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Isolation of Biofilm-Bioflocculant Producing Bacteria from Palm Oil Mill Effluent

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Abstract

A variety of flocculants have been used to treat wastewater by agglomerating various colloidal substances and cells. Due to the adverse effects and high cost of conventional flocculants, bioflocculant which are originally produced by microorganisms are gaining attention. Therefore, this study aimed to identify the biofilm-bioflocculant-producing bacteria which was isolated from palm oil mill effluent in Johor, Malaysia. The isolated bacteria were identified by their colony morphology, Gram-staining and 16S rRNA gene sequence analysis. All isolates were subjected to bioflocculant assay with kaolin clay suspension as a model of wastewater to assess the flocculation efficiency of the bacterial bioflocculants. Two different isolates were successfully identified as Gram-negative bacteria *Stenotrophomonas koreensis* TR6-01 and *Alishewanella agri* BL06. *S. koreensis* TR6-01 shows the highest flocculating activity at 82.79%, followed by *A. agri* BL06 with 78.52%, respectively. The strongest biofilm producer was shown by *S. koreensis* TR6-01 and thus, indicates good candidate for future wastewater treatment technology.

Keywords Biofilm, bioflocculant, palm oil mill effluent, bacteria

1.0 INTRODUCTION

Disruption of skin function or integrity due to burns, injuries, or diseases is defined as a wound and is often caused by infections, genetic disorders, surgery, trauma, and abrasions (Bhardwaj et al., 2018; Kamoun et al., 2017). Wounds can be classified as acute or chronic depending on the healing time and severity of the wound (Aderibigbe & Buyana, 2018). A chronic wound takes longer to heal because it involves more tissue loss, which affects vital components like nerves and joints, and heals completely within 8 to 12 weeks with little scarring (Aderibigbe & Buyana, 2018).

A wound can naturally heal by the regrowth of the missing and damaged tissue, but an efficient wound dressing is required to speed up the healing process (Boateng and Catanzano, 2015). The application of wound dressing is important to mediate the proper phase and create favourable conditions for wound healing (Summa et al., 2018). In general, an effective wound dressing should be able to absorb wound exudates, maintain a moist environment, allow adequate gas exchange, have no toxic effects, facilitate cell migration and proliferation, and regulate the release of growth factors (Bagher et al., 2020). In addition, the wound dressing must be easily removable without traumatizing the newly formed tissue to promote wound healing (Aderibigbe and Buyana, 2018).

Based on the above requirements, a natural polymer is a promising candidate that meets all the requirements to be used as a material for wound dressings. Biopolymers are usually used due to their water absorption capacity, mechanical strength, biocompatibility, biodegradability, readily available and low cost (Summa et al., 2018). Among the biopolymers available, alginate, derived from brown algae and composed of α-L-guluronic acids (G-chain) and β-D-mannuronic acids (M-chain), is

widely used in pharmaceutical and biomedical applications (Catanzano et al., 2015). Ionic interaction between guluronic acids and divalent cations such as Ca^{2+} ions leads to the formation of an "egg-box" structure in gel form (Aderibigbe and Buyana, 2018). This makes alginate dressings an ideal material for producing a wound dressing that can provide moisture to the wounded site (Boateng and Catanzano, 2015). Although alginate dressings can control the wound-healing process, proper and rapid wound healing is critical to prevent impaired or delayed wound healing (Summa et al., 2018).

In recent years, the design of advanced wound dressings has changed from a passive form that does not play an active role in wound healing (cotton and gauze) to an active form in which biological components are incorporated into the dressing (antibacterial agents or plant extracts) that have a specific function and play an active role in rapid wound healing (Boateng and Catanzano, 2015). Plants are used in traditional medicine to cure a variety of diseases. They are known to have less harmful effects, are naturally abundant, and are economical (Riaz & Chopra, 2018).

Extracts of roselle (*Hibiscus sabdariffa Linn.*) are known to have antibacterial properties against human pathogens and both Gram-positive and Gram-negative microorganisms (Alshami & Alharbi, 2014). The presence of flavonoids in HSL extract is attributed to their antimicrobial activity, in which they form complexes with the cell wall and cause the exit of ions from bacterial cells (Riaz & Chopra, 2018). The antibacterial property of HSL is important to protect the wound area from microbial infection during the healing process and to avoid prolonged infections that may increase the incidence of chronic wounds, leading to delayed wound healing (Summa et al., 2018). Therefore, the present work aimed to characterize the properties of alginate/gauze dressings with different alginate concentrations and preparations. Later, the survivability of human skin fibroblasts (HSF) in the alginate dressings infused with HSL extract was also observed.

2.0 EXPERIMENTAL

2.1 Materials

Nutrient agar (Sigma-Aldrich, USA) and nutrient broth (Merck, USA) were used to grow the isolated bacteria. Crystal violet, safranin and oil immersion were purchased from Sigma-Aldrich, UK.

2.2 Sampling Site

Palm Oil Mill Effluent (POME) anaerobic sludge sample was collected from an anaerobic digester at Felda Maokil Palm Oil Mill, Labis, Johor. The sample was collected at a temperature of 38.5°C and the collected sample was kept in a sterile Schott bottle at 4°C for further analysis.

2.3 Bacteria Isolation from POME

The bacteria were isolated following the methods by Abbas et.al. [4] with some modifications. In brief, serial dilution was carried out by adding 10% (v/v) of raw POME into sterile distilled water. A 0.1 mL serial diluted mixture was pipetted and spread onto nutrient agar (NA) and incubated at 37°C for 24 h. Bacterial colonies with distinctive colony morphology on the culture plate were re-streaked onto fresh plates (1 morphotype per plate) and further incubated at 37°C for 24 h. The re-streaking process was continued until pure colonies of distinctive morphologies were obtained. The newly fresh bacterial pure colonies were streaked on slant agar and incubated for 24 h at 37°C before being kept at 4°C for further use.

2.4 Morphological identification of the isolated bacteria

Morphological characteristics of isolated bacteria were performed by observing the differences of the isolated bacterial colonies on NA plates, based on their average size, colonial shape, colour, margin, elevation, and the surface of the colonies. For separating Gram-positive from Gram-negative microorganisms, the Gram staining method previously described by Aryal [6] was performed. A slide fixed with isolated bacterial culture (air-dried and heat-fixed) was flooded with crystal violet for 60 s before rinsing with sterile distilled water to prevent damage to the bacteria colony. Later, the washed slide was flooded with iodine solution for 1 min and rinsed again with sterile distilled water. The slide was washed using 95% ethanol and rinsed immediately with sterile distilled water. Then, the slide was stained with safranin for 60 s before being washed off with sterile distilled water. The slide was then air-dried in the laminar flow before being observed and examined using a 100X oil immersion objective. Gram-negative bacteria leave a red stain, while Gram-positive bacteria produce a purple tint.

2.5 Bioflocculant Assay

A loopful of the grown pure colony on a culture plate was inoculated into 100 mL of NB and shaken overnight at 180 rpm, 37°C. Following a 24-hour incubation period, 10 mL of the overnight culture was added to 90 mL of fresh NB, which was then shaken at 180 rpm and incubated at 37°C. The optical density of the culture was monitored during the incubation period at OD_{600nm} until it reached about 0.1. After that, 50 mL of the culture media was added into a 50 mL centrifuge tube and centrifuged for 30 min at 4°C, 8000 rpm. For further investigation, the supernatant was collected and kept at -20°C.

The flocculating activity of the isolated bacteria was done according to the procedure done by Ndejiko and Wan Dagang [7]. Briefly, 2 mL of cell-free supernatant and 3 mL of 1% calcium chloride ($CaCl_2$) were mixed into 100 mL of 4.0 g/L kaolin clay suspension in a 500 mL beaker. Using a 6-breaker jar tester (JLT6, VELP Scientifica, Italy), the mixture was first held still for 5 min before being agitated slowly for 5 min at 80 rpm in a flocculation tester. Utilizing a spectrophotometer, the absorbance of

the clearing upper solution was determined at OD_{550nm}. For the control, the 2 mL of cell-free supernatant was replaced with 2 mL sterile NB. The experiment was repeated three times for each isolated bacterium. The flocculating activity (%) was evaluated using **Equation 1**.

$$\text{Flocculating activity (\%)} = [(B - A) / B] \text{ X } 100 \quad (1)$$

where A represents the sample's $OD_{550\ nm}$ and B represents the control's $OD_{550\ nm}$.

2.6 Biofilm Assay

The isolated bacterial colonies were screened for biofilm formation ability using biofilm assay following the method by Saggu et al. [8] and Molobela et al. [9]. The NA plates were streaked with a loopful of isolated bacteria cultures, which were then incubated at 37°C for 24 hours. A few colonies of each isolated bacteria were inoculated into 100 mL of NB, shaken at 180 rpm and incubated at 37°C for 24 h. 10 mL of the overnight culture were added into 90 mL of fresh NB, shaken at 180 rpm and incubated at 37°C until each isolated bacteria grew into late exponential phase. A 200 µL of late exponential phase culture was pipetted into a 96-well microtiter plate and incubated at 100 rpm, 37°C for seven days. Sterile NB was used as a negative control. After the wells had been incubated for 7 days, the supernatant containing non-adherent cells was removed by washing them three times with 200 µL of sterile, distilled water. The plates were exposed to the hot air at 60°C for 60 min and allowed to dry overnight in an inverted position at room temperature. After that, each well was stained for 30 min with 200 µL of crystal violet. After that, sterile distilled water was used to cleanse the wells three times. To quantify the biofilm, 200 µL 30% (v/v) glacial acetic acid was added to the wells. The plates were analysed using a microtiter plate reader (ELISA) at OD_{600nm}. The 30% glacial acetic acid was used as a blank.

By comparing the final optical density (OD_{600nm}) result with the cut-off optical density (ODc, 600 nm) value, the potential of isolated bacteria to form biofilm can be evaluated [10]. The $OD_{c,\ 600\ nm}$ value was calculated using **Equation 2**.

$$OD_{c,\ 600\ nm} = OD_{avgNeg,\ 600\ nm} + 3SD \quad (2)$$

where $OD_{avgNeg,\ 600\ nm}$ is the average OD of the negative control, while 3SD is the standard deviation of the negative control. The OD negative control is the value of nutrient broth without bacteria. The final OD value of biofilm was calculated using **Equation 3**.

$$OD_{600\ nm} = OD_{avgStr,\ 600\ nm} - OD_{c,\ 600\ nm} \quad (3)$$

where $OD_{avgStr,\ 600\ nm}$ is the average OD of the bacterial strain. The potential of isolated bacteria to form biofilm was identified after the interpretation of the biofilm assay result. The interpretation of the biofilm assay result is based on **Table 1** below.

Table 1 Interpretation of biofilm production [10]

Final OD value	Biofilm production
$OD_{600\ nm} \leq OD_{c,\ 600\ nm}$	No
$OD_{c,\ 600\ nm} < OD_{600\ nm} \leq 2OD_{c,\ 600\ nm}$	Weak
$2OD_{c,\ 600\ nm} < OD_{600\ nm} \leq 4OD_{c,\ 600\ nm}$	Moderate
$4OD_{c,\ 600\ nm} < OD_{600\ nm}$	Strong

2.7 Molecular identification by using 16S rRNA sequence

The isolated bacteria were streaked on the NA plates and incubated for 24 h at 37°C. By using a sterile blade, the agar containing the colonies was cut for approximately 0.5 cm X 0.5 cm and put into a sterile 2.0 mL screw cap microcentrifuge tube. All isolated bacteria were done in triplicates. The tubes were sealed using parafilm, sprayed with 70% alcohol, put into zip-lock bags, and wrapped in bubble wrap before being sent to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for sequencing.

The following steps were done and provided by Apical Scientific Sdn. Bhd. The bacterial 16S rDNA, full-length 1.5 kb, was amplified using universal primers 27F and 1492R. The total reaction volume of 25 µL contained gDNA purified using an in-house extraction method, 0.3 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 400 µM each), 0.5 U of DNA polymerase, supplied PCR buffer and water. The PCR was performed as follows: 1 cycle (94°C for 2 min) for initial denaturation; 25 cycles (98°C for 10 sec; 53°C for 30 sec; 68°C for 1 min) for denaturation, annealing and extension of the amplified DNA. The PCR products were purified by standard method. The bi-directional sequencing of purified PCR products was done with universal sequencing primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The molecular identification of all bacterial isolates was carried out by using the full-length 16S rRNA sequence data and was analyzed by comparative studies for sequence homology using Basic Local Alignment Search Tool (BLAST) program from National Center for Biotechnology Information (NCBI). The phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis X (MEGAX) software. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed [11]. The estimation of evolutionary distances between sequences was done using Pairwise distance (p-distance) [12].

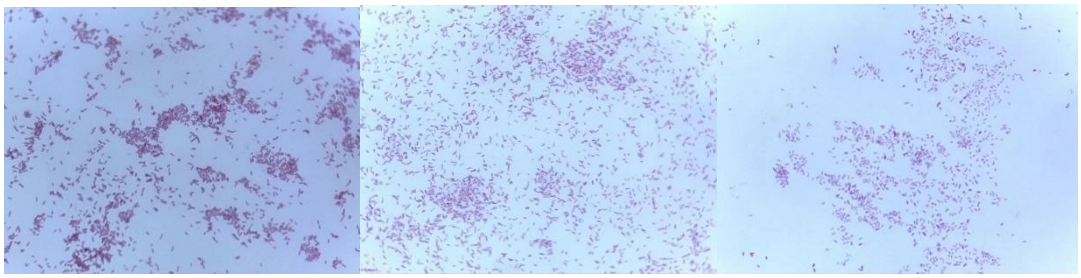
3.0 RESULTS AND DISCUSSION

3.1 Isolation of bacteria from POME

The morphological characteristics of pure isolated bacterial colonies from POME, labelled as P1, P2 and P3 are summarized in **Table 2**. P1 and P3 had an average size of 1.0 mm, while P2 had an average size of 1.3 mm. All the isolated bacterial colonies were small in size. According to Ahern [13], when the colony size is less than 1 mm, it is called punctiform, between 1-2 mm is small, between 3-4 mm is medium and more than 5 mm is large size. Based on the morphological characteristics of isolated bacterial colonies from POME, the colonies appeared in light yellow and light ivory on NA plates. Both P1 and P2 had a circular shape, entire margin, raised elevation and glistening surface. While for P3, the shape was circular, entire margin, flat elevation and glistening surface. All the isolated bacteria P1, P2 and P3 were Gram-negative bacteria as they appeared in pink stain under 100X magnification of light microscope, as shown in **Figure 1**. The same morphology of POME isolated bacteria was reported by Abbas et al. [4].

Table 2 Morphological characteristics of isolated bacterial colonies from POME

No.	Isolate code	Colony morphology						
		Gram	Average size (mm)	Shape	Colour	Margin	Elevation	Surface
1.	P1	Negative	1.0	Circular	Light yellow	Entire	Raised	Glistening
2.	P2	Negative	1.3	Circular	Light yellow	Entire	Raised	Glistening
3.	P3	Negative	1.0	Circular	Light ivory	Entire	Flat	Glistening



(a) (b) (c)

Figure 1 Gram-staining of pure isolated bacteria from POME: Gram-negative (a) P1, (b) P2, (c) P3

3.2 Bioflocculant Assay

A comparison of flocculating activity between P1, P2 and P3 indicates the difference in isolates' ability in flocculating the kaolin clay suspension. Increasingly, bioflocculant produced by P1 achieved the lowest flocculating activity towards kaolin clay suspension with 27.70%, followed by P3 (78.52%) and P2 with (82.79%). When compared to the other two isolated bacterial strains, P2's bioflocculant had the maximum flocculating activity toward a kaolin clay dispersion. Abbas et al. [4] recorded that *Bacillus nitratireducens* give the maximum flocculating activity when applied to a suspension of kaolin clay with 49.15% after 8 h of cultivation. This showed that bioflocculant produced by P2 and P3 can flocculate kaolin clay suspension more effectively than *B. nitratireducens* despite the difference in cultivation duration and the usage of $CaCl_2$ solution.

3.2 Biofilm assay

All of the isolated bacteria, P1, P2 and P3 were able to produce strong biofilm, where all of the final $OD_{600\ nm}$ was more than 4 times of OD_c values ($4OD_c = 0.564$), which were 0.976, 1.581 and 0.658, respectively. P2 recorded the highest final $OD_{600\ nm}$ of 1.581, indicating the strongest biofilm while P3 was the weakest biofilm producer among the isolated bacteria. As reported by Fazil [14], POME-isolated bacteria were among the bacteria biofilm producer. In addition, as suggested by Al-Amshawee et al. [5], the biofilm bioremediation process is an effective way to treat the POME wastewater, hence, bacterial biofilm isolated from POME itself could be a potential candidate for future approaches.

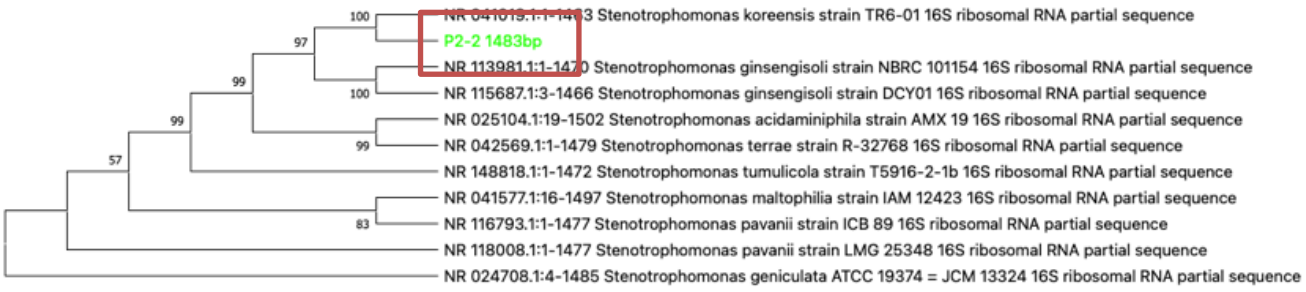
3.3 Molecular identification by using 16S rRNA sequence

Molecular identification showed that the bacterial strains were related to species of phyla *Proteobacteria.* **Figure 2** shows the phylogenetic tree and **Figure 3** shows the top 10 highest percentage similarity of isolated bacteria.

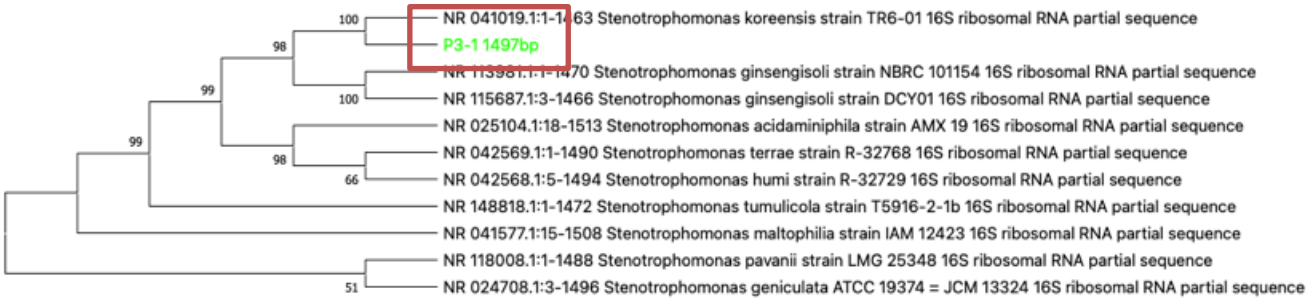
Molecular identification using 16S rRNA sequencing revealed that isolated bacteria P1 and P2 were both from the same genus *Stenotrophomonas*. Both of the isolated bacteria were *S. koreensis* strain TR6-01. Based on the top 10 best match

comparative studies for sequence homology using BLASTn, it showed that *S. koreensis* strain TR6-01 had the highest percentage identity of 99.73%, which meant that *S. koreensis* strain TR6-01 had the highest similarity with P1 and P2. Based on the construction of a phylogenetic tree using MEGAX software, the closest 16S rRNA gene sequence with P1 and P2 was *S. koreensis* strain TR6-01 (Accession number NR041019.1:1-1463). A similar strain of *S. koreensis* strain TR6-01 was discovered from cow dung and rice straw compost, near Daejeon city in South Korea [15].

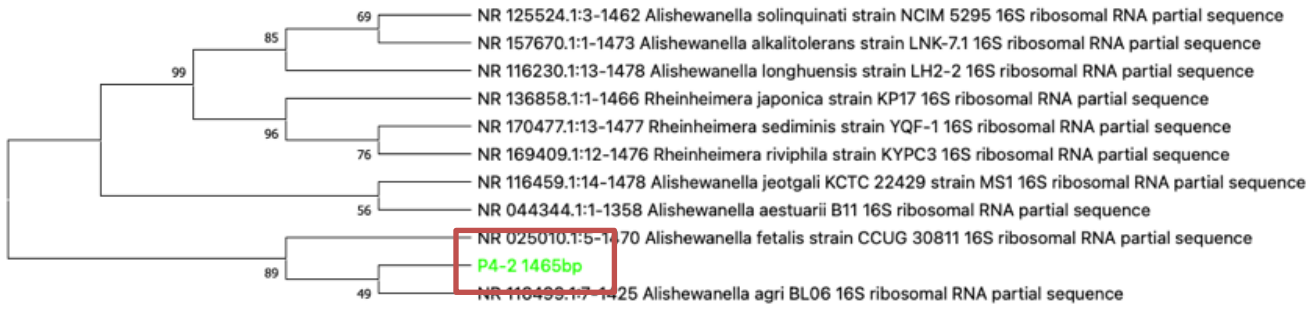
For P3, molecular identification using 16S rRNA sequencing revealed that the isolated bacteria P3 was from the genus *Alishewanella.* Based on the top 10 best match comparative studies for sequence homology using BLASTn, the result showed that *Alishewanella agri* strain BL06 had the highest percentage identity of 99.58%, which indicated that *A. agri* strain BL06 had the highest similarity with P3. Based on the construction of a phylogenetic tree using MEGAX software, the closest 16S rRNA gene sequence with P3 was *A. agri* BL06 (Accession number NR116499.1:7-1425). This strain has a close similarity with the strain found in landfill soil in Pohang, Korea [16]. However, Abbas et. al [4] isolated a different species from POME which indicates the varieties of microorganism can be found in this effluent. The four isolates, are *Pseudomonas alkaliphilic, Pseudomonas oleovorans subsp. lubricants, Pseudomonas chengduensis*, and *Bacillus nitratireducens*, are rod-shaped and Gram-negative bacteria. Moreover, *Bacillus* sp. genus and *Acinetobacter* sp. genus which are isolated from Artisanal palm oil mills [17] also have a similar morphology as reported here and Abbas et.al. [4].



(a) P1



(b) P2



(c) P3

Figure 2 Phylogenetic tree relatedness of bacterial isolated from POME: (a) P1, (b) P2, (c) P3

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Stenotrophomonas koreensis strain TR6-01 16S ribosomal RNA, partial sequence	Stenotrophomonas koreensis	2682	2682	98%	0.0	99.73%	1463	NR_041019.1
Stenotrophomonas ginsengisoli strain NBRC 101154 16S ribosomal RNA, partial sequence	Stenotrophomonas ginsengisoli	2573	2573	99%	0.0	98.23%	1470	NR_113981.1
Stenotrophomonas ginsengisoli strain DCY01 16S ribosomal RNA, partial sequence	Stenotrophomonas ginsengisoli	2555	2555	98%	0.0	98.16%	1467	NR_115687.1
Stenotrophomonas tumulicola strain T5916-2-1b 16S ribosomal RNA, partial sequence	Stenotrophomonas tumulicola	2507	2507	99%	0.0	97.42%	1472	NR_148818.1
Stenotrophomonas acidaminiphila strain AMX 19 16S ribosomal RNA, partial sequence	Stenotrophomonas acidaminiphila	2507	2507	100%	0.0	97.17%	1542	NR_025104.1
Stenotrophomonas terrae strain R-32768 16S ribosomal RNA, partial sequence	Stenotrophomonas terrae	2503	2503	99%	0.0	97.23%	1512	NR_042569.1
Stenotrophomonas maltophilia strain IAM 12423 16S ribosomal RNA, partial sequence	Stenotrophomonas maltophilia	2494	2494	100%	0.0	97.04%	1538	NR_041577.1
Stenotrophomonas pavanii strain LMG 25348 16S ribosomal RNA, partial sequence	Stenotrophomonas pavanii	2492	2492	99%	0.0	97.09%	1497	NR_118008.1
Stenotrophomonas pavanii strain ICB 89 16S ribosomal RNA, partial sequence	Stenotrophomonas pavanii	2490	2490	99%	0.0	97.09%	1483	NR_116793.1
Stenotrophomonas geniculata ATCC 19374 = JCM 13324 16S ribosomal RNA, partial seq...	Stenotrophomonas geniculata ATCC 193...	2488	2488	100%	0.0	96.70%	1497	NR_024708.1

(a) P1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Stenotrophomonas							1463	NR_041019.1
Stenotrophomonas ginsengisoli strain NBRC 101154 16S ribosomal RNA, partial sequence	Stenotropho...	2573	2573	98%	0.0	98.23%	1470	NR_113981.1
Stenotrophomonas ginsengisoli strain DCY01 16S ribosomal RNA, partial sequence	Stenotropho...	2555	2555	97%	0.0	98.16%	1467	NR_115687.1
Stenotrophomonas acidaminiphila strain AMX 19 16S ribosomal RNA, partial sequence	Stenotropho...	2523	2523	99%	0.0	97.13%	1542	NR_025104.1
Stenotrophomonas terrae strain R-32768 16S ribosomal RNA, partial sequence	Stenotropho...	2518	2518	99%	0.0	97.18%	1512	NR_042569.1
Stenotrophomonas maltophilia strain IAM 12423 16S ribosomal RNA, partial sequence	Stenotropho...	2510	2510	99%	0.0	96.99%	1538	NR_041577.1
Stenotrophomonas tumulicola strain T5916-2-1b 16S ribosomal RNA, partial sequence	Stenotropho...	2507	2507	98%	0.0	97.42%	1472	NR_148818.1
Stenotrophomonas pavanii strain LMG 25348 16S ribosomal RNA, partial sequence	Stenotropho...	2507	2507	99%	0.0	97.04%	1497	NR_118008.1
Stenotrophomonas geniculata ATCC 19374 = JCM 13324 16S ribosomal RNA, partial sequence	Stenotropho...	2505	2505	99%	0.0	96.66%	1497	NR_024708.1
Stenotrophomonas humi strain R-32729 16S ribosomal RNA, partial sequence	Stenotropho...	2503	2503	99%	0.0	96.98%	1494	NR_042568.1

(b) P2

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Alishewanella fetalis strain CCUG 30811 16S ribosomal RNA, partial sequence	Alishewanella...	2645	2645	100%	0.0	99.25%	1477	NR_025010.1
Alishewanella jeotgali KCTC 22429 strain MS1 16S ribosomal RNA, partial sequence	Alishewanella...	2623	2623	100%	0.0	98.98%	1490	NR_116459.1
Alishewanella agri BL06 16S ribosomal RNA, partial sequence	Alishewanella...	2591	2591	97%	0.0	99.58%	1425	NR_116499.1
Alishewanella longhuensis strain LH2-2 16S ribosomal RNA, partial sequence	Alishewanella...	2512	2512	100%	0.0	97.62%	1488	NR_116230.1
Alishewanella solinquinati strain NCIM 5295 16S ribosomal RNA, partial sequence	Alishewanella...	2490	2490	99%	0.0	97.47%	1462	NR_125524.1
Rheinheimera sediminis strain YQF-1 16S ribosomal RNA, partial sequence	Rheinheimer...	2473	2473	100%	0.0	97.14%	1485	NR_170477.1
Rheinheimera riviphila strain KYPC3 16S ribosomal RNA, partial sequence	Rheinheimer...	2440	2440	100%	0.0	96.73%	1500	NR_169409.1
Alishewanella aestuarii B11 16S ribosomal RNA, partial sequence	Alishewanella...	2438	2438	92%	0.0	99.04%	1358	NR_044344.1
Rheinheimera japonica strain KP17 16S ribosomal RNA, partial sequence	Rheinheimer...	2435	2435	100%	0.0	96.66%	1496	NR_136858.1
Alishewanella alkalitolerans strain LNK-7.1 16S ribosomal RNA, partial sequence	Alishewanella...	2420	2420	99%	0.0	96.48%	1485	NR_157670.1

(c) P3

Figure 3 The highest percentage similarity of bacterial isolated from POME: (a) P1, (b) P2, (c) P3

According to the findings of this study, P2 or *S. koreensis* TR6-01 generated the strongest biofilm and had the highest bioflocculant activity when compared to *A. agri* BL06 (P3). A recent study on the biofilm and bioflocculant properties of *S. koreensis* TR6-01 has not been publicly disclosed. The other species belong to the genus *Stenotrophomonas*, *Stenotrophomonas maltophilia* ZZC-06 produced bioflocculant MBF-06, achieved a flocculating rate of 95.29% for kaolin suspension and 81.43% for cadmium, which showed that MBF-06 can be considered as a viable alternative to removing cadmium from wastewater [18]. Azimi et al. [19] reported that *S. maltophilia* with the rpfF gene can produce strong biofilm formation and can form strong biofilm on polystyrene (PS) [20]. All 13 *S. maltophilia* isolates that were recovered from 13 patients with device-associated nosocomial infections formed strong biofilm on PS [20]. Bioflocculating activity is affected by several environmental factors including temperature, medium initial pH, inoculum size and carbon source [2]. Makapela et al. [2] recorded the optimum conditions for bioflocculant production by *Bacillus pumilus* strain ZAP 028 were 4% inoculum size, maltose as a carbon source, and an initial medium of pH7. The bacteria were originally isolated from Thyume River, South Africa. According to Peng et al. [1], standard medium exhibits the strongest bioflocculating activity by *Rhodococcus erythropolis* over

a wide pH range when compared to sludge/livestock wastewater medium. Jibrin and Dagang [21] indicated that the use of chicken viscera medium resulted in 91.8% of bioflocculating activity at optimum conditions using *Aspergillus flavus*. These suggest that the effectiveness of the produced bioflocculant is significantly influenced by various types of microbial strains under various controlled settings.

4.0 CONCLUSION

Biofilm-bioflocculant producer bacteria were successfully isolated from POME and identified morphologically and molecularly. Two bacterial isolates were identified as *S. koreensis* strain TR6-01 and *A. agri* strain BL06, where both of them were in *Proteobacteria* phyla and also Gram-negative bacteria. Isolated bacteria *S. koreensis* strain TR6-01 produced bioflocculant that have high flocculating activity towards kaolin clay suspension, as well as showed strong biofilm formation compared to *A. agri* strain BL06, indicated that *S. koreensis* strain TR6-01 could potentially be suggested as one of the safe bioflocculant producers to treat wastewater and waste removal due to its ability to produce strong biofilm and bioflocculant at the same time.

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