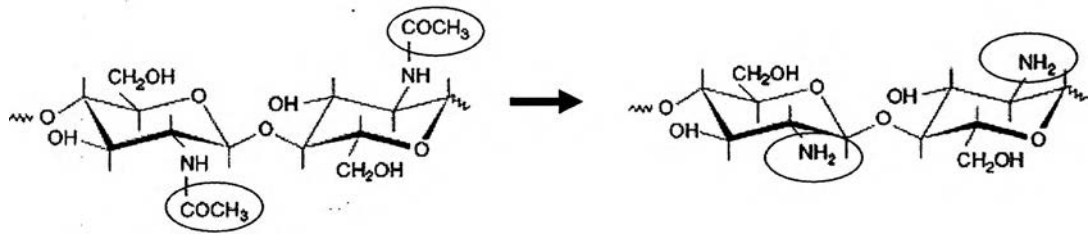
CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation and Characterization of Chitosan

4.1.1 Chitosan Production

Calcium carbonate and protein in the structure of shrimp shells were removed by solvent extraction, HCl and 4% NaOH, respectively. The remaining substance obtained is called chitin. Chitin was changed to chitosan by deacetylation process with 50% NaOH and small amount of $NaBH_4$ as a reducing agent. Deacetylation is the process that changed the acetamide group on chitin to the amino group on chitosan, which is more reactive for antimicrobial activity as shown below:



In the chitosan production, percentage yield of chitin was about 36% and percentage yield of chitosan was about 20% as shown in Table 4.1.

Table 4.1 Yield of chitin and chitosan production from shrimp shell

Product after the process	Yield*(%)
Dried shrimp shell	100.00
Decalcification	55.55
Deproteinization (chitin)	36.25
1st deacetylation chitosan	28.57
2nd deacetylation chitosan	25.44
3rd deacetylation chitosan	21.44
4th deacetylation chitosan	19.82

*dry weight basis

4.1.2 Degree of Deacetylation (%DD) of Chitosan

Infrared spectroscopic method was used to determine degree of deacetylation (%DD) of chitin and chitosan. From FTIR absorption spectra, the degree of deacetylation of partly deactylated chitin was 8%, based on measurement by Baxter *et al.* (1992), which was calculated from equation (4.1):

$$\%DD = 100 - 115\ (A_{1655}/\ A_{3450}) \qquad (4.1)$$

where A_{1655} and A_{3450} are absorbances at 1655 cm^{-1} and 3450 cm^{-1}, respectively.

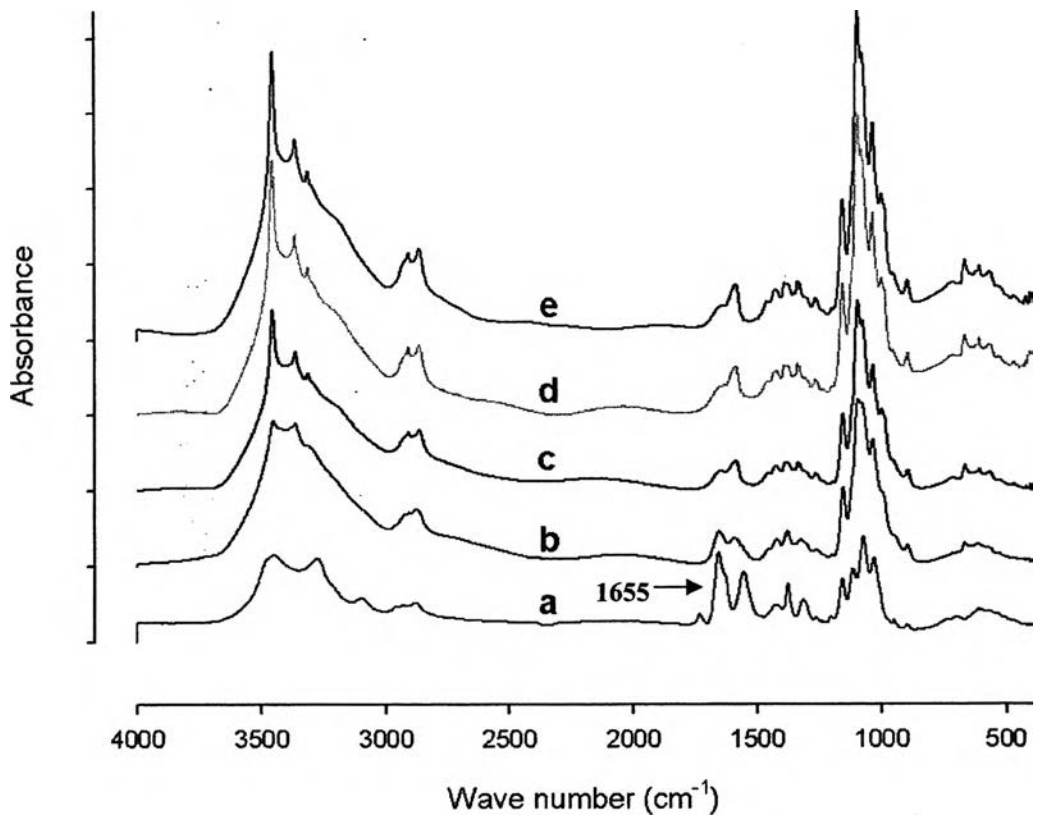


Figure 4.1 FTIR spectra of (a) chitin and chitosan after (b) 1st deacetylation, (c) 2nd deacetylation, (d) 3rd deacetylation, (e) 4th deacetylation.

Absorbance at 1655 cm^{-1} is the characteristic peak of amide (I) in chitin and absorbance at 3450 cm^{-1} is the characteristic peak of hydroxyl group which contain in structure of both chitin and chitosan was used as a reference peak. As shown in Figure 4.1, the amide peak decreased when increasing number of treating cycle, so degree of deacetylation was increased. The degree of deacetylation (%DD) of chitosan was 84%, 96% and it was become constant at 98% after three times of alkali treatment as shown in Figure 4.2.

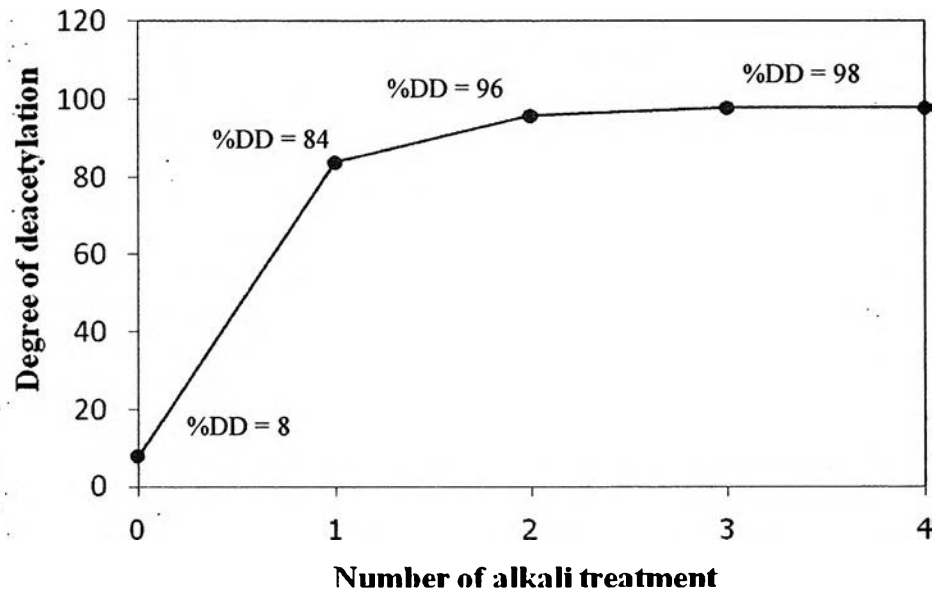


Figure 4.2 %DD of chitin and chitosan different number of treatment.

4.1.3 Viscosity-Average Molecular Weight of Chitosan

The molecular weight of chitosan was determined by Ubelohde viscometer based on the method of Wang *et al.* (1991). The viscosity-average molecular weight of chitosan was calculated via Mark - Houwing equation (4.2):

$$[\eta] = 6.59 \times 10^{-5} M_v^{0.88} \quad (4.2)$$

where $[\eta]$ = Intrinsic viscosity (ml/g)

M_v = Viscosity-average molecular weight.

The intrinsic viscosity was determined from the Y-intercept of the plot between $[\eta_{sp}]/c$ versus chitosan concentration (g/ml) and ln $[\eta_{rel}]/c$ versus chitosan concentration (g/ml).

Table 4.2 %DD and M_v of deacetylated chitosan samples

Number of alkali treatment	Degree of deacetylation (%DD)	Molecular weight (x 10^5 g/mole)
1	84	11.00
2	96	9.68
3	98	8.07
4	98	7.04

Table 4.2 illustrates the degree of deacetylation (%DD) and viscosity-average molecular weight (M_v) of chitosan samples with different number of alkali treatment. The results indicated that when increasing the number of alkali treatment the M_v of chitosan decreased whereas the degree of deacetylation of chitosan increased until constant after treatment for three times. So the chitosan after 3rd deacethylation was used in this research.

4.2 Preparation and Characterization of Bacterial Cellulose

4.2.1 Bacterial Cellulose Synthesis

Acetobacter xylinum (A. xylinum) strain TISTR 975 was used to produce bacterial cellulose. It is aerobic bacteria, which used oxygen to produce cellulose at the oxygen-rich air-liquid interface of the culture medium (Vandamme *et al.*, 1997) composed of 4% D-glucose and 1% yeast extract. For the initial stage, the formation of the bacterial cellulose took place on the upper site of the cellulose layer. As long as the system was kept in static condition, the disc-shape pellicle was suspended and steadily slided downwards as its thickness (Maneerung *et al.*, 2008). After cultured at 30°C for 4 days, leather-like white pellicle of cellulose was produced. Then the bacterial cellulose was purified by boiling in a diluted sodium hydroxide to eliminate bacteria cells, protein (a by-product during the bacterial

metabolism), and the components of the culture medium which entrapped within the bacterial cellulose network (Embuscado *et al.*, 1996).

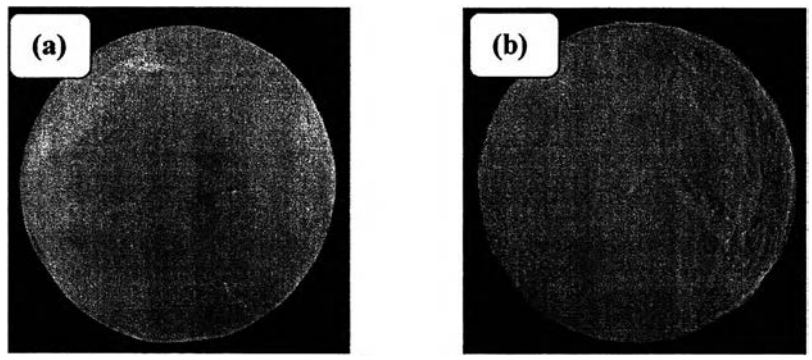


Figure 4.3 The pellicle of bacterial cellulose (a) white-transparent hydrogel pellicle, and (b) pellicle after freeze-drying.

After purification, the yellow-translucent pellicle became a white-transparent pellicle as shown in Figure 4.3(a). The estimated thickness and diameter of the pellicle were 4.4 ± 0.11 mm and 10.4 ± 0.19 cm, respectively. The bacterial cellulose was then freeze-dried, to eliminate water molecule without destroying a structure. The sponge-like pellicle approximately the same thickness and dimension as the hydrogel as shown in Figure 4.3(b). The solid content of the obtained freeze-dried BC is 1.65%.

4.2.2 Surface Modification of Bacterial Cellulose by DBD Plasma Treatment

The freeze-dried bacterial cellulose pellicle was then modified surface with dielectric barrier discharge (DBD) plasma, the conditions for plasma treatment are electrode gap distance 4 mm, applied voltage 40 kV, input frequency 325 Hz, treatment time 10 s in atmospheric pressure of air gas.

Chemical structure of bacterial cellulose was characterized by FTIR analysis. The FTIR spectra of the non-plasma treated and the plasma treated bacterial cellulose were shown in Figure 4.4. The specific wavenumber of FTIR signals with the possible functional groups are listed in Table 4.3.

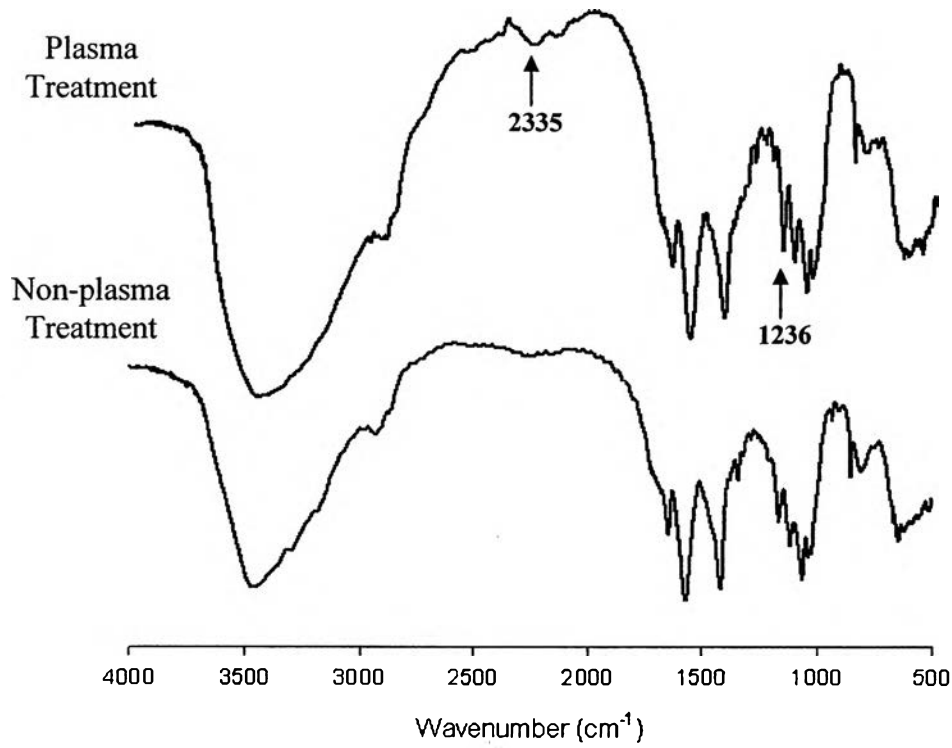


Figure 4.4 FTIR spectra of the non-plasma treated bacterial cellulose compare to the plasma treated bacterial cellulose.

Table 4.3 Main functional groups of the bacterial celluloses

Wavenumber (cm^{-1})	Vibration mode	Functional group
3600-3000	O-H stretching	Hydroxyl (-OH)
2980-2900	C-H stretching	CH_n unit
2335	O-H bending	Carboxyl (-COOH)
1236	C-O stretching	Carboxyl (-COOH)

The board peak at 3600-3000 cm^{-1} can be attributed to O-H stretching of hydrogen bonded hydroxyl groups which contain in structure of bacterial cellulose, was used as a reference peak. The results show that after air-plasma treatment, the peak intensity between 2980-2900 cm^{-1}, which is attributed to aliphatic C–H bond, slightly decreased. The peak appeared at 2335 cm^{-1} was associated with the O-H bending of the carboxyl group. In addition, the absorbance at 1236 cm^{-1} was increased, which can be attributed to C–O of the carboxyl groups (Laguadia *et al.,* 2005). From the results indicated that plasma treatment can increased the active species of oxygen, resulting in higher hydrophilicity of bacterial cellulose surface.

From XPS characterization, a DBD discharge under air at medium pressure can generate a wide range of active species, including atomic oxygen, ozone, nitrogen oxides, neutral and meta-stable molecules, radicals, and ultraviolet radiation. An air-plasma increases the surface energy of polymers and textiles by introducing oxygen-containing polar groups onto the polymer surface. Atomic oxygen is believed to be the main reactive species responsible for this oxygen inclusion (Kogelschatz, 2003). Atomic oxygen is formed because of the dissociation of O_2 molecules by electron impact. However, excitation and dissociation of nitrogen molecules also lead to a number of additional reaction paths that can produce additional atomic oxygen (Fang *et al.*, 2001). To investigate what functional groups were formed by plasma treatment, the surface characteristic of the untreated and of plasma-treated samples was examined by XPS.

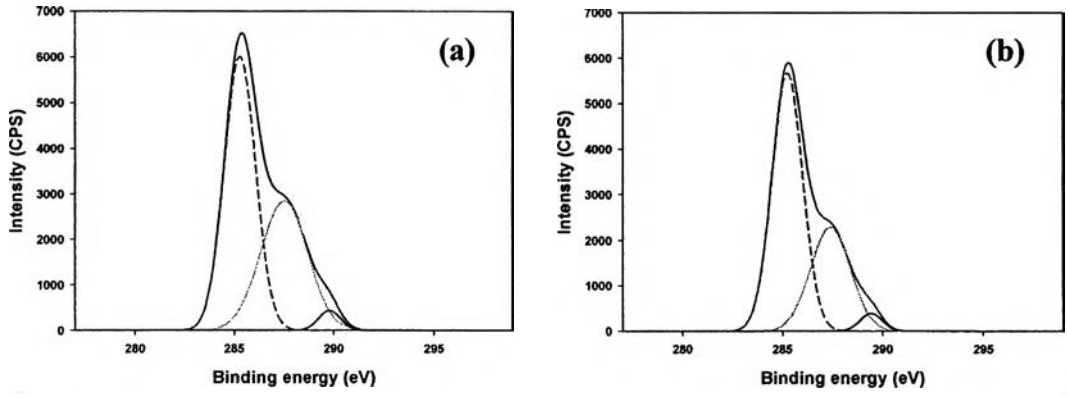


Figure 4.5 C1s XPS spectra of bacterial cellulose (a) before plasma treatment, and (b) after plasma treatment (electrode gap distance, 4 mm; treatment time, 10 s; input voltage, 40 V (low side); input frequency, 325 Hz).

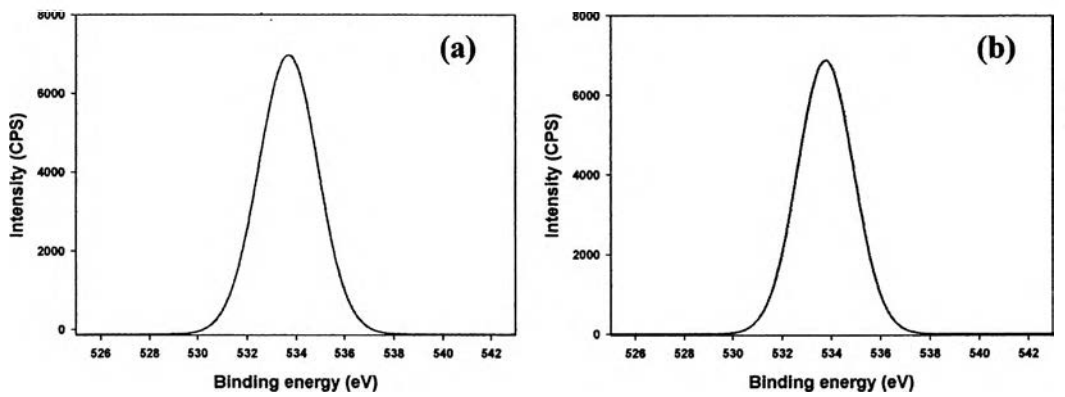


Figure 4.6 O1s XPS spectra of bacterial cellulose (a) before plasma treatment, and (b) after plasma treatment (electrode gap distance, 4 mm; treatment time, 10 s; input voltage, 40 V (low side); input frequency, 325 Hz).

Figure 4.5 and 4.6 show the C1s spectra and O1s spectra of bacterial cellulose surface, respectively. The C1s spectra of the bacterial cellulose were deconvoluted into 3 components: one at 285.0 eV due to the C–C and C–H groups, one at 286.7 eV due to the CH_2–O– group, and one at 289.0 eV due to the O–C=O group. From the figures, the C1s spectra and O1s spectra of bacterial cellulose after plasma treatment almost unchanged, duo to cellulose was composed of oxygen in

hydroxyl groups. However, when compare the chemical composition of bacterial cellulose before and after plasma treatment, the O/C ratio of the plasma treated bacterial cellulose was higher than that of the non-plasma treated one as shown in Table 4.4, the O/C ratios of the plasma treated and the non-plasma treated bacterial cellulose were 1.15 and 1.04, respectively. Accordingly, the XPS results are in good agreement with the FTIR spectra.

Table 4.4 Atomic composition of the plasma treated and the non-plasma treated bacterial cellulose as characterized by XPS

Condition	Atomic composition		
	O1s (%)	C1s (%)	O/C ratio
Without plasma	51	49	1.04
Plasma	53	47	1.15

Morphology of the bacterial cellulose surface was also studied. Results from scaning electron microscope analysis of the bacterial cellulose showed that its structure was a three dimensional non-woven network with many voids as shown in Figure 4.7(a). After plasma treatment for 10 s, there was no change in surface morphology of the bacterial cellulose observed as shown in Figure 4.7(b).

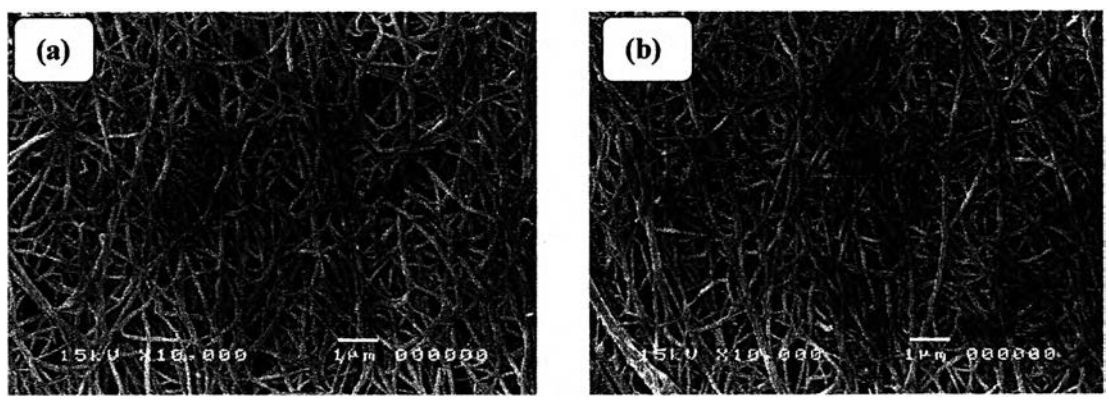


Figure 4.7 SEM images of BC (a) without and (b) with plasma treatment.

4.3 Chitosan Coating on Bacterial Cellulose Surface without Plasma Treatment

The original colorless freeze-dried bacterial cellulose pellicle was turned into more yellow color when increased % concentration of chitosan solution as shown in Figure 4.8, can implied that amount of chitosan coated on their surface was increased with an increasing chitosan concentration and chitosan was well dispersed throuout the bacterial cellulose surface.

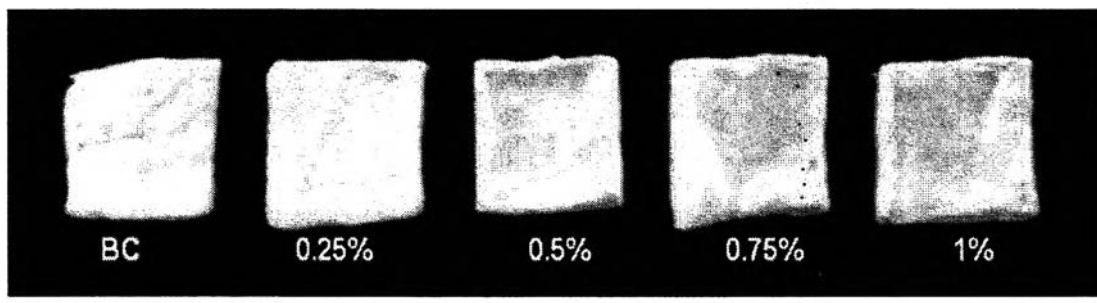


Figure 4.8 The freeze-dried bacterial cellulose pellicle and freeze-dried chitosan-coated bacterial cellulose pellicle prepared by immersing in different chitosan solution concentrations.

4.3.1 Surface Chemical State and Composition

The surface chemical state and composition of chitosan-coated bacterial cellulose was characterized by X-ray photoelectron spectroscopy (XPS) technique. Figure 4.9 shows the N1s spectra of the chitosan coating located at around binding energy 400 eV (Huh *et al.*, 2001), is attributed to the amino nitrogen ($-NH_2$) in chitosan. When increasing the chitosan concentration, the peak intensity of N1s was also increased.

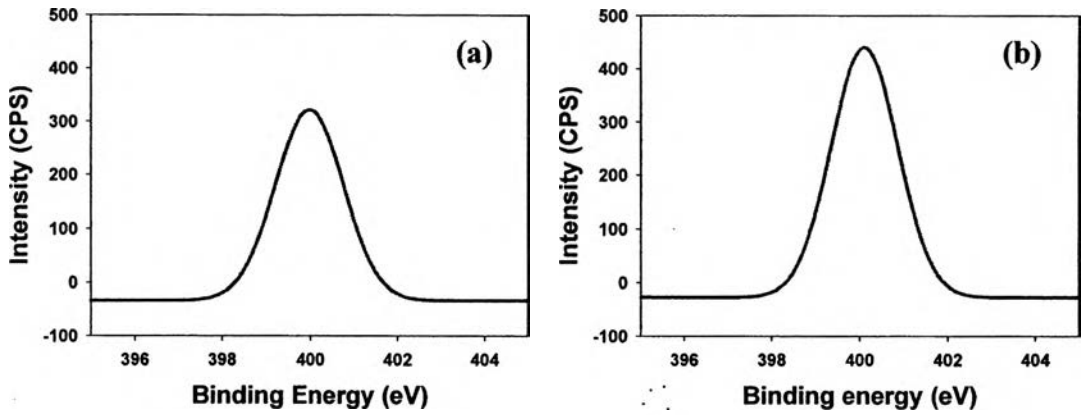


Figure 4.9 N1s XPS spectra of chitosan-coated bacterial cellulose (a) bacterial cellulose coated with 0.5% chitosan concentration, and (b) bacterial cellulose coated with 1.0% chitosan concentration.

4.3.2 Swelling Ratio

High swelling ratio of bacterial cellulose and chitosan-coated bacterial cellulose is resulted from their chemical and physical structure. Bacterial cellulose has high hydrophilicity as their multilayer linked together by hydrogen bonding. The freeze-dried bacterial cellulose pellicle has sponge-like properties or high porous structure to allow water molecule penetrated inside. The swelling ratios of bacterial cellulose and chitosan-coated BC were increased with an increasing immersion time in distilled water as shown in Figure 4.10. Moreover, due to the hydrophilic nature of chitosan, the swelling ratios were also increased with an increasing concentration of chitosan. The maximum swelling ratios of bacterial cellulose, 0.75% chitosan-coated bacterial cellulose and 1% chitosan-coated bacterial cellulose up to 21.04, 31.48 and 33.93, respectively were observed.

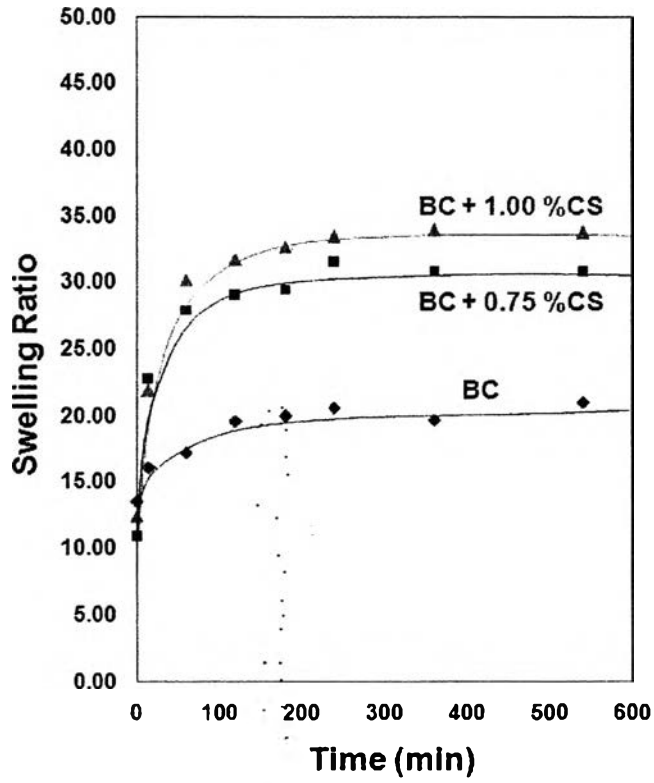


Figure 4.10 Swelling ratio of freeze-dried BC and chitosan-coated BC.

4.3.3 Dye Absorption Capacity

Amino black 10B, an anionic diazo dye used in biochemical research to stain for total amino group of chitosan on the surface of chitosan-coated bacterial cellulose. Protonated amino group of chitosan can act as cationic sites for anionic dyes. The absorption of dye molecules by chitosan was measured by the absorbance of amino black 10B at 618 nm using Shimazu UV-1800. Bacterial cellulose and chitosan-coated bacterial cellulose were immersed in an absorbent of dye at room temperature, sampling 1 ml of each solution at a specified immersion period ranging between 0 and 24 h, the concentration of the dye absorbed by the chitosan was then calculated. Figure 4.11 shows the percentage concentration of amino black 10B (mg/ 100 ml distilled water) absorbed by samples with different chitosan concentration. The results from the Figure 4.11 showed that the dye absorption capacity was increased with an increasing time. When increased chitosan concentration, the absorbed dye was also increased resulting from increased amount of chitosan coated on surface of samples.

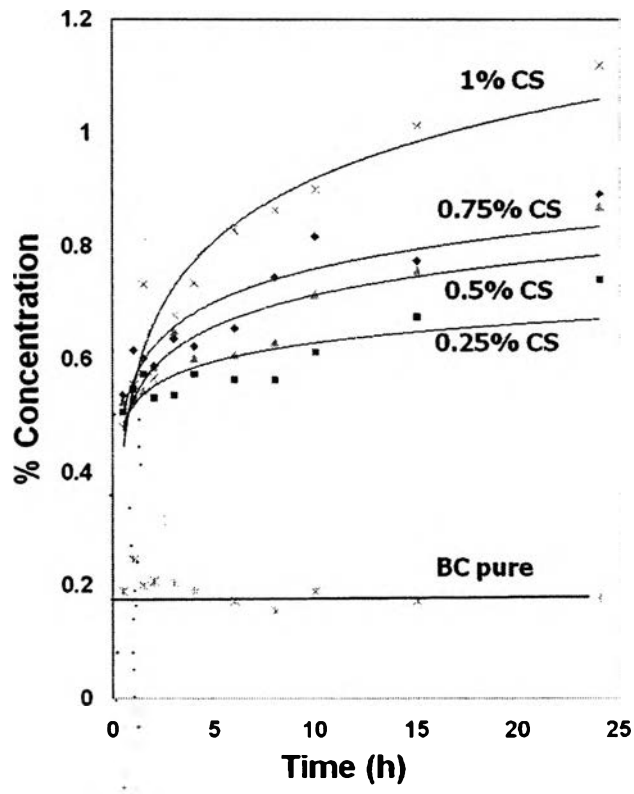


Figure 4.11 The percentage concentration of amino black 10B (mg/ 100 ml distilled water) absorbed by samples with different chitosan concentrations.

4.4 Chitosan Coating on Bacterial Cellulose Surface with Plasma Treatment

4.4.1 Surface Chemical State and Composition

From the previous XPS results of non-plasma and plasma treated bacterial cellulose, the plasma treatment could increase the amount of hydrophilic functional groups on the surface such as C-O and O-C=O. But after chitosan coating with 0.5% and 1.0% chitosan solution, these hydrophilic groups were decreased as evident by the disappearance of the peak in this region as seen in Figure 4.12, suggesting that These groups were used to interact with the protonated NH_2 of the chitosan, thus the surface of bacterial cellulose was covered with chitosan (Zhang *et al.*, 2003).

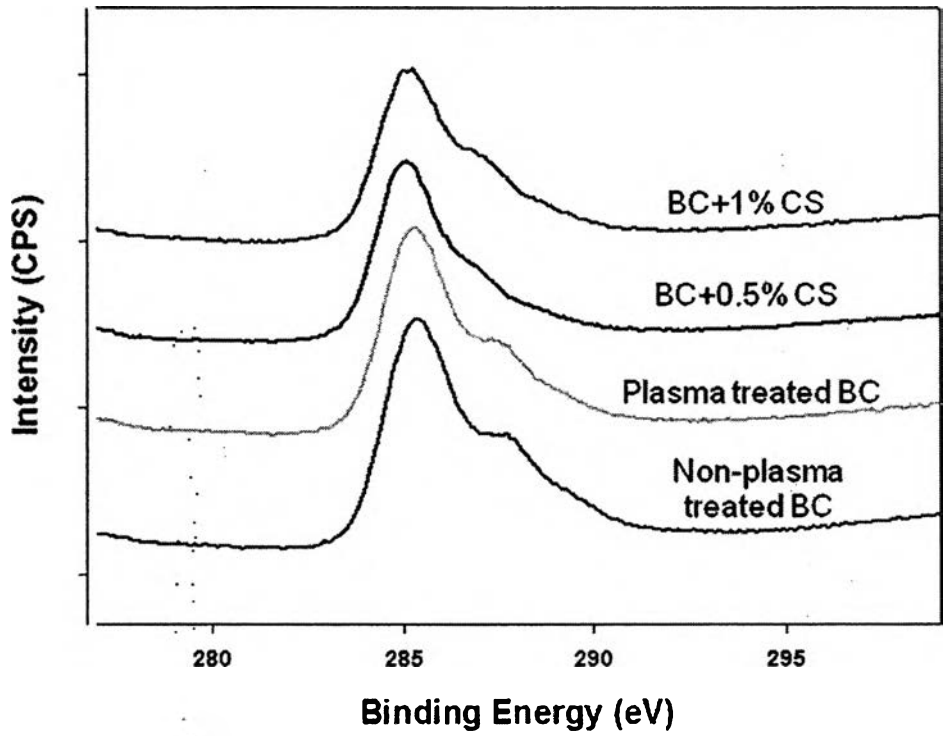


Figure 4.12 C1s XPS spectra of non-plasma treated bacterial cellulose, plasma treated bacterial cellulose, bacterial cellulose coated with 0.5% chitosan, and bacterial cellulose coated with 1.0% chitosan.

4.4.2 Surface Morphology

The three-dimensional non-woven network structure of bacterial cellulose from static culture (Figure 4.13(a)) was observed by scanning electron microscope (SEM). The ultrafine nanofibrils of bacterial cellulose was stabilized by hydrogen bonding and formed a dense network with high crystallinity index (above 60%) (Jonas and Farah, 1998). From the SEM images, at the same chitosan solution concentration (0.75% chitosan), higher amount of chitosan was observed on the surface of plasma-treated bacterial cellulose (Figure 4.13(c)) more than that of the non-plasma ttreated one (Figure 4.13(b)). This indicated that air-plasma treatment could improve the adhesion between chitosan and bacterial cellulose surface. Moreover, the structure of the chitosan-coated samples was denser than that of the

pure bacterial cellulose, since chitosan molecule penetrated into porous of the bacterial cellulose network.

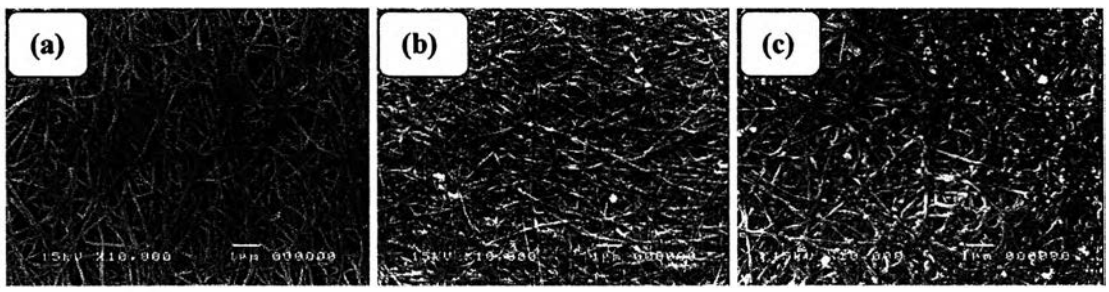


Figure 4.13 SEM images of (a) bacterial cellulose without chitosan coating, (b) non-plasma treated bacterial cellulose with 0.75% chitosan coating, and (c) plasma-treated bacterial cellulose with 0.75% chitosan coating.

4.4.3 Amount of Chitosan Coated on Bacterial Cellulose

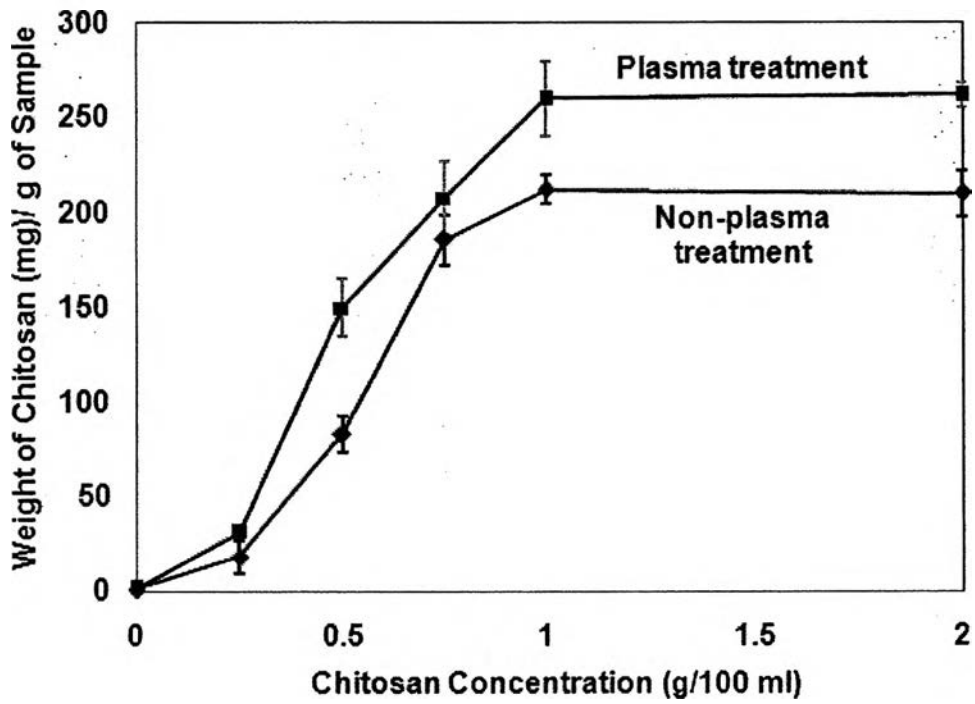


Figure 4.14 Amount of chitosan coated on non-plasma treated and plasma-treated bacterial cellulose with different chitosan concentrations.

The amount of chitosan coated on bacterial cellulose surface was determined by Kjeldahl method, the method for determining nitrogen based on the conversion of the bound nitrogen to ammonia (NH_3), which is then separated by distillation into trapping acid and determined by titration. The amount of ammonia was converted to nitrogen content and then chitosan content in the samples.

Figure 4.14 shows the amount of chitosan coated on non-plasma treated and plasma-treated bacterial cellulose. The amount of chitosan deposited on bacterial cellulose increased with increasing concentration of chitosan from 0.25% to 1.00% and then remained almost unchanged. At the same concentration, the amount of chitosan on the surface of plasma treated bacterial cellulose was relatively higher than that of the non-plasma treated one. The saturated amount of chitosan deposited on non-plasma treated and plasma-treated bacterial cellulose was approximately 209.4 and 261.7 mg/ g of sample, respectively.Therefore, after modifying of BC surface with DBD plasma treatment, the saturated amount of chitosan on the BC surface was increased up to 4.7% or approximately 50 mg/ g of sample.

4.4.4 Swelling Ratio

Swelling ratio of freeze-dried bacterial cellulose and chitosan-coated bacterial cellulose without and with plasma treatment shows in Figure 4.15(a) and 4.15(b), respectively. After plasma treatment, the swelling ratios of bacterial cellulose and chitosan-coated bacterial cellulose were increased resulting from higher hydrophilicity of the surface obtained from the treatment. However, the chitosan concentration had almost no effect on the swelling property of the chitosan-coated bacterial cellulose with plasma treatment. The maximum swelling ratios of bacterial cellulose, 0.75% chitosan-coated bacterial cellulose and 1% chitosan-coated bacterial cellulose after plasma treatment were 31.72, 43.63 and 44.99, respectively as shows in the Figure 4.15(b).

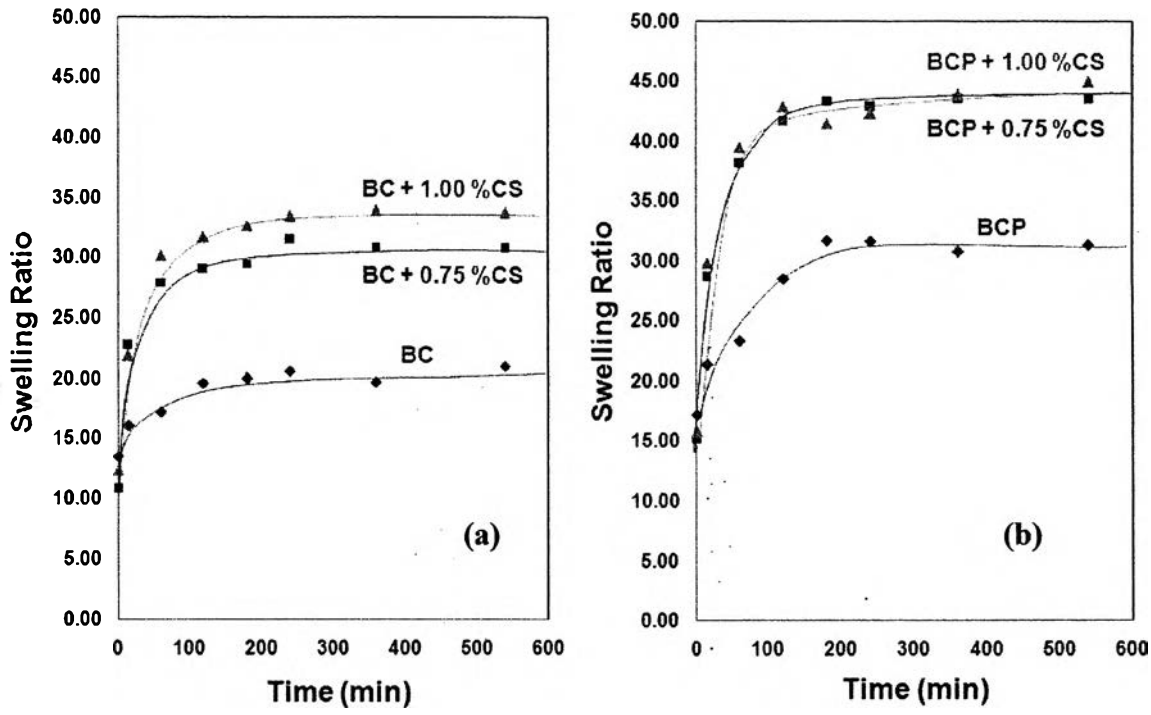


Figure 4.15 Swelling ratio of freeze-dried bacterial cellulose and chitosan-coated bacterial cellulose (a) without plasma treatment, and (b) with plasma treatment.

4.4.5 Release of Chitosan from Bacterial Cellulose

For determining the amount of chitosan released from the bacterial cellulose samples, the colorimetric determination method of Muzzarelli (1998) was used. This method is the rapid determination of unknown concentration of chitosan in aqueous solution. Chitosan was absorbed by a monochlorotriazine dye, Cibacron Brillient Red (CBR) 3B-A, the absorbance values were measured at 575 nm by Shimazu UV-1800, then the cumulative release of chitosan for each sample was calculated. The release characteristic of chitosan from the bacterial cellulose in phosphate buffered saline (PBS, pH = 7.4) and acetate buffer solution (pH = 5.5) was performed at temperature of 37°C and shaking speed of 50 rpm.

Figure 4.16 shows the cumulative release of chitosan from non-plasma treated and plasma treated bacterial cellulose samples in phosphate buffered saline (pH = 7.4) and acetate buffer solution (pH = 5.5). From the results, for all samples, chitosan was gradually released during 72 h of immersion. At the beginning,

the cumulative amounts of the chitosan released into the medium increased rapidly with the initial rate of 1.78% per hour (based on weight of chitosan in sample) during the first 5 h of immersion, resulting from the chitosan that absorbed on the outer surface of the bacterial cellulose. These results implied that surface erosion is the main mechanism for the release of chitosan in the first step (Watthanaphanit *et al.,* 2009). After 5 h of immersion, the chitosan was released at a slower rate until nearly constant within 33 hours. At 72 h of immersion, the final amounts of chitosan released from the DBD plasma treated bacterial cellulose were 30.90% and 29.54% (based on weight of chitosan in sample) in phosphate buffered saline and acetate buffer solution, respectively (Figure 4.16(c) and 4.16(d)), relatively lower than the non-plasma treated one, 35.29% and 41.97% (based on weight of chitosan in sample) in phosphate buffered saline and acetate buffer solution, respectively (Figure 4.16(b) and 4.16(a)). It clearly shows that the interaction between chitosan and bacterial cellulose surface was improved by plasma treatment.

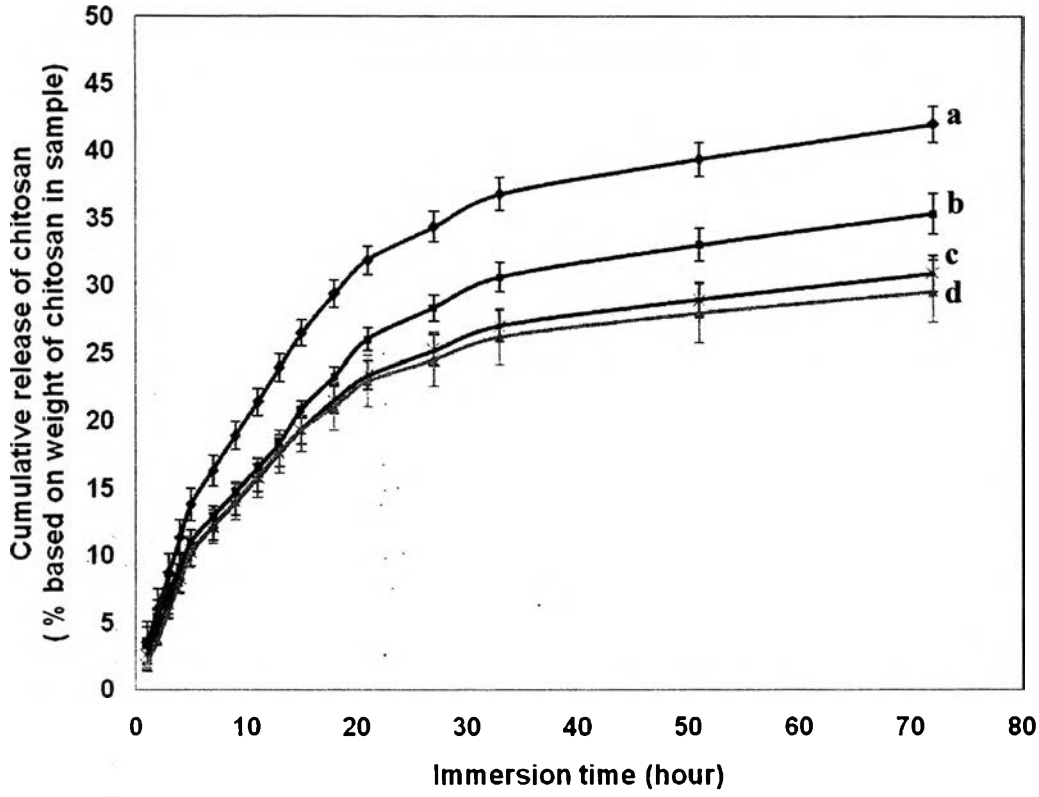


Figure 4.16 Cumulative amounts of the chitosan released from bacterial cellulose (BC) coated with 1.00% chitosan in acetate buffer solution (pH = 5.5) and phosphate buffered saline (pH = 7.4) at 37°C, 50 rpm; (a) without plasma treatment, pH = 5.5, (b) without plasma treatment, pH = 7.4, (c) with plasma treatment, pH = 7.4, and (d) with plasma treatment, pH = 5.5.

When considered the effect of pH of the medium, without plasma treatment, the amount of the chitosan released from bacterial cellulose in the acetate buffer solution (Figure 4.16(a)) was greater than those in the phosphate buffered saline (Figure 4.16(b)), because when immersing in the acetate buffer solution, chitosan could more protonated, thus the chitosan was more dissolved and released. For the plasma treated bacterial cellulose, pH of the medium shows no significant influence on the the release characteristic of chitosan (Figure 4.16(c) and 4.16(d)). However, the release characteristic of chitosan from plasma-treated bacterial cellulose in the acetate buffer solution (Figure 4.16(d)) slightly lower than that in the

phosphate buffered saline (Figure 4.16(c)) because the lower pH of the acetate buffer solution could stabilize the protonated amino groups of chitosan resulting in higher ionic interaction between plasma-treated bacterial cellulose and chitosan.

4.4.6 Antimicrobial Activity Testing

Antimicrobial property is important for wound dressing assay because wounds often provide favourable environments for colonization of microorganism, lead to infection and delayed healing (Watthanaphanit *et al.*, 2009). Chitosan is a natural biopolymer known for its antimicrobial ability. Even though the exact mechanism of antimicrobial action of chitosan is still imperfectly know, many different mechanism has been proposed and confirmed thieir antimicrobial property (Dutta *et al.*, 2009; Rebea *et al.*, 2003; Shahidi *et al.*, 1999).

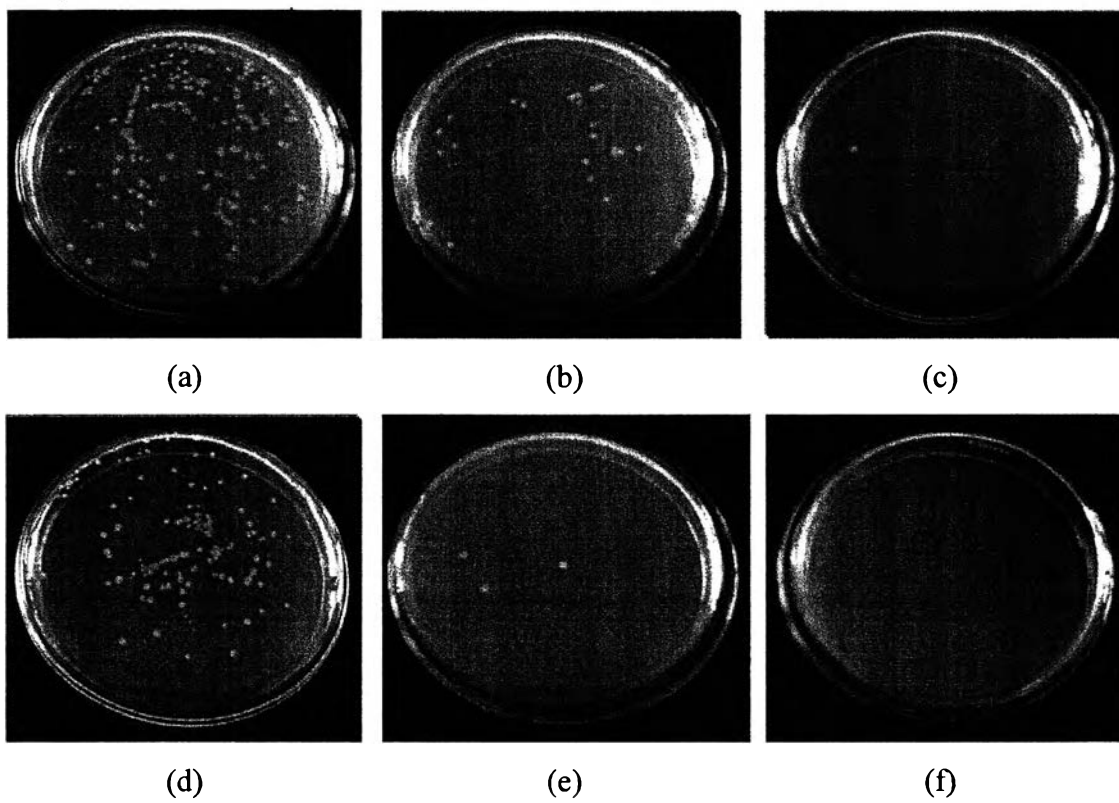


Figure 4.17 The colony forming unit of *E. coli* against non-plasma treated bacterial cellulose ((a) – (c)), and plasma treated bacterial cellulose ((d) – (f)) without chitosan ((a) and (d)), with 0.75% chitosan ((b) – (e)), and with 1.00% chitosan ((c) – (f)).

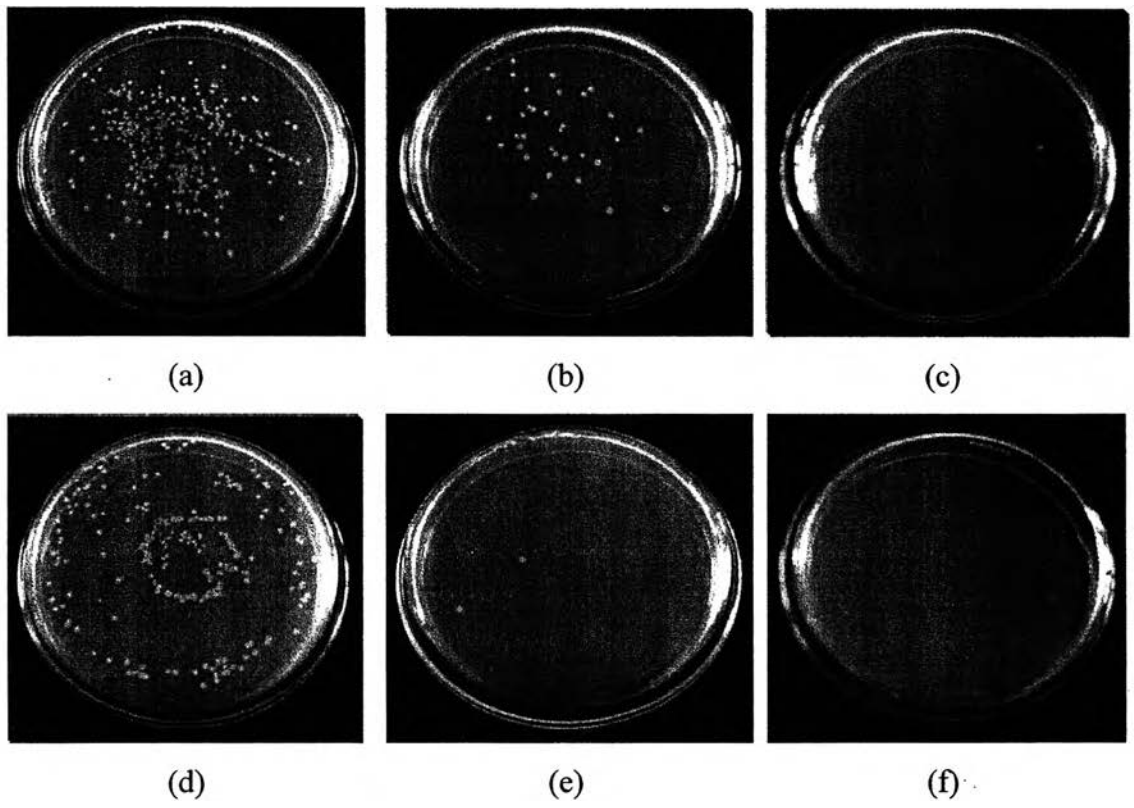


Figure 4.18 The colony forming unit of *S.aureus* against non-plasma treated bacterial cellulose ((a) – (c)), and plasma treated bacterial cellulose ((d) – (f)) without chitosan ((a) and (d)), with 0.75% chitosan ((b) – (e)), and with 1.00% chitosan ((c) – (f)).

In this work, the chitosan-coated non-plasma treated and plasma treated bacterial cellulose were also tested for antimicrobial activity against Gram-negative *E. coli* and Gram-posittive *S. aureus* by using the colony forming count method. After the samples had been in contact with the bacterial mixtures for 3 h, the numbers of the *E. coli* and *S. aureus* colonies for each type of sample were evaluated three times. The viable counts recovered from the blank control, non-plasma treated, plasma treated, with and without chitosan coating for *E. coli* and *S. aureus* are shown in Table 4.5 and Table 4.6, respectively. According to the tables, the viable cell counts against *E.coli* and *S.aureus* of non-plasma treated bacterial cellulose was increased as compared to those of the blank control (no sample added). This may results from both microorganisms could digest the bacterial cellulose as a carbon

source for growth. However, after plasma treatment, the increments of *E.coli* and *S.aureus* growth were not as high as the non-plasma treated bacterial cellulose. This supposed that plasma treatment promoted some physical changes on the bacterial cellulose surface, and obstructed the microorganisms to use the bacterial cellulose as their nutrient.

After 0.75% and 1.00% chitosan coating, the number of colonies of two microorganisms was significantly decreased from those of pure bacterial cellulose as shown in Figure 4.17 and 4.18. The bacterial reduction rate (BRR) values of the chitosan-coated bacterial cellulose increased with the increasing of chitosan concentrations and increased with the plasma treatment, resulting from an increasing amount of chitosan on the bacterial cellulose surface. Moreover, the effectiveness was slightly greater in the case of *S. aureus* than *E. coli*.

Table 4.5 Colony forming unit counts (cfu/ml) of non-plasma treated bacterial cellulose and plasma treated bacterial cellulose against *E. coli*

Sample		Blank (10^{-6})	BC	0.75% CS	1% CS
Non-plasma	CFU	275 ± 19.00	356 ± 24.34	14 ± 2.08	1 ± 0.58
	% reduction/ increment	-	29.45% increment	94.91% reduction	99.64% reduction
Plasma	CFU	275 ± 19.00	281 ± 3.46	3 ± 2.00	0 ± 0
	% reduction/ increment	-	2.18% increment	98.91% reduction	100% reduction

Table 4.6 Colony forming unit counts (cfu/ml) of non-plasma treated bacterial cellulose and plasma treated bacterial cellulose against *S.aureus*

Sample		Blank (10^{-5})	BC	0.75% CS	1% CS
Non-plasma	CFU	293 ± 10.60	382 ± 7.64	10 ± 2.65	1 ± 1.00
	% reduction/ increment	-	30.38% increment	96.59% reduction	99.66% reduction
Plasma	CFU	293 ± 10.60	303 ± 7.55	2 ± 1.00	0 ± 0
	% reduction/ increment	-	3.41% increment	99.32% reduction	100% reduction