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Interaction of silver sulfadiazine with bacterial cellulose via ex-situ modification method as an alternative diabetic wound healing

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ABSTRACT

The conventional silver dressing raises growing concerns amongst clinicians that this dressing could lead to increased morbidity and prolonged treatment period relating to uncontrolled wound bioburden. Bacterial cellulose (BC) is a good candidate of alternative treatment method for wound healing but it lacks of antimicrobial activity. Hence, this research was aimed to enhance the efficiency of wound healing by incorporating silver sulfadiazine (SSD) to the BC. BC pellicles was produced through static fermentation process in Hestrin Schramn medium. The BC-SSD membranes was prepared through ex-situ modification method by immersing BC pellicles in various concentration of SSD solution (0.2–1.0 %v/w) for 24 h. The properties of BC-SSD produced was characterized by SEM, FTIR, tensile strength, swelling test, release kinetic and antimicrobial activity. The presence of silver in the BC-SSD membrane was confirmed by the presence of sulfonamide, a basic structure of silver sulfadiazinee from the peaks obtained in FTIR spectrum. In addition, the tensile strength of the BC-SSD membranes increase proportionately with SSD concentration incorporated to the BC. The BC also have the capability of absorbing alkaline better compared to the acidic absorption. The release kinetic study shows that silver ions were efficiently released in 24 h and gradually reduced for the next 72 h. Ultimately, the BC-SSD had pronounced antibacterial activity against *Escherichia coli* and *Pseudomonas eruginosa*, the most commonly bacteria found in diabetic foot ulcer (DFU) which suggest its capability as alternative wound dressing for DFU.

1. Introduction

Bacterial cellulose (BC), a biosynthetic cellulose produced by strains of the Gram-negative bacterium *Acetobacter xylinum* (Wen et al., 2015) has been proven to be a remarkably versatile biomaterial and can be used in tissue-engineering products for both wound care and the regeneration of demand or diseased organs (Cairul et al., 2012; Laçin, 2014; Qiu et al., 2016; Wen et al., 2015). Due to its high purity, hydrophilicity, structure forming potential, chirality and biocompatibility BC offers a wide range of special applications. The high mechanical strength in the wet state, substantial permeability for liquids and gases and low irritation of skin indicated that the gelatinous membrane of bacterial cellulose was usable as an artificial skin for temporary covering of wounds (Keshk, 2014). BC also shows great stability, low toxicity, non-allergenicity, and can be safely sterilized. BC, however, does not possess antibacterial ability to prevent wound infection when used in wound treatment (Lin et al., 2013).

Diabetic patients with foot ulcer showed a 150-fold increase risk of amputation, in particular because of the shortcomings of the wound dressings (Pednekar et al., 2015). Up to date, there is no single dressing fulfills the requirements of a diabetic patient with an infected foot ulcer. Microbial infection is one of the main reason which lead to the delayed and failured of wound healing process. BC is able to control wound exudation and provide moisture to the skin which is crucial for wound healing (Petersen and Gatenholm, 2011). Unfortunately, BC do not have any antimicrobial properties to inhibit the growth of microorganisms. To overcome this problem, silver sulfadiazine (SSD) which has been using for many years for topical treatment on skin (Muangman et al., 2010) was incorporated to the BC. The ability of SSD as antimicrobial agent to inhibit the growth of several bacteria such as *P. aeruginosa, E. coli* and *S. aureus* was studied by (Luan et al., 2012). Hussain & Ferguson (2006) have reported that even though silver sulphadiazine cream exhibited evidence of antibacterial effect, there is no direct evidence of improved healing or reduced infection by topical skin treatment. They

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suggested the way forward may be by simply provide a clean, moist and undisturbed environment to allow healing. Moreover, other studies have shown that the healing of partial thickness burns is delayed with the use of SSD (Cho Lee and Hee, 2003; Stern, 1989), indicating the need for a better burn dressing (Beheshti et al., 2013).

Several parameters were studied in order to investigate the effect of BC-SSD ability as effective wound dressing specialized for wound healing process. SEM analysis also important to justify the physical characterictics of the deposition of SSD on bacterial cellulose. For the mechanical properties of the BC-SSD, the swelling test was conducted to investigate the stability of holding capacity of bacteria cellulose. The crucial part of this study, the release kinetic of the BC-SSD membrane was determined by atomic absorption spectrophotometry (AAS). Finally, the antimicrobial activity of BC-SSD was investigated by disc diffusion test to measure the susceptibility of diabetic ulcer bacteria such as *Staphylococcus aureus, Escherichia coli and Pseudomonas sp.* against the BC-SSD membrane.

2. Materials and methods

2.1. Production of bacterial cellulose

The culture medium of the bacterial cellulose producing bacteria, *Acetobacter xylinum 0416* (purchased from the Biotechnology Research Centre, MARDI, Serdang, Selangor, Malaysia) was prepared as follows (% w/v); 4% glucose, 0.5% peptone, 0.5% granulated yeast extract, 0.27% Na_2HPO_4 and 0.115% citric acid in 100 mL of distilled water. The culture medium was sterilized at 121 °C, 15 psi for 15 min. After the mixture was cooled to 28 ± 1 °C, 10% (v/v) of *A. xylinum* 0416 seed culture was aseptically transferred into the sterile culture medium. The static fermentation process was carried out for 2 weeks at room temperature (±28 °C).

2.2. Purification of bacterial cellulose

BC pellicles harvested from the fermentation medium was washed with deionized water and boiled in 0.1 M NaOH solution for 1 h at 80 °C using thermostatic water bath to remove the bacterial cells and other impurities adhered on the pellicles. After boiling process, the pellicles was removed and repeatedly washed with distilled water until the neutral pH was reached. Then, BC pellicles were stored in wet condition and dry condition for further analysis. The wet condition refers to the BC pellicles stored in deionized water where this condition is needed for disk diffusion test, tensile strength and swelling test. On the other hand, dry condition refers to the BC pellicles which was air-dried in laminar flow and stored in sterile condition. The dried BC is necessary for SEM and FTIR analysis.

2.3. Production of BC-SSD by ex-situ modification method

BC-SSD membrane was prepared by immersing the purified BC pellicles separately in different concentration of SSD compound (0.2%, 0.4%,0.6%, 0.8%, 1.0% w/v) for 24 h. The samples were dried at room temperature to remove excess moisture and stored in sterile condition for further studies.

2.4. Chararacterization of BC-SSD

2.4.1. Scanning electron microscopy (SEM)

The surface morphology of BC-SSD membrane produced with different concentration of SSD was observed by using Bruker scanning electron microscope (SEM) and compared with control membrane (BC). The cross section of native BC and BC-SSD were coated with thick carbon layer and examined under SEM. This method was done by using SEM Bruker model at Malaysia Palm Oil Board (MPOB).

2.4.2. Fourier-transform infrared spectroscopy (FTIR)

FTIR is an equipment which commonly used to detect the chemical compound associated within the BC-SSD sample. Both native BC and BC-SSD samples were frozen for one week and proceed to the freeze drying step for about 2 days. Then, the freeze dried samples were placed on the PerkinElmer FTIR spectrum 100 series disc which was then pressed. The FTIR analysis was carried out using FTIR spectrometer with the resolution of 4 cm^{-1} in the range of 4000 to 400 cm^{-1}.

2.4.3. Tensile strength

Both BC and BC-SSD were cut into 3 cm × 3 cm strips of length and width, respectively. The free films were evaluated for their mechanical properties by using TA.XT Texture analyzer model with 50 N load cell. The measurement were taken at a crosshead speed of 0.01 m/s, 25±2 °C and relative humidity of 60 ± 5%. Tensile strength of the samples were determined.

2.4.4. Swelling properties

The dry weight of BC discs (W_0) were measured before being soaked into phosphate buffer saline (PBS) solution at pH 2, pH 7 and pH 11, respectively. The soaking process was carried out for 15 h at room temperature to obtain equilibrium swelling. The swollen membranes were withdrawn and the wet weight of the swollen membranes (W_1) were measured after the disc were gently blotted using a filter paper to remove excess PBS solution. The swelling degree is calculated based on equation (1). W_0 was the initial weight of the dried sample and W_1 was the weight of sample after soaking in PBS solution.

$$\text{Swelling ratio} = \frac{W_1 - W_0}{W_0} \text{x } 100 \quad (1)$$

2.4.5. Silver release study

BC-SSD were cut into square pieces of 90 mm^2. These samples were then immersed in 50 mL of distilled water and kept in individual sealed containers at room temperature. The solution for each sample were analyzed by using PerkinElmer Atomic Absorption Spectrometer (AAS) model 100 to detect the amount of silver ion release at regular time intervals of 24 h, 48 h and 72 h. The percentage of silver ion released was calculated as below:

Amount of silver ion released (%) = Concentration of silver ion released × dissolution volume × 100

2.4.6. Antimicrobial activity

The antimicrobial activity of BC-SSD membranes were tested by disc diffusion method. The selected microorganisms from diabetic ulcer such as *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were spreaded on nutrient agar by using lawn culture method. Then, the BC-SSD membranes with different concentration of SSD (0.2%, 0.4%, 0.6%, 0.8% and 1.0%) were placed on the nutrient agar. Pure BC membranes without SSD acts as a control. The agar plates were incubated at 37 °C for 24 h. After incubation, the diameters of the inhibition zone were measured in mm. Statistical significance was analyzed by single factor analysis of variance (ANOVA) test. The data were reported as mean ± standard deviation. The significant difference was shown at $p < 0.05$ and its statistical analysis was calculated using Microsoft Excel Version 2013.

3. Results and discussion

3.1. SEM imaging

The morphological surface of the native BC and the modified BC were observed by using Scanning Electron Microscope (SEM). Fig. 1(A) shows the image of bacterial cellulose before the impregnation with

silver sulfadiazine. It was clearly observed that the structure of the native BC has many networks with porous structure around it. The networks are interconnected and formed fibrils that makes BC rigid. Besides, those porous makes BC capable to be impregnated with nanoparticles compound as it has large surface area to be incorporated with. As for BC-SSD, it is clearly seen from Fig. 1 (B, C, D, E, F) that silver sulfadiazine particles incorporated well with the denser network structure of BC. There is distinct deposition of silver compound on bacterial cellulose with different concentration of silver sulfadiazine. The higher the concentration of silver sulfadiazine, the higher the deposition of silver sulfadiazine filled the porous structure of bacterial cellulose. BC acts as stabilizing agent to control particle nucleation, avoid aggregation, produce silver particles at the nanoscale and due to its porosity and hydrophilicity (Picheth et al., 2017).

3.2. FTIR

The characterization of BC and modified BC incorporated with different concentrations of SSD were characterized and compared using FTIR spectrum. The FTIR spectrum characterizes BC with some bands and peaks, indicates that certain compounds are present in the BC structure as shown in Fig. 2. The presence of O–H intermolecular bond is indicated by the presence of band at 3344.63 cm^{-1} in pure BC (or BC-0% SSD), while peak 2987.88 cm^{-1} indicates the presence of stretching alkane in bacterial cellulose. Besides, the bending of methyl group associated at peak 1426.70 cm^{-1} and 1108.68 cm^{-1} shows the presence of stretching aliphatic ether. These peaks prove that the structure of bacterial cellulose consists of O–H molecular intermolecular bond, alkane, methyl group and aliphatic ether. Similar result was reported by Auta et al. (2017) where they found the structure of bacterial cellulose is comprised of O–H stretching, methyl group and the alkane structure. The presence of O–H stretching was detected at peak 3338.41 cm^{-1} while the alkane stretching was detected at 2917.85 cm^{-1}. Moreover, the methyl group compound was found at peak 1015.65 cm^{-1} where the compound is stretched at β-(1 → 4)-glycosidic bond.

Sulfonamide is the basic structure of silver sulfadiazine. The characterization of BC-SSD structure was done in order to determine the incorporation of silver sulfadiazine with the bacterial cellulose. The results obtained show that sulfonamide compound is associated with the bacterial cellulose structure based on the peaks obtained from each spectrum. The sulfonamide compound can be found at peak 1355.13 cm^{-1}, 1354.10 cm^{-1}, 1354.74 cm^{-1}, 1354.13 cm^{-1} and 1355.13 cm^{-1} for BC-SSD at the concentrations of 0.2%, 0.4%, 0.6%, 0.8% and 1.0%, respectively. Moreover, the silver sulfadiazine compound is only associated with the hydrogel structure without any chemical reaction (Jodar et al., 2015). This concludes that silver sulfadiazine will not change its physical properties and structure when it incorporates with bacterial cellulose.

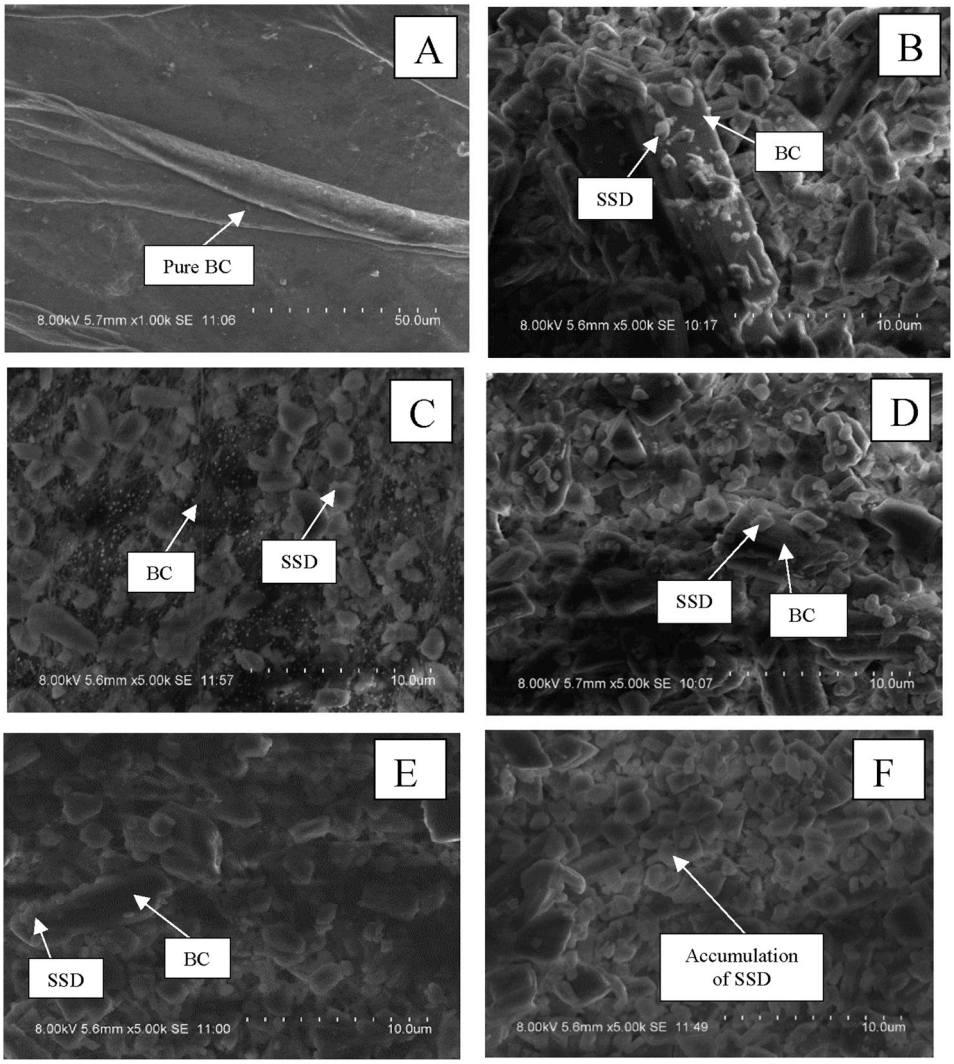

Fig. 1. Morphological structures of BC and BC-SSD under SEM; (A) native BC, (B) BC- 0.2% SSD, (C) BC-0.4% SSD, (D) BC-0.6% SSD, (E) 0.8% BC-SSD (F) BC-1.0% SSD.

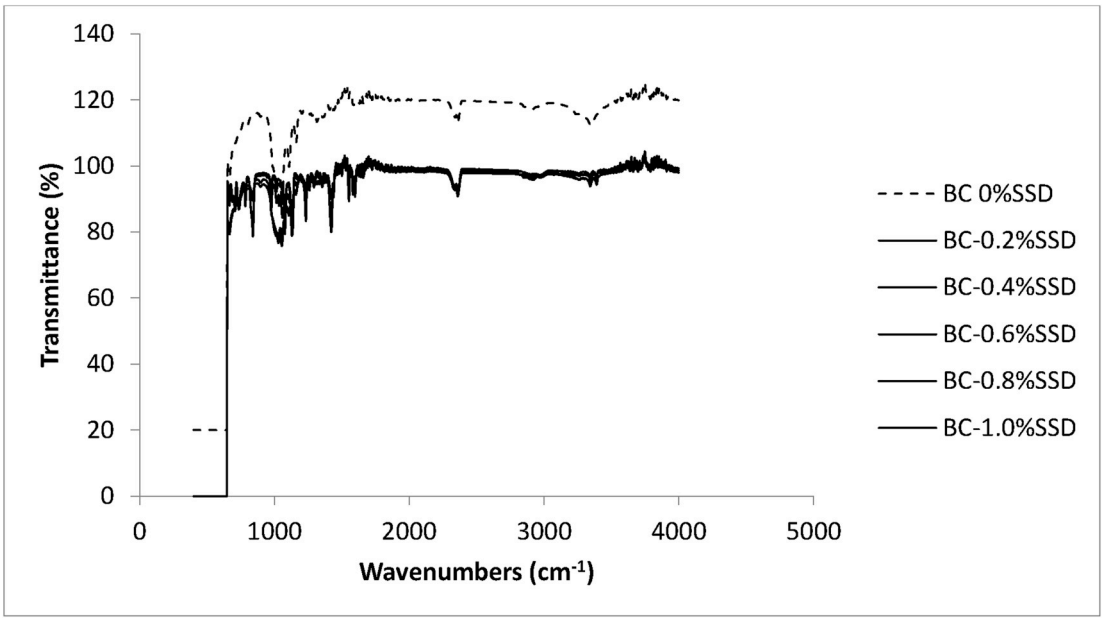

Fig. 2. FTIR spectrum of (A) native BC, (B) BC-0.2% SSD, (C) BC-0.4% SSD, (D) BC-0.6% SSD, (E) BC-0.8% SSD and (F) BC-1.0% SSD.

3.3. Tensile strength

The strength of BC and BC-SSD were tested by tensile strength in order to observe the robustness of both BC and BC-SSD as wound dressings. Based on Fig. 3, the tensile strength of BC-SSD are stronger than the native control BC. Moreover, the tensile strength of BC-SSD increases with the increment of silver sulfadiazine concentration as the compound strengthen the structure of BC. SSD partciles fills up the porous matrix of BC pellicles which directly reduced the porosity of the BC. Besides, it is also explainable that the native BC has the lowest tensile strength due to its high porosity which makes the structure of native BC weaker than BC-SSD. In another study, it was found that the tensile strength of the BC-composites increase by the addition of the additives due to the interaction between BC and the additives material such as entanglement, hydrophobic, electrostatic and hydrogen bonding interactions. Furthermore, the connectivity of BC-additive network increases as the structure reinforce by the interactions (Dayal and Catchmark, 2016).

3.4. Swelling test

Swelling test is crucial to evaluate the rehydration ability of the BC membrane. Swelling ability which is closely related to the hydrophilicity of the BC is one of the important dressings' characteristic (Zmejkoski et al., 2018). Whilst, moisture is one of the characteristics of wound dressing which maintain the higher permeability of water content which sustain the wound healing process (Moniri et al., 2017). Fig. 4 shows the swelling ratio of the BC pelllicles of the bacterial cellulose with pH 11

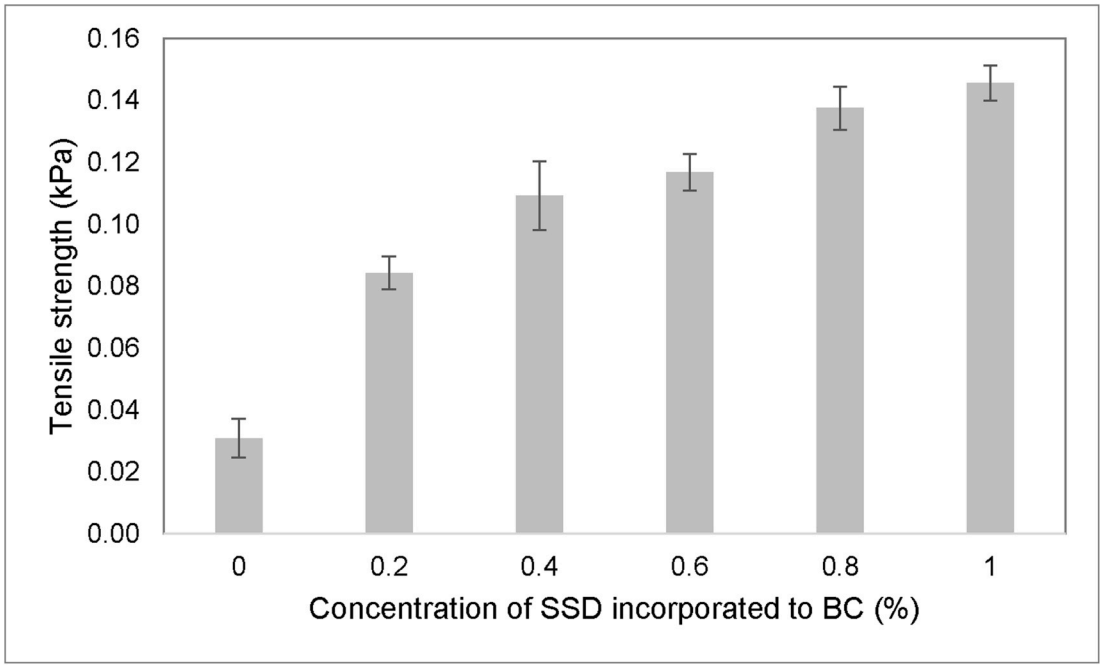

Fig. 3. Tensile strength of BC and BC-SSD with different concentrations.

gave the highest amount of water absorption compared to pH 3 and pH 7 of the test groups. Based on past studies, the ulcer fluid sample with pH 8.5 commonly found in isolates of *P.aeruginosa* compared to other microbes. Meanwhile, *E.coli* culture were found the most common in ulcer fluid with pH 8 and *S.aureus* were isolated the most from ulcer fluid sample of pH 7.5 (Mcardle et al., 2014). Hence, the swelling ratio data for this analysis gives significant properties to BC as it is proven to have the capability of absorbing alkaline fluid better compared to the acidic fluid.

3.5. Silver release study

Fig. 5 shows silver ion releasing behavior from the BC-SSD pellicles by using AAS analysis. There was a significant increased of the silver ions released proportional to the increment of the SSD concentration incorporated to the BC. The release rate of silver ions from all BC-SSD membranes are high at the first 24 h as compared to the next 48 h and 72 h. The SSD deposited at the surface and outer layer of BC networks influence the fast release of the silver ions from the BC at the first 24 h. Whilst, the silver ions impregnated at deeper BC networks were released gradually afterwards. This finding shows that the silver ions release is efficient in 24 h period which is crucial to prevent early phase of infection along the wound healing process. The early phase of wound healing process involves homeostasis and inflamation which starts at time of injury and lasts 4 to 6 days (O'Loughlin & O'Brien, 2011). Hemostasis begins at the onset of injury to stop the bleeding while inflammatory phase focuses on destroying bacteria and removing debris which essentially preparing the wound bed for the growth of new tissue. As the diabetic patient normally experiences of delayed wound healing as a results from dysregulation of the normal healings pathways, the antimicrobial compound should be added to the dressing in order to facilitate the wound healing process at these stages. On the other hand, the slow release of SSD as antimicrobial agent for the next 72 h will maintain antimicrobial capacity during the inflammatory stage. Similar trend was obtained by other researchers where the BC-dehydrogenative polymer of coniferyl alcohol (DHP) membrane undergone slow and continuous release of DHP in PBS solution. İt showed faster release rate of BC-DHP within 24 h and subsequently declined for the next 72 h (Zmejkoski et al., 2018).

3.6. Antimicrobial activity of BC-SSD film

The antimicrobial activity of BC-SSD was determined against *S. aureus, E.coli and P.aeroginosa* which commonly found in diabetic ulcer. The results obtained are statistically significant with P value < 0.05 (Fig. 6). However, the significant difference of the standard deviation may be due to the inconsistency of porosity and thickness of the bacterial cellulose produced. Further study on the optimization of the production of bacterial cellulose shall be carried out to enhance the deposition of the antimicrobial compound with bacterial cellulose via ex-situ method. The increment of porosity factor shall enhance the deposition of compound onto bacterial cellulose.

BC-SSD showed obvious inhibition zone on all tested bacteria but it works more effectively on *E.coli* and *P.aeruginosa* as compared to *S. aureus.* These results are in agreement with Luan et al. (2012) where BC-SSD exhibit strong antimicrobial activity against *P. aeruginosa, E.coli* and *S. aureus.* These bacteria are commonly isolated from diabetic foot ulcers with *Pseudomonas sp.* leads the number (16%) followed by *Escherichia coli* (14.6%) and *Staphylococcus aureus* (13.3%) (Shanmugam, 2013). The microbiology of the diabetic foot is unique as the infection can be caused by Gram-positive aerobic, and Gram-negative aerobic and anaerobic bacteria, singly or in combination (Edmonds, 2009). Gram positive bacteria, like *S. aureus* accumulates at the initial stage of infection and Gram negative bacteria such as *E.coli* and *Pseudomonas* species significantly high in later stage causes tissue damage in the deeper layer of skin (Simões et al., 2018). By taking this factor into account, it is suggested to introduce higher concentration of SSD at the later stage of treatment probably after 24 h or during the inflammatory phase of wound healing. Moreover, the proposed treatment by BC-SSD membrane can be explored for localized infection of diabetic foot ulcer.

4. Conclusion

BC-SSD has shown its ability as alternative wound dressing for

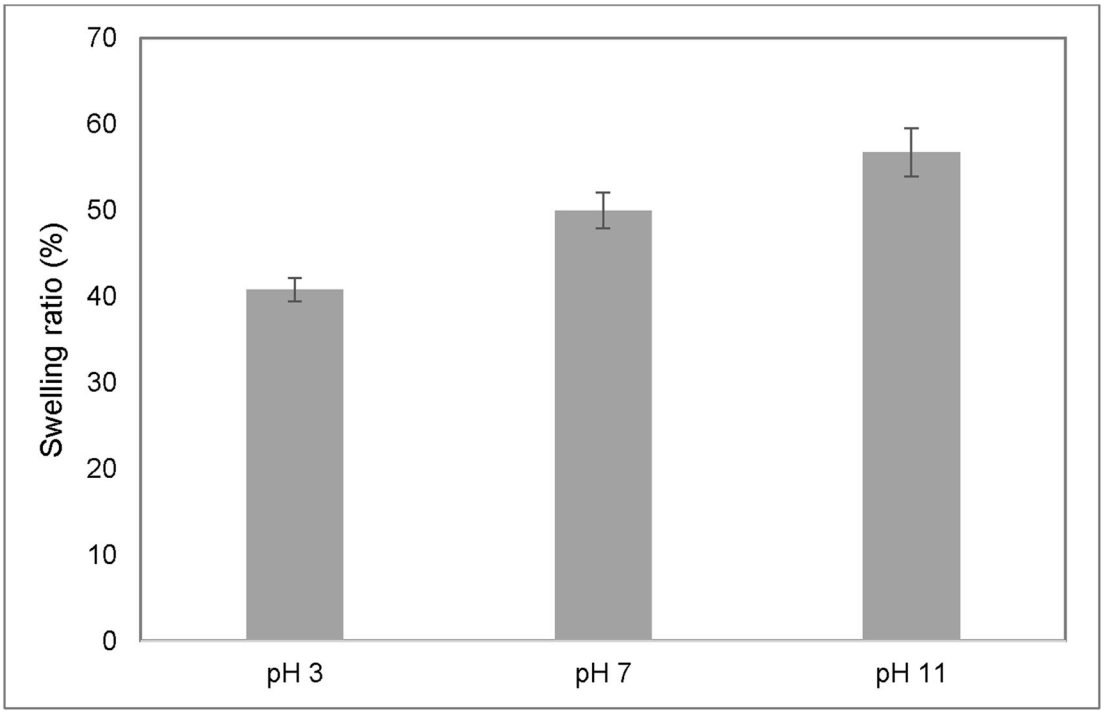

Fig. 4. Swelling ratio of BC in different pH solution.

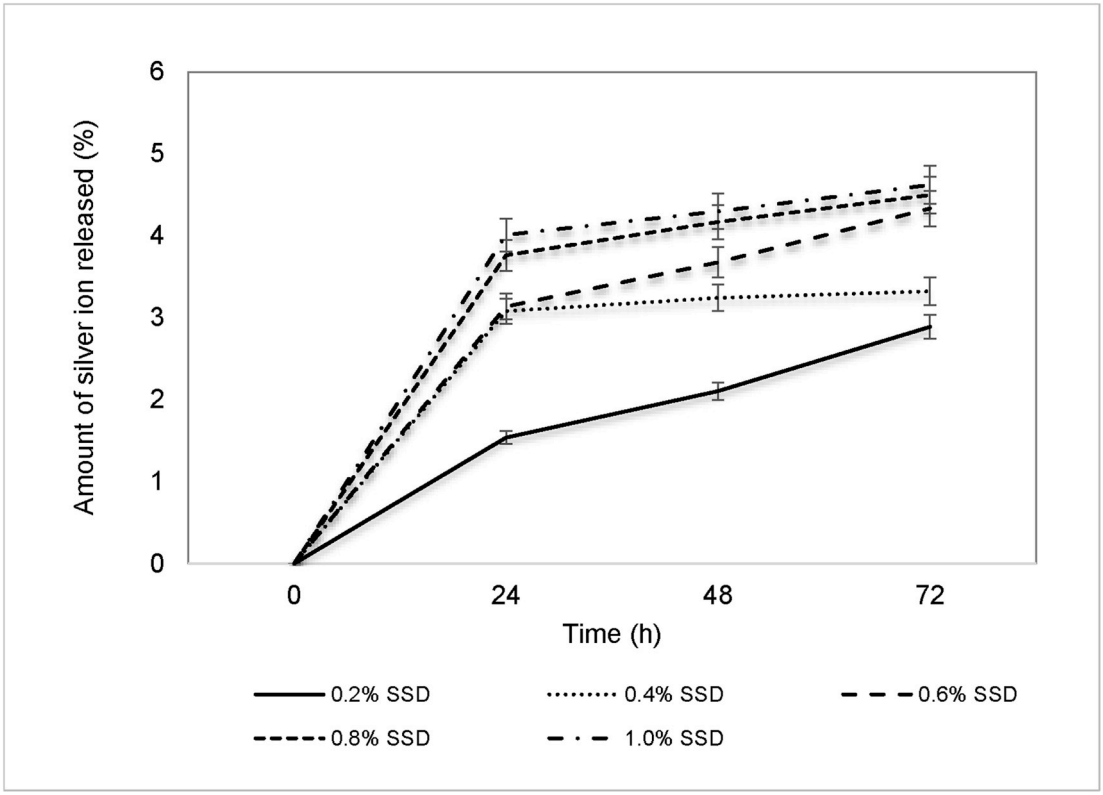

Fig. 5. The silver ion release from BC-SSD membrane.

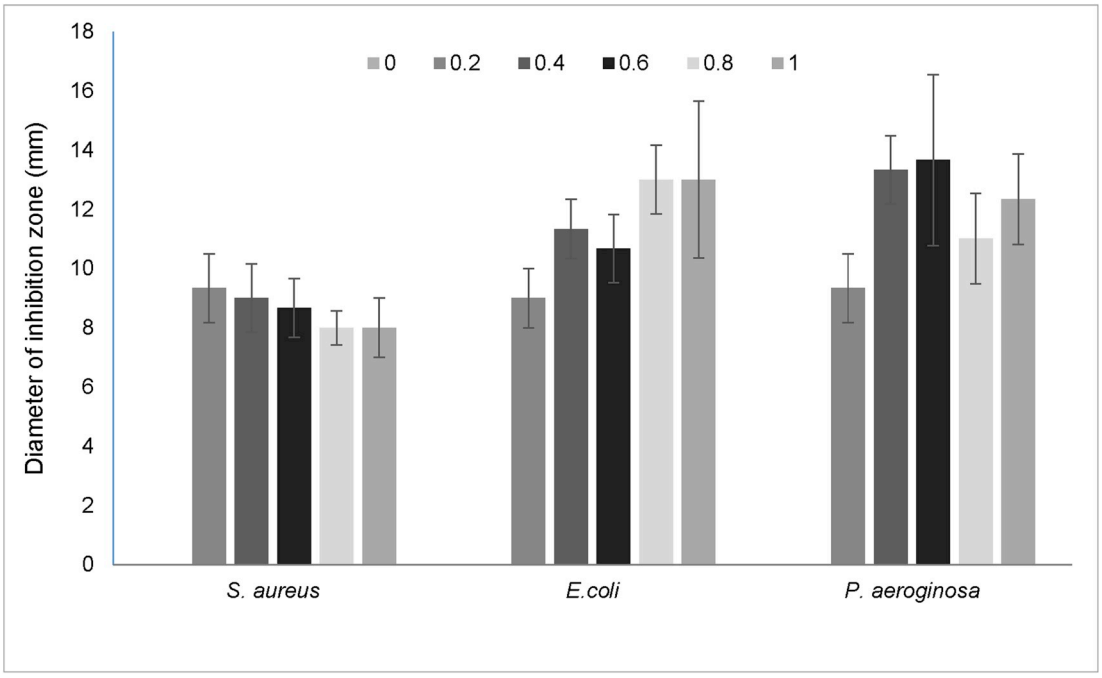

Fig. 6. Antimicrobial activity of BC-SSD against bacteria related to diabetic ulcer.

diabetic foot ulcers due to its promising antimicrobial activity against common bacteria found in diabetic foot ulcer. The deposition of SSD on bacterial cellulose was proved through SEM and FTIR analysis. The tensile strength of the BC-SSD membranes increase by the increment of silver sulfadiazine concentration incorporated to the BC. Moreover, the release of silver ions is efficient at the first 24 h and gradually decrease for the next 72 h. These properties are crucial to ensure the stability of BC-SSD as wound dressing and to prove the efficiency of the release of SSD and absorption of the SSD via atopic mechanism of wound healing process. The ultimate outcome of this research is BC-SSD offer alternative solution to the prolonged recovery of diabetic ulcer that became the major concern among diabetic patient.

Conflicts of interest

The authors have no conflicts of interest to declare.

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