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Characterisctics of Biosurfactant-Producing Bacteria Isolated from Palm Oil Mill Effluent

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ABSTRACT

Palm oil industries generate waste that is rich in organic matter and oil. Therefore, if not properly managed, this liquid waste can pollute the environment. One way to address this issue is by using biosurfactant-producing bacteria as bioremediation agents. The purpose of this study was to isolate, characterize, and identify biosurfactant-producing bacteria from palm oil mill effluent. This study was conducted using descriptive and identification methods through general screening using oil displacement, drop collapse, and hemolysis assays, morphological characterization, Gram staining tests, extraction, emulsification tests, and molecular identification of bacteria with the highest emulsification index value. In this study, 17 isolates were obtained, with two isolates having the highest oil-spreading results (SLP1.5 and SLP2.2). Both isolates showed negative hemolysis activity and were identified as gram-positive bacilli. The isolates were then cultivated for biosurfactant production and subsequent extraction. A crude biosurfactant extract was successfully obtained from isolate SLP2.2, with an emulsification index value of 36.93±0.57%. Molecular identification revealed that SLP2.2 is closely related to *Cytobacillus Kochii*. This study demonstrates the potential application of the isolated bacteria for hydrocarbon-contaminated environmental bioremediation and highlights the opportunity to utilize palm oil industry waste as an eco-friendly biosurfactant source.

Keywords: biosurfactant, *Cytobacillus kochii*, palm oil, surface tension

INTRODUCTION

Palm oil mill effluent (POME) is an organic agroindustrial waste composed of water, oil, and solids originating from the processing of fresh fruit bunches of oil palm to produce crude palm oil (CPO). The processing of oil palm into CPO, which involves the use of steam and boiling water, generates a substantial volume of liquid waste (Nasution 2004). To date, palm oil mill effluent remains one of the major environmental challenges that are difficult to manage. POME contains a high concentration of dissolved solids and appears brownish in color as a colloidal suspension, consisting of approximately 95–96% water, 0.6–0.7% oil, and 4–5% total solids (cellulose, proteins, and fats) (Ma *et al*. 2000). Owing to its high organic content, POME has the potential to harbor biosurfactant-producing bacteria and serve as a substrate for biosurfactant production (Imam *et al.* 2025).

Surfactants are complex compounds composed of a hydrophilic group, a polar part that dissolves in water, and a hydrophobic group, a non-polar part that dissolves in oil (Sipahutar *et al.* 2024). Biosurfactants are natural surfactants produced by microorganisms that can degrade oil in soil and water, utilizing it as an energy source (Reningtyas & Maherani 2015). The primary function of biosurfactants in microbial cells, particularly in contaminated environments, is to enhance the dispersion and emulsification of water-insoluble substrates, such as hydrocarbons, and facilitate their transport into the cell through micellar solubilization (Uzoigwe *et al.* 2015).

Several bacteria isolated from palm oil wastewater have been identified as biosurfactant producers, including *Bacillus* and *Clostridium* (Aswin & Rasyidah 2023), *Enterobacter aerogenes*, *Proteus vulgaris*, Proteus mirabilis (Firmansyah et al. 2021), and *Klebsiella variicola* (Andini *et al.* 2024), *Bacillus subtilis*, *Bacillus tequilensis* (Marajan *et al.* 2013), and *Acinetobacter calcoaceticus* (Chooklin *et al*. 2023). Further research is necessary to isolate and characterize biosurfactant-producing bacteria from palm oil mill effluent originating from PT Madinra Inti Sawit, as variations in processing methods, waste composition, and environmental conditions can result in significant differences in microbial community profiles and in the properties of biosurfactants. This study aimed to isolate, characterize, and identify biosurfactant-producing bacteria from this POME source that are safe and suitable for further application.

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METHODS

Sample Collection

Palm oil mill effluent samples were collected from two wastewater ponds at PT Madinra Inti Sawit, located in Sopura Village, Pomalaa Sub-district, Kolaka Regency, Southeast Sulawesi (Figure 1.).

Bacterial Isolation

Palm oil mill effluent samples were serially diluted to concentrations of 10^{-4}, 10^{-5}, and 10^{-6}, and 0.1 mL of each dilution was spread onto petri dishes with Mineral Salt Medium (MSM) containing (g/L): 20 g sucrose, 2 g NH4NO3, 3 g KH2PO4, 10 g Na2HPO4, 0,2 g MgSO4·7H2O, and 0.2 g yeast extract and POME (1.0%, w/v) (Sipahutar *et al.* 2024).The plates were incubated at room temperature (±27°C) for 24-48 hours. Bacterial colonies with distinct morphological characteristics were subsequently subcultured on fresh MSM plates to obtain pure isolates.

Screening of Biosurfactant Producing Isolates

Erlenmeyer flasks containing sterilized nutrient broth were inoculated with a loopful of bacterial culture (24 hour) and incubated in a shaking incubator at room temperature (±27°C) for 24 hours at 100 rpm. Subsequently, 10% (v/v) of the pre-culture was transferred into liquid sterilized Mineral Salt Medium (MSM) and incubated in a shaking incubator at room temperature for 48–72 hours at 100 rpm. After 3 days of incubation, the culture broth from each flask was centrifuged at 6000 rpm and 4°C for 30 minutes (Maherani *et al.* 2024). The selection of potential biosurfactant-producing isolates was performed using three assays: oil displacement and drop collapse using cell-free culture broth and hemolysis assay.

Oil Displacement Test

A total of 20 ml of distilled water was added to a petri dish, followed by the addition of 5 ml of palm oil to the surface of the water until a stable oil layer was formed. Then, 100 µl of cell-free culture broth was added to the oil surface. If biosurfactants are present in the cell-free culture broth, the oil will be displaced with an oil-free clearing zone (Gozan *et al.* 2014).

Drop Collapse Test

A 25 µl drop of cell-free supernatant was placed on a hydrophobic surface (parafilm). The shape of the drop was observed, where a flattened or collapsed drop indicated the presence of a surfactant in the sample (Tugrul & Cansuar 2005).

Hemolysis Assay

Bacterial isolates were streaked on the surface of Blood Agar and incubated at room temperature (±27°C) for 24 hours. Hemolysis activity was indicated by the presence of clear zones around the bacterial colonies.

Charachterization of Biosurfactant-Producing Bacteria

Colony observation of potential isolates was performed by growing bacterial isolates on Nutrient Agar (NA). The growing colonies were observed for their shape, color, elevation, and margin. The cell morphology and Gram type of each isolate were observed using the Gram staining method with a microscope at 1000× magnification.

Biosurfactant Extraction

Biosurfactant production was performed using a liquid MSM culture medium supplemented with 2% sucrose as the carbon source. A 100 mL pre-culture was prepared by inoculating a single colony from MSM agar into liquid LB medium and incubating it in a shaking incubator at room temperature (±27°C) for 24 hours. Subsequently, 10% (v/v) of the pre-culture was transferred into a 2 L Erlenmeyer flask containing 1 L of MSM liquid media. The culture was incubated in a shaking incubator at room temperature at 150 rpm for 48–72 hours. Following fermentation, the culture was centrifuged at 8000 g for 30 minutes at 4°C to obtain

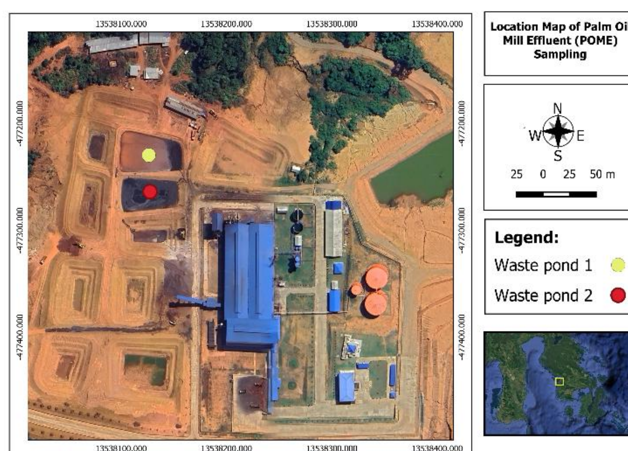

Figure 1 Location of palm oil mill effluent samples collection.

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the cell-free supernatant for biosurfactant extraction. The samples were precipitated using 6N hydrochloric acid (HCl), followed by extraction with a chloroform:methanol (2:1 v/v) solvent mixture at room temperature. The organic phase was then evaporated at 40°C to recover the biosurfactant (Maherani *et al.* 2024).

Physicochemical Charachterization of Biosurfactant Extract

The hydrophobic and hydrophilic properties of the biosurfactant molecules were evaluated by measuring the emulsification index (EI) of the culture supernatant. The assay was conducted by mixing equal volumes (1:1, v/v) of crude biosurfactant extract and oil, followed by vigorous mixing using a vortex for 2 minutes and allowing the mixture to stand undisturbed for 24 hours (Płaza *et al*. 2014). The emulsification index (EI24) was calculated using the following formula:

$$IE_{24} = \left(\frac{\text{Height of the emulsion layer (cm)}}{\text{Total height of the solution (cm)}}\right) \times 100\% \quad (1)$$

Identification of the Potential Bacterial Isolates

Potential isolates were incubated in Nutrient Broth culture for 24 hours. Genomic DNA was extracted from pure cultures using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, United States) following the manufacturer's instructions. The 16S rRNA gene sequences were amplified by PCR using universal primers (63F and 1387R) targeting the bacterial 16 rRNA gene. The obtained 16S rRNA gene sequences were visualized on agarose gels, purified, and sequenced. The obtained 16S rRNA gene sequences were compared with sequences in the NCBI database using a BLAST search to determine their identity. The sequences were aligned with their relatives using Clustal W, and a phylogenetic tree was constructed using the neighbor-joining method with the maximum parsimony algorithm in MEGA 11 software with 1000 bootstrap replicates.

RESULTS AND DISCUSSION

Bacteria Isolates from Palm Oil Mill Effluent

A total of 17 isolates with different colony morphologies were successfully isolated from two different palm oil mill effluent samples using mineral salt medium as the basal medium (given the code SLP1.1-SLP1.12 and SLP2.1-SLP2.5).

Biosurfactant Activity of Bacterial Isolates

Hemolysis assay of 17 bacterial isolates showed negative results (γ-hemolysis) after 48 hours of incubation (Figure 2A and 2B), indicated by the absence of clear zones (β-hemolysis) or greenish zones (α-hemolysis) around the colonies. These results were compared with those of the positive control, *Pseudomonas aeruginosa*, which produced a clear zone around the colony, indicating β-hemolysis (Figure 2A). The presence of clear or greenish zones indicates complete or partial lysis of the red blood cells, respectively.

A total of 12 isolates showed positive results in the oil displacement assay, indicated by the formation of oil dispersion zones of varying diameters after the addition of cell-free supernatant to the oil surface, while the remaining five isolates were negative. The two isolates with the largest dispersion zones were SLP1.5 and SLP2.2, with mean diameters of 1.45 ± 0.21 cm and 1.50 ± 0.28 cm, respectively (Figure 3). The drop collapse assay results showed that only 10 isolates were positive for biosurfactant production, with positive isolates displaying flat or spreading drops of supernatant (Figure 4). The screening results from the three assays indicated that 12 isolates possessed biosurfactant activity (Table 1). The two selected isolates, SLP1.5 and SLP2.2, were further characterized.

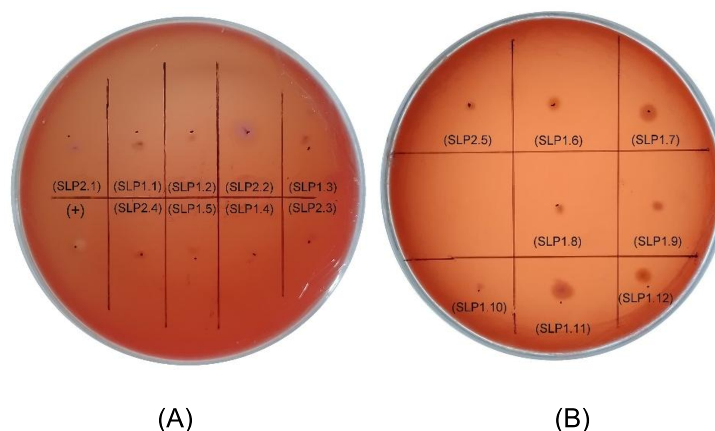

Figure 2 Hemolysis assay results after 48 hours measured at room temperature (±27°C): 17 isolates (SLP1.1-SLP1.12 and SLP2.1-SLP2.5) , and positive control *Pseudomonas aeruginosa* (+).

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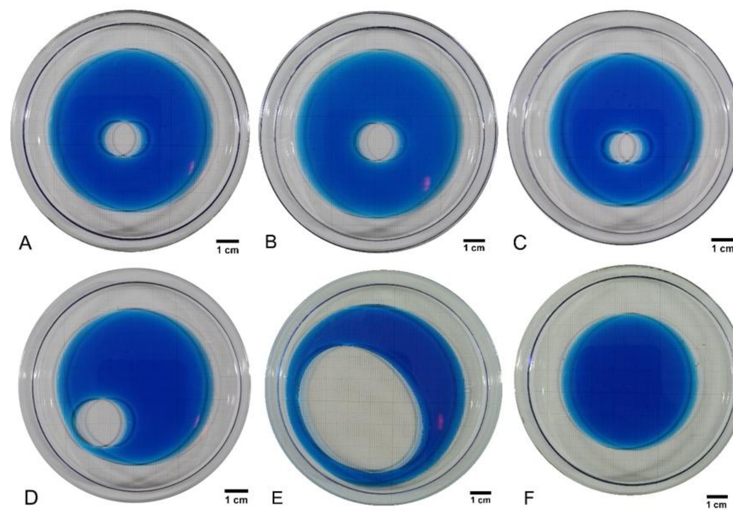

Figure 3 Oil displacement result of isolate SLP1.5 (1) (a) and (2) (b), isolate SLP2.2 (1) (c) and (2) (d), positive control (soap solution) (e), and negative control (distilled water) (f). The blue layer represents oil mixed with a blue hydrophobic dye.

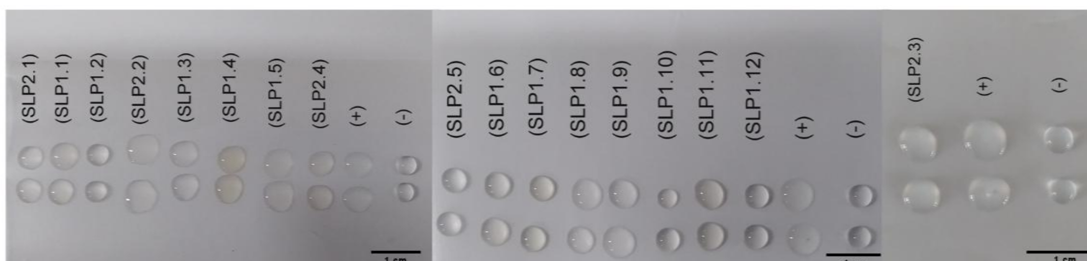

Figure 4 Drop collapse results of all bacteria isolates. Scale bar = 1 cm.

Table 1 Biosurfactant production

No	Isolate code	Assay				
		Oil displacement	Drop collapse	Hemolysis		
				α	β	γ
1	SLP1.1	0,8 ± 0,14	+	-	-	+
2	SLP1.2	0,5 ± 0,00	-	-	-	+
3	SLP1.3	0	+	-	-	+
4	SLP1.4	0,65 ± 0,07	+	-	-	+
5	SLP1.5	**1,45 ± 0,21**	+	-	-	+
6	SLP1.6	0,55 ± 0,07	+	-	-	+
7	SLP1.7	0	-	-	-	+
8	SLP1.8	0,55 ± 0,07	+	-	-	+
9	SLP1.9	0,45 ± 0,07	+	-	-	+
10	SLP1.10	0,6 ± 0,00	-	-	-	+
11	SLP1.11	0	-	-	-	+
12	SLP1.12	0	-	-	-	+
13	SLP2.1	0,10 ± 0,00	+	-	-	+
14	SLP2.2	**1,5 ± 0,28**	+	-	-	+
15	SLP2.3	0,60 ± 0,00	+	-	-	+
16	SLP2.4	0,55 ± 0,07	-	-	-	+
17	SLP2.5	0	-	-	-	+

Note: (+) = Tested positive and (-) = Tested negative. Oil displacement values are presented as mean ± standard deviation (SD) from two replications (n = 2).

Colony and Cells Morphology of the Bacterial Isolates

The morphological characteristics of the two bacterial isolates were observed macroscopically. Isolate SLP1.5 had a circular shape, yellow color, entire margin, and raised elevation (Figure 5A). Isolate SLP2.2 had irregularly shaped colonies that were white, with lobate margins and raised elevation (Figure 5B). The cell morphology of both isolates showed gram-positive bacteria with rod-shaped cells (Figure 6).

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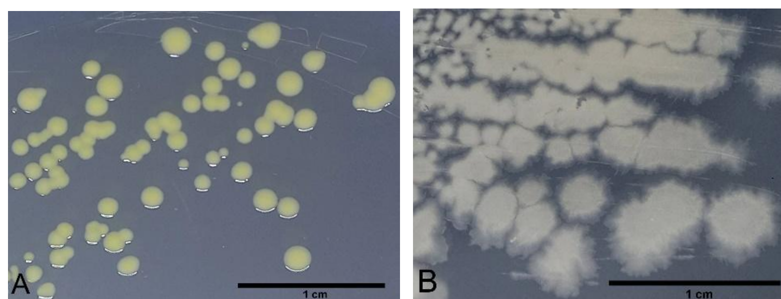

Figure 5 Morphological characteristic of the SLP1.5 (A) and SLP2.2 (B) bacterial isolates seen under observed under a light microscope at 1000× magnification.

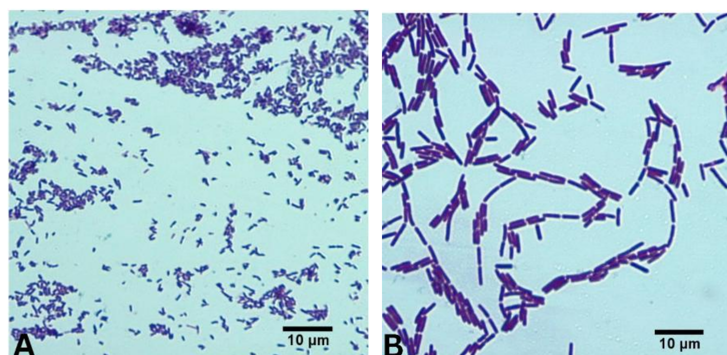

Figure 6 Gram staining result of the SLP1.5 (A) and SLP2.2 (B) bacterial isolates observed under a light microscope at 1000× magnification.

Table 2 Emulsification index of crude biosurfactant extract of SLP2.2

Isolate		Emulsification Index (%)	Average Emulsification Index ± SD (%)
SLP2.2	1	37.50	36.93± 0.57
	2	36.36	

Note: SD = Standard deviation

Characteristic of Crude Biosurfactant Extract of Selected Isolates

Biosurfactant production using a combination of acid precipitation and solvent extraction methods was successfully carried out for isolate SLP2.2, with a yield of approximately 0.08 g/L. In contrast, no extracts were obtained from the isolate SLP1.5. The emulsification index (EI_{24}) of the crude biosurfactant extract from isolate SLP2.2 at a concentration of 1000 ppm was 36.93 ± 0.57% after 24 hours of incubation (Table 2). The resulting emulsion layer appeared whitish and was formed above the biosurfactant layer (Figure 7).

Identification of The Potential Isolate

The 16S rRNA gene sequences of the isolate SLP2.2 were compared with sequences in the GenBank database. Phylogenetic analysis revealed that the 16S rRNA gene sequence of SLP2.2 exhibited 99,73% to *Cytobacillus kochii* 53644 (Figure 8).

DISCUSSION

Palm oil mill effluent (POME) can be utilized as a source of biosurfactant-producing bacteria and as a carbon source for their growth. According to Bala et al. (2018), POME provides a microbe-rich ecosystem that supports the growth of oil-degrading bacteria. Palm oil mill effluent, a waste stream rich in fats and various hydrocarbon compounds, promotes the adaptation of microbial populations to utilize such compounds for growth, thereby increasing the likelihood of biosurfactant production by bacterial cells (Viramontes-Ramos *et al.* 2010; Karlapudi *et al.* 2018).

Biosurfactants consist of many types based on their chemical nature, such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids, and neutral lipids (Rodrigues *et al.* 2006). Only one method was insufficient to detect biosurfactant-producing bacteria; therefore, a combination of various screening methods is required, thus using the combination of hemolysis assay, oil displacement test, and drop collapse test. The hemolysis test is generally carried out as a pre-elimination screening of bacteria to determine their ability to produce biosurfactants. The hemolytic activity of biosurfactants was first reported by Bernheimer and Avigad (1970), who found that surfactin, a biosurfactant produced by *Bacillus subtilis*, was capable of lysing red blood cells, suggesting a correlation between hemolytic

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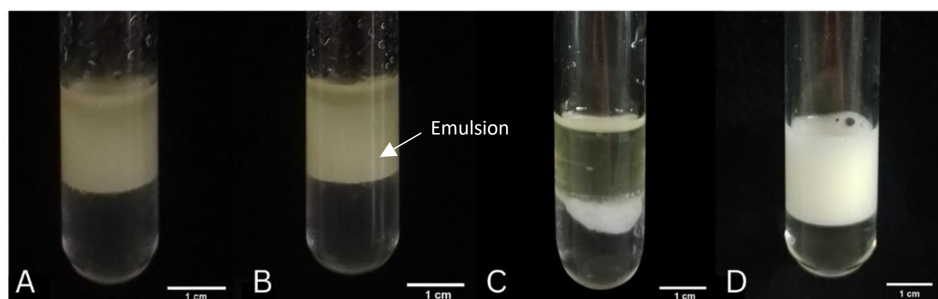

Figure 7 Emulsion formation (white layer) by the crude biosurfactant extract of the SLP2.2 isolate presented as two replicates: Rep 1 (A), Rep 2 (B), negative control (distilled water) (C), and positive control (soap solution) (D). Scale bar = 1 cm.

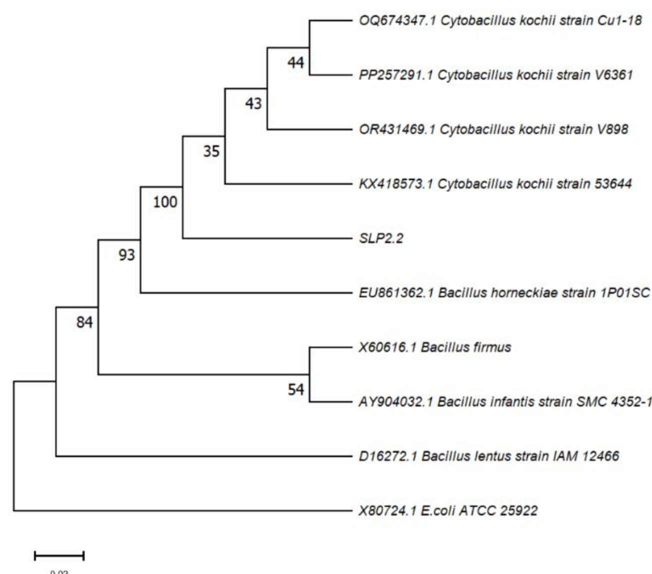

Figure 8 Phylogenetic tree of SLP2.2 with the related species of biosurfactant producing bacteria based on 16S rRNA sequence. The scale bar represents a 2% nucleotide sequence divergence.

activity and biosurfactant production. Therefore, hemolysis has become an important step in isolate screening. However, the relationship between hemolytic characteristics and biosurfactant production remains unclear. Several studies have reported that biosurfactant-producing isolates exhibit both β-hemolytic and γ-hemolytic traits. According to Youssef *et al.* (2004), 31 isolates tested positive for biosurfactant production through oil spreading and drop collapse assays, despite the absence of hemolytic activity. This is consistent with the hemolysis assay results of the present study, which showed that none of the isolates exhibited hemolytic activity (no formation of a clear or greenish zone around the bacterial colony), including the two promising biosurfactant-producing isolates, SLP1.5 and SLP2.2. Although the hemolysis assay does not serve as a direct indicator of biosurfactant activity, it provides valuable insights into the pathogenic potential of bacterial isolates, which is particularly important for their potential industrial applications.

Biosurfactant compounds were detected using cell-free supernatants through oil displacement and drop collapse assays. In the oil displacement test, the biosurfactant-producing isolates formed clear dispersion zones on the oil surface, with the diameter reflecting biosurfactant activity due to reduced surface tension between oil and water (Morikawa *et al.* 1993; Gozan *et al.* 2014). Twelve isolates produced positive results in this assay, forming distinct clear zones that spread across the oil surface. The drop collapse assay further confirmed the presence of biosurfactants, where biosurfactant-containing supernatants formed flattened or spreading drops on parafilm due to hydrophilic interactions that reduced surface tension (Riyanto *et al.* 2021). Among the 17 isolates tested, 10 exhibited positive drop-collapse results. A soap solution, representing a synthetic biosurfactant, was used as a positive control in both assays to validate the observed results. The discrepancy between the oil displacement and drop collapse test results was likely due to the varying sensitivities of each method.

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Crude biosurfactant extracts were obtained from the liquid cultures of the two selected isolates, considering the polarity of the target compounds. Lipopeptides are amphipathic molecules with hydrophilic head groups and hydrophobic tails (Santos *et al*. 2016); thus, extraction was performed using a combination of polar (methanol) and nonpolar (chloroform) solvents. Prior to extraction, surface-active peptides were precipitated from cell-free supernatants by acid precipitation, as biosurfactants are insoluble at low pH, enabling their separation from the aqueous phase (Mukherjee *et al.* 2009). This extraction method was successfully applied to isolate SLP2.2, with a yield of approximately 0.08 g/L. In contrast, no extracts were obtained from the isolate SLP1.5. This may be because the isolate SLP1.5 requires a more specific extraction method. The extract yield from isolate SLP2.2 was relatively low and could be further improved by optimizing the growth medium. Previous studies have successfully obtained extract yields of 1.32 g/L and 1.225 g/L from *Bacillus subtilis* SL and *Bacillus licheniformis* L20, respectively (Liu *et al.* 2022; Wu *et al.* 2022). A higher yield of 16 g/L was achieved from *Bacillus subtilis* UCP 1533 by optimizing the growth medium and culture conditions, including the C/N ratio, fermentation time, and carbon and nitrogen sources) (Albuquerque et al. 2025).

The emulsification index of the crude biosurfactant extract from isolate SLP2.2 at a concentration of 1000 ppm was 36.93 ± 0.57% after 24 hours of incubation. This assay aimed to determine the ability of biosurfactants to emulsify liquids with different polarities. An emulsification index value of less than 30% is considered low, equal to or greater than 30% is regarded as significant, and above 50% is categorized as high and potentially promising (Willumsen & Karlson 1997; Meena *et al*. 2021). Some potential bacterial isolates, such as *Bacillus altitudinis* MS16, *Pseudomonas putida* ICCF 391, and *Pseudomonas fluorescens* ICCF 392, have demonstrated high emulsification indices, with values of 95.4%, 75.4%, and 74.6%, respectively (Goswami & Deka 2019; Stoica et al. 2023). The emulsification index is related to the concentration of the biosurfactant; the lower the emulsification index, the lower the concentration (Walter *et al.* 2010).

Phylogenetic tree reconstruction of the selected isolate SLP2.2 revealed a close relationship with *Cytobacillus kochii* 53644. Patel and Gupta (2020) proposed the genus Cytobacillus as a new genus separated from *Bacillus* based on phylogenetic and taxonomic analyses. *Bacillus* is among the most frequently reported genera with biosurfactant activity and is the dominant producer of biosurfactants, particularly lipopeptide-type compounds (Reningtyas & Mahreni 2015). To date, there have been no reports on the isolation of this bacterium from palm oil mill effluent or any information regarding *Cytobacillus kochii* as a biosurfactant producer. Previous reports on this bacterium include the isolation of *Bacillus kochii* (now renamed as *Cytobacillus kochii*) strain PS17 from textile industry wastewater for the biodecolorization of azo dyes, and the use of *Bacillus kochii* (renamed *Cytobacillus kochii*) strain AHV-KH14 for the bioremediation of phenanthrene (PHE) in soil (Sampath *et al.* 2021; Feizi *et al.* 2020). Isolate SLP2.2 demonstrated novel potential as a biosurfactant-producing bacterium, supporting its prospective application in bioremediation and environmental management.

CONCLUSION

Palm oil mill effluent has potential as a source pf biosurfactant-producing bacteria. Among 17 isolates obtained that showed negative hemolytic activity, SLP2. showed the most promising biosurfactant activity and emulsification ability. Morphological and molecular identification revealed that isolate SLP2.2 belongs to *Cytobacillus kochii*. These findings indicate the potential application of SLP2.2 as a biosurfactant-producing bacterium for environmental and biotechnological applications.

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