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Comparison of Chemical Pretreatment for Recovery of Fermentable Sugars and Enzymatic Saccharification

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Pearl millet biomass represents an unused source for biofuel production. Pretreatment is required to break down complex structure and prepare the pearl millet biomass for enzymatic hydrolysis. The efficiency of chemicals (*Ortho*-phosphoric acid (H_3PO_4), alkaline H_2O_2 and lime) pretreatment on pearl millet was investigated in the present study. Acid (16 % of acid, 12.5 % of total solid loading, 121°C of temperature and 3 h of incubation time) pretreated biomass sample showed highest (0.418 g / g of biomass) sugar release, crystallinity index (36.43 %) and inhibitor concentration (4.02 %). The parameters were optimized by using Response Surface Methodology in a Optimal Custom Design. The studies confirmed that lime pretreatment was promising as it had high crystallinity index (70.18 %), fermentable sugar release (0.234 g/g of biomass) and minimal production of inhibitors, required less energy input and environmentally safe. Pretreated biomass was further characterised by SEM and X-ray diffraction (XRD).

Key words: Ortho-phosphoric acid, Alkaline H_2O_2, Lime, Pearl millet, Pre-treatment

Pearl millet (*Pennisetum glaucum* (L.) R. Br., Poaceae) is one of the most important millet crop in India (Bhanu Prasad Reddy *et al*., 2016). It is drought tolerant, warm season cereal grown on 26 mha in arid and semi-arid regions of South Asia and sub-Saharan Africa. It is one of the most important staple food crop for more than 500 million people. Pearl millet is mainly cultivated for food, forage, fuel, mulch crop and as building material. Biofuel is primarily derived from sugar, starch and lignocellulosic biomass. Lignocellulosic biomass composed of three major polymeric components such as carbohydrate (cellulose and hemicellulose) lignin along with minor quantities of ash and extractives.

Cellulose is a linear polymer of glucose units (more than 10^4 glucose units with 6-8 µm length) which are linked with β -1-4 linkage. The cellulosic chains are grouped together to form microfibrillar structure which contains 36 glucan units and is insoluble in native form which is stable up to 5-8 million years at 25°C (Zhang *et al*., 2006). Hemicellulose is a polymer which is consists of pentose and hexose sugar. These carbohydrate polymers are protected by a lignin matrix ultimately which leads to prevention of cell wall swelling and enzymatic saccharification but can be hydrolyzed by using chemical or enzymatic saccharification for maximum recovery of total reducing sugars. Lignin is an amorphous form of heteropolymer that can be associated with different types of phenyl propane units namely p-coumaryl, coniferyl and sinapyl alcohol. It is not involved in the biofuel production and binds with the enzymes that can makes them unproductive. The aim of the pretreatment is maximum recovery of reducing sugar with low cost.

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Material and Methods

Pearl millet biomass (ICMV 05222 samples were dried to less than 10 % level for easy milling to reduce the size to 180 to 850 µm. Similarly, the ash content of the samples was kept below 10 % by size reduction and sieving. Dried and milled sample was passed through the 2 mm sieve. The prepared sample were analyzed immediately or could be stored in airtight container or polyethylene bag to prevent the entry of moisture and other contaminants and kept at -20ºC until needed. The proximate analysis of the raw and pretreated sample *viz*., moisture content, ash, cellulose and lignin (acid soluble and in soluble lignin) content was carried out according to the Laboratory Analysis Protocol (LAP) of National Renewable Energy Laboratory (NREL), Colorado, USA (NREL, 2014). Cellulose and hemicellulose content were analyzed following the procedure described by Van Soest and Wine, (1967).

Chemical pretreatment

The pearl millet biomass was pretreated with different chemicals at varying concentrations *viz*., *ortho*-phosphoric acid (H_3PO_4) at 4, 8, 12, and 16 %, alkaline hydrogen peroxide (AHP) at 1.2, 1.8, 2.4, 3 % and lime at 0.7, 0.9 and 1.1 % using the solid loading of 7.5, 10 and 12.5 % subjected to different temperatures (for acid 100 and 121˚C lime and AHP 80, 100 and

121˚C, respectively). The treatment duration also varied for acid (60, 120 and 180 min), lime and AHP (60, 90 and 120 min). For AHP treatment, the pH was adjusted to 11.5 using NaOH. The biomass was treated based on the above conditions by maintaining temperature in an autoclave. The autoclaved sample was cooled and filtered to separate the solid and liquid fraction. Based on the experimental results, Response Surface Methodology (RSM) was followed for optimizing the pretreatment conditions with four different variables such as chemical concentration, solid loading, reaction time and temperature. The response was sugar release, cellulose increase (%) and lignin reduction. The optimization process has been done by using optimal custom design (OCD), Design Expert 9.0.6.2 (Stat-ease, Inc. Minneapolis, MN 55413) software version was used in this study. From this design, experimental and predicted values were found to be more or less similar, which confirms the optimization process.

Sugar analysis

The raw and pretreated biomass liquid sample fermentable sugars were analyzed by HPLC (Agilent 1200 series) and carbohydrate was calculated. The HPLC instrument was operated at 65ºC using 0.2 µm filtered 5Mm H_3PO_4 as a mobile phase at a flow rate at 0.6 mL. min^{-1} Standard curves were generated using glucose, xylose, arabinose and galactose. Pretreatment efficiency was calculated as the amount of total sugar release measured after each pretreatment. The pretreated pearl millet biomass hydrolysate sample sugar release was performed according to the method of Miller (1959).

The byproducts and degradation products formed during the pretreatment were analyzed by using spectrophotometric method and GC-MS. A gas chromatograph (Agilent Technologies 7890B) coupled with a mass spectrometer MSD (Agilent Technologies 5977A) equipped with a capillary column HP-5 MS (30m X 0.25 mm X 0.25µm) were used to analyze the furfural according to the modified method of (Andary *et al*., 2013). Total phenol content was measured in pearl biomass pretreated hydrolysate samples by a spectrophotometric determination with a method of Bray and Thorpe (1954).

Structural analysis of pearl millet biomass - SEM-Scanning electron microscope

The structure of raw and pretreated pearl millet biomass was evaluated by (SEM) (Amiri and Karimi, 2015). The SEM images were taken at 1000 X magnification using a 10 kv voltage with the working distance of 10.6 mm, spot size of 4.0 and the detector was (Everhart Thornley (QUANTA 250, FEI-Czec Republic).

Crystallinity measurement by using X-ray diffraction

The changes in crystallinity of the biomass due to chemical pretreatment was measured by using a Ultima IV diffractometer (Rigaku, PDXL software, Japan). The crystallinity index (CrI) was calculated according to the method of Yosidha *et al*. (2008) by using the following formula

$$\text{Cr I} = \frac{\text{Ioo2} - \text{Iam}}{\text{Ioo2}} \text{X } 100$$

Where I002 is the diffraction intensity at 2Ɵ=22.6˚ which represents both the crystalline and amorphous regions and Iam is the diffraction intensity at 2Ɵ=18.7˚, which represents the amorphous regions.

Enzymatic activity and saccharification

Filter paper activity (FPA) of cellulase and crude enzyme was measured according to the standard procedure recommended by the commission on Biotechnology, IUPAC (Ghose, 1987) and expressed in filter paper units (FPU). Enzymatic saccharification of raw and resultant residual pearl millet biomass obtained from each pretreatment was measured by standard procedure described in NREL/TP-510-42629 (Selig *et al*., 2008). The enzymatic digestibility was calculated by using the following formula

$$\text{Digestibility (\%)} = \frac{\text{Grams cellulose digested}}{\text{Grams cellulose added}} \text{ x100}$$

Results and Discussion

Lignocellulosic biomass consists of cellulose, hemicellulose, lignin, proteins, acids, salts and minerals. Chemical composition is also one of the important factors for biofuel production. Moisture content of raw pearl millet biomass was (8 %) and ash content (6.27 %) was obviously higher than that the literature report of 3.8 % (Dhabhai *et al*., 2012). The proximate analysis of pearl millet biomass revealed that moisture (8±0.32%), ash (6.27±0.08%), total solids (92±0.14%), water extractives (6.43±0.12), ethanol extractives (5.72±0.34), cellulose (41.6±0.16%), hemicellulose (22.32 ± 0.65), glucan (28.47±2.88%), galactan (17.24±0.46), arabinan (3.78±0.04), xylan (5.12±0.46), acid insoluble lignin (16.32±0.49) and acid soluble lignin (5.49±0.08%) (Table 1).The total extractives were 12.15 % in the pearl millet biomass showing that they were easy to dissolve into the liquid fraction (Kumar *et al*., 2016). Lignocellulosic biomass contains non-structural components, which could be easily extracted with water or ethanol.

The extractive free biomass could be used for the compositional analysis. Ethanol extraction was required to remove the waxy material. These waxy materials were precipitated during separation of liquid fraction which ultimately resulted in high lignin content. Water extractives were essential to remove the sugar acids, inorganic, nitrogenous and non-structural components. These components were interfering with total reducing sugar analysis. Cellulose and acid detergent lignin (ADL) was higher in triticale and wheat straw harvested at maturity stage than in premature stage but hemicellulose was high in pearl millet hay. The lignin content of the pearl

millet biomass ranged from 22.01 to 27.57 % (Chen *et al*., 2007) and the pearl millet biomass used in the study had 21.8 %. This result reflects a difference in pearl millet itself. The differences in composition of the biomass may vary with cultivar, season, location, soil type, plant to plant, maturity of the crop, method of harvest and storage, analytical procedure and size of the biomass.

Table 1. Compositional analysis of raw and pretreated pearl millet biomass Standard errors are shown in parenthesis

Composition	Raw biomass	Pretreatment / 100 g of biomass		
Biomass		PA (12 %)	AHP (3 %)	CH (1.1 %)
Moisture	8.0±0.32	7.48±0.04	8.90±0.05	8.05±0.02
Cellulose	41.6±0.01	70.67±0.06	60.35±0.01	62.14±0.05
Hemicellulose	22.32±0.65	7.67±0.04	10.82±0.02	10.39±0.04
Lignin	21.81±0.25	6.53±0.12	11.37±0.03	11.39± .05
Ash	6.27±0.08	7.65±0.02	8.56±0.02	8.03±0.05
Bulk density (kg/m_3)	72.66±0.41	78.25±0.04	73.09±0.02	78.73±0.07
Liquor				
Glucan (g/L)	28.47±2.88	19.89±0.03	8.40±0.19	10.89±0.12
Xylan(g/L)	5.12±0.46	14.76±0.04	6.23±0.15	8.08±0.09
Arabinan(g/L)	3.78±0.04	3.94±0.01	1.67±0.04	2.16±0.02
Galactan(g/L)	17.24±0.62	3.20±0.01	1.35±0.03	1.75±0.02
Total sugar (g/L)		41.80	17.65	23.40
Inhibitor		4.02±0.21	1.51±0.07	0.66±0.05
CrI(%)	60.43	36.43	65.04	67.25
Cellulase saccharification	32.42±0.01	66.61±0.01	69.40±0.01	71.34±0.01
Crude enzyme saccharification	32.42±0.01	58.37±0.01	62.54±0.02	64.34±0.01

PA - H_3PO_4,AHP - Alkaline H_2O_2, CA- $Ca(OH)_2$
Standard errors are shown in parenthesis

Chemical pretreatment

The variables of H_3PO_4 acid pretreatment (acid concentration, temperature, reaction time and solid loading) was evaluated by determining the amounts of released reducing sugars, lignin reduction and cellulose content increase. The optimized parameters were found to be 16% H_3PO_4 acid concentration, 12.5% total solids loading, 121°C and 3 h. The highest total sugar yield of 0.418g.g^{-1} of pearl millet biomass, 70.06% of lignin reduction and 70.67% of cellulose content increase was obtained for the optimized conditions. The biomass difference between raw and pretreated biomass loss was to solubilization of polymeric components to small dispersed fibers in the hydrolysate. The predicted responses were 0.416 g-g^{-1} of pearl millet biomass of sugar recovery, 70.68% cellulose content increase and 70.87% of lignin removal. A maximum lignin reduction of (70.87%) and increase of cellulose content (70.68 %) was evident from the optimized pretreatment conditions. The predicted value and experimental values were nearly similar to each other. In the present study, sugar recovery was high in acid pretreated hydrolysate which might be due to degradation of cellulosic fractions. The lignin fragments were then dissolved in the liquid phase and resulted in digestibility of the solid fraction. In case of dilute acid pretreated pearl millet biomass recorded 0.120 g.g^{-1} of biomass sugar recovery which was reported by Krishania *et al*., (2010).

The optimized parameters are found to be 1.1 % lime concentration, 12.5 % of total solids loading, 121°C and 2 h. The sugar yield of 0.234 g.g^{-1} of pearl millet biomass, 47.70% of lignin reduction and 49.37 % of cellulose increase was obtained for the optimized conditions. The experimental results are near to predicted value consequently it confirmed the results. Lime pretreated pearl millet biomass recorded a 47.70% of lignin loss which was higher than those of pretreatments carried out by Xu *et al*. (2010). In lime pretreatment, lignin loss was very low when compared to other treatment. Sugar recovery was increased with increasing reaction time and lime concentration. The pH of the pretreated slurry was found to be 12.5.

The optimized parameters were found to be 3% of AHP concentration, 10% total solids loading, 121°C and 2 h. The AHP pretreated liquid fraction recorded the total reducing sugar 0.177 g.$g^{-1}$of pearl millet biomass, which was in accordance with Cao *et al*. (2012) in sweet sorghum bagasse and 45.80% of lignin reduction 45.07% of cellulose increase was obtained for the optimized conditions. The total sugar release increased with increasing AHP concentration, total solid loading, incubation time and temperature. The biomass loss was observed after pretreatment which resulted in highly digested components present in the liquid fraction. Sugar recovery was increases with increasing reaction time

and lime concentration. The pH of the pretreated slurry was found to be neutral.

The pretreated liquid fraction contains complex innumerous compounds such as soluble lignin, total reducing sugars and inhibitor, which were increasing with solid loading, reaction time and temperature. The phenol contents in the optimized condition pretreated (H_3PO_4, lime and AHP) hydrolysate samples were 4.02, 0.48 and 1.51 % respectively. This result indicates that the partial removal of lignin and hemicellulosic fractions. Lignin fraction is oxidized and digested into formation of carboxylic acid and phenolic compounds (Martin *et al*., 2007). The H_2O_2 is under alkaline condition, pH 11.5, which separates into hydrogen and the hydroxyl anion. The anion group reacts with left over peroxide to form highly reactive hydroxyl radicals which can attack the lignin structure. This pretreatment is more effective in lignin degradation under alkaline pH.

GC-MS analysis of the acid pretreated hydrolysate showed acetic acid (6.95 %), formic acid (3.08 %), furfural (30.56 %) and hydroxymethylfurfural (HMF) (0.52 %). These (acetic acid, HMF, and formic acid) inhibitor components were formed from acetyl groups,

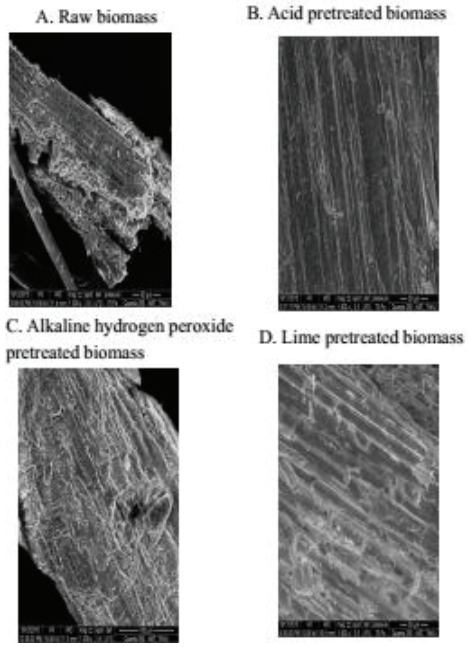

Fig.1. SEM micrographs of pearl millet biomass

hexose sugar and furfural compounds respectively. Acetic acid was high in lime pretreated sample (27.23 %) followed by alkaline H_2O_2 (21.33 %) and H_3PO_4 pretreated sample (6.95 %). Acetic acid is the most common organic compound present in the hydrolysate sample. This acid passes through the cytoplasm and collapse the cell wall structure ultimately leads to acidic pH (Cho *et al*., 2010). Furfural content was high in acid pretreated hydrolysate followed by AHP. Furfural compound may be produced from xylose and arabinose (pentose sugars) (Cai *et al*., 2014). These compounds are inhibiting the growth of yeast, which leads to reduce the ethanol production. Detoxification requires separate step which ultimately leads to increase of ethanol production cost. Lime has the additional advantage of low chemical cost and safety and recoverable from water as insoluble calcium carbonate ($CaCO_3$) by reaction with carbon dioxide. This $CaCO_3$ can be converted into lime by using the kiln technology.

Morphological analysis - SEM image

The raw biomass was observed under SEM and exhibited regular compact surface morphology and high fibrous structure before being pretreated (Fig. 1A). The cell wall surface morphological aberrations were observed in all the pretreated biomass samples which might be due to lignin and hemicellulose removal. H_3PO_4 pretreated biomass showed the expansion of the surface area, wrinkled, removal of fibrous structure and coarse or spherical shape particle were adhering to the surface (Xu *et al*., 2007) (Fig. 1B). Lignin fraction converted into liquids at elevated temperature and spread over the biomass. The pearl millet biomass pretreated with 3 % AHPat pH 11.5 exhibited peeled off appearance with higher porosity, hollow area of the interior parts was observed in the biomass (Fig.1C). The pretreated biomass showed swelling with stiffened structure might be due to removal of hemicellulose which is correlated to the water molecule absorptivity (Ramadevi *et al*., 2012). The lime pretreated biomass showed similar structural appearance as those observed due to other pretreatments as mentioned above (Fig. 1D). Raw pearl millet biomass samples were very hard and lime pretreated samples were softened in nature, which indicates the removal of lignin and acetyl groups. Compositional analysis was carried out before and after pretreatment showed that a significant fraction of lignin and hemicellulose removed during pretreatment.

***Structural characterization by crystallinity index** (CrI)*

The crystallinity index (CrI) of the raw and pretreated sample was calculated from the XRD data (Table 2 and Fig. 2.). The crystallinity index strongly influences the biomass composition.X-ray diffraction (XRD) measurement provided the information about qualitative and quantitative form of crystalline cellulosic components in a biomass. The raw biomass showed (60.43 %) the crystallinity index, because it has a higher content of hemicellulose and lignin, which are amorphous. In raw biomass consists of cellulose, hemicellulose and lignin. These components are tightly associated with each other which are unfavorable for enzymatic hydrolysis. Hence, the effective pretreatment is required for enhancing the productive binding and enzymatic hydrolysis rate. The crystallinity index was high (65.04 to 67.25 %) in all the pretreated biomass except in acid pretreated biomass which was in accordance with (Corredor *et al*., 2008). The high crystallinity of pretreated sample showed that the significant removal of lignin and hemicellulose fractions from pearl millet biomass.

H_3PO_4 pretreated sample showed lower crystallinity index (36.43 %), which indicates the profound changes between inter - and intra - chain hydrogen bonds in the cellulosic fibrils. H_3PO_4 promotes the digestion of cellulosic fractions and also having the capacity to absorb water molecules. Hence, the pretreated biomass was swollen in nature. The acid disrupts the crystalline structure of cellulose, which led to partially cellulose to amorphous parts. The acid hydrolysis of the biomass resulted in maximum recovery of fermentable sugars. These changes resulted in the surface area accessibility, which is correlated with findings of Nazarphour *et al*. (2013). AHP pretreated biomass recorded cellulose crystallinity (65.04 %) which was higher (60.59 %) than that of sugarcane bagasse (Guilherme *et al*., 2015). AHP pretreatment promotes the significant fraction of lignin removal due to the cleavage of glycosidic and ester bond in the pearl millet biomass. This pretreatment ultimately causes digestion and dissolving of biomass, redistribution and deposition of lignin polymer. Lime pretreated sample showed (67.25 %), which indicates the greater removal of amorphous substances. The subsequent enzymatic hydrolysis rate may be depending on the three structural characteristics such as lignin, crystallinity and acetyl group content.

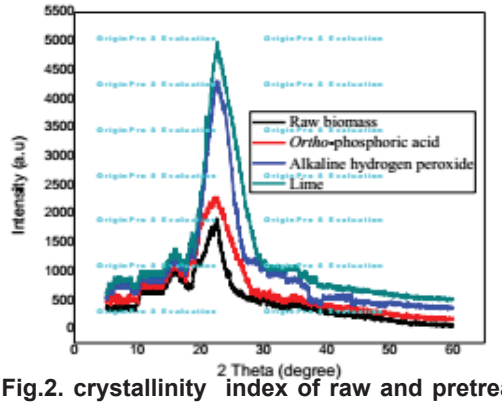

Fig.2. crystallinity index of raw and pretreated biomass

Enzymatic hydrolysis

The pretreatments efficiency was compared after enzymatic hydrolysis using a commercial cellulase and crude enzyme (IICT, Hyderabad). The activity of the cellulase and crude enzyme was determined as (123 FPU.mL^{-1}) and (185 FPU. mL^{-1}) with 1 unit of activity liberating 2 mg of reducing sugar expressed as glucose equivalent. In raw biomass cellulose, hemicellulose fractions are tightly packed with lignin matrix. The raw biomass showed much lower sugar yield (1.76-5.783 g.L^{-1}) and enzymatic hydrolysis (32.42%) because the complex structure of the biomass is unfavorable for enzymatic hydrolysis. The enzymatic hydrolysis of the pretreated biomass resulted more sugar release (4.83-17.14 g.L^{-1}) and enzymatic saccharification (58.37-64.34%). The accessible surface area is more in pretreated biomass. Amorphous form of cellulose is more accessible than in crystalline form of cellulose (Kumar *et al*., 2012). The hydrolysis rate is hindered by various factors such as substrates, composition, inhibitor concentration, reaction time, pH and enzyme activity.

Pretreatment efficiency

The cost-effective sugar recovery process is very difficult from the pearl millet biomass. So, the chemical pretreatment method has been focused on the conversion of biomass to fermentable sugars. The pretreatment process cost is still high but sugar release is low. Each method has its own advantages and disadvantages. The aim of the objective is water saving, time saving, environmental friendly and cost effective. H_3PO_4 is a weak acid (less aggressive) and produce less inhibitor when compared to strong acids. After acid pretreatment, both the process has been occurring, swelling of cellulose or expansion of biomass and dissolution of cellulose. Cellulose swelling is a process of uptake of H_3PO_4 and also maintains the residual cellulosic fibrous structure. Acid has to be removed from the biomass by filtration technique. Acid pretreated hydrolysate sample produced more reducing sugars it might be due to dissolution of cellulosic fraction. The lignin fragments were then dissolved in the liquid phase and resulted in increased digestibility of the solid fraction. The lime pretreatment is comparatively cheap, safe, environmental friendly and easily recyclable from pretreated slurry. AHP pretreated biomass showed more stable structure, redistribution, condensation and partial or selective removal of lignin, than in another pretreated biomass. It causes swelling of biomass ultimately which leads to expose the available surface area. All the chemical pretreatment methods are not having the capacity to produce enough fermentable sugar. To choose between alkaline hydrogen peroxide and lime pretreatment is not possible in this case because both of them release more or less the same quantity of fermentable sugars. These kinds of pretreatments indicate that enhancement of cellulosic accessible surface area leads to increased rate of enzymatic hydrolysis. Hence, the hydrolysis of fermentable sugars to ethanol (fermentation step) will be the deciding factor in future for choosing a specific chemical pretreatment. After pretreatment, the pearl millet biomass weight loss and sugar release are also varying according to the type of biomass, particle size, solid loading, chemical concentration, pH, residential time and temperature.

Conclusion

Forefficient enzymatic hydrolysis and ethanol production, pretreatment is an important technique. H_3PO_4 acid hydrolysis produced more sugar than others but requires special reactor, higher production of inhibitors, lot of water for neutralization process, high chemical cost and is hazardous. During pretreatment, AHP is unstable and neutralization process is unneeded. The overall optimized parameters were found to be lime pretreatment process with enzymatic

saccharification (60 FPU.g^{-1} of substrate, and 72 h). This should be recommended as a future energy source of the country as it is environmentally safe, requires low energy input and chemical cost.

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