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Optimization of enzymatic digestibility of sodium hydroxide-hydrogen peroxide oxidative pretreated siam weed for reducing sugar production

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ABSTRACT: *This study evaluated the enzymatic conversion of alkaline peroxide oxidative pretreatment of an invasive lignocellulosic biomass (siam weed) to reducing sugar, amenable to further microbial effects at the downstream processing. Using a statistical design of experiments approach (response surface methodology), optimum pretreatment conditions of 43.7 ^oC, 9.3 h, and 0.4% H_2O_2 , and enzymatic hydrolysis conditions of 25 FPU cellulase/g treated biomass, 50 ^oC hydrolysis temperature, 2% biomass loading, and 72 h hydrolysis period, 391.3 mg/g reducing sugar yield was achieved and validated. At the optimized pretreatment and enzymatic conditions, the conversion of treated biomass to untreated biomass was about a 6-fold increase.*

KEYWORDS: *enzymatic hydrolysis, oxidation, pretreatment, reducing sugar, response surface methodology*

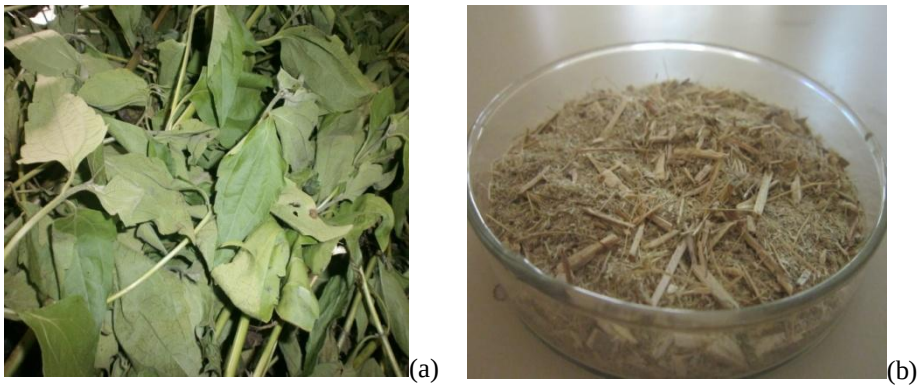
I. INTRODUCTION

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 [1]. However, a reduction of the ethanol production cost is desirable to improve the competitiveness. As the sugar and starch-containing feedstock's traditionally used for ethanol production represent the largest share of the total production cost [2], 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 [3,4]. 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 [5]. Pretreatment and enzymatic conversion of lignocellulosics are crucial steps to overcome lignocelluloses recalcitrance in the conversion to ethanol [6]. Lignocellulosic materials contain polymers (cellulose and hemicelluloses) needed to be broken down through hydrolysis (pretreatment and enzymatic) in other for the monosaccharides and other chemicals to be accessible. Alkaline peroxide oxidation pretreatment has been studied extensively for mostly agricultural residues and very few woody residues [7–14]. Scientific literature also reported the treatment of siam weed using different chemical methods as mild sulphuric acid, alkaline, and peracetic acid [15]. The hydrolysis of cellulolytic materials with diluted acids is well known, but this process generates toxic products of hydrolysis. Other negatives factors related to the acid hydrolysis are the corrosion and the high amounts of salts resulting from the acid neutralization. Enzymatic hydrolysis is preferred because of the higher conversion yields and less corrosive, less toxic conditions compared to an acid hydrolysis.

This study investigated the effect of alkaline sodium hydroxide under oxidative conditions (using hydrogen peroxide) on pretreatment of siam weed in order to cause appreciable enzymatic digestibility of treated biomass. Optimum conditions were predicted and validated for the enzymatic conversion of the alkaline peroxide oxidation pretreated siam weed. Furthermore, at the optimized enzymatic conditions, effect of the variation in hydrolysis temperature (at 45 ^{o}C) was also evaluated.

II. MATERIALS AND METHODS

Raw material: The Siam weed (*Chromolaena odorata*), is an invasive exotic weed [15], is typically a fast growing perennial herb. Raw material preparation from the field to the laboratory before compositional analysis was carried out by harvesting the shoots (leaves and stems) in late October, 2012 from an open fallow land around Ota town (6^{o}40'N 3^{o}08'E), South west, Nigeria **(**the growth period of the plant on the land was monitored to be 5 months). The leaves were chopped off from the branches manually. The stems were cut to 5±1 cm equal lengths and dried in an open space (35±2 ^{o}C) for 3 days (8 h each day)(Ayeni et al 2014; under review). Size reduction was further performed on the dried mass by knifing and milling. Samples were sieved to yield different size particles [16**],** and dried in a convection oven at 105 ^{o}C for 3 h to a dry matter content of 88%. Milled Siam weed stem was screened in the size range of 0.25 to 1 mm. The screened sample within the size range of 1 mm and 0.5 mm were retained while smaller particles were discarded because they corresponded mainly to sand. The bigger size fractions were manually mixed for 10 min to obtain an homogeneous equal proportions of sizes (Fig. 1). The raw biomass was stored in plastic bags and kept in a refrigerator until ready for use.



(a) (b)

Fig. 1: Harvested siam weed (a), and milled siam weed stem (b)

Experimentation: MINITAB 15 statistical software (PA, USA) was used for the design of the pretreatments (DOE) using response surface methodology (RSM)(2^{3}-central composite design (CCD) [17]. Design of experiments with MINITAB [18**]** was made up of 20 base runs (8 cube points, 4 centre points in cube, 6 axial points, and 2 centre points in axial, 2 base block, all in duplicate, resulting in a total of 40 experiments. The objective was to evaluate the influence of reaction temperature (X_1; Low level: 50 ^{o}C and High level: 70 ^{o}C), pretreatment time (X_2; Low level:4 h and High level: 8 h), and hydrogen peroxide concentration (X_3; Low level:1% and High level: 3%) on enzymatic digestibility of treated biomass. Table 1 shows the experimental design matrix.

Raw biomass pretreatment: 5 g of dried siam weed biomass were mixed with different concentrations of 100 mL hydrogen peroxide-water solution in a 500 mL beaker at pH 11.5. The distilled water contained H_2O_2 volume per volume distilled water of 0.32%, 1.00%, 2.00%, 3.00%, and 3.68%. The pH of solution was maintained to 11.5 by adding equivalent amount of sodium hydroxide pellets. Agitation of mixtures was made to occur by using a magnetic stirrer. Pretreatment occurred by varying the reaction temperature and reaction time. After each pretreatment time, the slurry was cooled to room temperature and separated into liquid and solid fractions by vacuum filtration. The solid part was washed with distilled water until it reached neutral pH. A portion of the solid was dried to a constant weight in a convention oven at 105 ^{o}C in order to estimate percent total solids [19]. The remaining wet treated materials were kept in the refrigerator for further determination of the extent of enzymatic hydrolysis and the optimum reducing sugar yields. Material balance for the residual total solids and solubilized fraction after pretreatment was evaluated. Each experiment was carried out in duplicate.

Compositional analysis of raw biomass: Extractives were determined by means of the Soxhlet extractor on 2.5 g of dry biomass using 150 mL acetone as solvent. The Soxhlet extractor was set up with the boiling flask

positioned on the heating mantle set at 70 ^{o}C. Each cycle on the extractor was maintained at 23 min for 4 h. At the end of each periods, the samples were air dried for few minutes at room temperature and further dried to constant weight at 105 ^{o}C in a convection oven. The extractives content was calculated as the difference in weight between the raw and extracted material [20–21]. The hemicellulose content was determined by weighing 1 g of dried biomass from the extractive analysis into a 250 mL Erlenmeyer flask and then 150 mL of 500 mol/m^3 NaOH solution was added. The mixture was boiled for 3 h and 30 min with distilled water [7, 22]. The hemicellulose content was obtained as the difference between the sample weight before and after boiling the extracted biomass with NaOH. Lignin composition was determined by weighing into glass test tubes 0.3 g of dry extracted biomass and adding 3 mL of 72% H_2SO_4. Acid hydrolysis was made to occur by keeping the samples at room temperature for 2 h with mixing of samples every 30 min. 84 mL of distilled water was added to each test tube after the 2 h acid hydrolysis step bringing the total volume to 87 mL. The samples were autoclaved for 1 h at 121 ^{o}C. After the second weak acid hydrolysis step, the hydrolyzates were cooled to room temperature and separated by vacuum filtration. The acid insoluble lignin was determined by drying the residue at 105 ^{o}C for 4 h and accounting for ash by burning the insoluble residue at 575 ^{o}C in a muffle furnace. The difference in weight of the acid insoluble residue when ash content was subtracted is the acid insoluble lignin [23]. The acid soluble lignin fraction was determined by measuring the absorbance of the acid hydrolyzed samples at 320 nm [23]. The lignin content was calculated as the summation of acid insoluble lignin and acid soluble lignin. The cellulose content was calculated by difference, assuming that extractives, hemicellulose, lignin, ash, and cellulose are the only components of the entire biomass [22]. The composition of the raw siam weed(wt.%) was estimated as; extractives content –4.82%, hemicellulose content –29.94%, acid insoluble lignin content –23.70%, soluble lignin content –0.52%, Ash content –0.97%, cellulose content –40.05%.

Enzymatic digestibility: The pretreated washed solid fractions were hydrolyzed by enzymes to determine the efficiency of substrate conversion. Enzymatic conversion was performed at 2% dry biomass content of total saccharification volume. 5 ml sodium citrate buffer at 0.1 M concentration and pH of 4.8 was added to the wet materials in 50 ml culture tubes. A preparation of *Trichoderma reesei* cellulase enzyme system with an activity of 57.8 filter paper unit (FPU)/ml was added at a loading of 25 FPU/g dry biomass. A total volume of 20 ml mixture was attained by adding an appropriate volume of distilled water to the citrate buffer and wet biomass. After an hydrolysis period of 72 h, 0.5 ml aliquot was sampled and analysed for reducing sugar. Experiments were conducted at 50 ^{o}C in a non-shaking incubator. To quench the hydrolysis, the samples were boiled for 15 min and then cooled in an ice bath. After hydrolysis, the samples were centrifuged at 4000 revolution/min for 5 min to remove residual solids. Fermentable sugars were estimated as reducing sugar with 3,5, dinitrosalicylic acid method [24] using glucose as standard. Reducing sugar yields from enzymatic hydrolysis was calculated based on mg equivalent glucose per g dry substrate (based on equivalent glucose in the hydrolyzed sample) [25].

$$Y = S \times D \times \frac{V}{W} \qquad \ldots(1)$$

where Y = reducing sugar yield (mg equivalent glucose/g dry biomass)
S = sugar concentration in diluted sample (mg equivalent glucose/mL)
D = dilution factor
V = working volume (mL)
W = weight of dry treated biomass (g)

Design of experiments: The objective was to evaluate the influence of reaction temperature (X_1), pretreatment time (X_2), and hydrogen peroxide concentration (X_3) on the APO process such that the pretreatment will enhance enzymatic hydrolysis of treated materials to reducing sugars. They were chosen for study as these parameters can influence the fractionation of the solid material. Table 2 shows the design matrix and both the experimental and predicted results of the reducing sugar. Temperature, time, and oxidation have been reported to have profound effects on ligno-cellulosic materials pretreatment [9,26]. The order in which the experiments were carried out was randomized. Each experiment in this study was replicated twice; reported results indicate the mean values of the replicated experiments.

The model generated as a function of X_1 (Temperature), X_2 (Time), and X_3 (% H_2O_2) variables (factors) on the predicted response of the reducing sugar yield (Y) is a second-order polynomial and is represented as follows:

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{1,1} X_1^2 + \alpha_{2,2} X_2^2 + \alpha_{3,3} X_3^2 + \alpha_{1,2} X_1 X_2 + \alpha_{1,3} X_1 X_3 + \alpha_{2,3} X_2 X_3 \qquad \ldots(2)$$

The predicted responses Y (reducing sugar yields) associated with each factor level combinations; α_0 to $\alpha_{2,3}$ are coefficients to be estimated from regression, they represent the linear, quadratic and cross-products of

X_1, X_2, X_3 on the responses. The MINITAB 15 (PA, USA) was used for regression analysis of experimental data, plotting of response surfaces and to optimize the process parameters. The coefficients in the second-order polynomial (equation 1) were calculated by multiple regression analysis, based on the experimentally obtained data, and then the predicted responses were obtained using equation (1). Analysis of variance (ANOVA) was used to estimate statistical parameters.

III. RESULTS AND DISCUSSION

Hydrolysis of treated biomass: The enzymatic digestibility of biomass is affected by the pretreated methods used and the structural modification of the biomass (e.g. lignin content, acetyl group content, and crystallinity) (27). The results of experiments obtained by utilizing a central composite design were analyzed by considering reducing sugar yields (RS) after enzymatic hydrolysis of pretreated siam weed samples. Table 1 shows the design matrix with the experimental and predicted RS yields for a 72 h terminal hydrolysis period. Reducing sugar yields did not follow a particular trend. The experimental maximum reducing sugar yield was 223 mg/g dry biomass. However, increasing temperature with longer pretreatment time produced more of the reducing sugar. For example, at temperature of 50 ^oC, 8 h, and 1%H_2O_2 (Run 20), reducing sugar yield was 205.92 mg/g. Also, at 60 ^oC, 6 h, and 0.4%H_2O_2, 223 mg/g reducing sugar was produced (Run 5), while at 70 ^oC, 4 h, and 3% H_2O_2, 194.34 mg/g RS was produced. In our previous work, comparable results were obtained on both screened and unscreened sugarcane bagasse under same pretreatment conditions (Ayeni et al 2014; under review), maximum reducing sugar attained was 285 mg/g for screened sugarcane bagasse (pretreatment conditions of 70 ^oC, 4 h, and 1%H_2O_2) and 297.56 mg/g for unscreened sugarcane bagasse (pretreatment conditions of 50 ^oC, 4 h, and 1%H_2O_2).

Optimization and validation of operating conditions: Following the result obtained from enzymatic hydrolysis of siam weed, the statistical software MINITAB 15 was also used to determine the coefficients of the second-order polynomial by multiple regression analysis as well as to build the quadratic model and the 3D response surface plots. The experimental results were analyzed by regression analysis consisting of the linear, quadratic and interaction effects which gave the following regression equation with reducing sugar yields (Y) as a function of pretreatment temperature, time and %H_2O_2.
The model equation generated for the enzymatic hydrolysis process is given as:

$$Y = 482.780 - 11.946X_1 + 30.577X_2 - 165.788X_3 + 0.160X_1^2 + 4.558X_2^2 + 49.111X_3^2 - 1.087X_1X_2 + 0.326X_1X_3 - 10.854X_2X_3$$

$$R^2 = 0.9568 \quad \ldots(3)$$

When the values from X_1 to X_3 were substituted in equation (3), the predicted responses were obtained (Table 1). The *P*-values (probability values) are used as tools to check the significance of each of the coefficients in the models, which in turn, may indicate the patterns of the interaction among the variables. The larger the magnitude of *T* and smaller the *P*-value the more significant is the corresponding coefficient. From Table 2, temperature is statistically significant on enzymatic hydrolysis (P = 0.049), hydrogen peroxide concentration is also significant (P = 0.002). All the square effects are significant while only the interaction effect between temperature and hydrogen peroxide concentration is not statistically significant.

Table 1: Design matrix of the experimental and predicted yields of reducing sugar of the pretreated siam weed

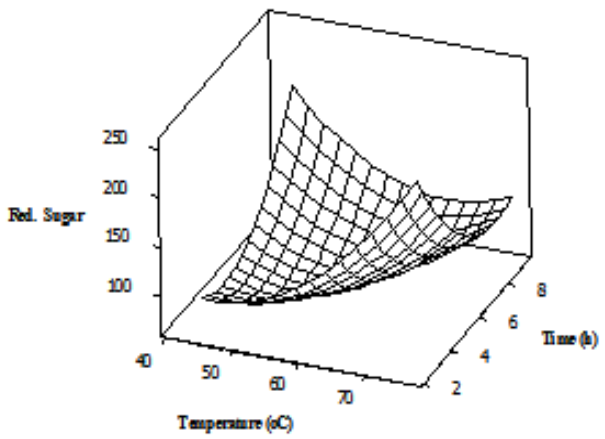
Run Order	Temperature (X_1), ^oC	Time (X_2), h	H_2O_2 (X_3)%(v/v)	Experimental (mg/g)	Predicted (mg/g)
1	43.7	6.0	2.0	89.85	92.07
2	60.0	6.0	2.0	67.35	71.98
3	60.0	6.0	3.6	187.78	178.60
4	60.0	9.3	2.0	95.89	115.26
5	*60.0*	*6.0*	*0.4*	*223.78*	*227.29*
6	76.3	6.0	2.0	145.10	137.21
7	60.0	6.0	2.0	75.15	71.98
8	60.0	2.7	2.0	150.98	125.94
9	60.0	6.0	2.0	69.75	71.98
10	70.0	8.0	1.0	190.95	177.49
11	60.0	6.0	2.0	73.95	71.98
12	50.0	4.0	3.0	109.34	126.58
13	50.0	4.0	1.0	118.95	119.50
14	70.0	8.0	3.0	107.55	110.78

15	50.0	8.0	3.0	132.50	120.09
16	60.0	6.0	2.0	71.30	71.98
17	70.0	4.0	1.0	167.89	184.08
18	60.0	6.0	2.0	72.50	71.98
19	70.0	4.0	3.0	194.34	204.20
20	50.0	8.0	1.0	205.92	199.84

Table 2: Estimated regression coefficients and their probability values

Term	Coefficient	T	P
Constant	482.780	2.455	0.034
X_1	-11.946	-2.245	0.049
X_2	30.577	1.461	0.175
X_3	-165.788	-4.207	0.002
X_1^2	0.160	3.878	0.003
X_2^2	4.558	4.420	0.001
X_3^2	49.111	11.907	0.000
X_1X_2	-1.087	-4.101	0.002
X_1X_3	0.326	0.615	0.552
X_2X_3	-10.854	-4.096	0.002

A



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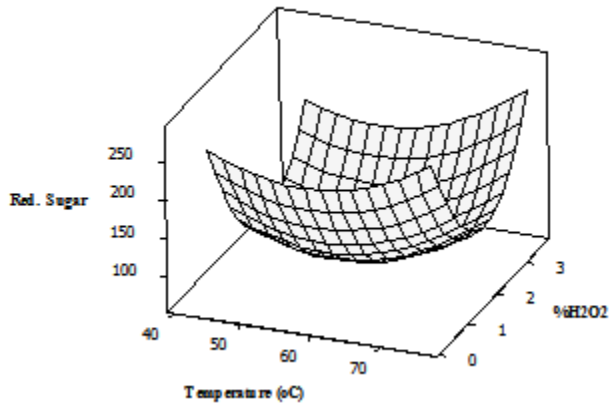

C

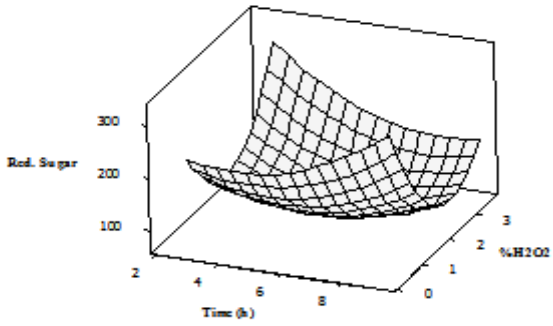

Fig. 2: Response surface plots of effects of operating variables on reducing sugar yields (mg/g dry biomass)A: Temperature and Time, B: Temperature and %H_2O_2, C: Time and H_2O_2.

The statistical significance of the model equation using confidence interval of 95% was validated by the *F*-value for analysis of variance (ANOVA), which showed that the regressions were statistically significant for the treated samples ($F = 24.62$, $P \leq 0.000$) (data not shown). The ANOVA of the models also showed the linear, square, and the interactive effects of factors on treatments to be statistically significant ($P \leq 0.004$). Analysis of variance (ANOVA) fitted for the model was required to test the significance and adequacy of the model. Temperature was reported to have significant effect on conversion for most lignocellulosic materials [9,28,29]. The coefficient of determination (R^2) of the model was 0.9568, indicating again that the model was suitable in establishing relationships among the reaction variables. The R^2 value explains that about 96% variability is attributed to the factors for the response Y (reducing sugar yield). This also means only 4% of the total variation is not explained by the model. The model equation for the response (equation 1) and the response surface plots (Fig. 2) were utilized in determining the optimum process conditions.

The influences of individual factors on on reducing sugar yield are shown in Fig. 2(A to C). These plots were obtained by holding the third variable at mid point value. Fig. 2 (A) shows the surface plot of Time and Temperature on sugar yields, indicating that the optimum reducing sugar yields should occur between the 40–50 ^oC with increasing time between 6–9 h. The surface plots also show that optimum hydrogen peroxide concentration should occur very closely to 1% and 2% (Fig. 2(B) and Fig. 1(C)). The response optimizer was set by maximizing with a target of 300. The upper limit was selected to be 300. Considering the minimum time, temperature, and %H_2O_2 set to 4 h, 50 ^oC, and 1% respectively, the optimum cumulative response was obtained at 43.7 ^oC, 9.3 h, and 0.4% H_2O_2. The optimized predicted response of the reducing sugar was 415.2 mg/g with a desirability of 1. The individual desirability evaluates how the settings optimize a single response. Desirability value of 1 represents an ideal case; zero indicates that one or more responses are outside their acceptable limit. A value close to 1 indicates that the settings are more effective at maximizing the response. Additional sets of experiments at these specific conditions were performed to validate the optimized conditions. The validated reducing sugar yields at optimized pretreatment conditions was obtained to be 391.3 mg/g dry biomass. The experimental and predicted responses were found to be in close agreement, thus confirming the optimization process.

Effects of variations of hydrolysis temperature at optimized conditions: A single optimum condition for enzymatic digestibility may be impossible because the optimum may shift due to factors such as dry solid content, pH, temperature, the desired residence time, and enzyme activity. Enzymes are inhibited by the end products, the build-up of any of these products negatively affects cellulose hydrolysis. The maximum cellulase activity for most fungal derived cellulases and β-glucosidase occurs at 50 ± 5 °C and a pH of 4.0–5.0 [30**]**. Treated to untreated biomass reducing sugar yield at 50 ^oC with same hydrolysis conditions was about 6-fold increase (Fig. 3). The untreated solid material was used as the control for comparing the enzymatic digestibility of the treated siam weed. This showed the efficiency of pretreatment process to cause disruption to the lignocellulosic complex. The digestibility of treated sample at 45 ^oC hydrolysis temperature (at optimized conditions; 43.7 ^oC, 9.3 h, and 0.4% H_2O_2) was also evaluated. Under the same digestibility conditions, (25 FPU/mL cellulase enzyme loading, pH of 4.8, time of 72 h, and 2% treated biomass loading), results showed a decrease of about 2-folds in the reducing sugar yield at 45 ^oC to 50 ^oC hydrolysis temperature (Fig. 3). This may not be unconnected to the mild pretreatment conditions on biomass and enzymatic hydrolysis conditions. Future studies will be directed at optimizing between the hydrolysis period, time, pH, and biomass loadings.

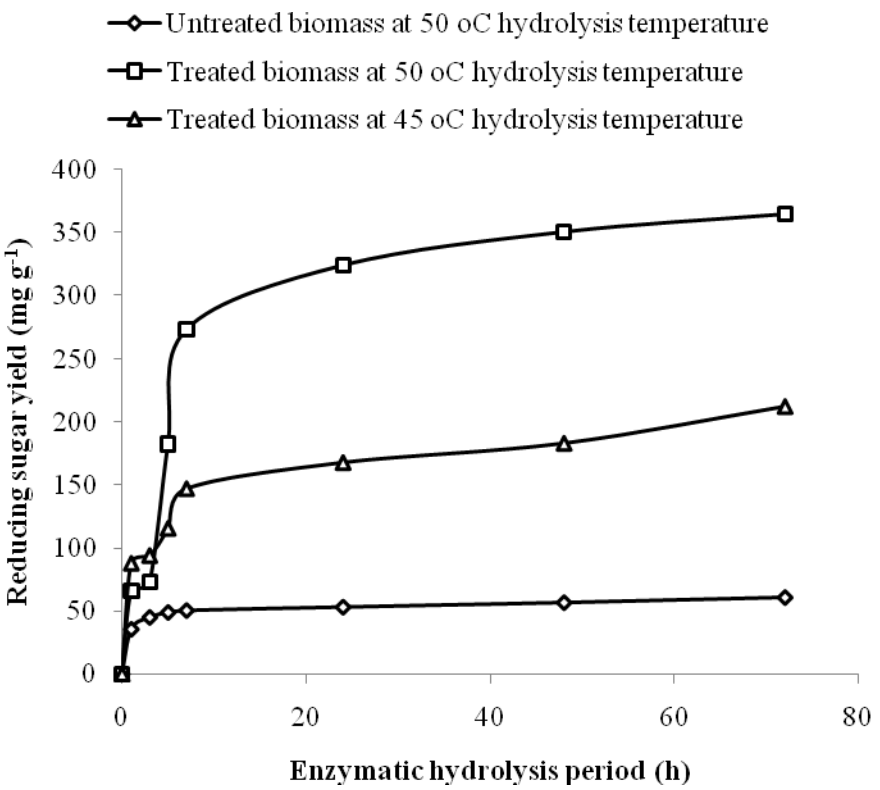

Fig. 3: 3-d Effect of temperature on treated and untreated biomass on sugar yields. Pretreatment conditions: 44 ^{o}C, 0.4%H_2O_2, and 9.3 h. Enzyme hydrolysis conditions: 25 FPU cellulase per g dry biomass, 45 ^{o}C and 50 ^{o}C hydrolysis temperatures, pH 4.8, 20 g kg^{-1} substrate concentration.

IV. CONCLUSIONS

The study explored the feasibility of using a suitable method (alkaline peroxide oxidation) pretreatment for the bioconversion of a lignocellulosic biomass (siam weed) to reducing sugar which may eventually be acted upon by microbes through fermentation techniques with the aim of producing ethanol. A 2^3 central composite design was used to determine the validated optimized pretreatment condition for the biomass as 43.7^{o}C, 9.3 h, 0.4% H_2O_2 so as to obtain 391.3 mg/g dry biomass reducing sugar yield. Enzymatic hydrolysis evaluated at the optimized conditions for the untreated biomass showed the efficiency of pretreatment on raw biomass. The reducing sugar yield of the treated to the untreated biomass was about a 6-fold increase.

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