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Formation of Enzymatic Recalcitrance during Bioethanol Production from *Pernnisetum Purpureum* with Three Pretreatment Methods

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Abstract
This research was aimed at observing the inhibiting effects of some substances that are said to be recalcitrant to enzymatic activities during the production of cellulosic ethanol, a source of liquid biofuels. The biomass used for this work was a perennial grass specie (*Pernnisetum purpureum* S.), which is a lignocellulosic biomass that is predominantly found in the South-South Zone of Niger Delta, Nigeria. The three pretreatment methods; Dilute acid, Sulphite Pretreatment to Overcome the Recalcitrance effects of Lignocellulose(SPORL) and Alkaline Wet Oxidation were used on three different biomass particle sizes. The saccharification reaction was carried out at 10 and 15FPUcellulase/gcellulose of enzyme loading. The fermentation reaction was carried out with *Saccharomyces cerevisiae* ($5.40X10^8$-$5.70X10^8$cell/ml) from yeast. Among the three organic inhibitors analyzed for in the cause of the reaction, two were generative as the reaction proceeded while one was deteriorating with time. The difference between the theoretical ethanol yield from the total amount of glucose converted (EY_2) and the theoretical ethanol yield from the initial amount of glucose before fermentation (EY_1) was 23.88% and 8.80% for enzyme loading of 10FPU/g and 15FPU/g respectively, which suggested the formation of side products (potential recalcitrance to enzymatic activities). This was further confirmed by the low values of enzymatic convertibility of cellulose and enzymatic convertibility of glucose; 28.92% and 25.92% respectively. Statistical analysis and optimization of data was done using ANOVA, Pearson correlation, Regression analysis, Duncan's multiple test, t-test for comparing means and response optimization.
Keywords: Cellulosic ethanol, Hydroxymethylfurfural, Acetic acid, Total Extractable Polyphenolics.

1. Introduction

The first generation bioethanol fuels as produced from mono- or disaccharides as well as starch are identified with less challenges of toxic substances interfering with enzymatic activities during production, compared to the second generation bioethanol (lignocelluloses biomass and agricultural residues used as substrates) which has an immense capital cost on establishing cellulosic ethanol biorefineries.

Some of these inhibitors to enzymatic activities are components of the plants biomass, while others are produced from side reactions in the cause of production. Lignin for instance has an inhibitive effect to the functions of enzymes and it is also a precursor to some other chemical substances, especially organic compounds. Lignin is a complex heterogeneous polymer, which results from the polymerization of phenols forming radicals, through the formation of dimer (a dehydro) by coupling at the β- positions (β-β, β-O-4 and β-5). It is also expected that during the pretreatment stage of production, certain reactions may produce other potential enzymatic inhibitors from the phenolic components of lignin (Argyropoulos *et al*., 2002; Ralph *et al.,* 2004; Wagner *et al.,* 2009).

Besides the sensitivity of enzymes to the physical conditions (temperature, surface area of substrates, pH, reaction time) of reaction systems, the operating conditions of the reaction routes from pretreatment to fermentation also determine the extents at which these enzymatic recalcitrance are formed and their effects to the enzyme used (Yang and Wyman, 2008; Harmsen *et al.,* 2010).

Ladisch *et al.,* (2009) observed that different phenolic compounds have variable toxic effects to enzymatic activities, such that three phenol compounds; vanillin > syringaldehyde>trans-cinnamic acid have their inhibitive effects as shown in increasing order. Phenols cause the partitioning of the cell membranes of the organism producing the enzyme, therefore reducing their cell growths as well as sugar assimilation.

During production, side products are also cable of forming; which may also be acting contrary to the performance of enzymes. Saha, De Sudipta and Dutta (2011), investigated and confirmed that disaccharides can be converted to 5-hydroxymethylfurfural (HMF) under favorable reaction conditions and the HMF formed which is a strong inhibitor to enzymatic activities could as well be decomposed to other toxic substances (levulinic acid and chloromethylfurfural). Furfural could also be formed from pentose sugar (xylose) if the condition of reaction permits such reaction. Also, acetic acid could be formed in the reaction system depending on the reaction conditions, either anaerobic or aerobic (reducing or oxidizing); the acetyl groups in hemicelluloses and ethanol available in the reaction system could also act as reactants for the formation of acetic acid.

The rate of generation of these toxic substances with respect to the method of pretreatment cannot be

disregarded as several authors have reported that these substances are formed more at high acidic conditions, especially at the pretreatment stage of production (Blanch *et al.,* 2011**;** Dale and Ong, 2012; Dussán *et al.,* 2014 and Ladisch *et al.,* 2014).

2. Materials and Methods

The chemical reagents used for the process of production, quantitative analysis as well as qualitative analysis in this work, were of analytical grades and ordered from Sigma-Aldrich, Germany and Megazyme, Ireland.

2.1 Biomass Handling

The grass biomass *(Pernnisetum purpureum)* used for this study was collected within the South-South Zone of Niger Delta, Nigeria. The actual life cycle of the biomass was not ascertained as samples were collected in the wild at their early life cycles.

The biomass was washed thoroughly and copiously with deionized water, chopped into size (1-2cm) and air dried in a greenhouse for six days.

The dried biomass was ground, then sieved to 0.15mm, 0.20mm, 0.35mm, 0.45mm and 0.71mm particle sizes using a DH-300T test sieve machine. The dried and sieved biomass was preserved in a cool and dry place at an average temperature of 12^0C.

2.2 Determination of cellulose, hemicellulose, lignin and total extractable phenols (TEPs)

The amounts of hemicelluloses, cellulose, lignin and total extractable phenolics were determined as reported by Ekpo *et al.,* (2016).

2.3 Pretreatment Processes

Three pretreatment processes; dilute acid (DA), sulphite pretreatment to overcome the recalcitrance effect of lignocelluloses (SPORL) and alkaline wet oxidation (AL) were used . All pretreatment occurred at the conditions of 0.2Mpa, 130^0C and residence time of 16mins in a pressure vessel. The biomass loading was 3%(v/w); dilute acid pretreatment utilized 1.1% (v/v) H_2SO_4 (Zhu *et al.,* 2011; Idrees *et al.,* 2013 and Dussán *et al.,*2014) While SPORL method used 1.0%(v/v) H_2SO_4 acid solution and 1.5mL of 3%(w/v) Na_2SO_3 (Zhu *et al.,* 2009, Zhu *et al.,*2011, Qureshi *et al.,* 2013 and) and alkaline wet oxidation was carried out with 0.019M Na_2CO_3 solution (Bjere, *et. al.,* 1996; Klinke *et al.,* 2002; Martin *et at.,* 2007 and Martin, Marcet and Thomsen, 2008). At the end of each pretreatment, the pretreated biomass were washed to bring the pH of solutions close to neutral value then filtered to obtain the residues for further reaction process and analyses.

2.4. Analyses of Reducing Sugars (Glucose and Xylose)

Analysis of glucose: Four standard solutions of glucose (0.63, 1.25, 2.50 and 5.00g/L) were prepared. 2mL of sample or glucose standard solution and 3mL of Dinitrosalicyli acid reagent were placed in boiling water for 5minutes then cooled to room temperature. Using distilled water as blank solution, the transmittance of both standard glucose solutions and samples were measured with a UV-Visible spectrophotometer (1801 UV-VIS) at 540nm wavelength. A calibration curve was prepared for the standard glucose solutions by plotting a graph of log% transmittance against concentration of the standard glucose solutions and the concentration of the samples were deduced from their transmittance.

Analysis of the amount of xylose was carried out with four prepared standard solutions of xylose (0.10, 0.25, 0.50 and 1.00g/L) used for producing the standard calibration curve. 3mL of standard solution or sample was measured into a beaker and 0.95mL of 6.37N HCl solution added to the beaker, followed by the addition of 5mL mixture of 0.5g phloroglucinol and glacial acetic acid then the solution boiled for 5mins, cooled and the absorbance read with a 1801UV/VIS series spectrophotometer at 554nm (Trinder, 1975; University of Nebraska Laboratory Manual, 2009).

2.5 Analysis of Ethanol

The analysis of ethanol was carried out by completely oxidizing ethanol present in the analyte to acetic acid using nicotinamide-adenine dinucleotide (NAD^+) as an oxidizing agent. The catalyst used for this analysis were alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (Al-DH). The principle of this analysis was derived from the stoichiometric relationship of 1:2 moles of ethanol to reduced nicotinamide-adenine dinucleotide (NADH) formed in the cause of the reaction as shown below:

$$C_2H_5OH + NAD^+ \xrightarrow{(ADH)} CH_3COH + NADH + H^+ \text{ ----------(1)}$$

$$CH_3COH + NAD^+ + H_2O \xrightarrow{(Al\text{-}DH)} CH_3CO_2H + NADH + H^+ \text{ ----------(2)}$$

The concentration of NADH was determined by measuring its absorbance at the wavelength of 340nm using a 1801UV/VIS series spectrophotometer (Beutler 1988and Megazyme 2014)

Calculation:

Conc. of Ethanol (g/L) = $\frac{V*MW}{\varepsilon * d*v*2}$ x ΔA ----------(3)

Where: V= final volume (mL)
MW= molecular weight of ethanol (g/mol)
ε (extinction coefficient of NADH at 340nm) = 6300 (l x mol^{-} x cm^{-})
d = light path (cm)
v = sample volume (mL)
ΔA = absorbance (NADH) differences for both sample and blank.
2 = 2moles of NADH produced for 1mole of ethanol

2.6 Analysis of Hydroxymethylfurfural

Standard solutions of 5-hydroxymethylfurfural (0.00125, 0.0025, 0.0050 and 0.0075mg/L) were used to prepare the standard calibration curve for quantitative analysis of this compound. Sample preparation prior to analysis was carried out by mixing 5mL of the solution of sample with 0.5g of activated charcoal in a boiling tube and boiled for 1min then filtered with Whatman filter paper 1. The absorbance of both samples and standards were detected with 1801UV/VIS series spectrophotometer at 280nm (White 1994; Zappal *et al.,*2004 ; Rocha *et al.,* 2004; Kmecl and Skerl, 2004 and Zhang *et al.,* 2013).

2.7 Analysis of Acetic Acid

The amounts of acetic acid in samples was determined using volumetric analysis.
0.20M sodium hydroxide solution was used to titrate against the sample using 3 drops of phenolphthalein indicator. The titration was carried out in triplicate and the average volume (VBav) evaluated (Pant, 2010). The equation of reaction and calculations are as shown below:

$HC_2H_3O_{2(aq)} + NaOH_{(aq)} \rightarrow NaC_2H_3O_{2(aq)} + H_2O(l)$ ----------(4)
1mole : 1mole

$C_A\,(mol) = \frac{C_B * V_{Bave} * N_A}{V_A * N_B}$ ----------(5)

C_A =Concentration of acetic acid (mol)
C_B = Concentration of NaOH (mol)
V_{Bave} = Average titire volume of NaOH (mL)
V_A = Volume of acetic acid at end point (mL)
N_A = Stoichiometric number of mole of acetic acid
N_B = Stoichiometric number of mole of NaOH

$\%\ \text{Acetic Acid} = \frac{\text{Mass of Acetic Acid in Solution}}{\text{Total Mass of Solution (Sample)}} \times 100$ ----------(6)

2.8 Enzymatic Hydrolyses of Cellulose

Substrate loading of 0.5%(w/v) and cellulase loading of 10 and 15FPU/g as well as some volume of viscozym (to prevent cellobiose accumulation) were used for the hydrolysis of cellulose at 50^0C, adjusted to pH of 4.8 and residence time of 12hours (Emmel *et al.,*2003; Martins *et al.,* 2008; Zhu, et. al.,2009; Blanch *et. al.,* 2011; Curvelo *et al.,*2012 and Wanderley *et al.,* 2014).

2.9 Fermentation

Strains of *Sacchromysis cerevisiae* were isolated from yeast cells and inoculated in a sterilized (121^0C for 15min) culture medium solution of 5g/L yeast extract, 4g/L peptone and 20g/L glucose, adjusted to a pH value of 7 and left for 24 hours at 34^0C, giving $40X10^8$-$6.50X10^8$cell/mL amounts of cells for fermentation. The medium was centrifuged and filtered to collect the S. cerevisiae strains then diluted to solution. A fermentation medium contained 50g/L glucose, 4g/L yeast extract, 2g/L $(NH_4)_2SO_4$, 2g/L KH_2PO_4 and 1g/L $MgSO_4$. The Inoculum, Fermentation medium and hydrolysate were combined in the volume ratio of 0.04 : 0.44 : 0.56(D'Amore *el al.,* 1988; Gibbons and Westby, 1989; Tahir *et al.,*2010; Wanderly *et al.,* 2014; Idrees *et al.,* 2013**).**

2.10 Statistical Analyses of Data

ANOVA, Pearson correlation, Regression analysis, Duncan's multiple test, t-test for comparing means and Minitab response optimizer software were used as statistical tools to analyze the data generated from analyses.

3.0 Results and Discussion

Table 1. Proximate analysis of Biomass Components

Biomass Component	Amount (%)
Neutral Detergent Fiber(NDF)	76.23±0.98
Acid Detergent Fiber(ADF)	36.9±1.51
Hemicellulose(HEM)	39.31±0.72
Cellulose(CEL)	34.04±0.24
Lignin(LIG)	5.62±0.09
Ash(ASH)	6.86±0.002
Total Extractable Polyphenolics (TEPs)	0.63±0.001

Table 1 shows the results for the proximate analyses of the crude grass biomass. These results are in conformance with the work of Chulalaksananukul *et. al.,* (2012).

Hemicelluloses are observed to be removed from biomass as the pH of the pretreatment medium increases toward acidity as shown in Table 2, this results in degradation of carbohydrates components in a similar order, as seen in Figure 4 and Table 6.

Table 2. Biomass composition (%) after different pretreatment methods at variable particle sizes.

Particle Size	**Dilute Acid**					**ǂSPORL**					**Alkaline Wet Oxidation**				
	HEM	**CEL**	**LIG**	**ASH**	**TEPs**	**HEM**	**CEL**	**LIG**	**ASH**	**TEPs**	**HEM**	**CEL**	**LIG**	**ASH**	**TEPs**
200µm	**4.52**	**60.96**	**4.14**	**2.44**	**1.40**	**31.12**	**46.24**	**3.46**	**2.32**	**0.64**	**32.88**	**40.88**	**1.52**	**1.34**	**0.55**
350µm	**5.02**	**67.50**	**2.68**	**2.92**	**2.16**	**36.86**	**46.66**	**2.66**	**2.90**	**1.48**	**36.76**	**46.02**	**1.30**	**1.52**	**1.24**
450µm	**5.66**	**69.48**	**1.03**	**2.28**	**1.55**	**35.76**	**54.10**	**1.00**	**2.82**	**1.36**	**36.36**	**49.48**	**1.24**	**2.50**	**1.26**

Values are mean of analysis carried out in triplicate.
ǂSPORL: Sulfite Pretreatment to Overcome the Recalcitrance effects of Lignocellulose.
HEM : Hemicellulose; CEL: Cellulose; LIG : Lignin
TEPs: Total Extractable Polyphenols

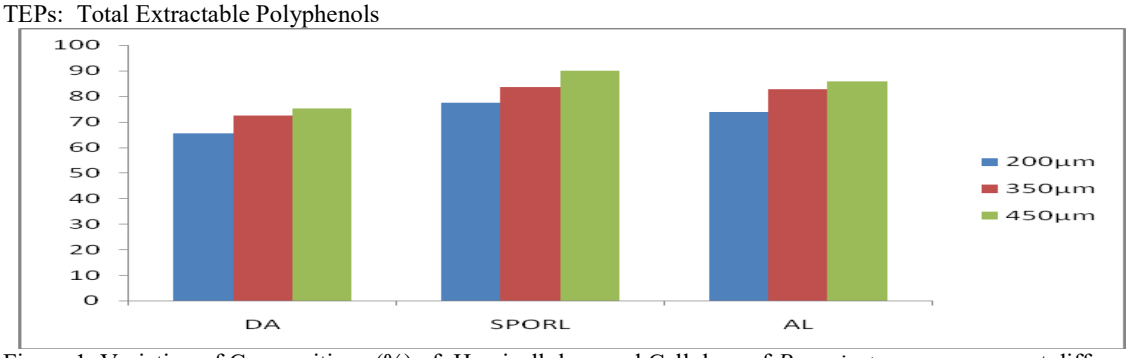

Figure 1. Variation of Compositions (%) of Hemicellulose and Cellulose of *Pernnisetum purpureum* at different Particle Sizes and Pretreatment Methods

The amounts of cellulose recovered during pretreatment processes follows a reverse order to that of hemicelluloses. Figure 1 shows that the amount of total available carbohydrate component (hemicellulose and cellulose) increased in the following order with pretreatment methods; DA<AL<SPORL. The regression analysis on Table 11, showed that the analyses of the amounts of carbohydrates components were significance at P≤0.05 and the amounts of cellulose produced was proportional to that of glucose and inversely proportional to hemicelluloses and xylose, while the amounts of hemicelluloses produced was directly proportional to xylose; these could imply that hexose sugars are the main components of cellulose while pentose sugars are the major components of hemicelluloses.

The pretreatment methods used in this work showed responsive effects to reduction in the amounts of lignin when compared to the amount of lignin in the crude grass biomass (LIG-Pp) and the increasing order of the effectiveness of this process is as shown on Figure 2; DA< SPORL < AL.

Table 3. Measurement of mean, showing comparison of quantities before and after pretreatments

HEM' (P)	HEM" (A)	CEL' (P)	CEL" (A)	LIG' (P)	LIG" (A)	ASH' (P)	ASH" (A)	TEPs' (P)	TEPs" (A)
39.31	24.99	34.04	53.48	5.62	1.81	6.86	2.34	0.63	1.29

‘ Prior to Pretreatment
“ After Pretreatment

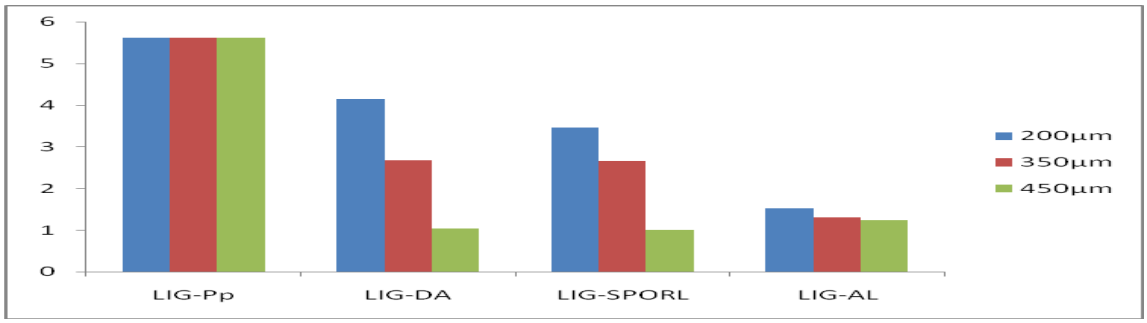

Figure 2. Variation of Compositions (%) of Lignin in *Pernnisetum purpureum* with Particle Sizes (µm) for different Pretreatment methods

All methods of pretreatment used in this study increased the amounts of total phenolics in the crude grass biomass (TEP-Pp), as shown in Figure3 and the increasing order of this substance with the pretreatment methods is, AL < SPORL < DA. The variations of lignin and total phenolics so far discussed, buttress the findings of other authors (Harmsen *et al.,*2010) that lignin is the precursor of phenolic compounds in the production routes of cellulosic ethanol. From the estimation of the mean amounts of substances in the crude biomass and after pretreatment processes(Table 3), substances are increased in their amounts before pretreatment process to after pretreatment process are cellulose and total phenolics, while substances that decreased are hemicelluloses, lignin and ash contents. The analyses of variance (ANOVA) on Table4, shows that among the components analyzed during pretreatment processes, the amounts of xylose and ash contents were not significant to the different sources of combination, that of xylose was supported by the Duncan’s multiple test on Figure10; which shows that the amounts of hemicelluloses obtained for alkaline wet oxidation and SPORL pretreatment methods were significantly different from that of dilute acid pretreatment, as hemicellulose is the major precursor of xylose. Concentrations of ethanol, converted glucose and unconverted glucose were significant to grass biomass and not to the combinations with other parameters (time, size, method); these may be traced to the effects of side products formed during the stages of production.

Table 4. Analysis of Variance (ANOVA) for non-significance measurements

Source	Dependent Variable	Type Sum of Square	df	Mean Square	F	Sig.
		PRETREATMENT				
grass	Xyl	0.00	2.00	0.00	0.81	0.46
size	ASH	0.93	2.00	0.47	3.13	0.06
grass *Size	ASH	0.93	4.00	0.23	1.56	0.21
size*method	Xyl	0.00	4.00	0.00	0.27	0.90
size*method	ASH	0.48	4.00	0.12	0.80	0.53
grass*size*method	Xyl	0.00	8.00	0.00	0.60	0.77
		HYDROLYSIS				
grass*size*time	Glu	6.767	8.00	0.846	1.902	0.071
size*method*time	Glu	4.304	8.00	0.538	1.209	0.304
grass*size*time*method	Glu	4.393	16	0.275	0.617	0.861
		FERMENTATION				
time	ETOH	34.216	2	17.108	0.748	0.490
size*time	ETOH	0.480	4	0.120	2.422	0.055
grass*size*time	UCG	0.727	8	0.091	1.815	0.086
grass*size*time	ETOH	0.574	8	0/072	1.448	0.190
size*method*time	ETOH	0.223	8	0.028	0.562	0.806
grass*size*time*method	ETOH	1.968	16	0.069	1.382	0.172

UCG: Unconverted Glucose, ETOH: Ethanol

The degrading effects on carbohydrate components by dilute acid pretreatment method was accounted for during hydrolysis, as the concentration of glucose that was highest in pretreatment liquor was lowest in hydrolysate (Table5) unlike the cases of SPORL and Alkaline Pretreatment methods. The low Concentration of

xylose between 0.19g/L to 0.30g/L from the fourth to twelfth hour of hydrolysis respectively is a clear indication that the hydrolysis of xylan was not of interest in this work as the required enzyme for its hydrolysis was not used.

Table 5. Comparison of the Amounts of Reducing Sugars between Pretreatment and Hydrolysis at 10FPUcellulase/g

P/Size(µm)	Pretreatment	GLU (P)	GLU(H)	XYL(P)	XYL(H)
200	ACID	13.11	4.36	0.11	0.08
200	SPORL	1.13	10.19	0.16	0.12
200	AL	1.08	9.03	0.18	0.08
350	ACID	10.01	3.86	0.07	0.05
350	SPORL	1.12	9.12	0.11	0.09
350	AL	0.92	6.93	0.11	0.05
450	ACID	9.52	2.60	0.09	0.05
450	SPORL	0.85	7.04	0.11	0.08
450	AL	0.64	5.85	0.19	0.03

Table 6. Concentration (g/l) of Reducing Sugar in Pretreatment Liquor

Particle Size	Dilute Acid		SPORL		Alkaline Wet Oxidation	
	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose
200µm	**10.32**	**0.14**	**1.65**	**0.18**	**1.64**	**0.23**
350µm	**9.85**	**0.06**	**1.46**	**0.08**	**0.90**	**0.12**
450µm	**9.75**	**0.05**	**0.93**	**0.09**	**0.88**	**0.10**

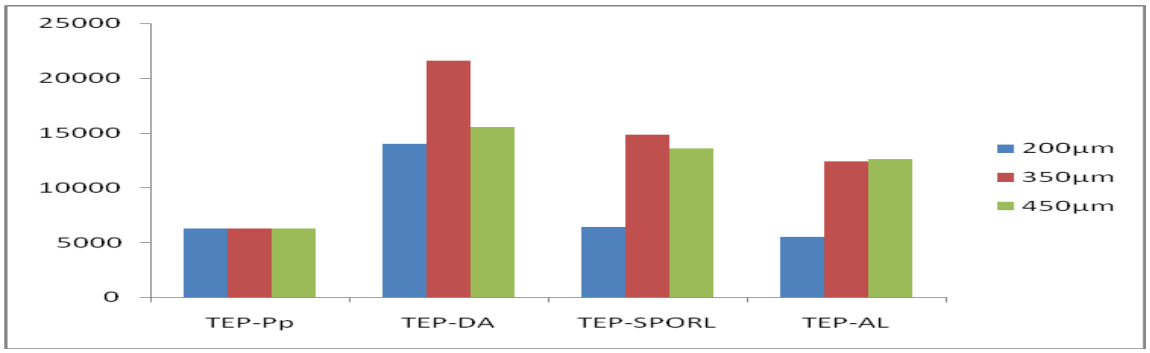

Figure 3. Variation of Amounts (ppm) of Total Extractable Polyphenolics in *Pernnisetum purpureum* with Particle Sizes (µm) for different Pretreatment methods

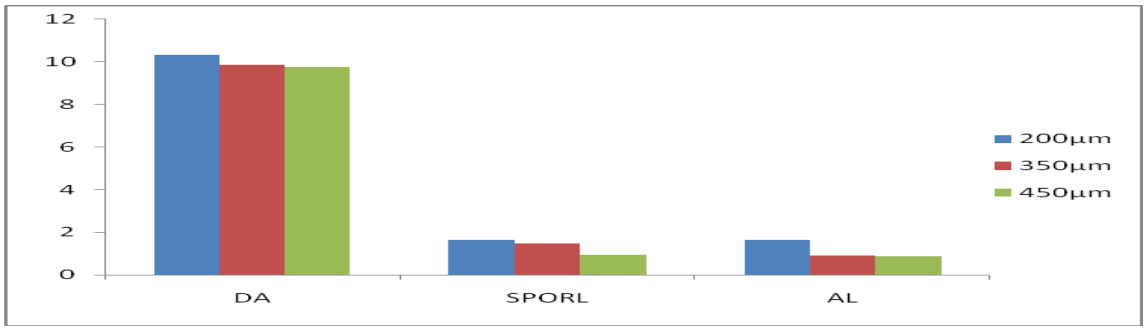

Figure 4. Variation of Amounts (g/L) of Glucose in *Pernnisetum purpureum* Pretreated Liquors with different Particle Sizes.

25.43g/L glucose was produced at the end of the 12hours residence time of hydrolysis and 44ml of 50g/l solution of glucose was used in the preparation of the fermentation medium. The fermentation of hydrolysate with 15FPUcellulase/g cellulose gave 8.75g/L, 10.55g/L and 13.88g/L concentrations of ethanol at the end of 24hours, 48hours and 72hours respectively. ANOVA on Table 4, showed that the amounts of unconverted glucose did not vary significantly with grass type, particle size and residence time of hydrolysis and the amount of ethanol formed was also insignificance particularly to sources combined with particle sizes and

residence time during fermentation; these could be due to the effects of enzymatic inhibitors, which are generated as reactions proceed and as well induced toxic effects to the enzymes.

Tables 7. Concentration (g/l) of Reducing Sugars during Hydrolysis as well as Ethanol and unconverted Glucose during Fermentation.

HYDROLYSIS			FERMENTATION		
Time	**Glucose**	**Xylose**	**Time**	**Glucose**	**Ethanol**
End of 4hrs	14.78	0.19	End of 24hrs	16.63	8.75
End of 8hrs	18.79	0.22	End of 48hrs	7.51	10.55
End of 12hrs	25.43	0.30	End of 72hrs	3.51	13.88

The difference between the ethanol yield EY_1 and EY_2 (EY_2>EY_1) for fermentation at 10FPU/g was 23.67% while that of 15FPU/g with stirring was 8.8%, these results indicated that fermentation is efficient at the higher enzyme loading with stirring. Aden *et al.*,(2002) reported that the higher enzyme loading gives higher yield as well as lower production cost. This was also confirmed with the amounts obtained for enzymatic convertibility of cellulose (ECC) and enzymatic convertibility of glucose (ECG) 20.88% and 18.72% respectively for hydrolysis at15FPU/g, while that of 10FPU/g gave 28.92% and 25.92% respectively.

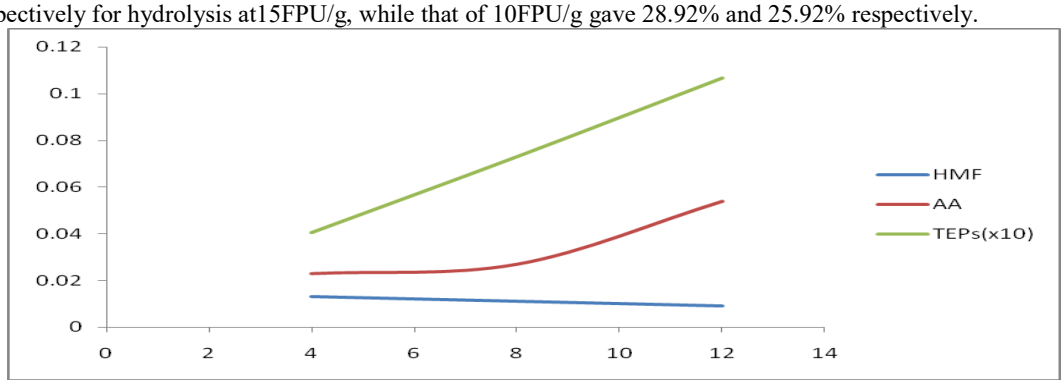

Figure 5. Concentration (g/L) of Enzymatic Recalcitrance against Time (hour) of Hydrolysis

Inhibitive effects of the enzymes in this study at the hydrolysis stage could be attributed to the presence of 5-hydroxymethylfurfural, total phenolics and acetic acid (Figure5 and Table8). 5-hydroxymethyl furfural decreases from the end of 4 hours (0.013g/L) during hydrolysis to an infinitesimal amount during the end of fermentation (72hours), unlike the case of TEPs and acetic acid which showed steady increased from pretreatment stage through fermentation stage. The inhibition of the enzyme from yeast is most likely due to the presence of phenolic compounds, this is because the amount of TEPs analyzed at the end of hydrolysis (12hours) was 1.066g/L, which is higher than the recommended amount of <0.1g/L. HMF and acetic acid at the end of hydrolysis were 0.009g/L and 0.0054g/L respectively and their maximum allowable concentration for ethanol fermentation are <0.25g/L and 3g/L respectively (Klinke and Thomson, 2004).

Tables 8. Amounts (g/l) of Recalcitrance during Hydrolysis as well as Fermentation Processes.

HYDROLYSIS				FERMENTATION			
Time	**HMF**	**AA**	**TEPs**	**Time**	**HMF**	**AA**	**TEPs**
End of 4hrs	0.013	0.023	0.404	End of 24hrs	0.007	0.198	0.871
End of 8hrs	0.011	0.027	0.729	End of 48hrs	0.005	0.220	1.181
End of 12hrs	0.009	0.054	1.066	End of 72hrs	ND	0.330	1.310

Total extractable phenolics increases from 0.63% in the crude grass biomass to 1.29% after pretreatment, during the hydrolysis stage of reaction the amounts of TEPs were 0.40g/L , 0.129g/L and 1.066g/L for the end of 4hours, 8hours and 12hours respectively. Further increased was observed during the fermentation stage of reaction as the amount at the end of retention time of 72hours was 1.310g/L. The amounts of acetic acid formed also followed steady increment from the hydrolysis stage across the fermentation stage as shown on Figure5 as well as Table8. Table12 shows a correlation between the amounts of these recalcitrance in both hydrolysis and fermentation stages of production; It was observed that in all cases, acetic acid was not correlated with the other two recalcitrance, while the amounts of hydroxymethylfurfural and total extractable polyphenolics correlated with each other at $p \leq 0.05$ and $p \leq 0.01$. This could be an indication that Lignin and hemicelluloses are the major precursors of organic inhibitors during cellulosic ethanol production.

Table 9. Evaluation (%) of Data Generated from Analyses

Evaluated Data	Amount (%)	
	10FPU/g	15FPU/g
Potential Glucose in Pretreated Biomass(PGP)	61.37	94.13
Theoretical ethanol yield from initial amount of glucose (EY_1)	65.57	78.26
Theoretical ethanol yield from converted amount of glucose (EY_2)	89.24	87.06
Enzymatic Convertibility of Cellulose (ECC)	20.88	28.92
Enzymatic Convertibility of Glucose (ECG)	18.72	25.92

$$^{1}PGP = \frac{\text{Amount of ethanol after fermentation}}{\text{Amount of cellulose after pretreatment}} \times 100\% \quad \text{----------(7)}$$

$$^{2}EY_1 = \frac{\text{Amount of experimental ethanol (g/L)}}{\text{Theoretical ethanol from the amount of glucose before fermentation (g/L)}} \times 100\% \quad \text{----------(8)}$$

$$^{3}EY_2 = \frac{\text{Amount of experimental ethanol (g/L)}}{\text{Theoretical ethanol from the amount of converted glucose during fermentation (g/L)}} \times 100\% \quad \text{----------(9)}$$

$$^{4}ECC = \frac{\text{(Conc. of final ethanol - Conc. of initial ethanol) g/L}}{\text{(Amount of cellulose after pretreatment X 0.57) g/L}} \times 100\% \quad \text{----------(10)}$$

$$^{5}ECG = \frac{\text{(Conc. of final ethanol - Conc. of initial ethanol) g/L}}{\text{(Amount of glucose after hydrolysis X 0.511) g/L}} \times 100\% \quad \text{----------(11)}$$

[[2and3] Manzanares *et al.,* 2003; [1] Stroeve *et al.,*2009 ; [4and5]Gouveia, Soares, and Wanderley, 2014]

Response optimization was carried out with Mintab Response optimization software and this gave results as shown on Tables 13, 14 and 15. On Table 13, both the amounts of glucose degraded during pretreatment processes (GlU) and the amounts of glucose hydrolyzed (GlU-H) were considered with other parameters by minimizing the former and maximizing the latter, the results obtained showed that the process is optimized with; SPORL pretreatment method, at 450µm particle size and 12hours of hydrolysis. Table 14 which excludes the amounts of glucose degraded (GlU) during hydrolysis, showed similar result for optimization and the combination of the data obtained at the final stage of production(fermentation reaction) in Table 15 still showed similar results.

Table 10. Duncan's Multiple Test showing the Significance of Lignocellulosic Biomass during Pretreatment.

HEMICELLULOSE				CELLULOSE					LIGNIN				
Duncan				Duncan					Duncan				
		Subset				Subset					Subset		
PRETREATMENT METHOD	N	1	2	PRETREATMENT METHOD	N	1	2	3	PRETREATMENT METHOD	N	1	2	3
ACID	18	8.54		AL	18	45.48			AL	18	0.97		
AL	18		38.55	SPORL	18		49.78		SPORL	18		1.99	
SPORL	18		38.58	ACID	18			61.68	ACID	18.			2.72
Sig.		1.00	0.94	Sig.		1.00	1.00	1.00	Sig.		1.00	1.00	1.00

Table 11. Regression Analyses with Equations to Predict the Relationship between variables at the Pretreatment Stage.

Regression Equation: CEL= 64.90 -0.4409HEM	Coefficients				
	Term	Coef	SE Coef	T-Value	P-Value
	Constant	64.90	1.72	37.70	0.000
	HEM	-0.4409	0.0533	-8.28	0.001
Regression Equation: XYL= 0.0729+0.001533HEM	Coefficients				
	Term	Coef	SE Coef	T-Value	P-Value
	Constant	0.0729	0.0152	4.81	0.000
	HEM	0.001533	0.000469	3.27	0.002
Regression Equation: GLU= 11.986 -0.2727HEM	Coefficients				
	Term	Coef	SE Coef	T-Value	P-Value
	Constant	11.986	0.467	25.68	0.000
	HEM	-0.2727	0.0144	-18.88	0.000
Regression Equation: XYL= 0.3427 -0.004320CEL	Coefficients				
	Term	Coef	SE Coef	T-Value	P-Value
	Constant	0.3427	0.0343	9.99	0.000
	CEL	-0.00432	0.000646	-6.68	0.000
Regression Equation: GLU=-14.20 + 0.3517 CEL	Coefficients				
	Term	Coef	SE Coef	T-Value	P-Value
	Constant	-14.20	2.61	-5.45	0.000
	CEL	0.3517	0.0491	7.16	0.000

Table 12. Correlations between Composition of Recalcitrance during Hydrolysis and Fermentation Stages at 15FPU/g cellulose.

		HMF-H	AA-H	TEPs-H	HMF-F	AA-F	TEP-F
HMF-H	Pearson Correlation	1	-.382	-.971**	.837**	-.417	-.840**
	Sig. (2-tailed)		.311	.000	.005	.264	.005
AA-H	Pearson Correlation	-.382	1	.427	-.409	.894**	-.004
	Sig. (2-tailed)	.311		.251	.274	.001	.991
TEPs-H	Pearson Correlation	-.971**	.427	1	-.890**	.452	.827**
	Sig. (2-tailed)	.000	.251		.001	.222	.006
HMF-F	Pearson Correlation	.837**	-.409	-.890**	1	-.359	-.698*
	Sig. (2-tailed)	.005	.274	.001		.343	.036
AA-F	Pearson Correlation	-.417	.894**	.452	-.359	1	-.065
	Sig. (2-tailed)	.264	.001	.222	.343		.869
TEPs-F	Pearson Correlation	-.840**	-.004	.827**	-.698*	-.065	1
	Sig. (2-tailed)	.005	.991	.006	.036	.869	

** Correlation is significant at the 0.01 level (2-tailed). ; –H During Hydrolysis.

* Correlation is significant at the 0.05 level (2-tailed). ;– F During Fermentation.

Table 13. Response Optimization for Pretreatment and Hydrolysis: GLU-H, TEP, ASH, GLU, XYL, CEL, HEM

Parameters					
Response	Goal	Lower	Target	Upper	Weight	Importance
GLU-H	Maximum	0.97		27.22	1	1
TEPs	Minimum	5.50		21.600	1	1
ASH	Minimum	1.34		3.940	1	1
GLU	Minimum	0.64		13.110	1	1
XYL	Minimum	0.04		0.230	1	1
CEL	Maximum	34.58		69.48	1	1
HEM	Maximum	4.52		51.82	1	1

Solution

Grass	Size	Method	Time	Loading	GLU-H Fit	TEPs Fit	ASH Fit	GLU Fit	XYL Fit	CEL Fit	HEM Fit	Composite Desirable
Pp	450	SPORL	Time3	15FPU	25.43	12.4000	1.52	0.85	0.11	54.1	35.76	0.744382

Table 14 . Response Optimization for Pretreatment and Hydrolysis: GLU-H, TEP, ASH, CEL, HEM

Parameters					
Response	Goal	Lower	Target	Upper	Weight	Importance
GLU-H	Maximum	0.97		27.22	1	1
TEP	Minimum	5.50		21.600	1	1
ASH	Minimum	1.34		3.940	1	1
CEL	Maximum	34.58		69.48	1	1
HEM	Maximum	4.52		51.82	1	1

Solution	Grass	Size	Method	Time	Loading	GLU-H Fit	TEPs Fit	ASH Fit	CEL Fit	HEM Fit	Composite Desirability
1	Pp	450	SPORL	Time3	15FU	25.43	12.4000	1.52	54.1	35.76	0.730387

Table15. Response Optimization for Pretreatment, Hydrolysis and Fermentation: TEPs-F, AA-F, HMF-F, ETH, U-GLU, TEPs, AA, HMF, GLU-F, TEP, ASH, GLU

Parameters					
Response	**Goal**	**Lower**	**Target**	**Upper**	**Weight**	**Importance**
TEPs-F	Minimum	0.871		3.298	1	3.0
AA-F	Minimum	0.189		3.000	1	3.0
HMF-F	Minimum	0.000		3.000	1	3.0
ETH	Maximum	0.731		15.930	1	4.0
GLU-U	Minimum	0.781		19.800	1	3.5
TEPs	Minimum	0.404		3.000	1	2.0
AA	Minimum	0.018		3.000	1	2.0
HMF	Minimum	0.006		3.000	1	2.0
GLU-H	Maximum	0.970		27.220	1	3.5
TEPs	Minimum	5.500		21.600	1	1.0
ASH	Minimum	1.340		3.940	1	1.0
GLU	Minimum	0.640		13.110	1	2.5
XYL	Minimum	0.040		0.230	1	2.5
CEL	Maximum	34.580		69.480	1	1.0
HEM	Maximum	4.520		51.820	1	1.0

Solution	Grass	Size	Method	Time	Loading	TEPs-F Fit	AA-F Fit	HMF-F Fit	ETH Fit	GLU-U Fit	TEPs Fit
1	Pp	450	SPORL	Time3	15FPU	1.331	0.33	0.00	13.88	3.51	1.066

Solution	AA Fit	HMF Fit	GLU-H Fit	TEP Fit	ASH Fit	GLU Fit	XYL Fit	CEL Fit	HEM Fit	Composite Desirability
1	0.054	0.009	25.43	12.40	1.52	0.85	0.11	54.1	35.76	0.851801

4.0 Conclusion

The dominant inhibitors to enzymatic activities in this study are organic compounds and three of them were identified; 5-Hydroxymethylfurfural, phenols and acetic acid. Phenolic compounds are the most challenging inhibitors from the use of this biomass for bioethanol production.

Sulphite pretreatment to overcome the effects of lignocellulose (SPORL) is likely to be most suitable for minimizing the formation of these recalcitrance and maximization of carbohydrates for this process.

Reducing biomass particle sizes did not have overall positive effect on the production and combination of biomass particle sizes with other sources at the hydrolysis and fermentation stages of reactions showed no significance difference to test of variation.

The evaluated amounts of potential glucose in pretreated Biomass(PGP) and theoretical ethanol yield from initial amount of glucose (EY_1); 94.13% and 78.26% respectively for production at enzyme loading of 15FPU/g and stirring during hydrolysis, showed that an ethanol biorefinery could be established in the study area.

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