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Biosurfactant Production by *Pseudomonas species* using Pre-treated Palm Oil Mill Effluent as Fermentation Medium.

Opurum C.C.[1],* Nwanyanwu C.E.[1], Nwaneri C. B. and Mbachu I.A.C[2].

[1]*Department of Microbiology, Federal University of Technology, P.M.B.1526, Owerri, Imo State, Nigeria.*

[2]*Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, Uli Campus, P.M.B. 02, Ihiala, Anambra State, Nigeria*

**Corresponding author's email: chrisopurum@gmail.com*

Abstract

In this study, pre-treated palm oil mill effluent (POME) was evaluated as a possible inexpensive, alternative and renewable medium source for the production of biosurfactant by *Pseudomonas fluorescens* and *Pseudomonas sp.* Different concentrations of 10, 20, 30, 40, 50 and 60 % (v/v) pre-treated POME was used in this study. The media were inoculated with 5 % of the inoculum standardized by adjusting the turbidity to OD comparable to 0.5 McFarland standards. The fermentation was carried out at 30^{O}C in a rotary shaker incubator at 160 rpm for 120 h. Biosurfactant production was screened based on haemolytic activity, tilted glass slide test (TGST), emulsification index (E24) and oil spreading properties. Results of the physicochemical analysis showed that besides carbohydrate, protein and oil, POME is remarkably rich in very valuable nutrient minerals particularly Ca, Mg, Cu, K, Na, Fe, Mn, Zn and P that could support the fermentative cultivation of bacteria for the production of biosurfactant. Although all the concentrations used appreciably supported bacterial growth, the highest cell count was recorded in 20 % (9.0 x 10^{8}- 13.40×10^{7} CFU/ml) between 72 –96 h incubation in *P. fluorescens* and 8.55 x 10^{7} – 6.47 x10^{8} CFU/ml between 48-72 h in *Pseudomonas sp.* Similarly, the peak of dehydrogenase activity was recorded in 20 % POME (38.08 µ/ml) in *Pseudomonas sp.* The biosurfactant produced by *P. fluorescens* and *Pseudomonas sp.* in 20 % POME exhibited maximum oil spreading properties of 1.9 and 1.4 cm, respectively. However, maximum emulsification activity was recorded in biosurfactant produced in 60 % POME by *P. fluorescens* (61 %) and *Pseudomonas sp.* (58.36 %). The properties exhibited by the biosurfactant suggest its potential for application in bioremediation, enhanced oil recovery, cosmetics and pharmaceutical industry. The findings in this study provide a strong indication that POME could be used as a veritable alternative and inexpensive medium source and holds a prospect in the large scale industrial production of biosurfactant by the *Pseudomonas*.

Keywords: Biosurfactant, effluents, palm oil, *P. fluorescens*, *P. Pseudomonas*

1. Introduction

A wide variety of microorganisms are known to produce biosurfactants that are valuable amphipathic surface active biomolecule capable of lowering the surface tension, interfacial

tension and forming microemulsion to enable mixing of two immiscible solutions (Auhim and Mohamed, 2013).

Biosurfactants are amphiphilic compounds that contain a hydrophobic and a hydrophilic moiety which confers on them the ability to reduce interfacial tension between different fluid phases (Fakruddin, 2012).They can be produced intracellularly and/or extracellularly in the culture medium. When produced intracellularly, their structure includes membrane lipids, and promotes the transport of insoluble substrates through the membrane. But when they are extracellular, the biosurfactants help in substrate solubilization and are usually a complex structure of lipids, proteins and carbohydrates (Akintokun and Balogun, 2017). Their uses and potential commercial application have been reported in several fields, such as microbial enhanced oil recovery, bioremediation of sites contaminated by recalcitrant organic compounds, composition for cosmetic industry and food processing (Nitshke and Pastore, 2006).

In recent times, biosurfactants have attracted an overwhelming attention and importance in industrial, medical and environmental applications (Chooklin, Phertmean, Cheirsilp, Maneerat and Saimmai, 2013). This is due some striking features demonstrated by biosurfactants over chemically synthesized surfactants. These include high biodegradability, low toxicity, environmental compatibility and greater stability at wide range of pH and temperature (Jamal, Nawawi and Alam, 2012; Auhim and Mohamed, 2013).

These salient characteristics have kindled considerable interest on biosurfactants as veritable alternative to chemical surfactants that pose threat to the environment. A number of industrial products such as toothpaste, pharmaceuticals and cosmetics contain surfactants and emulsifiers, which hugely increase their market demand. Due to the non-biodegradability, ability to accumulate and toxicity of chemically synthesized surfactants, the desire to find replacement with biosurfactants has emerged (Akintokun *et al.,* 2017).

However, the potentials of biosurfactants have not been largely exploited. The major challenge in the use of biosurfactants as an alternative to chemical surfactants is their high cost of production, expensive raw material and low yields in production (Saisa-Ard, Maneerat and Saimmai, 2013). These problems could be circumvented by the careful selection of efficient strains of biosurfactant- producing microorganisms, optimization of medium composition and other fermentation conditions and the formulation of fermentation medium using alternative and inexpensive renewable substrates or wastes (Saharan, Sahu & Sharma, 2011; Płaza, Zjawiony and Banat, 2011).

The choice of free-cost, inexpensive substrates or wastes as medium in the production of biosurfactant is very pertinent to the overall economy of the process and they account for about 50% of the final product cost and also minimize the cost of waste treatment (Nitschke *et al*., 2004).

The rising global demand for oil has resulted in a concomitant and rapid growth of palm oil processing industries, which has become the prime contributor to the economy of most tropical countries. Despite the high economic benefits, the liquid waste or palm oil mill effluent (POME) generation is also huge. It was estimated that for every tonne of fresh fruit bunch processed, between 0.5 and 0.75 tonne of POME is produced (Busu, Sulaiman, Hassan, Yoshihiro-Shirai, Abd-Aziz, Yocob and Wakisaka, 2010). However, these flourishing

palm oil industries have introduced unavoidable environmental challenges when very huge volumes of palm oil mill effluent (POME) are indiscriminately discharged into water bodies (Onyla, Uyu, Akunna, Norulaini and Omar, 2001).
Effluent from palm oil mills has been fingered by climate change authorities as being the second largest source of methane generator in Malaysia (38 %), followed by landfills (53 %) (Yahaya and Lau, 2013). This indicates that methane or biogas from palm oil mill effluent is therefore a major contributor to global warming.

During anaerobic digestion of POME, methane which is a greenhouse gas that is 25 times more potent than carbon dioxide in trapping heat is generated. Methane or biogas from palm oil mill effluent is therefore the major contributor to world global warming. A great deal of research and development as well as application has, during the last century, been devoted to new advance POME treatment technologies (PTT). The primary reason for such huge efforts is that POME generated from processing of fresh fruit bunch (FFB) has been declared as one of the major source of environmental pollution (Yahaya and Lau, 2013).

POME is an acidic (pH 4-5), brownish colloidal suspension that contains high concentration of organic matter, total solid and oil grease. The organic matter content is highly degradable and POME is considered non-toxic as no chemical was added during the oil extraction process (Cameotra, 2002). The characteristics of POME are dependent on the palm oil production processes in palm oil mills. Besides the organic content, POME is also rich in mineral content, particularly phosphorus (15.2 – 20.6 mg/L), potassium (2270 mg/L), magnesium (615 mg/L) and 439 mg/L of calcium (Nnaji, Okoye and Omotugba, 2016).

However, in recent decades interests are growing in the exploration of POME as a substrate in the fermentative production of various biomolecular compounds. This is because the presence of appreciably high concentrations of carbohydrate, proteins, nitrogenous compounds, lipids and minerals in POME, make it an excellent raw material for bioconversion through various biotechnological processes (Salihu and Alam, 2012).

This present study was therefore conceived to evaluate the suitability of POME as an alternative inexpensive and renewable source of medium for the production of biosurfactant by the Pseudomonads.

2. Materials and Methods

2.1 Sample Collection and Pre-treatment.

Palm oil mill effluent (POME) was collected from a palm oil processing plant at Umuagwo in Ohaji Local Government Area, Imo State, Nigeria. The sample was collected in a sterile four litre (4l) plastic container, and stored at 4^{O}C in the laboratory before pre-treatment. The POME was boiled at 100^{o}C for 90 min and filtered with layers of cheesecloth to remove debris. It was thereafter centrifuged at 4000 rpm for 30 min which gave a three layer formation. The first layer was decanted and the second layer was filtered with Whatman No.1 filter paper to obtain a clear brownish filtrate (Abas, Kader, Khalil, Hamid and Isa, 2013).

2.2 Physicochemical Analysis of Pre-treated POME

The physicochemical composition of the pre-treated POME was determined by the methods described by AOAC (2000). The percentage of oil, protein, carbohydrate and the mineral contents of Ca, Mg, K, P, Na, Fe, Mn, etc. were determined.

2.3 Culture media

The culture medium was prepared by diluting the pre-treated POME into different concentrations of 10, 20, 30, 40, 50 and 60 % (v/v) in 100 ml volume. Each concentration was supplemented with the mineral salts: K_2HPO_4, 0.16g; KH_2PO_4, 0.04g; Ca_2Cl, 0.01g; $MgCl_2$, 0.1g; $FeCl_2$,0.002g; $(NH_4)SO_4$,0.2g and NaCl,1.0g (Plaza, Pacwa-Plocinizak, Piotrowska-Seget, Jangid & Wilk, 2011).The control contains only the mineral salts. The media were sterilized by autoclaving at 121 ^{O}C for 15 min and the pH adjusted to 7.0.

2.4 Preparation and Standardization of Inoculum

The two bacterial isolates, *Pseudomonas fluorescens* and *Pseudomonas sp*. used in this study were obtained from Microbiology Laboratory Culture Collection Unit, School of Biological Sciences, Federal University of Technology, Owerri, Imo State, Nigeria. Three loopfuls of a 24 h culture of the isolates were inoculated onto previously sterilized nutrient broth. Standardization of the inoculum was performed as described by Santos, Ponezi & Fileti (2014). The flasks were incubated in a rotary shaker incubator for 24 h at 30^{O}C. After incubation, the cells were harvested by centrifugation at 4000 rpm for 20 min, and the supernatant decanted. The cells were washed thrice by centrifugation, and re-suspended in sterile distilled water. The optical density (OD) was measured at 520 nm. The inoculum was then adjusted to turbidity comparable to 0.5 MacFarland standards.

2.5 Fermentation and Biosurfactant Production

Production of the biosurfactant was carried out in seven 200 ml capacity Erlenmeyer flasks. Each flask contained 100 ml of the POME medium with the composition stated above. The medium was inoculated with 5% of the standardized inoculum. Incubation was carried out at 30^{O}C in a rotary shaker incubator at 160 rpm for 120 h. To monitor bacterial growth and activity, a volume of 2 ml of the fermenting broth was withdrawn at 24 h interval (24, 48, 72, 96, and 120 h) for viable bacterial count (Abas *et al.,* 2013). Determination bacterial activity in the broth was by dehydrogenase activity (DHA) assay as methods described by Nweke *et al.,* 2007.

2.6 Determination of Bacterial Growth by Viable Plate Count

One milliliter (1 ml) of each of the fermenting broth at every 24 h was serially diluted, and 0.1 ml aliquot of an appropriate dilution was inoculated onto standard plate count agar in replicates by spread plate technique. The plates were incubated at 30^{O}C for 24 h, and the number of bacterial colonies that developed was recorded and expressed in CFU/ml.

2.7 Determination of Growth of Pseudomonas *fluorescens* and *Pseudomonas sp* in the Different Percentages of POME by Dehydrogenase Activity Assay.

Determination of dehydrogenase activity was carried out using 2, 3, 5- triphenyl tetrazolium chloride (TTC) as the artificial electron acceptor, which is reduced to the red-coloured triphenylformazan (TPF) upon accepting electron.

One milliliter broth culture of the various percentages (10, 20, 30, 40, 50 and 60 %) of both *Pseudomonas fluorescens* and *Pseudomonas sp*. was incubated at 30^{O}C for 2 h with 0.2 ml of 2, 3, 5- triphenyltetrazolium chloride (TTC). After incubation, the coloured triphenylformazan (TPF) produced was extracted with 4 ml of butanol by shaking for 5 min and then centrifuged for 10 min at 3000 rpm, and the concentration determined spectrophotometrically at 500 nm (Nweke, Alisi, Okolo and Nwanyanwu, 2007).

2.9 Preparation of Crude Biosurfactant

After the fermentation, the brew was centrifuged for 30 min at 4000 rpm (Nasr, Soudi, Mehrnia and Sarrafzadeh, 2009), and the cell-free supernatant was then subjected to preliminary screening.

2.10 Screening of the Isolates for Biosurfactant-producing Potential

Biosurfactant-producing potential of *Pseudomonas fluorescens* and *Pseudomonas sp* were detected by using haemolytic activity, tilted glass slide test, oil spreading technique and emulsification activity.

2.11 Haemolytic Activity

The isolates were screened for haemolytic activity by streaking the 24 h culture on blood agar plates that contained 5 ml of human blood and incubated at 30^{O}C for 48 h. The plates were observed after incubation for α and β-haemolysis. Haemolytic activity was detected as the presence of clear zone of red blood cell haemolysis (beta and alpha haemolysis). Haemolysis was correlated with production of biosurfactant (Satpute, Bhawsar, Dhakephalkar and Chopade, 2008; Plaza, Zjawiony and Banat, 2006; Anandaraj and Thivakaran, 2010).

2.12 Oil Spreading Method

Some (50 ml) distilled water was added to petri dishes (25 cm diameter) in triplicates, 100 µl of crude oil was added to the surface of water. Then, 20 µl of cell-free culture broth was dropped on the surface of the crude oil, and it was compared to 20 µl of distilled water as negative control. The diameter of the clear zone was measured. A clear zone on oil surface indicated biosurfactant activity of the culture and diameter of the clear zone was proportional to the production of biosurfactant by the isolates (Patil, Aglave, Pethkar and Gaikwad, 2012).

2.13 Tilted Glass Slide Test

A discrete colony of the 24 h culture of the isolates was picked with sterile wireloop and thoroughly mixed with a droplet of normal saline (0.9% of aqueous solution of NaCl) on one end of a clean glass slide. The slide was titled and observed for the flow of water droplet over its surface (Balogun and Fagade, 2008; Satpute *et al.,* 2008).

2.14 Emulsification Activity

The emulsification capacity of the test isolates was evaluated by the emulsification index (E_{24}). The E_{24} of the broth culture of the bacterial isolates was determined by adding 2 ml of crude oil into 2 ml of the culture supernatant. The mixture was vortexed for 2 min and allowed to stand for 24 h. The E_{24} index was given as percentage of height of emulsified

layer (mm) divided by total height of the liquid column (Tambekar and Gadakh, 2012). The percentage of emulsification index was calculated using the equation of Surachai, Pimporn, Dammrong and Lumyong (2007).

$$E_{24} = \frac{Height\ of\ emulsification\ layer}{Total\ height\ of\ solution} \times 100$$

3. Results and Discussion

The physicochemical characteristics of the POME are shown in Table 1. The results indicate that POME is rich in organic (carbohydrate, protein and oil) and inorganic constituents that can support the growth and productivity of microorganisms. The Ca, K and Na content were found to be in high concentrations. These finding were in line with the reports of Okogbenin, Anisiobi, Okogbenin, Okunwaye and Ojieabu. (2014) and Abas *et al.* (2013) for the mineral contents and Chooklin *et al.* (2013) for total carbohydrate and protein contents.

Table 1: Physicochemical Composition of POME Used

Parameters	Concentration (mg/100ml)
pH	6.56 (28 ± 2^{O}C)
Total carbohydrate	1.05
Protein	1.16
Oil	12.00
P	2.74
Ca	117.0
Mg	0.033
Cu	0.16
K	23.03
Na	17.64
Fe	1.71
Mn	0.88
Zn	1.09
Moisture (%)	97.20

pH not in mg/100ml

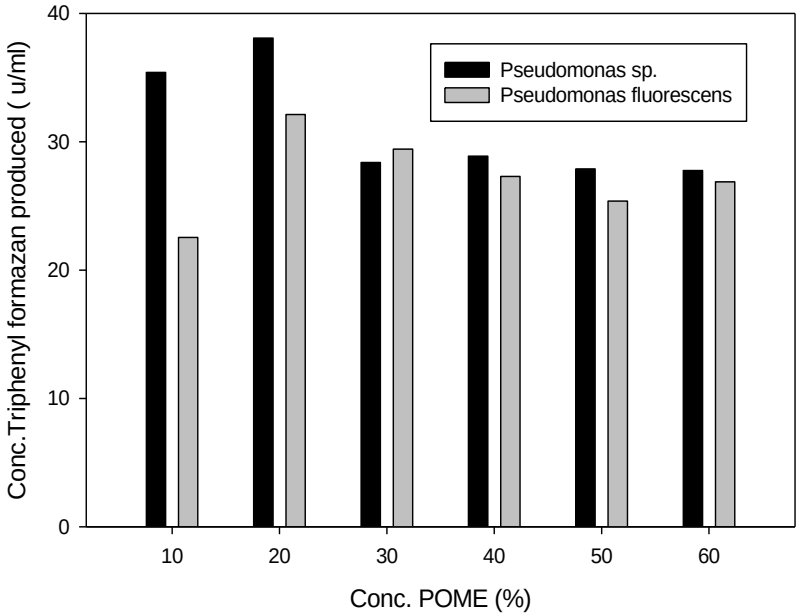

Fig.1 Total Dehydrogenase Activity of *Pseudomonas sp* and *P.fluorescens* in Different Percentages of POME.

The result of the viable plate count of the *Pseudomonas* in the fermenting broth is presented in Table 2. Although, all the percentages of POME (10, 20, 30, 40, 50 and 60 % (v/v)) used in the fermentation encouraged bacterial growth, 20% (v/v) remarkably supported and sustained high bacterial growth to 96 h. As evident from the result, in 10 and 20 % POME, peak of bacterial growth was recorded between 48 and 72 h. However, in *P. fluorescens*, the peak was observed between 72 and 96 h. This result agreed with the report of Abas *et al.* (2013). However, in their studies, 10% POME showed the fastest bacterial growth with doubling time (td) of 5.10 h, followed by 30 % POME (td = 5.32 h) and 50 % POME (td = 5.95 h).

. **Table 2. Cell count (CFU/ml) of *P. fluorescens* and *Pseudomonas* Species in Fermenting Broth**

	Time (h)				
Organism/POME (%)	24	48	72	96	120
P.fluorescens					
10	1.83×10^{7}	6.10×10^{8}	7.30×10^{7}	1.35×10^{8}	1.90×10^{8}
20	1.14×10^{7}	1.35×10^{7}	13.40×10^{7}	9.0×10^{8}	1.20×10^{7}
30	5.4×10^{7}	1.15×10^{7}	1.40×10^{7}	1.60×10^{9}	1.15×10^{9}
40	1.19×10^{7}	7.75×10^{6}	2.20×10^{7}	1.24×10^{9}	1.75×10^{9}
50	1.26×10^{7}	1.65×10^{7}	4.45×10^{8}	4.30×10^{5}	1.15×10^{5}
60	1.14×10^{7}	2.25×10^{6}	2.45×10^{8}	1.05×10^{8}	1.35×10^{9}
Pseudomonas sp.					
10	1.73×10^{7}	8.55×10^{7}	6.47×10^{8}	1.15×10^{9}	1.58×10^{9}
20	1.50×10^{7}	6.45×10^{6}	8.45×10^{7}	5.15×10^{9}	2.06×10^{9}
30	1.28×10^{7}	1.41×10^{7}	2.16×10^{8}	1.90×10^{9}	1.83×10^{9}
40	2.60×10^{6}	1.28×10^{7}	1.81×10^{8}	1.32×10^{9}	1.26×10^{9}
50	2.05×10^{6}	1.71×10^{7}	1.35×10^{7}	1.24×10^{9}	1.21×10^{9}
60	1.45×10^{6}	1.44×10^{7}	1.04×10^{8}	1.14×10^{9}	1.07×10^{9}
Control	2.05×10^{7}	2.17×10^{5}	5.0×10^{6}	1.73×10^{6}	1.4×10^{6}

Besides viable plate count, total dehydrogenase activities of the *Pseudomonas* in the different percentages of POME were evaluated (Figure 1). The result recorded the highest dehydrogenase activity in 20 %, followed by 10 % POME. This indicated the potential of POME, particularly 20% in the cultivation of bacteria for biosurfactant production. The dehydrogenase activity assay was based primarily on the fundamental principle that dehydrogenase enzymes are produced by all viable cells and the extent to which this enzyme group oxidized organic matter could be related to the number of live cells present (Burdock, Brooks and Ghaly, 2011).

Table 3: Percentage of Emulsification Activity (E_{24}) of Biosurfactants Produced by Microorganisms

	% Emulsification activity (E_{24})	
POME (%)	*P. fluorescens*	*Pseudomonas sp.*
10	Nil	42.51
20	43.15	54.49
30	Nil	Nil
40	56.00	Nil
50	46.00	Nil
60	61.00	58.36

Table 3 depicted the emulsification activity (E_{24}) of the biosurfactant produced *by P. fluorescens and Pseudomonas sp* in the different percentages of POME. The maximum emulsification activity recorded were 61.0 and 58.36 % in the biosurfactant produced by *P. fluorescens and Pseudomonas* sp. separately grown in 60 % POME. In both isolates, no emulsification was observed in the cell-free culture supernatant from 30 % POME; indicating the absence of biosurfactant. It is assumed that if cell-free culture supernatant contains biosurfactant, it will emulsify hydrophobic substrates and maintain stable emulsions for at least 24 h; this method therefore, gives an indication of the presence of biosurfactant (UmmulKhair & Hamzah, 2016). Isolates that are capable of releasing surfactant and emulsifying crude oil possess the potential for application in bioremediation of contaminated sites with crude oil (UmmulKhair and Hamzah, 2016).

The result of haemolytic activity test showed that both *Pseudomonas fluorescens* and *Pseudomonas sp* exhibited beta-haemolysis (complete) in blood agar, and positive result to tilted glass slide test (Table 4). The results were in consonance with the report of Satpute *et al.* (2008). The researchers assessed different screening methods for selecting biosurfactant-producing marine bacteria. About forty five (45) bacterial isolates were screened, 12 showed α-haemolytic activities in haemolytic assay and same isolates were positive to glass slide test.

Table 4. Tilted Glass Slide and Hemolytic Activity.

	P. fluorescens	*Pseudomonas sp.*
Tilted glass slide	+	+
Hemolytic Activity	Complete hemolysis (β)	Complete hemolysis (β)

The result of the oil spreading properties of the biosurfactants is shown in Table 5. The biosurfactants exhibited clear zone by the oil spreading method which is one of the efficient techniques for the prediction and detection of biosurfactants (Techaoei, Leelapornpisid, Santiarwarn and Lumyong, 2007). The oil spreading technique is dependent on the decrease in water-oil interfacial tension due to the presence of biosurfactant regardless of its structures (UmmulKhair and Hamzah, 2016).

The biosurfactant produced by *P. fluorescens* and *Pseudomonas sp* grown in 20% POME supports the report of Sarin, Khamsri and Sarin (2011) in which eight biosurfactant-producing bacterial isolates screened exhibited diameter of clear zone more than 1.5cm in the oil spreading test, and one of the isolates was identified as *P. fluorescens*. The effectiveness of this bacterial species in enhanced oil recovery is attributable to its biosurfactant production potential (Balogun, Fagade, Adegoke, Omole and Akaegobi, (2014).

Table 5: Oil Spreading Properties of the Biosurfactants Produced by the Microorganisms

POME %	Zone diameter (mm)	
	P. fluorescens	*Pseudomonas sp.*
10	6	7.5
20	19	14.0
30	4.5	9.5
40	6.5	6.0
50	10.5	7.5
60	6.5	7.5

The biosurfactant produced by *Pseudomonas flourescens and Pseudomonas sp* using POME exhibited high emulsification and oil spreading properties. The properties exhibited by the biosurfactant suggest its potential for application in bioremediation, enhanced oil recovery, cosmetics and pharmaceutical industry and holds a prospect in the large scale industrial production of biosurfactant by the *Pseudomonas*.

4. Conclusion

The results obtained from this study have expounded that besides carbohydrate, protein and oil, POME is remarkably rich in very valuable nutrient minerals, particularly Ca, Mg, Cu, K,

Na, Fe, Mn, Zn and P, and could serve as an excellent alternative and renewable low-cost source of medium for the production of biosurfactant; considering the high organic matter and mineral content.

References.

Akintokun, A. K., Adebajo, S. O. & Balogun, S. A. (2017). Production of biosurfactant by *Pseudomonas taenensis* using different solid and liquid wastes and energy sources. *International Journal of Current Research,* 9(05), 49724- 49728.

Abas, M. R., Kader, A. J. A., Khalil, M. S., Hamid, A. A. & Isa, M. H. (2013). Production of surfactin from *Bacillus subtilis* ATCC 21332 by using treated palm oil mill effluent (POME) as fermentation media. *2013 International Conference on Food and Agricultural Sciences IPCBEE. IACSIT Press, Singapore.*55, 87-93.

AOAC. (2000). Official methods of analysis, 17th Edition. Association of Official Analytical Chemists. Maryland, USA.

Anandaraj, J. & Thivakaran, P. (2010). Isolation and production of bio-surfactant producing organisms from oil spilled soil. *Journal of Bioscience Technology,* 3, 120-126.

Auhim, H. S. & Mohamed, A. I. (2013). Effect of different environmental and nutritional factors on biosurfactant production from *Azotobacter chrococcum. International Journal of Advances in Pharmacy, Biology and Chemistry,* 2(3), 477- 481.

Balogun, S.A. & Fagade, O.E. (2008). Screening for surface-active agent producing bacteria from diesel oil polluted tropical soil. World Applied Sciences Journal 3 (6), 930-933.

Balogun,S.A., Fagade, O. E., Adegoke, J. A. Omole, O & Akaegobi, I. M. (2014). Enhanced recovery of Bonny light crude oil from unconsolidated porous media by two *Pseudomonas* species. *Malaysian Journal of Microbiology*, 10(1), 1-6.

Burdock, T. J., Brooks, M.S. & Ghaly, A.E. (2011). A dehydrogenase activity test for monitoring the growth of Streptomyces Venezuelae in a nutrient rich medium. *Journal of Bioprocess and Biotechniques,* 1 (1), 1-10.

Busu, Z., Sulaiman, A Hassan, M. A., Yoshihito Shirai, Y., Abd-Aziz, S., Yacob, & Wakisaka, M.(2010). Improved anaerobic treatment of palm oil mill effluent in a semi-commercial closed digester tank with sludge recycling and appropriate feeding strategy. *Pertanika Journal of Tropical Agricultural Science*, 33 (1), 27 - 37

Cameotra, S.S. & Makker, R.S. (2002). Recent application of biosurfactant as biological and immunological molecules. *Current Opinion of Microbiology*, 7(3), 262-266.

Chooklin, C.S., Phertmean, S., Cheirsilp, B., Maneerat, S & Saimmai, A. (2013). Utilization of palm oil mill effluent as a novel and promising substrate for biosurfactant production by *Nevskiaramosa*NA3. Songklanakarin. *Journal of Science and Technoogy,* 35 (2), 167-176.

Fakruddin, M.D. (2012). Biosurfactant: Production and application. *Journal of Petroleum and Environmental Biotechnology.* 3(4) 1-5.

Jamal, P., Nawawi, W. M F. & Alam, M.Z. (2012). Optimum medium components for biosurfactant production by *Klebsiella pneumoniae* WMF02 utilizing sludge palm oil as a substrate. *Australian Journal of Basic and Applied Sciences*, 6(1), 100-108.

Nnaji J.C, Okoye, J.A. & Omotugba S.K. (2016).Soil quality in the vicinity of palm oil mills in Umuahia. *Nigeria. International Research Journal of Chemistry and Chemical Sciences,* 3 (1), 029-032.

Nasr, S., Soudi, M. R., Mehrnia, M. R. & Sarrafzadeh, M. H. (2009). Characterization of novel biosurfactant producing strains of *Bacillus* spp. isolated from petroleum contaminated soil. *Iran Journal Microbiology*, 1 (2), 54 – 61.

Nweke, C.O., Alisi, C.S., Okolo, J.C. & Nwanyanwu, C.E. (2007). Toxicity of zinc to heterotrophic bacteria of tropical river sediments. *Applied Ecoology and Envronmental Research,* 5(1), 123-132.

Nitschke, M., Ferraz, C. & Pastore, G.M. (2004). Selection of microorganisms for biosurfactant production using agro-industrial wastes. *Brazilian Journal of Microbiology*, 35, 81-85.

Nitschke, M. & Pastore, G. (2006). Production and properties of a surfactant obtained from *Bacillus substilis* grown on cassava wastewater. *Bio-resource Technology*. 97, 336-341.

Okogbenin, O.B., Anisiobi, G.E., Okogbenin, E.A., Okunwaye, T. & Ojieabu, A. (2014). Microbiological assessment and physiochemical parameters of palm oil mill effluent collected in a local mill in Ovia North East area of Edo State, Nigeria. *Herald Journal of Microbiology and Biotechnology*, 1 (1), 001 – 009.

Onyla, C.O., Uyu, A.M., Akunna, J.C., Norulaini, N.A. & Omar, A.K. (2001). Increasing the fertilizer value of palm oil mill sludge: Bioaugmentation in nitrification. *Water Science & Technology*, 44 (10), 157-162.

Patil, S. N., Aglave, B. A., Pethkar, A. V. & Gaikwad, V. B. (2012). *Stenotrophomonas koreensis*a novel biosurfactant producer for abatement of heavy metals from the environment. *African Journal of Microbiology Research*, 6 (24), 5173-5178.

Płaza, G. A., Pacwa-Płociniczak, M., Piotrowska-Seget, Z., Jangid, K., & Wilk, K.A. (2011). Agro-industrial wastes as unconventional substrates for growing of *Bacillus* strains and production of biosurfactant. *Environment Protection Engineering.* 37(3), 63-71.

Plaza, G. A., Zjawiony, I. & Banat, I.M. (2006). Use of different methods for detection of thermophilic bio-surfactant producing bacteria from hydrocarbon contaminated and bio-remediated soils. *Journal of Petroleum Science and Engineering.* 50, 71-72.

Saharan, B.S., Sahu, R.K. & Sharma, D. (2011). A review on biosurfactants fermentation, current developments and perspectives. *Genetic Engineering and Biotechnology Journal,* 29, 1-14.

Saisa-Ard, K., Maneerat, S & Saimmai, A. (2013). Isolation and characterization of biosurfactants-producing bacteria isolated from palm oil industry and evaluation for biosurfactants production using low-cost substrates. *Journal of Biotechnology, Computational Biology and Bionanotechnology*, 94(3), 275-284.

Salihu, A. & Alam, M. D.Z. (2012). Palm oil mill effluent: a waste or a raw material? *Journal of Applied Sciences Research,* 8(1), 466-473.

Santos, B. F. Ponezi, A. N. & Fileti, A. M. F. (2014). Strategy of using waste for biosurfactant production through fermentation by *Bacillus subtilis. Chemical Engineering Transactions,* 37, 727-732.

Sarin, S., Khamsri, B. & Sarin, C. (2011). Isolation of biosurfactant–producing bacteria with antimicrobial activity against bacterial pathogens. *Environment Asia*, 4 (1), 1-5.

Satpute, S. K., Bhawsar, B. D., Dhakephalkar, P.K. & Chopade, B.A. (2008). Assessment of different screening methods for selecting biosurfactant producing marine bacteria. *Indian Journal of Marine Sciences,* 37, 243-250.

Surachai, T., Pimporn, L., Dammrong, S. & Lumyong, S.(2007). Preliminary screening of biosurfactant producing microorganisms isolated from hot spring and garages in Northern Thailand. *KMITL Science and Technology Journal*, 7 (1), 38-43.

Tambekar, D. H & Gadakh, P. V. (2012). Isolation and characterization of biosurfactant-producing bacteria isolated from petroleum contaminated soil. *International Journal of Advance Pharmaceutical and Biological Sciences,* 2(2), 135-140.

Techaoei, S., Leelapornpisid, P., Santiarwarn, D. & Lumyong, S. (2007). Preliminary screening of biosurfactant-producing microorganisms isolated from hot spring and garages in Northern Thailand*. KMITL Science and Technology Journal,* 7 (1) 38-43.

UmmulKhair, M. S. & Hamzah, A. (2016). Determination of optimum conditions and stability study of biosurfactant produced by *Bacillus subtilis*UKMP-4M5. *Malaysian Journal of Analytical Sciences, 20(5), 986 - 1000*

Yahaya, S. M. & Lau, S. (2013). Palm oil mill effluent (POME) from Malaysia palm oil mills: waste or resource. *International Journal of Science, Environment and Technology*, 2(6), 1138 – 1155.