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Effect of prewashing silages of *Panicum virgatum* L. on carbohydrate composition, enzyme hydrolysis and yeast fermentation

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Abstract Ensilages of *Panicum virgatum* L. prewashed with hot water and limewater solution were evaluated for carbohydrates composition, efficiency of enzymes hydrolysis and products of yeast fermentation. Samples of three switchgrass varieties prewashed with and without limewater solution, rinsed with hot water, analysed for reducing sugar, glucans, xylan, arabinan and acid soluble lignin,, subjected to 1:4 ratio of Cellulase Cellobiase enzyme hydrolysis and fermentation by common yeast. Composition analysis revealed Bomaster variety contains reducing sugars and glucan significantly higher than varieties of Performer and Colony but it was equally comparable in xylan, arabinan and lignin contents. Enzyme hydrolysis converted xylan into solubles in silages prewashed with limewater solution with 62.21% efficiency compared to 48.47% in hot water washed silages. Fermentate of switchgrass produced low amount of ethanol which was attributed to lack of sufficient glucan, while the high yiled of acetate was attributed to high contents of xylan and arabinan. Prewashing silages with limewater washout and neutrilize silages, enhances enzyme hydrolysis pretreatment and impact yeast fermentation. Recommendation is to select yeast with ethanol fermentation from both glucan and xylan and arabinan, improvement in the conditions of fermentation of pentose sugars in the absence of sufficient glucan. Information generated in this study is useful in the process of prewashing as complement of ensiling as preservation of feedstock intended for ethanol production.

Keywords: *Panicum virgatum* L., Variety, Cellulose, Hydrolysis, Carbohydrates, Fermentation

Introduction

Among crops, corn entails higher demands as food and feed crops, that a UN moratorium favors the use of alternative or secondary feedstocks such as agriculture residues, cereals and grains and grass examples like wild switchgrass (Burns *et al*., 2008a; b).

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Genetic improvement of the wild specie *Panicum virgatum* L. lead to three new varieties namely; Bomaster (ST6-3E), Performer (ST6-3F) and Colony (ST6-I). Switchgrass is non-food commodity, adaptable to less fertile marginal land, can produce 12.6 oven dry Ton /ha/yr and sugar such as glucan average 30.95 % (Burns *et al.,* 2008a). It contains 20.60% lignin in lignocellulose that needs pretreatment to solubilize the carbohydrates in the grass. Pretreatment done such as the acid treatment of 1.5% sulfuric acid, steam treatment for 60 minutes, autoclaved at 121^0C with 18psi and the enzyme hydrolysis using Cellulases loaded at 30 FPU/g dry biomass and xylanase as supplements resulted into production efficiency of 91.8% of the enzyme pretreatment. The problem with the chemical pretreatment is furfural, a chemical by-product highly toxic to yeast. The incompletely neutralized chemical effluents disposal into soil and water prevented widespread industrial application (Mosier *et al*., 2007). Typical pre-treatments like physical, mechanical and chemicals or combinations aimed at input competitive process, environment friendly with production cost that should be less than 33% or cost reduction of at least 1US$ per gal cellulosic ethanol are practical goal of pretreatments like ensiling. Ensiling produces lactic acid that eventually reduced the pH to pH 4, a condition where acids that accumulated in the silages and acts as preservatives. Silage preservation can be accelerated by additives such as formic acid, or water soluble carbohydrates and cellulolytic enzymes that facilitate solubilisation of structural carbohydrates to be used by lactic acid bacteria. Specifically, digestibility is improved by silage cellulase enzymes (Muck *et al*., 2018). Distinctively, ensiling on bioethanol production was considered resolution to problem on bulk storage and preservation and transport of agriculture residues (Kitamoto *et al.,* 2011). Optimization of ensiling however, needs the removal of silages organic acids like lactic acid that can deactivate cellulases during subsequent enzyme hydrolysis and yeast fermentation activity. Hence, research on reducing the effect of acids on ensilage is gaining interest in the area of biofuel. This includes washing the silage with hot water to drain the acids prior to enzyme hydrolysis (Chen *et al*., 2007b).

This study aimed to mitigate the negative effect of lactic acid on switchgrass previously ensiled with cellulases with the end goal of developing ensiling process intended for alcohol production. Specifically, the study aimed to determine the effect of limewater, a weak solution of $Ca(OH)_2$ as alternative to hotwater as prewashing solution. Limewater is used to wash away and neutralize acids from the ensilages. The concept of limewater in prewashing is similar to the conceptual framework of alkali pretreatment recorded with the reduction of 30% lignin(Xu *et al.,* 2011a) when biomass treated with alkali

(NaOH) resulted in delignification and sub sequentially dissociated carbohydrates from lignocellulose prior to enzyme hydrolysis (Kaar and Holtzapple, 2000; Xu *et al*., 2011a).

According to Lee *et al*. (1999) using 0.1 to 0.2g NaoH/g biomass when combined with heat treatment of softwood, increase the amount of glucose and xylose in the proceeding enzymatic hydrolysis. Other studies on alkali pretreatment of lignocelluloses highlighted its used in animal feedstocks (Pacho *et al.*, 1977; Rabelo *et al.*, 2009) and shown positive impact on delignification, saccharification and fermentation for bioethanol production (Xu *et al.*, 2011b, Yang *et al.*, 2009). No study had tried using alkali such as lime water, a weak $Ca(OH)_2$ solution to prewash organic acids in ensilage and determine effect on biomass characteristics prior to enzymatic hydrolysis and fermentation.

The study aimed to determine the silages composition, enzymatic hydrolysis and yeast fermentation of switchgrass varieties silages. Specifically, the study had compared limewater, a weak solution of $Ca(OH)_2$ and hot water in pre washing silages and to determine the amount of reducing sugars, glucans, xylan and arabinans and soluble lignin contents of prewashed silages, the solubilized carbohydrates of enzyme hydrolysis and measurement of efficiency and fermentation by *Saccharomyces cerevisiea* (ATCC 24859).

Materials and methods

Samples of 30g silages from three switchgrass varieties previously treated with cellulase enzymes additives during process of ensiling (Chen *et al.*, 2007a) were chopped to small particles (1mm) then evenly sprayed with 500 ml wash solution. The limewater solution was prepared from mixing 200 ml of $Ca(OH)_2$ and 300 ml of deionized water. The alkali solution of $Ca(OH)_2$ solution was prepared using 2g of $Ca(OH)_2$ per liter of water. After prewashing, the biomass was rinsed with hot water several times until neutral pH. A separate batch of frozen silages and treated with hot water served as the control. All washed samples were stored at -80^0C for laboratory analysis and enzyme hydrolysis.

For the laboratory analysis, sub-samples of washed silages were analyzed for glucan, xylan and arabinan using HPLC and 3, 5 DNS assay for reducing sugars (Miller, 1959). Total solids of the washed silages were determined using 15g sample, vacuum dried overnight at temperature of 40^0C. Acid soluble lignin was analyzed following protocol for lignin (Sluiter *et al.*, 2006).

For the enzyme hydrolysis, 1g dry matter sample was placed in a 200 ml capacity serum bottle, diluted with 20ml citrate buffer with pH 4.8 and 1M concentration in order to come up with a solid to buffer ratio of 1:20. The buffered sample was added with 40µl of tetracycline hydrochloride and

deionized water and corrected final volume to 100 ml after enzyme loading. Cellulase enzyme (Novozyme, NS 50013, 75.5 FPU /g; specific density of 1.2) with desired activity of 15 FPU/g sample was added at the average of 234*ul* per bottle. After cellulase was loaded, enzyme Cellobiase (Novozyme, NS50010, 263.5 CBU/ml) was added at the average of 115 ul per bottle. The combination of 2 enzymes had at desired activity ratio of 1:4 ratio of cellulase to cellobiase enzymes. Immediately after enzymes loading, sealed the vial with rubber butyl cap and aluminium cap crimped in place. The vials were incubated for 72 hours in waterbath maintained at 55^0C and agitated at 150 rpm. After duration, 15 ml hydrolysates were collected for reducing sugar analysis, enzyme hydrolysis and fermentation. Conversion of carbohydrates to sugar was calculated using the formula;

$$\text{Conversion Efficiency (\%)} = \frac{\mathbf{C}(g/L) \times \mathbf{V}(L) \times DF}{\mathbf{M} \times \%\ \mathbf{G}\text{lucan} \times 1.11} \times 100 \quad (1)$$

Where: C is the concentration of glucose after enzymatic hydrolysis detected by DNS assay, V is the volume of hydrolysate; DF is the amount of aliquot of hydrolysates for sugar analysis; M is the weight of the dry biomass before hydrolysis and percentage of sample glucan and 1.11 is a multiplying factor to convert glucan to glucose.

For the yeast fermentation, sugar hydrolysates of enzymatic hydrolysis were centrifuged at 4000 rpm for 10 min using a refrigerated centrifuge pre-set at 4^0C. Pre-sterilized serum bottle was loaded with 75ml supernatant; pH was adjusted to 7.0 using 2N NaOH solution, then autoclaved for 1hr at 15 psi and 121^0C. After cooling down supernate temperature to 32^0C, 5 ml *Saccharomyces cerevisiea* culture was added. The concentration of dry yeast cell was 9g dry cell mass/liter. At 24 hr fermentation, additional ml of propagated yeast culture was added to fermentation vessel. The duration of fermentation was 72 hours in 32^0C incubator. At 72 hrs, fermentates were centrifuged at 4^oC, 4000 rpm for 10 minutes. Sample fermentate was analyzed for ethanol and acetate contents. The following equation was used in the calculation of ethanol yield.

$$\text{Percent ethanol yield} = \frac{\mathbf{C}(g/L) \times \mathbf{V}(L) \times DF}{\mathbf{A}\ (g) \times 0.511} \times 100 \quad (2)$$

Where: C is the concentration of ethanol detected by HPLC (g/L), V is amount volume of fermentate; A is the amount of aliquot for HPLC sugar analysis; Factor 0.511 representing the theoretical ethanol yield (g) per g fermented glucose by common yeast.

Yeast propagation

To prepare the culture media (Table 1), peptone water and serum bottle were sterilized in the autoclave, temperature of 121^0C, 15 psi and cycle duration of 30 minutes. Aseptically, a vial of *Saccharomyces cerevisiea* was inoculated into the 1L sterilized culture medium.

Table 1. Yeast propagation culture media

Ingredients	**Amount(g/L)**
Glucose	20 g
Yeast extracts	8.5g
NH4Cl	1.32g
MgSO4	0.11g
CaCl2	0.6g
Distilled water	1000 ml

This yeast cell suspension served as the source of inoculum for the yeast fermentation. For the propagation, the yeast culture was incubated at aerobic condition for 24 hours, sealed culture for anaerobic condition for another 24 hours. Incubation was done at 32^0C. Sample cell mass optical density (A_{540}) was monitored using a UV Vis spectrophotometer. At OD of 0.6, the yeast cell culture was centrifuged at 4000 rpm for 10 minutes using a refrigerated centrifuge. The yeast pellet was washed 4x with 0.1% peptone water, and then re-suspended in 50 ml of sterilized 1% peptone water. For the determination of dry cell mass concentration, 10ml of cell propagated culture was dried overnight at 105^0C temperature oven. The dry cell mass concentration of the propagated yeast was computed as follows:

$$\text{Dried Cell Mass (DCM)} = \frac{\text{weight of oven dried yeast cells}}{0.1(1000\text{ ml})}$$

Where: 10ml aliquot /1000ml = 0.1

Analysis of silage carbohydrates

For reducing sugar, 10 ml each of the hydrolysates of 72% sulfuric acid hydrolysis, cellulose cellobiase enzymatic hydrolysis, and yeast fermentation were centrifuged at 4000 rpm, in 4^0C for duration of 10 minutes. One ml of supernatant was collected for reducing sugar assay following the 3,5DNS procedure described by Miller (1959). To assay reducing sugar, 0.01 ml supernate was pipetted into 0.49 ml deionized water in 10 ml test tube. After mixing, reagents were added in the following in order; 1 ml of sodium acetate

buffer and 3 ml of DNS reagent. The reaction test tubes were place in beaker with water, heated and allowed 5 minutes at boiling point. After 5 min, the test tubes temperature was cooled down in 4^0C chiller for 5 min. prior to measurement of absorbance (A_{540}) using a spectrophotometer.

Standard glucose solution was prepared with the following sugar concentrations; 0g/L, 0.2g/L, 0.4g/L, 0.8g/L, 1.2g/L and 1.6g/L. Concentration of reducing sugar in sample aliquot was calculated from linear equation Y = 0.9516(X) where X was the data of Absorbance readings. The content of reducing sugar was calculated as follows;

$$Y \text{ (reducing sugars)} = C \text{ (g/L)} \times V\text{(L)} \times DF$$

$$\text{Where dilution factor} = \text{Sample aliquot /total volume of hydrolysis.}$$

For the assay of sugar monomers, 10 ml hydrolysate of the 4% sulphuric acid hydrolysis and 10 ml fermentate were centrifuged at 4^0C, 4000 rpm for 10 min. Two ml supernate was filtered through a 0.22 μm Millipore® membrane filter into a HPLC vial. Sugar calibration standards using glucose, xylose and arabinose were prepared at 1g/L, 2g/L, 2.5g/L and 4g/L of HPLC-ethanol water. The HPLC ethanol water grade solution with ethanol concentration of 16g/L was prepared by adding 20.28 mL of Ethanol (200 proof, density of 0.789) in 1L of HPLC grade water. This HPLC grade water solution was used in the preparations of ethanol concentrations of 8g/L and 4g/L diluents of glucose standard solutions.

For sugar monomer analysis, the HPLC (Shimadzu, Kyoto, Japan) was equipped with a refractive index detector (Shimadzu RID-10A), a Biorad Aminex HPX-87H column was maintained at a working temperature of 65℃ with a corresponding guard column. While in operation, the HPLC was maintained at flow rate of 0.6 ml/min using 5mM $H2SO_4$. After 24 hours, the concentrations of glucan, xylan, and arabinan in samples were directly uploaded in a computer attached to the HPLC.

Assay of acid soluble lignin

For the acid lignin analysis, 0.3g prewashed silages were hydrolyzed with 3ml of 72% sulfuric acid, then incubated at 30^0C for 2 hr duration. At duration,, rinsed the content with 50 ml water followed by 34 ml water to a final volume of 87 ml and attainment of 4% sulfuric acid concentration. The hydrolysis was incubated for another 2 hr at 30^0C. At duration, the bottles were steam sterilized at 121^0C, 15 psi for 60 min. Serum bottles were cooled down to room temperature, centrifuged at 4000 rpm, 4^0C for 10 minutes.

In the assay of acid soluble lignin, 10 ml hydrolysates were filtered using a vacuum pump. Sub-sample of 0.01 ml supernate was diluted with 0.49 ml of deionized water and the absorbance was measured at A_{205} nm using a UV visible spectrophotometer. A 4% sulfuric acid solution was prepared as reference blank. The percent acid soluble lignin of sample was calculated using the following equation;

$$\text{Acid soluble Lignin (\%)} = \frac{\text{Absorbance}_{205} \times 87 \text{ ml} \times \text{DF}}{\text{W} \times \%\text{ TS}} \times 100 \quad (3)$$

Where: Final Volume of Hydrolysis = 87 ml
Dilution factor = 0.5 ml aliquot/10 ml
W = the weight of sample
TS= % Total solids

Statistical analysis

Treatments data were analyzed following the General Lineal Model ANOVA approach in MINITAB 16.1.1 (State College, PA). Comparison of treatment means and interactions effects were done following Tukeys method at 95% confidence level.

Results

Carbohydrates composition

Table 2. Composition of prewashed silages of switchgrass varieties

	Switchgrass Varieties			Prewashing	
Composition (%)	**Bomaster**	**Performer**	**Colony**	**Water**	**Limewater**
Reducing sugars	29.08 *a*	26.67 *b*	27.12 *b*	27.89 *a*	27.37 *a*
Glucans	1.68 *a*	1.55 *b*	1.49 *b*	1.61 *a*	1.53 *a*
Xylan	22.73 *a*	21.92 *a*	19.79 *b*	21.56 *a*	21.40 *a*
Arabinan	2.37 *a*	2.38 *a*	1.97 *b*	2.21 *a*	2.31 *a*
Acid Soluble Lignin	2.95 *a*	2.96 *a*	2.99 *a*	3.00 *a*	2.93 *a*

Difference in letters within row indicate significance (p>0.5).

Composition of silages varies with switch grass varieties, contents of reducing sugars were highest in variety Bomaster compared with Performer and

Colony. Data on xylan, arabinan and soluble lignin were found not significantly different among varieties (Table 2). In prewashing solutions, use of both limewater and hot water did not differ in the composition of reducing sugars and other components of washed switchgrass silages.

Enzyme hydrolysis

Silage pH varies among varieties while, limewater washed silages had lower pH than hot water washed silages (Table 3**)**. Difference in pH between wash solution was significant ($p>0.05$). Comparison of means revealed that the limewater was effective in removing lactic acids than hot water alone. Further, limewater neutralizing effect had extended biomass cell wall breakdown causing the release of organic acid cell content during the hydrolysis.

Varieties vary in the amount of enzyme solubilized sugars (Table 3). In terms of prewashing, limewater washed silages showed 0.14g solubilized sugar/g biomass dry matter while 0.12g solubilized sugar/g biomass dry matter washed with hot water. Corresponding efficiency of the enzyme hydrolysis showed variations, that higher efficiencies were obtained with Performer and Colony than Bomaster. Limewater prewashed silages had higher conversion efficiency than hot water treatment. Significant difference in conversion efficiency was obtained between prewashing solutions ($p>0.05$). This could be attributed to the neutralizing factor effect of limewater and the stimulated enzyme activity of cellulases and cellobiase on intact silages composition.

Table 3. Products of hydrolysis of prewashed silages

Hydrolysis	**Switchgrass Varieties**			**Prewashing Solution**	
	Bomaster	**Performer**	**Colony**	**Water**	**Limewater**
Hydrolysates pH	5.09	5.17	5.17	5.96a	4.27b
Solubilized sugar. g/g Carb	0.13	0.12	0.14	0.12 *b*	0.14 *a*
Efficiency (%)	49.75b	50.60a	50.90a	*48.47b*	60.21a

Difference in letter within row indicate significance ($p>0.05$)

Fermentation yield

Fermentation product of ethanol varied within varieties of switchgrass (Table 4). Bomaster showed highest amount of ethanol with 0.39g ethanol/g sugar while very little ethanol yield from fermented sugars obtained from

Performer and Colony varieties. Statistically, ethanol yield among varieties was significantly different ($p<0.05$). Both limewater and hot water prewashing showed no significant difference in ethanol yield from fermented sugars of the prewashed silages.

For the acetate yield, all varieties had high concentrations of acetate. Acetate yield did not differ between solutions. The efficiency of the yeast fermentation was not significant among varieties. Using limewater as prewashing solution showed higher fermentation efficiency than using hot water alone. This means that limewater washed silages had higher soluble xylan sugars fermented by *S.cerevisiea* ATCC.

Table 4. Product of fermentation of prewashed silages of switchgrass

Products of	**Switchgrass Varieties**			**Prewashing**	
Yeast Fermentation	Bomaster r	Performer r	Colony y	Water r	Limewate r
Ethanol,g/g sugar	0.39 *a*	0.04 *b*	0.04 *b*	0.17 *a*	0.16 *a*
Acetate,g/g sugar	0.36 a	0.36 a	0.37 a	0.31 b	0.41 a
Efficiency(%) of ethanol fermentation	41.31*a*	4.75*b*	3.85*b*	18.60 *a*	16.09*a*

Difference in letters within rows indicate significance ($p>0.05$).

Discussion

Consequential loss of the significant amount of enzyme fermented glucans was due to prewashing treatment. According to earlier study of Chen *et al.* (2007b) on the impact of washing silages for biofuel, hot water prewashing had removed acids such as lactic acids. However, our study had shown prewashing compensatory impacted enzyme conversion efficiency, wherein silages prewashed with limewater were hydrolysed with efficiency of 60.21% than hot water with 48.47%. This 11.74% higher efficiency of the enzyme hydrolysis of limewater washed silages implied acid neutralization had occurred prior to enzyme hydrolysis that enzymes were not affected by lactic acids in washed silages. Further, the limewater solubilizing factor on polymers of lignin that dissociated xylan during the enzyme hydrolysis in limewater washed silages was in agreement with Van Soest (1982), who indicated the ability of alkali in solubilizing the lignin in grasses. As a result, limewater prewashing had improved enzymes cellulase and cellobiase activities on the prewashed silages biomass.

The Bomaster variety hydrolysate with high content of xylose and arabinose was fermented *S. cerevisiea ATCC 24859* with high ethanol in contrast with lack of ethanol in hydrolysates of fermented varieties of Performer and Colony. This suggested ethanolic fermentation from xylose and arabinose in the absence of sufficient glucose of the prewashed silages (Table 2). Acetate maybe was formed during deacylation of hemicellulose and lignin in cellulosic hydrolysates during pretreatment. It is inhibitory to yeast fermentation provided yeast have the ability to consume acetate into energy and ethanol as end product in the presence of sufficient co-factor NADH and ADH enzymes (Henningsen *et al*., 2015). Conversion of acetate to ethanol maybe is possible in yeast *S. cerevisiea* with functional xylose isomerase and the presence of high amount of xylose and arabinose (Kuyper *et al*., 2004) or xylitol which is an intermediate of xylose metabolism (Jeffries *et al.,* 2000).

In terms of ethanol fermentation efficiency, 41.31% in Bomaster compared to 4.75% in Performer and 3.85% in Colony indicated the significant impact of lack of glucans and high amount of xylan and arabinan in prewashed silages of all grasses. Our study shows that the presence of acetate as product of yeast fermentation indicated xylose to ethanol metabolism in *S. cerevisiea* ATCC 24859. Acetate from xylose was via the pentose phosphate pathway because of insufficient acetate maybe consumed by recombinant *S. cerevisiea* to produce ethanol in the presence of balance and sufficient co-factor NADPH and enzymes aldehydrogenase (Park *et al.,* 2016). The high efficiency of *S.cerevisiea* ATCC 24859 in Bomaster variety high concentrations of xylose, suggested conditions are met during yeast fermentation. Since fermentation of xylose to ethanol was dependent on the condition of fermentation like high temperature control (Cunha *et al*., 2019), condition of yeast fermentation of xylose and arabinose should be of major consideration aside from prewashing removal of acids and glucans.

Findings of our study on prewashing silages with alkali and its neutralizing acids in silages had influenced enzyme hydrolysis and yeast fermentation. The consequential losses of glucan in enzyme treated silages can be avoided by use of alternative silage additive that protects biomass from excessive solubilisation of carbohydrates since *P. virgatum* varieties have different digestion index (Burns *et al.,* 2008a; 2008b). Process prewashing may jeopardize carbohydrates leading to washout of solubilized carbohydrates and low ethanol yield due to lack of sufficient glucose during alcohol fermentation.

Conclusively, prewashing washout and neutralizes acids and can change composition in silages treated with cellulases, enhances solubilisation of carbohydrates by enzymes pretreatment hydrolysis and has impact yeast fermentation. Recommendation is to select silage additive that will prevent

significant loss of solubilized carbohydrates during prewashing of silages intended for biofuel production. Also, the recommendation is to consider selection of yeast with metabolism pathways for both glucose and xylose and arabinose. This includes determination of the factors affecting conditions of fermentation of xylan and other five carbon sugars in the absence of sufficient glucose. Information generated is vital in the optimization of prewashing ensilage as alternative feedstock intended for bioethanol production.

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