

Bioethanol Production From *Pennisetum* sp. Using Fed-Batch Simultaneous Saccharification and Co-Fermentation at High Solid Loadings and Its Life Cycle Analysis

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Abstract

Considerable progress has been achieved for production of bioethanol from lignocellulosic biomass. However, increasing the substrate concentration has shown to decrease the ethanol productivity. In the present study, *Saccharomyces cerevisiae* and *Pichia membraneferans* were used for ethanol production from glucose and xylose sugars respectively in optimized conditions. Further, co-culture fermentations were conducted in three different strategies for 13 g of sugar (10 g of glucose and 3 g of xylose) and the best strategy was further used for ethanol production from ultrasonication assisted NaOH (UA-NaOH) pretreated and enzymatically saccharified in batch and fed-batch fermentation conditions. Further, fed-batch fermentation was used for separate hydrolysis and co-fermentation (SHCF) and simultaneous saccharification and co-fermentation (SSCF) in shake flask conditions. The highest ethanol production of 12.2 and 7.9 (g/L) was observed for fed-batch SSCF denannath grass (DG) and Hybrid napier grass (HNG) (Palkonal MBW as the enzyme) biomass (80 g) respectively in shake flask conditions. However, increasing the biomass concentration to 270 g produced an ethanol concentration of 77.6 and 51.3 (g/L) for DG and HNG respectively in fed-batch SSCF conditions in bioreactor. Nuclear magnetic resonance studies of the residual biomass of both DG and HNG revealed presence of lower carbohydrate content, demonstrating the efficiency of the fermentation strategy. Further Life cycle Analysis (LCA) was also conducted to analyzed the effect of the ethanol on different environmental conditions

The substrate feeding strategy and the saccharifying enzymes play a major role for efficient bioethanol production with higher substrate loadings. Presence of lower carbohydrates and some lignin moieties demonstrating the efficiency of the SSCF strategy for maximum conversion of carbohydrates. Thus fed batch SSCF process can be considered as a promising technique for biorefinery based bioethanol production from *Pennisetum* sp. in the future.

Introduction

The rise in demand of fossil fuels due to the increase in population has led to emergence of exploring renewable alternatives such as biofuel which has the potential to meet the future energy demands (Kalyani et al. 2017). The annual production of biofuels such as bioethanol and biodiesel are approximately 89 billion litres and 24 billion litres respectively in the year 2015 with continuous increase in its production and demand in the present years. Though sugar and starch containing crops (1st generation feedstocks) have been the main source of bioethanol in the US and Brazil, their role as food crops limits their extensive use for bioethanol production in the future. On the other hand lignocellulosic biomass (2nd generation feedstocks) in the form of agricultural and woody residues or energy crops which do not interfere with food applications presents a near-term solution to supporting energy demands (Nguyen et al. 2017).

Lignocellulosic biomass is composed of celluloses, hemicelluloses and lignin which forms a network like structure and offers rigidity and recalcitrance nature to the cell wall. Pretreatment is an essential step for overcoming the recalcitrance nature of cell wall which eliminates most of the lignins with simultaneous

breakage of lignin-carbohydrate linkages. This eventually leads to easy accessibility of the enzymes to the cellulose and hemicellulose (Mohapatra et al. 2017). However, the efficacy of the pretreatment is greatly dependent on the type of pretreatment and biomass used. Subsequently, in the next step the enzymes saccharify the cellulose and hemicelluloses to glucans and xylans, which can further be fermented to ethanol by efficient fermentative organisms. Grasses which are generally referred as energy crops consist of lower concentrations of lignin although the linkage patterns for lignin-carbohydrate complexes are mostly similar as that of other lignocellulosic biomass (Xiros et al. 2014). Hence mild pretreatments like alkaline or alkaline pretreatments combined with ultrasonication have been observed to showcase higher delignification rates in grass biomass (Mohapatra et al. 2018). Nevertheless, the further steps of saccharification and fermentation can either be conducted separately (separate hydrolysis and fermentation) or simultaneously (simultaneous saccharification and fermentation) depending on the type of microorganism used for fermentation.

Appropriate selection of microorganism for the conversion of monomeric sugars to ethanol from biomass is the key point for all fermentation processes. The literature survey reveals many fermentative organisms such as bacteria, yeast and fungus that are efficient in conversion of these monosaccharides into ethanol. However, alike enzymes it is important to choose the microorganisms involved in the fermentation of lignocellulosic sugars into bioethanol (Singh et al. 2017) as the selection of the microorganisms is dependent on the substrate used. For example, *Zymomonas mobilis*, a gram negative bacterium has gained the attention of the scientific community as an efficient microorganism for conversion of hexose sugar to ethanol (Gu et al. 2015). However, owing to its restricted substrate accessibility, yeasts like *Saccharomyces cerevisiae* have been more preferred over bacterial strains. Several strains of *S.cerevisiae* have exhibited remarkable fermentation capabilities for sugary and starchy substrates which are rich in hexose sugar (Westman et al. 2017). But, lignocellulosic biomasses like grass feedstocks consist of pentose sugars, though in lower proportions, along with the hexose sugars. Hence, for utilization of these pentose sugars either the hexose fermenting strains have to be genetically modified or a co-culture system has to be selected in which both hexose and pentose fermenting yeasts can convert the sugars into ethanol. The later process appears to be more feasible from economical prospective of lignocellulosic bioethanol production as genetically modifying an organism is time consuming and the final outputs may or may not be encouraging. The majority of pentose fermenting yeasts belongs to *Pichia membraneferans*. which in the absence of glucose can solely use xylans or arabinoxylans as their carbon source. However, in the presence of glucose these microorganisms exhibit better preference towards the same.

To obtain maximum utilisation of the sugars those are produced during saccharification of grass biomass, the co-fermentation strategy need to be optimised. The first measure that has to be considered is the feed system which generally consists of batch or a fed batch strategy. Generally, batch systems involve the addition of all the feed (substrate), enzymes and microorganisms at a time whereas in feed-batch the substrate is fed in the reaction mixture at regular intervals with the enzymes and microorganisms given in the beginning of the process (Gao et al. 2014). Further, the feed system has to be integrated with the saccharification system i.e. a separate hydrolysis and co-fermentation (SHCF) or

simultaneous saccharification and co-fermentation (SSCF). Compared to SHCF, SSCF have been observed to potentially decrease the capital and operational costs with simultaneous decrease of end-product inhibition of enzymes and reduced contamination (Paulova et al. 2015; Amillastre et al. 2012). Different parameters affect the efficiency of fermentation processes, however temperature has a vital effect as compared to others. Maintaining the appropriate temperature for culture conditions, plays an important role in SSCF, as it has to be compromised between high temperatures at about 50 °C that are optimal for cellulolytic or xylanolytic enzymes and lower temperatures of 30–35 °C that are preferred by both hexose and pentose fermenting organisms. The fed-batch SSCF in contrast to fed-batch SHCF offers advantage in processes with high solid loadings due to less accumulation of glucose, wherein the sugars are continuously removed by fermentation (Olofsson et al. 2010).

With these insights the experiments in the present investigation were designed with the following objectives; a) screening and optimisation of single and co-fermentation conditions in pure hexose and pentose sugars, b) selection of the type of fermentation process for pretreated and saccharified DG and HNG biomass in shake flask conditions (the pretreatment and saccharification conditions were previously optimised by the authors) and c) scale-up of the best type of fermentation process in bioreactor. The quantification of ethanol that was produced in the above process was conducted using gas-chromatography while the structural variations in the fermented biomass were observed using nuclear magnetic resonance spectroscopy (NMR).

Results And Discussions

Microorganisms for ethanol production

Saccharomyces cerevisiae and *Pichia* sp. were selected for mono and co-culture ethanol production from grass biomass on the basis of optimisation results (data not shown). For the *S. cerevisiae* mono culture fermentation the optimum ethanol production of 3.7 g/L from was obtained at 72 h of incubation at 30 °C with 10 g/L of glucose as the substrate. For *Pichia* sp. the aforementioned parameters exhibited the highest ethanol production of 3.1 g/L from 10 g/L of xylose as the sole carbon source. The parameters optimized in the mono-culture fermentation i.e. incubation time 72 h, temperature- 30 °C, shaking velocity- 120 rpm, glucose: xylose concentration- 10:3 (g/L) and inoculum volume of 8 mL (6:2) were further used to screen co-culture fermentation conditions using *S. cerevisiae* and *Pichia* sp. Three conditions were considered (data not shown), among which the third condition (Condition III) wherein 13 g/L of substrate was first fermented by *S. cerevisiae* for 48 h, followed by centrifugation at 7000 rpm for separation of supernatant, followed by *Pichia* sp. for 24 h. The total sugar utilized in this fermentation process was 6.8 g/L and the final ethanol concentration was 3.0 g/L. The results from the co-culture experiments indicated that condition III was the most preferred condition for fermentation. However, although the results of co-culture fermentation were lower as compared to the monoculture system, the co-culture systems are preferred for various reasons. Co-culture systems for bioethanol production from lignocellulosic biomass are evidenced to be more advantageous as compared to single cultures mainly

because of the synergistic action of the metabolic pathways of the involved strains that helps in combating the stress and enhancing the ethanol productivity (Bader et al. 2010).

Pretreatment and Saccharification

The optimum pretreatment conditions of 60 W (ultrasonication watt), 40 °C for 40 minutes for Dennanath grass (DG) and 70 W, 40 °C with an incubation of 50 mins for Hybrid napier grass (HNG) revealed maximum lignin removal of 89.3 % and 86.7 % for DG and HNG respectively. In the same parametric conditions, the reducing sugars for DG and HNG were observed to be 227.2 ± 0.2 mg/g and 242.8 ± 0.6 mg/g of raw biomass respectively (Mohapatra et al., 2017). Palkonol MBW enzyme showed enhanced enzymatic hydrolysis with maximum total reducing sugar (TRS) release of 662.0 ± 0.5 mg/g and 433.4 ± 0.3 mg/g in DG and HNG variety respectively. The mixture of IC + xylanase revealed a maximum TRS of 421.3 ± 0.3 mg/g in DG samples while for HNG reduced TRS of 483.8 ± 0.5 mg/g was observed (Mohapatra et al., 2018). While the optimized parameters for saccharification conditions were maintained for separate hydrolysis and co-fermentation, the parameters such as temperature and incubation time were compromised for simultaneous saccharification and co-fermentation.

Batch and fed-batch fermentation of DG and HNG using co-culture of *S. cerevisiae* and *Pichia* sp.

Further co-culture system has to be integrated with the appropriate input of feed process. Therefore two feed strategies i.e. batch and fed-batch, were experimented for ethanol production from two grass varieties DG and HNG with the co-culture of *S. cerevisiae* and *Pichia* sp. Batch experiments were conducted using 20 g/L of UA-NaOH pretreated DG and HNG biomass as shown in **Table SII(a)**. For both DG and HNG the substrate concentration of 20 g/L and enzyme (Palkonal MBW) were taken in 2000 mL flask separately. The flasks were incubated for 48 h for saccharification and the glucose to xylose ratio was determined to be 10:3 and 6.5:2 (g) for DG and HNG respectively. The organisms (*S. cerevisiae* and *Pichia* sp.) were inoculated according to condition III of the screening experiments mentioned in the previous section. In case of DG, the total sugar concentration of 13 g/L resulted in 1.9 g/L of ethanol concentration. Similarly, in case of HNG a total of 1.5 g/L of ethanol was produced from 8.5 g/L of sugar.

Fed-batch experiments were conducted using 20 g/L of UA-NaOH pretreated and saccharified DG and HNG. The feed for fed-batch fermentation for both DG and HNG biomass was given for the total time of 8 h. The feed was increased rapidly from 2.2 to 8.2 (g/L) in the first 5 h after which the last feed was lowered to 5 g. Using this strategy the total ethanol concentration of 2.9 g/L was observed from 6.4 g/L of DG and 2.1 g/L from 5.2 g/L of HNG. The results from the co-culture batch and fed-batch experiments revealed that co-culture system integrated with fed-batch mode produced higher titers of bioethanol as compared to co-culture system integrated with batch mode. Batch mode fermentations though are simple and easier to control but exhibit a lot of bottlenecks. One example is the suppression of xylose fermentation in the presence of glucose specifically in the initial stages of fermentation (Grootjen et al., 1991). Further, most of the experiments conducted till date is either carried in co-culture batch or continuous fermentation mode. More recently, studies have been conducted using co-culture feed-batch mode for bioethanol production from wheat straw and have been observed to produce higher ethanol

yields (Nielsen et al., 2017). Co-culture fed-batch mode has the potential to affect culture growth and avoid overflow metabolism due to the feeding of a growth-limiting nutrient substrate. Furthermore, fed-batch mode of fermentation can also help in solving the problem that generally arises when the rising inhibitor concentrations adversely affects the ethanol production for pentose-fermenting yeasts by maintaining an optimum dilution rate (Cardona et al., 2007).

Fed-batch SHCF and SSCF processes for UA-NaOH pretreated DG and HNG biomass

Apart from the mode of fermentation, the process opted i.e. separate hydrolysis and co-fermentation (SHCF) or simultaneous saccharification and co-fermentation (SSCF) integrated with fed-batch mode is an important aspect for bioethanol production from lignocellulosic biomass (Koppram et al. 2013). UA-NaOH pretreated DG and HNG were subjected to SHCF and SSCF processes in fed-batch technique separately. The processes were conducted separately for combination of IC+Xyl (Isolated cellulase + commercial xylanase) and commercial enzyme (Palkonal MBW). The substrate loadings for DG and HNG were taken as 20, 40, 60 and 80 (g). The supply of feed rate (g of substrate/ time interval) for both SHCF and SSCF was fixed to 8 h. For SHCF process the pretreated DG and HNG were first enzymatically saccharified in the optimized conditions and then subjected to fed-batch co-culture fermentation. For SSCF experiments the initial feed was kept 20 % of the total feed and the total enzyme concentration was given with the initial feed concentration and incubated for 24 h at temperature of 50 °C. After 24 h the temperature was lowered to 35 °C and the organisms (*S.cerevisiae* and *P.membranifaciens*) were inoculated according to condition III. The efficiency of the SHCF and SSCF processes were evaluated on the basis of ethanol production and the best process was further chosen to conduct reactor scale fermentation using substrate concentration of 270 g of DG and HNG biomass.

Fed-batch SHCF of UA-NaOH pretreated DG and HNG using Palkonal MBW and IC+Xyl enzymes and co-culture of *S.cerevisiae* and *P.membranifaciens*

The glucose and xylose concentrations of DG and HNG before and after fermentation, the total sugar utilized and the ethanol concentration during fed-batch SHCF process using Palkonal MBW is as given in **Fig. 1(I)**. For DG, ethanol concentration of 2.9 g/L was obtained from 20 g of biomass which consisted 10 g of glucose and 3 g of xylose sugars. The total sugar utilized in the fed-batch SHCF process was 6.4 g. When the biomass concentration was increased to 40, 60 and 80 (g) the increase in ethanol concentration was observed to be only 18.3, 29.2 and 34 (%) respectively. The total sugar utilized for 40, 60 and 80 (g) of DG were 10.4, 12.4 and 15.8 (g) respectively. In case of HNG, fed-batch SHCF of 20 g of biomass resulted in 2.1 g/L of ethanol. Increase in biomass concentration to 40, 60 and 80 (g) resulted in an increase in ethanol concentration of 19.6, 19.2 and 33.3 (%) respectively as compared to 20 g of biomass concentration. However, ethanol concentration was observed to slightly decrease for 60 g of biomass as compared to 40 g of HNG using fed-batch SHCF. The results illustrated that when biomass concentration was increased, ethanol production from DG was higher as compared to HNG in fed-batch SHCF process using Palkonal MBW.

Fig. 1(II) depicts the glucose and xylose concentrations of DG and HNG before and after fermentation, the total sugar utilized and the ethanol concentration during fed-batch SHCF process using IC+Xyl enzyme. In DG, 20 (g) of UA-NaOH pretreated and saccharified biomass with glucose content of 5 g and xylose content of 4.4 g was fermented using fed-batch SHCF process to produce an ethanol concentration of 2.1 g/L. The total sugar utilized in the co-culture fermentation process was 5.3 g. Further, increasing the substrate concentration to 40, 60 and 80 (g) resulted in increased ethanol concentration of 20.7, 17.6 and 40 (%) respectively as compared to 20 g of biomass of DG. Nevertheless, the increase in substrate concentration from 40 to 60 (g) led to a reduced ethanol concentration in DG. In case of UA-NaOH pretreated and saccharified HNG, the glucose and xylose concentration were found to be 6 and 3.6 (g) respectively for 20 g of biomass. Increasing the substrate concentration to 40, 60 and 80 (g) resulted in an increase of 29.8, 34.4 and 44.4 (%) ethanol concentrations respectively. The results indicated that the combination of isolated cellulase and commercial xylanase was more beneficial for HNG as compared to DG in fed-batch SHCF process.

Fed-batch SSCF of UA-NaOH pretreated DG and HNG using Palkonal MBW and IC+Xyl enzymes and co-culture of *S.cerevisiae* and *P.membranifaciens*

The first set of fed-batch SSCF experiments were conducted for UA-NaOH pretreated DG and HNG biomass using Palkonal MBW enzyme as shown in **Fig. 2(I)**. Since, the enzymatic saccharification and fermentation occurs simultaneously the glucose and xylose concentrations in the fermented broth of DG and HNG after the completion of partial enzymatic hydrolysis (24 h) and after fermentation process was observed. For DG, ethanol concentration of 3.6 g/L was obtained from 20 g of biomass. The residual sugar in the fermentation medium after the fed-batch SHCF process was 4.8 g. When the biomass concentration was increased to 40, 60 and 80 (g) the increase in ethanol concentration was 26.5, 41.4 and 54.4 (%) respectively. The residual sugar for 40, 60 and 80 (g) of DG were 15, 19.8 and 26.3 (g) respectively. In case of HNG, fed-batch SHCF of 20 g of biomass resulted in 2.2 g/L of ethanol. Increase in biomass concentration to 40, 60 and 80 (g) resulted in an increase in ethanol concentration of 35.2, 45.0 and 56.4 (%) respectively as compared to 20 g of HNG biomass concentration. The residual sugar for 40, 60 and 80 (g) of HNG were 10.4, 13.9 and 16.6 (g) respectively. In fed-batch SSCF process using Palkonal MBW as the saccharifying enzyme, increase in biomass concentration led to higher ethanol production from HNG as compared to DG.

The second set of fed-batch SSCF experiments were conducted for UA-NaOH pretreated DG and HNG biomass using IC+Xyl enzyme as shown in **Fig. 2(II)**. Similar to the first set of fed-batch SSCF experiments, only the residual sugar (glucose and xylose) left in the fermented broth of DG and HNG was calculated. In DG, 20 (g) of UA-NaOH pretreated biomass produced an ethanol concentration of 2.9 g/L. The residual sugar concentration in the co-culture fermented DG broth was observed to be 3.9 g. Further, increasing the substrate concentration to 40, 60 and 80 (g) resulted in increased ethanol concentration of 27.5, 30.1 and 45.7 (%) respectively as compared to 20 g of biomass of DG. In case of UA-NaOH pretreated HNG, the residual sugar and ethanol concentration was found to be 3.4 g and 2.9 g/L respectively for 20 g of biomass. Increasing the substrate concentration to 40, 60 and 80 (g) resulted in

an increase of 2.3, 30.0 and 43.2 (%) ethanol concentrations respectively as compared to 20 g of HNG biomass. The results indicated that the combination of isolated cellulase and commercial xylanase was more beneficial for DG as compared to HNG in fed-batch SSCF process. Literature studies state that fed-batch SSCF process can prove to be efficient process in reduction of inhibitors which eventually interfere in the enzyme-substrate reaction (Almeida et al. 2007). Further, fed-batch SSCF also offers the advantage of using higher substrate loadings due to the ease of mixing the substrate which ultimately produces high ethanol concentrations (Zhang et al. 2010). Consequently, it also offers a possibility to maintain glucose at low levels allowing efficient co-fermentation of glucose and xylose (Ohgren et al. 2010).

Fed-batch SSCF of UA-NaOH pretreated DG and HNG using Palkonal MBW and IC+Xyl enzymes and co-culture of *S.cerevisiae* and *P.membranifaciens* in bioreactor

Reactor scale experiments were conducted for UA-NaOH pretreated DG and HNG biomass using Palkonal MBW and IC+Xyl enzymes separately in fed-batch SSCF process as given in **Table S III**. Since, the fed-batch SSCF experiments resulted in higher ethanol production both in DG and HNG as compared to fed-batch SHCF, reactor scale experiments were further conducted using the former process. A total of 270 g of UA-NaOH pretreated biomass each for DG and HNG were used in fed-batch SSCF experiments and the glucose and xylose concentrations in the fermented broth of DG and HNG after the completion of partial enzymatic hydrolysis (24 h) and after fermentation process was observed.

For UA-NaOH pretreated DG [**DG (A) in Table S III**] the glucose and xylose concentration after 24 h of partial hydrolysis (Palkonal MBW) were 72.1 and 19.3 (g) respectively. In the end of co-culture fermentation of 96 h (24 h for partial hydrolysis and 72 h for co-culture fermentation) the residual glucose and xylose concentrations were 1.8 and 0.2 (g) with an ethanol concentration of 77.6 g/L from 270 g of UA-NaOH pretreated DG. For the second enzyme (IC+Xyl), [**DG (B) in Table S III**] the glucose and xylose concentrations after 24 h of partial hydrolysis of UA-NaOH pretreated DG were 36.3 and 20.7 (g) respectively. The residual glucose and xylose concentrations after the fermentation cycle were 1.5 and 0.4 (g) respectively with a total ethanol concentration of 51.3 g/L from 270 g of UA-NaOH pretreated DG. The ethanol concentrations for DG saccharified with commercial enzyme (Palkonal MBW) were much higher as compared to the mixture of isolated cellulase and xylanase enzymes.

For UA-NaOH pretreated HNG [**HNG (A) in Table S III**] the glucose and xylose concentration after 24 h of partial hydrolysis (Palkonal MBW) were 33.4 and 15.3 (g) respectively. In the end of co-culture fermentation of 96 h (24 h for partial hydrolysis and 72 h for co-culture fermentation) the residual glucose and xylose concentrations were 1.5 and 0.7 (g) with an ethanol concentration of 49.6 g/L from 270 g of UA-NaOH pretreated DG. For the second enzyme (IC+Xyl), [**HNG (B) in Table S III**] the glucose and xylose concentrations after 24 h of partial hydrolysis of UA-NaOH pretreated HNG were 46.9 and 19.4 (g) respectively. The residual glucose and xylose concentrations after the fermentation cycle were 1.2 and 0.3 (g) respectively with a total ethanol concentration of 54.9 g/L from 270 g of UA-NaOH pretreated HNG. The ethanol concentrations for HNG saccharified with mixture of isolated cellulase and xylanase enzymes were slightly higher as compared to the commercial enzyme (Palkonal MBW).

NMR analysis

Solid state NMR of the residual solid fractions of the fed-batch SSCF DG and HNG biomass in bioreactor was conducted. The residual solid biomass that is left over after the extraction of ethanol primarily consists of lignin and unconverted sugars. The purpose of conducting NMR analysis in the present study was to observe the efficacy of the process for optimum utilization of carbohydrates during the fed batch simultaneous saccharification and fermentation process. The chemical shift assignments are based on the comparison of the ^{13}C NMR spectra from the switch grass and sugarcane bagasse (Watkins et al. 2015). The explanation for the chemical shifts that are depicted in Fig. S (I) are presented in three parts i.e. lignin, hemicellulose and cellulose in this section.

Lignin- Lignin related peaks were observed in all the samples of DG and HNG. While the peaks at 57.4 and 56.1 ppm were observed for Palkonal MBW saccharified and simultaneously co-fermented DG and HNG respectively, peaks at 56.3 and 57.1 ppm were observed for IC+ Xyl saccharified and simultaneously co-fermented DG and HNG respectively. The peaks belonged to the aliphatic lignin group which are related to oxygenated C α , C β , and C γ carbons of the phenyl propane in lignin.

Hemicellulose: The peaks related to hemicellulose were observed in 73.0 and 76.0 rpm for Palkonal MBW saccharified and simultaneously co-fermented DG and 73.0 and 75.0 ppm for Palkonal MBW saccharified and simultaneously co-fermented HNG. For IC+ Xyl saccharified and simultaneously co-fermented DG and HNG peaks at 74.7 and 75.0 ppm were observed respectively. These peaks are attributed to xylose units of hemicellulose and to carbon units of lignin.

Cellulose: No cellulose related peaks were observed for Palkonal MBW saccharified and simultaneously co-fermented DG. However Palkonal MBW saccharified and simultaneously co-fermented HNG exhibited only one peak at 84.7 ppm corresponding to C4 carbon of amorphous cellulose. Further peaks at 83.0, 84.0, 85.9 and 105.0 ppm were observed for IC+ Xyl saccharified and simultaneously co-fermented DG while for HNG only a single peak at 84.7 was observed. Peaks corresponding to 84.2-89.0 are attributed to C4 carbon of crystalline and non-crystalline cellulose regions. However, the same region is also contributed by signals from lignin and hemicellulose, but the latter one not contributing significantly in the same spectral regions.

It was evident from the results that, fed-batch SSCF DG biomass predominantly consisted more of lignin and hemicellulose while fed-batch SSCF HNG exhibited more unutilised cellulose peaks with few lignin peaks. Similar results as that for fed-batch SSCF DG biomass was obtained by Sannigrahi and Ragauskas (2011) using miscanthus and switchgrass residues that were fermented for bioethanol production. Although most of the dried solid stream (~40%) in bioethanol plants, after ethanol production is diverted to meet the thermal requirements of pretreatment and ethanol distillation (Watkins et al., 2015) still ~60 % of these residues are left out unutilized. Hence, characterization of residual biomass can be helpful for production of value added products and consequently better techno-economic evaluations for industrial bioethanol production..

LCA Analysis

The LCA analysis was conducted for the fermented DG and HNG samples that were obtained 828 in fed-batch SSCF experiments in the bioreactor **Fig. 3 (I)**. The results show that a total 829 ethanol yield of 26 % is obtained for DG biomass during the fed batch SSCF experiments in 830 bioreactor. Similar results can be obtained when the production is scaled up, i.e., 192 kg of 831 fermentation substrate would produce around 50 kg of ethanol. For the same, a total of 385 832 kg of raw biomass would be required. In case of HNG, a 20 % ethanol yield was observed. 833 From general mass balance, 250 kg of alkali pre-treated and sonicated biomass sample was 834 able to produce 50 kg of ethanol. For the same, a total raw biomass of 722 kg would be 835 required. Since HNG is a hybrid grass, its production process would have its own 836 environmental impact but since the raw material was directly purchased, the associated 837 impacts have been kept out of the scope of the study. As is clearly observed, for the same 838 amount of production of ethanol, we need more HNG than DG. Since the associated impacts 839 consider mostly the consumption of electricity and thermal energy (if any), obviously the 840 impacts associated with the production of ethanol from HNG would be more than that of DG.

The environmental analysis was carried out for the production of 50 kg of ethanol from the 842 DG and HNG biomass as given in Fig. 3(**II**). The method used to calculate the impacts 843 associated was ReCiPe calculation method. It is an end-point calculation method which 844 translates emissions and resource extractions into a limited number of environmental impact 845 scores by means of so-called characterisation factors. It expresses the aggregation of the midpoint indicators in terms of impacts on three main categories namely: effect on human 847 health (human toxicity), biodiversity (global warming potential/climate change) and resource 848 scarcity (water depletion) (Bai et al., 2010). The production process that used DG as the raw 849 biomass showed a total GWP of 261.76 (kg CO₂ equivalents), while that from HNG 850 showed 765.65 (kg CO₂ equivalents). This was mainly due to the increased electricity (and 851 thermal energy, if any) consumed for the treatment of a higher volume of biomass. Similarly, 852 for ethanol production from DG, the freshwater ecotoxicity was calculated to be 0.015 (kg 853 1,4-dichlorobenzene equivalents) while the same from HNG was found to be 0.074 (kg 1,4- 854 dichlorobenzene equivalents). Again for the production of ethanol from DG, the water 855 depletion was found to be 2.75 m³ while that from HNG was determined to be 8.56 m³. Since 856 the production from both the substrates used similar experimental arrangement, the results 857 followed the same pattern in terms of the impacts. The findings of our study show a common 858 trend in terms of impacts associated with individual unit processes. It was found that, for both 859 the substrates, ball milling and alkali pre-treatment constituted the major fraction of the total 860 impacts for every impact category.

It is evident from the studies that, the production of ethanol from DG had a higher yield as 862 well as lower environmental impact than HNG. The impacts associated with ethanol 863 production from HNG would have been higher had its production process been considered as 864 it is a hybrid variety. But this was excluded from the scope of the study as the raw material 865 was directly purchased from vendor. The common trend followed by the individual unit 866 processes in terms of environmental impacts is because of the fact that the unit processes 867 involved use primarily electricity and/or thermal energy as

inputs. Hence the process which 868 consumes more electricity and/or thermal energy contributes more to the overall impacts of 869 the process as a whole.

Conclusion

This study highlighted that co-culture fed batch fermentation strategy showed improved ethanol production for both the *Pennisetum* sp. as compared to co-culture batch fermentations. Further when fed batch strategy was used for simultaneous saccharification and co-fermentation (SSCF) in bioreactor, higher ethanol titers of 77.6 and 51.3 (g/L) from 270 g of DG and HNG biomass were obtained respectively. It is evident from the present study that the substrate feeding strategy and the saccharifying enzymes play a major role for efficient bioethanol production with higher substrate loadings. Furthermore, NMR studies revealed presence of lower carbohydrates and some lignin moieties demonstrating the efficiency of the SSCF strategy for maximum conversion of carbohydrates. Thus, the current work confirms that using of fed batch SSCF process can be considered as a promising technique for biorefinery based bioethanol production from *Pennisetum* sp. in the future.

Material And Methods

Microorganism

The ethanol producing strains of *Saccharomyces cerevisiae* and *Pichia membraneferans*. were previously isolated and characterized in Dept. of Biotechnology, CET (Dash et al., 2014). The organisms were grown at 30 °C and maintained on an agar medium containing yeast extract (10 g/l), peptone (20 g/l), dextrose (20 g/l) and agar (1.5 g/l) in sterile distilled water. The pH of the medium was adjusted to 6.0 using 1N NaOH solution. The cultures were maintained at 4 °C and sub-cultured freshly for each of the experiments.

Pretreatment and saccharification of DG and HNG biomass

Ultrasonication assisted NaOH (UA-NaOH) pretreatment was conducted in the conditions as previously described in Mohapatra et al., 2019. The pretreated DG and HNG biomass were further saccharified at optimized parameters using two different set of enzymes. The 1st set of enzymes consisted of Palkonal MBW (a mixture of cellulase- 12000 Maps cellulase unit (MCU) /mL and hemicellulose-5000 MHCU/mL) (Mohapatra et al, 2019) while the second set of enzymes consisted of Isolated cellulase (IC) + commercial xylanase (Xyl) [enzyme activity of the IC was 1.35 U/mL and 8450 U/ml for Xyl enzyme] (Mohapatra et al., 2018). Briefly, the optimised parameters for saccharification using the 1st set of enzymes (Palkonal MBW) were temperature-50 °C, pH-5.25, enzyme concentration-250 µl and incubation time of 30 h for DG and temperature 45 °C, pH-5, enzyme concentration-200 µl and incubation time of 40 h for HNG. Similarly, for the second set of enzymes (IC+Xyl) optimum conditions of temperature-35 °C, 6.25 pH, incubation time -48 h and enzyme ratio of 2.5 mL: 200 µl/g was kept for DG while temperature-35 °C, 7.0 pH, incubation time -30 h and enzyme ratio of 1.5 mL: 200 µl/g was maintained for HNG. While

the optimised parameters for saccharification conditions were maintained for separate hydrolysis and co-fermentation, the parameters such as temperature and incubation time were slightly compromised for simultaneous saccharification and co-fermentation.

Fermentation process for ethanol production from pretreated and saccharified DG and HNG biomass

The optimized [Denannath grass (DG) and Hybrid napier grass (HNG)] and enzyme saccharified grass biomass were used as fermentation substrates in the experiments. Commercial enzyme Palkonal MBW (Maps Pvt. Ltd) and mixture of Isolated cellulase + xylanase (IC+Xyl) were used in two separate sets for saccharification and fermentation of DG and HNG. The best condition obtained from the screening of co-culture fermentation i.e. Condition III was used for all the SHCF and SSCF experiments of DG and HNG biomass. Control experiments were conducted for batch and fed-batch fermentation systems in separate hydrolysis and co-fermentation (SHCF) conditions to evaluate the efficiency of fermentation category. As fed-batch fermentation was observed to exhibit better results as compared to batch process, all the further shake flask experiments were conducted in fed-batch process both for SHCF and simultaneous saccharification and co-fermentation (SSCF) conditions. Scale-up experiments in 5l reactor for DG and HNG biomass were then conducted for biomass using fed-batch SSCF conditions.

Batch fermentation using co-culture of *S.cerevisiae* and *P.membranifaciens*

Control experiments for batch fermentation in shake flask conditions were conducted using SHCF strategy for DG and HNG biomass separately in 250-mL flasks containing 0.05 M citrate buffer (pH 5.0). The substrate concentration was taken 20 g in terms of ratio of glucose and xylose percentage) for DG (10:3) and HNG (6.5:2). as per the optimized conditions of single culture. Saccharification was conducted using Palkonal MBW biomass prior to fermentation. After hydrolysis condition III of co-culture fermentation of pure sugars was imitated for co-culture fermentation of both the *Pennisetum* sp.

Fed-batch fermentation using SHCF process for ethanol production from different substrate concentrations of DG and HNG biomass

The fed-batch fermentations for different substrate concentrations [20, 40, 60 and 80 (g)] of DG and HNG biomass were conducted separately in shake flask conditions. The fed-batch SHCF experiments for separately saccharified (Palkonal MBW and IC+Xyl) DG and HNG biomass were conducted in the same procedure as mentioned in the above section. The initial and final glucose and xylose concentrations were measured in g for all the substrate concentrations of DG and HNG. The final ethanol concentration was measured in terms of g/L.

Fed-batch fermentation using SSCF process for ethanol production from different substrate concentrations of DG and HNG biomass

All the feed-batch SSCF experiments were started after an initial batch system (20 % of feed with the total enzyme for the experiment) for 24 h in order to maintain the high temperature (50 °C) that is required for the optimal activity of enzymes. The total enzyme concentration for Palkonal MBW (200 µl/g for DG and

250 µl/g for HNG) and IC+Xyl (2.5 mL: 200 µl/g for DG and 2 mL: 200 µl/g for HNG) was given with the initial feed. After 24 h the temperature was lowered to 35°C (temperature was compromised both for saccharifying enzymes and microorganisms) and condition III of co-culture fermentation of pure sugars was imitated by using *S.cerevisiae* and *P.membranifaciens* for both the *Pennisetum* sp.

Fed-batch fermentation using SSCF process for ethanol production from DG and HNG biomass in bioreactor

In the reactor scale fed-batch fermentation of DG and HNG biomass was conducted for a working weight of 270 g using SSCF process. The feed strategy was maintained as that of the shake flask condition (30 % of the feed in batch fermentation) in which all the enzymes were added at the beginning of the fermentation process. The concentration of 250 µl/g and 200 µl/g (DG and HNG respectively) of biomass [cellulase activity of 12000 Maps cellulase unit (MCU) /mL and hemicellulase activity of 5000 MCU/mL] was kept for Palkonal MBW while for the second set of enzyme IC+Xyl the concentration was kept in a ratio of 2.5 mL: 200 µl/g of DG and 1.5 mL: 200 µl/g of HNG. The total enzyme activity of the IC was 1.35 U/mL and 8450 U/ml for commercial xylanase enzyme. Subsequently, fed-batch fermentation was followed for 8 h with simultaneous addition of *S.cerevisiae*. After 48 h the substrate was centrifuged and to that *P. membranifaciens* was added. Cell loading was kept at 0.02 g cell/ g of biomass for *S.cerevisiae* and 0.01 g cell / g of biomass for *P. membranifaciens*.

Life cycle analysis

Life cycle analysis for dennanath and hybrid napier grass verities were conducted using GaBi software from Think step. This analysis uses the ReCiPe method which is an end-point calculation method.

Analysis of sugars and ethanol

Analysis of glucose was conducted using DNS reagent method while analysis of xylose was conducted using bial's reagent method (Baldwin et al. 1955). Ethanol was analysed using Clarus-680 gas chromatography which was operated at an initial oven temperature of 50 °C for 5 min, followed by 120 °C for 5 min with a ramp rate of 15 °C per min. The flow rate of the carrier gas was maintained at 2ml/min and 2-propanol was used as the internal standard. All samples were appropriately diluted and filtered through a 0.2µm filter before each chromatographic analysis.

Structural analysis using NMR spectroscopy

NMR samples were prepared with ground biomass packed into a 4-mm cylindrical Zirconia MAS rotor. Solid-state NMR measurements were carried out on a Bruker DSX 300 High Resolution Multinuclear FT-NMR Spectrometer. The spinning speeds were taken at different frequencies.

List Of Abbreviations

UA-NaOH: Ultrasonication assisted NaOH; SSCF: Simultaneous saccharification and co-fermentation; SHCF: Separate hydrolysis and co-fermentation; DG: Denannath grass; HNG: Hybrid napier grass; LCA: Life cycle Analysis; NMR: Nuclear magnetic resonance spectroscopy; MCU: Maps cellulase unit; IC: Isolated cellulase; Xyl: Commercial xylanase; TRS: Total reducing sugar

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: Available

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

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Authors' contributions: SM participated in the design of the experiments, performed the experiments and contributed to manuscript writing. BCB participated in the design of the experiments and manuscript writing. ANA and HT supervised the research. All authors contributed to scientific discussion. All authors read and approved the final manuscript.

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Figures

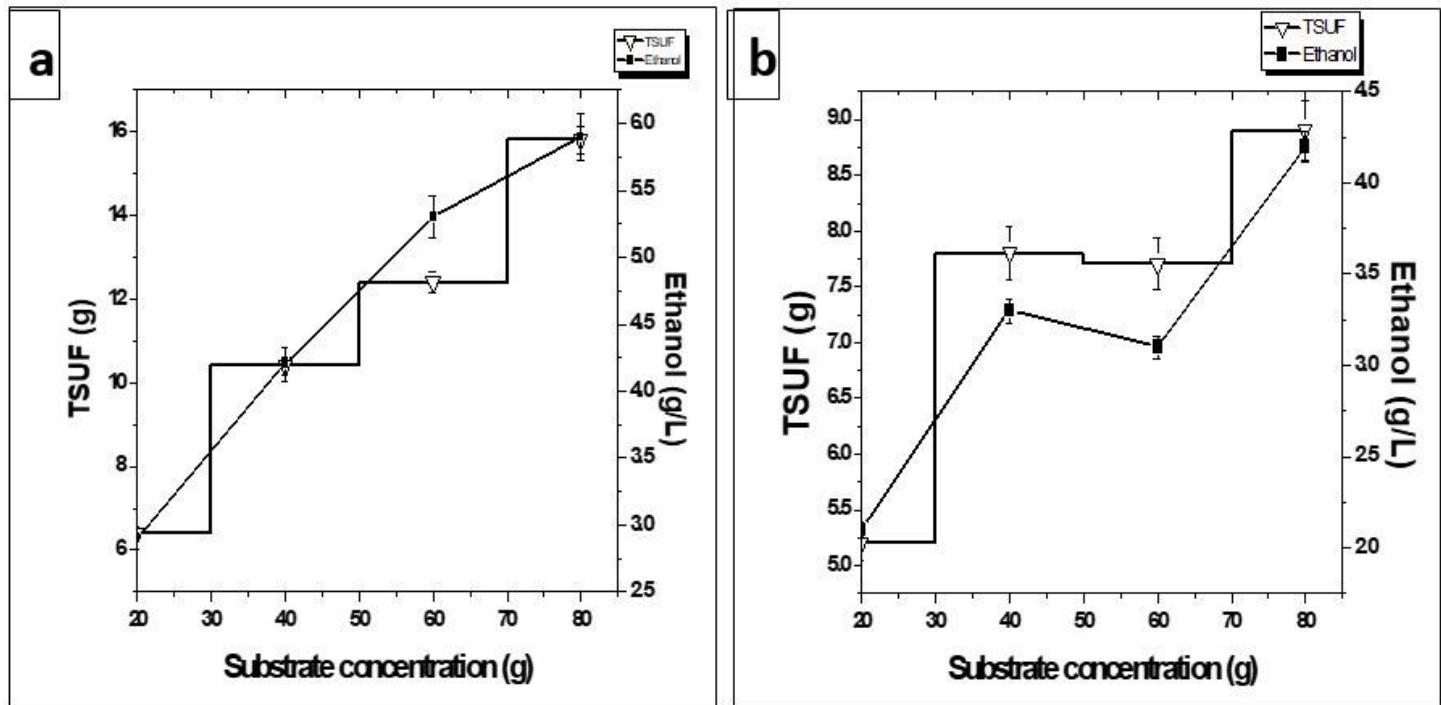


Figure 1

I. Fed-batch SHCF of UA-NaOH pretreated DG (A) and HNG (B) using Palkonal MBW. TSUF – Total reducing sugar utilized during fermentation.

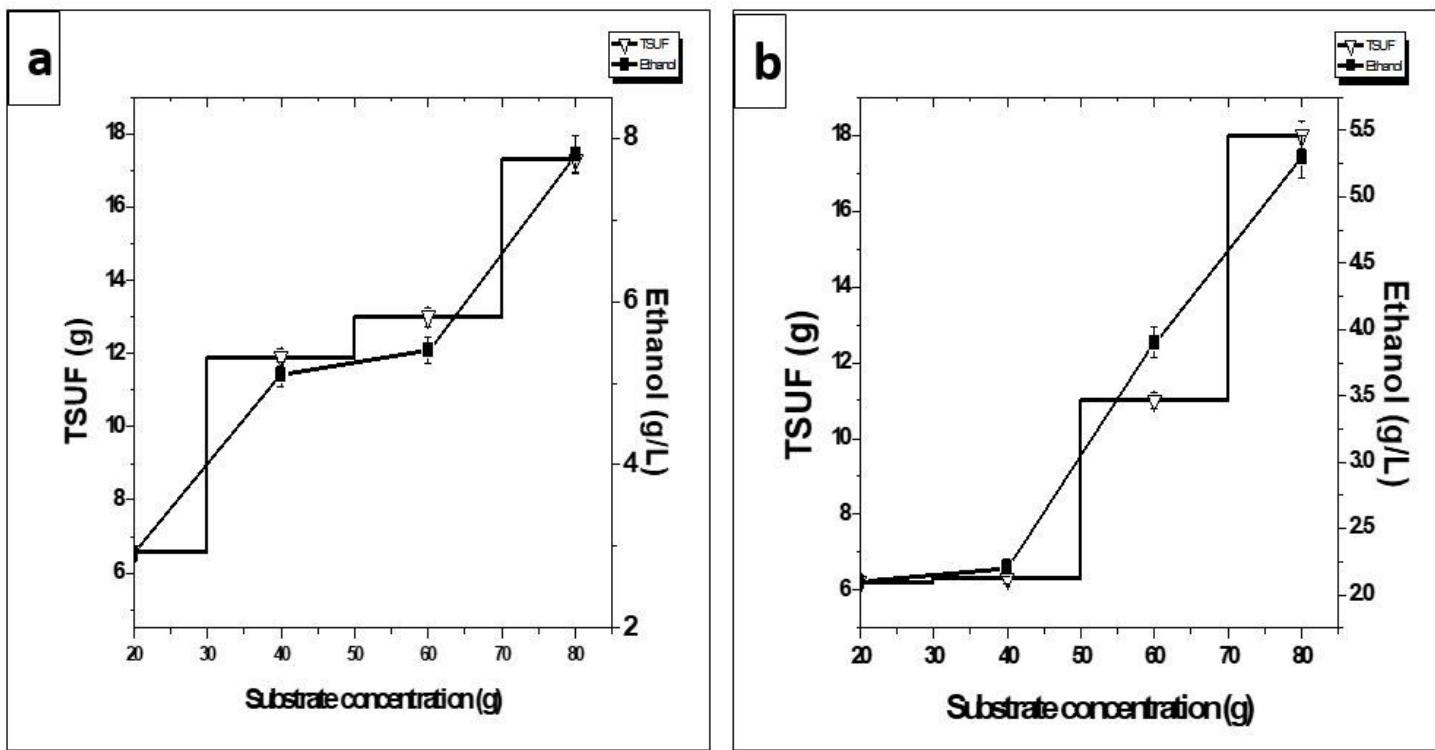


Figure 2

II. Fed-batch SHCF of UA-NaOH pretreated DG (A) and HNG (B) using IE+Xyl. TSUF – Total reducing sugar utilized during fermentation.

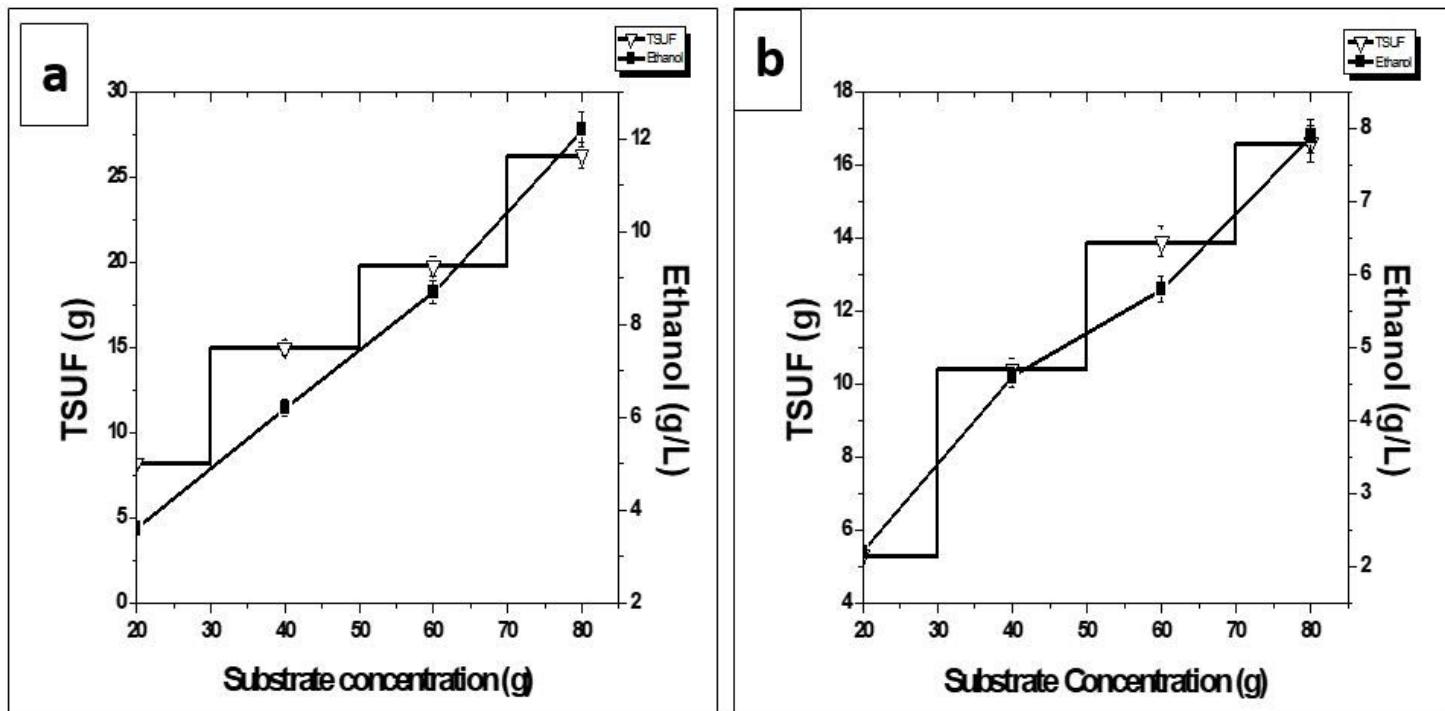


Figure 3

I. Fed-batch SSCF of UA-NaOH pretreated DG (A) and HNG (B) using Palkonal MBW. TSUF – Total reducing sugar utilized during fermentation.

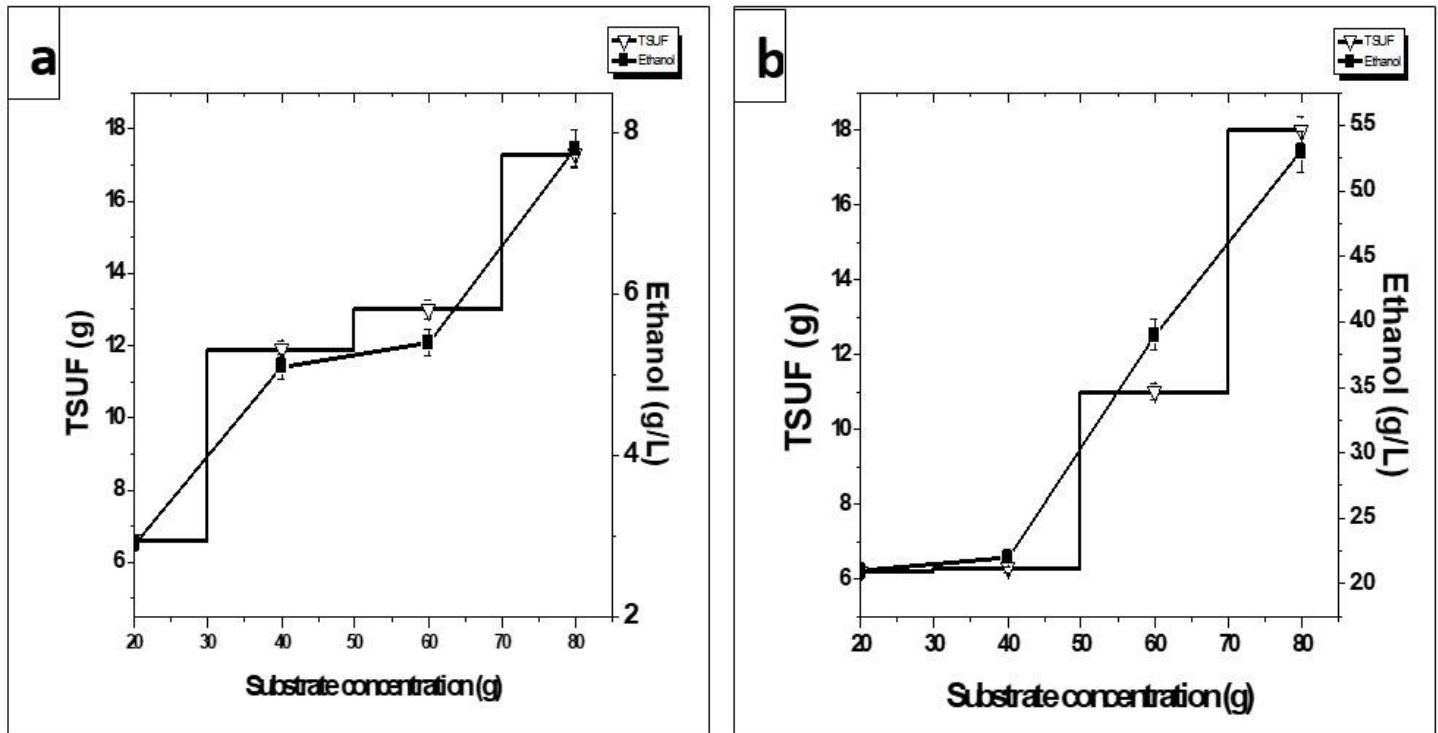


Figure 4

II. Fed-batch SsCF of UA-NaOH pretreated DG (A) and HNG (B) using IE+Xyl. TSUF – Total reducing sugar utilized during fermentation

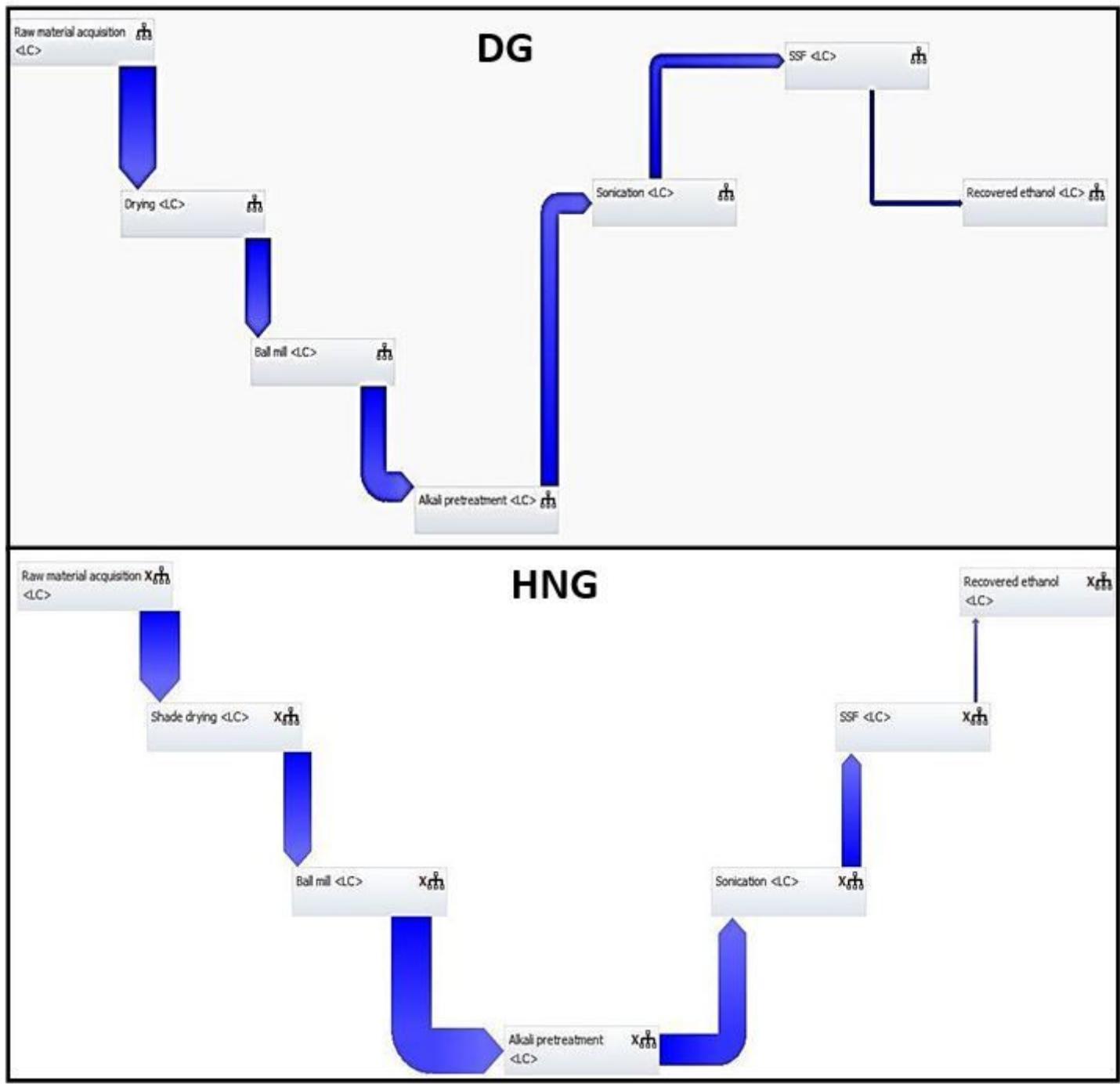


Figure 5

I. Life cycle flow diagram for the production of ethanol from DG and HNG.

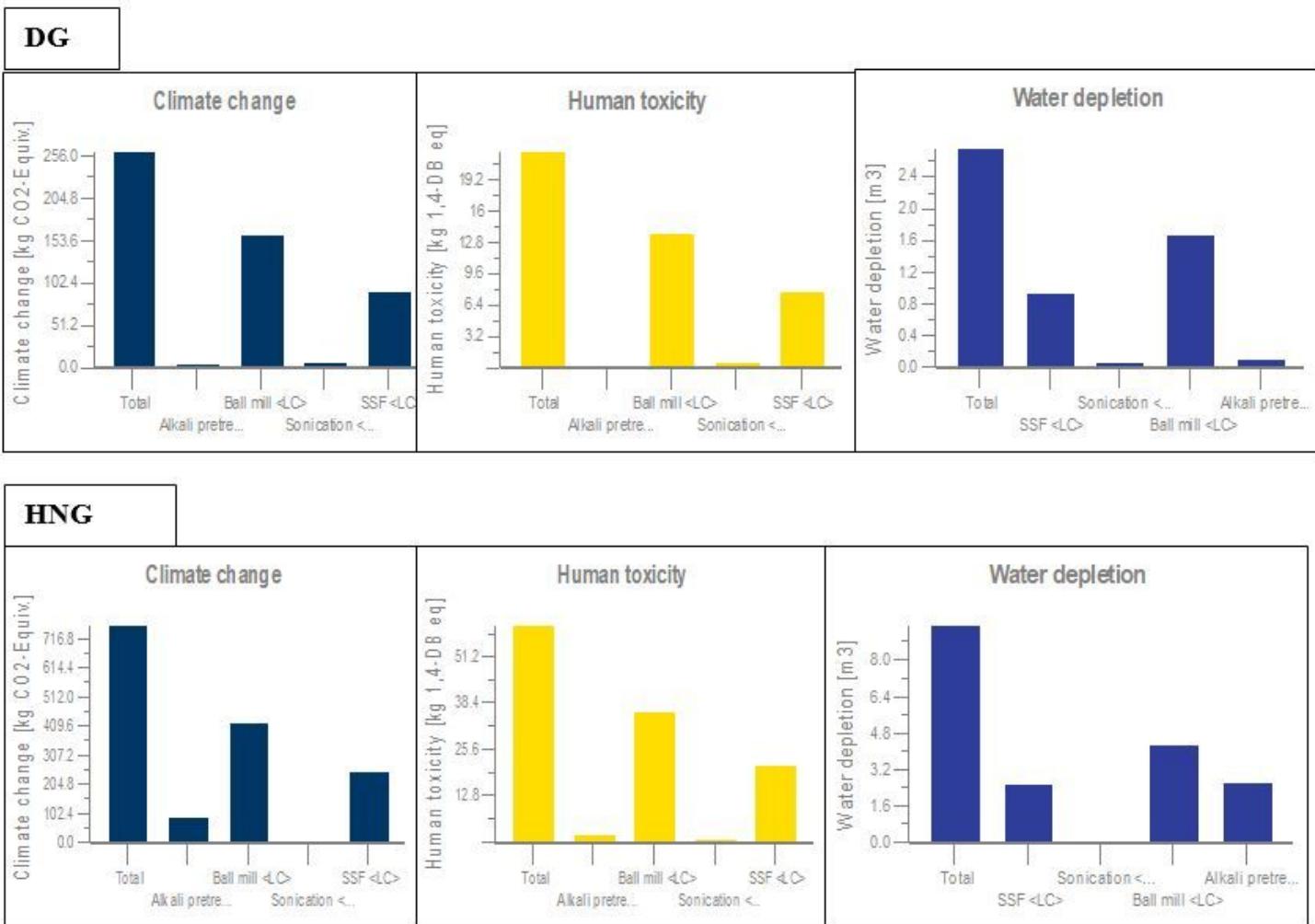


Figure 6

II. Carbon dioxide emission, human toxicity and water depletion footprints for ethanol production from DG and HNG

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