Full Report

"Green" Nanocomposites of Bacterial Cellulose Nanofibres Reinforced Biodegradable Polymer

By

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Year 2011

ACKNOWLEDGEMENT

First of all, we would like to express our appreciation to our diligent students for their hard work on this research project; Mr. Donnarit Meksri (Biotechnology), Ms. Kedmanee Somord, Ms. Siwaporn Duangsri, Ms. Ratchaneekorn Amkham, Ms. Pattira Pratumma (Applied Chemistry), and Mr. Nunin Aksonthong (Materials Science), School of Science, Mae Fah Luang University.

We are deeply grateful for the *Acetobacter aceti* sup sp. *xylinum* TISTR 975 used in this research which was kindly supplied by Dr. Sirirung Wongsakul, School of Agro-Industry, as well as a kind assistance in the statistical and data analysis from Mr. Nattawut Yodsuwan, School of Science, Mae Fah Luang University.

Special acknowledgement and recognition go to Ms. Nittaya Laosat and Ms. Atitaya Ngaokla for their assistances in bacterial cellulose production. Also, all of staffs in Physical Analysis Laboratory, Scientific and Technological Instrument Center, Mae Fah Luang University are gratefully acknowledged for their assistances in SEM, XRD, TGA, Tensile testing and compression molding machine.

Finally, we most gratefully thank Mae Fah Luang University for the financial support of this research work.

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November 2013

EXECUTIVE SUMMARY

A growing environmental awareness has inspired a deliberate attempt to develop bio-based composite materials. The combination of natural cellulosic fibres and biodegradable polymeric matrices has been driven by their potential positive ecological benefits with respect to ultimate disposability and renewability. The use of nano-scale fillers as reinforcement in bio-based composite materials is another technology that has been extensively investigated. With their nanometric size effect and extremely high specific-surface area, nano-fillers have the potential for significant reinforcement in composite materials at very small filler loadings as well as provide some unique outstanding properties as compared to their conventional microcomposite counterparts. Recently, nanometer-sized cellulose crystals commonly referred to as nanowhiskers or nanofibrils have gained interest to use as nanocomposite reinforcements. The cellulose nanowhiskers from renewable resources show high aspect ratio, large surface area, exceptional mechanical properties (high specific strength and modulus), and environmental benefits.

The aim of this research is firstly, to cultivate the bacterial cellulose from *Acetobacter xylinum* strain and use it as a raw material for preparation of bacterial cellulose nanowhiskers (BCNWs). In this part of work, the effect of type of carbon source and additional supplement in the cultured medium of bacterial cellulose on production yield, its structure and mechanical properties were also studied. Secondly, to prepare and characterize the bionanocomposites of starch-based reinforced with BCNWs. The effects of acid hydrolysis time, pH adjustment, and content of BCNWs on structure and properties of the resulting bionanocomposites were investigated. The starch/BCNWs bionanocomposites were prepared by film casting and characterized

by various techniques; i.e. x-ray diffraction (XRD), transmission electron microscopy (TEM), scanning electron microscopy (SEM), mechanical test, thermal gravimetric analysis (TGA) and moisture absorption technique. In summary, it can be concluded that BCNWs can be used as an effective reinforcement in the starch-based nanocomposite materials. However, the extent of reinforcing effect depends as well on the preparation, pretreatment (e.g. pH adjustment) and content of BCNWs.

The novel scientific knowledge from this research project has already been published in three academic papers; firstly, "Effect of carbon and nitrogen sources on bacterial cellulose production for bionanocomposite materials"; secondly, "Effect of additional supplements in the cultured medium of bacterial cellulose on the production yield"; and thirdly, "Effect of neutralization on structure and properties of cellulose nanowhiskers derived from bacterial". These three papers were published in the 1st Mae Fah Luang University International Conference 2012 (1st MFUIC 2012) proceeding. Furthermore, the other two papers in topics of "Starch-based bionanocomposites reinforced with bacterial cellulose nanowhiskers: Effect of pHs on morphological, thermal and mechanical properties" and "Effect of carbon source type in the bacterial cellulose cultivation on its structure and mechanical properties" are in preparation to publish in the peer-reviewed international scientific journals. Additionally, in economic viewpoints, the films which were prepared in this research are considered very interesting to further develop for food packaging application due to its high strength and biodegradable characteristic.

ABSTRACT

This research work is divided into two sections. The first section is focused on the study of the effect of type of carbon source and additional supplement in the cultured medium of bacterial cellulose (BC) on its production yield, structure, and properties. From the results, it was found that the type of carbon source significantly influenced not only the production yield of bacterial cellulose (BC) but also its structure and mechanical properties. Also, it can be summarized that except for glycerol ($p > 0.05$), other carbon sources, i.e. sucrose, glucose, fructose and mannitol, used in the culture media of BC resulted in the high mechanical properties of the BC sheets. In the study of the effect of additional supplements (i.e. pineapple and coconut juices) in the cultured medium of bacterial cellulose (BC) on the production yield, it was found that addition of optimum amounts of juices in the cultured medium of BC positively and significantly affected bacterial cellulose yield ($p > 0.05$). The addition of pineapple juice of 30% v/v and coconut juice of 50% v/v were the optimum amounts providing the highest cellulose productivity at approximately 2-fold increase in production yield as compared to 'the control medium' (no juice added medium). However, at the higher amounts of juice addition, the reduction in cell growth and cellulose production was found. Here, the added juices can create saturated carbon source environment that may directly inhibit cell activity and subsequently, decrease the cellulose yield. The results suggested that both supplements can effectively improve the cellulose yield but pineapple juice is a slightly more effective one.

In the second section, bacterial cellulose nanowhiskers (BCNWs) was prepared by acid hydrolysis of bacterial cellulose (BC) and then used to reinforce in the starch-based nanocomposite films. The effect of acid hydrolysis time and pH

adjustment on structure and properties of the obtained BCNWs was investigated. It was found that the 48 hours acid hydrolyzed BCNWs possessed the highest perfection of the crystal lattice or crystallinity. Transmission electron microscope (TEM) revealed that the continuous BC fibre network transformed into the isolated rod-like nanocrystals of the BCNWs with a diameter and length of averaged 28.18±2.0 nm and 637.61±147.10 nm, respectively. The sulfuric acid treatment leads to a decrease in the thermal stability of BCNWs confirmed by thermogravimetric analysis (TGA). This is due to the induced sulfate groups onto the BCNWs' surface after acid hydrolysis. Additional pH adjustment by NaOH can significantly improve the thermal stability of the BCNWs. The pH of BCNWs was adjusted to 3, 5, or 7 and, thereafter, bionanocomposites of starch-based reinforced with BCNWs of different adjusted pHs (at contents of 1, 5, 10 wt%) were prepared by film casting technique. With increasing BCNWs content, the bionanocomposites revealed a significant improvement in their crystallinity (confirmed by XRD), thermal stability (an increment of 20-30 ^{o}C, confirmed by TGA) and water resistance. The highest water resistance was observed in the bionanocomposite films reinforced with 10 wt% BCNWs of pH 7. However, the mechanical properties of the films reinforced with BCNWs of pH 3 and BCNWs of pH 7 were not found to be entirely enhanced because of a poor interaction between BCNWs of pH 3 and starch matrix as well as a formation of large aggregates of BCNWs of pH 7 in the bionaocomposite structures. Nevertheless, the films reinforced with BCNWs of pH 5 showed a noticeably improvement in the mechanical properties, the film stiffness in particular. Probably, the optimum dispersion of BCNWs and sufficient interfacial interaction in this system was obtained.

บทคัดย่อ

งานวิจัยนี้แบ่งเป็น 2 ส่วน ส่วนแรกมุ่งเน้นถึงการศึกษาผลกระทบของแหล่งคาร์บอนและอาหารเสริมที่ใช้ในการเลี้ยงแบคทีเรียเซลลูโลสต่อโครงสร้าง สมบัติ และปริมาณการผลิตแบคทีเรียเซลลูโลส จากผลการทดลองพบว่า ชนิดของแหล่งคาร์บอนมีผลต่อปริมาณการผลิตแบคทีเรียเซลลูโลส อีกทั้งยังมีผลต่อโครงสร้างและสมบัติเชิงกลของแบคทีเรียเซลลูโลสที่ผลิตได้อีกด้วย โดยชนิดของแหล่งคาร์บอนที่ใช้ในการเลี้ยงเชื้อแบคทีเรียอันได้แก่ ซูโครส กลูโคส ฟรุกโตส และแมนนิทอล ช่วยให้แผ่นแบคทีเรียเซลลูโลสที่เตรียมได้มีสมบัติเชิงกลที่ดี ยกเว้นแหล่งคาร์บอนจากกลีเซอรอลที่แผ่นแบคทีเรียเซลลูโลสแสดงสมบัติเชิงกลด้อยกว่าแหล่งคาร์บอนชนิดอื่นอย่างมีนัยสำคัญที่ระดับความเชื่อมั่นร้อยละ 95 ($p > 0.05$) สำหรับการศึกษาผลกระทบของการเติมอาหารเสริม ได้แก่ น้ำสับปะรด และน้ำมะพร้าวในอาหารเลี้ยงเชื้อนั้น พบว่าการเติมอาหารเสริมในปริมาณที่เหมาะสมจะส่งผลให้ปริมาณการผลิตแบคทีเรียเซลลูโลสเพิ่มขึ้นอย่างมีนัยสำคัญที่ระดับความเชื่อมั่นร้อยละ 95 ($p > 0.05$) ปริมาณการเติมน้ำสับปะรดและน้ำมะพร้าวที่เหมาะสมคือ ร้อยละ 30 และ 50 โดยปริมาตร ตามลำดับ โดยสามารถเพิ่มปริมาณการผลิตแบคทีเรียเซลลูโลสได้ถึง 2 เท่า เมื่อเทียบกับปริมาณการผลิตจากอาหารเลี้ยงเชื้อแบบมาตรฐาน อย่างไรก็ดีเมื่อปริมาณอาหารเสริมมากเกินไป การเติบโตและปริมาณการผลิตเซลลูโลสจะลดลง อันเนื่องมาจากสภาวะที่แหล่งคาร์บอนอิ่มตัวเมื่อมีปริมาณอาหารเสริมมากเกินไป ส่งผลให้กิจกรรมภายในเซลล์และปริมาณการผลิตแบคทีเรียเซลลูโลสลดลง จึงสรุปได้ว่า การเติมอาหารเสริมสามารถเพิ่มปริมาณการผลิตเซลลูโลสได้อย่างมีประสิทธิภาพแต่ต้องเติมในปริมาณที่เหมาะสม และน้ำสับปะรดมีประสิทธิภาพที่ดีกว่าน้ำมะพร้าวเล็กน้อย

ในส่วนที่สองของงานวิจัยนี้ ได้ทำการเตรียมเซลลูโลสนาโนคริสตัลด้วยวิธีการแยกสลายด้วยกรด (Acid hydrolysis) โดยใช้แบคทีเรียเซลลูโลสเป็นวัตถุดิบ จากนั้นนำมาใช้เป็นวัสดุเสริมแรงในแผ่นฟิล์มวัสดุผสมระดับนาโนที่มีแป้งเป็นวัสดุหลัก และทำการศึกษาถึงผลกระทบของเวลาที่ใช้ในกระบวนการแยกสลายด้วยกรด และการปรับค่าความเป็นกรด-ด่าง (pH) ที่ส่งผลต่อสมบัติของเซลลูโลสนาโนคริสตัลด้วย พบว่า เมื่อแยกสลายแบคทีเรียเซลลูโลสด้วยกรดเป็นเวลา 48 ชั่วโมง เซลลูโลสนาโนคริสตัลมีค่าความเป็นผลึกสูงที่สุด จากการศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องทะลุ (TEM) แสดงให้เห็นลักษณะของเส้นใยของแบคทีเรียเซลลูโลสที่เปลี่ยนจากแบบโครงข่ายไปเป็นเซลลูโลสนาโนคริสตัลที่มีเส้นผ่านศูนย์กลางเท่ากับ 28 นาโนเมตร โดยมีความยาวเฉลี่ยเท่ากับ 637.61 นาโนเมตร แต่เมื่อผ่านกระบวนการแยกสลายด้วยกรด ความเสถียรทางความร้อนของเซลลูโลสนาโนคริสตัลกลับลดลง ซึ่งยืนยันได้จากผลการ

วิเคราะห์เชิงความร้อน (TGA) ความเสถียรทางความร้อนที่ลดลงนี้เป็นผลมาจากหมู่ซัลเฟตที่เกิดขึ้นบนผิวของเซลลูโลสนาโนคริสตัลหลังจากการแยกสลายด้วยกรด การปรับค่าความเป็นกรด-ด่างของ เซลลูโลสนาโนคริสตัลโดยใช้โซเดียมไฮดรอกไซด์ (NaOH) สามารถปรับปรุงความเสถียรทางความร้อนของเซลลูโลสนาโนคริสตัลได้ ดังนั้นจึงปรับค่าความเป็นกรด-ด่างของเซลลูโลสนาโนคริสตัลเป็น 3 5 และ 7 จากนั้นจึงทำการเตรียมฟิล์มวัสดุผสมระดับนาโนโดยกำหนดปริมาณการเติมเซลลูโลสนาโนคริสตัล เป็นร้อยละ 1 5 และ 10 โดยน้ำหนัก จากผลการทดลองพบว่า เมื่อเพิ่มปริมาณเซลลูโลสนาโนคริสตัล ค่าความเป็นผลึก ความเสถียรทางความร้อนและการต้านทานน้ำของฟิล์มวัสดุผสมมีค่าดีขึ้น โดยฟิล์มวัสดุผสมที่ต้านทานน้ำได้ดีที่สุดคือฟิล์มที่เสริมแรงด้วยเซลลูโลสนาโนคริสตัลที่มีค่าความเป็นกรด-ด่าง เท่ากับ 7 ในปริมาณร้อยละ 10 โดยน้ำหนัก อย่างไรก็ตาม สมบัติเชิงกลของฟิล์มวัสดุผสมเสริมแรงด้วยเซลลูโลสนาโนคริสตัลที่มีค่าความเป็นกรด-ด่าง เท่ากับ 3 และ 7 ไม่ดีขึ้นในทุกด้าน เนื่องจากเซลลูโลสนาโนคริสตัลที่มีค่าความเป็นกรด-ด่าง เท่ากับ 3 ยึดเหนี่ยวกับแป้งได้ไม่ดี ส่วนเซลลูโลสนาโนคริสตัลที่มีค่าความเป็นกรด-ด่าง เท่ากับ 7 เกาะตัวเป็นก้อนในโครงสร้างของวัสดุผสม ในทางกลับกันฟิล์มวัสดุผสมที่เสริมแรงด้วยเซลลูโลสนาโนคริสตัลที่มีค่าความเป็นกรด-ด่าง เท่ากับ 5 ช่วยให้สมบัติเชิงกลมีค่าที่ดีขึ้น โดยเฉพาะค่าความแข็งแกร่ง ซึ่งคาดว่าน่าจะเกิดจากการที่เซลลูโลสนาโนคริสตัลกระจายตัวได้ดีในฟิล์ม ทำให้มีแรงยึดเหนี่ยวระหว่างองค์ประกอบที่มากพอ

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CHAPTER 1

INTRODUCTION

1.1 Statement and Significance of the Problem

In the last decade, a growing environmental awareness has inspired a deliberate attempt to develop bio-based composite materials (Heijenrath and Peijs 1996; Peijs et al. 1998; Peijs 2000; Singleton et al. 2003). The combination of natural cellulosic fibres and bio-based polymeric matrices has been driven by their potential positive ecological benefits with respect to ultimate disposability and the use of renewable resources. Nowadays, natural fibre reinforced composites are increasingly replacing glass fibre reinforced composites as a viable alternative in various applications particularly in automotive industry. (Averous and Boquillon ,2004; Bledzki and Gassan 1999; Lu et al. 2006; Mohanty et al. 2000; Nishino et al. 2003; Oksman et al. 2006; Samir et al. 2005; Soykeabkaew et al. 2004).

1.2 Objectives

1.2.1. Study the effect of type of carbon source in the cultured medium of bacterial cellulose on its structure and mechanical properties

1.2.2. Investigate the effect of additional supplements in the cultured medium of bacterial cellulose on its production yield

1.2.3. Preparation of bacterial cellulose nanowhiskers by acid hydrolysis of bacterial cellulose nanofibres

1.2.4. Characterization of bacterial cellulose nanowhiskers

1.2.5. Preparation of bionanocomposites of bacterial cellulose nanowhiskers reinforced biodegradable polymer

1.2.6. Characterization of bionanocomposites of bacterial cellulose nanowhiskers reinforced biodegradable polymer

1.3 Benefits

1.3.1. Knowledge and development in production of bacterial cellulose as a reinforcement in nanocomposites

1.3.2. Techniques for purification and characterization of bacterial cellulose nanofibres

1.3.3. Techniques for preparation and characterization of bacterial cellulose nanowhiskers

1.3.4. Preparation and characterization of bionanocomposites of bacterial cellulose nanowhiskers reinforced biodegradable polymer

1.4 Scope of Study

Part A

1. Study the effect of type of carbon source in the cultured medium of bacterial cellulose on its structure and mechanical properties
    - Various types of carbon source were used to prepare the medium for bacterial cellulose cultivation.
    - Structure and mechanical properties of the cultured bacterial cellulose from the various media were examined.
2. Investigate the effect of additional supplements in the cultured medium of bacterial cellulose on its production yield

- Supplements (i.e. pineapple and coconut juices) were additionally used to prepare the medium for bacterial cellulose cultivation.
- Production yield of bacterial cellulose from the various media were determined.

Part B

1. Preparation of bacterial cellulose nanowhiskers by acid hydrolysis of bacterial cellulose nanofibres
   - Acid hydrolysis of bacterial cellulose was performed using concentrated sulphuric acid to prepare bacterial cellulose nanowhiskers.
2. Characterization of bacterial cellulose nanowhiskers
   - Transmission electron microscopy (TEM), x-ray diffraction (XRD) and thermal gravimetric analysis (TGA) were used to characterize the bacterial cellulose nanofibres and nanowhiskers.
3. Preparation of bionanocomposites of bacterial cellulose nanowhiskers reinforced biodegradable polymer
   - Bionanocomposites of bacterial cellulose nanowhiskers reinforced starch matrix were prepared by film casting technique.
4. Characterization of bionanocomposites of bacterial cellulose nanowhiskers reinforced biodegradable polymer
   - The prepared bionanocomposites were characterized by using scanning electron microscopy (SEM), x-ray diffraction (XRD), thermal gravimetric analysis (TGA), tensile test and moisture absorption technique.

CHAPTER 2

LITERATURE REVIEWS

2.1 Biodegradable Materials

Nowadays, biodegradable materials present a number of promising properties in various applications, for instance, in packaging, automotive and biomedical fields. Specifically, thermoplastic biodegradable polymers, such as poly(lactic acid) (PLA), polyhydroxyalkanoates (PHA) and polycaprolactones (PCL), exhibit an excellent similar properties to conventional plastics, apart from being processable using conventional plastics machinery (Sanchez-Garcia et al., 2010). Biodegradable plastics are polymeric materials in which can be degraded (at least one step) in the presence of naturally occurring organisms. Under appropriate conditions of moisture, temperature and oxygen availability, biodegradation leads to disintegration of the plastics into carbon dioxide and water with no environmentally harmful residue. Biodegradable polymers can be classified into three categories according to their source (Sorrentino et al., 2007):

1. Polymers directly extracted or removed from biomass (i.e. polysaccharides, proteins, polypeptides, polynucleotides).

2. Polymers produced by classical chemical synthesis using renewable bio-based monomers or mixed sources of biomass and petroleum (i.e. polylactic acid or bio-polyester)

3. Polymers produced by micro-organism or genetically modified bacteria (polyhydroxybutyrate, bacterial cellulose, xanthan, curdian, pullan).

However, some problems related with the application of these biodegradable materials including their performance, processing, and cost have

presented. There is a requirement to improve some of their properties, so that they can compete with the current conventional plastics.

2.2 Bionanocomposites

The use of nano-scale fillers as reinforcement in bio-based composites is another technology that has been extensively investigated. With their nanometric size effect and extremely high specific-surface area, nano-fillers have the potential for significant reinforcement in composite materials at very small filler loadings and providing some unique outstanding properties as compare to their conventional microcomposite counterparts (e.g. natural fibre reinforced composites) (Grunert and Winter 2002; Samir et al., 2005). Studies incorporated clay, chitin or cellulose whisker as reinforcement into biodegradable polymers, polyvinyl alcohol (PVA), polylactic acid (PLA), polycaprolactone (PCL), polyvinyl acetate (PVAc), polyhydroxy butyrate (PHB), cellulose acetate butyrate (CAB), starch and aliphatic polyesters to create bionanocomposites have been reported (Garcia de Rodriguez et al., 2006; Jung et al., 2007; Lu et al., 2006; Oksman et al., 2006; Orts et al., 2005; Roohani et al., 2008; Samir et al., 2005; Wibowo et al., 2006; Wu et al., 2007; Yu et al., 2003).

More recently, biodegradable cellulose nanofibers have received a great deal of interests to incorporate within bio-based polymers since the additional value of generating fully bio-based materials, significant improvements in mechanical properties, thermal stability and permeability as well as retaining good transparency of the reinforced nanocomposite materials (Lagaron & Lopez-Rubio, 2011).

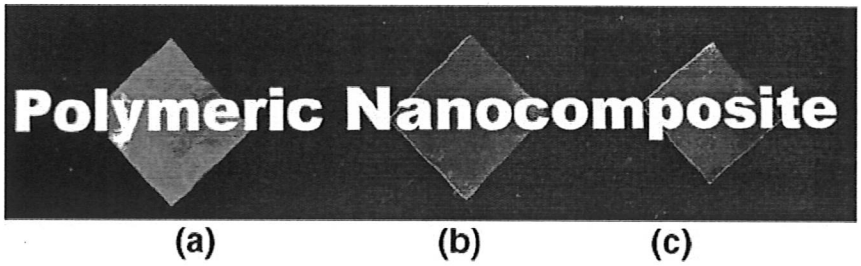

Figure 2 - 1 Photograph of (a) bacterial cellulose pellicle, (b) PLA/bacterial cellulose nanocomposite film, and (c) PLA film (Kim et al., 2009).

2.3 Cellulose

Cellulose is a ubiquitous and abundant structural polymer found in plants, hence, widely available and inherently low cost. The use of cellulose as reinforcing elements in composite materials present distinct advantages, when compared with their synthetic and inorganic counterparts, namely biodegradability, biocompatibility, low energy consumption, low density, high specific strength and modulus (with fibers possessing an adequate aspect ratio), high sound attenuation and comparatively easy processability (Lee et al., 2009; Martins et al., 2009; Pei et al., 2010). Two forms of nanoreinforcements obtained from cellulose have been currently focused – microfibrillated cellulose (MFC) and cellulose nanowhiskers (CNW) (Azeredo, 2009; Sanchez-Garcia & Lagaron, 2010; Tingaut et al., 2010).

Microfibrillated cellulose (MFC) is a form of expanded high-volume cellulose, moderately degraded and greatly expanded in surface area, achieved in the refining and homogenizing processes. Depending on the source of cellulose, individual fibrils can be about 5-10 nm in diameter and lengths varying from 100 nm to several micrometers (Nakagaito et al., 2009; Okubo et al., 2009). In the past few years, the use of MFC to reinforce in biodegradable polymers e.g. polylactic acid (PLA) have been introduced. Several previous studies have reported improved

performances of the prepared nanocomposites with incorporation of MFC including mechanical properties, thermal stability and reduction in time for polymer's crystallization process (Iwatake et al., 2008; Jonoobi et al., 2010; Nakagaito et al., 2009; Okubo et al., 2009; Suryanegara et al., 2010; Tingaut et al., 2010).

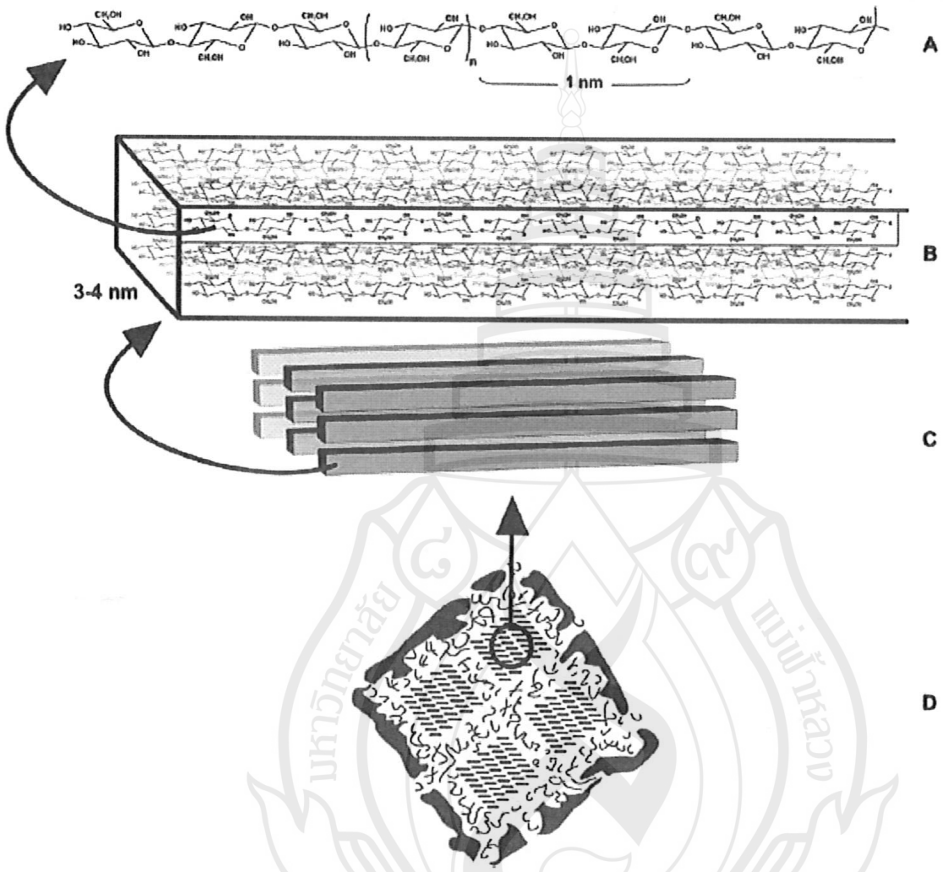

Figure 2 - 2 Internal structure of cellulose microfibril: (A) a cellulose chain; (B) an elementary fibril containing bundles of cellulose chains; (C) elementary fibrils; (D) microfibrils held together by hemicelluloses and lignin (Azeredo, 2009).

2.4 Bacterial Cellulose

Lately, bacterial cellulose, which presents a unique network structure of a random assembly of ribbon shaped nanofibres, has also drawn scientific attention as reinforcement for polymers. Bacterial cellulose has recently been incorporated in

hydroxyapatite (HAp), polylactic acid (PLA), polyvinyl alcohol (PVA), cellulose acetate butyrate (CAB) and also as a hybrid material in apple and radish pulp (Gea et al., 2007; Gindl and Keckes 2004; Millon and Wan 2006; Wan et al., 2006; Wan et al., 2007). An example of the high-strength and high transparency composites of bacterial cellulose sheets reinforced phenolic resin attaining an impressive Young's modulus of 28 GPa has as well been reported (Figure 2 - 3, Nakagaito et al., 2005).

Figure 2 - 3 A high transparency bacterial cellulose nanocomposite (Nakagaito et al., 2005).

Bacterial cellulose is synthesised by various bacteria belonging to the genera *Acetobacter*, *Rhizobium*, *Agrobacterium*, and *Sarcina* (Jonas and Farah, 1998) in different form such as extracellular pellicle cellulose ribbons, cellulose fibrils and amorphous cellulose. However, the most efficient producers are Gram-negative, acetic acid bacteria *Acetobacter xylinum* (Bielecki et al., 2004). In a culture medium containing carbon and nitrogen sources, cultivated bacteria produce extracellular cellulose, an ultrafine ribbons network structure in the form of a highly hydrated pellicle (Figure 2 - 2, Barud et al., 2007; Iguchi et al., 2000; Nakagaito et al., 2005).

Dimensions of the ribbons are roughly 3-4 nm (thickness) and 70-130 nm (width). They are made from cellulose chains aggregated to form sub-fibrils, which have a width of approximately 1.5 nm and then the sub-fibrils are crystallized into microfibrils, which subsequently form bundles, while the latter form ribbons (Bielecki et al., 2004; Jonas and Farah 1998; Yamanaka et al., 2000).

The culture condition of *A. xylinum* is an important step in cellulose production. Since the optimized condition has been investigated, this process can promote cell growth and cellulose synthesis. One of key factor is the composition of culture media. Here, various sources in culture media including carbon, nitrogen source and supplements have been shown the link to bacterial growth and bacterial cellulose production. For example, carbon source such as monosaccharide (glucose and fructose), disaccharides and alcohols highly showed effect on cellulose synthesis (Keshk and Sameshima, 2006). These studies indicated that monosaccharide and alcohol are potential carbon source to use in bacterial cellulose production (Ramana et al., 2000).

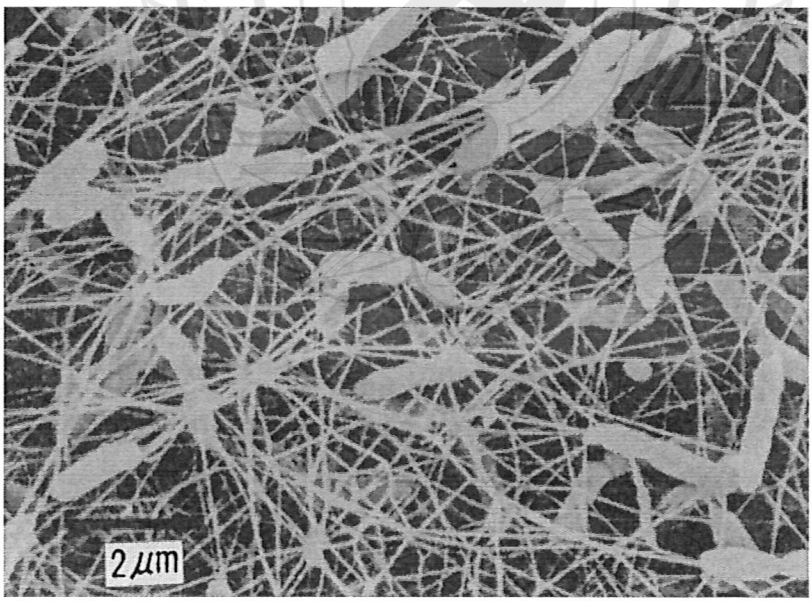

Figure 2 - 4 A scanning electron micrograph of freeze-dried surface of bacterial cellulose (gel) nano-fibre network (Iguchi et al., 2000).

In addition, nitrogen containing medium also promoted and showed different amount of cellulose yield when cultured *A. xylinum*, for instance, peptone, yeast extract, soybean meal, casein hydrolysate and ammonium sulphate (Dudman, 1959 and Chawla et al., 2009). This suggested that nitrogen is necessary for making component of cell, and enhance stability and activity of enzyme in cellulose synthesis (Embuscado et al., 1994). Finally, the addition of supplement e.g. fruit juices (such as coconut, orange, pineapple, organic acid and vitamins) is alternative that have been reported for the purpose of a yield increment in bacterial cellulose production (Kurosumi et al., 2000 and Budhiono et al., 1999).

The unique properties of bacterial cellulose; i.e. high purity, high crystallinity, high mechanical strength, high water-holding capacity, high porosity and good biocompatibility have made it find a multitude of applications in paper, textile, and food industries, and as a biomaterial in cosmetics and medicine (Bielecki et al., 2004; Jonas and Farah 1998; Zhou et al., 2007). An acoustic diaphragm of high fidelity loudspeakers and headphones marketed by Sony Corp. is another successful application that has reached the level of practical use (Iguchi et al. 2000). For this application, a high dynamic Young's modulus, close to 30 GPa, for sheets obtained from bacterial cellulose pellicles has been reported (Nishi et al., 1990; Yamanaka et al., 1989). This indicated that the ultrastructure of bacterial cellulose produced superior physical properties (Yamanaka et al., 2000). Recently, measurements using atomic force microscopy by Guhados and co-workers (2005) revealed a Young's modulus of 78 ± 17 GPa for bacterial cellulose ribbons with widths ranging from 35 to 90 nm. By using Raman spectroscopic technique, an estimation of the higher value of 114 GPa Young's modulus of a single filament bacterial cellulose is obtained (Hsieh et al., 2008). In fact, the crystal modulus of cellulose I (the type of cellulose

polymorphs for native cellulose including bacterial cellulose) in the direction parallel to the chain axis measured by X-ray diffraction was calculated to be 138 GPa (Nishino et al., 1995). Clearly, this information indicates to a high modulus which renders a promising candidate as reinforcement for bionanocomposites.

CHAPTER 3

METHODOLOGY

3.1 Research Methodology: Part A

3.1.1 Materials and equipments

- *Acetobater xylinum* *
- Sucrose
- Yeast extract
- $(NH_4)_2SO_4$
- $MgSO_4.7H_2O$
- KH_2PO_4
- Agar
- Acetic acid
- NaOH
- NaOCl
- Glucose
- Fructose
- Glycerol
- Mannitol
- Pineapple juice
- Coconut juice
- Incubator
- Compression machine
- Digital balance
- Universal testing machine
- Scanning electron microscope
- X-ray diffractometer

*Bacterial strain *Acetobacter xylinum* TISTR 975 was kindly supplied by Dr. Sirirung Wongsakul, School of Agro – Industry, Mae Fah Luang University. This isolate showed high potential in cellulose production.

3.1.2 Bacterial cellulose cultivation in the medium of various types of carbon source

In this present work, the control culture medium (adapted from Yamanaka et al., 2000) contains 50 g sucrose, 5 g yeast extract, 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4 and

0.05 g $MgSO_4.7\ H_2O$ in a litter of distilled water. In order to study the effect of type of carbon source in the cultured media of bacterial cellulose on its structure and mechanical properties, the medium of various types of carbon source as shown in Table 3 - 1 were prepared. It was then adjusted the pH to 5.0 by using acetic acid and sterilized at 121°C for 15 minutes.

Table 3 - 1 The media of various types of carbon sources for bacterial cellulose cultivation.

Media of various carbon sources	**Code**
Control; 50g/L of sucrose	Sucrose
50g/L of glucose	Glucose
50g/L of fructose	Fructose
50g/L of glycerol	Glycerol
50g/L of mannitol	Mannitol

Then, the strain of *A. xylinum,* 5.0 % (v/v), was inoculated into this culture medium. The growth condition is at 35°C in incubator with static condition for 7 days. Thereafter, the bacterial cellulose was harvested and purified by immersing in running water, 2% w/v NaOH, then 0.5% w/v NaOCl and finally running water, each step for 24 hours, respectively.

3.1.3 Preparation of disintegrated bacterial cellulose (dis-BC) sheet

In order to prepare disintegrated bacterial cellulose (dis-BC) sheet, firstly, the purified bacterial cellulose was cut into small cubes (~ 1 cm^3) and then disintegrated into a fibre suspension by using the kitchen blender (PHILIPS HR 2094)

at the maximum speed until homogeneous suspension was obtained, approximately for 10 minutes.

Next, disintegrated bacterial cellulose (dis-BC) sheets were prepared by vacuum fitration of a 0.2% wt/wt disintegrated bacterial cellulose (dis-BC) suspension. Prior to filtration, the suspension was stirred for 5 minutes to ensure a well dispersion of fibres. All dis-BC suspension were filtrated using a ceramic filter funnel (110 mm in diameter) on woven metal sheets (325 mesh) and Whatman filter papers, No. 1, England. After filtration, the wet dis-BC sheets were placed between woven metal sheets (325 mesh) and then dried at 55°C for approximately 5 days under about 1 kPa applied pressure. This resulted in dis-BC sheets with thicknesses in the range of 30-50 μm.

3.1.4 Tensile testing

Mechanical properties of the dis-BC sheets were assessed using a universal tensile tester, Instron 5566, equipped with a 1 kN load cell. The gauge length of the specimens with a width of 7 mm was 30 mm and a cross-head speed of 3 mm/min was utilized for the tests conducted at 25°C and a relative humidity of approximately 60%. The values of Young's modulus, tensile strength and elongation at break of the samples were evaluated and reported as the average values of five measurements of each material.

3.1.5 X-ray diffraction (XRD)

X-ray diffraction patterns were detected using Cu Kα radiation, generated with X'pertPro MPD (Philips, Netherlands) at 40 kV, 20 mA. The x-ray beam was

operated in reflection mode and the samples were examined over the angular range (2θ) of 5° to 35° with a step size of 0.02° and a count time of 4s per point.

3.1.6 Scanning electron microscopy (SEM)

The morphology and structure of the dis-BC sheets were observed using a scanning electron microscope, Leo 1450 VP, at an accelerating voltage of 10 kV. Prior to the examination, a surface of the specimen was sputter coated with a thin layer of gold.

3.1.7 Bacterial cellulose cultivation in the medium with additional supplements

The control culture medium (adapted from Yamanaka et al., 2000) contains 50 g sucrose, 5 g yeast extract, 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4 , and 0.05 g $MgSO_4.7\ H_2O$ in a litter of distilled water. In order to investigate the effect of additional supplements in the cultured medium of bacterial cellulose on its production yield, the supplements (i.e. pineapple and coconut juices) were used in replacement of the volume of distilled water which is normally used in the control culture medium. For example, in a litter of the 10% v/v pineapple juice medium, the volume of pineapple juice and distilled water used is 100 ml and 900 ml, respectively. The media of 10%, 20%, 30%, 50%, 70% and 100% v/v of both supplements were prepared. Then, the strain of *A. xylinum,* 5.0 % (v/v), was inoculated into this culture medium. The growth condition is at 35°C in incubator with static condition for 7 days. Thereafter, the bacterial cellulose was harvested and purified by immersing in running water, 2% w/v NaOH, then 0.5% w/v NaOCl and finally running water, each step for 24 hours, respectively.

3.1.8 Determination of yield of bacterial cellulose cultivation

In order to determine the dry weight and yield of the bacterial cellulose from the cultivation, the purified bacterial cellulose pellicle was dried into a sheet form by using a compression machine with temperature set at 115°C for 5 minutes. The yield (%) of the bacterial cellulose is calculated by the following equation:

$$Yield\ (\%) = \frac{BC}{CS} \times 100 \tag{3.1}$$

where *BC* is dry weight of the dried bacterial cellulose sheet (gram) and *CS* is weight of carbon source used in the culture medium (gram). The reported dry weight and yield (%) of the bacterial cellulose for each culture medium were averaged from 3 samples.

3.1.9 Statistical and data analysis

Microsoft excel 2010 was used for calculating ANOVA and Duncan's new multiple range test for independent samples. A p value ≤ 0.05 was considered statistically significant.

3.2 Research Methodology: Part B

3.2.1 Materials

The materials used in this work were bacterial cellulose (BC) sheet from cultivation of bacterial cellulose (*A. xylinum* TISTR 975). Deionized water was supplied by Mae Fah Luang University Laboratory (S2 building). Sulfuric acid (96% w/w) was purchased from Merck. Sodium hydroxide (NaOH) AR grade was purchased from Qrec, New Zealand. Corn flour brand Super-Find was purchased from

local supermarket, Chiang Rai, Thailand. Guar gum and glycerol (99.5 % w/v) was supplied from Sigma Aldrich and Analar Normapur, respectively.

3.2.2 Preparation of bacterial cellulose nanowhiskers by acid hydrolysis of bacterial cellulose nanofibres

Firstly, a bacterial cellulose (BC) sheet was prepared by compressing a bacterial cellulose pellicle sandwiched between woven metal sheets (325 mesh) at 115°C for 5 min using compression machine (Hydraulic hot press, Scientific LP-S-80, Labtech Engineering). The dried BC sheet was used as a raw material for preparation of bacterial cellulose nanowhiskers (BCNWs). The acid hydrolysis was performed using 50% (w/v) sulfuric acid, at a cellulose/acid ratio of approximately 8 g/L, shaking in water bath at 50°C for a fixed period of time (24, 48 and 72 hours). The BCNWs were obtained as a precipitate collected from 15 centrifugation cycles (Ultrasonic Centifuge Avanti j-30I) at 12,500 rpm and 15°C for 20 min. Flow chart of the BCNWs preparation is shown in Figure 3 - 1.

In order to determine yield (%) of BCNWs, the precipitate was dried in a hot air oven at 100°C for 2 hours. The yield (%) of the BCNWs is calculated by the following equation:

$$Yield\ (\%) = \frac{C}{G} \times 100 \tag{3.2}$$

where C is weight of the dried BCNWs (gram) and G is weight of the dried BC sheet (gram).

3.2.3 Preparation of Films

3.2.3.1 Preparation of Standard Films

All ingredients in Table 3 - 2 were pre-mixed in a beaker until a homogeneous mixture was obtained. The mixtures were then heated at 80°C using a hot plate for starch to gelatinize. It was continuously stirred for 20 min with heating. After that the mixture was degassed by sonification for 30 min. It was then poured onto a Petri dish and dried at 40°C for approximately 2 days. The preparation steps are summarized as shown in Figure 3 - 2.

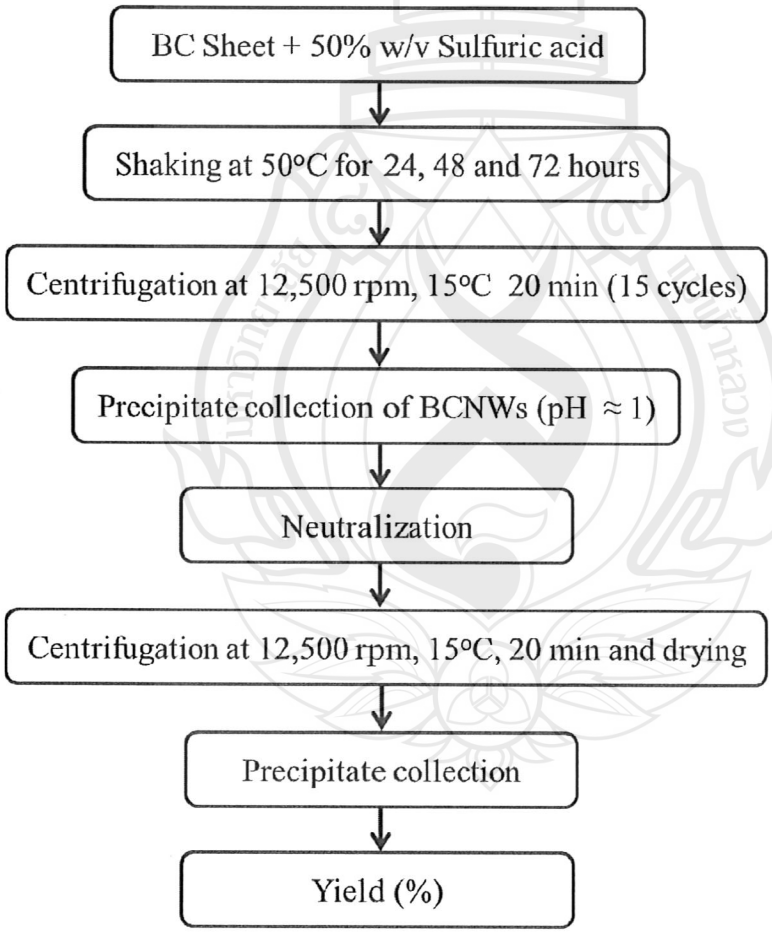

Figure 3 - 1 Flow chart of bacterial cellulose nanowhiskers (BCNWs) preparation.

Table 3 - 2 Ingredient for preparation of standard films.

Ingredient	**Weight ratio**
Corn starch	3.00
Guar gum	0.01
Glycerol (99.5%)	0.90
Deionised water	100.00

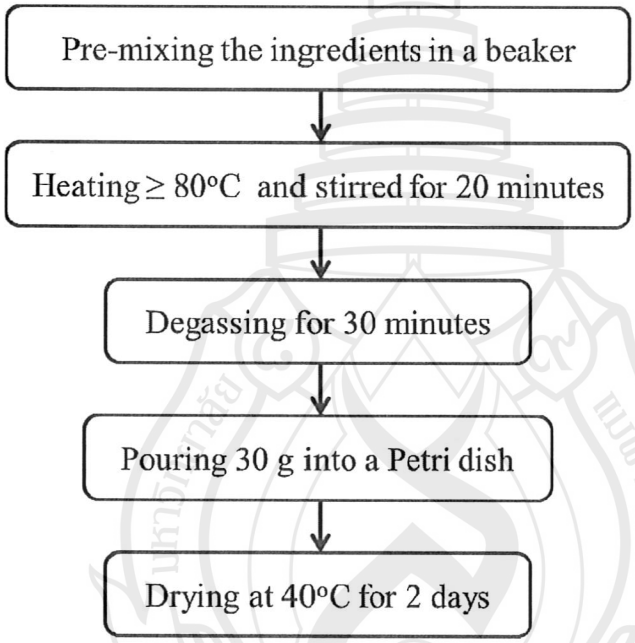

Figure 3 - 2 Flow chart of preparation of standard films.

3.2.3.2 Preparation of Bionanocomposite Films

The pH of the prepared BCNWs measured after the centrifugation was being around 1. All the BCNWs were then re-suspended in deionized water and adjusted pH to 3, 5 and 7 by using NaOH solutions of 0.5% and 5.0% (w/v) and subsequently centrifuged to obtain the BCNWs pH adjusted as a partially hydrated precipitate. To prepare the bionanocomposite films, BCNWs were pre-mixed with

deionized water (at contents of 1, 5 and 10 wt% based on starch weight) in a beaker until a homogeneous mixture was obtained. Then other ingredients in Table 3 - 2 were added into the mixture. After that, all ingredients were heated at 80°C using a hot plate for starch to gelatinize. It was continuously stirred for 20 min with heating. After that, the mixture was degassed by sonification for 30 min. Then, it was poured onto a Petri dish and dried at 40°C for approximately 2 days. The preparation steps are summarized as shown in Figure 3 - 3.

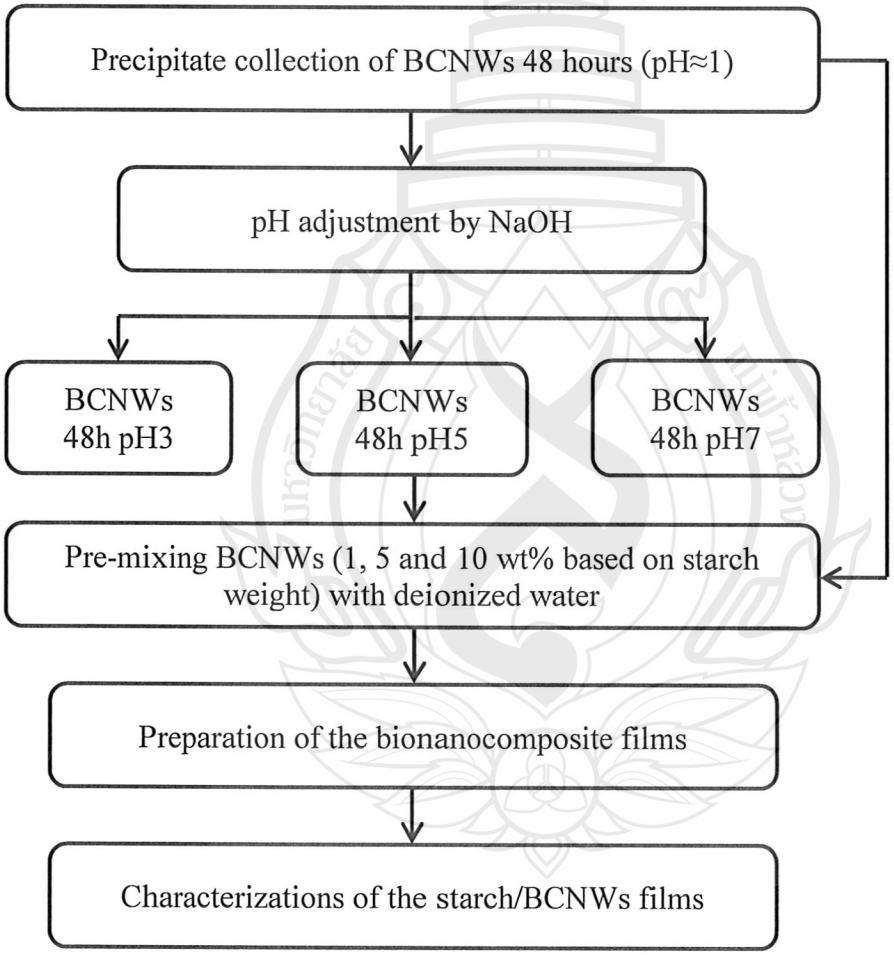

Figure 3 - 3 Flow chart of preparation of the bionanocomposite films.

3.2.4 Characterizations

Prior to all characterization, the samples were stored in condition of 50% relative humidity (RH) for 3 days (using a chamber of the saturated $Mg(NO_3)_2$ solution).

3.2.4.1 X-ray diffraction (XRD)

X-ray diffraction patterns were detected using Cu Kα radiation, generated with X'pertPro MPD (Philips, Netherlands) at 40 kV, 20 mA. The X-ray beam was operated in reflection mode and the samples were examined over the angular range (2θ) of 10° to 45° with a step size of 0.02° and a count time of 4s per point.

3.2.4.2 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was performed using a JEOL, model JEM-2010, equipped with a digital Bioscan (Gatan) image acquisition system at 80 kV. One drop (8 μL) of 0.002% aqueous suspension of BCNWs was allowed to dry on a carbon coated grid (200 mesh). The nanocrystals were stained with uranyl acetate. The reported dimension of BCNWs was averaged from measurements of several TEM micrographs.

3.2.4.3 Thermal gravimetric analysis (TGA)

Thermal analysis was carried out using a Mettler Toledo TGA/SDTA STAR 851e (Switzerland). Samples of approximately 5 mg were used. All the experiments were conducted using the constant heating rate of 5°C/min, from 25 to 600 °C, under a nitrogen atmosphere (flow rate of 50 ml/min). The peak degradation temperatures of all samples were determined.

3.2.4.4 Scanning electron microscopy (SEM)

Scanning electron microscopy micrographs of the fracture surfaces of the pure starch film and starch/BCNWs bionanocomposite films were taken by a scanning electron microscope JEOL model jms-5410 LV at an accelerating voltage of 10 kV. Prior to the examination, the surface of specimens was sputter coated with a thin layer of gold.

3.2.4.5 Mechanical test

Specimens with the dimension of 50 mm length and 7 mm width were cut from the films. The test was operated at a deformation rate of 3 mm/min using a load cell of 1 kN (Universal Testing Machine, INSTRON Model 5566) with an initial grip separation of 30 mm. The average values of tensile strength, Young's modulus, and elongation at break were calculated from 5 specimens.

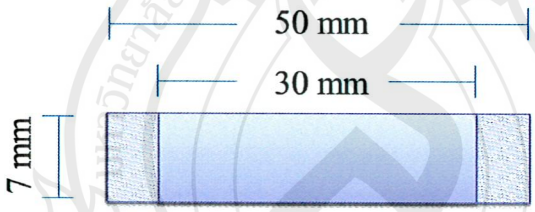

Figure 3 - 4 Illustration of tensile specimens.

3.2.4.6 Moisture absorption

Firstly, bionanocomposite specimens (dimension of 40 mm × 10 mm) were dried and then weighted (M_0). After that, the specimens were stored in condition of 75% RH (using a chamber of the saturated NaCl solution), and periodically removed and weighted (M_t). A minimum of four samples were tested for each film. Moisture absorption (M_a) at time t was calculated by the following equation:

$$M_a = \left(\frac{M_t - M_0}{M_0} \right) \times 100 \quad (3.3)$$

where M_0 is the specimen initial weight and M_t is the weight after a time t.

3.2.5 Statistical and data analysis

Microsoft excel 2010 was used for calculating ANOVA and Duncan's new multiple range test for independent samples. A p value ≤ 0.05 was considered statistically significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results and Discussion: Part A

4.1.1 Study the effect of type of carbon source in the cultured medium of bacterial cellulose on its structure and mechanical properties

In the previous work (the last year research in 2552-2553), the effect of type of carbon source in the cultured medium of bacterial cellulose (BC) on its production yield was investigated. This would suggest us which type of carbon suits in term of cellulose yield and the cost of production because carbon is key precursor in cell growth and bacterial cellulose synthesis. Here, we used monosaccharide (glucose and fructose), disaccharides (sucrose and lactose) and alcohol (glycerol and manitol) since they showed highly effect on bacterial cellulose production (Jonas and Farah 1998; Ramana et al. 2000; Bielecki et al. 2004; Panesar et al. 2009). For example, Ramana and his co-workers reported that among the carbon sources, mannitol was one that found to be suitable for optimum levels of cellulose production (Ramana et al. 2000).

Table 4 - 1 Yield (g/L of medium), relative yield, crystallinity index (%) and crystallite size (nm) of bacterial cellulose (BC) samples cultivated in medium of various types of carbon source.

Type of carbon source	Sucrose (control)	Glucose	Fructose	Glycerol	Mannitol
Yield (g/L of medium)	0.33	0.18	1.15	1.03	1.55
Relative yield (as compared to control)	1	0.57	3.53	3.16	4.76
Crystallinity index (%)	86.21	88.10	86.83	81.96	87.03
Crystalline size (nm)	6.70	7.01	6.70	6.86	6.86

In this result, it was found that the type of carbon source significantly influenced the production yield of bacterial cellulose (BC) as shown in Table 4 - 1. Mannitol, fructose and glycerol were proven to be the more efficient carbon source than sucrose and glucose in view of the production yield of bacterial cellulose (BC). Additionally, in this present work, the effect of type of carbon source in the cultured medium of bacterial cellulose on its structure and mechanical properties was examined.

The cultured BC pellicle from the medium of each carbon source was firstly cut and disintegrated by a kitchen blender and further fabricated into a sheet, so called 'disintegrated bacterial cellulose sheet' (dis-BC sheet). In order to examine the mechanical properties of the dis-BC sheet, the tensile test was performed and the tensile strength (MPa), Young's modulus (GPa) and elongation at break (%) of the dis-BC samples were then calculated as shown in Figure 4 - 1 – 4 - 3.

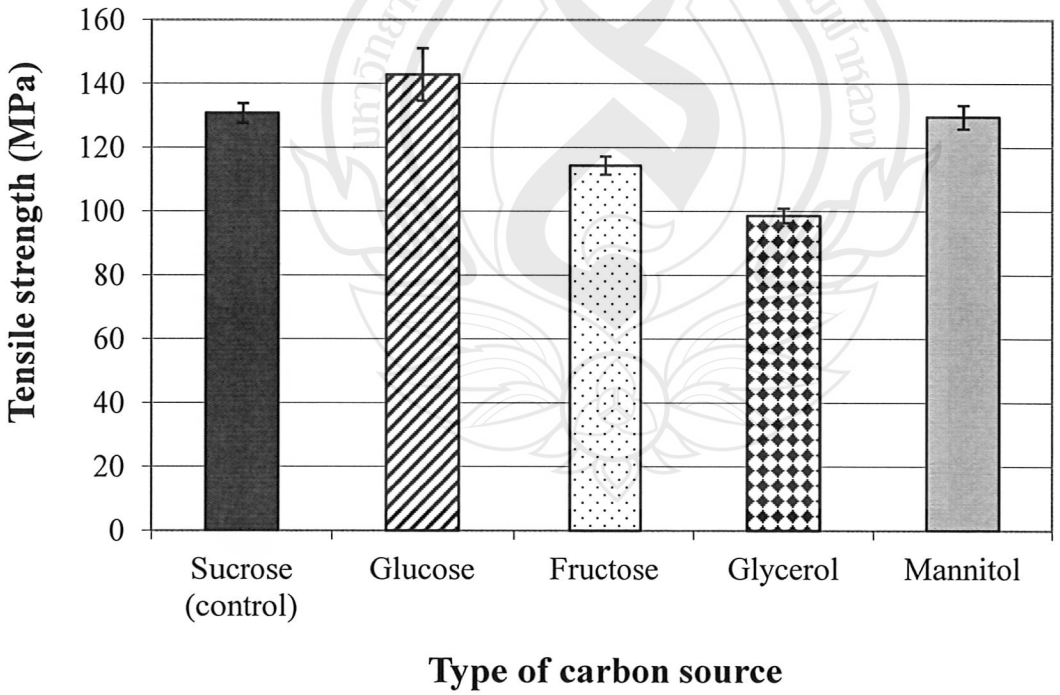

Figure 4 - 1 The effect of type of carbon source (used in the culture medium of bacterial cellulose) on tensile strength of the disintegrated bacterial cellulose sheets.

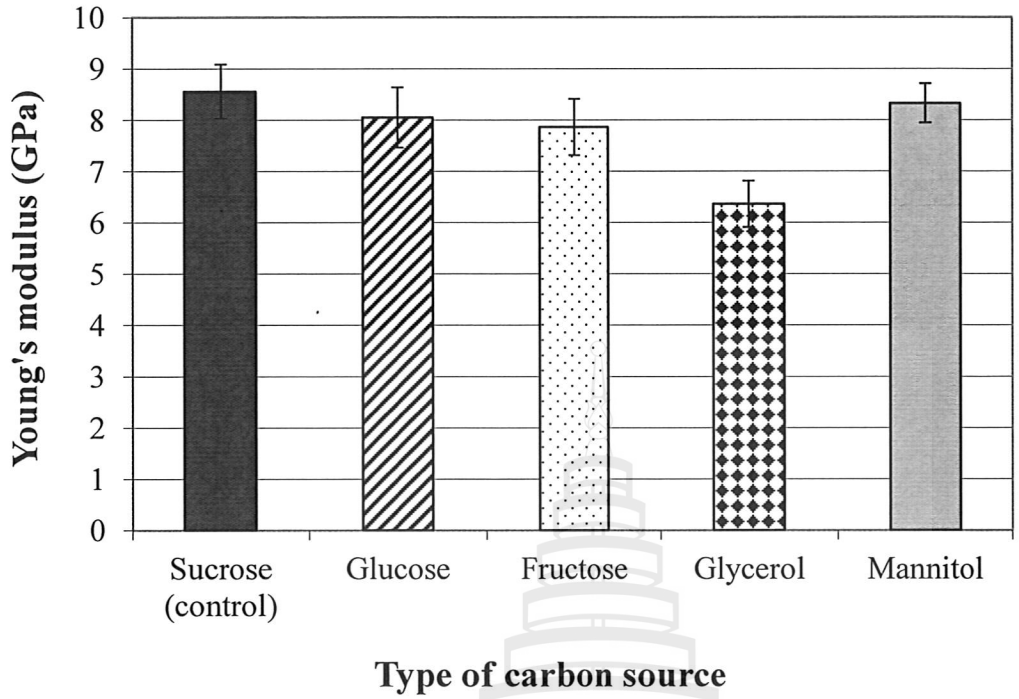

Figure 4 - 2 The effect of type of carbon source (used in the culture medium of bacterial cellulose) on Young's modulus of the disintegrated bacterial cellulose sheets.

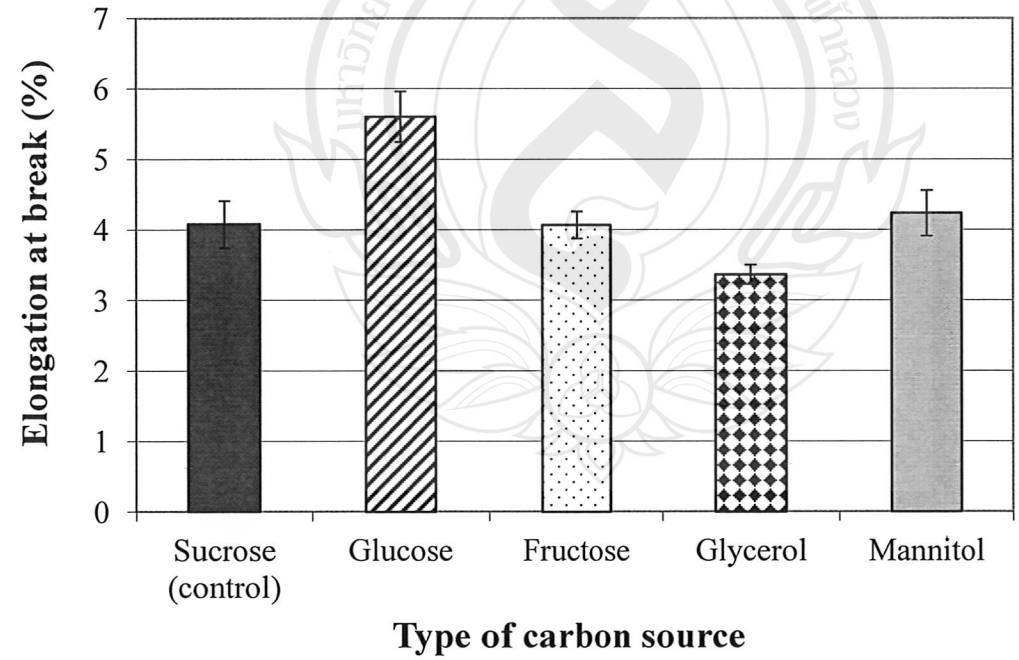

Figure 4 - 3 The effect of type of carbon source (used in the culture medium of bacterial cellulose) on elongation at break of the disintegrated bacterial cellulose sheets.

From the results, it can be concluded that the type of carbon source used in the cultured medium of bacterial cellulose (BC) had an influence on the mechanical properties of the dis-BC sheets. In Figure 4.1, the highest tensile strength was observed in the sheet prepared from BC that was cultured in the glucose medium ($p > 0.05$). But the sheet made from BC cultured in the glycerol medium was the weakest one. The statistical and data analysis also suggested that the sheets prepared from BC cultured in the sucrose and mannitol media were not significantly different in the tensile strength ($p < 0.05$). For the Young's modulus (Figure 4.2), the sheet prepared from BC cultured in the glycerol medium again showed the lowest value. The other sheets made from BC cultured in the sucrose, glucose, fructose and mannitol media have no significant difference in the Young's modulus ($p < 0.05$). As seen in Figure 4.3, the sheet prepared from BC cultured in the glycerol medium also exhibited that lowest elongation at break. The highest value was found in the sheet made from BC cultured in the glucose medium. No significant difference was observed in the elongation of the three other sheets prepared form BC cultured in the sucrose, fructose and mannitol media ($p < 0.05$). This difference in the mechanical properties of the dis-BC sheets implied to a possible difference existing in the BC fiber's structure and property which caused from the culture of BC in the media of different type of carbon sources.

SEM cannot distinguish differences such as amorphous regions from crystalline regions in the BC fibers. Nevertheless, the detail of their structural differences can be obtained from the X-ray diffraction (XRD) technique. The crystallinity index (%) and crystallite size (nm) of BC were calculated from each X-ray diffraction pattern of the dis-BC sheet prepared from BC cultured in the medium of each type of carbon sources (see Figure 4 - 5) and shown in Table 4 - 1. From the

data, it can be summarized that the type of carbon source used in the cultured medium of BC rather had an effect on the crystallinity index (%) and crystallite size (nm) of the resulting BC fibers. The values of crystallinity index (%) and crystallite size (nm) of BC evaluated in this work are similar to those reported in several previous studies (Castro et al., 2011; Shezad et al., 2010; Sheykhnazari et al., 2011).

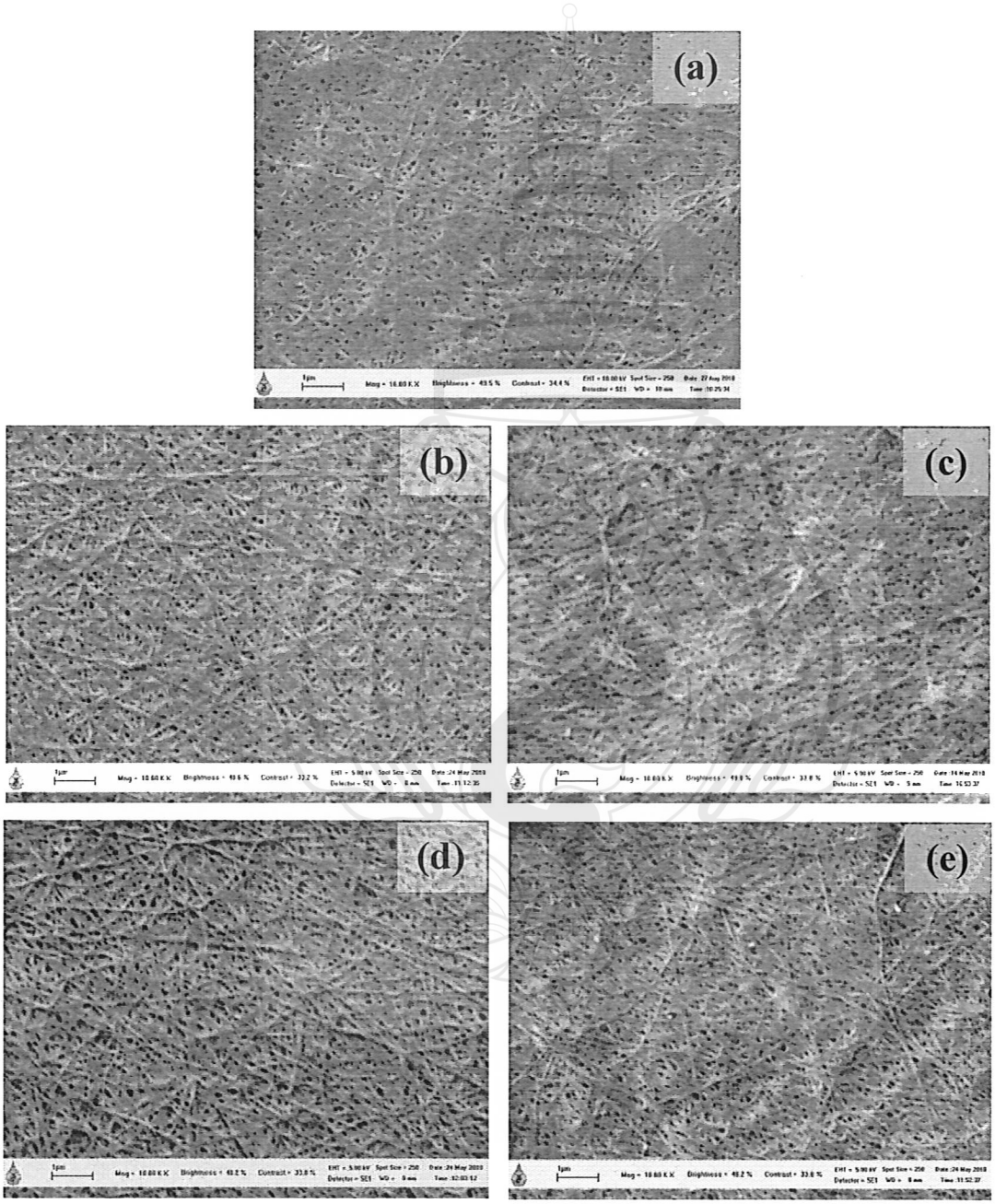

Figure 4 - 4 Scanning electron micrographs of the disintegrated bacterial cellulose sheets prepared from bacterial cellulose cultured in the medium of various carbon sources: (a) sucrose; (b) glucose; (c) fructose; (d) glycerol and (e) mannitol.

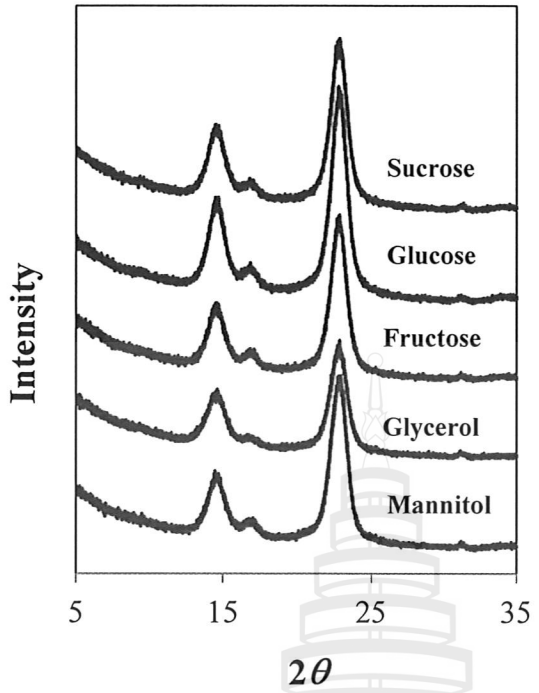

Figure 4 - 5 X-ray diffraction patterns of the disintegrated bacterial cellulose sheets prepared from bacterial cellulose cultured in the media of various carbon sources.

Comparing the XRD data (Table 4 - 1) together with the results of tensile testing (Figure 4 - 1 – 4 - 3), it can be observed that there is a strong relationship between the crystallinity index of BC and mechanical properties of the dis-BC sheets, especially in trends of tensile strength and elongation at break of the samples. The higher crystallinity index or the more structural order of BC fibers resulted in the better mechanical properties of the dis-BC sheets (Watanabe et al., 1998). Although, the relationship between crystallite size of BC and the mechanical properties of the dis-BC sheets cannot be clearly concluded.

4.1.2 Investigate the effect of additional supplements in the cultured medium of bacterial cellulose on its production yield

In this work, we interested to add pineapple or coconut juice in culture media because the composition of the juices have been reported that contains rich

component such as free sugars content (mono- and disaccharides), trace element (magnesium), vitamins (such as nicotinic acid, biotin and pyridoxin) and some hormones (Hui and Muhamad 2007). These compounds play a crucial role in cellulose synthase gene activity i.e. promoter and transcription factors (Heo and Son, 2002). However, other juices were also investigated but low yield and costly were found. So that both pineapple and coconut juice would be used as supplement rely on the complex of nutrient and cheaper compare to another one.

The effect of additional supplements (i.e. pineapple and coconut juices) in the cultured medium of bacterial cellulose on its production yield was investigated. *Acetobacter xylinun* were grown in culture media containing 50g of sucrose, 5g of yeast extract, 5g of $(NH_4)_2SO_4$, 3g of KH_2PO_4, 0.05g of $MgSO_4$ and supplied with either pineapple or coconut juice (varied from 10, 20, 30, 50, 70 and 100% (v/v)) at the initial adjusted pH of 5.0 and 30°C in static culture condition. After cultivation for 7 days, bacterial cellulose pellicle was collected, purified and dried in order to determine the production yield of bacterial cellulose from each culture medium. The relative yield of BC cultured from each medium was also calculated in comparison to the yield of BC obtained from 'the control media' (0% addition or no juice added media).

The effect of amount of added pineapple and coconut juices in the culture medium (%v/v) on relative yield of the BC production are shown in Figure 4 - 6 and 4 - 7, respectively. It was found that the addition of pineapple juice of 30% v/v and coconut juice of 50% v/v were the optimum amount to supply into the culture media and the highest bacterial cellulose productivity, at approximately 2-fold increase in yield as compared to 'the control media', was obtained ($p > 0.05$).

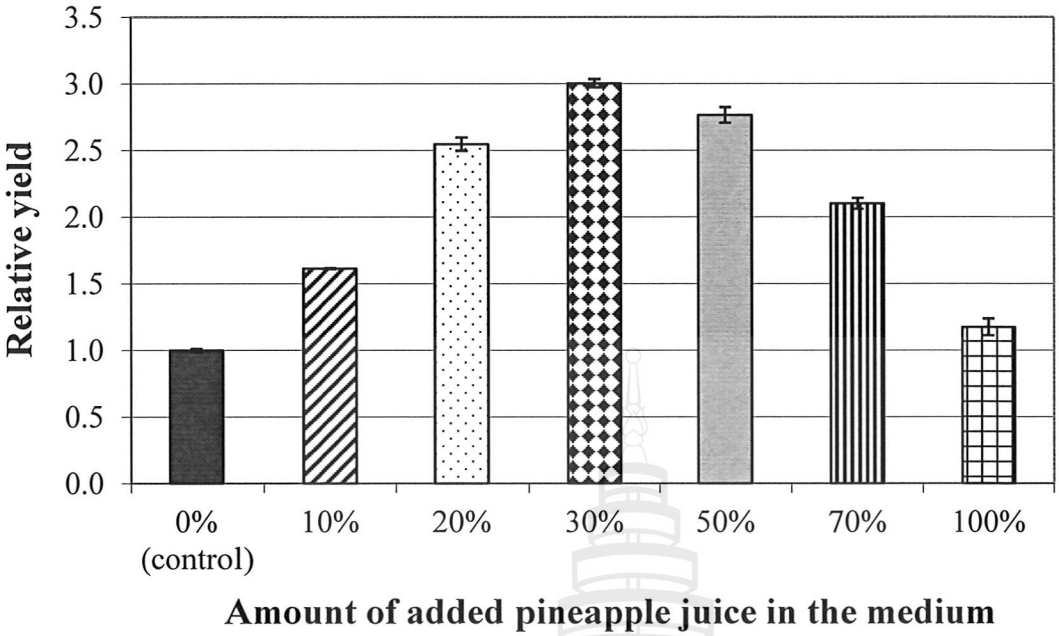

Figure 4 - 6 The effect of amount of added pineapple juice in the culture medium (%v/v) on relative yield of the bacterial cellulose (BC) production.

This result suggested that pineapple and coconut juices are the effective supplement sources that can be used in bacterial cellulose production. Since the composition of juices has been identified that they contain various carbon and nitrogen sources, trace elements (e.g. magnesium and manganese), vitamins and growth hormones, these would undoubtedly affect cell activity and cellulose yield in *A. xylinum* cultivation (Kurosumia et al., 2008). For example, magnesium (Mg^{2+}) or manganese (Mn^{2+}) ion as a cofactor is necessary for enzyme activity or control regulatory gene in cellulose biosynthesis e.g. glucosyltransferases and cellulose synthase enzymes (Ross et al., 1987; Jonas and Luiz, 1998; Vandamme et al., 1997).

In addition, the magnesium (Mg^{2+}) ion plays an important role in maintaining cellular metabolism for BC synthesis of *Acetobacter* strains (Heo and Son, 2002). The vitamins, pyridoxine, nicotinic acid and biotin are also the influence of cell growth and cellulose production. The other trace elements (e.g. acetate, citrate and succinate) showed highly effective in stimulating cellulose synthesis by *A.*

acetinenum in defined medium (Dudman, 1959). These compounds and other intermediates of the tricarboxylic acid cycle are all readily oxidized to CO_2 by washed suspensions of *A. xylinum*. The buffering property of these substances can maintain the cultural pH value within the optimum range for cellulose synthesis (Schramm et al., 1957).

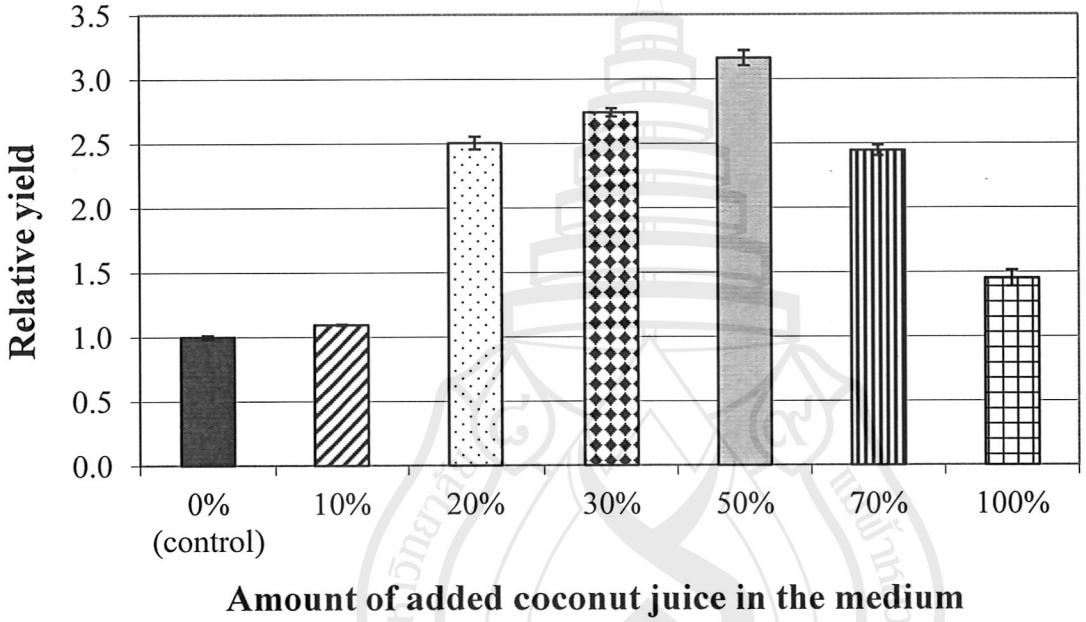

Figure 4 - 7 The effect of amount of added coconut juice in the culture medium (%v/v) on relative yield of the bacterial cellulose (BC) production.

Furthermore, since nitrogen is the main component of proteins and necessary in cell metabolism, additional nitrogen sources from juices could probably help to promote cell metabolism in cellulose synthesis by *A. xylinum* (Chawla et al., 2009). On the other hand, carbons source is a key component necessary for both cell growth and cellulose production. Because of additional various sugars in juices supplemented in culture media, this may increase cell activity and the synthesis to have more amount of a building block to produce cellulose. Consistently, this present

work showed that addition of optimum amounts of juices in the cultured medium of bacterial cellulose positively affected bacterial cellulose yield (i.e. addition of 10-30% v/v of pineapple juice and 10-50% v/v of coconut juice). In contrast, at high amount of juices, it caused the reduction in cell growth and cellulose production (see Figure 4 - 6 and 4 - 7). Here, more free sugars content (either mono or disaccharides) can create saturated carbon source environment that may directly inhibit cell activity (Kurosumi et al., 2009) and consequently, cellulose yield (i.e. the cultured medium supplied with pineapple juice of 50% v/v and higher and coconut juice of 70% v/v and higher). In addition, the statistical and data analysis suggested that the bacterial cellulose yields from the cultured medium supplied with coconut juice of 20% v/v and 70% v/v were not significantly different ($p < 0.05$).

In comparison between both supplements (pineapple and coconut juices), the results suggested that both supplements can effectively improve the bacterial cellulose yield but pineapple juice is a slightly more effective supplement because at low amount of juice addition (i.e. 10-30% v/v), the higher yield was obtained in this media. This described that pineapple juice may have richer and higher concentration of components including carbon and nitrogen sources, trace elements, vitamins and growth hormones, to promote cell growth and bacterial cellulose production by *Acetobacter xylinum*.

4.2 Results and Discussion: Part B

4.2.1 Effect of hydrolysis time on properties of bacterial cellulose nanowhiskers

In this research, bacterial cellulose nanowhiskers (BCNWs) were prepared by acid hydrolysis of the bacterial cellulose (BC) sheet using 50% (w/v) sulfuric acid

at 50°C. The effect of hydrolysis time of 24, 48 and 72 hours on the BCNWs' properties was studied. Figure 4 - 8 shows that yield (%) of the obtained BCNWs was decreased from 47.28±1.57% to 24.97±0.64% with increasing the hydrolysis time from 24 to 72 hours. The long the hydrolysis times, the more BC was hydrolyzed.

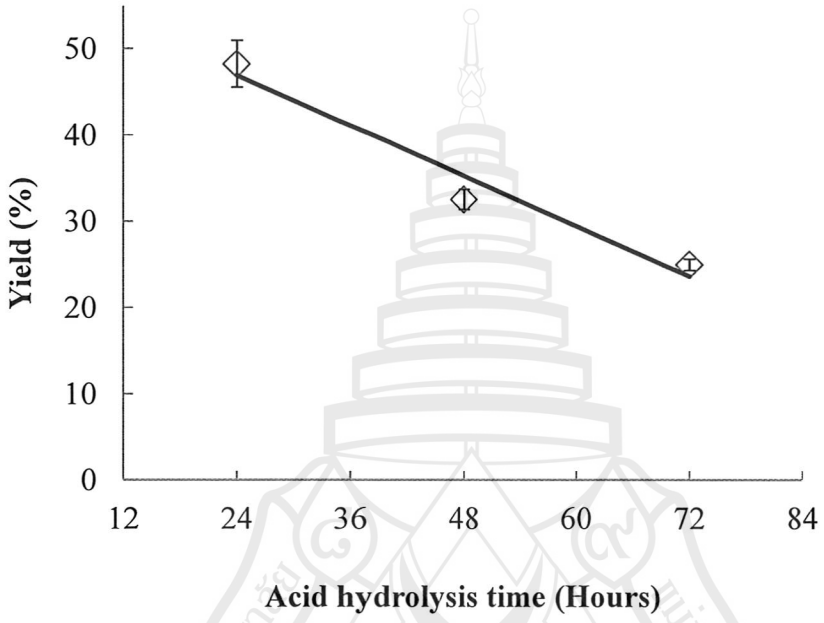

Figure 4 - 8 The effect of acid hydrolysis time on yield (%) of the bacterial cellulose nanowhiskers (BCNWs).

The effect of hydrolysis time on the crystallinity of the BCNWs is presented in Figure 4 - 9. The X-ray diffractions of the native BC and BCNWs after acid hydrolysis of 24, 48 and 72 hours (BCNWs 24h, BCNWs 48h and BCNWs 72h, respectively) show three cellulose I characteristic peaks at 2θ = 14.7°, 16.4°, and 22.5° (corresponding to 101, 10 $\bar{1}$ and 002 crystal planes, respectively) (Lu & Hsieh, 2010). After short acid hydrolysis time of 24 hours, the peaks of BCNWs 24h diffraction patterns are sharper than that of the native BC because some of the amorphous regions which are more accessible than crystalline regions have been

removed from the BC structure. As time passes to 48 hours the peaks of BCNWs 48h become sharper than BCNWs 24h.

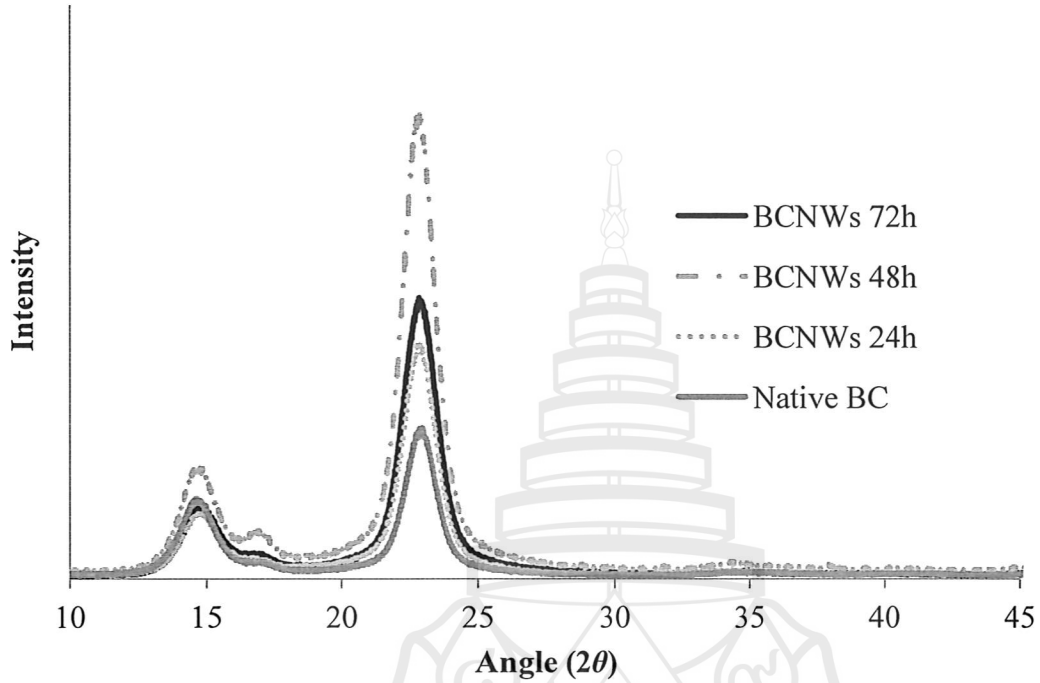

Figure 4 - 9 X-ray diffraction patterns of the native bacterial cellulose (BC) and the obtained nanowhiskers (BCNWs) after acid hydrolysis time of 24, 48 and 72 hours (BCNWs 24h, BCNWs 48h and BCNWs 72h, respectively).

This indicates that 24 hours are not long enough for the acid to extract crystalline domains on BC structure. As time passes to 72 hours the peaks of BCNWs 72h tend to decline. With long hydrolysis times, amorphous regions have been largely eliminated then the acid attacked further into the crystalline regions. From the results of this study, therefore, the acid hydrolysis time of 48 hours was chosen for the preparation of BCNWs to use as a reinforcement in the next part.

The morphology of the BC and BCNWs was studied by TEM. BC morphology showed continuous networks (Figure 4 - 10a). After acid treatment, morphology of BCNWs showed rod-like shapes as shown in Figure 4 - 10b. Diameters and lengths of BCNWs 48h were estimated from several measurements on

TEM micrographs. The averaged diameter and length of the BCNWs 48h were approximately 28.18±1.99 nm and 637.61±147.10 nm, respectively.

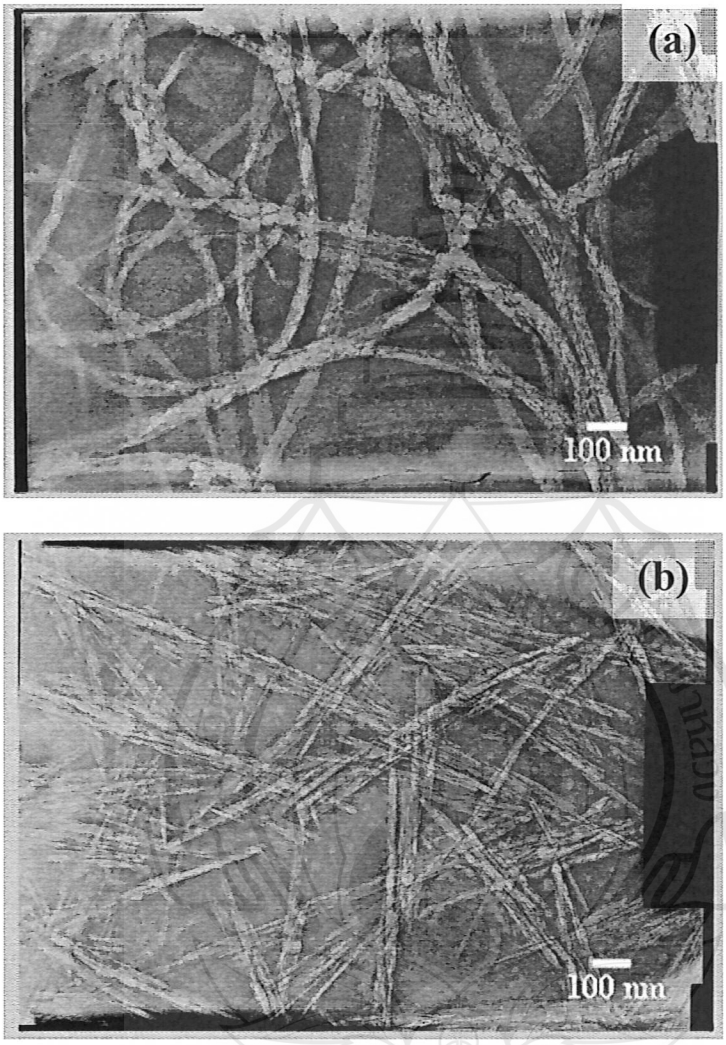

Figure 4 - 10 Transmission electron micrographs of (a) the native BC and (b) bacterial cellulose nanowhiskers after acid hydrolysis with 50% w/v sulfuric acid for 48 hours (BCNWs 48h).

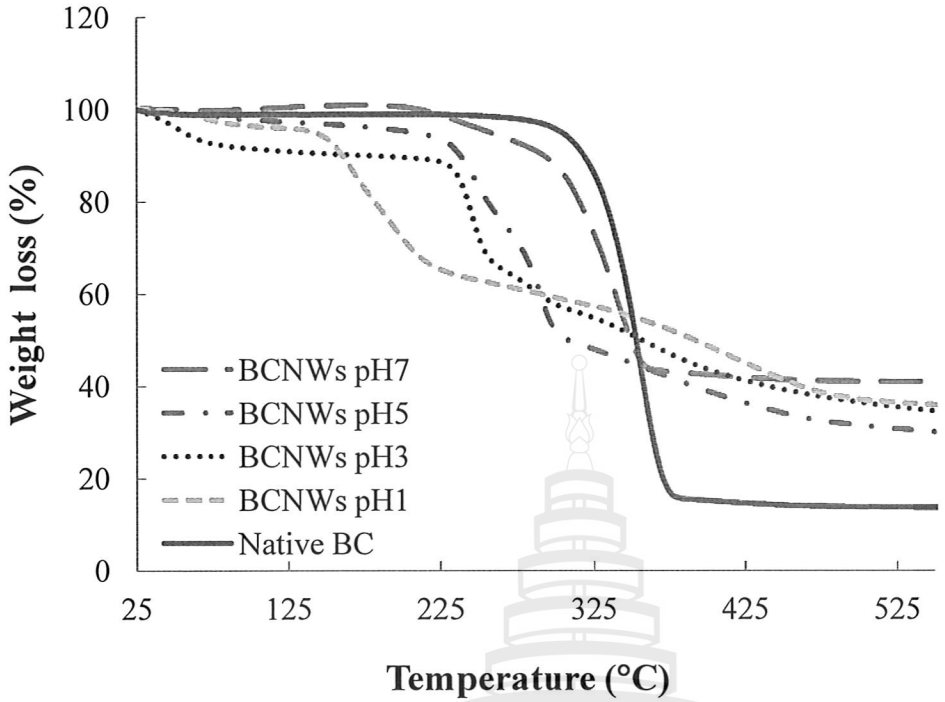

Figure 4 - 11 TGA curves of the native BC, BCNWs after acid hydrolysis time of 48 hours (BCNWs pH1) and BCNWs 48 hours with pH adjusted to 3, 5 and 7 (BCNWs pH3, BCNWs pH5 and BCNWs pH7, respectively).

Sulfuric acid introduced sulfate groups on the nanowhisker's surfaces due to the acid hydrolysis. The surface charges on BCNWs led to their effective separation for reinforcing in composites, however, this decreased thermal stability of BCNWs. Thermogravimetric analysis was carried out to investigate the effect of BCNWs' pH on their thermal stability. Figure 4 - 11 shows TGA curves of the native BC, BCNWs obtained after 48 hours of acid hydrolysis (BCNWs pH1) and BCNWs 48h with pH adjusted to 3, 5 and 7 (BCNWs pH3, BCNWs pH5 and BCNWs pH7, respectively).

After acid hydrolysis treatment of 48 hours thermal stability of BCNWs pH1 was greatly depressed. Sulfate group is a well-known decomposition catalyst of cellulose and also facilitates that formation of char residue (Kim, Nishiyama, Wada and Kuga, 2001). To improve thermal stability of BCNWs (Roman and Winter,

2004), pH of BCNWs was adjusted by NaOH in order to remove the sulfated group on surface of the BCNWs (Favier et al., 1995). After adjusting pH of BCNWs to 3, 5 and 7, thermal stability of the BCNWs was gradually increased. Table 4 - 1 shows the peak degradation temperatures of the native BC and BCNWs with different pHs obtained from the DTG curves. It confirmed the increase in thermal stability of BCNWs with degree of pH adjustment.

From Figure 4 - 12, the native BC and BCNWs pH7 shows approximately one step of their degradations. However, the degradation steps of BCNWs pH1, pH3 and pH5 are obviously divided into two steps. It was explained that the first step corresponds to the degradation of the more accessible regions (amorphous regions), which are highly sulfated, and the second step corresponds to the breakdown of the crystalline fraction, which has been attacked by sulfuric acid (Julien, Chornet and Overend, 1993; Sanz et al., 2011).

4.2.2 Effects of bacterial cellulose nanowhiskers' pH and content on bionanocomposite films

The X-ray diffraction patterns of the corn starch granule, pure starch film and bionanocomposite films are shown in Figure 4 - 13. The diffraction peaks of the corn starch granular at 2θ of 15°, 17°, 18° and 23° are related to its A-type crystalline structure. The peaks of starch film diffractograms are lower than the native corn starch because after gelatinization, crystallinity was decreased (Grande et al., 2009). For the bionanocomposite films, during preparation, it was found that the films with addition of BCNWs pH1 could not be obtained because all films were found to be cracked after drying.

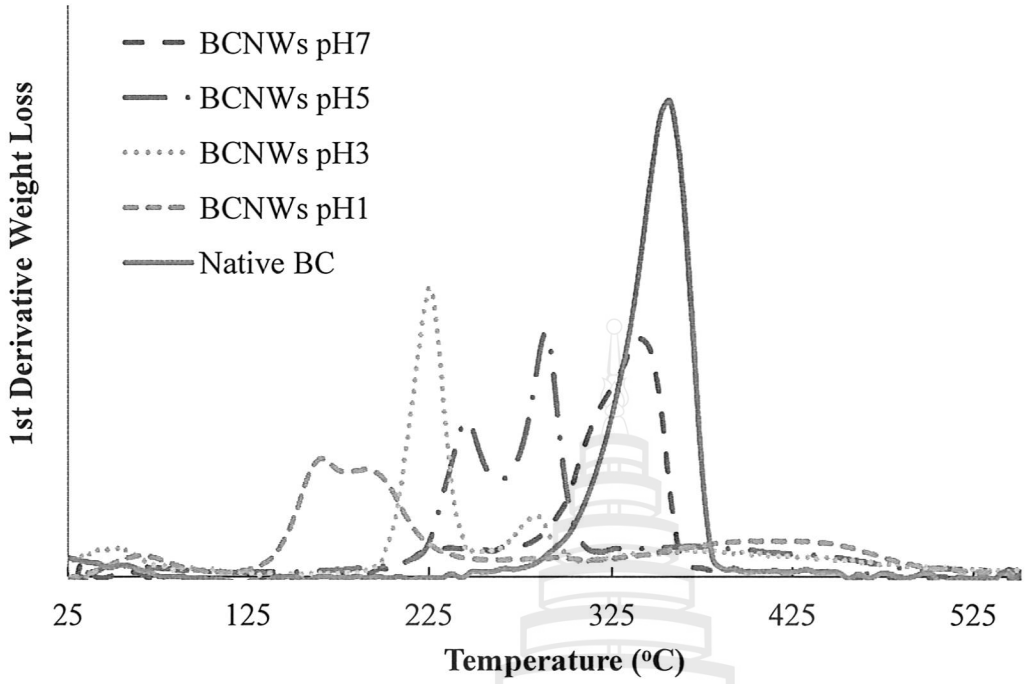

Figure 4 - 12 DTG curves of the native BC, BCNWs after acid hydrolysis time of 48 hours (BCNWs pH1) and BCNWs 48 hours with pH Adjusted to 3, 5 and 7 (BCNWs pH3, BCNWs pH5 and BCNWs pH7, respectively).

With addition of BCNWs pH3, BCNWs pH5 and BCNWs pH7, to the varied contents of 1, 5, and 10 wt%, their diffraction peaks obviously showed also three cellulose I characteristic peaks at 2θ = 14.5°, 16.4°, and 22.5°, the contents of 5 and 10 wt% in particular. This indicated that the crystalline structure of the BCNWs was well-preserved in the bionanocomposites films. With increasing BCNWs content the magnitude of the cellulose I peaks are observed to increase.

Figure 4 - 14 shows the smooth fracture surface of the pure starch film. For the starch/BCNWs pH3 films with increasing BCNWs content, the fracture surface becomes rougher. From the SEM images, a good dispersion of BCNWs on fractured surface of these bionanocomposite films was observed (Figure 4 - 15a, b and c).

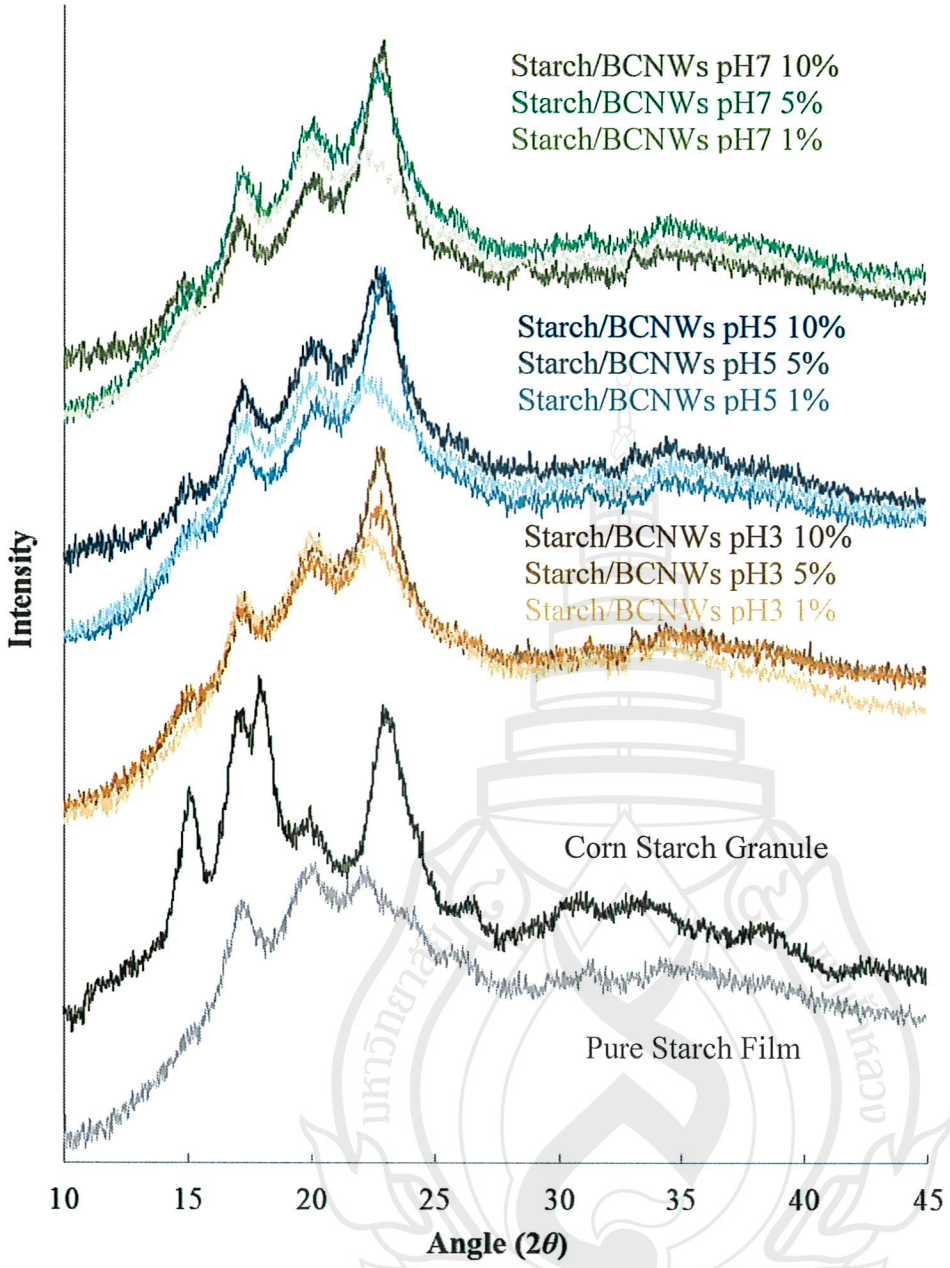

Figure 4 - 13 X-ray diffraction patterns of the corn starch granule, pure starch film and bionanocomposite films reinforced with BCNWs of pH 3, 5 and 7 (with varied contents of 1, 5 and 10 wt%).

The starch/BCNWs pH5 films with varied BCNWs contents of 1, 5 and 10 wt%, show rougher surface than the starch/BCNWs pH3 films. Formation of some aggregates BCNWs was found on the films; fracture surfaces (Figure 4 - 15a, b and c). For the starch/BCNWs pH7 films, a poor dispersion and high aggregation of

BCNWs was observed clearly at all BCNWs contents, particularly at 10 wt% (Figure 4 - 16a, b and c). It was obvious that the higher the pH, the lower degree of BCNWs dispersion in the bionanocomposite films was obtained. This due to the effect of removal of the sulfate group on surface of BCNWs. The dispersion of BCNWs in the matrix and their compatibility are important for a reinforcing capability and consequently, improvement in the properties of composite materials (Trovatti et al., 2012).

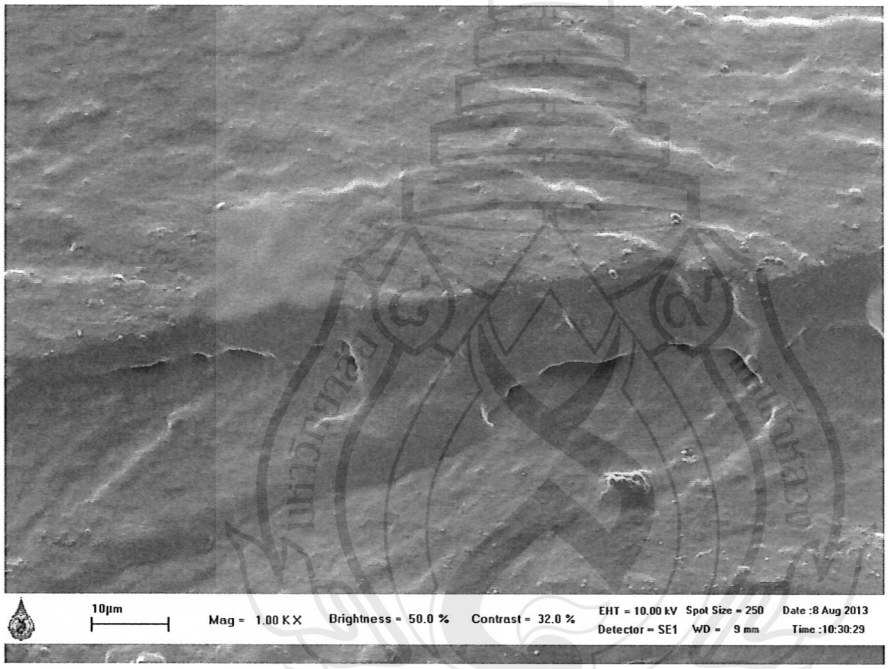

Figure 4 - 14 SEM image of fracture surface of the pure starch film.

Figure 4 - 18 shows the tensile properties of the pure starch film and bionanocomposite films reinforced with BCNWs of pH 3, 5 and 7 (with varied contents of 1, 5 and 10 wt%). It was found that the mechanical properties of the starch/BCNWs pH 3 which possessed a good dispersion of BCNWs was not improved in both strength and modulus as compared to the pure starch film ($p < 0.05$). This is possibly due to a poor interaction between the BCNWs and pure starch matrix which

is likely caused by the sulfate groups on the surface of BCNWs. On the other hand, the modulus of the starch/BCNWs pH 5 and starch/BCNWs pH 7 films with 5 wt% and 10 wt% contents were shown to significantly improve as compared to the starch film ($p > 0.05$). However, the strength of these films was not statistically different ($p < 0.05$). For the elongation at break, there were two films, the starch/BCNWs pH 3 with 10 wt% content and starch/BCNWs pH 7 with 10 wt% content, which exhibited significantly lower values than that of the starch film ($p > 0.05$). At high BCNWs content, the large agglomerations and poor dispersion of BCNWs within the pure starch matrix is likely to occur, leading to a premature failure of the materials. For mechanical properties improvement in composite systems both dispersion and interfacial adhesion are essential (Cao et al., 2008).

Though starch has been considered as one of the most promising materials for biodegradable plastics owing to its natural abundance and low cost, poor resistance to moisture absorption limits its wide applications. It is well known that addition of fillers is an effective way of decreasing its sensitivity to moisture and thus improving mechanical properties stability (Wan et al., 2009). Figure 4 - 19 shows the moisture absorption of the pure starch film and bionanocomposite films during conditioning in 75% RH as a function of time. The moisture absorption of pure starch film at equilibrium was 22.83%. With addition of BCNWs into the pure starch film, the moisture absorption at equilibrium was decreased as the BCNWs content increased from 1 to 10 wt%.

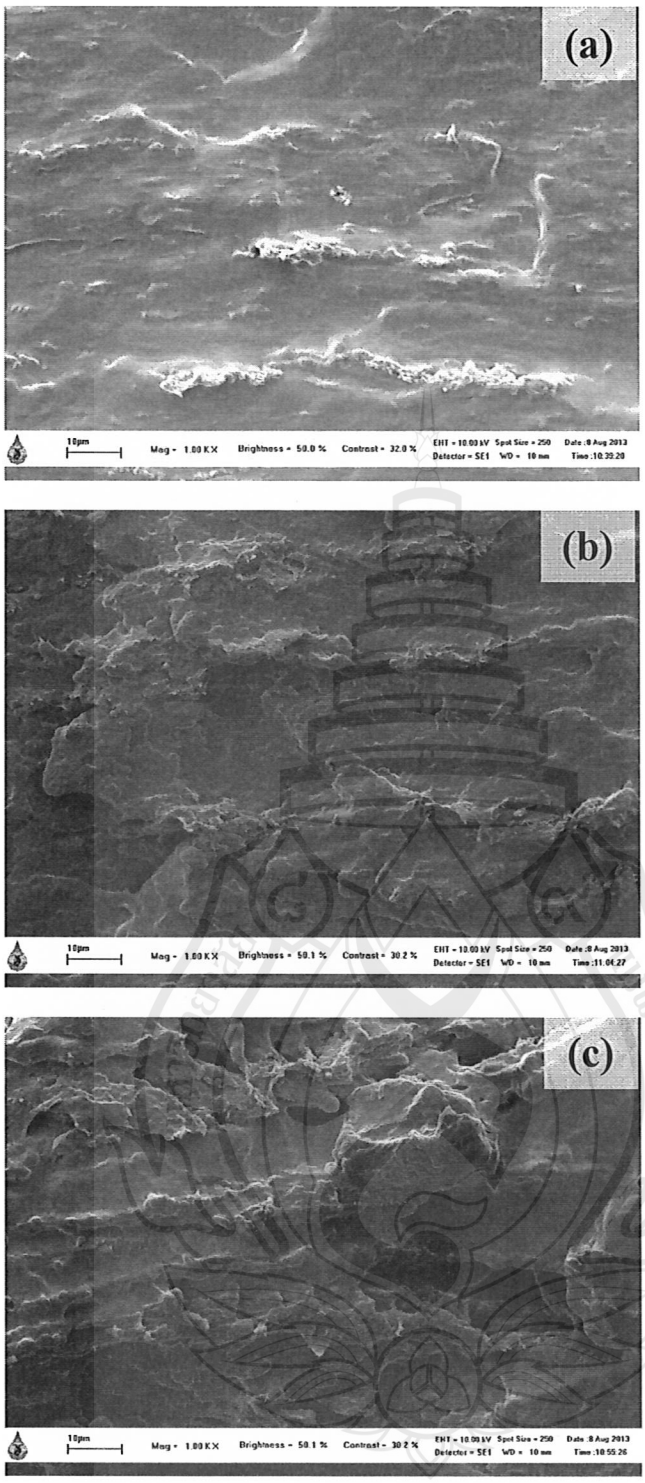

Figure 4 - 15 SEM images of fracture surfaces of bionanocomposite films with addition of 1 wt% (a), 5 wt% (b) and 10 wt% (c) of BCNWs pH3.

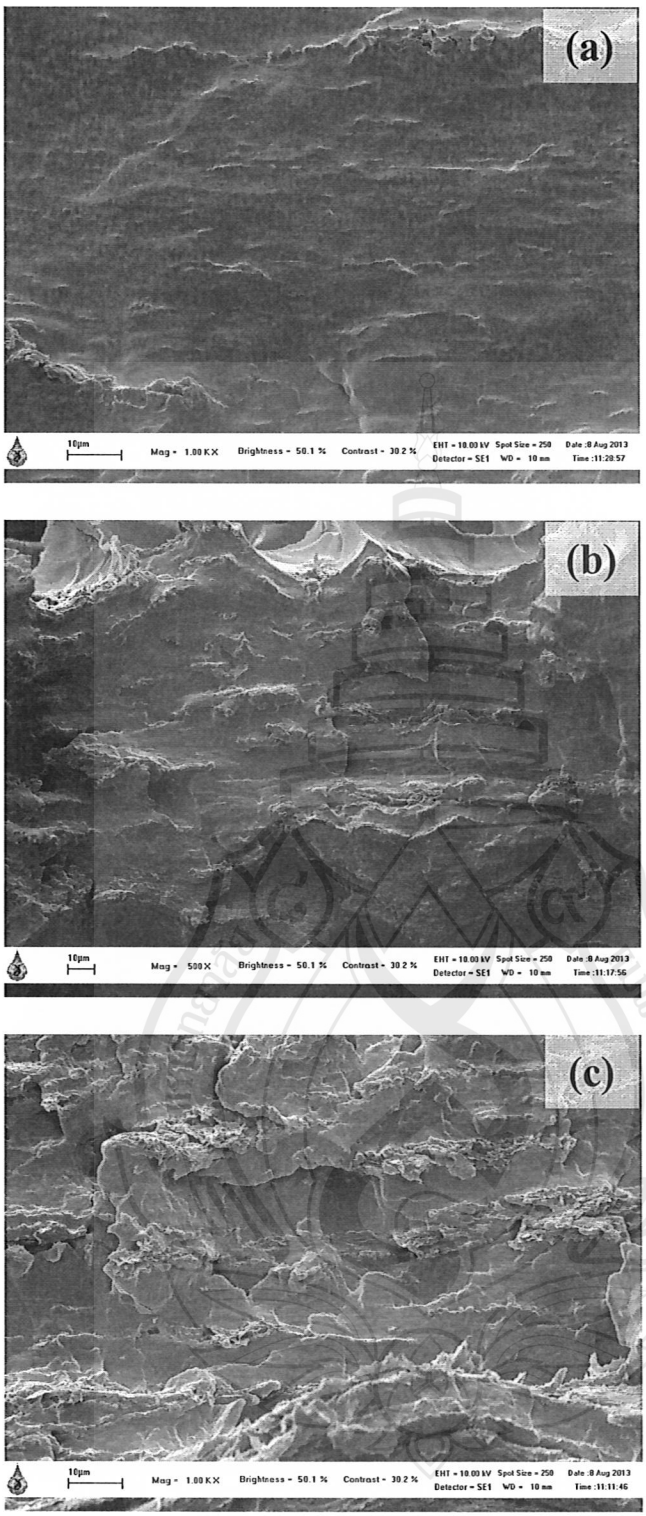

Figure 4 - 16 SEM images of fracture surfaces of bionanocomposite films with addition of 1 wt% (a), 5 wt% (b) and 10 wt% (c) of BCNWs pH5.

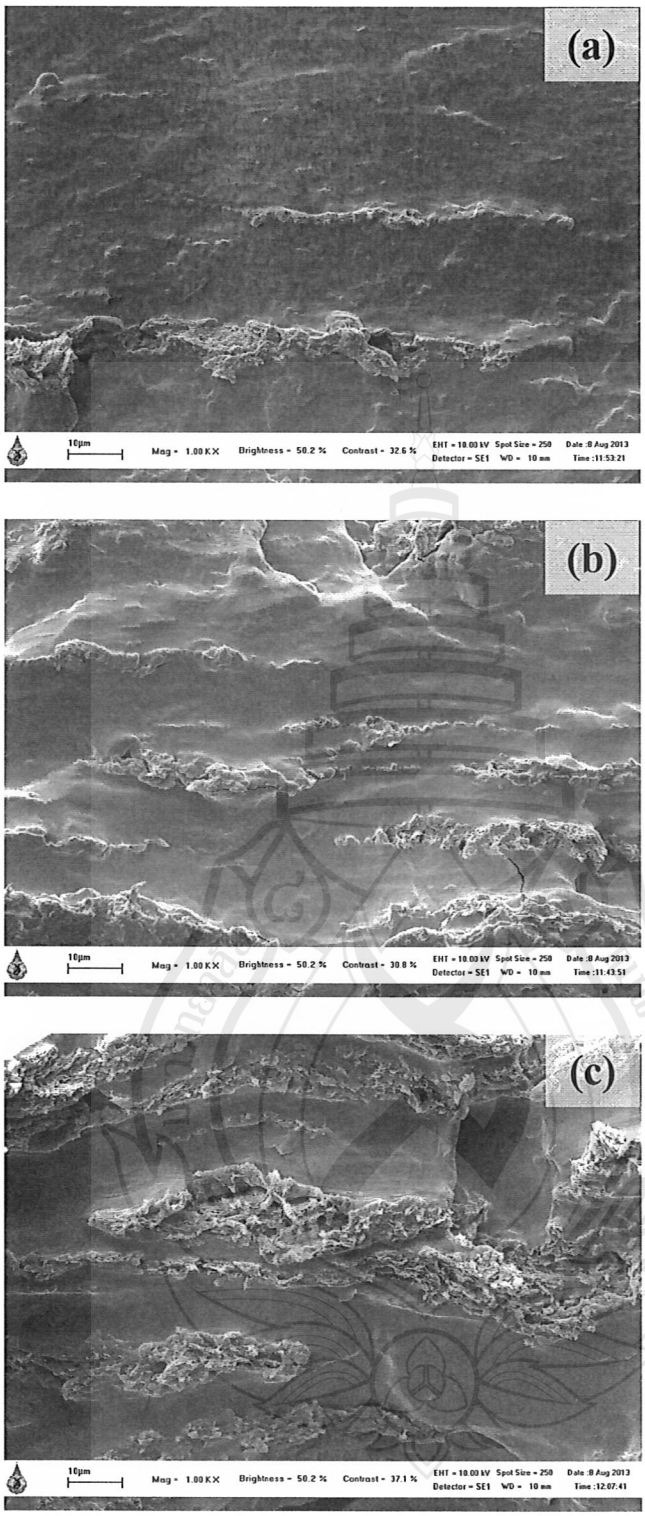

Figure 4 - 17 SEM images of fracture surfaces of bionanocomposite films with addition of 1 wt% (a), 5 wt% (b) and 10 wt% (c) of BCNWs pH7.

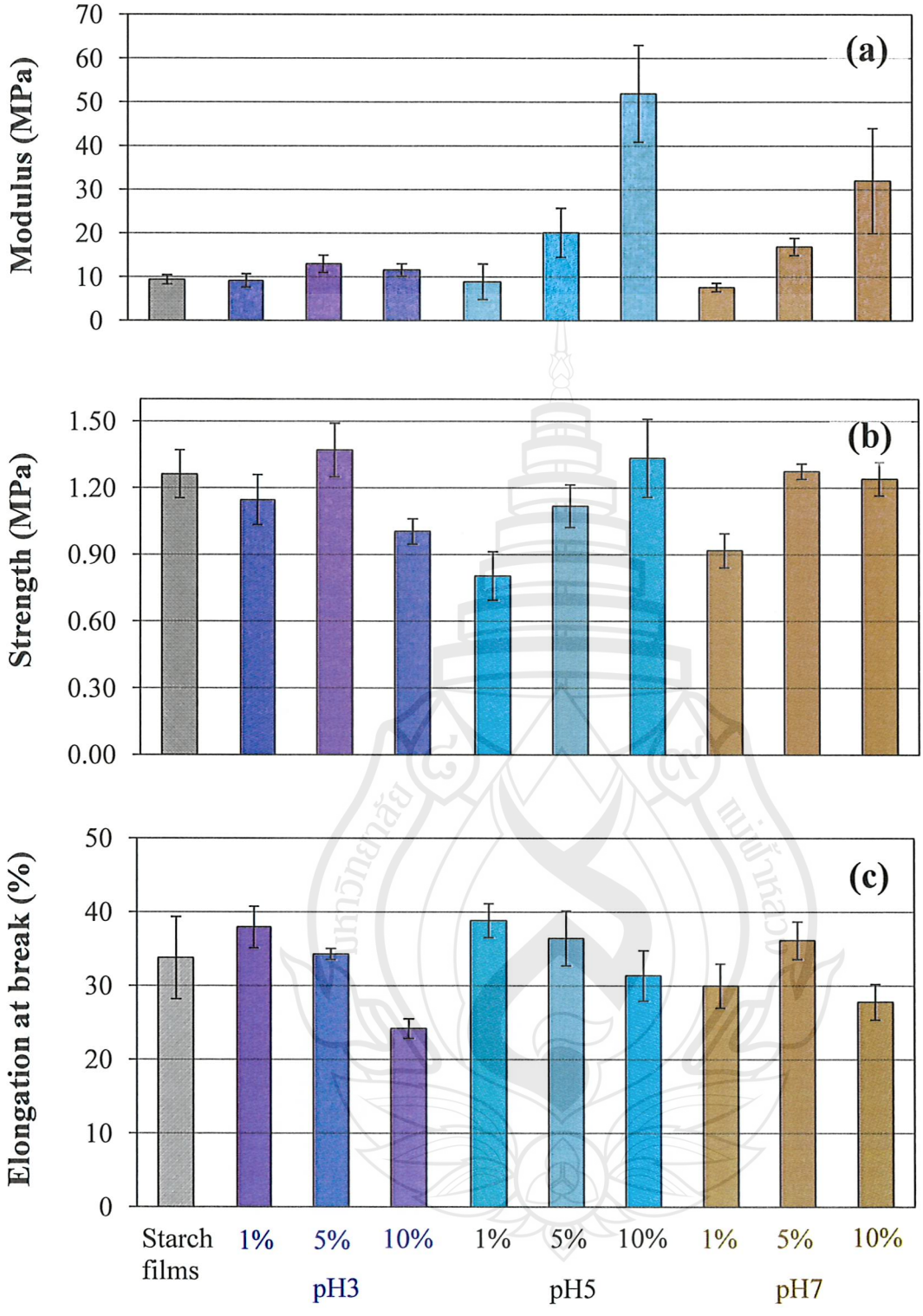

Figure 4 - 18 Young's modulus (MPa) (a), tensile strength (MPa) (b) and elongation at break% (c) of the pure starch film and bionanocomposite films reinforced with BCNWs of pH 3, 5 and 7 (with varied contents of 1, 5 and 10 wt%).

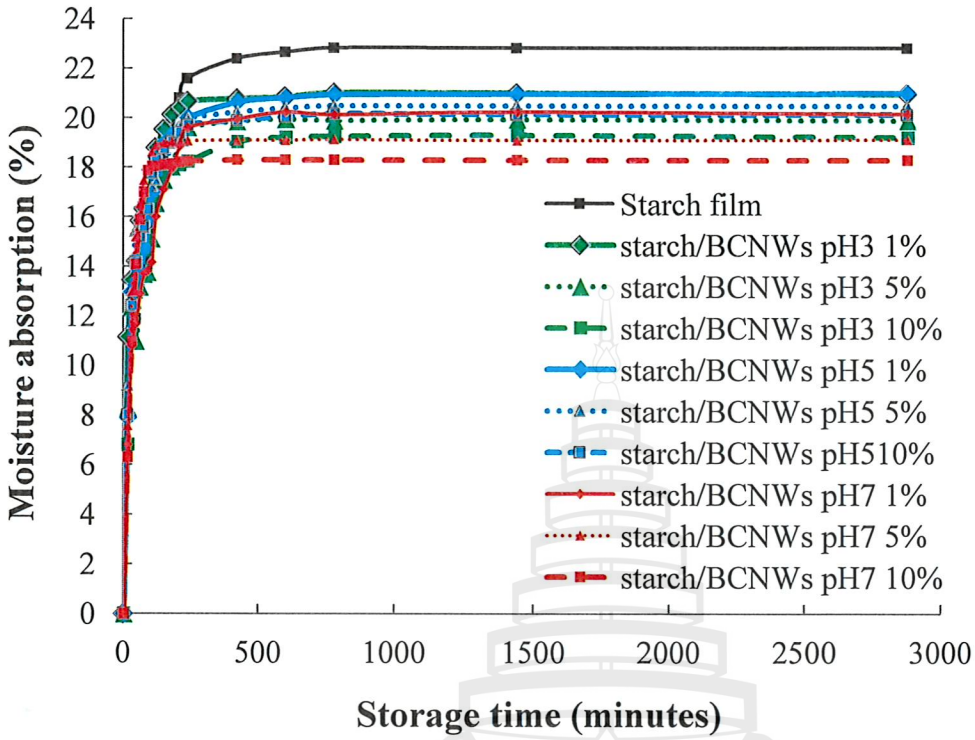

Figure 4 - 19 Moisture absorption (at 75% RH) as a function of storage time of the pure starch film, and bionanocomposite films reinforced with BCNWs of pH 3, 5 and 7 (with varied contents of 1, 5 and 10 wt%).

The moisture absorption of the films of the starch/BCNWs pH3 10 wt%, starch/BCNWs pH5 10 wt% and starch/BCNWs pH7 10 wt% were 19.22%, 20.14% and 18.05%, respectively. This suggests that a water resistance of all composite films greatly increased as compared to the pure starch film. The presence of BCNWs improved water barrier properties of the pure starch film because of firstly, the higher crystallinity of BCNWs and their low moisture absorption and secondly, hydrogen bonding that formed at the BCNWs-matrix interfaces. The water resistance of the starch/BCNWs pH7 films was higher than that of the starch/BCNWs pH3 and starch/BCNWs pH5 films because the sulfate group on the surfaces of the BCNWs,

pH3 and pH5 resulted in poor interactions with the starch matrix. Thus, there is possibly only poorly formed hydrogen bonding at the interfaces of these composites.

Thermogravimetric analysis (TGA) and differential thermogravimetry (DTG) curves of bionanocomposite films are shown in Figure 4 - 20 and 4 - 21, respectively. The peak degradation temperatures of all films are listed in Table 4 - 2. Firstly, TGA curves shows an intitial drop about 100 - 150 °C which corresponds to a mass loss of water and glycerol (Averous and Boquillo, 2004). With addition of BCNWs, the thermal stability of the bionanocomposite films were significantly improved about 20 - 30 °C as compared to the pure starch film. The peak degradation temperatures of the bionanocomposite films systematically increase with increasing BCNWs content from 1 to 10 wt%. Regardless to pH of the BCNWs. The improvement in thermal stability of the bionanocomposite films with addition of BCNWs can be possibly based on the fact that cellulose nanowhiskers have that are inherently good thermal stability and also the intensive hydrogen bonds formed between the starch matrix and BCNWs (Ahmad et al., 2012).

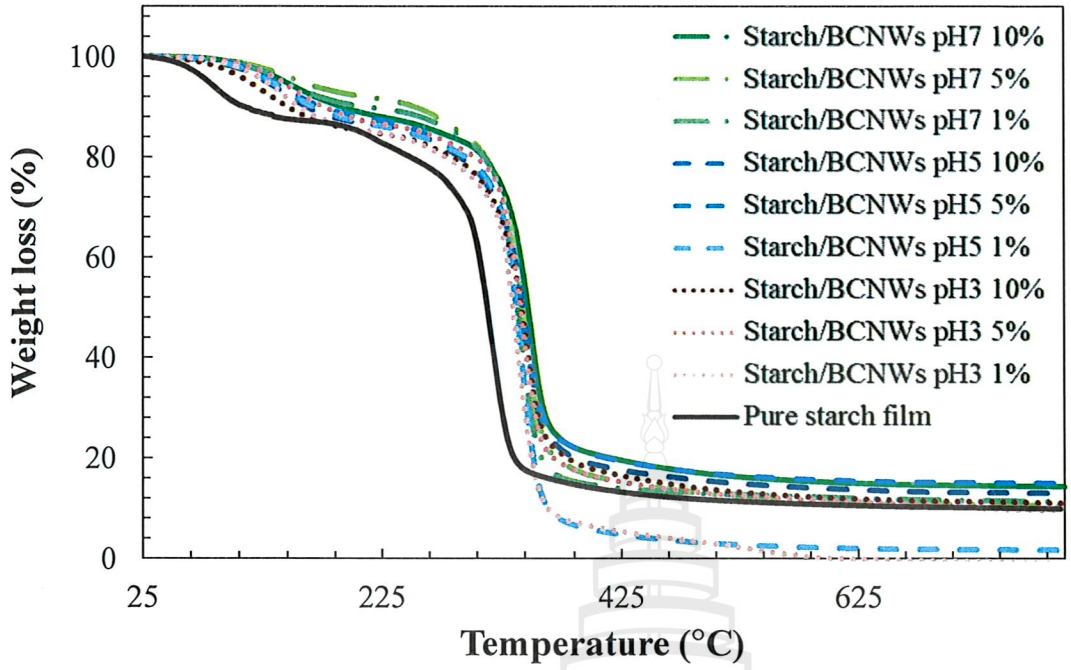

Figure 4 - 20 TGA curves of pure starch and bionanocomposite films reinforced with BCNWs of pH 3, 5 and 7 (with varied contents of 1, 5 and 10 wt%).

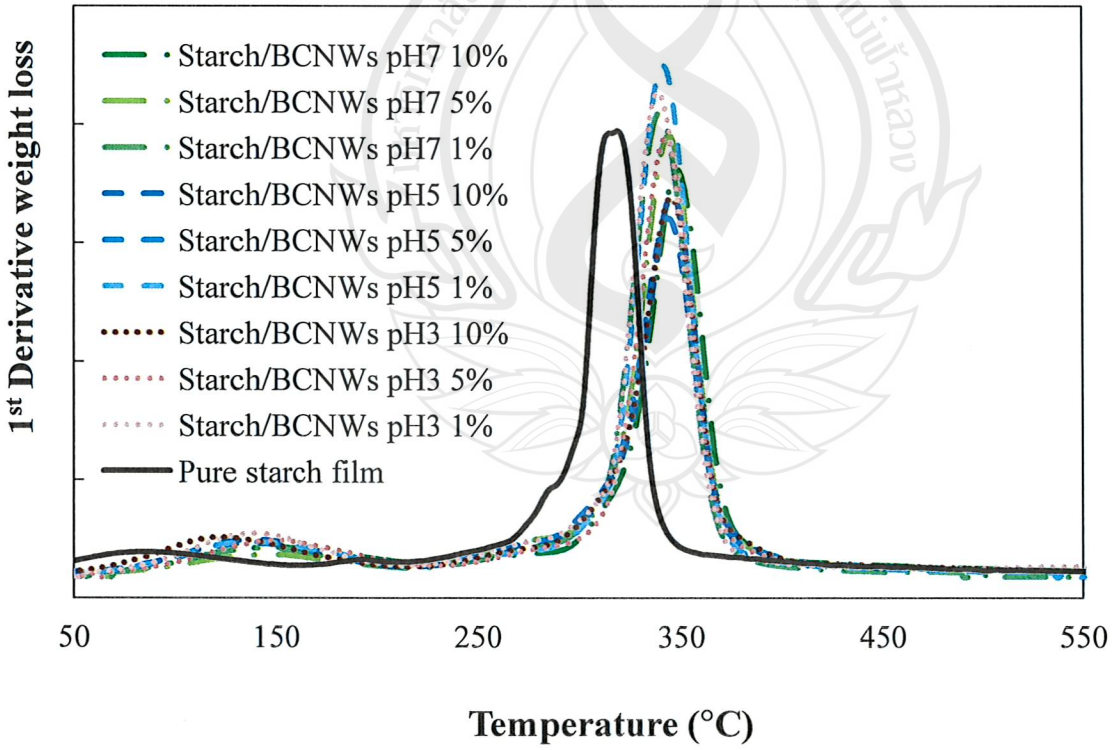

Figure 4 - 21 DTG curves of pure starch and bionanocomposite films reinforced with BCNWs of pH 3, 5 and 7 (with varied contents of 1, 5 and 10 wt%).

Table 4 - 2 Peak degradation temperatures of the native BC, BCNWs of pH 1, 3, 5 and 7, pure starch film and bionanocomposite films from their DTG curves.

Sample	Peak Temperature (°C)	
	1st Peak	2nd Peak
Native BC	358	-
BCNWs 48h pH1	165	191
BCNWs 48h pH3	226	280
BCNWs 48h pH5	248	286
BCNWs 48h pH7	340	-
Pure starch films	318	-
Starch/BCNWs pH3 1%	339	-
Starch/BCNWs pH3 5%	343	-
Starch/BCNWs pH3 10%	345	-
Starch/BCNWs pH5 1%	340	-
Starch/BCNWs pH5 5%	343	-
Starch/BCNWs pH5 10%	345	-
Starch/BCNWs pH7 1%	340	-
Starch/BCNWs pH7 5%	343	-
Starch/BCNWs pH7 10%	347	-

CHAPTER 5

CONCLUSIONS

5.1 Conclusions: Part A

5.1.1 Study the effect of type of carbon source in the cultured medium of bacterial cellulose on its structure and mechanical properties

In this present work, it was found that the type of carbon source significantly influenced not only the production yield of bacterial cellulose (BC) but also its structure and mechanical properties. The values of crystallinity index and crystallite size of each BC sample (cultured in the medium of different type of carbon sources) were evaluated by using X-ray diffraction (XRD) technique. It was found that the higher crystallinity index of BC fibers led to the higher mechanical properties of the dis-BC sheets. Although, the relationship between crystallite size of BC and the mechanical properties of the dis-BC sheets cannot be clearly concluded. Lastly, it can be summarized that except for glycerol, other carbon sources i.e. sucrose, glucose, fructose and mannitol, used in the culture media of BC resulted in the high mechanical properties of the prepared dis-BC sheets.

5.1.2 Investigate the effect of additional supplements in the cultured medium of bacterial cellulose on its production yield

The addition of optimum amounts of juices in the cultured medium of BC positively affected bacterial cellulose yield (i.e. addition of 10-30% v/v of pineapple juice and 10-50% v/v of coconut juice). This presumably due to various carbon and nitrogen sources, trace elements, vitamins and growth hormones which contain in the juices increase cell activity and consequently, cellulose yield. The addition of

pineapple juice of 30% v/v and coconut juice of 50% v/v were the optimum amounts and cellulose productivity at approximately 2-fold increase in production yield was obtained. At higher amounts of juice addition, the reduction in cell growth and cellulose production was found. Here, more free sugars content from the added juices can create saturated carbon source environment that may directly inhibit cell activity. In conclusion, the results suggested that both supplements can effectively improve the cellulose yield but pineapple juice is a slightly more effective one.

5.2 Conclusions: Part B

Bacterial cellulose nanowhiskers (BCNWs) were prepared by sulfuric acid hydrolysis at 50°C. It was found that 48 hours was the optimum treatment time to prepare BCNWs with the highest crystallinity in form of the isolated rod-like nanocrystals with diameter and length of approximately 28.18±1.99 nm and 637.61±147.10 nm, respectively. Then, the bionanocomposite films of starch reinforced with BCNWs of different pHs (3, 5 and 7) at varied contents of 1, 5 and 10 wt% were prepared by casting technique. With increasing BCNWs content, the bionanocomposites showed improvements in crystallinity, thermal stability and water resistance. Nevertheless, mechanical properties of the starch/BCNWs pH 3 and starch/BCNWs pH 7 films were not improved due to the poor interaction and presence of large BCNWs aggregation. Only in the film of starch/BCNWs pH 5, the overall mechanical performance was improved possibly because the optimum BCNWs dispersion and sufficient interaction were obtained in this system.

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