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Muhammad Nur Kholis
Major Program of Biotechnology, Graduate School, Institut Pertanian Bogor, Bogor 16680, Indonesia

Yopi
Biocatalyst and Fermentation Laboratory, Research Center for Biotechnology, Indonesia Institute of Science, Bogor 16911, Indonesia

Anja Meryandini
Major Program of Biotechnology, Graduate School, Institut Pertanian Bogor, Bogor 16680, Indonesia Research Center for Biological Resource and Biotechnology, Institut Pertanian Bogor, Bogor 16680, Indonesia Department of Biology, Faculty of Mathematics and Natural Science, Institut Pertanian Bogor, Bogor 16680, Indonesia, ameryandini@ipb.ac.id

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Xylooligosaccharide Production from Tobacco Stalk Xylan using Xylanase *Streptomyces* sp. BO 3.2

Muhammad Nur Kholis[1], Yopi[2], and Anja Meryandini[1,3,4*]

1. Major Program of Biotechnology, Graduate School, Institut Pertanian Bogor, Bogor 16680, Indonesia
2. Biocatalyst and Fermentation Laboratory, Research Center for Biotechnology, Indonesia Institute of Science, Bogor 16911, Indonesia
3. Research Center for Biological Resource and Biotechnology, Institut Pertanian Bogor, Bogor 16680, Indonesia
4. Department of Biology, Faculty of Mathematics and Natural Science, Institut Pertanian Bogor, Bogor 16680, Indonesia

[*]*E-mail: ameryandini@ipb.ac.id*

Abstract

Tobacco stalk (TS), which is one type of lignocellulosic material, has a xylan content of up to 21.9%. Lignocellulose can be used to produce xylooligosaccharides (XOs). XOs are dietary fibers that have prebiotic activity. This study aimed to produce XOs from tobacco stalk xylan using xylanase from *Streptomyces* sp. BO 3.2. After the TS was delignified, the xylan was extracted using the alkali method. The delignification process, which used 1% natrium hypoclorite (NaOCl), decreased the lignins from 32.93% to 18.15%. Xylan extraction was conducted using 10% natrium hydoroxide (NaOH); this extraction produced xylan of 15.53% (w/w). The xylanase produced by *Streptomyces* sp. BO 3.2 on a 0.5% TS medium had 5.92 U/mL of activity, with the optimum condition occurring at pH 5.5 and a temperature of 60 °C. The xylanase was stable, at temperature 4 °C and 30 °C for 120 hours. The xylanase *Streptomyces* sp. BO 3.2 was capable of hydrolyzing 2% TS xylan and 2% beechwood xylan during the first, third, sixth, and twelfth hours of incubation time; it also produced XOs with degrees of polymerization (DP) of 2.18 and 2.15, respectively. A Thin layer chomatography (TLC) analysis indicated that the hydrolysis products were XOs with the absence of xylose, glucose, and arabinose.

Abstrak

Produksi Xilooligosakarida dari Tangkai Tembakau Menggunakan *Streptomyces* sp. BO 3.2. Tangkai tembakau merupakan salah satu limbah lignoselulosa yang memiliki kandungan xilan sebesar 21,9%. Lignoselulosa dapat digunakan sebagai bahan baku untuk produksi xilooligosakarida. Xilooligosakarida (XOs) merupakan oligosakarida yang merupakan *dietary fiber* yang memiliki aktivitas prebiotik. Penelitian ini bertujuan untuk produksi xilooligosakarida dari xilan tangkai tembakau secara enzimatik menggunakan xilanase *Streptomyces* sp. BO 3,2. Xilan tangkai tembakau diekstraksi secara alkali. Deliginifikasi dilakukan dengan mengunakan natrium hipokorit (NaOCl) 1% mampu menurunkan kandungan lignin dari 32,93% menjadi 18,15%. Ekstraksi xilan tangkai tembakau dengan menggunakan natrium hidroksida (NaOH) 10% mampu mengasilkan rendemen kandungan xilan sebesar 15,53 %. Produksi xilanase *Streptomyces* sp. BO 3,2 pada media xilan tangkai tembakau 0,5% memiliki aktivitas sebesar 5,92 U/mL dengan kondisi optimum pada pH 5,5 dan suhu 60 °C. Xilanase *Streptomyces* sp. BO 3,2 stabil pada suhu 4 °C dan 30 °C selama 120 jam. Xilanase *Streptomyces* sp. BO 3.2 (4.40 U/mL) mampu menghidrolisis xilan tangkai tembaku 2% dan *xilan beechwood* 2% pada waktu inkubasi 1, 3, 5, dan 12 jam dan mampu menghasilkan xilooligosakarida dengan derajat polimerasi masing-masing 2,18 dan 2,15. Analisis *Thin layer chromatography* (TLC) menunjukkan produk hidrolisis berupa xilooligosakarida tanpa adanya xilosa, glukosa, dan arabinosa.

Keywords: Tobacco stalk, xylanase Streptomyces sp. *BO 3.2, xylooligosaccharides*

Introduction

Tobacco (*Nicotiana* sp.) is one of the most important agr**o**industry commodities in Indonesia. The growth production of tobacco in Indonesia from 2011 to 2012 was 5.68%; at end of 2012, production had reached 226.704 tons [1]. This growth in the agroindustry sector has increased the waste that results from the production

process and the raw material processing of tobacco. Tobacco stalk (TS) is not optimally used, and it is usually burned in tobacco fields. TS is composed of cellulose, hemicellulose, and lignin; these materials do not easily degrade naturally. This condition could damage the environment. The xylan content of TS can reach up to 21.9% [2], so has the potential for bioconversion into other materials with high economic value.

Xylan is a major component of hemicellulose. Xylan is a polysaccharides with a backbone formed by xylose subunit homopolymer. A partial breakdown of xylan can produce xylooligosaccharides (XOs), which are oligosaccharides composed of xylose units [3,4]. XOs are dietary fibers with prebiotic activity. They have the ability to improve bowel function, immunity, and antimicrobial activity in humans, among their other health benefits [5]. XOs taken from rice husks can enhance the growth of probiotic bacteria such as *Bifidobacterium adolescentis* CECT 5781, *Bifibacterium longum* CECT 4503, *Bifidobacterium infantis* CECT 4551, and *Bifidobacterium breve* CECT 4839 [6,7]. These XOs can also inhibit the growth of some pathogenic bacteria, including *Escherichia coli, Campylobacter jejuni*, and *Salmonella enteritidis* [8].

The production of XOs from xylan can be performed using the chemical method, the enzymatic method, or a combination of both methods. Enzymatic production of XOs using xylanolytic microbes has also been done [9,10]. Enzymatic hydrolysis using xylanase can increase the productivity of XOs, decreasing monomeric hydrolysis products and removing the need for special equipment [11].

Xylanase consists of 1,4-β endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-D-glucuronidase, acetyl xylan esterase, and phenolic acids (ferulic acid and acid koumarat) esterase [12]. Xylanase has been found to be produced by both bacterial and fungal microorganisms. Thermophilic xylanase has been isolated from *Bacillus* sp. YC335 [13]. *Bacillus* sp. TAR-1 has also produced xylanase, this xylanse kinds of thermostabel enzyme [14]. Many *Streptomyces* have been reported to produce xylanase, including *Streptomyces halstedii* JM8 [15], *Streptomyces* sp. SKK1-8 [16], *Streptomyces* sp. SKK1234P-16-8 [17], and *Streptomyces* sp. P12-137 [18]. The present study aimed to produce XOs from TS xylan using xylanase from *Streptomyces* sp. BO 3.2.

Materials and Methods

Material. The agricultural waste from Genjah variety TS was obtained from Boyolali, The Central of Java. *Streptomyces* sp. BO 3.2 was collected at the Laboratory of Animal Biotechnology and Biomedical, Research Center for Bioresources and Biotechnology (RCBB) from the Bogor Agricultural University Culture Collection.

Xylan extraction and physicochemical analysis. Xylan was extracted from tobacco plants using the alkali method [19] with slight modifications. The delignification of TS 60 mesh was carried out by using 1% NaOCl solution for 5 hours at room temperature. The soaked TS was rinsed with water and dried at 50 °C for 48 hours. Analyses of the TS components before and after delignification were conducted; the measured components included moisture content, ash, fat, protein, and fiber (lignin, cellulose, and hemicellulose). Xylan from the TS was extracted using 10% NaOH solution for 24 hours at 28 °C. The xylan-soluble fraction was neutralized using an hydrochloric acid (HCI) solution. The water-soluble xylan was separated by adding 95% ethanol (1 : 3 v/v). The precipitated xylan solution was obtained by centrifuging at 2.683 x g for 10 minutes. The xylan was then dried at 50 °C overnight.

Xylanase production. *Streptomyces* sp. BO 3.2 isolate was cultured on a 0.5% TS medium (0.5% TS, 0.02%, 0.0075% $MgSO_4$, 0.05% K_2HPO_4, 0.075% KNO_3, 0.05% K_2HPO_4, 0.002% $FeSO_4 \cdot 7H_2O$, 0.004% $CaCl_2$, 0.2% glucose, 0.1% yeast, and 1.5% agar) for 4 days at 28 °C. One sample crock borer (ϕ 1 cm) of *Streptomyces* sp. BO 3.2 isolate was cultured in 100 mL of 0.5% TS broth media at room temperature on a shaking incubator (150 rpm) for 7 days. A supernatant of the culture was taken daily to determine the optimum time for xylanase production. The optimum time was then used for the next production of xylanase.

Characterization of the xylanase. The optimum pH, temperature, and stability were characterized for the Xylanase *Streptomyces* sp. BO 3.2. pH levels were obtained using 50 mM of buffer citrate (pH 3.0-4.5), 50 mM of buffer phosphate (pH 5.0–6.5), and 50 mM of Tris HCl (pH 7.0–9.0). The effect of temperature on xylanase activity was tested at various temperatures (30–100 °C, with an interval of 10 °C) using the optimum pH. Xylanase stability was determined by incubating enzyme without its substrate at room temperature (28 °C) and at the optimum temperature (4 °C). A xylanase assay was carried out hourly at the optimum pH and temperature levels until the activity was lost.

Xylanase activity. The xylanase assay was conducted using the Dinitrosalisilic acid (DNS) method [20]. The reduction of sugar was measured at the 540 nm wavelength using a spectrophotometer. One unit of xylanase activity was defined according to the amount of enzyme which produces 1 μmol of xylose per minute under the specified condition.

Enzymatic hydrolysis tobacco stalk xylan. Before the xylanase was used in the hydrolysis process, it was purified using the dialysis method in 50 mM of phosphate buffer pH 5.5 to remove residual such as oligosaccharides, which was appeared during the enzyme production. TS and 2% beechwood (w/v) were hydrolyzed with xylanase for 5 different incubation times (0, 1, 3, 6, and 12 hours) at room temperature (28 °C). The total sugar level of the hydrolysis product was measured using the phenol-H_2SO_4 method [21] at a wavelength of 490 nm. The degree of polymerization (DP) of the hydrolysis product was determined using the ratio of total sugars to reduced sugar. A qualitative analysis of the hydrolysis products was determined using the thin layer chromatography (TLC) method.

Results and Discussion

A proximate analysis of the TS before and after delignification (Fig. 1) showed that the lignin content was decreased by 14.78% after delignification. The cellulose also showed a decreased value of about 9.28%, while the hemicellulose content increased from 10.62% to 12.32% (Table 1). The ash content (6.40%) and cellulose (44%) were higher in these samples than in other studies [2], while the hemicellulose was lower (19.9%) [23]. These differences in the content of the material were due to differences in the age and variety of the materials used.

The xylan extraction from the TS 80 mesh began with delignification (Fig. 1A). The delignification was performed using 1% NaOCl for breaking down the lignin, as the lignin was bound to the hemicellulose in a compact structure. A concentration of 1% NaOCl was able to eliminate the lignin content without making the material become soluble hemicellulose [19]. An NaOH solution worked to weaken the bond between the cellulose and hemicellulose, thus facilitating the extraction of the xylan [24]. Ethanol was used to precipitate the xylan to make the extraction process easier.

Extraction of the TS xylan gained a 15.53% yield (Fig. 1B). This xylan yield was lower when compared with other TS xylan extraction yields of 21.39% [2]. The TS, type, and growth of the tobacco plants used accounted for this difference in the the yield of extraction.

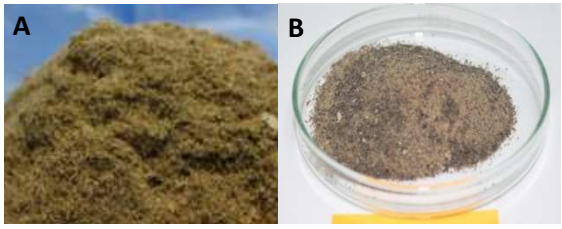

Figure 1. TS xylan substrate A). Biomass TS 80 Mesh, B). after TS Xylan Extraction

Production, characterization, and stability of the xylanase. The xylanase was produced using *Streptomyces* sp. BO 3.2. in 2% TS at room temperature and 150 rpm of agitation. The highest-activity xylanase was obtained on the 5th day at room temperature (28 °C) and with a pH of 7 (5.92 U/mL). The xylanase activity was much higher in *Streptomyces* 2340-16, which had activity of about 0.27 U/mL [10] at the same incubation time. However, the xylanase activity was lower in this sample when compared with *Streptomyces* sp. CD3[24]. These differences were due to the media and types of actinomycetes used in each sample.

The xylanase had an optimum activity level at a certain pH and temperature. The optimum pH and temperature helped enzymes to work effectively. The xylanase *Streptomyces* sp. BO 3.2 worked in a wide range of pH levels (pH 3–10) (Fig. 2). The highest xylanase activity was obtained at pH 5.5 (3.18 U/mL), and had decreased xylanase activity until at pH 7.5 but xylanase activity occurred at pH 8 rise again (1.46 U/mL). The xylanase Streptomyces sp. BO 3.2 had enzyme activity in a wide range of pH, and it was possible there is more than one kinds of enzyme to work as a complex enzyme.

Table 1. A Proximate Analysis of TS Delignification

Proximate analysis	Before delignification (%)	After delignification (%)
Water	10.11	9.92
Ash	6.89	3.76
Fat	1.50	0.90
Protein	9.16	6.07
Fibrous	32.93	42.15
- Lignin	23.48	18.15
- Cellulose	51.55	42.37
- Hemicellulose	10.62	12.32

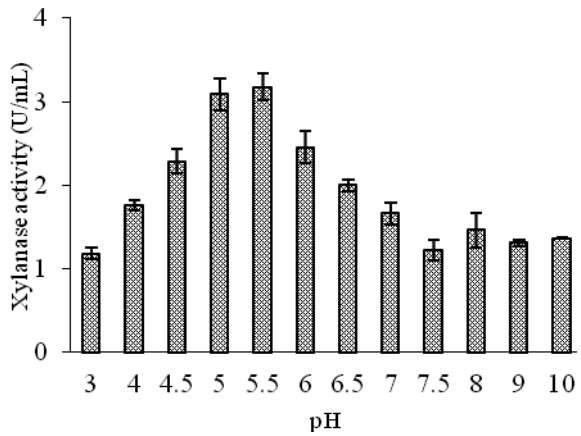

Figure 2. pH Effect of xylanase *Streptomyces* sp. BO 3.2 on 0.5% TS Medium at Room Temperature (28 °C)

The xylanase *Streptomyces* SKK1-8 had a wide range of optimum pH levels; it was active at pH levels between 4.5 and 7 [16]. *Streptomyces* spp. 234P-16 also had a wide range of acceptable pH levels. Between pH 3 and 10, it showed activity specifically in the xylanopiranoside and arabinofuranoside [17].

The xylanase from the *Streptomyces* sp. BO 3.2 had an optimum temperature of 60 °C with enzyme activity of about 8.99 U/mL (Fig. 3). As the temperature increased, the activity decreased. The xylanase almost lost its activity at 100 °C (0.4 U/mL). This is because temperature can structurally alter an enzyme, causing that enzyme to lose its activity. At a high temperature, enzymes are denatured and lose enzyme activity.

The xylanase from *Streptomyces* sp.BO 3.2 was in optimum condition at pH 5.5 and 60 °C. Thus, the enzyme was included in the acidostable enzyme and low temperature stable enzyme. The actinomycetes xylanase had an optimum pH range of 5.0 to 12.0 and an optimum temperature range of 40 to 80 °C [25]. S*treptomyces* sp KT23 and *Streptomysec exfoliates* had an optimum pH of pH 5.5 and an optimum temperature range of 50 to 60 °C [26,27]. The xylanase from the *Streptomyces* sp. BO 3.2 was stable at 4 °C and at room temperature (28 °C), but it was not stable at the optimum temperature (60 °C) (Fig. 4). The xylanase at 4 °C and room temperature (28 °C) still had 93% and 96% activity, respectively after 120 hours of storage time. The stability of the xylanase and the optimum pH level and temperature were used as parameters for the hydrolysis condition.

Xylooligosaccharide Production. Xylooligosaccharides were produced from the xylanase *Streptomyces* sp. BO 3.2. The product of the hydrolysis was analyzed based on the value of the DP.

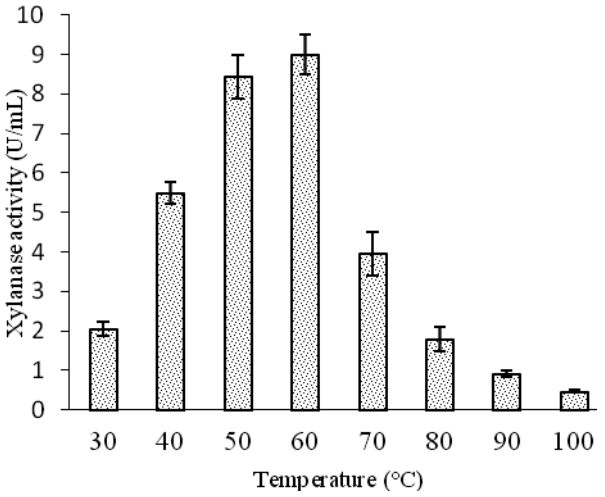

Figure 3. The Temperature Effect of Xylanase *Streptomyces* sp. BO 3.2 on 0.5% TS in 50 mM Buffer Phosphate pH 5.5

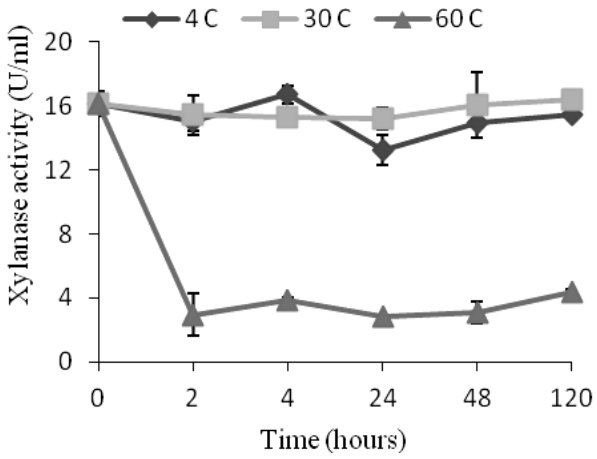

Figure 4. Stability of the Xylanase from *Streptomyces* sp. BO 3.2 on 0.5% TS using 50 mM Buffer Phosphate pH 5.5 at 4 °C, 28 °C, and 60 °C

A qualitative analysis was also performed using the TLC method. Product hydrolisis by xylanase could be measured by increasing the reducing-sugar and decreasing the DP value [10]. An increase in the reducing-sugar was caused by the xylanase action of cutting the complex xylan into a simpler form containing a xylose monomer and XOs. XOs is an oligomer of xylose. In general, XOs are formed from xylose units, which are linked to each other in the bond of β-(1–4) [11]. The number of xylose units can reach a total of 10 units depending on the type of XOs (i.e., xylobiose, xylotriose, etc.). XOs are used for food applications in the form of xylobiose (DP=2) [28].

The hydrolysis of the beechwood xylan using the xylanase from the *Streptomyces* sp. BO 3.2 had the same DP value (DP=2) for intervals of 1 hour and 3 hours. The incubation of the xylanase with the beechwood xylan for 6 hours caused a product hydrolysis with a DP value of about 1 (Table 2). The hydrolysis TS showed a DP value of about 2 for incubation times of 1, 3, 6, and 12 hours. This indicated that 1 hour is the most effective and efficient amount of time to hydrolyze TS into XO form.

The TLC analysis of the hydrolysis products with an incubation time of 1 hour indicated the presence of XOs (Fig. 5) by showing a spot below the xylose standard that was later assumed to be XOs. The hydrolysis product (i.e. XO) of the beechwood xylan was similar to that of the TS xylan. The TLC spot indicated that glucose and arabinose were absent in the hydrolysis products because the TS xylan as categorized as a linear homoxylan. Homoxylan is a xylan with xylose as its main chain; it has no glucose or arabinose as side chains [29].

Table 2. Reducing-sugar and DP of Xylan Hydrolysis Products Using Xylanase

Substrate	Time (hour)	Reducing-sugar	Total sugar	DP
Beechwood xylan 2%	1	5.68	14.69	2.59
	3	6.67	14.30	2.15
	6	7.60	14.25	1.87
	12	7.86	13.82	1.76
Xylan of TS	1	3.50	9.03	2.58
	3	4.36	9.50	2.18
	6	4.38	9.33	2.13
	12	4.54	9.33	2.06

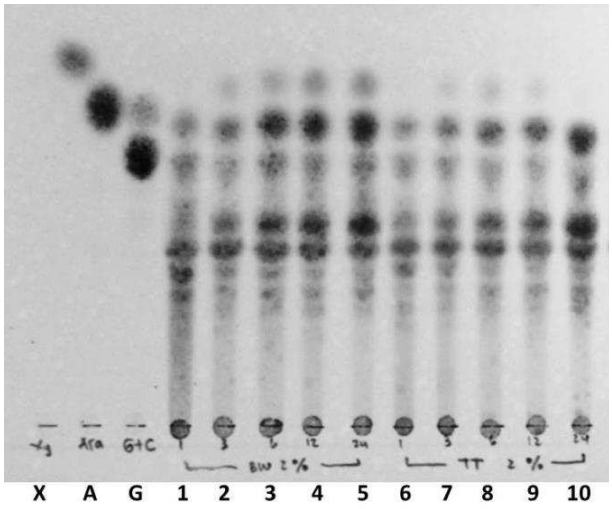

Figure 5. Analysis Product Hydrolysis Qualitatively by Thin Layer Chromatography

The TLC analysis of the hydrolysis product's TS xylan with xylanase *Streptomyces* sp. BO 3.2 indicated that the action of 1,4-β-end oxylanase was more dominant when compared with the action of the 1,4-β-exoxylanase. The 1,4-β-endoxylanase randomly hydrolyzed the xylan chain from inside the XOs, while the 1,4 β exoxylanase hydrolyzed the 1,4-β-D-XOs and the xylobiose, respectively, from their non-reducing ends to produce D-xylose [30].

Conclusions

Extraction of TS xylan using the alkali method produced a 15.53% (w/w) yield. The xylanase produced by the *Streptomyces* sp. BO 3.2 on 0.5% TS medium had 5.92 U/mL of activity, with optimum conditions occurring at pH 5.5 and a temperature of 60 °C. The xylanase was stable when between 4 °C and 30 °C for 120 hours. The xylanase *Streptomyces* sp. BO 3.2 was capable of hydrolyzing 2% TS xylan and 2% beechwood xylan during the first, third, sixth, and twelfth hours of incubation time. It also produced XOs with DPs of 2.18 and 2.15, respectively. The TLC analysis indicated that the hydrolysis products were XOs with the absence of xylose, glucose, and arabinose.

Acknowledgments

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