CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation of Bacterial Cellulose from *A. xylinum* Fermentation

After four days cultivation of *A. xylinum* strain TISTR 975, the yellow-translucent bacterial cellulose pellicle at the interface of culture medium was collected. In purification step, the bacterial pellicles were boiled in 1 %(w/v) sodium hydroxide (NaOH) in order to remove bacterial cells, protein, and the component of the culture medium which entrapped within the bacterial cellulose network. Figure 4.1 shows the yellow-translucent of NaOH untreated-bacterial cellulose pellicle became a white-translucent of bacterial cellulose pellicle after purification step.

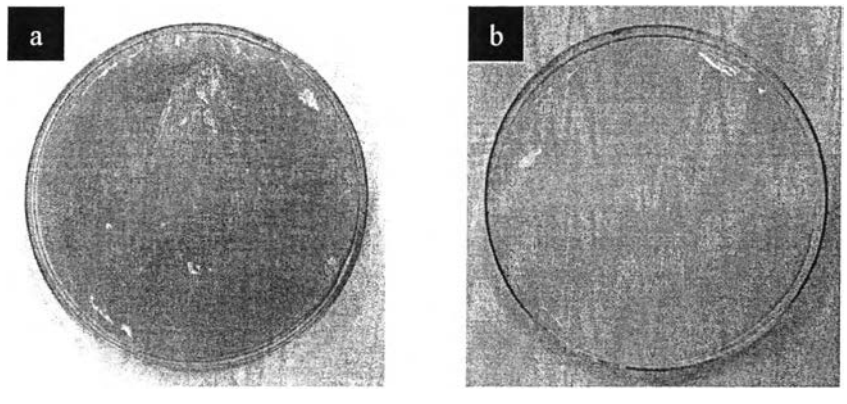


Figure 4.1 Appearance of bacterial cellulose: (a) NaOH untreated-bacterial cellulose pellicle (b) NaOH treated-bacterial cellulose pellicle.

4.2 Preparation of Chitosan and Silk Sericin-incorporated Bacterial Cellulose

Chitosan and sericin blend solution color is yellow color. After incorporation of chitosan and sericin blend solution in to bacterial cellulose, the color of bacterial cellulose pellicle was changed into yellow color. Acetic acid, used to dissolve chitosan, will be evaporated by autoclave process at 120 °C. Because of the melting point of acetic acid is 118.1 °C which less than the temperature of incorporated

process. This method could confirm that acetic acid was not remained in as-prepared sample and do not adversely affect to the skin when used.

4.3 Chemical Analysis

4.3.1 Chemical Structure of Bacterial Cellulose

The chemical structures of the bacterial cellulose, bacterial cellulose containing chitosan and sericin were investigated with the using of the FT-IR spectroscopy. As shown in Figure 4.2, the FT-IR spectra of bacterial cellulose show a broad band of the OH-stretching at 3343 cm^{-1}. The characteristics bands at 2900 and 1014 cm^{-1} represent the C-H stretching and C-O-C stretching of ether linkage, respectively. This result shows that the produced bacterial cellulose has the same chemical structure as β-1,4-glucan chain, as shown in Figure 4.3.

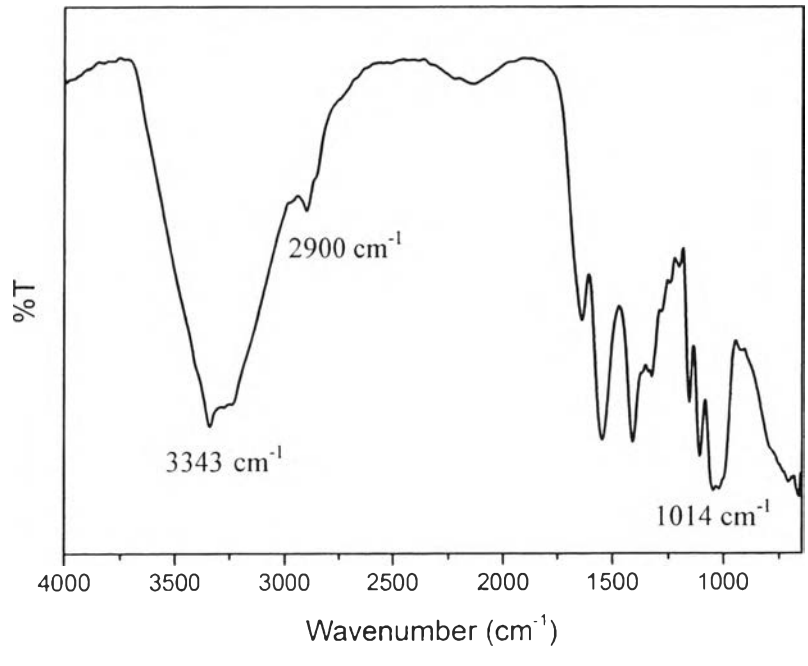


Figure 4.2 FT-IR spectra of pure bacterial cellulose.

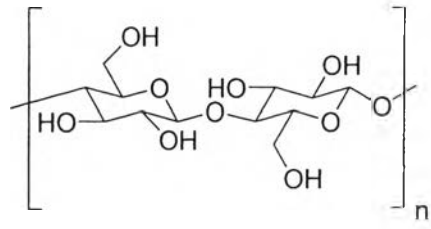


Figure 4.3 Chemical structure of cellulose.

4.3.2 Chemical Structure of Chitosan, Sericin and Chitosan and Silk Sericin-incorporated Bacterial Cellulose

The chemical structures of the chitosan, sericin, and chitosan and silk sericin-incorporated bacterial cellulose, as showed in Figure 4.4, 4.5, and 4.6, respectively, were investigated with the use of the FT-IR spectroscopy. For the FT-IR spectra of chitosan, a broad band located at 3363 $cm^{-1}$and 2878 $cm^{-1}$are assigned to the OH stretching band and CH stretching, respectively. The amino groups of chitosan show the adsorption peaks at 1637 cm^{-1} and 1557 cm^{-1} which corresponded to amide I (C=O stretching) and amide II (out of phase N-H stretching, C-N stretching, respectively. In case of the sericin, a broad band of the OH-stretching and NH stretchingin a wavenumber located at 3289 cm^{-1}. The characteristic bands of sericin at 1662 cm^{-1}, 1557 cm^{-1}, 1250 cm^{-1}, and 656 cm^{-1}, which attributed 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). The chitosan and silk sericin-incorporated bacterial cellulose, as shown in Figure 4.6, show a board band of hydroxyl group and ether linkage from bacterial cellulose together with the amide band of chitosan and sericin. Furthermore, the intensity of amide band, at wavenumber ranging from 1557 cm^{-1} to 1637 cm^{-1}, tended to increase with increasing sericin ratio. Therefore, all of these peaks indicated the incorporation of chitosan and sericin into bacterial cellulose pellicles. All the clearly shifts in wavenumber 3339 cm^{-1} indicate obvious interactions among the hydroxyl, carbonyl and amine groups of the two components. It can be attributed to the hydrogen bonding possibly formed between hydroxyl, carbonyl, amide groups in chitosan and carbonyl, hydroxyl, amine groups in sericin.

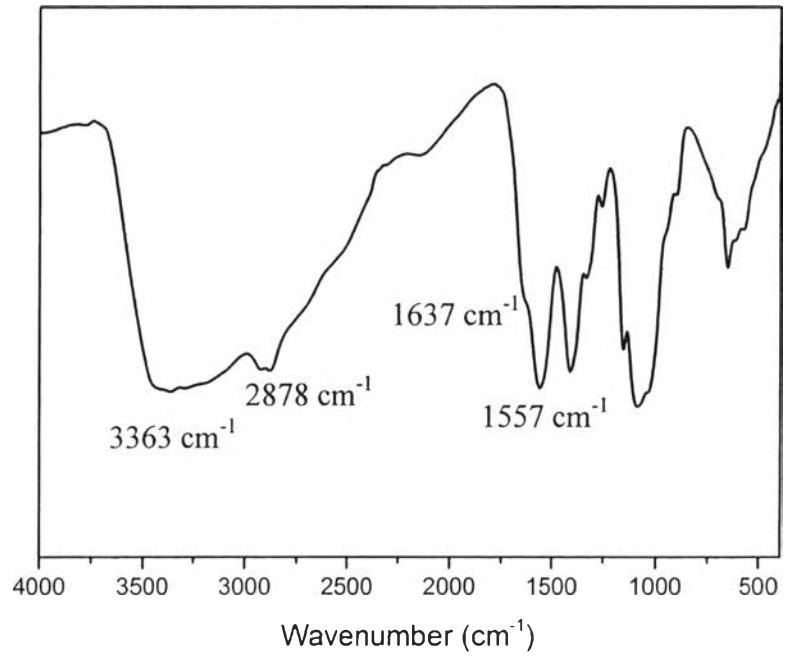


Figure 4.4 FT-IR spectra of chitosan.

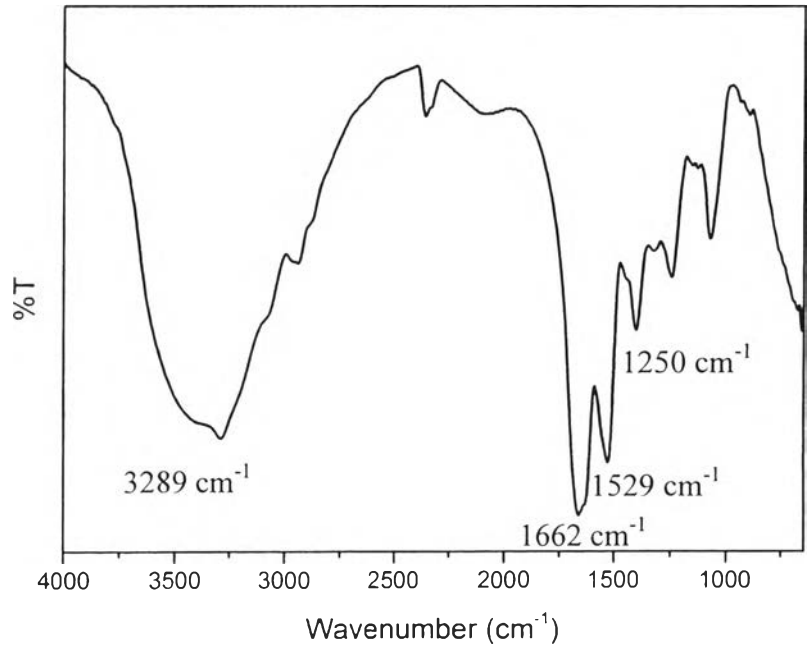


Figure 4.5 FT-IR spectra of silk-sericin.

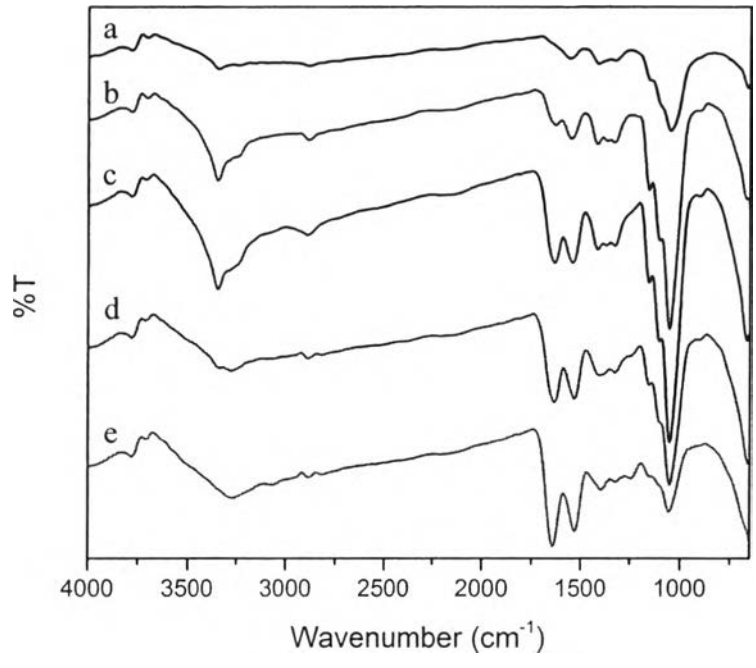


Figure 4.6 FT-IR spectra of chitosan and silk sericin-incorporated bacterial cellulose at different chitosan/sericin blend ratio: (a) 100/0, (b) 75/25, (c) 50/50, (d) 25/75, and (e) 0/100.

4.4 Morphological Analysis

4.4.1 Morphology of Bacterial Cellulose

The surface and cross-section morphology of freeze-dried bacterial cellulose and chitosan and silk sericin-incorporated bacterial cellulose samples were characterized by FE-SEM technique. Figures 4.7a and 4.7b illustrate the morphology of pure bacterial cellulose. The porous structure of the freeze-dried bacterial cellulose with three-dimensional non-woven structures of nanofibrils (50–100 nm) which are highly uniaxial oriented, as shown in Figure 4.7a, is observed on the surface of bacterial cellulose membrane. Whereas the multilayer of bacterial cellulose membranes linked together with the nanofibrils is observed in the cross-sectional morphology of bacterial cellulose, as shown in Figure 4.7b, due to in the process of bacterial cellulose pellicle growth, bacteria generate cellulose only in the

vicinity of culture surface. As long as the system is kept unshaken, bacterial cellulose pellicle is suspended by the cohesion to the interior wall of flask and slides steadily downwards as it thickens. These unique nano-morphology result in a large surface area that can hold a large amount of water (up to 200 times of its dry mass) and at the same time displays great elasticity, high wet strength, and conformability (Czaja *et al.*, 2006). In addition, the porous structure also allows drug loading or encapsulating of other compounds into this porous structure.

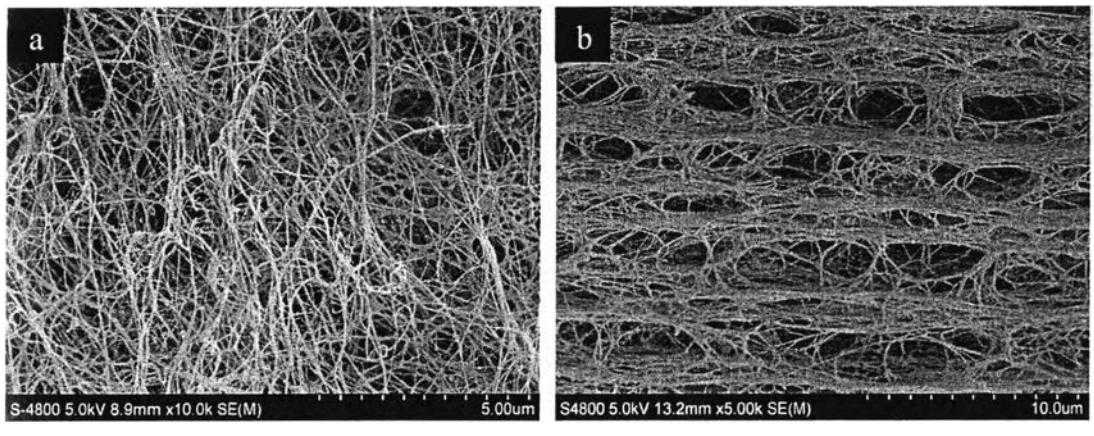
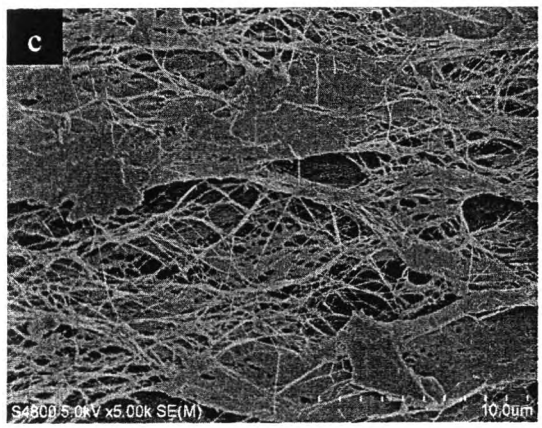


Figure 4.7 SEM images of (c) surface (left)and (d) cross-section (right) of pure bacterial cellulose at a magnification of 10,000 and 5,000, respectively.

4.4.2 Morphology of Chitosan and Silk Sericin-incorporated Bacterial Cellulose

After treated bacterial cellulose pellicle by chitosan and sericin solution, the surface morphology of bacterial cellulose was changed. The chitosan and sericin molecules filled the pores in bacterial cellulose pellicle, as shown in Figures 4.8c to 4.8l. From cross-sectional image, we can see that chitosan and sericin molecules can penetrate into bacterial cellulose and forms layers of bacterial cellulose/chitosan/sericin composite.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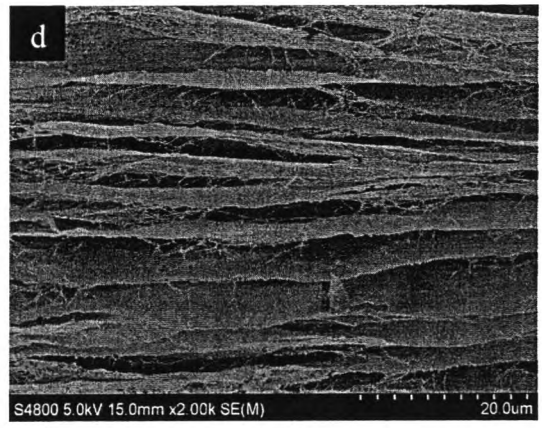
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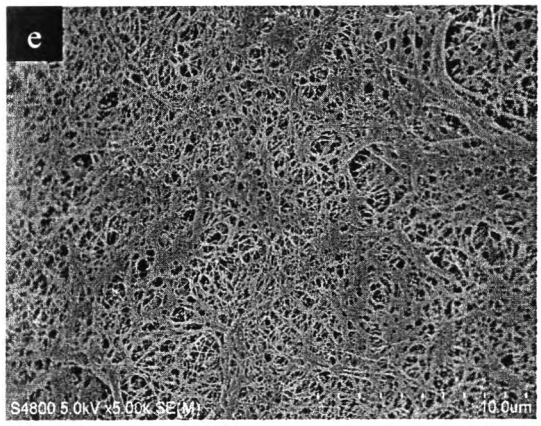
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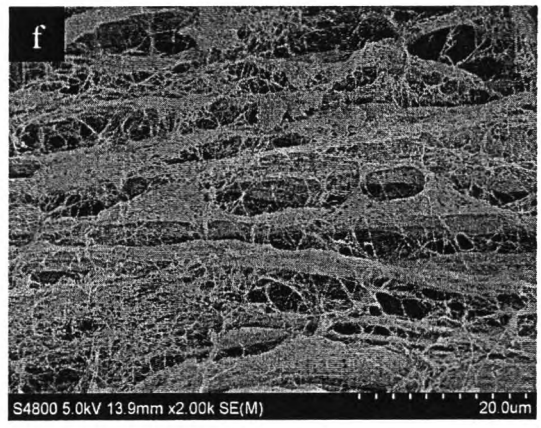
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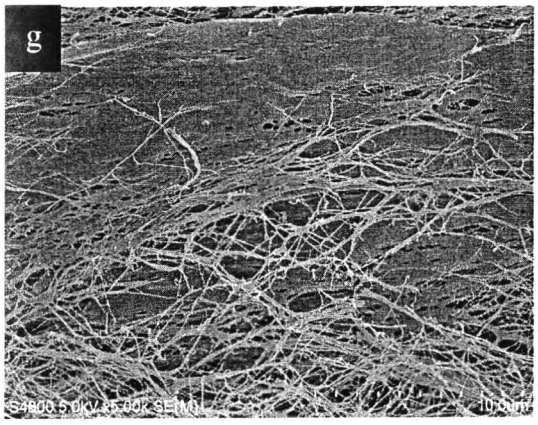
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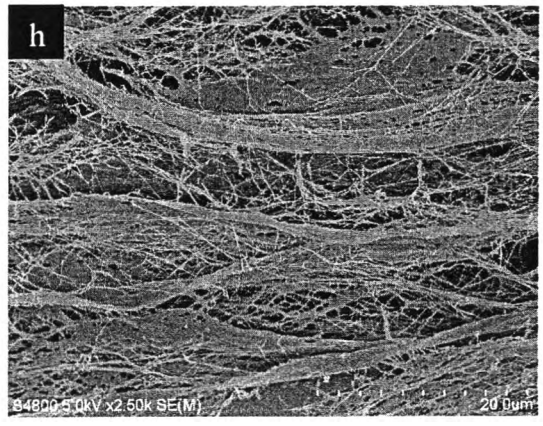
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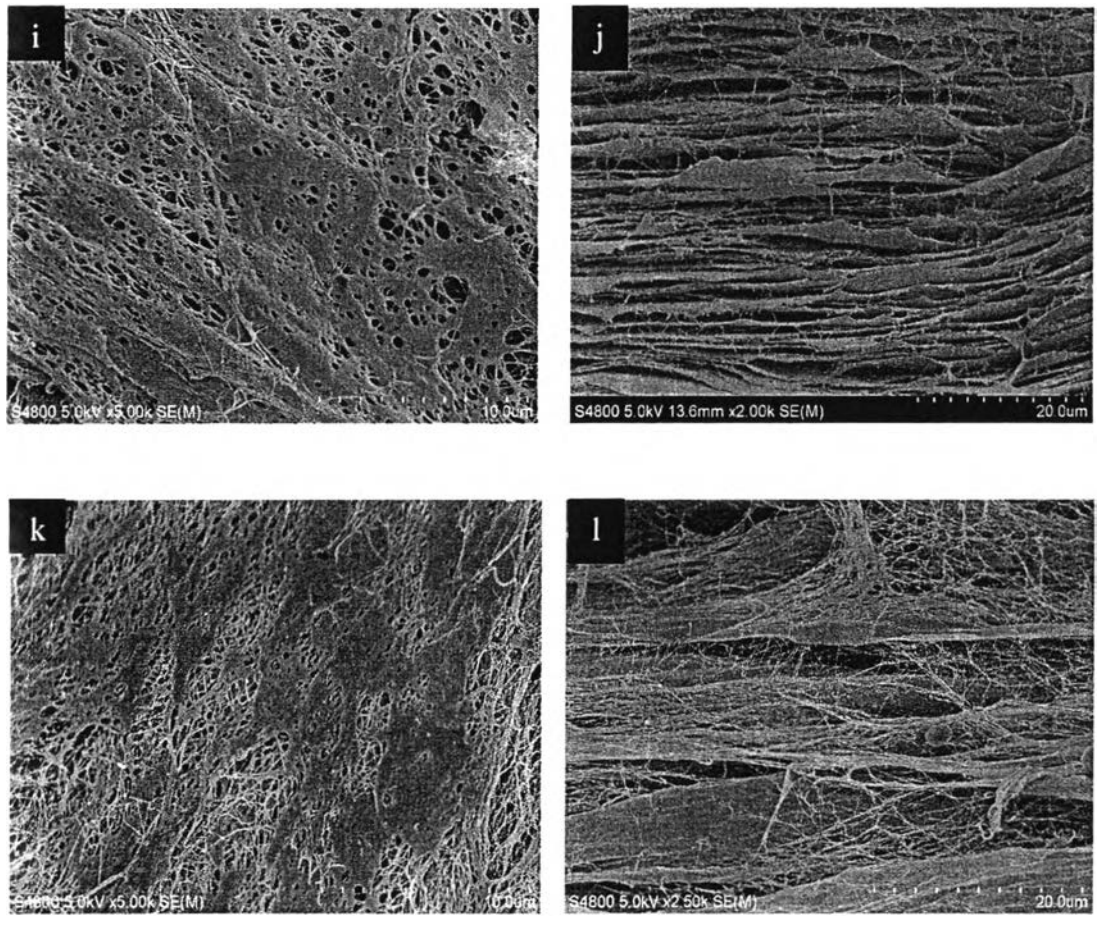


Figure 4.8 SEM images of surface (left) (× 5,000) and cross section (right) (×2,000) of chitosan and silk sericin-incorporated bacterial cellulose at the different chitosan/sericin blend ratio: (c,d) 100/0, (e,f) 75/25, (g,h) 50/50, (i,j) 25/75, and (k,l) 0/100, respectively.

4.5 Amido Black 10B Staining

To confirm as chitosan and sericin molecules have been incorporated into bacterial cellulose network, amino black 10B, is an amino acid staining diazo dye used in biochemical research to stain for total protein, was used to qualitatively determine of chitosan and sericin molecules in bacterail cellulose. An amino black 10B solution is black-blue color, when chitosan and sericin-incorporated bacterial cellulose pellicles were immersed in amido black 10B solution for 8-12 hours, the as-prepared samples color were changed into black colour as shown in Figure 4.9.

From this result, conclude that the incorporated chitosan and sericin was homogeneously distributed in the bacterial cellulose matrix.

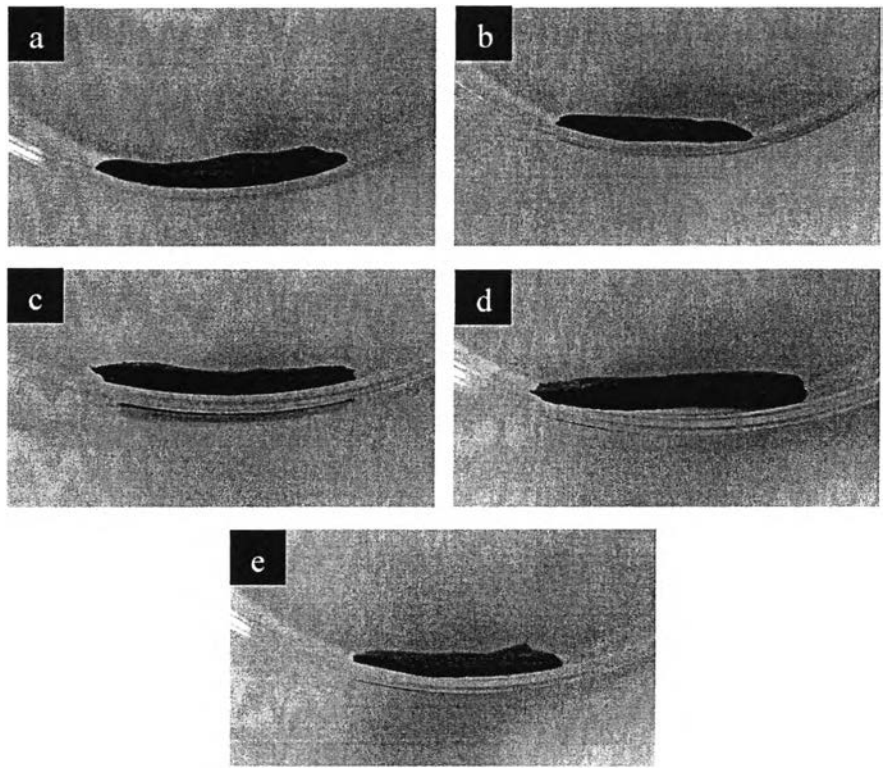


Figure 4.9 Appearances of chitosan and sericin-incorporated bacterial cellulose after amido black 10B staining at different chitosan/sericin blend ratio: (a) 100/0, (b) 75/25, (c) 50/50, (d) 25/75, and (e) 0/100.

4.6 Chitosan and Sericin Content Determination

Kjeldahl nitrogen analysis was used to determine nitrogen content of chitosan and sericin because bacterial cellulose does not contain nitrogen whereas nitrogen is presented only in chitosan and sericin component. The determined amounts of nitrogen content of chitosan and sericin blend solution before immerse into bacterial cellulose and chitosan/sericin-incorporated bacterial cellulose is showed in Figure 4.10. After the incorporation of chitosan and sericin into bacterial cellulose pellicle, the amount of nitrogen content increased with increasing a sericin blend ratio, as shown in Figure 4.10. The nitrogen content increased with increasing a sericin blend

ratio because chitosan has lower nitrogen atom per molecular mass unit than sericin, this reasons leads to sericin has higher nitrogen content than chitosan. From this result, the obtained samples have the nitrogen content similar to the chitosan/sericin blend solution. To confirm as-prepared sample have chitosan and sericin component, chitosan/sericin-incorporated bacterial cellulose pellicle were immersed in 1% NaOH in order to remove sericin embedded in the bacterial cellulose net. The chitosan was trapped in bacterial cellulose pellicles. The amount of chitosan in bacterial cellulose and sericin in NaOH solution were determined by using Kjeldahl analysis. The nitrogen content of chitosan and sericin is shown in Figure 4.10. The amount nitrogen content of chitosan and sercin corresponds to total nitrogen content in bacterial cellulose. The presence of an amount of nitrogen content suggested that chitosan and sericin were penetrated into bacterial cellulose pellicles.

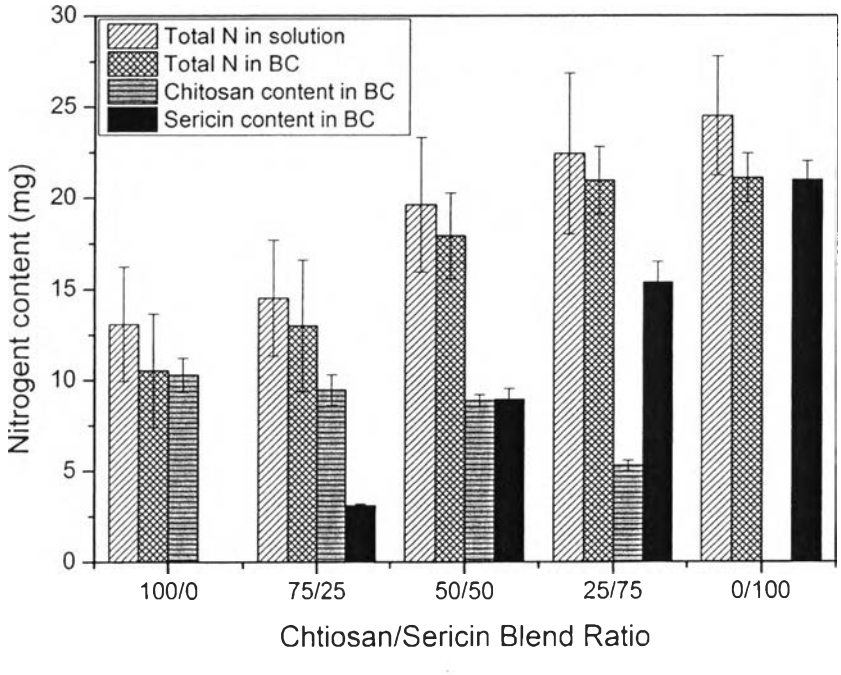


Figure 4.10 Nitrogen content of different chitosan/sericin blend ratio.

4.7 Water Vapor Transmission Rate

Water Vapor Transmission Rate Testing (WVTR) for primary wound dressing skin is the protector for internal stability and screening against external microorganism. Many infected cases are related to the functional loss of skin. So, water vapor transmission rate is an explicit factor that shows the capability of wound dressing in transmission of body liquid or wound exudates. If the WVTR is too high, this causes excessive dehydration which will generate dry condition around wound zone and produces scar formation. In the other hand, if the WVTR is too low, this may lead to the delay of the healing process and the increasing of bacterial growth because of the accumulation of wound exudates (Guptar, 2010). Therefore, water vapor transmission rate should be regulated within a suitable range. Normally, the WVTR of normal skin is 204.0 $g/m^2/day$. The WVTR value of pure bacterial cellulose is 2009.10 $g/m^2/day$. The incorporation of chitosan and sericin shows lower WVTR. The WVTR values of chitosan and sericin-incorporated bacterial cellulose pellicles having chitosan/sericin blend ratios varied from 100/0, 75/25, 50/50, 25/75, and 0/100 are 1618.00, 1518.32, 1450.46, 1375.72, and 1352.36 $g/m^2/day$, respectively, as shown in Figure 4.11. The larger pore size of pure bacterial cellulose than chitosan and sericin-incorporated bacterial cellulose induced water vapor transmission easier resulting in higher WVTR. The higher sericin composition shows lower WVTR because sericin has smaller molecules than chiotsan that tightly packing of the sericin to the bacterial cellulose which restricted the transmission ability of water. In comparison of chitosan A and chitosan B, chitosan B and sericin-incorporated bacterial cellulose having chitosan/sericin blend ratios varied from 100/0, 75/25, 50/50, and 25/75 are 1305.37, 1218.37, 1119.23, and 1070.80 $g/m^2/day$, respectively. The incorporation of chitosan B shown lower WVTR that chitosan A, because chitosan A have lower molecular weight than chitosan B, more hydroxyl group (-OH), as a consequence, water molecules could form more hydrogen bonds with CTSN-B resulting in lower WVTR. The WVTR values of chitosan and sericin-incorporated bacterial cellulose are in the range of WVTR of superficial injured skin from 800-1300 $g/m^2/day$ (Ching-Wen Lou, 2008).

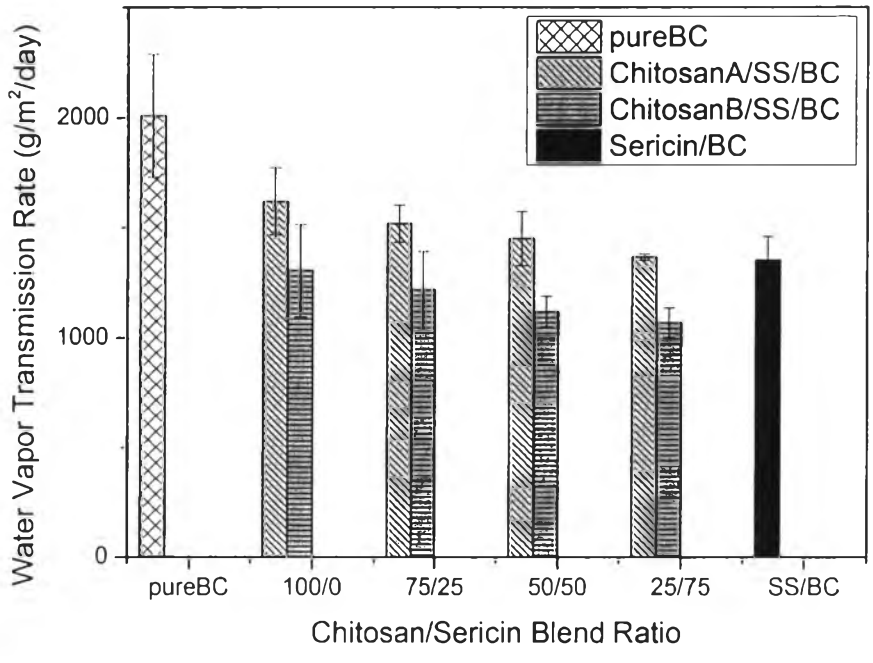


Figure 4.11 Water vapor transmission rate of pure bacterial cellulose and chitosan and sericin-incorporated bacterial cellulose.

4.8 Antioxidant Activity

In the inflammatory phase of wound healing process, the reactive oxygen species (ROS), e.g. hydroxyl and peroxyl radicals that are generated from which are generated during neutrophil accumulation since these compounds are unstable and reactive with substances in body by chain reaction, resulting in cell and tissue injury (Ilango and Chitra, 2010). ROS is produced in high amounts at the site of wound as a defense mechanism against invading bacteria. Nevertheless, the presence of increased numbers of neutrophils and ROS overwhelm the anti-protease substances that normally protect the tissue cells and the extracellular matrix. At high concentrations, ROS can induce severe tissue damage and even lead to neoplastic transformation decreasing the healing process by damages in cellular membranes, DNA, proteins and lipids. Fibroblasts may be killed and skin lipids will be made less flexible by excess ROS. Because of these, the overall role of antioxidants appears to

be significant in the successful treatment and management of wounds. Antioxidants reduce these adverse effects of wounds by removing products of inflammation. They counter the excess proteases and ROS often formed by neutrophil accumulation in the injured site and protect protease inhibitors from oxidative damage (Ipek Süntar *et al.*, 2012). DPPH is a common abbreviation for an organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. It is a dark-colored powder composed of stable organic free-radical molecules, which has been used for estimation of the antioxidant capacity (Tuanjai Noipai *et al.*, 2011) DPPH is a well-known radical and a trap ("scavenger") for other radicals. Thus, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. Because of a strong absorption band at about 520 nm, the DPPH radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at 520 nm of the DPPH. As a result, the solution changes colour stoichiometrically with the number of electrons consumed (Halliwell, 1991). The scavenging activity of chitosan and sericin-incorporated bacterial cellulose was performed with DPPH method. The results were shown in Figure 4.12, the result shown that chitosan and sericin have good antioxidant potential and the scavenging activity of chitosan and sericin in bacterial cellulose pellicle increases with increasing sericin ratio. 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 as shown in Figure 4.13 (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. In comparison of chitosan A and chitosan B, the scavenging activities of chitosan A and sericin-incorporated bacterial cellulose on

superoxide radical was more pronounced than that of chitosan B. Yin *et al.* (2002) estimated this result might be the effect of intra-molecular hydrogen bond. Chitosan owns a lot of hydrogen bonds on N_2–O_6 and O_3–O_5. High molecular weight chitosan had compact structures and the effect of their intra-molecular hydrogen bonds is stronger. Strong effect of intra-molecular hydrogen bonds caused activities of hydroxyl and amino groups to become soft. On the contrary, low molecular weight chitosan own incompact structure. It means to be the soft effect of intra-molecular hydrogen bonds. However, superoxide radical is a zwitter-ionic radical. It could react with free hydroxyl and amino groups in chitosan, and then superoxide radical was eliminated by this reaction. Because low molecular weight chitosan have high free hydroxyl and amino groups than high molecular weight chitosan, their scavenging activities on superoxide radical were more pronounced than that of high molecular weight chitosan (Ronge Xing *et al.*, 2005).

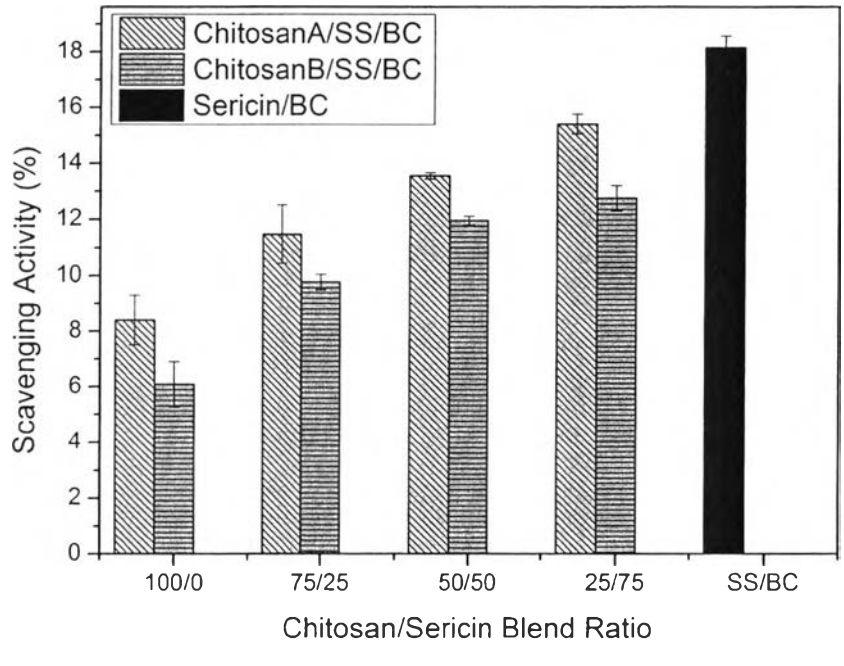


Figure 4.12 Antioxidant activity of chitosan and sericin-incorporated bacterial cellulose.

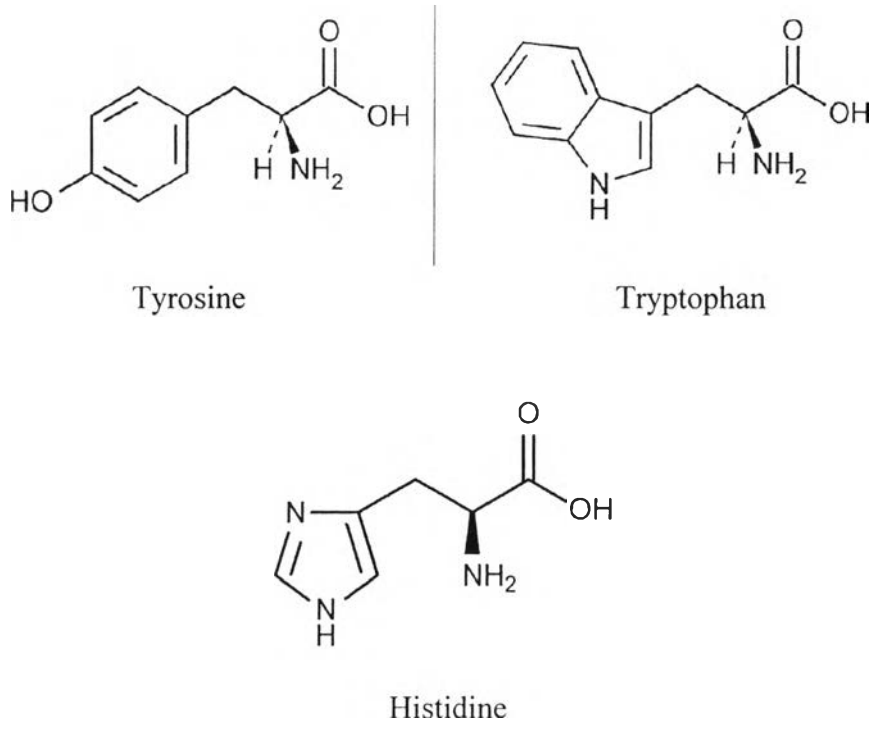
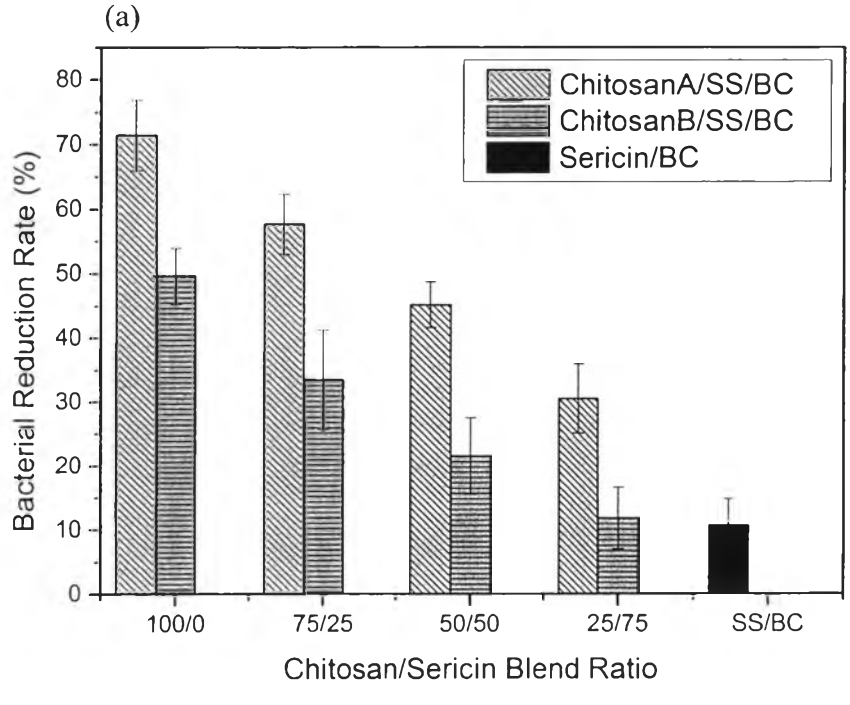


Figure 4.13 Aromatic amino acids.

4.9 Antibacterial Activity

The one desirable characteristics of ideal wound dressing is prevent infection to protect the wound from bacterial invasion, which prolongs the inflammatory phase and delays collagen synthesis, inhibits epidermal migration and induces additional tissue damage. Infected wounds can give an unpleasant odour (Joshua S. Boateng *et al.*, 2007). The development of antimicrobial wound dressing is interesting in order to decrease the amount of microbes on the wound. Since chitosan displays outstanding antimicrobial activities against a variety of microbes (Sakkawet Y. *et al.*, 2012), it is expected that the chitosan incorporating might introduce those biological properties to the bacterial cellulose. As shown in Figure 4.14, the bacterial reduction rate (BRR) was calculated to characterize the antibacterial activities of the chitosan and sericin-incorporated bacterial cellulose. The BRRs against *S. aureus* and *E. coli* of chitosan A and sericin-incorporated bacterial cellulose with a 100/0 chitosan/sericin blend ratio are 71.43 and 63.89 %, respectively. As bacterial reduction rate decrease with decreasing chitosan ratio, and the chitosan and sericin-

incorporated bacterial cellulose against *S. aureus* was greater than that against *E. coli*, perhaps due to a difference in the cell wall characteristics between the Gram-positive and -negative bacteria (Sakkawet Y. *et al.*, 2012). The cell wall of the Gram-negative consists of lipids, proteins and lipopolysaccharides (LPS) that provide effective protection against biocides whereas that of the Gram-positive does not consists of LPS (Thawatchai M. *et al.*, 2008). In comparison of molecular weight of chitosan, the low molecular weight chitosan (chitosan A) against *S. aureus* and *E. coli* greater than high molecular weight chitosan (chitosan B). Rejane C. Goy *et al.* (2009) reviewed that for lower molecular weight chitosan; greater is the observed effect on the reduction of microorganism growth and multiplication. The size and conformation appears to be fundamental to understand the effectiveness of low molecular weight chitosan. The movement, attraction and ionic interaction of short chains are easier than of long chain facilitating the adoption of an extended conformation and an effective binding to the membrane surface of microorganism. The results of antibacterial activity testing also suggest that the chitosan and sericin-incorporated bacterial cellulose is a promising approach to prepare wound dressing with appreciable antibacterial activities.



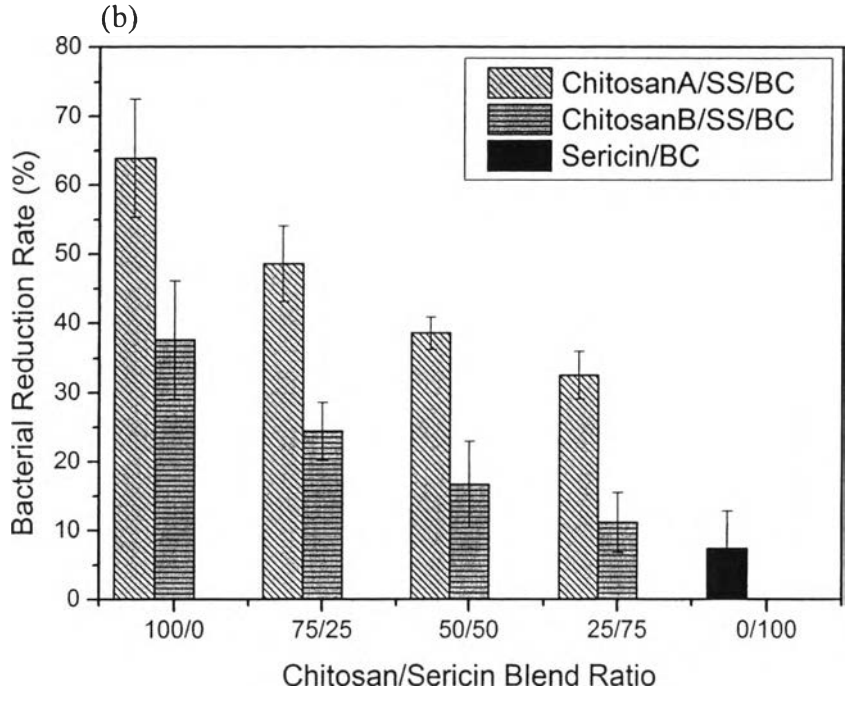


Figure 4.14 Bacterial reduction rate of chitosan and sericin-incorporated bacterial cellulose at various chitosan/sericin blend ratio against (a) *S. aureus* and (b) *E. coli.*

4.10 Chitosan and Sericin Releasing Behavior

The amount of chitosan and sericin released from chitosan and sericin-incorporated bacterial cellulose sample was determined by placing the sample on modified Franz diffusion cell containing acetic/acetate buffer pH 5.5, as shown in Figure 4.15. The hydrophilicity of chitosan and sericin contributed to the easily releasing of chitosan and sericin into an acetic/acetate buffer solution. Figure 4.16(a) demonstrates the releasing profile of sericin from the chitosan and sericin-incorporated bacterial cellulose pellicle. According to the releasing profile of sericin from bacterial cellulose pellicles is released rapidly during 0 h to 12 h. After that, the releasing amounts of sericin became stable. Moreover, a higher ratio of sericin exhibited a higher amount of released sericin from the as-prepared pellicle. The result of chitosan releasing from the chitosan and sericin-incorporated bacterial cellulose is shown in Figure. 4.16(b). The chitosan are rapidly released from chitosan

and sericin-incorporated bacterial cellulose pellicles during 0 h – 36 h. Afterward, chitosan releasing became stable. The amount of released chitosan from as-prepared sample increased with increasing chitosan ratio. The bacterial cellulose pellicle is still stable after placing in an acetic/acetate buffer solution for 72 hours. The three dimensional network structures of ultrafine nanofibrills contribute to the good stability during placing on wet area. 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. Moreover, the released chitosan is act as an antibacterial agent which, prevent infection to protect the wound from bacterial invasion. In the contrary, remained chitosan in bacterial cellulose matrixes was also valuable because chitosan can interact with water via hydrogen bonding to retain and create moist environment around the wound surface. Due to all of those properties, chitosan and sericin – incorporated bacterial cellulose would be useful and appropriate for wound dressing application.

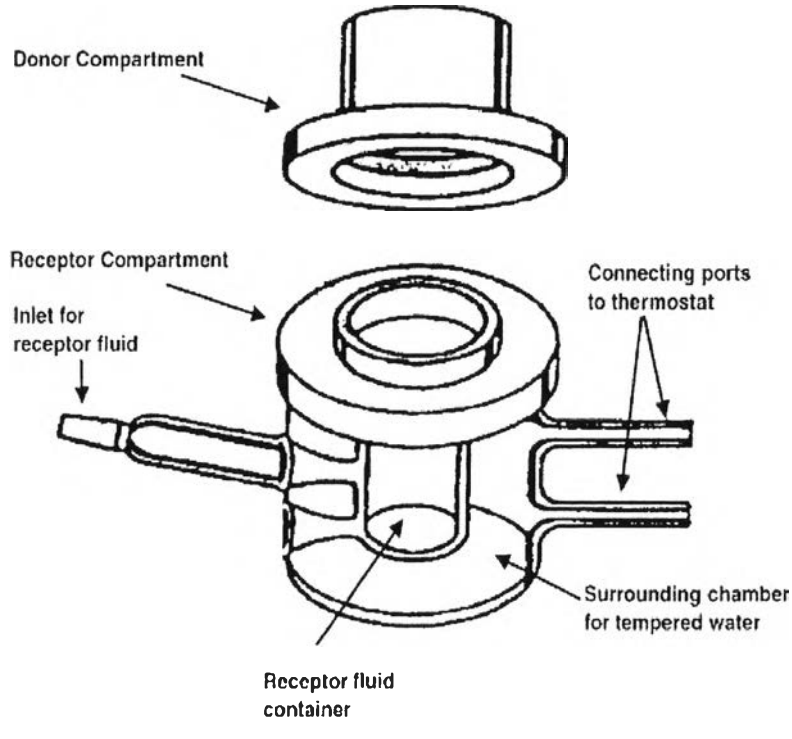


Figure 4.15 A modified Franz diffusion cell (A.O. Gamer *et al.*, 2006).

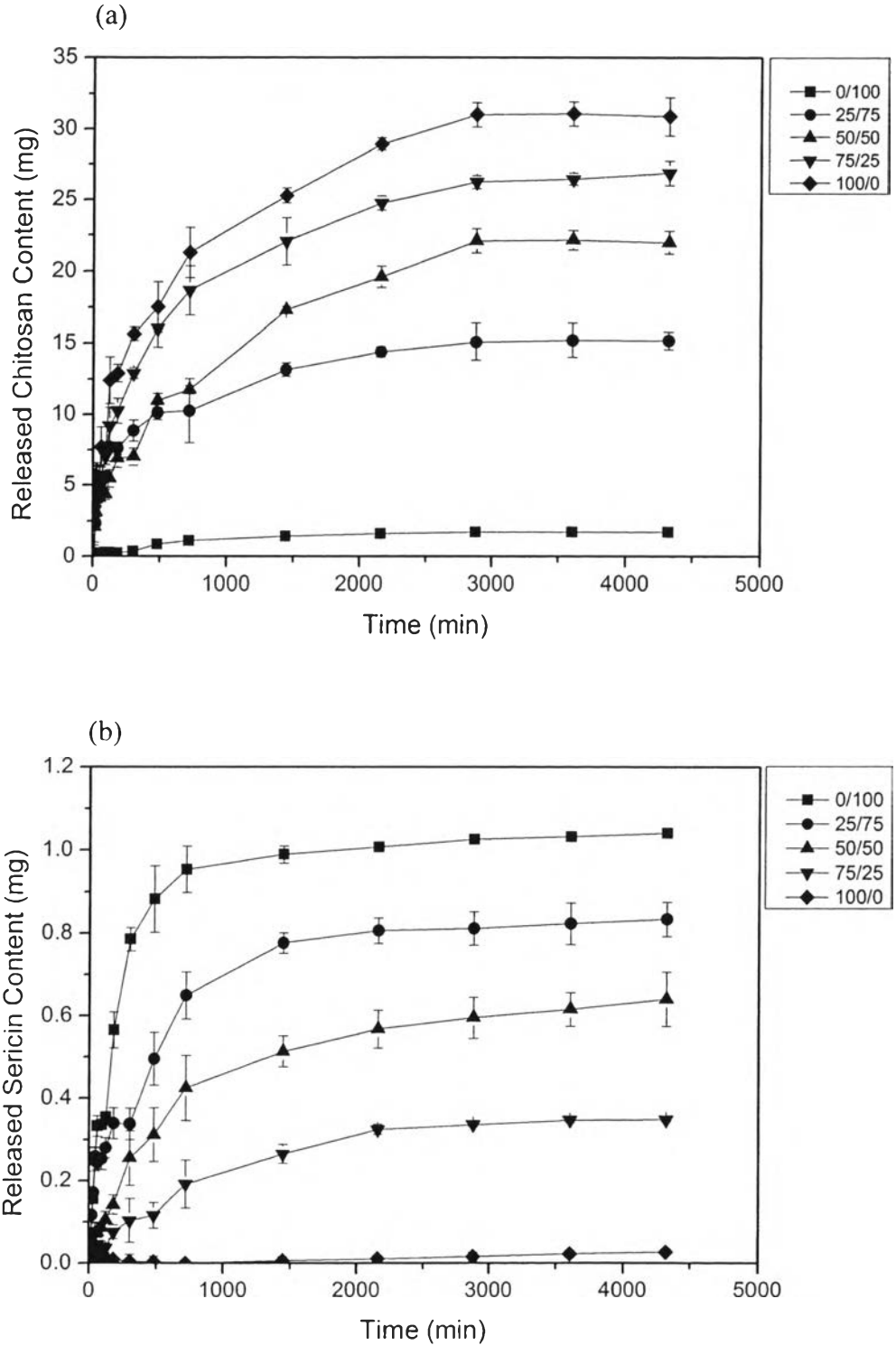


Figure 4.16 (a) Chitosan and (b) sericin releasing profile of chitosan and sericin-incorporated bacterial cellulose in acetic/acetate buffer at pH 5.5.