Statistical Modeling and Optimization of Enzymatic Pretreatment of Empty Fruit Bunches with Laccase Enzyme

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Laccase enzyme was used as a pretreatment agent to delignify empty fruit bunches (EFB) for sugar production. The degree of delignification of the biomass was assessed directly by the percentage of pre-pretreatment weight loss (%) after pretreatment and indirectly by the amount of total sugar produced after saccharification of the pretreated biomass with cellulase enzymes. Process parameters such as pretreatment time, temperature, enzyme concentration, substrate concentration, pH, and substrate size were studied using a one-factor-at-a-time (OFAT) analysis. The combined effect of temperature and pH on the pretreatment was studied using the face-centered central composite design (FCCCD) of response surface methodology (RSM). The optimized conditions for EFB pretreatment using laccase enzyme were achieved as follows: sample size, 2 mm; temperature, 25 °C; time, 4 h; substrate concentration, 5% (w/v); pH 5; and enzyme concentration, 20 IU/g of EFB. Although higher pretreatment was achieved with substrates of 1 mm size and at a temperature of 35 °C, these conditions were not considered energetically sustainable because of the need for energy during milling for sample size reduction and energy for temperature maintenance at 35 °C.

Keywords: Environment; Laccase enzyme; Pretreatment; Empty Fruit Bunch; Biofuel

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INTRODUCTION

Human activities, especially in the energy sector, have adversely affected the environment. The negative effects include the emission of greenhouse gasses that affect the integrity of our surroundings and adversely affect the quality of life. Presently, fossil fuel is the major source of energy for economic activities. Environmental contamination from oil and gas exploration activities are major concerns to environmental experts. Thus, it is necessary to pursue research for alternative energy sources and their conversion into suitable forms for transportation, storage, and use. Efforts are currently directed towards the development of environmentally sustainable energy sources. Green energy from bioethanol and biodiesel are the leading alternatives to fossil fuel. These fuels are largely produced from feedstocks after conventional pretreatment with chemical and physical agents for improved sugar production and a subsequent high yield of biofuel (Ukaegbu *et al*. 2014). The best established pretreatment methods using chemicals and energy are not considered environmentally friendly due to contribution to global warming through greenhouse gas emissions (Sun and Cheng 2002; Taherzadeh and Karimi 2008; Hendriks

and Zeeman 2009). However, effective pretreatment requires the disruption of cell wall integrity *via* the breakdown of lignocellulosic material for improved bioconversion using hydrolytic and fermentation agents. Good pretreatment process must also ensure low inhibitory products formation (Marièlle Bar 2001; Palamae *et al*. 2014) while posing little or no threat to the environment (Valery *et al*. 2011).

Addressing the challenges of the conventional pretreatment techniques requires research into ways of pretreatment that entirely eliminate the use of chemicals and are less dependent on energy as well (Saha and Cotta 2008; Pan and Saddler 2013). Given the knowledge of these problems and the need for environmental sustainability, this study was developed to provide an alternative pretreatment process in place of the conventional chemical and physical methods. 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 empty fruit bunches (EFB). EFB, obtained as a residue in the palm oil industry during the process of crude palm oil production, is abundant and readily available to source for biofuel production processes. Pretreatment of EFB with laccase enzyme was advocated in this study based on the role of laccase enzymes in phenolic compound oxidation (Archibald *et al*. 1997). Selective delignification, without threat to the environment, and the preservation of the sugar content of the biomass made the idea of enzymatic pretreatment interesting.

During the study, screening of various environmental and process factors that affect enzymatic activities such as time (duration) of pretreatment, the pH of the medium, the process temperature, the concentration of laccase enzyme in the reaction volume, and the size and concentration of the EFB were all studied using one-factor-at-a-time (OFAT) and response surface methodology (RSM). The rate of delignification was evaluated directly by the determination of the percentage of initial weight lost after pretreatment and also indirectly by determining the amount of total sugar produced after saccharification of the pretreated EFB with cellulase enzyme.

EXPERIMENTAL

Collection of EFB Biomass

EFB was collected from Dominion Square Sdn Bhd Oil Mill, Pahang, Malaysia. The sample was processed and milled to three different sizes in the laboratory located at the Faculty of Industrial Science and Technology (FIST), Universiti Malaysia Pahang, Malaysia.

Biomass Characterization

The cellulose, hemicellulose, lignin, and ash contents of EFB were estimated according to the method outlined by Datta (1981), with slight modifications as described below.

Hemicellulose content of EFB

A 1-g sample of dried EFB was suspended in 100 mL of 0.5 M H_2SO_4 in a conical flask and incubated for 2 h at 100 °C in a water bath. After 2 h, the content of the flask was filtered and dried in an oven at 90 °C until a constant weight was obtained. The loss in weight after drying was attributed to hemicellulose degradation.

Cellulose content of EFB

A total of 50 mL of 72% (v/v) H_2SO_4 was added to the dried residue from the hemicellulose treatment above in a conical flask and kept at 30 °C for 1 h on a rotary shaker at 200 revolutions per minute (rpm). After incubation, the mixture was diluted further with distilled water to 4% (v/v) H_2SO_4 and autoclaved at 1.06 kg/cm^2 for 40 min. The content of the flask was filtered and dried in a hot air oven at 90 °C to obtain a constant weight. The loss in weight after drying was attributed to cellulose degradation.

Lignin content of EFB

The remaining residue from the cellulose estimation was dried to a constant weight in an oven at 90 °C and then weighed. The weight balance was considered to be lignin content with ash.

Ash content of EFB

For estimating the residual ash content, 1 g of sample was kept at 550 °C for 5 h in a tare crucible, and after burning, the leftover ash was re-weighed to calculate the residual ash content

Determination of Total Carbohydrate Content of EFB

The total sugar analysis after saccharification of pretreated EFB was done using a modified phenol-sulfuric acid method described by Dubois *et al*. (1956). The reaction volume contained 100 µL of sample filtrates centrifuged at 5000 rpm for 5 min in a Hermle model Z206A centrifuge, 50 µL of 80% phenol, and 2 mL of 98% concentrated sulphuric acid. The reaction mixture was stood for 10 min at room temperature before reading of the absorbance of the developed color in a microplate reader, model Infinite pro 200 TECAN at 490 nm.

Determination of Laccase Enzyme Activity by ABTS Method

The laccase enzyme activity of Novozyme 51003 from *Myceliophthora thermophila* with pH stability range of 4 to 7 and redox potential of 0.5 to 0.8 V was determined using ABTS reduction method as described by Bourbonnais and Paice (1992). The reaction mixture contained 0.1 M sodium acetate buffer, pH 4.5 at 25 °C, and 0.4 mM ABTS solution 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 in a GENESYS™ 10S UV-Vis spectrophotometer for 5 consecutive minutes (Majcherczyk *et al*. 1998), and the readings expressed in International Units/mL (IU/mL). One unit of the enzyme was defined as the amount of the laccase enzyme that will oxidize 1 µmol of ABTS per minute. The temperature stability of the laccase enzyme was found to be within 25 ^oC and 40 ^oC.

Determination of Cellulase Enzyme Activity

Cellulase enzyme used in the study was purchased in powder form, and the activity was determined by using standard filter paper (1.0 x 6.0 cm) incubated with the enzyme at 50 ^oC for 1 h in a sodium acetate buffer of pH 5.0. The reducing sugar released by the enzyme was estimated using the DNS method of reducing sugar estimation. One unit of the enzyme was defined as the amount of enzyme that will liberate one micromole of

reducing sugar per minute, and the unit was expressed in International units (IU). The enzyme was reconstituted with deionized water to a concentration of 1 IU/µL before use.

Optimization of Pretreatment Parameters using OFAT

Six process parameters (time of pretreatment, medium pH, process temperature, concentration of the enzyme, biomass size, and concentration) were studied using OFAT. The degree of delignification was estimated directly by the percentage of the initial weight lost after pretreatment, and also indirectly by the saccharification of the pretreated biomass with cellulase enzyme at a concentration of 10 IU/g of EFB. The condition for the saccharification of the pretreated EFB was as follows: cellulase enzyme concentration 10 IU/g of EFB, EFB concentration 5 %(w/v), temperature 50 ^{o}C, pH 5, time 24 h, and agitation rate 150 rpm. The procedure for the study of the process parameters was as described below.

Laccase enzyme concentration

Five experiments were carried out in triplicates with laccase enzyme concentrations of 5, 10, 20, 30, and 40 IU/g of EFB, all with a substrate concentration of 5% (w/v). The pH and temperature of the reaction were maintained at 5 and 25 °C, respectively, while agitation was maintained at 150 rpm for a time of 4 h.

Substrate concentration

Four sets of experiment were carried out using substrate at varying concentrations of 5, 10, 15, and 20% (w/v) with a reduced laccase enzyme concentration of 5 IU/g of EFB. Laccase enzyme used at this stage of the study and subsequently was reduced to 5 IU/g to minimize enzyme consumption. The pH of the reaction and temperature were fixed at 5 and 25 °C, respectively, while the time and agitation were fixed at 4 h and 150 rpm, respectively.

Substrate (EFB) size

Three different EFB sizes of 1, 2, and 3 mm were studied for the effect of sample sizes on the pretreatment process. Reaction mixtures contained substrate concentration of 5% (w/v) and laccase enzyme concentration of 5 IU/g of EFB. The reaction was performed at 25 °C in an incubator shaker while the reaction pH was maintained at 5. The process was carried out for 4 h at an agitation rate of 150 rpm.

Time (duration) of pretreatment

The effect of time on the rate of EFB pretreatment with laccase enzyme was studied at three different periods of 2, 4, and 6 h. Reaction mixtures contained a substrate concentration of 5% (w/v) and a laccase enzyme concentration of 5 IU/g of EFB. The reactions were performed at 25 °C and at pH 5; agitation was maintained at 150 rpm.

Temperature of pretreatment

The effect of temperature on the pretreatment process was studied at 25 °C (room temperature (RT)), 35 °C, and 45 °C. Reactions contained a substrate concentration of 5% (w/v) and a laccase enzyme concentration of 5 IU/g of EFB. The pretreatment processes were maintained at pH 5 for a time of 4 h and agitation at 150 rpm.

pH of Medium

Six different sets of experiment were carried out at reaction pH of 2, 3, 4, 5, 6, and 7 to study the effect of medium pH on the EFB pretreatment process. The reaction mixtures contained a substrate concentration of 5% (w/v) and a laccase enzyme concentration of 5 IU/g of EFB. The pretreatment processes were performed for a time of 4 h at 25 °C and agitation at 150 rpm.

After studying the effect of the process parameters using OFAT, further study was carried out using FCCCD of the RSM to check the response of the pretreatment process due to the levels of interaction of pH and temperature.

Optimization of the EFB Pretreatment Process in FCCCD

The effect of the interaction of pH and temperature of the pretreatment process was studied in an experimental setup, which was designed with the Design-Expert software version 6.0.8. The Face Centered Central Composite Design (FCCCD) of the RSM was employed during the study design. The high and low points of pH and temperature were fixed at 3 and 7, and 25 °C and 45 °C, respectively, based on the nature of the effects of these parameters during OFAT studies. The reactions were performed in triplicates with a laccase enzyme concentration of 5 IU/g of EFB and a substrate concentration of 5% (w/v); all were incubated for a time of 4 h at the agitation of 150 rpm. Saccharification after pretreatment was performed with a cellulase enzyme concentration of 10 IU/g of EFB as described by Shah *et al.* (2016). Results were presented as the mean of the triplicates.

Validation of the Developed Model

After the optimization of the pretreatment process using RSM, the developed pretreatment models were validated by conducting five sets of experiments suggested by the models. The temperatures during the validation process were 44.67, 33.55, 35.00, and 26.00 °C; while the pH values were 4.22, 6.88, 5.00, 7.00, and 6.00. All the experiments were performed in triplicates, and the results were presented as the mean of the triplicates.

RESULTS AND DISCUSSION

Constituents of Characterized EFB

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, which intertwines with the sugar bases to provide strength to the biomass cell (Alvira *et al*. 2010). The results of this study were in agreement with the reports of Khalil *et al*. (2008) that characterized EFB and found that it contained more cellulose (49.6%) than hemicellulose (21.2%), with 18% lignin and 2% ash though it has been reported that biomass from different sources and environments differ in composition (Alvira *et al.* 2010).

Table 1. Constituents of Characterized EFB

EFB Condition	Cellulose (%)	Hemicellulose (%)	Lignin (%weight loss/g of EFB)	Ash (%)
Un-pretreated (control)	38.7	32.2	23.3	5.8
Pretreated at optimized condition	42.1	34.7	18.6	4.0
Percentage lignin loss			20.1	

n=3

OFAT Study of EFB Pretreatment Parameters

Effect of laccase enzyme concentration on the pretreatment of EFB

An increase in enzyme concentration increases the rate of enzymatic reactions (Choi *et al*. 2013). When the enzyme concentration of an enzymatic reaction is increased, the rate of reaction is rapid and increases proportionately with increases in enzyme concentration until a saturation point, when further increases in enzyme concentration have little or no effect on the rate of the reaction (Rashid 2011). The enzyme saturation is likely due to the lack of free substrate sites for the enzyme to bind with or may be as a result of product accumulation. The effect of laccase enzyme concentration during pretreatment of EFB was studied at five enzyme concentrations of 5, 10, 20, 30, and 40 IU/g of EFB. The results depicted in Fig. 1 revealed a steady increase in the rate of pretreatment when the enzyme concentration was increased from 5 IU/g to 10 IU/g of EFB, with sugar yield increasing from 2.41 mg/mL to 2.72 mg/mL, respectively. The increase in sugar yield was maintained until an enzyme saturation concentration of 20 IU/g of EFB was reached; at this point, a maximum sugar yield of 3.5 mg/mL was attained. Further increases in enzyme concentration to 30 IU/g and 40 IU/g of EFB had no appreciable influence on the rate of pretreatment. Weight loss after pretreatment also increased with increase in enzyme concentration until a saturation concentration when a further increase in enzyme concentration led to no appreciable loss in weight of the EFB. These findings agreed with the reports of Valls and Roncero (2009) where two enzyme concentrations were used to pretreat eucalyptus poplar, achieving 9% delignification with a laccase enzyme concentration of 0.4 IU/g of the substrate and 52.4% delignification with a laccase enzyme concentration of 10.5 IU/g of substrate.

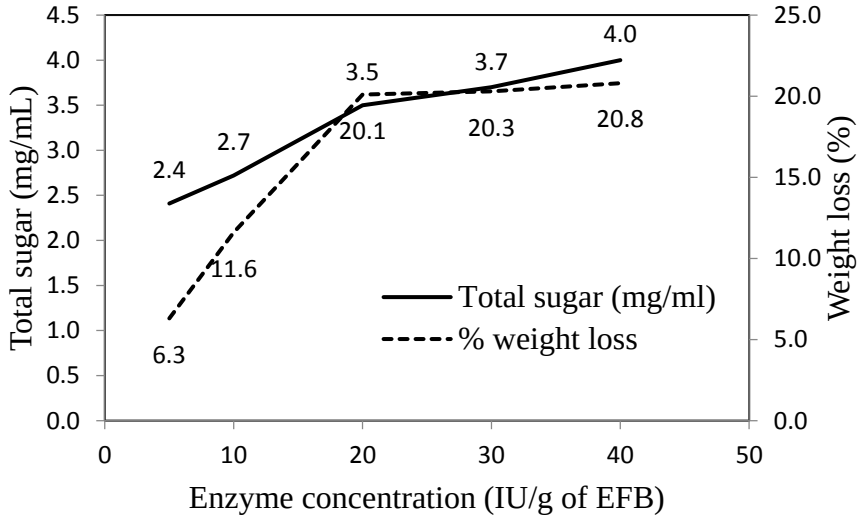

Fig. 1. Effect of laccase enzyme concentration on enzymatic pretreatment of EFB

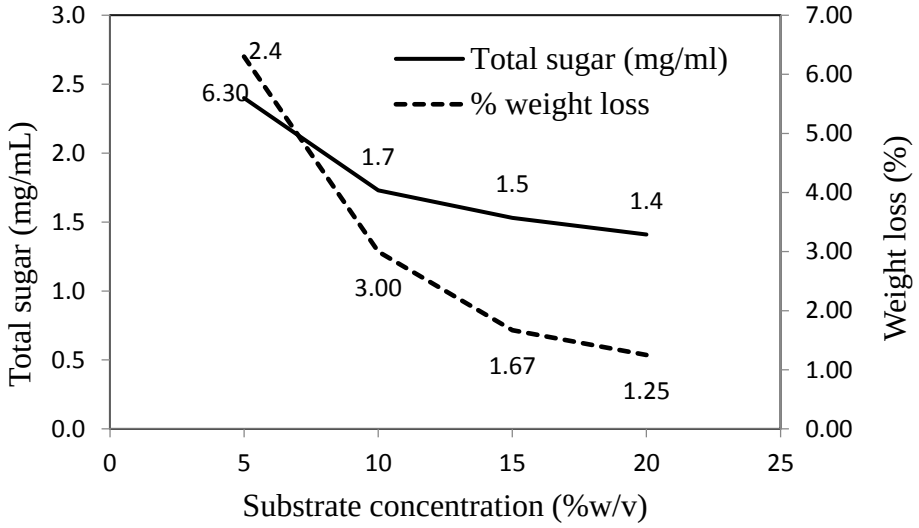

Fig. 2. Effect of substrate concentration on enzymatic pretreatment of EFB

Effect of substrate concentration on the enzymatic pretreatment of EFB

Substrate concentration affects the rate of enzymatic reactions because enzymatic activities are dependent on various factors, including the enzyme and substrate concentrations. However, at low substrate concentrations, the rate of reaction of enzymes increases proportionally with the enzyme due to the availability of enzyme active sites. But at high substrate concentrations, there is a maximum reaction rate (V_{max}), where no free active sites exist on the surface of the enzyme. V_{max} is directly related to the concentration of enzymes and its catalytic constant (Segel 1984). Accumulation of substrates at higher substrate concentration also leads to reduced action of enzymatic reactions because all enzyme sites are actively engaged with the substrate and there is no free enzyme to engage the introduced substrate.

In this study, pretreatment of EFB was performed using four different EFB substrate concentrations of 5, 10, 15, and 20% (w/v) and the same laccase enzyme concentration of 5 IU/g of EFB. Figure 2 reveals a steady decline in pretreatment performance as the substrate concentration was increased from 5% (w/v) to 20% (w/v). At a 5% (w/v) substrate concentration, total sugar produced after the saccharification of the pretreated EFB was 2.4 mg/mL. This was higher than the 1.73 mg/mL achieved when the concentration of substrate was increased to 10% (w/v). Further increase of the substrate concentration to 15 and 20% (w/v) witnessed a further decline in total sugar response (1.53 and 1.41 mg/mL), respectively. Weight loss after pretreatment also decreased consistently with an increase in substrate concentration. There is no current record of work done on the effect of substrate concentration on the rate of enzymatic pretreatment of biomass. Similar works on enzymatic saccharification of biomass reported by Han *et al*. (2012) showed that enzyme reactions are retarded when the concentration of substrate for an enzyme medium is exceeded, leading to a reduction in the product and accumulation of the substrate.

Effect of the substrate size on the enzymatic pretreatment of EFB

The rate of pretreatment of biomass is dependent on the size of the biomass used, as previously reported (Rashid 2011). In previous works, reported by Shah *et al.* (2011), different EFB sizes have been pretreated with NaOH. The results showed that samples with sizes of 0.5 mm yielded higher sugar after saccharification for 120 h, while samples with sizes of 1, 2, and 3 mm yielded lower sugar after saccharification for 120 h. In this study, pretreatment was performed with three different EFB sizes of 1, 2, and 3 mm (Fig. 3). At a lower substrate size of 1 mm, the rate of pretreatment was faster, which led to a higher total sugar production (2.74 mg/mL) after saccharification of the pretreated EFB. Substrate sizes of 2 and 3 mm showed reduced delignification compared with a sample size of 1 mm, since the total sugar produced after the saccharification, 1.61 and 1.15 mg/mL, respectively was lower than the amount of sugar produced after the saccharification of substrates of smaller size (1 mm). The same trend of responses was also observed in the weight lost after pretreatment. Lower sized biomass showed higher weight loss after pretreatment. In sum, the lower the substrate size, the higher the rate of pretreatment and subsequently, the higher the rate of sugar production, as previously noted (Shah *et al.* 2011). This higher sugar production was due to the reduction in crystallinity and the increase in surface area of the substrate, allowing for more enzyme to interact with the substrate from different contact surfaces.

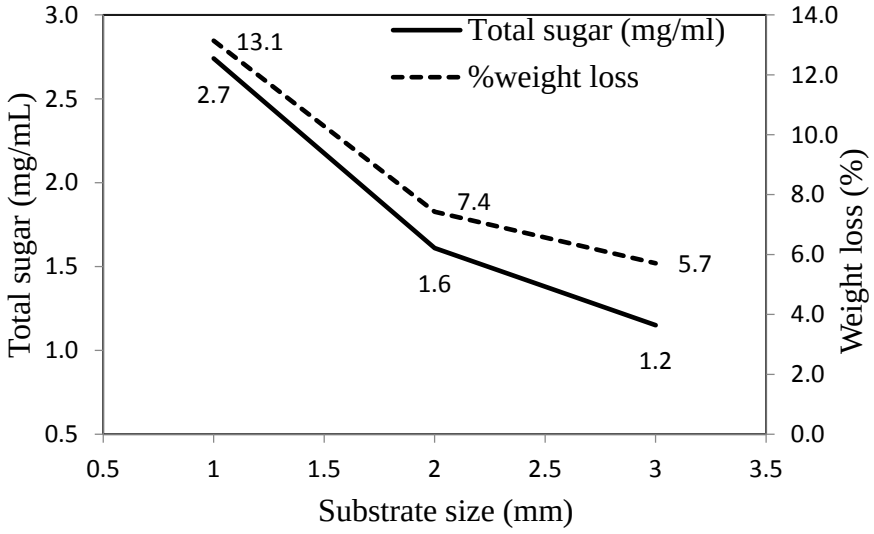

Fig. 3. Effect of substrate size on enzymatic pretreatment of EFB

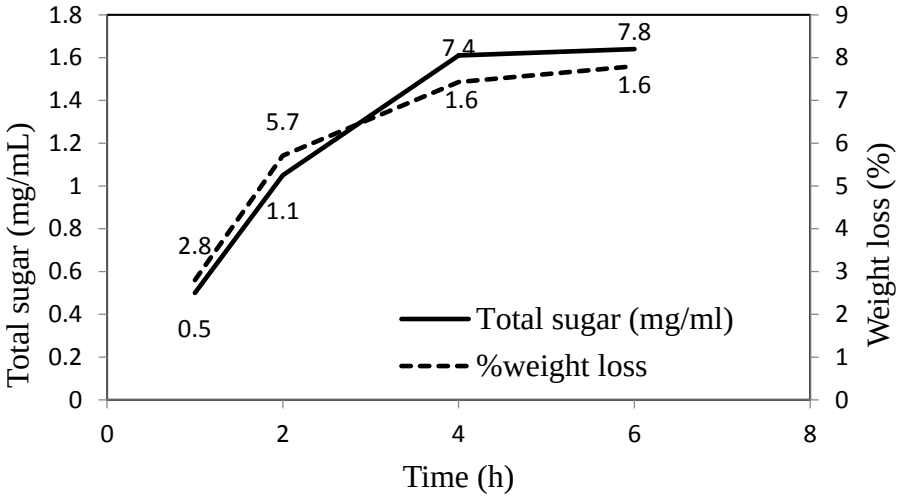

Fig. 4. Effect of time of pretreatment on enzymatic pretreatment of EFB

Effect of time on the enzymatic pretreatment of EFB

The effect of time was studied at three different periods of 2, 4, and 6 h. The rate of pretreatment tends to increase with time of contact between enzyme and substrate until the enzyme activity is exhausted. Figure 4 shows good progress in the pretreatment process between 2 h to 4 h, where the maximum rate was achieved. At 2 h, pretreatment was not completed, as the sugar released from the substrate (1.05 mg/mL) after saccharification was lower than the 1.61 mg/mL released after 4 h pretreatment. When prolonged to 6 h pretreatment, only a small difference was noted (1.64 mg/mL) because the enzyme activity was exhausted. The same trend of total sugar response was also observed in the weight lost after pretreatment, which showed a relationship between the rate of pretreatment and weight loss. The time of pretreatment had a positive effect on pretreatment when the enzymes were still in contact with the substrate; however, if all the enzymes have been utilized, prolonging the time of pretreatment makes no appreciable difference. Herpoël *et al*. (2002) used laccase enzyme to pretreat wheat straw for 4 and 6 h and recorded 47% and 64.6% delignification, respectively. Amin *et al*. (2010) also pretreated EFB with lignin peroxidase and manganese peroxidase and found maximum yields at 4 h and 3 h, respectively.

Effect of temperature on the enzymatic pretreatment of EFB

The effect of temperature on the pretreatment of EFB with the laccase enzyme was studied at 25 °C (RT), 35 °C, and 45 °C (Fig. 5). The temperature had a greater effect on the process. The thermal stability of the enzyme was between RT and 35 °C. Increasing the temperature to 45 °C resulted in a decline in the rate of the pretreatment process. This reduction was due to enzyme denaturation as the thermal tolerance limit of the enzyme was exceeded. Allowing enzymes at high temperature for a period of time changes the protein structure of the molecule and renders the enzyme inactive (Martinek 1969). After the saccharification of the pretreated EFB, 2.4 mg/mL and 2.9 mg/mL of total sugar were produced from EFB pretreated at RT and 35 °C, respectively. These results agreed with the findings of Amin *et al*. (2010), where EFB was pretreated with lignin peroxidase. Their

results demonstrated that the best delignification, approximately 70%, was achieved at room temperature. When EFB was pretreated at 45 °C, the sugar produced after saccharification had a concentration of 0.28 mg/mL, a result far lower than the responses of pretreatments conducted at RT and 35 °C. Weight loss after pretreatment was also decreased when the temperature was increased. Laccase enzymes from various sources have shown different tolerance to temperature, as shown in the findings of Woolridge, (2014). In that report, laccase enzyme from *Pycnoporus cinnabarinus* was used to pretreat wheat straw at 50 °C, and 35% delignification was obtained. However, Rico *et al*. (2014) pretreated eucalyptus feedstock with laccase enzyme from *T. versicolor* at 50 °C and obtained 18% delignification. Valls and Roncero (2009) also used laccase enzyme from *T. villosa* to pretreat eucalyptus kraft at 50 °C and obtained 41% delignification.

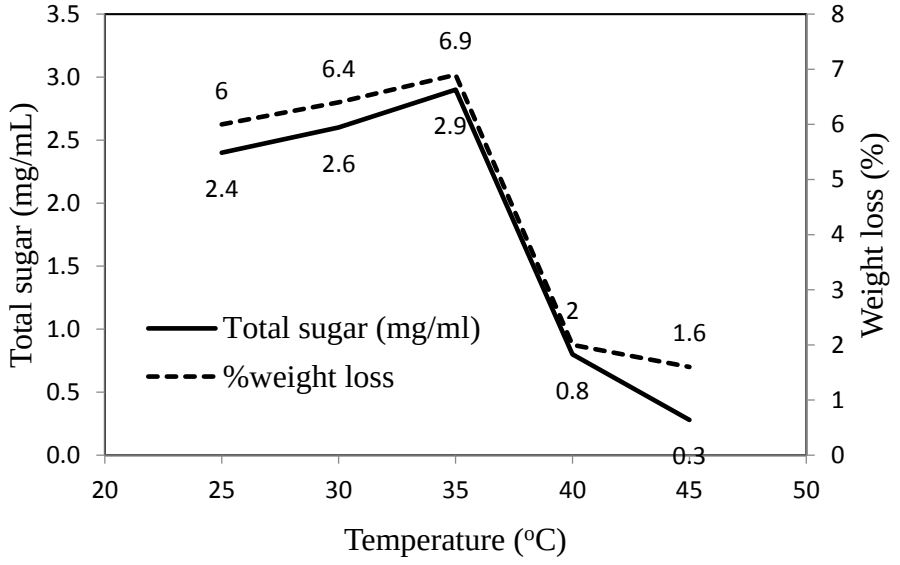

Fig. 5. Effect of temperature on the enzymatic pretreatment of EFB

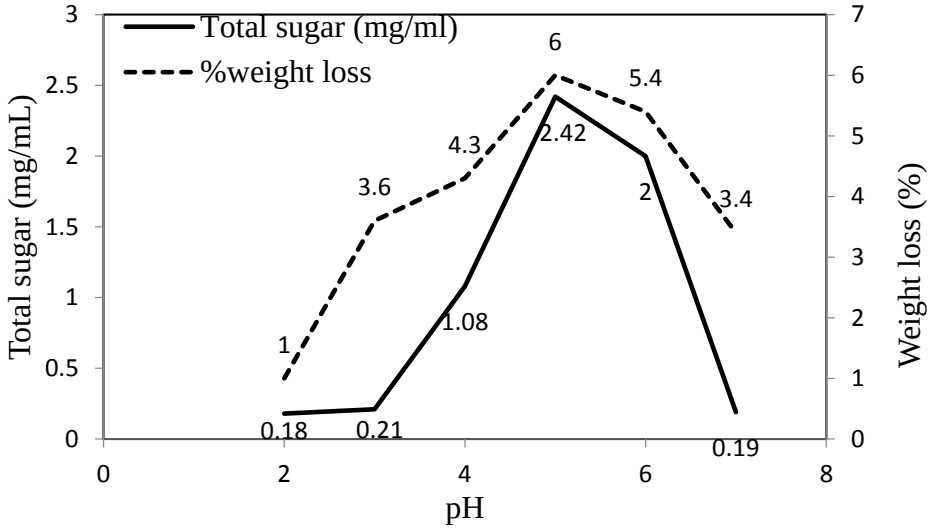

Fig. 6. Effect of pH on enzymatic pretreatment of EFB

Effect of pH on the enzymatic pretreatment of EFB

Enzymatic reactions are dependent on the pH of the reaction medium. Because all enzymes are protein, they are generally sensitive to the ionic concentration of the medium. All enzymes have a specific pH range at which they are most active due to the effect of pH on (i) the ionization of the substrate, (ii) the variation in the structure of the proteins, (iii) the binding of enzyme to substrate, and (iv) the catalytic activity of the enzyme. The pH is a measure of the acidity or alkalinity of a solution; as the pH changes from its optimum for an enzyme to the acidic range, the enzyme will tend to gain hydrogen ions. Similarly, when the pH of the medium tends to change to the alkaline, the enzyme looses hydrogen ions. Such changes, whether involving a gain or a loss of hydrogen ion, can lead to changes in the weak interactions that holds the shape of the enzyme molecule, resulting in denaturing the enzyme due to structural changes and a loss of the enzyme activity (Holum 1968).

The pretreatment of EFB with laccase enzyme was studied at pH 2, 3, 4, 5, 6, and 7 (Fig. 6). The results revealed a strong relationship between the rate of pretreatment and the pH of the medium. At pH 2, 3, and 4 which was highly acidic, the rate of pretreatment was slower; pH 5 was more favorable to the enzyme. At pH 7, enzyme activity was at the lowest, and this resulted in the little association of the enzyme with the substrate. The total sugar yields of 0.18, 0.21, 1.08, 2.42, 2.0, and 0.19 mg/mL were achieved after EFB pretreated at pH 2, 3, 4, 5, 6, and 7, respectively, was saccharified. The pH of the reaction medium has been studied by Bayındırlı (2010), who noted that all enzymes, depending on their group, have a specific pH range for optimum activity. Daas *et al.* (2016) studied the effects of pH, temperature, and some chemicals on polyphenol oxidase and peroxidase activity in harvested deglet, nour, and ghars dates. These authors concluded that at optimum temperatures and pH, peroxidase activity was high, but it decreased when both factors either increased or decreased.

Having studied the effect of the pretreatment process parameters using OFAT, further work was done using FCCCD of RSM to study the effect of the interaction of pH and temperature on the pretreatment process. Temperature and pH of the process were studied in RSM due to the pattern of their effects during the OFAT studies.

Experimental effects of factors (pH and temperature)

Enzyme-catalyzed reactions are dependent on the external environment, including the temperature and pH (Bayındırlı 2010) of the reaction. In this study, the effects of temperature and pH on the pretreatment of EFB using laccase enzyme were studied. The three-dimensional (3-D) response and contour plots showing the effects of both temperature and pH on the total sugar production after saccharification of the pretreated EFB and the weight loss after pretreatment are depicted in Figs. 7 and 8. Temperature and pH are two important factors that affect the rate of enzymatic reactions. Often, enzymes are active within a particular pH range, and their activities are influenced by the enzyme-substrate interaction, substrate ionization, and protein structure variation at extreme pH (Bayındırlı 2010). In the case of temperature, the activity of an enzyme can be influenced by temperature through thermal denaturation of the enzyme at high temperatures (Martinek 1969; Valls and Roncero 2009).

In this study, the effect of pH and temperature on the pretreatment of EFB was studied. The 3-D plots (Figs. 7(a) and 8(a)) with semi-spherical shapes displayed a trend of an increase in total sugar and weight loss with increasing temperature and pH. The increase was maintained until the levels when thermal denaturation and system isoelectric points were reached, and a decline in the responses was observed. The increasing trend was

expected because laccase enzyme activity is influenced by a temperature range of 25 to 50 °C and a pH range of 3 to 9. The total sugar production after saccharification increased from 0.12 mg/mL when the pretreatment was performed at 45 °C and pH 7 to a maximum of 2.9 mg/mL when pretreatment was performed at 35 °C and pH 5. This result showed that laccase enzymes can be denatured at a temperature that is higher than 45 °C. However, the observation from this study was consistent with the findings of Woolridge (2014), who used a laccase enzyme from *Pycnoporus cinnabarinus* to pretreat wheat straw at 50 °C and obtained 35% delignification. Also, Rico *et al.* (2014) pretreated eucalyptus feedstock with laccase enzyme from *Trametes versicolor* at 50 °C and obtained 18% delignification. However, Valls and Roncero (2009) used laccase enzyme from *T. villosa* to pretreat eucalyptus kraft at 50 °C and obtained 41% delignification. This showed that laccase enzymes from different sources have different tolerance to pH and temperature probably due to the type of ionization that exists within the proteins that made up the enzyme structure.

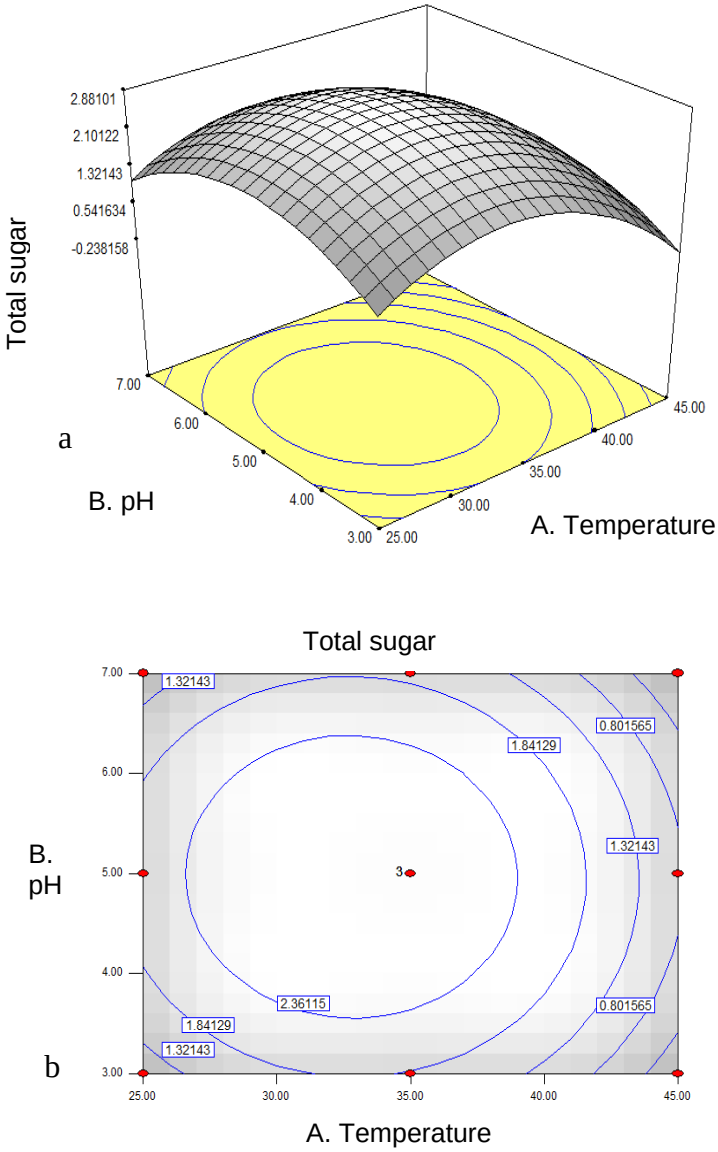

Fig. 7. The a) three-dimensional response and b) contour plot of the effects of temperature and pH on total sugar production

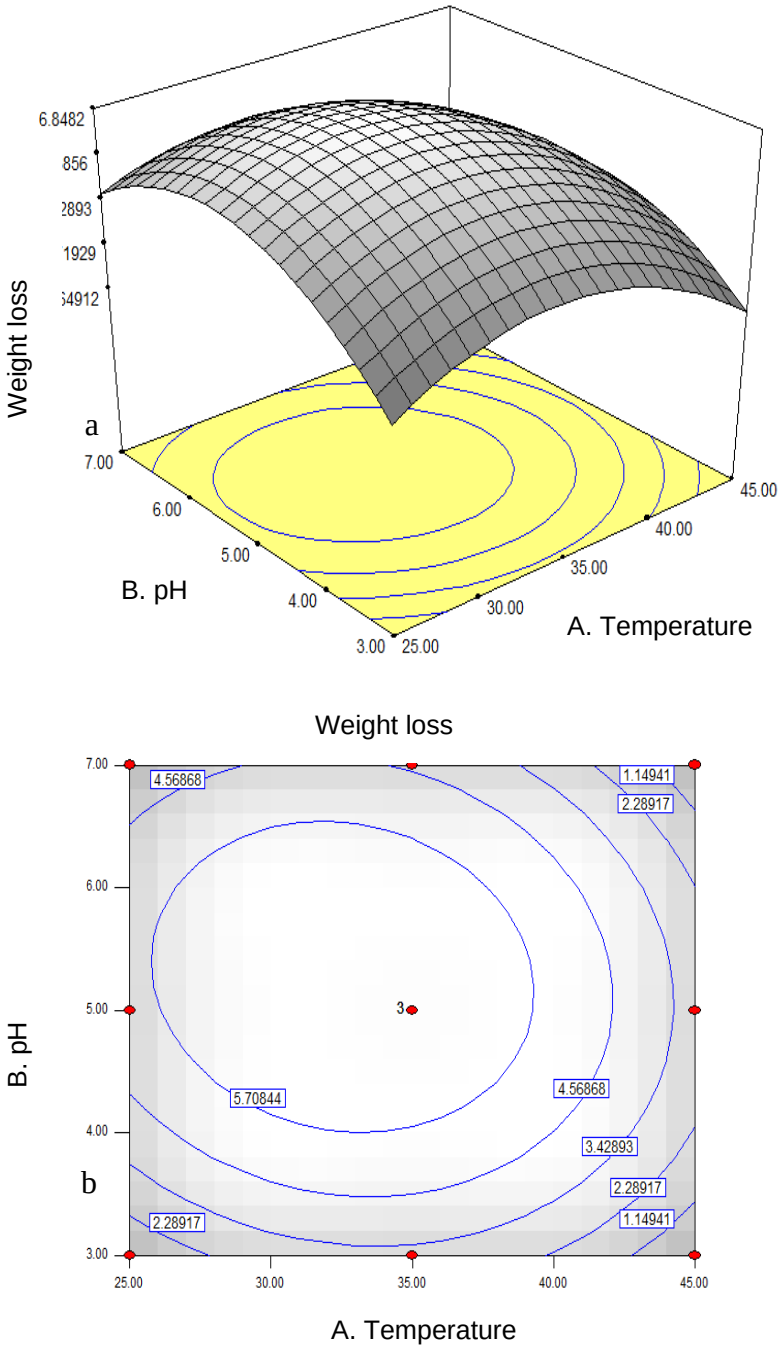

Fig. 8. The a) three-dimensional response and b) contour plot of the effects of temperature and pH on weight loss after pretreatment

The contour plots in Figs. 7(b) and 8(b) showed the effects of the interaction of temperature and pH on the rate of pretreatment. Temperature and pH interacted around the central points in an almost similar distribution, showing that the process was optimized at the central points, although the effect of temperature was more pronounced compared with the effect of pH. The experimental and predicted total sugar (mg/mL) yield and weight loss (%) tabulated as a mean of the triplicates are presented in Table 2. The analysis of variance (ANOVA) of the FCCCD for the total sugar and weight loss (%) are presented in Tables 3 and 4. From the ANOVA tables, it was observed that the temperature of the process had a more significant effect on total sugar and weight loss responses, which was more substantiated in the *P* values of 0.0159 and 0.025 in linear terms when compared with pH which has *P* values of 0.8194 and 0.1386. The total sugar response (TSR) and weight loss (WL) are represented by polynomial equations, where *T* is the temperature of the reaction.

$$TSR = -21.26235 + 0.60752 * T + 4.93599 * pH - 8.51172 * T^2 - 0.42494 * pH^2 - 0.015631 * TpH \quad (1)$$

$$WL = -28.630702 + 1.268991 * T + 6.239254 * pH0.020474 * T^2 - 0.599342 * pH^2 - 0.002500 * TpH \quad (2)$$

Based on the model *P*-values of 0.0053 and 0.0048, the model parameters obtained from RSM optimization using FCCCD were significant ($P<0.05$). The coefficient of determination (R^2) value of 0.935 and 0.938 for total sugar and weight loss obtained from the analysis of variance (ANOVA) showed a strong correlation between the factors (temperature and pH) and the responses (total sugar yield and weight loss) as shown in Table 5.

Table 2. Experimental Design using FCCCD showing Experimental and Predicted Responses

Standard	Temperature (°C)	pH	Total Sugar (mg/mL)			Weight Loss (%)		
			Experimental	Predicted	Error (%)	Experimental	Predicted	Error (%)
3	25	7	0.90	0.48	46.6	4.20	4.17	0.71
2	45	3	0.16	0.16	0.0	0.60	0.00	100
6	45	5	0.27	0.98	-262.9	1.60	2.66	-66.25
4	45	7	0.12	0.29	-75.0	1.00	0.53	47.0
10	35	5	2.90	2.88	0.68	6.90	6.48	6.08
1	25	3	0.80	0.59	26.25	3.60	3.43	4.7
9	35	5	2.91	2.88	1.03	6.90	6.48	6.08
7	35	3	1.80	1.73	3.88	3.00	3.00	0.0
11	35	5	2.90	2.88	0.68	6.90	6.90	0.0
5	25	5	2.42	1.74	28.09	6.00	6.00	0.0
8	35	7	1.50	1.61	-7.33	3.90	3.90	0.0

Note: n=3

Table 3. Analysis of Variance (ANOVA) for Total Sugar

Source	Sum of Squares	DF	Mean Square	*F*-value	*P*-value
Model	12.0481	5	2.4096	14.5412	0.0053
Temperature	2.1242	1	2.1242	12.8184	0.0159
pH	0.0096	1	0.0096	0.0579	0.8194
Temperature^2	4.5793	1	4.5793	27.6342	0.0033
pH^2	2.7373	1	2.7373	16.5184	0.0097
Temperature*pH	0.0049	1	0.0049	0.0296	0.8702
Residual	0.8286	5	0.1657		
Lack of Fit	0.8286	3	0.2762		
Pure Error	0.0000	2	0.0000		
Corrected Total	12.8767	10			

Table 4. Analysis of Variance (ANOVA) for Weight Loss (%)

Source	Sum of Squares	DF	Mean Square	*F*-value	*P*-value
Model	62.2543	5	12.4509	15.2302	0.0048
Temperature	8.1667	1	8.1667	9.9897	0.0251
pH	2.5350	1	2.5350	3.1009	0.1386
Temperature^2	16.1011	1	16.1011	19.6954	0.0068
pH^2	20.8821	1	20.8821	25.5436	0.0039
Temperature*pH	1.2100	1	1.2100	1.4801	0.2780
Residual	4.0875	5	0.8175		
Lack of Fit	4.0875	3	1.3625		
Pure Error	0.0000	2	0.0000		
Corrected Total	66.3418	10			

Table 5. Analysis of Variance Parameters of the Models Fitted for Total Sugar and Weight Loss

Term	Total Sugar	Weight Loss
F-value	14.5412	15.2302
P-value	0.0053	0.0048
Mean	1.5200	3.7300
R^2	0.9350	0.9380

Table 6. Validation of the Developed Model

No.	Temperature (°C)	pH	Total Sugar (mg/mL)			Weight Loss (%)		
			Experimental	Predicted	Error (%)	Experimental	Predicted	Error (%)
1	44.67	4.22	0.26	0.32	-23.08	1.58	1.62	-2.53
2	33.55	6.88	1.40	1.50	-7.14	3.70	3.10	16.22
3	35.00	5.00	2.90	2.88	0.69	6.90	6.90	0.00
4	35.00	7.00	1.50	1.61	-7.33	3.90	3.90	0.00
5	26.00	6.00	2.00	1.78	11.00	5.60	6.30	-12.50

Note: n=3

The parity plots of the experimental and predicted values of total sugar and weight loss are shown in Figs. 9 (a) and (b). It can be concluded from the plots that the model achieved a good predictability of the total sugar and weight loss, which are substantiated by the R^2 values of 0.906 and 0.965 obtained from the total sugar and weight loss plots, respectively.

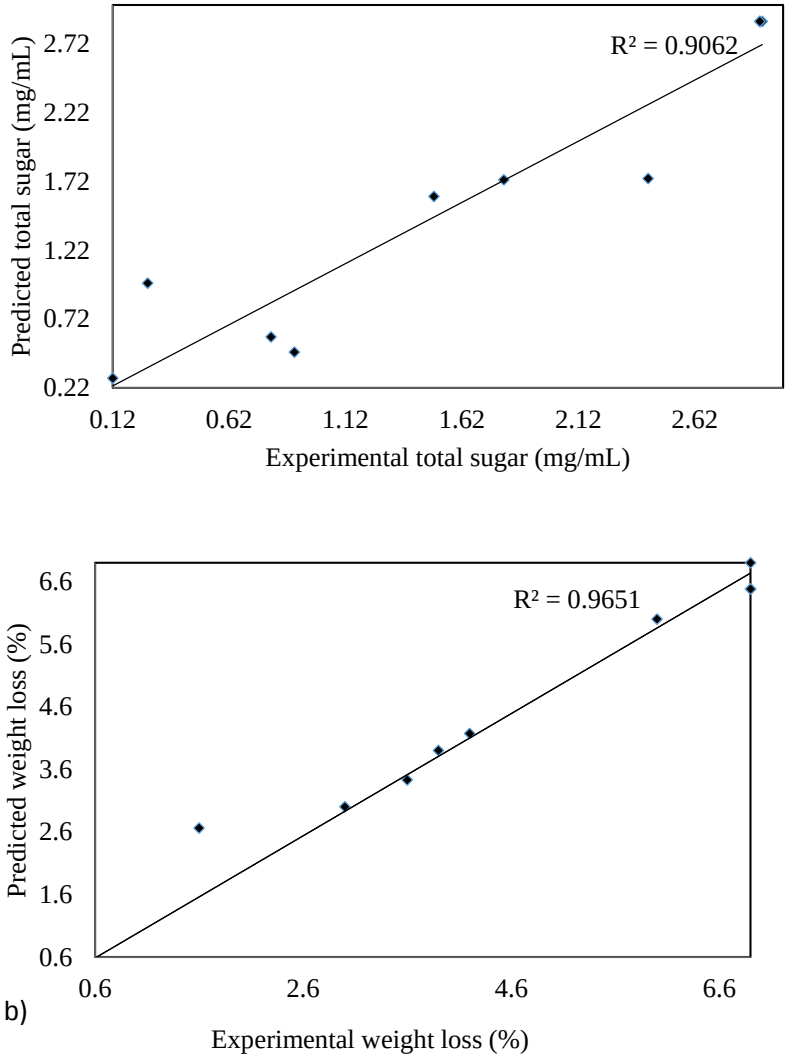

Fig. 9. Parity plot of the experimental and predicted values for the effects of temperature and pH on a) total sugar production and b) weight loss

Validation of the Developed Models

Five sets of experiment were carried out using the solutions suggested from the model to validate the predictability of total sugar production and weight loss using the developed model. The experimental and predicted responses, as well as the percentage errors of the five selected solutions, are presented in Table 6. Mean errors between the experimental and predicted yield of total sugar production and weight loss after pretreatment were 9.8% and 6.25%, respectively. Thus, the FCCCD model was statistically reliable up to 90.2% confidence for the prediction of the total sugar that will be produced

after pretreatment and 93.75% statistically reliable for the prediction of the weight loss after pretreatment with laccase enzyme.

CONCLUSIONS

1. The optimized process conditions for the pretreatment of EFB with laccase enzyme were: laccase enzyme concentration of 20 IU/g of EFB, substrate concentration of 5.0% (w/v), the temperature of 25 °C, pH 5, and time, 4 h; which yield a total sugar concentration of 3.5 mg/mL. These conditions had a significant effect on the pretreatment process.
2. The optimum conditions achieved here support previous studies on the use of ligninolytic enzyme approach towards pretreatment of biomass at room temperature because the laccase enzyme was relatively active at RT.
3. The temperature of the pretreatment process must be considered for successful pretreatment of EFB with laccase enzyme.

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