demonstrated in several pollution cases such as the Exxon-Valdez Wreck (Bragg *et al*., 1994). The second method consists of adding autochthonous microbial consortia (seeding), some which are commercially available and selected for their ability to biodegrade crude oil soil and its complex bacterial community can accept and degrade to some degree crude-petroleum into non-toxic forms. The immense potential of such microorganisms to

degrade petroleum hydrocarbons does not depend solely upon the wealth of catabolic enzymes that these organisms possess, but also upon their capacity for adaptive change (Sanni and Ajisebutu, 2003).

The two main approaches to oil spill bioremediation are bioaugmentation and biostimulation. Bioaugmentation involves the use of beneficial microbes that have an affinity towards a specific pollutant. Typically, these microbes are suspended by a stabilizing agent and lie dormant until activated in solution and applied together with micro-nutrients and stimulants. Bioaugmentation allows one to control the nature of the biomass. It ensures that the proper team of microbes is present in the spilled soil in sufficient type, number, and compatibility to attack the constituents effectively and break them into their most basic compounds (Venosa *et al.*, 1996; McKee and Mendelssohn, 1995). Biostimulation on the other hand involves aeration and the addition of selected micronutrients and sometimes topsoil in appropriate quantities (Swannell *et al.*, 1999; Shekwolo, 2005).

It is only effective when indigenous microbial populations, present in the substrate, are high enough to degrade the PHC, and when these microbes can readily adapt to foreign substrates. Mishra *et al.* (2001) argued that since microorganisms capable of degrading oil usually grow at the expense of one or more components of crude oil, and these bacteria are ubiquitous, there is usually no reason to add PHC biodegraders unless the indigenous bacteria are incapable of degrading one or more important components. The size of the hydrocarbon degrading bacterial population usually increases rapidly in response to oil contamination, and it is very difficult, if not impossible to increase the microbial population over that which can be achieved by biostimulation alone (Lin and Mendelssohn, 1998).

The carrying capacity of most environments is probably determined by factors such as predation by protozoan, the oil surface area, or scouring of attached biomass by wind activity that are not affected by bioaugmentation, and added bacteria seem to compete poorly with the

indigenous population. Therefore, it is unlikely that they will persist in a contaminated soil even when they are added in high numbers. Hence, bioaugmentation may not have any long term beneficial effects in clean up operations. Biostimulation involves the addition of rate-limiting nutrients to accelerate degradation by indigenous microbes. This assumes that every organism needed to accomplish the desired treatment-results is present (Mishra *et al.*, 2001). Though it is not certain that those organisms present are the most suitable to degrade the pollutant. When an oil spill occurs, it results in a huge influx of carbon into the impacted environment (Yakubu, 2007). Supplying enough nitrogenous nutrients to the cultures to enable their proliferation present serious problems, since sea, river, or lake water and soil have little nitrogen and phosphorus, rapid oil utilization will require external supplies of these elements for the oil-consuming microorganisms. Carbon is the basic structural component of living matter, and in order for the indigenous microorganisms to be able to convert this carbon into more biomass, they need significantly more nitrogen and phosphorous than is normally present in the soil (Wright *et al*., 1997). Both of these essential elements are ingredients of protein and nucleic acids of living organisms, and therefore should be adequately provided.

### 2.9.1 Use of Biosurfactants

These are surface-active-agents produced by microorganisms, which emulsify hydrocarbon in growth medium and reduce surface and interfacial tensions (Ilori *et al.*, 2005; Muthusamy *et al*., 2008; Mahmound *et al*., 2008; Ilori *et al*., 2008; Obayori *et al.*, 2009). Biosurfactants are compounds with vast potential for use in environment, petroleum, food, pharmaceutical and other Industries. Their range of possible application in oil industry processes includes cleaning up of oil spills (land and marine), removal of oily sludge from oil storage tanks through increased mobility, as well as general enhancement of oil recovery processes from reservoirs and the level of the production well.

The ability of a biosurfactant to emulsify hydrocarbon water mixtures have been widely reported (Borjana *et al*., 2001; Kvenvolden *et al*., 2000; Yakubu, 2007). These emulsification properties have also been demonstrated to enhance hydrocarbon degradation in the environment; hence making them potentially useful tools for oil spill pollution control (Cameotra and Pruthi, 1997).

Most surfactants currently being used are chemically derived from petroleum. Interest in microbial surfactants has however steadily increased in recent years due to desirable characteristics such as their selectivity, environmentally friendly nature, and stability at elevated temperatures, pH and salt concentrations (Borjana e*t al*., 2001). In addition, biosurfactants have increased versatility in comparison to many synthetic surfactants and are suitable for pollution control through biodegradation.

In any heterogenous system, such as microorganisms growing on water-insoluble hydrocarbons, boundaries are of fundamental importance to the behavior of the system as a whole. Therefore, it is not surprising that hydrocarbon-degrading microorganisms produce a wide variety of surface-active agents. The general topic of microbial surfactants has been reviewed by Zajic and Seffens (1984), Rosenberg (1986), and Desai and Desai (1992). Reviews are also available on the surface-active properties of *Acinetobacter* exopolysaccharides (Kaplan *et al.,* 1985), the biosynthesis of lipid surfactants (Zajic and Mahomedy, 1984) and biosurfactants in relation to environmental pollution (Mueller-Hurtig *et al.,* 1993).

Microbiologically derived surfactants can be divided into low and high molecular weight products. The low molecular weight surfactants are a mixture of glycolipids, fatty acids, phospholipids and lipopeptides. The high molecular weight surfactants are amphipathic polymers and complexes of hydrophobic and hydrophilic polymers. The production of acylated sugars, in which fatty-acid residues are bound directly to a monosaccharide or

disaccharide, is often associated with the growth of microorganisms on water-insoluble hydrocarbons.

It has been suggested that the natural role of emulsans is to enhance the growth of bacteria on petroleum by two mechanisms: (1) increasing the hydrocarbon surface area, and (2) desorbing the bacteria from 'used' oil droplets. The growth of microorganisms on water-insoluble hydrocarbons is restricted to the hydrocarbon/ water interface because the hydrocarbon oxygenases are always membrane-bound, never extracellular. After the bacteria adhere to the hydrocarbon surface, they begin to multiply on the surface. After a short time, the surface becomes saturated with bacteria, and growth becomes limited by the available surface.If the bacteria can split the oil droplets (emulsification), new surfaces become available for growth (Desai and Banat, 1997; Barkay *et al.*, 1999; *Rosenberg* and. *Ron*, 1999; Ron and Rosenberg, 2002; Salihu *et al.*, 2009; Ebrahimi *et al.*, 2012).

This is a large effect, as the surface area is inversely proportional to the square of the radius of the drop. Presumably, other microorganisms which can attach to hydrophilic surfaces and degrade aromatic and cyclic hydrocarbons continue the process of petroleum biodegradation. Surfactants are used widely in industry, agriculture and medicine. The materials currently in use are produced primarily by chemical synthesis, or as by-products of industrial processes. For a microbial surfactant to penetrate the market, it must provide a clear advantage over the existing competing materials. The major considerations are: (1) safety, i.e., low toxicity and biodegradability; (2) cost; (3) selectivity; and (4) specific surface modifications. Biosurfactants exhibit low toxicity and good biodegradability, properties that are essential if the surfactant is to be released into the environment. An emulsifying or dispersing agent not only causes a reduction in the average particle size, but also changes the surface properties of the particle in a fundamental manner. For example, oil droplets that have

been emulsified with emulsan contain an outer hydrophilic shell of spatially oriented carboxyl and hydroxyl groups. These emulsan-stabilized oil droplets bind large quantities of uranium (Zosim *et al.*, 1982).

In an elegant experiment, Pines and Gutnick (1984) demonstrated that emulsan on the surface of hexadecane droplets acts as a receptor for A. *calcoaceticus* bacteriophage ap3. Clearly, each bioemulsifier is going to alter, in a characteristic manner, the interface to which it adheres. Usually, 0.1-1.0% of a polymeric bioemulsifier or biodispersant is enough to saturate the surface of the dispersed material. Thus, small quantities of a dispersant can alter dramatically the bioremediation of petroleum contamination surface properties of a material such as its surface charge, hydrophobicity and, most interestingly, its pattern recognition, based on the three-dimensional structure of the adherent polymer.Surfactants enhance solubilization and removal of contaminants (Brusseau, *et al.*,1995; Barkay *et al.*,1997). Biodegradation is also enhanced by surfactants due to increased bioavailability of pollutants (Barkay *et al*., 1999). Bioremediation of oil sludge using biosurfactants has been reported by Cameotra and Singh (2008).

*Pseudomonads* are the best known bacteria capable of utilizing hydrocarbons as carbon and energy sources and producing biosurfactants (Beal and Betts, 2000; Rahman *et al*., 2003; Cameotra and Singh, 2008) Among *Pseudomonads*, *P. aeruginosa* is widely studied for the production of glycolipid type biosurfactants. However, glycolipid type biosurfactants are also reported from some other species like *P. putida* and *P. chlororaphis*. Biosurfactants increase the oil surface area and that amount of oil is actually available for bacteria to utilize it (Nikolopoulou and Kalogerakis, 2009).There are recent reports on biosurfactant production by different microorganisms. Biosurfactant can act as emulsifying agents by decreasing the surface tension and forming micelles. The microdroplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded.

### 2.9.2 Biodegradation of Petroleum Hydrocarbons by Immobilized Cells

Immobilized cells have been used and studied for the bioremediation of numerous toxic chemicals. Immobilization not only simplifies separation and recovery of immobilized cells but also makes the application reusable which reduces the overall cost. Wilsey and Bradely (1996) used free suspension and immobilized *Pseudomonas* sp. to degrade petrol in an aqueous system.

Hydrocarbon biodegradation can be significantly improved by immobilizing microbial cells. For example, using the bacterial consortium MPD-M isolated from sediments associated with Colombian mangrove roots can effectively degrade hydrocarbons in water with salinities varying from 0 to 180 g/L. However, the effectiveness was 4 and 7 times greater with immobilized cells on polypropylene fibers compared to free living cells (Diaz *et al*., 2002); Moslemy *et al.* (2002) demonstrated that an enriched bacterial consortium encapsulated in gellan gum micro beads (16-53 μm dia.) at the rate of 2.6 mg·cells/g bead degraded over 90% gasoline hydrocarbons within 5-10 days for the initial concentration of 50-600 mg/L. However, degrading the same amount of gasoline hydrocarbons by free cells at equivalent levels required as long as 30 days.

The improved effectiveness of encapsulation may result from potentially reducing biotic and abiotic stresses, providing a number of advantages, including protecting cells from the toxic effects of hazard-ous compounds (Bettmann and Rehm, 1984; Manohar and Karegoudar, 1998; Paje *et al*., 1998) and increasing their survival and metabolic activities (Hall *et al.*, 1998; Suzuki *et al.*, 1998). The study indicated that immobilization resulted in a combination of increased contact between cell and hydrocarbon droplets and enhanced level of rhamnolipids production. Rhamnolipids caused greater dispersion of water-insoluble n-alkanes in the aqueous phase due to their amphipathic properties and the molecules consist of

hydrophilic and hydrophobic moieties which reduced the interfacial tension of oil-water systems.

This resulted in higher interaction of cells with solubilized hydrocarbon droplets much smaller than the cells and rapid uptake of hydrocarbon in to the cells. Diaz *et al*. (2002) reported that immobilization of bacterial cells enhanced the biodegradation rate of crude oil compared to free living cells in a wide range of culture salinity. Immobilization can be done in batch mode as well as continuous mode. Packed bed reactors are commonly used in continuous mode to degrade hydrocarbons Cunningham *et al*. (2004) used polyvinyl alcohol (PVA) cryogelation as an entrapment matrix and microorganisms indigenous to the site. They constructed laboratory biopiles to compare immobilised bioaugmentation with liquid culture bioaugmentation and biostimulation. Immobilised systems were found to be the most successful in terms of percentage removal of diesel after 32 days. Rahman *et al*. (2006) conducted an experiment to study the capacity of immobilized bacteria in alginate beads to degrade hydrocarbons. The results showed that there was no decline in the biodegradation activity of the microbial consortium on repeated use. It was concluded that immobilization of cells is a promising application in the bioremediation of hydrocarbon contaminated site.

The use of free microorganisms for bioremediation of contaminated sites can fail because the inoculants must be able to overcome biotic and abiotic stresses in the environment in which they are introduced, and might cause other problems such as secondary pollution by the inoculants (Gentili, 2006). Immobilization of microorganisms offers several advantages over free cells, including protecting cells from the toxic effects of hazardous compounds and increasing their survival and metabolic activity in bioremediation systems (Moslemy, 2002), and therefore has been receiving increasing attention (Zhang *et al*., 2008; Liu *et al*., 2009).

## 2.10 Molecular Biology, Metabolic Engineering and Future Prospect.

Biologists are now to employ genetics to develop a simple, quick method for assessing the progress of environmental clean-up efforts at polluted sites. The principle involves screening soil for genes that reveal the presence of an enzyme produced by pollution-busting bacteria. If the enzyme is detected, that means bacteria probably are cleaning the soil. Information about the bacteria's presence and concentration might then be used to assess the progress of efforts to remove pollutant from the contaminated soil. It is like a direct biochemical method that takes the attendance of the bacterial organisms in the soil (Watanabe, 2002; Matthew *et al.*, 2000).The method could be used to test soil at oil contaminated sites within a few hours.

Conventional methods require that soil samples be taken to a laboratory, where bacteria are cultured on a growth medium. However, that can take days and is not always effective because some microorganisms will not grow under laboratory conditions (Yakubu, 2007). The technique works by detecting the enzyme catechol-2,3-dioxygenase, which several types of bacteria produce when soil is contaminated by petroleum-based compounds such as benzene, toluene and xylenes. DNA is first extracted from polluted soil samples and then specific genes that reveal the enzyme's presence are searched. Detecting the enzyme is made possible by using primer that matches the gene sequence indicating the enzyme's presence. The primer is specially designed to detect only catechol 2, 3- dioxygenase, which neutralizes the petroleum-based contaminants. After the primer finds and attaches to a matching piece of DNA, thousands of copies are produced through a well-known method known as polymerase chain reaction, which enables scientists to make billions of copies of a single strand of DNA (Watanabe, 2002). Catechol 2,3-dioxygenase is present in several types of bacteria that degrade environmental pollutants. The toxins typically get into soil, and they

are absorbed by the bacteria and broken down by enzymes into less toxic compounds, such as methyl catechol, which are further degraded into harmless chemicals by catechol 2,3-dioxygenase. While this is not the only enzyme used by microorganisms to neutralize petroleum based compounds, it is one of the key biochemical "pathways" through which the contaminants are degraded.

Despite all the advantages related to enzymatic bioremediation, high production costs, low yields, and enzymatic inhibition are some of the problems that must be overcome if much production improvements are necessary to avoid non-prohibitive processes. Therefore, molecular tools are being widely explored to provide competitive enzyme bioremediation products. Molecular tools allow one to detect genes related to degrading enzyme in environmental samples or isolates; thus serving as powerful tool for bioprospection. Furthermore, DNA engineering can considerably improve enzymes yield with low cost (Alcalde *et al.*, 2006).

Enzymatic bioremediation improved with molecular tools can be particularly suitable for situations where rapid remediation is required (Sutherland *et al.,* 2004). Alcalde *et al*. (2006) reported that studies of protein engineering, metagenomics, and proteomics have effectively contributed to cost reduction, minimizing chemical use and also improving cost benefit ratios. The use of molecular tools for biocatalyst applications can also help to solve the problems of GMO use in the production of a modified enzymes is performed in invitro, it is not necessary to introduce the modified organism into the natural environment.

Many PCR primers that target genes related to petroleum – degrading enzymes both in aerobic and anaerobic conditions, have been described. The utilization of these already – characterized primers may facilitate environmental screening of degrading abilities and may help to evaluate the potential of microbial isolates. More primers can be described for

specific pathways or to improve the comprehensiveness of known primers using available data-bases (Peixoto *et al*., 2011).

The benefits provided by molecular tools can open unlimited window of opportunity, as it is possible to detect genes from cultivate on non cultivable organisms (using metagenomics) and to express these genes in cultivable organisms, using enzymes that are no yet described. For instance, the use of fosmid and cosmid shotgun metagenomics libraries offers a great improvement to the bioprospection of new enzymes. The possibility of identifying and using genes from yet undescribed microorganisms increases possible enzyme targets from about 0.1% of microbial cells (consisting of cultivate microbial cells in environment samples), including all available DNA in that sample (Zeyaullah *et al.*, 2009).

Molecular tools also allow one to increases express levels manipulating not only physiochemical conditions (Optimal condition) but also the organisms at a genetic level, to improve enzyme production in many different conditions, for instance, improving the efficiency and speed of the petroleum degradation decreasing the time of the remediation process. Genetic manipulation would be also useful to allow or improve the petroleum degradation in extreme environment, such as cold or hypersaline sites (Peixoto *et al.*, 2011).

The use of free extreme enzymes would be advantageous in these environments, since it avoids some of the limitations of the bioremediation using whole cells in extreme conditions, such as microbial competitiveness. The advances in high – throughout "omics" techniques are improving the study of microbial ecology, including biodegradation processes, for instance, identifying and quantifying bacterial enzymes responsible for amount hydrocarbon metabolism (Kim *et al.*, 2009).

### 2.10.1 Microbial Degradative Genes

Numerous molecular biology techniques are now available to study microbial community, ecology by detecting and quantifying target microorganism on target genes in environmental sample. Specific gene probes and (or) specific oligonucleotide primers can be designed, based in existing DNA sequences, encoding different enzymes involved in degradation pathway. These molecular methods are advantages which may represent up to 99% of the naturally occurring cells in the environment can be analyzed rapidly and with increased detection sensitivity (Leahy and Colwel, 1990; Holben, 1992; Greer *et al.*, 1993; Gelsomimo *et al.*, 1999; Meger *et al.*, 1999). Furthermore, these same molecular approaches can help one to access the endemic and the cosmopolitan distribution of different catabolic genes in native communities. Microbial diversity and biogeographic studies can help identify genotypic characteristics are widely distributed or if they tend to be endemic only to specific areas (Staley, 1999; Staley and Gosink, 1999).

Mono-oxygenase and deoxygenize are key enzymes in the biodegradation of a wide range of environmental pollutants, and the genes encoding the enzymes have been characterized in *Pseudomonas putida* (formerly *P. oleovorans*) (Van Beilen *et al.*, 2001) ATCC 29347 (Pp alkB), *Rhodococcus spp.*, strain Q15 (Rh alkB1, Rh alkB2), *Acinetobacter* sp., ADP–1 (Ac alkM), *P. putida* ATCC 17484 (ndoB), *P. putida* F1 (todC1); *P. putida* ATCC 33015 (xylE, cat 23), and *P. pseudoalcaligenes* KF707 (bphA). Pp alkB, located on the OCT plasmid, a member of the IncP–2 family of plasmids, encoded alkane hydroxylase, a. membrane bond mon-oxygenase that is the first enzyme of the short-chain alkane ($C_6$-$C_{12}$) degradation pathway (Kok *et al.*, 1989; Van Beilen *et al.*, 1994).

Highly similar multiple alkane hydroxylase system have been isolated and characterized in *Rhodococcos erythropolis* NRRL B-16531 and *Rhodococcus* spp. strain Q15

(Whyte *et al.*, 2002), this gains and other closely related high %G+C Actimomycetes are considered important members of hydrocarbon degrading microbial population in contaminated soils (Warhurst,1994). Both organisms are psychrotroph, degrade a wide range of n- alkanes ($C_8$-$C_{32}$) and contain at least four alkane mono-oxygeniase homologues (Rh alk, alkB2, Rh alkB3 and Rh elkB4) when the Rhodococcal alkB2 was cloned into P. fluorescens KOB2, the recombinant strain was able to mineralize and grow on $C_{12}$-$C_{16}$ n-alkanes (Whyte *et al*., 2002), Ac alkM encodes alkane mono-oxygenase, one of the five essential genes encoding alkane utilization of $C_{12}$ – $C_{18}$ n-alkanes (Ratajczak *et al*., 1998). The natural diversity of the alkane hydroxylase system has been presented by Van Beilen *et al.* (2003). The ndo B gene, located on the NAH7 plasmid, is one of three genes that encode naphthalene deoxygenase, the first enzymes in the *P. putida* ATCC 17484 naphthalene degradation pathway (Zylstra and Gibson, 1989). The xyl E and cat 23 genes, located on the TOL plasmid of *P. putida* ATCC 33010, encode for catechol-2, 3-dioxygenase, and a key enzyme involved in the lower degradation pathway of aromatic compound, such as toluene and xylene (Nakai *et al.*, 1983; Moon, 1995; Okuta *et al*., 1998; Lara Mee *et al.*, 2000). The bph A gene, located on the chromosome of the polychlorinated biphenyl-degrading strain P. Pseudoalcaligenes KF707, encoding for bipharyldioxygenase, a multi-component enzymes responsible for the catalyst of the initial oxidation of biphenyl and chlorophenryls (Furukawa *et al.*, 1987; Asturias *et al.*, 1995).

## 2.11 Genetic control of the degradation of environmental pollutants

A broad definition of xenobiotics includes all compounds that are released in any compartment of the environment by the action of man and thereby occur at a concentration that is higher than natural (Top and Springael, 2003). Many environmental pollutants such as heavy metals, PAHs and oil derivatives (e.g. toluene) are included in this definition. There

are several mechanisms by which microbial populations can adapt to the presence of xenobiotics. Firstly, there can be an increase in the populations of the organisms that already possess catabolic genes that enable them to utilize the pollutant. Secondly, cells can adapt through mutations, such as single nucleotide changes and DNA rearrangements that result in resistance to or degradation of the compound. Thirdly, cells may acquire genetic information from related or phylogenetically distinct populations in the community by horizontal gene transfer (HGT). Eventually, the individual cells within the population that are best able to resist or degrade the xenobiotic will have a selective advantage and will proliferate until they constitute a majority of the microbial community (Top and Springael, 2003).

### 2.11.1 Horizontal transfer of functional genes between bacteria

Metabolic pathways and specific operon systems have been found in diverse but limited groups of microbes that are responsible for the transformation of xenobiotic compounds. Distinct catabolic genes are either present on mobile genetic elements, such as transposons and plasmids, or the chromosome itself that facilitates horizontal gene transfer and enhances the rapid microbial transformation of toxic xenobiotic compounds (Sinha *et al*., 2009). Gene clusters that code for the catabolism of aromatic compounds are frequently found in mobile genetic elements, as transposons and plasmids, which assist their horizontal gene transfer, and consequently enhance adaptation of specific bacterial genera to novel pollutants (Sinha *et al*., 2009).

Microbial communities possess a dynamic gene pool, and novel genetic combinations that arise due to horizontal gene transfer (HGT) act as a driving force to shape genomes (Barkay and Smets, 2005). With the exchange of existing genetic material between microorganisms, different DNA fragments can be combined together in new hosts and can either be incorporated or recombined, with the effect that new mosaic genetic structures are created. Upon successful expression of the new genetic mosaic, a metabolic pathway

composed of parts previously existent in different organisms can now appear within one individual. This may engender a strong growth advantage when the specific carbon source for the metabolic pathway is present (van der Meer and Sentchilo, 2003).

Mobile genetic elements support horizontal gene transfer more accurately via conjugation or transformation that can impart novel phenotypes or may modify existing genes through mutational processes. Typical catabolic plasmids are TOL, OCT, CAM, NAH etc. (Mishra *et al.*, 2001). Studies have confirmed that horizontal transfer of catabolic genes, mostly by means of plasmid-mediated conjugation, happens in soil microcosms, bioreactors and so on after inoculation of a donor strain containing natural catabolic genes (Fulthorpe and Wyndham, 1992). Comparing microbial genomes for evidence of HGT is a retrospective approach, detecting such events based on changes that HGT make on DNA sequences and on gene distributions, deletions and insertions. When supported by robust statistical analyses, the presence of a conflicting phylogeny for specific genes and a consensus phylogeny based on the majority of the genes, suggests inheritance of the former by HGT (Barkay and Smets, 2005).

Mobile genetic elements (MGEs) can be exchanged promiscuously between a broad spectrum of bacteria and contribute to bacterial genome plasticity and therefore contribute significantly to horizontal gene transfer. MGEs include insertion sequences, transposons, integrons, bacteriophages, plasmids and combinations of these elements (Sorensen *et al.*, 2005). Genomic islands are a wide range of large mobile (or potentially mobile) defined DNA segments that are found integrated into the chromosome or other replicons (Springael and Top, 2004). The role of MGEs in the distribution of catabolic pathways and in the adaptation of a microbial community to a pollutant stress has been demonstrated using microcosms, where donor bacteria carrying a catabolic MGE were introduced into an environment that was subsequently challenged with the xenobiotic specific for that MGE. In

many cases, transfer of the MGE to members of the indigenous community was shown. In some cases, this was accompanied by community changes and enhanced degradation of the xenobiotic by the 'adapted' microbial community (Springael and Top, 2004). It is evident that the presence of MGEs and the occurrence of HGT facilitate the evolution of bacterial populations to cope with oil and oil components. The genomes of several microbes relevant to biodegradation have been published e.g. naphthalene degradation by *Pseudomonas putida* G7 (Simon *et al*., 1993). This information provides an opportunity to gain insights into the potential of specific microorganisms to bioremediate polluted environments and might allow them to be exploited for oil recovery. This will be discussed in the applications of hydrocarbondegrading bacteria section.

## 2.12 Role of Plasmid in Bioremediation of Refinery Wastewater

Biofilms, flocs and other bio aggregates are self – organizing accumulations of microorganisms (Bathe and Hausner, 2010) Biofilms represent environmental niches characterized by high cell densities and hence are ideal environments for horizontal gene transfer to occur (Hausner and Wnertz, 1999; Sorenseen *et al*., 2005). The adaptation of microbes to survive in the polluted environment is mainly based on their genetic makeup (Thavasi *et al*., 2007).

Catabolic genes or those coding for antibiotics or metal resistance are often carried on plasmid (Bathe and Hausner, 2010).Plasmids appear to be versatile component for microorganisms to gain metabolic capacities for the exploitation of otherwise unavailable resources (Anthony *et al*., 2000). Catabolic potential may be acquired from either related or phylogenetically distinct populations in the community by horizontal gene transfer (HGT); this is mediated by mobile genetic elements e.g plasmids and transposons (Top and Sprigall, 2003).

One of the ways of accelerating bioremediation is bioaugmentation, the introduction of desired metabolic functions into an existing microbial community (Van Limbergen *et al*., 1998). However, that the previous work has shown that strains added to existing microbial populations may not survive under the new environmental conditions and disappear to levels below detection limit (Bouchez *et al*., 2000; Tchelet *et al*., 1999). However, bioaugmentation via insitu genetic manipulation (the introduction of catabolic genes into an existing indigenous community by means of HGT via conjugation or transformation may lead to a successful introduction of degradative genes into an existing microbial community (Bathe *et al*. 2000).

## 2.13 Homology Modeling and Molecular Docking of *Burkholderia cepacia* Catechol-2,3-dioxygenase involved in degradation of petroleum hydrocarbons

Catechol and its derivatives are key metabolic intermediates in the aerobic degradation of aromatic hydrocarbons by bacteria. Catechol dioxygenases thus play a major role in the metabolism of aromatic hydrocarbons. Catechol dioxygenases can be divided into two major groups: those that cleave the aromatic ring between the vicinal diols (the intradiol dioxygenase group) and those that cleave the ring to one side of the vicinal diols (the extradiol dioxygenase group) (Broderick, 1999). Catechol-2,3-dioxygenase ($C_{23}o$), also called meta-cleavage dioxygenase, belongs to the extradiol dioxygenase group and catalyzes the meta-ring cleavage of (substituted) catechols (Eltis and Bolin, 1996). A wide variety of $C_{23}Os$ have been reported from various bacteria. Most $C_{23}Os$ generally catalyze catechol more efficiently than substituted catechols including methylcatechols although there are rare exceptions. For example the $C_{23}O$ from a *Pseudomonas putida* strain able to grow on toluidine cleaves 3-methylcatechol at a higher rate than catechol (Wallis and Chapman, 1990).

Catechol-2,3-dioxygenase is present in several types of bacteria that degrade environmental pollutants.It is one of the key biochemical "pathways" through which the contaminants are degraded, this enzyme plays a key role, in the metabolism of aromatic compounds.are responsible for the cleavage of aromatic rings aerobic catabolism of compounds such as toluene and xylene, naphthalene or biphenyl derivatives, which are environmental pollutants of serious concern (Harayama and Rekik, 1989).

The Catechol-2,3-dioxygenase enzyme has potential uses in bioremediation of Petroleum hydrocarbon compounds However, the lack of characterization of the enzyme has limited its development as a practical biocatalyst .It is desirable to widen the substrate specificity of the enzyme to make it a more generic catalyst for breaking down these pollutants. The binding properties of petroleum hydrocarbon compounds along with identification of active site residues could be used for site directed mutagenesis in order to identify their role in activity and substrate specificity. Consequently, these enzymes were the subject of different studies and have been suitable targets for site, directed mutagenesis to improve their activities selecting the best mutant which had the best activity in oil degradation and use them in future for cleaning oil spills in the environment (Andujart and Santero, 2003).

The parental gene is first aligned against other genes with high homology or similar function. If crystal structures of homologous proteins are available, a homology model can be built and it can be used to identify key residues. Key residues can also be identified using sequence consensus. Site directed mutagenesis is then carried at these residues and the variants are screened to isolate improved variants.

## 2.14 Effects of oil Refinery Effluent

Refining process generates wastewater (0.4-1.6) times the volume of crude oil processed (Coelho *et al.*, 2006). Petroleum and its products are the dominant components of our modern industrial society. However, the refining of these fuels poses inevitable environmental risks (Peña-Castro *et al*., 2006). The refining process consumes large amounts of water. Consequently, significant volumes of wastewater are generated (Coelho *et al.*, 2006) and globally this wastewater has been recognized as hazardous industrial waste as it contains toxic substances such as phenols, hydrocarbons, metal derivatives, surface active substances, sulphides, naphthalene acids, and other chemicals (Suleimanov,1995; UNIDO, 2006; Zhao *et al.*, 2006; Ghulam *et al*., 2013).

A consequence of processing crude oil in petroleum refineries is the generation of these toxic effluents, which is estimated at 33.6 million barrels per day worldwide (Diya'uddeen *et al*., 2010). Petroleum refinery effluents (PRE) are wastes originating from industries primarily engaged in refining crude oil and manufacturing fuels, lubricants and petrochemical intermediates (Harry, 1995). These effluents are a major source of aquatic environmental pollution (Wake, 2005). The effluents are composed of oil and grease along with many other toxic organic compounds. The frequency of oil spill and untreated waste water discharge into water bodies and the attendant negative impact on the aquatic and terrestrial eco-systems is well known and well documentated over the years (Nduka and Orisakwe, 2009 ; Uzoekwu and Oghosanine, 2011).

Mbaneme *et al*. (2013) reported that direct effluent discharge from nearby Refining Company into aquatic ecosystem results into persistent organic pollutants such as petroleum aliphatic hydrocarbons to be among the most serious and important classes of pollutants.Therefore, discharge of untreated petroleum refining wastewater into water bodies

results in environmental and human health effects due to release of toxic contaminants (hydrocarbons, phenol and dissolved minerals (Muneron de Mello *et al*., 2000; Jo *et al*., 2008; Diya'uddeen *et al.*, 2011).

The refinery effluents could increase the potential of the receiving river to be contaminated with petroleum hydrocarbon pollutants including the aliphatic.Virtually every refinery processes, from primary distillation to final treatment, contains various fractions of oils and other hydrocarbon compounds in their waste waters. The oil and grease in this waste water may appear as free oil, dispersed oil, emulsified oil, soluble oil or as a coating or suspended matter (Singh *et al*., 2013). Other than oil and grease contamination, phenol and its derivatives are also among the most important contaminants present in the environment. Phenol is one of the major organic pollutants encountered in waste water produced by industrial and refinery activities. Phenols have been reported as highly toxic and hazardous to living organisms (Moschella *et al*., 2005).

Hydrocarbons (benzene, toluene, ethyl benzene and xylene (BTEX) are of serious concern due to their toxicity and as carcinogenic compounds (Irwin, 1997; Farhadiah *et al.*, 2007). High exposure for long periods to these compounds can cause leukemia and tumors in multiple organs (Muneron de Mello *et al*., 2000; Ishak *et al*., 2012). Phenol and dissolved minerals are also toxic to aquatic life and lead to liver, lung, kidney and vascular system infection (Baristein-Cardoso *et al*., 2000; Tang *et al*., 2008).Therefore, according to Environmental Protection Agency (USEPA,).Petroleum refining waste water have to to be sufficiently treated for quality to meet the established regulations (USEPA, 2008).However, Physical and chemical methods of treating Petroleum refining wastewater are costly due to excessive amounts of sludge production.Thus, biological methods are preffered due to simple , cheap and environmentally friendly operations (Muneron de Mello *et al*., 2000; Dania, 2010). Clearly, there is no universal approach to handling and treating refinery wastewater

streams. There have been reports from fishermen of tainted fish tissues development they attributed to possible pollution of the water body with improperly treated refinery effluents. Already, refinery effluents have been classified by several authors, including Garrettson (2001), Ogbuagu *et al*. (2011) and Okoli *et al*. (2011) as detrimental to public health, especially where they contain petroleum components such as the aliphatic hydrocarbons.

There are many different ways of testing the toxicity of different compounds but there are two main types of tests. Firstly, the acute lethal test which is usually lasts for 96 hours. The aim of this type of test is to find out the lethal concentration of a substance. Secondly, there are sub- lethal tests. These can take many forms but basically test for any sub-lethal reactions that a substance may cause a problem for the individual and / or the population over a long period of exposure (Wake, 2005).

Measurements of sub-lethal effects that are often used are respiration rate, growth rate, reproductive success and behavioural changes. Acute tests are the most common but sub-lethal tests are also important especially when looking at the impact of a chronic problem like refinery effluents. Many different species have been used to test for the toxicity of oil refinery effluents including species of fish, crustaceans, and algae.

The toxicity of oil refinery effluent is dependent on a number of factors. The volume, quality, salinity and variability of the discharge, the siting of the outfall, the physical and chemical conditions of the discharge area, the proximity of other effluents and pollutants, and the biological condition of the discharge area (Wake, 2005). Some of the different components of the refinery effluent such as pH, temperature, total dissolved solid, total suspended solids, turbidity, biological oxygen demand, phenol, ammonia, sulphides, nitrates and phosphate, phenol, total hydrocarbon concentration, heavy metals and faecal coliform can have varying effects and toxicities (Wake, 2005).

Toxicity from hydrocarbon exposure can be thought of as different syndromes, depending on which organ system is predominately involved. Organ systems that can be affected by hydrocarbons include the pulmonary, neurologic, cardiac, gastrointestinal, hepatic, renal, dermatologic, and hematologic systems (Mbaneme *et al*., 2013). The pulmonary system is the most commonly involved system (Lifshitz *et al*., 2003). Pulmonary complications, especially aspiration, are the most frequently reported adverse effect of hydrocarbon exposure. While most aliphatic hydrocarbons have little Gastro-intestinal absorption, aspiration frequently occurs, either initially or in a semi-delayed fashion as the patient coughs or vomits, thereby resulting in pulmonary effects. Once aspirated, the hydrocarbons can create a severe pneumonitis (Mbaneme *et al*., 2013).

The toxic action of phenol is always associated with loss of cytoplasmic membrane integrity. This results in disruption of energy transduction, disturbance of membrane barrier function, inhibition of membrane protein function and subsequent cell death (Collins and Daugulis, 1997; Yap *et al*., 1999).

Hydrocarbon pneumonitis results from a direct toxic affect by the hydrocarbon on the lung parenchyma. The type II pneumocytes are most affected, resulting in decreased surfactant production. This decrease in surfactant, results in alveolar collapse, ventilation-perfusion mismatch, and hypoxemia. Hemorrhagic alveolitis can subsequently occur, which peaks 3 days after ingestion (Gross *et al*., 1963). The end result of hydrocarbon aspiration is interstitial inflammation, intra-alveolar hemorrhage and edema, hyperemia, bronchial necrosis, and vascular necrosis. Rare pulmonary complications include the development a pneumothorax, pneumatocele, or bronchopleural fistula (Rodricks *et al*., 2003). Prolonged abuse of hydrocarbons can result in white matter degeneration (leukoencephalopathy) and atrophy (Borne *et al*, 2005). In addition, prolonged exposure to certain hydrocarbons (eg, n -

hexane or methyl-n -butyl ketone ([MnBK]) can result in peripheral neuropathy, blurred vision, sensory impairment, muscle atrophy, and Parkinsonism (Garrettson, 2001). Exposure to hydrocarbons can result in cardiotoxicity (Klein *et al*., 1986). Most importantly, the myocardium becomes sensitized to the effects of catecholamines, which can predispose the patient to tachydysrhythmias, which can result in syncope or sudden death. Many of the hydrocarbons create a burning sensation because they are irritating to the GI mucosa.

Vomiting has been reported in up to one third of all hydrocarbon exposures. The chlorinated hydrocarbons, in particular carbon tetrachloride, are hepatotoxic. Usually, the hepatotoxicity results after the hydrocarbon undergoes phase I metabolism, thereby inducing tree radical formation. These free radicals subsequently bond with hepatic macromolecules and ultimately cause lipid peroxidation. This metabolite creates a covalent bond with the hepatic macromolecules, thereby initiating lipid peroxidation.Aromatic compound is water soluble and highly mobile and as such is likely eventually to reach down stream drinking water sources where, even at very low concentrations, can cause severe odour and taste problems (Nwanyanwu and Abu, 2012).

The Presence of pollutants in normal water alters the quality and often poses serious threats to aquatic life. Various studies have shown positive correlation between pollution from petrochemical and refinery effluents and the health of aquatic organisms (Otukunefor and Obiukwu, 2005). Previous observations suggested a correlation between contaminants of water and sediments with aromatic hydrocarbon from refinery effluents and compromised fish health (Kuchn *et al*., 1995), as reported by Uzoekwu and Oghosanine (2011).

Due to the ineffectiveness of purification systems, wastewaters may become dangerous, leading to the accumulation of toxic products in the receiving water bodies with

potentially serious consequences on the ecosystems (Beg *et al.,* 2001; Beg *et al.,* 2003; Otokunefor and Obiukwu, 2005).

Waste water containing high concentration of pollutants or having low or high pH is always more difficult and highly expensive to treat (Obidiaku, 2005). This indicates the need for a more efficient, cheaper and eco-friendly method of cleaning up wastewater as suggested by Bako *et al.* (2008).

# CHAPTER THREE

# 3.0 MATERIALS AND METHODS

## 3.1 Study Area

The Kaduna Refining and Petrochemical Company (KRPC) is located to the west off Kachia Road in Kaduna. The Kaduna Refinery and Petrochemical Company (KRPC) occupies a land area of 2.89 square kilometres approximately 15km Southeast of Kaduna city (KRPC, 2004) (Shown in the map Fig. 3.1). Its location has an elevation of approximately 615m above mean sea level. Kaduna Refinery was constructed by the Chiyoda Chemical Engineering and Construction Company (now Chiyoda Corporation) and was commissioned in 1980 with an initial capacity of 100,000 barrels per stream day (BPSD) as the third Refinery in Nigeria in order meet the tremendous and growing demand for petroleum products . In December 1986, the design capacity of the fuels plants of the Refinery was successfully increased by an additional 60,000 BPSD to the initial 50,000 BPSD, bringing the total refinery installed capacity to 110,000 BPSD (KRPC,2004). The raw wastewater were collected from designated wastewater reservoir in the treatment plant areas as shown in Plates i, ii, iii and iv.

## 3.2 Collection of Samples

Refinery sludge was collected under the storage tank in Kaduna Refining and Petrochemical Company (KRPC) Kaduna State, Nigeria using sterile trowel into polyethylene bags, kept in sterile containers and transported in ice chests.Analysis commenced immediately upon arrival in the Department of Microbiology laboratory.

The wastewater samples from different concrete wastewater reservoirs in Kaduna Refinery and Petrochemical Company were collected into four litres plastic bottles and stored in an ice block cooler and transferred to the laboratory immediately for analysis (Department of Petroleum Resources, 1991; FEPA, 1991).

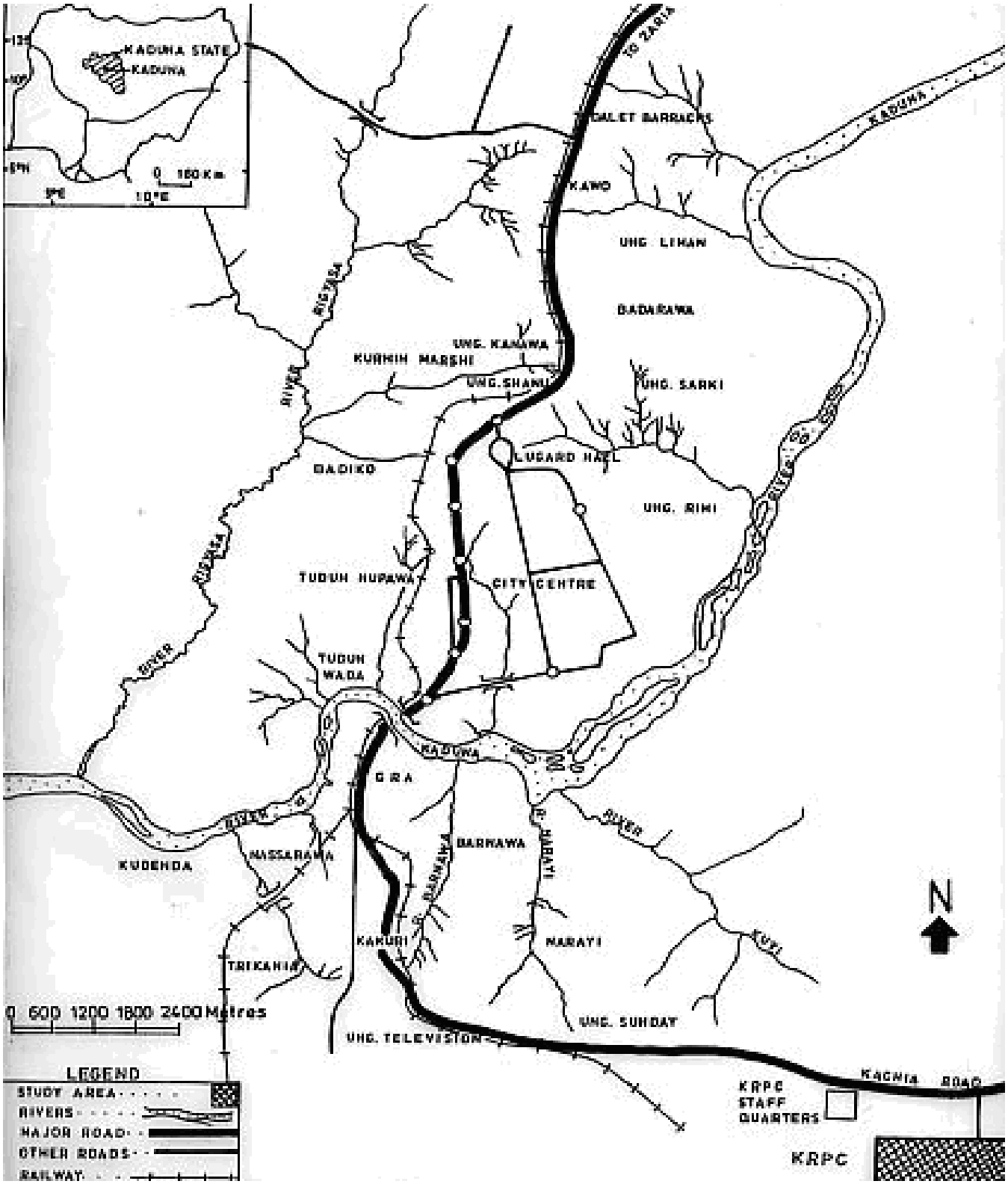



**Figure 3.1:** Map of Kaduna city (Bako *et al.*, 2008)

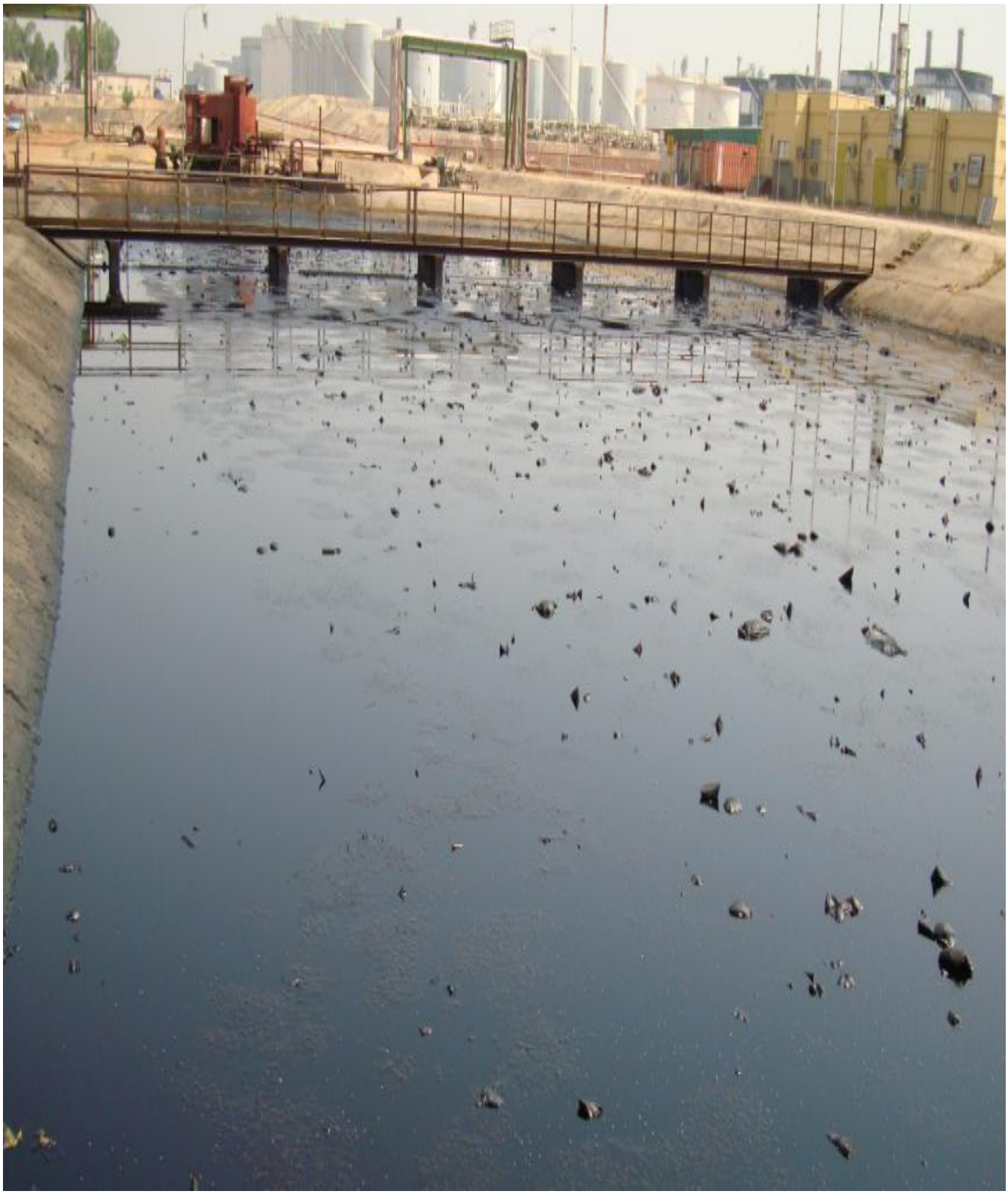


Plate I : Concrete wastewater reservoir in Kaduna Refining and Petrochemical Company (KRPC)



Plate II : Concrete wastewater reservoir in Kaduna Refining and Petrochemical Company (KRPC)

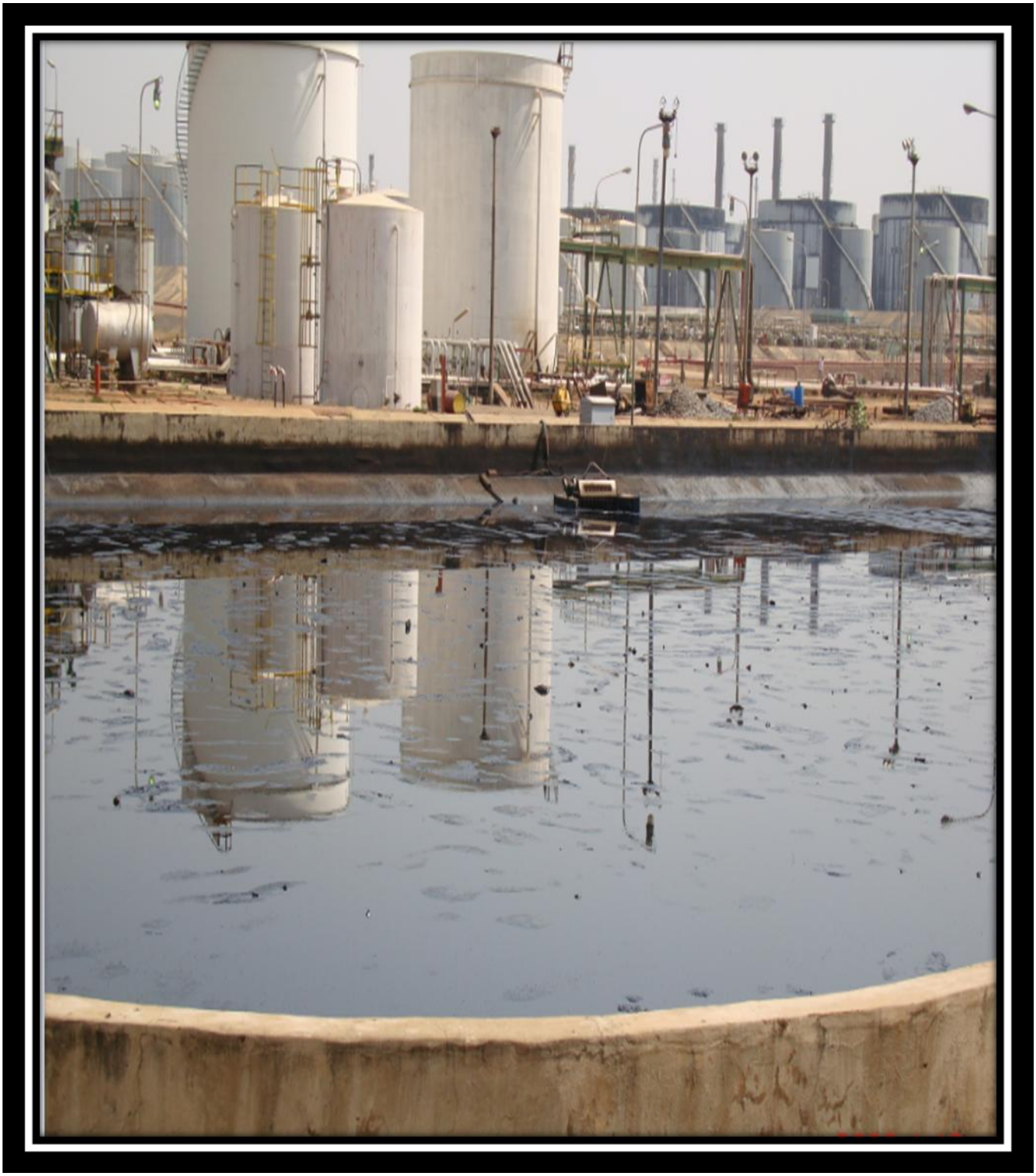


Plate III : Concrete wastewater reservoir in Kaduna Refining and Petrochemical Company (KRPC).

All the collected samples were preserved in accordance with guidelines and International Standards. All other quality assurance and quality control procedure relevant to samples collection, custody and analyses were strictly adhered to for the determination of physicochemical parameters (ASTM, 1979; APHA, 1995; Ademoroti, 1996; Joel and Amajuoyi, 2009).

### 3.3 Isolation of Bacteria

Selective enrichment techniques was used for the isolation of hydrocarbonoclastic bacteria from Kaduna refinery sludge.Bushnell-Haas broth was used as enrichment media supplemented with 5% (v/v) of Bonny light crude oil.Specifically, 10g of oil sludge was added to 185ml of Bushnell- Haas medium containing 5ml of crude oil in Erlenmeyer flasks. The flasks were incubated in a rotary shaker at room temperature at 150rpm for two weeks. Aliquot of 0.1ml from the set up was plated on the plate count agar and incubated at 30°C for 48hours. The two isolates that survived 2weeks selective enrichment technique were stored at $4^0$ C in a stock culture (Malatova, 2005; Mandri and Lin, 2007).

### 3.4 Identification of Isolates

The two isolates subjected to biochemical characterization. Identification of the isolate to the genus level followed the scheme in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).The Gram staining and some biochemical tests carried out are described below:

#### 3.4.1 Gram Staining Reaction

This was carried out as follows: A smear of the organism was prepared on a grease-free slide and fixed it was flooded with crystal violet and allowed to stand for 30seconds.Slide was placed at an angle downward and then flooded with Gram's iodine. It was allowed to stand

for 30sec and washed off with water,then flooded with acetone-alcohol (50:50v/v) and tilted from side to side until the color ceased to appear.It was counterstained with saffranin for 1min and washed off with water, allowed to air-dry and observed with oil immersion under the microscope. Gram positive organisms appeared purple while Gram negative organisms appeared red.

### 3.4.2 Indole Test

Indole test detects the ability of an organism to produce indole from the amino acid tryptophan.A speck of each isolate was inoculated onto 5 ml of sterile peptone water enriched with 1% tryptophan in test tubes and was incubated at 37% for 48hours, to the culture, 0.5ml kovac's indole reagent was added and gently shaken. In a positive test, indole (present in the culture) dissolves in the reagent which then becomes pink or red, and forms a layer at the surface of the medium. A yellow layer at the surface of the medium denotes a negative result (Singleton, 1997).

### 3.4.3 Methyl Red Test (MR Test)

MR test detects the ability of an organism growing in a phosphate buffered glucose-peptone medium to produce sufficient acid (from the metabolism of glucose) to reduce the pH of the medium from 7.5 to about 4.4 or below. A speck of each isolate was inoculated onto the glucose phosphate peptone water medium and was incubated at 37°C for 48hours. Few drops of methyl red were added to the culture. MR positive test was indicated by red colour formation while no change denote negative (Singleton, 1997).

### 3.4.4 Voges-Proskauer Test (VP Test)

A speck of each isolate was inoculated into glucose phosphate peptone water medium and incubated at 37°C for 48hours. 1.2ml of an ethanolic solution of 5% $\alpha$-naphtanol and 0.4m of 40% potassium hydroxide solution was vigorous and placed in a sloping position (for maximum exposure of the culture to air), and was examined after 30 to 60 minutes. The

evolution of red colour indicated a positive test for VP (Singleton, 1997; Cheesbrough, 2000; Fawole and Osho, 2004; Oyeleke and Manga, 2008).

### 3.4.5 The Citrate Test

This test detects the ability of an organism to use citrate as the sole source of carbon. A speck of each isolate was inoculated into Koser's citrate medium and incubated at 37°C for 72 hours. A positive citrate test was confirmed by formation of blue colour while the initial green colour denoted negative test (Singleton, 1997).

### 3.4.6 Urease Test

Urease is an enzyme which hydrolyzes Urea, ($NH_2$) to carbon dioxide and ammonia (Singleton, 1997). A speck of each isolate was inoculated onto christensen's Urea agar and incubated at 37°C for 42hours.formation of red colour indicated Urease-positive while initial yellow colour indicated negative.

### 3.4.7 Hydrogen Sulphide Production

A speck of each isolate was inoculated by streaking and stabbing into triple sugar iron agar and incubated at 37°C for 24 hours.Formation of blackening in the medium indicated a positive test while no blackening indicated negative result

### 3.4.8 Motility

This was done as describe by Singleton (1997). Motility can sometimes be inferred from the way an organism grows on solid media: Motile species may tend to spread outwards from the inoculated area as organism swim in the thin layer of surface moisture (Singleton, 1997). A speck of each isolate was stab into triple sugar iron agar and incubated at 37°C for 42 hours. Motility was observed by spread of the organism outwards from the stab area.

### 3.4.9 Gelatin Hydrolysis Test

This was done as described by Leboffe and Pierce (2010). A speck of each isolate was inoculated by streaking once across the surface of Frazier's gelatin agar and incubated at 37°C for 48hours. The culture plates were then flooded with 0.5ml of the test reagent (Mercuric Chloride solution). Hydrolyzed gelatin appeared as a clear zone while unhydrolyzed gelatin formed a white opaque precipitate.

### 3.4.10 Starch Hydrolysis Test

A speck of each isolate was inoculated on starch agar and incubated at 37°C for 24hours. The plates were then flooded with iodine solution. The absence of blue black colouration indicated starch hydrolysis while presence of blue black indicated that starch has not been hydrolyzed (Singleton, 1997).

### 3.4.11 Nitrate Reduction Test

This test detects the ability of an organism to reduce nitrate (Singleton, 1997)

A speck of each isolate was inoculated into nitrate broth, incubated at 37°C for 48hours and the medium was examined for evidence of nitrate reagent 'A and 0.5 ml of nitrite reagent B' were added to the culture, these reagents combine with any nitrite present to form a soluble red azodye. The absence of red coloration could mean that either (i) nitrite had not been reduced or (ii) nitrite was formed but had not been subsequently reduced e.g to nitrogen or ammonia. To distinguish between these two possibilities, the medium was tested for the presence of nitrate is present (i.e it has not been reduced by the test organism), the addition of zinc oil brings about a cell colouration since the newly-formed nitrite will react with the reagents present in the medium (Oyeleke and Manga, 2008).

### 3.4.12 Sugar Fermentation

The fermentation of glucose, sucrose and lactose was done using triple sugar iron agar. A speck of each isolate was inoculated by streaking and stabbing into the medium and incubated at 37°C for 24 hours. Fermentation of any of the sugar is indicate by change in colour from red to yellow and crack or raise of the medium indicates gas production.

Fermentation of fructose, galactose, lactose and mannitol was carried out by inoculating a speck of each isolate into the nutrient broth containing each of the sugars (fructose, galactose and mannitol) and incubated at 37°C for 24 hours. A positive test was indicated by formation of yellow colour while initial red colour denoted negative result. Gas production was observed in the Durham tubes (Oyeleke and Manga, 2008).

### 3.4.13 Biochemical Testing with the API System

The API system BioMerieux Vitek (Hazelwood, MO), API Coryne and API 20NE (bioMerieux, Marcy-I Etoile, France) was used to characterize two representative oil degrading bacteria isolated from the oil sludge. A pure culture of the suspected isolate was grown to achieve visual turbidity to a 0.5Mcfarland standard .The culture suspension (0.1ml) was distributed into each of the receptacles of the test strip, The NE strips (BioMerieux Vitek) were incubated at 28°C for 24 hours. Inoculation, addition of reagents, and scoring of the results were done according to the manufacturer's protocol.The enzyme activity of the isolates were determined with the API ZYM system.

## 3.5 Evaluation of Mineralization Potential of Isolated Bacteria

The ability of the bacterial isolates to utilize Bonny light crude oil as the source of carbon and energy was determined by the method of Okpokwasili and Okorie (1988); Okeretugba and Ezeronye (2003); Ojo (2006); Yakubu (2007) and Ganesh and Lin (2009) with some modifications.Each of the bacterial isolates was tested for their ability to mineralise crude oil using a liquid mineral medium, Bushnell-Haas (BH) medium (Bushnell

and Haas, 1941). A single colony each of the isolate was inoculated into 10ml nutrient broth (Merck) in different test tubes and incubated at 30°C overnight in a shaker operated at 160 rpm. The overnight culture was centrifuged for 10 min at 5000 rpm. The cell pellet was washed twice in phosphate buffer saline (pH 7.6) and re-suspended in BH medium until $OD_{600}$ nm was equivalent to 1. Ten millilitre each of bacterial inoculum (1=$OD_{600}$ nm equivalent) was added to 250 ml Erlenmeyer flasks containing 100 ml of BH medium supplemented with 2% Bonny light crude oil as carbon source. pH of the medium was adjusted to 7.0.Each of the flask contained pure culture of the isolate while mixed culture or co-culture was prepared by adding 5ml each of the pellet isolates into 100ml of the BH medium supplemented with 2% of Bonny light crude oil in 1:1. Incubated as described above Pure and mixed culture of the cells was cultivated separately. The control flasks contained the entire nutrient and the oil but no bacterial culture was used as control. All the flasks were incubated at $30^0$C for 18days on an orbital shaker at 120rpm.The following parameters were monitored in every 24hours in the culture filtrate:

(1) Dehydrogenase activities (2) Fluorescein diacetate (3) Total protein estimation (4) pH (5) $CO_2$ Production (6) Emulsification index (7) Optical density ($OD_{600}$) (8) Catechol-2,3-dioxygenase (9) Catechol-1,2-dioxygenase.

### 3.5.1 Dehydrogenase Activity

Microbial dehydrogenase activity (DHA) in bacterial culture was determined with 2,3,5-triphenyltetrazolium chloride (TTC) adopting an optimized method described by Casida (1977).The method is proceeded as follows: two ml of culture was added into test tubes and mixed with 2ml of TTC (Triphenyl Tetrazolium Chloride) solution. Test tubes were incubatedat $27^0$ C for 96hours. After incubation, 10ml of methanol were added to each tube and the tubes were shaken thoroughly for 30seconds and then further incubated at room

temperature for 3hours. The content was then transferred into vials, and the optical density was measured at 485nm. Controls were prepared with sterile broth and treated like other sample.

### 3.5.2 Fluorescein Diacetate (FDA) Assay (Microbial Activity Analysis)

Fluorescein Diacetate (FDA) assay was used to quantify microbial activity in the oil contaminated sediment intact cores. The FDA assay has been used to measure total heterotrophic soil microbial activity in a variety of ecosystems (Hunter, 2000; El-Tarabily, 2002). The FDA assay does not quantify microbial biomass, but it is useful for comparing microbial hydrolytic activity in similar soil ecosystems (Schnurer and Rosswall, 1982).

An FDA standard solution was made by dissolving 0.0399 g FDA in acetone and bringing the volume to 100 mL. Standards were made by adding 50 mL phosphate buffer and 10mL of bacterial culture to each of eight test tubes and then adding 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4mL of fluorescein standard to the flasks. The resulting solutions contained the equivalent of 0, 100, 200, 300, 400, 500,600 and 700µg FDA converted to fluoresceinper test tubes. Standards were incubated on a rotary shaker (120 rpm) for 3 hours and then 50 mL of acetone added. The solution was centrifuged for 10 minutes at 6000 rpm, filtered and filtrate absorbance values were measured spectrophotometrically (SHIMADZU UV-1201, 1-cm path length) at 490 nm. An FDA stock solution was made by dissolving 0.200 g fluorescein diacetate in acetone and bringing the volume to 100 mL with deionized water. Broth culture of 0.5ml of was incubated with 50 mL of 60 mM sodium phosphate buffer (pH 7.6). Then, 50 mL 0.1 M sodium phosphate buffer (pH 7.6) and 0.5 mL FDA stock solution was added and the tube capped and incubated on a rotary shaker at 120 rpm for 3 hours. After one hour, 50 mL acetone was added to terminate the FDA hydrolysis reaction. The solution was swirled by hand and 40ml decanted into a centrifuge tube. The solution was centrifuged for 10 minutes

at 6000 rpm and then filtered; the filtrate was measured on a spectrophotometer (SHIMADZU UV-1201, 1-cm path length cell) at a wavelength of 490 nm.Absorbance values were expressed as 1g fluorescein $mL^{-1}$ solvent $3h^{-1}$ by using a standard absorbance curve. Controls were performed with each culture filtrate sample analyzed to determine the color not derived from the hydrolysis of FDA and the color obtained from non-microbial hydrolysis of FDA. To perform controls, the procedure described for the FDA assay was followed, but 0.5 ml of sterile broth was used.

### 3.5.3 Total Protein Estimation

The method of Lowry *et al*. (1951) was used for the estimation of protein, taking Bovine serum albumin (BSA) as a standard. Four solutions were prepared with following composition:

**Solution A:**

| | |
|---|---|
| $Na_2\ CO_3$ | 2g |
| NaOH | 0.4g |
| Sodium Potassium Tartrate | 1g |
| Distilled water | 100 ml |

**Solution B:**

| | |
|---|---|
| $CuSO_4\ 5H_2O$ | 0.5 g/ 100 ml distilled water |

**Solution C:**

25 ml of solution A+ 0.5 ml of solution B (solution C should be freshly prepared)

**Solution D:**

Folin phenol and distilled water in 1:1 ratio; i.e. 1.5ml Folin phenol + 1.5 ml distilled water

**BSA Solution**

0.005 g/5 ml or 5mg/5 ml for standard curve. BSA solutions of different concentrations were prepared in the following ways:

Blank = 1000$\mu$l of distilled water

100$\mu l$ of BSA solution + 900 $\mu$l of distilled water = 100$\mu$g of BSA

120$\mu l$ of BSA solution + 880 $\mu$l of distilled water = 120$\mu g$ of BSA

140$\mu l$ of BSA solution + 860$\mu$l of distilled water =140$\mu g$ of BSA

160$\mu l$ of BSA solution + 840$\mu l$ of distilled water = 160$\mu$g of BSA

180$\mu l$ of BSA solution + 820$\mu$l of distilled water + 180$\mu$g of BSA

200$\mu l$ of BSA solution + 800$\mu l$ of distilled water + 200$\mu$g of BSA

In each test tube, 1ml of culture filtrate wasintroduced. Then 1ml of solution C was added to each test tube, after 10min. 0.1 ml of solution D was added. After 30min. it was read on a spectrophotometer at 650 nm wavelength.Graph was made for standard curve, then amount of sample protein was evaluated.

### 3.5.4 Determination of pH

This was determined according to the method in (America Society Testing and Materials) ASTM (2002) using Horiba M-13 pH meter.The pH meter and associated electrodes were standardized with phosphate buffer solutions pH 5 and 10. Five mililiter of the culture broth was tansferred into 20ml conical flask. pH electrode was inserted and left for 3mins before taking the readings.

### 3.5.5 Emulsification Index

In determining the emulsification index, 2ml of the culture was centrifuged at 3000 rpm for 15mins to separate the cells. The supernatant was collected in a test tube while the cells were discarded. The emulsification stability of each of the isolates was determined by adding 2ml of crude oil to the test tube containing the supernatant. The tubes were vortexed at high speed for 2min and allowed standing for 2hours. The emulsification index was

calculated as the height of the emulsification divided by the total height of supernatant with added oil multiplied by 100 (Abbass and Amiri, 2008).

### 3.5.6 $Co_2$ Production in a Biometric System

Bacterial inoculation of biometric flask (containing 48ml Bushnell-Haas medium) consist of 1.0ml of a 48-hour culture.Two percent of Crude oil was added (1.0ml).The sidearm of biometric flask was filled with 10ml of 0.1ml of KOH. Flasks were incubated at 23$^{o}$C, without shaking. Samples for measuring carbon dioxide were withdrawn by a syringe in scheduled time period from a sidearm of the flask. Evaluation of $CO_2$ during microbial utilization of the oil was determined by colorimetric titration according to Cornfield (1961), Katarina (2005); Obire and Nwaubeta (2001) method. The amount of trapped $CO_2$ in KOH was accurately titrated after addition of 1.0ml of saturated barium chloride and 0.1ml of phenolphthalein with 0.05M HCl until colourless solution was obtained.

The control sample contained 10ml of fresh KOH with 1.0ml of barium chloride and 0.1ml phenolphthalein. The volume of HCl needed for neutralization of the experimental KOH was subtracted from the amount of HCl needed for neutralization of the unexposed KOH. The difference in milliliters was converted into micromoles of the evolved $CO_2$ during microbial degradation of the oil.

### 3.5.7 Determination of Optical density

Aliquot of 0.2ml was diluted in 0.8ml of Bushnell-Haas medium. Absorbance was measured at 600 nm against blank (3ml of Bushnell-Haas medium) in a spectrophotometer.

## 3.5.8 Catechol-2,3-dioxygenase

The isolates grown on BH medium supplemented with 2% of crude oil for 72 hours were harvested by centrifugation and washed twice in 50mM phosphate buffer, pH 7.4, to a

cell density of 1 optical density at 600nm ($OD_{600}$) unit, which corresponds to $10^9$ CFU/ml. Total Reactions were initiated by adding cells (1 ml) to reaction mixtures containing 10mM catechol in 50 mM subjected to potassium phosphate, pH 7.4. Oxidation of Catechol to 2-hydroxymuconic semialdehyde was monitored continuously at 375 nm with a spectrophotometer at room temperature. A unit of $C_{23}O$ activity corresponds to 1μmol of catechol/min and values were expressed as units per minute per $10^8$ cells (Sahar, 2006).

### 3.5.9 Catechol-1,2-dioxygenase

The reaction condition was taken for catechol-1,2-dioxygenase same as catechol-2,3-dioxygenase assay except the formation of cis,cis-muconic acid which was monitored at 260nm.

### 3.5.10 Gas Chromatographic-Mass Spectrometry Analysis

Gas chromatographic analysis was made according to the methods of Michaud *et al.* (2004). After the incubation period, 5ml of the cultures were extracted with two 20 ml volumes of n-hexane as a solvent by using separatory funnels to remove cellular material. The residues were transferred to tarred vials and the volume of each extract was adjusted to 100ml by adding further n-hexane. The vials were kept at $4^oC$ until the gas chromatographic analysis. Uninoculated Bonny light crude oil was used as control.

Aliquot of one μl was then injected onto the column using a 1:20 split ratio. Mass spectra as well the retention times of standard mixture of hydrocarbons were used to quantify each analyte. The GC/MS analysis was performed using GCMS-QP2010 PLUS SHIMADZU, JAPAN. GC equipped with a cool-on-column inlet and capillary direct interface. The instrument conditions were the following: capillary column HP-1MS, 60m x 0.2 mm; Column Oven Temp $60^0$ C; Injection temp. 250 $^0$C helium column flow 1 ml/min; pressure 100.2 kPa and split ratio 1.0. The initial temperature was $70^0$ C kept for 5 minutes

with a temperature ramp of 14 $^{0}$C per minute and final temperature of 280 $^{0}$C kept for 10 minutes with total run time 35 minutes. A solvent delay was employed in order to prolong detector lifetime at 0-4.5 minutes. The solvent front reached the detector at 3.0 minutes and initial analyte retention time was approximately at 7 minutes, so there was no loss of resolution due to initial solvent delay. The solvent used in all analysis was mixture of hexanes.

## 3.6 Structural prediction of Catechol-2,3-dioxygenase ($C_{23}O$)

The three dimensional structure of Catechol-2,3-dioxygenase was generated using the homology modeling method. Protein sequence of $C_{23}O$ (314aa, UniProtKB accession number: Q7M0R6) from *Burkholderia cepacia* was retrieved from Uniprot (http://www.uniprot.org/). A Basic Local Alignment Structural Tool (BLASTp) search was made against the PDB database (PDB, http://www.rscb.org/pdb/) to spot the most appropriate template for homology modeling. Accordingly, the structure of Catechol-2,3-dioxygenase from *Pseudomonas putida (*Kita *et al.,*1999) was selected as best template based on their sequence percentage identity and query coverage.The alignment of both template and target was done using the align2D script in the Modeler$9_V8$. Then the aligned sequence was used for model generation using the model_ligand.py script in the Modeler$9_V8$ (Erramian *et al*., 2006; Eswar *et al.,* 2007). The coordinate information of the template FE2+ was also used for the $C_{23}O$ model generation. From the ten models the best model was chosen based on their Discrete Optimized Protein Energy (DOPE) and Modeler Objective Function (MOF) scores. The best model was validated using structural analysis and verification server (SAVES), which includes various tools like Ramachandran plot (Ramachandran,1963) ERRAT (Luthy, 1992) and VERIFY_3D (Colovos and Yeates, 1993).

### 3.6.1 Molecular Dynamics Simulation

The molecular dynamics simulation of Catechol-2,3-dioxygenase was carried out using GROMACS 4.5.4 (Vanderspoel *et al.,* 2005;Hess *et al.,* 2008) molecular dynamics package. The atoms of the molecule was treated with OPLS-AA/L all-atom force field Jorgensen *et al.*(1996) and solvated with TIP4P water model, which extended to 1.50 nm triclinic box from the molecule to edge of the box. In order to neutralize the system 10 Na2+ were replaced with SOL molecule. The 50,000 steps of steepest descent and conjugate gradient energy minimization algorithm was used to minimize the protein with a tolerance of 1000 kJ $mol^{-1}$ $nm^{-1}$. The PME Kawata and Nagashima (2001) method was used for long-range interaction with a 1.2 nm cut-off and 0.16 nm Fourier spacing also used. Then, the energy minimized solvated system was considered a sensible one in terms of geometry and solvent orientations and used further. The molecular bond angles and geometry of the water were constrained with LINCS (Hess *et al.,* 1997) and SETTLE (Miyamoto and Kollman, 1992) algorithm respectively. Consecutively to regulate the temperature (310 K) and pressure (1 atm) of the system, the V-rescale weak coupling and Parrinello-Rahman method Martonak *et al.*,(2003) were used respectively. Both NVT (constant number of particles, Volume and Temperature) and NPT (constant number of particles, Pressure and Temperature) position restraint method was used for 100 ps. These procedures indicated that, the system is well equilibrated in terms of temperature, pressure, density and total energy. In continuation with that, this pre-equilibrated system was subjected to 10000 ps (10 ns) production MDS with a time-step of 2 fs. In every 2 ps the structural coordinates were saved and analyzed.

### 3.6.2 Molecular Docking

The docking of substrate into the Catechol-2,3-dioxygenase was carried using Autodock 4.0 software package (Morris *et al.*,1998). This software contains AutoTor, AutoGrid and AutoDock, in which AutoTor defines the rotatable bonds and root to detect the rotation. AutoGrid generates grid map for each substrate, here the grid box was generated

based on the $FE^{2+}$ atom. The box size was set to 50 × 50 × 50 Å in the x, y, and z axes. The spacing between grid points was 0.375 Å. AutoDock menu helps for docking methodology. Lamarckian genetic algorithm (LGA) was used for the conformer searching in the grid box mentioned. This method was used to calculate the binding energy for each molecule as a scoring function. In docking protocol the number of individuals in each population is set to 150 and the maximum number of energy evaluations was set to 250,00,000, where as the maximum number of generations was set to 1000. The maximum number of top individuals, the rate of gene mutation and crossover were set as default value.

### 3.7 Physicochemical Characterization of Refinery Wastewater Samples

#### 3.7.1 Determination of Phenol Content of Refinery Waste water

The Phenol content was determined using indophenol method as described by Ademoroti (1996). Aliquot of 25ml of the sample was dispensed into 50ml volumetric flask, 1.0ml concentrated ammonia solution and 10ml of nitroprusside – buffer reagent were added. Alkaline hypochlorite reagent was promptly added. The content of the flask was mixed by inverting the bottle several times. Absorbance was measured at 635nm against reagent blank.

$$\mu g/l \text{ Phenol} = \frac{\text{Reading from circle x 1000 x D}}{\text{ml of sample used}}$$

Where D = dilution factor

#### 3.7.2 Determination of Oil and Grease of the Refinery Wastewater

Fifty millilitres of the sample was added to separating funnel of 250ml capacity. It was acidified with $H_2SO_4$ and mixed by inversion. Then 25ml of trichloro-trifluoro-ethane (T.T.E) was added to the content and shaking vigorously for two minutes. The solvent layer was transferred into the tared distilled flask. It was then distilled, on a water bath using reflux condenser, till 10ml was left. The remaining solvent was boiled at $45^0 \pm 2^0$C temperature. The flask was dried in a steam bath, cooled in desiccators and weighed.

$$\text{Oil or Grease (mg/l)} = \frac{\text{A} - \text{B x 1000}}{\text{ml sample}}$$

A = weight of tared distilling flask and oil and grease

B = weight of tared distilling flask alone.

### 3.7.3 Determination of Chemical Oxygen Demand (COD) of Refinery Wastewater

Anti-bumping granules were inserted into reflux flask. Ten milliliter of the sample was measured into the flask, 1ml of 20%m/V mercuric sulphate solution was added and swirled to mix, 5ml of 0.020833M potassium dichromate was added. Using a dispensing pipette, 15ml of 1% m/V silver sulphate was added. The contents in the flask was then fit to a condenser and boiled gently for 2hours. After 2hours, the flask was removed and allowed to cool for approximately ten minutes, and then 25ml of water was added. Two drops of Ferroin indicator was added to the contents in the flask and the residual dichromate was titrated with standardized ferrous ammonium sulphite.

The COD was obtained using the formula:

$$COD = 800\ M\ (V_b - V_a)\ mg/l$$

Where:

$V_b$ = average number of ml ferrous ammonium sulphate used in titrating the appropriate blank;

$V_a$ = number of ml ferrous ammonium sulphate used in titrating the sample.

M = molarity of standard ferrous ammonium sulphate solution.

### 3.7.4 Determination of Nitrates of Refinery Wastewater

A Ten millitres of the sample was introduced into a distillation flask1g of magnesium oxide and 1g devaralloy were added. The mixture was then introduced into the distillation apparatus. It was distilled for 30minutes and 30 – 35ml of the distillate was collected into 2%

boric acid with 2 – 3 drops of indicator. The mixture was then titrated against 0.025H $H_2SO_4$ and the titre value noted. Nitrate was calculated from the formula as follows:

$$\text{Nitrate (mg/l)} = \frac{0.014 \times 0.025 \times 100 \times \text{Titre value}}{10}$$

### 3.7.5 Determination of Sulphates of Refinery Wastewater

Fifty millilitres of waste water sample was measured and 10ml of nitric acid was added and the mixture digested on a hot plate until the solution reduced to about 5ml.It was allowed to cool after which the contents were added to 250ml volumetric flask and made up to 50ml with distilled water. The contents were shaken to form a homogenous mixture and then allowed to cool, 10ml of the content was introduced into a 50ml volumetric flask and the 10ml of distilled water was added followed by 1ml of gelatin barium chloride. The contents were made to mark (50ml) with distilled water and allowed to stand for 30 minutes. The colour intensity was read with spectrophotometer at 420nm wavelength and the corresponding sulphate concentration was obtained from a standard curve. The value of sulphate in the sample was obtained using the formula.

$$\text{Sulphate} = \frac{\text{Reading x volume of digest (50ml) x dilution factor (50)}}{\text{Volume of sample (50ml) x aliquot taken (10ml)}}$$

### 3.7.6 Determination of Phosphates of Refinery Wastewater Sample

Aliquots of 0.5ml of sample was measured into a 100ml beaker, 20ml of tannary acid was added to the sample and it was then placed on a sand bath and plugged to the mains at $60^0$C and gradually increased to $100^0$C.The sample was then digested at that temperature till brown fumes were produced followed by white dense fumes of perchloric acid. When the

samples became colourless and had reduced to about 5ml, the contents were transferred into a 50ml volumetric flask and made up to 50ml with distilled water.The contents were shaken to form a homogenous mixture and 10ml wasintroducedinto a 25ml volumetric flask, 5ml of phosphate developer was added and then distilled water to make up to 25ml.The mixture was shaken and allowed to stand for 30minutes for colour development. The colour intensity (absorbance or transmittance) was read from a spectrophotometer at 420nm wavelength. The value of phosphate (mg/l) corresponding to the absorbance was obtained from a standard curve.

### 3.7.7 Determination of Dissolved Oxygen (DO) of Refinery Wastewater

Two millitres of manganous sulphate ($MnSO_4$. $5H_2O$) was added to the sample in the titrating bottle well below the surface of the liquid and $2cm^3$ of alkaline iodide solution at the surface. The stopper was carefully replaced (to avoid inclusion of air bubbles) and the contents were thoroughly mixed by vigorously inverting and rotating the bottle several times. When the precipitate had settled in a portion of the bottle, the mixing was repeated and allowed to settle again, leaving a clear supernatant liquid. Then 4ml of 90% phosphoric acid was added, the stopper was replaced and the sample was thoroughly mixed to have a complete dissolution of the precipitate and 50ml of this solution was measured into250ml Erlenmeyer flask and immediately titrated with standard sodium thiosulphate using 2ml starch solution as indicator. The dissolved oxygen is given as:

$$BOD_5 = \frac{\text{Volume of thiosulphate x 101.6}}{Volume\ titrated}\ \text{mg/l}$$

$$BOD_5 \text{ in mg/l} = \frac{(D1-D2)\ X\ T}{V}$$

Where:

D1=DO of the sample before incubation in mg/l
D2= DO of the sample before incubation in mg/l
T= Titre value
V= volume titrated

### 3.7.8 Determination of Total Petroleum Hydrocarbon of Refinery Wastewater

Thirty milliliters of carbon tetrachloride ($CCL_4$) was added to one liter of the sample to extract the hydrocarbons. The mixture was shaken vigorously for one or two minutes. The mixture was poured into a separating funnel and shaken intermittently for two or three minutes. The funnel was then rinsed with twenty milliliters of carbon tetrachloride. The resulting oil was drained into a fifty milliliter volumetric flask through a funnel containing cotton wool to which had been added anhydrous sodium sulphate ($Na_2SO_4.7H_2O$) to remove traces of water from the oil. Fourier Transform Infrared Spectrophotometer (FTIR, genesis series) was calibrated with isooctane/octane in carbon tetrachloride. The TPH concentration in the samples was determined by using the stored calibration graph in the software of the equipment as a reference.

### 3.7.9 Determination of Percentage of Oil Degradation

Method of Sathiyamoorthi *et al*. (2008) was used with little modification. One hundred mililitres of incubation mixture of refinery waste water was extracted with 10ml of n – hexane. The aqueous phase was extracted with 10ml of n-hexane and the combined n-hexane fractions were filtered through whatman No.1 filter paper. The n-hexane was evaporated at $37^0$**C** to constant weight in a large petri dish. The rate of oil degradation was expressed in percentages.

Rate of oil degradation (Z) = x/y x 100

Rate of oil degradation (%) = Z%

X = periodical n-hexane extractable oil (mg)

Y = control n – hexane extractable oil (mg)

### 3.7.10 Determination of pH Value

A 50ml beaker was half-filled with the samples. Water was added to just sufficient depth to allow immersion of the electrode. Mixing was carried out using a shaker and stirred frequently for a few minutes. Then, allowed to stand for a further 15 minutes. The electrode of the pH meter was immersed into the sample waited for a while. The pH was recorded for each sample.

### 3.7.11 Determination of Heavy Metals

Fifty mililitres of the sample were transferred into a Kjeldahl flask, 20ml of concentrated nitric acid ($HNO_3$) was added and the sample pre-digested by heating gently for 20mins. More acid was thereafter added and digestion was continued for 30-40mins. Digestion was stopped when a clear digest was obtained.

The flask was cooled and the content transferred into a 50ml volumetric flask and made to the mark with distilled water. The resulting solution was analyzed for heavy metals using the Atomic Absorption Spectrophotometer Atomic Absorption Spectrometer (AAS, GBC 932 Plus).

## 3.8 Immobilization of Cells

Mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri* that showed degradative potential during mineralization of Bonny light crude oil was immobilised on Agar-agar as follows: Agar-agar solution and Inocula for immobilisation were prepared separately as thus; Fifty milliliter of each of the inoculum was inoculated into nutrient broth and incubated for 24 hours. A solution containing 3% bacteriological agar was dissolved in

0.9% sodium chloride (100ml) in a 250ml Erlenmeyer flask, sterilized and cooled to 40-45$^0$C. The inoculum with $OD_{600nm}$= 1.0 which corresponded to $10^9$ cfu/ml was added to the molten agar-agar maintained at 40$^0$C, shaken well for few seconds (without forming foam), poured into sterile flat bottom 4-inch-diameter Petri plates and solidification occurred after 10minutes.The solidified agar block was cut into equal sizes, fifteen pieces of cubes each of the cell was then added to sterile 0.1M phosphate buffer (pH 7.0), and kept in the refrigerator (1hour) for curing. Phosphate buffer was decanted and the cubes were washed with sterile distilled water three to four times before use (Adinarayana *et al.*, 2005).

### 3.9 Bioremediation Using Immobilized Mixed Culture

Two litres of the refinery waste water supplemented with Minimum Basal medium in g/l: NaCl (0.8), $MgSO_4.7H_2O$ (0.001); $KH_2P0_4$ (2); $NaN0_3$ (2); $CaCl_2.2H_20$ (0.5), $NaHP0_4.12H_20$ (2), was sterilized in an autoclave at 121$^0$C for 15mins. One litre of the sterile wastewater was dispensed into five liters bioreactor fitted with air sparger aseptically, fifteen pieces each of the immobilized bacterial cells were then added to the bioreactor (Margesin and Schinner, 2005). Physicochemical Parameters were determined at every five days intervals to monitor the progress of bioremediation for 20days.

### 3.10 Preparation of Crude Oil Degrading Transconjugant Culture

Method of Bathe and Hausner (2010) was modified and used for the preparation of crude oil degrading transconjugants. This was done to transfer catabolic plasmid from oil degrading host strains to a mixture of non-oil degrading indigenous strains in the oil sludge. Overnight donor cell of mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri* grown in Luria Bertani (LB) medium supplemented with 1% crude oil was prepared, also ten grams of oil sludge was added to 90ml of Bushnell-Haas medium and incubated at 30 $^0$C for 48hours to obtain oil sludge suspension.Two millilitres of donor strains

earlier prepared was mixed with 2ml of pre-grown suspension of oil sludge. Drop of 1ml of this mixture was plated on LB agar plates and incubated at room temperaturefor the conjugation to occur. The mating spot was scrapped off the agar surface and the cells were resuspended in 1ml of PBS (Phosphate Buffer Saline), centrifuged at 600 rpm for 20minutes and the supernatant was discarded. The pellet mixtures were spread on oil agar plates (BH medium supplemented with 2% crude oil) to select transconjugants.

Prior to mixing of donor and recipient indigenous suspension was unable to grow in oil agar. Transconjugant was selected, purified in PBS and later used for bioremediation process.The colonies on the oil agar were removed and transferred into 1ml of PBS, vortex vigorously, centrifuged for 10mins at 5,000rpm. Pellet was re-suspended again in PBS. The cell pellet was washed twice and resuspended with Bushnell-Haas medium until $OD_{600}$ was equivalent to 1.0.

### 3.10.1 Screening of Transconjugant for Bioremediation Capabilities

Ten grams of wood chips was added to five litres capacity bioreactor fitted with air sparger to serve as biofilm carrier material. One litre of refinery waste water supplemented with minimum basal medium was transferred to the bioreactor. The 10% of cells of crude oil degrading transconjugant with $OD_{600nm}$= 1.0 which correspond to $10^9$ cfu/ml was added to the bioreactor Control sample contained only the sludge inoculum without culture. Set up was left for 10 days. The following parameters were used for assessment: COD, Oil and Grease, phenol, % oil loss and sulphide.

## 3.11 Plasmid Profiling and Agar Gel Electrophoresis

Plasmid extraction was carried out using the method described by Ehrenfeld and Clewell (1987) with slight modification. Pure isolates were inoculated on LB broth and

incubated at $30^0$C for 24h. The grown cells were harvested and suspended in 200µl of solution A (100mM glucose-50mM Tris hydrochloride (pH 8)-10mM EDTA) containing 10 mg of lysozyme per ml and 10µg/ml mutanolysin and incubated at $37^o$C for 30 min in an incubator. 400µl of freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH was added and the samples were mixed by inverting tubes. 300µl of a 30% potassium acetate solution (pH 4.8) was added and the samples were mixed by vortexing. After incubating on ice for 5 minutes, the debris was removed by a 5-minute centrifugation in a centrifuge (model 5415R; Eppendorf).

The supernatant was removed and extracted once with a phenol – chloroform mixture (1:1) and precipitated with an equal volume of isopropanol. The plasmid DNA was then dissolved in 100µl of Tris-EDTA (TE) buffer. Electrophoresis of the DNA was carried out on a 0.8% agarose gel in a 0.5x concentration of Tris-Borate-EDTA (TBE) buffer. After boiling, the solution was allowed to cool and 10µl of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes and the comb was removed. 20µl of the plasmid DNA samples were then loaded into the wells after mixing with 2µl of bromophenol blue. A DNA molecular weight marker was also loaded into one of the wells. The gels were thereafter electropholesed in a horizontal tank at a constant voltage of 60v for about 1 hour 30 minutes. After electrophoresis, plasmid DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light transilluminator and the photograph were taken using a digital camera. The DNA bands were matched with those of lambda DNA hind III digest molecular weight marker in the range 0.1-23.1kb. The approximate molecular weight of each plasmid plots of molecular weight of marker against the distance traveled by the respective band.

### 3.12 Curing of Plasmid

Curing of plasmid was done to identify the catabolic plasmid encoded in the isolated bacteria. The modified methods of Ahrne *et al.* (1989) and Bhalakia (2006) were used. A stock solution of 10% SDS was prepared by the addition of 10g SDS to 100 ml of nutrient broth. The pH was adjusted to7.8 - 8.0. The mixture was steamed for 1 hour and kept aside as stock. The overnight cultures of selected isolates in LB were diluted 100- fold and 0.5 ml volume of each of the isolates was transferred aseptically into fresh 30 ml volume of nutrient broth. The cultures were incubated with shaking for 3 h and SDS stock solution was added to give the required final concentration (1%, w/v). The cultures were incubated at room temperature with mild agitation for 72 h. The cells were then tested for crude oil degradation: after transferring 0.5 ml sample of each of the isolates into nutrient broth supplemented with crude oil (2% , w/v), the plasmid content was determined by carrying out the plasmid isolation procedure described in section 3.11 of the present study.

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Isolation and Characterization of crude oil degrading bacteria from refinery sludge

Indigenous crude oil degrading bacteria were isolated from Kaduna Refinery Petrochemical Company (KRPC) wastewater after weeks of enrichment in crude oil medium. Adaptability of bacteria in oil sludge during enrichment resulted in the isolation of two bacterial colonies from oil sludge which were Gram positive and Gram negative. Both isolates were non capsulated, non spore former and non motile, but fermented glucose, lactose and mannitol.The isolated colonies were identified based on colonial morphology and biochemical characteristics .Automated test system vitek API 20NE and API Coryne. (BioMerieux, inc Hazelwood, Mo, USA) was used for the confirmation and authentication of the genera belonging to *Burkholderia cepacia and Corynebacterium kutscheri* as shown in Table 4.1.

The degradation pattern of the isolates revealed the occurrence of hydrocarbon degrading bacteria in the refinery sludge after two weeks of selective enrichment in the Bonny light crude oil. Two bacteria identified as *Burkholderia cepacia* and *Corynebacterium kutscheri* demonstrated the ability to survive and proliferate in the presence of high concentration of Bonny light crude oil and used it as sole source of energy and carbon. Though, enrichment technique selected only those indigenous bacteria that have been acclimated to degrade crude oil mutually.

### 4.2 Evaluation of Mineralization potential of the Isolates on Bonny light Crude oil

Biodegradation pattern of the isolated bacteria in mineral medium using Bonny light crude oil as source of carbon was evaluated as measured by Dehydrogenase activities, Fluorescein diacetate, Protein utilization, pH, $CO_2$ Production, Emulsification activities and Growth patterns.

**Table 4.1:** Morphological and Biochemical Characterization of Bonny Light Crude oil degrading bacteria

| Characteristics | **Isolate A** | **Isolate B** |
|---|---|---|
| Colonial Morphology | Small colonies, convex, regular margin, Translucent gelatinous | Cream, entire, convex and circular |
| Gram reaction | -ve rod | + rod |
| Spore/Capsule | - | - |
| Motility | - | - |
| Catalase | + | + |
| Oxidase | + | + |
| Urease | + | - |
| Indole | - | - |
| Gelatin hydrolysis | + | + |
| Methyl red | + | - |
| Voges – proskauer | - | + |
| Citrate utilization | + | + |
| Glucose fermentation | + | + |
| Lactose fermentation | + | + |
| Starch hydrolysis | - | - |
| Mannitol | + | + |
| Nitrate reduction | - | + |
| Maltose | + | + |
| Probable organisms | *Burkholderia cepacia* | *Corynebacterium kutscheri* |

### 4.2.1 Dehydrogenase Activities

The pattern of dehydrogenase activities in each of the isolates was basically the same and steady. The highest dehydrogenase activities of 63µg/TPF/h and 72µg/TPF/h was produced in *Burkholderia cepacia* and *Corynebacterium kutscheri* respectively after 216 h of incubation, while in their co-culture, there was gradual increase in dehydrogenase activities after 24hrs of incubation to achieve (68µg/TPF/h) and deep dropped at 96h to (35µg/TPF/h), before sharp increase to attain maximum dehydrogenase activities (210µg/TPF/h) at 168h and later decreased (80µg/TPF/h) in sinusoidal pattern as shown in Fig.4.1.

Dehydrogenase activities of both *Burkholderia cepacia* and *Corynebacterium kutscheri* were not statistically different from each other after 96h of incubation upto 144h ($P< 0.05$).Higher activities were observed in *Burkholderia cepacia and Corynebacterium kutscheri* after 192h of incubation and after 168h of incubation in their mixed culture.

### 4.2.2 Fluorescein Diacetate Activities

The fluorescein diacetate activities in the culture filtrate of *Burkholderia cepacia* and *Corynebacterium kutscheri* was 40µg fluorescein $mL^{-1}3h^{-1}$ and 46µg fluorescein $mL^{-1}solvent3h^{-1}$ respectively after 216h of incubation. Their co-culture increased after 96h of incubation to 111µg fluorescein $mL^{-1}solvent3h^{-1}$ and decreased slightly after 120hr of incubation, then later increased to reach the peak at 146 µg fluorescein $mL^{-1}solvent3h^{-1}$as shown in Fig. 4.2. Fluorescein diacetate activities between *Burkholderia cepacia* and *Corynebacterium kutscheri* were not statistically different from each other from 72h to 192h of incubation ($P< 0.05$), characterized by flunctuation.Duncan's multiple comparison showed that there were no significant differences in the activities of both isolates and their co-culture ($P< 0.05$) after 192h of incubation.

### 4.2.3 Protein Contents

Protein utilization in the *Burkholderia cepacia* and *Corynebacterium kutsheri* culture filtrate was determined. Protein production was slightly increased to between 24hours to 72hours of incubation to attain 2.75 mg/ml before steady increase between 96h to 162h of incubation to reach the peak of 7.5mg/ml and decrease progressively in their co-culture medium, while; while each of their axenic culture exhibited zig -zag pattern in the production of proteins shown in Fig.4.3. There was no significant difference between the two isolates throughout the incubation period. However, highest protein utilization was observed after 192h in both isolates.Ttheir mixed culture showed highest protein utilization after 168h ($P< 0.05$).

### 4.2.4 Changes in pH

The pH changes in the Bonny light crude oil medium are shown in Fig.4.3. The pH of the oil medium due to growth of both isolates tended to decrease progressively from 7.0 to 6.38 and 6.45 in *Corynebacterium kutscheri* and *Burkholderia cepacia* respectively while, in their co-culture, it gradually increased to 6.6 as shown in Fig.4.4.pH of the experimental medium were not statistically different from each other in both isolates and their mixed culture ($P < 0.05$).

### 4.2.5 $CO_2$ Production

The $CO_2$ production increased to 173µmol in 96h of incubation and later dropped to 100µmol after144h of incubation. There was sharp increase to reach the peak of 211µmol at 192h and later decreased progressively in co-culture of *Burkholderia cepacia* and *Corynebacterium kutscheri*, while, production of $CO_2$ in *Corynebacterium kutscheri* was slightly higher than in *Burkholderia cepacia* culture filtrate as shown in Fig.4.5. $CO_2$ production found to be significantly higher after 192h of incubation in each of the pure culture ($P< 0.05$).

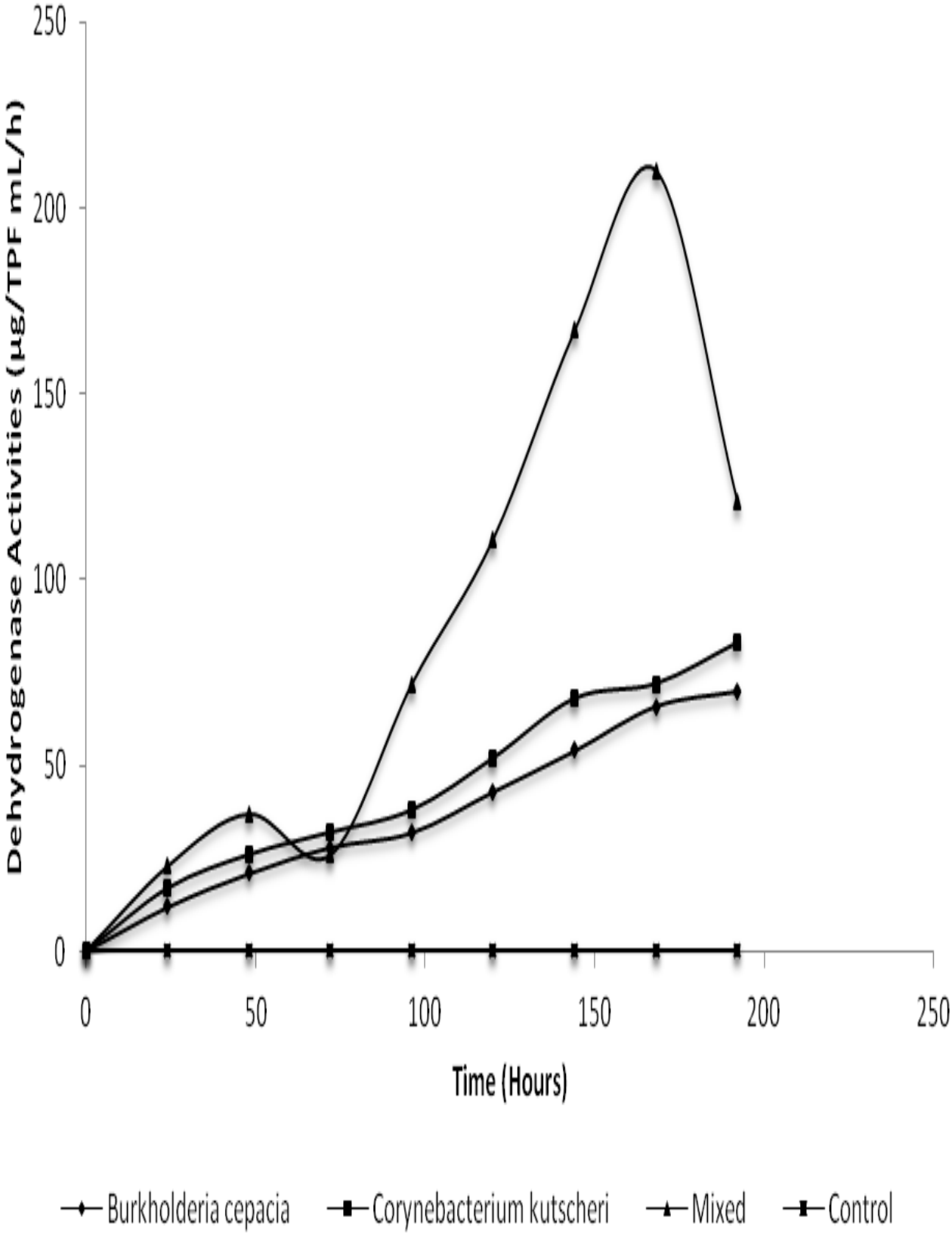



Figure 4.1: Changes in Dehydrogenase Activities during Mineralization of Crude oil by Pure and Mixed Culture of *Burkholderia cepacia* and *Corynebacterium kutsheri.*

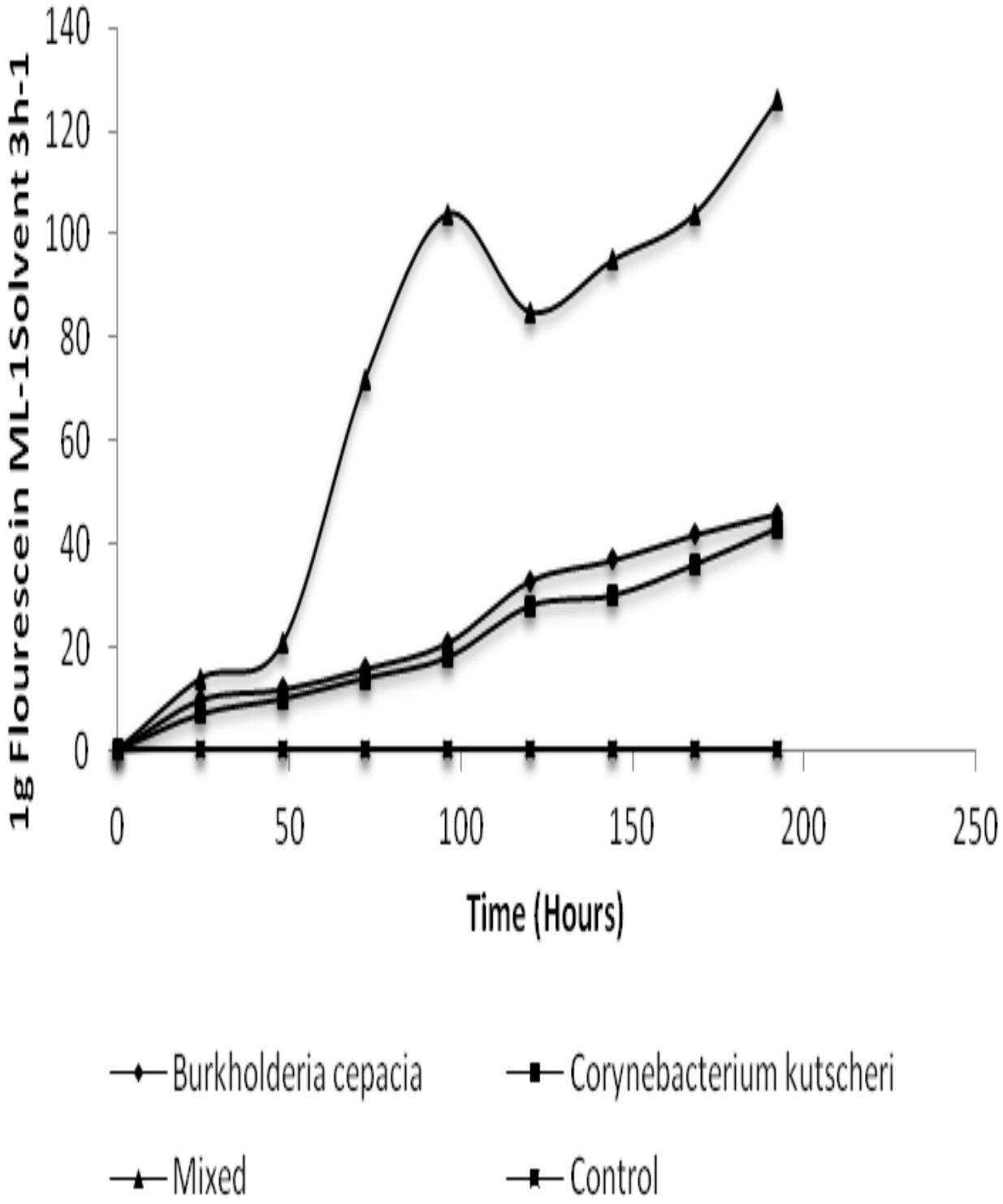



Figure 4.2: Changes in Fluorecein Diacetate Activities during Mineralization of Crude oil by Pure and Mixed Culture of *Burkholderia cepacia* and *Corynebacterium kutsheri*

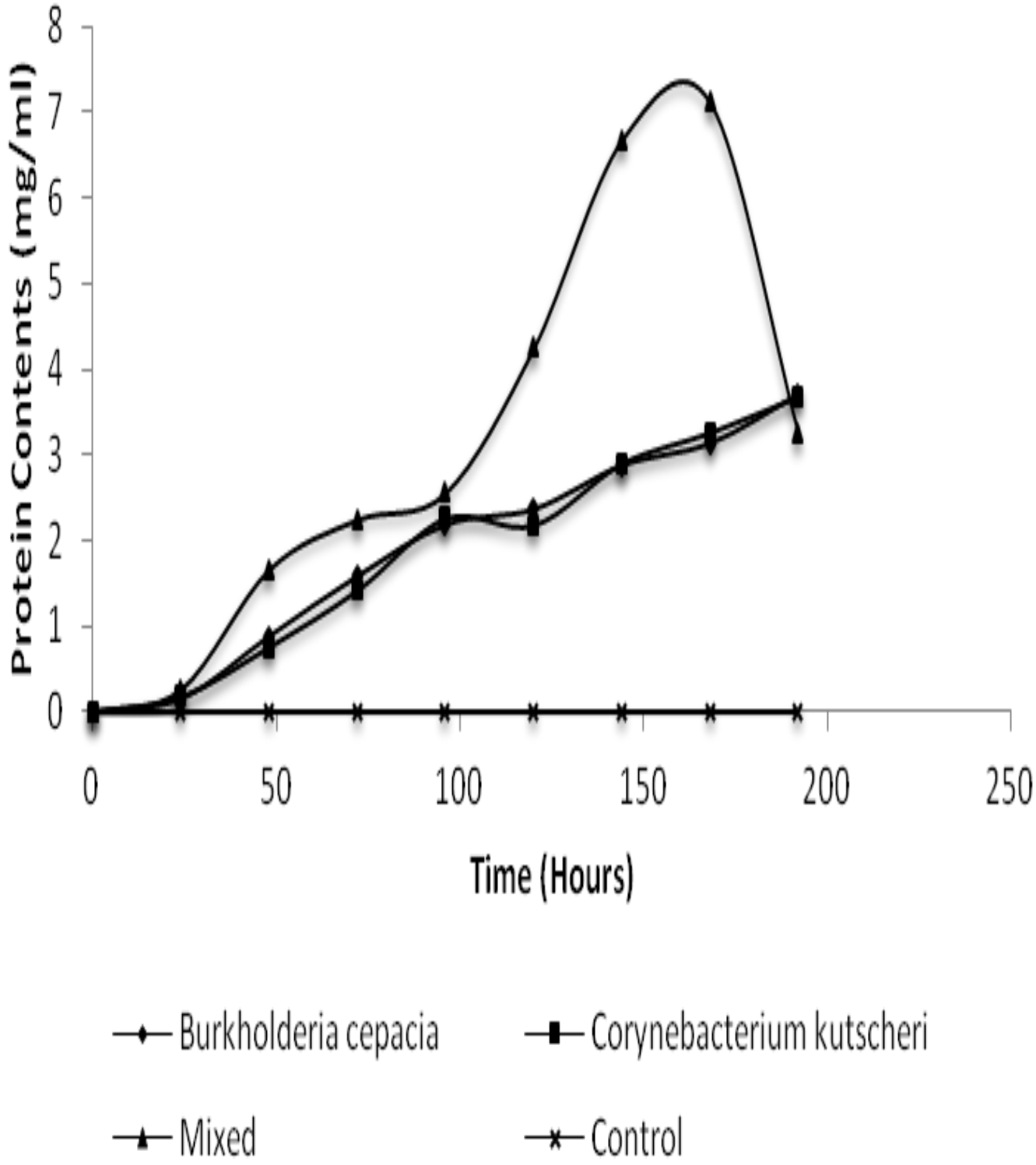



Figure 4.3: Changes in Protein contents during Mineralization of Crude oil by Pure and Mixed Culture of *Burkholderia cepacia* and *Corynebacterium kutsheri.*

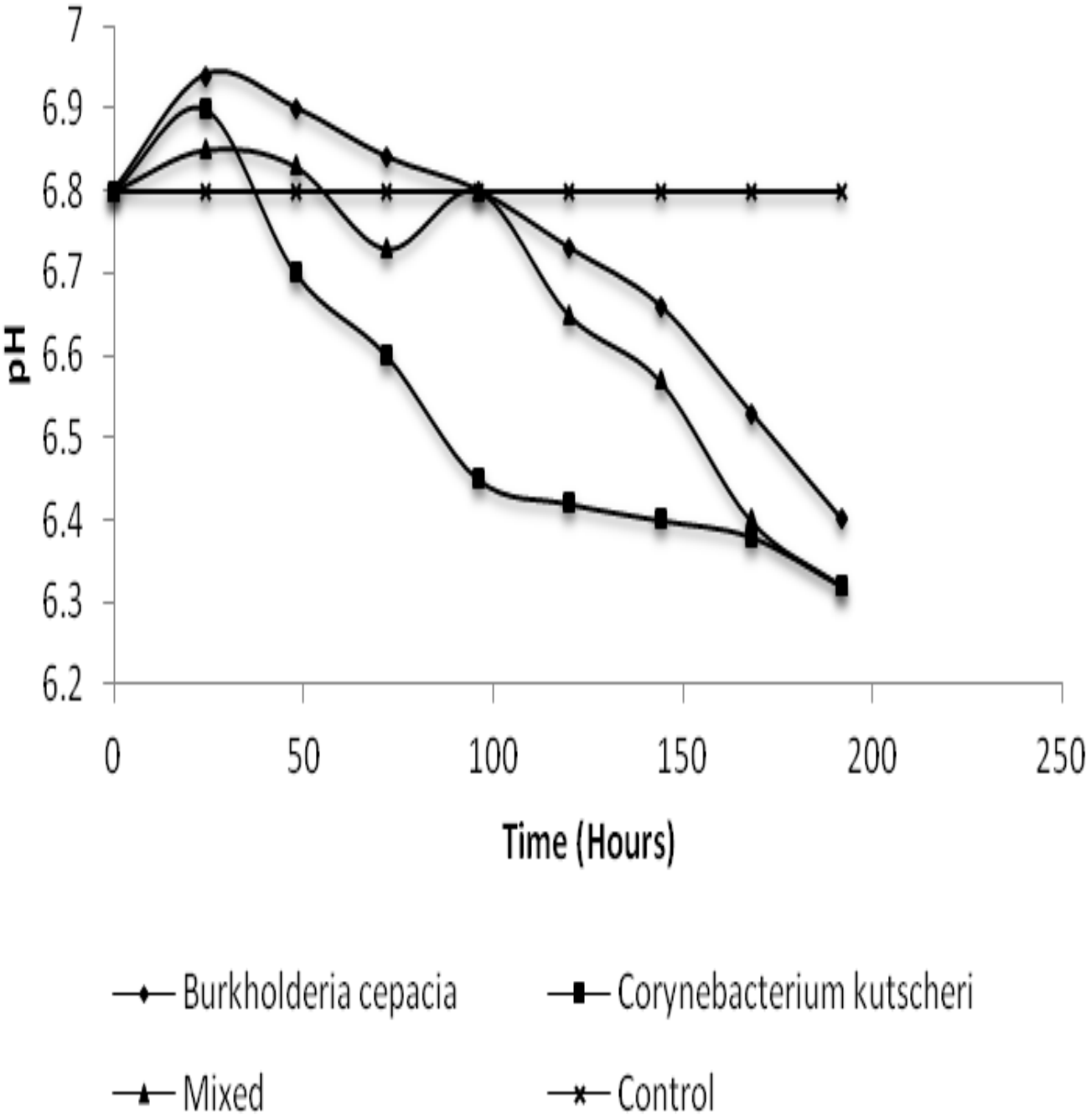



Figure 4.4: Changes in pH during mineralization of Crude oil by Pure and Mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri*

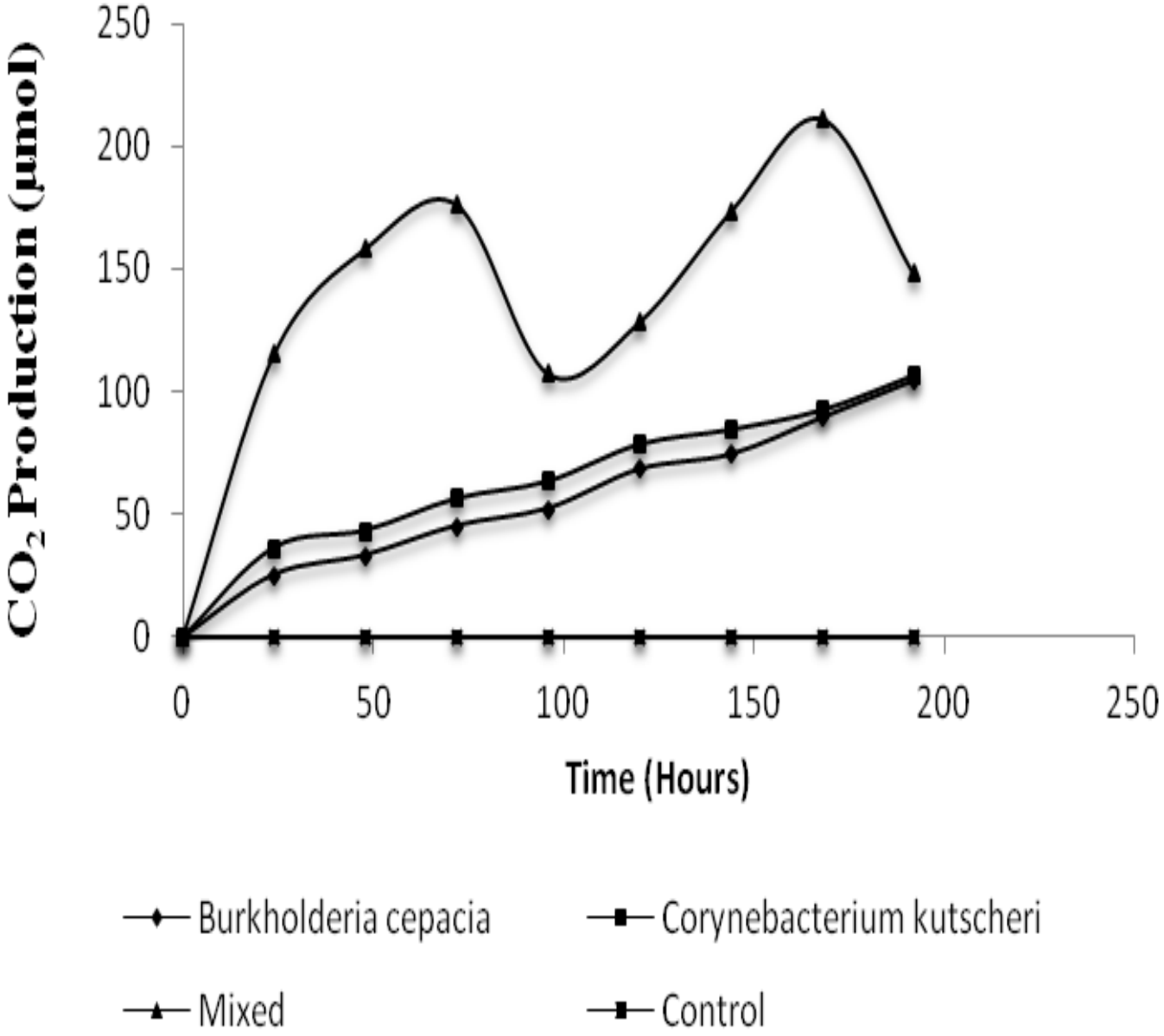



Figure 4.5: Changes in $CO_2$ Production during mineralization of Crude oil by Pure and Mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri*

### 4.2.6 Emulsification Activities

Emulsification activities decreased with increase in time in both pure culture and their co-culture.Emulsification index increased in both pure and mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri* to attained 70% in mixed culture, while, *Corynebacterium kutscheri* and *Burkholderia cepacia* attained 50% and 40% respectively before, being decreased progressively as shown in Fig.4.6. Emulsification ability of both pure and their mixed culture found to be higher after 48h and 24h in that order.Biosurfactants production were not statistically different in both isolates after 120h to 192h of incubation, while, there was no significant difference between the pure and mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri* between 168h to 192h of incubation ($P< 0.05$).

### 4.2.7 Growth Pattern

Growth pattern of *Corynebacterium kutscheri* and *Burkholderia cepacia* during mineralization of Bonny light crude oil showed gradual increase from 24h to 72h and little drop in 96hrs before sharp increase to a peak at 0.75nm and later decreased progressively in their co-culture while the growth pattern of *Corynebacterium kutscheri* was slightly higher than that of *Burkholderia cepacia*. However, both pure and mixed culture exhibited synchronous pattern and increased steadily as shown in Fig. 4.7.

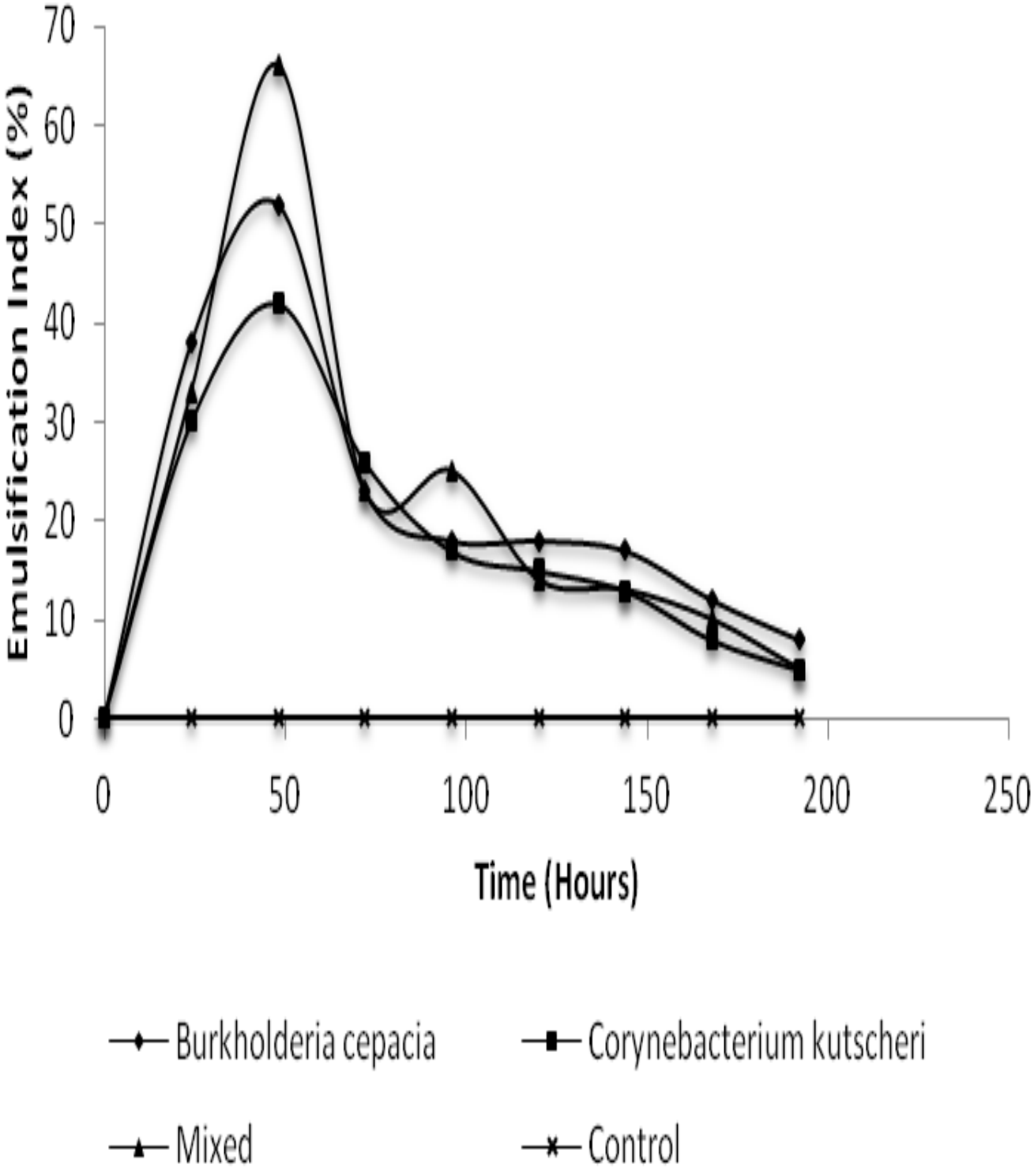



Figure 4.6: Changes in Emulsification index during mineralization of Crude oil by Pure and Mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri*

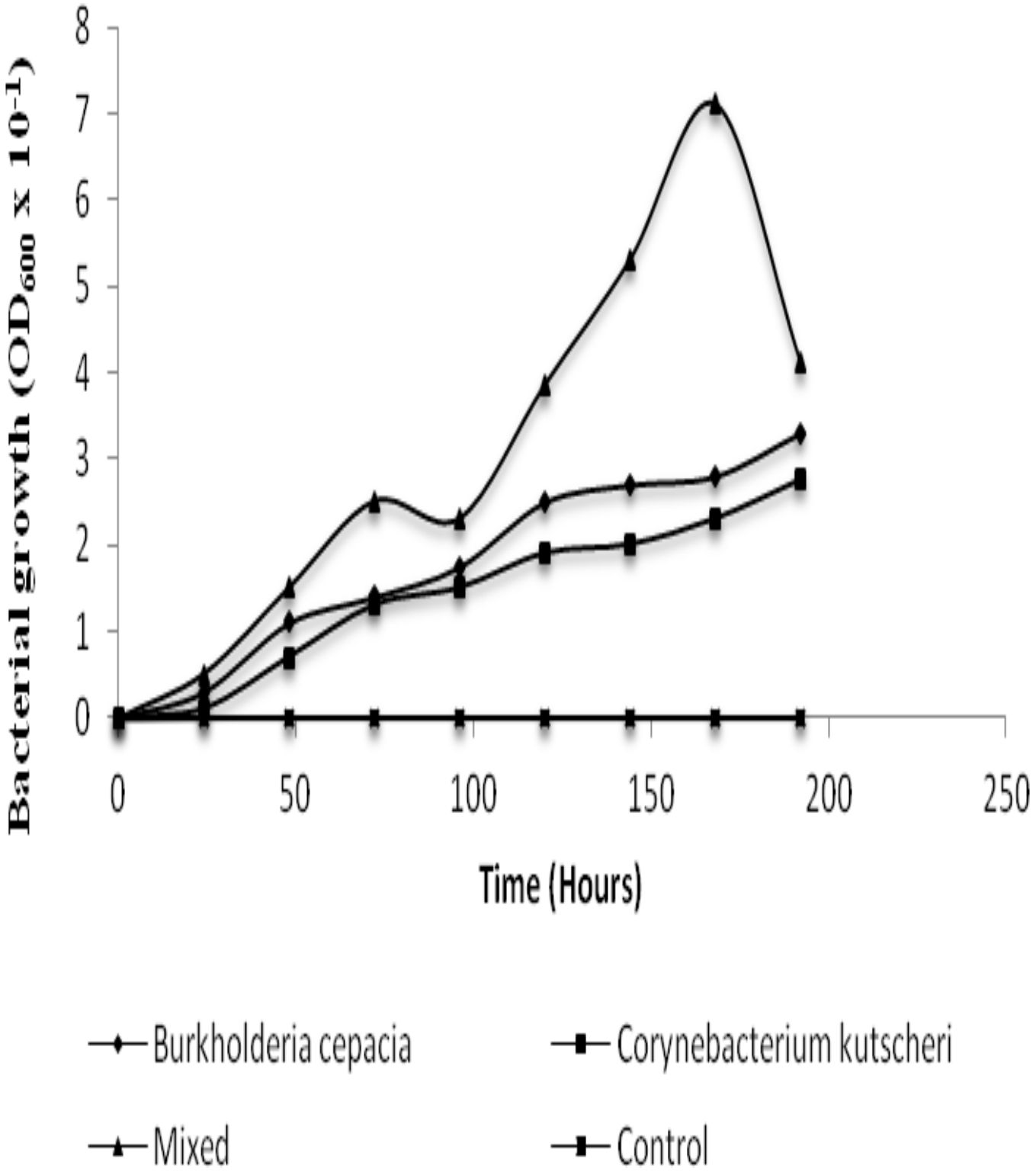



Figure 4.7: Changes in Bacterial growth during mineralization of Crude oil by Pure and Mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri*

### 4.3 Gas chromatographic analysis of Bonny light crude oil

Gas Chromatography mass spectrometry resolved and elucidated various hydrocarbon components of Bonny light crude oil. The GC peaks were found to range from n-$C_1$ to n-$C_{35}$ in the control samples as shown in Fig.4.8 as indicated by their GC- peak; n-$C_{34}$ and n-$C_{35}$ were observed to mineralize by *Burkholderia cepacia* after 5days of incubation as shown in Fig 4.9, while, n-$C_{28}$ to n-$C_{35}$were completely mineralized in the *Corynebacterium kutscheri* and other GC peaks were slightly reduced as shown in Fig.4.10. In Fig 4.11, Co-culture of *Burkholderia cepacia* and *Corynebacterium kutsheri* completely degraded quite number of hydrocarbon components of the crude oil as indicated in GC-peaks. All the GC peaks were greatly reduced after 5days of incubation, except n-$C_{13}$ and n-$C_{14}$. The number of peak in GC finger prints in oil recovered from flask inoculated with *Burkholderia cepacia* incubated after 10days was not reduced while their sizes were slightly reduced as shown in Fig.4.12. The GC peaks in *Corynebacterium kutscheri* culture filtrate after 10days of incubation showed that all the peaks were slightly reduced still present in the medium as shown in Fig 4.13. The n-$C_{13}$ and n-$C_{14}$ that were present in their co-culture filtrate after 5days of incubation disappeared while; n-$C_1$ to n-$C_{10}$ that were initially not found re-appeared. It was assumed that their co-culture prefferred n-$C_{13}$ and n-$C_{14}$ as a source of carbon and energy to n-$C_1$ to n-$C_8$ shown in Fig. 4.14.

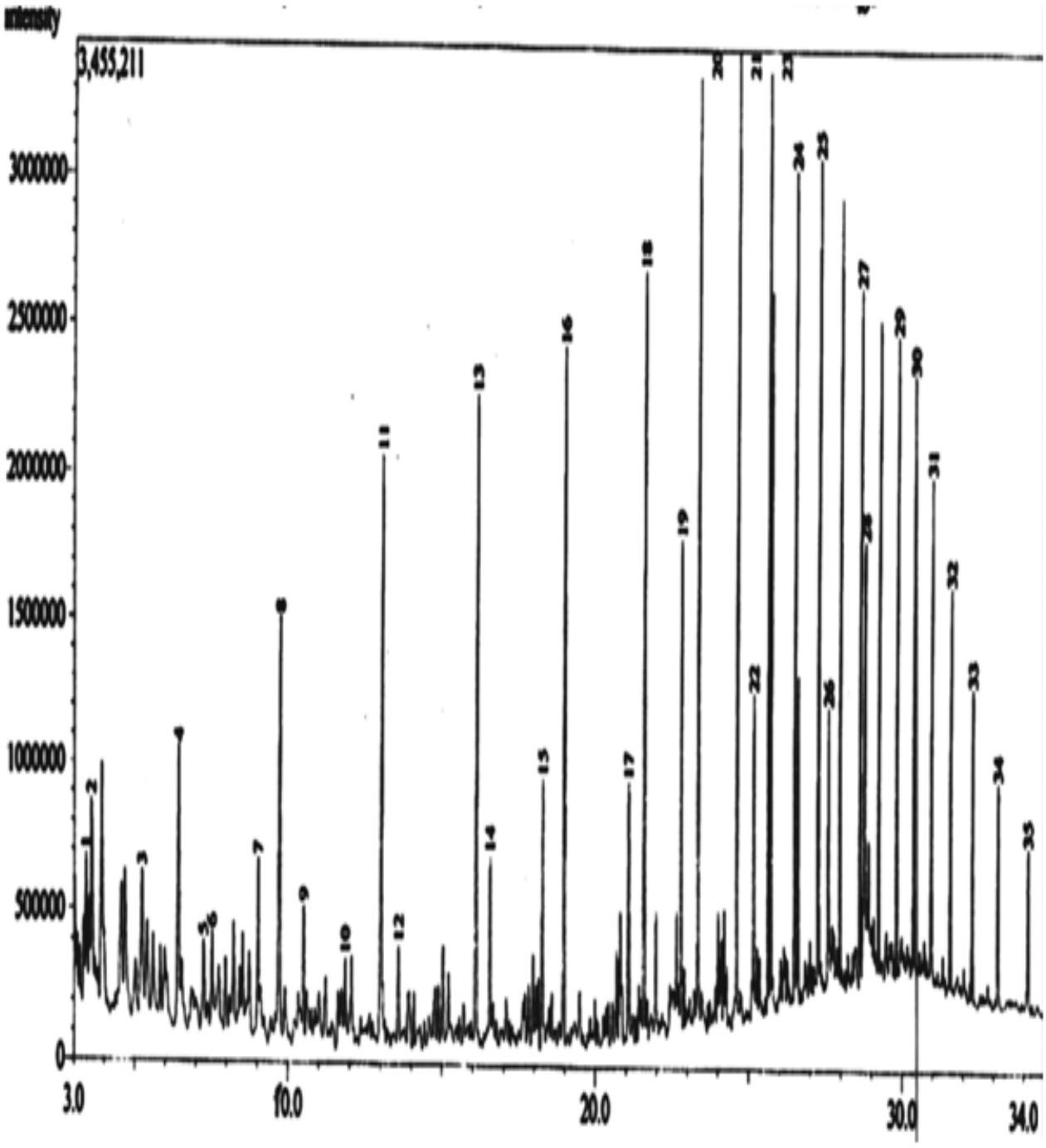



**Figure 4.8: Gas Chromatographic Fingerprints Bonny Light Crude oil recovered from Flasks Uninoculated with Bacterial Isolates (Control)**

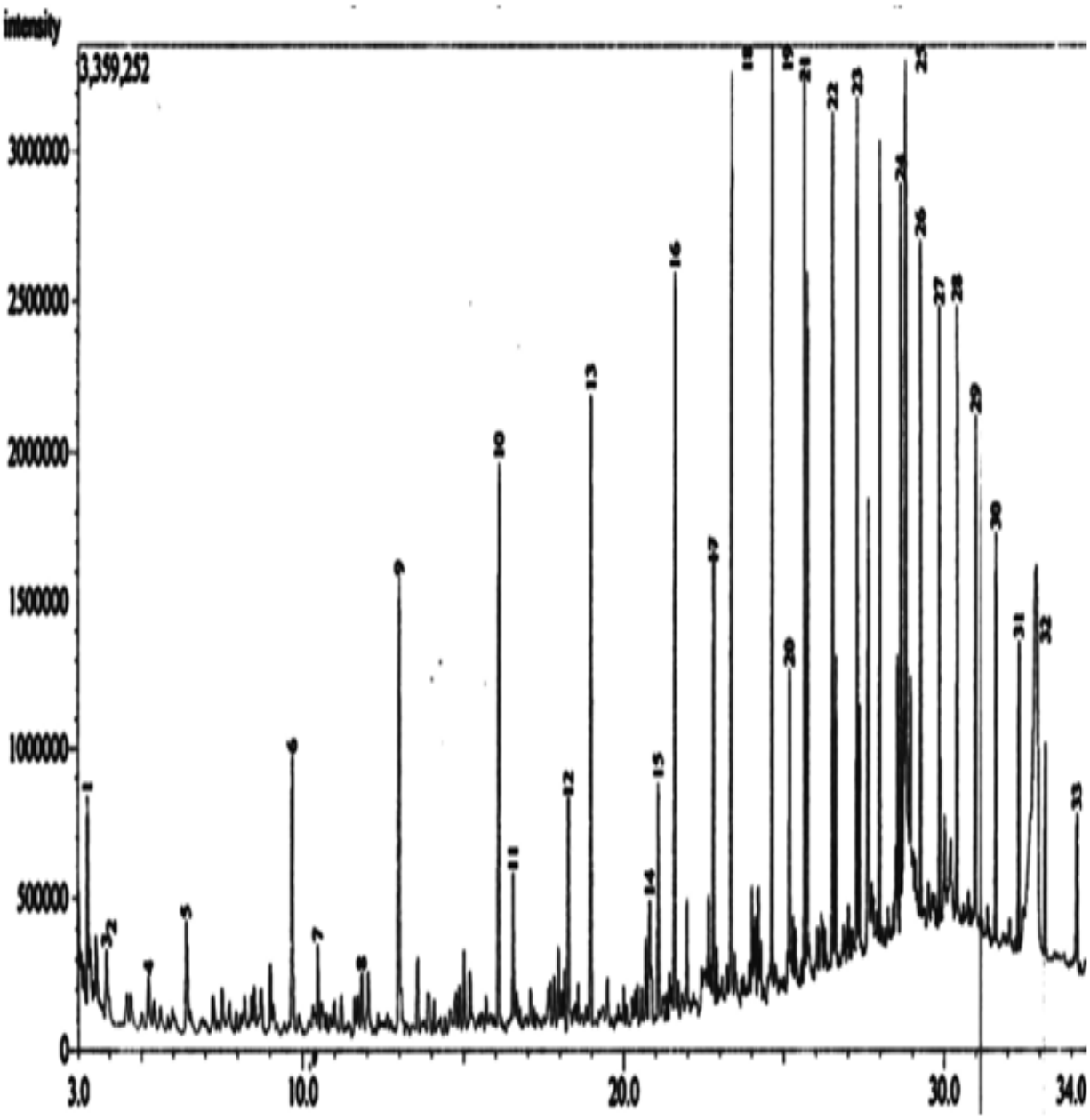



**Figure 4.9: Gas Chromatograhic Fingerprints of Bonny Light Crude Oil Recovered from Flasks Inoculated with *Burkholderia cepacia* after 5days of Incubation**

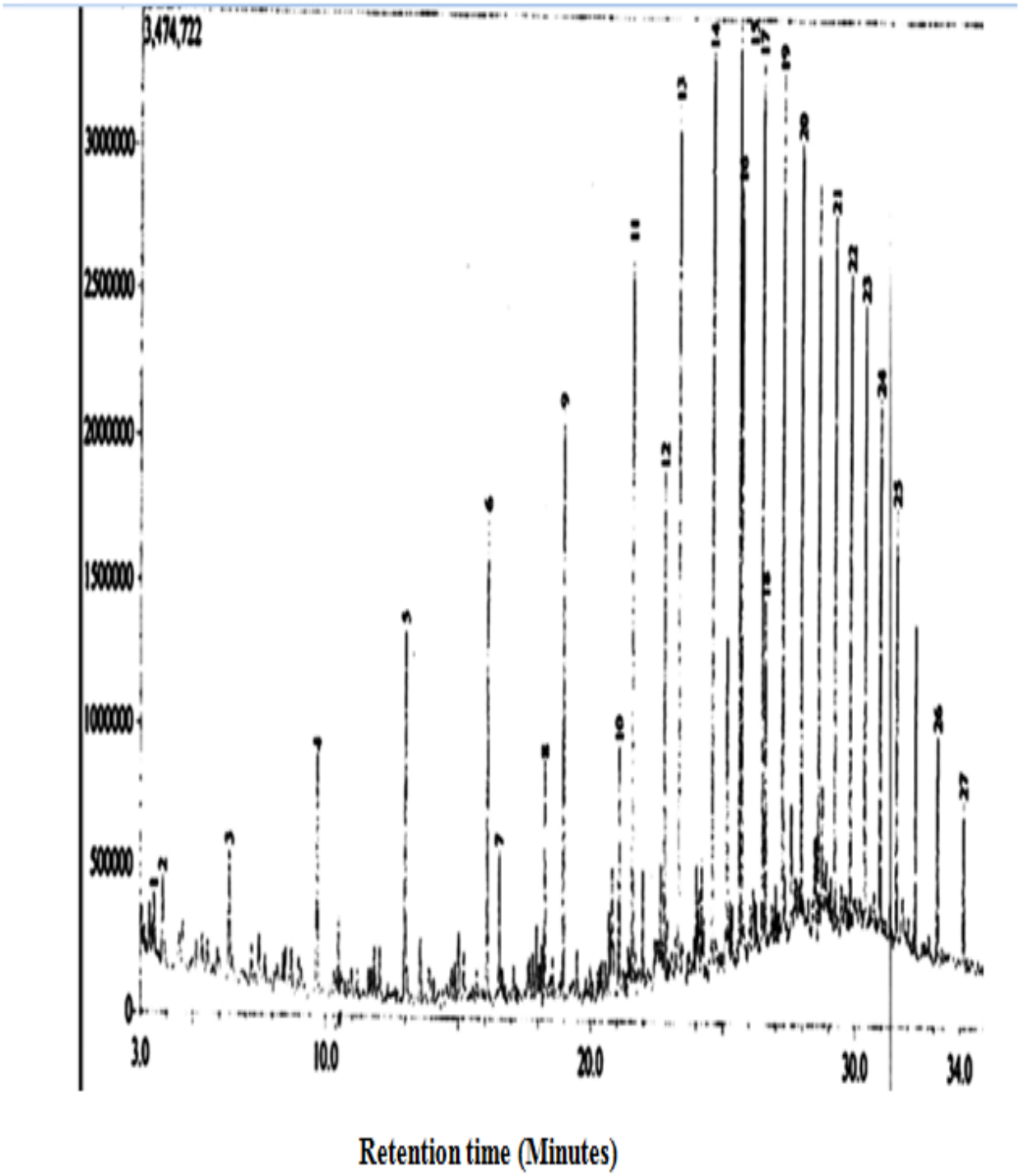



**Figure 4.10: Gas Chromatograhic Fingerprints of Bonny Light Crude Oil Recovered from Flasks Inoculated with *Corynebacterium kutsheri* after 5days of Incubation.**

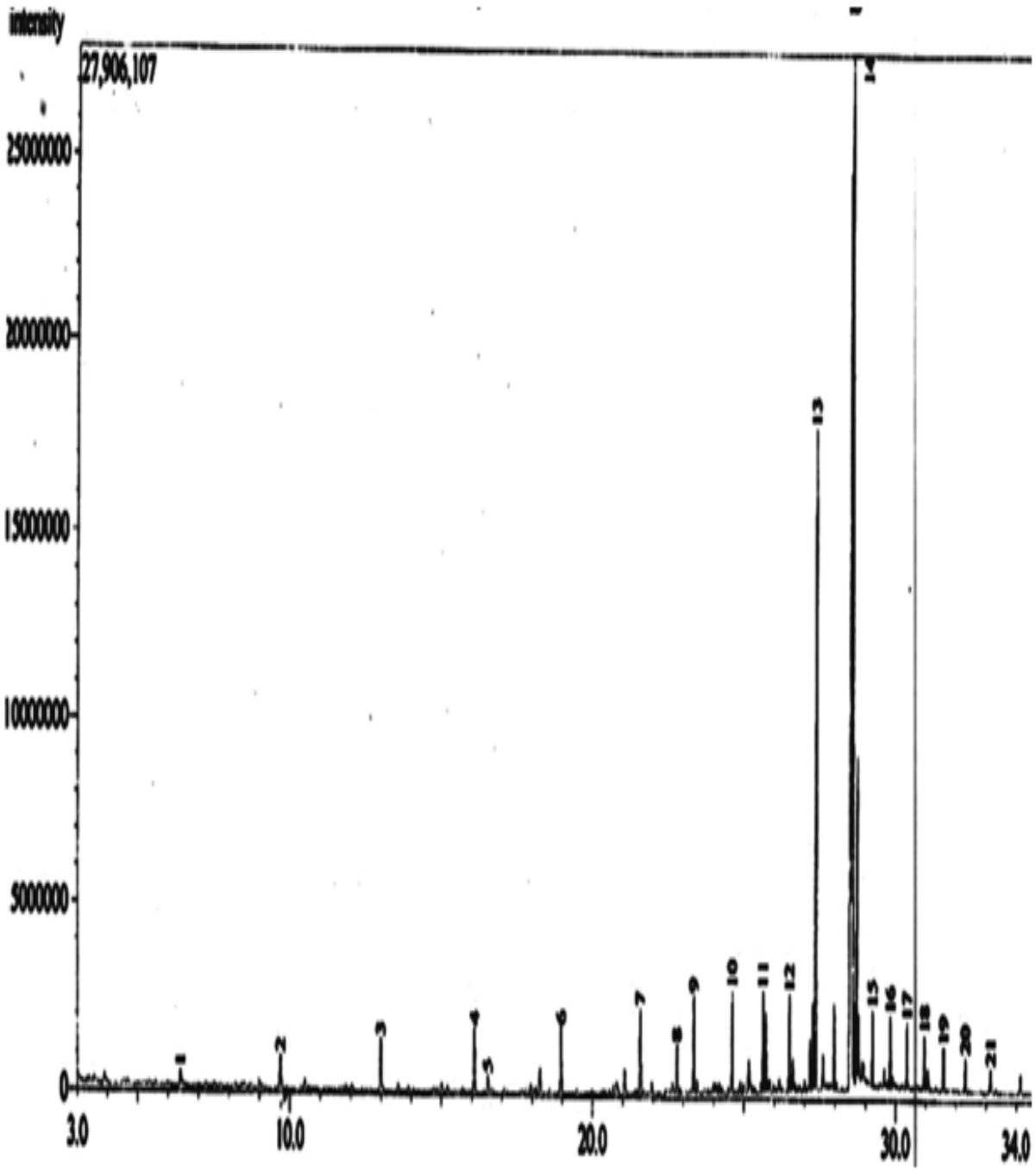



Figure 4.11: Gas Chromatograhic Fingerprints of Bonny Light Crude Oil Recovered from Flasks Inoculated with Mixed culture of *Burkholderia cepacia* and *Corynebacterium kutsheri* after 5days of Incubation.

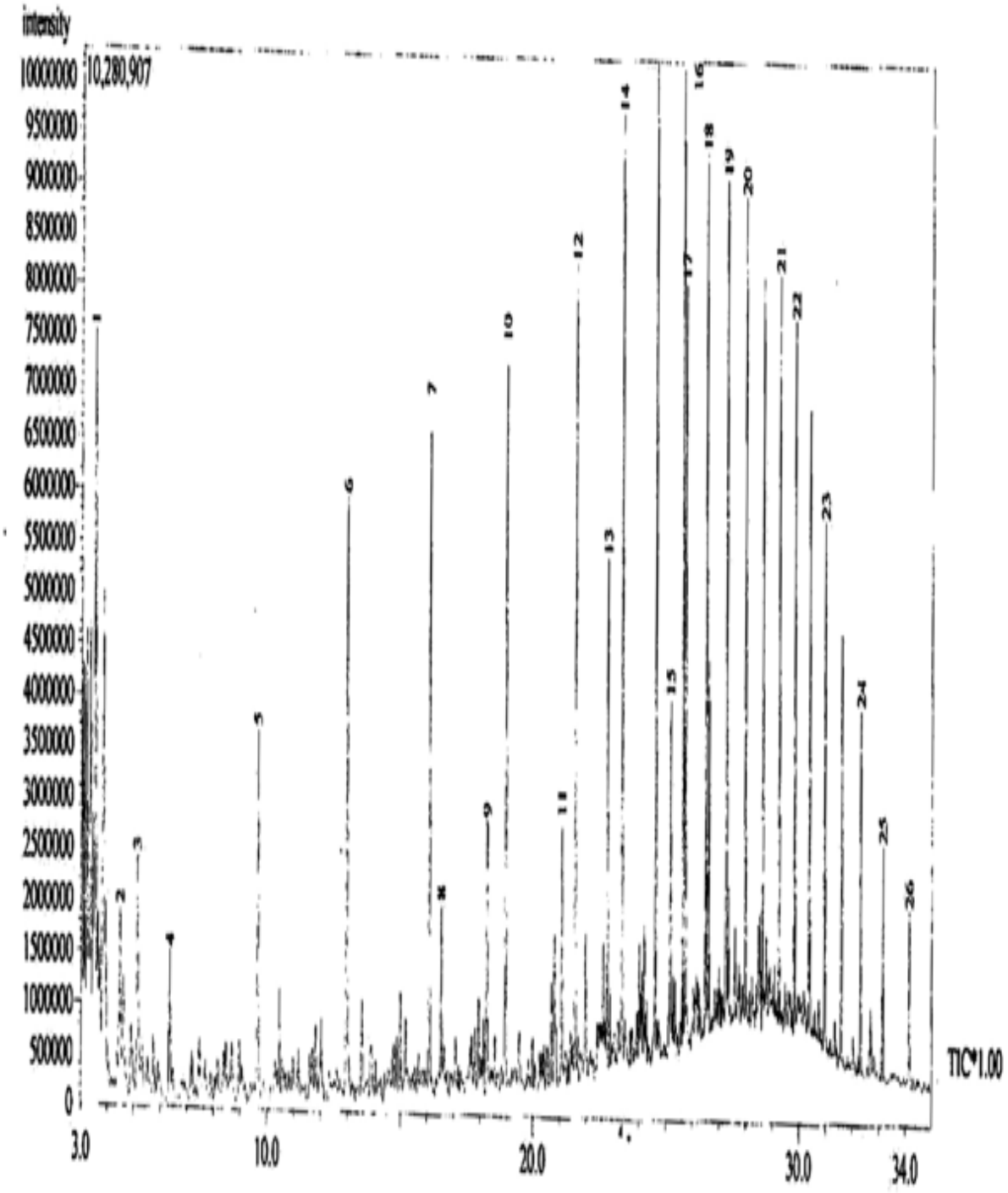



**Figure 4.12: Gas Chromatograhic Fingerprints of Bonny Light Crude Oil Recovered from Flasks Inoculated with *Burkholderia cepacia* after 10days of Incubation.**

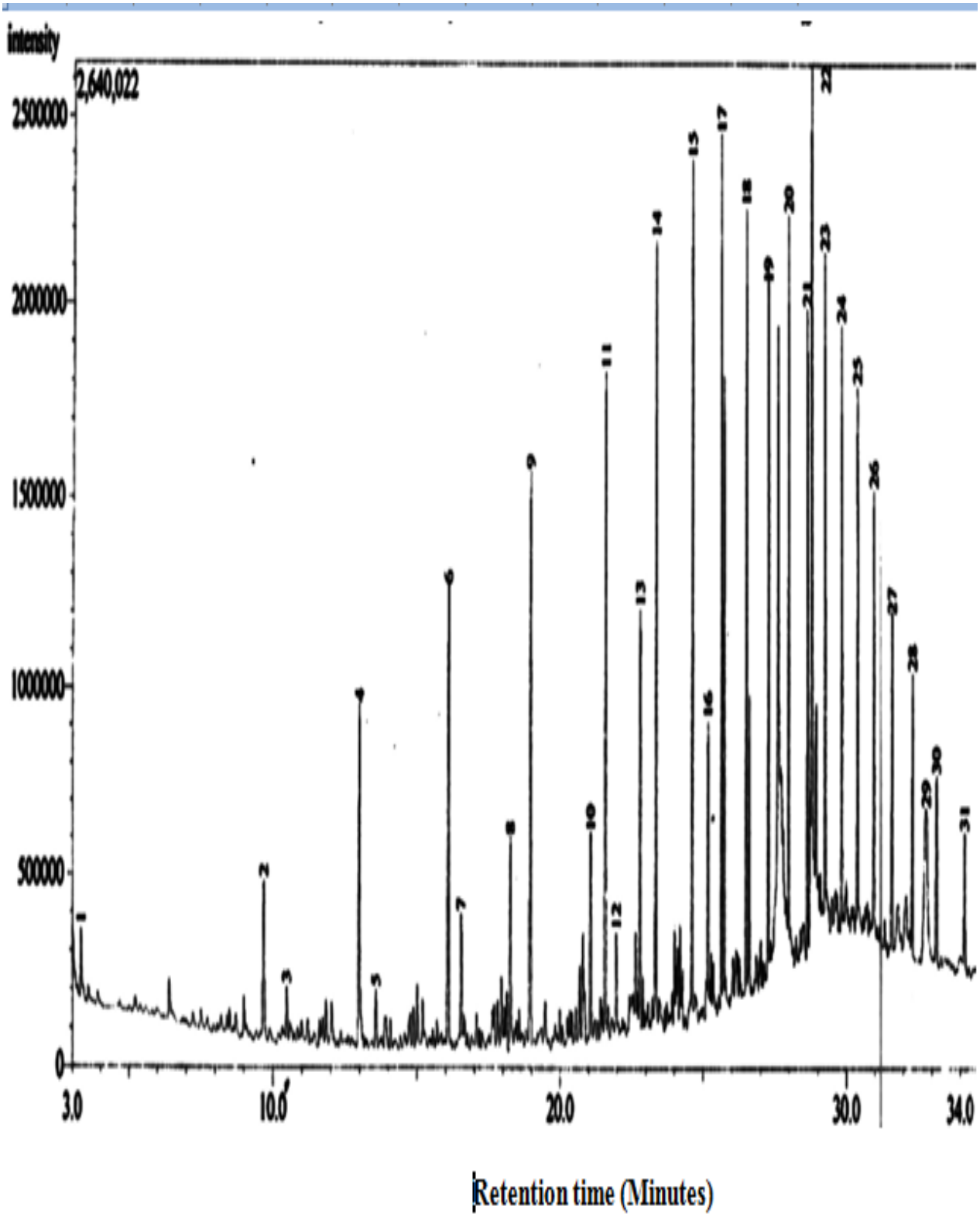



Figure 4.13: Gas Chromatograhic Fingerprints of Bonny Light Crude Oil Recovered from Flasks Inoculated with *Corynebacterium kutscheri* after 10days of Incubation.

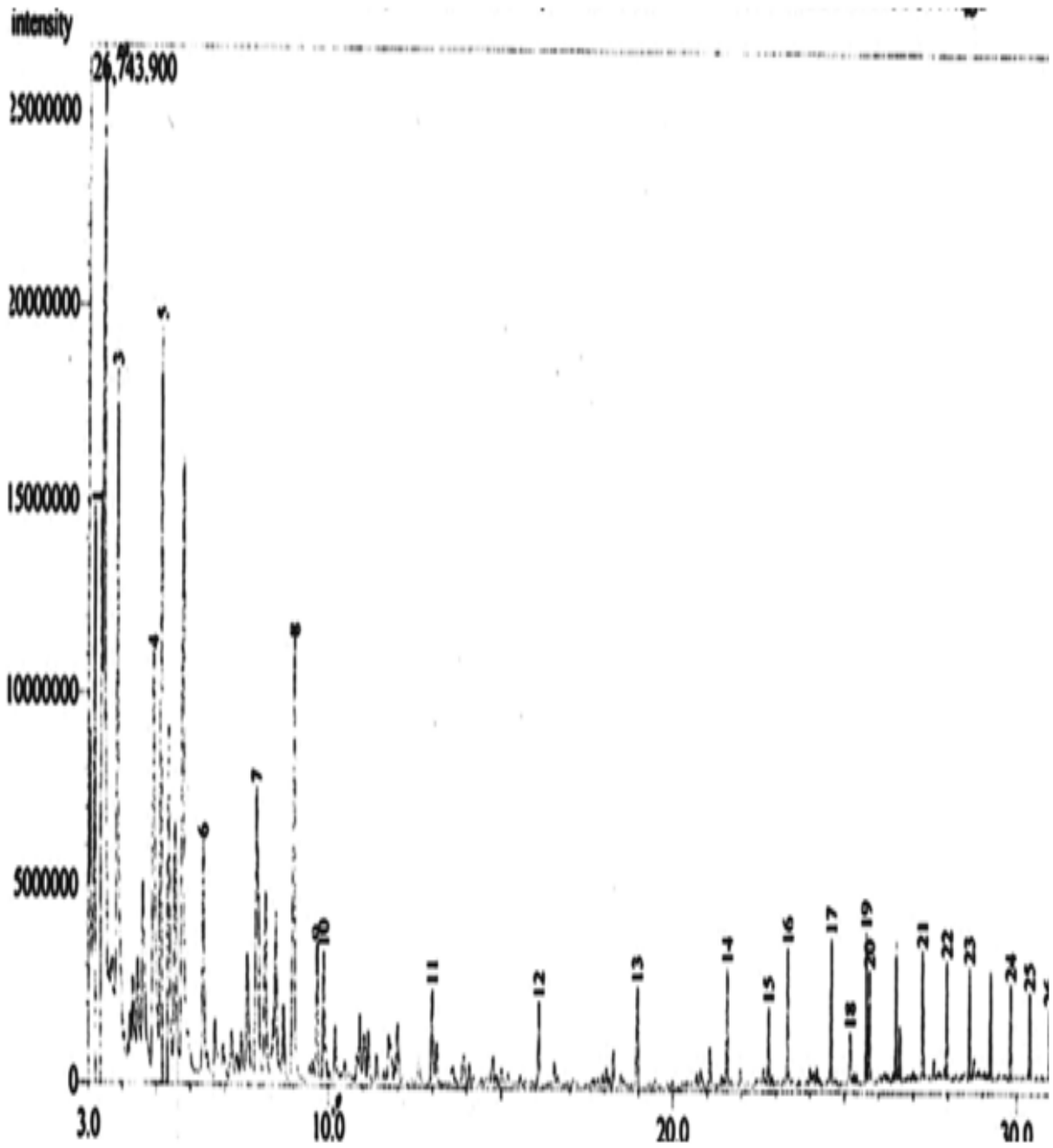



Figure 4.14: Gas Chromatograhic Fingerprints of Bonny Light Crude Oil Recovered from Flasks Inoculated with Mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri* after 10days of Incubation.

## 4.4 Dioxygenase Activities

To detect the presence of dioxygenase activity from the isolates and their consortium during biodegradation of bonny light crude oil.Whole-cell dioxygenases activities of the two isolates were determined by measuring the activity of catechol 1,2-dioxygenase as the formation of *cis,cis*-muconic acid. Additionally, by measuring the formation of 2-hydroxymuconic semialdehyde which is the product of catechol 2,3-dioxygenase activity.The result showed that the activity of catechol 1,2-dioxygenase was higher in *Burkholderia cepacia with specific activities* of 1.842 µmol $min^{-1}$ $mg^{-1}$ than in *Corynebacterium kutsheri* during degradation process while activity of catecho 1,2-dioxygenase was undetected in the shake flask medium.This indicated that the *Burkholderia cepacia* and *Corynebacterium kutscheri* utilize the *meta* pathway of catabolism Table 4.2.

## 4.5 Homology modeling and Molecular Docking

The sequence alignments between Catechol-2,3-dioxygenase from *Burkholderia cepacia* and *Pseudomonas putida* shows various conserved residues as shown in Fig.4.15 The symbol asterisks represents single fully conserved residues, colons: fully conserved strong groups, periods: fully conserved and dot: weaker groups. The results of Catechol 2, 3-dioxygenase alignment was validated using ERRAT and Verify3D (a**)** The overall quality factor predicted by ERRAT showed that the degree of compatibility was 81.311 % (b**)** The compatibility score for both template and target structure showed similar nature of the compatibility shown in Fig. 4.16. The list of substrates and their Hond Bond and Van derWaal interaction residues, free energy of Binding, estimated inhibition constant and substrate distance with Fe atom are given is shown in Table 4.3.

Table 4.2: Specific enzyme activity of crude oil degrading bacteria

| Isolates | Catechol-1,2-dioxygenase ($\mu mol\ min^{-1}\ mg^{-1}$) | Catechol-2,3-dioxygenase ($\mu mol\ min^{-1}\ mg^{-1}$) | Pathway |
|---|---|---|---|
| *Bulkholderia cepacia* | - | 1.842 | Meta |
| *Corynebacterium kutscheri* | - | 0.721 | Meta |

Fig. 4.17 shows three dimensional structure of Catechol-2,3-dioxygenase with catalytic Fe atom.( **a)** It contains 18 strands and 5 helices named from β1-β17 and α1-α5, in which the active site residues H150, H220 and E271 coordinating with Fe atoms are clearly pictured in the enlarged portion; (**b)** The four repeated βαβββ motifs are shown in each N and C-terminal domain. The molecular dynamics simulation results. (a**)** Potential energy graph illustrates the energetically stable nature of Catechol 2,3-dioxygenase; (b**)** RMSD profile explains the equilibration nature of the protein throughout the 10 ns MD simulation; (c**)** RMS fluctuation described the three different flexible regions during the production MD run shown in Fig.4.18. The molecular interaction of substrate with Catechol-2, 3-dioxygenase. **a)** Benzene **b)** O-xylene (c**)** Toluene (d**)** Fluorene (e**)** Napthalene and (f**)** Carbazole interacting with Fe atom in the active site of the enzyme. Fe atom shown in Spear format and the interacting residues are shown in stick format colored by heteroatom,whereas the substrates are shown in yellow color. The hydrogen bond formed between coordinated active siteof the enzyme and substrates are shown in Fig.4.19 and Fig. 4.20.

**4.6 Physicochemical Characterization and Bioremediation of Refinery Wastewater**

Physicochemical parameters of raw wastewater quality of Kaduna refinery including pH, $BOD_{5,}$ DO, TSS, TDS, BOD, COD, O&G and TPH were determined before and after treatment. Results indicated that the raw oily wastewater used during this study had extremely high levels of all the tested parameters. This makes it one of the strongest industrial effluents that has high pollution potential and dangerous effects on the receiving environments and also creates many difficulties in its treatment. The removal efficiencies of the bioremediation process were compared with Federal Environmental Protection Agency (FEPA-limit, Abuja, Nigeria) are shown in Table 4.4.

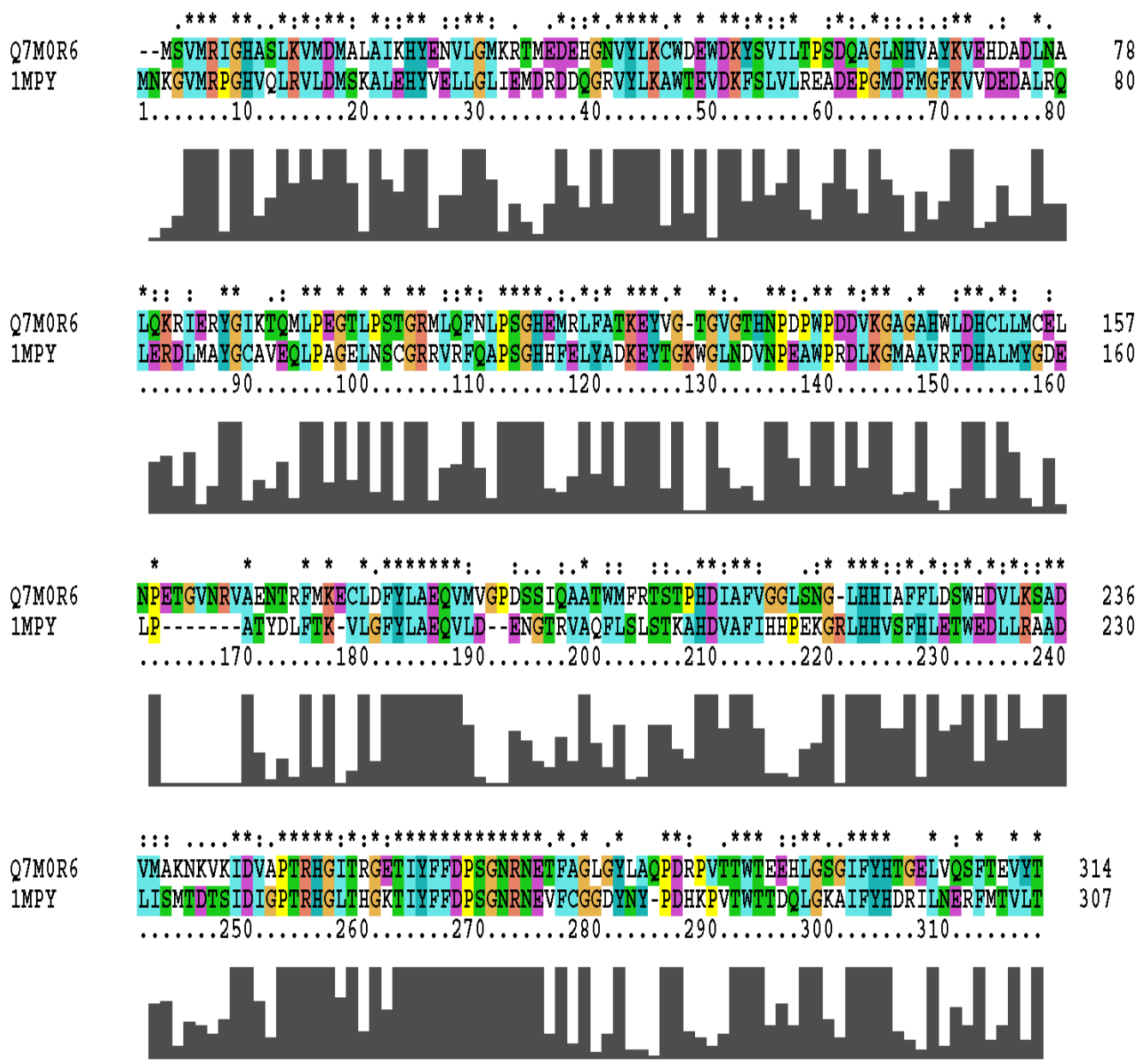



Fig. 4.15: The sequence alignments between Catechol-2,3-dioxygenase from *Burkholderia cepacia* and *Pseudomonas putida.*

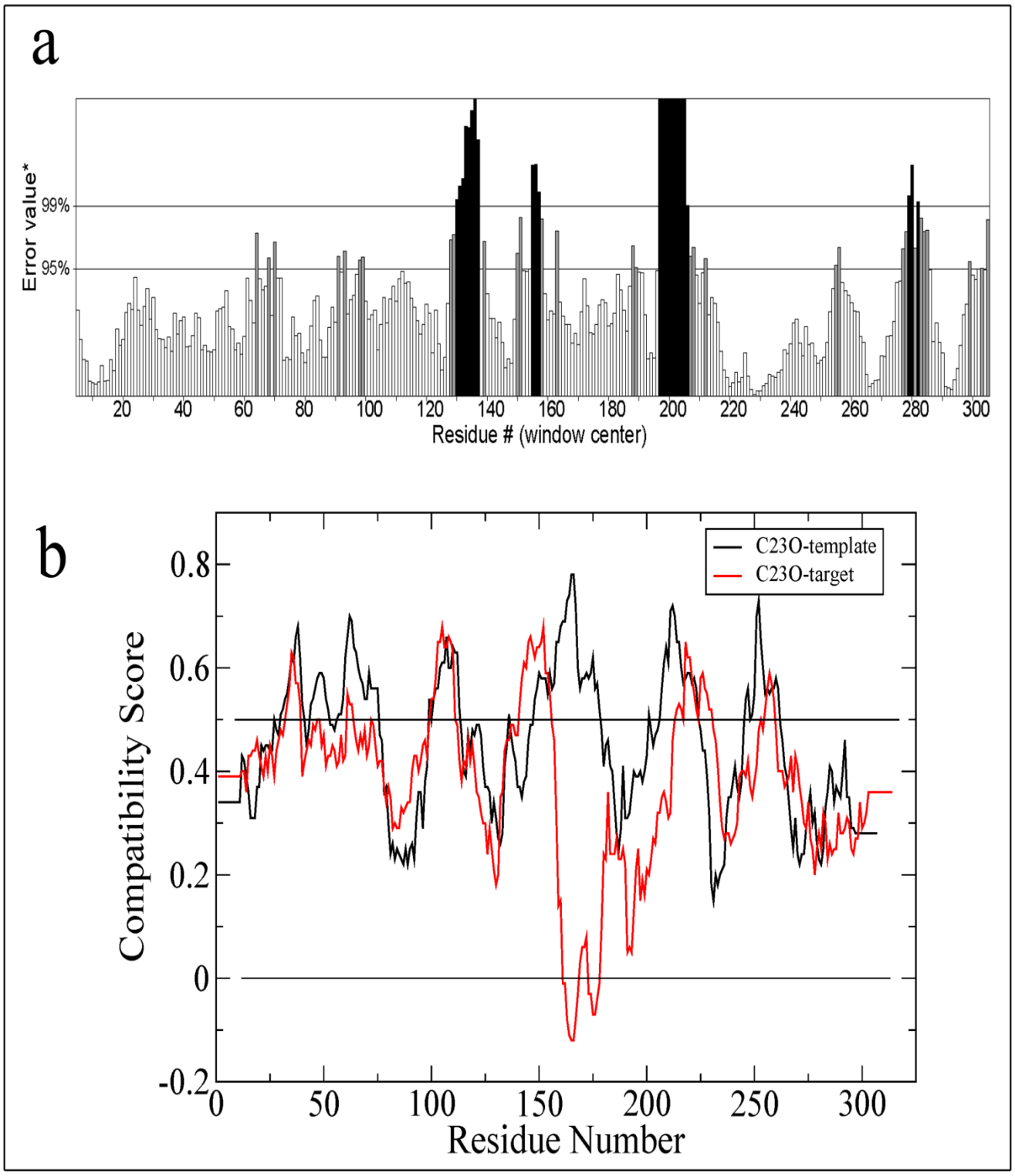



Fig. 4.16: The validation results of Catechol-2,3-dioxygenase using ERRAT and Verify3D.

**Table 4.3:** Molecular docking of substrate in the active site of Catechol-2,3-dioxygenase.

| C23O complex with substrate | HB and VdW interacting residues (Scaling factor 1Å) | Free energy of Binding (kcal/mol) | Estimated inhibition constant (Ki) | Substrate distance with FE2+ atom (Å) |
|---|---|---|---|---|
| Benzene | H150, W198, H206, H220, H252, I254, T255, Y261, E271 | -3.72 | 1.89 mM | 2.01 |
| O-xylene | H150, L152, W198, H206, H220, H252, I254, T255, Y261, E271 | -4.00 | 1.17 mM | 2.01 |
| Toluene | H150, L152, W198, H206, H220, H252, I254, T255, Y261, E271 | -4.21 | 822.96 uM | 1.99 |
| Fluorene | H150, L152, H206, H220, H252, I254, T255, Y261, E271, L276, I298, F309 | -4.57 | 450.47 uM | 1.79 |
| Naphthalene | H150, L152, W198, H206, H220, H252, I254, T255, Y261, E271, L276 | -4.63 | 405.72 uM | 1.75 |
| Carbazol | H150, L152, W198, H206, H220, H252, I254, T255, Y261, E271, L276 | -4.29 | 715.59 uM | 1.72 |
| Pyrene | H150, L152, V185, W198, H219, H220, V211, E271, I298, F309, Y313 | -3.93 | 1.31 mM | 7.54 |
| Dibenzothiophene | H150, L152, H206, H220, H252, I254, T255, Y261, E271, L276, I298, F309 | -3.50 | 2.73 mM | 1.77 |
| Anthracene | H150, L152, W198, H206, H220, H252, I254, T255, Y261, E271, L276, I298, F309 | -3.30 | 3.80 mM | 1.67 |
| Phenanthrene | H150, L152,W198, H206, H220, H252, I254, T255, Y261, E271, L276, I298, F309 | -2.14 | 27.04 mM | 1.76 |
| Biphenyl | H150, L152, W198, H206, H220,H252, I254, T255, Y261, E271, F273, L276 | -1.20 | 132.84 mM | 2.43 |
| Chrysene | H150, L152, W198, H220, H252, I254,T255, Y261, E271, F273, L276, I298, F309 | +6.61 | - | 1.8 |

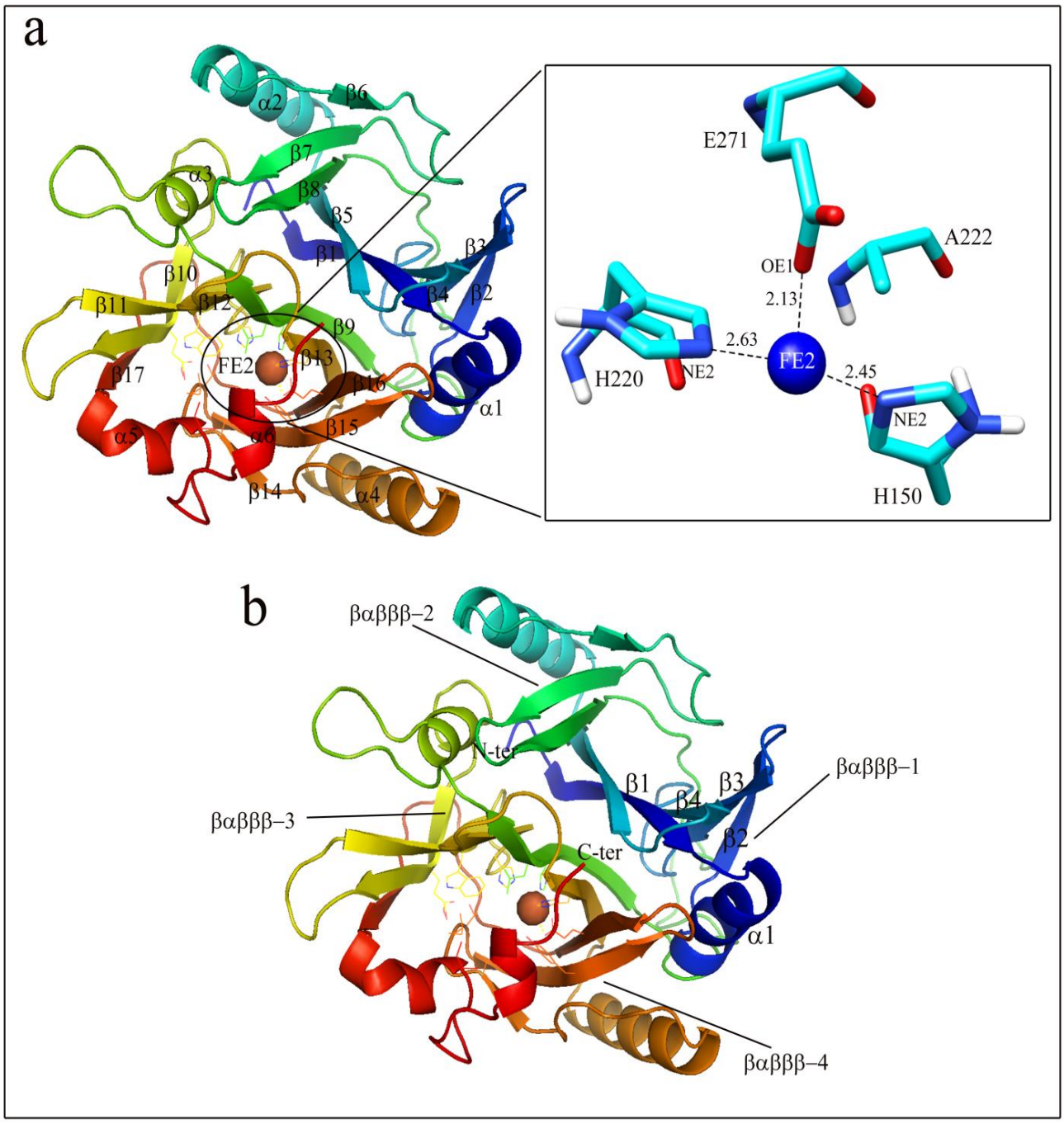



Fig.4.17: Three Dimensional Structure of Catechol 2,3-dioxygenase with Catalytic Fe atom

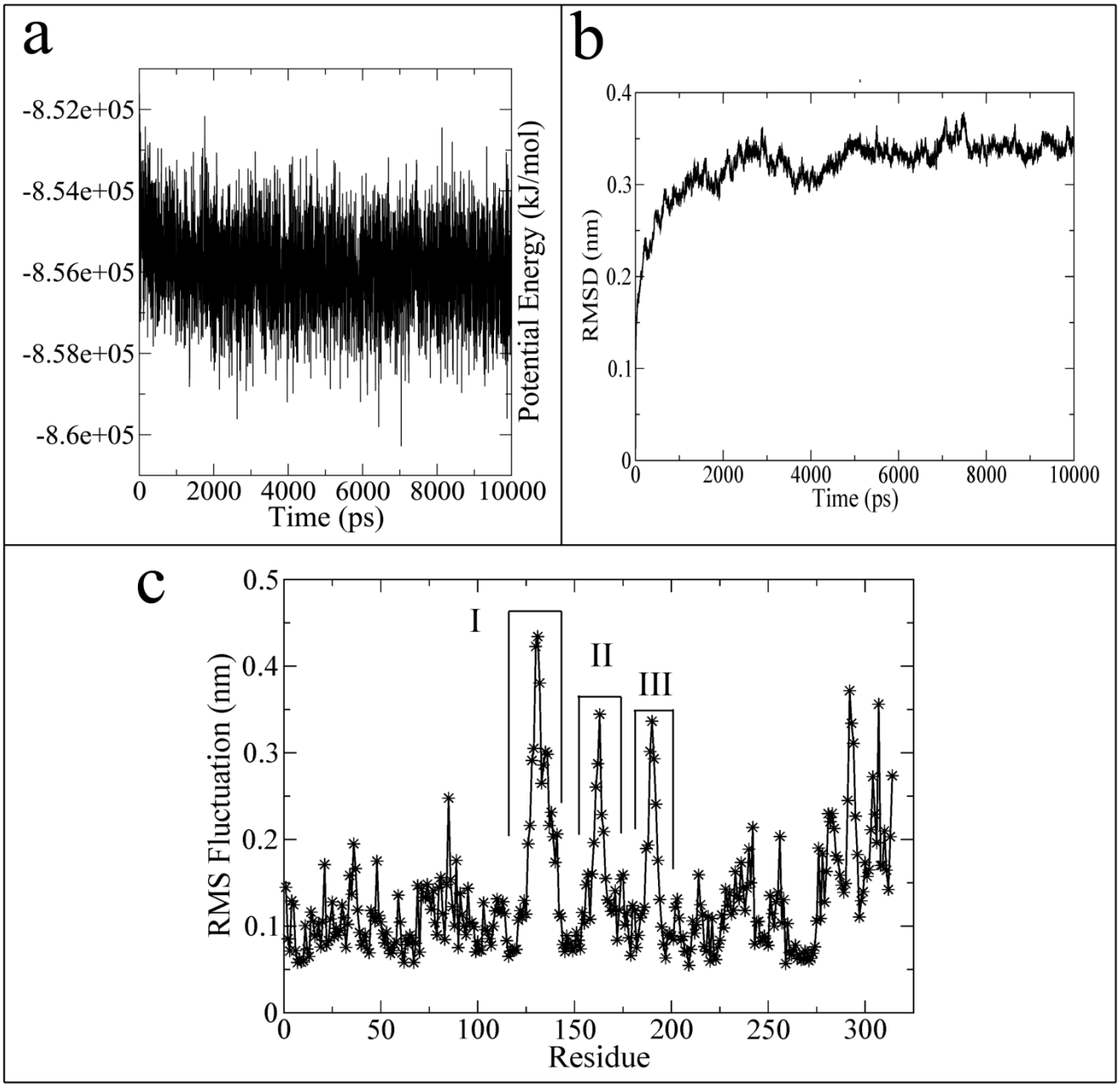



Fig. 4.18: The Molecular Dynamics Simulation Results

Keys:(a) Potential energy graph illustrates the energetically stable nature of Catechol 2,3-dioxygenase ( b) RMSD profile explains the equilibration nature of the protein throughout the 10 ns MD simulation. (c) RMS fluctuation describes the three different flexible regions during the production MD run.

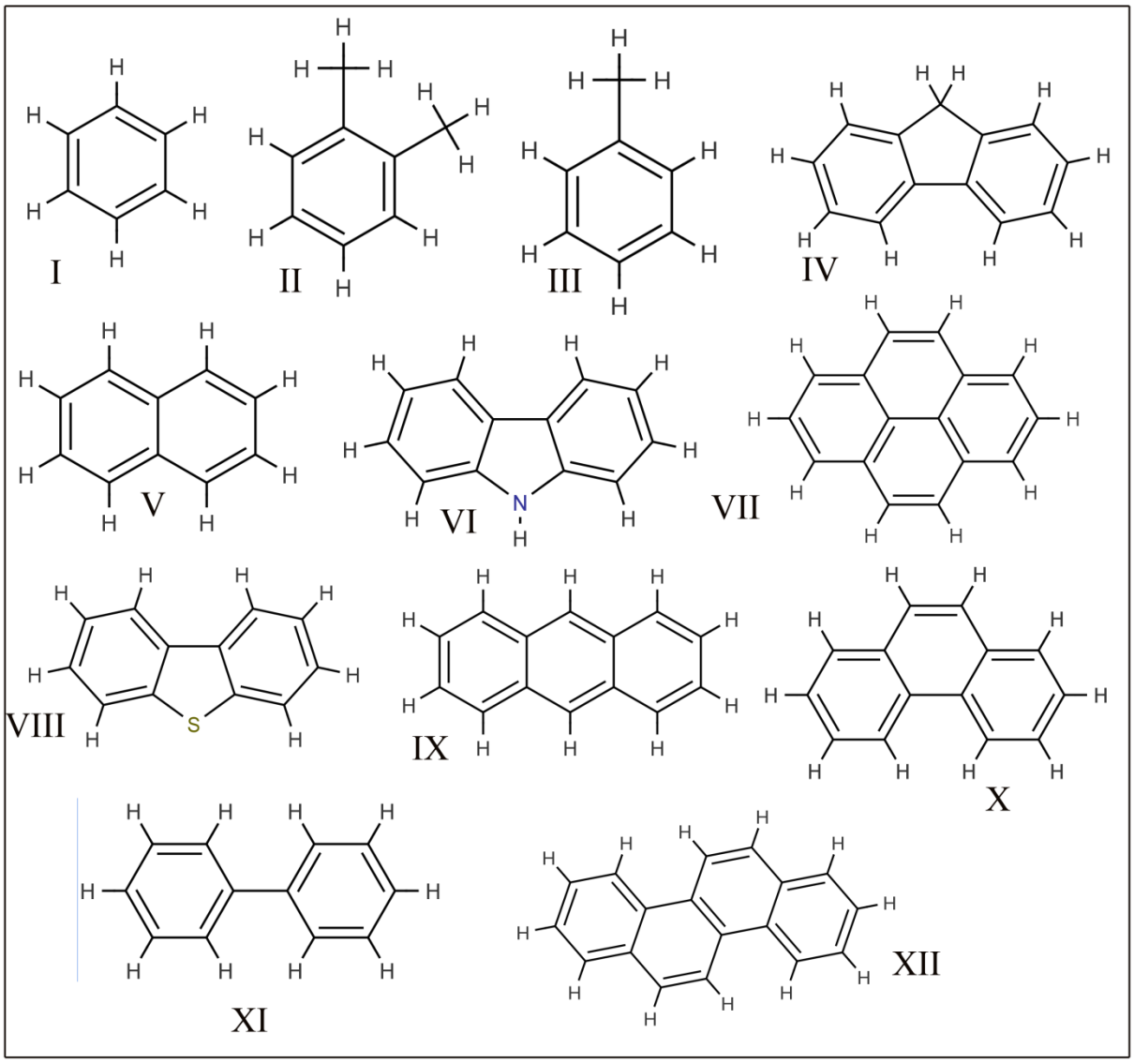



Fig. 4.19: The 2D Structure of Hydrocarbon Substrates used for Docking Analysis. Keys: (I) Benzene, ( II) O-xylene, (III) Toluene, (IV) Fluorene, (V) Naphthalene, (VI) Carbazol, (VII) Pyrene, (VIII) Dibenzothiophene, (IX) Anthracene, (X) Phenanthrene, (XII) Biphenyl and (XII) Chrysene.

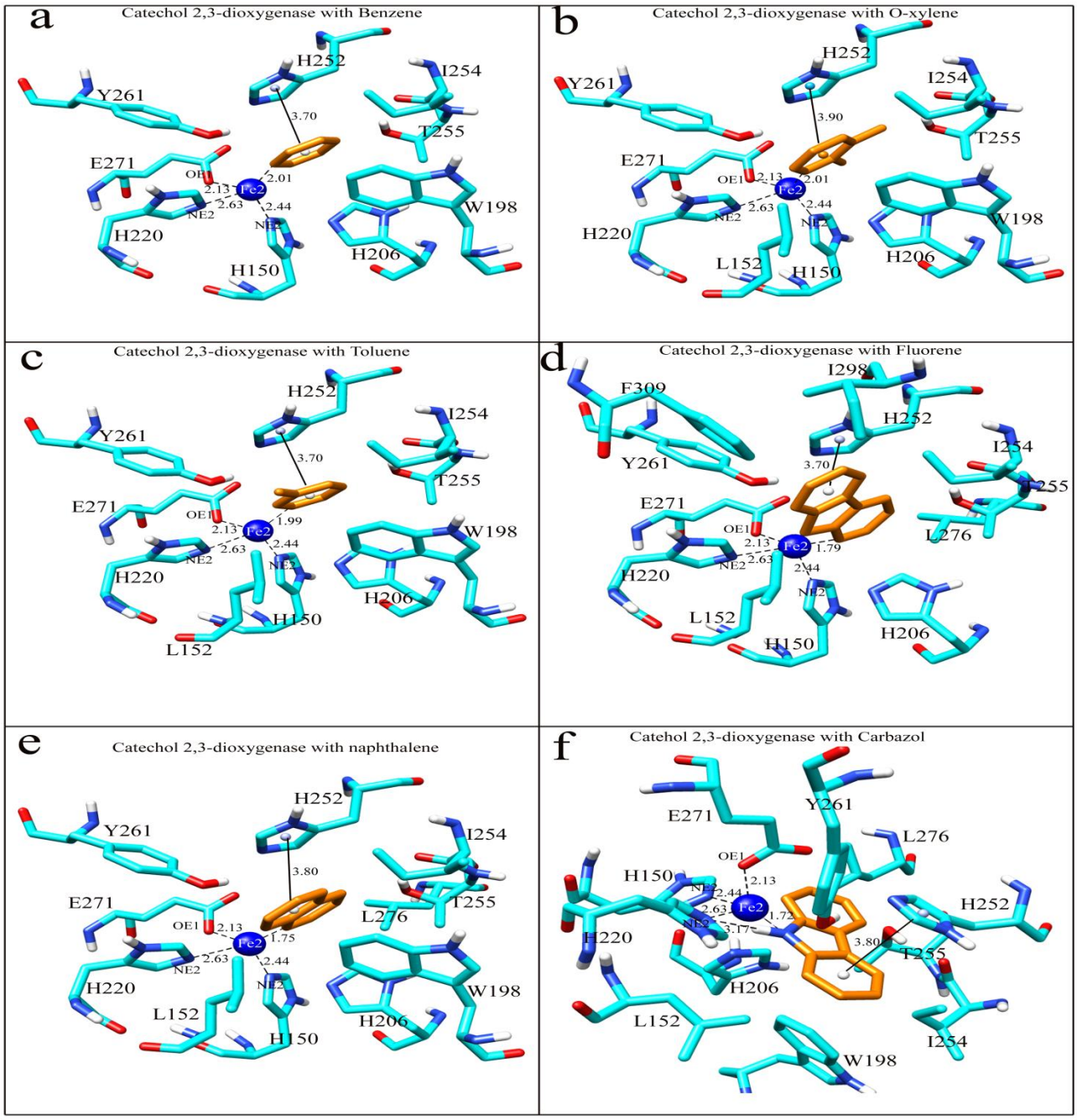



Fig.4.20: The Molecular Interaction of Substrate with Catechol-2,3-dioxygenase. Keys: (a) Benzene (b) O-xylene (c) Toluene (d) Fluorene (e) Napthalene and (d) Carbazole interacting with Fe atom in the active site.

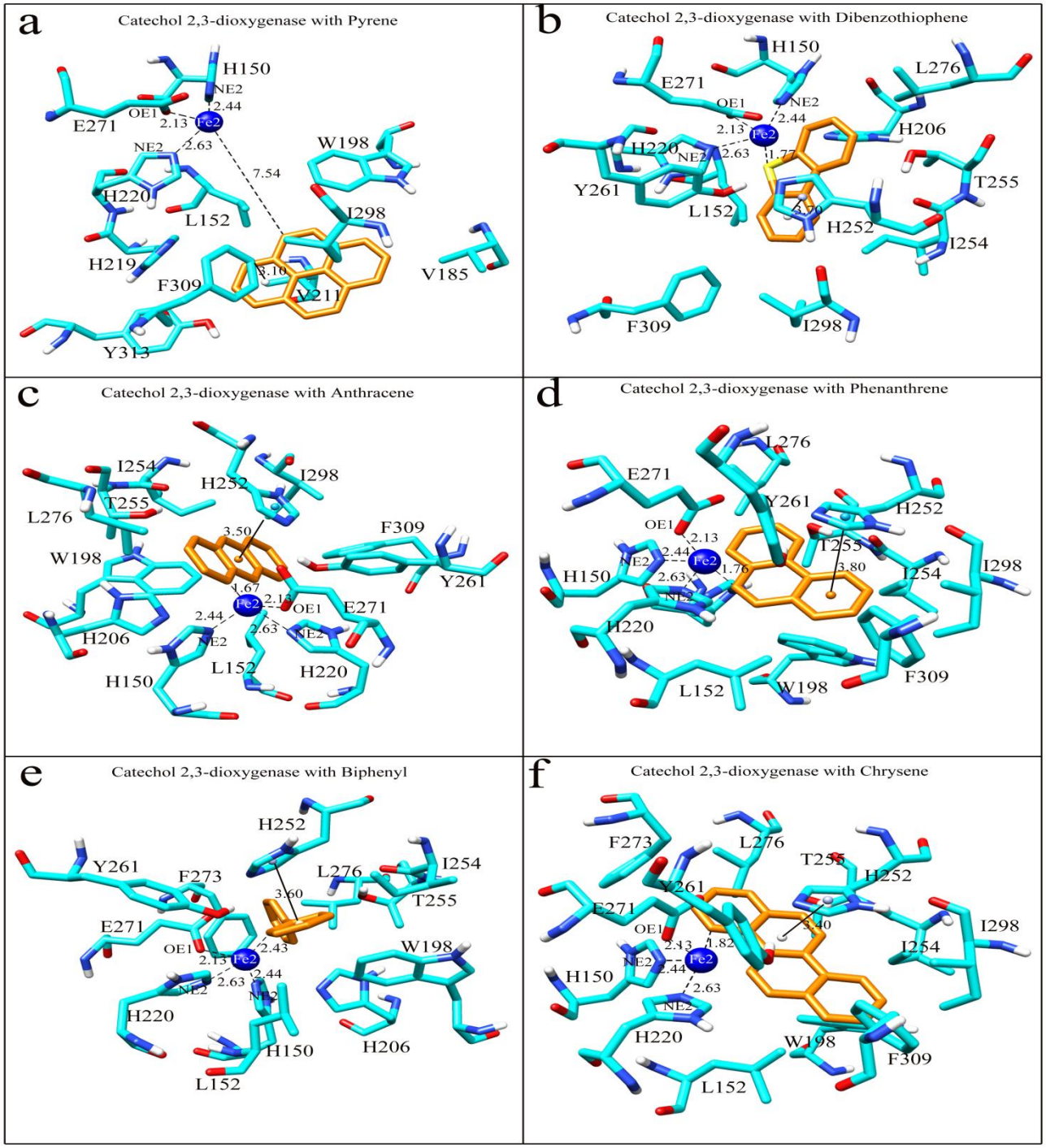



Fig.4.21:The Molecular Interaction of Substrate with Catechol-2,3-dioxygenase. Keys: (a) Pyrene, b) Dibenzothiophene, c) Anthracene, d) Phenanthrene, e) Biphenyl and f) Chrysene interacting with Fe atom in the active site.

**Table 4.4:** Physicochemical Characterization of Raw and Treated Wastewater of Kaduna Refining & Petrochemical Company (KRPC), Kaduna, Nigeria, compared with Federal Environmental Protection Agency (FEPA, Abuja, Nigeria)-Limit

| Parameters | Raw wastewater (mg/l) | 5 days | 10 days | 15 days | 20 days | FEPA-LIMIT |
|---|---|---|---|---|---|---|
| PH | 8.00 ± 0.25 | 7.55±0.62 | 7.23±0.46 | 6.72± 0.45 | 6.55± 0.61 | - |
| $BOD_5$ (mg/l) | 236 ± 0.40 | 176± 0.36 | 148± 0.32 | 96 ± 1.39 | 14 ± 0.23 | 10 |
| COD (mg/l) | 875 ± 3.92 | 211± 1.78 | 169± 1.62 | 111± 4.33 | 25.46±0.46 | 100 |
| TDS (mg/l) | 446.4 ± 5.25 | 384± 2.32 | 295± 1.43 | 201± 2.22 | 187 ± 1.58 | 200 |
| Phenol (mg/l) | 68.13 ± 0.36 | 42.16±0.92 | 21.71±0.43 | 10.81±1.12 | 0.32±0.03 | 10 |
| TPH (mg/l) | 4.12 ± 0.56 | 2.20 ± 0.36 | 2.01±0.082 | 1.67 ± 0.32 | 0.88 ± 0.12 | 10 |
| Oil & Grease (mg/l) | 43.20 ± 2.67 | 7.3 ± 0.52 | 6.12 ± 0.43 | 4.63 ± 0.65 | 2.53±0.94 | 10 |
| Nitrate | 1.87 ± 0.042 | 1.71 ± 0.63 | 1.68 ± 0.08 | 1.60 ± 0.10 | 0.62± 0.011 | 0.05 |
| Sulphate | 32.18 ± 0.61 | 24.61±0.52 | 19.62±0.94 | 14.21±0.51 | 8.13± 0.52 | 2.02 |
| Lead ($pb^{2+}$) | 0.04 ± 0.73 | 0.021±0.002 | 0.01 ± 0.10 | NIL | NIL | 0.05 |
| Iron ($Fe^{2+}$) | 4.30 ± 0.042 | 2.28±0.32 | 1.243±0.43 | 1.003±0.01 | 0.181±0.52 | |

FEPA-Limit, Federal Environmental Protection Agency; $BOD_5$, biochemical oxygen demand; TDS, total dissolved solids; TPH, total petroleum hydrocarbon; COD, chemical oxygen demand. Each value is a mean of 3 replicates±standard error.

Table 4.5: Comparison of mean values of the parameters from day zero (raw wastewater) to day 20.

| Duration (days) | Mean ± SEM | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **pH** | **BOD** | **COD** | **TDS** | **Phenol** | **TPH** | **Oil/Grease** | **Nitrate** | **Sulphate** | **Lead** | **Iron** |
| 0 | $7.99 \pm 0.08^{a}$ | $236.00 \pm 3.46^{a}$ | $873.00 \pm 9.29$ | $446.80 \pm 5.20^{a}$ | $66.43 \pm 3.30^{a}$ | $4.22 \pm 0.24^{a}$ | $43.31 \pm 1.45^{a}$ | $1.85 \pm 0.07^{a}$ | $32.28 \pm 0.27^{a}$ | $0.040 \pm 0.006^{a}$ | $4.30 \pm 0.01^{a}$ |
| 5 | $7.55 \pm 0.36^{ab}$ | $174.00 \pm 5.86^{b}$ | $209.67 \pm 7.54^{b}$ | $373.00 \pm 6.08^{b}$ | $40.33 \pm 1.00^{b}$ | $2.21 \pm 0.20^{b}$ | $1.77 \pm 0.27^{b}$ | $1.77 \pm 0.27^{a}$ | $24.53 \pm 0.37^{b}$ | $0.021 \pm 0.000^{b}$ | $2.25 \pm 0.16^{b}$ |
| 10 | $7.08 \pm 0.41^{ab}$ | $146.33 \pm 4.41^{c}$ | $167.67 \pm 1.86^{c}$ | $284.33 \pm 5.36^{c}$ | $21.67 \pm 0.20^{c}$ | $2.10 \pm 0.24^{b}$ | $6.17 \pm 0.21^{bc}$ | $1.66 \pm 0.05^{a}$ | $19.68 \pm 0.49^{c}$ | $0.013 \pm 0.003^{b}$ | $1.23 \pm 0.03^{c}$ |
| 15 | $6.76 \pm 0.23^{c}$ | $95.67 \pm 4.33^{d}$ | $103.67 \pm 4.06^{d}$ | $204.67 \pm 4.70^{d}$ | $10.88 \pm 0.12^{d}$ | $1.70 \pm 0.16^{b}$ | $4.59 \pm 0.41^{cd}$ | $1.62 \pm 0.04^{a}$ | $14.22 \pm 0.29^{d}$ | $0.000 \pm 0.000^{c}$ | $1.00 \pm 0.00^{d}$ |
| 20 | $6.54 \pm 0.36^{c}$ | $16.00 \pm 1.16^{e}$ | $24.25 \pm 0.68^{e}$ | $190.67 \pm 2.73^{d}$ | $0.47 \pm 0.11^{e}$ | $0.94 \pm 0.14^{c}$ | $2.62 \pm 0.02^{d}$ | $0.61 \pm 0.02^{b}$ | $8.17 \pm 0.27^{e}$ | $0.000 \pm 0.000^{c}$ | $0.18 \pm 0.01^{e}$ |
| F-ratio | 3.666 | 400.936 | 400.936 | 356.707 | 492.007 | 284.625 | 36.095 | 559.734 | 15.513 | 712.818 | 31.362 |
| p = value | 0.045* | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.001** | < 0.001** |

* = significant difference exists at p = 0.05.

** = significant difference exists at p = 0.01.

Mean values with different superscripts in the same column are significant different. Mean values were separated using Duncan Multiple Range Test (DMRT).

The Physicochemical characteristics of treated Kaduna refinery wastewater using immobilised mixed culture of *Burkholderia cepacia* and *Corynebacterium kutscheri* are presented in Table4.4

**4.6.1 pH**

The pH value in all the sampling stations as presented in Table 4.4 ranged from 6.55 ±0.61 (mg/l) to 8.00± 0.25(mg/l). Raw wastewater recorded the highest pH value 8.00±0.25 followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days with mean values of 7.55±0.62 (mg/l),7.23±0.46 (mg/l), 6.72± 0.45 (mg/l), 6.55± 0.61 (mg/l), respectively. Duncan Multiple Range Test (DMRT) comparison at ($P<0.05$) showed that the values were not significant in 5 and 10days treated wastewater but significantly diferent in 15days and 20 days treated wastewater. However the values were all within permissible limit as specified by FEPA except raw wastewater as shown in Table 4.5.

**4.6.2 Biological Oxygen Demand**

The mean Biological Oxygen Demand in both raw wastewater and all immobilized cell treated wastewater as shown in Table 4.4 ranged from 236 ± 0.40(mg/l) to 14 ± 0.23(mg/l). raw wastewater recorded the highest value of Biological Oxygen Demand with mean 236 ± 0.40(mg/l) followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days with mean values of 176± 0.36(mg/l),148± 0.32(mg/l),96 ± 1.39(mg/l) and 14 ± 0.23(mg/l), respectively.Mean values of $BOD_5$ found to reduce with increase in treatment duration.Duncan Multiple Range Test (DMRT) comparison at ($P<0.001$) showed that the mean values were statistically significant. However the mean values of raw wastewater and

immobilized cell treated cells were above permissible limit as specified by FEPA as shown in Table 4.5.

### 4.6.3 Chemical Oxygen Demand

The mean Chemical Oxygen Demand levels in both raw wastewater and all immobilized cells treated wastewater as shown in Table 4.4 ranged from 875 ± 3.92 (mg/l) to25.46±0.46 (mg/l). Untreated wastewater recorded the highest level of Chemical Oxygen Demand with mean value 875 ± 3.92 (mg/l) followed by followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days with mean values of 211±1.78 (mg/l), 169± 1.62(mg/l), 111± 4.33(mg/l) and 25.46±0.46 (mg/l), respectively. Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values were statistically significant shown in table 4.5.However the mean values of 20days immobilized cell treated wastewater was below permissible limit as specified by FEPA as shown in Table 4.5.

### 4.6.4 Total dissolved solids (TDS)

The mean values of total dissolved solids in raw wastewater and immobilized cells treated wastewater shown in Table 4.4 ranged from 446.4 ± 5.25(mg/l) to 187 ± 1.58 (mg/l). Untreated wastewater recorded the highest total dissolved solids mean values 446.4 ± 5.25 (mg/l) followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days of treatment with mean values of 384± 2.32 (mg/l), 295± 1.43 (mg/l), 201± 2.22 (mg/l) and 187 ± 1.58(mg/l) respectively. Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values were statistically significant shown in table 4.5.However the mean values in all stations were all below permissible limit as specified by FEPA as shown in Table 4.5.

### 4.6.5 Phenol

The mean phenol concentration in raw wastewater and immobilized cell treated wastewater as shown in Table 4.4 ranged from 68.13 ± 0.36 (mg/l) to 0.32±0.03 (mg/l). Untreated wastewater recorded the highest concentration of phenol with mean values of68.13 ± 0.36 (mg/l) followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days of treatment with mean values of 42.16±0.92 (mg/l), 21.71±0.43 (mg/l) 10.81±1.12 (mg/l) and 0.32±0.03 (mg/l), respectively. Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values were statistically significant shown in Table 4.5. However, the mean values for 20days immobilized cell treated wastewater were below permissible limit as specified by FEPA as shown in Table 4.5.

### 4.6.6 Total Petroleum Hydrocarbon (TPH)

The mean total petroleum hydrocarbon in raw refinery wastewater and immobilized cell treated wastewater as shown in Table 4.4 ranged from 4.12 ± 0.56 (mg/l) to 0.88 ± 0.12 (mg/l). Raw wastewater recorded the highest total petroleum hydrocarbon with mean values of 0.88 ± 0.12 (mg/l) followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days of treatment with mean values of 2.20 ± 0.36(mg/l), 2.01±0.082(mg/l),1.67 ± 0.32(mg/l) and 0.88 ± 0.12 (mg/l), respectively. Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values were statistically significant in 20days treated wastewater while, 5days, 10days and 15days streated wastewater were not statistically different from each other shown in Table 4.5. However, the mean value for 20days immobilized cell treated wastewater was below permissible limit as specified by FEPA as shown in Table 4.5.

### 4.6.7 Oil and Grease

The oil and grease concentration in raw refinery wastewater Table 4.4 ranged from 43.20 ± 2.67 (mg/l) to 2.53±0.94 (mg/l). Untreated raw wastewater recorded the highest concentration of oil and grease with mean values 43.20 ± 2.67 (mg/l) followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days of treatment with mean values of 7.3 ± 0.52(mg/l), 6.12 ± 0.43(mg/l),4.63 ± 0.65(mg/l) and 2.53±0.94(mg/l), respectively.Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values were statistically significant However the value for 20days treated wastewater was above permissible limit as specified by FEPA.

### 4.6.8 Nitrate (mg/l)

The mean levels of nitrate concentration in raw refinery wastewater shown Table 4.4 ranged from 1.87 ± 0.042 (mg/l) to 0.62± 0.011 (mg/l).Untreated wastewater recorded the highest concentration of nitrate concentration with mean value 1.87 ± 0.042 (mg/l) followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days of treatment with mean values of 1.71 ± 0.63(mg/l), 1.68 ± 0.08 (mg/l),1.60 ± 0.10 (mg/l) and 0.62± 0.011(mg/l), respectively. Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values of raw wastewater and other mean values up to 15days treated wastewater were significantly different. However the value for 20days treated wastewater was not also significantly high but still above permissible limit as specified by FEPA shown in Table 4.5.

### 4.6.9 Sulphate ($SO_4^{-2}$)

The mean levels of sulphate in all the raw wastewater and Immobilized cell treated wastewater shown ranged from 32.18 ± 0.61 (mg/l) to 8.13± 0.52 (mg/l). Raw wastewater recorded the highest concentration of nitrate with mean value 1.87 ± 0.042 (mg/l) followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days of treatment with mean values of 24.61±0.52 (mg/l), 19.62±0.94 (mg/l),14.21±0.51 (mg/l) and 8.13± 0.52 (mg/l), respectively. Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values were statistically significant. Meanwhile, mean value for 20days treated wastewater found above permissible limit as specified by FEPA shown in Table 4.5.

### 4.6.10 Lead

The concentration of lead in all the raw wastewater and Immobilized cell treated wastewater shown in Table 4.4 ranged from 0.04 ± 0.001 (mg/l) to 0.01 ± 0.001(mg/l). Raw wastewater recorded the highest concentration of lead with mean value of 0.04 ± 0.001 (mg/l) followed by Immobilized cell treated wastewater after 5days and 10days of treatment with mean values of 0.021±0.002(mg/l) and 0.01 ± 0.10 (mg/l), respectively.However, no lead was found after 15days and 20days of bioremediation process. Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values were statistically significant.

### 4.6.11 Iron (Fe)

The mean levels of iron in all the raw wastewater and Immobilized cell treated wastewater shown in Table 4.4 ranged from 4.30 ± 0.042 (mg/l) to 0.181±0.52 (mg/l). Raw wastewater recorded the highest concentration of nitrate with mean value 1.87 ± 0.042 (mg/l)

followed by Immobilized cell treated wastewater after 5days,10days,15days and 20days of treatment with mean values of 2.28±0.32(mg/l),1.243±0.43(mg/l), 1.003±0.01(mg/l) and 0.181±0.52 (mg/l)**,** respectively. Duncan Multiple Range Test (DMRT) comparison at P<0.01 showed that the mean values were statistically significant. Meanwhile, mean value for 20days treated wastewater found below permissible limit as specified by FEPA shown in Table 4.5.

**4.7 Bioremediation of refinery wastewater using Transconjugant**

Transconjugant of Burkholderia cepacia and Corynebacterium kutsheri obtained through triparental mating was used for bioremediation process of raw refinery wastewater for 10days.Oil and grease, Phenol, COD, % of oil degraded and sulphides were taken at every two days interval to asses the whole process.The mean values of varied between 4.27±0.31 to 31.1±1.21 ( mg/l), 1.06 ± 0.82 to 28.4±1.23( mg/l), 68±4.54 to 512±4.21( mg/l), 14±2.12 to 96±4.67 (%), 4.2±2.32 to 26±2.43( mg/l) in Oil & Grease , Phenol, COD, Oil degraded and sulphides, respectively between $2^{nd}$ and $10^{th}$ days of treatment.Duncan Multiple range test comparison at P<0.01 showed that the mean values for tested parameters were statistically significant.As the time of treatment increased, mean values of parameters reduced while, % of oil degraded increased shown in Table 4.6.

**4.8 Plasmid profiling of the isolates**

The gel electrophoresis result of the isolated plasmid-DNA from *Burkholderia cepacia* and *Corynebacterium kutscheri* showed the two isolates harboured mega plasmid each. Further investigation via curing of plasmids to confirm the position of catabolic traits in the isolates.Both isolates failed to grow on the oil agar indicating that their catabolic traits was located on the plasmid rather than being chromosomally borne as shown in Figure 4.22.

Table 4.6: Bioremediation of refinery wastewater using Transconjugants

| Time (days) | Oil & Grease (mg/l) | Phenol (mg/l) | COD (mg/l) | Oil degraded (%) | Sulphides ( mg/l) |
|---|---|---|---|---|---|
| Raw | 43.20±3.25[a] | 68.13±3.21[a] | 875±3.67[a] | NA | 26±2.43[a] |
| 2nd | 31.1±1.21[b] | 28.4±1.23[b] | 512±4.21[b] | 14±2.12[e] | 20 ±2.12[b] |
| 4th | 22.5±2.76[c] | 18.2±3.23[c] | 320±5.79[c] | 46±2.73[d] | 14±0.23[c] |
| 6th | 16.11±1.62[d] | 10.8±2.89[d] | 172±5.39[d] | 63±4.90[c] | 11±2.71[d] |
| 8th | 8.41±0.45[e] | 4.31±0.52[e] | 96±4.31[e] | 78±3.80[b] | 7.0±2.09[e] |
| 10th | 4.27±0.31[f] | 1.06 ± 0.82[f] | 68±4.54[f] | 96±4.67[a] | 4.2±2.32[f] |
| F-ratio | 506.284 | 797.432 | 12673.079 | 378.769 | 85.513 |
| P-value | <0.005** | <0.005** | <0.005** | <0.005** | <0.005** |

** = significant difference exists at p = 0.05.

COD, chemical oxygen demand; NA-: Not applicable

Values are presented as Mean ± SEM (n = 3). All groups are compared to each other at P < 0.05. Values with the same superscripts along the same column are not statistically different from each other.

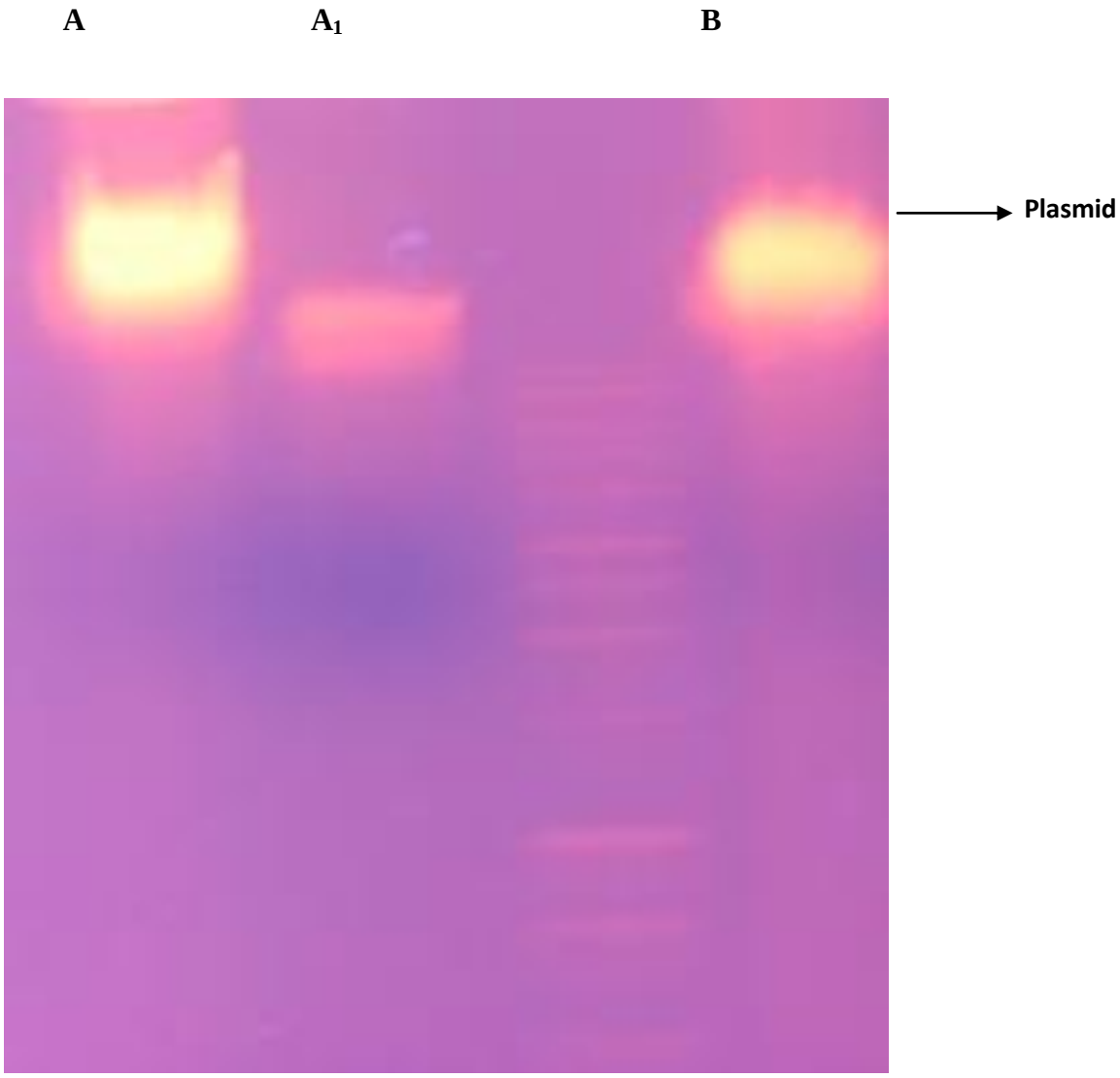



Fig. 4.22: Electrophoretic separation profiles of plasmids from cured and non-cured strains.
Lane A: *Corynebacterium kutscheri;* Lane A1: Cured *Corynebacterium kutscheri*; Lane B: *Burkholderia cepacia*

# CHAPTER FIVE

## 5.0 DISCUSSION

The presence or ability of the *Burkholderia cepacia* and *Corynebacterium kutscheri* to survive 5% concentration of crude oil during two weeks of enrichment technique is important and seemed to have led to the synthesis of enzymes involved in crude oil metabolism or changes in the genetic capacity of microbial species to maintain their ability to degrade crude oil (Leahy and Colwell, 1990). This finding showed that enrichment technique is useful for the isolation of various culturable oil degrading bacteria from contaminated sites for making consortia used in bioremediation (Okoh *et al*., 2001).The mechanisms employed by the autochthonous microorganisms to achieve this feat includes synthesis of inducible enzymes, mutations such as single nucleotide change or DNA re-arrangement that results in degradation of the compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-challenged community (Salam *et al*., 2011).

The occurrence of *Burkholderia cepacia* and *Corynebacterium kutscheri* in oil sludge is in line with the work of Okoh *et al. (*2001) who reported the degradation of heavy crude oil (maya) by *B. cepacia* strain RQI. There are other reports of *Burkhoderia* sp RQ1 capable of remarkable growth on aromatic fractions of crude oil (Nnamchi *et al., 2006; Somtrakoon et al., 2008;Obayori et al., 2009).* The biodegradation potentials of *Corynebacteium* sp have also been reported by Rahman *et al*. (2002); Thavasi *et al*. (2011) and Sathishkumar *et al*. (2008).

The slight increase after 72hours of incubation of Dehydrogenase activity in the culture filtrate can be attributed to the repression and induction of both inducible and constitutive enzymes involved in the biodegradation due to the presence of utilizable hydrocarbon substrates, while dip

drop in dehydrogenase activity 96hours may be as a result of non availability of utilizable hydrocarbon substrates that led to co-metabolism and increase in enzymatic activities.

As optical density and protein content increased dehydrogenase activity would also increase, this hypothesis is supported by the smaller increase in dehydrogenase which led to increase in bacterial growth and protein content.This is in agreement with the work of Bogan and Lamar (1996) who reported that the extracellular enzymes of organisms are produced in response to their growth phases. Wang *et al*. (2011) reported that consumption of easily-available organic-carbon in the experimental medium may directly result into high content of biomass and dehydrogenase activity.

A gradual increase in (FDA) observed in 96 hours can be attributed to high enzymatic activity, which may be as a result of the presence of non-degradable hydrocarbon compounds that led to decrease in both (DHA) and $CO_2$ production. Such increase in FDA can result into co-metabolism leading bioconversion of the substrate without releasing energy. It is reported that FDA is closely related to total C, total N, and total P and other nutrient indices in experimental medium and that it has close correlation between conversion status of organic matter and overall microbial activity (Schnurer and Rosswall, 1982; Bandick and Dick, 1999). Slight drop in 120hrs of incubation may be attributed to availability of utilizable hydrocarbon substrate as later increased progressively, such FDA production pattern not considered a representative measure of actual biodegradation because FDA did not decrease when all other parameters had decreased after mineralization, this is in agreement with work of Lee *et al*. (2008).

Protein content represents the total enzyme in the medium both inducible and constitutive enzymes. Sepahi *et al.* (2008) reported that the protein estimation is an effective method for monitoring the microbial population in an experimental medium.

It can be deduced that the growth dynamic of the isolate, dehydrogenase activities and protein contents are proportionate when there is availability of utilizable hydrocarbon compounds, therefore, the increase or decrease in protein concentration, Dehydrogenase activities and optical density during incubation period revealed that the isolates could utilize Bonny light crude oil as source of carbon and energy. Rahman *et al*. (2002), Sepahi *et al*. (2008) and Kumari *et al*. (2012) in their work, showed that the same pattern of relationship existed between the protein content and microbial growth during mineralization of crude oil.

The reduction in pH of the culture filtrate in the experimental flasks within the incubation period further confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes (Atlas and Bartha, 1971). It has been reported that microbial degradation of hydrocarbon often leads to production of organic acids and other metabolic products (Nwanchukwu and Ugodi, 1995; Okpowasili and James, 1995). Thus, the acids probably produced accounted for the reduction in the pH levels (Okpokwasili and Okorie,1988; Ijah and Ukpe,1992).

Biosurfactant biosynthesis occurred mainly between 24hours to 48hours of suggesting that biosurfactant is produced as primary metabolites accompanying cellular biomass formation which accounted for the flunctuation in pH during degradation, similar to Abu-Ruwaida *et al*. (1991). Ayanwu (2010) who reported that biosurfactant production is bacterial specific.

High emulsification index and low surface tension facilitate the bioavailability of hydrophobic compounds to bacteria cells and thus enhance crude oil degradation (Rosenberg and Ron, 1996;

Kumari *et al*., 2012).The rate of biodegradation is dependent on the chemico-physical properties of the biosurfactants and not by the effects on microbial metabolism (Franzetti *et al*., 2008; Franzetti *et al*., 2009; Saliu *et al*., 2009).

Malatova (2005) reported that the biometric flask experiments indicated that the $CO_2$ production rates correlated with GC data, which is somewhat complex and time-consuming to produce thus the $CO_2$ evolution rate, provided an essential criterion for evaluating the optional methods for determination of crude oil biodegradation. Gas-Chromatography and mass spectrometry (GC-MS) elucidated degradation pattern of Bonny light crude oil. Before the degradation the main peaks were $C_1$-$C_{35}$ in *Burkholderia cepacia* degraded n-$C_{34}$-n-$C_{35}$ over 5days while *Corynebacterium kutscheri* degraded n-$C_{28}$-n-$C_{35}$ and co-culture degraded n-$C_1$ to $_{n}$-$C_{12}$ n-$C_{22}$-n-$C_{35}$ and n-$C_{15}$ to n-$C_{21}$ within the same day. Little or no reduction in total petroleum hydrocarbon was noticed in both isolate while n-$C_{13}$ and n-$C_{14}$ were degraded to n-$C_1$ and n-$C_8$ in their co-culture filtrate. Bello (2007) and Sathiskumar *et al*. (2008) reported that microbes preferably degraded and metabolise n-$C_8$ to $_{n}$-$C_{15}$ n-akane followed by $C_{16}$-$C_{36}$ n-alkanes due to the simplicity of these hydrocarbon but this was contrary to this present finding, $C_{34}$-$C_{35}$ was observed to degrade within 5days while other lower hydrocarbons were still intact, this can be attributed to the autochthonous adaptation that allows microorganisms to be physically compatible with their habitat (Atlas and Bartha, 1998).

This present finding revealed that *Corynebacterium kutscheri* has slight higher ability to degrade Bonny light crude oil than *Burkhoderia cepacia* such difference in the rate of hydrocarbon degradation may be due to presence of different catabolic genes involved in hydrocarbon degradation in the bacterial species (Majid *et al*., 2008; Onuoha *et al*., 2011)

Britton (1984) and Adebusoye *et al.* (2007) also confirmed that individual organisms could only metabolize limited range of hydrocarbon substrates. This has led to the assertion that mixed culture exhibited superior degradative potential than pure culture strains (Leahy and Colwell,1990; Adebusoye *et al.,* 2007).This often results in adaptation of the autochthonous organisms to the pollutants due to selective pressure and acquisition of degradative abilities (Wackett and Hershberger, 2001; Salam *et al*., 2011)

Several reports (Obire, 1988; Amund and Nwakaye, 1993, Amund *et al*., 1993; Facundo *et al.,* 2001; Kulwadee *et al*., 2001) have confirmed microbial consortia as better degraders than pure isolates. In a culture, some species utilize intermediates of degradation of the original hydrocarbon produced by other members of the culture leading to a complete degradation of the oil (Atlas, 1981; Facundo *et al*., 2001). Thus, a mixed culture is a better innoculum for bioremediation. The observation of diauxic growth phenomenon in the complex mixture of petroleum is expected as petroleum consists of linear as well as aromatic compounds which usually required different enzymes and biodegradation pathways. In similar study, Rahman *et al*. (2002) also showed degradation of oil up to 78% after incubation of samples for 20 days inoculated with the bacterial consortium. Singh *et al*. (2013) also demonstrated 70% degradation of oil from the wastewater after 10 days of incubation.

The utilization of the oil resulted in increase in both Optical density ($OD_{6oo)}$, protein content and dehydrogenase activities evidenced from the reduction in pH of the culture confirmed by chemical changes of the hydrocarbon substrates which must have been caused by microbial enzymes and this agreed with various studies (Atlas and Bartha, 1971; Oboh *et al*.,2006; Sepahi *et al*., 2008).Dehydrogenase was found to be synthesized in proportionate to growth and protein content.The enzyme activity is regulated through product inhibition, covalent

modification and feedback inhibition (Brock and Madigan,1991). Biodegradation of petroleum hydrocarbon generally relies upon the cooperation of more than a single bacterial species. This is particularly true when complete mineralization of the hydrocarbons to $CO_2$ and $H_20$ is desired. A pure bacterial culture may not have the metabolic capability to readily degrade certain compounds or to have the biomass necessary to degrade the compounds rapidly enough. Consortium of mixed populations with overall broad enzymatic capacities may be required to achieve total degradation of the petroleum.

The higher biodegradation rate and microbial activity observed in the mixed culture may be related to shift in importance of one metabolic pathway over another possibly as a result of microbial synergism. The assumption that, more than one hydrocarbon compound degradation pathway exists in different microbial species and therefore, it is possible that individual bacteria able to degrade more than one aromatic substrate will have more than one pathway for their metabolism (Shringfellow and Artken, 1995). This assertion is contrary to the present phenomenon (quorum sensing). According to Williams (2007) who reported that *B.cepacia* produced N-acetylhomoserine lactone an inducer that can stimulate quorum sensing mechanism in response to environmental challenges. Since number of group of enzymes involved in complete mineralization of crude oil cannot be quantified, therefore, in this present finding the kind of synergism exhibited is neither protocooperation nor succession. GC- finger prints showed the same number of hydrocarbons was degraded throughout the experiment in their axenic culture while in their mixed culture degree of degradation was higher

However, the hypothesis that might explain this synergistical activities of these bacteria is quorum sensing phenomenon such that the *Corynebacterium kutscheri* was able to intercept and transduce the quorum sensing signal produced by *Burkholderia cepacia* to control physiological

interaction leading to gene expression that resulted into synthesis of various enzymes involved in mineralization of Bonny light crude oil.

Both isolates produced surface active agents which might have positively affected the uptake of hydrocarbons. For these reasons, mixed culture exhibited high degradation potential. There is no single strain of bacteria with the metabolic capacity to degrade all the components found within crude oil. In nature, biodegradation of a crude oil typically involves a succession of species within the consortia of microbes present (Malik and Ahmed, 2012; Battikhi, 2014). Degradation of petroleum involves progressive or sequential reactions, in which certain organisms may carry out the initial attack on petroleum constituents. This produce intermediate compounds that are subsequently utilized by a different group of organisms, in the process that results in further degradation (Karick, 1977).

A combination of bacterial strain with broad enzymatic capabilities is required for active extensive degradation of crude oil. Oil degrading mixed cultures can be constructed by combining a number of strains with known degradative capabilities (Komukai *et al*., 1996; Foght *et al*., 1996), from the same ecological niche such that all the interaction that exist between them in their ecosystem can be maintained to enhance mineralization.

The modeled Catechol-2,3-dioxygenase from *Burkholderia cepacia* comprises N-terminal (1-147) and C-terminal domains (148-314). Like Catechol-2,3-dioxygenase from *Pseudomonas putida* mt-2 (Kita *et al.*, 1999), these two domains are structurally similar to each other and separated by a local two fold axis. The N-terminal domain contains totally eight β stands (β1, β2, β3, β4, β5, β6, β7 and β8) and two α helices (α1 and α2), whereas, the C-terminal domain contains nine β stands (β9, β10, β11, β12, β13, β14, β15, β16 and β17) and four α helices

(α3, α4, α5 and α6). There is one Fe-binding site present in the β barrel of the C-terminal domain which is located at the end of a large pocket appropriately to accept the substrate molecules. The residues H150 Nε2, H220 Nε2 and E271 Oε1 coordinate the Fe atom by the distance of 2.45, 2.63 and 2.13 Å respectively (Fig.4.17a). These residues lie in the β9, β13 and β16 respectively and are conserved not only in Catechol-2,3-dioxygenase family but also in 2,3-dihydroxybiphenyl-1,2-dioxygenases. As like Catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 and 2,3 dihydroxy biphenyl 1,2-dioxygenases Han *et al.*(1995) and Senda *et al.* (1996) reported that this protein also has notable structural feature as each domain contains of an eight-stranded half-opened β barrel. The superimpose of template and target Catechol-2,3-dioxygenase shows that it has 0.173 Å RMSD and the C terminal loop region of N158-V166 is varied 3.78 Å from the template structure. The half-opened β barrel in each N and C-terminal domains consist of two repeated βαββββ motifs which is shown in Fig.4.17(b).

In order to evaluate the stability of the Catechol-2,3-dioxygenase from *Burkholderia cepacia* the molecular dynamics simulation of 10 ns was performed. The minimum potential energy (Fig. 4.18a) of the protein $-8.60324e^{+05}$ ($7038^{th}$ ps) proves that the modeled structure was energetically stable during the production MD run. The RMSD profile shows that the first 2000 ps of the protein backbone rise to equilibrate and remains quite stable until the $10^{th}$ ns of production MD with the maximum RMSD of 0.35 nm. This longest period of equilibration confirms the prolonged stability of the protein (Fig.4.18b). Moreover, the RMS fluctuation of Catechol-2,3-dioxygenase was also calculated and described in Fig.4.18c, showed it has three flexible regions. The first flexible region is a coil (124-140), which connects the N-terminal and C-terminal domain and fluctuate more with a maximum RMSD deviation of 0.45 nm. The second flexible region is the loop one (160-165), which connects the β9 and α3 with a maximum

RMSD ranges from 0.25 to 0.35 nm. Whereas the third one is very important β-hairpin motif which connects β10 and β11 (185-193), in combination with C-terminal tail region this motif covers the open side of the C-terminal domain bowl structure which offers a way for the substrate binding near to the Iron atom (Fig. 4.18c). This flexibility furnishes open and close conformation of the C-terminal domain bowl with the RMSD deviation of 0.35 nm.

The Fe atom containing Catechol-2,3-dioxygenase from *Burkholderia cepacia* has the cabability of degrading the hydrocarbons like Benzene, O-xylene, Toluene, Fluorene, Naphthalene, Carbazol, Pyrene, Dibenzothiophene, Anthracene, Phenanthrene, Biphenyl and Chrysene (Fig. 4.19) from crude oil. The present study described the catalytic mechanism of Catechol- 2,3-dioxygenase with its substrate through molecular docking analysis. Accordingly, the above mentioned substrates were prepared and docking with the active site of Catechol-2,3-dioxygenase and analyzed. From the Table 4.3, each Hydrogen Bond (HB) and Van der Waals interaction made between substrate and enzyme, along with Free energy of binding, estimated inhibition constant (Ki) and substrate distances with Fe atom were shown. The docking results revealed that the benzene, O-xylene, Toluene, Fluorene, Naphthalene, Carbazol, Pyrene, Dibenzothiophene, Anthracene, Phenanthrene, Biphenyl makes HB and VdW interaction with the active site residues of H150, L152, W198, H206, H220, H252, I254, T255, Y261, E271, L276 and F309 and shown the free energy binding of -3.72, -4.00, -4.21, -4.57, -4.63, -4.29, -3.93, -3.50, -3.30, -2.14 and -1.20 respectively. Chrysene shows positive energy of binding in the active site atom of Fe. Except Pyrene, all above-mentioned substrates made close contact with Fe atom by the distance ranges from 1.67 to 2.43 Å. In addition, the above-mentioned substrate except pyrene, all other made π-π stacking interaction with H252 by the distance of ranges from 3.40 to 3.90 Å (Fig. 20 and 21). The pyrene molecule is present 7.40 away from the Fe atom and

makes T-shaped stacking interaction with F309 with a distance of 3.10 Å. Moreover, the catalytic Fe atom retains their coordinate interaction with H150, H220 and E271 by the distance of 2.45, 2.63 and 2.13 Å respectively. All these docking results reveal that, except Chrysene, all other substrate has good free energy of binding to hold enough in the active site and makes strong VdW interaction with Catechol-2,3-dioxygenase. These results suggest that, the enzyme is capable of catalyzing the above-mentioned substrates.

The results of the analysis indicated that the Refinery wastewater was grossly polluted, and that if not properly treated before being discharged into receiving water bodies could be a major cause of pollutionif treatment plant is not efficient to remove pollutants to appreciable levels. Result from this study showed that pH values from both immobilized cell treated wastewater and raw wastewater were all below the permissible limit as specified by FEPA. Duncan multiple comparison at ($P<0.05$) showed that they were not statistically significant. In effect, continuous discharge could result in acid deposition in the recipient water body. pH values fall within the World Health Organization standard of 7.0 to 8.5 and the water quality ranges 6.5 to 8.5 for drinking water and water meant for full contact recreation, respectively (DWAF, 1996b; WHO, 1984; 1989). The EU also sets pH protection limits of 6.0 to 9.0 for fisheries and aquatic life (Chapman, 1996).

The levels of Biological Oxygen Demand were high in all stations than the permissible limit shown in Table 4.4; comparison of the mean values were also significant ($P<0.01$) using Duncan multiple comparison.This may be as a result of escape of organic matter from the biological treatment plant. Biological oxygen demand (BOD) test is useful in determining the relative waste loading and higher degree therefore indices the presence of large amount of organic pollutant and relatively higher level of microbial activities with consequent depletion of oxygen content. The

minimum amount of dissolved oxygen necessary for normal life in an aquatic environment is about 2mg/L (Attiogbe *et al*., 2007), and the discharge of waste water containing organic matter into water bodies results in the excess consumption of oxygen by the bacteria. This is in an attempt to oxidise the effluent, thus depleting oxygen from the water faster than it dissolves back into the water from the air (Attiogbe *et al*., 2007). This problem leads to the inadequate maintenance of higher life forms. In addition, oxygen availability is important because the end products of chemical and biochemical reactions in anaerobic systems often produce aesthetically displeasing colours, tastes and odours in water (Attiogbe *et al*., 2007).

The Chemical Oxygen Demand values recorded at all stations were all above permissible limits and standard set by FEPA (Table 4.4). Statistically the results were significant using Duncan multiple comparison at ($P<0.01$).All the mean values obtained for the period of 20days treatment.

Dissolved oxygen is an important factor used for water quality control. The effect of waste discharge on a surface water source is largely determined by the oxygen balance of the system and its presence is essential in maintaining biological life within a system (DFID, 1999). Dissolved oxygen concentrations in unpolluted water normally range between 8 and 10 mg/L and concentrations below 5 mg/L adversely affect aquatic life (DFID, 1999; Rao, 2005). DO standard for drinking purpose is 6 mg/L whereas for sustaining fish and aquatic life is 4-5mg/L (Rao, 2005). Both the BOD and COD tests are a measure of the relative oxygen-depletion effect of a waste contaminant. Both have been widely adopted as a measure of pollution effect. The BOD test measures the oxygen demand of biodegradable pollutants whereas the COD test measures the oxygen demand of oxidizable pollutants. The COD is a determinant of the level organic matter and carbon.

An indication of organic oxygen demand content of wastewater can be obtained by measuring the amount of oxygen required for its stabilization either as BOD and COD. Biological Oxygen Demand (BOD) is the measure of the oxygen required by microorganisms whilst breaking down organic matter. While Chemical Oxygen Demand (COD) is the measure of amount of oxygen required by both potassium dichromate and concentrated sulphuric acid to breakdown both organic and inorganic matters.

Higher levels of COD in the raw refinery waste water observed in this study is undesirable, continuous discharge of effluent has impact on the receiving water body to some extent and which may have negative effects on the quality of the freshwater and subsequently cause harm to the aquatic life especially fish, downstream (Morrison *et al.,* 2001). When this present result was compared with results of COD of the treated final effluent and receiving water bodies from developed countries, it was observed that the concentrations of COD differ as reported by UNEP (1993) and lower than permissible limit. According to Ogunfowokan *et al.* (2005), this increase in COD could be attributed to an increase in the addition of both organic and inorganic substance from the environment, as well as organic contaminant entering the systems from the municipal sewage treatment plants. In the same light, one observation agrees with the previous works of Fatoki *et al.* (2003) and Morrison *et al.* (2001) who reported that the contribution of COD to the effluent and receiving water bodies in Nigeria appears to be significant.

Total Dissolve Solid levels of untreated refinery wastewater and immobilized cell treated wastewater showed in (Table 4.4). Duncan's comparison at ($P<0.01$) showed that the values were significant. The high values of total dissolved solid in the untreated wastewater may be attributed to prolonged accumulation in the sewer without proper dilution.

Phenol contents in immobilized cell treated refinery wastewater were all below permissible limitafter 20days immobilized cell treated wastewater as specified by FEPA. Duncan's multiple comparison (at $P<0.01$) showed that the results were significant. However, Phenol is one of the major pollutants found in refinery effluents. It was found in large concentrations the raw wastewater. The major sources of phenol in the petroleum refinery wastewater are the thermal and catalytic cracking processes. The observed value for phenol was 68.13mg/L and 1.06mg/L in the Raw and Treated wastewater respectively. Its content in the treated sample was insignificantly higher than 0.5mg/l (FEPA, 1991) or 0.1mg/L (DPR, 1991) recommended for refinery effluents.

Phenols have been observed to be very toxic to fish and other aquatic organisms and has a nearly unique property of tainting the taste of fish if present in marine environment in concentration ranging from 0.1 to 1.0mg/l (de Bruin, 1976; Staples *et al.*, 1998). The toxic concentration for fishes may range from <0.1 to >100mg/l, depending on the chemical nature of the phenol, the fish species and the developmental stage, with embryo-larval stage being many times more susceptible than adults (De graeve *et al*.,1980).

Total petroleum hydrocarbons of untreated refinery wastewater and immobilized cell treated wastewater. The mean values varied between the 20days treated wastewater and Untreated wastewater $4.12 \pm 0.56$ (mg/l) to $0.88 \pm 0.12$ (mg/l). Duncan Multiple Range Test (DMRT) comparison at $P<0.01$ showed that the mean values were statistically significant in 20days treated wastewater. Petroleum hydrocarbons have been observed to be toxic to aquatic life. It has been observed (Pollino *et al*., 2003) that a water accommodated fraction of crude oil or dispersed crude oil water accommodated fraction increased the activity of gill citrate synthase LDH at a concentration of 14.5mg/l. lipophilic hydrocarbons have been observed to accumulate

in the membrane lipid bilayers of microorganisms and interfering with their structural and functional properties (Sikkema *et al.,* 1995). Briggs *et al.* (2002) concluded that oil containing hydrocarbons when discharged into water body can cause depletion of dissolved oxygen due to transformation of organic components into inorganic compounds, loss of that is important in food chain and eutrophication. Short term toxicity in fishes includes lymphocytosis, epidermal hyperplasia, haemorrhagic septicaemia (Beeby, 1993; Uzoekwe and Oghosanine,2011; Diya'uddeen *et al.*, 2011).

Oil and grease and TPH were found to be (13.20-4.27)mg/l and (4.12-0.88)mg/l in both raw waste water and treated waste water respectively after 20days of treatment. Joel and Amajuoyi (2009) reported that the high level of contamination with oil and grease poses a great concern and long term threat to all forms of life.

Oil and grease are sticky in nature; they tend to aggregate, clogging drain pipes and sewer lines, causing unpleasant odours and corroding sewer lines under anaerobic conditions (Chen *et al*., 1999; Xu and Zhu, 2004). They also interfere with unit operations in municipal wastewater treatment plants because they float as a layer on top of the water. They also stick onto pipes and walls consequently blocking strainers and filters (Xu and Zhu, 2004).

The most highly oxidized form of nitrogen compounds is commonly present in surface and groundwater because it is the end product of aerobic decomposition of organic nitrogenous matter. Unpolluted natural waters usually contain only minute amounts of nitrate (Jaji *et al.*,2007). In this study, the nitrate-N concentrations ranged between ranged from 1.87 ± 0.042 (mg/l) to 0.62± 0.011 (mg/l) significantly important ($P < 0.05$), as the mean values obtained were below the recommended limit for FEPA. Phosphate was also found to be low. However,

nutrient and phosphate are essential nutrients to plants life, but when found in excess quantities, stimulates excessive plant growth such as alga bloom (Igbinosu and Oko, 2009).

Heavy metal concentration such as iron and lead in the raw wastewater were reduced below the permissible limit after 20days of treatment as shown in Table 4.5. Higher concentration of iron and lead in the untreated wastewater could be attributed to slow dilution due to accumulation of the metals.Meanwhile, Lead usually arises from corrosion (Plumbosolvency) of pipes and lead solders of copper joints.Its solubility, governed by the formation of lead carbonates as pipe deposits, increases markedly as pH reduces below 8 due to decrease in equilibrium carbonate concentration (WHO, 2002).

The results obtained in the plasmid profiling showed that both isolates harbored plasmid. Thavasi *et al*. (2007) reported that the plasmids that have encoding the transformation of environmental pollutants are known as catabolic plasmids. The incidence of plasmids in oil degrading bacteria had been reported by many researchers. Mirdamadian *et al.*, (2010) demonstrated that the catabolic pathway which encode degradation routes of different aromatic and aliphatic hydrocarbons are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid.Similar research was carried out by Whyte *et al*. (1997) and Toledo *et al.* (2006) and who extracted plasmids from *Pseudomonas* and *Burkholderia* strains isolated from oil – contaminated soils in order to confirm whether the observed biodegradative potential in the isolates was plasmid or chromosomal mediated similar to the present work similar work was also carried out by Hardy (1993) Sheikh *et al*. (2003); Fuji *et al.* (1997) and Trevors (1986).

Failure of the cured isolates to grow on MBM supplemented with 2% crude oil after curing is an indication that the degrading gene is encoded in the plasmid not in the chromosomal DNA. The total loss of biodegradation ability after plasmid curing explains the role of plasmid in catabolic activity of hydrocarbon biodegradation, this indicates that the gene(s) responsible for crude oil degradation might have been cured, thus not allowing the colony to grow in oil agar (Kumar *et al.,* 2010). Our findings show that the abilities to mineralize crude oil by *Burkholderia cepacia* and *Corynebacterium kutscheri* were plasmid-encoded.Plasmidic DNA isolated from non-cured cultures showed that *Burkholderia cepacia* and *Corynebacterium kutscheri* harboured single plasmids. The total loss of biodegradation activity after plasmid curing over emphasizes the role of plasmids in catabolic activity of hydrocarbon biodegradation. Rheinwald *et al*. (1973) reported the loss of isobutyrate degradation after plasmid curing in *Pseudomonas putida*. Stuart-Keil *et al.* (1998) observed the loss of naphthalene degradation after plasmid curing in marine heterotrophic bacterial population. These findings supported the results of present study on loss of biodegradation ability after plasmid curing.Also Thavasi *et al*. (2007) reported in four species of hydrocarbonoclastic bacteria.

The ability to simulate plasmid transfer has been reported to have various applications in the studies of adaptive evolution, dissemination of antibiotic-resistant bacteria (Denap *et al.*, 2004), and the ability of microbial populations to degrade xenobiotics (Basta *et al*., 2004). However, transfer of a potentially catabolic plasmid from indigenous oil degrading bacteria to mixture of recipient strains from sludge, enhances the degradative capacity of transconjugants. Catabolic pathways, which encoded different aromatic hydrocarbon degradation routes, are

frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid (Coral and Karagoz, 2005).

There are alot of investigations on the effect of inoculation and subsequent transfer of catabolic genes on in situ biodegradation in soils and bioreactors as demonstrated in the present study, such as: diGiovanni *et al*. (1996) demonstrated transfer of the IncP-1L plasmid pJP4 from *Ralstonia eutropha* JMP134 to indigenous soil bacteria when very high concentrations of 2,4-D were added to the soil. In a similar study Bathe *et al.* (2004) showed the increase of 2,4-D degradation by conjugative transfer of pJP4 from an introduced donor strain to the bacterial community of a laboratory wastewater treatment system and demonstrated that adaptation of a microbial community to a xenobiotic compound can be accelerated by HGT of the respective catabolic genes.

On the whole, isolates used in this study were able to reduce the concentrating harzardous compounds associated with refinery waste water.This confirmes microbial consortia as better degraders than single culture as reported by several authors (Obire, 1988; Amund and Nwakaye, 1993; Facundo *et al*, 2001; Kulwadee *et al.*, 2001; Ndip *et al.*, 2008) and this microbialconsortium can be adopted in our refinery to reduce the incidence of hazardous pollutants in the river.

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# CHAPTER SIX

## 6.0 Summary, Conclusion and Recommendation

### 6.1 Summary

- This study demonstrated that the selective enrichment technique could be used for the isolation of hydrocarbon degraders from refinery sludge.
- Microbial activities and biodegradative potentials of the isolates confirmed that the measured parameters Dehydrogenase and Fluorescein diacetate synthesized in proportionate to total viable count and protein content which correlated with $CO_2$ evolution and GC chromatographs.
- The findings of this research work have also proven that the selected enzymes can serve as bioindicator for monitoring bioremediation of oil spills.
- This study demonstrated that the isolates harbour plasmid further investigation via curing of plasmids showed that the plasmid is the ultimate source of catabolic traits.
- Protein-ligand technique between Catecol-2,3-dioxygenase and hydrocarbons substrates revealed that, except Chrysene all other substrate have good free energy of binding to hold enough in the active site and made strong VdW interaction with Catechol-2,3-dioxygenase. These results suggested that the enzyme is capable of catalyzing the substrates.
- The mean values of physicochemical paramters of untreated refinery wastewater were all above the permissible limit, which was the major cause of worry and the treatment plant was not efficient enough to remove pollutants to acceptable limits which led to high concentrations of effluent in similar studies of refinery effluent .

- Immobilized cell treated wastewater and their transconjugants reduced the physicochemical parameters below permissible limit.
- It also revealed that refinery sludge harbours hydrocarbon degraders and that such organisms can be immobilised for reusable purposes and that they carry transmissible catabolic plasmid that can be acquired by other autochthonous bacteria with weak catabolic potentials.
- Furthermore, results showed that plasmid transfer occurred more readily between the donor organism and sludge-derived mixed culture recipients. This study also demonstrated that a stable array of indigenous plasmid hosts can be more effective remediators than an introduced inoculum.
- Plasmid mediated bioaugmentation resulted in the most rapid bioremediation of refinery wastewater.Therefore, bioaugmentation of wastewater using transmissible catabolic plasmid will enhance efficiency of the bioremediation by spreading the plasmid among indigenous microbial community either through horizontal gene transfer or transformation.
- In this particular study, augmentation of indigenous populations with catabolic genes was a more useful strategy than bioaugmentation with an introduced organism capable of carrying out the catabolic activity.

**6.2 Conclusion**

The results of the present study revealed that the organisms could be immobilised and employed in the bioremediation of KRPC wastewater to permissible limit prior to discharge into the receiving stream.The introduction of these strains in the process wastewater treatment plant

could be a plausible solution to the environmental pollution problem therein in the discharge of the KRPC effluent currently.

### 6.3 Recommendations

1. Careless disposal of untreated effluent into the observation pond without pretreatment should be discouraged.
2. The treatment plant should be efficient enough to remove pollutant to appreciable level.
3. Regulating agencies should impose direct charges on industrial effluents, as well as continuous monitoring and surveillance in order to ensure the protection of water resources from further degradation.

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# Appendices

**DEHYDROGENASE ACTIVITIES (1g fluorescein $ML^{-1}$ solvent $3hrs^{-1}$)**

| Hours | A | B | AB |
|---|---|---|---|
| 24 | $12.33\pm1.42^{f}$ | $17.00\pm1.73^{g}$ | $23.00\pm1.73^{g}$ |
| 48 | $21.00\pm1.73^{e}$ | $25.33\pm2.3^{f}$ | $37.33\pm2.03^{f}$ |
| 72 | $29.00\pm1.00^{d}$ | $31.67\pm2.03^{e}$ | $25.67\pm2.03^{g}$ |
| 96 | $32.00\pm1.73^{d}$ | $38.00\pm1.73^{d}$ | $72.00\pm1.73^{e}$ |
| 120 | $43.00\pm1.73^{c}$ | $52.00\pm1.73^{c}$ | $111.33\pm1.45^{d}$ |
| 144 | $54.00\pm1.73^{b}$ | $68.00\pm1.73^{b}$ | $167.33\pm2.03^{b}$ |
| 168 | $65.67\pm2.03^{a}$ | $72.00\pm1.73^{b}$ | $210.00\pm2.02^{a}$ |
| 192 | $70.33\pm2.03^{a}$ | $83.00\pm1.73^{a}$ | $121.00\pm1.73^{c}$ |
| F-ratio | 151.279 | 168.281 | 1381.696 |
| P-value | 0.000 | 0.000 | 0.000 |

Values are presented as Mean ± SEM (n = 3). All groups are compared to each other at $P < 0.05$. Values with the same superscripts along the same column are not statistically different from each other.

**Keys**: A-*Burkholderia cepacia*

B-*Corynebacterium kutscheri*

AB-Mixed culture

**FLUORESCIEN DIACETATE**

| Hours | A | B | AB |
|---|---|---|---|
| 24 | 10.33±2.03$^{e}$ | 7.33±2.02$^{f}$ | 14.00±2.31$^{g}$ |
| 48 | 12.00±2.31$^{e}$ | 10.00±2.31$^{ef}$ | 20.67±2.03$^{f}$ |
| 72 | 16.00±2.31$^{de}$ | 14.00±2.31$^{de}$ | 71.67±1.45$^{e}$ |
| 96 | 21.33±2.03$^{d}$ | 18.00±2.31$^{d}$ | 104.00±2.31$^{b}$ |
| 120 | 33.33±2.60$^{c}$ | 27.67±2.03$^{c}$ | 84.67±2.02$^{d}$ |
| 144 | 36.67±2.03$^{bc}$ | 30.00±2.31$^{bc}$ | 94.67±2.03$^{c}$ |
| 168 | 41.67±2.02$^{ab}$ | 36.00±2.31$^{ab}$ | 104.00±2.31$^{b}$ |
| 192 | 45.67±2.60$^{a}$ | 41.00±1.00$^{a}$ | 125.67±2.03$^{a}$ |
| F-ratio | 38.020 | 34.526 | 375.936 |
| P-value | 0.000 | 0.000 | 0.000 |

Values are presented as Mean ± SEM (n = 3). All groups are compared to each other at $P < 0.05$. Values with the same superscripts along the same column are not statistically different from each other.

**Keys**: A-*Burkholderia cepacia*

B-*Corynebacterium kutscheri*

AB-Mixed culture

**PROTEIN CONTENT**

| Hours | A | B | AB |
|---|---|---|---|
| 24 | $0.15\pm0.02^{h}$ | $0.18\pm0.02^{h}$ | $0.27\pm0.02^{h}$ |
| 48 | $0.88\pm0.02^{g}$ | $0.76\pm0.02^{g}$ | $1.67\pm0.02^{g}$ |
| 72 | $1.58\pm0.02^{f}$ | $1.42\pm0.02^{f}$ | $2.25\pm0.02^{f}$ |
| 96 | $2.18\pm0.02^{e}$ | $2.26\pm0.02^{e}$ | $2.56\pm0.02^{e}$ |
| 120 | $2.36\pm0.02^{d}$ | $2.18\pm0.02^{d}$ | $4.25\pm0.02^{c}$ |
| 144 | $2.88\pm0.15^{c}$ | $2.90\pm0.02^{c}$ | $6.68\pm0.02^{b}$ |
| 168 | $3.14\pm0.02^{b}$ | $3.26\pm0.02^{b}$ | $7.13\pm0.02^{a}$ |
| 192 | $3.70\pm0.04^{a}$ | $3.68\pm0.02^{a}$ | $3.26\pm0.02^{d}$ |
| F-ratio | 3882.782 | 4182.332 | 1383.491 |
| P-value | 0.000 | 0.000 | 0.000 |

Values are presented as Mean ± SEM (n = 3). All groups are compared to each other at $P < 0.05$. Values with the same superscripts along the same column are not statistically different from each other.

**Keys**: A-*Burkholderia cepacia*

B-*Corynebacterium kutscheri*

AB-Mixed culture

| pH | | | |
|---|---|---|---|
| Hours | A | B | AB |
| 24 | 6.80±0.02$^{e}$ | 6.88±0.02$^{e}$ | 6.90±0.02$^{b}$ |
| 48 | 7.10±0.02$^{d}$ | 6.90±0.02$^{e}$ | 7.22±0.02$^{ab}$ |
| 72 | 7.15±0.02$^{d}$ | 7.05±0.03$^{d}$ | 7.32±0.02$^{a}$ |
| 96 | 7.18±0.02$^{cd}$ | 7.12±0.02$^{c}$ | 7.52±0.33$^{a}$ |
| 120 | 7.25±0.03$^{bc}$ | 7.12±0.02$^{c}$ | 7.33±0.02$^{a}$ |
| 144 | 7.30±0.03$^{ab}$ | 7.26±0.02$^{b}$ | 7.33±0.03$^{a}$ |
| 168 | 7.31±0.03$^{ab}$ | 7.32±0.02$^{ab}$ | 7.31±0.02$^{a}$ |
| 192 | 7.25±0.02$^{bc}$ | 7.34±0.02$^{a}$ | 7.37±0.02$^{a}$ |
| 216 | 7.35±0.03$^{a}$ | 7.38±0.03$^{a}$ | 7.40±0.03$^{a}$ |
| F-ratio | 44.953 | 67.241 | 2.234 |
| P-value | 0.000 | 0.000 | 0.075 |

Values are presented as Mean ± SEM (n = 3). All groups are compared to each other at $P < 0.05$. Values with the same superscripts along the same column are not statistically different from each other.

**Keys**: A-*Burkholderia cepacia*

B-*Corynebacterium kutscheri*

AB-Mixed culture

| $CO_2$ PRODUCTION (µMOL) | | | |
|---|---|---|---|
| Hours | A | B | AB |
| 24 | 25.00±1.73$^{g}$ | 36.00±2.31$^{h}$ | 114.67±2.02$^{f}$ |
| 48 | 33.33±2.60$^{f}$ | 43.33±2.03$^{g}$ | 158.33±2.60$^{c}$ |
| 72 | 44.67±2.60$^{e}$ | 56.00±2.31$^{f}$ | 176.00±2.31$^{b}$ |
| 96 | 52.00±2.31$^{d}$ | 63.33±2.03$^{e}$ | 107.00±1.73$^{g}$ |
| 120 | 67.67±2.03$^{c}$ | 77.33±2.33$^{d}$ | 128.00±1.73$^{e}$ |
| 144 | 74.00±2.31$^{c}$ | 84.33±2.03$^{c}$ | 173.00±1.73$^{b}$ |
| 168 | 89.00±2.31$^{b}$ | 92.33±2.03$^{b}$ | 211.67±1.76$^{a}$ |
| 192 | 103.67±2.03$^{a}$ | 105.67±2.03$^{a}$ | 147.67±2.03$^{d}$ |
| F-ratio | 146.491 | 128.955 | 304.889 |
| P-value | 0.000 | 0.000 | 0.000 |

Values are presented as Mean ± SEM (n = 3). All groups are compared to each other at $P < 0.05$. Values with the same superscripts along the same column are not statistically different from each other.

**Keys**: A-*Burkholderia cepacia*

B-*Corynebacterium kutscheri*

AB-Mixed culture

**EMULSIFICATION INDEX**

| Hours | A | B | AB |
|---|---|---|---|
| 24 | 38.00±1.73$^{b}$ | 30.33±2.02$^{b}$ | 33.33±2.03$^{b}$ |
| 48 | 52.67±2.33$^{a}$ | 41.67±2.03$^{a}$ | 66.00±1.73$^{a}$ |
| 72 | 22.67±2.60$^{c}$ | 26.00±2.31$^{b}$ | 23.00±1.73$^{c}$ |
| 96 | 17.67±2.02$^{cd}$ | 17.33±2.03$^{c}$ | 25.00±1.73$^{c}$ |
| 120 | 18.00±1.73$^{cd}$ | 15.00±1.73$^{cd}$ | 14.00±2.31$^{d}$ |
| 144 | 17.33±2.02$^{cd}$ | 13.33±2.02$^{cd}$ | 13.00±1.73$^{de}$ |
| 168 | 12.00±2.31$^{de}$ | 10.67±1.33$^{de}$ | 10.00±2.31$^{de}$ |
| 192 | 8.33±2.02$^{e}$ | 6.67±1.67$^{e}$ | 7.33±1.45$^{e}$ |
| F-ratio | 48.594 | 37.151 | 100.795 |
| P-value | 0.000 | 0.000 | 0.000 |

Values are presented as Mean ± SEM (n = 3). All groups are compared to each other at $P < 0.05$. Values with the same superscripts along the same column are not statistically different from each other.

**Keys**: A-*Burkholderia cepacia*

B-*Corynebacterium kutscheri*

AB-Mixed culture

Appendice II

```
ONEWAY oil & grease BY days
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

Oilgrease

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Max |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1.00 | 3 | 43.2000 | 7.10000 | 3.24509 | 40.4674 | 45.9326 | 42.10 | |
| 2.00 | 3 | 31.1000 | 2.50000 | 1.20735 | 28.6159 | 33.5841 | 30.10 | |
| 4.00 | 3 | 22.5000 | 5.00000 | 2.76470 | 17.5317 | 27.4683 | 20.50 | |
| 6.00 | 3 | 16.1100 | 2.62000 | 1.61509 | 13.3774 | 18.8426 | 15.01 | |
| 8.00 | 3 | 8.4100 | .95000 | .44547 | 7.9132 | 8.9068 | 8.21 | |
| 10.00 | 3 | 4.2700 | .71000 | .30588 | 3.5993 | 4.9407 | 4.00 | |
| Total | 18 | 20.9317 | 18.88000 | 9.58358 | 14.1162 | 27.7472 | 4.00 | |

**ANOVA**

Oilgrease

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 3178.155 | 5 | 635.631 | 506.284 | .000 |
| Within Groups | 15.066 | 12 | 1.255 | | |
| Total | 3193.221 | 17 | | | |

## Post Hoc Tests

## Homogeneous Subsets

**Oil &Grease**

Duncan

| days | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 10.00 | 3 | 4.2700 | | | | | |
| 8.00 | 3 | | 8.4100 | | | | |
| 6.00 | 3 | | | 16.1100 | | | |
| 4.00 | 3 | | | | 22.5000 | | |
| 2.00 | 3 | | | | | 31.1000 | |
| 1.00 | 3 | | | | | | 43.2000 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY phenol BY days
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

Phenol

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Max |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1.00 | 3 | 68.1300 | 6.76807 | 3.21400 | 62.9926 | 73.2674 | 66.13 | |
| 2.00 | 3 | 28.4000 | 2.20000 | 1.23282 | 25.4190 | 31.3810 | 27.20 | |
| 4.00 | 3 | 18.2000 | 7.00000 | 3.23470 | 13.2317 | 23.1683 | 16.20 | |
| 6.00 | 3 | 10.8000 | 5.00000 | 2.89470 | 5.8317 | 15.7683 | 8.80 | |
| 8.00 | 3 | 4.3100 | 1.20000 | .51547 | 3.8132 | 4.8068 | 4.11 | |
| 10.00 | 3 | 1.0600 | 2.24000 | .81856 | .4638 | 1.6562 | .82 | |
| Total | 18 | 21.8167 | 24.40898 | 11.91025 | 10.2433 | 33.3900 | .82 | |

**ANOVA**

Phenol

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 9180.105 | 5 | 1836.021 | 797.432 | .000 |
| Within Groups | 27.629 | 12 | 2.302 | | |
| Total | 9207.734 | 17 | | | |

## Post Hoc Tests

## Homogeneous Subsets

**Phenol**

Duncan

| days | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 10.00 | 3 | 1.0600 | | | | | |
| 8.00 | 3 | | 4.3100 | | | | |
| 6.00 | 3 | | | 10.8000 | | | |
| 4.00 | 3 | | | | 18.2000 | | |
| 2.00 | 3 | | | | | 28.4000 | |
| 1.00 | 3 | | | | | | 68.1300 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY COD BY days
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN ALPHA(0.05).
```

# Oneway

**Descriptives**

Cod

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1.00 | 3 | 857.0000 | 3.67000 | 3.67118 | 847.8832 | 866.1168 | 853.33 | |
| 2.00 | 3 | 512.0000 | 4.21000 | 4.21064 | 501.5418 | 522.4582 | 507.79 | |
| 4.00 | 3 | 320.0000 | 5.79000 | 5.79286 | 305.6168 | 334.3832 | 314.21 | |
| 6.00 | 3 | 172.0000 | 5.39000 | 5.39192 | 158.6105 | 185.3895 | 166.61 | |
| 8.00 | 3 | 96.0000 | 4.00000 | 4.30940 | 86.0634 | 105.9366 | 92.00 | |
| 10.00 | 3 | 68.0000 | 4.54000 | 4.54117 | 56.7220 | 79.2780 | 63.46 | |
| Total | 18 | 337.5000 | 284.62558 | 67.08689 | 195.9590 | 479.0410 | 63.46 | |

**ANOVA**

Cod

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1376938.500 | 5 | 275387.700 | 12673.079 | .000 |
| Within Groups | 260.762 | 12 | 21.730 | | |
| Total | 1377199.262 | 17 | | | |

## Post Hoc Tests
## Homogeneous Subsets

**COD**

Duncan

| days | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 10.00 | 3 | 68.0000 | | | | | |
| 8.00 | 3 | | 96.0000 | | | | |
| 6.00 | 3 | | | 172.0000 | | | |
| 4.00 | 3 | | | | 320.0000 | | |
| 2.00 | 3 | | | | | 512.0000 | |
| 1.00 | 3 | | | | | | 857.0000 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY oildegraded BY days
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

Oildegraded

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Max |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 2.00 | 3 | 14.0000 | 2.00000 | 2.12470 | 9.0317 | 18.9683 | 12.00 | |
| 4.00 | 3 | 46.0000 | 1.00000 | 2.72735 | 43.5159 | 48.4841 | 45.00 | |
| 6.00 | 3 | 63.0000 | 3.00000 | 4.90205 | 55.5476 | 70.4524 | 60.00 | |
| 8.00 | 3 | 78.0000 | 3.00000 | 3.80205 | 70.5476 | 85.4524 | 75.00 | |
| 10.00 | 3 | 96.0000 | 4.00000 | 4.66940 | 86.0634 | 105.9366 | 92.00 | |
| Total | 15 | 59.4000 | 29.14937 | 7.52633 | 43.2576 | 75.5424 | 12.00 | |

**ANOVA**

Oil degraded

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 11817.600 | 4 | 2954.400 | 378.769 | .000 |
| Within Groups | 78.000 | 10 | 7.800 | | |
| Total | 11895.600 | 14 | | | |

## Post Hoc Tests
## Homogeneous Subsets

**Oil degraded**

Duncan

| days | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 2.00 | 3 | 14.0000 | | | | |
| 4.00 | 3 | | 46.0000 | | | |
| 6.00 | 3 | | | 63.0000 | | |
| 8.00 | 3 | | | | 78.0000 | |
| 10.00 | 3 | | | | | 96.0000 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY sulphides BY days
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

Sulphides

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Max |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1.00 | 3 | 26.0000 | 2.00000 | 2.43470 | 21.0317 | 30.9683 | 24.00 | |
| 2.00 | 3 | 20.0000 | 2.00000 | 2.12470 | 15.0317 | 24.9683 | 18.00 | |
| 4.00 | 3 | 14.0000 | .20000 | .22547 | 13.5032 | 14.4968 | 13.80 | |
| 6.00 | 3 | 11.0000 | 1.00000 | 2.70735 | 8.5159 | 13.4841 | 10.00 | |
| 8.00 | 3 | 7.0000 | 2.00000 | 2.09470 | 2.0317 | 11.9683 | 5.00 | |
| 10.00 | 3 | 4.2000 | 1.00000 | 2.31735 | 1.7159 | 6.6841 | 3.20 | |
| Total | 18 | 13.7000 | 7.77848 | 1.83340 | 9.8319 | 17.5681 | 3.20 | |

**ANOVA**

Sulphides

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1000.500 | 5 | 200.100 | 85.513 | .000 |
| Within Groups | 28.080 | 12 | 2.340 | | |
| Total | 1028.580 | 17 | | | |

## Post Hoc Tests
## Homogeneous Subsets

**Sulphides**

Duncan

| days | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 10.00 | 3 | 4.2000 | | | | | |
| 8.00 | 3 | | 7.0000 | | | | |
| 6.00 | 3 | | | 11.0000 | | | |
| 4.00 | 3 | | | | 14.0000 | | |
| 2.00 | 3 | | | | | 20.0000 | |
| 1.00 | 3 | | | | | | 26.0000 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY Fluorescein Diacetate BY hours
  /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

fluoresceinA

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 12.3333 | 2.51661 | 1.45297 | 6.0817 | 18.5849 | 10.00 | |
| 48.00 | 3 | 21.0000 | 3.00000 | 1.73205 | 13.5476 | 28.4524 | 18.00 | |
| 72.00 | 3 | 29.0000 | 1.73205 | 1.00000 | 24.6973 | 33.3027 | 28.00 | |
| 96.00 | 3 | 32.0000 | 3.00000 | 1.73205 | 24.5476 | 39.4524 | 29.00 | |
| 120.00 | 3 | 43.0000 | 3.00000 | 1.73205 | 35.5476 | 50.4524 | 40.00 | |
| 144.00 | 3 | 54.0000 | 3.00000 | 1.73205 | 46.5476 | 61.4524 | 51.00 | |
| 168.00 | 3 | 65.6667 | 3.51188 | 2.02759 | 56.9427 | 74.3907 | 62.00 | |
| 192.00 | 3 | 70.3333 | 3.51188 | 2.02759 | 61.6093 | 79.0573 | 67.00 | |
| Total | 24 | 40.9167 | 20.22249 | 4.12790 | 32.3775 | 49.4559 | 10.00 | |

**ANOVA**

fluoresceinA

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 9265.833 | 7 | 1323.690 | 151.279 | .000 |
| Within Groups | 140.000 | 16 | 8.750 | | |
| Total | 9405.833 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**Fluorescein Diacetate**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 24.00 | 3 | 12.3333 | | | | | |
| 48.00 | 3 | | 21.0000 | | | | |
| 72.00 | 3 | | | 29.0000 | | | |
| 96.00 | 3 | | | 32.0000 | | | |
| 120.00 | 3 | | | | 43.0000 | | |
| 144.00 | 3 | | | | | 54.0000 | |
| 168.00 | 3 | | | | | | 65.6667 |
| 192.00 | 3 | | | | | | 70.3333 |
| Sig. | | 1.000 | 1.000 | .232 | 1.000 | 1.000 | .071 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY Fluoresceinb BY hours /STATISTICS DESCRIPTIVES /MISSING ANALYSIS
/POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

Fluorescein b

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 17.0000 | 3.00000 | 1.73205 | 9.5476 | 24.4524 | 14.00 | |
| 48.00 | 3 | 25.3333 | 4.04145 | 2.33333 | 15.2938 | 35.3729 | 21.00 | |
| 72.00 | 3 | 31.6667 | 3.51188 | 2.02759 | 22.9427 | 40.3907 | 28.00 | |
| 96.00 | 3 | 38.0000 | 3.00000 | 1.73205 | 30.5476 | 45.4524 | 35.00 | |
| 120.00 | 3 | 52.0000 | 3.00000 | 1.73205 | 44.5476 | 59.4524 | 49.00 | |
| 144.00 | 3 | 68.0000 | 3.00000 | 1.73205 | 60.5476 | 75.4524 | 65.00 | |
| 168.00 | 3 | 72.0000 | 3.00000 | 1.73205 | 64.5476 | 79.4524 | 69.00 | |
| 192.00 | 3 | 83.0000 | 3.00000 | 1.73205 | 75.5476 | 90.4524 | 80.00 | |
| Total | 24 | 48.3750 | 23.16071 | 4.72766 | 38.5951 | 58.1549 | 14.00 | |

**ANOVA**

fluoresceinB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 12172.292 | 7 | 1738.899 | 168.281 | .000 |
| Within Groups | 165.333 | 16 | 10.333 | | |
| Total | 12337.625 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**fluoresceinB**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 24.00 | 3 | 17.0000 | | | | | | |
| 48.00 | 3 | | 25.3333 | | | | | |
| 72.00 | 3 | | | 31.6667 | | | | |
| 96.00 | 3 | | | | 38.0000 | | | |
| 120.00 | 3 | | | | | 52.0000 | | |
| 144.00 | 3 | | | | | | 68.0000 | |
| 168.00 | 3 | | | | | | 72.0000 | |
| 192.00 | 3 | | | | | | | 83.0000 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | .147 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY fluoresceinAB BY hrs /STATISTICS DESCRIPTIVES /MISSING ANALYSIS
/POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

fluoresceinAB

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | M |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 23.0000 | 3.00000 | 1.73205 | 15.5476 | 30.4524 | 20.00 | |
| 48.00 | 3 | 37.3333 | 3.51188 | 2.02759 | 28.6093 | 46.0573 | 34.00 | |
| 72.00 | 3 | 25.6667 | 3.51188 | 2.02759 | 16.9427 | 34.3907 | 22.00 | |
| 96.00 | 3 | 72.0000 | 3.00000 | 1.73205 | 64.5476 | 79.4524 | 69.00 | |
| 120.00 | 3 | 111.3333 | 2.51661 | 1.45297 | 105.0817 | 117.5849 | 109.00 | |
| 144.00 | 3 | 167.3333 | 3.51188 | 2.02759 | 158.6093 | 176.0573 | 164.00 | |
| 168.00 | 3 | 210.3333 | 3.51188 | 2.02759 | 201.6093 | 219.0573 | 207.00 | |
| 192.00 | 3 | 121.0000 | 3.00000 | 1.73205 | 113.5476 | 128.4524 | 118.00 | |
| Total | 24 | 96.0000 | 65.97364 | 13.46681 | 68.1418 | 123.8582 | 20.00 | |

**ANOVA**

fluoresceinAB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 99942.667 | 7 | 14277.524 | 1381.696 | .000 |
| Within Groups | 165.333 | 16 | 10.333 | | |
| Total | 100108.000 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**fluoresceinAB**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 24.00 | 3 | 23.0000 | | | | | | |
| 72.00 | 3 | 25.6667 | | | | | | |
| 48.00 | 3 | | 37.3333 | | | | | |
| 96.00 | 3 | | | 72.0000 | | | | |
| 120.00 | 3 | | | | 111.3333 | | | |
| 192.00 | 3 | | | | | 121.0000 | | |
| 144.00 | 3 | | | | | | 167.3333 | |
| 168.00 | 3 | | | | | | | 210.3333 |
| Sig. | | .325 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY PFD.A BY hrs
  /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

PFD.A

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 10.3333 | 3.51188 | 2.02759 | 1.6093 | 19.0573 | 7.00 | |
| 48.00 | 3 | 12.0000 | 4.00000 | 2.30940 | 2.0634 | 21.9366 | 8.00 | |
| 72.00 | 3 | 16.0000 | 4.00000 | 2.30940 | 6.0634 | 25.9366 | 12.00 | |
| 96.00 | 3 | 21.3333 | 3.51188 | 2.02759 | 12.6093 | 30.0573 | 18.00 | |
| 120.00 | 3 | 33.3333 | 4.50925 | 2.60342 | 22.1317 | 44.5349 | 29.00 | |
| 144.00 | 3 | 36.6667 | 3.51188 | 2.02759 | 27.9427 | 45.3907 | 33.00 | |
| 168.00 | 3 | 41.6667 | 3.51188 | 2.02759 | 32.9427 | 50.3907 | 38.00 | |
| 192.00 | 3 | 45.6667 | 4.50925 | 2.60342 | 34.4651 | 56.8683 | 41.00 | |
| Total | 24 | 27.1250 | 13.67738 | 2.79188 | 21.3496 | 32.9004 | 7.00 | |

**ANOVA**

PFD.A

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 4058.625 | 7 | 579.804 | 38.020 | .000 |
| Within Groups | 244.000 | 16 | 15.250 | | |
| Total | 4302.625 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**PFD.A**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 24.00 | 3 | 10.3333 | | | | |
| 48.00 | 3 | 12.0000 | | | | |
| 72.00 | 3 | 16.0000 | 16.0000 | | | |
| 96.00 | 3 | | 21.3333 | | | |
| 120.00 | 3 | | | 33.3333 | | |
| 144.00 | 3 | | | 36.6667 | 36.6667 | |
| 168.00 | 3 | | | | 41.6667 | 41.6667 |
| 192.00 | 3 | | | | | 45.6667 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Sig. | | .110 | .114 | .311 | .136 | .228 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY PFD.B BY hrs
/STATISTICS DESCRIPTIVES /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

PFD.B

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 7.3333 | 3.51188 | 2.02759 | -1.3907 | 16.0573 | 4.00 | |
| 48.00 | 3 | 10.0000 | 4.00000 | 2.30940 | .0634 | 19.9366 | 6.00 | |
| 72.00 | 3 | 14.0000 | 4.00000 | 2.30940 | 4.0634 | 23.9366 | 10.00 | |
| 96.00 | 3 | 18.0000 | 4.00000 | 2.30940 | 8.0634 | 27.9366 | 14.00 | |
| 120.00 | 3 | 27.6667 | 3.51188 | 2.02759 | 18.9427 | 36.3907 | 24.00 | |
| 144.00 | 3 | 30.0000 | 4.00000 | 2.30940 | 20.0634 | 39.9366 | 26.00 | |
| 168.00 | 3 | 36.0000 | 4.00000 | 2.30940 | 26.0634 | 45.9366 | 32.00 | |
| 192.00 | 3 | 41.0000 | 1.73205 | 1.00000 | 36.6973 | 45.3027 | 40.00 | |
| Total | 24 | 23.0000 | 12.27936 | 2.50651 | 17.8149 | 28.1851 | 4.00 | |

**ANOVA**

PFD.B

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 3252.667 | 7 | 464.667 | 34.526 | .000 |
| Within Groups | 215.333 | 16 | 13.458 | | |
| Total | 3468.000 | 23 | | | |

## Post Hoc Tests

## Homogeneous Subsets

**PFD.B**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 24.00 | 3 | 7.3333 | | | | | |
| 48.00 | 3 | 10.0000 | 10.0000 | | | | |
| 72.00 | 3 | | 14.0000 | 14.0000 | | | |
| 96.00 | 3 | | | 18.0000 | | | |
| 120.00 | 3 | | | | 27.6667 | | |
| 144.00 | 3 | | | | 30.0000 | 30.0000 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 168.00 | 3 | | | | | 36.0000 | 36.0000 |
| 192.00 | 3 | | | | | | 41.0000 |
| Sig. | | .387 | .200 | .200 | .447 | .062 | .115 |

Means for groups in homogeneous subsets are displayed.

```
ONEWAY PFD.AB BY hrs
  /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

PFD.AB

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | M |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 14.0000 | 4.00000 | 2.30940 | 4.0634 | 23.9366 | 10.00 | |
| 48.00 | 3 | 20.6667 | 3.51188 | 2.02759 | 11.9427 | 29.3907 | 17.00 | |
| 72.00 | 3 | 71.6667 | 2.51661 | 1.45297 | 65.4151 | 77.9183 | 69.00 | |
| 96.00 | 3 | 104.0000 | 4.00000 | 2.30940 | 94.0634 | 113.9366 | 100.00 | |
| 120.00 | 3 | 84.6667 | 3.51188 | 2.02759 | 75.9427 | 93.3907 | 81.00 | |
| 144.00 | 3 | 94.6667 | 3.51188 | 2.02759 | 85.9427 | 103.3907 | 91.00 | |
| 168.00 | 3 | 104.0000 | 4.00000 | 2.30940 | 94.0634 | 113.9366 | 100.00 | |
| 192.00 | 3 | 125.6667 | 3.51188 | 2.02759 | 116.9427 | 134.3907 | 122.00 | |
| Total | 24 | 77.4167 | 38.62182 | 7.88365 | 61.1081 | 93.7252 | 10.00 | |

**ANOVA**

PFD.AB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 34100.500 | 7 | 4871.500 | 375.936 | .000 |
| Within Groups | 207.333 | 16 | 12.958 | | |
| Total | 34307.833 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**PFD.AB**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 24.00 | 3 | 14.0000 | | | | | | |
| 48.00 | 3 | | 20.6667 | | | | | |
| 72.00 | 3 | | | 71.6667 | | | | |
| 120.00 | 3 | | | | 84.6667 | | | |
| 144.00 | 3 | | | | | 94.6667 | | |
| 96.00 | 3 | | | | | | 104.0000 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 168.00 | 3 | | | | | | 104.0000 | |
| 192.00 | 3 | | | | | | | 125.6667 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY Protein.A BY hrs
  /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

Protein.A

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | .1500 | .03000 | .01732 | .0755 | .2245 | .12 | |
| 48.00 | 3 | .8800 | .03000 | .01732 | .8055 | .9545 | .85 | |
| 72.00 | 3 | 1.5767 | .03512 | .02028 | 1.4894 | 1.6639 | 1.54 | |
| 96.00 | 3 | 2.1800 | .03000 | .01732 | 2.1055 | 2.2545 | 2.15 | |
| 120.00 | 3 | 2.3567 | .03512 | .02028 | 2.2694 | 2.4439 | 2.32 | |
| 144.00 | 3 | 2.8800 | .02646 | .01528 | 2.8143 | 2.9457 | 2.86 | |
| 168.00 | 3 | 3.1367 | .04041 | .02333 | 3.0363 | 3.2371 | 3.10 | |
| 192.00 | 3 | 3.7033 | .03512 | .02028 | 3.6161 | 3.7906 | 3.67 | |
| Total | 24 | 2.1079 | 1.13613 | .23191 | 1.6282 | 2.5877 | .12 | |

**ANOVA**

Protein.A

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 29.671 | 7 | 4.239 | 3882.782 | .000 |
| Within Groups | .017 | 16 | .001 | | |
| Total | 29.688 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**Protein.A**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 24.00 | 3 | .1500 | | | | | | | |
| 48.00 | 3 | | .8800 | | | | | | |
| 72.00 | 3 | | | 1.5767 | | | | | |
| 96.00 | 3 | | | | 2.1800 | | | | |
| 120.00 | 3 | | | | | 2.3567 | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 144.00 | 3 | | | | | | 2.8800 | | |
| 168.00 | 3 | | | | | | | 3.1367 | |
| 192.00 | 3 | | | | | | | | 3.70 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.0 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY Protein.B BY hrs
  /STATISTICS DESCRIPTIVES  /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

Protein.B

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | .1800 | .03000 | .01732 | .1055 | .2545 | .15 | |
| 48.00 | 3 | .7600 | .03000 | .01732 | .6855 | .8345 | .73 | |
| 72.00 | 3 | 1.4200 | .04000 | .02309 | 1.3206 | 1.5194 | 1.38 | |
| 96.00 | 3 | 2.2600 | .03000 | .01732 | 2.1855 | 2.3345 | 2.23 | |
| 120.00 | 3 | 2.1800 | .03000 | .01732 | 2.1055 | 2.2545 | 2.15 | |
| 144.00 | 3 | 2.9033 | .03512 | .02028 | 2.8161 | 2.9906 | 2.87 | |
| 168.00 | 3 | 3.2600 | .03000 | .01732 | 3.1855 | 3.3345 | 3.23 | |
| 192.00 | 3 | 3.6767 | .03512 | .02028 | 3.5894 | 3.7639 | 3.64 | |
| Total | 24 | 2.0800 | 1.16781 | .23838 | 1.5869 | 2.5731 | .15 | |

**ANOVA**

Protein.B

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 31.350 | 7 | 4.479 | 4182.332 | .000 |
| Within Groups | .017 | 16 | .001 | | |
| Total | 31.367 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**Protein.B**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 24.00 | 3 | .1800 | | | | | | | |
| 48.00 | 3 | | .7600 | | | | | | |
| 72.00 | 3 | | | 1.4200 | | | | | |
| 120.00 | 3 | | | | 2.1800 | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 96.00 | 3 | | | | | 2.2600 | | | |
| 144.00 | 3 | | | | | | 2.9033 | | |
| 168.00 | 3 | | | | | | | 3.2600 | |
| 192.00 | 3 | | | | | | | | 3.67 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.0 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY Protein.AB BY hrs
  /STATISTICS DESCRIPTIVES  /MISSING ANALYSIS  /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

Protein.AB

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | .2733 | .03512 | .02028 | .1861 | .3606 | .24 | |
| 48.00 | 3 | 1.6700 | .04000 | .02309 | 1.5706 | 1.7694 | 1.63 | |
| 72.00 | 3 | 2.2467 | .03512 | .02028 | 2.1594 | 2.3339 | 2.21 | |
| 96.00 | 3 | 2.5567 | .03512 | .02028 | 2.4694 | 2.6439 | 2.52 | |
| 120.00 | 3 | 4.2500 | .04000 | .02309 | 4.1506 | 4.3494 | 4.21 | |
| 144.00 | 3 | 6.6800 | .03000 | .01732 | 6.6055 | 6.7545 | 6.65 | |
| 168.00 | 3 | 7.1233 | .03512 | .02028 | 7.0361 | 7.2106 | 7.09 | |
| 192.00 | 3 | 3.2600 | .03000 | .01732 | 3.1855 | 3.3345 | 3.23 | |
| Total | 24 | 3.5075 | 2.28668 | .46677 | 2.5419 | 4.4731 | .24 | |

**ANOVA**

Protein.AB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 120.245 | 7 | 17.178 | 13834.491 | .000 |
| Within Groups | .020 | 16 | .001 | | |
| Total | 120.265 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**Protein.AB**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 24.00 | 3 | .2733 | | | | | | | |
| 48.00 | 3 | | 1.6700 | | | | | | |
| 72.00 | 3 | | | 2.2467 | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 96.00 | 3 | | | | 2.5567 | | | | |
| 192.00 | 3 | | | | | 3.2600 | | | |
| 120.00 | 3 | | | | | | 4.2500 | | |
| 144.00 | 3 | | | | | | | 6.6800 | |
| 168.00 | 3 | | | | | | | | 7.12 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.0 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY pH.A BY hrs
  /STATISTICS DESCRIPTIVES  /MISSING ANALYSIS  /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

pH.A

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 6.8033 | .03512 | .02028 | 6.7161 | 6.8906 | 6.77 | |
| 48.00 | 3 | 7.1000 | .04000 | .02309 | 7.0006 | 7.1994 | 7.06 | |
| 72.00 | 3 | 7.1467 | .03512 | .02028 | 7.0594 | 7.2339 | 7.11 | |
| 96.00 | 3 | 7.1767 | .03512 | .02028 | 7.0894 | 7.2639 | 7.14 | |
| 120.00 | 3 | 7.2467 | .04509 | .02603 | 7.1347 | 7.3587 | 7.20 | |
| 144.00 | 3 | 7.3000 | .05000 | .02887 | 7.1758 | 7.4242 | 7.25 | |
| 168.00 | 3 | 7.3133 | .05033 | .02906 | 7.1883 | 7.4384 | 7.26 | |
| 192.00 | 3 | 7.2500 | .04000 | .02309 | 7.1506 | 7.3494 | 7.21 | |
| 216.00 | 3 | 7.3500 | .05000 | .02887 | 7.2258 | 7.4742 | 7.30 | |
| Total | 27 | 7.1874 | .16301 | .03137 | 7.1229 | 7.2519 | 6.77 | |

**ANOVA**

pH.A

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .658 | 8 | .082 | 44.953 | .000 |
| Within Groups | .033 | 18 | .002 | | |
| Total | .691 | 26 | | | |

## Post Hoc Tests Homogeneous Subsets

**pH.A**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 24.00 | 3 | 6.8033 | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 48.00 | 3 | | 7.1000 | | | |
| 72.00 | 3 | | 7.1467 | | | |
| 96.00 | 3 | | 7.1767 | 7.1767 | | |
| 120.00 | 3 | | | 7.2467 | 7.2467 | |
| 192.00 | 3 | | | 7.2500 | 7.2500 | |
| 144.00 | 3 | | | | 7.3000 | 7.3000 |
| 168.00 | 3 | | | | 7.3133 | 7.3133 |
| 216.00 | 3 | | | | | 7.3500 |
| Sig. | | 1.000 | .051 | .061 | .095 | .192 |

```
ONEWAY pH.B BY hrs
  /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

pH.B

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 6.8767 | .03512 | .02028 | 6.7894 | 6.9639 | 6.84 | |
| 48.00 | 3 | 6.9033 | .03512 | .02028 | 6.8161 | 6.9906 | 6.87 | |
| 72.00 | 3 | 7.0500 | .05000 | .02887 | 6.9258 | 7.1742 | 7.00 | |
| 96.00 | 3 | 7.1200 | .04000 | .02309 | 7.0206 | 7.2194 | 7.08 | |
| 120.00 | 3 | 7.1767 | .03512 | .02028 | 7.0894 | 7.2639 | 7.14 | |
| 144.00 | 3 | 7.2600 | .04000 | .02309 | 7.1606 | 7.3594 | 7.22 | |
| 168.00 | 3 | 7.3200 | .04000 | .02309 | 7.2206 | 7.4194 | 7.28 | |
| 192.00 | 3 | 7.3433 | .04041 | .02333 | 7.2429 | 7.4437 | 7.30 | |
| 216.00 | 3 | 7.3767 | .03512 | .02028 | 7.2894 | 7.4639 | 7.34 | |
| Total | 27 | 7.1585 | .18150 | .03493 | 7.0867 | 7.2303 | 6.84 | |

**ANOVA**

pH.B

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .829 | 8 | .104 | 67.241 | .000 |
| Within Groups | .028 | 18 | .002 | | |
| Total | .857 | 26 | | | |

## Post Hoc Tests Homogeneous Subsets

**pH.B**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 24.00 | 3 | 6.8767 | | | | |
| 48.00 | 3 | 6.9033 | | | | |
| 72.00 | 3 | | 7.0500 | | | |
| 96.00 | 3 | | | 7.1200 | | |
| 120.00 | 3 | | | 7.1767 | | |
| 144.00 | 3 | | | | 7.2600 | |
| 168.00 | 3 | | | | 7.3200 | 7.3200 |
| 192.00 | 3 | | | | | 7.3433 |
| 216.00 | 3 | | | | | 7.3767 |
| Sig. | | .416 | 1.000 | .094 | .078 | .110 |

```
ONEWAY pH.AB BY hrs (1)
  /STATISTICS DESCRIPTIVES  /MISSING ANALYSIS  /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

pH.AB

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 6.9000 | .04000 | .02309 | 6.8006 | 6.9994 | 6.86 | |
| 48.00 | 3 | 7.2167 | .03512 | .02028 | 7.1294 | 7.3039 | 7.18 | |
| 72.00 | 3 | 7.3200 | .04000 | .02309 | 7.2206 | 7.4194 | 7.28 | |
| 96.00 | 3 | 7.5167 | .57951 | .33458 | 6.0771 | 8.9563 | 6.85 | |
| 120.00 | 3 | 7.3300 | .04000 | .02309 | 7.2306 | 7.4294 | 7.29 | |
| 144.00 | 3 | 7.3333 | .04509 | .02603 | 7.2213 | 7.4453 | 7.29 | |
| 168.00 | 3 | 7.3100 | .03000 | .01732 | 7.2355 | 7.3845 | 7.28 | |
| 192.00 | 3 | 7.3700 | .04000 | .02309 | 7.2706 | 7.4694 | 7.33 | |
| 216.00 | 3 | 7.3967 | .04509 | .02603 | 7.2847 | 7.5087 | 7.35 | |
| Total | 27 | 7.2993 | .23112 | .04448 | 7.2078 | 7.3907 | 6.85 | |

**ANOVA**

pH.AB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .692 | 8 | .086 | 2.234 | .075 |
| Within Groups | .697 | 18 | .039 | | |
| Total | 1.389 | 26 | | | |

## Post Hoc Tests Homogeneous Subsets

**pH.AB**

Duncan

| hrs | N | Subset for alpha = 0.05 |
|---|---|---|

| | | 1 | 2 |
|---|---|---|---|
| 24.00 | 3 | 6.9000 | |
| 48.00 | 3 | 7.2167 | 7.2167 |
| 168.00 | 3 | | 7.3100 |
| 72.00 | 3 | | 7.3200 |
| 120.00 | 3 | | 7.3300 |
| 144.00 | 3 | | 7.3333 |
| 192.00 | 3 | | 7.3700 |
| 216.00 | 3 | | 7.3967 |
| 96.00 | 3 | | 7.5167 |
| Sig. | | .064 | .119 |

```
ONEWAY CO2A BY hrs
  /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

CO2A

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | M |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 25.0000 | 3.00000 | 1.73205 | 17.5476 | 32.4524 | 22.00 | |
| 48.00 | 3 | 33.3333 | 4.50925 | 2.60342 | 22.1317 | 44.5349 | 29.00 | |
| 72.00 | 3 | 44.6667 | 4.50925 | 2.60342 | 33.4651 | 55.8683 | 40.00 | |
| 96.00 | 3 | 52.0000 | 4.00000 | 2.30940 | 42.0634 | 61.9366 | 48.00 | |
| 120.00 | 3 | 67.6667 | 3.51188 | 2.02759 | 58.9427 | 76.3907 | 64.00 | |
| 144.00 | 3 | 74.0000 | 4.00000 | 2.30940 | 64.0634 | 83.9366 | 70.00 | |
| 168.00 | 3 | 89.0000 | 4.00000 | 2.30940 | 79.0634 | 98.9366 | 85.00 | |
| 192.00 | 3 | 103.6667 | 3.51188 | 2.02759 | 94.9427 | 112.3907 | 100.00 | |
| Total | 24 | 61.1667 | 26.31360 | 5.37124 | 50.0554 | 72.2779 | 22.00 | |

**ANOVA**

CO2A

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 15680.667 | 7 | 2240.095 | 146.491 | .000 |
| Within Groups | 244.667 | 16 | 15.292 | | |
| Total | 15925.333 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**CO2A**

Duncan

| Hrs | N | Subset for alpha = 0.05 |
|---|---|---|

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| 24.00 | 3 | 25.0000 | | | | | | |
| 48.00 | 3 | | 33.3333 | | | | | |
| 72.00 | 3 | | | 44.6667 | | | | |
| 96.00 | 3 | | | | 52.0000 | | | |
| 120.00 | 3 | | | | | 67.6667 | | |
| 144.00 | 3 | | | | | 74.0000 | | |
| 168.00 | 3 | | | | | | 89.0000 | |
| 192.00 | 3 | | | | | | | 103.6667 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | .065 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY CO2B BY hrs
  /STATISTICS DESCRIPTIVES  /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

CO2B

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | M |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 36.0000 | 4.00000 | 2.30940 | 26.0634 | 45.9366 | 32.00 | |
| 48.00 | 3 | 43.3333 | 3.51188 | 2.02759 | 34.6093 | 52.0573 | 40.00 | |
| 72.00 | 3 | 56.0000 | 4.00000 | 2.30940 | 46.0634 | 65.9366 | 52.00 | |
| 96.00 | 3 | 63.3333 | 3.51188 | 2.02759 | 54.6093 | 72.0573 | 60.00 | |
| 120.00 | 3 | 77.3333 | 4.04145 | 2.33333 | 67.2938 | 87.3729 | 73.00 | |
| 144.00 | 3 | 84.3333 | 3.51188 | 2.02759 | 75.6093 | 93.0573 | 81.00 | |
| 168.00 | 3 | 92.3333 | 3.51188 | 2.02759 | 83.6093 | 101.0573 | 89.00 | |
| 192.00 | 3 | 105.6667 | 3.51188 | 2.02759 | 96.9427 | 114.3907 | 102.00 | |
| Total | 24 | 69.7917 | 23.43535 | 4.78372 | 59.8958 | 79.6875 | 32.00 | |

**ANOVA**

CO2B

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 12411.958 | 7 | 1773.137 | 128.955 | .000 |
| Within Groups | 220.000 | 16 | 13.750 | | |
| Total | 12631.958 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**CO2B**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 24.00 | 3 | 36.0000 | | | | | | | |
| 48.00 | 3 | | 43.3333 | | | | | | |
| 72.00 | 3 | | | 56.0000 | | | | | |
| 96.00 | 3 | | | | 63.3333 | | | | |
| 120.00 | 3 | | | | | 77.3333 | | | |
| 144.00 | 3 | | | | | | 84.3333 | | |
| 168.00 | 3 | | | | | | | 92.3333 | |
| 192.00 | 3 | | | | | | | | 105.66 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.0 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY CO2AB BY hrs
  /STATISTICS DESCRIPTIVES  /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

CO2AB

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | M |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 114.6667 | 3.51188 | 2.02759 | 105.9427 | 123.3907 | 111.00 | |
| 48.00 | 3 | 158.3333 | 4.50925 | 2.60342 | 147.1317 | 169.5349 | 154.00 | |
| 72.00 | 3 | 176.0000 | 4.00000 | 2.30940 | 166.0634 | 185.9366 | 172.00 | |
| 96.00 | 3 | 107.0000 | 3.00000 | 1.73205 | 99.5476 | 114.4524 | 104.00 | |
| 120.00 | 3 | 128.0000 | 3.00000 | 1.73205 | 120.5476 | 135.4524 | 125.00 | |
| 144.00 | 3 | 173.0000 | 3.00000 | 1.73205 | 165.5476 | 180.4524 | 170.00 | |
| 168.00 | 3 | 211.6667 | 3.05505 | 1.76383 | 204.0775 | 219.2558 | 209.00 | |
| 192.00 | 3 | 147.6667 | 3.51188 | 2.02759 | 138.9427 | 156.3907 | 144.00 | |
| Total | 24 | 152.0417 | 33.67812 | 6.87452 | 137.8206 | 166.2627 | 104.00 | |

**ANOVA**

CO2AB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 25892.292 | 7 | 3698.899 | 304.019 | .000 |
| Within Groups | 194.667 | 16 | 12.167 | | |
| Total | 26086.958 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**CO2AB**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 96.00 | 3 | 107.0000 | | | | | | |
| 24.00 | 3 | | 114.6667 | | | | | |
| 120.00 | 3 | | | 128.0000 | | | | |
| 192.00 | 3 | | | | 147.6667 | | | |
| 48.00 | 3 | | | | | 158.3333 | | |
| 144.00 | 3 | | | | | | 173.0000 | |
| 72.00 | 3 | | | | | | 176.0000 | |
| 168.00 | 3 | | | | | | | 211.6667 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | .308 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY Emulsification A BY hrs
  /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

EmulsificationA

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 38.0000 | 3.00000 | 1.73205 | 30.5476 | 45.4524 | 35.00 | |
| 48.00 | 3 | 52.6667 | 4.04145 | 2.33333 | 42.6271 | 62.7062 | 49.00 | |
| 72.00 | 3 | 22.6667 | 4.50925 | 2.60342 | 11.4651 | 33.8683 | 18.00 | |
| 96.00 | 3 | 17.6667 | 3.51188 | 2.02759 | 8.9427 | 26.3907 | 14.00 | |
| 120.00 | 3 | 18.0000 | 3.00000 | 1.73205 | 10.5476 | 25.4524 | 15.00 | |
| 144.00 | 3 | 17.3333 | 3.51188 | 2.02759 | 8.6093 | 26.0573 | 14.00 | |
| 168.00 | 3 | 12.0000 | 4.00000 | 2.30940 | 2.0634 | 21.9366 | 8.00 | |
| 192.00 | 3 | 8.3333 | 3.51188 | 2.02759 | -.3907 | 17.0573 | 5.00 | |
| Total | 24 | 23.3333 | 14.43627 | 2.94679 | 17.2374 | 29.4292 | 5.00 | |

**ANOVA**

EmulsificationA

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 4578.000 | 7 | 654.000 | 48.594 | .000 |
| Within Groups | 215.333 | 16 | 13.458 | | |
| Total | 4793.333 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**Emulsification A**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 192.00 | 3 | 8.3333 | | | | |
| 168.00 | 3 | 12.0000 | 12.0000 | | | |
| 144.00 | 3 | | 17.3333 | 17.3333 | | |
| 96.00 | 3 | | 17.6667 | 17.6667 | | |
| 120.00 | 3 | | 18.0000 | 18.0000 | | |
| 72.00 | 3 | | | 22.6667 | | |
| 24.00 | 3 | | | | 38.0000 | |
| 48.00 | 3 | | | | | 52.6667 |
| Sig. | | .239 | .083 | .120 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY Emulsification B BY hrs
  /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

EmulsificationB

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 30.3333 | 3.51188 | 2.02759 | 21.6093 | 39.0573 | 27.00 | |
| 48.00 | 3 | 41.6667 | 3.51188 | 2.02759 | 32.9427 | 50.3907 | 38.00 | |
| 72.00 | 3 | 26.0000 | 4.00000 | 2.30940 | 16.0634 | 35.9366 | 22.00 | |
| 96.00 | 3 | 17.3333 | 3.51188 | 2.02759 | 8.6093 | 26.0573 | 14.00 | |
| 120.00 | 3 | 15.0000 | 3.00000 | 1.73205 | 7.5476 | 22.4524 | 12.00 | |
| 144.00 | 3 | 13.3333 | 3.51188 | 2.02759 | 4.6093 | 22.0573 | 10.00 | |
| 168.00 | 3 | 10.6667 | 2.30940 | 1.33333 | 4.9298 | 16.4035 | 8.00 | |
| 192.00 | 3 | 6.6667 | 2.88675 | 1.66667 | -.5044 | 13.8378 | 5.00 | |
| Total | 24 | 20.1250 | 11.49031 | 2.34545 | 15.2731 | 24.9769 | 5.00 | |

**ANOVA**

EmulsificationB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 2860.625 | 7 | 408.661 | 37.151 | .000 |
| Within Groups | 176.000 | 16 | 11.000 | | |
| Total | 3036.625 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**EmulsificationB**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 192.00 | 3 | 6.6667 | | | | |
| 168.00 | 3 | 10.6667 | 10.6667 | | | |
| 144.00 | 3 | | 13.3333 | 13.3333 | | |
| 120.00 | 3 | | 15.0000 | 15.0000 | | |
| 96.00 | 3 | | | 17.3333 | | |
| 72.00 | 3 | | | | 26.0000 | |
| 24.00 | 3 | | | | 30.3333 | |
| 48.00 | 3 | | | | | 41.6667 |
| Sig. | | .159 | .148 | .180 | .129 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

```
ONEWAY EmulsificationAB BY hrs
  /STATISTICS DESCRIPTIVES  /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).
```

## Oneway

**Descriptives**

EmulsificationAB

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Ma |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 24.00 | 3 | 33.3333 | 3.51188 | 2.02759 | 24.6093 | 42.0573 | 30.00 | |
| 48.00 | 3 | 66.0000 | 3.00000 | 1.73205 | 58.5476 | 73.4524 | 63.00 | |
| 72.00 | 3 | 23.0000 | 3.00000 | 1.73205 | 15.5476 | 30.4524 | 20.00 | |
| 96.00 | 3 | 25.0000 | 3.00000 | 1.73205 | 17.5476 | 32.4524 | 22.00 | |
| 120.00 | 3 | 14.0000 | 4.00000 | 2.30940 | 4.0634 | 23.9366 | 10.00 | |
| 144.00 | 3 | 13.0000 | 3.00000 | 1.73205 | 5.5476 | 20.4524 | 10.00 | |
| 168.00 | 3 | 10.0000 | 4.00000 | 2.30940 | .0634 | 19.9366 | 6.00 | |
| 192.00 | 3 | 7.3333 | 2.51661 | 1.45297 | 1.0817 | 13.5849 | 5.00 | |
| Total | 24 | 23.9583 | 18.43550 | 3.76313 | 16.1737 | 31.7430 | 5.00 | |

**ANOVA**

EmulsificationAB

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 7643.625 | 7 | 1091.946 | 100.795 | .000 |
| Within Groups | 173.333 | 16 | 10.833 | | |

| | | | | | |
|---|---|---|---|---|---|
| Total | 7816.958 | 23 | | | |

## Post Hoc Tests Homogeneous Subsets

**EmulsificationAB**

Duncan

| hrs | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 192.00 | 3 | 7.3333 | | | | |
| 168.00 | 3 | 10.0000 | 10.0000 | | | |
| 144.00 | 3 | 13.0000 | 13.0000 | | | |
| 120.00 | 3 | | 14.0000 | | | |
| 72.00 | 3 | | | 23.0000 | | |
| 96.00 | 3 | | | 25.0000 | | |
| 24.00 | 3 | | | | 33.3333 | |
| 48.00 | 3 | | | | | 66.0000 |
| Sig. | | .062 | .177 | .468 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.