# CHAPTER IV
# RESULTS AND DISCUSSION

4.1 Preparation of Bacterial Cellulose Containing Sericin

Bacterial cellulose pellicles were synthesized by *Acetobacter xylinum* in culture medium which kept under static incubation at 30 °C for 4 days. During static incubation, *Acetobacter xylinum*, aerobic bacteria, synthesized cellulose on the oxygen-rich surface of culture medium in the form of pellicle. *Acetobacter xylinum* synthesized glucan chains as a linear row at the pores on the surface of bacterium. Then, glucan chains were assembled to cellulose subfibrils and crystallized to microfibrills and then tightly assembled to ribbon. During bacteria growth and bacterial cellulose production, bacterial cells were distributed and entrapped in the network of cellulose pellicles. Therefore, the advantage of cellulose production is to maintain these aerobic bacteria close to surface of culture solution. Thus, bacterial cellulose was produced on the oxygen-rich air-liquid interface of culture medium as shown in Figure 4.1. After that, purification step, the embedded bacterial cells and culture medium were removed. The obtained gelatinous like translucent pellicle is shown in Figure 4.2 (a). Silk sericin was extracted from yellow silk cocoons by boiling it in distilled water under pressure in an autoclave. After that, silk cocoons were filter out to obtain sericin solution with bright yellowish color. Figure 4.2 (b) and (c) illustrates the incorporation of sericin solution into bacterial cellulose pellicle which represented by bright yellowish color from silk sericin instead of the colorless of the original bacterial cellulose. In addition, the bright yellowish color seemed to homogeneously distribute in bacterial cellulose pellicle. Due to the hydrophilicity of both sericin and bacterial cellulose, the interaction between these two polymers should occur via the hydrogen bonding.
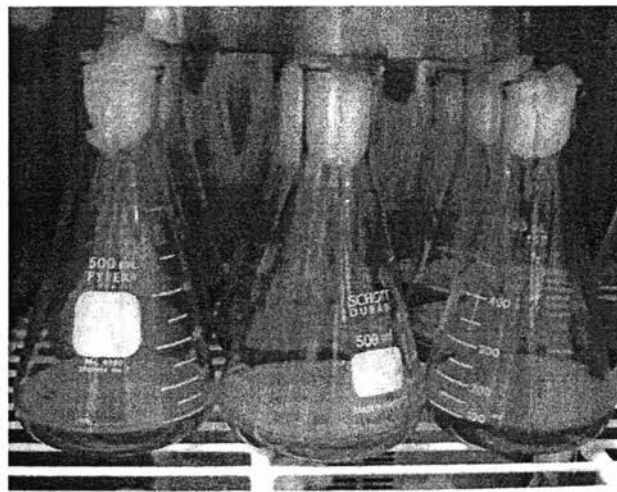
Figure 4.1 Bacterial cellulose pellicle appear on surface of culture medium.
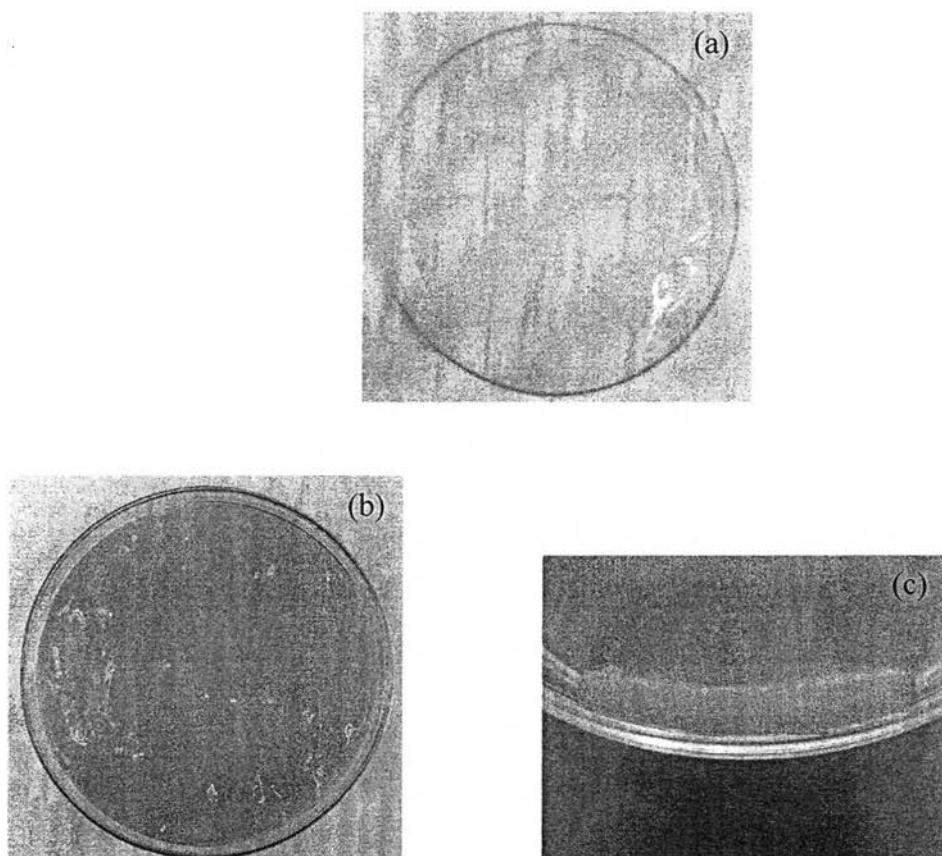
Figure 4.2 Appearances of (a) pure bacterial cellulose, (b) surface of bacterial cellulose containing sericin and (c) inside of bacterial cellulose containing sericin.

4.2 Extraction Time of Sericin

In general, sericin peptides are soluble in hot water. Therefore, the sericin was generally extracted by boiling silk cocoons in distilled water. In this study, the effect of extraction time on the yield of sericin was examined by measured the solid content of the extracted-sericin (1 g of silk cocoon: 10 ml of distilled water) in an autoclave at 121 °C at different time. Figure 4.3 shows the effect of extraction time on the solid content of sericin from 15 min to 4 h. Solid content of sericin increased with increasing extraction time and the solid content of sericin became stable at 2 h. The solid content of extracted-sericin was significantly increased with increasing the extraction time from 15 min to 30 min. However, the solid content of extracted-sericin was tended to reach equilibrium with further increasing the extraction time from 1 h to 4 h. Anyways, the sericin was mainly composed of many kinds of protein. In order to eliminate the effect of heat treatment on the chemical structure of sericin, the suitable condition for extraction sericin from silk cocoon was 30 min.
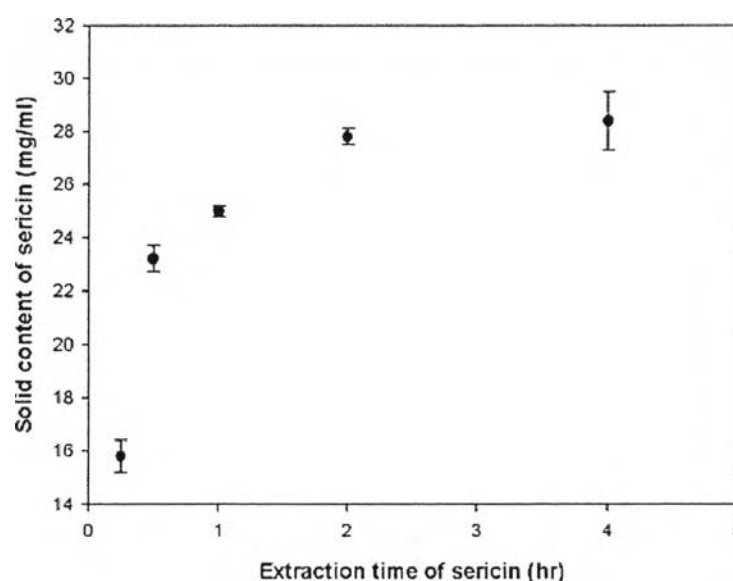
Figure 4.3 The effect of extraction time on the solid content of sericin.

**Table 4.1** The effect of extraction time of sericin by autoclaving method

Autoclave time (hr)	Solid content of sericin (mg/ml)	Percent increase (%)
0.25	15.8	-
0.5	23.2	46.8
1	25.0	58.2
2	27.8	75.9
4	28.4	79.7

4.3 Chemical Characterization of Bacterial Cellulose Containing Sericin

The FT-IR spectroscopy was used to identify the functional groups of the materials. The FTIR spectrum of bacterial cellulose, sericin and sericin incorporated-bacterial cellulose is showed in figure 4.4, 4.5 and 4.6, respectively. The FT-IR spectra of the bacterial cellulose shows a broad band at 3448 cm^{-1} which is attributed to OH-band. The characteristics bands at 2910 and 1060 cm^{-1} represent the C-H stretching and C-O-C stretching of ether linkage, respectively (Zhijiang and Guang, 2011). For the FT-IR spectra of sericin, a broad band located at 3433 cm^{-1} is assigned to the NH stretching and OH stretching band. The amino groups of sericin show the adsorption peaks at 1664 cm^{-1}, 1520 cm^{-1}, 1233 cm^{-1}, and 636 cm^{-1}, which corresponded to amide I (C=O stretching), amide II (out of phase N-H stretching, C-N stretching), amide III (in phase N-H stretching, C-N stretching), and amide V (out of plane N-H bending), respectively (Kong and Yu, 2007). In the case of bacterial cellulose containing sericin, the FT-IR spectrum exhibits the band of hydroxyl group and ether linkage from bacterial cellulose together with the amide band of sericin. In addition, the intensity of amide band tended to increase with increasing sericin concentration. Therefore, all of these peaks indicated the incorporation of sericin into bacterial cellulose pellicles.
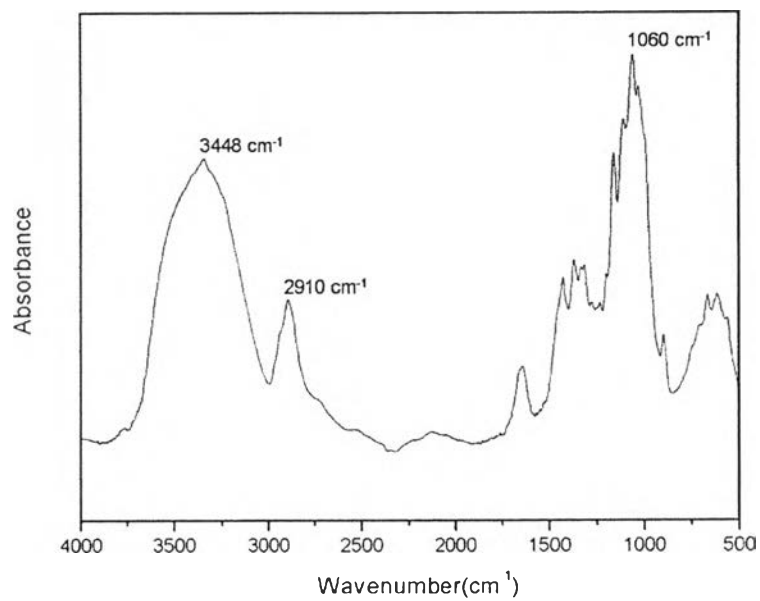
Figure 4.4 FTIR spectra of pure bacterial cellulose.
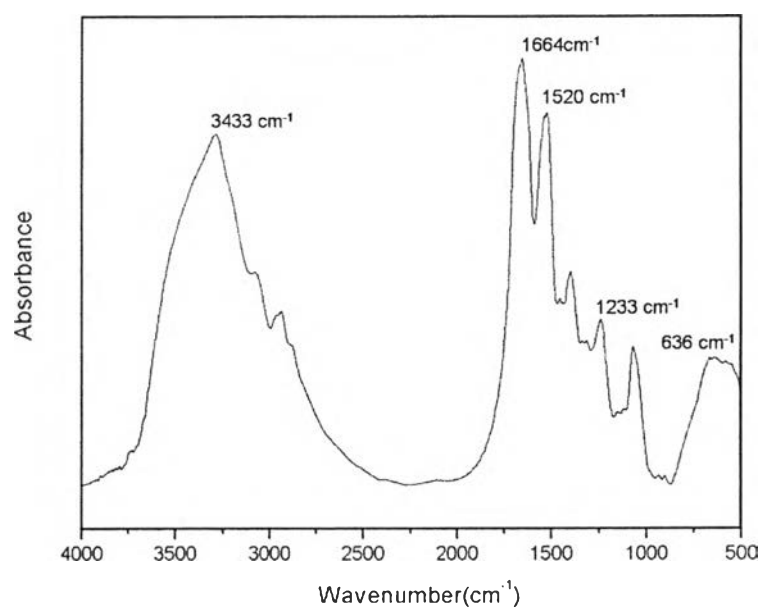

Figure 4.5 FTIR spectra of sericin.
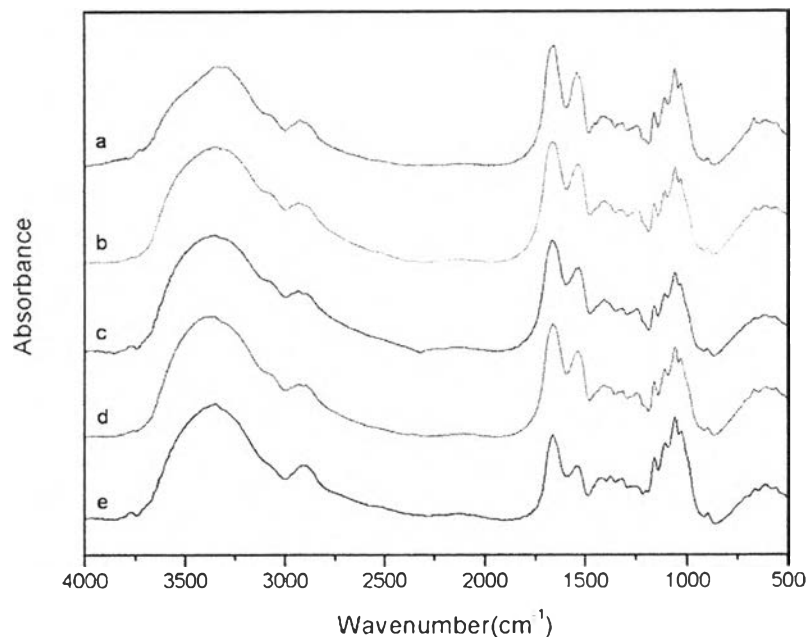
Figure 4.6 FTIR spectra of (a) BC/ sericin 50 mg/ml, (b) BC/ sericin 40 mg/ml, (c) BC/ sericin 30 mg/ml, (d) BC/ sericin 20 mg/ml, and (e) BC/ sericin 10 mg/ml.
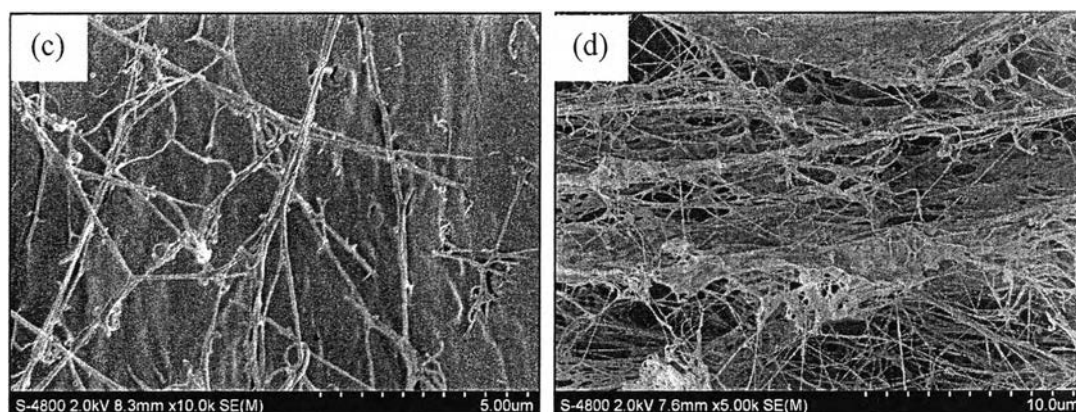
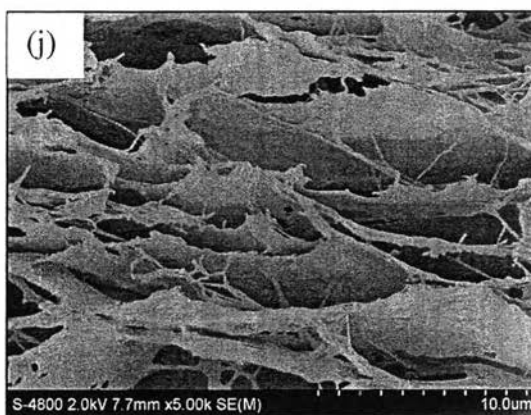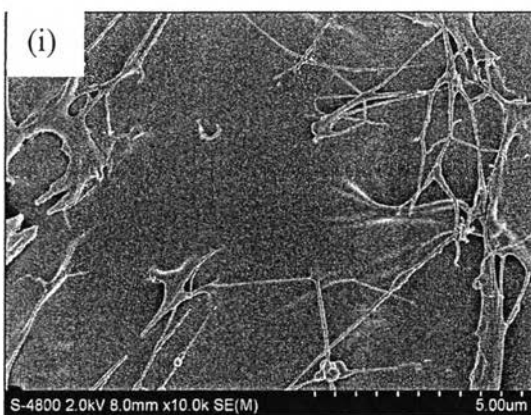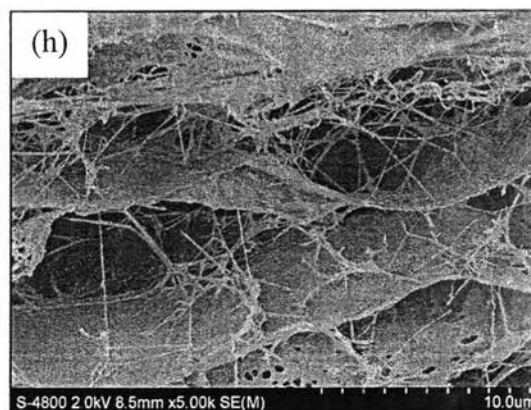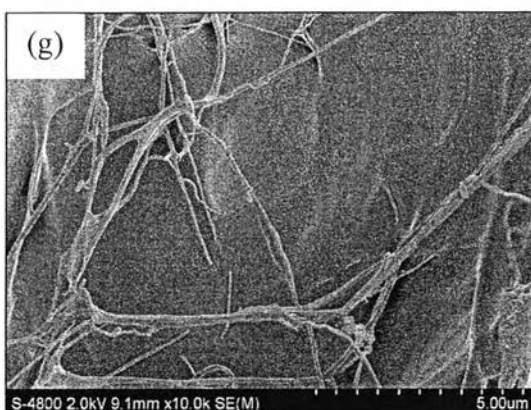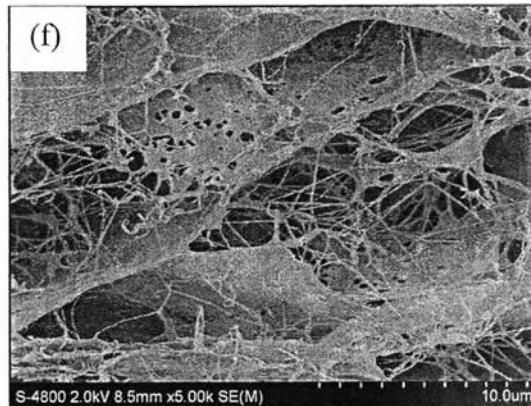## 4.4 Morphology of Bacterial Cellulose Containing Sericin

The SEM technique was used to analyze the surface and cross-sectional morphology of the freeze-dried bacterial cellulose samples. Figures 4.7a and 4.7b illustrate the morphology of pure bacterial cellulose. Obviously, the surface morphology of bacterial cellulose showed the random assembly of ultrafine nanofibrills in a three dimensional network structure consisting of porous morphology. The fibers diameters were around 30 nm to 40 nm and the porous size ranged from 2 μm to 5 μm. The cross sectional morphology showed the interconnection of fibrils linked between the multilayers of cellulose network structure. These unique structures resulted in a large surface area contributed to a good potential for using as a wound dressing, such as high exudate absorption, elasticity, and wet strength (Czaja *et al.*, 2006). The porous structure also allows drug loading or encapsulating of other compounds into this porous structure.

After the incorporation of sericin into bacterial cellulose pellicle, sericin solution filled the pores in bacterial cellulose pellicle, as shown in Figures 4.8c to 4.8l. The incorporated sericin was homogeneously distributed in the bacterial cellulose matrix due to the hydrophilic nature of both materials. Due to the high polar functional groups of bacterial cellulose and sericin, these two components were bonded together by hydrogen bonding.
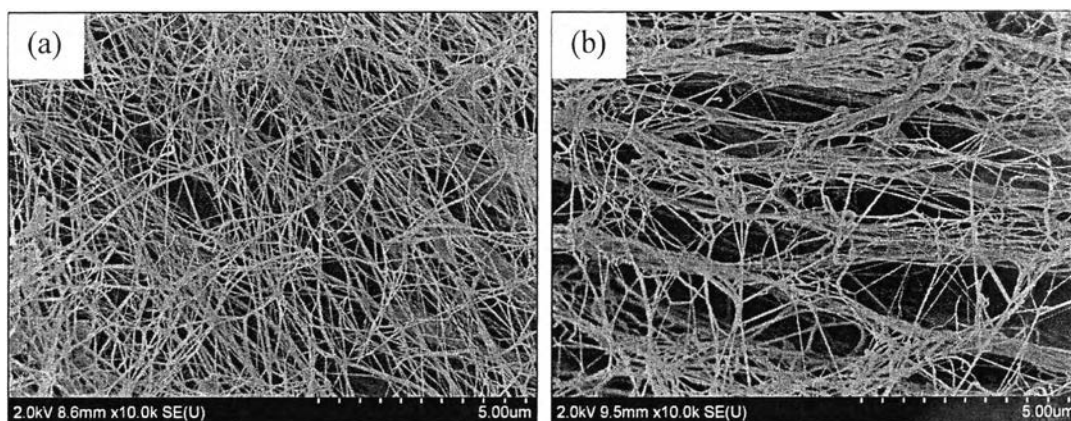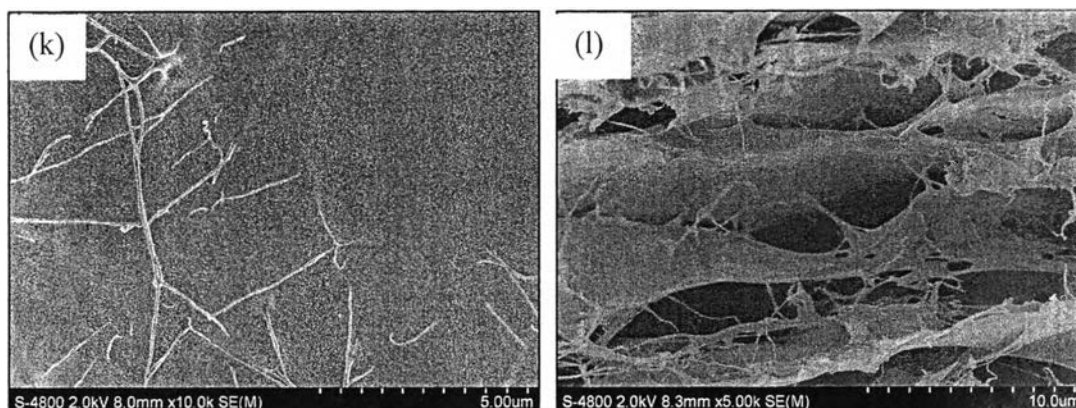

Figure 4.7 SEM images of (a) surface (left) and (b) cross section (right) of pure bacterial cellulose at a magnification of 10,000.
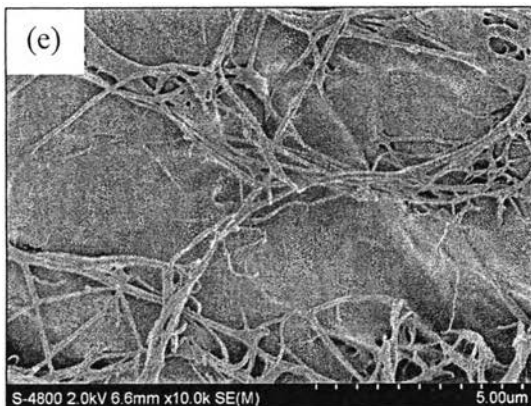
Figure 4.8 SEM images of surface (left) (× 10,000) and cross section (right)

(× 5,000) of bacterial cellulose containing sericin at the different sericin

concentration: (c, d) 10 mg/ml (e, f) 20 mg/ml (g, h) 30 mg/ml (i, j) 40 mg/ml and (k,

l) 50 mg/ml respectively.

4.5 X-Ray Diffraction Analysis (XRD)

The x-ray diffraction analysis was used to identify the crystalline structure of bacterial cellulose, bacterial cellulose containing different sericin concentrations of 10 mg/ml, 30 mg/ml, 50 mg/ml (after the removal of sericin from bacterial cellulose containing sericin samples by boiling with distilled water), and pure sericin samples. Figure 4.9a shows the three main peaks 2θ of bacterial cellulose at $14.52°$, $16.96°$ and $22.76°$ which are assigned to the $(1\overline{1}0)$, (110) and (200) planes of cellulose I (Tokoh *et al.,* 1998). For pure sericin, represented the broad diffraction peaks and the main peak is around $21.26°$. This pattern indicated that sericin is mainly amorphous and contains few of β structure (Tao *et al.,* 2005). For the removal of sericin from bacterial cellulose containing sericin samples, the position and intensity of diffraction peaks remain the same as pure bacterial cellulose sample (Figure 4.9b, c, and d). These results indicated that the crystal structure of bacterial cellulose remain unchanged when sericin was incorporated in bacterial cellulose (Baklagina *et al.,* 2005). This suggested that sericin is only coated around bacterial cellulose fibers not present inside the bacterial cellulose fibers.
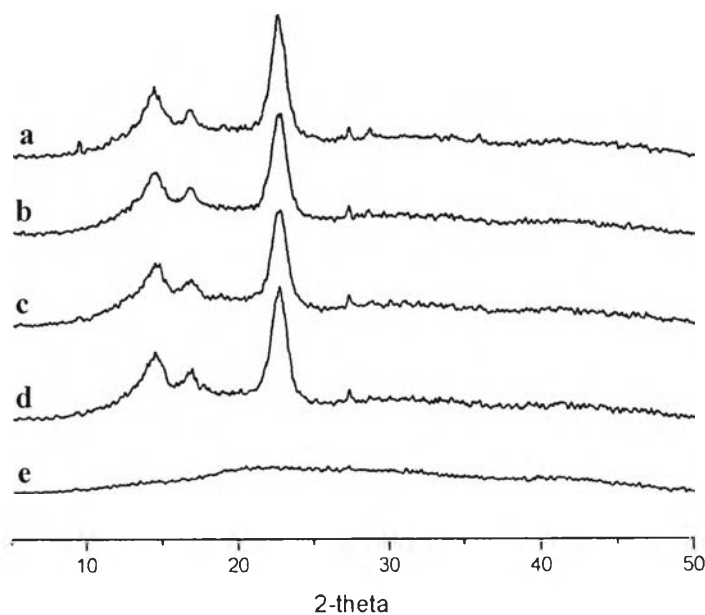
Figure 4.9 X-ray diffraction spectra of (a) pure bacterial cellulose, (b-d) bacterial cellulose containing different sericin concentrations of 10 mg/ml, 30 mg/ml, and 50 mg/ml after the removal of sericin from bacterial cellulose containing sericin sample by boiling with distilled water for 48 h respectively, and (e) pure sericin.

4.6 Sericin Content Determination

The amount of sericin in bacterial cellulose pellicle was determined by Kjeldahl nitrogen analysis because bacterial cellulose does not contain nitrogen whereas nitrogen is presented only in sericin component. The determined amount of nitrogen of sericin in bacterial cellulose is showed in Figure 4.10. After the incorporation of sericin into bacterial cellulose pellicle, the amount of nitrogen content increased with increasing a sericin concentration from 10 to 50 mg/ml, as shown in Figure 4.10. The successfully incorporation of sericin into bacterial cellulose was confirmed by the presence of nitrogen element in the as-prepared bacterial cellulose sample. The nitrogen content of the as-prepared sample was increased with the increasing of sericin concentration. The increasing in nitrogen content of the as-prepared samples with increasing of the concentration of sericin suggested the presence of an increasing amount of sericin in bacterial cellulose pellicle when higher sericin concentrations were used.
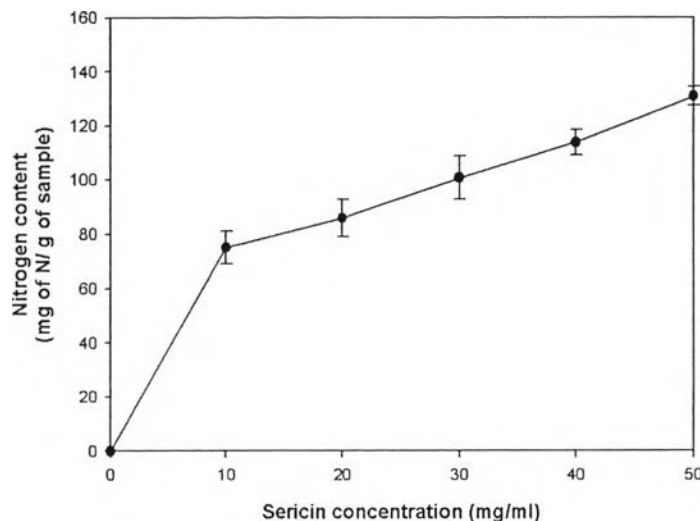
Figure 4.10 Nitrogen content of different sericin concentration in bacterial cellulose.

4.7 Swelling Behavior of Bacterial Cellulose Containing Sericin

Water absorption ability plays an important role in wound dressing application. The capacity of absorbing wound exudates and maintaining moist environment at wound surface are important factors in wound healing process. From figure 4.11, after immersing of freeze-dried bacterial cellulose sample in distilled water, a high water absorption capacity was observed due to the hydrophilicity and porous structure of nanofibril network structure of freeze-dried bacterial cellulose pellicles. The microporous structure of bacterial cellulose generated the capillary force which enhancing the water absorption capacity of the as-prepared sample. Moreover, the incorporation of sericin into bacterial cellulose pellicles were enhanced the water absorption capacity of the as-prepared sample as evidence by the higher water absorption capacity of bacterial cellulose containing sericin sample in comparing with pure bacterial cellulose. The water absorption capacity of the as-prepared bacterial cellulose containing sericin sample was firstly increased with increasing sericin concentration to 30 mg/ml. Then, the water absorption capacity of the as-prepared bacterial cellulose containing sericin sample was tended to decrease with further increasing of the sericin concentration to 40 and 50 mg/ml. The firstly increased in the water absorption capacity might result from the hydrophilicity of

sericin (hydroxyl, carboxyl and amino groups) together with bacterial cellulose. Whereas, the decreased in the water absorption capacity with further increasing of the sericin concentration might result from the tightly packing of the sericin to the bacterial cellulose which restricted the absorption ability.
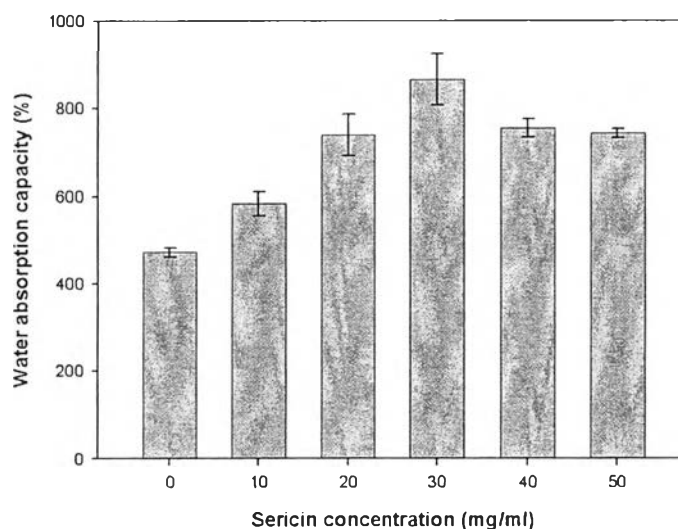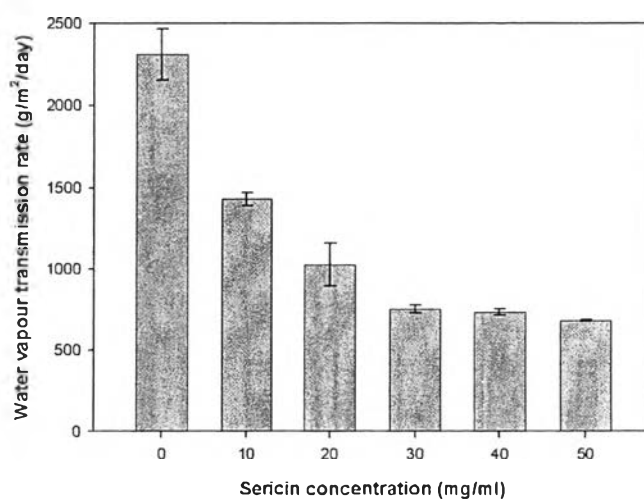

Figure 4.11 Water absorption capacity of pure bacterial cellulose and bacterial cellulose containing sericin.

4.8 Water Vapor Transmission Rate

Water vapor transmission rate (WVTR) of wound dressing should be controlled at an appropriate rate. If the WVTR is too high, this causes excessive dehydration which will create dry condition around wound area and produces scar formation. In contrast, if the WVTR is too low, this may lead to the delay of the healing process and the increasing of bacterial growth due to the accumulation of exudates (Guptar, 2010). Normally, the WVTR of normal skin is 204.0 $g/m^2/day$. The WVTR value of pure bacterial cellulose is 2310.54 $g/m^2/day$. The incorporation of higher sericin content show lower WVTR. The WVTR values of bacterial cellulose pellicles immersing in sericin solutions having concentrations varied from 10 to 50 mg/ml are 1430.07, 1025.41, 754.01, 734.17, and 683.43 $g/m^2/day$, respectively, as shown in Figure 4.12. The larger pore size of pure bacterial cellulose
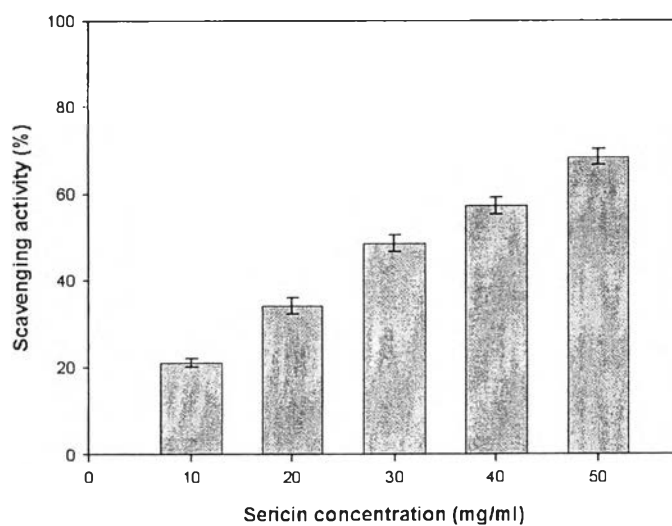
than bacterial cellulose containing sericin induced water vapor transmission easier resulting in higher WVTR. Due to the hydrophilicity of sericin, bacterial cellulose containing sericin could maintain moisture around wound surface resulting in lower WVTR. The WVTR values of bacterial cellulose containing sericin are in the range of the WVTR of commercial wound dressings such as Biofilm®, Comfeel®, Dermiflex®, Granuflex®, and etc. (Wu *et al.*, 1995). The evaporative water loss rate for injured skin can range from 800-1300 $g/m^2/day$ (Lou, 2008). The WVTR for first degree burn is 278.4 $g/m^2/day$, for second degree burn is 4274.4 $g/m^2/day$, and for third degree burn is 3436.8 $g/m^2/day$ (Witthayaprapakorn, 2011). Another study evaluated that WVTR values of dressing may predict rate of healing. Normally, wound dressing material having WVTR less than 840 $g/m^2/day$ defined as moisture retentive and encouraged in a rapid healing. The effects of moisture on epidermal regeneration include enhanced keratinocyte migration, proliferation, and differentiation. In addition, moisture promotes fibroblast proliferation, collagen synthesis, endothelial cell proliferation, new vessel formation (angiogenesis), and wound contraction. The effects of moisture on scar maturation (remodeling) include scar elevation, color, and inflammation (Brett, 2006).


Figure 4.12 Water vapor transmission rate of pure bacterial cellulose and bacterial cellulose containing sericin.

4.9 Antioxidant Activity of Bacterial Cellulose Containing Sericin

In the presence of reactive oxygen species (ROS), e.g. hydroxyl and peroxyl radicals which are generated during neutrophil accumulation in the inflammatory stage of wound healing process, causing delay in wound healing process since these compounds are unstable and reactive with substances in body by chain reaction, resulting in cell and tissue injury (Ilango and Chitra, 2010). Therefore, the antioxidant agent plays an important role in the treatment of wounds (Bharati et al., 2010). DPPH (2,2-diphenyl-1-picryhydrazyl) is a stable nitrogen-centered free radical having a purple colour which accepts an electron or hydrogen radical and converts to a stable diamagnetic molecules, displaying yellow colour of diphenyl picryl hydrazine. DPPH radicals react with suitable reducing agents so the electrons become pair off to form the corresponding hydrazine. As a result, the solution changes colour stoichiometrically with the number of electrons consumed (Halliwell, 1991). The scavenging activity of silk sericin was performed with DPPH method. From figure 4.13, the results show that the sericin has good antioxidant potential and the scavenging activity of sericin in bacterial cellulose pellicle increases with increasing sericin concentrations. Chen et al. (1996) claimed that Val or Leu at the N terminus end and Pro, His and Tyr in the sequences have been recognized as antioxidative peptide. In addition, sericin contains aromatic side groups such as His, Trp, and Tyr that have ability to donate hydrogen and stabilized free radicals by resonance delocalization to form stable products (Ajibola, et al., 2011), all of these amino acids are the composition of sericin protein. Moreover, serine and threonine composed mainly in sericin composition and the hydroxyl group of serine and threonine can be formed chelating trace elements, such as copper and iron, that is also responsible for the antioxidant action (Kato et al., 1998) because iron induced the production of reactive oxygen species via the Fenton reaction. Therefore, sericin was acted as the naturally effective antioxidant which was enhanced the wound healing process.

Figure 4.13 Antioxidant activity of bacterial cellulose containing sericin.

Tyrosine

Tryptophan

Histidine
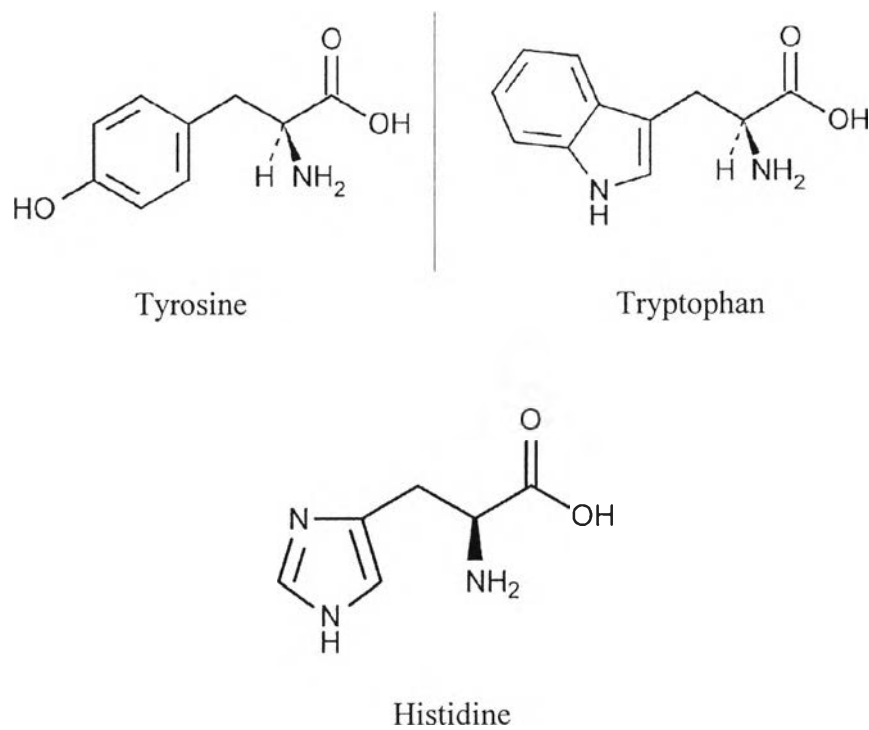
Figure 4.14 Aromatic amino acids.

4.10 Sericin Releasing Behavior

The amount of sericin released from bacterial cellulose containing sericin sample was determined by immersing the sample in a tris-HCl buffer solution. The hydrophilicity and amorphous structure of sericin contributed to the releasing of sericin into a tris-HCl buffer solution. In addition, the interaction between bacterial cellulose and sericin would be hydrogen bonding. Therefore, sericin released easily in water. Figure 4.15 demonstrates the releasing profile of sericin from the bacterial cellulose containing sericin pellicle. According to the releasing profile of sericin from bacterial cellulose pellicles released rapidly during 0 h to 12 h. After that, the releasing amounts of sericin became stable. The bacterial cellulose pellicle is still stable after immersing in a tris-HCl buffer solution for 72 h. The three dimensional network structures of ultrafine nanofibrills contribute to the good stability during immersing in water. Moreover, a higher concentration of sericin exhibited a higher amount of released sericin from the as-prepared pellicle. The amount of released sericin was crucial in terms of enhancing antioxidant capacity and promoting wound healing process. On the other hand, sericin remaining in bacterial cellulose pellicles was also beneficial because sericin can provide moisture and promote collagen production in wound area. Due to all of these properties, bacterial cellulose containing sericin should be useful and suitable for wound dressing application.
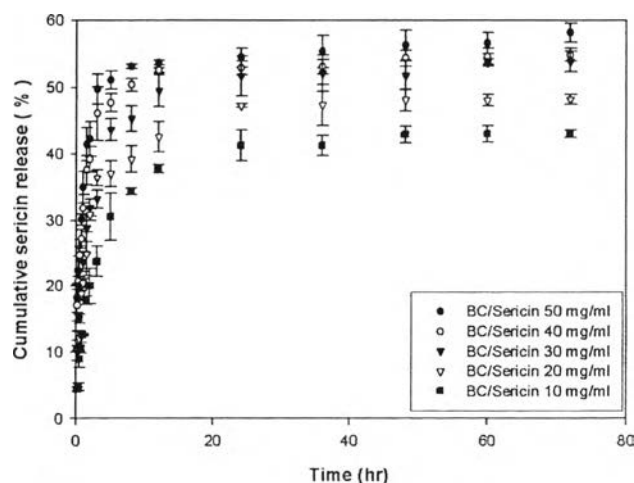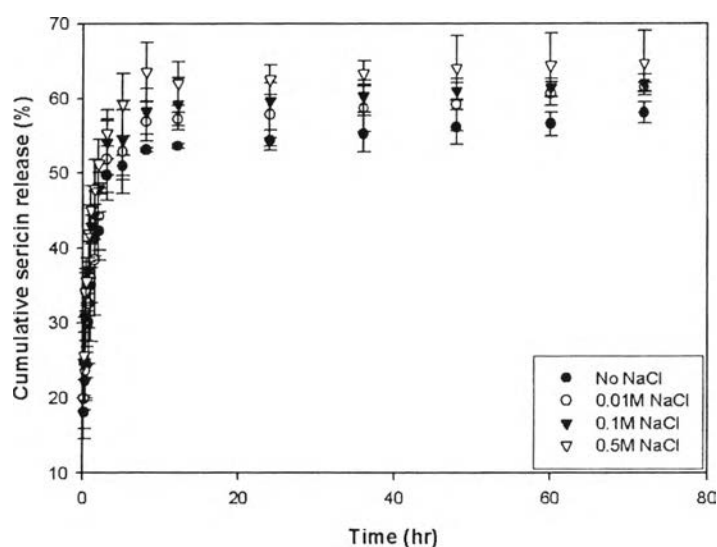

Figure 4.15 Sericin releasing profile of BC containing sericin in tris HCl.

4.11 The Effect of NaCl on Sericin Releasing Behavior

From the figure 4.16 illustrates the effect of NaCl on the releasing profile of sericin from the bacterial cellulose containing sericin. In the presence of NaCl in tris HCl buffer solution, NaCl could enhance sericin releasing resulting in a higher amount of sericin released. Due to the presence of sodium ion in buffer, could penetrate and break hydrogen bonding between bacterial cellulose and sericin because the ionic strength from sodium ion is stronger than the hydrogen bonding between bacterial cellulose and sericin. This might be implying that bacterial cellulose form hydrogen bonding with sericin.
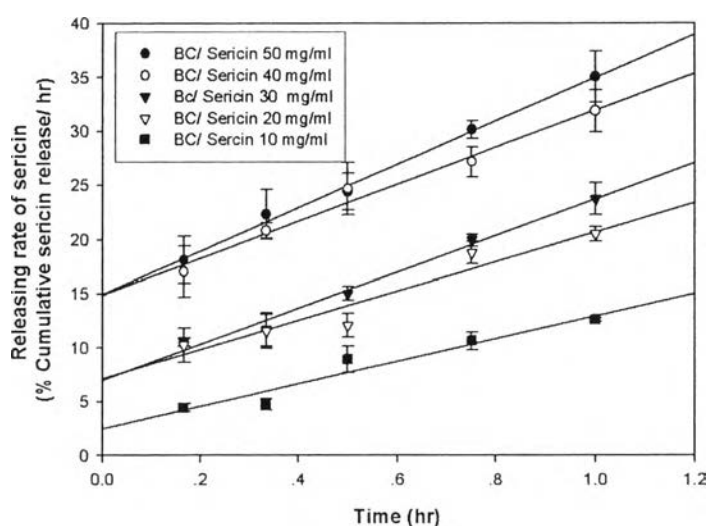

Figure 4.16 Sericin releasing profile of bacterial cellulose containing sericin in tris HCl containing NaCl.

4.12 Releasing Rate of Sericin

The releasing rate of sericin from the as-prepared bacterial cellulose containing sericin sample was determined by linear regression analysis. The zero order rates constant was obtained from the slope of straight line describing the sericin releasing rate which related with the concentration of sericin. As shown in

figure 4.17, the amount of releasing sericin at various concentration of sericin from 10 to 50 mg/ml was 10.50%, 13.55%, 16.73%, 17.05%, and 20.01 % cumulative sericin release/hr and the correlation coefficient (R^2) was 0.939, 0.927, 0.987, 0.981, and 0.995 respectively. The releasing rate of sericin increased with increasing sericin concentration. The higher sericin concentration exhibit more rapidly released due to the concentration of sericin much different with tris-HCl buffer solution.


Figure 4.17 Sericin releasing rate of bacterial cellulose containing sericin.

4.13 Mechanism of Sericin Release

The mechanism of sericin released was investigated by Korsmeyer-Peppus model. The relationship between the amount of sericin released at time t (M_t) and the amount of sericin release at time infinite (M_∞) can be expressed by Korsmeyer-Peppus equation, as follows: $M_t/ M_\infty = kt^n$. The n value is a diffusion release exponent which indicating of the releasing mechanism. The releasing mechanism can be defined as Fickian, non-Fickian (anomalous), linear (zero order), and super case II transport when n is equal to 0.5, 0.5<n<1.0, n=1 and n>1, respectively (Korsmeyer, 1983). From the plotted between $\ln(M_t /M_\infty)$ and ln time for determined the n values, The n values of bacterial cellulose immersing in sericin solutions

having concentrations varied from 10 to 50 mg/ml were 0.640, 0.481, 0.458, 0.351, 0.365 and the correlation coefficient (R^2) was 0.961, 0.951, 0.962, 0.994, 0.986 respectively. The n values of bacterial cellulose immersing in sericin solutions having concentrations varied from 20 to 50 mg/ml were identified as Fickian diffusion mechanism which is diffusion controlled release. At high sericin concentration exhibit the diffusion controlled release because the much difference in sericin concentration between sericin in the as-prepared sample and in tris-HCl buffer solution. Therefore, the as-prepared sample with high sericin incorporation exhibites the rapidly release of sericin into the tris-HCl buffer medium. However, the n value of as-prepared sample which prepared by using sericin concentration of 10 mg/ml was in the case of Anomalous transport which is the superposition of both phenomena (diffusion controlled and swelling controlled release). This may be due to the amount of sericin incorporation in sample prepared by using sericin concentration of 10 mg/ml is quite low, so the releasing mechanism cannot occur only through the diffusion mechanism by the difference in concentration gradient of sericin between inside and outside of the as-prepared bacterial cellulose containing sericin sample. Thus, the releasing mechanism of the as-prepared sample which prepared by using sericin concentration of 10 mg/ml was controlled by using both and diffusion controlled and swelling controlled release.
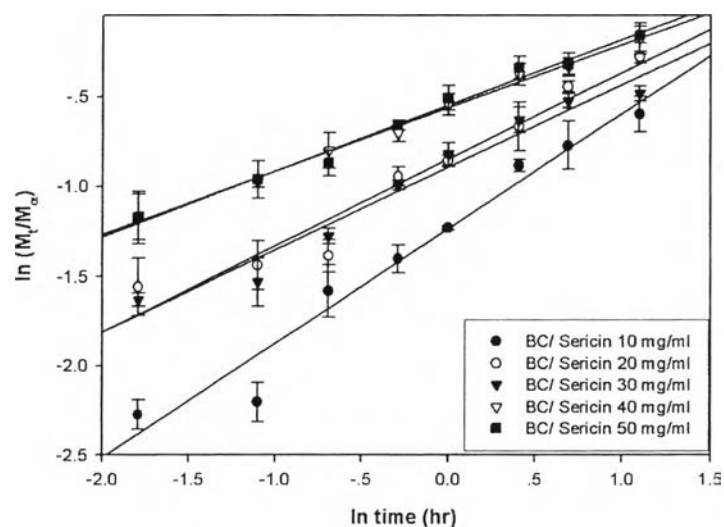
**Figure 4.18** Korsmeyer-Peppus model of mechanism of sericin release.

4.14 Cell Study

The biocompatibility is one of the important factors during wound repairing. MTT assay is colorimetric assay for measuring the activity of enzymes from metabolism of cell. The dehydrogenase enzymes, which secreting from the mitochondria of metabolically active cells, reduced the water soluble yellow tetrazolium salt to insoluble purple formazan crystals. The amount of these crystals can be determined by spectrophotometrically and serves as an estimate for the number of living cells (Twentyman and Luscombe, 1987). The indication of toxicity has been evaluated by monitoring the percent of cell viability after 24 hours of cultivation. At percent of cell viability exceeded 50%, the material was evaluated as a non toxicity. The cytotoxicity results of the pure bacterial cellulose, bacterial cellulose containing sericin 30 and 50 mg/ml are demonstrated in figure 4.19 which was evaluated for no toxicity to human dermal fibroblast cell lines.
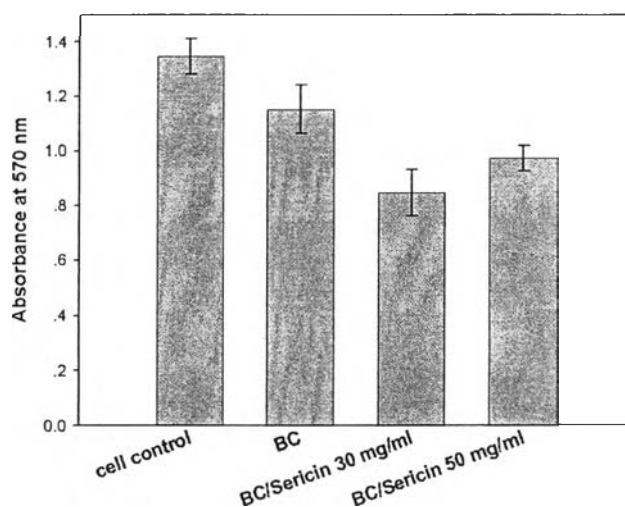

Figure 4.19 MTT assay of cell viability of human dermal fibroblast cell lines.

Figure 4.20 shows the morphology of cell growth for (a) BC, (b) BC/ Sericin 30 mg/ml, and (c) BC/Sericin 50 mg/ml after 24 hours of cultivation. The human dermal fibroblast cells were able to grow and spread on all samples. In addition, SEM observation revealed the elongate morphology of human dermal fibroblast cells growing on the all samples. This observation was similar to the study

of human dermal fibroblast cell on the collagen gel which showed the elongate cell shape. The formation of elongate cell shape is the organization of a cytoskeleton as shown in figure 4.21b (Nishiyama *et al.*, 1993). Whereas, Meyer *et al.* (2011) reported the effect of zinc oxide nanoparticles (ZnO NPs) on the induction of apoptosis in human dermal fibroblast cell. MTT results showed a significant decrease in the number of living cells after ZnO NPs exposure, and phase contrast image illustrated that the cells which treated with zinc oxide nanoparticles had a rounded morphology as shown in figure 4.21a. These evidences suggested that pure BC, BC containing sericin 30 and 50 mg/ml were non toxic, cytocompatible, and suitable for the growth of human dermal fibroblast cells. The results indicated that all samples have potentials for using as wound dressing materials.
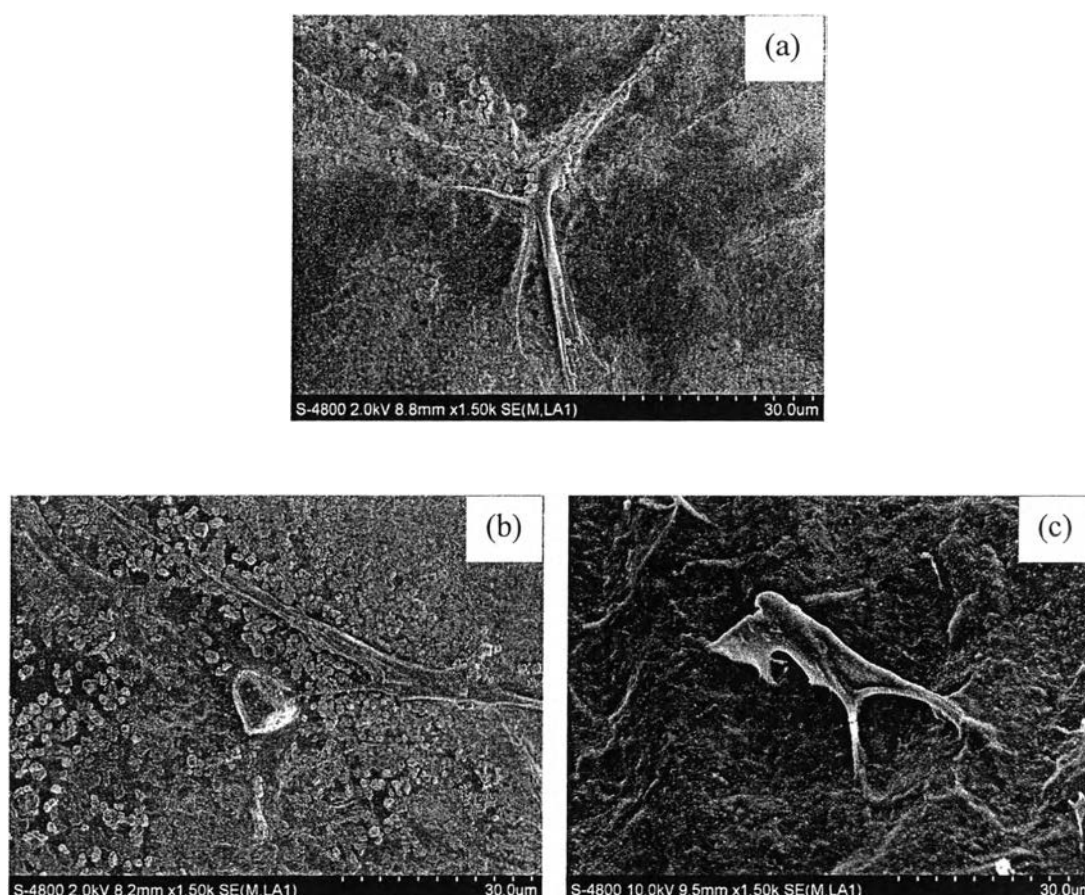
Figure 4.20 Morphology of cell growth (× 1,500) for (a) BC, (b) BC/Sericin30 mg/ml, and (c) BC/Sericin 50 mg/ml at 24 hours of cultivation.
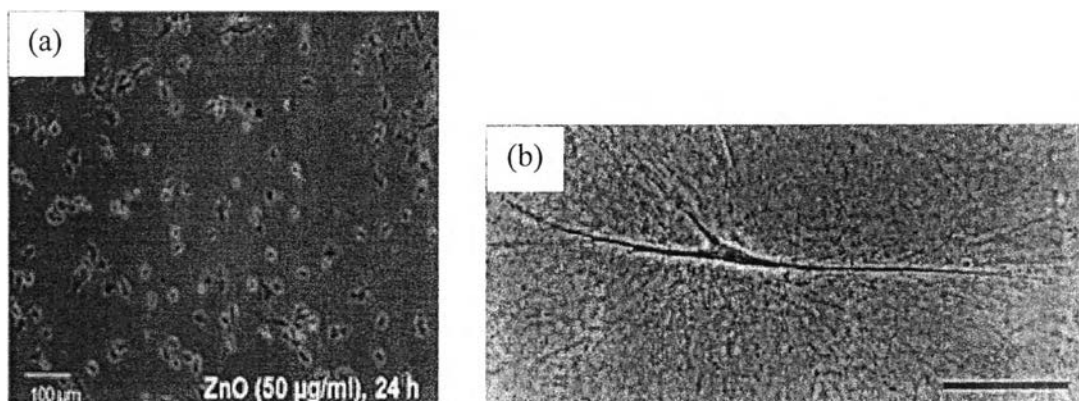
Figure 4.21 Phase contrast microscope of cell growth on (a) zinc oxide nanoparticles (50 µg/ml) for 24 h, and (b) collagen gel with a low dose of cytochalasin D (0.2 µM) for 15 h.