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Assessment of Bio-Surfactant Producing Microorganisms from Palm Oil Mill Effluents in Edo State, Nigeria

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ABSTRACT

Palm oil mill effluent (POME) is a wastewater generated from palm oil milling activities which requires effective treatment before being discharged into the watercourses due to its highly polluting properties. Hence this study was aimed at evaluating the biosurfactant-producing microorganisms from POME at different depths from large and small/medium scale enterprises in Edo State, Nigeria.

POME was aseptically collected using sterile bottles from various depths: top, middle and bottom in selected palm oil companies across Edo State, Nigeria. The companies were categorized into large-scale enterprises (L.S.E.), which included Okomu Oil Palm and NIFOR and small and medium-scale enterprises (S.M.E.), comprising Ovbiogie, Sapele Road and Aduwawa oil palm companies.

The bacteria isolated were Bacillus cereus, Pseudomonas aeruginosa, Bacillus amyloliqefaciens, Klebsiella aerogenes and Escherichia coli. Fungi like Aspergillus niger, Fusarium solani, Penicillium chrysogenum, Microsporum sp., Penicillium citrinum and Aspergillus flavus were also isolated from these samples using the Pour plate techniques. The bacterial species obtained in the pure culture of substrate were identified using standard bacterial and fungal techniques. Isolated organisms were screened for their ability to produce biosurfactants using oil spreading assay, hemolytic, and emulsification activity. The test of how susceptible the isolates were to antibiotics was conducted with the aid of the Kirby-Bauer disk diffusion assay. The data obtained were analyzed using Microsoft Excel 2019 and PhyloT software to establish the relationship between isolated microorganisms from POME.

The results of the total heterotrophic bacterial counts ranged from $\log_{10}$ 3.90±1.00 cfu/g (Small and Medium Scale- Sapele Road) to $\log_{10}$ 4.66±3.0 cfu/g (Large Scale Enterprise- Okomu). The total fungal counts ranged from $\log_{10}$ 3.78±1.00 cfu/g (Small and Medium Scale- Aduwawa) to $\log_{10}$ 4.34±2.00 cfu/g (Small and Medium Scale- Aduwawa). The difference in percentage reduction in the density of microbes between the top and bottom depth ranged from 43.18% (NIFOR) to 72.29 % (Okomu). Also, a significant difference ($p<0.05$) between the microbial diversity of large-scale and small-scale oil-producing enterprises was observed. The isolated bacteria included Bacillus cereus, Pseudomonas aeruginosa, Bacillus amyloliqefaciens, Klebsiella aerogenes and Escherichia coli.

The isolated fungi were Aspergillus niger, Penicillium citrinum, Penicillium chrysogenum, Aspergillus flavus, Microsporum sp. and Fusarium solani. Biosurfactant screening results revealed that most microbial isolates were potential biosurfactant producers, with Bacillus sp. showing the highest clear zone of oil spread assay. However, specific isolates like *E. coli* and *Microsporum* sp. did not produce any clear zone for oil spread assay. More so, *Bacillus* sp. was found to be the best biosurfactant producer due to its hemolytic activity and the assay with the highest zone (10mm) of displacement. POME is home to many microorganisms of importance to both industrial and environmental processes. This research has demonstrated that POME serves as a reservoir for microorganisms capable of producing biosurfactants.

Keywords: Palm Oil Mill Effluent (POME), Biosurfactant-producing microorganisms, Microbial diversity, Bacillus species, Oil spreading assay, Antibiotic susceptibility testing.

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INTRODUCTION

Biosurfactants are group of surface-active molecules that are synthesized by different microorganisms, and thus have uniquely diverse structure (Nitschke and Costa, 2007). They are amphiphilic in nature, possessing both water-loving (hydrophilic) and water-repelling (hydrophobic) polar properties. This unique structural feature makes them suitable surface tension reducing agent in different phases of fluid. They are very useful in many commercial processes that have been reported in different fields and these include the bioremediation of recalcitrant pollutants, microbial-enhanced oil recovery, food and cosmetic industries (Nitschke and Pastore, 2006). Biosurfactants have become very popular due to their inherent health, commercial and environmental benefits such as their low toxic levels, environmental preservation quality, easy sources and low cost of production including renewable materials or agricultural waste products (Saharan *et al.,* 2011). However, the application of biosurfactants in the wider industry is still limited due to economic and/or operational concerns (Saharan *et al.,* 2011).

Presently, biosurfactants have low potential benefits and competitive advantages when compared with chemical surfactants. This is mainly because its production output is low, relative to costs (Makkar and Cameotra, 2002). Efforts to address these challenges signaled the alternative use of agro-wastes or feed stocks obtained at little or no cost, as sources of biosurfactant-producing organisms, in many studies (Joshi *et al.,* 2008, Sobrinho *et al.,* 2008, Saimmai *et al.,* 2012). These approaches are unconventional and still have not addressed the challenges leading to the adoption of appropriate wastes as substrates (Nawawi *et al.,* 2010). The unavailability of waste substrates which have a uniform composition of important macromolecules such as carbohydrates and lipids, and therefore have the benefits of ensuring optimal microbial growth and biosurfactant outputs, is still a serious challenge for researchers (Makkar and Cameotra, 1999).

A wide range of waste products are usually generated in large quantities from oil extraction processes and these include residual oil that can potentially cause water and soil pollution (Singh *et al.,* 2011). However, oil residues can be absorbed, dispersed or made soluble by microorganisms which live in soil and water and survives by producing biosurfactants (Nerurkar *et al.,* 2009). Organic waste products from palm oil production are generally difficult to manage, raising serious environmental concerns in the areas where production takes place (Sulaiman *et al.,* 2011). Ameliorative actions would require approaches that are viable economically and also practical in their implementation (Puetpaiboon and Chotwattanasak, 2004). A general name for the waste product from palm oil extraction is palm oil mill effluent (POME). POME is a mixture of over 90% water, less than 1% oil and about 4.5% % total solids (Ma, 2000). Over 4,000 mg/l of oil and grease (which can occur as oil droplets in a water–oil emulsion) can be an important constituent of the colloidal suspension (Alhaji *et al.,* 2016). This study presents POME as a new and promising source for producing biosurfactants. Using agricultural wastes as substrates in the biotechnology industry has led to significant cost reductions in biosurfactant production and has also driven the development of innovative and effective waste management techniques (Banat *et al.,* 2010). Biosurfactants are produced by a diverse group of microorganisms which occur in diversity and are secreted or found on the cell surface of substrates that cannot mix with water, especially during the growth phase (Singh *et al.,* 2010). In this investigation, POME was utilized as a unique source for organisms capable of producing biosurfactants.

LITERATURE REVIEW

Introduction to Surfactants

Surfactants are compounds that reduce the surface tension of a liquid, making it easier for the liquid to spread or mix with other substances. Common examples include detergents, emulsifiers, wetting agents, foaming agents and dispersants. *(Rosen and Kunjappu, 2012).*

Definition and Composition of Biosurfactants

Surfactants of biological nature are those that are produced by microorganisms, and are therefore referred to as biosurfactants or microbial surfactants. These surfactants of biological origin can as well reduce the tension or force acting on the surface of a liquid or between two phases of liquids that do not form a uniform mixture

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(Mulligan and Catherine 2005). Their occurrence is as complex lipid macromolecules including phospholipids, fatty acids, glycolipids, and lipoproteins (Banat *et al.,* 2000).

Advantages of Biosurfactants over Synthetic Surfactants

Biosurfactants are not toxic, they are more effective, and poses no threat to the environment, unlike the alternative synthetic surfactants *(Ron and Rosenberg 2001)*. Also, biosurfactants could be produced from cheap agricultural materials unlike the chemical surfactants that are gotten from petroleum feedstock (Magalhaes *et al.,* 2018). Additionally, microbial surfactants are more stable at under extreme conditions of temperature, salinity, and pH; and this makes them more commercially viable than synthetic surfactants *(Gutnick and Bach, 2002; Mulligan and Catherine, 2005)*. These characteristics are very important in both food and non-food manufacturing processes including drug formulations, environmental bioremediation, and enhanced oil recovery.

Factors Affecting Biosurfactant Production

Historically, the production of surface-active molecules has gained increasing traction because of the potential benefits in food and non-food industrial processes. Some important factors that can affect the production of microbial surfactants include aeration, temperature, nitrogen, carbon, and other trace elements (Roy, 2017). Nevertheless, the type and quantity of biosurfactants generated typically rely on the specific biosurfactant-producing organism (Marchant and Banat 2012). The solubility of oil can also be increased by biosurfactants, and this can potentially increase their bioavailability as important sources of carbon and energy (Mulligan, 2009).

Biosurfactants are amphiphilic in nature, possessing both water-loving (hydrophilic) and water-repelling (hydrophobic) polar properties. This unique structural feature makes them suitable surface tension reducing agent in different phases of fluid (Nayak *et al.,* 2009).

Health, Economic and Environmental Significance

Biosurfactants have become very popular due to their inherent health, commercial and environmental benefits such as their low toxic levels, environmental preservation quality, easy sources and low cost of production including renewable materials or agricultural waste products, and possibly under extreme conditions of temperature, salinity and pH levels (Pansiripat *et al.,* 2010). Surfactants from microorganisms have very unique properties and are therefore a great fit for new applications. The evidence of this specificity has been adduced in many previous studies on the relevance of biosurfactants in industrial sectors (Perfumo *et al.,* 2010) and in environment protection (Das and Mukherjee, 2007), over the last decade.

Environmental Applications

Water repellent contaminants in petroleum, soil and water environments hinder microbial degradation. However, when these contaminants are solubilized, often through the action of biosurfactants, they become more bioavailable, facilitating easier and more efficient microbial breakdown *(Metcalfe et al., 2008).* Naturally-occurring surfactants are able to increase the surface area of water-repelling surfaces, and thereby increasing their solubility in water.

This property is particularly useful in environments contaminated with substances like pesticides in soil and water. For this reason, surfactants might contribute significantly to the degradation of polluting agents by microbes (*Murphy et al., 2005)*. The mechanisms or models for identifying and classifying microbial surfactants which are produced from different microorganisms have recently undergone broad review (*Ying, 2006*).

Various organic compounds serve as crucial carbon and energy sources for microbial proliferation, readily diffusing into cells with the assistance of microorganisms. This is essentially possible when the carbon and energy sources exists in a form that cannot dissolve in water, such as a hydrocarbon (CxHy); and thus their diffusion into the cell is aided by their production of biosurfactants. Usually, the CxHy active agents in the growth medium are blended by the release of ionic surface molecules (surfactants) by some fungi and bacteria, including yeast (Zheng *et al.,* 2008).

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Examples of Microbial Surfactants and Producing Organisms

Examples of these microbial surfactants include rhamnolipids or sophorolipids which are produced by various *Pseudomonas* sp. and *Torulopsis* sp., respectively (Walter *et al.,* 2010). Moreover, certain microorganisms possess an intrinsic capability to modify their cell wall structure through the production of macromolecules like nonionic surfactants. Examples include the cell wall binding lipopolysaccharides produced by *Candida lipolytica* and *Candida tropicalis,* followed by *Rhodococcus erythropolis* and several strains of *Mycobacterium* sp. and *Arthrobacter* sp. that creates nonionic trehalose corynomycolates (Tabatabaee *et al.,* 2005 and Makkar *et al.,* 2011). Similarly, Emulsan, derived from species of *Acinetobacter* and lipoproteins such as Surfactin and Subtilisin, are examples of some lipopolysaccharides that are produced by *Bacillus subtilis* (Gorkovenko *et al.,* 1997). Additional examples of microbial surfactants that demonstrate significant efficacy include:

(i) Mycolates and Corynomycolates, synthesized by *Rhodococcus sp., Corynebacteria sp., Mycobacteria sp.*, and *Nocardia sp.*
(ii) Ornithinlipids, derived from *Pseudomonas rubescens, Gluconobacter cerinus* and *Thiobacillus ferroxidans* (*Okoliegbe and Agarry, 2012)*.

Screening Methods for Biosurfactant-Producing Microorganisms

A variety of screening methods used in identifying biosurfactant-producing organisms exist, and examples include methylene blue assay/Centriamide test (CTAB), β haemolysis test, oil displacement test, drop collapsing and emulsification index test (Satpute *et al.,* 2010). However, it is not easy to precisely know the type of biosurfactant derived from the microorganisms by using only one method. This is basically as a result of their unique chemical and functional characteristics; which altogether makes it necessary to consider the application of a combination of screening methods to be able to sufficiently understand the biosurfactant-producing ability and mechanisms of individual microorganism. The research conducted by Satpute *et al.* (2010) also adduced evidence that the application of single screening method is not suitable for identifying all types of microbial surfactants, and thus recommended the adoption of a combination of (more than one) screening methods for the identification of potential biosurfactants-producing microbes.

Advantages of Biosurfactants

When compared to surfactants that are chemically synthesized, biosurfactants have the following advantages:

1. **They are Biodegradable**
   This is because they are not very toxic, their chemical structure is simple, they do not accumulate in the environment because they degrade easily.
2. **They are compatible with the biological environment and are easily digested (biocompactability and digestibility)**

   They are compatible with living systems and thus can be used in both food and non-food materials including additives, drugs and cosmetics; this is essentially because they are of biological origin.
3. **Raw materials abundance in supply**

   The raw materials for biosurfactants production are abundant in supply. The microbial production process can involve the separate or combined use of the carbon sources which include carbohydrates, lipids or hydrocarbons.
4. **Production economics of scales**

   Biosurfactants can typically be derived from industrial wastes which are potentially resourceful means of producing large quantities of microbial surfactants, when the intended use is permitted.
5. **Environmental control**

   Biosurfactants promotes bioremediation, control of oil spills, and are useful in biodegrading, detoxifying and stabilizing emulsions or effluents from industrial processes.

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6. **Specificity**

   Biosurfactants possess distinct functional groups that enable them to perform specific tasks, such as de-emulsifying industrial emulsions and detoxifying particular pollutants.

Other applications encompass the formulation of specialized cosmetics and their utilization in tailored pharmaceutical and food processing. The notable resilience of biosurfactants under extreme conditions of temperature, pH, and salinity underscores their significant characteristics (*Murphy et al., 2005)*.

Classification of Biosurfactants

The microbial origin of biosurfactants and what they are chemically composed of, are the basis of their classification. This is unlike chemical surfactants whose classification is based on the nature or type of polar groups they possess.

Biosurfactant Classification based on molecular weight

Biosurfactants can be categorized into two groups: low molecular mass molecules, which effectively reduce surface and interfacial tension, and large molecular-mass polymers, which serve as more efficient emulsion-stabilizing agents (Rosenberg and Ron, 1999). Important macromolecules such as phospholipids, lipopeptides, and glycolipids are the main classes of surfactants with low molecular mass (Mukherjee *et al.,* 2006). Biosurfactants majorly contain negative charges or they can be neutral. Similarly, the presence of compounds derived from fatty acids makes majority of biosurfactants have water-repelling structures, while the water-loving structures are usually composed of amino acid, phosphate, carbohydrate, or cyclic peptide (Banat *et al.,* 2014).

Low molecular weight biosurfactants:

These compounds effectively reduce surface and interfacial tension at the air/water interface. Low molecular-weight biosurfactants typically consist of glycolipids or lipopeptides. Among the extensively researched glycolipids are rhamnolipids, trehalolipids, and sophorolipids, which are disaccharides acylated with long-chain fatty acids or hydroxy fatty acids (Fracchia *et al.,* 2015).

High- molecular weight biosurfactants:

These are also known as bioemulsans, and are more efficient oil in water emulsions stabilizers. Their effective emulsifying property is based on their ability to show considerable specificity for substrate, as well as work at low concentrations (Uzoigwe *et al.,* 2015). Previous studies have reported that polymeric surfactants that are made up of large macromolecules such as polysaccharides, proteins, and lipopolysaccharides, are usually produced on the outer cell surface by a large number of bacterial species from different genera (Rosenberg and Ron, 1999).

Furthermore, biosurfactants can be classified based on the nature of their polar groups, resulting in either anionic or neutral characteristics. Their hydrophobic structure is determined by compounds derived from fatty acids or the presence of long-chain fatty acids. The hydrophilic region can include carbohydrates, amino acids, phosphates, or cyclic peptides. Generally, biosurfactants exhibit the following structural components: a hydrophilic moiety composed of amino acids or peptide anions or cations; a hydrophobic moiety comprising unsaturated, saturated, or derivative fatty acids and mono-, di-, or polysaccharides.

Classification Based On Chemical Composition

Glycolipids:

In biosurfactants, glycolipids make up the majority. Several carbohydrates, when paired with one or more aliphatic carboxylic acids, hydroxy fatty acids, or fatty alcohols, can produce glycolipids. These biosurfactant compounds hold significant potential for commercial applications due to their high production yields and capability to be synthesized from renewable substrates (Marchant and Banat 2012). Microbial species such as *Pseudomonas sp*. synthesise rhamnolipids, *Pseudozyma antarctica* synthesise mannosylerythriol lipids,

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Nocardia sp. and *Mycobacterium* sp., *Rhodococcus* sp. synthesises trehalose and Candida sp. synthesizes sophorolipids (Zheng *et al.,* 2008).

Rhamnolipids: The most widely researched type of lipids are glycolipids, which are made up of at least one or more than one rhamnose molecules bound to at least one or more than one β-hydroxydecanoic acid molecules (Abdel-Mawgoud *et al.,* 2010). One of the hydroxyl groups from the acid is involved in forming a glycosidic bond with the reducing end of the rhamnose disaccharide, while the second hydroxyl group from the acid is used to create ester bonds (Abdel-Mawgoud *et al.,* 2010). It was initially investigated the synthesis of glycolipid-containing rhamnose in Pseudomonas aeruginosa. Major glycolipids produced by P. aeruginosa are L-Rhamnosyl-Lrhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and Lrhamnosyl-β-hydroxydecanoyl-β-hydrtocydecanoate, also known as rhamnolipids 1 and 2 (Nitschke and Pastore 2006). It is believed that the phosphatidylethanolamine component in living membrane systems associate with the rhamnolipid molecular entity and as a result, according to Sanchez *et al.* (2006), they possess antibacterial action against both Gram positive and Gram negative microorganisms. Additionally, rhamnolipids have important use in beauty products, drugs, and food industries (Sánchez *et al*., 2006).

Trehalolipids: There are reports of several molecular variants of microbiological trehalolipid biosurfactants. Most taxonomic categories within *Mycobacterium*, *Corynebacterium* and *Nocardia* are associated with the disaccharide trehalose, which is linked to mycolic acid at positions C-6 and C-6 (Daffé and Draper, 1998). The long, α-branched chain, and β-hydroxy fatty acids molecular entity are called 2-alkyl, 3-hydroxy long-chain fatty acids (Brennan and Nikaido, 1995). The degree of branching or unsaturation, the total amount of carbon molecules, and molecular makeup of 2-alkyl, 3-hydroxy long-chain fatty acids in trehalolipids derived from different living organisms are all distinct. The surface energy and interfacial surface tension within the culture medium were reduced by trehalose lipids obtained from *Rhodococcus erythropolis* and *Arthrobacter* sp. (White *et al.,* 2013).

Mannosylerythritol lipids: The fungus *Pseudozyma antarctica* forms mannosylerythritol lipids (MEL) as a combination of four component parts: MEL-A and MEL-B consist of the principal derivatives, while MEL-C and MEL-D are the secondary derivatives (Konishi *et al.,* 2007). These complexes' foundation is a mannoseerythritol disaccharide, which happens to be acetylated to form short carbon molecules of two to eight or long carbon molecules of ten to eighteen fatty acid chain length (Kitamota *et al.,* 1990).

The variety of molecular properties exhibited by MEL, such as binding affinity of proteins to immunoglobulin G and adhesin and promotion of cell division in relation to distinct mammalian cells (Im *et al.,* 2001). They can also reduce the surface energy of water approximately to 35mN/m (Fischer and Zettl, 2000). Both pharmaceutical and medical industries are very interested in MELs due to their intriguing biological behavior.

Sophorolipids: Yeast like *Torulopsis bombicola*, *T. petrophilum,* and *T. apicola* are the dominant species that synthesizes glycolipids, and are made up of a hydroxyl fatty acid of long-chain length and a dimer of carbohydrate sophorose linked together by a glycosidic bond (Bajaj and Annapure, 2015). Sophorolipids are typically found in combination with macrocyclic lactones and free acid state (Hirata *et al.,* 2009). Evidence has shown that the sophorolipid's lactone state is crucial for a number of different purposes (Hu and Ju, 2001). A minimum of six to nine different hydrophobic sophorolipids are combined to form these biosurfactants (Hu and Ju, 2001).

Lipopeptides and lipoproteins: Typically, each of the molecules in this group of biosurfactants are made up of peptides that are cyclically connected to a fatty acid. These bactericidal-like compounds are produced by a number of microorganisms including *Bacillus subtilus* (Malfanova *et al.,* 2012). At 0.005 % level of concentration, this biosurfactant, which is among the strongest, reduces surface energy from 72.8 to 27.9 mN/m (Nguyen and Sabatini 2023).

Surfactin: One of the most notable biosurfactants is surfactin, a cyclic lipopeptide manufactured by *Bacillus subtilis*. It comprises a fatty acid chain linked to a cyclic structure of 7 amino acids through lactone bonding.

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Even in small concentrations as low as 0.005 %, it effectively reduces surface energy from 72 to 27.9 mN/m (*Singh and Cameotra, 2004)*.

Lichenysin: The bacterium *Bacillus licheniformis* generates a range of biosurfactants known for their remarkable balance in pH, temperature, and salt tolerance, working synergistically with each other. Their physical, chemical and structural characteristics are likewise comparable to those of surfactin. The surface energy of water can be lowered to 27 mN/m and the tension between it and its interface of water and n-hexadecane to 0.36 mN/m by the surfactants that are generated by *B. licheniformis* (Coronel-León *et al.,* 2015).

Neutral lipids, phospholipids and fatty acids: A significant quantity of phospholipid surfactants and fatty acids are produced by several bacteria and yeast during their growth phase on n-alkanes. The length of the hydrocarbon chain in each structure determines the equilibrium between affinity for water and affinity for lipids (HLB), which has directly proportionality. Phosphatidylethanolamine-abundant sacs that create optically transparent alkane solubilized oil are produced by *Acinetobacter* sp. Evidence has shown that the tension between the interface of hexadecane and water is lowered by phosphatidylethanolamine produced by *R. erythropolis* cultured on n-alkane, to a level below 1 mN/m, and an essential associated colloidal system concentration (Jorge *et al.,* 2018).

Polymeric biosurfactants: Alasan, liposan, lipomanan emulsan and a few additional protein–polysaccharide interactions are the most researched polymeric biosurfactants. The effective extracellular polyanionic amphipathics heteropolysaccharide bioemulsifier is produced by *Acinetobacter calcoaceticus* RAG-1. The molecules of hydrocarbon in water can be effectively emisfied by emulsan, regardless of minimal concentrations ranging from as 0.001 to 0.01% (Desai and Banat, 1997). *Candida lipolytica* produces liposan, an extracellular dispersible emulsifier that is 83% carbohydrate and 17% protein (Danyelle *et al.,* 2016).

Particulate biosurfactants: Microbial cells' absorption of alkanes is greatly aided by the microemulsion that is created when hydrocarbons are partitioned by extrinsic membrane compartments. *Acinetobacter* sp. compartments have a density of 1.158 cubic g/cm buoyancy, 20–50nm diameter, and a composition of lipopolysaccharide endotoxin, Phosphatidic acids, and protein (Makula *et al.,* 1975).

Characteristics of Biosurfactants

Due to the widening range of compounds that are becoming obtainable, biosurfactants are becoming more appealing for application commercially. When chemically juxtaposed with the inorganic, biosurfactants possess a number of advantageous superiorities. The following list includes a synopsis of each of the primary characteristics that set biosurfactants apart:

Surface and Frontier Activity

The tension in the interfacial space between water and hexadecane can be reduced from 40 to 1 mN/m while the surface energy of water from 72 to 35 mN/m by using a suitable surfactant. The *B. subtilis*-derived surfactin may lower the tension in the interfacial space of water and hexadecane to less than 1 mN/m and the surface energy of water to 25 mN/m (Gudiña, 2012). *P. aeruginosa* rhamnolipids reduce water's surface energy to 26 mN/m and the tension in the interfacial space of water and hexadecane to less than 1 mN/m (Mendes *et al.,* 2015). The tension across the interfacial space drops to 5 mN/m and the surface energy drops to 33 mN/m by *T. bombicola's* sophorolipids (Pakshirajan and Daverey 2010). Surfactants chemically produced takes a greater volume to achieve the highest reduction in surface energy; in contrast, biosurfactants generally has a greater degree of effectiveness, with a CMC that is roughly 10–40 times less (Sajadi *et al.,* 2024).

Temperature, Ph and Ionic Strength Tolerance

The temperature and pH levels of the surrounding environment have little effect on the surface activity of a variety of surfactants of biological origin. Evidence from the study carried out by Coronel-León *et al.* (2015) showed that the quantities of Ca (50g/l) and NaC (25 g/l), and temperature of up to 50^0C, had no effect on the lichenysin derived *B. licheniformis*. After subjecting to autoclave at 121°C and subsequently at six months at

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18^0C for 20 min, a lipopeptide derived from *B. subtilis* remained stable; Its surface activity remained consistent within a pH range of 5 to 11, and its effectiveness was unaffected by NaCl concentrations up to 20 %.

Biodegradability

Microbially derived surfactants, in contrast to chemical surfactants, are readily broken down and primarily used in ecological activities including oil spill dispersal and bio-remediation processes (Banat *et al.,* 2014).

Low Toxicity

Since they are typically regarded as safe by-product, biosurfactants can be used in food, beauty products and drugs production. Evidence from research proposed that a chemically synthesized anionic surfactant (Corexit) compared to *Photobacterium phosphoreum* derived rhamnolipids resulted in 50 % death of sample population (LC50), which is 10 times higher than rhamnolipids, indicating the higher lethality of the chemically synthesized surfactant (Nash *et al.,* 2014). The toxicological properties of six microbially derived surfactants, four chemical-based surfactants, and two industrial dispersing agents were evaluated. It became apparent that the majority of the microbially derived surfactants broke down more quickly, with the exception of a chemical-based emulsifier or sucrose-stearate, which broke down more quickly than biological derived glycolipids with displayed structural identity to glycolipids (Bhardwaj and Sharma, 2013). With respect to lethality and mutation causing capabilities, a chemically derived surfactant with frequent usage in industrial processes was juxtaposed with a biological surfactant derived from *P. aeruginosa* (Cooper and Cavalero, 2003). According to Vijayakumar and Saravanan (2015), surfactant derived from biological sources was found to be marginally non-toxic and non-mutagenic, while the surfactant derived from chemical sources showed mutagenicity and lethality of a greater degree in the two tests. It is possible to create persistent emulsifiers that degrade and form composites emulsion that persist for long period of time running into years. Surfactants of microbial origin can operate as emulsifiers or destabilizers, depending on the state of the emulsion. Overall, emulsifiers with higher molecular weight have superior performance than those with lower molecular weight surfactants of biological origin (Mnif and Ghribi, 2015). Although *T. bombicola*-produced sophorolipids can lower interfacial and surface energy, they are not effective emulsifiers (Cooper and Cavalero, 2003). Liposan, on the other hand, has been effectively employed in the emulsification of edible oils indicating inability to decrease surface energy (Paximada *et al.,* 2021). The fact that surfactants of polymer source cover tiny beads of oil to create steady emulsions gives them extra advantageous superiority: a characteristic that finds applicability in Making oil/water emulsions for food production and beauty products.

Chemical heterogeneity

Chemical heterogeneity refers to the diverse and complex molecular structures present in biosurfactants, which are surface-active compounds synthesized by various microorganisms, including bacteria, fungi, and yeasts (Dini *et al.,* 2024). This structural diversity arises from variations in the hydrophilic and hydrophobic moieties of biosurfactant molecules, such as differences in fatty acid chain lengths, sugar residues, amino acids or peptide structures. These molecular variations significantly influence the physicochemical properties of biosurfactants, including their surface tension reduction, emulsification capacity and critical micelle concentration. The chemical heterogeneity of biosurfactants is a key factor that enables them to function effectively under a wide range of environmental conditions and interact with a variety of substrates. Consequently, this feature enhances their applicability in numerous fields such as bioremediation, enhanced oil recovery, pharmaceuticals, cosmetics and food processing (Dini *et al.,* 2024). The ability to tailor biosurfactant properties by leveraging their inherent chemical diversity makes them a valuable alternative to synthetic surfactants in both sustainable and specialized applications.

Economical and Highly Promising Substrates

Like most biotechnology processes, the primary hurdle in the manufacture of biological surfactants is operational cost. The quantity of the input/raw resources and the type of resources can frequently have a significant impact on operational costs; as with other biotechnological processes, input/raw resources are thought to be responsible for between 10% and 30% of overall operational costs. The use of inexpensive input/raw resources to produce

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the appropriate biological surfactants is therefore preferable in order to lower the operational cost. A possible approach that has been thoroughly investigated is the use of inexpensive input/raw resources of agricultural source as substrates to produce biological surfactant. Production of biosurfactants can be aided by low-cost input/raw resources from sources such as oils obtained from plants and oil wastes. Numerous studies involving oils obtained from plants have demonstrated their potential as affordable and efficient input/raw resources for the synthesis of biosurfactants (Nitschke *et al.,* 2005).

factors affecting the production of biosurfactant

Composition of the growth

The composition of the growth, which include the type of carbon, nitrogen sources, and carbon itself, all affect the generation of biological surfactant in addition to the genetic variant of the producer. The total volume of biological surfactant derived as well as the type of bio-polymer formed are influenced by the nitrogen proportion, nutritional supplements constraints, and physical-chemical parameters like temperature, pH, aeration, divalent cations and salt (Ilori *et al.,* 2005).

Carbon sources for biosurfactant production: Many research investigations have employed a wide range of carbon sources to produce biological surfactant. Evidence suggesting a good supply of carbon substrate for the synthesis of biological surfactants includes; crude oil, diesel, glucose, sucrose, and glycerol, exist in the literature (Fagade *et al.,* 2009). Though its significance varies depending on the microorganism, it is clear that carbon substrate is essential to the derivation of biological surfactants. For example, in the case of *Pseudomonas* sp. the chemical makeup of the biological surfactant production was influenced by the various carbon sources in the medium, but the overall chain length of the fatty acid component parts in glycolipid was unaffected by the chain length of the substrate (Sari, 2019).

Nitrogen sources for biosurfactant production: A supply of nitrogen is necessary for the synthesis of biological surfactants. Nitrogen-containing medium is crucial for the growth of microorganisms since it is necessary for the synthesis of proteins and enzymes. Biological surfactants have been produced using a variety of nitrogen-rich sources, including meat extract, yeast extract, ammonium sulphate, ammonium nitrate, and sodium nitrate (Sari, 2018). *P. aeruginosa* produces biological surfactants mostly from nitrate-rich sources (Pacwa-Płociniczak *et al.,* 2011); ammonium salts and urea are favoured as source of nutrient for *Arthrobacter paraffineus* (Pacwa-Płociniczak *et al.,* 2011). Monosodium glutamate (MSG) otherwise known as yeast extract is a rather common source of nitrogen utilized for the synthesis of surfactants of biological origin. However, the amount of this nitrogen depends on the microbe and the medium used for cultivation. According to a report, during the stationary phase of cell growth, surface-active chemicals are frequently produced when the culture medium's nitrogen supply is reduced (Wu *et al.,* 2011).

Environmental factors: The total yield of the biological surfactant derived is highly dependent on environmental conditions. The biological surfactant production process must be optimized to derive substantial volume because variations in temperature, pH, air circulation speed, might have an impact on the final result (Saharan *et al.,* 2011). Although it is claimed that the vast majority of biological surfactant manufactures are carried out in the 25–30 °C temperature range, this range of temperatures modified the chemical makeup of the biological surfactant generated in *A. paraffineus* and *Pseudomonas* sp. variant DSM2874 (Pacwa-Płociniczak *et al.,* 2011). Zinjarde and Pant (2002) investigated the impact of pH on the amount of biological surfactant generated and found that optimal yield happened at pH 8.0, corresponding to the normal pH of seawater, which is the native ecosystem of *Y. lipolytica.* The generation of rhamnolipids by *Pseudomonas* sp. peaked at the pH range of 6 to 6.5 and declined precipitously above pH 7. Stirring and air circulation play a significant role in the synthesis of biological surfactants by facilitating oxygenation between the gaseous phase to the aqueous phase. Evidence in the literature suggests that bio-emulsifier or stabilizers synthesis can improve the dissolution of nutrients that are hydrophobic, hence facilitating the delivery of nutrients to microbial organisms, inferring that the biological significance of microbiological emulsifiers or stabilizers could be attributable to their ability to dissolve and deliver hydrophobic nutrients (Alizadeh-Sani *et al.,* 2018). The synthesis of biological surfactants is also significantly influenced by the salt concentration of a given medium or substrate. However, the results

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for certain biological surfactant derivatives showed that they were unaffected by salt levels as high as 10 % (weight/volume), despite having indicated that the CMC was slightly reduced (Mahmoud *et al.,* 2010).

Applications of Biosurfactants

Although, chemical synthesis typically used to create all surfactants before now. Interestingly, because of their wide spectrum of advantageous characteristics, and the various ways that microbes can synthesize them, biological surfactants have received a lot of research focus lately (Velioglu and Urek, 2016). The fact that they are less hazardous than chemically manufactured surfactants and readily biodegrade makes them environmentally acceptable, which is an especially important factor. Because of these special qualities, biological surfactants can be used in many industrial processes as substitutes for surfactants that are chemically manufactured (Zhao *et al.,* 2017). In addition, they can be used in biological remediation and treatment of wastewater, and are harmless on the environment. Removing heavy metals from polluted soil, hydrocarbon in water bodies, hexa-chloro cyclohexane decomposition, microbial enhanced oil recovery, and hydrocarbon decomposition in polluted soil are a few possible uses of biological surfactants in contamination control (Kumar and Mandal, 2017).

Ingredients in Food Formulations

Evidence that exists in the literature has shown that surfactants have the capacity for lowering surface energy and tension between surfaces, which makes emulsion derivation and stabilizing effect easier. Additionally, surfactants can serve a number of purposes in the manufacture of food (Nitschke and Costa, 2007). In this regard, to alter the viscoelasticity features of whole-wheat dough, stabilizing air-circulating systems, enhancing the whole-wheat and preservation period of yields of starch content, preventing accumulation of tiny aggregates of fat and enhancing the consistency and surface texture of calorie-dense food products (Wang *et al.,* 2024). Biological surfactants are stabilizers that help keep fats and oils from retrogradation while also allowing the flavor oils to be dissolved and regulation in ice cream and pastry recipes (Zhao *et al.,* 2017). Research investigation showed that it is possible to enhance the size, surface texture, uniformity of dough, and preservation of baked food products by including rhamnolipid surfactants (Wang *et al.,* 2024). In addition, rhamnolipids may be used to enhance the qualities of buttery cream and frosted food products (Wang *et al.,* 2024). Significant opportunity exists for L-rhamnose as a precursor to flavoring. Similar to furaneol, it is currently employed in industry as an intermediate for premium flavoring ingredients (Roscher *et al.,* 1997).

Adhesion-prevention agents

A colony of bacteria on a surface is referred to as a biofilm. In addition to the bacteria, the biofilm also comprises of any extrinsic material generated at the surface and any material substance confined in the structural matrix that has developed (Flemming and Wingender 2010). According to Srey *et al.* (2013), bacterial biofilms found on surfaces in the food manufacturing sector are probable causes of exposure that could cause food to deteriorate and spread illness (Srey *et al.,* (2013). Preventing bacteria from adhering to surfaces that come in touch with food is therefore, crucial to giving customers high-quality and healthy food products. Research have shown how biological surfactants affect microbial adherence to and disengagement from surfaces (Gudiña *et al.,* 2013). *Streptococcus thermophilus* produces a biosurfactant that has antimicrobial properties, including the ability to inhibit the growth of various thermophilic strains of *Streptococcus*. These strains are commonly used as fat and oil emulsifiers but may also contribute to the formation of foul odours. The biosurfactant from *S. thermophilus* is currently being applied in industrial settings, such as in pasteurizers, to reduce offensive odors by preventing bacterial spatter on heat-exchanger plates (Thando *et al.,* 2017) One novel approach to lessen adhesion has been proposed: the bio-treatment of surfaces via the application of surfactants of microbial origin (Thando *et al.,* 2017).

Therapeutic and biomedical applications and antimicrobial activity

Biological surfactants have shown microbicidal activity against various bacteria, algae, fungi, and viruses in numerous studies. Significant antimycotic action was demonstrated by the lipopeptide iturin derived from *Bacillus subtilis* (Rahman *et al.,* 2007). It has been shown that 80 mM surfactin inactivates enveloped viruses,

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including herpes and retrovRhamnolipids, at concentrations ranging from 0.4 to 10.0 mg/l, were found to hinder colony formation in *Heterosigma akashivo* and *Protocentrum dentatum*, both known toxic bloom algae (Wang *et al.,* 2005). Furthermore, a rhamnolipid mixture derived from *Pseudomonas aeruginosa* exhibited antifungal properties against *Chaetonium globosum*, *Penicillium crysogenum* and *Aspergillus niger* (at 16 mg/ml), *Aureobasidium pullulants* (at 32 mg/ml), as well as against the phytopathogens *Botrytis cinerea* and *Rhizoctonia solani*, with significant effectiveness (Goswami *et al.,* 2015). The rhamnolipid mixture exhibited antimicrobial activity against various bacterial species, including *Escherichia coli*, *Micrococcus luteus* and *Serratia marcescens*. It was also effective at concentrations of 32 mg/ml against *Alcaligenes faecalis*, 16 mg/ml against *Mycobacterium phlei* and 8 mg/ml against *Staphylococcus epidermidis* (Banat *et al.,* 2010). Potent antimycotic medium targeting plant and seed fungal pathogens were discovered to be rhamnolipids and sophorolipids (Chen *et al.,* 2020). Glycolipid surfactant mannosylerythritolcations lipid (MEL) derived from *Candida antartica* demonstrated significant microbicidal action against Gram positive bacteria such as *Staphylococci*, *streptococci* and some *listeria* species (Kitamoto *et al.,* 1993).

Anticancer activity

The impacts of 7 extracellular glycolipids synthesized by microorganisms, including mannosyl erythritol lipids-A, mannosyl erythritol lipids-B, rhamnolipid, polyol lipid, sophorose lipid, and others, have been investigated. With the exception of rhamnolipid, all these glycolipids were found to induce cell differentiation rather than cell growth in the HL60 human promyelocytic leukemia cell line (Isoda *et al.,* 1997). Specifically, sophorolipid and mannosylerythritol lipid notably promoted characteristic differentiation features in monocytes and granulocytes, respectively (Cameotra and Makkar, 2004). B16 cells exposed to MEL underwent chromatin condensation, DNA cleavage and sub-G1 arrest (Kitamoto *et al.,* 1993). This is the first demonstration that glycolipids can cause growth inhibition, cell death, and differentiation in malignant mouse melanoma cells (Briem *et al.,* 1999). Furthermore, MEL enhanced the activity of acetylcholine esterase and arrested the cell cycle at the G1 phase in PC12 cells, resulting in neurite outgrowth and partial cellular differentiation (Isoda *et al.,* 2000). MEL, a glycolipid surfactant sourced from *Candida antarctica*, additionally demonstrated antimicrobial effects against Gram-positive bacteria (Kitamoto *et al.,* 1993).

Anti-Human Immunodeficiency Virus and Sperm Immobilizing Activity

The significant occurrence of HIV/AIDS among women aged 15–49 years has underscored the necessity for a safe, efficient, and female-controlled vaginal microbicide. In response to this concern, researchers have investigated sophorolipid derived from *C. bombicola* and its derivatives for their potential to eliminate sperm, HIV and vaginal cells (Shah *et al.*, 2005). The sophorolipid diacetate ethyl ester derivative is the strongest spermicide and virucide of the sophorolipids examined. It has comparable effects to nonoxynol in inactivating HIV and human semen (Shah *et al.*, 2005). Nevertheless, it induced considerable damage to vaginal cells, raising doubts about its appropriateness for prolonged microbicidal contraceptive use (Shah *et al.*, 2005).

Agents for respiratory failure

Breathing difficulties in premature infants result from the absence of pulmonary surfactant, a combination of phospholipids and proteins (Nkadi *et al.*, 2009). To address this, the genes responsible for producing surfactant proteins can be extracted and inserted into bacteria, allowing for the proteins to be produced through fermentation for medical use (Shah *et al.*, 2005).

Agents that enhance the activity of skin fibroblast cells

Sophorolipids in lactone form contain a large amount of diacetyl lactones that can boost the metabolism of skin dermal fibroblast cells and the formation of new collagen, at a concentration of 0.01 parts per million (ppm) at 5 %) (w/w) of dry matter in formulation (Borzeix and Concaix 2003). This finds utility in both the fields of cosmetology and dermatology. The purified lactone sophorolipid compound holds significance in formulating anti-aging dermal products due to its ability to stimulate dermal cell activity (Borzeix and Concaix 2003). By encouraging the generation of fresh collagen fibers, purified lactone sophorolipids can help combat skin aging,

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and are utilized in various skincare products such as body creams, milks, lotions and gels (Borzeix and Concaix 2003).

Surgical antiadhesive agents: Surfactants derived from *S. thermophilus*, when applied to silicone rubber, effectively prevented 85 % of *C. albicans* adhesion (Busscher *et al.,* 1997). Similarly, surfactants obtained from *L. fermentum* and *L. acidophilus*, when applied to glass surfaces, reduced the attachment of uropathogenic *Enterococcus faecalis* cells by 77% (Velraeds *et al.,* 1996). Additionally, the biosurfactant sourced from *L. fermentum* inhibited *S. aureus* infection and adhered to surgical implants (Gan *et al.,* 2009). Furthermore, surfactin demonstrated a reduction in biofilm formation by *Salmonella typhimurium, S. enterica, Escherichia coli* and *Proteus mirabilis* on PVC plates and vinyl urethral catheters (Rodrigues *et al.,* 2006).

Palm Oil Mill Effluents

POME is recognized as one of the most environmentally harmful agro-industrial wastes, mainly because of its high organic content. Among the most polluting agro-industrial wastes due to its high organic content. From sterilization and clarification stages, palm oil mill effluent emerges as a dark brown, highly concentrated colloidal mixture of water, oil, and fine cellulose materials (Madaki and Lau 2011). POME constitutes a colloidal solution containing approximately 0.6 - 0.7 % oil, 95 – 96 % water and 4 – 5 % total solids (Ma, 2000). Oil palm production in Nigeria witnessed an increase of 0.8 million tonnes from 1990 to 2001, reaching 9 million metric tonnes (FAO, 2002). Of this production, approximately 43 – 45 % always remains as mill waste, comprising Empty Fruit Bunches (EFB), Shell, Fibre and Palm Oil Mill Effluent (POME) (Madaki and Lau, 2011). As production increases, these residues will continue to accumulate. Initiatives are underway to convert these waste materials into valuable resources for energy generation, animal feed, and organic fertilizers. The oil extraction procedure requires significant water usage for steam sterilization of palm fruit bunches and oil clarification. The resulting wastewater sludge, termed palm oil mill effluent, is a brown sludge containing approximately 4 – 5 % solids (predominantly organic matter), 0.5 – 1 % residual oil, and around 95 % water, with a high concentration of organic nitrogen (Onyia *et al.,* 2001). This effluent is a severe land and water pollutant when released directly into the environment. In addition to lipids and volatile compounds, the adverse impacts of palm oil mill effluent on living tissues may be attributed to water-soluble phenolic compounds (Radzia 2001, Perez *et al.,* 1992). The presence of ammonia in the effluent is undesirable as it contributes to high oxygen demand in water bodies. Although palm oil mill effluent is a pollutant for the palm oil industry, it has great potential for improving animal feed and soil quality (Binder *et al.,* 2002). The quality of the raw material and the palm oil production processes in the mills affect the characteristics of palm oil mill effluent. There are three main processing steps that result in the POME according to Sethupathi (2004). The sterilization process of fresh fruit bunches (FFB), clarification of crude palm oil (CPO), and the hydrocyclone separation of cracked kernel and shell mixture together account for around 36%, 60 % and 4 % of palm oil mill effluent (POME) respectively within the oil mills. According to Yacob *et al.* 2006), it is approximated that for every tonne of fresh fruit bunch processed, approximately 0.5 to 0.75 tonnes of palm oil mill effluent (POME) will be generated (Yacob *et al.,* 2006).

Attributes of Palm Oil Mill Effluent (Pome)

There is a lot of waste produced by the palm oil mill industry. POME primarily originates from oil extraction, washing, and purification processes within the mill. It comprises various components including cellulose material, fats, oils, and greases (Agamuthu, 1995). Additionally, POME is laden with solids, encompassing both suspended and dissolved particles, with concentrations ranging from 18,000 mg/L to 40,500 mg/L. These solids are called palm oil mill sludges (POMS). Newly generated effluents, according to Ma, (2000), is a warm, acidic liquid with a pH ranging from 4 to 5. It presents as a brownish colloidal suspension characterized by elevated levels of organic matter, substantial concentrations inclusive of COD (50,000 mg/L), total solids (40,500 mg/L), BOD (25,000 mg/L), and oil and grease (4,000 mg/L). Untreated or partially treated palm oil mill effluent (POME) is characterized by a substantial concentration of readily degradable organic substances. Since no chemicals are incorporated during the oil extraction process, POME is considered non-toxic. Nevertheless, in its untreated state, POME plays a considerable role in aquatic pollution by diminishing dissolved oxygen levels in water bodies (Khalid and Wan Mustafa, 1992). Conversely, it also contains significant amounts of essential nutrients such as nitrogen (N), magnesium (Mg), phosphorus (P), potassium (K), and calcium (Ca) (Habib *et al.,* 1997; Muhrizal *et al.,* 2006), which are crucial for plant growth and development. Because of its lack of toxicity

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and its potential as a fertilizer or alternative animal feed, POME can supply vital mineral nutrients. Additionally, Agamuthu, (1995) observed that the elevated organic nitrogen content in POME enhances its efficacy as a fertilizer.

According to Muhrizal, M., *et al.* (2006), POME has a higher aluminium (Al) concentration than composted sawdust and chicken dung. Habib *et al.,* (1997) stated that while lead (Pb) concentrations in palm oil mill effluent (POME) typically remain above sub-lethal levels (more than 17.5 µg/g) (James *et al.,* 1996), POME may contain dangerous elements. James *et al.,* (1996) explain that paints and glazing materials containing lead can contaminate plastic or metal pipes, tanks, and containers used in palm oil processing, which may result in lead leaching into the palm oil mill effluent (POME) (Okewole and Omin (2013).

Extraction of Crude Palm Oil

Harvested from oil palms, fresh fruit bunches (FFB) are processed in palm oil mills to produce crude palm oil and palm kernel. These mills, which are typically found inside plantations, allow fresh fruit bunches (FFB) to be moved about and processed. The primary process of palm oil milling primarily encompasses the physical extraction of palm products (Hii *et al.,* 2012).

The extraction of crude palm oil from FFB involves several processing stages. Sterilization is the initial step, wherein freshly harvested fruit bunches are subjected to high-pressure steam (120 to 140^0C at 40 psi or 275790 N/m²) promptly upon arrival at the mill. This procedure deactivates lipolytic enzymes accountable for oil hydrolysis and fruit degradation, simultaneously averting the formation of free fatty acids, and priming the fruit bunches for subsequent sub-processes (Igwe and Onyegbado, 2006). Bunch stripping follows, mechanically separating fruits from bunch stalks. The sterilized and separated fruits then undergo digestion, achieved by reheating them with steam to 80 - 90^0C. This stage aids in oil extraction by rupturing oil-containing cells in the mesocarp and separating the mesocarp from the nuts. The final stages involve oil extraction, clarification, and purification, where crude oil is extracted from the digested fruit mash using a screw press without damaging the kernel.

Initially, palm bunches are cut into quarters and left overnight to facilitate the separation of nuts from the spikelet. The fruits are then boiled for 1 - 1.5 hours, crushed in a mortar or mashed with feet in a canoe-like container, and water is added and thoroughly mixed. Subsequently, all nuts are meticulously removed by hand. The fibers are vigorously shaken in the sludge until oily foams emerge on the surface. The foam is carefully collected in a container until no more foam formation occurs. The collected foam is subsequently boiled for about 30 to 40 mins. The sludge sinks to the bottom, while the clean edible oil rises to the top. Occasionally, the oil extracted from the sludge pit is reclaimed and blended with fiber to produce a combustible mixture known as a fire starter cake, commonly referred to as flint. The sludge and the liquid waste, which is known as palm oil effluent, are sometimes thrown on the plants and soil around them (Wu *et al.,* 2007).

After extraction, the screw press separates the liquid from the nuts. However, the oil contains varying amounts of water, solids, and impurities that must be eliminated. Fiber particles are removed from the crude oil using a vibrating screen, while sand and dirt settle out. Water removal is accomplished through settling, centrifugation, and vacuum drying processes. The clarified crude oil retains approximately 0.1 - 0.25 % moisture, which aids in oxidative stability and prevents the formation of minute amounts of soluble solids known as gums (Shaaban *et al.,* 2004). The final product, crude palm oil, is either used locally or refined further (Igwe, 2006). Gunawan *et al.* (2009) reported that approximately 22 kg of palm fruit oil and 1.6 kg of palm kernel oil can be extracted from every 100 kg of fruit bunches. However, significant quantities of palm oil residues or pollutants are also generated simultaneously, potentially leading to severe environmental pollution (Hii *et al.,* 2012).

Effect of Palm Oil Mill Effluent on the Soil and River Quality

The cultivation and processing of oil palm, like many other agricultural and industrial activities, also contribute to environmental problems. During oil processing, significant water volumes are utilized within mills for extracting oil from palm fruits. Roughly 50 % of this water is transformed into palm oil mill effluent during extraction. Estimates suggest that for every 1 tonne of crude palm oil produced, approximately 5 - 7.5 tonnes of

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water result in POME (Okwute and Isu (2007). In Nigeria, oil palm processing is a widespread practice among many small-scale operators. In Nigeria's palm oil industry, little or no treatment is done for most of the palm oil mill effluent from small-scale traditional operators, and it is usually disposed off in the environment. Okwute and Isu (2007) in their study reported the potential for the palm oil mill effluent to pollute nearby streams, rivers, or surrounding land. As a result, river water often becomes brownish, emits unpleasant odors, and develops a slimy texture. This phenomenon leads to the death of fishes and other aquatic life, depriving local communities of access to clean water for household needs and fishing (Ezemonye *et al.*, 2008). When discharged untreated into local rivers or lakes, palm oil mill effluent (POME) emerges as a significant contributor to inland water pollution. Comprising of lignocellulosic wastes containing a blend of carbohydrates and oil, POME exhibits remarkably high levels of BOD and COD (Madaki and Lau, 2011). It is not uncommon for COD values to exceed 80,000 mg/L, a figure often associated with palm oil from the nut extracted insufficiently, which can significantly elevate COD levels (Oswal *et al.*,, 2002). The aquatic life is being disrupted by the higher COD value (Maygaonkar *et al.*, 2012).

The application of palm oil mill effluent to soil can yield several advantageous soil chemical and physical enhancements, including augmented levels of organic matter, organic carbon, major nutrients such as nitrogen and phosphorus, as well as improvements in water-holding capacity and porosity (Okwute *et al.*, 2023). However, it also causes undesirable changes such as decreases in pH, and increase 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 recognized as precocious and responsive markers of soil modifications, enabling the prediction of long-term trends in soil quality (Ros *et al.,* 2003). POME is rich in organic content, contains significant quantities of plant nutrients, and serves as a cost-effective source of these nutrients when subjected to fermentation processes (Onyia *et al.*, 2001). The detrimental impact of POME may be attributed to phenols and other acids that are organic in nature and have phytotoxic and anti bacterial properties (Pascual *et al.*, 2007). Over time, the polyphenolic fraction breaks down and partly changes into humic substances. There is limited understanding regarding the influence of POME on soil properties, particularly concerning biochemistry and microbiology. Research indicates that the effects of waste application to soil are predominantly observed during the initial weeks following the amendment (Binder *et al.*, 2002).

The composition and quality of oil mill effluent vary depending on factors such as seasonal changes, raw material standards, and current operational conditions. Typically, palm oil mill wastewater exhibits low pH levels, around 4-5, due to the presence of organic acids generated during fermentation. It additionally holds notably high total solids (40,500 mg/L), as well as oil and grease (4000 mg/L) (Ma, 2000). Moreover, the wastewater comprises dissolved components, such as elevated levels of proteins, carbohydrates, nitrogenous compounds, lipids, and minerals. These substances have the potential to be transformed into valuable materials through microbial activities (Alvionita *et al.,* 2019).

The release of untreated effluents from palm oil mills can present significant environmental concerns (Singh *et al.,* 2010). Therefore, resolving the challenge of converting palm oil mill effluent (POME) into a sustainable waste requires the implementation of efficient treatment and proper disposal methods.

Economic Importance of Palm Oil

Palm fruit oil ranks among the two most significant vegetable oils in the global oil and fats market, second only to soybeans. The oil palm (*Elaeis guineensis*) stands as the most productive oil-producing plant globally, with one hectare yielding between 10 and 35 tonnes of fresh fruit bunch (FFB) annually (Ma *et al.,* 1996). Even though palm trees can thrive for more than 200 years, their economic viability usually lasts between 20 to 25 years. The nursery phase typically spans 11 to 15 months, with the first harvest occurring 32 to 38 months after planting, and peak yield reached 5 to 10 years post-planting (Igwe and Onyegbado, 2007). Harvested fruit bunches yield oil extracted from the fleshy mesocarp, constituting at least 45 – 46 % of the total yield, while the kernel comprises approximately 40-50 %. The nutrient needs of the palm tree fluctuate considerably, mainly dictated by the genetic composition of the planting material and impacted by environmental factors like water availability, sunlight exposure, and temperature (Igwe and Onyegbado, 2007).

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Processes and Microorganisms Involved in Pome Treatment

The biological ponding system has witnessed substantial adoption as a prevalent treatment approach for palm oil mill effluent. Studies suggest that more than 85 % of palm oil mills exclusively utilize the biological ponding system for effluent treatment (Najafpour *et al.,* 2006). The typical components of this system include deoiling ponds, anaerobic ponds, facultative ponds, and aerobic ponds (Mohammad *et al.,* 2021). Successful operation of the ponding system requires extended retention times of over 20 days, with biogas released into the atmosphere. Yacob *et al.* (2006) reported that an average of 36 % methane gas is emitted into the atmosphere from open tank digesters. Similarly, Shirai *et al.* (2003) discovered that methane gas production from open tank digesters and lagoon systems is approximately 35 % and 45 %, respectively. A range of methods of treatment for the wastewater from palm oil mills were examined in order to meet strict rules on discharge into watercourses. According to Ahmad *et al.* (2003), treating the palm oil mill effluent with membrane technology in conjunction with physical-chemical pretreatment resulted in significant reductions in turbidity, COD, and BOD, with reductions of up to 100 %, 98.8 % and 99.4 %, respectively.

The effectiveness of various anaerobic treatment systems has been demonstrated through multiple studies. The two-stage up-flow anaerobic sludge blanket system, as reported by Borja *et al.,* (1996), can handle COD loading rates of up to 30 g COD/L/day, resulting in over 90% methane yield and COD reduction. Similarly, single-stage anaerobic tank digesters and anaerobic ponding systems, according to Ugoji (1997), achieve COD removal efficiencies exceeding 94 % after a 10-day retention time. Research by Borja and Banks (1995) showcases COD removal rates surpassing 90 % in both anaerobic filters and anaerobic fluidized bed reactors with an input rate of 10 g COD/L/day. Najafpour *et al.* (2005) reported COD removal rates of up to 88 % with a hydraulic retention time of 55 hours using attached growth on a rotating biological contactor. Additionally, Oswal *et al.* (2002) achieved a 95 % reduction in COD through treatment with tropical marine yeast within a retention time of 2 days. Anaerobic digestion systems are increasingly utilized in wastewater treatment, particularly within the agro-industry, due to their advantages over aerobic treatments. These benefits include the production of less waste sludge, reduced energy requirements, and simpler restart procedures following extended shutdowns (Beccari *et al.,* 1996). The possibility of producing methane, a by-product of biogas, adds to the method's appeal. Laboratory investigations show that the final result of anaerobic digestion of palm oil mill effluent is a biogas combination with 65 % CH_4, 35 % CO_2 and traces of H_2S, according to Yacob *et al.* (2005). It is anticipated that one tonne of palm oil mill effluent can yield about 28 m^3 of biogas.

Prokaryotes Involved in Pome Degradation

This study reviews two domains of prokaryotic organisms: eubacteria and archaeabacteria, also known as "ancient" bacteria. Both eubacteria and archaeabacteria are unicellular organisms, but archaeabacteria have distinct cellular chemistry. Overall, these prokaryotes play crucial roles in biological wastewater treatment processes. Archaeabacteria groups include halophiles, methanogens, and thermacidophiles (Gerardi, 2006).

Anaerobic Digestion

Suspended particles from dry plant matter and oil palm fruit debris make up the majority of organic components found in raw palm oil mill effluent (POME). The first stage of POME breakdown is anaerobic digestion, which entails removing bulk waste via a number of procedures. Anaerobic digestion is the process by which biodegradable components in wastewater are biologically converted, in the absence of oxygen, into carbon dioxide and methane (CH4) (Lam and Lee, 2011). The procedures encompass the breakdown of carbon compounds through hydrolysis, fermentation, acetate formation, and methane production, facilitated by diverse symbiotic microorganisms. The rich organic composition of POME, including cellulose, lignin, and residual oil, creates favorable conditions for hydrolytic bacteria. These bacteria secrete extracellular enzymes like cellulase, xylanase, and lipase to degrade carbon polymers into simpler compounds, thereby initiating the anaerobic digestion of POME (Hassan *et al.,* 2005). The outcomes of hydrolysis, such as monosaccharides, fatty acids, and amino acids (from triglycerides), act as the materials for acidogenesis or fermentation in the subsequent phase. During fermentation or anaerobic respiration, acidogenic bacteria break down carbohydrates and fatty acids into simpler organic acids, such as lactic, propionic, and butyric acids, along with the production of hydrogen gas (Chong *et al.,* 2009). This is why they are called acidogenic bacteria. The organic acids are then transformed

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into acetate by acetogenic bacteria. Acetogenic bacteria frequently engage in syntrophic relationships with certain methanogens, which utilize hydrogen gas to produce methane. Acetogenic bacteria and methanogens engage in a mutualistic relationship. Methanogens reduce the hydrogen partial pressure, facilitating the oxidation of organic acids into acetate, while relying on acetogenic bacteria to supply hydrogen gas for methanogenesis (Ahmad *et al.,* 2011). Additionally, certain acetogenic bacteria can reduce sulfate and utilize it as an electron acceptor to produce sulfide gas (Wong *et al.,* 2014). Finally, methanogens use the byproducts to produce methane, which completes the transformation of organic matter into biogas (Slonczewski and Foster 2014). Methanogens, classified as archaea, are typically categorized into two groups: acetotrophic and hydrogenotrophic methanogens, distinguished by their respective substrates for methanogenesis (Demirel and Scherer, 2008). Hydrogenotrophic methanogens utilize hydrogen gas as an electron acceptor during methanogenesis. In contrast, acetotrophic methanogens convert acetate into methane (Demirel and Scherer 2008).

Nitrification, Denitrification, and Phosphorus Accumulation

Numerous studies have highlighted the nutrient richness of POME, including nitrogen and phosphorus content (Chowdhury *et al.,* 2007). Important steps in the breakdown of POME include nitrification, denitrification, and phosphorus buildup, which remove phosphorus and inorganic nitrogen from wastewater.

Different nitrifiers carry out the oxidation of ammonium or ammonia to nitrate in two steps. *Nitrosomonas* sp. are the bacteria that catalyze the first step, which is the conversion of ammonia to nitrite. They do this by producing enzymes known as ammonia monooxygenase and hydroxylamine oxidoreductase (Hommes *et al.,* 2001). *Nitrobacter* bacteria are responsible for converting nitrite into nitrate using the enzyme nitrite oxidoreductase (Bartosch *et al.,* 1999). Denitrification occurs as denitrifying organisms reduce nitrate to nitrite, then to nitrogen gas. This process involves the enzyme nitrate reductase and utilizes nitrate or nitrite as an electron acceptor to generate energy, releasing nitrogen gas into the atmosphere (Daum *et al.,* 1998). Meanwhile, phosphorus removal from POME is facilitated by phosphorus-accumulating bacteria, which absorb excess orthophosphate in the wastewater and store it within their cells. The removal of biomass from the wastewater also eliminates the accumulated phosphorus (Bao *et al.,* 2017).

Eukaryotes Involved in Pome Degradation

Fungi, algae, protozoa, and animals (rotifers, worms – nematodes and flatworms) are some of the eukaryotic organisms that take part in POME treatment processes. Soil and water organisms infiltrate wastewater treatment plants via inflow and infiltration pathways (Gerardi, 2006). Fungi or yeast and algae are the two eukaryotic organisms that this review examines. They can be isolated from the POME, which is a liquid waste stream from palm oil mills. By secreting extracellular enzymes, most fungi from the POME can break down lignocellulose and lipids, which are complex polymers. Fungi play a vital role in breaking down lipids, not solely through the action of the enzyme lipase, but also by secreting biosurfactants, as seen in certain species like *Candida* sp. (Kim *et al.,* 1999). These biosurfactants reduce surface tension and interfacial tension between water and lipid phases, aiding in lipid degradation. *Geotrichium candidum*, for instance, can hydrolyze phenols and produce peroxidase enzymes capable of breaking down various color dyes (Coulibaly *et al.,* 2003). Similarly, *Aspergillus fumigatus* demonstrates colour removal from POME, albeit through bioadsorption (Neoh *et al.,* 2012). Additionally, *Chlorella pyrenoidosa* and *Chlorella vulgaris*, two algae species isolated from POME, are involved in nitrogen and phosphorus removal 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 dry biomass (Lam and Lee, 2011).

Anaerobic Digestion Process

Anaerobic digestion emerges as a highly effective treatment approach for palm oil mill effluent (POME). In this process, a diverse community of microorganisms orchestrates a series of complex biochemical reactions to degrade organic matter, resulting in the production of methane and carbon dioxide (Borja *et al.,* 1995). Achieving stability and efficiency in this process relies on various factors, including reactor configurations, hydraulic retention time, organic loading rates, pH levels, temperature, inhibitor concentrations, total volatile fatty acid

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(TVFA) levels, and substrate composition (Fikri *et al.,* 2020). Thorough investigation and meticulous control of these parameters are essential to prevent process failures or reduced efficiency, aiming to maintain them at or close to optimal conditions.

These anaerobic digestions are usually carried out at mesophilic (30 – 37^0C) or thermophilic (50 – 60^0C) temperatures. According to Najafpour *et al.* (2006), the effluent from palm oil milling is released at a high temperature of roughly 90^0C, which prepares the ground for the POME treatment at either mesophilic or thermophilic temperatures. In a semicontinuous anaerobic reactor operating in a mesophilic environment, Cail and Barford (1985) tested palm oil mill effluent, obtaining an approximate 75 % removal rate of chemical oxygen demand (COD) with an organic loading rate (OLR) of 12.6 g[COD]/L/day and a 5.6-day hydraulic retention period.

Similarly, employing a similar reactor design but operating under thermophilic conditions with a maximum OLR of 15.1 g[COD]/L/day and a hydraulic retention period of 4.3 days, Padilla and Banks (1993) achieved an 85 % removal of COD and a methane output of 295 ml/g[COD].

In comparison to running at 37^0C, Yu *et al.* (2002) found that operating at 55^0C resulted in a greater substrate degradation rate, biogas generation rate, and specific rate of aqueous product creation. According to research by De la Rubia *et al.* (2002), a reactor with OLRs of up to 2.19 kg m-3 d-3 COD and an operating temperature of 55^0C produced more gas than one running at 35ºC.

Furthermore, distillery waste digested at anaerobic digestion temperatures of 35 – 55^0C produced the highest amount of methane and total biogas at a digester temperature of 50^0C, according to Banerjee and Biswas, 2004). These results show that, depending on the temperature, anaerobic bacteria can produce more or less methane from organic waste. In actuality, if temperature rises are not controlled, biomass washout may occur, leading to an accumulation of total volatile fatty acids (Lau and Fang, 1997).

Anaerobic microbes help this multi-stage process happen in the absence of oxygen. Having been in use for almost a century, methanogenic anaerobic digestion of organic waste has a number of benefits over aerobic treatment techniques, such as high rates of organic waste removal, low energy needs, less sludge creation, and energy production (Choorit and Wisarnwan, 2007).

Each of the four main phases of anaerobic digestion hydrolysis, fermentation, acetogenesis, and methanogenesis involves a different population of microbes. Hydrolytic bacteria convert polymeric organic molecules into soluble monomers, such as glucose, fatty acids, and amino acids, during the hydrolysis stage (Menzel *et al.*, 2020). This procedure, which is essential for high levels of organic waste, could eventually become rate-limiting. After hydrolyzed products are transformed by acid-forming bacteria into alcohols, aldehydes, ketones, ammonia, carbon dioxide, water, and hydrogen, fermentation takes place. The result is the formation of organic acids such as valeric acid, propionic acid, butyric acid and acetic acid (Zhang *et al.*, 2020). However, methanogens are unable to directly use volatile fatty acids with chains longer than four carbons (Wang *et al.*, 1999).

In the acetogenesis stage that follows, obligatory hydrogen-producing acetogenic bacteria oxidize organic acids to acetic acid and hydrogen. During acetogenesis, carbon dioxide and hydrogen are also used to produce acetate. Acidogenesis and acetogenesis can occasionally coexist in a single stage (Aydin *et al.,* 2017). Lastly, there are two methods leading to the production of acetotrophic species convert acetate to carbon dioxide and methane, and hydrogenotrophic organisms reduce carbon dioxide with hydrogen (Demirel *et al.*, 2008).

Common methanogens found in biogas reactors comprise *Methanobacterium, Methanothermobacter, Methanobrevibacter, Methanosarcina,* and *Methanosaeta* (formerly known as Methanothrix) (Sekiguchi *et al.,* 2001).

Single Phase and Two-Phase Arrangement

Acidification and methanogenesis in the typical anaerobic digestion process take place in a single reactor system, or single-stage arrangement. However, because of their distinct physiologies, nutritional needs, development

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rates, and susceptibilities to environmental stimuli, acidogens and methanogens in such a system are difficult to keep in balance (Demirel and Yenigün, 2002).

Anaerobic Sequencing Batch Reactor (Asbr)

This system was manufactured as a solution for effectively managing effluents with high suspended solids content. Operating within a single reactor, the ASBR follows a four-step cycle (Angenent *et al.,* 2004). Initially, wastewater containing settled biomass is introduced into the reactor during the feeding stage. Subsequently, the wastewater and biomass undergo intermittent mixing during the reaction process. Following this, the biomass settles, and finally, the treated effluent is withdrawn from the reactor (Kannan and Singaram 2012).

According to Ratusznei *et al.* (2000), the ASBR system has a number of benefits, including better retention of solids, effective operational management, high organic matter removal efficiency, ease of use, and the lack of a settling tank.

Up-Flow Anaerobic Sludge Blanket (Uasb)

The Up-flow Anaerobic Sludge Blanket (UASB) reactor is a widely used system for anaerobic wastewater treatment, applied in about 60 % of full-scale anaerobic treatment facilities globally (Angenent *et al*., 2004). In this design, wastewater flows upward through a dense bed of anaerobic sludge granules, where microorganisms break down organic matter and produce biogas.

The system's efficiency largely depends on effective sludge retention. This is achieved through bacterial entrapment within or between sludge particles, as well as bacterial immobilization via natural mechanisms like biofilm formation and microbial aggregation within the sludge matrix (Lettinga, 1995).

MATERIALS AND METHODS

Study Area/Sample Collection

Samples Were Collected Aseptically Using a Calibrated Pipette from the Top, Middle and Bottom Layers (Each 5cm Apart) From Palm Oil Effluents. Sampling Was Conducted at Both Large-Scale (Okomu Oil Palm Company and The Nigerian Institute for Oil Palm Research) And Small-Scale (Ovbiogie, Sapele Road and aduwawa oil Mills) Palm Oil Mill Effluents In Edo State. The Collected Samples Were Then Transported Under Sterile Conditions to The Microbiology Laboratory at The University of Benin, Benin City, For Microbiological Analysis.

Preparation and Sterilization af Culture Media

The preparation of all culture media (Nutrient Agar, Tryptone Soya Agar and Potato Dextrose Agar) adhered strictly to the guidelines provided by the manufacturer. Sterilization procedures were conducted at 121^0C at 15 psi pressure for a duration of 15 mins.

Isolation and Enumeration of Bacteria/Fungi from Samples

A volume of 30.0 ml of the palm oil mill effluent was transferred under sterile conditions into a conical flask containing 270.0 milliliters of sterile distilled water. Subsequently, a tenfold serial dilution was conducted. An aliquot of 0.1 ml from the 10^-3 dilution tube was plated onto Nutrient Agar, Tryptone Soya Agar, and Potato Dextrose Agar (for fungal count). Each sample was inoculated in triplicate. The plates containing nutrient agar and Tryptone Soya Agar were then placed in an incubator at 37^0C for 24 hours, while the Potato Dextrose Agar plates were incubated at 28^0C for up to 5 days. Colony counts were determined for each plate, and the mean for each sample was calculated using the formula described in equation (1) provided by Willey *et al.,* (2008), indicating the mean colony forming unit (cfu) and spore forming units (sfu) per milliliter:

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$$cfu/ml = \frac{number\ of\ colonies\ x\ dilution\ factor}{volume\ of\ inoculum}$$

Morphological and Biochemical Characteristics of Bacteria

Gram stain

Slides were prepared for each isolate by making smears and heat-fixing them on clean, grease-free slides. Crystal violet, the primary stain, was applied to each smear for one minute, followed by rinsing with distilled water. Subsequently, the smears were submerged in iodine solution for approximately one minute. After rinsing the glass slide with distilled water, decolorization was carried out using a 95 % alcohol solution for 30 sec, followed by another wash with distilled water. Counterstaining of the smears on the slides was performed using Safranin solution for one minute. Finally, the slides were rinsed with distilled water, allowed to air dry, and examined under a microscope at 1000x total magnification (Willey *et al.,* 2008).

Motility /Test

Selected isolates were introduced into the medium (Motility Test Medium) using a sterile needle, which was inserted approximately halfway into the medium. The tubes were then left uncovered and subjected to incubation at 37^0C for 18 to 24 hrs. Fuzzy growth extending from the point of inoculation signifies the organism's motility, while growth confined strictly within the stab line signified non motility.

Biochemical Test

Catalase test

The purpose of this test is to determine the presence or absence of the catalase enzyme. Catalase facilitates the decomposition of hydrogen peroxide into oxygen gas and water. A few drops of freshly prepared 3 % hydrogen peroxide were added to the bacterial isolates smeared on a slide. The production of gas bubbles indicates a positive result for the catalase enzyme (Olutiola *et al.,* 1991).

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Oxidase Test

A diluted 1% solution of oxidase reagent, prepared according to standard protocols, was utilized. A small amount of culture obtained from Nutrient Agar plate using a sterilized platinum wire loop was smeared onto a moistened filter paper with an oxidase reagent. The appearance of a purple coloration indicates a positive result for the oxidase test (Cheesbrough, 2006).

Coagulase Test

Numerous microorganisms, including *Staphylococcus aureus*, produce an enzyme known as coagulase. This enzyme facilitates blood clotting by converting fibrinogen into fibrin. In the slide test method, a clean slide was divided into two sections. On one section, a small amount of the test organism was emulsified in a drop of water using a sterile wire loop; the other section contained only water and served as a negative control. A drop of human plasma was added to both sections, and the slide was gently rocked for a few minutes. Agglutination (clumping) observed only on the section with the test organism indicates a positive result for coagulase production. The control section showed no agglutination, confirming the validity of the test.

Urease test

The isolates were introduced into slants of urea medium and placed in an upright position, then incubated at 37^0C for 24-48 hours. Cultures testing positive for urease produced a red-pink coloration due to alterations in the indicator's colour (Cheesbrough, 2005).

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$NH_2\ CO.NH_2 + H_2O \rightarrow 2NH_3 + CO_2$

Indole Test

The indole test was conducted to determine the isolates capable of converting tryptophan to indole. This test is commonly employed to aid in distinguishing Gram-negative bacilli, particularly those belonging to the *Enterobacteriaceae* family. Peptone water was prepared, and approximately 3 ml of it was dispensed into Bijou tubes using a sterile pipette. Sterile loops were used to collect a well-isolated colony of bacteria, which was then inoculated into the Bijou tubes. Subsequently, the tubes were incubated at 37^0C for 48 hours. Following the incubation period, 0.5 ml of Kovac's Indole Reagent was added to each inoculated Bijou tube. The tubes were gently shaken and observed for the appearance of a red coloration in the surface layer within 10 mins. (Chessbrough, 2006). A red ring on the top of the tube indicated a positive indole reaction.

Citrate Utilization Test

The characteristic use of citrate as the only source of carbon, by some organisms, forms the basis of this test. The process involved the inoculation of the test organism in a medium which contained Simon's citrate, in a test tube. The incubation temperature was set at 37^0C for 24 to 48hours. The presence of a deep blue colour after incubation indicated a positive result (Chessbrough, 2006).

Sugar Fermentation Test

The isolates in the test medium were tested whether they could, alongside the production of acid or gas or only acid, ferment a sugar molecule. This test is based on the fact that most bacteria especially those of the Gram-negative strain, use a variety of sugar as carbon sources and energy, and are also able to produce either acid and gas or acid. Basically, this test serves as functionality test that helps to distinguish one bacteria strain from another. Peptone water prepared in conical flask containing the indicator phenol red, was the growth medium utilized in this study. Specialized tubes called Durhams tubes for the mixture were sterilized by an autoclave for about 15 mins and at a temperature of 121^0C. After preparing and sterilizing a 1 % sugar solution at 121^0C for approximately 10 mins, 5 ml of the solution was aseptically dispensed into tubes containing peptone solution and indicators (phenol red). Subsequently, the tubes were inoculated with a young culture (fresh bacterial culture) of the isolated organism and then incubated at approximately 37^0C. After a 24-hour incubation period, the production of acid and gas, or only acid, was observed. A change in the colour of the medium from light green to yellow indicated acid production, while the presence of gas in the Durham tube indicated gas production (Chessbrough, 2006).

Identification of Fungal Isolates

The identification of fungal isolates was carried out using the Lactophenol Cotton Blue (LPCB) staining technique and microscopic examination. A clean glass slide was prepared by placing a drop of lactophenol cotton blue stain in the center using a sterilized needle or dropper. A small portion of mycelium was carefully taken from the fungal culture using the same sterilized needle and transferred into the drop of stain on the slide.

Using the needle, the mycelium was gently spread out in the stain to ensure even distribution. A cover slip was then placed over the preparation, applying light pressure to eliminate air bubbles and secure the sample for viewing.

The slide was examined under a compound microscope, starting with the x10 objective lens for general orientation and then under the x40 objective lens for detailed observation.

Observation: Under The Microscope, Fungal Structures Such as Hyphae, Conidiophores, Spores and Other Morphological Features Were Observed. These Structures Were Compared with Standard Mycological References to Identify the Fungal Species Based On Their Shape, Arrangement and Reproductive Structures.

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Molecular Identification

DNA Extraction

DNA was extracted using the protocol described by (Sambrook and Russell, 2011). Briefly, single colonies grown on medium were transferred to 1.5ml of liquid medium, and cultures were grown on a shaker for 48 hours at 28^0C. After this period, cultures were centrifuged at 4600g for 5 mins. The resulting pellets were resuspended in 520 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37^0C, followed by the addition of 100 µl of 5 M NaCl and 80µl of a 10 % CTAB solution in 0.7 M NaCl, and vortexed. The suspension was incubated for 10 mins at 65^0C and kept on ice for 15 mins. An equal volume of chloroform:isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 mins and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube, and isopropanol (1:0.6) was added to precipitate the DNA at $–20^0$C for 16 hours. DNA was collected by centrifugation at 13000g for 10 mins, washed with 500µl of 70 % ethanol, air-dried at room temperature for approximately three hours, and finally dissolved in 50 µl of TE buffer

Polymerase Chain Reaction (PCR)

The PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM 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 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water, plus 8 µl of DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystems Inc., USA) with the following profile: initial denaturation at 94^0C for 5 mins; followed by 30 cycles consisting of 94^0C for 30 sec, 50°C for 60 sec and 72^0C for 1 min 30 sec; and a final termination at 72^0C for 10 mins, then held at 4^0C.

Gel Electrophoresis

The integrity of the amplified approximately 1.5 Mb gene fragment was checked on a 1 % agarose gel to confirm amplification. The buffer (1xTAE buffer) was prepared and subsequently used to prepare a 1.5 % agarose gel. The suspension was boiled in a microwave for 5 mins. The molten agarose was allowed to cool to 60^0C and stained with 3 µl 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 mins to form the wells.

The 1×TAE buffer was poured into the gel tank to barely submerge the gel. Two microliters of 10x blue gel loading dye were added to 4 µl 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 mins 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 run alongside experimental samples in the gel.

Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol-purified to remove the PCR reagents. Briefly, 7.6 µl of 3M Na acetate and 240 µl of 95 % ethanol were added to each approximately 40 µl PCR amplified product in a new sterile 1.5ml Eppendorf tube, mixed thoroughly by vortexing, and kept at $–20^0$C for at least 30 mins. Centrifugation for 10 mins at 13000g and 4^0C followed, with removal of the supernatant.

The pellet was washed by adding 150 µl of 70 % ethanol, mixed, then centrifuged for 15 mins at 7500g and 4^0C. Again, the supernatant was removed, and the tube was inverted on paper tissue to let it dry in the fume hood at room temperature for 10–15 mins. The pellet was then resuspended with 20 µl of sterile distilled water and kept at $–20^0$C prior to sequencing. The purified fragment was checked on a 1.5 % agarose gel run at 110V for about 1 hour, as previously described, to confirm the presence of the purified product and quantified using a nanodrop of 2000 spectrophotometer (Thermo Scientific).

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Sequencing Identification

All PCR products were purified with Exo sap and sent to Epoch Life science (USA) for Sanger sequencing. Sequencing were identified using Gen Bank's Basic Local Alignment Search Tool (BLAST) algorithm on National Centre for Biotechnology and Information website. The corresponding sequences were identified using the online blast search at (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Highly corresponding sequence were recovered from NCBI and subjected to multiple sequence alignment using Bio edit software.

Blood Hemolysin Production

This qualitative screening method was employed to preliminarily assess the potential for biosurfactant production by the organisms, utilizing blood agar as the culture medium. In this study, spot-inoculation of single colonies of isolates was performed on agar plates containing blood, followed by incubation for approximately 48 hours at 37^0C.

Following the incubation period, clear zones indicating the rupture of blood cells (hemolysis) were observed around the colonies. Absence of hemolysis indicated gamma hemolysis, while partial hemolysis indicated alpha hemolysis, and complete hemolysis indicated beta hemolysis of the blood culture medium (Satpute *et al.,* 2010).

The forms of hemolysis were differentiated based on the appearance of zones surrounding the colonies on blood agar plates after 48 hours of incubation at 37^0C:

- Beta (β) hemolysis: Characterized by a clear, transparent zone around the colony, indicating complete lysis of red blood cells.
- Alpha (α) hemolysis: Identified by a greenish or brownish discolouration around the colony, due to partial lysis and oxidation of hemoglobin.
- Gamma (γ) hemolysis: Indicated by no change in the medium around the colony, suggesting no hemolytic activity.

This visual assessment was done under normal lighting conditions on the incubated blood agar plates.

Screening of Biosurfactant Producing Bacteria

The various source materials for inoculation were standardized to an optical density of OD_{600} (= 1.0) and derived from selected bacterial cultures in nutrient broth. Approximately 5ml of the test bacterial cultures were transferred into 100 ml of a nutritionally rich solution composed of 2 % (w/v) glucose in MSM in 500 ml Erlenmeyer flasks. This solution served as the carbon source and was then incubated at 35^0C with shaking at 200 rpm.

Assay for Biosurfactant Activity Via Oil Displacement/ Spread Assay

This step involved introducing the surfactant-containing medium (cell culture supernatant) to the interface between oil and water. The test involved introducing a cell-free culture supernatant obtained by centrifuging microbial cultures grown in production medium at 8,000 rpm for 10 mins onto the interface of oil and water. The purpose was to observe the diameter of the clear zone formed as a result.

This was accomplished by adding 100 µl of kerosene to the surface of a Petri dish containing 15 ml of distilled water. Subsequently, the oil surface was inoculated with 20 µl of cell culture supernatant. The presence of an emulsified clear zone around the colonies served as a positive indication of biosurfactant production (Satpute *et al.,* 2010).

Data Analysis

Results were presented as mean of 3 replications. Analysis of variance were determined using the 1-way ANOVA. Duncan's multiple range tests were employed to assess mean differences ($P<0.05$) (Ogbeibu, 2014).

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RESULTS

Table 4.1 shows the total heterotrophic bacterial counts (THBC) observed in the POME. The THBC of POME derived from NIFOR ranged from 4.10 ± 1.39 to 4.34 ± 0.32 (Log10 cfu/ml). Similarly, bacterial counts of POME samples from Okomu Oil spanned from 4.06 ± 0.88 to 4.66 ± 0.83 (Log10 cfu/ml), whereas POME from Aduwawa exhibited total bacterial counts varying between 4.02 ± 1.00 and 4.52 ± 1.34 (Log10 cfu/ml). Moreover, Ovbiogie samples exhibited a range of 3.93 ± 1.09 to 4.33 ± 1.02 (Log10 cfu/ml), while Sapele Road samples ranged from 3.90 ± 1.49 to 4.18 ± 0.88 (Log10 cfu/ml). Noteworthy is that POME samples from Okomo Oil displayed the highest heterotrophic bacterial count (4.66 ± 0.83 Log10 cfu/ml), while those from Ovbiogie exhibited the lowest heterotrophic bacterial count (3.93 ± 1.09 Log10 cfu/ml). Statistical analysis revealed significant differences in bacterial density ($P < 0.05$) among the top, middle, and bottom POME samples collected from different locations.

The heterotrophic fungal count (THFC) of Palm Oil Mill Effluents is shown in Table 4.2. The heterotrophic fungal count of Palm Oil Mill Effluents (POME) from Aduwawa ranged from 4.34 ± 0.76 and 3.93 ± 1.70 Log_{10} sfu/ml, fungal counts of POME samples from NIFOR ranged 4.11 ± 1.57 to 4.29 ± 0.24, POME samples from Okomu oil ranged from 3.85 ± 0.23 to 4.26 ± 0.62 Log_{10} sfu/ml, while Sapele road samples ranged from 3.78 ± 0.58 to 4.08 ± 0.58 Log_{10} sfu/ml, Ovbiogie samples ranged from 3.74 ± 0.00 to 3.98 ± 0.26 Log_{10} sfu/ml. POME samples from Aduwawa had both the highest and least heterotrophic fungal count of 4.34 ± 0.76 and 3.93 ± 1.70 Log_{10} sfu/ml respectively. Top POME samples from NIFOR, Okomu Oil, Aduwawa and Sapele Road were not different significantly, while middle and bottom of POME samples from NIFOR, Aduwawa and Sapele Road were different significantly ($P<0.05$) from Okomu Oil and Ovbiogie POME samples.

Table 4.1: The Heterotrophic bacterial counts (THBC) of Palm Oil Mill Effluents

Sample depths	NIFOR (Log_{10} Cfu/ml)	Okomu Oil (Log_{10} Cfu/ml)	Aduwawa (Log_{10} Cfu/ml)	Ovbiogie (Log_{10} Cfu/ml)	Sapele Road (Log_{10} Cfu/ml)
Top	4.34 ± 0.32^{a}	4.62 ± 0.79^{a}	4.51 ± 0.71^{a}	4.33 ± 1.02^{a}	4.18 ± 0.88^{a}
Middle	4.28 ± 0.51^{a}	4.66 ± 0.83^{b}	4.52 ± 1.34^{b}	4.31 ± 1.10^{b}	3.95 ± 1.16^{b}
Bottom	4.10 ± 1.39^{b}	4.06 ± 0.88^{b}	4.02 ± 1.00^{b}	3.93 ± 1.09^{b}	3.90 ± 1.49^{b}

Key: NIFOR: Nigeria Institute for Oil Palm Research. Values are presented as mean ± SEM; n=3. Mean values with similar superscripts within a column across sampling depths are not significantly different, $P>0.05$.

Table 4.2: The Heterotrophic Fungal count (THFC) of Palm Oil Mill Effluents

Sample depths	NIFOR (Log_{10} sfu/ml)	Okomu Oil (Log_{10} sfu/ml)	Aduwawa (Log_{10} sfu/ml)	Ovbiogie (Log_{10} sfu/ml)	Sapele Road (Log_{10} sfu/ml)
Top	4.29 ± 0.24^{a}	4.26 ± 0.62^{a}	4.34 ± 0.76^{a}	3.98 ± 0.26^{b}	4.08 ± 0.58^{a}
Middle	4.23 ± 0.55^{a}	3.98 ± 0.90^{b}	4.23 ± 1.43^{a}	3.93 ± 0.33^{b}	4.02 ± 0.33^{a}
Bottom	4.11 ± 1.57^{a}	3.85 ± 0.23^{b}	3.93 ± 1.70^{a}	3.74 ± 0.00^{b}	3.78 ± 0.58^{a}

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Key: NIFOR: Nigeria Institute for Oil Palm Research, Values are presented as mean ± SEM; n=3. Mean values with similar superscripts within a column across sampling depths are not significantly different, P>0.05.

Table 4.3 shows the percentage bacterial and fungi reduction in small scale enterprise across the various depths in each location. Percentage bacterial reduction of samples from Sapele Road was 72.29 %, Aduwawa was 67.69 % and Ovbiogie was 60.47 %. Percentage fungal reduction of samples from Sapele Road was 61.36 %, Aduwawa was 42.11 % and Ovbiogie was 50.0 %. Samples from Sapele Road had highest bacterial and fungal reduction (72.29 % and 61.36 %) respectively while samples from Ovbiogie and Aduwawa respectively had least bacterial (60.47 %) and fungal (42.11 %) reduction respectively.

The percentage bacterial and fungi reduction in large scale enterprise is shown in Table 4.4. Percentage bacterial reduction of samples from Sapele Road and Okomo oil were 43.18 % and 72.29 % respectively while percentage fungal reduction of samples from Sapele Road and Okomo oil were 33.33 % and 61.11 % respectively with Okomu oil samples having the highest bacterial (72.29 %) and fungal reduction (61.11 %) and NIFOR recorded the least bacterial (43.18 %) and fungal reduction (33.33 %).

Table 4.5 shows the molecular identification of bacteriaL isolates obtained from palm oil mill effluents. Bacterial identified were *Bacillus cereus, Pseudomonas aeruginosa, Bacillus amyloliqefaciens, Escherichia coli* and *Klebsiella aerogenes.*

The distribution of bacterial isolates in large scale palm oil enterprise (LSE) is shown in Table 4.6. *Pseudomonas aeruginosa, Bacillus amyloliqefaciens, Bacillus cereus, Escherichia coli* and *Klebsiella aerogenes* were present in Okomu Oil (top) samples**.** *Pseudomonas aeruginosa* and *Bacillus amyloliqefaciens* were absent in NIFOR bottom, Okomu middle and Okomu bottom samples.

Table 4.7 shows the distribution of bacterial isolates in small and medium scale palm oil enterprise (SME). *Bacillus amyloliqefaciens, Escherichia coli* and *Klebsiella aerogenes* were absent in in Aduwawa and Ovbiogie (middle and bottom), Sapele Road (bottom) samples while *Bacillus amyloliqefaciens* was present in Aduwawa (bottom) and Ovbiogie (top and bottom) samples**.**

Table 4.3: Percentage bacterial and fungi reduction in small scale enterprise

Sample source	Bacteria (%)	Fungal (%)
Sapele Road	72.29	61.36
Aduwawa	67.69	42.11
Ovbiogie	60.47	50.0

The values above represent the average percentage reduction in bacterial and fungal counts from Palm Oil Mill Effluent (POME), calculated across three sampling depths (top, middle and bottom) in small and medium scale enterprises.

Table 4.4: Percentage bacterial and fungi reduction in large scale enterprise

Sample source	Bacteria (%)	Fungal (%)
NIFOR	43.18	33.33
Okomu	72.29	61.11

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The values above represent the average percentage reduction in bacterial and fungal counts from Palm Oil Mill Effluent (POME), calculated across three sampling depths (top, middle and bottom) in large scale enterprises.

Table 4.5: Molecular identification of bacterial isolates obtained from Palm Oil Mill Effluents

Sample Code	Bacterial identity	Query cover (%)	Percent identity (%)	Accession No.
LSE01	Bacillus cereus	99.0	99.80	CP053954.1
SME02	Pseudomonas aeruginosa	100.00	100.00	MK875779.1
SME03	Bacillus amyloliqefaciens	99.00	99.93	CP054415.1
LSE04	Escherichia coli	100.00	100.00	MK371829.1
LSE06	Klebsiella aerogenes	100.00	100.00	CP048598.1

Key:

LSE01: Bacillus cereus

SME02: Pseudomonas aeruginosa

SME03: Bacillus amyloliqefaciens

LSE04: Escherichia coli

LSE06: Klebsiella aerogenes

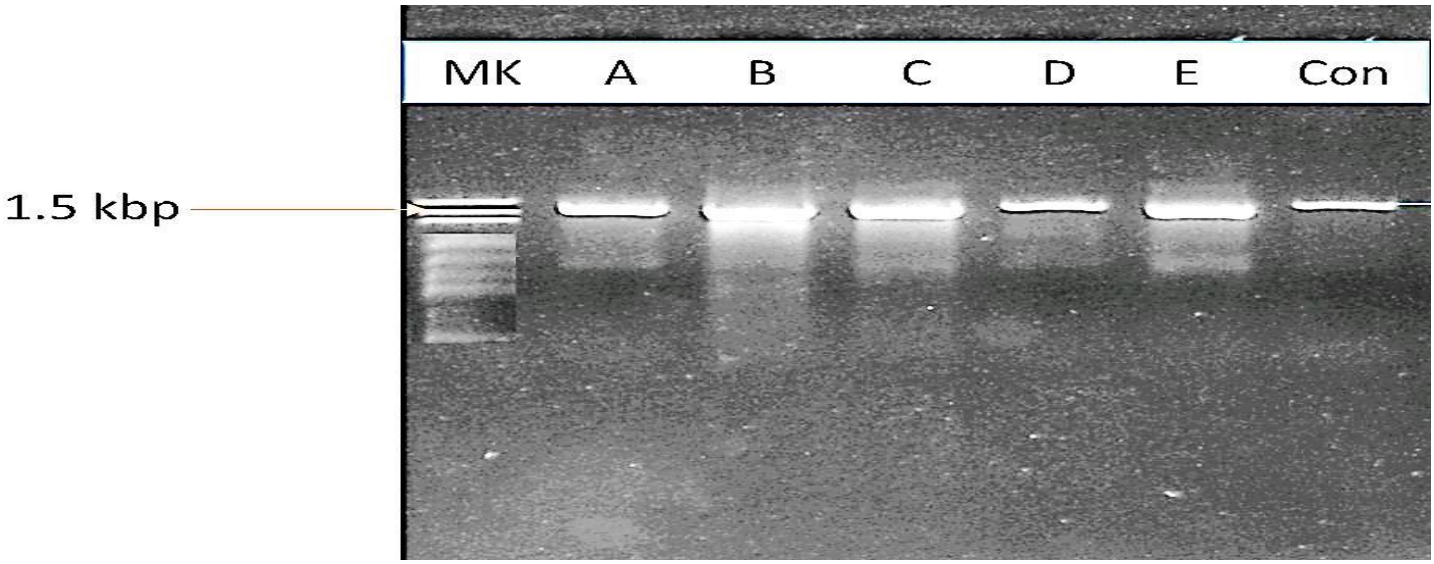

Plate 4.1 Agarose gel of 16s rRNA bacterial amplification of bacterial obtained from Palm Oil Effluents. Bands A, B, C, D and E indicates the genomic DNAs of Bacillus cereus, Pseudomonas aeruginosa, Bacillus amyloliqefaciens, Escherichia coli and Klebsiella aerogenes. "Con" indicates the control lane.

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Table 4.6: Distribution of bacterial isolates in large Scale Palm Oil Enterprise (LSE)

Sample depths	*Pseudomonas* sp.	*Bacillus amyloliqefaciens*	*Bacillus cereus*	*Klebsiella* sp.	*Escherichia* sp.
LSE 1 (NIFOR) Top	+	+	+	-	+
LSE 1(NIFOR) Middle	+	-	-	+	-
LSE 1 (NIFOR) Bottom	-	-	+	-	-
LSE 2 (OKOMU) Top	+	+	+	+	+
LSE 2 (OKOMU) Middle	-	-	-	+	+
LSE 2 (OKOMU) Bottom	-	-	+	-	-

Keys: + = present, - = absent

Top, middle and bottom indicates point of sample collection in the palm oil mill effluent

LSE 1 and LSE 2 represent the sampling depths.

Table 4.7: Distribution of bacterial isolates in Small and Medium Scale Palm Oil Enterprise (SME)

Sample depths	*Pseudomonas* sp.	*Bacillus amyloliqefaciens*	*Bacillus cereus*	*Klebsiella* sp.	*Escherichia* sp.
SME 1 (Aduwawa) Top	-	-	+	+	-
SME 1 (Aduwawa) Middle	+	-	-	-	-
SME 1 (Aduwawa) Bottom	-	+	-	-	-
SME 2 (Ovbiogie) Top	+	+	-	-	+
SME 2 (Ovbiogie) Middle	-	-	-	-	-
SME 2 (Ovbiogie) Bottom	-	+	-	-	-
SME 3 (Sapele Rd) Top	-	-	-	+	-
SME 3 (Sapele Rd) Middle	-	-	+	+	-
SME 3 (Sapele Rd) Bottom	-	-	-	-	-

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Key: + = present, - = absent

Top, middle and bottom indicates point of sample collection in the effluent

SME 1, SME 2 and SME 3 represent the sampling depths.

The distribution of fungal isolates in large scale palm oil enterprise (LSE) is shown in Table 4.8. *Penicillium chrysogenum* and *Penicillium citrinum* were present in NIFOR (Bottom) samples and absent in NIFOR (Middle), Okomu (Top and Middle) samples. *Aspergillus niger*, *Fusarium solani, Penicillium chrysogenum, Microsporum* sp.*, Penicillium citrinum* and *Aspergillus flavus* were absent in NIFOR (Middle) POME samples.

Table 4.9 shows the distribution of fungal isolates in small and medium scale palm oil enterprise (SME). *Aspergillus niger*, *Fusarium solani, Penicillium chrysogenum* and *Microsporum* sp.*,* were absent in Aduwawa (Bottom), Ovbiogie (Middle and Bottom) POME samples.

Table 4.10 shows the zones of oil spreading assay of biosurfactant producing microorganisms from palm oil effluents. *Pseudomonas aeruginosa, Bacillus amyloliqefaciens, Bacillus cereus, Aspergillus niger, Fusarium solani* and *Penicillium chrysogenum* produced positive Biosurfactant and Hemolytic activity at different zone of inhibitions (12.00mm, 15.00mm, 16.00mm, 14.00mm, 10.00mm and 7.00mm respectively) while *Escherichia coli* and *Microsporum* sp. showed negative Biosurfactant Production at 0.00mm zone of inhibition.

Table 4.11 shows the emulsification activity of biosurfactant-producing microorganisms isolated from Palm Oil Mill Effluents. The microorganisms were assessed for their emulsification ability at 610 nm, with results expressed as average values with standard deviations. *Bacillus amyloliquefaciens* exhibited the highest emulsification activity with a value of 1.01 ± 2.30, indicating strong biosurfactant production and emulsification capacity. *Bacillus cereus* followed with an emulsification activity of 0.91 ± 0.33, reflecting its comparable potential in biosurfactant production. *Pseudomonas aeruginosa* showed a slightly lower emulsification activity (0.644 ± 1.22), suggesting moderate emulsification potential. *Klebsiella aerogenes* and *Escherichia coli* exhibited the lowest emulsification activities, with values of 0.41 ± 0.50 and 0.39 ± 1.33, respectively.

Table 4.8: Distribution of fungal isolates in Large Scale Oil Enterprise (LSE)

Sample depths	***Aspergillus niger***	***Fusarium solani***	***Penicillium chrysogenum***	***Microsporum sp***	***Penicillium citrinum***	***Aspergillus flavus***
LSE 1 (NIFOR) Top	+	-	+	-	-	-
LSE 1 (NIFOR) Middle	-	-	-	-	-	-
LSE 1 (NIFOR) Bottom	-	-	+	-	+	-
LSE 2 (Okomu) Top	+	+	-	+	-	-
LSE 2 (Okomu) Middle	-	-	-	-	-	+
LSE 2 (Okomu) Bottom	-	-	+	-	+	-

Key: + = present, - = absent

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Top, middle and bottom indicates point of sample collection in the effluent

LSE 1 and LSE 2 represents the sampling depths.

Table 4.9: Distribution of fungal isolates in Small and Medium Scale Enterprise (SME)

Sample depths	*Aspergillus niger*	*Fusarium solani*	*Penicillium chrysogenum*	*Microsporum sp*	*Penicillium citrinum*	*Aspergillus flavus*
SME 1 (Aduwawa) Top	-	-	+	-	-	+
SME 1 (Aduwawa) Middle	-	-	-	+	-	-
SME 1 (Aduwawa) Bottom	-	-	-	-	+	-
SME 2 (Ovbiogie) Top	+	+	-	-	-	-
SME 2 (Ovbiogie) Middle	-	-	-	-	+	-
SME 2 (Ovbiogie) Bottom	-	-	-	-	-	-
SME 3 (Sapele Rd) Top	-	-	-	+	-	+
SME 3 (Sapele Rd) Middle	-	-	+	-	-	-
SME 3 (Sapele Rd) Bottom	-	+	-	-	-	-

Keys: + = present, - = absent

Top, middle and bottom indicates point of sample collection in the effluent

SME 1, SME 2 and SME 3 represents the sampling depths.

Table 4.10: Zones of Oil Spreading Assay of biosurfactant producing microorganisms from palm oil effluents

Isolates	Zones (mm)	Biosurfactant Production	Hemolytic activity
Pseudomonas aeruginosa	12.00	+	+
Bacillus amyloliqefaciens	15.00	+	+
Klebsiella aerogenes	7.00	+	-
Escherichia coli	0.00	-	+
Bacillus cereus	16.00	+	+
Aspergillus niger	14.00	+	+
Fusarium solani	10.00	+	+
Penicillium chrysogenum	7.00	+	+
Microsporum sp.	0.00	-	+

Keys: + = positive, - = negative

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Table 4.11: Emulsification activity of the microorganisms that produces biosurfactants obtained from Palm Oil Mill Effluents

ISOLATES	Emulsification activity at 610nm
Pseudomonas aeruginosa	0.644±1.22
Bacillus amyloliqefaciens	1.01±2.30
Bacillus cereus	0.91±0.33
Klebsiella aerogenes	0.41±0.50
Escherichia coli	0.39±1.33

Values are presented as mean ± SEM; n=3.

DISCUSSION

The management and disposal of wastewater, particularly from palm oil mills, pose significant environmental challenges for many villages reliant on small-scale palm oil production. While the practice of disposing untreated palm oil mill waste has been ongoing in these communities for years, heightened scrutiny from environmental regulatory bodies has brought attention to its adverse effects. The current study focused on identifying biosurfactant-producing microorganisms in palm oil mill effluents which revealed levels of both bacterial and fungal populations. Specifically, samples from Okomu oil exhibited the highest count of heterotrophic bacteria (4.66±0.83 Log10 cfu/ml). This finding aligns with previous research by Eno *et al.* (2017), who documented similarly high levels of total heterotrophic bacteria in palm oil mill effluents. Additionally, studies by Ibe *et al.* (2014), Ohimain *et al.* (2013) and Ohimain *et al.* (2012) have reported comparable microbial counts in POME samples, further supporting the findings of this investigation.

The elevated fungal count observed in the samples of POME utilized in this investigation suggests a stimulative effect of POME on fungal proliferation. This is consistent with prior research indicating that POME harbors metabolizable nutrients conducive to fungal growth (Nwago and Okolo, 2011). The variability in microbial populations detected in our study may stem from various factors such as composition of nutrient, mineral content, oxygen, temperature, acidity and the wastewater volume (Jeremiah *et al.,* 2018). The abundance of bacteria in POME could be attributed to potential contamination resulting from inadequate sanitation practices within the mills (Okechalu *et al.,* 2011), as well as the processing methods and environmental conditions prevailing in these facilities. As noted by Ohimain *et al.* (2013), POME serves as a favorable habitation for lipolytic and cellulolytic bacteria and fungi due to its nutrient-rich composition, including lipids and cellulose. The isolation of *Aspergillus niger* and *Aspergillus flavus* from POME samples suggests their potential involvement in the biodegradation of oily wastewaters, as previously reported in literature. However, further studies involving biodegradation assays are necessary to confirm their functional role. Additionally, the identification of *Penicillium chrysogenum* and *Penicillium citrinum* in POME aligns with previous research findings (Jeremiah *et al.*, 2018). Similarly, the isolation of *Fusarium solani* and *Microsporum* sp. in POME samples is consistent with the findings of Obire *et al.* (2011), who also reported the presence of these fungal species in POME.

The distribution of biosurfactant-producing microorganisms within effluent systems presents significant implications for both environmental management and microbial ecology. Observations indicate that these microorganisms are more concentrated at the top of the effluent compared to the bottom, a phenomenon that can be attributed to several interrelated factors (Wu *et al.,* 2007). One primary reason for this stratification is the differential availability of nutrients and oxygen in various layers of the effluent. The upper layers typically receive more sunlight and oxygen diffusion, creating a more favourable environment for aerobic biosurfactant-producing microorganisms, which thrive in such conditions (Wu *et al.,* 2007). Additionally, hydrodynamic factors play a crucial role in microbial distribution within effluents (Alvionita *et al.,* 2019). The agitation caused by inflow currents and turbulence tends to suspend lighter particles and microorganisms at higher levels, leading to an increased concentration of active biosurfactant producers near the surface. This hydrodynamic behavior

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can foster competitive advantages for these microorganisms as they exploit available organic substrates more efficiently than their counterparts residing deeper within the effluent column (Borja *et al.,* 1995).

Furthermore, the metabolic activity of biosurfactant-producing microbes contributes to their own proliferation at higher concentrations. As these organisms metabolize substrates and produce surfactants, they alter local microenvironments, enhancing their growth potential relative to other microbial populations that may not share such capabilities (Bajaj and Annapure, 2015). Consequently, this self-enhancing feedback loop further consolidates their presence at elevated levels within the effluent system.

The molecular identification of bacterial isolates obtained from palm oil effluents revealed the presence of various species, including *Bacillus cereus, Pseudomonas aeruginosa, Bacillus amyloliqefaciens, Klebsiella aerogenes* and *Escherichia coli*. Fungi like *Aspergillus niger*, *Fusarium solani, Penicillium chrysogenum, Microsporum* sp, *Penicillium citrinum* and *Aspergillus flavus* were also isolated from these samples. The presence of *Bacillus* species, including *Bacillus cereus*, in POME samples may suggest their adaptability to diverse environments, including those with high organic or lipid content (Imo and Ihejirika, 2021). While studies by Bala *et al.* (2018) and Mukesh *et al.* (2012) have reported the lipolytic abilities of *Bacillus* species, further assays would be necessary to confirm such activity under the conditions of this study.

Similarly, the isolation of bacteria like *Bacillus species*, *Pseudomonas* species*, Klebsiella* species*, Escherichia coli* and fungi including *Aspergillus niger, Fusarium solani, Penicillium chrysogenum, Microsporum* sp., *Penicillium citrinum* and *Aspergillus flavus* in POME samples is consistent with studies conducted by Jeremiah *et al.* (2014), Ohimain *et al.* (2012), and Okechalu *et al.* (2011). *Bacillus* spp were frequently isolated from effluent samples across different scales of palm oil enterprises, while *Penicillium chrysogenum* exhibited the highest frequency among fungal isolates. This aligns with previous findings by Ohimain *et al.* (2012) and Ibegbulam and Achi (2014), which reported similar occurrences of *Bacillus, Penicillium,* and *Aspergillus* species in palm oil effluent samples.

Furthermore, the study revealed that the isolated bacteria and fungi possess the capability to produce biosurfactants, as evidenced by inhibition zone assays. This finding is in corroboration by Kanokrat *et al.* (2013), who illustrated the biosurfactant-producing abilities of certain bacteria, including *Pseudomonas* sp. isolated from palm oil-contaminated soils. Biosurfactants plays a very significant role in reducing surface tensions and facilitating the desorption of POME pollutants from soil, as noted by Bustamante *et al.* (2012).

The presence of clear zones surrounding colonies on blood agar indicates the potential production of biosurfactants by selected isolates, as noted by Popoola *et al.* (2023). Previous studies, such as those of Rajni *et al.* (2016), have confirmed the ability to isolate fungal and bacterial strains capable of biosurfactant production using similar methodologies.

Pseudomonas aeruginosa, as highlighted by Okwute and Ijah (2014), is naturally associated with the degradation of palm oil and its derivatives, likely due to its capacity to metabolize oil as a carbon source. This species, along with other *Pseudomonas* strains, possess the ability for utilizing hydrogen as sources of carbon and energy, potentially leading to biosurfactant production Okwute *et al.* (2014).

The existence of *Klebsiella aerogenes* in POME samples is not unexpected, given its common occurrence in various environmental sources such as soil, water, and animals. Contamination during oil extraction processes could introduce *Klebsiella aerogenes* into POME samples.

This research sheds light on the pathogenic potential of bacterial isolates derived from palm oil mill effluents. The virulence of pathogenic bacteria, such as *Pseudomonas aeruginosa*, *Bacillus amyloliquefaciens*, *Escherichia coli* and *Bacillus cereus*, as well as fungal isolates including *Aspergillus niger*, *Fusarium solani*, *Penicillium chrysogenum*, and *Microsporum* sp. is often attributed to their hemolytic activity (Oliveira *et al.,* (2019). This activity contributes to the pathogens' ability to overcome host defense mechanisms, facilitating colonization and the establishment of infection (Oliveira *et al.,* (2019).

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Escherichia coli, known as one of the most prevalent human pathogens, exhibited hemolytic activity in this study. Its potential to cause various infections, from mild to severe, including food poisoning, underscores the significance of understanding its pathogenic traits, such as its ability to produce hemolysin, as emphasized by Sora *et al.* (2021).

The emulsification assay served as an indirect method for screening biosurfactant activity. In this study, *Bacillus amyloliquefaciens* exhibited the highest emulsification potential ($1.01 \pm 2.30\%$). Previous studies have demonstrated the emulsifying capabilities of various microorganisms, especially bacteria, in the treatment of palm oil mill effluent (POME), as reported by Sebiomo *et al.* (2011).

Similarly, hemolytic assay methods, as reported by Kawo *et al.* (2018) and Li *et al.* (2019), were utilized to detect clear zones on blood agar plates, indicating biosurfactant production by *Bacillus cereus* and *Aspergillus niger*, respectively. In contrast, *Escherichia coli* and *Microsporum* sp. did not exhibit biosurfactant-producing capabilities in this study. However, Muneer *et al.,* (2014) reported *Microsporum* sp. as a biosurfactant-producing fungus, which contrasts with the findings observed in this study. It is essential that biosurfactants be able to emulsify POME for the uptake and assimilation of hydrocarbons, suggesting that the isolates investigated in this study hold promise for hydrocarbon degradation and potentially serve as sources for bioremediation of oil-polluted environments.

Contributions to Knowledge

This study has contributed to knowledge in the following ways;

1. Palm Oil Mill Effluent is a repository for biosurfactant producing bacteria and fungi.
2. Biosurfactant-producing microbes are more concentrated at the top of the effluent compared to the bottom.
3. *Bacillus* spp. amongst other isolates possessed higher potential for biosurfactant production.

CONCLUSION AND RECOMMENDATION

Palm oil enterprises in Edo state present a promising opportunity for biosurfactant production, given the microorganisms found there and their capacity to produce biosurfactants. The presence of these microorganisms underscores the potential of palm oil mill effluents as significant reservoirs for harnessing biosurfactant-producing bacteria and fungi. Consequently, these isolates hold importance for synthesizing this valuable compound, which finds diverse applications across various industries. It is advisable to conduct further research to refine and optimize the production of biosurfactants by these organisms.

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