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Article

Used Palm Cooking Oil as Substrate for Optimization of Lipase Activity in *Aeromonas caviae* SS-2

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Abstract— **Palm oil is widely used for culinary applications, industrial formulations, and oleochemical processing. The increasing demand for palm oil for household and industrial use indirectly contributes to environmental pollution, particularly through the unethical disposal of palm oil waste. In this study, a sustainable biotechnological approach is explored to utilize this waste by exploiting the catalytic potential of bacterial lipases. Lipase-producing bacteria have been isolated from used palm oil-contaminated environments in Nilai, Negeri Sembilan, using the stab agar screening method. Among 90 potential isolates, *Aeromonas caviae* SS-2 demonstrates the highest lipase specific activity at 9.189 µmol/mL/min within just 9 hours of incubation at 40℃. *A. caviae* SS-2 indicates an optimum lipase production when it is cultivated in Tryptone Azolectin Tween (TAT) broth supplemented with 1% (v/v) of Tween 40 and 2% (v/v) of used palm oil. This enzymatic biotransformation efficiently hydrolyzed long-chain triglycerides into shorter, value-added carbon chains, demonstrating its potential for lipid production and industrial bio-conversion.**

Keywords— **Isolation, lipase, environmental pollution, substrate, used palm cooking oil.**

I. INTRODUCTION

Palm oil is a well-known and widely used vegetable oil worldwide, with global production and supply nearly 40% higher than that of other vegetable oils [1]. Since the 1960s, Malaysia and Indonesia have dominated palm oil plantations and the industry, and both countries have become the world's largest palm oil producers [2], [3]. Palm oil production in Malaysia has increased over the past 10 years, reaching a peak of 20,800 million metric tonnes in 2018 [4].

The increasing trend in palm oil production in Malaysia over the past 10 years reflects growing demand for palm oil for both industrial and household use. Without proper management of waste cooking oil, it will lead to severe environmental pollution. Surveys conducted by Kamaruzaman et al. [5] in Felda Lepar Hilir, Pahang, and by Daud et al. [6] in Pasir Gudang, Johor, revealed that Malaysian households' awareness of proper management of cooking oil waste remains low. Both surveys reported that 92% of residents discarded their used palm cooking oil into their local drainage system, ultimately polluting soil and water.

Furthermore, Beghetto [7] reported that, in addition to its use as biodiesel, waste cooking oil can be processed into high-value polymers for industrial use. Another approach is to utilize the waste cooking oil as a substrate for lipase production by microorganisms. This initiative is both cost-effective and environmentally friendly for industrial lipase production. Lipase has promising applications in several industries, including pharmaceuticals, foods, detergents, cosmetics, pulp and paper, perfumery, leather, and biosensors [8].

Notably, bacterial lipases are receiving more attention than fungal lipases due to their optimal activity in alkaline or neutral environments. Manipulations of genetics and growth environments are generally easier in bacteria because of their simple nutritional requirements, short generation times, and straightforward methods for screening for desirable traits. These manipulations can influence the cell biomass, extracellular and intracellular enzyme activities, or even lead to the generation of modified enzymes [9].

A study on optimal growth conditions for carbon sources and growth media is essential to optimize lipase production in bacteria. In this study, several potential high-lipase-producing bacteria were isolated from oil-contaminated water and soil and screened using the stab agar method. The selected isolate was optimized for culture conditions, including cultivation period, media composition, waste palm cooking oil concentration, and different types of oils, to enhance lipase production. Finally, the lipase activity of the identified isolate was studied at different reaction temperatures and times to determine the optimal conditions for lipase activity.

II. MATERIALS AND METHODS

A. *Sample Collection and Preparation of Bacterial Isolation*

Nine locations in Nilai, Negeri Sembilan, were selected, including riverbanks, oil-contaminated drains within palm plantations, and soils from oil-contaminated domestic and food stall areas. These locations were selected based on visible signs of oil contamination to maximize the likelihood of isolating lipase-producing microorganisms.

For soil samples, 5 g of each sample was accurately weighed and suspended in 45 mL of 1 M phosphate-buffered saline (Sigma Aldrich, USA). The mixture was thoroughly mixed and filtered to remove debris. The filtrate was diluted to a dilution factor of 10^{-3}, by transferring 1 mL of the filtrate into 9 mL of 1 M phosphate-buffered saline at each dilution. These dilutions were subsequently used for bacterial screening and isolation on selective media.

B. *Screening of Lipase Producing Bacteria*

Screening for lipase-producing bacteria was conducted using the spread-plate technique. Tryptic soy agar (TSA; Becton Dickinson) was used as the base medium, consisting of 1.5% (w/v) pancreatic digest of casein, 0.5% (w/v) papain digest of soybean, and 0.5% (w/v) sodium chloride. This base agar was further supplemented with 2% (v/v) used palm cooking oil as the substrate for screening lipase-producing bacteria, and 1% (v/v) Tween 40 was added as an emulsifying agent to facilitate oil-water dispersion. The medium pH was adjusted to 7.2 prior to autoclaving.

From each serially diluted water sample, 500 µL was pipetted and aseptically spread onto TSA plates. All of the plates were incubated at 30°C for 24 hours. Following incubation, colonies were observed for the formation of opaque zones surrounding the bacterial growth, a visual indicator of lipase activity [10].

As a positive control, *Bacillus subtilis* ATCC 6633, an established lipase producer, was used in the screening method.

C. *Isolation of Lipase Producer and Preparation of Glycerol Stock Solution*

Each bacterial colony with an opaque zone indicative of lipase activity was subcultured onto fresh TSA plates supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40 to obtain pure single colonies and confirm positive lipase production. These plates were incubated at 30°C for 24 hours.

Once isolated, individual colonies were inoculated into 4 mL of sterile Tryptone Azolectin Tween (TAT) broth (Becton Dickinson, USA) containing 2% (w/v) pancreatic digest of casein, 0.5% (w/v) soy lecithin, and 4% (w/v) Tween 20. The broth pH was adjusted to 7.2 prior to autoclaving. The cultures were incubated at 30°C overnight with agitation at 180 rpm.

Following incubation, 700 µL of each bacterial culture was mixed with 300 µL of sterile glycerol in a microcentrifuge tube to prepare bacterial stock solutions. These stocks were subsequently stored at -20°C for long-term preservation.

D. *Primary Selection by Stab Agar Method*

A simple primary selection method for identifying high lipase producers was adapted from Daouadji et al. [11], Kanlayakrit and Boonpan [12], and Gopinath et al. [13]. Specifically, lipase activity was assessed by the formation of a calcium salt precipitate around the bacterial colonies. The size or diameter of the opaque zone correlates with the level of extracellular lipase production.

To quantify lipase production, the ratio of the opaque zone diameter to the colony diameter was calculated and expressed as A: B, where A is the diameter of the opaque zone (mm) and B is the diameter of the bacterial colony (mm).

For this screening, each TSA plate supplemented with 2% (v/v) palm cooking oil and 1% (v/v) Tween 40 was divided into 4 quadrants. A single colony from each overnight culture was aseptically transferred using a sterile, sharp toothpick and stabbed into the middle of each quadrant of the TSA plates. All isolates had been incubated at 30°C for 24 hours. Moreover, following incubation, both the opaque zone and colony diameter of each lipase producer were measured. Isolates exhibiting the highest ratios were selected as high lipase producers for further study.

E. *Secondary Screening Using Rhodamine B-Olive Oil Agar Assay*

Kouker and Jaeger [14] described a sensitive and specific agar plate assay for detecting lipase producers using Rhodamine B-olive oil agar. Rhodamine B does not suppress the growth of the tested microorganisms or alter their physiological properties [15]. To prepare the medium, 5 mL of Rhodamine B solution (1.0 mg/mL in distilled water, filter-sterilized) was added to 500 mL of sterile TSA agar supplemented with 2% (v/v) olive oil and 1% (v/v) Tween 80.

Subsequently, the agar was allowed to cool to approximately 60°C before adding the Rhodamine B solution to prevent thermal degradation. The mixture was stirred vigorously to ensure homogeneity, then poured into sterile petri dishes and allowed to solidify, yielding Rhodamine B-olive oil agar that appeared opaque and pinkish.

Each previously selected high lipase-producing colony was streaked onto Rhodamine B-olive oil agar and incubated at 30°C for 24 hours. Lipase activity was indicated by the appearance of bright fluorescent orange halos around the colonies upon exposure under ultraviolet (UV) light at 350 nm, as described in previous studies [16], [17], [18].

F. Gram Staining and Microscopic Observation of Lipase-Producing Bacteria

A single colony of each high lipase-producing isolate was aseptically picked using a sterile inoculating loop and emulsified in a drop of sterile distilled water on a clean glass microscope slide. The bacterial smear on the slide was heat-fixed through a flame to adhere the cells to the slide.

Following this, the dried slide was first stained with crystal violet solution, the primary stain, for one minute, to allow all bacterial cells to take on a uniform violet colour. After staining, the slide was gently rinsed with running tap water.

The slide with the stained cells was then treated with a few drops of Mordant solution (iodine) and allowed to react for one more minute. The slide was then rinsed again with running tap water before decolourization with pure ethanol for no more than 10 seconds. In particular, this critical step distinguishes Gram-positive bacteria, which retain the crystal violet-iodine complex, from Gram-negative bacteria, which loses it due to their thinner peptidoglycan layer. Immediately after decolourization, the slide was rinsed with running tap water to halt the process.

Additionally, the smear was then counterstained with safranin for approximately 30 seconds, staining Gram-negative bacteria pink while Gram-positive bacteria remained a blueish colour. The slide was rinsed with running tap water, excess water was removed using blotting paper, and the slide was air-dried before microscopic examination.

G. 16S rRNA Sequence Identification

The seven highest lipase-producing isolates were selected based on the ratio of the diameter of the opaque hydrolysis zone to colony size. These selected isolates were submitted to 1st Base Laboratories Sdn. Bhd. (Serdang, Malaysia) for 16S rRNA gene sequencing to facilitate taxonomic identification.

H. Lipase Assay on Stab Agar using Different Carbon Sources

Fresh and used palm oil, soybean oil, and corn oil were selected as variable carbon sources to evaluate lipase activity among the isolates. TSA plates were supplemented with 2% (v/v) of each oil type, both fresh and used oils, added with 1% (v/v) Tween 40 to facilitate emulsification. Each plate was divided into four quadrants. A single colony from an overnight culture of each selected lipase producer was aseptically picked with a sterile, sharp toothpick and stabbed into the middle of each quadrant. The plates were then incubated at 30°C for 24 hours.

Following incubation, the diameter of the opaque hydrolysis zone and colony size were measured to assess the lipolytic activity of each isolate on the different types of carbon sources.

I. Preparation of Used Palm Cooking Oil Emulsion

The preparation of used palm cooking oil emulsion followed the protocols described by Agu et al. [19] and Rua et al. [20]. A 10% (w/v) gum Arabic solution was first prepared in distilled water. The oil emulsion was formulated by combining 25% (v/v) of used palm cooking oil into 75% (v/v) of the prepared gum Arabic solution. The mixture was thoroughly homogenized to achieve complete emulsification and subsequently stored at 4°C for no more than 2 weeks to maintain stability and prevent degradation.

J. Cultivation of Lipase Producers in TAT Broth

Further study on the selected isolates was conducted by cultivation in TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. A single colony of each isolate was transferred aseptically into a 50 mL conical flask containing 20 mL TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. The cultures were incubated at 30°C with agitation at 180 rpm until a turbidity of 0.5 at 600 nm (OD_{600}) was reached.

Subsequently, 500 µL of each pre-culture was transferred into 250 mL conical flasks containing 120 mL of TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. The cultures were incubated at 30°C for 72 hours with constant agitation at 180 rpm. Samples (20 mL) were collected in triplicate at 12-hour intervals throughout the incubation period. The collected cultures were centrifuged at 10000 rpm for 15 minutes at 4°C using sterile Falcon tubes. The supernatants were harvested and used as the crude enzyme extracts for subsequent lipase activity assays.

K. Lipase Assay by Titrimetric Method

Lipase activity in TAT broth cultures was quantified using a titrimetric assay based on the hydrolysis of used palm cooking oil, employing a substrate modification of the method described by Macedo et al. [21], which originally used olive oil. The reaction mixture consisted of 1.0 mL of 0.02 M phosphate buffer (pH 7.0), 0.5 mL of 0.03 M calcium chloride, 0.5 mL of 0.03 M calcium chloride (added twice, as per protocol), 0.5 mL of used palm cooking oil emulsion, and 0.5 mL of crude enzyme extract (supernatant).

The mixture was incubated in a water bath at 40°C for 120 minutes with constant agitation at 180 rpm, and the reaction was terminated by the addition of 10 mL of acetone: ethanol (1:1, v/v) solution. The free fatty acids (FFA) released during hydrolysis were titrated with 0.15 M sodium hydroxide (NaOH) using 5% (w/v) phenolphthalein as the pH indicator.

L. Determination of Lipase Activity

To generate a 72-hour lipase activity profile for each isolated lipase producer cultured in TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40, the amount of FFA liberated at each 12-hour interval was quantified. The method previously described by Pinsirodom and Parkin [22] was followed, in which lipase activity was determined by the amount of NaOH required to neutralize the liberated fatty acids during titration. The concentration of liberated fatty acids was calculated using the following equation:

$$\text{Fatty Acids Liberated} = \frac{[(\text{mL NaOH for sample} - \text{mL NaOH for blank}) \times M \times 1000]}{3 \text{ mL of the reaction volume}} \quad (1)$$

Where M represents the molarity of the NaOH titrant used, which in this study was 0.15 M.

Upon completion of data collection, a time-course graph was plotted to evaluate and select the highest lipase producer among the selected isolates, based on the peak value.

M. Aeromonas caviae SS-2 Growth Curve Study

To obtain the growth curve of *A. caviae* SS-2, the method previously described by Pepper and Gerba [23] was followed in this study. A single colony of *A. caviae* SS-2 was transferred aseptically into a sterile 50 mL TAT broth in a conical flask. The culture was incubated overnight at 30°C with agitation at 180 rpm. Following incubation, the culture reached approximately 10^9 colony-forming units per milliliter (CFU/mL).

A 100 µL aliquot of the overnight culture was then transferred into a new, sterile 250 mL flask containing TAT broth. The culture was thoroughly mixed, and 5 mL was immediately withdrawn from the flask. It was labeled the 0-hour time point, with an approximate concentration of 5×10^5 CFU/mL. The flask was then incubated at 30°C with agitation at 180 rpm, and 5 mL samples were collected every 3 hours over a 40-hour incubation period.

Each collected sample was diluted in 1 M phosphate-buffered saline to a 10^{-7} dilution factor. For each dilution factor, appropriate volumes were pipetted and plated in duplicate on TSA agar plates, then incubated at 30°C for 24 hours. After incubation, CFU/mL values were recorded by counting colonies on TSA agar plates.

The *A. caviae* SS-2 growth curve was plotted from CFU/mL values over time to identify the growth phases and generation time under the tested conditions.

N. Optimization of Culture Condition and Cultivation Time Using Different Media Broth

Four different media: TAT broth, modified Letheen broth (MLB), Luria-Bertani (LB) broth, and nutrient broth (NB), were selected to optimize the culture conditions and incubation time for lipase production by *A. caviae* SS-2. Each 100 mL of the selected media broth was supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40.

Furthermore, a 1 mL aliquot containing approximately 1.1×10^9 CFU/mL of *A. caviae* SS-2 culture, obtained after 9 hours of cultivation in TAT broth, was aseptically pipetted and transferred into triplicate flasks containing each type of media broth. The cultures were incubated at 30°C with agitation at 180 rpm. A 20 mL sample of each cultivation medium was withdrawn every 3 hours over a 24-hour cultivation period. Each sample was then centrifuged at 10000 rpm for 15 minutes at 4°C using sterile 50 mL centrifuge tubes. The supernatants were collected and used as crude enzyme extracts.

Lipase activity in each enzyme extract was determined by titrimetric assay, as previously described. The values from each 3-hour interval were used to plot a time-course graph to observe differences in lipase production across different media. The optimal culture medium and incubation time for maximal lipase production by *A. caviae* SS-2 were determined based on the highest lipase activity observed.

O. Optimization of Used Palm Cooking Oil Concentration in TAT broth

In this study, the influence of different concentrations of used palm cooking oil on lipase production by *A. caviae* SS-2 was investigated. Six concentrations of used palm cooking oil, ranging from 1% to 6% (v/v), were added to TAT broth, each containing 1% (v/v) Tween 40 to aid emulsification.

An inoculum of 1 mL containing approximately 1.1×10^9 CFU/mL, obtained from a 9-hour culture in TAT broth, was transferred into triplicate 250 mL conical flasks containing 100 mL of TAT broth supplemented with the respective used palm cooking oil concentrations. After 9 hours of incubation at 30°C with agitation at 180 rpm, 20 mL of culture was harvested from each flask. The samples were centrifuged at 10000 rpm for 15 minutes at 4°C. The resulting supernatants were collected and used as crude enzyme extracts.

Notably, these crude enzyme extracts were subjected to lipase assay by the titrimetric method, as previously described. This analysis allowed for the determination of the optimal concentration of used palm cooking oil for maximal lipase production by *A. caviae* SS-2.

P. Effect of Reaction Temperature on Lipase Assay

To determine the optimal temperature for *A. caviae* SS-2 lipase activity, four temperatures were selected for analysis: 30°C, 40°C, 50°C, and 60°C.

Crude enzyme extracts were obtained from the supernatant of a 9-hour culture grown in TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. Subsequently, triplicate reaction mixtures were prepared and incubated at each temperature with constant agitation. Lipase activity was then quantified using a previously described titrimetric assay. The results were used to identify the optimal temperature for lipase activity in *A. caviae* SS-2.

Q. Effect of Reaction Time on Lipase Assay and Lipase Specific Activity.

To identify the optimal reaction time for *A. caviae* SS-2 lipase, four incubation periods were tested: 30, 60, 90, and 120 minutes.

In this context, crude enzyme extracts were collected from the supernatant of a 9-hour culture cultivated in TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. Reaction mixtures were prepared in triplicate and incubated at 40°C with constant agitation for the specified durations. Lipase activity was then quantified using a titrimetric assay, as described previously.

A reaction progress curve was prepared by plotting the quantity of liberated fatty acids against reaction time to determine the enzyme's catalytic efficiency. The initial reaction velocity (V_0) was derived from the slope of the linear portion of this curve. Lipase specific activity was then calculated using the following equation, expressed in µmol of fatty acids released per minute per milliliter of protein (µmol/min/mL protein).

$$\text{Lipase Specific Activity} = \frac{V_0}{[0.5\text{ mL protein used}/\ 3\text{ mL reaction volume}]} \quad (2)$$

One lipase unit (U) was defined as the amount of the enzyme that released 1 μmol fatty acid per minute under standard assay conditions, as defined by Gombert et al. [24].

III. RESULTS AND DISCUSSION

A. Isolation of Lipase Producers from Environment

Of the nine sampling locations in Nilai, Negeri Sembilan, seven successfully isolated potential lipase producers. A total of 90 lipase-producing bacterial colonies were obtained after 24 hours of incubation on TSA agar supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. Colonies exhibiting lipase activity were identified by opaque zones surrounding them, indicative of enzymatic hydrolysis.

Figure 1 illustrates representative TSA plates with lipase-producing colonies, while TABLE 1 summarizes the distribution of potential lipase producers obtained from the environmental samples.

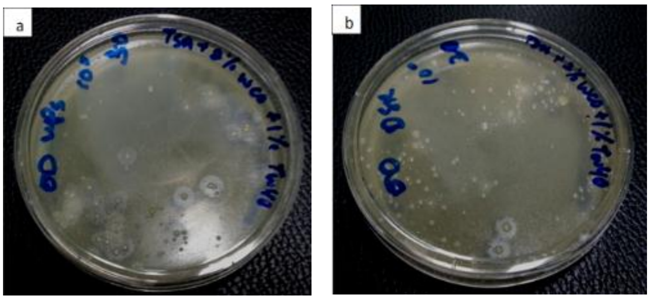

Figure 1. Evidence of lipase producers obtained from environmental samples after 24 hours of incubation on Tryptic soy agar (TSA) supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. The opaque zone formed around lipase-producing colonies was due to lipase activity.

TABLE 1. DISTRIBUTION OF LIPASE PRODUCERS OBTAINED FROM THE ENVIRONMENTAL SAMPLES AFTER 24 HOURS INCUBATION

Sampling Sites in Nilai, Negeri Sembilan		**Dilution Factor**	**Total Colonies**	**Lipase Producer Colonies**
Oil-contaminated Drain	Downstream Running Water (DSR)	10^{0}	129	8
		10^{-1}	6	1
	Downstream Stagnant Water (DSS)	10^{0}	(*)TNTC	11
		10^{-1}	36	1
	Upstream Stagnant Water (UPS)	10^{0}	78	20
		10^{-1}	12	3
	Upstream Drain Side Water (UPB)	10^{0}	63	10
	Soil (SOD)	10^{0}	(*)TNTC	12
		10^{-1}	(*)TNTC	11
		10^{-2}	21	2
Stall Soil (SS)		10^{-1}	(*)TNTC	2
		10^{-2}	(*)TNTC	5
		10^{-3}	(*)TNTC	4

(*)TNTC - Too numerous to count (colonies count exceed 250)

B. Primary Screening by Stab Agar Method

From 90 colonies of lipase producers isolated from the environment, a further morphological screening was conducted to eliminate the same species. Specifically, the morphological screening included shape, size, edge, and colour of the colonies. A final of 20 colonies with different colony morphological properties was selected for primary screening of the highest lipase producers using the stab agar method.

The extent of extracellular lipase production was evaluated by measuring the diameter of the opaque hydrolysis zone formed around each colony after 24 hours of incubation.

Lipase activity was expressed as a ratio of hydrolysis zone diameter to colony diameter (A: B), in which A is the diameter of the opaque zone (mm), and B is the diameter of the colony (mm). The results of this screening are illustrated as examples in Figure 2 and presented in detail in TABLE 2.

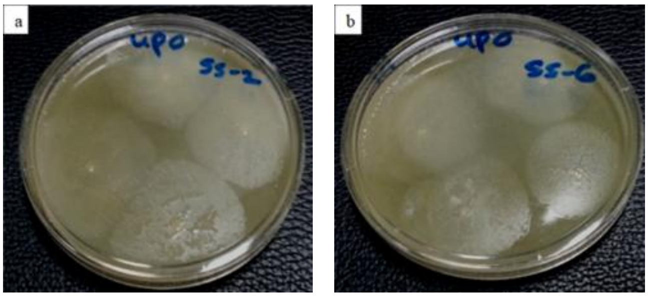

Figure 2. Evidence of opaque zone formation after 24 hours of incubation on TSA supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40 using the stab agar method for screening of highest lipase-producing isolates.

Based on the data presented in Figure 2 and TABLE 2, isolate SS-2, which was obtained from oil-contaminated stall soil, exhibited the highest opaque zone-to-colony size ratio at 20.00. Therefore, this finding indicates that SS-2 has the highest lipase activity among the tested isolates. The second-highest ratio was recorded for isolate SS-6, also derived from oil-contaminated stall soil, at 18.50.

TABLE 2. SCREENING OF THE HIGHEST LIPASE PRODUCERS USING STAB AGAR METHOD AFTER 24 HOURS OF INCUBATION

Isolates of Lipase-Producing Bacteria	**Ratio of Opaque Zone-to-colony Size**
DSR-1	5.63
DSR-2	2.67
DSR-7	3.25

DSS-1	1.76
DSS-3	1.95
DSS-5	9.83
DSS-6	2.53
DSS-8	2.79
DSS-9	5.13
UPS-1	4.00
UPS-2	2.47
UPS-4	8.67
UPB-1	13.13
UPB-3	3.13
UPB-6	2.25
SOD-3	1.83
SOD-7	16.63
SS-2	20.00
SS-3	2.36
SS-6	18.50

Isolate SOD-7, isolated from the soil of an oil-contaminated drain, demonstrated an opaque zone-to-colony size ratio of 16.63. In addition, isolated UPB-1, obtained from upstream drain-side water of an oil-contaminated drain, indicated a ratio of 13.13 while isolate DSS-5, obtained from downstream stagnant water of an oil-contaminated drain, indicated a ratio of 9.83. Therefore, the results reveal that oil-contaminated areas, such as stall soil and drain-associated sites, are promising sources of lipase-producing bacteria.

C. Screening by Observation of Lipase Activity Using Rhodamine B Agar Method

The five selected isolates exhibiting the highest lipase activity in the stab agar screening were further screened on Rhodamine B agar to confirm lipase production. Rhodamine B agar screening serves as a qualitative indicator of lipase activity, with fluorescent orange halos surrounding lipase-producing bacterial colonies when viewed under UV light at 350 nm.

All five selected isolates: SS-2, SS-6, SOD-7, UPB-1, and DSS-5 revealed fluorescent halos surrounding their colonies under the UV light exposure, after 24 hours of incubation. The results confirm that these isolates are promising lipase producers. Rhodamine B agar plates for the selected isolates, illuminated under UV light, are presented in Figure 3.

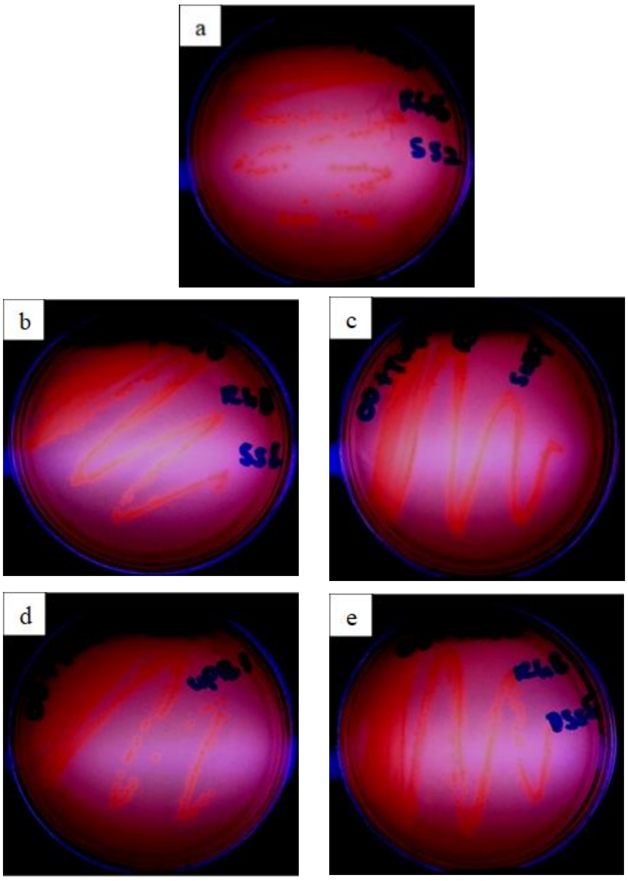

Figure 3. Observation of lipase activity on Rhodamine B agar supplemented with 2% (v/v) olive oil and 1% (v/v) Tween 80 under UV light. Fluorescent orange halos indicate lipase activity. Figure 3(a)-3(e) illustrate fluorescent orange halos formation around SS-2, SS-6, SOD-7, UPB-1, and DSS-5, respectively.

D. Identification and Characterization of Lipase-Producing Isolates

Gram staining confirmed that all five selected isolates belong to the Gram-negative, rod-shaped bacteria group. Moreover, further molecular identification via 16S rRNA gene sequencing conducted by 1st Base Laboratories Sdn. Bhd (Serdang, Malaysia) reported that all five lipase-producing isolates belong to the *Aeromonas* genus. Specifically, isolate SS-2 was identified as *A. caviae*, SS-6 as *A. dhakensis*, SOD-7 and UPB-1 as *A. hydrophilia,* and DSS-5 as *A. veronii*.

The genus *Aeromonas* is a group of Gram-negative, motile rods, facultative anaerobes, and is usually identified in fresh and brackish water. Among the identified species in this study, *A. caviae*, *A. veronii*, and *A. hydrophila* have previously been reported to harbour putative virulence factors in humans [25], suggesting their potential relevance to environmental and clinical microbiology studies.

Consequently, these results from the isolation, screening, and identification processes underscore the potential of oil-contaminated environments as rich sources of lipase-producing bacteria. The presence of lipid-based carbon sources, such as palm cooking oil, likely serves as a key selective pressure favouring the growth and spread of lipase-producing microorganisms. In addition to nutrient availability, other environmental factors, such as pH, soil composition, water, and water activity, also support microbial diversity and enzymatic activity in specific niches.

Lipase activity in the *Aeromonas* genus has been widely reported, and this study further confirms this observation by demonstrating its ability to hydrolyze used palm cooking oil. The ability of *Aeromonas* genus isolates in this study to liberate FFA from oil substrates demonstrates their potential for biotechnological applications, such as the bio-conversion of waste oil and lipid degradation.

E. Lipase Activity on Stab Agar using Different Carbon Sources

To study substrate specificity, four selected *Aeromonas* genus isolates (SOD-7, DSS-5, SS-2 and SS-6) were further assessed using both fresh and used palm cooking oil, soybean oil, and corn oil as carbon sources. The results were determined based on the opaque zone-to-colony size ratio on TSA agar plates supplemented with 2% (v/v) oil and 1% (v/v) Tween 40, as presented in TABLE 3.

TABLE 3. SUBSTRATE SPECIFICITY STUDY OF ISOLATED LIPASE-PRODUCERS AFTER 24 HOURS OF INCUBATION

Substrates / Isolates	Palm Oil		Soybean Oil		Corn Oil	
	Fresh	**Used**	**Fresh**	**Used**	**Fresh**	**Used**
A. hydrophilia SOD-7	9.0	16.6	3.1	19.3	10.3	12.2
A. veronii DSS-5	9.5	9.3	2.8	17.9	14.5	13.6
A. caviae SS-2	20.0	20.0	15.3	20.0	18.9	20.0
A. dhakensis SS-6	2.2	18.4	10.0	17.3	10.0	16.8

The results depicted that *A. caviae* SS-2 consistently demonstrated the highest lipase activity, regardless of oil type or condition (fresh or used), indicating that its lipase possesses broad substrate adaptability. On the other hand, *A. dhakensis* SS-6 lipase revealed a preference for thermally degraded or oxidized lipase substrates, as evidenced by higher lipase activity in used oils than in fresh oils.

Notably, *A. hydrophila* SOD-7 showed the highest opaque zone-to-colony ratio of 19.3 when cultivated on TSA agar supplemented with used soybean oil indicating a strong preference for the type of oil. However, its lipase activity was significantly inhibited when this isolate was cultivated in fresh soybean oil, with the ratio dropping to 3.1. *A. hydrophila* SOD-7 lipase activity however demonstrated relatively consistent activity between fresh and used corn oils, suggesting a moderate tolerance to variations in this substrate.

A. veronii DSS-5 recorded the highest lipase activity when it was cultivated in used soybean oil, with a ratio of 17.9. Simultaneously, a drastic decline was observed when it was cultivated in fresh soybean oil, with the ratio decreasing to 2.8. This isolate demonstrated relatively consistent lipase activity in both conditions (fresh or used) of palm cooking oil and corn oil.

In general, all isolates presented higher lipase activity when cultivated in TSA supplemented with used palm cooking oil as the substrate, except for *A. veronii* DSS-5. This finding aligns with a previous study by Awogbemi et al. [26], who suggested that enhanced lipase activity resulted from alterations in fatty acid composition during cooking. For example, used palm cooking oils contain about 93% saturated fatty acids and 7% monounsaturated fatty acids. The variations in the fatty acid profile between fresh and used oils play a significant role in regulating the enzymatic activity of lipase-producing microorganisms.

F. Lipase Activity Profile in TAT Broth Supplemented with 2% (v/v) Used Palm Cooking Oil and 1% (v/v) Tween 40

To evaluate the lipase profile of the selected isolates, each isolate was cultivated in TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. Lipase activity of each isolate was assessed at 12-hour intervals by the titrimetric method based on the quantification of released FFAs. This strategy enabled quantitative evaluation of lipase activity over time and facilitated identification of the highest lipase-producing isolate.

The data in TABLE 4 illustrate the mean progression values of lipase activity for each isolate from triplicate cultivations, underscoring differences in production rates and peak activity during the 72-hour incubation period.

TABLE 4. LIPASE ACTIVITY PROFILE OF EACH ISOLATE OVER A 72-HOURS INCUBATION PERIOD

Isolates	**Lipase Activity (U/mL Protein) at Various Time Intervals (Hours)**					
	12 h	**24 h**	**36 h**	**48 h**	**60 h**	**72 h**
(*)*B. subtilis* ATCC 6633	53.33	58.33	66.67	70.00	56.67	41.67
A. hydrophilia SOD-7	96.67	90.00	66.67	56.67	41.67	20.00
A. veronii DSS-5	48.33	51.67	45.00	38.33	30.00	11.67
A. caviae SS-2	105.00	101.67	93.33	75.00	60.00	41.67
A. dhakensis SS-6	98.33	91.67	75.00	63.33	48.33	28.33

(*)*B. subtilis* ATCC 6633 was used as positive control in this study

The results revealed that *A. caviae* SS-2 demonstrated the highest lipase activity among the selected isolates, with a peak at 12 hours of incubation, reaching 105 U/mL protein. A gradual decline in lipase activity was observed, with a value of 41.67 U/mL recorded at 72 hours.

Based on these findings, *A. caviae* SS-2 was selected as the most promising lipase-producing isolate and was subjected to further investigation in this study.

G. Aeromonas caviae SS-2 Growth Curve Study

The objective of the growth curve study is to identify the growth phases of *A. caviae* SS-2, including lag, exponential (log), stationary, and death, under standard cultivation conditions. The growth profile was established using colony-forming unit (CFU) counts from 10^{-6} dilution samples collected at 3-hour intervals for over a 40-hour incubation period.

The results as shown in Figure 4, illustrate a typical sigmoid growth curve, reflecting the dynamic changes in bacterial population density throughout the cultivation period.

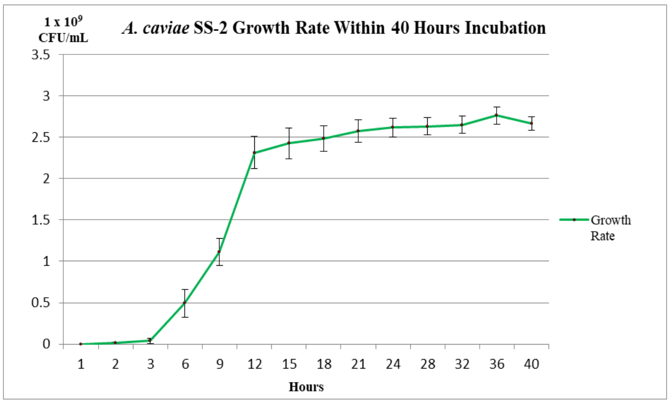

Figure 4. Sigmoid growth curve of *A. caviae* SS-2 for 40 hours of cultivation in TAT broth.

The lag phase of *A. caviae* SS-2 is from 0 to 3 hours of cultivation. After 3 hours of incubation, approximately 0.383 × 10^9 CFU/mL of lipase activity was obtained, a point where *A. caviae* SS-2 starts to grow exponentially and reaches 0.491 × 10^9 CFU/mL after 6 hours of cultivation, followed by 1.111 ×10^9 CFU/mL at 9 hours of cultivation.

At 12 hours of cultivation, the exponential phase had ended with 2.315 × 10^9 CFU/mL. Between 12 hours to 36 hours of incubation, *A. caviae* SS-2 had been in its stationary phase, and after 36 hours of cultivation, it starts to enter its death phase. The death phase after 36 hours may be due to exhaustion of the substrate and the culture broth. Based on the results summarized in TABLE 4, *A. caviae* SS-2 produced the highest lipase activity during its log growth phase, with 105 U/mL of protein produced at 12 hours of incubation.

H. *Optimization of Cultivation Time and Different Media.*

To determine the optimal culture medium and incubation time for maximal lipase production, *A. caviae* SS-2 was cultivated in four different broths: TAT, MLB, LB and NB. Each broth was supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. Samples were harvested every 3 hours for over 24 hours, and lipase activity was measured by titrimetric analysis. The incubation temperature, agitation speed, and culture concentration were kept constant in this study.

As depicted in TABLE 5, among the tested media, TAT broth indicated the highest lipase activity, reaching a maximum of 125.56 U/mL protein at 9 hours of cultivation. This finding was followed by NB (115.56 U/mL protein), MLB (95.56 U/mL protein), and LB (52.78 U/mL protein). In general, lipase activity increased progressively over the course of incubation hour, reaching a maximum at 9 hours in all media. After this time point, a gradual decline of lipase activity was observed until 24 hours of incubation in all media.

TABLE 5. LIPASE ACTIVITY OF *A. CAVIAE* SS-2 IN DIFFERENT BROTH MEDIA

Broth Types	**Lipase Activity (U/ml Protein) at Various Time Intervals (Hours)**				
	3 h	**6 h**	**9 h**	**12 h**	**24 h**
Modified Letheen Broth (MLB)	43.89	81.67	95.56	86.67	75.00
TAT Broth	65.56	103.33	125.56	113.89	103.33
Nutrient Broth (NB)	57.78	96.67	115.56	107.22	95.56
Luria Bertani Broth (LB)	18.89	43.33	52.78	38.89	28.33

Notably, lipase secretion by *A. caviae* SS-2 occurs during the log phase of growth, concurrent with active metabolic activity and cell division. As the culture progressed into the late stationary phase, a gradual decline in lipase activity was observed. This decreasing trend in lipase activity may be caused by several factors, including extracellular protease-mediated lipase degradation, cessation of enzyme production, or structural deactivation of the enzyme during prolonged incubation [27], [28].

I. *Optimization of Used Palm Oil Concentration in TAT Broth*

TAT broth supplemented with different concentrations of used palm cooking oil, ranging from 1% (v/v) to 6% (v/v), was assessed to further optimize lipase production in *A. caviae* SS-2. Cultures were incubated for 9 hours under previously established optimum conditions.

Upon completion of 9 hours of incubation, the culture supernatant was collected and used as the crude enzyme extract for the lipase activity assay by the titrimetric method. TABLE 6 displayed the results of lipase activity across the different oil concentrations.

TABLE 6. LIPASE ACTIVITY OF *A. CAVIAE* SS-2 IN DIFFERENT CONCENTRATIONS (V/V) OF USED PALM COOKING OIL IN TAT BROTH

	Lipase Activity (U/mL Protein) in Different Concentrations of Used Palm Cooking Oil in TAT Broth (v/v)					
	1%	**2%**	**3%**	**4%**	**5%**	**6%**
A. caviae SS-2	98.89	128.33	107.22	88.33	73.33	51.11

The highest lipase activity by *A. caviae* SS-2 was observed in TAT broth supplemented with 2% (v/v) of used palm cooking oil. Its lipase activity was 128.33 U/mL protein at 9 hours of incubation, suggesting this concentration as the optimal used palm cooking oil concentration for lipase production under the tested conditions.

At 1% (v/v) concentration of used palm cooking oil, lipase activity in *A. caviae* SS-2 was 98.89 U/mL protein, probably due to a lower enzyme-substrate interaction caused by the restricted availability of substrate, leading to an inadequate enzyme-to-substrate ratio.

In contrast, a decline in lipase activity by *A. caviae* SS-2 is observed when the culture media was supplemented with higher than 2% (v/v) of used palm cooking oil concentrations. This reduction may be influenced by several factors, such as substrate inhibition, reducing enzyme accessibility due to excess oil forming physical barriers. Additionally, the medium's potential toxicity at higher oil concentrations could adversely affect bacterial metabolism or lead to enzyme inactivation.

J. *Effect of Reaction Temperature on Lipase Assay*

Temperature plays a significant role in enzyme activity, with each enzyme functioning at its maximum efficiency at optimal temperature. Departures from the optimum temperature will impact enzyme structure, stability, and activity [29]. Thus, to determine the optimal temperature for lipase activity by *A. caviae* SS-2, assays were conducted at four different temperatures: 30°C, 40°C, 50°C, and 60°C.

Crude enzyme was obtained from culture supernatant of *A. caviae* SS-2 after 9 hours of incubation in TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. Lipase activity assays were performed at respective temperatures for 120 minutes with constant agitation.

Following incubation, the reactions were terminated, and the lipase activity was quantified by titrimetric analysis. The results of lipase activity at each temperature are summarized in TABLE 7.

TABLE 7. LIPASE ACTIVITY OF *A. CAVIAE* SS-2 AT DIFFERENT ASSAY TEMPERATURES

	Lipase Activity (U/mL Protein) at Different Temperatures (ºC)			
	30 ºC	**40 ºC**	**50 ºC**	**60 ºC**
A. caviae SS-2	90.00	130.00	104.44	53.89

Based on the results presented in Table 7, *A. caviae* SS-2 showed optimum lipase activity of 130.00 U/mL protein at 40°C. At elevated temperatures of 50°C and 60°C, a significant decline in enzyme activity was observed, with values of 104.44 U/mL protein and 53.89 U/mL protein, respectively. This reduction in activity at higher temperatures is likely due to thermal denaturation or structural degradation of the lipase, resulting in a loss of catalytic activity.

As a result, these findings suggest that the lipase produced by *A. caviae* SS-2 possesses moderate thermostability, with an optimal working temperature lower than that of some previously reported *A. caviae* lipases. Velu et al. [30] examined a higher thermostability profile for *A. caviae* lipase under similar assay conditions. According to them, this variation may reflect strain-specific differences or adaptations to environmental conditions.

K. Effect of Reaction Time on Lipase Assay and Lipase Specific Activity

To determine the optimal reaction time for lipase activity, assays were conducted using crude enzyme extracts from the supernatant of *A. caviae* SS-2 after 9 hours of incubation in TAT broth supplemented with 2% (v/v) used palm cooking oil and 1% (v/v) Tween 40. The lipase activity assay was performed at 40°C with constant agitation for four different reaction times: 30, 60, 90, and 120 minutes.

As presented in TABLE 8, *A. caviae* SS-2 lipase activity increased progressively with longer reaction time up to 90 minutes. At 30 minutes, the activity was 48.33 U/mL protein, rising to 89.44 U/mL protein at 60 minutes, peaking at 139.44 U/mL protein after 90 minutes of reaction. However, a decline in lipase activity to 128.33 U/mL protein was observed at 120 minutes of reaction time.

TABLE 8. LIPASE ACTIVITY OF *A. CAVIAE* SS-2 AT DIFFERENT REACTION TIMES

	Lipase Activity (U/mL Protein) at Different Reaction Times (Minutes)			
	30 min	**60 min**	**90 min**	**120 min**
A. caviae SS-2	48.33	89.44	139.44	128.33

These findings imply that 90 minutes is the optimal reaction time for *A. caviae* SS-2 lipase under the tested conditions. The decline in lipase activity at 120 minutes may be due to enzyme instability, reduced substrate concentration, or product inhibition during extended incubation.

IV. CONCLUSION

This study successfully isolated and characterized high-lipase-producing bacteria from oil-contaminated environments. The availability of carbon sources, along with conducive environmental conditions such as nutrients and optimal temperature, provides a suitable environment for the growth of lipolytic microorganisms. A cost-effective, direct screening method using the stab method on a modified TSA supplemented with used palm cooking oil effectively identifies lipase producers that can utilize it as a substrate.

Moreover, molecular identification proved that all selected isolates in this study belong to the *Aeromonas* genus, with *A. caviae* SS-2 identified as the most promising isolate. The predominance of the *Aeromonas* genus in these environments may be associated with its facultative anaerobic properties, which enable it to proliferate in low-oxygen, oil-rich ecosystems. In addition, the presence and accumulation of oils as a carbon source support their lipolytic and microbial cellular activities in such contaminated environments.

Optimization of culture conditions, including media composition, incubation time, temperature, and substrate concentration, identified the optimal growth conditions for *A. caviae* SS-2 and its lipase production.

Collectively, these findings emphasize *A. caviae* SS-2 potential for industrial-scale lipase production, particularly its ability to produce high levels of extracellular lipase within a short incubation time and using a low-cost substrate.

Notably, this study supports the sustainable use of used palm cooking oil, an agro-industrial waste, as an alternative carbon source for microbial enzyme production. Hence, by diverting used palm cooking oil from environmental discharge toward beneficial industrial applications, this approach benefits both economic efficiency and environmental preservation.

Additionally, public awareness on proper management of used palm cooking oil is essential to ensure the viability and expandability of this approach in the Malaysian context and beyond.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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