

Comparative analysis of bioethanol production involving saccharification by mixed recombinant clostridial enzymes using sugarcane leaves and kans grass as sustainable feed stocks from North-east India

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The present study involves bioethanol production from sugarcane (*Saccharum officinarum*) leaves (SL) and Kans grass (*Saccharum spontaneum*) (KG), under two separate approaches of fermentation, i) SHF (separate hydrolysis & fermentation) and ii) SSF (simultaneous saccharification & fermentation). The H₃PO₄-acetone pretreatment strategy was performed for effective lignin removal and enhanced porosity, confirmed by FT-IR (fourier transform infrared spectroscopy) and FESEM (field emission scanning electron microscopy). Saccharification was performed by involving recombinant cellulase GH5 (family 5 of glycoside hydrolase) and hemicellulase GH43 (family 43 of glycoside hydrolase) from *Clostridium thermocellum*, expressed and isolated from *Escherichia coli*. Successively, the bioethanol producers *Saccharomyces cerevisiae* (NCIM 3215) and *Candida shehatae* (NCIM 3500) were employed. Comparative SHF trials with 1% (w/v) pretreated KG and SL gave bioethanol titre of 0.52 and 0.64 g/L, respectively, whereas SSF experiments resulted in bioethanol titre of 0.8 and 1.0 g/L, respectively at 100 mL shake flask. An increased feedstock concentration of 5% (w/v) KG in shake flask resulted in increased bioethanol titre (4.2 g/L) in SSF. However, the increased substrate concentration of 5% (w/v) SL in individual shake flask resulted in 1.4 fold more bioethanol titre (5.7 g/L) in comparison to KG, and its scale up in bioreactor with 1 L working volume gave bioethanol titre of 11.4 g/L and yield of 0.267 g of bioethanol/g of pretreated SL.

Keywords: Bioreactor, cellulase GH5, hemicellulase GH43, Kans grass, SHF (Separate hydrolysis & fermentation), SSF (Simultaneous saccharification & fermentation), sugarcane leaves

Introduction

Clean and sustainable bioenergy production is a challenging task for substituting the fossil-based fuels, to get cleaner environment and also to reduce the dependency from other oil producing countries that creates the uncertainty in fuel prices¹. As an advancement of 21st century, the researchers are now shifting from non-renewable sources to renewable sources of energy and they have been getting successful by using lignocellulosic biomass as an alternative energy resource². It has become an important quest to search for alternative and renewable sources of energy, which can change or blend with the conventional fossil fuels³. Lignocellulosic biomass is a highly complex heterogeneous material made up of cellulose, hemicellulose and lignin. The structural carbohydrate content changes depending upon genetic, habitat and ambient niche conditions. The wastes of Agriculture, as one type of lignocellulosic resource,

can make up to 50% of agricultural production, and are regarded as accessible and promising feedstocks for the making of bioethanol because of its easy availability⁴. India accounts for the second largest production of sugarcane (*Saccharum officinarum*) after Brazil that accounts for more than 200 MMT⁵.

The cultivation of sugarcane is practiced over a large area owing to the favourable proportion of subtropical area (55%), which embraces various larger states like Maharashtra, Karnataka, Uttar Pradesh and many others. Kans grass (*Saccharum spontaneum*), a C-4 perennial grass, which also considered as “wasteland weed”, is the cultivar of switch grass and possesses fast growth ability with minimum economic input of water and land⁶. With improved ability of atmospheric CO₂ fixation for photosynthesis and scattered vascular bundles, these feedstocks show greater efficiency and faster life cycle than C-3 plants, thereby proved to be promising substrates for bioethanol production. Prospective of renewable feedstocks for bioethanol productions has recently been improved by the research on various pretreatment technologies, hydrolytic enzymes for

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saccharification and genetically modified microbes for enhanced conversion of biomass to bioethanol⁷. To facilitate commercial bioethanol production, research needs to be focused on pretreatment technologies at economical cost, which can integrate with biological conversion systems, without decreasing the fermentation efficiency. The presence of an advanced economical technology for extracting the cellulosic materials is hindered due to the occurrence of unorganized lignin, which combats for the production of bioenergy⁸. Therefore, lignin component must be separated for improved activity of hydrolytic enzymes⁹, and this is also affected by the per cent of crystallinity, the surface area accessible and degree or extent of polymerization in biomass¹⁰.

The biological progressions enduring the itemization of complex heterogeneous lingocellulosic feedstocks for improved production of bioethanol requires optimum pretreatment strategy for discharge of impregnated lignin content, thereby resulting in effective saccharification of accessible carbohydrate polymers, viz., cellulose and hemicellulose content, to release mixed pentose and hexose monomers, and further fermentation of these free sugars into bioethanol production. In order to analyse the morphological and structural alterations of the biomass produced by various pretreatments, FT-IR (Fourier transform infrared spectroscopy) and Electron microscopy have been employed^{11, 12}.

The cellulosomal complex formed by the *Clostridium thermocellum* (thermophilic) bacterium is a multienzyme assembly that has been reported to have an augmented specific activity with cellulose (crystalline form) up to 50-fold greater than the corresponding *Trichoderma reesei*¹³. Various cellulases and hemicellulases (hydrolytic enzymes) have been classified as glycoside hydrolases members of family 5 are known to show cellulose β -(1 $\rightarrow$ 4)-cellobiosidase (EC 3.2.1.91), cellulase (EC 3.2.1.4), licheninase (EC 3.2.1.73) and glucanase endo- β -(1 $\rightarrow$ 6)-glucosidase (EC 3.2.1.75); whereas family 43 glycoside hydrolase (GH43) displays activities of β -(1 $\rightarrow$ 3)-xylosidase (EC 3.2.1.-), β -xylosidase (EC 3.2.1.37), xylanase (EC 3.2.1.8), α -L-arabinofuranosidase (EC 3.2.1.55) and arabinanase (EC 3.2.1.99). *Saccharomyces cerevisiae* is a well known bioethanol producer, which utilizes a wide range of substrates. It also has high tolerance towards bioethanol and can endure metabolic inhibitions¹⁴. *Candida shehatae* encompasses enzymes (xylose

reductase & xylulose dehydrogenase), which are crucial for metabolic conversion of various pentose sugars to EtOH via the PP (pentose phosphate) pathway¹⁵. SSF (simultaneous saccharification & fermentation) process entails the combination of the enzymatic breakdown along with fermentation of the released sugars to bioethanol in a distinct process¹⁶. In SHF (separate hydrolysis & fermentation), it involves separate reactors used for the process of hydrolysis of biomass and fermentation, as its name suggests.

The present study involves the comparative bioethanol production from sugarcane leaves (SL) and Kans grass (KG) under two separate fermentation approaches (SHF & SSF).

Materials and Methods

Substrates, Chemicals and Reagents

SL and KG were collected from the agricultural land of Amingaon, North Guwahati, Assam. The leafy biomasses were individually cut, washed (3 times) with water and further dried separately at 55°C for 4 d. Subsequently, the dried biomass was grinded and allowed to pass through the sieve (pore size, 1 mm). The reagents and chemicals of analytical grade, viz., tryptone, glucose, peptone, Na-K tartrate, yeast extract, MgSO₄·7H₂O, NaCl, KH₂PO₄, Na₃AsO₄, Na₂CO₃, CuSO₄, K₂Cr₂O₇, Na₂SO₄, ampicillin, ammonium molybdate, agar-agar and kanamycin, were purchased from Hi-Media Pvt. Ltd., India. KBr, CMC (carboxy methyl cellulose), isopropyl- β -D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Sigma Aldrich (St. Louis, USA). Thin layer chromatography (TLC) plates (TLC Silica gel 60 F254 20×20 cm²) were procured from Merck, Germany. Rye arabinoxylan was procured from Megazyme International Ltd. (Ireland). H₃PO₄ and absolute alcohol were procured from Qualigens India Pvt. Ltd and Merck India Pvt. Ltd., respectively.

Microorganisms and Culture Conditions

The ORF regions of cellulase GH5 and hemicellulase GH43 from *C. thermocellum* were individually cloned in pET-21a(+) and pET-28a(+) vectors, respectively^{17,18}. The resulting recombinant plasmid of GH5 and GH43 were then transformed in host *Escherichia coli* BL21 (DE3) and *E. coli* BL21 (DE3) pLysS cells, receptively. The recombinant cellulase GH5 is presently commercially available at NZY Tech, Lisbon, Portugal. The *E. coli* cells comprising the recombinant plasmid were preserved and culture was maintained as 50% glycerol stock in

–80°C at the Carbohydrate Enzyme and Biotechnology Laboratory of Professor Arun Goyal at DBSBE, IIT Guwahati, India. The predominantly aerobic microbes, *viz.*, *C. shehatae* (NCIM 3500) and *S. cerevisiae* (NCIM 3215), for fermentation were procured from CSIR-National Chemical Laboratory, Pune, India. The cultures were grown and maintained separately in MGYB slants (2% agar, pH 5.0), comprising (g/10 mL): glucose (0.1); peptone, (0.05); yeast extract, (0.03) and malt extract (0.03)¹⁹. A loopful of these cultures from each slants were separately inoculated into Erlenmeyer flask (150 mL) with GYE medium of 50 mL (pH 5.5), comprising (g/50 mL): glucose (0.5), yeast extract (0.05), K₂HPO₄ (0.05), KH₂PO₄ (0.05), (NH₄)₂SO₄ (0.25) and MgSO₄·7H₂O (0.025); later the cultures were further incubated for 48 h, 120 rpm, 30°C prior to the fermentation trials. For the determination of the cell count of actively growing cultures (*C. shehatae* & *S. cerevisiae*), haemocytometer (counting chamber) was used at regular intervals.

Production of Recombinant Enzymes for Saccharification

The production of GH5 and GH43 recombinant enzymes were carried out in two separate 5 mL LB medium containing tubes supplemented with ampicillin (100 µg/mL) and kanamycin (50 µg/mL), respectively. About 50 µL of each *E. coli* BL21 (DE3) cells of GH5 and *E. coli* BL21 (plysS) cells of GH43 from glycerol stocks were inoculated in 5 mL LB medium and incubated at 37°C at 180 rpm for 20 h. Thereafter, 1% (v/v) inoculum (~2 × 10⁶ cells/mL) of the cultures were added to 100 mL of LB medium containing 50 µg/mL kanamycin or 100 µg/mL ampicillin and incubated at 37°C and 180 rpm until the culture achieved a mid-exponential phase (A_{600nm} ~ 0.6). After that, the cells were induced with 1 mM final concentration of IPTG (isopropyl-β-D-thio galactopyranoside) and further incubated at 24°C, 180 rpm for 18 h. The cells were harvested by centrifugation at 8000 g at 4°C for 10 min. About 15 mL of 20 mM sodium phosphate buffer, pH 6.0 was used for resuspending the cell pellets for sonication (On 5 sec/Off 10 sec pulse) and 33% amplitude for 10 min, by keeping the sample on ice-bath (Vibra Cell, Sonics, CT, USA). The sonicated cells were centrifuged at 12,000 g at 4°C for 40 min. The cell debris pellets were discarded and the supernatant containing the crude recombinant enzymes, *viz.*, cellulase GH5 and hemicellulose GH43 were used for SHF and SSF experimental trials.

Pretreatment Strategy and Estimation of Structural Carbohydrate

Owing to the considerable amount of cellulose and hemicellulose content in SL and KG, the samples were subjected to phosphoric acid (H₃PO₄)-acetone pretreatment²⁰. About 1 g of each, dried and powdered SL and KG were individually mixed with 8 mL of concentrated H₃PO₄ in 150 mL Erlenmeyer flask. The samples were incubated at 50°C and 120 rpm for 1 h. Then 24 mL chilled acetone was added into slurry and the mixture was centrifuged at 6,000 g for 10 min at room temperature. The supernatant was decanted and the pelleted sample was washed thrice with distilled water (dH₂O). Before the last wash, the pellet was resuspended in dH₂O and the pH was adjusted to 5.6 using 1 N NaOH. The drying of the sample was carried out at 50°C in a hot-air oven for 18 h.

Structural lignocellulosic content (cellulose, hemicellulose & lignin) of both the untreated and acid-acetone pre-treated SL and KG were estimated by following the standardized procedures of NREL, USA²¹. About 0.3 g of each untreated or pretreated SL and KG biomass were mixed with 27 N sulphuric acid (3 mL) and then incubated 30°C/1 h. About 84 mL dH₂O was further added in lowering the acidic concentration of it to 1.5 N. The samples were further autoclaved (121°C for 60 min). The samples were cooled to the room temperature (25°C) and both untreated and pretreated biomasses were filtered using a vacuum filtration unit. The remaining residue was acid insoluble lignin. The pH of the filtrate was neutralized with 1 M CaCO₃. At the end, the reducing sugar was determined from each filtrate and the cellulose content was determined. (1.1 g glucose=1 g cellulose). The leftover content was assessed to be hemicellulose. The pretreated samples were then taken for further studies.

FT-IR Spectroscopy and FESEM Analysis

The analysis of both the untreated and acid acetone pretreated SL and KG for functional groups was carried out by FT-IR (Spectrum-2 IR, Perkin Elmer, USA) spectroscopy. About 1 mg of each dried sample (50°C, 20 h) was mixed with 3 mg of KBr and the mixtures were pelleted by hydraulic press. Then 30 scans with data interval of 0.1 per cm and resolution (4 per cm) were performed for each sample with repetition. The samples were analysed in triplicates for the reproducibility of the results.

For field emission scanning electron microscopy (FESEM) analysis, 15 µL of each sample (0.04 g/L),

untreated and acid acetone pretreated SL and KG, was placed over a thin aluminium sheet and the sample was incubated at 50°C for 24 h. A double coating of gold film was performed over samples using a Polaron Sputter Coater, SC7620 “Mini” (Quorum Technologies, England). Each sample was then examined under FESEM (Carl Zeiss, SIGMA VP instrument, Germany). The images showing the structural topology were obtained for both untreated and pre-treated SL and KG.

Reducing Sugar Analysis by TLC and MS of Enzymatically Hydrolyzed Products from Pretreated SL

The qualitative analysis of the hydrolysed products by mixed enzymes (GH5 & GH43) from acid acetone pretreated SL (5% w/v) in SSF (simultaneous saccharification with fermentation) at bioreactor level was carried out using TLC on silica gel 60 F254 coated aluminium foil. Mass spectroscopy (MS) analysis of the enzymatically hydrolyzed products from pretreated SL was also performed using ESI-TOF MS (Agilent, Santa Clara, CA) to confirm the molecular mass of released reducing sugars.

The samples from bioreactor were collected at time intervals of 6, 12, 18, 24, 30 and 36 h for TLC analysis. The enzymatic reaction was stopped by keeping the samples in the boiling water bath for 2 min, followed by centrifugation at 13,000 g for 5 min. Standard, D-glucose and L-arabinose (each 1.0 mg/mL) along with samples were sequentially loaded over the TLC plate and further dried for 5 min. Then the plate was retained in the saturated developing chamber having the mobile phase, as reported earlier²². The migrated reducing sugars (the enzymatically hydrolyzed products) were determined by immersing the TLC plate in α -naphthol solution (α -naphthol 5.0 %, w/v & CH₃OH:H₂SO₄, 95:5, v/v). Then the TLC plate was kept at 80°C for 20 min in the hot air oven. The migrated hydrolysed products were spotted as dark (black brown) spots on the TLC plate.

The degree of polymerization (DP) of the released sugars was confirmed by mass spectrometer. The 1 mL sample was treated with CH₃OH (in the ratio 1:2) and centrifuged at 10,000 g for 10 min to remove the precipitate and the supernatant was filtered through 0.22 μ m membrane and subjected to MS analysis. The 36 h sample from SSF process with 5% (w/v) pretreated SL in bioreactor was analyzed by mass spectrophotometer (Agilent 6550 iFunnel Q-TOF-LC/MS, Agilent 1200 series, USA). ESI-MS was

performed in positive ionization mode and analyzed in TOF detector.

Comparative SHF Trials of Acid Acetone (1%, w/v) Pretreated SL and KG at Shake Flask Level

SHF process was performed by taking 1 g of each acid acetone pretreated SL and KG biomass individually in Erlenmeyer flask (250 mL) containing 100 mL 20 mM sodium acetate (pH 5.0) buffer. Thereafter, 0.5 mL each of recombinant, cellulase GH5 (5.9 U/mg, 0.51 mg/mL) and hemicellulase GH43 (3.9 U/mg, 0.35 mg/mL), was added and the saccharification was carried out at 55°C at 120 rpm in both the flasks for 36 h. The mixture was centrifuged at 8,000 g for 15 min at 25°C. The supernatants of both flasks were collected and additional supplements like yeast extract and peptone 0.1%, (w/v) was added to each for yeast fermentation medium. *S. cerevisiae* ($\sim 3.3 \times 10^6$ cells/mL) and *C. shehatae* ($\sim 3.7 \times 10^5$ cells/mL) were then added for fermentation with an inoculum volume of 0.5 mL. The fermentation was carried out at 35°C at 20 rpm for 3 d. The samples (2 mL) were withdrawn at a time interval of 6 h for estimation of bioethanol concentration (g/L), cell OD at 600 nm, specific activities (U/mg) of enzymes and reducing sugar (g/L). The experiment was run in triplicate for each SHF trial.

Comparative SSF Trials of Acid Acetone Pretreated SL and KG (1%, w/v)

SSF trials were performed with 1 g of each pretreated SL and KG biomass individually in 100 mL working volume in 250 mL flasks containing: 20 mM sodium acetate buffer, pH 5.0 supplemented with 0.1% (w/v) peptone and 0.1% (w/v) yeast extract. Later, 0.5 mL each of recombinant cellulase GH5 (5.9 U/mg, 0.51 mg/mL) and hemicellulase GH43 (3.9 U/mg, 0.35 mg/mL) enzymes isolated by sonication were added along with inoculum volume of 0.5 mL each of *S. cerevisiae* ($\sim 3.3 \times 10^6$ cells/mL) and *C. shehatae* ($\sim 3.7 \times 10^5$ cells/mL). The flasks were incubated at 35°C at 120 rpm in a shaker incubator. The sample collection was performed at an interval of every 6 h till 72 h, and the reducing sugar, bioethanol concentration, the cell OD at 600 nm and the specific activity (U/mg) of the enzymes were determined.

Comparative SSF Experiments Involving Mixed Enzymes and Mixed Cultures of Fermentative Microbes with 5% (w/v) SL and KG in Shake Flask

The concentration of both pretreated SL and KG substrate was increased to 5% (w/v), and were used

separately for SSF process. Further, the mixed recombinant enzymes and mixed cultures of fermentative microbes were involved in the process. For saccharification, 2.5 mL of each of recombinant GH5 (5.9 U/mg, 0.51 mg/mL) and recombinant GH43 (3.9 U/mg, 0.35 mg/mL) enzymes along with the mixed fermentative microbes, *viz.*, 2.5 mL each of *S. cerevisiae* ($\sim 3.3 \times 10^6$ cells/mL) and *C. shehatae* ($\sim 3.7 \times 10^5$ cells/mL), were added to 100 mL medium in batch SSF trials. The medium (100 mL) for fermentation also contained 20 mM sodium acetate buffer, pH 5.0 with the supplementation of 0.1% (w/v) peptone and 0.1% (w/v) yeast extract.

SSF Experiments Involving Mixed Enzymes and Mixed Cultures of Fermentative Microbes with 5% (w/v) SL in Bioreactor

The batch SSF cultivation was accomplished using bioreactor of 3 L capacity (Applikon, model Bio Console ADI 1025 make, Netherlands) with 1 L of working volume by involving mixed enzymes (GH5 and GH43) and mixed culture (*S. cerevisiae* & *C. shehatae*). About 50 g of acid acetone pretreated SL was taken in 1 L 20 mM sodium acetate buffer (pH 5.0) supplemented with 0.1% (w/v) peptone and 0.1% (w/v) yeast extract followed by autoclaving. Subsequently, after cooling to room temperature, 25 mL each of the crude recombinant GH5 (5.9 U/mg, 0.51 mg/mL) and GH43 (3.9 U/mg, 0.35 mg/mL) enzymes were added for saccharification. This was followed by addition of fermentative microbes, *viz.*, 25 mL each of *S. cerevisiae* ($\sim 3.3 \times 10^6$ cells/mL) and *C. shehatae* ($\sim 3.7 \times 10^5$ cells/mL) for bioethanol production. The temperature of fermentation was constantly monitored and maintained at 35°C, pH 5.0 was controlled and the agitation speed was kept at 120 rpm throughout the run. The aeration rate was maintained at 1 vvm using a mass flow controller to maintain DO (dissolved oxygen) level at a minimum 38% required for effective fermentative microbial growth, which was monitored through a spectrophotometer by taking OD at 600 nm (Varian Cary50, Australia). The continuous monitoring of various process parameters affecting the bioethanol yield like temperature (°C), pH and rpm (stirring speed) was carried out online and recorded at the periodic interval of time. Various factors, such as, specific activity (U/mg), reducing sugar (g/L), cell OD at 600 nm and bioethanol concentration (g/L) were examined at intervals of 6 h. The pH regulation was upheld to a fixed point of 5 by sustained and controlled addition of 1 N hydrochloric acid and 1 N

sodium hydroxide. Thus, pH changes of the fermentative organism below the fixed value were not allowed in response for its susceptibility towards growth for these changes.

Analytical Methods

Recombinant Cellulase GH5 and Hemicellulase GH43 Assay and Protein Content Determination

The activity of the recombinant cellulase GH5 assay was carried out in 100 μ L reaction volume containing 10 μ L enzyme, a final concentration of 1% (w/v) CMC in 20 mM sodium acetate buffer, pH 4.3 and by incubating at 55°C for 10 min. The samples were examined for the reducing sugar content as described earlier^{23,24}. The absorbance at 500 nm was measured using a UV-Visible spectrophotometer (Perkin Elmer, Model λ -45, USA). D-glucose was used as the standard. Similarly, hemicellulase GH43 activity was determined by taking 10 μ L enzyme in a 100 μ L reaction mixture containing 1% (w/v) rye arabinoxylan dissolved in 20 mM sodium acetate buffer, pH 5.4 and incubating at 50°C for 10 min. One unit (U) of enzyme activity (cellulase or hemicellulase) is the amount of enzyme required to release 1 μ mole of reducing sugar (glucose or arabinose) per min at mentioned assay conditions. The enzyme concentration was measured by Bradford method²⁵ using BSA as standard.

Bioethanol Content Determination Through Gas Chromatography

Gas chromatography attached with FID (Varian 450) (flame ionization detector) and the column packed with Porapak (Hayesep) Q (3000 mm $\times$ 0.002 m i.d., 80-100 mesh, manufactured by Varian), was used for detection of EtOH²⁶. N₂ acted as carrier gas at a persistent flow rate 53 cm³/min and the temperature of the oven was maintained isothermally, 150°C for a time period of 20 min. The volume of injection was 1 μ L and temperature at injector along with the detector was kept 170°C throughout the analysis. In the current study, the post fermentation determination of the cellulose and hemicellulose contents was not done, therefore the amount cellulose and hemicellulose uptake were not calculated.

Results and Discussion

Economic sustainability of the saccharification and fermentation process relies on the efficient pretreatment approaches and the cost effective saccharification methods for a competent biomass. This results in the effective release and subsequent

uptake of reducing sugars by fermentative microorganisms rendering higher production of bioethanol.

Composition Analysis of Untreated and H₃PO₄-Acetone Pretreated Feed Stocks (SL & KG)

The structural carbohydrate content of untreated SL revealed cellulose 34.2, hemicellulose 31.2 and lignin 28% (w/w); while KG showed cellulose 42.4, comparatively lower hemicellulose 21.6 and lignin 22.6% (w/w) (Table 1). This signified that both the substrates have the potential for production of bioethanol. After the pretreatment (H₃PO₄-acetone), the cellulose, hemicellulose and lignin contents for SL were 32.5, 29.5 and 15.5%, respectively, and for KG were 39.2, 19.4 and 12% (w/w), respectively, displaying significant post-pretreatment removal of lignin content in both SL and KG (Table 1). H₃PO₄-acetone pretreatment also showed the swelling of cellulosic content with decreased crystallinity²⁷, with minimal inhibitor production at increased concentration²⁰. Similar observation of pretreatment digestibility was also reported for poplar, bermuda grass and rapeseed stover^{20,28}.

FESEM and FT-IR Analysis of SL and KS

The FESEM analysis of the untreated SL and KG samples depicted less surface porosity and structural deterioration (Figs 1a & b) as compared with the pretreated SL and KG (Figs 1c & d). The pretreatment strategy enhances the structural porosity and intensification, imparting the accessibility of active sites present in biomass, for improved saccharification by hydrolytic enzymes. Similar observations were earlier reported from bamboo (*Dendrocalamus* sp.) with dilute acid pretreatment²⁹ and mixed microwave-assisted alkali and organosolv pretreated water hyacinth (*Eichhornia crassipes*)³⁰. Biomass selection and the effective pretreatment are the key factors in order to determine the efficiency of fermentation²⁹.

Table 1 — Structural carbohydrate content (% w/w) of untreated and pretreated feed stocks

Feedstock	Cellulose* (%, w/w)	Hemicellulose* (%, w/w)	Lignin* (%, w/w)
Untreated KG	42.4 ± 0.6	21.6 ± 0.4	22.6 ± 0.5
H ₃ PO ₄ -acetone treated KG	39.2 ± 0.4	19.4 ± 0.5	12.0 ± 0.2
Untreated SL	34.2 ± 0.1	31.2 ± 0.4	28.0 ± 0.4
H ₃ PO ₄ -acetone treated SL	32.5 ± 0.2	29.5 ± 0.1	15.5 ± 0.2

*Values are mean ± SE (n=3)

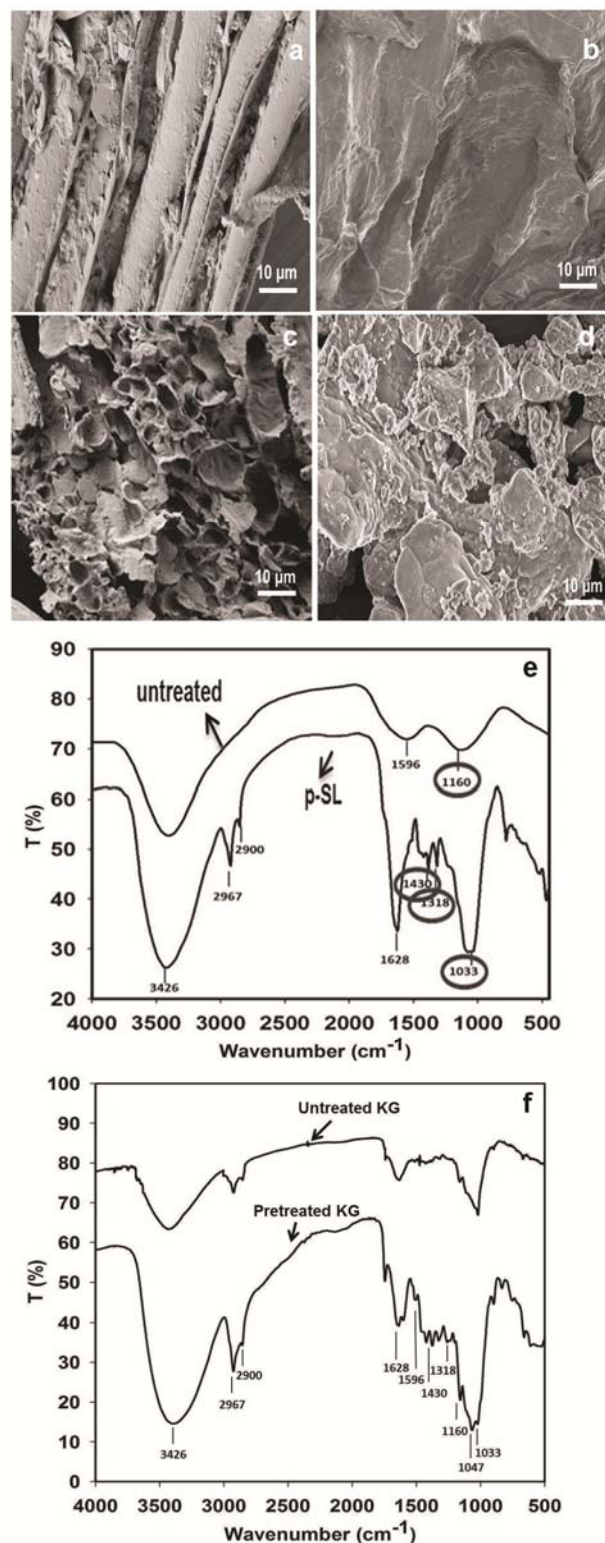


Fig. 1 (a-f) — FESEM images: (a) Untreated SL, (b) Untreated KG, (c) Pretreated (H₃PO₄-acetone) SL, (d) Pretreated (H₃PO₄-acetone) KG, (e) FT-IR spectroscopic analysis of untreated and pretreated SL, & (f) FT-IR spectroscopic analysis of untreated and pretreated KG.

The FT-IR spectral analysis of untreated and pretreated SL samples was carried out (Fig. 1e). The peaks assigned for carbohydrate specific FT-IR spectra for pretreated SL and KG are shown in Table 2. The OH stretching for hydrogen bond from 3416 cm^{-1} to 3426 cm^{-1} was predictive to be from glycosidic bonds in cellulose or various other groups (guaiacyl, hydroxyphenyl & syringyl) of lignin, released during the pretreatment. Due to the stretching of a bond (C-H) in the aliphatic structures, the peaks 2967 and 2900 cm^{-1} were observed, as also earlier reported by Cao and Tang³¹. A peak observed at 1628 cm^{-1} was probably due to the high affinity of hemicellulose towards H_2O and also because of bending mode of H_2O , and thereby rendering the easily hydrated and unordered structures³². Bands obtained at 1318 and 1430 cm^{-1} represent C-H wagging and C-H, OH bending, respectively. The peaks at 1160 and 1033 cm^{-1} observed in pretreated samples were ascribed to the twisting and stretching vibrations of C-C, C-O and C-OH that were arabinose and xylose moieties (from arabinoxylan) and glucose, respectively, which also corresponds to the earlier observations in substrates, such as, sugarcane bagasse, straw and sea buckthorn^{33,34}. The most significant peaks at 1160 and 1033 cm^{-1} in H_3PO_4 -acetone pretreated KG samples were corroborated with pretreated SL sample (Fig. 1f). Therefore, the pretreatment strategy was proficient for the release of

monomeric sugar and effective breakdown of lignin from complex SL and KG.

SHF Experiments Involving Acid-Acetone Pretreated 1% (w/v) SL and KG at Shake Flask Level

The saccharification trials were carried out using recombinant cellulase GH5 and hemicellulos GH43 with each acid-acetone pretreated (1%, w/v) SL and KG biomass and fermentative microorganisms, *viz.*, *S. cerevisiae* (for uptake of released hexose) and *C. shehatae* (for uptake of released pentose). With (1%, w/v) SL, the concentration of reducing sugar remained constant between 1.0 and 1.2 g/L up to 30 h and decrease rapidly to 0.36 g/L at 66 h (Table 3, Fig. 2a). The cell growth showed a mid-exponential growth and reached a maximum OD (600 nm) of 0.86 at 66 h and thereafter it declined (Fig. 2a). The production of bioethanol increased with the increase in cell concentration and showed an exponential rise between 36 - 60 h and showed 0.64 g/L at 60 h (Table 3, Fig. 2a). However, with pretreated KG, the maximum concentration of reducing sugar released by saccharification obtained was 1.10 g/L up to 12 h , which subsequently decreased after that reaching a minimum level of 0.23 g/L at 66 h (Fig. 2b, Table 3). The cell growth showed an exponential growth with maximum cell OD (600 nm) of 0.84 at 66 h (Fig. 2b), with the production of bioethanol 0.52 g/L at 60 h (Table 3, Fig. 2b). A 1.2-fold increase in bioethanol titre with 1% (w/v) pretreated SL (0.64 g/L) was obtained in SHF trial as compared with KG (0.52 g/L).

SSF Experiments Involving Acid-Acetone Pretreated 1% and 5% (w/v) SL and KG at Shake Flask Level

SSF with 1% (w/v) SL depicted the initial phase (0 - 6 h) as a steady increase in reducing sugar concentration and later a short accumulation phase (6 - 12 h) and then reaching a maximum of 1.8 g/L concentration at 36 h . Thereafter a decrease in concentration was observed (Table 3, Fig. 3a).

Table 2 — FT-IR analysis of H_3PO_4 -acetone pretreated SL and KG

Characteristic group name	Wave number (cm^{-1})
OH	3426
Hemicellulose (aromatic) ring stretch	1628
Aliphatic stretch (C-H)	2967, 2900
Antisymmetric stretching for cellulose C-O-C	1160
CH_2 -wagging vibrations in cellulose/hemicellulose	1318
Crystalline cellulose (C-H stretch)	1430
C-O stretch vibration (Glucose)	1033

Table 3—Comparison of fermentation experiments of SL and KG involving mixed enzymes and microbes *S. cerevisiae* and *C. shehatae*

Feedstock concentration/ Mode of fermentation	Reducing sugar (g/L)*	Bioethanol titre (g/L)*	Bioethanol yield ($\text{g ethanol/g substrate}$)
1% (w/v) KG, Shake flask/SHF	1.09 ± 0.03	0.52 ± 0.04	0.088
1% (w/v) SL, Shake flask/SHF	1.22 ± 0.04	0.64 ± 0.03	0.103
1% (w/v) KG, Shake flask/SSF	1.70 ± 0.05	0.80 ± 0.06	0.137
1% (w/v) SL, Shake flask/SSF	1.81 ± 0.02	1.00 ± 0.04	0.161
5% (w/v) KG, Shake flask/SSF	9.0 ± 0.04	4.20 ± 0.06	0.143
5% (w/v) SL, Shake flask/SSF	12.3 ± 0.05	5.70 ± 0.03	0.183
5% (w/v) SL, Bioreactor/SSF	23.5 ± 0.06	11.40 ± 0.03	0.267

*Maximum values of released reducing sugar and bioethanol concentration at a specific time; values are the mean $\pm$ SE (n=3)

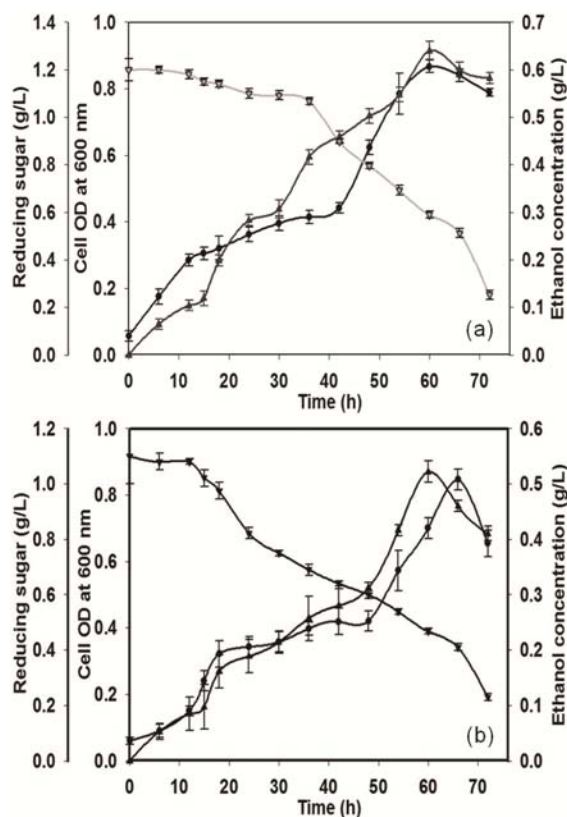


Fig. 2 (a & b) — 1% (w/v) SHF profile of (a) pretreated (H_3PO_4 -acetone) SL (b) pretreated (H_3PO_4 -acetone) KG in shake flask using recombinant cellulase GH5, hemicellulase GH43 along with *S. cerevisiae* and *C. shehatae*. [(●) Cell OD A_{600} , (▲) Bioethanol concentration (g/L), & (▼) Reducing sugar (g/L) with time (h). Fermentation was performed in 100 mL, initial pH 5, at 35°C and 120 rpm.]

Apparently, a slight decrease in bioethanol concentration was seen between 24–30 h, coupled with an abrupt rise in reducing sugar concentration (Fig. 3a). Thereafter, a steady increase in bioethanol concentration was observed, attaining a maximum of 1.0 g/L concentration at 66 h (Table 3, Fig. 3a). The SSF trail with 1% (w/v) KG showed steady increase in reducing sugar concentration after 12 h, reaching a maximum 1.7 g/L at 36 h and thereafter it decreased (Table 3, Fig. 3b). Simultaneously, the increase in bioethanol concentration was observed for initial 30 h and then it increased exponentially reaching 0.8 g/L at 66 h (Table 3, Fig. 3b). The decline in the concentration of cell biomass was observed after 66 h. The 1% (w/v) pretreated SL under SSF gave 25% higher ethanol titre than 1% (w/v) pretreated KG. A 1.6-fold escalation in bioethanol titre with 1% (w/v) pretreated SL and KG was obtained in SSF trial (0.8 & 1.0 g/L), respectively (Table 3) as compared with SHF process (0.52 and 0.64 g/L) involving the same

enzymes and the same fermentative microbes. The increase in the substrate concentration shows intensification in bioethanol concentration²⁸. Therefore, further experiments were performed with higher substrate concentration (5%, w/v) of both SL and KG in SSF mode.

The SSF profiles of 5% (w/v) pretreated SL showed the initial increase in cell biomass reaching a maximum cell OD (600 nm) of 2.6 at 66 h (Fig. 3c). The concentration of reducing sugar increased and reached a maximum 12.3 g/L at 36 h and thereafter it declined (Table 3, Fig. 3c). The bioethanol titre increased continuously till 66 h, attaining a maximum level of 5.7 g/L at 66 h with a yield of 0.183 g bioethanol/g substrate (Fig. 3c, Table 3). The cellulase activity decreased to 3.2 U/mg at 66 h (Fig. 3c). With 5% (w/v) KG, slightly different pattern was observed for enzyme activity but with the same 3.2 U/mg at 66 h of SSF (Fig. 3d) with maximum cell OD (600 nm) of 2.1 at 66 h (Fig. 3d). The maximum sugar concentration obtained was 9.0 g/L at 36 h and thereafter a deterioration in sugar concentration was recorded (Table 3, Fig. 3d). Moreover, the bioethanol concentration increased from 1.6 g/L at 36 h to a maximum of 4.2 g/L with and yield 0.143 g bioethanol/g substrate at 66 h (Fig. 3d, Table 3). On increasing the substrate concentrations of pretreated SL and KG from 1% to 5% (w/v) individually, 5-fold rise in 5.7 and 4.2 g/L bioethanol titre, respectively was attained at shake flask level (Table 3). In an earlier study, alkali pretreated KG was used for saccharification by crude enzyme from *Trichoderma reesei* and then fermentation using yeast strains of *S. cerevisiae* and *Pichia stipitis* resulted in bioethanol concentration of 2.89 and 3.29 (g/L), respectively³⁵. SL 5% (w/v) resulted 1.4-fold higher bioethanol titre (5.7 g/L) than 5% (w/v) KG (4.2 g/L) in SSF trial, involving the same pretreatment, enzymes and the fermentative microbes (Table 3).

Scale Up of SSF Involving 5% (w/v) SL at Bioreactor Level for Bioethanol Production

The scale up method from initial shake flask to bioreactor level demonstrates the capability to increase the productivity of fermentative microbes and simultaneously improving the bioethanol production²⁰. SSF process was scaled up to 1 L working volume in 3 L bioreactor level containing 5% (w/v) H_3PO_4 -acetone pretreated SL from 100 mL shake flask level. The SSF profile of the bioreactor run showed steady increase of

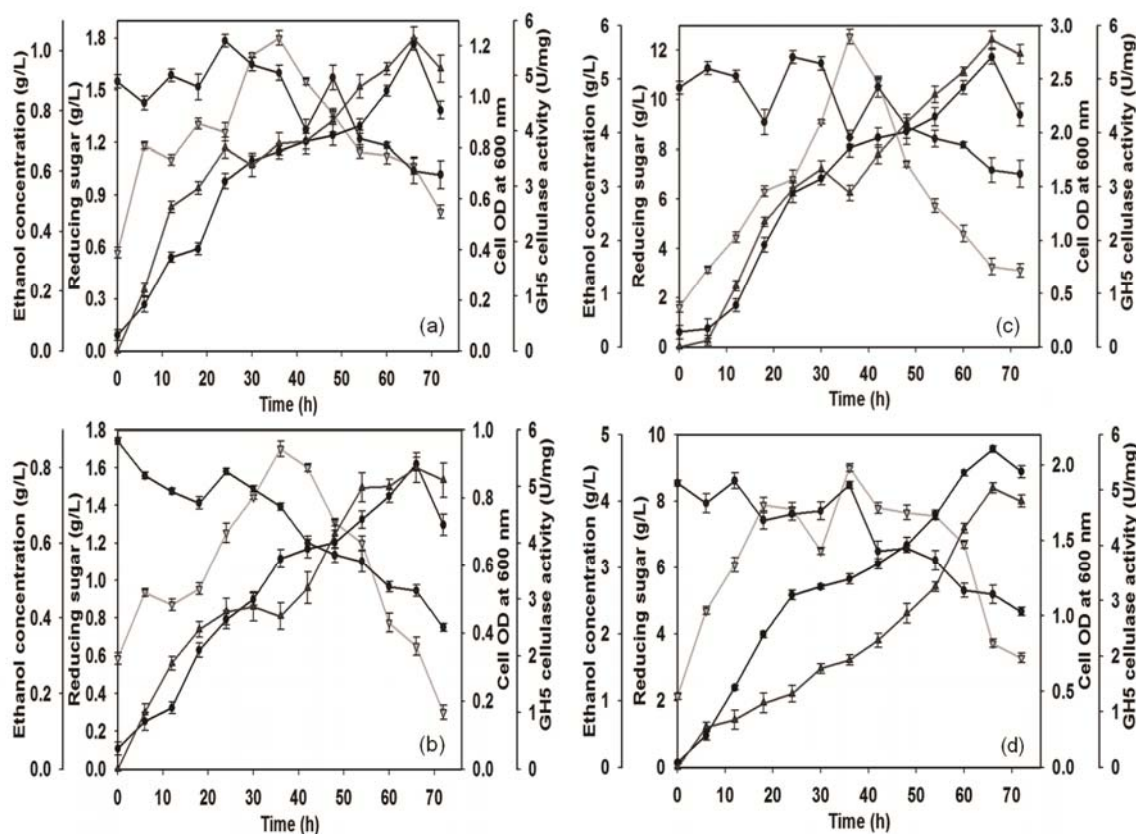


Fig. 3 (a-d) — SSF profile of pretreated (H_3PO_4 -acetone) 1% (w/v) SL (a), 1% (w/v) KG (b), 5% (w/v) SL (c) and 5% (w/v) KG (d) in shake flask using recombinant cellulase GH5, hemicellulase GH43 along with *S. cerevisiae* and *C. shehatae*. [(●) Cell OD A_{600} , (▲) Bioethanol concentration (g/L), (▼) Reducing sugar (g/L), & (○) Specific activity of GH5 (U/mg) with time (h). SSF was performed in 100 mL, initial pH 5, at 35°C and 120 rpm. Similar activity profiles were obtained for GH43 (data not shown). Values are mean $\pm$ SE (n=3).]

growth of fermentative microbes from a short initial lag phase with cell OD 0.8 at 6 h and later reaching a maximum at 6.8 at 66 h (Fig. 4). The concentration of reducing sugar increased progressively to 15 g/L at 12 h, and also the bioethanol concentration and cell biomass increased simultaneously. The reducing sugar reached at a maximum 23.5 g/L at 36 h (Fig. 4, Table 3). The recombinant cellulase GH5 activity displayed a sinusoidal behaviour with an initial value of 5.4 U/mg at 6 h, and then a decrease to 3.8 U/mg at 24 h, which might be owing to the inhibitory effect of increased concentration of reducing sugar (Fig. 4). A reverse effect on the enzyme activity was observed with an increase in content of reducing sugar (~20 g/L), as reported earlier³⁶. The bioethanol concentration showed an exponential increase till 50 h (9.2 g/L), and later a steady increase of bioethanol concentration of 11.4 g/L (maximum) at 60 h with a yield of 0.267 g bioethanol/g pretreated SL (Table 3, Fig. 4). The parameters, such as, pH and aeration rate that significantly affected the bioethanol concentration and the growth of

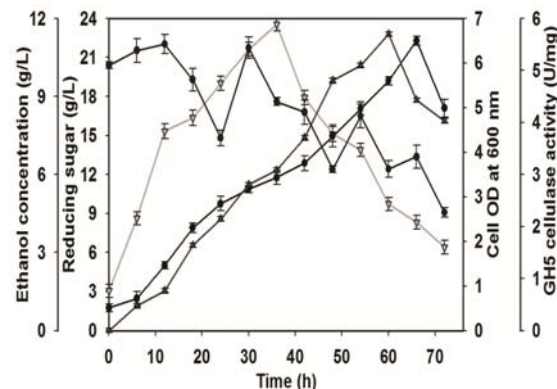


Fig. 4 — SSF profile of 5% (w/v) H_3PO_4 -acetone pretreated SL in 3L bioreactor with 1 L working volume, initial pH 5, at 35°C, agitation 120 rpm and aeration 1 vvm, showing variation of (●) Cell OD A_{600} , (▲) Bioethanol conc. (g/L), (▼) Reducing sugar (g/L), & (○) Specific activity of GH5 (U/mg) with time (h). Values are mean $\pm$ SE (n=3).

fermentative microbes were controlled. A threshold of 1 vvm aeration rate with $K_La=0.242 \text{ min}^{-1}$ maintained the proficient growth and improved bioethanol yield. The scale up of SSF in a bioreactor using 5% (w/v)

pretreated SL gave a 2-fold increase in the bioethanol titre (11.4 g/L) as compared with the SSF shake flask experiment that gave 5.7 g/L (Table 3, Fig. 4).

The time dependent qualitative analysis of the enzymatically hydrolysed products released from the 5% (w/v) pretreated SL showed the spot for mixed glucose (with major fraction) and arabinose (with smaller fraction) released during the SSF experiment (Fig 5a). The faint spot was observed at the 6 h of SSF, but after 12 h with the progress of hydrolysis, the spots became darker and concentrated at 18, 24, 30 and 36 h (Fig. 5a). A regular increase in the concentration of released sugar was observed with lowest at 6 h and highest at the 36 h of SSF (Fig. 5a). A standard of D-glucose and L-arabinose was run in parallel to compare the released sugars (S1 & S2, respectively). The MS analysis of sample of SSF from bioreactor was also carried out after 36 h, which displayed an m/z of 202.99 for glucose and 173 for arabinose (as Na adduct, $M + Na^+$) (Fig. 5b).

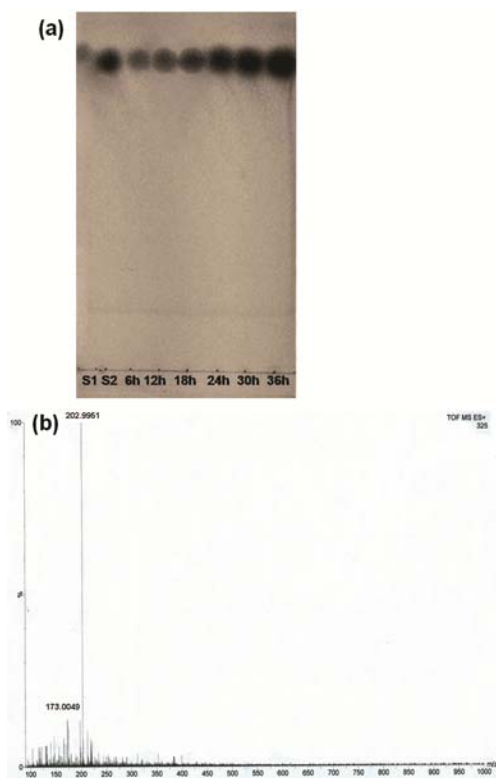


Fig. 5 (a & b) — (a) TLC analysis of the enzymatically (GH5+GH43) hydrolyzed product from pretreated SL during SSF in a bioreactor. Lane 1: Standard (S1) L-arabinose; lane 2: Standard (S2) D-glucose; lane 3: 6 h; lane 4: 12 h; lane 5: 18 h; lane 6: 24 h; lane 7: 30 h; lane 8: 36 h of SSF. (b) Mass spectra (positive ionization mode) of glucose and arabinose derived from pretreated SL on hydrolysis with GH5 and GH43 at 36 h of SSF in bioreactor.

The results showed the effectiveness of H_3PO_4 -acetone pretreated SL for improved saccharification using recombinant hydrolytic enzymes from *C. thermocellum*, while fermentation with *S. cerevisiae* and *C. shehata* resulted in 11.4 g/L of bioethanol concentration with 0.267 g of bioethanol/g of pretreated SL yield (Table 3, Fig. 4). The bioethanol concentrations accomplished in this study were similar to those reported earlier. The bioethanol titre of 11.3 g/L was reported from acid hydrolysed sugarcane tops³⁷. The chilled acetone usage during pretreatment resulted in the minimal release of inhibitors after the breakdown of lignin. At lower temperature, it is reported that various phenolic compounds, viz., quaiacyl, syringyl and furan derivatives, those mask the catalytic site of hydrolytic enzymes, are released minimally³⁸. Earlier studies showed the bioethanol titre of 4.71 g/L was achieved from dilute (1.5%, w/v) H_2SO_4 pretreated sugarcane leave tops with *S. cerevisiae* in SHF process after 24 h incubation³⁹. A 1.2-fold increase was observed in bioethanol titre (5.59 g/L) from acid pretreated sugarcane leaf hydrolysate from SSF using an improved strain of *K. marxianus* S1.17⁴⁰. SSF process showed improved bioethanol yield over SHF process by eliminating end product inhibition and also eliminating separate reactor's requirement.

Conclusion

The effectiveness of H_3PO_4 -acetone pretreated SL and KG under two separate fermentation modes (SHF & SSF), involving saccharification by clostridial recombinant hydrolytic enzymes, was assessed for the bioethanol production. A 1.6-fold higher bioethanol titre with 1% (w/v) pretreated KG and SL was obtained with SSF in comparison with SHF involving cocultivation of *S. cerevisiae* and *C. shehatae*. SSF exhibited a 5-fold higher bioethanol titre at 5% (w/v) concentration of SL and KG with respect to 1% (w/v) in SHF. SL resulted 5.7 g/L bioethanol titre, which was 1.4-fold higher than that obtained with KG (4.1 g/L). The scale up of pretreated 5% (w/v) SL from 100 mL volume under shake flask level to 1 L working volume bioreactor gave 2-fold increment in bioethanol titre (11.4 g/L) with yield coefficient of 0.267 (g/g). This opens up scope for large scale fermentation for production of bioethanol.

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