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Original Article

Antimicrobial electrospun fiber mat from gelatin and crude extract of lipopeptides

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Abstract

Lipopeptides from *Bacillus subtilis* represent a class of biosurfactants with increasing therapeutic and biotechnological interests**.** In this study, we added crude extract of lipopeptides into a gelatin solution to examine the effect of these crude lipopeptides using electrospinning. The addition of crude lipopeptides to a gelatin solution affected the solution viscosity and conductivity and resulted in a decrease in nanofiber diameter when the solution was electrospun to produce nanofibers. Crosslinking of nanofibers using saturated glutaraldehyde for 10 and 30 min improved the mechanical properties and water retention capacity of the mats formed from the nanofibers. Furthermore, the lipopeptides-loaded nanofiber mats showed antibacterial activity toward *Staphylococcus aureus* ATCC 6538P.

Keywords: biosurfactant, lipopeptides, gelatin, nanofiber, antimicrobial mat

1. Introduction

Biosurfactants are natural active compounds produced by microorganisms such as bacteria and fungi. Their structures contain hydrophobic and hydrophilic moieties that have the ability to accumulate fluid phases, resulting in the reduction of surface and interfacial tension (Muthusamy, Gopalakrishnan, Ravi, & Swachidambaram, 2008). They have advantages over chemical surfactants in biodegradability, effectiveness at extreme temperature or pH, and lower toxicity (Banat, Makkar, & Cameotra, 2000). Biosurfactants have been classified into six major groups based on their chemical structure: glycolipids, lipopeptides, phospholipids, fatty acids, polymeric surfactants, and particulate surfactants (Mukherjee, Das, & Sen, 2006).

Lipopeptide biosurfactants are produced by several *Bacillus* species. They consist of a peptide (hydrophilic moiety) linked to a fatty acid (hydrophobic component). Surfactin, one of most powerful lipopeptide biosurfactants produced by *B. subtilis* strains, consists of a common peptide loop of seven amino acids (Glu Leu Leu Val Asp Leu Leu) with a long hydrophobic fatty acid chain (Chen, Juang, & Wei, 2015). Aside from high surface activity, low toxicity, and high biodegradability and biocompatibility, surfactin also shows antimicrobial properties. These promising properties have led to applications in various industries (Gong *et al.*, 2009), for example pharmaceutical industry, cosmetics industry, and food industry (Mandal, Barbosa, & Franco, 2013). Recently, surfactin has acquired a role in nanotechnology as a preparation material for an antimicrobial

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nanoemulsion against food pathogens (Joe *et al.*, 2012) and a stabilizing agent for developing stable cadmium sulfide nanoparticles (Singh, Dwivedi, Al-Khedhairy, & Musarrat, 2011).

Electrospinning is a simple and cost-effective technique to produce continuous and uniform nanofibers with diameters between micrometers and nanometers via electrostatically driven jets of polymer solution. The nanofibers prepared by this technique have shown properties including very large surface area to volume ratios, and high porosity with small pore size (Bhardwaj & Kundu, 2010). Because of these advantages, nanofibers have been widely-applied in various fields. Dheraprasart, Rengpipat, Supaphol, and Tattiyakul (2009) found that a 22% (w/v) gelatin concentration in 70% (v/v) acetic acid was suitable for nanofiber formation. However, the gelatin nanofibers were water soluble and mechanically weak which limited their applications. Crosslinking treatment was able to improve both the water-resistance and mechanical properties of gelatin nanofibers (Zhang, Venugopal, Huang, Lim, & Ramakrishna, 2006). In addition, several researchers are interested in adding active compounds into nanofibers to add value to products. Kwak *et al.* (2014) reported that they successfully loaded *Phaeodactylum tricornutum*, which is a diatom that lives in marine water, into a gelatin dope solution to examine the antimicrobial activity using electrospinning. There are several reports of gelatin electrospun fiber mats that investigated the effectiveness of the mats to inhibit foodborne pathogens (Dheraprasart *et al.*, 2009). Sikareepaisan, Suksamrarn, and Supaphol (2008) reported that the methanolic crude extract of *Centellaasiatica* (L.) Urban, a plant widely known for its traditional medical applications including wound healing, was loaded into gelatin nanofiber mats. The results suggested the possible use of the nanofiber mats as wound dressings.

Roongsawang *et al.* (2002) reported that a lipopeptide biosurfactant, whose main components were identified as surfactin, bacillomycin L, and plipastatin, was produced by *Bacillus subtilis* BBK-1. However, the amount of lipopeptide biosurfactant was low. In our previous study, we scaled up the biosurfactant production in a 5-L batch bioreactor. As a result, we found that the aeration and agitation rates were highly correlated with the performance of lipopeptide biosurfactant and the optimum combination was 1.5 vvm (volume of air per volume of liquid per minute) and 300 rpm (Yoochang, Chanprateep Napathorn, & Thaniyavarn, 2015). Therefore, in the present study, we aimed to examine the antimicrobial activity of the lipopeptide biosurfactant. To integrate the functionality of the biosurfactant into nanofibers, we investigated the electrospinning conditions for gelatin in the presence of the biosurfactant. In addition, we examined the quality of prepared nanofibers, including their mechanical properties and antibacterial activity against *Staphylococcus aureus* ATCC 6538P.

2. Materials and Methods

2.1 Materials

Gelatin powder (type A; porcine skin) was purchased from Sigma-Aldrich (USA). Glutaraldehyde (50 vol% aqueous solution) was purchased from Fluka (Switzerland). Glacial acetic acid was from QRec (New Zealand) and methanol was from Labscan (Thailand). Yeast extract was purchased from Biospringer (France), Bacto Peptone was from Difco Laboratories (USA), ammonium nitrate was from Sigma, and iron sulfate heptahydrate, magnesium sulfate heptahydrate, and glucose were from Ajax Finechem (Australia). All chemicals were of analytical grade and used without further purification.

2.2 Biosurfactant production in a 5-L batch bioreactor

2.2.1 Bacterial strain and inoculum preparation

B. subtilis BBK-1, which produces biosurfactant lipopeptides and was previously screened and characterized by Roongsawang *et al.* (2002), was used in this study. From frozen stock, the bacterial strain was streaked on Lennox Luria-Bertani LB agar plates and incubated at 30 °C for 18–24 h. For seed culture preparation, *B. subtilis* BBK-1 was cultured in LB broth and incubated in an incubating shaker at 30 °C, 200 rpm, for 18 h. The OD_{600} was adjusted to 0.1–0.2. The inoculum size used in the fermenter was 4% (v/v).

2.2.2 Biosurfactant production

B. subtilis BBK-1 was cultured in modified production medium which consisted of 60 g glucose, 2 g NH_4NO_3, 5 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.15 g $FeSO_4 \cdot 7H_2O$ and 5 g NaCl per liter (pH 7.5). The production volume was 2 L in a 5-L batch bioreactor (FS01-5L Double Jacket, Winpact Bench-Top Fermentor) and batch cultures were incubated at 30 °C with an agitation rate of 300 rpm and an aeration rate of 1.5 vvm for 72 h.

2.2.3 Biosurfactant recovery

The biosurfactants were recovered using the method of Roongsawang *et al.* (2002). Cell-free broth was subjected to acid precipitation by adding 6 M HCl to achieve a final pH of 2 and allowing the precipitant to form at 4 °C overnight. The pellet was collected by centrifugation at 10000*g* for 15 min. The biosurfactants were extracted three times with methanol. The solvent was removed using a rotary evaporator under vacuum to obtain crude biosurfactant.

2.3 Evaluation of antimicrobial activity of biosurfactant

2.3.1 Microbial test strains

Antimicrobial tests were performed using test microbial strains procured from the American Type Culture Collection (ATCC), the Thailand Institute of Scientific and Technological Research (TISTR), the Department of Medical Sciences Thailand Culture Collection (DMST), and the Microbial Culture Collection, Department of Microbiology, Faculty of Science, Chulalongkorn University (MSCU). The test microorganisms included: i) Gram-positive bacteria *S. aureus* ATCC 6538P, *Micrococcus luteus* TISTR 884, *B. subtilis* ATCC 16633, *B. cereus* ATCC 11778, and *Listeria monocytogenes* DMST 17303; ii) Gram-negative bacteria *Escherichia coli* ATCC 8739 and *Salmonella typhimurium*

MSCU 0492; iii) molds *Aspergillus niger* MSCU 0361, *A. flavus* MSCU 0580, *Penicillium* sp. MSCU 0390; and iv) yeasts *Candida albicans* ATCC 10231 and *C. tropicalis* MSCU 0544.

2.3.2 Agar well diffusion test

Stock solutions of the biosurfactant were prepared by dissolution in Tris-HCl pH 8 to achieve concentrations of 100 and 500 mg/mL. Bacterial strains were cultured overnight in Mueller-Hinton broth (MHB) at 37 °C. Mold strains were grown on potato dextrose agar (PDA) at 25 °C for 3 days. Yeast strains were cultured overnight in yeast-mold (YM) broth at 30 °C. The bacterial cultures were adjusted to inocula of 10^8 colony forming units CFU/mL according to McFarland turbidity standards (Huang & Chang, 2003). The mold spores were adjusted to 10^5 spores/mL, and the yeast cultures were adjusted to inocula of 10^6 CFU/mL. Each tested strain was spread on 20 mL of Mueller-Hinton agar, PDA or YM agar for bacteria, mold, and yeast, respectively. Then, wells were made with a sterile cork borer (ø = 0.8 cm). Stock biosurfactant (50 µL) was added to each well. The plates were incubated at 37 °C for 18 h in the case of bacteria, 25 °C for 72 h in the case of mold, and 30 °C for 24 h in the case of yeast. Antimicrobial activities were determined by measuring the zones of inhibition in mm.

2.3.3 Determination of minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC)

The MIC of the tested strains was measured by a modified broth microdilution method in 96-well plates (Wiegand, Hilpert, & Hancock, 2008). Biosurfactant was diluted in the range 250–0.12 mg/mL (serial twofold dilution) with MHB for bacteria, PDB for mold, and YM broth for yeast. The bacterial inoculum concentration was 10^6 CFU/mL. The mold spore and yeast inocula were 10^5 spores/mL and 10^6 CFU/mL, respectively. Resazurin was used as a redox indicator to detect viable cells. The 96-well plates were incubated at 37 °C for 18 h in the case of bacteria, 25 °C for 72 h in the case of mold, and 30 °C for 24 h in the case of yeast. The lowest concentration in a well that did not change color was the MIC. For the determination of MBC/MFC, wells that did not change color were re-streaked and incubated in the same conditions to check the growth of bacteria and fungi.

2.4 Preparation of biosurfactant-gelatin solutions

A weighed amount of gelatin powder was dissolved in 70% (v/v) acetic acid aqueous solution to prepare a gelatin solution at a fixed concentration of 22% (w/v). After stirring for 3 h, biosurfactant was added to the gelatin solution to 12.5, 25, 50, and 75% (w/v) under constant stirring for 1 h. Prior to electrospinning, the prepared solutions were measured for pH, conductivity, viscosity and surface tension. The pH and conductivity of solutions were measured using a portable multiparameter meter (HACH: Sension 156, USA). The viscosity of the solutions was measured with a viscometer (Fungilab Model: Premium R, Spain). The surface tension of the solutions was measured with a tensiometer (Krüss: K6, Germany).

2.5 Electrospinning

The biosurfactant-loaded gelatin solutions were electrospun by loading each prepared solution in a glass syringe (10 mL), which was held on a stand and tilted about 45° from a horizontal baseline. The syringe needle was connected to the positive lead from a high-voltage power supply (Gamma High Voltage Research ES30P-5W, USA). A ground counter-electrode was connected to a rotating metal drum, which was used as the collector. The power supply was fixed at 15 kV, and the distance between the syringe tip and the collector was 20 cm. The electrospinning process was carried out for 3 days. Then, electrospun fiber mats were collected and stored in a desiccator for further use. Gelatin nanofibers were prepared as a control.

2.6 Crosslinking of electrospun nanofiber mats

Gelatin and biosurfactant-loaded gelatin nanofiber mats were crosslinked by saturated glutaraldehyde vapor as described in a previous study (Dheraprasart *et al.*, 2009). The process was performed by placing the dried nanofiber mats in a chamber containing 250 mL of aqueous glutaraldehyde solution, and crosslinking was by saturated glutaraldehyde vapor at 37 °C. The crosslinking time was 10 or 30 min. After crosslinking, the samples were placed in a fume hood for 1 h, followed by post-treatment at 60 °C for 1 h to remove residual glutaraldehyde.

2.7 Determination of nanofiber mat morphology

The morphologies of the nanofibers spum for 5 min by electrospinning were characterized by scanning electron microscopy (SEM; model JSM-5800 LV, JEOL, Japan). Diameters of the nanofibers were measured from the SEM images using Semaphore 5.21 software (JEOL CO., Finland). At least 100 measurements were analyzed for each sample type.

2.8 Determination of mechanical properties of the nanofiber mats

The thicknesses of the mat samples (20 by 40 mm) were measured using a digital micrometer (Mitutoyo: series 293, Japan). Tensile strength and elongation were determined with a texture analyzer (Stable Micro Systems: TA.XT plus, UK). The crosshead speed was 2 mm/s, and the gauge length was 10 mm. Tensile strength (T_s) and elongation (E) of the mats were calculated from these equations:

$$\text{Ts (MPa)} = \frac{\text{Fmax}}{\text{A}}$$

$$\text{E (\%)} = \frac{\Delta l}{l_0} \times 100$$

where Fmax is the maximum breaking force to pull the mat to the point where it breaks, A is the cross-sectional area of the mat (m^2), Δl is the change in length of the mat (mm), and l_0 is the initial length of the mat.

2.9 Determination of water retention capacity of the nanofiber mats

Nanofiber mat samples (circular discs about 1.65 cm in diameter) were submerged in an acetate buffer aqueous solution (pH 5.5) for 24 h at room temperature. Acetate buffer was chosen to simulate the human skin pH of 5.5 (Taepaiboon, Rungsardthong, & Supaphol, 2006). After 24 h, the samples were weighed immediately. The samples were then dried and reweighed. Water retention was determined using this equation:

$$\text{Water retention (\%)} = \frac{\text{M-Md}}{\text{Md}} \times 100$$

where *M* is the weight of each sample after submersion in acetate buffer for 24 h, and *M*d is the weight of the sample in its dry state after submersion in acetate buffer for 24 h and subsequent drying.

2.10 Antibacterial assay

The antibacterial activity of the gelatin and biosurfactant-loaded gelatin nanofiber mats was tested with *S. aureus* ATCC 6538P which is a skin pathogen. The procedure used was a modification of the method reported by Dheraprasart *et al.* (2009). Each mat sample (circular discs about 1.65 cm in diameter) was added to a test tube containing *S. aureus* ATCC 6538P (10^6 CFU/mL). The culture volume was 5 mL. The test tube was incubated in a shaker (180 rpm) at room temperature. A 100-µL sample was collected, then diluted and spread in three replications on tryptic soy agar and incubated at 37 °C until growth of *S. aureus* ATCC 6538P was observed. The number of colonies was counted and recorded in terms of log CFU/mL.

2.11 Statistical analysis

Data were collected in triplicate and are presented as mean±standard deviation (SD). Statistically significant differences were examined using one-way analysis of variance, followed by Duncan's multiple comparison test. The level of significance was set at $P \leq 0.05$. Statistical analysis was carried out using IBM SPSS statistics software, version 22 (IBM Corp., USA).

3. Results and Discussion

3.1 Evaluation of antimicrobial activity of biosurfactant

The crude biosurfactant (100 mg/mL) demonstrated antimicrobial activity against most of the tested microorganisms (Table 1). It had strong activity against Gram-positive bacteria such as *B. subtilis* ATCC 16633, *B. cereus* ATCC 11778, *M. luteus* TISTR 884, and *S. aureus* ATCC 6538P. It also showed potent inhibitory action against fungi such as *A. niger* MSCU 0361, *A. flavus* MSCU 0580, and *Penicillium* sp. MSCU 0390. However, 100 mg/mL biosurfactant could not inhibit *E. coli* ATCC 8739, *C. albicans* ATCC 10231, or *C. tropicalis* MSCU 0544. When the concentration of biosurfactant was increased to 500 mg/mL, it showed activity against *E. coli* ATCC 8739 and *C. tropicalis* MSCU 0544.

The MICs of the biosurfactant were found using the broth microdilution method. The MIC value for most of the tested microorganisms ranged from 17.5 to 25 mg/mL (Table 1). *E. coli* ATCC 8739, *C. albicans* ATCC 10231, and *C. tropicalis* MSCU 0544 were not inhibited by the biosurfactant at concentrations <250 mg/mL. The MBC/MFC values for most of the tested microorganisms ranged from 25 to 50 mg/mL (Table 1). However, biosurfactant at <250 mg/mL showed no bactericidal or fungicidal activity against *B. cereus* ATCC 11778, *E. coli* ATCC 8739, *C. albicans* ATCC 10231, or *C. tropicalis* MSCU 0544.

Singh and Cameotra (2004) also observed that lipopeptide N1, produced by *B. subtilis* C1, was active against several microorganisms, especially *S. aureus*. Several modes of action of lipopeptides have been proposed all of which depend on the fact that the hydrocarbon tail of the molecule can insert itself readily into the membranes of bacteria where it forms associations with the hydrophobic fatty acid chains of the phospholipids. Because of differences in the bacterial cell membrane and wall, lipopeptides were found to be more effective antimicrobial agents against Gram-positive bacteria than Gram-negative bacteria (Straus & Hancock, 2006). In addition, some research proposed that the antifungal activity of bacillomycin L may not be solely a consequence of fungal membrane permeabilization, but related to the interaction of it with intracellular targets (Zhang, Dong, Shang, Han, & Li, 2013).

3.2 Properties of biosurfactant-gelatin solution

The average pH of the samples was 2.25–2.85 (Table 2). The conductivity of the solution increased from 1.27 mS/cm at 0% (w/v) biosurfactant to 2.51, 2.46, 2.85, and 3.02 mS/cm at 12.5, 25, 50, and 75% biosurfactant (w/v), respectively. In contrast, the viscosity of the solution decreased from 384.34 cP (0% [w/v] biosurfactant) to 166.8 cP (75% [w/v] biosurfactant). The surface tension of the solution also decreased from 43.10 mN/m (0% [w/v] biosurfactant) to 36.00 mN/m (75% [w/v] biosurfactant).

The presence of biosurfactant in the gelatin solution had direct effects on the properties of the solution. This could be because the crude biosurfactant contains ionic substances such as NaCl. As the concentration of biosurfactant in the solution increased, the amount of NaCl would also increase, leading to a higher conductivity. Some reports also show that adding an ionic surfactant can alter solution properties such as viscosity, conductivity, and surface tension because the charges on the surfactant may bind to oppositely charged polymers via electrostatic attractive interaction forces (Kriegel, Kit, McClements, & Weiss, 2009).

3.3 Morphology of nanofiber mats

Nanofibers from gelatin solution blended with biosurfactant at different concentrations were spun for 5 min by electrostatic spinning. Scanning electron micrographs of the gelatin and biosurfactant loaded fibers are shown in Table 3. Except for the fibers formed from solutions containing 50% and 75% (w/v) biosurfactant, the nanofibers were continuous without beads and formed a non-woven fabric. Bhardwaj and

Kandu (2010) proposed that bead formation could occur due to viscosity and surface tension. At low viscosity and high surface tension, the solution jet, which forms the nanofiber, could not maintain its own shape at the end of the tip and thus small drops were formed among the fibers. This causes bead formation instead of nanofiber formation which reduces the uniformity of the nanofibers. This effect was observed here with 50% and 75% (w/v) biosurfactant.

The average nanofiber diameter was analyzed using Semaphore 5.21 software and SEM images (Figure 1), which showed that the average diameter of non-crosslinked nanofibers decreased from 304.9±48.2 nm in the control sample to 127.4±21.9, 128.8±32.7, and 127.2±50.3 nm with 12.5, 25, and 50% (w/v) biosurfactant concentrations, respectively. However, at 75% (w/v) biosurfactant, uniform nanofibers could not be produced so the diameter could not be measured. Because of the gelatin content, the nanofibers are water soluble and mechanically weak, which could limit their applications. Therefore, the nanofibers must be crosslinked for long-term biomedical application (Zhang *et al.*, 2006). In this study, the nanofibers were crosslinked using saturated glutaraldehyde vapor at 37 °C for 10 or 30 min. Figure 1 shows the average diameter of the electrospun fibers without crosslinking and those crosslinked for 10 and 30 min using glutaraldehyde vapor. The nanofiber diameter tended to increase with exposure time to glutaraldehyde vapor. However, there was no significant difference in the average diameter of biosurfactant loaded nanofibers which were crosslinked for 10 or 30 min. Since the nanofibers prepared from 50% and 75% (w/v) biosurfactant had a lot of bead formation, we selected 12.5% (w/v) and 25% (w/v) biosurfactant loading for subsequent experiments.

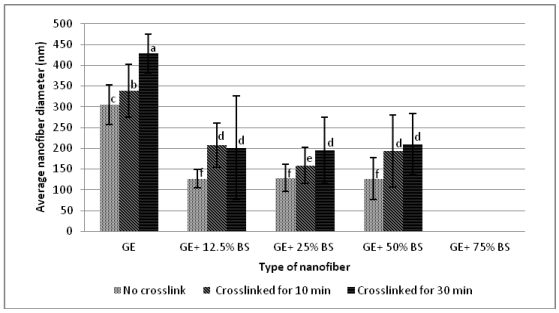


Figure 1. Average diameter of nanofibers which were electrospun from gelatin (GE) loaded with different biosurfactant (BS) concentrations. Statistically significant (P≤0.05) values are designated by different superscript letters.

3.4 Mechanical properties of nanofiber mats

Table 4 shows the thickness, tensile strength, and percentage elongation of non-crosslinked and crosslinked nanofiber mats made from gelatin with 12.5% (w/v) and 25% (w/v) biosurfactant which were spun for 72 h. Adding biosurfactant led to a reduced thickness of the nanofiber mats relative to no biosurfactant (P≤0.05). Crosslinking for 30 min could improve the tensile strength of the nanofiber mats relative to no crosslinking. The percentage elongation was not significantly different in any of the samples.

Table 1. Antibacterial and antifungal activities of the crude biosurfactant measured using the well diffusion method and minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of the crude biosurfactant using the modified broth microdilution method.

Microorganism	Zone of inhibition diameter (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)
Bacteria			
S. aureus ATCC 6538P	11.6±0.1	25.0±0.0	50.0±0.0
M. luteus TISTR 884	12.3±0.1	17.5±0.0	25.0±0.0
B. subtilis ATCC 16633	20.4±0.2	25.0±0.0	50.0±0.0
B. cereus ATCC 11778	14.0 ±0.0	15.63±0.0	>250±0.0
L. monocytogenes DMST 17303	7.6±0.1	25.0±0.0	25.0±0.0
E. coli ATCC 8739	12.0±0.2 *	>250±0.0	>250±0.0
S. typhimurium MSCU 0492	11.5±0.0	31.25±0.0	31.25±0.0
Fungi			
A. niger MSCU 0361	10.5±0.8	17.5±0.0	25.0±0.0
A. flavus MSCU 0580	9.5±0.3	25.0±0.0	25.0±0.0
Penicillium sp. MSCU 0390	10.0±0.2	25.0±0.0	25.0±0.0
C. albicans ATCC 10231	No inhibition *	>250±0.0	>250±0.0
C. tropicalis MSCU 0544	4.6±0.5 *	>250±0.0	>250±0.0

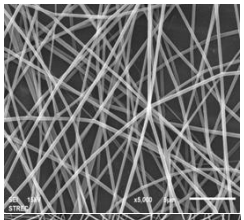
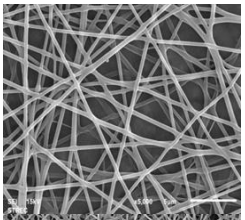
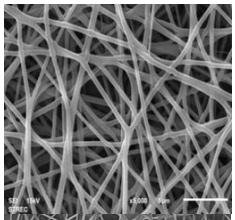
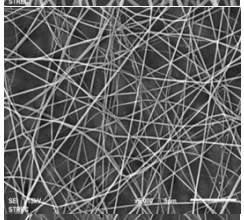
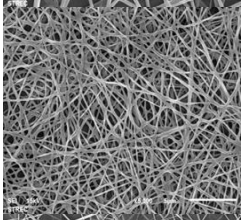
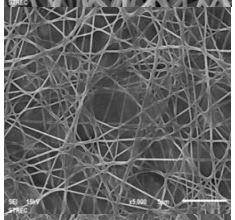
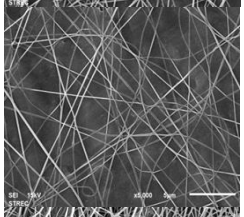
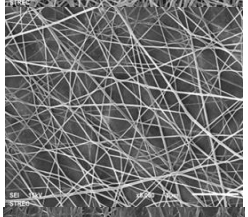
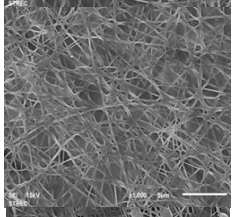
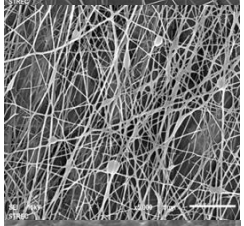
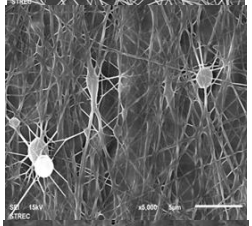
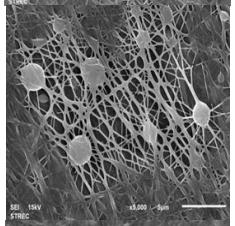
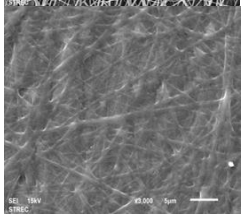
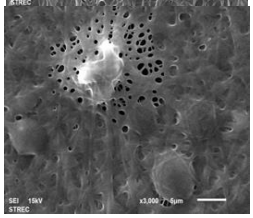
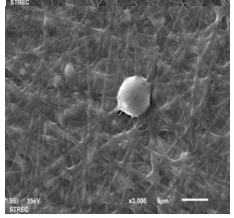
Note: *indicates that the microorganism was tested with 500 mg/mL of biosurfactant. Other strains were tested with 100 mg/mL of biosurfactant.

Table 2. Some properties of gelatin (GE) and biosurfactant (BS) loaded gelatin solutions.

Type of solution	pH	Conductivity (mS/cm)	Viscosity (cP)	Surface tension (mN/m)
GE	2.85±0.02^{a}	1.27±0.02^{d}	384.3±2.9^{a}	43.1±0.9^{a}
GE + BS 12.5% (w/v)	2.78±0.00^{b}	2.51±0.09^{c}	184.2±4.6^{b}	37.1±0.6^{b}
GE + BS 25 % (w/v)	2.75±0.03^{b}	2.46±0.02^{c}	183.3±1.3^{b}	36.2±0.5^{c}
GE + BS 50 % (w/v)	2.56±0.04^{c}	2.85±0.03^{b}	167.1±1.9^{c}	35.8±0.5^{c}
GE + BS 75 % (w/v)	2.25±0.03^{d}	3.02±0.16^{a}	166.8±1.3^{c}	36.0±0.0^{c}

Note: Statistically significant (P≤0.05) values are designated by different superscript letters. GE, gelatin; BS, biosurfactant

Table 3. Scanning electron micrographs of biosurfactant loaded nanofibers (12.5%–75% w/v biosurfactant concentration) crosslinked by glutaraldehyde.

Type of nanofiber	Crosslinking time		
	0 min	10 min	30 min
GE			
GE + BS 12.5% (w/v)			
GE + BS 25% (w/v)			
GE + BS 50% (w/v)			
GE + BS 75% (w/v)			

Note: All SEM photographs are 5,000× magnification except the photographs of GE + BS 75% (w/v), which are 3,000× magnification. GE, gelatin; BS, biosurfactant

Table 4. Average specimen thickness, tensile strength, and percentage elongation of non-crosslinked and crosslinked nanofiber mats spun for 72 h.

Type of nanofiber	Crosslinking time (min)	Thickness (μm)	Tensile strength (MPa)	% Elongation
GE	-	284 ± 0^{a}	1.3 ± 0.1^{bcd}	0.9 ± 0.3^{a}
GE	10	333 ± 0^{a}	1.9 ± 0.7^{b}	4.8 ± 2.1^{a}
GE	30	383 ± 0^{a}	4.5 ± 0.0^{a}	5.8 ± 0.0^{a}
GE + 12.5% BS	-	123 ± 0^{b}	0.9 ± 0.4^{cd}	0.9 ± 1.1^{a}
GE + 12.5% BS	10	169 ± 0^{b}	1.4 ± 0.2^{bcd}	3.4 ± 1.1^{a}
GE + 12.5% BS	30	308 ± 0^{a}	1.8 ± 0.3^{bc}	7.0 ± 6.2^{a}
GE + 25% BS	-	103 ± 0^{b}	0.6 ± 0.2^{d}	2.6 ± 3.1^{a}
GE + 25% BS	10	169 ± 0^{b}	1.1 ± 0.9^{bcd}	3.9 ± 3.2^{a}
GE + 25% BS	30	332 ± 0^{a}	1.4 ± 0.0^{bcd}	5.6 ± 1.9^{a}

Note: Statically significant ($P\leq0.05$) values are designated by different superscript letters. GE, gelatin; BS, biosurfactant

Crosslinking of collagenous materials with glutaraldehyde involves the reaction of free amino groups of lysine or hydroxylysine amino acid residues of polypeptide chains with the aldehyde group of glutaraldehyde (Zhang *et al.*, 2006). This resulted in an improvement in the mechanical properties of the nanofiber mats.

3.5 Water retention capacity of nanofiber mats

The water retention capacity of the non-crosslinked and crosslinked nanofiber mats was studied. After immersion of nanofiber mats in an acetate buffer solution at room temperature, it was observed that the non-crosslinked nanofiber mats were soluble immediately and could not maintain their appearance in the buffer solution. Conversely, nanofiber mats which were crosslinked for 10 or 30 min maintained their appearance for 24 h. Figure 2 shows the water retention capacity of the crosslinked nanofiber mats. The water retention capacity of crosslinked nanofiber mats loaded with 25% surfactant (w/v) was significantly greater ($P \leq 0.05$) compared with gelatin nanofiber mats and gelatin nanofiber mats loaded with 12.5% (w/v) biosurfactant. In addition, the water retention capacity of the nanofiber mats crosslinked for 30 min was lower than that of nanofiber mats crosslinked for 10 min.

3.6 Antibacterial activity of nanofiber mats

The effect of non-crosslinked nanofiber mats on the inhibition of *S. aureus* ATCC 6538P was compared with treatment without adding nanofibers (control) and with the effect of gelatin nanofiber mats containing no biosurfactant (Figure 3). It was observed that the biosurfactant at a higher concentration (25% [w/v]) showed 2.58 log reduction of *S. aureus* ATCC 6538P at 24 h. Further crosslinking of nanofiber containing 25% (w/v) biosurfactant did not affect the antimicrobial ability of the nanofiber (Figure 4).

4. Conclusions

The biosurfactant produced by *B. subtilis* BBK-1 showed potent antimicrobial activity against bacteria and fungi. Interestingly, we successfully prepared biosurfactant-loaded gelatin nanofiber mats with antibacterial activity. The 25% (w/v) biosurfactant-loaded gelatin nanofiber crosslinked for 30 min showed good mechanical properties, water retention capacity, and antibacterial activity against *S. aureus* ATCC 6538P. The antibacterial nanofiber mats may have good potential for application as active packaging for protection of food pathogens or a wound dressing material.

Acknowledgements

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References

Banat, I. M., Makkar, R. S., & Cameotra, S. S. (2000). Potential commercial applications of microbial surfactants. *Applied Microbiology and Biotechnology, 53*, 495-508.

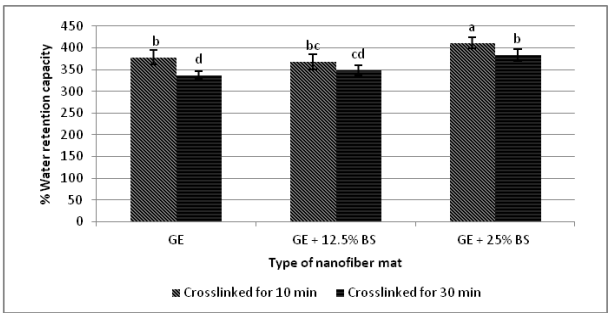

Figure 2. Water retention capacity of nanofiber mats crosslinked for 10 or 30 min then immersed in an acetate buffer solution at room temperature for 24 h. Statistically significant ($P \leq 0.05$) values are designated by different superscript letters. GE, gelatin; BS, biosurfactant.

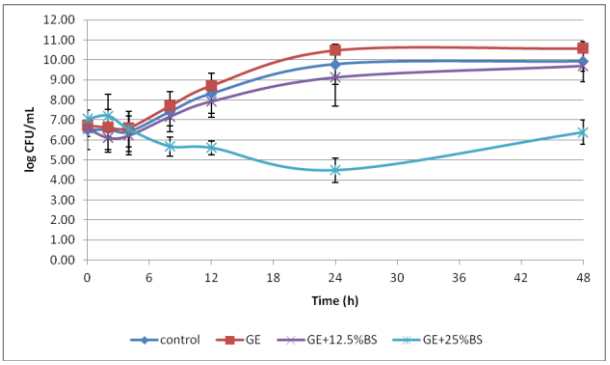

Figure 3. Effect of non-crosslinked nanofiber mats on *S. aureus* ATCC 6538P. GE, gelatin; BS, biosurfactant.

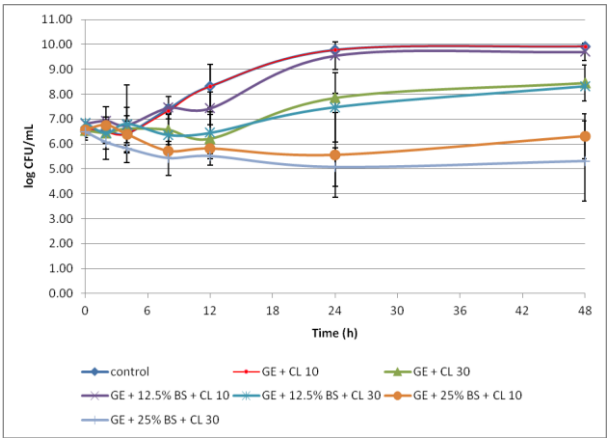

Figure 4. Effect of crosslinked nanofiber mats on *S. aureus* ATCC 6538P. GE, gelatin; BS, biosurfactant; CL, crosslinking time.

Bhardwaj, N., & Kundu, S. C. (2010). Electrospinning: A fascinating fiber fabrication technique. *Biotechnology Advances, 28*(3), 325-347. doi:10.1016/j.biotechadv.2010.01.004

Chen, W. C., Juang, R. S., & Wei, Y. H. (2015). Applications of a lipopeptide biosurfactant, surfactin, produced by microorganisms. *Biochemical Engineering Journal, 103*, 158-169. doi:10.1016/j.bej.2015.07.009

Dheraprasart, C., Rengpipat, S., Supaphol, P., & Tattiyakul, J. (2009). Morphology, release characteristics, and antimicrobial effect of nisin-loaded electrospun gelatin fiber mat. *Journal of Food Protection, 72*(11), 2293-2300.

Gong, G., Zheng, Z., Chen, H., Yuan, C., Wang, P., Yao, L., & Yu, Z. (2009). Enhanced production of surfactin by *Bacillus subtilis* E8 mutant obtained by ion beam implantation. *Journal of Biotechnology, 47*(1), 27-31.

Huang, C. F., & Chang, F. C. (2003). Comparison of hydrogen bonding interaction between PMMA/PMAA blends and PMMA-co-PMAA copolymers. *Polymer, 44*(10), 2965-2974. doi:10.1016/s0032-3861(03)00188-5

Joe, M. M., Bradeeba, K., Parthasarathi, R., Sivakumaar, P. K., Chauhan, P. S., Tipayno, S., . . . Sa, T. (2012). Development of surfactin based nanoemulsion formulation from selected cooking oils: Evaluation for antimicrobial activity against selected food associated microorganisms. *Journal of the Taiwan Institute of Chemical Engineers, 43*(2), 172-180. doi:10.1016/j.jtice.2011.08.008

Kriegel, C., Kit, K. M., McClements, D. J., & Weiss, J. (2009). Electrospinning of chitosan–poly(ethylene oxide) blend nanofibers in the presence of micellar surfactant solutions. *Polymer, 50*(1), 189-200. doi:10.1016/j.polymer.2008.09.041

Kwak, H. W., Kang, M. J., Bae, J. H., Hur, S. B., Kim, I. S., Park, Y. H., & Lee, K. H. (2014). Fabrication of *Phaeodactylum tricornutum* extract-loaded gelatin nanofibrous mats exhibiting antimicrobial activity. *International Journal of Biological Macromolecules, 63*, 198-204. doi:10.1016/j.ijbiomac.2013.11.004

Mandal, S. M., Barbosa, A. E., & Franco, O. L. (2013). Lipopeptides in microbial infection control: scope and reality for industry. *Biotechnology Advances, 31*(2), 338-345. doi:10.1016/j.biotechadv.2013.01.004

Mukherjee, S., Das, P., & Sen, R. (2006). Towards commercial production of microbial surfactants. *Trends in Biotechnology, 24*(11), 509-515. doi:10.1016/j.tibtech.2006.09.005

Muthusamy, K., Gopalakrishnan, S., Ravi, T. K., & Swachi dambaram, P. (2008). Biosurfactants: Properties, commercial production and application. *Current Science, 94*(6), 736-747.

Roongsawang, N., Thaniyavarn, J., Thaniyavarn, S., Kame yama, T., Haruki, M., Imanaka, T., . . . Kanaya, S. (2002). Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. *Extremophiles, 6*(6), 499-506. doi:10.1007/s00792-002-0287-2

Sikareepaisan, P., Suksamrarn, A., & Supaphol, P. (2008). Electrospun gelatin fiber mats containing a herbal-*Centella asiatica*-extract and release characteristic of asiaticoside. *Nanotechnology, 19*(1), 015102. doi:10.1088/0957-4484/19/01/015102

Singh, B. R., Dwivedi, S., Al-Khedhairy, A. A., & Musarrat, J. (2011). Synthesis of stable cadmium sulfide nanoparticles using surfactin produced by *Bacillus amyloliquifaciens* strain KSU-109. *Colloids and Surfaces B: Biointerfaces, 85*(2), 207-213. doi:10.1016/j.colsurfb.2011.02.030

Singh, P., & Cameotra, S. S. (2004). Potential applications of microbial surfactants in biomedical sciences. *Trends Biotechnology, 22*(3), 142-146. doi:10.1016/j.tibtech.2004.01.010

Straus, S. K., & Hancock, R. E. (2006). Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides. *Biochimica et Biophysica Acta, 1758*(9), 1215-1223. doi:10.1016/j.bbamem.2006.02.009

Taepaiboon, P., Rungsardthong, U., & Supaphol, P. (2006). Drug-loaded electrospun mats of poly(vinyl alcohol) fibres and their release characteristics of four model drugs. *Nanotechnology, 17*(9), 2317-2329. doi:10.1088/0957-4484/17/9/041

Wiegand, I., Hilpert, K., & Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols, 3*(2), 163-175. doi:10.1038/nprot.2007.521

Yoochang, T., Chanprateep Napathorn, S., & Thaniyavarn, J. (2015). *Optimization of lipopeptide biosurfactant production by Bacillus subtilis BBK-1 in 5-L batch bioreactor*. Paper presented at the The 27th Annual Meeting of the Thai society for Biotechnology and international Conference, Mandarin Hotel Bangkok, Thailand.

Zhang, B., Dong, C., Shang, Q., Han, Y., & Li, P. (2013). New insights into membrane-active action in plasma membrane of fungal hyphae by the lipopeptide antibiotic bacillomycin L. *Biochimica et Biophysica Acta, 1828*(9), 2230-2237. doi:10.1016/j.bbamem.2013.05.033

Zhang, Y. Z., Venugopal, J., Huang, Z. M., Lim, C. T., & Ramakrishna, S. (2006). Crosslinking of the electrospun gelatin nanofibers. *Polymer, 47*(8), 2911-2917. doi:10.1016/j.polymer.2006.02.046