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Original Article

Factors affecting enzymatic hydrolysis of oil palm frond bagasse using cellic HTec2 for xylooligosaccharides production

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Abstract

Enzymatic hydrolysis has become outstanding technology in converting lignocellulosic biomass to its xylose monomer for xylooligosaccharides (XOS) production. The present work involves an investigation on the effects of enzyme loading, agitation speed, substrate loading, temperature and hydrolysis time on enzymatic hydrolysis for XOS production. Pretreated oil palm frond bagasse by dilute nitric acid was used for enzymatic hydrolysis using Cellic HTec2. The effects of factors were analyzed by half fractional factorial design 2^{5-1} using Design Expert with Response Surface Methodology (RSM) to achieve maximum XOS production. The results revealed that the best enzymatic hydrolysis condition yielded 4.13 mg/L of XOS when conducted at 5% (w/v) of substrate loading, 50 U/mL enzyme loading with 200 rpm agitation speed and 55°C for 4 hours of hydrolysis time. Two factors that contributed to the highest production of XOS were substrate loading and enzyme loading. The model obtained in this present research is significant with p-value < 0.0001 and R-squared of 0.9545. It is recommended that model had a maximum point which is possible for the optimization process later. Overall, the findings of this study suggest that Cellic HTec2 is a suitable candidate for enzymatic hydrolysis of pretreated OPFB for higher XOS production.

Keywords: Oil palm frond bagasse, Enzymatic hydrolysis, Cellic HTec2, Xylooligosaccharides, Response surface methodology

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Introduction

Lignocellulosic biomass (LCB) can be easily found in large amount of waste throughout the year. Basically, it is mainly produced from agriculture and forestry sector such as palm oil, switch grass, rubber, sugarcane, kenaf and wood (forest residues). Most of these plant fibers were made up of cellulose, hemicellulose, lignin, waxes, ashes, pectin and water-soluble compounds. According to Rozario (2013) based on the National strategy, Malaysia's palm oil industry is expecting to produce high amount of solid biomass which up to 100 million dry tone by the year of 2020. The variety parts of palm oil byproduct that might be abundantly increase including oil palm trunk (OPT), oil palm fronds (OPF) and residues of

harvested fruit such as palm kernel shells (PKS), mesocarp fibres (MF) and empty fruit bunches (EFB). Therefore, to overcome huge amount of disposal problem, numerous studies have been done to substitute this biomass waste into alternative energy or useful-eco product without depleting the soil.

Oil palm frond (OPF) nowadays has become one of sustainable LCB sources which are commercial and important in agriculture industry. Currently, utilization of OPF into value added product has been reported as polyalcohol sugar (Abdul Manaf et al., 2018), bioethanol production (Farah Amani et al., 2017) and biofuel production through torrefaction (Yaacob et al., 2017). Xylooligosaccharides (XOS) are among useful valuable product that becomes attention by many researchers. XOS is an emerging prebiotic because it has various advantages to human body and nutraceutical industry. XOS has remarkable potential of being novel prebiotics, and their exceptional benefits include their role as antioxidants, improve the sugar and lipid on the type II diabetes mellitus, having cytotoxic effects on human leukemia cells, reducing the risk of colon cancer, enhancing the biological availability of calcium by improving its absorption, and improvement in bowel function (Jain et al., 2015). It was mainly produced from xylan-rich lignocellulosic biomass such as corn stover, hardwood, and OPF (Buruiana et al., 2016; Huang et al., 2016; Saleh et al., 2016).

Several methods such as autohydrolysis, chemical-enzymatic hydrolysis microwave-assisted hydrolysis, chemical process and enzymatic process have been used for XOS production. Among the methods, chemical with enzymatic hydrolysis is the frequently studied these days since the process could increase the efficiency and reduced the production cost (Jain et al., 2015; Otieno and Ahring, 2012). Acid or alkaline pretreatment is useful to extract xylan content prior to enzymatic hydrolysis. Enzymatic hydrolysis usually undergoes reaction through hemicellulase and cellulase enzyme in producing reducing sugar such as fructose, xylose and glucose. To date, most report used combination of two or more commercial enzymes to enhance XOS production. Alvira et al. (2011) was successfully observed higher XOS content from wheat straw using combination of Cellic Ctec and Accellerase 1500. Furthermore, in other studies conducted by Gonçalves *et al*. (2015) and Bowman et al. (2015), both Cellic CTec 2 and HTec2 enzymes from Novozymes were used to detect XOS from coconut waste and switchgrass, respectively.

However, there is no discussion so far about XOS production by using one commercial enzyme. The use of HTec2 alone could be useful to assist in converting hemicellulose to fermentable sugar such as xylose and xylobiose since pretreatment was earlier performed for xylan preparation. Cellic HTec2 will trigger off the reaction to completely hydrolyze the complex structure of xylan. Generally, endo-β-,4-xylanases degrade xylan by attacking the β-1,4-bond between xylose units to produce XOS (Qing et al., 2013). An attempt on the use of Cellic HTec2 for enzymatic hydrolysis for XOS production could give a new insight and at the same time could reduce the production cost. Besides, the use of experimental design developed by Design Expert Software is also useful to evaluate the effect of several factors simultaneously and determined the most significant factor through factorial design (Golshani et al., 2013). Unfortunately, no report has been carried out so far using factorial analysis with Response Surface Methodology (RSM) to study the factors effecting XOS production.

Thus, this research focused at the effects of five important parameters which are substrate loading, enzyme loading, agitation speed, temperature and hydrolysis time on enzymatic hydrolysis of pretreated OPFB to produce XOS using Cellic HTec2. The conditions of enzymatic hydrolysis were analyzed using statistical approach of two level fractional factorial analysis and RSM.

Material and Methods

Raw material

In this study, collection of oil palm frond (OPF) sample for substrate preparation was obtained from a local palm oil plantation at Felda Lepar, Gambang, Pahang. Sugarcane machine was used to get rid liquids from OPF by pressing it. Then, the oil palm fronds bagasse (OPFB) were sun dried for three days. It was mechanically shredded into pieces using grinder. Next, the grinded OPFB were sieved to obtain less than 2 mm of particle size. The dried OPFB was kept at room temperature in sealed plastic bag.

Acid pretreatment

Acid pretreatment process of OPFB was done in a 1000-mL Scott bottle. The OPFB sample was soaked in 0.1 M nitric acid (HNO_3) solution with ratio of 1:10 (g/mL). The sample undergoes treatment at 60°C for 12 hours in water bath. Then, the pretreated sample

was washed with tap water until neutral with followed by drying process of sample in the oven for overnight at 60°C. The dried sample was stored and used for enzymatic hydrolysis.

Enzyme assay

Enzyme activity of xylanase in Cellic HTec2 was measured throughout this study. The enzyme assay was done in 200 µL sodium citrate buffer with pH 5.3. The process including mixing of 1.8 mL 2% Birchwood xylan (Sigma) solution with 200 µL diluted enzyme (with respect Cellic HTec2 to ultrapure water). The enzymatic process was performed for 5 minutes in incubator shaker at 50°C. The reaction was stopped by adding 3, 5-Dinitrosalicyclic acid (DNS) solution followed by boiling process for 15 minutes. The samples then were analyzed using Ultraviolet-visible (UV-VIS) spectrophotometer at 575 nm wavelength to obtain graph of absorbance against concentration xylose. In this study, the detection of sugar concentration released from enzymatic hydrolysis process was done by DNS method as suggested by Miller (1959). Accordig to Bailey et al. (1992), one unit of xylanase enzyme is defined as the quantity of enzymes that liberates 1 µmol of xylose per minutes at 50°C.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed using Cellic HTec2 provided by Novozymes (Denmark) in 50-mL falcon tube at various parameter values according to Table 1. To hinder any microbial growth, the substrate was mixed with 0.02% sodium azide and 0.05M sodium citrate buffer (pH 5.3). The mixture was incubated at different agitation speed with certain temperature. Next, Cellic HTec2 was added to stimulate enzymatic hydrolysis where the enzyme loading was calculated based on xylanase assay done prior enzymatic hydrolysis. The residues were collected before subjecting to HPLC analysis.

Table 1. Parameters and their low and high value.

Factors	Coded	Units	Low value (-1)	High value (+1)
Substrate loading	A	%, w/v	1	5
Enzyme loading	B	U/mL	50	700
Temperature	C	°C	37	55
Hydrolysis time	D	hours	4	72
Agitation speed	E	rpm	50	200

Table 2. Experimental design of fractional factorial analysis with response.

Run	Coded values					Xylooligosaccharides (g/mL)
	A	B	C	D	E	
1	1	700	37	4	50	3.15
2	5	700	55	72	200	2.86
3	5	50	37	4	50	3.04
4	1	700	55	72	50	0.55
5	1	50	37	72	50	3.54
6	5	700	37	72	50	3.36
7	1	700	37	72	200	3.23
8	5	700	37	4	200	3.28
9	1	700	55	4	200	0.89
10	5	50	37	72	200	3.61
11	5	50	55	72	50	3.36
12	5	50	55	4	200	4.13
13	1	50	55	4	50	3.82
14	1	50	37	4	200	1.50
15	5	700	55	4	50	3.45
16	1	50	55	72	200	3.32

*A: Substrate loading; B: Enzyme loading; C: Temperature; D: Hydrolysis time and E: Agitation speed.

Experimental design of two-level fractional factorial analysis setup

Design Expert 7.0.0 (Stat-Ease Inc., USA) software was employed in this study to develop the experimental design for fractional factorial analysis. The five factors involved which were substrate loading (g/mL), enzyme loading (U/ml), agitation speed (rpm), temperature (°C) and hydrolysis time (hours) were analyzed using Response Surface Methodology (RSM). The screening process of the response on the effect on xylooligosaccharides (XOS) production were developed in fractional factorial designs of 2^{5-1} as shown in Table 2.

High performance liquid chromatography (HPLC) analysis

The analysis process to detect XOS in hydrolysate was done using Agilent 1260 HPLC (USA) system equipped with refractive index (RI) detector and Rezex Phenomenex RSO-Oligosachharides column. The temperature of the column was maintained at 85°C while the injection volume and flow rate were at 5 µL and 0.3 mL/min, respectively. The standard ranges between 0.4 mg/L to 1 mg/L which consists of pure xylose, xylobiose, xylotriose and xylotetraose were prepared to obtain the calibration curve.

Results and Discussion

Factors affecting enzymatic hydrolysis

This study employed two level fractional factorial design to evaluate the factors affecting production of XOS via enzymatic hydrolysis on pretreated OPFB. These five main factors include substrate loading, enzyme loading, temperature, hydrolysis time and agitation speed were analyzed with the assistance of Design Expert Software. The experimental design running sequences which consist of 16 run and response obtained are shown in Table 2. The table clearly shows the highest XOS production was obtained at 4.13 mg/L (Run 12) where the hydrolysis conditions at 5% (w/v) of substrate loading, 50 U/mL enzyme loading with 200 rpm agitation speed and 55°C for 4 hours of hydrolysis time were observed.

Model fitting

Pareto chart shown in Figure 1 represents the main and interaction effects of the enzymatic hydrolysis. The different color of bar symbolized the type of effect influenced the production of a process. Orange bar is referred as a factor that give a positive effect, while blue bar chart referred as negative effect on the enzymatic hydrolysis. From the chart, it is apparently illustrated that factor A and D contributed positive effect on XOS production. Positive effects of this chart can be translated with the increase of its high level will directly increase in the responses. In contradict, the negative effects were observed on factor B, C and B which indicated that the conversion to XOS will increase with lowest range value of factor used. The level of significance of each factor also clearly presented in the chart which evaluated by the effect of t-value based on the limit line provided. Both limit lines displayed are Bonferroni limit and t-value limit presenting the effect of t-value at 6.254 and 2.776. Factor which has the t-value effect above the Bonferroni limit portrayed a very strong significant effect on XOS product, while the factors are considered significant at confidence level of 95% when the effects of t-value lie between t-value limit and Bonferroni limit. The t-value of effect below the t limit line should be removed from the analysis because it is statistically insignificant (Banala et al., 2013). Therefore, the quick analysis through this chart determined that only the factors of A, B, BC, DE and CD were shown to be significant.

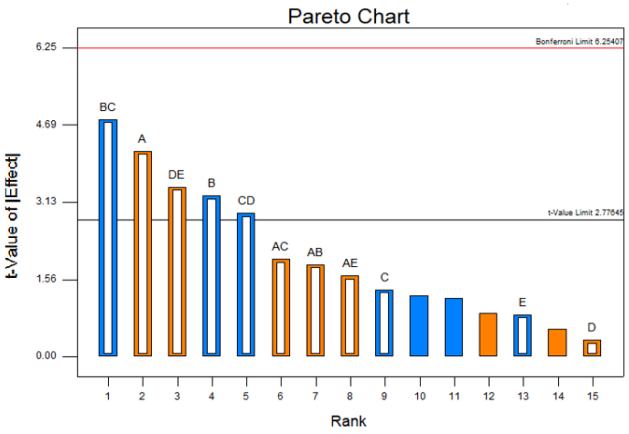

Fig. 1. Pareto chart for 2^{5-1} fractional factorial design

To optimize the enzymatic hydrolysis conditions, factorial analysis plays vital role in identifying the main factors that have the greatest influence on the response. Based on the chart, the main factors of substrate loading (A) and enzyme loading (B) are considered significant and useful to be used for optimization process. Pareto chart was obviously illustrated that the main factor of substrate loading contributed the highest effect to the enzymatic

hydrolysis. Generally, high substrate loading provides more advantages for enzymatic hydrolysis because larger surface could be exposed for enzyme active sites to attack the available substrate surface to form enzyme-substrate complex in the reaction medium (Wan Azelee et al., 2016). The result is consistent with finding of past study conducted by Hashim et al. (2017) on OPF as substrate which found that glucan loading significantly affected to the glucose production using a commercial cellulose enzyme, Sacchariseb C6. Furthermore, enzyme loading was revealed as the second highest contributor for the process of enzymatic hydrolysis. The addition of enzyme loading was observed could directly improve XOS production from hemicellulose. The present finding was supported by Mussatto et al. (2008) where the author concluded that enzyme loading had a highly significant effect on production of reducing sugar.

Analysis of variance (ANOVA)

The analysis of variance (ANOVA) of this experimental design was carried out to determine the significant effect of this enzymatic hydrolysis process model. Table 3 demonstrates that the p-value at 0.0322 and F-value at 7.64 implies that the model was significant and only 3.32% of chances that the model could not be significant due to noise. In this study, the R^2 value obtained was 0.9545 which resemble the model was well adapted to response while adjusted R^2 value of 0.8296 indicates a good fitting model with predicted value. The results of ANOVA of this model show it is valid for subsequent optimization process.

The independent and dependent variables were analyzed in terms of coded factors that presented production of XOS to obtain the following regression model:

$$Y = 2.94 + 0.44A - 0.35B - 0.15C + 0.036D - 0.091E + 0.20AB + 0.21AC + 0.17AE - 0.51BC - 0.31CD + 0.37DE$$

where *A, B, C, D* and *E* referred as the main effects while *AB, AC, AE, BC, CD* and *DE* are the interaction effects involved in the enzymatic hydrolysis process. Typically, for polynomial equation, synergistic effect represents by a positive sign while antagonistic effect indicates a negative sign on the system.

Table 3. Analysis of variance (ANOVA) for factorial analysis.

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value
Model	15.29	11	1.39	7.64	0.0322
A	3.15	1	3.15	17.32	0.0141
B	1.94	1	1.94	10.64	0.031
C	0.34	1	0.34	1.85	0.2451
D	0.02	1	0.02	0.11	0.7559
E	0.13	1	0.13	0.72	0.4439
AB	0.63	1	0.63	3.47	0.1359
AC	0.71	1	0.71	3.89	0.1199
AE	0.49	1	0.49	2.67	0.1775
BC	4.21	1	4.21	23.11	0.0086
CD	1.53	1	1.53	8.42	0.044
DE	2.15	1	2.15	11.8	0.0264
Residual	0.73	4	0.18		
Cor Total	16.02	15			
Std. Dev.	0.43				
Mean	2.94				
R-Squared	0.9545				
Adj R-Squared	0.8296				

Comparison of actual versus predicted graph

Evaluation of prediction model in this study was evaluated via predicted versus actual plot. Scatter plot is the common alternatives to observe the corresponding of the experimental point to the straight line in order to have a good fit. The fitness of the graph adversely affected by points that further from straight line either left or right of the plot (Piñeiro et al., 2008). Figure 2 illustrates the regression model of experimental values of the factors with observations on the XOS production. The closeness of experimental values observed around the straight line of predicted values presented the significance of this model at 95% of confidence level. Thus, good agreement between the predicted and experimental values implied in the range of operating factors (Pengilly et al., 2015). In terms of graph pattern, most of the points were more accumulated at the top side of the graph which differs from Khushairi et al. (2016) which study about production of ferulic acid using the same waste as current study.

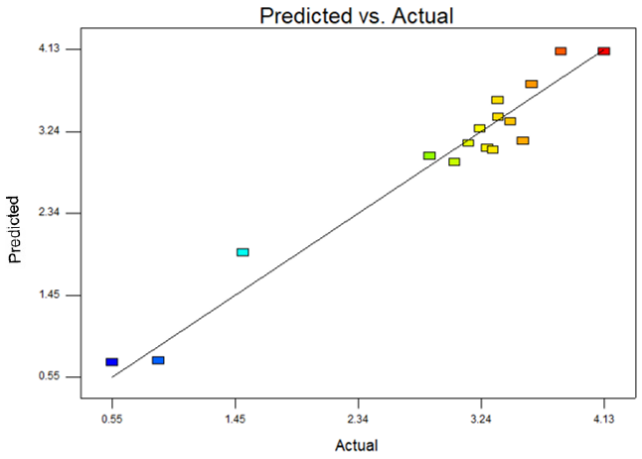

Fig. 2. Predicted versus actual regression model graph.

Validation of results

A validation experiment was carried out to validate the reliability of data obtained from the screening process. The best conditions suggested by Design Expert 7.0 were applied in this validation experiment with three replications. The condition at 5% w/v substrate loading, 50 U/mL of Cellic HTec2 at temperature of 55 °C with 200 rpm agitation speed were performed for 4 hours of hydrolysis time. The error values from each replicate was calculated from the predicted and experimental data. The error from these validations runs lie between 7.33% and 8.93% which indicates the validation process was successful. The low percentage of error which is less than 10% was signified that the model is reproducible and reliable. The analysis result indicates that the predicted values are in good agreement with experimental values thus verified the adequacy of the model.

Conclusion

This current study was designed to analyze the factors affecting enzymatic hydrolysis for XOS production using two level fractional factorial design. This study has shown that the best enzymatic hydrolysis condition throughout the experiment was at 5% w/v of substrate loading, 50 U/mL enzyme loading with 200 rpm agitation speed and 55°C for 4 hours of hydrolysis time. From the five factors, substrate loading and enzyme loading were discovered giving highest contribution to the process with 19.68% and 12.09% respectively. The model obtained throughout this study is significant based on the ANOVA with R^2 is 0.9545. Overall, the findings of this study suggest that Cellic HTec2 is a suitable candidate for enzymatic hydrolysis of pretreated OPFB for higher XOS production. In addition, the results of this study indicated that the proving design can be continued for optimization process for future studies.

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Contribution of Authors

Yaacob ASM: Data Collection, Data Interpretation, Manuscript Writing
Mazlan NA: Literature Search, Designed Research Methodology, Statistical Analysis
Samad KA: Manuscript Writing, Statistical Analysis
Saufi SM: Conceived Idea, Designed Research Methodology, Statistical Analysis, Data

Interpretation, Manuscript Final Reading and Approval
Yussof HW: Conceived Idea
Jahim J: Conceived Idea

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