Biomass Conversion and Biorefinery, Volume 14, April 2024, pp. 8483-8492
DOI:10.1007/s13399-022-02895-2

Comparative assessment of sugarcane bagacillo and bagasse at lab-scale for production of sugars and green chemicals via biochemical platform

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Abstract

The sugarcane-driven industry can exemplify sustainable waste management by valorizing its lignocellulosic streams and boosting the rural economy by product diversification. In this aspect, bagacillo is a promising yet untapped carbonaceous feedstock, representing fine fraction of sugarcane bagasse (SCB) with low bulk density. It is used either as a filter-aid for juice clarification, when added to mud or mixed with bagasse for cogeneration. This study explores bagacillo for the production of sugars and green chemicals using biochemical platform, with SCB as the benchmark. Its NaOH pretreatment preserved >90 and >70% glucan and xylan in solid fraction. Fed-batch hydrolysis with Cellic CTec3 at one kg scale liberated 167.4 ± 1.87 and 183.53 ± 1.23 g L^{-1} monomeric sugars in filtrates of bagacillo and SCB, respectively in 48h. Its high-ash content reduced glucan conversion yields by 16%, and led to glucan under-estimation in residual biomass during mass balance closure. Irrespective of feedstock type, within 18h *Saccharomyces cerevisiae* MTCC 180 and *Pachysolen tannophilus* MTCC 1077 produced ~5% (v/v) ethanol from 1.5L glucose-rich filtrates, with a ~18 fold enhancement in biomass accumulation. However, when *Bacillus coagulans* NCIM 5648 was assessed for high-temperature 2G lactic acid production, the obtained titre from bagacillo derived hydrolysate (33.6 ± 1.23 g L^{-1}) was lower than SCB (43.38 ± 1.89 g L^{-1}). The present study demonstrated that bagacillo is an equally amenable bioresource as ~506 g fermentable sugar was extracted from one kg raw biomass and its glucose-rich fraction showed feasibility for microbial transformation to bio-based platform chemicals.

Keywords: Sugarcane bagasse; Bagacillo; Cellic CTec3; Cellulosic ethanol; 2G-lactic acid

1. Introduction

Agro-industries can play an instrumental role in boosting the rural economy of any nation. In India, the sugar industry represents the second most well organized agro-based industry after textiles [1]. Conventionally, a variety of solid and liquid streams are generated from milling to sugar crystallization in a sugarcane-driven industry. These waste streams include cane trash, bagasse, press mud, fly-ash, spent wash or vinasse, sugarcane molasses etc. [1-3]. Some of these side streams are rich in carbohydrates and are sustainable bioresources. Upon valorization, they can produce industrially important bio-based chemicals and fuels, further benefitting the environment. Considering this fact, especially in the Indian context, the "Ethanol Blending Program" has taken centre stage. To reduce partial dependence on fossil fuels, foster production of green energy, generate newer employment, and lower greenhouse gas (GHG) emissions, the government of India is working aggressively to fulfil its ambitious target of 20% ethanol blending with gasoline by 2025. Owing to lucrative amendments in the National Biofuel Policy, the sugarcane cultivation area is likely to increase from 48.41 to 54.55 lakh hectares from 2019-20 to 2021-22 [4]. Even globally, sugarcane was the largest cash crop in 2020, with total production being 1907 million tonnes [5]. In this scenario, sugar industries have a vast scope for value addition of waste streams rich in organic carbon, thereby increasing their profitability and venturing into diversification by expanding their product portfolio [6]. Further, if sugar industries set forth an example, effective and sustainable waste management together with rural socio-economic development by other promising and related sectors can also be envisaged.

In this aspect, one of the waste solid streams from the sugar industry, namely bagacillo, can be suitable input materials. In general, sugarcane bagasse (SCB) is subjected to repeated milling process to maximize sugarcane juice yields thereby producing bagacillo. It represents fine particles (< 1mm) of SCB or sugarcane fibres with a density of ~605 kg/m^3 [7]. Owing to its fluffy nature and low particle size, separation of bagacillo is difficult and it often results in clogging equipments and centrifuges. In case of inappropriate storage conditions or mishandling, it can be a potential health hazard causing respiratory ailments as it generates suspended air particulate matter being dusty in nature [8]. Usually bagacillo is separated from SCB by screening or pneumatic separation and preferably used as a filter aid in rotary vacuum filters or for co-generation when mixed with mud and bagasse respectively [9]. During sugarcane juice clarification bagacillo is often mixed with press mud, which has certain disadvantages. For instance, bagacillo contains saccharetin whose contamination imparts yellow color and adversely affects sugar quality [10]. Further, the high ash content of the bagacillo increases the ash content in the raw sugar as well [11]. Yet like SCB, bagacillo contains a significant fraction of cellulose and hemicellulose as structural polysaccharides. Therefore, bagacillo valorization is worth exploring, where through biochemical platform its embedded sugars are extracted and transformed to bio-based products, rather than simply restricting its use for low-end applications. This biomass offers a competitive edge over SCB it can be directly subjected to pretreatment and energy is also saved as milling step is bypassed.

In the present study, optimized pretreatment and high-solids enzymatic saccharification (HSES) developed previously were replicated at a large scale with sugarcane bagacillo [12], and its potentiality was adjudged with SCB as the reference feedstock. Later, the feasibility of the high-gravity sugars, particularly the C6 stream obtained after Cellic CTec3 mediated hydrolysis of 20% solids was assessed for production of green platform chemicals. We

aimed this exquisite category, as these molecules contain reactive, multi-functional groups and serve as precursors for synthesis of marketable products with variegated industrial applications [13, 14]. The first targetted product was L (+) lactic acid (LA), with thermophilic *Bacillus coagulans* NCIM 5648 as the fermenting microbe [15]. Parallelly, the growth and fermentability of two yeasts, namely *Saccharomyces cerevisiae* MTCC 180 and *Pachysolen tannophilus* MTCC 1077 were assessed for 2G-ethanol production [16, 17]. These experiments confirmed the amicability of saccharified broths for direct valorization to bio-based products. Cellic CTec3 was chosen due to the enzyme's superiority over Cellic CTec2 and its availability in large quantities.

2. Materials and Methods

2.1. Raw Material and Enzyme

Raw SCB and bagacillo were kindly provided by our industrial collaborator, Dhampur Sugar Mills, Bijnor, India. Sugarcane bagacillo required no mechanical reduction whereas SCB was milled through a Restch cutting mill fitted with 2 mm mesh. Cellic CTec3 was generously gifted by Novozymes A/S (Bagsvaerd, Denmark) with Lot number being VDC12071. The protein content of Cellic CTec3 was determined using Bradford reagent kit from Sigma Aldrich and Bovine Serum Albumin (BSA) fraction V serving as standard reference [18]. The protein content was represented as mg BSA equivalents and came out to be 112.03 ± 5.81 mg BSA equivalents g^{-1} enzyme. The filter paper activity was conducted per the IUPAC protocol and was 117.37 ± 0.725 IU g^{-1} enzyme [19]. The enzyme was stored at 4°C in the cold room.

2.2. Alkali pretreatment and compositional analysis

Multiple pretreatment trials were conducted with bagacillo and SCB (400 g dry weight basis/batch) based on the method optimized previously [12]. 15% (w/v) raw bagacillo/SCB was mixed with 0.5 M sodium hydroxide (NaOH) and subjected to autoclaving at 121°C for 30 min. After pretreatment, the solids were separated by filtering and washed until a neutral pH was obtained. During pretreatment, ~10 kg and ~5kg of raw bagacillo and SCB were processed, respectively, whose batches were later pooled as two distinct pretreated feedstocks.

The composition of structural carbohydrates, lignin (acid-soluble and insoluble) and ash content in the biomass was analyzed before enzymatic hydrolysis as per the Laboratory Analytical Procedure (LAP), developed at National Renewable Energy Laboratory (NREL), United States [20, 21]. Likewise, after HSES trials, the residual biomass (RB) was dried, weighed, and subjected to compositional analysis. It helped in determining the weight residual xylan and glucan fraction in solid fraction, post-saccharification. Indirectly it gave an indication of hydrolyzed fraction of these fractions. Further, this experiment validated that residual polysaccharide content complemented the saccharification yields obtained during HSES and facilitated the mass balance closure.

2.3. HSES of alkali pretreated sugarcane bagacillo and its comparison with SCB

Before commencing the large-scale fed-batch trials, it was necessary to assess the performance of Cellic CTec3 at 52.5°C and 20% solids, as optimized in our earlier study using Cellic CTec2 [12]. In the preliminary investigation, 20g pretreated sugarcane bagacillo (dry wt. basis) was hydrolyzed with Cellic CTec3 dosed at 15 mg protein g^{-1} cellulose content. Fed-batch hydrolysis was started by adding with bagacillo at an initial conc. of 7.5 g and thereafter 7.5g and 5g substrate was added at 2nd and 4th hour respectively to finally reach 20% solids. The water and enzyme required for hydrolysis of 20% solids was added at the start of the reaction only. The pH of the reaction slurry was

adjusted to 4.5 ± 0.3 by adding 8N H_2SO_4. The pH of the reaction slurry was checked intermittently till 8h, wherein proper liquefaction could be observed.

After 48 h, the solid fraction was separated from the saccharified broth by centrifugation at 5752 *g* for 10 min at 4°C. The sugar-rich solution was termed "filtrate". Later, the residual solids were water washed to remove the adhered sugars from the biomass and were re-centrifuged. This liquid fraction was designated as "wash".

Once the preceding experiment established that ≥70% carbohydrate fraction was hydrolyzed, experiments were replicated with 1.0 kg (dry wt. basis) pretreated sugarcane bagacillo in four different batches. Equivalently, two sets with SCB were also run to assess the impact of substrate variation on the extent of carbohydrate depolymerization. Sugars in the filtrate and the wash were quantitatively estimated using high-performance liquid chromatography (HPLC). Simultaneously, acetic acid was also qualitatively determined by HPLC to understand the relative difference in the composition of hydrolysates derived from two feedstock's namely sugarcane bagasse and bagacillo, as it is considered as major inhibitor during microbial growth and fermentation.

Additionally, with the intent to reuse the enzyme at a later stage, we evaluated enzyme recovery by conducting a Bradford assay in the saccharified fractions (both filtrate and wash). Percentage enzyme recovery was calculated by the following formula as described earlier [16]:

$$(\%)\ \text{Enzyme Recovery in terms of protein} = \frac{(\text{Total protein recovered in the saccharified fraction})}{(\text{Initial protein dosed})} * 100$$

Wherein the protein content of the enzyme was reported as mg protein BSA equivalents

Later to obtain uniform sugar concentration for fermentation studies, the filtrate fractions of all the batches were pooled. Likewise, the wash fractions of all the batches were also pooled. They served as the carbon source for the fermentation and inoculum development, respectively.

2.4. Procurement of microbial cultures for production of green chemicals

Bacillus coagulans NCIM 5648 was purchased from the National Collection of Industrial Microorganisms (NCIM), Pune, India. Likewise, *Saccharomyces cerevisiae* MTCC 180 and *Pachysolen tannophilus* MTCC 1077 were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The bacterium and the yeasts were routinely maintained on nutrient agar and potato dextrose agar, respectively. These cultures were preserved as 25% glycerol stocks in -80°C deep freezer.

2.5. Fermentative production of green chemicals

In the present study, the suitability of the hydrolysate derived from bagacillo as appropriate fermenting medium was compared with SCB hydrolysate, which acted as a positive control as well. All the fermentation studies for LA and ethanol production were conducted in duplicates with 1.5L glucose-rich filtrate fortified with nutrients in 3L Erlenmeyer flasks under oxygen-deprived conditions as described previously [15,16].

In the present experiments, all the batch filtrates derived from two feedstocks were pooled separately and subjected to thermal inactivation of the enzyme followed by removal of coagulated protein via centrifugation. Further, these sugar-rich hydrolysates were stored at 4°C until fermentation trials were initiated. After adding nitrogen salt and other media ingredients, the initial glucose and xylose concentration was estimated in the saccharified broths obtained from both the feedstocks, which served as the fermentation media for all three microbial cultures.

For LA fermentation study, the pre-seed and seed cultures were prepared in the nutrient broth and modified CM5 medium, with sugars from wash fraction serving as carbon source, described previously. Since *B. coagulans* is a thermophilic bacterium, the temperature was maintained at 50°C, with the initial pH being 7.2 ± 0.2. However, the pH was restored to 6.5 ± 0.2 using 8N NaOH during the entire fermentation, when the pH dropped to 5.2 ± 0.2 due to acid production, as suggested earlier by Sun et al. [22]. Earlier, LA fermentation studies showed that 0.7g/L initial cell concentration on a dry wt. basis required merely 24h for complete glucose assimilation [15]. Hence, the present study was restricted to 42h, as inoculum concentration on dry wt. basis was ~0.35g/L.

Likewise, *S. cerevisiae* MTCC 180 and *P. tannophilus* MTCC 1077 were assessed separately for ethanol production under mesophilic conditions (32°C; pH-5.0 ± 0.2) from the saccharified broths of the two feedstocks as described earlier [16,17]. During cellulosic ethanol fermentation, the study was conducted until the residual glucose concentration reached < 5g/L. Furthermore, a "defined time limit" during fermentation was anticipated to clearly distinguish the impact of hydrolysate composition derived from two feedstocks on different microbial cultures' growth and fermentative ability.

2.6. Determination of microbial biomass accumulation at the end of the fermentation

At the end of all the fermentations, 10 ml of fermentation broth was aliquoted in pre-weighed 15 ml falcon tubes as triplicates and subjected to centrifugation at 10,000 RPM for 5 min. The clarified fermented broth was separated and the cell pellet in tubes was subjected to drying at 60°C till a constant weight was achieved. Thus, the final cell weight on dry weight basis was determined at the end of the fermentation.

2.7. Calculations for determining glucan and xylan hydrolysis

The following formula directly calculated the saccharification efficiency:

$$\text{Glucan hydrolysis (\%)} = \frac{\text{(Total glucose released in g)}}{\text{(Glucan content subjected to HSES)} * 1.11} * 100$$

$$\text{Xylan hydrolysis (\%)} = \frac{\text{(Total xylose released in g)}}{\text{(Xylan content subjected to HSES)} * 1.13} * 100$$

Where 1.11 and 1.13 represented depolymerization factor of glucan and xylan respectively.

Saccharification efficiency was calculated indirectly by residual biomass analysis (RBA) using the following formula:

$$\text{Glucan hydrolysis (\%)} = \frac{\text{(Initial glucan content (g) - Final glucan content (g) in residual biomass)}}{\text{(Initial glucan content in g)}} * 100$$

$$\text{Xylan hydrolysis (\%)} = \frac{\text{(Initial xylan content (g) - Final xylan content (g) in residual biomass)}}{\text{(Initial xylan content in g)}} * 100$$

2.8. Determination of sugars and fermentation metabolites by HPLC

Release of principal sugars from SCB and bagacillo was quantitatively measured by HPLC (Shimadzu make) equipped with Aminex HPX-87H (Bio-Rad, California, USA) column coupled with refractive index detector (RID-10A; Shimadzu Corporation Japan). Before injecting samples (post saccharification and fermentation) in the column, they were centrifuged at 10,000 RPM for 10 min and clarified through 0.22 µm PTFE membrane filters. The analysis was done at 55°C under isocratic conditions with 5mM H_2SO_4 as the mobile phase. The flow rate was

set at 0.55 mL min^{-1}, and the injection volume was 20 µL. All the sugars, including principal metabolites obtained after fermentation, were quantitatively assessed based on the standards graphs prepared in the range of 0.2 to 1 mg/ml.

3. Results and Discussion

3.1. Pretreatment trials with raw bagacillo and SCB

Table 1 depicts the chemical composition and mass yields obtained with SCB and bagacillo after alkali pretreatment. As shown, the glucan (40.49%), xylan (27.55%), and lignin (23.94%) content of the raw SCB was in close agreement to the earlier published state of the art as exclusively reviewed by Alokika et al [23].The average solid recovery for SCB and bagacillo were 62.95% and 73.18% respectively, after alkali pretreatment. The relatively high mass yields obtained in case of SCB compared to our previous study could be primarily attributed to the larger particle size (2-2.5 mm *vs* < 1mm) of the bagasse chosen for the present experimental set-up, which led to selective delignification and significant enrichment of structural polysaccharides [12]. Extensive mechanical reduction invariably results in higher defibrillation of biomass. Furthermore, it increases the vulnerability of biomass towards higher carbohydrate losses, especially the xylan fraction, during chemical pretreatment.

In the case of SCB, ~98% cellulosic fraction was preserved compared to ~90% in bagacillo. A similar trend was noticed in the case of xylan enrichment as well. However, the delignification in the two feedstocks ranged between 63- 69%. The results of lignin removal are in consensus with the earlier study where pretreatment of 10% SCB (w/w) with 1% NaOH for 60 min at 100°C resulted in 62.3% delignification [24]. The accuracy of deciphering actual acid-insoluble lignin (AISL) content in sugarcane bagacillo was difficult as the entire ash content cannot be accounted for in AISL during gravimetric analysis. A fraction of ash was extractable and hence was leached out by two-stage acid hydrolysis during compositional analysis.

Independent ash analysis revealed that, unlike raw SCB, where its content did not exceed >3.5%, the raw bagacillo contained ~10.5% ash. During NaOH pretreatment of bagacillo, high ash content likely contributed to higher sugar losses in black liquor when compared to SCB, owing to no chemical interactions with structural carbohydrates, unlike lignin. Alkali pretreatment further facilitated 44-47% deashing of both the feedstocks. Based on shreds of evidence demonstrated in state of the art, low glucan saccharification yields were anticipated with sugarcane bagacillo despite being pretreated by alkali, as its ash content (8.8%) was relatively higher compared to 2.47% in pretreated SCB [25, 26].

3.2. Preliminary studies involving CellicCTec3 mediated hydrolysis of alkali pretreated bagacillo

When the preliminary fed-batch trials were conducted at 52.5°C with the alkali pretreated bagacillo on 20 g dry weight basis using Cellic CTec3, the concentration of total monomeric sugars (glucose + xylose) in the filtrate and wash fraction was found to be 139.4 ± 1.1 g L^{-1} and 57.6 ± 1.0 g L^{-1} respectively. It led to >72.2% depolymerization of carbohydrate fraction. These trials further suggested that the efficacy of xylan hydrolysis (79.4 ± 1.5 %) by Cellic CTec3 was better than glucan hydrolysis (65.0 ± 0.8 %), unlike its predecessor Cellic CTec2 [12]. The most suitable explanation for this behaviour of Cellic CTec3 can be derived from the study performed by Sun et al. [27]. They have shown that both Cellic CTec2 and CTec3 displayed nearly identical activity in terms of FPU mg^{-1} protein. However, the β-xylosidase and CMC'ase activity of the latter was 4-fold higher (1.15: 0.28 U mg^{-1} protein Cellic

CTec3: Cellic CTec2) and 1.82-fold lower (1.12: 2.26 U mg^{-1} protein Cellic CTec3: Cellic CTec2) compared to the former commercial enzyme [27]. This experiment further validated that high ash content in the said feedstock severely impeded the glucose release from cellulosic fraction despite low lignin content, as demonstrated earlier with dilute acid pretreated corn stover and rice straw [25, 26].

3.3. Comparative assessment of sugarcane bagasse and bagacillo for HSES at 1kg scale

When saccharification trials were conducted in quadruplicates using one kg alkali pretreated bagacillo on dry wt. basis, the following results were obtained as shown in Table 2a. During HSES, the glucose content never exceeded 70% of the monomeric sugars in both the filtrate and wash, whereas its contribution was always >75% when the biomass was SCB [12]. In the Batch I and Batch II the average glucan and xylan conversion yields was 69.1± 2.3% and 90.94 ± 1.7%, respectively. Shifting from three-step substrate feeding (Batch I & II) to merely two-step (in Batch III) visually displayed no crucial effect on the fermentable sugar release from the alkali pretreated bagacillo. However, it impacted the final carbohydrate hydrolysis yields, which were reduced by ~11.5%. In an attempt to enhance glucan hydrolysis, an additional experimental variation was performed in Batch IV. The temperature was kept at 55°C till the substrate feeding was completed, envisaging an early onset of liquefaction. Henceforth, the saccharification was performed at 52.5°C till its termination. As a result, maximum carbohydrate depolymerization (74 and 94% glucan and xylan hydrolysis) and concentration (167.4 ± 1.87 g L^{-1} total monomeric sugars in filtrate) were attained in Batch IV only.

However, the glucan conversion yields were low compared to our previous study [12]. This kind of reduced glucose release can be corroborated with the earlier study where the inhibitory effect of exogenous ash and its fractionated components like ash, soluble and insoluble components, soluble salts and insoluble minerals was evaluated on wheat straw. It was observed that all these components retarded the cellulose digestibility of auto-hydrolysis pretreated deashed wheat straw significantly, but the most prominent effect was seen with insoluble mineral content [28]. When the HSES was terminated with bulk biomass, a lot of insoluble mineral content (mostly sand) was seen as the bottom of the flasks, suggesting it was an integral part of alkali pretreated bagacillo, despite extensive washing after pretreatment. Irrespective of different batches, the mass balance closure after RBA indicated that the total carbohydrate (glucan and xylan) hydrolysis was ≥ 90%. This discrepancy in the results was unfolded when the ash analysis was conducted with the residual biomasses. Nearly all the biomasses (Batch I to IV) showed a high ash accumulation decreasing the chances of adhered sugars to the biomass (Table 2b). As a result, lesser sugars were recovered in the wash fraction (Table 2a). In the case of Batch III, partial removal of ash content (22g recovered as silica on a dry wt basis) after saccharification also reduced the amount of RB content. It was also reflected in the final ash content obtained during RBA. The results are in consensus with the findings of Chambon et al. [29], where post-saccharification facile dissociation of silica was observed, which exists as both leachable and structural inorganic components [30].

Earlier, Sluiter et al. have given a compelling argument that the LAP procedure involving two-stage sulfuric acid hydrolysis is not suitable for biomasses having ash content >10%, as the minerals neutralize a part of acid, reducing its efficacy for complete hydrolysis of structural polysaccharides [31]. The theory was experimentally proven later by He et al., wherein corn stover with an ash content of 9.6% and 4.98% displayed a pH value of 4.15 and 2.78

respectively when impregnated with identical concentrations of sulfuric acid on a weight basis [25]. Very recently, an extensive review on biomass ash and, more particularly, sugarcane bagasse suggest that ash rich in alkali and alkaline earth metals have acid-neutralization potential [30, 32]. Thus there is a high probability that in the present case also, high ash content together with enriched basic metal oxides and hydroxides resulted in incomplete glucan hydrolysis and was the main culprit for its underestimation in the RB of all four batches. However, residual xylan content remained unaffected by the negative implications of ash content, the reason being less rigid polysaccharide compared to cellulose and easily prone to even dilute acid hydrolysis.

Despite all the odds, from one kg raw bagacillo, the first three batches extracted ~449 g of monomeric fermentable sugars. To the best of the authors knowledge this is the first ever report showing a maximum recovery of 506.35 g monomeric sugars from one kg raw bagacillo when the saccharification for the first four hours was conducted at 55°C followed by reducing temperature to 52.5°C for next 44h. However, we are optimisitic that a preliminary step of deashing may further enhance the extractability of sugars from sugarcane bagacillo. Nevertheless, it is likely to increase the cost economics of the pretreatment module simultaneously. Thus, the saccharification step proved that sugarcane bagacillo was a "viable and equally amenable feedstock option" to obtain high gravity sugar solutions. This study further opens avenue for the use of this under-exploited waste feedstock as presently in India, most of the efforts are focused towards efficient utilization of residual SCB [33].

The improved saccharification yields obtained Batch IV using alkali pretreated sugarcane bagacillo encouraged us to perform fed-batch hydrolysis of SCB (Batch A and B) with 0-4h set at 55°C and later reducing the temperature to 52.5°C (Table 2 a&b). Mass balance closure studies depicted that the bioconversion yields of polysaccharide fraction obtained during HSES were congruent with the RBA (Figure 1).

In Batch B, when the enzyme loading was reduced from 15 mg protein to 12.5 mg protein^{-1} glucan content, like Batch A >90% and >80% xylan and glucan conversion yields were observed (Figure 1). Ash analysis of residual SCB (~10%) revalidated our hypothesis that higher ash content in the case of sugarcane bagacillo (>30% ash) led to erroneous quantification of cellulose content in RB and resulted in its under-estimation (Table 2b).

Moreover, the monomeric fermentable sugar extraction from one kg raw SCB improved from 530 g to 601 ± 10.9 g [12]. This 13.5% enhanced sugar yield is primarily attributed to higher xylan content in the raw SCB and replacement of Cellic CTec2 with CTec3 that displayed better xylanase activities than its predecessor [27]. Thus in the present study, the initial SCB composition played a crucial role as it had <40% glucan and >27% xylan content. We also inferred that large particle size did not critically affect the hydrolysis of polysaccharides as SCB used in the present study was sieved and collected through a larger mesh size screen than our previous study. Earlier, a comprehensive review by Zhang et al. concluded that biomass particle size had a variable impact on cellulose hydrolysis, while crystallinity, specific surface area and biomass composition at micron level mattered more than particle size at macro-level [34].

Furthermore, protein estimation in the saccharified broth in the case of both bagacillo and SCB gave highly encouraging results. If 55.23 ± 3.27 % enzyme protein was retained in the saccharified broth obtained from bagacillo, in the case of SCB derived enzymatic hydrolysate, the protein recovery as per Bradford assay was 71.06 ± 5.75 %. This revelation broadens the scope of enzyme recycling through the 10kDa tangential flow filtration (TFF)

system when the process is upscaled at the pilot level. Earlier, the group has successfully illustrated the fractionation of glucose-rich broth in permeate and reuse of Cellic CTec2 obtained as enzyme retentate after hydrolysis of acid pretreated SCB using 10kDa centrifugal filters [16].

3.4. Feasibility check of high gravity sugar solutions derived from bagacillo and SCB for microbial biomass accumulation and biotransformation to green chemicals

Three microbial fermenting strains were used for evaluating the valorization potential of high-gravity sugar syrups derived from bagacillo and SCB to green chemicals. Fermentation studies revealed that the eukaryotic system was more robust than the prokaryotic system in the present case (Table 3).

During ethanol fermentation studies, nearly all the initial glucose present in the hydrolysates was assimilated by both the yeast strains. Irrespective of glucose-rich streams originating from two feedstocks, *S. cerevisiae* MTCC 180 and P. *tannophilus* MTCC 1077 showed a high glucose uptake rate and accumulated high biomass (~18 fold increase). The maximum cellulosic ethanol titres reached were 38.6 -42.6 g L^{-1} with productivity being ~2.2 gL^{-1} h^{-1}. The ethanol fermentation efficiency of both the yeasts was 72-77%, when the hydrolysates were derived from sugarcane bagacillo. However, in case of SCB-derived hydrolysates the ethanol fermentation efficiency was relatively lower (53-59%).

While performing LA production, we anticipated complete glucose uptake within 45h only using thermophilic bacterium. Still, at the end of 42h, ~37.5% and 45.5% glucose was left unconsumed in the glucose-rich filtrate derived from SCB and bagacillo, respectively. Our earlier study showed that LA fermentation was growth associated [15]. It is likely that during the present investigation, the required threshold cell concentration was not attained, which expedited glucose uptake and its complete biotransformation to LA. The LA yields of hydrolysates derived from SCB and bagacillo were 0.754 g/g and 0.544 g/g of glucose consumed, respectively. The LA yields with SCB hydrolysate were nearly identical to our earlier study [15].

Furthermore we anticipate that bagacillo derived hydrolysate was relatively more inhibitory than bagasse due to elevated levels of lignin-derived soluble aromatics, as visualized in Figure 2, which also imparted the darkness to hydrolysate after thermal inactivation of the enzyme (oxidation of phenolics). When the optical density of these hydrolysates was qualitatively measured at 280 nm, exploiting the ability of aromatic ring in phenolic compounds to absorb UV light [35], it came out to be 15.05 and 5.25 for bagacillo and SCB, respectively. It is likely that in contrast to yeasts, the chosen *B. coagulans* strain was less robust. It further substantiated that the bagasse derived hydrolysate was more conducive for biomass accumulation as compared to bagacillo, and hence *Bacillus* produced more LA in the former compared to the latter. This observation is in consensus with the study conducted by Chen et al [36]. Their group evaluated effect of water soluble phenolic compounds (WPC) obtained after four different pretreatments (alkali or AL, Liquid hot water or LHW, dilute acid or DA, ammonia fiber expansion or AFEX) of corn stover. They found that the growth and ethanol fermentation ability of *Zymomonas mobilis* 8b was severely inhibited by the presence of WPC at 4 g/L and followed the order: AL > LHW > DA > AFEX. Further chemical characterization of AL derived WPC showed predominance of monomeric phenolic aldehydes during FT-IR.

The present study suggests that besides choice of feedstock, the righteous selection of microbial cell factories involved in fermentation play an equally important role. Especially robustness of strain and its adaptability towards

real-time lignocellulosic feedstocks including presence of inhibitors (soluble and insoluble) and efficiently biotransforming a broad spectrum of biomass-derived carbon sources are some of the important criteria that need to be considered during scale-up, as reviewed comprehensively by Lu et al. [37]. Alternately, an appropriate low cost and effective detoxification strategy may also be devised for removal of lignin-derived soluble and insoluble phenolics from enzymatic hydrolysates before subjecting to fermentation.

As per the latest update, the total number of installed sugar mills in India is 732, which primarily process sugarcane juice for sugar production as its staple or main-stream product [38]. Besides it, these industries conservatively divert molasses for ethanol manufacturing while using bagasse for cogeneration. However, several other organic carbon-rich waste streams, for instance sugarcane bagacillo in the present case, offer ample opportunities by targeting multiple commercially-viable bio-based products.

4. Conclusion

The present investigation concluded with the fact that sugarcane bagacillo was an equally competitive feedstock, as it exhibited credibility towards cleaner production of concentrated sugar-syrups, favouring microbial growth and subsequent valorization to green chemicals. The authors are optimistic that after introducing deashing step during pretreatment, detoxifying sugar-rich hydrolysates and adopting fermentation intensification strategies at pilot-scale, the techno-economic feasibility of valorizing polysaccharide component of this lignocellulosic feedstock to green chemicals may ameliorate further.

Declaration of competing interest

The authors declare no personal or financial competing interests

Acknowledgements

The authors acknowledge Department of Biotechnology (DBT), India, Biotechnology and Biological Sciences Research Council (BBSRC) and Innovate UK for financially supporting the vWa Project (GAP 3513) under Indo-UK Industrial Waste 2017 grant.The authors are thankful to Dr Anjan Ray, Director CSIR-Indian Institute of Petroleum, for his valuable inputs and guidance. We are grateful to our industrial partner Dhampur Sugar Mills, India, for providing sugarcane bagasse and bagacillo for the said study.

Author's contribution

Pratibha Baral: Investigation, Methodology, Formal analysis, Writing-Original Draft; Arijit Jana: Investigation, Methodology, Data curation; Vinod Kumar: Writing- Review & Editing; Deepti Agrawal- Conceptualization, Supervision, Writing-Original Draft, Project Management and Fund acquisition.

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Figure Captions

Figure 1: Glucan and xylan hydrolysis yields from alkali pretreated SCB from Batch A and B, as per released sugars during saccharification and based on carbohydrate content in residual biomass

Figure 2: Sugar-rich filtrate fraction derived from (a) sugarcane bagacillo and (b) sugarcane bagasse after thermal inactivation of Celli CTec3 and its subsequent removal by centrifugation

Table 1: Compositional analysis of bagacillo and SCB before and after alkali pretreatment with 15% solids at 121°C for a duration of 30 min

Lignocellulosic feedstock	Solid recovery (%)	Biomass composition (%) of raw feedstock	Biomass composition (%) of alkali pretreated feedstock
Sugarcane Bagacillo	62.95 ± 1.13	Glucan: 42.21 ± 0.44 Xylan: 22.61 ± 0.45 Acid insoluble lignin: 22.83 ± 0.76 Acid soluble lignin: 2.62 ± 0.01	Glucan: 60.60 ± 1.8 Xylan: 26.27 ± 1.29 Acid insoluble lignin:10.9 ± 0.1 Acid soluble lignin: 1.08 ± 0.04
Sugarcane Bagasse	73.18 ± 0.89	Glucan: 40.49 ± 1.95 Xylan: 27.55 ± 0.31 Acid insoluble lignin: 21.91 ± 0.52 Acid soluble lignin: 2.03 ± 0.07	Glucan: 54.6 ± 1.72 Xylan: 29.78 ± 1.19 Acid insoluble lignin:10.4 ± 0.2 Acid soluble lignin: 1.19 ± 0.12

Note: Acid insoluble lignin represents lignin content without ash correction factor

Table 2a: Monomeric sugars released from alkali pretreated bagacillo and SCB during fed-batch HSES using Cellic CTec3

Feedstock Type	Batch	Substrate feeding (%)	Substrate feeding time (h)	Enzyme dosage (mg protein g^{-1} glucan)	Temp. during HSES (°C)	Total sugars (gL^{-1}) (glucose + xylose)	
						Filtrate	Wash
Sugarcane Bagacillo	I	7.5 + 7.5 + 5.0	0-2-4	15	52.5 (0-48h)	154.3 ± 1.5	32.5 ± 2.5
	II	7.5 + 7.5 + 5.0	0-2-4	15	52.5 (0-48h)	146.9 ± 2.8	37.1 ± 3.0
	III	10 +10	0-2	15	52.5 (0-48h)	142.2 ± 2.9	32.1 ± 1.9
	IV	7.5 + 7.5 + 5.0	0-2-4	15	55 (0-4h) 52.5 (4-48h)	167.4 ± 1.9	22.4 ± 1.8
Sugarcane Bagasse	A	7.5 + 7.5 + 5.0	0-2-4	15	55 (0-4h) 52.5 (4-48h)	181.4 ± 2.1	71.4 ± 1.1
	B	7.5 + 7.5 + 5.0	0-2-4	12.5	55 (0-4h) 52.5 (4-48h)	183.5 ± 1.2	74.8 ± 1.9

Note: The values are the average of HPLC runs at three different dilutions

Table 2b: Compositional analysis of residual alkali pretreated bagacillo and SCB obtained after fed-batch HSES using Cellic CTec3 with one kg initial biomass

Feedstock Type	Batch	Residual biomass (g)	(%) Composition of Residual Biomass			(%) Ash in residual biomass
			Glucan	Xylan	Acid insoluble lignin	
Sugarcane Bagacillo	I	232.9	17.8 ± 1.7	9.3 ± 0.7	53.6 ± 0.5	36.9 ± 0.5
	II	238.2	12.1 ± 0.1	9.0 ± 0.3	56.8 ± 3.0	36.1 ± 0.3
	III	195.7	14.1 ± 0.1	8.4 ± 0.4	58.4 ± 0.7	32.8 ± 0.9
	IV	225.7	10.9 ± 0.9	7.6 ± 0.5	66.5 ± 3.2	44.5 ± 0.3
Sugarcane Bagasse	A	215.4	34.8 ± 0.6	9.2 ± 0.3	46.7 ± 0.1	10.2 ± 0.8
	B*	222.3	35.9 ± 0.5	9.4 ± 0.5	45.7 ± 0.7	9.9 ± 0.6

Note: The values are the average of HPLC runs at three different dilutions while the acid-insoluble lignin content is without ash correction

Table 3: Comparative assessment of bagacillo and SCB derived sugars for microbial biomass accumulation and fermentative production of green chemicals

Sugar-rich filtrate from	Organism Used	Initial sugar conc. (gL^{-1})		Fermentation duration (h)	Microbial biomass (gL^{-1})		Final metabolites concentration (gL^{-1})		
		Glucose	Xylose		Initial	Final	Glucose	Xylose	EtOH/ LA
Bagasse	*S. cerevisiae*	133.7± 5.57	45.7± 2.18	18	0.362 ± 0.010	6.81 ± 0.04	0.88 ± 0.008	44.7 ± 2.03	39.4 ± 1.05
Bagacillo	MTCC 180	105.7± 4.19	40.1± 1.89		0.364 ± 0.008	6.82 ± 0.33	1.52 ± 0.015	39.3 ± 1.77	38.6 ± 0.97
Bagasse	*P. tannophilus*	131.1± 5.87	50.1± 2.67	18	0.372 ± 0.012	6.98 ± 0.65	0.70± 0.007	47.0 ± 2.47	39.8 ± 1.23
Bagacillo	MTCC 1077	108.5± 4.87	43.7± 1.65		0.375 ± 0.015	7.28 ± 0.32	NIL	40.5± 1.33	42.6 ± 1.07
Bagasse	*B. coagulans*	109.6 ± 5.02	42.1± 2.04	42	0.378 ± 0.007	6.25 ± 0.14	41.1 ± 1.2	45.2 ± 2.67	43.38 ± 1.89
Bagacillo	NCIM 5648	111.5± 5.33	40.0± 1.49		0.373 ± 0.012	4.78 ± 0.18	50.8 ± 2.11	40.6 ± 1.98	33.6 ± 1.79

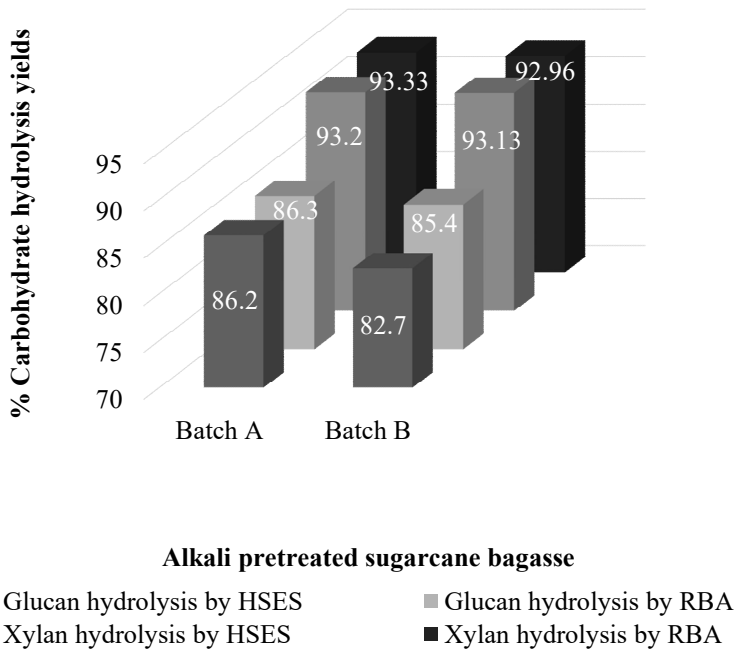
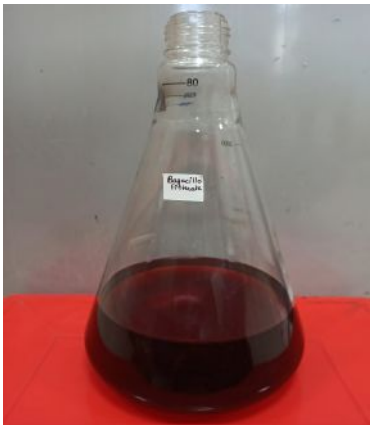

% Carbohydrate hydrolysis yields
95
90
85
80
75
70
86.2
86.3
93.33
93.2
82.7
85.4
93.13
92.96
Batch A
Batch B
Alkali pretreated sugarcane bagasse
Glucan hydrolysis by HSES
Glucan hydrolysis by RBA
Xylan hydrolysis by HSES
Xylan hydrolysis by RBA

Fig 1

(a) 

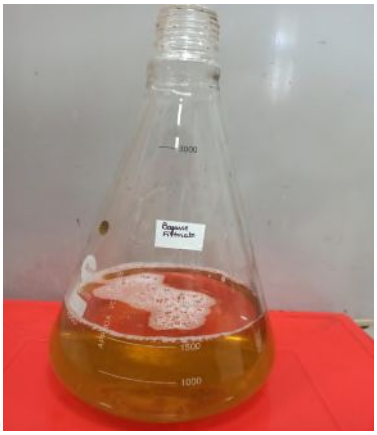
(b) 

Fig 2

Comparative assessment of sugarcane bagacillo and bagasse at lab-scale for production of sugars and green chemicals via biochemical platform

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2022-06-08

Baral P, Jana A, Kumar V, Agrawal D. (2024) Comparative assessment of sugarcane bagacillo and bagasse at lab-scale for production of sugars and green chemicals via biochemical platform, Biomass Conversion and Biorefinery, Volume 14, April 2024, pp. 8483-8492 https://doi.org/10.1007/s13399-022-02895-2