ลักษณะการดูดซึมและการปลดปล่อยของเคอร์คูมินในฟิล์มแบคทีเรียเซลลูโลส

นางสาว วรรณิภา วรหาญ

ABSORPTION AND RELEASE CHARACTERISTICS OF CURCUMIN IN BACTERIAL

CELLULOSE FILM
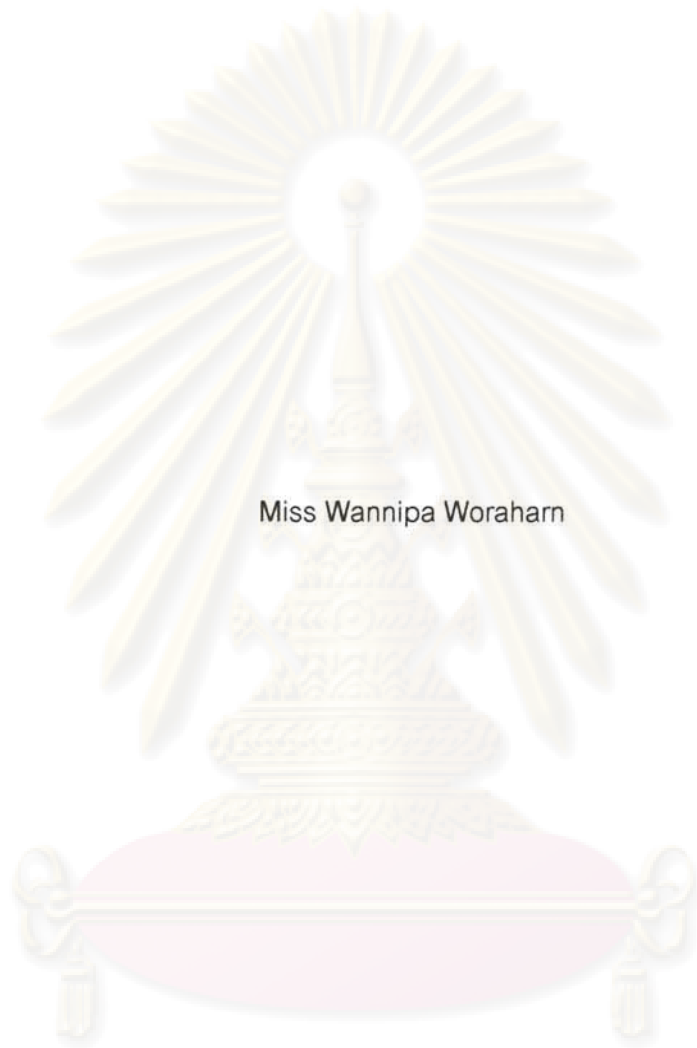
Miss Wannipa Woraharn

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Engineering Program in Chemical Engineering

Department of Chemical Engineering

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Thesis Title   ABSORPTION AND RELEASE CHARACTERISTICS OF CURCUMIN IN
                      BACTERIAL CELLULOSE FILM
By   Miss Wannipa Woraharn
Field of Study   Chemical Engineering
Thesis Advisor   Associate Professor Muenduen Phisalaphong, Ph.D.
Thesis Co-Advisor   Pongpun Siripong, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.... Dean of the Faculty of Engineering

(Associate Professor Boonsom Lerdhirunwong, Dr.Ing.)

THESIS COMMITTEE

.... Chairman

(Associate Professor Sarawut Rimdusit, Ph.D.)

.... Thesis Advisor

(Associate Professor Muenduen Phisalaphong, Ph.D.)

.... Thesis Co-Advisor

(Pongpun Siripong, Ph.D.)

.... Examiner

(Associate Professor Bunjerd Jongsomjit, Ph.D.)

.... External Examiner

(Chada Phisalaphong, Ph.D.)

วรรณิภา วรหาญ : ลักษณะการดูดซึมและการปลดปล่อยของเคอร์คูมินในฟิล์มแบคทีเรีย เซลลูโลส (ABSORPTION AND RELEASE CHARACTERISTICS OF CURCUMIN IN BACTERIAL CELLULOSE FILM. อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. เหมือนเดือน พิศาลพงศ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ผ่องพรรณ ศิริพงษ์, 78 หน้า

งานวิจัยนี้ได้ทำการศึกษา ลักษณะการดูดซึมและการปลดปล่อยของเคอร์คูมินในฟิล์ม แบคทีเรียเซลลูโลสที่สังเคราะห์ด้วยแบคทีเรียชนิด *Acetobacter xylinum* โดยการใช้สารละลาย เคอร์คูมินที่ความเข้มข้น 0.0, 0.2, 0.4, 0.6, 0.8 และ 1.0 (มิลลิกรัม ต่อ มิลลิลิตร) ดูดซึมลงในฟิล์ม แบคทีเรียเซลลูโลสแบบเปียก จากนั้นศึกษาถึงคุณสมบัติทางกายภาพของแผ่นแบคทีเรียเซลลูโลส-เคอร์คูมิน ที่พัฒนาขึ้นได้แก่ คุณสมบัติทางกล การบวมน้ำ และศึกษาคุณสมบัติทางชีวภาพ ได้แก่ คุณสมบัติการต้านเชื้อแบคทีเรียและเชื้อรา ด้วยวิธี Disc diffusion และคุณสมบัติการยับยั้งการ เจริญเติบโตของเซลล์มะเร็งผิวหนังของคน (B16 melanoma cells) ด้วยวิธี MTT assay ผลการวิจัย พบว่า แผ่นฟิล์มแบคทีเรียเซลลูโลส-เคอร์คูมินที่พัฒนาขึ้นนั้น มีสมบัติทางกลและความสามารถของ การบวมน้ำต่ำกว่าเมื่อเทียบกับแผ่นฟิล์มแบคทีเรียเซลลูโลส นอกจากนี้ จากผลการทดสอบด้วย อินฟราเรดทางโครงสร้างโมเลกุล แสดงให้เห็นถึงการเกิดปฏิสัมพันธ์ระหว่างโมเลกุลเซลลูโลสของ แบคทีเรียเซลลูโลสกับเคอร์คูมิน และจากการทดสอบคุณสมบัติการต้านเชื้อแบคทีเรียและเชื้อรา ของฟิล์มแบคทีเรียเซลลูโลส-เคอร์คูมินที่เตรียมโดยใช้สารละลายเคอร์คูมินที่ความเข้มข้น 0.5 และ 1.0 มิลลิกรัม ต่อ มิลลิลิตร พบว่ามีคุณสมบัติในการต้านเชื้อรา *Aspergillus niger* แต่ไม่พบ คุณสมบัติการต้านเชื้อแบคทีเรียทั้งแกรมบวก และแกรมลบ ส่วนการศึกษาคุณสมบัติการยับยั้งการ เจริญของเซลล์มะเร็งผิวหนังของคน พบว่าปริมาณของเคอร์คูมินที่ดูดซึมในแผ่นแบคทีเรียเซลลูโลส สามารถปลดปล่อยสู่อาหารเลี้ยงเซลล์ได้ซึ่งส่งผลในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งผิวหนัง ในระยะเวลา 3 ชั่วโมง ดังนั้นการพัฒนาคุณสมบัติของแผ่นฟิล์มแบคทีเรียเซลลูโลส-เคอร์คูมินนี้จึง เป็นประโยชน์ต่อการนำไปประยุกต์ใช้ในทางการแพทย์ต่อไป

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต......วรรณิภา วรหาญ......
สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก........
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KEYWORDS: BACTERIAL CELLULOSE / CURCUMIN/ ABSORPTION/ RELEASE

WANNIPA WORAHARN: ABSORPTION AND RELEASE CHARACTERISTICS OF CURCUMIN IN BACTERIAL CELLULOSE FILM. THESIS ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D., THESIS CO-ADVISOR: PONGPUN SIRIPONG, Ph.D., 78 pp.

In this research, absorption and release characteristics of curcumin in bacterial cellulose (BC) film by *Acetobacter xylinum* was studied using the curcumin solution at 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 (mg/ml) absorbed into the wet BC films. Then the physical properties of the developed bacterial cellulose-curcumin (BCC) films such as mechanical property and equilibrium water content and the biological properties such as antifungal ability, antibacterial ability (test by Disc diffusion) and the inhibition of B16 melanoma cells (test by MTT assay) were investigated. From the study, the mechanical properties and equilibrium water content of the BCC films were found to be relatively lower than those of the BC film. Fourier transform infrared spectroscopy analysis illustrated the interaction between BC fibrils and curcumin components. From the examination of antibacterial and antifungal properties, the BCC films with the loading of curcumin solution at concentration of 0.5 and 1.0 mg/ml displayed antifungal activity against *Aspergillus niger* with no antibacterial activity against Gram-positive and Gram negative bacteria. Characterization of inhibitory effect on growth of human melanoma cells showed that the amount of curcumin absorbed in the BCC films could be released into the cell culture medium and subsequently inhibited the growth of melanoma cancer within 3 hours. Therefore, the development of the BCC film will be useful in medical applications.
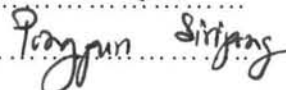
Department: Chemical Engineering   Student's signature..Wannipa Woraharn..

Field of Study: Chemical Engineering   Advisor's signature...

Academic Year: 2009   Co-Advisor's signature....Pongpun Siripong

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CHAPTER I

INTRODUCTION

Recent advances in the field of biomaterials and their medical applications have been interested. Cellulose has been used as biomaterial for many medical applications (Hoenich, 2006). However, plant cellulose is unpurified cellulose associated with other kinds of natural fiber like lignin and hemicellulose while bacterial cellulose (BC) is nearly-purified cellulose. Moreover, the production of plant cellulose product is facing some environmental problems (Saied et al., 2004).

The production of cellulose by *Acetobacter xylinum* was reported for the first time in 1886 by A. J. Brown. This nonphotosynthetic organism can procure glucose, sugar, glycerol, or other organic substrates and convert them into pure cellulose. Cellulose is synthesized by bacteria belonging to the genera Aceobacter, Rhizobium, Agrobacterium, and Sarcina. The *A. xylinum* was reported as the most efficient producer. BC displays advantages superior to the counterpart from plants with its physical and chemical properties. It has the unique properties such as high mechanical strength, high crystallinity, high hydrophilicity and ultra-fine network structure.

BC offers a wide range of applications. It has been used in the food industry (Iguchi, 2000), later in the fabrication of reinforced paper (Yamamoto, 1989) and recently it was investigated as material for medical applications. Studies carried out in vitro and in vivo have demonstrated its biocompatibility (Watanabe 1993 and Helenius 2006). Due to its good mechanical properties, water sorption capacity, porosity, stability and conformability, BC has been used in tissue engineering of

cartilage (Svensson, 2005), replacement of blood vessels in rats (Klemm, 2001), artificial skin for humans with extensive burns (Fontana *et al.*, 1990) and wound-dressing (Czaja *et al.*, 2006)

Curcumin is a naturally occurring compound found in the plant turmeric (*Curcuma longa L.*). Crude curcumin has a natural yellow hue and its components include curcumin, demethoxycurcumin, and bisdemethoxycurcumin, commonly called curcuminoids (Ahsan et al., 1999), which are polyphenols normally existing in at least two tautomeric forms, keto and enol. The enol form is more energetically stable, both in the solid phase and solution (Anonymous et al., 2007).

Curcumin has shown to suppress cellular proliferation in breast, colon, oral, other cancers and possesses a wide rage of pharmacological activity including anti-inflammatory, anti-oxidant, anti-hepatotoxic, anti-microbial, anti-depressant, chemopreventive, anticancer and anti-proliferation properties (Gafner et al., 2004, hidaka et al., 2002 and Sidhu et al., 1999)

The potential for use of the curcumin-loaded e-spun CA fiber mats and corresponding as-cast CA films as topical/transdermal patches or wound dressings was reported (Orawan et al., 2007). With curcumin incorporated collagen matrix, it was found that curcumin helped wound reduction and enhanced cell proliferation (Gopinath et al., 2004). Curcumin could enhance wound healing in rats and guinea pigs (Sidhu et al., 1999). The anticoagulation behavior of curcumin stents was improved significantly as the drug dose was increased (Pan et al., 2006).

In the present research, curcumin was loaded into a BC film synthesized under the static conditions by *Acetobacter xylinum*. It was then assessed for its potential use as a carrier for control release and transdermal delivery of curcumin. Various

properties such as surface morphology, physical properties of the curcumin-loaded BC film were investigated. Furthermore, the absorption and release characteristics of curcumin from the curcumin-loaded BC were examined.

Objectives

1. To develop and characterize curcumin-loaded BC films

2. To examine the absorption and release characteristics of curcumin from the curcumin-loaded BC

3. To evaluate the biological activity of the curcumin-loaded BC films

Research Scopes

1. Prepare BC film from biosynthesis under static conditions by *A. xylinum.*

2. Develop a procedure for loading curcumin into the BC film.

3. Examine the absorption and release characteristics of curcumin from the curcumin-loaded BC

4. Characterize physical properties of the developed BC-curcumin 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 the mechanical properties of the films.

5. Characterize biological properties of the developed BC-curcumin film:

   a. Antibacterial and antifungal activities

   b. Inhibitory effects on human melanoma cells

CHAPTER II

THEORY

2.1 Cellulose

Cellulose is composed of the homopolymer of β-1, 4-linked D-glucose. The degree of polymerization of cellulose varies from 100–15,000 glucose units with the crystallization of the long linear chains to form microfibrils of a single crystalline entity (Koyama et al., 1997). Cellulose molecule forms a straight, almost fully extended chain as shown in Figure 2.1.
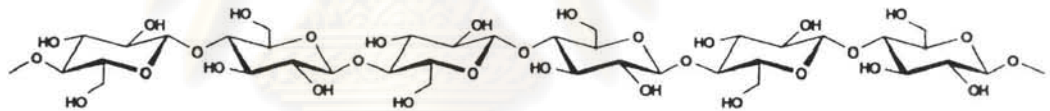
Figure 2.1 Structure of Cellulose (Gardner and Blackwell, 1974).

The cellulose's structure consist of crystalline or non-crystalline state. Crystalline cellulose has at least two distinct allomorphs, cellulose I and cellulose II. Both are found synthesized in nature; however, cellulose I is by far the most revalent. No eukaryotic cells are known to abundantly synthesize cellulose II in vivo.

Cellulose I is exclusively parallel chains. This has been re-affirmed by several independent approaches using electron diffraction, enzymatic degradation, and silver labeling of the reducing ends (Koyama, 1997). The cellulose I allomorph is the thermodynamically metastable form of cellulose (Ranby, 1952). Cellulose I can be converted directly to cellulose II; however, cellulose II cannot be directly converted to cellulose I. Cellulose II is the most thermodynamically stable allomorph of cellulose (Ranby, 1952). Glucan chain orientation within cellulose II is antiparallel. The chains are strong evidence.

2.2 Bacterial Cellulose (BC)

Cellulose is synthesized by bacteria belonging to the general Aceobacter, Rhizobium, Agrobacterium, and Sarcina (Jonas et al.,1998).The *A. xylinum* was reported as the most efficient producer. The production of bacterial cellulose could be carried out in either solid-phase cultivation or submerged culture. The methods of production process highly influence the quality and quantity of the cellulose and it is reflected directly on the process economics. Also, the microbial cellulose obtained via these methods is not completely pure and contains some impurities, such as culture broth components and whole *A. xylinum* cells. Some purification methods reduce the number of cells and coloration (Yamanaka et al., 1989).

The biosynthesis of bacterial cellulose was reviewed in detail by Ross et al., (1991). As shown in Figs. 2.2, the process includes the synthesis of uridine diphosphate-glucose (UDPGlc), which is the cellulose precursor, followed by glucose

polymerization into the b-1, 4-glucan chain and nascent chain association into the characteristic ribbon-like structure, formed by hundreds or even thousands of individual cellulose chains. The product that was excreted into the extracellular culture by a membrane protein complex known as cellulose synthase.

Glucose ---- Glucokinase ----> Glucose -6- Phosphate ---- Phosphoglucomutase ----> Glucose -1- Phosphate ---- UDP-Glucose Pyrophosphorylase ----> UDP-Glucose ---- Cellulose Synthase ----> Cellulose
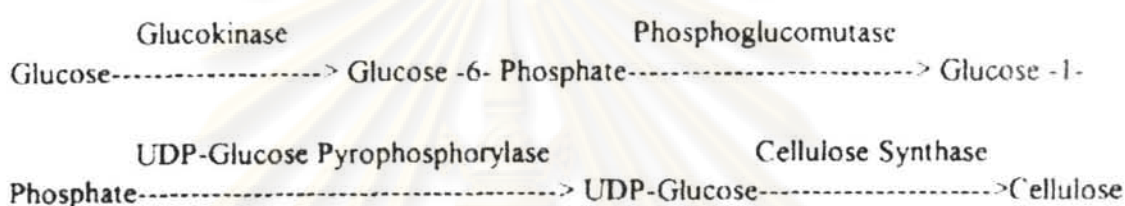
Figure 2.2 Proposed biochemical pathway for cellulose synthesis in *A. xylinum* (Ross et al., 1991).

Bacterial cellulose is composed of unique ribbon-shaped fibrils, its macromolecular structure and properties differ from the pulp cellulose (Fig. 2.3) (Iguchi et al., 2000).

Dimensions of the ribbon are 3.2x133nm, according to Brown et al.,(1976) 3–4 (thickness) x 70–80 nm (width), according to Zaar et al.,(1977) or 4.1x177 nm, according to Yamanaka et al.,(2000) whereas the width of cellulose fibers from pulping of birch or pine wood is larger (1.4–4.0x10x2 and 3.0-7.5x10x2 nm, respectively).
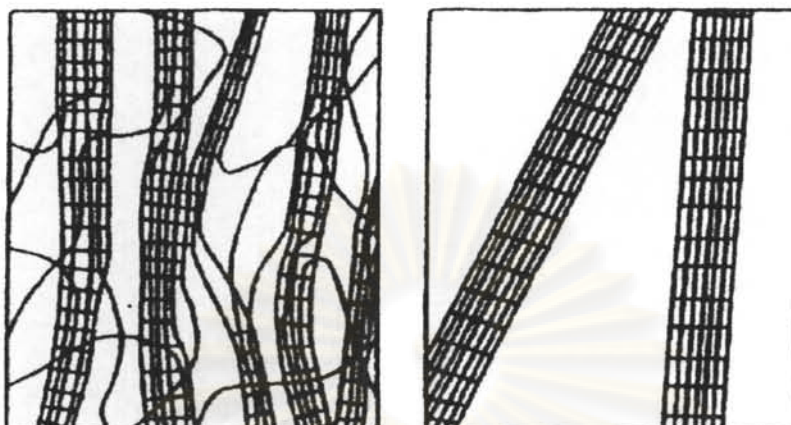
Figure 2.3 Schematic model of BC microfibrils (right) drawn in comparison with the fringed micelles; of PC fibrils (Iguchi et al., 2000).

The structural features of bacterial cellulose also were influenced by the kind of bacterial strain (Jonas et al., 1998).as in Table 2.1, and the additives in culture medium.

Table 2.1 Effect of microorganisms on cellulose producers (Jonas et al., 1998).

Organisms (genus)	Cellulose produced	Biological role
Acetobacter	Extracellular pellicle Cellulose ribbons	To keep in aerobic Environment
Achromobacter	Cellulose fibrils	Flocculation in wastewater
Aerobacter	Cellulose fibrils	Flocculation in wastewater
Agrobacterium	Short fibrils	Attach of plant tissues
Alcaligenes	Cellulose fibrils	Flocculation in wastewater
Pseudomonas	No distinct fibrils	Flocculation in wastewater
Rhizobium	Short fibrils	Attached to most plants
Sarcina	Amorphous cellulose	Unknown
Zoogloea	Not well defined	Flocculation in wastewater

Table 2.2 summarizes the distinguishing features of bacterial cellulose, as reported by

Krystynowicz and Bielecki et al., (2001)

Property	Description
Purity	– Cellulose is the only biopolymer synthesized – Absence of lignin or hemicelluloses – Completely biodegradable and recyclable, a renewable resource
Great mechanical strength	– High strength crystalline cellulose I – Consistent dimensional stability – High tensile strength – Light weight – Remarkable durability
Extraordinary absorbency in the hydrated state	– Remarkable capacity to hold water – Selective porosity – High wet strength – High surface-to-volume carrier capacity
Direct membrane assembly during biosynthesis	– Intermediate steps of paper formation from pulp unnecessary – Intermediate steps of textile assembly from yarn unnecessary – Extremely thin, submicron, optical clear membranes can be assembled

Property	Description
Direct modification of cellulose during assembly	– Delayed crystallization by introduction of dyes into culture medium – Control of physical properties of the cellulose during assembly (molecular weight and crystallinity)
Genetic modification of cellulose product	– Direct synthesis of cellulose derivatives (such as cellulose acetate, carboxymethyl cellulose, methyl cellulose, etc.) – Control of cellulose crystalline allomorph (cellulose I or cellulose II) – Control of molecular weight of cellulose

2.2 Curcumin

.Rhizomes of turmeric(*Curcuma longa L*, Zingiberaceae family) has been widely used for centuries in indigenous medicine for the treatment of a variety of inflammatory conditions and other diseases. Its medicinal properties have been attributed mainly to the curcuminoids and the main component present in the rhizome includes curcumin (diferuloylmethane)—(1,7-bis (4-hydroxy-methoxyphenyl)-1,6-hepadiene-3,5-dione) (Fig. 2.4).

CHEMICAL STRUCTURES OF CURCUMINOIDS

CURCUMIN   *Diferuloyl methane*

DEMETHOXY CURCUMIN *p-Hydroxy-cinnamoyl-feruloyl-methane*

BISDEMETHOXY CURCUMIN *pp'-Dihydroxy-dicinnamoyl-methane*
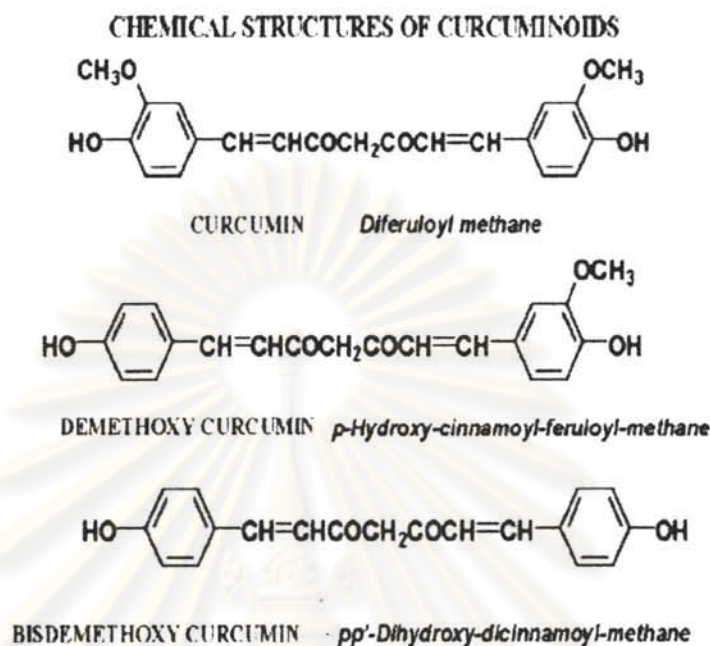
Figure 2.4 Chemical structure of curcuminoids curcumin, demethoxy curcumin and bisdemetohxy curcumin that have shown antioxidant and/or anti-inflammatory properties (Ammon and Wahl, 1991).

Curcumin (diferuloylmethane) is a low molecular weight polyphenol, first chemically characterized in 1910, that is generally regarded as the most active constituent of and comprises 2-8% of most turmeric preparations (Heath et al., 2004). Curcumin has been the subject of hundreds of published papers over the past three decades, studying its antioxidant, anti-inflammatory, cancer chemopreventive and potentially chemotherapeutic properties.

Chemical properties

Curcumin is a bis-a,b-unsaturated b-diketone. As such, curcumin exists in equilibrium with its enol tautomer. The bis-keto form predominates in acidic and neutral aqueous solutions and in the cell membrane (Wang et al., 1997). At pH 3-7, curcumin acts as an extraordinarily potent H-atom donor . This is because, in the keto form of curcumin, the heptadienone linkage between the two methoxyphenol rings contains a highly activated carbon atom, and the C–H carbon bonds on this carbon are very weak due to delocalisation of the unpaired electron on the adjacent oxygens (Fig. 2.5). In contrast, above pH 8, the enolate form of the heptadienone chain predominates, and curcumin acts mainly as an electron donor, a mechanism more typical for the scavenging activity of phenolic antioxidants (Jovanovic et al., 1999). Curcumin is relatively insoluble in water, but dissolves in acetone, dimethylsulphoxide and ethanol.
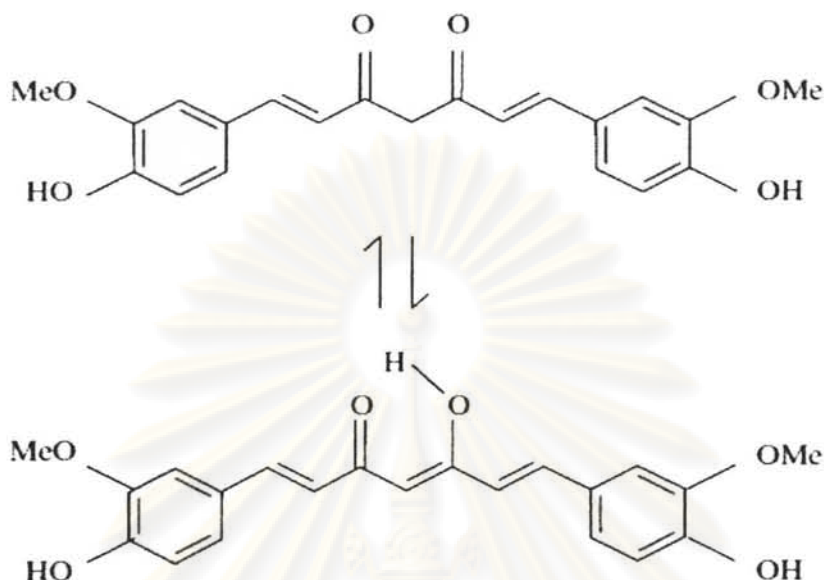
Figure 2.5 Tautomerism of curcumin under physiological conditions. Under acidic and neutral conditions, the bis-keto form (top) predominates, whereas the enolate form is found above pH 8 (Jovanovic et al., 1999).

Chemical Degradation of Curcumin

Curcumin is a yellow antioxidant substance. It seems that curcumin is sensitive to oxygen in aqueous solution, and that it is affected by UV under solar light exposure. Curcumin is unstable at basic pH, and degrades within 30 min to trans-6-(4'-hydroxy-3'-methoxyphenyl)-2, 4-dioxo-5-hexanal, ferulic acid, feruloylmethane and vanillin (Lin et al., 2000).

The degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices were reported (Wang et al., 1997). The presence of fetal calf serum or human blood, or addition of antioxidants such as

ascorbic acid, N-acetylcysteine or glutathione, completely blocks this degradation in culture media or phosphate buffer above pH 7.

Under acidic conditions, the degradation of curcumin is much slower, with less than 20% of total curcumin decomposed in 1 h (Wang et al., 1997). Other investigators have also found that curcumin is more stable in cell culture medium containing 10% foetal calf serum or in human blood, with less that 20% decomposition within 1 h compared to 90% within 30 min in serum-free medium. Based on mass and spectrophotometrical analysis, trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal was tentatively identified as a major degradation product while vanillin, ferulic acid and feruloylmethane were identified as minor degradation products (Fig. 2.6) (Wang et al., 1997).
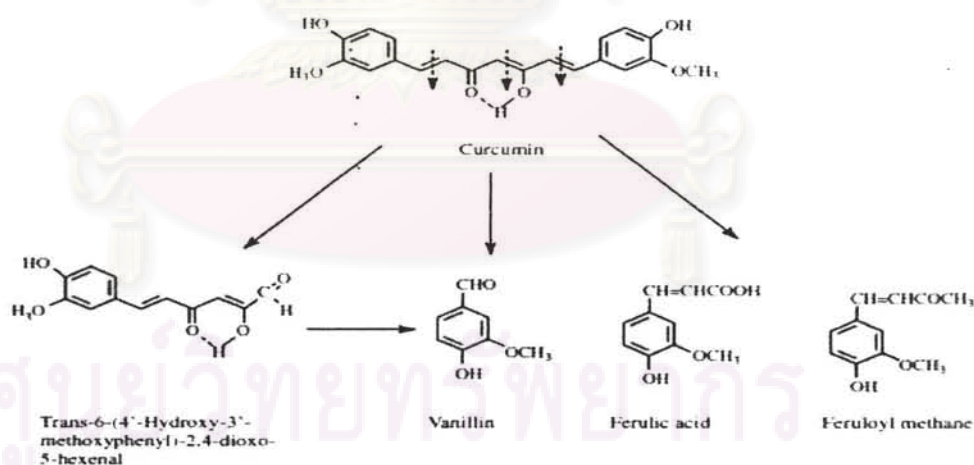
Figure 2.6 Degradation of curcumin in aqueous solution. The major degradation product of curcumin in 0.1 M phosphate buffer at pH 7.2 was tentatively identified as trans-6-(4'-hydroxy-3'-methoxyphenyl)-2, 4-dioxo-5-hexenal while the minor products were identified as vanillin, ferulic acid and feruloylmethane (Wang et al., (1997).

Since curcumin decomposes rapidly in serum-free medium, precautions must be taken during cell culture experiments. In addition, the biological effects caused by the degradation products of curcumin, especially vanillin, must be taken into consideration. Vanillin, a naturally occurring flavoring agent, has been reported to inhibit mutagenesis in bacterial and mammalian cells. It may act as an antimutagen by modifying DNA replication and DNA repair systems after cellular DNA damage caused by mutagens occurs. Vanillin is also a powerful scavenger of superoxide and hydroxyl radicals.

As a result of light sensitivity, samples containing curcumin should be protected from light. Above pH 7, curcumin's hue is less yellow and more red. Curcumin has a molecular weight of 368.37 and a melting point of 183 °C. Commercial grade curcumin contains the curcuminoids desmethoxycurcumin (MW 338; typically 10-20%) and bisdesmethoxycurcumin (MW 308; typically less than 5%, for structures see Fig. 2.7). On ultraviolet–visible spectrophotometric investigation, maximum light absorption of curcumin occurs at 420 nm. Studies in preclinical models of carcinogenesis have demonstrated that commercial grade curcumin has the same inhibitory effects as pure curcumin (Huang et al., 1995).
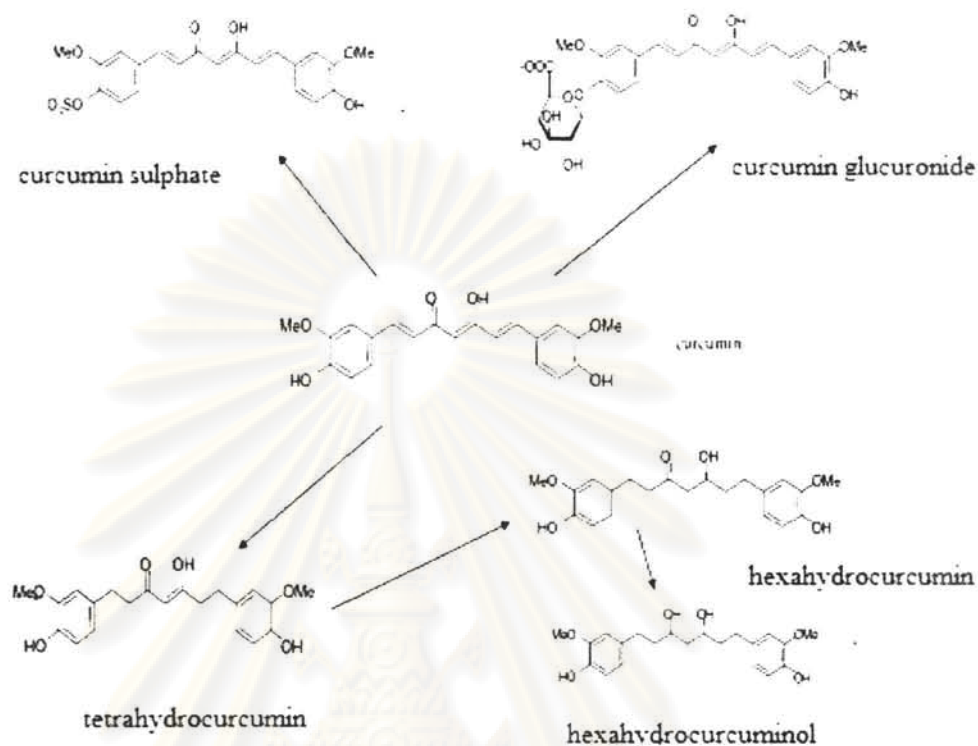
Figure 2.7 Chemical structures of major metabolites of curcumin in rodents and humans.

Pharmacokinetic properties

A study of high dose curcumin (2% in the diet, equating to approximately 1.2 g curcumin per kg body weight) for 14 days has shown that low nanomolar levels are detectable in plasma, with concentrations in liver and colon mucosal tissue ranging from 0.1 to 1.8 nmol/g tissue (Sharma et al., 2001). In a study of oral curcumin (2 g/kg) in rats performed in India, the investigators suggested that co-administration of piperine may increase systemic bioavailability following oral dosing by as much as 154%, potentially by inhibition of xenobiotic glucuronidation (Shoba et al., 1998).

Piperine is primarily found in the fruit of the pepper vine, piper nigrum, and can also be found in other vegetables and spices such as hot jalapeno peppers. In summary, curcumin exhibits low oral bioavailability in rodents and may undergo intestinal metabolism; absorbed curcumin undergoes rapid first-pass metabolism and excretion in the bile.

Curcumin in Mice

Pan et al., (1999) investigated the pharmacokinetic properties of curcumin in mice. After intraperitoneal adminstration of curcumin (0.1 g/kg) to mice, about 2.25 µg/ml of the curcumin appeared in the plasma during the first 15 min. One hour after administration, the levels of curcumin in the intestine, spleen, liver and kidneys were 177, 26, 27, and 7.5 µg/g, respectively. Only traces (0.41 µg/g) were observed in the brain at 1 h.

CHAPTER III

LITERATURE REVIEW

3.1 Application of *Bacterial Cellulose*

Bacterial cellulose offers a wide range of applications. This mainly is due to a high purity with a crystallinity structure and high water absorption capacity and mechanical strength in wet state. BC is highly regarded for it has the suitable properties especially for regenerative medicine (Czaja et al., 2007). Yet for biomedical application, properties such as thermal stability, strength, porosity, roughness, morphology and density are crucial (Rezwan et al., 2006). The main potential bacterial cellulose applications are the following.

Food Applications

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 et al., (1997) showed that Nata de Coco has the plasma cholesterol-lowering effect, in which the coconut milk was used as a carbon source. It is believed to protect against bowel cancer, artheriosclerosis, and coronary thrombosis, and prevents a sudden rise of glucose in the urine. Therefore, ''Nata de Coco'' is becoming increasingly popular. Another popular bacterial-cellulose–containing food product is 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 health improvement. The pellicle formed on the surface contains both cellulose and enzymes healthy for humans. Kombucha is believed to protect against certain cancers (Iguchi et al., 2000). Moreover, the bacterial cellulose has been applied as a functional food additive: a thickener, texturizer, and/or calorie reducer (ice cream, salad dressing, and weight-reduction base). The bacterial cellulose from an agitated culture has a much higher emulsifying effect than that from a static culture, because the former has a disordered structure (Hiroshi et al., 1997)

Paper and Paper Products

Microbial cellulose has been investigated as a binder in papers, and because it consists of extremely small clusters of cellulose microfibrils, this property greatly adds to strength and durability of pulp when integrated into paper. The Ajinomoto Co., along with Mitsubishi Paper Mills in Japan, is currently active in developing microbial cellulose for paper products (JP Patent 53295793). The disintegrated bacterial cellulose was found to have a remarkably high retention aid function for paper making. (Yamamoto et al., 1989). The filler granules are entrapped by ultrafine fibrils of bacterial cellulose. When the agitated bacterial cellulose and static bacterial cellulose were added at wet-end, both improved the tensile strength and filler retention of the produced handsheet; and the bacterial cellulose produced in an agitated culture has a higher retention aid function than that produced in static culture. It is thought that the bacterial cellulose from an agitated culture has a higher accessibility of the surface than that from a static culture, because the former has a

disordered structure (Hioki et al., 1995). Bacterial cellulose also is a valuable component of synthetic paper, (Iguchi et al., 2000) since nonpolar polypropylene and polyethylene fibers, providing insulation, heat resistance, and fire-retarding properties, cannot form hydrogen bonds. The amount of wood pulp in this type of paper is usually from 20% to 50% to achieve good quality.

Medical Applications

In 1980, Johnson and Johnson pioneered in exploratory investigations on the use of microbial cellulose as a liquid-loaded pad for wound care (U.S. Patent 4,655,758,4,588,400). Since that time, a company in Brazil, Biolfill Industries has continued to investigate the properties of microbial cellulose and is beginning to market specific microbial cellulose products in the wound care market. 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 (Fontana et al., 1990 and 1991). 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).

In Brazil, the purified gelatinous membrane of bacterial cellulose was developed and commercialized as an artificial skin (wound dressing) (Krystynowicz et al., 2000). A high mechanical strength in the wet state, substantial permeability for liquids and gases, and low irritation of skin, indicated that the gelatinous membrane of

the bacterial cellulose was superior to conventional quaze as an artificial skin for temporary covering of wounds. Since bacterial cellulose pellicles can have various dimensions, it is relatively easy to produce dressings for extensive wounds. Other commercial preparation of *A. xylinum* cellulose, such as Biofil® and Bioprocess® and Gengiflex® are realities in the health-care sector. Biofil® appeared to be excellent as skin transplants and in the treatment of second and third degree burns, ulcers and decubitus; while Gengiflex® was successfully applied in recovering periodontal tissues(Jonas et al., 1998). This biosynthetic cellulose also was successfully applied in experiments with dogs to substitute the duramater in the brain (Mello et al., 1997). Another very important advantage of bacterial cellulose is their mouldability in situ (White and Brown et al., 1990). By using special technologies, it is possible to prepare hollow fibers [bacterial synthesized cellulose (BASYC)-tubes], used as artificial blood vessels and ureters (Yamanaka et al., 1990). Artificial vessels are used to replace arteries or veins damaged as a result of tumors or accidents. High mechanical strength in wet state, enormous water retention values, low roughness of the inner surface, and a complete vitalization of BASYC microvessel- interpositions in rat experiments demonstrate the high potential of BASYC as an artificial blood vessel in microsurgery. Different methods for the formation of hollow fibers during biosynthesis also were investigated (Shirai et al., 1994).

Because the bacterial cellulose has high tensile strength, durability, and permeability to liquid and gases, it was applied as an additional membrane to protect immobilized glucose analyzers in biosensors used for assays of glucose levels. This bacterial cellulose membrane introduced the electrode stability. In human blood, the biosensor-coated commercial protecting membranes, as cuprophan (AKZO, England),

were stable for 3–4 hr, whereas bacterial cellulose membrane prolonged its stability to 24 hr. Cellulose gels containing immobilized animal cell were used for their culture to produce interferon, interleukin-1, cytostatic, and monoclonal antibodies (Iguchi et al., 2000). BC films are used to coat surface-treated medicalgrade polyesters for Vascular prosthetic device (to replace diseased arteries) (Charpentier et al., 2006). BC minimizes blood clotting and increases biocompatibility.

Kucharzewski *et al.* (2003) examined the treatment of non-healing venous leg ulcers with BC wound dressing in comparison to that with Unna's boot hydrocolloid dressing that was widely used in the therapy of these types of wounds. BC wound dressing was found more effective in the treatment of the chronic venous leg ulcers than Unna's boot.

3.2 Application of Curcumin

Anti-cancer effects of curcumin

Ozaki et al., (2000) studied the action of curcumin on rabbit osteoclast apoptosis and demonstrated that curcumin drastically inhibited bone resorption and stimulated apoptosis in the cancer cells. Since, cancer and bone inflammation are diseases that increase bone resorption, the researchers suggested that curcumin might be useful in the therapy of these diseases

Siwak et al. (2005) demonstrated that curcumin inhibited the growth and promoted cell death in three different melanoma cell lines. Curcumin appeared to work by suppressing the production of the proteins in the cancer cells that normally

protected the cells from cell death. All doses tested decreased cancer cell growth and triggered cell death. The higher the dose used, the more cancer cells died.

Curcumin triggered the death of head and neck squamous cell carcinoma in a recent study published in Clinical Cancer Research. This research indicated that the addition of curcumin to cultures of squamous cell carcinoma resulted in a dose-dependent growth inhibition of three cell lines. Curcumin was also applied as a noninvasive topical paste to the implanting squamous cell tumors in mice and demonstrated inhibitory effect on the tumor growth was observed (Lotempio et al., 2005).

Hidaka et al. (2002) demonstrated that curcumin could stop the growth of human pancreatic cancer cells. They found that curcumin inhibited the production of interlukin-8, a protein produced by white blood cells that contributed to tumor growth

Anti-inflammatory Effects of Curcumin

Curcumin inhibits enzymes which participate in the synthesis of inflammatory substances in the body. The natural anti-inflammatory activity of curcumin is comparable in strength to steroidal drugs, and some nonsteroidal drugs but does not have the same dangerous side effects (Mukhopadhyay et al., 1982). Inflammation results from a complex series of actions and/or reactions triggered by the body's immunological response to tissue damage. The processes of healing and infection fighting produce a moderate level of inflammation. Chronic inflammation leads to degenerative conditions like arthritis, arteriosclerosis, etc. Curcumin prevents the synthesis of several inflammatory prostaglandins and leukotrienes. Curcumin has

a similar action to aspirin except that curcumin does not cause vascular thrombosis as the way aspirin does. Curcumin's anti-inflammatory properties may be attributed to its ability to inhibit pro-inflammatory arachidonic acid, as well as neutrophil function during inflammatory states. (Mukhopadhyay et al., 1982 and Srivastava et al., 1989)

Antioxidant Effects of Curcumin

Free radicals can originate from environmental chemicals, tissue injury, infections and auto-immune processes. Antioxidants protect the body from damage caused by free radicals. Water- and fat-soluble extracts of turmeric and its curcumin component exhibit strong antioxidant activity, comparable to vitamins C and E. One study showed curcumin to be eight times more powerful that vitamin E in preventing lipid peroxidation. Taken in group arrangements such as C-complex, curcuminoids are three times as potent in neutralizing free-radical molecules (Toda et al., 1985).

Curcumin is reported to be a powerful antioxidant to repair both oxidative and reductive damage caused to proteins by radiation (Kapoor & Priyadarsini, 2001). Das et al., (2002) demonstrated that curcumin is a potent singlet oxygen quencher at physiological or pharmacological concentration. Additionally, singlet oxygen quenching by low concentration of curcumin in aqueous solutions is a physiologically relevant property of this compound, which can explain its effect in protecting skin against UV light. Singlet molecular oxygen is an electronically excited species of oxygen known to produce in mammalian cells under normal and pathophysiological conditions.

Antimicrobial Effects of Curcumin

Chopra et al. (1941) demonstrated that curcuma oil was tested against cultures of *Staphylococcus albus, Staphylococcus aureus and Bacillus typhosus* and the results showed the inhibition of growth of *S. albus* and *S. aureus* at different concentrations.

Bhavanishankar and Srinivasamurthy (1979) investigated the activity of turmeric fractions against some intestinal bacteria in vitro. Total inhibition of growth of lactobacilli in the presence of whole turmeric was reported (4.5–90 ml/100 ml).

Curcumin enhances wound healing

Tissue repair and wound healing are complex processes that involve inflammation, granulation and tissue remodeling. Injury initiates a complex series of events that involves interactions of multiple cell types, various cytokines, growth factors, their mediators and the extra-cellular matrix proteins (ECM). Local application of turmeric is a household remedy in India for several conditions such as skin diseases, insect bites and chicken pox (Nadkarni, 1976). Based on the ancient use of turmeric in wound healing, earlier studies evaluated the effect of curcumin on enhancement of wound healing. By using full thickness punch wound model to study its effect on wound healing, curcumin treated wound biopsies showed a large number of infiltrating cells such as macrophage, neutrophils and fibroblasts as compared to untreated wound. The presence of myofibroblast in curcumin treated wound demonstrated faster wound contraction (Sidhu et al., 1998).

Curcumin incorporated collagen matrix treatment showed increased wound reduction, enhanced cell proliferation and efficient free radical scavenging as compared with control and collagen treated rats (Gopinath et al., 2004).

Curcumin pretreatment enhanced the synthesis of collagen, hexosamine, DNA and nitrite. Histologic assessment of wound biopsy specimens showed improved collagen deposition and an increase in fibroblast and vascular densities suggesting that curcumin might be able to improve radiation-induced delay in wound repair (Jagetia and Rajanikant, 2005).

Suwantong et al. (2007) have incorporated curcumin into electrospun cellulose acetate fiber mats. The potential for use of the curcumin-loaded e-spun CA fiber mats and corresponding as-cast CA films as topical/transdermal patches or wound dressings was reported. Curcumin could enhance wound healing in rats and guinea pigs. (Sidhu *et al.*1999). Curcumin-eluting stents are superior stainless steel stents and PLGA-only-coated stents. The anticoagulation behavior of curcumin stents improved significantly as the drug dose was increased (Pan *et al.*, 2006).

CHAPTER IV

EXPERIMENTAL

4.1 Materials

4.1.1 Microbial Strains

The *Acetobacter xylinum* strain AGR60 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 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 (country)
Sucrose	Ajax Finechem (Australia)
Sodium acetate	Ajax Finechem (Australia)
Ammonium sulfate	Carlo Erba (Italy)
Sodium hydroxides	Carlo Erba (Italy)
Acetic acid	Carlo Erba (Italy)
Acetone	Carlo Erba (Italy)
Curcumin	Fluka (Switzerland)
N,N-dimethylacetamide	Sigma-Aldrich (Switzerland)

4.1.3 Equipments

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

-Fourier Transform Infrared (FT-IR) spectrometer (Perkin Elmer Spectrum One Massachusetts, USA).

-Instron testing machine (Instron 5567, NY, USA).

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

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

-UV-vis spectrophotometer (Shimadzu UV-2550, Tokyo, Japan).

-Oxygen permeation analyzer (Illinois Instruments, Model 8000, Johnsburg, IL)

4.2 Culture Media and Method

The medium for the inoculum was coconut-water supplemented with 5.0% sucrose , 0.5% ammonium sulfate $(NH_4)_2SO_4$ and 1.0% acetic acid. The medium was sterilized at 110 °C for 5 min. The 5%v/v precultures were prepared by transferring 25ml of a stock culture to 500 ml medium and incubated statically at 30 °C for 7 days. Afterwards the 5% (v/v) precuture broth was added to the main culture medium. The activated medium was inoculated at 30 ^{0}C for 7 days.

All sample films were purified by washing with DI water for 1 h and were then treated with 1%v/v NaOH at room temperature for 24 h to remove bacterial cells followed by rinsing with 1%v/v acetic acid and DI water until pH came to 7.

Afterward, the BC films were air-dried at room temperature (30 ^{0}C) and stored in plastic sheet at room temperature.

4.3 Preparation of curcumin loaded into BC films

Curcumin solutions were prepare by dissolving various concentrations of curcumin (>95% purity) in absolute ethanol with difference concentrations. For comparison, curcumin was loaded in BC films (square plate of 2.5×2.5 cm^2, with the thickness of 0.033 mm) that were prepared in forms of wet state (never dried film) and air-dried film by direct immersion of BC films into 12.5 ml the curcumin solution. Afterward, the BC-curcumin films were air-dried at room temperature (30 ^{0}C) and stored in dry film at room temperature.

4.4 Releasing of curcumin from curcumin loaded BC films

4.4.1. Preparation of acetate buffer

Acetate buffer was used to simulate the human skin pH condition of 5.5. For 1000 ml acetate buffer solution preparation, 150 g of sodium acetate was dissolved in 250 ml of distilled water and then exactly 15 ml of glacial acetic acid was added very slowly into the sodium acetate aqueous solution. Finally, distilled water was added into the solution to fill the volume.

4.4.2. Actual curcumin content

Actual amount of curcumin in the curcumin-loaded BC films was determined. Each specimen (square plate; 2.5 x 2.5 cm^2) was immersed in 4 ml of 2:1 v/v acetone/dimethylacetamide (DMAc) solution at room temperature (30°C). After that, 0.5 ml of the solution was added into 8 ml of the acetate buffer solution and the actual amount of curcumin content was measured by a Shimadzu UV-2550 UV-vis spectrophotometer at the wavelength of 420 nm.

4.4.3. Curcumin-release assay

The release characteristic of curcumin from the curcumin loaded BC films was investigated by total immersion. Because the limited of solubility of curcumin in the acetate buffer solution, the B/T/M releasing medium (96.5% v/v acetate buffer with 0.5% v/v Tween 80 and 3% v/v methanol) was used. Each specimen (square plate; 2.5 x 2.5 cm^2) was immersed in 30 ml of the medium at the temperature of 37 °C. At a specified immersion period ranging from 0 to 48 h (2880 min), either 1 ml of a sample solution was withdrawn and an equal amount of the fresh medium was refilled. The amount of curcumin in the sample solutions was determined using the UV-vis spectrophotometer at the wavelength of 420 nm.

4.5 Characterization of BC-curcumin Film

The BC-curcumin 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 and by MTT cell assay for evaluating the proliferative and cyctotoxic effects of the developed film.

4.5.1 Scanning Electron Microscope (SEM)

The surface properties of BC and BC-curcumin (BCC) films were examined 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 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 examination. SEM was obtained at 15 kV which is considered to be a suitable condition since too high energy can be burn the samples.

The specimens were examined at magnification 10,000X for surface morphology and both 10,000X and 3,500X for cross section.

4.5.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is used primarily to identify the chemical structure of the sample. FTIR spectra of the developed films were recorded with a Perkin Elmer (Spectrum One, Massachusetts, USA) in the region of 4000–500 cm^{-1} at Scientific and technological research equipment centre, Chulalongkorn University.

4.5.3 Mechanical Testing

The tensile strength and elongation at break of the dried BC and BCC films were measured by Instron Testing Machine (Instron 5567, NY, USA) with 1kN load cell at Polymer Engineering Laboratory, Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University. The film samples were cut into strip-shaped specimens 10 mm width and 10 cm length. The test conditions follow 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 10 mm/min. The tensile strength and break strain were the average value determined from at least five specimens.

4.5.4 Water Absorption Capacity (WAC)

Water absorption capacity (WAC) was determined by immersing the preweighted of dried BC and BCC films in distilled water at room temperature until equilibration. The films were then removed from the water. After excess water at the surface of the films was blotted out with Kimwipes paper, the weight of the swollen films 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 membrane, respectively.

4.5.5 The Oxygen Permeability Measurement

Oxygen transmission rate (OTR) of the dried BC and BCC films was determined with a 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 $23^{\circ}C$ 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. Testing was completed when the concentration of oxygen in the nitrogen side was constant.

4.5.6 The Water Vapor Permeability Measurement

Water vapor transmission rate (WVTR) of the BC and BCC films with area of $50.00 \ cm^2$, was measured at Thai packaging centre, Thailand Institute of Scientific and Technological Research. The test conditions follow ASTM E-96 with desiccant method. The determination of WVTR was done under the following conditions: temperature, 38 °C; % Relative Humidity, 98%. The test specimen is sealed to the open mount of test dish containing a desiccant, and the assembly placed in a controlled atmosphere. Periodic weighting is performed to determine the rate of water vapor movement through the specimen into the desiccant.

4.5.7 Antibacterial Test

The antibacterial test of BC-curcumin films against *Escherichia coli* Gram (-) and *Staphylococcus aureus* Gram (+) bacteria was determined at Microbiology

Laboratory, Department of Microbiology, Faculty of Sciences, Chulalongkorn University. The film samples were cut into 25 mm width and 50 mm length. Testing of antibacterial activity of the 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 by using UV irradiation for 20 min in each side. The incubation was 24 hours at 37 °C.

4.5.8 Antifungal Test

Testing antifungal activity of the BC-curcumin films was performed according to AATCC 39-1989(Assessment on Textile Materials: Mildew and Rot Resistance of Textile) at Microbiology Laboratory, Department of Microbiology, Faculty of Sciences, Chulalongkorn University. The film samples were punch into round-shaped sample of 3.8 cm diameter. The film samples used for the antifungal test were sterilized by using UV irradiation for 20 min in each side. *Aspergillus niger* inoculated on agar plates was exposed to the film specimens and incubated at 30 °C for 7 days.

4.5.9 Cell Proliferation Assay

Air- dried BC films were punched into round-shaped samples of 14 mm diameter. The samples were sterilized by autoclaving at 121°C for 15 min and transferred aseptically to 24-well culture plates. The experiments were conducted in triplicate. One milliliter of culture medium was added to each well to equilibrate the samples for 30 min before cell seeding. Inhibition of cell proliferation on BC and

BCC films were determined by MTT assay as previously described (Wasina et al., 2010). Briefly, cells were seeded into 24-well culture plates (Nunc, Rockford, IL) at an initial density of 3×10^4 cells per well on BCC and BC films. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 16 h. Then, the culture medium was removed and replaced for another 3 h by melanoma B16 complete growth medium and serum-free DMEM for the cultures of melanoma B16. The number of living cells was determined using the MTT assay.

4.5.10 MTT Assay The 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT) assay was based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed was proportional to the number of viable cells. First, the culture medium was aspirated and replace with 20 µl MTT solution [(3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyl-tetrazolium bromide), 5 mg/ml in PBS]. After that the plate was incubated at 37°C for 3 hours. After centrifugation at 1,400 rpm for 5 minutes at 4°C, the medium was aspirated and the formozan product was dissolved in 100 µl DMSO in each well. The absorbance was measured using a Microplate reader (Benchmark 550, Bio-Rad, USA) at 550 nm wavelength.

CHAPTER V

RESULTS AND DISCUSSIONS

5.1 Characterization of BC-curcumin membrane

The bacterial cellulose (BC) was produced in the form of a pellicle on the surface of a culture medium. Due to the unique biological properties of BC and curcumin, it was interesting to absorb curcumin into BC film as it might improve the biological properties of the developed BC-curcumin film. Therefore, in this work, the modification of the BC film was performed by loading curcumin into a BC film synthesized under the static conditions by *Acetobacter xylinum*. It was then assessed for its potential use as a carrier for control release and transdermal delivery of curcumin. The structure, morphology, mechanical, swelling, components, chemical structure and biological properties of the developed BC with the addition of curcumin were also examined and compared with the biosynthesized BC film with no curcumin content. Moreover the absorption and release characteristics of curcumin from the curcumin-loaded BC were also investigated

5.1.1 Surface morphology

In this research, Scanning electron microscopy (SEM) imaging were used to study the morphology of the BC and BC-curcumin(BCC) films. In definition, the BC refers to the BC film with no curcumin adding while the BC-curcumin refers to the BC with the addition of the curcumin. Figure 5.1 (a) presents SEM images of individual BC without the addition of curcumin. The upper SEM images show the surface morphology of dried (A) and reswollen (B) films at 10,000 magnifications, respectively. The lower ones are the cross section at 3,500 and 10,000 magnifications. As shown in the above figures, its surface structure was found ultrafine fiber network structure of micro fibrils below 0.05 μm. With hydrophilic property, after air-drying at room temperature and reswelling with water, the pore diameter of the swollen form was 0.2-1.0 μm, whereas the pore diameter of the dried film was less than 0.1 μm. The dimension of the dried BC fibers (0.01-0.1 μm) was comparable to an ultrathin membrane (Klemm *et al.*, 2001)

Figure 5.1(a) to (f) shows the analyzed SEM images of the BCC films with the adsorption using the curcumin solutions at the concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1 (mg/ml) absorbed into the wet BC films, respectively. The BC flim showed well-organized fibril network. With the loading of curcumin, curcumin could integrate into the BC fibril network. An observation on the surface morphology of the BCC films especially, the BCC film with 1(mg/ml) curcumin loading, illustrates' many curcumin particles inserted into BC fibril network.
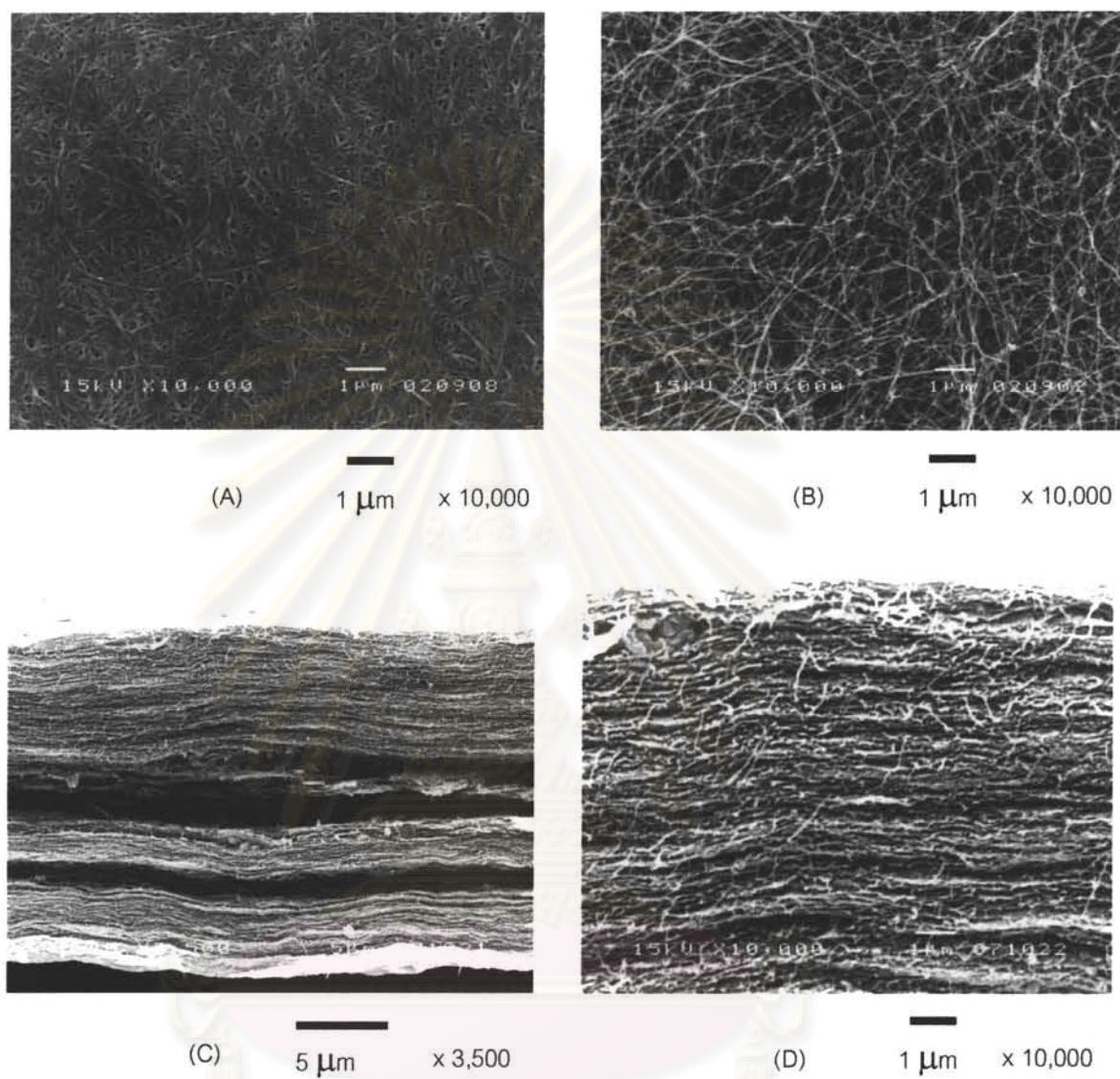
Figure 5.1 (a) SEM images of surface morphology of the dried films at none-curcumin supplement : (A) Top view; (B) swelling (C) Cross-section (x3,500)and (D) Cross-section(x10,000)
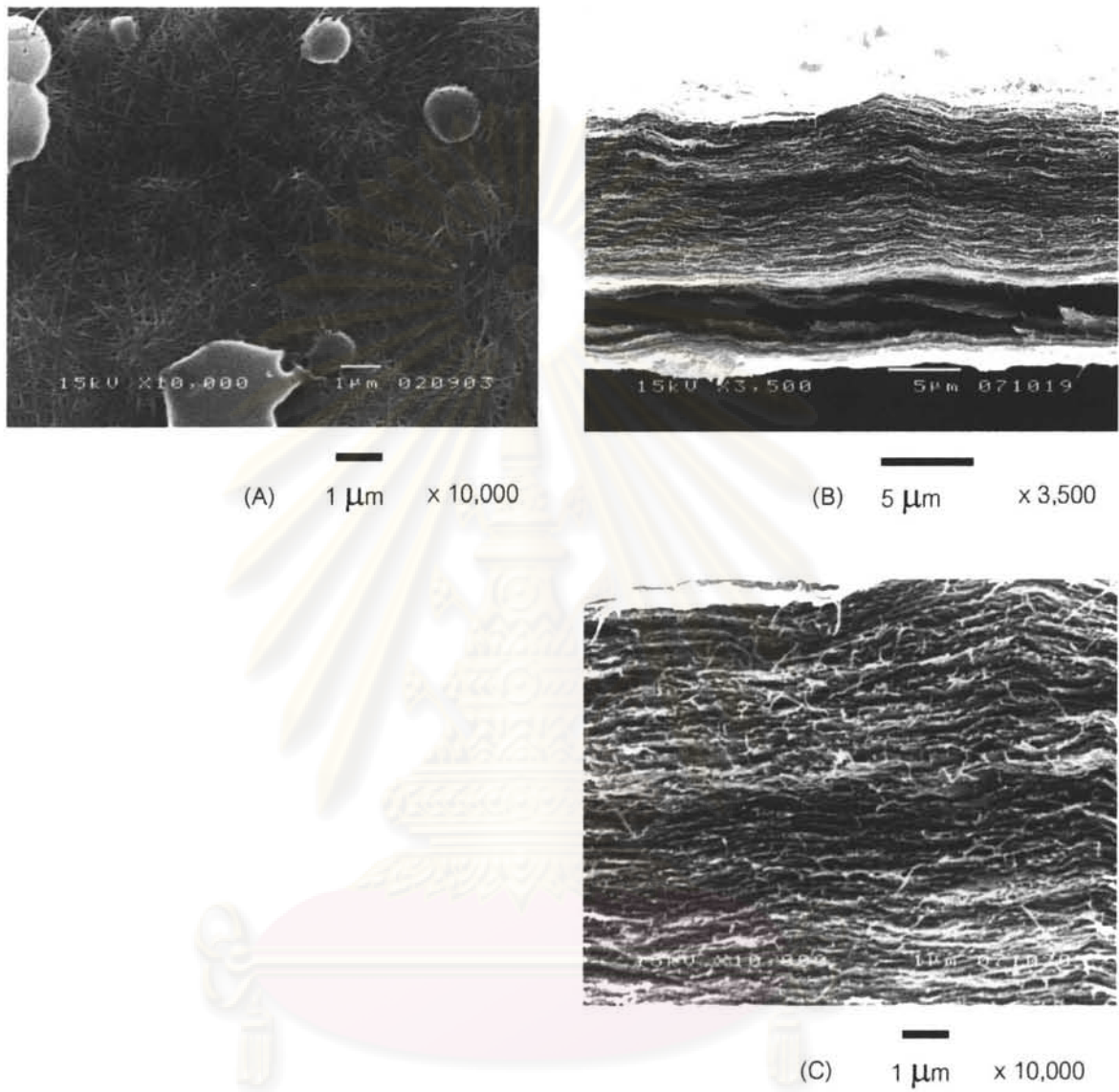
(A) 1 μm × 10,000

(B) 5 μm × 3,500

(C) 1 μm × 10,000

Figure 5.1 (b) SEM images of surface morphology of the dried films at 0.2 mg/ml

curcumin supplement: (A) Top view; (B) Cross-section (x3,500)and
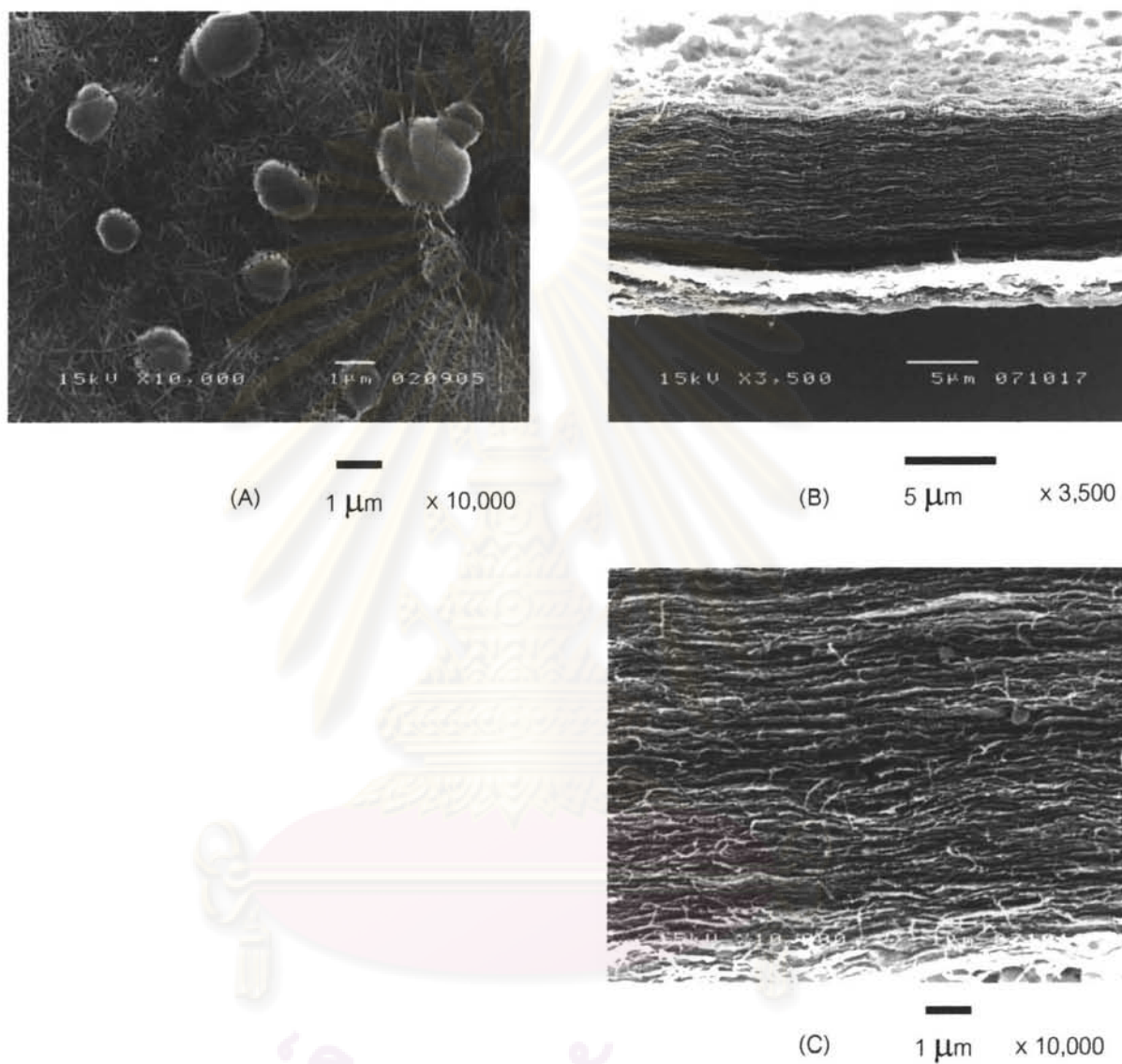
(C) Cross-section(x10,000)

(A) 1 μm x 10,000   (B) 5 μm x 3,500

(C) 1 μm x 10,000

Figure 5.1 (c) SEM images of surface morphology of the dried films at 0.4 mg/ml curcumin supplement: (A) Top view; (B) Cross-section (x3,500)and (C) Cross-section(x10,000)
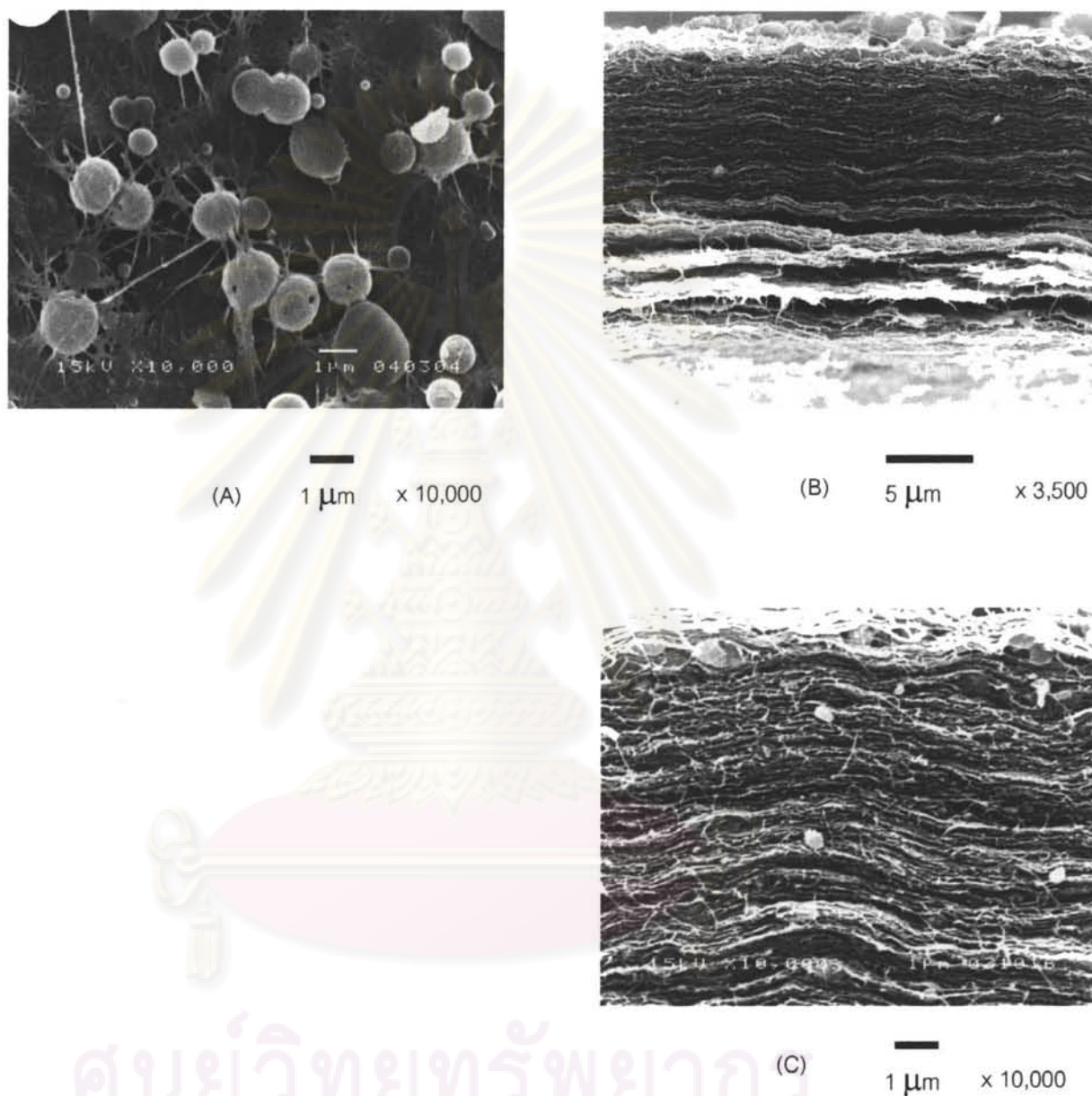
(A) 1 μm x 10,000

(B) 5 μm x 3,500

(C) 1 μm x 10,000

Figure 5.1 (d) SEM images of surface morphology of the dried films at 0.6 mg/ml

curcumin supplement: (A) Top view; (B) Cross-section (x3,500)and
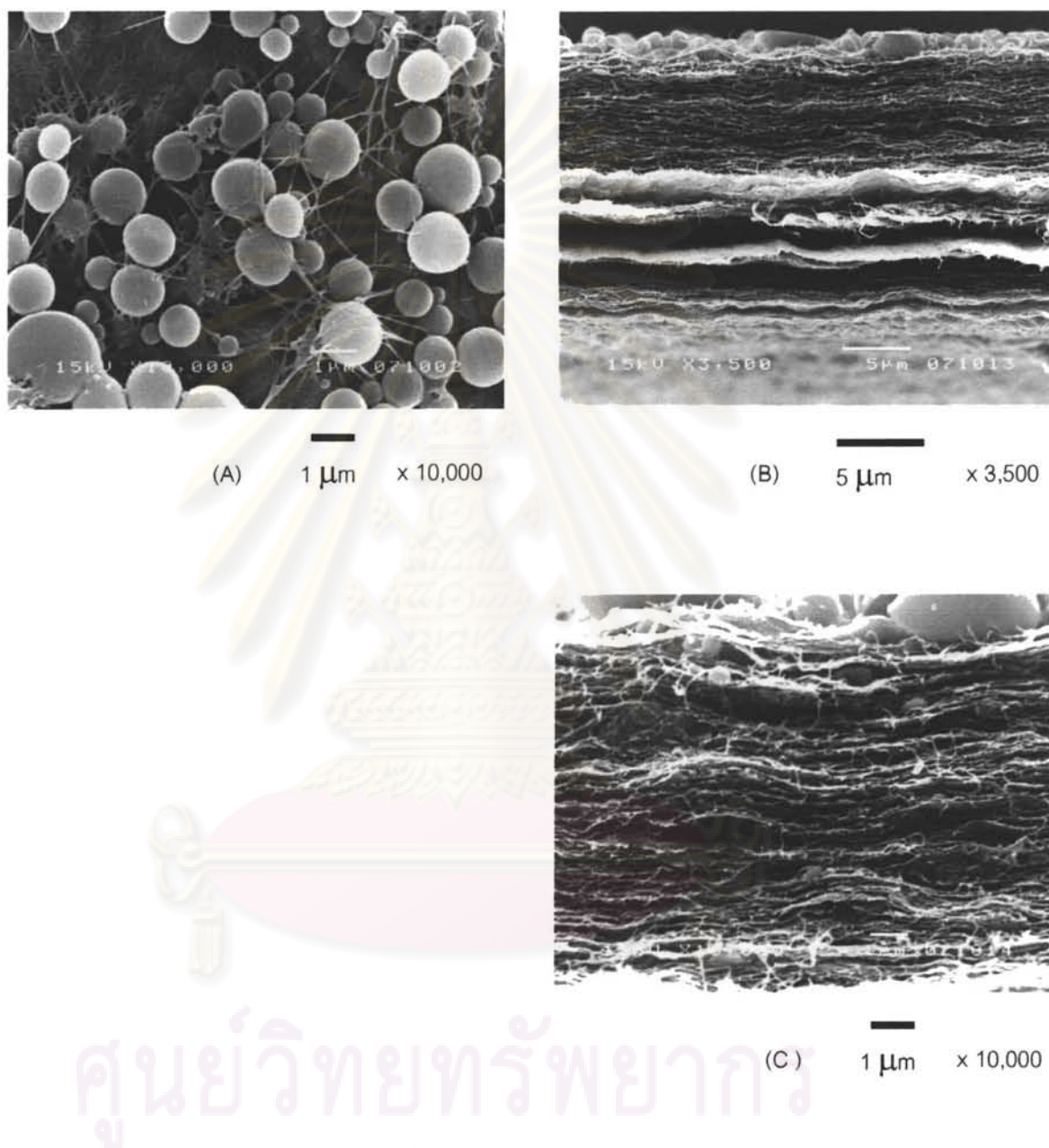
(C) Cross-section(x10,000)

(A)   1 μm   x 10,000   (B)   5 μm   x 3,500

(C)   1 μm   x 10,000

Figure 5.1 (e) SEM images of surface morphology of the dried films at 0.8 mg/ml

curcumin supplement: (A) Top view; (B) Cross-section (x3,500)and
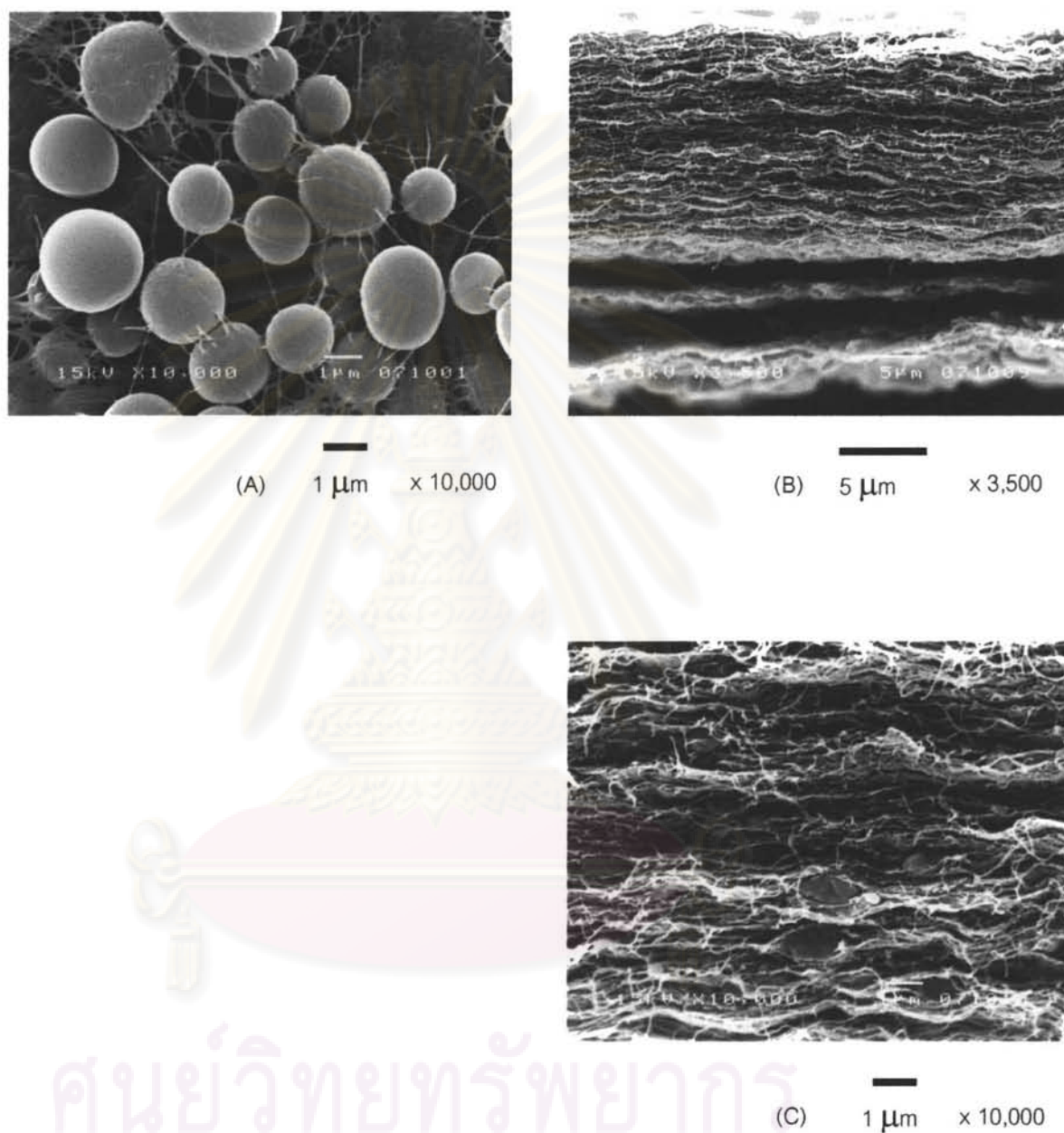
(C) Cross-section(x10,000)

(A)   1 μm   × 10,000

(B)   5 μm   × 3,500

(C)   1 μm   × 10,000

Figure 5.1 (f) SEM images of surface morphology of the dried films at 1 mg/ml

curcumin supplement: (A) Top view; (B) Cross-section (x3,500)and

(C) Cross-section(x10,000)

5.1.2 FTIR analysis

Fourier Transform Infrared (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 at., 1994). Therefore, in this research, the sample of BC and BCC films were analyzed by FTIR. The presence of peak at a specific wave number would indicate the presence of a specific chemical bound. As showed in Figure 5.2, the FTIR spectra of the BC, BCC films were measured at wave number ranging from 2000-1000 cm^{-1}. The BC film showed a band at 1639.38 cm^{-1} as in Figure 5.2 (a), which was attributed to carboxyl group of cellulose.

The strong band of curcumin at 1627.49 cm^{-1} is due to the enol carbonyl stretching vibration as showed in Figure 5.2 (f). All characteristic bands of the BC film was presented in the spectra of the BC-Curcumin films. However, the carboxyl group band for BCC films [Figure 5.2 (a-e)] were shifted from 1639.38 cm^{-1} to 1634.87, 1634.31, 1628.48 and 1628.35 cm^{-1}, respectively. The difference between absorption bands of the films could be attributed to interaction between the components of the biopolymers and curcumin.

The benzene group at 1509 and 1514 cm^{-1} were found in the spectra of curcumin films and the BCC films but not found in the BC film, indicating the existence of curcumin in the BCC films.
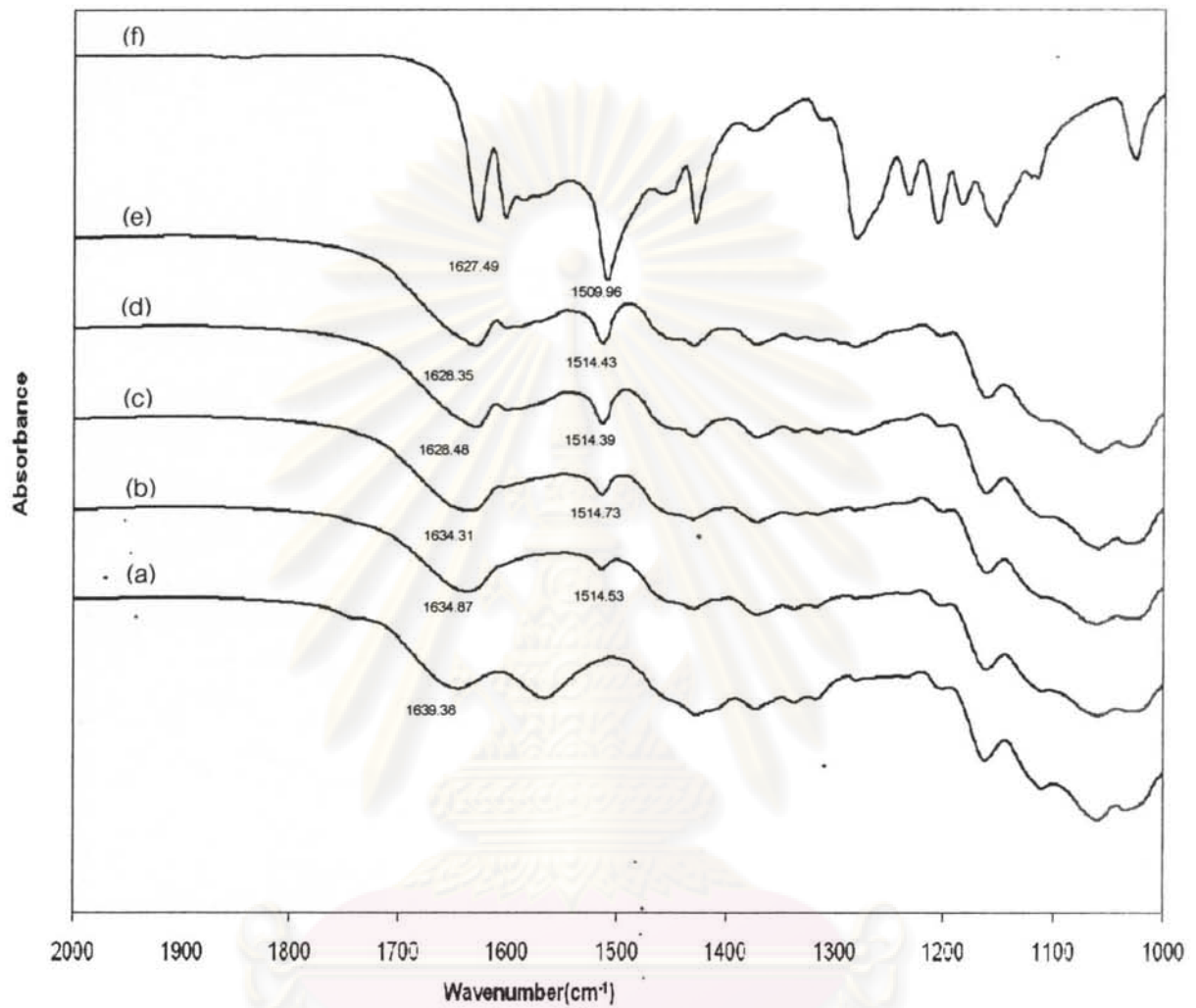
Figure 5.2 FTIR spectra of BC and BC-curcumin films in wave numbers ranging

from 2000 to 1000 cm⁻¹: (a) BC; (b) BCC-0.2 mg/ml; (c) BCC-0.4

mg/ml; (d) BCC-0.8 mg/ml; (e) BCC-1.0 mg/ml; and (f) curcumin.

5.1.3 Mechanical properties

Mechanical properties are often one of the most important properties because virtually all service conditions and the majority of end-use applications are involved some degree of mechanical loading. Therefore, in this study, the mechanical properties such as the tensile strength, Young's modulus and elongation at break were examined.
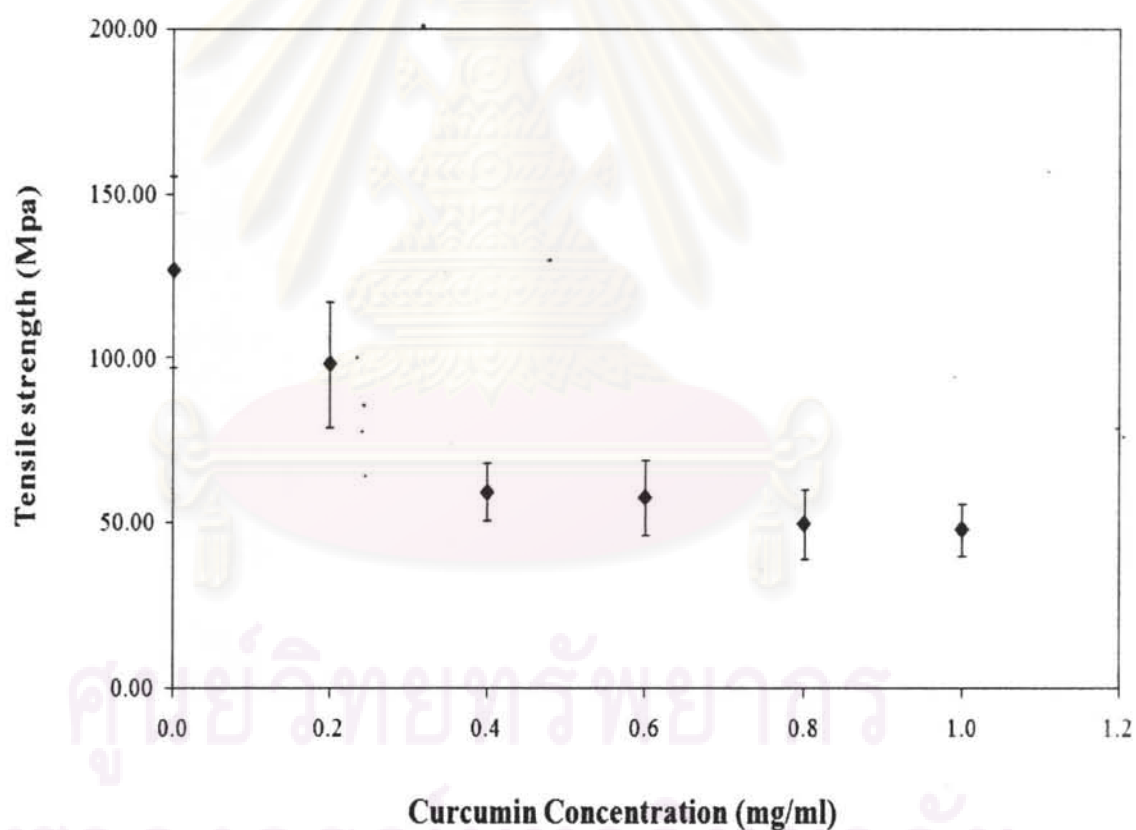

Figure 5.3 The tensile strength of the BC-curcumin films as a function of curcumin loading concentration.

Figure 5.3 illustrated the change of tensile strength of films as a function of curcumin content. The tensile strength of the BC film at the average thickness of 40 μm was 126.41 MPa. The tensile strength of films decreased from 126.41 to 47.91 MPa when the curcumin loading concentration was increased from 0.0 to 1.0 (mg/ml). It was found that the tensile strength decreased with the increase of the curcumin content.
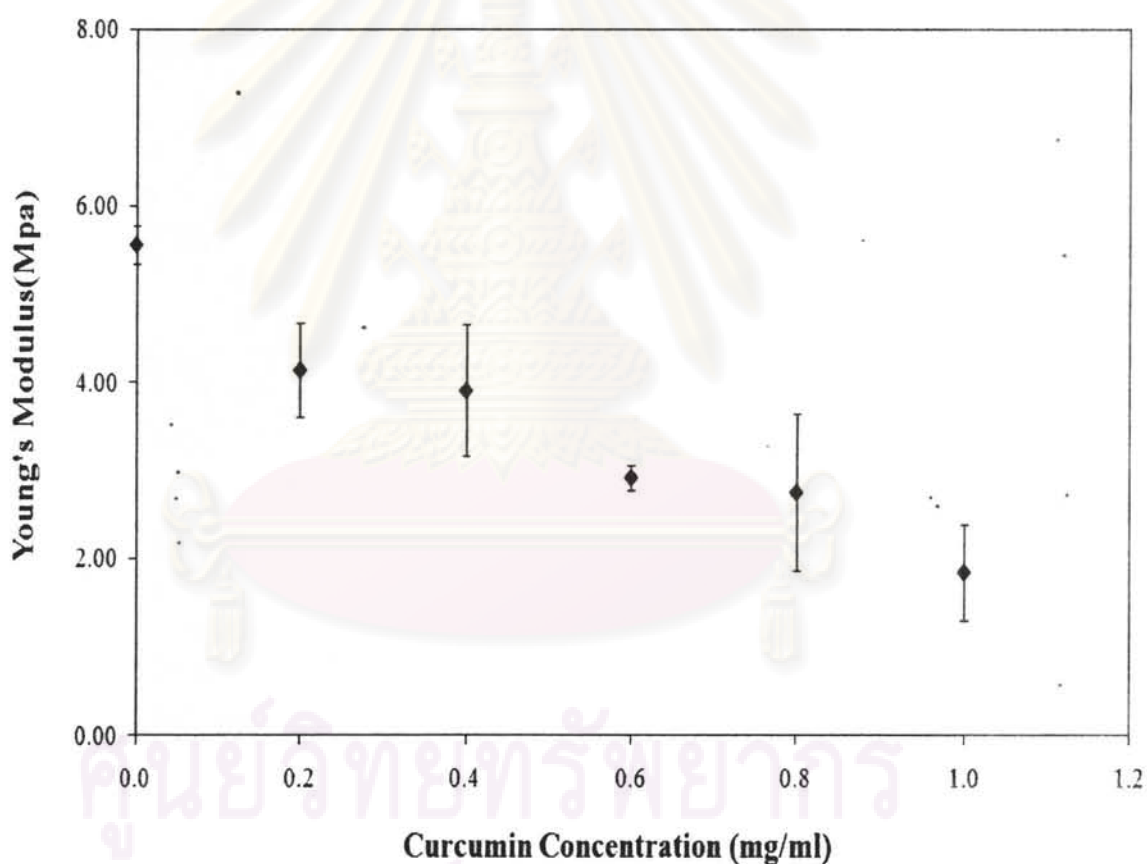

Figure 5.4 The young's modulus of the BC-curcumin membranes as a function of curcumin loading concentration.

Figure 5.4 shows the change of young's modulus of the BC-curcumin films as a function of curcumin content with the average thickness of 40 μm. The young's

modulus of the BC film was 5.56 MPa, which was the maximum observed value in the test. The minimum young's modulus was 1.84 MPa at the BCC loading with curcumin solution at concentration of 1.0 mg/ml.
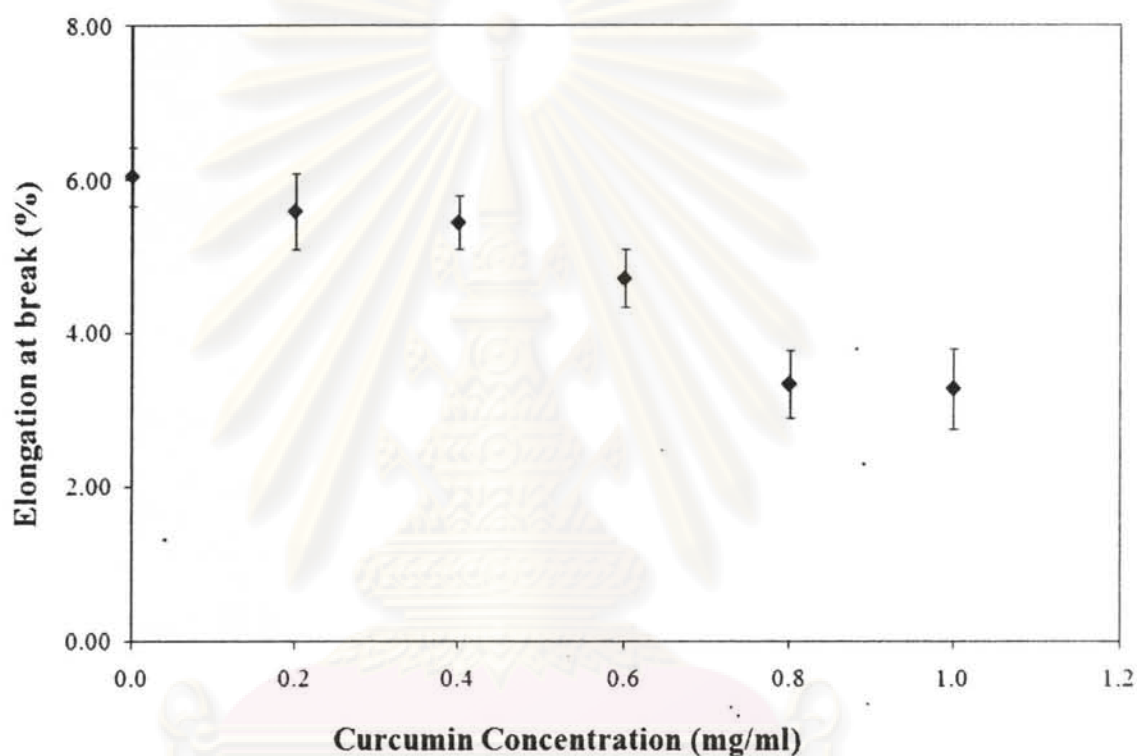

Figure 5.5 The Elongation at break of the BC-curcumin membranes as a function of curcumin loading concentration.

Figure 5.5 illustrated the change of elongation at break of membranes as a function of curcumin content. The elongation at break of BC membrane at the average thickness of 40 μm was 6.05%. The elongation at break of BCC membrane at 1% curcumin was 3.28% and it found that the elongation at break also decreased with the increased of curcumin content.

Overall the addition of curcumin resulted in BCC films with inferior mechanical properties in comparison to the BC film. The intermolecular bonds between the components of cellulose fibrils and curcumin could present relatively weak but rigid structure with less flexible motions. However, the tensile strength of the BCC films was still superior incomparison to other biopolymer films.
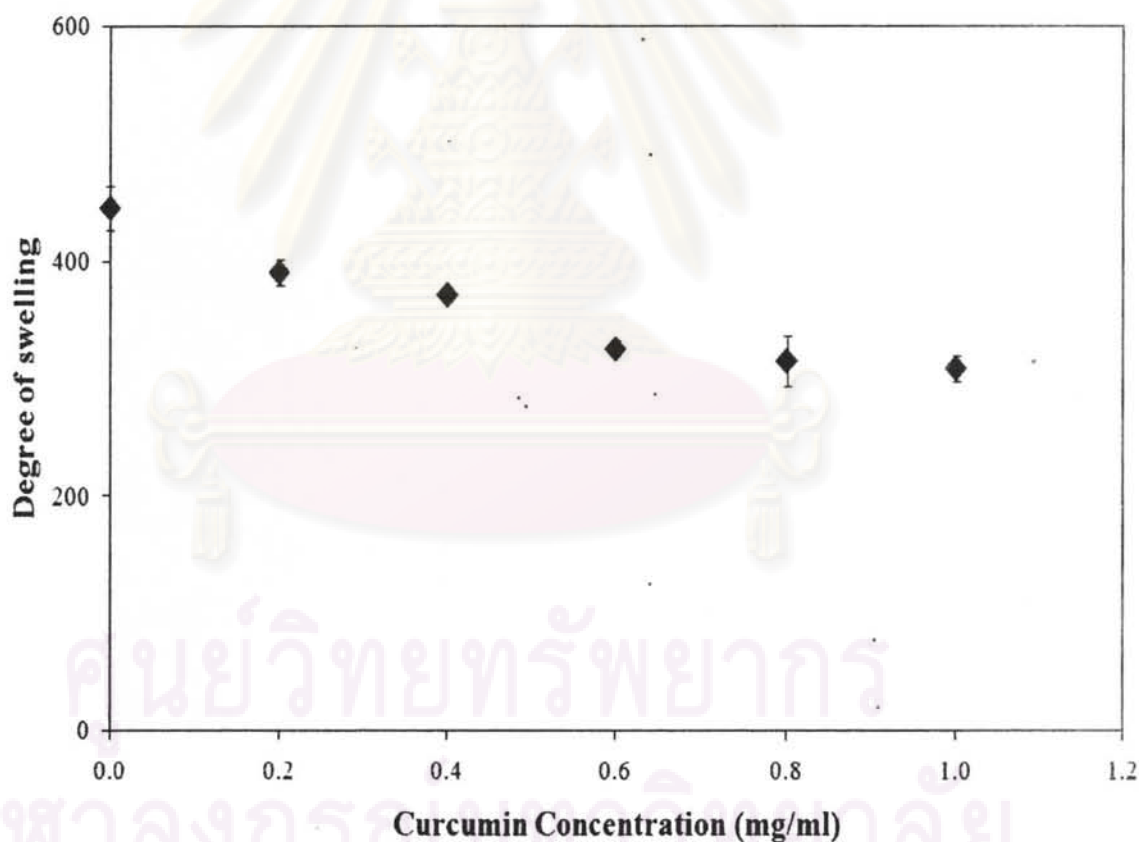
5.1.5 The degree of swelling

Figure 5.6 Degree of swelling of the BC-curcumin film as a function of curcumin loading concentration.

The BCC films were further characterized for the swelling ability after submersion in water for 48 h (see Figure 5.6). The degree of swelling of BC film was 444% in water. The swelling ability of the films was gradually decreased from 444 to 308% when curcumin loading concentration increased from 0.0 to 1.0 mg/ml. The degree of the swelling of the films decreased when increasing the curcumin content. The less water could be sorbed into the BCC films due to high hydrophobic property of curcumin (Jaruga et al.,1998).

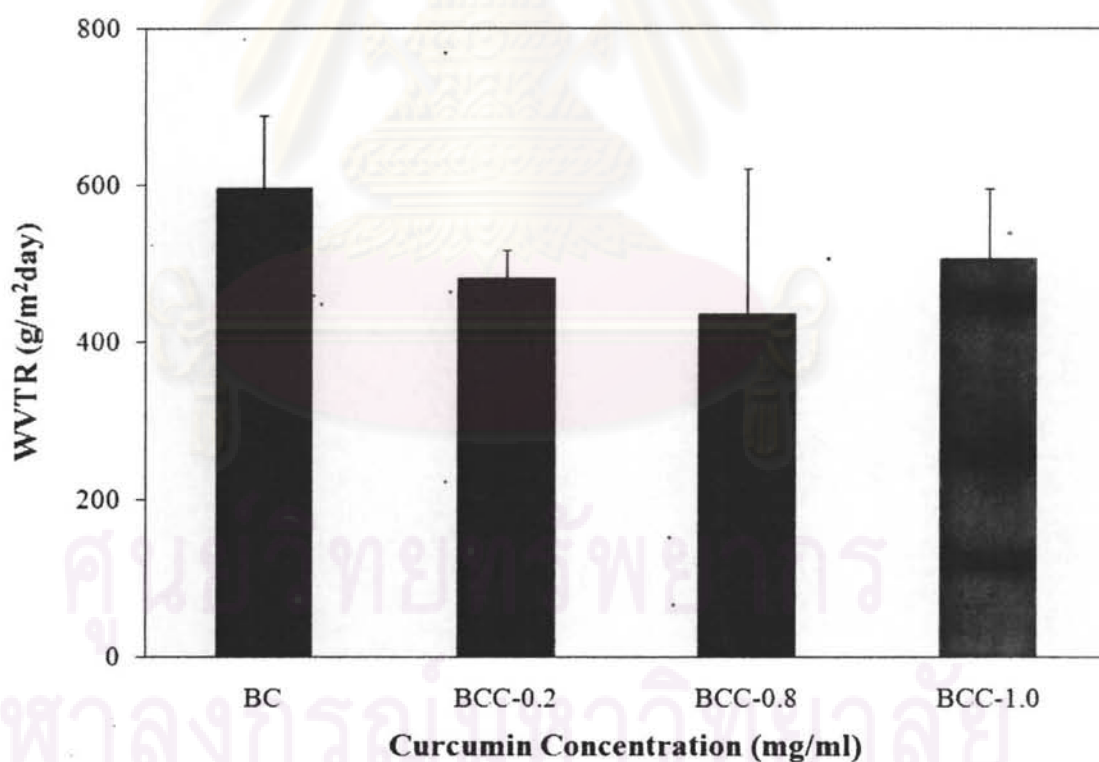## 5.1.6 Water vapor permeability test (WVTR)

Figure 5.7 The water vapor transmission rate of the BC-curcumin films as a function of curcumin loading concentration (mg/ml).

The water vapor transmission rate (WVTR) of the BC film was not significantly affected by curcumin loading at the concentration up to 1 mg/ml. The water vapor transmission rate of the BC-curcumin membranes were 435-597 g/m^2.day. It was found that the water vapor transmission rate of the BC-curcumin films were similar to commercial films such as Ultec® and Bioclusive® (Wu al et., 1995).
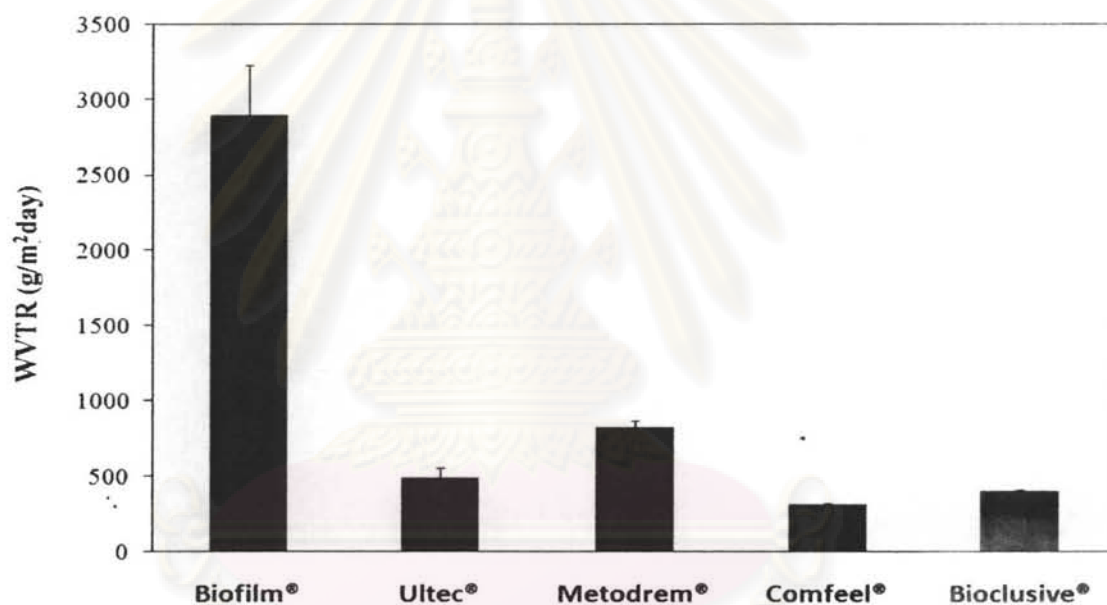

Figure 5.8 The water vapor transmission rate of the commercial films.

5.1.7 Oxygen permeability test (OTR)

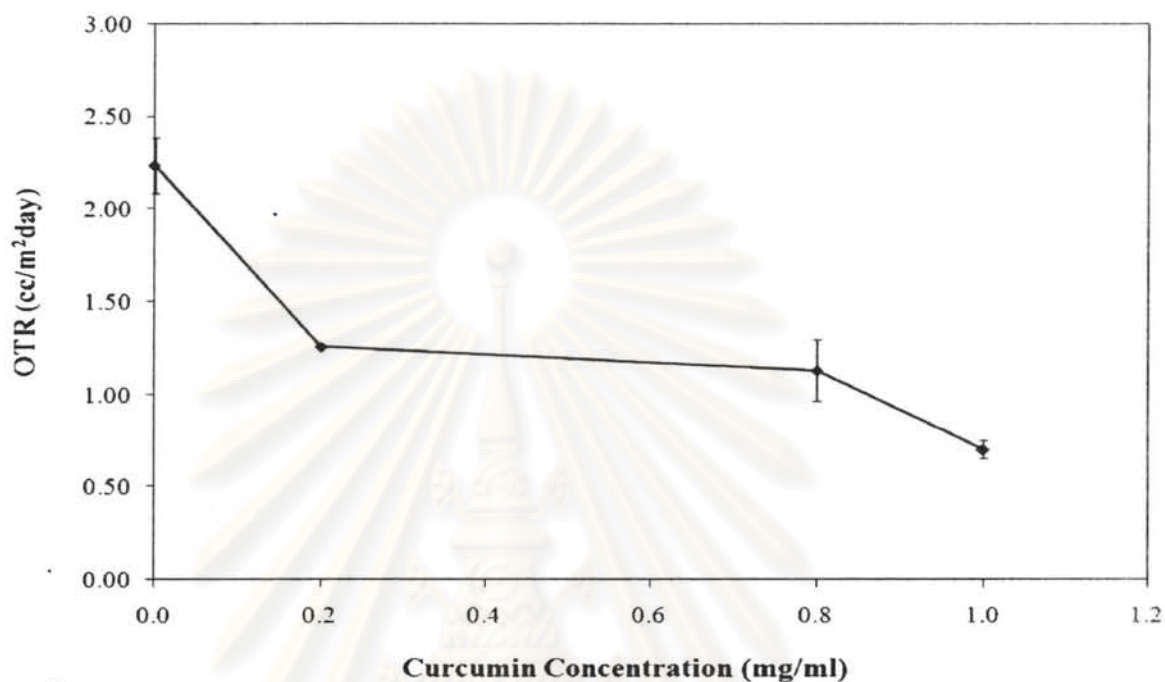**Figure 5.9** Oxygen transmission rate of the BC-curcumin membranes as a function of

curcumin loading concentration.

Figure 5.9 showed the rate of oxygen transmission (OTR) of the BC and BC-curcumin films. As increasing curcumin content, the OTR was decreased. Besides that, it was observed that the OTR was less in comparison to the WVTR. The denser structure could cause a greater reduction in diffusivity through the dried film.

5.1.8 Antibacterial Activity

In this study, both *Escherichia coli* and *Staphylococcus aureus* were used as the test bacteria to examine the antibacterial properties of BC and BC-curcumin films. A antimicrobial effect of curcumin in various loading concentrations was shown in Table 5.2, Figure 5.10 and Figure 5.11. The test with the BC, BCC 0.5 mg/ml curcumin and BCC 1 mg/ml curcumin exhibited no clear zone against all bacterial strains (*E. coli* and *S. aureus*). Only the inhibition of bacterial growth under the samples was observed.

However, it has been previously reported that curcumin at concentration of 2.5-50 mg/ml had antibacterial activity against *Staphylococcus aureus* but it had no effect on *Bacillus typhosus* (Chopra et al., 1941).

Test Microorganisms	Sample	Antimicrobial effect
Staphylococcus aureus	BC	-No clear zone -Inhibition of growth under the sample
	BCC-loading with 0.5 mg/ml curcumin solution	-No clear zone -Inhibition of growth under the sample
	BCC-loading with 1.0 mg/ml curcumin solution	-No clear zone -Inhibition of growth under the sample
Escherichia coli	BC	-No clear zone -Inhibition of growth under the sample
	BCC-loading with 0.5 mg/ml curcumin solution	-No clear zone -Inhibition of growth under the sample
	BCC-loading with 1.0 mg/ml curcumin solution	-No clear zone -Inhibition of growth under the sample

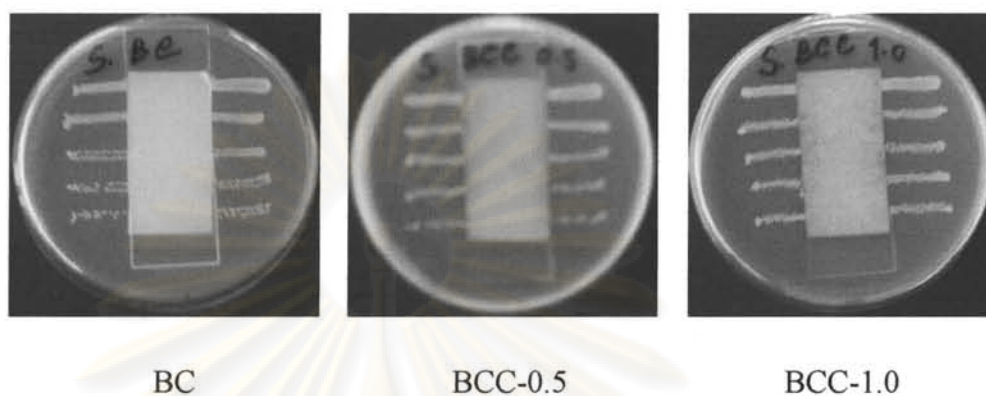**Table 5.1** The antimicrobial effect of BC and BC-Curcumin films.

BC   BCC-0.5   BCC-1.0

Figure 5.10 Inhibition tests of samples on *Staphylococcus aureus* for 24 h., incubated

at 37°C

BC   BCC-0.5   BCC-1.0
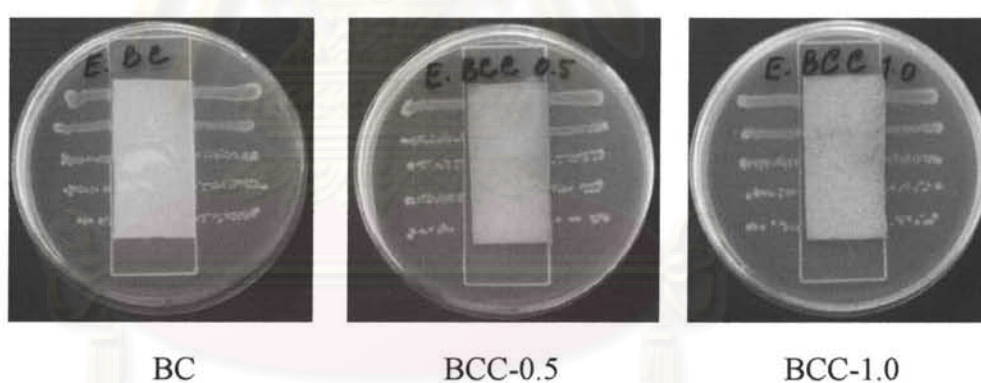
Figure 5.11 Inhibition tests of samples on *Escherichia coli* for 24 h., incubated at

37°C

5.1.9 Antifungal Ability

In this study, *Aspergillus niger* was used as the test fungai to examine the

antifungal activity of BC and BC-curcumin films. The antimicrobial effect of BC

films with loading curcumin in different concentration was shown in Table 5.3 and

Figure 5.10. The film samples of BCC-0.5 mg/ml curcumin and BCC-1 mg/ml curcumin films exhibited the significant effect on the growth of *Aspergillus niger*. On the basis of this observation, it could be proposed that the antifungal activity of BC film could be improved by the loading of curcumin solution of 0.5-1.0 mg/ml.

From the previous report, fresh juice and extract of *curcuma* inhibited the growth of *Aspergillus nige* and *Penicillium digitatum* (Kapoor et al., 1997).

Fungi	Sample	Observed growth Result
Aspergillus niger	BC	Heavy growth (more than 60%)
	BCC-loading with 0.5 mg/ml curcumin solution	Trace (less than 10%)
	BCC-loading with 1.0 mg/ml curcumin solution	Trace (less than 10%)

Table 5.2 The antifungal activity of BC and BCC films on *Aspergillus niger* activity after the incubation for 7 days at 30°C.
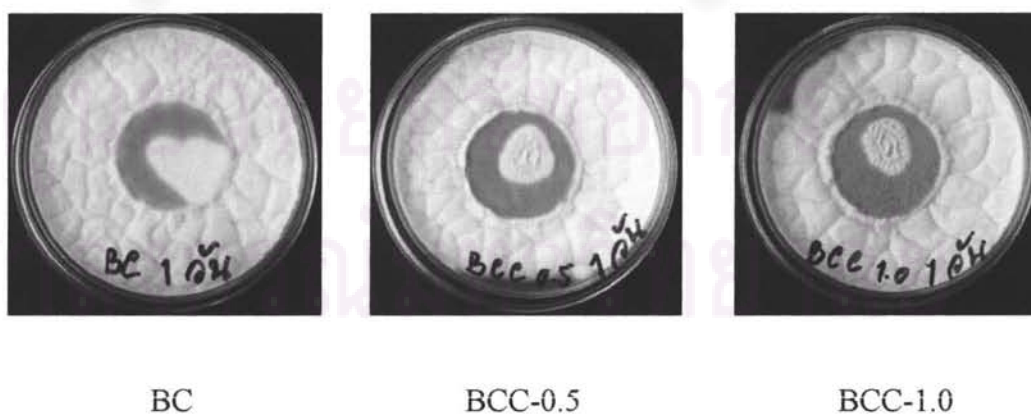

BC   BCC-0.5   BCC-1.0

Figure 5.12 The growth of *Aspergillus niger* on BC and BCC the specimens, at 30°C for 1 days.

BC BCC-0.5 BCC-1.0
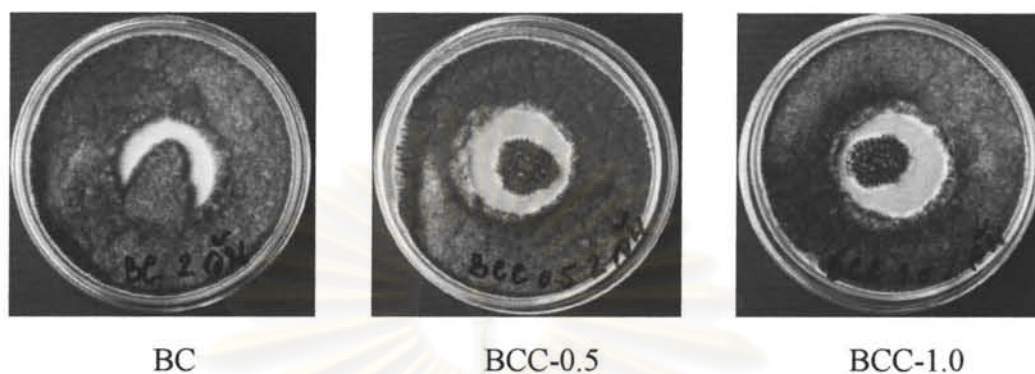
Figure 5.13 The growth of *Aspergillus niger* on the BC and BCC specimens, at 30°C

for 2 days.

BC BCC-0.5 BCC-1.0
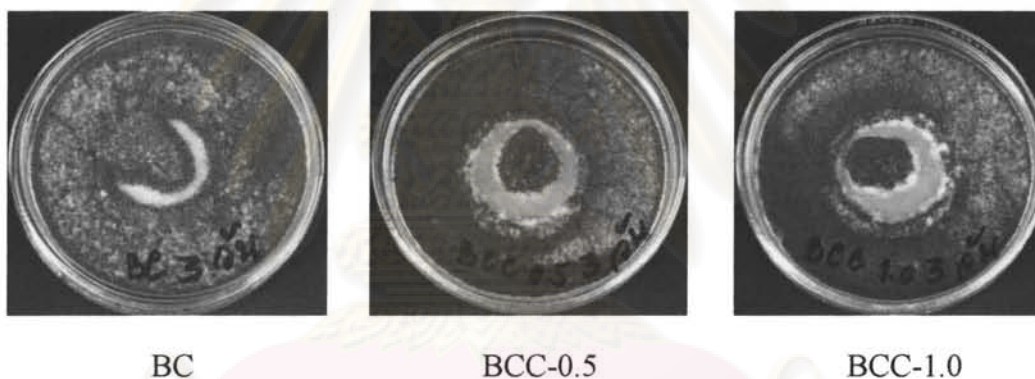
Figure 5.14 The growth of *Aspergillus niger* on the BC and BCC specimens, at 30°C

for 3 days.

BC BCC-0.5 BCC-1.0
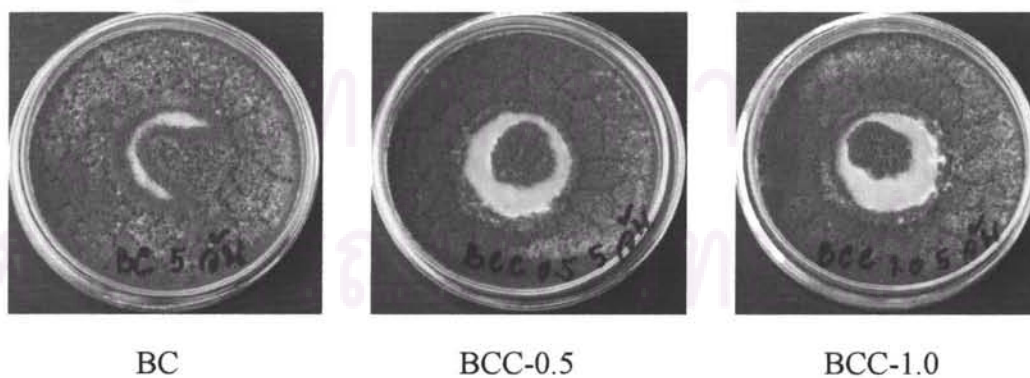
Figure 5.15 The growth of *Aspergillus niger* on the BC and BCC specimens, at 30°C

for 5 days.

BC   BCC-0.5   BCC-1.0

Figure 5.16 The growth of *Aspergillus niger* on the BC and BCC specimens, at 30°C

for 6 days.

BC   BCC-0.5   BCC-1.0
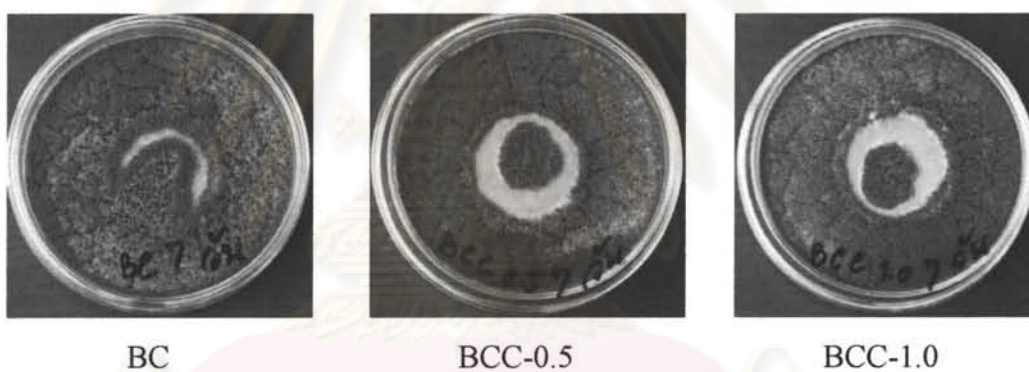
Figure 5.17 The growth of *Aspergillus niger* on the BC and BCC specimens, at 30°C

for 7 days.

5.2 Absorption and Release of curcumin from curcumin-loaded BC film

Absorption of actual amount curcumin in BC film need to be determined prior to investigate the release characteristic of curcumin from these films. Table 5.3 summarizes the actual amount of curcumin in these specimens (reported as mg of curcumin/cm^3 of BCC film.

curcumin concentration (mg/ml) loaded into BC film	Actual amount of curcumin (mg/cm^3) in BCC film	
	Using wet (never dried) film	Using dried film
0.2	47.52±0.26	0.57±0.04
0.4	78.55±1.24	1.96±0.02
0.6	98.91±0.14	2.27±0.01
0.8	124.61±0.07	2.42±0.18
1	176.97±0.36	6.08±0.1

Table 5.3 Actual amount of curcumin was absorbed into BC film in wet (never dried) state and dried state.

With the adsorption for 24 h using the curcumin solutions at the concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml absorbed into the wet BC films, the actual amount of curcumin in films were 47.52, 78.55, 98.91, 124.61 and 176.97 mg per cm^3 BCC film, respectively. Comparatively, much lower values were observed for

the curcumin loaded BC films that were prepared from the curcumin solutions absorbed into the dried BC films, where the actual amount of curcumin in films were 0.57, 1.96, 2.27, 2.42 and 6.08 mg per cm^3 BCC film, respectively. The superior porosity structure of the wet film should be the cause of the higher absorption capacity. From the previous report (Sanchavanakit et al.,2007), it was found that the equilibrium water content of the wet (never dried) film was 18.2 g water/ g dried film, which was more than 3- fold of the re-swollen film in water.

The release characteristics of curcumin from the curcumin-loaded BC films was carried out by the total immersion. The experiments were carried out using the acetate buffer solution containing 0.5% v/v Tween 80 and 3% v/v methanol (B/T/M medium) at 37°C. Previously, Suwantong et al.(2007) used a B/T/M medium to study the release characteristics of curcumin from curcumin-loaded e-spun CA fiber mats and corresponding as-cast CA films. Here, the cumulative release profiles of curcumin from the curcumin-loaded BC films were reported in two different manners, i.e., as the percentage of the weight of curcumin released divided by the actual weight of the specimens and as the percentage of the weight of curcumin released divided by the actual weight of curcumin in the specimens.

The curcumin- loaded BC film specimens showed a rapiod increase in the cumulative release of curcumin during 1200 min (20 hours) of the immersion in B/T/M medium after that the cumulative release of curcumin showed a gradual increase during 1200-3600 min (20-60 hours). As expected, the amount of curcumin released of the films increased with increasing the amount of curcumin loaded in the wet BC films. Specifically, for the BC-curcumin films that were prepared from the curcumin concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 (mg/ml), the amount of curcumin

after 60 hours released was ~5, ~8, ~11, ~15 and ~19 % (mg of curcumin/mg of specimen x 100), respectively. When reported as the percentage of the weight of curcumin released divided by the actual weight of curcumin in the specimens, the curcumin-loaded BC film specimens also showed a rapid increase in the total amount of curcumin released during 0-1200 min (0-20 hours) of the immersion and the slow release of curcumin was observed after that. However, the maximum amount of curcumin released in term of percentage based on actual amount of curcumin-loading slightly increased with increasing concentration of curcumin loaded in BC films. The BC-curcumin films that were prepared from the curcumin solutions of 0.2, 0.4, 0.6, 0.8 and 1.0 (mg/ml) gave the total amount of released curcumin at ~81, ~82, ~87, ~88 and ~91 % (based on actual amount of curcumin-loading), respectively.

The results showed that almost all of the curcumin absorbed in the curcumin-loaded BC specimens could be released into the B/T/M medium.

Figure 5.18 Cumulative release profiles of curcumin from curcumin-loaded BC films reported as the percentage of the weight of released curcumin divided by the actual amount curcumin.

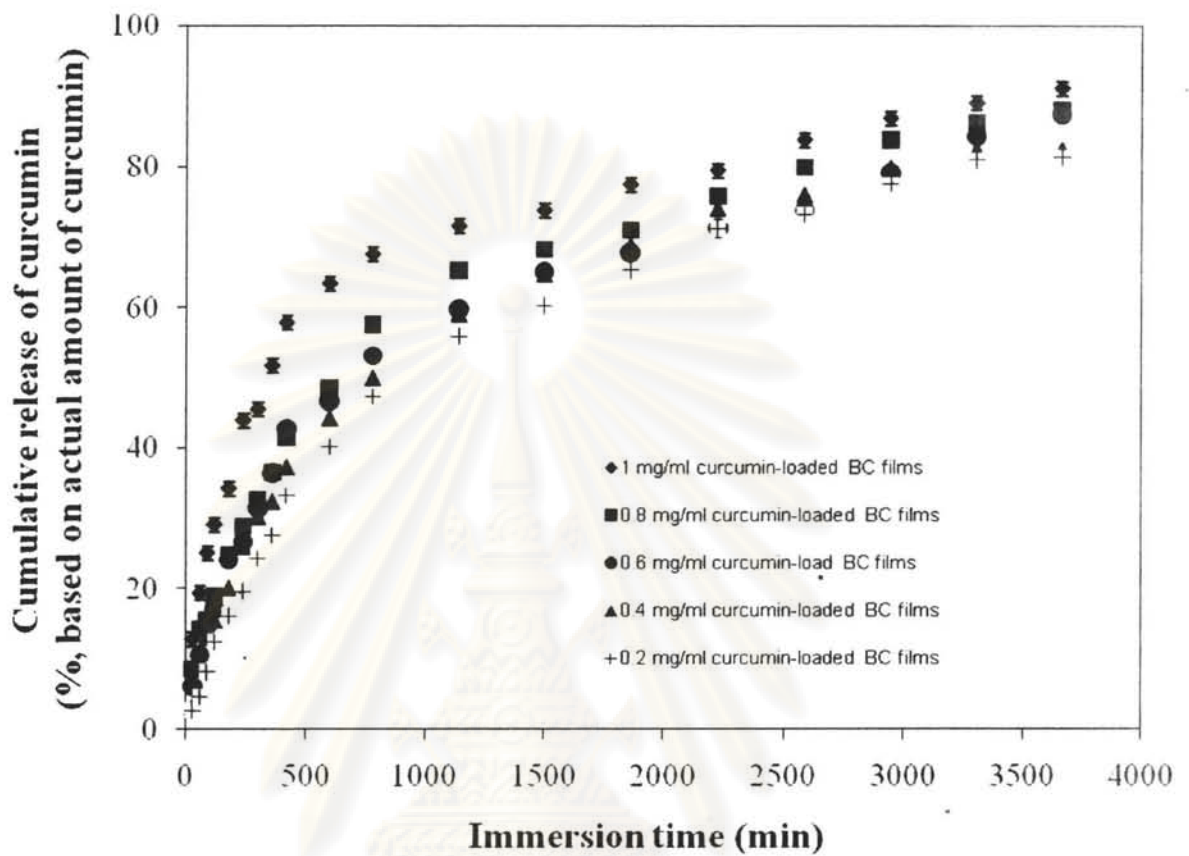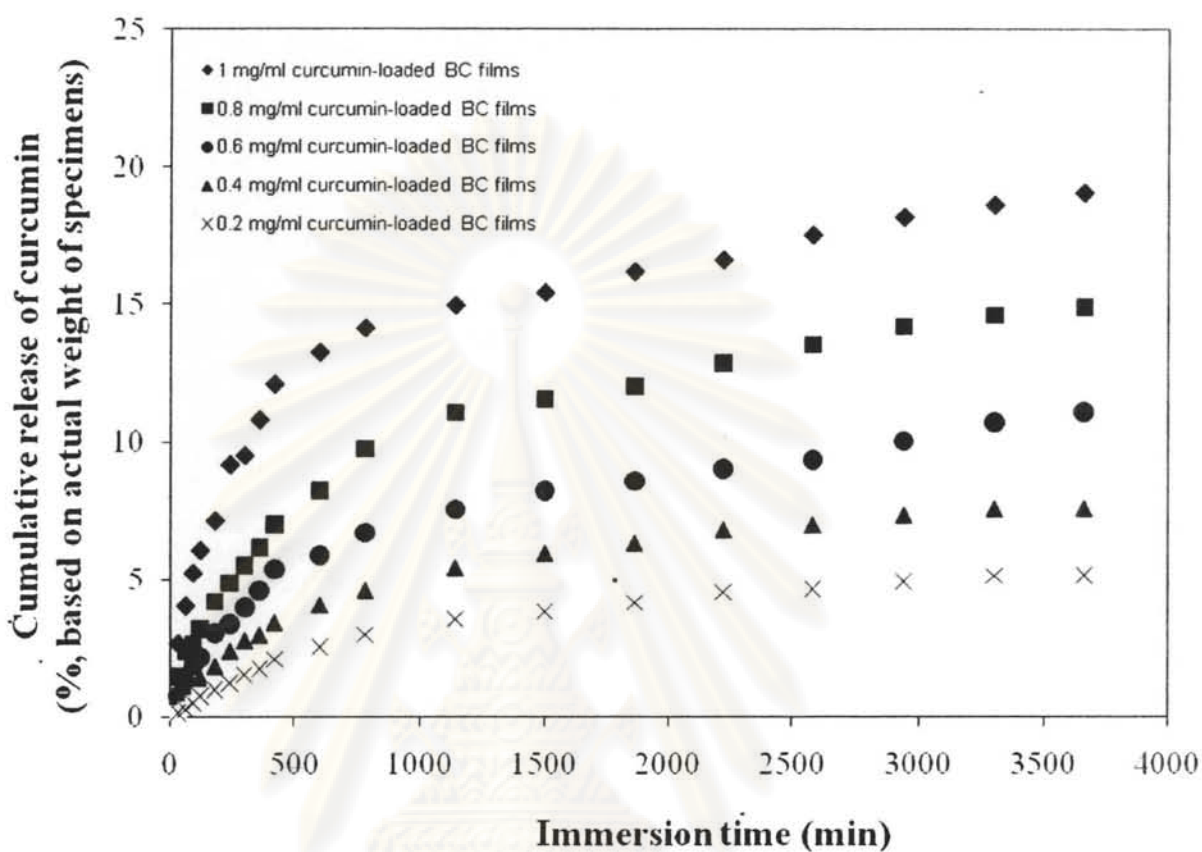**Figure 5.19** Cumulative release profiles of curcumin from curcumin-loaded BC films

reported as the percentage of the weight of released curcumin divided

by the actual weight of specimens.

5.3 Cytotoxic effects of BC-curcumin
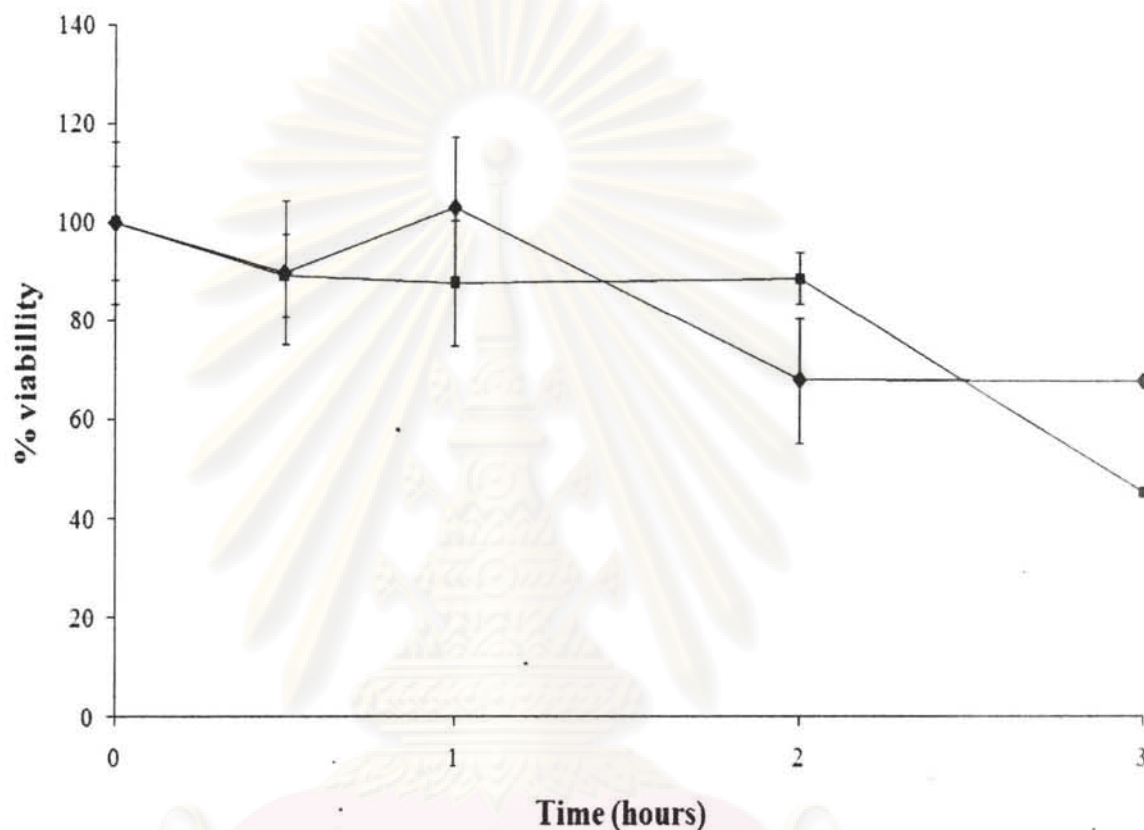
Figure 5.20 Cytotoxic effect of BC-curcumin against B16 melamoma cells.

♦ BCC film loading with 0.5 mg/ml curcumin solution

■ BCC film loading with 0.5 mg/ml curcumin solution

The viability of the human melanoma cells (B16) that were cultured with the extraction media of the BCC films in comparison to that with the extraction media of the BC film is illustrated in Figure 5.20. Both the extract of the BCC-0.5 and BCC-1.0 (mg/ml) significantly inhibited the viability of the dermal cancer cells within 3 hours. Figure 5.21 demonstrated the amount of curcumin that was released from BC-

curcumin film into water after the incubation. Within 3 hours, the amount of curcumin released from the BCC films which were prepared from the curcumin solutions at concentration of 0.5 and 1.0 (mg/ml) was 0.078 and 0.088 mg/ml, respectively. Curcumin has been reported for antioxidant, anti-inflammatory, cancer chemopreventive and anti-cancer effects (Ammon and Wahl, 1991; Ozaki et al., 2000; Hidaka et al,, 2002; Siwak et al., 2005). The result in this study indicated that the release of curcumin from the BCC film could suppress melanoma cancer proliferation and triggered cancer cell death. The extract of the film with higher curcumin content caused more cancer cell death.
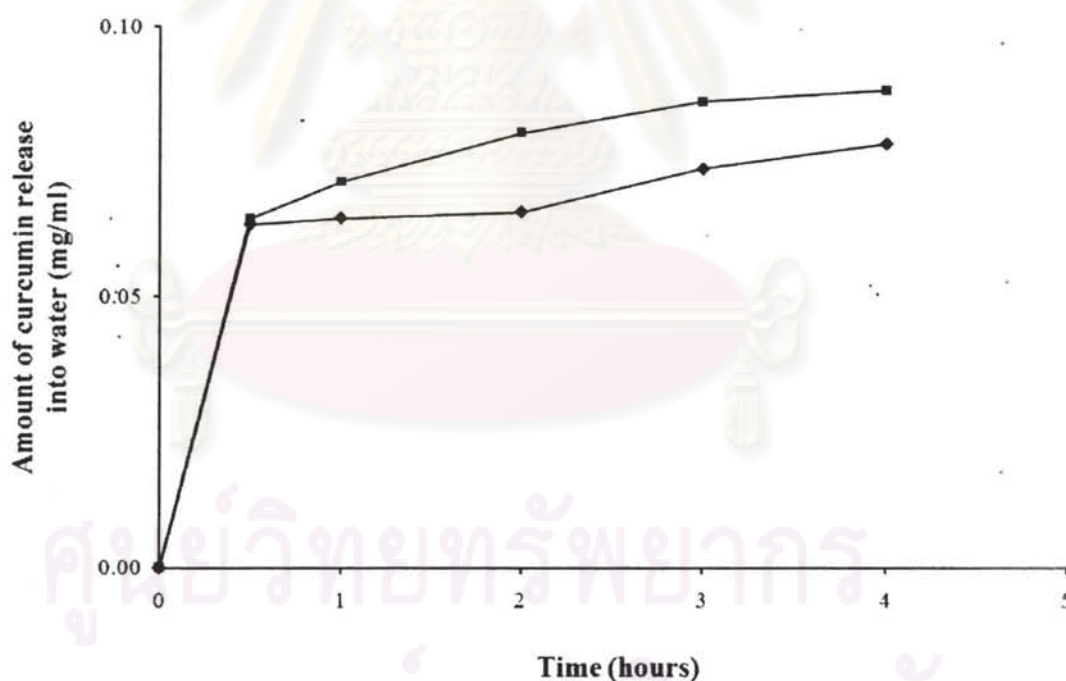

Figure 5.21 Actual amount of curcumin that was released into water after the

incubation.

♦BCC film loading with 0.5 mg/ml curcumin solution

■ BCC film loading with 1.0 mg/ml curcumin solution

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this study, a bacterial cellulose film was modified by loading with curcumin solution. The result of FTIR spectroscopy indicated that interactions might present between the hydroxyl groups of BC fiber and the carbonyl group of curcumin. The SEM micrograph of surface morphology demonstrated the decrease of average pore size when increasing curcumin content as curcumin particles were incorporated into BC fibril network.

The influence of the modification on the physical characteristics of the film was investigated. The tensile strength, Young's Modulus and Elongation at break of the bacterial cellulose-curcumin (BCC) films were found to be relatively lower than those of the BC film. The degree of swelling of the BCC film significantly decreased with the increase of curcumin content in the solution from 0.0-0.6 mg/ml. The decrease in OTR of the films with increasing curcumin content was observed. However, the WVTR of the films was not significantly affected by the curcumin loading. The WVTR of the BCC films were around 435-597 g/m^2.day.

From the examination of antibacterial and antifungal properties, the BCC films with the loading of curcumin solution at concentration of 0.5 and 1.0 mg/ml displayed antifungal activity against *Aspergillus niger*. However, the antibacterial test with the BCC films exhibited no clear zone against *Escherichia coli* and *Staphylococcus aureus*.

The loading concentration and film structure were found to be important factors that affected the absorption characteristics of curcumin in bacterial cellulose film. With the curcumin solution at concentration of 1 mg/ml loading into wet BC film, the actual amount of curcumin in the film at 177.0 mg/cm^3 was achieved. The examination of the release characteristic of curcumin by total immersion at 37°C demonstrated that 81- 91% of total loaded curcumin in the film could be released into the B/T/M medium.

Characterization of inhibitory effect on growth of human melanoma cells showed that the amount of curcumin absorbed in the BCC films could be released into the cell culture medium and subsequently inhibited the growth of melanoma cancer within 3 hours.

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 modifying bacterial cellulose by incorporated with other herbal extracts.

2. The study for more applications.

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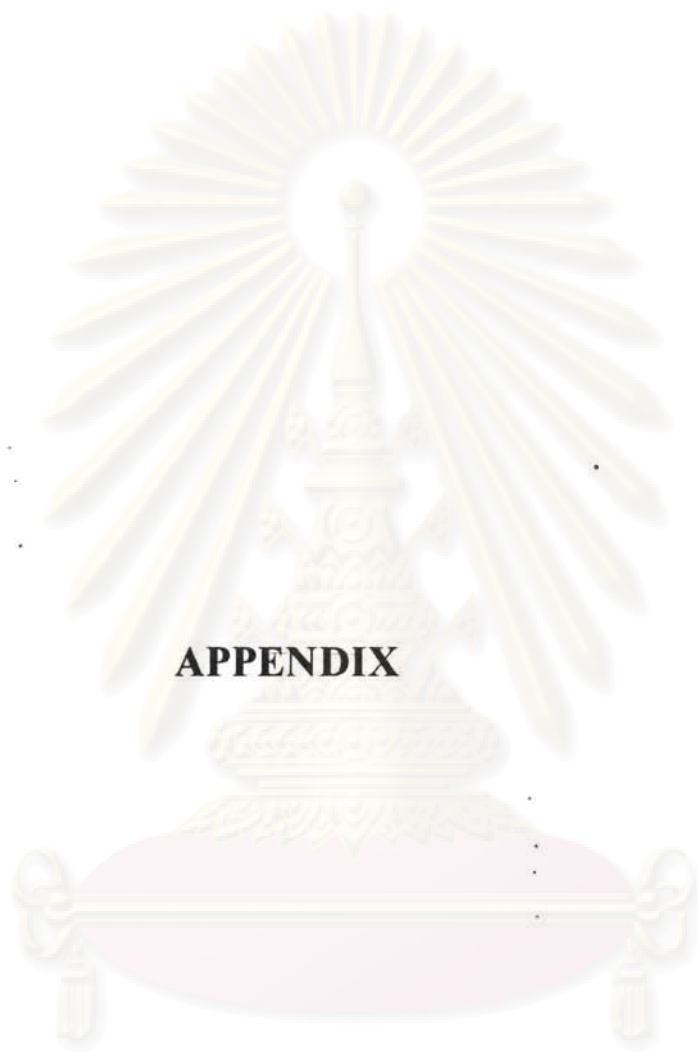
APPENDIX

APPENDIX

DATA OF MEMBRANE CHARACTERIZATION

Table A1 Data of Figure 5.4

Curcumin concentration(mg/ml)	Tensile strength (Mpa)						
	1	2	3	4	5	Average	S.D.
0	150.20	93.89	100.17	128.54	159.23	126.41	29.13
0.2	86.48	118.53	118.73	88.25	78.71	98.14	19.05
0.4	57.80	55.01	73.14	43.49	67.28	59.35	8.79
0.6	42.55	51.75	59.25	67.86	67.16	57.71	11.47
0.8	50.50	46.01	64.48	43.79	43.27	49.61	10.72
1	53.31	41.05	40.21	46.22	58.78	47.91	8.01

Table A2 Data of Figure 5.5

Curcumin concentration(mg/ml)	Young's Modulus(Mpa)						
	1	2	3	4	5	Average	S.D.
0	5.691	5.2375	5.4481	5.6933	5.7424	5.56	0.21
0.2	3.967	4.0501	5.0386	3.6377	4.0367	4.15	0.53
0.4	4.6608	3.4409	4.3428	4.2506	2.8643	3.91	0.74
0.6	2.85	3.0155	2.8657	3.1333	2.7692	2.93	0.15
0.8	2.0978	1.9953	3.9106	3.531	2.2604	2.76	0.89
1	2.0259	1.7291	0.9881	1.9898	2.4885	1.84	0.55

Table A3 Data of Figure 5.6

Curcumin concentration(mg/ml)	Elongation at break(%)						
	1	2	3	4	5	Average	S.D.
0	5.7333	6.4222	6.0122	6.4693	5.6162	6.05	0.39
0.2	5.9972	5.9992	5.0502	5.0745	5.8508	5.59	0.49
0.4	5.676	5.779	5.6212	5.0502	5.0955	5.44	0.34
0.6	4.6312	4.6798	4.1737	5.2318	4.86	4.72	0.38
0.8	3.5817	2.7912	3.6523	3.716	2.933	3.33	0.44
1	3.6928	3.246	3.8995	2.8787	2.6708	3.28	0.52

Table A4 Data of Figure 5.7

Curcumin concentration(mg/ml)	Degree of swelling (%) at pure water						
	1	2	3	4	5	Average	S.D.
0	467.35	428.30	461.22	436.73	428.00	444.32	18.68
0.2	386.76	380.82	398.46	381.54	405.89	390.70	11.05
0.4	366.22	368.06	381.48	372.98	368.88	371.52	6.09
0.6	333.13	321.74	328.79	315.87	325.76	325.06	6.61
0.8	295.77	332.81	324.24	331.69	286.57	314.22	21.54
1	309.59	290.93	308.11	308.06	322.22	307.78	11.13

Table A4 Data of Figure 5.8

Curcumin concentration(mg/ml)	WVTR (g/m^2/day)			
	1	2	Average	S.D.
BC	661.25	533.33	597.29	90.45
BCC0.2	506.38	456.73	481.56	35.11
BCC0.8	566.00	303.57	434.79	185.57
BCC1	569.57	442.73	506.15	89.69

Table A6 Data of Figure 5.9

Curcumin concentration(mg/ml)	OTR (cc/m^2/day)			
	1	2	Average	S.D.
0	2.34	2.13	2.24	0.15
0.2	1.25	1.27	1.26	0.01
0.8	1.25	1.02	1.13	0.16
1	0.74	0.67	0.70	0.05

VITAE

Miss Wannipa Woraharn was born on March 29th, 1986 in Khon Kaen, Thailand. She received the Bachelor Degree of Chemical Engineering from Faculty of Engineering, Thammasat University in April, 2008. She continued Master degree at Chulalongkorn University in June, 2008.

Her academic publication is as follow:

ABSORPTION AND RELEASE CHARACTERISTICS OF CURCUMIN IN BACTERIAL CELLULOSE FILM in the 19th Thailand Chemical Engineering and Applied Chemistry Conference (TICHE 19th) on October 26-27, 2008 at the Felix River Kwai Resort, Kanchanaburi, Thailand, pp.54-56.