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NOVEL TREATMENT OF HEAVILY OILED WASTEWATER USING PSEUDOMONAS AERUGINOSA NR.22 PRODUCING USABLE FREE FATTY ACIDS (FFA)

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Abstract

Heavily oiled wastewater treatment has been a great challenge for engineers to accomplish. Since too many water bodies are polluted with oil, chemical treatment could still give lots of negative side effects. We are recommending discharging the oil using our novel microbial approach. The strain Pseudomonas aeruginosa NR.22 (Ps.NR.22); an extracellular lipase producer isolated from a polluted lake in Malaysia has been proved to remove different oils from heavily oiled-wastewater with 59.0g/L oil concentration to about 70% lower in lesser than 48 hours with the addition of local source, mix grade 9.1g/L nitrogen compose. We measured the excess of oil using the standard Gravimetric method. Study of the factors affecting the percentage of oil removal have identified 45°C as the best temperature, 200rpm shaking rate and 11% (v/v) 24 hours inoculum with 5.0x10^{5}cells/mL. Lipase activities were significantly high and go all along with the values of oil removed. Oil removal was recorded as excellent using a combination of the best parameters; a value of 92.8±0.01% removed the oil with lipase activity about 29.8±0.08U/mL and a very positive sign of growth through biomass of 6.0±0.1g/mL. FTIR was used to study the oil characterization in the wastewater before and after the treatment and the findings supported the lipase-oil removal Gravimetric report where the oil was cut to simpler C-C bonds to 6 different types of free fatty acids (FFA), confirmed using GC-MS as lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), linoleic acid (C18:2), oleic acid (C18:1) and caprylic acid (C8:0). All of the listed FFA have great potential for biodiesel production.

Keywords*: Pseudomonas aeruginosa NR.22; Oil; Wastewater; Lipase; Free fatty acid (FFA)*

Introduction

The industrial development has caused the increment in the amount of oily wastewater generated into the system. According to *J. Coca-Prados et al*. [1], the large amount of oily wastewater comes from the petrochemical, oil refining, food and leather industries. There are several different methods that have been established to counter this problem. *L. Yu et al.* [2] has stated that the conventional methods such as flotation, coagulation, membrane separation technology and biological treatment are commonly used to treat the wastewater. However, there are still lots of negative side effects when using the conventional treatment methods such as economical and efficiency problem. Therefore, an alternative method to treat oily wastewater without causing too much negative side effects is crucial for these industries [3]. The *P.S.*

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Borkar et al. [4] have described the unicellular lipases, which were produced by various *Pseudomonas sp*., to be very useful in organic reactions and have been promoted in the whole cell water treatment. Thus, we are working on the treatment of oily wastewater through microbial approach by using the strain *Pseudomonas aeruginosa* NR.22 to convert the oil in the wastewater into useful free fatty acids and transform the dirty wastewater into useful clean water source [5-6].

Materials and Methods

Reagent, chemicals and pH.

All materials and chemicals were collected from various sources and quality as listed. Bacteriological peptone (Ultrapure, protein = Nx6.38 ≥ 76.5%), yeast extract (Y1625, Sigma-Aldrich), ammonium sulphate (A3920, ≥ 99.0% $(NH_4)_2SO_4$, Sigma), ammonium phosphate monobasic (79546, ≥ 98% $(NH_4)_2HPO_4$, Sigma-Aldrich), urea (U5378 powder, NH_2CONH_2, Sigma), ammonium nitrate (A9642 ≥ 99.0% NH_4NO_3, Sigma-Aldrich), ammonium carbonate (379999, 99.99% $(NH_4)_2CO_3$ Sigma-Aldrich), nitrogen compose (local source, mix grade), nitrogen soil (soil mixed with urea based fertilizer), nitrogen fertilizer (Carbamide, 46%N $(NH_2)_2CO$, local supplier), tributyrin (73105, analytical standard, Sigma-Aldrich), glycerol (Merck), Pseudomonas agar (Microbiology), nutrient agar (Merck), nutrient broth (Merck), olive oil (extra virgin, Bertolli, Italy), potassium dihydrogen phosphate (V90004, Vetec™ reagent grade, 99% KH_2PO_4), ammonium chloride (09718, ≥ 99.5% NH_4Cl, BioUltra, Sigma), poly vinyl alcohol ($[-CH_2CHOH-]_n$, Aldrich), calcium chloride (Sigma-Aldrich), acetone $(CH_3(CO)CH_3$ analytical grade, Sigma), ethanol (CH_3CH_2OH analytical grade, Sigma-Aldrich), sodium hydroxide (NaOH, Fluka) and hexane ($CH_3(CH_2)_4CH_3$, 95%, Sigma-Aldrich).

Pseudomonas species, enrichment medium and inoculum preparation.

Pseudomonas aeruginosa NR.22 (Ps.NR.22) was isolated from a lake in Selangor, Malaysia [7]. It is a Gram negative-rod shaped bacteria (Fig. 1B) and has been confirmed to carry a 16S rRNA gene, was isolated to be producing high lipase enzyme (23.44 U/mL) using tributyrin agar (Fig. 1C) [8- [9]. The strain was maintained at -20ºC in Pseudomonas glycerol agar. Figures 1A and 1D show the species on Pseudomonas agar (PA) and in the Erlenmeyer flask, respectively.

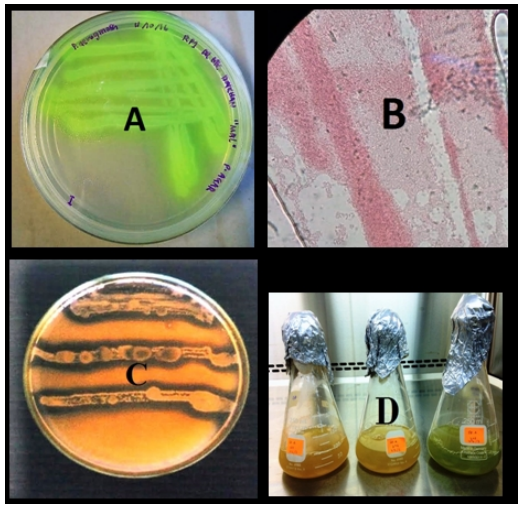

Fig. 1. Green pigmentation produced by of *Pseudomonas aeruginosa* NR.22:
A. After 24 hours incubation on Pseudomonas agar [7]; B. Gram negative rod species;
C. Positive lipase producer on tributyrin agar with clear zones [10]; D. Growth in Erlenmeyer flask

The enrichment medium was a modification of medium by *A. Adzharpour et al.* [11] contains 10-mL sample of the oily wastewater added to 100mL of nutrient broth containing 2.0% (v/v) of olive oil, 0.2% (v/v) potassium dihydrogen phosphate (KH_2PO_4) and 0.4% (v/v) ammonium chloride (NH_4Cl) in 250ml Erlenmeyer flask. The shaking rate was set at 150rpm, 35°C and 24 hours according to finding by *N.H. Nik Raikhan et al.* [7]. The 24 hours inoculum preparation was set up using nutrient broth (NB) at pH = 6.5, shaking rate at 150rpm and 35°C [8]. The amount of 8% (v/v) 24 hour cells at $5.0x10^5$cells/ml was used by modifying method from *N.H. Nik Raikhan* [8].

Estimation of Bacterial Cell Mass. The Optical Density (OD) of the culture broth at 540nm was measured periodically using Spectrophotometer and about 2.0mL sample of the culture was withdrawn from the flask and centrifuged at 10000rpm for 10 minutes for every 3 hours of sampling. The supernatant was poured out and the remaining cell was suspended in distilled water. The cell suspension was centrifuged again using the same parameters. The supernatant was again poured out and the remaining cell was washed slowly using distilled water on the cells suspension. Then, it was transferred to a falcon tube with a small opening, put into a desiccator at room temperature for overnight. After 24 hours, the falcon tube containing dry cell was weight. The difference between the final weight and pre-weight of the falcon tube were used to estimate Cell Dry Weight (CDW). This method was adapted and modified using method by *N.H. Nik Raikhan* [9].

Factors to conduct research. Survival of Ps.NR.22 was investigated using sub culturing method of samples after 36 hours of hydrolysis of oily wastewater. Apart from that, there should be no bacterial growth observed in the control sample. The effect of temperature on oil degradation by the Ps.NR.22 was studied using 35, 40 to 65°C. Ps.NR.22 degraded initial concentration of 9.1g/L oil from oily wastewater with pH = 6.5, 150rpm for 36 hours at 45°C.

Lipase assay. The method of *N.H. Nik Raikhan* [9] was modified. The bacteria's lipase activity was measured using polyvinyl alcohol and olive oil emulsion as substrate at 35°C. One unit of lipase activity was defined as the amount of enzyme required to release one micromole of fatty acid per minute under the test conditions.

Oily wastewater. The wastewater was collected from a cooking oil factory facility in Kelantan, Malaysia. Sampling was done with sterile containers. The wastewater was sterilized using autoclave at 121°C for 30 minutes under 15psi of pressure. This was done to prevent growth and activity of the other potential intervening biological agents. Characteristics of the oily wastewater have been determined and are presented in the Table 1.

Table 1. Characteristics of oily wastewater before the treatment

Parameters	Concentration	Parameters	Concentration
COD	10±0.1g/L	Viscosity	3.95±0.01mm^2/s
Ammoniac	0.8±0.1g/L	Total polar material TPM	8.22±0.01%
Phosphate	3.0±0.1g/L	Iodine value (IV)	5.38±0.03g I_2/100g
Various oils	59.0±0.03g/L	Peroxide value (PV)	3.44±0.01meq/kg
pH	6.4	Temperature	45°C

Oil concentration measurement. Oil concentration measurement was conducted using the standard method of gravimetric. The oil was extracted thrice with hexane. It was then transferred to a pre-weight distilling flask followed by evaporation of the hexane at 80°C. The flask was then reweighted. The oil concentration in g/L was calculated by getting the difference of weight of the flask [8].

Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography analysis (GC). Modification of method by [10] has been used for FTIR. GC analyses were carried out on HP 5890 or Agilent 4890D instruments equipped with FID detector and Hydrodex-β-PM 25m×0.25mm column (25m×0.25mm, 0.25µm film with permethylated-cyclodextrin; Macherey

& Nagel) or 0.25 or Betadex 225 fused silica capillary column (30m×0.25mm×0.25µm film; Supelco) using H_2 carrier gas (oven: 100°C, injector: 250°C, detector: 250°C, head pressure: 10 psi, 50:1 split ratio) [8].

Results and Discussion

Effect of different nitrogen sources added into the Erlenmeyer flask containing heavily-oiled wastewater was studied using 9.1g/L peptone, yeast extract, ammonium sulphate, ammonium phosphate, urea, ammonium nitrate, ammonium carbonate, nitrogen compose, nitrogen soil and nitrogen fertilizer. Figure 2 is depicting values of oil removal (%) and the lipase activity correlate to each of the values recorded from the wastewater containing the various nitrogen sources. We used 8% (v/v) of 24 hours Ps.NR.22 inoculum into 100mL sterile wastewater in 250mL Erlenmeyer flask. The best oil removal was achieved with the addition of sterile nitrogen compose with rate of oil removal of 82.1±0.01% with lipase activity valued at 18.8±0.01U/mL. According to Figure 2, urea, ammonium nitrate, yeast extract and peptone have given among the best values of oil removing, together with the three (3) groups of nitrogen sources; nitrogen compose, nitrogen soil and nitrogen fertilizer (78.7±0.03% - 82.1±0.01%). However the later was way cheaper and significant in bulky use if we were applying this at a pilot plant scale or at industrial level. We decided to concentrate this research using nitrogen compose since the price of this element is way cheaper than the rest of the other sources. We used wastewater without the addition of any nitrogen source as a standard and it was clearly seen that the ability to degrade oil in the wastewater by Ps.NR.22 was lower by 52.4% to compare with the highest degraded oil recorded with nitrogen compose (see Fig. 2A). Ps.NR.22 needs additional nitrogen sources to carry out critical biochemistry processes and to produce related enzymes including lipase.

The wastewater didn't contain enough nitrogen sources (only 2.2±0.1g/L, Table 1) to support the microbiological system, and lipase production was recorded very low in the system without additional nitrogen source (7.60±0.01U/mL, see Fig. 2A). On the other hand, the lipase activity was greatly correlated with the amount of degraded oil which confirmed the interaction between wastewater-nitrogen-lipase. For example the lowest oil degrading percentage of ammonium carbonate ($(NH_4)_2CO_3$) was 35.3±0.01% significantly correlated to only 11.6±0.02U/mL of lipase activity. Since the study resulted in nitrogen compose as the best nitrogen source that supported the growth and lipase production and therefore treated the oily wastewater the best, we have continued the study with nitrogen compose using different factors. Figure 2B portrayed the percentage of oil removal, lipase activity and cell biomass under the effect of several temperatures (35-65°C). Oil was removed above 70% at all tested temperatures suggesting that the lipase enzyme excreted by Ps.NR.22 is almost optimum at all those temperatures. However, the highest oil removal was achieved at 45°C with 80.9±0.01% removal conducted by lipase recorded at 23.45±0.01U/mL. The biomass gained from the same flask was 4.1±0.01g/L, the highest dry weight indicating that Ps.NR.22 was at its maximum rate of growth. Next, using the best temperature (45°C), we investigated the effect of different shaking rates to oil removal, lipase activity to the biomass. Figure 2C represents the findings. The best oil removal was recorded significantly at 200rpm (83.1±0.1%) followed by 150rpm (80.2±0.01%). Both rates were significantly great but as far as the 200rpm was better we have decided on the later. Lipases are enzymes that function at interfaces of oil-water thus high shaking rate will contribute to its great operating system. Nevertheless, 250rpm didn't help the hydrolysis yet the oil removal was shown to be 67.3±0.08%, a value almost the same as recorded at 100rpm. This scenario was caused by the shear factor over the active lipase protein ruptured to deactivation (see Fig. 2C). Among all, we considered that the effect of cell concentrations was the main player of this research since lipase was excreted to the surrounding. Initially we started with 8% (v/v/) of 24 hours inoculums containing

5.0x10^{5}cells/mL. In Figure 2D we present values of oil removal (%) and lipase activities as well as collected biomass using 6-14% (v/v) initial cells of 24 hour Ps.NR.22. Technically, one may disagree with very high initial cell concentrations (more than 10%, v/v), but since were dealing with heavily-oiled wastewater and the fact that cell survival was vital, our study was done out of norm. Surprisingly, Ps.NR.22 strain has performed the best hydrolysis at initial cell of 11% (v/v, 5.0x10^{5}cells/mL). The lipase activity was very high (31.2±0.02 U/mL) with biomass of 4.3±0.01g/L (see Fig. 2D).

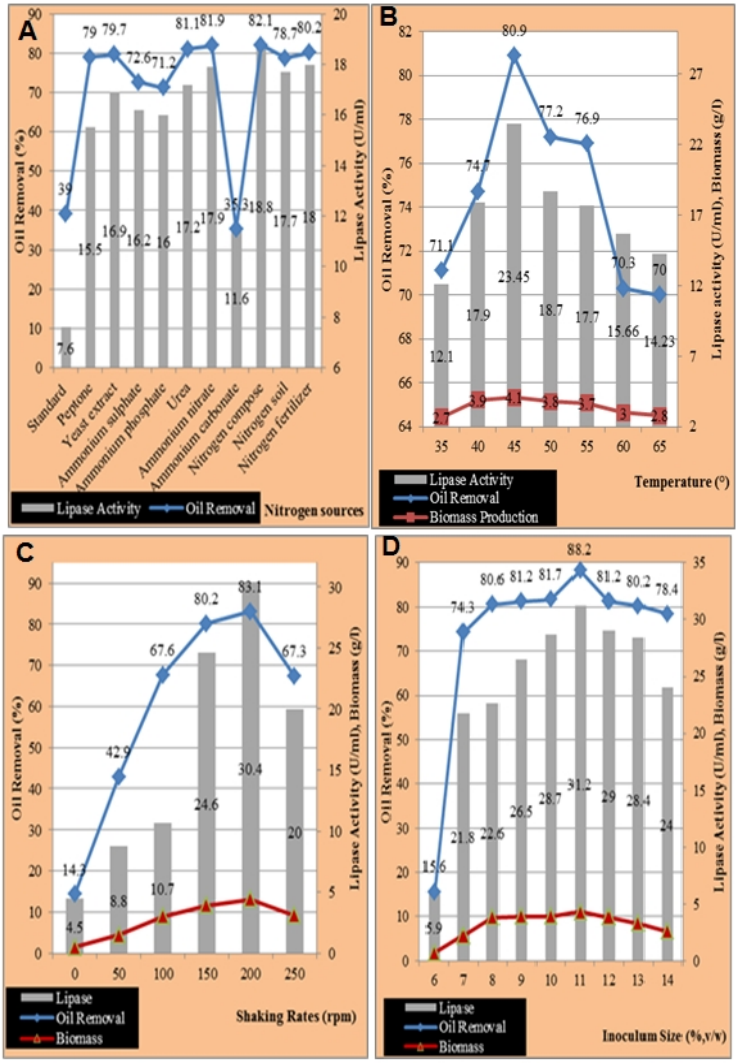

Fig. 2. Treatment of oil removal (%) with lipase Ps. NR.22: **A.** Percentage of oil removal by 9.1g/L of various nitrogen sources in oily wastewater using direct catalysis by Ps.NR.22 species after 36 hours of fermentation (35°C, 150rpm); **B.** Temperature effect on the oil removal, biomass production and extracellular lipase activity with 14.1g/L nitrogen compose in 36 hours fermentation with 8% (v/v) of Ps.NR.22 inoculum at 150rpm; **C.** Shaking rate effect on the oil removal, biomass production and extracellular lipase activity with 14.1g/L nitrogen compose in 36 hours fermentation with 8% (v/v) of Ps.NR.22 inoculum at 45°C; **D.** Cell concentration effect on the oil removal, biomass production and extracellular lipase activity with 14.1g/L nitrogen compose in 36 hours fermentation of Ps.NR.22 at 45°C, 200rpm

Next, the experimental and kinetic study was done using the optimum conditions (nitrogen compose, 45°C, 200rpm and 11%, v/v cells) with the running system took us 92 hours

before we stopped the hydrolysis with a confirm declination of both oil removal and lipase activity. But the kinetic and substrate utilization will be reported in a different academic report, we are focusing only on the wastewater treatment or the hydrolysis here. Figure 3A depicted the percentage of oil removal and lipase activity using 14.1g/L nitrogen composes for 92 hours using 11% (v/v) Ps.NR.22 cells. According to Figure 3A, oil removal achieved optimum values (> 88%) from 36th to 78th hours with maximum values obtained at 36th hours (92.8±0.01%), with lipase activity about 29.8±0.08U/mL and a very positive sign of growth through biomass of 6.0±0.1g/mL. The maximum value increased 32.6% after the optimization. Figure 3B shows the wastewater before and after 92 hours treatment.

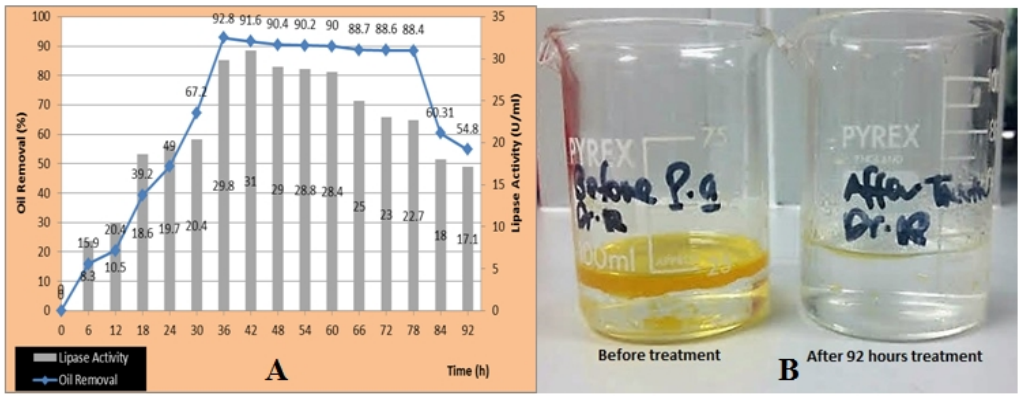

Fig. 3. Treatment of oil removal and its correlation with lipase activity: **A.** Percentage of oil removal and its correlation with lipase activity using 14.1g/L nitrogen compose for 92 hours using 11% (v/v) Ps.NR.22 cells (45°C, 200rpm); **B.** Wastewater; before and after treatment with removal of more than 90% of oil content from the system after 92 hours

In Figure 4, the FTIR spectra of oily wastewater sample before the treatment and after the 92 hours treatment using whole cells before and after treatment. In Figure 4A, it's clearly seen that the 2 blue blocks (1600-1800cm^{-1} and 2800-3200cm^{-1}) are showing spectrum of high C-C content related to the cooking oil in the wastewater and it was totally removed after 92 hours as seen in Figure 4B. Hydrolysis of the oil content in the wastewater has produced free fatty acids (FFA), mono- and diglycerides. Figure 5 reflects the GC-MS spectra of produced fatty acids in the treated oily wastewater. The hydrolysis has produced six types of unsaturated FFA namely lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), linoleic acid (C18:2), oleic acid (C18:1) and caprylic acid (C8:0). GC coupled with mass spectrometer (MS) or flame ionization detector (FID) is the most widely used technique for determination of FAs and TFA [12].

(C8:0).

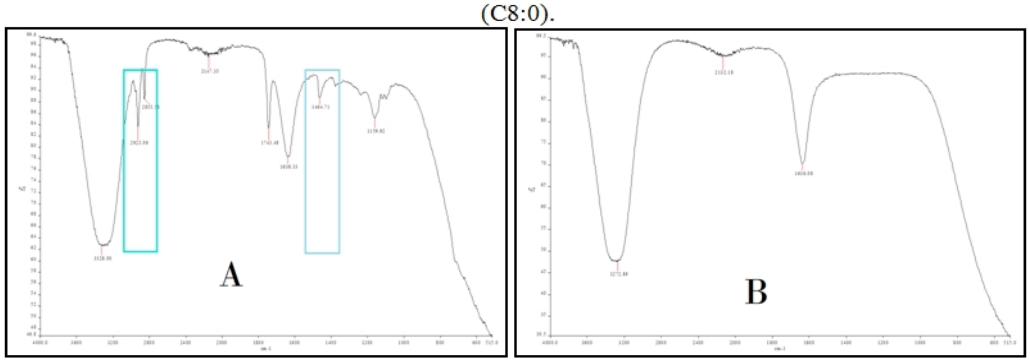

Fig. 4. FTIR spectra of oily wastewater sample: **A.** Before treatment; **B.** After treatment

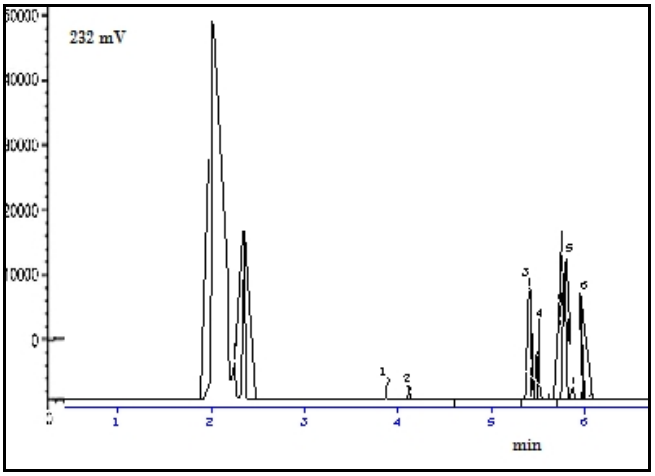

Fig. 5. GC-MS chromatogram on treated oily wastewater producing free fatty acids:
1 - lauric acid (C12:0); 2 - myristic acid (C14:0); 3 - palmitic acid (C16:0);
4 - linoleic acid (C18:2); 5 - oleic acid (C18:1); 6 - caprylic acid

Conclusion

In conclusion, *Pseudomonas aeruginosa* NR.22 is a great potential microbe for a safe oily water treatment but it needs some improvement in the system. This treatment produced safe usable unsaturated free fatty acids (FFA). Therefore, we are expanding this research using immobilized *Pseudomonas aeruginosa* NR.22 to treat higher level of C-C of the oil content while maintaining cell function, enzymes and microbial contamination in the environmental issue; and to detail out on the potential of the FFA for other vital purposes for example for biodiesel production.

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