Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-020-01123-z

ORIGINAL ARTICLE

Efficient bioconversion of sugarcane tops biomass into biofuel-ethanol using an optimized alkali-ionic liquid pretreatment approach

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Received: 27 August 2020 / Revised: 27 October 2020 / Accepted: 29 October 2020

Abstract
Sugarcane tops (SCT) may be an imperative lignocellulosic feedstock for production of renewable energy in view of ever-rising global energy demands. The current study presents the first ever report of combinative pretreatment of sugarcane tops biomass using ionic liquid (1-ethyl-3-methylimidazolium chloride) and alkali (ammonium carbonate) for effective and enhanced saccharification. The enzymatic hydrolysis of combinatorially pretreated SCT biomass resulted in a significantly higher reducing sugar yield (172.34 mg/g biomass) as compared to that obtained after standalone IL (85.9 mg/g biomass) or alkali pretreatment (102.6 mg/g biomass). The saccharification enzymes (cellulase/xylanase) used for hydrolysis of pretreated SCT biomass were *in-house* produced from an IL-tolerant, newly isolated fungus *Penicillium chrysogenum* VS4. Optimization of process variables for combined pretreatment was accomplished based on design of experiments, and enhanced reducing sugar yield was obtained. The experimental design for ascertaining optimal level of process variables, i.e., biomass loading (5.33%, *w*/*w*), temperature (100 °C), and time (4.50 h) was validated. Optimization of the process parameters resulted in 25.27% increased reducing sugar yield (215.89 mg/g biomass) as compared to that under unoptimized process. Ultrastructural analysis of SCT biomass after combined pretreatment was investigated by scanning electron microscopy and Fourier transform infrared spectroscopy which indicated that the biomass had undergone drastic alterations and substantial disintegration due to pretreatment. The current study highlights the potential of combinative pretreatment strategy for efficient conversion of SCT biomass. Such combined pretreatment approaches may be extended to other biomass feedstocks as well for developing successful biorefineries.

Keywords Bioethanol · Optimization · *Penicillium chrysogenum* VS4 · Pretreatment · Sugarcane tops

1 Introduction

Fossil fuel depletion rate coupled with deep environmental concerns serve as catalysts for intensification of research in exploring and developing renewable and sustainable sources of energy [1]. Among various renewable alternatives, lignocellulosic biomass (agricultural and forestry residues, energy crops, municipal solid waste, and others) represents a promising, profusely accessible, cost-effective, and sustainable energy resource for the future [2, 3]. Lignocellulosic biomass (LB) is constituted of mainly cellulose, hemicellulose, lignin, ash, and other extractives, in which the thickness and composition of each component may depend upon its type, growth, and climatic conditions [4]. Furthermore, inappropriate disposal of agro-industrial LB wastes negatively impacts the environment. However, the valorization of LB residues for production of energy, biofuels, or other products not only promises attenuation of its disposal problems but also offers judicious environmental, economic, and strategic advantages [5]. However, due to recalcitrant nature of LB, multifaceted physiochemical/biochemical pretreatments are prerequisite for destabilization of LB, and efficient enzymatic saccharification of polysaccharides [6]. Such pretreatments lower down the crystallinity of cellulose and reduce the overall recalcitrance of LB, thereby enhancing the accessibility of saccharifying enzyme(s) to the polysaccharides, and resulting in increased yield of fermentable sugars [7]. Several different pretreatments have been explored for LB to make it more amenable to enzymatic saccharification [8]. However, most of these pretreatment strategies are environmentally hazardous, expensive, inefficient, and

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Published online: 11 November 2020

energy intensive; cause sugar loss; and generate toxic wastes or by-products which may inhibit the saccharifying enzymes and/or fermentation microorganisms [1, 2, 6, 9].

Ionic liquids (ILs) have been recognized as proficient, green, non-toxic, less volatile, and biodegradable alternatives to conventional pretreatment solvents [4, 8]. Furthermore, ILs employ extraordinary molecular mechanisms to modify the supra-molecular structure of LB polysaccharides under mild pretreatment conditions, and promote their digestibility, thus promising the efficient bioprocessing of LB [1]. Imidazolium-based ILs, due to their excellent solubilizing properties, have been used extensively for pretreatment of LB to enhance the enzymatic digestibility of cellulose [7, 8, 10]. Although ILs are regarded as promising LB pretreatment agents, however, their high cost hinders the large-scale industrial application [6]. One of the approaches could be application of ILs in combination with other cost-effective pretreatment agents. Few attempts have been made wherein combined IL-alkali pretreatments have been investigated for biomass fractionation. It has been reported that such combined pretreatments not only improve the process effectiveness due to the synergistic impact of both pretreatment agents [10–12] but also make the process more economical.

Alkali pretreatment breaks the intermolecular ester bonds between lignin and hemicellulose polymers [13, 14], thus causing delignification/depolymerization and improving the digestibility of polysaccharides [15]. Therefore, a combined IL-alkali pretreatment may be designed that is effectual, economical, environmentally friendly, and even better than the available techniques for processing of LB [11]. However, inhibition of saccharification enzymes (cellulase/xylanase) by the IL residues left in the regenerated pretreated biomass, despite washing, may challenge the overall techno-economic feasibility of the process. Therefore, IL stable enzymes are desired for efficient enzymatic saccharification of IL pretreated biomass [6]. Enormous microbial diversity from various ecological niches especially the chemically polluted soils may be exploited to target microorganisms that are capable of producing IL stable enzymes [6]. It was hypothesized that the metabolic/biochemical machinery of the microorganisms thriving in the chemically adulterated environments gets acquainted to such hostile chemical milieu and therefore, their enzyme complement develops stability to harsh chemicals. In the current study, an IL stable fungal strain isolated from chemically polluted soil that produced IL stable saccharification enzymes (cellulase and xylanase) was used for *in-house* production of enzymes to be used for hydrolysis of pretreated biomass.

Considering LB as an imperative renewable resource, new LB resources are being investigated as prospective feedstocks for biofuel production [1, 12]. Sugarcane trash represented by sugarcane bagasse, sugarcane tops (SCT), and field left over may serve as a potent feedstock for biofuel-ethanol production. In India, annual production of sugarcane is approximately 348 million metric tonnes (MMT), and about one-third of this represents the sugarcane tops, i.e., 104.4 MMT [15]. SCT constitutes the leaves and top upper parts of a sugarcane plant which find no appropriate application, and is generally burnt in the field, thereby causing enormous air pollution [16]. However, the presence of high cellulosic (35%, *w*/*w*) and hemicellulosic (30.3%, *w*/*w*) content makes it an attractive feedstock for production of bioethanol-fuel or other commercial products [17]. Although sugarcane bagasse has extensively been used as a biomass resource for biofuel production [6, 18], but rare reports are available on utilization of sugarcane tops (SCT) as raw material for the production of biofuel/energy [15, 19]. To the best of our knowledge, no studies have yet been undertaken on the combined application of alkali and IL for pretreatment for SCT biomass, and the resultant ultrastructural aberrations in the pretreated biomass.

The current study presents the first ever report of combinatorial IL and alkali pretreatment of SCT biomass. Pretreatment process variables were optimized by design of experiments, and saccharification was realized with *in-house* developed IL stable cellulase/xylanase enzymes from a novel IL-resistant fungal strain *Penicillium chrysogenum* VS4. Physicochemical analysis of pretreated SCT biomass by SEM and FTIR was done to decipher the functional mechanisms that govern the structural, molecular, and organizational alterations in the biomass which might facilitate the enzymatic saccharification.

2 Materials and methods

2.1 Microorganisms, biomass, chemicals, and reagents

SCT biomass used in the study was obtained from a local vendor of Jammu, India. The biomass was washed properly, cut into small pieces, dried, homogenized, and sieved to particle size of < 4 mm. The powdered SCT biomass was stored in an airtight container at room temperature for further use. The yeast strains used in the current study for ethanol fermentation of sugar hydrolysate obtained from SCT biomass were *Saccharomyces cerevisiae* NCIM 3078 and *Pichia stipitis* NCIM 3497. Yeast strains were purchased from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL, Pune, India). Chemicals and reagents used in the study were of analytical grade.

2.2 Compositional analysis of SCT biomass

The composition of native sugarcane tops biomass was determined by a two-stage dilute acid hydrolysis protocol developed by National Renewable Energy Laboratory (NREL),

NREL/TP-510-42618 [20]. High-performance liquid chromatography (HPLC) (Shimadzu, Japan) was used to quantify the monomeric sugars of the hydrolysate. HPLC grade standards of glucose, xylose, mannose, and arabinose were used for analysis [6].

2.3 Cellulase and xylanase producing fungi and activity assay

Several fungal cultures isolated from decomposing lignocellulosic biomass especially from chemically polluted ecosystems such as soils/waters in the vicinity of industrial area of Jammu were screened for cellulolytic and xylanolytic activity by plate assay [6]. The selected isolates were subjected to submerged fermentation (SmF) for 5 days (120 h) at 28 °C in enzyme production medium (% *w*/*v*, wheat bran 2, yeast extract 0.5, peptone 0.5, $MgSO_4$ 0.02, NaCl 0.25, KH_2PO_4 0.1; pH 7) for production of cellulase and xylanase enzymes. The culture broth was centrifuged (10,000×*g*, 4 °C for 10 min), and the supernatant (crude enzyme) obtained was assayed for cellulase and xylanase activities. The cellulase activity was examined using carboxymethyl cellulose (CMC) as substrate, and the assay procedure was followed as described previously [6]. For xylanase activity, 0.5%, *w*/*v* beechwood xylan prepared in acetate buffer (50 mM, pH 5) was used as substrate. The crude enzyme preparation (0.1 ml) was incubated with xylan substrate (0.9 ml) at 50 °C for 10 min. The reducing sugars released were quantified by 3,5-dinitrosalicylic acid (DNSA) method using xylose as standard [21]. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose (for cellulase activity) or xylose equivalent (for xylanase activity) per min under standard assay conditions.

2.4 Ionic liquid stability of fungal isolate and cellulase/xylanase enzymes

Cellulase and xylanase producing fungal isolates were evaluated for their ionic liquid (IL) tolerance by analyzing their growth on IL-supplemented potato dextrose agar (PDA). The fungal isolates were spotted on PDA plates containing IL 1-ethyl-3-methylimidazolium chloride ([Emim]Cl; 5%, *w*/*v*). The plates were incubated for 4–5 days at 28 °C, and then visually observed for growth. The selected IL-tolerant fungal isolates were subjected to submerged fermentation in enzyme production medium for cellulase/xylanase production, and the enzyme preparation was analyzed for IL stability of cellulase/xylanase. The crude enzyme preparation was pre-incubated with [Emim]Cl (5%, *w*/*v*) at ratio of 1:1. Samples were withdrawn at different time intervals (0, 4, 5, 24, 48, 72, 96, 120, 144, 168, 192, and 216 h), and the residual cellulase/xylanase activity was assayed.

The fungal isolate (VS4) which produced the maximum IL-tolerant cellulase/xylanase enzymes was earmarked and identified based upon culture morphology, microscopic studies, and by sequence analysis of internal transcribed spacers (ITS) using PCR amplification of ITS regions. Genomic DNA from fungal isolate VS4 was isolated, and PCR amplification of ITS region was executed using universal ITS-based primers [10]. The PCR-amplified product was purified (QIAGEN) and sequenced (Agrigenome, Cochin). The sequence was put through BLASTn analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi), for revealing the closest homology of the organism. The phylogenetic tree was constructed using MEGA 6 software (https://www.megasoftware.net/), and the sequence was submitted to GenBank, NCBI (http://www.ncbi.nlm.nih.gov/).

2.5 Acetone concentration and properties of cellulase/xylanase enzymes

The fungal isolate VS4 was subjected to SmF, and the cultural broth was centrifuged to separate the supernatant (crude enzyme preparation). The crude enzyme preparation was incubated with chilled acetone in a ratio of 1:4 at − 20 °C for 60 min. After incubation, the acetone-enzyme mixture was centrifuged at 10,000*g* for 10 min and the pellet was dissolved in minimum amount of acetate buffer (50 Mm, pH 5). The concentrated enzyme preparation was lyophilized (Martin Christ, Germany) and used subsequently in various experiments. Enzymatic activities of cellulase/xylanase were assessed at various steps of enzyme concentration and lyophilization. Enzyme preparation was examined for various properties. For determining the effect of temperature and pH on activity, cellulase/xylanase assay was executed at different temperatures (40–90 °C) or pH (5–11). Thermal and pH stability of cellulase/xylanase was analyzed by pre-incubating the enzyme preparation at different temperatures (40–90 °C) or pH (5–11) for a particular time period (90 min), and then assaying the residual activity [10]. To study the effect of metal ions/additives, viz. K^+, Fe^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and cetyltrimethyl ammonium bromide (CTAB) on the activity of cellulase/xylanase enzyme, either of the metal ion or additive was included in the enzyme assay mixture at 10 mM concentration, and activity assay was executed [22].

2.6 Standalone IL or alkali and combined pretreatment of SCT

Pretreatment of SCT biomass was carried out using standalone ionic liquid ([Emim]Cl) at different concentrations (5–40%, *w*/*w*) with biomass loading of 5% *w*/*w*, in acetate buffer (50 mM, pH 5.0) at 121 °C for 15 min. The regenerated

biomass was recovered by precipitating with distilled water (5 times) followed by washing, and separation of solids through vacuum filtration. The solid content was dried at 60 °C for 24 h, and the filtrate was examined for sugar content. The dried biomass was then used to analyze enzymatic saccharification.

Similarly, standalone ammonium carbonate was used at different concentrations (5–30%, *w*/*w*) for pretreatment of SCT biomass (5%, *w*/*w* loading) in acetate buffer (50 mM, pH 5.0). The pretreatment was carried out at 121 °C for 15 min. The pretreated reaction content was filtered, and the solid residue was washed with deionized water until neutrality, and dried at 60 °C for 24 h, and then used for enzymatic saccharification.

Combined pretreatment of SCT biomass included simultaneously pretreating the biomass with both IL (25%, *w*/*w*) and ammonium carbonate (30%, *w*/*w*), in acetate buffer (pH 5.0) at biomass loading of 5% (*w*/*w*) for 15 min at 121 °C. The regenerated biomass was neutralized, by the addition of antisolvent (water), and subjected to vacuum filtration. Solid residues were thoroughly washed, dried, and examined for enzymatic saccharification.

2.7 Design of experiments for optimization of pretreatment variables

Statistical optimization of process variables for the combinatorial pretreatment of SCT biomass with IL ([Emim]Cl) and alkali (ammonium carbonate) was executed by central composite design (CCD) of response surface methodology (RSM). Three process variables, namely biomass loading, temperature, and time period of pretreatment, were selected for optimization. The process variables were used at lower and higher values, i.e., biomass loading *A* (4–7%, *w*/*w*), temperature *B* (55–85 °C), and time *C* (3–5 h), and reducing sugar yield was considered response (*Y*). The values of process variables at five coded levels ($-\alpha, -1, 0, 1, +\alpha$) are presented in Table 1. A set of 20 experiments generated by Design expert 6.0 (Stat Ease, Inc., Minneapolis, MN, USA) was executed, and the results were analyzed. The data was evaluated by multiple regression analysis, and a polynomial equation was derived. Statistical analysis of the model was done for evaluation of analysis of variance (ANOVA). The analysis included the Fisher's *F*-test (overall model significance), its associated probability *p* (*F*), correlation coefficient (*R*), and determination coefficient (R^2).

The optimal levels of the variables predicted by point prediction tool of the software were used to execute the experiments for validation of the process model. The predicted and experimental values were examined, and the response was depicted by the term "*Y*," i.e., reducing sugar yield. The results were analyzed using design expert software. The regression equation resulted in an empirical model that relates the measured response to the independent variables of the experiments. The quadratic model was represented as 3D response surface plots based upon the regression equation, which was used to predict the correlation between the response vis-a-vis experimental level of each variable, and the interaction between the variables. The response surface plots provided insights of the interaction between the variables.

Table 1 Range of independent variables at five coded levels used for combined [Emim]Cl and ammonium carbonate pretreatment of SCT

Variables	Coding	Unit	Levels				
			α	1	0	1	$+\alpha$
Biomass loading	*A*	%, *w*/*w*	2.98	4.00	5.50	7.00	8.02
Temperature	*B*	°C	44.77	55.00	70.00	85.00	95.23
Time	*C*	h	2.32	3	4	5	5.68

2.8 SEM and FTIR analysis of SCT biomass

The structural alterations induced in the SCT biomass due to the combinatorial pretreatment were studied by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). SEM analysis was carried out with the help of a scanning electron microscope JSM-6390LV (JEOL, Japan) at an accelerating potential of 20 KV to observe surface morphology of the native and the pretreated SCT biomass. FTIR analysis was used to study the profound chemical variations and functional group moieties of the biomass. The untreated and pretreated (standalone IL or alkali, or combined) samples were scanned within a range of 4000 to 400 cm^{-1} using an Avatar 370 spectrometer (Thermo Nicolet Corporation, Madison, WI) with a resolution of 4 cm^{-1}. Both SEM and FTIR techniques were performed at Sophisticated Test and Instrumentation Centre (STIC), SAIF, Cochin University of Science and Technology (CUSAT), Cochin.

2.9 Enzymatic saccharification of pretreated SCT biomass

Enzymatic saccharification of the variously pretreated SCT biomass, i.e., standalone ([Emim]Cl), or ammonium carbonate, or combined ([Emim]Cl and ammonium carbonate), was carried out. Pretreated biomass was immersed in acetate buffer (pH 5.0) at 5% (*w*/*w*) solid loading and subjected to hydrolysis with *in-house* produced IL stable enzyme preparation (cellulase at 50 FPU/g biomass, and xylanase at 330 IU/g biomass loading). The reaction mixture was incubated at 50 °C under shaking (150 rpm), and samples withdrawn at different time intervals (24–72 h) were examined for reducing sugar content

by dinitrosalicylic acid (DNSA) method [21]. In brief, 100 µl of sugar hydrolysate was mixed with 900 µl acetate buffer (pH 5.0). To this mixture, 1 ml DNSA reagent solution was added, and incubated the contents at 95 °C for 15 min in a water bath. After the incubation, 1 ml sodium potassium tartrate (Rochelle salt) was added to the mixture, and the absorbance was measured at 575 nm for sugar estimation.

2.10 Ethanol fermentation

The sugar hydrolysate obtained after saccharification of the aptly pretreated SCT biomass was subjected to ethanol fermentation using dual yeast culture, viz. *Saccharomyces cerevisiae* and *Pichia stipitis*. The yeasts *Saccharomyces cerevisiae* and *Pichia stipitis* were used at an inoculum size of 1.5%, *w*/*v* each (1:1 ratio). The ethanol production was carried at 30 °C for 72 h under static conditions as described previously [6]. Samples were examined for ethanol content (24–72 h) by HPLC (Shimadzu, Japan) using refractive index detector (RID). The samples were processed with liquid mobile phase (5 mM H_2SO_4) at a flow rate of 0.6 ml/min for 30 min (run time). A Bio-Rad Aminex HPX-87 column was fitted in the instrument with a column temperature of 50 °C. The system was operated at isocratic mode. Ethanol yield and fermentation efficiency (theoretical and experimental) were calculated using Eqs. 1, 2, and 3 as described previously [23].

$$\text{Ethanol from hexoses/pentoses (ml/g)} = \frac{1.1 \times 0.51 \text{ ethanol per g glucose}}{\text{Specific volume of ethanol (0.789 g/ml)}} \quad (1)$$

$$\text{Ethanol (g)} = \text{Ethanol (ml)} \times 0.798 \text{ (specific gravity of ethanol)} \quad (2)$$

$$\text{Fermentation efficiency (\%)} = \frac{\text{Experimental yield}}{\text{Theoretical yield}} \times 100 \quad (3)$$

3 Results and discussion

3.1 Polysaccharide composition analysis of SCT biomass

The composition analysis of native SCT biomass revealed that it had 23.59% of cellulose and 18.58% of hemicellulose content. In terms of monosaccharides content, SCT biomass consisted of 26.22% glucose, 19.6% xylose, and 3.66% arabinose (*w*/*w*). Sherpa et al. [24] biochemically analyzed raw sugarcane tops biomass and reported cellulose content of 37.64% (w/w) and hemicellulose content of 25.62% (*w*/*w*). However, Raghavi et al. [16] reported cellulose and hemicellulose content of 27.85% and 19.41%, respectively, in sugarcane trash. Similarly, another study reported the cellulose and hemicellulose content of about 29.85% and 18.85%, respectively, in SCT biomass [25]. The polysachharide composition of SCT biomass differs due to variations in crop variety, climatic, cultivational and environmental conditions, usage of different types of fertilizers, soil composition, harvesting time, and other related agronomic factors [6].

3.2 IL stable cellulase and xylanase from IL-tolerant fungal isolate VS4

The fungal isolates from chemical-polluted eco-niches were examined for their IL tolerance by cultivating them on PDA supplemented with IL 1-ethyl-3-methylimidazolium chloride ([Emim]Cl). The isolates which grew well were further subjected to submerged fermentation in enzyme production medium to produce cellulase and xylanase enzymes. The enzyme preparation was examined for IL stability against [Emim]Cl (5%, *w*/*v*). Of all the screened isolates, fungal isolate VS4 was found to produce highly stable cellulase/xylanase enzymes. Cellulase showed considerable residual activity after 5 h (97.6%) and even after 24 h of IL exposure (96.9%). Similarly, xylanase showed maximum residual activity of approximately 96.6% after 4 h of incubation with IL; however, after 24 h of IL exposure, residual xylanase activity was reduced to 71.4% (Fig. 1a). The ILs are considered inhibitory for enzymes and proteins, and prolonged exposure of enzymes to IL may cause partial or complete activity loss [23, 26]. The high salinity of ILs deters the enzyme activity by interfering with the protein folding, i.e., altering native three-dimensional structural confirmations of enzymes. Moreover, ILs disrupt various hydrogen bonds and hydrophobic interactions and separate the proteins from surrounding hydration shell, thus leading to loss of structure and enzyme activity [6].

The fungal isolate VS4 was identified based on culture morphology and microscopic and ITS sequence analysis. The isolate showed a dark green confluent and velvety growth on PDA with white periphery. After a few days, the growth of isolate appeared yellowish which could be due to release of certain pigments. The reverse morphology of the isolate was pale to slightly creamish. Microscopic analysis of the fungal isolate VS4 revealed septate hyphae, brush-like conidiophores, oval-shaped spores, and chained structures, which indicated that isolate may belong to genus *Penicillium*. Furthermore, the ITS sequence examination of fungal isolate VS4 showed 100% homology with that of other *P. chrysogenum* strains available in National Centre for Biotechnology Information (NCBI database). Thus, the fungal isolate VS4 was identified and designated as *Penicillium chrysogenum* VS4 (Fig. 1b). The ITS sequence has been submitted to GenBank under the accession number *MN911387*.

Microbial cellulase/xylanase enzymes from diverse sources, e.g., fungi, bacteria metagenomic sources, have been

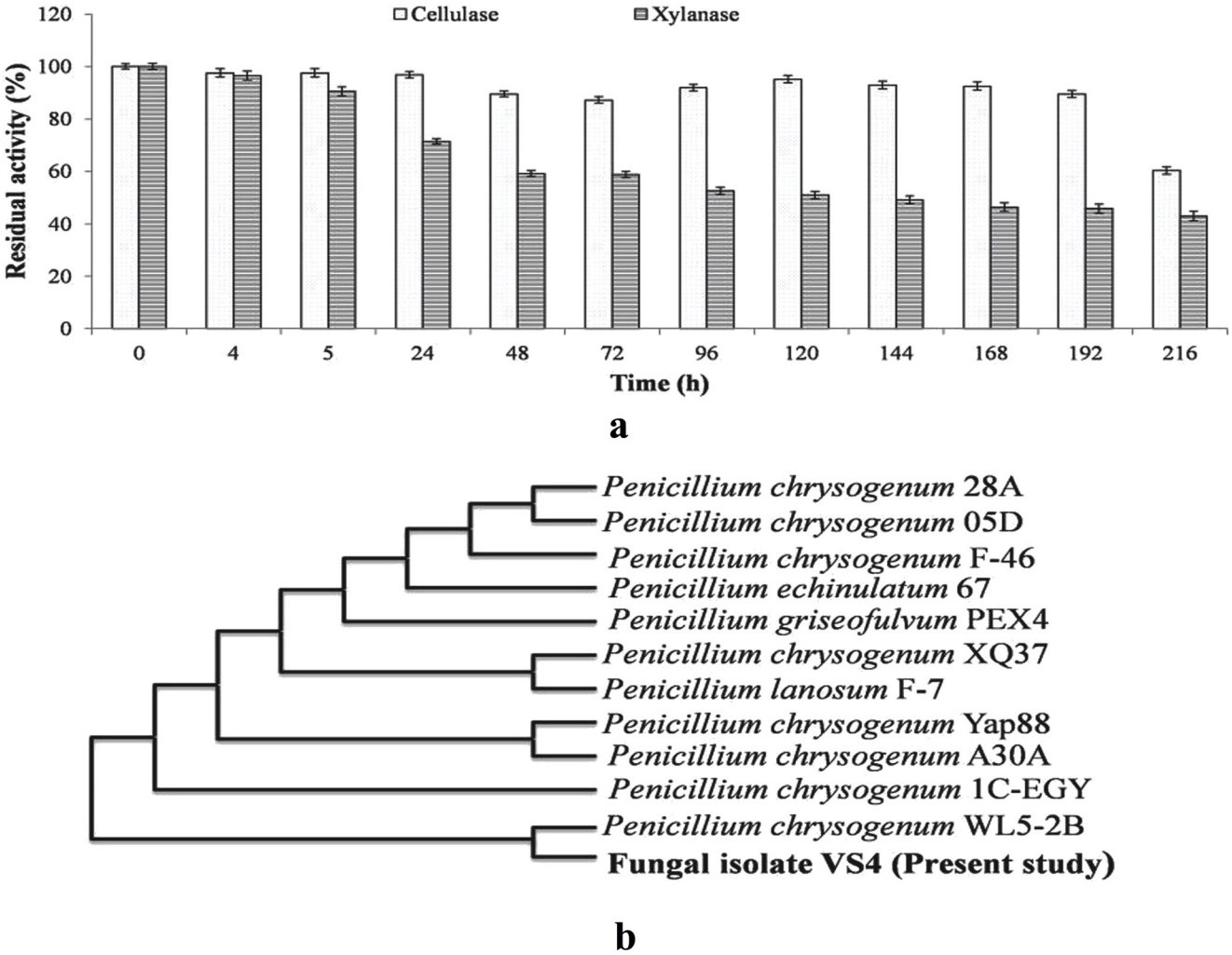

Fig. 1 Ionic liquid stability of fungal isolate VS4 cellulase and xylanase against 1-ethyl-3-methylimadizolium chloride ([Emim]Cl) (**a**). Phylogenetic homology analysis of fungal isolate VS4 based on ITS sequence (**b**)

reported to exhibit varying level of IL stability [6, 26]. Cellulase/xylanase enzymes from *Aspergillus assiutensis* VS34 [6] and *Aspergillus aculeatus* PN14 [23] isolates possessed adequate stability to several ionic liquids such as 1-butyl-3-methylimidazolium chloride ([Bmim]Cl), 1-ethyl-3-methylimidazolium chloride ([Emim]Cl), 1-ethyl-3-methylimidazolium methane sulfonate ([Emim][$MeSO_3$]), 1-ethyl-3-methylimidazolium dimethyl phosphate ([Emim][Dmp]), and 1-ethyl-methylimidazolium acetate ([Emim][CH_3COO]). Grewal et al. [27] studied the IL stability of free and immobilized cellulase from *Trichoderma reesei* to different concentrations of [Emim][Ac], and reported that IL stability of nanomatrix-immobilized cellulase was enhanced.

3.3 Some properties of cellulase/xylanase enzyme preparation

The crude enzyme preparation was concentrated through chilled acetone and enzymatic activities (cellulase and xylanase) were assessed. Acetone concentration of the crude enzyme preparation resulted in enhanced activities of both cellulase (17 IU/ml) and xylanase (73.1 IU/ml) by 2.23- and 1.73-fold, respectively. The concentrated enzyme preparation was then lyophilized, and the cellulase and xylanase activities were found to be 6.57 IU/ml and 55.72 IU/ml, respectively, per 10 mg of enzyme. The lyophilized enzyme preparation was used for further experiments. For various commercial applications in bio-based industries, it is highly desirable that microbial enzymes must be stable enough to withstand the relatively harsh process conditions such as elevated temperature, extremes of pH, and presence of general enzyme inhibitors. Cellulase and xylanase from *P. chrysogenum* VS4 exhibited activity over a broad temperature range (40–90 °C) with optima of 40 °C (14 IU/ml) and 50 °C (73.1 IU/ml), respectively. The thermostability analysis indicated that both cellulase and xylanase had adequate stability over a range of 40–90 °C and retained approximately 55–80% of the initial activity. High enzymatic stability at elevated temperature

could be attributed to various structural factors like increased molecular interactions, disulfide bonds, and cofactor binding, among others, which strengthen the enzyme structure and degree of stabilization [22]. Nargotra et al. [10] reported that *Penicillium oxalicum* PN8 produced a thermostable cellulase and xylanase which had substantial stability over 50 to 80 °C.

Cellulase and xylanase from *P. chrysogenum* VS4 exhibited maximal activity at pH 6 (16.2 IU/ml) and pH 7 (78.1 IU/ml), respectively. Also, both cellulase and xylanase enzymes displayed significant stability over a broad pH range (5–11) with maximum stability at pH 8 for cellulase (92.3% residual activity) and at pH 5 for xylanase (99.6% residual activity). The cellulase and xylanase enzymes retained more than 70% of the activity at pH 9 and 10, but activity loss was observed at pH 11. The altered enzyme activity at pH extremes could be accredited to changes in the ionic status of the enzymes, which results in alterations in the three-dimensional structural conformations, and therefore causing ineffective substrate binding and/or catalytic activity of the enzymes [10, 22]. Purified cellulase from *Aspergillus tubingensis* NKBP-55 exhibited optimum activity at pH 5.0 and retained more than 60% of the initial activity at pH 3.0 for 24 h [28].

Metal ions or other additives such surfactants, and chelators, may influence the activity of enzymes in multifaceted ways. Activity of cellulase from *P. chrysogenum* VS4 was significantly enhanced in the presence of Fe^{2+} (187.73%), and moderately increased in presence of Mn^{2+}, Co^{2+} (124.52%, 121.69%, respectively), however remained constant in the range of 70–95% in presence of all the metal ions/additives except Hg^{2+} (51.88%) which showed a significant inhibitory effect. Similarly, xylanase activity was slightly enhanced in presence of Fe^{2+} (107.01%) and Co^{2+} (105.11%), and remained constant in the range of 60–95% in presence of all metal ions. However, Hg^{2+} and Cu^{2+} had severe inhibitory effect on xylanase activity. Inhibition of enzymes in the presence of Hg^{2+} could be due to the presence of thiol groups of cysteine residues on their active sites or in their surroundings [29]. Metal ions interact with the amines or carboxylic acid group of the amino acids of enzymes, and therefore can have either stimulatory or inhibitory effect on the enzymatic activity [30]. Hamid et al. [31] reported the considerable rise in residual activity of β-xylanase enzyme by 55%, 26%, and 18% in the presence of Mg^{2+}, K^{+}, and Mn^{2+} metal ions.

3.4 Standalone ionic liquid or alkali pretreatment of SCT biomass

The pretreatment of SCT biomass with ionic liquid [Emim]Cl at different concentrations (5–40%, *w*/*w*) was followed by saccharification with IL stable enzyme preparation (cellulase/xylanase) obtained from an IL-tolerant fungus *P. chrysogenum* VS4. It was observed that with increased concentration of [Emim]Cl during pretreatment, an increased reducing sugar yield was obtained. Maximum reducing sugars were released (85.9 mg/g biomass) when biomass was pretreated at 25% (*w*/*w*) of [Emim]Cl. Reducing sugar released from untreated biomass (43.23 mg/g biomass) was considered control (Fig. 2a). The high [Emim]Cl concentration (25% *w*/*w*) probably exerts more severe and effective action on highly packed, compact, and crystalline biomass, and transforms it into more loosened and amorphous form, thus enhancing its accessibility to saccharification enzymes [10]. However, further increase in [Emim]Cl concentration resulted in a marginal decrease in the reducing sugar yield which might be due to the inhibitory effect of very high IL concentration on saccharifying enzymes despite their IL stability.

The ILs disrupt various hydrogen bonds and hydrophobic interactions, and interfere with neighboring hydration shell of the enzyme. This results in alterations in the structural conformation of three-dimensional native structure of the enzyme, and hence partial/complete loss of activity [6]. Also, high concentration of [Emim]Cl leads to an increase in the viscosity of the pretreatment reaction mixture, which may limit the proper mixing/dissolution of the biomass during pretreatment, and hence decrease the yield of total reducing sugars [23]. Therefore, a proper dose of IL is an absolutely essential requirement for efficient pretreatment of biomass to realize effectual saccharification. High reducing sugar yield from biomass ensures the overall biomass conversion efficiency and techno-economic feasibility of the process.

Recently, IL 1-ethyl-3-methylimidazolium acetate (2%, *w*/*v*) was used for pretreatment of long fiber cellulose, spruce sawdust, and oak sawdust at 45 °C to get a high yield of complex sugars [8]. Similarly, pretreatment of municipal solid waste with IL 1-ethyl-3-methylimidazolium chloride resulted in high yield of glucose (58%) and xylose (47%) at 160 °C and 120 °C, respectively [32]. IL 2-hydroxylethylammonium acetate–mediated pretreatment of cashew apple bagasse feedstock resulted in substantial lignin removal (95.8%) and increased glucose yield [33]. Thus, the results endorse that IL-based pretreatment is an effective and promising approach for successful biorefining of agricultural residues or other biomass resources to produce biofuels/chemicals.

Pretreatment of SCT biomass was also carried out by using ammonium carbonate (alkali) at varying concentrations (5–30%, *w*/*w*) followed by its enzymatic hydrolysis with *in-house* produced *P. chrysogenum* VS4 cellulase/xylanase enzymes. It was observed that a maximum reducing sugar yield of 102.6 mg/g biomass was obtained when SCT biomass was pretreated at 30% (*w*/*w*) concentration of ammonium carbonate (Fig. 2b). Alkali pretreatment effectively reduces the biomass recalcitrance by disrupting the compact biomass into fibers, removing lignin and hemicelluloses, thus increasing its surface accessibility to saccharification enzymes, and resulting in higher sugar yield [34]. Alkali-based pretreatment of biomass has been explored by different researchers [9, 10, 15].

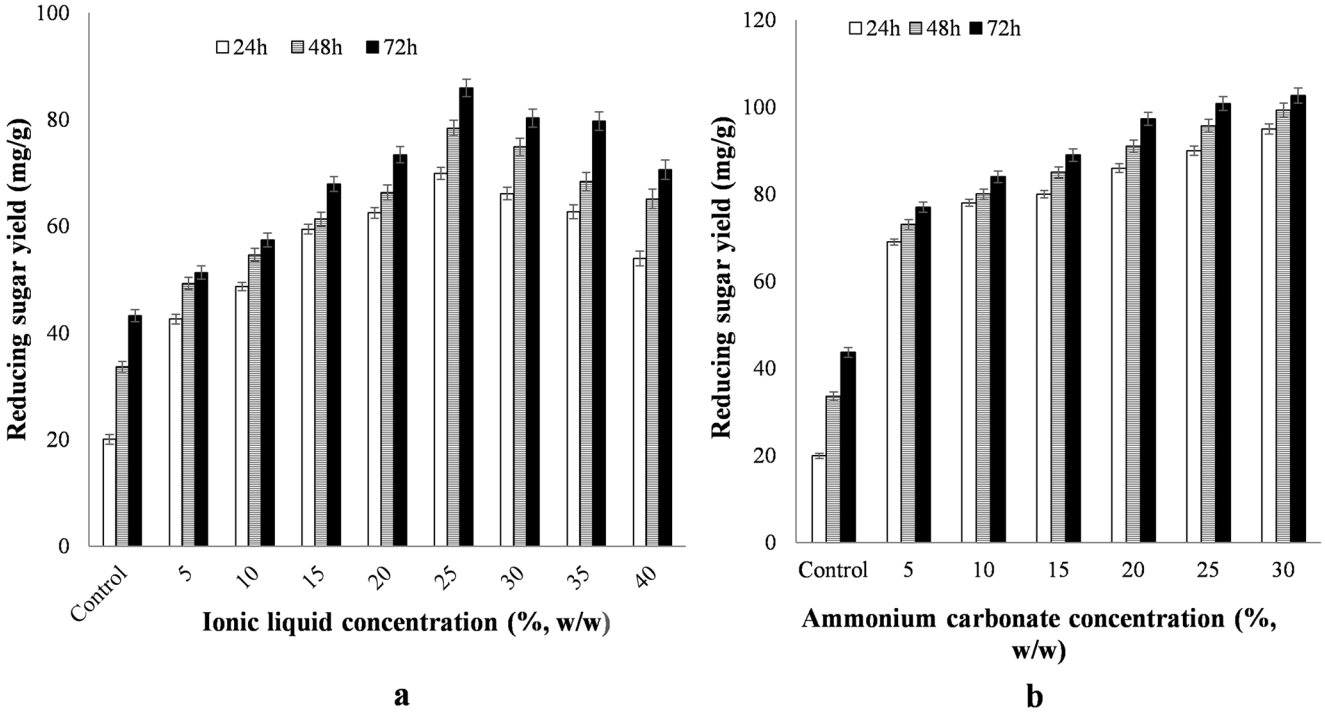

Fig. 2 Pretreatment of sugarcane tops biomass at varying concentrations of ionic liquid ([Emim]Cl) (**a**). Pretreatment of SCT with different concentration of ammonium carbonate (**b**)

A maximum sugar yield of 7.59 mg/g biomass was obtained after the pretreatment of pine needle biomass with 20% (*w*/*v*) ammonium carbonate [9]. Similarly, NaOH (0.5%, *w*/*v*) pretreatment of sunflower stalk biomass resulted in 97.38 mg/g biomass sugar yield upon enzymatic saccharification [10]. Due to partial removal of lignin and/or hemicellulose from biomass by alkali pretreatment, the structure of lignocellulosic matrix gets altered and becomes more prone to enzymatic hydrolysis. Alkali pretreatment though has been used quite extensively for biomass processing but only as a subsidiary technique. As standalone alkali pretreatment is not very effective, therefore, it is used along with some other major pretreatment approaches [10].

3.5 Combined pretreatment of SCT biomass with IL ([Emim]Cl) and ammonium carbonate

The combinatorial pretreatment of SCT biomass using [Emim]Cl (25%, *w*/*w*) and ammonium carbonate (30%, *w*/*w*) was executed, followed by enzymatic saccharification of solid residues. It was observed that combined ammonium carbonate and [Emim]Cl pretreatment resulted in significantly increased reducing sugar yield (172.34 mg/g biomass) as compared to standalone IL (85.9 mg/g biomass) or alkali (102.6 mg/g biomass) pretreatment. The enzymatic saccharification of untreated SCT biomass was used as control (43.23 mg/g biomass). The combined pretreatment enhanced the reducing sugar yield from SCT biomass by 100.62% (*w*/*w*) and by 67.97% (*w*/*w*), as compared to standalone [Emim]Cl and ammonium carbonate pretreatment, respectively. Thus, combinatorial pretreatment is more efficacious than either of the standalone pretreatments. Similar results have been reported previously [10] wherein combined IL ([Bmim]Cl) and alkali (NaOH) pretreatment of sunflower stalks was observed to increase the sugar yield by 51.29% (*w*/*w*) and 40.41% (*w*/*w*) as compared to that by standalone IL and alkali pretreatment, respectively.

Pretreatment of corn straw with IL (1-ethyl-3-methylimidazolium acetate) and NaOH [12] resulted in increased content of cellulose (85.69%) and hemicellulose (9.1%), and decreased lignin content (2.27%). The lignin removal efficiency up to 87.4% was achieved. Another study has shown that combined pretreatment approach of NaOH and IL reduces the crystallinity of the cellulose [11], therefore increasing the total sugar significantly (approximately 80%) as compared to standalone NaOH pretreatment. Therefore, combining alkali with IL-based pretreatment approach is not only imperative for enhancing the reducing sugar yield but involve less usage of IL, thus economizing the overall bioconversion of biomass process.

3.6 Optimization of process variables for combined IL and alkali pretreatment

Central composite design (CCD) of response surface methodology (RSM) was applied to deduce the optimum level of variables, i.e., biomass loading (*A*), temperature (*B*), and time period of pretreatment (*C*) for combined ([Emim]Cl and

ammonium carbonate) pretreatment of SCT biomass to get maximum possible response (sugar yield). Furthermore, the effect of individual variables and their interactive influence on the response (reducing sugar yield) was examined. A set of 20 experiments designed by the software, having varied level of variables, were performed, and the response (reducing sugar yield, mg/g biomass) was fed into the response column of the design (Table 2). A varied response (reducing sugar yield) in different experimental runs implied that the level of process variables is very important which influences the response differentially. Analysis of variance (ANOVA) was used to evaluate the fitness of the model. The model F value of 237.91 implied that the model was significant (Table 3). There was only 0.01% probability that this large model F value could be due to noise. Moreover, the lack of fit F value of 3.02 indicated its insignificance relative to the pure error, and the insignificant lack of fit value implied the strength of the model. Values of Prob < 0.05 indicated that model terms were significant. In this case, the model expressions A, B, and C were significant in linear terms, A^2 and C^2 were significant in squared terms, and the expression AB was significant in interactive terms.

Table 2 Experimental and predicted response for reducing sugar yield from combined [Emim]Cl and ammonium carbonate pretreatment of SCT based on response surface methodology

Runs	Experimental variables*			Reducing sugar yield (mg/g biomass)	
	A	*B*	*C*	Experimental	Predicted
1	5.50	70.00	4.00	170.7	175.72
2	5.50	44.77	4.00	130.3	129.93
3	4.00	55.00	3.00	79.3	79.12
4	4.00	55.00	5.00	126.2	124.70
5	5.50	70.00	5.68	170	167.80
6	7.00	55.00	3.00	85.4	86.98
7	5.50	70.00	4.00	175	175.72
8	7.00	85.00	3.00	124.9	129.33
9	4.00	85.00	5.00	175.4	176.76
10	5.50	70.00	2.32	112.1	110.15
11	8.02	70.00	4.00	78.6	72.74
12	4.00	85.00	3.00	142.7	142.53
13	7.00	85.00	5.00	149.2	152.31
14	5.50	70.00	4.00	178.3	175.72
15	5.50	70.00	4.00	176.5	175.72
16	2.98	70.00	4.00	85	86.70
17	5.50	70.00	4.00	177.2	175.72
18	5.50	95.23	4.00	213.1	209.32
19	7.00	55.00	5.00	118.2	121.31
20	5.50	70.00	4.00	175.9	175.72

*A, biomass loading (%, w/w); B, temperature (°C); C, time (h)

The coefficient of determination (R^2) was 0.9954, which indicated that the model could explain 99.54% variability of the response. The predicted R^2 of 0.9717 was in good agreement with the adjusted R^2 of 0.9912. The adjusted coefficient of determination ($R^2 = 99.12\%$) was found to be acceptable, and therefore established the significance of the model. Adequate precision estimates the signal to noise ratio, and its value of 51.41 indicates a suitable signal for the model. This model can also be used to navigate the design space. The relationship between the response and the independent variables could be explained by the polynomial regression Eq. (4) which is described as follows:

$$Y\ (\text{reducing sugar yield}) = +175.72 - 4.15A + 23.60B + 17.14C - 33.94A^2 - 2.16B^2 - 12.99C^2 - 5.26AB - 2.81AC - 2.84BC \quad (4)$$

where the coded terms A, B, and C represent biomass loading, temperature, and time, respectively.

Three-dimensional response surface plots were used to study the interaction of the significant variables and their combined effect on the reducing sugar yield. It was observed that the interactive influence of AB, i.e., biomass loading and temperature, on reducing sugar yield was negatively significant. It was obvious from the plot that the interaction between biomass loading (A) and temperature (B) had an inverse relationship with the response, i.e., reducing sugar yield (Fig. 3a). Moreover, individual biomass loading (A) was negatively significant, indicating that with increase in biomass loading up to a certain level, the reducing sugar yield increased but thereafter, increase in biomass loading resulted in decreased reducing sugar yield. On the contrary, increase in reducing sugar yield on increasing pretreatment temperature was suggestive of the positive significance of variable B (temperature). The perturbation plot depicts the effect of single variable on deviation from reference points when all other variables are kept constant. It was observed that temperature (B) had maximum positive influence on the reducing sugar yield on deviating from the reference point, whereas biomass loading (A) had the least effect (Fig. 3b).

Validation of the statistical model was performed by using the point prediction tool of RSM to identify the optimum values of all the three process variables. The process variables, i.e., biomass loading (A) 5.33 w/w, temperature (B) 100 °C, and time (C) 4.50 h, were predicted using the point prediction tool of the software, and the experiments were executed. The strength of the model was represented by the close proximity between the experimental (215.89 mg/g biomass) and the predicted reducing sugar yield (218.17 mg/g biomass). The DoE-based optimization resulted in a 25.27% augmentation in reducing sugar yield as compared to that obtained under unoptimized conditions.

Optimization of biomass pretreatment process parameters is an efficient approach that may increase the reducing sugar

Table 3 Analysis of variance (ANOVA) for reducing sugar yield after [EMIM][Cl] and ammonium carbonate pretreatment of SCT based on response surface methodology

Source	Sum of squares	Degree of freedom	Mean square	*F* value	Prob > *F*	
Model	30216.86	9	3357.43	237.91	< 0.0001	Significant
A	235.10	1	235.10	16.66	0.0022	Significant
B	7608.71	1	7608.71	539.15	< 0.0001	Significant
C	4012.01	1	4012.01	284.29	< 0.0001	Significant
A^2	16600.60	1	16600.60	1176.32	< 0.0001	Significant
B^2	66.95	1	66.95	4.74	0.0544	
C^2	2432.46	1	2432.46	172.36	< 0.0001	Significant
AB	221.55	1	221.55	15.70	0.0027	Significant
AC	63.28	1	63.28	4.48	0.0603	
BC	64.41	1	64.41	4.56	0.0584	
Residual	141.12	10	14.11			
Lack of fit	106.00	5	21.20	3.02	0.1253	Not significant
Pure error	35.12	5	7.02			
Cor total	30357.98	19				

*A, biomass loading (%, *w*/*w*); *B*, temperature (°C); *C*, time (h)

yield significantly, and therefore may be imperative for developing successful process for biorefining of biomass. An approximate 2.1-fold increase in reducing sugar yield (325 mg/g of biomass) was obtained after RSM-based optimization of ultrasound pretreatment of brewer's spent grain [35]. Similarly, optimization of process parameters for surfactant-assisted IL pretreatment of *Parthenium hysterophorus* biomass under consolidated bioprocessing (CBP) substantially increased the reducing sugar yield [23]. A combinatorial pretreatment regime involving IL [Bmim]Cl and polyethylene glycol (PEG-8000) was DoE optimized for sugarcane bagasse to attain 16.5% increase in the reducing sugar yield [6]. Also, DoE-based optimization of pretreatment parameters was reported to enhance the reducing sugar yield from pine needle biomass [9] and mixed softwood [7]. Thus, optimization of process parameters is a powerful tool to enhance overall efficacy and economic viability of the process.

3.7 SEM and FTIR analysis of raw and pretreated biomass

SEM is a powerful analytical technique to evaluate the impact of various pretreatments on the ultrastructure of lignocellulosic biomass. SEM images of untreated and pretreated SCT

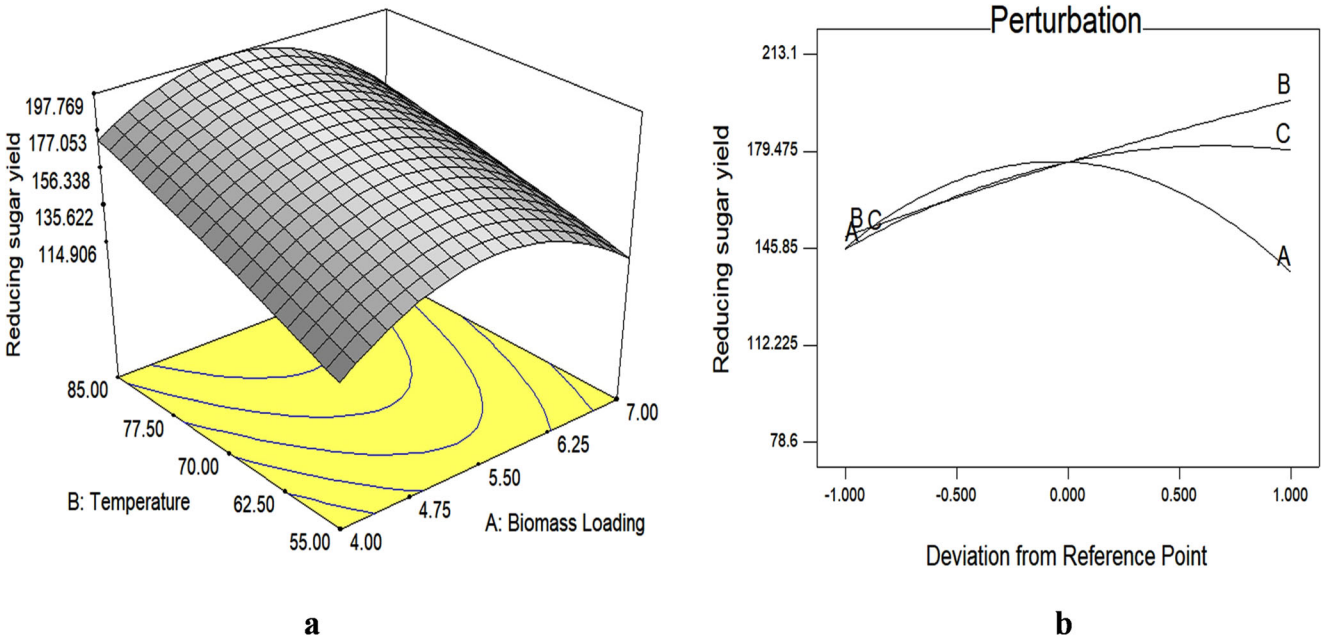

Fig. 3 Response surface plots showing the interactive effect of biomass loading and temperature on reducing sugar yield due to combined [Emim]Cl and ammonium carbonate pretreatment of sugarcane tops biomass (**a**) and perturbation plot (**b**)

biomass were taken at a magnification of 3000× (Fig. 4a and b). The images highlight the considerable irregularities in the structure of SCT biomass due to application of combined ionic liquid and alkali pretreatment. The analysis of native biomass revealed a compact, continuous, highly ordered, and smooth morphology of integrated lignocellulosic fibrils (Fig. 4a), whereas [Emim]Cl and ammonium carbonate pretreated SCT biomass showed a huge distortion in the structural rigidity as indicated by the development of holes, cracks, and grooves on the surface of biomass. Moreover, the combined pretreatment increased the pore volume and accessible surface area probably by removing the lignin and hemicelluloses network effectively (Fig. 4b).

Similar to the current study, enormous structural distortions were observed in bamboo biomass in response to alkali and [Bmim]Cl pretreatment [11]. According to a recent study by Sharma et al. [6], the surfactant (PEG-8000)-assisted IL ([Bmim]Cl) pretreatment of SCB biomass resulted in large structural aberrations and irregularities that led to an expanded surface area, reduction in the compactness of biomass framework, and increased accessibility to saccharification enzymes. SEM images depict the alterations in surface properties of biomass which indicate the severance and efficacy of a particular pretreatment approach. Thus, SEM analysis of pretreated biomass provides an understanding of the rigorousness of a particular pretreatment for a specific biomass type.

FTIR spectroscopy analysis of the untreated and pretreated SCT biomass was executed to gain the molecular insights into the structural and chemical alterations of the lignocellulosic biomass due to pretreatment (Fig. 5a and b). The transmittance peaks appearing at 2922 cm^{-1} and 2855 cm^{-1} in the spectra of both untreated (Fig. 5a) and pretreated samples (Fig. 5b) are attributed to the peaks of lignocellulosic biomass as they correspond to C–H stretching (methyl and methylene groups) indicating the aliphatic moieties in cellulose and lignin [23].

FTIR spectra revealed that the peaks appearing at 3417–3554 cm^{-1} of pretreated SCT biomass were broader and stronger as compared to that of untreated sample (3424 cm^{-1}) which signifies O–H stretching and C–H stretching in the polysaccharides of the biomass [23]. A strong peak at 1632 cm^{-1} in untreated sample becomes more narrower in pretreated samples and shifted to 1631 cm^{-1} which signifies the carbonyl stretching. The absorption peaks from 1600 to 1450 cm^{-1} indicate the aromatic skeletal vibrations in the lignin which were weakened in the pretreated samples indicating the disruption of chemical bonds and removal of lignin [11]. Absorption bands at 1251 cm^{-1} and 1159 cm^{-1} in the pretreated samples signify acetylated hemicelluloses and glycosidic linkages. Two most prominent changes observed in FTIR spectrum of combinatorially pretreated ([Emim]Cl and ammonium carbonate) SCT biomass were (1) the absence of peaks corresponding to lignin (1600–1450 cm^{-1}) and (2) shift in the band intensities of cellulose peaks (3417–3554 cm^{-1}). The results endorse that the combined pretreatment had a synergetic effect on the digestibility of SCT biomass, which reduced its recalcitrance and increased vulnerability to enzymatic hydrolysis.

3.8 Ethanol fermentation

The ethanol content was examined by HPLC after the fermentation of sugar hydrolysate derived through the enzymatic hydrolysis of combinatorially pretreated ([Emim]Cl and ammonium carbonate) SCT biomass. Maximum ethanol yield

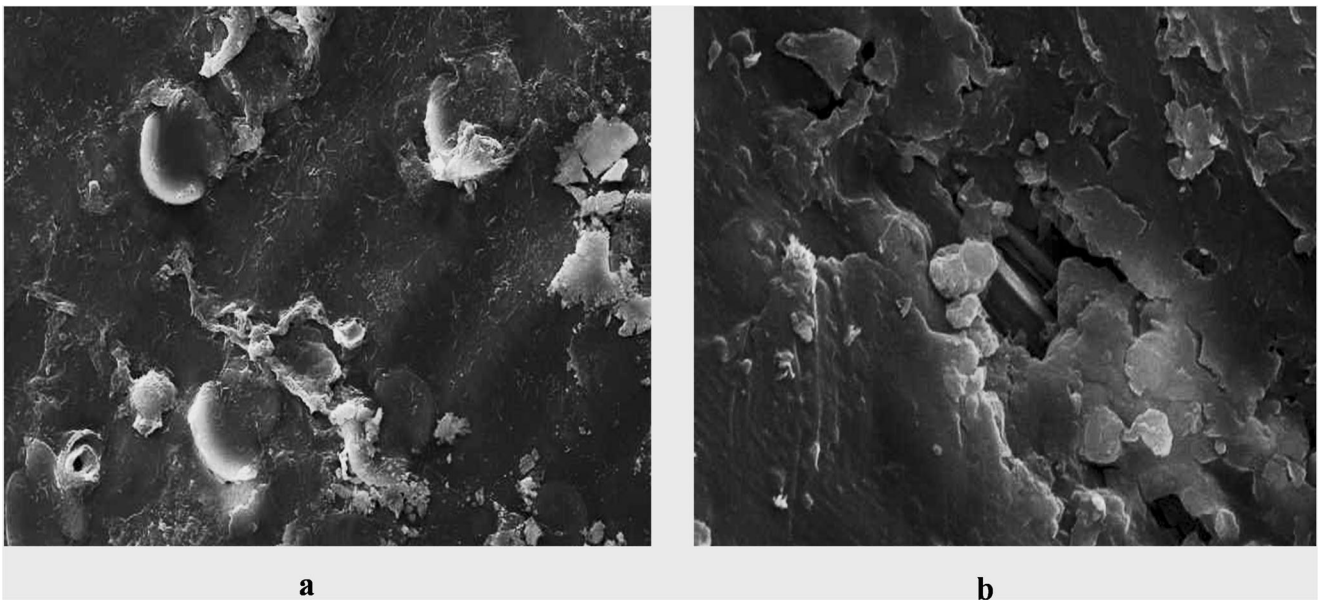

Fig. 4 Scanning electron microscopic analysis of sugarcane tops biomass. Untreated control (**a**). Combined [Emim]Cl and ammonium carbonate pretreated biomass (**b**)

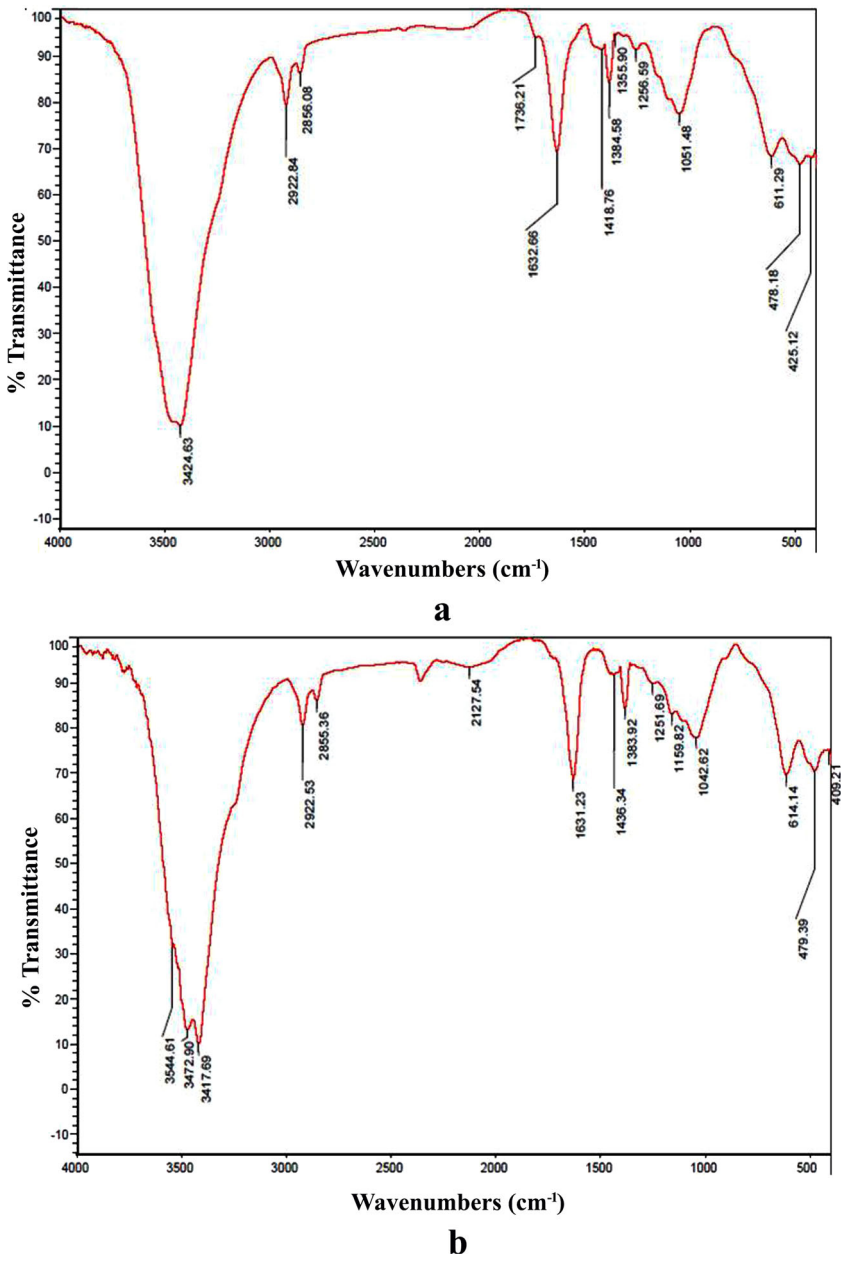

Fig. 5 Fourier transform infrared spectroscopic analysis of sugarcane tops biomass. Untreated control (**a**). Combined [Emim]Cl and ammonium carbonate pretreated biomass (**b**)

(102 mg/g biomass) was observed after 72 h of fermentation that corresponded to 42.85% of the theoretical yield. Ethanol production from a variety of lignocellulosics has been reported [6, 23]. Fermentation of sugar hydrolysate obtained through waste glycerol–assisted transition metal and alkali pretreatment of sugarcane trash resulted in an ethanol production of 31.928 g with a fermentation efficiency of 78.89% [16]. Amoah et al. [18] reported a high ethanol yield of 84.0% of the total fermentable sugars by the co-fermentation of xylose and glucose obtained from sugarcane bagasse after 1-butyl-3-methylpyridinium chloride pretreatment. According to Nargotra et al. [23], an ethanol yield of 81.5 mg/g biomass was achieved through the fermentation of sugar hydrolysate recovered from Tween-20 assisted [Emim][$MeSO_3$] pretreated *Parthenium hysterophorus* biomass.

4 Conclusion

The current study demonstrated that the combinatorial [Emim]Cl and ammonium carbonate pretreatment of SCT biomass is a proficient strategy for improving saccharification efficiency of the biomass. DoE-based optimization of process parameters increased the reducing sugar yield by 25.27% (215.89 mg/g biomass) as compared to that in unoptimized process (172.34 mg/g biomass). The physicochemical

analysis of SCT biomass by SEM and FTIR indicated that combined pretreatment induces substantial structural aberrations in the biomass which might enhance its accessibility to saccharification enzymes, thus resulting in increased sugar yield. However, there is a need for elucidating the precise mechanisms which induces structural deformities in the biomass which in turn enhances enzymatic hydrolysis. Molecular basis of IL stability of *Penicillium chrysogenum* VS4 and its enzyme complement must be deciphered completely to harness its biotechnological potential for biomass conversion.

Acknowledgments BKB acknowledges the following: Institute of Advanced Study, Durham University, UK, for providing COFUND International Senior Research Fellowship for research stay at Department of Biosciences, Durham University, Durham, UK., Commonwealth Scholarship Commission, UK, for providing Commonwealth Fellowship for research stay at Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Aberystwyth, UK, and Indo-US Science and Technology Forum (IUSSTF) for research stay at Ohio State University, USA. Authors thank the Director, School of Biotechnology, University of Jammu, Jammu for necessary laboratory facilities, and SAIF, STIC, Cochin University of Science and Technology, Kerala, India, for physicochemical analysis of samples.

Authors' contributions Dr. Bijender Kumar Bajaj (BKB) hypothesized and designed the research problem; Mr. Vishal Sharma (VS) and Ms. Parushi Nargotra (PN) designed and performed the experiments; PN, VS, and BKB analyzed and interpreted the data; Ms. Surbhi Sharma wrote the draft manuscript; BKB, PN, and VS corrected the MS; BKB submitted the MS. All authors read and approved the final manuscript.

Funding Financial support in the form of Research Projects to Dr. Bijender Kumar Bajaj (BKB) from funding agencies such as Department of Science and Technology (DST), University Grants commission (UGC), and Council of Scientific and Industrial Research (CSIR) is gratefully acknowledged. Mr. Vishal Sharma thankfully acknowledges Department of Science and Technology (Govt. of India) for providing Inspire fellowship for doctoral research. Ms. Parushi Nargotra acknowledges Rashtriya Uchchattar Shiksha Abhiyan (RUSA) for providing Ph.D. research fellowship. Ms. Surbhi Sharma is thankful to University of Jammu for providing University research scholarship.

Data availability All datasets used and analyzed in the current study are available from the corresponding author.

Compliance with ethical standards

Ethics approval and consent to participate Not applicable.

Consent for publication All authors agree to publish this manuscript.

Competing interests The authors declare that they have no competing interests.

References

1. Xie W, Ren Y, Jiang F, Liang J, Du SK (2020) Pretreatment of quinoa straw with 1-butyl-3-methylimidazolium chloride and physiochemical characterization of biomass. Renew Energy 146: 1364–1371. https://doi.org/10.1016/j.renene.2019.07.072
2. Nargotra P, Sharma V, Sharma S, Kapoor N, Bajaj BK (2020) Development of consolidated bioprocess for biofuel-ethanol production from ultrasound-assisted deep eutectic solvent pretreated *Parthenium hysterophorus* biomass. Biomass Convers Biorefin 1-16. https://doi.org/10.1007/s13399-020-01017-0
3. Saini JK, Gupta R, Verma A, Gaur P, Saini R, Shukla R, Kuhad RC (2019) Integrated lignocellulosic biorefinery for sustainable bio-based economy. In: Sustainable approaches for biofuels production technologies. Springer, Cham, pp 25–46. https://doi.org/10.1007/978-3-319-94797-6_2
4. Olugbade TO, Ojo OT (2020) Biomass torrefaction for the production of high-grade solid biofuels: a review. Bioenergy Res 13:1–17. https://doi.org/10.1007/s12155-020-10138-3
5. Carrillo-Nieves D, Alanis MJR, de la Cruz QR, Ruiz HA, Iqbal HM, Parra-Saldivar R (2019) Current status and future trends of bioethanol production from agro-industrial wastes in Mexico. Renew Sustain Energy Rev 102:63–74. https://doi.org/10.1016/j.rser.2018.11.031
6. Sharma V, Nargotra P, Bajaj BK (2019) Ultrasound and surfactant assisted ionic liquid pretreatment of sugarcane bagasse for enhancing saccharification using enzymes from an ionic liquid tolerant *Aspergillus assiutensis* VS34. Bioresour Technol 285:121319. https://doi.org/10.1016/j.biortech.2019.121319
7. Trinh LTP, Lee YJ, Lee JW, Lee WH (2018) Optimization of ionic liquid pretreatment of mixed softwood by response surface methodology and reutilization of ionic liquid from hydrolysate. Biotechnol Bioprocess Eng 23:228–237. https://doi.org/10.1007/s12257-017-0209-x
8. Alayoubi R, Mehmood N, Husson E, Kouzayha A, Tabcheh M, Chaveriat L, Gosselin I (2020) Low temperature ionic liquid pretreatment of lignocellulosic biomass to enhance bioethanol yield. Renew Energy 145:1808–1816. https://doi.org/10.1016/j.renene.2019.07.091
9. Vaid S, Bhat N, Nargotra P, Bajaj BK (2018) Combinatorial application of ammonium carbonate and sulphuric acid pretreatment to achieve enhanced sugar yield from pine needle biomass for potential biofuel–ethanol production. Energy Ecol Environ 3:126–135. https://doi.org/10.1007/s40974-018-0083-1
10. Nargotra P, Sharma V, Gupta M, Kour S, Bajaj BK (2018) Application of ionic liquid and alkali pretreatment for enhancing saccharification of sunflower stalk biomass for potential biofuel-ethanol production. Bioresour Technol 267:560–568. https://doi.org/10.1016/j.biortech.2018.07.070
11. Kassaye S, Pant KK, Jain S (2017) Hydrolysis of cellulosic bamboo biomass into reducing sugars via a combined alkaline solution and ionic liquid pretreatment steps. Renew Energy 104:177–184. https://doi.org/10.1016/j.renene.2016.12.033
12. Liu Z, Li L, Liu C, Xu A (2018) Pretreatment of corn straw using the alkaline solution of ionic liquids. Bioresour Technol 260:417–420. https://doi.org/10.1016/j.biortech.2018.03.117
13. Kim JS, Lee YY, Kim TH (2016) A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. Bioresour Technol 199:42–48. https://doi.org/10.1016/j.biortech.2015.08.085
14. Zhao C, Shao Q, Chundawat SP (2019) Recent advances on ammonia-based pretreatments of lignocellulosic biomass. Bioresour Technol 298:122446. https://doi.org/10.1016/j.biortech.2019.122446
15. Niju S, Nishanthini T, Balajii M (2020) Alkaline hydrogen peroxide-pretreated sugarcane tops for bioethanol production-a process optimization study. Biomass. Convers Bior 10:149–165. https://doi.org/10.1007/s13399-019-00524-z
16. Raghavi S, Sindhu R, Binod P, Gnansounou E, Pandey A (2016) Development of a novel sequential pretreatment strategy for the

production of bioethanol from sugarcane trash. Bioresour Technol 199:202–210. https://doi.org/10.1016/j.biortech.2015.08.062
17. Pathak P, Gupta A, Bhardwaj NK, Goyal A, Moholkar VS (2020) Impact of mild and harsh conditions of formic acid-based organosolv pretreatment on biomass fractionation of sugarcane tops. Biomass Convers Biorefin 1-14. https://doi.org/10.1007/s13399-020-00629-w
18. Amoah J, Ogura K, Schmetz Q, Kondo A, Ogino C (2019) Co-fermentation of xylose and glucose from ionic liquid pretreated sugar cane bagasse for bioethanol production using engineered xylose assimilating yeast. Biomass Bioenergy 128:105283. https://doi.org/10.1016/j.biombioe.2019.105283
19. Sindhu R, Kuttiraja M, Preeti VE, Vani S, Sukumaran RK, Binod P (2013) A novel surfactant-assisted ultrasound pretreatment of sugarcane tops for improved enzymatic release of sugars. Bioresour Technol 135:67–72. https://doi.org/10.1016/j.biortech.2012.09.050
20. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. Technical Report-NREL/TP-510–42618 National Renewable Energy Laboratory, Golden
21. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
22. Sharma S, Sharma V, Nargotra P, Bajaj BK (2018) Process desired functional attributes of an endoxylanase of GH10 family from a new strain of *Aspergillus terreus* S9. Int J Biol Macromol 115:663–671. https://doi.org/10.1016/j.ijbiomac.2018.04.096
23. Nargotra P, Sharma V, Bajaj BK (2019) Consolidated bioprocessing of surfactant-assisted ionic liquid-pretreated *Parthenium hysterophorus* L. biomass for bioethanol production. Bioresour Technol 289:121611. https://doi.org/10.1016/j.biortech.2019.121611
24. Sherpa KC, Ghangrekar MM, Banerjee R (2018) A green and sustainable approach on statistical optimization of laccase mediated delignification of sugarcane tops for enhanced saccharification. J Environ Manage 217:700–709. https://doi.org/10.1016/j.jenvman.2018.04.008
25. Niju S, Swathika M, Balajii M (2020) Pretreatment of lignocellulosic sugarcane leaves and tops for bioethanol production. In: Lignocellulosic biomass to liquid biofuels 301–324. Academic Press. https://doi.org/10.1016/B978-0-12-815936-1.00010-1
26. Vaid S, Bajaj BK (2017) Production of ionic liquid tolerant cellulase from *Bacillus subtilis* G2 using agroindustrial residues with application potential for saccharification of biomass under one pot consolidated bioprocess. Waste Biomass Valoriz 8:949–964. https://doi.org/10.1007/s12649-016-9626-x
27. Grewal J, Ahmad R, Khare SK (2017) Development of cellulase-nanoconjugates with enhanced ionic liquid and thermal stability for in situ lignocellulose saccharification. Bioresour Technol 242:236–243. https://doi.org/10.1016/j.biortech.2017.04.007
28. Prajapati BP, Suryawanshi RK, Agrawal S, Ghosh M, Kango N (2018) Characterization of cellulase from *Aspergillus tubingensis* NKBP-55 for generation of fermentable sugars from agricultural residues. Bioresour Technol 250:733–740. https://doi.org/10.1016/j.biortech.2017.11.099
29. Yegin S (2017) Single-step purification and characterization of an extreme halophilic, ethanol tolerant and acidophilic xylanase from *Aureobasidium pullulans* NRRL Y-2311-1 with application potential in the food industry. Food Chem 221:67–75. https://doi.org/10.1016/j.foodchem.2016.10.003
30. de Cassia PJ, Giese EC, de Souza Moretti MM, dos Santos Gomes AC, Perrone OM, Boscolo M, Martins DAB (2017) Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities. Enzyme Inhibit Activat:139–164
31. Hamid A, Aftab MN (2019) Cloning, purification, and characterization of recombinant thermostable β-xylanase Tnap_0700 from *Thermotoga naphthophila*. Appl Biochem Biotechnol 189(4):1274–1290. https://doi.org/10.1007/s12010-019-03068-0
32. Liang L, Li C, Xu F, He Q, Yan J, Luong T, Simmons BA, Pray TR, Singh S, Thompson VS, Sun N (2017) Conversion of cellulose rich municipal solid waste blends using ionic liquids: feedstock convertibility and process scale-up. RSC Adv 7:36585–36593 https://doi.org/10.1039/C7RA06701A
33. Reis CLB, e Silva LMA, Rodrigues THS, Felix AKN, de Santiago-Aguiar RS, Canuto KM, Rocha MVP (2017) Pretreatment of cashew apple bagasse using protic ionic liquids: enhanced enzymatic hydrolysis. Bioresour Technol 224:694–701. https://doi.org/10.1016/j.biortech.2016.11.019
34. Sun S, Sun S, Cao X, Sun R (2016) The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. Bioresour Technol 199:49–58. https://doi.org/10.1016/j.biortech.2015.08.061
35. Hassan SS, Ravindran R, Jaiswal S, Tiwari BK, Williams GA, Jaiswal AK (2020) An evaluation of sonication pretreatment for enhancing saccharification of brewers' spent grain. Waste Manage 105:240–247. https://doi.org/10.1016/j.wasman.2020.02.012