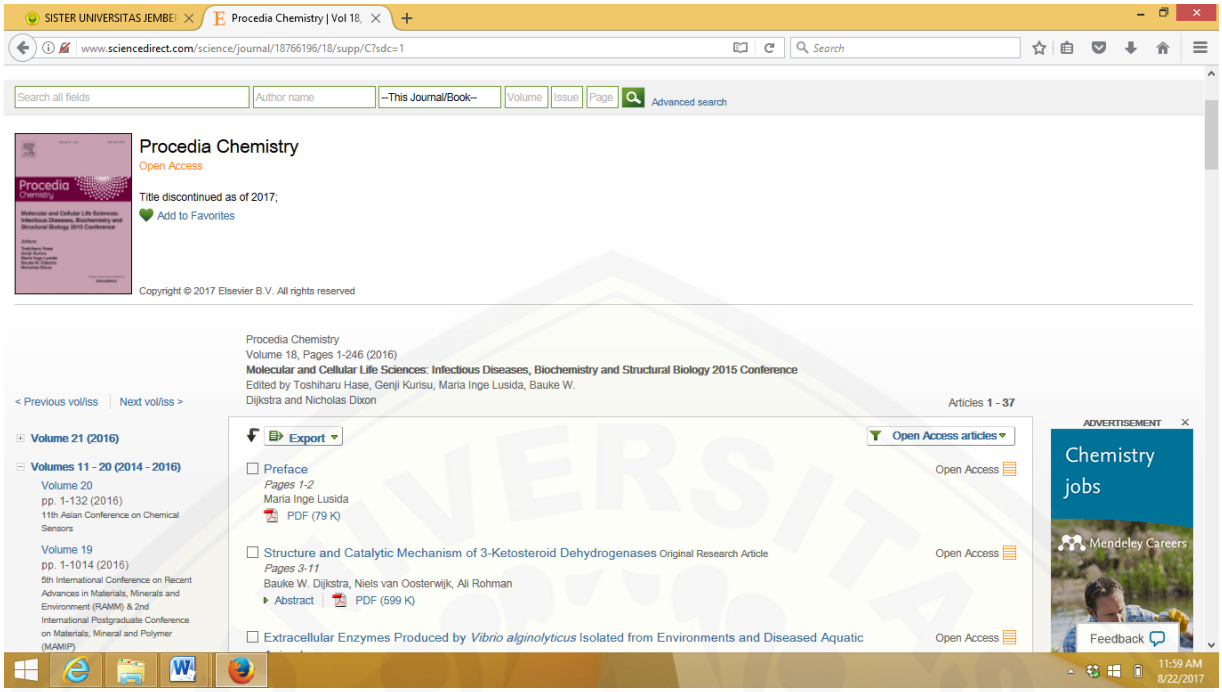

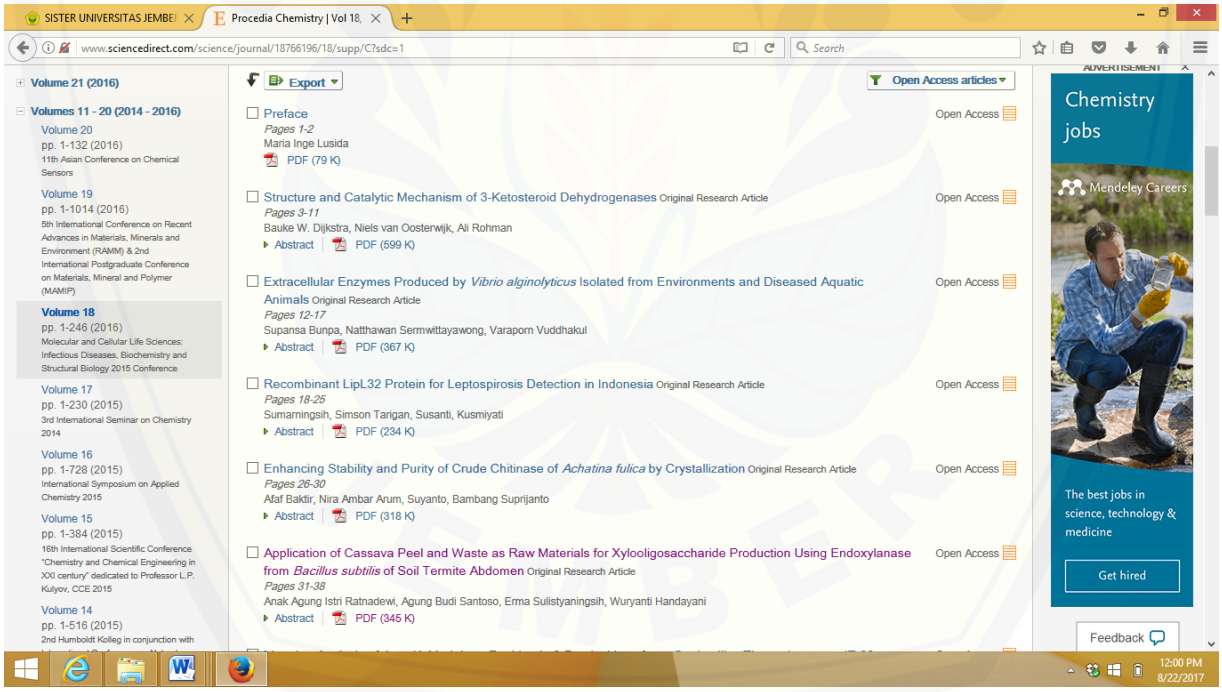

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Application of Cassava Peel and Waste as Raw Materials for Xylooligosaccharide Production using Endoxylanase from *Bacillus subtilis* of Soil Termite Abdomen

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Abstract

Xylooligosaccharides (XOS) are the sugars produced from xylan hydrolysis. XOS have a prebiotic characteristic by promoting the growth of probiotic microorganisms. Xylan containing agriculture wastes *e.g.* rice straw, sugarcane bagasse, corncobs, cassava peel and waste can be used to produce XOS by a consecutive process of alkali-pretreatment and enzymatic hydrolysis. In this study, we focused on enzymatic production of XOS from cassava peel and waste, which is a low cost material with a relatively high xylan content. The dried cassava peel and waste were ground and sieved to be <100 mesh size, and were then subjected to pretreatment with 0.5 % (w/v) sodium hypochlorite solution for 5 h to remove the lignin in the sample. In the next stage, the xylan was extracted by soaking in 10% sodium hydroxide (NaOH) for 24 h, followed by adjusting the pH to pH 7 by adding 5% (w/v) hydrochloric acid (HCl). Next, after centrifugation, the obtained filtrate was precipitated with ethanol (ratio 1:3) and dried at 80°C for 48 h. The NaOH pretreatment enabled almost 4.83% and 6.23 % recovery of the xylan that was present in the cassava peel and waste. Next, the xylan from cassava peel and waste was hydrolyzed using endoxylanase (2.21 U/mL) from *Bacillus subtilis* of soil termite abdomen at pH 5 and 50°C for 15 h. Analysis by TLC showed the production of XOS, with especially X5 as the major band. HPLC chromatography confirmed that the most abundant product was indeed X5. X3 and X4 were also found but no X2. The results were not so different from the hydrolysis of xylan from oat spelt xylan, but showed a relatively lower yield.

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Keywords: Xilooligosaccharides; Cassava peel and waste; Endoxylanase

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Nomenclature

h	hour
mM	millimolar
ppm	Parts Per Million
U	Unit Activity
µmol	Micromolar

1. Introduction

Cassava is an abundant product in Indonesia, especially in East Java. According to the Indonesian Statistical Board, in 2013 the production of cassava in Indonesia was 23,824,000 tonnes and in the East Java Province it was 3,600,000 tonnes. Around 16% of the weight of cassava is waste such as peel and fibre. These waste products contain almost 70% water and 30% dry weight. In the dry weight fraction, there is 3.5% protein, 10% crude fibre, 11% lignin, 14%, cellulose, and 27% hemicelluloses[1]. There is also a small amount of poisonous HCN present in cassava waste, which must be reduced to below 10 ppm to make it less poisonous. Cassava wastes are used for bioethanol and compost production, and for cattle feed and food. Hemicelluloses are the second highest component in cassava waste. Bioconversion of hemicelluloses gets high attention because of its benefit in many fields such as the generation of fuel and chemicals, delignification of paper pulp, clarification of juice, digestibility enhancement of animal feedstuffs in addition to the production of emerging prebiotics, i.e., xylooligosaccharides (XOS)[2-4]. Among the available list of prebiotics, only XOS can be produced from the lignocellulosic biomass, which is renewable, abundantly available and does not compete with human foods. XOS have demonstrated physiological benefits along with protection against several chronic and infectious diseases[5]. There is a great need to identify new raw materials for XOS production in accordance with the growing demands for prebiotics in the near future.

Xylooligosaccharides are sugar oligomers composed of 2-10 units of xylose; they are considered as non-digestible food ingredients[6]. XOS exhibit prebiotic effects when consumed as an agent to maintain and improve a balanced intestinal microflora for enhancing health and well-being, such as *Bifidobacteria* and *Lactobacillus,* and hence improves one's health[7-8]. XOS are produced from various xylan-rich agro-residues by physico-chemical and biological processes, or by a combination of processes. XOS are produced from biomass such as wheat straw, rice straw, corncobs, tobacco stalks, sunflower stalks etc. by various methods such as chemical, autohydrolysis, direct enzymatic hydrolysis of susceptible fractions, acid hydrolysis, or by a combination of these methods[9-11]. Generally, xylan exists as a xylan-lignin complex in the lignocellulosic biomass and is resistant to hydrolysis. Therefore, XOS production is carried out in two stages, alkaline extraction of xylan from lignocellulosic biomass followed by enzymatic hydrolysis[10,12].

In our research, we used the endoxylanase enzyme isolated from *Bacillus* sp of soil termite abdomen. Our previous research (unpublished) has tested this enzyme for hydrolysing oat spelt xylan to produce xylooligosacharides. The products of the enzyme are xylobiose X2, xylotriose X3, xylotetraose X4 and xylopentaose X5, with X5 as dominant product. Considering the potential market demand of XOS in the food and pharmaceutical industry, the present study aimed to produce XOS from cassava peel and waste employing an indigenous endoxylanase enzyme from *Bacillus subtilis* of soil termite abdomen. Xylan was extracted from cassava peel and waste by dilute alkali treatment and enzymatically hydrolysed for XOS production.

2. Methods

2.1. Materials

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

2.2. Microorganism

Bacillus subtilis isolated from microorganisms from the abdomen of the soil termite was used as an endo-xylanase producer and was maintained at -20°C as a glycerol stock.

2.3. Production and partial purification of endoxylanase

For endoxylanase production, the bacteria were cultured in LB (Luria Bertani) medium at 37°C. The crude enzyme was separated from the cell debris by centrifugation at 7,500 x *g* for 30 minutes at 4°C. Partial purification of endoxylanase was carried out by adding a calculated amount of solid ammonium sulfate (30 - 70%) to the crude endoxylanase with constant stirring at 10°C. Upon centrifugation at 8,500 x *g* for 20 minutes at 4°C, the precipitate was dissolved in a small volume of 50 mM sodium citrate buffer (pH 5.3). The enzyme solution was subjected to dialysis for about 18–24 h at 10°C against 50 mM sodium citrate buffer (pH 5.3). Endoxylanase activity and protein assays were carried out at each purification step. The samples were stored at 4°C until use.

2.4. Enzyme assays and protein estimation

Endoxylanase (E.C. 3.2.1.8) activity was measured using 1% oat spelt xylan solution as substrate[13]. The release of reducing sugars in 10 minutes at 50°C, pH 5.3 (50 mM sodium citrate buffer) was measured as xylose equivalents using dinitrosalicylic acid (DNS) method[14]. One unit of xylanase activity (U) is defined as the amount of enzyme liberating 1 µmol of xylose/min under assay conditions. The amount of soluble protein was determined by Folin's method using bovine serum albumin as a standard[15]. The protein concentration was determined by the Bradford method[16] using bovine serum albumin as a standard.

2.5. Extraction of xylan from cassava peel and waste

Cassava peel and waste were cut and immersed in water for many hours and rinsed several times to reduce the hydrogen cyanide (HCN) content. Reduction of the HCN content was also done by steaming after several rinses with water. The HCN concentration was analyzed in every step. The material was dried in an oven at 65°C until constant weight. Dry material was mashed in mortar to get powder. Lignin removal was done by the addition of 0.5% sodium hypochlorite (NaOCl) solution followed by filtration. Cassava peel and waste (100 g) were soaked in 250 ml of 0.5% NaOCl solution at room temperature for an hour to remove lignin and coloured materials. After washing with water, the solid material was dewatered by filtration. The delignified wet material was soaked in 10% NaOH at room temperature for 24 h to extract the xylan. After filtration, the filtrate was neutralized with 6 M HCl, and the xylan was precipitated using 3 volumes of ice-cold 95% ethanol. Then, the precipitated xylan was collected by centrifugation at 8,500 x *g* for 30 minutes at room temperature, the pellet was retained, dried in forced hot air oven at 65°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 percentage of xylan was derived from the percentage of true yield and the xylan (hemicellulose) content of the original cassava peel and waste. Hereafter, the best levels of alkali and its condition were followed to undertake the bulk xylan extraction, analysis, and XOS production.

2.6. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out in screw cap tubes containing xylan solution from cassava peel and waste with 2.65 U of partially purified endoxylanase (from procedure 2.3). Enzymatic hydrolysis was carried out by incubating the reaction system in a water bath at 40°C with mild shaking. Controls were kept for each reaction in which the enzyme was inactivated by heating. Samples were withdrawn at regular time intervals and subjected for qualitative analysis to thin-layer chromatography (TLC). Reducing sugars were quantified using the dinitrosalicylic acid method[14].

2.7. Analysis of XOS

The production of XOS during the enzymatic degradation of cassava peel and waste xylan was detected by TLC on silica plates. The solvent system for TLC comprised 1-butanol, acetic acid and water (2:1:1 v/v). After running the TLC plates for single ascent, XOS was detected through spraying with the α-naftol reagent. Xylose, xylobiose and XOS were used as the reference standards. The enzymatic hydrolysis products of the xylan from cassava peel and waste were analysed by high performance liquid chromatography (HPLC) using a Shim-pack SCR-101N (7.9 mm x 30 cm) column. Aliquots of filtered sample (20 µl) were injected through the manual injector. The XOS formed during the enzymatic conversion of xylan from cassava peel and waste were quantified after comparing the peak areas of XOS with that of standards and expressed as milligrams per ml.

3. Results and Discussion

3.1. HCN content

The content of HCN in wet, steaming and dry cassava peel and waste was detected qualitatively by picrate paper and then compared with *cyanide test indicator* as seen in Fig. 1. There are colour differences between wet, steaming and dry cassava. The HCN content was best reduced by steaming and rinsing several times with water, followed by drying. The picrate paper test is very simple, easy and does not need many instruments, so it can be applied well in practical processing. The concentration of HCN was reduced well after immersion and rinsing several times in day 1 till day 4. The concentration was still above the allowance limit for food grade. We are still exploring many processes to efficiently reduce the amounts of HCN.

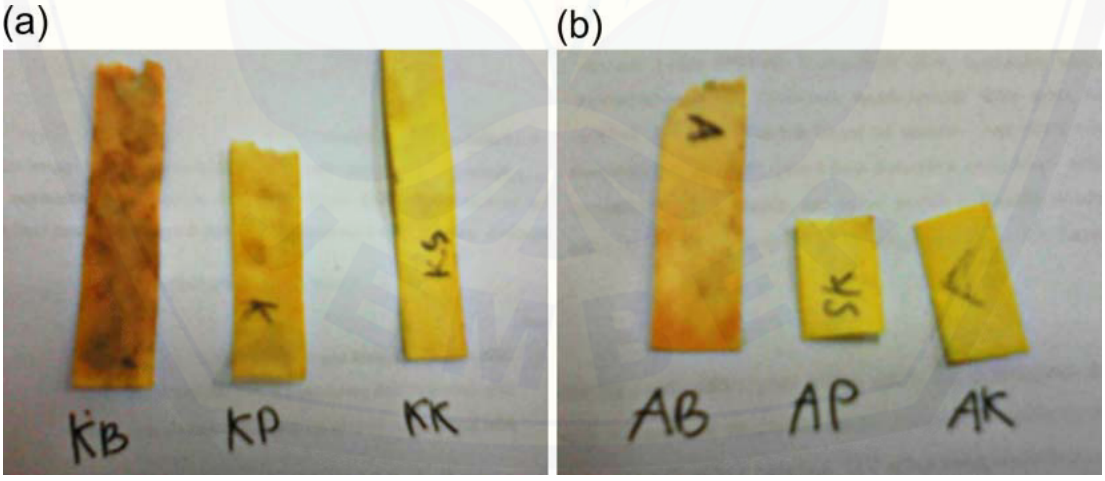

Fig. 1. (a) Qualitative analysis of HCN from Cassava Peel, KB (wet), KP (steaming) & KK (dry); (b) Qualitative analysis of HCN from Cassava Waste, AB (wet), AP (steaming) & AK (dry).

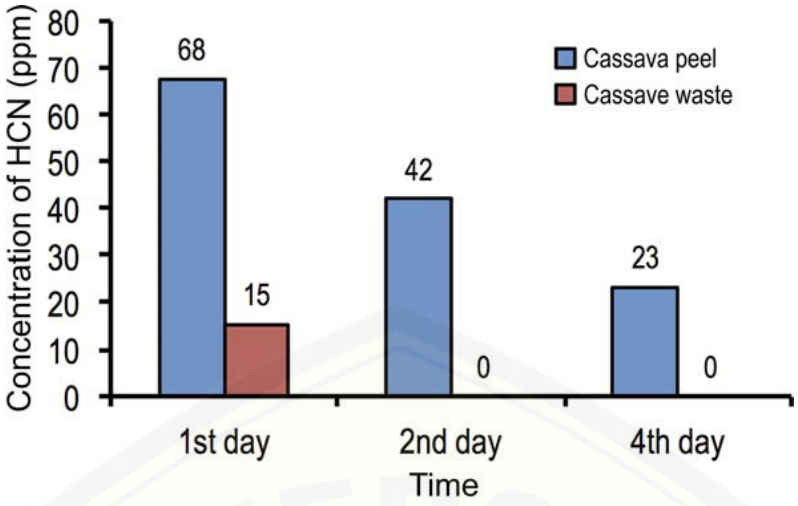

Fig. 2. Concentration of HCN after immersion in water for days 1 - 4

3.2. *Yield of xylan*

In this research, xylan from cassava peel and waste was extracted through application of NaOH followed by steam treatment or incubation at 25°C. The material was previously treated with 5% NaOCl to remove lignin. It can be seen that alkali is effective for separating the xylan from the lignocellulosic complex of cassava peel and waste (Table 1). After overnight incubation, NaOH treatment resulted in a yield that approaches 5-6% of total dry biomass. Xylan is the main hemicellulose found in higher plants. Its yield varies depending on the plant and extraction process applied, including the type of alkali, *i.e.*, sodium, potassium, barium, or calcium hydroxide. There are several methods (organic solvents, dilute acids, enzymes and water) for removal of xylan from lignocellulosic biomass. Application of NaOH was preferred because of its ability to dissolve hemicelluloses and because of its safety in food processing. The yield of xylan in this research was relatively low compared to the hemicellulose content of cassava which reached 27% of dry mass. From cassava peel, we obtained only 4.83% and from cassava waste we obtained 6.23% of xylan as seen in Table 1.The colour and texture of the isolated xylan are different between peel and waste of cassava as seen in Fig. 3. It means that there are impurities among them, at least the colour compounds.

Table 1. Recovery of xylan from cassava peel and waste

	Cassava Peel (21.10 g)	Cassava Waste (30.0 g)
Concentration of xylan	1.02 g (4.83%)	1.87 g (6.23 %)

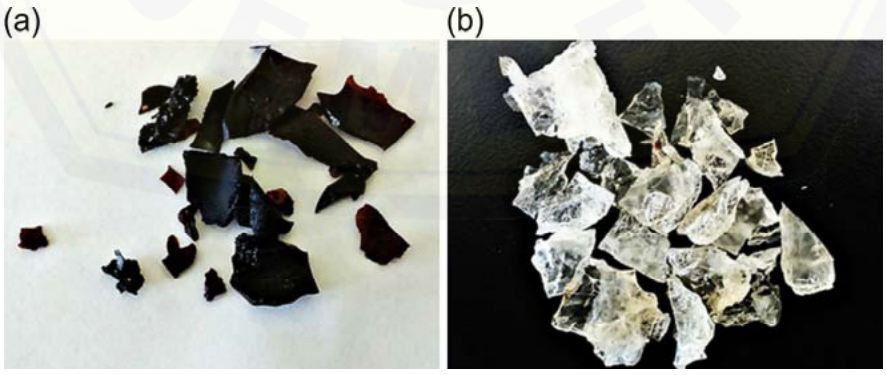

Fig. 3. Xylan product from (a) cassava peel; (b) cassava waste

3.3. Composition of XOS from xylan hydrolysis of cassava peel and waste

Xylooligosacharides are reducing sugars that can be measured quantitatively by the DNS method. In this research, we varied the concentration of the xylan substrate from cassava waste and peel. As seen in Table 2, the total yields of XOS as reducing sugar for many substrate concentrations of cassava waste were not significantly different. The production of XOS was conducted with 2.5 U/ml endoxylanase for 10 h at pH 5 and 50°C. At concentrations of 0.5 – 1.5% cassava waste, the total reducing sugar yields were around 14.30 mg/ml. It means that the concentrations over 0.5% have no effect on XOS production. We have not yet determined the highest substrate xylan concentration of cassava waste. The limitation of the XOS product yield at similar levels (around 14.3 mg/ml) indicates the presence of product inhibition of the responsible enzyme. Further research must be done to clarify and get the specific character of this inhibition. Production of XOS from cassava peel at concentrations of 0.5 – 1.5% increased from 5.63% to 13.69%. The XOS production from cassava peel was lower than that from cassava waste at similar substrate concentrations. This indicates that the xylan concentration in cassava peel is lower than that in cassava waste, or is more difficult to hydrolyse by the enzyme.

The TLC chromatogram presented in Fig. 4 shows the XOS profiles produced from cassava peel and waste. The predominant XOS product was X5 as seen as a thick spot at a similar retention time (RT) as the X5 standard. This result is consistent with previous research on oat spelt xylan, which also produced X5 as abundant product. There was also a spot in the tracks of cassava peel and cassava waste, which indicated a longer hemicellulose chain that was probably not cut well by the enzyme.

Table 2. Total Reducing Sugar from variation of the concentration of peel & cassava waste

Substrate	Concentration Substrate (%)	Total Reducing Sugar (mg/ml)
Cassava Waste	0.5	14. 28
	1.0	14.30
	1.5	14.31
Cassava Peel	0.5	5.63
	1.0	10.55
	1.5	13.69

Fig. 4. Thin layer chromatogram of hydrolysis products by endoxylanase with cassava peel (lane 3) and cassava waste (lane 4) at 40°C, pH 5. Lanes 1 and 2 represent xylose (X1) and xylooligosaccharides (X2 – X5), respectively.

3.4. XOS Production and Analysis

A HPLC analysis of the hydrolysis products of cassava peel and waste by the endoxylanase enzyme at 40°C, pH 5 for 10 h is shown in Table 3. The XOS formed was quantified by comparing the peak area of XOS with the standards (xylose, xylobiose, xylotriose, xylotetraose and xylopentaose). The HPLC chromatogram shows that the

amount of XOS includes X1, X3, X4 and X5. There was no X2 detected, neither in cassava peel nor in the waste. The dominant product is X5 as seen in Fig. 5. The chromatogram summarizes the concentrations of every XO from cassava peel and waste in Tables 3 and 4.

Table 3. HPLC Data on Cassava Peel

Xylooligosaccharides	Concentration (ppm)
Xylopentaose (X5)	5963.99
Xylotetraose (X4)	2.59
Xylotriose (X3)	65.55
Xylobiose (X2)	ND
Xylose (X1)	7.67

ND : Not Detectable.

Table 4. HPLC Data on Cassava Waste

Xylooligosaccharides	Concentration (ppm)
Xylopentaose (X5)	5591.15
Xylotetraose (X4)	35.17
Xylotriose (X3)	89.80
Xylobiose (X2)	ND
Xylose (X1)	7.43

ND : Not Detectable.

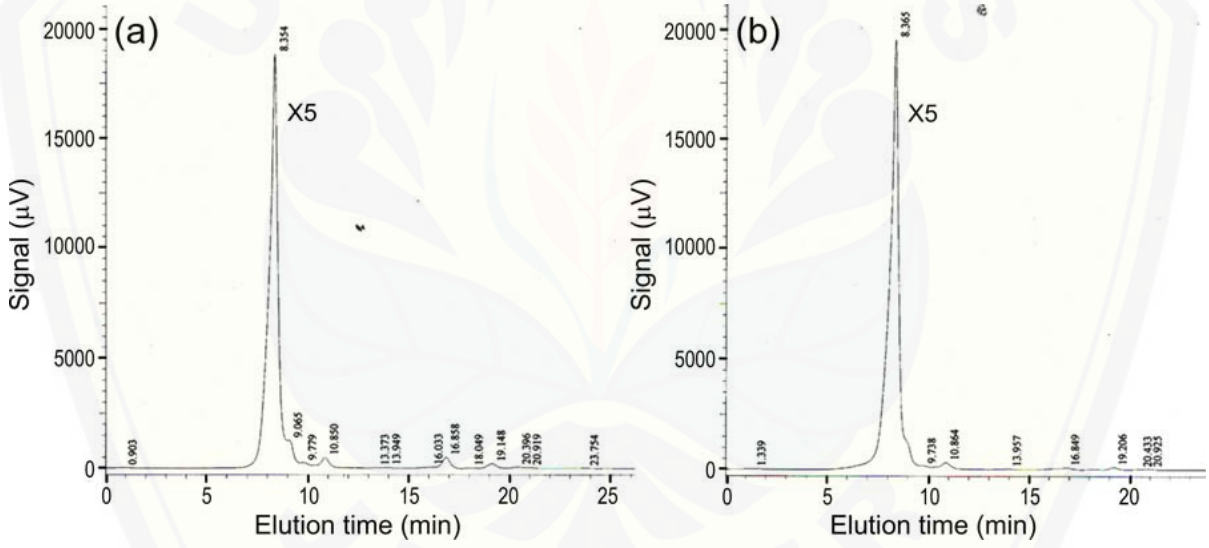

Fig. 5. HPLC chromatogram of the hydrolysis of xylan from (a) cassava peel; (b) cassava waste. The peak corresponds to the elution of standards of X1 xylose, X3 xylotriose, X4 xylopentaose and X5 xylopentaose)

4. Conclusion

In this study we show that xylan isolated from cassava peel and waste can be used as a raw material for XOS production. Endoxylanase from *Bacilus subtillus* of soil termite abdomen incubated with the xylan of peel and cassava waste generates XOS in different degrees of polymerization including X1, X3, X4 and specifically X5 as a dominant product. Further research will determine the optimal conditions, which include pH, temperature and time of incubation to obtain the maximum concentration of XOS and will furher elucidate their role as a gastrointestinal health guard.

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