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PRETREATMENT OF MILLET HUSK USING ALKALINE HYDROGEN PEROXIDE TO ENHANCE ENZYMATIC HYDROLYSIS FOR REDUCING SUGAR PRODUCTION

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ABSTRACT
The effectiveness of alkaline hydrogen peroxide as a suitable choice of pretreatment for the conversion of millet husk to reducing sugars using cellulase enzyme for hydrolysis and subsequent ethanol production was determined. The effects of three variables on reducing sugar production from millet husk were determined using one factor at a time (OFAT) method namely; peroxide concentration, pretreatment time and pretreatment temperature. From the results, it was observed that a significant *(P<0.05)* amount of reducing sugars were lost during pretreatment of millet husk. The untreated group which was only physically pretreated (milled) however yielded a significantly higher *(P<0.05)* reducing sugar concentration of 10.67mg/ml after enzymatic hydrolysis while the highest reducing sugar concentration of 4.82mg/ml was obtained using 0.375%v/v peroxide concentration for 60minutes at 25^0C. Therefore, pretreatment of biomass with alkaline hydrogen peroxide may be more suitable for feedstock with high lignin contents than millet husk.

Keywords: pretreatment, cellulose, hydrolysis, optimization, millet husk, hydrogen peroxide

INTRODUCTION

In order to improve the standard of living, there is a need to search for sustainable energy sources to meet up with the increased demand for energy worldwide. Fossil fuels have been used for a long time as the main sources of energy. However, its use has been associated with environmental pollution and global warming. As a result, a search for more environmentally favorable, renewable and sustainable source of energy is on the rise. Liquid bio-fuels contribute about 40% of energy consumption worldwide and have been said to contribute to a reduction in greenhouse gases, increased job creation and economic development (Mohd *et al*., 2017).

Lignocelluloses biomasses are renewable and abundant resources which are used for the production of bio based materials such as bioethanol. Lignocelluloses feed stocks are mainly made up of a complex structure of cellulose, hemicelluloses and lignin and a small extractive and ash fractions which are often resistant to enzymatic hydrolysis (Kim *et al*., 2016). Cellulose and hemicelluloses hydrolysis releases fermentable sugars for subsequent ethanol production. One of the best technologies used for the conversion of lignocellulosic biomass to fermentable sugars involves the use of enzymes due to its low energy requirement and less environmental pollution. One major problem however is the low accessibility of cellulose to cellulase enzyme because of the rigid association of cellulose with lignin. This often causes setbacks during the hydrolysis step. Therefore, an efficient way of disrupting the rigid structure of lignin to improve cellulose accessibility to enzymatic attack remains a critical aim of pretreatment (El-Naggar, 2014).

There are different types of pretreatments being employed which are; chemical, physical and biological methods or a combination of them (Dutra *et al*., 2017). In all, pretreatments have advantages and disadvantages. It is therefore important to create a criterion for the cost effective pretreatment given that this stage is one of the most expensive for lignocellulosic ethanol production (Dutra *et al*., 2017). For pretreatment efficiency, it is required that the following requirements are met: the production of reactive cellulose fibers, little or no loss of cellulose and hemicelluloses fractions, absence of possible inhibitors that may be generated during the hydrolysis and fermentation steps and least energy use (Dutra *et al*., 2017).

Most of the various the different pretreatment methods currently in use are conducted under high energy and/or pressure which may not be cost effective. More successful pretreatment methods which can be carried out at lower temperatures and pressure are still under investigation. This has caused an increased interest in using alkaline hydrogen peroxide (AHP) as a pretreatment choice for the pretreatment of different feed stocks. AHP is an oxidative pretreatment method. It exerts its effects by delignification of the lignocelluloses biomass thereby increasing the accessibility of cellulose to the hydrolyzing effects of cellulase enzyme. This pretreatment requires low-energy and does not generate inhibitors like hydroxymethyl furfural and furfural (Dutra *et al*., 20 17).

Therefore, for pretreatment efficiency to be achieved using alkaline hydrogen peroxide, the effects of the most important factors must be optimized. The main variables include: (i) the pretreatment time; (ii) H_2O_2 concentration in the pretreatment solution; (iii) pretreatment temperature (Dutra *et al*., 2017).

This research focused on determining the effects of: (i) H_2O_2 concentration in the pretreatment solution; (ii) pretreatment time; and (iii) pretreatment temperature on reducing sugar

production from millet husk using cellulase enzyme for hydrolysis.

MATERIALS AND METHODS

Preparation of raw biomass

Millet husks were collected from domestic wastes and sun dried. The samples were subjected to physical pretreatment (grinding) to reduce the size and increase the surface area of contact of the biomass. The sample was then used for compositional analysis and chemical pretreatment.

Compositional analysis of biomass

Chemical composition of yam peel (lignin, hemicelluloses and cellulose) was determined before and after pretreatment according to the method of Hernawan *et al*., (2017). 1g (a) of sample was added to 150ml of water and boiled for 1hour at 100°C. It was washed with 300ml of hot water, dried and weighed (b). To the dried residue, 150ml of 1N H_2SO_4 was added and boiled at 100°C for 1hour. It was then washed with 300ml of hot water, dried and weighed (c).10ml of 72% H_2SO_4 was added to the dried residue and soaked at room temperature for 4 hours and then filtered. 150ml of 1N H_2SO_4 was added to the residue and boiled in a water bath for 1 hour, filtered, washed with 800ml of water and dried at 105°C and weighed (d). The residue was ashed and weighed (e). The % of cellulose, hemicelluloses and lignin were determined using the formula below:

Lignin content = (d-e)/a*100

Cellulose content = (c-d)/a*100

Hemicellulose content = (b-c)/a*100

Optimization of some biomass pretreatment parameters using one factor at a time (OFAT) method

Millet husk was subjected to physical and chemical pretreatments. Physical pretreatment was achieved through grind milling, until a fine powder was obtained.

Pretreatment was carried out according to the method of Diaz *et al.,* (2013) with some modifications in the concentration of pretreatment solution, where lower concentrations were used. Millet husk (3g) was pretreated, in 50ml peroxide solutions at different concentrations (0.375, 0.75, 1.5, 3% v/v), depending on the experiment in 250ml flasks, adjusting the pH to11.5 with NaOH. Each experiment was carried out in triplicate.

Flasks were covered with aluminum foil and incubated in a water bath at 90°C for two (2) hours. The solid residue was collected by filtration and washed thoroughly until neutral pH of the filtrate was obtained. The residue was dried at 60°C overnight. After drying, it was weighed to determine mass loss, which corresponds to the lignin content and other solubilized compounds.

After each pretreatment, a liquid sample (pretreatment supernatant) was taken and tested for the presence of reducing sugars using DNS method. Before the analysis, samples were centrifuged for ten (10) minutes at 5000 rpm and the pH was neutralized by adding 2% v/v H_2SO_4. The same procedure as above was repeated for optimizations of pretreatment time (30, 60, 90 and 120 minutes) and temperature (25, 50, 75 and 90°C) where time was varied while concentration and temperature were kept constant. Likewise, temperature was varied and concentration and time were kept constant.

Analytical methods

To determine the chemical pretreatment efficiency, reducing sugar concentrations were determined colorimetrically after pretreatment (pretreatment supernatant) and after subsequent enzymatic hydrolysis (with cellulase enzyme) using dinitrosalicylic (DNS) method.

Enzyme Hydrolysis

Enzymatic hydrolysis was conducted according to the method of Karagoz and Ozkan (2014). Pretreatments were evaluated through the enzyme hydrolysis of pretreated millet husk by analyzing the reducing sugars produced after the hydrolysis of cellulose and hemicelluloses using DNS method. Cellulase enzyme (3mg/ml/g of dry substrate) was used for hydrolysis with citrate-Na_2HPO_4 buffer (0.05M, pH5.0) (Li *et al*., 2019). The washed and dried residues of millet husk obtained after pretreatment were hydrolyzed in 250ml flasks at a temperature of 50°C with a solid loading of 5% (w/v). Enzymatic hydrolysis was performed on an orbital shaker at 150 rpm.

Dinitro salicylic acid (DNSA) method (Miller, 1959).

Solution A: DNS (1g) was dissolved in 2M NaOH (20ml). Solution B: Sodium and potassium tartrate tetrahydrate (Rochelle salt) (30g) was dissolved in distilled water (50ml). Solutions A and B were mixed and heated in a water bath to homogenize. The volume was completed to 100ml with distilled water. The solution was stored in an amber bottle at 4°C.

Test tubes were labeled as blank and test**.** Dilutions of glucose standards with concentrations of 40, 80, 120, 160, 200 µg per 200 µl by transferring respective amounts of glucose from the standard glucose solution (1mg/ml) and adjusting it to a total volume of 200 µl by adding distilled water were made (for standard curve)**.** Samples obtained each from pretreatment supernatant and hydrolysis of millet husk were added (200 µl). DNSA reagent (1ml) was added to all the test tubes and mixed well. It was kept in a boiling water bath for 15minutes. Absorbance of the blank was read first at 540nm using 1ml cuvettes and it was made zero. The absorbance of all the tubes was read (Miller, 1959). The cuvettes were rinsed each time after taking the absorbance. A standard curve was plotted for absorbance at 540nm on Y axis versus concentration of glucose in µg/200µl on X axis. The value of unknown was recorded from the graph corresponding to the absorbance reading of the test sample. Sugar concentration was calculated using the following formula: Sugar concentration in Test sample = concentration of unknown in µg/200µl x 5µg/ml.

Table I: Effect of varied H_2O_2 concentration on the chemical composition and reducing sugar concentration (mg/ml) produced from millet husk pretreated at constant time (120 minutes) and temperature (90°C)

[H_2O_2] %v/v	Solubilized compounds (g)	Reducing sugar concentration (mg/ml) in pretreatment hydrolysate	Reducing sugar concentration (mg/ml) after enzymatic hydrolysis	Hemicellulose (%)	Lignin (%)	Cellulose (%)
Untreated	0.00$^{a, b, c, d}$	0.00 $^{a, b, c, d}$	10.67±0.05 $^{a, b, c, d}$	27.70 ± 0.1$^{a, b, c, d}$	20.35 ±0.07 $^{a, b, c, d}$	37.81 ± 0.12$^{a,b, c, d}$
0.375	2.29±0.02$^{a, e}$	4.17±0.18 $^{a, e}$	3.78±0.05 $^{a, e}$	19.53± 0.98$^{a, e}$	17.33± 0.20$^{a, e}$	31.03± 0.05$^{a, e}$
0.75	2.29±0.03$^{b, f}$	5.28±0.17 $^{b, e, f}$	2.76±0.02 $^{b, e, f}$	17.94± 0.1 $^{b, e, f}$	18.60± 1.56 $^{b, f}$	28.27± 0.2$^{b, e, f}$
1.5	2.40±0.02$^{c, e, f, g}$	5.59±0.07 $^{c, e, g}$	2.63±0.57 $^{c, e, f, g}$	12.71± 0.09 $^{c, e, f, g}$	16.12± 0.21 $^{c, f, g}$	24.74± 0.1 $^{c, e, f, g}$
3.0	2.46±0.02$^{d, e, f, g}$	6.61±0.13 $^{d, e, f, g}$	1.59±0.33 $^{d, e, g}$	10.11± 0.3 $^{d, e, f, g}$	14.07± 0.48 $^{d, e, f, g}$	15.71± 0.2 $^{d, e, f, g}$

Values in the Table represent mean ± SD, similar superscripts along columns indicate significant differences between groups at $P < 0.05$, n =3 for each group.

Table II: Effect of varied pretreatment time (minutes) on the chemical composition and reducing sugar concentration (mg/ml) produced from millet husk pretreated with constant H_2O_2 concentration (0.375% v/v) and temperature (90°C)

Time (minutes)	Solubilized compounds (g)	Reducing sugar concentration (mg/ml) in pretreatment hydrolysate	Reducing sugar concentration (mg/ml) after 48hrs enzymatic hydrolysis	Hemicellulose (%)	Lignin (%)	Cellulose (%)
Untreated	0.00$^{a, b, c, d}$	0.00 $^{a, b, c, d}$	10.67±0.05 $^{a, b, c, d}$	27.70 ± 0.1$^{a, b, c, d}$	20.35 ±0.07 $^{a, b, c, d}$	37.81 ± 0.12$^{a,b, c, d}$
30	1.29±0.03$^{a, e}$	2.21±0.10 $^{a, e}$	3.49±0.55 $^{a, e}$	26.33± 1.0$^{a, e}$	19.35± 0.39$^{a, e}$	34.56± 0.3$^{a, e}$
60	1.85±0.04$^{b, e, f}$	4.24±0.03 $^{b, e, f}$	3.74±0.41 $^{b, e, f}$	24.07± 0.07 $^{b, e, f}$	17.40± 0.3 $^{b, e}$	31.12± 0.25$^{b, e}$
90	2.32±0.03$^{c, e, f}$	4.59±0.04 $^{c, e, f, g}$	3.27±0.81 $^{c, e, f, g}$	22.71± 0.19 $^{c, e, f, g}$	17.22± 0.1 $^{c, e}$	31.00± 0.45 $^{c, e}$
120	2.28±0.03$^{d, e, f}$	4.71±0.06 $^{d, e, f, g}$	2.73±0.75 $^{d, e, f, g}$	20.50± 0.17 $^{d, e, f, g}$	17.30± 0.18 $^{d, e}$	30.86± 0.25 $^{d, e}$

Values in the Table represent mean ± SD, similar superscripts along columns indicate significant differences between groups at $P < 0.05$, n =3 for each group.

Table III: Effect of varied pretreatment temperature (°C) on the chemical composition and reducing sugar concentration (mg/ml) produced from millet husk pretreated with constant H_2O_2 concentration (0.375% v/v) and time (60 minutes)

Temperature (°C)	Solubilized compounds (g)	Reducing sugar concentration (mg/ml) in pretreatment hydrolysate	Reducing sugar concentration (mg/ml) after enzymatic hydrolysis	Hemicellulose (%)	Lignin (%)	Cellulose (%)
Untreated	0.00$^{a, b, c, d}$	0.00 $^{a, b, c, d}$	10.67±0.05 $^{a, b, c, d}$	27.70 ± 0.10$^{a, b, c, d}$	20.35 ±0.07 $^{a, b, c, d}$	37.81 ± 0.12$^{a, b, c, d}$
25	1.24±0.04$^{a, e}$	1.12±0.1 $^{a, e}$	4.81±0.08 $^{a, e}$	24.33± 0.50$^{a, e}$	19.90± 0.08 $^{a, e}$	35.62± 0.42$^{a, e}$
50	1.54±0.03$^{b, f}$	2.32±0.08 $^{b, e, f}$	4.59±0.11 $^{b, e, f}$	22.08± 0.09 $^{b, e, f}$	17.31± 0.32 $^{b, e}$	32.17± 0.31$^{b, e, f}$
75	2.15±0.04$^{c, e, f}$	3.65±0.09 $^{c, e, f, g}$	4.37 ±0.61 $^{c, e, f, g}$	21.90± 0.05 $^{c, e, g}$	17.33± 0.33 $^{c, e}$	31.12± 0.42 $^{c, e, f, g}$
90	2.36±0.05$^{d, e, f}$	4.21±0.08 $^{d, e, f, g}$	3.71±0.55 $^{d, e, f, g}$	20.43 ± 0.22 $^{d, e, f, g}$	17.30± 0.15$^{d, e}$	30.44± 0.44$^{d, e, f, g}$

Values in the Table represent mean ± SD, similar superscripts along columns indicate significant differences between groups at $P < 0.05$, n =3 for each group.

DISCUSSIONS

The effect of pretreatment with alkaline hydrogen peroxide on the chemical compositions and reducing sugar concentrations after 48 hours enzymatic hydrolysis of millet husk was determined.

Hydrogen peroxide (H_2O_2) concentration has an important role in improving the biomass accessibility to cellulase enzyme hydrolysis. The concentrations of hydrogen peroxide used for the pretreatment of lignocelluloses biomass may vary widely which depends on the nature of biomass, whether it contains more amorphous or crystalline cellulose. From the results of different studies, it was observed that concentrations of hydrogen peroxide used for pretreatment range between 1 to 10% (v/v). The main challenge encountered when using H_2O_2 as a pretreatment choice is associated with the adequate concentration that should be used. High concentrations have been reported to be more efficient at short periods of time (Rabelo *et al*., 2011). Saha and Cotta (2007) determined the effects of H_2O_2 concentration in the pretreatment of rice husks for increasing reducing sugar concentration using enzymes. They reported obtaining a high concentration of reducing sugars when 7.5% (v/v) H_2O_2 was used for pretreatment. At a concentration of 10% (v/v) however, they reported a decrease in the concentration of reducing sugars. It has also been reported that pretreatment of biomasses that have an initial sugar concentration with a high concentration of H_2O_2, such as microalgae, might result in the degradation of polysaccharides leading to subsequent byproducts and inhibitors production. Most of the pretreatment studies carried out were conducted using high concentrations of H_2O_2, therefore, it is important to determine the optimum pretreatment concentrations for different types of biomass, preferably using lower H_2O_2 concentrations, because this parameter plays an important role in determining the cost effectiveness of the pretreatment method (Juarez *et al*., 2016).

Table I shows the results of the chemical compositional analysis of millet husk before and after pretreatment with varied concentrations of alkaline hydrogen peroxide solution (pH11. 5) for 120minutes at 90^{o}C under static condition. In order to study the sole effects of pretreatment concentration, pretreatment time and pretreatment temperature on the chemical composition and reducing sugar production from millet husk, a control group which was only physically pretreated (milled) was also enzymatically hydrolyzed for 48hours.

It was observed that, the untreated millet husk group had significantly *(P<0.05)* higher concentrations of hemicelluloses, cellulose and lignin than the other pretreated groups (tables I, II and III). This corresponds with loss in weights of millet husk which was also observed to increase with increase in hydrogen peroxide concentration with the highest loss in weight observed in the group pretreated with 3.0%v/v H_2O_2. This finding corresponds with that of Diaz *et al.*, (2013) where rice husks were pretreated with alkaline hydrogen peroxide and the loss in weights of rice husks increased with increase in hydrogen peroxide concentration. However, in this study (table I), the untreated group was observed to release a significantly high *(P<0.05)* concentration of reducing sugars than the groups pretreated with varied concentrations of AHP. The high concentration of reducing sugars produced in the untreated group (table IV) may be due to no loss in cellulose and hemicelluloses fractions and subsequently no loss of reducing sugars during pretreatment, as was observed (table I). The significant *(P<0.05)* differences observed in the reducing sugar concentrations between the alkaline hydrogen peroxide pretreated groups and the untreated group may be due to loss of some fractions of cellulose and hemicelluloses during pretreatment and washing of millet husk (tables I and IV). Lignocellulosic biomass compositions of plants vary depending on their locality and seasonal changes (Joshi *et al*., 2018). Therefore, reducing sugars produced after enzymatic hydrolysis of biomass may also vary. The group pretreated with 0.375%v/v hydrogen peroxide concentration was however observed to liberate a significantly *(P<0.05)* higher reducing sugar concentration of 3.78±0.05mg/ml (table I) than the other groups after 48hrs enzymatic hydrolysis and was therefore used for further optimizations.

The time taken to pretreat a biomass which is the contact time of the biomass with the pretreatment solution has been reported to vary depending on the choice of pretreatment method. Pretreatment of lignocelluloses feed stocks with alkaline hydrogen peroxide have been reported to vary between 1 to 24hours. This depends on the biomass and the conditions being optimized. Saha and Cota, (2006) reported that wheat straw pretreated with 2.15% H_2O_2 (v/v) which had a total solid content of 8.6% (w/v), at a temperature of 24°C and pH of 11.5, an increase from 3 to 24 h in the pretreatment time yielded a higher concentration of reducing sugars after enzymatic hydrolysis.

In this study, pretreatment time was also observed to significantly *(P<0.05)* decrease the cellulose, hemicelluloses and lignin contents (table II). It was also observed that the concentration of reducing sugars liberated in the pretreatment solution increased with an increase in pretreatment time during the pretreatment. However, the group pretreated for 60minutes produced a significantly *(P<0.05)* higher reducing sugar concentration of 3.74±0.41mg/ml after enzymatic hydrolysis than the groups pretreated for 30, 60 and 90 minutes. This may be due to the longer contact time allowed between the biomasses and AHP which may have allowed for the sufficient oxidizing effects of H_2O_2 on the biomasses (Dutra *et al.*, 2017). The free radicals produced from the dissociation of H_2O_2 may also be responsible for the dissociation of cellulose and hemicelluloses fractions of and millet husk as observed in this study leading to higher reducing sugars concentration lost during pretreatment. Somilarly, Rabelo *et al.*, (2011) reported that pretreatment time was not significant in the release of sugars when high concentration of H_2O_2 was used for the pretreatment of sugarcane bagasse. However 60minutes pretreatment time was reported as the optimum. Nonetheless, Saha and Cotta, (2006) reported that wheat straw pretreated with 2.15% H_2O_2 (v/v) which had a total solid content of 8.6% (w/v), at a temperature of 24 °C and pH of 11.5, an increase from 3 to 24 hours in the pretreatment time yielded a higher concentration of reducing sugars after enzymatic hydrolysis. The best pretreatment time under which significantly high (*P<0.05*) concentration of reducing sugars was produced was 60minutes. Therefore, temperature optimization was carried out for 60 minutes.

The pretreatment temperature is an important factor which is directly associated with the cost effectiveness of the pretreatment method. Pretreatments using alkaline H_2O_2 were reported to be conducted at low temperatures ranging from 25-70°C. Pretreatment temperatures have been reported to have varying effects depending on the biomass being pretreated. In this study, pretreatment temperature was also observed to significantly *(P<0.05)* decrease the cellulose, hemicelluloses and lignin contents of millet husk pretreated at various temperatures (table III). It however significantly *(P<0.05)* increased the reducing sugar concentrations in the pretreated groups. High pretreatment temperature was observed to dissolve a significantly *(P<0.05)* higher mass of millet husk than the 25, 50 and 75°C pretreated groups. Thus, a significantly *(P<0.05)* high amount of reducing sugars may have been lost during pretreatment, recovery and washing of millet husk. After 48hrs of enzymatic hydrolysis, the highest reducing sugar concentration of 4.81±0.08mg/ml was released in the group pretreated for 25°C, which was nonetheless significantly *(P<0.05)* lower than the concentration produced in the untreated group (table III). This may be due to the oxidizing effects of H_2O_2 and the solubilizing effect of high temperatures on millet husk. The findings of Sun *et al*., (2000) supports that of this study, where it was reported that, at high pretreatment temperatures, 2% (w/v) H_2O_2 pH 11.5 and 12 h of reaction, rye straw biomass was observed to have an increased solubilization effect on the lignin and hemicelluloses fractions of the biomass. Contrarily, Rabelo *et al*., (2011) observed that high pretreatment temperatures had no effect in the release of reducing sugars when sugarcane bagasse was pretreated. The optimum conditions were reported as a temperature of 20°C, H_2O_2 concentration of 5% (v/v) H_2O_2, and 4% total solids (w/v) for 6 h.

This study supports the findings of Legodi *et al*., (2021), where it was reported that; chemical pretreatments increased the crystallinity and recalcitrance of feedstocks pretreated with varied chemicals, which lead to low release of sugars during enzymatic hydrolysis. Also, cellulose hydrolysis was reported to vary, based on the type of chemical pretreatment method employed (Legodi *et al*., 2021). According to Elzawawy *et al*., (2011), the concentration of reducing sugars produced during enzymatic hydrolysis was affected by the pretreatment and hydrolysis methods.

In all, the best conditions under which reducing sugars were produced from the chemically pretreated groups were; a hydrogen peroxide concentration of 0.375%v/v, pretreatment time of 60 minutes and pretreatment temperature of 25°C which was nevertheless significantly lower than the concentration produced in the untreated (milled) group.

CONCLUSION

From this work, it may be concluded that alkaline hydrogen peroxide had a negative effect when used as a pretreatment solution to enhance enzymatic hydrolysis of millet husk for reducing sugar production for subsequent ethanol production. This may be due to the chemical composition of millet husk and/or the oxidizing effects of hydrogen peroxide. Alkaline hydrogen peroxide may however be more effective in the pretreatment of feedstock that has a high lignin concentration.

Author's contributions

Zeenat Ibrahim Saulawa conducted the research.
Lawal Nura Co- supervised the research.
Muntari Bala Co- supervised the research.
Abdullahi Abdulkadir Imam Supervised the research.

Conflict of interest

No conflicts of interest were declared by the authors.

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