DISCUSSION

The extravagant energy demands of humans have put an enormous pressure on the fossil fuels, current major source of energy. At the current pace of utilization, the non-renewable fossil fuels will be exhausted soon. Moreover, concerns about greenhouse gas (GHG) emissions and increasing carbon foot print in environment have driven the researchers to explore a non-conventional, sustainable fuel (Kuhad et al., 2011a; Saini et al., 2016). Bioethanol is one such biofuel which when blended with petrol provides better thermal efficiency and less CO_2 emissions as compared to unblended petrol.

Bioethanol can be produced by utilizing lignocellulosic agricultural residue through microbial conversion. Though second generation bioethanol has numerous benefits, its production process is complex which involves pretreatment, enzymatic saccharification and fermentation. Each of these steps has its own technical challenges, some of which have been addressed in the present investigation in order to make the whole bioconversion process cost-effective.

Selection of lignocellulosic biomass

Lignocellulosic biomass is considered as the future feedstock for ethanol production because of its low cost and huge availability. The total LCB production in India alone exceeds 680 metric ton per annum which accounts for production of approximately 52 billion litres of bioethanol (Jain & Agrawal, 2018). The LCB biomass chosen in the present investigation was sugarcane bagasse, which is one of the major lignocellulosic biomass generated in large quantities. Sugarcane is one of the highly produced crops in India. Durimg 2019-20, sugarcane crop production was 376 metric ton, resulting in generation of nearly 100 million tons of residual bagasse which could be utilized for bioethanol production (Konde et al., 2021).

Screening and selection of thermotolerant ethanologenic yeasts

The isolation and screening of the efficient thermotolerant ethanol producing yeasts can be helpful in simultaneous saccharification and fermentation of SCB for biofuel production. During the study, 150 thermotolerant yeasts were isolated, among which 36 isolates were found to produce ethanol during glucose fermentation at 42 °C. Isolates *Pichia kudriavzevii* JKH 1 (54 g/L) and *Kluyveromyces marxianus* JKH 5 (55 g/L) were the most potential ethanol producers. Previously, several researchers have reported yeasts isolation from different environmental sources for cost-effective ethanol production. A study by Arora et al. (2015) reported ethanol production by *K. marxianus* at 45 °C. Kaewkrajay et al. (2014) isolated thermotolerant yeast from the soil samples collected from sugarcane, cassava and pineapple plantations. In a recent study by Gao et al. (2018), sugarcane bagasse was utilized for ethanol production by a thermotolerant yeast *K. marxianus*. The main advantage of using thermotolerant yeasts is the faster rate of ethanol production which is an industrial relevant feature.

Adaptive laboratory evolution of yeast for bioethanol

The inhibitors generated during physico-chemical pretreatment of lignocellulosic biomass (furfural, 5-HMF, acetic acid, formic acid, vanillin, etc.) make the process of bioethanol production challenging by reducing the growth of yeast strains and hence, fermentation efficiency while using unwashed pretreated biomass. The effect of three different inhibitors on growth and fermentation of the yeasts clearly showed that the inhibitors were toxic to yeast as evident from reduced specific growth rate and longer lag phase. The lag phase time and specific growth rate were chosen as selection parameters for monitoring the improvement in inhibitor tolerance of the strains during repetitive batch culture in adaptive laboratory evolution experiments (Çakar et al., 2005).

The adapted strains grew well with significantly reduced lag phase in the presence of inhibitors during fermentation. This was most apparent during the lag phase upon inoculation in inhibitors-containing media. *P. kudriavzevii* JKH1 and *K. marxianus* JKH5 both showed longer lag phase in the initial stages of evolutionary experiments. The developed yeast *K. marxianus* JKH5 C60 showed 3.3 folds higher specific growth rate and 56% reduced lag time as compared to parent strains in the presence of inhibitor cocktail. The detrimental effect of inhibitors could be due to inhibition of the enzymes of the central carbon metabolism and disturbance of the cells energy balance, following their accumulation inside the cell (Modig et al., 2002; Sárvári Horváth et al., 2003).

It is expected that the adaptation under medium supplemented with inhibitors and other stresses might introduce new features to the yeast strains, which are favorable for the bioethanol production process. Interestingly, both of the strains *P. kudriavzevii* JKH1 and *K. marxianus* JKH5 displayed almost comparable fermentation yields at 42 °C using glucose as carbon source. As shown in **Table 5.1** ethanol titer and productivity were better than the other previous studies in the presence of inhibitors. Additionally, the strains in the current study are thermotolerant and grown at higher concentrations of glucose and therefore, have multiple stresses to combat. In the present study, higher ethanol yields during fermentation were achieved at elevated temperature (42 °C). Wallace-Salinas and Gorwa-Grauslund (2013) developed *Saccharomyces cerevisiae* (ISO12) for tolerating cocktail of fermentation inhibitors. During the fermentation of spruce hydrolysate at 39 °C, the ethanol produced was 16 g/L. Another study by Narayanan et al. (2016) developed *Saccharomyces cerevisiae* TMB3500 strain tolerance against acetic acid, furfural, 5-HMF and vanillin. Under synthetic medium, the yield of ethanol produced was 0.45 g/g by fermenting glucose.

Table 5.1: Comparison of ethanol production by the adapted yeasts developed in the present study with previously reported yeasts

S. No.	Strain	Fermentation conditions				Ethanol		References
		Concentration Of inhibitors (g/L)	Temperature (°C)	Carbon source	Mode (Source of sugar)	Titer (g/L) and yield (g/g)	Productivity (g/L/h)	
1.	*Saccharomyces cerevisiae* F12	Acetic acid (5.2-6.8) Furfural (1.4-1.6) Vanillin (0.1)	30	Mixture of Xylose and Glucose	Fed-Batch SSF (Prehydrolysate of wheat straw)	27.4/n.d.	0.19	Tomás-Pejó et al. (2010)
2.	*Saccharomyces cerevisiae* (ISO12)	Acetic acid (5.8) Furfural (0.5) HMF (1.5)	39	Majorly Glucose	Batch Simultaneous saccharification and fermentation (Spruce hydrolysate)	16/0.38	0.3	Wallace-Salinas and Gorwa-Grauslund (2013)
3.	*Pichia stipitis* CBS 5776	Acetic acid (2.03) Furfural (0.10) HMF (0.15) Levulinic acid (0.12)	30	Xylose	Separate hydrolysis and fermentation (Prehydrolysate of corn strover)	15.92/ n.d.	0.66	Zhu et al. (2009)
4.	*Saccharomyces cerevisiae* S-adhE	Acetic acid (2)	30	Majorly Xylose	Separate hydrolysis and fermentation (Hydrolysate of corn strover)	41/0.414	0.51	Wei et al. (2013)
5.	*Saccharomyces cerevisiae* S-nc	Acetic acid (2)	30	Majorly Xylose	Separate hydrolysis and fermentation (Hydrolysate of corn strover)	40.0/0.390	0.5	Wei et al. (2013)
6.	*Pichia stipitis* strain NRRL Y-7124	Acetic acid (1.8) Furfural (0.2) HMF (0.3)	25	Mixture of Xylose and Glucose	Separate hydrolysis and fermentation (Hydrolysate of corn	40/ n.d.	0.23	Slininger et al. (2015)

					strover)			
7.	*Saccharomyces cerevisiae* TMB3500	Acetic acid (6) Furfural(1.5) HMF (0.5) Vanillin (1)	30	Glucose	Batch Fermentation (Synthetic medium)	n.d./0.45	n.d.	Narayanan et al. (2016)
8.	*K. marxianus* FIM1	Ethanol (10%, v/v)	30	Glucose	Batch Fermentation (Synthetic medium)	110/n.d	2.2	Mo et al. (2019)
9.	*K. marxianus* FIM1	Ethanol (10%, v/v)	45	Glucose	Batch Fermentation (Synthetic medium)	58/n.d	1.3	Mo et al. (2019)
10.	*K. marxianus* NIRE-K3.1	Xylose (30)	45	Xylose	Batch Fermentation (Synthetic medium)	15.7 (Xylitol) 4.67 (Ethanol)	0.22 and 0.1	Sharma et al. (2017)
11.	*Kluyveromyces marxianus* JKH5 C60	Acetic acid (3) Furfural (1) Vanillin (1)	42	Glucose	Batch Fermentation (Synthetic medium)	20.0/0.40	1.11	Current study
12.	*Pichia kudriavzevii* JKH1 C70	Acetic acid (3) Furfural (1) Vanillin (1)	42	Glucose	Batch Fermentation (Synthetic medium)	20.79/0.40	1.15	Current study

n.d = not determined, HMF – 5-hydroxy – 2 –methyl furfural

Sequential dilute acid-alkali pretreatment of sugarcane bagasse

Present study employed sequential dilute acid-alkali pretreatment of sugarcane bagasse (SCB) for enhancing its bioconversion to ethanol. Box-Behnken and D-optimal designs were used to optimise the process of dilute acid and alkali pretreatments sequentially, resulting in an optimum concentration of 3% (v/v) and 5% (w/v) for H_2SO_4 and NaOH with solid SCB loadings of 18 and 15% (w/w), respectively, for 30 min at 121 °C. The effectiveness of sequential pretreatment was supported by increased cellulose content (83%), drop in hemicellulose, lignin content of the pretreated bioimass. The obtained cellulose content after dilute acid pretreatment in this study was better than that reported in (R.G. et al., 2012), while it was slightly less as compared to obtained in the study by Aguiar et al. (2010) (Table 5.2). Therefore, to further enhance the cellulose content, delignifcation of dilute acid pretreated SCB was attempted using dilute alkali pretreatment method (Kaur et al., 2012). Thus overall sequential pretreatment lead to efficient removal of hemicellulose and lignin due to which higher cellulose content was obtained in comparison to the previous reports (Ahmadi et al., 2016; Binod et al., 2012; Talha et al., 2016) (Table 5.2).The characterization of SCB was done using techniques like FT-IR, XRD, TGA, SEM, SANS which revealed favorable structural changes in crystallinity, porosity, thermostability etc. after pretreatment. . The results of FT-IR analyses showed highly reduced peaks at 1386 cm^{-1} (C-O of syringyl) and 1268 cm^{-1} (C-O of guaicyl ring) in spectra of sequential dilute acid-alkali pretreated SCB were in agreement with previous reports (Pasma et al., 2013; Phitsuwan et al., 2017; Singh et al., 2005).

During SEM analysis, cell wall of sequentially pretreated SCB cell wall appeared ruptured and porous with piths on surface, and had detached fibers as a consequence of delignification. Simiar results have been been reported in previous study by (Zhu et al., 2016). TG analysis of SCB indicated difference in the pattern of thermal degradation of untreated and pretreated

biomass. The final decomposition stage for all samples was completed above 400 °C, which was in correlation with the results by Ávila-Lara et al. (2015). The values of T_{max} for untreated and dilute acid pretreated SCB were 493 and 495 °C, respectively. Contrastingly, the biomass after sequential pretreatment had significantly lower T_{max} (441°C), which could be attributed to its increased amorphous nature and hence, lowered thermostability. The results TG analysis of pretreated SCB were in agreement with Brugnago et al. (2011). Crystallinity is a crucial property of LCB which negatively affects its hydrolysis by lignocellulolytic enzymes. Generally, XRD or the wide angle X-ray scattering (WAXS) is used to reveal crystallinity of LCB after the pretreatment (Zhang et al., 2015). The diffraction pattern of SCB (untreated and pretreated) in the current study was similar to cellulose-I lattice as interpreted by three diffraction peaks, the main one at 22.18°, secondary one at 16.26° and smallest one at 34.64°. This indicated that the crystallnity was significantly decreased after pretreatment as reported in earlier reports (Cheng et al., 2015; Yuan et al., 2017).

SANS has recently emerged as a robust technique for characterization of porous materials and can measure total porosity in a range of 1 to 100 nm. SANS analysis of the pretreated biomass in the present investigation, indicated that the scattering power was proportional to the density of the pores and the porosity of the biomass was increased after pretreatment. The increased pore density favours better accessibility of cellulose to enzymes and hence, enhanced sugar yield (Pingali et al., 2017). Previously, SANS was utilized for assessing the relative porosity of eucalyptus, white poplar and pine samples after pretreatment by ionic liquids (Yuan et al., 2017). There are very limited reports on SANS analysis of pretreated biomass for its characterization and none of the previous studies has focused on SANS analysis of SCB. This is the first report on SANS analyses of SCB carried out in India.

Table 5.2: Compositional analysis of untreated and pretreated sugarcane bagasse

S.No.	Untreated SCB			Pretreated SCB			Method of pretreatment	References
	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)		
1	51.10±1.8	25.19±2.5	13.36±1.4	63.31	2.53	25.53	Dilute Acid	**Current study**
2	51.10±1.8	25.19±2.5	13.36±1.4	83.30	1.26	8.30	Sequential acid alkali	**Current study**
3	46.20	31.20	9.74	50.30	21.80	6.65	Dilute Alkali	Ahmadi et al. (2016)
4	43.20	25.20	22.90	57.50	6.60	32.50	Steam explosion	Rocha et al. (2012)
5	43.20	25.20	22.90	86.80	4.0	6.10	Sequential Steam explosion and Dilute Alkali	Rocha et al. (2012)
6	55.34	25.87	11.21	70.08	5.87	12.22	Dilute Acid	R.G. et al. (2012)
7	55.34	25.87	11.21	53.40	11.98	9.75	Dilute Alkali	Aguiar et al. (2010)
8	55.34	25.87	11.21	59.50	8.46	13.37	Hydrogen peroxide (alkaline)	Aguiar et al. (2010)
9	34.0	27.0	18.0	66.60	26.50	4.90	Microwave assisted alkali	Binod et al. (2012)
10	38.59	27.89	17.79	64.89	9.61	7.85	Acid and alkaline	Guilherme et al. (2017)
11	36.0	28.70	18.0	35.70	18.10	14.0	Alkaline	Carvalho et al. (2016)
12	35.60	32.20	22.50	40.10	8.70	4.70	Alkaline	Talha et al. (2016)

Enzymatic hydrolysis of pretreated sugarcane bagasse

The bioconversion of lignocellulosic biomass to fermentatble sugar is carried by enzymatic hydrolysis using cellulase. Cellulases are complexes of enzymes that work synergistically to bring about the breakdown of cellulose (Lynd Lee et al., 2002). Cellulase producing microbes include various fungi such as *Trichoderma reesei, T. koningii, T. lignorum, Penicillium funiculosum, P. chrysoporeum, P. oxalicum, Aspergillus wenti, A. niger, Fusarium solani,* and bacteria like *Clostridium sp, Psuedomonas sp., Bacillus sp., Serratia marscens* (Deswal et al., 2011; Kuhad et al., 2011c). Most of the cellulase producing microbes do not have complete cellulase system and therefore, could not efficiently hydrolyze lignocellulosic biomass (Ahmad et al., 2013; Hemansi et al., 2018). This shortcoming has been overcome by using commercial cellulase preparations, which have higher hydrolytic efficiency. Few of the commercial cellulases available in market include Spirizyme from Novozymes A/s, Cellic 2 from Novozymes A/s, SacchariSabC6 from Advance Enzymes, Ctec series from Novozymes, Cellulase from Zytex, Accelarase from Dupont, Cellulase blend and Cellulclast from Sigma-Aldrich etc. Though the commercial enzyme formulations are expensive, these are highly efficient in hydrolysis of LCB (Hung et al., 2018; Thite & Nerurkar, 2019).

In the current study, hydrolysis of pretreated SCB was investigated with both in-house cellulase and commercial cellulases. It was found that commercial enzyme (Cellulase blend from Sigma-Aldrich) was more efficient during hydrolysis of pretreated SCB (78% saccharification) than in-house cocktail cellulase (67% saccharification). The lower efficiency of the in-house cellulases of *Trichoderma* sp. *Penicillium* sp. and *Aspergillus* sp. might be due to lower Bgl/FPU ratio and low efficiency of cellobiohydrolase enzyme as has been reported earlier (Kuhad et al., 2011c). However, the commercial enzyme preparation (Sigma) are formulated to have better Bgl/FPU ratio resulting in better hydrolysis (Singhania et al., 2009). Hence, commercial cellulase enzyme was used for further hydrolysis and

fermentation experiments to obtain higher sugar yields and subsequently higher ethanol production.

Bioethanol production from sugarcane bagasse

Separate hydrolysis and fermentation (SHF) of pretreated SCB was performed at optimum conditions of the enzyme and yeast respectively, for hydrolysis and fermentation steps (Singhania et al., 2014) The optimal conditions for release of maximum reducing sugar and ethanol production were further utilized under simultaneous saccharification and fermentation process. In the present study ethanol titer obtained was 54.9 g/L during separate hydrolysis and fermentation (SHF) of SCB which was better than the titer of 33 g/L and 40 g/L reported by Méndez et al. (2019) and Slininger et al. (2015), respectively.

Simultaneous saccharification and fermentation of sugarcane bagasse

SSF process is considered better than SHF for bioethanol production due to use of a single vessel, low inhibition of enzyme by feedback mechanisms and overall better conversion efficiency. However, the hydrolysis temperature has to be compromised during SSF when using a mesophilic fermenting microorganism. Therefore, in order to achieve better ethanol production by alleviating the mismatch of the optimal temperature of the enzymes (near 45-55 °C) and that of the fermenting microorganisms (28-35 °C), a thermotolerant yeast *Kluyveromyces marxianus,* capable of fermentation above 40 °C was employed during current study. Previously, several thermotolerant yeasts belonging mainly to genera *Kluyveromyces* have been successfully used to produce higher ethanol ranging between 40 to 80 g/L under batch SSF (Choudhary et al., 2017; Hacking et al., 1984; Hughes et al., 1984). In the present study, the adapted yeast *Kluyveromyces marxianus* JKH5 C60 was employed for batch SSF of pretreated sugarcane bagasse (20 %, dry wt. SL) at 42 °C, resulting in maximum ethanol titer of 70.1 g/L. The titer reported here was higher than the titer of 63.15 g/L and 12.6 g/L obtained previously under similar conditions of fermentation by Gao et al.

(2018) and Ballesteros et al. (2002), respectively. The ethanol titers of 18 and 14.2 g/L reported by Hoyer and co-workers, (2010) during fermentation of spruce hydrolysate at 10 and 14% solid loading, respectively, were also lower than the current report.

Fed-batch simultaneous saccharification and fermentation of sugarcane bagasse for enhanced bioethanol production

SSF under fed-batch mode has the potential to improve ethanol production at high gravity of LCB. Various strategies have been followed in the past for feeding biomass, enzyme or/and inoculum at different time intervals in order to enhance the conversion efficiency and yield of ethanol (Liu et al., 2020; Zhang & Zhu, 2017). In the current study, strategy of feeding biomass and enzyme at 6 and 12 h produced maximum ethanol during SSF at 42 °C employing *K. marxianus* JKH5 C60. Under batch SSF ethanol production was 70.1 g/L, in comparison to 84.9 g/L under fed-batch SSF at 20 % (dry wt.) solid loading. The fermentation efficiency (~80%) was also increased at high solid loading during the fed-batch process. The enhancement in ethanol production during FBSSF of SCB was comparable with the previous study (Mukasekuru et al., 2018) employing feeding of enzyme and biomass (SCB). Another research by Darkwah et al. (2016) employed fed-batch SSF of sweet sorghum bagasse at variable solid loadings and reported higher ethanol titers and yields than that obtained in the batch process. In a recent study, Gao et al. (2018) also reported a higher concentration (75.57 g/L) of bioethanol during fed-batch SSF of SCB at high solid loading than the titer (62.65 g/L) obtained under batch SSF. The authors also reported that the use of alkali pretreated bagasse was an important factor in improving cost and efficiency of bioethanol production by improving the specific surface area and cellulose accessibility to enzymes, low water consumption, and energy usage. Similarly, in the current study, the sequential dilute acid-alkali pretreatment improved the digestibility of SCB, thereby, enhancing ethanol titer and yield during fermentation.

Under fed-batch SSF in the presence of inhibitors, the ethanol titer (73.4 g/L) was 88% of the maximum titer obtained when no inhibitor was present during fermentation. The reported ethanol production in presence of inhibitors was much higher than the similar studies reported in Table 5.3. For example, Kassim and co-workers (2016) reported an ethanol titer of 10.1 and 9.21 g/L, under batch and fed-batch SSF, respectively. Similarly, (Chang et al., 2012) also suggested higher ethanol yields during FBSSF (32 g/L) than batch SSF (23 g/L) at high solid loading. Comparison of the ethanol production under batch and fed-batch SSF indicates that fed-batch SSF is obviously a better option for large scale bioethanol production at higher solid loadings (>15%) and biomass containing inhibitor.

Thus, the yeast strain *K. marxianus* JKH5 C60 developed in this study can efficiently carry out the fermentation of unwashed biomass after pretreatment and can help decrease the overall cost, time, and wastewater generation during high titer bioethanol production.

Table 5.3: Comparison of Fed-batch and batch SSF processes for cellulosic ethanol production

Substrate	Microorganisms	Enzyme*	Ethanol Titer (g/L)		References
			Fed-Batch	Batch	
Recycled paper	*K. marxianus*	Celluclast	17.7	12.6	Ballesteros et al. (2002)
Spruce	*S. cerevisiae*	Celluclast	~19	~14.2	Hoyer et al. (2010)
Spruce	*S. cerevisiae*	Celluclast	~17	~18	Hoyer et al. (2010)
Newspaper waste	*S. cerevisiae*	Cellulase+Xylanase+ Novozyme 188	14.77	5.64	Kuhad et al. (2010)
Corn-cob	*S. cerevisiae*	Cellulase+ Novozyme 188	32.3	23.0	Chang et al. (2012)
Wheat straw	*S. cerevisiae*	Cellic CTec2 and Cellic HTec2+Laccase	32	19	Moreno et al. (2013)
Sugarcane bagasse	*S. cerevisiae*	Cellic CTec2	75.57	63.15	Gao et al. (2018)
Chlorella sp.	*S. cerevisiae*	Cellulase (Sigma)	10.1	9.21	Kassim et al. (2019)
Sago hampas	*S. cerevisiae*	Spirizyme® fuel HS	111.88	62.65	Muradi et al. (2020)
Sugarcane bagasse (shake-	*K. marxianus* JKH5	Cellulase blend	84.9 ± 2.5	70.1 ± 1.7	Current study
Sugarcane bagasse (fermenter)	*K. marxianus* JKH5	Cellulase blend	81.9 ± 3.4	72.4 ± 3.7	Current study

*The enzymes used in the study are commercially available in market.

Fermentation of pentose sugar present in acid hydrolysate for ethanol

Under biorefinery approach, biomass feedstock is converted into more than one useful product such as fuel and chemicals and there is near zero waste emission. Therefore, biorefinery approach is considered more sustainable for economic and efficient production of bio-based products. In the current study, biorefinery was employed to maximise ethanol production from both hexose (derived from cellulose) and pentose (derived from hemicellulose). The capability to efficiently ferment pentose sugars is not prevalent among microbes and only few yeasts have been reported to be promising pentose fermenters, such as *Candida* sp., *Pichia* sp., and *Pachysolen tannophilus* (Abbi et al., 1996; Gírio et al., 2010; Hahn-Hägerdal et al., 2007; Palmqvist & Hahn-Hägerdal, 2000).

In the current study, *Pichia stipitis* NCIM 3499, previously reported as a potent xylose fermenting yeast (Gupta et al., 2012), was utilised for producing ethanol from dilute acid hydrolysate of SCB. The ethanol titer obtained (6.8 g/L) in the present study, was less than 9.4 g/L from acid hydrolysate of rice straw reported by Kaur and Kuhad (2019) and 11.8 g/L from acid hydrolysate of corn cob reported by Gupta et al., (2012). However, the ethanol yield of 0.34 g/g in the present study was comparable to the yield of 0.37 g/g reported by da Silva et al. (2010). Our results were better than the study of Codato et al. (2018) and Martins et al. (2018) who reported ethanol titers of 5.9 g/L and 6 g/L while fermenting xylose.

Mass Balance

The primary objective of this study is to analyse the mass balance in each unit operations for sorghum biomass to ethanol conversion. Mass balance analysis is necessary for assessing the commercial feasibility of the process since loss of the biomass components occurs during each of the operational step and it is necessary to account for this loss (Akanksha et al. 2016). In the present study, during dilute acid pretreatment, hemicelluloses of SCB were hydrolysed. The acid hydrolysate consisted pentose sugars, majorly xylose, along with inhibitory compounds like acetic acid, furans (furfural and HMF) and phenolics. The cellulignin biomass remaining after acid pretreatment was subsequently pretreated with dilute alkali, which majorly removed the lignin fraction, thereby, enhancing amorphous cellulose content. The overall loss from the native biomass reported here is comparable to the study by Rocha et al. (2012). During the first step of pretreatment, the biomass recovery was 64% and the loss was majorly due to 92 % removal of hemicellulose. After the second step of pretreatment, the biomass recovery was 88.9% and the loss was majorly due to solubilisation of lignin. Thus, during sequential pretreatment, total solid biomass recovery was 54%. Considering all the components (including cellulose, hemicellulose, lignin and degradation products) in solid and liquid fractions, overall material which could be accounted for was 94.1% of the initial biomass, the remaining being lost during sugar degradation and washing while physico-chemical treatments. In a previous study, a total of only 76.4% of the material was accounted for during the mass balance (Rocha et al., 2012), indicating that overall pretreatment was better in our study in terms of less biomass loss. The pretreated biomass having 83% cellulose content was acted upon by cellulases during hydrolysis to release glucose. The glucose and xylose (derived from detoxified acid hydrolysate) were further fermented to ethanol separately, resulting in an overall ethanol production of 91.7 g/L after fermentation.

Pentose fermentation enhanced the overall ethanol yield during the process (Akanksha et al., 2016). Using the developed process during current study a total of 260.1 kg of bioethanol could be produced per tonne.