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REACTION OPTIMIZATION OF *Aspergillus niger* α-L-ARABINOFURANOSIDASE FOR IMPROVED ARABINOSE PRODUCTION FROM KENAF STEM

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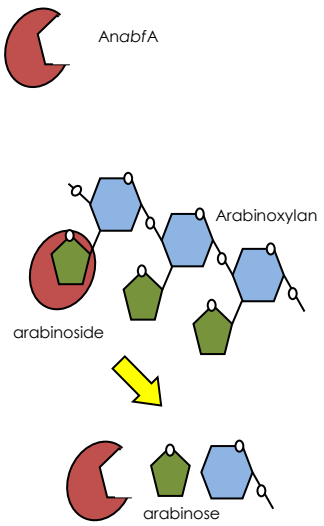
Graphical abstract



Abstract

There are abundant of lignocellulosic biomass readily available with varying compositions. Kenaf (*Hibiscus cannabinus*) is one of this lignocellulosic biomass that has a high content of hemicellulose. This particular hemicellulose is composed of high arabinoxylan, which is a xylan backbone with arabinofuranosyl branches. In order to hydrolyze arabinoxylan, a branching enzyme is needed. Therefore, a-L-arabinofuranosidase from *Aspergillus niger* ATCC120120 (An*abf*A) was used to hydrolyzed pre-treated kenaf and the reaction conditions were optimized using central composite design (CCD) to produce a significant amount of arabinose. There were 20 experiments conducted with 1.68 star points and 6 replicates at the centre points. The reaction conditions that were optimized are enzyme loading, substrate concentration and reaction time in which resulted with 88 U An*abf*A activity, 0.9% (w/v) and 48 h, respectively. These optimized conditions managed to increase the yield of arabinose with 47.17 mg/g arabinose produced.

Keywords: Arabinose, arabinofuranosidase, glycosyl hydrolase, kenaf, arabinoxylan, statictical analysis

Abstrak

Terdapat banyak biojisim lignoselulosa yang boleh didapati dengan komposisi berbeza-beza. Kenaf (*Hibiscus cannabinus*) adalah biojisim lignoselulosa dengan kandungan hemiselulosa yang tinggi. Hemiselulosa ini mengandungi banyak arabinoxilan, iaitu tulang belakang xilan dengan cabang arabinofuranosil. Untuk menghidrolisis arabinoxylan, enzim pencabang diperlukan. Oleh itu, a-L-arabinofuranosidase dari *Aspergillus niger* ATCC120120 (An*abf*A) telah digunakan untuk menghidrolisis kenaf pra-rawat dan keadaan tindak balas dioptimumkan menggunakan reka bentuk komposit pusat (CCD) untuk penghasilan arabinosa yang banyak. Terdapat 20 ujikaji yang dilakukan dengan 1.68 titik bintang dan 6 replikasi pada titik tengah. Keadaan tindak balas yang dioptimumkan adalah penambahan enzim, kepekatan substrat dan masa tindak balas yang dikenalpasti pada 88 U aktiviti An*abf*A, 0.9% (w/v) dan 48 h, masing-masing. Pengoptimuman tindak balas berjaya meningkatkan hasil arabinosa dengan 47.17 mg/g arabinosa dihasilkan.

Kata kunci: Arabinosa, arabinofuranosida, hidrolasa glikosil, kenaf, arabinoxilan, analisis statistik

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1.0 INTRODUCTION

Natural fibers have gain interest compared to synthetic fibers due to lower tool wear, lower density, cheaper cost and availability [1]. It is a good candidate for the reinforcement fiber of high performance biodegradable polymer composites [2]. *Hibiscus cannabinus* (kenaf) is one example of natural fiber crops. It serves as a quality animal feed because of its high content of structural carbohydrate (~89%). Previous analysis carried out for 3.5–4 months old hardwood kenaf shows that the composition of holocellulose, cellulose and lignin are (71–89 %), (31–64 %) and (14–34 %), respectively [3]. Raw kenaf has 51.83% (w/v) dry weight composition of hemicellulose, which consist of 37.11% (w/v) xylan and 14.11% (w/v) arabinose [4].

Until now, kenaf has been rarely utilized as raw material in bioconversion processes as it is still in the research stage. Each type of lignocellulosic material is slightly different in its composition and results a unique challenges in the conversion process. Kenaf that undergone two stage pre-treatment process was used. It is reported that pre-treatment of kenaf removes 59.25% (w/v) of lignin while maintaining 87.72% (w/v) and 96.17% (w/v) of hemicellulose and cellulose, respectively [4]. Substrate with a low lignin content is important because lignin on the surface of lignocelluloses slows hydrolysis rates and decrease digestibility [5].

Arabinoxylan is a L-arabinofuranosyl side chains that substituted on xylan backbone. The complexity of xylan backbone is the main impediment for arabinose production. In order to produce arabinose, a-L-arabinofuranosidase (EC 3.2.1.55) acts as an accessory enzyme that is important for the hydrolysis of a-L-arabinofuranosyl residues at the non-reducing ends of oligosaccharides and polysaccharides. The debranching enzymes is extensively studied as it facilitates a more complete hydrolysis of arabinoxylan and arabinan containing feedstock [6]. Previous study shows that purified a-L-arabinofuranosidase can be used to hydrolyse arabinan, debranched arabinan and arabinoxylans for production of L-arabinose [7], which is the second most abundant pentose beside D-xylose. Recent study also demonstrated their key role in softening the lignocellulosic material and results a higher efficiency and significant reduction of costs in the conversion of plant biomass [8]. The use of a single accessory enzyme for partial or specific modification of lignocelluloses [9] might offer new interesting options for the utilization of this low cost material for arabinose production.

The monosaccharide L-arabinose is an aldopentose that is normally found in nature as D-form. However, L-arabinose is a rare exception to this rule and found mainly in its furanose form as a component of plant hemicellulose and pectin [6]. Production of arabinose from various sources of commercial arabinoxylan such as wheat-arabinoxylan and rye-arabinoxylan has been reported by many research groups. The production of arabinose at industrial scale is carried out from lignocellulosic biomass rich in arabinoxylan either by chemical or physical process such acid hydrolysis [10] and enzyme hydrolysis [11]. However, only a few attempts have been performed for the production of arabinose using in-house produced arabinofuranosidase, especially when hemicellulosic kenaf is used as the substrate.

The ability of enzyme to release arabinose from arabinoxylan makes the enzyme a promising candidate for use in the production of fermentable sugars from hemicellulosic biomass [7] such as kenaf. Beside the role as a reducing sugar for food and biofuel industries, L-arabinose also selectively inhibits intestinal sucrase in non-competitive manner, leading to decrease plasma glucose levels after sucrose ingestion [12]. L-arabinose are also considered as a potential prebiotics [13] which gives effects when consumed as a part of diet. Due to all advantages, this study is conducted to increase arabinose production by optimizing its hydrolysis reaction parameters.

2.0 METHODOLOGY

2.1 Recombinant Enzyme (AnabfA) Preparation

a-L-arabinofuranosidase used in this work was from *Aspergillus niger* ATCC 120120 (An*abf*A). It was cloned and expressed into *P.pastoris* X-33 in a previous work conducted by Izawati *et al.,* [14]. The glycerol stock of *P. pastoris* integrant containing recombinant An*abf*A gene was grown on YPD plate under non-induced condition. After 3 days, one colony of *P. pastoris* transformant was inoculated in 10 ml of BMGY medium in a 100 ml baffled flask and grown until the culture reaches an OD_{600} = 2-6. This 10 ml culture was used to inoculate 1250 ml of BMGY medium in a 5 L baffled flask. The flasks were incubated at 28-30°C, 250 rpm in a shaking incubator until the culture absorbance reaches an OD_{600} = 2-6 (approximately 16-18 h). The cells were harvested by centrifugation (6000 rpm for 5 min at 4°C) before adding 250 ml of BMMY medium to the pellet. The medium was then transferred in 1 L baffled flask and shaken at 250 rpm, 30°C for 4 days. Absolute methanol was added routinely (every 24 hours) to a final concentration of 2% (v/v) in order to maintain induction.

The cells were harvested at fourth day and enzyme's activity was assayed based on modified procedure assay of a-L-arabinofuranosidase [15]. A total of 500 ml enzyme was concentrated using Minimate™ Tangential Flow Filtration Systems. Then, 50 ml of the partially purified enzyme was subjected

for purification using ÄKTAprime plus chromatography.

2.2 Enzyme Purification

Purification was performed using HisTrap™ HP Columns (GE Healthcare) based on the interaction between Ni^{2+} and His-Tag of An*abf*A. The purification process was done via ÄKTAprime plus chromatography system (GE Healthcare). The method used for purifying protein using ÄKTAprime plus was carried out according to the manufacturer's protocol (GE Healthcare).

2.3 Substrate Preparation

The substrate used in this study was kenaf, which has been pre-treated with alkaline-acid pre-treatment method [4]. A two-stage pre-treatment steps using $Ca(OH)_2$ and Peracetic acid (PPA) was used to remove 59.25% of lignin while maintaining most of the hemicellulose composition (87.72%). Kenaf was washed extensively before enzymatic hydrolysis to ensure the substrate is free from any contaminants. Total arabinose from hemicellulose was determined using two stages hydrolysis process. The final dimension of kenaf between 40–60 mesh were collected for hydrolysis [4].

2.4 Enzymatic Hydrolysis of Kenaf

Effects of several hydrolysis parameters on arabinose production from kenaf were carried out in a batch system. The reaction was conducted in a 20 ml of 50 mM sodium acetate buffer (pH 5.0), 200 rpm water bath shaker and 50°C temperature, unless mentioned otherwise. These parameters were kept constant as optimized previously [14]. All reactions mixtures were incubated in a shaking water bath with An*abf*A enzyme. Twenty microliter of tetracycline hydrochloride was added into every reaction mixtures to prevent microbial contamination. The supernatants were harvested for every 2 hours to analyze a reducing sugar. All reactions were repeated three times and assayed in triplicate.

The effects of enzyme loading, substrate concentration and hydrolysis time on An*abf*A hydrolysis were optimized using central composite design (CCD) in the Design Expert software (2002) version 6.0.8 (Stat-Ease, Minneapolis, US). The response of the experiment design was based on the reducing sugar released. The process consisted of a total of 20 experiments trials which involves 1.68 star points and 6 replicates at the centre points. The reaction conditions investigated are enzyme concentration (X_1), substrate concentration (X_2) and time of reaction (X_3) with the arabinose production as dependent variable/response (Y). Low and high levels were coded as -1 and +1; the centre point was coded as 0. Details of coded levels of the variables are given in Table 1. Experiments were conducted as per design matrix that was arranged by Design Expert software. The significance among the groups of the experimental data was analysed using the ANOVA test and a *P*-value. Adequacy of the model developed was further analysed.

Table 1 Reaction conditions and the levels studied in the optimization design

Factors	Notation	Unit	Low star point	Centre point	High star point
			- a	0	+ a
Enzyme loading	X_1	U	36.36	70	103.64
Substrate loading	X_2	%	-0.01	1.25	2.51
Reaction time	X_3	h	-0.27	30.00	60.27

2.5 Analytical Methods

Reducing sugar concentration was determined according to Miller (1959) using a modified DNS reagent [16]. To determine the content of reducing sugar, the reaction mixtures were injected through high performance liquid chromatography (HPLC). The HPLC was equipped with a Rezex RSO-Oligosaccharides Ag^+ 4% guard column (60 mm x 10.00 mm, Phenomenex) in line with a Rezex RSO-Oligosaccharides Ag^+ column (200 mm x 10.00 mm, Phenomenex analytical column) coupled to a Waters Associates refractive index (RI) detector. The reaction mixture was eluted at 80°C with deionised water flowing at 0.25 mL/min.

3.0 RESULTS AND DISCUSSION

3.1 Gene Expression

The gene encoding a-L-arabinofuranosidase from *Aspergillus niger* (An*abf*A) was successfully expressed in *P. pastoris* strain X-33 expression system [14]. The enzyme was concentrated using Minimate™ Tangential Flow Filtration Systems before purified using ÄKTAprime plus chromatography. The purification process of An*abf*A was summarized in Table 2 below. The purification in this study showed an improvement in yield compared to previous study [14]. A pure enzyme with 9.21% yield and 77.17 purification fold was used to avoid any impurities that can interfere with the characterization process.

Table 2 Purification table for An*abf*A enzyme

	Total Activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	2204.45	13.16	1.00	100.00
Concentrated	347.56	32.70	2.48	15.77
Purified	203.10	1015.50	77.17	9.21

The purified recombinant An*abf*A was resolved as a single band by SDS-PAGE (Figure 1a) and Western Blot (Figure 1b). The molecular weight was estimated to be 83 kDa. It is higher with the predicted molecular weight of recombinant An*abf*A calculated from CLC Sequence Viewer (Version 6.0.1) software, which is 65.54 kDa. A larger molecular weight resolved with compared to theoretical molecular mass is due to *N*-linked or/and *O*-linked oligosaccharides in the recombinant protein produced from *P. pastoris* X-33. Previous study proved the recombinant An*abf*A was *N*-glycosylated [14].

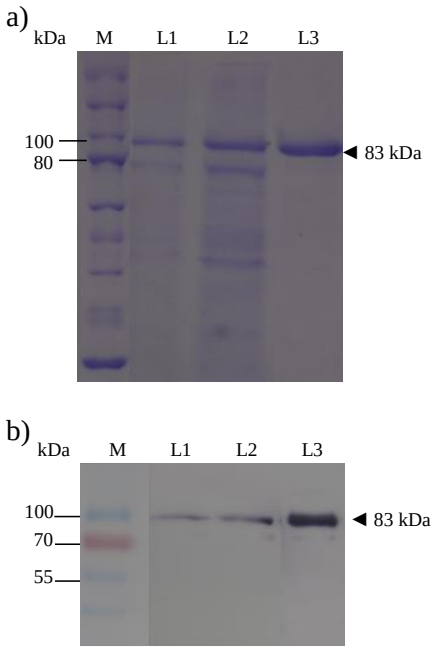

Figure 1 Analysis of crude and purified An*abf*A by (a) SDS-PAGE and (b) corresponding western blot. M: protein marker, L1: crude enzyme, L2: concentrated enzyme, L3: purified enzyme. Size of glycosilated enzyme predicted was 83 kDa and marked as (◀).

3.2 Effect of AnabfA Loading on Hydrolysis

Efficiency of reactions of An*abf*A on kenaf was determined using several enzyme loadings which were evaluated in a range of 40-90 U. Enzyme loading in hydrolysis can vary over a wide range of unit activities depending on the type of substrate, concentration of substrate and on the protocol used [17]. Hence, it is important to choose the optimum concentration of An*abf*A for maximum arabinose conversion. The values of kenaf concentration, time and temperature were initially kept constant at 1% (w/v), 24 hours, 50°C and pH 5.0, respectively. The amounts were fixed according to the common values used by previous research [18].

The efficiency of enzymatic hydrolysis of kenaf arabinoxylan is shown by the amounts of arabinose released during the reaction (Figure 2). It shows that the arabinose yield was effectively enhanced by the addition of higher An*abf*A loadings. From Figure 2, it was found that the hydrolysis efficiency of kenaf increased sharply at the initial enzyme loading range (40-80 U). At 80 U of An*abf*A loading, more than 30 mg/g arabinose was hydrolyzed from kenaf stem. A study on hydrolysis of alkali pre-treated sugarcane bagasse using xylanase also reported that increment of enzyme concentration up to 120 U/g will increase a hydrolysis efficiency [19]. A similar trend reported for production of glucose from BSG cellulose. A glucose yield increased when enzyme concentration increase up to 45 FPU/g substrate, but higher enzyme loading did not favor more cellulose conversion because at this point, all active site of enzyme are saturated with substrate [20].

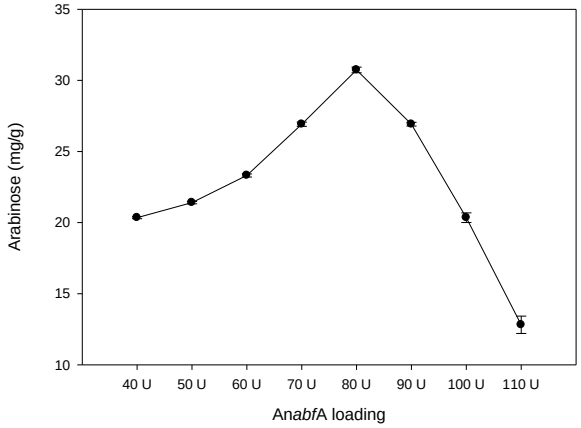

Figure 2 Effect of different An*abf*A loading on arabinose production

However, the hydrolysis efficiency gradually reduced when the enzyme concentration exceeded a certain level (> 80 U). At a certain limit, hydrolysis rate is constant since the velocity of the reaction cannot be altered [20]. Similar enzymatic hydrolysis on sugarcane bagasse using xylanase also indicated a decrease in the sugar production when using enzyme concentration higher than 40 U/g [19]. It is assumed that the initially adsorbed enzyme on the fiber surface forms a single layer in such a way that the excess of enzyme molecules would be absorbed forming an additional multiple layers. This superficially adsorbed enzyme on the first layer of the fiber would play a significant role in the hydrolysis and restrict the diffusion process of enzyme through the structure of the substrate [21]. Therefore, an excess of An*abf*A formed a multiple layers at kenaf's surface area nevertheless only the first layer An*abf*A attached will participate in hydrolysis. High enzyme loadings will overcrowd accessible hemicellulose chains and it is believed that this reduction in percentage of hemicellulose hydrolysis is due to the lack of available binding sites on the hemicellulose [22].

The decreases of hydrolysis rate could be also due to the modification that occurred on substrate

structure during reaction [19] and substrate particle size [20]. Large substrate particle size will give a low total surface area and limit the penetration of enzyme into substrate. As kenaf has a large and complex complex structure (particle size between 40–60 mesh), it is presumed to limit the penetration of enzyme and reduce the capability of hydrolysis [23]. To ensure higher hydrolysis to occur, a good contact between enzyme and substrate for reaction should be optimized. All of kenaf's surface area should be fully covered by enzyme to ease the enzyme attack and consequently, increase hydrolysis efficiency.

3.3 Effect of Kenaf Concentration on Hydrolysis

Kenaf in a range between 0.5-3.0% (w/v) were added at the initial of experiment and the result is shown in Figure 3. Hydrolysis of An*abf*A towards kenaf gives significant increment of arabinose production when kenaf was increased from 0.5 to 1.0 % (w/v). The increment was likely due to the high substrate to enzyme loading ratio. This is clearly understood that when more substrates present in the reaction medium, a higher surface areas are available for the enzyme active sites to attack. The efficiency of an enzymatic hydrolysis increases with the increased in substrate concentration. Reaction of 80 U of An*abf*A and 1.0% (w/v) kenaf provide highest arabinose yield. At this condition, the mixture provided a good combination of fast initial hydrolysis and high conversion yield. Adequate enzyme to substrate ratio ensure good contact between substrate and enzyme, thus promote heat and mass transfer within reaction vessel [24].

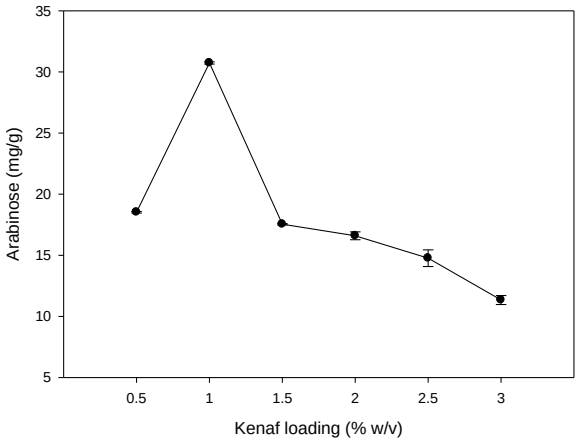

Figure 3 Effect of different kenaf loading on arabinose production

However, increased substrate concentrations for insoluble substrate (up to a certain concentration) decreased hydrolysis rate. Further enhancement in the substrate concentration of kenaf (>1.5% (w/v)) resulted a decreased arabinose. Addition of more than 2.0% (w/v) pre-treated kenaf (0.4 g kenaf in 20 ml reaction solution) will give a slurry effect to the reaction mixture. The corresponding yield decreased in a trend that cannot be explained by current model and knowledge of enzyme-substrate interactions [25]. One theory suggested that high substrate concentration will create a "high solid enzymatic hydrolysis phenomenon." This phenomenon explains higher substrate concentrations will cause a lower amount of free water and limits the high solid enzymatic hydrolysis. It was clearly explained by Ehrhardt *et al.,* (2010), where a higher solid concentration results in a higher viscosity and therefore higher diffusion times, especially for substrates that cannot be dissolved completely or insoluble in water [26].

3.4 Effect of Reaction Time on Hydrolysis

One of the challenges to hydrolyze a complex and insoluble substrate includes long hydrolysis time. Theoretically, the longer time of enzyme incubated with its substrate; the greater the amount of product will be formed. However, the rate of formation of a biological product is not a simple linear function of incubation time. Therefore, arabinose production at different hydrolysis time was studied because sufficient time is important for complete enzymatic hydrolysis. The experiment conditions were fixed at 1% (w/v) kenaf and 80 U An*abf*A as obtained in previous screening. Figure 4 shows arabinose produced is directly proportional with time of reaction. Maximum arabinose produced at 96 h and the amount reduced when longer reaction time used (> 96 h).

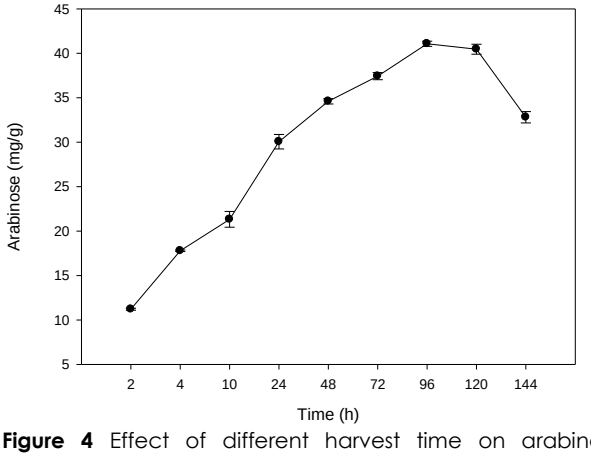

Figure 4 Effect of different harvest time on arabinose production

The time taken for hydrolysis of kenaf arabinoxylan was shorter than hydrolysis of sugar cane bagasse (96 h) [19] but slower than hydrolysis of hemicellulose extracted bagasse, which takes 48 h of reaction [27]. The time difference is because all substrates have a different structure and composition. In this study, a pre-treated kenaf with low amount of lignin was

used. In contrast, untreated samples has very poor enzymatic hydrolysis due to complex, compact and unopened structure which limits the access of enzymes to breakdown its carbohydrate polymers [4].

3.5 Statistical Analysis Optimization by ANOVA

Enzymatic hydrolysis can be greatly influenced by concentration of enzyme, concentration of substrate and time of reaction. Statistical design has proved efficient for optimizing the lignocellulosic hydrolysis [28]. A rotatable central composite design (CCD) was applied to predict the optimum reaction conditions to achieve maximum arabinose production. The objectives are to reduce the amount of enzyme loading, substrate loading and reaction time; and at the same time increase the arabinose yield. A model was adjusted simultaneously involving reaction parameters (X_1-X_3) as stated in Table 1. Arabinose production was collected as a response (actual value) and the results is shown in Table 3.

Table 3 Central composite design matrix for arabinose production

Std	Reaction parameters			Arabinose (mg/g)	
	X_1 (U)	X_2 (%)	X_3 (h)	Actual value, *Y*	Predicted value, *Z*
1	50.00	0.50	12.00	24.52	27.58
2	90.00	0.50	12.00	38.06	33.91
3	50.00	2.00	12.00	32.24	36.46
4	90.00	2.00	12.00	37.99	41.51
5	50.00	0.50	48.00	43.00	40.05
6	90.00	0.50	48.00	45.55	40.91
7	50.00	2.00	48.00	28.47	32.20
8	90.00	2.00	48.00	41.29	37.80
9	36.36	1.25	30.00	48.96	44.55
10	103.6	1.25	30.00	47.05	52.06
11	70.00	0.01	30.00	0	5.55
12	70.00	2.51	30.00	12.81	7.86
13	70.00	1.25	-0.27	40.06	35.90
14	70.00	1.25	60.27	35.98	40.74
15	70.00	1.25	30.00	39.02	37.23
16	70.00	1.25	30.00	41.10	37.23
17	70.00	1.25	30.00	32.12	38.73
18	70.00	1.25	30.00	37.98	38.73
19	70.00	1.25	30.00	24.67	32.69
20	70.00	1.25	30.00	42.52	32.69

The response was analysed using the analysis of variance (ANOVA) and the details are shown in Table 4. The *P*-value for the model source, each model terms and interactions were detailed in the ANOVA. The *P*-value of 0.0158 indicates that there is only 1.58% chance that the model failure occurs due to noise. Model terms were said to be a significant factors that influence the arabinose production if it has low *P*-value. The *P*-value for term 'Lack of Fit' was calculated to be 0.076, which is greater than 0.05 indicates that the 'Lack-of-Fit' of model is insignificant. The value also implies there is a 7.60% chance that the 'Lack of Fit F-value' this large could occur due to noise. In other words, the model is fit with the responses data collected and is desirable for the following experiment.

An actual model is developed to predict the optimum hydrolysis conditions to maximize the arabinose production. The final equation in term of coded factor was shown in Equation 1. All terms regardless of their significance are included in the equation, where *Y* is the response and X_1-X_3 are the actual values of the test variables: An*abf*A loading (U), kenaf loading (% w/v) and reaction time (h), respectively.

$$Y = + 37.67 + 2.23X_1 + 0.69X_2 + 1.44X_3 + 4.99X_1^2 - 9.72X_2^2 + 1.46X_3^2 + 0.43X_1X_2 - 0.61X_1X_3 - 3.43X_2X_3$$

(Equation 1)

Table 4 ANOVA for response surface model for the arabinose production

Source	Sum of squares	DOF	Mean Square	*F*-value	*P*-value
Model	92.66	2	46.33		0.0158
X_1	67.93	1	67.93	1.46	0.2612
X_2	6.49	1	6.49	0.14	0.7184
X_3	28.21	1	28.21	0.61	0.4584
X_1^2	358.48	1	358.48	7.71	0.0240
X_2^2	1360.04	1	1360.04	29.26	0.0006
X_3^2	30.66	1	30.66	0.66	0.4402
X_1X_2	1.51	1	1.51	0.032	0.8617
X_1X_3	3.01	1	3.01	0.065	0.8054
X_2X_3	94.05	1	94.05	2.02	0.1927
Residual	371.88	8	46.48		
Lack of fit	340.78	5	68.16	6.58	0.076
Pure error	31.09	3	10.36		
Correlation	2595.02	19			

The fitness of the model was expressed by the R^2 value which is 0.8514; indicates 85.14% of the variability in the response can be explained by the model. This value demonstrates a good agreement between the predicted and experimental value of arabinose production. Regression model applied to calculate the predicted values and the usefulness of the model. The predicted values are closely matched with the experimental values or in the other words; the model obtained is applicable to predict the optimum reaction conditions to maximize the

arabinose production. Two-dimensional contour plot response surface were used as the graphical representations of the regression equation (Equation 1). Figure 5 and 6 shows a contour plot of model on how production of arabinose varies with enzyme loading, substrate loading and reaction time, in which the model is valid.

The ANOVA shows that substrate loading (X_2^2) gave the most significant effect to the arabinose production with the lowest *P*-value (*P*=0.0006). Theoretically, higher kenaf loading will produce a high sugar yield. This significant effect can be easily observed from Figure 5. It reveals maximum arabinose hydrolyzed was at 0.9% kenaf loading where at this point, the ratio between An*abf*A and kenaf is optimum for reaction. Further addition of substrate to the reaction mixture will reduce the arabinose production.

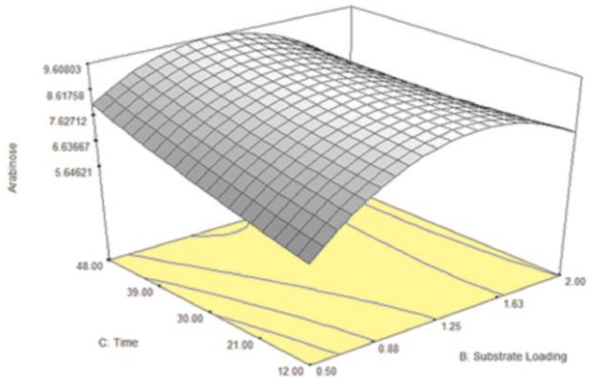

Figure 5 Response surface plot of arabinose production: kenaf loading vs. reaction time with constant level of enzyme loading

Enzyme loading (X_1^2) also gives significant effect to the arabinose production with the second lowest *P*-value (*P*=0.0240). The effect can be observed in Figure 6 where high enzyme loading enhances kenaf hydrolysis and thus produce more arabinose. Increasing enzyme loading will generate more available active site for hydrolysis and produce higher arabinose. Figure 6 reveals that 88 U of An*abf*A is optimum enzyme require for high yield of arabinose. At high enzyme loading, it will overcrowd accessible arabinoxylan before the hydrolysis takes place [22]. During the enzymatic hydrolysis by An*abf*A, non-reducing a-1,2- and a-1,3-linked arabinofuranosyl side chain are broken down from arabinoxylan (in hemicellulose) to form an arabinose. The higher enzyme loading (from 40 U up to 80 U) resulted in higher arabinose. As stated previously, reaction with 80 U of enzyme resulted in a decreasing arabinose. This was possibly due to retardation of carbohydrate hydrolysis, which responds the enzyme aggregation at higher loadings. However, statistical analysis using RSM shows the optimum enzyme loading required after considering all parameters to be 88 U.

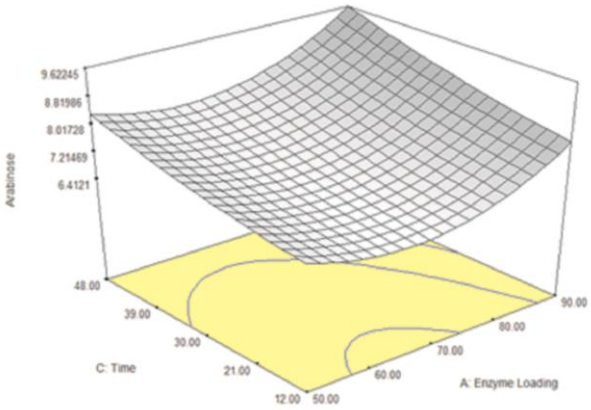

Figure 6 Response surface plot of arabinose production: enzyme loading vs. reaction time with constant level of kenaf loading

Previous study conducted by Musatto and Roberto (2005) had optimize the hydrolysis of brewer's spent grain by statistical analysis, with glucose and cellulose as a respond [28]. From their findings, enzyme loading gives highly significant effect with a positive signal, indicating that glucose yield and cellulose conversion were increased when the enzyme loading was increased. Their next study proved that the effect of enzyme loading was the highest when compared to other variables such as agitation speed and substrate concentration [20]. Nevertheless, the enzyme dosage required to achieve a complete conversion of hemicellulose vary to each raw material.

The reaction time (X_3^2) is insignificant (*P*=0.4402) in influencing the arabinose production. The equilibrium is only crucial for parameters X_1^2 and X_2^2 in hydrolysis reaction as both parameters shown a significant effect in arabinose production. A production of arabinose is lower at shorter incubation time due to kenaf's complex structure and insoluble characteristic. Although longer hydrolysis time (> 48 h) will enhance the arabinose yield, it is no worth in term of economic and production perspective at upscale production [29]. In order to maintain a high yield of arabinose with more economical value, shorter hydrolysis time with significant sugar yield must be chosen as the best hydrolysis time. Therefore, the optimum 48 h time was selected as one of the optimized hydrolysis conditions.

After optimization, the arabinose production was found to be optimum at 88 U An*abf*A loading, 0.9% (w/v) kenaf loading at 48 h of reaction time. Experiments with the reaction conditions proposed by the model had been carried out and a final arabinose yield shows that 47.17 mg of arabinose was hydrolysed from every gram of arabinose in kenaf hemicellulose (arabinoxylan) used. The rest of orabinose was assumed to remain attached to the

xylan backbone. The final arabinose yield obtained (47.17 mg/g) was close to the predicted value (48.76 mg/g).

The initial and optimized parameters for optimum kenaf hydrolysis were detailed in Table 5. Interestingly, in order to get the optimum arabinose yield, it requires lower amount of substrate and less reaction time. Although longer time increases kenaf hydrolysis, the increment was not significant and not economically feasible for an upscale process. The HPLC result for both conditions was shown in Figure 7. Based on the result, the arabinose yield was successfully improved.

Table 5 Summary of the optimized parameters for arabinose production from kenaf

Parameter	Before optimization	After optimization
AnabfA loading (U)	80	88
Kenaf loading (% w/v)	1.0	0.9
Reaction time (h)	72	48
Arabinose (% yield)	67	77

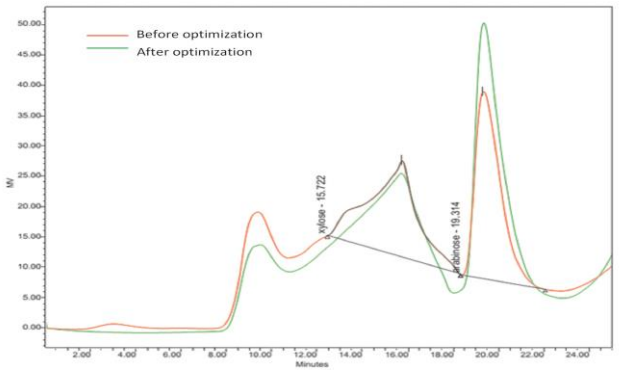

Figure 7 HPLC chromatogram show an arabinose detected where noise, xylose and arabinose

4.0 CONCLUSION

Using statistical methodology, two reaction variables that give significant influence towards enzymatic hydrolysis of kenaf are identified as An*abf*A loading and kenaf loading. Optimization using Central Composite Design (CCD) successfully improves arabinose production while reducing hydrolysis time and kenaf loading. From the findings, it reveals that kenaf is a very potential lignocellulosic biomass for the utilization into value added product, such as arabinose. For future direction, designing mutants that can fit the substrate would greatly benefit the entire bioconversion process.

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