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Effects of pH, Temperature, Enzyme and Substrate Concentration on Xylooligosaccharides Production

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***Abstract*—**Agricultural residue such as oil palm fronds (OPF) is cheap, widespread and available throughout the year. Hemicelluloses extracted from OPF can be hydrolyzed to their monomers and used in production of xylooligosaccharides (XOs). The objective of the present study was to optimize the enzymatic hydrolysis process of OPF hemicellulose by varying pH, temperature, enzyme and substrate concentration for production of XOs. Hemicelluloses was extracted from OPF by using 3 M potassium hydroxide (KOH) at temperature of 40°C for 4 hrs and stirred at 400 rpm. The hemicellulose was then hydrolyzed using *Trichoderma longibrachiatum* xylanase at different pH, temperature, enzyme and substrate concentration. XOs were characterized based on reducing sugar determination. The optimum conditions to produced XOs from OPF hemicellulose was obtained at pH 4.6, temperature of 40°C , enzyme concentration of 2 U/mL and 2% substrate concentration. The results established the suitability of oil palm fronds as raw material for production of XOs.

***Keywords*—**Hemicellulose, oil palm fronds, *Trichoderma longibrachiatum*, xylooligosaccharides.

I. Introduction

OIL palm industry is the important contributor to Malaysian economy with over 5.00 million hectares of planted areas in 2011, increase by 3% against 4.85 million hectares in 2010 [1],[2]. Due to the rapid growth of oil palm production in Malaysia, oil palm sector generated largest amount of biomass for about 80 million dry tones in 2010 [3]. The types of biomass from oil palm industry included empty fruit bunch (EFBs), palm oil mill effluent, fiber, shell, wet shell, palm kernel, fronds and trunks [4]. Among these wastes, oil palm fronds (OPF) is the most abundant agriculture wastes in Malaysia with 44.84 million tones reported in 2009 [5]. Oil palm fronds does not only created improper disposal problem, but also mostly the wastes are left to rot on the fields and caused environmental pollution [6],[7]. However, the enormous amounts of agriculture waste produced which contain lignocellulosic materials were potentially to be used as renewable material for production of value added and healthy products [8],[9]. Agricultural waste depending on lignocellulosic materials comprises of the major portion of plant cell walls and mainly composed of three components which are cellulose, hemicellulose and lignin, beside extractives and ashes. Since OPF contain of hemicellulose (11-37 % dry weight), they are can be used as starting material to produce xylose, xylitol and xylooligosaccharides [7].

XOs are xylose-based oligomers that can be obtained from hemicellulose-rich lignocellulosic materials during hydrolysis process and also can be recognized as oligomers, oligosaccharides, substituted oligosaccharides, or xylo-oligomers [10]. XOs are not only having satisfactory sweetness and acceptable odour, but also have low-calorie and non-carcinogenic characteristic which could be used as anti-obesity dietary. Moreover, they are stable at wide range of pH (2.5-8.0) and temperature (up to 100°C) compared to the others non-digestible oligosaccharides such as fructooligosaccharides. XOs have favorable application especially in food, pharmaceutical, cosmetics, feed and agriculture product [11]. The most important features of XOs are they have prebiotics effect which stimulating the growth of microflora in the gastrointestinal tract such as *Bifidobacteria* and *Lactobacillus*, and thus enhance health [9]. XOs can be used for prevention and treatment of several health disorders due to their antioxidant activities. Instead of act as precursor in antiviral and antitumor drugs, XOs can introduce as active agents against skin, hair disorder and osteoporosis [12].

Several methods have been used for producing of XOs from hemicellulose-rich lignocellulosic materials, including direct enzymatic treatments, chemical treatments, combination of chemical and enzymatic treatments, autohydrolytic treatments and combination of autohydrolytic and enzymatic treatments [13]. Production of XOs from lignocellulosic materials is carried out in two stages by combination of chemical and enzymatic method, where hemicelluloses is extracted with alkaline such as potassium hydroxide, sodium hydroxide or calcium hydroxide followed by conversion of XOs by xylanase enzyme containing low exo-xylanase or β-xylosidase activity to prevent xylose production which can inhibit production of XOs [14]. Other than that, XOs also can be produced by autohydrolysis treatment and acid hydrolysis of lignocellulosic biomass. However, this method can yield high amount of monosaccharide and undesirable product such as soluble lignin [9]. Compared to the others, enzymatic hydrolysis are more

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preferable because it does not produce high undesired by-product, high quantity of monomers or does not used any equipments [14].

In this study, xylanase from *Trichoderma longibrachiatum* was selected for endoxylanase production due to their widely used in industry and commercially available. The aim of this study was to optimize the enzymatic hydrolysis process of OPF hemicellulose by varying pH, temperature, enzyme and substrate concentration for production of XOs.

II. Materials and Methods

A. Materials

Oil palm fronds were obtained from local farmers in Selangor. The samples were chipped, dried, ground and sieved into size of less than 1.0 millimetres (mm) and stored at room temperature in the plastic bag prior to use. The moisture content of OPF was about 7.79% in untreated OPF [13]. A commercial enzyme from *Trichoderma longibrachiatum* (X2629) containing enzyme activity of 1.0 Unit/milligrams (U/mg) was purchased from Sigma Chemical Co., USA. All chemicals used were of analytical grade unless otherwise stated.

B. Extraction of Hemicellulose

Prior undergoes enzymatic hydrolysis, OPF were pretreated by alkaline extraction with slight modification [15]. Extraction was performed at solid to liquid ratio of 1:10 in the 3 M potassium hydroxide (KOH), temperature of 40^{O}C and stirred at 400 rpm with mechanical heat stirrer (HS-30D, WiseStir, Germany) for 4 hrs. After filtration, the filtrate was mixed with 50 % (v/v) acetic acid until pH 4.8 ± 0.1 was reached then allowed to stand for 24 hrs at 4 ^{O}C. The extract was centrifuged (Centrifuged 5702, Eppendorf, USA) at 3,500 rpm for 15 min and filtered. Then, four volumes of 95% ethanol was added to the supernatant and kept at 4 ^{O}C for overnight. Hemicellulose pellet was obtained after filtered and dried in the oven (UFB 500, Memmert, Germany) at 40^{O}C for 4 hrs and used as a hemicellulose source for further analysis while the supernatant was discarded.

C. Enzymatic Hydrolysis

Xylanase activity was determined according to procedure recomended by Bailey *et al*. [16]. One unit of xylanase activity release 1µmol of reducing sugar as xylose equivalents from xylan per minute at pH 4.5 at 30°C.

The hydrolysis conducted by mixing 1 mL of 2 U/mL of xylanase from *Trichoderma longibrachiatum* with 10 mL of 2% hemicellulose in 0.05M citrate buffer at pH 4.6. The mixtures were incubated at 40^{O}C for 48 hrs with shaking at 150 rpm in an incubator shaker (IKA® KS 4000i Control, China). The samples were taken out at different period of time (0, 2, 8, 24, 48 hrs) and boiled at temperature of 100ºC for 5 min to stop the enzyme activity and cooled down before assayed for reducing sugar analysis. The pH (3.6, 4.6, 5.6), temperature (30, 40, 50, 60^{O}C), enzyme concentrations (2, 4, 6 units/mL) and substrate concentration (1%, 2%, 3%) were varied to optimize the hydrolysis process which lead to the production of XOs. The hydrolysis were carried out for 48 hrs to observed the maximum yield of XOs.

D. Reducing Sugar Analysis

The hydrolysates were quantified by DNS method [17]. An amount of 1 mL sample was added to 1 mL of DNS reagent and 2 mL of deionized water. The mixture was boiled in a water bath (Memmert, Germany) at 100°C for 5 min. The mixture was cooled immediately with ice cubes and then reducing sugar was analyzed using UV-Vis spectrophotometer (Perkin Elmer Precise, 35 Lambda, U.S.A). The absorbance of the mixture was read at 503 nm. The concentration of reducing sugar was quantified by using a standard curve of xylose and expressed as milligrams per milliliter (mg/mL).

E. Statistical Analysis

The experimental data was analyzed by using one-way *Anova* and the significant differences between mean was performed by Tukey HSD test, where $p < 0.05$ was considered statistically significant. The SPSS (Statistical Package for Social Science) Version 16.0 was used to analyze the data (SPSS, Inc., USA).

III. Results and Discussion

The yield of hemicellulose of 15.83% (dry weight) was obtained from alkaline extraction of OPF.

A. Effect of pH

Fig. 1 shows the effect of different pH (3.6, 4.6 and 5.6) on XOs production. It was observed that reducing sugar production rates increased with the increasing of time.

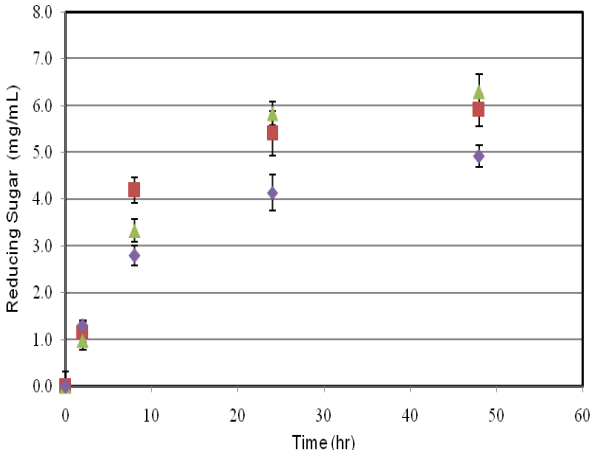

Fig. 1 Effect of pH on XOs production from oil palm fronds hemicellulose with xylanase from *Trichoderma longibrachiatum:* pH 3.6, ■ ; pH 4.6, △ ; pH 5.6, ◇; Each data point is the average of four replicate determination, and error bars show the data ranges

In the range of different pH studied, shows that pH 5.6 was significant with pH 3.6 and pH 4.6 ($p < 0.05$), but there was no significant difference between pH 3.6 and pH 4.6 ($p > 0.05$). However, since the production rate of XOs with pH 4.6 was faster than pH 3.6 and pH 5.6, therefore pH 4.6 was used in all further experiments. Literature value of optimum pH for *Trichoderma longibrachiatum* revealed at range of pH 5 to 6

[18]. However, the results were similar to the previous studies carried out using other agricultural wastes which hydrolyzed by *Trichoderma longibrachiatum* xylanase [9],[14],[19]. Ref. [20] recently reported that optimum pH for enzymatic hydrolysis with other *Trichoderma* species which was *Trichoderma viridae* was at pH 5. It was differ from this study might be due to the use of different agriculture wastes.

B. *Effect of Temperature*

The effect of temperature on XOs production was investigated at pH 4.6 by varying the temperature (30°C, 40°C, 50°C and 60°C) for 48 hours (Fig. 2). The production of XOs at 40°C was significantly higher than the production of XOs at 30°C, 50°C and 60°C ($p < 0.05$). Previous study on wheat straw, cotton stalk and tobacco stalk showed that the most suitable temperature for enzymatic hydrolysis by *Trichoderma longibrachiatum* was obtained at 50°C [19]. However, an optimum temperature was found out to be 40°C in a study carried out with *Trichoderma viride* [13],[21]. The utilization of mesophilic xylanase which having temperature optimum between 40-60°C results in time consuming enzymatic hydrolysis process in the production of XOs [22],[14],[23],[24]. Therefore, the temperature of 40°C at pH 4.6 was chosen in further experiment.

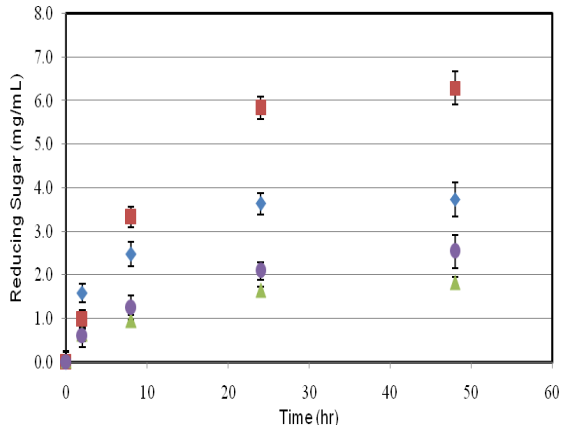

Fig 2 Effect of temperature on XOs production from oil palm fronds hemicellulose with xylanase from *Trichoderma longibrachiatum:* 30°C, ◆ ; 40°C, ■ ; 50°C, △ ; 60°C, ● ; . Each data point is the average of four replicate determinations, and the error bars show the data ranges

C. *Effect of Enzyme Concentration*

Different concentrations of enzyme (2 U/mL, 4 U/mL and 6 U/mL) were used to optimize the enzymatic hydrolysis of OPF hemicellulose at pH 4.6 and temperature of 40 °C for 48 hrs. Fig. 3 illustrates XOs production from OPF hemicelluloses with xylanase from *Trichoderma longibrachiatum*. XOs production with xylanase was characterized by rapid increased in reducing sugar.

As observed from hydrolysis progress of *Trichoderma longibrachiatum* xylanase, the hydrolysis rate of hemicelluloses was very fast up to 8 hrs periods and slowly stopped increasing after 24 hrs. Statistical analysis showed that there were significant differences between 2 U/mL, 4 U/mL and 6 U/mL ($p < 0.05$). The results showed that the enzyme concentration of 2 U/mL yielded significantly higher amount of XOs than 4 U/mL and 6 U/mL for all the reaction time periods. Considering the rate of XOs production and cost of enzyme, enzyme concentration of 2 U/mL was chosen in further parts of the study

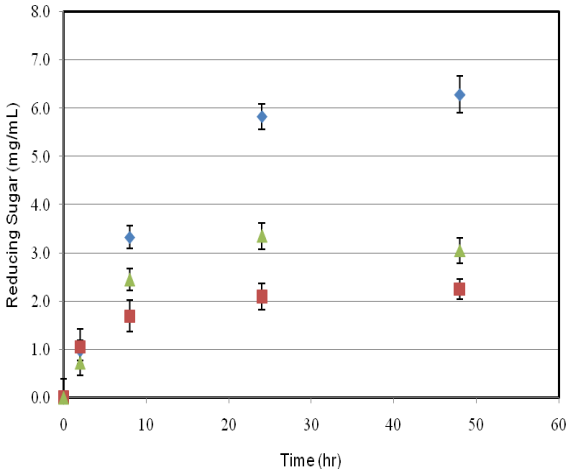

Fig. 3 Effect of enzyme concentration on XOs production from oil palm fronds hemicellulose with xylanase from *Trichoderma longibrachiatum:* 2 U/mL, ◆ ; 4 U/mL, ■; 6 U/mL, △ . Each data point is the average of four replicate determination, and the error bars show the data ranges

Statistical analysis showed that there were significant differences between 2 U/mL, 4 U/mL and 6 U/mL ($p < 0.05$). The results showed that the enzyme concentration of 2 U/mL yielded significantly higher amount of XOs than 4 U/mL and 6 U/mL for all the reaction time periods. Considering the rate of XOs production and cost of enzyme, enzyme concentration of 2 U/mL was chosen in further parts of the study.

D. *Effect of Substrate Concentration*

The effect of different substrate concentration on production of XOs was determined through measurement of reducing sugar as shown in Fig. 4. Reaction was carried out at various substrate concentrations in a range of 1-3%. The quantity of reducing sugar per miligram of substrate was highest at concentration of 2%, which indicated high yield of XOs. Statistical analysis showed that significant differences were existed between 1%, 2% and 3% concentration of substrate ($p < 0.05$). Results of similar experiments shown by other researcher reveal that utilization of hemicelluloses at concentration higher than 2% are difficult to handle and decreased XOs production, mostly due to enzyme inhibition by the present of impurities and increases in the viscosity [14]. Moreover, high concentration of substrate might reduced the water content in reaction mixture which lowered pentose yield [25] and also can lower the rate of hydrolysis as shown in hydrolysis progress in Fig. 4. Low substrate concentration can cause in an increase of the XOs yield and reaction rate of hydrolysis [26]. A substrate concentration of 2% was used in all further experiments, considering high rate of the production of XOs.

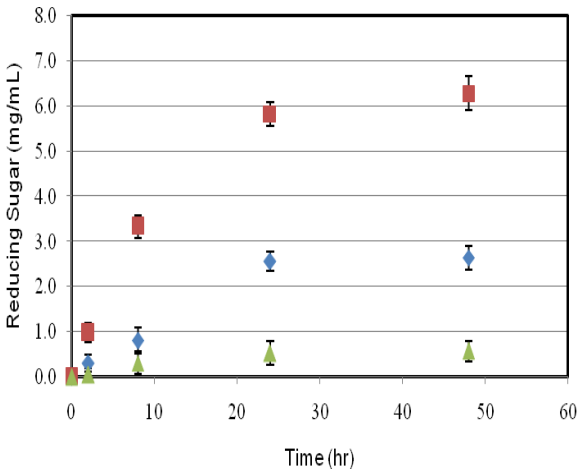

Fig. 4 Effect of substrate concentrations on XOs production from oil palm fronds hemicelluloses with xylanase from *Trichoderma longibrachiatum* : 1%, ◆ ; 2% , ■ ; 3%, △. Each data point is the average of four replicate determinations, and the error bars show the data ranges

IV. Conclusion

It was found that hydrolysis process of OPF hemicellulose by *Trichoderma longibrachiatum* can be performed under optimum condition at pH 4.6, temperature of 40°C, enzyme concentration of 2 U/mL and 2% substrate concentration. Thus, this study indicated that OPF can be utilized to produce XOs.

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