Evaluation of pre-treatment methods for *Lantana camara* stem for enhanced enzymatic saccharification

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Abstract

This study evaluates certain pre-treatment methods for *Lantana camara* stem for efficient conversion to fermentable sugars. The composition analysis of *L. camara* stem showed 66.8% (w/w) holocellulose, 34.9% (w/w) cellulose and 17% (w/w) hemicellulose. Comparative analysis of various chemical, physical or physico-chemical pre-treatments on *L. camara* stem was performed. Of all pretreatment methods used, pre-treatment with 1% (v/v) H₂SO₄ assisted autoclaving gave maximum total reducing sugar yield 132.7 mg/g (13.2 g/L) of raw biomass in pretreated hydrolysate. Major contribution to total reducing sugar was from hemicellulosic fraction, because total pentose sugar yield was 119.4 mg/g of raw biomass whereas, glucose released was only 10 mg/g of untreated biomass. The enzymatic saccharification of pre-treated *L. camara* stem by 1% (v/v) H₂SO₄ assisted autoclaving was performed with partially purified carboxymethylcellulase from *Bacillus amyloliquefaciens* SS35. Enzymatic saccharification at 30 °C for 48 h gave total reducing sugar yield, 63.3 mg/g of pre-treated biomass in the hydrolysate, while untreated biomass gave 43.3 mg/g of untreated biomass. The total sugar yield i.e. the sum of pre-treated biomass hydrolysate total reducing sugar (132.7 mg/g of raw biomass) and enzymatic hydrolysate total reducing sugar (63.3 mg/g of pre-treated biomass) was 196.0 mg/g of raw biomass, indicating the effectiveness of pre-treatment method. Field emission scanning electron microscopy, Fourier transform infrared and X-ray diffraction analyses displayed enhanced porosity, removal of non-cellulosic sugars and increased cellulose crystallinity, respectively, in pre-treated *L. camara* stem, showing the effectiveness of acid assisted autoclaving pre-treatment.

Keywords *Lantana camara* stem · Pre-treatment · Carboxymethylcellulase · Enzymatic saccharification

Abbreviations

LCS	<i>Lantana camara</i> Stem
TRS	Total reducing sugars
CMC	Carboxy methyl cellulose
FESEM	Field emission scanning electron microscopy
FTIR	Fourier transform infrared
XRD	X-ray diffraction
CrI	Crystallinity index
TS	Total sugar yield (sum of pre-treated hydrolysate TRS and enzymatic hydrolysate TRS)

EH	Enzymatic hydrolysis
BM	Biomass
PT	Pre-treatment

Introduction

Human population increased phenomenally in the last few decades (Balat 2011). Non-renewable resources such as fossil fuels have been over-exploited, which led the societies to look for an alternative energy sources from renewable energy resources (Serrano-Ruiz et al. 2012). Biofuels such as a bioethanol offer realistic solution to the issues of energy safety and weather change risks (less greenhouse gas emission). Bioethanol can be mixed with gasoline and used as a fuel for dedicated engines (Hahn-Hägerdal et al. 2006). Bioethanol produced from lignocellulosic biomasses, for example forest and agricultural residues, paper and wood industries waste, etc., is called as the second-generation bioethanol. These biomasses have several advantages over

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other sources, such as low cost, sustainable supplies and non-competence with food crops (Zhu et al. 2009). Lignocellulosic biomass consists mainly of cellulose, hemicellulose and lignin. Among these cellulose and hemicellulose are the most significant ingredients to produce bioethanol. Strategies in second-generation bioethanol production mainly focus on following processes: pre-treatment, enzymatic hydrolysis, fermentation and distillation (Aditiya et al. 2016). Pre-treatment of raw biomass prior to bioethanol production is necessary for efficient conversion of lignocellulosic biomass into fermentable monosugars (hexose and pentose). It modifies the structural arrangement of cellulosic biomass and hence, increases the accessibility of cellulosic enzyme to convert the cellulosic polymers into fermentable monosugars (Mosier et al. 2005). Screening of efficient pre-treatment process for a selected biomass helps in reducing the total cost of biofuel production (Ranjan and Moholkar 2013). The dilute sulfuric acid treatment can lead to high reaction rates by significantly improving the hydrolysis of hemicelluloses as compared to cellulose (Ishizawa et al. 2007).

Invasive weeds (lignocellulosic waste biomass) can be used as a feedstock for producing bioethanol because of its high holocellulose content (Borah et al. 2016). These invasive weeds are major threat to non-arable and agricultural land and results in destroying the natural habitats of native species. So, bioconversion of invasive weeds in bioethanol can help in controlling the damage to ecosystem and health. *Lantana camara* being an invasive weed has taken over millions of hectares of pasture (Day et al. 2003). It is the 100 most devastating and noxious plants in the world has high potential for bioethanol production because of its high cellulose content, adaptability and availability. This plant is prevalent in most tropical and subtropical regions of India. *L. camara* stem (LCS) has a potential to be used as a feedstock for producing bioethanol because of its high holocellulose content (61% w/w raw biomass), easy availability and non-competitive nature with the food (Kuhad et al. 2010). They optimized the pre-treatment method for LCS using 1–5% (v/v) H_2SO_4 at 120 °C for 45 min resulting a total reducing sugar (TRS) yield of 19.1 g/L. The pre-treated biomass after enzymatic saccharification using commercial enzymes under optimized conditions gave TRS yield, 777 mg/g of pretreated LCS. They studied only the optimization of acid pre-treatment of LCS, though there are several other methods which could also be explored. Pasha et al. (2007) studied the dilute acid (1% H_2SO_4) pre-treatment of LCS for 18 h, followed by autoclaving at 121 °C for 20 min and obtained TRS, 66 g/L. The acid treatment of the biomass for more than 18 h is not feasible at industrial level. Gupta et al. (2011) performed the acid pre-treatment on LCS using 3% (v/v) acid autoclaving for 45 min and found that pretreated LCS contains 54% (w/w) holocellulose and 38% (w/w) lignin content and

leading to 92% (w/w) hemicellulose solubilization. They did only the saccharification of residual biomass without focusing on the release of pentose sugars due to hemicellulose solubilization. However, the potential of a biomass to be used as a feedstock for bioethanol production shall be determined by analyzing the pentose sugars released during the pre-treatment as well as the hexose sugars released after the saccharification.

In the present study, the potential of LCS as a feedstock for bioethanol production was explored as only few reports are available on its pre-treatment. In this study, 20 different pre-treatments (physical, chemical and physico-chemical) on LCS were employed to assess the complete potential of LCS as a feedstock for bioethanol industry for the production of fermentable sugars. The best pre-treated biomass was subjected to enzymatic saccharification using in-house produced microbial CMCase. The concentration of acid, alkali and other oxidizing agents were optimized. The TRS yields of hydrolysates after acid pre-treatment of LCS and also after the enzymatic hydrolysis were determined and compared.

Materials and methods

Biomass collection and preparation

The plant biomass *L. camara* was collected from the campus of Indian Institute of Technology Guwahati, Assam, India. Only LCS was used as a substrate (biomass) for pre-treatment. It was slashed and washed with tap water and dried at 60 °C for overnight before pre-treatment. The dried biomass of LCS was powdered by chopping and milling, using a mixer grinder (Philips, India) and further passed through 2 mm sieve to obtain a particle size of 1–2 mm.

Carbohydrate composition analysis of LCS

The moisture content and carbohydrate composition analysis of powdered LCS for holocellulose, cellulose, hemicellulose and lignin content was carried out before the pre-treatment and after the best pre-treatment by TAPPI protocols (1991).

Pre-treatment of LCS

Physical pre-treatment

The physical pre-treatment used was uncatalyzed thermal explosion/autoclaving at neutral pH without any chemical. This pre-treatment under mild environment is the promising and simple pre-treatment technique which makes enzymatic hydrolysis more accessible (Sharma et al. 2007; Ibrahim et al. 2011; Das et al. 2013). In this process, steam was applied on biomass under high pressure for few minutes

and the reaction was stopped by sudden decompressing it at atmospheric pressure. This allows the lignocellulosic biomass to expand which will separate into individual fibres (Balat et al. 2008). Recent reports on this pre-treatment showed that during this pre-treatment solubilisation of hemicellulosic fraction was occurred (Singh et al. 2014a).

In this pre-treatment, 1 g of dried raw biomass was soaked in 10 mL of water in solid to liquid ratio of 1:10 and autoclaved at 121 °C under 15 psi pressure for 20 min. The pressure was quickly released after the pre-treatment to generate the expansion of biomass. The TRS released in the hydrolysate after pre-treatment was analysed by methods of Nelson (1944) and Somogyi (1945).

Chemical pre-treatment

The LCS was pre-treated with H_2SO_4 and NaOH, and released TRS in pre-treated hydrolysate was determined. In each condition 10% (w/v) LCS in aqueous solution system was used according to the method as described earlier (Singh et al. 2014a).

Acid pre-treatment The dried raw biomass was soaked in 1%, 2%, and 3% (v/v) of H_2SO_4 solution in solid to liquid ratio of 1:10 (biomass: acid) and heated in a hot air oven at 120 °C for 20 min.

Alkaline pre-treatment The biomass was treated with 1%, 2% and 3% (w/v) of NaOH solution in solid to liquid ratio of 1:10 and heated in a hot air oven at 120 °C for 20 min.

Physicochemical pre-treatment

Acid assisted autoclaving LCS was mixed with 1%, 2%, and 3% v/v of H_2SO_4 solution in solid to liquid ratio of 1:10 and autoclaved at 121 °C, 15 psi for 20 min.

Alkali assisted autoclaving Effectiveness of alkali pre-treatment process mainly depends on its pre-treatment conditions and biomass used (Sharma et al. 2007; Zhao et al. 2008). The biomass was treated with 1%, 2% and 3% (w/v) of NaOH solution in solid to liquid ratio of 1:10 and autoclaved at 121 °C, 15 psi for 20 min.

Oxidizing agent assisted autoclaving H_2O_2 pre-treatment delignifies the biomass along with hemicelluloses hydrolysis (Hendriks and Zeeman 2009). The biomass was mixed with 1%, 2% and 3% (v/v) of H_2O_2 solution in solid to liquid ratio of 1:10 and autoclaved at 121 °C, 15 psi for 20 min.

Ammonia assisted autoclaving This pre-treatment is similar to physical pre-treatment (thermal explosion) with addition of liquid ammonia (Singh et al. 2014a). The biomass was soaked in 1% (v/v) ammonia solution in solid to liquid ratio of 1:10 and autoclaved at 121 °C, 15 psi for 20 min.

After each pre-treatment process, biomass was filtered through a muslin cloth and washed with the tap water until it reached a neutral pH. The hydrolysed liquid portion obtained

after pre-treatment were used for reducing sugar analysis. The biomass residue was dried in a hot air oven at 70 °C to constant weight and used for further analysis such as structural, functional group composition, crystallinity index analysis and enzymatic hydrolysis.

Carboxymethylcellulase (CMCase) production and assay

Carboxymethylcellulase (CMCase) from *Bacillus amyloliquefaciens* SS35 was used for enzymatic saccharification of untreated and pre-treated LCS biomass. The medium composition and culture conditions used for CMCase production from *B. amyloliquefaciens* SS35 followed were as described earlier Singh et al. (2014b). The extracellular CMCase present in cell-free supernatant produced from 200 mL culture of *B. amyloliquefaciens* SS35 was partially purified by ammonium sulphate (up to ~90% saturation) precipitation method. The precipitated enzyme was resuspended in 10 mL of 50 mM sodium acetate buffer (pH 5.0). The desalting of this 10 mL enzyme was performed using a 10 kDa dialysis membrane and dialysing against 2 L of 50 mM sodium acetate buffer (pH 5.0) with 5 buffer changes every 2 h. The protein concentration for the partially purified CMCase was estimated by the Bradford method using bovine serum albumin (BSA) as standard (Hammond and Kruger 1988). The enzyme assay was carried out, by incubating 10 µL of dialysed enzyme (7 U/mg, 0.2 mg/mL) in 100 µL reaction volume containing 1% (w/v) CMC in 50 mM sodium acetate buffer, pH 5.0 incubated at 65 °C for 5 min.

Enzymatic saccharification of biomass

The initial screening of best pre-treatment was done by enzymatic saccharification of all the pre-treated as well as raw biomass. The raw and pre-treated biomass (3%, w/v biomass loading) was soaked in 50 mM sodium acetate buffer, pH 5.0. The partially purified CMCase from *B. amyloliquefaciens* SS35 was added at loading, 10 U/g of biomass for saccharification reaction. The experiments were performed with 1.5 mL reaction volume in 2 mL microcentrifuge tube. The microcentrifuge tubes were incubated in a shaking incubator at 30 °C, 150 rpm for 10 min and the TRS yield was determined by the method as mentioned earlier (Nelson 1944; Somogyi 1945). Further, the enzymatic saccharification of raw LCS and the best pre-treated (1%, v/v H_2SO_4 assisted autoclaving) LCS was carried out in 20 mL reaction volume in 100 mL conical flask. Both biomasses were separately soaked in 50 mM sodium acetate buffer, pH 5.0 and the partially purified CMCase at 10 U/g of biomass was added. To avoid bacterial contamination, sodium azide (0.005%, w/v) was added to the reaction mixture. The conical flasks were

incubated in a shaking incubator at 30 °C, 150 rpm for 60 h. 100 µL sample (hydrolysate) was collected at regular intervals and estimated for TRS release by method as mentioned earlier (Nelson 1944; Somogyi 1945).

Analytical procedures

The total reducing sugar (TRS) in the hydrolysate after each pre-treatment and after enzymatic saccharification were analysed by standard protocol described earlier by Nelson (1944) and Somogyi (1945). The concentration of hexose and pentose sugars present in the hydrolysate of each pre-treatment was estimated by high performance liquid chromatography (Agilent technology, 1220 Infinite LC, USA) attached with RI detector. The analysis of mono-saccharides was carried out using the HPLC column [Phenomenex Rezex ROA (H+) (300×7.8 mm)] along with a guard column (50×7.8 mm). The mobile phase used was 0.05 N H₂SO₄ at 0.5 mL/min flow rate. The hydrolysate samples used were passed through a syringe filter (pore size, 0.45 µm), prior to loading onto HPLC. The change in structure, functional group and crystallinity index of untreated and only acid assisted autoclaving pre-treated LCS was performed because only these pre-treatments showed the comparable results described later.

Structure analysis of untreated and pre-treated LCS by FESEM

The structure analysis of the untreated and acid assisted autoclaving pre-treated LCS was done by field emission scanning electron microscopy (FESEM) according to the protocol described earlier (Singh et al. 2014a). Samples were dried at 70 °C for 48 h. The dried biomass was mounted on the carbon tape, on the surface of stub. Approximately, 10 nm gold coating was sputtered on the samples in a sputter chamber. The stub was placed in the instrument, vacuum was applied before the examination of the samples, and image was collected.

Functional groups composition analysis of untreated and pre-treated LCS by FTIR spectroscopy

Change in the functional groups of untreated and acid assisted autoclaving pre-treated LCS was analyzed by FTIR spectroscopy according to the protocol described earlier (Singh et al. 2014a). Biomass and KBr were dried at 70 °C for 24 h. The samples were prepared by mixing the biomass and KBr in the ratio of 1:100 (w/w). The pellets were prepared by a hydraulic press and analysed in the FTIR spectrometer (Perkin-Elmer, Spectrum Two). The spectra were

recorded at 400–4000 cm⁻¹ using ~20 mg of biomass in the pellet with ~2 g of KBr.

Crystallinity index analysis of untreated and pre-treated LCS by X-ray diffractometer

The effect of various acid assisted autoclaving pre-treatment on crystallinity of biomass was observed by X-ray diffractometer (XRD) according to the protocol described earlier (Singh et al. 2014a). The samples were dried at 70 °C for overnight before analysis. The XRD was set to radiation Cu Kα (λ = 1.54 Å), 40 kV and 40 mA. The samples were measured in the degree range, 2θ = 5–30° with a step size of 0.05°. Crystallinity index (CrI) of the samples was determined by the formula given by Segal et al. (1962).

$$\text{CrI (\%)} = \frac{I_{\text{crystalline}} - I_{\text{amorphous}}}{I_{\text{crystalline}}} \times 100,$$

where, $I_{\text{crystalline}}$ is the intensity of a crystalline peak at a degree, 2θ = 22° and $I_{\text{amorphous}}$ is intensity of an amorphous peak at a degree, 2θ = 18°.

Results and discussion

Carbohydrate analysis of LCS

The untreated LCS had 66.8% (w/w) holocellulose, which consist of 34.9% (w/w) cellulose and 17.0% (w/w) hemicellulose, 17.0% (w/w) insoluble lignin and 2.1% (w/w) moisture content. The presence of high holocellulose content (66.8%) and less insoluble lignin 17.0% makes it a potential substrate for bioethanol production. Kuhad et al. (2010) also reported the 61% w/w holocellulose content in LCS. After dilute acid pre-treatment (1%, v/v H₂SO₄ assisted autoclaving), amount of holocellulose, cellulose, hemicellulose and insoluble lignin in pre-treated biomass was 70% (w/w), 54.11% (w/w), 9.0% (w/w) and 24% (w/w), respectively. The decrease in hemicellulose content to 9% (w/w) suggested mainly the hemicellulose content was hydrolysed by the dilute acid pre-treatment. Later analyses of acid assisted autoclaving pre-treated hydrolysate by HPLC (Table 1) also corroborated the same observation.

Effect of pre-treatment methods

The results of different pre-treatment methods on LCS are summarized in Table 1. The release of total reducing sugar (TRS) during pre-treatment was expressed as relative value in mg/g biomass and as true value in g/L.

Table 1 TRS released and monosugars analysis in pretreated hydrolysate after different pre-treatments

Pre-treatment methods	Conditions	TRS released (g/L) ^{a,b}	TRS yield (mg/g of raw BM) ^b	^c Glucose conc. (mg/g) ^a	^c Arabinose conc. (mg/g) ^a	^c Xylose conc. (mg/g) ^a	^c Total pentose sugar (mg/g)
Physical pre-treatment							
Uncatalysed thermal explosion	Approx. 1 mm particles size LCS was expose to 15 psi pressure at 121 °C, 20 min	0.29 ± 0.7	2.9	—	—	1.0	1
Chemical pre-treatment							
Acid	1% (v/v) H ₂ SO ₄ , 120 °C, 20 min	3.2 ± 0.7	32.0	6.2	9.9	10.8	20.7
Acid	2% (v/v) H ₂ SO ₄ , 120 °C, 20 min	4.9 ± 0.1	49.8	6.6	10.0	21.8	31.8
Acid	3% (v/v) H ₂ SO ₄ , 120 °C, 20 min	5.5 ± 0.4	55.0	8.4	7.5	29.3	36.8
Alkali	1% (w/v) NaOH, 120 °C, 20 min	0.3 ± 0.9	3.0	—	—	—	0
Alkali	2% (w/v) NaOH, 120 °C, 20 min	0.62 ± 0.6	6.2	—	—	—	0
Alkali	3% (w/v) NaOH, 120 °C, 20 min	0.28 ± 0.4	2.8	—	—	—	0
Physicochemical pre-treatment							
Acid + autoclaving	1% (v/v) H ₂ SO ₄ , 121 °C, 15 psi, 20 min	13.2 ± 0.2	132.7	10.2	25.7	93.7	119.4
Acid + autoclaving	2% (v/v) H ₂ SO ₄ , 121 °C, 15 psi, 20 min	27.6 ± 0.4	156.5	12.1	27.5	101.1	128.6
Acid + autoclaving	3% (v/v) H ₂ SO ₄ , 121 °C, 15 psi, 20 min	5.8 ± 0.3	57.8	16.9	2.5	20.5	23
Alkali + autoclaving	1% (w/v) NaOH, 121 °C, 15 psi, 20 min	1.0 ± 0.3	10.1	—	—	3.9	3.9
Alkali + autoclaving	2% (w/v) NaOH, 121 °C, 15 psi, 20 min	7.6 ± 0.1	76.6	—	—	5.0	5.0
Alkali + autoclaving	3% (w/v) NaOH, 121 °C, 15 psi, 20 min	1.3 ± 0.5	13.4	—	—	8.1	8.1
Oxidizing agent + autoclaving	1% (v/v) H ₂ O ₂ , 121 °C, 15 psi, 20 min	0.2 ± 0.2	2.1	—	—	—	0
Oxidizing agent + autoclaving	2% (v/v) H ₂ O ₂ , 121 °C, 15 psi, 20 min	0.7 ± 0.5	7.9	1.3	—	4.1	4.1
Oxidizing agent + autoclaving	3% (v/v) H ₂ O ₂ , 121 °C, 15 psi, 20 min	0.8 ± 0.1	8.6	1.6	—	5.8	5.8
Ammonia + autoclaving	Ammonia (2 g/g of biomass) + water (2 g/g of biomass), 121 °C, 15 psi, 20 min	0.5 ± 0.4	5.2	—	—	5.6	5.6

Biomass loading = 10%

TRS total reducing sugar, BM biomass

^aValues are mean of SE (n = 3)^bTRS yield was determined by the method described earlier by Nelson (1944) and Somogyi (1945)^cHPLC analysis

Physical pre-treatment

Autoclaving or uncatalysed thermal explosion

In this pre-treatment, TRS yield was 2.9 mg/g of the raw biomass and HPLC analysis of pre-treated hydrolysate showed that no hexose sugar was released, only 1 mg/g of raw biomass xylose sugar was released (Table 1). This indicated that, in the absence of any chemical, this pre-treatment may have broken down the complex carbohydrate structure of biomass to very low extent. Nevertheless, this process may not efficiently hydrolyse the lignocellulosic structure of biomass. Singh et al. (2014a) also reported that TRS yield for *Parthenium hysterophorus* was very low 8.3 mg/g of raw biomass by autoclaving pre-treatment.

Chemical pre-treatment

Acid pre-treatment

The dried residue obtained after LCS pre-treatment with 1%, 2% and 3% (v/v) H_2SO_4 , oven heating at 120 °C for 20 min on analysis by reducing sugar estimation method gave TRS yield 32.0, 49.8 and 55.0 mg/g of raw LCS biomass, respectively by method as mentioned earlier (Nelson 1944; Somogyi 1945). The TRS (glucose + pentose) analysis of 1, 2 and 3% (v/v) acid pre-treated hydrolysate by HPLC showed 26.9, 38.4, 45.2 mg/g of raw LCS (Table 1). Both analyses showed that 3% (v/v) acid gave higher TRS value. The increase in TRS yield from 1 (v/v) to 3% (v/v) acid concentration was mainly due to the increased hydrolysis of hemicellulosic fraction because the pentose sugar released (36.8 mg/g) was 4.3-fold higher than the glucose released at 3% (v/v) acid pretreatment of LCS (Table 1). Kuhad et al. (2010) reported that with 3% (v/v) H_2SO_4 pre-treatment of LCS at 120 °C for 45 min produce maximum TRS 187 mg/g of pre-treated LCS. Moutta et al. (2012) also reported that 2.9% (v/v) H_2SO_4 was the optimum concentration for pre-treatment of sugarcane leaf straw at 130 °C for 30 min producing 56.5 g/L of pentose sugars in the pretreated hydrolysate. In acid pre-treated LCS (Gupta et al. 2011) and *P. hysterophorus* (Singh et al. 2014a), the hemicellulose content decreases, because the acid solubilizes the hemicellulosic sugars present in the biomass.

Alkali pretreatment

The alkaline pre-treatment of LCS was done by 1%, 2% and 3% (w/v) NaOH solution under oven heating at 120 °C for 20 min that gave TRS yield 3.0, 6.2 and 2.8 mg/g of raw biomass, respectively (Table 1). The low TRS yield indicated that inefficient hydrolysis of cellulose and hemicellulose. However, several authors have reported the removal

of lignin could be achieved by alkali pre-treatment (Mosier et al. 2005; Gupta et al. 2011; Kim et al. 2016). HPLC analysis of pre-treated hydrolysate also showed no hydrolysis of hemicellulosic fraction in LCS because release of pentose sugars was almost negligible (Table 1). This insignificant hydrolysis of cellulosic and hemicellulosic fraction can be explained by the fact that the alkali pre-treatment mainly causes disruption of the lignin fibres by saponification of intermolecular ester bonds crosslinking the xylan and lignin (Singh et al. 2014a).

Physicochemical pre-treatment

Acid assisted autoclaving

The acid, 1%, 2% and 3% (v/v) H_2SO_4 assisted autoclaving pre-treatment of LCS gave volumetric TRS yield 13.2, 27.6 and 5.8 g/L of raw biomass, respectively in the pre-treated hydrolysate, that corresponded to TRS yield, 132.7, 156.5 and 57.8 mg/g of raw biomass, respectively (Table 1). The TRS yields in the hydrolysates of 1% (v/v) and 2% (v/v) acid assisted autoclaving pre-treatment were 4.1-fold and 3.1 higher than those obtained with 1% (v/v) (32 mg/g of raw biomass) and 2% (v/v) acid pre-treatment (49.8 mg/g of raw biomass) (Table 1). The HPLC analysis of pre-treated LCS hydrolysate by 1% and 2% (v/v) acid assisted autoclaving showed that the total pentose sugar released was between 120 and 130 mg/g of raw LCS and was significantly higher than 20–32 mg/g of raw LCS obtained with only 1% or 2% (v/v) acid pre-treatment (Table 1). In the acid assisted autoclaving, the increase in acid concentration from 1 (v/v) to 2% (v/v), the TRS yield increased, but, the total pentose sugar release (128 mg/g of raw biomass) was almost the same as in 1% (v/v) (~120 mg/g of raw biomass) acid treatment. Kuhad et al. (2010) also reported that the major contribution to the TRS in the acid pre-treated hydrolysate is of pentose sugars (xylose and arabinose). Further, the increase in acid concentration to 3% (v/v) resulted in decreased TRS yield to less than half (57.8 mg/g raw biomass) as compared with 1% (v/v) acid pre-treatment (Table 1). This decrease in TRS could be explained by the fact that, at higher acid concentration, the pentose sugars in the hydrolysate are converted into inhibitory products such as acetic acid, furfural, hydroxy methyl furfural and phenolics (Mosier et al. 2005; Chandel et al. 2007). Therefore, it was concluded that among all acid pre-treatments, 1% (v/v) H_2SO_4 acid assisted autoclaving was the best pre-treatment method for LCS.

Alkali assisted autoclaving

The alkali, 1%, 2% and 3% (w/v) NaOH assisted autoclaving pre-treatment of LCS resulted in TRS yield, 10.1, 76.6 and 13.4 mg/g of raw biomass, respectively (Table 1). The

TRS yields obtained by these conditions were much higher than the oven heating alkali pre-treatment where maximum TRS 6 mg/g of raw biomass was obtained. Moreover, these values are also far lesser than that obtained with 1% (v/v) acid assisted autoclaving pre-treatment (132 mg/g of raw biomass) (Table 1). Singh et al. (2014a) reported the TRS yield 82 mg/g from pre-treated *P. hysterophorus* biomass by 2% (w/v) NaOH assisted autoclaving and further that the TRS yield obtained by alkali pre-treatment was much lower than the acid assisted autoclaving pre-treatment. The hydrolysates of 1%, 2% and 3% (w/v) NaOH pre-treated LCS gave only 3.9, 5.0 and 8.1 mg/g of raw biomass xylose, respectively, on HPLC analysis (Table 1). This xylose yield was very less than that from the acid assisted autoclaving pretreatment (120 mg/g). It is also reported earlier that the alkali pre-treatment might be helping in only delignification of the biomass by distorting the linkage between lignin and hemicellulose and not contributing to its hydrolysis (Sun and Cheng 2002).

Oxidizing agent assisted autoclaving

Autoclaving assisted H_2O_2 pre-treatment (3%, v/v H_2O_2 assisted autoclaving) of LCS gave highest reducing sugar yield 8.6 mg/g of raw biomass which was 15 times lesser than 1% (v/v) acid assisted autoclaving (Table 1). The reason for low TRS yield might be the strong oxidizing nature of H_2O_2 , which can oxidise the lignin and pentose sugars into inhibitors such as soluble aromatic compounds and acetic acid (Hendriks and Zeeman 2009).

Ammonia assisted autoclaving

The TRS yield after ammonia assisted autoclaving pre-treatment was only 5.2 mg/g of raw biomass (Table 1), this might be due to the high lignin content of biomass. According to the earlier report (Sun and Cheng 2002), biomasses having high lignin content are not suitable for ammonia pre-treatment.

Structure analysis of untreated and pre-treated LCS by FESEM

The surface morphology of untreated and acid assisted autoclaving pre-treated biomass of LCS was shown in Fig. 1. The surface of raw LCS showed compact and rigid structure of highly ordered fibres which restrains the entry of hydrolytic enzymes (Fig. 1a). The surface of 1% (v/v) H_2SO_4 assisted autoclaving pre-treated LCS showed irregular cracks with pores of different sizes and disruption of fibres structure (Fig. 1b). In Fig. 1c, d showed surface of 2% (v/v) H_2SO_4 assisted autoclaving and 3% (v/v) H_2SO_4 assisted autoclaving pre-treated LCS respectively, exhibited well broken

and disrupted structure and smooth surface with smaller pores. The pre-treatment involving 1% (v/v) H_2SO_4 assisted autoclaving resulted in more fibre bundles may be due to a partial breakdown of hemicellulose and lignin structure, thereby increasing the accessibility of hydrolytic enzymes. Singh et al. (2014a) for 1%, 2% and 3% (v/v) and Borah et al. (2016) for 1% (v/v) acid assisted autoclaving pre-treatment for *Parthenium* and LCS respectively, reported the similar observation in their studies.

Functional groups composition analysis by FTIR spectroscopy

The functional group changes in lignocellulosic biomass after the acid assisted autoclaving pre-treatments was analysed by FTIR. The FTIR spectra of LCS before and after acid pre-treatments are shown in Fig. 2. An increase in the relative intensity of hemicellulose peak can be seen at band position 1378 cm^{-1} . The peaks for ester linkage at 1720 cm^{-1} between lignin and hemicellulose and the C–O–C stretching in the acetyl group of hemicellulose at 1245 cm^{-1} as also reported earlier (Kumar et al. 2009) disappeared in the acid assisted autoclaving pre-treatments as against untreated LCS. This explained the fact that the increase in pentose sugars content is mainly due to the hydrolysis of hemicelluloses (Table 1). High concentration of acid in 3% (v/v) H_2SO_4 assisted pre-treatment resulted complete disappearance of the peak, which could explain the fact of getting less TRS yield and suggested that pentose sugars release due to hemicellulose breakdown was converted into inhibitors. The absorption bands at $1300\text{--}1600\text{ cm}^{-1}$ related to lignin as also reported earlier (Kumar et al. 2009) also decreased in acid assisted autoclaving pre-treatment as against the raw biomass. This decrease indicated that in acid assisted autoclaving pre-treatment xylan and lignin content was reduced. The O–H stretching at 3330 cm^{-1} and C–H stretch at 2900 cm^{-1} associated with cellulose were stronger after 1% (v/v) H_2SO_4 assisted autoclaving pre-treatment as compared with raw biomass and other acid assisted autoclaving pre-treatment. Singh et al. (2014a) explained that stronger stretching of O–H and C–H bonds at 3330 cm^{-1} and 2900 cm^{-1} respectively, showed the most exposed cellulose fibrils to cellulolytic enzymes. This depicts that 1% H_2SO_4 assisted autoclaving pre-treatment of LCS was the best pre-treatment among all acid assisted autoclaving pre-treatment.

X-ray diffraction analysis of untreated and pre-treated LCS

XRD analysis was performed to measure the crystallinity index (CrI) of untreated and acid assisted autoclaving pre-treated LCS, respectively (Fig. 3). CrI of 1% (v/v) H_2SO_4 assisted autoclaving, 2% (v/v) H_2SO_4 assisted autoclaving and 3% (v/v) H_2SO_4 assisted autoclaving pre-treated

Fig. 1 **a** FESEM image of untreated LCS. FESEM images of pre-treated LCS; **b** after 1% (v/v) H_2SO_4 at 121 °C and 15 psi for 20 min treatment; **c** after 2% (v/v) H_2SO_4 at 121 °C and 15 psi for 20 min treatment; **d** after 3% (v/v) H_2SO_4 at 121 °C and 15 psi for 20 min treatment

LCS biomass were 61.31%, 60.73% and 53.59%, respectively as compared to the CrI of untreated LCS biomass of 43.63%. An increase in CrI to 1.4-fold after 1% and 2% (v/v) H_2SO_4 assisted autoclaving pre-treatment as compared with untreated LCS suggested that the pretreated biomass is composed of almost pure cellulose. This result corresponds to the total pentose sugar release after 1% and 2% (v/v) H_2SO_4 assisted autoclaving pre-treatment was maximum i.e. 120–128 mg/g of raw biomass (Table 1) suggested the efficient solubilisation of hemicellulosic fraction in pretreated LCS. The CrI of LCS decreases with the increase in acid percentage because higher concentration of acid solubilises cellulosic fraction in addition to the hemicellulosic fraction as 3% (v/v) H_2SO_4 assisted autoclaving pre-treatment released maximum hexose sugar (Table 1). It was earlier reported that, the CrI increases after the acid pre-treatment of switch grass (Samuel et al. 2010) and sugarcane tops (Sindhu et al. 2014) respectively, due to the solubilisation of hemicellulosic fraction and exposure of the cellulosic fraction. The 1% (v/v) H_2SO_4 assisted autoclaving pretreated

LCS showed maximum enzymatic hydrolysis discussed later in section enzymatic saccharification. As crystallinity of cellulose has been regarded as one of the primary factors that have a significant impact on the rate of enzymatic hydrolysis (Hall et al. 2010; Cao and Tan 2005).

Enzymatic saccharification of pre-treated LCS

Of all the pre-treatments, the 1% (v/v) acid assisted autoclaving gave the maximum TRS of 11 mg/g of pre-treated LCS upon saccharification by partially purified CMCase for 10 min (Table S1). Therefore, the saccharification of 1% (v/v) acid assisted autoclaving pre-treated LCS was carried out for 60 h (Fig. 4). This gave maximum saccharification yield of 63.3 mg/g pre-treated LCS at 48 h (Fig. 4). Further, the saccharification yield at 48 h from 1% (v/v) acid assisted autoclaving pre-treated LCS was compared with untreated LCS. This showed that 1% (v/v) acid assisted autoclaving pre-treated LCS gave 1.5-fold (46%, w/w) higher saccharification yield than that

Fig. 2 FTIR spectrum of untreated and acid assisted autoclaving pre-treated LCS

Fig. 3 Diffractogram from XRD showing the crystallinity index of untreated and acid assisted autoclaving pre-treated LCS

obtained with the untreated biomass (43.3 mg/g of raw biomass). The saccharification rate of pre-treated LCS was 0.79 mg/g/h. In lignocellulosic biomass the seal of lignin and hemicellulose prevents the penetration of cellulase

molecules to the cellulosic fibres (Sindhu et al. 2014). The pretreatment of biomass breaks this lignin and hemicellulosic seal and results in the increase of saccharification yield. Gupta et al. (2011) reported 25% (w/w) increase in

Fig. 4 Enzymatic saccharification profile of 1% (v/v) H_2SO_4 assisted autoclaving pre-treated LCS

saccharification yield for pre-treated LCS using cellulase as compared with untreated LCS.

Assessment of bioethanol production potential

Total reducing sugar released in hydrolysate after 1% (v/v) acid assisted autoclaving pre-treatment for 20 min was due to the hydrolysis of hemicellulosic fraction, as total pentose (arabinose and xylose) sugars release was 119.4 mg/g as compare to the hexose sugars 10 mg/g of raw biomass (Table 1). Compositional analysis of pre-treated LCS also confirmed that hemicellulose was reduced after above mentioned pre-treatment. A moderate value of crystallinity index of 61.3% after 1% (v/v) H_2SO_4 with autoclaving showed that the pre-treated biomass almost comprises of ~96% of cellulose. According to the earlier report (Borah et al. 2016) after pre-treatment 61.3% of crystallinity index indicated almost pure cellulose. So, after enzymatic hydrolysis mainly hexose sugars will release in enzymatic hydrolysate. The total sugar yield i.e. the sum of pre-treated biomass hydrolysate TRS and the enzymatic hydrolysate TRS in 1% (v/v) acid assisted autoclaving (20 min) pre-treatment was 196 mg/g of raw biomass (Table 2). Therefore, theoretical ethanol yield in above mentioned pre-treatment will be 0.1 g/g of glucose (Table 2). These results showed that LCS has a potential to be a feedstock for bioethanol production.

Conclusion

In this study, the potential of LCS as a substrate for the production of bioethanol was demonstrated. The economy of bioethanol will depends on the type of biomass used as a substrate and its carbohydrate content. The higher cellulose content of LCS makes it a potential candidate for bioethanol production. Different pre-treatment methods such as physical, chemical and physiochemical were

Table 2 Total sugar release from LCS after 1% (v/v) acid assisted autoclaving pre-treatment and after subsequent enzymatic hydrolysis

Pre-treatment	TRS ^a (PT) (mg/g of raw BM)	TRS ^a (EH) (mg/g of pre-treated BM)	TS yield (mg/g of raw BM)	Theoretical Ethanol yield (mg/g of raw BM) ^b
1% (v/v) H_2SO_4 assisted autoclaving	132.7	63.3	196	100

TRS total reducing sugar, PT pre-treatment, EH enzymatic hydrolysis, BM biomass, TS yield total sugar yield (sum of pre-treated hydrolysate TRS and enzymatic hydrolysate TRS)

^aTRS concentration was determined by the method described earlier by Nelson (1944) and Somogyi (1945)

^bMaximum theoretical yield for ethanol from hexose as well as pentose sugars is 0.51 g/g of glucose

employed to disrupt the structure of lignocellulosic biomass. Among all the pre-treatment methods used, 1% (v/v) H_2SO_4 assisted autoclaving for 20 min was the most efficient that yielded the maximum reducing sugar of 132.7 mg/g raw biomass. This pre-treated biomass after enzymatic saccharification by partially purified CMCase from *B. amyloliquefaciens* SS35 gave the TRS yield of 63.3 mg/g of pretreated biomass. Therefore, the maximum TS yield was 196 mg/g of pretreated LCS, which will result in the production of 0.1 g/g of theoretical bioethanol yield. These results showed that LCS has the potential to be used as a substrate for bioethanol production. The detoxification of hydrolysate and the optimization of saccharification conditions will further improve the TS yield and thus the efficiency of the bioethanol production process. LCS opens an additional possibility for the low-priced and easily available renewable substrate for cost-effective bioethanol production.

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Author contributions AG conceived the idea and designed the objectives. AK and SS together performed the pretreatment, XRD, FESEM and HPLC studies and data analysis, VR performed the FTIR and HPLC studies. AG, SS and AK wrote the paper.

Compliance with ethical standards

Conflict of interest There are no conflicts of interest to declare.

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