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Xylanase Activity of *Streptomyces violascences* BF 3.10 on Xylan Corncobs and its Xylooligosaccharide Production

W. Salupi[a], Yopi[b], & A. Meryandini[c,d,*]
[a]Major Program of Biotechnology, Graduate School, Bogor Agricultural University
[b]Biocatalyst and Fermentation Laboratory, Research Center for Biotechnology, Indonesian Institute of Science
Jalan Raya Bogor Km. 46 Cibinong, Indonesia
[c]Biology Department, Faculty of Mathematics and Natural Science, Bogor Agricultural University
[d]Research Center for Bioresources and Biotechnology
Kampus IPB Dramaga, Bogor 16680, Indonesia
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ABSTRACT

Corn is one of the important carbohydrate sources in Indonesia that is mainly used for food and industrial materials. In addition, the byproducts of corn, such as corncobs, have been reported as xylan-containing materials that can be utilized as substrate in xylooligosaccharides (XOS) production. XOS are natural prebiotic fibers that can enhance the performance of animal's digestive system. The main objective of this study was to exploit xylan from corncobs to produce XOS. The research consisted of extraction and production of xylan from corncobs and the synthesis of XOS from corncob-produced xylan. The corncob and *Streptomyces violascens* BF 3.10 xylanase is a collection of PPSHB IPB Laboratory. Corncobs xylan extracted by using alkaline method and reducting sugar was analyzed by dinitrosalicylic acid method. The xylan extraction from corncobs could produce 7.93% (w/w) of xylan. The activity of *S. violascens* BF 3.10 xylanase on the substrate of concorb-produced xylan was 6.4 U/mL at the optimum temperature of 60 °C in 50 mM phosphate buffer with pH 5.5. The thin layer chromatography analysis indicated that 1% (w/v) corn-cob xylan could produce XOS with degree of polymerization (DP) 3.92. XOS, with DP ranging from 2-4, could be used as a livestock feed mixture to stimulate the growth of normal microbes in the gastrointestinal tract of livestock.

Key words: corncobs, Streptomyces violascens BF 3.10, xylan, xylanase

ABSTRAK

Jagung merupakan sumber karbohidrat penting di Indonesia, khususnya untuk makanan dan bahan baku industri. Tongkol jagung adalah limbah prospektif yang mengandung xilan tinggi sehingga dapat digunakan sebagai substrat untuk memproduksi xilooligosakarida (XOS). XOS adalah serat prebiotik alami yang dapat membantu kesehatan saluran pencernaan. Penelitian ini bertujuan untuk memanfaatkan xilan dari limbah tongkol jagung untuk menghasilkan XOS. Penelitian ini meliputi ekstraksi xilan tongkol jagung, produksi xilanase dan produksi XOS dari xilan tongkol jagung. Tongkol jagung dan xilanase *Streptomyces violascens* BF 3.10 merupakan koleksi dari Laboratorium PPSHB IPB. Ekstraksi xilan tongkol jagung menggunakan metode alkali dan kadar gula dianalisa menggunakan metode *dinitrosalicylic acid*. Ekstraksi xilan dari tongkol jagung menghasilkan rendemen xilan 7,93%. Aktivitas *Streptomyces violascens* BF 3.10 xilanase pada xilan tongkol jagung sebesar 6,4 U/mL pada suhu optimum 60 °C dalam 50 mM bufer fosfat pH 5.5. Hasil analisis kromatografi lapis tipis, 1% xilan tongkol jagung pada kondisi optimum menghasilkan xilooligosakarida dengan nilai derajat polimerisasi (DP) 3,92. Xilooligosakarida (dengan DP antara 2-4) dapat digunakan sebagai campuran pakan ternak untuk merangsang pertumbuhan flora normal dalam saluran pencernaan ternak.

Kata kunci: Streptomyces violascens BF 3.10, tongkol jagung, xilan, xilanase

*Corresponding author:
E-mail: ameryandini@yahoo.com

INTRODUCTION

Corn is one of the carbohydrate sources in Indonesia especially for feed and industrial raw materials. Based on its sugar composition, hemicellulose is classified as xylan, mannan, arabinoxylan, and arabinan. Corncobs consist of 38.99% (w/w) crude fiber with the highest content of xylan (12.4%) as compare to other agricultural by products such as rice straw, oil palm kernel, bagasse, cotton stalk, sorghum, tobacco stalk and soybean kernel (Richana *et al*., 2004). Xylan, which is the most abundant hemicellulose in monocotyledonous plants and hard wood, is also reported to interact with cellulose. Compared to xyloglucan, information about its critical backbone length required for interaction with cellulose, as well as about the influence of xylan substituents on this interaction is limited (Kabel *et al*., 2007). Xylan from corncobs can be used as a carbon source for the growth of xylanase producing bacteria.

Xylanase is a xylan hydrolytic enzyme. It can also hydrolyze the polymer of either xylose or xylooligosaccharides (XOS). Several actinomyces that have been reported to produce xylanase are *Streptomyces* (*S*.) *galbus* (Kansoh & Nagieb, 2004), *S. albus*; *S. chromofuscu* (Rifaat, 2005), *S. cyaneus SN32* (Ninawe& Kuhad 2005), *Streptomyces* sp. 451-3 (Meryandini *et al*., 2007), *S. lividans* (Arias *et al*., 2007), *S. bangladehienis* (Al-Bari *et al*., 2007), *Streptomyces* sp. *OM 09* (Ray, 2010), *S. megasporus* DSM 41476 (Qiu *et al*., 2010), *Streptomyces* sp. RCK-2010 (Kumar *et al*., 2010), *Streptomyces* sp. SWU10 (Deesukon *et al*., 2011), *S. rameus* (Bhosale *et al*., 2011), *S. chartreusis L1105* (Zhu*et al*., 2012), *S. griseorubens* LH-3 (Cheng *et al*., 2013) and *Streptomyces* sp. ESRAA-301097 (El-Gendy & El-Bondkly, 2014).

The products of hydrolyzing agricultural waste by xylanase are *xylooligosaccharides*. *Xylooligosaccharides* (XOS) have high economic value as food additive. XOS consumption in piglet can improve fermentation in the digestive tract so that it improves the growth of normal flora and production of short-chain fatty acid (Moura *et al*., 2008). In addition, XOS are able to function as fiber, so it might acts as a prebiotic (Yang *et al*., 2005). Fiber plays a role as a trigger for the increasing of beneficial bacteria, such as *Lactobacillus* and *Bifidobacteria,* in the intestine tracts. A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health (Slavin, 2013). The ingested prebiotic stimulates the growth of the whole indigenous population of bifidobacteria, and the larger that population, the larger is the number of new bacterial cells appearing in feces (Roberfroid, 2007). Prebiotics can also suppress the growth of pathogen bacteria and decrease the level of ammonium in the feces of livestock (Yusrizal, 2012).

XOS resulted from degradation of xylan has an advantage for the growth of intestinal microbes. Previous research showed that XOS could suppress the growth of pathogen microbes and decrease ammonium in the feces of livestock (Yusrizal, 2012). The objective of this study is to hydrolyze corncobs as either xylan substrate or agricultural waste to produce XOS by using xylanase produced by *S. violascens* strain BF 310. *S. violascens* BF 3:10 are local isolates isolated from soil in National Park Bukit Dua Belas Jambi. Exploration of local isolates and local corn cob substrate contributes greatly to the utilization of agricultural waste. XOS produced from corn cobs can be used as a dietary supplement to improve the health of the digestive tract in cattle.

MATERIALS AND METHODS

Microorganism

The *S. violascens* BF 3.10 is a collection of PPSHB IPB Laboratory isolated from Bukit Dua Belas, Jambi. This isolate was used as a xylanase producing bacteria. For this purpose, *S. violascens* BF 3.10 was grown on the xylan media containing 0.5% xylan substrate from corncobs, 0.2% (w/v) yeast extract; 0.5% (w/v) $MgSO_4$; 0.05% (w/v) K_2HPO_4; 0.075% (w/v) KNO_3; 0.002% (w/v) $FeSO_4.7H_2O$; 0.004% (w/v) $CaCl_2$; 0.1% (w/v) glucose.

Xylan Extraction

Corncobs used in this experiment were "Silangan Dramaga 3". Delignification of corncob was performed by immersing 40 mesh corncobs flour in 1% (w/v) NaOCl for 5 h at room temperature and the decanted was rinsed with aquadest and filtered. The solid part was a delignified corncob. The delignified corncobs were dried under the sun for 48 h. Chemical analyzes were conducted to determine water, lignin, dry weight, hemicellulose and cellulose levels.

Corncobs xylan extraction was conducted by using a modified method of Richana *et al.* (2007). The dried delignified flour was immersed in 15% (w/v) NaOH for 24 h at room temperature and filtered. The filtrate was neutralized with 37% (w/v) HCl which was then centrifuged at 6000 rpm for 30 min. Ninety five percent (v/v) ethanol was added to the pellet with the proportion of pellet and ethanol was 1:3 and centrifuged at 6000 rpm for 30 min to obtain pure xylan. The xylan was dried by using oven at 50°C for 48 h and crushed to the size of 80 mesh.

Xylanase Activity and Xylanase Assay

The 96 h culture of *S. violascens* BF 3.10 was inoculated in 100 mL of liquid medium containing 0.5% (w/v) xylan corncobs by using crock borer (1 cm in diameter). The culture was incubated for 96 h at room temperature with agitation of 150 rpm (Stuart orbital incubator s1500, Staffordshire, United Kingdom) (Meryandini *et al*., 2008). The enzymatic activity of the supernatant (assumed as a crude enzyme) was measured by using dinitrosalicylic (DNS) method (Meryandini *et al*., 2008; Akpinar *et al*., 2009; Utami *et al*., 2013) with xylose as a standard. Xylanase activity was tested by incubating crude enzyme in 0.5% beechwood xylan (50 mM phosphate buffer pH 6). The reaction was stopped by immersing the tubes in boiling water for 20 min. Reducing sugar produced was measured at a wavelength of 540 nm (Hitachi, U-3900H, Tokyo, Japan). One unit xylanase activity was defined

as the amount of enzyme producing 1 μmol xylose per minutes.

pH and Temperature Dependencies of Xylanase Activity

Effect of pH on xylanase activity of the crude enzyme was examined at pH 3-4.5 (50 mM sodium citrate buffer), pH 5-6.5 (50 mM sodium phosphate buffer) and pH 7-10 (50 mM glycine NaOH buffer). Meanwhile, the effect of temperature on the xylanase activity was tested by reacting the enzyme with substrate at temperature ranging from 30-100 °C, at the obtained-optimum pH, for 30 min. The xylanase activity was measured by DNS method (Miller, 1959). The stability of crude enzyme was analyzed by incubating the crude enzyme without substrate at different temperatures (4 °C, 30 °C and obtained-optimum temperature) for 0, 3, 24, 72, and 96 h.

Xylanase Production

Production of xylanase was induced by cultivating one crock borer (1 cm diameter, 96 hours old) into 100 mL 0.5% xylan-containing corncobs liquid. The culture was incubated with agitation append of (Stuart orbital incubator s1500, Staffordshire, United Kingdom) at room temperature for 96 h. The crude enzyme was separated from its pellet by centrifugation at 12000 rpm 4 °C for 20 min. The enzyme activity assay was analyzed by using DNS method (Meryandini *et al*., 2008).

Xylan Hydrolysis Using Xylanase

Enzymatic hydrolysis was performed by adding xylan corncobs 1% (w/v) with 12.8 U xylanase *S. violascens* BF 3.10 and incubated at room temperature with agitation append of 150 rpm. One milliliter of sample produced from hydrolysis was collected at 0, 3, 6, 12, 24 h. The total sugar content was measured by Fenol H_2SO_4 method (Dubois *et al*., 1956) and the reducing sugar was measured by DNS method (Miller, 1959), respectively. Polymerization degree was calculated according to the proportion of the total sugar content and reducing sugar.

Table 1. Composition of corncobs fiber before and after delignification

Composition	Before delignification (%)	After delignification (%)
Dry weight	89.58	91.67
Crude fiber	25.15	26.09
Cellulose	33.10	34.07
Hemicellulose	17.90	37.92
Lignin	21.00	16.70
Ash	2.59	1.46
Water	7.99	5.11

Note: Analysis in Laboratory of Feed Science and Technology, Department of Nutrition and Technology, Faculty of Animal Science, Bogor Agricultural University and Research Centre for Bioresources and Biotechnology, Institute of Research and Community Empowerment, Bogor Agricultural University.

Thin Layer Chromatography

Hydrolytic product was detected on chromatography paper (silica gel 60F254 Merck Art 20-20 cm, Darmstadt, Germany). The eluents used were n-butanol, acetic acid and aquadest with the proportion of 2:1:1, respectively. The sugar content was detected by heating the plate at 120 °C for 10 min after spraying with diphenilamine, anillyn, acetone and phosphoric acid (DAP).

Statistical Analysis

The data were analyzed descriptively to describe the effect of pH and temperature on xylanase activity.

RESULTS AND DISCUSSION

Xylan Extraction

The chemical compositions before and after delignification are shown in Table 1. The water content observed in this study was less than that reported by Richana *et al*. (2007) which was 6.43% (w/w). Meanwhile, ash content found in this study was higher than that reported by Koswara (1991), which was 1.33% (w/w). Water content was influenced by drying process, longer drying process yielded in little water content. Ash content could be influenced by mineral content. Ash content decreased after delignification process showed that the delignification process can reduce the mineral content.

Richana *et al*. (2004) stated that fiber content in corncobs was approximately 25% to 39% (w/w), while in this study the fiber content was 26.09% (w/w). The difference in fiber content in this study was related to the variety and the harvesting time of corn.

Delignification by using 1% (w/v) NaOCl as a solvent could cleave carbon linkage on lignin structure. It could also open the linkage between lignin and polysaccharide so that the bacteria are able to use xylan easier (Lehninger, 1982). In this study, the level of hemicellulose after delignification increased to 37.92% (w/w). The use of NaOCl in delignification process was able to dissolve hemicellulose that finally increased its delignification concentration (Richana *et al*., 2007). Fifteen percent of NaOCl concentration could degrade cell wall structure and increase hemicellulose solubility. Compare to other solvents such as hot water, cold water, or HCl, NaOH has been proved to have ability to dissolve xylan at high concentration. Beside NaOCl and NaOH, ethanol can also increase hemicellulose content after delignification. The concentration ratio of 1:3 (supernatant : ethanol) could produce the highest yield (Richana *et al*., 2007).

In this study, the extraction of 500 g corn cobs produced 39.65 g of xylan (7.93%). When compared with the fiber content, which was 26.09% (w/w) (Table 1) so that the ratio of pure xylan was 29.47% (w/w). The result found in this study was similar to that reported by Thu & Preston (1999) who found the ratio of corn cob xylan was 28% (w/w). The ratio of corn cob xylan may vary depends on the extraction process, the age of maize and maize varieties.

Xylanase Production from *S. violascens* BF 3.10

Daily production of xylanase from S. *violascens* BF 3.10 and its activity measured at pH 5.5 under room temperature incubation is presented in Figure 1. Exponential growth of *S. violascens* BF 3.10 was observed until 48 h followed by the stationary phase which was ended after 72 h. Moreover, S. *violascens* BF 3.10 secreted extracellular xylanases at a low rate in the exponential phase and achieved the maximum xylanase production at the end of the stationary phase (72 h, Figure 1). These enzymes were optimally expressed at the end of the exponential phase. but after the stationary phase (72 h), the xylanase activity was gradually decreased. The decreased xylanase activity could be due to the hydrolysis by autologous protease in the decline phase as were reported previously in *Paenibacillus* sp. strain NF1 (Zheng, 2014), *Fomitopsis pinicola* KMJ812 (Shin *et al*., 2010), *Paenibacillus campinasensis* G1-1 (Zheng *et al*., 2012) and *Streptomyces* spp. SKK1-8 (Meryandini *et al*., 2006).

The enzyme activity of xylanase from *S. violascens* BF 3:10 was optimum at 96th hour, while the biomass of *S. violascens* BF 3:10 cells reached the highest level at 72nd hour. These results showed that the optimum activity was not always at the time of optimum cell growth. Enzyme production of xylanase *S. violascens* BF 3:10 reached the optimum level when the growth rate and metabolic activity were in balance. The reduced carbon and nutrients availabilities promoted the dead of cells.

Effects of pH and Temperature on Xylanase Activity

The effect of pH on the xylanase activity produced by *S. violascens* BF 3.10 is shown in Figure 2. The xylanase showed a remarkable activity on a wide range of pH, from 4 to 10, in which the optimum activity was observed at pH 5.5. The effect of temperature on the enzyme activity was observed by incubating the enzyme with the substrate at several temperature ranges for 30 minutes and optimum pH 5.5 (Figure 3). Xylanase produced by *S. violascens* BF 3.10 displayed a remarkable activity at temperature range from 30 to 80 °C with maximum activity observed at 60 °C. The highest enzyme activity was reached at pH 5.5 and temperature of 60 °C. and his condition was similar to that reported by Ratanakhanokchau *et al*. (2009). The difference in enzyme activity at several temperature and pH levels could occur due to the variety of chemical interactions on protein (Bataillon, 2000).

Thermal stability was important in characterization of an enzyme. Thermal stability of xylanase produced by *S. violascens* BF 3.10 examined by incubation at 4 °C, 30 °C, and 60°C are shown in Figure 4. The enzyme was relatively stable upon incubation at 4 °C and 30 °C for 96 h. However, the increased incubation temperature to 60 °C resulted in a decrease in activity up to 20%. The rapid decrease in enzyme activity when it was incubated at 60 °C was caused by the inactivation of the enzyme by high temperature. Xylanase activity was unstable in preservation temperature more than 50 °C (Chapla *et al*. 2010). Protein stability is influenced by environmental conditions such as pH and temperature. Appropriate pH

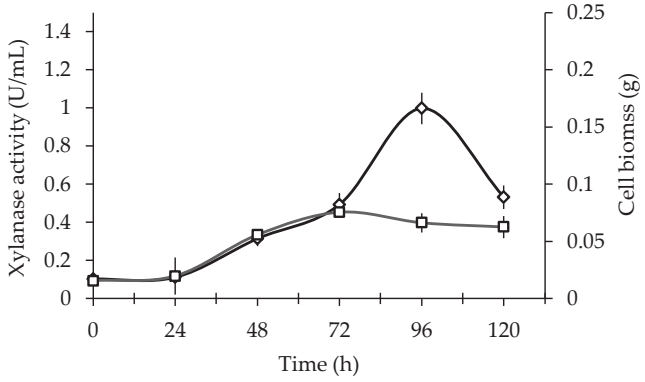

Figure 1. Xylanase activity (–◊–) and cell biomass curve of *S. violascens* BF 3.10 (–□–) on 0.5% corncob xylan medium with 150 rpm agitation at room temperature

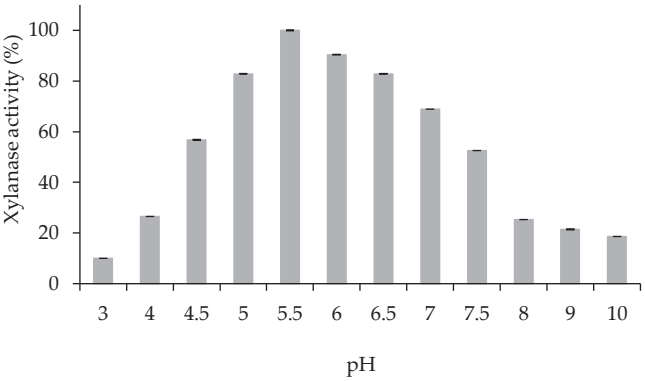

Figure 2. The effect of pH on *S. violascens* BF 3.10 xylanase activity at room temperature. pH 3-4.5 (buffer citrate 50 mM), pH 5-6.5 (buffer phosphate 50 mM) and pH 7-10 (buffer Tris HCl 50 mM). The optimum activity of 2.60 U/mL was observed at pH 5.5 and adjusted as 100%.

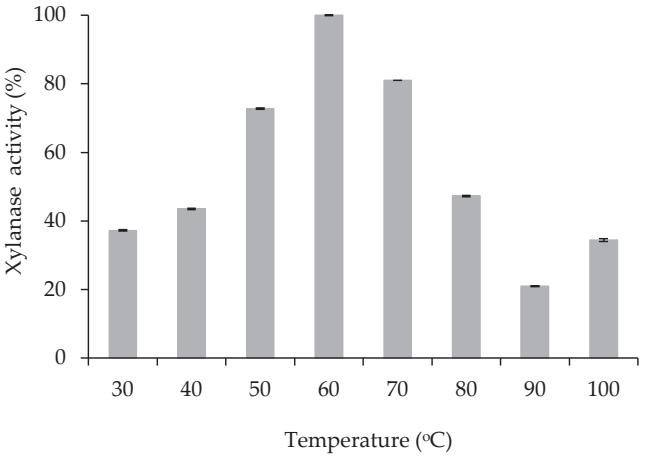

Figure 3. The effect of temperature on *S. violascens* BF 3.10 xylanase activity measured at pH 5.5. The optimum temperature activity was obtained at pH 5.5. The optimum activity of 6.4 U/mL was observed at 60 °C and adjusted as 100%.

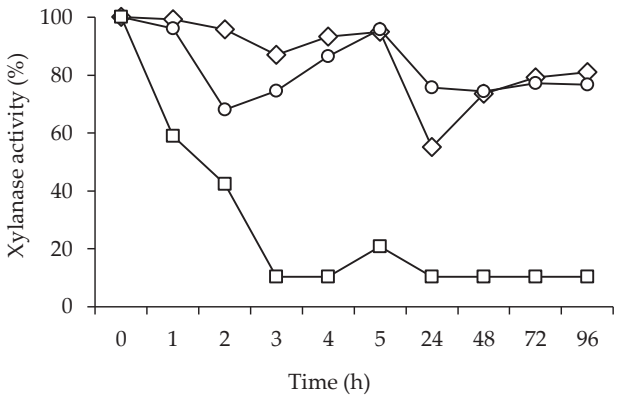

Figure 4. Stability curve of *S. violascens* BF 3.10 xylanase at various temperatures. The enzyme activity was measured at pH 5.5 and 4 °C (–◊–), 30 °C (–o–) and 60 °C (–□–). The initial activity was 8.72 U/mL and adjusted as 100%.

and temperature will increase the interaction between the enzyme proteins that will prevent them from denaturation (Nath & Rao, 2001).

Hidrolysis of Xylan

The pattern of xylan hydrolysis could be qualitatively seen on thin layer chromatography (TLC). The TLC plat showed that most of the hydrolysis product was XOS with spot located under the standard xylose (Figure 5). The result also indicated that XOS was produced at least after 3 hours of hydrolysis reaction. However, little amount of xylose was also produced. The increasing amount of the hydrolysis product was observed as indicated by thicker spots after 3 h incubations as compared to that of shorter incubation time. Following 3 h incubation, short xylooligosaccharide was started to be produced and thickened along with the addition of the incubation time up to 24 h. The spots observed in our TLC showed a variety of short xylooligosaccharide chains produced upon the hydrolysis. This result might be due to multiple substrate binding sites of the enzymes, as reported by Collins *et al* (2005), to produce several polysaccharides chains.

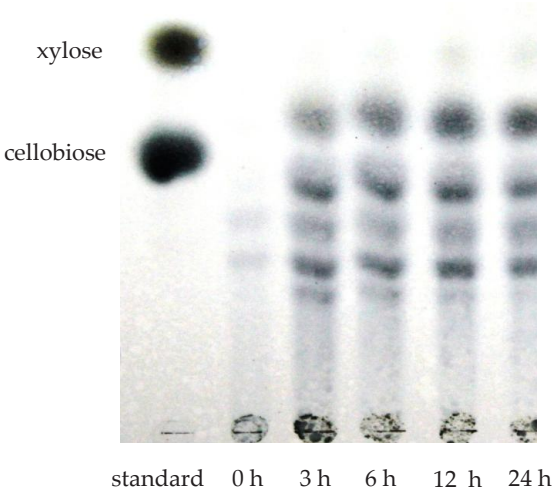

Figure 5. Thin layer chromatography analysis of corncobs hydrolysis product. The reaction was done by using 1% of corncobs with 1.6 U/mL enzyme *S.violascens* BF 3.10 at various incubation times (0, 3, 6, 12, and 24 h).

Based on the results reported by Chapla *et al.* (2012), using different concentrations of substrate at different incubation times played important role in enzymatic hydrolysis during XOS production. Increased xylan concentration in the reaction, from 1 % (w/v) to 3% (w/v), did not increase the production of XOS. However, reduction of substrate concentration from 1.0% (w/v) to 0.1% (w/v) decreased the production of XOS. The reduction of XOS production at high substrate concentration (more than 3%) is probably due to saturation of the binding sites of xylanase occupied by the substrates and the decreased number of water molecules assisting hydrolysis reactions. In addition to substrate and hydrolysis time, the possible factors affecting hydrolysis process were bacterial properties and environmental condition.

The degree of polymerization (DP) expressed the number of polysaccharide chains that can be cleaved into monosaccharide. Smaller number of DP showed that the polysaccharides were depolymerized into shorter chain compounds. After 3 h of incubation the DP did not decrease. Longer hydrolysis time up to 24 h did not affect the DP (Table 2).

According to Wang (2009), production of xylooligosaccharides as a major product of the hydrolysis indicates that the enzyme is very active to digest xylose chain. These properties are characteristic of the endotype xylanase enzyme (endoxylanase). There are different characteristics of xylanase from variety of microorganisms, the xylanase of *Bacillus* sp. X13 has the capacity to produce cellobiose (Aygan & Arikan, 2009).

Corncobs as an agricultural by product that could be utilized as a substrate for xylanase produced by *S.violascens* BF 3.10 to produce XOS. According to Richana *et al.* (2007), corncob is considered as a prospective raw material for either xylan industry or agricultural waste management. As xylan production from corncobs is considerably high, it is also promising to be developed for several oligosaccharide products, including prebiotic. The application of this product as a prebiotic was reported in cattle. This is because microbes in the cattle's rumen were able to produce xylanase that had the same characters as those obtained in this experiment, i.e. the xylanase optimum pH was 6-7, and optimum 50-60 °C (Budiansyah *et al*., 2010).

Tabel 2. Degree of polymerization of hydolysis product of corncobs by *S. violascens* BF 3.10

Time (h)	Total sugar (mg/mL)	Reducting sugar (mg/mL)	Degree of polymerization
0	12.75	0.82	15.40
3	10.82	2.30	4.70
6	9.50	1.89	5.00
12	10.83	2.50	4.30
24	6.41	1.63	3.90

CONCLUSION

Utilization of xylan as a substrate for xylanase to produce XOS is an interesting field to be explored. In this research, 500 g of corncobs could produce 39.65 g of xylan with 7.93% (w/w) yield. Further, xylan extracted from corncobs can be used as a substrate for the growth of *S. violascens* BF 3.10. The optimum time of xylanase activity of *S. violascens* BF 3.10 (pH 5.5, 60 °C) to hydrolyze 1% (w/v) corncobs substrate was 24 hour with the hydrolysis product is xylooligosaccharides (XOS) with polymerization degree of 3.92. Since XOS is considered as a prebiotic, xylan corncobs can be used as substrates to produce this prebiotic in a larger scale.

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