Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(6):26-36

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Lipase Production and Optimization from Bioremediation of Disposed Engine Oil

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ABSTRACT

Engine oil is one of the several refined products of crude oil made up of long- chain saturated hydrocarbon (base oil) additives. There are several ways engine oil can enter the environment, such as leaking oil tanks, cleaning of tanks by merchants, warships carrying engine oil, as well as the activities of auto mechanics. Engine oil is a common industrial waste that has harmful environmental and health effects. This study aims to produce lipase enzyme from disposed engine oil (DEO) using a microbial degradation approach. The hydrocarbon-degrading bacterium, GS-3 was isolated from an oil-contaminated area in Kuantan, the capital city of Pahang, a state in Malaysia. Upon GC-MS analysis, it was revealed that GS-3 was able to produce methyl-3, 4, 5-trimethoxy-2, 6-dinitrobenzoate, an organic acid from the DEO. Besides, GS-3 recorded the highest lipase activity of 0.097 ± 0.007 U/ml/min within the first 24 h of activity, using DEO as the only carbon source. The FTIR data revealed the presence of a new broadband at 3421 cm^{-1} wavelength attributed to the stretching of the O-H group, though a new band occurred at 3424 cm^{-1} and 1645 cm^{-1} wavelength after the bioremediation process. The subsequent optimization of the process parameters for the production of lipase revealed that the bacteria perform optimally when the DEO was used as the sole carbon source at the concentration of 4% (v/v), with pH and temperature values of 7.0 and 30°C, respectively. Urea was the best source of nitrogen within the first 24 h of incubation; the addition of Tween 80, a surfactant, enhanced the production of lipase. In conclusion, lipase enzyme production through a microbial process could be a better way to manage DEO and other oil-related environmental contaminants.

Keywords: Bioremediation; Disposed engine oil; Lipase production; Optimization

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INTRODUCTION

Engine oil (lubrication oil) is one of the products of base oil produced through the distillation of petroleum. It is a non-volatile liquid and acts as a friction reducer between two moving metal parts; playing a key role in the maintenance of internal combustion engines. After being drained from an automobile or generator engine, it is commonly referred to as disposed engine oil (DEO). According to the Environmental Quality (Scheduled Wastes) Regulations 2005, it is mandatory to recycle or dispose used engine oils at a licensed facility to prevent pollution. Despite the enforcement of these rules and regulations, cases of indiscriminate disposal of DEO remain prevalent. The DEO is composed mainly of long-chain hydrocarbons and some amount of heavy metals [1]. A precise description of the chemical composition of DEO is not feasible due to its complex nature [2]. The indiscriminate disposal of DEO could cause environmental problems especially when it finds its way into the soil, resulting in the depletion of the nutrients that are necessary for plant growths such as nitrogen and phosphorus, and thereby,

affecting the physical properties of the soil [3]. These DEOs are recalcitrant pollutants, containing long chain saturated and aromatic hydrocarbon. An effective biodegradation of DEO, therefore, requires a concerted effort of a consortium of microorganisms, be it prokaryotes or eukaryotes. Moreover, the presence of heavy metals and polycyclic aromatic hydrocarbons might hinder the microbial degradation process as well [4]. It is, therefore, challenging to isolate a single microorganism that can degrade the wide range constituents that made up the DEO. Some bacterial species such as include *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Staphylococcus* have been reported as excellent oil degraders [5-9].

Several studies have reported the negative impact of DEOs on the environment and their potential threats to public health. There are reports suggesting that DEOs are toxic and have considerable adverse effects on the growth of plants [10]. The presence of DEOs in the soil has also been said to inhibit the growth of seedlings and the inhibition effect on the seedlings actually depend on the concentration of DEO in the soil [10]. When disposed into the soil, there is a considerable decrease in the aeration of the soil due to the absorption of the soil particles by the oil [11]. When spilled on the land, DEO can be rapidly transferred to the aquatic environments by rain and runoff waters. This study, therefore, aims to degrade DEO through the use of a hydrocarbon-degrading bacterium (GS-3) isolated from an oil-contaminated area of Kuantan, the capital city of Pahang, a state in Malaysia.

EXPERIMENTAL SECTION

Sample Collection

Soil samples were collected from an oil-contaminated area of an automobile workshop located in Kuantan. The soil samples were collected from a trench of about 10 cm deep dug from the surface of the soil to avoid contamination and stored in sterile bottles. The soil samples were homogenized using a mortar and pestle and sieved through a sieve with a pore size of 2 mm to remove unwanted debris. The sieved soil samples were kept tightly closed in polyethylene bags and stored at 4 ± 1°C.

Media Preparation

The isolation of the engine oil-degrading bacteria used in this study was done in Bushnell-Haas (BH) broth media [12]. The media composition was as follows: $MgSO_4.7H_2O$ 0.2 (g/L), K_2HPO_4 1.0 (g/L), KH_2PO_4 1.0 (g/L), $FeCl_3$ 0.05 (g/L), NH_4NO_3 1.0 (g/L), and $CaCl_2$ 0.02 (g/L) with a final pH of 7.2. The prepared media were sterilized in an autoclave (MO-GR) at 121°C for 15 minutes. About 50 mL of the autoclaved media was transferred into 250 mL Erlenmeyer flasks and supplemented with 2% (v/v) DEO as the sole carbon source for microbial growth.

Isolation of the Hydrocarbon-Degrading Bacteria

The isolation of the bacteria used in this study was carried out using the method described by [13]. One gram of the oil-contaminated soil sample was suspended in 10 mL of sterilized distilled water and vigorously shaken. Then, 1 mL of the mixture was transferred into 50 mL of BH medium and incubated at 30°C for 7 days at an agitation rate of 170 revolutions per minute (rpm). After the incubation period, 1 mL of the fermentation broth was withdrawn and diluted with 6 dilution series, each of 10^{-1} fold using sterile distilled water. A 100 µL aliquot of each fifth and sixth dilution series was taken and plated onto the BH agar enriched with 1% DEO, and nutrient agar supplemented with 1% DEO. The bacterial colonies with different morphological characteristics were isolated and purified to on nutrient agar plates supplemented with 1% DEO using the streaking technique to obtain single colonies.

Screening of Extracellular Lipase Producing Bacteria

The screening of the lipase producing bacteria was done using two types of screening agar namely: Tributyrin agar (TBA) and Olive oil with phenol red agar. The TBA medium is comprised of Tributyrin (1% w/v), $CaCl_2$ (0.1% w/v), and agar (2% w/v). The pH of the prepared agar was adjusted to 7.0 before been autoclaved at 121 °C for 15 minutes. The Olive oil agar plates contained phenol red (0.01% w/v), Olive oil (0.1% v/v), $CaCl_2$ (0.1% w/v), and agar (2% w/v). The pH of the olive oil plate was adjusted to 7.3 before been autoclaved at 121°C for 15 minutes. All the isolated bacteria were screened for extracellular lipase enzyme activity qualitatively and quantitatively.

Qualitative Screening Method

The qualitative screening of the organisms was done by streaking the isolated bacterial onto TBA and Olive oil media [14]. The culture plates were incubated at 30°C for 24 h. According to [15], the formation of clear halo zone around the TBA colonies indicates the production of extracellular lipase enzyme; but for the olive oil agar plates, the production of lipase enzymes will be indicated by the formation of a yellow coloration. The positive colonies for

lipase enzyme production were then, inoculated on agar slants and subsequently incubated at 30°C for 24 h before storing at 4°C as stock cultures.

Quantitative Screening Method

For the quantitative screening of the isolates for lipase production, 2 loops of a 24 h culture were inoculated into 50 mL of nutrient broth (NB) supplemented with 2% DEO. The culture was incubated for 24 h at 30°C and agitation of 150 rpm in an incubator shaker (Benchtop incubator). After incubation, the overnight culture was suspended in 0.9% (w/v) sodium chloride (NaCl) solution to obtain an initial cell density of 0.5 McFarland standards.

The Preparation of McFarland Standards

Submerged microbial culture was incubated in 250 mL Erlenmeyer flasks containing 50 mL of BH broth supplemented with 2% DEO before inoculation with 1 mL of the bacterial suspension. After 24 h of incubation, the culture was centrifuged at 10,000 rpm for 20 minutes at 4°C to obtain the cell-free filtrate used as the crude enzyme. The quantitative assay was carried out by determining the lipase activity of the crude enzyme using titrimetric assay [16]. The reaction mixture contained 5 mL of an Olive oil emulsion, 4 mL of 100 mM sodium phosphate buffer (pH 7), and 1 mL of the crude enzyme. The reaction mixture was incubated at 30°C with agitation of 250 rpm for 2 h. The control mixture contained all the necessary components but the crude enzyme was pre-heated at 100°C to be inactivated before adding into a reaction mixture. The reaction was stopped by adding 5 mL of 1:1 (v/v) acetone–ethanol mixture after 2 h of incubation. The amount of fatty acid liberated after the incubation period 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 to release 1 µmol of fatty acid per minute under the assay conditions. The enzyme activity was expressed as units (U) per milliliter of enzyme extract and was calculated using Equation 1.

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

Selection of the Most Potent Bacteria

The most potent bacteria were selected based on the size of the halo zone on the TBA agar and the yellow zone on the Olive oil agar. The partial identification of the bacterial isolates was carried out through the Gram differential staining technique to identify the Gram reaction of the chosen isolates [17].

Crude Enzyme Recovery

The product of the fermentation process was extracted using a liquid-liquid extraction technique previously described in the literature [18]. A 50 mL of Trichloromethane was added to the broth culture into the conical flask and thoroughly shaken. The mixture was then, transferred into a separating funnel and allowed to stand for 2 h. The layer containing the organic phase and the residual oil was emptied into a beaker. After that, the crude extracts were subjected to GC-MS and FTIR analysis for the identification of its components.

Protein Determination

The extracellular protein content of the crude extract was determined according to an already established method [19]. Bovine serum albumin (BSA) was used as the protein standard during the preparation of the protein standard curve. The protein standard curve was prepared over a range of BSA concentrations (0.0 - 0.8 mg/ml).

Optimization of Lipase Enzyme Production Parameters

Engine oil concentration:

The effect of the concentration of DEO on the production of lipase enzyme was studied at different concentrations ranging from 1 to 4%. During the study of the concentration effect, all the other parameters were kept fixed.

Nitrogen source:

The effect of the nitrogen source on lipase enzyme production was studied by replacing ammonium nitrate with other three nitrogen sources (urea, peptone, and ammonium sulfate) at the concentration of 1% (w/v). Cultures with ammonium nitrate as the nitrogen source served as the control.

Different surfactants:

The effect of adding different surfactants on lipase enzyme production was studied using three different surfactants, namely Tween 80, Tween 20, and Triton X-100. These surfactants were tested individually using 50 µL of each in the BH medium supplemented with 2% disposed engine oil. The culture plates without surfactants served as the controls.

Incubation temperature:
The effect of the incubation temperature on lipase enzyme activity was studied within the range of 30 to 50°C. The microbial isolates were inoculated on the BH media and incubated in batches at 30, 40, and 50°C. The lipase activity of each batch was measured every 24 h for 4 days. During this study, all other parameters were kept unchanged.

Medium pH:
The effect of the initial pH of the medium was studied by varying the pH of the BH medium from 6 to 9 while keeping all the other parameters unaltered.

Trace elements:
The effect of the level of trace elements on lipase enzyme activity was carried out by individually removing the trace element ($FeCl_3$, $MgSO_4$, and $CaCl_2$) from the production media. The controls were established by using the original BH medium without the removal of any ingredient. All the other parameters were kept unchanged. After the incubation period of each parameter, the lipase activity and extracellular protein content were measured every 24 h until the end of the experiment.

RESULTS AND DISCUSSION

Isolation and Screening of Hydrocarbon-Degrading Bacteria
A total of 10 oil-degrading bacteria designated GS-1 to GS-10 was successfully isolated from soils collected from different locations of study. Only 2 (GS-2 and GS-3) out of the 10 isolates produced extracellular lipase enzyme. The qualitative screening of the isolates showed that the two isolates had the same level of lipase activity. However, the titrimetric assay showed that GS-3 performed better as a lipase producer as can be seen in Table 1.

Table 1: 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 ± 0.010 0.097 ± 0.007

The growth of the microorganisms was relatively slower on BH agar plate when compared to that on nutrient agar supplemented with 1% DEO. About 4 days of incubation was required for the colonies to be visible on the BH agar, while distinctive colonies were visible after just 48 h on the nutrient agar plate supplemented with 1% DEO. This result suggested that the growth of the microorganism was slower when DEO was used as the sole carbon source as it is not a favorable carbon source for microorganisms. This result agreed with a previous which reported that *Pseudomonas spp* required 9 days to reach the stationary phase of growth when DEO was used as the sole carbon source [20]. It was also shown that *Pseudomonas fluorescenes* reached the stationary phase of growth after 5 days of growth [21]. Generally, DEO is composed of a broad range of hydrocarbons, with varying chain lengths (C16 - C32) [1]. Besides, it has low solubility and high hydrophobicity, resulting in their recalcitrant nature towards biodegradation. Therefore, the microorganism can exhibit a slow growth rate when DEO is utilized as the sole carbon source. A partial identification of the isolates showed that GS-3 is a Gram-negative bacteria.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis
The data shown in Figures 1 and 2 revealed the presence of a new and broadband which is related to the O-H stretching formed at 3421 cm^{-1}. Besides, a new band was also observed at 1706 cm^{-1} and 1649 cm^{-1} corresponding to the formation of carboxylic acid after 2 weeks of biodegradation.

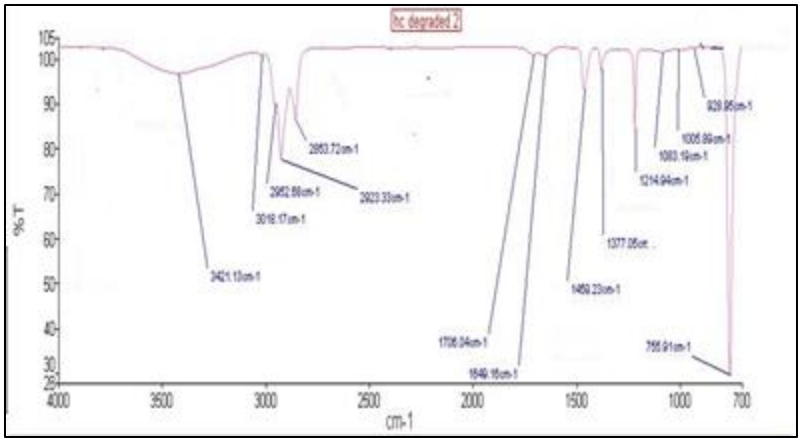

Figure 1: FTIR analysis profile of degraded engine oil after 14 days of incubation

This result corresponds with other DEO remediation reports where carboxylic acid was reported as one of the intermediates which can be produced along the biodegradation process [22]. New bands at 3424 cm^{-1} and 1645 cm^{-1} corresponding to the carboxylic group have also been reported after bioremediation processes [22,23].

Gas Chromatography - Mass Spectrometry (GC-MS) Analysis

The GC-MS analysis revealed that the crude extract recovered from the DEO after biodegradation contained 3 major compounds which are hydrocarbons, alcohols, and carboxylic acids (Table 2). The major component formed was benzoic acid with an observed formation level of 89%.

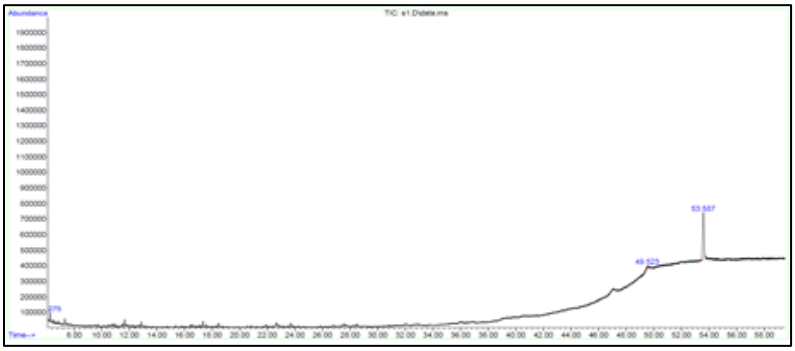

Figure 2: GC-MS chromatogram of recovered crude extract which are hexadecanoic acid and octadecanoicacid were the major component formed during biodegradation of diesel oil

The subsequent GC-MS analysis confirmed the presence of carboxylic acid in the degraded DEO. According to the results in Table 2, the GC-MS analysis showed that the extracted product contained a high percentage of methyl 3, 4, 5-trimethoxy-6, 7-dinitrobenzoate, a small amount of complex alcohol, and aliphatic hydrocarbon of nona-decane sources.

Table 2: 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-trimethyoxy- 2,6- dinitro- methyl ester	89

Two bacteria with engine oil-degrading ability (GS-2 and GS-3) were successfully isolated from the oil contaminated soil. However, both isolates possessed low lipase activities of 0.058 and 0.097 U/mL/min respectively. This is likely due to the fact that lipase activity is usually low when hydrocarbons or petroleum is used as carbon sources during bacterial growth. Studies have reported a successful production of a maximum of 25 U/mL/h of extracellular microbial lipase using n-hexane (analytical grade) as the sole carbon source for *Pseudomonas* species G6 [24]. Also, *Penicillium chrysogenum* has been reported as the only bacteria that exhibited the highest lipase activity of 68 U/mL/h within 7 days of incubation [25]. Up to date, only a few studies have reported lipase activity

during the biodegradation of DEO. Over the years, studies on the production of lipase enzyme have focused on the use of lipids such as olive, coconut, or castor oils, all of which are proven to be good substrates for the production of extracellular lipase. Only a few studies have focused on the production of lipase enzyme using non-lipid substrates such as DEO. A comparison of the current study to previous studies is, therefore, limited and cannot be extensively done. The GS-3 isolate was selected over GS-2 for extracellular lipase enzyme production study because it recorded a higher lipase activity of 0.097 ± 0.007 U/mL/min on the first day. Besides, the extracellular protein content of GS-3 also showed a similar trend, with the highest concentration been achieved within the first 24 h of incubation. However, the protein content did not precisely reflect the total extracellular lipase content because other enzymes could also have been secreted during the degradation process. Oxidase, catalase, lipase, and peroxide have been reported as some of the common extracellular enzymes which can be excreted during biodegradation of engine oil [26]. Further study needs to be done to confirm the secretion of other extracellular lipase enzymes by the GS-3 isolate besides lipase.

Optimization of Lipase Enzyme Production Parameters

Extracellular lipase activity of GS-3- derived lipase over 96 h of incubation:

A protein standard curve was constructed before the initiation of this study. The GS-3 isolate was able to produce the highest lipase activity of 0.097 ± 0.007 U/mL/min after 24 h of incubation and a protein content of 0.4651 ± 0.010 mg/mL (Figure 3).

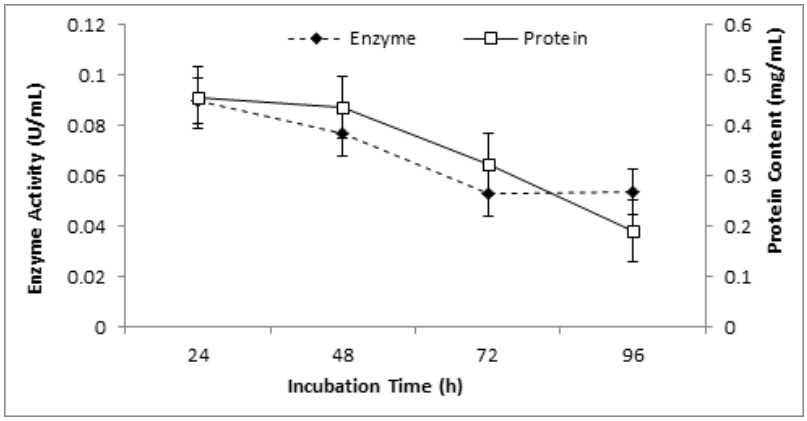

Figure 3: Extracellular lipase activity of lipase production by GS-3 over 96 hours of incubation

DEO concentration:

The results shown in Figure 4 illustrated that the lipase activity of GS-3 isolate was highest when 4% (v/v) of the DEO was used as the carbon source, achieving a lipase enzyme activity of 0.161 ± 0.067 U/mL/min and protein content of 0.850 ± 0.031 mg/mL during the first 24 h of incubation. The effect of different concentrations of the DEO as the carbon source on lipase activity was studied and the results revealed that GS-3 isolate was able to produce extracellular lipase enzyme when the DEO was used within the range of 14%, suggesting no inhibitory effect on the enzyme production within this range. During the first 24 h, the lipase activity was highest (0.161 ± 0.067 U/mL/min) when the oil was used at 4%, followed by 3%, and 2%; 1% trailed the most, registering only 0.079 ± 0.003 U/mL/min of lipase activity. This data supported a previous theoretical statement where lipase activity was opined could be induced and favored at a high petroleum concentration [27].

Different nitrogen sources:

The data presented in Figure 5 showed urea as the best nitrogen source for extracellular lipase production during the first 24 h of incubation; achieving a lipase activity of 0.240 ± 0.063 U/mL/min and protein content of 0.722 ± 0.001 mg/mL. Protease peptone was seen as a poor nitrogen source for GS-3 during lipase production of GS-3 isolate as the lipase activity was extremely low, recording only 0.010 ± 0.001 U/mL/min on the first day. Urea as a nitrogen source enhanced lipase production, achieving 0.240 ± 0.063 U/mL/min during the first 24 h of incubation, approximately 1-fold higher than that of the control sample where ammonium nitrate was used as the nitrogen source. Comparing to ammonium sulfate and ammonium nitrate, urea seemed to be an unsuitable nitrogen source for the GS-3 isolate since ammonium nitrate and ammonium sulfate produced lipase activities within the range of 0.80 to 0.125 U/mL/min.

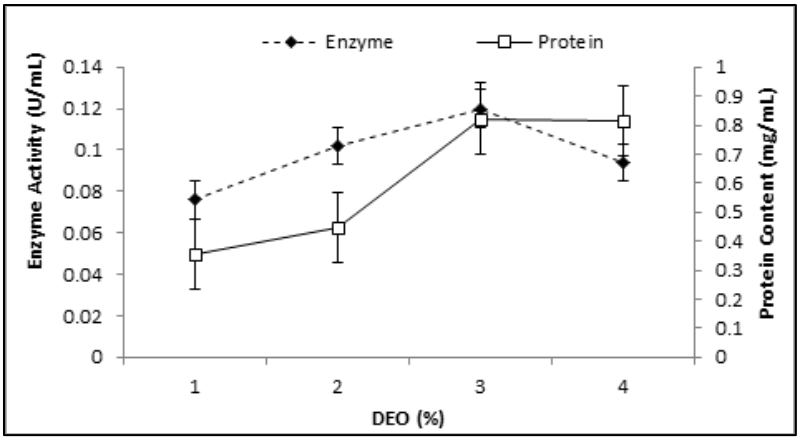

Figure 4: Effect of different disposed engine oil concentration on lipase activity over 24 hours of incubation period

On the other hand, protease peptone was found to be a poor nitrogen source for the GS-3 isolate for lipase production as it was only able to give a lipase activity as low as 0.010 U/mL/min on the first day. Therefore, this study concludes that inorganic nitrogen sources performed better than the organic nitrogen sources used in this study for lipase production. In contrast, it has been reported that urea can inhibit lipase production at concentrations above 0.5 mg/L as it is toxic to the culture [28]. This might as well explain the drastic decrease in lipase activity after 4 days when urea was used as the nitrogen source. The current study also contradicted to other studies which reported that organic nitrogen sources were generally better than inorganic sources. Organic sources have been reported to be better than inorganic sources because ammonium salts create acidic condition due to the liberation of free acids after the utilization of ammonium ions which might eventually inhibit or interfere with lipase secretion [29]. However, the choices of nitrogen sources for optimal enzyme activity depend on the microorganism employed [30].

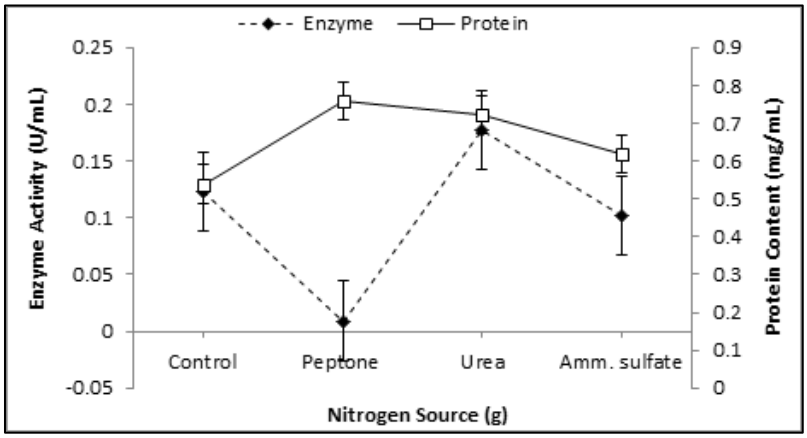

Figure 5: Effect of different nitrogen sources on lipase activity over 24 hours of incubation period

Initial pH:

The data presented in Figure 6 showed the lipase activity of GS-3 isolate over different ranges of initial pH. The results showed that pH 7 was the optimal initial pH for lipase production, achieving a lipase activity of 0.095 ± 0.002 U/mL/min and protein content of 0.665 ± 0.086 mg/mL after 24 h of incubation.

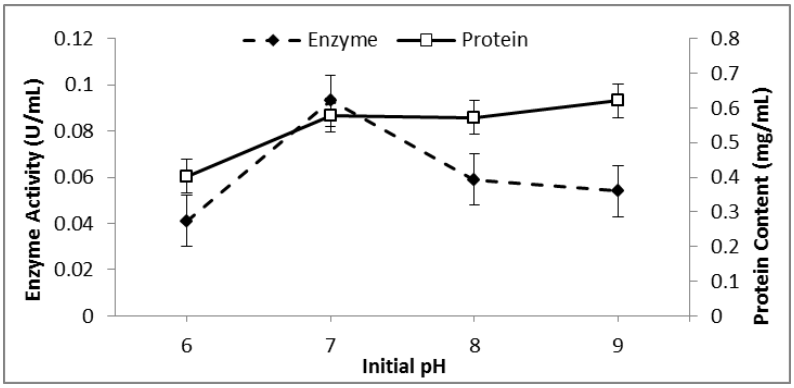

Figure 6: Effect of different initial pH on lipase activity over 96 hours of incubation period

Like temperature, pH can also affect the metabolic activity of microorganisms. Based on Figure 4, different initial pH affected the extracellular lipase activity of the GS-3 isolate. The results showed that lipase production was stable from pH 6 to 9, with the highest (0.095 ± 0.002 U/mL/min) activity achieved at pH 7, when after the first 24 h of incubation, suggesting that GS-3 is a neutrophilic organism. When GS-3 was incubated in a production medium with initial pH of 9, the enzyme activity decreased. The main problem with using DEO as a carbon source is the insolubility of engine oil. A reasonable level of lipase enzyme production has been opined to be achievable at pH 6.17 [31].

Incubation temperature:

The data presented in Figure 7 showed that after 24 h of incubation at 30°C, the lipase activity had already reached the highest yield of 0.125 ± 0.013 U/mL/min while the lowest enzyme activity (0.051 ± 0.007 U/mL/min) was observed at 50ºC within the same period of incubation.

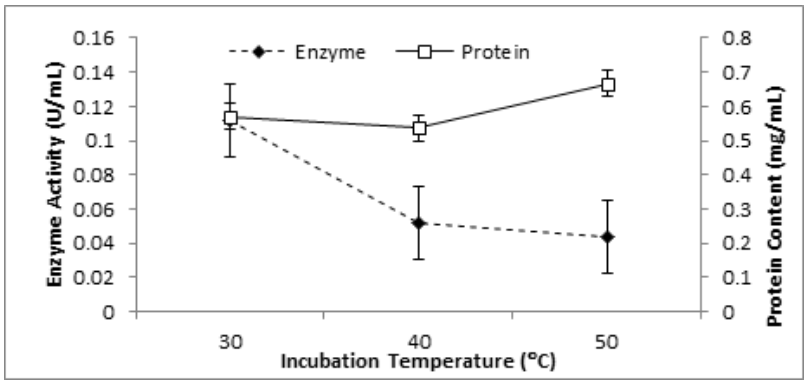

Figure 7: Effect of incubation temperature on lipase activity

From Figure 7, a temperature of 30°C was shown as the optimal temperature for lipase production as the activity was highest (0.125 ± 0.013 U/mL/min) at 30°C compared with other temperatures, suggesting that GS-3 is a mesophilic organism. The optimal temperature favored the metabolic and enzymatic activities of the organism throughout the incubation period. Often, the optimal cultivation temperature varies among microorganism. However, 30°C has been found to be the optimal temperature for lipase enzyme production from many microorganisms. For example, 30°C was reported as the best temperature for *Bacillus spp* MPTK 912 during the production of lipase enzyme from oil mill effluent [32]. Besides, *Pseudomonas spp* was also found to have the highest extracellular lipase activity at the same temperature when olive oil was used as carbon sources [33].

Different surfactants:

Figure 8 presents the lipase activity achieved with the addition of different surfactants. Tween 80 maximally enhanced lipase activity after 24 h of incubation, with a reported activity of 0.221 ± 0.001 U/mL/min. The protein content was also found to be 1.907 ± 0.031 mg/mL.

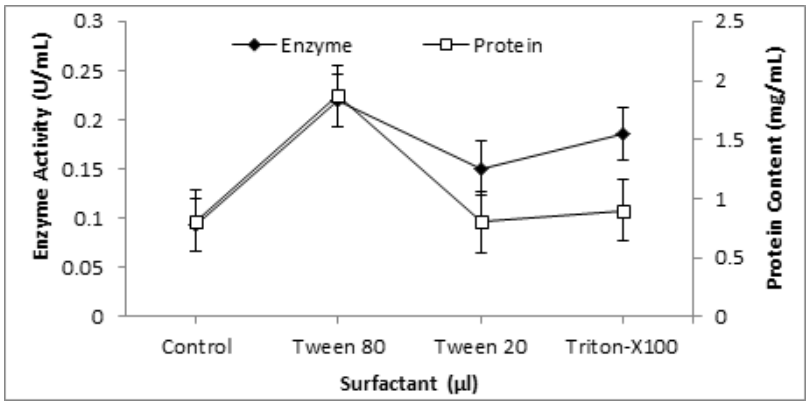

Figure 8: Effect of addition of different surfactants on lipase activity over 24 hours of incubation period

Engine oil is hydrophobic in nature and is not miscible with water. The addition of surfactant into the production medium would improve the solubility of engine oil, which in turn increases the bioavailability of the substrate to the bacteria [34]. The commonly used surfactants are Triton X-100, Tween 80, and Tween 20, all of which were used in this study to investigate their effect on extracellular lipase enzyme production. The results revealed that all the surfactant could enhance lipase production significantly within 24 h of incubation. From Figure 8, all the three surfactants enhanced lipase production by approximately 2 folds, with Tween-80 presenting as the most promising surfactant with a lipase activity of 0.221 ± 0.001 U/mL/min during the first 24 h. Triton X-100 was the next performer with a lipase activity of 0.189 ± 0.003 U/mL/min, and then, Tween-20 recording 0.179 ± 0.028 U/mL/min within the same period. Tween-80 plays a double role on the biodegradation of engine oil; it can induce the production of lipase enzyme besides playing the role of an emulsifier which increases the solubility of engine oil in a medium, rendering them more accessible for biodegradation [35].

A higher level of lipase production has also been reported to be achieved when Tween-80 was incorporated into a medium containing a lipid substrate, where it acted as an emulsifier, subsequently lowering the interfacial tension between oil and water, and resulting in increased cell permeability, thus, possibly facilitating enzyme secretion [36]. From Figure 8, the lipase activity was lower in the medium supplemented with Tween-20 and Triton X-100 during 48 and 72 h of incubation when compared with the control samples that had no surfactant. This might be due to the inhibition effect of the surfactant on lipase production. Cells have been reported to be disrupted when exposed to Triton X-100 over a long period due to the alteration of the hydrogen bonds present within the cell's lipid bilayer, leading to the destruction of the integrity of the lipid membrane [37].

Trace element withdrawal/removal from the production medium:

The effect of trace element on lipase activity was studied. The withdrawal of $FeCl_3$ from the production medium had a positive effect on the lipase activity, with a reported activity of 0.142 ± 0.048 mg/mL after 2 h of incubation; while the withdrawal of $MgSO_4$ and $CaCl_2$ adversely impacted the lipase activity.

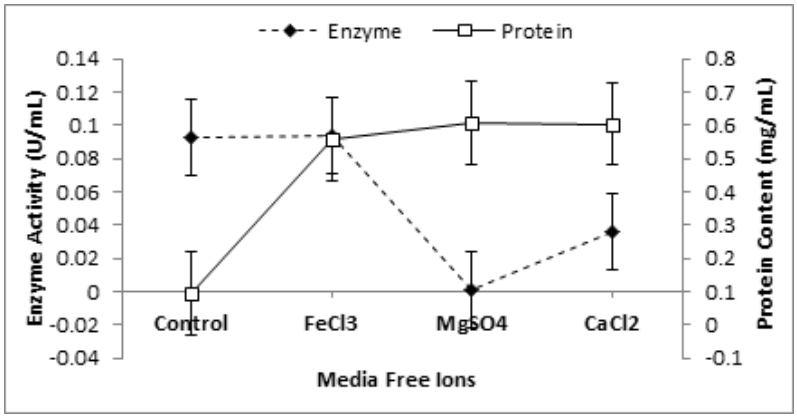

Figure 9: Effect of ingredient removal from production medium on lipase activity over 24 hours of incubation period

As previously discussed, lipase activity was generally low when DEO was used as the sole carbon source. Besides, the exact chemical composition of DEO used as the substrate in this study remains unknown. Therefore, it was

worth to identify the compounds in the production media which can inhibit the lipase activity, especially the trace element such as like Fe^{3+} and Ca^{2+}. The data presented in Figure 9 showed that the withdrawal of $FeCl_3$ from the production medium could enhance lipase activity. The production medium without $FeCl_3$ had a lipase activity of 0.142 ± 0.048 U/mL/min, approximately1.5 fold higher than the control sample (0.095 ± 0.002 U/mL/min) within 24 h of incubation. The data, therefore, revealed that $FeCl_3$ inhibited lipase enzymes of the GS-3 isolate. Besides, $MgSO_4$ and $CaCl_2$ were essential nutrients for the GS-3 isolate as their withdrawal from the production medium significantly reduced the lipase activity. Without $CaCl_2$, the lipase activity halved as compared with the control sample throughout the incubation period. Calcium ion has been reported as a factor for structural stabilization of lipase enzymes [38]. Up to date, there is no reported trend on the effect of metal ions on lipase activity [39]. $FeCl_3$ was reported to drastically inhibit lipase activity from *Aspergillus niger* at low concentrations [40]. Besides, Fe^{3+} ion also inhibited the lipase activity from *Bacillus subtilis* [41] and *Staphylococcus spp* at 200 ppm [42]. However, some studies showed contradictory results. According to [31], maximum lipase was reported when using $MgSO_4$ at the concentration of 0.03%. For lipase enzymes, metal ions play important role in their structural property rather than catalytic property. It has been suggested that metal ion can inhibit lipase enzyme activity by reacting with the –SH groups of the lipase enzyme molecule through oxidation [43,44].

The presence of –SH groups in the lipase molecules have also been remarked as the cause of their intrinsic instability. When metal ions react with –SH groups in the side groups of cysteine residues, the tertiary structure of enzymes will be altered subsequently. The shape of the active site will be altered and thus, the enzyme will be deactivated. Therefore, just as nitrogen sources, the effect of trace elements on microbial lipase activity varies across species.

CONCLUSION

This study successfully isolated a hydrocarbon-degrading bacterium (GS-3) from the oil-contaminated soil. The isolate was able to produce complex alcohols and organic acids from DEO, and also secreted extracellular lipase, an industrially important enzyme. This study also demonstrated that the optimization of the process parameters during the production of the lipase enzyme enhanced the secretion of the enzyme from the isolate. Urea was the best nitrogen source for maximum lipase production during the first 24 h though it had a negative effect when the duration of the process was prolonged. The withdrawal of $FeCl_3$ from the production medium led to an increase in the enzyme production (0.142 ± 0.048 U/mL/min), suggesting that $FeCl_3$ inhibited the lipase enzyme production. The addition of surfactants also enhanced lipase enzyme production. Besides, the optimum pH and temperature for the maximum production of lipase enzyme from the isolated GS-3 were found to be 7 and 30 °C, respectively.

ACKNOWLEDGMENT

The authors gratefully acknowledge Universiti Malaysia Pahang (UMP), Malaysia for the financial supported by grants RDU160333 and PGRS170314 that enables the authors to accomplish this work.

REFERENCES

[1] A Kupareva; P Mäki-Arvela; H Grénman; K Eränen; R Sjöholm; M Reunanen; DY Murzin. *Energ Fuel*. **2012**, 27(1), 27-34.
[2] MM Amro. *Microbiol Rev.* **2004**, 45, 180-209.
[3] A Amadi; DA Samuel; M Anthony. *J Water Air Soil Pollut.* **1994**, 67(1-3), 28-30.
[4] SH Lee; BI Oh; J Kim; *Bioresour Technol*. **2008**, 99:2578-2587.
[5] OS Obayori; LB Salam; OS Ogunwumi. *J Bioremed Biodeg*. **2014**, 5, 213.
[6] D Borah; RNS Yadav. *Arab J Sci Eng*. **2014**, 39(7), 5337-5345.
[7] E Stephen; OE Emmanuel; OS Okpanachi; S Emmanuel; OT Temola; K Musa; IP Ebiloma. *Nat Sci*. **2013**, 11(10): 40-44.
[8] R Thavasi; S Jayalakshmi; IM Banat. *Bioresource Technol*. **2011**, 102(3), 3366-3372.
[9] AJ Thatheyus; D Ramya. *Sci Int.* **2014**, 2(1), 76-81.
[10] J Kayode; O Olowoyo; A Oyedeji. *Res J Soil Biol.* **2009**, 1(1): 15-19.
[11] B Alloway; DC Ayres. Chemical principles of environmental pollution. CRC press, **1997**.
[12] M Bhattacharya; D Biswas; S Sana; S Datta. *Biotech*. **2015**, 3, 1-11.
[13] P Sivapuram. *Adv Environ Biol.* **2011**, 5(6), 1051-1057.

[14] LP Lee; HM Karbul; M Citartan; SC Gopinath; T. Lakshmipriya; TH Tang. *BioMed Res Int.* **2015**, 15(1), 1-10.
[15] K Sagar; Y Bashir; MM Phukan; BK Konwar. *Int J Sci Technol Res.* **2013**, 2(10), 3-10.
[16] A Mustranta. *Appl Microbiol Biotechnol*. **1992**, 38(1): 61-66.
[17] R Tiwari; S Buse; C Herstatt. Innovation via global route: Proposing a reference model for chances and challenges of global innovation processes. **2007.**
[18] KIT Eniola; OA Opasola. *J Environ Sci Toxicol Food Technol.* **2014**, 8(5), 66-70.
[19] OH Lowry; NJ Rosebrough; AL Farr; RJ Randall. *J Biol Chem*. **1951**, 193(1), 265-275.
[20] S Gagandeep; DK Malik. *Int J Chem Anal Sci*. **2013**, 4(2), 80-84.
[21] A Pandey. *J Drug Discov Therap.* **2013**, 1(11), 23-69.
[22] E Dominguez-Rosado; J Pichtel. In Proceedings of the Indiana Academy of Science, **2003**, 112(2), 109-116.
[23] Z Sadouk; A Tazerouti; H Hacene. *World J Microbiol Biotechnol*. **2009**, 25(1), 65-70.
[24] L Kanwar; BK Gogoi; P Goswami. *Bioresource Technol.* **2002**, 84(3), 207-211.
[25] V Balaji; P Arulazhagan; P Ebenezer. *J Environ Biol.* **2014**, 35(3), 521.
[26] GO Adams; P Tawari-Fufeyin; E Igelenyah; E Odukoya. *Int J Environ Bioremediation Biodegradation.* **2014**, 2(2), 84-92.
[27] R Margesin; G Feller; M Hämmerle; U Stegner; F Schinner. *Biotechnol Lett.* **2002**, 24(1), 27-33.
[28] K Sujatha; K Dhandayuthapani. *J Pharm Bio Sci*. **2013**, 4(2), 645-652.
[29] S Gupta; DR Lehmann; JA Stuart. *J Marketing Res*. **2004**, 41(1), 7-18.
[30] JFM Burkert; F Maugeri; MI Rodrigues. *Bioresource Technol.* **2004**, 91(1), 77-84.
[31] P Zhao; Y Yuan; Y Wang; Y Zhang; J Li. *J Chem Pharm Res*. **2013**, 5(12), 418-424.
[32] KDJ Mukesh; R Rejitha; S Devika; MD Balakumaran; AIN Rebecca. *Adv Appl Sci Res*. **2012**, 3, 930-938.
[33] G Narasimha; AP Kumar; D Subramanyam. *An Indian J*. **2011**, 5(1).
[34] JL Li; BH Chen. *Materials*. **2009**, 2(1), 76-94.
[35] G Immanuel; A Jwbadhas; A Palaresam. *Food Technol Biotechnol*. **2008**, 46 (1), 60-65.
[36] HS Wu; MJ Tsai. J Enzmictec. **2004**, 35(6), 488-493.
[37] D Koley; AJ Bard. Proceedings of the National Academy of Sciences, **2010**, 107(39), 16783-16787.
[38] JWF Simons; MD van Kampen; I Ubarretxena-Belandia; RC Cox; CM Alves dos Santos; MR Egmond; H M Verheij. *Biochemistry*. **1999**, 38(1), 2-10.
[39] JJ Shangguan; YQ Liu; FJ Wang; J Zhao; LQ Fan; SX Li; JH Xu. *Appl Biochem Biotechnol.* **2011**, 165(3-4), 949-962.
[40] S Ali; Z Huang; SX Ren; MH Bashir; M Afzal; L Tong. *Pak J Zool*. **2009**, 41, 341-347
[41] J Ma; Z Zhang; B Wang; X Kong; Y Wang; S Cao; Y Feng. *Protein Expres Purif*. **2006**, 45(1), 22-29.
[42] VR Tembhurkar; LB Dama; NP Attarde; PS Zope. *Trends Biotechnol Res*. **2012**, 1, 36-41.
[43] Ü Açikel; M Erşan; YS Açikel. *Turk J Biol*. **2011**, 35(1), 35-44.
[44] K Liebeton; A Zacharias; KE Jaeger. *J Bacteriol.* **2001**, 183(2), 597-603.