BIODEGRADATION OF PALM OIL MILL EFFLUENT BY INDIGENOUS IMMOBILIZED BACTERIAL ISOLATES

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ABSTRACT

the production of palm oil resulted in the formation of vast amounts of polluting wastewater known as palm oil mill effluent (POME).). This study was designed to determine the biodegradation potential of indigenous immobilized bacteria isolated from palm oil mill effluent. *Bacillus subtilis, Pseudomonas aeruginosa, Bacillus megaterium*, and *Bacillus cereus* were isolated from the POME. The immobilized bacteria in form of bead were taken with the use of sterile spatula for biodegradation of the effluent. The experimental set-up was for four (4) days interval. Day zero, day four (4), day eight (8), day twelve (12), day sixteen (16), day twenty (20) and day twenty-four (24). Results revealed that percentage degradation for *Bacillus subtilis* recorded the highest percentage of degradation of 43.85% followed by *Bacillus megaterium* with 42.02%, *Pseudomonas aeruginosa* recorded 22.94% while *Bacillus cereus*recorded the lowest degradation of 16.88%. This study suggests that the degrading ability of the indigenous immobilized bacteria isolated from POME is *a* clear indication that these bacteria could be used for POME biodegradation

TABLE OF CONTENTS

Contents **Page**

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

LIST OF TABLES

Table

Page

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND TO THE STUDY

Palm oil has grown in importance as a worldwide agricultural product for food and non-food uses, value-added product manufacture, and, more recently, as a viable feedstock for biofuel production (Aluyor *et al.*, 2009). In various regions of the world, such as Malaysia, Indonesia, Thailand, Columbia, and Nigeria, the oil palm business is an important agricultural sector (Izah and Ohimain, 2016). Currently, there are about five million hectares of palm planted in the world, representing 16 million tons of annual production (The United States Department of Agriculture (USDA 2019). Much of this oil is obtained from the African oil palm (*Elaeis guineensis*) and hybrids with other species as well.

About 50 percent of the global traded vegetable oil comes from palm oil. The palm oil industry is a major agro-based enterprise especially in the southern part of the world where palm oil trees are found both in the wild and plantations (Nwaugo, *et al.*,2008). Palm oil industries release Palm Oil Mill Effluent (POME) in colossal amounts with its attendant pollution impending. POME has unfavourable environmental ramifications including land and aquatic ecosystem contamination and loss of biodiversity and increase in Chemical Oxygen Demand COD and Biochemical Oxygen Demand BOD in environment (Singh, *et al.*,2009). The penetration of palm oil mill has been considered due to the ran off of its effluents into the waterways and ecosystems remaining a meticulous concern towards the food chain interference and water consumption (Cheng J., *et al.*, 2010) This can cause considerable environmental problems if the effluents are discharged without effective treatment as they pollute land and effectively suffocate other aquatic life. Thus, palm oil mills are required to treat their POME prior to discharging it into rivers and streams. In the process of palm oil milling, POME is mainly generated from sterilization and clarification of palm oil in which a large amount of steam and hot water are used.

Palm oil Mill Effluent (POME) is a thick brownish liquid that is composed of high concentrations of total solids, oil and grease, chemical oxygen demand (COD), and biological oxygen demand (BOD). The processes leading to the production of palm oil generate a number of waste products. These include empty fruit bunches, palm oil mill effluent (POME) and palm oil mill sludge (POMS). These wastes are produced in very huge quantities. Out of these wastes, POME is of great significance because it is considered harmful when it is discharged into the environment untreated (Rupani *et al.,* 2010).

It has been estimated that for every tonne of crude palm oil produced, 5-7.5 tonnes of water are used and that about half of this water ends up as palm oil mill effluent (POME) (Oviasogie and Aghimien, 2011). The palm oil mill industry thus produces huge quantities of waste which if not properly managed, can cause significant environmental problems. Excessive generation of reactive oxygen species (ROS) is an integral part of many stress situations. ROS include hydrogen peroxide, hydroxyl radical and superoxide. The levels of these ROS can be controlled by the activities of antioxidant scavenging enzymes such as superoxide dismutase, catalase and peroxidase. Biodegradation of POME is essential to avoid environmental pollution (Ohiman *et al.,* 2012)

In general, there are four types of treatment systems adopted by the palm oil industry, which are as follows: Waste stabilization ponds, activated sludge system, Closed anaerobic digester and Land application system (Rupani *et al.,* 2010). An interesting option for the management of POME and other liquid wastes relies on biological treatment, which allows the degradation of the organic contaminants present in effluents, while generating organic sludge that can be later exploited for the production of compost (Roig *et al.*, 2006). Unfortunately, in developing countries like Nigeria, it is very difficult to manage the pollution caused by POME due to ignorance, use of crude milling equipment/facilities and lack of implementation of discharged standards, therefore POME produced by small scale producers undergoes very little or no treatment before being discharged to the environment.

Immobilization is the method of entrapping/attaching the microbial or plant cells in a suitable matrix. Different methods such as encapsulation, gel entrapment, covalent bonding, cross-linking and adsorption are carried out to prepare immobilized cells. When compared to enzyme immobilization, cell immobilization is regarded as a convenient method because of its low cost and improved stability. The cost of isolation and purification is very high in enzyme immobilization. These immobilized cells are mainly used in the areas of biotechnology and industrial production (Elakiya *et al.,* 2016).

Microbial immobilization has been defined as the physical confinement or localization of viable microbial cells to a certain defined region of space in such a way as to limit their free migration and exhibit hydrodynamic characteristic which differ from those of the surrounding environment while retaining their catalytic activities for repeated and continuous use (Dervakos and Webb, 1991; Freeman and Lilly, 1998; Covizzi *et al.,* 2007; Amim *et al.,* 2010).

Biodegradation is the process by which organic substances are broken down into smaller compounds by living microbial organisms (Marinescu *et al.,* 2009). When biodegradation is complete, the process is called "mineralization". Several microorganisms, including fungi, bacteria and yeasts are involved in biodegradation process. Algae and protozoa reports are scanty regarding their involvement in biodegradation (Das and Chandran 2011). Biodegradation processes vary greatly, but frequently the final product of the degradation is carbon dioxide (Pramila *et al.,* 2011).

So far, some investigations have been carried out on degradation leading to reduction in organic waste embedded in Palm oil mill effluent (POME) (Wu *et al.,* 2010), despite that it was still observed that some physicochemical property such as Total Suspended solids (TSS) which is a chief cellulite entity of wastewaters was still present even after several chemical and physical treatment. This poses a problem that needs to be resolved (Das and Chandran 2011). Introduction of microorganisms as a biological option in the treatment of wastewaters offers an answer to the reduction of TSS, COD, oil and grease and BOD of POME (Bala *et al.,* 2016).

Biological treatment of waste water consists of mixed communities with a wide range spectrum of microorganisms including bacteria, rotifers, protozoa and even algae. In addition, the mixed microbial combination consortium showed the maximum percentage reduction of organic load. Combination of those mixed cultures display metabolic versatility and superiority to pure cultures. Sathishkumar (2008) reported that a microbial consortium containing a number of microorganisms is considered to be well suited for the degradation of industrial wastewaters. Sathishkumar (2008) used a combination of bacteria and yeast to degrade olive oil mill wastewater (OMW). The yeast, *Yarrowia lipolytica*, reduced 80% of COD from OMW. Oswal *et al.,* (2002) has used the combination of *Yarrowia* with a consortium of bacteria and algae developed from garden soil, achieving 95% of COD reduction for the treatment of POME. Other investigators have reported reduction of organic load (COD, BOD, TSS and O & G) from POME and bakery wastewater with cultures of microorganisms. *Acinetobacter* sp. (KUL8), *Bacillus* sp. (KUL39), and *Pseudomonas sp. (KLB1* (Bala *et al.,* 2014b) and *Trichoderma harzianum and Penicillium* (Abdul aziz *et al.,* 2007). Microbial degradation of organic wastes in wastewaters using microorganisms such as bacteria, molds and yeasts had shown to be capable of completely degrading organic matter in oily wastewaters (Bala *et al.,* 2016). Microbial degradation of oily wastewaters involves the application of variety of microorganisms which has demonstrated effective degradation of oil in wastewater (Wu *et al.,* 2006). Similarly, treatment of wastewater containing high concentrations of oil and grease matter with photosynthetic bacteria from food and agricultural wastewater has been reported to achieve remarkable reduction of COD and oil and grease (Tambekar *et al.,* 2010).

There has been very scarce or no work has been done to the best of my knowledge on the use of immobilized bacteria on the degradation of Palm Oil Mill Effluent using indigenous. The immobilized indigenous bacteria were isolated from the POME and in turn the POME was used as the substrate in this study. The cellulose which constitutes about 50% of the POME organic matter is the total suspended solids (Choi *et al.,* 2013).

1.2 Statement of the Research Problem

Suspended solids have been identified as one of the major contributors of water contamination. Organic matter is defined as suspended solids in POME (Iwuagwu *et al.*, 2014). The total suspended solids (TSS) in the POME sample, which reflect the cellulosic components, have been scarcely investigated to the best of my knowledge. To treat agricultural industrial effluents, a variety of physical and chemical treatment techniques have been developed, however, the issue of chemical residues and total suspended solids (TSS), which persist after treatment, needs to be addressed further (Aliyu *et al.,* 2010).

As a result, TSS limits the amount of light that reaches a water body, limiting algae's capacity to create food and oxygen (Alade *et al.*, 2011). Suspended solids obstruct proper drinking water treatment and lead to water turbidity, or cloudiness (Jameel and Olanrewaju, 2011).

In Nigeria the business of palm oil extraction is dominated by peasant farmers who used mainly the semi-mechanized method of extraction (Orji, 2006). Consequently, the POME generated is discharged into available land near the mill. When the POME has accumulated considerably in the area due to continued deposition, the site is abandoned due to pollution of the site by the POME and fresh space is located. The ecological and pollution hazards associated with this disposal method in streams have severally been reported (Salahaldin *et al.,* 2021). POME physicochemical study reveals that most parameters are significantly beyond Environmental Health and Safety (EHS) requirements, and the heavy load of microorganisms confirms the lack of any effluent treatment. The implications of releasing

POME without treatment are far-reaching since the community's health is jeopardized (Verla *et al.*, 2014).

The presence of unrecovered palm oil contributes to the high content of degradable organic matter in raw or partially treated POME (Ahmad *et al.,* 2003). POME that hasn't been treated has a lot of fatty acids, proteins, carbohydrates, and other plant elements, which have the potential to change environmental factors like Biochemical Oxygen Demand (BOD), Carbon/Nitrogen ratio and Chemical Oxygen Demand (COD) level (Okwute *et al.,* 2007, Bala *et al.,* 2014). Due to oxygen depletion, land use, and other related impacts, this highly polluting POME may pollute streams. POME discharged into aquatic ecosystems renders the water dark, stinky, and slimy, potentially killing fish and other aquatic species, and denying human residents of such areas access to safe drinking water.

1.3 Justification for the Study

Several palm oil extraction disposal sites have been studied; however, there is scarce information available for biodegradation of palm oil mill effluent (POME) by indigenous microorganisms isolated from POME disposal site of Dekpor Irruan of Boki Local Government Area, Northern Cross River, Cross River State, and South South Nigeria.

Previous studies have shown the use of microorganisms to degrade POME with the microbes either isolated from it, or gotten from other source isolated (Bala *et al.,* 2014; Okwute *et al.,* 2015; Panida *et al.,* 2015). There is scarce literature on the use of immobilized indigenous microorganism isolated from the POME to degrade the POME. Therefore, the use of immobilized microorganisms will allow for, More efficient operation by reducing the more productive growth phase, Easy separation of the biomass from the liquid, Specific product creation, Immobilization protects the cells from shear forces and imparts a special stability to the microorganism against environmental stress like pH, temperature, organic solvents, salts inhibiting the substrates and products, poisons and self-destruction, The cells can be maintained for a long time period, which facilitates continuous cultivation processes and results in better operational stability, Cell washout is avoided even at high dilution rates of the continuous mode, Immobilized cells can be handled more easily and recovered from the solution without difficulty

Furthermore, cell immobilization is a promising tool in the treatment of toxic pollutant in industrial waste water (Claudia *et al.,* 2013). As a result, the degradation of organic matter in palm oil mill effluent (POME), specifically the basic physicochemical contaminants in POME, was investigated using their indigenous (autochthonous) immobilized isolates in this work.

1.4 Aims and Objectives

This study is aimed at the biodegradation of Palm Oil Mill Effluent by Indigenous Immobilized bacteria isolated from Palm Oil Mill Effluent while the objectives of this study are to:

I. Isolation and molecular identification of bacteria from POME
II. Immobilization of bacteria cells
III. Determination of the physicochemical properties of the POME.
IV. Determination of the biodegradation and percentage reduction of selected physicochemical properties of POME.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Palm Oil Industry

Palm oil industry has become one of the main Agro industries in Malaysia. Palm oil mills release POME in colossal amounts with its attendant polluting impending. POME has unfavourable environmental ramifications effects including land and aquatic ecosystem contamination and loss of biodiversity and increase in COD and BOD in environment (nee'Nigan and Pandy, 2009; Wu, *et al.,* 2010). Today, the penetration of palm oil mill has been considered due to its effluents into the waterways and ecosystems remaining a meticulous concern towards the food chain interference and water consumption (Wu, *et al.*, 2010). This can cause considerable environmental problems if discharged without effective treatment by polluting land and effectively suffocating other aquatic life (Cheng *et al.,* 2010). Thus, palm oil mills are required to treat their POME prior to discharging it into rivers and streams. In the process of palm oil milling, POME is mainly generated from sterilization and clarification of palm oil in which a large amount of steam and hot water are used (Rupani *et al.,* 2010). POME is a thick brownish liquid that is compiled with high concentrations of total solids, oil and grease, chemical oxygen demand (COD), and biological oxygen demand (BOD) (Poh and Chong 2009). The biological treatment depends enormously on consortium of microorganism's activities, which operate the organic substances present in the POME as supplements and eventually degrade these organic matters into simple by-product such as methane, carbon dioxide and hydrogen supplied, and water. The biological treatment process requires large pond to hold the

POME in place for the effective biodegradation, which regularly takes a few days relying upon the sort and native of the microorganisms (Wu, *et al.,* 2010). Besides, so as to enhance the effectiveness of this medication process, powerful mono or combined culture of feasible fungi and bacteria in biodegradation treatment of POME waste. Therefore, the challenge of converting POME into an environmentally friendly waste demanded an efficient treatment and effective disposal technique.

Palm oil mill effluent (POME), as well known as Palm oil effluent (POE), Palm oil slurry (POS), or Palm oil sludge (POS), is a perennial plant with one of the most common species "Elaeis guineensis" grown extensively in West Africa's humid tropical and subtropical countries, in which it came into existence (Bambang *et al.,* 2012; Alam *et al.,* 2007). (Ohimain & Izah, 2014; Izah, Angaye, & Ohimain, 2016).

The palm oil industry is one of the major agro-industries in Malaysia. The production of palm oil, however, results in the generation of large quantities of polluted wastewater commonly referred to as palm oil mill effluent (POME). The most significant pollutant from palm oil mils is POME (Poh and Chong, 2009). Typically, 1 tonne of crude palm oil production requires 5–7.5 t of water; over 50% of which ends up as POME. This wastewater is a viscous, brownish liquid containing about 95–96% water, 0.6–0.7% oil and 4–5% total solids (including 2– 4% SS, mainly debris from the fruit). It is acidic (pH 4–5), hot (80–90 0C), nontoxic (no chemicals are added during oil extraction), has high organic content (COD 50,000 mg/l, BOD 25,000 mg/l) and contains appreciable amounts of plant nutrients (Singh *et al.,* 2010). POME contains about 4000–6000 mg/l of oil and grease (Ahmad *et al.,* 2005). The composition of POME are mainly water, oil, suspended solid, dissolved solid and sand (Ibrahim *et al.*, 2013), total suspended solids (TSS), as well as cellulose wastes (Rupani *et al.,* 2010), vegetative matter, colloidal slurry of water and solids including about 2% suspended solids originating mainly from cellulose fruit debris, that is, palm mesocarp (Poh and Chong, 2009). The suspended solids in POME which are the cellulolytic material derived from palm mesocarp are organic in nature and considered as organic matter (Clarens *et al.,* 2010) and constitute about 50% of the POME (Ho *et al.,* 1983; Ho *et al.,* 1984). Treatment and disposal of oily wastewater, such as palm oil mill effluent is presently one of the serious environmental problems contributors. Palm oil mill wastes have existed for years but their effects on environment are at present more noticeable. The oily waste has to be removed to prevent interfaces in water treatment units, avoid problems in the biological treatment stages, and comply with water-discharge requirements (Ahmad *et al.,* 2005). Palm oil mill effluent (POME) is an important source of inland water pollution when released into local rivers or lakes without treatment. POME contains lignocellulolic wastes with a mixture of carbohydrates and oil (Oswal *et al.,* 2002). Recently, various physical and chemical treatment processes have been designed to treat POME, however, the problem of chemical residues and total suspended solids (TSS) which is still present after the treatment process remain to be resolved further (Abdul aziz *et al.,* 2007). The use of microorganisms in biological treatment of POME in this present study offers an alternative solution to reduce the TSS and organic load content of the effluent (Alam *et al.,* 2009). Palm oil industries are facing tremendous challenges to meet the increasingly stringent environmental regulations (Najafpour *et al.,* 2006). Thus, it is obvious that the presence of high levels of fat, oil and grease in wastewater induces serious problems not only to the receiving water but also to treatment plants and waste collecting systems. Although oil is not generally thought of as a material which is discharged into Land Rivers, it can and does reach these waters; not only as tin coloured films but also in sufficient volumes to necessitate the closing of abstraction points (Panida *et al.,* 2015). It is therefore essential that the potential danger from oil pollution is fully appreciated. The various effluent treatment schemes which are currently used by the Malaysian palm oil industry are listed in descending order:

(a) anaerobic/facultative ponds (Wong *et al.,* 1980),

(b) Tank digestion and mechanical aeration,

(c) Tank digestion and facultative ponds,

(d) Decanter and facultative ponds, and

(e) Physico-chemical and biological treatment (Amim 2010).

The current methods adapted for the treatment of palm oil mill effluent (POME) in most of the mills in Malaysia is the ponding system in which about 85% of the mills practice (Poh and Chong, 2009). This is not very effective in treating the pollutants in the POME to the stringent standards required (Jameel and Olanrewaju, 2011); The status and concentration of the oily matter/oil residue (oil and grease) after the treatment process is given less attention and this suggest that this approach employed is not sustainable to minimize the environmental impact of oil and grease in POME. Moreover, the range of concentration of oil and grease in POME is relatively higher than those obtained in toxic wastewater (Jameel and Olanrewaju, 2011). Thus, the need for effective treatment process for POME. The anaerobic digestion treatment of POME using various types of bioreactors by researchers and the ponding systems in the mills uses undefined microbial populations (McHugh *et al.*, 2003) to reduce the polluting power of wastes and wastewaters. This involves a consortium of undefined microorganisms catalysing a complex series of biochemical reactions that mineralize organic matter producing methane and carbon dioxide. These microorganisms are not established and hence the substrate they degrade and utilize is not ascertained. This led to poor effluent discharge into the environment as the performance of the microorganisms with regards to the rate of reduction and removal of oily waste cannot be monitor since they are not known. The present study will use defined/known microorganisms isolated from POME to inoculate the POME and monitor the percentage removal/reduction of the physicochemical parameters with a view to enhance treatment. This emphasizes the originality of the study and hence, this has therefore attracted the interest of this study. Furthermore, since several researchers based their findings on the overall COD removal, methane production and not the individual microorganisms (using undefined microbial population) utilizing and degrading the components in POME making up the COD and BOD, tailored to the fact that, no work has been done on the isolation of different individual microorganisms breaking down and utilizing the different components in POME making up the COD and BOD in order to remove or reduce the organic load level. Therefore, this research to the best of our knowledge can be listed as a novel study. Very few investigations have been conducted on aerobic digestion process for the treatment of oil and grease present in POME (Wu *et al.*, 2010). The major problems lie in the establishment of the most suitable microbial population for POME waste to be treated (Yacob *et al.*, 2006; Poh and Chong, 2009). Some aerobic treatment approaches include: degradation of POME using a tropical marine yeast (Yarrowia lipolytica) NCIM 3589 in a lagoon (Oswa *et al.,* 2002), trickling filter (TF) (Norulaini *et al.,* 2001) and rotating biological contactors (RBC) (Najafpour *et al.,* 2005). Organisms used for these aerobic treatments by the investigators are isolated from different source while this present study will isolate indigenous organisms from POME for the treatment. Microbial degradation of oil wastewater is a concern in recent years. A variety of microorganisms such as bacteria, molds, and yeasts, have been shown to be capable of completely degrading oil wastewater (Erguder *et al.,* 2000; Kissi *et al.,* 2001 Ettayebi *et al.,* 2003; Dhouin *et al.,* 2006). Therefore, using of microorganisms for treatment and bioremediation purposes affords a very efficient tool for purifying contaminated effluents and natural water (Glazer & Nikaido 1995). Using bacterial strain that possesses high efficiency in accumulating toxic contaminants or biodegradation of persistent biodegradable matter has potential in the use of the treatment system to remove pollution such as oil and grease or heavy metals from any polluted aquatic effluent (Campere *et al.,*1993). The application of microorganisms such as *Trichoderma viride spores, T. viride mycelium, Yarrowia lipolytica* and *Saccharomyces cerevisiae* for the treatment of POME have not been extended to the removal of oil and grease (Jameel and Olanrewaju, 2011) despite their high potential in removing COD from POME. This may be due to the fact that these microorganisms are not indigenous to POME. This therefore offer researchers a greater opportunity to investigate the removal of oily matter/oil residue (oil and grease) from POME using microorganisms isolated from POME (Jameel and Olanrewaju,2011). This is the focus and emphasis of the present study and it is design for this purpose. Hence, this has therefore attracted the interest of this study. The main aim of the present study was to evaluate the biodegradation potential of bacteria isolated from POME and to find the most suitable strain(s) for a biological treatment technology of POME.

2.2 Palm Oil Management

Around 44 million m^3 of POME were produced in year 2013 to yield 19.66 million tons of total crude palm oil. Around 85% of palm oil mills have treated raw POME using biological treatment (Tong and Jaafar, 2006). The biological treatment of POME is a series of pond systems, including anaerobic, facultative and aerobic pond systems (Ahmad *et al.,* 2003). However, the final treatment by aerobic pond system is struggling to achieve the discharge standards because of inefficient operational design (Parthasarathy *et al.,* 2016). The final effluent of the treated POME must comply with the discharge standards set by the Department of Environment (DOE), Malaysia. There is a requirement for a sound and effective management system in the treatment of these by-products in such a way that will assist to protect the environment and check the deterioration of air and river water quality. Treatment of POME is vital to avoid environmental contamination (Kamyab *et al.,* 2013).

2.3. Wastes Generated from Palm Oil Mills

Concomitant production of huge wastes always results from processing of oil palm fresh fruit bunches (FFB) primarily for palm oil. Prasertan and Prasertan (1996) reported that during processing in palm oil mills, more than 70% (by weight) of the processed FFB is usually left over as oil palm wastes. The wastes products from oil palm processing consist of oil palm trunks (OPT), oil palm fronds (OPF), palm oil mill effluent (POME), empty fruit bunches, Palm Oil Mill Effluent (POME) From Malaysia Palm Oil Mill, palm press fibre (PPF), shell palm oil mill sludge (POMS), and palm kernel cake (PKC) (Aziz & Abdul, 2007; Singh, Hakimi & Esa, 2010).

According to Pleanjai et al., 2004), fibre, shell, decanter cake and EFB accounts for 30, 6, 3, and 28.3% of FFB respectively. Palm kernel oil (white palm oil) is obtained from the seed known as kernel or endosperm. When oil has been extracted from the kernel, what remains is known as palm kernel cake (PKC). This is rich in carbohydrate (48%) and protein (19%) as reported by Onwueme and Sinha (1991).

POME; "Waste or Resource' which is the focus of this research is generated mainly from oil extraction, washing and cleaning processes in the mills. POME consists of water-soluble components of palm fruits as well as suspended cellulosic materials like palm fibre, fat, grease and oil residues (Agamuthu, 1995). However, (Rupani *et al.*,2010) also argued that among the wastes that are generated from processing of oil palm fruits, POME is considered the most harmful waste to the environment if discharge untreated.

2.4. Palm Oil Mill Effluent (POME)

POME is a colloidal suspension originating from mixture of sterilizer condensate, separator sludge and hydro cyclone wastewater in a ratio of 9:15:1 respectively (Wu *et al.*,2010). According to Borja & Bank (1994); Ma & Ong (1985), about 2.5-3.0 tonnes of POME per tonnes of produced crude oil is obtained in the extraction processes. POME, when fresh, is a thick brownish colloidal mixture of water, oil and fine suspended solids. It is hot (80-90oc) and possess a very high Biochemical Oxygen Demand (BOD) which is non-toxic as no chemicals are added to the extraction process (Khalid & Mustafa, 1992; Ma *et al.*,1993), and also acidic with a PH of around 4.5 as it contains organic acids in complex forms that are suitable to be used as carbon sources (Md Din *et al.*,2006). However, Ho and Tan, (1983) reported that the suspended solids or particulate fraction of the effluent contribute with less than 50% to the total pollutant level.

In view of Ng *et al.,* (1987), POME may vary considerable for different batches, days and factories, processing techniques and the age or type of fruit. While Ahmad *et al*., 2006), reported that nature of POME may as well depends on the discharge limit of the factory, climate and condition of the palm oil processing. Ahmad *et al.*, (2003) noted that raw or partially treated POME has an organic matter which is due in part to the presence of unrecovered palm oil. This highly polluting wastewater according Ahmad *et al.*, 2003), can cause pollution of water –ways due to oxygen level in rivers leads to anaerobic conditions and the release of noxious gases, particularly hydrogen sulphide. Thus, the natural ecology of the rivers is destroyed (Khalid & Wan Mustafa, 1992). Raw or untreated POME is characterized by high BOD often in the range of 25,000gm/l or higher.

In particular, POME is a basic expression referring to the effluent from the last phases of palm oil manufacture in the mill. It incorporates different fluids, dirt, leftover oil and suspended Palm Oil solids. POME in its untreated shape is a high-quality waste, relying upon the operation of the procedure. POME is generated mainly from oil extraction, washing and cleaning processes in the mill, and these contain cellulosic material, fat, oil and grease, and so on (Bala *et al.,* 2014). POME also contains substantial quantities of solids; both suspended solids and total dissolved solids in the range of 18,000 and 40,500 mg/L, respectively. Oil palm is the most productive oil producing plant in the world, with 1 ha of oil palm producing between 10 and 35 tons of fresh fruit bunch (FFB) per year (Bala *et al.*, 2014). During processing of oil palm, more than 70% by weight of the fresh fruit brunch was left over as waste (Chavalparit *et al.*, 2006). Usually, the harvested part is the fruit whereby oil is obtained from the fleshy mesocarp of the fruit. Despite the importance of the edible oil and fats extracted from the palm fruits, the POME contains residual oil which affects the environment cannot be ignored. Treatment and disposal of oily wastewater such as POME is presently one of the serious environmental problems. Palm oil mill wastes have existed for years but their effects on environment are at present more noticeable (Bala *et al.,* 2014). The oily waste has to be removed to prevent problems which are considered as hazardous pollutants particularly in the aquatic environments because they are highly toxic to the aquatic organisms. Discharging the effluents or by-products on the lands or release to the river may lead to pollution and might deteriorate the surrounding environment. In order to conserve the environment, an efficient management system in the treatment of these by-products is needed (Kamyab *et al.,* 2014). Treatment of POME is essential to avoid environmental pollution (Kamyab *et al.,* 2016). POME wastes are the fiber free non-oil components obtained from the clarification zone of an oil mills. The significant contamination comes out of the fresh fruit brunch (FFB). In fact, every ton of FFB is composed of 230–250 kg of empty fruit bunches (EFB), 130–150 kg of fibers, 60–65 kg of shell and 55–60 kg of kernels and 160–200 kg of unrefined oil. POME contains high amounts of oil and grease (4000 mg/L) and COD (50,000 mg/L). Although the effluent is nontoxic, it has a very high concentration of biochemical oxygen demand (BOD) (i.e., 25,000 mg/L) which becomes a serious threat to aquatic life when discharged in relatively large quantities into watercourses. The high number of total solids (40,500 mg/L) contributes to the large amount of nutrients available in the wastewater, hence possible algae bloom. In general, there are four types of treatment systems adopted by the palm oil industry, which are as follows:

a. Waste stabilization ponds,

b. Activated sludge system,

c. Closed anaerobic digester and

d. Land application system.

The most proper secondary treatment for POME is natural assimilation with the blend of anaerobic and aerobic ponds. Right now, the management of POME has developed from treatment of waste for transfer to gainful use of assets. POME contains generous amounts of significant plant supplement that shift as indicated by the level of treatment to which it is subjected. The potential utilization of recovery of water and natural issues from POME has been applied for different applications (Kamyab *et al.*, 2015). Commercial trials and applications of these Palm Oil Mill Effluent as an Environmental Pollutant are currently underway, especially conversion of the solid residual materials into saleable value-added products.

2.4.1 Composition of Palm Oil Mill Effluent

Composition of POME depends mainly on raw material quality; season and the particular operations being used at any given time. As stated earlier, POME when fresh is a thick brownish colloidal mixture of water, oil and fine suspended solids. It is hot (80-90oc) and possesses high amounts of total solids (40,500gm/l), very high BOD of 25,000gm/l, Chemical Oxygen Demand (COD) of 50,000gm/l, oil and grease of 4,000gm/l (Ma, 2000). Khalid & Wan Mustafa, (1992) and Ma et al., (1993) all reported that POME is non-toxic, as no chemicals are added during extraction process. However, POME is low in PH because of the organic acids produced in the fermentation process; it is acidic with PH of about 4.5 as it contains organic acids in complex forms that are suitable to be used as carbon source (Md Din *et al.*,2006). Ugoji, (1997) confirmed that POME consist of water-soluble components of palm fruits as well as suspended materials like oil residues, short palm fibre, cell walls, organelles, a variety of carbohydrates ranging from cellulose to simple sugars, a range of nitrogenous compounds from proteins to amino acids, free organic acids and assembly of minor organic and mineral constituents.

Nutrients contains in POME as reported by Habib *et al.*, (1997) and Muhrizal *et al.*, (2006), are nitrogen, phosphorus, potassium, magnesium and calcium, which are all vital nutrients elements for plant growth. High content of A1 in POME as compared to chicken manure and composted sawdust was also reported by Muhrizal, (2006). According to Habib *et al.*, (1997), toxic metals such as lead can also be found in POME, but James *et al.*, (1996), argued that lead concentration are usually below sub lethal levels (> 17.5 ug/g) and in their view lead is found in POME as a result of contamination from plastic and metal pipes, tanks and containers where lead is widely used in paints and glazing materials.

POME, despite its biodegradability, cannot be discharged without first being treated because it is acidic and contains residual oil that cannot be easily separated using conventional gravity-based systems. Basically, this oily mix needs a lot of oxygen before it can decompose completely, and this phenomenon is called having high biochemical oxygen demand, and raw POME can sometimes have BOD of up to 100 times higher than that of domestic sewage. Because POME still contains a significant amount of organic matter even when treated, still imposes a demand on the environment. Microbes in water take in dissolved oxygen as they digest organic matter. This demand for oxygen known as BOD is usually measured in milligrams per litre (mg/l) and is normally used as an indication of the organic quality or the degree of organic pollution of water. Basically, a higher BOD means poorer quality, and the inverse holds true as well. Consequently, it has been observed that the microbial population increases in proportion to the amount of food available. In such conditions, the microbial action will consume dissolved oxygen faster than atmospheric oxygen can dissolve in the water. Apparently, fish and other aquatic organism might die because the water body has been depleted of its oxygen.

2.4.2. Palm Oil Mill Effluent as a Waste

POME is a by-product of a processed FFB to obtain mainly palm oil and other major components. According to one of the definitions of wastes by Pongrácz and Pohjola, (1999), waste is a man-made thing, which in a given time and place, in its actual structure and state, is not useful to its owner, or an output that does not have any owner. Clearly, because millers in the past have not completely found useful purpose for POME, it is seen and considered as a waste and a burden to palm oil industry Pongrácz and Pohjola (2004), stated in their view that, depending on the nature of a given waste, owners may be restricted in their ability to freely give up ownership. This lends further insight in to reasons why palm oil mills are restricted or regulated by environmental regulatory authorities when it comes to discharge or disposal of POME by palm oil mills. This is obviously due to the pollutant nature of the raw or untreated POME.

Although, new methods and technologies have been developed to find approachable solutions for POME management yet, palm oil mills are still struggling to meet up with more stringent limits of effluent discharge allowed by Department of Environment (DOE) Malaysia. However, these challenges faced palm oil mills can be overcome if POME can be re-defined as a secondary raw material as supported by Jacobs (1997), assertion that a "by-product or residual product does not constitute waste if it is destined for direct re-use in a further process. To this end Pongrácz (2000), defined non-waste as an object that has been assigned a purpose by its (or a potential) owner, and this owner will either use it for that purpose, or by adjustment of state or structure to ensure that the object is able to perform in respect to the assigned purpose. According to Pongrácz and Pohjola (2004), non-waste definition introduced by Pongrácz (2002) was necessary to avoid obstacles to resource conservation due to materials being considered waste. Pongrácz and Pohjola (1997), argued that waste is created as an unwanted but not avoided output with no purpose. In their view, waste is process-specific and can be avoided or minimised by changing the process performance and therefore most industrial processes that are aiming at a specified output often produce undesired by-products that we call waste (Pongrácz and Pohjola, 2004).

The new agenda for waste management focuses upon the development of more appropriate, sustainable definitions of waste, so that what is now commonly perceived as being waste will in fact be increasingly seen as resource-rich, "non-waste" (Pongrácz and Pohjola, 2004). Under the present definition of waste, which POME is also categorized, recoverable material or substance like POME is seen more as a potential pollutant rather than as a potential raw material or resource. The emergent of the new biotechnological advances and POME treatment technologies, like POMETHANE, has changed the status of POME from being a waste to non-waste or resource. Consequently, the use of current advance technologies to manage POME by mill operators will not only turn POME to resource but also means of achieving zero discharge concept been champion by environmentalist worldwide.

2.4.3 Palm Oil Mill Effluent as a Resource

Resource according to Wikipedia is a source or supply from which benefit is produced. Typically, resources are materials, money, services, staff or other assets that are transformed to produce benefit and, in the process, may be consumed or made unavailable. The question is can POME, a by-product of a processed FFB fit into above definition of resources? This paper explores the potentials of POME as a resource rather than just a by-product or waste.

What is required for POME to achieve this feat, according to Wu., (2009) is to adopt an international trend of promoting pollution prevention through cleaner production, which is based on the 5R policy. Therefore, the introduction of the emerging 5R policy namely; reduction, replacement, reuse, recovery and recycling into POME management through environmentally sound biotechnologies will change the status of POME from waste to resource.

The use of biotechnological advances and advance POME treatment technology in the sustainable reuse and recovering of POME has redefined and changed POME from being a waste to a valuable resource of different forms. Wu., (2006) and Suwandi (1991) pointed out the possibility of recovering and concentrating the available bio resources in POME by an ultra-filtration process in order for the concentrated bio resources to be reused more effectively as fermentation media, fertilizers and animal feeds. POME has also been successfully converted to energy through an anaerobic thermophilic digestion process which maximizes the yield of biogas production. The process captures the methane from POME to run a gas engine to generate electricity or alternatively turn the biogas in a boiler to generate steam and hot water.

2.4.4 Fermentation Media from Palm Oil Mill Effluent

Palm Oil Mill Effluent and its derivatives have been exploited as fermentation media to produce various products or metabolites such as antibiotics, bio insecticides, solvents (Acetone-Butanol ethanol; ABE), polyhydroxyalkanoates (PHA), organic acids as well as enzymes to varying degree of success Wu., (2007). The hydrogen production from POME during anaerobic treatment has also been intensively studied (Atif *et al.*,2005;

Vijayaraghavan & Ahmad 2006). In addition, it has been reported that POME also contains certain powerful water-soluble antioxidants phenolic acids and flavonoids (Wattanapenpaiboon & Wahlqvist, 2003).

According to Hwang et al., (1980); Phang (1990) and Habib *et al.*, (1997), the possibility of reusing POME as fermentation media is largely due to the fact that POME contains high concentration of carbohydrate, protein, nitrogenous compounds, lipids and minerals. Results of various studies on fermentation media such as antibiotics, bio insecticides, PHA, ABE, organic acid, enzymes and hydrogen from POME have been reported by; Lin., (2005); Uzel., (2005); Jamal., (2005); Takriff., (2005); Md Din., (2006); Masngut., (2007); Hipolito., (2007).

2.4.5 Palm Oil Mill Effluent as a Fertilizer

The potential for using POME as a cheap organic fertilizer that may offer an alternative to the excessive application of chemical fertilizers was reported by Wu *et al.*, (2009). Oviasogie & Aghimien (2011), in their work confirmed that a proper use and safe disposal of POME in the land environment is lead to improved soil fertility and contribute to environmental sustainability. Their results showed an enrichment of soils with regards to phosphorus. Copper, iron and lead were said to be predominant in their organic forms, while zinc was particularly present in its exchangeable form.

Wu *et al.*, (2009) reported that biologically treated POME has been widely used in the oil palm plantations for irrigation purposes and can be employed as a liquid fertilizer. It is estimated according to *Wu et al.*, (2009), that each 15 million tonnes of POME is have a fertilizer value of RM 95.41 million ($31.80 million)

However, Wood (1979) reported that an application of POME at 4.5 x 1061 per applied hectare was estimated to represent a fertilizer application of about 30kg ammonium sulphate, 7kg rock phosphate, 52kg potash and 18kg kieserite per palm per year.

According to Chan (1980), the use of POME has been shown to improve soil productivity and increase the yield of crops as well as contribute to better root health by improving the soil structure. Results of many other works on POME as fertilizer was also reported by; Muhrizal *et al.*, (2006); Guo (2007).

2.4.6 Use of Pome as a Live Food for Animals and Aquaculture Organisms

According to Wu *et al.*, (2009), POME as a dietary substitute for pigs, poultry and small ruminants as well as aquaculture organisms is gaining importance. Devendra (2004) reported that in Colombia, POME has been fed with good results directly to pigs (10-12 1/head/day) together with palm oil and other ingredient. The Malaysian Agricultural Research Development Institute (MARDI) proved that waste from the palm oil industry (such as oil palm sludge and palm press fibre) alone or in combination, dried to moisture contents of 7% could be used as supplementary food for sheep (Devendra and Muthurajah, 1976). Hutagalung *et al.*, (1977) in their work investigated the use of POME as animal feed for growing-finishing pigs, in which case two types of "meals" known as censor tk8 (35% palm oil sludge, 32.5% cassava root meal, 32.5% palm kernel cake) and tk9 (32% palm oil sludge, 34% cassava root meal, 17% palm kernel cake, 17% grass meal) were used. They argued that it was economical to replace 50% maize (which is the regular diet constituent) with a POME-based animal feed, thus saving up to RM 0.02 ($0.01) per pig per day. This lends further support to the view that animal feed production from palm oil waste can replace at least half of the amount of imported maize for poultry diets and up to 100% for pig diet. The nutritive values of a POME product known as "Prolima" as a protein source in broiler chicken diets were investigated by Yeong *et al.,* (1980). They observed that the amino acid content of palm kernel cake and palm oil sludge were somewhat close to cereal by-products and that of "Prolima" was between soybean meal and peanut meal, in which case the overall percentage of amino acid availability for palm kernel cake, palm oil sludge and "Prolima" were 74.4%, 24.8% and 71.0% respectively. It was also argued in the foregoing that, the concentrations of "Prolima" up to 30% could be included in broiler diets as a replacement for soybean meal without causing any adverse effect on the growth performance of the chickens (Yeong *et al.,*1980). According to Habib *et al.,* (1997), POME could also be reused as a food source by aquatic organisms such as chironomid larvae known as "bloodworms". They reported that production of chironomid larvae was significantly higher in POME (580g/20l POME) than in algal cultures (35g/20l algal culture). These chironomid larvae, in turn, present valuable live food for fish or cultured invertebrates (Shaw and Mark, 1980; Yusoff *et al.,*1996).

2.4.7 Palm Oil Mill Effluent as an Energy Source

Ponding or open lagoon system for treating POME in Palm oil mills in Malaysia have been in existence for a very long time. The pond systems have been applied in Malaysia for POME treatment since 1982 and they are classified as waste stabilization pond (Onyia *et al.,*2001). Parveen *et al.,* (2010), reported that more than 85% of palm oil mills exclusively use ponding. Therefore, ponding system was the most conventional method for treating POME (Khalid & Wan Mustafa, 1992). Then, there was an introduction of close aerobic and anaerobic digestion tank or treatment in the management of POME to meet up with the discharge limits allowed. According to Perez *et al.,* (2001), anaerobic process is a suitable treatment method due to the organic characteristics of POME.

However, all the methods that are employed by mills for digestion of POME always produce or generate biogas as a by-product. But amount of biogas generated largely depends on the particular method used. In open tank digestion system, Yacob *et al.,* (2005) reported that every tonne of treated POME, an average of 5.5kg of methane (or approximately 36% of biogas) is emitted from open digesting tanks. This value is significantly lower than what was reported by Ma *et al.,* (1999), that is 65%. In total, an average of 5.4 1/min m2 biogas was recorded and total methane emission per open digesting tank was 518.9kg/day.

According to Ng *et al.,* (2011), it is estimated that 1 tonne of FFB processed will generate 0.67 tonne of POME, and each tonne of POME is able to produce 28m3 of methane. Organics, a company involved in project design to recover energy from POME also published that a typical mill rated at 40 tonnes per hour FFB can produce between 1 and 2 Mega Watt (MW) of electricity from the biogas that can be generated in an anaerobic digester. Biogas, a by-product of anaerobic degradation of POME consists of about 65% methane, 35% carbon dioxide, and trace of hydrogen sulphate. Ahmad *et al.,* (2011), reported that based on a study on Clean Development Mechanism (CDM), potential in the waste sectors (for energy source), it was found that the most potential is where anaerobic degradation takes place within the municipal landfills and POME ponds.

Therefore, POME is another potential energy source from which the methane gas or biogas released during anaerobic digestion can be collected for power generation (Wendy *et* *al.,*2012). To date; there are 411 local palm oil millers with the potential to be Independent Power Producers (IPPs) of sustainable green energy through utilization of methane from POME. Based on these potentials, it has long been suggested that Sabah could look at its palm oil mills as a source for electricity to overcome the power shortage supply especially in its east coast. Regrettable, more than 90% of the palm oil mills in Malaysia have been wasting this essential resource by its emission into the atmosphere, there by polluting our environment and contributing to global warming. However, complete lack of technology by palm oil mills to harness this biogas was the major reason for this unacceptable phenomenal in the oil industry.

Consequently, the development and introduction of new advance POME treatment technology for management of POME, has presented a new platform to change the trend. It is now possible for palm oil mills to generate, collect and utilize methane as energy source while treating POME. Many companies involved and specialized in liquid waste treatment have lunched the new advance POME treatment technology. Some of these companies, especially within the Southeast Asia have already entered into project agreement with some of the palm oil mills in Malaysia. Camco Southeast Asia, a regional clean energy company has completed arrangement to build a 2 MW biogas plant that will make use of methane pollutants generated from anaerobic digestion of POME. The $4 million project is part of 13 years “build-own-operate-transfer” agreement between Camco and a palm oil mill in Malaysia. Under the term of agreement, the miller owner will provide adequate POME feedstock for free for the entire duration of the contract.

In a related development, Kubota Corporation has also received a turnkey order for biogas recovery plant and POME treatment plant from a new palm oil mill in Bintulu, Sarawak, Malaysia. In 2012, Felda Global Ventures Holdings announced its readiness to install POME to-biogas plant. The RM 8 million (€2 million) pilot plant according to Felda is built in Kota Tinggi, Johor by Weida Bhd. The biogas is used to generate 2 MW of electricity that is sold to Tenega Nasional Bhd. In addition, Felda has also chosen Veolia Water Solutions and Technology to deliver a turnkey installation consisting of a biogas plant to treat POME generated from Serting Hilir palm oil mill. The biogas plant as reported by the company will generates about 1.2MW electricity per hour, and the electricity generated is supplying the mill's production and will also be connected to the grid of the local energy provider (Tenaga Nasional Bhd). Also taking advantage of this new technology for POME management through CDM projects are Alambumi palm oil mill Sdn Bhd Miri and Rinwood Pelita palm mill Mukah.

2. 5 Characteristics of Palm Oil Mill Effluent and Utilization by Microalgae

POME contains high content of degradable organic matter, which is due in part to the presence of uncovered palm oil (Ahmad *et al.*,2011). The discharge of improperly treated POME creates adverse impact to the environment. However, the substances in POME are able to support the growth of microalgae. Microalgae naturally exist in many palm oil mill processes, phenomena known as "algae bloom," hence declining the water quality. Because POME consists of large number of organic compounds and inorganic compounds which is hazardous to environmental health, microalgae have been suggested as a potential candidate to remove these pollutants and able to breakdown the organic compounds present in it (KaMunoz and Guieysse., 2006; Kamyab *et al.*, 2016).

Alternatively, culturing microalgae in wastewater offers an economy, which is alternative to the traditional types of wastewater treatment (Hoh *et al.*,2016). In the meantime, microalgae can apply the nitrogen and phosphorus compound in wastewater to produce microalgae biomass for various kinds of lipid generation, which can serve as a substrate for biofuel production (Kamyab *et al.,* 2016).

POME is a colloidal suspension, starting from the blend of sterilizer condensate, separator sludge and hydrocyclone wastewater in a proportion of 9:15:1, respectively (Wu *et al.,*2009). In total, about 2.5–3.0 tons of POME for huge amounts of produced crude palm oil is obtained in the extraction procedure (Ma *et al.,* 2000). Fresh POME is a thick brownish colloidal blend of water, oil and fine-suspended solids. It is hot (80–900°C) and has a high BOD, which is 100 times as contaminating as domestic sewage (Wu *et al.*,2009). The effluent is not hazardous, as no chemicals are added to the extraction procedure (Wu *et al.*,2009), and also acidic with a pH around 4.5 as it contains organic acids in complex forms that are suitable to be used as carbon sources (Din *et al.*,2006). Palm oil mill effluent is a high-strength pollutant with low pH due to the organic and free fatty acids arising from partial degradation of palm fruits before processing. The characteristics of POME depend on the quality of the raw material and the production processes (Aliyu *et al.,* 2012).

POME while fresh is hot acidic and pH range between 4 and 5, brownish colloidal suspension containing high concentrations of natural matter, high quantities of total solids (40,500 mg/L), oil and grease (4000 mg/L) COD (50,000 mg/L) and BOD (25,000 mg/L) (Bala *et al.*,2014). However, it also contains appreciable amounts of N, P, K, Mg and Ca which are the vital nutrient elements for plant growth (Kamyab *et al.*,2016). According to

Kamyab *et al.,* (2016), the raw or partially treated POME has an extremely high content of degradable organic matter.

However, it has nontoxic nature and has fertilizing properties, POME can be used as fertilizer or animal feed substitute, in terms of providing sufficient mineral requirements. The Malaysian government provides an effort to reduce the effluent of palm oil through licensing system, which mainly consists of effluent standards and effluent charges. According to POME characteristic and standard discharge limit in Environmental Quality Act (EQA) 1974, the palm oil industry faces the challenge of balancing the environmental protection, its economic viability and sustainable development. The year 1978 witnessed the enactment of the Environmental Quality Regulations detailing POME discharge standards. Normally, the characteristics of POME may vary considerably for different batches, days and factories, depending on the processing techniques and the age or type of fruit as well as the discharge limit of the factory, climate and condition of the palm oil processing (Ahmad A.L., *et al.,*2003). Occasional oil palm cropping and activities of the palm oil will also impact those quality and quantity of the discharged POME, thus influence the ecological treatment procedure of POME (Yacob S. *et al.,* 2005). Hence, the variation of the characteristics of POME, in terms of its quality and quantity, is the main reason that causes selection in the treatment of POME in the palm oil industries (Wang *et al.,* 2009).

2.6 Wastewater Treatment Technology

The wastewater treatment technologies are expensive, dependent on skilled personnel and hard to carry out, as the volume of contaminated wastewater is huge (Ahmad *et al.,*2003). Furthermore, the common conventional treatment is unable to meet the regulations set by the Department of Environment (DOE) with the level of BOD at 100 mg/L. According to Ahmad *et al.,* (Ahmad *et al.,* 2003), large quantities of water are used during the extraction of crude palm oil from the fresh fruit bunch, and about 50% of the water results in POME. The disposal of this very contaminating effluent is turning into a noteworthy issue assuming that it may be not continuously treated appropriately as well as a severe standard boundary obligatory set by the Malaysian Department of Environment for the discharge of effluent. A POME treatment system based on membrane technology shows high potential for decreasing the ecological issue, and also, this alternative treatment system offers water reusing (Ahmad A.L., *et al.,*2003). The utilization of wastewater for the microalgal growth is considered beneficial for limiting the utilization of freshwater, dropping the cost of supplement option, expelling nitrogen and phosphorus from wastewater and generating microalgal biomass as bioresources for biofuel or value-added by-products. Three primary sources of wastewater are municipal (domestic), agricultural and industrial wastewater which included a variety of elements. Some elements in the wastewater, such as nitrogen and phosphorus, are valuable components for microalgal cultures (Chiu *et al.,*2015).

2.7 Characteristics of Palm Oil Meal Effluent as Nutrients Source to Culture Microalgae

A life cycle assessment on microalgae cultivation has underlined that 50% of energy use and greenhouse gas emissions are associated with fertilizer (nutrients) (Clarens A.F. *et al.,* 2010). In general, culturing of microalgae on a large scale required high nitrogen and other related chemical fertilizers, which driven the process toward non-environmentally friendly.

On the other hand, culturing microalgae can actually play an important role as a self-purification process of natural wastewaters (Lam *et al.,* 2009).

Utilizing POME as supplements source to culture microalgae is not another scenario in Malaysia. Most palm oil millers favour the culture of microalgae as a tertiary treatment before POME is released because of practically low cost and high impact. Consequently, vast majority of the nutrients such as nitrate and ortho-phosphates that are not detached during anaerobic digestion is additionally treated in a microalgae pond. Thus, the cultured microalgae are used as a food nutrition for live feed culture (Lam *et al.,* 2009). Meanwhile, nitrogen source (usually appears in nitrate form) plays an important role in promoting microalgae growth. In order to grow microalgae effectively, the basic nitrate concentration required is in the range of 200– 400 mg/L. Other's minerals such as Fe, Zn, P, Mg, Ca and K that are required for microalgae growth are also present in POME. Thus, POME emerged to be an alternative option as a chemical remediation to grow microalgae for biomass production and simultaneously act as a part of wastewater treatment process (Lam *et al.,* 2009).

These days, there is an incredible and nonstop increment in industrialization, foundation and urban expansion in Asia, which has added to the critical wastes demand and water deficiency because of water contamination (Lam *et al.*, 2009). Industry in particular agro-based industry is one of the significant divisions releasing extensive amount of wastewater yearly influencing the other water sources and human life. The palm oil industry in Malaysia is generating the biggest amount of natural contamination loads into rivers (Arif *et al.*,2001). POME is a highly polluted waste having unpleasant odour. There is a greater need to find alternative way to utilize these organic pollutants for the good benefit of both human beings and the environment (Kamyab *et al.,* 2015). Microalgae cultivation in POME offers an alternative to conventional forms of tertiary wastewater treatments and spontaneously utilizes organic compounds present in POME to generate microalgae biomass for algae oil production (Lam *et al.,* 2009). There are several environmental and operational factors, which can affect the microalgae growth in order to make the cultivation fruitful. The natural effluent discarded from palm oil mill might be colloidal, dark and viscous, which should be considered prior media preparation for culturing the microalgae (Bello *et al.,* 2013). Vairappan and Yen 2008 had found that for the marine *Isochrysis sp*., the concentration of POME at 5% dilution is the best concentration for culture media due to properties of POME. This dilution procedure will then enhance the light penetration into media for the algal growth in wastewater (Olguin *et al.,*2003). As described in Table 5, limited growth conditions are required for the growth of microalgae using palm oil mill effluent. The concentrated nutrients (i.e., C, N, P, carbohydrate, lipid, protein and minerals) in POME are highly applied in biotechnology studies for growing microalgae (Kamyab *et al.,*2016). The concentration extend about POME in various accepting water body may give high effect on the aquatic environments if the release surpasses the limit of standards set by Malaysia Environmental Quality Act.

Numerous species of microalgae exist in freshwater, seawater or brackish make them appropriate to be grown in great scale reactor on unfertile lands. The usage of macroalgae and microalgae in the utilization or remediation of the excess nutrients and CO_2 present in natural water resources, lagoons and ponds is called as phycoremediation (Olguin *et al.,* 2003). This biological remediative treatment was introduced about 40 years ago when it was usually used in tertiary wastewater treatment (Rawat *et al.,* 2011). Kamyab *et al.*, 2013) have done their studies by focusing on the nutrient's reduction in POME, lipid production and microalgae growth. Meanwhile, it can be found that other researchers have not focused much on nutrient reduction, which is to be considered more important in relation to the growth of microalgae.

Malaysia is the biggest generator and exporter of palm oil. Palm oil processing is achieved in palm oil mills where oil is removed from a palm oil fruit bunch. Expansive amounts of water are utilized throughout the extraction of crude palm oil from the fresh fruit bunch, and around half of the water consequences in POME, which is a highly polluting wastewater that pollutes the environment if discharged directly due to its high COD and BOD concentration. In conclusion, the research was carried out mainly to investigate the influence of discharging POME from the treatment plant especially in tropical region like Malaysia and the effect on microalgae growth efficiency in POME. In other words, a combination of wastewater treatment and renewable bioenergy's production is an added advantage to the palm oil industry.

With the rising global demand for fats and oil, the palm oil industry has been growing rapidly thus becoming a major contributor to the economy of several tropical countries, including Malaysia. As one of the world's largest palm oil producers, Malaysia yields more than ten million tons of palm oil each year (Sheil *et al.,* 2009). The flourishing palm oil industry, however, has also brought along inevitable environmental problems when a massive volume of industrial effluent is discharged into the water sources. Palm oil mill effluent (POME) is a brown slurry of organic solids (4-5%), residual oil (0.5-1.0%) and water (95%) which is generated by the palm oil mill during the multiple processing steps of crude oil production (Onyla *et al.*, 2001). It is estimated that 5-7.5 tons of water are used during the process of palm oil production, and half of the water ends up as POME (Rupani *et al.*, 2010). With its rich organic content, high biological oxygen demand (BOD) and chemical oxygen demand (COD), POME is known to cause environmental problems such as eutrophication and water pollution (Lam and Lee, 2011). Many reported the release of untreated POME into the environment also causes loss of biodiversity and soil fertility (Awotoye *et al.*, 2011). As the awareness concerning public health and environmental sustainability begins to arise, there is a surging demand for efficient managing strategies to protect the environment from deterioration by POME.

In Malaysia, concerted research activities have been accentuated on the treatment of POME via various high-end processes. In order to establish successful pollution abatement, extensive studies have been carried out to investigate the characteristics of POME, ranging from its constituents, pH, toxicity to the thermal effect as well as its odour (Poh *et al.*, 2010). This allows wastewater treatment to be designed and developed precisely based on the extensive studies of POME. The efforts so far have welcome tremendous success, proving that a well-developed understanding on POME characteristics is the key of success to the wastewater treatment. While physical and chemical characteristics of POME are being well-studied, the biological aspect is often overlooked. Due to its richness in food resources such as hydrocarbons, nitrogenous compounds, lipids and inorganic minerals, POME is inhabited by a diverse microbial community (Hassen-Aboushiba *et al.,* 2013) which plays a crucial role in the natural degradation of POME. These microorganisms release hydrolytic enzymes such as cellulase, xylanase and lipase to break down the complex polymers in POME (Wong *et al.,* 2008). Some of the microorganisms are responsible for the removal of nitrogen and phosphorus from POME through bioaccumulation (Bao *et al.,* 2007). Other roles of microorganisms include phenol removal and decolourization of POME (Ayed *et al.,* 2002). Seeing the importance of microorganisms in biodegradation, it is believed that the performance of wastewater treatment can be promoted by developing a better understanding on the role of microorganisms associated with the degradation of POME. In this paper, microorganisms that inhabit POME are classified and reviewed in order to provide an insight of microbiological characteristics of POME so that removal efficiency of wastewater treatment can be enhanced through combining microbiological and physiochemical aspects of POME.

2.8. Classification of Microorganism in Characteristics of Palm Oil Mill Effluent

2.8.1. Prokaryotes

The eubacteria or "true" bacteria and archaebacteria or "ancient" bacteria are two domains of prokaryotic organisms reviewed in this paper. Both eubacteria and archaebacteria are unicellular organism while the archaebacteria have unique cellular chemistry. In general, these prokaryotes play vital roles in biological wastewater treatment processes. The archaebacteria groups can be represented by the halophiles, methanogens, or thermoacidophiles (Gergouri *et al*., 2006). In the subsequent review, these prokaryotes are classified in accordance to their respective roles in biodegradation and the processes they are associated with, the anaerobic digestion, the nitrification process, the denitrification process, and phosphorus accumulation.

A) Anaerobic Digestion Raw POME contains suspended solids (SS) which are mainly organic materials originating from oil palm fruit debris and dry plant matter. The first step in POME degradation is the removal of the bulk from the waste through a series of processes known as anaerobic digestion. Anaerobic digestion refers to the biological conversion of biodegradable constituents in wastewater into methane (CH4) and carbon dioxide in the absence of oxygen (Lam *et al.,* 2009). This collection of processes includes hydrolysis of carbon compounds, fermentation, acetate formation and methanogenesis, each of them assisted by a wide variety of microorganisms which existed in a symbiotic relationship,

The high organic content of POME in the form of cellulose, lignin and residual oil greatly favours the inhabitation of hydrolytic bacteria. This group of bacteria secrete extracellular enzymes such as cellulase, xylanase and lipase to hydrolyse carbon polymers into simpler substances, initiating the anaerobic digestion of POME (Hassan., 2002). The products of hydrolysis including simple sugars, fatty acids and amino acids (triglycerides) serve as the substrates for acidogenesis or fermentation in the subsequent step. As suggested by the name, acidogenic bacteria further degrade carbohydrates and fatty acids into simpler organic acids such as lactic acids, propionic acid and butyric acid as well as hydrogen gas (Cheng *et al.,* 2010). This process may take place through fermentation of anaerobic respiration. Organic acids produced are then utilized by acetogenic bacteria to form acetate.

Acetogenic bacteria often work in syntropy with some methanogens that consume hydrogen gas to yield methane. This is a mutualistic relationship as acetogenic bacteria rely on methanogens to keep the hydrogen partial pressure low so that oxidation of organic acids to acetate can take place, while methanogens need acetogenic bacteria to provide hydrogen gas as the substrate of methanogenesis (Ahmad *et al.,* 2011). Some acetogenic bacteria possess the ability to reduce sulphate and use it as electron acceptor to form sulphide gas (Wong *et al.,* 2009). Ultimately, methanogens utilize the end-products from the previous processes to form methane, completing the conversion of organic matter into biogas. Methanogens are archaea and generally divided into acetotrophic and hydrogenotrophic methanogens, differentiated by their substrate of methanogenesis. Hydrogenotrophic methanogens use hydrogen gas as electron acceptor during methanogenesis. Acetotrophic methanogens, on the other hand, cleave acetate to form methane.

B) Nitrification, Denitrification, and Phosphorus Accumulation Many have reported the richness of POME in nutrients such as nitrogen and phosphorus (Chowdhury *et al.,* 2006). Nitrification, denitrification and phosphorus accumulation are crucial processes in POME degradation that remove inorganic nitrogen and phosphorus compound from the wastewater.

During nitrification, ammonium or ammonia is oxidized to nitrate in two steps, each governed by one type of nitrifier. Nitrosomonas sp. are responsible for the oxidation of ammonia to nitrite as the bacteria secrete ammonia monooxygenase and hydroxylamine oxidoreductase to catalyse the process (Hommes *et al.,* 2001). Nitrobacter sp. will in turn oxidize nitrite to nitrate using the enzyme nitrite oxidoreductase (Bartosch *et al.,* 1999). Denitrification takes place when denitrifiers reduce nitrate into nitrite and then to nitrogen gas. This process requires enzyme nitrate reductase and employs nitrate or nitrite as electron acceptor in energy generation, liberating nitrogen gas into the atmosphere (Daum *et al.,* 1998). Phosphorus, on the other hand, is removed from POME by phosphorus-accumulating bacteria which take up excess orthophosphate in the wastewater and store it within their cells. The removal of biomass from wastewater will get rid of the accumulated phosphorus as well (Bao *et al.,* 2007).

2.8.2. EUKARYOTES

In wastewater treatment processes, several groups of eukaryotic organisms, i.e., fungi, algae, protozoa, and animals (rotifers, worms – nematodes and flatworms) can be found. They enter wastewater treatment plants through inflow and infiltration as soil and water organisms (Gerardi, 2006). Fungi or yeast and algae are two selected groups of eukaryotic organisms in this review that can be isolated from the POME. They tend to exhibit distinct functions in wastewater.

Generally, most fungi isolated from the POME exhibit hydrolytic property as they secrete extracellular enzymes to break down complex polymers such as lignocellulosic biomass and lipids. Fungi play a significant role in lipid degradation, not just attributed to the enzyme lipase, but also the secretion biosurfactant by some species such as Candida sp. (Kim *et al.,* 1999). Biosurfactant minimizes the surface tension and interfacial tension between water and lipid phase, facilitating the breakdown of lipids. *Geotrichium candidum* is known to hydrolyze phenols and secrete a peroxidase enzyme that break down a wide range of color dyes (Coulibaly *et al.,* 2004). The same ability to remove color from POME is observed on Aspergillus fumigates though it takes place through bio adsorption (Neoh *et al.,* 2013).

Chlorella pyrenoidosa and *Chlorella vulgaris* are two species of algae isolated from POME. Both of them are involved in the removal of nitrogen and phosphorus from the wastewater. Chlorella sp. rapidly takes up nitrogen and phosphorus from POME for their growth and proliferation (Safi *et al.,* 2014). These nutrients are used to build up phospholipids and glycolipids which make up approximately 30% of their weight of dry biomass (Lam *et al.,* 2011).

2.9 Current state of Palm Oil Mill Effluent treatment

Indonesia and Malaysia are the two biggest oil palm manufacturing nations and is rich in various endemic and forest dwelling species (Shafiqah and Nasir., 2013). Malaysia has a tropical atmosphere and is Palm Oil prosperous with regular assets. Oil palm as of now involves the biggest real acreage of cultivated land in Malaysia (Hansen S.2007, Yusoff S. and Hansen S.B.,2007). The total oil palm acreage from 1970 to 2000 has expanded from 320 to 3338 ha. In the year 2003, there were more than 3.79 million ha of land under palm oil cultivation, occupying more than 33% of the total developed area and 11% of the total land area of Malaysia (Hansen., 2007). Palm oil, edible oil, is derived from the meaty mesocarp of the fruit of oil palm (Elaeis guineensis). One hectare of oil palm produces 10–

35 tons of fresh fruit bunches (FFB) per year (Abdullah A.Z *et al.,* 2009, Singh B.P. *et al.*,2010). Malaysia also accounts for the highest percentage of global vegetable oils and fats trade in the year 2005 (Sumathi., 2008). The oil palm has the expectancy of over 200 years, whereas the economic life is about 20–25 years. The nursery period is 11–15 months for plants, and first harvest is done after 32–38 months of planting. It takes 5–10 years for palm oil plant to reach the highest yield. The yield is approximately 45–56% of FFB, and the fleshy mesocarp of the fruit is used to get oil. The yield of oil from the kernel is about 40–50% (Kittikun *et al.,* 2000). Both mesocarp and kernel of fruit produce about 17 t ha^{-1} yr^{-1} of oil (Abdul *et al.,* 2007). Starting with 5.8 ton of FFB about 1 ton of crude palm oil (CPO) is produced (Singh *et al.,* 2010).

While the oil palm industry has been recognized for its contribution toward economic growth and rapid development, it has also contributed to environmental pollution due to the production of huge quantities of by-products from the oil extraction process (Singh *et al.,* 2010). Although, palm oil mill effluent (POME) is not the only waste generated during processing of fresh fruit bunch (FFB). But it is the most expensive and difficult waste to manage by mill operators. This is because large volumes in tonnes are generated at a time. The palm oils industry still considers POME treatment a burden rather than as part of the production process, one a profit centre (Ma., 1999). For these obvious reasons, raw POME or partially treated POME is still being discharged into nearby rivers or land, as this is the easiest and cheapest method for disposal. However, excessive quantities of untreated POME deplete a water body of its oxygen and suffocate aquatic life. Many small and big rivers have been devastated by such discharge as people living downstream are usually affected. This was evident in the frustration of Keningau and Murat villagers who repeatedly reported the case of pollution to press to tell the world that palm oil mills are polluting the Ongom, International Journal of Science, Environment. Murat villagers complained that oil and dirt pollution from a relatively new mill had crippled a water source for residents in the plains of Pegalan River. Beyond obvious water pollution problems, is the use of both aerobic and anaerobic digestion by palm oil mills in treating POME. Methane, a greenhouse gas, 25 times more potent than carbon dioxide in trapping heat is generated during anaerobic digestion of POME. Palm oil mills are fingered by climate change authorities as being the second largest source of methane generator in Malaysia, (38%), next to landfills (53%). Methane or biogas from palm oil mills is therefore chief contributor to world global warming. During the last century, a great deal of research and development as well as application has been devoted to new advance POME treatment technologies (PTT). The major reason for such huge efforts are that POME generated from processing of FFB has been declared as one of the major source of environmental pollution. Even with the advent of the new biotechnology advances and PTT like POMETHANE. Palm oil mills are still struggling to meet up with more stringent limits of effluent discharge allowed. By 1984, law on effluent discharge in Malaysia limits the Biochemical Oxygen Demand (BOD) to 100 parts per million (PPM). However, in environmentally sensitive areas of Sabah and Sarawak like Kinabatangan River, Department of Environment (DOE) Malaysia imposed a more stringent condition of 20ppm since 2006. For new mills, a 20ppm BOD requirement coupled with land irrigation has been imposed in Sabah. In very sensitive areas, the DOE has even imposed a zero-discharge requirement. Over the last decades, the management of POME has evolved from treatment of the "POME for disposal" to beneficial, utilisation of either treated POME and or its by-products (Ma, 1999). This paper aims to investigate and present the potentials of POME as a resource that can contribute to world economic and sustainable development. Secondly how the palm oil mill operators are taking advantage of the emergent of the new PTT in changing the status of POME from waste to resource and at the same achieving zero discharge concepts in palm oil industry.

2.10 Biodegradation

Biodegradation has long been applied in the treatment of wastewater. However, the current biological treatment processes often involve consortia of undefined microorganisms. This gives rise to poor performance of the treatment processes as the actual biochemical reactions taking place are not clearly defined due to the lack of knowledge regarding the microbiological aspect of wastewater. By acknowledging and characterizing the microbial populations in wastewater, it is believed that performance of the working microorganisms can be better monitored thus enhancing the removal efficiency of the treatment.

A variety of microorganisms have been investigated to be capable of biodegrading oil wastewater with high profits. Anaerobic and aerobic treatments are the great and capable biological methods for POME treatment. However, the suspended and colloidal components are neither effectively decomposed biologically nor by other conventional means because their floating on the surface of the wastewater has an impact on the microbial cycle (Poh and Chong, 2009). It is the major problem to cause failure of treatment system. Particularly, the major component in biological digestion, microorganism plays an important role and core factor of the system to control reactor performance and stability. This research intended to investigate the viability of microorganisms present in POME under conditions for treatment. The process is investigated in terms of COD removal and decolourization (ADMI) with pH tolerance. The experimental work carried out on the activated sludge processes is give some accepting treatment of the microbial behaviour as well as the substrate removal performances and evaluation of the application of an existing palm oil waste on digestibility of POME condition.

2.10.1 Role of Microorganisms in Biodegradation of Pollutants

Biodegradation is described associated with environmental bioremediation. Therefore, biodegradation is nature's way of recycling wastes, or breaking down organic matter into nutrients that can be used and reused by other organisms. In the microbiological sense, "biodegradation" means that the decaying of all organic materials is carried out by a huge assortment of life forms comprising mainly bacteria, yeast and fungi, and possibly other organisms. Bioremediation and biotransformation methods endeavour to harness the astonishing, naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (like oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), radionuclides and metals (Seo *et al.,* 2008).

2.10.2. Some Biodegradable Pollutants

In the last few decades, highly toxic organic compounds have been synthesized and released into the environment for direct or indirect application over a long period of time. Fuels, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pesticides and dyes are some of these types of compounds (Diez., 2010). Some other synthetic chemicals like radionuclides and metals are extremely resistant to biodegradation by native flora compared with the naturally occurring organic compounds that are readily degraded upon introduction into the environment.

Bioremediation of pollutants utilizing biodegradation abilities of microorganisms include the natural attenuation, although it may be enhanced by engineered techniques, either by addition of selected microorganisms (bioaugmentation) or by biostimulation, where nutrients are added. Genetic engineering is also used to improve the biodegradation capabilities of microorganisms by GEM. Nevertheless, there are many factors affecting the efficiency of this process and risks associated to the use of GEM in the field.

Biodegradation: Involved Microorganisms and Genetically Engineered Microorganisms

2.11 Hydrocarbons: are organic compounds whose structures consist of hydrogen and carbon. Hydrocarbons can be seen as linear linked, branched or cyclic molecules. They are observed as aromatic or aliphatic hydrocarbons. The first one has benzene (C_6H_6) in its structure, while the aliphatic one is seen in three forms: alkanes, alkenes and alkynes (Seo *et al.,* 2008). Polycyclic aromatic hydrocarbons (PAHs): are important pollutants class of hydrophobic organic contaminants (HOCs) widely found in air, soil and sediments. The major source of PAH pollution is industrial production (Mrozik *et al.,* 2003). They have been studied with increasing interest for more than twenty years because of more findings about their toxicity, environmental persistence and prevalence (Seo *et al.,* 2008). PAHs can sorb to organic-rich soils and sediments, accumulate in fish and other aquatic organisms, and may be transferred to humans through seafood consumption (Mrozik *et al.,* 2003). The biodegradation of PAHs can be considered on one hand to be part of the normal processes of the carbon cycle, and on the other as the removal of man-made pollutants from the environment. The use of microorganisms for bioremediation of PAH-contaminated environments seems to be an attractive technology for restoration of polluted sites. Polychlorinated biphenyls (PCBs): are mixtures of synthetic organic chemicals. Due to their non-flammability, chemical stability, high boiling point, and electrical insulating properties, PCBs were used in hundreds of industrial and commercial applications including electrical, heat transfer, and hydraulic equipment; as plasticizers in paints, plastics, and rubber products; in pigments, dyes, and carbonless copy paper; and many other industrial applications. Consequently, PCBs are toxic compounds that could act as endocrine disrupters and cause cancer. Therefore, environmental pollution with PCBs is of increasing concern (Seo *et al.*, 2008). Pesticides: are substances or mixture of substances intended for preventing, destroying, repelling or mitigating any pest. Pesticides which are rapidly degraded are called non persistent while those which resist degradation are termed persistent. The most common type of degradation is carried out in the soil by microorganisms, especially fungi and bacteria that use pesticides as food source (Seo *et al.*, 2008). Dyes: are widely used in the textile, rubber product, paper, printing, color photography, pharmaceuticals, cosetics and many other industries (Diez., 2010). Azo dyes, which are aromatic compounds with one or more (–N=N–) groups, are the most important and largest class of synthetic dyes used in commercial applications (Diez., 2010). These dyes are poorly biodegradable because of their structures and treatment of wastewater containing dyes usually involves physical and / or chemical methods such as adsorption, coagulation-flocculation, oxidation, filtration and electrochemical methods (Seo *et al.*, 2008). The success of a biological process for colour removal from a given effluent depends in part on the utilization of microorganisms that effectively decolorize synthetic dyes of different chemical structures. Radionuclides: a radionuclide is an atom with an unstable nucleus, characterized by excess energy available to be imparted either to a newly created radiation particle within the nucleus or via internal conversion. During this process, the radionuclide is said to undergo radioactive decay, resulting in the emission of gamma ray(s) and/or subatomic particles such as alpha or beta particles (Diez., 2010).

2.11.1 Heavy Metals: unlike organic contaminants, the metals cannot be destroyed, but must either be converted to a stable form or removed. Bioremediation of metals is achieved through biotransformation. Mechanisms by which microorganisms act on heavy metals include biosorption (metal sorption to cell surface by physicochemical mechanisms), bioleaching (heavy metal mobilization through the excretion of organic acids or methylation reactions), biomineralization (heavy metal immobilization through the formation of insoluble sulfides or polymeric complexes), intracellular accumulation, and enzyme-catalysed transformation (redox reactions) (Seo *et al.*, 2008).

2.11.2 Bacterial Degradation

There are many reports on the degradation of environmental pollutants by different bacteria. Several bacteria are even known to feed exclusively on hydrocarbons (Leitae., 2009). Bacteria with the ability to degrade hydrocarbons are named hydrocarbon-degrading bacteria. Biodegradation of hydrocarbons can occur under aerobic and anaerobic conditions, it is the case for the nitrate reducing bacterial strains Pseudomonas sp. and *Brevibacillus sp*. isolated from petroleum contaminated soil (Mrozik *et al.,* 2003). However, data presented (Seo *et al.*, 2008) suggest that the anaerobic biodegradation may be much more important. 25 genera of hydrocarbon degrading bacteria were isolated from marine environment (Seo *et al.*, 2008). Furthermore, among 80 bacterial strains isolated by Kafilzadeh (Pramila *et al.*, 2012) which belonged to 10 genera as follows: *Bacillus, Corynebacterium, Staphylococcus, Streptococcus, Shigella, Alcaligenes, Acinetobacter, Escherichia, Klebsiella and Enterobacter, Bacillus* was the best hydrocarbon degrading bacteria. Bacterial strains that are able to degrade aromatic hydrocarbons have been repeatedly isolated, mainly from soil. These are usually gram-negative bacteria, most of them belong to the genus Pseudomonas. The biodegradative pathways have also been reported in bacteria from the genera *Mycobacterium, Corynebacterium, Aeromonas, Rhodococcus and Bacillus* (Mrozik *et al.*, 2003). Although many bacteria are able to metabolize organic pollutants, a single bacterium does not possess the enzymatic capability to degrade all or even most of the organic compounds in a polluted soil. Mixed microbial communities have the most powerful biodegradative potential because the genetic information of more than one organism is necessary to degrade the complex mixtures of organic compounds present in contaminated areas (Seo *et al.*, 2008). Both, anaerobic and aerobic bacteria are capable of biotransforming PCBs. Higher chlorinated PCBs are subjected to reductive dehalogenation by anaerobic microorganisms. Lower chlorinated biphenyls are oxidized by aerobic bacteria (Diez., 2010). Research on aerobic bacteria isolated so far has mainly focused on Gram-negative strains belonging to the genera *Pseudomonas, Burkholderia, Ralstonia, Achromobacter, Sphingomonas and Comamonas*. However, several reports about PCB-degrading activity and characterization of the genes that are involved in PCB degradation indicated PCB-degrading potential of some Gram-positive strains as well (*genera Rhodococcus, Janibacter, Bacillus, Paenibacillus and Microbacterium*) (Seo *et al.,* 2008). Aerobic catabolic pathway for PCB degradation seems to be very similar for most of the bacteria and comprises four steps catalysed by the enzymes, biphenyl dioxygenase (BphA), dihydrodiol dehydrogenase (BphB), 2, 3-dihydroxybihenyl dioxygenase (DHBD) (BphC) and hydrolase (BphD) (Seo *et al.,* 2008). Successful removal of pesticides by the addition of bacteria had been reported earlier for many compounds, including atrazine (Das and Chandran., 2011). Recent findings concerning pesticide degrading bacteria include the chlorpyrifos degrading bacterium *Providencia stuartii* isolated from agricultural soil (Alexanda 1994) and isolates Bacillus, Staphylococcus and Stenotrophomonas from cultivated and uncultivated soil able to degrade dichlorodiphenyltrichloroethane (DDT) (Leitae., 2009). Researches on bacterial strains that are able to degrade azo dyes under aerobic and anaerobic conditions have been extensively reported (Seo *et al.,* 2008). Based on the available literature, it can be concluded that the microbial decolourization of azo dyes is more effective under anaerobic conditions. On the other hand, these conditions lead to aromatic amine formation, and these are mutagenic and toxic to humans requiring a subsequent oxidative (aerobic) stage for their degradation. In this context, the combined anaerobic/aerobic biological treatments of textile dye effluents using microbial consortia are common in the literature (Fritsche and Hofrichter., 2008). For example, Chaube *et al.,* (1980) Bonnet *et al.,* 2002) have used the mix consortia of bacteria consisting of *Proteus sp., Pseudomonas sp. and Enterococcus sp.* in biodegradation and decolourisation of dye. However, several researchers have identified single bacterial strains that have very high efficacy for removal of azo dyes, it is the case of *Shewanella* discolorations (Bonnet *et al.,* 2002). In contrast to mixed cultures, the use of a pure culture has several advantages. These include predictable performance and detailed knowledge on the degradation pathways with improved assurance that catabolism of the dyes will lead to nontoxic end products under a given set of environmental conditions. Another advantage is that the bacterial strains and their activity can be monitored using culture-based or molecular methods to quantify population densities of the bacteria over time. Knowledge of the population density can be extrapolated to quantitative analysis of the kinetics of azo dye decolouration and mineralization (Seo *et al.,* 2008). Heavy metals cannot be destroyed biologically (no "degradation", change in the nuclear structure of the element, occurs) but are only transformed from one oxidation state or organic complex to another (Seo *et al.,* 2008). Besides, bacteria are also efficient in heavy metals bioremediation. Microorganisms have developed the capabilities to protect themselves from heavy metal toxicity by various mechanisms, such as adsorption, uptake, methylation, oxidation and reduction. Reduction of metals can occur through dissimilatory metal reduction (Fritsche and Hofrichter., 2008), where bacteria utilize metals as terminal electron acceptors for anaerobic respiration. In addition, bacteria may possess reduction mechanisms that are not coupled to respiration, but instead are thought to impart metal resistance. For example, reduction of Cr(VI) to Cr(III) under aerobic (Pramila *et al.,* 2012) or anaerobic conditions (Seo *et al.,* 2008), reduction of Se(VI) to elemental Se (Marinescu *et al.,* 2009), reduction of U(VI) to U(IV) (McMurry., 2000) and reduction of Hg(II) to Hg (0) (Vargas 1975). Microbial methylation plays an important role in heavy metals bioremediation, because methylated compounds are frequently volatile. For example, Mercury, Hg (II) can be biomethylated by a number of different bacterial species *Alcaligenes faecalis, Bacillus pumilus, Bacillus sp., P. aeruginosa and Brevibacterium iodinium* to gaseous methyl mercury (Seegar *et al.,* 2010). In addition to redox conversions and methylation reactions, acidophilic iron bacteria like *Acidithiobacillus ferrooxidans* (Alexande., 1994) and sulfur oxidizing bacteria (Marinescu *et al.,* 2009) are able to leach high concentrations of As, Cd, Cu, Co and Zn from contaminated soils. On the other hand, metals can be precipitated as insoluble sulfides indirectly by the metabolic activity of sulphate reducing bacteria (Lesley and Penny 2012). Sulphate reducing bacteria are anaerobic heterotrophs utilizing a range of organic substrates with SO_4^{2-} as the terminal electron acceptor. Heavy metal ions can be entrapped in the cellular structure and subsequently biosorbed onto the binding sites present in the cellular structure. This method of uptake is independent of the biological metabolic cycle and is known as biosorption or passive uptake. The heavy metal can also pass into the cell across the cell membrane through the cell metabolic cycle. This mode of metal uptake is referred as active uptake. Pseudomonas strain, characterized as part of a project to develop a biosorbent for removal of toxic radionuclides from nuclear waste streams, was a potent accumulator of uranium (VI) and thorium (IV) (Menn *et al.,* 2008).

Most works on pollutants bioremediation uses pure microbial cultures. However, the use of mixed microbial cultures is undoubtedly advantageous. Some of the best examples of enrichment cultures comprising several specific consortia involve the bioremediation. In the case of heavy metals removal, Seegar *et al.,* (2010) have used an environmental bacterial consortium to remove Cd, Cr, Cu, Ni and Pb from a synthetic wastewater effluent. For Cr (VI) removal we reported that the survival and stability of bacteria are better when they are present as a mixed culture, especially, in highly contaminated areas and in the presence of more than one type of metal (Alexande., 1994). Indeed, the indigenous bacteria enriched from chromium contaminated biotopes, were able to remove Cr (VI) successfully in multi-contaminated heavy metal solution (Marinescu *et al.,* 2009). A microbial consortium consisting of three bacterial Pseudomonas species originally obtained from dye contaminated sites was capable of decolorizing textile effluent and dye faster than the individual bacteria under static conditions (Marinescu *et al.,* 2009).

2.11.3 PGPR and PGPB Degradation

Plant associated bacteria, such as endophytic bacteria (non-pathogenic bacteria that occur naturally in plants) and rhizospheric bacteria (bacteria that live on and near the roots of plants), have been shown to contribute to biodegradation of toxic organic compounds in contaminated soil and could have potential for improving phytoremediation (Alexande., 1994). Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion (Lesley and Penny., 2012). Some plants can release structural analogs of PAHs such as phenols, to promote the growth of hydrocarbon degrading microbes and their degradation on PAHs. For such plant/microbe systems, an important class of bacteria is Pseudomonas spp., have PGPR activity and hydrocarbon degrading capacity (Lesley and Penny., 2012). Furthermore, the rhizosphere of vegetation in contaminated field contains higher diversity of population of PAH-degrading bacteria, among which two Lysini bacillus strains were isolated (Alexande., 1994). Culturable PCB degraders were also associated with both the rhizosphere and root zone of mature trees growing naturally in a contaminated site, they were identified as members of the genus *Rhodococcus, Luteibacter* and *Williamsia,* which suggest that biostimulation through rhizoremediation is a promising strategy for enhancing

PCB degradation in situ (Lesley and Penny., 2012). Also, the free-living nitrogen fixer Azospirillum lipoferum generally found in the rhizoplane of the crop plants was used for Malathion degradation which is one of the largest organo phosphorus insecticides in the world (Vargas 1975). Results from the literature suggest that heavy metals may be removed from contaminated soils using plant growth promoting rhizobacteria. The use of soil bacteria (often plant growth promoting bacteria (PGPB)) as adjuncts in metal phytoremediation can significantly facilitate the growth of plants in the presence of high (and otherwise inhibitory) levels of metals (Lesley and Penny., 2012). To increase the efficiency of contaminants extraction, it is interesting to apply plants combined to some microorganisms; such technique is called rhizoremediation (Vargas 1975).

2.12 BIOREMEDIATION AND BIODEGRADATION

The application of bioremediation as a biotechnological process involving microorganisms has become a crescent study field in microbiology, because of its increasing potential of solving the dangers of many pollutants through biodegradation. Microorganisms might be considered excellent pollutant removal tools in soil, water, and sediments, mostly due to their advantage over other bioremediation procedures (Vargas., 1975). Moreover, bioremediation using biodegradation represents a high impact strategy, but still a low-cost way tool of removing pollutants, hence a very viable process to be applied. The principles of bioremediation are based on natural attenuation, bioaugmentation and biostimulation (Raffi *et al.*, 1997). The simplest method of bioremediation is natural attenuation, in which soils are only monitored for variations in pollution concentrations to ensure that the pollutant transformation is active (Menn *et al.*, 2008). Bioaugmentation is usually applied in cases where natural active microbial communities are present in low quantities or even absent, wherein the addition of contaminant degrading organisms can accelerate the transformation rates (Lesley and Penny., 2012). In such cases, the adaptation of exogenous strains that exert highly efficient activities for pollutant transformation to new environments is a key challenge in implementation (McMurry., 2000). The capacity of a microbial population to degrade pollutants can be enhanced also by stimulation of the indigenous microorganisms by addition of nutrients or electron acceptors (Okere and Semple., 2012).

2.13 Natural Attenuation

Natural attenuation or bioattenuation is the reduction of contaminant concentrations in the environment through biological processes (aerobic and anaerobic biodegradation, plant and animal uptake), physical phenomena (advection, dispersion, dilution, diffusion, volatilization, sorption/desorption), and chemical reactions (ion exchange, complexation, abiotic transformation). Terms such as intrinsic remediation or biotransformation are included within the more general natural attenuation definition (Vargas., 1975). Although, one of the most important components of natural attenuation is biodegradation, the change in form of compounds carried out by living creatures such as microorganisms. Under the right conditions, microorganisms can cause or assist chemical reactions that change the form of the contaminants so that little or no health risk remains. Natural attenuation occurs at most polluted sites. However, the right conditions must exist underground to clean sites properly. If not, clean-up will not be quick enough or complete enough. Scientists monitor these conditions to make sure natural attenuation is working. This is called monitored natural attenuation or (MNA). So, monitored natural attenuation is a technique used to monitor or test the progress of natural attenuation processes that can degrade contaminants in soil and groundwater. It may be used with other remediation processes as a finishing option or as the only remediation process if the rate of contaminant degradation is fast enough to protect human health and the environment. Natural processes can then mitigate the remaining amount of pollution; regular monitoring of the soil and groundwater can verify those reductions (Seegar *et al.,* 2010). When the environment is polluted with chemicals, nature can work in four ways to clean it up (Marinescu *et al.,* 2009):

1) Tiny bugs or microbes that live in soil and groundwater use some chemicals for food. When they completely digest the chemicals, they can change them into water and harmless gases.

2) Chemicals can stick or sorb to soil, which holds them in place. This does not clean up the chemicals, but it can keep them from polluting groundwater and leaving the site.

3) As pollution moves through soil and groundwater, it can mix with clean water. This reduces or dilutes the pollution.

4) Some chemicals, like oil and solvents, can evaporate, which means they change from liquids to gases within the soil.

If these gases escape to the air at the ground surface, sunlight may destroy them. If the natural attenuation is not quick enough or complete enough, bioremediation is enhanced either by biostimulation or bioaugmentation.

2.13. Biostimulation

Biostimulation involving the addition of soil nutrients, trace minerals, electron acceptors, or electron donors enhances the biotransformation of a wide range of soil contaminants (Raffi *et al.,* 1997). There are many examples of biostimulation of pollutants biodegradation by indigenous microorganisms. Trichloroethene and perchloroethene are reported to be completely converted to ethane by microorganisms in a short span of time with the addition of lactate during biostimulation (Vandevivere *et al.,* 1998). Electron shuttles, such as humic substances (HS), may play a significant stimulation role in the anaerobic biotransformation of organic pollutants through enhancing the electron transfer speed. Anthraquinone-2,6-disulfonate (AQDS) from the category of HS can serve as an electron shuttle to promote the reduction of iron oxides and transformation of chlorinated organic contaminants (Verma and Madamwac., 2003). Petrucci *et al.,* (2002) reported that the biostimulation of indigenous microbial communities by the addition of lactate and AQDS led to the enhanced rates of Pentachlorophenol PCP dichlorination by the dechlorinating and iron reducing bacteria in soils. Among various nutrient media, glycerol appeared to show the most favourable metabolic characteristics against phenol toxicity on the indigenous *Rhizobium Ralstonia taiwanensis*, leading to better degradation efficiency of the toxic pollutant (Bonnet *et al.,* 2002). Marinescu *et al.,* (2009) observed that biostimulation was more efficient when compared to natural attenuation of biodiesel in contaminated soils. However, the comparative study of Alexande., (1994) revealed that bioaugmentation showed the greatest degradation potential and natural attenuation was more effective than biostimulation of soils contaminated with diesel oil. Results obtained by Seo *et al.,* (2008) indicate that autochthonous microbes may interact and even compete with the enriched consortium during polycyclic aromatic hydrocarbons biodegradation and the natural attenuation appeared to be the most appropriate way to remedy fluorene and phenanthrene contaminated mangrove sediments while biostimulation was more capable to degrade pyrene contaminated sediments.

2.14. Bioaugmentation

We can define bioaugmentation as the technique for improvement of the capacity of a contaminated matrix (soil or other biotope) to remove pollution by the introduction of specific competent strains or consortia of microorganisms (Marinescu *et al.,* 2009). The basic premise for this intervention is that the metabolic capacities of the indigenous microbial community already present in the biotope slated for clean-up is increased by an exogenously enhanced genetic diversity, thus leading to a wider repertoire of productive biodegradation reactions (seo *et al.,* 2008). Moreover, genetically engineered microorganisms (GEMs) exhibiting enhanced degradative capabilities encompassing a wide range of aromatic hydrocarbons have also potential for soil bioaugmentation (Seo *et al.,* 2008). It is thought that bioaugmentation approach should be applied when the biostimulation and bioattenuation have failed (Marinescu *et al.,* 2009). Many studies have shown that both abiotic and biotic factors influence the effectiveness of bioaugmentation, the most important abiotic factors are temperature, moisture, pH and organic matter content, however, aeration, nutrient content and soil type also determine the efficiency of bioaugmentation. Biotic factors, including competition between indigenous and exogenous microorganisms for limited carbon sources as well as antagonistic interactions and predation by protozoa and bacteriophages, also play essential roles in the final results of bioaugmentation (Bonnet *et al.,* 2002). The combination of bioaugmentation and biostimulation might be a promising strategy to speed up bioremediation. Both indigenous and exogenous microorganisms could benefit from biostimulation by the addition of energy sources or electron acceptors (Bonnet *et al.*, 2002). Bioaugmentation assisted phytoextraction using PGPR or AMF is also a promising method for the cleaning-up of soils contaminated by metals (Alexande., 1994).

2.15 Factors Affecting Microbial Degradation

Microorganisms can degrade numerous of organic pollutants owing to their metabolic machinery and to their capacity to adapt to inhospitable environments. Thus, microorganisms are major players in site remediation. However, their efficiency depends on many factors, including the chemical nature and the concentration of pollutants, their availability to microorganisms, and the physicochemical characteristics of the environment (Fritsche and Hofrichter., 2008). So, factors that influence the rate of pollutants degradation by microorganisms are either related to the microorganisms and their nutritional requirements (biological factors) or associated to the environment (environmental factors).

2.16 Biological Factors

A biotic factor is the metabolic ability of microorganisms. The biotic factors that affect the microbial degradation of organic compounds include direct inhibition of enzymatic activities and the proliferation processes of degrading microorganisms (Alexande., 1994). This inhibition can occur for example if there is a competition between microorganisms for limited carbon sources, antagonistic interactions between microorganisms or the predation of microorganisms by protozoa and bacteriophages (Alexande., 1994). The rate of contaminant degradation is often dependent on the concentration of the contaminant and the amount of "catalyst" present. In this context, the amount of "catalyst" represents the number of organisms able to metabolize the contaminant as well as the number of enzymes(s) produced by each cell. Furthermore, the extent to which contaminants are metabolized is largely a function of the specific enzymes involved and their "affinity" for the contaminant and the availability of the contaminant. In addition, sufficient amounts of nutrients and oxygen must be available in a usable form and in proper proportions for unrestricted microbial growth to occur (Alexande., 1994). Other factors that influence the rate of biodegradation by controlling the rates of enzyme catalysed reactions are temperature,

Biodegradation: Involved Microorganisms and Genetically Engineered Microorganisms pH and moisture. Biological enzymes involved in the degradation pathway have an optimum temperature and will not have the same metabolic turnover for every temperature (Das and Chondran., 2011). Indeed, the rate of biodegradation is decreased by roughly one-half for each 10°C decrease in temperature (Das and Chondran., 2011). Biodegradation can occur under a wide-range of pH; however, a pH of 6.5 to 8.5 is generally optimal for biodegradation in most aquatic and terrestrial systems. Moisture influences the rate of contaminant metabolism because it influences the kind and number of soluble materials that are available as well as the osmotic pressure and pH of terrestrial and aquatic systems (Alexande., 1994).

2.18 Environmental Factors

Soil type and soil organic matter content affect the potential for adsorption of an organic compound to the surface of a solid. Absorption is an analogous process wherein a contaminant penetrates into the bulk mass of the soil matrix. Both adsorption and absorption reduce the availability of the contaminant to most microorganisms and the rate at which the chemical is metabolized is proportionately reduced (Alexande., 1994). Variations in porosity of the unsaturated and saturated zones of the aquifer matrix may influence the movement of fluids and contaminant migration in groundwater. The ability of the matrix to transmit gases, such as oxygen, methane and carbon dioxide, is reduced in fine grained sediments and also when soils become more saturated with water. This can affect the rate and type of biodegradation taking place (Fritsche and Hofeichter., 2008). The oxidation-reduction potential of a soil provides a measurement of the electron density of the system. Biological energy is obtained from the oxidation of compounds in which electrons are transferred to various more oxidized compounds referred to as electron acceptors. A low electron density (Eh greater than 50 mV) indicates oxidizing, aerobic conditions, whereas high electron density (Eh less than 50 mV) indicates reducing, anaerobic conditions (Alexande., 1994).

2.19. Degradation by Genetically Engineered Microorganisms

As mentioned above, bioaugmentation and biostimulation are methods that can be applied to accelerate the recovery of polluted sites. In the late 1970s and early 1980s, bacterial genes encoding catabolic enzymes for recalcitrant compounds started to be cloned and characterized. Soon, many microbiologists and molecular biologists realized the potential of genetic engineering for addressing biodegradation (Alexande., 1994). A genetically engineered microorganism (GEM) or modified microorganism (GMM) is a microorganism whose genetic material has been altered using genetic engineering techniques inspired by natural genetic exchange between microorganisms. These techniques are generally known as recombinant DNA technology. Genetically engineered microorganisms (GEMs) have shown potential for bioremediation of soil, groundwater and activated sludge, exhibiting the enhanced degrading capabilities of a wide range of chemical contaminants (Leitae 2009). As soon as the prospect of releasing genetically modified microorganisms for bioremediation became a reality, much of the research effort in the field was aimed at biosafety and risk assessment (Leitae., 2009). There are at least four principal approaches to GEM development for bioremediation application (Seo *et al.,* 2008). These include:

1) Modification of enzyme specificity and affinity;

2) Pathway construction and regulation;

3) Bioprocess development, monitoring and control;

4) Bioaffinity bioreporter sensor applications for chemical sensing, toxicity reduction and end point analysis.

2.20 Genetically Engineered Microorganisms

Molecular biology offers the tools to optimize the biodegradative capacities of microorganisms, accelerate the evolution of "new" activities, and construct totally "new" pathways through the assemblage of catabolic segments from different microbes (Diez., 2010). Genes responsible for degradation of environmental pollutants, for example, toluene, chlorobenzene acids, and other halogenated pesticides and toxic wastes have been identified. For every compound, one separate plasmid is required. It is not like that one plasmid can degrade all the toxic compounds of different groups. The plasmids are grouped into four categories:

1) OCT plasmid which degrades, octane, hexane and decane;

2) XYL plasmid which degrades xylene and toluenes,

3) CAM plasmid that decompose camphor and

4) NAH plasmid which degrades naphthalene (Diez., 2010).

The potential for creating, through genetic manipulation, microbial strains able to degrade a variety of different types of hydrocarbons has been demonstrated (Seo *et al.*, 2008). They successfully produced a multiplasmid-containing Pseudomonas strain capable of oxidizing aliphatic, aromatic, terpenic and polyaromatic hydrocarbons. Pseudomonas putida that contained the XYL and NAH plasmid as well as a hybrid plasmid derived by recombinating parts of CAM and OCT developed by conjugation could degrade camphor, octane, salicylate, and naphthalene (Diez., 2010) and could grew rapidly on crude oil because it was capable of metabolizing hydrocarbons more efficiently than any other single plasmid

(Diez., 2010). This product of genetic engineering was called as superbug (oil eating bug). The plasmids of P. putida degrading various chemical compounds are TOL (for toluene and xylene), RA500 (for 3, 5-xylene) pAC 25 (for 3-cne chlorobenxoate), pKF439 (for salicylate toluene). Plasmid WWO of P. putida is one member of a set of plasmids now termed as TOL plasmid. It was the first living being to be the subject of an intellectual property case. At that point, it seemed that molecular techniques, either through plasmid breeding or sheer genetic engineering, could rapidly produce microbes with higher catalytic abilities, able to basically degrade any environmental pollutant (Seo *et al.,* 2008). Reports on the degradation of environmental pollutants by genetically engineered microorganisms are focused on genetically engineered bacteria using different genetic engineering technologies: Pathway modification, modification of substrate specificity by *Comamonas testosteroni* VP44 (Seo *et al.,* 2008). The application of genetic engineering for heavy metals removal has aroused great interest. For example, *Alcaligenes eutrophus* AE104 (pEBZ141) was used for chromium removal from industrial wastewater (Seo *et al.,*2008) and the recombinant photosynthetic bacterium, *Rhodopseudomonas palustris,* was constructed to simultaneously express mercury transport system and metallothionein for Hg2+ removal from heavy metal wastewater (Seo *et al.,* 2008). For polychlorinated biphenyls degradation, chromosomally located PCB catabolic genes of *R. eutropha* A5, *Achromobacter sp*. LBS1C1, *and A. denitrificans* JB1 were transferred into a heavy metal resistant strain *R. eutropha* CH34 through natural conjugation (Das and Chandran., 2011). Genetic engineering of endophytic and rhizospheric bacteria for use in plant associated degradation of toxic compounds in soil is considered one of the most promising new technologies for remediation of contaminated environmental sites (Seo *et al.,* 2008). To select a suitable strain for gene recombination and inoculation into the rhizosphere, there are three criteria that has been recommended: first, the strain should be stable after cloning and the target gene should have a high expression, second, the strain should be tolerant or insensitive to the contaminant; and third, some strains can survive only in several specific plant rhizosphere (Seo *et al.,* 2008). Many bacteria in the rhizosphere show only limited ability in degrading organic pollutants. With the development of molecular biology, the genetically engineered rhizobacteria with the contaminant-degrading gene are constructed to conduct the rhizoremediation (Seo *et al.,* 2008). Examples about the molecular mechanisms involved in the degradation of some pollutants such as trichloroethylene (TCE) and PCBs has been studied (Leitae., 2009). For heavy metals, Sriprang *et al.,* (1980) Mrozik *et al.,* 2003) introduced Arabidopsis thaliana gene for phytochelatin synthase (PCS; PCSAt) into Mesorhizobium huakuii subsp. rengei strain B3 and then established the symbiosis between M. huakuii subsp. rengei strain B3 and Astragalus sinicus. The gene was expressed to produce phytochelatins and accumulate Cd2+, under the control of bacteroid specific promoter, the nifH gene (Seo *et al.,* 2008). Finally, the use of GEM strains as an inoculum during seeding is preclude the problems associated with competition between strains in a mixed culture. However, there is considerable controversy surrounding the release of such genetically engineered microorganisms into the environment, and field testing of these organisms must therefore be delayed until the issues of safety and the potential for ecological damage are resolved (Seo *et al.,* 2008).

2.21 Obstacles Associated with the use of Gem in Bioremediation Applications

While genetic engineering has produced numerous strains able to degrade otherwise intractable pollutants in a Petri dish or in a bioreactor, the practical translation of this research into actual in situ bioremediation practices has been quite scanty (Diez., 2010). One major issue in this respect is the growing realization that the strains and bacterial species that most frequently appear in traditional enrichment procedures are not the ones performing the bulk of biodegradation in natural niches and may not even be any good as bioremediation mediators. The use of stable isotope probing (SIP) and equivalent methods in microbial ecology have revealed that *Pseudomonas*, *Rhodococcus*, and the typical aerobic fast growers that are widely favoured as hosts of biodegradation related recombinant genes are far less significant under natural conditions (Pramila *et al.,* 2012). Furthermore, using fast-growers as agents for biodegradation is the inevitable build-up of unwelcome biomass. As an alternative, the optimal clean-up agent is the one that displays a maximum catalytic ability with a minimum of cell mass. The expression of biodegradation genes can be artificially uncoupled from growth with the use of stationary phase promoters or starvation promoters (Pramila *et al.,* 2012). In addition, the recent advances in the area of recombinant DNA technologies have paved the way for conceptualizing “suicidal genetically engineered microorganisms” (S-GEMS) to minimize such anticipated hazards and to achieve efficient and safer decontamination of polluted sites (Pramila *et al.,* 2012). In some cases, whether the introduced bacterium is recombinant or not makes little difference, because the problem is that of implantation of foreign microbes in an unfamiliar territory. The introduction of bacterial biomass in an existing niche may create a palatable niche for protozoa that prevents the bacterial population to grow beyond a certain level (Mrozik *et al.,* 2003). Ingenious approaches have been developed to circumvent this problem, including encapsulation of the inoculums in a polymeric matrix or protection in plastic tubing. The efficacy of a desired in-situ catalytic activity (biodegradation or otherwise) depends first on its presence in the target site. One key enzyme may not be there, or it may pre-exist in the site but not be manifested. Alternatively, it can be hosted by just a very minor part of the whole microbial population, so that its factual expression in the site might not be significant (Seo *et al.,* 2008). A field release of *P. fluorescens* HK44 for bioremediation application has been successfully conducted on moderately large-scale and controlled field condition (Seo *et al.,* 2008). However, the future application of genetically engineered bacteria for pollution remediation will not be free from the risks associated with their release in the environment. The future risk regarding use of other engineered bacteria is still unclear. Therefore, the future perspectives of engineered bacterial strains under the field conditions is the focus of review, which may help us to assess the obstacles related with application of genetically engineered bacteria in environmental bioremediation. The major problem encountered in successful bioremediation technology pertains to hostile field conditions for the engineered microbes. Besides, the molecular applications are mainly confined to only few well characterized bacteria such as *E. coli*, *P. putida, B. subtilis,* and so on. Other bacterial strains need to be tried for developing the engineered microbes. The specific characteristic of open biotechnological applications has clearly necessitated the development of engineered bacterial strains to meet the new challenges. The main concern is to construct GE bacteria for field release in bioremediation with an adequate degree of environmental certainty. Efforts should be made to examine the performance of engineered bacteria in terms of their survival, potential of horizontal gene transfer, which may affect the indigenous microflora within a complex environmental situation. Often the novel scientific researches always give rise to still more fascinating questions pertaining to public concern. In the majority cases, the bacteria designed for bioremediation processes have been designed for specific purpose under the laboratory conditions, ignoring the field requirement and other complex situations. However, there is no evidence that the deliberate release of GE bacteria for bioremediation has caused a measurable adverse impact on the natural microbial community. At least the overstated idea of risk appraisal has fuelled so much debate and triggered so many research efforts, which have immensely contributed a lot in the field of environmental microbiology. However, survival of the GE bacteria in complex environmental situations is still a big question, needs to be addressed in the light of latest findings (Seo *et al.,* 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out at Depkor Irruan of Boki Local Government area, of Cross River State, South South, Nigeria. Coordinates: 6°16’26’’N 9°00’36’’E. It has a total of 1,070 square metre (2,771km square), and a population of 186,611 and a density of 174/square metre. They speak Bokyi language. The region has a contiguous border with the Republic of Cameroon and is known for Agricultural commodities such as Cocoa, Coffee, Timber and Palm products (Achived copy 2014)

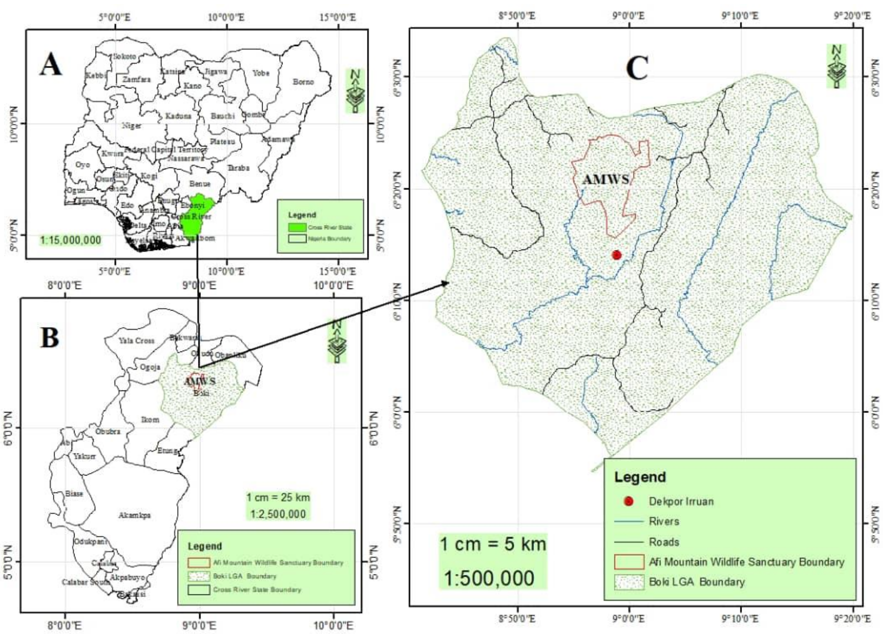

Figure 1: Map showing the location and coordinates of Depkor Irruan of Boki Local Government Area, Cross River state. Nigeria

3.2 Collection of Samples

Raw palm oil mill effluent (POME) was collected from POME disposal site of Depkor Irruan of Boki Local Government Area, Cross River State in a sterile container and brought to the Federal University of Technology laboratory Minna in an Ice Box. Raw POME collected aseptically from the mill in a sterile microbiological container (4L) enclosed in an icebox at 4°C in order to prevent the POME from undergoing biodegradation due to microbial action (APHA, 2005) and brought to the laboratory of the Department of Microbiology Federal University of Technology Minna.

3.3 Sample Preservation

This is achieved by preserving the sample at temperature 4°C, in an ice box but above the freezing point in order to prevent the wastewater from undergoing biodegradation due to microbial action according to American Public Health Association (APHA, 2005).

3.4 Growth Media Preparation

3.4.1 Peptone Water

1.5 g of peptone water base powder was weighed and added to 100ml of distilled water which was shaken thoroughly and then heated to dissolve the medium completely. Five millilitre (5 mL) each of the medium is then dispensed in clean test tubes and sterilized by autoclaving at 121℃ for 15 minutes.

3.4.2 Nutrient Agar

The 2.8 g of nutrient agar powder was weighed and added to 100 mL of distilled water, shaken thoroughly, heated to dissolve the medium completely and sterilized by autoclaving at 121ºC for 15 minutes

3.5 Cultural Identification of Bacteria

A loopful of the bacteria culture is streaked on nutrient agar plate, and incubated at 37^{o}C for 24 hours and the shape, colour and structure of the colonies was examined after appropriate growth.

3.5.1 Isolation of Bacteria from Palm Oil Meal Effluent

One millilitre (1mL) of each sample was processed under aseptic conditions by suspending in 9ml of 0.15% Peptone water and homogenized. One millilitre (1mL) of each thoroughly mixed sample is serially diluted up to 10^{-6} and 10^{-3} used to inoculate Nutrient agar plates supplemented with 100 mg/l fluconazole. Each plate is incubated at 37^{o}C for 48 hours and observed for the growth of colonies.

3.6 Identification of Bacterial Isolates

Colonies with distinct morphological characteristics was randomly selected and purified by repeat streaking on the antibiotic supplemented Nutrient agar. Distinct isolates were subjected to morphological identification aided by using the following parameters: Colour, Shape, Texture, Elevation, Margin and Opacity (Cappuccino and Sherman, 2010).

Biochemical characterization: oxidase test, catalase test, citrate test, urease test and indole test were carried out. The bacterial isolates were identified based on the Bergy's Manual of Systemic

3.7 Biochemical Identification of Bacterial Isolates

3.7.1 Oxidase: oxidase test was performed on the organism. filter paper is moistened with 3-4 drops of oxidase reagent (tetra methyl-p-phenylenediamine dihydrochloride solution) and placed over a colony and then observed for violet colour after 10-15 seconds. The appearance of violet coloration indicates the presence of cytochrome oxidase in the test colony.

3.7.2 Catalase: small amount of 24 hours old bacterium colony was transferred to the surface of clean, dry, grease free glass slide using a sterile wire loop and a drop of 3% hydrogen peroxide (H_2O_2) was placed on the slide and mixed. A positive result shows the appearance of air bubbles within 5 to 10 seconds while a negative result shows little or no bubbles

3.7.3 Citrate: Simmons citrate agar slant is inoculated and incubated at 37^{o}C for 18 to 24 hours. The colour change from green to blue indicates a positive test.

3.7.4 Urease: the surface of urea agar slant is inoculated with the test organism and incubated at 37^{o}C for 24 hours. The colour change from yellow to pink indicates a positive test.

3.7.5 Indole: tryptophan broth is inoculated with the test organism and incubated at 37^{o}C for 24 hours. After 24 hours, 0.5 mL of Kovac's reagent is added to the broth culture and observed the formation of pink ring. Appearance of pink coloration after the addition of Kovac's reagent shows positive result.

3.8 Molecular Characterization of the Isolates

After examining the morphological and biochemical characters of the bacteria isolates, molecular characterization was done. DNA extraction, Polymerase Chain Reaction (PCR) gel electrophoresis and DNA sequencing was performed to ascertain the identity of the bacteria according to the method of Nikunjkumar (2012).

3.8.1 DNA Extraction

DNA was extracted using the protocol stated by Trindade *et al.,* (2007). Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

3.8.2 Polymerase chain reaction

PCR preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at

94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds ; and a final termination at 72°C for 10 mins. And chill at 4°C.

3.8.3 Integrity (Agarose Gel Electrophoresis) of PCR Product

The integrity of the amplified 1.5Mb gene fragment was checked on a 1% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

3.8.4 Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Aqueous Sodium, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube Eppendorf, mix thoroughly by vortexing and keep at -20°C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet was washed by adding 150 µl of 70% ethanol and mix then centrifuge for 15 min at 7500 g and 4°C. Again, remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 min. then resuspend with 20 µl of sterile distilled water and kept in -20oC prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific.

3.8.5 Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis

3.9 Determination of the Physicochemical Properties of the Palm Oil Mill Effluent Sample

The following physicochemical properties of the POME, contaminated and uncontaminated soil was determined. Temperature of the POME is being measured in situ with a mercury thermometer. The thermometer is being allowed to remain in the soil until a constant temperature is noted. pH of the soil was determined at ambient temperature using glass electrode pH and conductivity meter in 1:1 water to soil ratio. Nitrogen and particle size distribution (percentage of sand, clay and silt) is determined by the method of

Ibitoye (2006). Phosphorus is determined by the method of Murphy and Riley (1962) while the organic matter content is determined by the ignition method of Akinsanmi (1975) and total organic matter is determined by the method proposed by Walkley and Black (1934). The dry weight method is used to determine the moisture content of the soil sample. Approximately 5g of fine soil sample is weighed in a pre-weighed beaker and dried overnight at 105oC until constant weight is obtained. it is cooled in a desiccator and reweighed.

The moisture content in wt% (m/m) is Equation (1)

$$\text{Moist (wt \%)} = \frac{C_{raw} - C_{degraded}}{C_{raw}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

3.10 Determination of the Physicochemical Properties of the POME Sample

The physicochemical characteristics of the sample are determined in accordance with the standard methods published by the American Public Health Association (APHA, 1995; APHA, 2005; APHA 2012).

3.11 Immobilization of Bacterial cells

Immobilization was achieved using polysaccharide gel entrapment in which Sodiun Alginate was as an efficient immobilization approach to prevent the cells from diffusing into the surrounding. Cell suspension of the isolates was mixed with 4% Sodium alginate solution separately in the ratio of 1:2. Then the mixture is extruded into 0.2 M $CaCl_2$ solution using syringe to form alginate beads (Mostafa *et al.*, 2017)

3.12 Biodegradation of Palm Oil Meal Effluent Using Immobilized Bacteria

The biodegradation was carried out by putting the POME sample into 4 conical flasks where the physicochemical analysis and oil recovery was done. The immobilized bacteria in beads were introduced to the samples and physicochemical analysis were carried out on for days interval for 24 days.

3.13 STATISTICAL ANALYSIS

The analyses are performed in triplicates. The mean values and standard deviation (mean ± SD) is calculated and tested. Statistical analysis of variance (ANOVA) is performed on all values and tested for $p < 0.05$ for significance.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1 Palm Oil Mill Effluent (POME) characteristics

The POME sample recovered from the palm oil mill was thick, dark brownish in colour, colloidal suspension, greasy and viscous, and odourless. According to Agustin *et al.,* (2008), Bala *et al.,* (2016); and Ma *et al.,* (2020); the dark brown colour of palm oil mill effluent is made up of organic substances such anthocyanin and carotene pigment removed from fresh fruit bunches during the sterilizing process (Bala *et al.,* 2016).

The high values of the selected parameters obtained from raw POME in this study indicate that POME has a high polluting potential and has negative environmental consequences. The discharge of untreated palm oil mill effluent (POME) into the environment has been linked to an alarming increase in environmental pollution, according to researchers (Grace MA *et al.,* (2020); Bala *et al.,* (2016); Cheng *et al.,* 2010; Wu *et al.,* 2010; Lam and Lee, 2011; Alade *et al.,* 2011; Ibrahim *et al.,* 2012. Maygaonkar *et al.,* 2012; Francis *et al.,* 2012; Abass *et al.,* 2012; Mohammed *et al.,* 2014; Soleimaninanadegani and Manshad, 2014). The pH value in this study was 4.74, but Ohimain *et al.,* (2013) reported a pH of 6.56. The POME's low pH indicates that it is acidic, and it should be treated to get a pH of 7–7.5, which suggests plant compatibility. The corrosion of iron utilized in processing may have influenced the acidic character of POME (Soleimaninanadegani and Manshad, 2014). Jameel et al., (1980)2011) and Din et al., (1980)2006) POME's acidic nature is attributed to the presence of organic acids, according to reports. In a similar investigation, Oswal et al., (1980)2002) discovered that POME is acidic (pH 5).

In Table 4.1 it was observed that the raw POME has the lowest volume of manganese compared to the uncontaminated and contaminated but it was observed that the contaminated soil contains more than three times the volume of manganese could it be that the action of the POME on soil causes increase in the volume of Manganese in the soil? But it was also observed that there was no zinc present in the raw POME but there is a reduction of zinc in the soil after being contaminated by POME this was observed for copper, Lead, Nickel, Magnesium and Potassium. But it was opposite for Manganese, Calcium, Sodium and Iron. This suggests that the mixture of POME to the soil has effects in the volume of some hard metals.

Table 4.1: Heavy Metal Analysis of Raw POME in comparison with Contaminated and uncontaminated soil of POME Site

Parameters	**Raw POME (mg/L)**	**Uncontaminated soil (mg/L)**	**Contaminated soil (mg/L)**
Manganese	0.1393	2.6224	10.1965
	0	0	0
Zinc	0	4.6926	4.1782
Cadmium	0	0	0
Copper	0.1509	2.0524	1.4356
Lead	0	1.9608	1.6484
Iron	1.8510	62.3723	64.7820
Nickel	0	0.1547	0.0585
Calcium	1.1648	53.2566	4.2576
Sodium	2.1651	3.9327	20.2706
Cobalt	0	0	0
Magnesium	10.5563	0	0.7881
Potassium	13.3378	29.1917	24.3640

4.2 Physicochemical Analysis of Pome

Table 4.2 shows the physicochemical parameters of POME, the parameters of the raw POME show a high potential which was above the standard for discharge which suggest increase potential for environmental pollution of the POME. The pH was 4.20±0.02 which was more acidic to the environment and the COD of 488.000±0.622, BOD of 120.000±0.880. After introduction of the immobilized cells and observed according to design at 4 days interval. A progressive increase in degradation was recorded as the days go by. After 24 days, the degraded POME was taken for another physicochemical analysis and the result in table 4 was recorded which brought almost all the parameters to almost standard limit for discharge, the pH value drifted to almost neutral 6.5±0.02, Dissolved Oxygen 9.45±0.012, Biochemical Oxygen Demand 108±0.880, Chemical Oxygen Demand 250±0.622 was still above the standard discharge limit but there was a tremendous degradation. For oil and grease the degradation did not get close to the standard limit for discharge although the total suspended solid wasn't an issue since it was okay from the beginning.

POME, on the other hand, has a significant concentration of organic matter, with a COD of 61,200 -75,900 mg/L, a BOD5 of 30,500 - 34,393 mg/L, and oil and grease 145 - 191 Mg/L, according to Bala et al., (1980)2016). Other researchers have observed similar 65,000 mg/L, a BOD5 of 48,000 mg/L, and oil and grease greater than 2000 mg/L, according to Chin et al., (1980)1996), BOD5 (25,000-43,750mg/L), COD (50,000-55,775mg/L), TSS (16,500-18,479mg/L), oil & grease (130-8020mg/L), and pH values to the current study (4.20±0.02). When compared to the results of previous researchers who achieved higher values for oil and grease (4,000-11,000 mg/L), the present study's oil and grease content was low (Ahmad *et al.*, 2003; Najafpour *et al.*, 2005; Wanna and Pompan, 2007; Vijayaraghavan *et al.*, 2007; AbdulKarim *et al.*, 2011; Mohammed *et al.*, 2014; Soleimaninanadegani and Manshad, 2014).

Although, similar to the current investigation, Lam and Lee (2011) showed low levels of 130 mg/L for oil and grease in raw POME before treatment, which surpassed the discharge standard limit. The variation could be attributed to variances in oil palm species, degree of oil extraction during milling, and extraction process.An issue to consider is the amount of water utilized during the milling process. Furthermore, due to mill operations and seasonal cropping, the chemical characteristics of POME vary greatly during the year, according to Yacob et al., (1980)2006).

Table 4. 2 Physicochemical analyses of POME

Parameters	Raw Pome	Degraded Pome	Standard Limit for Discharge
pH	4.20±0.02	6.5±0.02	5-9
Dissolved Oxygen (DO) (mgL^{-1})	11.59±0.012	9.45±0.012	10
Biochemical Oxygen Demand (BOD) (mgL^{-1})	120.000±0.880	108±0.880	100
Chemical Oxygen Demand (COD) (mgL^{-1})	488.000±0.622	250±0.622	150
Oil and Grease	284±0.577	177±0.577	50
Total Suspended Solid	18.855±0.005	18.001±0.055	20
Total Solids	50.200±0.200	42.200±0.200	38
Phosphorus (PPM)	22.000±0.200	17.557±0.200	
Sodium %	0.029±0.000	0.020±0.000	
Calcium	3.97%	2.00%	
Magnesium	3.20%	1.59%	
Sodium	1.67%	1.10%	

All parameters are in mg/L except pH.

4.3 Microbial populations of Palm Oil Mill Effluent sample

All samples 1A, 1B, 2A, 2B and 2C underwent serial dilution and 10^{-1}, 10^{2}, 10^{-3}, 10^{-4} and 10^{-5}. 10^{-3} was plated in duplicates 1A, 1B, 2A and 2B then 3A and 3B, 4A and 4B. Six (6) samples of palm oil mill effluent were collected from the site and spread method was used for bacteria isolation using nutrient agar. Sample four (4) and six (6) recorded the highest growth of $16x10^{3}$ cfu/ml and $11x10^{3}$ cfu/ml respectively while sample one (1) and three (3) recorded the lowest growth of $3x10^{3}$cfu/ml and $5x10^{3}$cfu/ml respectively. The bacteria were isolated, characterized as shown in table 1 and molecularly identified Table 2 and figures 1 and 2 as Bacillus subtilis, Pseudomonas aeruginosa, Bacillus megaterium and Bacillus cereus. All the isolates were immobilized by mixing with 4% sodium alginate solution separately in the ratio of 1:2. Then the mixture is extruded into 0.2 M $CaCl_2$ solution using syringe to form alginate beads (Claudia *et al.*, 2014) for palm oil mill effluent degradation

Bala *et al.,* (2016) found 9.5 x 10^{5}-7.9 x 10^{6} cfu/mL in a comparable study, and Okwute and Isu (2007a) and Okwute and Isu (2007b) found total bacterial populations of 9.6 x 10^{8}, 1.64 x 10^{9}, and 1.07 x 10^{9} in a microbiological investigation of POME samples. Furthermore, in a microbiological investigation of raw POME, Okwute (2013) found a bacterium population of 4.0 x 10^{9} cfu/mL, 2.6 x 10^{3} cfu/mL, and 2.6 x 10^{3} cfu/mL. Serikovna et al., (1980)2013) reported 10^{8} cfu/mL, 10^{7} cfu/mL, and 2 x 10^{8} cfu/mL from oily wastewaters, and Wu et al., (1980)2009) found 6.65 x 10^{6} cfu/mL.Total bacteria populations varied from 7.4 x 105 to 2.0 x 10^{6} cfu/mL, according to Ohimain et al., (1980)2012a). Similar numbers have been found in pharmaceutical wastewater by Bala et al., (1980)2012). This proves that bacteria can be found in wastewater.

The above-mentioned study's results revealed some variances in microbial numbers. Many factors influence the diversity of microbial populations, including nutritional, mineral, temperature and oxygen levels, acidity, wastewater volume (Okereke *et al.,* 2007), and the content of oil and grease and carbohydrates in the POME.The high population of bacteria in the POME could be linked to pollution in the mills due to inadequate sanitation and inconsistent disinfection (Okechalu *et al.,* 2011). It could also be a result of the handling procedure and the mill's current environmental conditions. POME's high content of carbohydrates, proteins, nitrogenous substances, lipids, minerals, cellulose, hemicelluloses, and lignin may be linked to the presence and proliferation of viable bacteria and fungus (Hii *et al.,* 2012). POME microbial species have the ability to breakdown the carbon source contained in the POME. The saprophytic capacity of bacteria to grow on and breakdown carbon sources in industrial effluents is linked to biodegradation (Haimann, 1995). The findings demonstrate that the microbial species identified are identical to those found in petroleum hydrocarbon-polluted environments. Okwute *et al.,* (2007). Because it is high in nutrients like lipids (oil) and cellulose, POME is a possible habitat for lipolytic and cellulolytic bacteria and fungi (Ohimain *et al.,* 2012a; b). From POME obtained from oil palm processing locations, Ohimain et al., (1980)2012a) recovered lipase and cellulase generating Bacillus sp. In POME, these enzymes are in charge of breaking down cellulose and oil (Wong *et al.,* 2008). The occurrence of these microorganisms (bacteria) in POME may be due to their ability to utilize oil and cellulose as their carbon source which has been reported by Ojumu et al., (1980)2005). The utilization of POME as a carbon source by these microorganisms has been reported by Sira *et al.*, (2010) and Wu *et al.,* (2007). *Bacillus megaterium* MZ379521, *Bacillus cereus* MZ379522, *Bacillus subtils* MZ379523, and *Pseudomonas aeruginosa* MZ379524 were found in the POME sample, indicating that they are capable of biodegrading oily wastewaters (Bala *et al.,* 2016, Okwute *et al.*, 2014, Ohimain *et al.,* 2012a; b; c; Nwuche and Ogbonna, 2011).

POME-derived microorganisms have been employed to treat wastewaters such as POME and olive oil mill effluent to reduce COD levels (Bala *et al.,* 2016, Oswal *et al.,* 2002; Ohimain *et al.,* 2012a; Kamal *et al.,* 2011; Neoh *et al.,* 2013; Nawawi *et al.,* 2010; Ahmad *et al.*, 2011). In their work, the aforementioned authors isolated microbes from different sources such as marine habitat, soil, and sewage sludge, whereas bacteria were isolated from POME and immobilized in the current investigation. Because of the unrecovered oil present in the effluent, which acts as a carbon source, the oily environment in POME may create a favourable habitat for lipolytic microbes to thrive. The presence of these bacteria in POME, on the other hand, is beneficial for decomposing hazardous chemicals in wastewaters, such as bioremediation of crude oil spills. Soleimaninanadegani and Manshad, 2014; Ohimain *et al.,* 2012a).

Table 4.3: Cultural characteristics and Gram Reaction of bacteria isolated from POME

characteristics	Gram's reaction	Presumptive Organisms
Large, irregular, opaque colonies. Smooth and moist colonies, whitish to cream.	Positive rod	*Bacillus cereus*
Colonies are concave, smooth, and milky white, convex, and opaque with a brownish center.	Positive rod	*Bacillus megaterium*
Greenish blue, translucent opaque and bacterial colonies have a smooth appearance.	Negative rod	*Pseudomonas aeruginosa*
Dry, flat, and irregular, with lobate margins; colonies round or irregular; surface dull; become thick and opaque; whitish.	Positive rod	*Bacillus subtilis*

Table 4.4: Biochemical Characteristics of POME Bacteria

Isolate	Gram	Shape	Catalas	Coagul	Starch	Utilizat Citrate	MSA	MR	VP	Glucos	Oxidas	Presum ptive
1	+	Rods	+	-	+	+	-	+	-	+	-	*B. megaterium*
2	+	Rods	+	-	+	-	-	-	+	-	-	*B. cereus*
3	+	Cocci	+	+	-	+	+	+	-	+	-	*S. aureus*
4	+	Rods	+	-	+	+	-	+	-	+	-	*B.cereus*
5	-	Rods	+	-	-	+	-	+	-	+	+	*P. aeriginosa*

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Table 4.5: Molecular identification of isolates Based Blast Analysis

sample ID	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
sample1	*Bacillus megaterium*	2669	2669	100%	0	100.00%	1449	MZ379521
sample2	*Bacillus cereus*	2684	2684	100%	0	100.00%	1453	MZ379522
sample3	*Bacillus subtilis*	2747	2747	100%	0	99.80%	1496	MZ379523
sample4	*Pseudomonas aeruginosa*	2647	2647	100%	0	100.00%	1433	MZ379524

Table 4.4: Blast analysis showing the relationship between isolate sequence and the most closely related as present in the NCBI database

Figure 2:

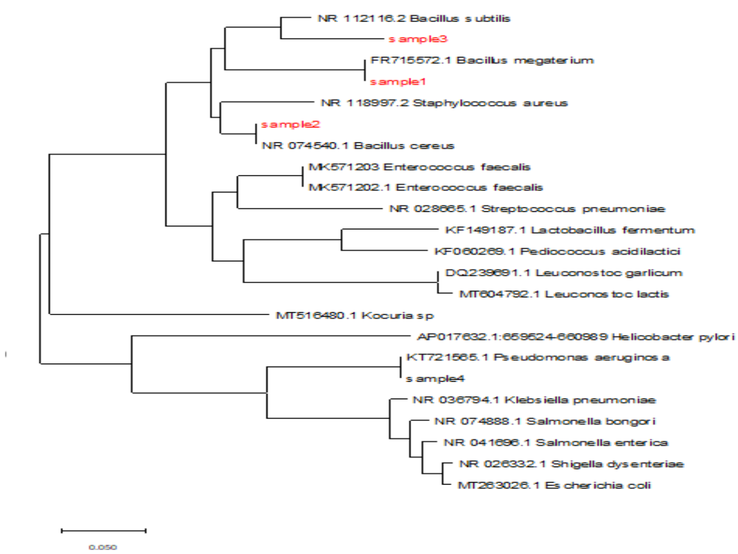


Figure 2: Dendrogram showing the sequence relationship on the Bacteria isolates and selected sequences from the NCBI database

4.4 Reduction and Biodegradation of Palm Oil Meal Effluent

Individual bacterial strains were used for determination of the percentage (%) reduction and degradation of BOD, COD, TSS, and O&G in raw POME samples as shown in Table 4.6. The following percentage reduction in for all physicochemical properties was observed using *Bacillus subtilis* MZ379523 DO 18.46%, BOD 10%, Oil and Grease (O&G) 37.68%, TSS 4.53 and COD 48.77%. It was discovered that the percentage reduction for COD was the highest compared to the other parameters followed by Oil and Grease (O&G), DO, BOD and TSS being the least reduced. This trend was observed for all the other organisms as shown in figures: 3,4,5 and 6 respectively. *Bacillus subtilis* MZ379523 was the most effective, with DO reduction rates of 18.46 percent, BOD 10%, O&G 37.68 percent, TSS 4.53, and COD 48.77 percent, when compared to other isolates. *Pseudomonas aeruginosa* MZ379524, *Bacillus cereus* MZ379522, and *Bacillus megaterium* MZ379521 were the least effective.

In agreement with Serikovna *et al.,* (2013). This shows that our indigenous isolates are successful at reducing physicochemical properties of POME and also suggest that isolated indigenous immobilized bacteria have some significant effects since they were originally from the POME and also have adaptive features. Serikovna et al., (1980)2013) observed that bacteria must be pre-adapted in the contaminant-containing medium in order to be more competitive, which is in line with their findings. This also demonstrated that our isolates in the sterile POME sample can be combined or mixed since they are already pre-adapted from POME. This study will contribute to our understanding of bacteria's role in biological wastewater treatment from POME oil production.

In this present study, comparisons of physicochemical percentage reduction by isolated indigenous bacteria from POME found that the percentage reduction was similar to that reported in earlier publishers from oily industrial wastewaters. (Abduil Kaiirim *et al.,* 2011; Abass *et al.,* 2012; Mohamed *et al.,* 2014; Soleimaninianadegani and Mainshad, 2014)

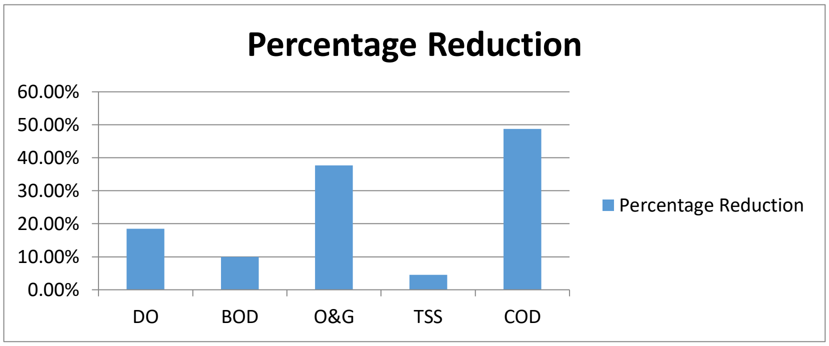


Figure: 3 Degradation and Reduction of POME by *Bacillus subtilis* MZ379523

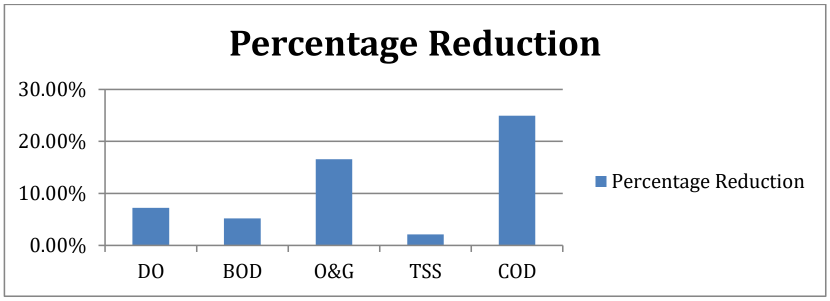


Figure: 4 Figure: 3 Degradation and Reduction of POME by *Pseudomonas aeruginosa* MZ379524

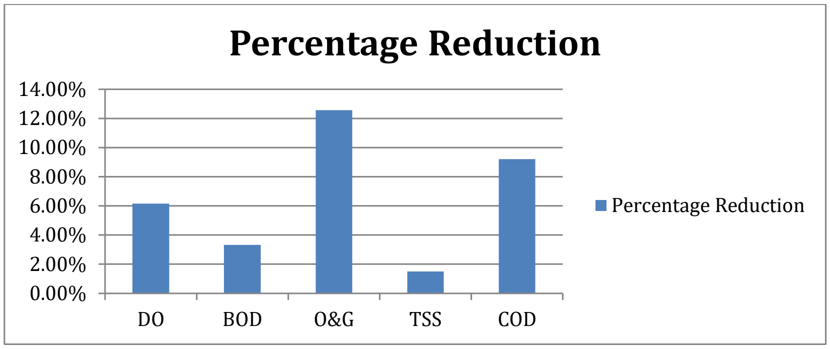
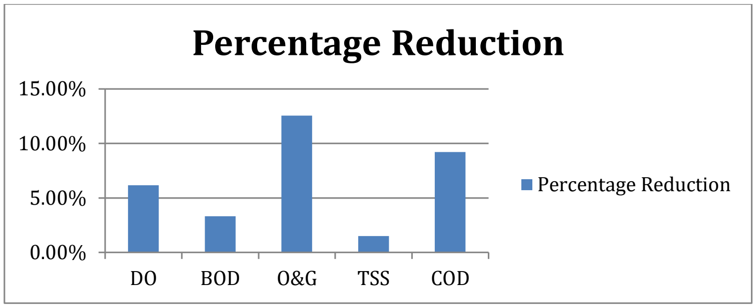


Figure: 5 Degradation and Reduction of POME by *Bacillus megaterium* MZ379521



Degradation and Reduction of POME by *Bacillus cereus* MZ3795227

Table 4.6 Oil and Grease content of POME Inoculated with Immobilized Bacteria

DAYS	ISOLATE 1	ISOLATE 2	ISOLATE 3	ISOLATE 4
-4	3.05	2.54	2.70	4.64
-8	7.70	4.58	4.64	11.16
-12	13.92	5.20	10.81	21.46
-16	26.31	10.91	21.21	29.52
-20	41.86	11.52	22.85	42.78
-24	42.02	16.88	22.94	43.85

All parameters are in Percentage (%)

Key

Isolate 1 = *Bacillus megaterium*

Isolate 2= *Bacillus cerus*

Isolate 3 = *Pseudomnoas earoginosa*

Isolate 4 = *Bacillus subtilis*

Table 4. And figure shows the percentage degradation of the various isolated organisms. The experimental set-up was for four (4) days interval. Day zero, day four (4), day eight (8), day twelve (12), day sixteen (16), day twenty (20) and day twenty-four (24). Results revealed that percentage degradation for *Bacillus subtilis* recorded the highest percentage of degradation of 43.85% followed by *Bacillus megaterium* with 42.02%, *Pseudomonas aeruginosa* recorded 22.94% while *Bacillus cereus* recorded the lowest degradation of 16.88%.

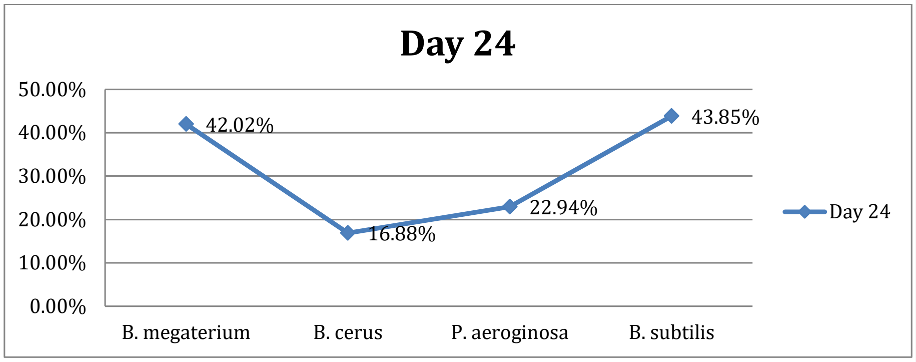


Figure 7. Clearly showing the degradation on day 24 of all the organisms

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study suggests that isolated bacteria cells from POME also have ability to degrade their original source and immobilizing them confers some more ability to carry out the degradation process. The degrading ability of the indigenous immobilized bacteria isolated from POME was *a* clear indication that these bacteria can be used for biodegradation of POME even in a larger scale.

The isolated indigenous immobilized bacteria show efficiency in their degradation potential of some physicochemical parameters such as DO, TSS, TS, COD, BOD, pH and inadvertently oil and grease which can enhance POME treatment.

Bacillus substilis MZ379523 demonstrated 43.85% of the biodegradation potential of indigenous immobilized bacteria isolated from POME, indicating that it is the best degrader of all contaminants in the POME based on this study

5.2 Recommendation

I will suggest that further research be carried on how to mass produce and preserve indigenous immobilized microorganisms for industrial use since they are appearing to be more efficient.

More so, these immobilized organisms can be patented and sold to local Palm Oil manufacturers for handy use in order to reduce the menace caused by POME pollution in the environment

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Plate 1. Showing the effect of Palm Oil Mill Effluent in Irruan Boki LGA Cross River State, Nigeria. Photo by Egbe Otor, 2019.

>sample 1 *Bacillus megaterium*

GTTCCGCATGCAAGTCGAGCGAACTGACGTTTCAAGCTTGCGTTCTTTGACGT
TAACACGTTGGACGGGATGAGTAACCCGTGGGCCACCTGCCTGTAACACTGG
GATAACTTCGGGAAACCGAAGCTAATACCGGATATGATCTTCTCCTTCATGG
GAAATGATTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACAGARCAA
CGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAA
GAACAAGTACAAGAGTAACTGCTTGTACCTTGACGGTACCTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA
TCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGT
GAAAGCCCACGGCTCAACCGTGGAGGGTCATTTGAAAACTGGGGAACTTGAG
TGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGAT
GTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGA
GGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGC
TAAACGCATTAAGCACTTCGCCTGGGGAGTACGGTCGCAAGACTTAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAA
GCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGAT
AGAGCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT
CTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA
CACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCGAGGTCAAG
CCAATCCCATAAAACCATTCTCAGTTCGGATTTTAGGCTGCAACTCGCCTACA

TGAAGCTGGAATCGCTATTAATCGTGGATCAGCATGCCGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCTCGTCACACCACGAGAGATTGTACCTCCCGA
AGTCGTTGGAGCCGTAAGGAGYAGCAGTGTAGCTATAGAGTTTTT

>sample 2*Bacillus cereus*

CGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGA
AGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGG
GATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGG
TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAA
GAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
ATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGT
GAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGT
GCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATA
TGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAG
GCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTT
AACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAG
GGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTA
GTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTA
ATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGA

AGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGT
CGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATT

>sample 3 *Bacillus subtilis*

GTAACTACAAGAAAAGGGACGGAGGAAGAATAAAGAACCTTTTCTCCCTAA
AATAATTAGGCGGGGGACCGGGGGGGATTACCCACTGGGGGTAACCCTGCCC
CTATAAAGACTGGGGATAAATTCCGGGGAAAACCGGGGGCTTAAATCCCGGG
ATGATTTTTTTGAACCCGCCAGGGTTTCAAACATAAAAAGGGGGGTTTGGGG
TTTCCACTTTACGAGTGGGACCCCCCGGTGGCATTTAGCTAAGTTGGTGAAGG
TAAAGGGTTCACCCAAGGGAAAGGATGGGTTAGCCGACCCTGAGAGGGTGA
TCCGGCCCCCACTGGGGTTGAGACCCCGGGCCCGAATTCCTACGGGAAGGCA
ACCAGTAGGGAATTTTTCCGCAATGGGACGAAAATTCTGACCGAGCAAACCC
CGCGTGAGTGATGAAAGGTTTTCGGATCGTAAAAATTCTGTTGTTAGGGAAG
ACCAAGTACCTATTGAATAAGGCGGTACCTTGACGGTACCTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG
TCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTG
AAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTG
CAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT
GGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGGGGGTTTCCCCCCCTTAGTGCTGCAGCTA
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GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGG
ACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGT
TGCCATCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACAC
GTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTGAGCCAAT

CCCACAAATCCGTTCTCAGTTCGGATTGCAGTCTGCAACTCGCCTGCGTGAAG
CTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCG
GTGAGGTAACCTTTTGGAGCCAGCCGCGAATGGGGCCCTT

>Sample 4 *Pseudomonas aeruginosa*

GGCCTTCAACACATGCAAGTCGAGCTTATGAAGGGAGCTTGCCTTGGATTCA
GCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAAC
GTCCGGAAACGGCCGCTAATACCGCATACGTCCTGAGGGAGAAAGTCGGGG
ATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG
GGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCA
GTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATTGCCGCGTGTGTGA
AGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGT
TAATACCTTGCTGTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTG
CCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGCGCGTAAGTGGTTCAGCAAGCTTGATGTGAAATCCCCGGGCTCA
ACCTGGGAACTGCATCCAAAAGCTACTGAGCTAGAGTACGGTAGAGGTGGTA
GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGG
CGAAGGCGACCACCTGGACTGTACTGACACTGAGGTGCGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAG
CCGGTTGGGATCCTTGAGATCTTAGTGGCGCACGTAACGCGATAAGTCGACC
GCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAA
CAGAGACACAGGTGCTGGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
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GTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
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TCGGTACAAAGGGTTGCCAAGCCGCGAGTGGGAGCTAATCCCATAAAACCGA

TCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTA
GTAATCGTGAATCAGAATGTCACGGTGAATACGTCCCCGGGCCTTGTACACA
CCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCG
CAAGGGGGACGGTTACCACGGAGTGATTCAT