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## BIOREMEDIATION OF DISPOSED ENGINE OIL FOR LIPASE PRODUCTION

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### ABSTRACT

Engine oil is one of the several refined products of crude oil. It is composed of long chain saturated hydrocarbons (base oil) additives. Generally, engine oil can enter into the environment through leak of oil tankers, cleaning of tanks by merchants, warship carrying engine oil, and operations by motor mechanics which is a common industrial waste that is harmful to environment and human health. This research aims to produce lipase enzyme using microbial degradation of disposed engine oil. In this study hydrocarbon-degrading bacterium, GS-3 was successfully isolated from oil contaminated area. GC-MS analysis revealed that this isolate was able to produce organic acid, methyl-3,4,5-trimethoxy-2,6-dinitrobenzoate from disposed engine oil. Besides, GS-3 isolate produced highest lipase activity, achieving  $0.097 \pm 0.007 \text{ U ml}^{-1} \text{ min}^{-1}$  during first 24 hrs when disposed engine oil (DEO) was used as carbon source. Data revealed new and broad band which is related to O-H stretching formed at  $3421 \text{ cm}^{-1}$ , though new band occurred at  $3424 \text{ cm}^{-1}$  and  $1645 \text{ cm}^{-1}$  after bioremediation. Subsequent lipase optimization parameters revealed that this bacterial isolate could produce highest lipase activity when the 4% (v/v) DEO was used as carbon source. The best nitrogen source was urea. Addition of surfactant Tween 80 could also enhanced lipase production. Optimal pH value and temperature was 7.0 and  $30^\circ\text{C}$  respectively.

**Keywords:** Biodegradation, Disposed engine oil, Lipase, Optimization

## INTRODUCTION

Engine oil (lubrication oil) is one of the products from base oils produced through distillation of petroleum. It is non-volatile liquid and functions to reduce friction between two moving parts, playing key role in the maintenance of motor and internal combustion engines. After being drained out from automobile or generator engine, it is then referred to as disposed engine oil. According to Environmental Quality (Scheduled Wastes) Regulations 2005, it is mandatory to recycle or dispose used engine oil at licensed facilities to prevent pollution. Despite the enforcement of rules and regulation, case of indiscriminate disposal of used engine oil remains prevalent because it is cheaper than transporting it to recycling facility. Disposed engine oil is mainly composed of long chain hydrocarbon and some amount of heavy metal (Kupareva et al, 2012). Precise description of the chemical composition of disposed engine oil is not practicable due of its complexity (Amro, 2004). Indiscriminate disposal of used engine oil will cause environmental problem as it will infiltrate into the soil, resulting in depletion of nutrients necessary for growth of plants (especially nitrogen and phosphorus) and affect physical properties of soil Amadi et al., (1993). Disposed engine oil is recalcitrant pollutant. It contains long chain saturated and aromatic hydrocarbon. Therefore, effective biodegradation of disposed engine oil requires concerted effort of a consortium of microorganism, be it prokaryote or eukaryote. Moreover, the presence of heavy metals and polycyclic aromatic hydrocarbon might hinder the microbial degradation process Lee et al. (2008). So, it is challenging to isolate a single microorganism that can degrade wide range 7 constituents of the disposed engine oil. Bacteria that have been reported to be good engine oil degrader include *Pseudomonas*, *Enterobacter*, *Bacillus*, *Staphylococcus*, and many others (Obayori et al., 2014; Borah and Yadav, 2014; Stephen et al., 2013; Thavasi et al., 2011; Thatheyus and Ramya, 2014). Several studies had confirmed the negative impacts of disposed engine oil to the environment and its potential threat to public health. Kayode et al. (2009) reported that used engine oil is toxic and has considerable adverse effect on the growth of plant. He proposed that presence of used engine oil in the soil would inhibit the seedlings and the extent of growth inhibition in the seedlings is proportional to the concentration used oil pollutants. When it is disposed of into the soil, the aeration of soil would be dramatically impacted because the oil absorb recalcitrantly to the soil particles (Alloway and Ayres, 1997). Spilled engine oil on the land could be transferred rapidly to aquatic environments by rain and runoff waters. This study aims to degrade hydrocarbon- bacterium GS-3 through isolating it from oil contaminated area at Kuantan – Pahang/ Malaysia.

## MATERIALS AND METHODS

### Sample Collection

Oil contaminated soil samples were collected in the vicinity of automobile workshop at Kuantan Pahang Malaysia. A soil sample was collected 10 cm depth from the surface of the soil to avoid sample contamination, and being stored in sterile bottles. Soil samples were crushed using mortar and pestle, thoroughly mixed and sieved through a 2 mm pore size sieve

to remove unwanted large debris. The sieved soil was kept in polyethylene bag, closed tightly and stored at  $4 \pm 1$  °C.

### **Media Preparation**

Bushnell Haas (BH) broth medium was used to isolate engine oil-degrading bacteria (Bhattacharya et al., 2015). The medium composition was as follows: (g/L)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2,  $\text{K}_2\text{HPO}_4$  1.0,  $\text{KH}_2\text{PO}_4$  1.0,  $\text{FeCl}_3$  0.05,  $\text{NH}_4\text{NO}_3$  1.0,  $\text{CaCl}_2$  0.02 with final pH of 7.2. The prepared medium WAS sterilized using autoclave at 121°C for 15 minutes. about 50 mL of autoclaved BH broth was prepared in 250 mL Erlenmeyer flask and supplemented with 2% (v/v) disposed engine oil as sole carbon source for microbial growth.

### **Isolation of Hydrocarbon-Degrading Bacteria**

Isolation of disposed engine oil-degrading bacteria was carried out using the method of Sivapuram (2011). One gram of oil-contaminated soil sample was suspended in 10 mL sterilized distilled water and vigorously shaken. Then, 1 mL of mixture was transferred into 50 mL of BH medium and incubated at 30°C at 170 rpm for 7 days. After incubation period, 1mL of fermentation broth was withdrew and diluted with 6 dilutions series of each  $10^{-1}$  fold using sterile distilled water. A 100  $\mu\text{L}$  aliquot of each fifth and sixth dilution series was taken and plated onto BH agar enriched with 1% disposed engine oil and nutrient agar supplemented with 1% disposed engine oil respectively. Bacterial colonies that showed different morphological characteristics were picked and purified to nutrient agar supplemented with 1% disposed engine oil by streaking technique until single colony was obtained.

### **Screening of Extracellular Lipase Producing Bacteria**

Two types of screening agar, namely tributyrin agar (TBA) and olive oil with phenol red agar were used to screen extracellular lipase enzyme PRODUCTON. Tributyrin agar medium comprised of tributyrin (1% w/v),  $\text{CaCl}_2$  (0.1% w/v), and agar (2% w/v). The prepared agar was adjusted to pH 7.0 before autoclaved at 121°C for 15 minutes. The olive oil agar plates were prepared by incorporating phenol red (0.01% w/v), olive oil (0.1% v/v),  $\text{CaCl}_2$  (0.1% w/v), and agar (2% w/v). The prepared agar was adjusted to pH 7.3 before autoclaved at 121°C for 15 minutes. All isolated oil-degrading bacteria were screened for extracellular lipase activity using qualitative and quantitative assay.

### **Qualitative Screening Method**

Qualitative assay was done by streaking the bacterial isolates onto TBA and olive oil with phenol red agar respectively (Lee et al., 2015). Culture plates were incubated at 30°C for 24 hours. According to Sagar et al. (2013), formation of clear halo zone around the colony on TBA indicates extracellular lipase enzyme production. While for the olive oil with phenol red agar, lipase-producing bacteria would turn the red dye into yellow color. These positive colonies for lipase enzyme production were then inoculated on agar slants and subsequently incubated at 30°C overnight before storing at 4°C as stock culture for further use.

## Quantitative Screening Method

Two loops of 24 hours-incubated petri dish culture was inoculated into 50 mL nutrient broth (NB) supplemented with 2% of disposed engine oil. The culture was incubated for 24 hours at 30°C and 150 rpm in the incubator shaker. Overnight culture was suspended in 0.9% (w/v) Sodium Chloride (NaCl) solution to obtain initial cell density of 0.5 McFarland standards. The preparation of McFarland Standard could be referred to Appendix C. Submerged microbial culture was incubated in 250 mL Erlenmeyer flasks containing 50 mL of BH broth with 2% disposed engine oil and inoculated with 1 mL with bacterial suspension. After 24 hours of incubation, the culture was centrifuged at 10,000 rpm for 20 minutes at 4°C. Cell free filtrate obtained was used as crude enzyme. Quantitative assay was carried out by determining lipase activity using titrimetric assay as per Mustranta (1992). The reaction mixture containing 5 mL olive oil emulsion and 4 mL of 100 mM pH 7 sodium phosphate buffer. After that, 1 mL of crude enzyme was added into the reaction mixture and incubated at 30°C and 250 rpm for 2 hours. A control was established by using 1 mL of crude enzyme, which is pre-heated at 100°C before added into a reaction mixture. The reaction was stopped by the addition of 5 mL of acetone–ethanol mixture (1:1 v/v) after 2 hours of incubation. The amount of fatty acid liberated was titrated with 0.05 N NaOH solutions to a final pH of 10.0. One lipase enzyme unit was defined as the amount of enzyme required for the release of 1 µmol fatty acid per minute under the assay conditions. Enzyme activity was expressed as units (U) per milliliter of enzyme extract and could be calculated using Equation 1.

$$\text{Lipase Activity (U/mL/min)} = \frac{N[\text{NaOH}] \times \text{Volume NaOH titrated} \times 1000}{\text{time of incubation}} \quad (1)$$

## Selection of Most Potent Bacteria

The selection of most competent bacteria was based on largest halo zone on TBA agar, largest yellow zone olive oil agar, and showed highest extracellular lipase activity was used in the preset study. Partial identification of bacterial isolates was subjected to differential staining as described by Tiwari et al. (2007) to identify gram reaction of the chosen bacterial isolates.

## Recovery of Crude Extract

The fermentative product was extracted using liquid-liquid extraction technique as described by Eniola and Ogasola (2014). A 50 mL of trichloromethane was added to broth culture in conical flask and shaking thoroughly. The mixture was then transferred into separating funnel and allowed to stand for two hours. The layer containing the organic phase and residual oil was emptied into a beaker. After that, the crude extracts were subjected to GC-MS and FTIR analysis for identification of component in the crude extract.

## Protein Determination

The extracellular protein content was determined according to (Lowry *et al.*, 1951). Bovine serum albumin (BSA) was used as protein standard. The protein standard curve was carried out in dark environment. Different concentrations of BSA were prepared from range of 0.0 to 0.8 mg/ml.

## Optimization of Parameters for Lipase Production

The effect of concentration of disposed engine oil as carbon sources for lipase production was studied different concentrations of disposed engine oil from 1% to 4% whereas other parameters unchanged. Effect of nitrogen sources on the lipase activity was studied by replacing the original nitrogen source, ammonium nitrate with three other nitrogen sources, namely urea, peptone, and ammonium sulfate at concentration of 1% (w/v). Culture using the original nitrogen source, ammonium nitrate was established and served as control. Effect the addition of different surfactants on lipase enzyme production was analyzed by using three different surfactants, namely Tween 80, Tween 20, and triton X-100. These surfactants were tested individually at amount of 50  $\mu$ L in the BH medium with 2% disposed engine oil. Culture without surfactant was served as control. Other parameters remained unchanged. The effect of incubation temperature on lipase activity was studied in range of 30 to 50°C. The microbial isolate was grown on BH medium at temperature of 30, 40, and 50°C respectively. The lipase activity was measured every 24 hours for 4 days. Other parameters were remained unchanged. The effect of initial incubation pH was performed by varying pH of BH medium from 6 to 9 whereas other parameters remain unaltered. The effect of trace element on lipase activity was carried out by individually removing trace element, FeCl<sub>3</sub>, MgSO<sub>4</sub>, and CaCl<sub>2</sub> from the production media. Control was established by using original BH medium without any ingredient removal. Other parameter remains unchanged. After incubation period of each parameter the lipase activity and extracellular protein content was measured every 24 hours until the end of experimental period.

## RESULTS AND DISCUSSION

### Isolation and Screening of Hydrocarbon-Degrading Bacteria

A total number of 10 disposed engine oil-degrading bacteria were successfully isolated from soils at different locations were symbolized as GS-1 to GS-10 isolates. Only 2 out of 10 hydrocarbon-degrading bacteria produced extracellular lipase enzyme. were GS-2 and GS-3 respectively. Qualitative isolates screening method showed that both isolates had same lipase activities. However, titrimetric assay showed that GS-3 was better lipase producer according to higher lipase activity (Table 1 and 2).

Table 1: The diameter of zone formed on two difference screening agar

Screening Media	Bacterial isolates zone diameter (mm)	
	GS-2	GS-3
Tributylin agar	5	5
Olive oil with phenol red agar	5	5

Table 2: Lipase activity of bacterial isolate GS-2 and GS-3

Incubation time (hour) of Bacterial isolates	Lipase Activity (U/mL/min)	
	GS-2	GS-3
24	0.058 $\pm$ 0.010	0.097 $\pm$ 0.007

The growth of microorganism was relatively slower on BH agar plate as compared to that on nutrient agar supplement with 1% disposed engine oil. About four days of incubation were required for colony to be visible on BH agar. On the other hand, distinguished colony could be observed after 48 hours when nutrient agar with 1% disposed engine oil was used to grow microorganism. This result suggested that growth of microorganism is slow when disposed engine is used as sole carbon sources as it is not a favorable carbon source for microorganism. This result was agree with Gagandeep and Malik (2013) reported that *Pseudomonas* sp. required 9 days to reach stationary phase of growth when engine oil was used as sole carbon source, also Pandey (2013) showed that *Pseudomonas fluorescenes* reached stationary phase at 5th day. Generally, disposed engine oil is composed of broad range of hydrocarbon, with chain length varied from C16 to C32 (Kupareva et al., 2012). Besides, it possesses properties of low solubility and high hydrophobicity, resulting them recalcitrant for biodegradation. Therefore, microorganism exhibits slow growth rate when engine oil is utilized as carbon sources partial identification of bacterial isolate gs-3 using gram-staining revealed that belongs to Gram negative bacteria.

### Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Data represented in Figure 1 and 2, revealed new and broad band which is related to O-H stretching formed at  $3421\text{ cm}^{-1}$ . Besides, new band could also be observed at  $1706\text{ cm}^{-1}$  and  $1649\text{ cm}^{-1}$ , which was associated occurrence of these bands, signified the formation of carboxylic acid after 2 weeks of biodegradation.

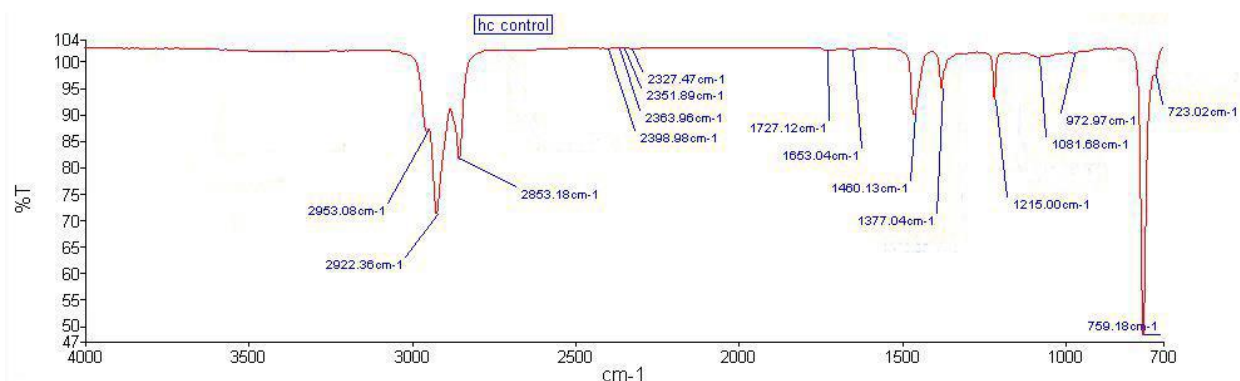


Figure 1: FTIR analysis profile of control sample

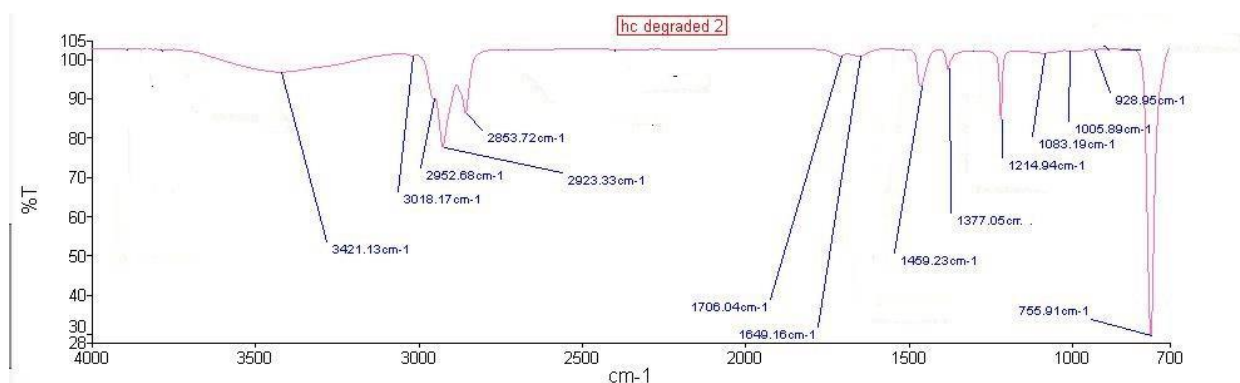


Figure 2: FTIR analysis profile of degraded engine oil after 14 days of incubation

This result is corresponding to that of Dominguez-Rosado and Pichtel (2003), who remediated disposed engine oil utilizing phytoremediation technique and remarked that carboxylic acid is one of the intermediates which were produced along the biodegradation process. They reported that new band occurred at  $3424\text{ cm}^{-1}$  and  $1645\text{ cm}^{-1}$  after bioremediation. Besides, Sadouk et al. (2009) also reported that carboxylic acids.

### Gas Chromatography - Mass Spectrometry (GC-MS) Analysis

GC-MS analysis revealed that crude extract recovered from disposed engine oil after biodegradation contained 3 major compounds: hydrocarbon, alcohol, and carboxylic acid (Table 3). The major component formed was benzoic acid with 89%.

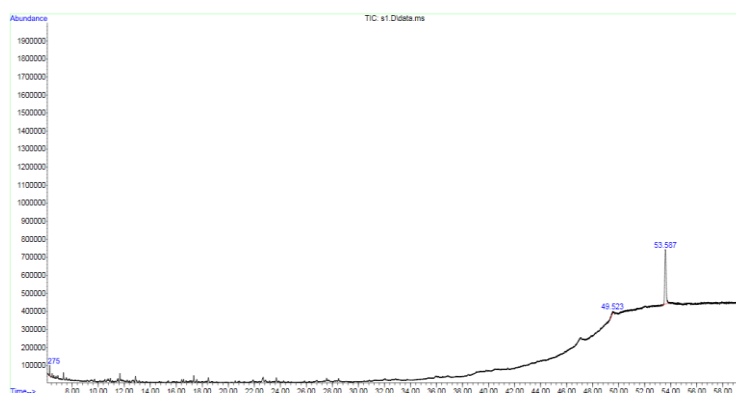


Figure 3: GC-MS chromatogram of recovered crude extract

Figure 3: GC-MS chromatogram of recovered crude extract which are hexadecanoic acid and octadecanoic acid were the major component formed during biodegradation of diesel oil. Subsequent GC-MS analysis confirmed the presence of carboxylic acid in the degradative engine oil. according to data recorded in Table 3, GC-MS analysis showed that extracted product contains high percentage of methyl 3,4,5-trimethoxy-2,6-dinitrobenzoate, small amount of complex alcohol, and aliphatic hydrocarbon of nonadecane sources by GC-MS analysis.

Table 3: Chemical structure of crude extract produced by GS-3 grown on disposed engine oil as sole carbon

Peak	Retention time (minute)	Name of Compound	Area (%)
1	6	Nonadecane	6.11
2	49.5	3,3,7,11 – Tetramethyltricyclo-[5.4.0.0 (4,11)]-undecan-1 –ol	4.88
3	53.6	Benzoic acid, 3, 4, 5-trimethoxy- 2,6- dinitro-methyl ester	89.00

Two engine oil-degrading bacteria, GS-2 and GS-3 were successfully isolated from soil. However, both of the isolated bacteria possess low lipase activity, exhibited only 0.058 and

0.097 U/mL/min respectively after 24 hours of incubation. This is understandable because lipase activity is generally low when hydrocarbon or petroleum is used as carbon sources. Kanwar et al. (2002) only successfully produced maximum 25 U/mL/h of extracellular microbial lipase using analytical grade of *n*-hexadecane as the sole carbon source for *Pseudomonas* species G6. Balaji et al. (2014) also reported that *Penicillium chrysogenum* only exhibited highest lipase activity of 68 U/mL/h during 7 days of incubation. Up to date, only few accessible literatures studied the lipase activity during biodegradation of used engine oil. Over years, researches regarding production of lipase enzyme have been focusing on using lipidic substances such as olive, coconut, or castor oils, all of which are proven to be good substrates for extracellular lipase production. Only few researches have been focusing on production of lipase enzyme using non-lipidic substrates of hydrocarbon. Therefore, comparison of current study to others cannot be done holistically. GS-3 was chosen over GS2 for extracellular lipase enzyme production because it had higher lipase activity, achieving  $0.097 \pm 0.007$  U/mL/min on first day. Besides, the extracellular protein content also showed the similar result when protein content WAS highest during first 24 hour of incubation. However, the protein content did not precisely reflect the total extracellular lipase content because other enzyme would also be secreted along the degradation process of disposed engine oil. Adam et al. (2014) reported that oxidase, catalase, lipase, and peroxide were the common extracellular enzymes which would be excreted during biodegradation of engine oil. Further study needs to be carried out to confirm other extracellular lipase enzyme that secreted by GS-3 isolate besides lipase.

### Optimization of Lipase Production

#### *Extracellular lipase activity of lipase production by GS-3 over 96 hours of incubation*

Protein standard curve was constructed before this experiment Bacterial isolate GS-3 was able to produce highest lipase activity after 24 hours of incubation period, recording  $0.097 \pm 0.007$  U/mL/min with protein content of  $0.4651 \pm 0.010$  mg/ml Figure 4.

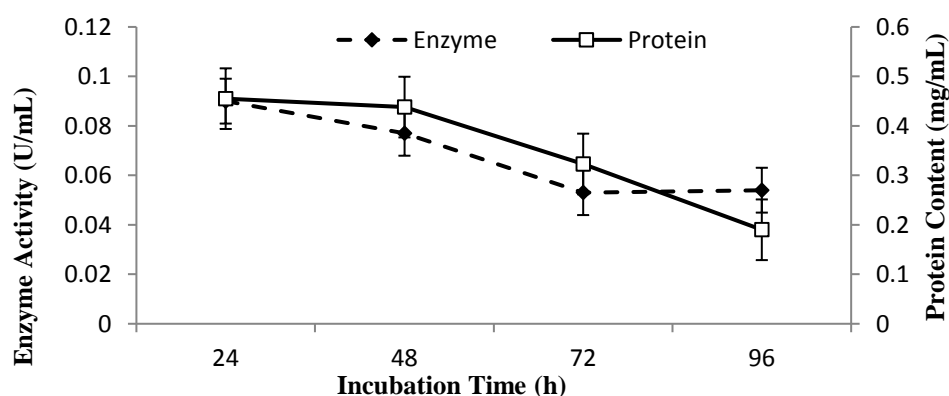


Figure 4: Extracellular lipase activity of lipase production by GS-3 over 96 hours of incubation

#### *Effect of disposed engine oil concentration*

Data represented in Figure 5 illustrated that lipase activity of GS-3 bacterial isolate was highest when 4 % ( v/v) disposed engine oil was used as carbon source, achieving  $0.161 \pm 0.067$  U/mL/min during first 24 hours of incubation. The protein content was also measured to be



0.850± 0.031 (mg/ml). The effect of different concentrations of disposed engine oil as carbon source on lipase activity was studied in this research and revealed that Gs-3 isolate was able to produce extracellular lipase enzyme in range of 1 until 4% of disposed engine oil, suggesting no inhibition effect of disposed engine oil on enzyme production within this range. During first 24th hour, lipase activity WAS highest at 4% recording  $0.161 \pm 0.067$  U/mL/min, followed by 3 and 2%, whereas 1% trailed the most, registered only  $0.079 \pm 0.003$  U/mL/min of lipase activity. This data supported the theoretical statement of Margesin et al. (2002), who opined that lipase activity could be induced and was favored at high petroleum concentration.

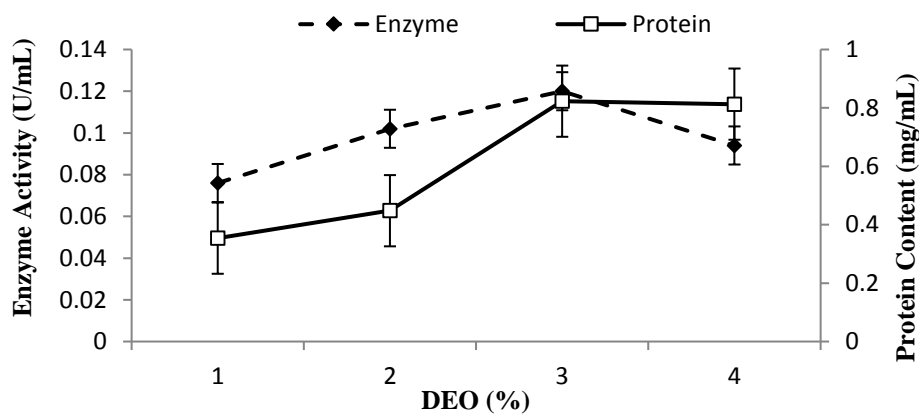


Figure 5: Effect of different disposed engine oil concentration on lipase activity over 24 hours of incubation period

#### Effect of different nitrogen sources

Data represented in Figure 6 showed that urea was the best nitrogen source for extracellular lipase production during first 24 hour of incubation, achieving lipase activity of  $0.240 \pm 0.063$  U/mL/min and protein content up to  $0.722 \pm 0.001$  mg/ml. While protease peptone was a poor nitrogen source for lipase production of GS-3 isolate as the lipase activity was extremely low, recorded only  $0.010 \pm 0.001$  U/mL/min on first day. Urea as nitrogen source enhanced lipase production up to  $0.240 \pm 0.063$  U/mL/min during 24th hour of incubation, approximately 1-fold higher than that of control sample which used ammonium nitrate as nitrogen source. Comparing to ammonium sulfate and ammonium nitrate, urea seemed to be unstable nitrogen sources for extracellular lipase enzyme produced BY GS-3 isolate. Although ammonium nitrate and ammonium sulfate as nitrogen sources could not produce high lipase activity as urea did on first day. Both of them produced lipase activities that varied within range of 0.80 to 0.125 U/mL/min. On the other hand, protease peptone was found out to be poor nitrogen sources for GS-3 isolate for lipase production as it was only able to give lipase activity as low as 0.010 U/mL/min on first day. Therefore, This study showed that inorganic nitrogen sources were better than organic nitrogen sources for lipase production BY GS-3. In contrast Sujatha and Dhandayuthapani (2013) reported that urea inhibited lipase production at concentration above 0.5 mg/L as it is toxic to the culture as high concentration of ammonia produced during urea degradation is toxic to bacteria. This might as well explain the drastic decrease of GS-3 isolate's lipase activity over 4 days when urea was used as nitrogen source. The current also contradicted to other researches which reported that organic nitrogen source was generally better than inorganic source. Gupta *et al.* (2004) reported that organic sources were better than inorganic sources because ammonium salt creates acidic condition due to liberation of free acid

after the utilization of ammonium ions, which might eventually inhibit or interfere the lipase secretion. However, the choice of nitrogen sources for optimal enzyme activity was various among microorganism (Burkert et al., 2004)

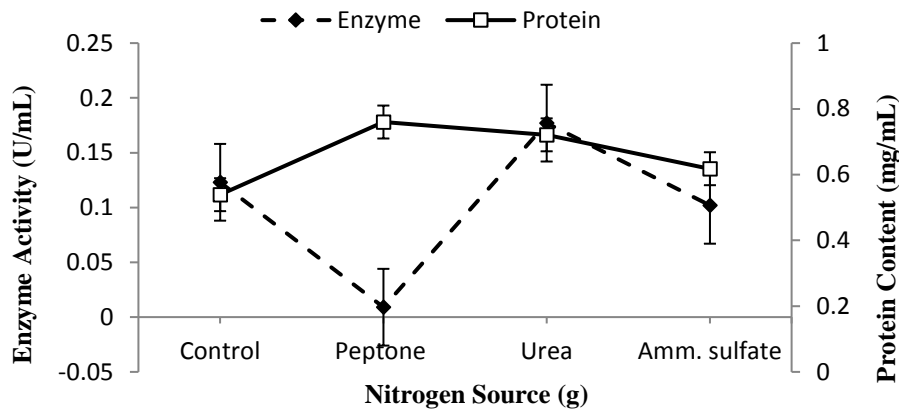


Figure 6: Effect of different nitrogen sources on lipase activity over 24 hours of incubation period

#### *Effect of initial pH on lipase activity*

Data represented in Figure 7 showed the lipase activity of isolate GS-3 over different range of initial pH. Result showed that pH 7 was the optimal initial pH for lipase production achieving  $0.095 \pm 0.002$  U/mL/min and protein content up to  $0.665 \pm 0.086$  mg/ml after 24 hours of incubation.

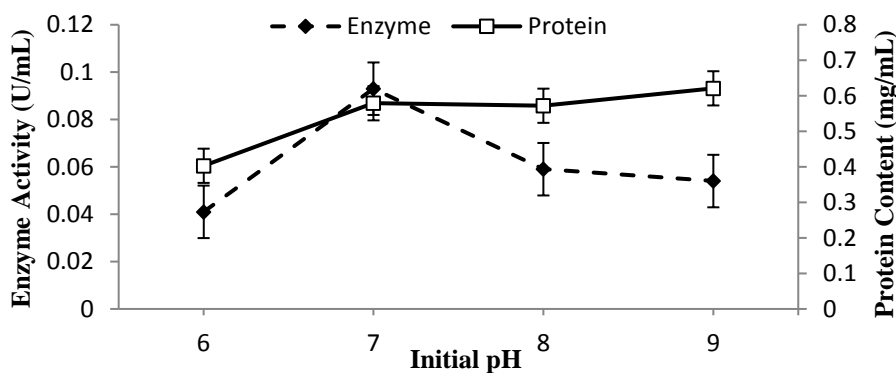


Figure 7: Effect of different initial pH on lipase activity over 96 hours of incubation period

Like temperature, pH was also affecting the metabolic activities of a microorganism. Referring Figure 4, different initial pH affected the extracellular lipase activity of GS-3 isolate. The result showed that lipase production was stable from pH 6 to 9, with highest extracellular lipase activity achieved at pH 7, when  $0.095 \pm 0.002$  U/mL/min lipase activity WAS obtained at first 24th hour of incubation. This suggested that GS-3 is a neutrophilic microorganism. When GS-3 was incubated in production medium of initial pH 9, the main problem of using disposed engine oil as carbon source was the insolubility of engine oil.

### Effect of incubation temperature

Data represented in Figure 8 showed that after 24 hours of incubation, the lipase activity reached the highest yield of  $0.125 \pm 0.013$  U/ml/min at 30 °C of incubation temperature while lowest enzyme activity observed at 50 °C, recorded as  $0.051 \pm 0.007$  U/mL/min during the same period. With protein content up to  $0.580 \pm 0.010$  mg/ml.

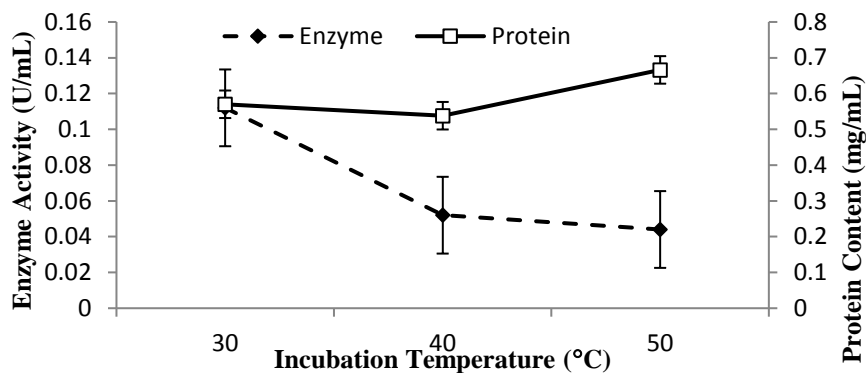


Figure 8: Effect of incubation temperature on lipase activity

Referring Figure 8, data revealed that 30°C was the optimal temperature for lipase production as lipase activity was the highest compared with incubation temperature of 40 and 50°C. GS-3 isolate produced highest extracellular lipase activity at 30°C of first 24h incubation, achieving  $0.125 \pm 0.013$  U/mL/min. This data also showed that GS-3 isolate was mesophilic microorganism. Optimal temperature favoring metabolic and enzymatic activities throughout incubation period. Often, optimal cultivation temperature varies among microorganism. However, 30°C was found out to be the optimal temperature for lipase enzyme production of many other microorganisms. For example, Mukesh et al. (2012) reported that 30°C was the best temperature for *Bacillus* sp. MPTK 912 for production of lipase enzyme from oil mill effluent. Besides, Narasimha et al. (2011) also found out that *Pseudomonas* sp. has highest extracellular lipase activity at the same temperature when olive oil was used as carbon sources.

### Effect of different surfactants

Figure 9 represent the lipase activity was achieved with addition of different surfactants. Tween 80 could enhance the lipase activity to the highest on 24 hours of incubation, reporting  $0.221 \pm 0.001$  U/mL/min. The protein content was also measured and recorded in as  $1.907 \pm 0.031$  mg/ml.

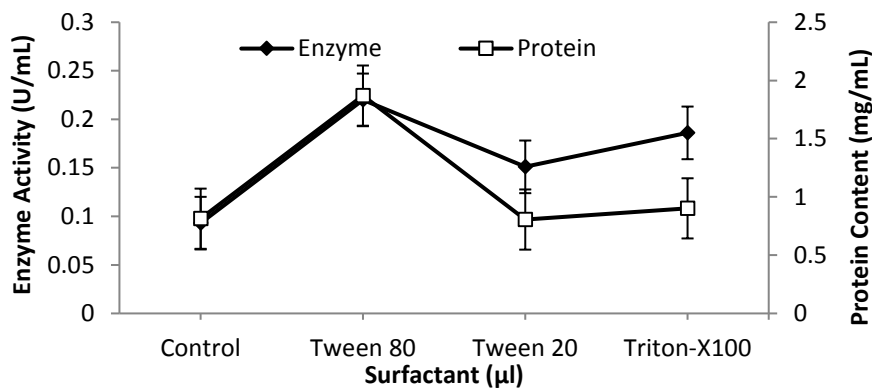


Figure 9: Effect of addition of different surfactants on lipase activity over 24 hours of incubation period

Engine oil is hydrophobic in nature and is not miscible with water. Addition of surfactant into the production medium would improve the solubility of engine oil, which in turn, increases the bioavailability of substrate to bacteria (Liand, 2005). The commonly used surfactants are Triton X-100, Tween 80, and Tween 20, all of which were used in this study to investigate its effect on extracellular lipase enzyme production. The result revealed that all the surfactant used could enhance lipase production significantly at 24 hour of incubation. Referring Figure 9, all three surfactants enhanced the lipase production for approximately 2-fold, with the utilization of Tween-80 provided most promising result when it increased lipase activity to  $0.221 \pm 0.001$  U/mL/min during 24 hour, followed by Triton X-100, registered  $0.189 \pm 0.003$  U/mL/min and Tween-20, recording  $0.179 \pm 0.028$  U/mL/min at the corresponding period. According to Immanuel et al. (2008), Tween-80 plays double role on biodegradation of engine oil. It can induce the production of lipase enzyme besides playing role as an emulsifying agent which increases the solubility of engine oil in medium, rendering them more accessible for biodegradation. Wu and Tsai (2004) also reported that higher level of lipase production could be achieved when Tween-80 was incorporated into a medium with lipidic substrate because it can emulsifying the oil, subsequently lower the interfacial tension between oils and water and resulting increase of cell permeability, thus possibly facilitates enzyme secretion. Referring to Figure 9, lipase activity was lower in the medium supplemented with Tween-20 and Triton X-100 during 48 and 72 hour of incubation as compared with control sample, which had no surfactant. This might be due to the inhibition of surfactant on lipase production. Koley and Bard (2010) reported that if cells are subject to prolonged exposure to Triton X-100, this chemical compound would disrupt hydrogen bonding present within the cell's lipid bilayer, leading to the destruction of the integrity of the lipid membrane.

#### *Effect of ingredient removal from production medium*

Removal of  $\text{FeCl}_3$  from production medium could enhance the lipase activity up to  $0.142 \pm 0.048$  mg/ml after 2 hours of incubation while removal of  $\text{MgSO}_4$  and  $\text{CaCl}_2$  would adversely impact the lipase activity. With protein content up to  $0.572 \pm 0.014$  mg/ml.

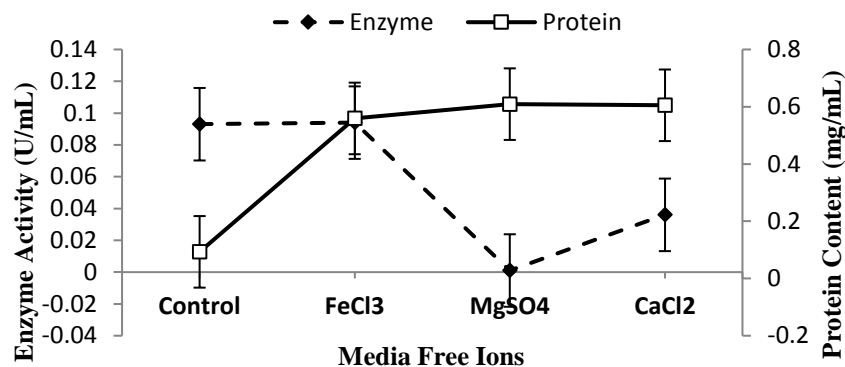


Figure 10: Effect of ingredient removal from production medium on lipase activity over 24 hours of incubation period

The effect of trace element on lipase activity was studied. As previously discussed, lipase activity WAS generally low when disposed engine oil is used as sole carbon source. Besides, the exact chemical composition of disposed engine oil used as substrate for this study remains unknown. Therefore, it was worth to identify the compound in the production media that inhibit the lipase activity, especially trace element like Fe<sup>3+</sup> and Ca<sup>2+</sup>. DATA represented in Figure 10, showed that removal of FeCl<sub>3</sub> from production medium could increase lipase activity. The production medium without FeCl<sub>3</sub> was 0.142±0.048 U/mL/min, approximately 1.5-fold higher than control sample during 24 hour incubation, which produced lipase activity of 0.095±0.002 U/mL/min. Therefore, data revealed that FeCl<sub>3</sub> inhibited lipase enzyme of GS-3 isolate. Besides, MgSO<sub>4</sub> and CaCl<sub>2</sub> were essential nutrients for GS-3 isolate as removal of them from production medium significantly reduced the lipase activity.. Without CaCl<sub>2</sub>, lipase activity halved as compared with control sample throughout the incubation period. Simons et al. (1999) reported that calcium ion was important for structural stabilization of lipase enzyme. Up to date, there is no consistent trend has been reported regarding the effect of metal ion on lipase activity (Shangguan et al., 2011). According to Ali et al. (2009), FeCl<sub>3</sub> drastically inhibit lipase activity FROM *Aspergillusniger* at low concentration. Besides, Fe<sup>3+</sup> ion also inhibited the lipase activity from *Bacillus subtilis* (Ma et al., 2006) and *Staphylococcus* sp at 200 ppm as per Tembhurkar et al. (2012). However, some researches showed contradictory result. For lipase enzyme, metal ion plays important role in structural property rather catalytic property. Acikel et al. (2011) suggested that metal ion will inhibit lipase enzyme activity by reacting with –SH groups of lipase enzyme molecule through oxidation. Liebeton et al. (2001) also remarked that presence of –SH groups in the lipase molecules resulting them to be intrinsically unstable. When metal ions react with –SH groups in the side groups of cysteine residues, tertiary structure of enzyme will be altered subsequently change .The shape of the active site and thus the enzyme becomes deactivated. Therefore, like nitrogen sources, the effect of trace elements on lipase activity varies among bacteria.

## CONCLUSION

This research project was successfully being carried out. Hydrocarbon-degrading bacteria, namely GS-3 was isolated from oil-contaminated soil. It was able to produce complex alcohol and organic acid from disposed engine oil. It is able secrete extracellular lipase, which is an industrially important by-enzyme. This research also demonstrated that optimization of

production medium was very important in enhancing lipase activity. The best nitrogen sources for maximum lipase production during first 24 hours were urea. Removal of FeCl<sub>3</sub> from production medium could increase lipase production to 0.142±0.048 U/mL/min, approximately 1.5-fold higher than control, suggesting that FeCl<sub>3</sub> inhibit the lipase enzyme production. Addition of surfactant, tween 80 could enhance lipase production. Besides, optimal cultivation pH and temperature of isolate GS-3 for maximum lipase production was revealed to be pH 7 and 30°C respectively.

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