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Expeditious production of concentrated glucose-rich hydrolysate from sugarcane bagasse and its fermentation to lactic acid with high productivity

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Abstract

Sugarcane bagasse (SCB) is anticipated to emerge as a potential threat to waste management in India on account of cheap surplus energy options and lower incentives through its co-generation. Through biotechnological intervention, the efficient utilization of SCB is seen as an opportunity. The present study aimed towards expeditious production of concentrated glucose-rich hydrolysate from SCB. Alkali pretreated biomass was chosen for hydrolysis with a new generation cellulase cocktail, Cellic CTec2 dosed at 25mg g^{-1} glucan content. A two-step (9% + 9%) substrate feeding strategy was adopted with a gap of an hour, and saccharification was terminated in three different ways. Irrespective of the methods employed for termination, ~84.5% cellulose was hydrolyzed releasing ≥100 g L^{-1} glucose from 18% biomass. Direct use of glucose-rich filtrates yielded 69.2 ± 2.5 g L^{-1} of L (+) lactic acid (LA) using thermophilic *Bacillus coagulans* NCIM 5648. The best-attained glucose and LA productivities during separate hydrolysis and fermentation (SHF) in the present study were 5.27 and 2.88 g L^{-1} h^{-1}, respectively. A green and sustainable process is demonstrated for the production of industrially relevant sugars from SCB at high productivity and its valorization to bio-based LA.

Keywords: Cellic CTec2; alkali pretreated sugarcane bagasse; high-solids saccharification; glucose-rich filtrate; productivity; lactic acid

Highlights

- Saccharification of 18% alkali pretreated sugarcane bagasse attempted
- Three strategies adopted for termination of the batch hydrolysis
- Cellic CTec2 released ≥100 g L^{-1} glucose in 24 h with 5.27 g L^{-1} h^{-1} productivity
- *B. coagulans* mediated fermentation yielded 69.2± 2.5 g L^{-1} L (+) lactic acid

1. Introduction

Disruptive technological innovations in sustainable and profitable management of agricultural wastes may be highly rewarding for the emerging economies like India. Besides being cheap, abundant and easily available these lignocellulosic biomasses (LCB's) are the natural source of renewable carbon. Successful transformation of the embedded carbon in these feedstocks into versatile products such as bio-based chemicals, biofuels, bio-power etc. can benefit any nation. Besides contributing towards the self-reliance and energy security, such technologies can generate new employment opportunities, foster the rural economy and curtail down the green-house gas (GHG) emissions as well (Guragain *et al.*, 2016; Wenger and Stern, 2019; Vera *et al.*, 2020). Particularly cellulose, which represents a significant fraction of LCB, is an insoluble structural homo-polysaccharide and inexhaustible source of glucose. However, for industrial feasibility, the glucose release from cellulose in "concentrated form" is highly desirable. Once the sugar syrup is obtained, several products can be synthesized at high-titres, using various chemical and biotechnological routes. There are multiple advantages of getting concentrated input (sugar) and output carbon products (fuels, chemicals etc), as they reduce the capital and operational expenditure (CAPEX and OPEX) involved in their production, downstream processing, waste disposal etc. (da Silva *et al.*, 2020).

Presently in India, the annual production of sugarcane bagasse (SCB) from various sugar industries and distilleries account for ~80 million metric tonnes. This inexpensive and potential agricultural waste is anticipated to loom as the biggest problem in the next five years by the “Sugar Technologists Association of India” (STAI). Earlier, the industries used SCB for cogeneration. But easily accessible and cheaper surplus energy options such as hydro, thermal and solar etc. has reduced its usage for the production of heat and electricity (Patel., 2019). Hence the industries are exploring lucrative options for efficient utilization of SCB where commercially viable bio-based products can be manufactured. Profit margin of any product is generally governed by its applications, market size, production costs, consumer and industrial demand etc. (Guragain *et al.*, 2016; Wenger and Stern, 2019). Thus, there is tremendous scope for the researchers and technocrats to leverage upon promising biorefining processes targeting specific bio-based products. By coupling techno-economic analysis (TEA) with life cycle assessment (LCA), these processes can be further vetted and can be easily integrated with 500+ operating sugar mills in India as per the data of Indian Sugar Mills Association (ISMA) (https://www.indiansugar.com/Statics.aspx).

One of the straightforward strategies for attaining industrially relevant sugar yields from SCB could be its high-solids enzymatic saccharification (HSES). In this green process, there is no availability of free water on the commencement of the hydrolysis (Da Silva *et al.*, 2020). Several variable and inter-related factors impede the process of HSES. Process conditions, namely water constraints, mass transfer limitations and rheological characteristics of the whole slurry are the primary culprits. But intrinsic properties of enzymes such as inhibition due to lignin, water-soluble and insoluble degradation products, oligomeric and monomeric sugars also impact biomass liquefaction (Da Silva *et al.*, 2020; Fockink *et al.*, 2017; Liu *et al.*, 2020).

State of the art suggests that most of the batch HSES studies with different types of pretreated LCB's are carried out for a minimum period of 72 h which may further extend up to 144 h, as reviewed earlier (Modenbach and Nokes, 2013; Chen and Liu, 2016). The constraint of a single feeding substrate regime is overcome by adopting fed-batch strategies. But in such cases, to ensure complete hydrolysis of LCB, time is often stretched, as demonstrated by various co-workers (Gao *et al.*, 2014; Mukasekuru *et al.*, 2018). One of the noteworthy advancement to improve the efficacy of HSES has been the use of new generation cellulase cocktails. These unique cocktails are less prone to inhibitors. Further, they have an arsenal of accessory hydrolytic and non-hydrolytic enzymes such as xylanase, β-glucosidase, swollenin, cellobiose dehydrogenase (CDH), lytic polysaccharide monooxygenase (LPMO), which synergistically enhance cellulose hydrolysis (Ekwe *et al.*, 2013; Lopez *et al.*, 2018; da Silva *et al.*, 2020).

Taking into account the future opportunities and acknowledging the challenges with HSES, an investigation was undertaken where hydrolysis of 18% partially delignified SCB was carried out. The study targeted the release of a minimum 10% glucose at high productivity from its cellulosic fraction using Cellic CTec2, a known new generation commercial cellulase (Cannella and Jørgensen., 2013). In our earlier study, substrate loading beyond 12.5% drastically reduced the glucose productivity and led to incomplete cellulose hydrolysis during shorter incubation time (Nalawade *et al*., 2020). Therefore to overcome the drawback of batch hydrolysis arising due to poor mixing, in the present study a two-stage (9% + 9%) substrate feeding strategy was adopted within a gap of one hour. Later, the concentrated glucose-rich hydrolysate was directly valorized to L (+) lactic acid (LA). Separate hydrolysis and fermentation (SHF) with thermophilic *Bacillus coagulans* NCIM 5648 was inspired by the findings of Müller *et al.* (2017). They suggested that Cellic CTec2 outperformed in terms

of LA productivity under this condition as against simultaneous saccharification and fermentation (SSF) due to the absence of competition from microbes for dissolved oxygen (2017).

LA was chosen as the targeted product as presently the commercial production of LA is dominated via fermentation (Tarraran and Mazzoli, 2018). Moreover, this versatile platform chemical finds numerous applications in various sectors such as food and beverages, chemical industry, cosmetics, pharmaceuticals, textiles etc. with poly-lactic acid (PLA) being the most demanding biodegradable polymer product flourishing its market demand (Cubas-Cano *et al.,* 2018). More recently Rosales-Calderon and Arantes have reviewed LA as one of the most promising commodity chemical to reduce the cost of cellulosic ethanol production (2019) whereas Mandegari *et al.,* have proposed LA to be more advantageous the cellulosic ethanol in terms of LCA (2017).

2. Materials and methods

2.1. Raw Material and Enzyme

Raw SCB and Cellic® CTec2 were kindly gifted by Dhampur Sugar Mills, India and Novozymes A/S, Denmark respectively. Cellic CTec2 contained 93.89 ± 0.99 mg BSA equivalents protein g^{-1} enzyme as assayed by the method of Bradford (1976). The enzyme activity in terms of filter paper units (FPU) was found to be 125.8 ± 2.6 IU/g, measured as per IUPAC protocol (Ghosh, 1987).

2.2. Alkali pretreatment and compositional analysis

Alkali pretreatment was conducted as per the method described previously with slight modifications (Nalawade *et al.*, 2020). The previous study revealed when the water bath was set at 80°C the difference between the water bath and the slurry temperature was ~15°C. Hence 5% raw SCB was suspended in 0.5 M NaOH, and the beaker was placed only when the water bath reached at 95°C. To ensure uniform mixing, a homogenizer (Remi Motors RQ124A; Mumbai, India) set at 200 rpm was used and when the slurry temperature reached 76 ± 2°C, the delignification of the SCB was allowed for 120 min. After pretreatment, the solids were separated by filtering and then washed until a neutral pH was obtained.

The compositional analysis as per the procedure of National Renewable Energy Laboratory (NREL) revealed that alkali pretreated biomass contained 63.74± 1.38% cellulose, 17.05 ± 0.42% xylan, 12.19 ± 0.27% acid-insoluble lignin (AISL), 1.28 ± 0.01% acid-soluble lignin (ASL) and 2.93 ± 0.13% ash (Sluiter *et al.*, 2012; Sluiter *et al.*, 2008). This biomass served as the starting feedstock for HSES.

2.3. HSES of alkali pretreated SCB

Saccharification study was carried out at 18% (w/w) substrate loading in a non-buffered medium (pH 4.5) containing PEG 6000 (0.2g/g AISL content) placed in an incubator shaker set at 50°C, 180 rpm as described previously (Baral *et al.*, 2020). A two-step substrate feeding strategy (9% + 9%) within a gap of one hour was adopted to mitigate the problem of poor mixing as observed previously (Nalawade *et al.*, 2020).

Cellulose hydrolysis was expedited by adding Cellic CTec2 (25 mg protein g^{-1} glucan content) in bulk at the very start of the hydrolysis, corresponding to 18% substrate. Time course profile was studied during hydrolysis by intermittent removal of flasks to decipher the duration where it attained product saturation. SCB hydrolysis was terminated in three different ways, as shown in Figure 1.

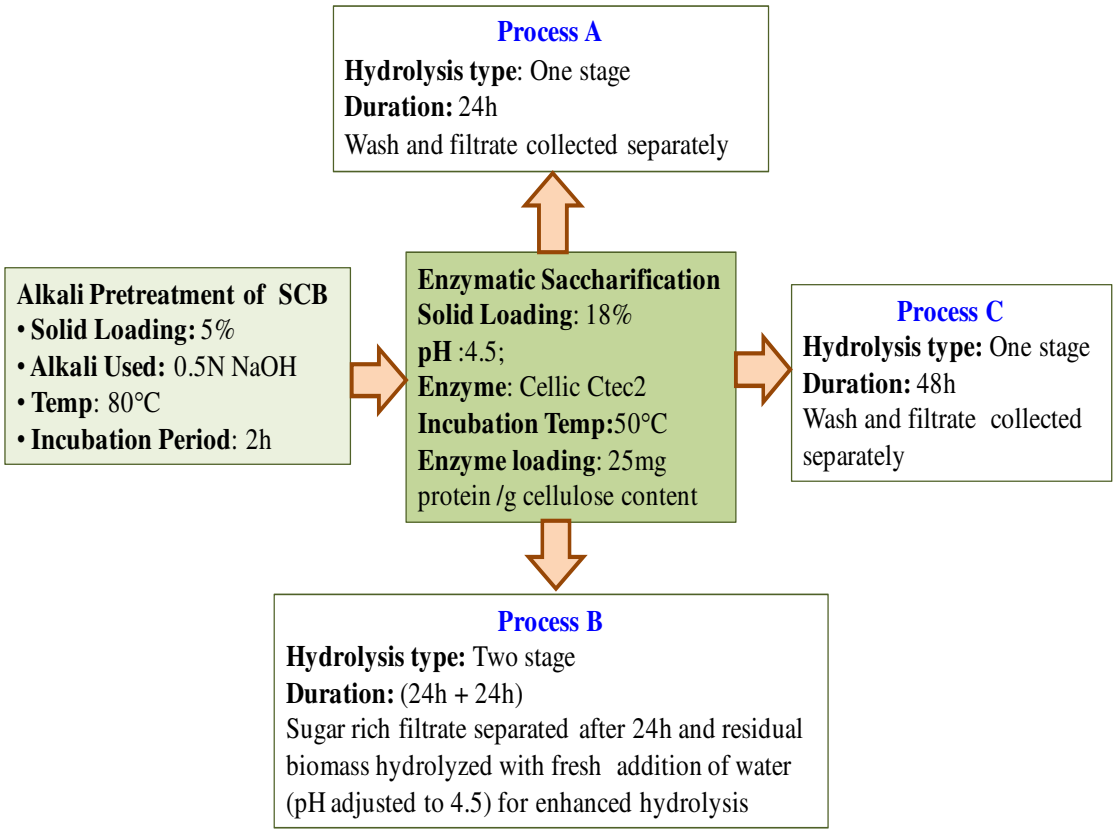

Fig.1 Schematics of terminating Cellic CTec2 aided saccharification conducted with alkali pretreated

In Process A and C, batch hydrolysis was conducted for 24 and 48 h respectively, whereas in Process B, discontinuous batch hydrolysis was attempted. After 24 h hydrolysis, the reaction was arrested, and sugar-rich filtrate was removed and water washed. Based on the fermentable sugars obtained in the filtrate and wash fraction, the amount of hydrolysed

biomass was calculated and second round of hydrolysis was carried out for next 24 h by adding appropriate amount of acidified water (pH 4.5) in residual biomass. Thus in discontinuous mode also 18% solids concentration was maintained and hydrolytic potential of Cellic CTec2 adsorbed to the biomass was explored.

When hydrolysis was completed, the residual solid fraction was separated from the saccharified broth by centrifugation at 5752 *g* (equivalent to 7000 rpm) for 10 min at 4°C. This concentrated sugar-rich solution was termed as "filtrate". Washing step was found inevitable to recover the adhered sugars from the biomass in our earlier study (Nalawade et al., 2020. Therefore, the residual biomass was water washed (equivalent to 50% filtrate volume) and said sugar solution was termed as "wash".

Filtrate and wash fractions were subjected to high performance liquid chromatography (HPLC) for analysis of all hydrolysed products released from alkali pretreated SCB during saccharification.

Before proceeding for LA fermentation, enzyme activity was thermally inactivated in the glucose-rich filtrates and washes. The coagulated protein was removed by centrifugation, and pH was neutralized using 25% liquor ammonia. They were stored at 4°C until used for LA fermentation. The main fermentation studies were carried out with the filtrate fraction whereas wash was used for inoculum preparation. All experiments were performed in duplicates, and the average values with standard deviations are presented.

2.4. Inoculum preparation and LA fermentation by B. coagulans NCIM 5648

The glucose-rich filtrates obtained after hydrolysis were valorized LA using a thermophilic bacterium *B. coagulans* NCIM 5648. The pre-seed and seed cultures were prepared in the modified CM5 medium with wash samples as a carbon source (Nalawade *et al.*, 2020). After 8 h, the culture was centrifuged at 5752 *g* to remove the residual sugars and LA formed from the actively growing cells of *B. coagulans*. The centrifuged cells were resuspended in physiological saline to achieve a dense inoculum, and optical density (OD) was measured at 600 nm. The LA production medium was prepared in the similar way as described in the preceding lines except that the carbon source was the "glucose-rich filtrate. It was fortified with (gL^{-1}) Na_2HPO_4- 2.17; KH_2PO_4 – 0.26; $(NH_4)_2$ SO_4 -2; $MgCl_2$-0.2; $CaCl_2$-0.025, yeast extract- 10 and Bromo-cresol purple -50mg with initial pH being 7.2 ± 0.2.

Fermentation was initiated by adding 2.5 ml (70 mg) of an active and highly dense inoculum (OD_{600nm} 82.2 ± 0.47) in 100 ml medium, and pH drop was monitored visually by change in dye-color (violet to yellow). The pH was restored to 7.0 ± 0.2 using 8N NaOH intermittently.

All the experiments were performed in duplicates. Samples were withdrawn periodically and processed for HPLC determination of residual glucose and xylose with the simultaneous production of LA. Metabolites other than LA were also qualitatively detected if present in traces and quantified if emerged as other primary products other than LA.

2.5. HPLC determination of sugars and fermentation products

All the sugars (glucose, xylose, cellobiose, arabinose, fructose, sucrose) and possible fermentation products (lactic acid, succinic acid, acetic acid, 2,3 butanediol, ethanol, xylitol) anticipated to be generated during SHF were analyzed by HPLC system (Shimadzu make) equipped with Aminex HPX-87H (Bio-Rad, California, USA) column coupled with refractive index detector (RID-10A; Shimadzu Corporation Japan). The analysis was done at 55 °C under isocratic conditions with 5mM H_2SO_4 as the mobile phase at a flow rate of 0.55 mL min^{-1} with an injection volume of 20 µL. Based on the retention time and calibration curves (0.2-1.0 mg mL^{-1}) using standards, all the sugars and metabolites were determined both qualitatively and quantitatively. The optical purity of LA being an "L" isomer was already confirmed through our earlier study (Nalawade *et al.,* 2020) using chiral column.

The glucose productivity during enzymatic saccharification was calculated by dividing the concentration of glucose obtained in the filtrate fraction with the time of hydrolysis and expressed as g L^{-1} h^{-1}.

3. Results and Discussion

3.1. HSES of alkali pretreated SCB

In any second generation or LCB-based biorefinery, saccharification represents the rate-limiting step. The time course studies revealed that the cellulose saccharification efficiency was 20.99 ± 1.15% and 64.62 ± 2.23% respectively in 8h and 16h. With 85.7% efficiency, it peaked in 24h and attained saturation when hydrolysis was continued further for 48h (Fig S1). However, in the case of xylan, 16% improved xylose yields were seen when hydrolysis was extended from 16h to 48h. A target of >100 g L^{-1} of glucose in the filtrate fraction was achieved in the first 24h only (Fig 2) with >30 g L^{-1} xylose as well. Besides glucose and xylose, acetic acid, arabinose and cellobiose were also detected qualitatively by HPLC but their concentrations were far below detectable limit.

Further water washing extracted the adhered sugars, which remained adsorbed to the residual biomass. The high standard deviation observed in Fig 2 could be attributed to different volumes of filtrates and washes obtained, which was nullified when total sugar released was estimated on a weight basis, as shown in Table S1

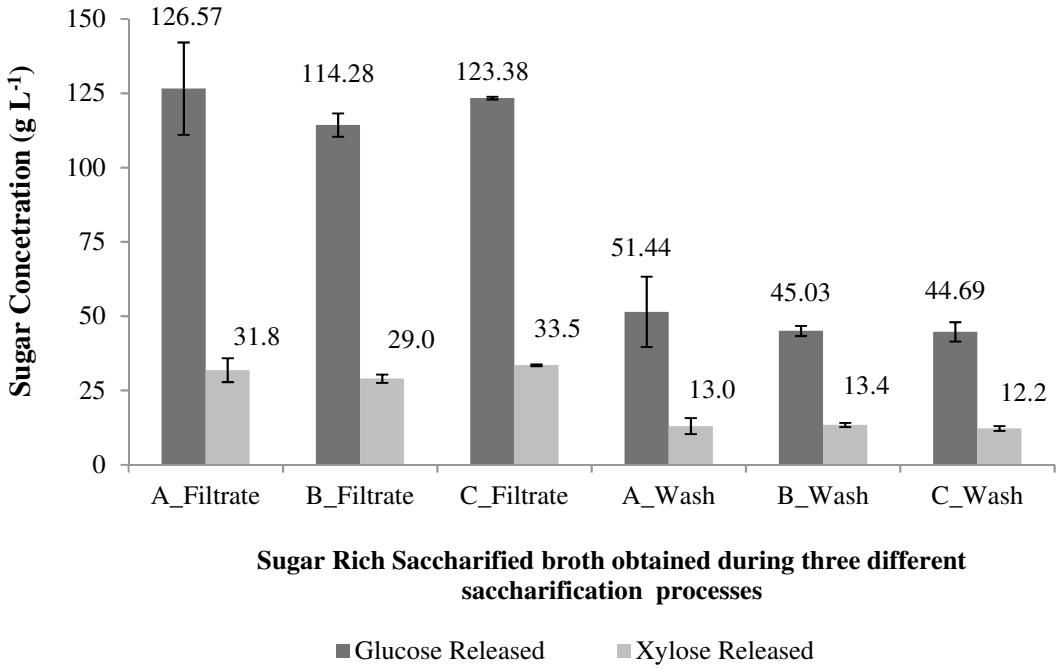

Fig. 2 Sugars (glucose and xylose) released during Cellic CTec2 mediated alkali pretreated sugarcane bagasse hydrolysis as seen in wash and filtrates using three different saccharification processes

Irrespective of the strategy employed for terminating saccharification, ≥80% and 75% cellulose and xylan hydrolysis was achieved respectively, as shown in Table S1. In the present study when the glucose-rich broth was removed from the residual biomass as in Process B to circumvent the problem of product inhibition, the enzyme bound to the bagasse could not contribute to a higher release of sugars. The low performance of the adsorbed Cellic CTec2 may be due to enzyme deactivation (by soluble, insoluble lignin and biomass-derived inhibitors) or inhibition by the unrecovered sugars (Hodge *et al.*, 2008; Puri *et al.*, 2013).

During our previous studies, increasing the substrate loading from 12.5 to 20% showed a sharp decline in cellulose hydrolysis from 77% to 68% (Nalawade *et al.*, 2020). Two-step substrate feeding regime (9+9) with a gap of one hour was able to overcome the drawbacks successfully. Considering Process A, a nearly two-fold increase in glucose concentration was observed in the filtrate fraction, and its productivity improved remarkably from 2.65 to 5.27 g L^{-1} h^{-1} (Nalawade *et al.*, 2020). Thus, this improved method of feeding favoured high substrate loading without comprising on glucose yields and productivity. The results also indicated that the glucose accumulation of >120 g L^{-1}severely impaired the rate of hydrolysis. The impedance in sugar release could be attributed to end-product inhibition of Cellic CTec2

and its successor CTec3 as discussed earlier (Hseih *et al.*, 2014; da Silva *et al.*, 2016). Therefore, further increasing the duration of hydrolysis from 24h (Process A) to 48h (Process C) did not enhance the glucose yields and saccharification efficiency was constant (~83%) in both the cases. A similar observation was made during fed-batch hydrolysis of 15% corn stover, where cellulose hydrolysis improved from 76.4 to 76.9% when residence time of Cellic CTec2 was increased from 72h to 120h (Geng *et al.*, 2015). Kadhum *et al.* also noticed a marginal improvement in glucose release (149.1 to 151.6 g L^{-1}) when the Cellic CTec2 mediated batch hydrolysis of 30% pretreated wheat straw was extended from 48 to 96h (2019).

The present results of achieving glucose concentration of 126.57± 4.03 g L^{-1} (filtrate fraction) and 85.7 ± 4.8 % cellulose hydrolysis in merely 24 h by adopting Process A is far superior to the finding of Liu *et al.* (2020). They chose fed-batch strategy for the Cellic CTec2 mediated hydrolysis of 30% alkali pretreated SCB and obtained 86.11 g L^{-1}glucose after 96 h. Earlier, Gao *et al.* also could get 129.5 g L^{-1} of glucose in 120h when they opted for fed-batch hydrolysis of alkali pretreated SCB with Cellic CTec2 enzyme (2014). Even Ramos *et al.* attained 69.2% cellulose hydrolysis with Cellic CTec2 in a batch process with 20% solids after 72h with 76.8 g L^{-1} glucose concentration (2015).

Table 1 highlights the glucose concentration and productivity attained by different researchers during HSES of SCB, pretreated by different strategies.

Table 1: Comparison of enzymatic saccharification of pretreated SCB at high-solids in different studies

Type of Pretreatment	Type of hydrolysis	Total Solid Loading (w/v)	Cellulase and accessory enzymes used	Time (h)	Sugar released (g L^{-1})	% cellulose hydrolysis	Glucose productivity (g L^{-1} h^{-1})	Reference
Alkali	Fed Batch	33% (12% initial followed by 7% after every 6h till 24h)	Cellic CTec2 @ 15.82 FPU/g glucan	120	glucose: 129.5 xylose: 56.03	60	1.07	Gao et al, 2014
Alkali and glycerol	Fed batch	20% (8% initial followed by 4% after every 6h till 18h) ; Additives: 40mg/g Tween 80, 10 mg/g tea saponin + 20 mg/g BSA	Cellic CTec2 @ 5.26 FPU/g glucan Accessory enzymes: 2.4 mg/g endo-xylanase and 1 mg/g AA9	72	glucose: 105 xylose: 51	83	1.45	Mukasekuru *et al.*, 2018
Alkali	Fed batch	22% (10% initial followed by 5, 4 and 3% after 8,12 and 16h); Additives: Whey protein -25 mg + Tween 80-40 mg + sophorolipid -25 mg + calcium lignosulfonate -10mg	Cellic CTec3 @ 11.54 mg/g glucan + 150 U hemicellulase/g dry matter and 60 mg β-glucosidase/g dry matter	72	glucose: 127 xylose: 44	80	1.76	Xu et al, 2019
H_3PO_4 impregnated steam-treated	Batch	20%	Cellic CTec2 @ 8.26 FPU/g glucan	72	glucose: 76.8	69.2	1.06	Ramos *et al.*, 2015
Hydrothermal	Batch	20%	Cellic CTec2 @ 20FPU/g glucan	72	-	69		da Silva *et al.*, 2016
Steam	Batch	20%	Cellic CTec3 @ 38.6 FPU/g glucan + Cellic HTec3	72	glucose: 120	-	1.66	Fockink *et al.*, 2017
Alkali	Batch	30%	Cellic CTec2 @ 16.61 FPU/g glucan	96	glucose: 86.1 xylose: 36.6	76.4	0.89	Liu *et al.*, 2020
Hydrothermal	Batch	20%	Cellic CTec2 @ 16.27 mg protein /g glucan	144	glucose: 95.37	70.27	0.66	Godoy *et al.*, 2019
Acid -alkali	Batch	20%	Cellic CTec2 @ 13.95 mg protein /g glucan	144	glucose: 115.5	73	0.80	Godoy *et al.*, 2019
Alkali	Batch Process A	18% ; Additive: PEG 6000 (0.2g/g AISL content)	Cellic CTec2 @ 25mg protein/g glucan	24	glucose: 126.6 xylose: 31.8 (Filtrate)	85.7	5.27#	This work
Alkali	Batch Process C	18% ; Additive: PEG 6000 (0.2g/g AISL content)	Cellic CTec2 @ 25mg protein/g glucan	48	glucose: 123.4 xylose: 33.5 (Filtrate)	83.4	2.57#	This work

 # refers to glucose productivity in the supernatant fraction excluding wash

Based on the obtained results, the authors feel that extending the time of hydrolysis beyond 24h was futile and unprofitable. Glucose productivity, in particular, decreased from 5.27 to 2.57 g $L^{-1}h^{-1}$, when the duration was extended to 48h (Fig.3). This result is in concurrence with the findings of Xu *et al.*, wherein stretching the Cellic CTec3 mediated hydrolysis of alkali pretreated SCB from 48h to 72h decreased the productivity of glucose by 30% (2019). If the results of the present study are extrapolated to one kg biomass (Figure 3), the minimum sugar yield obtained after pretreatment and saccharification correspond to 492 ± 2.71 g/kg SCB. These yields are far superior to our earlier results (Nalawade *et al.*, 2020) and cross the threshold benchmark of 400 g/kg biomass deciphered by Mark *et al.* for economically beneficial and favourable production of the biofuels (2020).

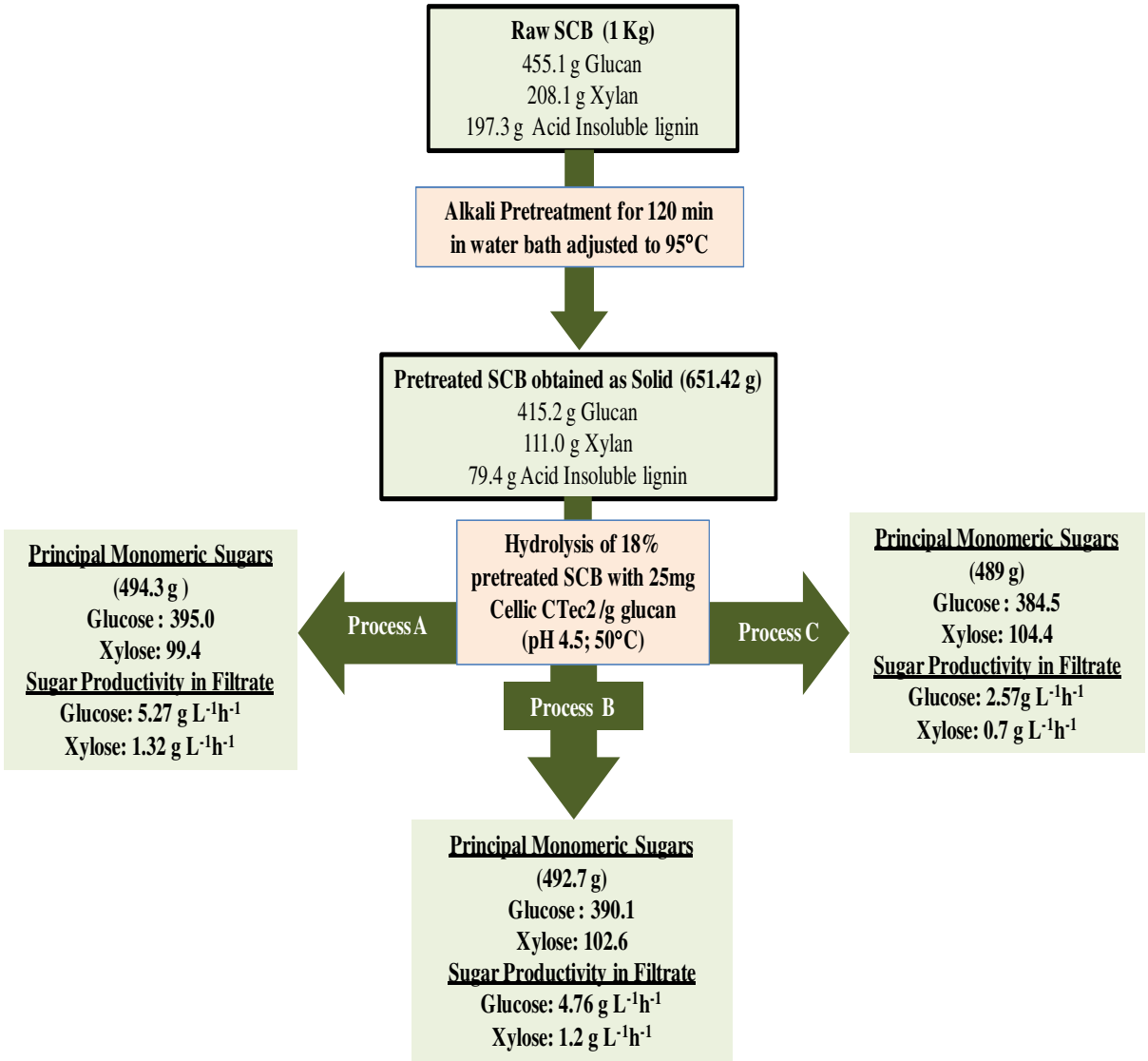

Fig. 3 Overall schematics of sugars flow during pretreatment and saccharification from one kg raw sugarcane bagasse based on the results of the present study and sugar productivities from different saccharification processes.

The present study shows that even shorter duration of HSES can generate concentrated sugar solution with uncompromised productivities. However, an appropriate selection of pretreatment and saccharification strategy is imperative. Pretreatment methods which lead to delignification often tend to reduce biomass recalcitrance and facilitate high digestibility by cellulases (Nguyen *et al.*, 2017; Chi *et al.*, 2019). Considering saccharification aspect, there is a strong need to break the traditional approach of loading new generation cellulase cocktails like their earlier versions. Also, their true hydrolytic potential should be harnessed with an entirely new perspective. The authors are of the view that longer duration with these enzymes is only recommended when loadings are extremely low (5-7.5 FPU or 7.5-10 mg protein/g cellulose content). There is immense scope for improving the saccharification process by identifying the maximum tolerance limit of these generation enzyme cocktails for their end products and inhibitors. Later these features can be correlated with the type of pretreatment rather than solely concentrating on developing newer pretreatment strategies.

Even during fed-batch hydrolysis, feeding regimes should be restricted to 8h as it represents the onset of the exponential phase of cellulose liquefaction. Once the hydrolysis enters this phase, the sugars released tend to impede the hydrolysis of fresh fed substrate, reducing the overall efficiency of the process. Moreover, substrate loading beyond 25% needs to be forbidden as by loading beyond this threshold value researchers are compelled to extend hydrolysis time for complete extraction of valuable fermentable sugars from biomass.

3.2. Valorization of released sugars to LA using fermentative B. coagulans NCIM 5648

The time course profile of glucose consumption and LA production by *B. coagulans* under the pH-stat condition at 50°C and 150 rpm are shown in Figure 4. Besides "L" isomer of LA as the primary product of fermentation, succinic acid and 2,3 BDO were also detected in HPLC after 12h, with their cumulative concentration's not exceeding beyond 1%.

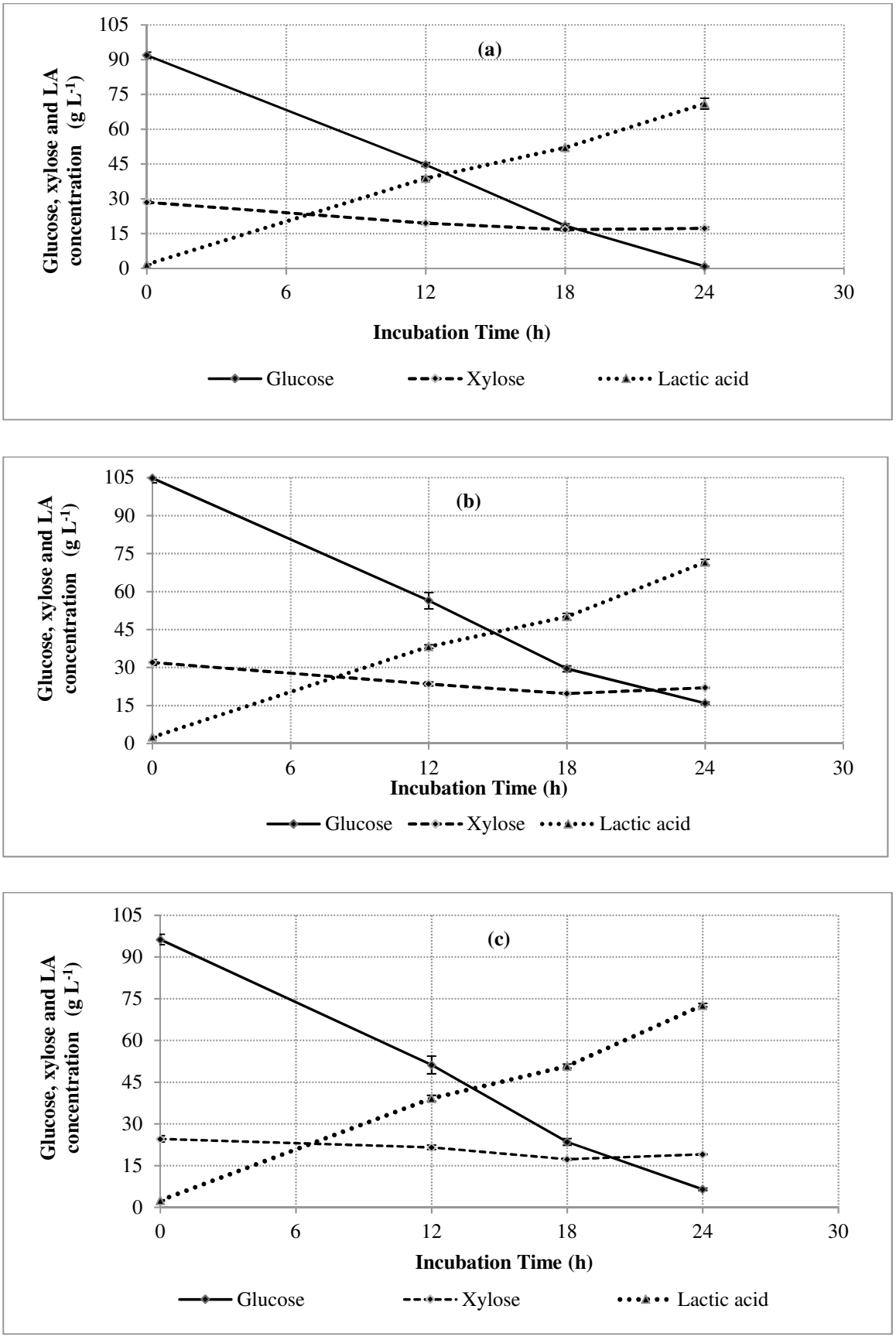

Fig. 4 Time course profile of glucose & xylose consumption and LA production by *B. coagulans* under pH stat conditions at 50°C and 150 rpm in filtrates obtained by (a) Process A (b) Process B (c) Process C.

As reported earlier, glucose was the preferred carbon source and xylose invariably remained unutilized. After 24 h fermentation, 71, 72.7 and 71.6 g L^{-1} of LA were produced from the filtrates obtained by Process A, B and C, respectively. Intermittent opening of flasks for pH restoration disfavored maintenance of complete anaerobic conditions. As a result, 20% glucose was diverted towards biomass accumulation which rose significantly (~9 fold). An LA productivity of $\geq$2.88 g $L^{-1}h^{-1}$ compared to 1.75 $gL^{-1}h^{-1}$ in our previous study confirmed that *B. coagulans* mediated fermentation was primarily dependent on the initial glucose concentration and undetectable inhibitors produced during SCB hydrolysis at high-solids had no impact on the performance of the bacterium (Nalawade *et al.*, 2020). These results are highly encouraging when compared to the duration taken and LA yields by various researchers for the production of L (+) lactic acid from different lignocellulosic feedstocks using SSF approach (Table 2).

Table 2: Summary of HSES carried out with different types of pretreated lignocellulosic biomass and subsequent LA fermentation

Biomass and pretreatment	Hydrolysis and Fermentation	Conditions during Enzymatic saccharification and fermentation	Total Duration (h)	LA formed ($g\ L^{-1}$)	LA yield	Reference
NH_3-H_2O_2-pretreated corncob	Fed Batch SSCF[$]	Total Solid Loading: 16% with initial loading 8% and 4% loading after 18h and 24h Enzyme: Cellic CTec2 @ 75 FPU/g glucan Conditions: pH stat-6.0; Temp-50°C; 100 rpm Organism used: *Bacillus coagulans* LA 204	90	118.6	0.74 g/g of total stover (glucose and xylose)	Zhang *et al.*, 2016
Dilute acid pretreated corn stover	Batch SSF*	Total Solid Loading: 27% in 50L bioreactor Enzyme: Accellerase 1000 @ 15 FPU/g substrate Conditions: pH stat-5.5; Temp-48°C; 150 rpm Organism used: *Pediococcus acidilactici* DQ2	8[a] + 96 (104)	101.9	0.77 g/g glucose consumed	Zhao *et al.*, 2013
NaOH pretreated sweet sorghum bagasse (SSB)	Batch SSF*	Total Solid Loading: 15% Enzyme: Cellulase from Tianfeng Bioengineering Corporation, China @ 48.38 FPU/g glucan Conditions: pH stat-5.6; Temp-50°C Organism used: *Bacillus coagulans* LA1507	~70	74	0.59 g/g SSB	Wang *et al.*, 2016
Dilute acid pretreated corn stover	Batch SSF*	Total Solid Loading: 30% Enzyme: Youtell #6 @ 28 mg protein/g glucan content Conditions: pH stat-5.5; Temp-45°C; 150 rpm Organism used: *Pediococcus acidilactici* TY 112	6[a] + 72 (78)	87.8	0.62 g/g glucose consumed	Liu *et al.*, 2015
Dry acid pretreated and bio-detoxified corn stover	SSCF[$]	Total Solid Loading: 30% Solids containing 33.3% cellulose, 3.35% xylan and 11.5% xylose as principal components and 12.6 % other soluble sugars. Enzyme: Cellic CTec2 @ 15 mg protein/g glucan Conditions: pH stat-5.5; Temp-42°C; 150 rpm Organism used: *Pediococcus acidilactici* ZY271	6[a] + 72 (78)	130.2		Han *et al.*, 2018
Beechwood organosolv hydrolysate	Fermentation of hydrolysate	Prehydrolysate containing (g/L) nearly 82.25 glucose; 21.81 xylose and 27.23 cellobiose Conditions: pH stat-6.0; Temp-52°C; Organism used: *Bacillus coagulans* DSM *2314*	30	79.4± 2.1	0.6 g/g xylose and glucose consumed	Glaser G and Venus J., 2018
NaOH pretreated corn stover	Fed Batch SSCF[$]	Total Solid Loading: 14.4% with initial loading 8% and rest within 18-20h Enzyme: Cellic CTec2 @ 53.6 FPU/g glucan content Conditions: pH stat-6.0; Temp-50°C; 100 rpm Organism used: *Bacillus coagulans* LA 204	60	97.59	0.68 g/g of total stover (glucose and xylose)	Hu *et al.*, 2015

NaOH pretreated sugarcane bagasse	Batch SHF[#]	Total Solid Loading: 18% Enzyme: Cellic CTec2 @ 25mg protein/g glucan Conditions: pH stat-7.0; Temp-50°C; 150 rpm Organism used: *Bacillus coagulans* NCIM 5648	24[b] + 24 (48)	69.2 ± 2.5	0.759 g/g glucose consumed	This study

*SSF refers to simultaneous saccharification and fermentation; SHF[#] refers to Separate hydrolysis and fermentation; SSCF[$] refers to simultaneous saccharification and co-fermentation of two sugars namely glucose and xylose; [a] refers to pre-hydrolysis without fermenting microbe; [b] refers to enzymatic saccharification in batch mode

This investigation gains more significance in the light of recent findings by Kadhum *et al.*, where the targeted product was ethanol (2019). During their study, SHF was compared to SSF with 30% dilute acid pretreated wheat straw using Cellic CTec2. They inferred that lower processing times during saccharification and fermentation not only had a lower environmental impact (during sensitivity analysis) but it also played an instrumental role on Return on Investment (ROI). Even Müller *et al.* also reported 26-32% more LA yields in SHF set up as compared to SSF while carrying out hydrolysis of 10% steam-exploded birch with Cellic CTec2 in combination with lactic acid bacteria (2017).

With the existing productivities of glucose and LA obtained in the present SHF study, the authors believe that sugar industries can intervene and collaborate with research groups working on similar lines to evaluate the proposed scheme for efficient SCB valorization and process integration. Holistic utilization of entire SCB is envisaged by feeding the dewatered lignin-rich black liquor (obtained after alkali pretreatment) to the boilers for steam generation. A significant improvement in the LA yields and productivity is foreseen; when the same experiments are shifted from shake flasks to bioreactor wherein the microaerophilic environment, including temperature and pH conditions, can be controlled tightly. Xylose which presently remains unutilized can be removed by sequential hydrolysis with acid or hydrothermal treatment followed by alkali. We envisage to go for process integration wherein xylose can be transformed to valorized products like succinic acid and xylitol, as demonstrated by our collaborators (Prabhu *et al*., 2020a; Prabhu *et al*., 2020b).

In the future, we target to reduce the enzyme dosage by at least 1.5 times by optimizing feeding strategy, using in-house developed enzymes in combination and working towards process conditions or working towards enzyme recycling and reuse.

4. Conclusion

The present study showed that the expeditious production of glucose was possible from 18% alkali pretreated SCB, with unprecedented glucose titers (~126 g L^{-1}) in 24 h and its productivity being 5.27 g L^{-1} h^{-1}. Its direct valorization led to the production of 69.2± 2.5 g L^{-1} L (+) lactic acid within 24 h with LA productivity being 2.88 g L^{-1} h^{-1}. This study proves that there is an enormous scope to maximize and exploit the efficiency of new generation cellulases, thereby sustainably producing sugar syrups from LCB. An environmentally benign process is driven by these enzymes for cellulose hydrolysis, which can be transformed into any commercially viable product, LA being a representative in the present case.

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Figure Captions:

Fig.1 Schematics of terminating Cellic CTec2 aided saccharification conducted with alkali pretreated sugarcane bagasse

Fig. 2 Sugars (glucose and xylose) released during Cellic CTec2 mediated alkali pretreated sugarcane bagasse hydrolysis as seen in wash and filtrates using three different saccharification processes

Fig. 3 Overall schematics of sugars flow during pretreatment and saccharification from one kg raw sugarcane bagasse based on the results of the present study and sugar productivities from different saccharification processes.

Fig. 4 Time course profile of glucose & xylose consumption and LA production by *B. coagulans* under pH stat conditions at 50°C and 150 rpm in filtrates obtained by (a) Process A (b) Process B (c) Process C.

Fig. S1 Cellulose and xylan saccharification efficiency during Cellic CTec2 mediated alkali pretreated sugarcane bagasse hydrolysis at different time points.

Supplementary information

Fig S1: Cellulose and xylan saccharification efficiency during Cellic CTec2 mediated alkali pretreated sugarcane bagasse hydrolysis at different time points.

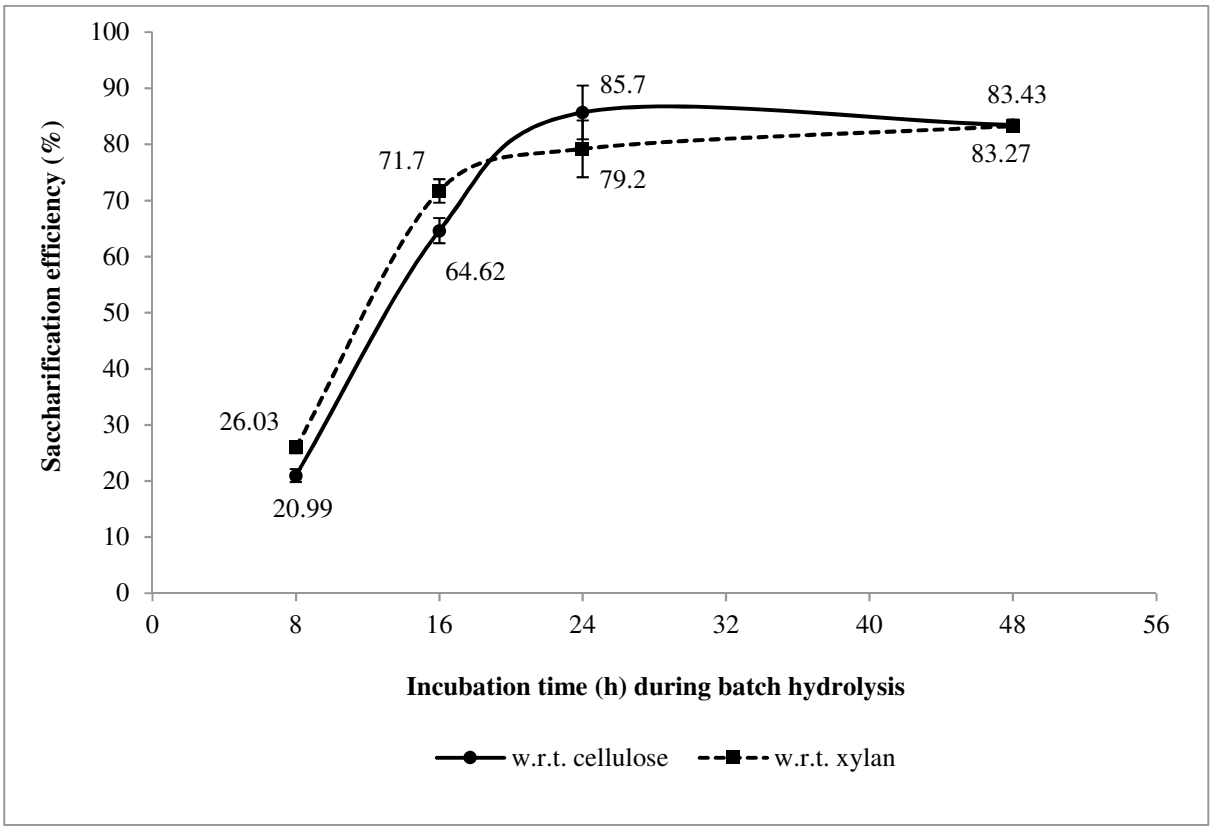

Table S1: Sugars released (g) and saccharification efficiency (%) from 18g alkali pretreated SCB by Cellic CTec2 at 50°C and pH 4.5

Process conditions	Total sugars released (g)		Saccharification efficiency (%)	
	glucose	xylose	w.r.t cellulose	w.r.t. xylan
A	10.91 ± 0.61	2.75 ± 0.17	85.69 ± 4.8	79.21 ± 5.06
B	10.78 ± 0.70	2.84 ± 0.22	84.64 ± 5.5	81.82 ± 6.36
C	10.62 ± 0.12	2.89 ± 0.02	83.43 ± 1.0	83.27 ± 0.71