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Xylooligosaccharides produced from sugarcane leaf arabinoxylan using xylanase from *Aureobasidium pullulans* NRRL 58523 and its prebiotic activity toward *Lactobacillus* spp.

Sorawit Na Nongkhai [a,b], Phitchayakon Piemthongkham [a,b], Wichanee Bankeeree [b], Hunsa Punnapayak [b,c], Pongtharin Lotrakul [b,**], Sehanat Prasongsuk [b,c,*]

[a] *Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[b] *Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[c] *Department of Biology, Faculty Science and Technology, Airlangga University, Surabaya 60115, Indonesia*

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ABSTRACT

In an attempt to enhance the value of sugarcane leaf, xylan was extracted and used for xylooligosaccharide (XO) production via enzymatic hydrolysis using xylanase from the black yeast *Aureobasidium pullulans*. The xylan was extracted from sugarcane leaf using alkali extraction according to the response surface methodology. The highest xylan yield (99.42 ± 4.05 % recovery) was obtained using 14.32 % (w/v) NaOH, 13.25:1 liquid: solid ratio, at 121 °C and 15 lb. in^2 for 32 min. Sugar composition and FTIR spectrum analyses confirmed its structure as arabinoxylan. The extracted arabinoxylan had a relatively high molecular weight compared to previous studies. Crude endoxylanase from *A. pullulans* NRRL 58523 was selected for enzymatic hydrolysis of the xylan. The enzyme hydrolyzed well at 50 °C, pH 4.0 and was relatively stable under this condition (87.38 ± 1.26 % of the activity remained after 60 h). XOs, especially xylobiose and xylotriose, were obtained at the maximum yield of 237.51 ± 17.69 mg/g xylan via endoxylanase hydrolysis under the optimum conditions (50 °C, pH 4.0, 65.31 U/g xylan, 53 h). XOs exhibited species-specific prebiotic activity toward three strains of *Lactobacillus* spp. but not toward *Bifidobacterium* spp.

1. Introduction

In the sugar industry, only the stalks of sugarcane (*Saccharum officinarum*) are desirable materials, while their leaves are considered as waste [1]. Burning of sugarcane fields before harvesting is a common practice in many developing countries to reduce the labor cost of leaf removal. However, this practice has also caused significant air pollution, especially particulate matters with diameter less than 2.5 μm (PM 2.5) and carbon dioxide emission [2–4]. To reduce such a harmful effect, conversion of unwanted sugarcane leaves into value-added products is necessary [5]. Although a number of studies have shown the promising potential of sugarcane biomass [6–8], only a few reports have emphasized sugarcane leaves [9–15].

* Corresponding author. Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
** Corresponding author.
E-mail addresses: pongtharin.l@chula.ac.th (P. Lotrakul), sehanat.p@chula.ac.th (S. Prasongsuk).

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The major heteropolysaccharide in sugarcane leaves is xylan, which has been highlighted as the precursor for production of xylooligosaccharides (XOs) [16–18]. XOs are oligomers of two to ten xylose units which are non-digestible and applicable as functional food additives since they have been reported to enhance the growth of beneficial probiotic bacteria and suppress harmful pathogens in the gastrointestinal tract [19–21]. XOs can be produced through xylan hydrolysis using either an enzymatic or chemical process. Enzymatic hydrolysis is considered more feasible than acid hydrolysis since it is more specific and does not produce toxic byproducts, such as furfural, which would adversely affect the downstream processes [22,23]. Even though enzymatic hydrolysis is somewhat costly compared to acid hydrolysis due to enzyme production expenses, its high specificity and the product quality outweigh this disadvantage. Moreover, other measures such as using cheap or no-value substrates (i.e. agricultural wastes) can be employed to compensate these expenses. Many bacteria and fungi can produce xylanolytic enzymes, especially xylanase (endo-1,4-β-xylanase, E. C.3.2.1.8), with a wide range of yields and properties. Members of the *Aureobasidium* genus, a group of polymorphic ascomycetous black yeasts commonly found worldwide, have been reported as good xylanolytic enzyme producers, especially the so-called color variant strains [24,25]. In Thailand, a number of xylanase-overproducing, color variant strains have been isolated and their enzymes have shown promising potential in industrial applications such as hemicellulose digestion in food and pulp industries [26–31]. Therefore, it is of interest to explore the potential of using sugarcane leaves as the source of sugars for valued compound production such as prebiotic XOs, especially through environmentally friendly enzymatic hydrolysis. The specific objectives of this study were to: (1) screen xylanases from these tropical *Aureobasidium* strains and select one that was suitable for XOs production, (2) optimally extract xylan from sugarcane leaf and determine its structure and sugar composition, (3) use the crude *Aureobasidium* xylanase for sugarcane xylan hydrolysis under the optimal condition as determined by Response Surface Methodology (RSM), and (4) evaluate the prebiotic activity of the obtained XOs toward six strains of *Lactobacillus* and *Bifidobacterium*. Results obtained from this study can be further used for development of large scale XOs production from sugarcane leaves, which would add the value to the otherwise agricultural waste.

2. Materials and methods

2.1. Microbial strains and sugarcane sample

Sugarcane leaves (SLs) were provided by Department of Agronomy, Kasetsart University: Kamphaeng Saen Campus, Nakhon Pathom province, Thailand. The leaves were chopped, oven dried at 60 °C until the constant weight was obtained and kept in airtight plastic bag until further used. For xylanase screening, 36 strains of *Aureobasidium* spp. were selected from the culture collection of Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand (Supplementary data I) [26,27,31]. All strains were cultured in yeast malt (YM) broth at room temperature (28 ± 2 °C) with 150-rpm agitation for 3 days. For short-term storage, the yeasts were maintained on YM agar and kept in a refrigerator. For long-term storage, the cells were lyophilized in 15 % (w/v) skim milk and stored at −20 °C. For probiotic bacteria, *Lactobacillus brevis* TISTR 868, *Lactobacillus casei* TISTR 390, *L. casei* subspecies *rhamnosus* TISTR 047, *Bifidobacterium longum* subspecies *longum* TISTR 2195, *Bifidobacterium breve* TISTR 2130 and *Bifidobacterium animalis* subspecies *animalis* TISTR 2194 were purchased from the Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand, and maintained on de Man Rogosa Sharpe (MRS) agar at 4 °C. For long-term storage, the probiotic bacteria were lyophilized in 15 % (w/v) skim milk and stored at −20 °C.

2.2. Xylan extraction

Biomass composition of SLs including cellulose, hemicellulose and lignin was determined according to the method of Goering and Van Soest [32]. Sugarcane leaf xylan (SLX) was extracted by using 12 % (w/v) NaOH at a liquid to solid ratio of 10:1 according to Samanta et al. [33]. After alkaline treatment, the supernatant was collected by centrifugation at 6,000×*g* for 10 min and neutralized with glacial acetic acid to pH 7.0 for delignification. The lignin pellets were removed by centrifugation at 6,000×*g* for 10 min. Then, 3 vol of 95 % (v/v) ethanol were added for xylan precipitation. After centrifugation at 6,000×*g* for 10 min, the xylan pellets were collected and dried in hot air oven at 60 °C until the constant weight was obtained. Xylan yield and relative xylan recovery were calculated via Equations (1) and (2):

$$\text{Xylan yield (\%)} = [\text{Dry weight of extracted xylan (g)}/\ \text{Dry weight of sample (g)}] \text{ x } 100 \quad (1)$$

$$\text{Relative xylan recovery (\%)} = [\text{Xylan yield (\%)}/\ \text{Hemicellulose content in sugarcane leaf (\%)}] \text{ x } 100 \quad (2)$$

where hemicellulose content in sugarcane leaf (%) was obtained from the biomass composition analysis as described above. To optimize the xylan extraction, Box-Behnken design with three variable factors including NaOH concentrations (12, 15, 18 % (w/v)), steaming times (30, 45, 60 min) in autoclave (121 °C, 15 lb/in^2) and liquid-to-solid ratios (5:1, 10:1, 15:1, mL/g) was carried out with three replicates. Response surface methodology (RSM) was employed to predict the highest relative xylan recovery under the suggested optimal condition. Independent xylan extraction was also conducted based on the predicted values to validate the RSM prediction. Student's t-test was employed for comparison between the predicted and observed values.

2.3. Structural analysis and properties of SLX

The structure of obtained SLX was analyzed by Fourier Transform Infrared (FTIR) Spectrophotometer (Nicolet 6700, Thermo Nicolet Corp, Madison) in the range of 4000-400 cm^{-1} using a KBr that was mixed with a sample at a ratio of 10:1 (by weight)

comparing with that of commercial beechwood xylan (Megazyme, Ireland) [34]. For analysis of sugar composition, SLX was completely hydrolyzed with 4 % (v/v) H_2SO_4 according to Peng et al. [35]. The sugar type and content were determined by High-Performance Liquid Chromatography (HPLC, Alliance, USA) using refractive index detector and Varian hydrogen ion-exchange column at 40 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Each sugar was quantified according to the standard sugars (xylose, glucose, arabinose, galacturonic acid and glucuronic acid) (Sigma-aldrich, USA) [35].

The molecular weight of SLX was determined by Gel Permeation Chromatography (GPC) using Shodex PLgel column that was operated at 40 °C and eluted with distilled water at a flow rate of 0.5 mL/min (Showa Denko, Japan). The sample was dissolved in distilled water at a concentration of 0.1 % (w/v) and pullulans (Sigma-aldrich, USA) were used as polysaccharide standards for calibration. The degree of polymerization (DP) of SLX was calculated via Equation (3):

$$DP = M_w / M_o \quad (3)$$

Where M_w is the average molecular weight of xylan and M_o is the molecular weight of xylose repeating unit (132 g/mol, [36]). The viscosity of SLX was measured by rotational rheometry using Brookfield Synchro-Lectric Viscometer (RVF model, Brookfield, USA). The measurement was conducted in a water bath at 50 °C [37].

2.4. Xylanase production

For screening of high potential strain in xylanase production, each of *Aureobasidium* strain was cultivated in YM broth for seed culture preparation before transferred (0.1 % v/v) to xylanase production medium containing corncob (1 % w/v) as the sole carbon source according to Bankeeree et al. [30]. Supernatants were harvested by centrifugation at 6,000×*g* for 10 min and used as the crude enzymes. The xylanase activity was then assayed using beechwood xylan (Megazyme, Ireland) as the substrate and the releasing of reducing sugars was determined by 3,5-dinitrosalicylic acid (DNS) method. The reaction mixture (500 μL) contained 125 μL of 1 % (w/v) beechwood xylan, 50 μL crude enzyme, 75 μL distilled water and 250 μL 50 mM sodium phosphate buffer (pH 5) was incubated at 50 °C for 15 min. Then, 750 μL DNS was added and the mixture was boiled for 5 min before 1.5 mL distilled water was added to each tube. The absorbance at 540 nm was detected and the amount of released reducing sugar was calculated according to the xylose standard curve [38,39]. One unit (U) of xylanase was defined as the amount of enzyme that catalyzes the release of 1 μmole xylose equivalent per min [24,40]. The strain with the highest xylanase activity was selected for the following enzyme characterization.

2.5. Xylanase characterization

The effects of pH and temperature on xylanase activities were assayed using 5 different buffers (50 mM): sodium citrate buffer (pH 3 to 4), sodium acetate buffer (pH 4 to 5), sodium phosphate buffer (pH 5 to 8), tris buffer (pH 8 to 9) and Na_2HPO_4–NaOH buffer (pH 9 to 11) and at temperatures ranging from 40 to 80 °C [41]. The relative activity was calculated as the percentage of the maximum activity. To determine the thermostability, the crude enzyme was incubated in the optimal buffer and pH at 40, 50, 60, 70 and 80 °C in the absence of substrate. Samples were taken every 6 h from 0 to 60 h and assayed under the optimal condition. The remaining activity was calculated as the percentage of enzyme activity before incubation (0 h).

2.6. XOs production

Condition for SLX hydrolysis was performed according to the optimum pH and temperature of selected xylanase. The hydrolysis mixture (10 mL in 20 mL Erlenmeyer flask) containing 1 % (w/v) SLX in suitable buffer (the optimum pH) and the crude enzyme was incubated at the optimum temperature (obtained from 2.5) with 150-rpm agitation for 24 h. After incubation, the reaction mixture was boiled for 10 min to stop the enzyme reaction [42–44]. The content of XOs including xylobiose (X2) and xylotriose (X3) in the reaction was determined by HPLC (Alliance, USA) using refractive index detector and Varian hydrogen ion-exchange column at 40 °C with 5 mM H_2SO_4 as mobile phase at a flow rate 0.6 mL/min. To optimize the XOs production, CCD was employed with two variables: xylanase dosage (25, 50, 75 U/g xylan) and incubation time (24, 48, 72 h). RSM was employed to predict the optimal condition that yielded the highest XOs content. The predicted condition was validated in an independent experiment, and Student's t-test was employed for comparison between the predicted and observed values. The experiment was conducted in triplicate.

2.7. Prebiotic property

The seed cultures were prepared by cultivation of each probiotic bacterium in MRS broth at 37 °C under a static condition in an anaerobic container flushing with nitrogen gas for 24 h. Then, the absorbances (OD_{600}) of seed cultures were adjusted to 0.1 with sterile medium and transferred (1 % v/v) individually into the MRS broth supplemented with glucose and oligosaccharides including produced XOs, commercial fructooligosaccharide (FO; Vista Cafe, Thailand), and commercial inulin (Krungthepchemi, Thailand) at 2 mg carbon/mL. Culture without carbon source addition was used as the negative control. The cultures were incubated under the same condition until the mid-log phase of each strain was reached (according to glucose-containing medium): *L. brevis* (10 h), *L. casei* (11 h), *L. casei* subspecies *rhamnosus* (9 h), *B. longum* subspecies *longum* (15 h), *B. breve* (16 h) and *B. animalis* subspecies *animalis* (14 h). The growth of probiotic bacteria was determined using the standard plate count technique under anaerobic condition [44,45].

2.8. Statistical analysis

The results were expressed as mean values with standard deviation derived from three replications. The SPSS statistical computer package (SPSS Inc., USA) was used to analyze the experimental data using One-Way ANOVA and Duncan's multiple range test (DMRT) or Student's t-test when appropriated. Significant difference was determined at $p \leq 0.05$.

3. Results and discussion

3.1. Xylan extraction

Alkaline extraction has been reported as an efficient method for hemicellulose extraction since it can solubilize hemicellulose and lignin from biomass without significantly breaking down the hemicellulose chain compared to acid extraction [33,46–48]. Samanta et al. [33] reported that NaOH solution at 12 % (w/v) efficiently solubilized xylan from *Sehima* grass and the obtained xylan was readily digested to XOs. Therefore, NaOH at this concentration was chosen for preliminary xylan extraction from SL. The yield and relative xylan recovery of SLX extracted by 12 % (w/v) NaOH were 25.33 ± 0.80 % and 77.51 ± 1.89 %, respectively, according to the SL composition that was found to consist of cellulose, hemicellulose, lignin, ash, and others at 32.79 ± 0.45 %, 32.69 ± 1.03 %, 8.29 ± 0.11 %, 1.71 ± 0.02 % and 24.52 ± 0.69 % (w/w), respectively. Previous reports have shown that xylan can be effectively extracted from the dry biomass of fast growing weeds and grass crops [16,44,49]. However, such extraction protocols might not always be feasible for large scale production, especially when feedstock availability is considered in terms of logistic practicality and transportation cost. For example, although vetiver grass has been shown to be a good source of xylan, it is not an economic crop and therefore not extensively grown [50]. Moreover, only 14.37 g/m^2 of vetiver biomass can be harvested, as reported by Ng et al. [51]. Napier grass, another plant suitable for xylan extraction, is an economic crop used for animal feed and often grown in large areas. However, at 700.00 g/m^2 [52], its biomass availability might not be attractive for xylan extraction in terms of profit as it has more value as animal feed. The solution to this limitation is the biomass wastes of economic crops since they have no value, need to be removed and are available in large quantities annually. SL is one such agricultural waste available in large quantities in many countries, including Thailand. Currently SL has no profitable application, unlike sugarcane bagasse. Jusakulvijit et al. [53] estimated that SL could be harvested at 1780.00 g/m^2. Since sugarcane is often grown in vast areas [54], the logistic is pragmatic as SL would be readily collected at the same time as sugarcane stalks. Therefore, SL was chosen from potential candidates of non-feedstock agricultural wastes as the material for xylan production. Based on the biomass composition analysis, SL was found to contain high amounts of hemicellulose, up to one-third of its dry weight, which was similar to the previous report by Forsan et al. [13].

To enhance the relative xylan recovery, a Box-Behnken design with 3 levels of 3 variable factors was used and the response values are presented in Table 1. The highest value of 98.73 ± 5.90 %, which was found in trial no.8, was not significantly different from those of trial no.13 (98.42 ± 3.09 %) and 14 (97.38 ± 0.88 %). The statistical significance of the model was calculated by ANOVA, as shown in Supplementary data II, and the data were fitted into a second order polynomial equation (Equation (4)) as follows.

$$Y = 29.39X1 + 3.01X2 + 130.85X3 - 0.05 \times 1 \times 2 + 1.99 \times 1 \times 3 - 0.71 \times 2 \times 3 - 0.98X12 - 0.01X22 - 26.44X32 - 364.20 \quad (4)$$

where Y was the relative xylan recovery (%), X_1 was the NaOH concentration (% w/v), X_2 was the steaming time (min) and X_3 was the liquid-to-solid ratio (mL/g). Based on an R^2 value of 0.99, the equation could determine 99 % of the observed response. The model was significant at $p = 0.0001$, whereas lack of fit was not significant ($p = 0.07$), suggesting that the regression model could accurately predict the response. Moreover, the low coefficient of variation (CV = 2.85 %) indicated the uniformity of data. For independence,

Table 1
Box-Behnken design -RSM used for optimization of xylan extraction from SL.

Run	Actual level			Relative xylan recovery (% of hemicellulose content)	
	NaOH concentration (X_1; %w/v)	Steaming time (X_2; min)	Liquid-to-solid ratio (X_3; mL/g biomass)	Observed[a]	Predicted
1	12 (−1)	30 (−1)	10 (0)	76.99 ± 0.88	75.52
2	18 (+1)	30 (−1)	10 (0)	90.60 ± 1.04	90.56
3	12 (−1)	60 (+1)	10 (0)	87.55 ± 3.66	87.59
4	18 (+1)	60 (+1)	10 (0)	93.15 ± 2.79	94.62
5	12 (−1)	45 (0)	5 (−1)	37.73 ± 2.34	39.80
6	18 (+1)	45 (0)	5 (−1)	38.24 ± 4.59	38.88
7	12 (−1)	45 (0)	15 (+1)	74.31 ± 1.09	73.67
8	18 (+1)	45 (0)	15 (+1)	98.73 ± 5.90	96.66
9	15 (0)	30 (−1)	5 (−1)	32.47 ± 0.74	31.86
10	15 (0)	60 (+1)	5 (−1)	63.40 ± 2.86	61.29
11	15 (0)	30 (−1)	15 (+1)	96.93 ± 9.51	99.05
12	15 (0)	60 (+1)	15 (+1)	85.14 ± 0.88	85.75
13	15 (0)	45 (0)	10 (0)	98.42 ± 3.09	97.56
14	15 (0)	45(0)	10 (0)	97.38 ± 0.88	97.56
15	15 (0)	45 (0)	10 (0)	96.87 ± 5.79	97.56

[a] Mean ± SD derived from three replicates.

Durbin and Watson test showed insignificant positive autocorrelation (1.75) indicating the precision and reliability of the experiments [55]. The response surface plots are shown in Fig. 1A–C. The RSM predicted that the maximum relative xylan recovery (99.61 %) could be achieved when the extraction was carried out in 14.32 % (w/v) NaOH for 32 min with a liquid-to-solid ratio of 13.25:1. Independent extraction of SLX was performed under the suggested conditions and a relative xylan recovery of 99.42 ± 4.05 % was obtained, which was not significantly different from the predicted value ($p = 0.95$), indicating that the equation was accurate. Using these optimized conditions, the SLX recovery (99.42 ± 4.05 %) was 1.28-fold higher than that of the referenced condition (77.51 ± 1.89 %). In this study, nearly complete hemicellulose recovery was achieved after optimized alkaline extraction, which was higher than that of the previous report (69.70 %) on hemicellulose extraction from SL [13]. The difference was likely due to the extraction method as Forsan et al. [13] used alkaline-peroxide extraction at 60 °C that had originally been optimized for xylan extraction from bagasse [56]. The lower NaOH concentration and temperature might have contributed to the lower hemicellulose yield. Compared to the xylan recovery percentages from SL, those reported from sugarcane bagasse were lower and in a range between 53.00 and 72.10 % [13,57,58], which was probably due to the high lignin content that blocked the hemicellulose release [59]. The optimal NaOH concentration, incubation time and liquid to solid ratio for xylan extraction in this study fell in similar ranges previously reported for other agricultural residues, namely, 12–15 % (w/v) NaOH, a 20–45 min incubation time and a liquid to solid ratio of 10:1, which resulted in relative xylan recovery in a range between 54 and 98 % [33,43,58,60,61]. Alkali-based ammonia fiber expansion (AFE) has been used for cellulose and hemicellulose extraction from SL [9,11]. Although high sugar conversion percentages were reported (70–90 %), the processes require severe conditions (up to 140 °C, 650 psi), high ammonia loading (1:1 ratio or higher) and specific equipment [9,11]. Therefore, a simpler and less severe condition of NaOH extraction might be more attractive in comparison.

3.2. Structural analysis and properties of the extracted SLX

The structure of the obtained SLX was determined by comparing its FT-IR with that of commercial xylan. The spectra are shown in Fig. 2A and B. The typical peaks of xylans at 3437, 2920, 1407, 1046, 897, 639 and 530 cm^{-1} were observed in both samples, which indicated the similarity between their structures. The spectra peaks of SLX at 3437 and 2920 cm^{-1} corresponded to the stretching of the H-bond in the OH group and C–H stretching [62]. The peak at 1407 cm^{-1} was the stretching of C–H; moreover, the peak at 1046 cm^{-1} revealed the C–O, C–C stretching and C–OH bending of the xylose pyran ring [54]. The spectra band at 897 cm^{-1} corresponded to the stretching vibration in C–*O*–C to indicate the β-1,4-xylosidic linkage of the xylan structure [63]. Moreover, the absorption bands at 639 and 530 cm^{-1} revealed the result of the stretching or bending of C–C–H or C–*O*–C [64]. The presence of arabinose was also apparent at 989 cm^{-1} [62].

From the sugar analysis, xylose (81.50 %) was found to be the dominant sugar in SLX, followed by arabinose (18.50 %), which was exhibited as a major constituent of arabinoxylan. Similar to previous reports which found that the major component of sugarcane bagasse was xylan, the present study found that the major component of SLX was arabinoxylan [65]. However, Morais de Carvalho et al. [66] reported that xylan from SLs was acetylated glucuronoarabinoxylan. This difference was probably due to the different extraction method, which was performed with dimethyl sulfoxide [66].

The DP of SLX was analyzed and widely varied in value from 13.20 (oligosaccharides) to 30,508.40 (very long chain polymers). This result was related with its average molecular weight, which was between 1737 and 4,027,110 g/mol the presence of XOs was probably caused by alkaline extraction, as suggested by previous reports [17]. The average molecular weight of SLX fell in a broader range than those of previous reports on sugarcane bagasse (65,000–212,000 g/mol) [67], wheat bran (470,000–600,000 g/mol) [68] and switch grass (47,700–64,300 g/mol) [69]. The difference in molecular weights was affected by xylan types and extraction methods that had an impact on xylan structure and degree of substitution [57]. The high molecular weight/DP xylan would allow a wider range

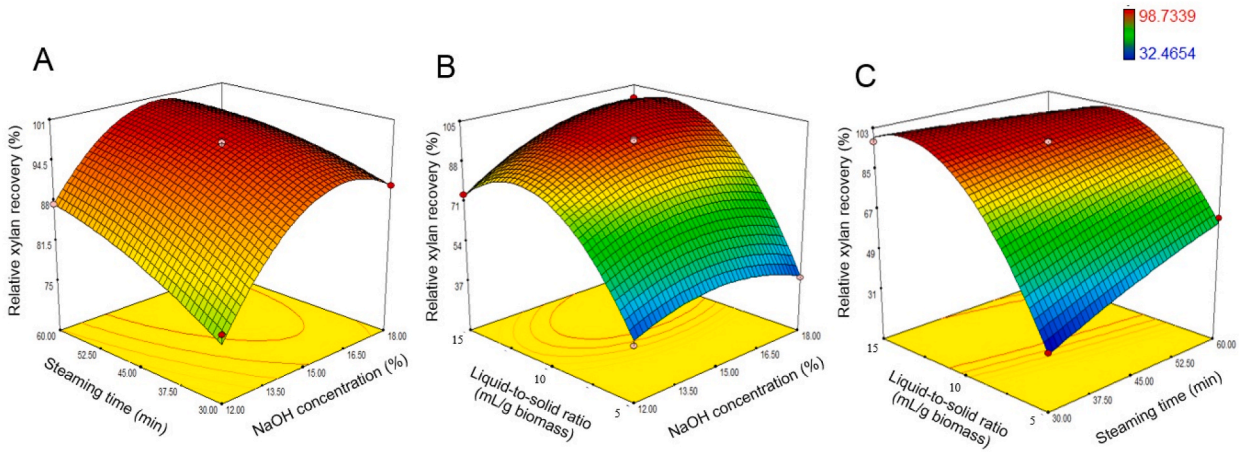

Fig. 1. Response surface plot for optimal condition of the relative xylan recovery from SL. (A) Effect of NaOH concentration and steaming time, (B) Effect of NaOH concentration and liquid-to-solid ratio and (C) Effect of steaming time and liquid-to-solid ratio.

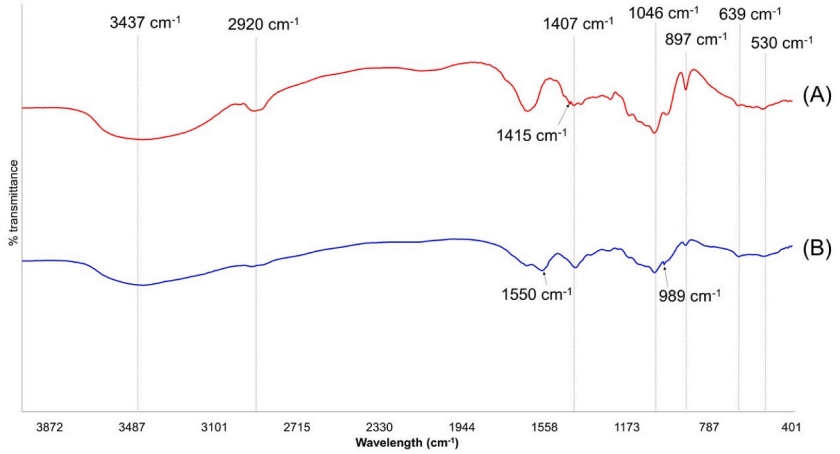

Fig. 2. The FT-IR spectra pattern of xylan: (A) commercial beechwood xylan (B) SLX.

of applications, including packaging files and gels that require high molecular weight xylan [70].

At a spindle speed of 5 rpm, the highest recorded viscosity value of SLX was 18.93 mPa s and this value rapidly decreased to 11.33 mPa s when the spindle speed was increased to 50 rpm (Fig. 3). However, the viscosity was quite stable at speeds ranging from 50 (11.33 mPa s) to 100 rpm (10.33 mPa s). With regard to SLX viscosity, the xylan was exhibited as a pseudoplastic whereby increasing the spindle speed or shear rate force damages the network structure of xylan and its derivatives to reduce the viscosity [71]. The viscous behavior of the xylan depended on the plant biomass type, molecular weight and structure [72]. The xylan viscosity of 11.33 mPa s (shear rate of 100 s^{-1}, spindle speed at 50 rpm) fell in a moderate range, similar to the viscosity of xylan from wheat, cotton and sugarcane bagasse (6.00–20.00 mPa s) [37,71,73]. However, the SLX viscosity was lower than that of xylan with a similar structure, such as sugarcane bagasse xylan. Heavily branched xylans reportedly had lower viscosity than linear or sparsely branched ones [74]. The SLX viscosity reported here showed potential for certain applications, including the improvement of paper strength [71,73].

3.3. Xylanase production from Aureobasidium spp.

In this study, 36 Thai strains of three *Aureobasidium* species, including *A. pullulans*, *A. thailandense* and *A. melanogenum*, were used to explore the prospect of xylanase production. The xylanase activities were detected in the range of 0.56–22.43 U/mL (Supplementary data I). The best xylanase producer was *A. pullulans* NRRL 58523. This species had the highest activity at 22.43 ± 0.27 U/mL, which was not significantly different from those of *A. pullulans* NRRL 58519 (21.64 ± 0.55 U/mL) and NRRL 58536 (21.84 ± 0.44 U/mL). This strain, NRRL58523, was selected for further determination of xylanase properties, including optimum pH, optimum temperature and thermostability.

In most enzymatic hydrolyses of hemicellulose previously reported, a very large dose of enzyme (>50 U/g) and a long incubation time (>24 h) were frequently employed [42,75,76]. Therefore, enzyme over-producing organisms and enzymes highly stable under an

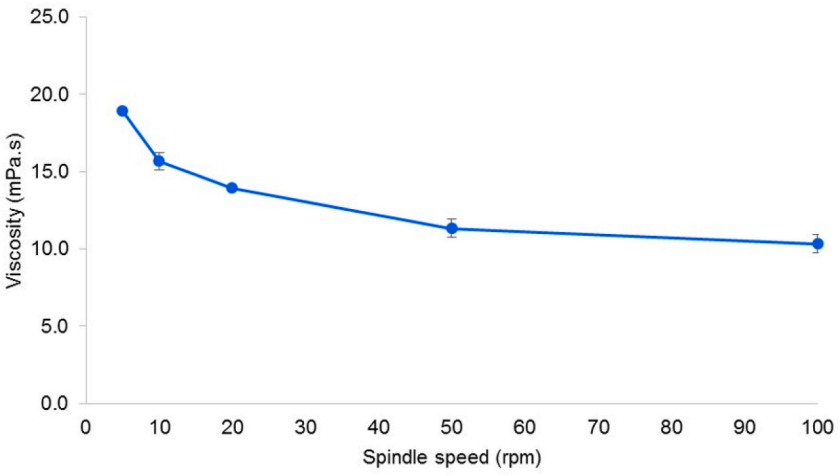

Fig. 3. The measured viscosity of SLX as function of spindle speed. Bars indicated one standard deviation derived from three replicates.

incubation condition are required [77]. *Aureobasidium* spp. have been reported as good xylanase producers [24] and the enzymes they produced exhibited a wide range of properties [26,30,44]. The selected strain, *A. pullulans* NRRL 58523, produced xylanases with an activity, which was higher than those of the previous reports, including those of xylanases from *A. melanogenum* PBUAP46 (5.19 U/mL; [44]), *A. pullulans* SN 090 (2.73 U/mL; [29]) and A. pullulans CBS 135684 (4.10 U/mL; [30]).

3.4. Xylanase properties

The optimum pH and temperature of crude xylanase from *A. pullulans* NRRL 58523 were pH 4.0 in 50 mM sodium citrate buffer and 50 °C, respectively (Fig. 4). The enzyme also functioned relatively well from pH 3.0 to 5.0 at temperatures from 40 to 60 °C. The enzyme lost most of its activity at pH values higher than 5 and did not function at all under neutral and alkali conditions. At 70 °C, the crude xylanase showed moderate activity (around 50 % of its maximum activity) and lost most of activity at 80 °C (Fig. 4). The crude xylanase from *A. pullulans* NRRL 58523 was highly stable at 40 °C and 50 °C with 91.99 ± 2.00 % and 87.38 ± 1.26 % of its initial activity remaining after 60 h, respectively. Exposure to temperatures of 60 °C and higher completely diminished the enzyme activity within 6 h (Fig. 5).

The crude enzyme from *A. pullulans* NRRL 58523 optimally functions at 50 °C in 50 mM sodium citrate buffer (pH 4.0), which is similar to a number of fungal xylanases, such as *Thermomyces lanuginosus*, *Trichoderma reesei* and *Aspergillus fumigatus* [78–80]. Interestingly, the fungal xylanases that hydrolyze well at room temperature (28 °C) and higher than 50 °C and milder pH (pH 6.0–7.0) reportedly have lower efficiency of xylan hydrolysis [33,44,75,81] than those which function well at temperatures between 40 and 50 °C, and moderately acidic pH (pH 4.0–5.0) [42,82,83]. In addition to the optimal temperature and pH, a thermostability test was conducted to prove that the crude enzyme still had its hydrolytic activity after a long incubation period, and the results showed that this enzyme was highly stable under its optimal conditions (50 °C in 50 mM sodium citrate buffer, pH 4.0) with over 87 % of its initial activity remaining. Most fungal xylanases reported to date are much less stable under optimal conditions, such as the enzymes from *A. pullulans* CBS 135684 [30], *A. flavus* [84] and *A. awamori* [85], with between 35 and 60 % of their initial activity remaining after 2–5 h. Therefore, the crude xylanase from *A. pullulans* NRRL 58523 has promising potential for the hydrolysis of SLX under optimal conditions.

3.5. Hydrolysis of SLX for XOs production

In general, XOs with low DP, such as X2 and X3, have had higher market values than those with higher DP since they show higher prebiotic properties and are commercialized as functional food products [86]. Therefore, the combined amount of X2 and X3 was chosen as the desired response of hydrolysis optimization. In the preliminary study, SLX was converted to XOs at 21.01 ± 0.42 mg/g xylan by using the crude NRRL 58523 xylanase (20 U/g xylan) in 50 mM sodium citrate buffer (pH 4.0) and incubating at 50 °C for 24 h (data not shown). Further optimization of XOs production was performed according to the CCD with 5 levels of 2 variable factors based on preliminary experiments (data not shown) and its thermostability profile. The response values (combined X2 and X3 contents) with predicted values are presented in Table 2. The combined yields of X2 and X3 contents were in the range of 20.71–252.88 mg/g xylan and the highest yield was found in trial no.6. The statistical significance of the model was calculated by ANOVA (Supplementary data III), and the actual data were fitted into a second-order polynomial equation (Equation (5)) as follows:

$$Y = 11.74A + 7.93B + 0.01AB - 0.09A^2 - 0.07B^2 - 366.67 \quad (5)$$

where Y was the combined yield of X2 and X3 contents (mg/g xylan), A was xylanase dosage (U/g initial xylan) and B was incubation time (h). Based on an R^2 value of 0.98, the equation could determine 98 % of the observed response. Although the coefficient of variation was relatively high (CV = 9.54 %), the significant model ($p = 0.0002$), and insignificant lack of fit ($p = 0.09$) suggested that the regression equation could accurately predict the response. For independence, Durbin and Watson test showed insignificant positive autocorrelation (1.66) indicating the precision and reliability of the experiments [55]. The response surface plots were plotted from the

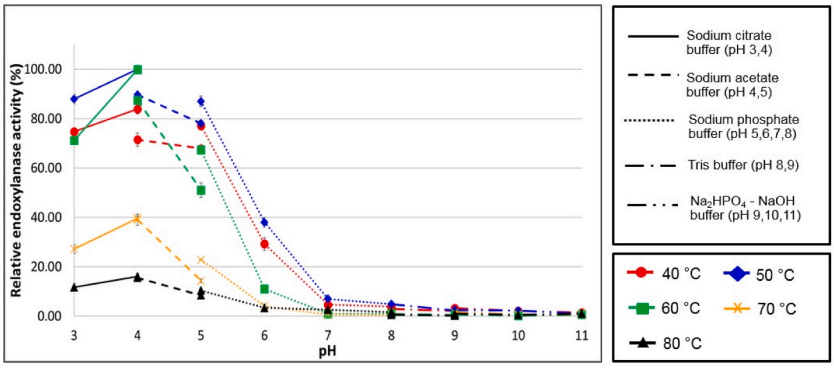

Fig. 4. Optimum pH and temperature of *A. pullulans* NRRL 58523 xylanase. Bars indicated one standard deviation derived from three replicates.

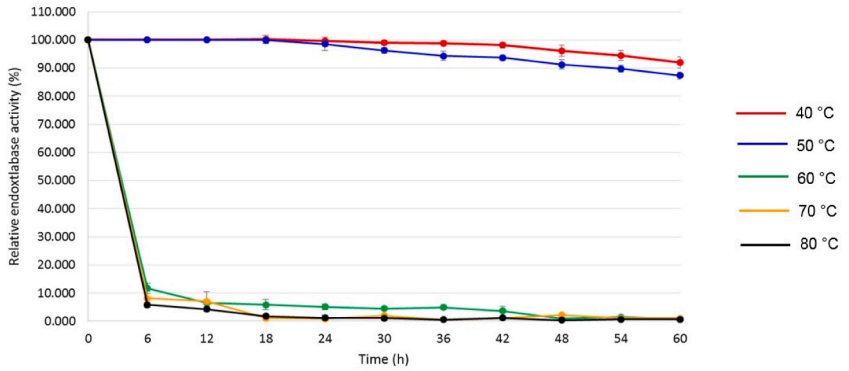

Fig. 5. Xylanase activity thermostability of *A. pullulans* NRRL 58523 endoxylanase. Bars indicated one standard deviation derived from three replicates.

Table 2
CCD-RSM used for optimization of XOs production.

Run	Actual level		Yield of X2 and X3[a] (mg/g xylan)	
	Xylanase dosage (A; U/g initial xylan)	Incubation time (B; h)	Observed[b]	Predicted
1	25 (−1)	24 (−1)	29.40 ± 0.74	26.63
2	75 (+1)	24 (−1)	180.71 ± 5.19	180.82
3	25 (−1)	72 (+1)	85.39 ± 3.51	101.35
4	75 (+1)	72 (+1)	250.90 ± 10.40	269.73
5	15 (−1.414)	48 (0)	20.71 ± 2.81	18.06
6	85 (+1.414)	48 (0)	252.88 ± 9.04	243.87
7	50 (0)	14 (−1.414)	97.44 ± 4.00	102.27
8	50 (0)	82 (+1.414)	239.63 ± 10.97	218.18
9	50 (0)	48 (0)	245.34 ± 9.51	238.76
10	50 (0)	48 (0)	238.66 ± 7.07	238.76
11	50 (0)	48 (0)	232.29 ± 9.07	238.76

[a] X2 = xylobiose and X3 = xylotriose.
[b] Mean ± SD derived from three replicates.

combined yields against a combination of variables (Fig. 6). The RSM assessment predicted that the maximum yield of X2 and X3 contents at 274.68 mg/g xylan could be achieved when incubating SLX with 65.31 U crude xylanase/g xylan at 50 °C for 53 h. The predicted yield was validated in an independent hydrolysis under the suggested conditions that produced a yield of 237.51 ± 17.69 mg/g xylan, which was not significantly different from the predicted value. The increment in XOs yield was up to 11.30-fold compared with the reference condition.

In comparison, enzymatic hydrolysis of xylan gives XOs yields (11.40–67.90 %) in similar, albeit slightly lower, ranges to those of acid hydrolysis (13.00–76.50 %) [13,76,87,88]. However, enzymatic hydrolysis yields more XOs with lower DP (X2-X3) than acid hydrolysis [76]. After optimization, the crude xylanase from *A.pullulans* NRRL 58523 yielded XOs at 24 % (1 % substrate concentration and 53 h incubation time) which was moderately efficient compared to other previously reported enzymatic hydrolysis (from 13.2 to 18.1 % with substrate concentrations between 2 and 15 % (w/v) and incubation time between 12 and 92 h) [33,44,75,89]. However, higher XOs yields, 30.6–69.7 % with substrate concentration between 0.1 and 0.3 % (w/v) and incubation time between 6 and 24 h, were also reported in endoxylanases from *Bacillus subtilis* [90], *Escherichia coli* [91], *A. versicolor* [13], *Pichia stipitis* [92] and transgenic *Pichia pastoris* [93]. In this study the substrate concentration (1 % w/v) was not optimized since previous reports suggested that lower substrate concentrations provided higher hydrolysate products than those of higher substrate concentrations and without end-product inhibition problem [94,95]. Moreover, undesirable product, such as furfural that was usually formed during acid hydrolysis, was not detected in the hydrolysates in this study (data not shown). The furfural-free XOs are more desirable since additional purification step is not required and subsequent microbial activity is not inhibited [96].

Previous studies have applied mixed SL glucan and xylan through complete saccharification, and the obtained monosaccharides were fermented to ethanol [9,11]. Up to 28 L (22.12 kg) ethanol were calculatedly obtained from 100 kg SL biomass [9]. Roughly, the ethanol production efficiency from mixed SL sugars seems to be comparable to that of the XO production in this study (23.7 %) although simultaneous study must be conducted to reach the finite conclusion. Methane was also obtained from either glucose alone or mixed monosaccharides after chemical pretreatment and enzymatic saccharification [14,15]. However, high conversion efficiency was obtained only when glucose alone was fermented but not mixed monosaccharides [14,15].

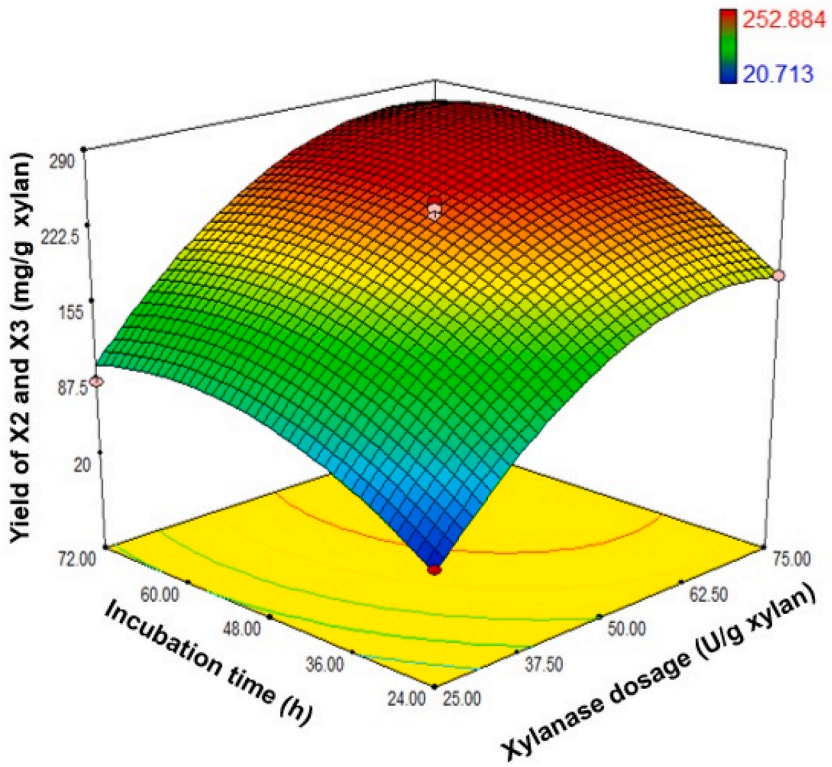

Fig. 6. Response surface plot showing the effect of xylanase dosage and incubation time for optimal condition of SLX hydrolysis.

3.6. Prebiotic property of XOs

Both positive controls, inulin and commercial FO, showed significant prebiotic activities toward all tested strains of probiotic bacteria in this study (Table 3). The enriched XOs were able to significantly stimulate the growth of all *Lactobacillus* strains tested at 7.67 ± 0.19 log CFU/mL for *L. casei* subsp. *rhamnosus*, 7.86 ± 0.03 log CFU/mL for *L. brevis* and 7.74 ± 0.23 log CFU/mL for *L. casei*, which was comparable to those of commercial FO. However, it was shown that XOs failed to activate the growth of every *Bifidobacterium* strain tested as growth was lower than that of glucose. The enriched XOs obtained in this study significantly enhanced *Lactobacillus* growth in a species-specific manner compared to glucose. No significant prebiotic activity was observed in *Bifidobacterium*. Such species-specific prebiotic activity toward *Lactobacillus* has been reported previously in XOs from corn fiber autohydrolysis [97]. *Lactobacillus* spp. are reportedly the major probiotic bacteria in the market [98], and these *Lactobacillus*-containing products have shown strong potential for the prevention of diseases and diarrhea [99]. Moreover, *Lactobacillus* spp. are more stable in the gastrointestinal tract than *Bifidobacterium* spp [100]. Therefore, XOs obtained from SLX via *A. pullulans* NRRL 58523 xylanase hydrolysis have high potential as commercial functional foods. Similar prebiotic activity toward *Lactobacillus* spp. has also been reported in XOs from vetiver grass and sugarcane bagasse [44,58].

4. Conclusions

Previous studies on sugarcane biomass utilization have, for the greatest part, focused on sugarcane bagasse. Only a few have centered on SL, and they were mainly for biofuel fermentation. This study shows that alternate products with higher market value such as those in functional food industry can also be produced from SL through an environmentally friendly process. This study showed the high potential of SL as the xylan source. SLX could be extracted using a simple protocol that yielded a nearly complete xylan recovery. Xylanase overproduction from *A. pullulans* NRRL 58523 also showed it had high potential as a source of cheap and highly stable xylanase. Although a moderate XOs yield was obtained under optimal conditions, it was free of toxic byproducts. The SL XOs also showed species-specific prebiotic activity toward *Lactobacillus* spp., thus they have high potential in functional food supplementation. Overall, this study showed that SL value could be enhanced if SL was used as the raw substrate for XOs production. Further upscale optimization could enhance the XOs yield and make it more cost efficient.

Table 3
Growth of *Lactobacillus* spp. and *Bifidobacterium* spp. in MRS medium with various carbon supplements (2 mg carbon/mL).

Carbon Supplement	Bacterial growth (log CFU/mL)*					
	B. animalis	B. longum	B. breve	*L. casei* subsp. *rhamnosus*	L. brevis	L. casei
None	6.48 ± 0.03^{d}	6.42 ± 0.11^{d}	6.63 ± 0.06^{c}	6.51 ± 0.07^{d}	6.36 ± 0.02^{d}	6.52 ± 0.06^{d}
Glucose	8.38 ± 0.06^{b}	8.33 ± 0.07^{b}	8.44 ± 0.04^{a}	7.42 ± 0.03^{c}	7.46 ± 0.15^{c}	7.50 ± 0.06^{c}
Inulin	8.65 ± 0.16^{a}	8.61 ± 0.02^{a}	8.49 ± 0.02^{a}	7.98 ± 0.07^{a}	8.10 ± 0.05^{a}	8.12 ± 0.08^{a}
FO	8.63 ± 0.06^{a}	8.36 ± 0.09^{b}	8.54 ± 0.06^{a}	7.84 ± 0.06^{ab}	7.97 ± 0.03^{ab}	7.89 ± 0.11^{ab}
XOs	7.68 ± 0.14^{c}	7.76 ± 0.14^{c}	7.93 ± 0.08^{b}	7.67 ± 0.19^{b}	7.86 ± 0.03^{b}	7.74 ± 0.23^{b}

* Mean ± SD derived from three replicates, different superscript letters in the same column indicate a significantly different values (ANOVA and DMRT, $p \leq 0.05$).

Declarations

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Data availability statement

No data was used for the research described in the article.
No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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