EXTRACTION, CHARACTERIZATION, AND ANTIMICROBIAL ACTIVITY OF F7 BIOSURFACTANT FROM *BACILLUS CLAUSII* AGAINST ORAL PATHOGEN KEY PLAYERS

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Abstract
Biosurfactants are surface-active molecules produced by various microorganisms. It holds exceptional properties, including its capacity to lower surface tension, antiadhesive activity, non-toxicity, biodegradability, and antimicrobial activity. **Objectives:** This study investigated the production, characteristics, and antimicrobial potential of biosurfactants extracted from *Bacillus clausii,* isolated from a crude oil sample obtained from a natural oil reservoir. **Methods:** Biosurfactant was extracted using the chloroform-methanol extraction method. Characterizations were assessed through Fourier-Transform Infrared Spectroscopy (FTIR), determination of surface tension, Critical Micelle Concentration (CMC), and emulsification index. Antimicrobial activity was determined by the Minimum Inhibitory Concentration (MIC) evaluation against *Streptococcus mutans* ATCC 25175, *Enterococcus faecalis* ATCC 24212, and *Candida albicans* ATCC 14503 using the microdilution method. **Results:** The biosurfactant extraction yielded at 1.6 mg mL^{-1}, and was labelled as F7 biosurfactant. FTIR analysis revealed that the F7 biosurfactant belonged to the lipopeptide group, as evidenced by the presence of an aliphatic chain (CH_3 and CH_2). It exhibited a surface tension of 12.0 mN m^{-1}, a CMC of 157.5 mg L^{-1}, and an emulsification index of 56.5%. The MIC for each tested organism was 0.2 mg/mL^{-1}, 0.4 mg/mL^{-1}, and 0.8 mg/mL^{-1} with inhibition percentages of 3.20%, 4.03%, and 7.87% against *S. mutans*, *E. faecalis*, and *C. albicans*, respectively. **Conclusions**: The antimicrobial activities of the F7 biosurfactant demonstrated dose-dependent. These findings suggest that increasing the F7 biosurfactant concentration could lead to a more effective antimicrobial effect, making it a potential antimicrobial agent for oral applications.

Keywords: Antimicrobial Agents, *Bacillus clausii*, Biosurfactants, Minimum Inhibitory Concentration, Oral Pathogen

Introduction

Biosurfactants are amphiphilic compounds produced by various microorganisms (1). Biosurfactants have numerous advantages over chemical surfactants, including less toxicity, higher biodegradability, environmentally friendly, higher foaming capability, highly selective, and specific activity at extreme pH, temperature, and salinity (2, 3). Biosurfactants have been recognized as having a wide range of potential applications in various industries, including agriculture, food, cosmetics, pharmaceuticals, and petroleum (4). *Bacillus clausii*, isolated from a crude oil sample obtained from a wellhead of an oil reservoir in Sumatra, Indonesia, produced a type of biosurfactant labeled as F7 biosurfactant with superior properties and obtainability (5). The F7 biosurfactant is highly stable across a wide pH range, high temperature, and salinity. It also possesses strong emulsifying properties in various conditions and was found to be efficient against soil and bio-corrosion-causing bacteria (6). Biofilm formation is related to a lot of chronic illnesses and persistent

infections, particularly in oral and dental diseases. *Streptococcus mutans*, *Enterococcus faecalis*, and *Candida albicans* are some of the most frequent oral pathogenic microorganisms (7, 8). These pathogens produce microbial biofilms and attach to solid surfaces as a mechanism to shield themselves from a hazardous environment (9). Several lines of study have shown that biosurfactants can be more effective than many classic biofilm inhibitions and/or disruption strategies under particular testing conditions (10). Thus, this study aims to study the efficiency of F7 biosurfactant against *Streptococcus mutans*, *Enterococcus faecalis*, and *Candida albicans* that are commonly found in oral infections and to elucidate the potential of F7 biosurfactant for further research and practical applications.

Methodology

Strains and Control Preparation

The *Bacillus clausii* which has been isolated and identified in our previous research was inoculated and incubated in 15 mL of sterile Nutrient Broth (NB) (OXOID, United Kingdom) for 24 h at 37°C in a shaker incubator at 150 rpm (6). *Streptococcus mutans* ATCC 25175, *Enterococcus faecalis* ATCC 24212, and *Candida albicans* ATCC 14503 were inoculated and incubated in 15 mL of sterile Brain Heart Infusion (BHI) broth, Tryptic Soy Broth (TSB), and Sabouraud Dextrose Broth (SDB) (OXOID, United Kingdom) respectively for 24 hours at 37°C in a shaker incubator at 150 rpm. The agar used in this study were Brain Heart Infusion (BHI) agar for *S. mutans*, Tryptic Soy Agar (TSA) for *E. faecalis*, and Sabouraud Dextrose Agar (SDA) for *C. albicans* (OXOID, United Kingdom).

Extraction of F7 Biosurfactant

Bacillus clausii was activated in 50 mL Nutrient Broth (NB) then incubated at 37°C and agitated at 125 rpm for 24 hours. The second activation was performed by inoculating 10% (v/v) culture in 50 mL NB incubated at 50°C and agitated at 125 rpm for 24 hours. The activated bacterial culture was then adapted twice to a Stone Mineral Salt Solution (SMSSe) medium with molasses incubated at 50°C and agitated at 125 rpm for 24 and 84 hours respectively. The production media was centrifuged at 4°C and agitated at 7,500 rpm for 15 minutes then extracted using chloroform: methanol (2:1). The phase at the bottom was evaporated to be further dried in acid air flow until it became a precipitate and formed a dry extract of biosurfactant. The dry extract will be mixed with deionized water for further usage (6).

Characterization of F7 Biosurfactant

Fourier Transform Infrared Spectroscopy (FTIR) Assay

Various bonds and functional groups present in the biosurfactants were identified using Fourier transform infrared spectroscopy (FTIR) (Nicolet 6700, Thermo Scientific). One drop of biosurfactant in liquid form was directly added and recorded in the 4000–600 cm-1 range. Peaks were analyzed using the infrared absorption frequency database (1).

Determination of Surface Tension and Critical Micelle Concentration (CMC)

Surface tension was determined using The Du-Nouy-Ring method. Rings must be free from contaminants before use and accomplished by flaming the platinum ring (2). The CMC was determined with a tensiometer by measuring the surface tension of a series of concentrations. The CMC is obtained from the intersection of the linearly dependent region regression line and the straight line through the plateau (3).

Determination of Emulsification Index

Crude oil was added to the biosurfactant in a 1:1 ratio. The mixture was vortexed for 2 minutes and let sit idle for 24 hours. The emulsification index will be calculated as the height of the emulsion layer (mm) divided by the total height of the liquid column (mm) and multiplied by 100 (2).

Antimicrobial Activity of F7 Biosurfactant

The initial exploration of the antimicrobial efficacy of the F7 biosurfactant involved assessing the inhibition zone through a well diffusion method on an agar plate, using a cork borer with a diameter of 6 mm (11). The Minimal Inhibitory Concentration (MIC) was measured by the broth microdilution method (12). The assay was initiated by adding 100 µL of broth for each microorganism followed by 100 µL of F7 biosurfactant with a concentration of 1.6 mg mL−1. The contents of the well were homogenized using a pipette, and then 100 uL was transferred from the initial well to the next well to create two-fold serial dilutions. The final concentration of F7 biosurfactant was 0.1 mg mL^{-1}, 0.2 mg/mL^{-1}, 0.4 mg/mL^{-1}, and 0.8 mg/mL^{-1}. Then aliquot 20 µL of bacterial and fungal strain (10^6 CFU mL^{-1}) to the wells. The total volume of

each well was 120 µL (13). Chlorhexidine 0.2%, NaOCl 2.5%, and Nystatin oral suspension were used as positive controls for *S. mutans*, *E. faecalis*, and *C. albicans* respectively, and deionized water was used as negative control. The resulting suspensions were then incubated at 37°C for 24 hours. The optical density was read at wavelength 600 nm for *S. mutans* and *E. faecalis*, and 550 nm for *C. albicans* of 0.144 which is equivalent to 10^6 cells/mL or to #0.5 McFarland standard. The MIC value was the minimum concentration of the F7 biosurfactant that inhibits the growth of the tested microorganism (14). The percentage inhibition of each concentration of biosurfactants was calculated using the formula below (15):

$$\% \textbf{ Growth Inhibition } = [\mathbf{1} - (\mathbf{Ac/Ao})] \mathbf{\ x\ 100} \quad \textbf{(1)}$$

Ac denotes the absorbance of the well containing a biosurfactant concentration, while Ao signifies the absorbance of the negative control well.

Results

The F7 biosurfactant extraction yielded at 1.6 mg mL-1 and exhibited a surface tension of 12.0 mN m-1, a CMC of 157.5 mg L-1, and an emulsification index of 56.5%. FTIR analysis revealed that the F7 biosurfactant belonged to the lipopeptide group, as evidenced by the presence of the bands 3333.06 cm-1 (NH stretching mode) and 1634.08 cm-1 (CO–N stretching mode) assigned to peptides as seen in Figure 1 (16).

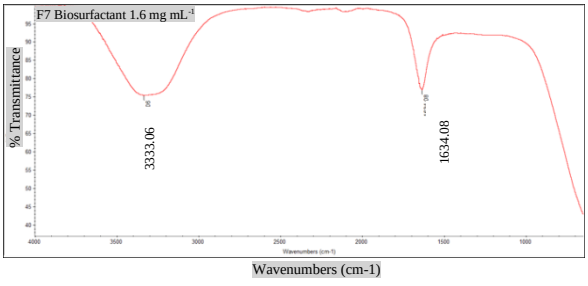

Figure 1: FTIR analysis of F7 biosurfactant with concentration 1665 µg mL^{-1}.

As shown in Figure 2, the agar diffusion test did not exhibit a distinct clear zone around the F7 biosurfactant at any concentration against all tested species.

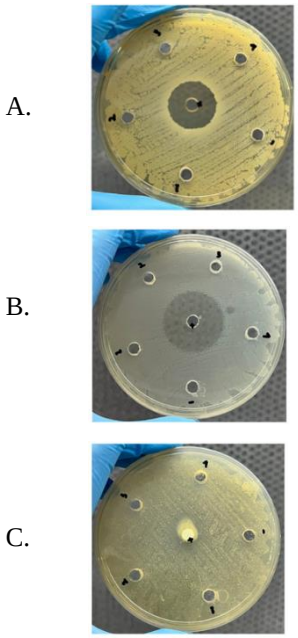

Figure 2: Agar well diffusion assay of four different concentrations of F7 Biosurfactant against each microorganism tested. (**A)** *S. mutans* with chlorhexidine 0.2% as a positive control in the center of the agar. (**B)** *E. faecalis* with NaOCl 2.5% as a positive control in the centre of the agar. **C.** *C. albicans* with nystatin as a positive control in the center of the agar.

To further confirm the antimicrobial effect of the F7 biosurfactant, MIC tests were conducted. The MIC value was determined as the minimum concentration of the tested antimicrobial agent that hinders the observable growth of the tested microorganism (14). MICs for F7 biosurfactant were determined for all microorganisms examined in this study by the broth microdilution method (Table 1).

Table 1: Growth inhibition activity of F7 biosurfactant against *E. faecalis*, *S. mutans*, and *C. albicans.*

Strains	Percentage Inhibition (%) of Various F7 Biosurfactant Concentrations			
	0.1 mg mL-1	0.2 mg mL-1	0.4 mg mL-1	0.8 mg mL-1
S. mutans	-	3.20 ± 0.008	7.14 ± 0.016	17.81 ± 0.008
E. faecalis	-	-	4.03 ± 0.003	10.00 ± 0.004
C. albicans	-	-	-	7.87 ± 0.125

*Data expressed as mean ± SD

Table 1 shows the results of the MIC evaluation. Each species showed a different MIC, indicating varying effectiveness of the biosurfactant. Notably, the biosurfactant was more effective against *S. mutans*, requiring a minimum concentration of 0.2 mg/mL^{-1}, while higher concentrations were needed to initiate inhibition in *E. faecalis* (0.4 mg/mL^{-1}) and *C. albicans* (0.8 mg/mL^{-1}). Higher concentrations showed greater inhibition percentages.

Discussion

Currently, the market for commercially accessible biosurfactants is still restricted, featuring only a few selections, including surfactin, sophorolipids, and rhamnolipids. Consequently, there is a pressing need to intensify the search for novel biosurfactant-producing microorganisms by employing a wide array of screening techniques (2). *Bacillus sp.* has been known as one of the bacteria capable of producing biosurfactants which exhibit its highest rate during the stationary growth phase (17). Our present study used *Bacillus clausii* isolated from oil reservoirs as biosurfactant producers. Our study showed that the F7 biosurfactant had an emulsification and surface tension-reducing ability. The F7 biosurfactant falls under the lipopeptide category. Other known lipopeptide biosurfactants are surfactin, fengycin, and iturin. They are the best-known antimicrobial lipopeptides produced by *B. subtilis* (18). The agar well diffusion method is widely recognized as one of the efficient methods for evaluating the antimicrobial properties of a compound. In the initial test using this method, the F7 biosurfactant has no inhibition zone observed.

Several factors can contribute to this outcome, such as susceptibility of the test medium, cell concentration and growth rate of the test organisms, concentration of the tested material, diffusion characteristics of the material in the agar, and the susceptibility of the organism to the material (19). It is plausible that Biosurfactant F7 might not effectively diffuse through the agar, preventing it from reaching bacteria growing above the agar. MIC assays are commonly referred to as quantitative assessments, while diffusion tests are considered as qualitative evaluations (19). Therefore, for further investigation, we conducted the MIC assay to determine the minimum concentration of the F7 biosurfactant of the tested microorganism. In this method, the biosurfactant is mixed directly with the microorganism suspension, eliminating the need for the biosurfactant to diffuse through the agar. This can yield different results compared to the previous method. The MIC assay showed that the biosurfactant did have inhibition activity against all tested microorganisms, with varying sensitivity observed in each microorganism. The minimum concentration of F7 Biosurfactant was 0.2 mg/mL-1 for *S. mutans*, 0.4 mg/mL-1 for *E. faecalis*, and 0.8 mg/mL-1, for *C. albicans*. The inhibition percentage increased with a higher concentration of the biosurfactant. Further exploration with higher concentrations to achieve a higher inhibition percentage should be carried out. These findings demonstrate the potential of F7 biosurfactant as an antimicrobial agent.

The antimicrobial effects result from the capacity of these biosurfactants to integrate into and disrupt the cell membrane of pathogens, leading to their disintegration. Additionally, biosurfactants have been found to induce cell death by interfering with protein synthesis (1, 3). Biosurfactants are also known as potent anti-biofilm agents, through decreasing the adhesion of microorganisms to surfaces. Therefore, it could also be beneficial as an anti-biofilm agent in oral biofilm infection (7, 8). The antibiofilm activity of F7 biosurfactant is currently being investigated. In line with other studies, biosurfactants showed dose-dependent characteristics. Biosurfactants obtained from *Lactobacillus acidophilus* DDS-1, *Lactobacillus rhamnosus* ATCC 53103, and *Lactobacillus paracasei* B21060 exhibited substantial inhibition of adhesion and biofilm formation on titanium surfaces by *S. mutans* and *S. oralis* in a dose-dependent manner. This was evident from the significant reduction in cfu/ml values and biomass production (20).

Conclusion

The antimicrobial activities of the F7 biosurfactant have demonstrated a dose-dependent characteristic. Higher F7 biosurfactant concentrations showed greater inhibition percentages. These findings suggest that increasing the F7 biosurfactant concentration could lead to a more effective antimicrobial effect, making it a potential antimicrobial agent for oral applications.

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Competeing interest

The authors declare that they have no conflicts of interest.

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