CHAPTER 4

DISCUSSION

1. Production and evaluation of bacterial cellulose film

It is difficult to control the fix BC production. In this study, BC production was used in medical applications such as wound dressing or topical drug carrier into wound. Dry weight, thickness and % yield were studied for the purpose of fix BC production. In this study, *A. xylinum* TISTR 975 was used to produce. The conditions of this culture were controlled such as the volume of air contacted surface of medium, the composition of culture medium except the carbon sources, the pH of medium, the temperature and the period time of culture. From 7 sugars i.e., glycerine, mannitol, glucose, sucrose fructose, lactose and arabinose, *A. xylinum* TISTR 975 can produce BC film from the first five sugars but not from lactose and arabinose. However, in lactose and arabinose contained medium, the growth of bacteria was observed but no BC formation. It is indicated that this bacteria cannot convert these two sugars into cellulose. The BC films from different sugars were produced in the form of gelatinous films or pellicles and floating on the media surface. They can hold a large amount of water and at the same time display great elasticity. Keshk and Sameshima, (2005) demonstrated that *A. xylinum* ATCC 10245 produced BC from various kinds of monosaccharides, oligosaccharides, alcohol and organic acids. This bacterium also produced BC from lactose. It is indicated that *A. xylinum* TISTR 975 can produce BC from special kinds of monosaccharide and disaccharide event it can use such sugar for growth. To compare with 3 sugars glycerine, glucose and fructose,

A. xylinum TISTR 975 as well as *A. xylinum* ATCC 10245 produced the highest yield of BC from glycerine. Whereas glucose and fructose produced similar yield which was much lower than that of glycerine. *A. xylinum* TISTR 975 produced the higher BC yield from glycerine than glucose. The similar results also observed by Oikawa *et al*., (1995) that the amount of BC produced by *A. xylinum* TISTR 975 from D-mannitol was more than 3 times as much as that from D-glucose under the same culture conditions. D-mannitol added to the medium was converted into D-fructose, and then incorporated into the cellulose biosynthesis pathway of this organism. Ramana *et al*., (2000) reported that the efficient production of cellulose by the bacteria depends on its ability to synthesize glucose from various carbon sources substrates and glucose polymerization to cellulose. These results indicate that the resulted BC is the product of very complicated reactions. The thickness of BC produces from different sugars was correspondence with the weight of BC dry films. The highest thickness was from glycerine followed by mannitol, sucrose glucose and fructose, respectively.

2. Observation of bacterial cellulose fiber under scanning electron microscope (SEM)

The SEM micrographs of BC dry films from *A. xylinum* TISTR 975 showed the ultrafine network of cellulose nanofibres (3-8 nm) which are highly uniaxially oriented. These results similar to the finding of Czaja *et al*., (2004) on 3-D structure of BC produced by *A. xylinum*. Czaja *et al*., (2006) mentioned that the size of BC fibrils is about 100 times smaller than that of plant cellulose. Furthermore, the BC nanofibres form tutorial nano-pores with the pore size approximately 50-200 nm.

Wanichapichart *et al*., (2003) found that the porosity of BC film from *A. xylinum* TISTR 975 vary from 1.4% to 2.4%, with the average pore size 0.08 μm. Dammstrom *et al*., (2005) studied the morphology of BC fibrils had a sandwich-like, laminar structure consisting of a completely random network of fibrils. The nano-porous of BC allows for the potential transfer of antibiotics or other medicines into wound, while as the same time serving as an efficient physical barrier against any external infection. Klemm *et al*., (2001) studied the treatment of the cellulose pellicle with 0.1 N NaOH, they determined a reduction of impurities and the thickness of fibrils approximately 100 μm. Ross *et al*., (1991) described the production of BC by *A. xylinum* that during the process of actual biosynthesis, various compounds of the nutrition medium are utilized by the bacteria, then polymerized into single, linear β-1-4 glucans chains and finally secreted outside the cells through a linear row of pores located on their outer membrane. The subsequent assembly of the β-1-4 glucans chains outside the cell is a precise, hierarchical process. Initially they form subfibrils (consisting of an 10-15 nascent β-1-4 glucans) then later microfibrils, and finally bundles of a loosely wound riboon, which is comprised of about 1000 individual glucan chain.

However, the appearances of films from various sugars have some differences. The films from mannitol, sucrose and glucose have dense fibrils and the surfaces of fibrils are not smooth. The film from glycerine shows very smooth and clean fibrils and they are not broken. The film from fructose has different size of fibrils. On the top of the film surface it has the big ones but they are small underneath. The *A. xylinum* TISTR 975 cells were not detected since they were dissolved during

washing with strong base. From the SEM monographs, the film from glycerine is interesting compared with others.

3. Crystallinity of bacterial cellulose by X-ray diffractometer (XRD)

Crystallinity of fibrils is one of the characteristic of bacterial cellulose. The BC films obtained from this study have the similar XRD spectra. All films have similar XRD-spectra. Each of them has two diffraction dominant peaks located at A1 and A2. A1 peak located at between 11.03º to 19.38º and A2 peak located at between 19.38º to 25.98º. Mannitol has the highest % crystallinity, following by sucrose, glucose, fructose and glycerine. The results are similar to the research of Czaja *et al*., (2006), they reported that BC has approximately 60 to 80 % crystallinity but in this study, mannitol gave the higher % crystallinity than their report. Keshk and Sameshima, (2005) found that XRD pattern of BC films produced from glucose, fructose, inositol and glycerine have not much different in crystal structure.

Atalla and VanderHart (1984) reported that most native celluloses crystallized as a mixture of two polymorphic forms, termed Iα and Iß. Cellulose Iα predominated in various algal and bacterial cellulose and cellulose Iß predominated in the cellulose of higher plants. Sugiyama *et al*., (1991) confirmed that theoretical models of cellulose Iα and Iß, they indicated different hydrogen-bonding patterns. Electron diffraction patterns show type Iα have a triclinic unit cell and type Iβ have a monoclinic unit cell and cellulose Iβ is more stable than cellulose Iα and the conversion from type Iα to Iβ is irreversible. Barud *et al*., (2007) investigated the composite membranes were prepared from BC and sodium polyphosphate solution, two dominant diffraction peaks at 15° and 22.5° are assigned to the cellulose Iα and Iß

phases ($100_{1\alpha}$, $110_{1ß}$ and $010_{1ß}$ planes at 15° and $110_{1\alpha}$ and $200_{1ß}$ planes at 22.5°). Their patterns were similar to in this study, two dominant diffraction peaks at between 11.03° - 19.38° and 19.38° – 25.98° are assigned to the cellulose Iα and Iß phases.

Magne *et al*., (1947) reported that water molecules in the amorphous region of BC production have a strong relation with the bound water content. Nakamura *et al*., (1981) reported that bound water content decrease with increasing % crystallinity and they indicated that only the amorphous region in cellulose molecules can be regarded as the adsorption site of water molecules. The amorphous index was calculated according to the following 100 - % crystallinity. In this study, mannitol gave the highest % crystallinity (84.38%) and glycerine gave the highest % amorphous (35.16%) which related to their fibrils morphology. The fibrils from mannitol are dense and less porosity when compared with glycerine.

4. Tensile strength test of bacterial cellulose production produced from different carbon sources

Because of one property required of BC production for wound dressing is the ability of high mechanical strength, elasticity and conformability. BC production for wound dressing is essential to adhere to the wound sites very well and its elastic properties allowed an excellence molding to all facial contours, displaying a high degree of adherence even to the moving parts such as eyelids, nose, mouth, elbow, etc. So in this study, the tensile strength of BC production from different carbon sources was evaluated. BC from mannitol gave the highest tensile strength and maximum load but BC from glycerine gave the highest extension at break. To observe that mannitol gave the highest tensile strength and maximum load which relate to the

highest % crystallinity. The results were similar to the study of Krystynowicz *et al*., (2002), they studied the tensile strength and the % crystallinity of BC produced by *A. xylinum* E 25 under stationary condition and horizontal bioreactor. They found that BC from stationary condition gave the highest tensile strength and % crystallinity while BC production from horizontal bioreactor gave the lower tensile strength than under stationary condition and not gave % crystallinity. Wu *et al*., (2004) compared the tensile strength between bacterial cellulose membrane and chitosan, the tensile strength of bacterial cellulose membrane and chitosan are 63 and 34 N because bacterial cellulose membrane forms crystal where intra-molecular and intra-strand hydrogen bonds hold the network (Isogai and Atalla, 1992).

In this study, the maximum load and tensile strength related to % crystallinity and the fibril morphology. Mannitol gave the highest maximum load (56.98 N) and tensile strength (126.63 kN/m^2) which related to its % crystallinity is the highest (84.38%) and its fibrils are dense. Glycerine gave low maximum load (11.31 N) and tensile strength (26.26 kN/m^2) which related to its % crystallinity is the lowest (64.84%) and its fibrils are less dense when compared with mannitol. In this study, BC film from glycerine is interesting because it has the highest extension at break (8.563 mm). So, it is indicated the high elasticity which suitable to develop as wound dressing to use adherence even to the moving parts.

5. The nitrogen adsorption isotherm and pore size distribution of bacterial cellulose films

This study investigated the nitrogen adsorption isotherm and the pore size distribution of BC film from five carbon sources. According to the BET

classification, nitrogen adsorption isotherm of BC films belongs to the isotherm of type II describing the process of physical adsorption of nitrogen (Zhao, 2005). The isotherm of type II, nitrogen gas was multilayer adsorbed when increased the pressure.

In this study, the total pore volume and the pores size distribution of BC films were calculated using BJH method. All BC films from five sugars have the majority pore diameter 6-80 nm. At the pore size 20 to 80 nm, they have the highest pore volume. Among them, the film from glycerine has the highest pore volume 0.06995 ml/g. Cuperus *et al*., (1992) classified the size of pore into three groups, i.e., the micropores volume (<2 nm), the mesopores volume (2 to 50 nm) and the macropore volume (>50 nm). According to their definition, most of pore size of BC films from five sugars can be classified as mesopores and macropores. Saibuatong *et al*., (2007) studied the pores size of BC film produced by *A. xylinum* strain isolated from nata de coco and found that the pore diameters of BC films in the dry and swollen form were 6 nm and 20 nm, respectively. Wanichapichart *et al*., (2002) reported that the average pore size of BC film produced by *A. xylinum* TISTR 975 from coconut juice was 80 nm. Their finding support the result of this study. The SEM images of BC film from previous study also show the diameter of pore size approximately 50 to 200 nm. BC film with highly nano-pore has the high potential carry some drug such as antibiotics or disinfectants for topical wound treatment.

6. Percent of water loss and percent water readsorption of bacterial cellulose wet film

During the cultivation, *A. xylinum* produces cellulose as a high swollen fiber network. The hydrophobicity of the cellulose film is explained by the presence of pore structures and tunnels within the wet film and depends on the extensive interior surface area of the interstitial spaces of the never dry matrix (White and Brown, 1989). BC fibrils are about 100 times smaller than that of plant cellulose (Czaja, *et al*., 2005). This unique nano-morphology results in a large surface area that can hold a large amount of water. The demonstration of the water retention of never dried BC leads to values in the range of 1000% while the water retention values of typical plant cellulose like cotton linters are amounted under comparable conditions to values about 60% (Klemm *et al*., 2001).

When cellulose has the very high amount of water contain, it can adsorb only small amount of water. If such a film is used to absorb exudates or pus from the wound it cannot work efficiently. On the opposite way, when the film is totally dry it cannot swell very well and cannot absorb much water. The results form the study with BC film from glycerine showed that at 91.68% water lose it can reabsorb the highest water (348.92%) compared with lesser or higher % water lose. The results indicated that the highest efficiency of water absorption of BC film occurs when the certain amount of water still remain in the film. When drying the BC at 50^{o}C for 24 h and reswelling with water at 30^{o}C for 1 h, the water retention capacity was drastically decreased and comparable with those of plant cellulose. This result is comparable with that of Klemm *et al*., (2001). The results suggest that when using BC film for exudates absorption purpose it should be get rid water about 90-91% w/w

before use and the dry BC in is not suitable for the wound exudates absorption purpose.

7. The moisture adsorption isotherm of bacterial cellulose

Moisture absorption ability of BC films depends on the amount of water remaining in the film. From preliminary experiment, BC dry film could absorp very small amount of moisture event storage in 97% RH atmosphere for 24 h. The dry film cannot adsorp much water when soaked in the water for hours. It is indicated the dry BC film is not suitable to control moisture balance of the wound and cannot be used as wound exudate adsorption. To use BC film for wound dressing, it should contain suitable amount of water. From the early experiment, it was found that when the wet film was removed 90% of water, it has the highest water readsorption ability. For these reason, the wet film which 90% contained water remove was used to study moisture adsorption isotherm. The moisture adsorption isotherm of all BC films has the same pattern in any % RH atmosphere. The adsorption increase when they stay longer in humidified atmosphere. BC film from glycerine has the highest adsorption isotherm. Dammstrom *et al*., (2005) found that % moisture content of BC film produced by *A. xylinum* subsp *sucrofermentans* BPR 2001 increased when time increased. Their BC films have % moisture content 12.7 at 90% RH but in this study, five BC films have the % moisture content higher than 12.7 at 97% RH. Glycerine gave the highest % moisture content at all % RH when time increased. Valentine, (1958); Nakamura *et al*., (1981) reported that the water molecule are adsorbed around the polar groups in the amorphous regions of the BC. Nakamura *et al*., (1981) reported that bound water content decrease with increasing % crystallinity and they

indicated that only the amorphous region in cellulose molecules can be regarded as the adsorption site of water molecules. Yoshinaga *et al*., (1997) implied that the low water uptake of BC is related to high crystallinity (approximately 70%). From the previous study, glycerine gave the lowest % crystallinity (64.84%) which related to this study. If the BC film from glycerine is used as wound dressing it should protect the wound from drying better than other films and it can balance wound moisture content leading to the fast wound healing.

8 Antimicrobial activity of chlorhexidine digluconate

Chlorhexidine digluconate is a good antiseptic with a broad activity spectrum. It is used as antibacterial and antifungal in human and veterinary. In evaluation of its' antimicrobial activity by agar diffusion method, the paper disks contained chlorhexidine digluconate show inhibition against Gram-positive bacteria i.e., *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *S. epidermidis* ATCC 12228 and Gram-negative bacteria i.e., *E. coli* ATCC 25922 and one yeast i.e., *C. albicans* NCPF 3153. Estrela *et al*., (2003) showed that chlorhexidine digluconate could inhibit *S. aureus* AGPC, *P. aeruginosa* AGNR and *C. albibans* 18, 24 and 16 mm. The means of inhibition zone were similar in this study but the strains were different.

BC films from glycerine were tested for antibacterial activity by agar diffusion method. The chlorhexidine digluconate film showed inhibition activity against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *S. epidermidis* ATCC 12228 and *C. albicans* NCPF 3153. BC chlorhexidine digluconate

film has ability to release chlorhexidine digluconate to inhibit the tested microorganisms and high potential to be developed for topical drug carrier.

The MIC and MBC of chlorhexidine digluconate of tested reference strains are between 2-4 μg/ml except MIC and MBC of *P. aeruginosa* ATCC 27853 are 2 μg/ml and 32 μg/ml, respectively. It is indicated that *P. aeruginosa* ATCC 27853 is more resistance to chlorhexidine digluconate than other tested bacteria. Koljalg *et al*., (2002) studied the chlorhexidine MIC and MBC of Gram negative and Gram-positive bacteria and they found hat MIC and MBC values were wider in Gram-negative bacteria, it varied from 1 to 64 μg/ml but Gram positive bacteria varied from 0.25 to 8 μg/ml.

9. Chlorhexidine digluconate content in the film and chlorhexidine digluconate releasing study

After preparation of BC films containing 0.2% w/w chlorhexidine digluconate, they were assayed for drug content. BC film from glycerine has the highest drug content (87.79%) followed by the films from mannitol (84.79%), fructose (82.56%), glucose (79.57%) and sucrose (76.74%). Some of the drug may loss during application to the films because it may not adsorp on the fibrils or in the pores. During preparation of samples for drug content assay, some of drug in the film may not release into the test solution and it cannot be detected. The film form glycerine has the highest drug content. It may be due to the drug like to absorp to the fibrils from glycerine and the large amount of drug fill in the its' pores. However, the films should be developed for the higher drug content.

In drug releasing study, the releasing of chlorhexidine digluconate from BC films from various sugars has the same pattern. They have the high rate of drug releasing during the first 12 h. After that the rate of drug release was slow. The film from glycerine showed the highest drug release profile compared with others and most of drug content release after 24 h. This film is more suitable than others if they are used to treat the topical wound for microbial infection since it has the best efficiency drug release. In contrast, the film from fructose showed the lowest drug release and drug content, it should not be suggested to be used as topical drug carrier. There are many researches studied the releasing of chlorhexidine digluconate from different films formulation to improve the releasing of drug but not available to study the releasing of chlorhexidine digluconate from BC films. Youn Lee *et al*., (2005) evaluated the suitable of using chitosan, poly (lactide-co-glycolide) (PLGA) and polymethyl methacrylate (PMMA) to control the release of chlorhexidine digluconate from a prototype of controlled release drug for root canal disinfection. They found that non-coated polymer group, the drug released was very fast, and all loaded chlorhexidine digluconate was released within 2 h.