

LACCASE AS BIO-PRETREATMENT STEP OF SAWDUST FOR ETHANOL PRODUCTION: OPTIMIZATION AND STATISTICAL MODELING

Amani M. Hashabra

Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
amanimohamma@gmail.com*

Essam A. Makky

Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
essam22001@gmail.com

Samiur Rashid Shah

Faculty of Industrial Sciences & Technology,
Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
samiur@ump.edu.my

ABSTRACT

For a successful refining of lignocellulosic biomass in production of bioethanol, it needs a delignification step to increase production of sugar. In this study, we use Laccase enzyme (as environment-friendly pretreatment) to pretreat sawdust and evaluate the degree of delignification after pretreatment in terms of weight loss percentage (%) and Total sugar produced (mg/dL). The parameters used in the optimization process was temperature (°C), pH, time of pretreatment (hrs), enzyme concentration (IU/g), substrate concentration (%), substrate size (mm) and speed of agitation (RPM). In order to achieve best condition of pretreatment, first, we use the one-factor-at-a-time (OFAT) analysis to see the significance of parameters then the Face-Centered Central Composite Design (FCCCD) of Response Surface Methodology (RSM) is used to study the combined effect of temperature and pH parameters. Our experimental results show that optimized conditions for sawdust pretreatment are: temperature, 35°C; pH 5; time, 10 hrs; enzyme concentration, 20 IU/g of sawdust; substrate concentration, 5% (w/v); sample size, 1 mm and Agitation, 150 rpm. The experiments also show a high pretreatment result considering energy sustainability which can be achieved by using sample Size of 2 mm, temperature of 25 °C and 4 hrs.

Keywords: Environment; Laccase enzyme; Pretreatment; sawdust; Bioethanol

INTRODUCTION

Concerns about exhaustion of the world's reserves of fossil fuels and about the negative impacts, such as greenhouse gas emissions associated with the combustion of these fuels have resulted in an increasing worldwide interest in using fuels from renewable resources, for instance ethanol. However, a reduction of the ethanol production cost is desirable to improve the competitiveness. As the sugar and starch-containing feedstocks traditionally used for ethanol production represent the largest share of the total production cost, the use of cheaper and more abundant raw materials is desirable for increasing the production. In recent years, the worldwide trends toward scientific and technological advances in the field of new fuels point to the importance of more efficient utilization of cellulosic feedstock's (agro-industrial and other residues) as raw material in the ethanol production process. Lignocellulosic biomass (cellulosic biomass) is favourable because of its high abundance, low cost, and high-energy potential. Lignocellulose consists of three major components: cellulose, hemicellulose, and lignin. These components are contained within the primary and secondary cell walls of plants. A huge diversity of lignocellulosic wastes is available around the world. Sugarcane bagasse, rice hulls, peanut shells, and cassava stalks are agricultural and agro-industrial residues that could be considered for bioconversion in tropical countries. These lignocellulosic residues are available on a renewable basis as they are generated during harvesting and processing of agricultural and forest products; sugar cane, rice, peanuts, cassava, wood residues (including sawdust and paper mill discards), grasses, waste paper, straws of different grains, stover, peelings, cobs, stalks, nutshells, non food seeds, domestic wastes (lignocelluloses garbage and sewage), food industry residues, municipal solid wastes. Pretreatment and enzymatic conversion of lignocellulosics are crucial steps to overcome lignocelluloses recalcitrance in the conversion to ethanol.

Laccase enzyme pre-treatment has been studied extensively for few woody residues. Interest into the role of laccases on lignin degradation has developed due to reports of some white rot basidiomycetes that are found to degrade lignin or lignin model compounds in the absence or deficiency of LiP and/or MnP. The degradation mechanisms by other enzymes such as laccase or aryl-alcohol oxidase (AAO) can predominate in lignin depolymerisation. . From an economical and environmental standpoint, the filtration and washing steps should be avoided since they increase operational costs and generate wastewater. For these reasons, enzymatic pretreatment using laccase has been explored (Moreno et al. 2012; Rico et al. 2014).

The main aim of the study is to provide a rapid, feasible, industrially achievable, and environmentally sustainable pretreatment process that is reliable and efficient in the delignification of lignocellulosic biomass, such as sawdust.

RSM is very useful in identifying the important parameters and interactions between two and more parameters in a few experiments. The main reason for doing OFAT is to screen out the parameters for further study by response surface methodology and to determine the best optimal conditions for the production of sugars.

MATERIALS AND METHODS

Biomass Collection

Sawdust was collected from the residues of different wood mills, Pahang, Malaysia, air dried, milled, then sieved to obtain four different sizes of sawdust. This done by using instruments inside the laboratory of Faculty of Industrial Science and Technology (FIST), Universiti Malaysia Pahang, Malaysia.

Biomass Characterization

The total biomass contents (moisture, lignin, cellulose, hemicellulose and ash content) will be fractionated sequentially using slight modified methods of Datta (1981).

Water soluble content estimation

Weight of 1g of dried sawdust (W1) was suspended in 100 ml distilled water, and kept in a water bath at 100°C for 2 hours, Then filtered on a crucible; the residue was oven dried at a temperature of 80°C until a constant weight is achieved (W2). Loss in weight was considered as water soluble part of the lignocellulose and can be calculated using the following equation 1.

$$WSC = \frac{\text{weight 1} - \text{weight 2}}{\text{weight 1}} \times 100 \quad (1)$$

Hemicellulose content estimation

The dried Weighed residue from above treatment was suspended in 100 ml of 0.5 M H₂SO₄ and kept in a water bath for 2 hours at 100°C. After two hours, the contents filtered, dried at 80°C and weighed (W3). The loss in weight was considered as hemicellulose content. Refer to equation 2.

$$HC = \frac{W2 - W3}{W2} \times 100 \quad (2)$$

Cellulose and lignin content estimations

10 ml of 72% (v/v) sulphuric acid was added to the dried residue from above treatment and kept for 1 hour on a rotary shaker (200 rpm) at 30°C. After incubation, the mixture was diluted with distilled water down to 4% (v/v) of sulphuric acid and autoclaved at 1.06 kg/cm² (at which temperature is 121 °C) for 40 min. The contents filtered, dried at 80°C and weighed again (W4). The loss in weight considered as cellulose, and the leftover residue was counted as lignin content of sawdust. Refer to equation 3.

$$CC = \frac{\text{weight 3} - \text{weight 4}}{\text{weight 3}} \times 100 \quad (3)$$

Ash Content Estimation

Tube Furnace was used to estimate ash content of 1 g of sawdust at 550 °C for 5 h in a tare crucible. After incineration, crucible cooled and the ash test result was expressed as % ash, calculated (equation 4) from the weight of the ash (mash) and the initial weight of the sample.

$$\text{Ash \%} = \frac{W \text{ of ash}}{W \text{ of sample}} \times 100 \quad (4)$$

Determination of Total Carbohydrate Content

By using optimized phenol–sulfuric acid method in microplate format described by T. Masuko et al. (2005) we can measure the total sugars in sawdust filtrate before and after pretreatment process. Then sugar concentration of samples could be read from a ready-made standard graph. Centrifuge samples at 5000 rpm for 5 min. to get the sample filtrates then in a microplate wells 150µL of concentrated sulfuric acid was added to 50 µL of sample filtrate followed immediately by 30 µL of 5% phenol After incubating for 5 min at 90 °C then the plate was cooled to room temperature for 5 min and wiped dry to measure A 490 nm by Infinite® 200 PRO multimode microplate reader.

Determination of Laccase Enzyme Activity by ABTS Method

The laccase enzyme activity of Novozyme 51003 from *Myceliophthora thermophila* was determined using ABTS reduction method as described by Bourbonnais and Paice (1992). The reaction mixture consisted of 0.1 M sodium acetate buffer, 0.4 mM ABTS solution, and pH 4.5 at 25 °C in a reaction volume of 0.6 mL, made up of 580 µL of ABTS-buffer solution and 20 µL of laccase enzyme. The experiment was maintained at 25 °C in a 1-cm light path cuvette. Absorbance was read at 420 nm, and the readings expressed in International Units/mL (IU/mL). The laccase enzyme stability was within 25 °C and 40 °C. One unit of the enzyme was defined as the amount of the laccase enzyme that will oxidize 1 µmol of ABTS per minute.

Optimization of the Bio-pretreatment Process (OFAT)

Pretreatment of the sawdust by biological catalysts (laccase) was carried out in lab-scale which conducted in the shake flasks. The optimization methods used was one-factor-at-a-time (OFAT) followed by FCCCD design of the Response Surface Methodology (RSM). Different parameters such as (biomass size and concentration, Enzyme concentration, Reaction time, pH of medium, agitation and temperature) was studied in this statistical optimization.

Delignification was evaluated by the percentage of weight loss, and by total sugar content after pretreatment. The process parameters was studied as described below.

Laccase enzyme concentration

Pretreatment carried out using Laccase enzyme concentration, 5, 10, 20, 30 and 40 IU/g with 5% substrate concentration, pH 5, Temperature RT, 10 ml reaction volume and the Substrate size 1 mm for 4 hrs on rotating shaker at 150 rpm.

Biomass (sawdust) size

Pretreatment carried out using Laccase enzyme concentration 5 IU/g with 5% substrate concentration, pH 5, Temperature 25, 10 ml reaction volume and the Substrate size was optimized using ≤ 5 mm, 1 mm, 2 mm, ≥ 2 mm for 4 hrs on rotating shaker at 150 rpm.

Biomass (sawdust) concentration

Pretreatment carried out using Laccase enzyme concentration 5 IU/g with 1, 5, 7 and 10 % sawdust concentration, pH 5, Temperature RT, 10 ml reaction volume and the Substrate size 1 mm for 4 hrs on rotating shaker at 150 rpm.

Time of pretreatment

Pretreatment carried out using Laccase enzyme concentration, 5 IU/g with 5% substrate load, pH 5.0, Temperature RT, 10 ml reaction volume and the Substrate size 1 mm for 4, 6, 8, 10 and 12 hrs on rotating shaker at 150 rpm

Temperature of pretreatment

The effect of temperature on the pretreatment process was studied at 25 °C, 37 °C, and 50 °C. Reactions contained a substrate concentration of 5% (w/v) and a laccase enzyme concentration of 5 IU/g of sawdust. The pretreatment processes were maintained at pH 5 for a time of 4 h and agitation at 150 rpm..

pH of medium

Pretreatment carried out using Laccase enzyme dose 5 IU/g with 5% substrate load, pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8. Temperature RT, 10 ml reaction volume and the Substrate size 1 mm for 4 hrs on rotating shaker at 150 rpm.

Agitation rate

Pretreatment carried out using fixed Laccase enzyme concentration, 5 IU/g with 5% substrate concentration, pH 5, Temperature RT, 10 ml reaction volume and the Substrate size 1 mm for 4 hrs on rotating shaker speed at 100, 135, 150 and 200 rpm.

After using OFAT to study the effect of each process parameters, further study using FCCCD of the RSM was carried out to check the interaction of pH and temperature and their effect on the pretreatment process.

Optimization of the Sawdust Pretreatment Process in FCCCD

The Face Centered Central Composite Design (FCCCD) of the RSM were applied using Design-Expert software version 6.0.8 to design the interaction effect of pH and temperature on the pretreatment process. The low and high points of pH and temperature were fixed at 3 and 7, and 25 °C and 45 °C, respectively according to results obtained from OFAT studies. Laccase enzyme concentration of 5 IU/g of sawdust and substrate concentration of 5% (w/v); all were incubated at the agitation of 150 rpm for 4 hrs. The reactions were performed in triplicates and the results were presented as the mean of the triplicates.

RESULTS AND DISCUSSION

Constituents of Characterized Sawdust

The EFB constituents were characterized before and after pretreatment with laccase enzyme at optimized conditions using the method of Datta, (1981), and the results were as shown in Table 1. Lignocellulosic biomass consists mainly of cellulose and hemicellulose, with an appreciable amount of lignin, that forms rigidity to biomass cell along with the sugar bases. Cellulose is higher than that found in Meranti wood sawdust by I.S.M. Rafiqul, (2012) of percentage 41.06. Where water soluble content 5.9% is less than 7.15 % that found by Y. Huang et al. (2015).

Table 1: Constituents of characterized sawdust

	Water soluble part (%)	H-cellulose (%)	Cellulose (%)	Lignin (%)	Ash content (%)
Control/ non pretreated	5.9	14	50	4.9	10.4
Pretreated	-	21	61	3.1	-

OFAT Study of Sawdust Pretreatment Process Parameters

Effect of laccase enzyme concentration

The rate of enzymatic reactions increased with increasing enzyme concentration (Choi et al. 2013). The effect of laccase enzyme concentration at 5, 10, 20, 30, and 40 IU/g of sawdust was studied. The results illustrated in Figure 1 shown an increase in the rate of pretreatment when the enzyme concentration was increased from 5 IU/g to 10 IU/g, with sugar yield increasing from 2.6 mg/mL to 4.0 mg/mL and weight loss from 4.5 to 6.2 % respectively. At point of enzyme concentration of 20 IU/g, a maximum sugar yield of 6.3 mg/mL and weight loss of 9.1 were reached. Further increases in enzyme concentration to 30 IU/g and 40 IU/g of sawdust had no significant influence on the rate of pretreatment. These findings agreed with the reports of Rico et. al. (2014) treatments with laccase decreased the lignin content about 12% and 20% when using laccase doses of 10 U/g and 50 U/g, respectively.

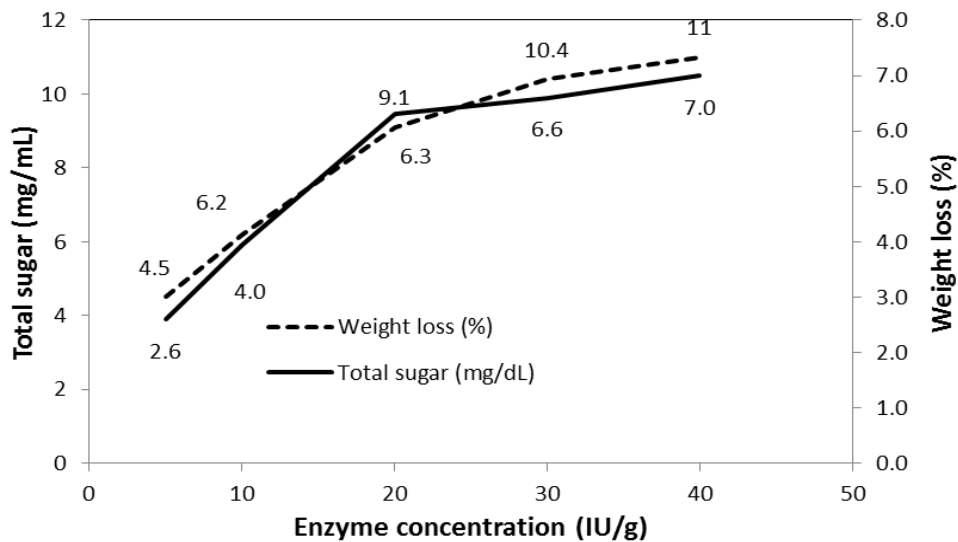


Figure 1: Effect of laccase enzyme concentration on enzymatic pretreatment of sawdust

Effect of the substrate size

The rate of biomass pretreatment is so far reliant on the size of the biomass used, (Rashid 2011). In this study, pretreatment was performed with four different SD sizes of ≤ 5 mm, 1 mm, 2 mm, ≥ 2 mm (Figure 2). At the substrate size of ≤ 5 mm, the percentage of weight loss was high this mainly due to loss of substrate during washing and filtration after pretreatment process as the part < 0.5 mm can't be maintained during filtration process. Where size of 1 mm was considered as the ideal biomass size for pretreatment process as more total sugar and high weight loss percentage of 5.1 mg/mL and 4.8 % respectively were achieved. Results obtained from higher substrate sizes were less valuable as larger surface area were less affected by enzyme and needs more time and enzyme concentration to release more sugar by enzyme degradation.

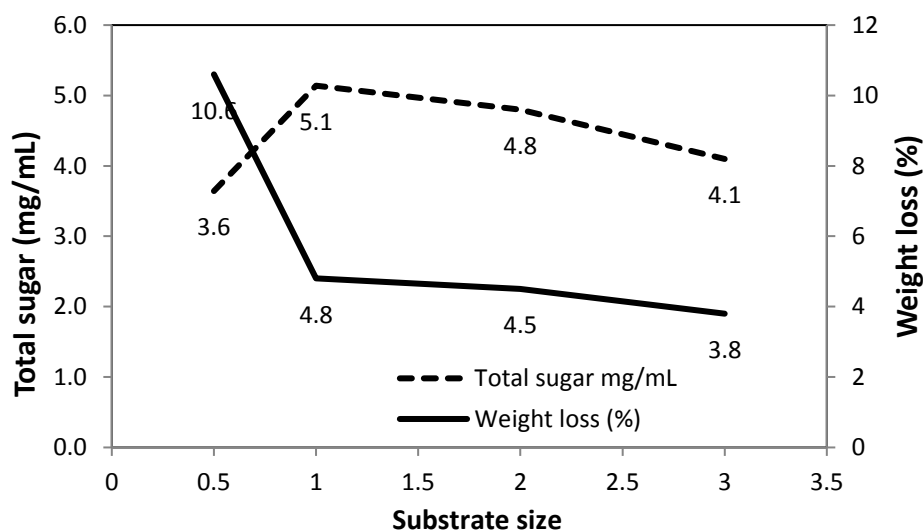


Figure 2: Effect of substrate size on enzymatic pretreatment of sawdust

Effect of substrate concentration

The rate of enzymatic reactions depends largely on enzyme and substrate concentrations where enzyme active sites were occupied by the substrate evenly. Higher substrate concentration reduce rate of reaction and lower substrate concentration increase enzymatic reaction rate. In this study, the influence of substrate concentration was determined by varying the substrate concentrations as 1, 5, 7 and 10 % (w/v) with constant enzyme concentration of 5 IU/g of sawdust. At 1 % (w/v) in (fig. 3) the total sugar produced 1.7 that mean insufficient substrate to be engaged with enzyme but at substrate concentration of 5% (w/v) higher total suger produced of 3.4 mg/mL and 6.0 % weight loss. The reaction rate reduced with 7 and 10 % (w/v) substare concentrations. Similar findings by Ishmael et al. (2016) where a steady decline in pretreatment of EFB as the substrate concentration was increased from 5% (w/v) to 20% (w/v), at 5 % substrate concentration 2.4 mg/mL total sugar were produced and 1.73 mg/mL at 10 % (w/v) EFB as shown in Figure 3.

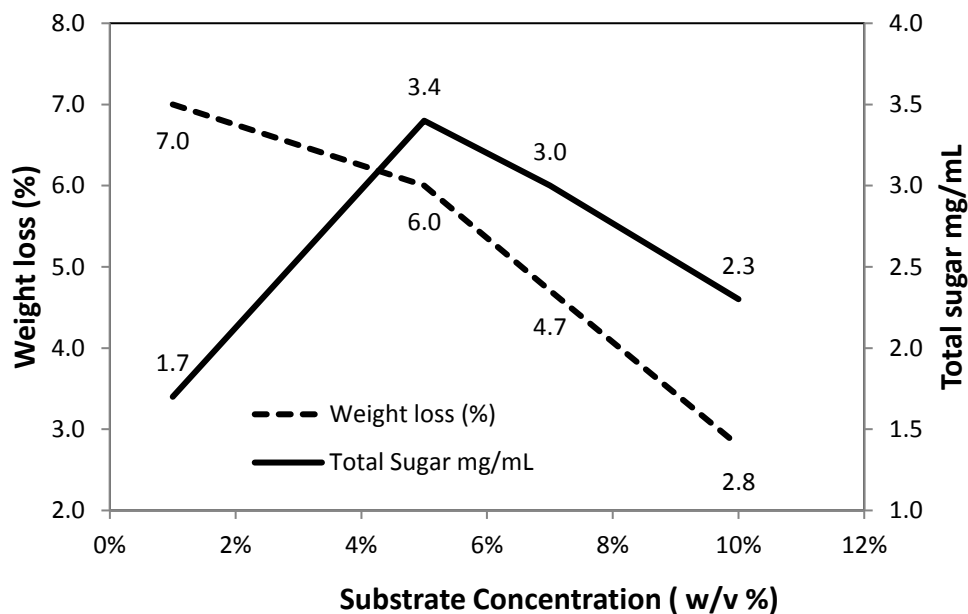


Figure 3: Effect of substrate concentration on enzymatic pretreatment of sawdust

Effect of time of reaction

Different levels of reaction time ranging from 2 to 12 hrs were tested to determine the possible optimum level to maximize total sugar production. The highest total sugar concentration of 3.7 mg/mL and 3.9 mg/mL, respectively, were obtained by conducting pretreatment for 8 and 10 hrs (Figure 4). The reduction in total sugar concentration at longer time (>10 hrs) resulted from the degradation of these compounds to monosaccharrides and low total sugar after short time (2 hrs) is because insufficient time for enzyme reaction to make biomass degradation to produce sugar. So far it meets study by Ishmael et al. (2016) that good progress in the pretreatment process of EFB between 2 h to 4 h, where the maximum rate was achieved with total sugar of 1.61 mg/mL released after 4 h pretreatment.

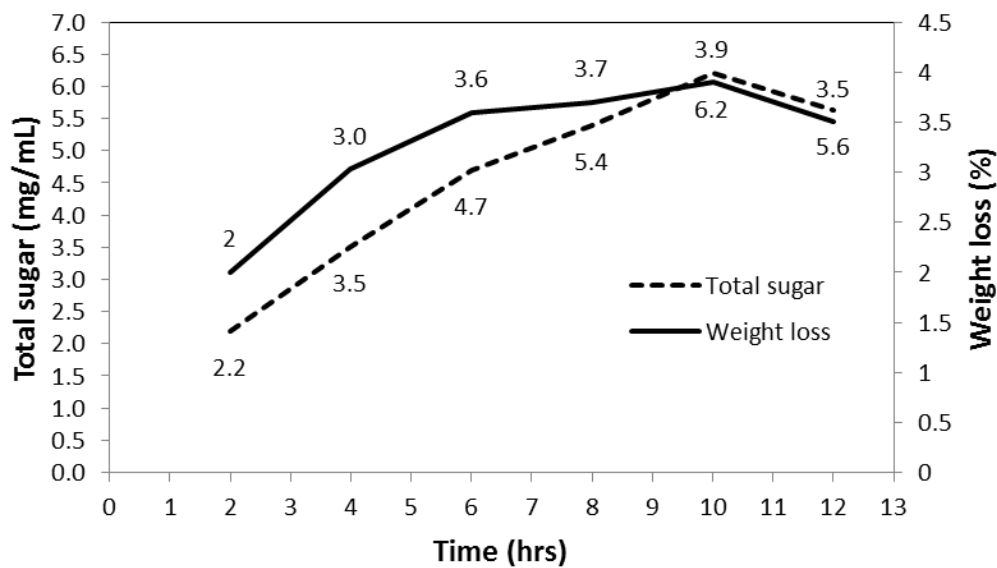


Figure 4: Effect of reaction time on enzymatic pretreatment of sawdust

Effect of temperature

In order to evaluate the effect of temperature on the pretreatment process, three temperature levels 25 °C, 37 °C, and 50 °C were employed. Figure 5 demonstrates the effect of temperature on the laccase pretreated sawdust where production of sugar and weight loss increased with increasing temperature and reached to a maximum value of 3.7 mg/mL and 2.8 % respectively at 37 °C. But at higher temperature degrees 50 °C the enzyme reaction rate dropped with low sugar production 2.4 mg/mL. A previous study by Rico et al. (2014) pretreated eucalyptus feedstock with laccase enzyme from *T. versicolor* at 50 °C and obtained 18% delignification.

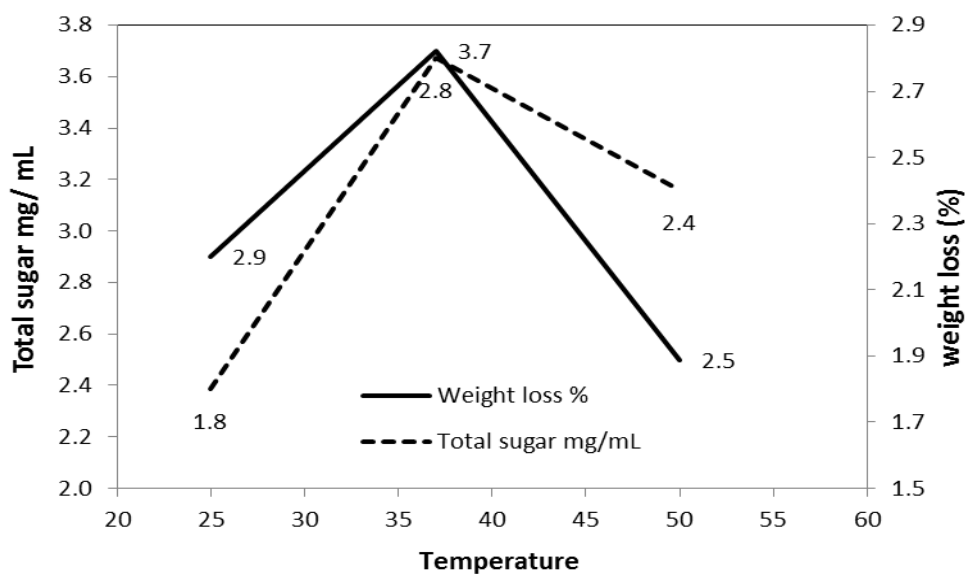


Figure 5. Effect of temperature on enzymatic pretreatment of sawdust

Effect of pH of medium

All enzymes have a specific pH range at which they are most active due to the effect of ionization of proteins (enzymes) in medium. Pretreatment carried out using pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8 (Figure 6). High sugar produced were of pH 5 and 6, 5.2 and 5.5 mg/mL respectively. This result parallel with many previous studies Ishmael et al. (2016) findings where pretreatment of EFB with laccase enzyme was studied at pH 2, 3, 4, 5, 6, and 7. The total sugar yields of 0.18, 0.21, 1.08, 2.42, 2.0, and 0.19 mg/mL were achieved. The results revealed that pH 2, 3, and 4 the rate of pretreatment was slower; pH 5 was more favorable to the enzyme.

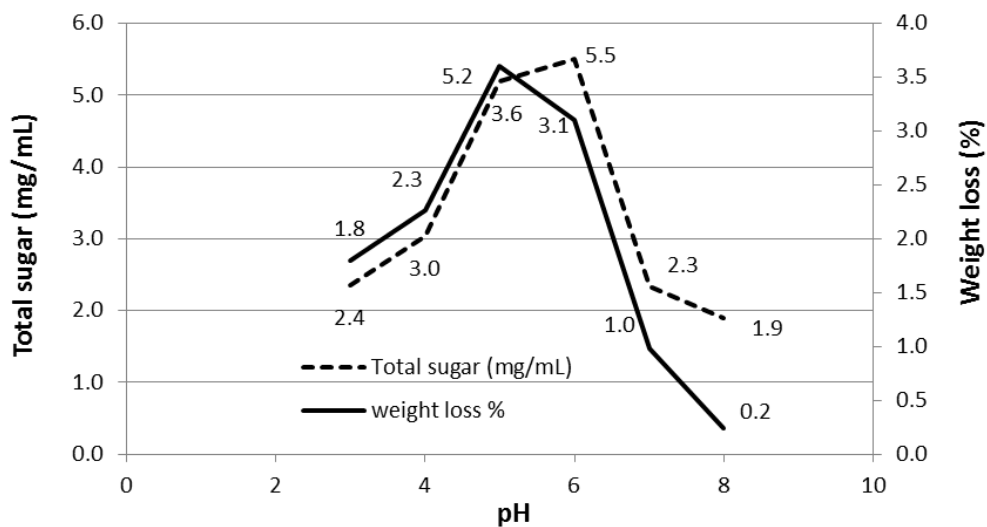


Figure 6: Effect of medium pH on enzymatic pretreatment of sawdust

Effect of Agitation rate

Speed of shaker usually affect the protein structure (e.g. enzymes) and may cause loss of their chemical binding structure. In this study agitation at 100, 135, 150 and 200 rpm were done, resulted in high total sugar and weight loss 3.6 mg/mL and 5.8 % respectively on speed of 150 rpm otherwise speed of 200 rpm cause decline in pretreatment rate with both low sugar and weight loss 2.5 mg/mL and 1.7 mg/mL (Figure 7). Kasetsart J. (2008) in his Optimization of Agitation Conditions for Maximum Ethanol Production by Coculture at the agitation rate of 0, 50, 100, 150 and 200, respectively, he suggested that the agitation rate of 50 rpm was suitable for ethanol production by the coculture from the mixed sugars.

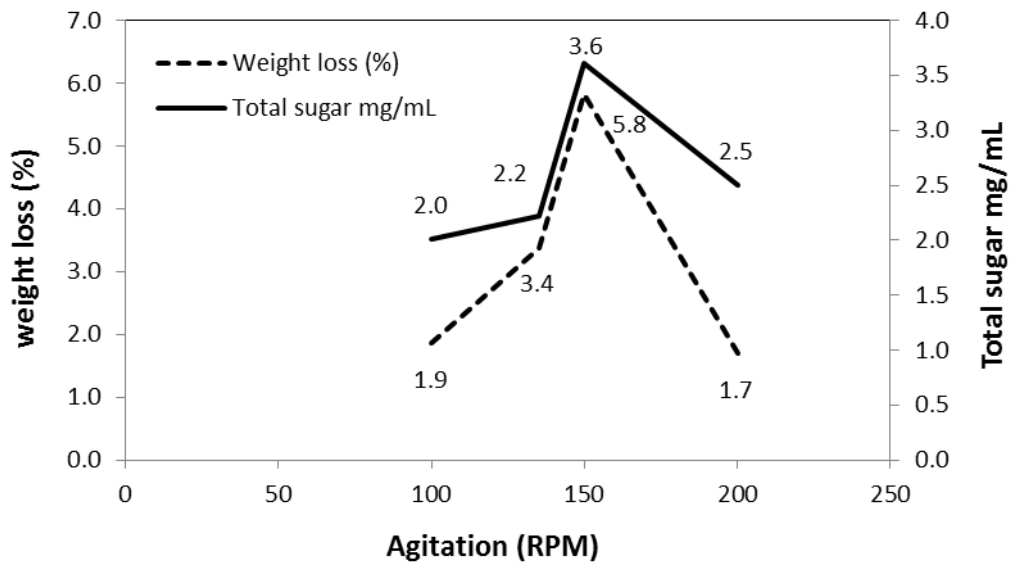


Figure 7: Effect of Agitation on enzymatic pretreatment of sawdust

Experimental effects of pH and temperature factors

Temperature and pH are two important factors that can affect the rate of enzymatic reactions. As high and low values could change protein structure and alter their ionization in solutions. The three-dimensional (3-D) response (Figure 8) and contour plots (Figure 9) show the effects of both temperature and pH on the weight loss (%) of substrate. The experimental and predicted weight loss (%) results presented as a mean of the triplicates in Table 2. The analysis of variance (ANOVA) of the FCCCD for the weight loss (%) are presented in Table 3. From the ANOVA tables, it was observed that the process temperature had a more significant effect on weight loss response, which was more demonstrated in the P values of 0.000 in linear terms when compared with pH which has P values of 0.477. The weight loss (WL) was represented by polynomial equation 5, where T is the temperature of the reaction.

$$\text{Weight loss} = -3.74612 + 0.14592 * \text{Temperature} + 3.13305 * \text{pH} - 3.96552\text{E-}003 * \text{Temperature}^2 - 0.3366 * \text{pH}^2 + 5.00000\text{E-}003 * \text{Temperature} * \text{pH} \quad (5)$$

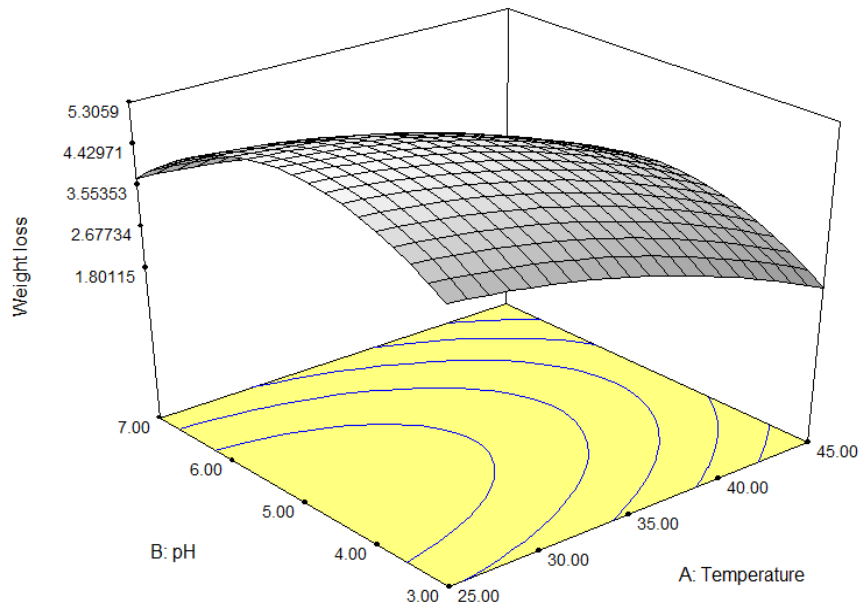


Figure 8: Three-dimensional response of the effects of temperature and pH on weight loss

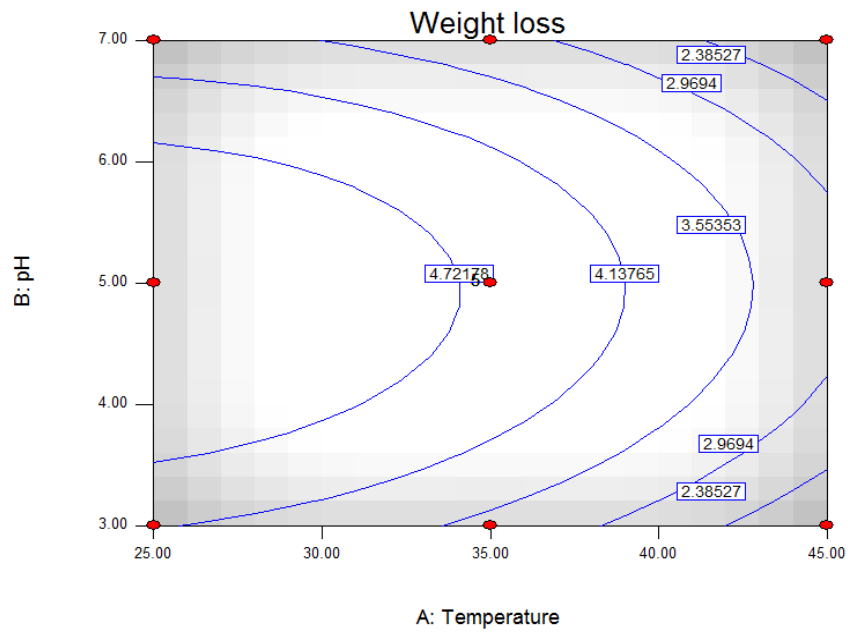


Figure 9: Contour plot response of the effects of temperature and pH on weight loss

Table 1: . The experimental and predicted weight loss (%) RSM optimization results

STD	Temperature	pH	Weight loss (%)	
			Practical	Predicted
1	25	3	4.4	4.2
2	45	3	1.6	1.8
3	25	7	3.8	3.7
4	45	7	1.4	1.8
5	25	5	5	5.3
6	45	5	3.8	3.2
7	35	3	3.4	3.4
8	35	7	3.5	3.2
9	35	5	4.7	4.6
10	35	5	4.6	4.6
11	35	5	4.3	4.6
12	35	5	4.5	4.6
13	35	5	4.7	4.6

Table 3: Analysis of variance (ANOVA) for weight Loss (%) response

Source	Sum of squares	DF	Mean square	F-Value	P-Value
Model	14.629	5	2.926	20.193	0.001
Temperature	6.827	1	6.827	47.115	0.000
pH	0.082	1	0.082	0.564	0.477
Temperature 2	0.434	1	0.434	2.998	0.127
pH2	5.008	1	5.008	34.563	0.001
Temperature* pH	0.040	1	0.040	0.276	0.616
Residual	1.014	7	0.145		
Lack of Fit	0.902	3	0.301	10.741	0.022
Pure Error	0.112	4	0.028		
Corrected Total	15.64	12			

Validation of the developed model

The developed pretreatment RSM optimized models were validated by suggesting five sets of experiments of the models. As shown in Table 4, the temperatures during the validation process were 37.29, 39.87, 36.78, 28.56 and 26.25°C; while the pH values were 3.55, 5.44, 6.49, 5.7, and 6.04. All the experiments were performed in triplicates, and the results were presented as the mean of the triplicates

Table 4: Validation of the developed model

Temperature	pH	Weight loss(%)	
		Predicted	Practical
37.29	3.55	3.7	3.2
39.87	5.44	3.9	4.1
36.78	6.49	3.6	3.7
28.56	5.7	4.9	4.5
26.25	6.04	4.8	4.7

CONCLUSION

The optimized process conditions for the pretreatment of sawdust with laccase enzyme were: laccase enzyme concentration of 20 IU/g of sawdust, substrate concentration of 5.0% (w/v), the temperature of 37 °C, pH 6, and time, 10 h. These conditions had a significant effect on the pretreatment process.

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