การพัฒนาและวิเคราะห์คุณลักษณะของฟิล์มไคโตซานผสมแบคทีเรียเซลลูโลส

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DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN BLENDED BACTERIAL CELLULOSE FILM

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กัมพล อินทสร: การพัฒนาและวิเคราะห์คุณลักษณะของฟิล์มไคโตซานผสมแบคทีเรียเซลลูโลส. (DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN BLENDED BACTERIAL CELLULOSE FILM) อ.ที่ปรึกวิทยานิพนธ์หลัก: รศ.ดร.เหมือนเดือน พิศาลพงศ์, 75 หน้า.

การพัฒนาบรรจุภัณฑ์อาหารที่สามารถย่อยสลายได้ทางชีวภาพซึ่งผสานคุณสมบัติในการยับยั้งเชื้อจุลินทรีย์ โดยใช้ฟิล์มองค์ประกอบผสมไคโตซานกับแบคทีเรียเซลลูโลส(CBC) เตรียมโดยการผสมไคโตซานในสารละลายกรดอะซิติกและสารละลายข้นของแบคทีเรียเซลลูโลส ฟิล์มองค์ประกอบผสมทางชีวภาพนี้ถูกเตรียมด้วยวิธีการตากแห้ง และ ศึกษาคุณลักษณะทางกายภาพ, ทางกล, ตลอดคุณสมบัติในการยับยั้งเชื้อจุลินทรีย์

พบว่าคุณสมบัติทางกายภาพและทางกลของฟิล์ม CBC ถูกปรับปรุงดีขึ้นเมื่อเปรียบเทียบกับฟิล์มไคโตซานบริสุทธิ์ ซึ่งขึ้นอยู่กับน้ำหนักโมเลกุลของไคโตซานและปริมาณการเติมสารละลายข้นของแบคทีเรียเซลลูโลส ผลจากการทดสอบด้วยอินฟราเรดทางโครงสร้างโมเลกุลพบว่า โมเลกุลของไคโตซานและแบคทีเรียเซลลูโลสมีปฏิสัมพันธ์ระหว่างกัน ความเป็นผลึก และการดูดซึมไอน้ำเพิ่มขึ้นในอัตราส่วนประมาณ 10:6 ถึง 10:8 ของไคโตซานต่อแบคทีเรียเซลลูโลสโดยน้ำหนัก ฟิล์ม CBC ยังสามารถแสดงถึงการยับยั้งเชื้อแบคทีเรียชนิดแกรมบวก *สแตปฟิโลคอคคัส ออเรียส* นอกจากนี้ได้แสดงให้เห็นว่ามีการปรับปรุงโครงสร้างของฟิล์ม CBC และมีการเกิดปฏิสัมพันธ์เชื่อมโยงระหว่างไอออนของไคโตซานกับโครงสร้างตาข่ายเส้นใยของแบคทีเรียเซลลูโลสซึ่งมีประสิทธิภาพในการควบคุมการปลดปล่อยไคโตซานออกจากฟิล์ม CBC ดังนั้นการพัฒนาฟิล์มไคโตซานโดยการผสมแบคทีเรียเซลลูโลสได้แสดงคุณลักษณะที่จะพัฒนาเป็นวัสดุบรรจุภัณฑ์ทางอาหารได้

ภาควิชา.....วิศวกรรมเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....วิศวกรรมเคมี..... ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2555.....

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KAMPOLE INTASORN: DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN BLENDED BACTERIAL CELLULOSE FILM. ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D.75 pp.

In order to develop a bio-degradable food packaging film combined the preservative functions of antimicrobials; the chitosan/bacterial cellulose (CBC) composite film was developed by blending the chitosan in acetic acid solution and bacterial cellulose (BC) slurry. The composite bio-films were prepared by casting method and were characterized for physical, mechanical, and antimicrobial properties.

It was found that the physical and mechanical properties of CBC films were improved in comparison to those of the chitosan pure film, which was remarkably dependent on molecular weight of chitosan and the amount of BC content. The FTIR result indicated the intermolecular interaction between chitosan and BC. The crystallinity and the water absorption capacity were also enhanced with the BC content up to the weight ratio of chitosan: BC around 10:6-10:8. The CBC film exhibited the antimicrobial activity against the gram positive bacteria, *Staphylococcus aureus*. It was shown that the modified CBC film structure and interactions in ionic-crosslinked chitosan combined with interactions with BC fibril networks were effective for the control and limit of chitosan release from the CBC films. The modified chitosan films by blending with BC show the benefit properties for further development as food packaging material.

Department :…Chemical Engineering.. Student's Signature ……
Field of Study :…Chemical Engineering.. Advisor's Signature ……
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CHAPTER I
INTRODUCTION

1.1 Introduction

Currently, over 100 million tons of plastics are produced annually worldwide. Consumption and production of plastics have been continuously rising, so as to increase the amount of plastic waste. The management of plastic waste associated with serious environmental pollution from waste disposal and undegraded polymers (Shalleh et al., 2007). To reduce the problem of plastic waste disposal, biodegradable polymers or biodegradable plastics have to be developed as substitutes for non-biodegradable plastics.

In food packaging, natural polysaccharides have been widely applied to produce degradable films. In particular, there has been a growing interest in starch, cellulose and their derivatives (Weber, 2002) with an abundant agricultural surplus raw material since it can be produced at low cost with a large scale, non-allergic and thermal processability (L. Famá et al., 2004). A packaging material with extensively antimicrobial properties is also required and desirable for general use to improve the storage stability of various foods. For this purpose, the integration of antimicrobial substances in packaging material can be useful. Because of good film forming and antimicrobial properties of chitosan, chitosan-based materials have been developed for antimicrobial packaging films (Ban et al., 2005).

Chitosan is a natural biopolymer derived by deacetylation of chitin, which is composed of (1,4)-linked 2- amino-deoxy-β-D-glucan (Darmadji et al, 1994). It is a major component of the shells of crustacean such as crab, shrimp and crawfish. It has received much attention for commercial applications in food and biomedicine. Chitosan is non-toxic, biodegradable, biofunctional and biocompatible (Knorr, 1984; Jayakumar et al, 2005). Moreover, it also has antibacterial properties (Kendra et al., 1984; Sudarshan et

al., 1992). Nevertheless, uses for chitosan films have been limited because of its weak mechanical properties. Many works focused on improvement of properties of chitosan films. The properties of chitosan films could be improved by addition of other polymers (Hima et al., 2010). The effect of chitosan concentration and type of solvent on the mechanical properties of chitosan has been studied (Kienzle-Sterzer et al., 1982).

Bacterial cellulose (BC) is cellulose, biosynthesized by *Acetobacter xylinum* using glucose as a substrate. It has been recognized as a biologically appropriate property allowing for biomedical and tissue engineering applications (Andrade et al., 2010; Czaja et al., 2007). It has been reported for its high purity, strength and moldability (Jonas et al., 1998). BC has high mechanical strength, high water absorption capacity, high crystallinity, and highly pure fiber network structure (Vandamme et al.,1998).

In this study, a novel antimicrobial packaging film made with chitosan-BC is developed. Chitosan-BC based films were prepared by casting method and were characterized for physical, mechanical and antimicrobial properties.

1.2 Objectives

To develop chitosan/BC composite film (CBC) and characterize for its physical, mechanical and antimicrobial properties.

1.3 Research scopes

1. Chitosan/BC (CBC) composite films are fabricated by casting method at room temperature (30°C±5°C).
2. Characterization of CBC is as follows:

   2.1 Scanning Electron Micrographs (SEM) for preliminary investigation of morphology.

2.2 Fourier Transform Infrared (FT-IR) spectrometer for identification of chemical structures

2.3 Universal testing machine for determining mechanical properties.

2.4 X-ray diffraction (XRD) for measurements of crystallinity index (CI)

2.5 Oxygen permeation tester for measurements of oxygen transmission rate (OTR).

2.6 Water vapor permeation tester for measurements of water vapor transmission rate (WVTR).

2.7 Water absorption capacity

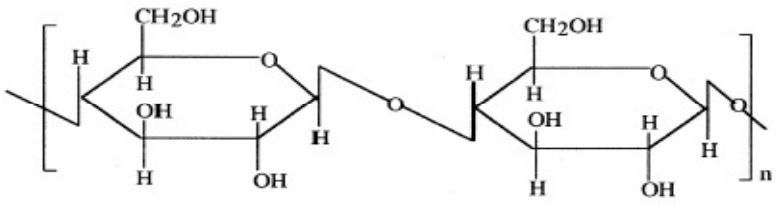
2.8 Antimicrobial ability.

3. Application of CBC as food film packaging and bio-preservation in extending the shelf-life of food.

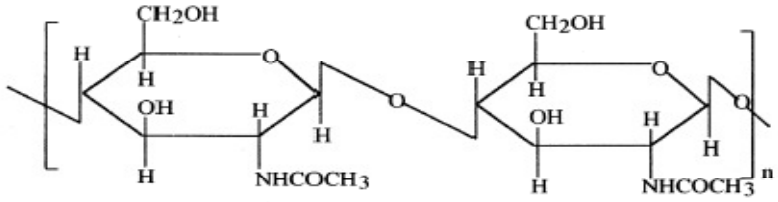
CHAPTER II
THEORY

2.1 Chitosan

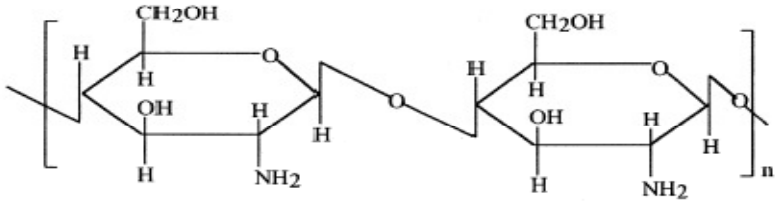
Chitin, an abundant natural polysaccharide which is a major supporting material structure of crustaceans, is consisted of (1,4)-linked 2- acetamido-deoxy-β-D-glucose. It may be regarded as cellulose with hydroxyl group at position C-2 replaced by an acetamido group . Chitosan was derived by deacetylation of chitin, which composed of (1,4)-linked 2- amino-deoxy-β-D-glucan.The structure of chitin ,chitosan and cellulose are shown in Figure 2.1(Muzzarelli.,1973; Zikakis., 1984).



Cellulose



Chitin



Chitosan

Figure 2.1 Structures of cellulose, chitin and chitosan (Muzzarelli.,1973; Zikakis., 1984).

Chitosan is a polycationic biopolymer with a specific structure and properties. It is obtained from chitin by alkaline deacetylation(Figure 2.2). Recently, the production of chitosan is associated with advance fermentation process technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan (Teng et al.,2001; Pochanavanich et al ., 2002).

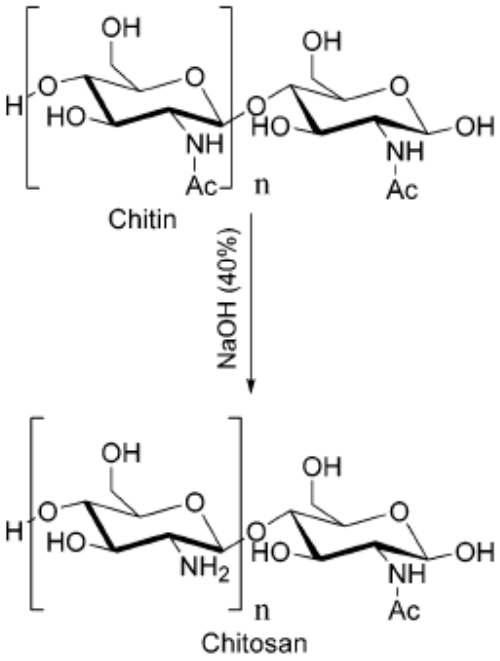


Figure 2.2 Preparation of chitosan from chitin (Muzzarelli., 1977).

The characteristics of chitosan may be varied as required for a particular application are the degree of deacetylation and the molecular weight. It is soluble in dilute organic acids such as acetic acid, formic acid, succinic acid, lactic acid and malic acid. The viscosity of solutions containing chitosan is affected by the degree of deacetylation, the molecular weight, the concentration, the ionic strength, the pH, and the temperature. Generally, to increase in temperature causes a decrease in the viscosity of the solution. The effect of the pH on the viscosity depends on the particular acid used. (Rabea et al., 2003).

Chitosan and chitin are interesting compounds which consisted of their high nitrogen content (6.89%) compared to synthetically substituted cellulose (1.25%). Thus chitosan was a useful chelating agent, cause most of the polymers are synthetic materials, their biocompatibility and biodegradability are much more limited than those of natural polymers such as cellulose (Rabea et al., 2003).

2.1.1 Antimicrobial of chitosan

As the result of the positive charge on the C-2 of the glucosamine monomer below pH6, chitosan is more soluble and has a better antimicrobial activity than chitin molecule. The Interaction between positive charged of chitosan molecules and negative charged of microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents (Chen et al,1998;Seo et al,1992; Hadwiger et al,1999).

Chitosan mostly acted on the outer surface of the bacteria. At a lower concentration (<0.2 mg/mL), the cationic of chitosan molecule does presumably bind to the negatively charged bacterial surface to cause agglutination. While at higher concentrations, the larger number of positive charges may have imparted a net positive charge to the bacterial surfaces to keep them in suspension. However, chitosan shows its antibacterial activity only in an acidic medium because of its poor solubility above pH 6.5 (Sudarshan et al., 1992; Papineau et al.,1991).

Chitosan with a molecular weight ranging from 10,000 to 100,000 would be helpful in inhibiting the growth of bacteria. Moreover, chitosan with an average molecular weight of 9300 was effective in restraining *E. coli*, while that with a molecular weight of 2200 accelerated growth(Tokura et al.,1994).In addition, the antibacterial activity of chitosan is influenced by its degree of deacetylation, its concentration in solution, and the pH of the medium(Rabea et al., 2003).

2.1.1.1 Fungicidal applications of chitosan.

The fungicidal effect of *N*-Carboxymethyl Chitosan (NCMC) is also different in vegetable as compared to graminea hosts. In addition, chitosan oligomers have a better antifungal effect than larger units.The antimicrobial of chitosan activity is more immediate on fungi and algae than on bacteria (Savard et al., 2002).

Chitosan has been shown to be fungicidal against several fungi in Table 2.1 .The minimum inhibitory concentrations (MICs) reported for specific target organisms range from 0.0018% to 1.0% which are influenced by a multitude of factors such as the pH of the growth medium, the degree of polymerization of chitosan, and the presence or absence of interfering substances such as lipids and proteins (Liu et al .,2001).

Table 2.1 MIC of Native Chitosan against Fungi (Liu et al .,2001).

Fungi	**MIC[a](ppm)**
Botrytis cinerea	10
Furarium oxysporum	100
Drechstera sorokiana	10
Micronectriella nivalis	10
Piricularia oryzae	5000
Rhizotonia solani	1000
Trichophyton equnium	2500

[a]MIC = minimum growth inhibitory concentration.

2.1.1.2 Bactericidal applications of chitosan.

Chitosan has several advantages over other type of disinfectants cause it possesses a higher antibacterial activity, abroader spectrum of activity, a higher killing rate, and a lower toxicity toward mammalian cells (Franklin et al.,1981; Takemono et al .,1989).

Chitosan in acetic acid medium is stronger than that in water. Their antibacterial activity increased as the concentration of acetic acid is increased. It was also found that the antibacterial activity against *E. coli* and inhibited the growth of a wide variety of bacteria (Table 2.2)

Table 2.2 MIC of Chitosan against Bacteria (Liu et al .,2001).

Bacteria	**MIC[a](ppm)**
Agrobacterium tumefaciens	100
Bacillus cereus	1000
Conrinebacterium michiganence	10
Erwinia sp.	500
Erwinia cartovora subsp.	200
Escherichia coli	20
Micrococcus luteus	20
Pseudomonas fluorescens	500
Staphylococcus aureus	20
Xanthomonas campestri	500

[a]MIC = minimum growth inhibitory concentration.

2.1.1.3 Antiviral Activity of chitosan.

Antiviral activity of chitosan depends on the average degree of polymerization, the degree of *N*-deacetylation, the positive charge value, and the character of the chemical modifications of the molecule. (Chircov et al.,2002; Pospieszny et al .,1997 Kochkina et al.,1995 ; Liu et al.,2002)

Most factors of suppressing phage infections by chitosan are phage particle inactivation and inhibition of bacteriophage reproduction at the cellular level. Obviously, Chitosan may be used for induction of phagoresistance in industrial microorganism cultures to prevent undesirable phagolysis caused by inoculum contamination by virulent bacteriophages or by spontaneous prophage induction in lysogenic culture. Chitosan possesses an antiviral activity by its ability to induce resistance toward viral diseases in plants, to inhibit viral infections in animal cells, and to prevent the multiplication of bacteriophages in infected cultures of microorganisms (Chircov et al.,2002).

The ability of chitosan to suppress viral plant infections does not depend on the virus type.Chitosan affects the plant itself inducing resistance to the viral infection in plants. Imitating the contact of the plant with a phytopathogen, which induces a wide spectrum of protective reactions in the plant, which limit a systemic spread of the viruses and viroids over the plant and reach to the development of the systemic acquired resistance.Chitosan applied by spraying or inoculating leaves protected various plant species against local and systemic infection caused by alfalfa mosaic virus (ALMV), tobacco necrosis virus (TNV), tobacco mosaic virus (TMV), peanut stunt virus (PSV), cucumber mosaic virus (CMC), and potatovirus X (PVX). The efficiency of chitosan to inhibit viral infections depends on the host-viruscombination, chitosan concentration, and application method (Pospieszny et al .,1997).

2.2 Bacterial Cellulose

Cellulose is the fundamental material of all plants substance that is the basic structure of the cell wall of almost all plants, many fungi and some algae. Cellulose is a linear polymer of D-glucose residues joined by β-1, 4-glycosidic linkages as polysaccharides (Crawford et al.,1981;Updegraffet et al.,1969),which is an organic compound with the formula $(C_6H_{10}O_5)_n$ (Figure 2.3).

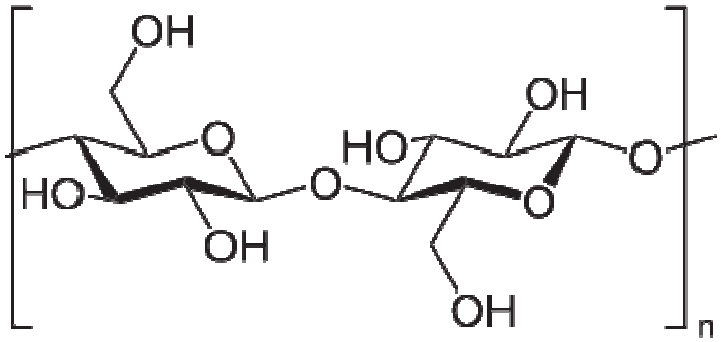


Figure 2.3 Structure of Cellulose (Nishiyama et al .,2002).

Cellulose which derived from plant is unpurified cellulose associated with other types such as lignin and hemicelluloses, while bacterial cellulose is nearly-purified cellulose (Phisalaphong and Jatupaiboon., 2008).

2.2.1 The synthesis of BC

Bacterial cellulose (BC) is a natural cellulose produced from certain types of bacteria is presented in Table 2.3, attention has been given to the bacteria *Acetobacter xylinum* due to its cellulose's mechanical properties and applications to biotechnology, microbiology, and materials science(Iguchi et al, 2000).Bacteria cellulose has a higher water holding capacity and hydrophilicity, greater tensile strength caused a larger amount of polymerization, ultrafine network structure (Klemm, D.,2001).

Table2.3 Bacterial cellulose producers (Jonas and Farah, 1998).

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

The synthesis of cellulose by *Acetobacter xylinum* occurs between the outer membrane and cytoplasmic membrane by a cellulose-synthesizing which is related to the porous surface of the bacteria (Figure 2.4).

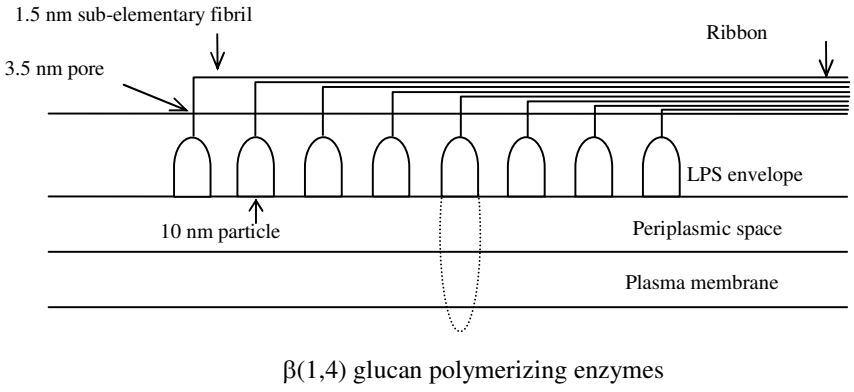


Figure 2.4 The formation of bacterial cellulose (Jonas and Farah, 1998).

Cellulose synthase is the most important enzyme in this process. . The biochemical pathway from glucose to cellulose was proposed (Figure 2.5), which should be linked to cell growth as well as to cellulose formation., caused nanofibrils leave the pore and from together with many synthesized fibrils a ribbon of crystalline cellulose. A self-assembly process should be responsible for the crystallization after the polymerization of the fibrils was completed (Jonas and Farah, 1998).

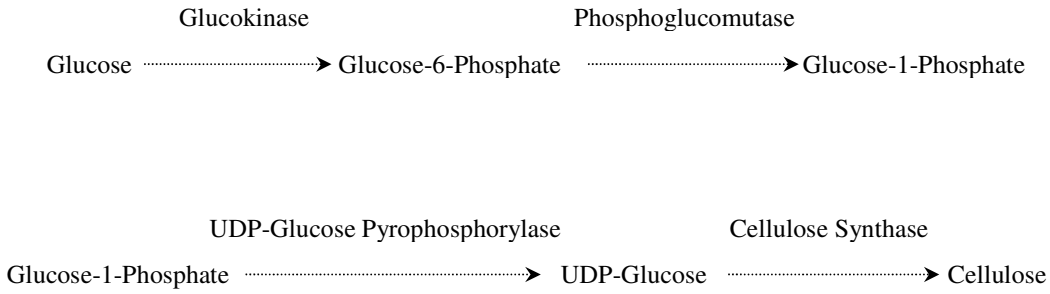


Figure 2.5 The biochemical pathway for cellulose synthesis in *Acetobacter xylinum* (Jonas and Farah, 1998).

The microscopic structure of BC depends on the growing culture condition. For a static culture, a leather-like pellicle of overlapping and intertwined ribbons formed (Jonas and Farah, 1998) ,displays particularly properties, including high mechanical strength, high water absorption capacity, high crystallinity, and highly pure fiber network structure(Vandamme, E.J. et al,1998).BC has been suggested to have a structure like a ‘cage' which protects the foreign heavy metal ions, while still allowing nutrients to be supplied easily by diffusion , as shown in Figure 2.6 (Lynd, L. et al, 2002) .Moreover, one of the most important features of BC is its chemical purity and stable towards chemicals at high temperatures (Sun, D.et al 2010).

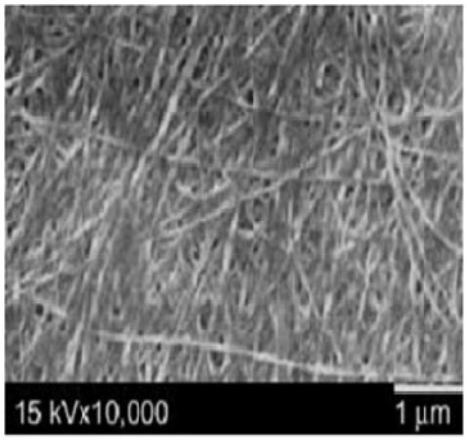


Figure 2.6 SEM micrograph of structure of BC (Phisalaphong,et al., 2008)

To compare of diameter of BC fibrils in natural and artificial fiber materials represent in Figure 2.7. The thinnest fiber is a chemically synthesis fiber having size approximately 1 µm, while the size of the BC fibril is approximately one tenth of that.

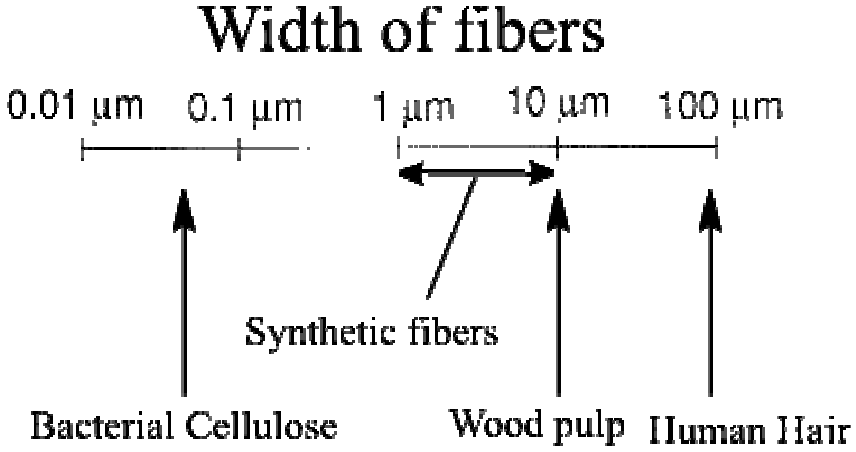


Figure 2.7 Size of synthetic and naturally occurring fiber (Yoshinaga et al, 1997).

CHAPTER III
LITERATURE REVIEW

3.1Chitosan

3.1.1 Chitosan for food application.

Chitin and its deacetylated form (Figure 3.1) have been of interest in the past few decades caused their potential broad range of industrial applications (Ornum et al ., 1992; No et al ., 1995).

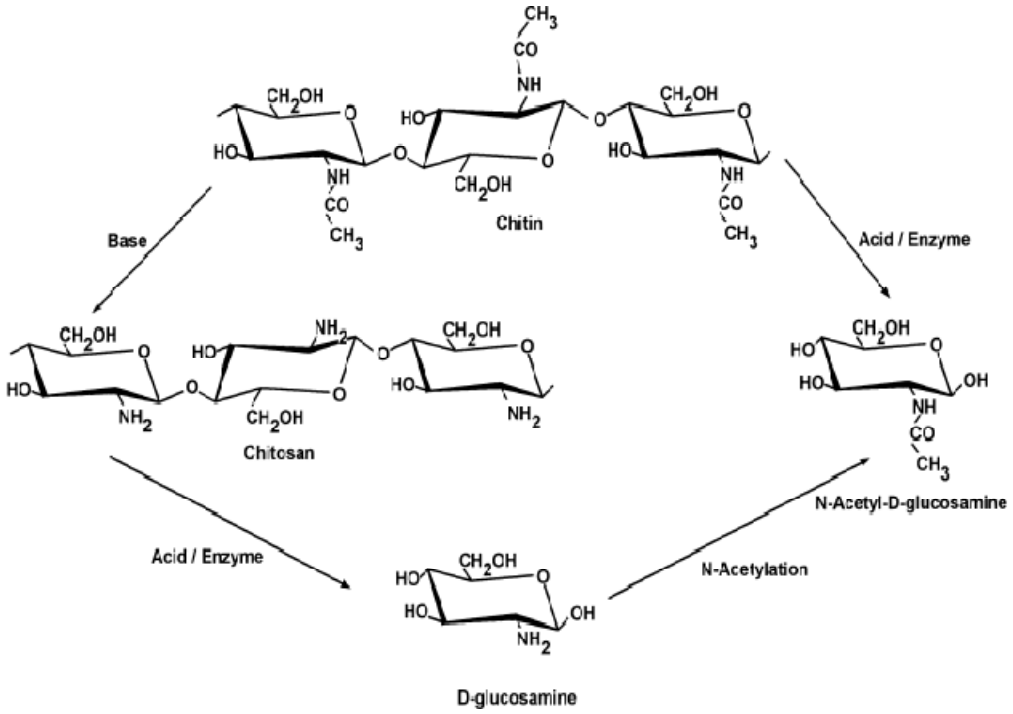


Figure 3.1 Preparation of chitin and derivative from chitin (Furusaki et al.,1996)

Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2,C-3 and C-6 positions, respectively. These functional groups have provided various useful materials in different field of application (Furusaki et al.,1996).(Figure 3.2)

The change of processing discards into valuable by-products and alternative specialty materials has been identified as a timely challenge for food research and development associated with various applications for chitosan biopolymers. In that way, these biopolymers offer a wide range of unique applications including bioconversion for the production of value-added food products(Shahidi et al.,1991; Carroad et al.,1978; Revah-Moiseev et al.,1981).

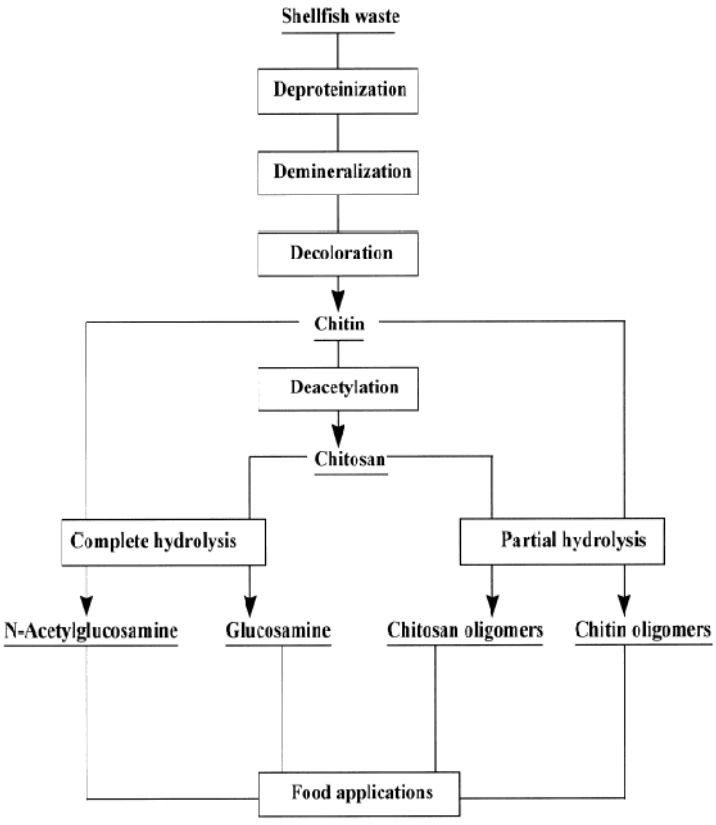


Figure 3.2 Flow sheet for preparation of chitin, chitosan, their oligomers and monomers from shellfish waste.

The preservation of foods from microbial deterioration, formation of biodegradable film , recovery of waste material from food processing discards , purification of water and clarification and deacidification of fruit juices (Shahidi et al.,1999,See Table 3.1)

Table 3.1 Food application of chitin,chitosan and their derivatives in food industry. (Shahidi et al.,1999)

Application	Examples
Antimicrobial agent	-Bactericidal -Fungicidal -Measure of mold contamination in agricultural commodities
Edible film industry	-Controlled moisture transfer between food and surrounding environment
Edible film industry	-Controlled release of antimicrobial substances -Controlled release of antioxidants -Controlled release of nutrients, flavours and drugs -Reduction of oxygen partial pressure -Controlled rate of respiration Temperature control -Controlled enzymatic browning in fruits -Reverse osmosis membranes
Additive	-Clarification and deacidification of fruits and beverages. -Natural flavour extender -Texture controlling agent -Emulsifying agent -Food mimetic -Thickening and stabilizing agent

Application	Examples
Nutritional quality	-Dietary fibre -Hypocholesterolemic e€ect -Livestock and fish feed additive -Reduction of lipid absorption -Production of single cell protein

3.1.2Chitosan in the edible film industry

The usage of edible films and coatings to extend shelf life which improve the quality of fresh, frozen and fabricated foods has been examined during the past few years (Kester and Fennema.,1986; Labuza and Breene.,1989).

Kittur et al (1998) studied the functional packaging of chitosan film and reported that chitosan film which has an eco-friendly and biodegradable nature. These outer layers films can provide supplementary and sometimes essential means of controlling physiological, morphological and physicochemical changes in food products. In addition, chitosan films have moderate water permeability values and could be used to increase the storage life of fresh produce and foodstuffs with higher water activity values.

Labuza et al (1989) reported that there are many mechanisms to involve in extending shelf life of food by coating films. These include controlled moisture transfer between food and surrounding environment, controlled release of chemical agents like antimicrobial substances, antioxidants, reduction of oxygen partial pressure in the package that results in a decreased rate of metabolism, controlled rate of respiration, high impermeability to certain substances like fats and oils,temperature control, structural reinforcement of food and coat flavor compounds and leavening agents in the form of microcapsules.

Davies et al (1989) demonstrated the film-forming properties chitin and chitosan have been successfully used as food wraps caused use of N ,O carboxy- methylchitin films to preserve fruits over long periods has been approved in both Canada and the USA .Owning to its ability to form semipermeable film, chitosan coating can be expected to

modify the internal atmosphere as well as decrease the transpiration loss and delay the ripening of fruits .

Kester and Fennema(1986) proposed that there has been several approaches used to form these edible films or coatings, including simple coacervation, where a single hydrocolloid is transfering from aqueous suspension or caused to change its phase by evaporation of the solvent. Moreover, complex coacervation, which two solutions of oppositely ionized hydrocolloids are united, causing interaction and precipitation of the polymer complex as well as simple cooling of a warm hydro- colloid suspension to bring about a sol-gel transformation has been practiced.

Butler et al (1996) investigated chitosan films which are tough, long-lasting, flexible and very difficult to tear. Most of these mechanical properties are comparable to many medium-strength commercial polymers.

Wong et al (1992) observed chitosan film that has an extremely good barrier to permeation of oxygen, while exhibiting relatively low vapour barrier character- ristics. By incorporating fatty materials, hydrophobicity can be increased thereby producing composite films resistant to water transmission

Du et al(1997) and El Ghaouth et al(1991) studied the effect of chitosan coating on storability and quality of fresh fruits and reported that the extension of the storage life and better control of decay of peaches, Japanese pears and kiwifruits by
application of chitosan film has been documented Similarly, cucumbers, and bell peppers could be stored for long periods after coating with chitosan. These results may be attributed to decreased respiration rates, inhibition of fungal development and delaying of ripening due to the reduction of ethylene and carbon dioxide evolution.

Chen et al. (1996) have observed that the packaging film prepared from methylcellulose, chitosan and preservatives possesses antimicrobial activity. In addition, Chitosan and chitosan-laminated films containing antimicrobial agents provide a type of active package such that the preservatives released from the film deposit on the food surface and inhibit the microbial growth.Torres et al.(1985) evaluated the sorbate-loaded edible barrier for mold inhibition on food surfaces, and Field et al. (1986) advocated the

use of glucose oxidase/glucose as a dip for extension of shelf life of fish. The presence of preservatives in chitosan films reduces the inter-molecular electrostatic repulsion in the chitosan molecules and facilitates formation of intramolecular hydrogen bonds (Rinaudo et al.,1989).

Spagna and Rwan et al. (1996) observed that chitosan has a good affinity for polyphenolic compounds such as catechins,proanthocyanidins, cinnamic acid and their derivatives that can change the initial straw-yellow colour of white wines into deep golden-yellow colour due to their oxidative products. By adding chitosan to grapefruit juice at a concentration of 0.015 g/mL, total acid content was reduced by about 52.6% due to decreasing the amount of citric acid, tartaric acid, l-malic acid, oxalic acid and ascorbic acid, by 56.6, 41.2, 38.8, 36.8 and 6.5%, respectively .

3.2 Bacterial Cellulose (BC)

3.2.1 BC applications

BC has a variety of properties such as its remarkable mechanical properties in both dry and wet states, porosity, water absorbency, moldability, biodegrability excellent biological affinity and consequently it has a wide range of potential application. For BC to be appropriate for these many applications have been shown in Table 3.2

Table 3.2 Bacterial cellulose applications (Brown, 2007)

Applications	Product Processing	Ideal/Obtained Properties
Vascular prosthetic device	-BC films are used to coat surface-treated medical grade polyesters.	-Minimizes blood clotting and increases biocompatibility. -Has high mechanical strength in wet state, substantial permeability to water and gases, high water retention and low surface roughness.

Applications	Product Processing	Ideal/Obtained Properties
Wound care product	-BC sheets were impregnated with drugs. -Never-dried BC sheets are immersed in chitosan solution.	-Highly nanoporous, allowing transfer of antibiotics or medicines while serving as a physical barrier against external infections. -Wound healing accelerated. -High mechanical properties in wet state
Artificial blood vessel in microsurgery	-BC grown in a static culture molded in BASYC® tubes	-High mechanical strength in wet state enormous water retention values, low surface roughness of inner surface. -Highly moldable in situ. -Can sustain a mean tensile force of 800mN.
Tissue-engineered blood vessels	-BC grown in a tubular shaped mold.	-Young modulus should match carotid arteries, about 3MPa. -Inner side of tubular BC must be smoother compared to the outside.
Substrate for mammalian cell culture	-BC membrane grown statically and electrically charged.	-High permeability
Cation-exchange membrane for industrial waste water treatment	-BC membranes are modified with cation exchangeable acrylic acid	-Tensile strength of 12MPa and elongation of 6%
Electronic paper	-BC sheets are doped with conductors.	-Paper has high reflectivity and contrast.

3.2.2Bacterial cellulose for Food application

BC proposes a wide range of applications owning to its high purity and special physicochemical characteristics. Because of its high water absorption capacity and high mechanical strength (Vandamme et al.,1998). Therefore, it can be used in the food field.

The potential applications of BC in the food depend on its price and accessibility. Therefore, strains and cultivation medium must be optimized. Previous studies on biosynthesis, structural features and properties, and factors affecting the production of BC (El-Saied et al.,2004).

Nata de Coco, which is the dessert, has been a cottage industry in the Philippines (Lapuz, M.M et al,1969). The import of Nata from the Philippines into Japan has significant impact on the global expansion outlook of microbial cellulose production. In 1992, a fad originated in Japan with the introduction of microbial cellulose into diet drinks. As a result of production of Nata de Coco through G. xylinus(Kuwana.,1997).

Moreover BC food product , Chinese Kombuchar or Manchurian Tea, obtained by growing yeast and *Acetobacter* in a medium containing tea extract and sugar. There is growing use of the fermented extract for healthy. The pellicle formed on the surface contains both cellulose and enzymes healthy for humans. Kombucha is believed to protect against certain cancers(Iguchi, M et al, 2000).

Another application of BC has been applied as a functional food additive: a thickener, texturizer, and/or calorie reducer (ice cream,salad dressing, and weight-reduction base), from an agitated culture has a much higher emulsifying effect than that from a static culture(Hiroshi et al,1997).

3.2.3Film or membrane application

Among various applications, BC pellicles have a uniform fiber distribution and a high tensile strength. they can serve as acoustic membranes or conductive membranes. The large accessible surface area, high durability, and superior adsorptive properties

means that bacterial cellulose can be applied as a carrier for immobilization of biocatalysts (Hiroshi et al,1997).

Stevanic et al (2011) synthesized food film packaging by renewable source which is xylan film with improved strength properties with the addition of BC. By mixing of BC with xylans in a high-pressure microfluidiser resulted in optically transparent nano composite films. For this purpose, the result showed that the adding of BC has increased stiffness and strength of the films.

Trovatti et al (2012) reported the composite BC and pullulan were prepared by a casting water based suspensions. The incorporation of BC into the pullulan matrix increases considerably the mechanical performance and also the thermal stability of the nanocomposite films. The use of glycerol as plasticizer increases the flexibility of the films which is an important parameter in several applications. These novel sustainable nanocomposite films could find applications in several fields such as in dry food packaging, transparent organic electronics and also in biomedical applications.

Stoica-Guzun et al (2012) developed the biodegradable packaging material from poly vinyl alcohol composites with BC which obtained by casting method. They reported that the main role of a food packaging material is to protect food from the environment in order to enhance storage life and food safety, which was interacted with ultraviolet (UV) radiation .This purpose of the study was investigated the effect of UV-irradiation on composite material. From this paper found that the situation of irradiation of pre-packaged foods were considered using UV radiation doses for different periods of time. The investigated properties of all the composites are influenced by UV radiation. From the raw materials bacterial cellulose is more sensitive to UV radiation as it was expected. For this reason the films which contain more bacterial cellulose are also more UV sensible.

George et al (2012) studied on synthesis and characterization edible film which is Gelatin based edible nano composite films were prepared by incorporating bacterial cellulose nanocrystals (BCNC) at various concentrations. Nanocrystals of cellulose were isolated from edible BC fibers by acid hydrolysis.The results showed that the

incorporation of BCNC has appreciably reinforced the gelatin matrix and also improved the moisture sorption and water vapor permeability behavior, which attributed to the enhanced mechanical properties. Therefore, bacterial cellulose nanocrystals obtained from BC was found to be a better reinforcing agent in improving the properties of edible films.

CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Chitosan:Seafresh Chitosan Powder at 85% DAC, Molecular weight of 30,000 and 200,000 purchased from Seafresh Chitosan (Lab) Company Limited Bangkok 10500,Thailand.

4.1.2 Bacterial Cellulose (BC):Supplied by PramoteTammarate, the Instituteof Food Research and Product Development, Kasetsart University,Bangkok, Thailand.

4.1.3 Glycerol: Glycerol 99.5% v/v, Density 1.2570@ 25^{o}C AR,purchased fromAjax Finechem Pty Ltd,Bangkok 10210 Thailand.

4.2 Preparation of Bio-composite films

Chitosan solution (1% w/v) was prepared by dissolving chitosan powder in 1% v/v acetic acid solution and stirring overnight. The pH of solution was adjusted to 5.0 with 1N NaOH. Then the chitosan solution was mixed with BC slurry at various weight ratios(Chitosan: BC): 10:0, 10:2, 10:4, 10:6, 10:8 and 10:10 respectively. Glycerol (2%v/v) was used as a plasticizer for the mixture. The mixture was stirred until homogeneous. After that, it was poured into Petri plate and dried in an incubator at 35 °C for 5 days. Finally, Chitosan/BC (CBC) film was obtained.

4.3 Characterization

4.3.1 Scanning Electron Microscopy

The examination of the film morphology was performed by scanning electron microscopy (SEM) (JSM- 5410LV, JOEL, Tokyo, Japan) at Scientific and Technological Research Equipment Centre, ChulalongkornUniversity. The CBC films were sputtered with gold in a Balzers-SCD 040 sputter coater (Balzers, Liechtenstein) 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

Fourier transform infrared (FTIR) spectroscopy (Spectrum One, Perkin Elmer, Massachusetts, USA).is used primarily to identify the chemical structure of the samples (chitosan film, BC and CBC).FTIR spectra of the developed film were recorded with Perkin Elmer (Spectrum One, Massachusetts, USA) in the region of 4000–400 cm^{-1} at Scientific and Technological Research Equipment Centre, Chulalongkorn University.

4.3.3 X-Ray diffraction

X-ray diffraction (XRD) patterns of the bio polymers and CH/BC-composite were determined with a diffactometer (Bruker AXS Model D8 Discover, USA). 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:

$$C.I.(\%) = \frac{\left(I_{020} - I_{am}\right)}{I_{020}} \times 100$$

Where I_{020} is the maximum intensity of the lattice diffraction, and I_{am} was the intensity at $2\theta = 18^{o}$.

4.3.4 The mechanical property testing

In this study, the tensile strength, Young's modulus and elongation at break of CBC dried films were measured by Universal Testing Machine (Hounsfield H 10 KM, Redhill, England) at Scientific and Technological Research Equipment Centre, Chulalongkorn University. The load cell capacity was 1 kN. The film samples were cut into strip-shaped specimens 10 mm width and 10 cm length. The test conditions followed ASTM D882 as a standard test method for tensile elastic properties. Two ends of the specimens were placed between the upper and lower jaws of the instrument, leaving a length of 6 mm of film in between the two jaws. Extension speed of the instrument was 2 mm/min. The tensile strength and break strain were the average value determined from at least five specimens.

4.3.5 The water absorption capacity

The water absorption capacity (WAC) was determined by immersing the pre-weighted of dried samples in distilled water at room temperature until equilibration. The sample was then removed from the water. After excess water at the surface of the sample was blotted out with Kimwipes paper, the weight of the swollen sample 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:

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

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

4.3.6 The water vapor permeability measurement

The water vapor transmission rate (WVTR) of the CBC film with area of 50.00 cm^2, was measured at Thai packaging centre, Thailand Institute of Scientific and Technological Research. The test conditions followed ASTM E-96 with desiccant method. The determination of WVTR was done at 38^oC and 98% relative humidity. The test specimen was sealed to the open mount of test dish containing a desiccant, and the assembly placed in a controlled atmosphere. Periodic weighting was performed to determine the rate of water vapor movement through the specimen into the desiccant.

4.3.7 The oxygen permeability measurement

The oxygen transmission rate (OTR) of the CBC film was determined with an oxygen permeation analyzer: Illinois Instruments (Johnsburg, IL) Model 8000 at Thai packaging centre, Thailand Institute of Scientific and Technological Research. The test condition followed ASTM D3985. The determination of OTR was done at 23 ^oC and 0 % relative humidity. The film was held in such a manner that it separated two side of test chamber. One side was exposed to a nitrogen atmosphere, while the other side was exposed to an oxygen atmosphere. A. Testing was completed when the concentration of oxygen in the nitrogen side was constant.

4.3.8 Antimicrobial ability testing

The antimicrobial properties of chitosan andCBC films were examined against 2 types of bacteria such as *Escherichia coli* and *Staphylococcus aureus*. The films were cut into round-shaped sample of 0.5 grams according to the method described by ASTM E2149-10.For each product to be tested, 50 mL of the standardized microbial culture was placed into 250 mL Erlenmeyer flask.The initial microbial concentration of the solution (o hour) was determined by performing serial dilutions and standard plate count

techniques. Then the test and the control specimen were placed in the individual flasks on the wrist-action Shakerand were shaken at maximum stroke for 3 hours. Then microbial concentrations in all flasks were determined once again. Concentrations of microorganisms in the flask that contained the antimicrobial product (test specimen) were compared to the flask that contained the control specimen. For plate count techniques, all the Petri dishes were incubated for 24 hours at 35°C and then the colonies in the Petri dish werecounted. The recorded the value is the average number from the triplicate Petri dish numbers and was converted to the colony-forming unit per milliliter (CFU/mL). The percent reduction of organisms from treated sample (A) directly compared to the untreated control (C).

$$\text{Reduction},\%(\text{CFU/mL}) = \frac{\text{C-A}}{\text{C}} \times 100$$

$$\text{Log}_{10} \text{ bacteria reduction} = \text{Log}_{10}(\text{C}) - \text{Log}_{10}(\text{A})$$

Where

A = CFU per milliliter for the flask containing the treated substrate after the specified contact time.

C =CFU per milliliter for the flask containing the untreated substrate after the specified contact time.

CHAPTER V
RESULTS AND DISCUSSIONS

The antimicrobial films have gained more interest to apply for food packaging. Especially, cationic polymers have being used since positive charges interact with negative charges on the bacterial cell membrane leading to leakage of intracellular constituents (Goldberg et al., 1990). Chitosan which is a cationic biopolymer has been used as a coating to protect fresh vegetables and fruits from fungi and bacteria, because it may act as a barrier between the nutrients contained in the product and microorganisms (Cuq et al., 1995).

In addition, mechanical properties, such as tensile strength and elongation at break are very important to be considered for food packaging films. Because its use would not be satisfied if the product is damaged. Other properties or functions that are also important to be concerned for food packaging films include extending food's shelf life, water vapor transmission rate (WVTR) and oxygen transmission rate (OTR). Chitosan has good antimicrobial properties without toxicity to humans however the uses for chitosan films have been limited because of its weak mechanical properties. It is interesting to add bacterial cellulose (BC) to chitosan matrix as it may improve the physical property of the developed chitosan/BC (CBC) film.

In this study, chitosan film modified by BC slurry supplement was used as a bio-polymer to develop films for food packaging. The CBC composite films that obtained from casting techniques were characterized for the physical, mechanical and antimicrobial properties. The influence of low and high molecular weight of chitosan (M.W. 30,000 and 200,000, respectively) and the effects of BC content were determined.

5.1 Morphology

In this section, the morphology of the films incorporated BC into the chitosan matrix was studied using a Scanning Electron Microscopy (SEM) technique. The SEM investigation on the films represented the structure of surface morphology and cross-sectional of chitosan and CBC films (Figures 5.1-5.6). In definition, chitosan refers to chitosan film with no BC slurry adding, whereas the CBC refers to the chitosan with the addition of BC. The ratio of chitosan: BC was varied at 10:2, 10:6 and 10:10.

The SEM of the chitosan M.W. 30,000 film presented the fine textured film (Figure 5.1(a)), whereas the more coarse-textured film of chitosan M.W. 200,000 was observed as shown in Figure 5.4(a), which should be according to the lower particle size of the low M.W. chitosan. This result was supported by the report of Huang et al (2003) that the mean size of the chitosan nanoparticles decreases with decreasing polymer M.W. in the range of 213 to 17 kDa, which could be attributed to shorter polymer chains giving rise to smaller nanoparticles. Moreover, these films have no the fiber network structure.

The surface morphology of CBC films with the addition of BC slurry at various weight ratio of 10:2, 10:6 and 10:10 as shown in Figure 5.2(b-d) and 5.5(b-d)) demonstrates that with the increase of BC supplement, the composite CBC films display increasing nano fibril networks of BC distributed throughout chitosan matrix. Refer to the previous report (Czaja et al., 2007), the BC supplement showed well-organized fibril network. This combination causes the reduction in the hollow space of film

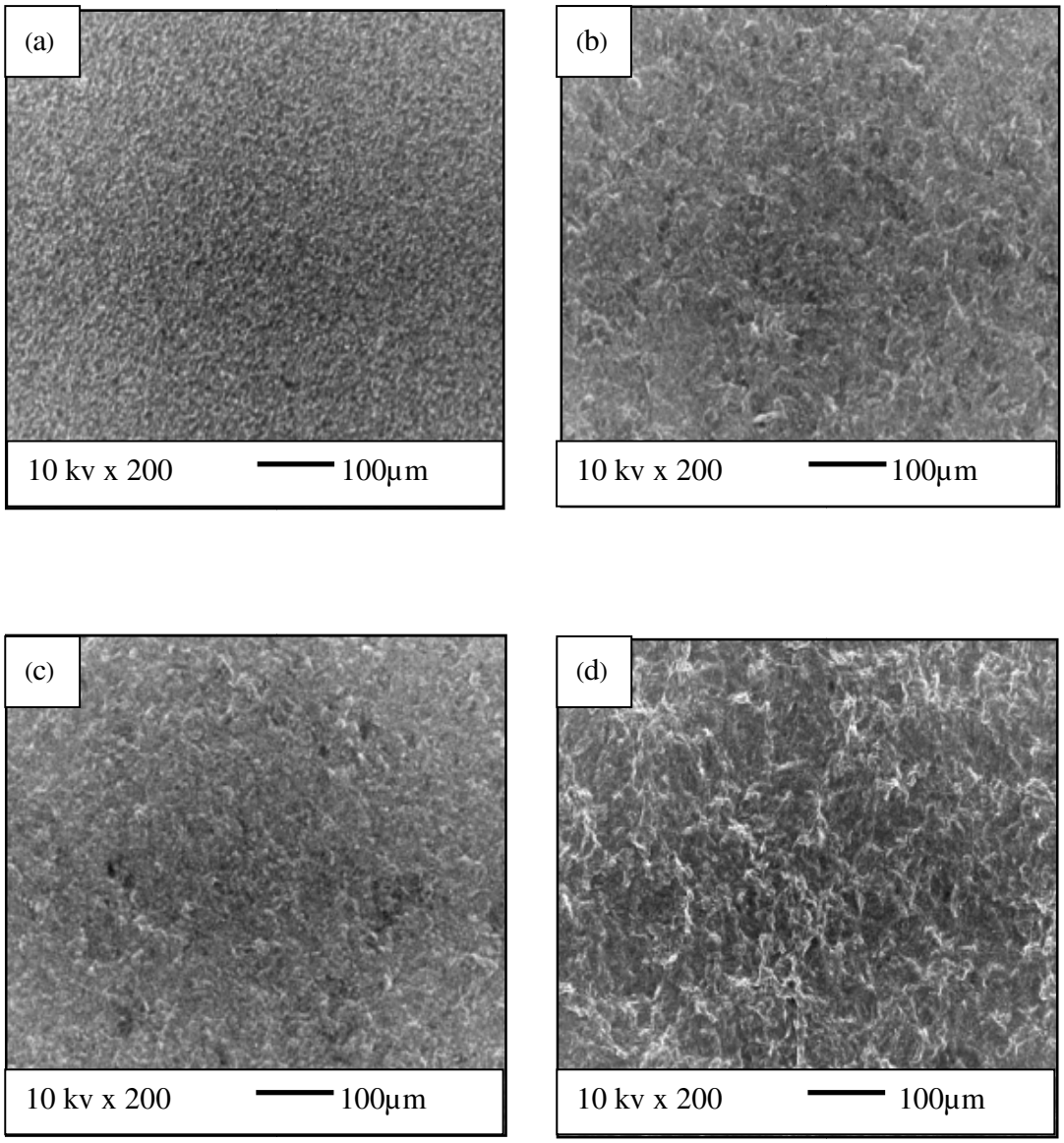


Figure 5.1 SEM images of surface morphology of the dried films (chitosan M.W.30,000) at 200X magnification: (a) Chitosan ,(b) CBC 10:2 ,(c) CBC 10:6 and (d) CBC 10:10

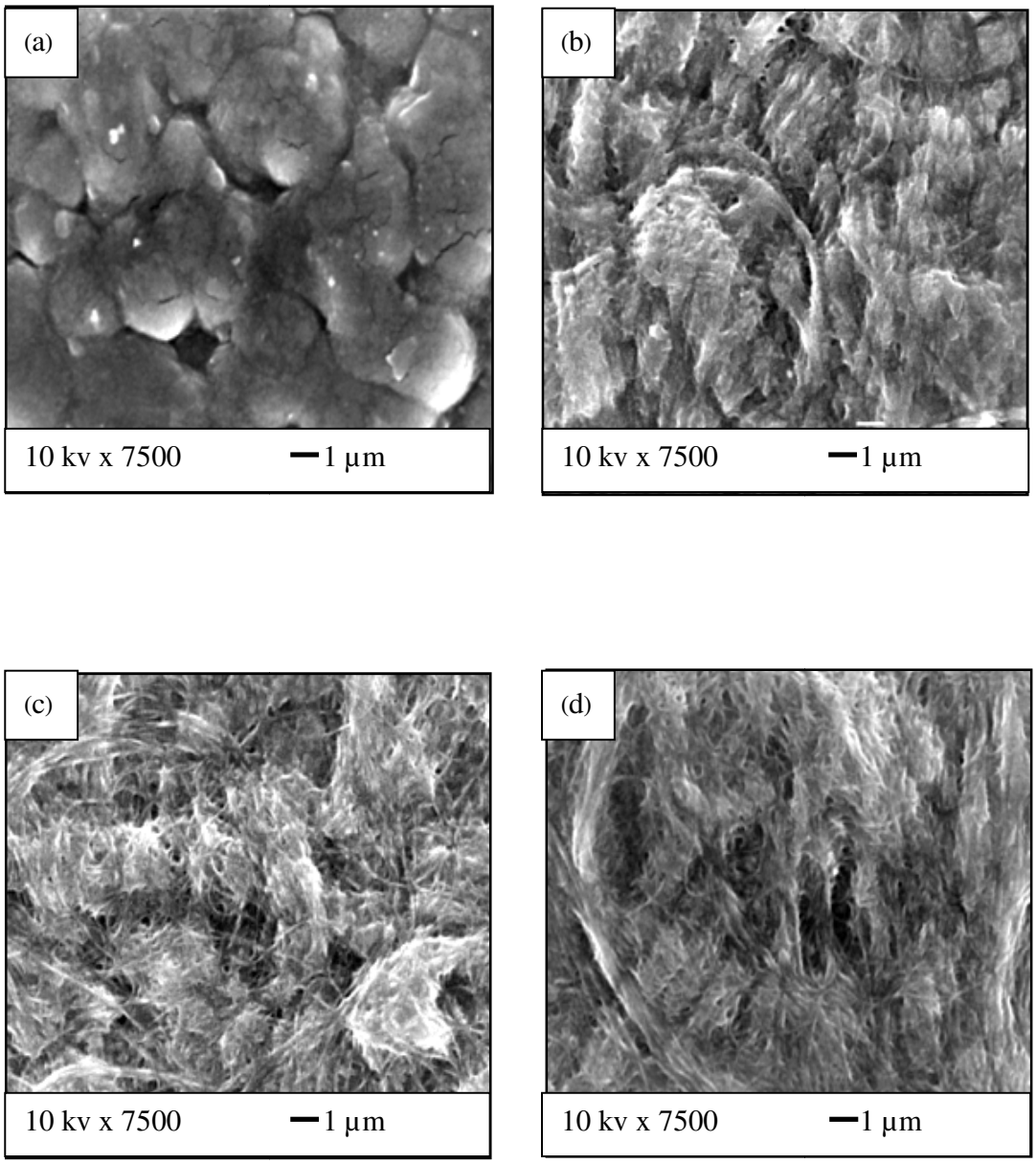


Figure 5.2 SEM images of surface morphology of the dried films(chitosan M.W.30,000) at 7500X magnification: (a) Chitosan ,(b) CBC 10:2 ,(c) CBC 10:6 and (d) CBC 10:10

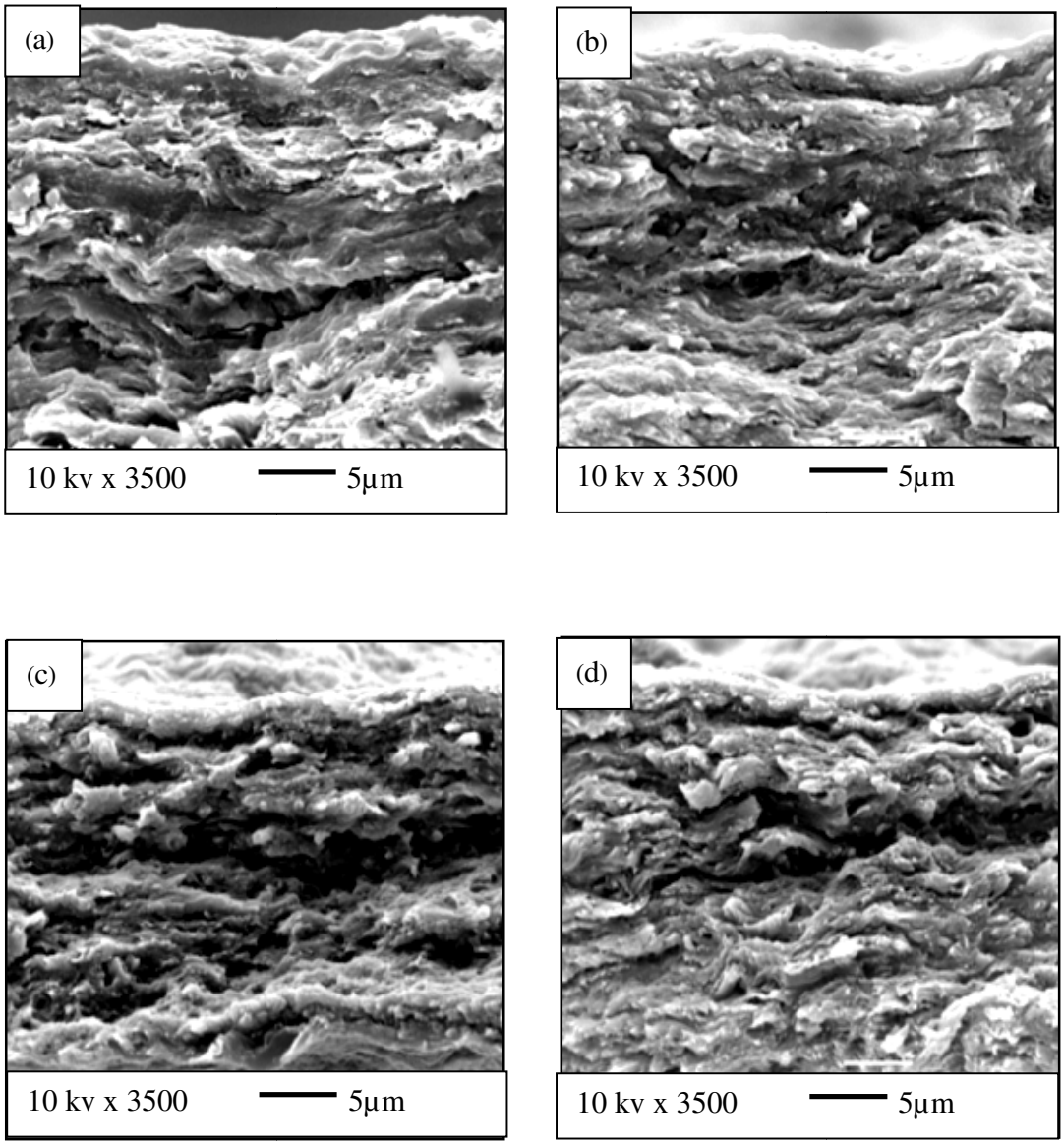


Figure 5.3 SEM images of cross sectional morphology of the dried films at 3500X (chitosan M.W.30,000) magnification: (a) Chitosan ,(b) CBC 10:2 ,(c) CBC 10:6 and (d) CBC 10:10

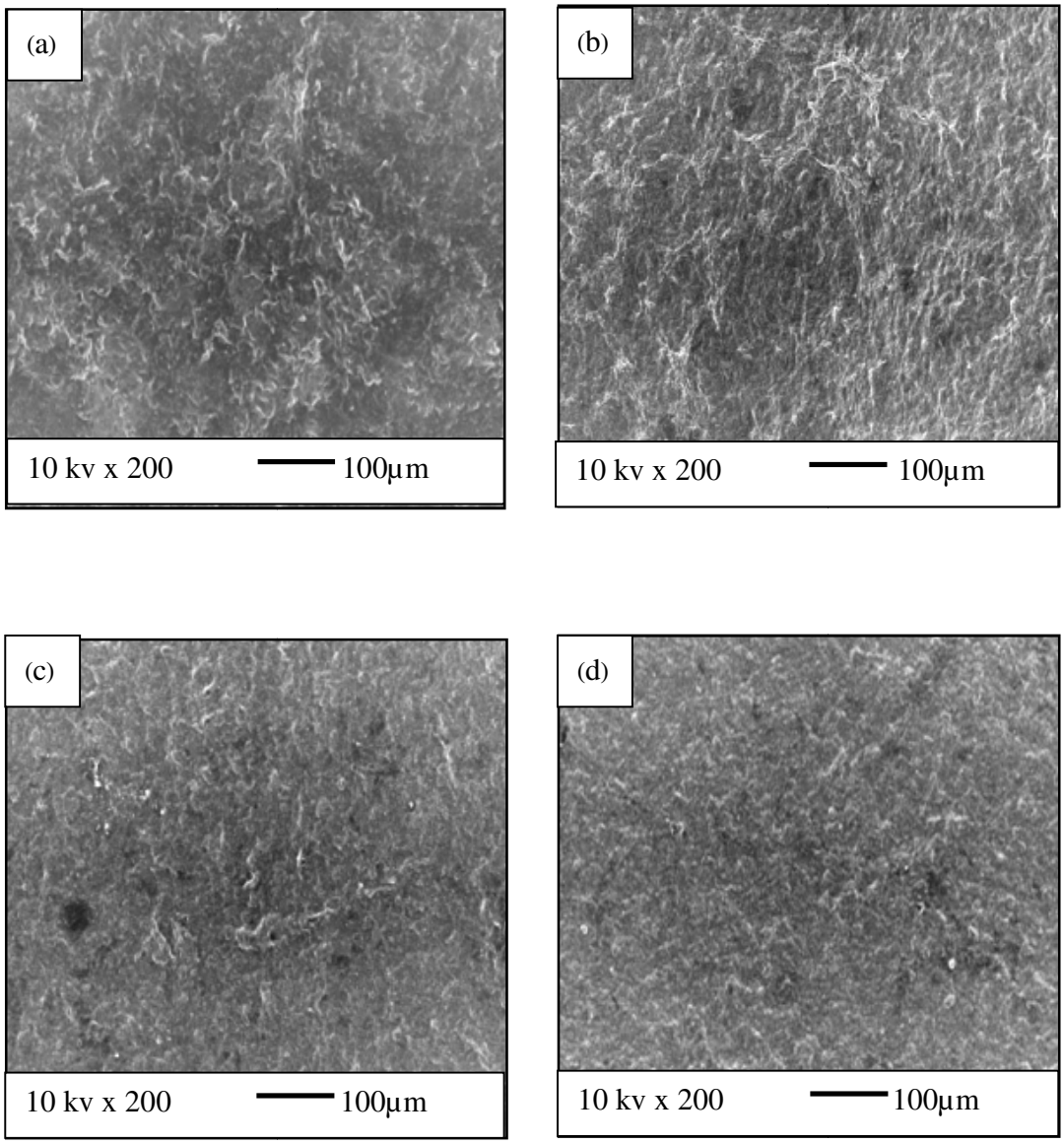


Figure 5.4 SEM images of surface morphology of the dried films(chitosanM.W.200,000) at 200X magnification:(a) Chitosan ,(b) CBC 10:2 ,(c) CBC 10:6 and (d) CBC 10:10

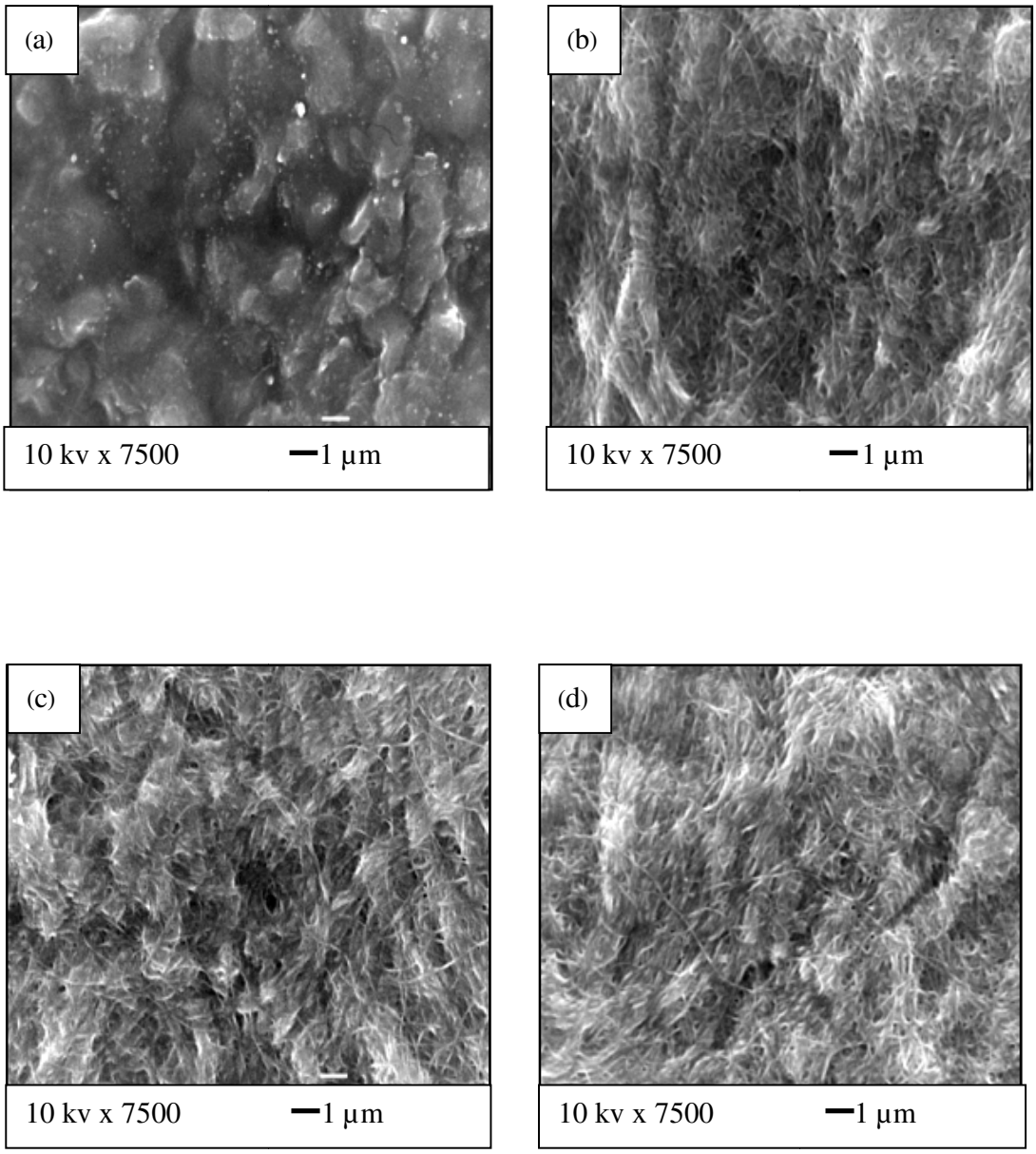


Figure 5.5 SEM images of surface morphology of the dried films(chitosanM.W.200,000) at 7500X magnification:(a) Chitosan ,(b) CBC 10:2 ,(c) CBC 10:6 and (d) CBC 10:10

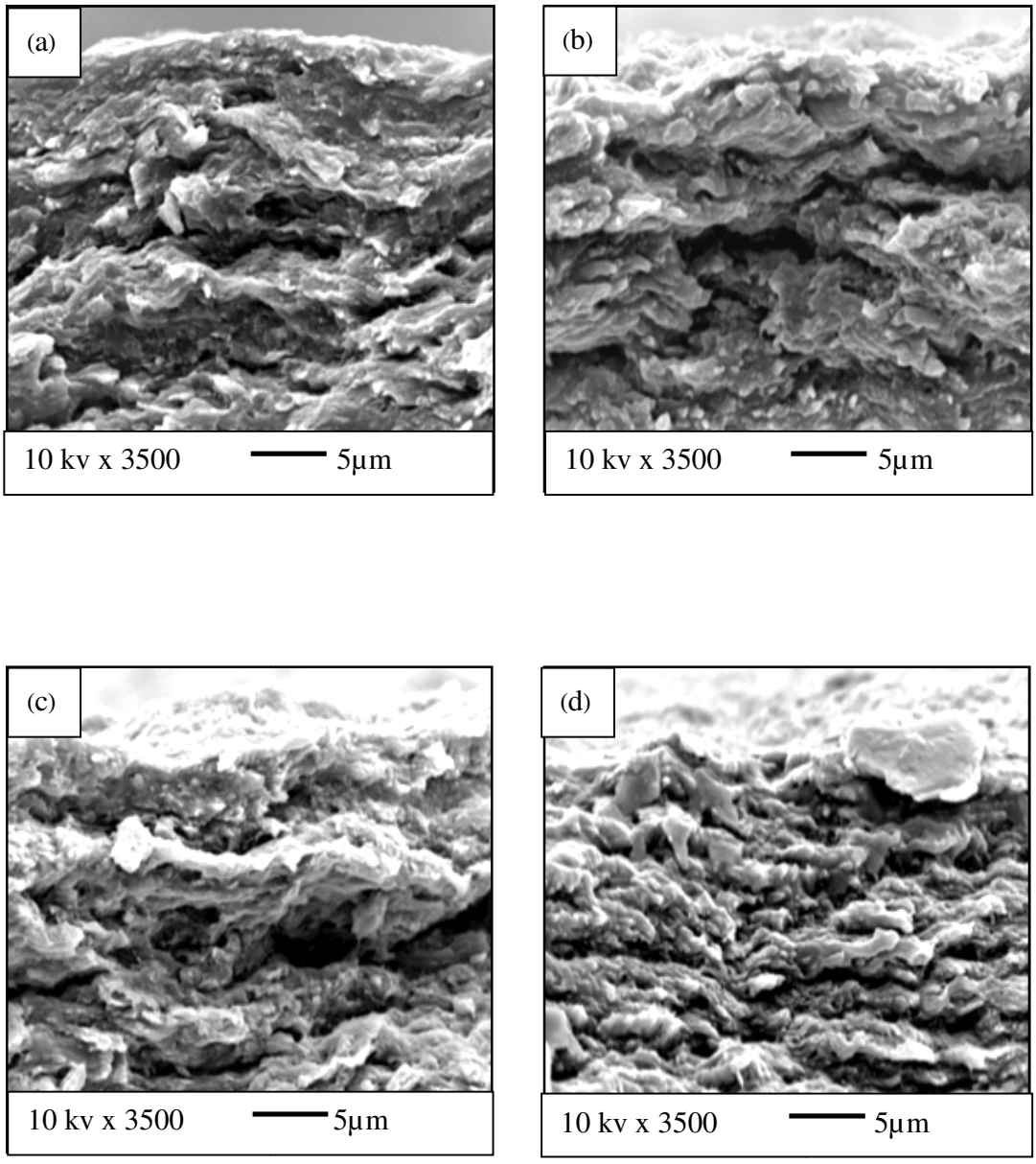


Figure 5.6 SEM images of cross sectional surface morphology of the dried films (chitosanM.W.200,000) at 3500X magnification:(a) Chitosan ,(b) CBC 10:2 ,(c) CBC 10:6 and (d) CBC 10:10

The cross-section of the bio-films was dense and very rough as represented in Figure 5.3 and 5.6.

Yin et al. (2006) studied the structural morphology of blended chtitosan with methylcellulose film compared with chitosan/hydroxypropyl methylcellulose. They reported that the morphology of the blends of chitosan and cellluliose was homogeneous, because chemical structure of chitosan and cellulose were similar. Chitosan derived by deacetylation of chitin, which composed of (1,4)-linked 2- amino-deoxy-β-D-glucan. It may be regarded as cellulose with hydroxyl group at position C-2 replaced by an amino group (Muzzarelli.,1973; Zikakis., 1984).

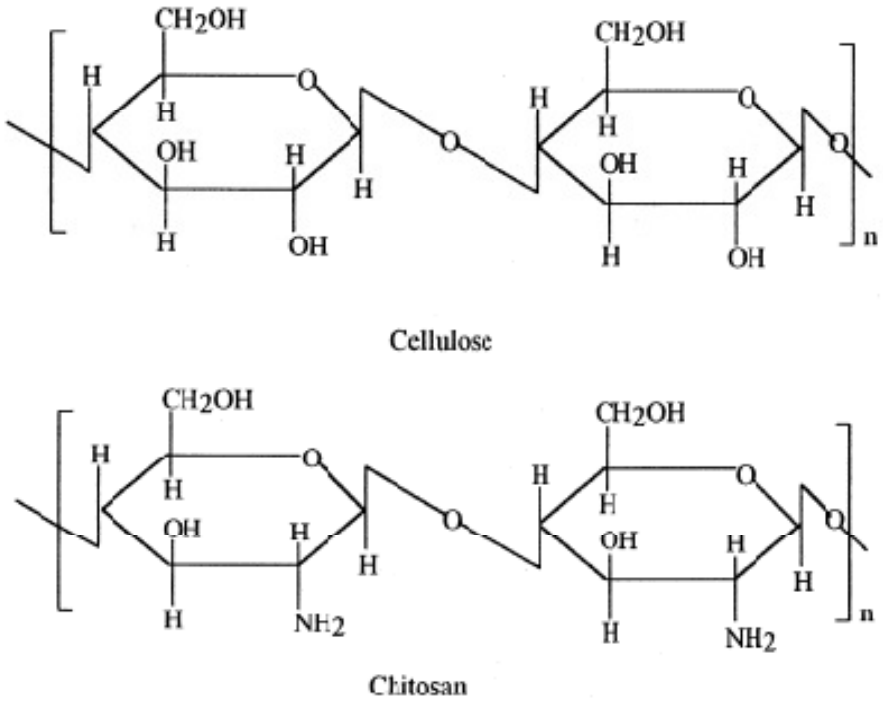


Figure 5.7 Structures of cellulose and Chitosan (Muzzarelli.,1973; Zikakis., 1984).

5.2. Mechanical property

In this study the mechanical properties of CBC films were analyzed in order to investigate the effects of BC slurry supplement and M.W. of chitosan on the composite CBC films. Figures 5.8 and 5.9 show the tensile strength and % elongation at break of CBC films, respectively with the average thickness of 83µm for CBC-M.W 200,000 and 56 µm for CBC-M.W 30,000.

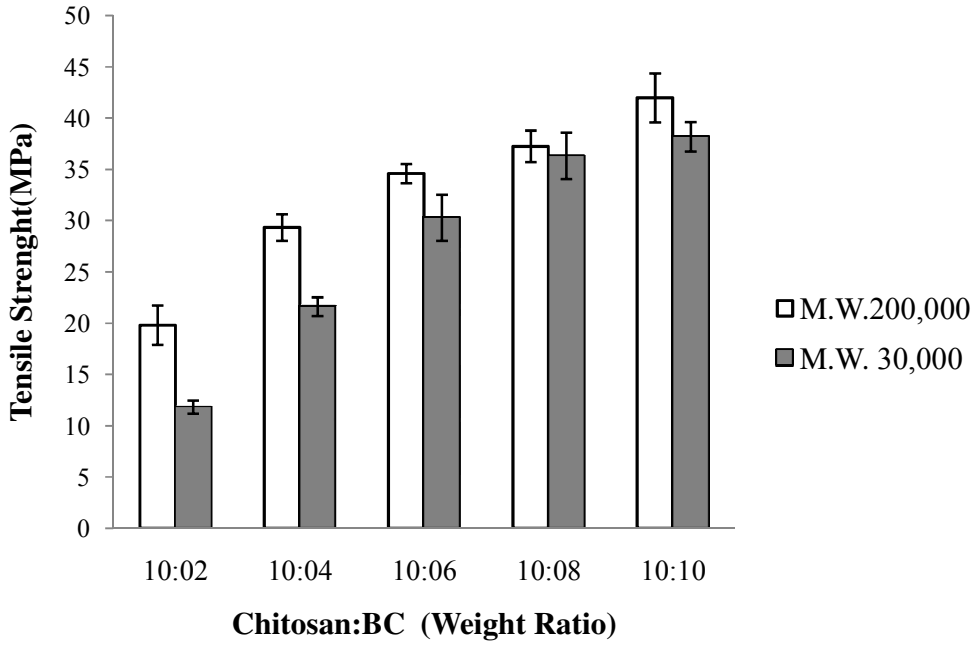


Figure 5.8 Tensile strength of CBC films in dry form

Figure 5.8 shows the effect of weight ratio of BC on tensile strength of the CBC films. The CBC- M.W. 200,000 film with the ratio of chitosan : BC at 10:2 and 10:10 has tensile strength at 19.82±1.9 to 41.97±2.4MPa, respectively and the CBC-M.W.30,000 with the ratio of chitosan:BC at 10:2 and 10:10 has tensile strength at 11.82±0.6 to 38.14±1.4MPa, respectively. The tensile strength property of the CBC dried films were significantly improved with the increase of weight ratio of BC. Since BC has

high mechanical strength (Vandamme et al.,1998), the incorporation of BC into the chitosan matrix considerably increased the overall mechanical performance of the composite films. The CBC-M.W. 200,000 has relatively higher tensile strength compared to that of the CBC-M.W. 30,000. This result is supported by the report of Liu et al in 2012, that the higher M.W. chitosan is stronger than the lower one.

Hosokawa et al (1991) reported that only trace amounts of carbonyl group can play an important role in cross-linking chitosan. So the amount of cellulose increased, the tensile strength tended to increase, probably due to some interaction between cellulose and chitosan molecule resulting in more cross-linking. The results of mechanical properties are also supported by the SEM, XRD and FTIR results. In general, chitosan contains amine groups which can cross-link with another anionic compound, whereas BC is expected to act as material supporting mechanical strength of films (Hosaokawa et al., 1992)

Chitosan with higher molecular weights may provide more reactive groups within one chain for interaction with BC via hydrogen bonds and hydrophobic interactions, leading to film strengthening. As a result, the better inter-connection between chitosan and BC was more pronounced.

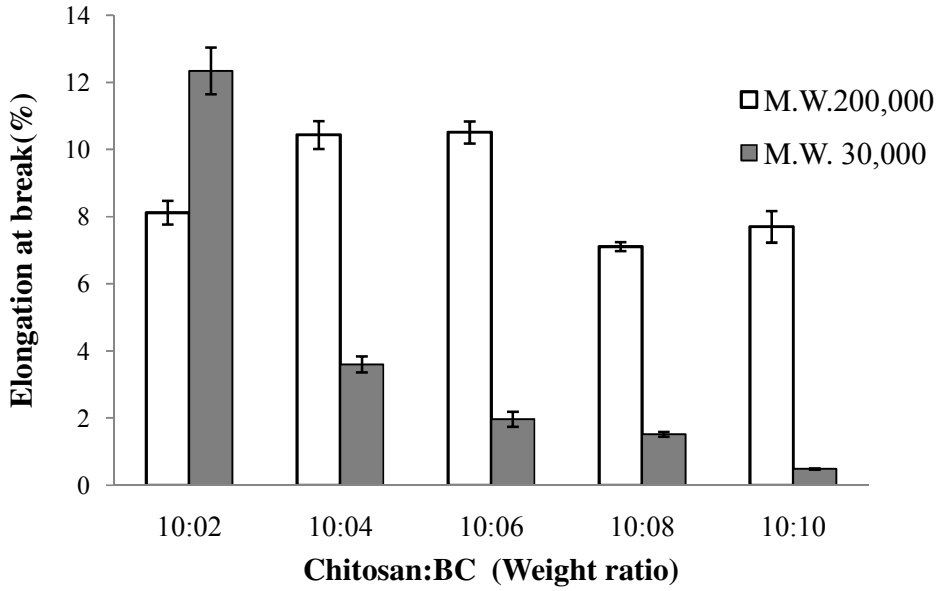


Figure 5.9 Elongation at break (%) of CBC in dry form

Figure 5.9 shows the effect of weight ratio of BC on the percentage of elongation at break. It was found that the composite film of CBC-M.W.200,000 exhibited the highest value of elongation at break (10.51±0.3%) at the weight ratios of chitosan: BC of 10:6, whereas the film of CBC-M.W.30,000 showed the maximum elongation at break (12.34±0.7%) at chitosan: BC of 10:2. In case of chiosan M.W. 200,000, the increased BC content higher than the chitosan: BC of 10:6 reduced the flexibility of the CBC film (the elongation at break was reduced to about 70-80% of the maximum value). On the other hand, the increased BC content more than the chitosan: BC of 10:2 considerably reduced the film flexibility (the elongation at break was reduced to about 10-30% of the maximum value). With high supplement of BC, the chitosan chain and BC is more cross-linking leading to form the composite film with structure of complex network. Consequently, the structure of high complex networks could increase film stiffness but lower film flexibility. In case of the CBC- M.W. 30,000, it was found that the increase in BC supplement tended to decrease in the value of % Elongation at break.

5.3 Water absorption capacity

The effect of the BC slurry supplement on the water absorption capacity (WAC) of the composite CBC films is shown in Figure 5.10. The maximum percentage of WAC of CBC-M.W. 200,000 at 548.2±28% was obtained at the weight ratio of chitosan: BC of 10:6 which was about 2 folds compared to that at the weight ratio of 10:2. Because BC has higher water holding capacity and hydrophilic character (Klem et al., 2001), the incorporation of BC into chitosan matrix for a certain amount resulted in the increase in WAC. However, at very high levels of BC supplement, it reduced the pore size of the composite films, resulting in lower WAC. The results agree with the SEM image observation. In case of CBC-M.W. 30,000, it was found that the water absorption capacity was lower than that of CBC-M.W.200,000. This result shows similar trend to that of the composite BC-Chitosan films prepared by immersing wet BC pellicle in chitosan solution (Kingkaew et al., 2011).

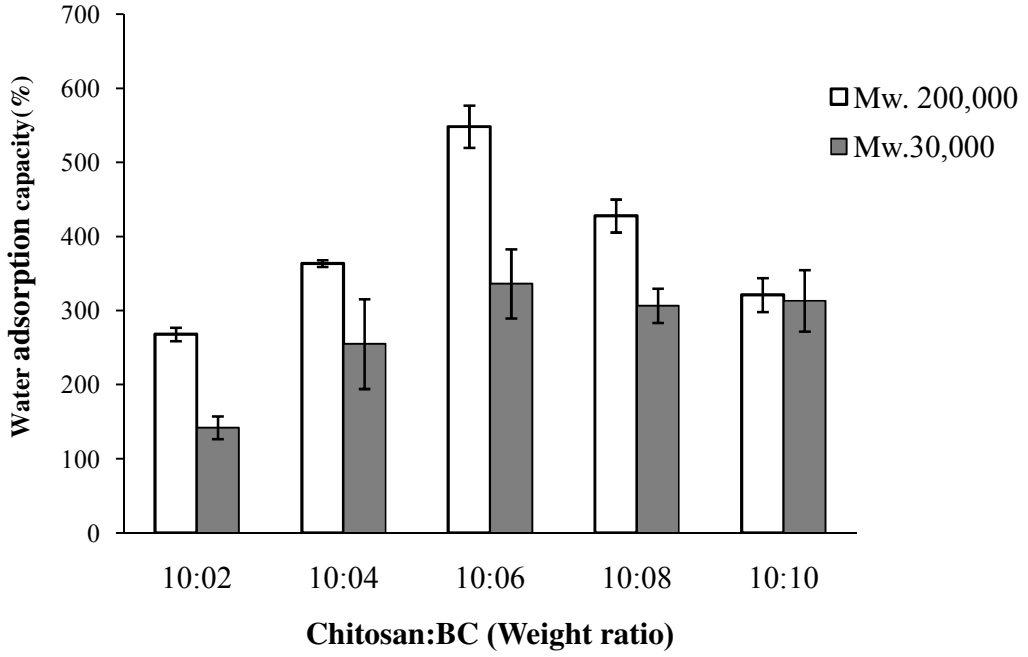


Figure 5.10 The water absorption capacity of CBC films

Kingkaew et al (2011) modifies the properties of bacterial cellulose (BC) with chitosan various M.W. between 30,000, 80,000 and 200,000. The composite films were developed by immersing the purified BC pellicle in chitosan acetic acid solution. They explained that the incorporation of chitosan, especially at high MW could enhance hydrophilic property of the film. This study implied that the chitosan M.W. 200,000 has more hydrophilic property than M.W. 30,000 that affects to the higher value of % WAC.

5.4 Antimicrobial ability

Antimicrobial ability is a very important feature of the food packaging. It can provide an increased shelf life and safety for fresh food (Pardini, 1987). Chitosan film itself has an antimicrobial activity (Elsabee et al., 2013) but BC alone does not. The incorporation of BC in chitosan film affected its antibacterial ability as presented in Table 5.1.

Table 5.1: Antimicrobial activities of chitosan and CBC films against *E. coli* and *S. Aureus* under dynamic contact conditions (Method : ASTM2149-10, % Reduction).

Test microorganism	**Sample**		**%Reduction**
E.coli	Chitosan M.W.30,000 Chitosan:BC	10:0	99.99
		10:4	0.00
		10:6	0.00
	Chitosan M.W. 200,000 Chitosan:BC	10:0	7.89
		10:4	0.00
		10:6	0.00
S.aureus	Chitosan M.W.30,000 Chitosam:BC	10:0	95.00
		10:4	25.00
		10:6	37.50
	Chitosan M.W. 200,000 Chitosan:BC	10:0	30.95
		10:4	53.12
		10:6	37.50

The summary of antimicrobial activities of chitosan and CBC films against *E. coli* and *S. aureus* under dynamic contact conditions determined by ASTM 2149-10 Standard Test method is shown in table 5.1. This test method maintains good contact between the microbial and the test substrate by constant agitation of the test specimen in a suspension during the test period. The antibacterial test of the chitosan films of M.W. 30,000 resulted in 99.99 % reduction of *E. coli* and 95% reduction of *S. aureus*, whereas, 7.89 % reduction of *E.coli* and 30.95% reduction of *S. aureus* were for the chitosan films of M.W. 200,000. Refer to the study of Zheng et al (2003), the antimicrobial effect on *S. aureus* was strengthened as the chitosan M.W increased. In contrast, the effect on *E. coli* was weakened. The analysis showed that CBC had no inhibitory effect on *E. coli*. This

result is similar to the study of No et al. (2002), who reported that chitosan showed stronger antibactericidal effects for gram-positive bacteria than gram-negative bacteria. Martinez-Camacho et al (2010) reported the antimicrobial abilities of chitosan with three proposed mechanisms. Firstly, the positive charges in the molecule of chitosan interact with the negative charges from the membranes of microbial cells, interfering with the nutrient exchange between the exterior and interior of the cells. Secondly, chitosan acts as a chelating agent which creating compounds from traces of metals essential to the cells. Lastly, the low molecular weight chitosan is capable of entering the cell's nucleus itself; interacting with the DNA affecting the synthesis of proteins processes of cells. In addition, the antibacterial activity of chitosan is also influenced by its degree of deacetylation, its concentration and the pH of the medium (Rabea et al., 2003).

Table 5.2 Antimicrobial activities of CBC(M.W. 200,000) films against *Escherichia coli* and *Staphylococcus aureus* under static conditions (Method: AATCC TM 39-198, Clear zone).

Microorganisms	**Sample**	**Clear zone (mm)**
E.coli	Chitosan:BC 10:4	0
	Chitosan:BC 10:6	0
S. aureus	Chitosan:BC 10:4	0
	Chitosan:BC 10:6	0

The results of the Agar diffusion tests are shown in Table 5.2. No antibacterial clear zone was observed around the CBC films on the test with *E. coli* and *S. aureus*. According to the method of the Agar diffusion test, the antimicrobial compound in the film has to release from the film and diffuse well through the agar. This method is a poor test for antimicrobial compounds with large M.W or the one that is trapped within the film, since no microbial inhibition can be detected if the antimicrobial compound cannot

diffuse through the agar effectively. This result implied that chitosan in the CBC films hardly released into the agar. Because the CBC films were prepared by ionic crosslinking combined with the complex fibril networks of BC, the release of chitosan was limited. For consumer satisfaction with food products, chemical components in packaging should not release or contaminate into the products.

5.5 Fourier transform infrared spectroscopy

In this section, Fourier transform infrared (FTIR) spectroscopy of the chitosan M.W.200,000, BC and CBC (10:6) films was carried out to detect any peak shift that could be attributed to interactions between chitosan and BC. Normally, FTIR spectroscopy has often been utilized as the useful equipment in determining chemical bond or functional group that exists in a material

Figure 5.11 presents the spectra of chitosan M.W.200,000, BC and CBC(10:6) films measured from 4000–400 cm^{-1}. The chitosan film's spectra showed a broad band at wave number about 1579 cm^{-1}, which represented N-H stretching vibration. The spectra of BC film shows a main band at wave number 3418 cm^{-1}, that represents O-H stretching vibration, a band at 2895 cm^{-1} represented the aliphatic C-H stretching vibration (Kim et al., 2010) and a band at around 1641 cm^{-1} was represented for the glucose carbonyl of cellulose (Phisalaphong and Jatupaiboon, 2008). It was shown that the band representing N-H group of CBC film has shifted from 1579 cm^{-1} to 1572 cm^{-1}.

The shift of the absorption bands of the films could be attributed to the interaction between the amino group of chitosan and the hydroxyl group of BC. The results indicated that the intermolecular hydrogen bonding might take places between chitosan and BC, leading to a good miscibility film. The FTIR result supported the results from the previous analysis of surface morphology (SEM) and mechanical properties (Universal Testing Machine).

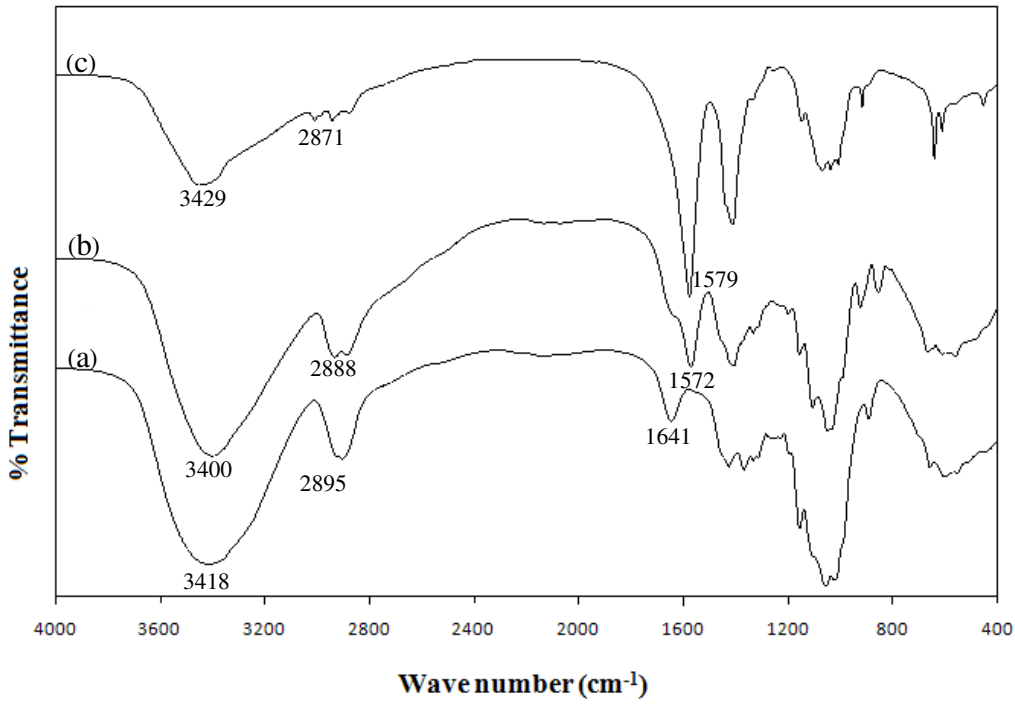


Figure 5.11 The FTIR spectra in wave number ranging from 4000 to 400 cm^{-1} of (a) BC film (b) Chitosan: BC 10:6 (c) Chitosan film Mw.200,000.

5.6 X-ray diffraction

Generally, X-Ray Diffraction (XRD) is used for structure measurement of polymer. In this research, the diffraction patterns of CBC films are shown in Figures 5.8. The pattern of CBC exhibits three main peaks at $2\theta = 14.2^{o}$, 16.6^{o}, and 22.4^{o} (Cai et al., 2010). The diffraction patterns of all chitosan: BC weight ratios of CBC films demonstrated that the three main peaks slightly shifted to wider angle to the pattern of BC.

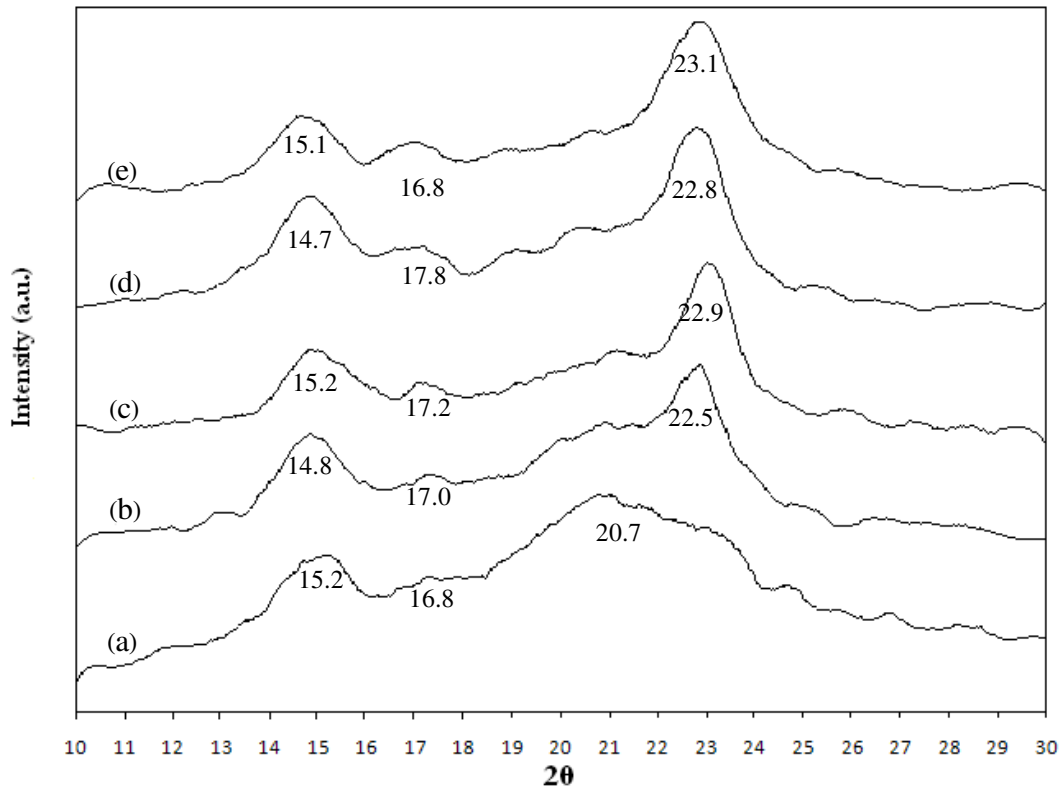


Figure 5.12 The XRD pattern of (a) CBC 10:2 (b) CBC 10:4 (c) CBC 10:6 (d) CBC 10:8 and (e) CBC 10:10

The crystalline indices (% C.I.) values of the CBC films at chitosan: BC of 10:2, 10:4, 10:6, 10:8, and CBC 10:10 were 23.14, 33.51, 40.51, 53.64, and 46.98, respectively. Overall, the supplement of BC slurry to chitosan based film showed effect of increase in %C.I. value. However, at the very high level of BC supplement (CBC 10:10), the slightly reduction of the C.I. value was observed, which were in a similar trend to the values of mechanical strength of the CBC films. The %C.I. considerably increased from 23.14 to 53.64 with the increase of BC content from the weight ratio of chitosan: BC at10:2 to 10:8. The result might imply that with the addition of BC slurry at the weight ratio of chitosan: BC of 10:8, the developed CBC film was more orderly arranged in uniform and firmly fiber network than that at weight ratio chitosan: BC of 10:10.

5.7 Water vapor transmission rate

The water vapor transmission rate (WVTR) is a major factor of characteristic of food packaging which controls evaporative fluid loss from fresh food and maintains a high humidity. In general, some kind of food requires not only permeation of unfavorable gas from the inside but also maintenance of oxygen barrier (C.M.D. Man et al., 2000). In this present work, The WVTR of the CBC film at the chitosan: BC of 10:6 (M.W. 200,000) was analyzed following the ASTM E 96-00 Standard and the results are represented in Table5.3.

Table 5.3 Water vapor transmission rate of CBC 10:6 (M.W.200,000) and commercial food packaging (*C.M.D Man et al.,2000 ,**Kingkaew et al.,2011and ***Sangsuwan et al.,2011)

Sample	Water vapor transmission rate ($g/m^2/day$)
Chitosan film***	28
Bacterial Cellulose**	1049
CBC 10:6	2,559
PVC*(unplasticized)	20
HDPE*	2
OPP*(biaxially oriented polypropylene)	1.5
LDPE*	5

In Table 5.2, the WVTR of the selected CBC film was significantly higher compared to those of commercial food plastic packagings, such as PVC (unplasticized), HDPE, OPP and LDPE (C.M.D Man et al., 2000). The comparison with the WVTR value of the chitosan film indicated that the supplement of BC in the composite film enhanced the WVTR of the films.

Hosokawa et al. (1990) reported that chitosan films had low WVTR. Similar result was reported by Wong et al. (1992), who produced chitosan films using formic acid aqueous solutions as a solvent. The water vapor permeability coefficient (WVPC) was about 0.3 g/m·day·atm for unplasticized films of unspecified thickness. Furthermore, Sansuwan et al (2011) modified chitosan film (M.W. in the range between 900,000-1,300,000) by incorporating plasticizer at various concentrations and reported the value of WVTR of the chitosan film without plasticizer at 28 $g/m^2/day$ (Table 5.2). According to the previous observation of the SEM images as shown in Figure 5.2(a) and 5.5(a), the unmodified chitosan film has a very dense structure comparing to the modified films. Usually, the permeability of a gas or vapor through a dense polymeric film is very low.

BC is composed of cellulose fibrils in form of ultrafine networks, giving the unique high porous structure. The combination of chitosan and BC could enhance hydrophilic property of the film and also increase hollow spaces of the composite film as represented in Figure 5.5(c). Consequently, the incorporation of BC slurry into chitosan matrix leads to the increase of WVTR of the modified films.

5.8 Oxygen transmission rate

In food packaging, oxygen is a major cause of oxidation reaction, which causes food spoilage. In this study, the oxygen transmission rate (OTR) test was analyzed following the ASTM D3985-05 Standard. Table5.3 shows the results of the OTR of the CBC at the weight ratio of chitosan: BC of 10:6 in comparison to those of commercial food plastic films.

The OTR of the selected CBC film was significantly higher than those of commercial food plastic films, chitosan film and BC film.

According to the previous reports by Conca Yang, (1993) and Anker, (1996), the chitosan film is of interest as a potential edible film component because of its excellent oxygen barrier properties. Sansuwan et al (2011) reported that the chitosan film without plasticizer has the value of OTR at 5.7 $cm^3/m^2/day$ (Table 5.4).

Table 5.4 Oxygen transmission rate of CBC 10:6 (M.W.200,000) and commercial food plastic packaging (*C.M.D. Man et al.,2000 and **Kingkaew et al.,2011)

Sample	**Oxygen transmission rate ($cm^3/m^2/day$)**
Chitosan film***	5.7
Bacterial Cellulose**	2.65
CBC 10:6	6,050
PVC*(unplasticized)	200
HDPE*	1,400
OPP*(biaxially oriented polypropylene)	1,600
LDPE*	8,000

The explanation was reasonably similar to the result of WVTR. The supplement of BC increased hollow spaces of the composite film. As a result, the OTR of the CBC was enhanced. Besides, the addition of glycerol as the plasticizer for the formation of the CBC films could play an important role in the increase of the OTR value. Previously, Sumitra et al (2004) reported that the amount of glycerol affects to OTR and WVTR. They explained that glycerol was a small molecule which could be inserted between polymer chain in order to reduce inter molecular and hydrogen bonding. Therefore, polymer has more hollow space leading to higher OTR and WVTR.

Generally, the OTR value of food packagings should be low to prevent oxidation in foods, however, there are some applications which required film packaging with high OTR value (7,000-10,000($cm^3/m^2/day$)), such as cold-smoked process for fresh fish etc. (C.M.D. Man et al.,2000)

5.9 Release of chitosan from CBC films

The cumulative releases of chitosan M.W. 200,000 (mg/cm^{3}of dried film) from the CBC film at the weight ratio of chitosan: BC at 10:6 in the buffer pH 5.5 and pH 7.4 were presented in Figure 5.13. This result showed that the maximum release amounts of chitosan were 0.137± 0.05 mg/cm^3 and 0.03±0.003 mg/cm^3 of dried films in the buffer solutions of pH 5.5 and 7.4, respectively. The actual content of chitosan in CBC films is 2.29 mg/cm^3.

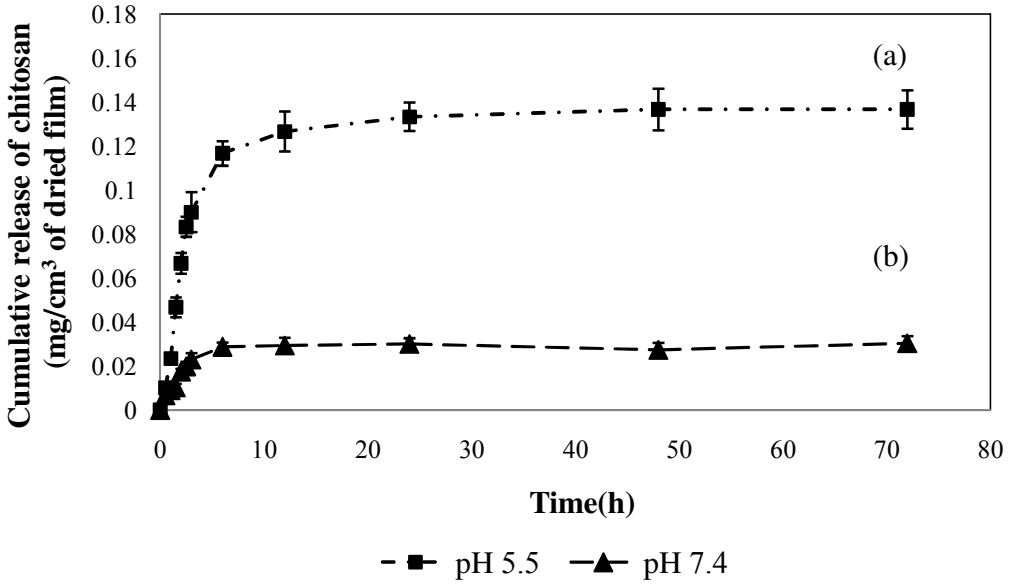


Figure 5.13 Cumulative release profile of chitosan from CBC 10:6 (M.W.200,000) films in (a) buffer pH5.5 and (b) buffer pH 7.4

In buffer solution pH 5.5 or 7.4, The CBC film at chitosan: BC of 10:6 showed a rapid increase in releasing chitosan during the initial 3 h. Afterwards, the release rate of chitosan was relatively slow and became constant at around 72 of the immersion time. The releasing rate of chitosan in the buffer solution pH 5.5 was higher than that of pH 7.4 because chitosan can be dissolved in agents which have pH below 6. The solubility of chitosan is very poor above pH 7 (Rabea et al., 2003).

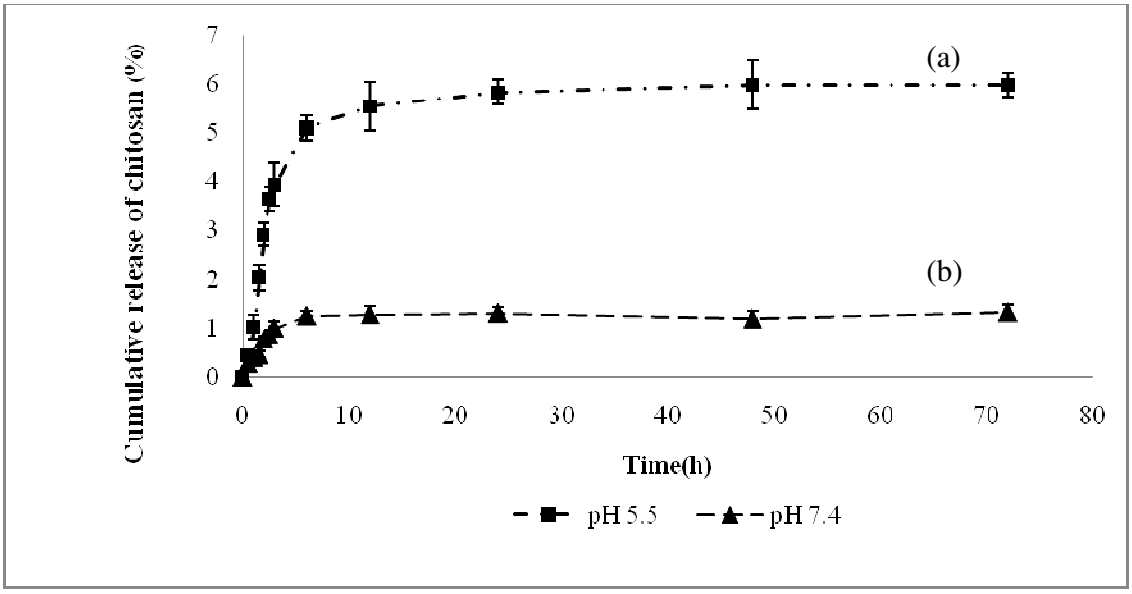


Figure 5.14 Cumulative release profile of chitosan (%) from CBC 10:6 (M.W.200,000) films in (a) buffer pH5.5 and (b) buffer pH 7.4

Figure 5.13 represented the cumulative release profile of chitosan from the CBC film at chitosan: BC ratio of 10:6 (M.W.200,000) in buffer solution pH 5.5 and 7.4 as a function of time. The value of cumulative released of chitosan in buffer pH 5.5 was higher than pH7.4. The cumulative release profile was similar to that of Figure 5.14. There were 2 main factors that could possibly affect the release rate of chitosan. The first one was the effect of pH of buffer solutions. The second one, which was considered very important, was the effect of film structure and interactions in ironically crosslinked chitosan combined with interactions with BC fibril networks, which were found to be effective for the control and limit of chitosan release from the CBC films.

Putivaranat et al (1996) reported that the network structure of chitosan affected the release of chitosan. In general, chitosan is well dissolved in acetic solution because the interaction between molecule of chitosan and acetic is higher than the interaction between chitosan molecules. But the crosslinked chitosan leads to strength covalence bond between chitosan chains which affects to poor solubility.

CHAPTER VI
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In order to improve properties of chitosan films, chitosan (85% DAC , M.W. 30,000 and 200,000) in acetic acid aqueous solution was blended with BC slurry at various weight ratios and chitosan-BC (CBC) films were prepared by casting method. Glycerol at 2% v/v was used as a plasticizer inorder to increase the flexibility of the composite films. The results demonstrated that the mechanical properties of the CBC films depended on the weight ratio of BC and molecular weight of chitosan. The FTIR spectra of the modified films revealed the intermolecular interaction between amino groups of chitosan and hydroxyl groups of cellulose. The significant improvement of mechanical properties, water absorption capacity (WAC), and % crystalline index were apparently obtained by the supplement of BC at the weight ratio of chitosan: BC at 10:6. However, the increased BC content higher than 10:6 could decrease the elongation at break of the composite film. Based on the overall mechanical and physical properties, we suggested using the formulation containing the weight ratio of chitosan: BC at 10:6 for the fabrication of thin films for food packaging. It was also showed that the CBC films had inhibitory effect on *S. aureus* growth.

Moreover, the CBC composite film at weight ratio chitosan: BC at 10:6 was characterized for WVTR and OTR. The release of chitosan (%) in buffer pH 5.5 and 7.4 was evaluated. It was shown that the modified CBC film structure and interactions in ionic-crosslinked chitosan combined with interactions with BC fibril networks were effective for the control and limit of chitosan release from the CBC films.

6.2 Recommendations for future studies

Based on this study, further works for the improvement of the chitosan composite films are recommended as following.

1. Searching the methodology to improve CBC films for achieving the optimal oxygen transmission rate (OTR) and water vapor transmission rate (WVTR). Multi-layer coating films for the reduction of OTR and WVTR is recommended.
2. To study the method to improve antimicrobial properties, for example by integration of natural antimicrobial agents.
3. Modifying the CBC film for other applications, for example by incorporating other herbal exacts which derived from natural products such as Gallic acid to be used as wound dressing material.

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APPENDICES

APPENDIX A

A.1 In vitro released studies

The release characteristics of chitosan content from the CBC films were investigated by total immersion method (Suwantong et al., 2007). The relevant buffer-simulated gastric fluid acetate buffer pH 5.5 and phosphate buffer pH 7.4 were used as releasing medium. Each specimen (square plate; 2.5 x 2.5 cm^2) was immersed in 30 ml of the releasing medium at the temperature of 37 ^oC. At a specified immersion period ranging from 0 to 72 h, either 1ml of a sample solution was withdrawn and an equal amount of the fresh medium was refilled. The amounts of chitosan content in the sample solutions were determined using the ninhydrin method (Leane et al., 2004).

A.2 Ninhydrin method

Chemicals

1. Chitosan M.W. 30,000, and 200,000 with DAC. > 85%
2. Lithium acetate dihydrate
3. Glacial acetic aicd
4. Ninhydrin
5. Hydrindantin
6. Ethanol

Ninhydin reagent preparation

To prepare 10 ml of lithium acetate buffer by dissolving 4.08 grams of lithium acetate dehydrate in 6 ml of DI water. The pH of solution was adjusted to 5.2 using glacial acetic acid and the volume adjusted to 10 ml with DI water. The ninhydrin reagent was prepared by adding 10 ml of lithium acetate buffer to 0.8 g ninhydrin and 0.12 g hydrindantin in 30 ml DMSO.

Ninhydrin assay

0.5 ml of ninhydrin reagent was added to 0.5 ml of the sample in a glass scintillation vial. The vials were immediately capped, briefly shaken and heated in boiling water for 30 min to allow the reaction to proceed. After cooling, 1 ml of a 50:50 ethanol:water mixture was added to each vial. The vials were then vortexed for 15 s in order to oxidise the excess of hydrindantin. The absorbance of each solution was measured on a UV spectrophotometer at 570 nm and the concentration of chitosan in the sample calculated from a standard calibration curve.

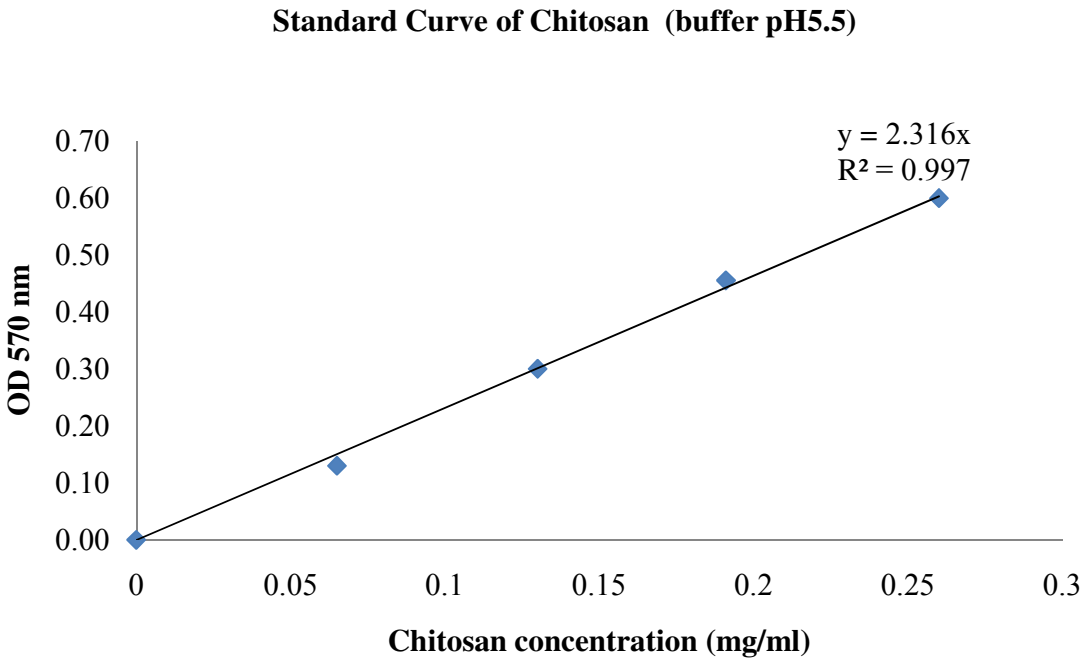

Figure A1 Chitosan standard curve for release analysis (buffer pH 5.5)

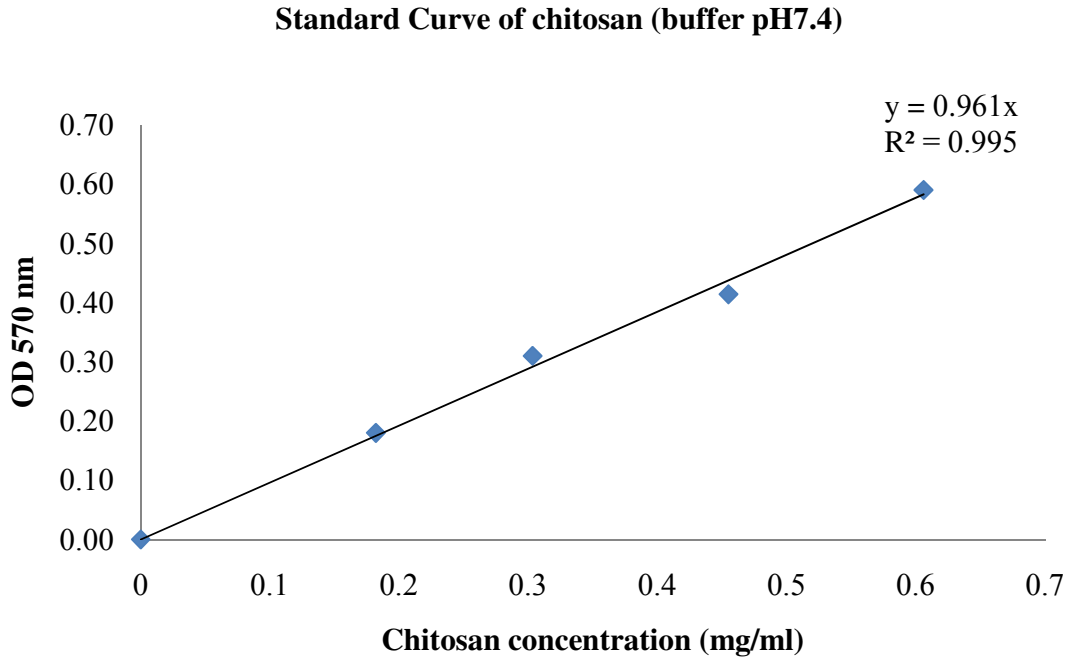

Figure A2 Chitosan standard curve for release analysis (buffer pH 7.4)

A.3 Method of hydrolysis of CBC films

Chemicals

1. CBC at weight ratio chitosan:BC at 10:6
2. 37% HCl
3. 20% NaOH

Each 2.5 x 2.5 cm^2 of sample was hydrolyzed by 37% HCl at the temperature 100 ^oC for 30 min. After that, 20% NaOH was added in order to neutralize the solution and then the sample solution was centrifuged at 2500 rpm for 30 min. The amount of chitosan content in the supernatant was determined using the ninhydrin method.

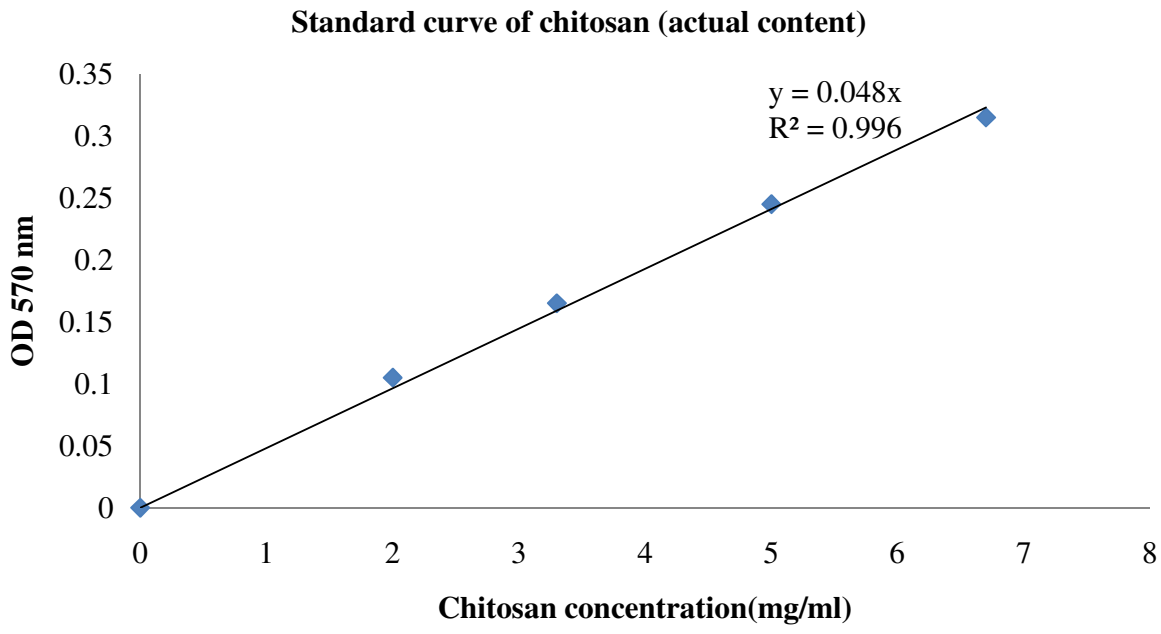

Figure A3 Chitosan standard curve for actual content

Appendix B

Table B1 Data of Figure 5.8

Chitosan Mw.30,000 Chitosan:BC	**Tensile strength(MPa)**				
	1	**2**	**3**	**Average**	**SD.**
10:2	12.46	11.19	11.81	11.82	0.64
10:4	21.76	20.64	22.45	21.61	0.66
10:6	32.35	27.90	30.58	30.28	2.24
10:8	36.77	34.06	38.55	36.46	2.26
10:10	38.07	36.80	39.66	38.18	1.42

Table B2 Data of Figure 5.8

Chitosan Mw.200,000 Chitosan:BC	**Tensile strength(MPa)**				
	1	**2**	**3**	**Average**	**SD.**
10:2	17.90	19.80	21.74	19.81	1.92
10:4	31.04	29.48	28.46	29.66	1.30
10:6	34.78	35.41	33.57	34.59	0.94
10:8	37.31	35.69	38.75	37.25	1.53
10:10	44.32	42.01	39.57	41.97	2.38

Table B3 Data of Figure 5.9

Chitosan Mw.30,000 Chitosan:BC	**Elongation at break (%)**				
	1	**2**	**3**	**Average**	**SD.**
10:2	12.00	11.89	13.14	12.34	0.69
10:4	3.56	3.86	3.39	3.6	0.24
10:6	2.04	2.15	1.71	1.97	0.23
10:8	1.60	1.46	1.50	1.52	0.72
10:10	0.50	0.48	0.48	0.49	0.01

Table B4 Data of Figure 5.9

Chitosan Mw.200,000 Chitosan:BC	**Elongation at break (%)**				
	1	**2**	**3**	**Average**	**SD.**
10:2	8.45	8.15	7.76	8.12	0.69
10:4	10.03	10.86	10.43	10.44	0.24
10:6	10.15	10.60	10.79	10.51	0.23
10:8	7.11	7.24	6.97	7.11	0.72
10:10	7.16	7.97	7.97	7.7	0.01

Table B5 Crystallinity Index of CBC

Chitosan Mw. 200,000 Chitosan:BC	**$2\theta = I_{max}$**	**$2\theta = I_{am}$**	**C.I. (%)**
10:2	389	299	23.14
10:4	388	258	33.51
10:6	207	123	40.57
10:8	371	172	53.64
10:10	341	172	49.41

Table B6 Data of Figure 5.10

Chitosan Mw.30,000 Chitosan:BC	**Water absorption capacity (%)**				
	1	**2**	**3**	**Average**	**SD.**
10:2	133.33	160	133.33	142.22	15.4
10:4	220	220	325	255	60.62
10:6	300	320	388.89	336.29	46.63
10:8	320	320	280	306.67	23.09
10:10	360	300	280	313.33	41.63

Table B7 Data of Figure 5.10

Chitosan Mw.200,000 Chitosan:BC	**Water absorption capacity**				
	1	**2**	**3**	**Average**	**SD.**
10:2	278.26	260.87	265.21	268.12	9.05
10:4	359.18	359.09	363.63	363.63	4.54
10:6	516.67	572.22	555.56	548.15	28.51
10:8	450	405.56	427.78	427.78	22.22
10:10	310.52	347.37	305.26	321.05	22.94

Table B9 Data of Table 5.3 and 5.4

Chitosan Mw.200,000 Chitosan:BC	**Water vapor transmission rate($g/m^2/day$)**			
	1	**2**	**Average**	**SD.**
10:6	2549.76	2568.48	2559.12	13.24
Chitosan Mw.200,000 Chitosan:BC	**Oxygen transmission rate($cm^3/m^2/day$)**			
	1	**2**	**Average**	**SD.**
10:6	6139	5961	6050	125.86

Table B10 Data of Table 5.1

Test Microorganism	Sample		% reduction
E.coli	Chitosan Mw.30,000	10:0	99.99
	Chitosan:BC	10:4	0.00
		10:6	0.00
	Chitosan Mw.200,000	10:0	7.89
	Chitosan:BC	10:4	0.00
		10:6	0.00
S.aureus	Chitosan Mw.30,000	10:0	95.00
	Chitosan:BC	10:4	25.00
		10:6	37.50
	Chitosan Mw.200,000	10:0	30.95
	Chitosan:BC	10:4	53.12
		10:6	37.50

Table B11 Data of Figure 5.13

Time (h)	**Chitosan concentration (mg/cm^3 of dried film) (Buffer pH 5.5)**				
	#1	**#2**	**#3**	**Average**	**SD.**
0	0	0	0	0	0
0.5	0.01	0.01	0.01	0.01	0
1	0.02	0.02	0.03	0.023	0.005
1.5	0.05	0.04	0.05	0.046	0.005
2	0.07	0.06	0.07	0.067	0.005
2.5	0.08	0.08	0.09	0.083	0.005
3	0.09	0.08	0.1	0.090	0.010
6	0.11	0.12	0.12	0.116	0.011
12	0.14	0.12	0.12	0.126	0.005
24	0.14	0.13	0.13	0.133	0.011
48	0.13	0.13	0.15	0.136	0.011
72	0.14	0.14	0.13	0.136	0.005

Table B12 Data of Figure 5.13

Time (h)	Chitosan concentration (mg/cm^3 of dried film) (Buffer pH 7.4)				
	#1	**#2**	**#3**	**Average**	**SD.**
0	0	0	0	0	0
0.5	0.006	0.006	0.007	0.006	0.0006
1	0.009	0.008	0.010	0.009	0.0011
1.5	0.010	0.012	0.009	0.010	0.0015
2	0.017	0.017	0.019	0.018	0.0011
2.5	0.018	0.020	0.022	0.020	0.0025
3	0.023	0.026	0.020	0.023	0.0030
6	0.028	0.027	0.031	0.029	0.0020
12	0.029	0.026	0.033	0.029	0.0035
24	0.029	0.033	0.028	0.030	0.0020
48	0.025	0.031	0.026	0.027	0.0032
72	0.028	0.034	0.029	0.030	0.0032

Table B13 The amount of actual chitosan content of CBC films

Actual chitosan concentration (mg/cm^3 of dried film)				
#1	**#2**	**#3**	**Average**	**SD.**
2.32	2.27	2.29	2.29	0.025

VITAE

Mr. Kampole Intasorn was born on September 15th, 1986 in Singburi, Thailand. He received the Bachelor Degree's in Chemical Engineering from Kasetsart University in May, 2008. He continued Master degree in Chemical engineering at Chulalongkorn University in June, 2010.

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