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Isolation And Hydrolysis Xylan From Soybean Waste With Endo-β-1,4-D-Xilanase Of *Bacillus* sp. From Soil Termite Abdomen

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Abstract

Soybean waste is by product in production of tofu and soymilk. Soybean waste commonly dumps directly to the water sewer and made environmental problems like eutrotification. Soybean waste contains 23% hemicellulose, 16% cellulose dan 28% protein. Xylan rich hemicellulose is potential source of xylooligosacharides (XOS). XOS was known as functional food with prebiotic activities. In this research, xylan was isolated from soybean waste then hydrolyzed by endoxylanase enzyme of *Bacillus sp* from soil termite abdominal. At the beginning, Soybean waste was processed by reflux to exclude lipid compound. Then process continued by alkaline treatment using NaOH 4-18% to separate the xylan from lignocellulose complex. Isolated xylan was hydrolyzed by endo-β-1,4-D-xylanase to get XOS. Hydrolised product analysed by TLC and HPLC. TLC analysis shown thick spot confirmed as xylopentaose (X5) compare to the Rf of the standard compound. HPLC data supported these analyses with the result of X5 as highest component (6959,88 ppm) beside the other XOS such as xylobiosa (X2) 6,34 ppm and xylosa (X1) 7,1 ppm. With the same enzyme, the result of hydrolyzed of soybean wastes xylan is not different with hydrolysed of oat spelt xylan, the common xylan source.

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Keywords: soybean waste, xylooligosacharides, endo-β-1,4-D-xylanase

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1. Introduction

Soybean waste is a byproduct of tofu and soybean milk production. This solid waste always raises a significant problem on its waste treatment every year. In fact, a dry-based soybean waste contains 23% of hemicellulose, 16% of cellulose and 28% of dissolved protein (Heck et al., 2002). Commonly, soybean waste is used for animal feeds which cost so inexpensive for the farmer. Another use of soybean waste is for making of soybean-based flour. Hemicellulose, which rich on xylan as a heteropolysaccharide containing a backbone of xylose units (Saha, 2003), can be potentially used as a raw material of oligosaccharide-based prebiotic containing xylooligosaccharides (XOS). Since XOS is known to have a great activity as a prebiotic, then it can be included in functional food group. XOS cannot be hydrolyzed and absorbed by digestion organs in human. However, XOS can stimulate the growth of probiotic bacteria in duodenum.

Xylanase enzymes group hydrolyses 1,4-glycosidic bonding in xylan. Kind of xylanase enzymes are endoxylanase (1,4-D-xylohydrolase, EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) and esterase, which cuts on the side chain of the xylan backbone (Akpinar and Bostaner, 2009). Endo-β-1,4-D-xylanase is a xylanolitic enzyme catalyzing endohydrolysis reaction on β-1,4-D-xyloside bonding on hetero- or homoxylan and resulting xylooligosaccharides and xylose. In the future, endo-β-1,4-D-xylanase have a great prospect on food, animal feed, health, pulp and paper industry. Biochemistry research group in Departement of Chemistry, University of Jember, has succeeded isolate and characterize endo-β-1,4-D-xylanase from bacteria in abdominal of termites (Ratnadewi et al., 2007). The analysis shows that hydrolysis product of oat spelt xylan by endo-β-1,4-D-xylanase produce XOS with polymerization degree of 2, 3, 4 and 5.

The exploration of utilization of agroindustrial waste is still low and results no significant value added. In fact, more than a million ton of agroindustrial residues have been directly removed without any plans for further utilization and result in a big problem in environment. As described before that those wastes may contain xylan, a source of XOS production. XOS as a functional food has a high economic value nowadays. The commercially XOS is expensive since it uses a commercial substrate as well. Some previous research on soybean waste (terms okara) are including isolation of xylanase and cellulose from soybean waste (Heck et al., 2002), enzymatic process on okara/soybean waste (Kasai et al., 2004), composition of polypeptide in soybean waste (Li et al., 2012), and prebiotic product and okara ability to stimulate the growth of probiotic bacteria Lactobacillus acidophilus, Bifidobacterium (Martos et al., 2009; Bedani et al., 2013). According to this background, this research has a main aim to utilize a soybean solid waste as a source of xylan which then it will be hydrolyzed using endo-β-1,4-D-xylanase from *Bacillus sp* of termite abdomen.

2. Materials and Methods

All the reagents, media and chemicals used in this research were analytical grades (Qualigens, Hi-media, Merck, Sigma). Xylan from Oat spelt xylan was procured from Sigma. Standard xylooligosaccharides (X2,X3,X4 and X5) were purchased from Megazyme, Ireland. Thin-layer chromatography plates (TLC plates) of silica gel 60 F254 were obtained from Merck, Germany. Agro wastes like soybean waste were procured from local industry.

2.1. Microorganism

Bacillus sp from microorganisms abdomen soil termite was used as an endo-β-1,4-D-xylanase producer and was maintained at -20 ^{o}C in gliserol stock.

2.2. Production and partial purification of endo-β-1,4-D-xylanase

Endo-β-1,4-D-xylanase production, the cultured bacteria in LB (Luria Bertani) medium were incubated at 37 °C. The crude enzyme was separated from debris cells by centrifugation at 7,500×g for 30 min at 4 ^{o}C. Partial purification of endo-β-1,4-D-xylanase was carried out by adding calculated amount of solid ammonium sulfate (30 - 70%) to the crude endo-β-1,4-D-xylanase with constant stirring at 25 ^{o}C. Upon centrifugation at 8500 g for 20 min at 4 ^{o}C, the precipitates were dissolved in small volume of 50 mM sodium citrate buffer (pH 5.3). Enzyme solution

was subjected to dialysis for about 18 – 24 h at 4 ^{o}C against 50 mM sodium citrate buffer (pH 5.3). Endo-β-1,4-D-xylanase and protein assay were carried out at each step of purification. The samples were stored at 4 ^{o}C until use.

2.3. Enzyme assays and protein estimation

Endo-β-1,4-D-xylanase (E.C. 3.2.1.8) activity was measured using 1% oat spelt xylan solution as substrate (Bailey,P *et.al.*, 1997). The release of reducing sugars in 10 min at 50 ^{o}C, pH 5.3 (50 mM sodium citrate buffer) was measured as xylose equivalents using dinitrosalysilic acid (DNS) method (Miller, 1959) . One unit of xylanase activity (U) is defined as the amount of enzyme liberating 1 µmol of xylose/min under assay condition.. The soluble protein was determined by Folin's method using bovine serum albumin as standard (Lowry, 1951) . Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.4. Extraction of xylan from soybean waste

Soybean waste was cleaning up with water then drying till constant weight. Delignitation was done by the addition of 0.5% sodium hypochlorite (NaOCl) solution then filtration. Soybean waste (5 g) were soaked in 100 ml of 0.5% NaOCl solution at room temperature for an hour to remove lignin and colored materials. After washing with water, the solid material was dewatered by filtration. The delignified wet material was soaked in 4 – 18% NaOH at room temperature for 24 h to extract the xylan. After filtration, the filtrate was neutralized with 6 M HCl, the xylan was precipitated using 3 volume of ice-cold 95% ethanol. Then, the precipitated xylan was collected by centrifugation at 8,500 g for 30 min at 10 ^{o}C at room temperature, the pellet was retained, dried in forced hot air oven at 65 ^{o}C until constant weight. The pellet was weighed and powdered in mixer and stored at room temperature for furher analysis.

The true yield of xylan was calculated using the following formula:

$$\text{True yield (\%)} = \frac{\text{Dry weight of extracted xylan (g)} \times 100}{\text{Weight of the sample (g)}}$$

The relative yield percent of xylan was derived from percent of true yield and xylan (hemicelluloses) contents of original soybean waste. Hereafter, the best levels of alkali and its condition were followed to undertake the bulk xylan extraction, analysis, and XOS production.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out in the screw cap tubes containing xylan solution from soybean waste with 4.59 U/mg of partially purified endo-β-1,4-D-xylanase (from procedure 2.3). Enzymatic hydrolysis was carried out by incubating the reaction system in water bath at 40 ^{o}C with mild shaking. Controls were kept for each reaction in which the enzyme was inactivated by heating. Samples were withdrawn at regular interval of time and subjected for qualitative analysis by Thin-layer chromatography (TLC) and reducing sugars was quantified using dinitrosalysilic acid method (Miller, 1959).

2.6. XOS Production and Analysis

The production of XOS during the enzymatic degradation of soybean waste xylan were detected by TLC on silica plates. The solvent system for TLC was comprised of 1-butanol, acetic acid, and water (2:1:1 v/v) (Kubata et al., 1994). After running of TLC plates for single ascent, XOS was detected through spraying of α-naftol reagents. Xylose, xylobiose, and XOS were used as the reference standards. The enzymatic hydrolysis originated from xylan of soybean waste was analyzed by High performance liquid chromatography (HPLC) using Shim-pack SCR-101N (7,9 mm X 30 cm) colomn. Aliquots of filtered sample (20 µl) were injected through the manual injector. The XOS

formed during the enzymatic conversion of xylan soybean waste were quantified after comparing the peak areas of XOS with that of standards and expressed as milligrams per milliliter.

3. Results and Discussion

3.1. Pretreatment of soybean waste

Soybean waste was cleaning up with water then drying till constant weight. Contain of lipid and protein was measured by reflux in *n*-hexane and Kjehdahl methods. Lipid and protein content of dry soybean was 11 and 26% of 5 gram dry sample was delignification with 0,5 % NaOCl for 5 h then filtered and dried.

3.2. Xylan extraction

Non lignin dried soybean waste sample was immerge in NaOH varied from 4 – 18 % for 24 hours. Sample then filtered with Buchner and the filtrate neutralize with HCl 6 N. After addition of ethanol (1:3 v/v) filtrate was centrifuge. Sediment was xylan which drying until constant weight. NaOH treatment will separate the xylan from lignocellulose complex (Samanta et al., 2013). Xylan is the most abundant component of hemicellulose in plants. Xylan will be solved by NaOH, separating it from other components of hemicellulose. Xylan then precipitated with ethanol after centrifugation. Result of NaOH variation concentration was the optimal concentration with highest xylan yield at 16% NaOH (33% of dry weight). Samanta *et.al* 2013 had isolated xylan from peagen pea stalk with various concentration of alkali (NaOH and KOH) and got optimal xylan recovery 96% for NaOH 12%. Xylan and xyloglucans are the main type of hemicelluloses in the cell wall of soybean plant (Huisman, 2000; Mateos-Aparicio, Redondo-Cuenca & Villanueva-Suárez, 2010; Villanueva-Suárez et al., 2013).

We found that delignification process reduce the xylan yield compare to without pretreatment. It maybe because in no delignification, there were lignin also isolated with xylan as impurities. Total reduction sugar analyses will confirm this deduction. Complete result of Xylan isolation from soybean waste with various NaOH concentration for delignification and without could be seen in Table 1 and Figure 1 illustrate the isolate of xylan from soybean waste in the delignification and non delignification process.

Tabel 1. Effect of various concentration of NaOH for non and delignification pretreatment on true recovery of xylan from soybean waste

Pre-treatment	Actual recovery of % xylan (g/10 g of raw materials)					
	Levels of alkali					
	4%	8%	12%	14%	16%	18%
No delignification	2.8	3.0	11.8	29.2	33.0	24.8
Delignification	2.3	4.2	8.4	16.7	25.9	22.8

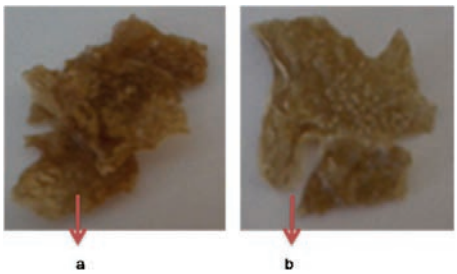

Figure. 1 (a) The xylan isolated from soybean waste with of delignification, (b) The xylan isolated from soybean waste without treatment of delignification

3.3. Enzymatic hydrolyses of xylan from soybean waste by endo-β-1,4-D-xylanase

TLC chromatogram in Fig. 2 shows the profile of the hydrolyses product of xylan from soybean waste by endo-β-1,4-D-xylanase. Enzymatic hydrolyses conducting under condition of 40℃, pH 5 for 16 h. These are the optimum condition for enzymatic hydrolyses by similar endo-β-1,4-D-xylanase for oat spelt xylan as substrate from former research. The TLC showed some spots of several oligosaccharide under xylose standard for the hydrolyzed sample. But the control sample which the enzyme was inactivated shows no spot at all (Ka, Kb,Kc, Kd and Ke). It mean the enzyme was working well in substrate of xylan from soybean waste. In chromatogram there are thick spot at the start point which mean not all xylan substrate were hydrolyzed and probably need more time for the hydrolyses process.

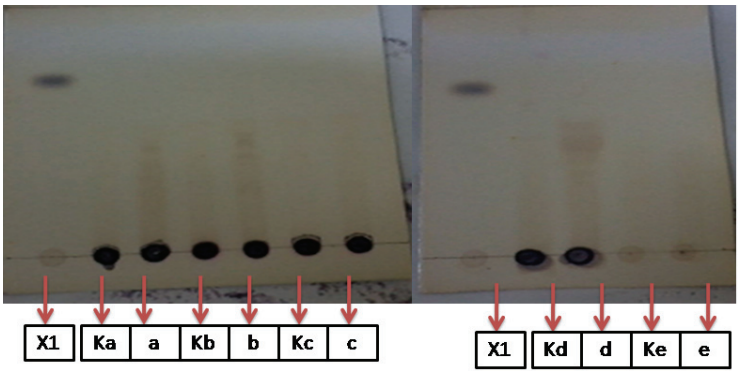

Figure 2. TLC chromatogram of enzymatic hydrolyses products of xylan from soybean waste by endo-β-1,4-D-xylanase at 40 ^{0}C, pH 5. (X1 xylose standard, K, control, Ka, Kb, Kc, Kd, Ke without active enzyme. a, sample with lipid and protein elimination also delignification, b, sample with lipid and protein elimination without delignification c sample without lipid and protein elimination but delignification, d. sample without lipid and protein elimination also without delignification, e soybean waste.

3.4. XOS production and analyses

HPLC analyses shows composition of oligosaccharide from enzymatic hydrolyses endo-β-1,4-D-xylanase originally of *Bacillus sp.* Isolated from soil termite abdominal with xylan from soybean waste as substrates. HPLC chromatogram shows distribution of monosaccharide and oligosaccharides (xylooligosaccharides, XOS). XOS product was quantitatively measured by comparing the wide of XOS peak area with the peak area of standard xylose (X1), xylobiose (X2), xylotriose (X3) and xylopentaose (X5). HPLC chromatogram showed the XOS products were X1, X2 and X5 with the dominant product was X5. The result is similar with hydrolyses oat spelt xylan as substrate with similar enzyme which dominant product is X5 (Table 2, Figure 3).

Tabel 2. Quantitative calculation of HPLC chromatogram of hydrolyses xylan from soybean waste

Kind of XOS	Retention time (minute)	Wide of the peak	Concentration (ppm)
Xylose (X1)	13,966	4577	7,10
Xylobiose (X2)	11,384	3077	6,34
Xylopentaose (X5)	8,414	7221724	6595,88

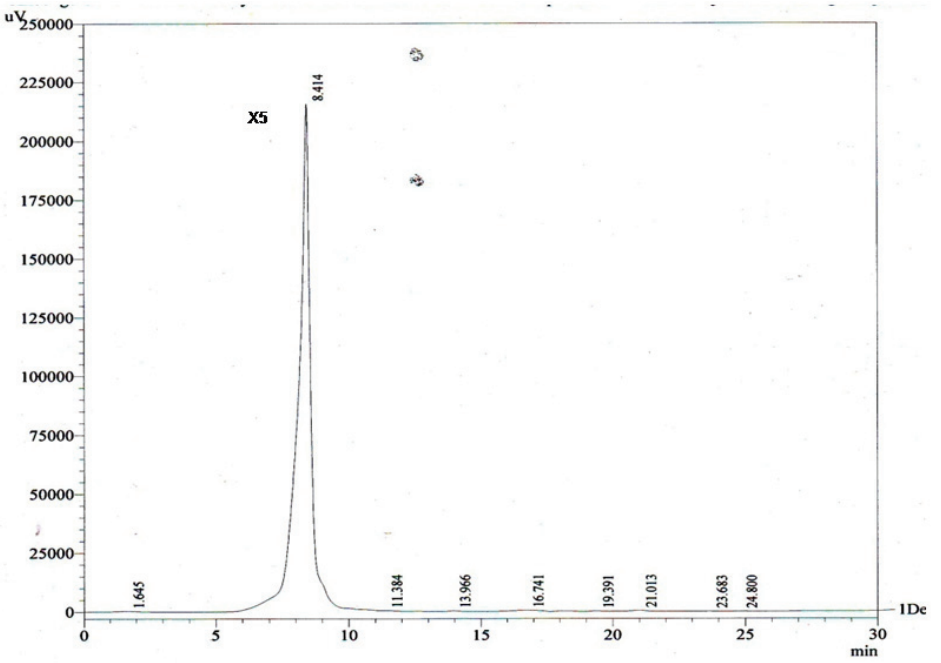

Figure 3. HPLC chromatogram of hydrolyses xylan from soybean waste

4. Conclussion

The research succesfully isolated xylan from soybean waste with 16% NaOH as the optimal concentration to get highest xylan yield (33%). The xylan from soybean waste succesfully hydrolyzed by endo-β-1,4-D-xylanase of *Bacilus sp* originally from soil termite abdominal as shown in TLC spot analyses then confirmed by HPLC analyses. HPLC analyzes shows the product of hydrolyses are X1, X3 and X5. Quantitative analyses from the wide of the peak in HPLC get the dominant product is X5. This result is similar with former research when the source of xylan hydrolized was oat spelt xylan.

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