Optimization of Laccase Enzyme Pretreatment Process Parameters of Empty Fruit Bunches (EFB) Using One-Factor-At-a-Time (OFAT)

Ukaegbu Chinonso Ishmael
Faculty of Industrial Science and Technology, University Malaysia Pahang
Lebuhraya Tun Razak, 26300, Gambang, Kuantan Pahang Darul Makmur
chinoreal1456@yahoo.com

Shah Samiur Rashid
Faculty of Industrial Science and Technology, University Malaysia Pahang
Lebuhraya Tun Razak, 26300, Gambang, Kuantan Pahang Darul Makmur
tsamiur@yahoo.com

Nik Sharifah Suhaiza Binti Nik Wan Ahmad
Faculty of Industrial Science and Technology, University Malaysia Pahang
Lebuhraya Tun Razak, 26300, Gambang, Kuantan Pahang Darul Makmur
ns_suhaiza@yahoo.com

Abstract— Global warming due to increasing in carbon dioxide emission worldwide is generating much concern recently. The unsustainability of mans' activity in the generation of energy is a major cause of these changes in the environment. Production of energy carriers from petroleum and the fossil is a major contributor to challenges currently experienced in the world today. Sustainable biofuel from lignocellulosic biomass ensures sustainability of all processes of production. However, alternative sources of energy have been developed and green energy from lignocellulose biomaterials proved to be the ultimate alternative. Conversely, production of biofuels such as bioethanol from biomass needs pretreatment of the biomass for efficient saccharification of the sugar content of the biomass. Pretreatment has been conventionally done with chemicals such as sulphuric acid or sodium hydroxide but a critical review of these pretreatment process showed that the problem of greenhouse gas emission and generation of inhibitory substances such as furfural and hydroxyl methyl furfural still exists. In other to produce biofuels in a sustainable and environmental friendly way, this study adopted laccase enzyme for the pretreatment of empty fruit bunches (EFB). The degree of delignification after pretreatment of the EFB was assessed directly by the percentage of the pre-pretreatment weight lost after pretreatment, and also indirectly through the saccharification of the pretreated biomass with cellulase enzyme to check the quantity of sugar produced by altering pretreatment process parameters. Pretreatment parameters such as pretreatment time, temperature, enzyme and substrate concentrations, pH, and substrate size were studied using OFAT design. Results showed sample size of 2 mm, temperature at 35 ^{o}C, time of 4 h, substrate concentration of 5 % (w/v), pH 5 and enzyme concentration of 20 IU/g of EFB as optimized pretreatment process conditions. Room temperature (25 ± 3 °C) could also be used during the pretreatment process based on the fact that similar results were obtained when pretreatment was done at room temperature and 35 ^{o}C.

Keywords— *Laccase enzyme, Pretreatment, Total sugar, EFB*.

1. INTRODUCTION

Generation of energy from fossil and petroleum sources has adversely affected the environment due to pollution and greenhouse gas emission that occurs at alarming rates. Energy from fossil fuel is the major energy carrier for most economic activities currently. Environmental pollution arising from oil and gas exploration is a major concern to environmental experts. This necessitated research into alternative energy carriers and the availability of its raw materials. Efforts are currently ongoing towards the development of pretreatment process that is not only feasible but also environmentally sustainable during biofuel

production process. Green energy carriers such as bioethanol from lignocellulose materials and biodiesel from oily materials are the major sources of high prospects as alternative energy carriers. Bioethanol is majorly produced from feed stocks after pretreatment with chemical and physical agents to enhances sugar production and subsequent high yield of bioethanol while biodiesel has been produced through esterification of lipids and other oil materials [1]. Conventional pretreatment methods are considered non-environmentally sustainable owing to their contribution to the building up of greenhouse gasses that contributes to global warming and also the low fermentation rate of the sugar produced from these processes [2];[3];[4].

Good pretreatment process ensures that cell wall of lignocellulose materials is adequately altered for improved saccharification using hydrolytic enzymes. Pretreatment must also ensure low inhibitory products formation [5];[6], and contributes little or no threat to the environment [7]. Research into other ways of pretreatment needs to be carried out to address the challenges of the conventional pretreatment methods. Alternative pretreatment methods must also avoid the use of chemicals, and be less dependent on energy [8];[9]. Having identified the problems and the need for addressing them, this study was developed to provide an alternative biomass pretreatment strategy for the production of simple sugar from biomass for bioethanol production. The main aim of this paper is to provide a fast, achievable, and environmentally sustainable EFB pretreatment process which is dependable enough for industrial application.

Pretreatment of EFB with laccase enzyme was modeled and used in this study due to the knowledge of the role of laccase enzymes in phenolic compounds oxidation [10]. Selective attacks on lignin and preservation of cellulose and hemicellulose content of EFB made the idea of enzymatic pretreatment interesting. Effect of environmental and experimental conditions which alters enzymatic activities was studied using OFAT. Some of the factors studied during the pretreatment process are; time of pretreatment, pH of pretreatment buffer, the temperature of the pretreatment environment, the concentration of the laccase enzyme, and concentration and size of the EFB in the pretreatment medium [1]. The degree of delignification of the EFB was assessed directly by the percentage of the pre-pretreatment weight lost after pretreatment, and also indirectly through the saccharification of the pretreated biomass with cellulase enzyme to check the quantity of sugar produced after various pretreatment process parameter combinations.

2. MATERIALS

EFB Collection

About 20 kg of EFB was collected from Dominion Square Sdn Bhd Oil Mill, Gambang Kuantan, Malaysia and washed with water several times to remove mud, dust, and other substances, then dried in an electric oven at 80 ^{o}C for 48 h to a constant dry weight. The dried EFB fibers were manually cut to approximated uniform size of 2 cm before milling and separating to various sizes of 1, 2 and 3 mm using a sieve shaker and sieves with various gauges.

Chemicals and Reagents

All chemicals used in this study were purchased from Sigma-Aldrich (USA), Novozymes (Denmark), Nacalai tesque (Japan), Bendosen (Malaysia), HMBG (Germany) and Qrec (Malaysia).

Equipment and Instruments

Equipment and instruments used included incubator shaker, pH meter, magnetic stirrer, microplate reader, hot air oven, incubators, water bath, fume hood, measuring balance, desiccators, micropipettes, and laminar air flow cabinet.

3. EXPERIMENTAL METHODS

Determination of Total Carbohydrate Content of EFB.

The determination of total sugar was carried out using a modified phenol-sulfuric acid method of Dubois *et al.* (1956). A total of 100 µL of sample centrifuged at 5000 revolutions per minute (rpm) was added to glass test tubes, followed by the addition of 50 µL of 80 % phenol solution. The tube was vortexed for 30 s and 2 mL of 98 % concentrated sulphuric acid was added in a stream and the tubes were further vortexed for 30 seconds. The tubes were allowed to stand for 10 minutes at room temperature and after 10 min; the absorbance was read at 490 nm using a microplate reader model Infinite pro-TECAN.

Determination of Laccase Enzyme Activity by ABTS Method.

The laccase enzyme activity of the Novozym 51003 solution was determined using 0.4 mM 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) method in a 0.1 M sodium acetate buffer pH 4.5. The ABTS enzyme assay method was as described by [5]. ABTS is a non-phenolic dye which is oxidized by laccase to a cation radical that is more stable and preferred state of the compound. The intensity of the developed blue color of the cation radical is related to the concentration

of the cationic radical formed by the laccase, and co-related to the enzyme activity [11] and expressed in international units (IU). The blue color develops within minutes and keeps forming as long as the enzyme remains active. Absorbance was monitored at 420 nm while the reaction was maintained at 25 ^{o}C. One unit of enzyme was defined as the amount of the laccase that oxidized 1 μmol of ABTS substrate per minute.

Optimization of Enzymatic Pretreatment Process of EFB Using OFAT

Effect of Laccase Enzyme Concentration on the Pretreatment of EFB

Five (5) experiments were done in triplicates with enzyme concentrations of 5, 10, 20, 30 and 40 IU/g of EFB and a substrate concentration of 5 % (w/v). The pH and temperature of the process were maintained at 5 and 35 ^{o}C, respectively. Citrate-phosphate buffer was used at the molarities of 0.3 M / 0.4 M citrate to phosphate, respectively. Agitation was maintained at 150 rpm. After 4 h of pretreatment, saccharification of the pretreated EFB was done with cellulase enzyme at the concentration of 10 IU/g of EFB to correlate sugar release with the rate of delignification.

Effect of Substrate Concentration on the Pretreatment of EFB

Here, four (4) sets of experiments were carried out using four different substrate concentrations of 5, 10, 15, and 20 % (w/v) at a reduced laccase enzyme concentration of 5 IU/g of EFB. The enzyme concentration was reduced to minimize much consumption of laccase enzyme during the study. Other process conditions were same as described during enzyme concentration study, but the substrate concentration was varied.

***Effect of Substrate Size on the Pretreatment of EF*B**

At this stage, a substrate concentration of 5 IU/g of EFB was used and three (3) substrate sizes 1, 2, and 3 mm were used to check the effect of the size of the biomass on its delignification. Other process parameters were the same as described during the study of the effect of enzyme concentration, but the sizes of the EFB in the different flasks were varied.

Effect of Time on the Pretreatment of EFB

Time of the pretreatment process was also studied to check how long it takes to achieve maximum delignification of the biomass. Effect of time was studied at three different periods of 2, 4 and 6 h. The reaction mixture was made up as already discussed during the study of the effects of enzyme concentration except that the time of pretreatment was varied.

Effect of Temperature on the Pretreatment of EFB

The effect of temperature of the reaction on the rate of delignification of EFB was studied at three (3) different temperatures- 25 ± 3 ^{o}C (room temperature (RT)), 35 ^{o}C and 45 ^{o}C. The same reaction conditions were maintained as described above only that the temperatures of the process were varied.

Effect of Buffer pH on the Pretreatment of EFB

Six (6) experiments were carried out at 6 different citrate-phosphate buffer pH of 2, 3, 4, 5, 6 and 7 to check the effect of pH on the rate of EFB delignification. The reaction was performed under the same conditions as when studying the effect of enzyme concentration only that the pH of the different combinations were varied.

Validation of optimum pretreatment condition

After the screening of the various pretreatment process parameters using OFAT, an experiment was performed using the optimum conditions, comprising of Laccase enzyme concentration 20 IU/g, EFB concentration of 5 % (w/v), temperature of 35 ^{o}C, time of 4 h, pH of 5 and agitation of 150 rpm. The responses of total sugar (mg/mL) and weight loss (%) were determined and compared with the results of the previous studies.

4. RESULTS AND DISCUSSION

The results of the OFAT studies of the process parameters for the pretreatment of EFB with laccase enzyme are discussed in the subsections underneath.

Effect of Enzyme Concentration on the Pretreatment of EFB

The increase in enzyme concentration increases the rate of enzymatic reactions [12]. When the enzyme concentration of an enzymatic reaction is increased, the rate of reaction is rapid and increases proportionately with the increases in enzyme concentration until a saturation point, when further increases in the enzyme concentration have little or no effect on the rate of the reaction [13]. 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 delignification 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 the enzyme concentration. These findings agreed with the reports of Valls and Roncero *et al*.[14] when 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.

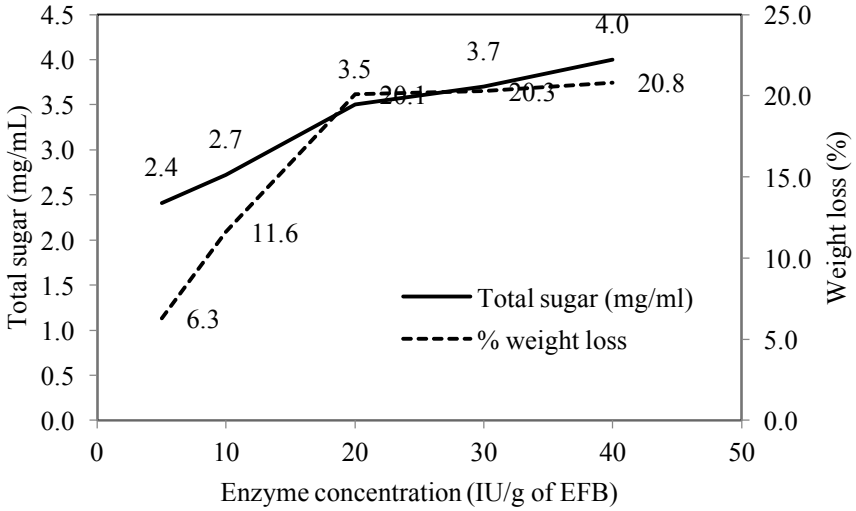

Figure 1*:* Effect of laccase enzyme concentration on the pretreatment of EFB

Effect of Substrate Concentration on the 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 [15]. 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 enzyme 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. Accumulation of substrates at higher concentration also leads to reduced action of enzymatic reactions because all enzyme sites were actively engaged with the substrate and there was no free enzyme to engage the introduced substrate. In this study, pretreatment was performed at four different EFB concentrations of 5, 10, 15, and 20 % (w/v) and the same laccase enzyme concentration of 5 IU/g of EFB. The result as shown in Fig. 2 reveals a sharp 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 saccharification was 2.4 mg/mL. It was higher than the 1.73 mg/mL achieved when the concentration of the 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.*[16] showed that enzyme reactions are retarded when the concentration of the substrate for an enzyme medium is exceeded, leading to a reduction in the product and accumulation of the substrate.

Effect of Substrate Size on the Pretreatment of EFB

The rate of delignification was dependent on the size of the substrate used, as previously reported Rashid [13]. In previous works, different EFB sizes were pretreated with chemicals (NaOH). The results showed that samples with sizes of 0.5 mm yielded higher sugar after saccharification for 120h, while samples with sizes of 1, 2, and 3 mm yielded lower sugar after

saccharification for 120 h [17]. 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, delignification was higher, which led to a higher total sugar production (2.74 mg/mL) after saccharification. Substrate sizes of 2 and 3 mm showed reduced pretreatment compared with a sample size of 1 mm, as the total sugar produced after their saccharification was 1.61 and 1.15 mg/mL, respectively. The same trend was also observed in the weight loss after pretreatment. Lower sized biomass showed higher weight loss after pretreatment. In sum, the lower the substrate size, the higher the degree of delignification and subsequently, the higher the rate of sugar production, as previously noted [17]. This higher sugar production was due to the reduction in crystallinity and the increase in surface area of the substrate, allowing for more enzyme contact.

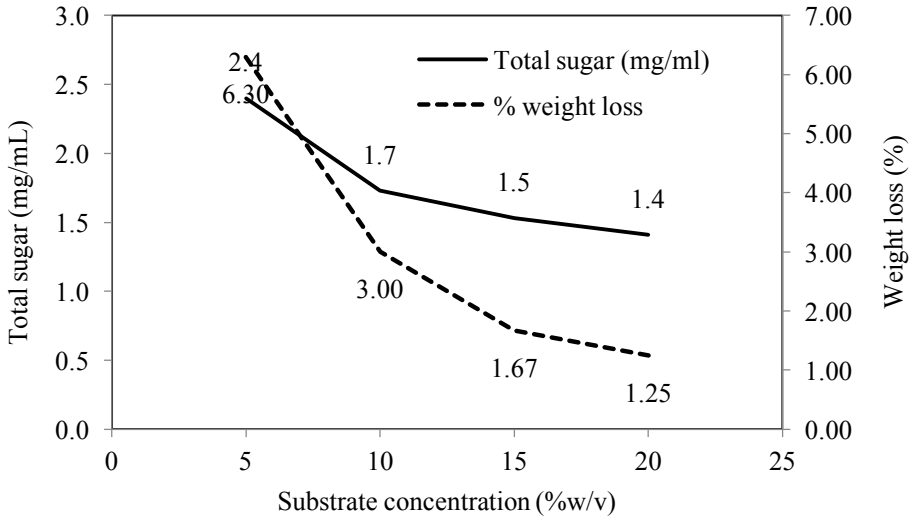

Figure 2: Effect of substrate concentration on enzymatic pretreatment of EFB

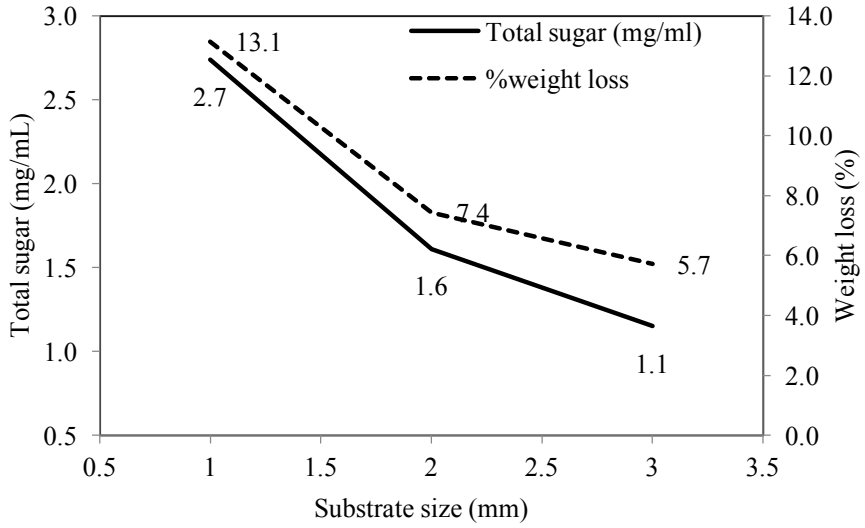

Figure 3: Effect of substrate size on enzymatic pretreatment of EFB

Effects of Pretreatment Time on the Pretreatment of EFB

The effect of time was studied at three different times of 2, 4, and 6 h. Delignification tends to increase with time of contact between enzyme and substrate until the enzyme activity is exhausted. Fig. 4 shows a 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 the1.61 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 loss after pretreatment, which showed a relationship between the rate of pretreatment and weight loss [15]. The time of pretreatment had a positive effect on delignification when

enzymes were still in contact with the substrate; if all enzymes have been utilized; prolonging the time of pretreatment makes no appreciable difference. Valls and Roncero [14] used laccase enzyme to pretreat wheat straw for 4 and 6 h and recorded 47 and 64.6 % pretreatment, respectively. Amin *et al.*[18] pretreated EFB with lignin peroxidase and manganese peroxidase and found maximum yields at 4 h and 3 h, respectively.

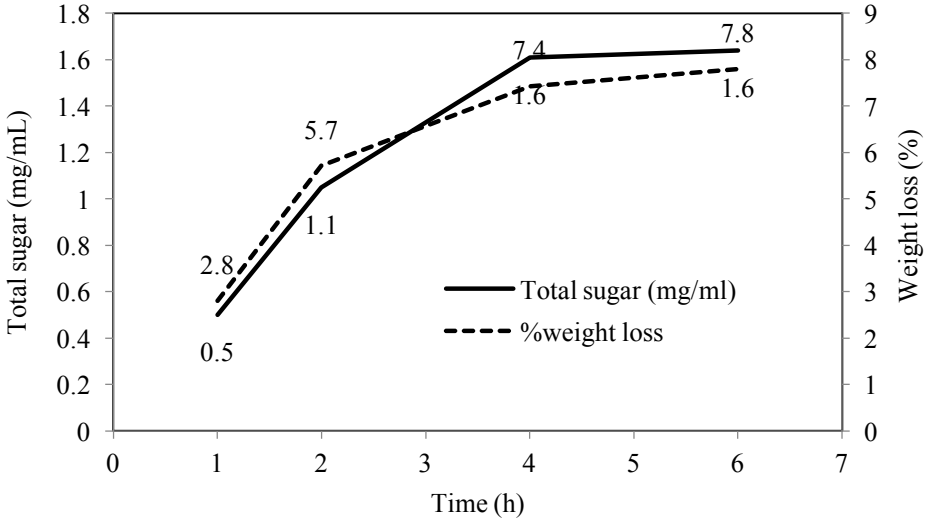

Figure 4*:* Effect of time of pretreatment on enzymatic pretreatment of EFB

Effects of Temperature on the Pretreatment of EFB

The effect of temperature on the pretreatment of EFB with the laccase enzyme was studied at 25 ± 3 °C, (RT) 35 °C, and 45 °C (Fig. 5). The temperature of pretreatment 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 sharp decline in the pretreatment process. This reduction is due to enzyme denaturation as the thermal tolerance limit of the enzyme was exceeded. Allowing the enzyme at high temperature for a period of time changes the protein structure of the molecule and renders the enzyme inactive. Assessing the extent of delignification after saccharification, 2.4 mg/mL and 2.9 mg/mL of total sugar were produced when EFB was 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 RT. When EFB was pretreated at 45°C, the sugar produced after saccharification had a concentration of 0.28 mg/mL, a result lower than the responses of pretreatment conducted at RT and 35 °C. Weight loss was also decreased by increased temperature. Laccase enzymes from various sources have shown different tolerance to temperature, as shown in the findings of Wooldridge [19]. In that case, laccase enzyme from *Pycnoporus cinnabarinus* was used to pretreat wheat straw at 50 °C, and 35 % delignification was obtained. However, Rico *et al.*[20] pretreated eucalyptus feedstock with laccase enzyme from *T. versicolor* at 50 °C and obtained 18 % delignification. Valls and Roncero [14] also used laccase enzyme from *T. villosa* to pretreat eucalyptus kraft at 50 °C and obtained 41 % delignification.

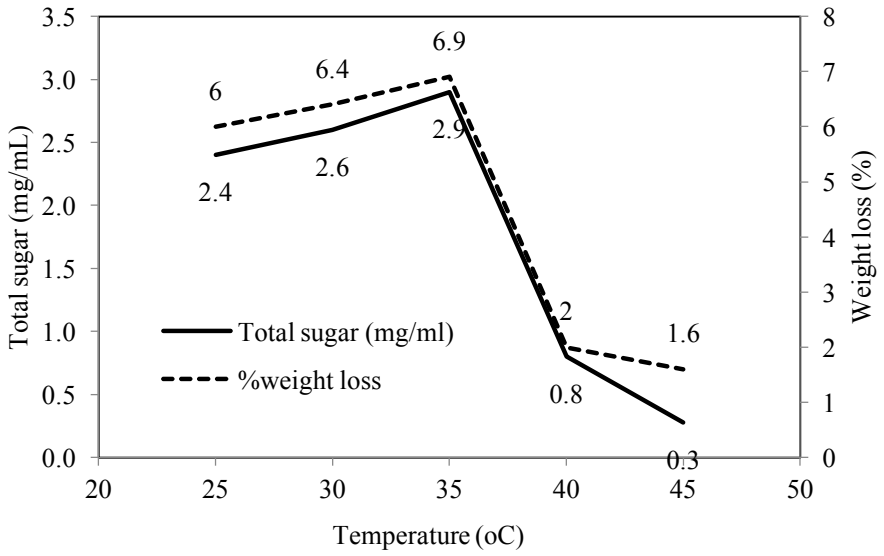

Figure 5*:* Effect of temperature on enzymatic pretreatment of EFB

Effect of pH on the 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 results of 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. pH is a measure of the acidity or alkalinity of a solution which as it changes from optimum for an enzyme to the acidic range, the enzyme will tend to gain hydrogen ions. Similarly, if the pH of the medium tends to change to the alkaline, the enzyme will lose hydrogen ions. Whether gain or loss of hydrogen ion, it leads to changes in the weak interactions that holds the shape of the enzyme molecule resulting in the denature of the enzyme due to structural changes and a loss in activity.

The pretreatment of EFB with laccase enzyme was studied at pH 2, 3, 4, 5, 6 and 7 (Fig. 6) using a citrate-phosphate buffer. The results revealed a strong relationship between the rate of delignification and the pH of the medium. At pH 2, 3, and 4 which was highly acidic, the rate of delignification was minimal; 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 produced after EFB pretreated at pH 2, 3, 4, 5, 6, and 7 respectively, were saccharified. The pH of the reaction medium has been studied by Bayindirli [21], who noted that all enzymes, depending on their group, have a specific pH range for optimum activity. Daas *et al.*[22] 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.

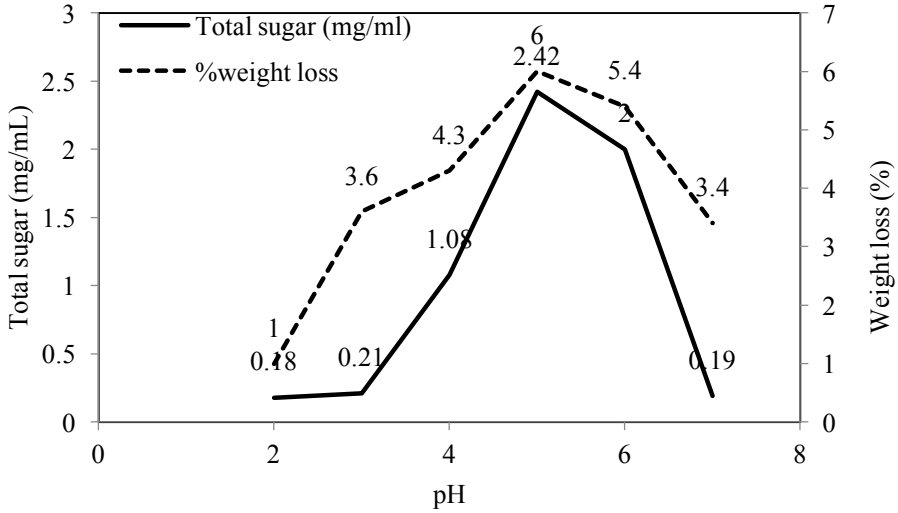

Figure 6*:* Effect of pH on enzymatic pretreatment of EFB

Pretreatment process validation

The pretreatment process was validated by carrying out a set of experiment using the OFAT determined optimum conditions. The results of the study were as shown in the Table 1. The difference in the results when compared with the results from combination of parameters during screening and found to be statistically significant at $p < .05$ level of significance.

Table 1: Optimum pretreatment parameter combination and the responses.

Enzyme concentration	**EFB concentration**	**Temperature**	**pH**	**Time**	**EFB size**	**Total sugar**	**Weight loss**
(IU/g)	**% (w/v)**	**(^{o}C)**		**(h)**	**(mm)**	**(mg/mL)**	**(%)**
20	5	35	5	4	2	3.8	22

5. CONCLUSION

Pretreatment of EFB, a lignocellulosic biomass with laccase enzyme was investigated using OFAT. The responses of the weight loss and total sugar showed that a good recovery of mono and polysaccharides is achievable with the use of laccase enzyme as pretreatment agent. A sample size of 2 mm, temperature at 35 ^{o}C, time of 4 h, substrate concentration of 5 % (w/v), pH 5 and enzyme concentration of 20 IU/g of EFB was achieved as optimized pretreatment conditions. Room temperature (25 ± 3 °C) could also be used during the pretreatment process based on the fact that similar results were obtained when pretreatment was done at room temperature and 35 ^{o}C. This study, in conclusion, has therefore demonstrated that laccase enzymes can be used as pretreatment agent for EFB. It could be used to pretreat biomass when the safety of the environment is of great concern.

REFERENCES

[1] Ukaegbu, C. I., Shah, S. R., Esmail, A. M. B. (2014). "Chemical pretreatment methods, sugar yield, and economic costs: A review," *Journal of Biotechnology Science Research*, 1(2), 30-38.

[2] Hendriks, T. W. M., and Zeeman, G. (2009). "Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*," 100(1), 10-8. DOI:10.1016/j.biortech.2008.05.027

[3] Sun, Y., and Cheng, J. (2002). "Hydrolysis of lignocellulosic materials for ethanol production: A review," *Bioresource Technology*, 83(1), 1-11. DOI:10.1016/S0960-8524(01)00212-7

[4] Taherzadeh, M. J., and Karimi, K. (2008)."Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review," *International Journal of Molecular Sciences* 9, 1621-1651.

[5] Mariëlle Bar. (2001). "*Kinetic and physiochemical properties of white rot fungal laccases*," M.sc thesis submitted to the university of the free state, Bloemfontein.

[6] Palamae, S., Palachum, W., Chisti, Y., and Choorit, W. (2014). ScienceDirect Retention of hemicellulose during delignification of oil palm empty fruit bunch (EFB) fiber with peracetic acid and alkaline peroxide. *Biomass and Bioenergy*, *66*, 240-248. doi:10.1016/j.biombioe.2014.03.045

[7] Valery, B., Agbor, C. N., Sparling, R., Berlin, A., and Levin, D. B. (2011). "Biomass pretreatment: fundamentals toward application," *Biotechnology Advances*, 29(6), 675-685.

[8] Pan, X., and Saddler, J. N. (2013). Effect of replacing polyol by organosolv and kraft lignin on the property and structure of rigid polyurethane foam. *Biotechnology for Biofuels*, *6*(1), 12. doi:10.1186/1754-6834-6-12

[9] Saha, B. C., and Cotta, M. A. (2008). "Lime pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol," *Biomass and Bioenergy*, 32(10), 971-977. DOI:10.1016/j.biombioe.2008.01.014

[10] Archibald, F. S., Bourbonnais, R., Jurasek, L., Paice, M. G., and Reid, I. D. (1997). "Kraft pulp bleaching and delignification by *Trametes versicolor", Journal of Biotechnology*, *53*, 215-236.

[11] Majcherczyk, A., Johannes, C., and Hu, A. (1998). "Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of Trametes versicolor," *Enzyme and Microbial Technology*, 0229(97), 335-341.

[12] Choi, W. I., Park, J. Y., Lee, J. P., Oh, Y. K., Park, Y. C., Kim, J. S., Lee, J. S. (2013). "Optimization of NaOH-catalyzed steam pretreatment of empty fruit bunch", *Biotechnology for Biofuels*, *6*(1), 170. DOI:10.1186/1754-6834-6-170

[13] S. S. Rashid, "Cellulase enzyme production from palm oil mill effluent, characterization and application," 2011.

[14] C. Valls and M. B. Roncero, "Using both xylanase and laccase enzymes for pulp bleaching.," *Bioresour. Technol.*, vol. 100, no. 6, pp. 2032–9, 2009.

[15] Rashid, S. S., Ukaegbu, C. ., Jaya, V. P., Fazli M. F., and Suhaiza S. A. (2016). "Optimization of the Enzymatic Saccharification Process of Empty Fruit Bunch Pretreated with Laccase Enzyme," *Bioresources*, vol. 11, no. 2, p. In press.

[16] Han, L., Feng, J., Shuangxi Z., Zhiqing, M., Yonghong, W., and Xing, Z. (2012). "Alkaline pretreatment of wheat straw and its enzymatic hydrolysis", *Brazillian Journal of Microbiology*, 2012: 53-61.

[17] Shah S. R., Md. Zahangir A., Karim, M. I. A., and Salleh, M. H. (2011). "Development of pretreatment of empty fruit bunches for enhanced enzymatic saccharification," *African Journal of Biotechnology*, 10(81), 18728-18738.

[18] Amin, N. A. S., Ya'ini, N., Misson, M., Haron, R., and Mohamed, M. (2010). "Enzyme pretreated empty fruit bunch for Biofuel production", *Journal of Applied Sciences,* 10(12), *1181-1186*.

[19] Woolridge, E. (2014). "*Mixed Enzyme Systems for Delignification of Lignocellulosic Biomass*," *Catalysts* 4, doi:10.3390/catal4010001

[20] Rico, A., Rencoret, J., Del Río, J. C., Martínez, A. T., and Gutiérrez, A. (2014). "Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock," *Biotechnology for Biofuels*, 7(1), 6. DOI:10.1186/1754-6834-7-6

[21] Bayındırlı, A. (2010). "*Enzymes in Fruit and Vegetable Processing chemistry and engineering application",* In the book″ Introduction to enzymes. CRC press ISBN 9781420094336.

[22] Daas Amiour, S., and Hambaba, L. (2016). "Effect of pH, temperature and some chemicals on polyphenol oxidase and peroxidase activities in harvested Deglet Nour and Ghars dates", *Postharvest Biology and Technology* 111, 77-82. DOI:10.1016/j.postharvbio.2015.07.027