CHAPTER 4

Purification and Characterization of Thermostable Endo-Xylanase from *Streptomyces thermovulgaris* TISTR1948 and Its Application on Xylooligosaccharide Production

4.1 Introduction

Xylans are the main carbohydrate contained in hemicellulose, which constitute 30–35% of the lignocellulosic materials (LCMs) (Collins et al., 2005). They are an essential hydrolytic substrate for production of various value-added products (Sedlmeyer, 2011). These processes require several types of enzyme to completely hydrolyze. Among these enzymes, xylanases are regarded as an important enzyme. The role of xylanases is the catalyzing the of 1,4-β-D-xylosidic linkages in the xylan chain to generate the xylooligosaccharides (XOs) (Javier et al., 2007).

Nowadays, xylanases are applied in numerous biotechnological industries, including food, animal feed, pulp and paper, textile as well as biofuel industries (Bajpai, 2014b). Because of the high demand for xylanases, those from the glycoside hydrolase (GH) family, especially the GH10 and GH11 xylanases of microorganisms, have been investigated in detail (Bajpai, 2014a). Besides that, thermostable enzymes active at high temperatures have greater applicability than mesophilic enzymes. Among the xylanase producers, *Streptomyces* spp. show an attractive advantage on the high level of extracellular activity of these enzymes, their thermal stability (50–85°C) and their stability across a broad pH range (pH 3–13) (Deesukon et al., 2011; G.C. et al., 2013; Mander et al., 2014; Nascimento et al., 2002; Qiu et al., 2010; Yan et al., 2009). Recently, xylanases have been purified and characterized from *Streptomyces* sp. strain AMT-3 (Nascimento et al., 2002), *Streptomyces* sp. CS624 (Mander et al., 2014) and *S. megasporus* DSM 41476 (Qiu et al., 2010). In addition, their industrial scale production

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright by Chiang Mai University
All rights reserved

has potential applications in the brewing industry (Qiu et al., 2010) and in the biodegradation of agroindustrial by-products (Nascimento et al., 2002) and agricultural residues (Mander et al., 2014).

XOs are realized as noticeable value-added products in foods, pharmaceuticals, cosmetic and animal feed. More importantly than other prebiotics, they can resist the heat up to 100°C and are stable over a wide pH range of pH (2.5–8.0) (Deutschmann and Dekker, 2012). In the previous report, *S. thermovulgaris* TISTR1948 have been isolated and employed as a in-house thermostable endo-xylanase producer (Chaiyaso et al., 2011). After that, the optimization of XO production from corncob by crude xylanases from strain TISTR1948 via a statistical design as well as the prebiotic property of corncob-XOs were investigated. The result showed that corncob-XOs showed the prebiotic activity because the growth of probiotic lactobacilli strain was enhanced. (Boonchuay et al., 2014)

Despite the apparent utility of the xylanases from strain TISTR1948 as a XOs-producing biocatalyst, its properties have not been fully investigated. Therefore, in this study, the xylanase of strain TISTR1948 was purified and characterized. After that, the purified xylanase was applied to produce high-purity corncob-XOs and the prebiotic property of high-purity corncob-XOs were examined using probiotic *Lactobacillus plantarum*.

4.2 Materials and Methods

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

4.2.1 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 lactic acid bacteria strain was kindly given by the culture collection section of the TISTR. The probiotic *L. plantarum* TISTR1465

was used for prebiotics activity study. All of microorganisms were maintained at –20°C in glycerol stock.

4.2.2 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 and cool place at 4°C until used.

4.2.3 Chemicals

Oat spelt xylan, beech wood xylan (average molecular weight approximately 20,000 Da (Bastawde, 1992), dinitrosalicylic acid (DNS), bovine serum albumin, lactic acid, xylose (X1) and arabinose were purchased from Sigma, USA. Xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylopentaose (X5) were purchased from Megazyme, Ireland. Folin-Ciocalteu's phenol reagent was purchased from Merck, Germany. DEAE-Toyopearl 650M and Toyopearl HW-55 were from Tosoh, Japan. Other chemicals used in this study were of analytical grade.

4.2.4 Corncob morphology analysis

KOH-treated corncob samples hydrolyzed by a crude xylanase, partially purified xylanase and purified xylanase 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.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

4.2.5 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).

4.2.6 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 product 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.

4.2.7 Xylanase production

Crude 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 30 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).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

4.2.8 Enzyme assay and kinetic parameters of purified xylanase

A reaction mixture (0.1 mL) consisting of an appropriate concentration of xylanase, 0.1 M potassium-phosphate buffer (pH 6.5) and 1.0% (w/v) beech wood xylan was incubated at 55°C for 10 min. The concentrations of the released reducing sugars were measured using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of xylanase activity (U) was defined as the amount of enzyme liberating 1.0 μmol of reducing sugar (as xylose) per min (Chaiyaso et al., 2011) (Appendix A-4). The protein concentration was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard (Appendix A-8).

The K_m and V_{max} of the purified xylanase were measured in the presence of 0.5–20 mg beech wood xylan/mL (25–1,000 μM) in 0.1 M potassium-phosphate buffer (pH 6.5) at 60°Cand determined by Lineweaver-Burk plot (Appendix A-9).

4.2.9 Purification of xylanase

The culture supernatant (1,500 mL) from strain TISTR1948 (xylanase_1948) was concentrated to approximately 150 mL in a rotary evaporator (Rotavapor® R-3, Büchi Labortechinik, Switzerland) and then dialyzed overnight against 10 mM Tris-HCl buffer (pH 8.5). The dialysate was applied to a DEAE-Toyopearl column (2.4×20 cm) and the proteins were eluted with a linear gradient of NaCl (0–0.7 M) in 50 mM Tris-HCl buffer (pH 8.5) at a flow rate of 40 mL/h. The active fraction was concentrated to approximately 1.0 mL using a 10 kDa membrane filter (Amicon Ultra-15 Centrifugal Filter Units, Merck, Germany). The concentrated sample was loaded onto a Toyopearl HW-55 column (3.2×60 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl. Proteins were eluted with the same buffer at a flow rate of 25 mL/h. The purified enzyme was concentrated by membrane filtration and used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme characterization.

4.2.10 SDS-PAGE

The purified enzyme was loaded onto a 12.5% polyacrylamide gel with 0.1% SDS and subjected to SDS-PAGE at 20 mA (Laemmli, 1970). The bands were visualized by staining the gel with Coomassie brilliant blue R-250 and then decolorized according to a previously reported method (Takenaka et al., 2015).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

4.2.11 Xylanase characterization

4.2.11.1 Effect of pH on xylanase activity and stability

The effect of pH on xylanase activity was studied by incubating 12.5 μL of the purified protein (0.63 μg) with 87.5 μL of 1.0% (w/v) beech wood xylan dissolved in different buffer solutions (0.1 M, pH 3.0–12): sodium-citrate (pH 3.0–6.0), potassium-phosphate (pH 6.0–8.0), Tris-HCl (pH 7.5–9.0), glycine-NaOH (pH 8.5–10.5),

bicarbonate (pH 9.5–11.0) and sodium-phosphate (pH 11.0–12.0). Xylanase activity was then measured at 60°C. For the pH-stability study, one volume of xylanase (0.63 µg protein) was mixed with three volumes of each buffer and incubated at 4°C for 12 h; after which the remaining activity of the enzyme was measured at pH 6.5 and 60°C.

4.2.11.2 Effect of temperature on xylanase activity and stability

The effect of temperature on the xylanase activity of the purified protein was determined over a temperature range of 35–85°C. Thermostability was measured by pre-incubating the enzyme (50 µg protein/mL) at 50–70°C for 180 min, after which the activity of 2.5 µg protein of the respective xylanase preparation at 60°C was tested.

4.2.11.3 Effect of metal ions and chemical reagents on xylanase activity

One volume of purified xylanase was mixed with three volumes of metal ions, chelating agents, or surfactant solutions (Table 4.2). The final concentration of the added reagents was 5.0 mM and the pH of the reaction was 6.5. After a 60-min incubation at 60°C, the activity of 0.63 µg protein of the respective xylanase treated with the respective reagent was determined as described above.

4.2.11.4 Substrate specificity

The substrate specificity of the purified xylanase was examined with carboxymethyl cellulose, xanthan gum and *p*-nitrophenyl-β-D-xylopyranoside, besides xylan from beech wood. Among tested substrates, the xylanase showed the hydrolytic activity towards the xylan, only.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

4.2.12 Enzymatic hydrolysis of KOH-treated corncob and prebiotic activity testing of the produced xylooligosaccharides

The KOH-treated corncob samples were hydrolyzed using crude xylanase from the culture supernatants, xylanase partially purified by ion-exchange chromatography and purified xylanase at an enzyme concentration of 130 U/g of substrate; the reaction was carried out at 53.8°C and pH 6.17 as previously reported (Boonchuay et al., 2014). Samples collected after 0, 1, 3, 6, 9, 12, 18 and 24 h were subjected to HPLC analysis as described above.

Corncob-XO powders were prepared from each reaction mixture and their prebiotic activities were tested using *L. plantarum* TISTR1465 according to previously reported procedures and conditions (Boonchuay et al., 2014). The dried corncob-XO powder (final concentration: 30 g/L as reducing sugar) was added to replace glucose as the carbon source in MRS medium. Strain TISTR1465 was anoxically cultivated at 30°C. The culture broth was sampled after 0, 6, 12, 18, 24, 36 and 48 h. The growth of strain TISTR1465 was measured based on viable cell counts (CFU/mL) and XO utilization was monitored by HPLC.

4.3 Results and Discussion

4.3.1. Xylanase purification

The xylanase from *S. thermovulgaris* TISTR1948 (xylanase_1948) was purified in a 4-step procedure. Purification profile of xylanase from *S. thermovulgaris* TISTR1948 on ion exchange chromatography (DEAE-Toyopearl) and gel filtration chromatography (Toyopearl HW-55) were shown in Figure 4.1 and 4.2, respectively. After the final step, a 15-fold purification of the extracellular xylanase and a recovery yield of 13%, were determined (Table 4.1). On SDS-PAGE, the final enzyme preparation migrated as a single protein band with a molecular mass of 46.2 kDa (Figure 4.3). Microbial xylanases can be divided into low and high molecular mass (<30 kDa and >30 kDa, respectively) xylanases (Paës et al., 2012). Therefore, the xylanase_1948 is of the high molecular mass type.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Table 4.1 Summary of the protocol used to purify xylanase from *Streptomyces thermovulgaris* TISTR1948.

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Concentrated cultural supernatant	3,050	420	7.3	100	1.0
DEAE-Toyopearl	2,100	78	27	70	3.7
Toyopearl HW-55	380	3.5	110	13	15.0

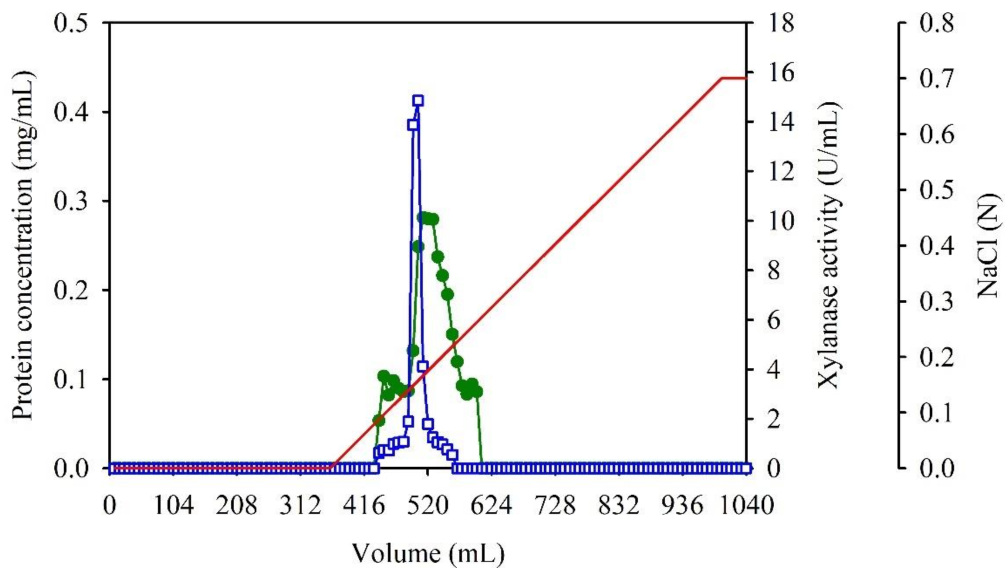


Figure 4.1 Purification profile of xylanase from *Streptomyces thermovulgaris* TISTR1948 on ion exchange chromatography (DEAE-Toyopearl) (Protein concentration (●), xylanase activity (□) and NaCl concentration (—)).

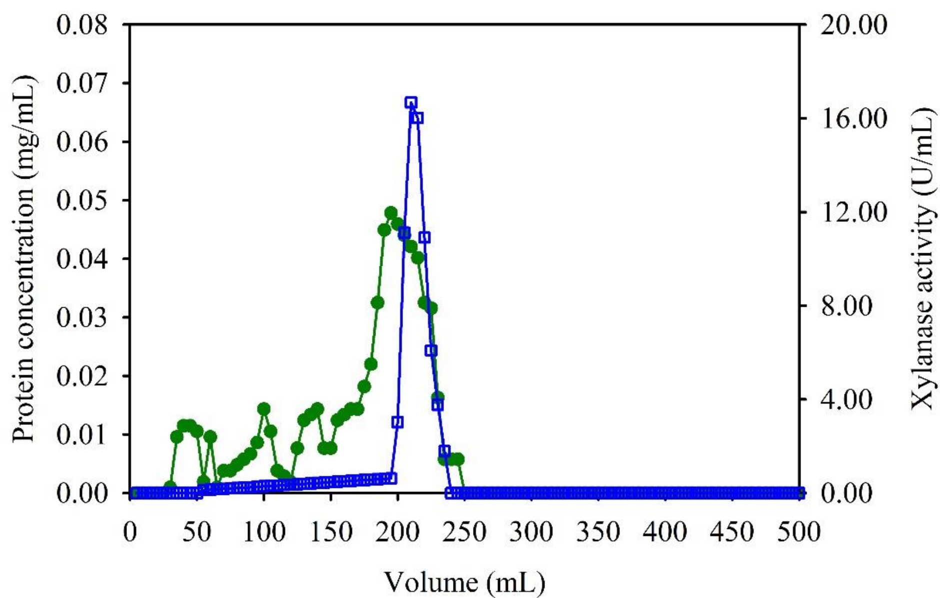


Figure 4.2 Purification profile of xylanase from *Streptomyces thermovulgaris* TISTR1948 on gel filtration chromatography (Toyopearl HW-55) (Protein concentration (●) and xylanase activity (□)).

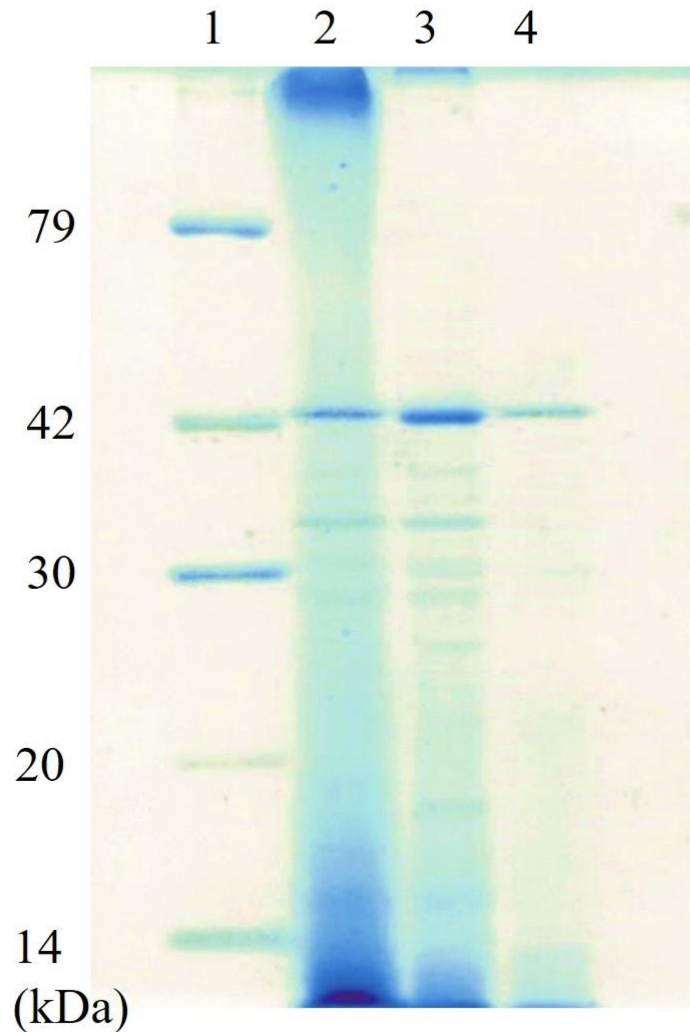


Figure 4.3 SDS-PAGE of xylanase from *Streptomyces thermovulgaris* TISTR1948. Lanes 1: middle-range molecular markers (Wako Pure Chemicals, Osaka, Japan); 2: culture supernatant; 3: DEAE-Toyopearl fraction (7.8 µg); 4: Toyopearl HW-55 fraction (0.5 µg protein).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

4.3.2 Xylanase characterization

4.3.2.1 Kinetic parameters

The K_m and V_{max} values of purified xylanase_1948 for beech wood xylan were 0.75 mg/mL (37.3 µM) and 13.85 U/$mg_{protein}$ (µmol/min/$mg_{protein}$), respectively (Figure 4.4). The catalytic ability (V_{max}/K_m) was 18.55. Other *Streptomyces* xylanases and their kinetic parameters have been reported: *Streptomyces* sp. FA1 (substrate, beech wood

xylan; K_m, 3.45 mg/mL; V_{max}, 0.0007 U/mg; V_{max}/K_m, 0.0002; (He et al., 2014)), *S. megasporus* DSM 41476 (oat spelt xylan; 1.68 mg/mL; 0.0004 U/mg; 0.0002; (Qiu et al., 2010)), *Streptomyces* sp. CS428 (beech wood xylan; 102.30 mg/mL; 3.23 U/mg; 0.032; (G.C et al., 2013)) and *Streptomyces* sp. CS624 (beech wood xylan; 5.61 mg/mL; 0.075 U/mg; 0.013; (Mander et al., 2014). While, purified xylanase_1948 showed high affinity and hydrolytic activity toward beech wood xylan.

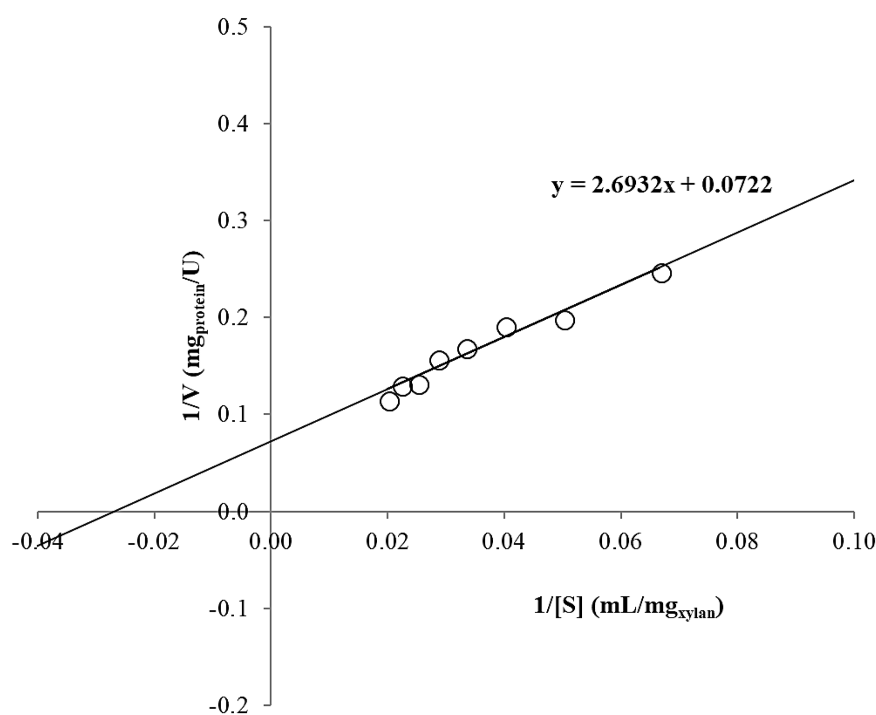


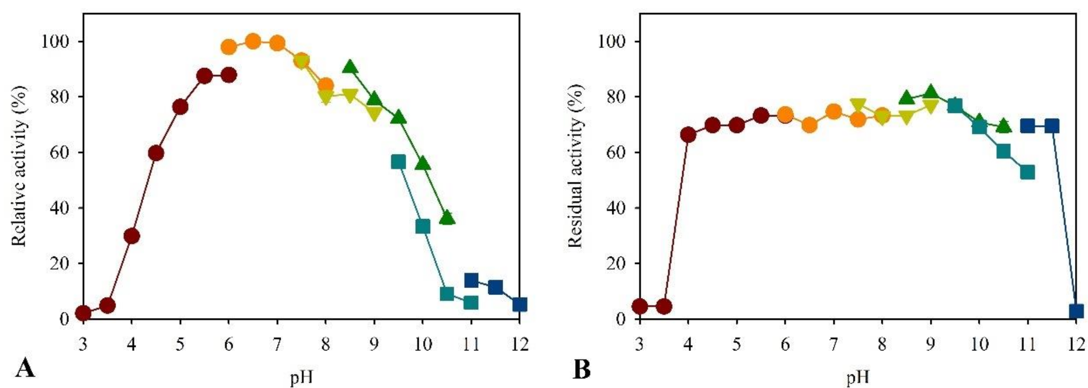
Figure 4.4 Lineweaver-Burk plot of the purified xylanase from *Streptomyces thermovulgaris* TISTR1948 using beech wood xylan as substrate.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

4.3.2.2 Effect of pH and temperature on xylanase activity and stability

The purified xylanase_1948 was stable across a broad pH range (4.0–11.5) but the optimum pH was 6.5 (Figure 4.5A and B). Enzyme activity was highest at 65°C, pH 6.5 (Figure 4.5A) but was retained for 3 h at 50–65°C (Figure 4.5B). The highest activities of the previously reported *Streptomyces* xylanases were at pH 5.0–7.0 (Deesukon et al., 2011; G.C et al., 2013; He et al., 2014; Mander et al., 2014; Nascimento et al., 2002; Qiu et al., 2010; Yan et al., 2009) and 65°C (Nascimento et al., 2002; Yan et al., 2009).

Incubation of the KOH-treated corncobs with crude xylanase_1948 showed that enzyme activity was highest at 53.8°C and pH 6.17 (Boonchuay et al., 2014). Since the crude solution may contain compounds from the culture that inhibit or destabilize xylanase_1948, use of the purified enzyme would be more appropriate for XO production at high temperature. The properties of purified xylanase_1948, including its stability at high temperature (<65°C), recommend the use of this enzyme in many industrial applications in which it is important to avoid microbial contamination, enhance the reaction rate, and increase substrate solubility (An et al., 2015). Moreover, the stability of xylanase_1948 across a broad pH range makes this enzyme suitable for numerous industrial processes carried out at different pH conditions, such as wood and pulp bio-bleaching (alkaline conditions), bread-making (acidic conditions), xylitol production (acidic conditions) and XO production (neutral conditions) (Chi et al., 2013).



ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Figure 4.5 (A) Effect of pH on the activity of the purified xylanase_1948. The activity of the purified enzyme was measured using various buffers, including; ●: sodium-citrate, ●: potassium-phosphate, ▼: Tris-HCl, ▲: glycine-NaOH, ■: bicarbonate and ■: sodium-phosphate. (B) pH stability of the xylanase purified from *Streptomyces thermovulgaris* TISTR1948.

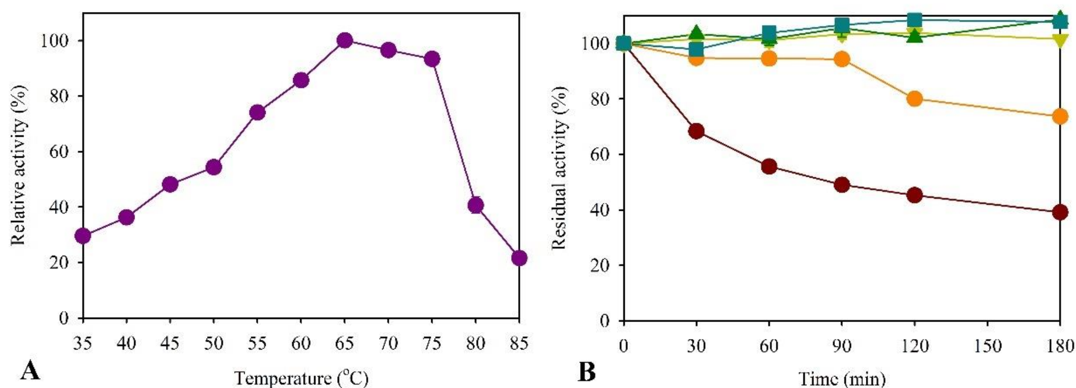


Figure 4.6 (A) Effect of temperature on the activity of the purified xylanase_1948. The activity of the purified enzyme was measured over a temperature range of 35–85°C. (B) Thermal stability of the purified xylanase_1948. The purified xylanase was pre-incubated at ■: 50°C, ▲: 55°C, ▼: 60°C, ●: 65°C and ●: 70°C, after which the remaining activity was measured at pH 6.5.

4.3.2.3 Effects of metal ions and chemical reagents on xylanase activity

Xylanase activity was enhanced in the presence of metal ions, Ca^{2+}, Co^{2+} and Mn^{2+} (Table 4.2). Ca^{2+} concentrations as high as 10 mM enhanced xylanase activity whereas the same concentrations of Co^{2+} and Mn^{2+} were inhibitory. Generally, calcium ions are probably required for the structural maintenance of the enzyme. Xylanase from *Pseudomonas fluorescens* retains the activity at the temperature <65°C in the presence of Ca^{2+}, whereas it easily loses the activity after removal of Ca^{2+} (Spurway et al., 1997). Structural and site-directed mutagenesis studies have suggested that Ca^{2+} confers stability by binding to an extended loop of the enzyme (Spurway et al., 1997). In *Bacillus* xylanases, Co^{2+}, Ca^{2+} and Mg^{2+} strongly stimulate activity (Gaur et al.; Mamo et al., 2006). Meanwhile, the activity of xylanase_1948 was almost completely inhibited by Hg^{2+}, Pb^{2+} and SDS (Table 4.2). On the other hand, the activity of xylanase_1948 was not affected by the chelating agent EDTA.2Na at concentrations up to 10 mM (94±0.94%). By contrast, the xylanases from *Caulobacter crescentus* and from *Paenibacillus* sp. NF1 are inhibited by 2 mM and 1 mM EDTA.2Na, respectively (Graciano et al., 2015; Zheng et al., 2014).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Table 4.2 Effect of metal ions and chemical reagents on xylanase activity.

Reagents	Relative activity (%)
	5 mM
Control	100±0.32
$MgSO_4.7H_2O$	87±1.37
$CaCl_2.2H_2O$	105±2.56
NaCl	94±2.89
KCl	94±0.37
$FeSO_4.7H_2O$	68±0.68
$CoCl_2$	112±0.55
$CuSO_4.5H_2O$	88±0.85
$MnSO_4.5H_2O$	120±1.00
$HgCl_2$	9±0.83
$ZnCl_2$	70±1.52
$AgNO_3$	76±1.10
$Pb(CH_3CO_2)_2$	0
$(NH_4)_2S_2O_8$	98±1.53
EDTA.2Na*	85±2.05
SDS**	9±2.39

*EDTA.2Na, Ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt; **SDS, sodium dodecyl sulfate.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

4.3.3 Xylooligosaccharides production by crude, partially purified and purified xylanase_1948

Oligosaccharides derived from cell-wall xylan are an important source of dietary fiber (Broekaert et al., 2011) and they have raised considerable interest regarding their use as prebiotics (Michlmayr et al., 2013). XOs from corncob exhibit prebiotic properties when tested in several *Lactobacillus* spp. and that they enhance the growth of

L. plantarum TISTR1465 (Boonchuay et al., 2014). XOs produced from corncob xylan using a crude xylanase preparation and a partially purified xylanase from *Aspergillus foetidus* MTCC 4898 have also been investigated for their prebiotic effects in known probiotic strains. However, the crude xylanase preparation contained a slight amount of β-xylosidase activity which probably inhibited XO production (Chapla et al., 2012).

In this study, XO production in reactions using the crude xylanase preparation, partially purified xylanase and purified xylanase_1948. The products of the three reaction mixtures were analyzed by HPLC and TLC for their content of total XOs (Figure 4.7 and 4.8). Steady-state total XO production was reached in the following order: crude xylanase, partial purified xylanase and purified xylanase. The final concentrations of total XOs and the production of the individual XOs were similar in all three reactions (Figure 4.8A, B and C). Specifically, xylobiose (X2), xylotetraose (X3) and xylopentaose (X5) were the main products and xylotriose (X3) and xylose (X1) were minor products. Surprisingly, the concentration of arabinose was lowest in the reaction mixture containing purified xylanase (Figure 4.8C). As a principle, corncob xylan is a β-(1,4) linked polymer of D-xylose with D-glucuronic acid or L-arabinose substituents (arabinoxylan). In addition, the results from SEM analysis of the enzyme-treated corncobs showed that those hydrolyzed by the crude xylanase preparation had many more pores and a rougher surface than corncobs hydrolyzed with the other two enzyme preparations (Figure 4.9). Hence, the crude and partially purified xylanase preparations probably contained other xylanolytic enzymes which able to hydrolyze the side chain of arabinoxylan in corncob.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

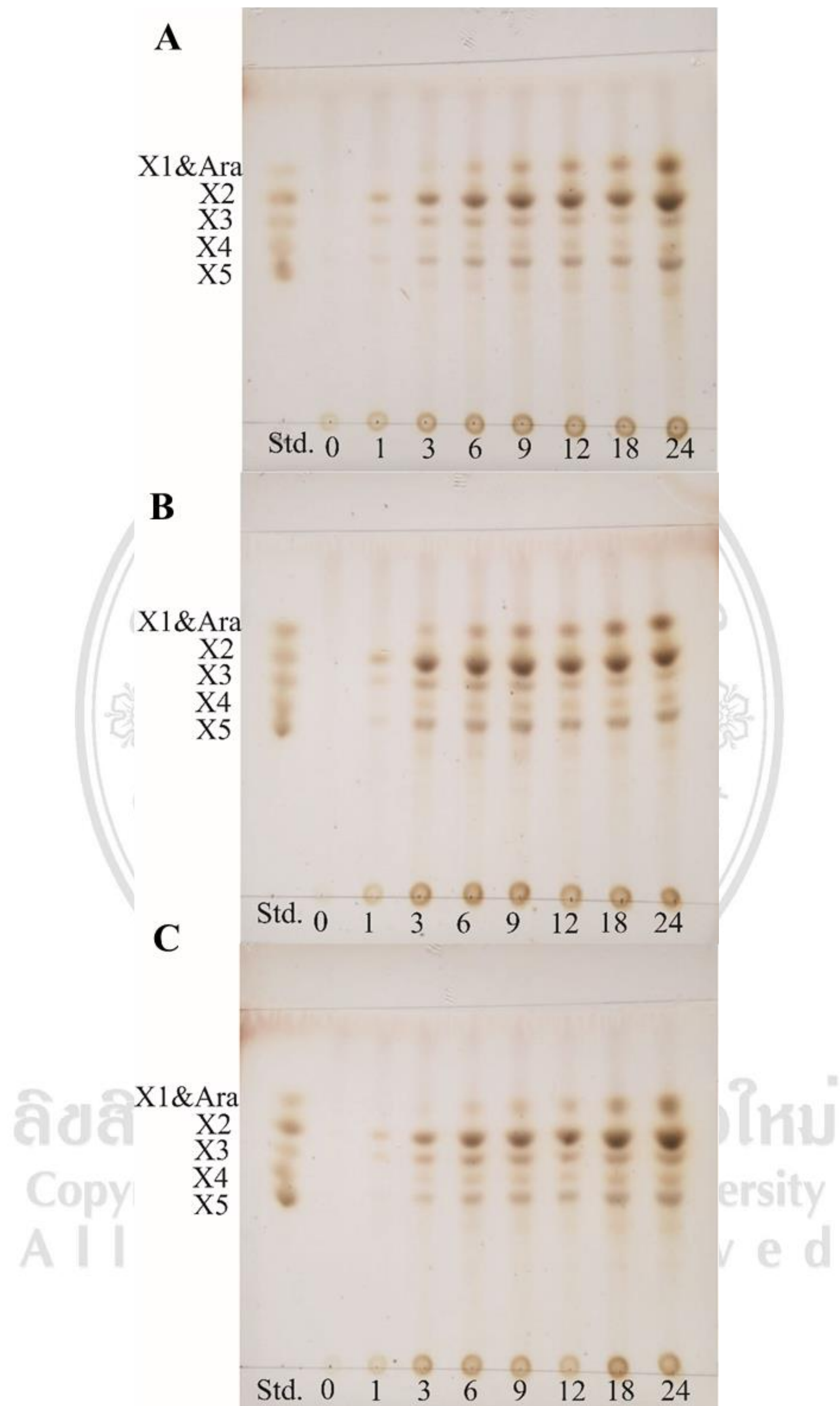
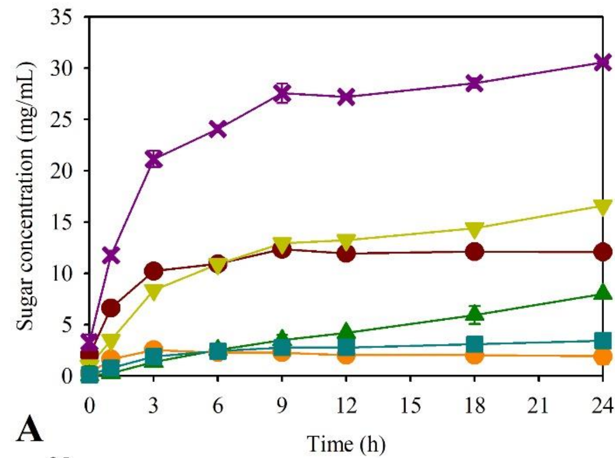
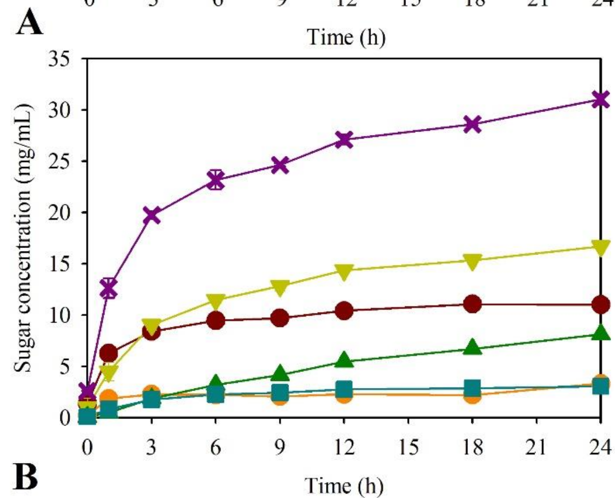


Figure 4.7 TLC chromatogram of the time course of xylooligosaccharides production from KOH-treated corncob by (A) the crude xylanase, (B) partially purified xylanase and (C) purified xylanase_1948. (X5: xylopentaose; X4: xylotetraose; X3: xylotriose; X2: xylobiose; X1: xylose; and Ara: arabinose)





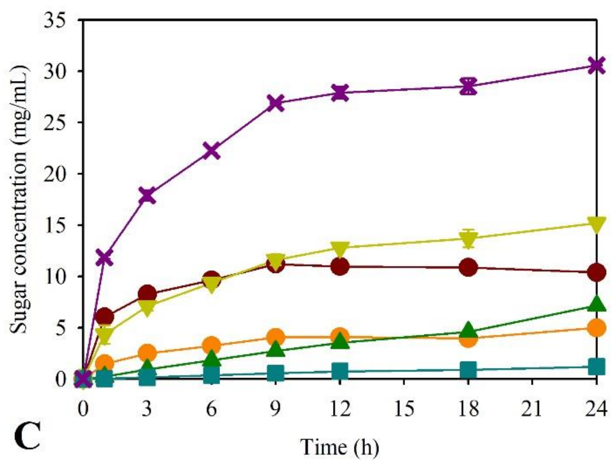


Figure 4.8 HPLC analysis of time course of xylooligosaccharides production from KOH-treated corncob by (A) the crude xylanase, (B) partially purified xylanase and (C) purified xylanase_1948. (●: xylopentaose (X5) and xylotetraose (X4) ●: xylotriose (X3); ▼, xylobiose (X2); ▲, xylose (X1); ■, arabinose (Ara); and ×, total XOs.

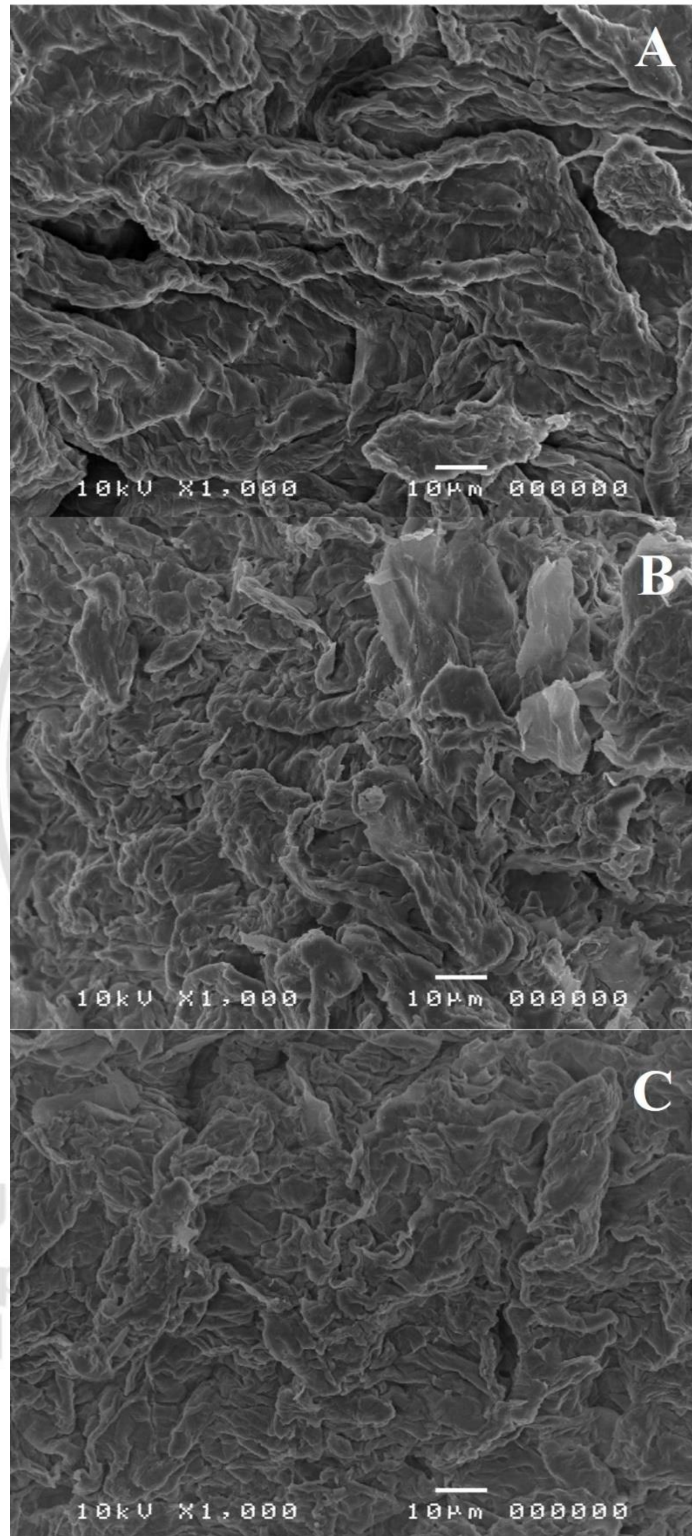


Figure 4.9 Scanning electron microscope (SEM) photomicrograph of the surface of KOH-treated corncob hydrolyzed by (A) the crude (B), the partially purified and (C) purified xylanase_1948 (C) at 1,000×.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright by Chiang Mai University
All rights reserved

From the results, purified xylanase_1948 would be useful for the hydrolysis of corncob to obtain XOs and arabinooligosaccharides which are a significant and valuable compound in the nutraceutical and pharmaceutical industries (Michlmayr et al., 2013). The design of a xylanase over-expression system would facilitate the production of these compounds. The initial content of oligo- and monosaccharides in each XO mixture is presented in Table 4.3. In the reaction mixture containing purified xylanase, the product contains minor amounts of arabinose. Besides that, the crude and partially purified xylanase solutions probably contained other xylanolytic enzymes able to hydrolyze the side chain of arabinoxylan in corncob (Sutay Kocabaş et al., 2015). Therefore, the purified xylanase_1948 can be used when a high yield of XOs are of interest, as it will minimize unwanted arabinose production.

Table 4.3 The composition of xylooligosaccharides and monosaccharides generated by crude, partially purified and purified xylanase_1948 at 12 h of reaction time.

XOs and monosaccharides (% (w/w))	**Crude xylanase**	**Partially purified xylanase**	**Purified xylanase**
Xylotetraose and xylopentaose	36.64±1.40	34.99±2.44	39.40±3.61
Xylotriose	5.33±1.10	6.32±1.29	11.04±0.51
Xylobiose	37.30±3.13	34.92±2.85	35.08±1.07
Xylose	13.90±0.57	17.07±1.76	11.41±1.30
Arabinose	6.83±0.32	6.71±0.36	3.06±0.29

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

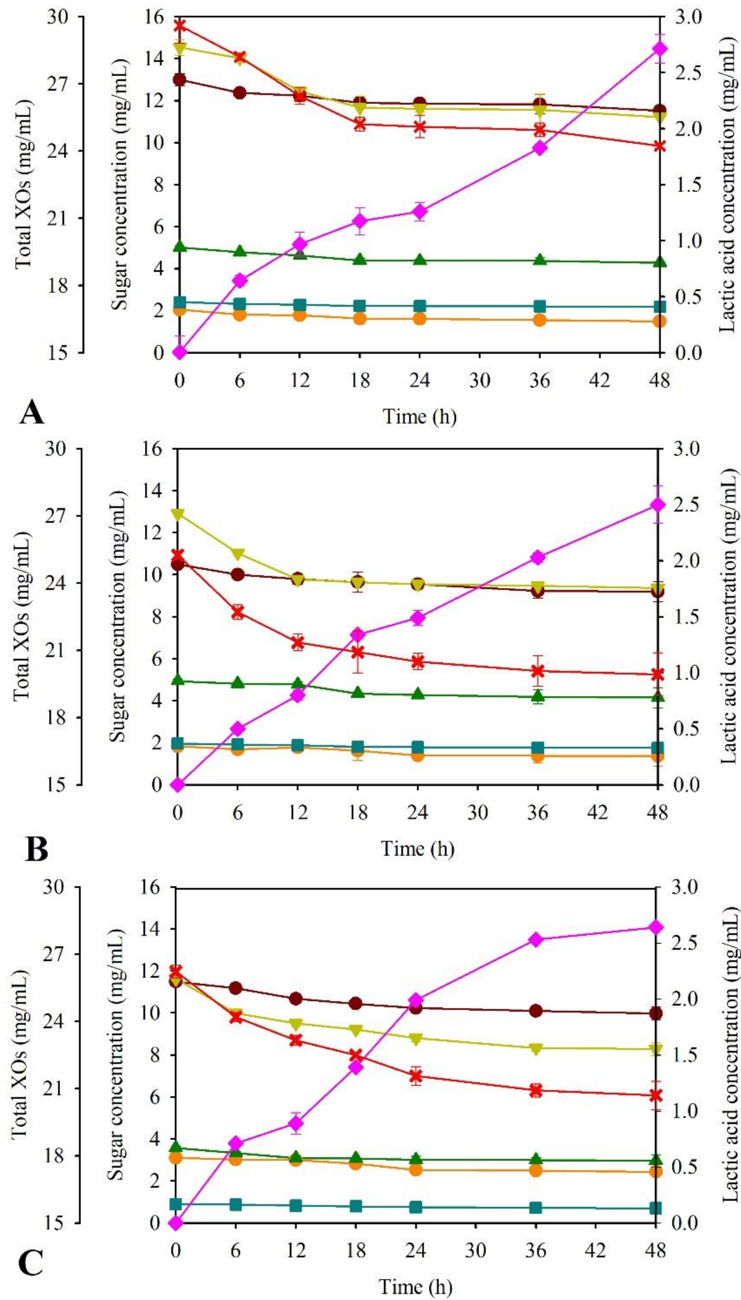
4.3.4 Prebiotic effect of xylooligosaccharides on fermentation by *Lactobacillus*

In vitro fermentation of three XO preparations were compared with control experiment (without sugar). The decreasing of XO content, increasing of viable cell count, increasing of lactic acid concentration (Figure 4.10) and β-xylosidase activity (data not shown) may indicate the prebiotic properties of all XO preparations. Among the XOs generated from three xylanases, the XOs generated by the purified xylanase promoted the growth of *L. plantarum* TISTR1465 and its production of lactic acid than others (Figure 4.10A-C). Strain TISTR1465 was able to use xylobiose (X2) and

xylopentaose (X5) as carbon sources. While, the prebiotic activity of XOs derived from wheat bran was better than XOs derived from Bengal gram husks during fermentation by lactic acid bacteria and bifidobacteria. This difference can be ascribed to the relatively high arabinose content in the Bengal gram husks-XOs (Madhukumar and Muralikrishna, 2012). In comparison, commercially obtained arabinoxylan oligosaccharides were shown to have a prebiotic effect in bifidobacteria (Rivière et al., 2014). From this study, HPLC analysis of the XO content of medium sampled during fermentation by *L. plantarum* TISTR1465 showed similar patterns of consumption of the three XO preparations (Figure 4.10). During fermentation by this bacterium, the concentration of xylobiose (X2) in MRS medium supplemented with XOs produced by the crude xylanase, partially purified xylanase and purified xylanase decreased to approximately 3.33 (from 14.55 to 11.22), 3.55 (from 12.91 to 9.36) and 3.35 (from 11.63 to 8.28) mg/mL, respectively.

The concentration of XOs with a higher degree of polymerization (DP), i.e., xylotriose (X3), xylotetraose (X4) and xylopentose (DP≥5), as well as xylose and arabinose decreased only slightly. According to the study of Moura et al. (2008), *Lactobacillus* spp. is also able to metabolize XOs mixture mainly composed by xylobiose and xylotriose. As a principle, the arabinose to xylose ratio is one of an important factor that influence on fermentation pattern of XOs by bacteria (Chapla et al., 2012) and the prebiotic effect of XOs (Singh et al., 2015). From Figure 4.10, the XOs produced by the purified xylanase resulted in a higher viable cell count than the XOs from crude xylanase. In the latter cultures, there was a sharp decrease in viable cell counts after 36–48 h of incubation (Figure 4.10). This finding suggests that the accumulation of large amounts of xylose and arabinose (6.47±0.01 g/L) in MRS supplemented with XOs produced by the crude xylanase preparation inhibited the growth of strain TISTR1465 during its long-term cultivation. While, the concentration of arabinose and xylose in MRS supplemented with XOs produced by purified xylanase was much lower (3.66±0.03 g/L). Thus, purified xylanase_1948 is suitable for XO production from corncob under conditions in which xylose and arabinose production is undesirable such as functional food production.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

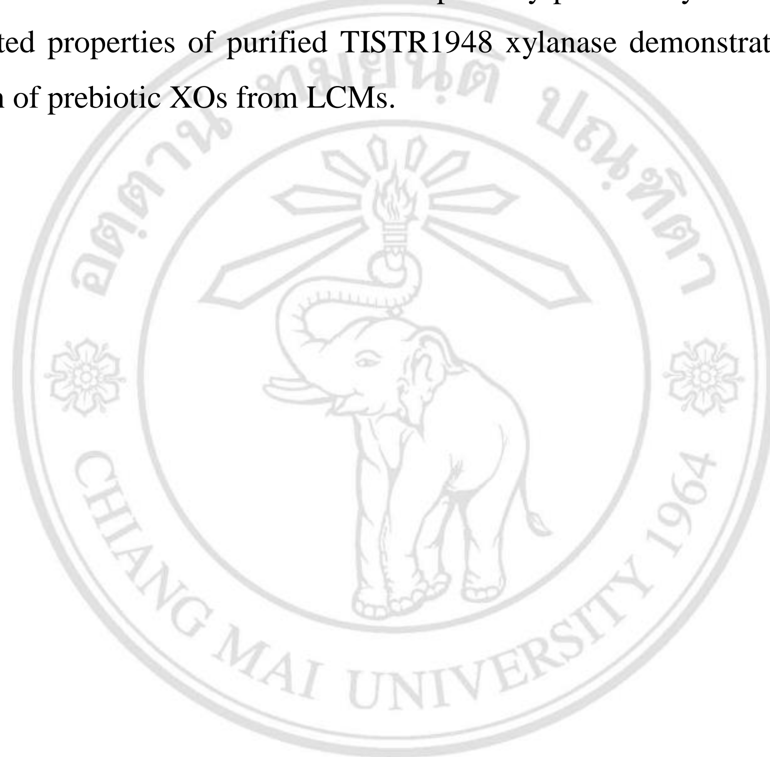


All rights reserved

Figure 4.10 Time course of xylooligosaccharide fermentation by *Lactobacillus plantarum* TISTR1465. XOs produced by the (A) crude xylanase preparation, (B) partial purified xylanase and (C) purified xylanase were added as a sole carbon source to MRS medium inoculated with *L. plantarum* TISTR1465. The culture broth was periodically sampled and analyzed for the growth of strain TISTR1465 (sugar utilization (×: total XOs; ●: xylopentaose and xylotetraose; ●: xylotriose; ▼: xylobiose; ▲: xylose; and ■: arabinose) and lactic acid production (◆)).

4.4 Conclusion

Xylanase from *S. thermovulgaris* TISTR1948 showed maximum activity at 65°C and pH 6.5. The enzyme was stable over broad pH (3.5–11.5 and temperature (50–70°C) ranges. While, XOs produced from KOH-treated corncobs using the purified enzyme resulted in very low concentrations of xylose and arabinose. Furthermore, these XOs enhanced the growth of the probiotic lactic acid bacterium *L. plantarum* TISTR1465 than XOs from either the crude or partially purified xylanase preparations. The investigated properties of purified TISTR1948 xylanase demonstrate its utility in the production of prebiotic XOs from LCMs.



ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved