CHAPTER 3

Production of Xylooligosaccharides from Corncob Using a Crude Thermostable Endo-Xylanase from *Streptomyces thermovulgaris* TISTR1948 and Prebiotic Properties

3.1 Introduction

Xylooligosaccharides (XOs) are non-digestible carbohydrate made up from xylose linked with β-(1,4) bond with degree of polymerization from 2–6 (DP 2–6) which cannot be absorbed and used by human (Samanta et al., 2015). The health benefits of XOs are selective stimulating the growth and activity of one or a limited number of intestinal beneficial bacteria (Carvalho et al., 2013). Moreover, recent studies though *in vitro* and *in vivo* of XOs reported that XOs can promote the growth of probiotics such as *Bifidobacterium* spp. (Gobinath et al., 2010; Hsu et al., 2004; Manisseri and Gudipati, 2010; Rycroft et al., 2002), *B. adolescentis* (Chapla et al., 2012; Moura et al., 2007; Moura et al., 2008), *B. bifidum* (Chapla et al., 2012), *Lactobacillus brevis* (Moura et al., 2007; Moura et al., 2008), *L. fermentum* (Chapla et al., 2012) and *L. acidophilus* (Chapla et al., 2012).

XOs, promising prebiotics, are produced from xylan containing lignocellulosic materials (LCMs) by various methods such as chemical hydrolysis, enzymatic hydrolysis and enzymatic hydrolysis combined with chemical methods (Aachary and Prapulla, 2011). Enzymatic hydrolysis is a method of choice because of the specificity and controllability of the reaction. However, the production of XOs by enzymatic method requires alkali or acid pretreatment prior hydrolysis step for limiting a generation of unwanted products from LCMs, and increasing a hydrolysis rate (Romaní, et al., 2012).

An alkali pretreatment can solubilize hemicelluloses and celluloses, meanwhile generates inhibitor less than other processes. Thus, this method is suitable for the process that hemicellulose is desirable product. Moreover, it is more effective for LCMs that have low lignin content (<26%) such as corn stover, switchgrass, sugar cane bagasse, wheat bran, softwood and rice straw (Brodeur et al., 2011). Various types of alkali can be used to pretreat LCMs prior to XOs production e.g. NaOH, KOH, $Ca(OH)_2$ and NH_4OH (Jayapal et al., 2013; Samanta et al., 2012a). In this study, we found that KOH was a choice chemical for corncob pretreatment that gave high recovery yields of 43.69±1.30% (w/w) more than a conventional pretreatment using NaOH (38.77±1.47% (w/w)).

Endo-xylanase, a key enzyme for xylan hydrolysis is a glycoside hydrolase that can randomly hydrolyze internal linkages in xylan to produce XOs (Javier et al., 2007). Recently, enzymatic production of XOs from various alkali-treated LCMs by numerous xylanases have been reported such as corncob (Aachary and Prapulla, 2009; Ai et al., 2005; Li et al., 2012; Teng et al., 2010; Uçkun Kiran et al., 2013), wheat bran (Manisseri and Gudipati, 2010; Wang and Lu, 2013), natural grass (Samanta et al., 2012a), oil palm frond fiber (Sabiha-Hanim et al., 2011) and sugarcane bagasse (Bian et al., 2013; Jayapal et al., 2013). *Streptomyces* spp. is one of dominant xylanolytic bacteria that mainly produce endo-type xylanase (Li et al., 2009). While, *Streptomyces thermovulgaris* TISTR1948 was previously reported as a thermostable endo-xylanase producer. A high level of xylanase activity of 274.49 U/mL or 10,000 U/g was obtained when rice straw was used as the sole carbon source (Chaiyaso et al., 2011).

Thailand generates more than 59 million tons of agricultural waste annually especially rice straw, corn stover, corncob, sugarcane bagasse and rubber saw dust. Accordingly, there is no market demand for these materials, most of farmers tend to dispose them by burning (Department of alternative energy development and efficiency, Ministry of Energy, 2018). Corncob is one of high xylan content-LCMs and to manipulate this agricultural waste, it could be used as a potential raw material for XO production (Samanta et al., 2012b). Thus, the main purposes of the present study were to investigate the chemo-enzymatic process for XO production from KOH-treated corncob using the in-house thermostable endo-xylanase from *S. thermovulgaris*

TISTR1948 and to examine the prebiotic activity of that produced corncob-XOs. For optimization purpose, effect of three independent variables, namely, enzyme concentration, pH and temperature on XO production were studied using a response surface methodology (RSM) via central composite design (CCD). Moreover, the prebiotic property of corncob-XOs was also investigated using three probiotic lactic acid bacteria (LAB) strains.

3.2 Materials and Methods

3.2.1 Microorganisms

S. thermovulgaris TISTR1948 was used as the in-house thermostable endo-xylanase producer. The isolated strain *S. thermovulgaris* TISTR1948 was identified by 16S rDNA gene sequence analysis (1,390 bp with 99.0% identity matching of *S. thermovulgaris* NRRL B-12517T (Accession number DQ442547.1). The identified strain was deposited to the Thailand Institute of Scientific and Technological Research (TISTR) as the TISTR1948. The probiotic LAB strains were kindly given by the culture collection section of the TISTR. Three strains, including, *Lactobacillus casei* TISTR1463, *L. lactis* TISTR1464 and *L. plantarum* TISTR1465 were used for prebiotics activity study. All of microorganisms were maintained at –20°C in glycerol stock.

3.2.2 Raw materials

Corncob and rice straw were kindly given by the local farmers in Chiang Mai and Phayao provinces, Thailand in December of 2012. They were sun dried and cut to 10–cm length. Both of dried materials were further ground by a Hammer mill (Munson, USA), sieved by 100 mesh sieving, and kept in dry place at 4°C until used.

3.2.3 Chemicals

Oat spelt xylan, beech wood xylan, dinitrosalicylic acid (DNS), xylose (X1) and arabinose were purchased from Sigma, USA. Xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentaose (X5) were purchased from Megazyme, Ireland. Other chemicals used in this study were of analytical grade.

3.2.4 Corncob composition and morphology analysis

The cellulose (TAPPI T-203-cm-99), hemicellulose (TAPPI T-203-cm-99), lignin (TAPPI T-222-om-02) and ash (T-211-om-02) contents of those materials were determined by the TAPPI method which was analyzed the Animal Nutrition Laboratory, Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University (Boonchuay et al., 2014; Romaní et al., 2012) (Appendix A-10, A-11, and A-12). Dried samples of each step were mounted on stubs, placed on conductive carbon tape and coated with gold using a sputter coater (JFC-1200, JEOL) at 15 mA for 150 s. Then, gold-coated samples were viewed under a scanning electron microscope (SEM; JEOL 5410-LV, JEOL, Japan) to observe the morphology, surface area and physical structure.

3.2.5 Enzyme assays

Xylanase activity was measured using 1.0% (w/v) beech wood xylan (Sigma, USA) solution in 0.1 M potassium-phosphate buffer (pH 6.5) as substrate. The clear supernatant from strain TISTR1948 culture medium was diluted in 0.1 M potassium-phosphate buffer (pH 6.5) and incubated at 55°C with 1.0% (w/v) beech wood xylan for 10 min (Appendix A-4). The release of reducing sugars was measured using dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of xylanase activity (U) is defined as the amount of enzyme liberating 1.0 µmol of reducing sugar (as xylose) per min under assay condition (Chaiyaso et al., 2011).

3.2.6 Analysis of xylooligosaccharides, other sugars and lactic acid

Samples were filtered through a membrane filter (0.2 µm Nylon membrane, FiltrEX, USA) and subjected to HPLC analysis (SCL-10Avp; Shimadzu, Kyoto, Japan) with an Aminex HPX 87H column (300×7.8 mm; Bio-Rad, Hercules, USA). The mobile phase consisted of 5.0 mM H_2SO_4 as an eluent at a flow rate of 0.45 mL/min (Boonchuay et al., 2014). The column thermostat was set at 40°C. XOs, other sugars and lactic acid were detected using RI detector (refractive index detector RID-10A) in a linear gradient over 25 min (Appendix A-2).

3.2.7 Alkali pretreatment of corncob

The dried corncob powder was subjected to alkali pretreatment by soaking in 5.0–10.0% (w/v) KOH at 90°C for 1 h, followed by adjustment of the pH to 7.0 by addition of 5.0% (w/v) H_2SO_4. The neutralized corncob was washed with tap water, filtered through a muslin cloth. Then, the filtrate was dried at 80°C in a hot air oven (UN110; Memmert, Germany) until the constant weight was obtained. The KOH-treated corncob powder was kept in dry and cool place at 4°C.

3.2.8 Thermostable endo-xylanase production

Crude thermostable endo-xylanase production was carried out in the basal medium which contained, yeast extract 5.42 g/L, K_2HPO_4 1.0 g/L, KH_2PO_4 0.5 g/L, $(NH_4)_2SO_4$ 1.0 g/L, NaCl 0.2 g/L, $MgSO_4.7H_2O$ 0.1 g/L, $CaCl_2.2H_2O$ 0.1 g/L, Tween 80 0.1 g/L and rice straw 27.45 g/L as sole carbon source. Initial pH was adjusted to 7.11 with 1.0 N NaOH or 1.0 N HCl before autoclaving at 121°C for 20 min (Appendix B-2). Strain TISTR1948 was cultivated in 250-mL Erlenmeyer flask containing 50 mL of basal medium and incubated in a shaking incubator (LSI-3016R, Labtech, Korea) at a shaking speed of 250 rpm at 50°C for 96 h (Chaiyaso et al., 2011).

3.2.9 Productions of xylooligosaccharides

The KOH-treated corncob was used as a substrate for XO production. The substrate (15.0% (w/v)) was then subjected to enzymatic hydrolysis by mashing with 10 mM potassium-phosphate buffer pH 6.5. Then, 100 $U/g_{substrate}$ of crude thermostable endo-xylanase from *S. thermovulgaris* TISTR1948 was added, and the reaction was carried out at 55°C under static conditions for 24 h. Samples from KOH-treated corncob, oat spelt xylan and beech wood xylan were periodically taken and analyzed by thin-layer chromatography (TLC, silica gel 60 F_{254}, Merck, Germany) (Kubata et al., 1994) compared with the standard xylose (Sigma, USA), xylobiose, xylotriose, xylotetraose and xylopentaose (Megazyme, Ireland) (Appendix A-1).

3.2.10 Optimization of xylooligosaccharide production using response surface methodology

The optimal conditions for XO production from KOH-treated corncob was studied by RSM via a CCD by the Design Expert® software version 6.0.10 (Stat-Ease Inc., Minneapolis, USA). The range and center point values of three independent variables, namely, X_1: xylanase concentration (U/$g_{substrate}$), X_2: pH of buffer and X_3: temperature (°C), were presented in Table 3.1. The CCD contained an imbedded factorial or fractional factorial matrix with center points and star points around the center point that allowed estimation of the curvature. The distance from the center of the design space to a factorial point was ±1 unit for each independent variable, and the distance from the center of the design space to a star point was ±α, where |α|>1. The precise value of α depended on certain properties needed for the design and on the number of factors used (in this case α = 1.68). The star points represent new extreme values (low and high) for each independent variable in the design (Haaland, 1989). The data obtained from CCD were analyzed and the significant values of the model were evaluated by Fisher's test as expressed in term of the *F*-ratio:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad (1)$$

Where Y represents the response variable, β_0 is the interception coefficient, β_i the coefficient for the linear effect, β_{ii} the ij^{th} coefficient of the interaction effect, and $X_i X_j$ are input variables that influence the response variable Y. The response variable in each trial was the average of the three replicates.

A range of enzyme concentration between 100–150 U/$g_{substrate}$ with a boundary of 82–168 U/$g_{substrate}$, pH level 5.5–7.5 with a boundary of 4–9 for ±α, and temperature between 45–65°C with the boundary of 38–72°C for ±α were selected in the experimental design (Table 3.1). The CCD generated a total of 17 experiments with three trials for center points. The total of 17 experiments generated from CCD was carried out. After that, the samples from each experiment were periodically taken at preset times (0, 6, 12, 18 and 24 h), centrifuged (10,000 rpm) at 4°C and analyzed by HPLC (Appendix A-2).

The validation of CCD optimization model was carried out by the hydrolysis of KOH-treated corncob under the suggested optimal conditions.

Table 3.1 Experimental codes, ranges and levels of independent variables in the response surface methodology (RSM).

Variables	Units	Symbol codes	Levels				
			-α	**Low (-1)**	**Center (0)**	**High (+1)**	**+α**
Enzyme concentration	$U/g_{substrate}$	X_1	82.96	100.00	125.00	150.00	167.04
pH	-	X_2	4.82	5.50	6.50	7.50	8.18
Temperature	°C	X_3	38.18	45.00	55.00	65.00	71.82

3.2.11 Preparation of corncob-xylooligosaccharides powder

After enzymatic hydrolysis under suggested optimal condition from CCD, the crude XOs were separated by filtering through a filter paper (Whatman No. 4). The liquid hydrolysate was demineralized by mixing with 10% (w/v) of DEAE-cellulose (Sigma, USA) at 4°C for 30 min, then, centrifuged at 6,000 rpm for 15 min at 4°C. After that, the hydrolysate was 5-times concentrated by a rotary vacuum evaporator (Rotavapor® R-3, Büchi Labortechinik, Switzerland) at 55ºC. After the concentration process, 5.0% (w/v) maltodextrin was added before processing by a spray drying (Spray dryer, JCM Engineering Concept Co., Ltd., Bangkok, Thailand) to recover corncob-XOs in a powder form.

3.2.12 *In vitro* fermentation of corncob- xylooligosaccharides

The probiotic LAB, *L. casei* TISTR1463, *L. lactis* TISTR1464 and *L. plantarum* TISTR1465 were used for study of prebiotics activity. The different carbon sources, including, glucose, xylose, maltodextrin, commercial XOs and corncob-XOs were used. The MRS media was used containing of protease peptone 10.0 g/L, beef extract 10.0 g/L, yeast extract 5.0 g/L, tween 80 1.0 g/L, ammonium citrate 2.0 g/L, sodium acetate 5.0 g/L, $MgSO_4$ 0.1 g/L, $MnSO_4$ 0.05 g/L, K_2HPO_4 2.0 g/L, and supplemented either

with glucose or xylose or maltodextrin (Sigma, USA) or commercial XOs (Wako, Japan) or corncob-XOs (20.0 g) as a sole carbon source (Appendix B-3).

The inoculum was prepared by pre-culture of each three LAB strains in MRS medium (Chapla et al., 2012) at 30°C under anaerobic condition in anaerobic jar (Schuett-Biotec GmbH, Germany) for 24 h. After that, the pre-cultured probiotic LAB was inoculated to MRS media containing each carbon source (glucose or xylose or maltodextrin or commercial XOs or corncob-XOs). The probiotic cultures were grown at 30°C under anaerobic condition. The culture broth was periodically taken at 0, 6, 12, 18, 24, 36 and 48 h, and monitored the growth and utilization of carbon source by a viable count on MRS plate. Colony forming units (CFU) were counted in plates containing 30 to 300 colonies and cell concentration was expressed as log CFU/mL.

3.3 Results and Discussion

3.3.1 Alkali pretreatment of corncob

The recovery yield after alkali pretreatment by 10% (w/v) KOH was 43.69±1.30% (w/w); the major components of raw corncob and KOH-treated corncob are shown in Table 3.2. After alkali pretreatment, hemicellulose and lignin content in KOH-treated corncob were decreased because the alkali solution may cause swelling leading to an increase in internal surface area, reduce the degree of polymerization (DP) and crystallinity, and disrupt the crystalline structure by separation of structural linkages between lignin, hemicellulose and cellulose (Brodeur et al., 2011). Therefore, the amount of hemicellulose and lignin soluble in the alkaline solution resulted an increase of cellulose content in KOH-treated corncob. Although alkali pretreatment resulted in decreasing hemicellulose content, the alkali pretreatment helped the enzymatic hydrolysis reaction by making LCMs a suitable substrate for enzymatic hydrolysis and more accessible to the endo-xylanase (Brodeur et al., 2011).

3.3.2 Productions of xylooligosaccharides

After periodically taking the samples, hydrolysis products from KOH-treated corncob were analyzed by TLC (Figure 3.1) and compared with the hydrolysis products of commercial xylan, namely, oat spelt xylan (Figure 3.2) and beech wood xylan

(Figure 3.3). The results showed that the product from enzymatic hydrolysis of KOH-treated corncob contained several types of XOs, including, xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentaose (X5). The optimum hydrolysis time for XO production from the KOH-treated corncob was 12 h, when xylobiose was found as the main product, while the xylose content was low. Similar to the results of Lee et al. (2009) who reported that the mainly components of the hydrolysis products from birch wood xylan catalyzed by recombinant endo-xylanase from *Saccharomyces cerevisiae*, were xylobiose and xylotriose (Lee et al., 2009).

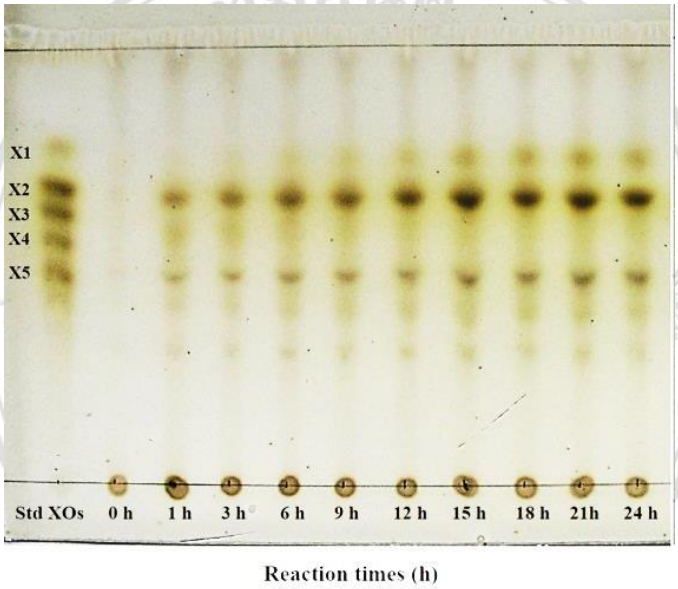

Figure 3.1 TLC chromatogram of the time course for xylooligosaccharide production from KOH-treated corncob catalyzed by endo-xylanase from *Streptomyces thermovulgaris* TISTR1948 (xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentose (X5))

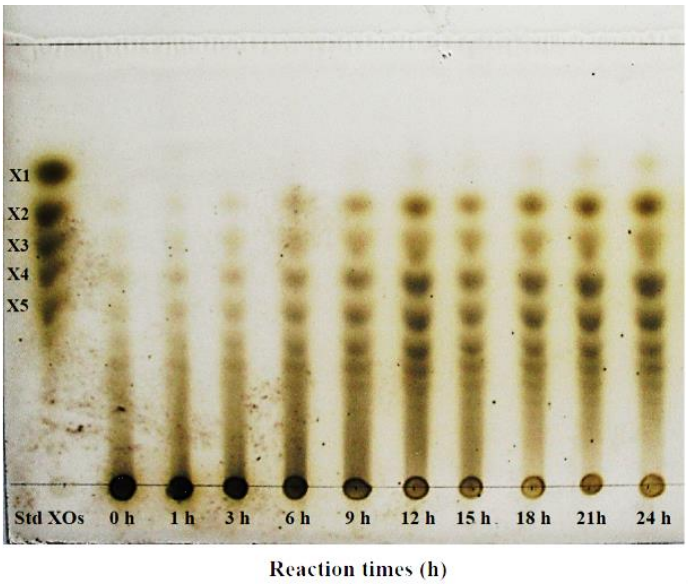

Figure 3.2 TLC chromatogram of the time course for xylooligosaccharide production from oat spelt xylan catalyzed by endo-xylanase from *Streptomyces thermovulgaris* TISTR1948 (xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentose (X5))

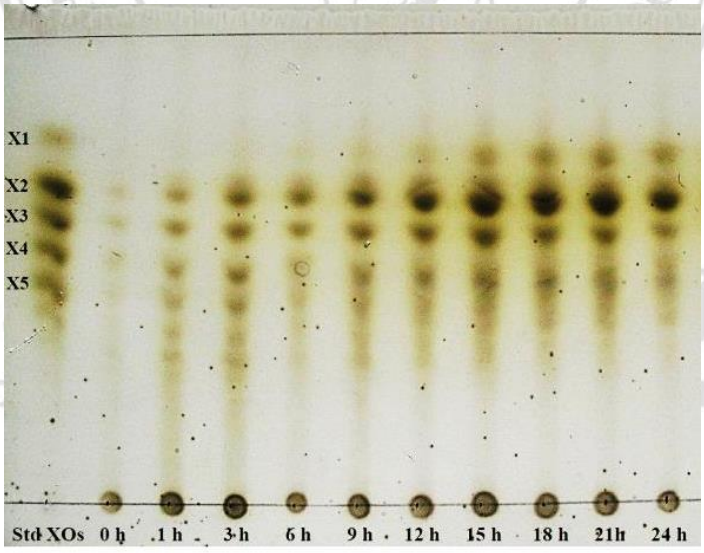

Figure 3.3 TLC chromatogram of the time course for xylooligosaccharide production from beech wood xylan catalyzed by endo-xylanase from *Streptomyces thermovulgaris* TISTR1948 (xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentose (X5))

3.3.3 Analysis of corncob composition

The compositions of raw corncob, KOH-treated corncob and hydrolyzed KOH-treated corncob were analyzed by the method of TAPPI as shown in Table 3.2. The results showed that hemicellulose content in xylanase hydrolyzed KOH-treated corncob was 5.09% and this decreased from 24.67% in KOH-treated corncob, indicating that hemicellulose was enzymatic hydrolyzed to XOs. The XO content obtained from the optimal condition was 162.99 $mg/g_{substrate}$ or 752.15 $mg/g_{hemicellulose}$ content which obtained by the calculation from hemicellulose content in KOH-treated corncob. Moreover, the results also revealed that the XO content was about 16.30% and other products of xylose and arabinose were about 3.28% (by weight of hemicellulose content in KOH-treated corncob) which was similar to the decreased content of hemicellulose in enzymatic hydrolyzed KOH-treated corncob (19.58%).

Table 3.2 The composition of raw corncob, KOH-treated and hydrolyzed KOH-treated corncob.

Composition (%)	**Raw corncob**	**KOH-treated corncob**	**Hydrolyzed KOH-treated corncob**
Cellulose	40.38±1.02	65.21±1.41	84.81±1.10
Hemicellulose	41.45±1.23	24.67±0.71	5.17±0.17
Lignin	7.26±1.17	4.29±0.40	5.27±0.25
Ash	1.37±0.03	0.47±0.03	0.04±0.03
Other components	9.54±1.61	5.36±0.64	4.71±0.39

3.3.4 The optimal conditions for xylooligosaccharide production

In this study, three independents variables, namely, enzyme concentration, pH and temperature were selected for the optimization of XOs production. The central composite design (CCD) experiment led to a total of 17 sets of experiments. The low, center and high levels of each variable and the experimental design and experimental results are shown in Table 3.3. The results obtained by the CCD were analyzed by

ANOVA (Table 3.4). The CCD generated a quadratic equation for XO production yield (Y) as follows:

$$Y = +150.58+4.39X_1–13.90\ X_2–8.15X_3–3.47X_1^2–16.34\ X_2^2–18.74X_3^2–6.83X_1X_3 \quad (2)$$

The experimental values, predicted values and XOs after 6, 12, 18 and 24 h of reaction time in 17 conditions are shown in Table 3.3. Various statistical data (standard error of estimate, sum of squares of the error, F statistic and p-value) were examined, as shown in Table 3.4. The quality of the model was expressed in terms of the R^2 value. The predicted values match the experimental values, at values of R^2 = 0.9842, adj-R^2 = 0.9640 and pred-R^2 = 0.8810. The results showed that all three factors were significant and influenced the yield of XOs.

Table 3.3 Experimental and theoretically predicted values for xylooligosaccharide production (mg/$g_{substrate}$) from KOH-treated corncob.

Run order	Code levels			Xylooligosaccharide yields (mg/g $_{substrate}$)				
	X_1*	X_2**	X_3***	Predicted values (12 h)	Actual values			
					6 h	12 h	18 h	24 h
1	82.96	6.50	55.00	133.4	91.2	138.0	109.8	101.7
2	125.00	6.50	38.18	111.3	75.1	112.0	94.7	92.8
3	100.00	7.50	65.00	95.2	37.0	92.0	88.7	86.1
4	100.00	5.50	65.00	117.4	42.1	119.0	105.5	78.7
5	167.04	6.50	55.00	148.2	90.4	143.0	139.7	132.3
6	150.00	7.50	45.00	114.7	66.2	114.0	84.9	79.4
7	125.00	6.50	55.00	150.6	93.6	150.0	130.7	118.9
8	100.00	7.50	45.00	98.3	60.1	98.0	72.8	63.4
9	125.00	4.82	55.00	127.8	99.3	126.0	109.9	99.5
10	150.00	7.50	65.00	84.3	64.0	89.0	87.3	75.6
11	150.00	5.50	65.00	118.6	85.6	120.0	84.2	82.2
12	125.00	6.50	55.00	150.6	92.8	149.0	103.8	91.6
13	125.00	8.18	55.00	81.0	58.7	81.0	78.6	74.1
14	125.00	6.50	55.00	150.6	87.6	154.0	145.4	142.9
15	150.00	5.50	45.00	148.1	106.4	153.0	153.1	148.9
16	100.00	5.50	45.00	119.6	40.2	115.0	88.5	85.5
17	125.00	6.50	71.82	83.9	41.8	82.0	69.0	69.2

*X_1: Enzyme concentration (U/$g_{substrate}$); **X_2: pH; ***X_3: Temperature (°C)

Table 3.4 Analysis of variance (ANOVA) of the quadratic model for response variable.

Probability	**SS**	**DF**	**MS**	***F*-value**	***p*-value**
Original model	**9927.43**	**9**	**1103.05**	**48.59**	**<0.0001[a]**
Linear effect					
X_1*	263.71	1	263.71	11.62	0.0113[a]
X_2	2640.40	1	2640.40	116.31	<0.0001[a]
X_3	906.48	1	906.48	39.93	0.0004[a]
Quadratic effect					
X_1^2	135.77	1	135.77	5.98	0.0444[a]
X_2^2	3010.21	1	3010.21	132.60	<0.0001[a]
X_3^2	3959.57	1	3959.97	174.42	<0.0001[a]
Interaction effect					
X_1X_2	74.04	1	74.04	3.26	0.1139[b]
X_1X_3	373.66	1	373.66	16.46	0.0048[a]
X_2X_3	0.40	1	0.40	0.018	0.8981[b]
Residual	158.91	7	22.70		
Lack of Fit	145.67	5	29.13	4.40	0.1955[b]
Pure Error	13.24	2	6.62		
Cor Total	10086.34	16	1407.57		
Coefficient of determination					
R^2					0.9842
Adj R^2					0.9640
Modified model	**9852.99**	**7**	**25.93**	**54.29**	**<0.0001[a]**
Residual	233.36	9	31.44		
Lack of Fit	220.11	7	6.62	4.75	0.1849[b]
Pure Error	13.24	2			
Cor Total	10086.34	16			
R^2					0.9769
Adj R^2					0.9589

X_1: Enzyme concentration ($U/g_{substrate}$); X_2: pH; X_3: Temperature (°C)

[a] Significant at $p \leq 0.05$

[b]Not significant at $p \leq 0.05$

From Table 3.4, the probability *p*-value of the model was relatively low (<0.0001), indicating a significant model. The coefficient of variation for the original model (R^2 = 0.9842) and modified model (R^2 = 0.9769) were represented and they

implied a high correlation between the experimentally observed. So, both models were used in the prediction of XO production from KOH-treated corncob using thermostable endo-xylanase from *S. thermovulgaris* TISTR1948. The interaction term between the enzyme concentration and temperature (X_1X_3) had a relatively low *p*-value less than 0.05 at 0.0048. From the *p*-value, it was deduced that the xylanase concentration and temperature levels influenced on XO production.

According to Table 3.3, the run orders of 7, 12 and 14 could enhance XOs production to the levels of 149.81, 148.61 and 153.55 $mg/g_{substrate}$, respectively. From the Figure 3.4, it obvious that XO production yield was strongly affected by enzyme concentration and temperature levels. The XO production yield increased when thermostable endo-xylanase concentration was elevated from 137.98 $mg/g_{substrate}$ at 82.96 $U/g_{substrate}$ (low point $-\alpha$) to 153.55 $mg/g_{substrate}$ at 125.00 $U/g_{substrate}$ (center point). The results indicated that thermostable endo-xylanase from *S. thermovulgaris* TISTR1948 had potential as an effective enzyme for XO production from corncob. However, at an enzyme dosage higher than 150 $U/g_{substrate}$, XO production yield was decreased. Similar result was observed by Jayapal et al. (2013), the concentrations of xylobiose were decreased from 1.18 to 0.83 mg/mL when xylanase dosages were increased from 2.65 to 13.25 U. Moreover, Brienzo et al. (2010) reported that an increasing of *Thermoascus aurantiacus* xylanase dosage higher than 120 U/g, the XO yield decreased rapidly. The possible reason for this effect is the reduction of DP of xylan generates high amount of xylose from using higher enzyme dosage (Uçkun Kiran et al., 2013).

Temperature is one of the important factors for xylanase activity (Techapun et al., 2003). As shown in Table 3.3 and Figure 3.4, high temperature (71.82°C) significantly decreased XO production yield because of inactivation of enzyme at higher temperature during long reaction time (Chapla et al., 2012). The maximum XO production yield of 153.55 mg/g substrate was obtained at the center point (55°C).

To confirm the applicability of the CCD optimization model, the XO production was carried out by the hydrolysis of KOH-treated corncob under the suggested optimal conditions; endo-xylanase dosage of 129.43 $U/g_{substrate}$, and pH 6.17 at 53.80°C for maximum XO yield. From the experimental results, a yield of XOs of 162.97 $mg/g_{substrate}$ or 752.15 $mg/g_{hemicellulose}$ was obtained, a value higher than predicted by 5.10%. This result indicated that the model generated by CCD could be used to predict the maximum yields of XOs.

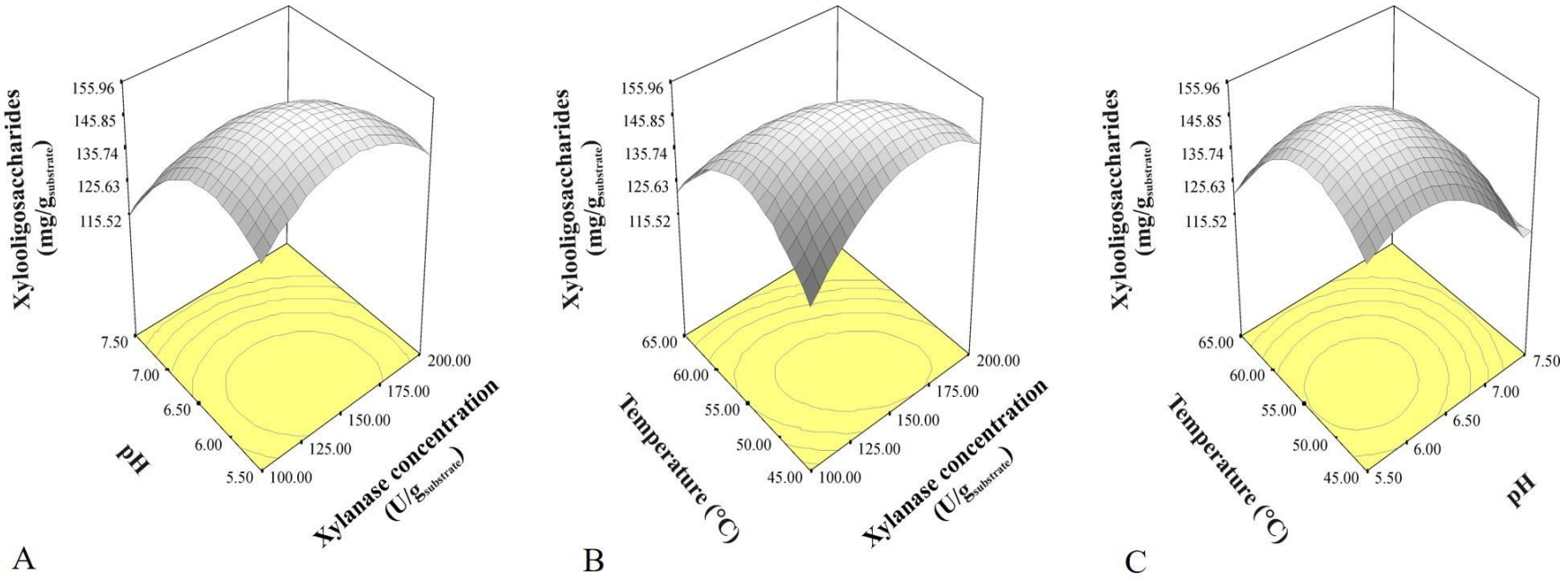

Figure 3.4 Xylooligosaccharide production from KOH-treated corncob in three-dimensional graphic for quadratic response surface optimization. The comparison was made between (A) enzyme concentration and pH value of buffer (X_1X_2), (B) enzyme concentration and temperature (X_1X_3) and (C) pH value of buffer and temperature (X_2X_3).

3.3.5 Time course of xylooligosaccharide production from CCD validation model

The time course of XO production in validation of the CCD experiment model is shown in Figure 3.5. As a general trend, the XO concentrations increased sharply in 12 h of reaction time (Figure 3.5A). The HPLC chromatogram (Figure 3.5B) at appropriate reaction time (12 h) show the maximum XO yield of 162.97±2.85 mg/$g_{substrate}$ with concentration of xylobiose (85.15±1.18 mg/$g_{substrate}$), xylotriose (19.34±0.55 mg/$g_{substrate}$), xylotetraose (23.58±1.62 mg/$g_{substrate}$) and xylopentaose (34.90±0.31 mg/$g_{substrate}$), were obtained, while a low xylose content of 17.70±0.80 mg/$g_{substrate}$ and arabinose content of 15.14±1.55 mg/$g_{substrate}$ was observed. After 12 h of reaction time, XO contents continuously decreased, the XO yield dropped to 143.76±1.54 mg/$g_{substrate}$ at 24 h of reaction time, while the xylose content reached to 41.16±1.65 mg/$g_{substrate}$. From these results, as well as the report of Javier et al. (2007) revealed that endo-xylanase is still active on xylooligomers with a degree of polymerization higher than 2. Hence, endo-xylanase can hydrolyze xylotriose to one molecule of each xylobiose and xylose under the late of the reaction time.

The XO production yields between chemo-enzymatic conversion using the thermostable endo-xylanase from strain TISTR1948 and other methods have been compared. Li et al. (2012) reported that the purified endo-xylanase from *S. rameus* L2001 could hydrolyze NaOH-treated corncob to produce XOs with the yield of 150 mg/g_{xylan}, and Ai et al. (2005) revealed that immobilized xylanase from *S. olivaceoviridis* E-86 produced XOs from the same substrate with the yield of 387.5 mg/g_{xylan}. However, the XOs production yield obtained from both *Streptomyces* strains were relatively low compared to the TISTR1948 (752.15 mg/$g_{hemicellulose}$). Moreover, Jayapal, et al. (2013) reported a chemo-enzymatic process for conversion of sugarcane bagasse to XOs by NaOH pretreatment and enzymatic hydrolysis by a commercial xylanase from *Trichoderma viridae*. A total reducing sugar yield of 367.79 mg/g_{xylan} under optimum conditions, was obtained. While, using of commercial xylanase (Shearzyme 500L and Veron 191) could produce XOs only 143 mg/g_{xylan} when KOH combined with $NaBH_4$-treated corncob was used as a substrate (Uçkun Kiran et al., 2013). In this study, a higher XO content of 162.97 mg/$g_{substrate}$ or 752.15

mg/$g_{hemicellulose}$ was obtained at 12 h of reaction times, with only small amounts of xylose. Moreover, thermostable endo-xylanase from *S. thermovulgaris* TISTR1948 showed high capability to hydrolyze xylan (>75% xylan content), thermostability of enzyme (50–70^{o}C) and this enzyme was produced from low cost agricultural waste (rice straw). According to the abovementioned points, our in-house thermostable endo-xylanase had an advantage on economical production cost over other xylanases.

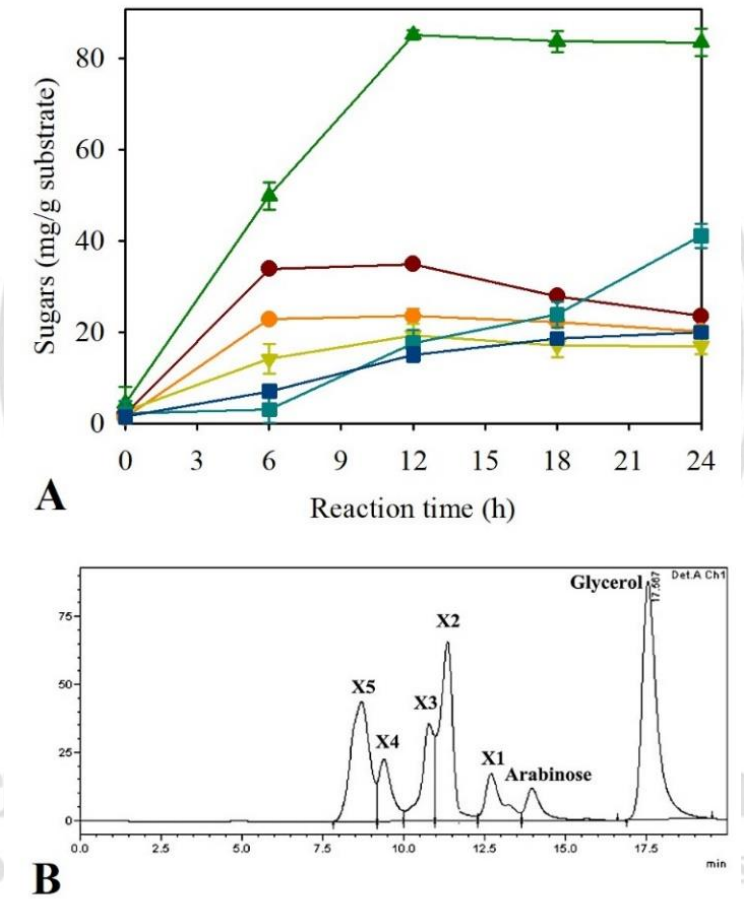

Figure 3.5 (A) Time course of xylooligosaccharide production from KOH-treated corncob in validation of CCD experiment model (▲: xylobiose, ●: xylopentaose, ●: xylotetraose, ▼: xylotriose, ■: xylose and ■: arabinose) and (B) HPLC chromatogram of xylooligosaccharide from alkali-pretreated corncob at 12 h of reaction time.

3.3.6 Scanning electron microscope analysis of corncob samples

The results of scanning electron microscope (SEM) showed that different corncob samples had different surface morphologies. Raw corncob was rigid with a smooth lignocellulose structure and no pores (Figure 3.6A and B). Meanwhile, KOH-treated corncob (Figure 3.6C and D) showed a flaky and rough surface. Thus, KOH increased the surface area accessibility of the enzyme, breakdown of the rigid structure, and partial decrystallization of the corncob. After enzymatic hydrolysis using the thermostable endo-xylanase from strain TISTR1948, the corncob surface had more surface area, a porous structure, and a rough surface (Figure 3.6E and F). Thus, endo-xylanase could breakdown the amorphous structure of hemicellulose to release XOs and other sugars, and retain cellulose in the rough corncob.

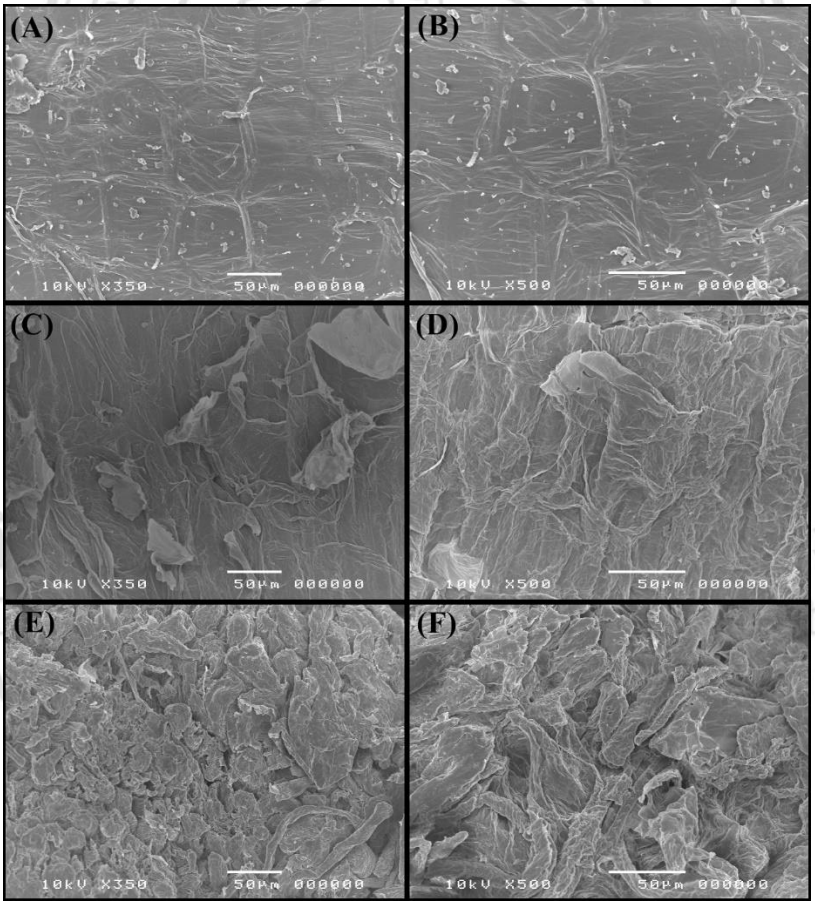

Figure 3.6 Scanning electron microscope (SEM) photomicrograph of the surface of raw corncob at (A) 350× and (B) 500×, KOH-treated corncob at (C) 350× and (D) 500×, and hydrolyzed KOH-treated corncob at (E) 350× and (F) 500×.

3.3.7 *In vitro* fermentation of corncob-xylooligosaccharides

In this study, three probiotics lactobacilli strains were able to utilize corncob-XOs obtained from enzymatic hydrolysis of KOH-treated corncob and showed the growth as evident from increasing in the viable cell count on MRS. The results in the Figure 3.7 showed the growth of *L. casei* TISTR1463, *L. lactis* TISTR1464 and *L. plantarum* TISTR1465 in MRS medium supplemented either with different carbon sources. During a fermentation period of 48 h, it can be observed that *L. casei* TISTR1463, *L. lactis* TISTR1464 and *L. plantarum* TISTR1465 exhibited the highest capacity to grow on MRS medium supplemented with glucose. Generally, glucose is an energy source for cell metabolism in most microorganisms. Similar results were observed by previous report of Madhukumar and Muralikrishna (2012) and Rycroft et al. (2001). Surprisingly, all three described lactobacilli strains of *L. casei* TISTR1463, *L. lactis* TISTR1464 and *L. plantarum* TISTR1465 were able to use corncob-XOs for growth, based on an increase in viable cell counts on MRS medium. Maximum viable cell counts for *L. casei* TISTR1463, *L. lactis* TISTR1464 and *L. plantarum* TISTR1465 on corncob-XOs were higher than for a control (without a carbon source) at 1.50, 1.94 and 1.08 log CFU/mL, respectively. The different efficiencies for use of corncob-XOs may depend on specificity of oligosaccharide use mechanisms for each strain (Holt et al., 2005). Similar to the results for other *Lactobacillus* spp., such as *L. fermentum* (Moura et al., 2007), *L. acidophilus* (Chapla et al., 2012), *L. fermentum* (Chapla et al., 2012), *L. brevis* (Madhukumar and Muralikrishna, 2012) and *L. plantarum* (Madhukumar and Muralikrishna, 2012), the lactobacilli strains used in these studies could not use XOs and glucose with similar degrees of efficiency. Kontula et al. (1998) reported that *L. plantarum* can use oat bran-XOs. Moreover, Manisseri and Gudipati (2010) and Madhukumar and Muralikrishna (2012) reported that *L. plantarum* NDRI strain 184 is able to use XOs from wheat bran but grows poorly on Bengal gram husk-XOs. The novel results from this study showed that *L. lactis* TISTR1464 had a greater ability to use corncob-XOs than *L. plantarum* TISTR1465. Moreover, corncob-XOs, was a good carbon source for *L. casei* TISTR1463. The β-xylosidase activity (data not shown) and growth characteristics of *L. casei* TISTR1463, *L. lactis* TISTR1464 and *L. plantarum* TISTR1465 on corncob-XOs were comparable to commercial XOs demonstrating a prebiotic property of corncob-XOs.

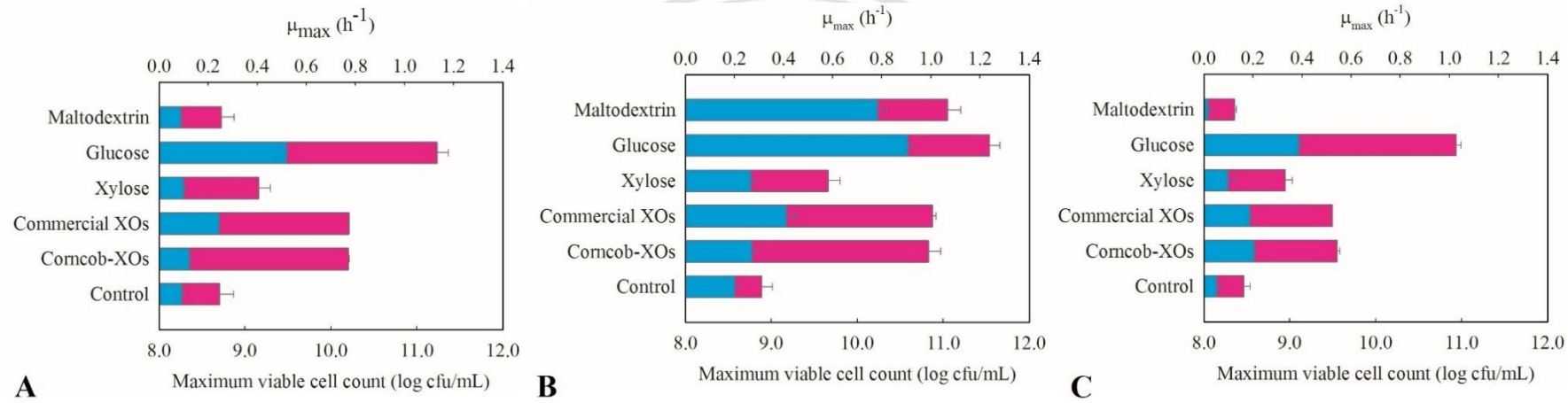

Figure 3.7 ■: Maximum viable cell counts and ■: maximum specific growth rates of (A) *Lactobacillus casei* TISTR1463, (B) *L. lactis* TISTR1464 and (C) *L. plantarum* TISTR1465 in MRS medium supplemented with different carbon source.

3.4 Conclusion

Corncob, one of an abundant agricultural waste, has been shown to be a good cost-effective raw material for XO production using a thermostable endo-xylanase from *S. thermovulgaris*. Moreover, the prebiotic properties of corncob-XOs were comparable with commercial XOs based on similar enhancement of the growth of probiotic lactobacilli strains.