Production, Characterization and Application of Polymers from *Rhizopus microsporus* ST4 and Oil Palm Wastes

Hatsalinda Binma-ae

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

Prince of Songkla University

2018

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การผลิต คุณลักษณะ และการประยุกต์ใช้พอลิเมอร์จาก *Rhizopus microsporus* ST4 และวัสดุเศษเหลือจากปาล์มน้ำมัน

ผู้เขียน นางสาวหัสลินดา บินมะแอ

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บทคัดย่อ

งานวิจัยนี้ศึกษาการผลิต คุณลักษณะและการประยุกต์ใช้พอลิเมอร์จากเชื้อรา *Rhizopus microsporus* ST4 และวัสดุเศษเหลือจากปาล์มน้ำมัน

จากการศึกษาสภาวะในการผลิตพอลิเมอร์จากเชื้อรา *R. microsporus* ST4 ในอาหารเลี้ยงเชื้อที่มีองค์ประกอบของกลูโคส ร้อยละ2 yeast extract ร้อยละ 0.05 กรดกลูตามิคร้อยละ 5 และ $MgSO_4 \cdot 7H_2O$ ร้อยละ 0.05 ปรับพีเอชเป็น 5.8 เลี้ยงเชื้อที่อุณหภูมิ 45 องศาเซลเซียส เป็นเวลา 3 วัน บนเครื่องเขย่า (200 รอบต่อนาที) พบว่า *R. microsporus* ST4 สามารถผลิตพอลิเมอร์ได้ 12.33 กรัมต่อลิตร เมื่อศึกษาองค์ประกอบทางเคมีของพอลิเมอร์ดังกล่าว พบว่ามีกรดอะมิโนทั้งหมด ร้อยละ 99.3 ซึ่งเป็นกรดอะมิโนที่มีกรดกลูตามิก ปริมาณมากที่สุด รองลงมา คือ ไลซีน กรดแอสปาติกอะลานีน และ ทรีโอนีน ตามลำดับ ดังนั้น พอลิเมอร์ที่ผลิตจาก *R. microsporus* ST4 คาดว่าเป็น พอลิเมอร์ชนิดกลูตามิกแอซิด เมื่อศึกษาน้ำหนักโมเลกุลด้วยวิธี SDS-PAGE พบว่ามีน้ำหนักโมเลกุลประมาณ 80 กิโลดาลตัน และสามารถละลายได้ดีในน้ำกลั่น

จากการศึกษาการเก็บเกี่ยวพอลิเมอร์และคุณสมบัติของพอลิเมอร์จากวัสดุเศษเหลือปาล์มน้ำมัน ได้แก่น้ำทิ้งจากโรงงานสกัดน้ำมันปาล์ม และลำต้นปาล์มที่โค่นทิ้ง (อายุ > 25 ปี) โดยการสกัดพอลิเมอร์จากน้ำทิ้งโรงงงานสกัดน้ำมันปาล์ม ได้แก่ น้ำนึ่งปาล์ม และน้ำทิ้งดีแคน-เตอร์ นำมากำจัดของแข็งทั้งหมดโดยการหมุนเหวี่ยงตัวอย่าง และนำสารละลายส่วนใสมาตกตะกอนด้วยเอทานอลร้อยละ 95 ปริมาตร 5 เท่า ได้ผลผลิตพอลิเมอร์ร้อยละ 2.16 และ 2.47 (น้ำหนักต่อปริมาตร) เมื่อศึกษาน้ำหนักโมเลกุลด้วยวิธี gel permeation chromatography (GPC) ของพอลิเมอร์ดังกล่าว พบว่ามีน้ำหนักโมเลกุลสองขนาดได้แก่ น้ำหนักโมเลกุล 42.19 และ 3.57 กิโลดาลตัน และ 36.34 และ 3.61 กิโลดาล ตัน ตามลำดับ และทั้งสองตัวอย่างสามารถละลายได้ดีในน้ำ สำหรับลำต้นปาล์ม นำมาลดขนาดในช่วง 0.6>OPT<2 มิลลิเมตร แล้วนำมาสกัดพอลิเมอร์โดยใช้วิธีทางเคมีด้วย alkaline peroxide (AP) แล้วนำสารละลายส่วนใสมาตกตะกอนด้วยเอทานอลร้อยละ 95 ปริมาตร 3 เท่า ได้ผลผลิตพอลิเมอร์ ร้อยละ 26.25 (น้ำหนักต่อน้ำหนัก) มี

น้ำหนักโมเลกุล 67.57 กิโลดาลตัน และสามารถละลายได้ดีใน dimethylsulfoxide (DMSO) เมื่อศึกษาคุณสมบัติของพอลิเมอร์พวกไซแลนทางการค้าที่ผลิตจากไม้บีช พบว่ามีน้ำหนักโมเลกุลสองขนาดได้แก่ โมเลกุลขนาดเล็กมีน้ำหนักโมเลกุล 21 กิโลดาลตัน และขนาดใหญ่มีน้ำหนักโมเลกุล 930 กิโลดาลตัน และสามารถละลายได้ดีในน้ำ

การศึกษาผลิตไฮโดรเจลจากพอลิเมอร์น้ำนึ่งปาล์ม (HSC) พอลิเมอร์น้ำทิ้งดีแคน-เตอร์ (HDE) พอลิเมอร์ลำต้นปาล์ม (PHC_{CM}) และไซแลนทางการค้า (CXB) ใช้ปฏิกิริยาทางเคมีในการเชื่อมต่อพอลิเมอร์กับ maleic anhydride (MA) มี DMSO เป็นตัวทำละลาย ที่อุณหภูมิ 50 องศาเซลเซียส เวลา 2 ชั่วโมง ตกตะกอนด้วย isopropanol ปริมาตร 3 เท่า และอบแห้งที่อุณหภูมิ 50 องศาเซลเซียส และนำพอลิเมอร์ดังกล่าวทำปฏิกิริยากับ polyvinyl alcohol (PVA) เป็นสารเชื่อมไขว้เพื่อเกิดโครงสร้างสามมิติในโครงสร้างของไฮโดรเจล ได้ไฮโดรเจลน้ำนึ่งปาล์ม (HSC:H1-H12) พอลิเมอร์น้ำทิ้งดีแคนเตอร์ (HDE:H13-H24) ไซแลนทางการค้า (CXB:H25-H36) และพอลิเมอร์ลำต้นปาล์ม (PHC_{CM}:H1-H12) พบว่า พอลิเมอร์จากน้ำทิ้งจากเครื่องดีแคนเตอร์ (HDE:H17) ที่ทำปฏิกิริยากับ MA ในอัตราส่วน 1.5:1 และ PVA ในอัตราส่วน 1:0.55 ให้ค่าการบวมน้ำสูงสุดร้อยละ 395.9 และ พอลิเมอร์จากน้ำทิ้งดีแคนเตอร์ (HDE:H20) ที่ทำปฏิกิริยากับ MA ในอัตราส่วน 1:1 และ PVAในอัตราส่วน 1:0.55 ความต้านทานแรงดึงสูงสุดเท่ากับ 21.12 เมกะปาสกาล เมื่อนำไฮโดรเจลที่มีคุณสมบัติบวมน้ำดี และความต้านทานแรงดึงดูดสูง ได้แก่ ไฮโดรเจลที่ผลิตจากพอลิเมอร์น้ำนึ่งปาล์ม (H11) ไฮโดรเจลที่ผลิตจากพอลิเมอร์น้ำทิ้งดีแคนเตอร์ (H17) ไฮโดรเจลที่ผลิตจากไซแลนทางการค้า (H35) และไฮโดรเจลที่ผลิตจากลำต้นปาล์ม (H10) มาศึกษาความเป็นพิษต่อเซลล์หนูปกติ (L929) พบว่าไฮโดรเจลทั้งหมดมีความเป็นพิษต่อเซลล์ปกติ ส่วน H17 และ H35 มีฤทธิ์ในการยับยั้งเชื้อ *Staphylococcus aureus* ATCC 6538 จึงมีความเป็นไปได้ในการใช้ทางการแพทย์ต่อไปได้

การประยุกต์ใช้พอลิเมอร์จากวัสดุเศษเหลือจากปาล์มน้ำมันและไซแลนทางการค้ามาผลิตไซโลโอลิโกแซ็กคาไรด์ โดยใช้เอนไซม์ endo-(1,4)-β-xylanases (EC 3.2.1.8) จำนวน 4 ยูนิตต่อ 100 มิลลิกรัมของพอลิเมอร์ ที่อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 12 ชั่วโมง พบว่าไซแลนทางการค้าสามารถผลิตไซโลไบโอสมากที่สุด (ร้อยละ 0.05) รองลงมา คือ พอลิเมอร์จากลำต้นปาล์ม สามารถผลิตไซโลไบโอส (ร้อยละ 0.03) และนำไซโลโอลิโกแซ็กคาไรด์ที่ผลิตจากพอลิเมอร์ลำต้นปาล์ม พบว่ามีฤทธิ์ต้านอนุมูลอิสระ ที่มีค่า IC_{50} เท่ากับ 19,610 ไมโครกรัม/มิลลิลิตร

Thesis Title	Production, Characterization and Application of Polymers from *Rhizopus microsporus* ST4 and Oil Palm Wastes
Author	Miss Hatsalinda Binma-ae
Major Program	Biotechnology
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ABSTRACT

This study aimed to produce, characterize and apply biopolymers from *Rhizopus microsporus* ST4 and oil palm wastes.

The condition for production of biopolymer from *R. microsporus* ST4 were: 2% glucose, 0.05% yeast extract, 5% glutamic acid and 0.05% $MgSO_4 \cdot 7H_2O$ with pH of 5.8 at 45 °C for 3 days. Under this condition, the maximum concentration of biopolymer was 12.33 g/L. Analysis of amino acid profile revealed that the biopolymer ST4 contained 99.3% of glutamic acid with a trace amount of lysine, aspartic acid, alanine and threonine. Therefore, the biopolymer from *R. microsporus* ST4 was considered to be polyglutamic acid (PGA). The molecular size of PGA (estimated by SDS-PAGE) was about 80 kDa and soluble in water.

The recovery and characterization of biopolymers from oil palm wastes were investigated using palm oil mill effluent (POME) and oil palm trunk (OPT). For POME, the biopolymer from sterilizer condensate (SC) and decanter effluent (DE) were extracted with 5 folds (v/v) of 95% ethanol and obtained the precipitated hemicellulose from SC (HSC) and DE (HDE) of 2.16% and 2.47% (w/v), respectively. There were two sizes of Mw from each source; 42.19 kDa and 3.57 kDa from HSC, with 36.34 and 3.61 kDa from HDC were determined with a gel permeation chromatography (GPC). The maximum solubility of HSC and HDE in water were 96.82% and 99.05% respectively. Polymer from the selected OPT (0.6>OPT<2 mm) was extracted using alkaline peroxide (AP) pretreatment, then precipitated using the 3-fold volume of absolute ethanol. Under the optimal condition, the maximum concentration of the precipitated hemicellulose from OPT (PHC_{CM}) was 26.25% (w/w). The PHC_{CM} had a molecular weight (Mw) of 67.57 kDa and the

maximum solubility in DMSO. The commercial xylan from beechwood (CXB) was found to have also two size of Mw 930 kDa and 21 kDa. The maximum solubility of CXB in water was 84.62%.

Hemicellulose-maleic anhydride (MA)/polyvinyl alcohol (PVA) hydrogels were prepared by modifying from HSC (H1-H12), HDE (H13-H24), CXB (H25-H36) and PHC_{CM} (H1-H12). HDE-MA was prepared by reacting HDE with MA (ratio 1.5:1) using dimethyl sulfoxide (DMSO) as a solvent. The reactions were performed and stirred at 50 °C for 2 h and precipitated with 3 folds (v/v) of isopropanol for 48 h and then dried in an oven at 50 °C. The dried HDE-MA were blended with PVA (ratio 1:0.55). This condition (H17) gave the highest degree of swelling (395.9%). Under the optimum conditions (H20;HDE-MA 1:1/PVA1:0.55), the highest compressive strength could reach to 21.12 MPa. Evaluation of the cytotoxicity of the hydrogels revealed that the cell viability of L929 cells (Mouse Fibroblast Cells) and antimicrobial tests on the selected hydrogel which was high swelling and strength including from HSC (H11), from HDE (H17), from commercial xylan (H35) and from PHC_{CM} (H10), hydrogel suggested that the hydrogel was cytotoxic and had a potential for inhibition of cancer cell. The hydrogel from HDE (H17) and hydrogel from CXB (H35) exhibited inhibition zones of *Staphylococcus aureus* ATCC 6538 could be the probability in the field of biomedical application.

The xylooligosacharide (XOS) production from HSC, HDE, CXB and PHC_{CM}, was using the enzymatic method. Under the optimal conditions of CXB (4 U for 12h at 40 °C), the highest xylobiose (oligosaccharide) could reach to 0.05% following this polymer from OPT (4 U for 12h at 40 °C), the highest xylobiose (oligosaccharide) could reach to 0.03%. Moreover, XOS from PHC_{CM} exhibited IC_{50} inhibition at 19,610 µg/mL of antioxidant activity test.

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Hatsalinda Binma-ae

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	=	Degree celcious
MDW	=	Mycelia dry weight
Da	=	Dalton
kDa	=	Kilo Dalton
g	=	Gram
mg	=	Milligram
g/L	=	Gram per liter
h	=	Hour
µL	=	Microliter
µg/mL	=	Microgram per milliliter
mL	=	Milliliter
Mw	=	Molecular weight
rpm	=	Revolutions per minute
in	=	Inch
cm		Centimeter
spore/mL	=	Spore per milliliter
v/v	=	Volume by volume
w/w	=	Weight by weight
w/v		Weight by volume
mm	=	Millimeter
OD	=	Optical density
MPa	=	Megapascal

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Currently, a majority of polymers is produced from petrochemical substances which extracted from coal, oil and natural gas such as polyethylene (PE), polyvinyl chloride (PVC), polypropylene (PP) and polystyrene (PS) that are non-biodegradable polymers (Ahmed *et al*., 2018). As per global estimation, about 300 million tons of synthetic polymers is generated annually and an annual increase trend (Drzyzga *et al*., 2015). The increasing population will increase the demand for polymers and other plastics which generally takes more than 300-400 years to degrade. The accumulation of non-biodegradable polymers and water soluble synthetic polymers in wastewater is a major problem of environmental pollution (Rao *et al*., 2011). With incorrect burning or disposal, it will create toxic residues in the environment also. Moreover, the limited resources of petroleum, supply security, greenhouse gas emissions, feedstock costs and a long time to degrade in nature, the area of polymers from biological sources or biopolymers have been increasing of interest owing to the excessive use of petrochemical polymers can cause health and environmental problems. The degradable biopolymers will take only a few months to degrade. Biopolymers are macromolecules of biological origin such as microorganism and plant. They are biodegradable, eco-friendly and renewable.

Microbial extracellular polymers produced by various strains of microorganism with different properties, production of polysaccharide bioflocculants from *Halomonas* sp. OKOH (Mabinya *et al*., 2011), production of protein flocculant from *Bacillus licheniformis* (Shih *et al*., 2001) and *Rhodococcus erythropolis* (Tadeka *et al*., 1991), producing of glycoprotein bioflocculant from *Halobacillus* sp. Mvuyo (Cosa *et al*., 2012) and production of poly-γ-glutamate (PGA) from *Bacillus subtilis, B. licheniformis, and B. sonorensis* (Chettri *et al.,* 2016). Exopolysaccharides (EPS) can be produced by fungi including *Ganoderma lucidum*, *Agaricus blazi*, *Cordyceps* sp., *Lentinus edodes*, and *Grifola frondosa* (Mahapatra and

Banerjee, 2013). Fungal EPS have several applications in food, feed, medicine, cosmetics and pharmacy (Mahapatra and Banerjee, 2013). EPS produced by *Morchella crassipes* has applications in pharmaceuticals (He *et al*., 2012). *Ganoderma lucidum*, *Phellinus rimosus*, *Pleurotus florida* and *Pleurotus pulmonaris* possessed profound antioxidant and antitumor activities (Ajith and Janardhanan, 2007). The biopolymer from *Rhizopus oryzae* ST29 was found to possess the anticancer activity (oral human epidermal carcinoma) (Suyala, 2007). Pullulan which produced from *Aureobasidium pullulans* can be used as a viscosity stabilizer in food industry (Mahapatra and Banerjee, 2013). In addition, fungal biopolymers possessed flocculating activity such as the bioflocculant PM-5 from *Aspergillus niger* cultivated in palm oil mill effluent (POME) (Aljuboori *et al*., 2014). EPS produced by the *A. fumigatus* could remove Cu(II) and Cd(II) (Yin *et al*., 2010). The intracellular polysaccharides of *Lepista sordid* had potent antioxidant and antiaging activity (Zhong *et al*., 2010). The advantage of microbial polymer production is the possibility to use fermentation processes to obtain high concentrations of pure product and independent of seasonal variations (Selbmann *et al*., 2003). Thus, research focused on producing newer biopolymer from fungal are growing in importance.

Generally, plant biomass is abundant and distributed widely in nature. Because plants contain 45-55% cellulose, 25-35% hemicellulose and 20-30% lignin (Deutschmann and Dekker, 2012). Lignocellulosic biomass is abundant renewable source for production of various biorefinery products. One of the most important biomass resources, Oil palm (*Elaeis guineensis*) is monocotyledonous species in the same group with Cocos under the tribe Cocoideae. The dispersion of oil palm is restricted to tropical regions of the world and has mainly been cultivated in Indonesia, Malaysia and Thailand, Nigeria in Africa, Colombia and Ecuador in South America and Papua New Guinea in Oceania (UNEP, 2011).

Oil palm is an important economic plant particularly in the south of Thailand (Krabi, Satun, Suratthani and Chumphon). In 2009, the biggest producer, with a 47.6% share in production, was Indonesia, followed by Malaysia (38.8%) and Thailand (2.9%) (Dallinger, 2011). The dramatic increase in demand for the plantation area was around 10% with an average annual growth rate of 11% from 1981 to 2000 and 9% from 2001 to 2010. The Thai government plans to increase the

oil palm plantation area to 5 million rai (or 0.8 million ha) in order to have an adequate supply of raw material for biodiesel production (Sattayasamitsathit, 2011). Standard or wet process is commonly employed for palm oil extraction in the palm oil mill that resulted in the generation of large quantity of palm oil mill effluent (Madaki, *et al*., 2000). The biomass from oil palm residue include the oil palm trunk (OPT), oil palm frond (OPF), kernel shell, empty fruit bunch (EFB), palm pressed fiber (PPF) and palm oil mill effluent (POME). The sterilizer condensate (SC) and decanter effluent (DE) are two of the three major sources of effluent from palm oil mill. It had a brownish colloidal suspension and characterized by high organic content. In addition, a 25 years, the oil palm trees are no longer considered of an economic value and need replantation. Thailand has estimated 100,000 ha of planting area covered with >25 years old trees (Dallinger, 2011). Oil palm waste is a reliable resource owing to its availability, continuity and capacity for a renewable energy solution. Furthermore, in nowadays situation, the presence of oil palm wastes has created a major disposal problem, thereby, affect the environment. The technological, economic, energy balance and environmental considerations must be kept at a balance to meet the best solution of utilization oil palm wastes (Abdullah and Sulaiman, 2013). The OPT and POME were rich lignocellulosic biomass contain 40–55% cellulose, 25–40% hemicellulose and 15–35% lignin on a dry basis (Puhpamalar *et al.,* 2006; Noparat *et al.,* 2013). The cellulose and hemicellulose consist up to two third of the lignocellulosic. Hemicelluloses are heteropolysaccharides and xylans are the most abundant of the hemicelluloses found in the cell walls of land plants also. Especially, hemicelluloses could be utilized for producing xylitol in food industry (Prakash *et al.,* 2011), xylooligosaccharides (Hanim *et al*., 2011; Pedraza *et al.,* 2014; Ho *et al*., 2014), bioethanol in energy industry (Velmurugan and Muthukumar, 2011) and hydrogel (Tanodekaew *et al*., 2006; Wu *et al*., 2012; Dai *et al.,* 2016). Therefore, research on the utilization of OPT and POME should be undertaken, particularly on the fractionation of hemicellulose for the production of monosaccharides and oligosaccharides such as xylooligosaccharides (XOS) and xylan-based hydrogels with new promising applications in biotechnology environmental chemistry and biopolymeric materials with properties desired for medical applications.

The aims of this study are to produce biopolymer from thermotolerant fungi, *Rhizopus microsporus* ST4, and the biopolymer recovered from palm oil mill wastes and extracted from oil palm trunk, then characterize to find the interesting properties for potential application.

Literature Reviews

1. Biopolymers from microorganisms

1.1 Type of biopolymers from microorganisms

Type of biopolymers from microorganisms could be classified on the three criteria, as follows: biosynthesis, electrical charge property and chemical composition as shown in Table 1.1.

1.2 Biopolymers from fungi

The fungal polymers are currently of interest because they are a biologically rich source of various bioactive substances. During the last two decades, most of the researchers have reported that polysaccharides production from fungi such as *Rhodotorula glutinins, Ganoderma lucidum*, *Phellinus rimosus, Aureobasidium pullulans* etc. A few reports exist on the protein-bound polysaccharides production from fungi (Young *et al*., 2008). Microorganisms constitute polysaccharides form intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides (Nehad and Shamy, 2010). The intracellular polysaccharides extracted from the mycelium of *Lepista sordid* grown in basal medium exhibited the antioxidant activity *in vitro* demonstrated that intracellular polysaccharides (CLSP) had significant free radical-scavenging activity on hydroxyl, superoxide anion and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radicals and could be explored as a potential dietary supplement to retard aging and attenuate age-related diseases in humans (Zhong *et al*., 2013). The polysaccharide from *Agaricus bisporus* was extracted with hot water and purification using DEAE-Sepharose gave molecular weight of $5.17 x 10^4$ Da and its main components were D-glucose and D-mannose (He *et al*., 2014). *Lepista sordida* was extracted polysaccharide with absolute alcohol at 75 °C for 3 times, 2 h for each time and then extracted with distilled water at 80 °C for 3 times 2 h for each time, centrifuged and precipitated with three volumes of ethanol had molecular $4 x 10^4$ Da (Luo *et al*., 2012).

Table 1.1 Type of biopolymers from microorganisms

Criteria of classification	Type of biopolymers	Examples
Biosynthesis	A. Extracellular polymers, sometimes called exopolymers, are synthesized inside the cells and excreted outside the cell well in two forms, the capsule is attached to the cell well and slime are released into the surrounding environment.	Polysaccharide Glycoprotein Poly-glutamate
	B. Intracellular polymers are synthesized inside the cells and require cell extraction for their isolation and purification.	DNA Protein Enzyme
Electrical charge property	A. Anionic or acidic polymers contain acidic groups.	Xanthan
	B. Cationic, sometimes called basic polymers, are mainly found in nature.	Chitosan
	C. Naturally neutral is composed of only neutral sugars and without any uronic or other ionizable groups.	Scleroglucan Pullulan Curdlan
Chemical composition	A. Homopolymers contain only one type of monomer repeating units side chain.	Cellulose Polyglutamic acid
	B. Heteropolymers contain two or more dimer repeating units side chain.	Xanthan Hyaluronic

Source: Sutherland,1998; Takagi and Kadowaki,1985;Matsuyama *et al*., 2013; Kunioka, 1997

Extracellular polysaccharides or exopolysaccharide (EPS) that have been recognized as high-value biomacromolecules are pullulan produce by *Aureobasidium pullulans* (Singh *et al*., 2008; Ravella *et al*., 2010; Wu *et al*., 2012), scleroglucan produce by *Sclerotium rolfsii*, and botryosphaeran produce by *Botryosphaeria rhodina* (Mahapatra and Banerjee, 2013). The molecular weight variations and sugar compositions of the fungal EPS mainly depend on the physical

conditions during fermentation and type of medium components used in the production process.

A large number of fungi especially higher basidiomycetes, lower filamentous fungi and various yeasts were known EPS to produce (Table 1.2). Generally, the fungi favored temperature for EPS production within the range of 22°C to 30°C, only a few researchers reported that fungi produced EPS most effective at 20°C. There are three factors affecting EPS production; physical conditions, medium composition, mode and methods of fermentation (Mahapatra and Banerjee, 2013). The physical conditions include temperature, pH of culture medium, oxygen level, incubation time etc. Medium composition contains a carbon source, inorganic and/or organic nitrogen sources, phosphate source, ionic salts and special additives. The mode and methods of fermentation refer to agitated cultures, static culture and submerged culture.

For the production of EPS from *Rhodotorula glutinins*, the medium contained glucose (3%), yeast extract (0.3%), 7.5 % inoculums size 168 h incubation period and pH 6.0 at 25 °C. The HPLC analysis indicated that EPS is composed of mannose, glucose, and arabinose with the following relative molar ratio of 3.2: 1.0: 0.8, respectively (Ibrahim *et al*., 2012). The effect of various carbon sources for the EPS production by *Morchella crassipes* in shake flask cultures for 7 days at 28°C with initial pH 6 was studied. Sucrose, glucose, fructose, maltose and xylose were used instead of glucose in the GP medium at a concentration of 30 g/L. Maltose was the most suitable for both cell growth (11.2 g/L) and EPS production (3.5 g/L) (He *et al*., 2012). For exobiopolymer production by *Cordyceps militaris*, sucrose gave the maximum specific growth rates (μ_{max}) (0.142 h^{-1}) and yield coefficient ($Y_{P/S}$) of 0.29 (Park, *et al*., 2001). Among the carbon sources tested, the highest mycelial growth and exobiopolymer levels from *Auricularia polytricha* were obtained from 6 days cultivation in sucrose (10 g/L) medium giving of 8.26 and 0.81 g/L, respectively (Xu and Yun, 2003). After 6 days cultivation *Paecilomyces tenuipes* C240, the highest mycelial growth of 8.60 and exobiopolymer production of 0.82 g/L were obtained in the basal medium (Xu *et al*., 2003). Maltose medium gave higher EPS production

Table 1.2 List of some fungi that are reported to produce exopolysaccharides in laboratory culture techniques.

Organism	Organism	Organism	Organism	Organism
Absidia corymbifera	*Cladosporium herbarum*	*Hansenula minuta*	*Peniophora cinerea*	*Ripartitella* cf. *brasiliensis*
Absidia cylindrospora	*Cladosporium tricoides*	*Helotium* sp.	*Perenniporia piperis*	*Sarcodon aspratus* (Berk)
Acremonium charticela	*Claviceps purpurea*	*Hirsutella* sp.	*Pestalotia* sp. 815	*Schizophyllum commune*
Acremonium diospyri	*Climacodon pulcherrimus*	*Hymenostilbe* sp.	*Pestalotiopsis* sp. KCTC 8637P	*Sclerotium glucanicum*
Acremonium persicinum	*Collybia maculate*	*Hypocrella tamurai*	*Phanerochaete chrysosporium*	*Sclerotium rolfsii*
Agaricus nevoi HAI610	*Cookenia tricholoma*	*Hypsizigus marmoreus*	*Phellinus baumii*	*Selenotila peltata*
Agaricus xanthodermus	*Cordyceps dipterigena*	*Inonotus levis* HAI796	*Phellinus gilvus*	*Shiraia bambusicola*
Agrocybe cylindracea	*Cordyceps militaris*	*Isaria farinose* BO5	*Phellinus igniarus* HAI795	*Sorangium cellulosum*
Agrocybe platensis	*Cordyceps nipponica*	*Lachnum* sp. YM261	*Phellinus robustus* HAI531	*Sporobolomyces salmonicolor* AL1
Akanthomyces pistillariiformis	*Cordyceps sinensis*	*Lentinus edodes*	*Pholiota nameko*	*Sporothrix schenkii*
Alternaria alternate	*Cordyceps sphecocephala*	*Lipomyces starkeyii*	*Phomopsis foeniculi*	*Stemphylium* sp.
Alternaria mellea	*Cordyceps taii*	*Lyophyllus decastes*	*Phytocordyceps* sp.	*Stereum sanguinolentum*
Alternaria solani	*Coriolus (Trametes) versicolor*	*Melanoporia nigra*	*Pichia mucosa*	*Stropharia rugosoannulata*
Antrodia camphorate	*Coriolus hirsutus*	*Metarhizium anisopliae* var. *majus*	*Platymonas* sp.	*Syncephalastrum racemosum*
Antrodia cinnamomea	*Cryphonectria parasitica*	*Moniliella pollinis*	*Plectania occidentalis*	*Thamnidium elegann*
Antrodiella ginestae	*Cryptococcus albidus* 16-1	*Morchella crassipes*	*Pleurotus cornucopiae*	*Torrubiella tenuis*
Armillaria luteo-virens Sacc.QH	*Cryptococcus elinorii*	*Mucor circinelloides*	*Pleurotus dryinus* IBB903	*Torulopsis melibiosum*
Armillaria mellae	*Cryptococcus laurentii*	*Mucor hiemalis*	*Pleurotus eryngii*	*Torulopsis pinus*
Aschersonia samoensis	*Cyttaria harioti*	*Mucor mucedo*	*Pleurotus flabellatus*	*Trametes versicolor*
Aspergillus alliaceus	*Drechslera spicifera*	*Mucor racemosus*	*Pleurotus floridanus*	*Trametes villosa*
Aspergillus fumigates	*Elsinoe leucospila*	*Mucor rouxii*	*Pleurotus ostreatoroseus*	*Tremella brasiliensis*
Aspergillus niger	*Epicoccum nigrum*	*Nigrospora oryzae* var. *glucanicum*	*Pleurotus ostreatus*	*Tremella encephala*
Aspergillus parasiticus	*Flamnulina velutipes*	*Nothopanus hygrophanus*	*Pleurotus pulmonarius*	*Tremella foliacea*
Aspergillus sp. Y16	*Fomes fomentarius*	*Oligoporus* sp.	*Pleurotus sajor-caju*	*Tremella fuciformis*
Aureobasidium pullulans	*Fomitopsis pinicola*	*Oudemansiella canarii*	*Pleurotus tuber-regium*	*Tremella mesenterica*
Auricularia fuscosuccinea	*Fusarium coccophilum*	*Oudemansiella radicata*	*Polyporus fomentarinus*	*Tremella subanomala*
Beauveria bassiana	*Fusarium oxysporium* Dzf 17	*Paecilomyces japonica*	*Polyporus ignarius*	*Trichaptum byssogenum*
Botryosphaeria rhodina	*Fusarium solani*	*Paecilomyces lilacinus*	*Polyporus tuberaster*	*Tricholoma crassum*
Botrytis cinerea	*Ganoderma applanatum*	*Paecilomyces sinclairii*	*Psilocybe castanella*	*Trichosporon asahii*
Byssochlamys nivea	*Ganoderma lucidium*	*Paecilomyces tenuipes*	*Psilocybe subcubensis*	*Trogia buccinalis*
Calvatia cyathiformis	*Ganoderma resinaceum*	*Panaeolus papilionaceus*	*Pullularia pullulans*	*Tuber sinense*
Candida bogoriensis	*Gibellula pulchra*	*Penicillium charlesii*	*Pycnoporus sanguineus*	*Tyromyces pseudolacteus*
Candida boidinii	*Gliomastic gueg*	*Penicillium citrinum*	*Rhinocladellia elatior*	*Ustilago maydis*
Cephalosporium serrae Maffei	*Glomerella cingulata*	*Penicillium islandicum*	*Rhizomucor pusillus*	*Zygosporium masonii*
Cephalosporium stiehmeri	*Gomphidius rutilus*	*Penicillium luteum*	*Rhizopus nigricans*	
Cephalosporium subverticillatum	*Grifola frondosa*	*Penicillium paraphergal*	*Rhizopus stolonifer*	
Ceratocystis stenoceras	*Hansenula capsulate*	*Penicillium varians*	*Rhodotorula glutinis*	
Cerrena maxima IBB681	*Hansenula holstii*	*Penicillium vermiculatum*	*Rigidoporus microporus*	

Source : Mahapatra and Banerjee, 2013

(30 g/L) from *P. japonica* than that in sucrose medium (25 g/L) (Bae *et al*., 2001). When *Cordyceps militaris* NG3 were grown in the sucrose medium, both mycelial growth and EPS production indicated the highest yields of 30 and 40 g/L, respectively (Kim *et al*., 2003). *Rhodotorula glutinins* was cultivation in several carbon sources in basal medium (glucose, xylose, galactose, sucrose and rhafinose) indicated that glucose and xylose in basal medium gave the high EPS production of 1.7 and 1.7 g/L, respectively (Ibrahim *et al*., 2012). *Rhizopus oryzae* ATCC 46242 grown in the PGY salt medium with glucose 20 g/L gave 10.04 g/L biomass and 0.08 g/L chitosan (Hu *et al*., 2004). Thermotolerant fungus *R. oryzae* ST29, exhibited mycelial growth (3.29 g/L) and EPS production (5.58 g/L) after 4 days in POME at 45 °C (Suyala *et al*., 2003). The production of chitosan from *Rhizopus oryzae* TISTR3189 using potato chip processing wastes (trimmed potato, potato peel and substandard potato chips) the fermented potato peel had the highest cell growth (> 100 g/kg substrate) and chitosan production (>4 g/kg substrate) after 5 days of fermentation (Kleekayai and Suntornsuk, 2011). POME at pH 6 was a good carbon source for bioflocculant (PM-5) production by *A. niger* with the flocculating rates up to 80% after cultivation (Aljuboori *et al*., 2014).

The nitrogen source is a requirement for cell growth, the enzyme synthesis and product formation. These can be divided into organic and inorganic nitrogen. Inorganic nitrogen such as $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 and organic nitrogen such as yeast extract, peptone, glutamic acid, polypeptone or urea are most often add in the medium. Among nitrogen source tested, at 32°C for 3 days glutamic acid was efficiently used in the production of PM-5 with the flocculating rate up to 81%. Although yeast extract and urea were favorable nitrogen sources for biomass growth, the flocculating rate showed not higher than 65%. Moreover, the low production of PM-5 and biomass growth were observed when inorganic nitrogen sources : $(NH_4)_2SO_4$, NH_4NO_3 and $NaNO_3$ were used. EPS production by *Morchella crassipes* added tryptone, as nitrogen sources, in GP medium for 7 days at 28 °C with initial pH 6 was the best for maximum EPS production (2.5 g/L) (He *et al*., 2012). Meat peptone (2 g/L) was favorable for the mycelial growth and exobiopolymer production of *P. tenuipes* C240, giving the yields of 13.23 and 0.51 g/L respectively. For inorganic nitrogen sources, potassium nitrate gave the highest mycelial growth

(7.38 g/L) and exobiopolymer production (0.61 g/L) (Xu *et al*., 2003). Studies on the effect of several nitrogen sources; $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_3PO_4$, peptone, yeast extract and trypton (2.5 g/L) on exopolysaccharide production by *Rhodotorula glutinins*, the highest mycelial growth (12.08 g/L) and exobiopolymer production (1.2 g/L) were obtained in yeast extract medium after 168 h at 22 °C (Ibrahim, *et al*., 2012). Cultivation of by *Cordyceps military* in several nitrogen sources (organic nitrogens: corn steep liquor, corn steep powder, casein peptone, martone A-1, meat peptone, tryptone, polypeptone, soy peptone and yeast extract and inorganic nitrogens: ammonium nitrate, ammonium chloride, ammonium phosphate and sodium nitrate) in the medium shown that corn steep powder in the medium gave the highest exobiopolymer yield of 1.97 g /L (Park *et al*., 2001). Among seven nitrogen sources (10 g/L) was examined, yeast extract was the most effective for mycelial growth (6.09g/L) and exobiopolymer production of *Auricularia polytricha* (1.27 g/L) (Xu *et al*., 2003). Among 10 different nitrogen sources, corn steep powder was favorable for the mycelial growth (14.2 g/L) and exobiopolymer production *Cordyceps militaris* NG3 (3.1 g/L) (Kim *et al*., 2003). For chitosan production from *R. arrhizus* UCP 402 in culture media consisting of corn steep liquor (6%) and honey (13.24%) 96 h of fermentation gave the yield of mycelial mass (11.71 g/L) and the highest yield of chitosan (29.30 g/L) (Cardoso *et al*., 2012)

Consequently, the optimum medium for the production of fungal biopolymers depends on different fungal strains which are shown in Table 1.3.

Table 1.3 Optimum culture conditions for maximum biopolymers production by different fungal strains.

Organism	Carbon (g/L)	Nitrogen (g/L)	Salts and phosphates	Additives	pH	Temp. (°C)	Days	EPS (g/L)	Ref.
Agrocybe cylindracea	Maltose (60)	Martone A-1 (6)	$CaCl_2$, $MgSO_4 \cdot 7\ H_2O$	–	6.0	25	10	3.0	Kim *et al*., 2005
Alternaria alternata	Glucose (40)	Yeast extract (20)	KH_2PO_4, $MgSO_4 \cdot 7H_2O$	–	3.0	30	9	4.5	Nehad *et al*., 2010
Antrodia cinnamomea	Glucose (50)	Calcium nitrate (5)	$FeSO_4$	Nicotinic acid (0.1%)	5.5	28	14	0.49	Lin *et al*., 2006
Armillaria luteo-virens Sacc.	Glucose (31.26)	Yeast extract (1.06)	KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$	–	–	23	5	5.40	Jiao *et al*., 2008
Aspergillus niger	POME	Glutamic acid (1)	$CaCl_2$	–	6.0	32	3	7	Aljuboori *et al*., 2012
Aureobasidium pullulans CJ001	Sucrose (50)	Yeast extract (2)	KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, $(NH_4)_2SO_4$	–	6.0	22	4	31.25	Wu *et al*., 2012
Collybia maculate	Glucose (30)	Martone A-1 (20)	K_2HPO_4, $CaCl_2$	–	5.5	20	5	2.4	Lim *et al*., 2004
Cordyceps militaris NG1	Sucrose (40)	Corn steep powder (5)	–	–	8.0	30	16	5.05	Kim *et al*., 2005
Cordyceps militaris NG3	Sucrose (30)	Corn steep powder (10)	–	–	8.0	30	15	3.4	Kim *et al*., 2003
Cordyceps sinensis	Sucrose (20)	Corn steep powder (25)	$CaCl_2$, $MgSO_4 .7\ H_2O$	–	4.0	20	16	4.15	Kim *et al*., 2005
Cordyceps sphecocephala	Sucrose (40)	Yeast extract (6) and polypeptone (2)	KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7\ H_2O$	–	4.0	25	11	2.5	Oh *et al*., 2007

Table 1.3 Optimum culture conditions for maximum biopolymers production by different fungal strains (Cont.)

Organism	Carbon (g/L)	Nitrogen (g/L)	Salts and phosphates	Additives	pH	Temp. (°C)	Days	EPS (g/L)	Ref.
Cordyceps taii	Xylose (31.27)	$(NH_4)_2SO_4$ (0.15) and soybean steep liquor (4.85)	KH_2PO_4, $FeSO_4$, $MgSO_4 \cdot 7H_2O$	Vitamin–A and Vitamin–D (0.01 g/L)	5.5	28	6	43.87	Xiao *et al*., 2010
Coriolus (Trametes) versicolor	Glucose (15)	Malt extract (3), yeast extract (6) and peptone (5)	–	-	5.5	28	9	0.64	Tavares *et al*., 2005
Cryptococcus laurentii AL100	Sucrose (40)	Yeast extract (1)	KH_2PO_4, $MgSO_4 . \cdot 7H_2O$, NaCl, $CaCl_2$	–	5.3	22	4	6.4	Pavlova *et al*., 2011
Fomes fomentarius	Glucose (50)	Yeast extract (3)	$CaCl_2$, $MgSO_4 \cdot 7H_2O$	Silk worm chrysalis (3 g/L)	6.0	25	8	3.64	Chen *et al*., 2008
Fomitopsis pinicola	Glucose (40)	Yeast extract (5) and malt extract (1)	K_2HPO_4, $MgSO_4 \cdot 7H_2O$	–	6.0	25	11	4.4	Choi *et al*., 2007
Ganoderma applanatum	Glucose (60) also maltose	Yeast extract (2)	KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeCl_3$, $MnSO_4$	Glutamic acid(1g/L), biotin (0.5mg/L), thiamine (0.1 g/L)	4.5	25	12	1.35	Lee *et al*., 2007
Gomphidius rutilus	Sucrose (30)	Soybean meal (3)	K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4$, $FeSO_4$	–	8.0	25	6	0.54	Gao *et al*., 2012

Table 1.3 Optimum culture conditions for maximum biopolymers production by different fungal strains (Cont.).

Organism	Carbon (g/L)	Nitrogen (g/L)	Salts and phosphates	Additives	pH	Temp. (°C)	Days	EPS (g/L)	Ref.
Lentinus edodes	Glucose (15.78)	Yeast extract (5.86)	KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$	–	4.48	28	8	0.751	Feng *et al*., 2010
Lepista sordida	Glucose (20)	Peptone (2)	–	–	6.0	28	4	–	Zhong *et al*., 2013
Lyophyllum decastes	Glucose (30)	Yeast extract (20)	–	–	7.0	25	10	2.46	Pokhrel *et al*., 2007
Morchella crassipes	Maltose (44.79)	Tryptone (4.21)	–	–	6.0	28	7	9.67	He *et al*., 2012
Mucor rouxii	Beet-molasses	–	–	–	3.5	28	2	–	Abdel *et al*., 2012
Nigrospora oryzae var. *glucanicum*	Glucose (120)	Yeast extract (1) and urea (0.1)	KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl	–	5.6	28	5	4.5– 5.3	Sudhakaran *et al*., 1988
Oudemansiella radicata	Sucrose (39.3)	Peptone (3.16)	KH_2PO_4, $MgSO_4 \cdot 7H_2O$	–	–	28	5	2.65	Zou *et al*., 2005
Paecilomyces sinclairii	Sucrose (60)	Corn steep powder (10)	KH_2PO_4, K_2HPO_4	–	6.0	30	9	7.4	Kim *et al*., 2002
Paecilomyces teunuipes C240	Glucose (3)	KNO3 (0.4)	KH_2PO_4, $MgSO_4 \cdot 7H_2O$	–	6.0	28	8	2.36	Xu *et al*., 2003
Rhodotorula glutinins	Glucose (30)	Yeast extract (3)	–	–	6.0	25	7	2.06	Ibrahim *et al*., 2012
Tremella fuciformis	Glucose (20)	Tryptone (2)	KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$	–	8.0	28	5	3.05	Cho *et al*., 2006

Microbial biopolymers can be produced by bacteria, yeast, fungi and actinomycetes. Fungal polymers have various applications, produced in large quantities within a short time, easy to isolate and purify. Moreover, upstream and downstream processing of the fungal polymers are easier and shorter time when compared to plant or seaweed polysaccharide production. The fungal EPS have several applications in the food, feed, pharmacy, medicine and cosmetics (Mahapatra and Banerjee, 2013). Fungal bioactive polysaccharides deriving mainly from the Basidiomycetes family (and some from the Ascomycetes) and medicinal mushrooms have been well known and widely used in far Asia as part of traditional diet and medicine, and in the last decades have been the core of intense research for the understanding and the utilization of their medicinal properties in naturally produced pharmaceuticals (Giavasis, 2014).

Polysaccharides exist as a structural component of the fungal cell wall. The fungal cell wall is divided into two groups of polysaccharides: one is a rigid fibrillar of chitin (or cellulose) and the other one is a matrix-like β-glucan, α-glucan and glycoproteins (Zhang *et al*., 2007). Extraction of polysaccharides can be further purified using a combination of techniques such as ethanol precipitation, fractional precipitation, acidic precipitation with acetic acid, ion-exchange chromatography, gel filtration and affinity chromatography. The ethanol precipitation excludes the impurities from the polysaccharides. The separation of acidic and neutral polysaccharides can be achieved by anion-exchange chromatography on a DEAE-cellulose column (Zhang *et al*., 2007).

Several fungal biopolymers showed the antibacterial and viral infections *in vitro* or *in vivo*, owing to the stimulation of phagocytosis of microbes by neutrophils and macrophages (Giavasis, 2014). Also, in another study with fruit body extracts from different *Agaricus* species, a significant antibacterial activity was observed against *S. aureus*, *B. subtilis*, and *B. cereus* (Barros *et al*., 2008). Anti-microbial activity from the biopolymer of *Rhizopus oryzae* ST29 cultivated in POME synthetic medium for 4 days at 45°C gave positive result to antibacterial activity (*Mycobacterium tuberculosis* H37Ra) and antiyeast activity (*Candida albicans* ATCC 90028) (Suyala, 2007). The β-glucan prodution by *Pleurotus tuberregium* was

separated into a number of fractions using hot alkali extracts named HAE. The HAE fractions was used in the *in vitro* experiments using human tumor cell lines HL-60(leukemic cells) and HepG2 (liver cancer) experiments, HAE-17 fraction inhibited the proliferation of the two tumor cell lines with the highest inhibition ratios of 44.5% (at 50 μg/mL) and 63.4% (at 100 μg/mL) for HL-60 and HepG2 cell lines, respectively (Zhang *et al.*, 2001). The biopolymer from *Rhizopus oryzae* ST29 was found to possess the anticancer activity (oral human epidermal carcinoma) after 4 days cultivation at 45 °C in palm oil mill effluent (POME) (Suyala, 2007). EPS from *Rhodotorula glutinins* was used in the preliminary test of anti-tumor using Human hepatocarcinoma cell line (Hep G2), colon carcinoma cells (HCT-116) and lymphoblastic leukemia cells (1301). The effect of the exopolysaccharide on the growth of colon carcinoma HCT-116 cells revealed that the exopolysaccharide had a strong cytotoxic effect on these cells as indicated by its low IC50, 14.7 µg/mL (Ibrahim *et al.*, 2012).

2. Polyglutamate polymer

Polyglutamate (PGA) or poly-γ-glutamic acid is a biodegradable, water soluble and a polyanionic polymer that may be composed of only D (poly-γ-D-glutamate; PDGA), only L (poly-γ-L-glutamate; PLGA) or both glutamate enantiomers (poly-γ-L-D-glutamate; PLDGA). Unlike proteins, which display α-amino like proteins, PGA displays γ-linkage of its component glutamate residues (Candela and Fouet, 2006). PGA is therefore resistant to proteases, which cleave only α-amino bonds. PGA also differs from proteins in being stained with methylene blue, but not different with Coomassie blue. Specific antibodies are required for the sensitive detection of PGA (Candela and Fouet, 2005). PGA can be differentiated into two isoforms, α-PGA and γ-PGA, depending on the attachment of the carboxy group (α and γ, respectively). α-PGA is synthesized chemically by nucleophile-initiated polymerization of the γ-protected N-carboxy anhydride of L-glutamic acid (Figure. 1.1) (Ogunleye *et al.*, 2015).

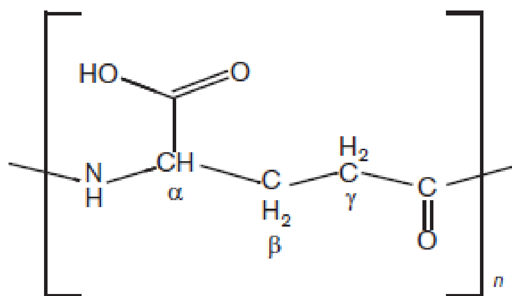


Figure 1.1 Structure of γ-PGA.
Source : Ogunleye *et al*., 2015

PGA is an exocellular capsular polymer produced by several bacteria (all gram-positive), one archaea and one eukaryote. PGA is produced mostly by Gram-positive bacteria. There are the genus *Bacillus*. Among, members of the genus *Bacillus*, *B. subtilis* (Ho *et al*., 2006; Chettri *et al*., 2016;) *B. licheniformis* (Mabrouk *et al*., 2011; Kongklom *et al*., 2012; Chettri *et al*., 2016) and *B. sonorensis.* (Chettri *et al*., 2016). *Staphylococcus epidermidis* also synthesizes surface-associated PGA (Kocianova *et al*., 2016). *Planococcus halophilus* (Kandler *et al*., 1983), *Sporosarcina halophile* (Kandler *et al*., 1983) and the only archaea is *Natrialba asiatica* was isolated from hypersaline soil close to Aswan city (Egypt) which produce extracellular polymer of L-glutamic acid 85% (w/w). The purification of PGA divided into three steps: the centrifugation or filtration for removal of cells, then precipitation with ethanol, methanol or 1-propanal and the dialysis of low molecular (Shih *et al*., 2001). The pure PGA was characterized by molecular mass determination, amino acid analysis, NMR spectroscopy and functional group using Fourier Transform. PGA with a range of molecular masses of PGA produced by Bacillus species ranges from 10kDa to 1,000 kDa (Ashiuchi and Misono, 2002). The production of γ-PGA from *Bacillus licheniformis* in E medium (L-glutamic acid 75 g/L) at pH 6.5, 30 °C for 72h gave the molecular size of γ-PGA band at the size over 98kDa by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Kongklom *et al*., 2012).
The molecular weight of PGA from *Bacillus licheniformis* NCIM 2324 in medium supplemented with 0.5 mM L-glutamine and 10 mM α-ketoglutaric at 37±2 °C for 96h was 5.3×10^5 Da and 5.6×10^5 Da, respectively by gel permeation chromatography (GPC) (Bajaj and Singhal 2009). The γ-(D,L)-Polyglutamic acid produced by

Bacillus subtilis (natto) had the IR absorption spectrum from Fourier transform-IR spectroscopy technique exhibited strong amide absorption at 1620–1655 cm^{-1} , a weaker carbonyl at 1394–1454 cm^{-1}, a strong hydroxyl at 3400–3450 cm^{-1} , a characteristic strong C–N groups at 1085 – 1165 cm^{-1} aliphatic N–H stretching at 2800-2900 cm^{-1} and amide groups, two position, at 1600–1660 and 1390–1450 cm^{-1} (Ho *et al*., 2006). A few reports exist on fungi able to grow on PGA. *Myrothecium* sp. was isolated from a soil can grow on Czapek -Dox agar medium agar with 0.5% PGA pH 4.5 at 25 °C (Tanaka *et al*., 1993). *A. niger* was grown in the medium with glutamic acid (7.92 g/L) at 32 °C for 3 days gave the high biomass (7 g/L) (Aljuboori *et al*., 2012). The γ-PGA have several applications in the food industry, medical, cosmetics, membrane and wastewater treatment (Shih and Van, 2001).

3. *Rhizopus* sp.

The *Rhizopus* group is *Mucorales, Zygomycota*. Colony morphology of *Rhizopus* spp. is rapidly growing, the sporangium morphology is variation in size with species and variety, the apophysis is small, the sporangiospore morphology is round to oval, often 4–6 mm wide (may be larger); ridged, the rhizoids is abundant, often well developed, the sporangiophore morphology is predominantly unbranched; occur above rhizoidal as showm in Figure 1.2, the zygospores is heterothallic and most species is good growth at 37°C and some species able to grown at 50°C (Ribes *et al*., 2000).

The *Rhizopus microsporus* group (*Zygomycota, Mucorales, Mucoraceae*) is a complex group of taxa, which are hard to differentiate morphologically even for a trained observer. The group consists of five species, of which *R. microsporus* has six varieties (Jennessen *et al*., 2008). The differentiating features of the *R. microsporus* group and related small *Rhizopus* species are shown in Table 1.4.

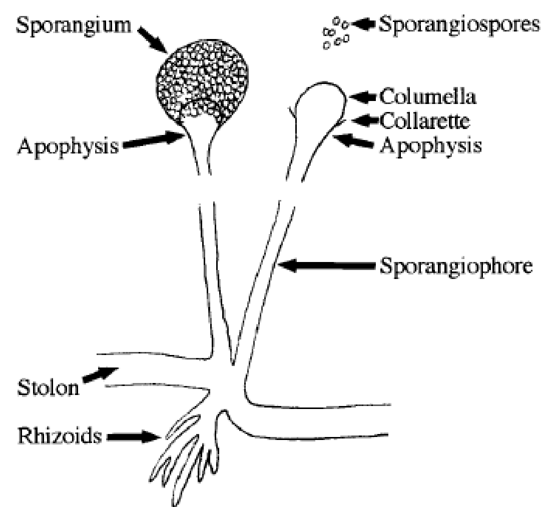


Figure 1.2 Schematic diagram labeling the morphologic structures seen in the sporangium-producing *Rhizopus* spp (not drawn to scale).

Source: Ribes *et al*., 2000

Table 1.4 Differentiating features of the *Rhizopus microsporus* group and related small *Rhizopus* species

Organism name	Sporangiophore grouping and morphology	Sporangiospore morphology	Rhizoid morphology	Zygospore morphology	Other characteristics
R. microsporus var. *rhizopodiformis*	Occur in singly or in clusters of up to 4	Subglobose to rhomboid shape; regular size 4–6 mm; striated and spiny	Rudimentary to intensely ramified	Not known	Good growth at 50°C
R. microsporus var. *microsporus*	Occur singly, in pairs, or clusters of 3	Rhomboidal shape; regular size 5–6 mm; distinctly striated	Simple	Seen with mating	No growth at 50°C
R. microsporus var. *oligosporus*	Generally, occur in clusters	Pleomorphic shapes; variable size 7–10 mm; almost smooth	Simple, with blunt ends	Not known	No growth at 50°C
R. azygosporus	Generally, occur in pairs or clusters	Round to oval shape; regular size 4–7 mm; faintly striated	Simple	Azygospores formed readily within a single isolate; no true zygospores produced; chlamydospores usually produced in abundance	Glabrous growth without sporulation seen at 50°C; optimal sporulation and a zygospore formation between 25 and 37°C
R. schipperae	Occur in large clusters of up to 10	Subglobose to an oval shape; regular size 5 by 7 mm; faintly striated	Simple, rarely branched	Not known	No growth at 50°C; produces abundant Chlamydospores and fails to sporulate on routine fungal media

Source: Ribes *et al*., 2000

Rhizopus microsporus var. *rhizopodiformis* was isolated from soil of the Brazilian ''cerrado'' and produced high levels of amylase activity in Carvalho Peixoto (CP) medium at 45 °C for 72h gave the highest specific activity of amylase (88.25 U/mg protein) (Peixoto *et al*., 2003). Production 1,3-1,4-β-glucanase from *Rhizopus microsporus var. microsporus* in liquid culture medium containing chitin (0.5%) at 40 °C for 48h exhibited the highest activity 8.5 ($U.mL^{-1}$) (Celestino *et al*., 2006).

Like other members of the Zygomycetes class, the filamentous fungus *R. arrhizus* have chitosan polymers in the structure of its cell wall. *R. arrhizus* UCP 402 produce chitosan using corn steep liquor and honey as agroindustrial nitrogen and carbon sources. The results obtained showed higher biomass (11.71 g/L) and chitosan yields (29.3 mg/g) (Cardoso *et al*., 2012). Thermotolerant *R. oryzae* ST29 produce polymer in palm oil mill effluent (POME) medium only at 45°C. The effect of temperature on separation of the biopolymer by incubating the culture broth in a water bath (room temperature to 70 °C) was studied and found no influence on the biopolymer yields (5.58-5.78 g/L) and biomass yields (2.90-3.29 g/L) (Suyala *et al*., 2008). *R.oryzae* produced chitosan in the potato chip processing waste of trimmed potato under the cultivation condition of chitosan obtained from *R. oryzae* was the optimum condition for a peel size of less than 6 mesh, 70% moisture content and a pH of 5 (Kleekayai and Suntornsuk, 2011). Furthermore, the best extraction condition was using 46% sodium hydroxide at 46 °C for 13 h followed by 2% acetic acid at 95 °C for 8 h, giving the maximum chitosan yield of 10.8 g/kg substrate. Fungal chitosan properties were found to be 86–90% degree of deacetylation, the molecular weight of 80–128 kDa and viscosity of 3.1–6.1 mPa.

4. Biopolymers from palm oil mill effluent (POME) and oil palm trunk (OPT)

Oil palm (*Elaeis guineensis*) is the important domestic plant in the south of Thailand. The dramatic increase in demand for the plantation area with an average annual growth rate of 10% during 10 years. Thai government plans to increase the oil palm plantation area to 5 million rai (or 0.8 million ha) in order to have an adequate supply of raw material for biodiesel production (Sattayasamitsathit, 2011). Oil palm plantation was the vegetable oil production which is cooking and many other alternations such as biodiesel. In 2010 production of crude palm oil (CPO) in Thailand reached 1,287,509 tons of which made up 5.1% of total production in 2010. (Dallinger, 2011). Basically, the milling process in Thailand is categorized into a dry process and wet process (standard process) (Prasertsan and Prasertsan, 1996). Palm oil milling process in the wet process generated wastewater in the range 0.44-1.18 m3/tonne fresh fruit bunches (FFB) with the average figure of 0.87 m3/tonne FFB (Salmiati and Salim, 2010). Lignocellulosic biomass which is produced from the oil palm industries includes oil palm trunks (OPT), oil palm fronds (OPF), empty fruit bunches (EFB) and palm pressed fibers (PPF), palm shells and palm oil mill effluent (POME). The solid wastes could be estimated as 386,930 tons of EFB, 165,830 tons of PPF and 110,550 tons of palm kernel shell (PKS) (Prasertsan and Prasertsan, 1996). Therefore, lignocellulosic biomass can be supplied on a large scale basis from low cost raw materials in oil palm industrial wastes.

Lignocellulosic biomass refers to plant material that is mainly composed of three biopolymer; cellulose, hemicellulose and lignin. In nature, plants contain 45-55% cellulose, 25-35% hemicellulose and 20-30% lignin (Deutschmann and Dekker, 2012). Hemicellulose is the second most abundant biopolymer in the plant cell walls. Hemicellulose is composed of several heteropolymers. There are xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan, mannan and xyloglucan and their representatives are different as a function of the plant species (Isikgor and Becer, 2015). Nowadays, hemicellulose is an increasing interest to develop new application in raw materials for chemical industry, food and pharmaceutical industries (Figure 1.3). For example, xylitol that is important applications in pharmaceuticals and food industries produce from hydrolyzed hemicellulose fraction of sugarcane bagasse using activated charcoal and then, the detoxified hydrolysate was used for

xylitol production in batch culture by *Debaryomyces hansenii* cells gave the highest xylitol (13.5g/L) at 60 h of fermentation (Prakash *et al.,* 2011). The xylooligosaccharides (5%) from wheat bran had a beneficial effect on controlling body weight, improving blood glucose and lipid levels and increasing antioxidant status (Wang *et al.,* 2011). Moreover, glucuronoxylan isolated from the seeds of quince for hydrogel production shown the potential to design oral formulations for broader applications in targeted drug release (Ashraf *et al.,* 2017).

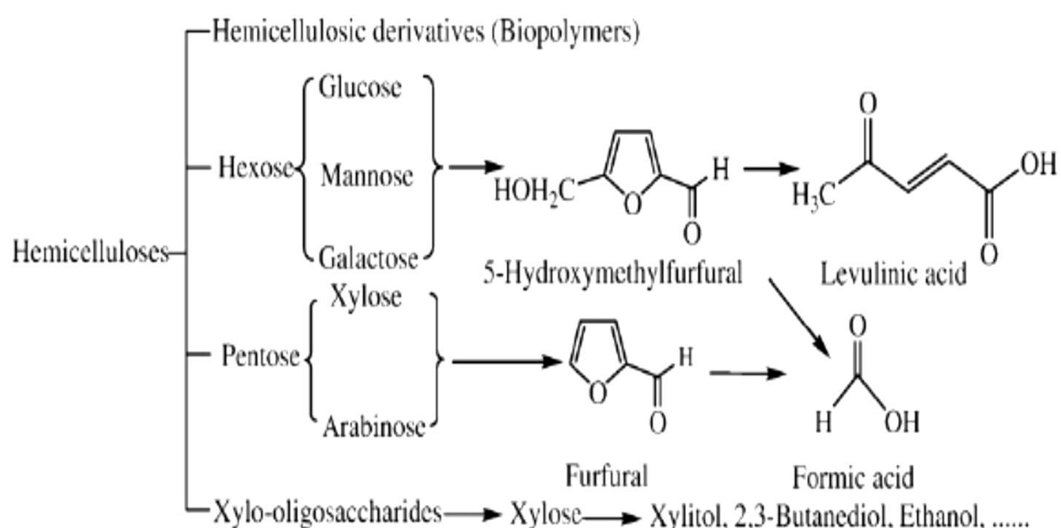


Figure 1.3 The potential products from hemicelluloses
Source: Peng *et al*., 2012

4.1 Palm oil mill effluent (POME)

For every ton of fresh fruit bunches (FFB), it generates abundant amount of POME (28%), palm empty fruit bunch (EFB) (23%), palm oil (21%), fiber (15%), palm kernel (7%) and shell (6%) (Husain *et al.,* 2002; Abdullah and Sulaiman, 2013) and also 0.87 ton of effluent which is very high in organic matter and causes serious problem to the natural water (Husain *et al.,* 2002; Abdullah and Sulaiman, 2013). The liquid waste is generated from an extraction of palm oil of a wet process in a decanter or separator. This liquid waste combined with the wastes from cooling water and sterilizer is called palm oil mill effluent (POME). During POME digestion,

odor released into surrounding air, thus, reduces air quality in the surrounding lagoons area (Abdullah and Sulaiman, 2013). The chemical characteristics of the POME, sterilizer condensate effluent and decanter effluent are shown in Table 1.5.

POME could be converted into useful material using microbial process. Based on these nutrients and acidic condition, the POME could be used as medium for growth of polymer-producing fungi. Biopolymers have various properties such as prebiotic, bioactive compound (Suyala, 2007; Pensawat, 2009). The biopolymer was produced from *Bacillus subtilis* WD161 and *Bacillus* sp. WD79 using decanter effluent (65,442 mg/L COD and 126 mg/L nitrogen) at pH 7.0 and 30ºC for 72h. The biopolymers were identified to be glycoprotein with viscosity of 1214 and 982 cp, respectively. Moreover, they could decrease the organic matter with COD removal of 65.0 and 54.5%, respectively. In addition, biopolymer from the decanter effluent (without cultured) was polysaccharide. There was the molecular weight of 7,499 Da (Chaisorn *et al.,* 2016). Production *Rhizopus oryzae* ST29 cultivated in palm oil mill effluent (POME) at 45 °C for 4 days, crude polymer contain glucose and protein and gave a positive result to antibacterial activity (*Mycobacterium tuberculosis* H37Ra) and antifungal activity (*Candida albicans*) (Suyala, 2007). The sterilizer condensate which is palm oil mill effluent was extracted hemicellulose with 2 folds (v/v) of 95% ethanol gave the high hemicellulose yield of 64.2 g/L (Prasertsan and Oi, 2001).

Table 1.5 Characteristics of POME, sterilizer condensate and decanter effluent from palm oil mill

Parameter	POME[a]	Sterilizer condensate[b]	Decanter effluent[c]
pH	4.2-4.5	5.05	4.3
BOD	22,00-54,300	-	45,275
COD	75,200-96,200	120,000	65,442
Total solids	35,00-42,000	71,900	66,247
Suspended solids	8,500-12,000	43,300	29,480
Oil & grease	8,300-10,600	-	-
Total nitrogen	83-920	800	126
Phosphorus	14.7-21	-	-

Unit in mg/L except for pH

Source a: O-Thong *et al*. (2008), b: Pensawat (2009), c: Chaisorn (2007)

4.2 Oil palm trunk (OPT)

In southern Thailand, old oil palm trees with over 25 years old that need to be cut down for replantation as the yield keeps decreasing about 100,000 hectare (ha) of planting area (Dallinger, 2011) and also cannot be used as timber as is. OPT is a solid waste obtained in large quantities after the felling of oil palm trees estimate 50,000 ha (Dallinger, 2011). OPT is classified as lignocellulosic residues that typically contains 41.02-45.81% cellulose, 17.74-29.12% hemicellulose, and 24.49-24.51% lignin (Khalil *et al.,* 2008; Ang *et al.,* 2013). The composition of OPT was 31.28-42.85% cellulose, 19.73-25.56% hemicellulose, 10.74-18.47% lignin, 1.63-2.25% protein, 1.60-1.83% fat, 1.12-1.35% ash and with trace amounts of mineral content (0.01-0.40%) (Table 1.6) (Noparat *et al.,* 2013).

Table 1.6 Composition analysis at various heights and positions of oil palm trunk (% dry weight)

Composition	Top (%)	Middle (%)	Bottom (%)
Cellulose	42.85 ±2.5	40.65 ±2.8	31.28±3.5
Hemicellulose	25.56±3.4	22.12 ±1.5	19.73 ± 2.1
Lignin	10.74 ±1.8	15.32 ±2.2	18.47 ±1.6
Protein	2.25 ± 0.05	1.84 ± 1.2	1.63 ± 0.4
Fat	1.83±0.02	1.61±0.02	1.60 ±0.01
Ash	1.12 ±0.03	1.17 ±0.03	1.35±0.02
N	0.26±0.05	0.25±0.01	0.23±0.01
P	0.06±0.01	0.06±0.01	0.06±0.01
K	0.40±0.05	0.40±0.01	0.23±0.01
S	0.10±0.01	0.10±0.01	0.10±0.01
Ca	0.12±0.01	0.12±0.01	0.15±0.01
Mg	0.13±0.01	0.13 ±0.01	0.11±0.01
Fe	0.03 ±0.01	-	-
Zn	0.0.1±0.01	-	-

Results are means ±SD of three determinations.

Source: Noparat *et al.,* 2011

OPT could be used for production of many value-added products. Ethanol and lactic acid could be produced not only from a hydrolysate of OPT but also from oil palm sap (OPS) squeezed from OPT due to its high glucose content. The glucose concentration of the OPS from the inner part was 85.2 g/L and decreased towards the outer part. Other sugars found in relatively low concentrations were sucrose, fructose, galactose, xylose, and rhamnose (Kosugi *et al*., 2010). In addition, OPS was found to be rich in various kinds of amino acids, organic acids, minerals and vitamins. It was used to produce ethanol using the sake brewing yeast strain, *Saccharomyces cerevisiae* Kyokai no.7 without the addition of nutrients, at a comparable rate and yield to the reference fermentation on YPD medium with glucose as a carbon source (Norhazimah and Faizal, 2014). Lactic acid, a promising material for bio-plastics, poly-lactate, was produced from the sap using the homolactic acid bacterium *Lactobacillus lactis* ATCC19435 (Kosugi *et al*., 2010). The optimum condition for production of cellulases and xylanase by *A. fumigatus* SK1 using untreated oil palm trunk (OPT) as a carbon source under solid-state fermentation (SSF) was found to be 80% moisture level and initial pH 5.0. The enzymes activities obtained were 54.27, 3.36, 4.54 and 418.70 U/g substrates for endoglucanase (CMCase), exoglucanase (FPase), β-glucosidase and xylanase respectively (Ang *et al*., 2013).

5. Application of the biopolymers

The biopolymers have several applications in the food, pharmacy, feed, medicine, cosmetics and industries owing to their diversity in composition, structure, and physical properties (Mahapatra and Banerjee., 2013).

5.1 Hydrogel

Hydrogels are hydrophilic polymers with three-dimensional networks that can absorb water without dissolving. Several methods have been reported for the preparation of hydrogels *via* co-polymerization or crosslinking reactions by the use of a functional group of monomers which act as crosslinking agents. A chemical initiator is used to start the polymerization reaction. The key feature of hydrogels with network structures is their ability to store a lot of water and biological fluid. Subsequently, the

hydrogels can be used in many applications, such as the absorption of heavy metals, drug delivery systems and materials for wound healing.

The hydrogel products can be divided on different bases as detailed of samples. For example, hydrogels can be classified into two groups into natural or synthetic origins based on source while it can be divided into two categories based on the chemical or physical nature of the cross-link junctions.

Preparation of hydrogel as three-dimensional crosslinked hydrophilic polymer networks can be designed with controllable two responses include physical; temperature, electric or magnetic field, light, pressure, and sound, and chemical stimuli; pH, solvent composition, ionic strength, and molecular species (Ahmed 2015).

Hydrogels can be prepared from synthetic polymers or natural polymer because they have precise chemical structure and can be designed at molecular level (Liu *et al*., 2012). Especially, hydrogel from natural such as chitosan (Liu *et al*., 2012; Wu *et al*., 2012) and hemicellulose (Tanodekaew *et al*., 2006; Wu *et al*., 2012) have a safe use, biodegradability and low toxicity. Preparation of hydrogel from xylan from beechwood was modified with maleic anhydride (MA) and blended polyvinyl alcohol (PVA) showed the high swelling degree (350%) while low compressive strength (2N) at 1:1 weight ratio of xylan to MA with 0.5 of PVA (weight fraction) and the gel was noncytotoxic and had a potential for biomedical application (Tanodekaew *et al*., 2006). Polyvinyl alcohol/polyvinyl pyrrolidone (PVA/PVP) hydrogel has been prepared by using γ-irradiation technique found that the PVA/PVP hydrogel (0.0/100 wt.%) possess the highest swelling > 20 g/g at 20 kGy of irradiation dose and the ratio of PVA/PVP under condition 100/0, 90/10, 80/20, 70/30, 50/50, 0/100 (wt.%) at 40 kGy of irradiation dose exhibited antimicrobial activity (*Bacillus subtilis*) was very sensitive (Abd El-Mohdy and Ghanem, 2009).

Some functional features of an ideal hydrogel material can be listed as follows: the highest absorption capacity (maximum equilibrium swelling), the lowest price, the highest durability and stability in the swelling environment and during the storage, the highest biodegradability without formation of toxic species following the degradation, pH-neutrality after swelling in water, colorlessness, odorlessness and re-

wetting capability (if required) the hydrogel has to be able to give back the imbibed solution or to maintain it;depending on the application requirement (Ahmed 2015). In fact, a hydrogel sample not achieves altogether mentioned required features. Consequently, Ahmed (2015) suggested of hydrogel production, in practice, the production reaction variables must be optimized such that an appropriate balance between the properties is achieved.

5.2 Xylooligosaccharide (XOS)

XOS is sugar oligomers made up of xylose units that contain 2 to 10 xylose molecules linked by β 1-4 bonds in Figure 1.4. XOS can be produced at industrial scale from xylan-rich materials. Hemicellulose is rich in xylan, a polysaccharide used to develop technology for producing alcohol, xylose, xylitol and XOS.

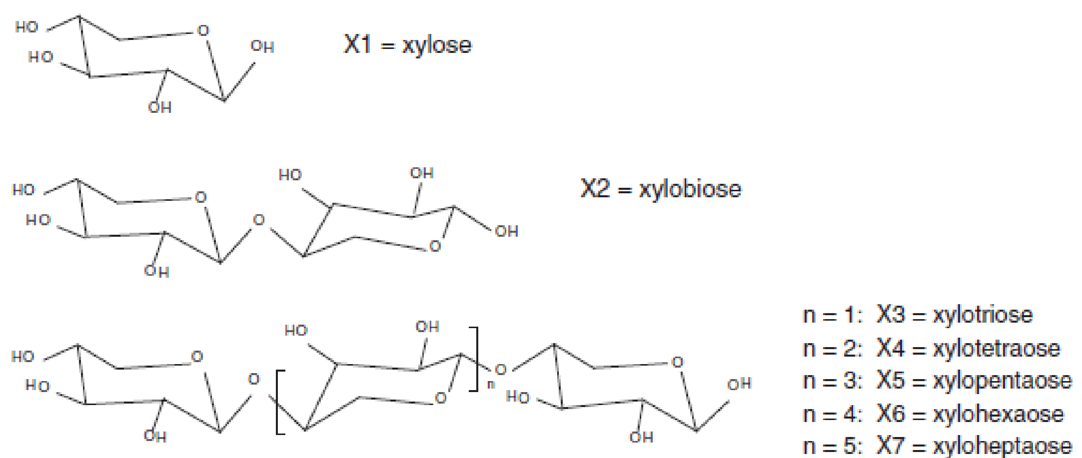


Figure 1.4 Schematic structure of xylose and xylooligosaccharides.
Source: Carvalho *et al*., 2013

Xylooligosaccharide were reported to exhibit prebiotic properties which acted as a food supplement in the functional food industry (Crittenden and Playne, 1996; Vazquez *et al*., 2000). To characterize any biopolymer, enzymatic or chemical decomposition of the biopolymer to their monosaccharide building blocks should be carried out followed by identification and quantification of each component by gas chromatography (GC) or high-performance liquid chromatography (HPLC). Characterizing oligosaccharide released during biomass pretreatment or enzymatic

hydrolysis can reveal important structural information about hemicellulose polysaccharides in plant cell walls (Qing *et al*., 2013).

Xylooligosaccharides are naturally present in fruits, vegetables, bamboo, honey and milk and can be produced at industrial scale from xylan-rich materials. Of particular interest are those sources of residual origin, such as forestal, agricultural or industrial wastes of lignocellulosic nature. Processing of residual vegetable biomass as a raw material offers economic and ecological benefits since it is a biorenewable, widely distributed and abundant resource. The growing commercial importance of these nondigestible oligosaccharides is based on their beneficial health properties, particularly the prebiotic activity (Gupta *et al*., 2012). Xylooligosaccharides also have excellent potential for applications in pharmaceutical, agriculture, and food industry (Qing*et al*., 2013).

Hemicellulose from oil palm frond fibres was treated autohydrolysis method using an autoclave at 121°C for 60 min and then, using enzymatic treatment of the hemicellulosic fraction with 8 U of *Trichoderma viride* endo-(1,4)-b-xylanases (EC 3.2.1.8) per 100 mg of autohydrolysate at 40 °C for 24 h gave 17.5% xylooligosaccharides which were contributed mainly by xylobiose (13.89%) and 25.6% xylose (Hanim *et al*., 2011). Oil palm empty fruit bunches (OPEFB) fibre was heated to final temperatures between 150°C and 220°C in a non-isothermal autohydrolysis treatment gave the highest XOS concentration (17.6 g/L) at 210°C (Ho *et al*., 2014). The xylan from *Sehima nervosum* grass was treated 12% NaOH and steam and then enzymatic hydrolysis of the alkali solubilized xylan by endoxylanase from *Trichoderma viridae* to produce XOS and response surface model (RSM) was used to optimize the XOS. It was reported that the highest xylobiose yield (11 g/100g xylan) at pH 5.03, temperature 45.19 °C, reaction time 10.11 h with enzyme dose 17.41 U and xylotriose yield, ideal hydrolysis conditions were pH 5.11, temperature 0.33 °C, reaction time 16.55 h with enzyme dose 13.20 U (Samanta *et al*., 2012). Production of XOS from garlic straw xylan that extracted with alkali by 12U/g of purified xylanase from *Bacillus mojavensis UEB-FK* was carried out at 50 °C, shaking at 100 rpm after 8h hydrolysis gave the maximum XOS yield (29 ±1.74%) and their composed of xylobiose, xylotriose, xylotetrose and together with a small amount of xylopentaose and xylohexose (Kallel *et al*., 2015).

Pedraza, *et al.* (2014) studied the prebiotic activity of xylooligo-saccharides from xylan. Xylan was obtained through thermochemical pretreatment of the corncob and its solution was analyzed by HPLC. Among the tested species of Lactobacilli, the growth of *L. acidophillus* in the presence of xylooligosaccharides showed the most notable effect. For *L. rhamnosus,* the population remained active at 24 and 48 h when XOS are added to the media culture. The other strains do not modify their behavior with supplementation of prebiotics. The co-culture of *L. brevis, L. plantarum, L. acidophilus, L. rhamnosus* and *Escherichia coli* showed that the growth of the challenge microorganism was inhibited. The same result was found with the XOS obtained from the reagent grade xylan or from the corncob.

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation reactions can produce free radical, which start a chain reaction that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. The antioxidant activity of xylooligosaccharides was studied XOS from xylan-rich hemicelluloses isolated by using potassium hydroxide from sugarcane bagasse and then, hydrolysis with crude xylanase secreted from *Pichia stipitis* showed the antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Bain, *et al.* 2011).

6. Objectives

1. Selection of synthetic medium and production of biopolymer from *Rhizopus microsporus* ST4
2. Extraction of the biopolymers from *Rhizopus microsporus* ST4, palm oil mill effluent (POME) and oil palm trunk (OPT)
3. Characterization of the biopolymers from *Rhizopus microsporus* ST4, palm oil Mill effluent (POME), oil palm trunk (OPT) and commercial xylan
4. Application of the biopolymers to produce hydrogel and xylooligosaccharied

CHAPTER 2

PRODUCTION AND CHARACTERIZATION OF BIOPOLYMER FROM *RHIZOPUS MICROSPORUS* ST4

2.1 Abstract

Six synthetic media were use to screen for polymer production from *Rhizopus microsporus* ST4 cultivated at 45 °C under shaker condition (200 rpm). The highest biopolymer yield (12.56%) was obtained from medium A, followed by medium D (0.70%), B (0.25%), E (0.17%) and medium C (0.08%), respectively, and no polymer production in medium F. Studies on the effect of cultivation temperature at room temperature (28 ±2ºC) and 45ºC on biopolymer production from *R. microsporus* ST4 indicated that the strain produced by the biopolymer only at 45ºC (12.33 g/L) but not at room temperature (28 ±2ºC) despite the cell growth (7.08 g/L). Analysis of amino acid profile revealed that the biopolymer ST4 contained 99.3% of glutamic acid with a trace amount of lysine, aspartic acid, alanine and threonine. Therefore, the biopolymer from *R. microsporus* ST4 was considered to be polyglutamic acid. The molecular size of the biopolymer ST4 was band at the size 80 kDa using SDS-PAGE method Solubility tests on the biopolymer ST4 showed that DW was the best to solubilize.

2.2 Introduction

The biopolymers have received increasing interest, mainly because of their useful physiochemical features and uncomplicated biodegradability in the natural environment. Microbial extracellular polysaccharides (EPS) are polymers produced by various strains with different properties. *Bacillus* sp. I-47 (Kumar *et al*., 2004), *Vagococcus* sp. W31 (Jie *et al*., 2006) and *Halomonas* sp. OKOH (Mabinya *et al*., 2011) produce polysaccharide bioflocculants. *Nocardia amarae* YK-1 (Koizumi *et al*., 1991), *Bacillus licheniformis* (Shih *et al*., 2001) and *Rhodococcus erythropolis* (Takeda *et al*., 1991) produced protein flocculant. *Arcuadendron* sp. TS-4 (Lee *et al*., 1995), *Arathrobacter* sp. (Wang *et al*., 1995), *Halobacillus* sp. Mvuyo (Cosa *et al*., 2012) produce glycoprotein bioflocculant whereas *Bacillus* strains produced poly-γ-glutamate (PGA) (Ashiuchi and Misono, 2002). PGA producing bacteria are mainly *Bacillus subtilis, B. licheniformis, and B. anthracis* (Chettri *et al.,* 2016; Candela *et al.,* 2005). Tanaka *et al.* (1993) reported that *Myrothecium* sp. TM-4222 was able to grow on PGA medium.

Biopolymers produce mostly from bacteria with less common among yeasts and fungi. Types of fungal biopolymers mainly depend on the type of strain used, physical conditions during fermentation and type of medium components used for the production. The fungal EPS have been recognized as high-value biomacromolecules with several applications in industries, pharmaceuticals, medicine, foods, environmental etc (Mahapatra and Banerjee, 2013). For example, the EPS was produced from *Rhodotorula glutinins* in glucose (3%) and yeast extract (0.3 %) at 25℃ h and its showed the biological activity as antioxidant, antiviral and anti-tumor (Ibrahim *et al*., 2012). *Ganoderma lucidum*, *Phellinus rimosus*, *Pleurotus florida* and *Pleurotus pulmonaris* possessed profound antioxidant and antitumor activities (Ajith and Janardhanan, 2007). A yeast-like fungus *Aureobasidium pullulans* (Singh *et al*., 2008; Ravella *et al*., 2010; Wu *et al*., 2012) produced a large amount of extracellular polysaccharide so-called pullulan. The pullulan can be used as food science, health care and also pharmacy fields. *Rhizopus arrhizus* UCP 402 produce chitosan (29.3 mg/g) using corn steep liquor and honey as agroindustrial nitrogen and carbon sources at 28 °C (Cardoso *et al*., 2012). Besides *Ganoderma lucidum*, *Agaricus blazi*, *Cordyceps* sp. and *Lentinus edodes* (Mahapatra and Banerjee, 2013), *Morchella*

crassipes (He *et al*., 2012) and *Grifola frondosa* (Tao *et al*., 2018) also produce EPS. Most of the fungal strains produced maximum EPS within a temperature range 22 °C to 30 °C (Mahapatra and Banerjee, 2013). Only a few reports on EPS or other biopolymers produced from fugal at a higher temperature. The thermotolerant polymer producing *Rhizopus* sp. ST4 and *Rhizopus* sp. ST29 was isolated from a palm oil mill were previously studied by Muneesri (1995) and found to COD removal in POME under aseptic condition *Rhizopus* sp. ST29 exhibited higher COD removal (66%) than *Rhizopus* sp. ST4 (53.4%) (Pechsuth *et al*., 2001). The thermotolerant fugal isolate *Rhizopus* sp. ST29 was identified to be *R. oryzae* ST29 and found to produce biopolymer in POME medium at 45 °C (Suyala *et al*., 2008). The thermotolerant *R. microsporus* var. *rhizopodiformis* was isolated from soil and produced amylase at 45 °C in liquid Carvalho Peixoto (CP) medium gave the highest specific activity 88.25 U/mg protein (Peixto *et al*., 2003). The taxonomic of *Rhizopus* group is *Zygomycota* phylum, *Zygomycetes* class, *Mucorales* order, Mucoraceae family and its can growth between 25°C and 50°C (Ribes *et al*., 2000). Nowadays, the information on fungal biosynthesis is scarce (Mahapatra and Banerjee, 2013). On the other hand, the research for new fungal species can produce novel EPS is still needed. Thus, the fungal species *Rhizopus* sp. was considered as great candidates for the production of biopolymer at a high temperature.

This study aims to assess the effect of synthetic media and temperatures on growth and production of extracellular biopolymer from thermotolerant *R. microsporus* ST4, then characterized the biopolymer.

2.3 Materials and methods

2.3.1 Microorganism and culture condition

R. microsporus ST4 was isolated from palm oil mill effluent (The Southern Palm (1978) Co., Ltd.) by Kaewthong (1995). The strain was identified at National Center for Genetic Engineering and Biotechnology (BIOTEC) by PCR and nucleotide sequencing technique based on nuclear ribosomal DNA (rDNA), amplification and sequencing of the internal transcribed spacer (ITS) regions of rDNA were performed using universal primers (ITS5/ITS4) (Prasertsan *et al*., 2015, unpublished data). The strain is maintained on PDA slant at 4°C and subcultured every month.

2.3.2 Culture media

The composition of each medium was as follow:

2.3.2.1 Medium A contain 2% glucose, 0.05% yeast extract, 5% glutamic acid and 0.05% $MgSO_4 \cdot 7H_2O$ (Yokoi *et al*., 1995) with pH 5.8 (Cardoso *et al*., 2012).

2.3.2.2 Medium B contain 1% glucose, 0.2% yeast extract, 0.1 % NH_4CI, 0.01 % $MgSO_4 \cdot 7H_2O$ and 0.05 % $MnSO_4 \cdot 4H_2O$ (Lee *et al*., 1995) with pH 5.8 (Cardoso *et al*., 2012).

2.3.2.3 Medium C contain 1% glucose, 0.05% urea, 0.05% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCI (Kurane *et al*., 1986) with pH 5.8 (Cardoso *et al*., 2012).

2.3.2.4 Medium D contain 2% glucose, 1% polypeptone, 0.05% yeast extract, 0.2% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (Yokoi *et al*., 1997) with pH 5.8 (Cardoso *et al*., 2012).

2.3.2.5 Medium E contain 1% glucose, 0.4% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.6% Na_2HPO_4 (Takeda *et al*., 1992) with pH 5.8 (Cardoso *et al*., 2012).

2.3.2.6 Medium F contain 2% glucose, 2% citric acid and 1% $(NH_4)_2SO_4$ (Kunioka *et al*., 1997) with pH 5.8 (Cardoso *et al*., 2012).

In medium preparation glucose was autoclaved at 115°C for 15 min, urea was sterilized by using syringe filter and for preparation of agar plate, 1.5% agar was added.

2.3.3 Growth and crude biopolymer yield analysis

The dry cell weight (DCW) was determined by filtering the fungal mycelium through a cheese cloth until a clear filtrate was obtained. The mycelia were washed twice with distilled water and dried overnight at 103 °C in a hot air oven until constant weight was obtained, then cooled in a desiccator and weighed (Adapted from Feng *et al*., 2010).

The clear supernatant obtained after centrifugation (3,635 x g) at 4 °C for 20 min (Pensawat, 2009) was used for extraction of the crude biopolymers by precipitation with 4 volume of cold 95 % ethanol (4°C) overnight, then centrifugation (10,000 rpm) at 5°C for 15 min. The obtain precipitate was dried at 105°C in a hot air

oven for 24 h, then weigh to constant weighted after cooling in a desiccator (Demlim, 1999).

2.3.4 Effect of culture medium, cultivation temperature and time on growth and biopolymer production from *R. microsporus* ST4

2.3.4.1 Effect of culture medium on growth and biopolymer production

Spore suspension was prepared by cultivation of *R. microsporus* ST4 on PDA at 45 °C for 3 days and adding 10 ml of 0.1% Tween 80, then adjusting the suspension to reach 2.4×10^6 spore/mL (Adapted from Karim and Kamil, 1989). The 10 % spore suspension of *R. microsporus* ST4 was inoculated into 250 ml Erlenmeyer flask containing 100 ml each of following media: A, B, C, D, E and F broth and incubated at 45°C for 3 days on a rotary shaker (200 rpm). Samples were measured for growth (DCW) and crude biopolymer yield. For preparation of agar plate, 1.5% agar was added in each media and samples were indicated by growth zone to colony every day. The medium giving the highest crude biopolymer concentration was selected for further studies.

2.3.4.2 Effect of cultivation temperature on growth and biopolymer production from *R. microsporus* ST4

The selected culture medium (result from 2.3.4.1) was used to study on the effect of cultivation temperature on growth and biopolymer production from *R. microsporus* ST4. The experiment was carried out as above but incubated at room temperature (28 ±2ºC) and 45ºC on a shaker (200 rpm) for 3 days. The samples were measured for growth (DCW) and crude biopolymer yield.

2.3.4.3 Effect of cultivation time on growth and biopolymer production from *R. microsporus* ST4

The experiment was conducted as described above using the selected culture medium and cultivation temperature (from 2.3.4.2) on a shaker (200 rpm) for 7 days to determine the optimum cultivation time for growth and polymer from *R. microsporus* ST4. Samples were taken every day to determine for pH, growth (DCW) and crude biopolymer yield.

2.3.5 Characterization of the biopolymer from *R. microsporus* ST4

2.3.5.1 Preparation of the biopolymer from *R. microsporus* ST4

R. microsporus ST4 was cultivated in the selected culture medium at the optimum temperature and time (result from 2.3.4.3) to obtain the highest biopolymer yield. The biopolymer was extracted (as described in 2.3.3). After centrifugation (10,000 rpm) at 5°C for 15 min, the precipitate biopolymer was dissolve in deionized water and centrifuged to remove insoluble materials. The supernatant was dialyzed (molecular weight cutoff 8,000 Da) against deionized water at 4 against deionized water at 4 °C for 24 h with 2 times of fresh deionize water and then lyophilized to obtain partially purified polymers (socalled biopolymer ST4) (Chaisorn *et al*., 2016).

2.3.5.2 Amino acid profile of the biopolymer from *R. microsporus* ST4

The amino acid profile of the biopolymer from *R. microsporus* ST4 (socalled biopolymer ST4) was quantitatively analyzed by HPLC (Waters Alliance 2695) using Hypersil gold column and Jasco FP2020 fluorescence detector at Central Instrument Facility in Mahidol University, Bangkok, Thailand. The biopolymer ST4 (0.6 g) was hydrolyzed by adding 5 mL of 6N HCl and putting it in a heating block at 110°C for 22 h. The internal amino acids standard was added in the hydrolysate and diluted with deionized water, then filtered through a 0.45 μm membrane filter. Each filtrate was mixed with AccQ-fluor reagent to derivatize, then heated at 55 °C for 10 min in heating block and injected into HPLC (Bosch *et al*., 2006).

2.3.5.3 Functional group analysis

The biopolymer ST4 (1 mg) was homogenized with 99 mg of KBr for 1 min and pressed into a disc. The FT-IR spectra of the sample was recorded with 16 scans at the resolution of 4 cm^{-1} between the wavenumbers of 400 and 4000 cm^{-1} using Fourier Transform Infrared Spectrometer (FTIR) (Vertex70, Bruker, Germany).

2.3.5.4 Molecular size estimation

The molecular size of the biopolymer ST4 was measured by the mobility on the gel. It was estimated by using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 10% acrylamide and standard Perfect Protein™ Makers (Meack, USA) which consists of 10-225 kDa. The

biopolymer ST4 was then visualized by Coomassie blue Stain. (Adapted from Kongklom *et al*., 2012).

2.3.5.5 Solubility test

The biopolymer ST4(30 mg) was tested for its solubility in 15 ml of the following: distilled water (DW), dimethylsulfoxide (DMSO), ethanol, methanol, acetone, ethylacetate, acetonitrile, benzene, chloroform, hexane, hexadecane and isooctane, in a closed container under constant agitation (200 rpm) at room temperature (28±2°C) overnight (Marques., 2014). The sample was centrifuged at 850×g for 15 min (Noor *et al*., 1999). The percentages solubility was calculated.

$$S = \frac{Wo - Wr}{Wo} \times 100 \qquad (1)$$

where S is the solubility degree, Wr is the weight of undissolved biopolymer residue, and Wo is the original weight of biopolymers.

2.3.5.6 Thermal stability

Thermal behavior of the biopolymer ST4was performed using thermogravimetry-differential thermal analysis (TGA-DTA) in a Simultaneous Thermal Analyzer (STA8000, perkin Elmer, USA). The sample weighed between 4 and 20 mg was heated from room temperature to 700°C at a rate of 10ºC/min in nitrogen.

2.4. Results and Discussion

2.4.1 Effect of culture medium, cultivation temperature and time on growth and biopolymer production from *R. microsporus* ST4

2.4.1.1 Effect of culture medium on growth and biopolymer production

R. microsporus ST4 was cultivated in several synthetic media (A, B, C, D, E and F) on agar plate at 45°C for 3 days. Growth was observed every day and recorded qualitatively (+,-) (Table 2.1 and Figure 2.1). The strain showed very good growth (≥9 cm) in the A, B, C and D agar plate medium, good growth (7-9 cm) in the medium E and no growth in the medium F (Figure 2.1).

Cultivation of *R. microsporus* ST4 in several synthetic media in broth (A, B, C, D, E and F) at 45°C for 3 days indicated that A medium gave the highest mycelial growth (5.95 g/L) and biopolymer production (12.56 g/L) (Figure 2.2). Growth was much lower in the medium D (2.16 g/L), B (3.14 g/L), C (1.61 g/L) and

E (0.84 g/L), respectively, and no growth in the medium F. Besides medium A, the strain ST4 produced very low amount biopolymer in the medium D (0.70 g/L), B (0.25), E (0.17 g/L) and C (0.08 g/L), respectively. The main factors affecting fungal biopolymer production are divided into three groups: medium composition, physical conditions and mode and methods of fermentation (Mahapatra and Banerjee, 2013). The medium composition includes the carbon source, nitrogen source, different salts and special additives and their concentration. Different types of carbohydrate are used as carbon source in the culture medium such as glucose, sucrose and maltose. The source and concentration of carbon depend on microorganism. In this study, 2% glucose was used in medium A, D and F while 1% glucose was used in the medium B, E and C. The medium A, B, C, D, E and F were lower concentration of glucose than that the cultivation of *R. glutinins* (3% glucose) but 6 folds higher maximum EPS (2.6 g/L) (Ibrahim *et al,*. 2012). Similar results was obtained from the EPS production from *R. mucilaginsa* CICC 33013, (2% glucose) and 6.2 g/L EPS (Ma *et al.,* 2018). Nitrogen source is an important addition needed for biopolymer production from fungi for cell growth, the enzyme synthesis and product formation. The nitrogen source can be classified into organic and inorganic nitrogen. The organic nitrogen include yeast extract, peptone, urea and polypeptone and inorganic nitrogen consist of $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 are most often added in medium. The organic nitrogen was used in the medium A and D containing yeast extract and polypeptone. The medium B, E and C used both organic and inorganic nitrogen including yeast extract, urea, $(NH_4)_2SO_4$ and NH_4Cl, whereas medium F used only inorganic nitrogen; $(NH_4)_2SO_4$. It can be clearly seen that *R. microsporus* ST4 need organic nitrogen in the medium A, B, C, D and E than inorganic nitrogen (medium F) for mycelial growth and biopolymer production.

Table 2.1 Effect of several synthetic media on mycelial growth of *R. microsporus* ST4 on agar plate during 3 days cultivation at 45 °C.

Medium formulas*	Growth (Days)		
	1	2	3
A	+	++	++++
B	+++	++++	++++
C	++	++++	++++
D	+++	++++	++++
E	++	+++	+++
F	-	-	-

Remarks ++++ = very good growth (≥9 cm)

+++ = good growth (7-9 cm)

++ = medium growth (3-6 cm)

\+ = little growth (0.1-2 cm)

\- = no growth

* (Medium A contain 2% glucose, 0.05% yeast extract, 5% glutamic acid and 0.05% $MgSO_4 \cdot 7H_2O$; Medium B contain 1% glucose, 0.2% yeast extract, 0.1 % NH_4CI, 0.01 % $MgSO_4 \cdot 7H_2O$ and 0.05 % $MnSO_4 \cdot 4H_2O$; Medium C contain 1% glucose, 0.05% urea, 0.05% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCI; Medium D contain 2% glucose, 1% polypeptone, 0.05% yeast extract, 0.2% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$; Medium E contain 1% glucose, 0.4% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.6% Na_2HPO_4 and Medium F contain 2% glucose, 2% citric acid and 1% $(NH_4)_2SO_4$).

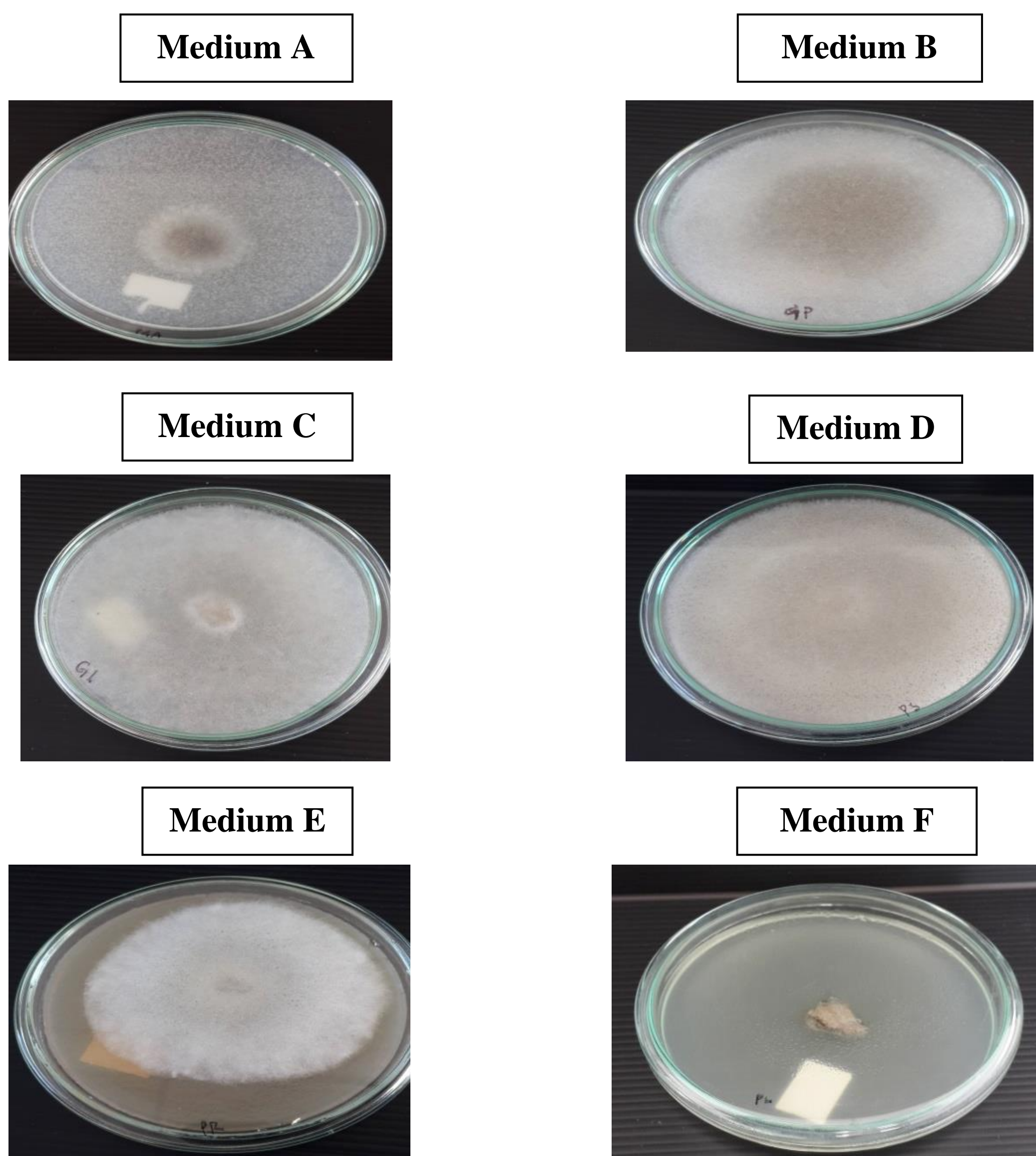


Figure 2.1 Comparison on the growth of *R. microsporus* ST4 cultivated in different synthetic media on agar plate at 45°C for 3 days. Medium A contain 2% glucose, 0.05% yeast extract, 5% glutamic acid and 0.05% $MgSO_4 \cdot 7H_2O$; Medium B contain 1% glucose, 0.2% yeast extract, 0.1 % NH_4CI, 0.01 % $MgSO_4 \cdot 7H_2O$ and 0.05 % $MnSO_4 \cdot 4H_2O$; Medium C contain 1% glucose, 0.05% urea, 0.05% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCI; Medium D contain 2% glucose, 1% polypeptone, 0.05% yeast extract, 0.2% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$; Medium E contain 1% glucose, 0.4% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.6% Na_2HPO_4 and Medium F contain 2% glucose, 2% citric acid and 1% $(NH_4)_2SO_4$.

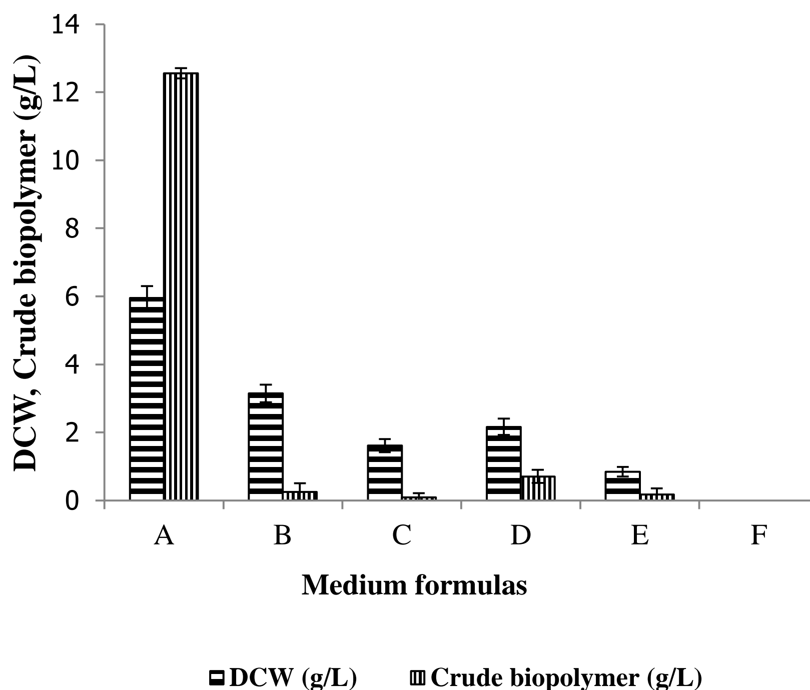


Figure 2.2 Effect of synthetic media on mycelial growth and biopolymer production from *Rhizopus microsporus* ST4 after cultivation at 45 °C for 3 days on a shaker (200rpm). Medium A contain 2% glucose, 0.05% yeast extract, 5% glutamic acid and 0.05% $MgSO_4 \cdot 7H_2O$; Medium B contain 1% glucose, 0.2% yeast extract, 0.1 % NH_4CI, 0.01 % $MgSO_4 \cdot 7H_2O$ and 0.05 % $MnSO_4 \cdot 4H_2O$; Medium C contain 1% glucose, 0.05% urea, 0.05% yeast extract, 0.5% K_2HPO_4, 0.2% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCI; Medium D contain 2% glucose, 1% polypeptone, 0.05% yeast extract, 0.2% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$; Medium E contain 1% glucose, 0.4% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.6% Na_2HPO_4 and Medium F contain 2% glucose, 2% citric acid and 1% $(NH_4)_2SO_4$.

The special additives in medium included glutamic acid, nicotinic acid, biotin and vitamins were also studied (Lin and Sung, 2007; Lee *et al*., 2007and Xiao *et al*., 2010). Glutamic acid, biotin, and thiamine in culture medium are required for EPS production (1.25 g/L) from *Ganoderma applanatum* (Lee *et al*., 2007). Production of a bioflocculant (PM-5) from *A. niger* used glutamic acid (0.79%) in the medium and cultivated for 3 days at 32 °C. Glutamic acid was the most favorable for PM-5 production and gave higher biomass (up to 6 g/L) and flocculating rate (up to 81%) (Aljuboori *et al.,* 2014). In this case, *R. microsporus* ST4 gave the highest mycelial growth and biopolymer production in medium A that contained glutamic acid. Thus, medium A was chosen for the next experiments.

In this experiment, to study the evaluation of growth and biopolymer production by *R. microsporus* ST4 in medium A which modified medium A. Four media as described in Table 2.2 were incubated at 200 rpm and 45°C for 3 days.

Table 2.2 The formulas of modified medium A for growth and biopolymer production by *R. microsporus* ST4

Formulas	**Medium**		
	A1	**A2**	**A**
5% glutamic acid	-	/	/
2% glucose	/	-	/
0.05% yeast extract	/	-	/
0.05% $MgSO_4 \cdot 7H_2O$	/	-	/
R. microsporus ST4	/	/	/

Medium A1 and A2 were used for growth and biopolymer production by *R. microsporus* ST4 in comparison with medium A (control). The result showed that *R. microsporus* ST4 could be growth (3.9 g/L DCW) and produce biopolymer (12.2 g/L) outstanding in medium A consist with 5% glutamic acid, 2% glucose, 0.05% yeast extract and 0.05% $MgSO_4 \cdot 7H_2O$ (Figure 2.3) while medium A1 and A2 gave the mycelial growth (0.3-1.6 g/L DCW) and

biopolymer production (0.04-1.2 g/L). Poly glutamic acid (PGA) is synthesized by several bacteria, especially those of *Bacillus* species and a few reports exist on the eukaryote. Generally, the function of organism produce PGA depends on the organism producing it and the environment the organism inhabits and the function of peptidoglycan bound which may help confer virulence or it can act as a source of glutamate under conditions of starvation or released into the environment, PGA can help the organism survive under adverse conditions (Ogunleye *et al*., 2015). Many reports had been described the nutrient requirements, PGA producing bacteria are categorized into require and not require glutamic acid in the medium (Shih and Van, 2001). Therefore, a source of energy in medium A including glutamic acid, glucose, yeast extract and $MgSO_4 \cdot 7H_2O$ are required for *R. microsporus* ST4 growth and its biopolymer production. The glutamic acid biosynthetic pathway is derived into two sources. There are either endogenously or exogenously. L-glutamic acid, as a carbon source in medium, requires conversion via acetyl-CoA and α-ketoglutaric acid from the TCA cycle serves as a direct precursor of glutamic acid synthesis for endogenous production while the exogenous production of L-glutamic acid can be converted to L-glutamine that is a precursor of PGA using enzyme glutamine synthase (Ogunleye *et al*., 2015).

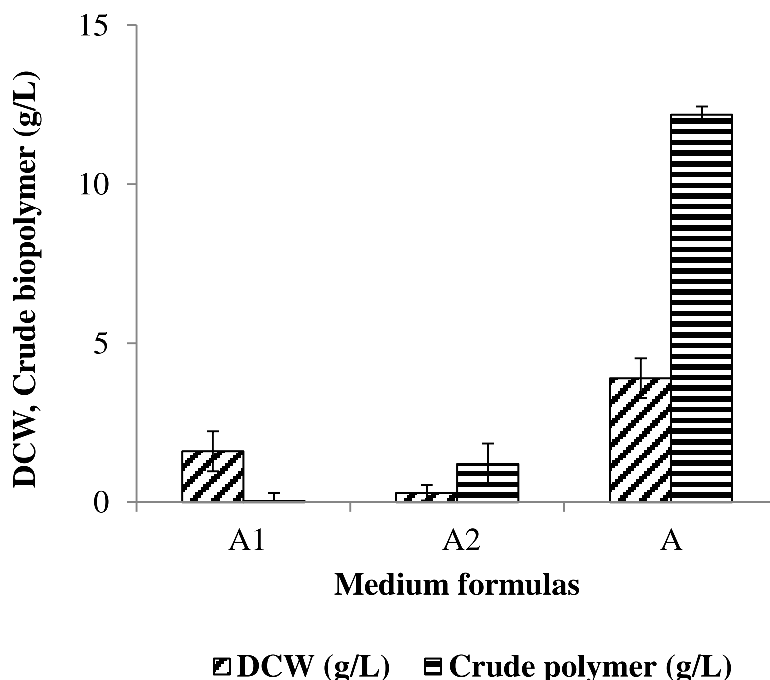


Figure 2.3 Effect of various medium formulas on growth and biopolymer production from cultivation *Rhizopus microsporus* ST4 on a rotary shaker (200 rpm) at 45 °C

2.4.1.2 Effect of cultivation temperature on growth and biopolymer production from *R. microsporus* ST4

Temperature is a physical condition that is one of main factors for growth and production of biopolymer from fungi. Biopolymer production from *R. microsporus* ST4 was cultivated in the medium A at room temperature (28 ± 2 °C) and 45°C for 3 days on a rotary shaker (200 rpm). The maximum mycelial growth (2.04 g/L DCW) and biopolymer production (12.47 g/L) was obtained at 45°C, It was noted that *R. microsporus* ST4 could grow (7.08 g/L DCW) at room temperature (28 ± 2 °C), but no production of biopolymer. The fungi that grow only within the temperature range of 20 °C to 50°C or higher are considered to be thermophilic fungi (Cooney and Emerson, 1964). *R. microsporus* ST4 is thermophilic fungi because of it able to grow at 28 °C and 45°C. The effect of temperature on biopolymer production from *R. microsporus* ST4 was in agreement with *R. oryzae* 29 that produced

biopolymer (5.58-5.78 g/L) only at 45 °C in POME medium (Suyala *et al*., 2008). The growth of *R. microsporus* in the medium A at 45 °C was the optimum condition on growth and biopolymer production from *R. microsporus* ST4.

2.4.1.3 Effect of cultivation time on growth and biopolymer production from *R. microsporus* ST4

Time course on growth and biopolymer production from *R. microsporus* ST4 cultivated in the medium A at 45°C was conducted (Figure 2.4). Growth was occurred within 1 day, as indicated by the increase of DCW and declined of pH due to the metabolism of sugar and increased slightly thereafter. *Rhizopus* spp. to show germination of spores, mycelial growth and eventual spore formation within 15 h (Sankpal *et al.,* 2001). As seen from the figure, while the growth of *R. microsporus* ST4 continues slightly after 1 day cultivation, biopolymer was produced rapidly and reached the highest yield of 12.33 g/L after cultivation for 3 days. During the growth, the strain simultaneously produced the biopolymer and excreted it into the culture broth. This optimum cultivation time (3 days) of *R. microsporus* ST4 for biopolymer production was in agreement with that of *R. oryzae* for chitosan production in potato peel under 70% moisture content at 30°C (for 3 days) (Kleekayai and Suntornsk, 2011). Therefore, medium A and cultivation at 45 °C for 3 days was the optimum condition of biopolymer production from *R. microsporus* ST4 and used for further studies.

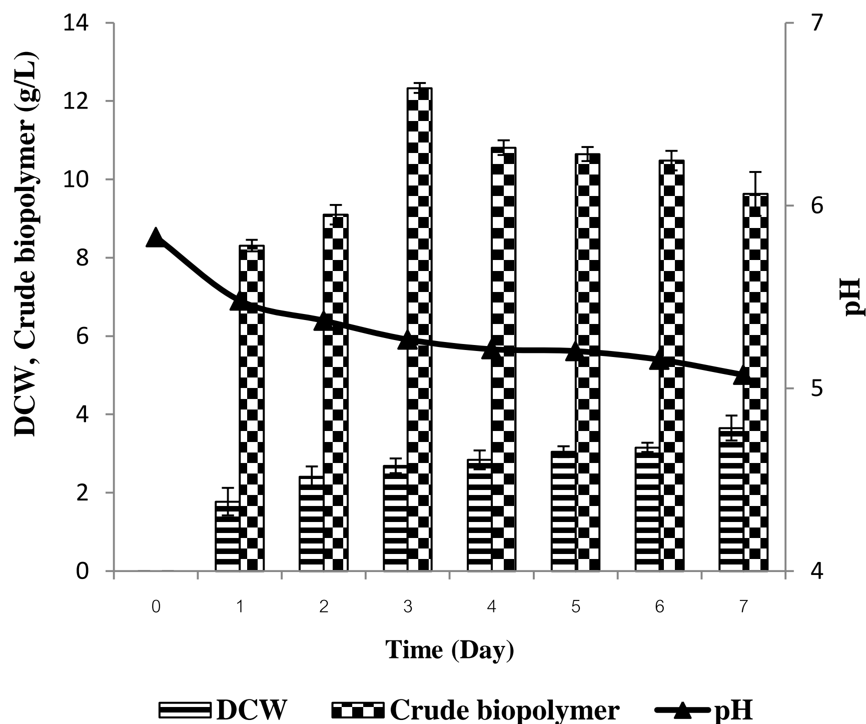


Figure 2.4 Time course on growth and biopolymer production from cultivation *Rhizopus microsporus* ST4 in the medium A on a rotary shaker (200 rpm) at 45 °C

2.4.2 Characterization of the biopolymer from *R. microsporus* ST4

The supernatant (without cell) from *R. microsporus* ST4 was precipitation with 4 volume of cold 95 % ethanol (4°C) overnight, then centrifugation, dissolve in deionized water and dialyzed by dialysis bag (molecular weight cutoff 8,000 Da) against water at 4 °C for 24 h to remove the low molecular (lower than 8,000 Da). The solution from dialysis was lyophilized to obtain partially purified polymers to be called biopolymer ST4 (Figure 2.5). The biopolymer ST4 was white.

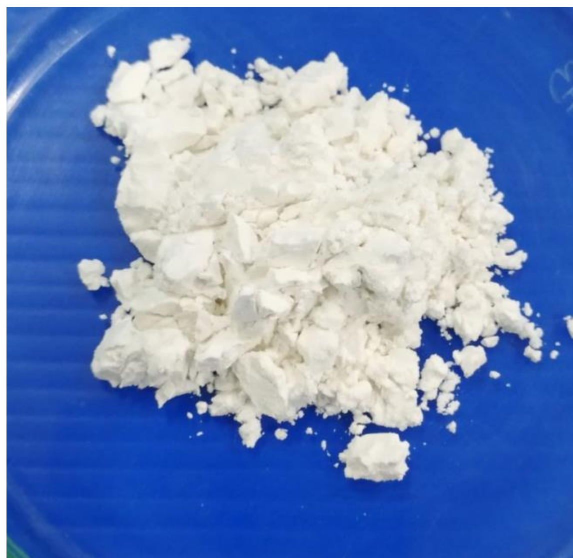


Figure 2.5 The biopolymer production from cultivation *Rhizopus microsporus* ST4 in the medium A on a rotary shaker (200 rpm) at 45 °C for 3 days after freeze drying

Table 2.3 showed amino acid profile of the biopolymer from *R. microsporus* ST4 cultivated in the medium A on a rotary shaker (200 rpm) for 3 days at 45 °C. Glutamic acid was the major component (75 mg/100mg polymer) with trace amount of lysine, aspartic acid, alanine and threonine (0.19, 0.18, 0.12 and 0.07 mg/100mg polymer, respectively. The amount of glutamic acid was calculated to be 99.3% of the total amount of total amino acid in the biopolymer ST4. Similar result was also reported by Chaisorn *et al*. (2016) when she was studied the biopolymer production from *B. subtilis* WD161. The puirified biopolymer includes glutamic acid, aspartic and lysine of 5.29, 3.1 and 2.01 mg/100mg polymer, respectively.

Table 2.3 Amino acid composition of biopolymer from *Rhizopus microsporus* ST4 cultivation in the medium A on a shaker (200 rpm) at 45 °C for 3 days

Amino acids	Amino acid concentration (mg/100 mg polymer)
Aspartic acid	0.18
Serine	-*
Glutamic acid	75.32
Glycine	-*
Histidine	-*
Arginine	-*
Threonine	0.07
Alanine	0.12
Proline	-*
Tyrosine	-*
Valine	-*
Lysine	0.19
Isoleucine	-*
Leucine	-*
Phenylalanine	-*

Remarks: * = not detected

The functional groups of the biopolymer ST4 were illustrated in the FT-IR absorption spectra (Figure 2.6) which was similar and comparable to that of the commercial glutamic acid. The peak in each spectrum corresponds to a specific bond of the compound. The FT-IR absorption spectra of the biopolymer ST4 displayed the characteristics of strong hydroxyl absorption peaks at 3411 cm^{-1}, a weaker carbonyl absorption of 1398 cm^{-1} and C-N groups absorption at 1075 cm^{-1} (Ho *et al*., 2006). The 1515 band corresponds to the $–NH_3$ symmetric deformation, at 1456 cm^{-1} is due to the symmetric stretching of the deprotonated R-carboxylate group and at 1515 and

1353 cm^{-1} correspond to the $–NH_3$ and $–CH_2$ deformation, respectively (He *et al.*, 2000). The absorption peaks at 1627 cm^{-1} shows strong amide while at 2935 cm^{-1} was characteristics of aliphatic N–H stretching (Ho *et al.*, 2006). Base on their similarity of the FT-IR spectra, the biopolymer produced by *R. microsporus* ST4 was identified to be polyglutamic acid (PGA) (Figure-Appendix B2).

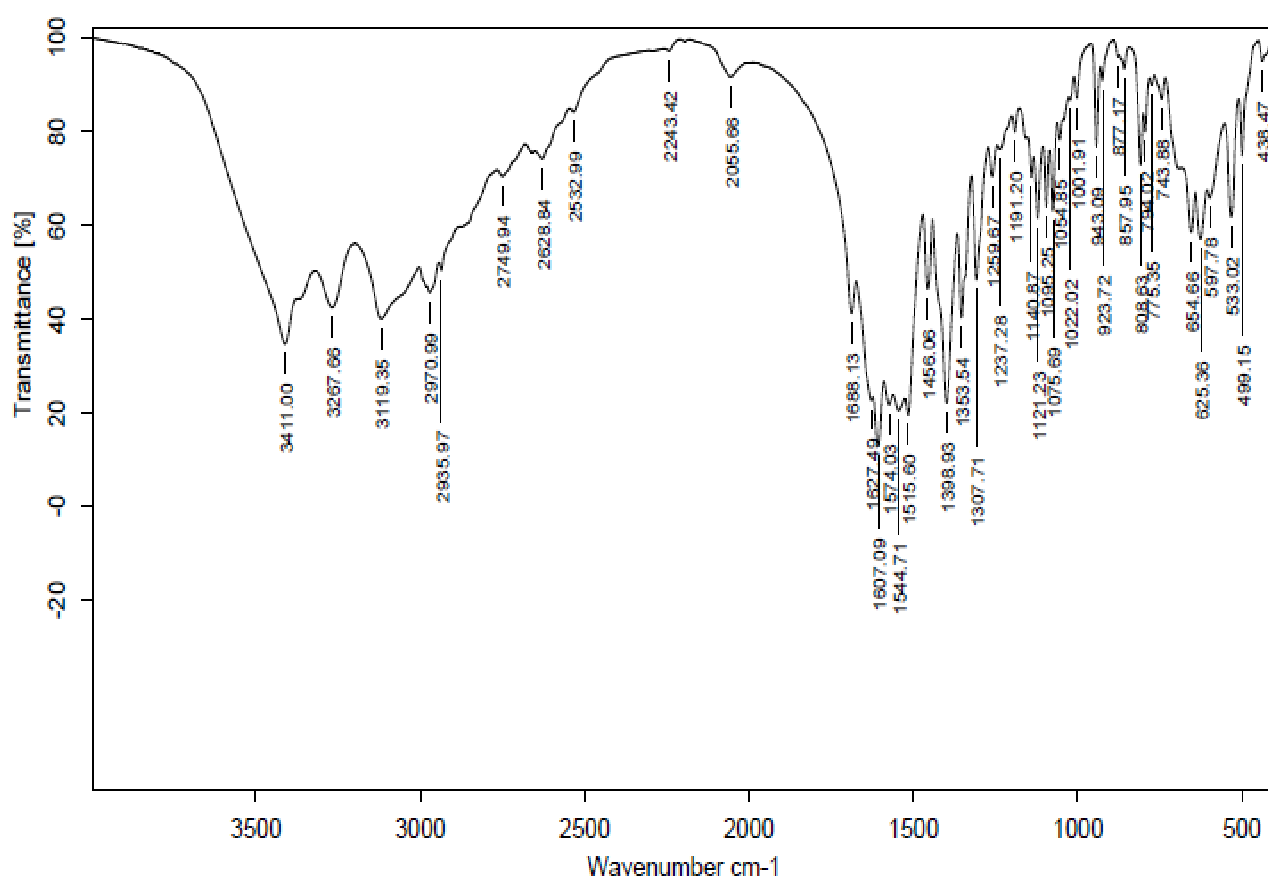


Figure 2.6 FT-IR spectra of biopolymer production from cultivation of *Rhizopus microsporus* ST4 in the media A on rotary shaker (200 rpm) at 45 °C and for 3 days

The molecular size of the biopolymer ST4 in the medium A on a rotary shaker (200 rpm) at 45 °C for 3 days was estimated by the SDS-PAGE method. The bands of PGA at the various initial concentrations (1-2 g/mL) were shown in Figure 2.7. The molecular size of biopolymer was determined by comparison of marker proteins. The results showed that at 1, 1.5, 2 g/mL gave the PGA bands at the size over 75 kDa (~ 80 kDa). It can be clearly seen that the concentrations had no affect on the molecular size of the biopolymer ST4. Moreover, the concentrations at 1.5 and 2 mg/mL showed apparently longer bands. The γ-PGA from *B. licheniformis* ATCC®9945™ gave the bands at the size over 98 kDa (estimated by SDS-PAGE method) (Kongklom *et al*., 2012). The molecular size of biopolymer from *B. subtilis* WD161 was 1.54x10^6 Da. (Chaison *et al*., 2016). In general, The molecular weight of γ-PGA produced by *Bacillus* species ranges from 100-1,000 kDa (Ogunleye, *et al*., 2015). The PGA from eukarya *Hydra* with the Mw betwen 3 and 25 kDa (Weber, 1990) and from halophilic archaeon *Natrialba aegyptiaca* with the Mw of >1000 kDa were reported (Ashiuchi and Misono, 2002). Thus, the molecular weight of γ-PGA produced by several organisms in the range of 3 - >1000 kDa.

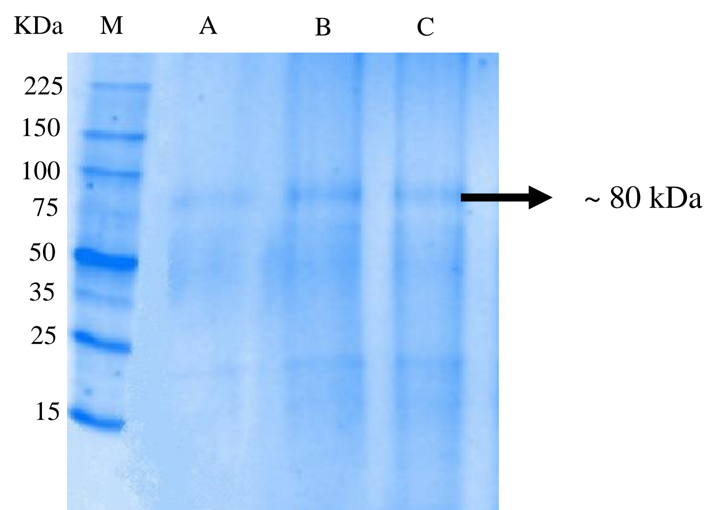


Figure 2.7 SDS PAGE of biopolymer production from cultivation of *Rhizopus microsporus* ST4 in the medium A on a rotary shaker (200 rpm) at 45 °C and for 3 days. land M: standard protein maker, land A:1 mg/mL, land B: 1.5 mg/mL and land C: 2 mg/mL

The relation between the solute and solvent and polarity are important in determining solubility. The polar solutes usually tend to dissolve best in polar solvents while non-polar solutes tend to dissolve best in non-polar solvents. In addition, the dielectric constants also indicated the solubility. Solvents are classified into three groups according to their dielectric constants as polar (> 50), semi-polar (20 -50) and non-polar (1-20). The distilled water as polar showed the highest in dielectric constants of 80 that was effected well in the solubility. Among 12 solvents tested, the biopolymer ST4 was best soluble in distilled water (DW) (87.93%) followed by methanol (85.67%) and DMSO (81.25%) (Figure 2.8). The polymer from *Akanthomyces pistillariiformis* BCC2694, *Beauveria bassiana* BCC2692, *Cordyceps dipterigena* BCC2073, *Hymenostilbe* sp. BCC2146, *Torrubiella tenuis* BCC1056 and *Zygosporium masonii* BCC7543 were also soluble in distilled water and DMSO at high levels (Madla *et al*., 1999).

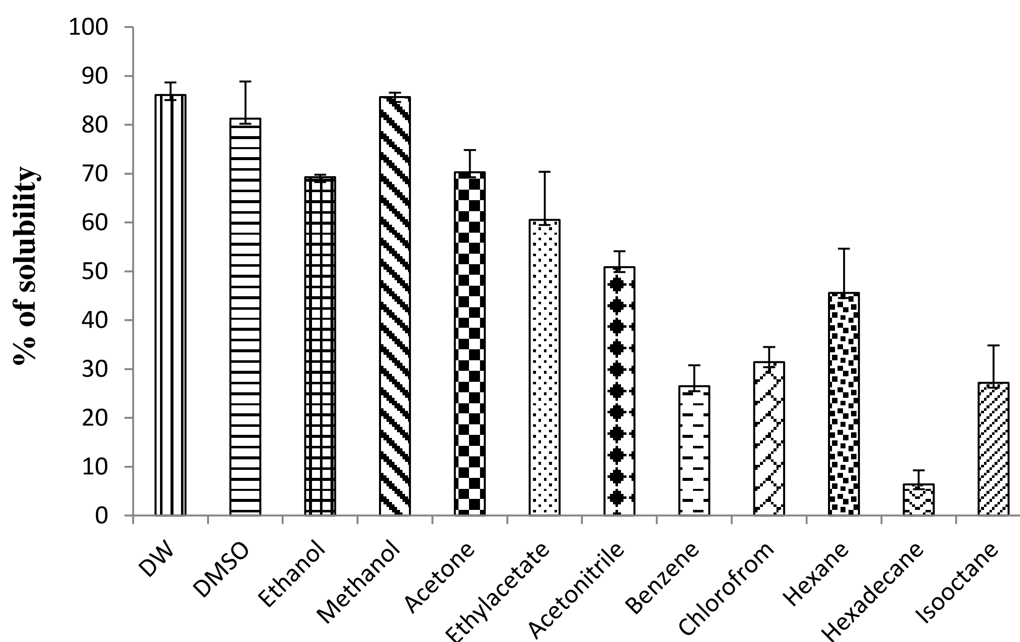


Figure 2.8 The solubility of the biopolymer from *Rhizopus microsporus* ST4 cultivated in the medium A on rotary shaker (200 rpm) at 45 °C for 3 days

Thermal properties of the biopolymer from *R. microsporus* ST4 were investigated using the thermogravimetry- differential thermal analysis (TGA-DTA) technique. TGA is used for detecting the missing weight volume because of the disintegration of composites in the samples when temperature changes in the range of 50 to 1300 °C. It is real-time measurement and analysis of sample weight change while subjecting it to a controlled continuously of the optimization in temperature time and atmosphere. An application is characterizing inorganics, analyzing polymers mineral or coal. DTA are used for analyzing the physical property of materials such as boiling point, melting point. Both equipments can analyze plastic, polymer, drugs, animal protein, clay etc. The changing temperature point of DTA ranges from 50 to 1300 °C. The TGA curves of the pyrolysis process can be divided into three phases. The first stage is in the range of 0◦C -210◦C (loss of water), the second stage is between 210◦C and 350◦C (main pyrolyze) and last stage is charring (> 350◦C) (Luo *et al*., 2012; Peng *et al*., 2014; Su *et al*., 2014). The TGA, DTG and DTA curves of the biopolymer ST4 are shown in Figure 2.9. The thermograms of biopolymer ST4 are classified into three stage. loss of water stage (< 186 °C), main pyrolysis stage (186 to 341.9 °C), and charring (> 341.9 °C) and the temperatures appeared at 702.5°C has the weight of solid residues left <35%. The thermal analysis was concerned with chemical transformation, melting, decomposition temperatures represented morphological changes and stability of the biopolymer. The thermal characterization of the biopolymer is the evaluation of its properties and therefore its potential areas of application.

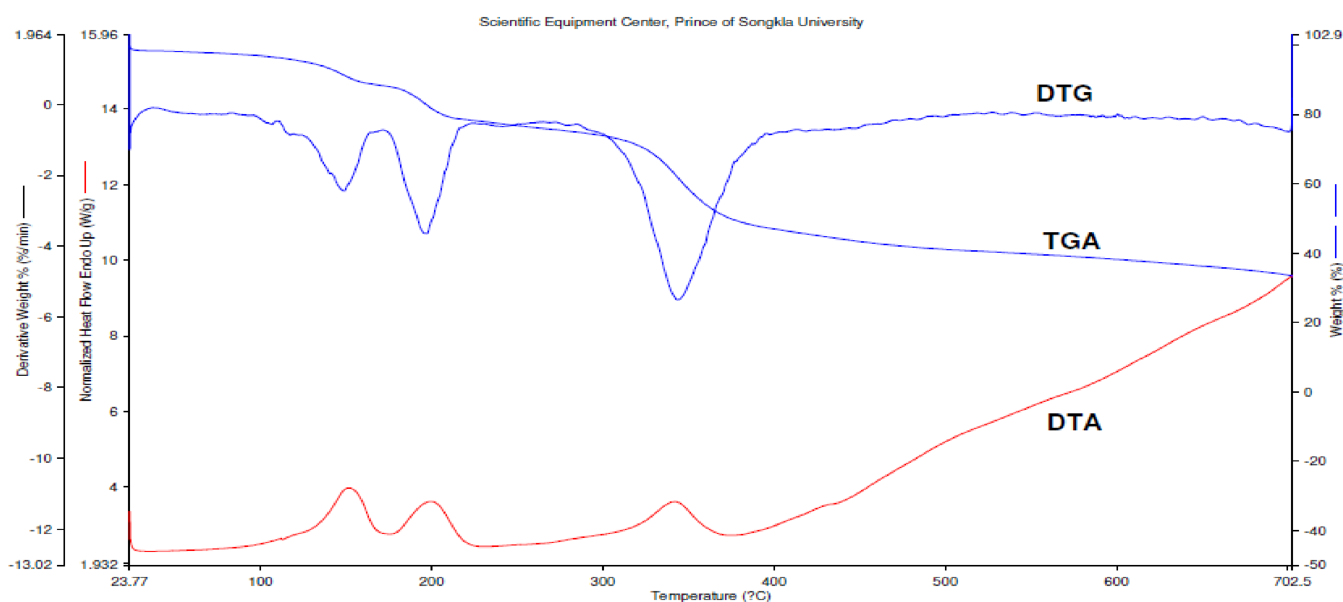


Figure 2.9 Thermograms of biopolymer from *Rhizopus microsporus* ST4

DTG: Derivative thermogravimetry, TGA: Thermogravimetric analysis

DTA: Differential Thermal Analysis

2.5 Conclusion

The biopolymer production from *R. microsporus* ST4 was cultivated in the medium A (2% glucose, 0.05% yeast extract, 5% glutamic acid and 0.05% $MgSO_4$ $.7H_2O$ with pH adjusted to 5.8) at 45 °C for 3 days gave the highest yield of biopolymer. Characterization of the partially purified biopolymer ST4 reached its major component was glutamic acid (75.3 mg/g polymer or 99.3% of the total amino acids) follow by lysine, aspartic acid, alanine and threonine and it was water soluble compound although it could be solubilized in many solvents; methanol, DMSO and acetone. The molecular size of biopolymer was estimated to be over 75 kDa.

CHAPTER 3

RECOVERY, CHARACTERIZATION AND APPLICATION OF HEMICELLULOSE FROM PALM OIL MILL EFFLUENT

3.1 Abstract

Recovery and characterization of the biopolymer from palm oil mill wastes were investigated. The biopolymer from sterilizer condensate (SC) and decanter effluent (DE) generated during palm oil extraction process were recovered by precipitation with 5 folds (v/v) of 95% ethanol. SC and DE gave the total biopolymer yields of 2.16% and 2.47% (w/v) respectively, with the high content of hemicellulose 82.98±0.85% and 82.75±0.39%, respectively. Characterization of the biopolymers from SC (HSC) and DE (HDE) revealed that they were hemicellulose based on their major monosaccharide compared to the standard commercial xylan from Beech wood (CXB) were xylose (66.9%, 71.5% and 79.34%, respectively). These three sources of hemicellulose (HSC, HDE and CXB) revealed that there were two sizes of Mw for each source; 42,191 Da and 3,577 Da from HSC, 36,348 and 3,613Da from HDE and 930,000 Da and 21,000 Da from CXB. Solubility tests on hemicellulose samples showed that they are water soluble (96.82%, 99.05 % and 84.62%). The HSC, HDE and CXB began to decompose at 209, 238 and 221°C, respectively and at 698, 697.7 and 698.8°C appeared residue weight of the decomposition (<40%). For biological properties, the biopolymers from HSC and HDE had no activity for antimicrobial test, no inhibition on any cancer cells tested; the human breast cancer and oral cavity cancer and small cell lung cancer and noncytotoxicity in HDE while HSC exhibited cytotoxicity against human hepatocarcinoma (HepG2) ATCC HB-8065.

These biopolymers were used for preparation of hydrogel by reacting with maleic anhydride (MA) and polyvinyl alcohol (PVA), respectively. The highest degree of swelling of 382.05±1.84%, 395.9±3.26% and 269.23±1.14% were obtained from the formulation H12 of HSC (H:MA=1:1.5 and PVA: (H:MA) = 1:1), the formulation 17 of HDE (H:MA = 1.5:1 and PVA: (H:MA) = 1:0.5) and the

formulation H36 of CXB (H:MA=1:1.5 and PVA: (H:MA) = 1:1), respectively. Tensile strength of HSC and HDE were obtained from HSC fraction H4HSC fraction H4 (18.31 MPa), HDE formulation H20 (21.12 MPa) and CXB formulation H34 (19.53 MPa). The hydrogels possessing both high swelling and strength: the formulations H11 from HSC, H17 from HDE and H35 from CXB, were selected for cytotoxicity test for normal cells and antimicrobial tests. The HSC hydrogel (formulation H11) exhibited the least viability of L929 (Mouse Fibroblast Cells, ATCC CCL1, NCTC 929. of Strain L) at 3% recommended dose. This indicated that the hydrogel was cytotoxic and had a potential application for cytotoxicity activity against cancer cells. The HDE hydrogel (formulation H17) and hydrogel from CXB (formulation H35) showed antibacterial activity against *Staphylococcus aureus* ATCC 6538 and had potential to be applied in the biomedical field. Application of the three hemicellulose (HSC, HDE and CXB) was conducted for production of xylooligosaccharide (XOS) using the enzymatic method. The XOS prepared from HSC and HDE was cut with 8U of endo-(1,4)-β-xylanases (EC 3.2.1.8) for 12h at 48 °C. The both sample possessed xylopentaose (0.001-0.002 g/L) and xylobiose from HSC and HDE was not detected. Under the optimum conditions for xylooligosaccharide (XOS) production was prepared from commercial xylan at 4 U of endo-(1,4)-β-xylanases for 12h at 40 °C, the highest xylobiose could reach to 0.5g/L.

3.2. Introduction

Oil palm (*Elaeis guineensis*) is an important economic crop for food and energy (biodiesel) source. The dispersion of oil palm is restricted to tropical regions of the world and has mainly been cultivated in Indonesia, Malaysia and in the south of Thailand (UNEP, 2011). The dramatic increase in demand for the plantation area was around 10% with an average annual growth rate of 11% from 1981 to 2000 and 9% from 2001 to 2010 (Dallinger, 2011). The Thai government plans to increase the oil palm plantation area to 5 million rai (or 0.8 million ha) in order to have adequate supply of raw material for biodiesel production (Sattayasamitsathit *et al.*, 2011). Standard or wet process is commonly employed for palm oil extraction in the palm oil mill that resulted in the generation of large quantity of palm oil mill effluent (POME) with 0.87 ton of effluent per tonne of fresh fruit bunch (FFB) in Thailand (H-Kittkun *et al.*, 1994) or average 2.5-3.0 tonnes of POME per tonnes of produced crude oil in Malaysia (Husain *et al.*, 2013). There are three major sources of POME generated during oil palm extraction process; sterilizer condensate (SC) decanter effluent (DE) and purification effluent. The amount of effluent from sterilizer condensate was 200 L per 10 tonne of FFB or 2.5-3 folds of oil (Cheah *et al.*, 1988). POME had acidic pH (pH 4.2-4.5) and contained high organic matter (22-54.3 g/L BOD, 75.2-96.2 g/L COD), solids (35-42 g/L total solids (TS), 8.5-12 g/L suspended solids (SS)), oil & grease (8.3-10.6 g/L) but low nitrogen (0.083-0.92 g/L) (O-Thong *et al.*, 2008). These characteristics of POME indicated that it can cause serious problem to the natural water if not property treated. During POME digestion, odor could be released and thus reduced the air quality in the surrounding lagoons area of the wastewater treatment system (Abdullah and Sulaiman, 2013). To mitigation the environment problem caused by POME, the organic matter would be better utilized for production of some valuable product such as enzyme cellulase and xylanase (Prasertsan *et al.*, 1997). The biomass in POME contain three major components; cellulose, hemicellulose and lignin. Recovery of hemicellulose from POME was first reported by extraction using 2 folds (v/v) of 95% ethanol and tested for xylan content compared to the commercial xylan by using as substrate for determination of xylanase(Prasertsan and Oi, 2001). In a wet process, fresh fruit bunch was sterilized (at 120-130 °C, 40 lb/in^2 for 45 min) and hemicellulose was

extracted into soluble matters in the sterilizer condensate (Prasertsan and Oi, 2001). The hemicellulose recovered from the sterilizer condensate by precipitation with 5 folds (v/v) of 95% ethanol gave the highest hemicellulose yield of 1.2 ±0.35% (w/v) (Pensawat, 2009). Among plant polymers, the hemicelluloses are second most abundant renewable polymers after cellulose in lignocellulosic materials. Hemicelluloses have a very wide variety of applications such as food (sugar and xylitol), medical or functional food (xylooligosaccharides), chemical industry (furfural), additives, thickeners, emulsifiers, gelling agents, adhesives and adsorbents. However, recent research has been made in the biomedical field (polymeric materials; film, hydrogel and carrier). For example, hemicelluloses have been shown to work as dialdehyde hemicelluloses /chitosan/Ag hydrogel (Guan *et al*., 2015) and hemicellulose-chitosan hydrogel (Li *et al*., 2011) possessing antimicrobial activity agains *Staphylococcus aureus* and *Escherichia coli* and xylan-chitosan conjugate endowed antioxidant (Li *et al*., 2011). This study aims to produce hemicellulose by recovery from the two sources of palm oil mill effluent, then characterize to find out the interesting properties. The extracted hemicelluloses were used for preparation of the complex hydrogels and characterized for further application.

3.3. Materials and methods

3.3.1 Materials

3.3.1.1 Palm oil mill effluent (POME)

Two sources of POME, sterilizer condensate (SC) and decanter effluent (DE) were provided by Nam Hong Palm Oil Co., Ltd., at Krabi Province, Thailand. The characteristics and chemical composition of SC and DE were determined for pH, chemical oxygen demand (COD), total solid (TS) and suspended solid (SS) according to the methods as described in the Standard Methods for the Examination of Water and Wastewater (APHA AWWA and WEF, 1998).

3.3.1.2 Xylan

Xylan from beech wood with over 90% xylose residues was purchased from Sigma–Aldrich (Steinheim, Germany).

3.3.2 Recovery of the hemicellulose from POME

The hemicellulose from sterilizer condensate (SC) and decanter effluent (DE) were recovered in two steps (modified from Pensawat, 2009). First, solids and oil in the SC and DE were removed by centrifugation (3632xg) at 4°C for 15 min, then the two supernatant were centrifuged again. The supernatants of SC and DE were filtered through Whatman No.4 filter paper. The filtrates precipitated with 5 folds (v/v) of 95% ethanol at room temperature (28± 2°C) and left for 8 h, then centrifuged (3632xg) at 4°C for 20 min and lyophilized to obtain the dried hemicellulose from SC (HSC) and DE (HDE).

3.3.3 Characterization of the hemicellulose from POME and commercial xylan

The HSC, HDE and standard commercial xylan from beechwood (CXB) were passed through a 60 mesh screen, the samples powder were dried in an oven at 50 °C for 12 h prior to use. Each sample was characterizied as following:

3.3.3.1 Chemical composition

The hemicellulose from SC and DE (HSC and HDE) and CXB were determined by Lin, *et al.* (2010). Acid insoluble lignin and ash were determined according to the methods of the National Renewable Energy Laboratory (NREL/TP-510-42618, TP/-510-42622) (Sluiter *et al.*, 2008; Sluiter *et al.*, 2008). The cellulose was determined as described by Wright and Wallis (1998). The sugar composition was determined using with a High-Performance Liquid Chromatography (HPLC) under the condition as described by Yoon, *et al.* (2016).

3.3.3.2 Molecular weight determination

Average molecular weight (MW) of the HSC, HDE and CXB were determined by a gel permeation chromatography (GPC, Waters 600E, Milford, USA) (Sun *et al.*, 2010; Peng *et al.*, 2013) using ultrahydrogel linear 1 column (Waters 600E, Milford, USA) with a refractive index (RI) detector (Waters 240, Milford, USA). The analysis was carried out using a GPC (MW resolving range from 1,000 to 20,000,000). The universal calibration log (Mp the peak molecular weight) versus V_R (retention volume) was obtained by using pullulan (polysaccharide) standards with molecular weights ranging from 5,900 to 708,000. The injection volume was 20 µl and the flow rate of the mobile phase (0.05M sodium bicarbonate buffer pH 11) was 0.6 ml/min at 30 °C (Madla *et al.*, 2005).

3.3.3.3 Functional group analysis

The HSC, HDE and CXB (1 mg each) were homogenized with 99 mg of KBr for 1 min and pressed into a disc. The FT-IR spectra of these samples were recorded with 16 scans at the resolution of 4 cm^{-1} between the wavenumbers of 400 and 4000 cm^{-1} using Fourier Transform Infrared Spectrometer (Vertex70, Bruker, Germany).

3.3.3.4 Solubility tests

The HSC, HDE and CXB (30 mg each) were tested for their solubility in 15 ml of the following solvents: distilled water (DW), dimethylsulfoxide (DMSO), 0.1M NaOH and toluene, in a closed container under constant agitation (200 rpm) at room temperature (28±2°C) overnight (Marques., 2014). The sample was centrifuged at 850×g for 15 min (Noor *et al*., 1999). The percentages solubility was calculated.

$$S = \frac{Wo - Wr}{Wo} \times 100\% \qquad (1)$$

where S is the solubility degree, Wr is the weight of undissolved biopolymer residue, and Wo is the original weight of biopolymers.

3.3.3.5 Thermal stability

Thermal behavior of the HSC, HDE and CXB were performed using thermogravimetry-differential thermal analysis (TGA-DTA) in a Simultaneous Thermal Analyzer (STA8000, perkin Elmer, USA). The sample weighed between 4 and 20 mg was heated from room temperature to 700°C at a rate of 10ºC/min in nitrogen.

3.3.3.6 Biological properties

The HSC and HDE were filtered through a 0.22 µm filter (Onyango *et al*., 2010) before *in vitro* testing for antimicrobial test (anti- *Bacillus cereus* (Clinical and Laboratory Standards Institute, 2006), anti- *Klebsiella pneumoniae* (Clinical and Laboratory Standards Institute, 2006), anti-*Mycobacterium tuberculosis* (anti-TB test) (Changsen *et al.,* 2003; Collins *et al.,* 1998) and anti- *Magnaporthe grisea*(rice blast disease pathogen) (Haugland, 2002; Josep *et al*., 1998)), three anti-cancer test by Brien, *et al*. 2000; MCF7-breast cancer KB-Oral cavity cancer, NCI-H187-small cell lung cancer, two cytotoxicity test by Brien, *et al*. 2000 (against

human hepatocarcinoma (HepG2) ATCC HB-8065 and human caucasian colon adenocarcinoma (Caco2) ATCC HB-37). The concentration in the range of 5-50 µg/mL of the two biopolymers were used for testing which were analyzed at Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park.

3.3.4 Application of the three hemicellulose as source of hydrogel production

The HSC, HDE and CXB were made to reacted with maleic anhydride (MA) with the weight ratios of hemicellulose to MA at 1:0, 1.5:1, 1:1 and 1:1.5 (Table 3.1), using dimethyl sulfoxide (DMSO) as a solvent. The reactions were performed and stirred at 50 °C under acid catalysis for 2 h. The resulting derivative, HSC-MA and HDE-MA, were precipitated with 3 folds (v/v) of isopropanol for 48 h at room temperature (28 ± 2 °C) followed by centrifugation (3,500 rpm, 10 min). Then, the samples were dried in an oven at 50 °C. Their chemical structure were analyzed by FTIR (Tanodekaew *et al*., 2006). To prepare HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogels, the dried HSC-MA, HDE-MA and CXB-MA were blended with polyvinyl alcohol (PVA, molecular weight ~100,000) at various PVA weight contents under acidic condition (Table. 3.1). The blends were stirred and heated at 70 °C for 4 h. Then, each sample was placed on a tray and dried at 50 °C for 8 h (Tanodekaew *et al*., 2006).

Table 3.1 The composition of hydrogel-forming from hemicelluloses from HSC,HDE and commercial xylan with maleic anhydride (MA) and polyvinyl alcohol (PVA)

Formulation	Type of hemicelluloses	Ratio of concentration (g)	
		Hemicellulose:MA	PVA: Hemicellulose -MA
H1	HSC	1:0	1:0.25
H2	HSC	1:0	1:0.5
H3	HSC	1:0	1:1
H4	HSC	1.5:1	1:0.25
H5	HSC	1.5:1	1:0.5
H6	HSC	1.5:1	1:1
H7	HSC	1:1	1:0.25
H8	HSC	1:1	1:0.5
H9	HSC	1:1	1:1
H10	HSC	1:1.5	1:0.25
H11	HSC	1:1.5	1:0.5
H12	HSC	1:1.5	1:1
H13	HDE	1:0	1:0.25
H14	HDE	1:0	1:0.5
H15	HDE	1:0	1:1
H16	HDE	1.5:1	1:0.25

Table 3.1 The composition of hydrogel-forming from hemicelluloses from HSC,HDE and commercial xylan with maleic anhydride (MA) and polyvinyl alcohol (PVA) (cont.)

Formulation	Type of hemicelluloses	Ratio of concentration (g)	
		Hemicellulose:MA	PVA: Hemicellulose -MA
H17	HDE	1.5:1	1:0.5
H18	HDE	1.5:1	1:1
H19	HDE	1:1	1:0.25
H20	HDE	1:1	1:0.5
H21	HDE	1:1	1:1
H22	HDE	1:1.5	1:0.25
H23	HDE	1:1.5	1:0.5
H24	HDE	1:1.5	1:1
H25	CXB	1:0	1:0.25
H26	CXB	1:0	1:0.5
H27	CXB	1:0	1:1
H28	CXB	1.5:1	1:0.25
H29	CXB	1.5:1	1:0.5
H30	CXB	1.5:1	1:1
H31	CXB	1:1	1:0.25
H32	CXB	1:1	1:0.5
H32	CXB	1:1	1:1
H34	CXB	1:1.5	1:0.25
H35	CXB	1:1.5	1:0.5
H36	CXB	1:1.5	1:1
H37	PVA	-	1:0

The HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogels were characterized as following:

3.3.4.1 Functional group analysis

The dried HSC-MA, HDE-MA and CXB-MA (1 mg) was homogenized with 99 mg of KBr for 1 min and pressed into a disc. The samples were determined for chemical structure using FTIR as described above.

3.3.4.2 Swelling test

Swelling test of HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogels were determined by the method adapted from Tanodekaew *et al*. (2006). The preweighed crosslinked HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogels (1 cmx1cmx0.03cm) were immersed in distilled water at room temperature (28±2°C) for 5 min. At certain time, the swollen gels were removed from the water, quickly wiped to remove excess water on the surface and weighed. The degree of swelling (DS) is calculated as follows:

$$DS = (Ww - Wd) / Wd \times 100 \quad (2)$$

where Ww and Wd are weights of wet and dry blends, respectively.

3.3.4.3 Surface morphology

The HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogels were mounted on the aluminum stub, placed in the sample holder and metalized with a thin layer of gold. The samples were examined using scanning electron microscope (Quanta 400, FEI, Czech Repubic).

3.3.4.4 Tensile strength

Tensile strength of the dumbbell shaped HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA of 2.54 cm width and 11.43 cm length were measured by universal testing machine (UTM, Lloyd Instruments, UK) at room temperature (28±2 ˚C) under American Society for Testing and Material (ASTM) D638 standards test methods were followed to measure tensile strength. The average value of the tensile strength was calculated using at least three samples (Adapted from Hassan *et al*., 2010).

3.3.4.5 Cytotoxicity test for normal cells

The HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogels were used for MTT cytotoxicity assay (Based on ISO 10993-5) which was analyzed by staff at Biological *In vitro* Testing for Biomaterials Service, MTEC, NSTDA, Thailand. Cell suspension of $1X10^5$ cell/mL L929 (Mouse Fibroblast Cells, ATCC CCL1, NCTC 929. of Strain L) in MEM completed medium was seed into the 96-well plate. It was incubated at 37±1 °C, 0.1% CO_2 and 95±5% relative humidity for 24±2 h to obtain confluent monolayers of cells prior to test. The MEM completed medium was replaced with the extracts of blank (the media without test specimen). Thermanox (Nunc) coverslip was used as a negative control. Polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC):RM-A as positive control was used as a positive control material. The negative control, positive control, HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogel were extracted at surface area to volume extraction ratio of 6, 3, 3 and $3cm^2$/mL, respectively. All of them were extracted at 37±1 °C for 24±2 h. The extracts of the tested specimen were diluted to 2X before testing. The cells were incubated further for 24±2 h. After incubation, the viable cells were stained with MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated further for 2 h. Then MTT was removed and DMSO was added in each well. The absorbance was measured using microplate reader (EASYS, UVM340 S/N ASY54180) at 570 nm. The % viability was calculated as follows:

$$\% \text{ viability} = 100x \ OD_{570C} / OD_{570b} \qquad (3)$$

OD_{570C} = the mean value of the measured optical density of the 100% extracts of the test sample

OD_{570b} = the mean value of the measured optical density of the 100% extracts of the blanks

If viability is reduced to $< 70\%$ of the blank, it has a cytotoxic potential.

3.3.4.6 Test of antimicrobial activity

Antimicrobial activity of the HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogels was determined by agar diffusion assay (Yadollahi *et al*., 2015). It was analyzed by staff at Biodiversity Research Centre, Thailand Institute of Scientific and Technology Research, Thailand. Two bacteria (*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739) and one yeast (*Candida albicans* ATCC 10231) as test microorganisms were grown on Mueller Hinton agar and YM agar, respectively by streak plate technique and incubated at 37 ºC for 18-24 h. A few single colonies were selected and inoculated in either Mueller Hinton broth or YM broth and incubated on a shaker (200 rpm) at 37 ºC for 18-24 h. The inoculum was prepared by adjusted turbidity of the overnight culture with normal saline 0.85% to match that of McFarland No. 0.5 standard solution. The inoculum should be used within 30 min. Twenty milliliters of either Mueller Hinton agar or YM agar was poured into each plate and left to solidify for preparation of basal medium. The inoculum (2.5 ml) was thoroughly mixed with 100 ml medium soft agar (Mueller Hinton broth or YM broth with 0.75% agar) maintained at 45 ºC and it should be used within 15 min for preparation of seed medium. Each piece (10 mmx10 mmx3 mm) of the test sample was placed over the surface of the basal medium and gently pressed for firm contact. Then 4 ml of seed medium were overlaid to the plate which was tilted until the seed agar was completely covered the surface of the test sample and the basal medium. After incubation at 37 ºC for 18-24 h, the diameters of the inhibition zone observed around each test sample was measured visually using a ruler displaying their antibacterial activity (Guan *et al*., 2015). Amoxycillin disk (6 mm) and nystatin disk (6 mm) as antibacterial and antiyeast, respectively were used as controls.

3.3.5 Application of the three hemicelluloses for production of xylooligosaccharide (XOS)

The freeze-dried samples of the three hemicellulose; HSC, HDE and CXB, were prepared for XOS using enzymatic method (Hanim *et al*., 2011).

3.3.5.1 Effect of hydrolysis time on XOS production from the three hemicelluloses

Enzyme xylanase (endo-(1,4)-β-xylanases: EC3.2.1.8) from *Trichoderma viride* X3876 (Sigma–Aldrich) concentration of 8 U per 100 mg of substrate was added in 0.05 M phosphate buffer, pH 7.0, in 250-mL conical flasks. The hydrolysis reaction was carried out at 40 °C with orbital shaking at 150 rpm for 0, 12, 24, 36 and 48 h. The reaction was stopped by holding the samples in boiling water for 5 min (Hanim *et al*., 2011). The XOS contents was determined by HPLC. The xylobiose was quantitatively analyzed by HPLC (Agilent 1200 series, USA) using BioRad-Aminex HPX-87P (300 mm x 7.8 mm) and Refractive index detector. The mobile phase used was water and the flow rate was 0.6 mL/min at 80 °C.

3.3.5.2 Effect of enzyme concentration on XOS production from the three hemicelluloses

The selected hydrolysis time (result from 3.3.5.1) was used to study on the effect of enzyme concentration on XOS production from the three hemicelluloses. The experiment was carried out as above but the enzyme concentration at 0,4, 8, 12 and 16 U per 100 mg of substrate was added in 0.05 M phosphate buffer, pH 7.0, in 250-mL conical flasks. The hydrolysis was carried out at 40 °C with orbital shaking at 150 rpm for selected time (from 3.3.5.1). The reaction was stopped by holding the samples in boiling water for 5 min (Hanim *et al*., 2011). The samples were measured for XOS contents was determined by HPLC (as above).

3.3.6 Statistical Analysis

The experiment design was a completely random design with three replications. The data were subjected to analyses variance (ANOVA) using a computer program SPSS 20 for windows. Statistical significance was evaluated using Duncan´s Multiple Range Test (DMRT) and a p <0.05 was considered to demonstrate a significance difference.

3.4. Results and discussion

3.4.1 Recovery of the hemicelluloses from POME

The sterilizer condensate (SC) and decanter effluent (DE) is the liquid effluent that generated during the palm oil extraction in the wet process. The characteristics of the SC and DE were determined. The pH value of SC and DE was in the range of 4.5-5 which is the typical acidic pH and brown color of the palm oil mill effluent (POME). In general, the SC contained 1.2-1.4 times lower values of organic matter (COD; 72.63 and 89.75g/L) total solids (58.20 and 84.60g/L) and protein (0.62 and 0.75%) with about 53 times higher oil and grease (0.04 and 2.14g/L) than the DE. The interesting point was that the total phenol of SC was much higher than the DE (5.04 and 0.0018g/L) which resulting from the hydrolysis of lignin during the first step of sterilization process of fresh fruit bunches. Both sources contained trace amounts of mineral content (Table 3.2). Similar results were obtained in previous studies (Pensawat, 2009 and Chaisoorn, 2007). They reported that the characteristics of the SC taken from a palm oil mill (Permkiat Palm Oil Co., Ltd., Satun, Thailand) and DE taken from a palm oil mill (Pure Palm Oil Co., Ltd., Songkhla, Thailand) showed the acidic pH (5.1 and 4.3), the high COD (120 and 65.44 g/l) and high total (71.92 and 66.24g/l), respectively. The composition of POME characteristics could vary depending on many factors that are season, raw material, the extraction process, the efficiency of the process and the sampling time (Rupani *et al*., 2010)

In general, sterilizer in a wet process was used for sterilization of EFB at 120-130 °C, 40 lb/in^2 for 45 min. The soluble matters from sterilizer condensate contain hemicellulose as they are extracted during steaming at 40 lb/in^2 (Prasertsan and Oi, 2001). Recovery of the hemicellulose from the SC and DE were carried out by extracting and precipitated with 5 folds (v/v) of 95% ethanol at room temperature and left for 8 h. The yields of the precipitated hemicellulose from SC and DE were 2.16±0.56% and 2.47±0.89%, respectively. In a similar study, the sterilizer condensate precipitated with 5 folds (v/v) of 95% gave the highest hemicellulose yield of 1.2 ±0.35% (w/v) (Pensawat, 2009).

Table 3.2 Characteristics of sterilizer condensate and decanter effluent

Parameter	Sterilizer condensate		Decanter effluent	
	This study	Pensawat, 2009	This study	Chaisorn, 2007
pH	5	5.1	4.5	4.3
Color	Brown	Brown	Brown	-
COD (g/l)	72.63	120	89.75	65.44
Total solids (g/l)	58.20	71.90	84.60	66.24
Oil & grease (g/l)	0.04	-	2.14	-
Total phenol (g/l)	5.04	1.67	0.0018	-
Protein (%)	0.625	-	0.75	-
P (ppm)	337.27	200	241.82	-
K (%)	0.45	0.37	0.51	-
Ca (%)	0.05	0.6	0.07	-
Mg (%)	0.08	0.8	0.09	-
Fe (ppm)	194.04	149.45	99.27	-

3.4.2 Characterization of the hemicelluloses from POME and commercial xylan

3.4.2.1 Chemical composition

The HSC, HDE and CXB contain mostly hemicellulose (82.98±0.85, 82.75±0.39% and 90.84±0.46% respectively), with small amount of cellulose (6.40±0.53, 7.01±0.25 and 3.21±0.64%) and lignin (10.62±0.93, 10.24±0.58% and 5.95±0.13%, respectively) (Figure 3.1). The major monosaccharide in the HSC was xylose (66.98±045%) which was 3.1 times higher than glucose (21.35±0.94%). The HDE contained higher xylose (71.5±0.83%) with slightly lower glucose (20.31±078%) than the HSC. As the beech wood was a pure commercial xylan (Sigma Co, Ltd) hence it contained a higher amount of xylose (79.34±0.12%). Thus, the HSC and HDE composition were similar to the commercial beechwood xylan.

Figure 3.1 Hemicellulose from sterilizer condensate (HSC) (A), decanter effluent (HDE) (B) of palm oil mill waste and commercial xylan from beech wood (CXB) (C)

3.4.2.2 Molecular weight determination

The HSC had two sizes of weight-average of molecular weights (Mw) with 42,191 Da (so called large sized HSC: HSC-L) and 3,577 Da (small sized HSC: HSC-S) while the Mw of HDE were 36,348 Da (HDE-L)and 3,613 Da (HDE-S) (Table 3.3). The commercial xylan had two sizes with 930,000 Da (CXB large size: CXB-L) and 21,000 Da (CXB small size: CXB-S) also. The molecular weights of polysaccharide polymers were vary depending on many factor including the harvesting method, solvent quality and chain aggregation (Xu *et al*., 2007). The polydispersity is equal to 1, shown each polymer contains molecules all of the same size (Hanim *et al*., 2011). In this study, the polydispersity values obtained 1.04-1.76 which are equal to 1.

Table 3.3 Molecular weight and polydispersity of hemicellulose from POME and commercial xylan

Source of hemicellulose	Mn* (Da)	Mw ** (Da)	Mp*** (Da)	Polydispersity
Sterilizer condensate (SC)				
- HSC-L	24,134	42,191	20,002	1.74
- HSC-S	3,428	3,577	3,606	1.04
Decanter effluent (DE)				
- HDE-L	20,755	36,348	15,554	1.75
- HDE-S	3,468	3,613	3,724	1.04
Commercial xylan from beech wood				
- CXB-L	553,875	928,861	1,446,390	1.67
- CXB-S	11,800	20,879	33,442	1.76

*Mn: Number average of molecular weights
**Mw: Weight average of molecular weights
***Mp: The peak of molecular weights

3.4.2.3 Functional group analysis

The functional groups of the hemicellulose from POME (HSC and HDE) and commercial xylan from beech wood (CXB) were determined by the FT-IR (Figure 3.2). Among them, the three bands at 3432-3443 cm^{-1} showed the absorbance for the hydroxyl stretching vibrations of the hemicelluloses and water (Sun *et al.*, 2004). The peak at 2919-2925 cm^{-1} may be assigned to C-H stretching of $-CH_2-$ (Peng *et al.*, 2012). The absorption at 1620-1635 cm^{-1} was attributed to the bending mode of the absorbed water (Liu *et al.*, 2006). The absorbances at 1419-1421, 1305-1320 and 1250-1262 cm^{-1} were associated with hemicelluloses which represented the C-H, C-C and C-O bending or stretching frequencies (Peng *et al.*, 2012; Xu *et al.*, 2007). The absorbances at 3432-3443, 2919-2925, 1419-1421, 1149-1168, 1046-1080, 1030-1046, 893-897 cm^{-1} were associated with the typical values of celluloses (Liu *et al.*,

2006; Su *et al*., 2005). The absorption at 1419-1421 cm^{-1} assigned with the aromatic rings in lignin (Palamae *et al*., 2017; Su *et al*., 2014).

3.4.2.4 Solubility tests

Solubility is defined as the maximum quantity of a substance that can be completely dissolved in a given amount of solvent at equilibrium and their characterization displays a fundamental in fields of research that are chemistry, physics, food science, pharmaceutical and biological sciences. Solvents are classified into three groups according to their dielectric constants as polar (> 50), semi-polar (20 - 50) and non-polar (1 - 20). The solubility tests on HSC, HDE and CXB showed that distilled water (DW) was the best to solubilize hemicellulose at 96.82%, 99.05 % and 84.62%, respectively (Figure 3.3). In addition, toluene partially dissolved HSC, HDE and CXB. In this case, distilled water (DW) gave the highest solubility owing to the high dielectric constants (80.1) that is polar (Murov, 2016). In common, the polar solutes tend to dissolve best in polar solvents while non-polar solutes tend to dissolve best in non-polar solvents. The dielectric constants of DW was 78.84 (polar), NaOH was 57.5 (polar), DMSO was 47 (semi-polar) and toluene was 2.35 (non-polar). Strong polar which the high dielectric constants attractions equate to greater solubility while weak polar attractions equate to lesser solubility. Thus, the dielectric constants also have indicated with solubility. Gomes *et al*. (2015) reported that the xylan from beechwood (Sigma–Aldrich) gave high solubility in deionized water of 98.8%.

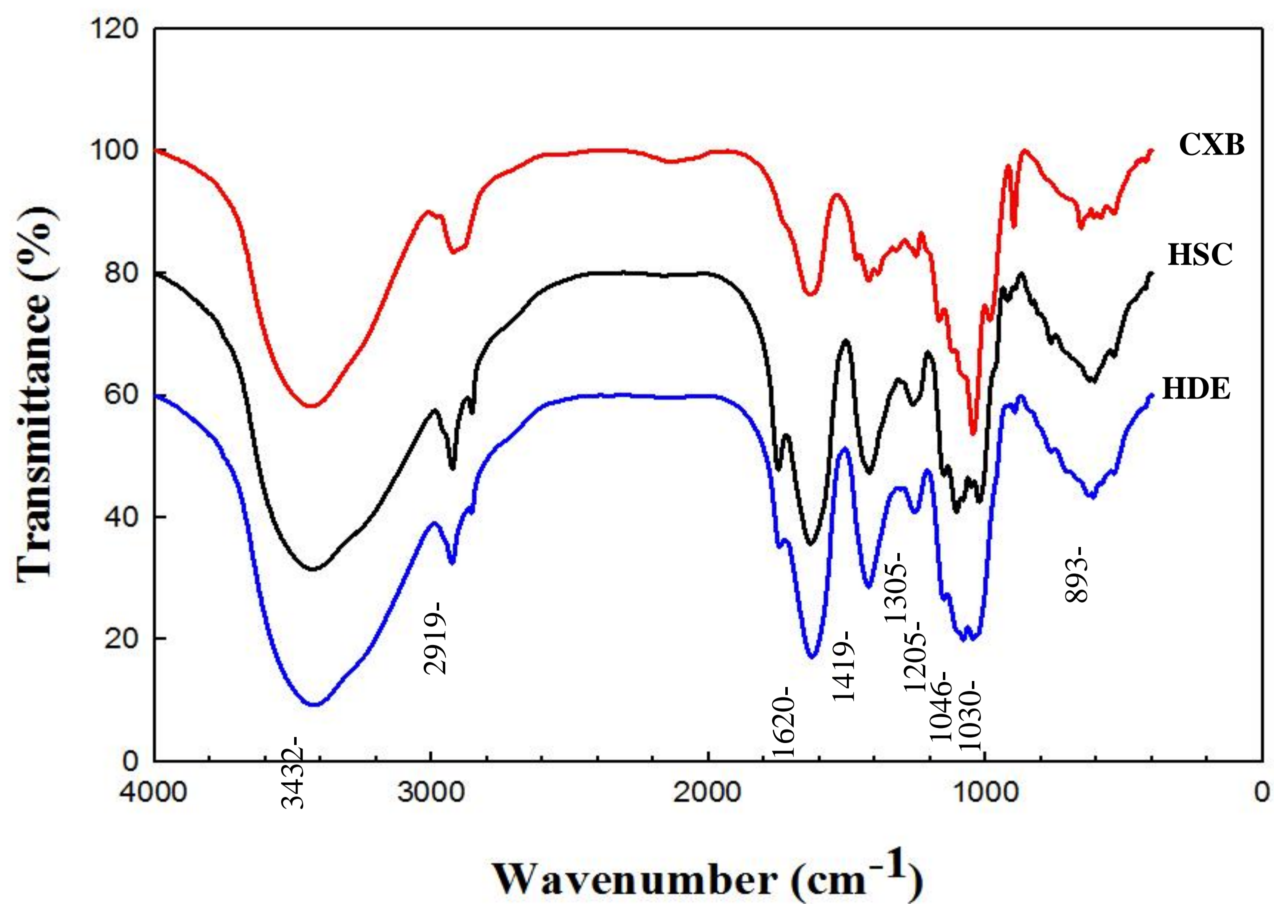


Figure 3.2 FT-IR spectra of the commercial xylan (CXB) and the hemicellulose from sterilizer condensate (HSC) and decanter effluent (HDE) of palm oil mill

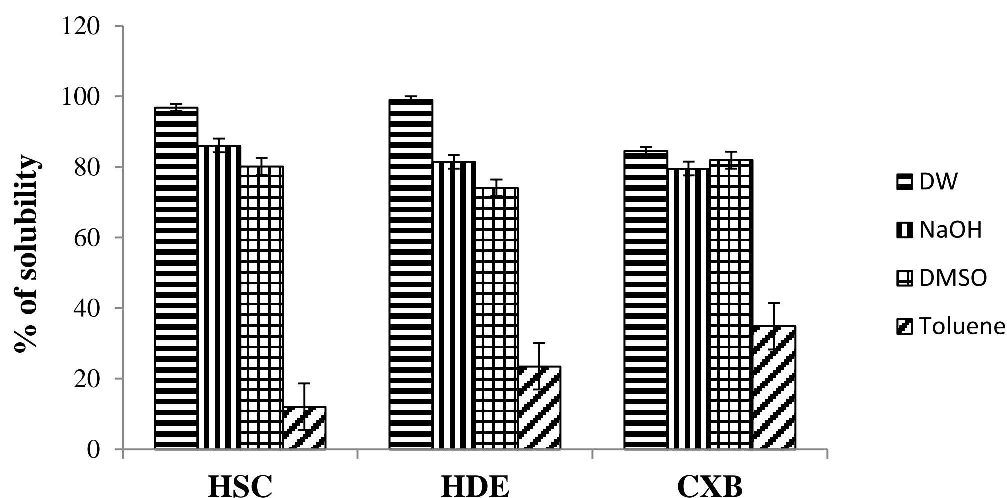


Figure 3.3 The solubility of polymers from sterilizer condensate (HSC), decanter effluent (HDE) of palm oil mill and commercial xylan from beech wood (CXB)

3.4.2.5 Thermal stability

The thermal properties of the hemicelluloses were analyzed to investigate the physicochemical properties of hemicellulose for application use the thermogravimetriy–differentaial thermal analysis. The thermal properties of the hemicellulose were analysed by Thermogravimetric analysis (TGA) and Derivative thermogravimetry (DTG) between room temperature (25±2°C) and 600°C. TGA–DTG analysis was determined weight loss allows us to describe the distinct thermal events recorded during the process, in this case, hemicelluloses were decomposed to monomer sugars, furfural, furan, aldehyde and acetic acid (Peng *et al.,* 2011). The thermograms of the TGA and DTG curves for HSC and HDE compared to commercial xylan were shown in Figure 3.4. The pyrolysis of hemicelluloses form *Phyllostachys pubescens* Mazel are divided into three stages: loss of water stage (< 190 °C), main pyrolysis stage (186 to 330 °C), and charring (> 330 °C) (Luo *et al.,* 2012). The TGA curves of the HSC and HDE began to decompose at 209 and 238°C, respectively while commercial xylan was at 221°C. Beyond these temperatures, thermal degradation takes place. At 697.7°C, 698°C and 698.8°C, respectively, of the three samples appeared residue weight (<40%) of the decomposition. The peaks at 290°C in the DTG curves correspond to the thermal decomposition of the

hemicellulose (Su *et al.,* 2014). However, the different thermal stability of fractions depended on many factors such as crystalline and amorphous regions, molecular weights, linear and branched structure of hemicelluloses (Peng *et al.,* 2011).

3.4.2.6 Biological properties

HSC and HDE had no antimicrobial activity tested (anti- *Bacillus cereus*, anti- *Klebsiella pneumoniae*, anti-*Mycobacterium tuberculosis* (anti-TB test) and anti- *Magnaporthe grisea* (rice blast disease pathogen)) and no inhibition against cancer cells (the human breast cancer (MCF7), Oral cavity cancer (KB) and small cell lung cancer (NCI-H187)). Moreover, the HSC and HDE had no-cytotoxicity against human caucasian colon adenocarcinoma (Caco2) ATCC HB-37 while HSC exhibited cytotoxicity against human hepatocarcinoma (HepG2) ATCC HB-8065 with the IC_{50} of 7.28 mg/mL and noncytotoxicity in HDE (Table 3.4). Consequently, the HSC and HDE have potential on several applications in the food, pharmacy, feed, medicine, cosmetics and industries owing to their diversity in composition, structure, and physical properties (Mahapatra and Banerjee. 2013)**.**

Table 3.5 shown the summarization of hemicellulose production by various sources. The several kinds of the plants have been used as extraction for produce hemicellulose. The 8% NaOH extraction of hemicellulose from sugar cane bagasse shown the highest hemicellulose of 2.8% (Peng *et al.,* 2010). The extraction with 90% dioxane also recovered high yield of hemicellulose (2.2%) (Sun *et al.,* 2010). In addition, The extraction with KOH, NaOH and ethanol were also able to extraction and recovered hemicellulose (Peng *et al.,* 2013, Peng *et al.,* 2011, (Peng *et al.,* 2010 and Peng *et al.,* 2011). This study has shown that SC and DE from POME is another good candidate for hemicellulose production with 95% ethanol (2.16 and 2.47%, respectively). Moreover, the hemicellulose from SC and DE gave high hemicellulose (82.98±0.85 and 82.75±0.39%), two sizes of molecular weights, decompose at 209 and 238 °C and residue weight (<40%) at 698 and 697.7 °C, respectively. Moreover, HSC showed cytotoxicity against human hepatocarcinoma (HepG2) ATCC HB-8065. Therefore, the characterization of HSC and HDE this species provides fundamental information for their potential application in the field of hydrogel material.

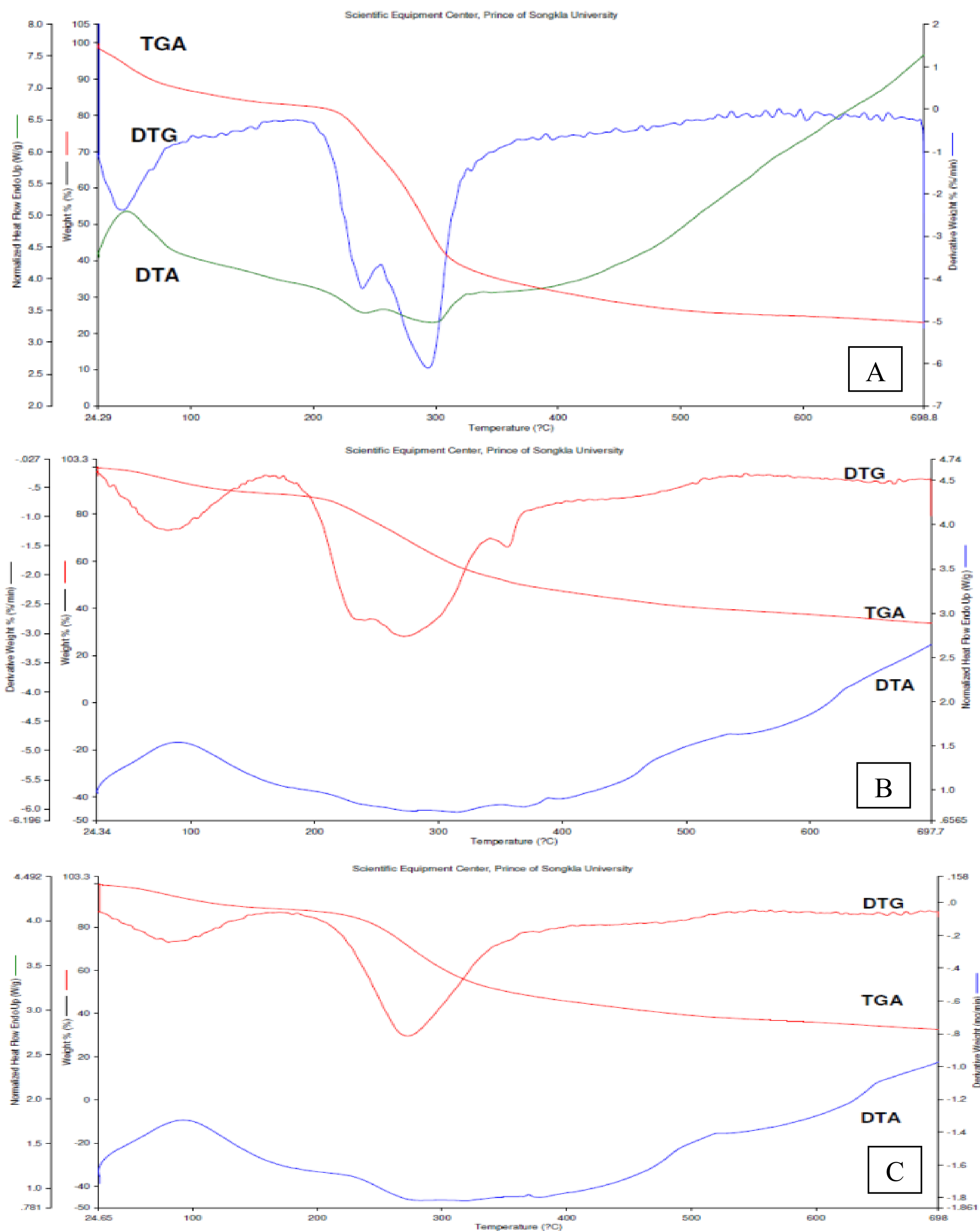


Figure 3.4 Thermograms of commercial xylan from beech wood (CXB) (A) and hemicellulose from sterilizer condensate (HSC) (B) and hemicellulose from decanter effluent (HDE) (C)

Table 3.4 The biological activity of hemicellulose from sterilizer condensate (HSC) and decanter effluent (HDE)

Test	Result (% inhibition)		Positive control (μg/ml)
	HSC (50μg/ml)	HDE (50μg/ml)	
Antimicrobial test			
- anti-*B. cereus*	Inactive	Inactive	Vancomycin (MIC= 2.00)
- anti-*K. pneumonia*	Inactive	Inactive	- Amikacin (MIC = 0.50) - Ofloxacin (MIC = 1.00)
- anti-*M. tuberculosis*	Inactive	Inactive	-Rifampicin(MIC = 0.0125) - Ofloxacin (MIC = 0.391) - Streptomycin (MIC = 0.313) - Isoniazid (MIC = 0.0469) - Ethambutol (MIC = 1.88)
- anti-*M. grisea*	Inactive	Inactive	- Amphotericin B (MIC = 3.13)
Anti-Cancer			
- NCI-H187 Small cell lung cancer	Inactive	Inactive	- Doxorubicin (IC_{50} 0.134) - Ellipticine (IC_{50} = 2.72)
- MCF7-breast cancer	Inactive	Inactive	- Tamoxifen (IC_{50}= 7.34) - Doxorubicin (IC_{50}= 8.52)
- KB-Oral cavity cancer	Inactive	Inactive	- Ellipticine (IC_{50}= 2.95) - Doxorubicin (IC_{50}= 0.755)
Cytotoxicity against human hepatocarcinoma (HepG2) ATCC HB-8065	cytotoxic	Non-cytotoxic	- Ellipticine (IC_{50}= 2.31)
Cytotoxicity against human Caucasian colon adenocarcinoma (Caco2) ATCC HB-37	Non-cytotoxic	Non-cytotoxic	- Ellipticine (IC_{50}= 9.15)

Table 3.5 The hemicellulose production by various sources

Sources	Pretreatment hemicellulose extraction	Hemicellulose extraction yield (%)	Reference
Maize stem	90% dioxane	2.2	Sun *et al*. 2010
Olea europaea L.	10%KOH	1.7	Peng *et al*. 2013
Bamboo	0.5% NaOH	1.85	Peng *et al*. 2011
Bamboo	75% ethanol	1.5	Peng *et al*. 2011
Sugar cane bagasse	0.5% NaOH	2.8	Peng *et al*. 2010
Sterilizer condensate	95% ethanol	2.16	This study
Decanter effluent	95% ethanol	2.47	This study

3.4.3 Hydrogel from the hemicelluloses from POME and commercial xylan from beech wood

Hemicellulose is polysaccharide polymer that cannot form stable hydrogel alone. Therefore, the production of stable polymeric hydrogels has been suggested to form stable hydrogels made by blending natural and synthetic polymers (Kamoun *et al*., 2015). In this study, the modification hemicellulose reacted with maleic anhydride (MA) and then, crosslinked between polyvinyl alcohol (PVA) and hemicellulose-MA to from blend hydrogel. The results showed that the modification of HSC, HDE from POME and commercial xylan from beech wood (CXB) was done by reaction with MA. The modification hemicelluloses were verified by FT-IR to support the existence of the ester linkages by esterification under a thermal treatment. The esterification of the commercial xylan resulted in the formation of a new peak at 1727 cm^{-1}, due to the stretch of carboxylic acid (C=O) produced by the cross-linking reaction of xylan with maleic anhydride (Tanodekaew *et al*., 2006) (Figure 3.5A) while the original commercial xylan did not exhibit that peak. In contrast, Figure 3.5B and 3.5C

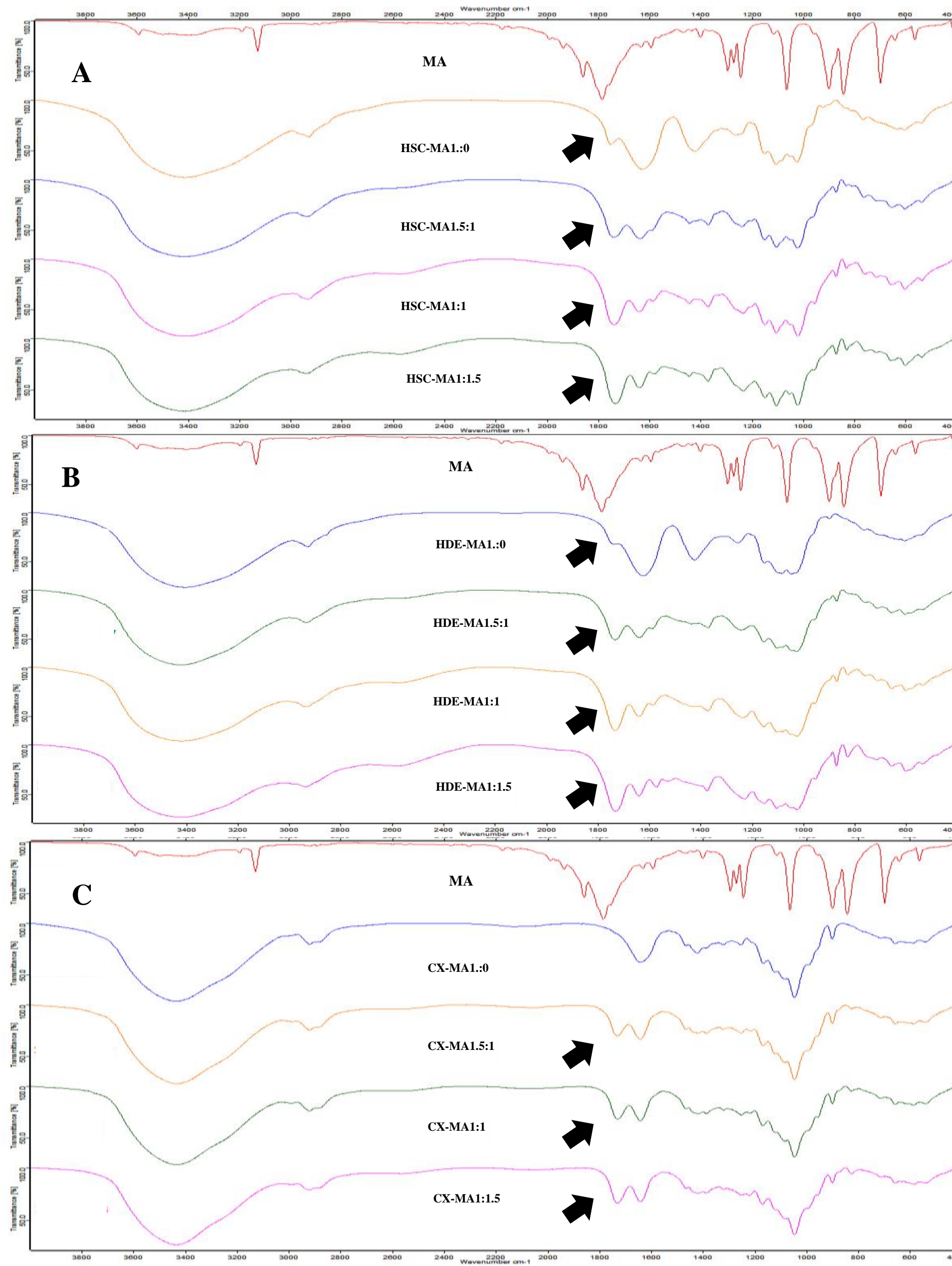


Figure 3.5 FT-IR spectra of the maleic anhydride (MA) and various ratio of HSC-MA (A), HDE-MA (B) and CXB-MA (C). Arrow indicate peak at 1727-1748 cm^{-1} in the modified from HSC-MA, HDE-MA and CXB-MA.

showed the original HSC and HDE assigned to ester bonds at 1743 and 1748, respectively (Peng *et al*., 2012). As a result, the combination between xylan the hemicellulose samples and MA effect seem to be dissolved in water than original and also promoted the hydrophilicity of the derivative (Tanodekaew *et al*., 2006). Then, each dried HSC-MA, HDE-MA and CXB-MA was blended with PVA at various PVA concentration under a thermal treatment.

In this case, the 37 hydrogels were elegant, flexible, thin and smooth surface in HSC-MA/PVA and HDE-MA/PVA but not smooth surface on the hydrogel in CXB-MA/PVA. The surface morphology appearance of hydrogels was detected by SEM (Figure 3.6). The PVA has hydrophilic character which is poor stability in water (Figure 3.6A, 3.6C and 3.6E). To above this problem, the PVA should be insolubilized by cross-linking with another polymer and these processes may reduce the hydrophilic character of PVA (Abd El-Mohdy and Ghanem, 2009). The HSC, HDE and CXB were done by cross-linking with PVA which spread in the hydrogel (Figure 3.6B, 3.6D and 3.6F). This structure is an advantage for improving the swelling of hydrogel also.

The HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogel were immersed in distilled water and their swelling behaviors were examined, as shown in Table 3.6, 3.7, 3.8 and Figure 3.7A, 3.7B and 3.7C. PVA hydrogel was used as a control. Table 3.6, 3.7 and 3.8 showed that without and with MA and weight ratio of hemicellulose to MA was effective. Increasing the MA concentration at PVA:hemicellulose-MA ratio of 1:1 surprisingly increased the swelling of HSC-MA/PVA, HDE-MA/PVA and CXB-MA/PVA hydrogel from 136.21±3.99 to 382.05±1.84%, from 150.84±3.1 to 238.23±2.63% and from 141.02±5.63 to 269.23±1.14%, respectively. Hence, the modification hemicellulose with MA in the first step and then, blend with PVA gave more hydrophilic polymers with three-dimensional networks that can absorb water without dissolving than without MA and PVA hydrogel (H37) which was without MA and hemicellulose. The PVA hydrogel has very limited hydrophilicity characteristics which restrict its use alone (Kamoun *et al*., 2015). Among 37 formulations test, the hydrogel that prepared from H17 of HDE gave the highest swelling behaviors than those samples (395.9±3.26%). It should be noted that the hydrogels from HSC-MA/PVA (H1-H12) and HDE-

MA/PVA (H13-H24) gave high swelling than CXB-MA/PVA (H25-H36). This may be due to the fact that the original HSC and HDE from POME appeared to have ester bands at 1743 and 1748 cm^{-1}, respectively to support the ester linkages.

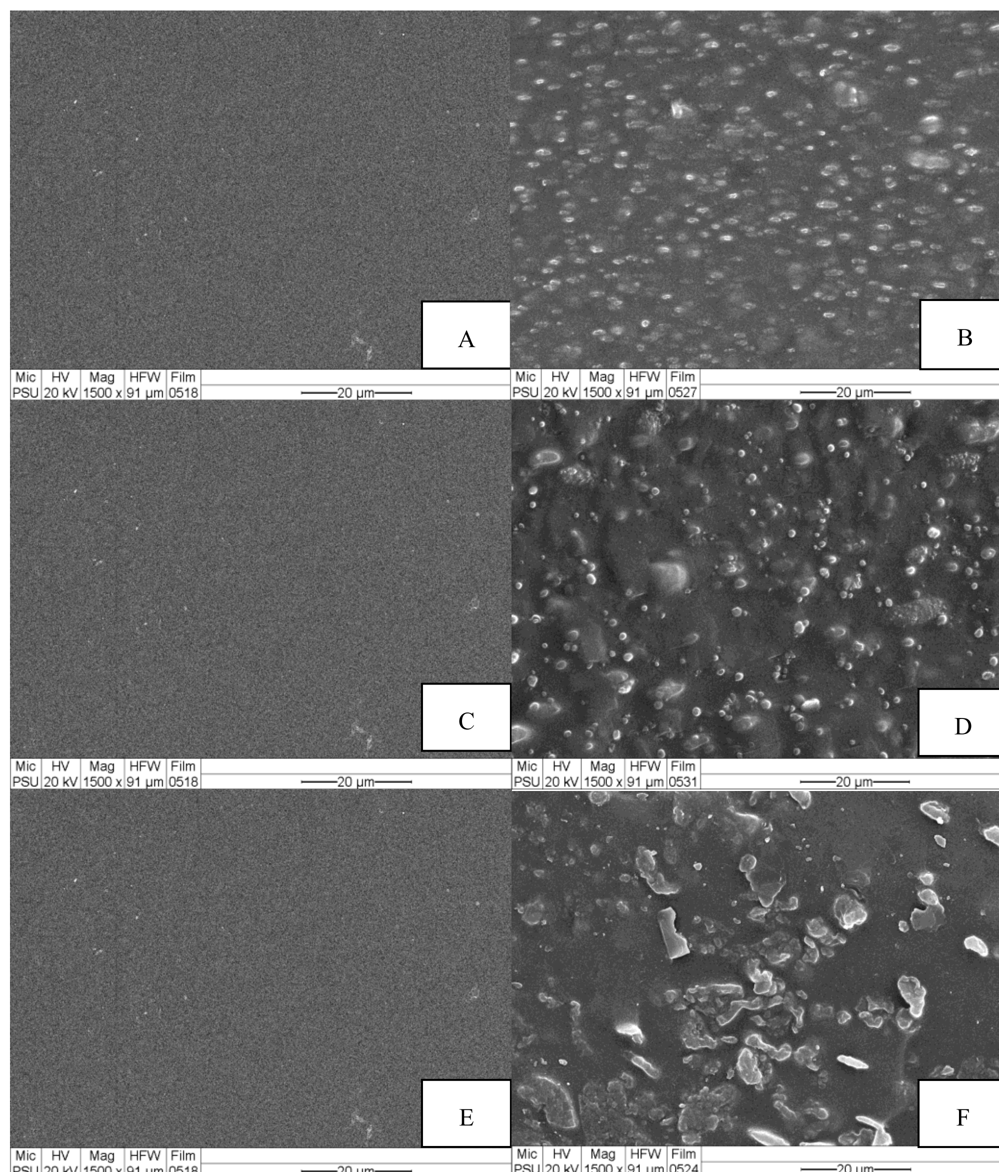


Figure 3.6 SEM of hydrogels prepared at various MA/PVA compositions; A. PVA hydrogel (H37), B. PVA/HSC-MA (H12), C. PVA hydrogel (H37), D. PVA/HDE-MA (H17), E. PVA hydrogel (H37), F. PVA/CXB-MA (H36) at 70°C. Magnification: 1500X

Table 3.6 The effect of MA and PVA contents on swelling degree and tensile strength of HSC-MA/PV hydrogels

Formulation	Type of hemicellulose	Ratio of concentration (g)		Degree of swelling (%)	Tensile strength (MPa)
		hemicellulose:MA	PVA: hemicellulose -MA		
H1	HSC	1:0	1:0.25	180.87±3.5^{B}	10.56±0.94^{CD}
H2	HSC	1:0	1:0.5	196.28±4.79^{B}	16.76±1.30^{AB}
H3	HSC	1:0	1:1	136.21±3.99^{B}	3.96±0.33^{E}
H4	HSC	1.5:1	1:0.25	215.82±3.95^{AB}	18.31±1.08^{A}
H5	HSC	1.5:1	1:0.5	231.04±1.73^{AB}	14.07±1.34^{ABC}
H6	HSC	1.5:1	1:1	235.61±1.58^{AB}	12.72±1.59^{ABCD}
H7	HSC	1:1	1:0.25	223.08±1.74^{AB}	15.7±0.75^{ABC}
H8	HSC	1:1	1:0.5	274.41±2.83^{AB}	7.95±1.11^{DE}
H9	HSC	1:1	1:1	284.59±4.15^{AB}	10.99±0.72^{CD}
H10	HSC	1:1.5	1:0.25	199.43±1.76^{B}	14.07±2.76^{ABC}
H11	HSC	1:1.5	1:0.5	284.33±2.44^{AB}	11.51±1.54^{BCD}
H12	HSC	1:1.5	1:1	382.05±1.84^{A}	10.44±2.02^{CD}
H37	PVA	-	1:0	134.61±1.46^{B}	12.35±1.46^{AB}

* The data in columns was presented as mean values with standard deviations, with significance threshold $p<0.05$

Table 3.7 The effect of MA and PVA contents on swelling degree and tensile strength of HDE-MA/PVA hydrogels

Formulation	Type of hemicellulose	Ratio of concentration (g)		Degree of swelling (%)	Tensile strength (MPa)
		hemicellulose:MA	PVA: hemicellulose-MA		
H13	HDE	1:0	1:0.25	229.35 ± 4.33^{B}	17.14 ± 1.46^{AB}
H14	HDE	1:0	1:0.5	175.96 ± 3.71^{B}	3.57 ± 1.23^{C}
H15	HDE	1:0	1:1	150.84 ± 3.11^{B}	8.95 ± 1.76^{B}
H16	HDE	1.5:1	1:0.25	182.69 ± 1.22^{B}	15.87 ± 1.84^{AB}
H17	HDE	1.5:1	1:0.5	395.9 ± 3.26^{A}	15.07 ± 3.77^{AB}
H18	HDE	1.5:1	1:1	280.32 ± 3.42^{AB}	15.94 ± 1.67^{AB}
H19	HDE	1:1	1:0.25	258.84 ± 3.33^{B}	15.82 ± 2.41^{AB}
H20	HDE	1:1	1:0.5	253.44 ± 2.36^{B}	21.12 ± 1.66^{A}
H21	HDE	1:1	1:1	211.48 ± 2.26^{B}	12.84 ± 2.54^{AB}
H22	HDE	1:1.5	1:0.25	226.56 ± 3.62^{B}	18.64 ± 3.33^{A}
H23	HDE	1:1.5	1:0.5	225.43 ± 4.62^{B}	14.26 ± 1.89^{AB}
H24	HDE	1:1.5	1:1	238.23 ± 2.63^{B}	10.02 ± 1.86^{AB}
H37	PVA	-	1:0	134.61 ± 1.46^{C}	12.35 ± 1.46^{B}

* The data in columns was presented as mean values with standard deviations, with significance threshold $p<0.05$

Table 3.8 The effect of MA and PVA contents on swelling degree and tensile strength of CXB-MA/PVA hydrogels

Formulation	Type of hemicellulose	Ratio of concentration (g)		Degree of swelling (%)	Tensile strength (MPa)
		hemicellulose:MA	PVA: hemicellulose-MA		
H25	CXB	1:0	1:0.25	191.45±4.38^{B}	10.74±1.55^{BCDE}
H26	CXB	1:0	1:0.5	163.36±2.43^{B}	4.22±1.46^{DE}
H27	CXB	1:0	1:1	141.02±5.63^{B}	19.01±1.55^{A}
H28	CXB	1.5:1	1:0.25	210.25±2.88^{B}	12.49±0.51^{BCD}
H29	CXB	1.5:1	1:0.5	192.27±1.32^{B}	5.59±1.38^{E}
H30	CXB	1.5:1	1:1	183.77±1.99^{B}	5.12±3.08^{E}
H31	CXB	1:1	1:0.25	168.82±4.52^{B}	16.62±2.84^{AB}
H32	CXB	1:1	1:0.5	208.28±1.32^{B}	4.4±1.48^{E}
H33	CXB	1:1	1:1	157.77±1.48^{B}	6.65±1.93^{DE}
H34	CXB	1:1.5	1:0.25	221.92±0.64^{A}	19.53±0.60^{A}
H35	CXB	1:1.5	1:0.5	241.57±2.46^{A}	14.92±0.62^{ABC}
H36	CXB	1:1.5	1:1	269.23±1.14^{A}	8.32±1.62^{CDE}
H37	PVA	-	1:0	134.61±1.46^{C}	12.35±1.46^{BCD}

* The data in columns was presented as mean values with standard deviations, with significance threshold $p<0.05$

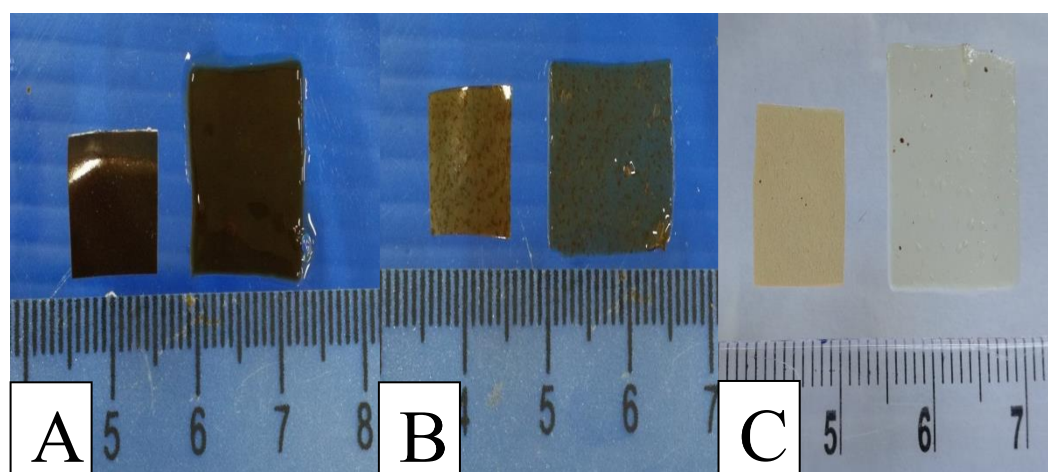


Figure 3.7 The before and after swelling degree of hemicellulose-MA/PVA hydrogels (A) SC, (B) DE and (B) commercial xylan

during esterification under a thermal reaction with MA. The results showed that the swelling in 37 formulations increased with increasing MA content. Under the optimal conditions of HSC (H12), HDE (H16), and CXB (H36), the highest swelling test could reach to 382.05±1.84%, 395.9±3.26 and 269.23±1.14%, respectively. The swelling of xalan in hemicellulose from beech wood hydrogel which reacted with MA and blened xalan-MA with PVA was depending upon the contents of MA and PVA in the xylan-MA/PVA hydrogels (Tanodekaew *et al*., 2006).

In this study, PVA was selected for hydrogel-forming properties in the second step, due to its good water-soluble, biodegradable, noncarcinogenic, biocompatible characters and can blended with synthetic and natural polymers (Kamoun *et al*., 2015).The mechanical testing specifically tensile properties of hydrogels are important parameters, as they can limit hydrogels application. The tensile strength data is shown in Table 3.6. The tensile strength of all samples (H1-H36) ranged from 3.57±1.23 to 21.12±1.66 MPa. The PVA hydrogel (H37) as a control was 12.35±1.46 MPa. Apparently, the number of hydrogel preparing from HDE (H13-H24) gave the higher average tensile strength than those of HSC (H1-H12) and CXB (H25-H36), respectively. Under the optimal conditions of H20 from HDE, the highest tensile strength could reach to 21.12±1.66 MPa which was due to higher crosslink density of PVA in the hydrogel-forming.

From the result, the optimal hydrogels which possessing high swelling and high tensile strength were selected for further study. These include the formulation H11 from HSC, H17 from HDE and H35 from CXB for cytotoxicity test for normal cells and antimicrobial tests.

The cytotoxicity of the hydrogels from formulations H11, H17 and H35 were evaluated. The objective of this test was to evaluate cellular response with the test specimen extracts by MTT cytotoxicity assay (Based on ISO 10993-5) using Microplate reader (EASYS, UVM340 S/N ASY54180). The % viability of L929 (Mouse Fibroblast Cells, ATCC CCL1, NCTC 929. of Strain L) cells adjacent to the negative and positive controls as well as the hydrogel is shown in Figure 3.8. All of them were reduced viability to < 70% of the blank. They have a cytotoxic potential. It was noticeable that the formulation H11from HSC hydrogel had the least viability of L929 at 3%. The formulation H35 and H17 hydrogel also have a cytotoxic potential respectively. There

is the viability of L929 at 9% and 13%, respectively. Consequently, the behavior of the viability of L929 cells on the formulation H11 from HSC hydrogel indicated that the gel was cytotoxic and had a potential application for cytotoxicity activity against cancer cells.

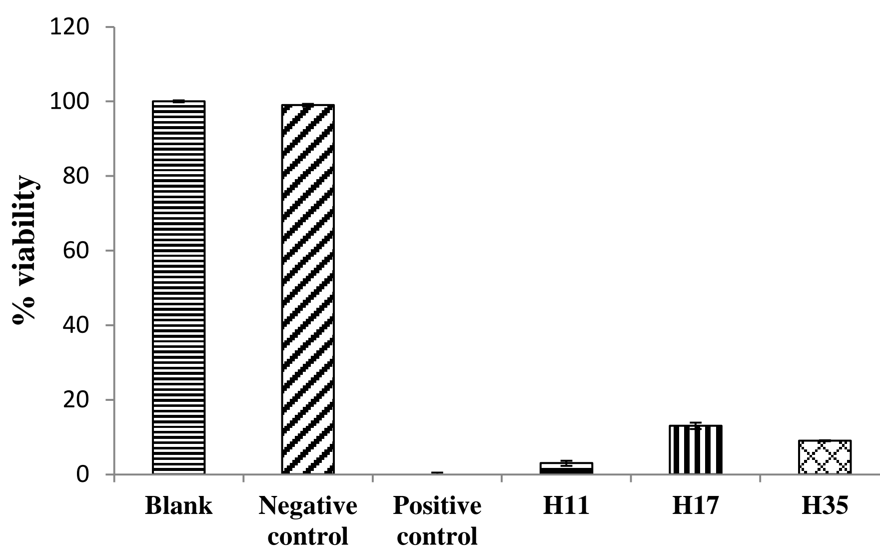


Figure 3.8 The percentage of cell viability of the formulation H11:HSC-MA/PV, the formulation H17:HDE-MA/PVA and the formulation H35:CXB-MA/PVA hydrogels. The celled viability was assessed by MTT assay

The antimicrobial activity of the hydrogel is the probability in the field of biomedical application. It was performed by zone inhibition methods after incubation for 24 h at 37 °C of Gram-positive bacterial *S. aureus* ATCC 6538, a Gram-negative bacterial *E. coli* ATCC 8739 and a yeast strain *C. albicans* ATCC 10231. The inhibition of antimicrobial activity of the formulation H11, H17 and H35 hydrogels is depicted in Table 3.7 and Figure 3.9, 3.10 and 3.11. The results showed that the formulation H17 from HDE and the formulation H35 from CXB hydrogels exhibited the inhibition zones (31 mm. and 30 mm., respectively) of *S. aureus* ATCC 6538. On the other hand, the formulation H11 from HSC hydrogel had no found inhibition zone. No antibacterial activity of *E. coli* ATCC 8739 and antiyeast activities (*C. albicans* ATCC 10231) of all biopolymer-MA/PVA compound were detected. Nevertheless, changing to use other stains of bacteria and fungi for antimicrobial activity test may find a potential for biomedical application such as *Klebsiella pneumonia* (Vimala *et al.*, 2010), *Bacillus subtilis* (Abd El-Mohdy and Ghanem, 2009) and *Penicillium italicum* (Abd El-Mohdy and Ghanem, 2009). The antibacterial properties of HDE-MA/PVA and CXB-MA/PVA hydrogels to *S. aureus* were successfully presented by observing the inhibition zones surrounding the samples.

Table 3.9 Antimicrobial activities of the formulation H11:HSC-MA/PV, the formulation H17:HDE- MA/PVA and the formulation H35:CXB-MA/PVA hydrogels

Formulation	The average diameter of inhibition zone (mm)		
	Staphylococcus aureus ATCC 6538	*Escherichia coli* ATCC 8739	*Candida albicans* ATCC 10231
H11 (10mmx10mmx3mm)	0	0	0
H17 (10mmx10mmx3mm)	31	0	0
H35 (10mmx10mmx3mm)	30	0	0
Ampicillin*	45	24	-
Nystatin**	-	-	26

*The diameter of the inhibition zones of amoxicillin of *Staphylococcus aureus* ATCC 6538 and *E. coli* ATCC 8739 as antibacterial controls were made.

**The diameter of the inhibition zone of nystatin of *Candida albicans* ATCC 10231 as antiyeast control was made.

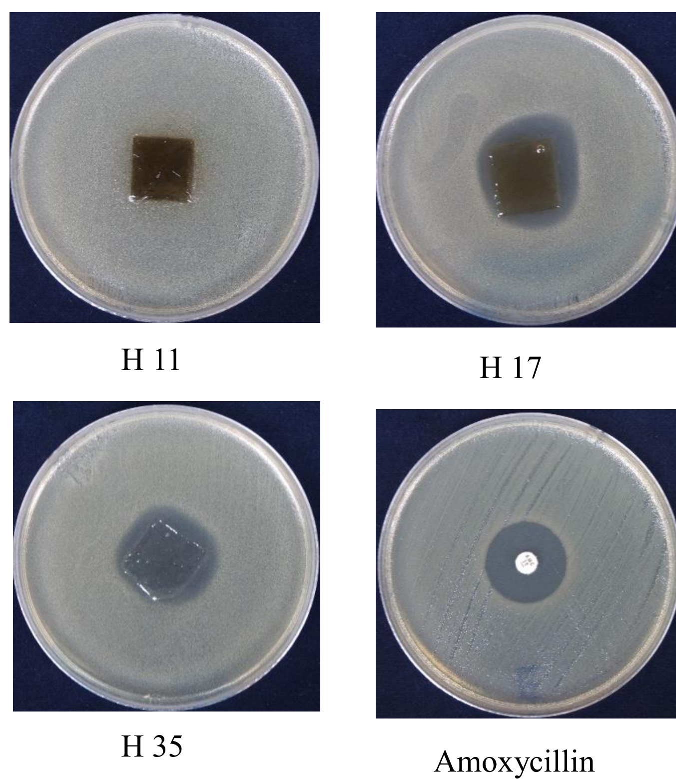


Figure 3.9 Antimicrobial activities of the H11:HSC-MA/PV, H17: HDE- MA/PVA and H35:CX-MA/PVA hydrogels against *Staphylococcus aureus* ATCC 6538. The amoxicillin was used as a control.

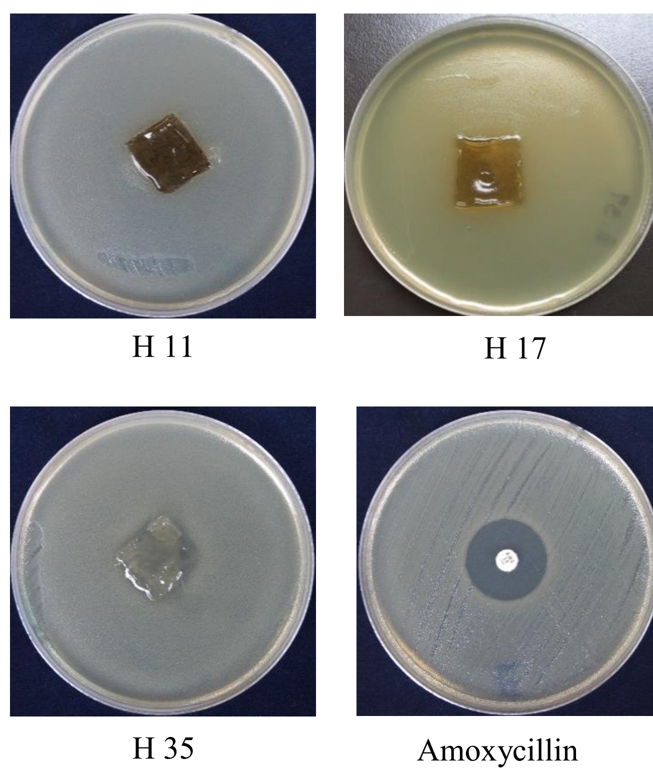


Figure 3.10 Antimicrobial activities of the H11:HSC-MA/PV, H17: HDE- MA/PVA and H35:CX-MA/PVA hydrogels against *Escherichia coli* ATCC8739. The amoxicillin was used as a control.

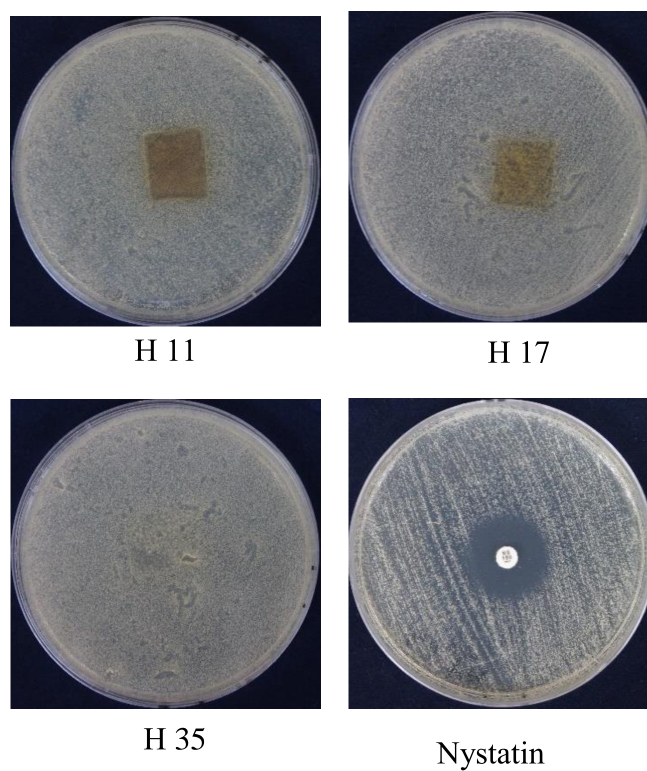


Figure 3.11 Antimicrobial activities of the H11:HSC-MA/PV, H17: HDE- MA/PVA and H35:CX-MA/PVA hydrogels against *Candida albicans* ATCC10231. The nystatin was used as a control.

3.4.4 Production of xylooligosaccharide (XOS) from hemicellulose from sterilizer condensate (HSC), hemicellulose from decanter effluent (HDE) and commercial xylan from beech wood (CXB)

3.4.4.1 Effect of incubation time on XOS production from the three hemicelluloses

The XOS production from the HSC, HDE and CXB was carried out by using endo-(1,4)-β-xylanases (EC 3.2.1.8). Table 3.8 shows the effects of incubation time on the production of XOS from HSC, HDE and CXB. The xylobiose from HSC, HDE was not detected. In contrast, the xylobiose from commercial xylan increased from 0.50±0.43g/L to a maximum of 0.77±0.12 g/L with the increase of reaction time from 0 h to 48 h when 8 U of xylanase was added to 100 mg of substrate and incubated at 40 °C. However, the concentration of xylobiose at 48 h did not significantly (*p*>0.05) different from that at 12 h. The results found that the xylanase concentration of 8 U incubated at 40 °C had low concentration of xylopentaose in all samples. In point of this, the production of XOS from commercial xylan was able to produce at the short time. Thus, The production of XOS from commercial xylan at 12 h was selected for further studies.

Table 3.10.Enzymatic hydrolysis of HSC, HDE from POME and commercial xylan as functions of hydrolysis time

Time (h)	HSC		HDE		CXB	
	Xylobiose (g/L)	Xylopentaose (g/L)	Xylobiose (g/L)	Xylopentaose (g/L)	Xylobiose (g/L)	Xylopentaose (g/L)
0	ND	ND	ND	ND	ND	ND
12	ND	$0.001±0.03^{A}$	ND	$0.001±0.13^{A}$	$0.50±0.43^{A}$	$0.01±0.03^{A}$
24	ND	$0.002±0.03^{A}$	ND	$0.003±0.03^{A}$	$0.60±0.95^{A}$	$0.02±0.03^{A}$
36	ND	$0.002±0.04^{A}$	ND	$0.004±0.45^{A}$	$0.76±0.55^{A}$	$0.03±0.04^{A}$
48	ND	$0.002±0.04^{A}$	ND	$0.005±0.22^{A}$	$0.77±0.12^{A}$	$0.03±0.04^{A}$

The data in columns was presented as mean values with standard deviations, with not significance threshold *p*>0.05

ND = not detected

3.4.4.2 Effect of enzyme concentration on XOS production from commercial xylan

Table 3.9 shows the effects of xylanase concentration on hydrolysis of commercial xylan from beech wood 12h. Increasing the xylanase concentration from 4 U to 8, 12, and 16 U/100 mg substrate, produced xylobiose from 0.50 ±0.02 g/L to 0.67±0.02, 0.57±0.04 and 0.49±0.04 g/L, respectively and also low concentration of xylopentaose in all samples. In this study, increasing the xylanase activity from 4 U to 16 U did not significantly (p>0.05) increase the xylobiose production. In fact, the enzyme activity was limited by the availability of the substrate. Under the optimum conditions of using commercial xylan to produce XOS at 4 U for 12h at 40 °C, the highest xylobiose could reach 0.5 g/L.

Table 3.11 Enzymatic hydrolysis of commercial xylan from beech wood as functions of enzyme concentration at 12h

Enzyme conc. (U)	Xylobiose (g/L)	Xylopentaose (g/L)
0	ND	ND
4	0.50 ± 0.02^{A}	0.02 ± 0.01^{A}
8	0.67 ± 0.02^{A}	0.02 ± 0.03^{A}
12	0.57 ± 0.04^{A}	0.03 ± 0.04^{A}
16	0.49 ± 0.04^{A}	0.03 ± 0.04^{A}

The data in columns was presented as mean values with standard deviations, with not significance threshold p>0.05

ND = not detected

3.5 Conclusions

1. The hemicelluloses recovered from sterilizer condensate (HSC) and decanter effluent (HDE) gave the yield of 2.16±0.56% and 2.47±0.89% (w/v), respectively.

2. Chemical composition of the HSC and HDE gave high hemicellulose (82.98±0.85 and 82.75±0.39%) and commercial xylan from beech wood (CXB) also gave high hemicellulose content (90.84±0.46%). Each HSC, HDE contained two sizes of molecules with higher Mw of HSC-L than the HDE-L (43 kDa vs 36 kDa) while the Mw of HSC-S and HDE-S was about the same (3.56 kDa vs 3.61 kDa). CXB contained two sizes of molecules with higher Mw 930 kDa and 33kDa, respectively. The major monosaccharide of the HSC and HDE was xylose (about 67% and 71.5%, respectively) followed by glucose (21.4% and 20.3%, respectively) while in CXB was xylose only (79%). They were characterized on thermochemical and biological properties. The HSC and HDE had no antimicrobial activity against various microorganisms tested and found not to inhibit the cancer cells. In addition, only the HSC showed positive result on the cytotoxicity test against human hepatocarcinoma (HepG2) ATCC HB-8065.

3. Hemicellulose-MA/PVA hydrogels was prepared by modified HSC, HDE and CXB with maleic anhydride (MA) and then blended with polyvinyl alcohol (PVA). With various formulations, the hydrogel with high swelling and tensile strength; the formulations H11 from HSC and H17 from HDE, were selected for characterization studies. The formulation H11 of HSC-hydrogel possesed cytotoxicity activity against cancer cells. In addition, the formulation H17 of HDE and H35 of CXB hydrogel exhibited antibacterial activity against *Staphylococcus aureus* ATCC 6538

4. Production of xylooligosaccharide (XOS) from HSC, HDE and CXB was conducted using the enzymatic method. Under the optimum conditions for XOS production prepared from commercial xylan at 4 U for 12h at 40 °C gave the highest xylobiose oligosaccharide of 0.5 g/L.

CHAPTER 4

EXTRACTION, CHARACTERIZATION AND APPLICATION OF HEMICELLULOSE FROM OIL PALM TRUNK

4.1 Abstract

Extraction and characterization of the hemicellulose from oil palm trunk (OPT) were investigated. The OPT was cut, debarked and reduced its size, namely OPT_R. To obtain a fraction rich in OPT_R, the OPT_R was mixed with a mass ratio of solid to distilled water at 1:1 (w/v). The filter cake, designated as $OPT_{RT,}$ was grinded into smaller pieces using knife mill. The size of OPT_{RT} (0.6>OPT <2 mm), called OPT_{RT1}, was treated with alkaline peroxide (AP) solution, then the liquid fraction was precipitated with 3-fold volume of absolute ethanol. Under the optimal conditions, the maximum concentration of precipitated hemicellulose (PHC_{CM}) was 26.25% (w/w) of OPT_{RT1}. The PHC_{CM} had a molecular weight (Mw) of 67.57 KDa. The major monosaccharide in PHC_{CM} was glucose and xylose. Solubility tests on hemicellulose sample showed that DMSO was the best solvent to solubilize hemicellulose. The TGA curves of the PHC_{CM} began to decompose at 261°C.

PHC_{CM} -MA was prepared by reacting PHC_{CM} with maleic anhydride (MA) under the ratio of PHC_{CM} -MA (1:1.5 (w/w)), and blended with polyvinyl alcohol (PVA) to make hydrogel. The hydrogel prepared from PVA: PHC_{CM} -MA at 1:0.25 (H10) is the best ratio to achieve the highest degree of swelling (308.32%). Under the optional conditions (PHC_{CM}-MA1:0.67/PVA1:0.25;H4), the highest tensile strength could reach to 15.39 MPa. The hydrogel that have high strength and swelling was selected for the cytotoxicity test. The formulation H10 exhibited the least viability of L929 (Mouse Fibroblast Cells, ATCC CCL1, NCTC 929. of Strain L) at 14% recommended dose and no antimicrobial activity.

The xylooligosaccharide (XOS) were produced from PHC_{CM} using the enzymatic method. Under the optimum conditions, 4 U for 12h at 40 °C, the high XOS production (48%) and xylobiose (oligosaccharide) could reach to 0.25 g/L. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay showed that the XOS from PHC_{CM}

exhibited antioxidant activity. The IC_{50} as the XOS from PHC_{CM} showed 50% inhibition at 19,610 μg/mL.

4.2. Introduction

Currently, oil palm is an important economic plant particularly in the south of Thailand (Krabi, Satun, Suratthani and Chumphon). Standard or wet process is commonly employed for palm oil extraction in the palm oil mill that resulted in the generation of large quantity of a liquid waste, average 2.5 tonnes of palm oil mill effluent are generated for every ton of crude palm oil production (Madaki and Seng, 2013). Apart from the liquid waste, the solid waste such as the oil palm trunk (OPT), oil palm frond (OPF), kernel shell, empty fruit bunch (EFB) and presses fruit fibre (PFF) are generated from the mill/plantation area.

In addition, the economic live span of an oil palm tree is between 25 to 30 years. After this period the oil palm trees are no longer considered of an economic value. They need to be replaced with new trees. Presently, Thailand has estimated 100,000 ha of planting area covered with >25 years old trees (Dallinger J, 2011). The attraction towards utilization of natural fibres as a reinforcement of polymer-based composites is mainly due to their various advantages over synthetic fibres such as are low density, lower cost, biodegradability, and non abrasiveness (Khalil *et al*., 2012).

OPT is a solid waste obtained in large quantities after felling of oil palm trees. It is consists of lignocellulosic material and its biomass is utilized in the production of many products. OPT is classified as lignocellulosic residues that typically contain 29-45.81% cellulose, 12-29.12% hemicellulose, and 18-24.51% lignin (Ang *et al*., 2013; Khalil *et al*., 2008; Khalil *et al*., 2012) and quite significant amount starch (12.19-17.17%) (Hasshim, *et al.* 2011). Hemicellulose is the second component most abundant after cellulose. Omar *et al.* (2011) reported the percentage of starch content at different portions of oil palm trunk were 2.93-10.86%, which distribute within oil palm trunk, in which it was increasing from base upwards and from the outer inwards. The OPT rich starch and hemicellulose content are promising feed stock. Therefore, research on the utilization of OPT should be undertaken, particularly on the fractionation of starch and hemicellulose for the production useful products. Xylan is the major component of hemicellulose. It has biodegradable and biocompatibility properties (Sun *et al*., 2016). Thus, xylan has become a very hopeful raw material for preparing novel materials.

Hydrogels are hydrophilic polymers with three-dimensional networks structure. Several methods have been reported for the preparation of hydrogels *via* co-polymerization or crosslinking reactions. Hydrogels were able to preparation from synthetic polymers or biopolymers that can be prepared from the hybrids of a synthetic polymer and a biopolymer, two different biopolymers, or two different synthetic polymers (Liu *et al*., 2012) such as polyvinyl alcohol/polyvinyl pyrrolidone (Abd El-Mohdy and Ghanem, 2009), cellulose/chitosan (Liu *et al*., 2012), chitosan/polyvinyl alcohol (Maji *et al*., 2013), xylan/chitooligomer–zinc (Wu *et al*., 2012) and xylan/poly(methacrylic acid) (Sun *et al*., 2016). The key feature of hydrogels with network structures is their ability to store a lot of water and biological fluid. Subsequently, the hydrogels can be used in many applications, such as the absorption of heavy metals, drug delivery systems and materials for wound healing. Tanodekaew, *et al*. (2006) modified with maleic anhydride (MA) to obtain xylan-MA and then blended with polyvinyl alcohol (PVA) that the gel was noncytotoxic and had a potential for biomedical.

Xylooligosaccharides are sugar oligomers made up of xylose units. XOS are naturally oligomers sugar present in fruits, vegetables, bamboo, honey and milk and can be produced at industrial scale from xylan-rich materials. Of particular interest are those sources of residual origin, such as forestal, agricultural or industrial wastes of lignocellulosic nature. Processing of residual vegetable biomass as a raw material offers economic and ecological benefits since it is a biorenewable, widely distributed and abundant resource. It can be produced at industrial scale from xylan-rich materials. Hemicellulose is rich in xylan, a polysaccharide used to develop technology for producing alcohol, xylose, xylitol and XOS. XOS also have excellent potential for applications in pharmaceutical, agriculture, and food industry (Carvalho, *et al*., 2013; Jain, *et al*., 2015; Va´zquez, *et al*., 2000).

The aims of this study are to produce hemicellulose from the palm oil palm trunk, then characterize and to find the interesting properties. For examples, molecular weight was determined with a gel permeation chromatography (GPC); structural characterization was investigated by scanning electron microscopy (SEM),the chemical functional group identification was done by Fourier Transmission Infrared spectroscopy (FTIR); the thermal stability of hemicellulose was investigated

by Thermogravimetric analysis (TGA). Finally, the extract hemicellulose was used for the productions of hydrogel and xylooligosaccharide.

4.3 Materials and methods

4.3.1. Oil palm trunk and raw material preparations

Twenty-five years old of oil palm (*Elaeis guineensis*) trunk (OPT) with 47 cm in diameter, 9 m long, and 1,786 kg in weight was provided from oil palm plantation in Phangnga Province, southern Thailand. OPT was cut along horizontal line into 3 parts. The first part from top end was 3 m, middle was 2 m and the last 4 m in lengths, respectively. The middle part was debarked and cut a long vertical line into thick slabs with 1.5 in thick x 3 in width x78.74 in length. Then, they were ground into smaller pieces using a hammer mill (Model, SCL-KR, Mitsubishi electric Co., Ltd, Bangkok, Thailand.). The resulting material was particles in the range of 1-8 cm to be called $OPT_{R.}$, and were brought for starch extraction according to method presented by Daiuto *et al.* (2005) with slightly modification. The OPT_R was triturated with distilled water at a ratio of 1:1 (w:v) for 5 min at room temperature (28±2 ºC). After that this mixture was filtrated through a 0.6 mm sieve. The liquid suspension passed through a 0.6 mm sieve was kept in a cold room (7±2 °C) overnight for sedimentation process. Then, the supernatant liquid was discarded. The precipitated was placed on trays and left in an oven at 50 °C for 48h. The dried starch was ground in a knife mill (Somboonsub marketing Co., Ltd, Bangkok, Thailand.) driven by a 1.8 kW electric motor. The rotational speed of the mill was 25,000 rpm. The dried starch powder was passed through a 250 µm sieve, namely $OPT_{RS.}$

The filter cake which remained on the filter membrane, designated as OPT_{RT}, was leaved for 48 h at 50 ºC in hot air oven till obtained 5% moisture content. Then, it was reduced particle size using the knife mill (as described above). The dried OPT_{RT} was sieved to obtain particle sizes, >0.6-<2 mm (= OPT_{RT1}), <0.6 mm (= OPT_{RT2}) and < 250 µm (=OPT_{RT3}).

4.3.2 Pretreatment of OPT_{RT1}

4.3.2.1 Chemical pretreatment

Alkaline peroxide (AP) reagent with slightly modified from Palamae *et al.*, 2017 was prepared. It was prepared by mixing 10% of sodium hydroxide, 2%

of hydrogen peroxide and added 1.5% of magnesium sulfate. Nitric acid and acetic acid solution was prepared by diluting nitric acid (90 mL) and acetic acid (732 mL) to 1 L with deionized water (Wright and Wallis, 1998). Anthrone solution was prepared by adding 0.2 g of anthrone in 100 mL of 72% sulphuric acid (Bra´nyikova´ *et al.*, 2011).

The OPT_{RT1} was mixed with AP solution at a ratio of 1:20 (w/v). The flask was incubated at 45 °C on a rotary shaker (90 rpm) for 3 h, then, the OPT_{RT1} mixture was filtered through the nylon mesh with pore size 45 µ and retained the filtrate. The solid fraction was neutralized to final pH of 5-5.5 with 6 M hydrochloric acid. Lastly, the OPT_{RT1} fibers were washed with distilled water and dried in a hot air oven at 50 °C for 48 h. The filtrate fraction was adjusted to pH 5.5 with 37% hydrochloric acid. The filtrate precipitated with various volumes of ethanol (95%) at room temperature (28 ± 2 °C) for 48 h and then centrifuged (3,500 rpm) at 4 °C for 10 min. The precipitated fraction was washed with 70% ethanol and dried at 50 °C in a hot air oven for 24 h, namely precipitated hemicellulose (PHC_{CM}). The solid fraction (OPT_{RT4}) and PHC_{CM} were kept at room temperature (28 ± 2 °C) for further used. The samples were measured for hemicellulose, cellulose and acid insoluble lignin.

4.3.2.2 Autohydrolysis pretreatment

Twenty grams of OPT_{RT1} and 200 ml distilled water were placed in a 500-mL autoclavable bottle, and the resulting mixture was heated in an autoclave (121 °C and 15 psi for 60 min). After autoclaving, the sample was recovered by centrifugation (2380xg, 15 min). The supernatant of OPT_{RT1} was filtered through Whatman no. 4 filter paper (Hanim *et al.*, 2011). The supernatant (hemicellulose fraction) was precipitated with 3 folds (v/v) of 95% ethanol at room temperature and left 48 h, then centrifugation (10,000 rpm) at 5°C for 15 min and then lyophilized to obtain a precipitated hemicellulose (PHC_{AH}). The solid fraction and precipitated hemicellulose fraction were determined for hemicellulose, cellulose and acid insoluble lignin.

From the processed described above, precipitated hemicellulose was obtained by chemical and thermal treatments. PHC_{CM} and PHC_{AM} stand from precipitated hemicellulose being chemical and thermal treatments, respectively.

Application of the selected PHC depended on the yield of hemicellulose production after pretreatment. PHC would be applied as hydrogel and xylooligosaccharide.

4.3.3 Application of the precipitated hemicellulose by chemical treatment (PHC_{CM}) of hydrogel production

Preparation of hydrogel from PHC_{CM} was carried out into 2-step. Firstly, the PHC was reacted with maleic anhydride (MA) with the weight ratios of PHC to MA at 1:0, 1.5:1, 1:1 and 1:1.5 (Table 4.1), using dimethyl sulfoxide (DMSO) as a solvent. The mixture was continuously stirred at 200 rpm at 50 °C under acid condition (pH 3) for 2 h. The resulting derivatives of PHC_{CM} -MA were precipitated with 3 folds (v/v) of isopropanol for 48 h at room temperature. They were recovered using centrifugation (3,500 rpm, 10 min). Then, the samples were dried in an oven at 50 °C. The PHC_{CM} -MA products were determined for chemical structure using FTIR.

Secondly, PHC_{CM} -MA/PVA hydrogel were prepared as following: the PHC_{CM}-MA production were dried before blending with polyvinyl alcohol (PVA, molecular weight ~100,000) at 1:0, 1:0.25, 1:0.5 and 1:1 (Table 4.1), using distilled water as a solvent. The mixture was continuously stirred at 90 rpm at 70 °C for 4 h, then, pouring on a tray and dried at 50 °C for 8 h (Tanodekaew *et al*., 2006). The samples measured for swelling test, tensile test, cytotoxicity test for normal cells and antimicrobial.

4.3.4 Application of the hemicelluloses (PHC) for production of xylooligosaccharide (XOS)

4.3.4.1 Effect of hydrolysis time on XOS production from PHC

Enzyme xylanase (endo-(1,4)-β-xylanases:EC3.2.1.8) from *Trichoderma viride* X3876) concentration of 8 U per 100 mg PHC was added in 0.05 M phosphate buffer, pH 7.0, in 250-mL conical flasks. The hydrolysis was carried out at 40 °C with orbital shaking at 150 rpm for 0, 12, 24, 36 and 48 h. The reaction was stopped by holding the samples in boiling water for 5 min (Hanim *et al*., 2011). The XOS contents was determined by HPLC. The xylobiose was quantitatively analyzed by HPLC (Agilent 1200 series, USA) using Biorad-Aminex HPX-87P (300 mm x 7.8 mm) and

Refractive index detector. The mobile phase used was water and the flow rate was 0.6 mL/min at 80 °C.

Table 4.1 The composition of hydrogel-forming from precipitated hemicellulose by chemical treatment (PHC_{CM}) with maleic anhydride (MA) and polyvinyl alcohol (PVA)

Formulation	Ratio of concentration (g)	
	PHC:MA	PVA:PHC-MA
H1	1:0	1:0.25
H2	1:0	1:0.5
H3	1:0	1:1
H4	1.5:1	1:0.25
H5	1.5:1	1:0.5
H6	1.5:1	1:1
H7	1:1	1:0.25
H8	1:1	1:0.5
H9	1:1	1:1
H10	1:1.5	1:0.25
H11	1:1.5	1:0.5
H12	1:1.5	1:1
H13	-	1:0

4.3.4.2 Effect of enzyme concentration on XOS production from PHC_{CM}

The results as obtain from the experiment of section 4.3.4.1 were selected for studying on the effect of enzyme concentration on XOS production. The experiments were carried out as above but enzyme concentrations at 0,4, 8, 12 and 16 U per 100 mg PHC_{CM} were added in 0.05 M phosphate buffer, pH 7.0, in 250-mL conical flasks, in separated experiment. The hydrolysis was carried out at 40 °C with orbital shaking at 150 rpm. The reaction was stopped by holding the samples in

boiling water for 5 min (Hanim *et al*., 2011). The samples were measured for XOS contents was determined by HPLC (as above).

4.3.4.3 Production of XOS from PHC_{CM}

In this section, the selected conditions that are time for reaction and suitable enzyme concentration (see sections 4.3.4.1 and 4.3.4.2) were used for XOS production. After the reaction was stopped by putting a sample in boiling water for 5 min (Hanim *et al*., 2011), then the sample was brought for centrifugation (1120 x g) at 4 °C for 5 min and filtered through a 0.2 µm filter (Bian *et al*., 2013) unhydrolyzed hemicellulose retained as solid form while the XOS was dissolved in the solvent. The XOS was precipited with 3 volume of cold 95 % ethanol (4°C) overnight (Kallel *et al*., 2015), then centrifugation (8,000 rpm) at 4°C for 15 min. Ethanol was removed from the filtrate by rotary evaporation (RV 10 digital, IKA, Malaysia) at 45 °C and lyophilized. The XOS powder from PHC_{CM} measured for antioxidant activity.

4.3.5 Analysis

4.3.5.1 Chemical composition of raw materials

The samples of OPT_R, OPT_{RT}, OPT_{RS}, OPT_{RT1}, OPT_{RT2}, OPT_{RT3}, OPT_{RT4}, PHC_{CH} and PHC_{AH} were determined for hemicellulose (Lin *et al*., 2010) and cellulose contents (Wright and Wallis, 1998). The samples of OPT_R, OPT_{RT}, OPT_{RS}, OPT_{RT1}, OPT_{RT2}, OPT_{RT3}, OPT_{RT4}, PHC_{CH} and PHC_{AH} were determined for acid insoluble lignin contents (Sluiter *et al*., 2008). The samples of OPT_R, OPT_{RT}, OPT_{RS}, OPT_{RT1}, OPT_{RT2},OPT_{RT3} and OPT_{RT4} were determined for ash (Sluiter *et al*., 2008). The samples of OPT_R, OPT_{RT}, OPT_{RS}, OPT_{RT1}, OPT_{RT2},OPT_{RT3} and OPT_{RT4} were determined for moisture content (A.O.A.C. 1999). The samples of OPT_R, OPT_{RT}, OPT_{RS}, OPT_{RT1}, OPT_{RT2} and OPT_{RT3} were determined for starch content (Bra´nyikova´*et al.,* 2011). The samples of PHC_{CH} and PHC_{AH} were determined for sugar composition content with a High-Performance Liquid Chromatography (HPLC) (Agilent 1200 series, USA) using Biorad-Aminex HPX-87P (300 mm x 7.8 mm) and Refractive index detector. The mobile phase used was water and the flow rate was 0.6 mL/min at 80 °C. The samples were prepared by hydrolyzing the PHC_{CH} and PHC_{AH} (100 mg) using 1 mL of 72% sulfuric acid at 30 °C for 2 h. 28 mL of deionized water was added and treated at 121 °C for 1 h using an autoclave. The hydrolysate samples were filtered through Whatman No.1. The liquid fractions were adjusted the initial pH

to 5-6 with 15N NaOH. Then, adjusting the initial volume to 40 mL and through filters with a 0.45 µm and determined type and concentration of sugar.

4.3.5.2 Surface morphology

The OPT_{RS}, OPT_{RT1}, OPT_{RT3}, OPT_{RT4}, PHC_{CM}-MA/PVA hydrogel and PVA hydrogel samples were mounted on the aluminum stub, placed in the sample holder and metalized with a thin layer of gold. The samples were examined using scanning electron microscope (Quanta 400, FEI, Czech Repubic).

4.3.5.3 Molecular weight of PHC

Average molecular weight of the PHC sample and commercial xylan were determined with a gel permeation chromatography (GPC). The analysis was carried out using a GPC, Waters 600E system equipped with a refractive index (RI) detector and an Ultrahydrogel linear 1 column and guard column (MW resolving range from 1,000 to 20,000,000). The universal calibration between the peak molecular weight standard versus its relative velocity (V_R) was obtained by using pullulan (polysaccharide) standards with molecular weights ranging from 5,900 to 708,000. The injection volume was 20 µl and the flow rate of the mobile phase (0.05 M sodium bicarbonate buffer pH 11) was 0.6 ml/min at 30 °C (Madla *et al*., 2005).

4.3.5.4 Solubility tests of PHC_{CM}

The PHC_{CM} sample and commercial beech wood xylan (30 mg) were tested for its solubility in 15 ml of the following: distilled water (DW), dimethylsulfoxide (DMSO), dimethyl sulfoxide (DMSO), 0.1M NaOH and toluene, in a closed container under constant agitation (200 rpm) at room temperature (28±2) overnight (Marques., 2014). The sample was centrifuged at 850×g for 15 min (Noor *et al*., 1999). The percentages solubility was calculated.

$$S = \frac{Wo - Wr}{Wo} \times 100\% \qquad (1)$$

where S is the solubility degree, Wr is the weight of undissolved biopolymer residue, and Wo is the original weight of biopolymers.

4.3.6.5 Thermal stability of PHC

Thermal behavior of PHC and commercial beech wood xylan were performed using thermosgravimetry differential thermal analysis on a Simultaneous

thermal analyzer, STA8000, perkin Elmer, USA. The sample weighed between 4 and 20 mg was heated from room temperature to 700 °C at a rate of 10 ºC/min in nitrogen.

4.3.5.6 FT-IR spectra

The $OPT_{RS,}$ OPT_{RT3}, commercial beech wood xylan, PHC_{CM} and various ratio of PHC_{CM}-MA samples to be measured FT-IR spectra 1 mg was homogenized with 99 mg of KBr for 1 min and pressed into a disc. The FT-IR spectra of the sample were recorded with 16 scans at the resolution of 4 cm^{-1} between the wavenumbers of 400 and 4000 cm^{-1} (Fourier Transform Infrared Spectrometer, Vertex70, Bruker, Germany).

4.3.5.7 Swelling test

Swelling test of PHC_{CM}-MA/PVA hydrogel was measured according to Tanodekaew *et al.* (2006). The preweighed PHC_{CM}-MA/PVA blended (1cmx1cmx0.03cm) before suspending in distilled water at room temperature for 5 min. At certain time, the swollen gels were removed from the water, quickly wiped to remove excess water on the surface, and weighed. The degree of swelling (DS) was calculated as follows:

$$DS = (Ww - Wd) / Wd \times 100 \qquad (2)$$

where *Ww* and *Wd* are weights of wet and dry blends, respectively.

4.3.5.8 Tensile test

The PHC_{CM}-MA/PVA hydrogel was cut to dumbbell shaped with size of 2.54 cm width and 11.43 cm length. The tensile tests were carried out on universal testing machine (UTM, Lloyd Instruments, UK) at room temperature (28±2 ˚C) under American society for testing and material (ASTM) D638 standards test methods. The average value of the mechanical properties was calculated using at least samples. (Adapted from Hassan *et al*., 2010).

4.3.5.9 Cytotoxicity test

The PHC_{CM}-MA/PVA hydrogel was used for cytotoxicity testing. Cell suspension of $1X10^5$ cell/mL L929 (Mouse Fibroblast Cells, ATCC CCL1, NCTC 929. of Strain L) in MEM completed medium was seed into the 96-well plate. It was incubated at 37±1 °C, 0.1% CO_2 and 95±5% relative humidity for 24±2 h to obtain confluent monolayers of cells prior to test. The MEM completed medium was replaced

with the following extracts ; the blank was the media without hydrogel sample, the negative control was Thermanox'(Nunc) material, the positive control was polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC) and the hydrogel sample. All of them were extracted at 37±1 °C for 24±2 h. The cells were incubated further for 24±2 h. After incubation, the viable cells were stained with MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated further for 2 h. Then MTT was removed and DMSO was added in each well. The absorbance was measured using microplate reader (EASYS, UVM340 S/N ASY54180) at 570 nm. The % viability was calculated as follows:

$$\% \text{ viability} = 100\text{x } OD_{570C} / OD_{570b} \quad (3)$$

OD_{570C} = the mean value of the measured optical density of the 100% extracts of the test sample

OD_{570b} = the mean value of the measured optical density of the 100% extracts of the blanks

If viability is reduced to < 70% of the blank, it has a cytotoxic potential.

These testing were analyzed by staff at Biological *In vitro* Testing for Biomaterials Service, MTEC, NSTDA, Thailand.

4.3.5.10 Test of antimicrobial activity

Two species of bacteria (*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739) and one species of yeast (*Candida albicans* ATCC 10231) were used as the test microorganisms in this study. Antimicrobial activity was determined by agar diffusion assay. Bacteria and yeast were grown on Mueller Hinton agar and YM agar, respectively by streak plate technique. Incubation was done at 37 ºC for 18-24 h. A few single colonies were selected and inoculated in either Mueller Hinton broth or YM broth. Incubation was done with 200 rpm at 37 ºC for 18-24 h. The inoculum was prepared by adjusted turbidity of the overnight culture with normal saline 0.85% to match that of McFarland No. 0.5 standard solution. The inoculum should be used within 30 min. Twenty milliliters of either Mueller Hinton agar or YM agar was poured into each plate and left to solidify for preparation of basal medium.

The inoculum (2.5 ml) was thoroughly mixed with 100 ml soft agar (Mueller Hinton broth or YM broth with 0.75% agar) maintained at 45 ºC and it should be used within 15 min for preparation of seed medium. Each piece (1cmx1cmx0.03cm) of the test sample was placed over the surface of the basal medium and gently pressed for firm contact. Then 4 ml of seed medium were overlaid to the plate. The plate was tilted until the seed agar was completely covered the surface of the test sample and the basal medium at 37 ºC for 18-24 h. After incubation, the diameters of the inhibition zone observed around each test sample was measured visually using a ruler displaying their antibacterial activity (Guan *et al*., 2015). Amoxycillin disk (6 mm) and nystatin disk (6 mm) as antibacterial and antiyeast, respectively were used as controls.

These testing were analyzed by staff at Biodiversity Research Centre, Thailand Institute of Scientific and Technology Research, Thailand.

4.3.5.11 Antioxidant activity of the XOS

Antioxidant activity of XOS are measured by the ability of scavenging 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH radical) according the method adapted from Subhasree *et al*. (2009). The sample solutions (12 mg/mL) of XOS from PHC_{CM} was prepared by dissolving in deionized water (DI). The XOS solution was diluted to 2-time before placing in a 96 well-plate, and 0.1 mL of $6x10^{-5}$ M of DPPH was added. The mixture is shaken vigorously and kept for 30 min in the dark at room temperature (28±2°C). The absorbance is measured at 517 nm. The control is carried out with DI water instead of the sample solution (1:1 dilution of DPPH), while ethanol is used as the blank. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula of Subhasree *et al*. (2009):

$$\% \text{ Inhibition } = \{(A_{ctr} - A_{sanple}) / A_{ctr} \times 100 \qquad (5)$$

A_{ctr} = Absorbance of control- Absorbance of control blank

A_{sanple} = absorbance of sample- Absorbance of sample blank

The concentration of XOS required to quench 50% of the initial DPPH radical is defined as IC_{50}.

4.3.6 Statistical Analysis

Data of triplication were subjected to analyses variance (ANOVA) using a computer program SPSS 20 for windows. Statistical significance was evaluated using Duncan´s Multiple Range Test (DMRT) and a p <0.05 was considered to demonstrate a significance difference.

4.4 Results and discussion

4.4.1 Characterization of the raw materials

Investigation was made on matured oil palm tree of about 25 years old collected from an oil palm plantation in Phangnga Province, southern Thailand. Oil palm trunk (OPT) was debarked, cut into small chips and then crushed to separate parenchyma from vascular bundles. The OPT_R has a moisture content of 5.63± 0.15%. Cellulose was 21.60±0.01%, hemicellulose was 73.93±1.60%, and starch was 53.17±3.78%, levels of lignin and ash as illustrated (Table 4.2). The OPT_R was yellowish color (Figure 4.1A). The OPT_R can be divided into 2 different sections, namely parenchyma and vascular bundles, as shown in Figure 4.1A. Noor *et al.*, 1999 reported the oil palm trunk starch is stored inside the parenchymatic cells. It can be said that parenchyma tissue served as food storage for oil palm plant (Noor *et al.*, 1999). Tomimura, *et al.* (1992) found that the OPT estimated 25 years old contained starch markedly high in the parenchyma (55%) and a few percent of vascular bundles (2.4%). Many reports had been described the extraction of starch from fresh oil palm trunks. Noor *et al.* (1999) received 7.15% of oil palm trunk starch by soaking a fresh oil palm trunks in an aqueous solution containing 0.5 % sodium metabisulphite for 60 second. The extraction from 20 mesh power size of OPT soaked with 0.2 % sodium metabisulphite for 36h. Then, the samples was soak with a mixture of 0.5% lactic acid and 0.2 % sodium metabisulphite solutions (1:5w/w) gave the highest starch yield of 1.7% (H´ng *et al.*, 2011). Omar, *et al.* (2011) reported that starch extraction of OPT was the traditional grating-dissolving method in water gave the starch from 12.87% to 25.6% from the base upwards and from the outer inwards of OPT. In this study, the OPT_R and OPT_{RT} were extremely high hemicellulose due to these fractions consisted mainly of starch (51.73-71.61%) which is soluble in water and NaOH at 80°C during measured for hemicellulose content. The result obtained was in agreement with

Noor, *et al.* (1999) found that the solubility of oil palm starch in water at 80 °C was higher at 30%. Probably, the temperature at 80 °C used in this study for determination hemicellulose was affected to increase the solubility of starch. To mitigation the starch problem caused by parenchyma which stored starch in plant cells, the starch would be separated from sample such as sectioned into small pieces, granulated by pounding in a mortar and then using a screen (3 mm) can separated parenchyma and vascular bundle from OPT (Prawitwong *et al.*, 2012). In this study, the OPT_{RT1} after reducing size using knife mill and a screen (0.6-2 mm) showed lower starch (2.24%) than those other samples.

Then, starch was extracted from OPT_R by grating and dissolving water process. There are 2 fractions, namely fibrous dregs (OPT_{RT}) (Figure 4.1B) and starch slurry (OPT_{RS}), were obtained (Figure 4.1C). The chemical composition of OPT_{RT} result showed that moisture content of 5.54± 0.10%, cellulose was 26.90± 0.89%, hemicellulose was 68.51± 2.90%, starch was 51.73± 2.24% and lignin was 4.59± 0.35% (Table 4.2). The OPT_{RS} fraction contained starch (71.61±6.50%) higher than that found in OPT_{RT} fraction. Cellulose was 4.54±0.48%, hemicellulose was 93.60±0.55% and a few of lignin (Table 4.2).

The OPT_{RT} was further process by size reduction using knife mill. After sieving, the OPT_{RT} with size of >0.6 - <2 mm (OPT_{RT1}), <0.6 mm (OPT_{RT2}) and <250µm (OPT_{RT3}) were collected (Figure 4.1D,E). The chemical composition of OPT_{RT1} result showed that starch was 2.24±0.46%, cellulose was 43.71±0.77%,

Table 4.2 Chemical composition of the raw materials obtained from oil palm trunk (OPT) after physical fractionation

Composition	OPT_R	OPT_{RT}	OPT_{RS}	OPT_{RT1}	OPT_{RT2}	OPT_{RT3}	OPT_{RT4}
Cellulose* (%)	21.60±0.01	26.90±0.89	4.54±0.45	43.71±0.77	16.55±0.26	13.25±0.89	64.43±3.45
Hemicellulose* (%)	73.93±1.60	68.51±2.90	93.60±0.55	35.96±0.4	79.44±0.08	79.44±2.90	26.80±0.35
Lignin* (%)	4.47±0.13	4.59±0.35	1.86±0.12	17.33±0.51	4.32±0.36	7.31±0.35	8.77±0.24
Moisture content (%)	5.63±0.15	5.54±0.10	5.92±1.43	5.36±0.13	5.42±0.40	4.59±0.35	5.62±0.18
Ash* (%)	1.66±0.02	1.25±0.2	0.32±0.01	2.64±0.06	1.57±0.06	1.25±0.2	2.65±0.06

*dry basic

OPT_R = oil palm trunk with particle size of 1-8 cm, OPT_{RT} = filter cake of OPT after triturated with distilled water, OPT_{RS} = liquid suspension after OPT triturated with distilled water, precipitated and then dried, OPT_{RT1} with particle size of >0.6-<2 mm, OPT_{RT2} with particle size of <0.6 mm, OPT_{RT3} with particle size of <250μm and OPT_{RT4} = the OPT_{RT1} was treated with AP pretreatment

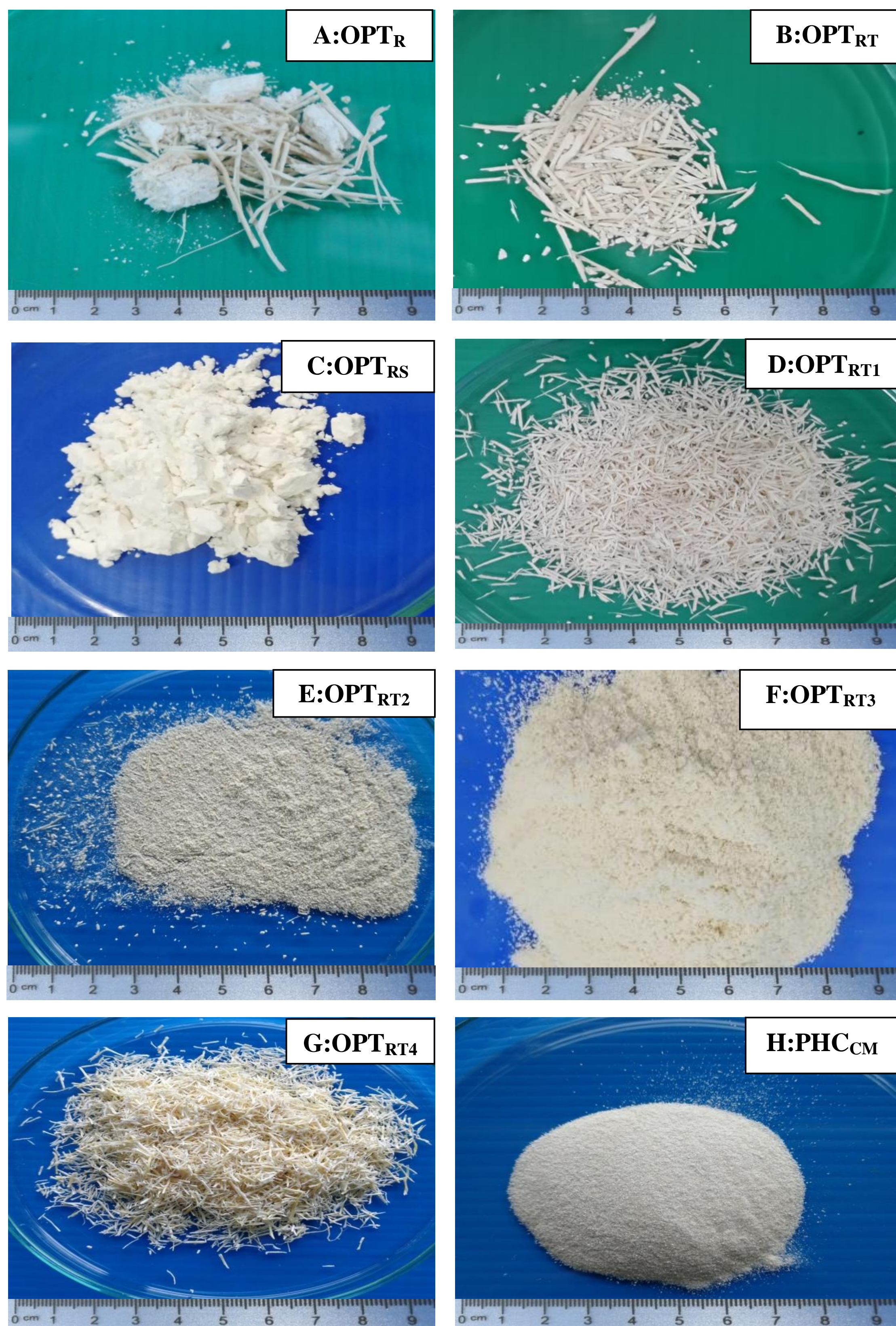


Figure. 4.1 Oil palm trunks samples used for experiments

hemicellulose was 35.96±0.40% lignin was 17.34±0.51% and a few of ash (Table 4.2). In this study, the higher results were obtained in previous studies (Noparat *et al.,* 2013). They reported that the middle part of OPT was 40.65±2.8% cellulose, 22.12±1.5% hemicellulose, 15.32±2.2% lignin and 1.17±0.03% ash. The chemical composition of OPT_{RT2} result showed that starch was 58.53±1.83%, cellulose was 16.55±0.20%, hemicellulose was 79.13±0.08% and a few of lignin and ash (Table 4.2). The chemical composition of OPT_{RT3} result showed that starch was 58.12±1.83%, cellulose was 13.25±0.20%, hemicellulose was 79.44±0.55% and a few of lignin and ash (Table 4.2). The chemical compositions in term of starch, cellulose and hemicellulose found in OPT_{RT1} and OPT_{RT2} were differed. The ratio of OPT_{RT1} and OPT_{RT2} in the OPT_{R} was 35.13:64.86 (% dry weight).

OPT tissue contains 2 mains component parenchyma and vascular bundles tissues. Parenchyma contains a considerable amount of starch up to 46.7 % w/w (Prawitwong *et al.*, 2012) and storage of starch granules is frequently observed in the parenchyma tissue (Marques *et al.*, 2014). Lang, *et al.* (2006) reported that the highest sago starch which is accumulated in the pith core of the stem content of 41% in sago palm (*Metroxylon* spp.) which is 12.5-13 years old. The vascular bundles tissues function in the supply of water and nutrients to the oil palm leaves. Its cells are long and slender which is similar to pipes form, as observed in OPT $_{RT1}$ (Figure 4.1D).

Photographs of OPT_{RS} and OPT_{RT3} samples show in Figure 4.2. Their shape are ovoid to elliptical with a truncated end (Figure 4.2 A,C). Their size from 7 to 14.7 μm, with an average of 10.69 μm (Figure 4.2B,D) and the size of most of the granules is within the range of 10–10.99 μm. Noor *et al.* (1999) reported granule size of oil palm starch from oil palm trunk ranged from 3 to 37μm, with an average of 14.6 μm. The granule size of OPT_{RF} and OPT_{RT3} is smaller than that of sago starch (28.50–45.35μm)**.** In order to investigate the functional groups of the OPT_{RF} and OPT_{RT3} samples, the FT-IR spectra were measured (Figure 4.3). The comparison of the infrared spectra standards showed that band intensities can be related to the potato gnocchi and grits, respectively. These two products could be alternative sources of the field of food.

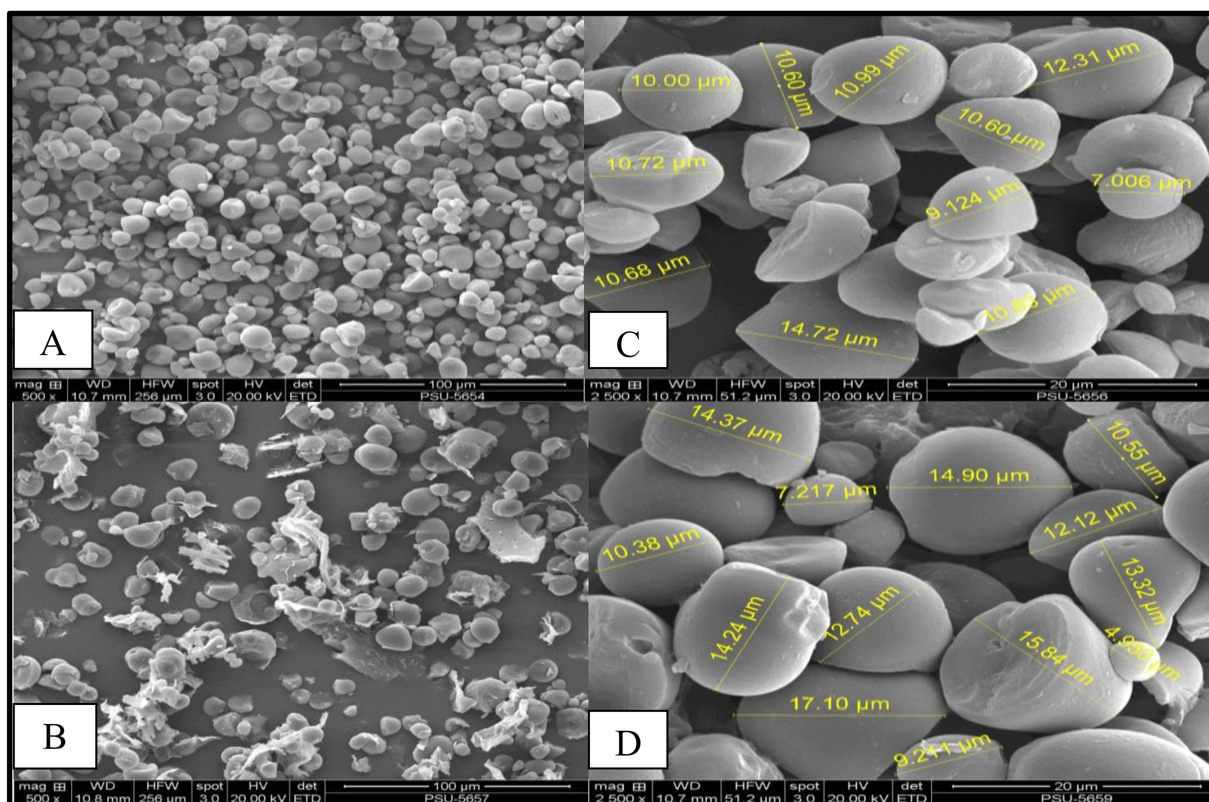


Figure 4.2 Scanning electron micrographs of OPT_{RS} (A) and OPT_{RT3} (B) and granule sizes of OPT_{RS} (C) and OPT_{RT3} (D). Magnification folds 500X for A and B and 2500X for C and D.

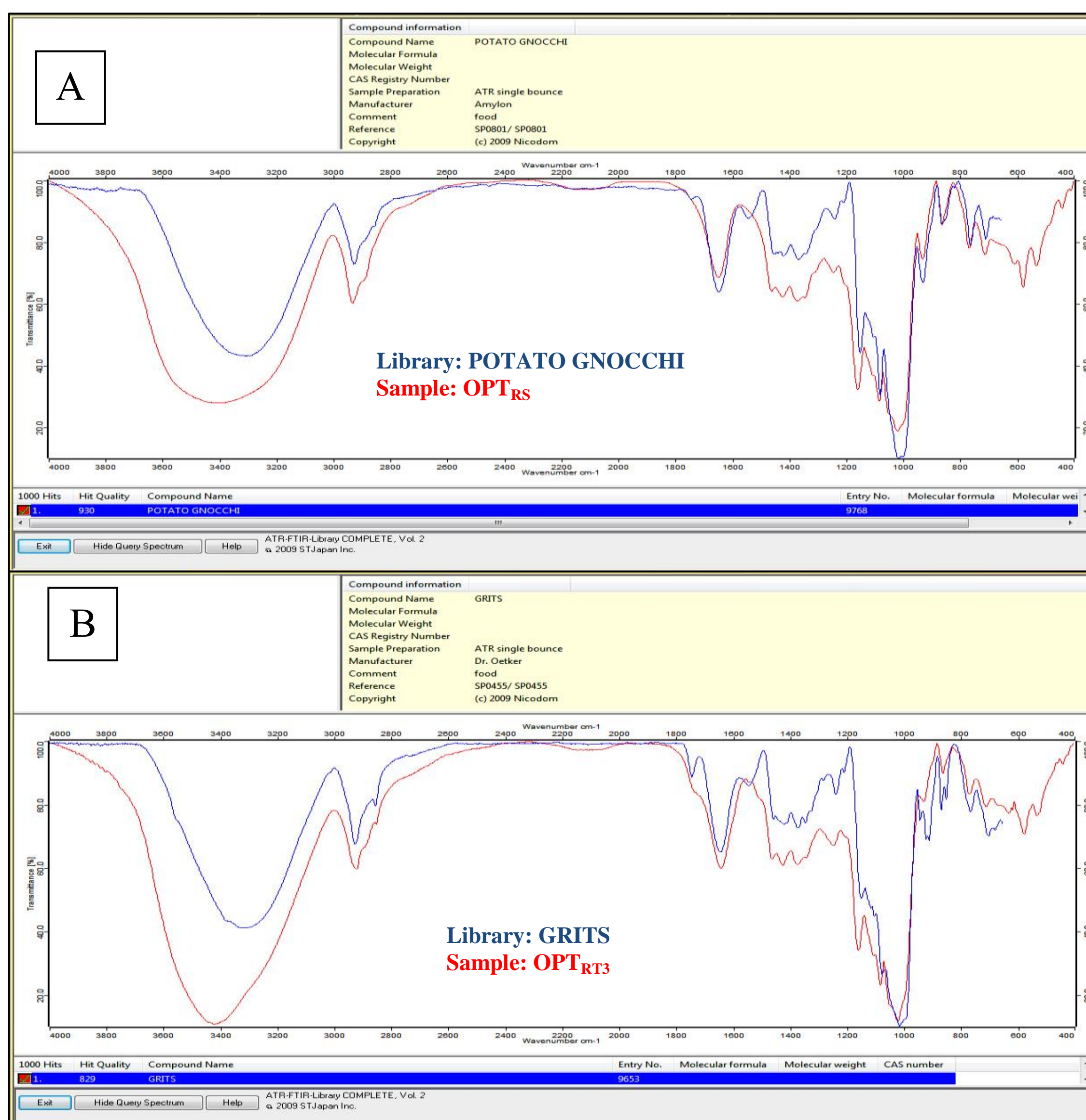


Figure 4.3 FT-IR spectra of the OPT_{RS} (A) and OPT_{RT3} from OPT

4.4.2 Extraction of the hemicellulose from OPT_{RT1}

4.4.2.1 Chemical pretreatment

AP pretreatment effectively increased the surface area by swelling the biomass particles, breaking the bonds between lignin and carbohydrates, solubilizing some of the lignin, do not require specialized equipment and as the alkaline reagents typically used do not cause corrosion like dilute acids (Modenbach and Nokes, 2012). There are two fractions obtained after the AP treatment of OPT_{RT1}: the solid fraction (OPT_{RT4}) and the precipitated fraction (PHC_{CM}). Chemical composition of the solid fraction was as follows: hemicellulose was 26.80±0.35%, cellulose was 64.43±% and a few of lignin Table 4.3.

The SEM images of the OPT_{RT1} and the AP treated OPT_{RT1} (OPT_{RT4}) samples were shown in (Figure 4.4). The untreated OPT_{RT1} fibers had a smooth surface (Figure 4.4A) while the AP treatment considerably altered the OPT_{RT4} fiber morphology (Figure 4.4B). Therefore, the AP pretreatment extracted the hemicellulose from OPT $_{RT1}$ and opened up spaces between them by removing some of the interfiber material (Palamae *et al.,* 2017). Table 4.3 also showed results of PHC_{CM} yield and its chemical composition after precipitation of AP solubilized fraction at various concentration of ethanol. The best PHC_{CM} yield (26%) was achieved at 70% ethanol. Similar results were obtained in hemicellulose from *Olea europapea* L. were extracted with KOH and fractionation with graded ethanol that condition gave the total yield of hemicelluloses (23.5%) based on the dewaxed material (Peng *et al*., 2013). The pretreatment of empty fruit bunch (EFB) fiber of oil palm by two-step treatment. The first step involved the treatment with peracetic acid at 35 ◦C for 9 h and then extraction with alkaline peroxide (8% NaOH) at 20 °C for 12 h gave the highest precipitated fraction of 17.4±0.7% (Palamae *et al.,* 2017). The increase of ethanol concentrations resulted in the precipitation of the more branched hemicelluloses. In point of this, hemicelluloses rich in backbone structure could be precipitated at low ethanol concentrations, while with increasing ethanol concentrations, hemicelluloses with some side chains and more complex structure were obtained. This observation also revealed that the higher the degree of substitution of the backbone, the higher the solubility of hemicelluloses in aqueous solution, which required a much higher ethanol concentration to precipitate them from

the solution (Peng *et al*. 2013). Thus, PHC_{CM} which is precipitated with 70% ethanol was chosen for the next experiments.

4.4.2.2 Autohydrolysis pretreatment

Two fractions obtained after the treatment of OPT_{RT1}. There are the residual solids and the filtrate of the treatment process. The chemical composition of solid fraction using OPT_{RT1} was determined. The result showed that solid fraction has cellulose content of 47.90±0.10% hemicellulose was 32.42±0.45% and lignin was 19.68±0.36% (Table 4.4). The precipitate fraction after treated gave PHC_{AH} (10.01±0.35%) and also give high hemicellulose (88.11±0.54%) (Table 4.4).

Table 4.3 Chemical composition of solid and precipitated fraction of OPT_{RT1} treated with AP pretreatment

Solid fraction			Precipitated fraction				
Hemicellulose (%)	Cellulose (%)	Lignin (%)	Precipitated with ethanol (%)	% Yield	Hemicellulose (%)	Cellulose (%)	Lignin (%)
26.80±0.35	64.43±3.45	8.77±0.24	30%	10.52±0.26[a]	79.25±1.97	10.06±1.55	10.69±1.86
			50%	15.60±0.41[b]	86.58±2.45	6.30±1.96	7.12±2.10
			70%	26.25±0.24[C]	88.93±0.53	4.73±1.47	6.34±0.13

* The data in columns was presented as mean values with standard deviations, with significance threshold $p<0.05$

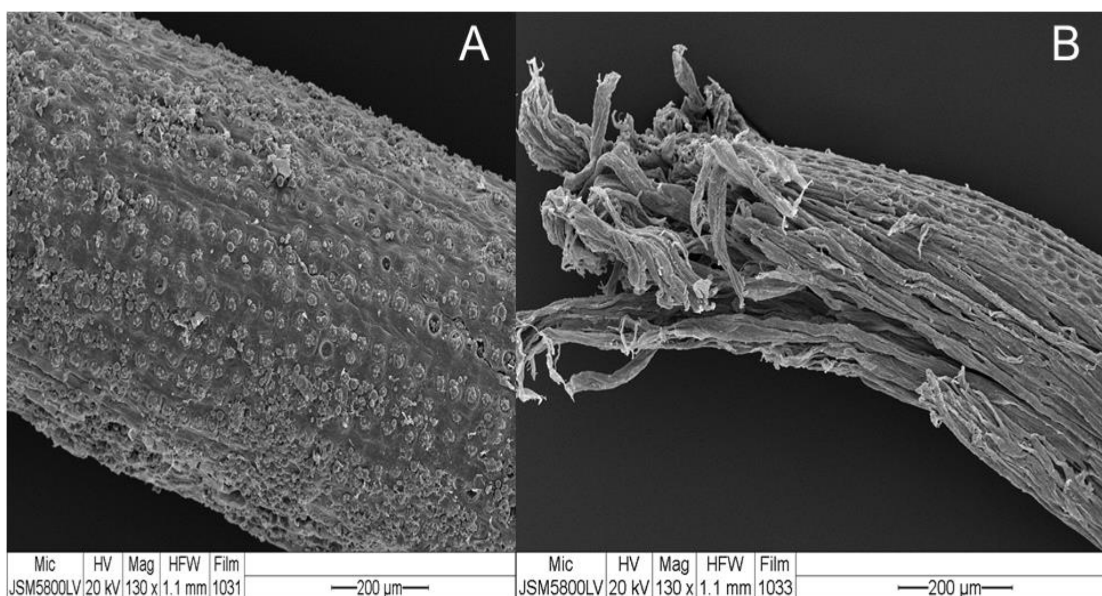


Figure 4.4 Scanning electron micrographs of: OPT_{RT1} (A) and OPT_{RT4} (AP treated OPT_{RT1}). Magnification of the photographs was 130X.

Table 4.4 The chemical composition of solid fraction and precipitate fraction using OPT_{RT1} treated with autohydrolysis pretreatment

Sample	Yield (%)	Composition of solid fraction		
		Cellulose (%)	Hemicellulose (%)	Lignin (%)
Solid fraction	84.23±0.24	47.90±0.10	32.42±0.45	19.68±0.36
Precipitate fraction	10.01±0.35	3.01±0.36	88.11±0.54	8.88±0.12

4.4.3 Characterization of the hemicellulose from OPT_{RT1}

The selection hemicellulose for characterization was depended on the yield of the production. Thus, the hemicellulose from OPT_{RT} was treated with chemical pretreatment (PHC_{CM}) which is precipitated with 70% ethanol and commercial xylan used in the next experiments.

4.4.3.1 Chemical composition of the hemicellulose from OPT_{RT1}

The chemical component of PHC_{CM} from OPT_{RT1} was hemicellulose (79.25-88.93%), cellulose (4.73-10.06%) and lignin (6.34-10.69%) (Table 4.3). The sugar composition was monitored with HPLC. The major monosaccharide in the PHC_{CM} was glucose (56%) and xylose (44.69%) appeared as the second major sugar constituent. As the beech wood was a pure commercial xylan obtained from Sigma company, it contained a higher amount of xylose (79.34%).

4.4.3.2 Molecular weight determination

The gel permeation chromatography (GPC) technique was conducted to determine the molecular weight distribution of the re-solubilized precipitate hemicellulose. Table 4.5 were displayed the weight-average (Mw) and number-average (Mn) molecular weights and the polydispersity of these subfractions. The result showed that the Mw of PHC_{CM} was 67,570 Da., Mn was 24,784 Da and the polydispersity was 2.7. The commercial xylan from beech wood had a moderate Mw, two sizes with 930,000 Da and 21,000 Da and polydispersity was 1.7and 1.8 respectively. The molecular weights of polysaccharide polymers depend on the method, solvent quality, and chain aggregation which are may be partially responsible for such a wide variation (Xu *et al.*, 2007). The extraction of *Olea europaea* L. with alkali pretreatment (10% KOH containing 1% H_3BO_3 at 25°C for 10h) and precipitation at ethanol vary concentration that condition gave the Mw of 16640-83610 Da (Peng *et al.*, 2013). The polydispersity is the ratio of Mw to Mn and revealed the structural heterogeneity of the subfractions. When polydispersity is equal to 1, shown the polymer mixture contains molecules all of the same size. The polydispersity values obtained in this study (polydispersity = 2.7) show that the into a few different sizes of molecule with a narrow molecular weight. The PHC_{CM} are comparable to produced hemicellulose which treatments of maize stems, rye straw

and rice straw with 1 M NaOH at 30 °C for 3 h gave the polydispersities of 1.74–2.03 (Xiao *et al.,* 2001).

Table 4.5 Molecular weight and polydispersity of the precipitated hemicellulose by chemical pretreatment (PHC_{CM}) from OPT_{RT1} and commercial xylan

Source of hemicellulose	Mn* (Da)	Mw** (Da)	Mp** (Da)	Polydispersity
PHC_{CM}	24,784	67,570	21,751	2.72
Commercial xylan from beech wood				
- CXB-L	553,875	928,861	1,446,390	1.67
- CXB-S	11,800	20,879	33,442	1.76

*Mn: Number average of molecular weights

**Mw: Weight average of molecular weights

***Mp: The peak of molecular weights

4.4.3.3 Functional group analysis

The FTIR spectra of the PHC_{CM} and commercial xylan were shown in (Figure 4.5). The result showed that as expected, the two spectral profiles and relative intensities of the most bands were rather similar, indicating a similar structure of the two hemicellulosic samples. The absorbances at 3443, 2919-2921, 1635-1639, 1386-1388, 1320 and 1248-1250 cm^{-1} were associated with hemicelluloses (Palamae *et al.,* 2017; Peng *et al*., 2012; Peng *et al*., 2013; Su *et al*., 2014; Xu *et al*., 2007)The absorbances at 1166-1120, 1043-1044 and 1166-1120 cm^{-1} were associated with celluloses (Liu *et al*., 2006; Palamae *et al.,* 2017). The absorption at 1421-1422 cm^{-1} assigned with the aromatic rings in lignin (Palamae *et al*., 2017; Su *et al*., 2014).

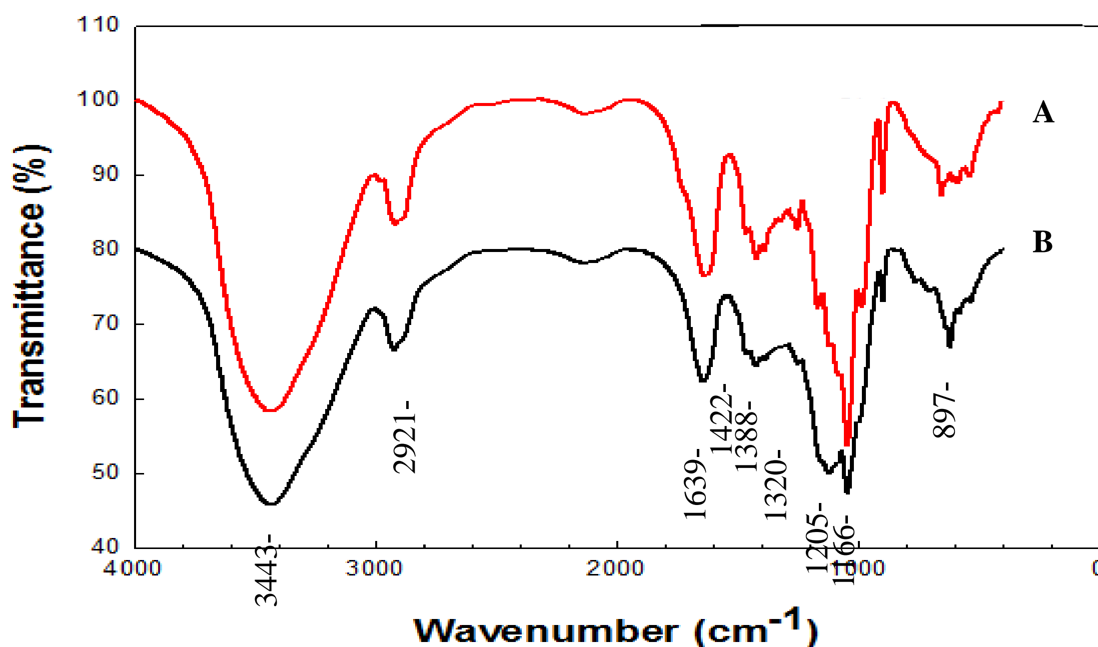


Figure 4.5 FT-IR spectra of commercial xylan (A) and the precipitated hemi-cellulose by chemical pretreatment (PHC_{CM}) (B)

4.4.3.4 Solubility tests

The solubility of the PHC_{CM} sample in these solvents was classified as good or bad depending on the maximum or minimum quantity of a substance dissolved in a given amount of solvent at equilibrium. In this study, the solubility tests on PHC_{CM} from OPT showed that DMSO (94.93%) and DW (78.54%) are the best solvents to solubilize hemicellulose. While xylan from beech wood, slightly higher solubility were obtained in DW. The present of solubility reached the maximum solubility tests of 84.62%. On the other hand, toluene partially dissolved xylan from beech wood and hemicellulose from OPT_{RT} (Figure 4.6)

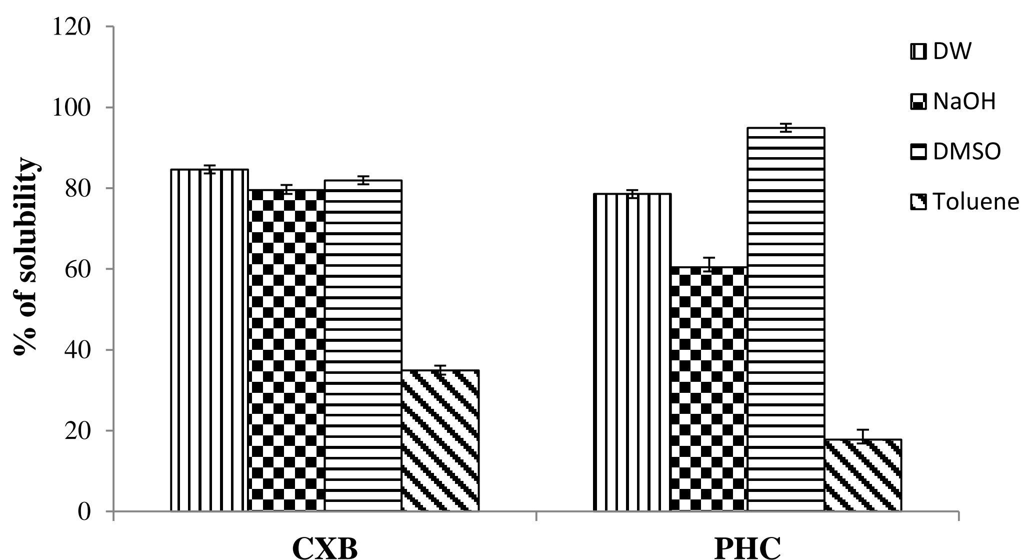


Figure 4.6 The solubility of PHC_{CM} from OPT_{RT1} and commercial xylan (CXB)

4.4.3.5 Thermal stability

The thermal property of the hemicellulose was analysed by TGA and DTG(Figure 4.7). gives the thermograms of xylan from beech wood and PHC_{CM}. As can be seen from the TGA curves of the xylan from beech wood and PHC began to decompose at 221 and 261°C, respectively. Beyond these temperatures, thermal degradation takes place. Similarly, at <40% weight loss the decomposition temperatures of the four polymer samples appeared at 412 and 428°C, respectively. The peaks at 290°C in the DTG curves correspond to the thermal decomposition of the hemicellulose (Su *et al.,* 2014). Thermal degradation of biomass, and in particular decomposition of carbohydrates, is a complex process and it includes many different reactions occurring simultaneously, e.g. dehydration (<190ºC), depolymerization (186 to 330ºC), fragmentation, rearrangement, repolymerization, condensation and carbonization (>330ºC). The pyrolysis process results in a char residue (fixed carbon and ash) and gaseous products (Marques, 2014).

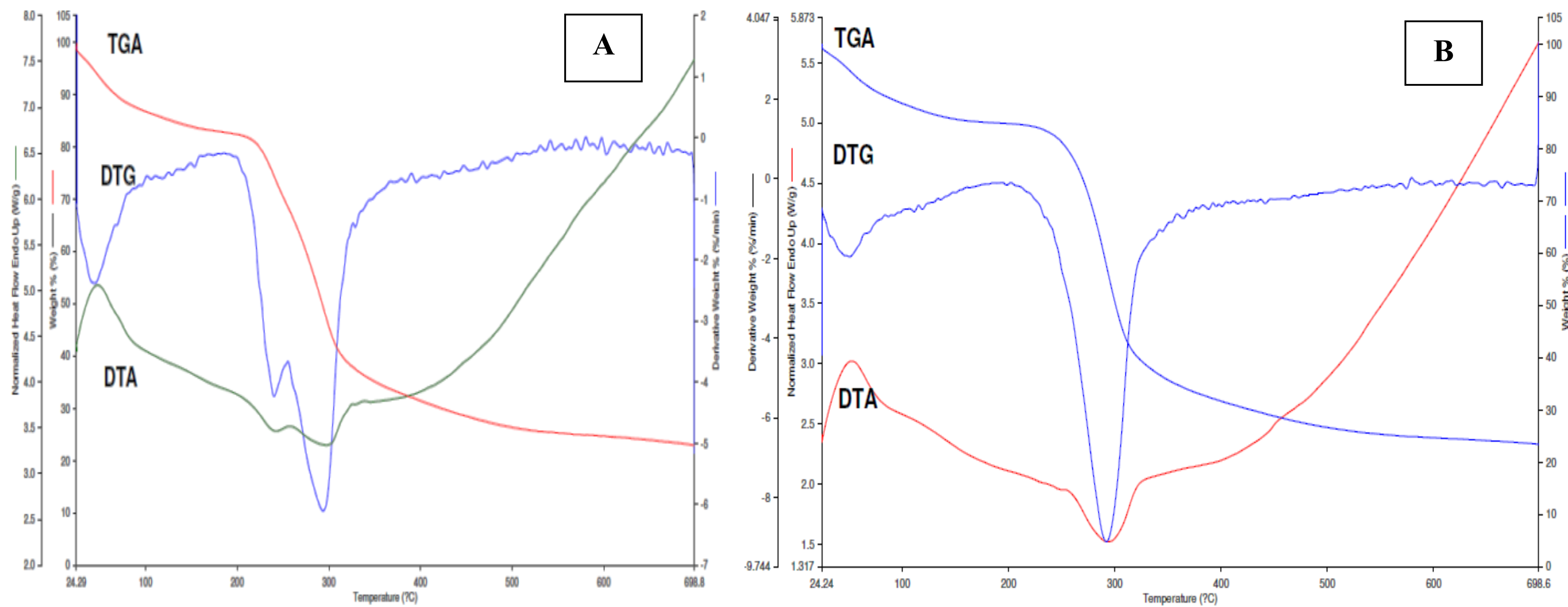


Figure 4.7 Thermograms of commercial xylan from beech wood (CXB)(A) and precipitated hemi-cellulose by chemical pretreatment (PHC_{CM})(B)

Table 4.6 summarizes the previous reports of hemicellulose production. Su *et al.* (2015) reported the dried corncob sizes 80 mesh was pretreated with alkaline hydrogen peroxide (AHP) gave hemicellulose of 24.3%. In the study by Peng *et al.* (2013), the hemicellulose from *Olea europaea* L. followed by 10% KOH containing 1% H_3BO_3 at 25 °C for 10h and precipitated with 30% ethanol. They found that the yield of hemicellulose of 10.8%. The hot water, 2%KOH and then, 0.5% NaOH extraction of hemicellulose from bamboo (*Phyllostachys pubescens* Mazel) stem shown the yield hemicellulose of 19.8% (Peng *et al.,* 2012). The treatments of barley straw using 90% dioxane, 80% acidic dioxane, 100% dimethyl sulfoxide and then, 8% NaOH recovered high a total yield of hemicellulose (32.1%) (Sun *et al.,* 2011). In this study, the results showed that the OPT pretreated with alkaline peroxide (AP) is the good candidate for hemicellulose production. In addition, the PHC_{CM} had a moderate Mw of 67.57 KDa, and DMSO was the best solvents to solubilize hemicellulose. Therefore, the characterization of PHC_{CM} was established in order to improve hydrogel accessibility.

Table 4.6 Summary and comparisons of hemicellulose production in different substrates pretreated by different methods

Substrates	Pretreatment methods	Hemicellulose yield (%)	Reference
Corncob	Alkaline hydrogen peroxide	24.3	Sun *et al*. 2015
Olea europaea L.	Alkaline	10.8	Peng *et al*. 2013
Bamboo (*Phyllostachys pubescens* Mazel) stem	Hot water, 2%KOH and 0.5% NaOH	19.24	Peng *et al*. 2012
Bamboo (*Phyllostachys bambusoides* f. shouzhu Yi)	Hot water and 8% NaOH	5.1	Peng *et al*. 2011
Oil palm trunks	Autohydrolysis	10.01	This study
Oil palm trunks	Alkaline peroxide	26.25	This study

4.4.4 Hydrogel from the hemicellulose

Hydrogel from PHC_{CM} was prepared into 2-step.To begin with the PHC_{CM} was reacted with maleic anhydride (MA). The modified PHC_{CM} from OPT with MA was also verified by FTIR to support the existence of the ester linkages by esterification under a thermal treatment. The success of those reactions was evidenced by the FTIR spectrum (Figure 4.8 and 4.9). Figure 4.8 showed the presence of a new peak at 1725 cm^{-1} in the modified hemicellulose from OPT while original PHC_{CM} no exhibits that pack. The PHC_{CM}/MA1.5:1, PHC_{CM}/MA1:1, PHC_{CM}/MA1:1.5 described the stretch of carboxylic acid (C=O) produced by the cross-linking reaction at 50 °C for 2 h of hemicellulose (xylan) with maleic anhydride while PHC_{CM}/MA1:0 no peak at 1725 cm^{-1} in the FT-IR spectrum (Figure 4.9). As a result, the incorporation between xylan in hemicellulose (PHC_{CM}) and MA derivative was found easier to be dissolved in water than original xylan and also promoted the hydrophilicity of the derivative (Tanodekaew *et al.*, 2006). Lastly, the dried PHC_{CM}-MA was blended with PVA at various PVA. The hydroxyl groups of PVA were esterified with the carboxylic acid groups of PHC_{CM}-MA under a thermal treatment.

The surface morphology of PVA hydrogel and PHC_{CM}-MA/PVA hydrogels was detected by SEM (Figure 4.10). The PVA has hydrophilic character which is poor stability in water (Figure 4.10 A). The solution to this problem the PVA should be insolubilized by cross-linking with another polymer and these processes may be reduce the hydrophilic character of PVA (Abd El-Mohdy and Ghanem, 2009). In this study, the PVA hydrogel was done by cross-linking with PHC_{CM} which dispersed in the hydrogel (Figure 4.10 B). This structure is an advantage for improving the swelling of hydrogel also.

All the prepared samples were elegant and strenuous in appearance flexible, thin and not smooth surface was found on the hydrogel. The PHC_{CM}-MA/PVA hydrogel from OPT (H1-H12) and PVA hydrogel (H13), as a control, were immersed in distilled water and their swelling behaviors were examined, as shown in Figure 4.11 and Table 4.7. We found that the without or with MA in the hydrogel before blend with at various PVA were did not significantly differ to the swelling degree ($p>0.05$). Amount 13 formulations test, the hydrogel that prepared from H12

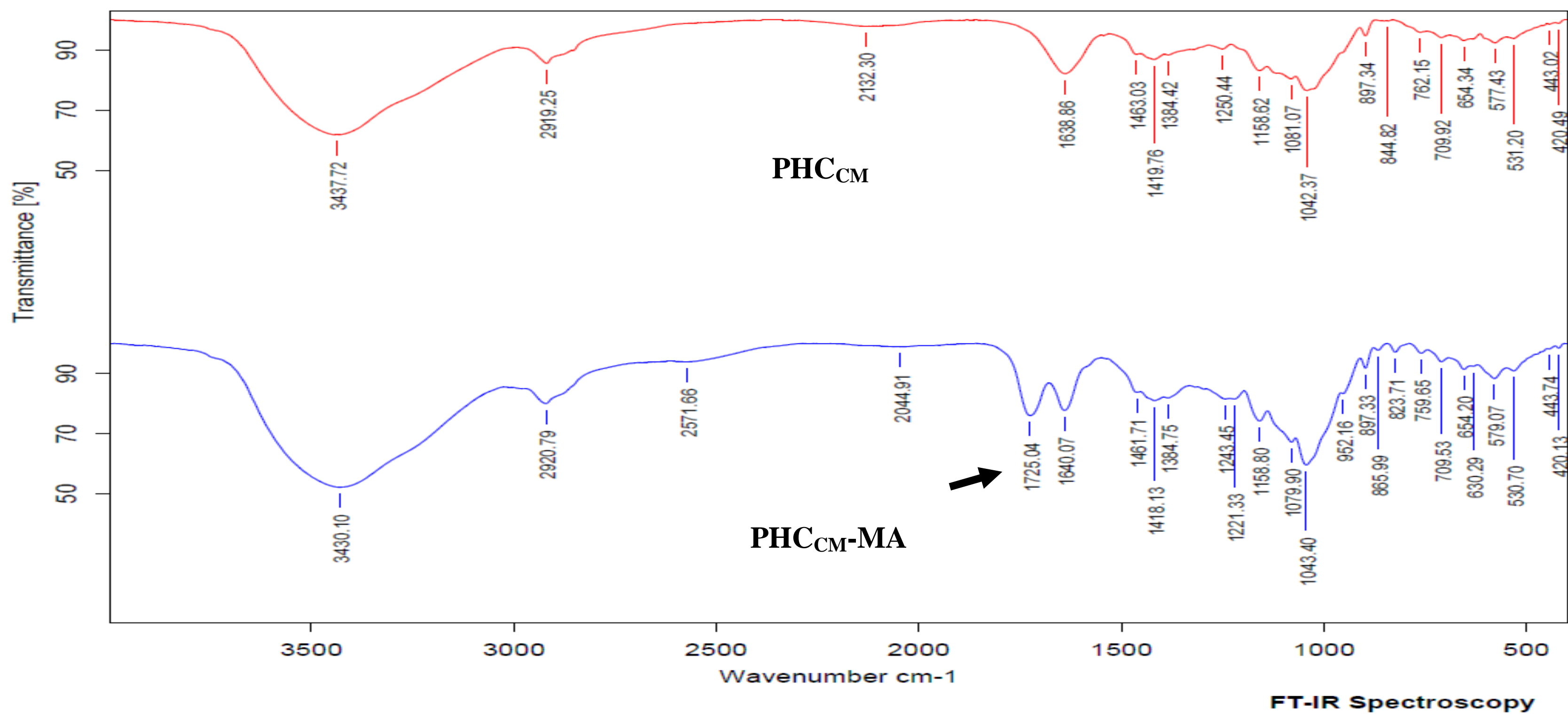


Figure 4.8 FT-IR spectra of the PHC_{CM} and PHC_{CM}-MA from OPT before and after made to react with maleic anhydride (MA)

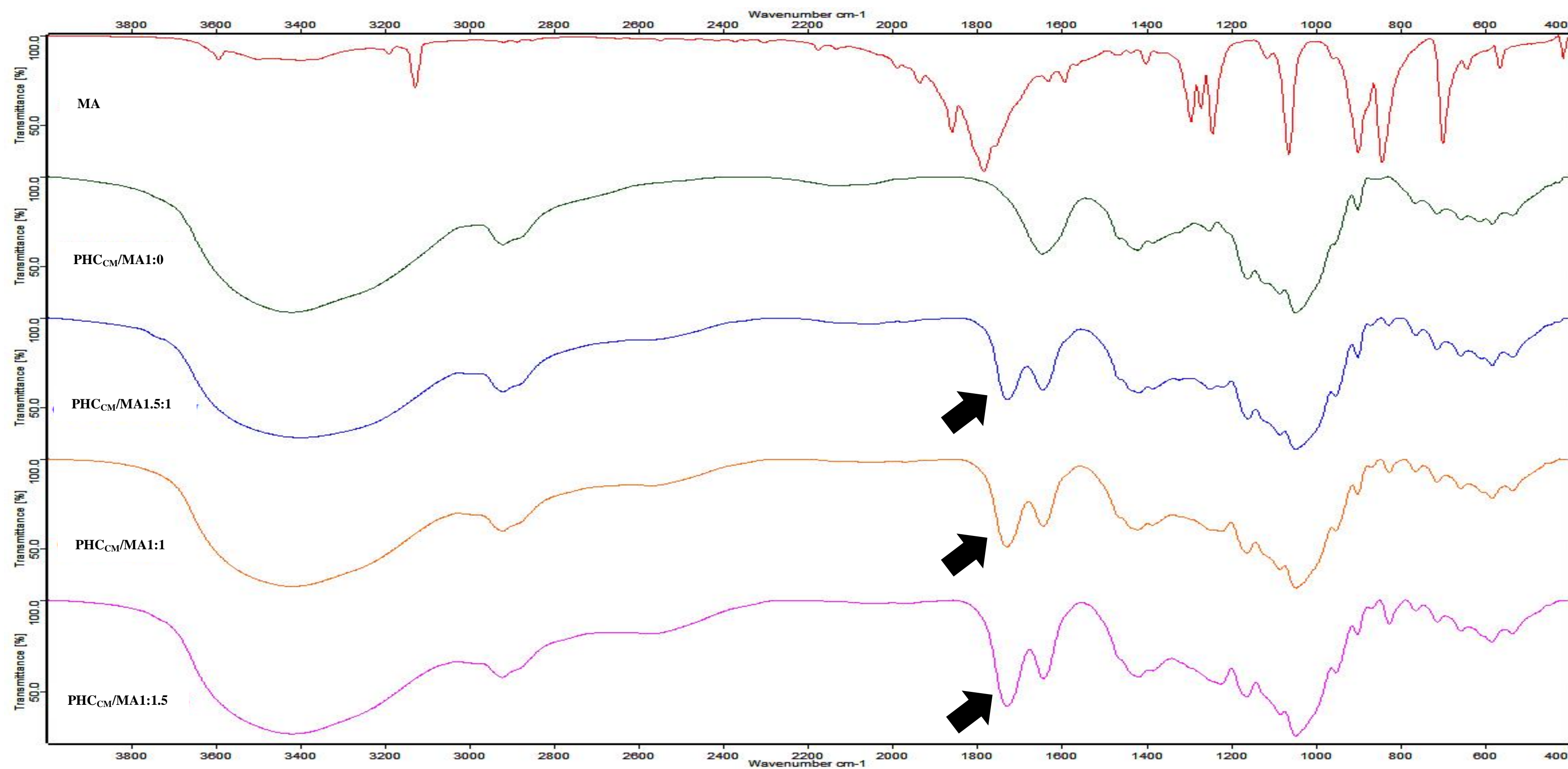


Figure 4.9 FT-IR spectra of the maleic anhydride (MA) and various ratio of PHC_{CM}-MA from OPT. Arrow indicate new peak at 1725 cm^{-1} in the modified hemicellulose from OPT with MA

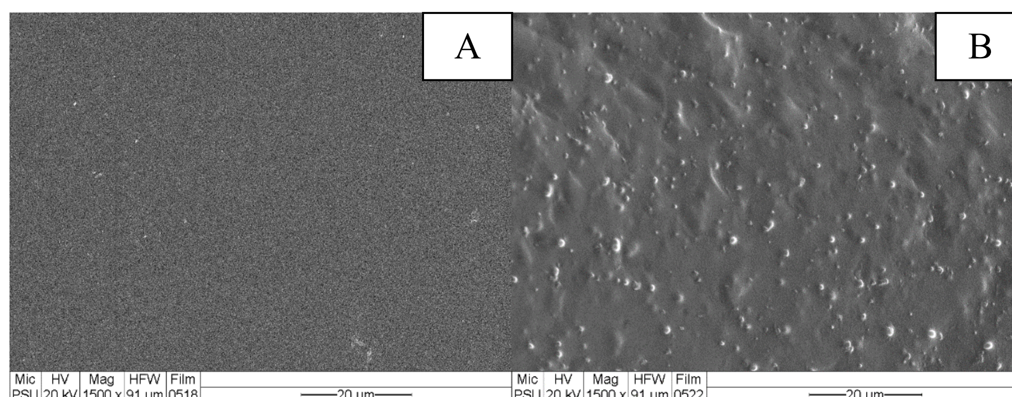


Figure 4.10 SEM of PHC_{CM}-MA/PVA hydrogels prepared at various PHC_{CM}-MA/PVA compositions; a PVA hydrogel (H13), b PVA/PHC_{CM}-MA1(H10) at 70°C. Magnification: 1500X

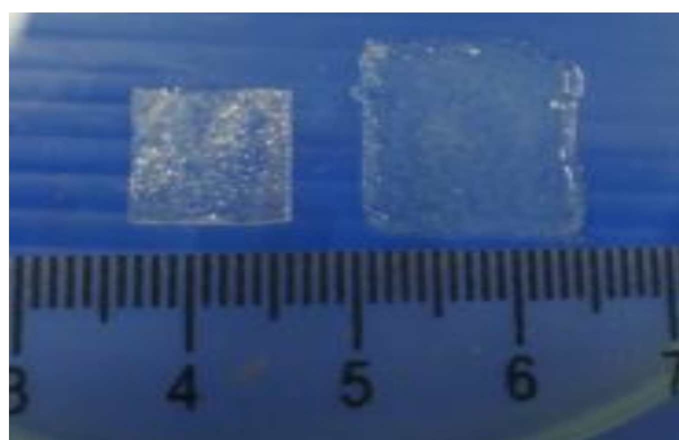


Figure 4.11 The before and after swelling degree of PHC_{CM}-MA/PVA hydrogel

Table 4.7 The effect of MA and PVA contents on swelling degree and strength of PHC_{CM}-MA/PVA hydrogel.

Formulation	Ratio of concentration (g)		Degree of swelling (%)	Tensile strength (MPa)
	PHC_{CM}:MA	PVA:PHC_{CM}-MA		
H1	1:0	1:0.25	238.87 ± 6.71^{A}	4.94 ± 1.55^{BC}
H2	1:0	1:0.55	263.14 ± 0.70^{A}	7.11 ± 0.51^{B}
H3	1:0	1:1	301.53 ± 1.22^{A}	4.79 ± 1.38^{BC}
H4	1.5:1	1:0.25	291.85 ± 3.15^{A}	15.39 ± 3.08^{A}
H5	1.5:1	1:0.55	227.77 ± 4.5^{A}	11.61 ± 1.46^{A}
H6	1.5:1	1:1	206.89 ± 3.17^{A}	2.91 ± 1.48^{C}
H7	1:1	1:0.25	261.30 ± 2.36^{A}	13.48 ± 1.93^{A}
H8	1:1	1:0.55	212.83 ± 7.80^{A}	5.12 ± 0.60^{BC}
H9	1:1	1:1	242.08 ± 4.46^{A}	4.25 ± 0.62^{BC}
H10	1:1.5	1:0.25	308.32 ± 5.37^{A}	12.25 ± 1.03^{A}
H11	1:1.5	1:0.55	291.28 ± 8.84^{A}	7.89 ± 0.79^{B}
H12	1:1.5	1:1	216.20 ± 4.38^{A}	4.83 ± 1.52^{BC}
H13	-	1:0	134.61 ± 6.14^{B}	12.35 ± 1.46^{A}

* The data in columns was presented as mean values with standard deviations, with significance threshold $p<0.05$

gave the higher swelling behaviors than those samples. Under the optional conditions (H12), the highest swelling test could reach to 308.32±5.37.9%. In contrast, increasing the PVA concentration at PHC_{CM}-MA/PVA hydrogel surprisingly increased the tensile strength of 1.5:1 of PHC_{CM}-MA, 1:1 of PHC_{CM}-MA and 1:1.5 of PHC_{CM}-MA hydrogel from 2.91±1.48 MPa to 15.39±3.08 MPa, from 4.25±0.62 MPa to 13.48±1.93 MPa and from 4.83±1.52 MPa to 12.35±1.46 MPa, respectively. The tensile strength of all samples (H1-H12) and PVA hydrogel (H13), as a control, ranged from 2.91±1.48 to15.39±3.08 MPa. Under the optimal conditions (H4), the highest compressive strength could reach to 15.39±3.08 MPa. Similar results were obtained in previous studies (Maji *et al.,* 2013). They reported that the maleic

anhydride cross-linked with chitosan-polyvinyl alcohol hydrogel has shown highest maximum tensile strength than the other formulations when increased the PVA concentration in hydrogel. This may be due to the presence of crosslinking density of PVA in hydrogels.

In this research, the priority of property of hydrogel for the selected hydrogel to study in the next experiment was the highest swelling and high tensile strength. From the result, the optimal hydrogel including H10 from OPT was selected for cytotoxicity test for normal cells and antimicrobial tests.

The cytotoxicity of the hydrogels from H10 was evaluated. The objective of test to evaluate cellular response with the test specimen extracts by MTT cytotoxicity assay. The % viability of L929 cells adjacent to the negative and positive controls as well as the hydrogel is shown in Figure 4.12. All of them were reduced viability to < 70% of the blank. They have a cytotoxic potential. It is noticeable that the H10 hydrogel has the least viability of L929 at 14%. Consequently, the behavior of the viability of L929 cells on the H14 from OPT recommend that the gel was cytotoxic and had a potential for cytotoxicity activity against to cancer cells application.

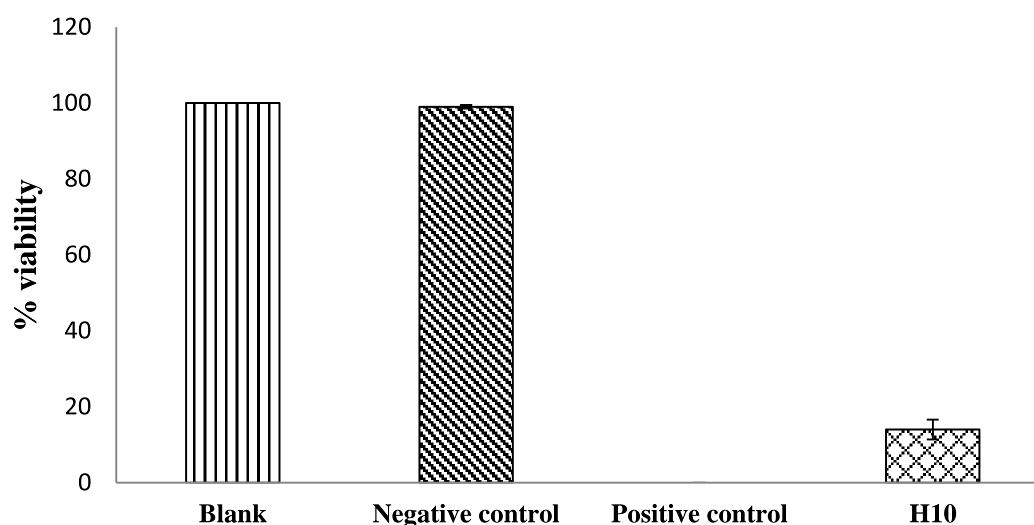


Figure 4.12 The percentage of cell viability of PHC_{CM}-MA/PVA hydrogel. The celled viability was assessed by MTT assay

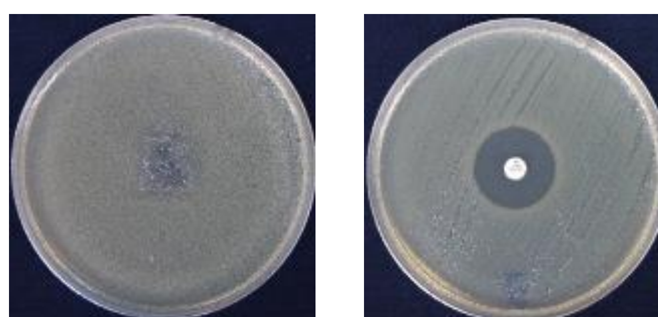
The antimicrobial activity of the PHC_{CM}-MA/PVA hydrogel will be the most valuable property in the field of biomedical application. Antimicrobial activity was performed by zone inhibition methods. A piece of samples after 24 h on Petri dishes inoculated with a Gram-positive bacterial strain *S. aureus* ATCC 6538, a Gram-negative bacterial strain *E. coli* ATCC 8739 and a yeast strain *C. albicans* ATCC 10231. The plates were incubated for 24 h at 37 °C. The inhibition of antimicrobial activity of PHC_{CM}-MA/PVA hydrogel is depicted in Table 4.8 by the method of antibacterial zone diameter. The results showed the no antibacterial activity of H10 PHC_{CM}-MA/PVA hydrogel (Figure 4.13, 4.15 and 4.15). In this study, the behavior of the antimicrobial activity in the PHC_{CM}-MA/PVA hydrogel suggested that should change another stain of bacterial and fungi for antimicrobial activity test and may find a potential for biomedical application. For instance, *Bacillus subtilis* (Abd El-Mohdy and Ghanem, 2009), *B. megaterium* (Wu *et al.*, 2013), *Klebsiella pneumonia* (Vimala *et al.*, 2010), *Aspergillus fumigatus* (Abd El-Mohdy and Ghanem, 2009) and *Penicillium italicum* (Abd El-Mohdy and Ghanem, 2009).

Table 4.8 Antimicrobial activities of the PHC_{CM}-MA/PVA hydrogel.

Formulation	The average diameter of inhibition zone (mm)		
	Staphylococcus aureus ATCC 6538	*Escherichia coli* ATCC 8739	*Candida albicans* ATCC 10231
H10(10mmx10mmx3mm)	0	0	0
Ampicillin*	45	24	-
Nystatin**	-	-	26

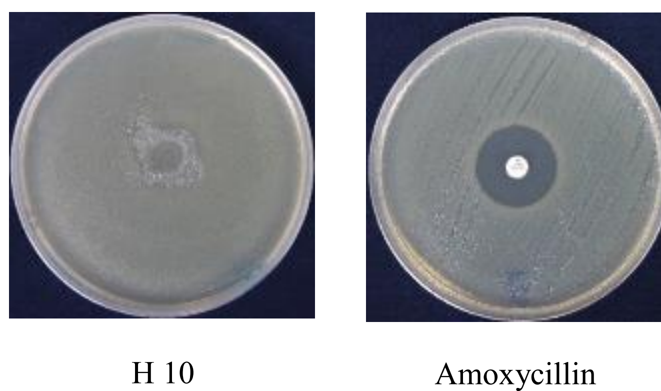
*The diameter of the inhibition zones of amoxicillin of *Staphylococcus aureus* ATCC 6538 and *E. coli* ATCC 8739 as antibacterial controls were made.

**The diameter of the inhibition zone of nystatin of *Candida albicans* ATCC 10231 as antiyeast control was made.



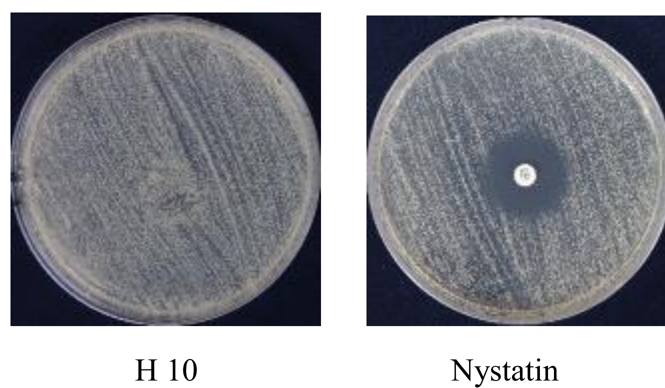
H 10 Amoxycillin

Figure 4.13 Antimicrobial activities of the H10:PHC-MA/PVA hydrogel against *Staphylococcus aureus* ATCC 6538. The amoxicillin was used as a control.



H 10 Amoxycillin

Figure 4.14 Antimicrobial activities of the H10:PHC-MA/PVA hydrogel against *Escherichia coli* ATCC 8739. The amoxicillin was used as a control.



H 10 Nystatin

Figure 4.15 Antimicrobial activities of the H10:PHC-MA/PVA hydrogel against *Candida albicans* ATCC 10231. The nystatin was used as a control.

4.4.5 Production of xylooligosaccharide (XOS) from precipitated hemi-cellulose by chemical pretreatment (PHC_{CM})

4.4.5.1 Effect of incubation time on XOS production from PHC_{CM}

The XOS from the selected and prepared from OPT was cut using endo-(1,4)-β-xylanases (EC 3.2.1.8). Table 4.9 shows the effects of incubation time on the PHC_{CM} under investigation. The xylobiose from PHC_{CM} increased from 0.18±0.01 g/L to a maximum of 0.30±0.04 g/L with the increase of reaction time from 0 h to 48 h when 8 U of xylanase was added to 100 mg of PHC_{CM} and incubated at 40 °C. However, the concentration of xylobiose at 48 h was did not significantly (p>0.05) different from that at 12 h. In point of this, the production of XOS from PHC_{CM} able to produce at the short time. Thus, The production of XOS at 12h was selected for further studies (Figure 4.16).

Table 4.9 Enzymatic hydrolysis of PHC_{CM} from OPT as functions of hydrolysis time

Time (h)	Xylobiose (g/L)	Xylopentaose (g/L)
0	ND	ND
12	0.18 ± 0.01^{A}	0.007 ± 0.01^{A}
24	0.26 ± 0.03^{A}	0.007 ± 0.03^{A}
36	0.27 ± 0.04^{A}	0.005 ± 0.04^{A}
48	0.30 ± 0.04^{A}	ND

The data in columns was presented as mean values with standard deviations, with not significance threshold p>0.05

ND = not detected

Figure 4.16 The appearence of XOS production from PHC use enzymatic method.

4.4.5.2 Effect of enzyme concentration on XOS production from PHC_{CM}

Table 4.10 shows the effects of xylanase concentration at 12h. Increasing the xylanase concentration from 4 U to 8, 12, and 16 U/100 mg substrate, produced xylobiose from 0.25 ±0.04 g/L to 0.25±0.03, 0.31±0.02 and 0.30±0.02 g/L, respectively. In this study, increasing the xylanase activity from 4 U to 16 U did not significantly ($p>0.05$) increase the xylobiose production. In fact, the enzyme activity was limited by the availability of the substrate. Thus, hydrolysis with 4U of xylanase at pH 5 and 40 °C for 12 h, at this condition, a maximum of xylobiose production (0.25 g/L) was achieved.

Table 4.10 Enzymatic hydrolysis of PHC_{CM} from OPT as functions of enzyme concentration at 12h

Enzyme conc. (U)	Xylobiose (g/L)	Xylopentaose (g/L)
0	ND	ND
4	0.25 ± 0.04^{A}	0.078 ± 0.01^{A}
8	0.25 ± 0.03^{A}	0.007 ± 0.03^{A}
12	0.31 ± 0.02^{A}	0.008 ± 0.04^{A}
16	0.30 ± 0.02^{A}	0.04 ± 0.04^{A}

The data in columns was presented as mean values with standard deviations, with not significance threshold $p>0.05$

ND = not detected

4.4.5.3 Production of XOS from PHC

The XOS was prepared from PHC_{CM} using enzyme xylanase (endo-(1,4)-β-xylanases:EC3.2.1.8) from *Trichoderma viride* X3876 concentration of 4 U per 100 mg PHC_{CM} at 40 °C with orbital shaking at 150 rpm for 12h and then, XOS recovery process. The yield of XOS during enzymatic hydrolysis are gave 48% (w/w). Bian, *et al.* (2013) reported that XOS were prepared from hemicelluloses extracted with alkali (potassium hydroxide) from sugarcane bagasse by hydrolysis with crude xylanase secreted by *Pichia stipites* at 25 U g^{-1} for 12 h gave a maximum yield of 31.8%. Thus, this study showed that the condition for XOS production gave high yield of XOS in low concentration of enzyme and short time.

4.4.5.4 Antioxidant activity of the XOS from precipitated hemicellulose by chemical pretreatment (PHC_{CM})

The antioxidant activity of the XOS from PHC_{CM} was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH (Subhasree *et al*., 2009). The scavenging ability of XOS obtained from the 12 h enzymatic hydrolysis is demonstrated in Figure 4.17. The results found that the concentration of XOS at 3,000, 6,000, and 12,000 µg/mL, the scavenging effect of XOS were 4.48%, 14.89% and 29.78% respectively. It found that the XOS exhibited concentration dependent antioxidant activity. Similar results were obtained in Rashad *et al*., 2016. They reported that the antioxidant activities of the XOS produced from orange peels which are agricultural lignocellulosic wastes by crude xylanase from *Bacillus amyloliquifaciens* NRRL B-14393 increased antioxidant activity when increas concentration of XOS. Many reports had been described the similarly behavior for other XOS, from varieties of cereal and millet brans (Veenashri and Muralikrishna, 2011) and sugarcane bagasse (Bian *et al.,* 2013). The IC_{50} showed 50% inhibition at 19,610 µg/mL. The above results indicated that XOS from OPT can be exploited in the potential resource of natural antioxidants for health foods.

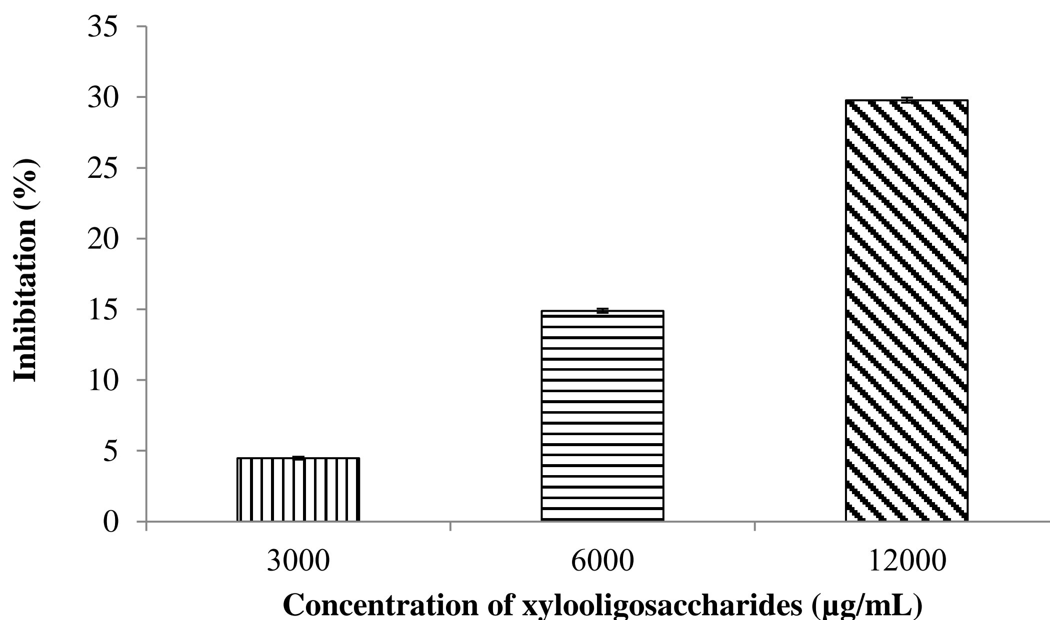


Figure 4.17 Antioxidant activity against DPPH of xylooligosaccharide from PHC_{CM} obtained by enzymatic hydrolysis for 12 h.

4.5. Conclusions

The OPT_R raw material was prepared using cutting, grinding, grating and dissolving water process. Extraction of the hemicellulose from OPT_{RT1} (0.6>OPT<2 mm) used chemical and autohydrolysis pretreatment. Hemicellulose from OPT_{RT1} was extracted with Alkaline peroxide (AP) pretreatment and fractionation with graded ethanol, gave the highest total yield of the precipitated hemicellulose (PHC_{CM}) increased from 10.52-26.25% when increase ethanol concentration from 30% to 70%, also give high hemicellulose. The sugar composition was monitored with HPLC. The major monosaccharide in PHC_{CM} was glucose and xylose. PHC_{CM} had a moderate Mw of 67.57 KDa. Solubility tests on samples from PHC_{CM} showed that DMSO are the best solvents to solubilize hemicellulose. On the other hand, toluene partially dissolved hemicellulose from OPT. The TGA curves of PHC_{CM} began to decompose at 261°C. The peaks at 290°C in the DTG curves correspond to the thermal decomposition of the hemicellulose. Preparation of PHC_{CM}

-MA/PVA hydrogels, the PHC_{CM} from OPT was first modified using crosslinking method with maleic anhydride (MA) to obtain PHC_{CM} -MA and then blended with polyvinyl alcohol (PVA) to generate PHC_{CM} -MA/PVA hydrogel. The PHC_{CM} -MA/PVA hydrogel were immersed in distilled water and their swelling behaviors were examined. The PHC_{CM} -MA/PVA hydrogel, the swelling degree increases as the MA content in the hydrogel increases. The strength of all samples ranged from 2.91 to15.39 MPa. Under the optional conditions (PHC_{CM}-MA1.5:1/PVA1:0.25), the highest compressive strength could reach to 15.39 MPa. The condition that gave high strength and swelling was selected for the cytotoxicity test for normal cells and antimicrobial test. In this research, the priority of property of hydrogel for the selected hydrogel to study in the next experiment was high strength and swelling. From the result, The H10 hydrogel was used. The cell viability of L929 cells on the H10 hydrogel suggested that the gel was cytotoxic and possibly had a potential for inhibition of cancer cell. The results showed the no resistance of *S. aureus* ATCC 6538, *E. coli* ATCC 8739 and *C. albicans* ATCC 10231 to H10 PHC_{CM}-MA/PVA hydrogel. Production of xylooligosaccharide (XOS) from PHC_{CM} was cut using the enzymatic method. Under the optimum conditions of PHC_{CM} (4 U for 12h at 40 °C) gave high xylobiose and XOS production of 0.25 g/L and 48%, respectively. Moreover, the XOS from PHC_{CM} showed the antioxidant activity by DPPH assay.

CHAPTER 5

SUMMARY AND SUGGESTIONS

1. The optimum condition values of polymer from *R. microsporus* ST4 in medium A with pH adjusted to 5.8 at 45 °C for 3 days. Under the optimum condition, *R. microsporus* ST4 gave high glutamic acid, lysine, aspartic acid, alanine and threonine. The molecular size of PGA was estimated by the SDS-PAGE method. The results showed the PGA bands at the size over 75 kDa. Solubility tests on PGA showed that DW was the best to solubilize.

2. Polymer from two sources of POME, sterilizer condensate (SC) and decanter effluent (DE) of a palm oil mill were recovered and precipitation with 5 folds (v/v) of 95% ethanol, obtaining a total yield of 2.159% and 2.47%, respectively, also gave high hemicellulose. The HSC and HDE had a moderate Mw, two sizes with 42,191 Da and 3,577 Da of SC. DE had a moderate Mw, two sizes with 36,348 and 3,613Da.

The commercial xylan (CXB) from beech wood exhibited two size of Mw, 930,000 Da and 21,000 Da

3. Extraction of the hemicellulose from OPT_{RT} size 0.6>OPT<2mm was extracted two methods with alkaline peroxide (AP) pretreatment and fractionation with graded ethanol, obtaining a total yield of the precipitated hemicellulose from oil palm trunk (PHC_{CM}) increased from 10.52-28.68% when increased ethanol concentration from 30% to 80%, also give high hemicellulose (82.74-89.44%). The PHC_{CM} had a moderate Mw of 67,570 Da. The second method is hydrothermal method. The result showed that precipitate fraction of OPT_{RT} after treated with hydrothermal and then freeze-dried gave high precipitated fraction (PHC_{AH})10.1% and also give high hemicellulose (97.80%).

The PHC_{CM} was selected for next experiments because the yield of the production high than PHC_{AH}.

4. The PHC_{CM}, HSC and HDE from palm oil mill and CXB were characterized as follows: sugar composition analysis, the solubility of biopolymer and Thermal stability. The sugar composition was monitored with HPLC. The major monosaccharide in hemicellulose from PHC_{CM}, HSC and HDE from palm oil was glucose and xylose. As the beech wood was contained a higher amount of only xylose. Solubility tests on PHC_{CM} showed that DMSO was the best solvents to solubilize hemicellulose. While hemicellulose from SC, DE and beech wood, slightly higher solubility were obtained in DW. On the other hand, toluene partially dissolved hemicellulose from OPT, SC and DE and beech wood. The TGA curves of the xylan from beech wood, PHC_{CM}, HSC and HDE began to decompose at 221, 261, 209 and 238 °C, respectively. At <40% weight loss, the decomposition temperatures of the four polymer samples appeared at 412, 428, 592 and 606 °C, respectively. The peaks at 290◦C in the DTG curves correspond to the thermal decomposition of the hemicellulose.

For biological properties, the biopolymers from HSC and HDE had no activity for antimicrobial test, not inhibit the human breast cancer, oral cavity cancer and small cell lung cancer and non-cytotoxicity for human caucasian colon adenocarcinoma . The HDE showed non-cytotoxicity human hepatocarcinoma (HepG2) ATCC HB-8065 against while HSC presented cytotoxicity in test.

5. Preparation of polymers-MA/PVA hydrogels, the modified polymers from OPT, SC and DE from palm oil mill were also verified by FTIR resulted in that reaction was evidenced by the FTIR spectrum. The presence of a peak at 1726-1735 cm^{-1} in the modified hemicellulose from OPT, SC and DE and commercial xylan. The polymers -MA/PVA hydrogel from four biopolymers were immersed in distilled water and their swelling behaviors were examined. The swelling degree of hydrogel ranged from 134.61% to 395.9%. Under the optional conditions (H17 of HDE), the highest swelling test could reach 395.9%. The strength of all samples ranged from 2.91 to19.29 MPa. Under the optional conditions (H20 of HDE), the highest compressive strength could reach to 21.12 MPa. The cell viability of L929 cells on the H11 from HSC, H17 from HDE and H35 from commercial xylan and H10 from PHC_{CM}, hydrogel suggested that the gel was cytotoxic and had a potential for inhibition of cancer cell. The H17 from HDE and H35 from CXB hydrogels exhibited

inhibition zones of *S. aureus* ATCC 6538 in agar plates. Consequently, the HDE-MA/PVA and CXB-MA/PVA hydrogel with an antibacterial property could be used to treat attainable wounds to protect or kill the existing infection.

6. The xylooligosacharide (XOS) from PHC_{CM}, HSC, HDE and CXB was using the enzymatic method. The CXB under the optimum conditions at 4 U for 12h at 40 °C gave the highest xylobiose could reach to 0.5 g/L follow by polymers from OPT (4 U for 12h at 40 °C), the highest xylobiose could reach to 0.3 g/L. In addition, XOS from PHC_{CM} exhibited IC_{50} inhibition at 19.61 mg/mL of antioxidant activity test.

Thus, the hydrogel preparation and XOS production for application of biopolymers can be used instead the commercial xylan from beech wood.

Suggestions

1. For the hydrogel preparation, the hemicellulose SC and DE from palm oil mill can produce high capability hydrogel. To improve of the hydrogel production should be optimized as well as designed with other controllable condition.
2. For the xylooligosaccharide production, the PHC_{CM} from OPT able to used instead of the commercial xylan and antioxidant also. Up-scale of the XOS production should be optimized condition such as pH and type of enzyme.

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APPENDIXS

APPENDIX A

1. Chemical composition

1.1 Hemicellulose (Lin *et al*., 2010)

To determine the amount of hemicellulose, 10 ml 0.5 mol/L of sodium hydroxide solution was added to 1 g of extractive-free dried biomass, and the temperature was held at 80 °C for 3.5 h. After that, the sample was washed using DI water until the pH value of the solution approach 7, then it was dried to a constant weight. The difference between the sample weight before and after this treatment is hemicellulose content.

1.2 Acid insoluble lignin (NREL/TP-510-42618)

The sample 0.5g was tested in 72% Sulfuric acid incubate 30 ± 3 °C. And autoclaving at 121 ± 3 °C. Filtration setup, equipped with a vacuum source and vacuum adaptors for crucibles. The difference between the sample weight before and after this treatment is lignin content.

1.3 Cellulose (Wright and Wallis, 1998)

The sample 1 g and nitric acid-acetic 25 ml (prepared by diluting nitric acid 90ml and acetic acid 732ml to 1L with water) and the mixture were refluxed at 120 °C for 30 min. After cooling, the residue was filtered under vacuum into preweight sintered porous crucibles and washed with hot water 500 ml and then ethanol 25 ml. Dry for 3h at 105°C. The difference between the sample weight before and after this treatment is cellulose content.

1.4 Ash (NREL, TP/-510-42622)

1.4.1 Analytical balance, accurate to 0.1 mg.

1.4.2 Desiccator containing a desiccant

1.4.3 Ashing crucibles, 50 mL, porcelain, silica, or platinum

1.4.4 Porcelain markers, high temperature, or equivalent crucible marking method

1.4.5 Ashing burner, ignition source, tongs, and clay triangle with stand

1.4.6 Convection drying oven, with temperature control of 105 ± 3°C, optional

1.4.7 Oven dry weight (ODW) the weight of biomass mathematically corrected for the amount of moisture present in the sample at the time of weighing.

1.5 Moisture (A.O.A.C., 1999)

Dry the empty dish and lid in the oven at 105°C for 3h and transfer to a desiccator to cool. Weight the empty the dish and lid.

1.5.1 Weight about 3 g of the sample to the dish. Spread the sample with spatula.

1.5.2 Place the dish with the sample in the oven. Dry for 3h at 105°C.

1.5.3 After drying, transfer the dish with a partially covered lid to the desiccator to cool. Reweight the dish and its dried sample.

Moisture(%) = (W1-W2)/W1x100

W1=weight (g) of the sample before drying

W2= weight (g) of the sample after drying

1.6 Starch Analysis (Bra´nyikova´, 2011)

The samples were extracted three times using 4mL of 80% ethanol for 15 min at 68 °C and centrifuge the samples at 1370 x g for 15 min and discharge the ethanol supernatant. For total hydrolysis of starch, 3.3 mL of 30% perchloric acid was added to the sediment, stirred for 15 min at 25°C and centrifuged. This procedure was repeated three times. The extracts were combined and made up to 10 mL. Thereafter, 0.5mL of the extract was cooled to 0°C; 2.5 mL of anthrone solution (2 g of anthrone in 1 L of 72% (v/v) H_2SO_4) were added and stirred. The mixture was kept in a water bath at 100 °C for 8 min. It was then cooled to 20 °C, and the absorbance was measured at 625 nm. Calibration was carried out simultaneously using glucose as the standard. The values measured for glucose were multiplied by 0.9 to obtain a calibration curve for starch determination.

2. Test of antimicrobial activity

2.1 Anti- *Bacillus cereus* (Clinical and Laboratory Standards Institute, 2006)

The gram positive strain; *B. cereus* ATCC 11778 (TISTR 687) is grown on tryptic soy agar (TSA) at 37 °C for overnight. A single colony is inoculated in 5 mL of tryptic soy broth (TSB) and incubated in a rotary shaker 200 rpm at 37°C for 30 min (OD_{600} ~ 0.1). The culture is then diluted 200 folds in 20 mL TSB and incubated at 37ºC, 200 rpm for 3 h. Afterward, cell suspension is adjusted to OD_{600} of 0.4-0.5 (equivalent to 1 x 10^8 CFU/mL) and further diluted to 3.53 x10^5 cfu /mL with Mueller Hinton broth (MHB) prior to assay. This assay is performed in 384-well plate in triplicate. Briefly, test wells are added with 7.5 μL of compound, 42.5 μL of cell suspension and 25 μL of resazurin solution (0.0625 mg/mL in PBS). Wells without cells is set as blank. Plates are then incubated at 37°C for 2 h. Fluorescence is measured at excitation and emission wavelengths of 530 and 590 nm, respectively. The signal of test wells is subtracted with that of blank before calculation.

Percent of bacterial inhibition is calculated by the following equation:

$$\% \text{ Inhibition} = [1- (FU_T/ FU_C)] \times 100$$

Where FU_T and FU_C represent the mean fluorescence unit of cells treated with test compound and that treated with 0.5% DMSO, respectively.

A threshold of 90% inhibition is used as a cut off for antimicrobial activity of compound, which can be classified by these criteria:

If % inhibition is < 90%, the activity is reported as "Inactive".

If % inhibition is ≥ 90%, the activity is reported as "Active".

MIC value is defined as the lowest concentration of compound exhibiting 90% inhibition of bacterial growth after exposure incubation.

2.2 Anti- *Klebsiella pneumonia* (Clinical and Laboratory Standards Institute, 2006)

The Gram-negative bacteria; *K. pneumoniae* (ATCC 700603) is grown. In TSA at 37°C for overnight. A single colony is inoculated in 5 mL Mueller hinton broth (MHB) and incubated in a rotary shaker 200 rpm at 37 °C for 2 h. Cell suspension is adjusted to OD_{600} of 0.2 (equivalent to 1 x 10^8 CFU/mL) and further

diluted to 1 x10^4 CFU/mL with MHB prior to assay. This assay is performed in 384-well plate in triplicate. Each well is added with 5 µL of sample (or positive or negative control agents) and 45 µL of cell suspension. Blank wells are added with 5 µL of 5% DMSO and 45 µL media. Plate is then incubated at 37 °C for 14 h. Bacterial growth is observed by OD_{600} measurement using microplate reader. The percent of bacterial inhibition is interpreted as above. The OD units of test wells are subtracted with mean OD units of blank wells before calculation.

Percent of bacterial inhibition is calculated by the following equation:

$$\% \text{ Inhibition} = [1- (OD_T / OD_C)] \times 100$$

Where OD_T and OD_C represent the mean OD unit of cells treated with test compound and that treated with 0.5% DMSO, respectively.

2.3 Anti-*Mycobacterium tuberculosis* (anti-TB test) (Changsen *et al.,* 2003; Collins *et al.,* 1998)

Green fluorescent protein (GFP) expressing *M. tuberculosis* H37Ra strain is established by Changsen, *et al* (2003). H37Ra *gfp* is cultivated on 7H10 agar containing 30 µg/mL kanamycin at 37°C for 4 weeks or until growth is observed. For starter cultivation, 2-3 single colonies are inoculated into 10 ml of 7H9 broth supplemented with 0.2% (v/v) glycerol, 0.1 % (w/v) of casitone, 0.05% (v/v) Tween 80, 10% (v/v) Middlebrook OADC enrichment solution and 30 µg/mL of kanamycin. The culture is then incubated in a rotary shaker 200 rpm at 37 °C for weeks until the optical density at 550 nm (OD_{550}) is between 0.5-1. For batch cultivation, the starter cultures are transferred at the rate of 1/10 volume to the 7H9 broth and incubated in shaking condition until the OD_{550} is approximately 0.5 to 1. Then, cells are pelleted by centrifugation (8,000 rpm) for 10 minutes, washed twice and suspended in PBS buffer, sonicated for 15 seconds each of 8 times to disperse the clumps. TB suspension is then aliquoted and stored at -80°C for 2 to 3 months. Working suspension of TB is prepared at approximately 1x10^6 cfu/mL prior to assay. Test materials are assayed in 384-well plate in quadruplicate. Briefly, 45 µL of TB suspension is exposed to 5 µL of compound at 37°C for 10 days. Fluorescence is measured at excitation and emission wavelengths of 485 and 535 nm, respectively. The percent of TB inhibition is interpreted as above (3.1.1). The signal of day-10 is subtracted with that of day-0 before calculation.

Percent of TB inhibition is calculated using the formula below:

$$\% \text{ Inhibition} = [1- (FU_T / FU_C)] \times 100$$

Where FU_T and FU_C represent the mean fluorescent unit of cells treated with test compound and that treated with 0.5% DMSO, respectively.

2.4 Anti- *Magnaporthe grisea* (rice blast disease pathogen) (Haugland, 2002; Josep *et al*., 1998)),

M. grisea Mat 1-1 strain THL1156 (BCC 10261) is grown in PDA and incubated at 25°C for 7 days. Then, the fungal spores are induced under black light for 3-4 days. After that, spores are harvested with 0.8% (v/v) Tween 20, centrifuged (10,000 rpm) for 5 minutes and re-suspended with sterilized water. Spores are then counted and diluted to 1.2 x10^5 spores/mL in 20 % (v/v) minimum salt medium (MM) (3 g/L NH4NO3, 20 g/L glucose, 0.5 g/L KH_2PO_4, 0.5 g/L $NaHPO_4.H_2O$, 0.5 g/L $MgSO_4.7HO_2$, 0.5 g/L $CaCl_2$ and 1 g/L yeast extract). This assay is performed in 384-well plate in triplicate. Each well is filled with 25 µL spore-suspension and left at room temperature for 2-2.5 hours to allow the sporeadhesion to wells. Subsequently, 25 µL of test compound (or positive or negative control agents) are added. Blank wells are added with 25 µL of medium and 25 µL of 10%DMSO. Plate is then incubated at 25°C for 16 - 18 h. After incubation period, plate is added with 25 µL of distilled water and 2 µL of 0.9 mg/ml CFDA in 70% DMSO, and kept in dark for 5-10 minute. Plate is then washed with tap water and blotted dry on paper towels and filled with 25 µL of distilled water to each well. Fluorescence measurement is detected at 485 nm excitation and 535nm emission wavelengths by using the bottom-reading mode of fluorometer. The percent of fungal inhibition is interpreted as above (3.1.1). The signal of test wells is subtracted with that of blank wells before calculation.

Percent of spore-germination inhibition is calculated by the following equation:

$$\% \text{ Inhibition} = [1- (FU_T / FU_C)] \times 100$$

Where FU_T and FU_C represent the mean fluorescence unit of spores treated with test compound and that treated with 0.5% DMSO, respectively.

3. Test of anti-cancer activity

3.1 Anti-human breast adenocarcinoma cancer tests (Brien *et al*., 2000).

MCF-7 cell line (ATCC HTB-22) is derived from breast adenocarcinoma. Cells are grown and maintained in a complete medium MEM supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 0.01 mg/ml insulin, 1X non-essential amino acid and 1.5 g/L sodium bicarbonate), and incubated at 37°C humidified incubator with 5% CO_2. Cells at a logarithmic growth are prepared to 3.3 x10^4 cells /mL in complete medium prior to assay. This assay is performed in 384-well plate in triplicate. Each well is added with 5 µL of test compound and 45 µL of cell suspension. Plate is then incubated at 37°C in a humidified incubator with 5% CO_2 for 3 days. After that, 12.5 µL of 62.5 µg/mL resazurin solution is filled to each well and the plate is further incubated at 37°C for 4 h. Fluorescence is measured at 530nm excitation and 590nm emission wavelengths by using the bottom-reading mode of fluorometer. The signal is subtracted with blank before calculation.

The percentage of cytotoxicity is calculated by the following equation:

% Cytotoxicity = [1-(FUT/ FUC)] x100

Whereas FU_T and FU_C are the mean fluorescent unit from cells treated with test compound and that treated with 0.5% DMSO, respectively.

A threshold of 50% cytotoxicity is used as a cut off for cytotoxic activity of compound, which can be classified by these criteria:

If % cytotoxicity is < 50%, the activity is reported as “Non-cytotoxic”

If % cytotoxicity is ≥ 50%, the activity is reported as “Cytotoxic”.

The IC50 value is derived from dose-response-curve that is plotted between % cytotoxicity versus the sample concentrations by using SOFTMax Pro software (Molecular Devices, USA).

3.2. Anti-human oral cavity carcinoma cancer tests (Brien *et al*., 2000).

KB cell line (ATCC CCL-17) is derived from epidermoid carcinoma of oral cavity. Cells are grown and maintained in a complete medium MEM supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 0.01 mg/ml insulin, 1X non-essential amino acid and 1.5 g/L sodium bicarbonate) and incubated at 37°C humidified incubator with 5% CO_2. For cell suspension preparation, cells at a logarithmic growth are harvested and diluted to 2.2x10^4

cells /mL in complete medium prior to assay. This assay is performed in 384-well plate in triplicate. Briefly, each well is added with 5 μL of test compound and 45 μL of cell suspension. Plate is then incubated at 37°C in a humidified incubator with 5% CO_2 for 3 days. After that, 12.5 μL of 0.0625 mg/mL resazurin solution is filled to each well and the plate is further incubated at 37°C for 4 h. Fluorescence is measured at 530nm excitationand 590nm emission wavelengths by using the bottom-reading mode of fluorometer. The percent of cytotoxicity is interpreted as above (3.1). The signal is subtracted with blank before calculation.

The percentage of cytotoxicity is calculated by the following equation:

% Cytotoxicity = [1-(FU_T/ FU_C)] x100

Whereas FU_T and FU_C are the mean fluorescent unit from cells treated with test compound and that treated with 0.5%DMSO, respectively.

3.3 Anti-human small-cell lung carcinoma cancer tests (Brien *et al.*, 2000).

NCI-H187 cell line (ATCC CRL-5804) is derived from small cell lung carcinoma. Cells are grown and maintained in a complete medium (RPMI-1640 supplemented with 15% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2.5 g/L Glucose and 2.2 g/L sodium bicarbonate), and incubated at 37°C humidified incubator with 5% CO_2. Cells at a logarithmic growth are harvested and diluted to 6.7x104 cells /ml in complete medium prior to assay. This assay is performed in 384-well plate in triplicate. Each well is added with 5 μL of test compound and 45 μL of cell suspension. Plate is then incubated at 37°C in a humidified incubator with 5% CO_2 for 5 days. After that, 12.5 μL of 0.0625 mg/mL resazurin solution is filled to each well and the plate is further incubated at 37°C for 4 h. Fluorescence is measured at 530nm excitation and 590nm emission wavelengths by using the bottom-reading mode of fluorometer. The percent of cytotoxicity is interpreted as above (3.1). The signal is subtracted with blank before calculation.

The percentage of cytotoxicity is calculated by the following equation:

% Cytotoxicity = [1-(FU_T/ FU_C)] x100

Whereas FU_T and FU_C are the mean fluorescent unit from cells treated with test compound and that treated with 0.5%DMSO, respectively.

4. Cytotoxicity test

4.1 Cytotoxicity against human hepatocarcinoma (HepG2) (Brien *et al*., 2000).

HepG2 cell line (ATCC HB-8065) is isolated from human epithelial Hepatocellular carcinoma cells. Cells are grown and maintained in a complete medium MEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 unit/ml penicillin and 100 µg/mL streptomycin) and incubated at 37 °C humidified incubator with 5% CO_2. Cells at a logarithmic growth are harvested and diluted to $2x10^4$ cells/ml in complete medium prior to assay. This assay is performed in 96-well plate in four replicate wells. First, plates are seeded with 200 µL of cell suspension or blank medium into well, and incubated at 37 °C humidified incubator with 5% CO_2 for 48 h. Subsequently, culture medium was replaced with 200 µL of fresh medium containing 100 µg/mL of testcompounds or 1%DMSO, and plates are further incubated for 24 h. After incubation period, the plates was added with 50 µL of 125 µg/mL resazurin solution and incubated at 37 °C humidified incubator with 5% CO_2 for 4 h. Fluorescence is measured at 530 nm excitation and 590 nm emission wavelengths by using the bottom-reading mode of fluorometer. The percent of cytotoxicity is interpreted as above (3.1). The signal is subtracted with blank before calculation.

The percentage of cytotoxicity is calculated by the following equation:

% Cytotoxicity = $[1-(FU_T/ FU_C)]$ x100

Whereas FU_T and FU_C are the mean fluorescent unit from cells treated with test compound and that treated with 1%DMSO, respectively.

4.2 Cytotoxicity against human caucasian colon adenocarcinoma (Caco2) ATCC HB-37 (Brien *et al*., 2000)

Caco2 cell line (ATCC HTB-37) is isolated from human epithelial colorectal adenocarcinoma cells. Cells are grown and maintained in a complete medium MEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 0.1 IU/ml Insulin-Transferrin-Selenium-X, 1.5 g/L sodium bicarbonate, 100 unit/ml penicillin and 100 µg/mL streptomycin) and incubated at 37 °C humidified incubator with 5% CO_2. Cells at a logarithmic growth are harvested and diluted to $2x10^4$ cells /mL in complete medium prior to

assay. This assay is performed in 96-well plate in four replicate wells. First, plates are seeded with 200 µL of cell suspension or blank medium into well, and incubated at 37 °C humidified incubator with 5% CO_2 for 48 h. Subsequently, culture medium is replaced with 200 µL of fresh medium containing test-compounds or 1% DMSO, and plates are further incubated for 24 h. After incubation period, the plates is added with 50 µL of 125 µg/mL resazurin solution and incubated at 37 °C humidified incubator with 5% CO_2 for 4 h. Fluorescence is measured at 530nm excitation and 590nm emission wavelengths by using the bottom-reading mode of fluorometer. The percent of cytotoxicity is interpreted as above (3.1). The signal is subtracted with blank before calculation.

The percentage of cytotoxicity is calculated by the following equation:

$$\% \text{ Cytotoxicity} = [1-(FU_T/ FU_C)] \times 100$$

Whereas FUT and FUC are the mean fluorescent unit from cells treated with test compound and that treated with 1%DMSO, respectively.

APPENDIX B

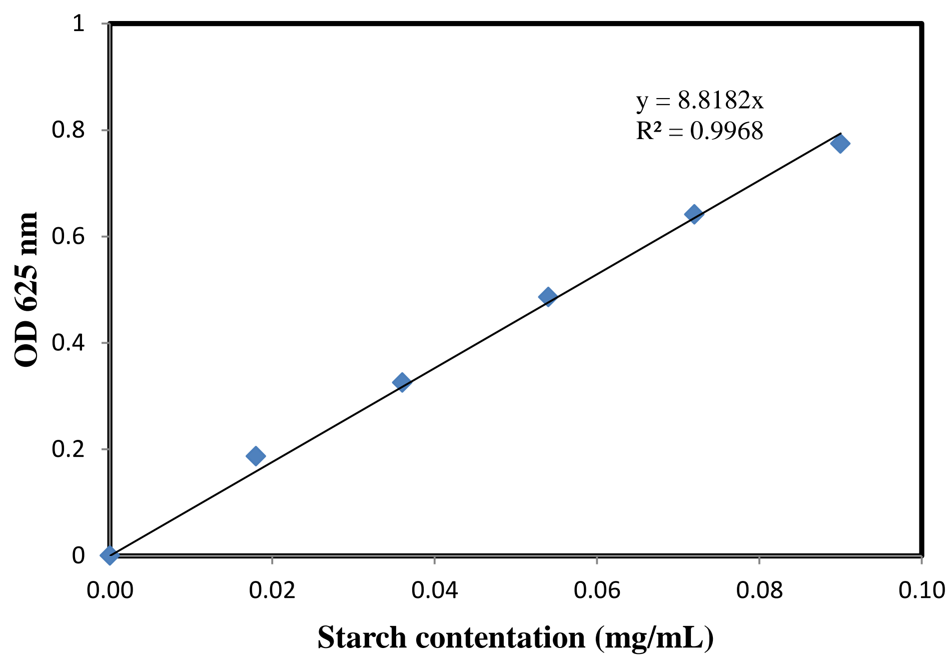


Figure-Appendix B1. Standard curve of starch

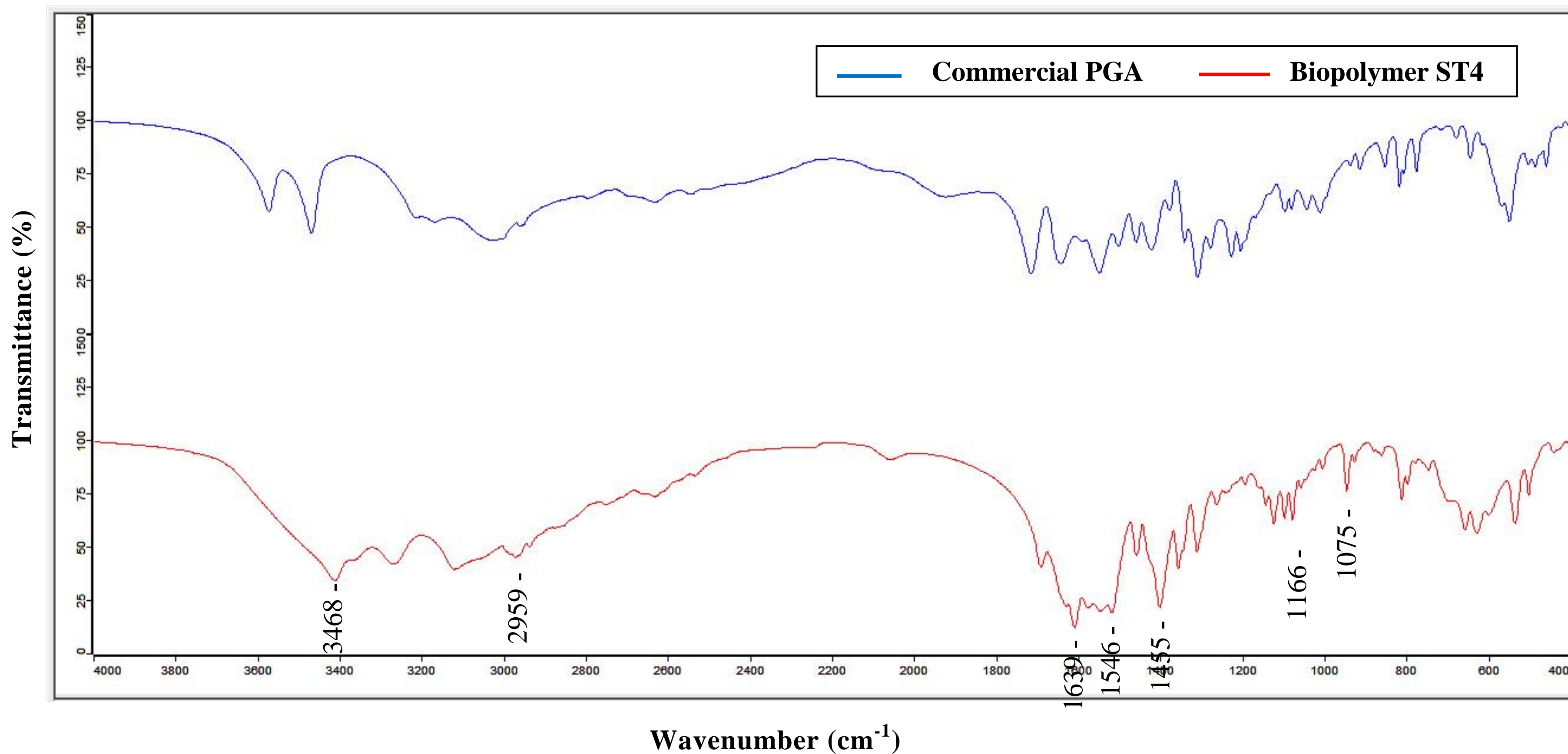


Figure-Appendix B2. FT-IR spectra of biopolymer production from cultivation of *Rhizopus microsporus* ST4 in the medium A on rotary shaker (200 rpm) at 45 °C and for 3 days compared to the spectra of a commercial PGA sample

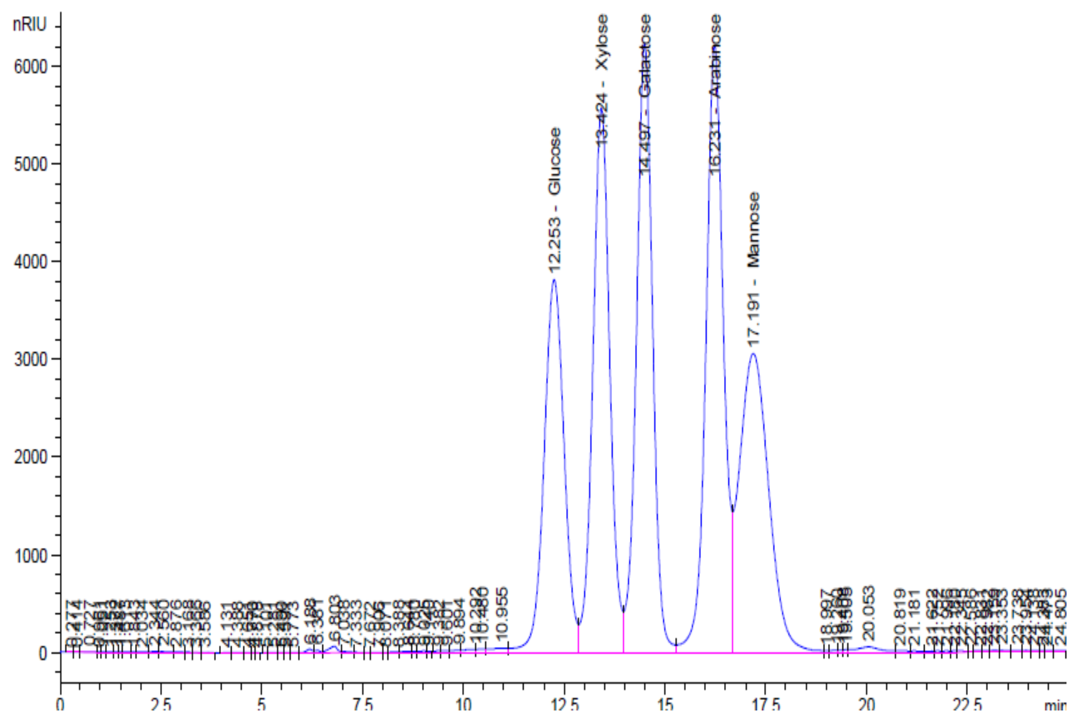


Figure-Appendix B3. HPLC chromatogram of standard sugars

nRIU
4000
3500
3000
2500
2000
1500
1000
500
0
6.195
7.223
6.774
4.742
8.485 - Xylopentaose
8.760
Area: 1619.09
9.788
12.493
13.445
14.166
15.575
16.963
19.789
3.164
3.748
min

Figure-Appendix B4. HPLC chromatogram of enzymatic hydrolysis from the hemicellulose decanter effluent (HDE) of palm oil mill with 8U of xylanase at 40 °C for 48h

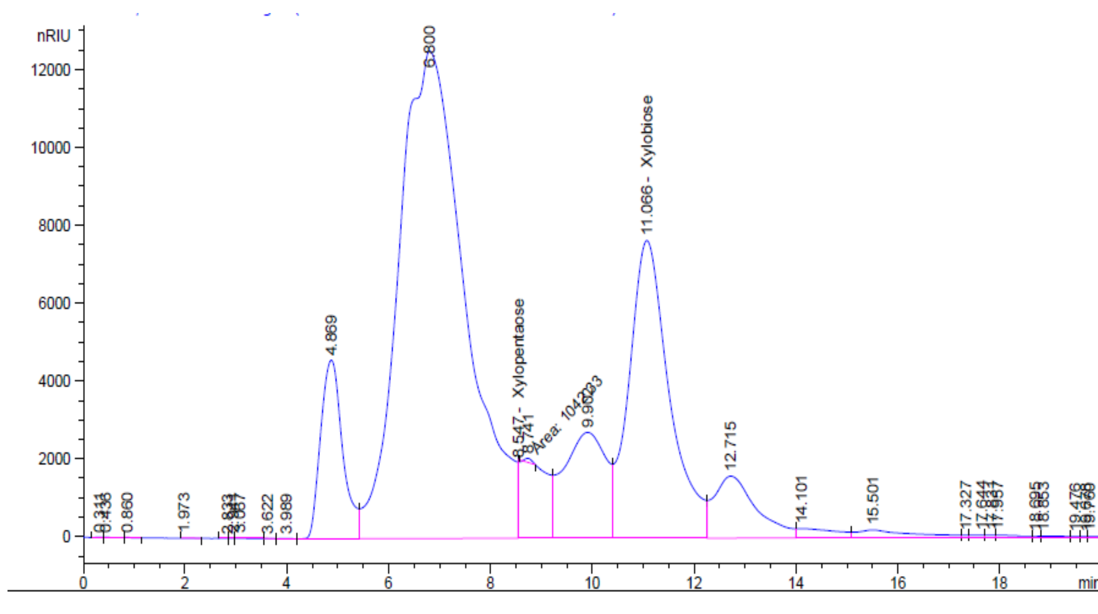


Figure-Appendix B5. HPLC chromatogram of enzymatic hydrolysis from the commercial xylan from beech wood (CXB) with 4U of xylanase at 40 °C for 12 h

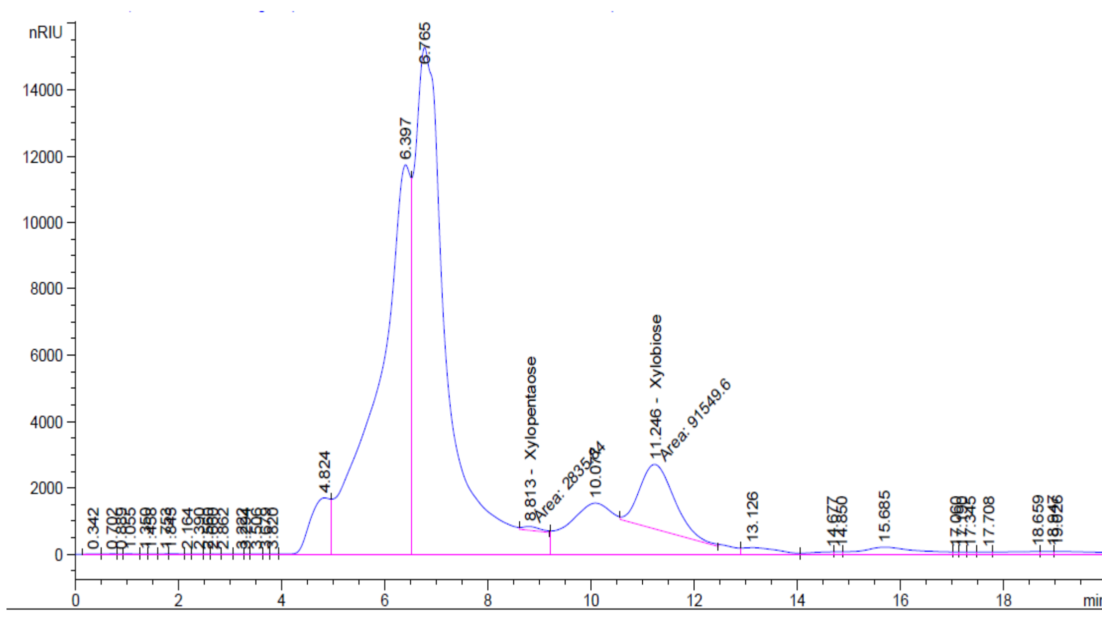
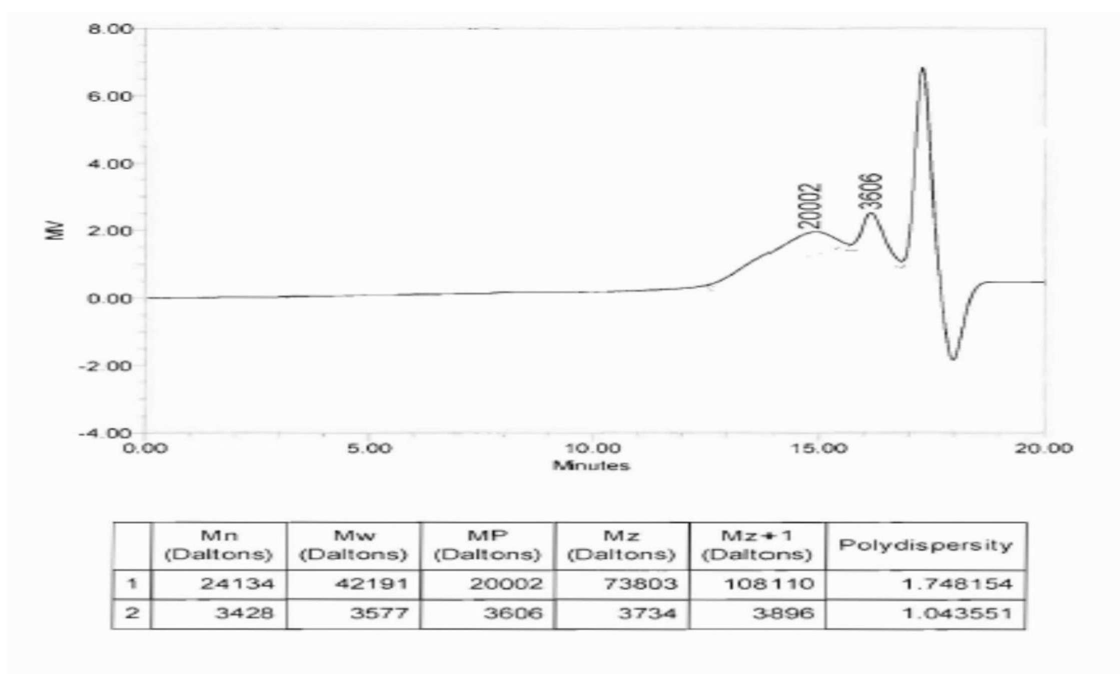
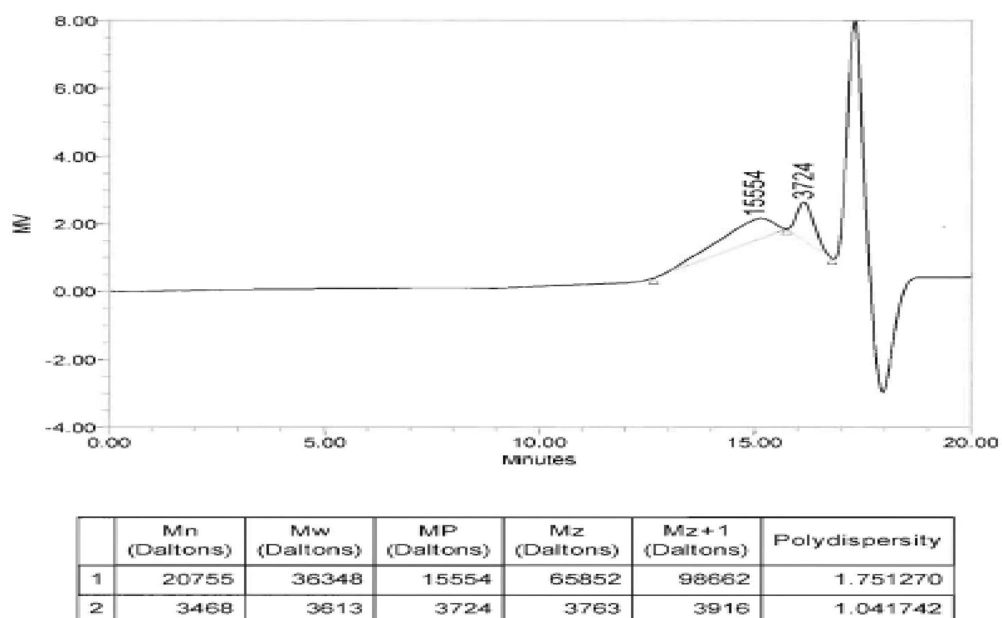


Figure-Appendix B6. HPLC chromatogram of enzymatic hydrolysis from the hemicellulose by chemical pretreatment (PHC_{CM}) from OPT with 4U of xylanase at 40 °C for 12h



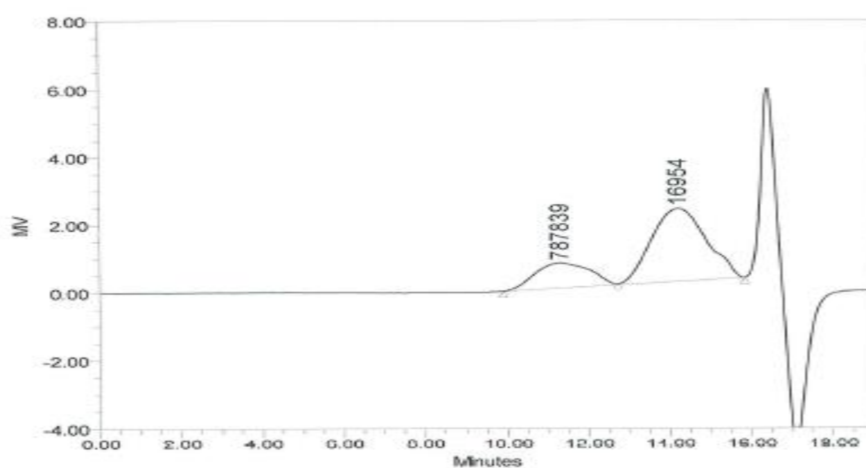
	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity
1	24134	42191	20002	73803	108110	1.748154
2	3428	3577	3606	3734	3896	1.043551

Figure-Appendix B7. GPC chromatogram of hemicellulose from sterilizer condensate (HSC) of palm oil mill



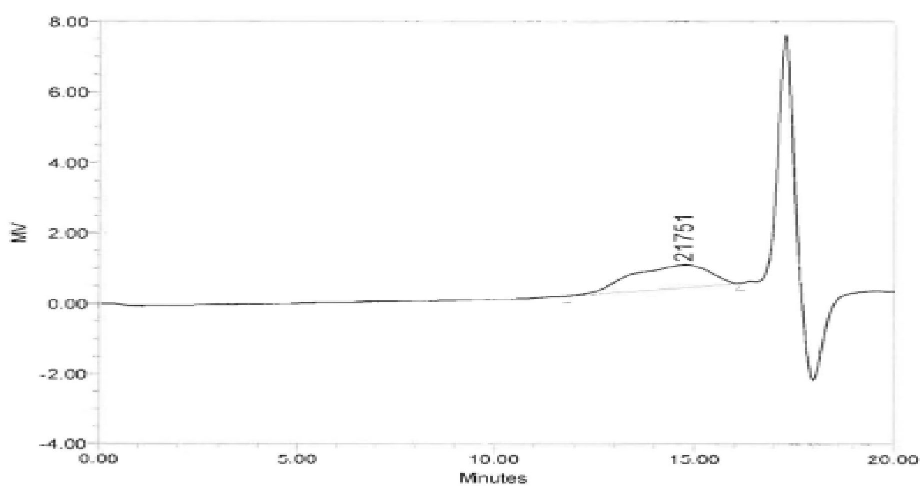
	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity
1	20755	36348	15554	65852	98662	1.751270
2	3468	3613	3724	3763	3916	1.041742

Figure-Appendix B8. GPC chromatogram of hemicellulose from decanter effluent (HDE) of palm oil mill



	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	% Area
1	553875	928861	787839	1446390	1978951	1.677022	25.72
2	11800	20879	16954	33442	46777	1.769363	74.28

Figure-Appendix B9. GPC chromatogram of commercial xylan from beech wood (CXB)



	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity
1	24784	67570	21751	159529	270489	2.726358

Figure-Appendix B10. GPC chromatogram of the precipitated hemicellulose by chemical pretreatment (PHC_{CM}) from OPT

VITAE

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List of Publications and Proceedings

Binma-ae, H. and Prasertsan, P. 2018. Production and characterization of biopolymer from *Rhizopus microsporus* ST4 (Manuscript preparation).

Binma-ae, H. Prasertsan, P. and Choorit, W. 2018. Preparation and characterization of complex hydrogels form hemicelluloses recover from palm oil mill effluent (Manuscript preparation).

Binma-ae, H., Choorit, W. and Prasertsan, P. 2018. Preparation and characterization of chemical crosslinked hemicellulose from oil palm trunk /poly (viny alcohol) complex hydrogels (Manuscript preparation).