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Master Thesis

自小花蔓澤蘭以膜分離及酵素水解生產木寡糖

Production of xylooligosaccharides from *Mikania micrantha* by membrane separation and enzymatic hydrolysis

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摘要

小花蔓澤蘭(*Mikania micrantha*)為外來入侵蔓藤植物，已對海拔一千公尺以下之植物造成嚴重的影響，有「綠癌」之稱，目前農林單位已全力加以剷除中。然而其極高的生產力及廣泛的分布性，極易成為生質能源的良好原料。

木寡糖是一種機能性食品，能在較寬的 pH 值、溫度範圍內保持穩定，與其他寡糖相比較，木寡糖最難被人體消化;木寡糖能選擇性地增加直腸道中的益菌，改善腸道菌相。在日本，木寡糖被認為是最有前途的功能性寡糖之一，並已得到廣泛的應用。

本研究利用 12% NaOH 持溫 60 ºC 與 150rpm 反應 16 小時進行小花蔓澤蘭粗聚戊醣萃取後，再以酒精沉澱法、奈米過濾法和酒精超膜過濾法製備三種小花蔓澤蘭木聚糖(EMX、NMX 與 EUMX)。結果顯示酒精沉澱法所得到的木聚糖純度較高但聚戊醣總量較少;利用膜過濾法可得到純度較低但較多的聚戊醣總量。

三種不同的小花蔓澤蘭木聚糖與商用樺木木聚糖(聚戊醣含量為 0.9%)，以實驗室所分離的 Paenibacillus campinensis BL 11 木聚糖酶，在 10、50、100 U/mL 三種酵素劑量，持溫 60 ºC 與 150 rpm 反應 0-96 小時的條件下進行水解，並以 HPLC 檢測木寡糖含量。

結果顯示奈米過濾法製備的小花蔓澤蘭木聚糖在 50 U/mL 木聚糖酶的條件下水解 24 小時後，與商用樺木木聚糖相較之下，可產出較多的木寡糖，其木寡糖產率分別為 68%與 59%。奈米過濾法之小花蔓澤蘭木聚糖水解後，主要產出的木寡糖為木六糖(X6)、木五糖(X5)及木二糖(X2)，含量分別為 43%、21%及 21%。

關鍵字:小花蔓澤蘭、木寡糖、纖維素酶、膜過濾、奈米過濾

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Abstract

Mikania micrantha (*M. micrantha*), “green cancer”, is one of the invasive vine plants; it already causes the serious effect of plants which are after an elevation of 1000 meters. Nowadays, the agricultural and forest organizations are making efforts to eradicate the alien plant. Because of its vigorous, fast and rampant growth habit, *M. micrantha* has the potential to be a good biomass raw material.

Xylooligosaccharides (XOs), one of the functional foods, it has a wide range of pH and temperatures stability. XOs is more non digestible than other oligosaccharides; it can proliferate beneficial bacteria and improve microflora in human gut. In Japan, XOs is considered as one of the most potential functional oligosaccharides, and already has rather extensive application.

In this study, extracted crude *M. micrantha* xylan(MX) with 12 %(w/v) NaOH solution at 150 rpm, 60 ºC for 16 h., and then collected different kind of MX by ethanol-precipitation(EMX), nanofiltration(NMX) and ethanol-precipitation and ultrafiltration(EUMX). The result showed that ethanol -precipitation method could get purer xylan but less total pentosan weight, membrane filtration method was opposite to ethanol -precipitation method.

In XOs production, three kind of MX and commercial birchwood xylan(BX)

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(pentosan content: 0.9%) were hydrolyzed by adding 10, 50 and 100 U/mL Paenibacillus campinensis BL 11 xylanaes at 60 ºC and shake (150 rpm) for 0-96 h; analyzed XOs by the HPLC.

The result showed that NMX which was hydrolyzed by 50 U/mL for 24h, could get more XOs than commercial BX, the yield of XOs were 68 and 59%, respectively. The major XOs products of NMX were xylohexaose(X6), xylopentaose(X5) and xylobiose(X2); the yields were 43, 21 and 21%.

Key word: *Mikania micrantha*, Xylooligosaccharides, Paenibacillus, Membrane filtration, Nanofiltration

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Chapter 1 Introduction

There are many alien aggressive climbers has infested many lowland forests and waste areas in Taiwan, especially *Mikania micrantha* (*M. micrantha*), one of the top 10 worst weeds in the world (Holm et al., 1977). It not only interferes with the growth and development of trees and crops, but also reduces the density of wild grass species. In this strained circumstance, the government strongly hoped the national pay attention to focusing on control this vicious invader to alleviate an imminent disaster of land ecology system. The fastest and efficient way to eliminate *M. micrantha* is cutting (Kuo et al., 2002). Because of the fast-growing and widespread character, *M. micrantha* have the potential to be a good biomass raw material.

By the development of economic and technology, nowadays people pay attention to “How to eat more healthily?” In this sense, non-digestible oligosaccharides (NDOs) as the” functional food” has been use to improve health. NODs present important physicochemical and physiological properties beneficial to the health of people. Xylooligosaccharides (XOs), one of the NODs, are showing potential for practical applications in a variety of fields, including pharmaceuticals, feed formulations, agricultural purposes and food applications. They have various physiological important actions such as reducing cholesterol, maintaining gastrointestinal health, and improving the biological availability of calcium (Voragen, 1998).

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XOs are naturally present in fruits, vegetables, bamboo, honey and milk and can be produced at industrial scale from xylan-rich materials such as forest, agricultural or industrial wastes. Processing of residual biomass as raw material offers the economic and ecological benefits, since it is a bio-renewable, widely distributed and abundant resource (Domínguez *et al*., 2003).

Three different methods have been used for XOs production from these feedstocks: (Vazquez *et al*., 2000)

A. Enzyme treatments of native, xylan-containing lignocelluloses materials (LCM).

B. Chemical fractionation of a suitable LCM to isolate (or to solubilize) xylan, with further enzymatic hydrolysis of this polymer to XOs.

C. Hydrolytic degradation of xylan to XOs by steam, water or dilute solutions of mineral acids.

To produce XOs with chemical and enzymatic methods, xylan is generally extracted with an alkaline such as KOH or NaOH. Extracted xylan is converted to XOs by xylanase enzyme having low exo-xylanase and/or β-xylosidase activity. XOs produced by enzymatic hydrolysis and autohydrolysis (Akpinar *et al*., 2007). Enzymatic method is more desirable than autohydrolysis method, because it does not produce undesirable byproducts or high amount of xylose and does not require special equipment. Therefore, there are many papers that describe production of XOs by

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enzymatic hydrolysis xylan from oat spelt (Chen et al., 1997), beechwood (Freixo *et al*., 1997), corn cob (Pellerin et al., 1991), wheat straw (Zilliox *et al*., 1998) and hardwood (Nishimura *et al*., 1998).

There were the volumes of demand and average price of oligosaccharides in Japan (Wang, 2008) (Table 1). XOs not only have great potential to be the commercial product but also improve the economic development.

Table 1. The volume of demand and average price of oligosaccharides in Japan (Wang, 2008).

Oligosaccharides	Volume of demand (ton/year)	Average price (yen/kg)
Fructooligosaccharide	3000	390
Galactooligosaccharide	4600	560
Xylooligosaccharide	700	2500
Lactosucrose	2000	700
Raffinose	230	2000
Lactulose	2800	1000
Isomaltooligosaccharide	11000	150
Nigerooligosaccharide	700	350
Gentiooligosaccharide	700	350

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Chapter 2 Literature Reviews

2.1 *Mikania micrantha* H. B. K

2.1.1 Description

Mikania micrantha, an herbaceous perennial climber of the family Asteraceae, is a twining herb with 5-ribbed branches, pubescent or glabrous; internodes are 7.5-21.5 cm long. Leaves are opposite, ovate-deltoid, 6-15 x 3-9 cm, base cordate, apex acuminate, margins are coarsely dentate, crenate or sub-entire, glabrous on both sides, minutely glandular beneath and 3-5 nerved from the base; the petiole is 3-7 cm long. The inflorescence is axillary panicle corymb; capital is cylindrical, 1.5 mm across; there are 4 flowers per capitulate; 4 bracts, oblong to obovate, acute, green in color, 1-3 mm long with a fifth smaller one that is 1-2 mm long; the corolla is 5-lobed, white, often with a purple tinge, 4-5 mm long; achenes 2-3 mm long, narrowly oblong, 4-angled, black, glabrous; connate at base, 3 mm long, white at first, becoming reddish-brown (Sankaran, 2007).

In Taiwan, flowers appear during October and the fruits ripen during the middle ten days of November with prolific seed production of 0.17 million seeds per square meter of ground. Wind, water and animals are the common agents for the dispersal of seeds. *M. micrantha* is not an annual plant, during the winter season most of the leaves will be shed. New leaves will sprout in the next spring (kuo *et al*., 2002). Except spread by

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seeds, *M. micrantha* can also reproduce from stem fragments which root easily at the nodes and from vegetative remits arising from rosettes. Most local spread results from vegetative propagation (Wen *et al*., 2000).

2.1.2 History of spread of *Mikania micrantha* in Taiwan

According to Chiang *et al.,* (2002), the first occurrence of *M. micrantha* in Taiwan was recorded in 1986 in Wan Luan, Pingtung County. Nowadays, *M. micrantha* spread over 130 towns' agricultural lands of 17 countries. Total areas are 13,206 hectares, south Taiwan is the most serious (6,982 hectare), and central Taiwan is the second serious (4,078 hectare), the north Taiwan is less serious (43 hectare). The alien plant is commonly found on abandoned land, in rarely managed orchards and betel nut plantations, in forest clearings, along the edge of forests and along roadsides (Hwang *et al.,* 2003).

2.1.3 Control of *Mikania micrantha* in Taiwan

Spraying chemicals may be the easiest way to control *M. micrantha*. In fact, many farmers in Taiwan prefer to use herbicides to control this vine in open fields. But chemicals would cause the environmental contamination and hurt human health. Consideration for environmental safety and practical effectiveness, the Forest Bureau of Taiwan has been using the consecutive-cutting method since 2001 to control *M. micrantha*. In 2001, this invasive vine was controlled on more than 900 ha of forest.

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The vines are cut manually for the first time 20 cm above the ground. Three weeks later they are cut for a second time, to remove all the new sprouts. After another three weeks, the area is checked and all the remaining *M. micrantha* sprouts are cut. About 50% of the cut vines have been sprouted if the vines were cut only once in summer or autumn. Cutting the vines twice resulted in 90% mortality of the vines. Three consecutive cuttings eliminated 92-98% of the vines (Table 2). However, this method was less effective during winter and spring (kuo *et al*., 2002).

Table 2. Cumulative mortality of Mikania micrantha after various stem cuttings in different seasons (kuo et al., 2002)

Season	Date of first cutting	Cumulative mortality (%)		
		1st cutting	2nd cutting	3rd cutting
Summer	June 22, 2000	50	88	98
Autumn	Sept. 28, 2000	50	92	92
Winter	Dec. 30, 2000	30	44	60
Spring	Mar. 29, 2001	4	24	52

The growth rate of *M. micrantha* is fast, and the government's publicity and prevention has continued for about 20 years, the 2018 survey data shows the distribution area of *M. micrantha* has been reduced from about 51,852 ha in 1990 to 5,347 ha, and the distribution area has been reduced by 90%, which shows that the control effect is good. (Huang *et al*., 2003; Huang *et al*., 2019).

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2.2 Xylooligosaccharides

2.2.1 Non-digestible functional oligosaccharides

Oligosaccharides are derived from the Greek word *oligos*, meaning "a few", and from the Latin/Greek word *sacchar* which means "sugar". Consequently, oligosaccharides are low molecular weight carbohydrates. According to IUB-IUPAC nomenclature, oligosaccharides are defined as saccharides containing between 3 and 10 sugar moieties (Voragen, 1998).

The carbohydrates can be classified as digestible or non-digestible. The concept of non-digestible oligosaccharides (NDOs) originates from the observation that the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their glycosidic bonds non-digestible to the hydrolytic activity of the human digestive enzymes (Roberfroid and Slavin, 2000). The main categories of NDOs presently available or in development as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose and/or xylose. Table 3 shows 13 classes of non-digestible functional oligosaccharides that present bifidogenic functions, and are commercially produced. NDOs are made from one, two, three different types of monosaccharides (Sako *et al*., 1999). Although oligosaccharides are composed at least three sugar moieties, Lactulose is a disaccharide, and xylobiose is a compound of polymerization degree = 2, both of them are considered

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to be NDOs because they present similar properties to the oligosaccharides (Crittenden and Playne, 1996; Vazquez *et al*., 2000). Table 4 shows the physicochemical and biological properties of non-digestible Functional oligosaccharides (Nakakuki, 2005). These properties make the NDOs suitable for using in sweet, low-caloric diet foods, and for consumption by individuals with diabetes. (Mussatto and Mancilha, 2007)

Table 3. Non-digestible oligosaccharides with bifidogenic functions the commercially available (Sako *et al*., 1999).

Compound	Molecular structure[a]
Cyclodextrins	$(Gu)_n$
Fructooligosaccharides	$(Fr)_n$–Gu
Galactooligosaccharides	$(Ga)_n$–Gu
Gentiooligosaccharides	$(Gu)_n$
Glycosylsucrose	$(Gu)_n$–Fr
Isomaltooligosaccharides	$(Gu)_n$
Isomaltulose (or palatinose)	$(Gu–Fr)_n$
Lactosucrose	Ga–Gu–Fr
Lactulose	Ga–Fr
Maltooligosaccharides	$(Gu)_n$
RaYnose	Ga–Gu–Fr
Soybean	oligosaccharides $(Ga)_n$–Gu–Fr
Xylooligosaccharides	$(Xy)_n$

[a] Ga, galactose; Gu, glucose; Fr, fructose; Xy, xylose.

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Table 4. The functional properties of oligosaccharides (Nakakuki, 2005).

Physicochemical property	Sweetness, Bitterness, Hygroscopicity, Water activity, Stabilization of active substances (protein, flavor, color, etc.) Inclusion capability, Reinforcement agent for drinks, etc.
Biological property	Non-digestibility, Non-cariogenicity, Anti-cariogenicity, Bacteriostatic action, Selective proliferation of bifidobacteria, etc. Improvement of serum lipids and blood glucose, Effects on the absorption of minerals, Superoxide dismutase-like activity, Improvement of hepatic sufficiency, Improvement of atopic dermatitis, Immunopotentiating activity, Apoptosis induction, Reduction of allergic reaction, etc.
Other properties	Specific substrate for enzyme, Enzyme inhibitor, Elicitor, etc.

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2.2.2 The applications of xylooligosaccharides and their effects on health

XOs are showing potential for practical applications in a variety of fields, including pharmaceuticals, agricultural purposes and food applications. Fig. 1 shows several purposes of XOs (Vazquez *et al*., 2000).

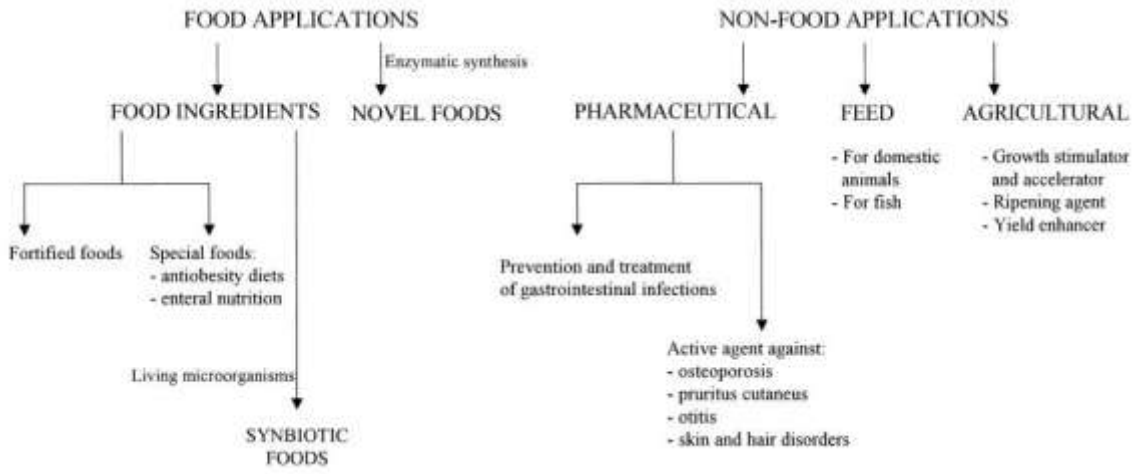

Fig 1. Applications of xylooligosaccharides (Vazquez *et al*., 2000).

XOs are stable over a wide range of pH (2.5-8.0, an advantage compared with other non-digestible oligosaccharides such as fructooligosaccharides (FOs), particularly in the acidic range, even at the relatively low pH value of the gastric juice) and temperatures (up to 100^{o}C). As food ingredient, XOs have an acceptable odour, and are non-cariogenic and low-calorie, allowing their utilization in anti-obesity diets (Vazquez *et al,* 2000). For food applications, the sweetness of xylobiose (X2) is equivalent to 30%

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that of sucrose. In food processing, XOs has steady resistance to both acids and heat, allowing their utilization in low-pH juices and carbonated drinks (Modler, 1994).

One of the most important features of XOs as food ingredients is their ability to stimulate the growth of intestinal *Bifidobacteria*. In comparison with other NDOs, *Bifidobacterium* spp. preferred xylooligomers, raffinose and fructooligomers to hexoses. Xylooligomers were almost as effective as raffinose and better than FOs to enhance the *in vitro* growth of these strains. Reported beneficial effects of *Bifidobacteria* on human health include (i) suppressing activity of entero putrefactive bacteria, preventing the formation of products such as toxic amines, (ii) repression of the proliferation of pathogenic bacteria due to the production of short-chain organic acids (such as lactic acid or acetic acid) leading to decreased pH in the gastro-intestinal tract and (iii) promotion of the digestion and absorption of nutrients (Yuan *et al*, 2004).

2.2.3 Structure of xylooligosaccharides

Hemicelluloses are branched polysaccharides consisting of the pentose D-xylose and L-arabinose, and the hexoses D-mannose, D-glucose, D-galactose and uronic acids (Saka, 1991). The main component of plant hemicellulose is xylan, the second most abundant polysaccharide in nature, accounting for approximately one-third of all renewable organic carbon on earth (Prade, 1996). Xylan is a *β*-1, 4 linked polymer of D-xylose with D-glucuronic acid or L-arabinose substituents (Yuan *et al*., 2004). XOs

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are containing two to seven molecules of xylose with *β*-1,4 linkages (Fig. 2), which can be substituted via ether or ester bonds (for example with *α*-D-glucopyranosyluronic acid or its 4-*O*-methyl derivative, acetyl groups and acids (Fig. 3) (Vazquez *et al.*, 2000).

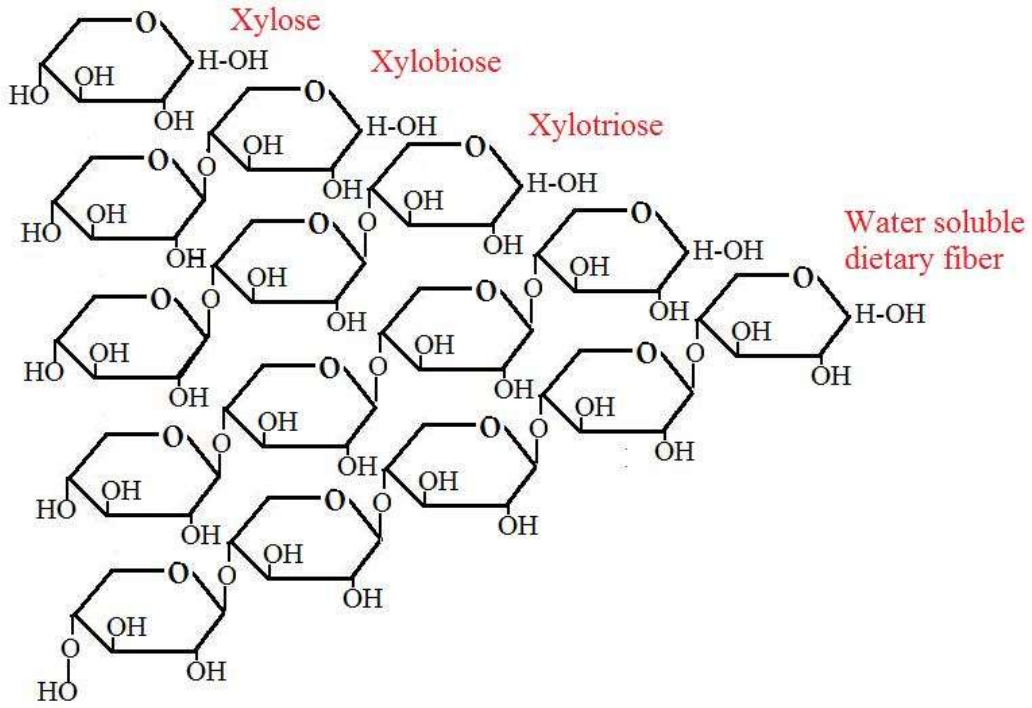

Fig 2. Structure of xylose and XOs (Hsiao, 2006).

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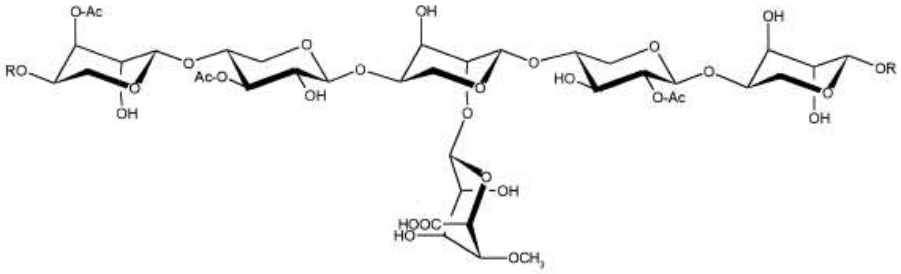
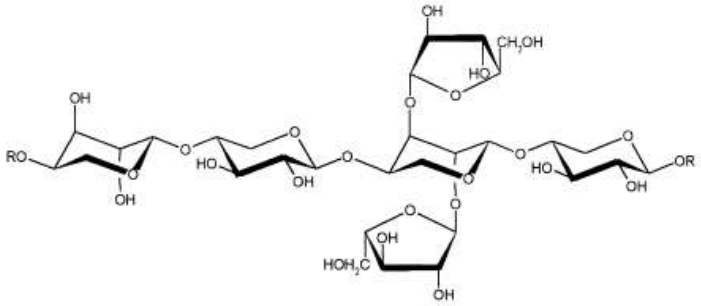
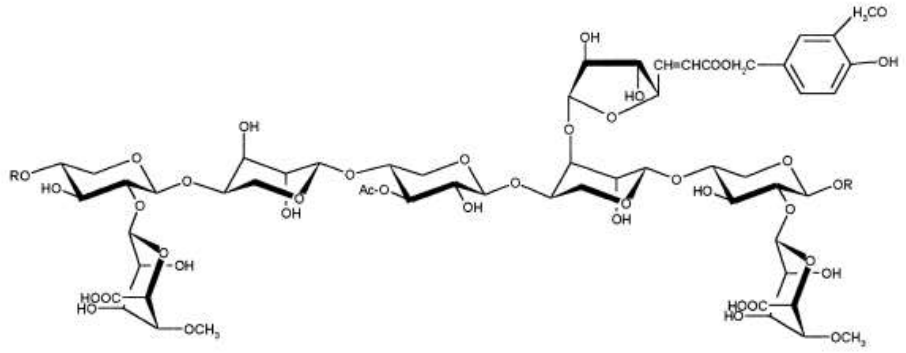
O-acetyl-4-O-methylglucuronoxylan from hardwood



Arabino-xylo-oligosaccharide from wheat bran hemicellulose



Arabinoxylan from grasses



Arabino-4-O-methylglucuronoxylan from softwoods

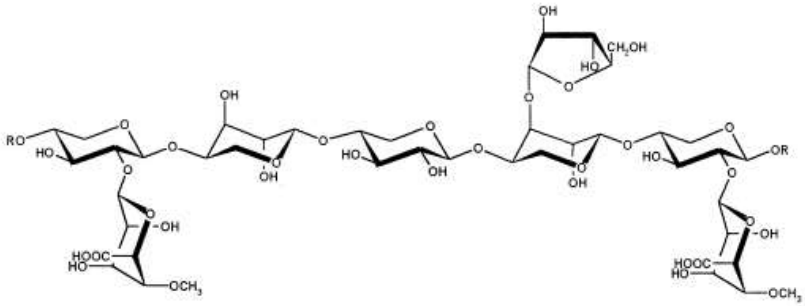


Fig 3. Chemical structure of xylans from several source. (Vazquez *et al.*, 2000)

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2.3 Production of xylooligosaccharides

2.3.1 The source of materials

Xylooligosaccharides (XOs) are naturally present in fruits, vegetables, bamboo, honey and milk. XOs production at an industrial scale is carried out from the polysaccharide xylan, which is extracted from lignocelluloses materials (LCM) which are mainly composed of cellulose, hemicellulose and lignin (Mussatto and Mancilha, 2007). Typical raw materials for XOs are hardwoods, corn cobs, straws, bagasse, hulls, malt and bran. Of particular interest are those sources of residual origin, such as forestall, agricultural or industrial wastes. Corncob, birchwood and rice shell contain relatively high levels of xylan (35–40%, 24–32%, and 24–32% xylan content, respectively) (Yuan *et al*., 2004). Processing of residual biomass as raw material offers the economic and ecological benefits, since it is a bio-renewable, widely distributed and abundant resource (Domínguez *et al*., 2003).

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2.3.2 Xylan degradation

Production of XOs from xylan-rich lignocellulosic materials generally includes chemical methods, enzymatic methods, and a combination of these methods. The production of XOs with chemical methods can be accomplished by steam, diluted solutions of mineral acids, or alkaline solutions. Extraction of xylan with steam or acid produces large amounts of monosaccharides and their dehydration product. Steam or hydrolytic degradation of xylan, known as autohydrolysis, involves the deacetylation of xylans to produce acetic acid, which hydrolyzes the hemicellulose. This method eliminates the use of corrosive chemicals for the extraction of xylan. However, it requires special equipment that can be operated at high temperature. The production of XOs with direct enzymatic treatment of xylan-containing materials is the only suitable method for susceptible materials such as citrus peels.

To produce XOs with chemical and enzymatic methods, xylan is generally extracted with an alkaline such as KOH or NaOH from suitable lignocellulosic materials. Extracted xylan is converted to XOs by xylanase enzyme having low exo-xylanase and/or β-xylosidase activity. In contrast to autohydrolysis, this method is more desirable, because it does not produce undesirable byproducts or high amount of monosaccharides and does not require special equipment. Therefore, there are many papers that describe production of XOs by enzymatic hydrolysis xylan from oat spelt,

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beechwood, corn cob, wheat straw and hardwood (Domínguez *et al*., 2003). Total biodegradation of xylan requires endo-*β*-1, 4-xylanase, *β*-xylosidase, and several accessory enzymes, such as *α*-L-arabinofuranosidase, *α*-glucuronidase, acetylxylan esterase, ferulic acid esterase, and p-coumaric acid esterase, which are necessary for hydrolyzing various substituted xylan. Table 5 lists the enzymes involved in the degradation of xylan and their modes of action. The endo-xylanase attacks the main chains of xylans, and *β*-xylosidase hydrolyzes xylooligosaccharides to xylose. The *α*-arabinofuranosidase and *α*-glucuronidase remove the arabinose and 4-O-methyl glucuronic acid substituents, respectively, from the xylan backbone. The esterases hydrolyze the ester linkages between xylose units of the xylan and acetic acid (acetylxylan esterase) or between arabinose side chain residues and phenolic acids, such as ferulic acid (ferulic acid esterase) and p-coumaric acid (p-coumaric acid esterase) (Saha and Bothast, 1999).

The complex structure of xylan needs different enzymes for its complete hydrolysis. Endo-1, 4-*β*-xylanases (E.C.3.2.1.8) depolymerize xylan by the random hydrolysis of the xylan backbone and 1, 4-*β*-D-xylosidases (E.C.3.2.1.37) split off small oligosaccharides. The side-groups present in xylan are liberated by *α*-L-arabinofuranosidase, *α*-D-glucuronidase, galactosidase, and acetyl xylan esterase (Beg *et al*., 2001) (Fig. 4).

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Table 5. Enzymes involved in the hydrolysis of complex heteroarabinoxylans (Saha and Bothast, 1999).

Enzyme	Mode of action
Endo-xylanase	Hydrolyzes mainly interior *β*-1,4-xylose linkages of the xylan backbone
Exo-xylanase	Hydrolyzes *β*-1,4-xylose linkages releasing xylobiose
β-Xylosidase	Releases xylose from xylobiose and short chain xylooligosaccharides
α-Arabinofuranosidase	Hydrolyzes terminal nonreducing a-arabinofuranose from arabinoxylans
α-Glucuronidase	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	Hydrolyzes feruloylester bonds in xylans
ρ-Coumaric acid esterase	Hydrolyzes *p*-coumaryl ester bonds in xylans

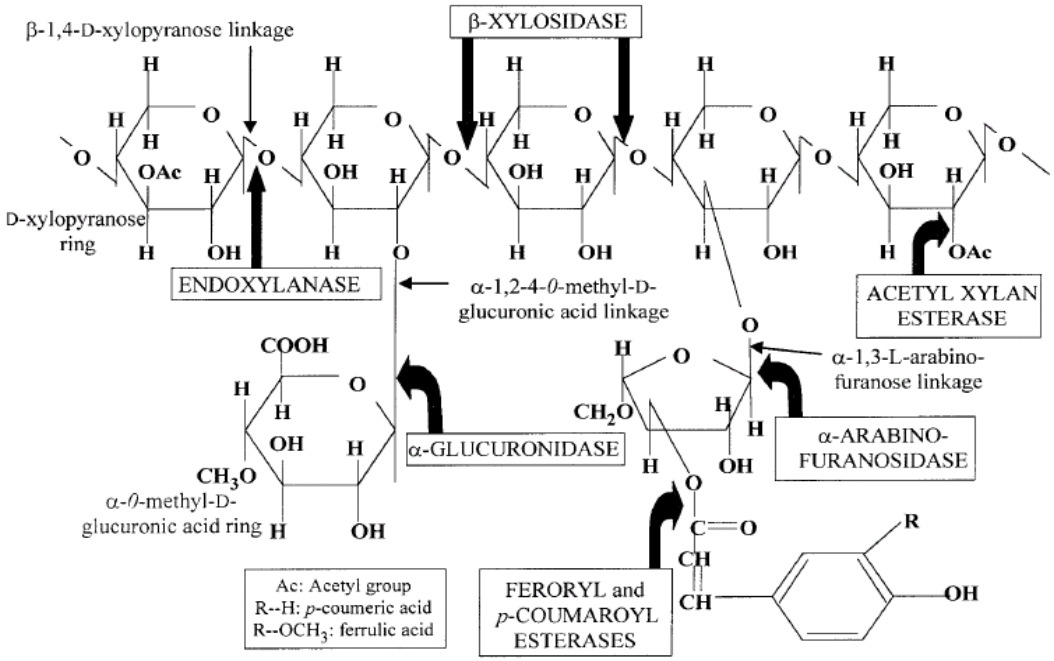

Fig 4. A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases (Beg *et al*., 2001).

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2.4 Microbial xylanases

2.4.1 *Paenibacillus* sp. isolate BL11

A xylanase-producing bacterial strain was isolated from the thermo alkaline black liquor (58ºC, pH 9.83) collected from pulping machine of Taiwan Pulp and Paper Company. The phylogenetic relationship of this strain with other bacteria was identified by 16S rDNA sequencing (16SrDNA accession number DQ 232773). The 16S rDNA sequence of this strain showed high similarity (99%) to those of the *Paenibacillus* spp. The isolate has been designated as *Paenibacillus* spp. isolate BL11 (Tsai, 2005).

Paenibacillus sp. isolate BL11 possesses several kinds of polysaccharide hydrolases those include xylanase, CMCase, pectinase, amylase, and β-glucanase. The xylanase was analyzed in zymogram and showed a molecular mass of about 41 kDa. The zymographic studies revealed that the BL11 xylanase was not only secreted to the extracellular medium but also remained cell-associated. The optimum temperature and pH for crude xylanase activity were 60ºC and pH 7, respectively. The highest activity of crude enzyme under optimal conditions (pH 7, 60ºC) was 23 IU/mg. In comparison with other microorganisms, the BL11 xylanase is not higher than fungi or yeast, however, its stability is better than others (Tsai, 2005).

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2.5 Membrane separations

Membrane separation processes, as developed starting from the pioneering research work by Loeb and Sourirajan (1963), have recently begun to be recognized as efficient, economical and reliable separation processes. The main applications of membranes are in the dairy industry, followed by beverages and egg products. Other fields are emerging: fruit and vegetable juices and concentrates, waste streams and technical fluids like brines and cleaning-in-place solutions.

Membrane processes can be distinguished as dialysis, electrodialysis, gas separation, pervaporation, and filtration processes, depending upon the driving force used to affect species separation. Filtration processes are further classified as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO), depending on membrane pore size, feed cross flow velocity, transmembrane pressure difference applied and permeation flux.

The advantages of membrane processes are multiple. Unlike other mechanical separations units like centrifugation, conventional filtration, screening or sedimentation with coagulants, membrane separation processes involve relatively low energy requirements, no additives, generally mild operating conditions and easy scaling-up rules. Membranes can be specifically tailored to match practically any end user requirement and this allows not only the generic solute to be recovered, but also a

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practically zero total organic carbon effluent to be formed and reused or disposed of without further treatment.

The most important parameters in filtration processing are the membrane itself. To characterize the filtration capability of a given membrane, especially with UF membranes, it is customary to refer to the molecular weight cut-off (MWCO), which represents the molecular weight of a test solute that is rejected by the membrane under standard conditions. The MWCO rating should be regarded just as a rough guide, since it depends on the form and chemical structure of the macromolecules involved. At constant molecular weight, linear molecules will have a greater probability of permeating through the membrane than globular ones; on the contrary, molecules tending to fold will be more easily rejected. Therefore, an ideal membrane would retain all molecules larger than its MWCO and allow all molecules smaller than its MWCO to pass through it, but such an ideal membrane does not exist. In practice, a 10-fold difference in molecular size is needed for complete separation of two compounds. For MF membrane, the filtration capability can also express in terms of mean pore diameter, which ranges from 0.1-1.5 μm.

The terminology used to describe pressure-driven membrane processes was reviewed by Gekas (1988). The main operating variables are: feed flow rate, solute concentration, transmembrane pressure difference, temperature, and pH, as well as

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suspended solid concentration. Any membrane process gives rise to two streams of permeate and retentate (or concentrate), that is the feed fractions passing through or rejected by the membrane.

Ultrafiltration and nanofiltration, well-known membrane separation processes, are the most promising methods for refining and concentrating oligosaccharides. The size-dependent selection of mechanism of the membrane process results in the various concentrations of molecules with different molecular weights. Membrane separations have been used for the preparation of several oligosaccharides such as fructooligosaccharides, chitooligosaccharides, pectic oligosaccharides, soybean oligosaccharides, and maltooligosaccharides. Recently, membrane technologies have been used successfully for processing XOs produced by enzymatic hydrolysis and autohydrolysis.

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Chapter 3 Materials and methods

3.1 Research framework

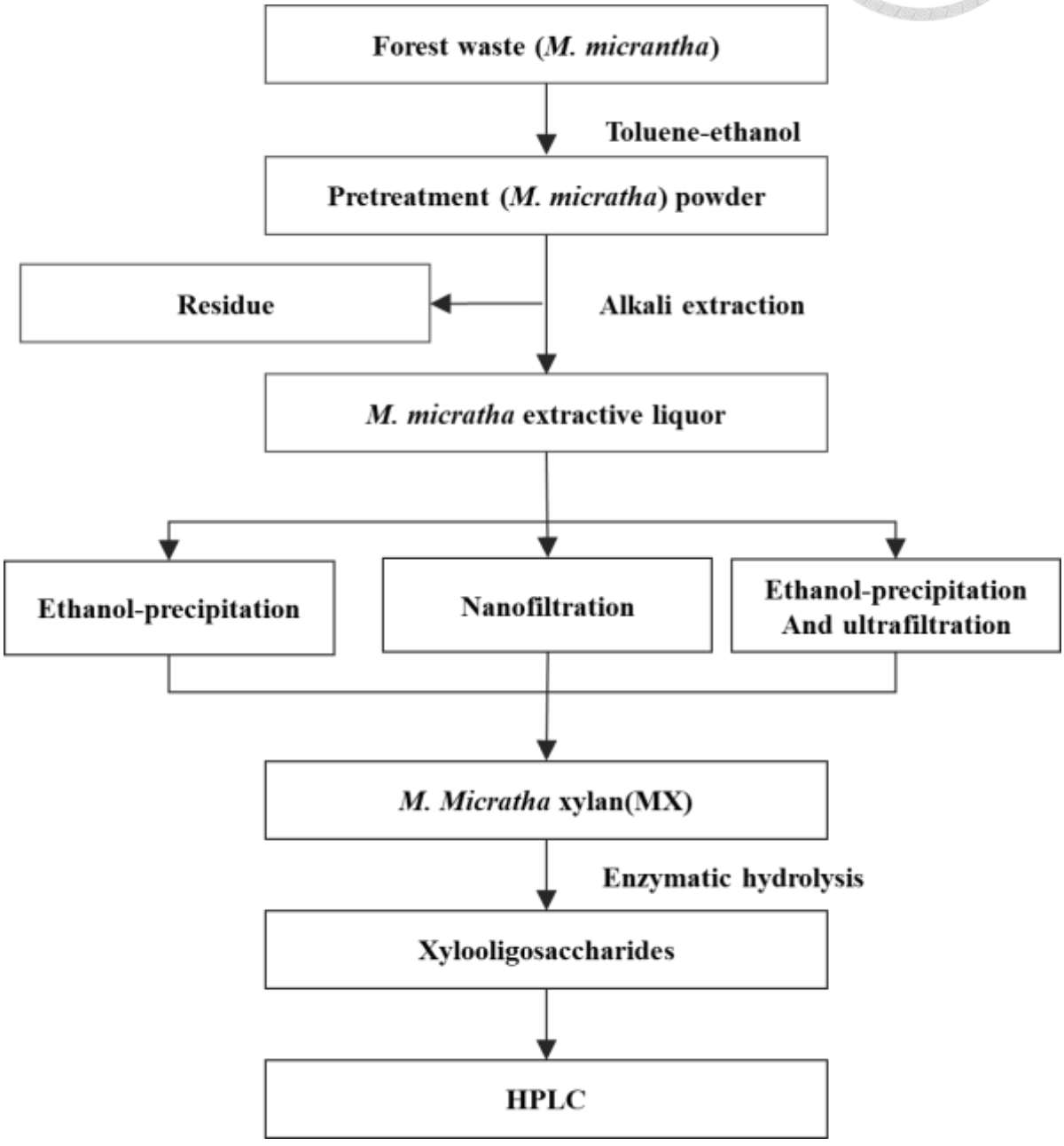

Fig 5. Research framework

doi:10.6342/NTU202001954

3.2 Raw material and analysis

The aboveground samples of *M. micrantha* were collected in January 2009 at Lu-Ku township, Nan-Tou county, central Taiwan. *M. micrantha* samples were collected from a low-elevation site in Lu-Ku, Nantou county, central Taiwan. Samples were air dried, milled to collect portions 40 and 60 mesh screen, and homogenized to a single lot for further experiments. The average composition of materials determined according to standard methodology: moisture content (CNS 452), ash content (CNS 3084), alcohol-toluene solubility (CNS 4713), holocellulose (CNS 3085), pentosan (CNS 7749), Klason lignin (CNS 14907)..

3.3 Pretreatments of *M. mikania* samples

Milled *M. mikania* powders were extracted with a 2:1 (v/v) toluene-ethanol mixture using a soxhlet extractor. Solvent extracted *Mikania* powders (250 g air-dried powder in 2.5 L solution) were further extracted, using an Erlenmeyer flask, with 12% (w/v) NaOH solution at 150 rpm, 60 ^{o}C for 16 h. The extracts were centrifuged for 15 min at 12000 rpm, 4^{o}C in a high-speed refrigerated centrifuge (HITACHI, HIMAC CR21GⅡ). After the treatments, the residue was centrifuged after washing one time with distilled water, air-dried to ensure quantitative non-extracted xylan by pentosan analysis. The supernatants above were mixed and neutralized with HCl to a final pH 7.

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3.4 Extraction of *M. micrantha* xylan (MX) from various treatments

Three different treatments have been used for MX extraction:

a. Ethanol- precipitation (EMX)

The pH of the supernatant was adjusted to 6 by adding drops of 1N HCl. The above mixture was allowed to settle for one day at room temperature with 1.5 times of 95% (w/w) ethanol. Then the precipitate was collected after centrifugation at 12,000 rpm, 4^{o}C for 15 min. Repeat washed the precipitate with 70 and 95% ethanol for twice. The collected precipitate was vacuum-dried at room temperature. The dried solids were defined as EMX and stored in 4^{o}C.

b. Nanofiltration (NMX)

A nanofiltration process was employed to concentrate and to remove the mineral salts in the raw MX solutions. The pH of the supernatant was adjusted to 7 by adding drops of 1N HCl, and then concentrated MX and removed NaCl by nanofiltration. Each experiment filtration run was setup with a pressure gauge before the filtration unit and a back pressure regulator after the filtration unit to fine tune the trans-membrane pressure. Membrane filtration runs were conducted in a batch recirculation mode. Influent reservoir, containing raw MX suspension in a beaker, was constantly mixed with a magnetic stirrer. One filtration run with 10 kD MWCO

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ultrafiltration membrane and four runs with thin-film composite nanofiltration membrane were conducted. Four TFC membrane runs were conducted using same membrane without change. Permeate flux was monitored using a volumetric cylinder. A filtration run was terminated after steady-state fluxes was reached. The concentrated MX liquid was vacuum-dried at room temperature. Final obtained solid was defined as NMX.

c. Ethanol- precipitation and ultrafiltration (EUMX)

This method was combining the above-mentioned ways. The pH of the supernatant was adjusted to 6 by adding drops of 1N HCl. The mixture was allowed to settle out for a day at room temperature with 1.5 times of 95% ethanol. Instead of precipitation and centrifugation steps, the mixture was subjected to staged-ultrafiltration processes to concentrate and to remove NaCl. The set-up and operation principles were the same as described in the previous section. Successive filtration runs with 5, 10, and 30 kD MWCO ultrafiltration membranes were conducted. Membranes with larger pore size were used in later stages to accelerate separation processes. Four consecutive 5 kD membrane runs were conducted using the same membrane without change. The final concentrated MX liquor was vacuum-dried at room temperature. The obtained solid was defined as EUMX.

Extracted NMX and EUMX were used membrane filtration to remove out NaCl

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and low molecular weight saccharides. Na^+ was detected on an Ion Chromatography; total sugar recovery was measured by total sugars assay. The pentosan contents of EMX, NMX and EUMX were determined according to the method provided by CNS 7749.

3.5 Membrane filtration

Fig. 6 was the schematics of a cross-flow filtration module and the filtration cell. A gear pump: Model 75211-10, 50-5000 rpm, 0.07 HP, Cole-Parmer Instrument Company. The way to filtrate effluent is cross-flow filtration and filtration area is 0.0084 m^2, room temperature, pH 6-7, operation pressure is 45 psi. Table 6 was the specification of membranes which has been used in this experiment.

Fig 6. The schematics of a cross-flow filtration module and the filtration cell.

Table 6. The specification of membranes

Material	Operation pH range	Max. Temp. (^oC)	Operation pressure (psi)	Normal MWCO
PES	2-11	50	50-200	5,000 D
PES	2-11	50	80-135	10,000 D
PS	0.5-13	50	30-120	30,000 D
TFC	1-11	50	70-400	> 100 D

* PES: Polyethersulfone
* PS: Polysulfone
* TFC: Thin-Film Composite

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3.6 Xylan Hydrolysis

Hydrolysis of xylan was conducted by mixing 1 mL of 50.0 units/mL of xylanase from *Paenibacillus* sp. isolate BL11 with birchwood xylan, EMX, NMX and EUMX which pentosan contents content were 0.9% in 0.1 M tris-buffer, pH 7, and allowing the incubation to proceed at 60ºC and shake (150 rpm) for 24 h.

In order to optimize enzyme concentration and reaction time on production of XOs, the reaction was performed using the method described above with different enzyme dosage (10, 50, 100 U/mL) and reaction time (in the range of 0-96 h); the substrate concentration was using 1% birchwood xylan (pentosan content: 0.9%) to substitute for three *M. micrantha* xylan (EMX, NMX, EUMX). At 0, 1, 2, 3, 6, 12, 24, 48, 72 and 96 h, a 1 mL sample was taken at regular interval and heated to 100ºC for 5 min to inactivate the enzyme, centrifuged (10 min, 3500 rpm) analyzed by the HPLC method.

3.7 HPLC separation of XOs

XOs were chromatographed on a (Transgnomic) HPLC system equipped with a UV detector (RI-810) and column oven (Col Box). Before injection, samples were filtered through a 0.45 μm filter. Aliquots of filtered sample (20 μl) were injected onto the HPLC system. XOs were eluted using 0.0085 N H_2SO_4 as the mobile phase. The column (300 mm × 7.8 mm) (ICSep ICE-ION-300) was used at 70ºC and a flow rate of 0.4 mL/min. A complete analysis of XOs was carried out in 30 min.

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3.8 Total sugars assay

Phenol, in a 5% (w/v) solution is added to a glass test tube containing a clear sample solution. Concentrated sulfuric acid is added in a rapid stream directly to the surface of the liquid in the test tube. The mixture is thoroughly combined using a vortex mixture and then permitted to stand a sufficient time to allow for color development. The solution absorbance is read at 480 nm using a spectrophotometer, depending on the type of sugar present. Mixing and standing time should be kept the same for all samples to assure reproducible results (Dubois *et al*., 1956).

3.9 DNSA (dinitrosalicylic acid) assay

The simple, robust and highly reproducible method according to König *et al.* was used to estimate the activity of xylanase, *β*-glucanase, and cellulose. The method is based on measurement of reducing sugars released from xylan, *β*-glucan, or carboxymethylcellulose (CMC) by the enzymes digestion. Xylanase activity was measured by the increase in reducing sugars, as given by the dinitrosalicylic acid method using xylose as a standard. One unit of xylanase activity was defined as the amount of enzyme producing 1 mol of reducing equivalents per min under the assay conditions. The effects of temperature and pH on the stability of the recombinant enzymes activity were determined.

Reducing sugar that resulted from the enzymatic reaction was determined by measuring

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the absorbance at 540 nm using xylose as the standard after the reaction mixture cooled down. One unit (IU) is defined as the amount of enzyme that releases 1 μmol of reducing sugar per min. Enzyme was tested for its ability to hydrolyze the variety of substrates, including 1.5% birchwood xylan (Sigma X0502). For the pH and temperature, the enzyme activity was assayed in reaction Buffers: 0.1 M Tris buffer adjust to pH 7, 20 mM $CaCl_2$ (E. Merck TA144482), 0.04% (v/v) Tween 20 (Bio Basic D0560) under temperatures 60^{o}C at 20 min.

3.10 Ion Chromatography

Na^+ was detected on a Ion Chromatography (DKK-TOA, IA-300) (IC) analyzer. Before injection, samples were filtered through a 0.45 μm filter. Aliquots of filtered sample (20 μL) were injected onto the IC system. Na^+ was eluted using 6 mM methanesulfonic acid as the mobile phase. The column (250 mm × 4.6 mm) (PCI-322) was used at 40^{o}C and a flow rate of 0.8 mL/min. A complete analysis of Na^+ was carried out in 18 min.

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Chapter 4 Results

4.1 Chemical compositions of *M. micrantha*

The experimental data, the average value of three replicate analyses, were expressed as weight percent of wood (oven dried basis) in ash, Klason lignin, holecellulose, extractives, and pentosan as 3.08 ± 0.34, 23.54 ± 0.89, 56.04 ± 0.86, 10.54 ± 0.56 and 14.05 ± 0.18%, respectively.

4.2 Extraction of xylan

4.2.1 Pentosan content of *M. micrantha* xylan

250 g air-dried *M. micrantha* powders which were pretreatment by alcohol-toluene (1: 2) had 36.71 g pentosan. After alkaline extraction (12% NaOH) for 16 h at 150 rpm and 60ºC, the solid residues were air-dried to measure quantitative pentosan; there was still 5.11 g pentosan in the residues. The result showed that alkaline extraction could extract 86.08% pentosan yield from *M. micrantha* powder.

In order to refine MX, the crude alkaline extractive divided into three equal segments. Table 7 compared the pentosan content in *M. micrantha* xylan (MX) from different treatments: ethanol-precipitation method (EMX), NF-desalination method (NMX) and combine both methods with slight modification (ethanol-precipitation and UF-desalination) (EUMX). After refining the MX by three methods, the xylan weights (oven-dry) of EMX, NMX and EUMX were 10.51, 54.84 and 47.86 g. The pentosan

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contents of EMX, NMX and EUMX were 47.15 ± 2.36, 9.89 ± 0.40 and 9.68 ± 0.77%, respectively. The result showed the purity of EMX was the highest; NMX was as lower as EUMX, because ethanol-precipitation and centrifuged method could remove almost whole NaCl and other low molecular weight saccharides. The total pentosan weights of EMX, NMX and EUMX were 4.95, 5.42 and 4.63 g; the result showed that concentrated MX by NF without ethanol-precipitation treatment could get the highest pentosan weight. The total pentosan yields of the alkaline crude extractives were 17.81, 19.50 and 16.66%. Extracted MX from various treatments, NF-desalination method was the best way to extracted *M. micrantha* xylan in this study.

Table 7. Pentosan contents in *M. micrantha* xylan prepared from various treatments.

Treatment	Xylan weight (g)	Pentosan content (%)	Pentosan weight (g)	Total pentosan yield (g/100g dry alkaline extractive)
EMX	10.51	47.15 ± 2.36	4.95	17.81
NMX	54.84	9.89 ± 0.40	5.42	19.50
EUMX	47.86	9.68 ± 0.77	4.63	16.66

EMX: Xylan from *M. micrantha* purified by ethanol precipitation and centrifuged.
NMX: Xylan from *M. micrantha* purified by nanofiltration.
EUMX: Xylan from *M. micrantha* purified by ethanol precipitation and ultrafiltration.

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4.2.2 Flux reduction of various pretreatments

There were two kind of membrane used to extract MX with or without ethanol-precipitation. UF was used to concentrate MX, and remove NaCl and ethanol. NF was used to concentrating MX and removing NaCl. The condition to filtrate effluent is cross-flow filtration and filtration area is 0.0084 m^2, room temperature, pH 6, operation pressure is 45 psi.

Fig. 7 showed the batch flux reduction of UF when extracted EUMX. According to Fig. 7A was the flux reduction of 5 kD membranes, the initial flux of 5 kD 1st, 2nd, 3rd, and 4th were 0.55, 0.47, 0.45 and 0.40 mL/min; and the equilibrium flux of 5 kD 1st, 2nd, 3rd, and 4th were 0.06, 0.06, 0.05 and 0.04 mL/min when the time pass by about individual 48 h. The initial flux has been getting slower and slower because the liquor has become more concentrated. (Declines of initial fluxes for the first four 5 kD MWCO runs were observed due to membrane fouling and higher influent total xylose concentrations. The fouling occurred faster after each later run.)

Fig. 7B showed the flux reduction of 10 kD and 30 kD membranes, the initial flux of 10 kD and 30 kD were 0.7 and 1.2 mL/min; and the equilibrium flux of 10 kD and 30 kD were 0.15 and 0.30 mL/min when the time pass by about 8 and 12 h. Steady-state fluxes for the 30 kD filtration run were reached earlier than that of the 10 kD filtration run. The decline of flux was depended on the MWCO of the membrane. Compared Fig 7A and

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7B, the flux of 5 kD membranes decreased smoothly, the flux of 10 kD and 30 kD membranes decreased seriously, and the initial flux was 30 kD > 10 kD > 5 kD.

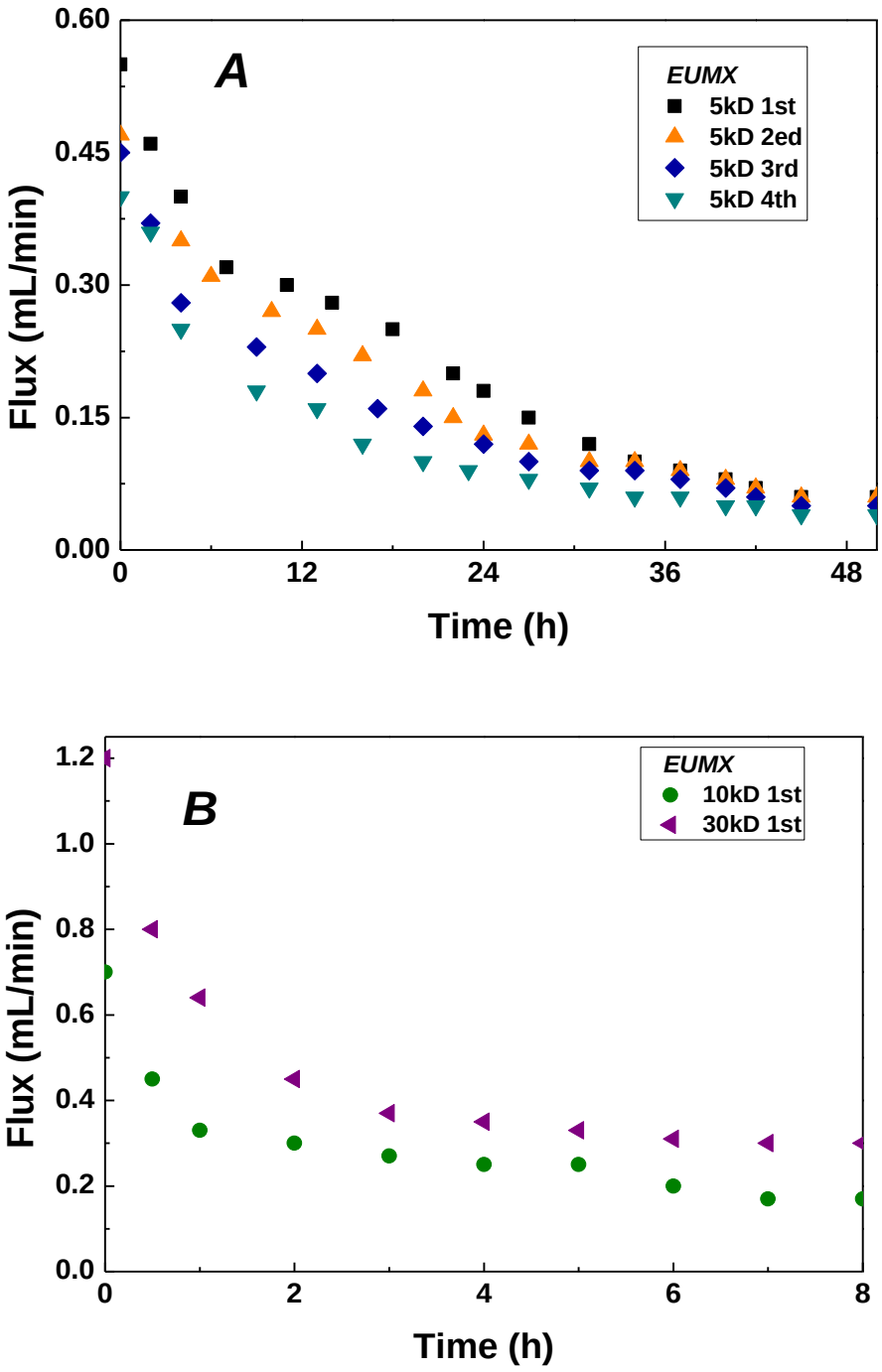

Fig 7. Flux reductions during sequential UF membrane separations.

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Fig. 8 showed the batch flux reduction of NF (TCF) and UF (10 kD) when extracted NMX. The way to filtrate effluent is cross-flow filtration and filtration area is 0.0084 m^2, room temperature, pH 7, operation pressure is 45 psi.

The initial flux of 10 kD was 0.24 mL/min; and the stop flux was 0.07 mL/min (11 h). Initial flux of the 1st, 2nd, 3rd, and 4th TFC runs were 0.15, 0.13, 0.08, and 0.04 mL/min, respectively; and the steady-state fluxes of the 1st, 2nd, 3rd and 4th TFC runs were 0.06, 0.06, 0.03, and 0.01 mL/min, respectively. Fluxes of runs reached steady-state after 36 hours among all four consecutive TFC membrane. Declines of fluxes for TFC membrane runs were observed due to membrane fouling and higher dissolved solid concentrations of influents. The result showed that the flux of 10 kD membrane decreased seriously, the flux of TFC membranes decreased smoothly.

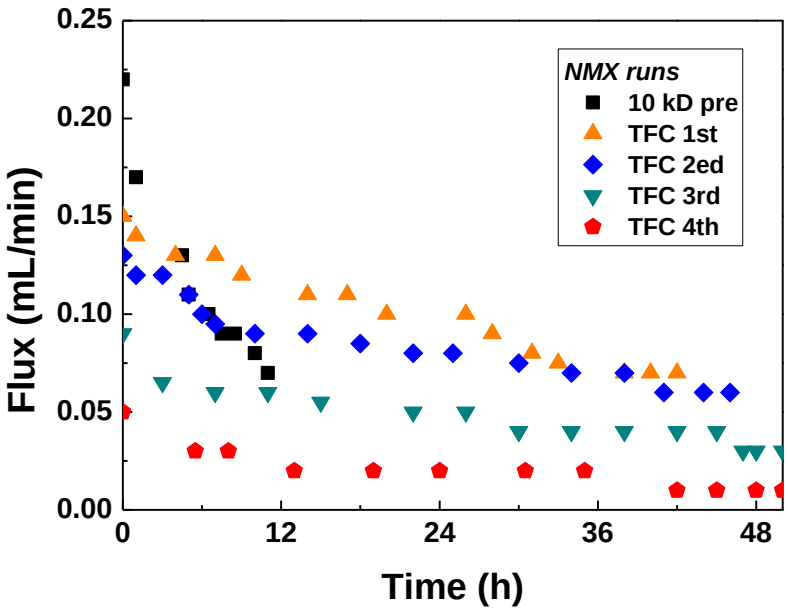

Fig 8. Flux reduction during sequential NF membrane separation.

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4.2.3 Desalination and total sugars recovery from UF and NF

NMX and EUMX were concentrated and removed NaCl by NF and UF. Fig. 9 and 10 showed the desalination of Na^+ and total sugars recovery of NMX and EUMX. Desalination was detected on an Ion Chromatography; total sugar recovery was measured by total sugars assay.

The mass balance of desalination (upper panel) and total sugars recovery (lower panel) after sequential UF membrane separation runs are shown in the upper and lower panels of Fig. 9.

The original retentate volume of EUMX liquor was 3000 mL, after batch UF processing by 5 kD 1st, 2nd, 3rd, 4th, 10 kD 1st and 30 kD 1st for 50, 50, 50, 50, 15 and 8h, the permeate volumes were 514, 425, 419, 350, 180 and 183 mL. The total permeate volume was about 2071 mL for 223 h. The original retentate Na^+ was 43.50 g, after batch UF processing by 5 kD 1st, 2nd, 3rd, 4th, 10 kD 1st and 30 kD 1st for 50, 50, 50, 50, 15and 8 h, the permeate Na^+ were 7.12, 5.66, 5.66, 4.87, 2.51, and 2.55 g. The above values corresponded to 16.3, 15.5, 18.4, 19.4, 12.4, and 14.3% for sodium ions removals for each stage. Total desalination yield was about 65.2% corresponded well with volume reduction of 69%.

According to the original total sugar content of Fig. 9 was 11.4 g, after batch UF processing by 5 kD 1st, 2nd, 3rd, 4th, 10 kD 1st and 30 kD 1st for 50, 50, 50, 50, 15

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and 8 h, 0.14, 0.11, 0.11, 0.12, 0.16, and 0.35 g of total sugars were lost after each stage of separation. 91.2% of total sugars was retained in the system.

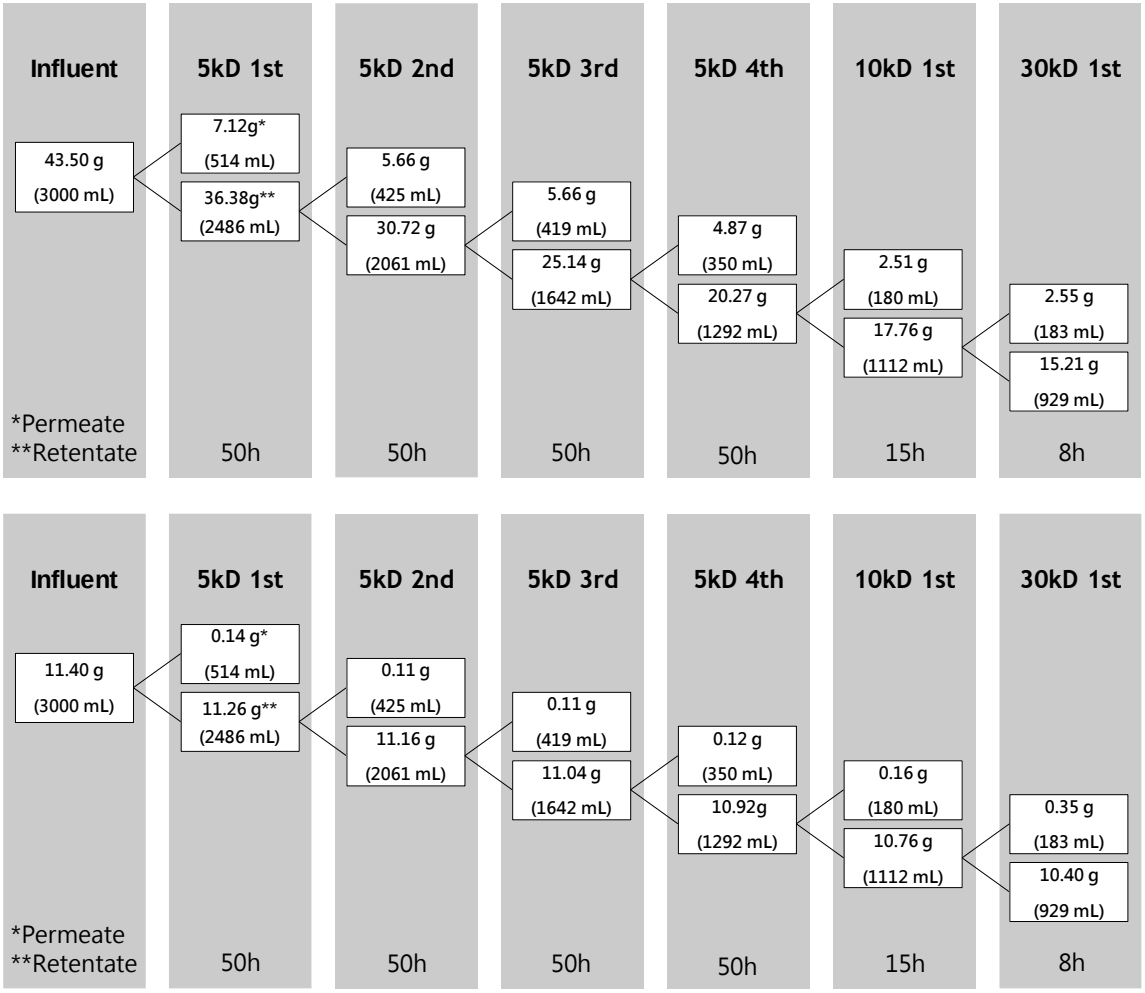

Fig 9. Mass balance of desalination (upper panel) and total sugars recovery (lower panel) by UF membrane after extracted xylan from *M. micrantha*.

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Mass balance of desalination (upper panel) and total sugars recovery (lower panel) after sequential 10 kD and four TFC filtration runs are presented in the upper and lower panels of Fig. 10.

The original retentate volume of NMX liquor was 1120.0 mL, as shown in Fig. 10, after batch UF and NF processing by 10 kD 1st, TFC NF 1st, 2nd, 3rd and 4th for 11, 42, 46, 119 and 124 h, the permeate volumes were 80.7, 251.5, 224.4, 240.0 and 92.7 mL. Final retentate volume was 230.7 mL, while 899.3 mL of liquor was rejected by the NMX process for 342 h. The original retentate Na^+ was 42.26 g, after batch filtration processing by 10 kD 1st, TFC NF 1st, 2nd, 3rd and 4th for 11, 42, 46, 119 and 124 h, the permeate Na^+ were 3.00, 9.25, 8.74, 9.36, and 3.52 g. Total permeate Na^+ was 33.85 g, Desalination yield was 73.18% corresponded well with volume reduction of 79.4%.

Xylose is soluble in water, but XOs and xylan are insoluble. Hence, different removal patterns for total xylose from sodium ions can be expected. According to the original total sugar content of Fig. 9 was 12.3g, after batch filtration processing by 10 kD 1st, TFC NF 1st, 2nd, 3rd and 4th for 11, 42, 46, 119 and 124 h, 0.17, 0.02, 0.03,0.07 and 0.05 g of total sugars were lost after each stage of separation.

Larger pore opening of 10 kD MWCO UF membrane did contribute to more xylose-containing substances passing through UF membrane during a shorter period,

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11 hours. Because 10 kD is larger than xylose and XO's with DP 2 to 6. Total sugars recoveries were 98.62, 98.48, 98.26, 97.70, and 97.26% for each stage of separation, while the losses were also in proportion to filtration times. Low mass loss for total sugars suggested there was only few xylose monomers in this system.

These results demonstrated that TFC is very efficient for retaining total xylose for every stage of the NMX process. Concentrations of total sugars were raised from 10.98 to 51.85 mg/mL after NMX process, while 73.18% of sodium ions was removed.

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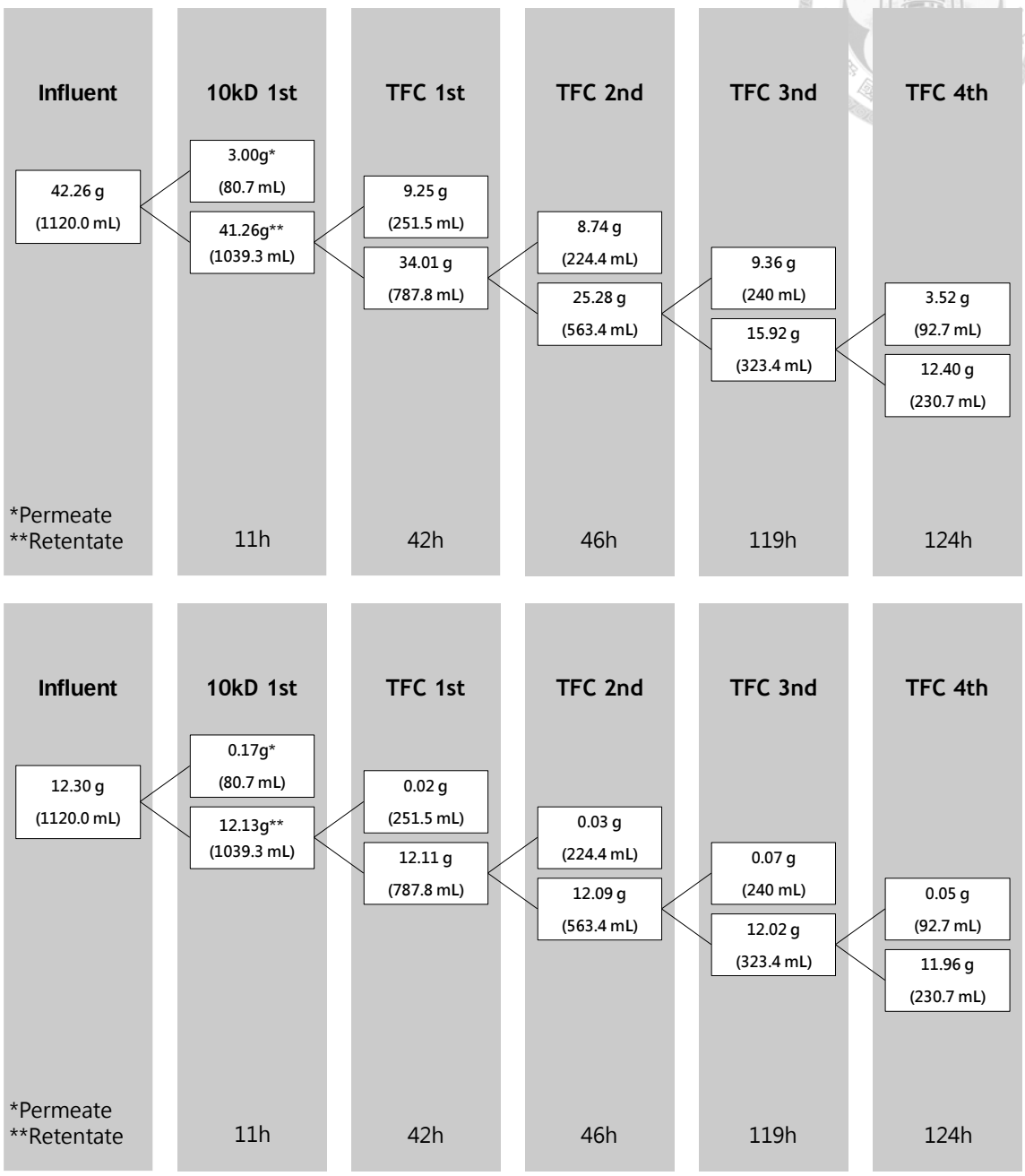

Fig 10. Mass balance of desalination (upper panel) and total sugars recovery (lower panel) by 10 kD UF and TFC NF membrane after extracted xylan from *M. micrantha*.

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4.3 Hydrolysis 1% birchwood xylan (pentosan content: 0.9%)

4.3.1 Effect of xylanase dosage on hydrolysis of 1% birchwood xylan

Different dosage xylanase of heated xylanase (10, 50 and 100 U/mL) was adding in the mixture. The reaction mixture of 1% birchwood xylan, which pentosan content was 0.9% in 0.1 M tris-buffer, pH 7, and allowing the incubation to proceed at 60ºC and shake (150 rpm) for 0-96 h. Samples were withdrawn periodically, centrifuged at 13,000 rpm for 5 min, and analyzed by HPLC to measure the extent of hydrolysis.

Fig. 11 represented the extent of hydrolysis of effect of birchwood xylan for various xylanase dosages (10, 50 and 100 U/mL). As the xylanase dosage was increased, both the XOs yield and rate also increased. There was no significant effect of enzyme dosage 50 and 100 U/mL. As observed from hydrolysis progress of 1% birchwood xylan was fast up to 6 h period and stopped increasing after 24 h. A suitable enzyme concentration should be selected depending on economic analysis of the process.

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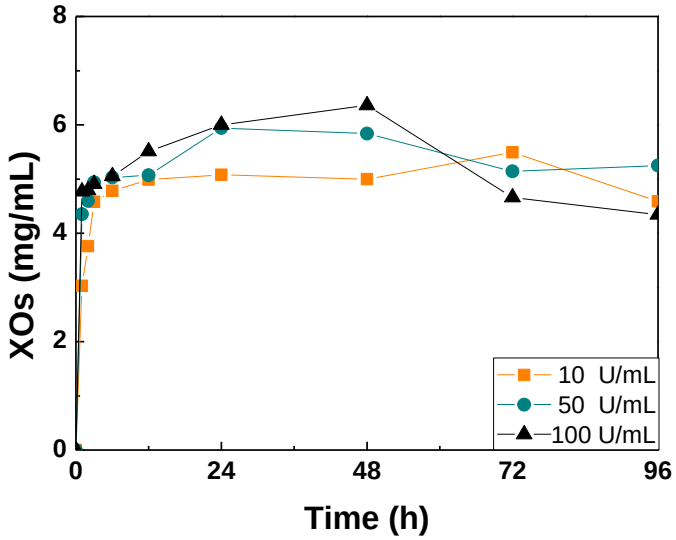

Fig 11. Impact of different xylanase dosage on hydrolysis of 1% birchwood xylan.

4.3.2 Time course for production of XOs from hydrolysis of 1% birchwood xylan

The reaction time would affect the composition of hydrolysate. Generally, the DP of XOs becomes smaller when the reaction time was longer. In Fig 12, the reaction mixtures consisted of 1% Birchwood xylan (petosan content: 0.9%), pH 7, and 10, 50 and 100 U/mL of heat-treated xylanase. The reaction conditions were as follows: reaction time was 96 h; shaking speed, 150 rpm; reaction temperature, 60°C. The XOs yield was defined as the sum of X2, X3, X4, X5, and X6 produced by hydrolysis divided by total xylose contents of starting material on a dried weight basis.

Fig. 12 showed the time course of the composition of XOs and XOs yield. In the upper panel of Fig. 12, X6, X5, X3, and X2 were increased fast up to 6 h, and then X6

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and X3 were decreased by the reaction time; X5 and X2 were increased smoothly until 48 h and increased seriously at 72 h. X4 was increased fast up to 3 h and then totally decreased pass through the reaction time. The longer hydrolysis times, the more xylose produced. When finished the hydrolysis for 96 h, the major XOs were X5 and X2, the XOs yield was about 46%. But the XOs yield was stopped increasing after 24 h, the major XOs were X6, X5, and X2, the XOs yield was about 51%.

The mid panel of Fig. 12 was as same as the upper one. X6, X5, X3 and X2 were increased fast up to 6 h, and then X6 and X3 were decreased by the reaction time; X5 and X2 were still increased pass through the times. X4 was decreased faster than the upper one. When finished the hydrolysis for 96 h, the major XOs were X5 and X2, the XOs yield was 53%. But the XOs yield was stopped increasing after 24 h, the major XOs were X5, X3 and X2, the XOs yield was about 59%.

In the lower panel of Fig. 12, X6 was increased fast up to 6 h and then decreased slowly pass through the reaction time. X3 increased up to 12 h and then decreased slowly pass through the reaction time. X5 and X2 were increased up to 48 h, and then decreased. X4 was decreased very fast. When finished the hydrolysis for 96 h, the major XOs were X5 and X3, the XOs yield was 43%. But the XOs yield was stopped increasing after 48 h, the major XOs were X6, X3, and X2, the XOs yield was about 64%.

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In Fig 12, when xylanase dosage was 10, 50 and 100 U/mL, xylose was production after hydrolysis 24 h, 12 h and 6 h. The result showed that as the xylanase dosage was increased, xylose was production earlier; and xylose was increased by the reaction time.

A suitable reaction time should be select depending on economic analysis and XOs yield of the process. The result showed the suitable reaction time of hydrolysis of 1% birchwood xylan with 10, 50 and 100 U/mL xylanase was 24 h. The XOs yields were 51, 59 and 60 %. The best condition of hydrolyzed 1% birchwood xylan was mixing 1 mL of 50 U/mL of xylanase in 0.1 M tris-buffer, pH 7, and allowing the incubation to proceed at 60^{o}C and shake (150 rpm) for 24 h.

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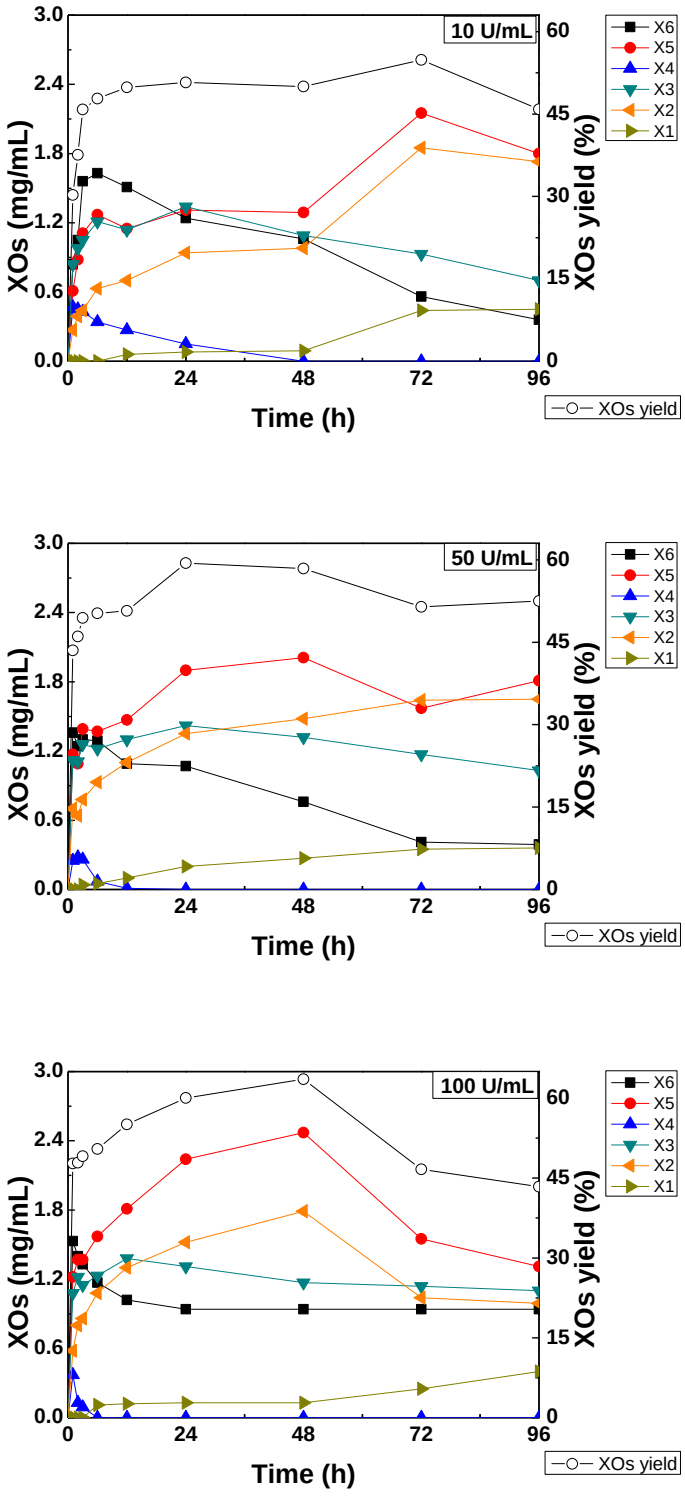

Fig 12. Production of XOs from hydrolysis of Birchwood xylan (1%)

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4.4 Hydrolysis various MXs (pentosan content: 0.9%)

In order to produce XO in its final form, dried EMX, NMX, and EUMX solids were subjected to hydrolysis by Paenibacillus xylanase at the dosages of 10, 50, and 100 U/mL. Total xylose contents were set to 0.9 % (w/v) in all reaction mixtures. HPLC was employed to analyze hydrolysis products. Concentrations and yields of xylooligosaccharides. (XO) during hydrolysis of EMX, NMX, and EUMX solids are shown in Fig. 13-15. The XOs yield was defined as the sum of X2, X3, X4, X5, and X6 produced by hydrolysis divided by total xylose contents of starting material on a dried weight basis.

4.4.1 Effect of xylanase dosage on hydrolysis of various MXs

In order to realize the hydrolysis activity and xylanase dosage of three different treatments MXs, the reaction mixture of MX, which pentosan content was 0.9% (like the situation of birchwood xylan) in 0.1 M tris-buffer, pH 7, and allowing the incubation to proceed at 60ºC and shake (150 rpm) for 0-96 h. Different dosage xylanase of heated xylanase (10, 50 and 100 U/mL) was adding in the mixture. Samples were withdrawn periodically, centrifuged at 13,000 rpm for 5 min, and analyzed by HPLC to measure the extent of hydrolysis.

A suitable enzyme concentration should be selected depending on economic analysis of the process. The effects of xylanase dosage (10, 50 and 100 U/mL) on

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different MXs were given in Fig. 13. In the upper panel of Fig. 13, EMX significantly higher amount of XOs when add 50 and 100 U/mL xylanase. As the xylanase dosage was increased, the XOs yield also increased. There was no significant effect of enzyme dosage 50 and 100 U/mL, so the suitable xylanase dosage was 50 U/mL. In the mid panel of Fig 13, adding 50 U/mL xylanase could get the suitable hydrolysis efficiency. In the lower panel of Fig. 13, adding 100 U/mL xylanase could get the suitable hydrolysis efficiency.

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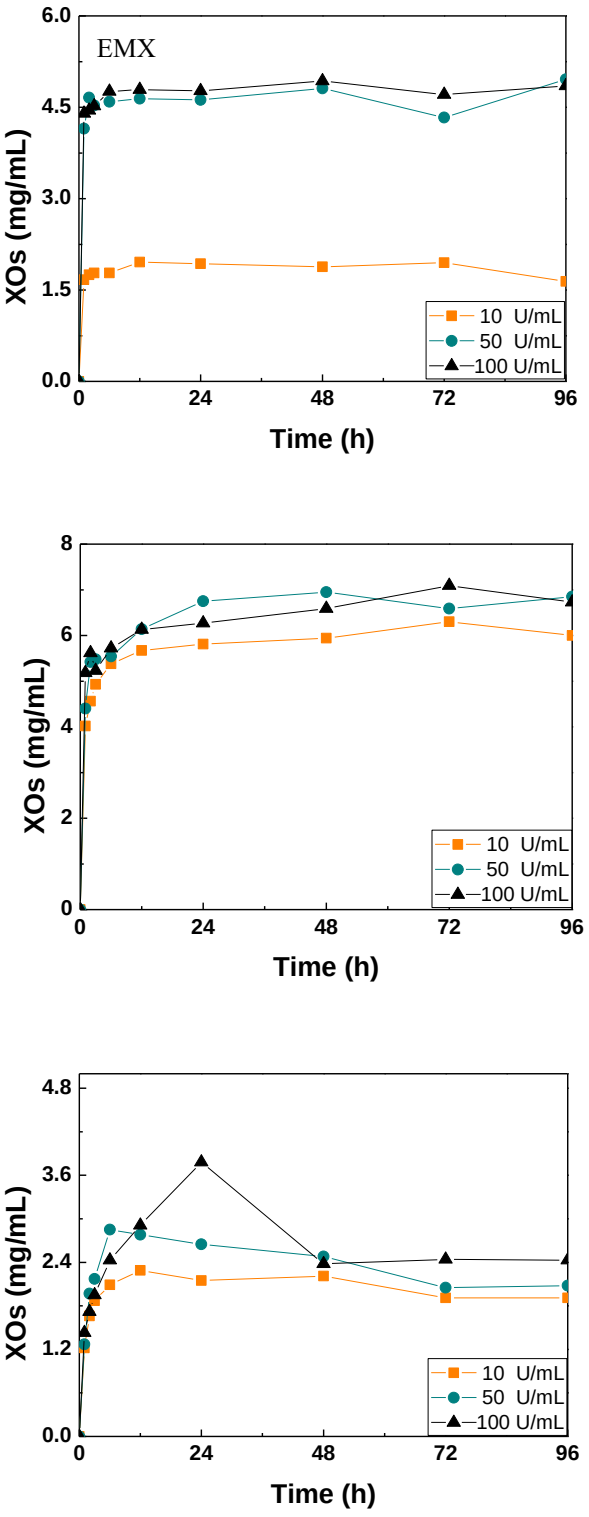

Fig 13. Impact of different xylanase dosage on hydrolysis of various MXs.

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4.4.2 Time course for production of XOs from hydrolysis of various MXs

In order to realize the hydrolysis activity and xylanase dosage of three different treatments MXs, the reaction mixture of MXs which pentosan content were 0.9% (like the situation of birchwood xylan) in 0.1 M tris-buffer, pH 7, and allowing the incubation to proceed at 60^{o}C and shake (150 rpm) for 0-96 h. Different xylanase dosage of heated xylanase (10, 50 and 100 U/mL) was adding in the mixture. Samples were withdrawn periodically, centrifuged at 13,000 rpm for 5 min, and analyzed by HPLC to measure the extent of hydrolysis.

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Fig. 14 illustrated the time course for production of XOs of EMX; xylanase dosage was 10, 50 and 100 U/mL. In the upper panel of Fig 14, the concentrations of XOs were rapidly increased during hydrolysis by 10 U/mL Paenibacillus xylanase in the first 3 h and then remained unchanged. The optimal XOs yield was 19%, occurring at the 12th h. Major XOs were X6, X3, and X4, in the order of quantity. X6 were hydrolyzed after the 12th h, as shown by its reduced concentration. There was very little xylose (X1) present in this hydrolysis.

The concentrations of XOs during hydrolysis by 50 U /mL are shown in the mid panel of Fig. 14. Major resulting XOs were X5, X3, and X2, in the order of quantity. The concentrations of XOs were rapidly increased during xylanase hydrolysis in the first 3 h and then they remained unchanged. The optimal XOs yield reached about 50% at the 3rd h. X5 and X3 were partially hydrolyzed during the 3rd to 12th h, while the increase of X2 concentration was observed. There was very little xylose (X1) present in this hydrolysis. The XOs yield did not increase after the 3rd to 24th h, and the XOs yield was 46% at the 24th h. The major XOs remained to be X5, X3, and X2 at the later stage of hydrolysis. The fluctuating of the XO yields after the 3rd h might due to the simultaneous degradation of XO and generation of XO from EMX mixture.

The concentrations of XOs during hydrolysis by 100 U /mL are shown by the lower panel of Fig. 14. Major resulting XOs were X5, X3, X2, and X6, in the order of

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quantity. The concentrations of XOs were rapidly increased during xylanase hydrolysis in the first 6 h and then remained unchanged. The optimal XOs yield reached about 49% at the 6th h, then X5, X3, and X6 were partially hydrolyzed. And the increase of X2 concentration was observed. There was more xylose (X1) observed in this hydrolysis then in those with 10 and 50 U/mL. The XOs yield did not increase after the 72nd h, the XOs yield was about 46% at the 24th h. XO yields from enzymatic hydrolysis showed EMX solid was very sensitive to the increase from 10 to 50 U/mL of xylanase dosage applied. But the increase of xylanase dosage from 50 to 100 U/mL in reaction mixture did not further increase XO yields.

A suitable xylanase dosage should be selected depending on economic analysis of the process. The best condition of the hydrolysis of EMX was conducted by mixing 1 mL of 50 U/mL of xylanase in 0.1 M tris-buffer, pH 7, and allowing the incubation to proceed at 60ºC and shake (150 rpm) for 24 h.

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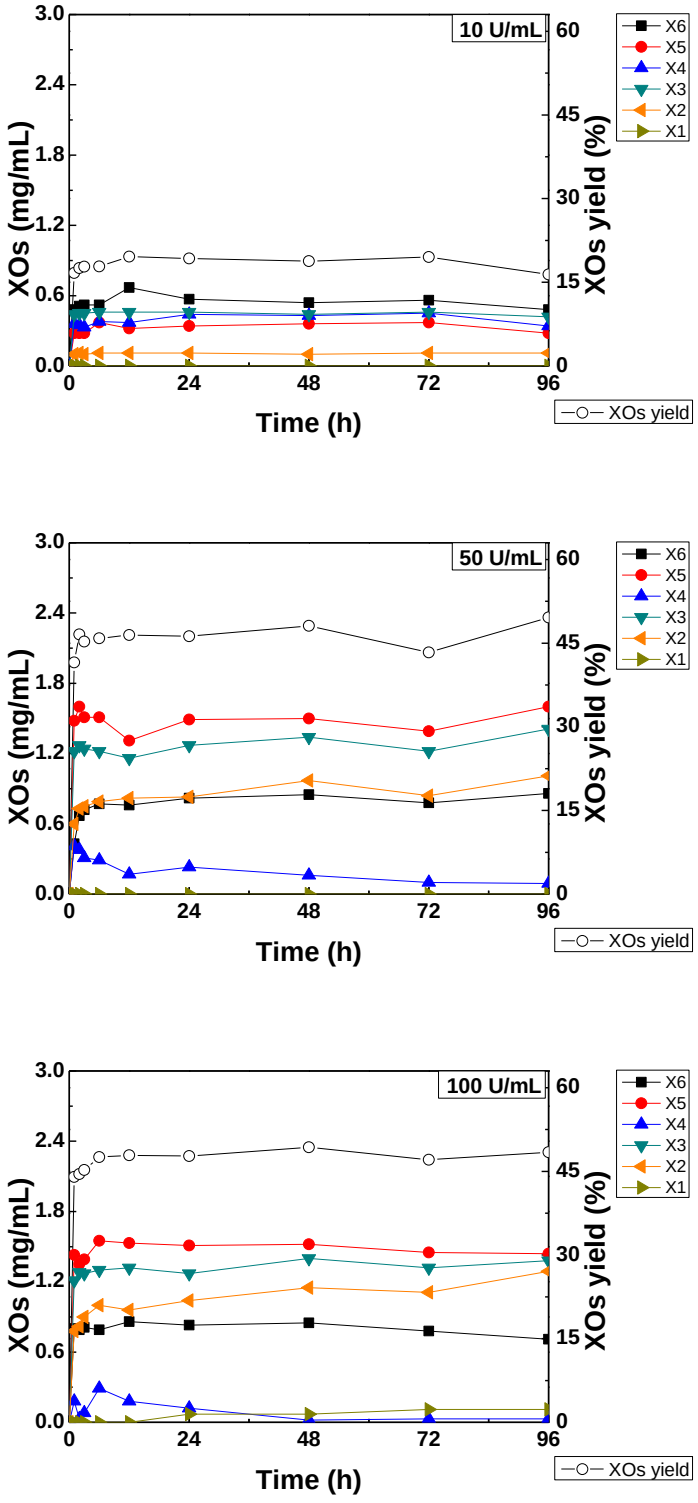

Fig 14. Production of XOs from hydrolysis of EMX

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Fig. 15 illustrated the time course for production of XOs of NMX; xylanase dosage was 10, 50 and 100 U/mL. In the upper panel of Fig 15, the concentrations of XOs were increased during hydrolysis by 10 U/mL Paenibacillus xylanase in the first 12 h, reached to 59%, and then slowly increased. The optimal XOs yield was 63%, which occurred at the 72th h. Major XOs were X6, X5, X2, and X3, in the order of quantity. X6 were slightly hydrolyzed after the 12th h, as shown by its reduced concentration. There was very little xylose (X1) present in this hydrolysis.

The concentrations of XOs during hydrolysis of NMX by 10 U/mL are shown by the mid panel of Fig. 5. Major resulting XOs were X5, X6, and X2, in the order of quantity. The concentrations of XOs rapidly increased during xylanase hydrolysis in the first 3 h and still increased up to the 48th h. The optimal XOs yield reached about 69% at the 48th h, but it already reached about 68% at the 24th h. X6 were partially hydrolyzed during the 12th to 24th h, but the simultaneous increase of X5 concentration was observed. There was around 0.3 mg/mL xylose (X1) present in this hydrolysis. The XOs yield did not increase after the 48th h, due to the hydrolysis of X5. The major XOs remaining were X5, X6, and X2 at the later stage of hydrolysis.

The concentrations of XOs during hydrolysis by 100 U /mL are shown by the lower panel of Fig. 15. Major resulting XOs in the order of quantity were X5, X6, X2, and X3 before the 12th to 24th h, and X5, X2, X6, and X3 after the 24th h. The

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concentrations of XOs were rapidly increased during xylanase hydrolysis in the first 6 h and then remained unchanged. The XO yield reached about 63% at the 24th h. The optimal XO yield reached about 67% at the 72nd h, but then X6 were partially hydrolyzed. And the increase of X2 concentration was observed. Since X6 is regarded as a more valuable product, the 24th h was indeed a better reaction time for a better refining process. Lower reaction times would require a smaller reactor, and implicates improvement the process economics.

XO yields from enzymatic hydrolysis showed NMX solid was already significant at 10 U/mL of xylanase dosage applied. NMX solid was easier to be hydrolyzed than EMX solid. Although both NMX and EMX solids were thoroughly dried prior to

hydrolysis, traced ethanol left might hinder enzymatic hydrolysis. The raising of xylanase dosage to 50 U/mL in reaction mixture did increase some XO yields. But the increase to 100 U/mL did not increase much of XOs yield.

A suitable xylanase dosage should be selected depending on economic analysis of the process. The best condition of the hydrolysis of NMX was conducted by mixing 1 mL of 50 U/mL of xylanase in 0.1 M tris-buffer, pH 7, and allowing the incubation to proceed at 60^{o}C and shake (150 rpm) for 24 h.

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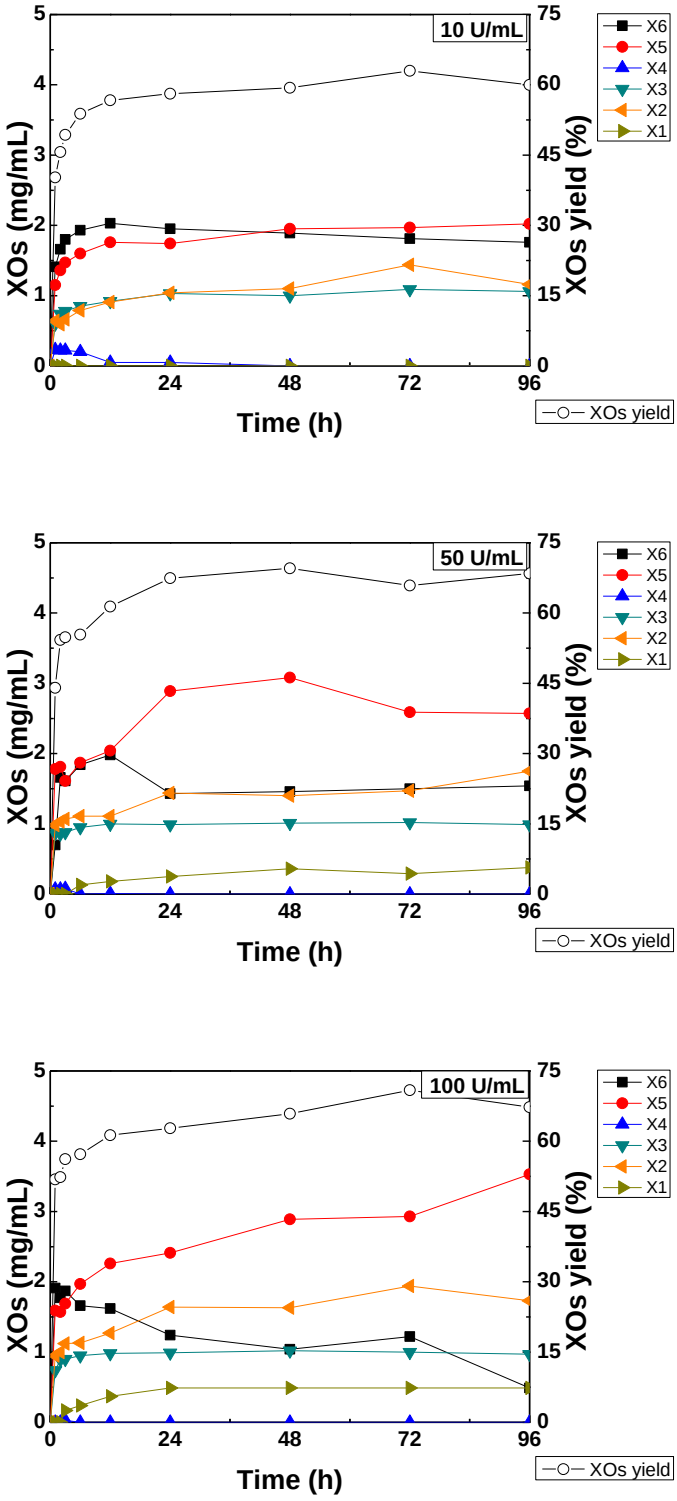

Fig 15. Production of XOs from hydrolysis of NMX

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Fig. 16 illustrated the time course for production of XOs of EUMX; xylanase dosage was 10, 50 and 100 U/mL. In the upper panel of Fig. 16, the concentrations of XOs were increased during hydrolysis by 10 U/mL Paenibacillus xylanase in the first 12 h, reached to 23%, and then slowly increased. Major XOs were X6, X5, X2, and X3, in the order of quantity. X6 were heavily hydrolyzed after at the 12th h, as shown by its reduced concentration. There was some X1 present in this hydrolysis.

The concentrations of XOs during hydrolysis of EUMX by 50 U/mL are shown by the mid panel of Fig. 5. Major resulting XOs were X6, X5, and X2, in the order of quantity. The XOs yield reached about 29% at the 6^{th} h, and gradually reduced to 21% at the 48^{th} h. The concentrations of XOs rapidly increased during xylanase hydrolysis in the first 6 h, but the hydrolysis of X6 and X5 after the 24^{th} h contributed to decline of the XO yield. At the later stage of hydrolysis, X2 and X6 were the major products of hydrolysis.

The concentrations of XOs during hydrolysis of EUMX by 100 U/mL are shown by the lower panel of Fig. 6. Major resulting XOs in the order of quantity were X6, X5, X2, and X3 before the 24^{th} h. The XOs yield reached about 37% at the 24^{th} h, but rapidly declined to 24% at the 48^{th} h. The hydrolysis of X6 and X5 also occurred after the 24^{th} h and contributed to decline of the XO yield.

A suitable xylanase dosage should be selected depending on economic analysis of

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the process. The best condition of the hydrolysis of EUMX was conducted by mixing 1 mL of 100 U/mL of xylanase in 0.1 M tris-buffer, pH 7, and allowing the incubation to proceed at 60^{o}C and shake (150 rpm) for 24 h.

Some increase of XO yields for enzymatic hydrolysis of EUMX solid was observed by higher xylanase dosage applied at 50 and 100 U/mL, especially at the first 24 hr. Composition of XOs for EUMX hydrolysis was different from those of EMX and NMX. X6 was the most abundant XO present during EUMX hydrolysis. XO yields of the EUMX process were just slightly higher than those of EMX at 10 U/mL. And XO yields of EUMX process was even lower than those of EMX at 50 and 100 U/mL. Since all MWCO pore size opening of UF membrane used in the EUMX were larger than molecular weights of all XO and low-molecular xylans, the low-molecular xylan loss during UF separation might lead XO yields lower than ones of EMX process. Enzymatic hydrolysis of EUMX might be also be affected by traces of ethanol present as well as that on EMX. Hence XO yields of EUMX hydrolysis under higher dosages were lower than ones of EMX hydrolysis. Hence, overall efficiencies for EUMX hydrolysis were inferior to ones of NMX and EMX.

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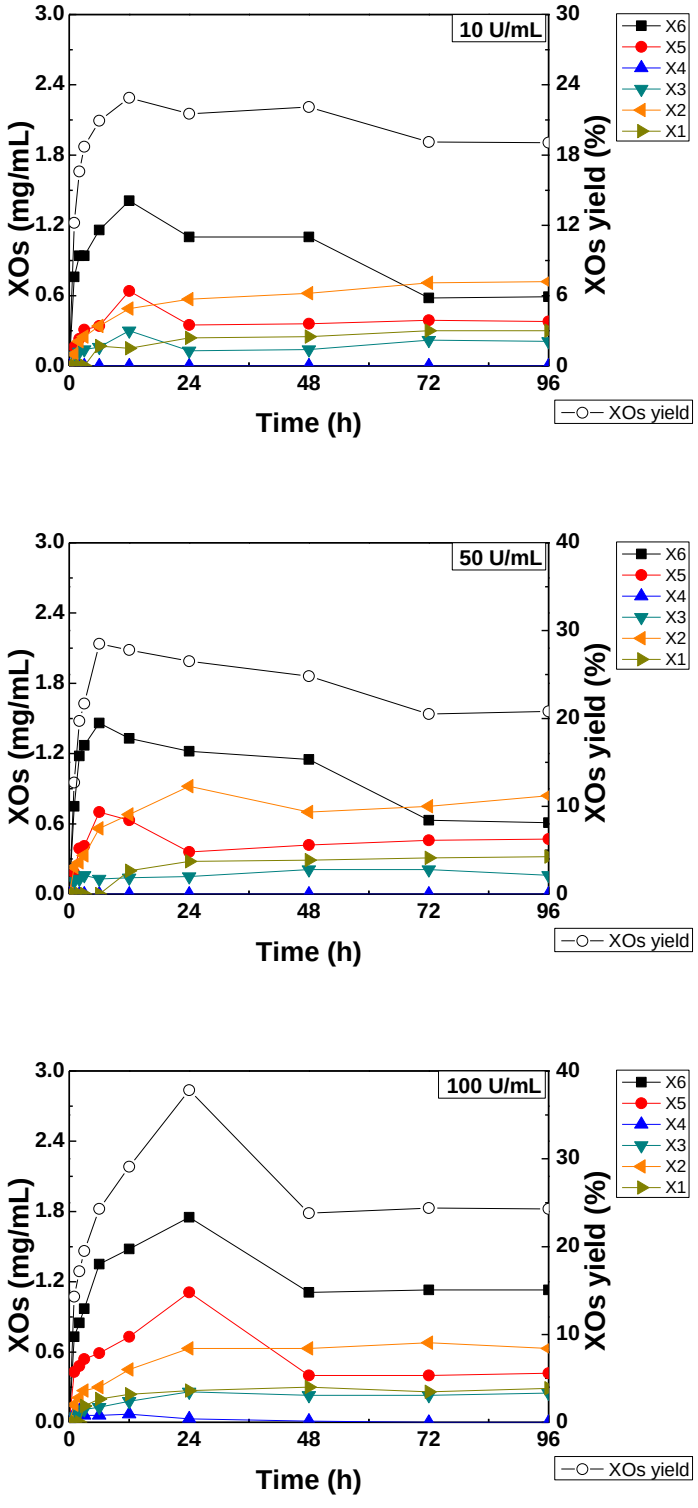

Fig 16. Production of XOs from hydrolysis of EUMX

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Chapter 5 Conclusions

The nature pentosan content in *M. micrantha* was about 14%; about 86% pentosan was extracted after treatment of alkaline extraction for 16 h at 150 rpm and 60ºC. Alkaline extraction was efficiency to extract xylan. In this study, at the same condition, ethanol-precipitation method could produce the more purity MX; nanofiltration could collect more pentosan.

Compare the hydrolysis efficiency of various treatments *M. micrantha* xylan (EMX, NMX and EUMX) with commercial birchwood xylan (BX) (pentosan content: 0.9%) by adding 10, 50 and 100 U/mL xylanase at 60ºC and shake (150 rpm) for 0-96 h. The result showed that the best hydrolysis period of BX, EMX, NMX and EUMX were at 24 h, xylanase dosages were 50, 50, 50 and 100 U/mL, and the yields of XOs were about 59, 46, 68 and 37%. Generally, ethanol – precipitation was the common method to produce xylan. The hydrolysis efficiency of four xylans were NMX ＞ BX ＞ EMX ＞ EUMX. The major products of NMX were X6, X5 and X2; the yields were 43, 21 and 21%.

Concentrated xylan by nanofiltration without ethanol-precipitation was a suitable way to produce MX. And the hydrolysis efficiency was better than commercial birchwood xylan.

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Chapter 6 Abbreviation

MX	*M. micrantha* xylan.
BX	Birchwood xylan.
EMX	Xylan from *M. micrantha* purified by ethanol precipitation and centrifuged.
NMX	Xylan from *M. micrantha* purified by nanofiltration.
EUMX	Xylan from *M. micrantha* purified by ethanol precipitation and ultrafiltration.
MF	Microfiltration
UF	Ultrafiltration
NF	Nanofiltration

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