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Effect of dilute acid pretreatment of wild rice grass (*Zizania latifolia)* from Loktak Lake for enzymatic hydrolysis

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A R T I C L E   I N F O

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A B S T R A C T

Zizania latifolia commonly known as wild rice grass which is available in huge quantities in Loktak Lake is a major concern as it occupies a large area of the Lake and causing a several environmental problems. The investigation of present study was to evaluate possibilities of using *Zizania latifolia* as feed stock for bioethanol production. The method involved the pretreatment with dilute acid or alkali followed by enzymatic hydrolysis with commercial cellulase. Acid pretreatment was performed with 10% biomass loading with different concentration of acids ([illegible] v/v) and alkali ([illegible]1.5% w/v). Maximum sugar release of 457 mg/g was obtained from 10% biomass loading with 2% w/v of acids. Alkali pretreatment is not effective for this grass. Physicochemical characterization of untreated and treated biomass was carried out by XRD, FTIR, SEM and corresponding alteration in the chemical composition were also monitored. Results showed the feasibility of this grass as biofuel (bioethanol) feed stock and can be potential approach to address the sustainable utilization phumdi grass of Loktak Lake for the production of value added product.

1. Introduction

Production of biofuels from lignocellulosic based biomass could be an alternative sustainable solution to minimize dependency on fossil fuels. The conversion of various products from lignocellulosic material is significantly blocked by the structural and chemical complexity of raw material, which makes cellulosic biofuel economically unfit. Generally lignocellulosic materials contain cellulose, hemicelluloses, lignin as well as other components. Cellulose and hemicellulose are chain carbohydrates that can be broken down or modified into fermentable sugars and wide variety of other products (Chatterjee et al., 2015). Lignin is strongly connected and bonded with carbohydrates structure through covalent or non-covalent bonds which can be exploited as source of high value aromatic products (Zeng et al., 2014). Therefore pretreatment is an important step to unlock the lignocellulosic complex structure to produce monomeric sugars from their respective polysaccharides. In this context, acid pretreatment is the most widely used and proven to be a fast as well as cost effective method (Behera et al., 2014).

The pretreatment step itself is one of key costly process to the economics of biofuels while it also influencing the cost effectiveness of the downstream processes (Shirkavand et al., 2016). There are different pretreatment approaches that have been studied for fractionizing the lignocellulosic material. Dilute acid pretreatment is commonly used for solubilizing hemicellulosic part of the biomass; lignin present in biomass can block the cellulase binding of biomass in further saccharification process (Yan et al., 2017). For further increase in the process yield, delignification is needed. Alkaline pretreatment helps to solubilize the lignin part of biomass without significant cellulose degradation (Sun et al., 2016). Ester linkages present in lignin as well as hemicelluloses are broken during alkali pretreatment (Sun et al., 2016). Different lignocellulosic feedstocks, such as *Miscanthus* grass (Li et al., 2014), para grass (Sahoo et al., 2017) chili post harvest residue (Sindhu et al., 2017), sugarcane bagasse (Zhu et al., 2016) and rice straw (Zhang et al., 2018) has been treated by combined acid and alkaline pretreatment.

Zizania latifolia locally known as 'Kambong' and commonly called as wild rice grass is belonging to the family Poaceae. It is perennial

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aquatic, tall erect herb up to 2.5 m with well-developed stolons and fibrous roots generally grown in swampy areas and wetlands. The plant grows luxuriously in the wetlands of Manipur state, North–Eastern part of India (lies in the Indo-Burma biodiversity "hotspot" zone) more prominently at Loktak Lake which is one of the 'Ramsar sites' of global significance. There is no literature available for the utilization of this grass for biofuel application. This grass has over grown as an aquatic weed associated with the floating vegetation of the Lake called phumdis, which a major concern as it occupies a large area of the Lake and causing a several environmental problems. Therefore, effective addressing of this issue is utilizing aquatic biomass from the Lake for commercial application such as bio-ethanol and other platform chemicals for the socio-economic benefit of the region.

The objective of the present study was to evaluate potential of wild rice grass as bio-ethanol feed stock by dilute acids and alkali pretreatment process. Effect of pretreatment on biomass was evaluated by digestion of samples for releasing fermentable sugars which was carried out with commercial cellulase and alteration in the structure of biomass was monitored through FTIR, XRD, SEM along with its compositional analysis.

2. Materials and methods

2.1. Feedstock

Wild rice grass was collected from Loktak Lake (Manipur, India). The biomass were dried under sunlight and milled using a knife mill in order to get a particle size less than 1 mm. Milled sample was mixed well and stored at room temperature (26–30 °C). Native biomass contains 12.7% moisture content.

2.2. Compositional analysis

The compositional analysis of the sample was analyzed according [illegible] National Renewable Energy Laboratory (NREL) analytical methods for biomass (Sluiter et al., 2011). In a brief, 300 [illegible] of biomass was weighed after oven drying and first hydrolyzed with H[illegible] with a concentration of 72% (v/v) for 1 h at 30 °C. Hy[illegible] diluted to 4% (v/v) with distilled water [illegible] second hydrolysis of the reaction mixture was performed by autoclaving at 121 °C [illegible] h. Further steps are identical to our previous re[illegible] component analysis of para grass (Sahoo et al., 2017).

2.3. Dilute acid and alkali pretreatment

Pretreatment experim[illegible] was conducted in 500 ml beaker with 10% biomass loading with 0.25[illegible] (w/v) [illegible]H and H_2SO_4 loading of 0.4–2% (w/v)[illegible] were autoclaved at 121 °C for 60 min. Pretreated samples were neutralized up to pH 4.8 with 1 N NaOH and 1 N H_2SO_4 respectively.

2.4. Physico chemical characterization of biomass

2.4.1. SEM analysis

In order to view the morphological changes happening in the surface of pretreated and native biomass samples, scanning electron microscopy analysis was done for viewing the surface structure. The analysis of SEM was performed using a scanning electron microscope (JEOL JSM5600LV, Germany).

2.4.2. FTIR and XRD analysis

Fourier transform infrared spectra of native and pretreated biomass were studied using a spectrometer (Shimadzu, Japan). It helps to track the changes in the functional groups and bonding of the lignocellulosic molecular structure of the biomass. FTIR and XRD analysis of the samples was done according to Sahoo et al. (2017).

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of samples was performed in 150 ml conical flask by adding 2% (w/w) of biomass with commercial cellulase (Zytex Pvt. Ltd. India). The enzyme was loaded at 20 FPU/g of pretreated samples with 200 µl of 10x solution of antibiotic (Penicillin Streptomycin mix Himedia) and surfactant (Tween-80) 0.1% (w/w) was added. The reaction volume was made up to 20 ml with citrate buffer (0.1 M). The reaction mixtures were incubated at 50 °C in a shaking water bath at 120 rpm for 48 h. Reducing sugar analysis of hydrolyzed sample was carried out by 2, 5-dinitrosalicylic acid method (Miller, 1959).

3. Results and discussion

3.1. Surface characterization of biomass

Morphological changes of native and pretreated material was analyzed by scanning electron microscopic analysis. Acid pretreated biomass showed that fi[illegible] are [illegible] completely and many places are disconnected from the fibers which helps the exposing active site of the biomass to hydrolytic enzymes such as cellulase that helps in releasing the fermentable sugars in the further hydrolytic process. While alkali pretreated biomass fibers are not much affected compared to acid pretreatment. Native biomass is intact and organized surface compared to pretreated biomass. After pretreatment, the fibers became ruptured and thinner which could be due to hemicellulose removal. Similar observations are earlier reported in the case of elephant grass as well as para grass (Santos et al., 2018; Sahoo et al., 2017). These alterations in structure of biomass ultimately assist in hydrolysis of cellulose by exposing the available active site of cellulose fibers, [illegible] improving the accessible cellulose surface for cellulase attack during the hydrolytic step.

3.2. Compositional analysis

Biomass chemical composition characterization is the important step in the establishment of energy conversion processes, as it helps to detect the variability of raw material, optimization processes and assist in setting up of quality parameters. Composition of raw material was analyzed for evaluating the cellulose, hemicelluloses and lignin content. Table 1 shows the chemical composition of wild rice grass before and after pretreatment. Raw biomass consists of 28.5% cellulose, 13% hemicelluloses and 31% lignin. Cellulosic content in acid pretreated sample was 38%, hemicelluloses is 6% and lignin content 36% respectively. Dilute Acid pretreatment is normally used for hemicellulosic solubilization that improves the biomass digestibility (Santos et al., 2018). While in the case of alkali pretreated sample contained 39% cellulose, 15.5% hemicelluloses and 13% lignin respectively. 85% of the solid was recovered after pretreatment. Alkali pretreatment causes the ligno-cellulosic surface to be engorged and it is more rough in nature compared to acids which might increase the internal surface area which further helps accessibility of polysaccharides for enzymatic hydrolysis (Udeh and Erkurt, 2017; Wang et al., 2017; Sahoo et al., 2017).

Table 1
Compositional analysis of wild rice grass.

Composition (%)	Native biomass	Alkali Pretreated	Acid Pretreated
Cellulose	28.5 ± 1.8	39 ± 0.6	38 ± 4
Hemicellulose	12.9 ± 0.5	15 ± 0.3	6 ± 0.5
Lignin	31 ± 3.2	13 ± 0.8	36 ± 2
Ash	3 ± 0.8	13 ± 1.5	5 ± 0.3
Extractives	18.9 ± 1.2	14.4 ± 3.2	10 ± 2

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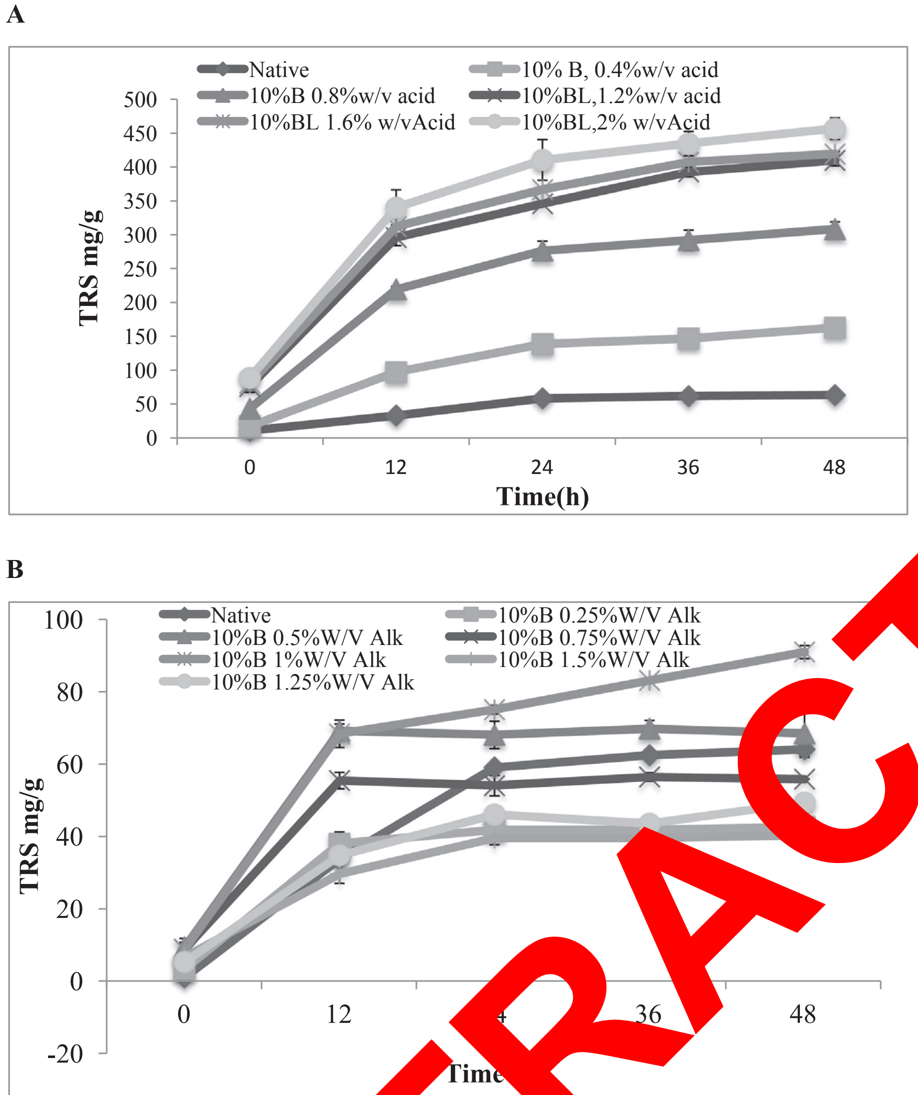

Fig. 1. Hydrolysis of pretreated biomass of wild rice grass. (A) Acid pretreated (B) Alkali pretreated.

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3.3. XRD and FTIR analysis

Cellulose crystallinity [illegible]ex (CrI) [illegible] an importan[illegible] process parameter in the subsequent step [illegible]drolys[illegible] cellulose and it is directly related with the enzyme-cellulos[illegible]bility. I[illegible]e target of pretreatment reaction is to re[illegible] ligni[illegible]d hem[illegible]ulose, higher crystallinity would show [illegible]er [illegible]ct (Zha[illegible]., 2018; Wang et al., 2017). Whereas, i[illegible] target [illegible]o break th[illegible]ellulose crystallization zone of the raw mate[illegible] lowe[illegible] would indicate better pretreatment effect (Wang [illegible] 2017). [illegible]D pattern of CrI is the best option to evaluate the in[illegible] of chemical pretreatment on crystallinity of biomass. CrI of this st[illegible] was calculated according to Segal et al., (1959). In this study degree [illegible] crystallinity was 47.8% in the native biomass while biomass pretreated with both acid and alkali was 57.5% and 52.6% respectively. It showed that pretreatment is affecting the amorphous structure of biomass. The degree of crystallinity is more on pretreatment indicates that pretreatment effect on amorphous zone was higher than the crystalline zone. Identical observations were earlier reported in the case of elephant grass and *Phoenix canariensis* (Santos et al., 2018; Udeh and Erkurt, 2017).

The changes in functional groups and biomass chemical nature directly influence the properties and play a vital role in hydrolytic process. FTIR analysis helps to find out the structural changes happening in the biomass during pretreatment reaction. A significant reduction in intensity at 897 cm^{-1} band corresponding information regarding β-D-cellulose linkages (Bodirlau et al., 2010). The band at around 1031 cm^{-1} was attributed to C–O, C=O stretching of hemi cellulose, or cellulose (Chung et al., 2004). The band at 1161 cm^{-1} corresponds to β-1,4-glycosidic linkages cellulose and hemicelluloses (Binod et al., 2012) peak intensities are higher for acid pretreated samples. The band at 1321 cm^{-1} and 1512 cm^{-1} shows the C–O stretching of syringyl ring of lignin and C=O stretching of aromatic rings of lignin. Band at 2916 cm^{-1} and 2848 cm^{-1} gives the information related with C–H stretching of lignin (Chung et al., 2004). The chemical changes in the structure of biomass were clearly observed in the acid pretreated biomass compared to alkali and native. Alkali pretreatment has not much affected the structure of wild rice grass biomass. This might be due to alkali pretreatment not enough to break the strong bond present in lignin hemicellulosic matrix.

3.4. Enzymatic saccharification

Enzymatic hydrolysis of cellulose is a vital step in lignocellulosic biomass processing for bio-ethanol industry. The fermentable sugar yield from pretreated raw material typically depends on biomass hydrolyzing enzymes such as cellulases and their activities. These qualities are mostly determined by loading of enzymes as well as hydrolytic durations which further determine the overall economics of the process

(Sukumaran et al., 2010).

After pretreatment of 10% wild rice grass biomass loading with different concentration of dilute acid and alkali, the biomass was neutralized and washed with tap water and used for hydrolytic experiment. Hydrolysis was carried with cellulase (Zytex) 20 FPU/g of pretreated biomass incubated at 50 °C with 120 rpm. Reducing sugar release was increased as the concentration of dilute acids increases in the pretreatment process. Maximum Total Reducing Sugar (TRS) was increased from 163 mg/g in 0.4 (%w/v) dilute acids treatment to 457 mg/g at 48 hrs of incubation in 10% biomass loading and 2% (w/v) of acid, untreated native biomass produced TRS concentration of 64 mg/g respectively (Fig. 1A). This results is supporting with the previous studies on dilute acid pretreatment of bamboo and sorghum biomass (Sindhu et al., 2014; Akanksha et al., 2014). Whereas evaluation of biomass digestibility of dilute alkali pretreatment showed that alkali pretreatment has no effect on hydrolysis process from this biomass compared to dilute acids pretreatment, which is evident in the structure of biomass by FTIR analysis that showed alkali pretreatment does not much affect the in the structure of biomass compared to dilute acids pretreatment (Fig. 1B). Maximum reducing sugars of 92 mg/g was obtained in 10% biomass with 1% w/v of alkali pretreatment. We have repeated the same alkali pretreatment under same condition and we got almost identical result. This indicated that pretreatment of this grass with alkali at higher temperature (121 °C) not effective for breaking the strong bonding of lignin and hemicellulosic structure. Therefore our future research on this grass is focusing on alternative pretreatment approach for getting maximum sugar yield for bio-ethanol production.

4. Conclusion

Wild rice grass available in the phumdis of Loktak Lake cou[illegible]ploit as a feed stock for bioethanol without using land energy [illegible]. Pretreatment results showed that dilute acids pretreatment is effec[illegible] for this particular variety of grass for producing fermentable sugars [illegible] ethanol production compared to alkali pretreatmen[illegible] showe[illegible] the feasibility of this grass as biofuel (bioethanol) [illegible] stock [illegible]d it can be potential approach to address the sustainab[illegible]ilizatio[illegible] Loktak Lake phumdi grasses for the production of [illegible]lue [illegible]

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