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Preparation and Characterization of Bacterial Cellulose– Chitosan Composite as Antimicrobial Material

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Abstract

Bacterial cellulose (BC) has grown an increasing trend for applications in biomedical field due to its conformability, high water absorption capacity and biocompatibility. Nata de coco is a local food product with the main component of BC. Chitosan (Ch) is a polysaccharide with intrinsic antibacterial property considered for biomedical solicitations. For this work, BC and BC-Ch membranes was formed from nata de coco. The produced BC membrane was subjected to ex-situ modifications by immersing them in 1% chitosan solution. The purity of the BC membrane produced and the incorporation of chitosan in the BC-Ch composite was confirmed through infrared spectroscopy. Scanning electron microscope (SEM) was utilized to evaluate the surface morphology of the membranes. The SEM pictures showed that the BC-Ch composite owned a solider fibril network compared to the BC membrane. Mechanical properties of BC and BC-Ch membranes exhibited no significant variation. The BC-Ch composite membrane exhibited superior water holding capacity and water retention ratio compared to the BC membrane. The antibacterial tests revealed that the introduction of chitosan in the membrane imparted antimicrobial properties to the composite membrane. The produced BC-Ch membrane from nata de coco can be contemplated for antimicrobial wound dressing material.

Keywords: chitosan, bacterial cellulose, nata de coco,

1. INTRODUCTION

Bacterial cellulose (*BC*) is a biopolymer created by bacteria which exhibits superior properties than plant cellulose. Bacterial cellulose is formed by fermentation of carbon compounds from a nutrition medium in the presence of the *Acetobacter xylinum* producing ultrafine network of cellulose nanofibers [1]. Bacterial cellulose presents high purity, great elasticity, conformability, biocompatibility, transparency, good water holding capacity, unique mechanical property, good permeability, resistance to degradation, non-toxicity and sterilization is achievable deprived of characteristics changes [2]. Although *BC* has unique characteristics, development of polymer composites enhances the physical and biological properties of *BC* further enhancing the effectiveness of the desired application. Applications of bacterial cellulose in different fields have increased through synthesizing composites using bioactive polymers, nanomaterials and solid clay particles [3]. Synthesis of *BC* composites grant desirable properties to *BC* such as antibacterial, antiviral, antifungal, increase in tensile and mechanical strength, thermal stability, wicking, water retention ability and water absorbency [4]. Applications of bacterial cellulose composite extends from water purification membranes, headphone membranes, tires, high grade paper, high performance speaker diaphragms, food packaging, and textiles as scaffolds for tissue engineering, drug delivery system and wound dressing materials [3,4]. Reinforcement materials used to improve the performance of bacterial cellulose for wound dressing application is by promoting antimicrobial activity to *BC* using chitosan, vanillin, benzalkonium chloride, montmorillonite and silver nanoparticles [5].

Nata de coco, a locally made product and known dessert in the Philippines, is a outcome of fermentation culture of *A. xylinum* in coconut water medium. The use of nata de coco is being

extensively used in the food industry and as well as in the biomedical field as an open wound dressing material [6]. Halib [7], determined whether pure cellulose can be extracted from food grade nata de coco, and results showed that nata de coco is a good source of *BC*, and suitable for research regarding pure cellulose. However, there is only little literature about synthesizing bacterial cellulose composites from nata de coco which suggests the need for further investigation. For wound dressing purposes, bacterial cellulose with an antibacterial activity is more desirable to prevent wound infections upon healing [8]. Chitosan, an inherent antimicrobial agent, has been successfully used for bacterial cellulose composite synthesis. Hence, the application of chitosan as a reinforcement material on bacterial cellulose derived from nata de coco was studied.

The objectives of this study include the synthesis of bacterial cellulose-chitosan composite membrane using bacterial cellulose derived from nata de coco via ex-situ method and characterization of the membranes in terms of surface morphology, bonding mechanisms of chitosan on bacterial cellulose from nata de coco, mechanical properties, water holding capacity, water retention property and antimicrobial activity.

This study will be a significant endeavor in justifying the possibility of synthesizing bacterial cellulose from nata de coco and using it for synthesizing *BC-Ch* composite to enhance its properties such as the introduction of antimicrobial activty.This study can increase the possible applications of nata de coco resulting to larger demand of the native product.

The study was limited to the synthesis of *BC* and *BC-Ch* composite from food grade nata de coco and the characterization of the *BC* and *BC-Ch* composite. Characterization method to be performed included structural characteristics using Scanning Electron Microscopy (SEM), bonding mechanisms of chitosan on bacterial cellulose derived from nata de coco using Fourier Transform Infrared (FTIR) spectroscopy, mechanical properties using Universal Testing Machine (UTM), water holding capacity, water retention property and antimicrobial activity using *Escherichia coli* and *Staphylococcus aureus.* The economic viability of the study was not studied.

2. EXPERIMENTAL PROCEDURE

Food grade nata de coco was purchased from Udiong Trading in the form of uncooked slabs. Chitosan with ≥75% degree of deacetylation was obtained from Sigma Aldrich. Analytical grade acetic acid from Harnwell Chemical Corps was used.

2.1 *BC* derived from nata de coco

Nata de coco slabs were washed initially and soaked with distilled water. The distilled water was changed daily until the pH is neutral (6 – 7) which required two weeks. Nata de coco slabs were dried in a conventional oven (Memmert incubator/ UM-200) at 60°C for two days with the resulting membrane termed as *BC*.

2.2 Characterization *of BC and BC-Ch*

BC and *BC-Ch* sheets were described in terms of surface morphology, bonding mechanism of chitosan to bacterial cellulose, water holding capacity, water retention ratio, mechanical properties and antimicrobial activity.

2.3 Surface morphology

Scanning electron microscope (Hitachi/ TM3000) was utilized for surface morphology analysis in 4000 and 6000 magnification. Samples of BC and BC-Ch membranes with dimensions 1 cm x 1 cm

were brought to University of Santo Tomas Research Center for the Natural and Applied Sciences – Analytical Services Laboratory as well as samples were placed on a carbon tape before inserting into the instrument.

2.4 Fourier transform infrared spectroscopy

The chemical structure of *BC* and *BC–Ch* was examined using Fourier Transform infrared spectroscopy (Perkin Elmer/ Spectrum 100) in absorption mode in the range 4000-1000 cm^{-1}. Triplicate scans per sample were implemented to inaugurate accurateness.

2.5 Mechanical Properties

Testing machine (Shimadzu/ AGS-5kNX) was utilized to determined the mechanical properties of the specimens. Three samples each of *BC* and *BC-Ch* membranes were brought at the University of the Philippines (UP) Diliman Department of Chemical Engineering Analytical Laboratory. The dimensions of the membrane was 25 x 200 mm with thickness of 1.0 and 0.8 mm for *BC* and *BC-Ch*, respectively.

2.6 Water holding capacity and water retention ratio

The water holding capacity (WHC) and water retention ratio (WRR) of *BC* and *BC-Ch* membranes were determined by gravimetric method. The membranes were initially cut into 2 cm x 2 cm squares. Individual specimen was submerged in a storage container containing 30 mL of distilled water and incubated at room temperature for 24 hrs. Tweezers were utilized to handle the sheets. The wet sheets were streaked with filter paper to remove the surface water and then weighed, W_{wet} using analytical balance (Mettler Toledo/ AB04-S).

2.7 Antimicrobial assay

The antimicrobial assays of the *BC* and *BC-Ch* membranes were investigated against two test organisms: *Escherichia coli* (UPCC 1195), a Gram negative bacilli model bacteria and *Staphylococcus aureus* (UPCC 1143), a Gram positive cocci model bacteria. The chosen test organisms are the most commonly found bacteria in wounds. Samples of *BC* and *BC-Ch* membranes with dimensions 4 cm x 4 cm were brought at UP Diliman Natural Sciences Research Institute - Microbiological Research and Services Laboratory for testing. The *BC* and *BC-Ch* samples cut into squares that would fit a circle with approximately 13 mm diameter were placed at three equidistant points on the agar plate.

3. RESULT AND DISCUSSION

3.1 Surface Morphology

For *BC* and *BC-Ch* membranes, the surface arrangement were analyzed by SEM at 4000 and 6000 magnification as shown in **Figure 1**. For the bacterial cellulose membranes, an arbitrarily arranged fibrils and diverse range of void space in between were noticed. Formation of pores with varied diameter on the surface were resulted from the arrangement of fibrils and was seen throughout the whole matrix of the *BC* sheets [10]. The culture medium bacteria polymerize and crystallize the glucose molecules for the formation of nanofibrous structure of the *BC* in which these nanofibrils have cross sectional dimensions in the nm range which then aggregates to form microfibrils [11].The findings for the *BC* membrane were coherent to the reported literature [11].The surface morphology of *BC-Ch* membranes were more denser. The existence of chitosan

had contributed to this as it has filled the void spaces from the original *BC* membranes. The chitosan penetrated into the pores of the bacterial cellulose and interacted with the microfibrils, affected the physico-chemical properties [12]. The presence of OH moieties in bacterial cellulose chains results in a hydrogen bonding with the chitosan [3]. Due to the porosity of *BC*, chitosan solution can easily adhere and attach to the matrix. The original structure of the *BC* remained completely unchanged but is only added by the structure of chitosan through ex situ modification. The results suggested that chitosan was incorporated within the microfibrils of *BC* membrane, that enabled compact arrangement of network structure and reduced the void spaces within the *BC* network.

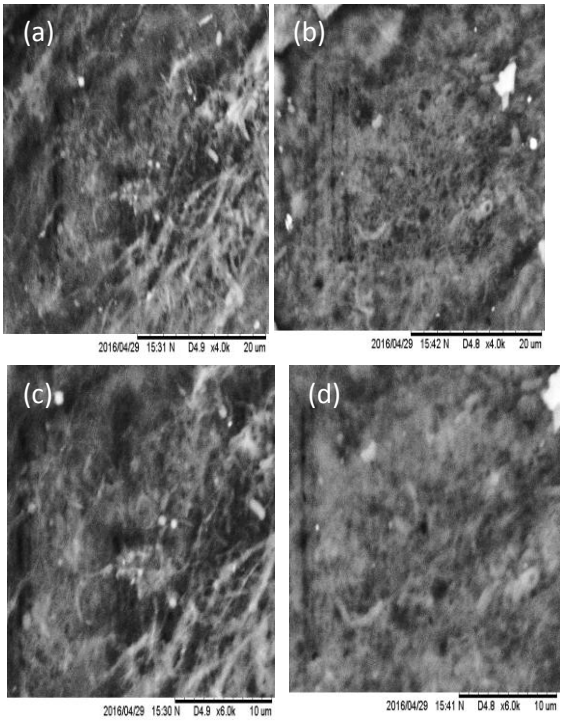

Figure 1. Surface morphology at 4000x magnification (a) *BC* (b) *BC-Ch* and surface morphology at 6000x magnification (c) *BC* (d) *BC-Ch*

3.2 Fourier transform infrared spectroscopy

Figure 2 shows the FTIR spectra of *BC* and *BC-Ch* membranes in the wave numbers 4000 to 1000. For pure *BC* membrane, a extensive distinctive peak at 3344 cm^{-1} corresponded to intermolecular hydrogen bonding and O-H stretching vibration. The peak at 2895 cm^{-1} was attributed to aliphatic C-H stretching vibration. The concentrated absorption in the spectrum of the cellulose was the band at 1638 cm^{-1} which was frequently consigned to glucose carbonyl of cellulose. Another intense peak located at 1053 cm^{-1} was ascribed to C-O-C pyranose ring skeletal stretching vibration. The peaks found in the FTIR spectra were very similar to the peaks found in bacterial cellulose in previous studies. The result of the FTIR spectra shows the purity of the *BC* membrane derived from nata de coco.

The FTIR spectra of the *BC-Ch* membrane is very similar to the spectra of the pure *BC* membrane. The characteristic bands of pure *BC* membrane matrix remained in the spectrum since the original

structure of the *BC* remained unchanged after the ex situ modification. The characteristic peaks of chitosan was observed in absorption bands at 1551, 1420, 1371, 1161 cm^{-1} indicated the C=O stretching in secondary amide (amide I), C-N stretching in secondary amide (amide II), -C-O stretching of primary alcoholic group ($-CH_2$ -OH) and free amino group ($-NH_2$) at C2 position of glucosamine, respectively. The FTIR spectra verified the presence of chitosan molecules in *BC-Ch* membrane proving that chitosan was successfully incorporated into the *BC* membrane via ex situ modification.

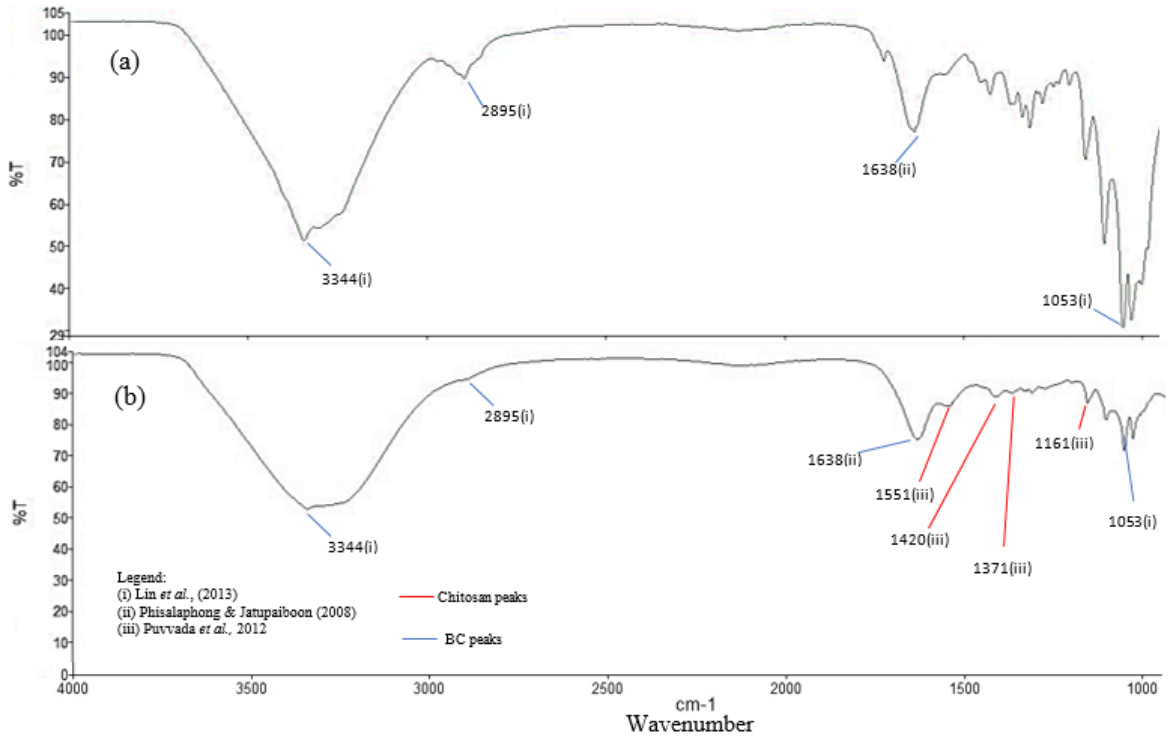

Figure 2: FTIR spectra of *BC* and *BC-Ch* membranes (a) *BC* (b) *BC-Ch*

3.3 Mechanical Properties

The Young's modulus for *BC* and *BC-Ch* membranes determined from the study and compared to the literature range was presented on **Table 1**. The results obtained were lower than that of the literature value range. The Young's modulus of *BC-Ch* membrane was higher compared to that of *BC* which was due to the presence of chitosan increasing Young's Modulus of the *BC* membrane. The same trend for Young's Modulus of *BC* and *BC-Ch* membrane had been observed by the study of [12].

The addition of chitosan has made cellulose fiber to be thick, which has resulted to a compact fiber structure that enhanced the membrane's strength.

The tensile strength for *BC* and *BC-Ch* membranes determined from the study and compared to the literature range was presented on **Table 2**. The results were within the range when compared with other literature. The tensile strength of *BC-Ch* membrane showed lower value compared to that of *BC* membrane. Blending of chitosan with the cellulose normally results to the increase in

tensile strength due to entanglement of molecular chain of chitosan with the cellulose changing the structure and shape of membranes. However, chitosan has poor tensile strength when wet resulting to a decrease in the value which was observed in the study. The *BC-Ch* membrane upon further drying for more than four hours resulted to a thin rubbery membrane that can no longer be reinflatable for good water holding capacity and retention.

Table 1 Young's Modulus of *BC* and *BC-Ch*

Sample	Value (MPa)	Literature Value
BC	4.61 ± 0.97[a]	4 [2] 33.57 [12]
BC-Ch	5.90 ± 1.28[a]	10 [2] 132.19 [12]

Value with different letters are significantly different at p = 0.05

Table 2 Tensile strength of *BC* and *BC-Ch*

Sample	Value (MPa)	Literature Value
BC	2.86 ± 0.88[b]	1.5 [2] 1 [13] 14.77 [12]
BC-Ch	1.85 ± 0.64[b]	2 [2] 10 [13] 10.26 [12]

Value with different letters are significantly different at p = 0.05

The elongation at break for *BC* and *BC-Ch* membranes determined from the study and compared to the literature range is presented on **Table 3** The results are generally lower than that of the literature value. The elongation at break of *BC-Ch* results to a slight decrease compared to the *BC* membrane opposite with Young's Modulus. The reduction in the elongation at break was due to the chitosan's inherent poor elongation properties. The value of the elongation at break for both *BC* and *BC-Ch* (24-26%) designated satisfactory durability allowing the potential application for wound coverings and may fit the designated area of wound for treatment.

Table 3 Elongation at break of *BC* and *BC-Ch*

Sample	Value (%)	Literature Value
BC	26.92 ± 2.87[c]	4 [2] 32.17 [12]
BC-Ch	24.72 ± 3.16[c]	2 [2] 28.54 [12]

Value with different letters are significantly different at p = 0.05

3.4 Water holding capacity and water retention capacity

On the surface, water molecules are imprisoned physically and the inside of the three dimensional matrix of the *BC* membranes. The water holding capacity (WHC) of the samples is shown on **Table 4**. The results show that the *BC* membrane absorbed 79.94 times its dry weight of water while the *BC-Ch* had a significantly higher WHC which was 87.33 times of its dry weight. The water retention ratio (WRR) of the *BC* and *BC-Ch* membranes were determined for a period of 48 hours as shown in **Figure 3**.

Table 4 Water holding capacity of *BC* and *BC-Ch*

Sample	WHC (g water/ g dry sample)
BC	79.94 ± 2.65[a]
BC-Ch	87.33 ± 1.00[b]

Means with different letters are significantly different at p = 0.05

3.5 Antimicrobial activity

Bacterial infection greatly affects wound healing. Since chitosan is a known antimicrobial agent, the incorporation of chitosan in the *BC* membrane should impart antimicrobial properties to the *BC-Ch* composite membrane. The antimicrobial properties of the membrane were evaluated via direct contact with the test organisms. The results from the antimicrobial assay test using *E. coli.* and *S. aureus* on *BC* and *BC-Ch* membranes are shown in **Figure 4** and **Table 5**.

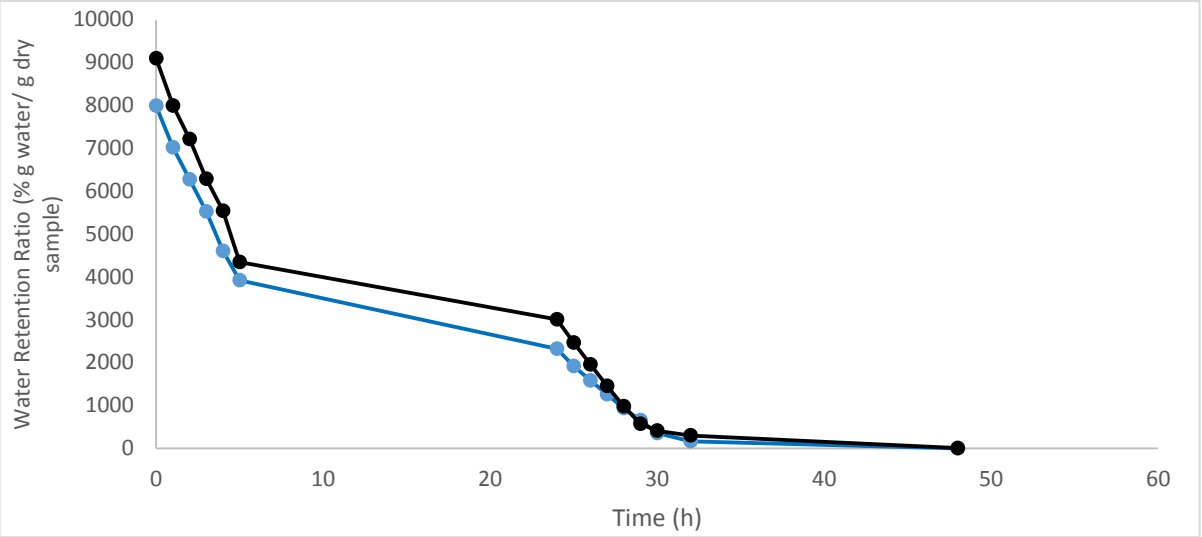

Figure 3: Water retention ratio of *BC* (blue) and *BC-Ch* (black)

Table 5 Antimicrobial Assay of *BC* and *BC-Ch*

Test Organism	Sample	Clearing zone, mm			Antimicrobial Index (AI)
		1	2	3	
Escherichia coli	*BC*	-[a]	-	-	0
	BC-Ch	14	14	15	0.1
	Ciproflaxin[b]	25			3.2
Staphylococcus aureus	*BC*	-	-	-	0
	BC-Ch	20	20	20	0.5
	Ciproflaxin[b]	25			3.2

[a]No clearing zone; no inhibition of growth of the test organism observed
[b]Contains 1µg ciprofloxacin, 6mm disc

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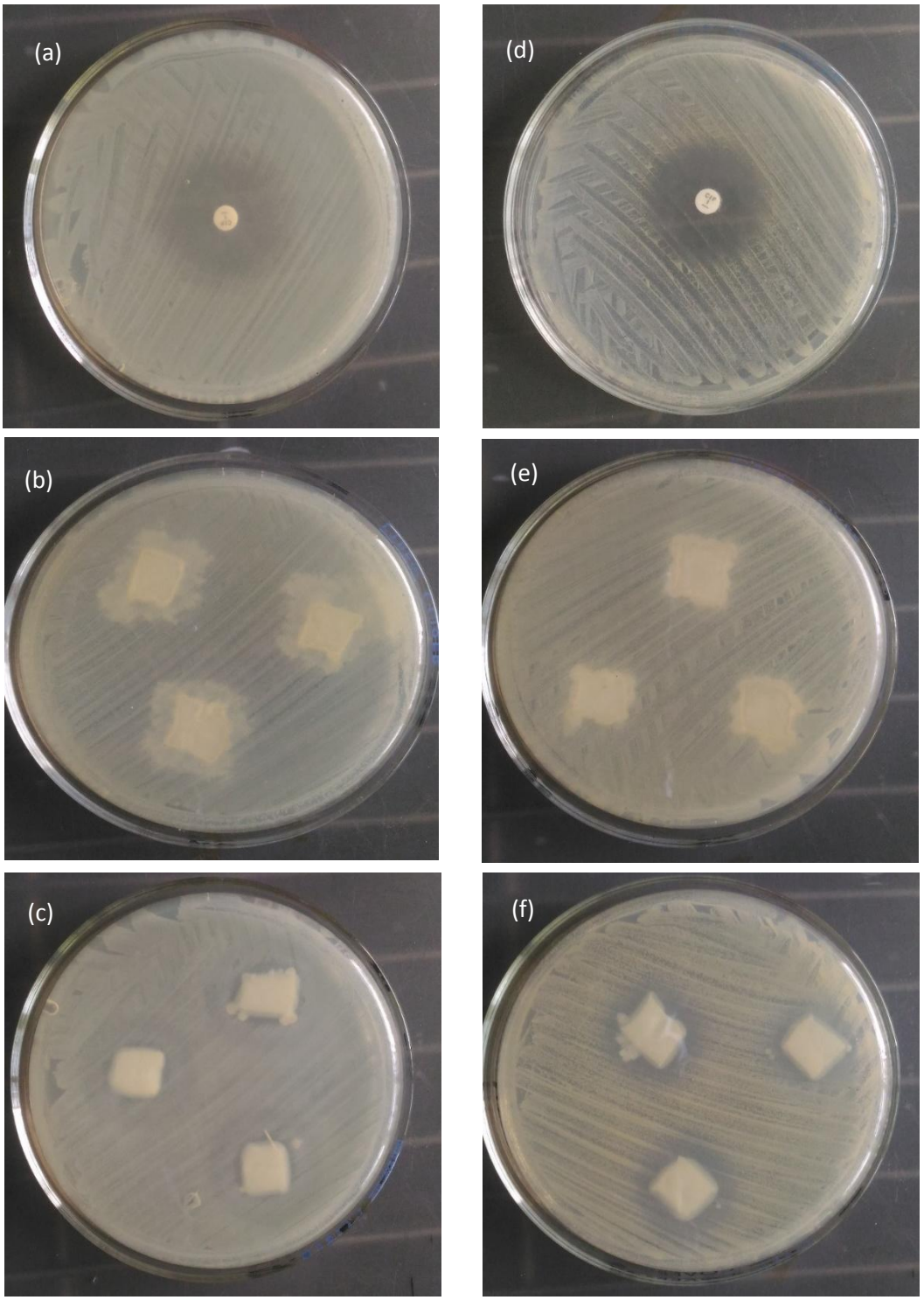

Figure 4: Antimicrobial activity against *E. coli* (a) Ciproflaxin (b) *BC* (c) *BC-Ch* and antimicrobial activity against *S. aureus* (d) Ciproflaxin (e) *BC* (f) *BC-C*

The *BC* sample did not show antimicrobial activity against *E. coli.* and *S. aureus*. Clearing zones under and around the *BC* were not observed. The *BC-Ch* sample showed antimicrobial activities against *E. coli.* and *S. aureus* with AIs of 0.1 and 0.5, respectively. Clearing zones were observed both under and around the *BC-Ch* sample for both test organisms. The results reveal that the addition of chitosan in the *BC* membrane imparted antimicrobial properties to the composite membrane. Various studies have exposed that chitosan has wide dimension of actions alongside microorganisms [14].

Ciproflaxin is a known antibiotic used to treat different bacterial infections. The *BC-Ch* samples had smaller clearing zone and antimicrobial index compared to ciproflaxin. Zone of clearing tests is not classically quantitative and antimicrobial agents that easily leach out into the agar matrix show better results compared to antimicrobials that are affixed or impregnated to a matrix such as the *BC* membrane.

Lin [5] assessed the antimicrobial behaviors of cellulose, chitosan/cellulose and chitosan/cellulose -AgNPs composite films against *E. coli.* and *S. aureus*. The results of their study showed similar results to this study; no clearing zones were observed in the cellulose films and the chitosan/cellulose films had a smaller clearing zone compared to the chitosan/cellulose -AgNPs composite films since silver ions from the silver nanoparticles can easily be released and diffused into the agar matrix.

The smaller clearing zone observed in the *BC-Ch* membrane against E. *coli* and S. *aureus* strongly suggests that chitosan may be strongly united in the *BC* matrix which prevents the antimicrobial section from diffusing to the surrounding area.

4. CONCLUSIONS

In this study, *BC* and *BC-Ch* membranes were effectively made from nata de coco. Infrared spectroscopy confirmed the clarity of the *BC* produced from nata de coco and the combination of chitosan in the *BC-Ch* composite membranes. The SEM images showed that the *BC* membranes consisted of randomly arranged fibril networks. The incorporation of chitosan resulted to a more denser fibril network with lesser void spaces. The mechanical properties of *BC* and *BC-Ch* membranes showed no significant varioation.The *BC-Ch* composite membrane showed improved water holding and water retention properties due to the introduction of chitosan. The water retention study also shows that the *BC* and *BC-Ch* membranes can maintain a high moisture content for a period of 24h. The antimicrobial test showed that the antimicrobial property of chitosan was successfully incorporated in the *BC-Ch* membrane. The *BC-Ch* membrane showed successful growth inhibition against *E. coli* and *S. aureus*. The results of the study demonstrated that the *BC-Ch* composite membrane produced from nata de coco had improved characteristics compared to the *BC* membrane. The improved properties made the *BC-Ch* composite membrane a

good candidate as an antimicrobial wound covering agent.

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