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BIODEGRADATION OF FATS, OIL AND GREASE USING MICROORGANISMS ISOLATED FROM PALM OIL MILL EFFLUENT

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ABSTRACT: The biodegradation of fat, oil, and grease (FOG) is important in water pollution control and wastewater management. In this study, the viability of FOG-degrading microorganisms on palm oil biodegradation was assessed. Seven strains capable of degrading FOG were isolated from palm oil mill effluent (POME). The potential bacterial strains were selected based on Tween-80-degrading ability. *Micrococcus lylae* strain DSM 20315 showed the highest growth compared to the other strains. Hence, it was selected for FOG degradation test. The biodegradability was performed as a function of pH (6, 7, 8), initial oil concentration (1, 3, 5% v/v), and inoculum concentration (2, 6, 10% v/v). Optimization of these parameters of palm oil degradation was studied using 2-level factorial design. The maximum oil degradation was 68%, obtained at pH 6, initial oil concentration 1 % v/v, and bacterial inoculum concentration of 10 % v/v. The lowest oil degradation obtained was 22%. The initial oil concentration followed by bacterial inoculum concentration enhanced the removal efficiency of FOG, but the pH level did not significantly promote the degradation rate. As a result, the optimum process conditions for maximizing oil degradation were at pH 6, initial oil concentration 1 %v/v, and bacterial inoculum concentration of 10 %v/v.

ABSTRAK: Biodegradasi lemak, minyak, dan gris (FOG) adalah penting dalam kawalan pencemaran air dan rawatan air buangan. Kajian ini adalah berkenaan kebolehhidupan organisma pengurai-FOG dalam biodegradasi minyak kelapa sawit. Tujuh strain berkeupayaan mendegradasi FOG diasingkan daripada cairan buangan minyak kelapa sawit (POME). Strain bakteria yang berpotensi telah dipilih berdasarkan keupayaan degradasi-Geladak-80. Strain *Mikrokokus lilae* DSM 20315 menunjukkan pertumbuhan tertinggi berbanding strain lain. Oleh itu, ia dipilih bagi ujian degradasi FOG. Keupayaan biodegradasi telah dihasilkan berdasarkan fungsi pH (6, 7, 8) ketumpatan awal minyak (1, 3, 5% v/v) dan ketumpatan inokulum (2, 6, 10% v/v). Parameter optimum degradasi minyak kelapa sawit dikaji menggunakan reka bentuk faktorial 2-tahap. Nilai maksimum degradasi minyak adalah sebanyak 68%, terhasil pada pH 6, berketumpatan awal 1% v/v, dan ketumpatan inokulum bakteria 10% v/v. Degradasi minyak terendah pula adalah sebanyak 22%. Ketumpatan awal minyak diikuti ketumpatan bakteria inokulum meningkatkan kecekapan penyingkiran FOG, tetapi level pH tidak ketara dalam membantu kadar degradasi. Sebagai kesimpulan, keadaan optimum bagi degradasi minyak maksimum adalah pada pH 6, ketumpatan awal minyak 1% v/v dan ketumpatan bakteria inokulum sebanyak 10% v/v.

KEYWORDS: *FOG; degradation; POME; oil; optimization; wastewater*

1. INTRODUCTION

Wastewater with high fat, oil, and grease (FOG) concentrations is attracting more attention because of population growth and industrialization. FOG accumulation in sewer systems has a negative impact on both health and the environment, thus effective strategies for controlling FOG deposition are critical. Triglycerides, or triglycerides linked to glycerol molecules, are the building blocks of fat, oil, and grease. The physical properties of fat, oil, and grease vary depending on the type of fatty acids that comprise the triglycerides and their physical state.

FOG is produced by the dairy industry, slaughterhouses, food processing plants, and other industries that process fatty substances, in addition to private residences [1]. When wastewater carrying fat, oil, and grease flow in sewer pipes, FOG will deposit in the pipes by forming layers of lipid-rich materials [2]. Sewer blockage causes over 25,000 flooding events in the United Kingdom each year. FOG is thought to account for more than half of all sanitary sewer overflows (SSOs) [3]. The American Environmental Agency (EPA) estimates that between 10,350 and 36,000 sanitary sewer spills occur in the United States each year, with FOG accounting for approximately 47% of the total. Furthermore, Indah Water Konsortium (IWK), the wastewater municipality in Malaysia, reported that up to 45% of SSOs may be caused by FOG [4]. Due to their prevalence in sewer systems (such as pipelines, pump stations, wet wells, etc.) and subsequent wastewater treatment facilities, FOG deposits have an impact on health and the environment. They often result in sanitary sewer overflows (SSOs) by reducing sewer diameter or totally blocking the pipelines. The ensuing sewage discharge increases water contamination and pathogen exposure. FOG can also attract vermin such as rats, and sloughed deposits can clog pumping stations and sewage treatment plants [5]. To implement effective control measures, it is necessary to have a complete understanding of the properties and impacts of FOG.

Approaches to controlling FOG can be categorized as physical, chemical, or biological. Long chain fatty acids are introduced to wastewater during the chemical hydrolysis of FOG, which may limit microbial activity and disrupt the wide variety of microorganisms present [6], resulting in a decline in wastewater treatment efficacy and recurrent production of intolerable odors.

Physical methods use FOG's reduced density in comparison to water. Grease traps (also known as grease interceptors) are one of the most frequently used physical-based FOG removal devices used prior to entering wastewater systems. When FOG-containing wastewater enters a grease trap, lower density FOG floats to the surface, allowing virtually FOG-free water to exit the grease trap and enter the sewer system. The FOG that has floated to the surface will be manually removed. Tilted plates (TP) are a modified grease trap. TP are parallel gravity separators that offer a large surface area while taking up less than 10% of the volume of a typical grease trap [7]. During dissolved air foliation, micro-bubbles bind to FOG particles, allowing them to float to the water's surface and be swiftly removed. To clear the accumulating FOG layers, physical approaches require human intervention. The increased demand for human resources raises the expense of FOG cleaning and the maintenance of related facilities, which already cost millions of dollars per year. Furthermore, these techniques are ineffective at reducing FOG, which can eventually limit the oxygen transfer rate, compromising biological treatment [8]. Additionally, it was shown that microbial activity in grease traps generated large amounts of long-chain fatty acids, which, when released into sewer systems, aid in the production of FOG deposits [9].

Biological FOG treatment is gaining traction due to its ability to treat FOG at the source and prevent it from entering sewer systems. FOG is biologically treated by bacteria capable

of secreting enzymes that speed up the hydrolysis process or by direct use of enzymes such as lipases and commercial additives. Isolating such bacterial strains and determining the optimal parameters and overall process. Teixeira et al. isolated bacteria from activated sludge and wastewater effluent to biodegrade triolein and oleic acid [10]. *Aeromonas* sp. and *Staphylococcus cohnii* were shown to be the best degraders. However, in a 7-day assessment of FOG content, the elimination of oleic acid and/or triolein was 37.9% and 19.1%, respectively. Phong et al. identified bacteria from wastewater collected from restaurants and canteens containing vegetable oil [11]. Because of its strong lipid degradation ability, *Acinetobacteria soli* strain AL3 was selected as a potential for FOG biodegradation. Similarly, strains belonging to the genera *Acinetobacter*, *Bacillus*, and *Pseudomonas* demonstrated the ability to efficiently degrade FOG [12]. The FOG removal efficiency of *Pseudomonas* sp. strain D2D3 were 94.5% and 94.4% for olive oil and animal fat, respectively, whereas sunflower oil had the lowest percentage at 62% [2]. It is beneficial to isolate the bacteria from a medium that is rich in FOG and where the microorganisms have had time to acclimate to ensure a high degree of biodegradation.

Raw palm oil mill effluent (POME) contains high concentrations of organic substances, including oil and grease, which lead to elevated levels of COD and BOD. POME treatment in open oxidation ponds will eventually reduce these amounts significantly [13]. This suggests that POME would accommodate and sustain bacteria capable of metabolizing palm oil during the natural decomposition process in the ponds. Many studies have demonstrated the potential of isolating lipolytic bacteria from POME [14]. Some strains, however, were unable to use palm oil directly as a carbon source. As a result, the isolation and screening procedures were modified to select bacterial strains capable of using palm oil as a carbon source. POME is a preferred source for isolating lipid-degrading bacteria due to its nature and potential.

The objective of this study is to isolate bacteria from palm oil mill effluent and screen them for lipolytic activity on Tween 20. The high-performing bacteria will then be used to biodegrade cooking palm oil, which is a popular oil with a fatty acid composition similar to that of FOG. The selected bacteria's growth conditions will then be optimized.

2. MATERIALS AND METHODS

2.1 Screening and Isolation of Lipolytic Bacteria

POME that was serially diluted from 101 to 107 was used to isolate bacterial strains. A sample of each dilution was plated on LB agar and incubated overnight at 37 °C [15]. To obtain the pure strains, the colonies that formed on the agar plates were sub-cultured on new agar plates. More than 20 pure strains were obtained. The lipase activity was seen using Kanmani et al.'s agar plate assay [16]. Tween 20 (Sigma-Aldrich) was utilized as a substrate since it is suitable for the simple and rapid detection of microbial lipolytic activity on an agar plate. The pure colonies were streaked on Tween 20 agar plates. Lipase producers have a zone of white-like precipitate surrounding the lipase-active colony. The first 7 strains that appeared on agar were selected for further screening.

2.2 Characterization of the Isolated Strains

Individual bacteria isolates were identified beforehand using classical tests such as cell shape, Gram staining, and colony morphology on solid nutrient media. First BASE Laboratories Sdn. Bhd., Malaysia, performed the genetic identification of isolates by determining the nucleotide sequence of the 16S rRNA gene.

2.3 Growth Profile of the Isolated Strains

The lipolytic strains were added to a 250 mL Erlenmeyer flask containing 1 mL of palm oil as a carbon source and 100 mL of minimal salt medium (MSM) media. The author of [15] describes how to make an inoculum in LB broth medium. The inoculum was added to 100 mL of MSM containing 2.5 g/L NaCl, 4.74 g/L K_2HPO_4, 0.56 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L $CaCl.6H_2O$, and 0.5 g/L NH_4NO_3 in dH_2O. The media (100 mL) were poured into a 250 mL Erlenmeyer flask and autoclaved for 15 minutes at 121 °C. All samples were inoculated with a starter culture (1% v/v) produced in LB medium (OD600=1.0) [15]. Before inoculation, the starter culture was centrifuged (13,000 rpm, 10 minutes, 4°C) and rinsed a couple of times in a physiological salt solution to avoid the transfer of LB organic compounds into fresh media. Bacteria were omitted from the control samples. All the samples underwent a two-week incubation period in a rotary shaker (150 rpm, 25 °C). On a UV-Visible spectrophotometer every 6 hours, the growth was measured at absorbance of 600 nm [17].

2.4 Optimization of FOG-Degradation

Biodegradation of palm oil was performed to determine the highest concentration of palm oil that could be degraded using bacterial strains in 250 mL Erlenmeyer flasks that were stoppered with cotton wool to promote oxic conditions. Each flask held 100 mL of MSM, supplemented with a predetermined oil concentration, inoculum concertation, and pH level. 1N NaOH or HCl were used to adjust the pH. Before inoculation, samples were autoclaved. The experiment lasted two weeks at 37 °C and 150 rpm. Experiments with bacterial samples and controls were carried out in duplicate.

Using Design Expert 6.0.8 software, biodegradation optimization runs were designed applying 2-Level Factorial Design (2LFD) with one replicate and three center points. The optimized parameters were the pH of the MSM medium (A), the concentration of oil (B), and the inoculum concentration (C). These variables were assessed at 3 different levels: pH (6, 7, 8), oil concentration (1, 3, 5% v/v), and inoculum concentration (2, 6 and 10% v/v). The software developed a matrix containing 11 experiments. Under predetermined conditions, oil degradation was carried out as designed. Following the incubation period, the oil was degraded using gravimetric methods. The software was used to enter and analyze these data. ANOVA (standard analysis of variance) and a contour plot were produced.

The partition gravimetric technique was implemented to estimate the medium's residual oil content [18] The residual oil content was strained numerous times with 5 mL of hexane such that there was no oil layer in the aqueous phase and the solvent phase was clear. In a water bath at 70°C, the mixed solvent extract was completely evaporated, and the amount of residual oil was calculated to determine the percent decrease [3].

3. RESULTS AND DISCUSSION

3.1 Growth Profile of the Isolated Strains

To construct an efficient oil-containing wastewater treatment system, oil-degrading microorganisms were isolated from POME. A sample of palm oil mill effluent was serially diluted and screened. In the initial screening, twenty POME isolates were obtained. The oil-degrading ability of the selected strains was then tested using Tween 20 agar medium. Seven strains that displayed the highest precipitate formation around the colonies in Tween 20 agar plates were selected for subsequent FOG biodegradation experiments. The growth profiles of the seven strains were screened using optical density, and the strain with the highest cell

growth rate was investigated for oil degradation optimization. Figure 1 depicts the growth curve for the selected seven. Strain X3 demonstrated the highest growth, hence it was selected for further investigation.

3.2 Characterization of Isolated Bacteria

Based on gram staining of the isolated strains, Table 1 indicates that two strains belong to gram negative category, while the other five strains belong to gram positive bacteria. Table 2 shows the phenotypical characteristics of the seven isolates when grown on LB agar.

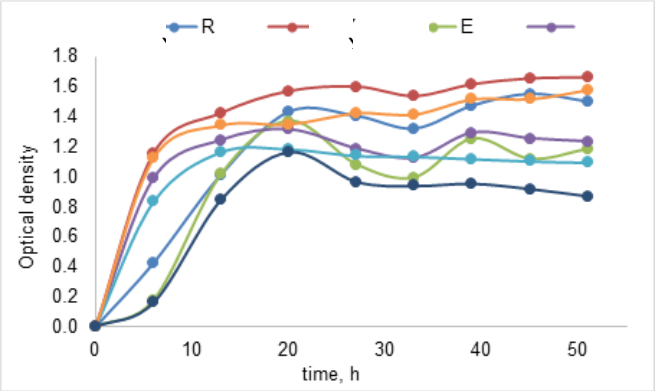

Fig. 1: The growth curve for the seven selected strains.

Table 1: Identification of the isolated strains

Sample	Gram staining	Identification of the strains
(X1, X2,X3)	Gram positive	*Micrococcus lylae* strain DSM 20315
X4	Gram positive	*Corynebacterium aurimucosum* strain H2456
X5	Gram negative	*Lysinibacillus boronitolerans* strain 10a
X6	Gram positive	*Staphylococcus hominis* subsp. novobiosepticus strain GTC 1228
X7	Gram negative	*Bacillus drentensis* strain LMG 21831

Table 2: Phenotypical characteristics of the seven isolates when grown on LB agar

Isolate	(X1, X2, X3)	X4	X5	X6	X7
Form of colony	Irregular	Circular	Circular	Circular	Circular
Elevation	Convex	Convex	Flat	Flat	Flat
Margin	Entire	Entire	Entire	Entire	Entire
Pigmentation	Yellow	White	Red	White	Red
Texture	Rough	Smooth	Smooth	Smooth	Smooth
Optical properties	Opaque	Opaque	Opaque	Opaque	Opaque
Appearance	Dull	Shiny	Shiny	Shiny	Shiny

Strain X3, which showed the highest degradation capability, was identified using morphological tests and 16S RNA sequence analysis (Fig. 2). Strain X3 was gram-positive bacteria capable of producing lipase. The 16S ribosomal RNA (16S rRNA) gene was sequenced and the generated sequences were analyzed using BLAST to reveal that X3 belongs to the genus *Micrococcus*. The 16S rRNA (500bp) sequence analysis of strain X3 revealed a significant similarity (more than 99%) to that of the genus *Micrococcus*. Based on these traits and its 16S rRNA sequence, strain X3 was identified as *Micrococcus lylae* strain DSM 20315.

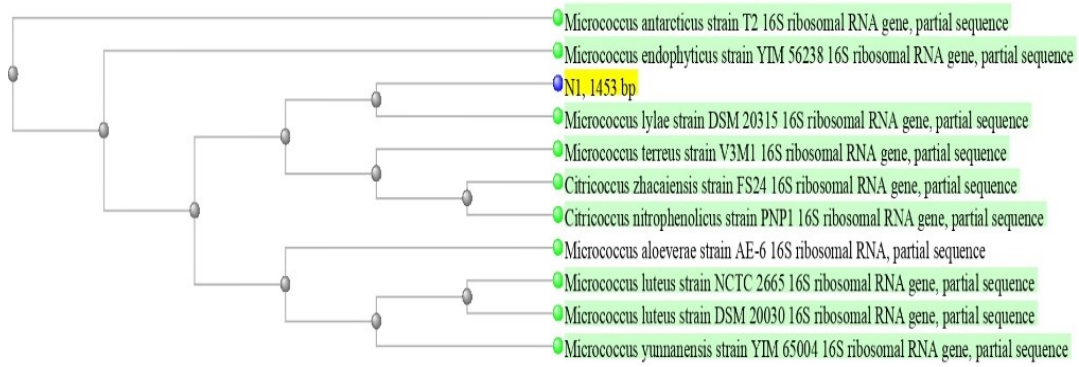

Fig. 2: Phylogenetic tree of X3 strain.

In the present study, *Micrococcus lylae* strain DSM 20315 was shown to have lipolytic activity. To the knowledge of the researchers, this study was the first time *Micrococcus lylae* strain DSM 20315 was used as a potential lipid-degrading strain. This supports the hypothesis that POME is a potential source for oil-degrading bacteria [5].

Table 3: Two LFD Experimental design setup and response

Run no.	A pH	B Oil conc. % (v/v)	C Inoculum conc. % (v/v)	Response Oil biodegradation %		Std
				Exp'l	Pred.	
1	7.0	3.0	6.0	35.3	44.9	6.79
2	8.0	5.0	10.0	25.4	22.8	1.84
3	8.0	1.0	2.0	58	55.4	1.84
4	6.0	5.0	2.0	38.6	36.0	1.84
5	7.0	3.0	6.0	33	44.9	8.41
6	8.0	1.0	10.0	53	55.6	1.84
7	7.0	3.0	6.0	37	44.9	5.59
8	6.0	1.0	10.0	68	65.4	1.84
9	6.0	5.0	10.0	29.2	31.8	1.84
10	8.0	5.0	2.0	37	39.6	1.84
11	6.0	1.0	2.0	50	52.6	1.84

3.3 Statistical Optimization of FOG-Degradation

The highest biodegradation achieved following the experimental design setup (Table 3) was 68% at pH 6, 1% (v/v) oil concentration, and 10% (v/v) inoculum concentration (run no. 8). Lipid-degrading bacteria such as *Bacillus thermoleovorans* IHI-91 was found to be able to degrade palmitic acid, steric acid, lanoline, olive oil, sunflower seed oil, soya oil, and fish oil as sole carbon source [19]. [2] isolated and identified *Pseudomonas* sp. strain D2D3 that is capable of degrading fat, oil, and grease. They also found that the degradation rate ranged from (62.0% -94.5%) based on the substrate. [20] investigated the lipolytic activity of *Raoultella planticola* bacterial strain and found its efficiency to degrade a mixture of lipids substances with maximum degradation level of 91.9% for oleic acid.

ANOVA for the 2LFD model (Table 4) revealed an F-value of 11.84, indicating that the model is significant and that there is only a 3.4% likelihood that such a big F-value could occur due to noise. Model terms are significant when "Prob > F" is less than 0.05. In this case, B (oil concentration) and BC are significant model terms. Values larger than 0.10

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suggest that the model terms are not significant. Generally, large magnitudes of t and F, as well as smaller p-values, indicate that the associated coefficient terms are significant. R^2 indicates how much of the observed response's variability may be attributed to the experimental factors and their interactions. R^2 is a measure of how well a model can predict a response, and the closer it is to 1, the stronger the correlation between experimental and predicted values are. A ratio larger than 4 is preferred when measuring the signal to noise ratio with adequate precision. With a ratio of 10.975, it is clear that there are sufficient signals and models to help one move about the design space. Lack of fit > 0.05 is insignificant, and because the lack of fit test reflects how well the model fits the experimental data, this model could be used to make predictions.

The final equation terms of coded factors are:

$$\text{Oil biodegradation} = +44.9 - 1.55 * A - 12.35 * B - 1.00 * C + 0.2 * A * B - 3.15 * A * C - 4.25 * B * C \quad (1)$$

The equation can be used to make predictions about the outcomes, for given amounts of each of the factors. It can also be used to ascertain the relative influence of factors by comparing the factor coefficient.

Table 4: ANOVA of oil biodegradation

source	Sum of squares	Df	Mean square	F-value	p-value Prob >F	
Model	1471.6	6	245.2667	11.839	0.034	significant
A-pH	19.22	1	19.22	0.928	0.407	
B-Oil conc.	1220.18	1	1220.18	58.896	0.005	
C-Inoculum conc.	8	1	8	0.386	0.578	
AB	0.32	1	0.32	0.015	0.909	
AC	79.38	1	79.38	3.832	0.145	
BC	144.5	1	144.5	6.975	0.078	
Pure Error	8.0726	2	4.0363			
Lack of Fit	54.08	1	54.08	13.398	0.067	not significant

Figure 3 shows the predicted versus actual plot values. The data points are evenly distributed across the 45° line, indicating agreement between the actual data and that produced by the model.

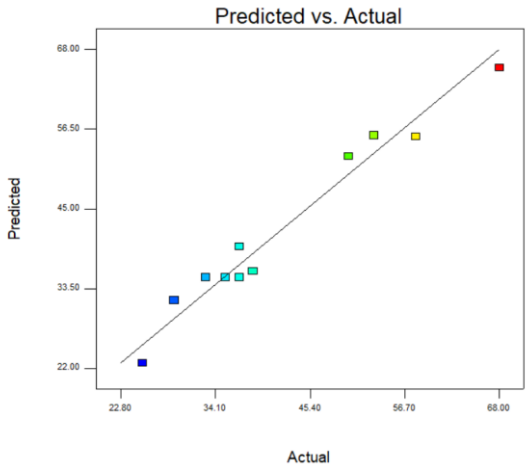

Fig. 3: Predicted versus actual plot of oil biodegradation percentage.

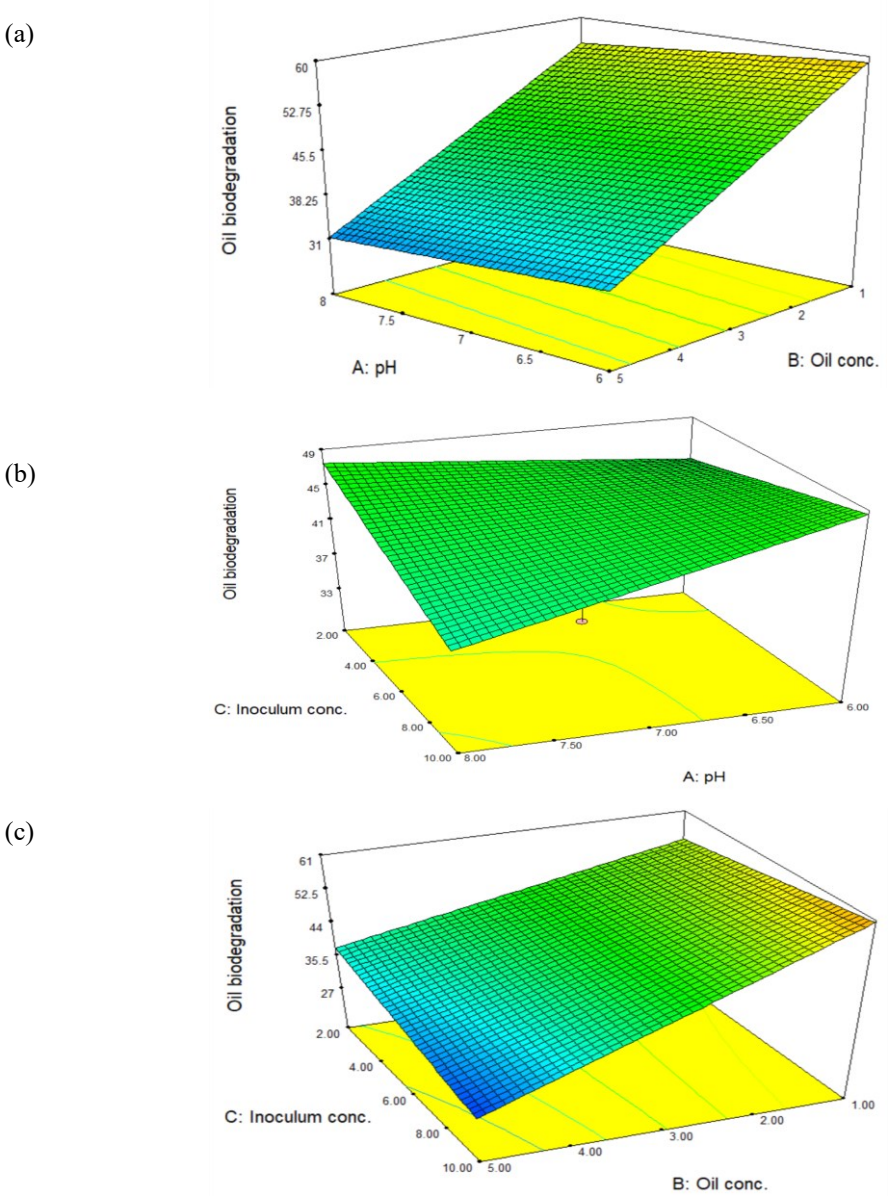

Fig. 4: 3D response surface showing the effects of (a) pH and oil concentration (b) pH and inoculum concentration and (c) oil concentration and inoculum concentration on oil degradation.

The 3D response surface plots are a graphical approach to expressing the regression equation. They illustrate how the variables interact and are used to identify the optimal value of each factor for a suitable response. The plots for the interactions between pH and oil concentration (A-B), pH and inoculum concentration (A-C), and oil concentration and inoculum concentration (A-C) are shown in Fig. 4 a, b, and c, respectively (B-C).

Figure 4a shows a decrease in oil biodegradation with an increase in oil concentration while pH did not show any effect. Figure 4b depicts the interaction between pH and inoculum concentration. Increased pH resulted in a slight increase in oil biodegradation, whereas increased inoculum concentration resulted in an increase in oil biodegradation at lower pH. In Fig. 4c, the interaction between oil concentration and inoculum concentration shows that oil degradation is highest when the oil concentration is lowest, and the inoculum concentration is highest. This is consistent with what is stated by [21]. Increased bacterial enzyme accessibility to substrate will result in improvement of the hydrolysis reaction rate.

This study revealed that while microbial concentration does not have significant effect as a single factor (Table 4) its interaction with oil concentration has a significant effect on the amount of degraded oil. Microbial concentration is important as it provides more bacterial cells and thus more enzyme secretion to degrade the oil and therefore increase the rate of degradation [22].

By applying the 2-Level Factorial Design of Oil degradation, a maximum oil degradation was obtained at a pH of 6, oil conc. 1% (v/v), and 10% (v/v) of bacterial inoculum.

4. CONCLUSIONS

Seven microorganisms isolated from POME were studied for the degradation of FOG. *Micrococcus lylae* strain DSM 20315 was identified as the largest growing microorganism through morphological tests and 16S rRNA sequence analysis. This strain was used to optimize the degradation process. The optimum condition that achieved the highest biodegradation was at pH 6, oil concentration 1% (v/v), and 10 % (v/v). The results revealed that microorganisms isolated from POME are capable of biodegrading FOG, which could be attributed to the two wastes' similar properties.

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