Statistical Screening of Factors Affecting Production of Fermentable Sugars from Sugarcane Bagasse under Solid-state Conditions

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A Plackett-Burman design (PBD) combined with a steepest ascent approach is a powerful technique to screen the important operating parameters for the production of reducing sugars from sugarcane bagasse (SB). In this study, the most significant parameters ($p< 0.05$), as identified by PBD, were as follows: pretreatment duration, pH of pretreatment process, loading of enzyme cellulase, SB loading, and moisture content of SB. Analysis of variance (ANOVA) results showed that the model of reducing sugar productivity was able to provide a high correlation between the response and its parameters. Thus, the path of steepest ascent (PSA) method was used to assess the optimal region of variables for improved reducing sugar productivity from SB. The PSA analysis revealed that by treating 7.25 g of SB (with 84% moisture content) for 82.0 minutes at a pH of 8.8, followed by the addition of 34.0% v/w of cellulase, a reducing sugar productivity of 0.03 g/L could be achieved per hour during the enzymatic saccharification process.

Keywords: Plackett-Burman design; Path of steepest ascent; Statistical screening; Reducing sugar; Sugarcane bagasse; Solid-state fermentation

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INTRODUCTION

Agricultural commodities are the main raw material available to mankind for the sustainable production of various industrial and non-food consumer products. However, their use has had a large impact on agricultural waste residues. These wastes are produced in quantities of millions of tons annually and are usually disposed of without further use (Shaikh *et al.* 2009).

Among the various agricultural crop residues, rice straw, wheat straw, corn straw, and sugarcane bagasse are the four main agricultural wastes that serve as important bio-product feedstocks due to their global availability throughout the year (Sarkar *et al*. 2012). The majority of corn straw and sugarcane bagasse is produced in America (North America, Central America, and South America), while rice straw and wheat straw are mainly produced in Asia (Kim and Dale 2004). As one of the most important agricultural countries in the world, Malaysia also produces a large quantity of lignocellulosic biomass from agricultural waste (Goh *et al*. 2010). The largest portion of total agricultural waste in Malaysia comes from oil palm fields, followed by paddy straw, rice husk, banana residue, and sugarcane bagasse (Ahmad 2001; Misson *et al*. 2009).

Agricultural wastes are mainly lignocellulosic materials. These wastes are cost-effective, renewable, and abundant (Sarkar *et al*. 2012). Hence, instead of merely disposing of them, these wastes can be utilized as the major substrate for the production of fuel alcohol, chemicals, and protein for food and feed purposes (Amartey *et al*. 1999; Kuhad and Singh 1993; Kuhad *et al*. 1997; Herrera 2004; Saxena *et al*. 2009; Girio *et al*. 2010; Sarkar *et al*. 2012).

Lignocellulosic materials are composed of carbohydrate polymers (cellulose and hemicellulose), lignin, and a small fraction of extractives and minerals packed in an intricate structure that is recalcitrant to deconstruction. Cellulose and hemicellulose typically account for up to two-thirds of lignocellulosic material. Cellulose is a homopolymer composed of repeating glucose sugar units connected by β-1,4glycosidic bonds. It is linear and crystalline. Hemicellulose is a short and highly branched polymer which is composed of D-xylose, D-arabinose, D-glucose, D-galactose, and D-mannose. Meanwhile, lignin is insoluble in water and is tightly bound to both cellulose and hemicellulose. Its main function is to protect cellulose and hemicellulose from microbial attack (Lee 1997; Peiji *et al*. 1997).

The production of fermentable sugars from lignocellulosic materials involves two steps: (i) pretreatment of lignocellulosic materials, and (ii) enzymatic saccharification of cellulose and hemicellulose to produce reducing sugars. Lignin, which is a non-carbohydrate component, can be processed for other useful applications (Balat *et al*. 2008). The pretreatment process can remove hemicellulose, reduce cellulose crystallinity, and increase the porosity of the material (Sun and Cheng 2002), while enzymatic saccharification can convert complex carbohydrates into simple sugars (Ferreira *et al*. 2009).

Optimizations of sugar production from lignocellulosic materials focusing on the effect of various pretreatment techniques and saccharification conditions on its production, using a conventional method 'change-one-factor-at-a-time' approach, have been reported. This is an experimental method in which a single factor is varied while other factors are kept at a specific set of conditions. This method may lead to unreliable results and wrong conclusions, and is inferior to the factorial design method (Logothetis and Wynn 1989). This approach does not guarantee the true optimum due to the interaction among variables (Adinarayana and Ellaiah 2002; Kim *et al*. 2005; Raza *et al*. 2012). Thus, improvement of yield and overall productivity are essential for the development of a commercially feasible fermentation process for large-scale production. Various methods of optimization, such as experimental design, mathematical methods, and kinetics models can be used to improve fermentation processes. The experimental design constitutes an efficient tool and is well adapted for treating problems with a large number of variables (Hounsa *et al*. 1996). Furthermore, the statistical experimental designs allow simultaneous, systematic, and efficient variation of all components.

To date, a literature survey revealed that the application of statistical experimental design in optimizing production of fermentable sugar from lignocellulosic materials has still remained scarce. Thus, the aim of this work was to investigate the application of Plackett-Burman design in screening and selecting the potential operating parameters for the production of fermentable sugars from sugarcane bagasse. Following that, the Path of Steepest Ascent (PSA) method was employed to search for a region of improved response.

EXPERIMENTAL

Preparation of Sugarcane Bagasse

The lignocellulosic material used in this study was sugarcane bagasse, collected from Restaurant Lian Heng, located in Subang Jaya, Selangor Darul Ehsan, Malaysia. The skin of the sugarcane bagasse (SB) had been removed by the supplier, while the interior part of SB was collected and used as the main substrate throughout the whole project. Upon collection, the interior parts of SB were cut into smaller pieces to improve the drying efficiency. The raw materials were washed thoroughly with deionised water to remove dirt and adhered particles. Then, the raw materials were oven-dried until they reached a constant weight. The dried sugarcane bagasse was ground and sieved to three particle sizes (300 to 600 µm, 600 to 850 µm, and 850 to 1400 µm). The bagasse was then stored in sealed plastic bags with silica gel at room temperature until it was used in the experiments.

Methods

Plackett-Burman design

The Plackett-Burman design was introduced in this study as a first optimisation step to identify which factors had a significant effect on both pretreatment and enzymatic hydrolysis. This design was based on a first-order model (Eq. 1),

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i \quad (1)$$

where Y is the studied response (productivity of reducing sugar), β_0 and β_i are the model intercept and linear coefficient, respectively, and X_i is the level of the independent variable.

Seven important variables were tested, *i.e.*, pretreatment temperature, pretreatment duration, pH of pretreatment process, particle size of SB, enzyme cellulase loading, SB loading, and moisture content of the solid medium. For screening, Design Expert version 7.1.3 (State-Ease, Inc., USA) was used to generate the experimental design matrix of the Plackett-Burman factorial design; each variable was examined at two levels: low (-1) and high (+1) (Plackett and Burman 1946). This design was used to screen and evaluate the most important variables that influence the response, rather than considering the interaction effects among variables. In this study, seven assigned variables, three centre points (for standard deviation estimation), and four unassigned variables, commonly referred to as dummies, were screened in 15 experimental designs. All measurements were carried out in triplicate, and the average reducing sugar productivity was taken as the response. Table 1 illustrates the factors under investigation as well as the levels of each factor used in the experimental design. Table 2 represents the design matrix with 15 runs of PBD for method optimisation.

Pretreatment of lignocellulosic materials

A combination of physical, chemical, and thermal pretreatment was employed in this study to investigate their significance on productivity of reducing sugar. The first run was conducted using acid, physical, and thermal pretreatment technique (Table 2). Pretreatment at pH 2 was classified as acid pretreatment, and sulphuric acid was used to pretreat the sugarcane bagasse. Firstly, dried sugarcane bagasse (solid-liquid ratio of 3% w/v) with a particle size of 850 to 1400 μm^2 was pretreated with sulphuric acid in a water

bath at 35 °C for 120 min. After that, the particles were separated from the solution. Then, the particles were washed with distilled water until the pH became neutral and were dried in an oven at 50 °C until constant weight. The remaining experimental runs were conducted according to the designed conditions, as shown in Table 2.

Enzymatic saccharification of lignocellulosic material

Enzymatic saccharification of pretreated SB were conducted in Erlenmeyer flasks, each containing 0.5, 2.75,or 5.0 g of pretreated sugarcane bagasse, and were moistened with the appropriate amount of sterilised distilled water and cellulase enzyme (Novozymes A/S, Bagsværd, Denmark) to reach 70%, 80%, or 90% (v/w) moisture content (Table 2). Next, the flasks were left under solid-state conditions for the complete saccharification to proceed. Sampling was performed every 24 h. During the sampling process, a considerable amount of distilled water was used as an extractant and added to the respective flask. The flask was well shaken. Following this, the pH of the solution was measured. The mixture was centrifuged at 9000 rpm for 10 min. The supernatant was used for the analysis of reducing sugars.

Table 1. PBD Factor Levels (-1/+1)* and Centre Point Conditions (0) for Seven Potential Factors for the Production of Reducing Sugar

Code	Independent variable	Unit	Low level (-1)	Zero level (0)	High level (+1)
A	Pretreatment temperature	°C	35	80	125
B	Pretreatment duration	min	30	75	120
C	pH of pretreatment process	-	2	7	12
D	Particle size of SB	µm	300	850	1400
E	Cellulase loading**	% (v/w)	20	35	50
F	SB loading	g	0.5	2.75	5.0
G	Moisture content of SB	%	70	80	90

*Selection of low and high levels of the factors was based on the literature survey of saccharification-related researches.

**Carboxymethyl-cellulase (CMCase) activity = 3880 U/mL; Filter-paperase (FPase) activity = 42 U/mL.

Path of steepest ascent

Following the identification of significant factors by PBD, estimation of the optimal factor conditions was conducted using the method of steepest ascent. The method of steepest ascent is a simple and efficient procedure for moving along the experimental region of a response towards the direction in which the response rises most rapidly by increasing or decreasing the values of the significant parameters (He and Tan 2006; Chen *et al*. 2009). The zero level (0) of PBD was considered the origin of the path. Experiments were conducted along the steepest ascent path until there was no further increase in the response.

Table 4 illustrates the experimental design and response of the PSA experiments. Assuming that the point $x_1 = x_2 = \ldots = x_k = 0$ is the base or origin point, the step size can be calculated using Eq. 2 (Meyer *et al*.2009),

$$\Delta x_j = \frac{b_j}{b_i / \Delta x_i} \quad (2)$$

where b is the regression coefficient, j = 1, 2, …., k, and $i \neq j$.

Determination of reducing sugar concentration

Sampling was performedevery 24 h until the saccharification process reached equilibrium stage. During the sampling process, a considerable amount of distilled water was added to the collected flask and the mixture was mixed well. The pH of the solution was measured before it was centrifuged at 9,000 rpm for 10 min. Then, the supernatant was used for analysis of thetotal reducing sugars with the 3,5-dinitrosalicylic acid (DNS) test (Miller 1959).

X-ray diffraction (XRD) analysis

The crystallinity of native and treated sugarcane bagasse was measured using an X-ray diffraction diffractometer with monochromatic Cu-Kα radiation (λ = 1.54 Å), generated at a voltage of 40 kV and a current of 40 mA. Samples were scanned over an angle of diffraction (2θ) range between 2° and 30° at a 1.25°/min scanning rate. The crystallinity index (CrI) was calculated as follows (Segal *et al*. 1959),

$$CrI\ (\%) = \frac{I_{002} - I_{am}}{I_{002}} \quad (3)$$

where I_{002} is highest peak intensity at an angle of diffraction (2θ) of ~ 22° and I_{am} is the peak for the amorphous region at an angle diffraction (2θ) of ~ 18°.

Scanning electron microscopy (SEM) analysis

Physical changes in the native and treated sugarcane bagasse were observed using scanning electron microscopy (SEM). Images of the surfaces of the untreated and treated sugarcane bagasse were taken at magnification 750× using aTESLA BS-340 scanning electron microscope. The specimens were placed on conductive tape, coated with gold palladium using a PELCO SC-6sputter coater, and observed using a voltage of 15 kV.

RESULTS AND DISCUSSION

Response: Maximum Productivity of Reducing Sugar

Table 2 shows the independent variables and their respective low and high coded values in thePlackett-Burman design, with productivity of reducing sugar recorded as the response. From Table 2, reducing sugar productivity was observed to be the highest in run 9, with a value of 0.025 g/L/h, and the lowest in run 7, with a value of 0.006 g/L/h.

Table 3 illustrates the ANOVA analysis of the productivity of reducing sugar. The *p*-value of the model was significant (<0.0001) with an F-value of 23279.20. In addition, the suitability of the model was confirmed by a confidence level (R^2) above 99%. The lack of fit with an F-value of 0.62 implied an insignificant lack of fit relative to the pure error. As observed in Table 3, five of the seven factors had a significant effect on the productivity of reducing sugar from pretreated sugarcane bagasse. These parameters (B-pretreatment duration; C- pH of pretreatment process; E- amount of enzyme cellulase

used; F- loading of SB; and G- moisture content of solid medium) had a *p*-value below 0.05. On the other hand, the temperature of the pretreatment process and particle size of SB were found to be insignificant (p>0.05) and were both excluded from Table 3. The corresponding first-order equation fitted to the data obtained from PBD for each individual factor is shown in Eq. 4,

$$Productivity\ of\ Reducing\ Sugar = 0.015 + 5.348*10^{-4}B + 1.234*10^{-3}C - 2.326*10^{-4}E + 6.899*10^{-3}F + 1.508*10^{-3}G + 6.756*10^{-4} \quad (4)$$

where productivity of reducing sugar is the response of the study.

Equation 4 determined the predicted response of each experiment, as tabulated in Table 2. It can be seen that the actual results were very close to the predicted values. The coefficient of determination, R^2, was then used to check the goodness of fit of the model. The value of R^2, predicted R^2, and adjusted R^2 were 1.0000, 0.9993, and 0.9999, respectively, which showed that there was a high correlation between the observed values and predicted values. Moreover, the relationship between the process variables (factors) and the response (productivity of reducing sugar) was explained well by the regression model. Hence, the model was statistically sound. Furthermore, the coefficient of variation (CV) in the present study was 0.54%, implying a high degree of reliability.

The half-normal probability plot for productivity of reducing sugar (Fig. 1) clearly shows that the pretreatment temperature (A) and particle size of SB (D) were located on the near-zero line (red line), indicating that they were unimportant. On the other hand, SB loading (F) was found to be an important factor due to its location, followed by moisture content of SB (G), pH of pretreatment process (C), pretreatment duration (B), and enzyme cellulase loading (E).

Table 2. Plackett-Burman Design Variables (in Coded Levels) with Productivity of Reducing Sugar as Response

Run order	A	B	C	D	E	F	G	Productivity of reducing sugar (g/L/h)	
								Actual	Predicted
1	-1	1	-1	1	1	-1	1	0.0083	0.0092
2	1	1	-1	1	1	1	-1	0.0203	0.0200
3	1	-1	-1	-1	1	-1	1	0.0066	0.0082
4	-1	-1	-1	1	-1	1	1	0.0225	0.0225
5	1	1	1	-1	-1	-1	1	0.0128	0.0122
6	-1	1	1	1	-1	-1	-1	0.0077	0.0092
7	-1	-1	-1	-1	-1	-1	-1	0.0055	0.0057
8	1	-1	1	1	-1	1	1	0.0236	0.0249
9	-1	1	1	-1	1	1	1	0.0248	0.0255
10	0	0	0	0	0	0	0	0.0157	0.0156
11	0	0	0	0	0	0	0	0.0156	0.0156
12	-1	-1	1	-1	1	1	-1	0.0209	0.0215
13	0	0	0	0	0	0	0	0.0155	0.0156
14	1	-1	1	1	1	-1	-1	0.0073	0.0077
15	1	1	-1	-1	-1	1	-1	0.0189	0.0205

Table 3. Analysis of Variance for Plackett-Burman Design Model

Source	Effect	Sum of squares	DF	Mean square	F-value	P-value
Model	-	6.196×10^{-4}	4	1.549×10^{-4}	23279.20	<0.0001
B: Pretreatment duration	0.22	3.431×10^{-6}	1	3.431×10^{-6}	515.67	<0.0001
C: pH of pretreatment process	0.27	1.827×10^{-5}	1	1.827×10^{-5}	2744.91	<0.0001
E: Cellulase loading	-0.16	6.491×10^{-7}	1	6.491×10^{-7}	97.55	0.0006
F: SB loading	2.02	5.711×10^{-4}	1	5.711×10^{-4}	85821.05	<0.0001
G: moisture content of SB	0.57	2.730×10^{-5}	1	2.730×10^{-5}	4102.24	<0.0001
Curvature		1.095×10^{-6}	1	1.095×10^{-6}	164.61	0.0002
Residual		2.662×10^{-8}	4	6.654×10^{-9}		
Lack of fit		1.017×10^{-8}	2	5.083×10^{-9}	0.62	0.6180
Pure error		1.645×10^{-8}	2	8.225×10^{-9}		
Cor.total		6.208×10^{-4}	9			

R^2 = 1.0000, adjusted R^2 = 0.9999, predicted R^2 = 0.9993

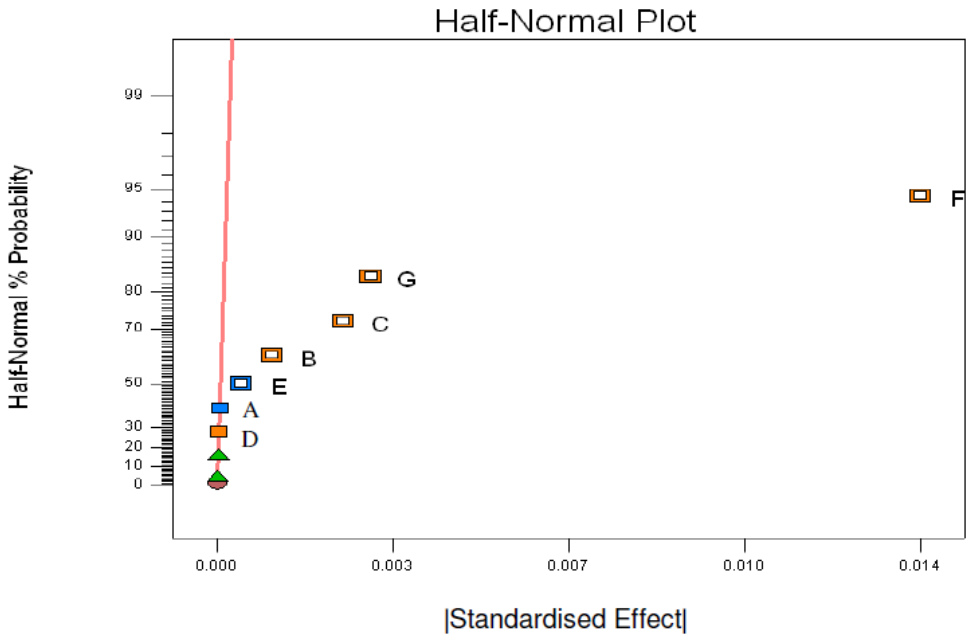

Fig. 1. Half-normal probability plot of the standardized effect for productivity of reducing sugar. Symbols: Green triangle – error from replicates; orange colour – positive effect; blue colour – negative effect

A Pareto chart enables identification of factors that produce positive or negative effects on the productivity of reducing sugar. In the Pareto chart (Fig. 2), the maximal effects are depicted in descending order, from left to right. A factor that had a positive effect on the reducing sugar productivity is given as an orange bar, where as a negative effect is given as a blue bar. Additionally, two horizontal lines in red and blue can be observed across the Pareto chart. The red line, which is located at a higher level, represents the Bonferroni limit, while the blue line represents the *t*-value limit. The Bonferroni limit and *t*-value limit are statistically based acceptance limits (similar to 95% confidence intervals) for each bar in the Pareto chart (Wilkinson 2006). Parameters

above the Bonferroni limit were more significant than those above the *t*-value limit. In statistics, Bonferroni correction is used (i) to counteract the problem of multiple comparisons; (ii) to control the false discovery(s) rate, and (iii) to eliminate the possibility of eliminating correct null hypotheses (known as a type I error) (Abdi 2007). Parameters that were above the *t*-value limit had 95% significance; parameters that were below the *t*-value limit were not likely to be significant. Of all seven factors evaluated, substrate loading (F) appeared to be the most important factor affecting reducing sugar concentration, followed by moisture content of solid medium (G), pH of pretreatment process (C), pretreatment duration (B), enzyme loading (E), and pretreatment temperature (A). Particle size (D) was the least important factor, located below the *t*-value limit.

The effect of each contributing factor on the productivity of reducing sugar is presented in Fig. 3, and the productivity of reducing sugar was very much dependent on these significant parameters. Substrate loading was the most significant parameter that positively affected the response, followed by moisture content of SB, pH of pretreatment process, and pretreatment duration. On the other hand, cellulase loading was significant, with a negative effect on the productivity of reducing sugar from pretreated sugarcane bagasse, in which a lower enzyme loading was more desirable for higher productivity of reducing sugar.

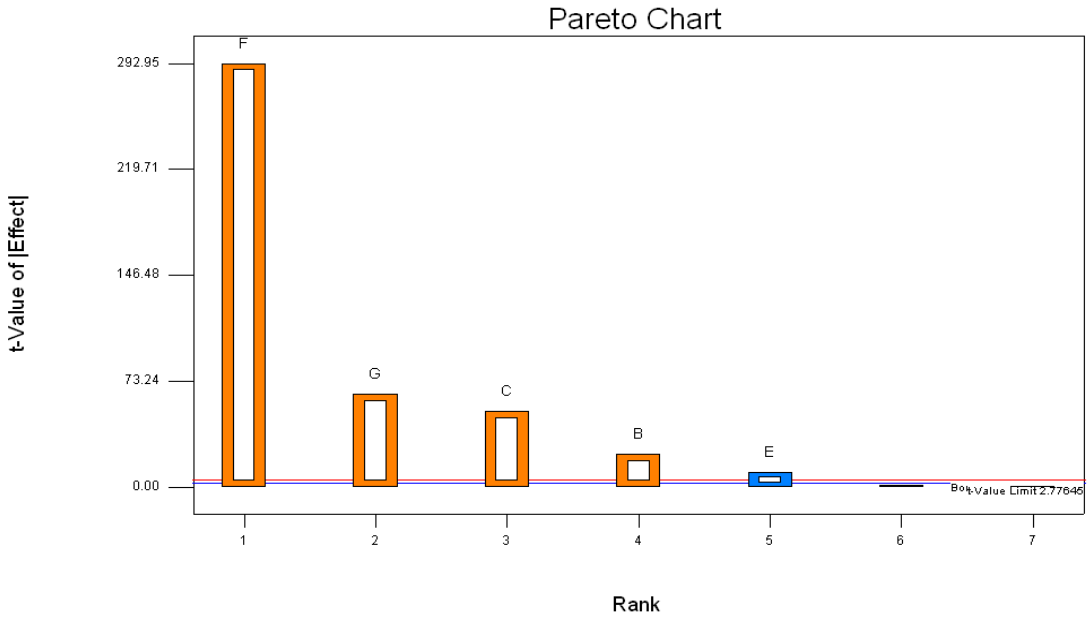

Fig. 2. Pareto chart from thePlackett-Burmandesign. Red horizontal line represents Bonferroni limit and blue horizontal line represents *t*-value limit.

Path of Steepest Ascent

The PBD results (Fig. 3 and Eq. 3) indicated that pretreatment duration (B), pH of pretreatment process (C), loading of SB (F), and moisture content of SB (G) had a positive coefficient, while enzyme loading (E) had a negative coefficient. This implies that increasing elements B, C, F, and G while decreasing E should result in a higher level of reducing sugar productivity.

The estimated coefficients from Eq. 3 were used as the steps along the path of steepest ascent. Experiments were performed based on the zero level of the Plackett-

Burman design (Table 1). The non-significant factors screened out by PBD (A- pretreatment temperature; D- particle size of SB) were fixed at their respective zero levels throughout the optimisation process. The steepest ascent experimental design and the corresponding results are illustrated in Table 4. The productivity of reducing sugar peaked on the third experimental run, and no further improvement could be achieved in the response. The reducing sugar productivity would be maximized under the conditions near to those of runs 2 and 3 (Table 4), which could be chosen for further optimisation study.

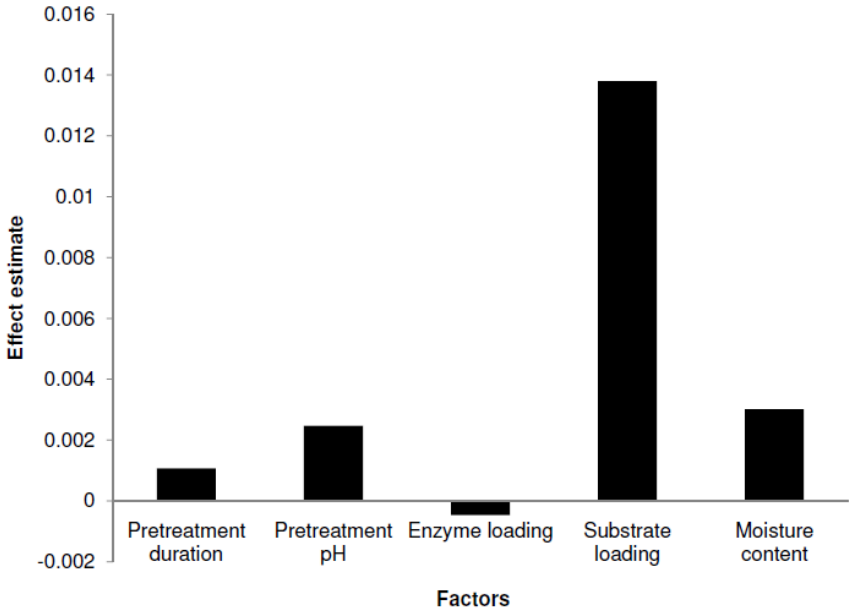

Fig. 3. Main effects of the contributing factors on reducing sugar productivity, based on the Plackett-Burman experimental results

Table 4. Experimental Design of the Steepest Ascent Method and the Corresponding Response

Experimental run	B (min)	C pH	E (% v/w)	F (g)	G (%)	Productivity of reducing sugar (g/L/h)
0	75.0	7.0	35.0	2.75	80.0	0.0156
0 + 1Δ	78.5	7.9	34.5	5.00	82.2	0.0240
0 + 2Δ	82.0	8.8	34.0	7.25	84.4	0.0280
0 + 3Δ	85.5	9.7	33.5	9.50	86.6	0.0246

B: pretreatment duration (min), C: pH of pretreatment process, E: enzyme loading (%v/w), F: SB loading (g), G: moisture content of SB (%)

Effect of Individual Parameters

Substrate loading

As shown in Fig. 3, sugarcane bagasse had the greatest positive effect on reducing sugar productivity. The productivity of reducing sugar increased three-fold when the substrate loading was raised from 0.5 g (run 12) to 5.0 g (run 14). The PSA method also

showed a further increase in productivity when the substrate loading was set in the range of 5.00 g to 7.25 g (Table 4).

As a greater amount of substrate was loaded into the medium, more cellulose could attach itself to the active sites of cellulase enzymes. Hence, this led to a higher saccharification rate, which in turn enhanced the productivity of reducing sugar. At low substrate levels, an increase of substrate concentration usually leads to an increase of the yield and hydrolysis reaction rate. When substrate loading is excessively high, substrate inhibition may arise. This phenomenon would considerably decrease the rate of hydrolysis (Cheung and Anderson 1997). As justified by the results of the PSA (Table 4), the productivity of reducing sugar began to decrease when substrate loading was increased to 9.70 g. According to Sun and Cheng (2002), the degree of substrate inhibition is dependent on the ratio of the total substrate to total enzyme.

Moisture content

Following substrate loading, moisture content was the next most influential parameter that had a positive effect on the productivity of reducing sugar. The moisture content indicates the amount of water to be added to the sugarcane bagasse during solid-state enzymatic hydrolysis. Table 2 shows that run 5 (90% moisture content) had higher reducing sugar productivity than that of run 6 (70% moisture content). The volume of water added to run 5 was nearly four times more than that of run 6. Results of these two runs indicated that increasing the moisture content of sugarcane bagasse during solid-state saccharification process would promote the higher productivity of reducing sugar.

Enzymatic hydrolysis of cellulose into reducing sugar is performed via the breakdown of glycosidic bonds (Felby *et al*. 2008). It is a two-substrate reaction, in which both cellulose and water are involved. Water also serves as a solvent that allows enzymes to function, enhances the contact between enzymes and the substrate, and facilitates the transportation of products (Zaccai 2004; Kristensen *et al*. 2009). With high initial moisture content in solid particles, enzymatic transport mechanisms throughout the saccharification process became smoother and hence enhanced the interaction between enzymes and the substrate. This would, in turn, increase the overall productivity of reducing sugar. In addition, analysis through the steepest ascent method (Table 4) demonstrated that an initial moisture content as high as 84% is favorable for the high level of reducing sugar productivity from pretreated sugarcane bagasse.

Pretreatment pH and pretreatment time

As illustrated in Fig. 3, both pretreatment duration and pH of the pretreatment process had positive effects on reducing sugar productivity. A pH value of 12 (run 6) indicated an alkali pretreatment, whereas a pH value of 2 (run 7) represented an acid pretreatment. Higher reducing sugar productivity was achieved when the native sugarcane bagasse was treated with an alkaline solution (pH 12) for a longer period of time (Table 2). The PSA method also depicted a further increase in the productivity of reducing sugar when pretreatment pH and pretreatment time were set in the range of 7.9 to 8.8 and 78.5 to 82.0 min, respectively (Table 4). The reducing sugar productivity of run 6 was 40% higher than that of run 7. As the pretreatment time increased, more lignin and hemicellulose in the biomass was reduced, resulting in an increase of surface area. This would allow more water molecules to access the inner layers of biomass, breaking the bonds between hemicellulose and lignin-carbohydrate (Balat *et al*. 2008).

The physical structure and chemical composition of the substrate, as well as the treatment conditions, are the three key factors that affect the efficacy of alkali pretreatment (Chen *et al*. 2013). Alkali pretreatment was found to be more effective in increasing the hydrolysis rate of hardwoods and agricultural waste, which have lower lignin contents than softwood (Millet *et al*. 1976; Bjerre *et al*. 1996). Hence, higher reducing sugar productivity could be expected when sugarcane bagasse (agricultural waste) was treated under alkaline conditions. Han *et al*. (2012) studied the alkali pretreatment of wheat straw and its enzymatic hydrolysis. It was reported that, as the alkali pretreatment duration increased, the saccharification efficiency improved considerably. However, when treated for a prolonged period, the cellulose and hemicellulose were destroyed, resulting in a lower concentration of reducing sugar.

Enzyme loading

By comparing the results of runs 2 and 15, it was determined that a higher loading of enzyme cellulase did not significantly improve the saccharification rate (Table 2). In theory, a higher enzyme loading would hydrolyse more cellulose into monomeric sugar, which would increase the rate of hydrolysis. The presence of a limited amount of substrate in the medium (5 g) might be one of the reasons for the low productivity of reducing sugar. Enzyme loading and substrate loading have a close relationship to each other. If the substrates are saturated in the medium, neither the saccharification rate nor the reducing sugar yield will be improved. This is due to insufficient active sites for enzymes on the substrates. Moreover, if enzyme loading is higher than substrate loading, the enzyme-substrate reaction will not occur, which in turn increases the waste of valuable enzymes.

As illustrated in Fig. 3, cellulase loading was significant, with a negative effect on the productivity of reducing sugar from pretreated sugarcane bagasse, in which decreasing the amount of enzyme cellulase loading would lead to better productivity of reducing sugar. Leaustean *et al*. (2010) reported that an extremely low input of enzyme would lead to incomplete saccharification, as the substrates would remain unconverted due to enzyme saturation, while with too high loading of enzyme, some enzymes may remain unused. Some studies reported that increasing the enzyme loading in an enzymatic saccharification process can help to improve the yield and rate of hydrolysis; however, this would significantly raise the cost of a process, making it unprofitable (Sun and Cheng 2002).

X-Ray Diffraction (XRD) Analysis

One of the important factors affecting enzymatic hydrolysis is the crystallinity of cellulose (Sun and Cheng 2002; Kuila *et al*. 2011). X-ray diffraction is one of the most commonly used methods for the determination of the crystallinity of lignocellulosic materials (Sun *et al*. 2011). According to the data presented in Table 5, the CrI value of the untreated sample was 34.14%, while the CrI was in the range of 46 to 53% for the treated samples. In general, treated samples had a higher CrI than the untreated sample, which might be due to the effective removal of both lignin and hemicellulose during the pretreatment process. As pretreatment progressed, more of the amorphous region (lignin and hemicellulose) of the samples was eliminated as that of the crystalline region (Wanitwattanarumlug *et al*. 2012), leading to an increase in crystallinity of the pretreated sample. According to Sun *et al.* (2011), enzymatic hydrolysis possesses significant effect on crystallinity of lignocellulosic materials. Higher values of CrI could be achieved if

XRD analysis was conducted before saccharification of pretreated lignocellulosic materials. In the present study, XRD analysis was conducted after the saccharification process took place. The crystalline cellulose in the sample would have been hydrolysed into simple sugar and hence reduce the crystallinity of pretreated sugarcane bagasse.

In addition, if only the effect of pH was considered, the crystallinity index of alkali-pretreated samples (run 5, run 6, run 8, run 9, run 12, run 14) was generally higher than that of acid-pretreated samples (run 1, run 2, run 3, run 4, run 7, run 15). Alkaline pretreatment has been proven to be a better option than acid pretreatment in increasing the CrI of lignocellulosic biomass (Chang and Holtzapple 2000; Kasahara *et al*. 2001; Mosier *et al*. 2005). Acid pretreatment may give rise to inhibitory products, lowering the effectiveness of lignin and hemicellulose removal, which would reduce the crystallinity of the samples, resulting in a lower CrI value.

Scanning Electron Microscope (SEM) Analysis

Two main morphological features can be obtained from SEM of the surfaces of the ground raw bagasse. Figures 4A and 4B illustrate the fiber and pith structure of raw bagasse, respectively. The fiber structure was formed by parallel stripes, and this structure was partially protected by residual material. The pith, on the other hand, was a fragmented structure that was more breakable than the fiber structure. This structure contained pits, which are tiny pores joining neighbouring cells on the surface of the walls (Rezende *et al*. 2011). Pith residues (Fig. 4B) were formed by soft walls from parenchyma cells, which possessed less resistance towards acid treatment. In contrast, the fiber structure (Fig. 4A) was formed by vascular bundles surrounded by sclerenchyma. Because the fiber structure is a lignified tissue, it was relatively resistant to an acidic environment. A similar result was reported by Artschwager (1940). Thus, acid treatment would be more effective in treating the pith structure, while alkali treatment would be more effective in treating the fiber structure.

Table 5. Crystallinity Index of Untreated and Treated Samples After Enzymatic Saccharification

Samples	Crystallinity index (CrI) (%)
Untreated	34.14
Run 1	48.93
Run 2	50.54
Run 3	50.70
Run 4	49.00
Run 5	52.26
Run 6	52.07
Run 7	49.64
Run 8	52.20
Run 9	53.10
Run 10	47.71
Run 12	51.86
Run 14	51.00
Run 15	46.74

Prior to pretreatment and saccharification processes, a complete and compact lignocellulosic structure was observed (Fig. 4A). However, pretreatment and saccharification completely disrupted the structure of the sugarcane bagasse (Figs. 5A and 5B). More holes were observed in Fig. 5B (run 8- treatment at pH 12) than in Fig. 5A (run 1- treatment at pH 2). The increased formation of holes increased the surface area of accessible cellulose, which in turn enhanced the productivity of reducing sugar. Hence, this explained the higher CrI obtained in run 8 than in run 1 (Table 4).

Two types of tissue, sclerenchyma (S) and parenchyma (P), can be found in the structure of sugarcane bagasse (Fig. 6). The images shown in Figs. 6A and 6B were the results of acid and alkali pretreatments, respectively. The cell wall seemed to peel off from the structure in Fig. 6B, while no significant change was observed on the cell wall of the bagasse shown in Fig. 6A. One of the main reasons for this might be the removal of lignin fractions from the inner parts of the wall as a result of the alkaline reaction (Rezende *et al*. 2011).

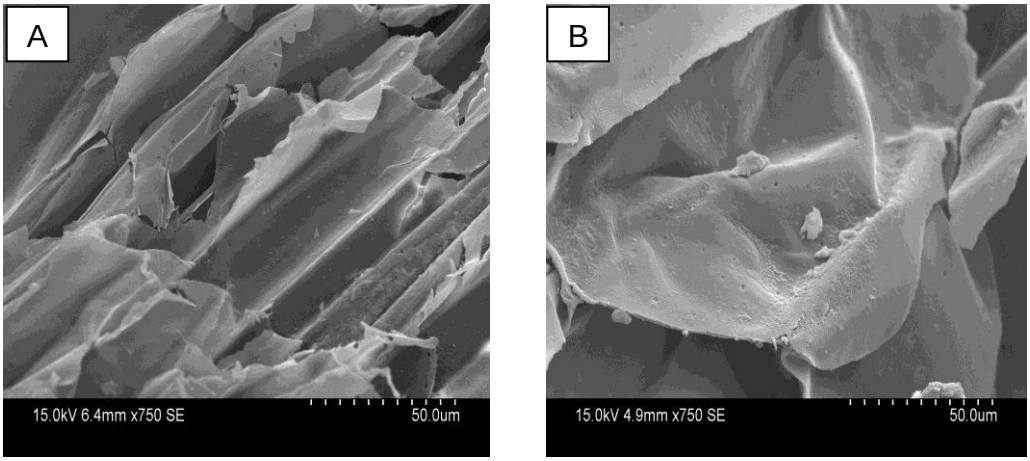

Fig. 4. SEM images of the fiber (A) and pith (B) structures of untreated sugarcane bagasse

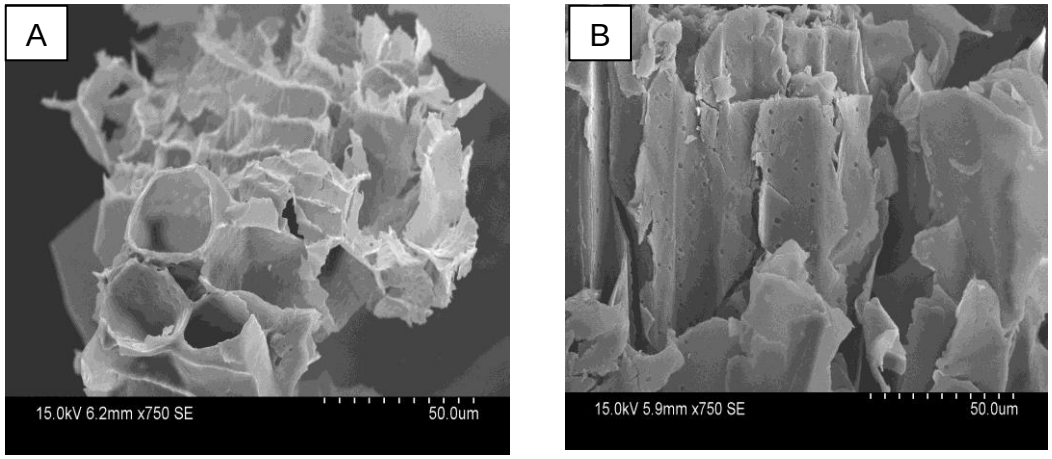

Fig. 5. SEM images of treated sugarcane bagasse:run 1 (A) and run 8 (B), after enzymatic hydrolysis

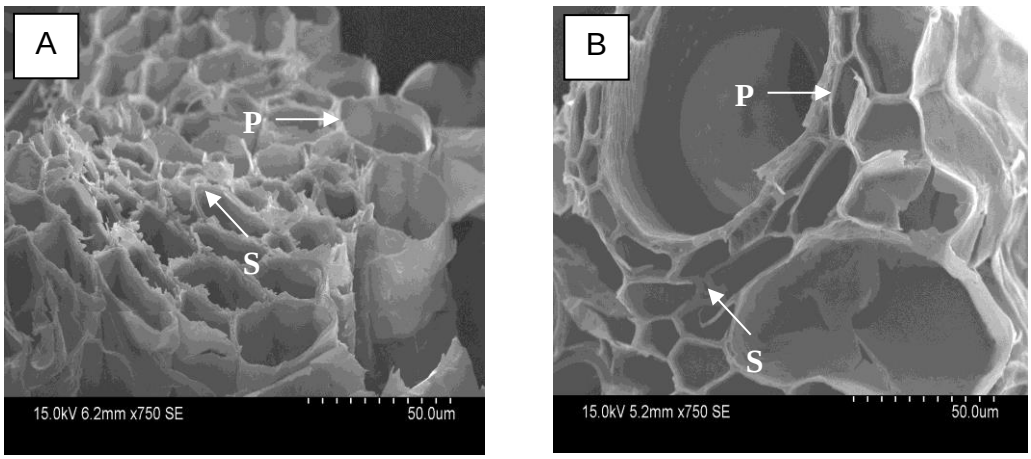

Fig. 6. SEM images of the structure of sugarcane bagasse with sclerenchyma and parenchyma: run 3- acid treatment (A) and run 6- alkali treatment (B)

CONCLUSIONS

1. Based on PBD analysis, five of the seven variables, *i.e.*, pretreatment duration, pH of pretreatment process, loading of substrate, enzyme cellulase loading, and moisture content of substrate, were observed to have a significant effect on the productivity of reducing sugar from pretreated sugarcane bagasse. ANOVA results showed that the model of reducing sugar productivity provided a high correlation between the response and its parameters.
2. The steepest ascent method effectively and efficiently approached the experimental design space for the optimal productivity of reducing sugar from sugarcane bagasse. The optimum point of each parameter was as follows: pretreatment duration of 82.0 min, pretreatment pH of 8.8, enzyme loading of 34.0% (v/w), substrate loading of 7.25 g, and moisture content of 84.4%.
3. The significant parameters affecting reducing sugar productivity were successfully screened with the aid of a statistical design approach.

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