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Evaluating the Role of Ultrasonication-Assisted Alkali Pretreatment and Enzymatic Hydrolysis on Cellwall Polysaccharides of *Pennisetum* Grass Varieties as Potential Biofuel Feedstock

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Production of renewable fuel like bioethanol from plant biomass and agro wastes will be the future energy source to combat the depletion of fossil fuels. The first detailed profile of the non-cellulosic cell wall polysaccharides of native, ultrasonication assisted alkaline (NaOH) pre-treated and enzyme hydrolysed *Pennesitum* grass varieties viz. hybrid Napier grass and denanath grass, were identified using glycome profiling. The best pre-treatment conditions resulted in 89.3% and 86.7% delignification of denanath grass (DG) and hybrid Napier grass (HNG) respectively. In the same conditions, 227.2 mg/g and 242.8 mg/g of total reducing sugar was achieved for DG and HNG respectively. Comparative assessment of a new enzyme i.e. Palkonal MBW with the conventional combination of Celluclast 1.5 L+ xylanase was undertaken. The amounts of glucose and xylose released with Palkonal MBW saccharified DG and HNG were 662.0 mg/g and 431.2 mg/g, which were significantly higher as compared to the conventional enzyme cocktail. The glycome profiling results showed that pectic arabinoxylan and arabinogalactan backbones were significantly less in DG samples and they do play a major role in enhancing enzymatic hydrolysis. These reports can provide a good insight in designing potential perennial feedstocks for bioethanol production in bio refinery concepts. Furthermore, the underutilised DG variety may also be exploited owing to its promising cell wall characteristics that can produce higher bioethanol yields.

Introduction

In recent years, modern commercialization and industrial development have led to the continuous depletion of coal and petroleum. There is an urgent need for developing new technologies to achieve a steady environmentally sustainable source of renewable energy to meet the energy crisis.[1] In this regard the lignocellulosic biomass, the most abundant renewable raw material available, has a great potential to replace fossil fuel.[2] Among different plant biomass grasses such as switchgrass, Miscanthus, Napier grass, sugarcane and maize are regarded as important potential sources of lignocellulose for the production of bioenergy.[3] For example, use of various grass varieties like wild grasses (Bamboo) and *Miscanthus* sp. have been witnessed to represent a more sustainable alternative to food stocks for the creation of fuel bioethanol.[4–6] Grass varieties like denanath grass (*Pennisetum pedicellatum*) and hybrid Napier (*P.purpureum*) grass C0-3 variety which are perennial in nature also hold great potential as lignocellulosic biomass for bioethanol production. Denanath grass, which is tolerant to high drought conditions[7] and salinity has an annual yield of 82.1-128.5 t/ha/yr.[8] Since, the protein content of the grass variety accounts to a maximum of 9% it is therefore not considered as a potential biomass for grass fodder. Further, an attempt to use this species as fuel has been made by Misra in 1960.[9] In case of hybrid Napier grass (CO-3 variety), the high biomass yield of 150–200 t/ha has attracted many researchers for using the grass variety as a biomass for bioethanol production. The biomass holds similarity with denanath grass in its low protein content and resistance to drought conditions. Thus, these biomasses with the added advantages to grow in almost any soil conditions with very little water, can be explored for their potential as a bioethanol feedstock.

In the conversion of lignocellulosic biomass to biofuels, main challenges that need to be addressed are the reticular connection of lignin with the carbohydrate, the uncertainty on

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conformation, structure and composition of the biomass components like cellulose, hemicellulose, lignin and finally the structural heterogeneity resulting in low thermal conductivity of biomass reaction systems.[10,11] The lignin-carbohydrate complexes are thought to significantly contribute to biomass recalcitrance necessitating harsh conditions and specific solvents for the degradation of lignin and its complexes, which not only makes the processing costly but also results in environmental problems. Similarly, varied constituents of wax, proteins and organic compounds interfere with the downstream processing and so they have to be removed by necessary pre-treatment or separation steps.[12] Specifically, the reduction in waxes and lignin had been shown to improve the bioethanol production from grasses.[13] Apart from these, low thermal conductivity creates a barrier for heat and mass transfer in the biomass leading to insufficient catalyst and reactant contact, thus resulting in poor biomass conversion.[12] To avoid this condition the selection of the biomass becomes an important criterion to obtain good bioethanol yield. The selection of the biomass mainly depends on high dry matter yield, high percentage of cellulose, total content and relative abundance of monolignols and their linkages and the plant developmental stage.[4,14,15] Apart from this, the free phenolics present in the biomass also forms an advantageous regulatory factor in determining the efficacy of pre-treatment, as these provide opening sites for alkali to solubilize the lignin by the mechanism of deprotonation of phenolic hydroxyls at a lower pH as compared to aliphatic hydroxyls.[16]

The pre-treatment step greatly affects the structural form and heterogeneity of the biomass leading to an increase or decrease in enzymatic hydrolysis.[17] The technique of using alkali for pre-treatment has shown to exhibit comparatively higher enzymatic digestibility of biomass than acid or organosolvic pre-treatments in different grass varieties.[17–19] To enhance the efficacy of this pre-treatment, the use of ultrasound assisted alkaline pre-treatment, a nonconventional technique, has been gaining interest in the recent past.[20–22] Ultrasound assisted alkaline pre-treatment has the advantage of lesser use of alkali and reduction in the processing time and has been reported to be extremely beneficial for delignification and subsequent enhancement of enzymatic digestibility in cellulosic biomasses.[23,24] Better enzymatic hydrolysis in grasses is observed to happen concurrent with the decrease of S/G ratio [**syringyl**/guaiacyl], during the pre-treatment process.[17,25]

This can be accounted to the pressure wave created by ultrasonication which creates regions of high pressure (compression zone) and low pressure (rarefaction zone) in the reaction medium. This leads to contraction and expansion of the reaction medium in the regions of high and low pressure respectively. Small cavities are formed due to the pulling apart of the molecules due to the expansion and this leads to the formation of a critical radius, releasing large amount of energy. The enormous amount of energy released forms active free radicals that help in higher delignification and thus enhanced enzymatic hydrolysis.[26] As the practice of using ultrasound assisted treatments is still in a growing stage,[23] it becomes important to investigate its effects on the important biomass feedstocks like grasses. Though not heavily studied, the major advantages that have been observed with ultrasound assisted pre-treatments is the decrease in the total pre-treatment time and minimum inhibitor production with similar sugar yields like that of other pre-treatment techniques.[27–29] Although, the process is not economical as compared to acid or steam explosion pre-treatments, the inhibitory compounds formed during these pre-treatment processes simultaneously causing corrosions in the reactor vessel makes ultrasonication assisted pre-treatment a preferred choice against the above mentioned pre-treatment techniques.[29] Nevertheless, literature studies also suggest that, ultrasonication assisted pre-treatments are much more suitable for delignification compared to acid pre-treatments.[1] Further ultrasonication–assisted alkali treated biomass have also been seen to produce higher ethanol titres as compared to the conventional techniques.[30] This clearly indicates the technology to be one of the major areas of interest, specifically as an add-on step with the existing processes, with least alterations and minimal enzymatic usage. Therefore, in the present investigation, NaOH which showed good delignification in the two *Pennisetum* sp i.e. hybrid napier grass and denanath grass in our previous studies,[31] was subjected to a combined pre-treatment with ultrasonication and the processes were optimised for the power supply, temperature and duty cycle of the ultrasonicator to obtain best delignification and reducing sugar yield.

For enhancing the enzymatic digestibility of the biomass an efficient optimisation tool is required. Response surface methodology (RSM) is a multivariate statistical technique which allows the determination of multivariate equations for the experimental data to give an optimized experimental design. Hence, the best system performance is achieved as the evaluation and management of each variable of the experiment is simultaneously carried out by RSM.[32] The advantage of using RSM over other conventional methods is maximum information can be obtained with a minimum number of runs.[33] This is an important factor owing to the economical aspect of the process optimisation, keeping in view the high costs of the enzymes available for hexose and pentose sugar hydrolysis and the minimum use of the lignocellulosic biomass. In this study, the best optimised ultrasonication assisted alkaline pre-treated samples were further optimised for enzymatic hydrolysis using RSM. A total number of thirty-six runs were evaluated for each of the grasses and tested for the optimum glucose and xylose optimisation using high performance liquid chromatography (HPLC). The study of composition and extractability of most major non-cellulosic cell wall glycans was conducted using glycome profiling. Glycome profiling is a powerful tool that involves sequential extraction of plant biomass/cell walls using increasingly harsh reagents.[34,35] The sequential extractions allow isolation of most major non-cellulosic plant cell wall glycans based on the relative tightness with which they are bound to the cell walls. The extracts are subsequently screened using a large and diverse group of monoclonal antibodies (mAbs) for detecting and monitoring most major non-cellulosic polysaccharide epitopes that are found in the cell walls of plants.[34,36] The technique is broadly

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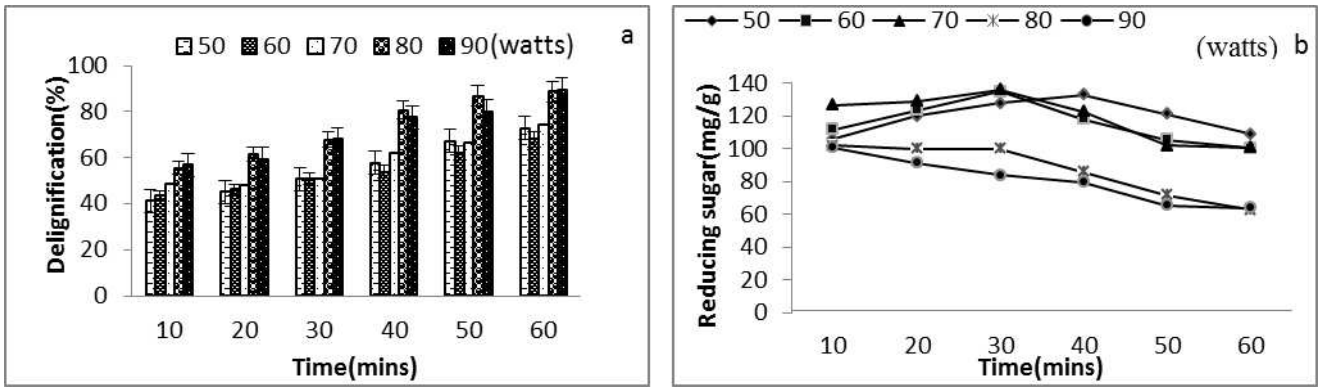

Figure 1. Effect of ultrasonication power on delignification (a) and reducing sugar (b) of DG

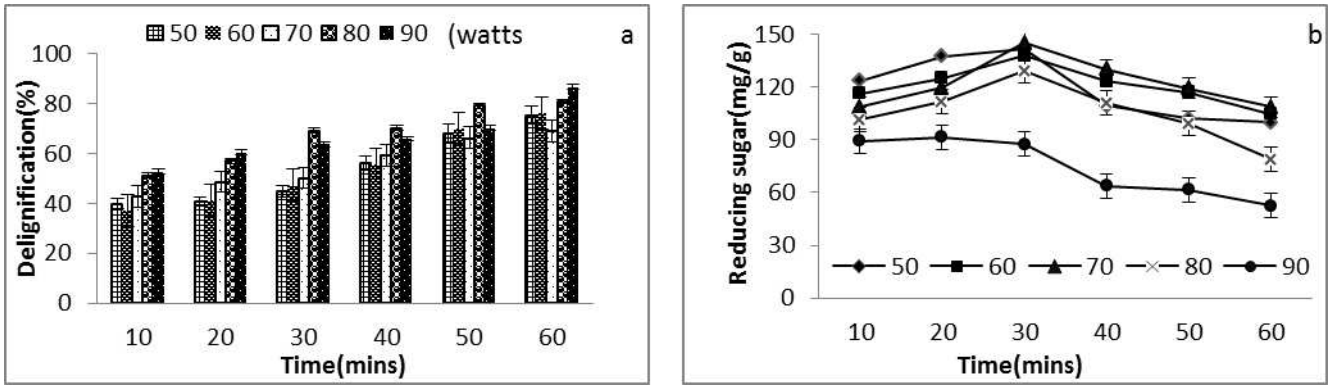

Figure 2. Effect of ultrasonication power on delignification (a) and reducing sugar (b) of HNG

used in detecting and monitoring plant glycan structures allowing in-depth biomass analyses assisting on characterizing any modifications occurring to plant biomass non-cellulosic glycans.

3. Results and Discussion

The results of the experiments with different parameters like power supply, duty cycle and temperature of ultrasono assisted NaOH (1%) treated DG and HNG biomass was optimised for maximum delignification and reducing sugar production and is given in **Figure 1 and 2**. The ultrasono assisted alkali pre-treatment will be denoted as UA-NaOH in rest of the sections. The optimised UA-NaOH pre-treated biomass was further optimised for enzymatic hydrolysis using RSM for commercial enzymes applications i.e. Palkonal MBW (an enzyme with both cellulolytic and hemicellulolytic activity) and a combination of Celluclast 1.5 L (cellulolytic activity) + Xylanase (hemicellulolytic activity) as shown in **Table S1 and S2** (Supplementary data). The efficiency of enzymatic hydrolysis was evaluated on the basis of the increase in TRS content from that of the TRS obtained after pretreatment, using HPLC and RSM design for each of the factors (temperature, incubation time, enzyme dosage, pH and substrate concentration). The structural differences of untreated, UA-NaOH treated and enzyme treated samples were investigated through glycome profiles with a view to understand the effect of pre-treatment and enzymatic hydrolysis towards production of enhanced sugar production from grass biomass.

3.1. Parametric investigation of UA-NaOH pre-treatment on production of reducing sugar and delignification.

Ultrasonication assisted alkali treatment can reduce the reaction processing intensity, the reaction time and the amounts of alkali required for delignification process which is however dependent on various parameters of ultrasonication.[37] Hence effect of ultrasonication was studied under different parametric conditions for pre-treatment of grass biomass (DG and HNG) to enhance delignification with higher reducing sugar production.

3.1.1. Effect of ultrasonication power on NaOH pre-treated samples

Ultrasound power plays a major role in degrading macro polymers like crystalline cellulose, hemicellulose and lignin.[37] In the present study maximum delignification of 88. 8% and 86.7% was attained for DG and HNG respectively, with power

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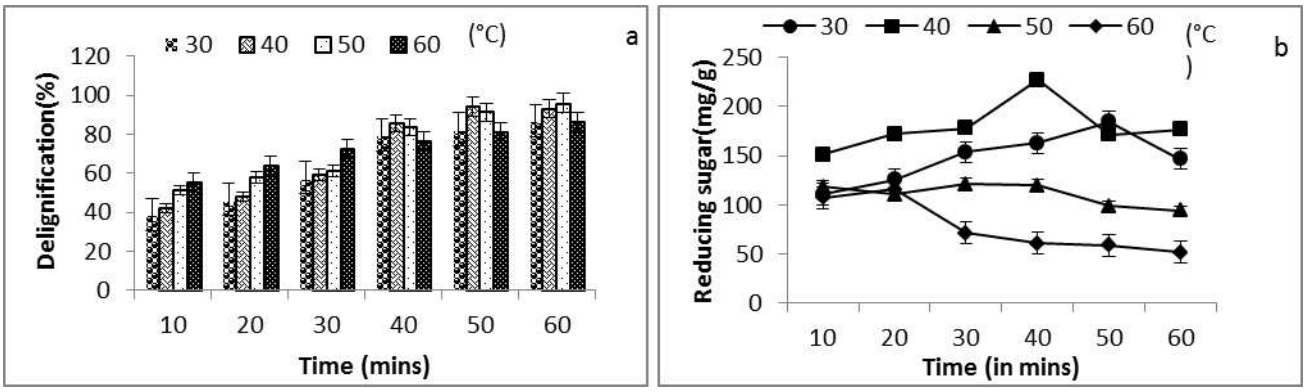

Figure 3. Effect of ultrasonication temperature on delignification (a) and reducing sugar (b) of DG

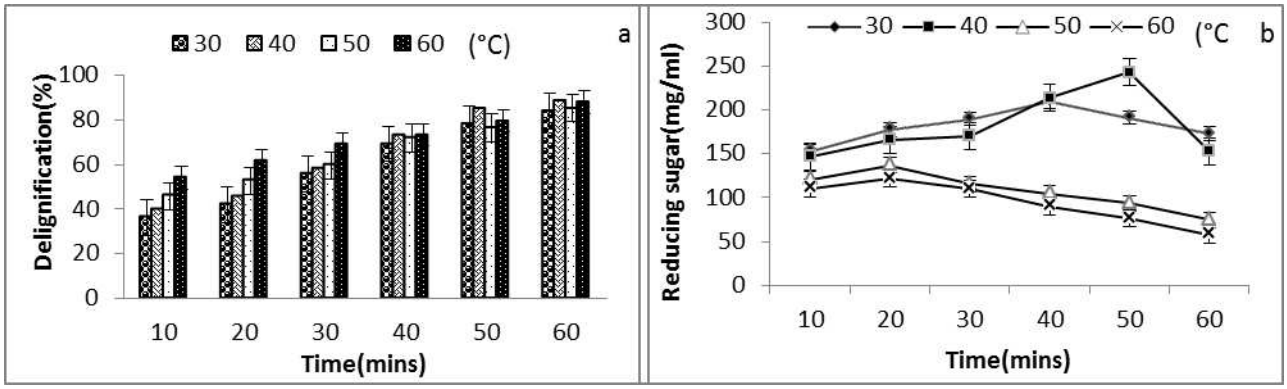

Figure 4. Effect of ultrasonication temperature on delignification (a) and reducing sugar (b) of HNG

supply of 90 W at 60 minutes as given in **Figure 1 (a and b)** and **2 (a and b)**. The temperature and duty cycle in this condition were maintained at 30 °C and 50% respectively. Subhedar et al. obtained 80.16% delignification at 100 W, for waste newspaper using ultrasono assisted alkaline pre-treatment.[24] But, significant loss of reducing sugar after pretreatment was the major drawback at greater power supply. The presence of significantly lower amounts of reducing sugar can be attributed to the principle of ultrasonication method which states that low-power supply and high-intensity ultrasonic energy can cut the chemical bonds like β-1,4-glycosidic bonds which bind the xylan branches to glucan, by increasing vibratory energy of material particles, thereby reducing the molecular weight and increasing water solubility.[38] The highest reducing sugar yield of 166.8 mg/g was achieved for DG (60 W for 40 mins) whereas for HNG, 145.0 mg/g of reducing sugar was achieved at 70 W for 30 mins. It has been reported that, in a specified sonication pre-treatment, the power given during the sonication and the time required are inversely proportional.[38] The release of reducing sugars in the medium during ultrasonication process mainly occurs due to the ultrasonication power which allows the degradation of the polymer chain by a process called depolymerisation. In this process cuts near the midpoint of the polymer chain are produced, thereby leading to release of reducing sugars.[28] In the above-mentioned parameters for achieving highest reducing sugar yields the delignification was restricted to 69.2% in DG and 50.3% for HNG.

3.1.2. Effect of Ultrasonication temperature on NaOH pre-treated samples.

It is observed in **Figure 3 (a and b)** for DG and **Figure 4 (a and b)** for HNG that the delignification and reducing sugar produced from biomasses using UA-NaOH pre-treatment is highly effective as compared to untreated and only alkali (1% NaOH) pre-treated samples. In case of untreated DG and HNG the acid soluble lignin and acid insoluble lignin (ASL + AIL) was 19.0% and 19.8% respectively. The 1% NaOH pre-treatment enhanced the delignification by reducing the ASL from 2.95% and 2.79% to 1.03 and 1.04% for DG and HNG respectively.[31] The AIL was found to be reduced from 16.05% and 17.01% to 11.52% and 12.28% for DG and HNG respectively during alkaline pre-treatment. Though alkali hydrolysis is normally utilised in biomasses like sugarcane bagasse, rice husk, corn stover etc., for breakage of ester bonds that form the cross-linkage in lignin and xylan, the crystallinity of the cellulose is not much altered.[30,39] The maximum cellulose content in

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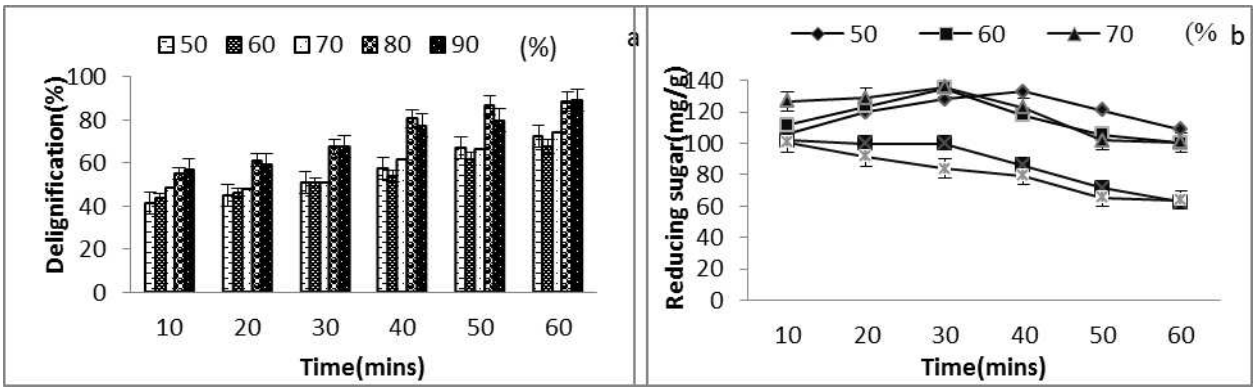

Figure 5. Effect of ultrasonication duty cycle on delignification(a) and reducing sugar (b) of DG

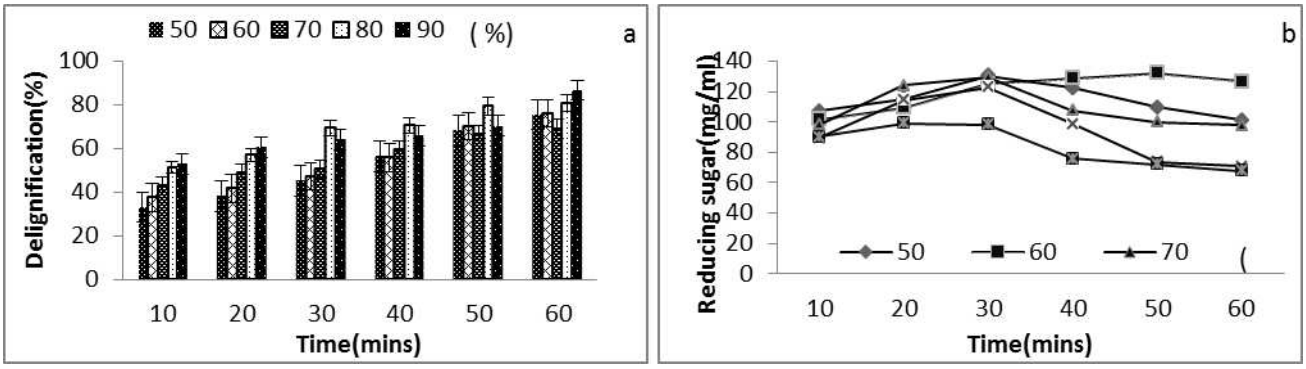

Figure 6. Effect of ultrasonication duty cycle on delignification (a) and reducing sugar (b) of HNG

untreated DG and HNG was 161 mg/g and 172.4 mg/g respectively. Similarly, the total hemicellulose content was observed to be 298.6 mg/g and 323.3 mg/g for untreated DG and HNG respectively.[31] Combination of alkaline pre-treatment with ultrasonication has been evaluated in lignocellulosic biomasses like sugarcane bagasse with greater modifications in cellulose structure (approximately 50%) leading to enhanced reducing sugar production.[40] In the present study, for the UA-NaOH pre-treatment the data for ASL and AIL is given as total lignin reduction (ASL + AIL) from the alkali pre-treated DG and HNG. While the temperature and incubation time were varied, duty cycle of 30% was kept constant. In case of DG, maximum delignification of 95.9% was achieved at 50 ^{0}C with an incubation time of 60 mins. But, at the maximum delignification parameters, the reducing sugar production were considerably low i.e. 62.7 mg/g. Hence, the parameter (40 °C for 40 mins) at which highest reducing sugar of 227.2 mg/g (after pretreatment) and delignification of 85.7% was achieved was considered for the subsequent enzymatic hydrolysis step. For HNG, the highest delignification and reducing sugar of 85.1% and 242.8 mg/g respectively was attained at 40 °C with an incubation of 50 mins. Kim et al. 2012, also carried out similar investigations on rice straw and the results exhibited that ultrasonication assisted alkali pre-treatment was more effective in delignification and cellulose conversion as compared to only alkali pre-treated biomass.[32]

3.1.3. Effect of ultrasonication duty cycle on NaOH pre-treated samples

Unnecessary ON time in ultrasonication leads to undue heating of the biomass and consumption of electrical energy. Hence, optimising the ultrasonication duty cycle (i.e. ON and OFF time) controls the length of each pulse in a sonicator when not in a continuous mode.[41] The present investigation was performed for 50%, 60% 70%, 80% and 90% duty cycles as shown in **Figure 5 (a and b)** and **Figure 6 (a and b)**. The temperature was maintained at 40 °C both for DG and HNG. With the increase in the duty cycle to 70% at 60 mins, 89.3% and 86.7% delignification was achieved for DG and HNG respectively. Subhedar et al., 2015 reported 80.0% delignification of cellulosic newspaper at 70% duty cycle, which agrees with the present study.[24] But, the increase in duty cycle and time of incubation beyond 50% and 30 mins was observed to be inversely proportional to the reducing sugar yield for both DG and HNG. The highest reducing sugar yield at 50% duty cycle and 30 mins was 135.8 mg/g in DG. In the same conditions reducing sugar yield of HNG was 129.7 mg/g which was lower as compared to DG. It is reported that ultrasound

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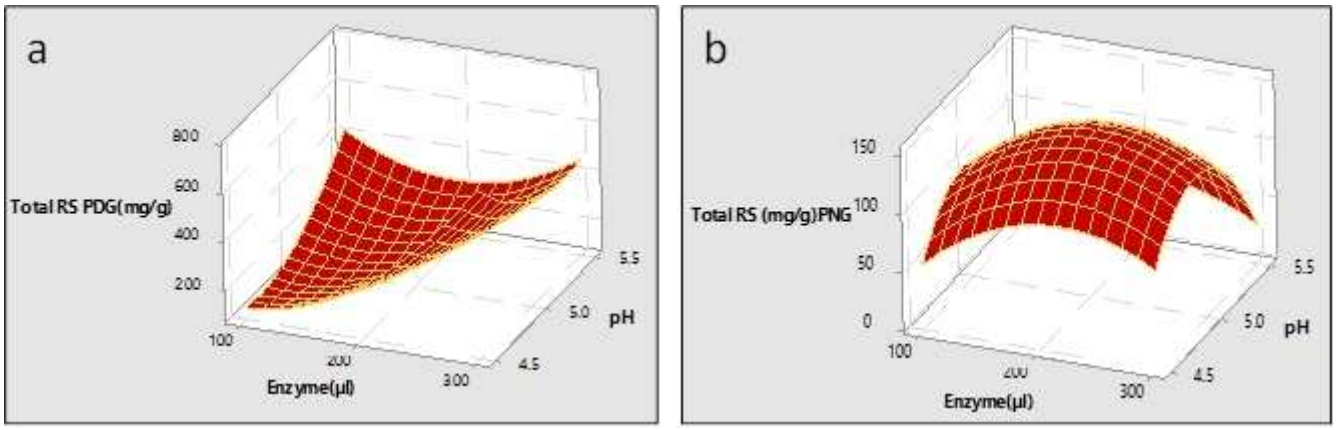

Figure 7. (a).3-D Surface plot representing optimized outputs of total sugar with respect to enzyme loading and pH(Enzyme loading and pH were considered for the 3D plots as these were the two parameters which significantly affected the release of reducing sugars from Palkonal MBW hydrolysed DG biomass). (b). 3-D Surface plot representing optimized outputs of total sugar with respect to enzyme loading and pH. Enzyme loading and pH were consideredfor the 3D plots as these were the two parameters which significantly affected the release of reducing sugars from Palkonal MBW hydrolysed HNG biomass) [Hold Values were Temperature: 45 °C, Substrate Loading: 1 g, Incubation Time: 32 hrs]

duty cycle does not strongly affect sugar release in lignocellulosic biomass.[20] But contrary reports have been given by Eblaghi et al., 2016 who reported that ultrasound pre-treatment of bagasse prior to hydrolysis step resulted in increased sugar yield as compared to sole alkaline pre-treatment.[42] The ultrasound-assisted alkaline (at 3% NaOH) pre-treatment exhibited maximum sugar yield of 33.73 g sugar in 100 g biomass (337.3 mg/g). Ultrasonication assisted NaOH pre-treatment in *Parthenium hysterophorus* (carrot grass) resulted in maximum reducing sugar yield of 30.84% after 84 h of alkaline hydrolysis.[43]

3.2. Response surface methodology for designing of enzymatic hydrolysis of UA-NaOH pre-treated biomass using commercial enzymes.

UA-NaOH pre-treated DG and HNG were subjected to enzymatic convertibility to determine the efficiency of pre-treatment using a new commercial enzyme Palkonal MBW and a conventional combination of Celluclast1.5 L + Xylanase. Enzymatic digestibility is an excellent probe that strongly correlates with pre-treatment effectiveness and the accessibility of cellulose to depolymerisation catalysts.[44] Though many studies consider in house production of enzymes using solid state or submerged fermentations[45] for saccharification of lignocellulosic biomass, but the present study focuses on the commercially available enzymes and the effects of combinations of the same. The experiments (thirty-two combinations) were designed according to CCD, in two separate experiments for each of the biomass and the response in terms of glucose and xylose was evaluated.

In the first experimental setup, Palkonal MBW was evaluated against DG and HNG taking different parameters like temperature, pH, substrate concentration, enzyme concentration and incubation time into consideration as given in **Table S1**(Supplementary data). The 3-D plot for the best parameter for enzymatic hydrolysis of DG with total reducing sugar yield of 662.0 mg/g at temperature-50 ^{0}C, pH-5.25, substrate concentration-2.5 g, enzyme concentration-250 µl and incubation time of 30 h is given in **Figure 7 (a)**. Similarly **Figure 7 (b)** signifies the total sugar outputs of Palkonal MBW enzyme treated HNG. In case of HNG the highest reducing sugar yield of 331.2 mg/g was obtained at temperature 45 ^{0}C, pH-5, substrate concentration-0.5 g, enzyme concentration-200 µl and incubation time of 40 h. The surface plots were considered based on optimized **Eq S1** and **Eq S2** for removal of insignificant terms. Enzyme loading and pH had significant effects over the course of experiments. In **Figure 7 (a)** it is observed that the surface is concave in nature which suggests that higher reducing sugar values were found at extremum. As per the model, further optimization studies can be considered to evaluate the performance of the enzyme at higher pH and substrate concentrations. In case of **Figure 7 (b)** a convex surface is observed which shows the optimum enzyme concentration and pH conditions for high total sugar production. According to **Eq S1** and **Eq S2**, substrate concentrations can be further studied as optimum enzyme and pH conditions have been found. Since this is the first report for enzymatic hydrolysis by Palkonal MBW enzyme, therefore no comparative reports have been found. DG was found to have a higher reducing sugar yield with greater substrate loading and less incubation time as compared to HNG. The reduction in total reducing sugar yield in HNG is because of the significantly low amount of xylose sugar (107.6 mg/g) that is produced during the enzymatic hydrolysis. In case of DG though the xylose sugar concentration was also less (201.8 mg/g), but the higher glucose yield (374 mg/g) resulted in higher production of total reducing sugar. The results clearly indicate that, the enzyme Palkonol MBW has a greater cellulolytic activity, but is also efficient in hydrolysing the pentosans. From an economic prospective the enzyme can be of great potential for hydrolysing lignocellulosic biomasses.

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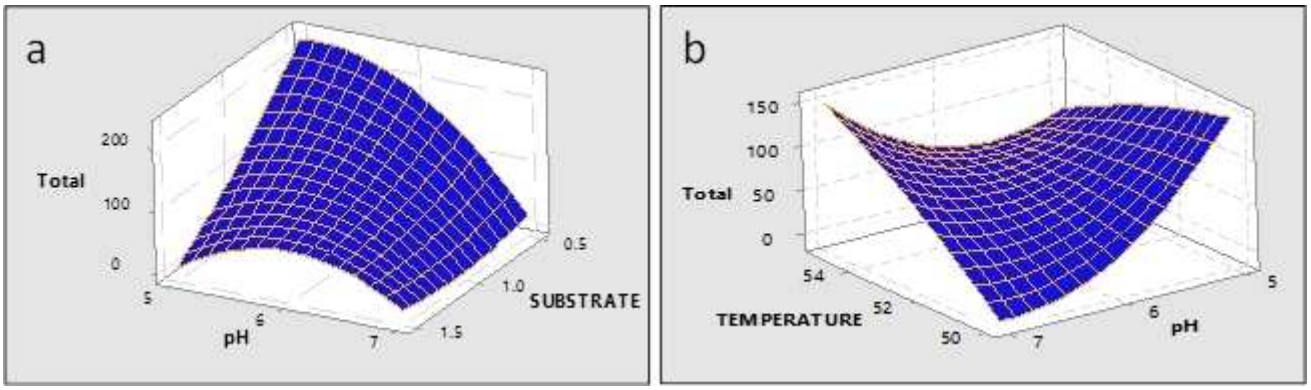

Figure 8. (a) 3-D Surface plot representing optimized outputs of total sugar with respect to pH and substrate loading (pH and substrate loading were considered for the 3D plots as these were the two parameters which significantly affected the release of reducing sugars from Celluclast1.5 l-Xylanase enzyme hydrolysed DG biomass) **(b)** 3-D Surface plot representing optimized outputs of total sugar with respect to to incubation temperature and pH (to incubation temperature and pH loading were considered for the 3D plots as these were the two parameters which significantly affected the release of reducing sugars from Celluclast1.5 l-Xylanase enzyme hydrolysed HNG biomass) [Hold Values were Temperature: 52.5 °C, Xylanase per 200 μl Cellulase: 4 μl, Incubation Time: 42 hrs]

In the second experimental setup, i.e. Celluclast1.5 L + Xylanase, the concentration of Celluclast1.5 L was kept constant (200 μl) and the concentration of xylanase was varied for all the thirty-two experiments designed by CCD for both DG and HNG as given in **Table S2**(Supplementary data). Although pre-treatment eliminates most of the xylan content, even low amounts of residual xylan can restrict the extent and efficiency of cellulose hydrolysis by cellulases. This limitation can be eradicated by addition of xylanases that solubilizes xylan in the substrates.[46,47] Thus, xylanases play an important role in efficient hydrolysis of xylan-containing lignocellulosic materials. For the second experimental setup, **Figure 8(a)** represents the 3-D plots which show the concave plots suggesting best results at extreme points of the parameters like substrate loading and pH for achieving the highest total reducing sugar yield of 189.46 mg/g in case of DG. The glucose concentration at the optimum conditions of temperature-51 °C, pH-5.5, substrate concentration-2.5 g, xylanase/celluclast 1.5 L ratio- 2.5/200 (μl), and incubation time of 30 h was 138 mg/g while the xylose concentration was 51.46 mg/g. 3-D plots presented that the results for total reducing sugar yield in DG was in agreement with the earlier reports of enzyme hydrolysed alfalfa, switch grass and reed canary grass with total reducing sugar yields of 201.0 mg/g, 207.0 mg/g and 197.0 mg/g respectively.[48] However, in case of HNG temperature and pH **(Figure 8 b)** were the important factors, responsible for the highest reducing sugar yield of 148.7 mg/g, with the substrate loading of 1%, which was significantly low as compared to DG. This decrease in reducing sugar may have occurred because of the presence of hemicellulose in the biomass that can impede the accessibility of enzyme molecules to cellulose leading to lesser hydrolysis.[49] Here also the 3-D plots were concave suggesting that at higher substrate concentrations, the effect of other parameters is greatly reduced and does not result in higher yields. In comparison to HNG, DG plots show basis of improvement of yield with lower incubation time while maintaining the optimum enzyme loading and incubation temperature. Though, xylanase has an activity for hydrolysis of hemicellulose but in the present experiment the xylanase activity seems to be negligible for all the HNG biomass. In recent studies xylose, xylo-oligosaccharides (XOs) and xylans have been seen to impart a negative impact during hydrolysis of cellulose with cellulase.[50–52] Another factor that has also been seen to be partly responsible for the negative impact of XOs on cellulases efficiency is the property of competitive inhibition.[53,54] Though studies have shown that supplementation of a cellulase with xylanase improved glucan conversion from corn stover, recent studies have reported that the improvement of enzymatic cocktails largely depends on the substrate used for hydrolysis.[55] Reports of cellobiohydrolases(CBH) in cellulase having higher affinity, to lignin's than endoglucanases (EG) have also been cited for low reducing sugar productions from biomass.[56] A phenomenon called as adsorption/desorption of lignocellulose takes place during the enzymatic hydrolysis step, which determines the rate of enzymatic hydrolysis. At this stage if more amount of lignin is present CBH having more affinity towards lignin will undergo nonspecific adsorption to the lignin and can play a major role in insufficient hydrolysis of the lignocellulosic substrate. Hence, appropriate pre-treatment conditions which will lead to biomass properties like smaller particle size, lower lignin content and less crystalline cellulose are preferred conditions to avoid unspecific binding of CBH.[57] The difference seen in the reducing sugar yield between the two grass biomass was notable and a hence a comparative assessment of the total reducing sugar of saccharified DG and HNG was conducted in comparison to the literature studies done with different grass biomass as shown in **Table 1**. Since, Palknonal MBW treated DG and HNG showed the highest reducing sugar yield as compared to Celluclast 1.5 L + xylanase treated DG and HNG, ANNOVA (Analysis of Variance) was performed only for the Palknonal MBW treated DG and HNG.

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Table 1. Summary of Literature on the enhancement of reducing sugar after enzymatic hydrolysis using ultrasonication assisted alkali pretreatmemt of biomass

Grass variety	Parameters maintained in Pretreatment	Reducing sugar produced after pretreatment (mg/g)	Enzymes used for saccharification of pretreated biomass	Reducing sugar produced after saccharification(mg/g)	References
Parthenium hysterophorus	Sono assisted alkali pretreatment	NM	Carboxymethylcellulase (CMCase, 1.0 U/ mg), produced by *Bacillus amylolique-faciens* and β- glucosidase from Novozyme 188	308.4	[49]
Sugarcane bagasse	Sono assisted alkali pretreatment	362.2 mg/g	Accellerase 1500 (550 U/g)	434.2	[43]
Rice straw	Sono assisted alkali pretreatment	310.0 mg/g	Accellerase 1500 (550 U/g)	441.4	[43]
Sugarcane bagasse	Sono assisted alkali pretreatment	337.0 mg/g	Cellulase (NS22086 cellulase complex) and cellobiase (NS22118 β-glucosidase)	504.5	[42]
Sugarcane bagasse	Sono assisted alkali pretreatment	471.6 mg/g	0.46 CBU/g cellulose of commercially available cellulase and b-glucosidase(SRL India)	924.6	[40]
Switchgrass	Ultrasonication assisted pretreatment (15 mins, 180 °C)	400 mg/g	Accellerase 1500(0.5 ml/g)	480	[37]
Rice straw	Ultrasonication assisted pretreatment (15 mins, 180 °C)	550 mg/g	Accellerase 1500(0.5 ml/g)	610.5	[37]
Hybrid Napier grass	Ultrasound assisted alkali (50 mins,40^{0}C,)	242.8 mg/g	Palkonal MBW(250 U/g)	431.2	Present study
			Celluclast1.5 l + Xylanase(Novozymes)	370.8	
Dennanath grass	Ultrasound assisted alkali (40 mins, 40^{0}C)	227.8 mg/g	Palkonal MBW()	662.0	Present study
			Celluclast1.5 l + Xylanase(Novozymes)	433.4	

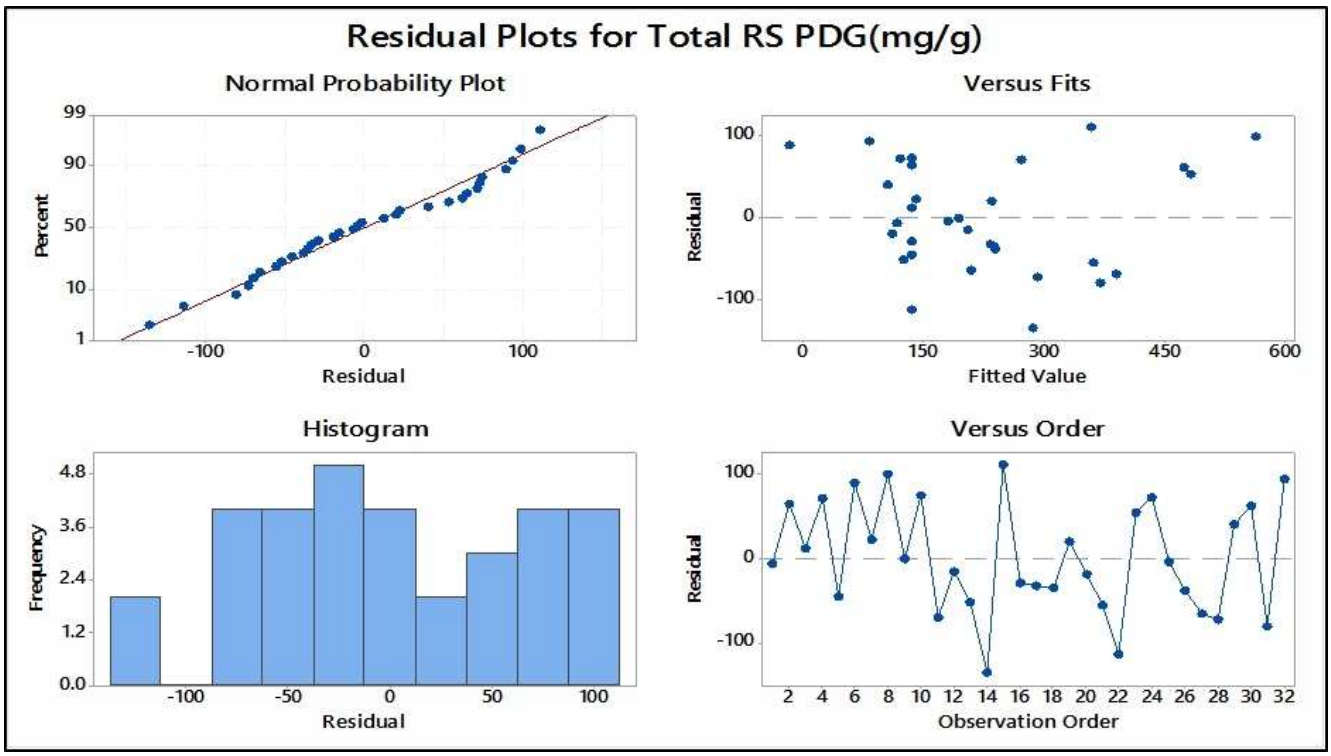

Figure 9. Residual plots presenting the probability plots, the fitted values and the variations in the experimentation data are given for the total reducing sugar release after enzymatic hydrolysis. The denotations are given as total reducing sugar (total RS) release using Palkonal MBW for DG (PDG)

ANNOVA was carried out to evaluate the effects of the five continuous factors and their possible interactions.

Residual plots of total sugar yield of Palkanol MBW for both DG [**Figure 9**] and HNG [**Figure 10**] suggested that the observed yields do not have a greater difference from the

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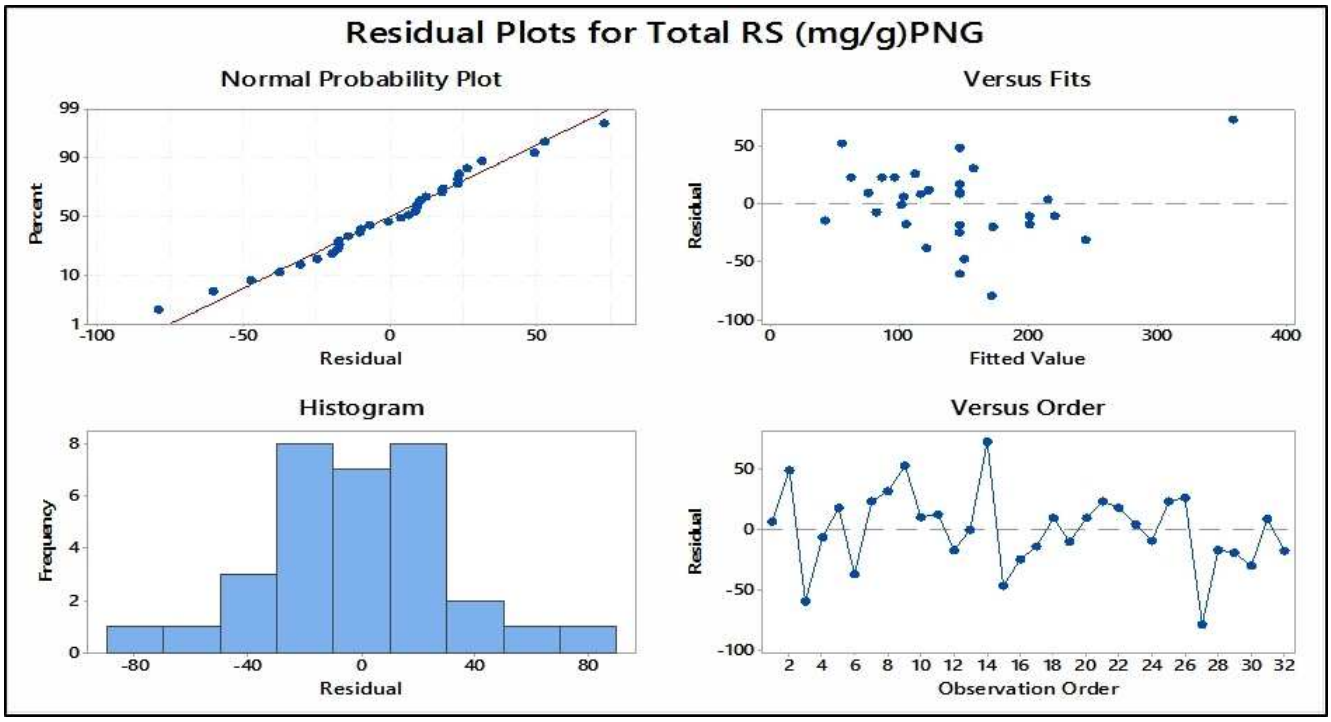

Figure 10. Residual plots presenting the probability plots, the fitted values and the variations in the experimentation data are given for the total reducing sugar release after enzymatic hydrolysis. The denotations are given as total RS (total RS) release using Palkonal MBW for HNG (PNG)

Table 2. Mass balance of total reducing sugar and residual lignin content. The T.R.S represents total reducing sugar in case of pretreated samples.

Grass cell wall component	Untreated sample		Alkali treated sample		Ultra-sonicated sample		Enzyme hydrolysed sample (PalkonalMBW)		Enzyme hydrolysed sample (Celluclast 1.5 L + Xylanase)	
	DG	HNG	DG	HNG	DG	HNG	DG	HNG	DG	HNG
Solid Fraction (% of substrate)	100	100	54.82	55.49	68.86	70.85	15.05	33.51	24.14	38.73
Cellulose (% of Retentate)	32.94	37.34	46.50	47.94	60.18 T.R.S	61.74 T.R.S	59.29	48.63	51.08	45.11
Hemicellulose (% of Retentate)	23.41	18.82	11.69	12.94			18.54	16.18	6.05	15.40
Lignin (% of Retentate)	19	19.8	12.55	13.32	5.85	9.84	–	–	–	–
Liquid Fraction (% of substrate)	0	0	45.18	44.51	31.14	29.15	84.95	66.49	75.86	61.27

predicted values in the RSM equation. But, in case of Celluclast 1.5 L + Xylanase combination for DG [**Figure 11**), the values strayed out more from the residual plot line. In case of HNG [**Figure 12**] values were similar for both Palkanol MBW and Celluclast 1.5 L + Xylanase combination. It showed that in comparison to Celluclast 1.5 L + Xylanase enzyme combination, Palkanol MBW had more significant effect with better results. The optimum values for DG and HNG also showed that DG had higher total sugar yields at optimum conditions. The structural and functional characterization was analyzed in Mohapatra et al., 2017.[58] The mass balance report is as shown in **Table 2** which represents the efficiency of ultrasono-assisted alkali pretreatment followed by enzymatic hydrolysis in extraction of reducing sugars along with high delignification. The cellulose and hemicellulose composition in untreated and alkali treated samples were noted as per Mohapatra et. al., 2015.[31] It was observed that there is a significant loss in sample amount in alkali pretreatment which could be attributed to the higher degradation of pentosans as compared to glucans. However, in the proceeding step of ultrasonication, interestingly there was minimal mass loss in both DG and HNG, accrediting to the removal of lignins rather than carbohydrates. Further, in enzymatic hydrolysis, a greater biomass loss was observed for both the grass biomass treated with Palkonal MBW and Celluclast 1.5 L + Xylanase. Nevertheless, DG samples exhibited higher biomass reduction for both the enzymes attributing to the higher saccharification of cellulose and hemicellulose to glucans and xylans and its release in the liquid fractions. The results in **Table 2** also demonstrated that PalkanolMBW showed higher affinity for DG than HNG as greater reductions in DG biomass was observed.

3.4. Glycome profiling

The study conducted in this manuscript is evaluating the roles of cell wall polysaccharides including major non-cellulosic glycans in *Pennisetum* grass feedstocks under untreated and pretreated conditions. Glycome profiling, a powerful technique,

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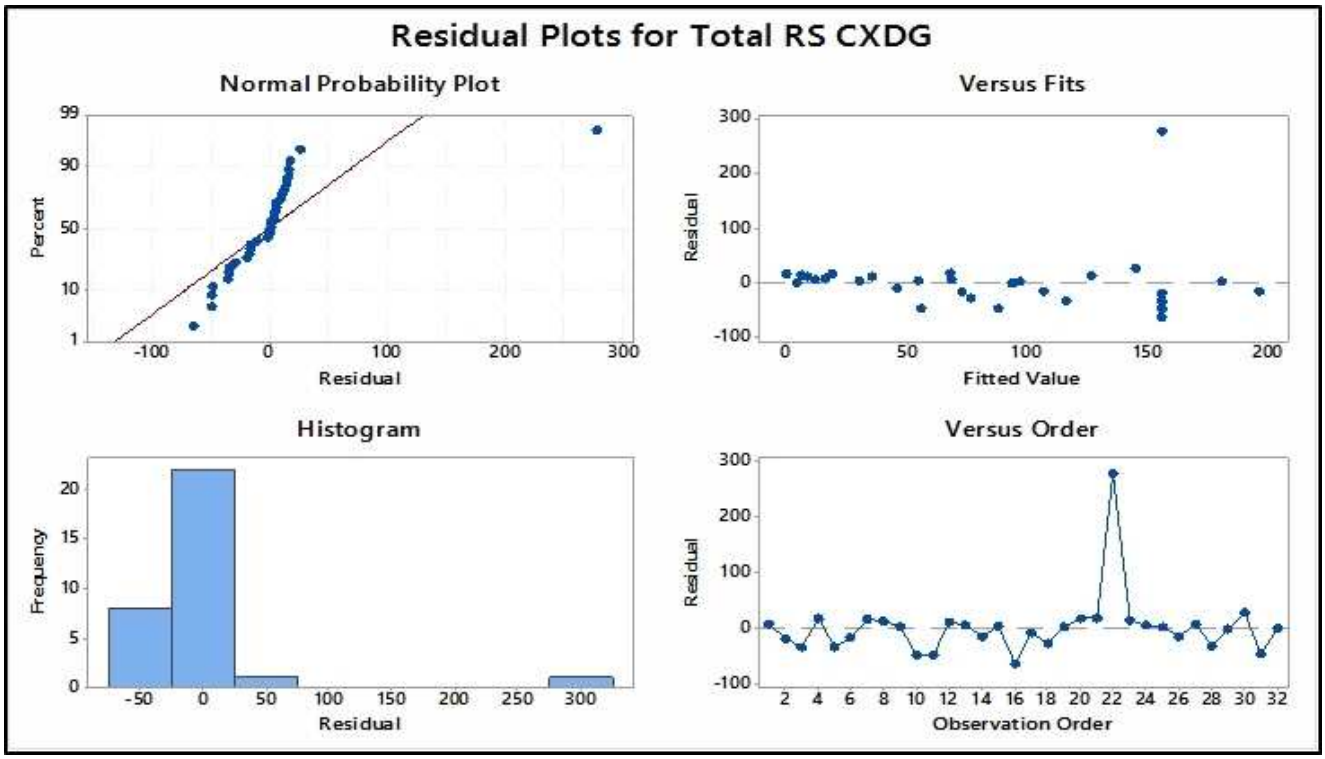

Figure 11. Residual plots presenting the probability plots, the fitted values and the variations in the experimentation data are given for the total reducing sugar release after enzymatic hydrolysis. The denotations are given as (a) total reducing sugar using Celluclast1.5 L + Xylanase (total RS CX) for DG (CXDG).

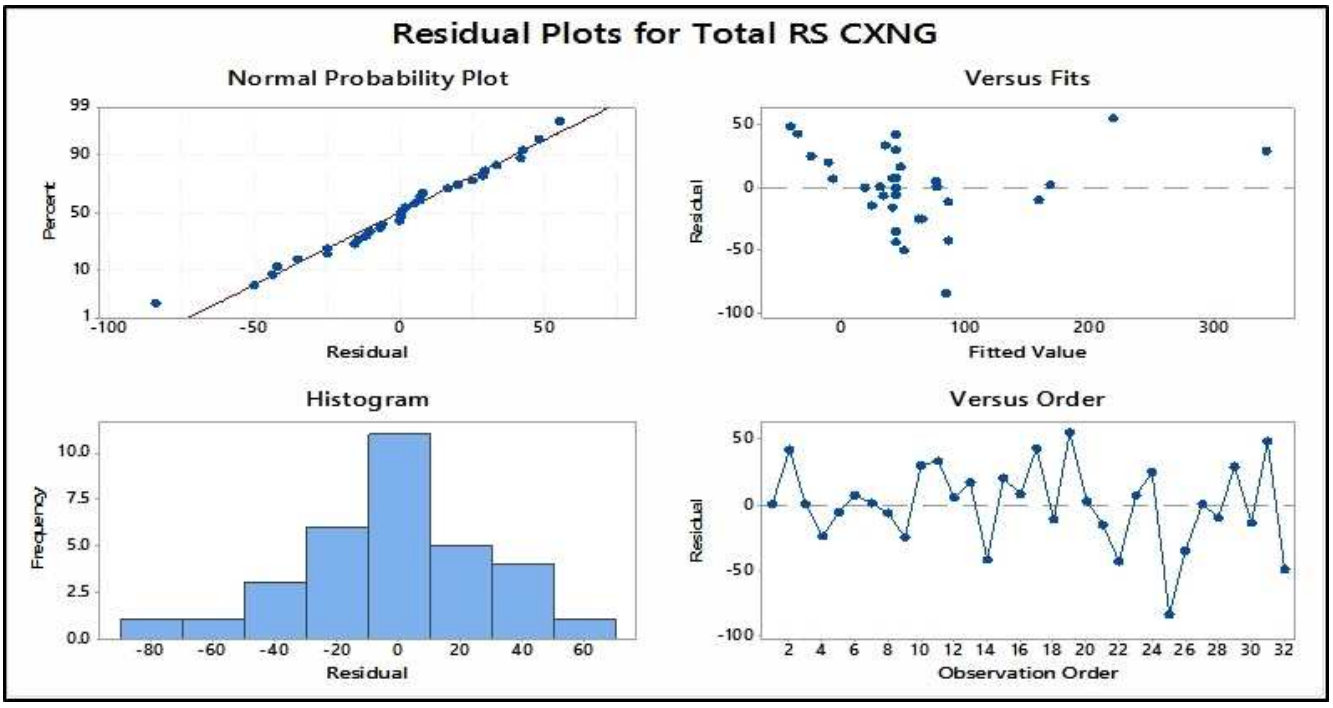

Figure 12. Residual plots presenting the probability plots, the fitted values and the variations in the experimentation data are given for the total reducing sugar release after enzymatic hydrolysis. The denotations are given as total RS release using Celluclast1.5 L + Xylanase for HNG(CXNG).

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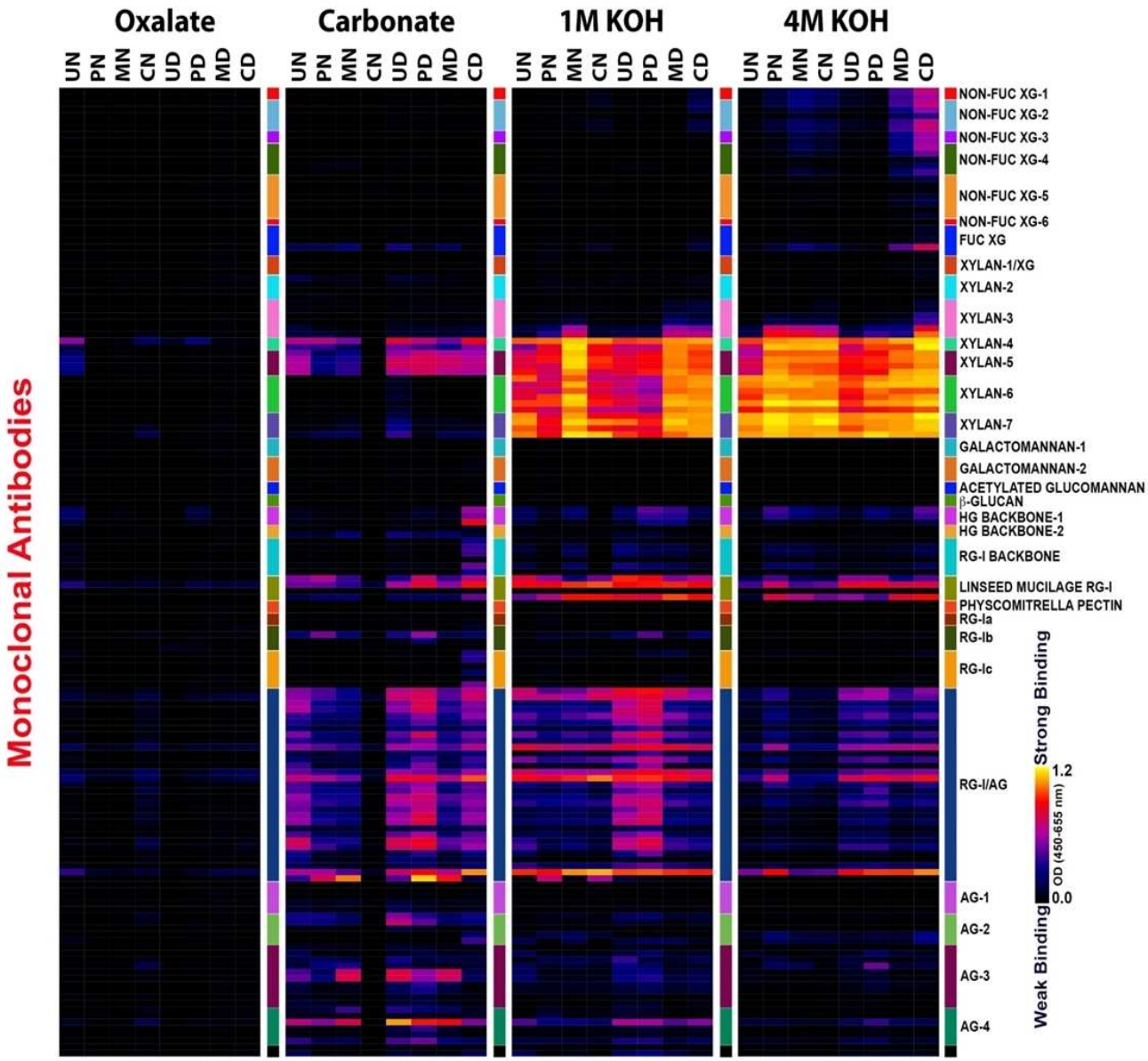

Figure 13. Glycome profiles of various fractions of DG and HNG to elucidate and correlate the overall non-cellulosic matrix cell wall glycan structures and compositions. The annotations for different fractions (oxalate, carbonate, 1 M KOH and 4MKOH) are as follows- UN- Untreated hybrid napier grass, PN- Pretreated (ultrasonication-alkali) hybrid napier grass, MN- Palkonal MBW enzyme hydrolysed hybrid napier grass, CN- Celluclast1.5 l + xylanase hydrolysed Hybrid napier grass, UD- Untreated dennanath grass, PD- Pretreated (ultrasonication-alkali) dennanath grass, MD- Palkonal MBW enzyme hydroysed dennanath grass, CD- Celluclast1.5 l + xylanase enzyme hydrolysed dennanath grass.

allows comprehensive characterization of composition and extractability of most major non-cellulosic glycans and reveals how tightly these glycans are integrated in the pretreated plant biomass compared to untreated controls (an indirect measure of biomass recalcitrance). The method is an advanced approach that takes advantage of the availability of worldwide collection of plant cell wall glycan epitope-directed monoclonal antibodies (that could monitor most major non cellulosic glycan structures in plant biomass) for rapid and reliable characterization of non-cellulosic glycans comprised in plant cell walls. Several previous studies have employed glycome profiling in plant biomass based bio-fuel research for reaching important scientific conclusions and such studies have been comprehensively reviewed earlier.[35,59]

We conducted glycome profiling to evaluate the fate of most major non-cellulosic cell wall glycans in *Pennisetum* grass feedstocks that include hemicelluloses and pectin's (**Figure 13**). Oxalate extracts, overall, showed significantly reduced abundance of non-cellulosic glycan epitopes across all samples. Pectic arabinogalactan epitopes were present in trace levels in oxalate extracts from most samples analysed. Interestingly, a detectable abundance of substituted (arabinosylated) xylan

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epitopes (as denoted by the binding of xylan-4 and 5 groups of mAbs) was observed in the oxalate extract from untreated HNG (UN) samples. Carbonate extracts revealed more variation dynamics in the abundances of non-cellulosic glycan epitopes among all samples analysed. Carbonate extracts from all samples with the exception of enzyme hydrolysed (Celluclast1.5 l + xylanase) HNG (denoted in the **Figure 13** as CN, which showed no detectable presence of any non-cellulosic glycans in it) exhibited significant abundance of substituted xylan epitopes (as denoted by the binding of xylan-4 and 5 groups of mAbs). This pattern was similar in the case of pectic arabinogalactan epitopes as well (as denoted by the binding patterns of linseed mucilage RG–I, RG–I/AG and various AG groups of mAbs) where in significant abundances of these epitopes were noted in all samples except enzyme hydrolysed (Celluclast1.5 l + xylanase) HNG (denoted in the **Figure 13** as CN, which showed no detectable presence of any non-cellulosic glycans in it). Interestingly, in carbonate extract of enzyme hydrolysed (Celluclast1.5 l + xylanase) DG (denoted in the figure as CD) showed an enhanced abundance of pectic backbone epitopes (as denoted by the increased abundance of pectic epitopes detected by HG-backbone-1 and RG–I backbone groups of mAbs). Overall, the patterns of extractability of most non-cellulosic glycan epitopes were similar in 1 M KOH and 4 M KOH extracts among most samples wherein they exhibited significant abundances of substituted and unsubstituted xylan and pectic arabinogalactan epitopes. However, some interesting differences were noted in the 1 M KOH extracts from both the enzyme hydrolysed DG samples (denoted as MD and CD in **Figure 13**) where in a significant reduction in the pectic arabinogalactan epitopes was noted in comparison to corresponding untreated and pre-treated DG samples (denoted as UD and PD in **Figure 13**). These results demonstrate that enzymatic hydrolysis does induce changes in the overall compositions of DG biomass used. Recently, Damm et al., 2017, also highlighted the presence of pectin polysaccharides in cell wall of seda (a potential bioenergy crop) to have negative influence on the enzymatic hydrolysis.[60] The reduction in pectin polysaccharides also indicates the greater efficiency of delignification in DG samples. Similarly, glycome profile studies on cell wall components of Miscanthus have revealed the tight association between pectin and lignin and its reduction to be directly proportional to delignification.[61] In general, 4 M KOH extracts from all samples exhibited high abundance and proportion of xylan (both substituted and un-substituted xylans) epitopes with most extract showing monocot/grass specific xylan-3 detected epitopes.[35] Again, 4 M KOH extracts from both enzyme hydrolysed DG (denoted as MD and CD in Figure 11) were marginally different from corresponding untreated and pre-treated DG samples (denoted as UD and PD in Figure 11) in that they contained higher abundance of xyloglucan epitopes (denoted by relatively increased binding of non-cellulosic XG-1 through 4 groups of mAbs). Overall, glycome profiling studies allowed delineating overall structures, compositions and extractabilities of most major non-cellulosic glycans in the samples studied here and, even though subtle, emphasized variations among them. Further, glycome profiling revealed that no significant change exists in the overall composition and extractability of most matrix cell wall glycans among untreated and pre-treated samples. However, some changes do get induced after enzymatic hydrolysis as revealed above in the case of DG samples. It is quite interesting to note that xylan and pectic polysaccharide extractability varies with the maturity of the grass biomass, with harsher pre-treatments to more mature biomasses.[62] These results also indicated that enzymatic hydrolysis conditions need to be further optimized to achieve a complete biomass conversion deconstructing the otherwise unhydrolyzed non-cellulosic matrix polysaccharides (as revealed by above glycome profiling results).

4. Conclusion

The information presented in this study can be helpful for understanding the effect of ultra-sonication assisted alkali pre-treatment and subsequent enzymatic hydrolysis on the cell wall polysaccharides of the two *Pennisetum* grass varieties. These insights can help in building genetically modified grass feedstocks with targeted modifications for lower pectin related polysaccharides in the cell wall. Further, this can be utilised for higher release of fermentable sugars, without harsh pre-treatment strategies and costly enzymatic applications. Interestingly, denanath grass proved to be a potential biofuel candidate which demonstrated promising cell wall polysaccharides to that of bio refinery based perennial feedstocks. Overall, the present study gives a holistic picture of the importance of the cell wall components on the mechanism of the pre-treatment and enzymatic hydrolysis of a low-cost biomass for efficient sugar production which will subsequently lead to sustainable bioethanol generation in future

Supporting Information Summary

The supporting information consists of the experimental section of the present investigation. It also consists of Table S1 and S2 which represent RSM Design for enzymatic hydrolysis of hybrid napier grass (HNG) and denannath grass (DG) using Palkonal MBW enzyme and Celluclast1.5 L (cel) –Xylanase (xyl) enzyme mixture.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: *Pennisetum* species · Response surface methodology · Enzymatic hydrolysis · Glycome profiling.

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