Production of Xylooligosaccharides from Forest Waste by Membrane Separation and *Paenibacillus* Xylanase Hydrolysis

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Xylooligosaccharides (XO), derived from the alkaline (NaOH) extractant of *Mikania micrantha*, were produced using multiple staged membrane separation and enzymatic xylanolysis. Staged nanofiltration (NMX), ultrafiltration (EUMX), and centrifugation (EMX) processes for the ethanol precipitates were conducted. NMX recovered 97.26% of total xylose and removed 73.18% of sodium ions. Concentrations of total xylose were raised from 10.98 to 51.85 mg/mL by the NMX process. Recovered xylan-containing solids were hydrolyzed by the recombinant *Paenibacillus* xylanase. 68% XO conversions from total xylose of NMX was achieved in 24 hours. Xylopentaose (DP 5) was the major product from NMX and EMX hydrolysis. Xylohexaose (DP 6) was the major product from EUMX hydrolysis. Results of the present study suggest the applicability for XO production by nanofiltration, as NMX gave higher XO yields compared to those from a conventional ethanol-related lignocellulosic waste conversion process.

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INTRODUCTION

Mikania micrantha has been well recognized as one of the most rampant invasive species worldwide (Lowe *et al.* 2001). It has been widespread throughout the Asia-Pacific regions, especially in southeast China since 1980 (Zhang *et al.* 2004). It was introduced into Taiwan in the 1990s to provide an additional plantation cover for barren landslide sites (Chiang *et al.* 2002). However, it has spread uncontrollably in forests in a range below 1000 m of elevation (Kuo 2003). Since it causes extensive stress on local terrestrial ecosystems by strangling trees, this invasive species has raised immense public attention and forced the government to seek control. The most efficient measure to control *Mikania micrantha* has been periodic cutting (Kuo *et al.* 2002). Because of its fast-growing and widespread character, *Mikania micrantha* has good potential to serve as a source of biomass. Since significant xylans are present in dicot biomass (Saha 2003), it is reasonable to expect that *Mikania* biomass could be applied for the mass production of xylooligosaccharides (XO).

XO's, defined as xylooligomers with degrees of polymerization at 2 to 6, are produced during the hydrolysis of xylan. The xylans are complex heteropolysaccharides consisting of a backbone chain of 1,4-β-D-xylopyranose units with a variety of side linkages (Sjöström 1993). XOs, categorized as non-digestible oligosaccharides (NDOs), possess unique properties such as reducing cholesterol, maintaining gastrointestinal health, and improving the biological availability of calcium (Voragen 1998; Mussatto and Mancilha 2007). Hence, XOs are claimed to behave as prebiotics. XOs could be produced at industrial scale from xylan-rich materials such as forest or agricultural wastes. Reutilizing of such waste as raw material can offer additional environmental, economic, and ecological benefits (Alonso *et al.* 2003).

Xylan-rich lignocellulosic materials are generally employed to produce XOs by using chemical methods (Moure *et al.* 2006; Teng *et al.* 2010). Steam and diluted acids have been employed to pretreat biomass and to produce XOs. However, extraction of xylan with steam and acid could hydrolyze the hemicellulose and their side chains (Yang *et al.* 2005) and generate large amounts of furfurals (Garrote *et al.* 2002; Kabel *et al.* 2002). The processes mentioned above also require steam generators and equipment capable of enduring high-temperature operations.

Several processes, such as vacuum evaporation, solvent extraction, or chromatographic methods, have been employed to refine XO mixtures from other carbohydrates (Alonso *et al.* 2003). However, these methods are either time- or energy-consuming for the scale-up production of food-grade xylooligosaccharides. On the other hand, direct and sole enzymatic treatment have failed to significantly produce XOs from larger varieties of plant biomass (Alonso *et al.* 2003).

Alkaline agents such as KOH or NaOH have been used to extract xylan from raw lignocellulosic materials, and then the extracted xylan was converted to XOs by xylanase (Akpinar *et al.* 2007; Teng *et al.* 2010). The above process has been employed to produce XOs from different genres of biomass, including corn cob (Pellerin *et al.* 1991), oil-palm residue (Sabiha-Hanim *et al.* 2011), wheat straw (Zilliox and Debeire 1998), and hardwood (Freixo *et al.* 2002; Nishimura *et al.* 1998; Yang *et al.* 2011).

Membrane separations, such as ultrafiltration and nanofiltration, has been shown very promising methods for refining and concentrating several oligosaccharides (Akpinar *et al.* 2007). Membrane technologies have been applied successfully in conjunction with enzymatic hydrolysis following steam hydrolysis for processing XOs (Nabarlatz *et al.* 2007; Vegas *et al.* 2008).

Since the utilization of *Mikania micrantha* for XO production has not been reported, the objective of this study was to investigate the feasibility of using *Mikania micrantha* biomass for XO preparation. Alkali extracted *Mikania micrantha* biomass was prepared and further processed by multi-staged nanofiltration (NMX), and the ethanol-mixture with ultrafiltration (EUMX). Ethanol precipitation (EMX) was also conducted as a control. Since extracted xylan and xylo-oligomers are presented as a suspension in a reaction mixture, the multi-staged membrane processes could preferentially separate sodium ions from alkali-extracted *Mikania* xylan mixtures. Newly developed recombinant xylanase derived from mesophilic *Paenibacillus campinensis* BL 11 (Ko *et al.* 2007; Ko *et al.* 2010) were employed to refine the above crude xylan mixtures and to produce xylooligomers.

EXPERIMENTAL

Materials

The aboveground samples of *M. micrantha* were collected in January 2009 at Lu-Ku township, Nan-Tou county, central Taiwan. The voucher specimen was identified by Dr. Kuo-Fang Chung, School of Forestry and Resource Conservation, National Taiwan University. *M. micrantha* samples were collected from a low-elevation site in Lu-Ku, Nantou county, central Taiwan.

Recombinant xylanase derived from mesophilic *Paenibacillus campinensis* BL 11 was prepared based on published protocols (Ko *et al.* 2010). Ultrafiltration membranes with molecular weight cut-off (MWCO) 5,000 and 10,000 were made of polyether-sulphone (PES). Ultrafiltration membrane with MWCO 30,000 was made of polysul-phone. PES, PS ultrafiltration flat sheet membranes, as well as thin film composite (TFC) nanofiltration flat sheet membranes, were purchased from GE Osnomics, Minnetonka, MN, USA. MWCO stands for molecular weight cut-off.

Xylooligosaccharide standards for chromatographic calibration, including xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexose (X6), were obtained from Megazyme, Bray, Ireland. All other chemicals were of analytical grade, and obtained from Sigma Chemical Co, St. Louis, MO, USA.

Pretreatment of *Mikania* Samples

Samples were air dried, milled to collect portions between 40 and 60 mesh screens, and homogenized to a single lot for further experiments. Milled *Mikania* powders were extracted with a 2:1 (v/v) toluene-ethanol mixture using a soxhlet extractor. Solvent extracted *Mikania* powders were further extracted, using an Erlenmeyer flask, with a 12% (w/v) NaOH solution at 150 rpm, 60°C for 16 h. The extracts were centrifuged for 15 min at 12,000 rpm, 4°C, and neutralized to pH 7. Precipitates were collected, dried, and denoted as the raw *Mikania* xylan (MX). A polyethylene (PE) squeeze bottle containing DI water was used to transfer the precipitates out of centrifuge tubes for the further experiments. Pentosans and total xylan contents of dried MX were analyzed.

Extraction of Xylan

Ethanol-precipitation (EMX)

The pH of the raw MX was adjusted to 6 by adding drops of 1N HCl. Then 95% (w/w) ethanol was added at 15 mL per gram of solid raw MX. The above mixture was allowed to settle for one day at room temperature. Then the precipitate was collected after centrifugation at 12,000 rpm, 4°C for 15 min. The above procedure was repeated twice. The collected precipitate was vacuum-dried at room temperature. The dried solids were defined as EMX and stored in 4°C.

Nanofiltration (NMX)

A nanofiltration process was employed to concentrate and to remove the mineral salts in the raw MX solutions. Membranes were held in a cross-flow module with 0.0084 m^{2} filtration area (Sterlitech, Seattle, WA, USA). A gear pump (Model 75211-10, Cole-Parmer Instrument Company, Vernon Hills, USA) was employed to drive the system. Each experiment filtration run was conducted based on a reported setup (Ko and Chen 2008) 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 ultrafiltration membrane and four runs with thin-film composite nanofiltration membrane were conducted. Permeate flux was monitored using a volumetric cylinder. A filtration run was terminated after steady-state fluxes was reached. 3 kg/cm^2 trans-membrane pressure was used in the 10 kD MWCO ultrafiltration run of NMX filtration; 7 kg/cm^2 was used in next four TFC nanofiltration runs. Four TFC membrane runs were conducted using same membrane without change. The concentrated MX liquid was vacuum-dried at room temperature. Final obtained solid was defined as NMX.

Ethanol-precipitation and ultrafiltration (EUMX)

The pH of the raw MX was adjusted to 6 with drops of 1N HCl. Then 95% (w/w) ethanol was added at 15 mL per gram of raw MX. Instead of precipitation and centrifugation steps, the mixture was subjected to staged-ultrafiltration processes to concentrate and to remove the mineral salts in raw MX solution. 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. Consecutive 4 kg/cm^2 membrane pressure was used in all EUMX filtration runs. 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.

Xylan Hydrolysis

Hydrolysis of xylan in EMX, NMX, and EUMX solutions was conducted by using recombinant *Paenibacillus* xylanase (Ko *et al.* 2010) at different dosages from 10, 50 to 100 U/mL in reaction mixtures at 0 to 96 h. The reaction mixtures contained 0.9% w/v equivalent total xylose and 0.1 M tris-buffer. This translated to 1.91%, 9.1%, and 9.3% w/v equivalent total xylose for EMX, NMX, and EUMX in reaction mixtures, respectively. The mixtures were incubated in a shake-flask setup at 60°C and 150 rpm. Concentrations of XO were monitored by HPLC analysis with respect to time.

Analytical Procedures

The compositions of *Mikania* powders and/or processed MX solids were determined according to the following TAPPI protocols: moisture content (TAPPI T 258 om-06), ash content (TAPPI T 211 om-85), solvent extractive of wood and pulp (TAPPI T204 cm-07), holocellulose (TAPPI T 249-um75), pentosans (TAPPI T223-cm10), and Klason lignin (T222-om98). Reducing sugars were determined with dinitrosalicylic acid assays (DNSA) (Miller 1959). The sum of dissolved xylose, suspended XO's and other xylan containing suspension of MX were denoted as the total xylose in the present study. Total xylose were determined with phenol/sulfuric acid assays using a calibration curve constructed by known xylose concentrations (Dubois *et al.* 1956). An Eppendorf pipette were employed to collect a 0.5 mL aliquot from well mixed influent reservoir containing suspended xylan. Xylanase activities were measured by the published protocol using xylose as a standard (König *et al.* 2002). XOs were assayed using a HPLC system with a Jasco RI-810 RI detector. An ICSep ICE-ION-300 (300 mm × 7.8 mm) column was

eluted by 0.0085 N H_2SO_4, at 0.4 mL/min, 70°C. Na^+ was analyzed on a IA-300 ion chromatographer (DKK-TOA). A 250 mm × 4.6 mm column (DKK-TOA) was eluted using 6 mM methanesulfonic acid as the mobile phase at 0.8 mL/min, 40°C. Sodium ion concentrations were analyzed in the permeate at the end of each filtration runs. Organic portion of dried MX was estimated by deduction of ashes.

RESULTS AND DISCUSSION

Chemical Compositions of the Raw Material and Pretreated Products

Major chemical compositions of the raw *M. micrantha*, pentosans, holocellulose, and lignin were measured as 56.04 ± 0.86%, 14.05 ± 0.18%, and 23.54 ± 0.89%. Then, the 86.06% of pentosan was retained in the organic portion of oven dried alkaline extracts (MX). 90.3 % of total xylose was measured over oven-dried MX .

Sequential NF Separation

The nanofiltration process was employed to remove the sodium ions from the raw MX solution. NMX influent, 1120 mL, containing 42.26 g sodium ion and 12.3 g total xylose, was prepared by dispersing MX solid into the influent reservoir. Fluxes of the pre-filtration run with 10 kD MWCO ultrafiltration membrane and four consecutive runs with TFC nanofiltration membrane are shown in Fig. 1. Fluxes of the first filtration run with 10 kD MWCO decreased from 0.24 to 0.07 mL/min from the first to the eleventh hour. Initial fluxes 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. Repeated passages of incoming fluxes caused significant reduction for the steady-state fluxes after each filtration runs. Here, TFC membrane showed its susceptibility toward repeated high solid influxes.
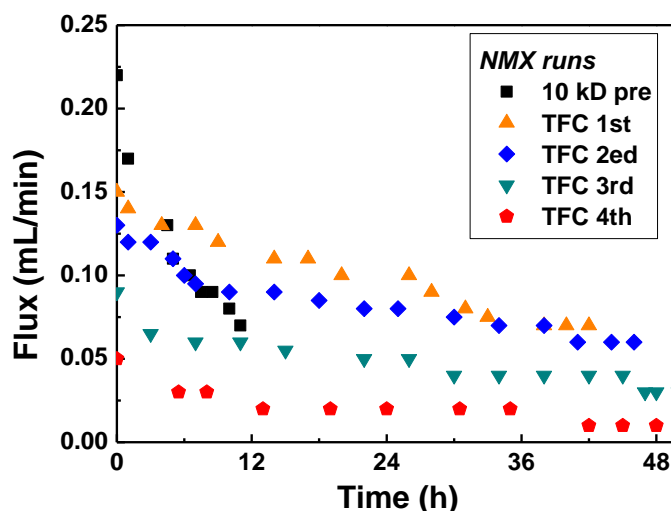

Fig. 1. Flux reduction during sequential NF membrane separation

Mass balance of sodium ions (upper panel) and total xylose recovery (lower panel) after sequential 10 kD and four TFC filtration runs are presented in the upper and lower panels of Fig. 2. Membrane processes were employed to reject sodium ion from the permeate and to retain xylans (measured as total xylose) in retentate. The volume of influent liquor was 1120.0 mL, as shown in Fig. 2. Permeate volumes were 80.7, 251.5, 224.4, 240.0, and 92.7 mL for each respective filtration run. Final retentate volume was 230.7 mL, while 899.3 mL of liquor was rejected by the NMX process. Although the 10 kD MWCO UF membrane had the largest pore opening, lower trans-membrane pressure and a shorter operation time could lead a lower volume passing through the membrane. Influent amount of 7.27 mL/hr was removed by the 10 kD MWCO UF membrane.

The sodium ion of influent NMX liquor was 42.26 g. There were 6.52, 22, 25.6, 37, and 23.4% (w/w) values for Na^+ removals for each stage. The mass of removed sodium ion for each stage, shown by the upper panel of Fig. 2, corresponded well with the permeate volumes of each respective stage. The above trend was expected, since sodium ion was well dissolved in the influent.
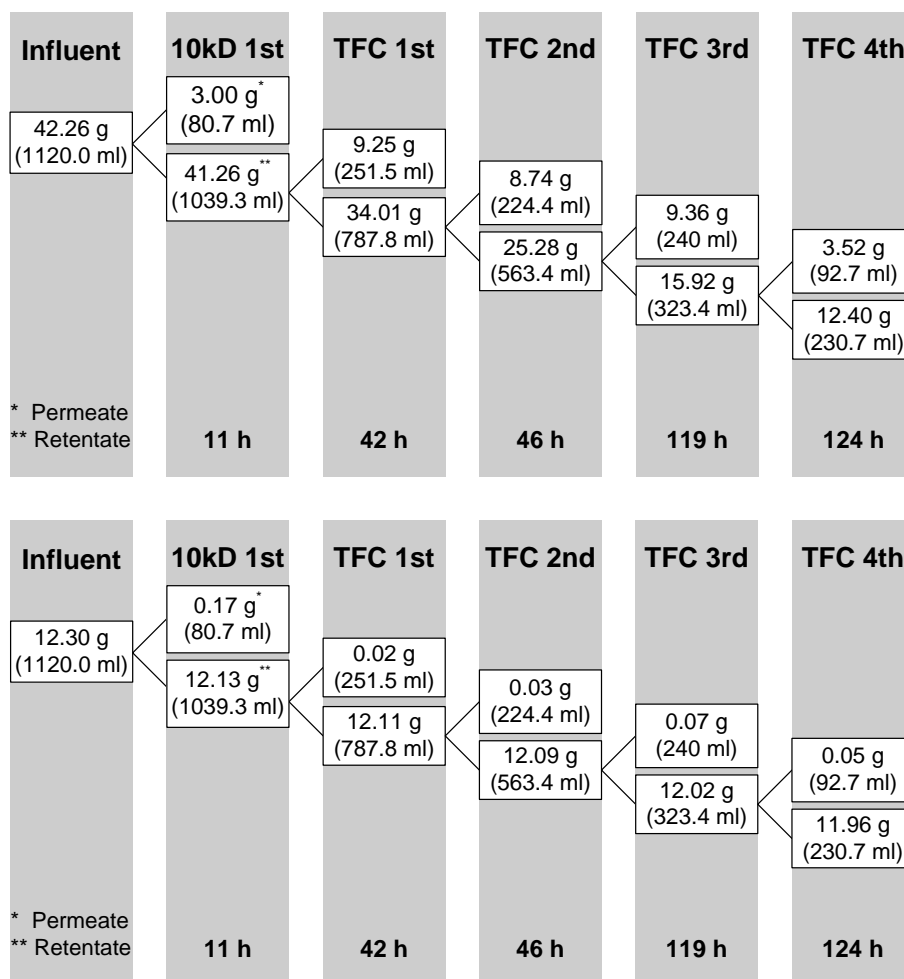

Fig. 2. Mass balance of sodium ions (upper panel) and total xylose recovery (lower panel) after a sequential 10 kD UF and TFC NF membrane separation

Figure 2 shows that the third TFC filtration run removed 37 % (w/w) of Na^+ and 42 % v/v of influent for that stage. An operating time of 119 hours contributed the

highest liquid separation in that stage. However, due to fouling on the membrane and higher incoming concentration of total xylose, the averaged flux for each NF stage dropped significantly. Removal of 5.99 and 0.75 mL/hr influent occurred in the first and last TFC stage, respectively. The total Na^+ removed was 33.85 g, corresponding to an overall separation efficiency of 73.18% for NMX process.

Xylose is soluble in water, but XOs and xylan are insoluble. Hence, different removal patterns for total xylose from sodium ions can be expected. The total xylose content of influent liquor was 12.30 g. As shown in Fig. 2, 169.25, 17.57, 26.64, 69.27, and 54.32 mg of total xylose 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, 11 hours. Because 10 kD is larger than xylose and XO's with DP 2 to 6. Total xylose 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 xylose suggested there was only few xylose monomer 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 xylose were raised from 10.98 to 51.85 mg/mL after NMX process, while 73.18% of sodium ions was removed.

Sequential UF Separation

Mixtures of MX water/ethanol solution were subjected to successive ultrafiltration processes to concentrate and to remove the sodium ions. Volumes, masses of total xylose and sodium ions of the first four 5 kD MWCO membrane filtration runs and two additional runs with 10 and 30 kD MWCO UF membranes are represented in the upper and lower panel of Fig. 3. NMX influent, 3000 mL, containing 43.5 g sodium ion and 11.4 g total xylose, was prepared by dispersing MX solid into the influent reservoir.

Initial fluxes of the first four 5 kD MWCO runs were 0.55, 0.47, 0.45, and 0.40 mL/min; and the steady-state fluxes of the first four 5 kD MWCO runs were 0.06, 0.06, 0.05, and 0.04 mL/min. 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. Fluxes of all four consecutive TFC membrane runs similarly reached steady-state after 36 hours. Steady-state flux remained for repeated 5 kD filtration runs, since there was only one-third influent concentration of insoluble XO's and xylan for EUMX than for NMX runs. Figure 3B shows the flux reductions for the 10 kD and 30 kD MWCO UF membrane separations: the initial fluxes were 0.7 and 1.2 mL/min; and the steady-state fluxes were 0.15 and 0.30 mL/min, respectively. Steady-state fluxes for the 30 kD filtration run were reached earlier than that of the 10 kD filtration run. The above finding may be attributed to the higher influent solid concentration. The steady-state fluxes observed for 5 KD, 10 kD, and 30 kD MWCO UF membranes were increased with increasing pore sizes, even with higher influent solid concentration.

The mass balance of sodium ions (upper panel) and the total xylose recovery (lower panel) after sequential UF membrane separation are shown in the upper and lower panels of Fig. 4. It is clearly shown that the EUMX process was able to remove sodium ions in permeate and to retain xylans in retentate. The volume of influent EUMX liquor was 3000 mL, and the permeate volumes were 514, 425, 419, 350, 180, and 183 mL after six stages of separation of 50, 50, 50, 50, 15, and 8 h are shown by Fig. 4. The volume of final retentate was 929 mL, and 2071 mL of permeate was rejected. The mass of sodium

ions of influent EUMX liquor was 43.5 g, and 7.12, 5.66, 5.66, 4.87, 2.51, and 2.55 g of sodium ions were removed after each stage of separation. 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.
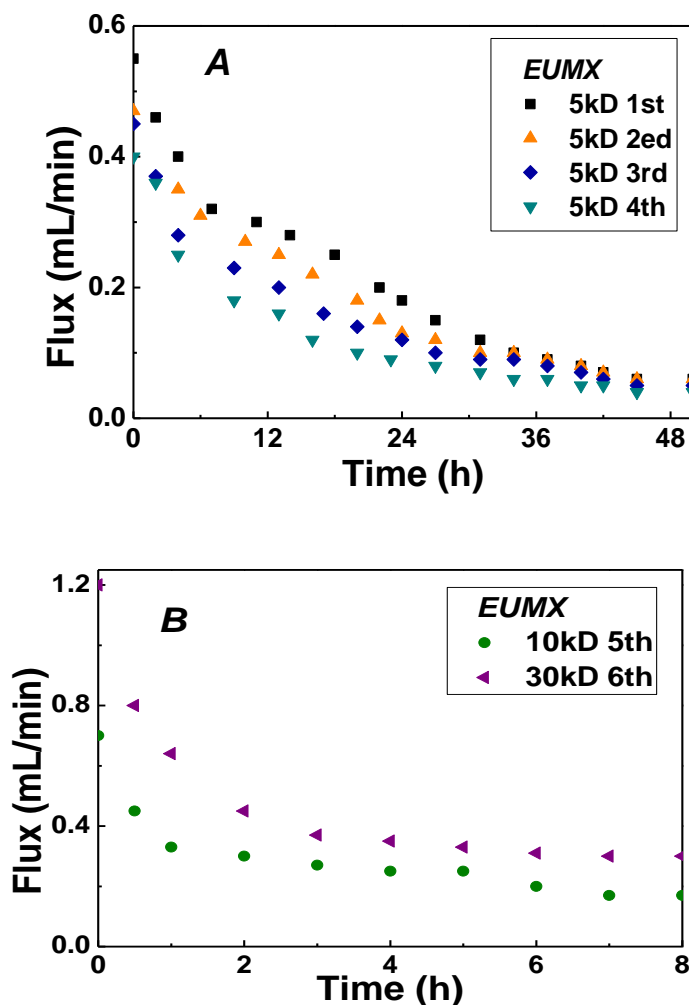

Fig. 3. Flux reductions during a sequential four 5 kD (upper panel), 10 and 30 kD (lower panel) UF membrane separation

Total sodium ion removal at 65.2 % corresponded well with volume reduction of 69%. Fouling still occurred during four consecutive 5 kD filtration runs, as shown by the drop of averaged flux for each 5 kD filtration stage drops. 10.28 and 7 mL/hr influent were removed in the first and last 5 kD filtration stage, respectively. Higher average flux and less significant fouling for EUMX runs could be attributed to larger UF membrane pore size and one-third influent concentration for sodium ion and xylans, compared the results of NMX runs shown by Figs. 1 and 2. The sodium ion removal at 65.2% for the EUMX process was found to be lower than 73.18% for NMX. Total time for EUMX runs was 223 hours, but total time for NMX runs was 342 hours. Fifty percent more filtration time for NMX runs did not proportionally increase sodium removals, compared to those of the EUMX runs. On the other hand, larger pore size of UF membranes and one-third influent concentration did not result much higher fluxes for EUMX runs. Concentration polarization of sodium ions and insoluble xylans accumulated in the areas adjacent to membrane surfaces might have played a role here (Bhattacharjee *et al.*, 1999).
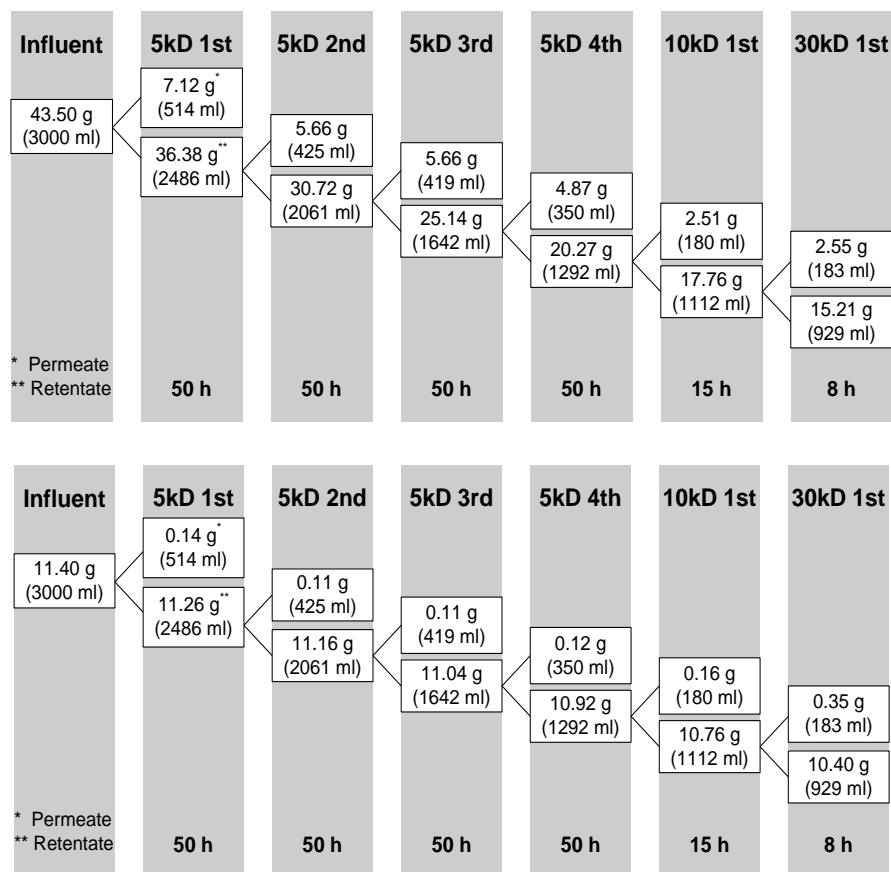
Fig. 4. Mass balance of sodium ions (upper panel) and total xylose recovery (lower panel) after sequential UF membrane separation

The total xylose content of influent liquor was 11.4 g, and 0.14, 0.11, 0.11, 0.12, 0.16, and 0.35 g of total xylose were lost after each stage of separation. 91.2% of total xylose was retained in the system, while total xylose concentrations were increased from 3.78 to 11.18 mg/mL. These results indicate that staged UF membrane separation are also very efficient at retaining total xylose. Higher total xylose losses during staged UF runs were expected, since XO molecular weights are less than 5 kD. In addition, the losses of total xylose by 30 kD membranes filtration runs were also found to be higher than that of 10 kD filtration runs, even with higher influent total xylose concentrations at this stage. Hence, it was suggested that the membrane pore size affected the most of total xylose recovery.

Hydrolysis of Xylan

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. 5. 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.

The left panel of Fig. 5 depicts the *Paenibacillus* xylanase hydrolysis of EMX. The upper-left panel of Fig. 5 shows results corresponding to the dosage of 1.1 U xylanase/mg equivalent total xylose. 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 xylanase /mL are shown in the mid-left panel of Fig. 5, with the dosage of 5.5 U xylanase/mg total xylose. 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 about 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 xylanase /mL are shown by the low-left panel of Fig. 6, with the dosage of 11.1 U xylanase/mg total xylose. Major resulting XOs were X5, X3, X2, and X6, in the order of 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. 2.28 g of XO were optimally produced from EMX solid with 4.95 g equivalent total xylose. 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.

The central panel of Fig. 5 corresponds to the *Paenibacillus* xylanase hydrolysis of NMX. As shown by the upper-middle panel of Fig. 6, 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-central panel of Fig. 6. 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 of NMX by 100 U/mL are shown by the lower-middle panel of Fig. 5. 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 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, with higher X6 content. 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. 3.69 g of XO were optimally produced from NMX solid with 5.42 g equivalent xylose.

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.
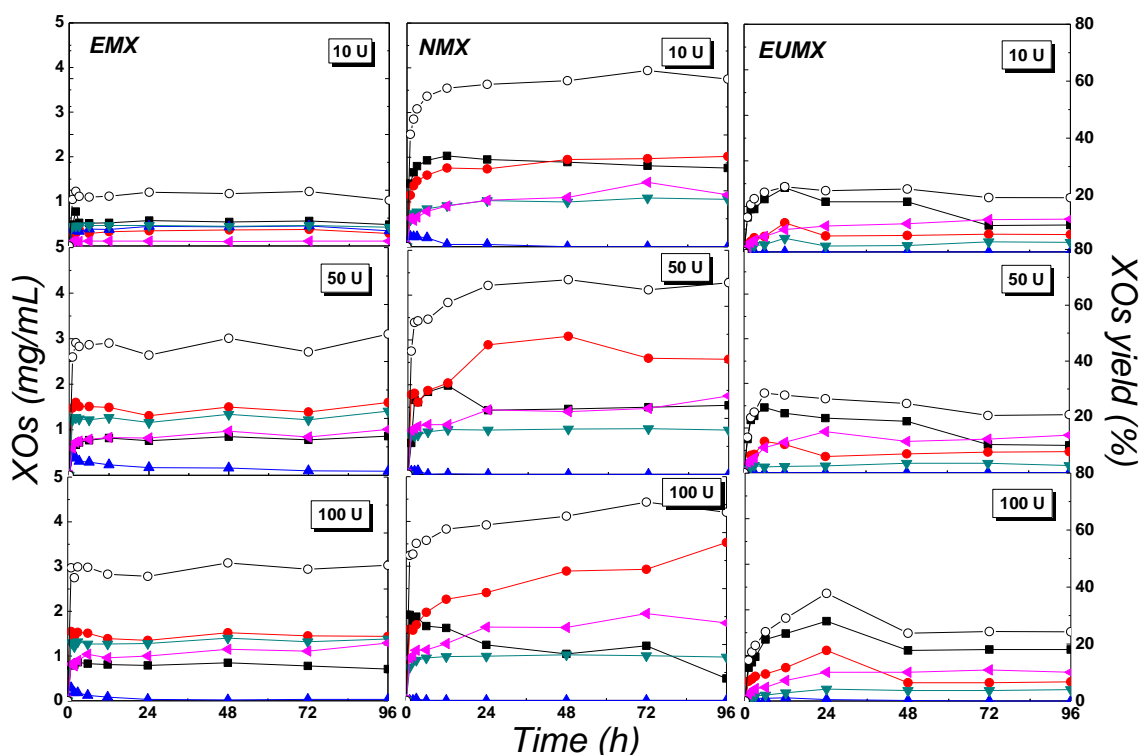

Fig. 5. Concentration and yields of xylooligosaccharides (XO) from hydrolysis of EMX, NMX, and EUMX mixtures. Figure legends are: XOs' yield (○); X2 (◀); X3 (▼); X4(▲); X5(●); X6(■)

The right panel of Fig. 5 represents the *Paenibacillus* xylanase hydrolysis of EUMX. As shown by the upper-right panel of Fig. 5, 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-right 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 6th h, and gradually reduced to 21% at the 48th h. The concentrations of XOs rapidly increased during xylanase hydrolysis in the first 6 h, but the hydrolysis of X6 and X5 after the 24th 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-right panel of Fig. 6. Major resulting XOs in the order of quantity were X6, X5, X2, and X3 before the 24th h. The XOs yield reached about 37% at the 24th h, but rapidly declined to 24% at the 48th h. The hydrolysis of X6 and X5 also occurred after the 24th h and contributed to decline of the XO yield. 1.71 g of XO were optimally produced from EUMX solid with 4.63 g equivalent total xylose.

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.

Efficiencies of XO production

XOs yields were 46, 68, 37 % (w/w) from total xylose contents for EMX, NMX and EUMX processes, respectively. Based on the experimental results, schematics for XO production mass balance from *M. micrantha* xylan after three processes was derived and showed in Fig. 6. This showed that nanofiltration membrane separation processing could yield more XO than those yielded by ethanol extraction and UF separation process.
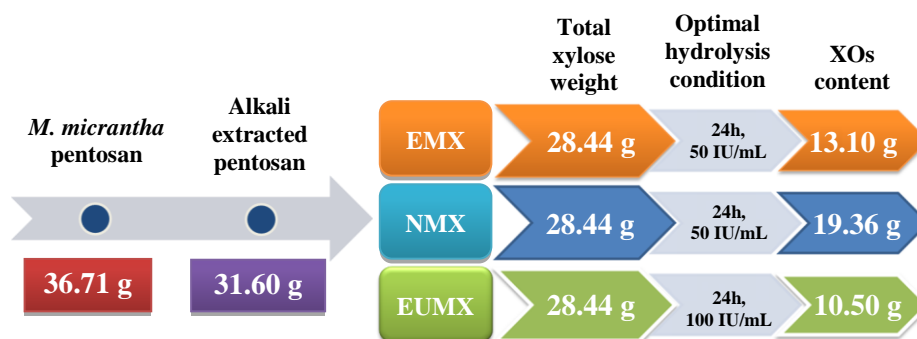

Fig. 6. Schematics for XO production mass balance from *M. micrantha* xylan after three processes

In our previous work (Ko *et al.* 2011), recombinant *Paenibacillus* xylanase was employed to hydrolyze commercially prepared (Sigma) oat spelt xylan and yielded 48.1% (w/w) XOs by using 10 U/mL for 72 h. Even counting the difference between total xylose and pentosan contents of MX, NMX solids was shown to be better hydrolyzed than commercial xylan.

Results of the present studies were compared with data obtained from several recent studies converting agricultural waste to more valuable XO products (the final yields of XO) with various combinations of membrane filtration and enzymatic hydrolysis. Table 2 summarizes the results of this compilation. The enzymatic hydrolysis of extracted xylan contents is always superior to chemical- or auto- hydrolysis alone (Sabiha-Hanim *et al.* 2011), but purification of xylanase by membrane filtration would enhance the final yields of XOs.

The applicability of membrane separation for XO production was demonstrated as being able to produce add-value XO products. Yields of XOs by enzymatic hydrolysis from agricultural waste biomass with multi-staged nanofiltration filtration processes were shown to be superior to the most results derived from other treatment processes. Although autohydrolysis and gel filtration could achieve matching XO yields to those of the present study, the setup of membrane separation could make it unnecessary to undertake boiler construction, operation, and maintenance and be faster than gel filtration.

Table 2. Finally Yield of XOs from Agricultural Waste with Various Membrane Separation processes and Enzymatic Hydrolysis

Sources	Membranes	Hydrolysis Conditions	Yields of XOs (%)	Reference
Cotton stalks	Ultrafiltration	Enzyme, 40°C, 24 h	53.0	Akpinar *et al.* 2007
Almond shells	Ultrafiltration	Autohydrolysis, 179°C, 23 min	54.2	Nabarlatz *et al.* 2007
Rice husk	-	Autohydrolysis, 205°C & Enzyme, 40°C	51.8	Vegas *et al.* 2008
	Nanofiltration		72.8	
Tobacco, cotton, and sunflower stalk, and wheat straw	-	Alkali extraction & acid hydrolysis, 30 min	8.0-13.0	Akpinar *et al.* 2009
Corncobs	Gel filtration	Alkali extraction & enzyme hydrolysis, 50°C, 24 h	79.2	Aachary & Prapulla 2009
Corncobs	Gel filtration	Steam explosion, 196°C, 5 min & enzyme hydrolysis, 70°C, 2.5 h	28.6	Teng *et al.* 2010
Oil palm (*Elaeis guineensis* Jacq.)	-	Autohydrolysis, 121°C, 60 min & Enzyme 40°C, 24 h	17.5	Sabiha-Hanim *et al.* 2011
Populas tomentosa	-	Chemical & Enzyme, 50°C, 14 h	36.8	Yang *et al.* 2011
Mikania micrantha	EMX NMX EUMX	Alkali extraction & Enzyme, 60°C, 24 h	46.0 68.0 37.0	This study

CONCLUSIONS

This research demonstrated that *M. micrantha*, a significant forest waste and invasive species, can be converted to more valuable XO products by alkaline extraction, staged-nanofiltration, and enzymatic hydrolysis. Staged-nanofiltration were demonstrated to offer more benefits for enzymatic hydrolysis over the ethanol-based fractionation:

generating more higher value-added X5 and X6, while requiring less xylanase dosages and reaction times.

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REFERENCES CITED

Aachary, A. A., and Prapulla, S. G. (2009). "Value addition to corncob: Production and characterization of xylooligosaccharides from alkali pretreated lignin-saccharide complex using *Aspergillus oryzae* MTCC 5154," *Bioresour. Technol.* 100, 991-995.

Akpinar, O., Ak, O., Kavas, A., Bakir, U., and Yilmaz, L. (2007). "Enzymatic production of xylooligosaccharides from cotton stalks," *J. Agr. Food. Chem.* 55, 5544-5551.

Akpinar, O., Erdogan, K., and Bostanci, S. (2009). "Production of xylooligosaccharides by controlled acid hydrolysis of lignocellulosic materials," *Carbohydr. Res.* 344, 660-666.

Alonso, J. L., Domínguez, H., Garrote, G., Parajo, J. C., and Vázquez, M. J. (2003). "Xylooligosaccharides: properties and production technologies," *Electron. J. Environ. Agric. Food. Chem.* 2, 230-232.

Bhattacharjee, S., Kim, A. S., and Elimelech, M. (1999), "Concentration polarization of interacting solute particles in crossflow membrane filtration, "*J. Colloid Interface Sci.* 212, 81-99.

Chiang, M. Y., Hsu, L. M., and Chen, F. Y. (2002). "Confirmation on the presence of *Mikania micrantha* Kunth in Taiwan," *Plant Prot. Bull.* 44, 61-62. (In Chinese with English abstract).

Dubois, M., Gills, K. A., Hamilton, J. K., Robert, F. A., and Smith, F. (1956), "Colorimetric method for determination of sugars and related substances," *Anal. Chem.* 28, 350-356.

Freixo, M. R., and Pinho, M. D. (2002). "Enzymatic hydrolysis of beechwood xylan in a membrane reactor," *Desalination* 149, 237-242.

Garrote, G., Dominguez, H., and Parajo, J.C. (2002). "Autohydrolysis of corncob: Study of non-isothermal operation for xylooligosaccharide production," *J. Food. Eng.* 52, 211-218.

Kabel, M. A., Acrvalheiro, F., Garrote, G., Avgerinos, E., Koukios, E., Parajo, J. C., Gino, F. M., Schols, H. A., and Voragen, A. G. J. (2002). "Hydrothermally treated xylan rich by-products yield different classes of xylo-oligosaccharides," *Carbohydr. Polym.* 50, 47-52.

Ko, C. H., and Chen, S. S. (2008). "Enhanced removal of three phenols by laccase polymerization with MF/UF membranes," *Bioresour. Technol.* 99, 2293-2298.

Ko, C. H., Chen, W. L., Tsai, C. H., Jane, W. N., Liu, C. C., and Tu, J. (2007). "*Paenibacillus campinasensis* BL11: A wood material-utilizing bacterial strain isolated from black liquor," *Bioresour. Technol.* 98, 2727-2733.

Ko, C. H., Tsai, C. H., Tu, J., Lee, H. Y., Ku, L. T., Kuo, P. A., and Lai, Y. K. (2010). "Molecular cloning and characterization of a novel thermostable xylanase from *Paenibacillus campinasensis* BL11," *Process Biochem.* 45, 1638-1644.

König, J., Grasser, R., Pikor, H., and Vogel, K. (2002). "Determination of xylanase, *ß*-glucanase, and cellulase activity," *Anal. Bioanal. Chem.* 374, 80-87.

Kuo, Y. L. (2003). "Ecological characteristics of three invasive plants *Leucaena leucocephala*, *Mikania micrantha*, and *Stachytarphetar jamaicensis* thriving in southern Taiwan," In: Oka, M., Matsui, M., Shiomi, T., Ogawa, Y., Tsuchiya, K. (eds.), *Biological Invasions- Environmental Impacts and the Development of a Database for the Asian-Pacific Region*, Proceedings of the NIAES-FFTC joint international seminar, Tsukuba, Japan, 177-191.

Kuo, Y. L., Chen, T. Y., and Lin, C. C. (2002). "Using a consecutive-cutting method and allelopathy to control the invasive vine, *Mikania micrantha* H.B.K.," *Taiwan J. Forest Sci.* 17, 171-181. (In Chinese with English abstract).

Lowe, S., Browne, M., Boudjelas, S., and Poorter, M. D. (2001). *100 of the World's Worst Invasive Alien Species: a Selection from the Global Invasive Species Database*, The International Union for Conservation of Nature -Invasive Species Specialist Group (IUCN-ISSG), Gland, Switzerland .

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.* 31, 426-428.

Moure, A., Gullón, P., Domínguez, H., and Parajó, J. C. (2006). "Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals," *Process Biochem.* 41, 1913-1923.

Mussatto, S. I., and Mancilha, I. M. (2007). "Non-digestible oligosaccharides: A review," *Carbohydr. Polym.* 68, 587-597.

Nabarlatz, D., Torras, C., Garcia-Valls, R., and Montané, D. (2007). "Purification of xylo-oligosaccharides from almond shells by ultrafiltration," *Sep. Purif. Technol.* 53, 235-243.

Nishimura, T., Ishihara, N., Ishii, T., and Kato, K. (1998). "Structure of neutral branched xylooligosaccharides produced by xylanase from in situ reduced hardwood xylan," *Carbohydr. Polym.* 308, 117-122.

Pellerin, P., Gosselin, M., Lepoutre, J., Samain, E., and Debeire, P. (1991). "Enzymatic production of oligosaccharides from corncob xylan," *Enzyme Microb. Technol.* 13, 617-621.

Sabiha-Hanim, S., Noor, M. A. M., and Rosma, A. (2011). "Effect of autohydrolysis and enzymatic treatment on oil palm (*Elaeis guineensis* Jacq.) frond fibres for xylose and xylooligosaccharides production," *Bioresour. Technol.* 102, 1234-1239.

Saha, B. C. (2003). "Hemicellulose bioconversion," *J. Ind. Microbiol. Biotechnol.* 30, 279-291.

Sjöström, E. (1993). *Wood Chemistry: Fundamentals and Applications*, 2nd Ed., Academic Press, New York.

Teng, C., Yan, Q., Jiang, Z., Fan, G., and Shi, B. (2010). "Production of xylooligosaccharides from the steam explosion liquor of corncobs coupled with enzymatic hydrolysis using a thermostable xylanase," *Bioresour. Technol.* 101, 7679-7682.

Vegas, R., Alonso, J. L., Domínguez, H., and Parajó, J. C. (2008). "Enzymatic processing of rice husk autohydrolysis products for obtaining low molecular weight oligosaccharides," *Food Biotechnol.* 22, 31-46.

Voragen, A. G. J. (1998). "Technological aspects of functional food-related carbohydrates," *Trends Food Sci. Tech.* 9, 328-335.

Yang, R., Xu, S., Wang, Z., and Yang, W. (2005). "Aqueous extraction of corncob xylan and production of xylooligosaccharides," *LWT−Food Sci. Technol.* 38, 677-682.

Yang, H., Wang, K., Song, X., and Xu, F. (2011). "Production of xylooligosaccharides by xylanase from *Pichia stipitis* based on xylan preparation from triploid *Populus tomentosa*," *Bioresour. Technol.* 102, 7171-7176.

Zhang, L. Y., Ye, W. H., Cao, H. L., and Feng, H. L. (2004). "*Mikania micrantha* H. B. K. in China – An overview," *Weed Res.* 44, 42-49.

Zilliox, C., and Debeire, P. (1998). "Hydrolysis of wheat straw by a thermostable endoxylanase: Adsorption and kinetic studies," *Enzyme Microb. Technol.* 22, 58-63.