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ORIGINAL PAPER

Influence of pH on the biodegradation efficiency of fats, oils, and grease by biosurfactant-producing bacterial consortia

Jia-Ying Wong · Ngui-Sing Ngieng · Ahmad Husaini · Rosmawati Saat · Hasnain Hussain

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Abstract The accumulation of fats, oils, and grease (FOG) in wastewater systems presents major environmental challenges, necessitating the development of effective bioremediation strategies. Biosurfactant-producing bacteria are promising for FOG degradation; however, their efficacy is highly pH-dependent, affecting microbial metabolism and biosurfactant stability. This study evaluates the impact of pH on FOG biodegradation by locally isolated biosurfactant-producing bacterial consortia to identify optimal pH conditions. Two highly efficient biosurfactant-producing bacterial isolates, identified via 16S rRNA sequencing as *Pseudomonas aeruginosa* and *Bacillus velezensis*, were cultured in Bushnell Haas (BH) medium to form a bacterial consortium. The consortium was then inoculated into fresh BH medium, adjusted to pH values from 4 to 9, and supplemented with 1% FOG (w/v). Samples were monitored at six-day intervals for 30 days under continuous shaking at 130 rpm. After 30 days of biodegradation, the solid FOGs in pH 6 disappeared while flocs were observed in both pH 4 and 5. Despite greater floc formation at pH 6, GC–MS analysis revealed that pH 4 achieved the highest degradation rate, displaying the fewest FOG peaks and the lowest area under peaks, indicating the most substantial FOG reduction. Notably, the consortium achieved the highest FOG removal at pH 4, an acidic condition under which most long-chain FOG components were completely degraded or transformed into shorter chains. This finding reveals an unexpected optimum pH 4 for FOG bioremediation by two efficient biosurfactant-producing bacteria combined into a synergistic consortium, highlighting a potential strategy to enhance grease waste treatment.

Keywords FOG biodegradation · Biosurfactant-producing bacteria · pH · Bacterial consortia

J.-Y. Wong · N.-S. Ngieng (✉) · A. Husaini · R. Saat · H. Hussain
Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
e-mail: snngui@unimas.my

Introduction

FOGs are pervasive pollutants, primarily originating from food processing and restaurant waste, but also significantly contributed by domestic kitchen discharges and certain industrial operations (e.g., slaughterhouses and dairy processing). Even household cooking and dish washing activities can introduce substantial FOG into municipal sewers, compounding the buildup of greasy deposits (Yusuf et al. 2023). These substances accumulate in drainage networks, where they solidify into solid deposits that restrict the flow of wastewater (Ahmad et al. 2023). The consequences of FOG build-up are severe, including sanitary sewage overflows (SSO), environmental contamination, and infrastructural damage. Additionally,

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FOG-related blockages also lead to unpleasant odors, public health hazards due to untreated wastewater exposure, and substantial financial burdens for the maintenance and replacement of wastewater systems (Yusuf et al. 2023).

Conventional FOG removal strategies, including mechanical scraping, chemical treatments, and enzymatic degradation, are inefficient and often pose secondary environmental risks. While enzymatic treatments offer a more targeted approach by breaking down FOG components into smaller molecules, their cost and stability under varying wastewater conditions remain significant challenges. Mechanical removal processes offer temporary relief but fail to address the root cause of FOG accumulation, while chemical treatments risk introducing hazardous by-products into the environment (Wallace et al. 2017). These limitations underscore the need for alternative strategies to manage FOG in wastewater systems. The rising demand for sustainable and effective solutions has led to the exploration of microbial bioremediation as a viable alternative.

Bioremediation, particularly using biosurfactant-producing bacteria, offers a promising and environmentally friendly approach for FOG degradation. Biosurfactants, which are amphiphilic compounds produced by bacteria, lower surface and interfacial tension, enhancing the emulsification of hydrophobic substances like FOGs (Malkapuram et al. 2021). Studies have demonstrated that biosurfactants not only enhance the bioavailability of hydrophobic pollutants but also significantly accelerate their biodegradation, as observed in biodiesel-contaminated soil remediation (Decesaro et al. 2021). However, the role of biosurfactants extends beyond emulsification. They play a crucial role in disrupting the structural integrity of FOG, making these lipophilic substances more accessible to microbial enzymatic attack (Gaur and Manickam 2021). Studies have demonstrated that biosurfactants can significantly enhance the rate of biodegradation by increasing the surface area of hydrophobic compounds, facilitating greater interaction between the bacterial cells and FOGs. Furthermore, biosurfactant production is often associated with the bacteria's ability to form biofilms, which further enhances FOG degradation by improving bacterial adhesion to the substrate and ensuring persistence in challenging environmental conditions (Malkapuram et al. 2021; Venkatesan et al. 2023).

While biosurfactant-producing strains have shown efficacy in hydrocarbon degradation, further investigation is needed to understand how environmental factors, such as pH, influence their activity in the specific context of FOG biodegradation. The pH of the environment is a crucial factor affecting microbial metabolism, biosurfactant stability, and enzymatic activity during FOG biodegradation (Mishra et al. 2021). Changes in pH can alter the physical properties of biosurfactants, including their ability to emulsify fats, and affect the stability and activity of enzymes such as lipases, which are essential for breaking down triglycerides into more easily metabolizable components. Furthermore, changes in pH also affect interactions within bacterial consortia as it may favour the growth of only certain bacterial species within the consortium. Optimizing pH conditions is therefore critical to enhance the efficiency of FOG biodegradation.

Unlike previous studies that largely focused on single strains or did not specifically examine pH effects (e.g., often reporting optimal performance near neutral pH in monocultures), our study uniquely investigates a defined two-species bacterial consortium and systematically evaluates a range of pH conditions. By doing so, we bridge a knowledge gap on how environmental pH influences FOG degradation efficacy in mixed cultures. We introduced biosurfactant-producing isolates into a complementary team and identified an unusually acidic optimal pH (pH 4) for FOG removal, an outcome not commonly reported in earlier works. For instance, Ali et al. (2021) observed optimal biosurfactant activity for FOG degradation at alkaline pH 8, highlighting the novelty of our acidic optimum. This study therefore aims to investigate the impact of pH on FOG degradation by locally isolated biosurfactant-producing bacterial consortia to determine the optimal pH conditions for enhanced biodegradation. By focusing on a single, co-cultured consortium rather than multiple consortia, we demonstrate how synergy between two biosurfactant-producing strains can yield a distinctive pH profile for effective FOG breakdown, thus extending current literature on FOG bioremediation. The results of this study will provide valuable insights into the potential application of biosurfactant-producing bacterial consortia for sustainable wastewater management and offer a new perspective on applying acidic conditions to improve FOG removal.

Materials and methods

Sample collection

The fats, oil, and greases (FOGs) containing samples were collected at the Student Pavilion of University Malaysia Sarawak because the drains at the selected places have been clogged with FOGs and with oil surface. Samples were collected in sterile bottles and stored at 4 °C until further use. All samples were analysed within 48 h to ensure consistency in sample conditions.

Enrichment of the microbial culture

Indigenous bacteria from the collected FOGs were first enriched through a series of subculturing in liquid media. The FOGs containing samples were first transferred at a volume of 5 mL into 95 mL of sterile Phosphate buffered saline (PBS) (g/L: 0.2 KCl, 8 NaCl, 1.44 Na_2HPO_4, and 0.24 KH_2PO_4, pH 6.90) solution in a 250 mL Erlenmeyer flask. The flask was then incubated at 37 ºC on a shaker at 130 rpm for 12 h. After 12 h, 5 mL of each suspension was inoculated into three different 250 mL Erlenmeyer flask containing 50 mL of Minimal salt media (MSM) supplemented with 1% (v/v) cooking oil. The MSM used is consisting of (g/L) 0.28 Na_2HPO_4, 1.14 KH_2PO_4, 3 NH_4NO_3, 0.36 K_2S0_4, 0.154 $MgSO_4{\cdot}7H_2O$, 0.023 of $CaCl_2{\cdot}2H_2O$, 0.038 $FeSO_4{\cdot}7H_2O$, 0.10 NaCl, 0.5 yeast extract, and 1 mL of trace element stock solution containing (g/L) 0.83 KI, 0.048 $CoCl_2{\cdot}6H_2O$, 0.048 $Na_2MoO_4{\cdot}2H_2O$, 0.125 $CuSO_4{\cdot}5H_2O$, 0.01 H_3BO_3, 0.223 $MnSO_4{\cdot}4H_2O$, and 0.287 $ZnCl_2$. The pH of the medium was adjusted to 6.90. The inoculated flasks were then cultured at 37 °C on a shaker at 130 rpm for 48 h. A 5 mL aliquot of the growing bacterial culture was then transferred into 50 mL of fresh MSM similarly as mentioned above and repeatedly grow for another 2 more cycles using fresh MSM to enrich microbial cultures (Nayarisseri et al. 2018). Enrichment progress was monitored by observing turbidity increases and FOG emulsification in the culture over successive transfers.

Isolation of biosurfactant producing bacteria

After three continuous cycle of enrichment (visual disappearance of oil slicks and formation of emulsions/flocs), 50 µL of growing culture was spread onto MSM agar plate containing 2% agar and 1% cooking oil and incubated at 37 °C for 5 days. All growing bacteria was isolated and purified as they appear, with repeated streaking onto fresh MSM agar plate. Isolates were differentiated based on their distinctive morphological growth appearance on agar plate and under microscope after Gram staining. Pure bacterial cultures were stored as 25% glycerol stock and kept at − 20 °C after snap freezing with liquid nitrogen. Working culture of the isolates were maintained by repeating streaking of the culture on fresh oil containing MSM agar plate on a weekly basis (Yu and Huang 2011).

Screening of biosurfactant producing bacteria

Preparation of supernatant/ cell free culture broth

All isolated bacteria were screened for their ability to produce biosurfactant. The bacteria were grown from a single colony in Luria–Bertani (LB) medium to a density of 10^7 colony forming units (CFU/mL) for 24 h. After 24 h, 0.5 mL of each bacterial culture were added into 20 mL of liquid Bushnell Hass (BH) media supplemented with 1% (w/v) cooking oil as the sole carbon source. The BH liquid medium used contained (g/L): 2.38 $(NH_4)_2SO_4$, 1.36 KH_2PO_4, 10.69 $CaCl_2{\cdot}7H_2O$, 0.25 $MgSO_4{\cdot}7H_2O$, 1.42 Na_2HPO_4, and 0.00028 $FeSO_4{\cdot}7H_2O$ (Nzila et al. 2021). The culture was then incubated on a rotary shaker at 130 rpm for 7 days at 37 °C. After incubation, the culture broth was centrifuged at 6000 rpm for 15 min at 4 °C to separate the bacterial cells. The supernatant was then collected and filtered through a 0.45 µm pore-size membrane filter (Millipore) and used for both oil spreading assay and emulsification index (E24) assay to screen for biosurfactant activity. All screening experiments were conducted in triplicate unless otherwise specified, and the mean values were reported as the results.

Oil spreading assay

In this procedure, 25 mL of distilled water was added to a petri dish. Then, 500 µL of cooking oil was added to the water surface followed by 100 µL of the cell free supernatant was added to the centre of the oil surface. Supernatant that causes the

displacement of oil with the formation of a clearing zone on the oil layer was considered as biosurfactant positive and the diameter of clearing zone was measured. This procedure was repeated for all supernatant prepared from each isolated bacterial strain and performed in triplicates (Nayarisseri et al. 2018). Bacterial isolates that produced clear zone on the oil layer was further tested for emulsification index (E24) test.

Emulsification index (E24) test

The cell free supernatant of strains that are positive for the oil spreading assay was further tested with Emulsification index (E24) test. To carry out the test, 3 mL of the cell free supernatant was added to equal volume of cooking oil in a test tube. Then, the mixture was mixed vigorously with a vortex for 2 min and was left undisturbed for 24 h. After 24 h, the height of the emulsified layer and total height of the mixture were measured. An equal volume of uninoculated broth (cell free) was also used in place of the cell free supernatant as negative controls for the E24 test. The procedure was carried out in triplicate and the emulsification index (E24) of the supernatant was calculated using formula (1) (Nayarisseri et al. 2018).

$$\text{Emulsification Index (E24)} = \frac{\text{Height of emulsified layer (cm)}}{\text{Total height of mixture (cm)}} \times 100\% \quad (1)$$

Identification of biosurfactant producing bacteria using 16S rRNA technique

Overnight bacterial colonies

The two best biosurfactant-producing bacterial isolates were selected for molecular identification via 16S rRNA sequencing based on their highest oil spreading assay clearing zones and emulsification index (E24) values. Genomic DNA was extracted using a modified boiling method based on Shahzad et al. (2024). Briefly, two colonies from overnight cultures were suspended in 100 µL of Tris–EDTA (TE) buffer in a 2 mL Eppendorf tube. The suspension was boiled at 96 °C for 10 min, followed by immediate cooling in an ice bath for 5–10 min. After cooling, the tubes were centrifuged at 10,000 rpm for 2 min. The supernatant, containing the extracted bacterial DNA, was transferred to a fresh 1.5 mL Eppendorf tube and stored at − 20 °C until further PCR amplification.

Polymerase chain reaction (PCR)

The extracted DNA from the biosurfactant-producing bacteria was used as template for the amplification of the bacterial 16S rRNA gene through polymerase chain reaction (PCR) using both pA (5′-AGAGTTTGATCC TGGCTCAG-3′) and pH (5′-AAGGAGGTGATC CAGCCGCA-3′) primers. The PCR mixture was prepared using GoTaq® G2 Green Master Mix (Promega, USA) consisting of 5.0 µL of the DNA supernatant, 25.0 µL of 2X GoTaq® G2 Green Master Mix buffer, 0.5 µL of each primer (10 pmol/µL), and top up with 19 µL of Nuclease-free water to a final volume of 50 µL. The thermal cycling conditions was set up with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. A final extension step was performed at 72 °C for 7 min.

The resulting amplicons were purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA) according to the manufacturer's protocol prior to sequencing by First BASE Laboratories Sdn Bhd, The sequences obtained for each isolate were used as query sequences and aligned using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI, USA) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely matching sequences in the GenBank database. The DNA sequences were deposited in GenBank, and the corresponding accession numbers were obtained.

Biodegradation of FOG using biosurfactant producing bacteria

Bacterial consortia preparation

Two different bacterial isolates with the highest biosurfactant activity were selected for further FOG

biodegradation. The bacteria were grown separately in LB medium to a density of 10^7 colony forming units (CFU/mL) for 24 h. After 24 h, 0.5 mL of each bacterial culture were added into 20 mL of liquid BH supplemented with 1% (w/v) cooking oil (Nzila et al. 2021). After that, the bacterial consortia were cultured for 24 h at 37 °C with continuous 130 rpm shaking until they reach 10^6 colony forming units (CFU/mL).

Lab scale biodegradation of FOGs (Effect of different pH)

FOG biodegradation by the prepared consortia was carried out in 50 mL Falcon tubes to mimic the condition of a piping setting. Falcon tubes containing 20 mL of BH medium and 1% (w/v) of FOG collected from The Hills food court was inoculated with 2 mL of the overnight grown bacterial consortia. In order to examine the effect of different pH on FOG degradation by the prepared bacterial consortia, the BH medium was adjusted to different pH ranging from 4 to pH 9 using buffered solution. The pH range of 4 to 9 was chosen to cover moderately acidic through alkaline conditions while maintaining viability of the mesophilic bacteria. pH values below 4 or above 9 were not tested due to expectations that such extremes would severely inhibit bacterial activity (and are less common in environmental scenarios). To maintain the pH of 4 to 6, citrate–phosphate buffer was used to replace water in the media preparation (Sathishkumar et al. 2008). For pH 7 and 8, phosphate buffer was the best buffer to maintain the pH while carbonate-bicarbonate buffer was used to maintain pH 9 (Sathishkumar et al. 2008).

The final pH in each Falcon tubes was adjusted with hydrochloric acid (HCl) and sodium hydroxide (NaOH) respectively. The pH in the tubes were set from 4 to 9 at the beginning of the experiment and all flasks of different pH were prepared in triplicate. Negative control was prepared with only FOGs but no bacterial consortia. The mixtures were then placed into an incubator at 37 °C with continuous 130 rpm shaking for 30 days. Periodically within the 30 days, pictures were taken to observe for possible physical change to the FOGs. The remaining FOGs after 30 days of biodegradation were determined according to the method of Sathishkumar et al. (2008) via solvent extraction using n-hexane.

Determination of residue FOGs

Gravimetric analysis

The partition-gravimetric technique was used to determine the remaining FOGs. Residual FOGs in the tube were extracted three times using n-hexane at a ratio of 1:1. Extraction was carried out with vigorously shaking for two nights using a water-bath shaker at a speed of 130 rpm. Recovery check via spiking a known amount of oil into a blank sample and extracting it under the same protocol showed a recovery of > 95% of the spiked oil. The upper layer solvent extract was then dehydrated using anhydrous sodium sulphate before solvent removal via rotary evaporation. The weight of the residual oil was then determined gravimetrically (Tzirita et al 2019).

Fatty acid derivatization and gas chromatography analysis

The solvent extracted residue FOGs were also analysed using gas chromatography after derivatization of the fatty acids using sulphuric acid derivatization method (Wenming et al. 2024). The dried extract was mixed with 0.15 mL of methanol: sulphuric acid (90:10 v/v) mixture and then heated at 95 ℃ for several minutes in a shaking water bath. The mixtures were then extracted again with 1 mL of hexane and dried before adding 1 mL of dichloromethane prior to gas chromatography (GC) analysis. GC was performed using Shimadzu GCMS-QP2010 Plus (Japan) equipped with a single quadrupole mass filter and a non-polar cross-linked capillary column, BPX-5 (30.0 m × 0.25 mm × 0.25 µm) which was coupled with a series of mass selective detector. The instrument was initially set to a temperature of 50 °C and held for 5 min. Following this, the oven temperature was ramped up to a final temperature of 280 °C at a rate of 10 °C/min and maintained for an additional 10 min. The injection port and detector temperatures were both set to 300 °C. The system pressure was stabilized at 53.5 kPa, with helium used as the carrier gas at a flow rate of 1 mL/min. The ionization voltage was 70 eV. Subsequently, 1 µL of the extract was injected in splitless mode. The mass spectral scan range was set between 45 and 1000 m/z, with a scan speed of 3333. Identification of bioactive compounds in the extracts was achieved by comparing

the mass spectra with the National Institute of Standards and Technology (NIST 14) mass spectral libraries (Jamal et al. 2022). Identification of compound was only considered reliable when the match quality was > 90%. To quantify the extent of FOG degradation, the percentage reduction in total peak area of the chromatograms from treated samples was calculated relative to the untreated control. This was performed by integrating the area under all detectable peaks, and the percentage reduction was determined using formula (2).

$$\text{Percentage reduction } (\%) = \frac{\text{Area Control} - \text{Area Treatment}}{\text{Area Control}} \times 100\% \quad (2)$$

Statistical analysis

All experiments were performed in triplicate, and data are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was conducted to determine significant differences among treatments, with statistical significance defined at $p < 0.05$. When appropriate, Tukey's post-hoc test was applied to identify specific pairwise differences. The raw data obtained from all the triplicate experiments conducted were collected and the mean and standard deviation values of the data were calculated.

Results and discussion

The enrichment of the FOGs sample in MSM supplemented with 1% (v/v) cooking oil resulted in the successful isolation of seven morphologically distinctive bacterial cultures labelled F1 through F7. All these isolates were stored as glycerol stock and screened for their ability to produce biosurfactants using oil spreading assay and emulsification (E24) test. Cooking oil was used in the oil spreading and E24 assays to closely mimic the nature of FOG present in real wastewater (which often comprises used vegetable oils). In the oil spreading assay, the cell-free supernatant from three of the seven isolates displaced the oil layer on the surface of a 9 cm Petri dish, forming a visible clearing zone (Fig. 1). Among the isolates, F3 exhibited the largest clearing zone diameter at 4.6 ± 0.2 cm, followed by F6 at 4.0 ± 0.1 cm, and F2 at 2.7 ± 0.2 cm. Since the diameter of the oil clearing zone is directly correlated with biosurfactant quality (Sumiardi et al. 2018), the larger clearing zone produced by isolate F3 suggested that the isolate produced better biosurfactant activity compared to the other isolates.

Further emulsification assays were conducted on the seven bacterial isolates to screen for biosurfactant production. The emulsion height formed was measured and expressed as a percentage of the total height of the oil and cell-free supernatant mixture, referred to as the emulsification index (E24). The cell free supernatant was presumed to contain biosurfactant if it would emulsify the added cooking oil. A higher emulsification index indicates greater biosurfactant production, as biosurfactants enhance the stability of oil-in-water emulsions by reducing surface and interfacial tension and therefore leading to a more stable emulsion layer (Al-Sakkaf and Onaizi 2024).

Among the seven isolates tested, two demonstrated emulsification indices exceeding 30% while the remaining isolates displaying values between 12 and 26% (Fig. 2). The negative control prepared showed no emulsification, thus indicating that the positive results are due to biosurfactant production

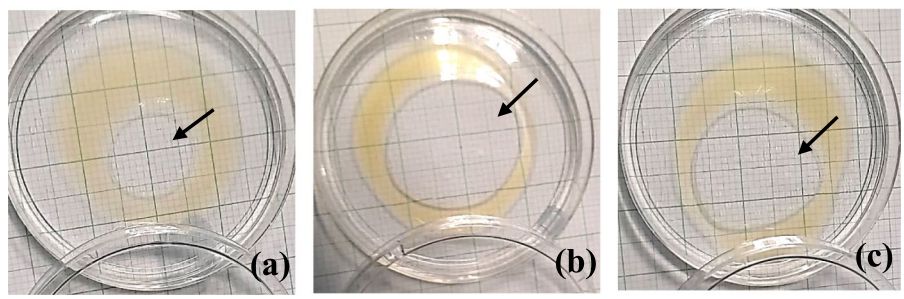


Fig. 1 Oil spreading assay using cell free supernatant produced by isolate **a** F2, **b** F3 and **c** F6 resulting in the displacement of oil layer and formation of a clearing zone (arrows)

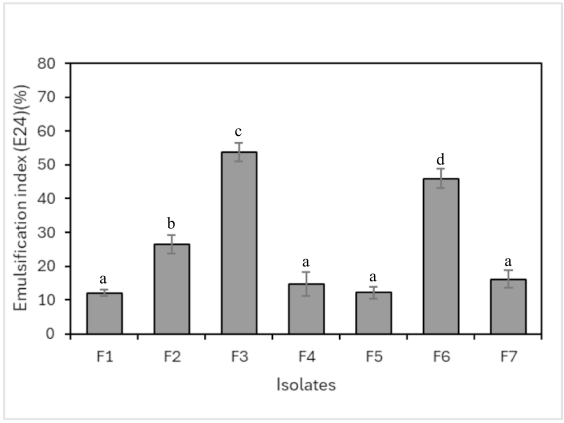


Fig. 2 Emulsification index (E24) of the seven bacterial isolates after 24 h of standing at room temperature. Different letters above each bar represent significant differences at $p < 0.05$ probability level

by the isolates. The highest emulsification index was observed from the cell free supernatant of isolate F3 with 53.8% and followed by isolate F6 with 46%. Both isolates produced stable emulsions that persisted for over 24 h (Fig. 3), thus indicating the potential ability of these isolates in producing biosurfactant. The stability of the emulsion layer is an important characteristic, as it correlates with the quality and quantity of biosurfactants produced; stable emulsions indicate effective surfactant activity, which has significant applications in various industries, including bioremediation and enhanced oil recovery (Jahan et al. 2020). These findings align with previous studies indicating that bacterial isolates with emulsification indices above 50% are typically considered strong biosurfactant producers with enhanced biotechnological potential. For instance, a study by Ali et al. (2021) reported that four bacterial isolates with high emulsification indices as promising agents in hydrocarbon degradation due to their ability to form stable emulsions that enhance hydrocarbon availability for microbial degradation.

Bacterial identification

The two biosurfactant producing bacteria, isolate F3 and F6 were selected for further identification via comparison of its 16S rRNA gene with sequences from GenBank database. PCR amplification of both the isolated bacterial genomic DNA resulted in the formation of a single PCR product DNA band of approximately 1.5 kb in length when visualized on an agarose gel (Fig. 4). Further BLAST analysis of the 1.5 kb PCR product showed that the 16S rRNA gene of isolate F3 shared up to 98.75% sequence similarity with *Pseudomonas aeruginosa* while that of isolate F6 showed 99.75% sequence similarity with *Bacillus velezensis*. The 16S rRNA gene of isolate F3 was deposited into GenBank under the accession number PQ279204 (Strain MGL 1) while that of isolate F6 was deposited with the accession number PQ279027 (Strain MGL 2).

The identification of both the biosurfactant producing isolates as *P. aeruginosa* and *B. velezensis* is particularly relevant, as both species are recognized for their robust biosurfactant production capabilities, which are critical for effective FOG degradation (Eslami et al. 2020; Meena et al. 2021; Rehman et al. 2021). *P. aeruginosa* is famous for its ability in

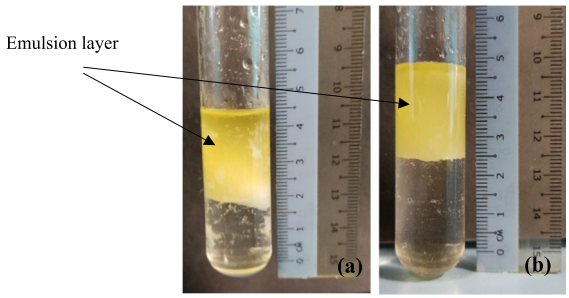


Fig. 3 Emulsion layer produced by the cell free supernatant of **a** isolate F3 and **b** isolate F6 in from the emulsification index (E24) test

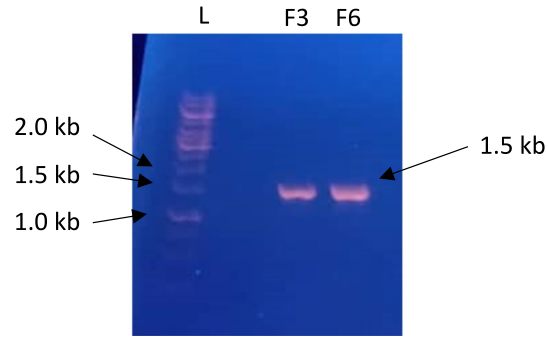


Fig. 4 Agarose gel electrophoresis of both isolate F3 and F6 16S rRNA gene of approximately 1.5 kb in size. L: Promega 1 kb DNA Ladder

producing rhamnolipids biosurfactant that are highly effective at emulsifying hydrophobic compounds like oils and fats, thereby enhancing bioavailability and microbial access to FOG substrates (Zhao et al. 2020). *B. velezensis*, on the other hand, has been reported of its ability to synthesize lipopeptide biosurfactants, such as surfactin which was reported to be responsible for the degradation of engine oil and showed a high emulsification index of 56.2% when mixed with engine oil (Meena et al. 2021). Biosurfactants produced by *B. velezensis* MHNK1 have also been reported to show high emulsification index of 85.2% and capable of biodegrading atrazine up till 87.1% within just 5 days (Jakinala et al. 2019). These biosurfactants not only aid in the initial breakdown of FOGs but also create a microenvironment favorable for enzymatic action by increasing the surface area of lipid molecules (Hernández et al. 2023).

Biodegradation of FOGs by bacteria consortia at different pH

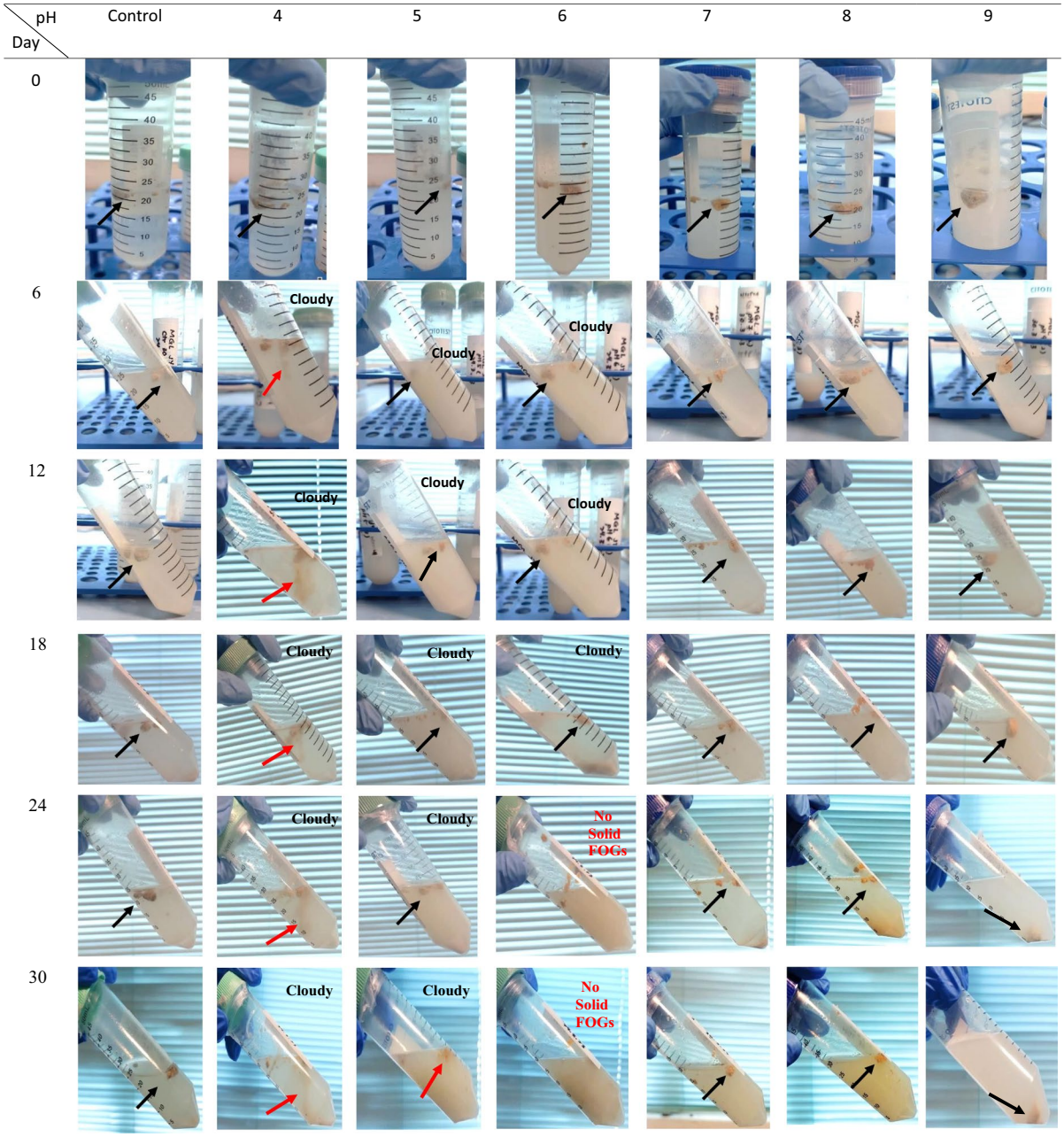
The biodegradation of FOGs by the bacterial consortium of *P. aeruginosa* F3 and *B. velezensis* F6 was evaluated across a pH range from 4 to 9, using Bushnell Haas (BH) medium. The choice of BH medium, which lacks hydrocarbons, promotes the use of FOGs as the sole carbon source for these bacteria. This setup simulates conditions in real-world environments where FOG accumulates in areas with variable pH, such as kitchen pipes that often have a pH of 3 to 4 due to acidic waste.

The concentration of bacterial consortia was standardised using spectrophotometer at reading of 0.4 (OD600). Biodegradation was carried out at the temperature of 37 °C and with shaking at 130 rpm. Shaking is needed because both *P. aeruginosa* and *B. velezensis* are aerobic bacteria which need oxygen for their growth. Incubation of the bacterial consortia at a high revolutions per minute (rpm) will increase the amount of oxygen and prevent the bacterial consortia from clumping together. According to Sakil Munna et al. (2014), shaking speed of 100 rpm is best for bacterial growth but too high speed of 200 rpm however, could lead to morphological impairment of the bacteria cells as well as generating potential oxidative stress. On the other hand, low oxygen mass transfer occurs when shaking is done using low rpm which limits the bacteria cell growth and eventually resulting in low biomass production (Chung et al. 2020).

The changes for the FOGs biodegradation in each pH treatment throughout the 30 days were observed and recorded at the interval of 6 days. On day 0, the bacterial consortia and 2 g of FOGs were introduced into each treatment tubes of different pH. As can be seen in Table 1, the FOGs were solid lumps that float on the BH media solution (Black arrow). On day 0, the solution in all the Falcon tubes were all clear. Starting from the 6th day of incubation, it was observed that the liquid media in pH treatment 4,5 and 6 started to turn cloudy while the liquid media in control, treatment pH 7, 8 and 9 remained clear. For the rest of the incubation period until day 30, the liquid media in pH treatment 4,5 and 6 remained cloudy while the control, treatment pH 7, 8 and 9 on the other hand remained clear (Table 1). Throughout the 30-day incubation, sustained microbial activity was evident from the persistent FOG emulsification and floc formation in the pH 4–6 treatments. At the end of the 30 days incubation period, all samples were plated onto MSM agar plate containing 1% cooking oil. Colonies of both *P. aeruginosa* and *B. velezensis* were successfully recovered, confirming the survival of both strains under the culture conditions except for pH 9. However, it should be noted that bacterial abundance was not quantified over time, as no CFU counts were conducted beyond this viability check.

The FOGs in all tubes were observed to be still in solid lumps except for FOGs in treatment pH 4 which started to form floc starting from day 6 (Red arrow). The flocculation of the FOGs in pH 4 remained until the last day of incubation (Day 30). On day 12, the flocs observed in pH 4 were bigger compared to Day 6. The size of the solid FOGs lumps in pH 5 and 6 however, were observed to be getting smaller as they were broken down into smaller pieces starting from day 12. Flocs formation was similarly observed in pH 5 on Day 30. Interestingly, in Falcon tube of pH 6, there were observable powdered solid that sink at the bottom of the tubes while the observable solid lumps of FOGs floating on the solution became smaller on Day 18. Surprisingly, no more observable solid FOG lump can be found at pH 6 on both day 24 and day 30 (Table 1). No observable change however can be found for FOGs in pH 7, 8 and 9 except for some minor yellowish colour formation at pH 8 on day 24 onwards (Table 1).

Table 1 Observable physical changes on the FOGs at different pH for a period of 30 Days (black arrow: solid FOGs; red arrow: flocs)



Analysis of residual FOGs via *n-hexane extraction*

After 30 days of biodegradation by the bacterial consortia, n-hexane was used to extract the residual FOGs that is still present and quantified via standard gravimetric method according to Tzirita et al. (2019). As the FOGs were also found to contain additional solid material that were not extractable using n-hexane, the residual visible solid were also weight and recorded. Both the n-hexane extractable FOGs and non-extractable residual solid were totaled up and result obtained recorded into Fig. 5. As can be seen

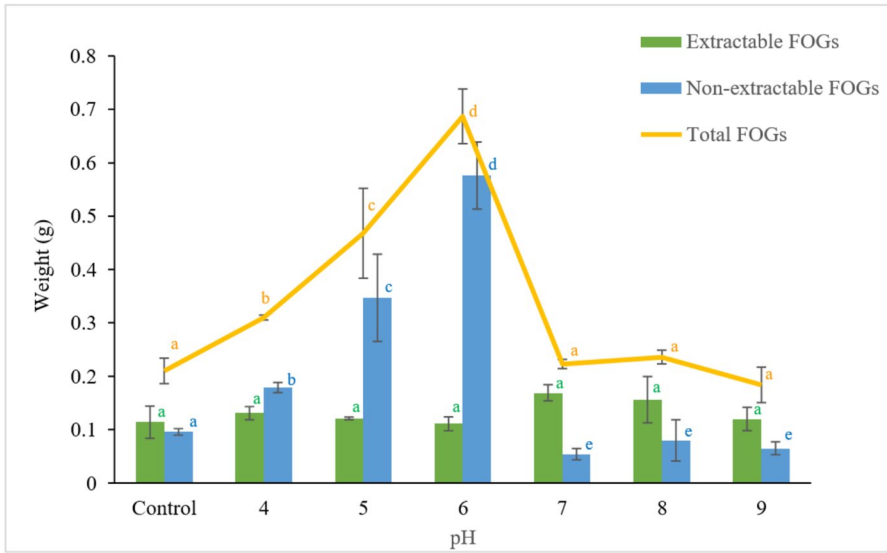


Fig. 5 Residual FOGs after 30 days of biodegradation by bacterial consortia at different pH. Different letters represent significant differences at $p < 0.05$ probability level

in the figure, total FOGs (represented by the yellow-colored line) increased as compared with the control from pH 4 and peaked at pH 6.

The total FOGs found in pH 7, 8 and 9 however remained similar with the total FOGs found in the control. The increased total FOGs observed at pH 4–6 especially at pH 6 is mainly attributed from the increased n-hexane non extractable residual FOGs (blue bar) at these pH range respectively (Fig. 5). The n-hexane non-extractable residual FOGs at pH 6 were at least 5 times higher than the n-hexane non-extractable residual FOGs that was found in the control. However, no similar result can be observed in pH 7–9. N-hexane extractable FOGs throughout the different pH (pH 4—pH 9) were also similar to the control with no significant difference ($p > 0.05$).

The increased n-hexane non-extractable residual at pH 4–6 could be explained by the observed flocculation formation at these range of pH. Bacteria have the ability to produce extracellular polymeric substances (EPS) that aid in the flocculation process (Álvarez et al. 2020). EPS are complex mixtures of polysaccharides, proteins, and nucleic acids which serve several functions which include providing structural integrity to biofilms, protecting bacterial cells, and aiding in the adhesion of cells to surfaces (Álvarez et al. 2020). In the presence of oil, the EPS produced by bacteria can act as a binding agent. The EPS molecules can interact with both the oil droplets and the bacterial cells, causing them to aggregate or flocculate together. This aggregation brings the oil and bacterial cells into closer proximity which therefore enhances the efficiency of oil degradation. The EPS effectively facilitates the physical clustering of oil and bacterial cells, creating larger flocs that can be more easily separated or removed from the surrounding environment.

The flocculation process can have practical implications in oil spill remediation and bioremediation strategies. By promoting the formation of flocs, bacteria can help facilitate the removal or containment of oil from water or soil. The larger flocs can be more effectively removed through processes like settling, flotation, or filtration (Emamjomeh and Sivakumar 2009). Furthermore, the formation of flocs can also enhance the accessibility of bacteria to the oil, improving the overall efficiency of oil degradation. This therefore explained the flocculation that was observed at pH 4–6 tubes could indicate the degradation of the FOGs by the bacteria consortia. The FOGs at pH 6 are broken down into small fragments which then cause its appearance in the n-hexane non extractable FOGs.

The research by Álvarez et al. (2020) also claims that pH can affect the production of microbial flocculants. Each microbial flocculant has its own sensitivity degree towards pH value. The surface charge and state of charge of flocculant molecules and suspended particles in liquid can have a direct impact on the flocculation effect due to electrical neutralisation.

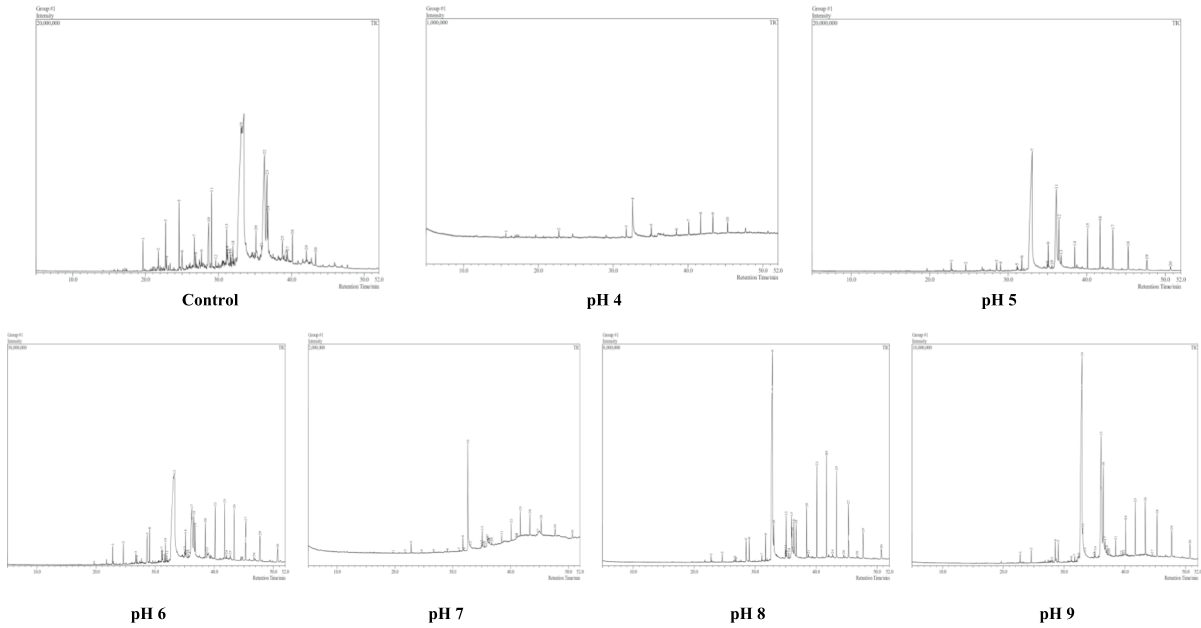


Fig. 6 GCMS Chromatogram of n-hexane extractable FOGs after 30 days of biodegradation at different pH (control, pH 4–9)

The surface charge and charged state of the colloid particles are both affected by the changes in the pH of flocculation system, which later has an impact on their capacity to neutralize charges (Zhang et al. 2013). The research by Zhang et al. (2013) also reported that flocculants perform better in acidic environment which align with our present findings where pH 6 provided the best conditions for FOG degradation by enhancing EPS-mediated flocculation. Similarly, Fujita et al. (2000) discovered that the optimal pH for bacterial growth was between pH 7.2 and 10.0, whereas the pH range for efficient flocculation is between pH 2.0 and pH 6.0.

Gas chromatography mass spectrometry (GCMS) analysis

The n-hexane extractable FOGs were further subjected to GCMS analysis to examine the possible changes to the FOGs after 30 days of remediation at different pH by the bacterial consortia. Although according to Table 1, it shows that pH 4 and 6 were the best pH for flocculation activities to occur. pH 6 might most likely be the best pH for breaking down the solidified FOGs into smaller pieces as can been seen in Table 1. However, based on GCMS analysis, pH 6 does not seem to aid in FOG degradation. Quantitative analysis of peak area reduction revealed that pH 4 and pH 5 resulted in the most significant decreases in residual hydrocarbons, with 92.5% and 89.2% peak area reductions respectively, compared to the control (0%). Subsequent reductions were observed at pH 7 (83.2%), pH 8 (61.3%), pH 9 (57.7%), and pH 6 (54.1%). Statistical analysis using one-way ANOVA showed a highly significant difference in FOG reduction across the different pH conditions with p value less than 0.0001. Further post hoc analysis using Tukey's HSD test also confirmed that the reductions at pH 4 and pH 5 were significantly greater than those observed at higher pH levels, including pH 6 and the untreated control.

Peak reduction and disappearance from chromatogram as compared to the control is the most direct sign to indicate the decomposition and dismantling of the compounds or hydrocarbon by the bacterial consortia. At pH 4, many original long-chain FOG compounds disappeared from the chromatogram, and only trace peaks of shorter-chain hydrocarbons (e.g., C18 fatty acids) were detected, suggesting these may be intermediate breakdown products (Fig. 6). The disappearance of major peaks at pH 4, combined with only minimal

detection of shorter-chain derivatives, suggests that the consortium largely degraded those compounds beyond detectable intermediates. This points to near-complete utilization of the fatty acids through β-oxidation pathways (Jimenez-Diaz et al. 2017). In this context, pH 4 is therefore the most suitable pH for the degradation of the FOGs by the bacterial consortia. Alnuaimi et al. (2020) similarly reported reduction of chromatography peaks when the bacteria *Bacillus megaterium* degraded various hydrocarbons in crude oil.

Five compounds that can be found in the control and all 6 different pH treatments, tetrapentacontane ($C_{54}H_{110}$), dotriacontane ($C_{32}H_{66}$), eicosane ($C_{20}H_{42}$), octadecanoic acid ($C_{18}H_{36}O_2$) and 2,4-di-tert-butylphenol ($C_{14}H_{22}O$) were selected for further determination of their degradation after 30 days at different pH. Among the 5 compounds, the longest carbon compound found in the n-hexane extractable FOGs was found to be a C54 carbon called tetrapentacontane.

Tetrapentacontane is a saturated hydrocarbon compound that is widely used as the model compound in research studies due to its relatively unreactive and stable characteristic. The percentage degradation of this longest carbon compound was monitored throughout the different pH tested. It was found that tetrapentacontane was 100% removed in pH 4 and 5 based on the graphs shown in Fig. 7a. The long carbon chain of tetrapentacontane can said to be all broken down into shorter carbon chain. The removal of tetrapentacontane is also quite effective in pH 7, pH 8 and pH 9 as their removal percentages are 99.05%, 95.69% and 97.73% respectively. The tetrapentacontane removal is not productive in pH 6 as the percentage of removal is only 1.53%. Most of the long carbon chains in tetrapentacontace is not being broken down into shorter carbon chain thus indicating the non- suitability of pH 6 for the degradation of tetrapentacontane by the bacteria consortia. Tetrapentacontane (C54) is a high molecular weight hydrocarbon that is not easily degraded (Swetha et al. 2020). The reduction of this long hydrocarbon after 30 days at all different tested pH (except pH 6) thus further provide evidence of the

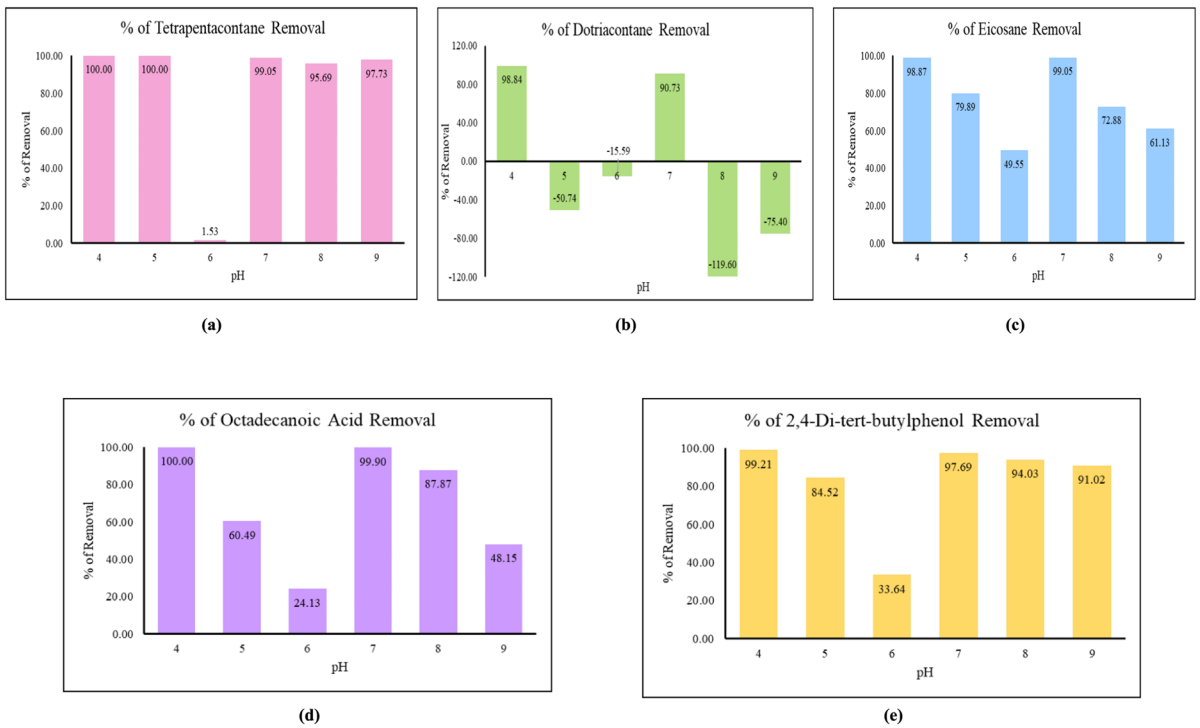


Fig. 7 Percentage removal of **a** tetrapentacontane, **b** dotriacontane, **c** eicosane, **d** octadecanoic acid, **e** 2,4-di-tert-butylphenol

FOG biodegradation ability of the bacteria consortia (Fig. 6).

Dotriacontane is a 32-carbon alkane. It is a hydrocarbon lipid molecule that is largely insoluble in water, very hydrophobic, and moderately neutral. As seen in Fig. 7b, the percentage removal of dotriacontane is not satisfied except for pH 4 and 7. Up to 98.84% of dotriacontane has been removed at pH 4 while 90.73% of dotriacontane were removed in pH 7. The area of dotriacontane increased in other pH treatments. It was believed that the increase in area is due to breaking down from compounds that have longer carbon chain such as tetrapentacontane. During degradation, high molecular weight compounds have been reported to be converted into lower molecular weight compounds which therefore explained the increased in the C32 carbon (Swetha et al. 2020). Even so, pH 4 is still the most efficient pH for hydrocarbon degradation as the percentage of dotriacontane removal is still the highest at that pH.

Eicosane is a saturated alkane with long chain hydrocarbon of 20 carbons (Fig. 7c). Eicosane can be found in foods such as lemon balms, all spices, papaya, coconut, and linden. The most effective pH for eicosane removal would be pH 7, which removed 99.05% of it. Following by pH 4 where 98.89% of eicosane was removed in the treatment. The least effective eicosane removal happened at pH 6, it only removes 49.55% of eicosane. The percentage degradation of eicosane by our tested bacterial consortia is comparable to oil degradation ability of bacteria such as *Enterococcus mundtii* which has been reported to degrade 65.17% of eicosane (Patel and Shah 2023).

A C18 straight-chain saturated fatty acids (Fig. 7d) which is octadecanoic acid is also detected based on the GCMS result. This fatty acid is commonly found in animals and vegetable lipids. No octadecanoic acids however can be found in pH 4. It is believed that octadecanoic acid is fully broken down into a shorter carbon chain compound at the particular pH. pH 7 and pH 8 are also suitable for the removal of octadecanoic acid. The percentages of octadecanoic acid removal were up to 99.90% for pH 7 while 87.87% for pH 8. pH 6 has the least removal percentage which indicates that pH 6 is not favorable for the bacterial consortia to remove octadecanoic acid.

2,4-Di-tert-butylphenol is a compound with 14 carbons and has two tert-butyl substituted at positions 2 and 4 (Fig. 7e). It is an alkylbenzene which belongs to the phenol family. It is utilized industrially as an antioxidant for hydrocarbon-based product and UV stabilizer for fuel addictive. pH 4 showed the highest percentage of 2,4-di-tert- butylphenol removal by bacterial consortia with up to 99.21% of the phenol compound being removed. The least removal of 2,4-di-tert-butylphenol compound is at pH 6 which only removed 33.64% of the compound.

As a whole, the results obtained demonstrated the capability of the bacterial consortia to degrade FOGs effectively across most tested pH levels, particularly at pH 4. Acidic pH conditions were observed to promote better flocculation; however, extensive flocculation at pH 6 did not translate to efficient degradation. At pH 6, substantial extracellular polymeric substance (EPS) production led to large floc formation and significant physical sequestration of FOGs into bacterial aggregates. This physical sequestration likely limited further enzymatic breakdown, as FOG embedded in extensive flocs may have become less accessible to bacteria or oxygen, thereby hindering complete biodegradation despite initial enhanced physical dispersion (Huang et al. 2022).

In contrast, pH 4 supported fewer but more metabolically active flocs, facilitating more comprehensive FOG degradation. Several mechanisms may underpin this enhanced degradation observed at pH 4. Firstly, acidic environments at pH 4 may catalyze partial abiotic hydrolysis of FOG substrates, breaking triglyceride ester bonds and liberating free fatty acids and glycerol. These hydrolysis products, particularly fatty acids, are more readily accessible and efficiently metabolized by bacteria, thus increasing substrate bioavailability for microbial assimilation (Prasad et al. 2024). Additionally, the bacterial consortium might have upregulated acid-stable lipases or stress-response pathways, enabling *P. aeruginosa* and *B. velezensis* to maintain metabolic activity and continue biosurfactant production under acidic conditions (Mozaheb et al. 2023). At pH 4, the enzymatic activity can also be enhanced due to the protonation of substrate molecules, increasing their hydrophilicity and susceptibility to enzymatic attack. Lipases, the primary enzymes responsible for FOG degradation, can exhibit altered conformational stability under acidic conditions, potentially increasing their catalytic efficiency. For instance, lipases produced by *Bacillus* species, have showed stable activity under mildly acidic conditions (Duan et al. 2023).

Biosurfactants, particularly rhamnolipids produced by *P. aeruginosa*, play a crucial role by reducing surface tension and emulsifying hydrophobic substrates (Sharma et al. 2019). At pH 4, these biosurfactants exhibit enhanced stability and activity due to protonation of their functional groups, improving their interaction with lipid molecules. *P. aeruginosa* produces rhamnolipids in response to acidic conditions, enhancing emulsification and facilitating FOG degradation. While rhamnolipids maintain stability across a wide pH range, their emulsification activity peaks under mildly acidic conditions like pH 4 (El-Housseiny et al. 2020). Furthermore, biosurfactants such as rhamnolipids from *P. aeruginosa* and lipopeptides from *B. velezensis* likely retained their activity or were produced in greater quantities at low pH, counterbalancing any expected loss of biosurfactant stability due to acidic conditions. Stable biosurfactant-mediated emulsification at pH 4 ensured sustained FOG availability for microbial uptake, while optimal enzyme induction (such as acid-tolerant lipases and hydrolases) supported efficient hydrocarbon degradation. Moreover, mild acidic conditions might have facilitated abiotic hydrolysis of certain FOG components (e.g., triglyceride ester bonds), further enhancing biodegradability.

The consortium likely employs established hydrocarbon degradation pathways. Initially, lipases secreted primarily by *B. velezensis* hydrolyze triglycerides into glycerol and free fatty acids. Glycerol is then funneled into central metabolism through glycolysis, while fatty acids undergo β-oxidation to produce acetyl-CoA units, which enter the tricarboxylic acid (TCA) cycle, leading to complete oxidation to CO_2 and water. For aromatic compounds (e.g., 2,4-di-tert-butylphenol), specialized pathways involving ring-hydroxylating dioxygenases followed by ring-cleavage enzymes are employed, notably by *Pseudomonas* species, known for phenolic compound degradation. The complementary roles of *B. velezensis* and *P. aeruginosa* within the consortium are critical for the observed synergistic effects. *B. velezensis*, known for potent lipase production, likely hydrolyzes complex FOG molecules into more manageable fatty acids and glycerol, which are subsequently oxidized by *P. aeruginosa*. Concurrently, the biosurfactants from *P. aeruginosa* enhance FOG solubilization and bioavailability, benefiting the enzymatic action of *B. velezensis*. This cooperative interaction, particularly effective under acidic conditions, explains the notable FOG degradation efficiency observed at pH 4. Recent literature supports these findings, suggesting microbial consortia with diverse biosurfactant profiles typically exhibit enhanced degradation efficiencies compared to single-strain cultures due to their broader functional adaptability across varying pH environments (Elumalai et al. 2021; Rizzo et al. 2018). This comprehensive understanding of consortium synergy and the beneficial effects of acidic conditions provides valuable insights for optimizing FOG biodegradation strategies using biosurfactant-producing bacterial consortia.

To facilitate real-world application of this system in wastewater treatment, a simple and effective scale-up strategy would be the development of a two-stage bioreactor system. In this setup, the first stage could be maintained at or near pH 4 to optimize the FOG degradation performance of the bacterial consortium, followed by a neutralization step before discharge or further biological treatment. This approach mirrors the optimal lab-scale conditions identified in this study while addressing downstream treatment compatibility (Amha et al. 2019). Alternatively, bioaugmentation of grease traps in food-processing or restaurant wastewater systems could be employed (Witharana et al. 2018). Given that these environments naturally accumulate FOGs and can tolerate mild acidification, introducing the consortium, potentially along with controlled acid dosing, could enhance in situ FOG breakdown before the wastewater enters larger municipal treatment systems. This consortium that works best at acidic condition can be useful in degrading FOG effluents that are acidic in nature (Sultana et al. 2022).

This research aligns with sustainable waste management efforts that focus on using biological methods to reduce FOG buildup, an area that is gaining growing attention in wastewater treatment. Future studies should verify these findings using real wastewater matrices and assess the scalability of this system. Additionally, integrating this system with existing infrastructure, such as grease trap pre-treatment, should be explored. Other important areas for future research include examining the long-term stability of the consortium, characterizing the dominant strains and EPS composition within the flocs formed under different pH conditions, and determining whether synergistic interactions persist under varying

environmental stresses. Furthermore, evaluating the emulsification index of the consortia on a broader range of oil types would help determine the spectrum of biosurfactant activity. Although this study did not include temporal analysis of emulsion stability, such work would be valuable in future investigations aimed at characterizing the performance and durability of emulsified layers over time.

Conclusion

Two biosurfactant-producing bacterial strains were successfully isolated and identified as *Pseudomonas aeruginosa* and *Bacillus velezensis*. When combined into a consortium, these strains demonstrated superior removal of long-chain hydrocarbons from fats, oils, and grease (FOG), particularly under acidic conditions, with pH 4 emerging as the optimal degradation environment. This finding contrasts with several previous studies where neutral or alkaline pH conditions were reported as more favorable for lipid degradation (Jiang et al. 2023; Nzila et al. 2017). Our result suggests that the unique combination of *Pseudomonas* and *Bacillus*, along with their biosurfactant production, can function effectively in more acidic regimes than traditionally expected. This observation aligns with emerging research emphasizing that tailored microbial consortia can outperform individual strains under specific stress conditions. Notably, a cooperative degradation of fats by *Bacillus* and *Pseudomonas* strains was also observed in earlier studies under neutral pH conditions (Ke et al. 2021; Tzirita et al. 2019). Our work extends this concept to acidic environments. It also contributes to the growing trend of utilizing biosurfactant-producing consortia for environmental remediation, demonstrating that operational parameters like pH can drastically alter biodegradation performance.

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Author contributions Jia-Ying Wong and Ngui-Sing Ngieng performed the experiment and drafted the manuscript. Ahmad Husaini, Rosmawati Saat and Hasnain Hussain conceptualised and proposed the study, assisted with data analysis and critically revised the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article. Sequence data that support the findings of this study have been deposited in the NIH genetic sequence database, GenBank. Isolate F3 was deposited into GenBank under the accession number PQ279204 (Strain MGL 1) while that of isolate F6 was deposited with the accession number PQ279027 (Strain MGL 2).

Declarations

Conflict of interest The authors declare that no conflict of interest or competing interests.

Ethical approval Is not applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

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