ENZYMATIC PRODUCTION OF XYLOOLIGOSACCHARIDES FROM CORNCOB USING ENDO-XYLANASE FROM *Streptomyces thermovulgaris* TISTR1948

Pinpanit Boonchuay,[1] and Thanongsak Chaiyaso,[2]*

[1] Biotechnology in Agro-Industry Program, Graduate School, Chiang Mai University, Chiang Mai 50200, Thailand
[2] Division of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand
*e-mail thachaiyaso@hotmail.com

Abstract

Xylooligosaccharides (XOs) are the sugar oligomers produced from xylan hydrolysis. XOs have a characteristic of prebiotic by promoting the growth of probiotic microorganisms. Xylan containing agricultural wastes e.g. rice straw, sugarcane bagasse and corncob could be applied to produce XOs by a consecutively process of alkali-pretreatment and enzymatic hydrolysis. In this study, we emphasized on enzymatic production of XOs from corncob, a low cost raw material and relatively high xylan content. The dried corncob was ground and sieved to be <100 mesh size, then subjected to alkali-pretreatment by soaking in 10.0% (w/v) KOH solution at 100°C for 1 h, followed by adjusting pH to 7.0 by adding 5.0% (w/v) H_2SO_4, washed by tap water, filtered through a filter cloth and dried the filtrate at 80°C for 48 h. The recovery yield after KOH-pretreatment was 43.69±1.30% (w/w) and the major components of KOH-pretreated corncob were; cellulose (68.21±1.41%), hemicellulose (21.67±0.71%) and lignin (4.29±0.40%). The KOH-pretreated corncob was then subjected to enzymatic hydrolysis by mashing with 10 mM K-P buffer pH 6.5 (15.0% solid). Then, 100 U/g substrate of endo-xylanase from *Streptomyces thermovulgaris* TISTR1948 was added, the reaction was carried out at 55°C under static condition for 24 h. The samples were periodically taken and analyzed by a thin-layer chromatography (TLC). The results revealed that the suitable reaction time for XOs production was 12 h, which the xylobiose was found as the main product while few xylose contents were obtained. The optimal conditions for XOs production was studied by the response surface methodology (RSM) via the central composite design (CCD). The factors of pH and temperature (°C) were investigated with endo-xylanase concentration (U/g substrate). The results showed that all factors were significant and influenced on the quantity of XOs in term of reducing sugar content (mg/g substrate). The optimal conditions to achieve maximum yield of reducing sugar were; endo-xylanase concentration 142.70 U/g substrate at 53.56°C and pH 6.51. Using this experimental design, the reducing sugar content increased from 109.89±1.87 to 130.83±1.4 mg/g substrate or 603.74±6.49 mg/g xylan content in KOH-pretreated corncob which was 19.09% higher than un-optimized condition.

Keywords: xylooligosaccharides, corncob, endo-xylanase, *Streptomyces thermovulgaris* TISTR 1948

Introduction

Xylooligosaccharides (XOs) are sugar oligomers produced from xylan containing lignocellulosic materials by various methods such as chemicals hydrolysis, enzymatic hydrolysis and enzymatic hydrolysis combined with chemicals methods (Aachary and Prapulla, 2011). Endo-xylanases are the key enzyme of xylan biodegradation is one type of glycoside hydrolase enzyme that can hydrolyse internal linkages in xylan by a random attack mechanism to cleave the xylan backbone into XOs (Pastor *et al*., 2007). XOs are non-digestible carbohydrate made up from xylose linked with β-(1,4) bond with degree of polymerization from 2-4 (DP 2-4) which can't absorb and used by human (Mäkeläinen *et al*., 2009). The health benefits of XOs are selective stimulating the growth and activity of one or a limited number of bacteria in the colon that have the potential to improve host (human) health. Moreover, recently, *in vitro* and *in vivo* studies of XOs reported that XOs can promoted the growth of probiotics such as *Bifidobacterium* spp. (Gobinath *et al*., 2010; Manisseri and Gudipati, 2010 and Hsu *et al*., 2011), *B. adolescentis* (Moura *et al*., 2007; Moura *et al*., 2008 and Chapla *et al*., 2011), *B. bifidum* (Chapla *et al*., 2011), *Lactobacillus* spp., *L. brevis* (Moura *et al*., 2007 and Moura *et al*., 2008), *L. fermentum* (Chapla *et al*., 2011), *L. acidophilus* (Chapla *et al*., 2011).
Thailand generates the agricultural wastes more than 29 million tons per year. Moreover, most farmers tend to get rid of these wastes by burning them because these materials are not in market demand. From database of carbon dioxide emission by burning debris, weeds and wildfire in the forest indicates the impact of agriculture on global warming. For example, in one acre of corn fields release 429.41 kg of CO_2 from sowing to harvesting and burning them. As the mentioned data, that makes a potential for value addition to the corncob to produce the valuable XOs and reduction the CO_2 emission. So, in this study we report the chemo-enzymatic process for XOs production from KOH-pretreated corncob by endo-xylanase from *Streptomyces thermovulgaris* TISTR1948.

Methodology

Microorganism

Streptomyces thermovulgaris TISTR1948 was used as an endo-xylanase producer and was maintained at -20°C in glycerol stock.

Raw materials

Corncob and rice straw were kindly given by the local farmers in Chiang Mai and Phayao provinces, Thailand. They were sun dried, cut to 10.0 cm length and kept at 4°C until used.

Alkali-pretreatment of corncob

The alkali-pretreatment used in this study was modified from the study of Chaiyaso *et al*. (2010). The dried corncob was ground and sieved to be <100 mesh size, then subjected to alkali-pretreatment by soaking in 10.0% (w/v) KOH at 100°C for 1 h, followed by adjusting pH to 7.0 by adding 5.0% (w/v) H_2SO_4, washed by tap water, filtered through a filter cloth and dried the filtrate at 80°C for 48 h.

Endo-xylanase production

The basal medium (Chaiyaso *et al*., 2011) used in this experiment contained, per liter, yeast extract 5.42 g, K_2HPO_4 1.0 g, KH_2PO_4 0.5 g, $(NH_4)_2SO_4$ 1.0 g, NaCl 0.2 g, $MgSO_4.7H_2O$ 0.1 g, $CaCl_2.2H_2O$ 0.1 g, Tween 80 0.1 g and rice straw (mesh size <1 mm) 27.45 g 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. The strain TISTR1948 was cultivated in 250-mL Erlenmeyer flask contained 50 mL of culture medium and incubated in a shaking incubator

(Kühner, Switzerland) at a shaking speed at 200 rpm at 50°C for 96 h. The highest endo-xylanase activity of *S. thermovulgaris* TISTR1948 was 149.41 U/mL under optimum conditions.

Enzyme assays

Xylanase activity was measured using 1.0% (w/v) oat spelt xylan (Sigma, USA) solution in 0.1 M K-P buffer (pH 6.5) as substrate (Techapun *et al*., 2001). The supernatant from culture medium was diluted in 0.1 M K-P buffer (pH 6.5) and incubated at 55°C with 1.0% (w/v) oat spelt xylan for 10 min. The release of reducing sugars was measured by using dinitrosalysilic acid (DNS) method (Miller, 1959). One unit of xylanase activity (U) is defined as the amount of enzyme liberating 1 μmol of reducing sugar per min under assay condition.

Productions of XOs

The KOH-pretreated corncob was used as a substrate for XO production. The substrate was then subjected to enzymatic hydrolysis by mashing with 10 mM K-P buffer pH 6.5 (15.0% w/v). Then, 100 U/g substrate of endo-xylanase from *S. thermovulgaris* TISTR1948 was added, the reaction was carried out at 55°C under static condition for 24 h. The samples from KOH-pretreated corncob, oat spelt and beechwood xylan were periodically taken and analyzed by thin-layer chromatography (TLC) (Kubata *et al*., 1994) and the commercial XOs (Wako, Japan) were used as standard. The hydrolysis products were monitored by measuring the reducing sugar content by the DNS method (Miller, 1959).

Analysis of corncob composition

Analysis of composition of raw corncob, KOH-pretreated corncob and hydrolyzed corncob; cellulose, hemicellulose, lignin by using methods from TAPPI (2002) and ash by using methods from AOAC (2000)

The optimal conditions for XOs production by response surface methodology

The optimal conditions for XOs production from KOH-pretreated corncob was studied by the response surface methodology (RSM) via the central composite design (CCD) by the statistical software package Design Expert®, version 6.0.10 (Stat-Ease Inc., Minneapolis, USA). Table 1 shows the CCD which was applied with three design factors namely: *A*: endo-xylanase concentration (U/g substrate), *B*: pH value of buffer and *C*: temperature (°C) for XOs production in term of reducing sugar content (mg/ g substrate)

Table 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**
Endo-xylanase concentration	U/g substrate	*A*	82.96	100.00	125.00	150.00	167.04
pH	-	*B*	4.82	5.50	6.50	7.50	8.18
Temperature	°C	*C*	38.18	45.00	55.00	65.00	71.82

Result and Discussion

Alkali-pretreatment of corncob

The connection between hemicellulose and lignin in raw corncob provides a protective sheath around cellulose (Hamelinck *et al*., 2005) and associated in the form of bundle or microfibrils (Brodeur *et al*., 2011). From the mention above, raw corncob is not suitable substrate for enzyme hydrolysis. Alkali-pretreatment of lignocellulosic substrate may cause swelling, leading to increase in internal surface areas, reduce the degree of polymerization (DP) and crystallinity, disrupt the crystalline structure by separation of structural linkages between lignin and carbohydrates as well as disruption or degradation of lignin, thereby alkali-pretreatment helps in easy recovery of xylan from lignocellulosic substrates and more accessible to the endo-xylanase (Harmsen *et al*., 2010). The recovery yield after alkali-pretreatment by 10% KOH (w/v) was 43.69±1.30% (w/w) the major components of raw corncob and KOH-pretreated corncob are shown in Table 2.

Productions of XOs

Optimum hydrolysis time of the KOH-pretreated corncob (Figure 1A) compared with commercial xylans namely; beechwood (Figure 1B) and oat spelt xylan (Figure 1C) investigated by thin layer chromatography (TLC) (Kubata *et al*., 1994). The results revealed that the suitable reaction time for XOs production was 12 h, which the xylobiose was found as the main product while few xylose contents were obtained. The analysis of products that, obtained from endo-xylanase hydrolyzed KOH-pretreated corncob contained different types of XOs e.g. xylobiose, xylotriose, xylotetraose and xylopentaose, respectively.

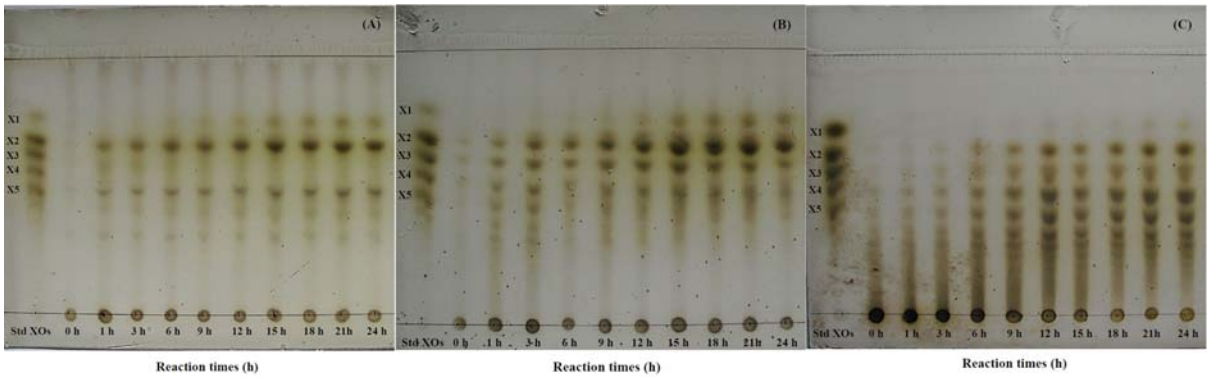

Figure 1 TLC chromatogram of the time course for XOs production from (A) corncob, (B) beechwood xylan and (C) oat spelt xylan, catalyzed by endo-xylanase from *Streptomyces thermovulgaris* TISTR1948; xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentose (X5)

Analysis of corncob composition

The compositions of corncob were analyzed by the method of TAPPI (2002). The results showed that, hemicellulose content in hydrolyzed corncob was 3.17% which decreased from 21.67% in KOH-pretreated corncob indicating that hemicellulose was hydrolyzed to be XOs or reducing sugar. The reducing sugar content that obtained from the center point experimental was 109.89 mg/g substrate or 507.11 mg/g xylan which obtained by the calculation from xylan content in KOH-pretreated corncob. Moreover, the results also revealed that the reducing sugar content was about 11.00% (by weight of xylan content in KOH-pretreated corncob) which was lower than the decreasing of xylan content in

hydrolyzed corncob (18.5%). This might be that the reducing sugar content in XOs analyzed by DNS method was lower than the exactly XOs content in the hydrolysis products. So, the exactly XOs content has to be analyzed by high performance liquid chromatography (HPLC).

Table 2 The composition of raw corncob, KOH-pretreated and hydrolyzed corncob

Composition (%)	Raw corncob	KOH-pretreated corncob	Hydrolyzed corncob
Cellulose	40.38±3.02	68.21±1.41	84.81±1.10
Hemicellulose (xylan)	41.45±2.23	21.67±0.71	3.17±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 (by difference)	9.54±1.61	5.36±0.64	6.71±0.39

The optimal conditions for XOs production

The optimal conditions for XOs production was studied by the response surface methodology (RSM) via the central composite design (CCD). The factors of pH and temperature were investigated with endo-xylanase concentration. The CCD generated a quadratic equation of XOs production in term of reducing sugar (mg/g substrate) as follow:

$$\text{Response (reducing sugar content, mg/g substrate)} = -686.17029 + 0.85770A + 95.79182B + 16.06779C - 5.38197E\text{-}003A^2 - 6.89203B^2 - 0.16120C^2 - 0.025453AB + 0.016532AC - 0.13362BC$$

After 12 h of reaction time in 17 conditions, the experimental, predicted values and XOs in term of reducing sugar were shown in Table 3 and Figure 2 (A), (B) and (C). Various statistic data (standard error of estimate, sum of squares of the error, *F* statistic and *p* value) were examined, as shown in Table 4. The quality of the model was expressed in terms of the R^2 value. The predicted values match the experimental values, a values of $R^2 = 0.9749$, adj-$R^2 = 0.9427$ and pred-$R^2 = 0.8118$. The results showed that all of three factors were significant and influenced on the quantity of XOs. To confirm the applicability of the CCD optimization model, the XOs production was carried out by the hydrolysis of KOH-pretreated corncob under the suggested optimal conditions; 142.70 U/g substrate endo-xylanase concentration, pH 6.51 at 53.56°C for a maximum XOs production yield. From the experimental results, a yield of XOs production in term of reducing sugar of 130.83±1.4 mg/g substrate or 603.74±6.49 mg/g xylan (by calculation) was obtained with a higher than the predicted value by 16.41% and the un-optimized condition by 19.09%, respectively. This result indicated that the model generated by CCD could be used to predict the maximum yields of XOs production.

Table 3 Experimental and theoretically predicted values for XOs production (reducing sugar content; mg/g substrate) from KOH-pretreated corncob

Run order	Code level			Reducing sugar (mg/g substrate)	
	Factor *A* Endo-xylanase concentration (U/g substrate)	Factor *B* pH	Factor *C* Temperature (°C)	Actual Value	Predicted Value
1	82.96	6.50	55.00	88.65	89.43
2	125.00	6.50	38.18	72.89	71.96
3	100.00	7.50	65.00	64.18	63.11
4	100.00	5.50	65.00	75.58	73.18
5	167.04	6.50	55.00	108.87	110.96
6	150.00	7.50	45.00	86.26	86.63
7	125.00	6.50	55.00	110.66	109.71
8	100.00	7.50	45.00	86.26	83.37
9	125.00	4.82	55.00	99.02	97.51
10	150.00	7.50	65.00	88.34	82.90
11	150.00	5.50	65.00	94.66	95.52
12	125.00	6.50	55.00	107.31	109.71
13	125.00	8.18	55.00	78.54	82.93
14	125.00	6.50	55.00	111.66	109.71
15	150.00	5.50	45.00	94.87	93.90
16	100.00	5.50	45.00	84.70	88.09
17	125.00	6.50	45.00	52.46	56.28

Table 4 Analysis of variance (ANOVA) for XOs production (reducing sugar content, mg/g substrate) of RSM fitted to the quadratic equation

Source	SS	DF	MS	*F*-value	*p*-value	
Model	4298.97	9	477.66	30.22	< 0.0001	***Significant***
A	559.45	1	559.45	35.40	0.0006	
B	256.68	1	256.68	16.24	0.0050	
C	296.92	1	296.92	18.79	0.0034	
A^2	127.56	1	127.56	8.07	0.0250	
B^2	535.49	1	535.49	33.88	0.0006	
C^2	2929.46	1	2929.46	185.34	< 0.0001	
AB	3.24	1	3.24	0.21	0.6645	
AC	136.65	1	136.65	8.65	0.0217	
BC	14.28	1	14.28	0.90	0.3735	
Residual	110.64	7	15.81			
Lack of Fit	100.25	5	20.05	3.86	0.2186	***Not significant***
Pure Error	10.39	2	5.20			
Core Total	4409.60	16				

R^2= 0.9749, Adj R^2= 0.9427, Pred R^2= 0.8118

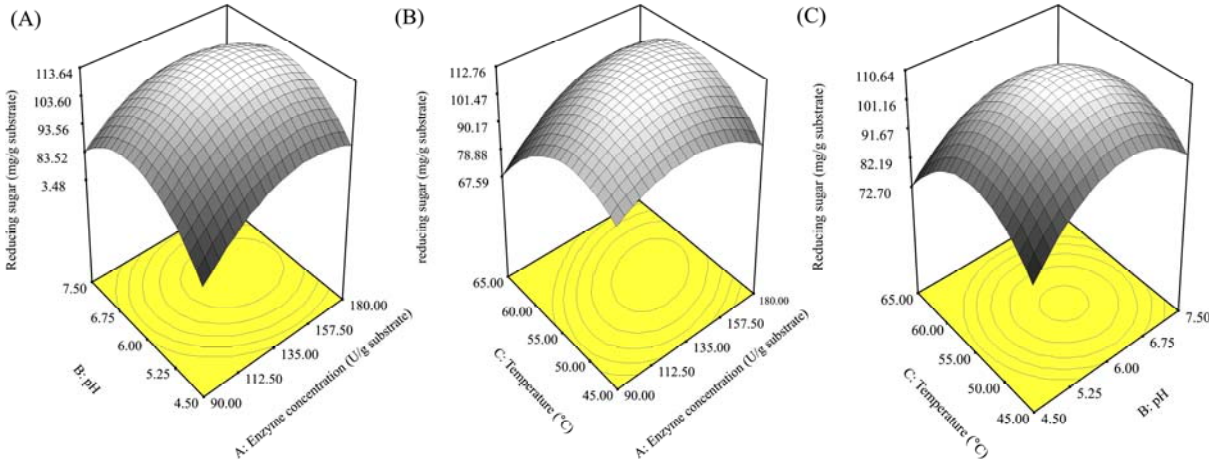

Figure 2 The three dimensional graphic of XOs production (reducing sugar content; mg/g substrate) for quadratic response-surface optimization. The comparison was made between (A) pH value of buffer and endo-xylanase concentration, (B) temperature and endo-xylanase concentration and (C) temperature and pH value of buffer

The comparison of XOs production yield between chemo-enzymatic conversion using the thermostable endo-xylanase from *Streptomyces thermovulgaris* TISTR1948 and other methods is shown in Table 5. The study of Samanta *et al*. (2012) who reported the chemo-enzymatic process conversion of *Sehima nervosum* grass to produced XOs by NaOH pretreatment and hydrolysis by commercial xylanase from *Trichoderma viridae* (Sigma, USA) found that under optimum condition the total reducing sugar of 180.60 mg/g xylan (110 mg xylobiose and 0.706 mg xylotriose) was obtained. While, Sabiha-Hanim *et al*. (2011) reported that oil palm frond fibers were used as the substrate for XOs by autohydrolysis pretreatment and follow by commercial xylanase from *T. viridae* (Sigma, USA) hydrolysis. The results showed that the highest XOs yield in term of reducing sugar was 403 mg/g substrate at 24 h of reaction time, however, the hydrolysis product contained 25.6% xylose (256 mg/g substrate) and only 17.5% XOs (175 mg/g substrate). Our study, higher reducing sugar content of 130.83 mg/g substrate or 603.74±6.49 mg/g xylan content in KOH-pretreated corncob with a few amounts of xylose was obtained. So, it seemed to be better than mentioned report because of the advantages in economically production by using crude xylanase and the shorter reaction time (12 h) of enzymatic hydrolysis process.

Table 5 Comparison of XOs yields in term of reducing sugar between chemo-enzymatic conversion using the endo-xylanase from *Streptomyces thermovulgaris* TISTR1948 and other xylanases

Substrate	Methods	Conditions	XOs yields (reducing sugar; mg/g xylan)	References
Wheat bran soluble polysaccharides	Purified Ragi malt xylanase	50°C, pH 5.0 2.5 h	105.0	Manisseri and Gudipati., 2010
Autoclaved-oil palm frond fibers	Commercial xylanase (*Trichoderma viridae,* Sigma, USA)	40°C, pH 5.0 24 h	256.0	Sabiha-Hanim *et al*., 2011
NaOH and stream –pretreated *Sehima nervosum* grass	Commercial xylanase (*Trichoderma viridae,* Sigma, USA)	45.19°C, pH 5.03, 10.11 h	180.6	Samanta *et al*., 2012
KOH-pretreated corncob	Crude xylanase (*S. thermovulgaris* TISTR1948)	53.56°C, pH 6.51, 12 h	603.7	This study

Conclusion

The recovery yield after alkali-pretreatment was 43.69±1.30% (w/w) and the components of KOH-pretreated corncob were; cellulose (68.21±1.41%), hemicellulose (21.67±0.71%) and lignin (4.29±0.40%), respectively. The TLC analysis of the products obtained from KOH-pretreated corncob hydrolyzed by endo-xylanase from *Streptomyces thermovulgaris* TISTR1948 revealed that the suitable reaction time for XOs production was 12 h and xylobiose was found as the main product while few xylose contents were observed. The optimal conditions to achieve maximum yield of reducing sugar from using experimental design were; endo-xylanase concentration 142.70 U/g substrate at 53.56°C and pH 6.51. Using this experimental design, the reducing sugar content increased from 109.89±1.87 to 130.83±6.4 mg/g substrate or 603.74±6.49 mg/g xylan content in KOH-pretreated corn cob, which was 19.09% higher than un-optimized condition.

References

1. Aachary A.A, Prapulla S.G (2011) Xylooligosaccharides (XOs) as an emerging prebiotic: Microbial synthesis, utilization, structural characterization, bioactive properties and applications: Compr. Rev. Food. Sci. F 10: 1-15.
2. AOAC (2000) Official Method of Analysis of AOAC International. (17thed). Washington D.C., USA: The Association of Official Analytical Chemists.
3. Brodeur G, Yau E, Badal K, Collier J, Ramachandran K.B, Ramakrishnan S (2011) Chemical and Physicochemical Pretreatment of Lignocellulosic Biomass: A Review. Enzyme Research.

4. Chaiyaso T, Kantiya A, Techapan C, Leksawasdi N, Seesuriyachan P, Hanmoungjai P (2011) Optimization of cellulose-free xylanase production by thermophilic *Streptomyces thermovulgaris* TISTR1948 through Placket-Burman and Response surface methodological approaches. Biosci. Biotechnol. Biochem 75(3): 531-537.
5. Chapla D, Pandit P, Shah A (2012) Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. Bioresource Technol. 115: 215–221.
6. Gobinath D, Madhu A.N, Prashant G, Srinivasan K, Prapulla S.G. (2010) Beneficial effect of xylo-oligosaccharides and fructo-oligosaccharides in streptozotocin-induced diabetic rats. Br. J. Nutr. 104: 40–47.
7. Harmsen P.F.H, Huijgen W.J.J, Bermúdez López L.M, Bakker P.R.C (2010) Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Wageningen UR, Food & Biobased Research.
8. Hsu C.K, Liao J.W, Chung Y.C, Hsieh C.P, Chan Y.C (2004) Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. J. Nutr. 134: 1523-1528.
9. Kubata B.K, Suzuki T, Horitsu H, Kawai K, Takamizawa K (1994) Purification and characterization of *Aeromonas caviae* ME-1 xylanase V, which produces exclusively xylobiose from xylan. Appl. Environ. Microbiol. 60: 531-535.
10. Manisseri C, Gudipati M (2010) Bioactive xylo-oligosaccharides from wheat bran soluble polysaccharides. LWT-Food. Sci. Technol. 43: 421-430.
11. Miller G.C (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar: Anal. Chem. 31: 426-428.
12. Moura P, Barata R, Carvalheiro F, Gírio F, Loureiro-Dias M.C, Esteves M.P (2007) *In vitro* fermentation of xylo-oligosaccharides from corn cobs autohydrolysis by *Bifidobacterium* and *Lactobacillus* strains. LWT- Food. Sci. Technol. 40: 963-972.
13. Moura P, Carvalheiro F, Esteves M.P, Gírio F.M (2008) Prebiotics xylo-oligosaccharides as a high-value co-products on an integrated biorefinery approach from lignocellulosic feedstock: International Conference and Exhibition on Bioenergy.
14. TAPPI. Technical Association of the Pulp and Paper Industry (2002): On compact disc, [CD ROM]. Available: TAPPI Press. (30th August 2007)

Acknowledgements

This work was financial supported by the Faculty of Agro-Industry, Chiang Mai University and Graduate School of Chiang Mai University, Thailand.