

**MICROBIOLOGICAL ANALYSIS OF LOCAL AND MECHANIZED PALM OIL MILL
EFFLUENT.**

BY

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MAT NO: PG/LSC01714670**

**UNIVERSITY OF BENIN
BENIN CITY**

OCTOBER, 2019.

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**A PROJECT REPORT SUBMITTED TO THE SCHOOL OF POSTGRADUATE
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CERTIFICATION

This is to certify that this project work was carried out by **Chineme Mayfair, AKPANGENE (Mrs)** in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City under my supervision.

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APPROVAL

This project work is accepted in partial fulfillment for the award of Postgraduate Diploma (P.G.D.) in Microbiology, Department of Microbiology, University of Benin, Benin City.

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DEDICATION

This work is dedicated to God Almighty who gave me the strength, wisdom and understanding to carry out this work.

ACKNOWLEDGEMENTS

My greatest gratitude and thanks go to the Almighty God who has kept me all through this period and has also given me the grace to complete my seminar work.

The success story of this work will not be complete if I fail to give credit to whom credit is due.

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ABSTRACT

Palm oil mill effluent (POME) is an acidic, viscous, and voluminous colloidal suspension formed during palm oil processing from the mixed stream of sterilizer condensate, separator sludge, and hydrocyclone wastewater. The aim of this study was to investigate the microbiological analysis of localized and mechanized palm oil mill effluents. Palm oil mill effluent (POME) were collected aseptically with sterile microbiological bottles from mechanized (NIFOR) and Local palm oil mill in Benin City, Edo State, Nigeria. The samples were immediately transported to the laboratory for analysis. The standard method of isolating microorganisms was adopted. Microorganisms were cultured on Nutrient Agar and Antimicrobial analysis was done using the agar well diffusion method. Local palm oil mill effluent had the highest bacteria count (13.00×10^3 cfu/ml) while mechanized palm oil mill effluent had the least bacteria count (3.0×10^3 cfu/ml). Bacteria isolated were *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Shigella dysenteriae*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella enterica* and *Staphylococcus aureus*. *P. aeruginosa* (18.%) and *E. coli* (18.8%) were the most occurring bacterial isolates while *S. dysenteriae* (7.8%) and *P. mirabilis* (7.8%) were the least occurring bacterial isolates. *B. subtilis*, *S. aureus* and *S. dysenteriae* were susceptible to rocephin, septrin and resistant to gentamycin, zinnacef and ciprofloxacin. *E. coli*, *P. aeruginosa* and *S. enterica* were resistant to augmetin and chloramphenicol and susceptible to pefloxacin and ofloxazin. *P. aeruginosa* was the most resistant bacteria with multiple antibiotic resistance index of 0.7. *E. coli* was the least antibiotic resistant bacteria with multiple antibiotic resistance index of 0.2%. Proper hygiene practice by palm oil workers and sanitation of the palm oil facilities should be constantly carried out to avoid transmission of potentially infectious agents to patients. Also, use of fertilizers to grow palm trees should be reduced to minimize the incidence of drug resistance.

CHAPTER ONE

1.0 Introduction

Palm oil processing is carried out using large quantities of water in mills where oil is extracted from the palm fruits. During the extraction of crude palm oil from the fresh fruits, about 50% of the water results in palm oil mill effluent (POME). It is estimated that for 1 tonne of crude palm oil produced, 5 - 7.5 tonnes of water ends up as palm oil mill effluent (Ahmad *et al.*, 2003). The solid waste products that result from the milling operation are empty fruit bunches, palm fibre, and palm kernel. In both traditional and modern milling settings, these solid waste products are all put to economically useful purposes such as fuel material and mulch in agriculture. It is the palm oil mill effluent that is usually discharged into the environment, either raw or treated.

Palm oil mill effluent (POME) is an acidic, viscous, and voluminous colloidal suspension formed during palm oil processing from the mixed stream of sterilizer condensate, separator sludge, and hydrocyclone wastewater (Said *et al.*, 2013). Depending on the method of processing, it is composed of 95-96% water, 0.6-0.7% oil, and 4-5% total solids. POME is a highly polluting wastewater and causes considerable deterioration of soil and water quality when discharged untreated into the environment (Eze *et al.*, 2013). Recently, the enforcement of stringent laws pertaining to waste effluent discharges in some palm oil producing communities in Nigeria have challenged researchers to investigate new approaches for the effective management of POME.

Raw palm oil mill effluent consisting of complex vegetative matter is thick, brownish, colloidal slurry of water, oil and solids including about 2% suspended solids originating mainly from cellulose fruit debris, that is, palm mesocarp (Ahmad *et al.*, 2006). The raw or partially treated palm oil mill effluent has an extremely high content of degradable organic matter, which is due in part to the presence of unrecovered palm oil (Ahmad *et al.*, 2003).

It has been observed that most of the palm oil mill effluent produced by the small-scale traditional operators undergoes little or no treatment and is usually discharged into the surrounding environment (Awotoye *et al.*, 2011). The local palm fruit processing method to produce palm oil is very long and laborious. Firstly, the palm bunches are quartered (cut into four) and left overnight for easy separation of nuts from the spikelets. The fruits are boiled for 1-1½ h, pounded in a mortar or macerated with feet in a canoe-like container, water is added and it is well mixed up. All nuts are then carefully removed by hand. The fibres are well shaken over in the sludge, until oily foam comes up to the surface of the sludge. The foam is collected in a container until the operation is completed when there is no more foam formation. This foam is later boiled for about 30 to 40 min. The clean edible oil then collects on the surface leaving the sludge at the bottom. Sometimes the oil in the sludge pit is recovered and mixed with fibre to make a fire starting cake called flint. At other times, the sludge is poured onto the surrounding bushes and soil together with the liquid waste known as palm oil mill effluent (POME).

Large and medium scale mills produce copious volumes of liquid waste (POME) from the processing lines, (sterilizers, clarifying centrifuges and hydro cyclones) when palm oil mill effluent is produced on a large and commercial level. However effective the system of oil recaptured from the sludge may be; the palm oil mill effluent discharged from an oil mill is objectionable and could pollute streams, rivers or surrounding land (Mohammad *et al.*, 2007). While mills were comparatively few and mostly beside large fast flowing rivers, the problem was not a serious one but the situation in many countries including Nigeria is quite different with much attention being recently given to the subject of effective disposal and protection of the environment.

The constituents of raw palm oil mill effluent have been reported to be a colloidal suspension of 95 - 96% water, 0.6 - 0.7% oil and 4 - 5% total solids including 2 - 4% suspended solids (Ahmad *et al.*, 2003). The raw palm oil mill effluent has an extremely high content of degradable organic matter, which is due in part to the presence of unrecovered palm oil, thus, palm oil mill effluent should be treated before discharge to avoid serious environmental pollution (Ohimain *et al.*, 2013).

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 (Onyia *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.

Palm Oil Mill Effluents (POME) in Nigeria are mostly from palm oil processing and refining mills. Palm oil is extracted from palm oil fruit mesocarp (Ohimain *et al.*, 2013). The two principal effluent (liquid wastes from palm oil mills are the clarification sludge and the sterilizer condensate, which are discharged as oily, brown/liquids at temperatures between 75°C and 85°C

(Said *et al.*, 2013). Palm oil mill effluents are high volume liquid wastes that have unpleasant odour. They are predominantly organic in nature and are highly polluting. In practice, these effluents contain both dissolved and fine suspended matter, some being colloidal, and others residual oil. Its Biochemical Oxygen Demand (BOD) is fairly high, slightly acidic in reaction and consists of around 95 – 99% water, 1–4% material variously in solution and suspension and 0.5 – 1% residual oil (Eze *et al.*, 2013). These effluents are difficult to treat because the organic matter is essentially made up of lipids and cellulosic materials, which are not readily biodegradable. In the past, the effluent had been simply discharged into the sea or river without treatment (Embrandiri *et al.*, 2012). Effluents affect the soil properties, thereby inhibiting crop growth. The higher amount of salts and high concentration of sodium and alkalinity in the wastewater generated from the industries increase the exchangeable sodium percent (ESP) of the soil to a harmful level during land disposal. The effect of high ESP is manifested by soil permeability (Ere *et al.*, 2011).

In Nigeria, palm oil effluent is discharged into the environment in its raw form especially by small-scale operators. Palm oil mill effluent in the effluent dumpsite has negative impact on some physicochemical properties, pH, soil texture, water holding capacity, organic carbon, total nitrogen, e.t.c. of the soil. Numerous organisms invade and grow in palm oil mill effluent breaking down complicated molecules into simple ones. The high organic matter in palm oil effluent, may have a major role in the proliferation of aerobic microorganisms (Ohimain *et al.*, 2012).

Soil productivity is decreased as the population of various microorganisms (bacteria, Actinomycetes and free-living N-fixing bacteria) decrease due to irrigation by well waters polluted with discharge of industrial effluents (Okereke *et al.*, 2007). Once the microbial

population is affected, the soil productivity decreases resulting in reduced nutrient availability, plant production and the soil becomes sterile. Nitrogen nutrition of plant is affected due to the inhibition of Nitrogen- fixing organisms activities and nitrification rate (Okwute and Isu, 2007). Industrial wastewaters are essential habitat for diverse microbes. Generally, some of the microorganisms have been used for biotreatment of wastewaters (Abdel-Raouf *et al.*, 2012; Bala *et al.*, 2014, Bala *et al.*, 2015, Bala 2016). Microorganisms domiciled in diverse wastewaters can also cause diseases such as tuberculosis, cholera, typhoid, dermatomycosis, hepatitis and dysentery (Shaaban *et al.* 2004). The physicochemical properties of POME are well documented. Conversely, the microbiological aspect is overlooked; as such there seem to be dearth of information on the microbiota been documented proving that a well-developed understanding of these is needed

1.1 Aim and Objectives

Aim

This study examined the microbiological analysis of localized and mechanized palm oil mill effluents.

Objectives

The specific objectives of this study is to:

- 1) Isolate microorganisms from localized and mechanized palm oil mill effluents
- 2) Determine the distribution of microbial isolates in localized and mechanized palm oil mill effluents
- 3) Determine the antibiotic susceptibility pattern of microbial isolates

CHAPTER TWO

LITERATURE REVIEW

2.1 Composition of Wastewaters

Water receive pollution from many different sources, which vary both in strength and volume. The composition of wastewater is a reflection of the life styles and technologies practiced in the producing society (Blodgette, 2001). It is a complex mixture of natural organic and inorganic materials as well as man-made compounds. Organic and inorganic substances which are released into the environment as a result of domestic, agricultural and industrial water activities lead to organic and inorganic pollution. Three quarters of organic carbon in wastewaters are present as carbohydrates, fats, proteins, amino acids, and volatile acids (Abdel-Raouf *et al.*, 2012). The inorganic constituents include large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulphur, phosphate, bicarbonate, ammonium salts and heavy metals (Lim *et al.*, 2010). Different sources of pollutants include discharge of either raw or treated wastewaters from towns and villages; discharge from manufacturing or industrial plants; run-off from agricultural land; and leachates from solid waste disposal sites (Eze *et al.*, 2013). These sites of pollution have problems so that a solution is sought. Scarcity of water, the need for energy and food are forcing us to explore the feasibility of wastewater recycling and resource recovery (Ahmad *et al.*, 2006). Wastewater environment is an ideal media for a wide range of microorganisms specially bacteria, fungi, viruses and protozoa. The majority is harmless and can be used in biological wastewater treatment, but some wastewaters also contain pathogenic microorganisms (Abdel-Raouf *et al.*, 2012). Bacteria which cause cholera, typhoid and tuberculosis; fungi which cause dermatophytosis; viruses which cause infectious hepatitis; protozoa which cause dysentery and

the eggs of parasitic worms are all found in various wastewaters depending on their sources (Shaaban *et al.*, 2004).

2.2 Sources of Oily Wastewaters

The largest source of wastewater is produced during oil extraction processes in most oil mills and the mill effluents such as palm oil mill effluent (POME) may be categorized as an oily wastewater (Ahmad *et al.*, 2005). Oil contaminated wastewaters comes from variety of sources such as palm oil mills, olive oil mills, crude oil production, oil refinery, petrochemical industry, metal processing, compressor condensates, lubricant and cooling agents, car washing, restaurants (Lan *et al.*, 2009). Oily wastewaters contain toxic substances such as phenols, petroleum hydrocarbons, polyaromatic hydrocarbons, which are inhibitory to plant and animal growth, equally, mutagenic and carcinogenic to human being. Similarly, oily wastewaters contain high oil content, chemical oxygen demand (COD) and colour. The increase in global demand for edible oils has in the last few decades resulted in a tremendous increase in the cultivation of oil seeds, particularly of soybean and oil palm (Yacob *et al.*, 2006). The oil seeds are usually processed to obtain the oil contents which are subsequently processed for human consumption and industrial applications. Thus the vegetable oil industries are, equally, associated with oil extraction, refining, transportation, uses and reuses. However, these industries have been linked with environmental pollutions resulting from oil spill, oily effluent discharge into water bodies and oily sludge discharge into the environment indiscriminately, untreated or in conditions below the standard discharge limits (Alade *et al.*, 2011)

2.3 Palm Oil Mill Effluents

Palm oil mill effluent is considered as one of the most polluting agro-industrial residues due to its high organic load. Palm oil mill effluent is in the form of highly concentrated dark brown

colloidal slurry of water, oil and fine cellulose materials from sterilisation and clarification stages. POME is a colloidal suspension of 95-96% water, 0.6-0.7% oil and 4-5% total solids (Ma, 2000). Oil palm production in Nigeria has risen from 8.2 million tonnes in 1990 to 9 million metric tonnes in 2001 (FAO, 2002). About 43-45 % of this is always a mill residue in the form of Empty Fruit Bunches (EFB), Shell, Fibre and Palm Oil Mill Effluent (POME). These residues will continue to accumulate with increasing production. Efforts are geared towards converting these waste materials into useful products in energy production, animal feedstock and organic fertilizer.

The process to extract the oil requires significant large quantities of water to steam sterilize the palm fruit bunches and clarify the extracted oil. The separated wastewater sludge commonly referred to as palm oil mill effluent is a brown slurry, which is composed of 4-5 % solids, (mainly organic), 0.5-1% residual oil and about 95% water and high concentration of organic nitrogen (Onyia *et al.*, 2001). This effluent is a serious land and aquatic pollutant when discharged immediately into the environment. Besides the presence of lipids and volatile compounds, the inhibitory effects of palm oil mill effluent on living tissues, could also be due to presence of water-soluble phenolic compounds (Radzia 2001; Perez *et al.*, 1992).

The discharge of effluent containing ammonia is undesirable because it causes excessive oxygen demand in the receiving waters. Although palm oil mill effluent is a pollutant as far as the palm oil industry is concerned, it has enormous potentials for animal feed improvement and soil amendment (Binder *et al.*, 2002).

Characteristics of palm oil mill effluent depend on the quality of the raw material and palm oil production processes in palm oil mills. The extraction of crude palm oil from fresh fruit bunches (FFB) requires huge amounts of water (Rupani *et al.*, 2010; Mohammed *et al.*, 2014; Ma, 1999;

Ahmad *et al.*, 2003). Sethupathi (2004) has categorized three major processing operations responsible for producing the POME. Sterilization of FFB, clarification of the extracted crude palm oil (CPO), hydrocyclone separation of cracked mixture of kernel and shell hydrocyclone contributes about 36, 60 and 4% of POME respectively in the mills. Yacob *et al.* (2006) estimated that about 0.5- 0.75 tonnes of POME will be discharged from mill for every tonne of fresh fruit bunch

2.4 Extraction of Crude Palm Oil

Fresh fruit bunches (FFB) harvested from the oil palms are processed in palm oil mills and turned into crude palm oil and the palm kernel. Generally, palm oil mills are located in the plantations to facilitate the transportation and processing of the FFB. The palm oil milling process basically involves the physical extraction of the palm products (Hii *et al.*, 2012).

There exists several processing stages in the extraction of crude palm oil from fresh fruit bunches. The first stage is sterilization. This involves subjecting freshly harvested fruit bunches brought to the mill to a high pressure steam (120 to 140°C at 40 psi (275790 Newton / square meter [N/m²])) with a minimal delay so as to inactivate the lipolytic enzymes that causes oil hydrolysis and fruit deterioration. This stage also prevent further formation of free fatty acids due to enzyme action and preparing the fruit bunches for the subsequent sub processes (Igwe and Onyegbado, 2006; Hii *et al.*, 2012). The next stage is called bunch stripping. This offers a means of separating the fruits from the bunch stalks by mechanical stripping. The separated and sterilized fruits thereafter undergo a process of digestion. This is achieved by reheating the fruits using steam to a temperature of 80-90°C. This prepares the fruits for oil extraction by rupturing the oil bearing cells in the mesocarp and loosening the mesocarp from the nuts. Oil extraction followed by

clarification and purification are the last processes of oil extraction. The crude oil is extracted from the digested fruit mash by the use of the screw press without kernel breakage.

The extracted liquid and nuts are discharged from the screw press. However, the extracted oil contains varying amounts of water, solids and dissolved impurities that must be removed. The fiber particles from the pressed crude oil are first removed by passing the oil over a vibrating screen; sand and dirt are allowed to settle. Water is removed by settling or centrifuging and finally by vacuum drying. It is worthy to note that the moisture content of the clarified crude oil is still about 0.1-0.25% of moisture (Shaaban *et al.*, 2004). This helps in maintaining oxidative stability and also prevents the deposition of small amounts of soluble solids known as gums. The final product is consumed locally as crude palm oil or can further be refined (Igwe, 2006). For every 100 kg of fruit bunches, typically 22 kg of palm oil and 1.6 kg of palm kernel oil can be extracted (Gunawan *et al.*, 2009). However, enormous and large amounts of palm oil residues or pollutants are produced at the same time, which might result in serious environmental pollution (Hii *et al.*, 2012).

2.5 Impact of Palm Oil Mill Effluent on the Quality of Receiving Soil and River

Oil palm cultivation and processing, like other agricultural and industrial activities, also raises environmental issues. Palm oil processing is carried out using large quantities of water in mills where oil is extracted from the palm fruits. During the extraction process, about 50% of the water results in palm oil mill effluent. It is estimated that for every 1 tonne of crude palm oil produced, 5-7.5 tonnes of water end up as palm oil mill effluent (POME) (Okwute and Isu, 2007; Wu *et al.*, 2009).

The process of oil palm processing in Nigeria is a routine procedure for most small scale operators. First the palm bunches are quartered (cut into four) and left overnight for easy

separation of the nuts from the spikelet. The fruits are boiled for 1-1.5 hours, pounded in a mortar or macerated with feet in a canoe-like container; water is added and is well mixed up. All nuts are then carefully removed by hand. The fibres are well-shaken over in the sludge, until oily foams come to the surface of the sludge. The foam is collected in a container until there is no more foam formation. The collected foam is later boiled for about 30 to 40mins. The clean edible oil then collects on the surface leaving the sludge at the bottom. Sometimes the oil in the sludge pit is recovered and mixed with fibre to make a fire starting cake called flint. At other times, the sludge is poured on the surrounding bushes and soil together with the liquid waste known as palm oil effluent (Wu *et al.*, 2007).

In Nigeria's palm oil industry, most of the palm oil mill effluent produced by small scale traditional operators undergo little or no treatment and are usually discharged in the surrounding environment. This palm oil mill effluent could pollute streams, rivers or surrounding land (Okwute and Isu, 2007). River water consequently turns brown, smelly and slimy. Often fish and other aquatic organisms get killed and local people are denied of the availability of local water sources for domestic uses and fishing (Ezemonye *et al.*, 2008).

Palm oil mill effluent (POME) is an important source of inland water pollution when released into local rivers or lakes without treatment. POME contains lignocellulosic wastes with a mixture of carbohydrates and oil. Chemical oxygen demand (COD) and biochemical oxygen demand (BOD) of POME are very high and COD values greater than 80,000 mg/L are frequently reported. Incomplete extraction of palm oil from the palm nut can increase COD values substantially (Oswal *et al.*, 2002). The high COD value is responsible for distraction of aquatic life (Maygaonkar *et al.*, 2012)

Palm oil mill effluent application to soil can result to some beneficial soil chemical and physical characteristics, such as increases in organic matter, organic carbon, major nutrients (e.g N,P), water-holding capacity and porosity (Mantzavinos and Kalogerakis, 2005). However, it brings about undesirable changes such as decreases in pH, and increases in salinity etc. (Onyia *et al.* 2001). These effects occur very slowly and need many years to provide significant results. Soil microbiological and biochemical properties have been considered early and sensitive indicators of soil changes can be used to predict long term trends in the quality of soil (Ros *et al.*, 2003).

Palm oil mill effluent contains high organic load, substantial amounts of plant nutrients and represent a low cost source of plant nutrients when fermented (Onyia *et al.* 2001). It is generally believed that the toxicity effect of POME is due to presence of phenols and other organic acids which are responsible for phytotoxic effect and antibacterial activity (Pascual *et al.* 2007). However, the polyphenolic fraction degrades with time and partially transforms in humic substances. Little information is known on the impact of POME on the biochemical and microbial properties of soil. Studies show that effects of wastes supplied to soil occurred mainly in the first weeks after amendment (Binder *et al.* 2002).

Wastewater composition depends mainly on the season, raw matter quality and the particular operations being conducted at any given time. Typically, palm oil mill wastewater is low in pH because of the organic acids produced in the fermentation process, ranging about 4-5. It also contains large amounts of total solids (40,500 mg/L), oil and grease (4000 mg/L) (Ma, 2000). Wastewater includes dissolved constituents such as high concentration of protein, carbohydrate, nitrogenous compounds, lipids and minerals, which may be converted into useful materials using microbial processes. The effluents from palm oil mill can cause considerable environmental problems, if discharged untreated (Singh *et al.*, 2010). Therefore, the challenge of converting

POME into an environmental friendly waste requires an efficient treatment and effective disposal technique. 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°C), 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.*, 1999). Palm oil is one of the two most important vegetable oils in the world’s oil and fats market following soya beans. Oil palm (*Elaeis guineensis*) is the most productive oil producing plant in the world, with one hectare of oil palm producing between 10 and 35 tonnes of fresh fruit bunch (FFB) per year (Ma et al., 1996). The palm has a life of over 200 years, but the economic life is 20-25 years (nursery 11-15 months, first harvest is 32-38 months from planting and peak yield is 5-10 years from planting) (Igwe and Onyegbado, 2007). Usually, the harvested part is the fruit “fruit bunch “whereby oil is obtained from the fleshy mesocarp of the fruit. Oil extraction from flesh amounts to at least 45-46% while kernel accounts for at least 40-50%. The palm has a highly varied nutrient demand which depends mainly on the yield potential determined by the genetic make-up of the planting material and on yield limit set by climatic factors such as water, effective sunshine and temperature (Igwe and Onyegbado, 2007)

2.6 Classification of Microorganism in POME

2.6.1 Prokaryotes

The eubacteria or “true” bacteria and archaeobacteria or “ancient” bacteria are two domain of prokaryotic organisms reviewed in this paper. Both eubacteria and archaeobacteria are unicellular organism while the archaeobacteria have unique cellular chemistry. In general, these prokaryotes

play vital roles in biological wastewater treatment processes. The archaeobacteria groups can be represented by the halophiles, methanogens, or thermacidophiles (Gerardi, 2006).

2.6.2 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 and Lee, 2011). 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 favors the inhabitation of hydrolytic bacteria. This group of bacteria secrete extracellular enzymes such as cellulase, xylanase and lipase to hydrolyze carbon polymers into simpler substances, initiating the anaerobic digestion of POME (Hassan *et al.*, 2005). 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 (Chong *et al.*, 2009). 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 syntrophy 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.*, 2014). 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

2.6.3 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 catalyze 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).

2.6.4 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.

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 bioadsorption (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 and Lee, 2011).

2.7 Biodegradation and bioremediation of Palm Oil Mill Effluent

Biodegradation and bioremediation has been successfully used in the clean-up wastewaters (Okonko and Shittu, 2007) and palm oil mill effluent (Oswal *et al.*, 2002). The benefits of bioremediation and biodegradation are enormous. It saves cost; it is ecofriendly, ecologically simple, destroys contaminants (not moving them from one place to another) as well as treating wastes on site (Nadeau *et al.*, 1993). The application of bioremediation will be an important aspect of waste management now and into the future as more is learned about this technology. El- Masry *et al.* (2004) and Bhumibhamon *et al.* (2002) have reported bioremediation of oily polluted industrial wastewater with combination of bacterial isolates for the reduction of COD and O & G. While El- Bestawy *et al.* (2005) also reported the combination of *Pseudomonas* sp. and *Pseudomonas. diminuta* as mixed cultures for reducing COD in contaminated industrial effluents.

Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Mueller *et al.*, 1996). By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms (Okonko and Shittu, 2007). It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment (Takeno *et al.*, 2005). The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site (Vidali, 2001). Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes (Okonko and Shittu, 2007).

Bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali, 2001).

The use of microorganisms to destroy, or reduce the concentration of hazardous waste on a contaminated site is called bioremediation. Such a biological treatment system has various applications, including, clean-up of contaminated sites such as water, soils, sludges, and wastewater streams (Boopathy, 2000). Bioremediation methods use microorganisms that occur naturally in the environment and degrade (mineralize) contaminants to less toxic or harmless products like carbon dioxide and water. Biological processes or microbial processes have been used successfully to remediate soils contaminated with petroleum hydrocarbons and their derivatives (Mulligan *et al.*, 2001).

Bioremediation technologies can be broadly classified as ex situ and in situ. Ex situ technologies are those treatments which involve the physical removal of the contaminated material for treatment process. In contrast, in situ techniques involve treatment of the contaminated material in place. Some of the examples of in situ and ex situ bioremediation are as follows:

Land farming: Solid-phase treatment system for contaminated soils: may be done in situ or ex situ.

Composting: Aerobic, thermophilic treatment process in which contaminated material is mixed with a bulking agent; can be done using static piles or aerated piles.

Bioreactors: Biodegradation in a container or reactor; may be used to treat liquids or slurries.

Bioventing: Method of treating contaminated soils by drawing oxygen through the soil to stimulate microbial activity.

Biofilters: Use of microbial stripping columns to treat air emissions.

Bioaugmentation: Addition of bacterial cultures to a contaminated medium; frequently used in both in situ and ex situ systems.

Biostimulation: Stimulation of indigenous microbial populations in soils or ground water by providing necessary nutrients. **Intrinsic bioremediation:** Unassisted bioremediation of contaminant; only regular monitoring is done. **Pump and treat:** Pumping ground water to the surface, treating, and reinjecting (Boopathy, 2000).

Bioremediation is a natural process and is therefore perceived by the public as an acceptable waste treatment process for contaminated material such as soil. Microbes able to degrade the contaminant increase in numbers when the contaminant is present; when the contaminant is degraded, the biodegradative population declines. The residues for the treatment are usually harmless products and include carbon dioxide, water, and cell biomass. Generally, bioremediation is useful for the mitigation of pollution load in the environment. Many compounds that are legally considered to be hazardous can be transformed to harmless products. This eliminates the chance of future liability associated with treatment and disposal of contaminated material. Instead of transferring contaminants from one environmental medium to another, for example, from land to water or air, the complete destruction of target pollutants is possible. Bioremediation can often be carried out on site, often without causing a major disruption of normal activities. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise during transportation. Bioremediation can prove less expensive than other technologies that are used for clean-up of hazardous waste. Bioremediation is limited to those compounds that are biodegradable.

Not all compounds are susceptible to rapid and complete degradation. There are some concerns that the products of biodegradation may be more persistent or toxic than the parent compound. Biological processes are often highly specific. Important site factors required for success include the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants. It is difficult to extrapolate from bench and pilot-scale studies to full-scale field operations. Research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment. Contaminants may be present as solids, liquids, and gases. Bioremediation often takes longer than other treatment options, such as excavation and removal of soil or incineration. Regulatory uncertainty remains regarding acceptable performance criteria for bioremediation. There is no accepted definition of “clean”, evaluating performance of bioremediation is difficult, and there are no acceptable endpoints for bioremediation treatments (Vidali, 2001).

2.8 Characteristics of Palm Oil Mill Effluent (POME)

Large amount of waste are produced in the palm oil mill industry. The process of oil extraction results in generation of liquid waste commonly named as palm oil mill effluent (POME) (Rupani *et al.*, 2010; Mohammed, 2014). Palm oil mill effluent is generated mainly from oil extraction, washing and cleaning processes in the mill and these contains cellulosic material, fat, oil and grease etc (Agamuthu, 1995). Palm oil mill effluent also contains substantial quantities of solids, both suspended solids and total dissolved solids in the range of 18,000 mg/L and 40,500 mg/L respectively. These solids are commonly named palm oil mill sludges (POMS). The solid waste that are produced in the process of extraction are the leaves, trunk, decanter cake, empty fruit bunches, seed shells and fiber from the mesocarp (Rupani *et al.*, 2010). Fresh POME is a hot,

acidic (pH between 4 and 5), brownish colloidal suspension containing high concentrations of organic matter, high amounts of total solids (40,500 mg/L), oil and grease (4,000 mg/L) COD (50,000 mg/L) and BOD (25,000 mg/L) (Ma, 2000). The characteristics of typical POME are given in Table 2.1. According to Vairappan and Yen (2008), 66.8 million tonnes of POME was generated in year 2005. The raw or partially treated POME has an extremely high content of degradable organic matter. As no chemicals were added during the oil extraction process, POME is considered as non toxic, but it is identified as a major source of aquatic pollution by depleting dissolved oxygen when discharged untreated into the water bodies (Khalid and Wan Mustafa, 1992). However it also contains appreciable amounts of N, P, K, Mg and Ca (Habib *et al.*, 1997; Muhrizal *et al.*, 2006), which are the vital nutrient elements for plant growth. Due to the non toxic nature and fertilizing properties, POME can be used as fertilizer or animal feed substitute, in terms of providing sufficient mineral requirements. Agamuthu *et al.* (1995) has also reported the increase of organic nitrogen leading to the production of a better fertilizer in POME. Muhrizal *et al.* (2006) reported that POME contains high content of Al as compared to chicken manure and composted sawdust. According to Habib *et al.* (1997) toxic metals, such as Pb, can also be found in POME, but their concentrations are usually below sub lethal levels (greater than 17.5 µg /g) (James *et al.*, 1996). According to James *et al.* (1996), Pb is found in POME as a result of contamination from plastic and metal pipes, tanks and containers where Pb is widely used in paints and glazing materials.

2.9 Palm Oil Mill Effluent Treatment

The biological ponding system or the lagoon system has developed rapidly as a typical palm oil mill effluent treatment system. More than 85% of the palm oil mill use solely lagoon system for palm oil mill effluent treatment (Yeoh, 2004; Tong and Jaafar, 2004; Najafpour *et al.*, 2006).

This system consists of deoiling ponds, anaerobic, facultative and aerobic ponds. The ponding system normally requires long retention time in excess of 20 days and the biogas is released into the atmosphere. According to Yacob *et al.* (2005), an average of 36% methane gas is emitted into the atmosphere from open tank digester. The methane gas produced by open tank digester and lagoon system is 35% and 45% respectively as studied by Shirai *et al.* (2003).

Various palm oil mill effluent treatments were studied in order to meet the stringent water course discharge regulation. Palm oil mill effluent treatment using membrane technology with physical chemical pretreatment showed a reduction in turbidity, COD and BOD up to 100%, 98.8% and 99.4% respectively (Ahmad *et al.*, 2003). Two-stage up-flow anaerobic sludge blanket system could work efficiently up to 30 g COD/L/day whilst methane yield and COD reduction greater than 90% (Borja *et al.*, 1996). COD removal efficiencies greater than 94% obtained in single stage anaerobic tank digester and single stage anaerobic ponding system after 10 days of retention time (Ugoji, 1997). A work carried out by Borja and Banks (1995) showed COD removals higher than 90% in both anaerobic filter and anaerobic fluidized bed reactor at loading of 10 g COD/L/day. COD removal up to 88% was obtained with 55h hydraulic retention time using attached growth on a rotating biological contactor (Najafpour *et al.*, 2005). A 95% COD reduction was achieved using treatment of tropical marine yeast with 2 days retention time (Oswal *et al.*, 2002).

The anaerobic digestion systems are being increasingly used in wastewater treatment especially in agro-industry because they do not require high energy demanded as in aerobic biological treatment, produce less waste sludge and they can be easily restarted after months of shut down (Beccari *et al.*, 1996). The potential of producing methane, a biogas as a by-product make this method is even more attractive. As cited by Yacob *et al.* (2005), the end product of the anaerobic

digestion of palm oil mill effluent is a mixture of biogas (65% CH₄, 35%CO₂ and traces of H₂S) from laboratory studies and approximately 28m³ of biogas can be obtained from 1 tonne of palm oil mill effluent.

2.9.1 Anaerobic Digestion Process

Anaerobic digestion is considered to be an effective treatment process for palm oil mill effluent (POME). This involves a consortium of microorganisms catalysing a complex series of biochemical reactions that mineralize organic matter producing methane and carbon dioxide. The key factors to successfully control the stability and efficiency of the process are reactor configurations, hydraulic retention time (HRT), organic loading rates (OLR), pH, temperature, inhibitor concentrations, concentrations of total volatile fatty acid (TVFA) and substrate composition. In order to avoid a process failure and/or low efficiency, these parameters require an investigation so that they can be maintained at or near to optimum conditions.

Generally, these anaerobic digestions are conducted at either mesophilic (30-37°C) or thermophilic (50-60°C) temperatures. In a palm oil mill processing system, the wastewater is discharged at relatively high temperatures (80-90°C) (Najafpour *et al.* 2006), making it feasible to treat the palm oil mill effluent at either mesophilic or thermophilic temperatures. With palm oil mill effluent added at an OLR of 12.6 g[COD]/L/day and a hydraulic retention time of 5.6 days under mesophilic temperature, Cail and Barford (1985) using a semicontinuous anaerobic reactor achieved a chemical oxygen demand (COD) removal of around 75%. Using a similar configuration, of a semi-continuous anaerobic reactor, but operating with thermophilic conditions and a maximum organic loading rates of 15.1 g[COD]/L/day and a hydraulic retention time of 4.3 days they achieved a COD removal of 85%, and a methane yield of 295 ml/g[COD] (Padilla and Banks, 1993). Yu *et al.* (2002), found that the operation at 55°C achieved a higher

substrate degradation rate, biogas production rate, and specific rate of aqueous product formation than when operated at 37°C. De la Rubia *et al.* (2002) concluded that a reactor operating at a lower hydraulic retention time and 55°C produced more gas than at 35°C with OLR's of up to 2.19 kg m⁻³ d⁻³ COD. The digestion of a distillery waste at anaerobic digestion temperatures ranging from 35-55°C, gave a maximum total biogas and methane yield at a digester temperature of 50°C (Banerjee and Biswas, 2004). According to these data, temperature is an important parameter that modifies the effectiveness of the anaerobic bacterial consortium to produce methane from organic matter. In practice, failure to control temperature increases can result in biomass washout with a resulting accumulation of total volatile fatty acid (Lau and Fang, 1997). The anaerobic digestion is a versatile biochemical process and is being increasingly used to treat agricultural wastes. Anaerobic digestion is a multistage biochemical process in which complex organic substances are fermented by microorganisms in the absence of oxygen and the presence of anaerobic microorganisms. Methanogenic anaerobic digestion of organic waste has been performed for about a century and is advantages over aerobic treatment process because of its high organic removal rates, low energy requirement, low sludge production and energy production (Wanna and Pornpan, 2007). In general, anaerobic digestion occurs in four major stages, hydrolysis, fermentation, acetogenesis and methanogenesis. Each step involves different microbial population.

In the first stage of hydrolysis, the polymeric organic materials are hydrolysed to monomers such as glucose, fatty acids and amino acids by hydrolytic bacteria. The hydrolysis process is of significant importance in high organic waste and may become rate limiting. Solubilisation involves hydrolysis process where the complex organic matter is hydrolysed into soluble monomers. Fats are hydrolysed into fatty acids or glycerol; proteins are hydrolysed into amino

acids or peptides while carbohydrates are hydrolysed into monosaccharides and disaccharides. The hydrolysis reaction can be expressed as below (Gray, 2004).

In fermentation stage, the hydrolysed products are converted to volatile fatty acids, alcohols, aldehydes, ketones, ammonia, carbon dioxide, water and hydrogen by the acid-forming bacteria. The organic acids formed are acetic acid, propionic acid, butyric acid and valeric acid. Volatile fatty acids with more than four-carbon chain could not be used directly by methanogens (Wang *et al.*, 1999). Those organic acids are further oxidised to acetic acid and hydrogen by obligatory hydrogen-producing acetogenic bacteria through a process called acetogenesis. Acetogenesis also includes acetate production from hydrogen and carbon dioxide by acetogens and homoacetogens. Sometimes the acidogenesis and acetogenesis stage is combined together as one stage. Finally the methane gas is produced in two ways. One is conversion of acetate to carbon dioxide and methane by acetotrophic organisms and another is reduction of carbon dioxide with hydrogen by hydrogenotrophic organisms (Verma, 2002; Hutňan *et al.*, 1999). Predominant methanogens in biogas reactors is limited to *Methanobacterium*, *Methanothermobacter*, *Methanobrevibacter*, *Methanosarcina* and *Methanosaeta* (formerly *Methanotrix*) (Sekiguchi *et al.*, 2001).

2.9.2 Single-stage and Two-stage System

In conventional anaerobic digestion process, acidification and methanogenesis take place in a single reactor system (single-stage) and there is a delicate balance between acidogens and methanogens because both groups differ widely in terms of physiology, nutritional needs, growth kinetics and sensitivity to environmental conditions (Demirel and Yenigün, 2002). The aim for two-phase anaerobic digestion process is to separate the acid and methane fermentation phases for determining and satisfying optimum environmental conditions for each type microbial

population in two separate reactors (Demirel and Yenigün, 2006). A major limitation of anaerobic digestion of fruit and vegetable wastes in one stage system is a rapid production of volatile fatty acids attributed to pH decreased during acidification which stressed and inhibited the activity of methanogenic bacteria (Bouallagui *et al.*, 2004). Conditions that are favorable to the growth of acid-forming bacteria such as short hydraulic retention time and low pH are inhibitory to the methanogens. A two-phase process can optimise the condition for hydrolytic acidogenic groups of bacteria as well as for the acetogenic and methanogenic groups (Ince, 1998). The pH during the acidogenic phase usually maintained at 5.5 to 6.0 and HRT less than 5 days while in the methanogenic phase the pH was maintained at pH greater than 7.0 (Ince, 1998; Raynal *et al.*, 1998; Demirel and Yenigün, 2002; Bouallagui *et al.*, 2004). As a consequence, higher treatment efficiency and better process stability can be attained by two-phase process with an overall organic matter removal greater than 87% at 17 days hydraulic retention time (Raynal *et al.*, 1998), 96% of the total COD was converted to biomass and biogas (Bouallagui *et al.*, 2004).

2.9.3 Anaerobic Sequencing Batch Reactor

Anaerobic sequencing batch reactor was developed to better handle high suspended solids wastewater. The Anaerobic sequencing batch reactor bioreactor operates in four-step cycle in a single reactor (Ratusznei *et al.*, 2000; Zaiat *et al.*, 2001; Angenent *et al.*, 2004). The first stage is the feeding process where wastewater is fed into the reactor with settled biomass. The wastewater and biomass are mixed intermittently in reaction process. This follow by settling of biomass and lastly effluent is withdrawn from the reactor. Advantages of the Anaerobic sequencing batch reactor system are better solids retention, efficient operating control, high organic matter removal efficiency, simple operation and absence of settling tank (Ratusznei *et al.*,

2000). A very short start-up and 86% COD removal was observed in Ratusznei *et al.* study (2000) treating 0.5 L synthetic substrate containing 485 mg/L COD per cycle with 8 hours each cycle.

2.9.4 Up-flow Anaerobic Sludge Blanket (UASB)

Approximately 60% of the thousands anaerobic full-scale treatment facilities worldwide are now based on the up-flow Anaerobic Sludge Blanket design concept (Angenent *et al.*, 2004). The excellent sludge retention in UASB system is based on bacterial sludge entrapment in or between sludge particles and bacterial immobilisation by a mechanism of bacteria self agglomeration and bacteria attachment to support material present in the sludge (Lettinga, 1995).

2.9.5 Attached-growth System

Low growth rate of anaerobic microorganisms has encouraged the development of various techniques for their immobilisation within bioreactors so as to retain the viable bacteria biomass in the reactor. Buffering capacity is greater in the reactors with support material (Björnsson *et al.*, 1997). Attached-growth reactors developed include fluidised bed, fixed-bed, fixed-film, and anaerobic biofilter. The anaerobic fixed-film reactor treating a synthetic wastewater with 3000 mg/L COD could recover from the inhibition due to shock loading and resumed normal operation within eight days (Chua *et al.*, 1997). The anaerobic fixed-film reactor is packed with 1cm in diameter of fire expanded clay spheres. COD removal up to 98.1% was achieved in the study at 5 days hydraulic retention time. Fluidised-bed reactors offer many advantages to the anaerobic digestion process. Microbial biomass was attached on the support thus provided high concentration of biomass as consequence been not easily washed out from the reactor. Anaerobic fixed-film reactor enables high mass transfer, no plugging, channeling or gas hold-up (Borja *et al.*, 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Sample

Palm oil mill effluent (POME) were collected aseptically with sterile microbiological bottles from mechanized (NIFOR) and Local palm oil mill in Benin City, Edo State, Nigeria. The effluent was mixed thoroughly before being fetched into clean plastic containers, tightly screwed and transported to the laboratory in an ice box. When not in use, the POME was stored in a refrigerator at 4⁰C.

3.2 Preparation and Sterilization of Culture Media

All culture media were prepared according to the manufacturer's instructions. Sterilization was at 121°C at 15psi for 15 min unless otherwise stated by manufacturer.

3.3 Media Preparation

Media for microbiological analysis was weighed according to the manufacturer's specifications.

3.3.1 Nutrient agar

Twenty-eight grams (28 g) of nutrient agar were dissolved in 1000 ml of distilled water in a conical flask corked with cotton wool and foil paper and allowed to dissolve in 1000 ml of distilled water in a conical flask. The medium was the placed in an autoclave to sterilize it for 15 minutes at 121 °C. After sterilization, the flask was allowed to cool.

3.3.2 McConkey Agar

A 52.0g of MacConkey agar (MAC) was weighed and poured into a serial conical flask. 1000ml of distilled water was added and the mixture was sterilized at 120°C for 15 minutes at 15 pis in the autoclave for even distribution of the ager. It was poured in to petridishes in 20ml amounts and allowed to set at room temperature.

3.3.3 Eosine-methylene Blue

Eosine-methylene blue agar was prepared by dissolving 500ml of Eosin is a dye in 250mls of distilled water and boil to completely dissolve agar. The autoclave weresterilized for 15 minutes at 15 psi (121°C). It were then cool to 60°C and before pouring into sterile Petri dishes.

3.3.4 Salmonella Shigella Agar

Sixty-three gram (63 g) of Salmonella-Shigella Agar powder was dissolved in 1 litre of distilled water in a conical flask covered with cotton wool and aluminium foil paper. It was mixed thoroughly and sterilized by autoclaving at 121°C for 15 minutes. The medium was cooled to 45-50°C and then dispensed aseptically into sterile Petri dishes.

3.4 Enumeration and Isolation of Bacteria from Samples

A stock of 30 ml of the effluent was aseptically transferred into a conical flask containing 270 ml sterile distilled water. A tenfold serial dilution was thereafter made for the respective palm oil mill effluent obtained by pipetting 1 ml of the stock into test tubes containing 9 ml sterile distilled water. From the aforementioned 10 ml suspension tube, 1 ml was also removed and transferred to another tube containing 9 ml of diluent. The process was repeated until the fourth test tube containing 9 ml of diluent received 1 ml from the preceding tube. Thereafter, an aliquot of 1 ml of the sample from the second serially diluted test tubes was aseptically measured and transferred into a petri dish to which molten nutrient agar (supplemented with 2% w/v of fluconazole) was poured into the petri dish (pour plate method). The agar plates were incubated for 24 h. at 37 °C. It should be noted following serial dilution, plating or culture was done in triplicate colonies were counted and enumerated using the formula in equation (1) below given by Willey *et al.* (2008).

$$\frac{cfu}{ml} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of inoculum}} \quad (1)$$

3.5 Morphological and Biochemical Characteristics of Bacteria

Gram Stain

Thin smears of the isolates were made on glass slides using a wire loop, were heat-fixed, and allowed to cool. The smears were stained with crystal violet stain for 60 seconds and then washed off immediately with clean water. Then the smears were covered with Lugol's iodine for 30-60 sec and immediately washed off with water. The smears were decolorized rapidly with alcohol and washed rapidly with clean water after 5 seconds. Then the smears were stained with safranin for 60 seconds and immediately washed off. The stained smears were allowed to air-dry after which a few drops of oil immersion were dropped on the smears after which they were viewed under the optical microscope using the 100 x objective lens. The Gram-positive organisms were viewed as purple cells while the Gram-negative organisms were viewed as pink or red cells.

Biochemical Tests

These tests were conducted to determine the ability of the bacterial isolates to produce enzymes such as catalase, oxidase, and urease. Other biochemical tests were carried out to determine the ability of the bacteria to utilize sugars or produce a compound (see appendix for biochemical tests carried out).

3.6 Antibiogram Characterization

The suspected bacterial isolates, which were presumptively, identified employing standard culture techniques. The isolates were thereafter subjected to standard antibacterial susceptibility testing to determine their resistance or sensitivity to different antibiotics standard discs manufactured by Oxoid, Basingstoke UK. Standard disc diffusion method (Kirby Bauer test) was employed for this antibiogram assay. Briefly, the isolated bacteria were grown between 18 and 24 h. on Mueller-Hinton agar. The inoculum were streaked using sterile loops into Mueller-

Hinton agar plates before antibiotics discs were carefully added using sterile forceps for susceptibility determination. The results were recorded after 24 h. of incubation at 37 °C. Using the directions of standard antimicrobial susceptibility testing established by the Clinical Laboratory Standards Institute (2017), the zone of inhibition in diameter around each disc was evaluated and interpreted on the basis of Resistant (R), Intermediate resistant (I) or Sensitive (S).

3.7 Multiple Antibiotic Resistance (MAR) Index

The (MAR) index is a good tool for health risk assessment, which identifies if the isolates are from a region of high or low antibiotic use. A MAR index of 0.2 and above indicates a ‘high-risk’ source of contamination (Davis and Brown, 2016). The MAR index was determined for each isolate using the methods delineated by Chitanand *et al.* (2010) and calculated as (MAR) index (a/b) where a = Number of antibiotics an isolate is resistant to and; b = Total number of antibiotics used in the study. MAR index greater than 0.2 implicates a bacterium that originate from high-risk source of contamination or worse still, where antibiotics have often been used or abused (Krumpernam, 1983).

CHAPTER FOUR

RESULTS

The total heterotrophic bacterial count of effluent samples is presented in Figure 1. Local palm oil mill effluent had the highest bacteria count (13.00×10^3 cfu/ml) while mechanized palm oil mill effluent had the least bacteria count (3.0×10^3 cfu/ml).

Table 1 shows the cultural, morphological and biochemical characteristics of bacterial isolates. Bacteria isolated include *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Shigella dysenteriae*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella enterica* and *Staphylococcus aureus*.

The percentage frequency of occurrence of bacterial isolates found in palm oil mill effluents is presented in Table 2: *P. aeruginosa* was the most occurring bacteria isolate (22.9%) while *P. mirabilis* was the least occurring bacteria isolate (2.9%) in Local palm oil mill effluent. *S. aureus* was the most occurring bacteria isolate (27.6%) while *K. pneumoniae* was the least occurring bacteria isolate (3.4%) in mechanized palm oil mill effluent respectively.

Table 3 shows the antibiotic susceptibility of isolated bacteria from effluent samples. *B. subtilis*, *S. aureus* and *S. dysenteriae* were susceptible to rocephin, septrin and resistant to gentamycin, zinnacef and ciprofloxacin. *E. coli*, *P. aeruginosa* and *S. enterica* were resistant to Augmetin and Chloramphenicol and susceptible to Pefloxacin and Ofloxazin. *K. pneumoniae* and *P. mirabilis* were susceptible to Gentamycin, Pefloxacin, Sparfloxacin, Ciprofloxacin and resistant to Ofloxazin. *P. aeruginosa* was the most resistant bacteria with multiple antibiotic resistance index of 0.7. *E. coli* was the least antibiotic resistant bacteria with multiple antibiotic resistance index of 0.2% (Figure 2).

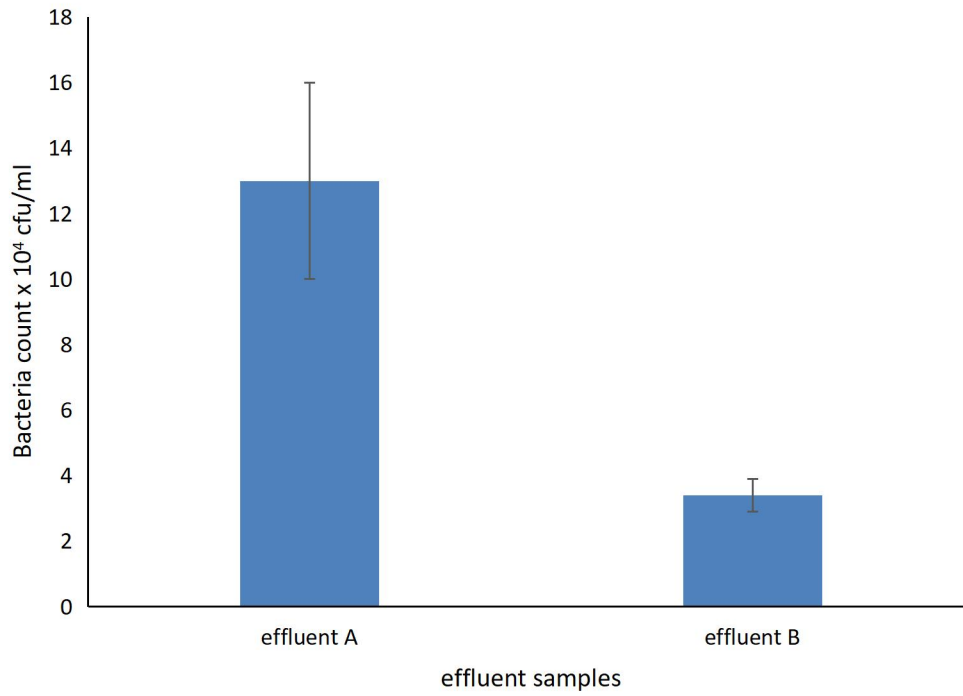


Figure 1. Total heterotrophic bacterial count of effluent samples

Key:

Effluent A = Local palm oil mill effluent

Effluent B = Mechanised palm oil mill effluent

Table 1: Cultural, Morphological and Biochemical Characterization of Bacteria Isolates

Parameters	A	B	D	E	F	G	H	I
Cultural characteristics								
Shape	Circular	Irregular	Circular	Circular	Circular	Irregular	Circular	Irregular
Colour	Milky	Milky	Milky	Creamy	Yellow	Creamy	Milky	Creamy
Size	Large	Large	Large	Large	Small	Large	Entire	Entire
Wet/dry	Dry	Dry	Wet	Wet	Wet	Dry	Dry	Dry
Elevation	Flat	Flat	Flat	Raised	Flat	Flat	Flat	Flat
Transparency	Opaque	Translucent	Opaque	Opaque	Opaque	Translucent	Translucent	Translucent
Morphology								
Gram stain	-	-	+	+	-	-	-	+
Cell type	Cocci	Curve Rod	Rod	Slim Rod	Thick Rod	Short Rod	Bacilli	Cocci
Cell arrangement	Single	Single	Pair	Single	Pairs	Single	Pairs	Cluster
Biochemical test								
Citrate utilization	-	+	+	+	-	+	-	+
Spore forming	-	-	+	-	-	-	-	-
Catalase production	-	+	-	+	+	+	+	+
Indole	+	+	-	-	+	+	+	+
Motility	+	-	+	+	-	+	-	-
Coagulase test	-	-	-	-	+	-	+	+
Oxidase test	+	-	-	+	-	-	-	-
Fermentation test								
Lactose	+	+	+	+	+	-	+	+
Glucose	-	-	+	+	-	-	+	+
Galactose	+	+	-	-	-	+	+	-
Maltose	-	+	+	+	+	+	-	-
Manitol	+	+	+	+	+	+	+	+
Probable isolates	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella pneumoniae</i>	<i>Bacillus subtilis</i>	<i>Shigella dysenteriae</i>	<i>Escherichia coli</i>	<i>Proteus mirabilis</i>	<i>Salmonella enterica</i>	<i>Staphylococcus aureus</i>

Key:

+ = positive

- = Negative

Table 2: Percentage frequency of occurrence of bacterial isolates found in palm oil mill effluents

Organism	Local (%)	Mechanized (%)	Local + Mechanized (%)
<i>Pseudomonas aeruginosa</i>	8(22.9)	4(13.8)	12(18.8)
<i>Klebsiella pneumoniae</i>	5(14.3)	1(3.4)	6(9.4)
<i>Bacillus subtilis</i>	3(8.6)	4(13.8)	7(10.9)
<i>Shigella dysenteriae</i>	3(8.6)	2(6.9)	5(7.8)
<i>Escherichia coli</i>	7(20)	5(17.2)	12(18.8)
<i>Proteus mirabilis</i>	1(2.9)	4(13.8)	5(7.8)
<i>Salmonella enterica</i>	6(17.1)	1(3.4)	7(10.9)
<i>Staphylococcus aureus</i>	2(5.7)	8(27.6)	10(15.6)
Total	35(100)	29(100)	64(100)

Table 3: Antibiotic susceptibility of Gram positive isolated bacteria from effluent samples

Gram +ve	PEF	CN	APX	Z	AM	R	CPX	S	SXT	E	MARI
<i>Bacillus subtilis</i>	19 (S)	12(R)	20(S)	13(R)	11(R)	21(S)	11(R)	10(R)	19(S)	13(R)	0.6
<i>Staphylococcus aureus</i>	13(R)	9(R)	22(S)	12(R)	23(S)	19(S)	13(R)	16(I)	22(S)	16(I)	0.4
<i>Shigella dysenteriae</i>	20 (S)	13(R)	13(R)	10(R)	16(I)	19(S)	12(R)	20(S)	21(S)	20(S)	0.4

PEF: Pefloxacin, CN: Gentamycin, APX= Ampiclox, Z= Zinnacef, CPX= Ciprofloxacin,
 S= Streptomycin, SXT= Septrin, E= Erythromycin, AM = Amoxicillin, R = Rocephin,
 CH = Chloramphenicol, SP = Sparfloxacin, AU = Augmetin, OFX = Ofloxazin

MARI = Multiple Antibiotic Resistance index.

MAR index \geq 0.2 (public health significance)

Table 4: Antibiotic susceptibility of Gram negative isolated bacteria from effluent samples

G-ve	AU	CN	PEF	OFX	S	SXT	CH	SP	CPX	AM	
<i>Klebsiella pneumoniae</i>	19(S)	20(S)	19(S)	13(R)	16(I)	12(R)	13(R)	18(S)	18(S)	20(S)	0.3
<i>Proteus mirabilis</i>	12(R)	21(S)	26(S)	16(I)	13(R)	19(S)	16(I)	21(S)	23(S)	12(R)	0.3
<i>Escherichia coli</i>	13(R)	16 (I)	19(S)	21(S)	18(S)	11(R)	15(I)	18(S)	21(S)	19(S)	0.2
<i>Pseudomonas aeruginosa</i>	11(R)	9(R)	19(S)	20(S)	12(R)	21(S)	13(R)	11(R)	12(R)	10(R)	0.7
<i>Salmonella enterica</i>	13(R)	21(S)	18(S)	20(S)	23(S)	12(R)	13(R)	13(R)	20(S)	12(R)	0.5

PEF: Pefloxacin, CN: Gentamycin, APX= Ampiclox, Z= Zinnacef, CPX= Ciprofloxacin,

S= Streptomycin, SXT= Septrin, E= Erythromycin, AM = Amoxicillin, R = Rocephin,

CH = Chloramphenicol, SP = Sparfloxacin, AU = Augmetin, OFX = Ofloxazin

MARI = Multiple Antibiotic Resistance index.

MAR index \geq 0.2 (public health significance)

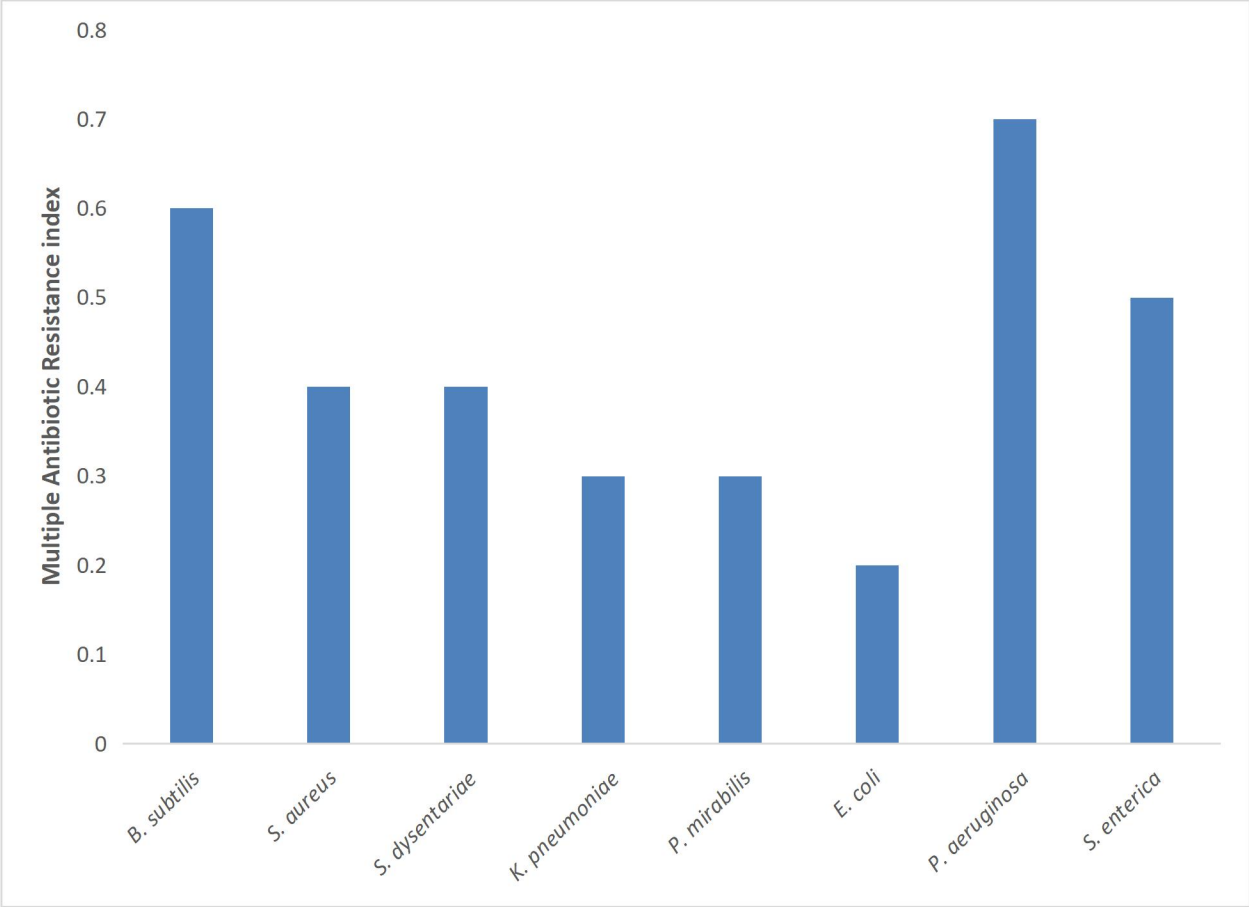


Figure 2: Multiple Antibiotic Resistance index of bacteria isolates

CHAPTER FIVE

5.0 Discussion

The total heterotrophic bacterial counts in the localized palm oil mill effluent was high compared to the mechanized palm oil mill effluents. This increase may be due to the high hydrocarbon content of the palm oil mill effluent. This is similar to the report of Kar *et al.* (2015). The difference could also be attributed treatment of effluents by mechanized palm oil mill compared to localized palm oil mill that discharge effluents directly to the environment without treatment.

This result also showed palm oil mill wastewater from all the location were contaminated with *Bacillus subtilis*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis* and *Salmonella enterica*. The most prevalent bacteria species observed in this study were *P. aeruginosa*, *E. coli* and *S. aureus*. The bacteria isolated are common and have the ability to grow on palm oil mill effluents (Williams, 2008). The high count may also be due to the fact that, the palm oil effluent may contain many growth factors that could be easily utilized by the organisms. It may also be attributed to the destabilization of the soil ecological balanced as a result of the contamination due to the discharge of the wastewater into the soil ecosystem (Eze *et al.*, 2013). In addition, the frequency of isolation of *B. subtilis* might be connected with the abundance of cellulose in the effluent. Cellulose is dominant in plant materials found in POME composts and both organisms have been shown to be good cellulase producers (Sethi *et al.*, 2013).

The presence of *Klebsiella pneumoniae* in this study is in line the with the study of Abass *et al.* (2012) who reported that bacteria such as *Klebsiella pneumoniae* and *S. dysenteriae* were present in the palm oil mill effluent with *Klebsiella pneumoniae* being one of the most prevalent coliform bacteria. *Klebsiella pneumoniae* has been reported to naturally occur in the soil and

surfaces (Hassen *et al.*, 2013). *Klebsiella pneumoniae* have been implicated in food borne disease and are health concerns as regards food safety globally (Berger *et al.*, 2010). Improper hand washing before handling of food may lead to contamination of food by *Klebsiella pneumoniae*.

Staphylococcus aureus is known to be a permanent and ubiquitous colonizer of human skin, *S. aureus* is not usually pathogenic but patients with compromised immune systems are often at risk for developing an infection. *Staphylococcus aureus* is one of the most common causes of both community and hospital acquired infections (Ekhaise *et al.*, 2008). *Staphylococcus aureus* is known to be the causative pathogen in a range of diseases: impetigo, folliculitis, septic arthritis, osteomyelitis, septicaemia, pneumonia and meningitis (Kozitskaya *et al.*, 2005). Palm oil effluent may have been contaminated with *Staphylococcus aureus* either by human contact with palm nuts or by contacts with contaminated processing tools (Okwute and Isu, 2007).

Bacillus cereus isolate in this study have been implicated as promoting food borne disease and are health concerns as regards food safety globally (Clarence *et al.*, 2009). *Bacillus* species are emetic toxins producers that cause serious illness and fatalities in human consequent to consuming food contaminated with their toxins (Uriah, 2004). Improper hygiene practice by palm oil millers before handling of food may lead to contamination of food by *Bacillus* sp.

The presence of *Escherichia coli* in this study may be due to closeness of some of these oil mills to toilet. This agrees with the report of Russell and Jarvis (2001), stating that *E. coli* is expelled into the faecal matter. The bacterium grows massively in fresh faecal matter under aerobic conditions for 3 days, but its numbers decline slowly afterwards (Russell and Jarvis, 2001). *E. coli* has also been reported to be harmless and part of normal flora but can cause serious food poisoning in their hosts, and is occasionally responsible for product recalls due to food contamination (Vogt and Dippold, 2005). Faecal–oral transmission is the major route through

which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination (Uraih, 2004).

The presence of *Pseudomonas aeruginosa* in this study is in agreement with the studies of Serikovna *et al.* (2013) who reported 46.1% cases of *Pseudomonas aeruginosa* from oil mill effluents. Bala *et al.* (2014) also in their work have reported the role played by oil mill effluents in the transmission of diseases. Poor sanitation of oil milling equipment and poor hygiene practice by oil mill workers may contribute to proliferation of microorganisms in palm oil industries. Microbial flora may contaminate surfaces of objects, devices and materials which subsequently contact susceptible body sites (Ohimain *et al.*, 2012).

The high levels of faecal contamination in the effluent are therefore an indication of the contamination of the effluent with faecal material and possibly pathogenic organisms from palm oil wastewater discharged untreated; similar findings have early been reported (Rupani *et al.*, 2010).

Antibiotic susceptibility profile of bacterial isolates from palm oil effluent showed that Gram positive bacteria such as *B. subtilis*, *S. aureus* and *S. dysenteriae* were resistant to gentamycin, zinnacef and ciprofloxacin. Gram negative bacteria *K. pneumoniae*, *P. mirabilis*, *E. coli*, *P. aeruginosa* and *S. enterica* were the most susceptible bacterial to pefloxacin and ciprofloxacin. This shows that most of the antibiotics were not effective in the treatment of the bacterial isolates. This result is not in concord with the studies of Aminu *et al.* (2014) who reported that antibiotic susceptibility potentially of *P. aeruginosa*, *S. epidermidis*, *K. pneumoniae*, *P. mirabilis* and *E. coli* were resistant to pefloxacin, amoxicillin, ciprofloxacin, augumentin and erythromycin. This study is in agreement with the studies of Gaber *et al.* (2018) who revealed that *S. epidermidis*, *E.*

coli, *P. aeruginosa*, *M. luteus*, *K. pneumoniae* and *Corynebacterium* species were susceptible to ofloxacin, pefloxacin, ciprofloxacin and erythromycin. The resistance of the bacterial isolates to some of the antibiotics could be due to chromosomally or plasmid mediated resistant genes in the bacteria genetic make-up. This is associated with indiscriminate usage of antibiotics (Manisha *et al.*, 2017).

Multiple antibiotic resistance to more than four (4) antibiotics was observed for *P. aeruginosa*, *B. subtilis* and *S. enterica* with *P. aeruginosa* being the most resistant bacteria with multiple antibiotic resistance of 0.7. This finding was supported by Adetunji *et al.* (2014) and Mohammed *et al.* (2014) who reported the existence of multidrug resistant *E. coli*, *B. subtilis* and *P. aeruginosa*. This corroborates the findings of Igbinosa *et al.* (2014) who also noted that multidrug resistant phenotypes have been spread widely among some Gram-negative and positive bacteria. In general, the development of drug resistant *P. aeruginosa* isolates and other Gram-negative bacteria can be linked to various aspects including the practice of indiscriminate use of fertilizers growing crops/plants (Enabulele and Uriah, 2009) and due to the selective pressure to extensive use of fertilizers in agriculture (Mohammed *et al.*, 2014). There are reports demonstrating the role played by industrial and human activities on the antibiotic resistance distribution of bacterial isolates in the environment (Adetunji *et al.*, 2014). Onuoha (2018) from his study suggested that the antibiotic resistance patterns of bacterial isolates may be due to factors that are not linked to faecal pollution based on weak correlation between antibiotic resistance and faecal coliforms.

5.1 Conclusion

The result of this study indicated that palm oil effluents were highly contaminated by bacteria. The chances of contracting infections from these effluents due to microbial contaminations are very high if necessary hygiene treatment steps and good hygiene practice are not taken, hence, it is imperative that the government create awareness to people involved in palm oil processing on the requirement for proper treatment and disposal of effluent. Palm oil workers should imbibe the culture of proper hygiene to minimize the proliferation of microorganisms. Also, use of fertilizers to grow palm trees should be reduced to minimize the incidence of drug resistance.

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APPENDIX I

CULTURE MEDIA AND USES

***Salmonella-Shigella* Agar (SSA)**

Salmonella-Shigella agar (SSA) is a moderately selective and differential medium, which was used for the isolation, cultivation and differentiation of *Salmonella* species and some strains of *Shigella* species. It was prepared by suspending 60 g of the powdered commercially prepared SSA in 1 litre of distilled water and dissolved thoroughly. It was then aseptically dispensed into sterile petri dishes.

Nutrient Agar (MHA)

Nutrient agar is a non-selective, non-differential medium that is used majorly for antimicrobial susceptibility testing. It is the standard medium for the Kirby Bauer method. It was prepared by suspending 38g of the medium in 1 liter of distilled water and was dissolved properly to get a homogenous mixture. It was then autoclaved at 121°C for 15 min and was cooled to 45°C. The molten agar was aseptically dispensed into sterile petri dishes.

MacConkey Agar

A differential medium for the detection, isolation and enumeration of coliforms and intestinal pathogens in water, dairy products and biological specimens. MacConkey Agar corresponds to the medium recommended by the World Health Organization for the bacteriological examination of water. Although principally used for coliforms, this medium may also be employed for the differentiation of other enteric bacteria (including pathogens) and is suitable for the differentiation of *Pasteurella* species. It was prepared by suspending 52 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Dry the surface of the gel before inoculation.

Eosin Methylene Blue Agar

The medium was prepared by suspending 37.5 g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 60°C and shake the medium in order to oxidize the methylene blue (i.e. restore its blue colour) and to suspend the precipitate which is an essential part of the medium.

APPENDIX II

CLSI	Antibiotics	Short code	Disk content µg	Diameter Zone of inhibition			Organisms
				S	I	R	
1.	Meropenem	MEM	10	≥23 ≥19 ≥18 - 10 - - 10 -	20-22 16-18 14-17 - - - - - 8	≤19 ≤15 ≤13 - - - - - ≥16	Enterobacteriaceae <i>Pseudomonas aeruginosa</i> <i>Acinetobacter spp.</i> Non-enterobacteriaceae <i>Haemophilus influenzae</i> & <i>H. parainfluenzae</i> <i>Streptococcus pneumoniae</i> <i>Streptococcus spp. β – Hemolytic Group</i> <i>Streptococcus spp. viridans Group</i> <i>Neisseria meningitidis</i> Anaerobes
2.	Gentamycin	CN	10	≥15 ≥15 ≥15 - ≥15	13-14 13-14 13-14 - 13-14	≤12 ≤12 ≤12 - ≤12	Enterobacteriaceae <i>Pseudomonas aeruginosa</i> <i>Acinetobacter spp.</i> Non-enterobacteriaceae <i>Staphylococcus spp.</i>
3.	Vancomycin	VA	- 30	- ≥17 ≥17 ≥17 ≥17	- 15-16 - - -	- ≤14 - - -	<i>Staphylococcus spp.</i> <i>Enterococcus spp.</i> <i>Streptococcus pneumoniae</i> <i>Streptococcus spp. β – Hemolytic Group</i> <i>Streptococcus spp. viridans Group</i>
4.	Ciprofloxacin	CIP	5	≥31 ≥21 ≥21 - ≥21 ≥21 ≥21 ≥41 ≥35	21-30 16-20 16-20 - 16-20 16-20 - 28-40 33-34	≤20 ≤15 ≤15 - ≤15 ≤15 - ≤27 ≤32	Enterobacteriaceae <i>Pseudomonas aeruginosa</i> <i>Acinetobacter spp.</i> Other Non-Enterobacteriaceae <i>Staphylococcus spp.</i> <i>Enterococcus spp.</i> <i>Haemophilus influenzae</i> & <i>H. parainfluenzae</i> <i>Neisseria gonorrhoeae</i> <i>Neisseria meningitidis</i>

5. Azithromycin	AZM	15	≥13 ≥12 ≥18 ≥18 ≥18 ≥18 ≥20	- - 14-17 14-17 14-17 14-17 -	≤12 - ≤13 ≤13 ≤13 ≤13 -	Enterobacteriaceae <i>Haemophilus influenzae</i> & <i>H. parainfluenzae</i> <i>Streptococcus pneumoniae</i> <i>Staphylococcus spp</i> <i>Streptococcus spp. β – Hemolytic Group</i> <i>Streptococcus spp. viridans Group</i> <i>Neisseria meningitidis</i>
6. Sulfamethoxazole / Trimethoprim	RL	23.75 /1.25 - 23.75 /1.25	≥16 ≥16 ≥16 ≥16 - ≥16 ≥19 ≥16 ≥30	11-15 11-15 11-15 11-15 - 11-15 16-18 11-15 26-29	≤10 ≤10 ≤10 ≤10 - ≤10 ≤15 ≤10 ≤25	enterobacteriaceae <i>Acinetobacter spp</i> <i>Burkholderia cepacia complex</i> <i>Stenotrophomonas maltophilia</i> Other Non-Enterobacteriaceae <i>Staphylococcus spp</i> <i>Streptococcus pneumoniae</i> <i>Haemophilus influenzae</i> & <i>H. parainfluenzae</i> <i>Neisseria meningitidis</i>
7. Ceftriazone	CRO	30 - 30	≥23 ≥21 ≥26 - ≥27 ≥34	20-22 14-20 - - 25-26 -	≤19 ≤13 - - ≤24 -	Enterobacteriaceae <i>Acinetobacter spp</i> <i>Haemophilus influenzae</i> & <i>H. parainfluenzae</i> <i>Streptococcus pneumoniae</i> <i>Streptococcus spp. viridans Group</i> <i>Neisseria meningitidis</i>
8. Amoxicillin/Clavulanic acid	AMC	20/10 - 20/10 -	≥18 - ≥20 ≥4/2	14-17 - - 8/4	≤13 - ≤19 ≤16/8	Enterobacteriaceae <i>Streptococcus pneumoniae</i> <i>Haemophilus influenzae</i> & <i>H. parainfluenzae</i> Anaerobes

9. Erythromycin	E	15	≥23 ≥23 ≥21 ≥21 ≥21	14-22 14-22 16-20 16-20 16-20	≤13 ≤13 ≤15 ≤15 ≤15	<i>Staphylococcus spp</i> <i>Enterococcus spp</i> <i>Streptococcus pneumoniae</i> <i>Streptococcus spp. β – Hemolytic Group</i> <i>Streptococcus spp. viridans Group</i>
10. Ceftazidime	CAZ	30 - - 30	≥21 ≥18 ≥18 ≥21 - - ≥26 ≥31	18-20 15-17 15-17 18-20 - - - -	≤17 ≤14 ≤14 ≤17 - - - -	Enterobacteriaceae <i>Pseudomonas aeruginosa</i> <i>Acinetobacter spp</i> <i>Burkholderia cepacia complex</i> <i>Stenotrophomonas maltophilia</i> Other Non-Enterobacteriaceae <i>Haemophilus influenzae & H. parainfluenzae</i> <i>Neisseria gonorrhoeae</i>

Azithromycin (15 µg)

Gentamicin (10 µg)

Vancomycin (30 µg)

Ciprofloxacin (5 µg)

Ceftriazone (30 µg)

Sulfamethoxazole/Trimethoprim (25 µg)

Meropenem (10 µg)

Amoxicillin/Clavulanic acid (30 µg)

Ceftazidime (30 µg)

Erythromycin (15 µg)

APPENDIX III

GRAM STAINING AND BIOCHEMICAL REAGENTS

STAIN AND REAGENT

Gram stain

The Gram stain was prepared using two stains (crystal violet and safranin or carbol fuchsin), Gram's iodine, and a decolorizing agent (ethyl alcohol).

A. Gram crystal violet

Solution A

Crystal violet - 2.0 g

Dissolved in ethanol (95%) - 20.0 ml

Solution B

Ammonium oxalate - 0.8 g

Distilled water - 80.0 ml

Gram iodine

Iodine (crystalline) - 1.0 g

Potassium - 2.0 g

Distilled water - 300.0 ml

3.0g of medium was dissolved in 300.0 ml of distilled water.

It is very important to note that; crystalline iodine, potassium and distilled water were combined to produce iodine solution and that Gram's iodine solution was stored in a dark bottle and protected from light so that it does not degrade.

Decolorizer

95 % ethyl alcohol was used.

Gram safranin

Safranin-O (certified) - 0.25 g

Ethyl alcohol (95 %) - 100.0 ml

Working solution:

Safranin stock solution – 10.0ml

Distilled water – 90.0 ml

Biochemical reagents

Indole medium

Peptone – 20.0 g

Sodium chloride – 5.0 g

Distilled water – 1000 ml

pH – 7.4

25.0 g of indole medium was dissolved in 1000 ml of distilled water and autoclaved for 15 min at 121 °C and dispensed aseptically into sterile test tubes.

Oxidase reagent (Kovac's oxidase)

Amul-alcohol – 15.0 ml

p-dimethyl-aminobenzaldehyde – 0.5 ml

Concentrated HCl – 50ml

Small quantity of Kovac's reagent was prepared by dissolving the aldehyde into alcohol and adding the acid slowly and then kept inside the refrigerator.

Catalase test

3% Hydrogen peroxide 7