Antibacterial Activity of Surfactin Produced by *Bacillus subtilis* MSH1

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ABSTRACT Surfactin is one of the most powerful lipopeptide biosurfactants produced by various strains of *Bacillus subtilis*, having exceptional surface activity as well as antiviral, antibacterial and antitumor properties. In this study, fermentations in shake flasks were conducted to assess the ability of *B. subtilis* MSH1 to produce surfactin in Cooper's media. Investigation of antibacterial activity of surfactin against *Shigella dysenteriae* and *Staphylococcus aureus* by using well diffusion method, minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) shows surfactin having potent bacteriostatic and bactericidal properties which potentially could be utilized for commercial antibiotic formulations with medical and pharmaceutical purposes.

KEYWORDS: *Bacillus subtilis*; Surfactin; Minimum inhibitory concentration; Minimum bactericidal concentration (MBC)

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INTRODUCTION

Biosurfactants known as microbial surfactants are surface-active biomolecules produced by microorganisms, and is regarded to be extremely important in various applications due to lower level of toxicity, higher degree of biodegradability and various biological characteristics (Mukherjee *et al*., 2006). One of the most intensive works on biosurfactants producing bacteria rests on *Bacillus subtilis*, which is considerably the most efficient biosurfactant producer (Peypoux *et al*., 1999). *B. subtilis* brings out a great spectrum of bioactive compounds including surfactin, fengycin, iturin, mycosubtilins, and bacillomycins. Surfactin was discovered by Arima *et al*. (1967) in the culture broth of *B. subtilis* and due to its exceptional surfactant activity it was named surfactin (Peypoux *et al*., 1999). Surfactin is a cyclic lipopeptide consisting of a heptapeptide head group with the sequence of Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu linked to a lactone ring by a $C_{14\text{-}15}$ β-hydroxyl fatty acid produced by various strains of *B. subtilis* (Heerklotz & Seelig, 2007). Surfactin was initially identified as a strong inhibitor of fibrin clot formation and subsequently found to lyse protoplasts, erythrocytes and spheroplasts. It has the ability to reduce the surface tension of water from 72 to 27 mN m^{-1} at a trace concentration as low as 0.005% (Yeh *et al*., 2005). Various reports have indicated that surfactin has haemolytic, antiviral, antibacterial, anti-tumour and hypocholesterolemic properties (Peypoux *et al*., 1999; Isa *et al*., 2007; Isa *et al*., 2008) and can be used in medicine, biotechnology, agriculture and environmental applications (Mukherjee *et al*., 2006). Currently, nearly all surfactants in industrial use are chemically derived from petroleum. Recently, interest in biosurfactants (including surfactin) has been steadily rising as a result of their environmentally-friendly nature, diversity, the possibility of their production by fermentation, potential applications in environmental protection, crude oil recovery, food-processing and health-care (Isa *et al*., 2007).

Surfactin is a suitable alternative to synthetic antibiotics and antibacterial agents and can be used as a safe and effective therapeutic agent. The antibacterial activity of surfactin is determined by

the lipid chain length and charge of its hydrophilic head group which is a characteristic of surfactin isoforms chemical structure make up. In addition, types of bacteria (e.g. gram positive and gram negative) respond differently to surfactin. In this study, the antibacterial activity of surfactin produced by *B. subtilis* MSH1 was evaluated against two pathogenic bacteria, *Shigella dysenteriae* and *Staphylococcus aureus.*

METHODOLOGY

Investigation of surfactin antibacterial activity

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of surfactin

The MIC was determined by culturing the two pathogenic bacteria of *S. dysenteriae* and *S. aureus* in Mueller Hinton broth and incubated overnight at 37 ^{o}C. The bacterial cultures were diluted to attain a cell density of 1.5× 10^8 CFU/ml. A 100 μL of surfactin (50, 100, 150, 200, 250 mg/L) recovered from culture broth of *B. subtilis* MSH1 was added into 96-well microtitre plate. Following overnight incubation, the 100 μL bacterial inoculum was added into the wells. The microtiter plates were then incubated at 37ºC for 24 h. The MIC is the lowest concentration of surfactin needed to stop microbial growth. Each sample from the test wells were streaked on nutrient agar (NA) plates and observation was carried out after the incubation period. The MBC values were taken as the lowest concentration of the sample that did not have any visible bacteria colony on agar plate after the incubation period.

Well diffusion method

The antibacterial activity of surfactin can also be assessed by using the well diffusion technique. 100 μL (10^5 CFU/ml) of *S. dysenteriae* and *S. aureus* were spread onto Muller-Hinton agar plates and various concentrations of surfactin (50, 100, 150, 200, 250 mg/L) recovered from culture broth of *B. subtilis* MSH1 were placed in the wells created in the agar (sterile cork borer). Streptomycin (0.10%, w/v) and 10% dimethyl sulfoxide (DMSO) were used as the positive control and negative control, respectively. Plates were incubated at 37°C for 24 h and the zones of inhibition were then measured.

Bactericidal Activity

Cell rupture evidence

Bacterial strains were grown overnight in Mueller Hinton broth at 37°C, harvested and washed and resuspended with PBS solution, respectively. The bacterial strain was tested with various concentrations of surfactin (50, 100, 150, 200, 250 mg/L) and incubated for 30 min at 37°C. Streptomycin (0.10%, w/v) was used as a positive control. Both treated and untreated samples were centrifuged at 9000 rpm for 5 min and then the pellet was resuspended in crystal violet (10 μg/mL) solution prepared in PBS (pH 7.4) and incubated at 37°C for 10 min. The percentage of crystal violet dye uptake in the samples was calculated according to Vaara (1981), as described in eq. (1)

$$OD_{590} \text{ value of the sample}/OD_{590} \text{ value of the crystal violet solution} \times 100 \quad (1)$$

Bacterial cell disintegration analysis

Bacterial strains were grown in Mueller Hinton broth at 37°C for 12 h and later were centrifuged at 5000 rpm for 10 min at 4°C. The cell pellet was washed twice with PBS and then resuspended in PBS. The resuspended bacterial strains were treated with various concentrations of surfactin (50, 100, 150, 200, 250, mg/L) and 0.10% w/v streptomycin were applied as positive control. These samples

were later incubated at 37°C for 1 h and optical densities were measured at 260 nm (Zhou *et al*., 2008). The optical densities of the samples supernatant were considered as a percentage of the extracellular UV-absorbing materials released after treatment with surfactin.

RESULT AND DISCUSSION

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of surfactin

The antibacterial activity of surfactin produced by *B. subtilis* MSH1 was assessed by determination of MIC and MBC values. Table 2 shows MIC of surfactin produced by *B. subtilis* MSH1 was found to be at 150 mg/L and 200 mg/L against *S. dysenteriae* and *S. aureus*, respectively. The growth of *S. dysenteriae* and *S. aureus* were completely inhibited with treatment of 200 mg/L and 250 mg/L of surfactin, respectively as shown in Table 2, and these concentrations were determined as the MBC. MIC values of surfactin obtained in this study were lower than MBC and these findings suggested that surfactin were bacteriostatic at lower concentrations and were bactericidal at higher concentration. The ratios of MBC/MIC of surfactin produced by *B. subtilis* MSH1 were 1.33 and 1.25 against *S. dysenteriae* and *S. aureus*, respectively. According to Oussou *et al.* (2008), an antimicrobial substance with ratio of MBC/MIC≤4, can be considered as bactericidal. Based from the results obtained we can conclude that surfactin produced by *B. subtilis* MSH1 is bactericidal against *S. dysenteriae* and *S. aureus*.

Table 2. MIC and MBC of surfactin produced by *B. subtilis* MSH1 against *S. dysenteriae* and *S. aureus*.

Surfactin concentration (mg/L)	*S. dysenteriae*		*S. aureus*	
	MIC	MBC	MIC	MBC
50	+	+	+	+
100	+	+	+	+
150	–	+	+	+
200	–	–	–	+
250	–	–	–	–

+ = growth; - = no growth after 24 h incubation at 30 °C.

Results obtained in this study show B. subtilis MSH1 were able to produce surfactin which were bacteriostatic and bactericidal against the two pathogenic bacteria tested. Evaluation on the effectiveness of antibacterial agents depends on the mechanism of their activity, which involves the inhibition of cellular processes such as expression of genes; synthesis of vital cellular biomolecules and their transport. In addition, the strength of antibacterial activity also depends on the sensitivity of the bacterial strains towards specific types of antibacterial agent.

Well diffusion

Zones of inhibition for various concentrations of surfactin produced by *B. subtilis* MSH1 against *S. dysenteriae* and *S. aureus* are shown in Table 3. For *S. dysenteriae*, the inhibition zone diameter was 2.0±0.2 mm at 50 mg/L of surfactin and increased up to 13.0±0.2 mm with 250 mg/L of surfactin. For *S. aureus*, inhibition zones were 2.0±0.17 mm at 50 mg/L and increased up to 10.5±0.1 mm when treated with 250 mg/L of surfactin. By comparison, streptomycin (positive control) inhibition zones were 22 ±0.4 mm and 20 ±0.5 mm for *S. dysenteriae* and *S. aureus*, respectively, and 0 for DMSO (negative control).

Table 3. Zones of inhibition for various concentrations of surfactin produced by *B. subtilis* MSH1 against *S. dysenteriae* and *S. aureus*.

Surfactin concentration (mg/L)	Inhibition zone (mm) *S. dysenteriae.*	Inhibition zone (mm) *S. aureus*
Streptomycin	22.0 ± 0.30	20 ±0.24
50	2.0 ± 0.170	2.0 ± 0.17
100	6.0 ± 0.10	5.0 ± 0.26
150	8.5 ± 0.10	7.0 ± 0.17
200	10.0 ± 0.20	8.0 ± 0.20
250	13.0 ± 0.17	10.5 ±0.10
DMSO	0	0

Diameter Mean ± Standard Deviation of inhibitory zone (mm) after 24 h incubation at 30 °C.

Results obtained in Table 3 suggested a linear relationship between surfactin concentration and the diameter of zones of inhibition. Previous study was reported, bacterial zone of inhibition (diameters) for surfactin was in the range of 10 mm to 30 mm (Yakimov *et a*l., 1997; Fernandes *et al.*, 2007; Bechard *et al.*, 1998). Variations in the diameters of zones of inhibition could be due to the structure of surfactin isoforms and the susceptibility of pathogenic bacterial strains to various antibacterial agents. The results shown in Table 3 also indicated the ability of surfactin to inhibit the growth of *S. aureus* and *S. dysenteriae*, although the activity of surfactin against *S. aureus* was lower than *S. dysenteriae*.

Determination of bactericidal activity

Alteration of membrane permeability

Figure 3 shows the uptake of crystal violet by *S. dysenteriae* increased of up to 79 (±3)% when treated with 250 mg/L of surfactin.

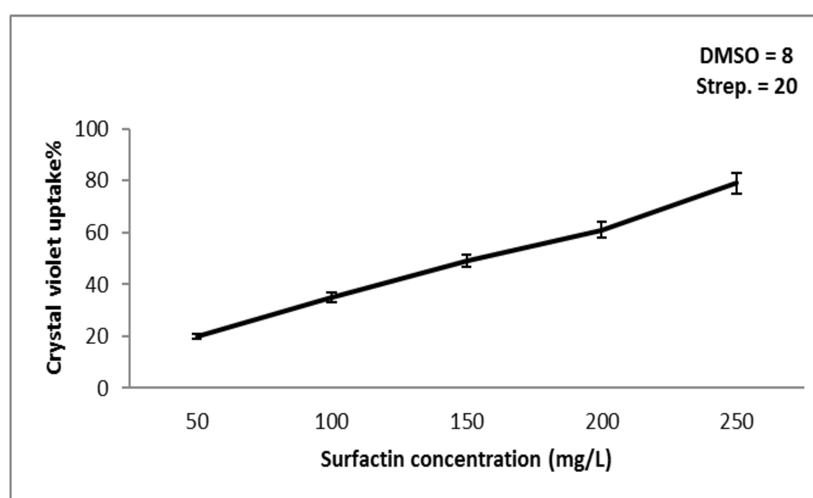

Figure 3. Alteration of membrane permeability of *S. dysenteriae* with various concentrations of surfactin.

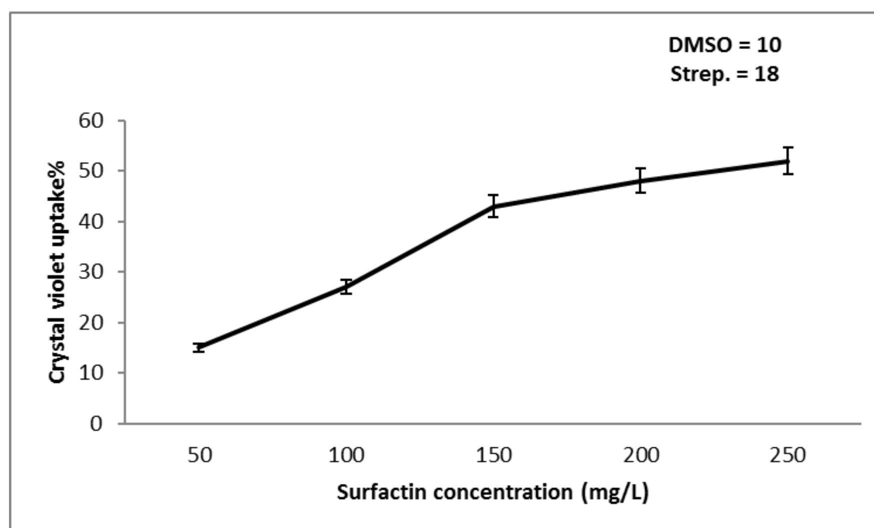

Figure 4. Alteration of membrane permeability of *S. aureus* with various concentrations of surfactin

Figure 4 shows the uptake of crystal violet by *S. aureus* increased up to 52 (±2)% when treated with 250 mg/L of surfactin. *S. dysenteriae* and *S. aureus* treated with positive control (streptomycin) exhibited uptake values of 20 (±1) % and 18 (±0.5)%, respectively and indicates no significant involvement of streptomycin with the alteration of bacterial membrane cell wall permeability. *S. dysenteriae* and *S. aureus* without surfactin treatment resulted in 8.0 (±1) % and 10 (±0.5)% uptake of crystal violet, respectively. Overall, results obtained showed the ability of surfactin to alter membrane cell wall structure of S. aureus was lower than *S. dysenteriae*. These results confirmed the properties of the surfactin molecule which having both hydrophobic and hydrophilic groups that could insert its fatty acid tail into the cell membrane and considerably altered the cell structure (Carillo *et al.*, 2003). Furthermore, treatment of surfactin for both bacterial strains tested in this study had caused a significant increase in the uptake of crystal violet in comparison to the control cells. Such results clearly justify the direct impact of surfactin on the cell membrane of the tested bacteria and the alteration which increased the permeability for crystal violet dye uptake.

CONCLUSION

B. subtilis MSH 1, isolated from oil contaminated soils at various locations in Kajang (Selangor, Malaysia) were able to produce competitive amount of surfactin at 30 ^{0}C with mineral medium containing 4% (w/v) glucose. Furthermore, surfactin produced by *B. subtilis* MSH1 was bactericidal and bacteriostatic towards the tested pathogenic microorganisms of *S. dysenteriae* and *S. aureus*. Such activity of surfactin could potentially be used as an additive in the formulation of antibiotic and other antibacterial compound for enhancing the effectiveness of chemotherapeutics. Discovery of high yield of surfactin producer of *B. subtilis* could be one of the key factors in reducing the overall cost of surfactin upstream and downstream processing, which later will assist in enhancing commercial use of surfactin in medical, pharmaceutical, cleaning agents, emulsifiers and bioremediation.

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REFERENCES

[1] Arima, K., Kakinuma, A. & Tamura, G. (1967). Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. *Biochemical and Biophysical Research Communications*, **31**(3), 488-494.

[2] Bechard, J., Eastwell, K., Sholberg, P., Mazza, G. & Skura, B. (1998). Isolation and partial chemical characterization of an antimicrobial peptide produced by a strain of *Bacillus subtilis*. *Journal of Agricultural and Food Chemistry*, **46**(12), 5355-5361.

[3] Carrillo, C., Teruel, J. A., Aranda, F. J. & Ort, A. (2003). Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochimica et Biophysica Acta*, **1611**(1-2), 91-97.

[4] Fernandes, P., de Aruda, I. & dos Santos, A. (2007). Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug bacteria. *Brazilian Journal of Microbiology*, **38**(4), 704-709.

[5] Heerklotz, H. & Seelig, J. (2007). Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. *European Biophysics Journal*, **36**(4-5), 305-

[6] Isa, M. H. M, Frazier, R. & Jauregi, P. (2008). A further study of the recovery and purification of surfactin from fermentation broth by membrane filtration. *Journal of Separation and Purification Technology*, **64**(2), 176-182.

[7] Isa, M. H. M., Coraglia, D., Frazier, R. & Jauregi, P. (2007). Recovery and purification of surfactin from fermentation broth by a two-step ultrafiltration process. *Journal of Membrane Science*, **296**(1-2), 51-57.

[8] Mukherjee, S., Das, P. & Sen, R. (2006). Towards commercial production of microbial surfactants. *Trends in Microbiology*, **24**(11), 509-515.

[9] Oussou, K., Yolou, S., Boti, B., Guessend, N. & Kanko, C. (2008). Chemical and Activity Study Antidiarrhal activity of essential oils of two aromatic plants pharmacopoeia of Ivory Coast. *European Journal of Scientific Research*, **24**(1), 94-103.

[10] Peypoux, F., Bonmatin, J. & Wallach, J. (1999). Recent trends in the biochemistry of surfactin. *Applied Microbiology and Biotechnology*, **51**(5), 553–563.

[11] Vaara, M., Vaara, T., Jensen, M., Helander, I., Nurmin, M., Rietschel, E. & Mäkelä, P. H. (1981). Characterization of the lipopolysaccharide from the polymyxin-resistant pmrA mutants of *Salmonella typhimurium*. *FEBS Letters*, **129**(1), 145–149.

[12] Yakimov, M., Amro, M. & Bock, M. (1997). The potential of *Bacillus licheniformis* strains for in situ enhanced oil recovery. *Journal of Petroleum Science Engineering*, **18**(1-2), 147-160.

[13] Yeh, M. S., Wei, Y. H. & Chang, J. S. (2005). Enhanced production of surfactin from *Bacillus subtilis* by addition of solid carriers. *Biotechnology Progress*, **21**(4), 1329-1334.

[14] Zhou, K., Zhou, W., Li, P., Liu, G., Zhang, J. & Da, Y. (2008). Mode of action of pentocin 31-1: An antilisteria bacteriocin produced by *Lactobacillus pentosus* from Chinese traditional ham. *Food Control*, **19**(8), 817–822.