ฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซนจากการสังเคราะห์โดยจุลินทรีย์ *ACETOBACTER XYLINUM*

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BACTERIA CELLULOSE-CHITOSAN FILM FROM MICROBIAL SYNTHESIS BY *ACETOBACTER XYLINUM*

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อ. ที่ปรึกษา: ผศ. ดร. เหมือนเดือน พิศาลพงศ์, 64 หน้า.

เนื่องจากความเหมือนกันระหว่างโครงสร้างทางเคมีของแบคทีเรียเซลลูโลสและไคโตแซน จึงเป็นที่น่าสนใจว่าไคโตแซนที่เติมเข้าไปในอาหารเลี้ยงเชื้อของแบคทีเรียเซลลูโลสจะสามารถปรับปรุงคุณสมบัติทางกายภาพและทางชีวภาพของฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซน การเติมไคโตแซนที่มวลโมเลกุลของไคโตแซน 30,000 และ 80,000 ณ ความเข้มข้นมากกว่า 0.75 เปอร์เซ็นต์ของน้ำหนักไคโตแซนต่อปริมาตรในอาหารเลี้ยงเชื้อจะยับยั้งการสร้างแผ่นฟิล์มแบคทีเรียเซลลูโลสอย่างมากในระหว่างการเลี้ยงเชื้อ ฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซนแสดงการรวมตัวกันของโพลีเมอร์และแสดงคุณสมบัติทางกลได้ดีที่สุดที่ความเข้มข้น 0.75 เปอร์เซ็นต์ของน้ำหนักไคโตแซนต่อปริมาตรในอาหารเลี้ยงเชื้อ ในงานวิทยานิพนธ์นี้ได้ทำการศึกษาถึงคุณสมบัติทางกล การบวมน้ำ การแพร่ผ่านของน้ำ โครงสร้างที่เป็นรูพรุน การต่อต้านแบคทีเรีย การต่อต้านเชื้อรา ดัชนีความเป็นผลึก และการเจริญเติบโตในเซลล์คนบนแผ่นฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซน

ฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซนมีความแน่นและหนาเพิ่มขึ้นหลังจากที่ใส่ไคโตแซน ลักษณะรูพรุนของฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซนมีขนาดเล็กกว่าฟิล์มแบคทีเรียเซลลูโลส ในขณะที่พื้นที่ผิวไม่เพิ่มขึ้นมากนัก ขนาดรูของฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซนจะลดลงเมื่อเพิ่มปริมาณไคโตแซน ขนาดรูของฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซนที่มวลโมเลกุล 30,000 และ 80,000 มีค่า 151 และ 121 อังสตรอม ตามลำดับ ผลการทดสอบด้วยอินฟราเรดทางโครงสร้างโมเลกุลพบว่า มีการมีปฏิสัมพันธ์กันระหว่างโมเลกุลของแบคทีเรียเซลลูโลสและไคโตแซน คุณสมบัติทางกลและความสามารถในการบวมน้ำของฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซน มีค่าสูงกว่าฟิล์มแบคทีเรียเซลลูโลส นอกจากนี้ ฟิล์มแบคทีเรียเซลลูโลส-ไคโตแซนไม่เป็นพิษต่อและส่งเสริมการเจริญเติบโตของเซลล์ผิวหนังมนุษย์

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สาขาวิชา วิศวกรรมเคมี ลายมือชื่ออาจารย์ที่ปรึกษา.....
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THESIS ADVISOR: ASST. PROF.MUENDUEN PHISALAPHONG, Ph.D., 64 pp.

Due to the similarity between the chemical structures of BC and chitosan, it is interesting to add chitosan to BC culture medium as it might improve the physical and biological properties of the developed BC-chitosan film. The addtion of chitosan of MW 30,000 and 80,000 more than 0.75 percent (w/v) in the culture medium strongly inhibited the formation of BC biosynthesis. BC-chitosan film showed the best polymer miscibility and mechanical properties at 0.75 percent (w/v) in the culture medium. In this thesis, mechanical property, equilibrium water content, water vapor permeability, porous structure, antibacteria ability, antifungal ability, crystallinity index, and the growth of human skin cells on the BC-chitosan film were investigated.

The BC-chitosan films were denser and thicker after the addition of chitosan. The BC-chitosan film had the pore sizes much lessen than that of BC while the surface area was not slightly increased from the latter. The average pore size of the films decreased with the increase of chitosan supplementation. The pore sizes of the films of BC-chitosan MW30000 and MW80000 were 151 A° and 132 A°, respectively. The FTIR result indicated the intermolecular interaction between BC and chitosan. The mechanical properties of BC-chitosan film were relatively improved. The equilibrium water content of BC-chitosan film was higher than that of the BC film. Additonally, BC-chitosan film had no toxicity and supported cell proliferation.

Department: Chemical Engineering Student's signature Nirun Jatupaiboon

Field of study: Chemical Engineering Advisor's signature

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CHAPTER I

INTRODUCTION

Cellulose, the basic material of all plant substance, is the most abundant polysaccharide found in nature. Cellulose derived from plant has been known as unpurified cellulose associated with other kinds of natural fiber like lignin and hemicellulose while bacterial cellulose (BC) is nearly-purified cellulose. In general, BC was extracellularly synthesized into nano-sized fibrils by the bacteria *Acetobactor Xylinum,* with coconut water being used as liquid medium. Plant-derived cellulose and BC have the same chemical structure. BC displayed advantages superior to the counterpart from plants with its physical and chemical properties: such as high mechanical strength, crystallinity, hydrophilicity, ultra-fine network structure, and purity.

There have been several applications of BC in medical fields such as artificial skin for humans with extensive burns (Fontana *et al.*, 1990), artificial blood vessels for microsurgery (Klemm, 2001), scaffold for tissue engineering of cartilage (Svensson *et al.*, 2005) and wound-dressing (Czaja *et al.*, 2006). Especially in the wound dressing application, BC is eligible because of its outstanding properties. The excellent dressing should be maintained the wound in a wet condition, inexpensive, lightweight, flexible, and impermeable to microorganisms (Czaja *et al.*, 2006). BC shows high water content, good sorption of liquids, non-allergenic, and can be safely sterilized without any change of its characteristics. The innovative wound dressing still has been developed continuously in a wide range of good candidate materials such as alginate, polyurethane, and chitosan.

Chitosan, like BC, has been reconized for application in various fields including biomedical area. It has been known for its absorption of exude, anti-fungal, anti-microbial, anti-viral and wound-healing properties. Chitosan is useful as a wound management aid to reduce scar tissue. Moreover, chitosan may be used to inhibit fibroplasia in wound healing, and to promote both tissue growth and differentiation in tissue culture (Muzzarelli *et al.*, 1999).

Therefore, this study aims to prepare BC-chitosan film from microbial synthesis under static conditions by *Acetobacter xylinum*. in coconut-water. Microstructure and mechanical properties of the BC films were characterized. Furthermore, the growth of human skin HaCat on BC films were examined. The present study provided indications for the chitosan concentration in BC-chitosan film for using in therapy of skin wound.

Objectives

1. To develop BC-chitosan film from biosynthesis by *Acetobacter xylinum*.
2. To investigate the effect of chitosan in BC-chitosan film on the water absorbtion, porous structure, mechanical property, antibacteria activity, antifungal ability, crystallinity index, and effect on the growth of human skin cells.

Research Scopes

1. Prepare BC film from biosynthesis under static conditions by *Acetobacter xylinum.*
2. Examine effects of chitosan content in the range of 0 – 0.75% wt/vol in the culture medium. Chitosan MW was used both 30,000 and 80,000.
3. Characterizing the developed BC-Chitosan film by
    a. Scanning electron micrographs (SEM) for preliminarily investigating morphology.
    b. Fourier Transform Infrared (FT-IR) spectrometer for identifying the chemical structure.
    c. Universal testing machine for determining stress-strain curve.
    d. X-ray diffraction (XRD) for finding crystallinity index.
    e. Brunauer-Emmett-Teller (BET) for identifying the pore size, porosity, and pore size distribution.
    f. Antibacteria ability.
    g. Antifungal ability.

4 In vitro study of human skin HaCat on the developed film in Petri-dish.

Overview

This present work is organized as follows:

Chapter I present an introduction of this study.

Chapter II contains background theory of cellulose, BC, and chitosan.

Chapter III is consisted of the literature review: medical application of BC, chitosan for wound dressing, and cellulose-chitosan blend.

Chapter IV states the details of the experimental procedures and techniques of this research.

Chapter V reviews the experimental results of the characterization of BC and BC-chitosan films.

Chapter VI contains the overall conclusion obtained from this research. Future work and recommendations are also stated.

Finally, the additional data of the experiments which had emerged from this study are included in appendixes at the end of this thesis.

CHAPTER II

THEORY

2.1 Cellulose

Cellulose is considered as the majority of biopolymer material. It is the main structural component of the plant cell walls in form of semi-crystalline microfibrils. Cellulose is a linear polymer composed of D-glucose residues joined by ß-1,4-glucosidic bonds (Brown, 1983). Cellulose molecule forms a straight, almost fully extended chain as shown in Figure 2.1. The cellulose chains are organized in a crystalline or semi-crystalline lattice, thus giving rise to microfibrils with a high tensile strength. The chemical formula of cellulose normally is $(C_6H_{10}O_5)_n$. Cellulose is an insoluble structure. In general, the advantages of cellulose include high specific strength and good thermal stability.

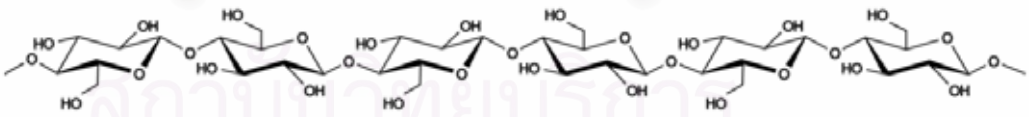


Figure 2.1 Structure of Cellulose (Gardner and Blackwell, 1974).

The cellulose's structure can be defined traditionally as two major types: cellulose I and cellulose II. Both of them have a difference in polarity of the cellulose chains. The backbone conformations of the chains themselves are essentially identical. Cellulose I has

parallel chains whereas cellulose II has alternating antiparallel chains (Gardner and Blackwell, 1974).

2.2 Bacterial Cellulose (BC)

Bacterial cellulose (BC) has generally been fermented by *Acetobacter xylinum* (Schramm *et al*., 1957). *A. xylinum* is a simple Gram-negative bacterium which has an ability to synthesize a large quantity of high-quality cellulose organized as twisting ribbons of microfibrillar bundles (Czaja *et al*., 2006). It can be produced from many different substrates such as Nata de pina and Nata de coco, synthesized by using *A. xylinum* with pineapple water and coconut water as medium, respectively. Morphologically, the reproducible pellicle is obtained by controlling parameters of bacterial growth, for instance, the composition of the culture media, pH, temperature, and oxygen tension.

Glucose is employed as a common substrate. Nonetheless, other simple carbohydrates, alcohols, or polyalcohols can be considered as carbon sources (Brown, 1991). During the process of actual biosynthesis, many carbon compounds of the nutrition medium are utilized by the bacteria, then polymerized into single, linear ß-1,4-glucan chains and finally secreted outside the cells through a linear row of pores located on their outer membrane. Bacteria build bacerial cellulose (BC) and confine themselsves in it to protect themselves from enemies and heavy-metla ions while nutirents can be supplied by diffusion (Sangrungraungroj, 2003).

BC traditionally originates as a white gelatinous pellicle on the surface of the liquid medium in a static culture. These bacteria produce cellulose nanofibrils of 3-8 nm diameters. Together, the mesh of these fibrils forms a gelatinous membrane. The size of BC fibrils is about 100 times smaller than that of plant cellulose shown in Figure 2.2

(Czaja *et al*., 2006). This unique nano-structure results in a larger surface area. Consequently, BC have oustanding tensile strength, high crystallinity, pure fiber network structure, and especially remarkable water holding capacity. It is extremely hydrophilic, absorbing 60 to 700 times its weight in water (Suwanmajo, 2006).

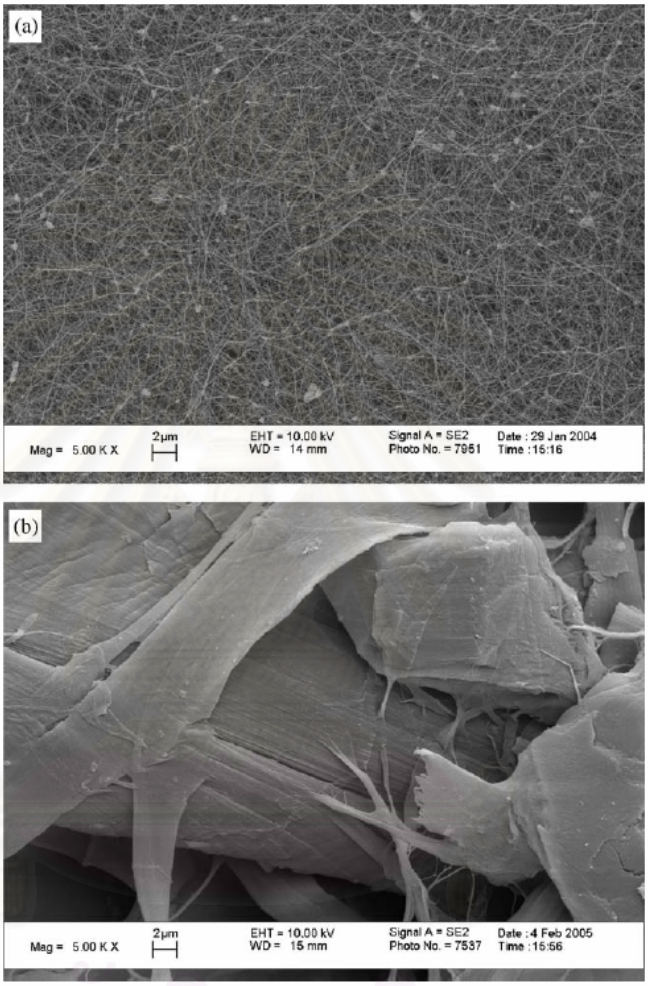


Figure 2.2 A comparison of microfibrillar organization between BC (a) and wood pulp (b).

Many kinds of BC producers is presented in Table 2.1 The polymer structure depends on the organism. *Acetobacter xylinum* is the most representative BC producer.

Genus	Cellulose structure
Acetobacter	extracellular pellicle composed of ribbons
Achromobacter	fibrils
Aerobacter	fibrils
Agrobacterium	short fibrils
Alcaligenes	fibrils
Pseudomonas	no distinct fibrils
Rhizobium	short fibrils
Sarcina	amorphous cellulose
Zoogloea	not well defined

Table. 2.1 BC producers (Jonas and Farah, 1998).

All strains of *Acetobacter xylinum* produce cellulose extracellularly in the form of flat, twisting ribbons. They have been used ordinarily for the production of vinegar from wine, and utilized for the production of gluconic acid, ketogluconic acids and sorbose as well.

Nata de coco is one of the commercially well-known products of BC. There have been a wide range of applications in numerous areas. For example, in the acoustic speaker diaphragms, BC is used to create a sound transducing membrane for meeting the strict requirements. In the field of paper, adding disintegrated BC to paper pulp was able to create a stronger paper. In the food industry, BC is used as a food additive for a chocolate drink in place of xanthan gum. In dialysis membrane, BC shows a significantly higher permeation rate and a greater molecular weight cut-off relative to a commercial dialysis

membrane (regenerated cellulose membrane) (Yamanaka *et al*., 1994; Brown, 1991; Shibazaki *et al.,* 1993).

Svensson *et al*. (2005) demonstrates bacterial cellulose is a promising material for tissue engineering of cartilage and is looked up on as the biomedical applications. Biomedical applications include a bacterial cellulose skin substitute, the replacement of blood vessels, gingiva, and the dura mater during in-vivo animal testing. Moreover, the BC composite material may be used as a biomaterial in orthopedic applications. For example, osteoblasts were used for the in-vitro evaluation of the compatibility of the calcium-deficient hydroxyapatite-BC matrix (Fontana *et al*., 1990).

2.3 Chitin &Chitosan

Chitin is recognized as the second most myriad of polysaccharide after cellulose. Chitin is a white, hard, inelastic, and nitrogenous polysaccharide. Chitin is recognized as one of the main components in the exoskeleton animals. For commercial utilization, most chitin's resources are the crustaceans like crab, prawn, lobster, and shrimp shell waste. The major sources of surface pollution in coast come clearly from chitin.

Chitosan is a natural biopolymer derived from chitin. Chitin and chitosan do not occur in higher plants and higher animals. Chitin is widely distributed in marine like invertebrates, insects, fungi, and yeast. Both of them are a non-toxic, biocompatible, biodegradable, bacteriostatic, and fungistatic substance. Their structure is very much similar to cellulose structure. The structure of chitosan composes of a homopolymer of ß-(1→4)-linked *N*-acetyl-D-glucosamine. Given chemical structures, chitin and chitosan have nearly similar feature as shown in Figure 2.2 (Majeti and Kumar, 2000). That is, chitin is comprised of a linear chain of acetylglucosamine groups whereas chitosan is

obtained by extracting enough acetyl groups (CO- CH_3): this process is called deacetylation.

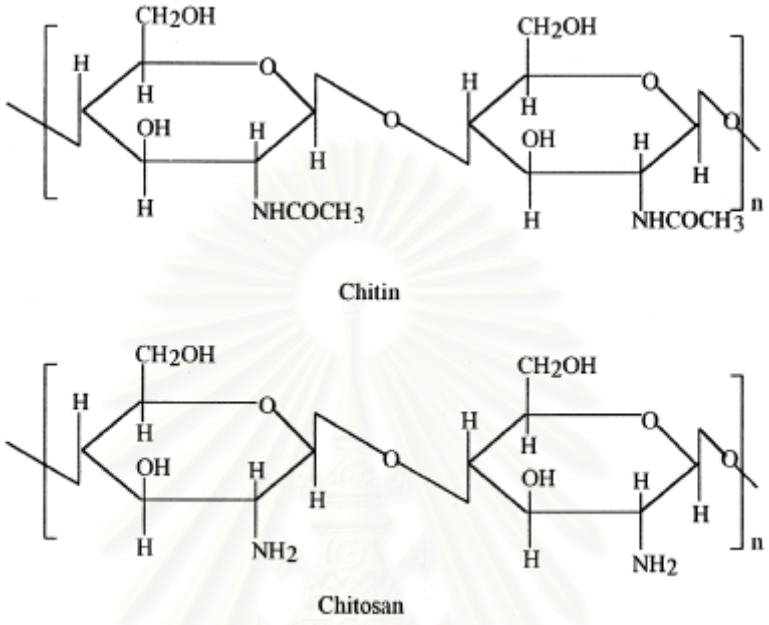


Figure 2.3 Structure of chitin and chitosan.

Like cellulose, chitin functions normally as a structural polysaccharide, but differs from cellulose in its properties. Both chitin and chitosan have unique properties, including polyoxysalt formation, ability to form films, chelate metal ions and optical structural characteristics (Majeti and Kumar, 2000). Chiin is highly crystalline, hydrophobic, and insoluble. Nevertheless, it can be soluble in some organic solvents, for example, hexafluoroisopropanol, hexafluoroacetone, and chloroalcohols. In contrast, chitosan can dissolve in dilute acids such as acetic acid, formic acid, and so on. Hence, chitosan have well chemical and biological qualities that can be used in many medical applications (Whistler, 1983).

The applications of chitosan have been found in many fields, for instance, the wastewater treatment, the photography, the cosmetics, the opthalmology, the agriculture, the paper finishing, the drug-delivery systems, the food industry, and etc (Majeti and Kumar, 2000).

In an artificial skin, chitosan has many distinctive biomedical properties. It reports chitosan, having structural characteristics similar to glycosamino glycans, could be considered for developing substratum for skin replacement (Sandford and stinnes, 1991). Moreover, chitosan may be used to inhibit fibroplasia in wound healing, and to promote both tissue growth and differentiation in tissue culture (Muzzarelli *et al.*, 1999).

Chitosan is useful as a wound management aid to reduce scar tissue. Other applications have also been discovered in controlled release of lactic acid bacteria during butter and cottage cheese production by encapsulating the bacteria in chitosan beads (Majeti and Kumar, 2000).

CHAPTER III

LITERATURE REVIEW

3.1 Medical Application of BC

Usually the characteristics of the modern wound care dressing material are non-toxic, non-pyrogenic, and biocompatible. They are also able to provide barrier against infection, to control fluid loss, to reduce pain during treatment, and to maintain a moist environment in the wound (Czaja *et al*., 2006). BC displays high potential as a new wound healing system. It shows high mechanical strength, purity, uniformity, and the never-dried membrane. The biological dressing of BC is mentioned many advantages: immediate pain relief, good and close adhesion to the wound bed, good barrier against infection, easiness of wound inspection, faster healing, improved exudates retention or reduced time of treatment, as well as reduced costs (Fontana *et al*., 1990). The advantage of the BC includes its transparency, which allows for continuous clinical observation of the healing progress.

Fontana *et al*. (1990) first reported the application of BC as temporary skin substitutes. The product, called Biofills, can provide non-woven, shaped objects in medicine such as artificial arteries, vessels, skin, and etc. It has been still utilized for several skin injury treatments such as basal cell carcinoma/skin graft, severe body burns, facial peeling, sutures, dermabrasions, skin lesions, chronic ulcers, and both donor and receptor sites in skin grafts (Czaja *et al*., 2006).

Mayall *et al*. (1990) used a Biofill skin substitute in the treatment of trophic ulcerations of the limbs and showed that this material was very effective by

shortening the cicatrisation time, reducing the contamination, and saving the cost of treatment.

Artificial skin's BC for burn and skin injuries treatment displays dramatic clinical results such as immediate pain relief, diminished post-surgery discomfort, faster healing, reduced infection rate and reduced treatment time and cost (Fontana *et al*., 1990). In addition, the wound healing effects of never-dried BC are fully biocompatible and also successfully protected burn wounds from excessive external fluid loss, thus accelerating the entire process of healing (Czaja *et al*., 2006).

Alvarez *et al*. (2004) demonstrated the use of BC in the form of a hydrated membrane in the treatment of chronic venous ulcers. BC was more effective than a standard protocol (non-adherent cellulose acetate gauze) in the process of autolytic debridment.

Kucharzewski *et al.* (2003) showed two methods of treating non-healing venous leg ulcers were compared. That is, the experimental group of patients was treated with BC wound dressing (Bioprocess), whereas the control group was treated with Unna's boot hydrocolloid dressing that is widely used in the therapy of these types of wounds. The authors inferred BC wound dressing was more effective in the treatment of the chronic venous leg ulcers than Unna's boot.

3.2 Chitosan for Wound Dressing

Chitosan has been studied widely as a wound dressing material, and known in many medical applications: for instance, implantation, topical ocular, transparent membrane, and wound-healing. It was employed for tissue reconstruction and wound healing. Biochemistry and histology of chitosan in wound healing has been reviewed

by Feofilova *et al*. (1999). Application of chitosan wound dressing made the chronic ulcers heal faster. The graft take was excellent and the re-epithelialization process was faster. It was extremely satisfactory particularly at donor site with painless healing. Chitosan could used to inhibit fibroplasia in wound healing, to promote tissue growth, and to differentiate in tissue culture (Muzzarelli *et al.*, 1999). Moreover, it has an aptitude to stimulate cell proliferation and histoarchitectural tissue organization (Muzzarelli, 1989).

The biological properties like bacteriostatic and fungistatic properties are particularly useful for wound treatment. The antimicrobial properties are particularly useful for wound treatment. Chitosan possess the antimicrobial activity, coming from various factors, such as molecular weight, degree of deacetylation, pH-value, and tempertature. However, the main factors affecting the antibacterial activity of chitosan are molecular weight (MW) and concentration (Liu *et al*., 2006).

Chitosan is effective in inhibiting growth of bacteria. Wang (1992) examined the antibacterial effects of chitosan in vitro against a variety of bacteria typical in food. Chitosan was most effective against *S. aureus, S. typhimurium, E. coli* and *Y. entemlitica.*

Jeon *et al*. (2001) studied the antibacterial effects of three different molecular weight chitosans. Bactericidal activity against all bacteria decreased with decreasing molecular weight.

Fang *et al.* (1994) reported that the growth of *Aspergillus niger* was inhibited by chitosan. Chitosan at the concentration of 5.0 mg/mL induced considerable leakage of UV-absorbing and proteinaceous materials from A. niger at pH 4.8.

Kendra and Hadwiger (1984) examined the antifungal effect of chitosan oligomers on *Fusarium solani* f. sp. *pisi* and *Fusarium solani* f. sp. *phaseoli*. In the

assessment of the minimum concentration (μg/mL) at which no fungal growth was detected, the antifungal activity was found to increase as the polymer size increased.

3.3 Cellulose-chitsoan Blend

The blending of polymers, for new materials with improving chemical and physical properties, has been received considerable attention of many researchers in the past several decades. The final properties of the blends are determined by the miscibility of the polymers, which is greatly favored by formation of intermolecular hydrogen bonds between the component polymers (Yin *et al.*, 2006).

Because of the similar structure between cellulose and chitosan, there may be sufficiently similar to facilitate the formation of homogeneous composite films. It has been reported that the hydrophilic property of chitosan could be modified via blending with PEG and PVA (Kweon and Kang, 1999; Zhang *et al.*, 2002).

Suto and Ui (1996) diplays the chemical cross-linking of chitosan/hydroxypropyl cellulose blends with glyoxal and glutaraldehyde. Based on the similarity of the backbones of chitosan (CHI) and hydroxypropylcellulose (HPC) they assumed that these polymers are miscible in the blend. The cross-linked films were shown to be amorphous, although the uncross-linked films retained cholesteric liquid crystalline order. Chitosan and cellulose blends were prepared using trifluoroacetic acid as a co-solvent (Wu *et al*., 2004)**.** Cellulose-chitosan blends were not well miscible. However, the membranes used as a wound dressing may prevent wound from excessive dehydration. The chitosan-cellulose blend membranes demonstrate effective antimicrobial capability against *Escherichia coli and Staphylococcus aureus*. These results indicate that the chitosan-cellulose blend

membranes may be suitable to be used as a wound dressing with antibacterial properties. Also blending cellulose with chitosan is expected to be a useful method to improve the mechanical properties of chitosan.

Yang *et al.* (2001) reported their composite chitosan–cellulose membranes were prepared by coating chitosan on filter paper. They found the tensile strength of the composite membranes coated with different chitosan concentrations increases with increasing chitosan concentration. The obtained composite membrane provided tensile strength larger than the plain cellulose support, in both wet and dry states.

CHAPTER IV

EXPERIMENTAL

4.1 Materials

4.1.1 Microbial Strains

The *A. xylinum* strain was isolated from *nata de coco*. The stock culture was kindly supplied by Pramote Thammarad, the Institute of Research and Development of Food Product, Kasetsart University, Bangkok, Thailand.

4.1.2 Chitosan

Chitosan -the deacetylated from 85 % to 90%, MW 30000, 80000- was purchased from Seafresh Chitosan (Lab) company limited. Chitosan were prepared by dissolving chitosan in acetic acid (1%, wt/v) solution before taking into culture medium.

4.1.3 Other Chemicals

The details of chemicals used in this experiment are shown in Table 4.1

Table 4.1 The chemicals used in this experiment

Chemical	Supplier
Sucrose	Ajax Finechem
Ammonium sulfate $(NH_4)_2SO_4$	Carlo Erba
Sodium hydroxides (NaOH)	Carlo Erba
Acetic acid	BDH

4.1.3 Equipments.

-Scanning electron microscopy, SEM (JOEL JSM-5410LV, Japan).

-Fourier Transform Infrared (FT-IR) spectrometer (Nicolet SX-170, USA).

-Universal testing machine (LLOYD 2000R, UK).

-X-ray diffraction(Bruker AXS Model D8 Discover,USA).

-Brunauer-Emmett-Teller (BET) surface area analyzer (Model ASAP 2020,USA).

-Autoclave**(**Model Tomy Autoclave SS-325, Ner ima-ku, Tokyo, Japan**).**

4.2 Culture Media and Method

The medium for the inoculum was coconut-water supplemented with 5.0% sucrose, 0.5% ammonium sulfate $(NH_4)_2SO_4$, 1.0% acetic acid, and different chitosan concentration. The experiment was designed to test effects of molecular weight chitosan and chitosan percentages (0, 0.25, 0.5 and 0.75). Adding chitosan in medium was sterilized at 110 ^{0}C for 5 min. Precultures were prepared by a stock culture to 500 mL, and incubated statically at 30 ^{o}C for 7 days. The preculture broth was added to the main culture in medium. The activated medium was inoculated to 75 mL in a Preti-dish and kept at 30 ^{0}C for 7 days.

All sample films were first purified by washing with DI water and then was treated with NaOH at room temperature to remove bacterial cells followed by a rinse

with DI water until pH came to 7. Afterward, the BC film was air-dried at room temperature ($30\ ^0$C) and stored in plastic film at room temperature.

4.3 Characterization of BC-chitosan Film

The BC-chitosan films were characterized by Scanning electron micrographs (SEM) for investigating morphology, by Brunauer-Emmett-Teller (BET) for finding the pore size, porosity, and pore size distribution, by universal testing machine for determining stress-strain curve, by Fourier transform infrared (FT-IR) spectrometer for identifying the chemical structure**,** by X-ray diffraction(XRD) for finding crystallinity index, and also in vitro study of human skin cells (Keratinocytes) on the developed film in individual wells of falcon twenty-four-well plates.

4.3.1 Scanning Electron Microscope (SEM)

The examination of the surface properties was performed by scanning electron microscopy (SEM). Scanning electron micrographs were taken with JOEL JSM-5410LV microscope at Scientific and technological research equipment centre, Chulalongkorn University. The BC films were frozen in liquid nitrogen, immediately snapped, and vacuum-dried. Then, the films were sputtered with gold and photographed. The coated specimens were kept in dry place before experiment. SEM

was obtained at 15 kV which is considered to be a suitable condition since too high energy can be burn the samples.

4.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is used primarily to identify the chemical structure of the sample. FTIR spectra of the membranes were recorded with a Nicolet FT-IR Spectrometer (SX-170) in the region of 4000–500 cm^{-1} ,at Polymer Engineering Laboratory, Chulalongkorn University.

4.3.3 Mechanical testing

In this study, the tensile strength of the film was measured by Instron Testing Instron (5567,NY,USA) at Polymer Engineering Laboratory, Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University. The test conditions follow ASTM D882. The determination of tensile property was done under BC film was cut into strip-shaped specimens 10 mm in width and 10 cm in length. At least five specimens were used for each blend composition.

4.3.4 BET Surface Analysis

Pore size and surface area of the membranes were measured by a Brunauer-Emmett-Teller (BET) surface area analyzer (Model ASAP 2020). The samples were placed in the sample cell, which was then heated up to 75 °C and held at this temperature for 2 hours. The samples were cooled down to room temperature and ready to measure the surface area. There were three steps to measure the surface area: adsorption step, desorption step and calibration step.

4.3.5 Equilibrium Water Content (EWC)

Equilibrium water content (EWC) was determined by immersing the preweighted of dried membrane in distilled water at room temperature until equilibration. The membrane was then removed from the water. After excess water at the surface of the membrane was blotted out with Kimwipes paper, the weight of the swollen membrane was measured and the procedure was repeated until there was no further weight change. Water content was determined by gravimetric method (Kim *et al*., 1996) and calculated using the following formula:

$$EWC(\%) = \frac{W_h - W_d}{W_d} x100$$

Where W_h and W_d denoted the weight of hydrate and dry membrane, respectively.

4.3.6 The Water Vapor Permeability Measurement

Water vapor transmission rate (WVTR) of the BC film and the cellulose-chitosan film with area of 50.00 cm^2, were measured on water vapor permeation tester; Lyssy L80-4000 (at Thailand institute of scientific and technological research). The test conditions follow ISO 15106-1. The determination of WVTR was done under the following conditions: temperature, 38 °C; % Relative Humidity, 90%. The principle of this electronic tester is similar to that of conventional method. One side of the membrane is exposed to the water vapor. As water solubilizes into the membrane and permeates through the sample material, nitrogen sweeps the opposite side of the film and transports the transmitted water vapor molecules to the calibrated infrared sensor. The response is reported as a transmission rate.

4.3.7 Antibacterial Test

The antibacterial test of modified bacterial cellulose against *Escherichia coli* Gram (-) and *Staphylococcus aureus* Gram (+) bacteria was determined. Testing of antibacterial activity of the BC-chitosan films was performed according to the method described by AATCC TM 147-1998 (Anti-bacterial Activity Assessment of Textile Materials: Parallel Streak Method). The samples used for the antibacterial assay were sterilized at 121 ^oC by an autoclave over a period of 30 min. The incubation was 24 hours at 37 ^oC.

4.3.8 Antifungal Test

Testing antifungal activity of the chitosan membranes was performed according AATCC 39-1989(Assessment on Textile Materials: Mildew and Rot Resistance of Textile). The samples used for the antibacterial assay were sterilized at 121 ^{o}C by an autoclave over a period of 30 min. The incubation was used AGAR plate with *Aspergillus niger* for a week of inoculation.

4.3.9 X-Ray Diffraction

X-ray diffraction patterns of the polymers and BC-chitosan film were determined with a diffactometer (Bruker AXS Model D8 Discover). The operation conditions were as follows: power 40 kV and 30 mA. The crystallinity index (C.I.) was calculated from the reflected intensity data using the Segal et al. method, and calculated using the following formula:

$$C.I. = (I_{020}-I_{am})/ I_{020}$$

Where I_{020} is the maximum intensity of the lattice diffraction, and I_{am} was the intensity at 2θ =18◦.

4.3.10 Cell Study

The cytotoxicity and tissue compatibility of BC, chitosan, and BC-chitosan film were kindly evaluated by Jirun khewkan, Department of chemical engineering, faculty of engineering and Assist. Prof. Dr.Neeracha Sanchavanakit, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University. Tissue compatibility was evaluated by growth and spreading of keratinocytes on each material. The test samples were punched into round-shaped samples of 14 mm diameter. The samples were sterilized by autoclaving at 121 °C for 20 min, and transferred aseptically to 24-well culture plates. Proliferations of cells on the films were determined by MTT assay. The experiments were conducted in triplicate. The number of living cells was determined using MTT assay.

CHAPTER V

RESULTS AND DISCUSSIONS

5.1 Cultivating BC-chitosan Film

In static conditions, bacterial cellulose (BC) was synthesized in the form of a pellicle on the surface of a culture medium (Schramm *et al*., 1957). Due to the similarity between BC and chitosan, it was interesting to add chitosan to BC culture medium as it might improve the physical and biological properties of the developed BC-chitosan film. The result of our preliminary test demonstrated that the addition of chitosan of MW 30,000 and 80,000 more than 0.75 percent (w/v) in the culture medium strongly inhibited the formation of BC biosynthesis. Therefore, the experimental study was limited to test the effects of 30,000 and 80,000 MW chitosan supplementation in BC culture medium in the concentration range of 0 – 0.75 % (w/v). The structure, pore morphology, tensile strength, components, chemical structure and biological properties of the developed BC with the addition of chitosan are then investigated and compared with the biosynthesized BC film without chitosan supplementation.

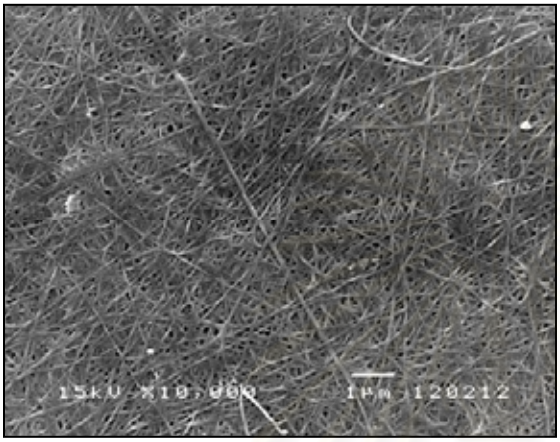
5.2 Surface Morphology

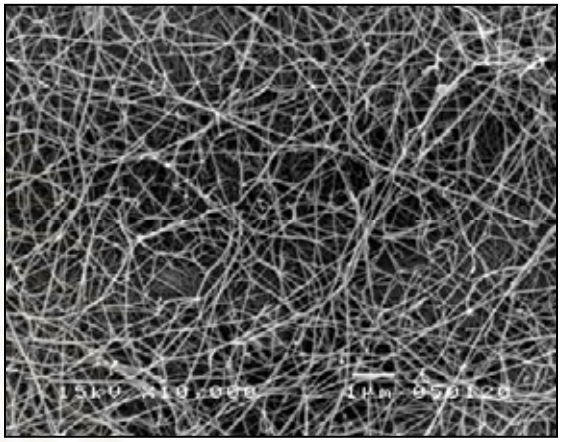
The surface structure of BC and BC-chitosan films was analyzed by scanning electron microscopy. The SEM investigation of the films showed the difference between dried film and swollen film in water. In this study, BC and BC-chitosan film referred to BC film without and with addition of chitosan in culture medium, respectively. MW30000 and MW80000 referred to sample film with the supplementation of chitosan at MW of 30000 and 80000, respectively.

All SEM photographs obtained in static culture were presented in Figure 5.1. Generally, BC shown the well-organized fibril network (Czaja *et al*., 2006). Adding chitosan seemed to be well-bonded into BC fibril network. The pore size of BC-chitosan film decreased with increasing the percent of chitosan. In the same percent of chitosan, the addition chitosan of MW80000 film seem to thicker than that of MW30000.

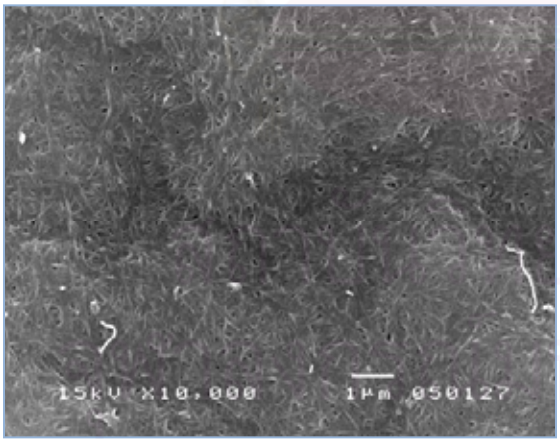
The similar results were described by Yang *et al.* (2002). Their composite chitosan–cellulose membranes were prepared by coating chitosan on filter paper. They concluded the cellulose fiber becomes thicker after adding with chitosan.

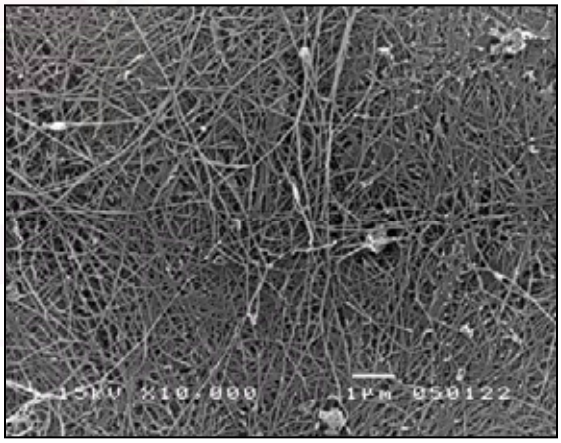
Figure 5.1 The micrograph of surface morphology of dried films (left) and reswollen film (right) with/without addition of chitosan at different MW and concentration.



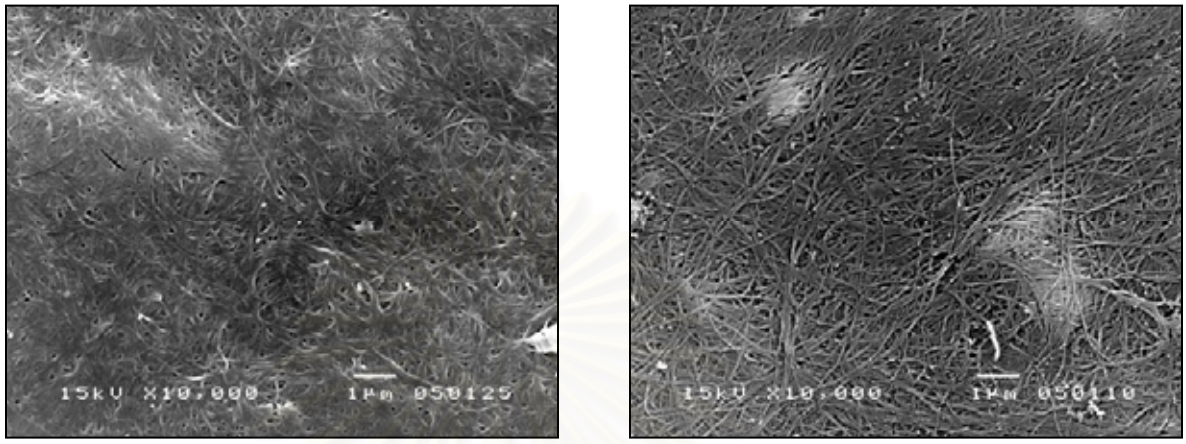


BC from biosynthesis at 0 % Chitosan.

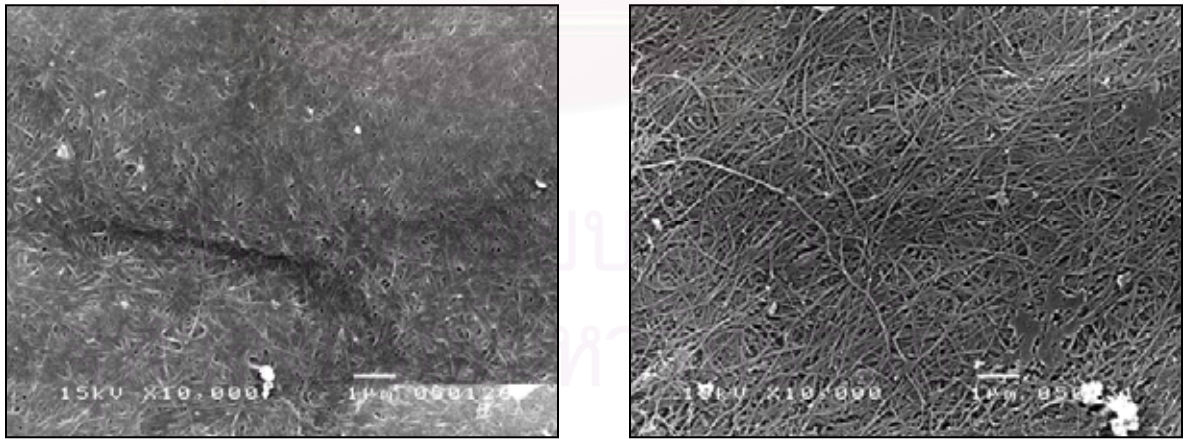




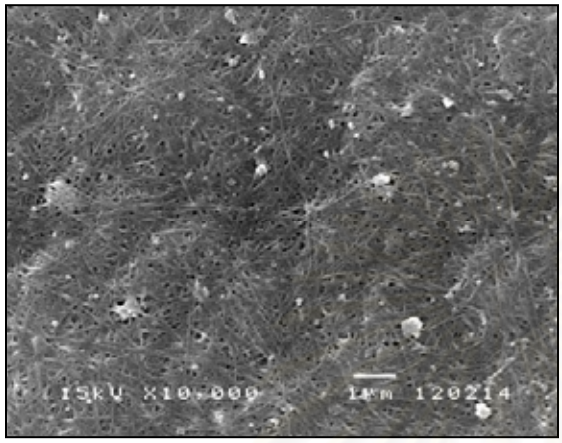
At 0.25% Chitosan of MW30000.

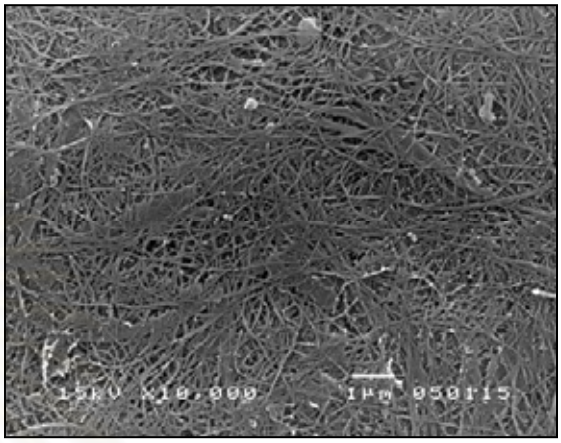


At 0.50% Chitosan of MW30000.

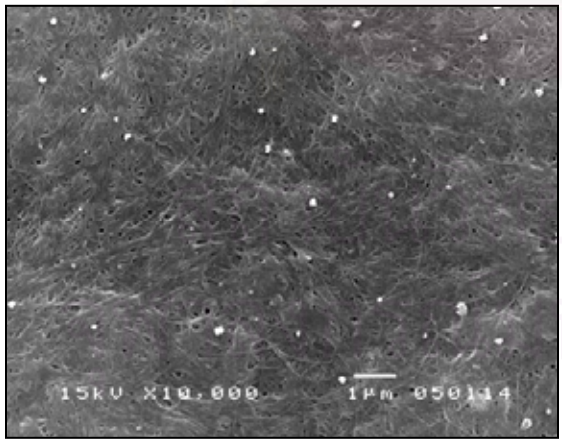


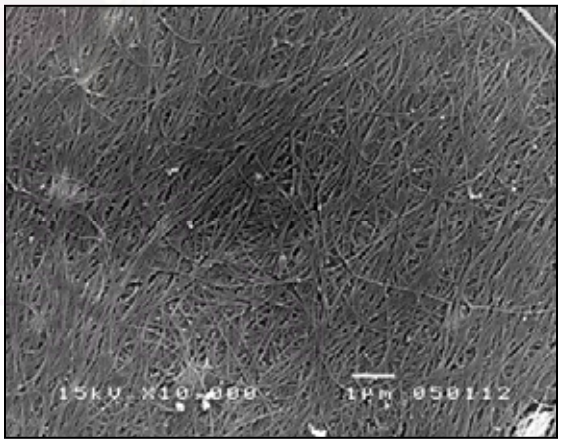
At 0.75% Chitosan of MW30000.



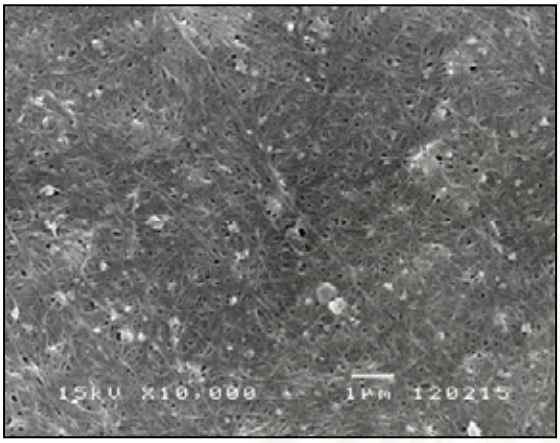


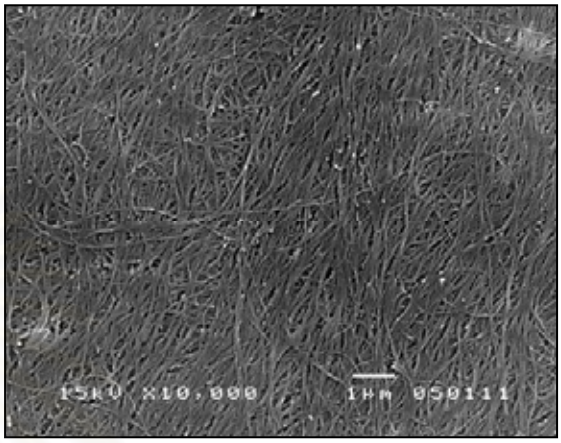
At 0.25% Chitosan of MW80000.





At 0.50% Chitosan of MW80000.





At 0.75% Chitosan of MW80000.

5.3 FTIR Analysis

In this research, the samples of BC and BC-chitosan films were analyzed by FTIR spectroscopy. FTIR spectroscopy has often been utilized as the useful tool in determining specific functional groups or chemical bonds that exist in a material (Lee *et al.*, 1994). As shown in Figure 5.2, the FTIR spectra of all samples was detected at wave number ranging from 1800 to 1500 cm^{-1}. BC-chitosan films demonstrated the adsorption bands at

around 1639.1 cm^{-1} and around 1556 cm^{-1}. The intense absorption in the spectrum of the cellulose was the band at 1639.1 cm^{-1}, which was mostly assigned to glucose carbonyl of cellulose as shown in figure 5.2(a). The characteristic absorption of the chitosan was the band at 1556 cm^{-1}, which was assigned to the amino groups of chitosan as shown in figure 5.2(h). The results indicated that strong intermolecular hydrogen bonding interaction take placed between cellulose and chitosan from biosynthesis culture, leading to a good miscibility film; Namely, the amino groups of chitosan (Figure 5.2(h-b)) were shifted from 1556 cm^{-1} to 1559.8 ,1558.6, 1557.4, 1559.5, 1557.5, and 1557.2, respectively. The similar observation previously was described by Yin *et al.* (2006) in blends of chitosan with two cellulose ethers—hydroxypropylmethylcellulose and methylcellulose by casting from acetic acid solutions. This result shifted to the interaction between the component polymers confirming their molecular miscibility.

Additionally, this result was in good agreement with the conclusion from SEM. In addition, the intensity of this band increased gradually when chitosan concentration in BC-chitosan film increased.

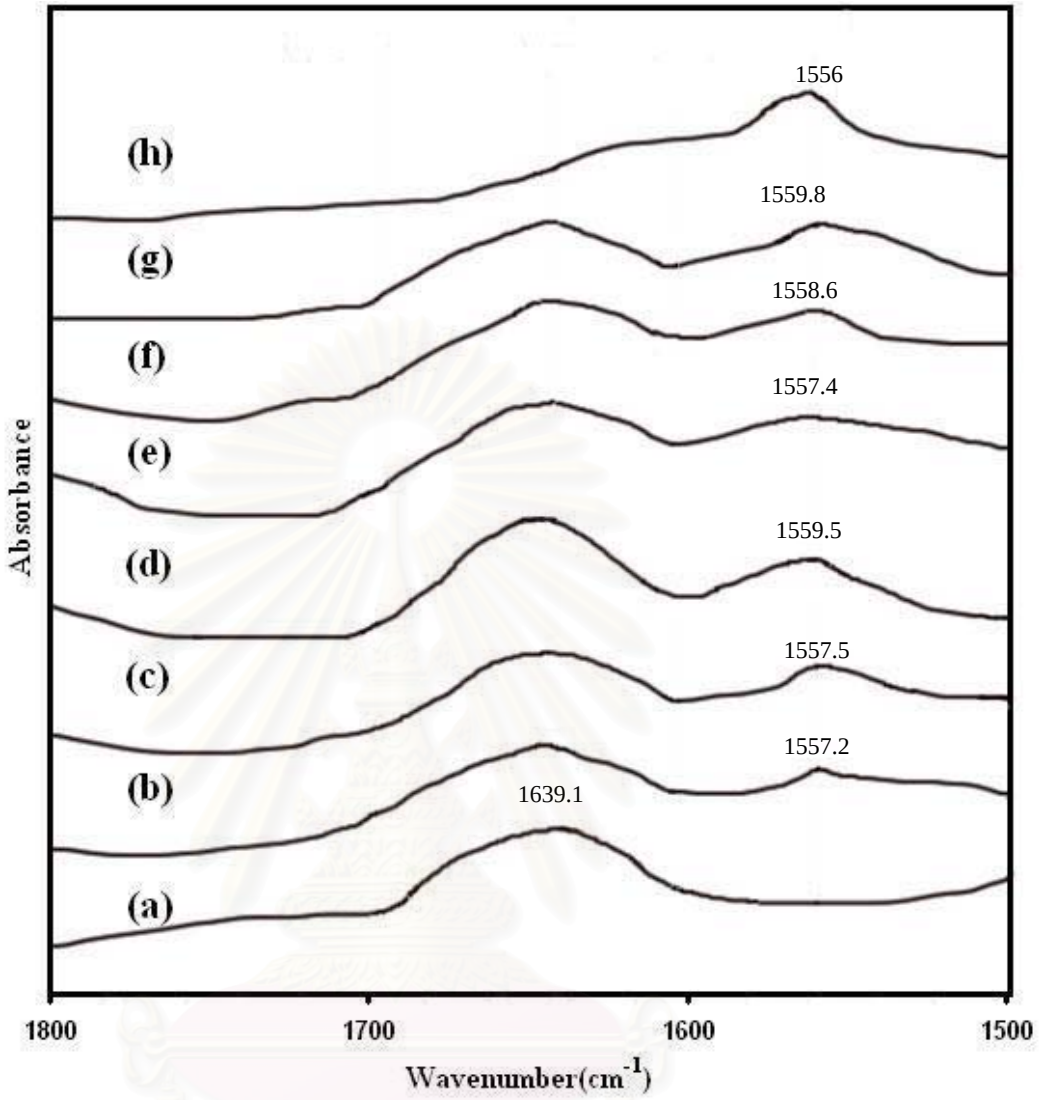


Figure 5.2 The FTIR spectra of BC and BC-chitosan films: (a) BC; (b) 0.25% chitosan of MW30000; (c) 0.50% chitosan of MW30000; (d) 0.75% chitosan of MW30000; (e) 0.25% chitosan of MW80000; (f) 0.50% chitosan of MW80000; (g) 0.75% chitosan of MW80000; (h) chitosan at 80000 molecular weight.

5.4 Mechanical Property

From mechanical analysis of the BC film with average thickness of 0.037 mm, the average tensile strength, Young's modulus, and break strain were 5.30 MPa, 151.71 MPa, and 3.78%, respectively. However, after the film was reswollen in DI water, the average tensile strength, Young's modulus, and break strain became 1.62 MPa, 20.54 MPa, and 8.13%, respectively.

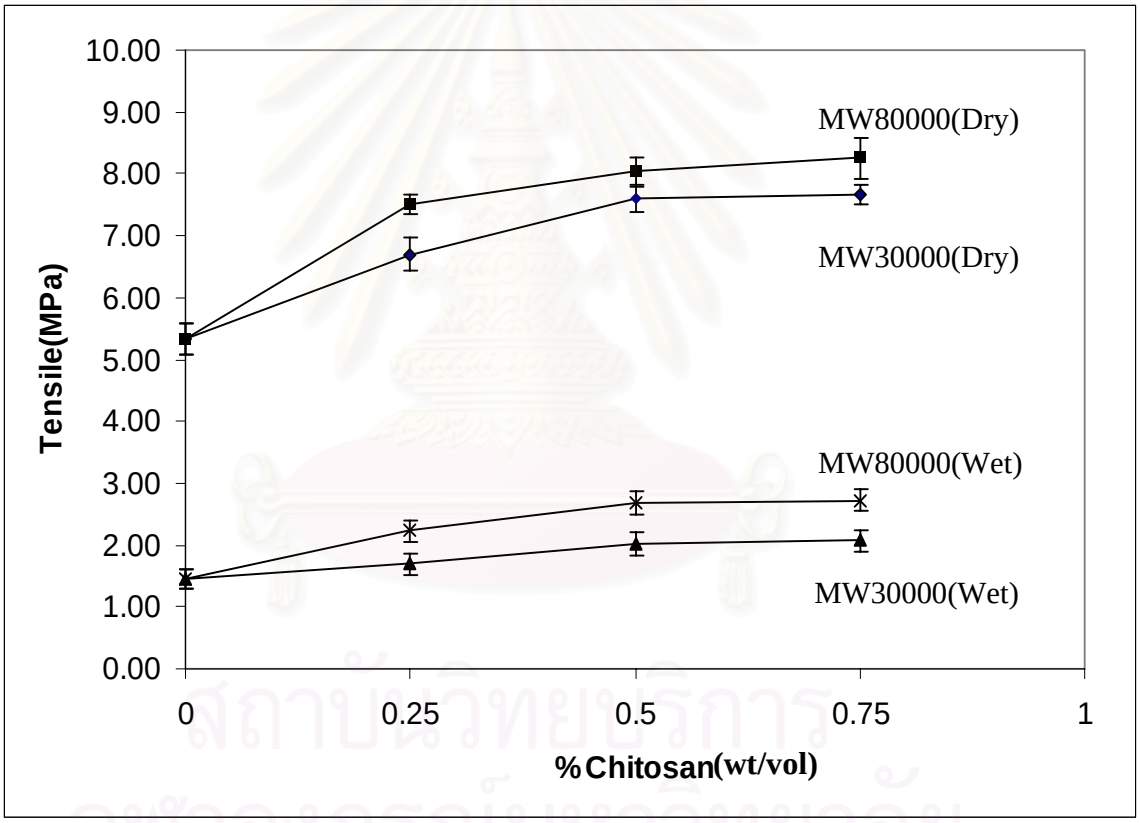


Figure 5.3 Tensile strength of BC-Chitosan film as a function of chitosan content in culture medium.

For dry sample films, Figure 5.3 showed the addition effects of chitosan on the tensile strength. With the thickness of 0.030-0.042 mm, the tensile strength increased with an increase of chitosan content. The maximum average value recorded was 7.66 and 8.26 MPa at addition of 0.75 wt% chitosan both MW30000 and MW80000, respectively.

For wet sample films, Figure 5.3 showed the tendency of tensile strength somewhat corresponded to dry sample. The tensile strength in wet state was lower than that in dry state. The maximum average value recorded was 2.16 and 2.34 MPa at 0.75 wt% chitosan both MW30000 and MW80000, respectively. The tensile strength of the addition of MW80000 was rather higher than that of MW30000.

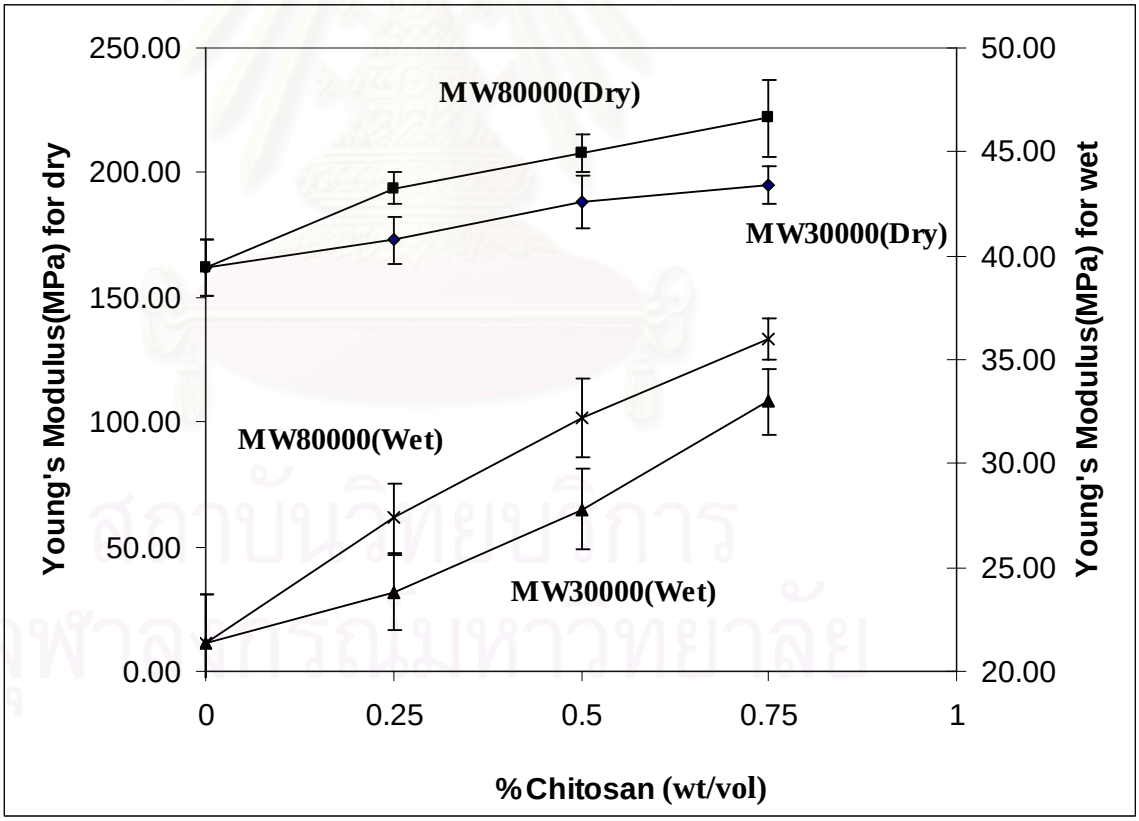


Figure 5.4 Young's modulus of BC-Chitosan film as a function of chitosan content in culture medium.

For dry sample films, Figure 5.4 showed the addition effects of chitosan on the Young's modulus. With the thickness of 0.030-0.042 mm, the Young's modulus increased when the chitosan concentration increased. The maximum average value recorded was 195.0 and 221.8 MPa at 0.75 wt% chitosan both MW30000 and MW80000, respectively.

For wet sample films, Figure 5.4 showed the tendency of the Young's modulus corresponded to dry sample. The young's modulus in wet state was much lower than that in dry state. The maximum average value recorded was 33 and 36 MPa at 0.75 wt% chitosan both MW30000 and MW80000, respectively. Young's modulus of MW80000 was a little higher than that of MW30000.

This result was rather similar to Yang *et al.* (2002). They found the tensile strength of the composite membranes coated with different chitosan concentrations increased with increasing chitosan concentration. They also found the cellulose fiber became thicker after being coated with chitosan, which improves mechanical properties. The fiber in the composite membrane would then withstand a stronger pull force than the cellulose fiber alone. In addition, chitosan network was formed between cellulose fibers in the composite membrane; the pull force on the cellulose fiber would be distributed on the chitosan network. They proved the tensile strength in wet state was much lower than that in dry state due to the swelling of cellulose fiber and chitosan in aqueous solution.

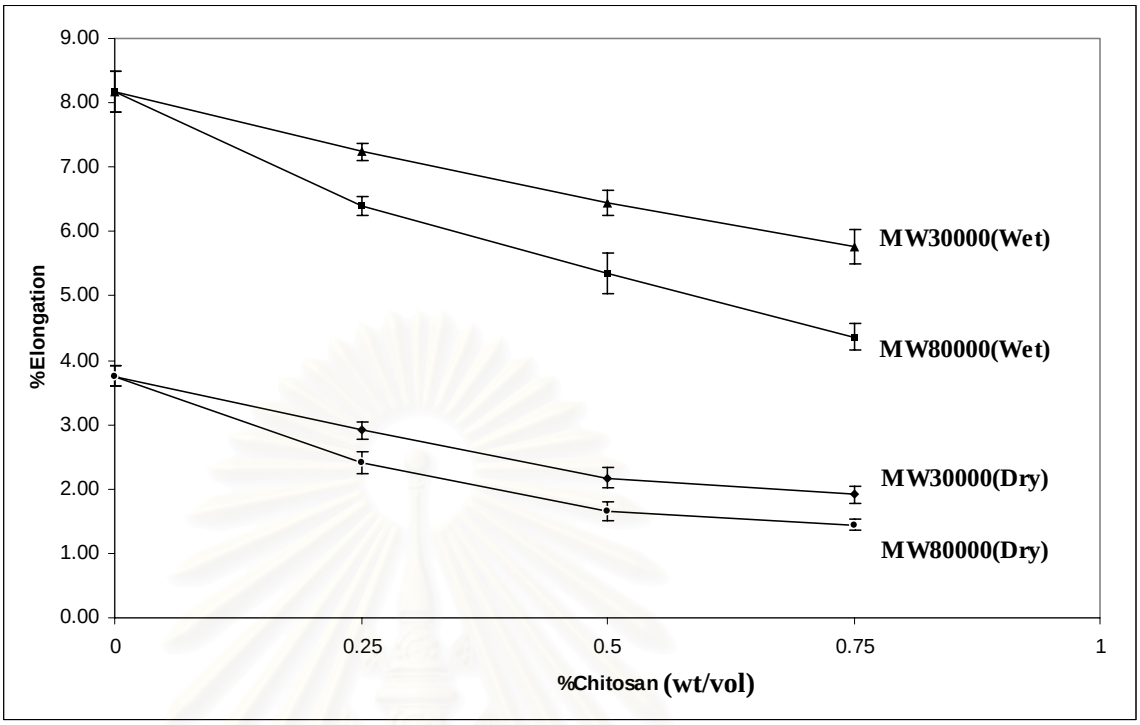


Figure 5.5 The elongation at break of the BC-Chitosan films as a function of chitosan content in culture medium.

For dry sample films, Figure 5.5 showed the addition effects of chitosan on the percentage of elongation at break. With the thickness of 0.030-0.042 mm, the percentage of elongation at break decreased when the chitosan concentration increased. The minimum value of the percentage of elongation at break recorded was 1.91 and 1.44 at 0.75 wt% chitosan both MW30000 and MW80000, respectively.

For wet sample films, Figure 5.4 showed the percentage of elongation at break corresponded to dry sample. We also observed that the percentage of elongation at break in wet state was higher than that in dry state. The minimum value of the percentage of

elongation at break recorded was 5.76 and 4.36 in 0.75 wt% chitosan both MW30000 and MW80000, respectively.

Apparently, chitosan had a good impact on the mechanical properties of BC-chitosan films. Increasing tensile strength and Young's modulus indicated good elasticity of the bacterial cellulose, which was very important from the medical point of view. Elastic dressing which fits the wound site well was good protection against external infection.

5.5 Equilibrium Water Content (EWC)

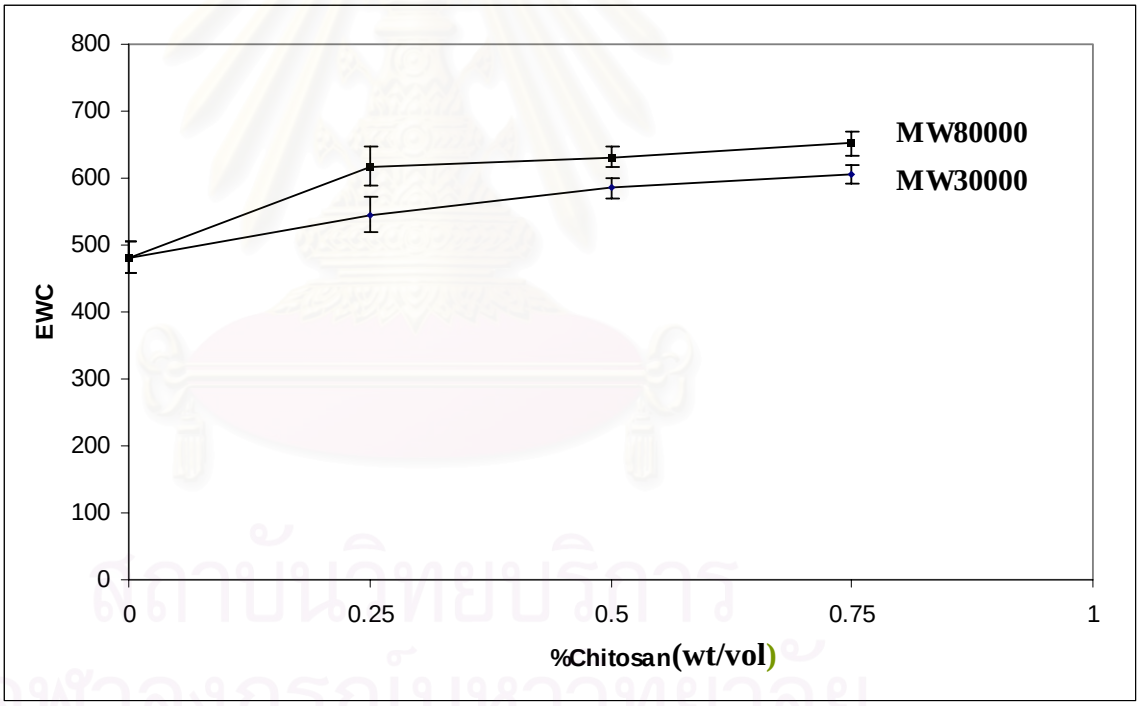


Figure 5.6 The equilibrium water content (EWC) of the BC-Chitosan films as a function of chitosan content in culture medium.

From Figure 5.6, the equilibrium water content of BC was 482%. EWC increased when the chitosan concentration increased. At supplementation of 0.75% both MW30000

and MW80000 showed the maximum increase in equilibrium water content, 606 and 651 %, respectively. This indicated increasing the EWC of the BC-chitosan films depended on the amount of chitosan content because of chitosan provide hydropilic and can incoporate with cellulose fiber well. Overall the equilibrium water content of MW80000 was a little higher than that of MW30000.

5.6 XRD (X-Ray Diffraction)

The XRD pattern of BC and BC-chitosan films was shown in Figure 5.7. Generally, the film XRD pattern of BC demonstrated that the peaks observed at 14.4°, 16.6° and 22.4° were attributed to the BC cultured in static circumstance The broad diffraction peaks observed for BC because BC was not a completely crystalline material (Hong *et al.,* 2005). The diffractograms of MW30000 and MW80000 showed nearly no obvious difference from that of BC.

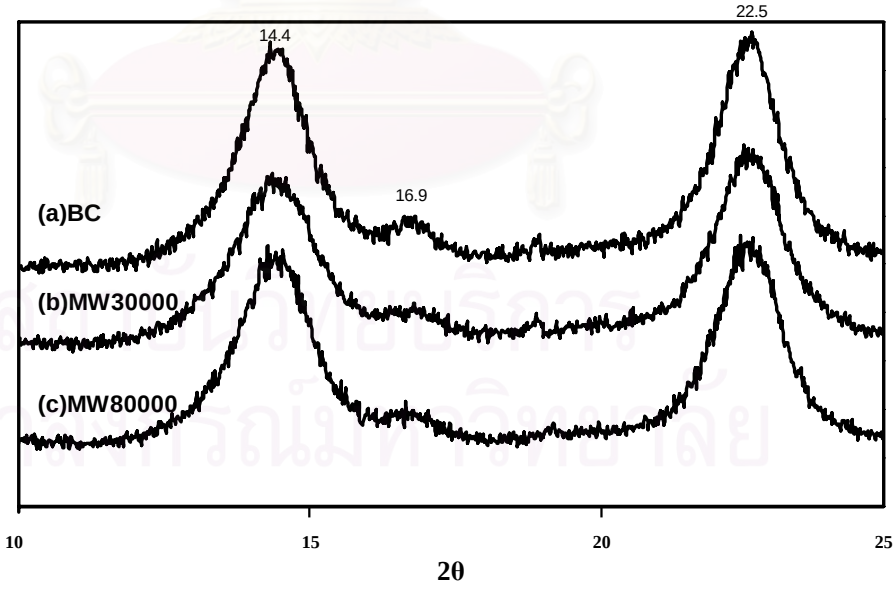


Figure 5.7 X-ray pattern of BC and BC-chitosan films; (a) bacterai cellulose, (b) BC-chitosan film at MW30000, (c) BC-chitosan film at MW80000.

The average crystallinity index value of BC film was slightly higher the that of BC-chitosan film. The average crystallinity index value of the BC-chitosan MW30000, BC-chitosan MW80000, BC was 73.04, 74.86, and 75.16, respectively.

Li *et al.* (2002) prepared cellulose-chitosan film by mixing cellulose with chitosan aqueous solution. They concluded the diffraction peaks of cellulose and cellulose-films showed almost no difference. This proved that small amount of chitosan existing did not influence on the crystallinity of cellulose-chitosan film.

5.7 Porosity

BET is one of the most widely used for finding the surface area of material by physical adsorption of gas molecules (Brunauer, Emmett and Teller, 1938). Surface area and porosity are important characteristics, capable of affecting the quality and utility of many materials.

Sample	Pore diameter(A^o)	Surface area(m^2/g)
BC (Sungruangroj *et al.*, 2006)(dry form)	224	12.6
MW30000 at 0.75% chitosan(dry form)	151	14.2
MW80000 at 0.75% chitosan(dry form)	132	14.8
BC(reswollen form)	612	55.2
MW30000 at 0.75% chitosan(reswollen form)	486	82.3
MW80000 at 0.75% chitosan(reswollen form)	401	98.1

Table 5.1 Surface area and pore diameter of the BC and BC-chitosan analyzed by BET analyzer.

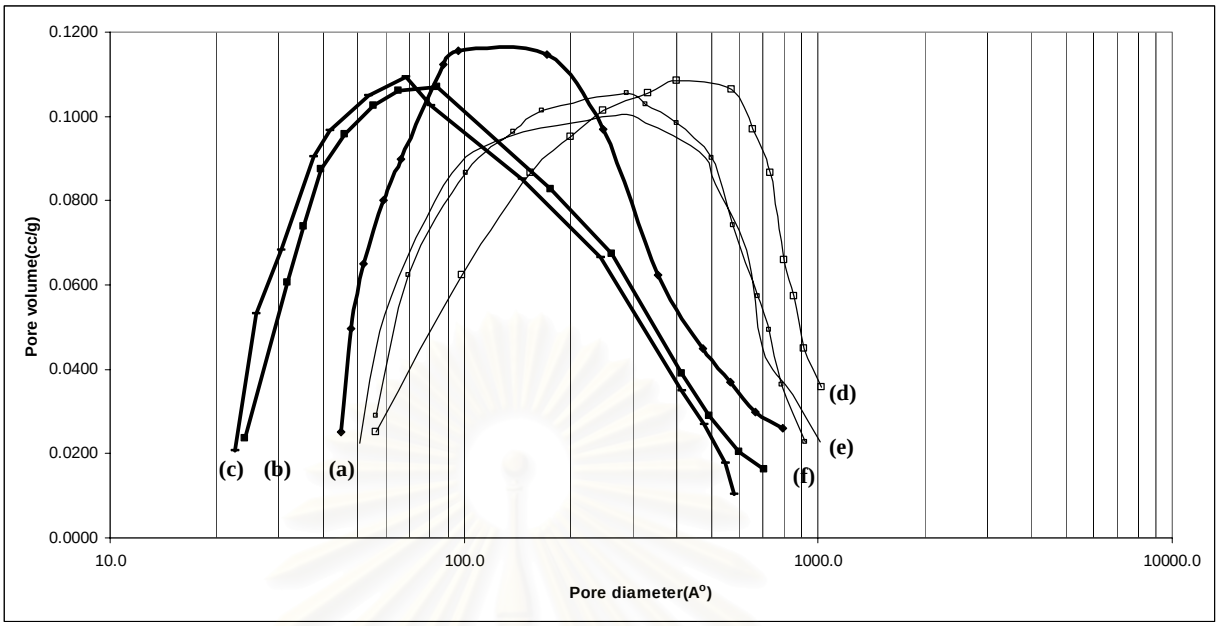


Figure 5.8 The typical pore size distribution of BC and BC-chitosan films; (a) bacterai cellulose, (b) BC-chitosan film at MW30000, (c) BC-chitosan film at MW80000, (d) bacterai cellulose (drying film), (e) BC-chitosan film at MW30000 (drying film) , (f) BC-chitosan film at MW80000 (drying film).

The total surface area and average pore size of the BC film determined by BET were 12.62 m^2/g and 224 Å (Sungruangroj et al., 2006), respectively. As shown in Figure 5.8 and Table 5.1, the result data was shown that BC-chitosan film had the pore sizes much lessen than that of BC while the surface area was slightly increased from the latter. The pore size of the MW30000 and MW80000 was 151 A° and 132 A°, respectively.

For the reswollen form, the total surface area and average pore size of the BC film determined by BET were 55.2 m^2/g, and 612 Å, respectively. The pore size of the

MW30000 and MW80000 was 486 A° and 401 A°, respectively. This result was in good agreement with the conclusion from SEM micrograph.

Yang *et al.* (2002) suggested more chitosan resulted in the formation of denser matrix networks, higher surface area, and smaller pore diameter.

5.8 Water Vapor Transmission Test

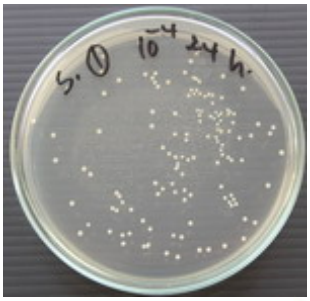
The water vapor transmission rate(WVTR) of BC was 1593 g/m^2 day. The water vapor transmission rate of the MW30000 and MW80000 was 1578 g/m^2 day, and 1564 g/m^2 day, respectively. Therefore, it can conclude, from SEM photograph, that the new nextwork of chitosan and BC had no impact on the water vapor transmission rate of BC-chitosan film.

5.9 Antibacterial Ability

In this study, both *Escherichia coli* and *Staphylococcus aureus* were used as the test bacteria to examine the antibacterial properties of BC and BC-chitosan films. The antimicrobial effect of chitosan in different concentration was shown in Table 5.2 and Figuer 5.9. The antibacterial properties of BC, BC-chitosan MW30000 and BC-chitosan MW80000 had no the impact on *Escherichia coli* and *Staphylococcus aureus*.

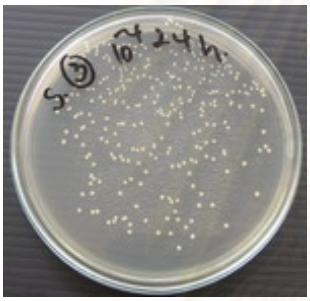
Test Microorganisms	Sample	Antimicrobial effect
Staphylococcus aureus	BC	No effect
	MW30000 at 0.50%chitosan	No effect
	MW30000 at 0.75%chitosan	No effect
	MW80000 at 0.50%chitosan	No effect
	MW80000 at 0.75%chitosan	No effect
Escherichia coli	BC	No effect
	MW30000 at 0.50%chitosan	No effect
	MW30000 at 0.75%chitosan	No effect
	MW80000 at 0.50%chitosan	No effect
	MW80000 at 0.75%chitosan	No effect

Table 5.2 The antimicrobial effect of BC and BC-Chitosan films.

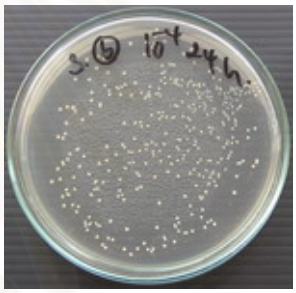


BC on *S.aureus*

BC on *Escherichia col*

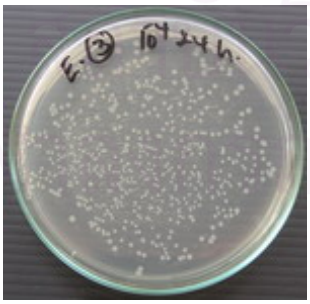


MW30000 at 0.50%chitosan



MW80000 at 0.50%chitosan

on *S.aureus*

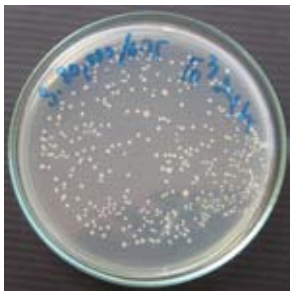


MW30000 at 0.50%chitosan

MW80000 at 0.50%chitosan

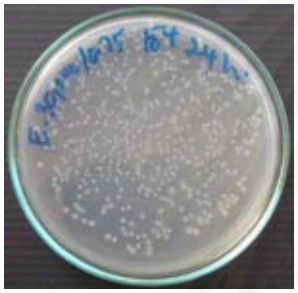
on *Escherichia col*

MW30000 at 0.75%chitosan



MW80000 at 0.75%chitosan

on *S.aureus*



MW30000 at 0.75%chitosan

MW80000 at 0.75%chitosan

on *Escherichia col*

Figure 5.9 The number of bacteria from samples at one day incubated at 37^{o}C.

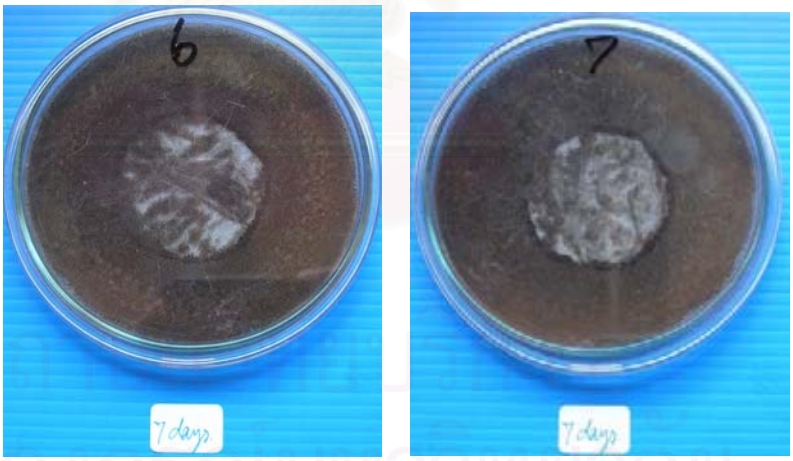
5.10 Antifungal Ability

In this study, *Aspergillus niger* was used as the test fungai to examine the antifungal activity of BC and BC-chitosan films. The antimicrobial effect of chitosan in different concentration was shown in Table 5.3 and Figure 5.10. BC-chitosan film, at MW80000 in 0.75% chitosan, only inhibited the growth of *Aspergillus niger*. On the basis of this observation, it could be proposed that the antifungal activity of BC-chitosan film slightly improved by using at MW80000 in 0.75% chitosan content.

Fungi	Sample	Observed growth
		Result
Aspergillus niger	BC	Medium growth
	BC-chitosan MW30000 at 0.50% chitosan	Medium growth
	BC-chitosan MW30000 at 0.75% chitosan	Medium growth
	BC-chitosan MW80000 at 0.50% chitosan	Medium growth
	BC-chitosan MW80000 at 0.75% chitosan	Light growth

Table 5.3 The antifungal activity of BC and BC-chiotsan film on *Aspergillus niger* activity at the end of the incubation 7 days.

BC MW30000 at 0.50% chitosan MW30000 at 0.75% chitosan



MW80000 at 0.50% chitosan MW80000 at 0.75% chitosan

Figure 5.10 The growth of *Aspergillus niger* on the specimens, at 30^{o}C at the end of the incubation 7 days.

5.11 Cell Study

We evaluated the effect of BC and BC-chitosan films on cell proliferation. For HaCat, the percentage of living cells after seeding on BC film for 0, 24 and 48 h was comparable to the cells cultured on the polystylene culture plate(control) as shown Figure 5.11

Our results indicated that the BC-chitosan film had no toxicity and supported cell proliferation. BC supported the most growth and spreading of HaCat. MW30000 and MW80000 slightly decreased the growth of HaCat when comapared to BC counterpart. However, the normal cell proliferation and spreading of MW30000 is slightly better than that of MW 80000.

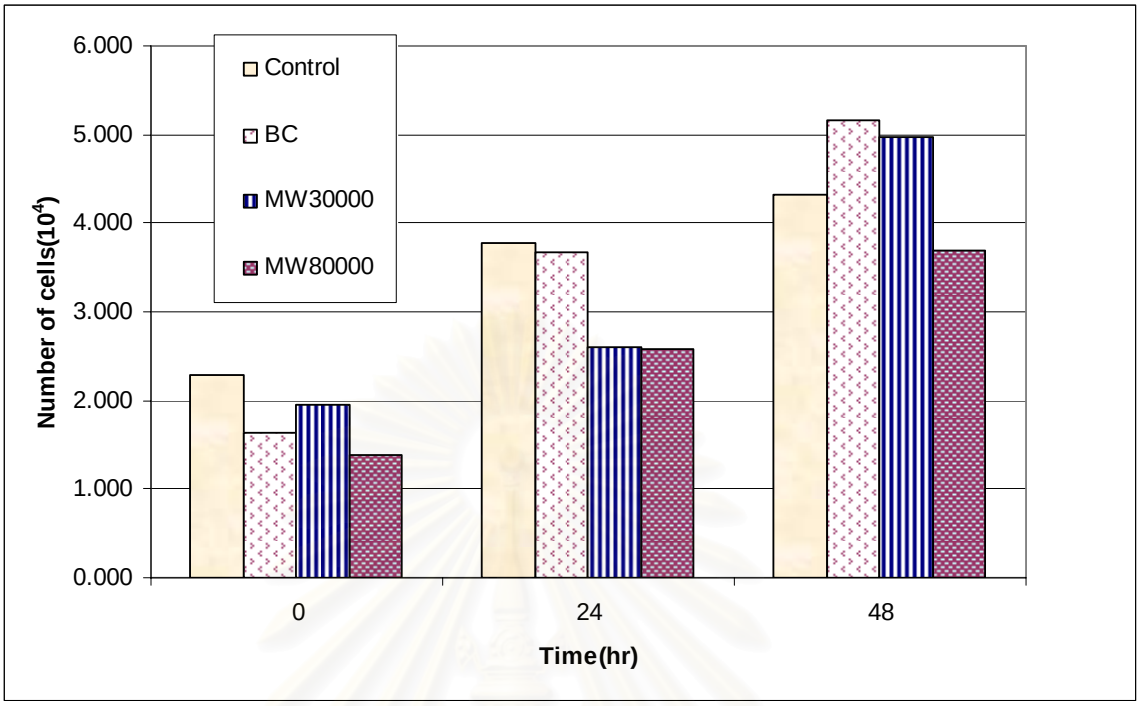


Figure 5.11 Proliferations of HaCat on control, BC, chitosan, MW30000, MW80000 film at 0, 24, and 48 hr of culture by MTT assay.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Modifying bacterial cellulose with chitosan (BC-chitosan) during its biosynthesis resulted a number of valuable features: high mechanical properties in wet and dry state, high the equilibrium water content (EWC), and high average surface area. From SEM micrograph of surfaces, we found at 0.75 % chitosan, both MW30000 and MW80000 were the homogenious and optimize properties.

However, BC-chitosan film had not impact significantly on some properties: the antibacterial ability, water vapor transmission rates, average crystallinity index, and antifungal ability.

FTIR spectroscopy was used to evaluate the interaction between BC fiber and chitosan molecules. This result indicated that interactions presented between the hydroxyl groups of BC fiber and the amino groups of chitosan.

For BC film, the tensile strength, Young's modulus, the elongation at break, the equilibrium water content(EWC), the average crystallinity index, and the average pore diameter of BC film were 5.30 MPa, 151.71 MPa, 3.78%, 482%, 75.16 A^o, and 224 A^o, respectively.

For MW30000 at 0.75% chitosan , the tensile strength, Young's modulus , the elongation at break, the equilibrium water content(EWC), the average crystallinity index, and the average pore diameter of BC film were 7.66 MPa, 195 MPa, 1.91%, 606%, 73.04 A^o, and 151A^o, respectively.

For MW80000 at 0.75% chitosan , the tensile strength, Young's modulus , the elongation at break, the equilibrium water content(EWC), the average crystallinity index, and the average pore diameter of BC film were 8.26 MPa, 221 MPa, 1.44%, 652%, 74.86 A^o, and 132 A^o, respectively.

In the preliminary cell study, our results indicated that the BC-chitosan film had no toxicity and supported cell proliferation. That is, BC supported the most growth and spreading of HaCat. MW30000 and MW80000 slightly decreased the growth of HaCat when comapared to BC counterpart. However, the normal cell proliferation and spreading of MW30000 is slightly better than that of MW 80000.

6.2 Recommendations for future studies.

Based on this study, further studies for the improvement of bacterial cellulose film are recommended.

1. The study of the method of impregnation silver nanoparticles into bacterial cellulose for achieving an antimicrobial activity.

2. The study of modifying bacterial cellulose by synthesizing with other polysaccharides such as alginate, pectin, amylose, strach, xanthan, emulsan, carrageenan, agar and so on.

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APPENDICES

Appendix A

Supercritical Drying Method

In this study, supercritical drying method was used for the preparation of porous BC and BC-chitosan film. Firstly, the film were dipped in distilled water for 24 hours. After that, to replace water with ethanol, the swollen films were immersed in 10, 30, 50, 70 % (w/v) ethanol for 30 mins in each step and in 100 % (w/v) ethanol for 1 h, respectively. Lastly, the swollen films were dried by using supercritical drying method.

In supercritical drying method, the films were placed in a vessel inside the high-pressure cell with inner diameter 10 cm. The cell was immediately filled with supercritical CO_2 and controlled at temperature = 40 °C and pressure = 1200 Psi (the critical point of carbon dioxide; P_c = 1072 Psi and T_c = 31 °C). Temperature and pressure were selected such that the CO_2 and ethanol inside the films were fully miscible. Subsequently, the cell was flushed by adding fresh CO_2 at the same conditions of pressure and temperature in order to replace the residual ethanol inside. The addition was performed for 2 hours and then the system was slowly depressurized at a constant rate of 150 psi/min to remove CO_2.

APPENDIX B

Table B1 Data of Figure 5.3.

Chitosan MW30000 content (%wt)	Tensile strength (MPa) for dry film						
	1	2	3	4	5	Average	SD
0	5.63	5.53	5.32	5.02	5.12	5.32	0.26
0.25	6.85	6.89	6.75	6.21	6.81	6.70	0.28
0.5	7.93	7.55	7.46	7.52	7.49	7.59	0.19
0.75	7.66	7.89	7.61	7.48	7.66	7.66	0.15

Table B2 Data of Figure 5.3.

Chitosan MW80000 content (%wt)	Tensile strength (MPa) for dry film						
	1	2	3	4	5	Average	SD
0	5.63	5.53	5.32	5.02	5.12	5.32	0.26
0.25	7.52	7.77	7.41	7.34	7.5	7.51	0.16
0.5	8.18	7.82	8.05	8.35	7.86	8.05	0.22
0.75	8.33	8.23	8.77	7.86	8.11	8.26	0.33

Table B3 Data of Figure 5.3.

Chitosan MW30000 content (%wt)	Tensile strength (MPa) for wet film						
	1	2	3	4	5	Average	SD
0	1.31	1.63	1.44	1.62	1.26	1.45	0.17
0.25	1.88	1.81	1.75	1.62	1.44	1.70	0.17
0.5	1.77	2.05	2.23	2.15	1.86	2.01	0.19
0.75	2.11	1.93	2.24	1.85	2.22	2.07	0.17

Table B4 Data of Figure 5.3.

Chitosan MW80000 content (%wt)	Tensile strength (MPa) for wet film						
	1	2	3	4	5	Average	SD
0	1.31	1.63	1.44	1.62	1.26	1.45	0.17
0.25	2.21	2.41	2.01	2.01	2.48	2.22	0.22
0.5	2.77	2.98	2.51	2.78	2.42	2.69	0.23
0.75	2.48	2.61	2.66	2.87	2.99	2.72	0.21

Table B5 Data of Figure 5.4.

Chitosan MW30000 content (%wt)	Young's Modulus(MPa) for dry film						
	1	2	3	4	5	Average	SD
0	168	153	175	148	165	161.80	11.08
0.25	172	188	162	174	169	173.00	9.54
0.5	190	171	188	195	198	188.40	10.50
0.75	201	197	182	194	201	195.00	7.84

Table B6 Data of Figure 5.4.

Chitosan MW80000 content (%wt)	Young's Modulus(MPa) for dry film						
	1	2	3	4	5	Average	SD
0	168	153	175	148	165	161.80	11.08
0.25	192	201	184	195	197	193.80	6.38
0.5	205	198	210	208	218	207.80	7.29
0.75	221	204	242	232	210	221.80	15.56

Table B7 Data of Figure 5.4.

Chitosan MW30000 content (%wt)	Young's Modulus(MPa) for wet film						
	1	2	3	4	5	Average	SD
0	25.22	20.13	19.24	22.85	21.53	21.79	2.36
0.25	26.23	22.82	25.56	22.78	24.53	24.38	1.57
0.5	30.25	27.26	29.24	25.78	28.11	28.13	1.73
0.75	32.56	31.53	35.83	34.48	33.78	33.64	1.67

Table B8 Data of Figure 5.4.

Chitosan MW80000 content (%wt)	Young's Modulus(MPa) for wet film						
	1	2	3	4	5	Average	SD
0	25.78	20.82	19.24	22.75	21.44	22.01	2.46
0.25	30.12	26.97	25.89	28.12	26.44	27.51	1.68
0.5	35.14	32.56	33.41	30.98	30.11	32.44	1.99
0.75	34.59	36.82	37.55	36.55	35.12	36.13	1.23

Table B9 Data of Figure 5.5.

Chitosan MW30000 content (%wt)	Elongation at break (%) for dry film						
	1	2	3	4	5	Average	SD
0	3.78	3.99	3.68	3.55	3.77	3.75	0.16
0.25	2.77	2.88	2.81	2.98	3.1	2.91	0.13
0.5	2.02	2.16	2.33	2	2.33	2.17	0.16
0.75	2.11	1.96	1.72	1.9	1.86	1.91	0.14

Table B10 Data of Figure 5.5.

Chitosan MW80000 content (%wt)	Elongation at break (%) for dry film						
	1	2	3	4	5	Average	SD
0	3.78	3.99	3.68	3.55	3.77	3.75	0.16
0.25	2.53	2.43	2.41	2.56	2.12	2.41	0.17
0.5	1.66	1.88	1.55	1.63	1.52	1.65	0.14
0.75	1.42	1.55	1.32	1.41	1.51	1.44	0.09

Table B11 Data of Figure 5.5.

Chitosan MW30000 content (%wt)	Elongation at break (%) for wet film						
	1	2	3	4	5	Average	SD
0	8.42	7.89	8.22	8.52	7.79	8.17	0.32
0.25	7.22	7.39	7.22	7.33	7.03	7.24	0.14
0.5	6.51	6.66	6.25	6.56	6.21	6.44	0.20
0.75	5.32	5.99	5.88	5.88	5.72	5.76	0.26

Table B12 Data of Figure 5.5.

Chitosan MW80000 content (%wt)	Elongation at break (%) for wet film						
	1	2	3	4	5	Average	SD
0	8.42	7.89	8.22	8.52	7.79	8.17	0.32
0.25	6.21	6.31	6.55	6.44	6.51	6.40	0.14
0.5	5.11	5.38	5.88	5.09	5.31	5.35	0.32
0.75	4.32	4.67	4.29	4.1	4.44	4.36	0.21

Table B13 Data of Figure 5.6.

Chitosan MW30000 content (%wt)	Equilibrium Water Content (%)						
	1	2	3	4	5	Average	SD
0	476	502	470	450	510	482	24
0.25	555	538	542	510	583	546	27
0.5	591	582	570	572	609	585	16
0.75	612	599	586	625	607	606	15

Table B14 Data of Figure 5.6.

Chitosan MW80000 content (%wt)	Equilibrium Water Content (%)						
	1	2	3	4	5	Average	SD
0	476	502	470	450	510	482	24
0.25	608	666	590	612	612	618	29
0.5	638	622	610	637	651	632	16
0.75	658	662	620	652	666	652	18

Table B15 Data of Figure 5.11.

Number of Cell *10^4					
Type of film		Plastic	BC	MW30000	MW80000
0 hr	1	2.283	1.510	1.865	1.427
	2	2.303	1.608	2.004	1.371
	3	2.269	1.781	1.962	1.336
	Average	2.285	1.633	1.944	1.378
24 hr	1	3.612	3.730	2.770	2.735
	2	3.431	3.688	2.380	2.380
	3	4.273	3.605	2.672	2.596
	Average	3.772	3.674	2.607	2.570
48 hr	1	4.308	4.509	4.426	4.370
	2	3.486	5.539	5.581	4.217
	3	5.164	5.442	4.920	2.498
	Average	4.319	5.164	4.976	3.695

VITAE

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