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Development of a novel sequential pretreatment strategy for the production of bioethanol from sugarcane trash

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Abstract

A novel sequential pretreatment strategy using biodiesel industry generated waste glycerol assisted transition metal and alkali pretreatment of sugarcane trash were developed for the production of bioethanol. Various process parameters affecting pretreatment as well as hydrolysis were optimized by adopting a Taguchi design. This novel method was found to be superior when compared to conventional pretreatment strategies like acid and alkali in removing hemicelluloses and lignin and the hydrolyzate is devoid of major fermentation inhibitors like organic acids and furfurals. Physico-chemical changes of the native and the pretreated biomass were evaluated by scanning electron microscopy (SEM), X-ray diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR) analysis. Under optimized hydrolysis conditions 0.796 g of reducing sugar (pentoses and hexoses) per g of dry biomass after saccharification was produced. Fermentation of the non-detoxified hydrolyzate using *Saccharomyces cerevisiae* produced 31.928 g of bioethanol per g of dry biomass with an efficiency of 78.89%.

Keywords: pretreatment; biomass; bioethanol; hydrolysis; fermentation

1. Introduction

Increase in accumulation of greenhouse gases and depletion of fossil fuels lead to search for sustainable alternative strategies of energy. The production of second generation biofuels from lignocellulosic biomass seems promising since it is the most abundant organic material in nature. Some of the advantages of utilizing lignocellulosic biomass are it promotes rural economy, enhance energy security and decrease greenhouse gas emissions. Various agro-residues, forest residues and bioenergy crops can be exploited for the production of second generation biofuels.

For the lignocellulosic biorefinery it requires three sequential operations involving pretreatment, enzymatic saccharification and fermentation. Due to recalcitrant nature of the biomass, a pretreatment process is essential to increase cellulose conversion efficiency for biofuel production. The first and the most critical step in lignocellulosic biorefinery is pretreatment since it strongly influence downstream costs involving detoxification, enzyme loading, waste treatment demands and other variables. Pretreatment constitutes for more than 40% of the total processing cost. Enzymatic saccharification of lignocellulosic biomass is limited by various factors like degree of polymerization, crystallinity of cellulose, available surface area as well as lignin content. An ideal pretreatment process must improve the sugar yield after enzymatic saccharification, minimal effluent generation, reduction of the degradation of carbohydrates and formation of inhibitors for hydrolysis and fermentation, and low energy demand and low capital and operational cost requirement.

In India sugarcane is cultivated over a large area. Annual generation of sugarcane in the country was calculated from the Department of Agriculture statistics for 2013 using conversion factors as per Ravindranath et al., 2005 as 139.148 MMT. Sugarcane trash (SCT) is the surplus available biomass in India and is usually burnt in the field after harvesting. Utilization of this surplus available underexploited biomass for the production of biofuels and other value added products seems promising. SCT includes leaves and top portion of sugarcane plant.

Though several pretreatment strategies were available, only a few seems to be promising. These pretreatment methods include dilute acid, dilute alkali, hydrothermal, alternative strategies, physical, ammonia fiber explosion etc. Few reports were available on pretreatment

of sugarcane tops or trash. This includes acid (Sindhu et al., 2011; Srinorakutara et al., 2014), alkali (Sindhu et al., 2014a; Srinorakutara et al., 2014), lime impregnation and steam explosion (Saska and Gray, 2006), (surfactant assisted acid (Sindhu et al., 2012), surfactant assisted ultrasound pretreatment (Sindhu et al., 2013), alkali based AFEX pretreatment (Krishnan et al., 2010), biological pretreatment (Singh et al., 2008) and Microwave pretreatment (Maurya et al., 2013).

Conventional pretreatment using acid or alkali removes either hemicelluloses or lignin. Transition metals play an important role in hemicelluloses removal and organosolvents like glycerol removes lignin. Glycerol serves as a potential agent in organosolv pulping for fractionation of lignocellulosic biomass and for improving enzymatic hydrolysis of cellulose. Delignification increased proportionally with increase of glycerol concentration (Martin et al., 2011). At high concentration of glycerol, it selectively removes lignin than xylan.

Sun and Chen, 2008a reported aqueous glycerol pretreatment of lignin for effective delignification of wheat straw. Aqueous glycerol organosolv pulping of wood biomass has been reported by Demirbas et al., 1998 and exhibited high delignification and negligible cellulose degradation for wood biomass. Hence an integrated approach exploiting the potential of transition metals, biodiesel industry generated waste glycerol streams as well as sodium hydroxide were evaluated for better hemicelluloses as well as lignin removal.

The objective of the present study was to develop a novel sequential pretreatment strategy to exploit the surplus available biomass in India- the sugarcane trash for the production of bioethanol as well as to optimize various process parameters affecting pretreatment and hydrolysis.

2. Materials and Methods

2.1. Feed stock

Sugarcane trash received from Godavari Biorefineries, Mumbai, Maharashtra, India was used in this study. Sugarcane trash consists of dry leaves and tops of sugarcane plant. The feed stock was dried and was milled in a knife mill. The compositional analysis of native and pretreated sample was evaluated by two stage dilute acid hydrolysis method following NREL protocol.

2.2. Screening of various transition metals for crude glycerol assisted pretreatment of Sugarcane trash

Pretreatment was carried out in a 150 ml stoppered conical flask with a biomass loading of 10% w/w. Biomass of mixed particle size were used for pretreatment, since it minimizes wastage of biomass. Biodiesel industry generated crude glycerol (3% v/v) in presence of 1% NaOH and different transition metals at a concentration of 1% w/w (manganese sulphate, cadmium acetate, nickel chloride, zinc sulphate, ferrous sulphate and ferric chloride) were used for the initial screening. Control experiments were carried out without any reagents (water alone) and with crude glycerol and NaOH alone. Pretreatment was carried out in a laboratory autoclave at 121°C for 60 min. After pretreatment the pretreated samples were neutralized, washed and dried at room temperature (30 ±2°C).

2.3. Optimization of various process parameters affecting sequential crude glycerol assisted transition metal and alkali pretreatment of sugarcane trash

Various process parameters affecting sequential pretreatment of sugarcane trash was carried out by adopting a Taguchi design. The experimental set up is shown in Table 1. The variables selected were biomass loading, ferric chloride concentration, crude glycerol concentration, sodium hydroxide concentration and incubation time. These five variables were selected at four levels and a total of 16 runs were used in this study. After pretreatment the samples were neutralized and washed with tap water followed by air drying at room temperature.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis of sequential pretreated sugarcane trash was carried out in a 150 ml stoppered flask with 10% w/w of biomass loading with commercial cellulase (Zytex India Private Limited, Mumbai, India). Surfactant (0.1% w/w of Tween 80) and Penicillin-Streptomycin antibiotic cocktail (Hi-Media, India) were used. The total reaction volume was made up to 20 ml with 0.1 M citrate buffer (pH 4.8) and incubated in a shaking water bath at 50°C for 48 hrs. After incubation the samples was centrifuged to remove the unhydrolyzed residue and the hydrolyzate was used for reducing sugar analysis by 2, 5 dinitrosalicylic acid method (Miller, 1959).

2.5. Optimization of various process parameters affecting hydrolysis of sequential pretreated sugarcane trash

Various process parameters affecting hydrolysis of sequential pretreated sugarcane trash were optimized by adopting a Taguchi design. The experimental set up is shown in Table 2. The variables selected were biomass loading, enzyme loading, surfactant concentration and incubation time. These four variables were selected at four levels and a total of 16 runs were used in this study.

2.6 Inhibitor analysis of the hydrolyzate

The hydrolyzate was centrifuged to remove the solids and filtered through 0.2µm PES membrane filters (Pall, USA) and the filtrate was analyzed by HPLC. The inhibitors were analyzed using a photodiode array detector kept at 55°C. Other conditions of operation were Rezex ROA column (Phenomenex) with an injection volume of 10µl and flow rate was maintained at 0.6 ml/ min. The concentrations of inhibitors were analyzed using the standard curve.

2.7. Characterization of native and pretreated sugarcane trash

To investigate changes in biomass physical and chemical features after pretreatment, characterizations of native and pretreated samples were carried out by scanning electron microscopy visualizations, FTIR and XRD spectrum.

2.7.1. Scanning electron microscopy

Scanning electron micrographs were taken using a scanning electron microscope (JEOL JSM -1600) to visualize the morphological differences between the native and the pretreated sugarcane trash. The images of native and pretreated samples were acquired with an accelerating voltage of 15kV with a magnification of 500X.

2.7.2. X-ray diffraction

X-ray diffraction analysis was performed using a PANalytical (Netherlands), X-pert pro diffractometer with a step size of 0.03° using a Cu-Kα radiation X-ray (k= 1.54A°) at a voltage of 40kV and current of 30mA.

2.7.3. FTIR analysis

In FTIR spectroscopy, infrared radiation is passed through a sample it absorbs some radiation and some radiation is transmitted and the resulting spectrum is a molecular finger print of the sample. This will detect changes in functional groups that may have been caused during

pretreatment. The FTIR spectrum was recorded between 4000 and 400 cm^{-1} using a Shimadzu Spectrometer with detector at 4 cm^{-1} resolution and 25 scan per sample. Discs were prepared by mixing 3 mg of dried samples with 300 mg of spectroscopic grade KBr in an agate mortar and the resulted mixture was pressed at 10 Mpa for 3 min (Sindhu et al., 2014a).

2.8. Fermentation

The hydrolyzate obtained after enzymatic saccharification were centrifuged to remove the unhydrolyzed solid residues. The supernatant was used for fermentation. Fermentation was carried out in a screw capped vials with 10 ml of the non-detoxified hydrolyzate and was inoculated with 18 h old *Saccharomyces cerevisiae* at 30°C for 72 hrs. After fermentation the samples were centrifuged and filtered through a 0.4µ filters (Pall, USA) and analyzed by Gas Chromatography (Chemito, India) set at an oven temperature 150°C, injector temperature 175°C and detector temperature 250°C . Nitrogen with flow rate 30 ml per minute was used as carrier gas. The ethanol concentrations of the fermented samples were calculated based on the calibration curve drawn with known concentrations of external standard. The analyses were done in triplicate and the mean values were presented.

3. Results and discussion

3.1. Compositional analysis of native and pretreated sugarcane trash

Compositional analyses for native, control samples (crude glycerol, ferric chloride and sodium hydroxide alone) and sequentially pretreated samples were carried out for cellulose, hemicellulose and lignin. Mass balance analysis shows that there was 45% loss after pretreatment. Native biomass contains 27.85% cellulose, 19.41% hemicellulose and 27.11% lignin for dry biomass. Crude glycerol pretreated samples contains 47.82% cellulose, 18.37% hemicelluloses and 12.39% lignin. Sodium hydroxide pretreated samples contains 48.97% cellulose, 17.32% hemicelluloses and 10.78% lignin. Ferric chloride pretreated samples contains 51.78% cellulose, 10.11% hemicelluloses and 25.91% lignin. The sequential pretreated sample contains 67.8% cellulose, 9.22% hemicellulose and 5.77% lignin. The results indicate that this novel method is effective in lignin and hemicelluloses removal compared to conventional pretreatment using acid or alkali. Crude glycerol and sodium hydroxide pretreated samples showed a better lignin removal while the ferric chloride pretreated samples showed better hemicellulose removal. Transition metals are known to play a good role in hemicelluloses removal (Liu et al., 2009). The exact mechanism is not known

whether they alter the lignocellulosic matrix and improves the saccharification rate. Alkali as well as crude glycerol plays an important role in delignification. Hence by exploiting the potential of hemicelluloses and lignin removal agents made the pretreatment more effective in terms of lignin and hemicelluloses removal. This is evident from the compositional data. Our previous studies adopting alternative strategies of pretreatment like surfactant assisted acid pretreatment of sugarcane tops (Sindhu et al., 2012) and surfactant assisted ultrasound pretreatment of sugarcane tops (Sindhu et al., 2013) were found to be effective in lignin and hemicelluloses removal, this sequential pretreatment was found to be superior in terms of lignin and hemicelluloses removal. In the present study there was 50% removal of hemicelluloses. Liu et al., 2009 reported ferric chloride pretreatment of corn stover where there was 100% removal of hemicelluloses when the pretreatment was carried out at higher temperatures (160°C). The low hemicelluloses removal in this study may be due to pretreatment carried at lower temperature (121°C). Carrying pretreatment at higher temperature makes the process energy intensive as well as generation of inhibitors from sugar degradation which will affect further fermentation. High lignin removal in the sequential pretreatment was comparable with aqueous glycerol pretreatment of wheat straw reported by Sun and Chen, 2008a. The delignification process in the present study took only 45 min but the delignifying process reported by Demirbas et al., 1998 for wood chips took a long time of 8hrs and there was a significant degradation of cellulose.

3.2. Primary Screening

Among the different transition metals screened for sequential pretreatment of sugarcane trash, ferric chloride pretreatment was found to be superior in terms of reducing sugar yield (0.25 g of reducing sugar yield per g of dry biomass, g/g) followed by ferrous sulphate (0.15 g/g), nickel chloride (0.145 g/g), cadmium sulphate (0.13 g/g) and manganous sulphate (0.11 g/g). Hence ferric chloride was selected for further optimization of sequential pretreatment of sugarcane trash. Control experiments with crude glycerol alone (0.036 g/g), ferric chloride alone (0.045 g/g), sodium hydroxide pretreatment (0.149 g/g) as well as water alone (0.05 g/g) gave low reducing sugar yield. An identical observation was earlier reported by Liu et al., 2009 for pretreatment of corn stover.

3.3. Effect of different process parameters on sequential crude glycerol assisted transition metal and alkali pretreatment of sugarcane trash

The results were presented in Table 1. Maximum reducing sugar (0.721 g/g) was observed with Run number 13 where the pretreatment conditions were biomass loading of 10% w/w, crude glycerol concentration of 6%, sodium hydroxide concentration of 5%, ferric chloride concentration of 1% and incubation time of 45 min. Run number 6 gave reducing sugar yield of 0.706 g/g where the conditions of pretreatment were biomass loading of 15 % w/w, crude glycerol concentration of 4% (Yanthra Fintech Ltd, India), sodium hydroxide concentration of 2%, ferric chloride concentration of 2% and incubation time of 45 min. Since the reagent concentrations were lower and with high biomass loading the conditions of Run number 6 was selected for further studies.

Fig. 1a shows interactions between biomass loading and incubation time on reducing sugar yield. At low level of incubation time (10-35 min) and low levels of biomass loading (5-8% w/w), the reducing sugar yield is low (0.5 g/g). Reducing sugar yield increases with increase of biomass loading and incubation time. Maximum reducing sugar yield (0.7 g/g) was observed with biomass loading of 10% w/w and with incubation time of 40-50 min. Anwar et al., 2012 observed maximum reducing sugar yield at higher levels of incubation time (50 min) for dilute acid pretreated rice polish.

Fig. 1b shows interaction between ferric chloride concentration and biomass loading on reducing sugar yield. At low levels of biomass loading (8.5-10.5% w/w) and low levels of ferric chloride concentration (1.0 – 1.5% w/w) the reducing sugar yield is high (0.7 g/g). With increase of biomass loading and ferric chloride concentration there was a decrease in reducing sugar yield. The decrease in reducing sugar yield with increase of biomass loading may be due to poor penetration of the pretreatment agent at high biomass loading. Similar observation was earlier reported by Sindhu et al., 2012 for organosolvent pretreatment of rice straw.

Fig. 1c shows interaction between sodium hydroxide and ferric chloride concentration on reducing sugar yield. Maximum reducing sugar yield (0.7 g/g) was observed with low levels of sodium hydroxide concentration (2.0 – 2.5%) and low levels of ferric chloride concentration (1.5 – 2.0%). With increase of sodium hydroxide concentration as well as ferric chloride concentration there was a decrease in reducing sugar yield. Sodium hydroxide concentration of 3% was reported as the best condition for alkali pretreatment of sugarcane trash (Sindhu et al., 2014a).

Fig. 1d shows interaction between incubation time and crude glycerol concentration on reducing sugar yield. At low levels of incubation time (20 – 35 min) the reducing sugar yield

is low (0.5 g/g). Reducing sugar yield increases with increase of incubation time. Maximum reducing sugar yield (0.7 g/g) was observed with high levels of incubation time (40 – 50 min) and low levels of crude glycerol concentration (3.0 – 4.0 %).

Due to highly polar polyalcohol structure, glycerol can easily penetrate into the biomass and provides an effective reaction medium for delignification of lignocellulosic biomass. Sun and Chen, 2008a carried out atmospheric aqueous glycerol pretreatments of wheat straw where the optimum conditions of pretreatment were 70% crude glycerol with a liquid –solid ratio 200/10.

Role of acidified aqueous glycerol in the removal of hemicelluloses and lignin has been reported by Zhang et al., 2013. Utilization of aqueous glycerol for delignification has been earlier reported by Demirbas, 1998. Contrary observations were earlier reported by Sun and Chen, 2008b for crude glycerol pretreatment of wheat straw though it enhanced delignification and improved enzymatic saccharification rate, the lipophilic compounds from crude glycerol formed pitch deposition in the pretreatment process. Hence pretreatment processes of crude glycerol must be carried out before using for pretreatment of lignocellulosic biomass. In the present study the biodiesel industry generated waste glycerol was found suitable for pretreatment of sugarcane trash without any processing. Our earlier report shows that this crude glycerol without any processing can serve as sole carbon source for the production of biopolymer, poly-3-hydroxybutyrate (Sindhu et al., 2011).

3.4. Effect of different process parameters affecting hydrolysis of sequential crude glycerol assisted transition metal and alkali pretreated sugarcane trash

The results were presented in Table 2. Maximum reducing sugar yield (0.796 g/g) was observed with Run number 4 where the conditions of hydrolysis were biomass loading of 5% w/w, enzyme loading of 80 FPU, surfactant concentration of 0.25% w/w and incubation time of 48 hrs. Run number 12 gave a reducing sugar yield of 0.738 g/g where the conditions of hydrolysis were biomass loading of 10% w/w, enzyme loading of 80 FPU, incubation time of 24 hrs and surfactant concentration of 0.05% w/w. Hence Run number 12 was selected for further studies since the biomass loading was high and the incubation time was lower which will make the process economically viable. Performing enzymatic hydrolysis with higher

solid loading offers many advantages like increased sugar and ethanol yields and decreased operational and capital costs there by making the process economically viable.

Contour plots showing interaction between various process parameters affecting hydrolysis were presented in Fig. 2 a-f. The interaction between enzyme loading and biomass loading is presented in Fig. 2a. At low levels of biomass loading the reducing sugar yield was high. Reducing sugar yield decreases with increase of biomass loading. At low levels of enzyme loading (20 – 50 FPU) the reducing sugar yield was low. Maximum reducing sugar yield (0.78 g/g) was observed with high levels of enzyme loading (60 – 80 FPU) and low levels of biomass loading (5-7%w/w). Contrary observations were reported by Sindhu et al., 2014b for hydrolysis of acid pretreated bamboo were maximum reducing sugar yield was observed with low levels of enzyme loading (20 – 30 FPU). The decrease in reducing sugar yield at high biomass loading is due to lack of available free water at high biomass loading which limits the enzymatic saccharification rate. The hydrolysis efficiency was higher at low biomass loading since more enzymes were available per unit biomass to be hydrolyzed. Maurya et al., 2013 reported higher hydrolysis efficiency at low biomass loading for microwave pretreated SCT.

The interaction between biomass loading and incubation time on reducing sugar yield is presented in Fig. 2b. At low to middle levels (10 – 35hrs) of incubation time the reducing sugar yield is low. Reducing sugar yield increases with increase of incubation time and maximum reducing sugar yield (0.78 g/g) was observed with high levels of incubation time (40 – 45hrs) and low to middle levels of biomass loading (5 - 8% w/w). One of the major limitations for enzymatic hydrolysis at high biomass loading is the lack of available free water which limits mass transfer and lubricity. Other limitations with high biomass loading include difficulty in mixing and handling as well as increased concentration of inhibitors. Most of the reports on enzymatic hydrolysis at high solid loading were carried out by employing batch addition of substrates as well as enzymes.

Fig. 2c shows interaction between biomass loading and surfactant concentration on reducing sugar yield. At low levels of biomass loading and low level of surfactant concentration the reducing sugar yield was low. Reducing sugar yield increases with increase of biomass loading and surfactant concentration. Maximum reducing sugar yield (0.78 g/g) was observed with high levels of surfactant concentration (0.2-0.25% w/w) and low levels of biomass loading (5 – 7% w/w). Surfactant plays an important role in non-productive adsorption of enzymes by lignin. Surfactant will change the nature of the substrate either by increasing the

available surface or by removing inhibitory lignin. The low conversion of glucans at high biomass loading is due to feed back inhibition of enzymes by the accumulated monomeric and oligomeric sugars during the enzymatic saccharification process as well as due to mass transfer problems derived from high biomass loading.

Fig. 2d shows interactions between enzyme loading and incubation time on reducing sugar yield. At low levels of incubation time and low levels of enzyme loading the reducing sugar yield is low. Reducing sugar yield increases with increase of incubation time and enzyme loading. Maximum reducing sugar yield (0.78 g/g) was observed with high levels of enzyme loading (40-80 FPU) and with high levels of incubation time (40 – 45 hrs). Contrary observations was earlier reported by Maurya et al., 2013 where maximum reducing sugar yield was observed with middle levels of enzyme loading and high levels of incubation time for microwave pretreated SCT. This might be due to feed back inhibition of enzyme by the product.

Fig. 2e shows interactions between enzyme loading and surfactant concentration on reducing sugar yield. At low levels of enzyme loading (20-40 FPU) and low levels of surfactant concentration (0.05-0.15) the reducing sugar yield is low (0.66 g/g). Reducing sugar yield increases with increase of enzyme loading and surfactant concentration. Maximum reducing sugar yield (0.78 g/g) was observed with high levels of enzyme loading (40-80 FPU) and with high levels of surfactant concentration (0.2 – 0.25% w/w). Eriksson et al., 2002 observed that major obstacle in the enzymatic saccharification of lignocellulosic biomass is the adsorption of significant amounts of enzymes to exposed lignin surfaces. The dominating effect of surfactant adsorption to lignin prevents the unproductive binding of cellulases to lignin. Surfactant also stabilizes enzymes from denaturation during hydrolysis and increase positive interaction between enzymes and substrate (Kaar and Holtzapple, 1998).

Fig. 2f shows interaction between incubation time and surfactant concentration on reducing sugar yield. It was observed that at low levels of incubation time (10 – 20hrs) and low levels of surfactant concentration (0.05 – 0.15% w/w) the reducing sugar yield was low. Maximum reducing sugar yield (0.78 g/g) was observed with middle to high levels of incubation time (20 – 45hrs) and middle to high levels of surfactant concentration (0.15 – 0.25% w/w). Surfactant plays a positive role in affecting the rheological properties of the slurry by reducing the viscosity. Non-ionic surfactants were more suitable for improving enzymatic hydrolysis by hydrophobic interaction with lignin and release the non-productive adsorbed

enzymes (Errikson et al., 2002). Other possible mechanisms for improving enzymatic saccharification rate are by increasing enzyme stability, increasing accessibility of the substrate etc. Higher concentration of surfactant may not always lead to higher increment in cellulose conversion and this can be due to occupying all binding sites on substrate by surfactant irrespective of the ability of the substrate to un-specifically bind enzymes (Kristensen et al., 2007).

Analysis of variance data for reducing sugar yield after sequential pretreatment and hydrolysis of sugarcane trash is presented in Table-3. *p* value was used as a tool to check the significance of each of the coefficients. Smaller the *p* value greater is the correlation with the corresponding coefficient. In this model biomass loading, enzyme loading and incubation time were found to be the significant factors. The regression coefficient was found to be best with incubation time (*p* value 0.006).

3.5. Inhibitor profile of the hydrolyzate

Inhibitor analysis of the hydrolyzate revealed that it was devoid of major fermentation inhibitors like furfural and 5-hydroxymethyl furfural and other organic acids like acetic acid, propionic acid, succinic acid, formic acid and oxalic acid. Hence the hydrolyzate can be used for fermentation without any detoxification. Studies by Krishnan et al., 2010 showed that it is not necessary to supplement external nutrients or detoxification prior to fermentation for mild AFEX pretreatment of cane leaf hydrolysate. Earlier reports by Lau et al., 2008 showed that the cost of hydrolyzate detoxification can go up to 22% of the total ethanol production cost. Elimination of detoxification step in the biomass to ethanol process makes the process more economically viable.

3.6. SEM, XRD and FTIR profile of native and pretreated sugarcane trash

Scanning electron micrographs of native and pretreated sugarcane trash was carried out. Native biomass showed a compact and highly ordered structure while the pretreated biomass showed a highly distorted structure with removal of some external fibers and with more roughness and surface area. Identical observations were earlier reported for dilute acid and dilute alkali pretreatment of sugarcane tops (Sindhu et al., 2011; Sindhu et al., 2014a) and aqueous glycerol pretreatment of wheat straw (Sun and Chen, 2008). After pretreatment the cell walls of sugarcane trash got altered showing development of numerous pores and the inner parts were exposed showing a network structure with multiple pores opened during

pretreatment which resulted in an improved enzymatic conversion of the pretreated sample. The porosity in the pretreated biomass greatly increased the enzyme-accessible surface area. This helped in the increase of enzyme accessibility to biomass and hence high reducing sugar yield.

X-ray diffractogram of native and pretreated samples were presented in Supplementary Fig. S1. There is difference in the diffraction pattern for native and pretreated samples. Pereira et al., 2011 reported that peaks at 15º and 22º provide evidence on the effectiveness of treatment on fibers for acetic acid and sodium chloride pretreated sugarcane bagasse. This occurs due to disorder of components in the lignocellulosic biomass after pretreatment. It indicates an increase in interplanar distance of the untreated sample in relation to treated fibers. Filho et al., 2007 earlier reported projections of substituting groups along the axis are associated with an increase in the interfibrillar distance. Native samples were less crystalline than the treated samples. The crystallinity index of native sample was 45% while that of sequential pretreated sample is 57%. Increase in crystallinity index indicates that amorphous components like lignin and hemicelluloses were removed which in turn increase the crystallinity index. Similar observations were earlier reported for microwave assisted pretreatment of sugarcane bagasse (Binod et al., 2012) surfactant assisted acid pretreated sugarcane tops (Sindhu et al., 2012), surfactant assisted ultrasound pretreated sugarcane tops (Sindhu et al., 2013) and acetic acid and sodium chloride pretreated sugarcane bagasse (Pereira et al., 2011). Contrary observations for decrease in crystallinity index for biomass pretreatments like COSLIF and ionic liquid based pretreatment where there was a decrease in crystallinity index by disrupting highly ordered hydrogen bonds in crystalline cellulose fibers have been reported by Lee et al., 2009.

FTIR spectrums of native and pretreated samples were presented in Supplementary Fig. S2. The bands at 1000 – 1200 cm^{-1} corresponds to structural features of cellulose (Langkilde and Svantesson, 1995). After pretreatment the absorption peaks at 1000 – 1200 cm^{-1} region were enhanced indicating there was an increase in cellulose content after pretreatment. Sequential pretreatment removes the hemicelluloses and lignin fraction from the raw material which in turn increase the cellulose content. The broad absorption band at 3000 – 3500 cm^{-1} region was due to stretching of –OH groups and absorption at 2900 cm^{-1} region is related to $–CH_2$ groups. An identical observation was earlier reported by Jahan et al., 2011 for microcrystalline cellulose of jute straw and by Rosa et al., 2012 for cellulose from rice husk. Sun et al., 2003

reported lignin corresponds to adsorption peaks at 1509, 1464 and 1422 cm^{-1}. The reduction in band intensity at these regions indicates lignin removal. The band widening at 2800 cm^{-1} corresponds to CH_2 stretching and this region is distinguished feature of cellulose (Parker, 1971).

3.7. Fermentation

Fermentation of the hydrolyzate with *Saccharomyces cerevisiae* yielded 31.928 g of ethanol with a fermentation efficiency of 78.89%. This is without any optimization of various process parameters affecting fermentation. The increase of fermentation efficiency is due to absence of inhibitors. Similar observation was earlier reported by Srinorakutara et al., 2014 for sodium hydroxide followed by acid pretreated sugarcane trash where the maximum ethanol concentration is 30.40 g/l. In our previous study for fermentation using acid pretreated and hydrolyzed sugarcane tops the ethanol yield was 11.365g with a fermentation efficiency of 50%. This may be due to presence of inhibitors generated during acid pretreatment (Sindhu et al., 2011).

4. Conclusions

The effectiveness of sequential pretreatment in improving enzymatic saccharification of sugar cane trash was evaluated in this study. The study revealed that the hydrolyzate obtained after enzymatic saccharification is devoid of major fermentation inhibitors like organic acids and furfurals and can be used for fermentation without any detoxification. To the best of our knowledge this is the first report on sequential pretreatment of sugarcane trash using crude glycerol assisted ferric chloride and sodium hydroxide pretreatment and was highly effective in removing hemicelluloses and lignin and the hydrolyzate serves as a good source for bioethanol production by *Saccharomyces cerevisiae*.

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Table 1: Taguchi design for optimization of various process parameters affecting sequential pretreatment of sugarcane trash

Run No	Crude glycerol (% w/w)	Ferric chloride (% w/w)	Sodium hydroxide (% w/w)	Biomass loading (% w/w)	Incubation time (min)	Reducing sugar yield (g/g)
1	3	1	2	7.5	15	0.651
2	3	2	3	10	30	0.665
3	3	3	4	12.5	45	0.670
4	3	4	5	15	60	0.680
5	4	1	3	12.5	60	0.631
6	4	2	2	15	45	0.704
7	4	3	5	7.5	30	0.559
8	4	4	4	10	15	0.585
9	5	1	4	15	30	0.494
10	5	2	5	12.5	15	0.682
11	5	3	2	10	60	0.557
12	5	4	3	7.5	45	0.586
13	6	1	5	10	45	0.721
14	6	2	4	7.5	60	0.673
15	6	3	3	15	15	0.613
16	6	4	2	12.5	30	0.507

Table 2: Taguchi design for optimization of various process parameters affecting hydrolysis of sequential pretreated sugarcane trash

Run No	Biomass Loading (% w/w)	Enzyme Loading (FPU)	Incubation Time (hrs)	Surfactant Conc. (%w/w)	Reducing sugar yield (g/g)
1	5	20	12	0.05	0.617
2	5	40	24	0.125	0.709
3	5	60	36	0.2	0.683
4	5	80	48	0.25	0.796
5	7.5	20	24	0.2	0.663
6	7.5	40	12	0.25	0.682
7	7.5	60	48	0.05	0.738
8	7.5	80	36	0.125	0.681
9	10	20	36	0.25	0.663
10	10	40	48	0.2	0.732
11	10	60	12	0.125	0.651
12	10	80	24	0.05	0.738
13	12.5	20	48	0.125	0.681
14	12.5	40	36	0.05	0.712
15	12.5	60	24	0.25	0.708
16	12.5	80	12	0.2	0.681

Table 3: Analysis of variance data for reducing sugar yield after sequential pretreatment of sugarcane trash

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Biomass loading	3	0.0232407	0.0232407	0.0077469	19.05	0.019
Enzyme loading	3	0.0439062	0.0439062	0.0146354	35.99	0.007
Surfactant Concentration	3	0.0005973	0.0005973	0.0001991	0.49	0.714
Incubation time	3	0.0520781	0.0520781	0.0173594	42.69	0.006
Error	3	0.0012199	0.0012199	0.0004066		
Total	15	0.1210423				
S= 0.0201649	R-Sq = 98.99%		R-Sq (adj) = 94.96%			

S- Square of the root mean square; R2 – Co-efficient of determination; Seq SS – Sequential Sum of Squares; DF- Degree of Freedom; Adj SS – Adjusted sums of squares; Adj MS – Adjusted Mean Square; F- F test; P- Probability; PRESS – Predicted Residual Sum of Squares

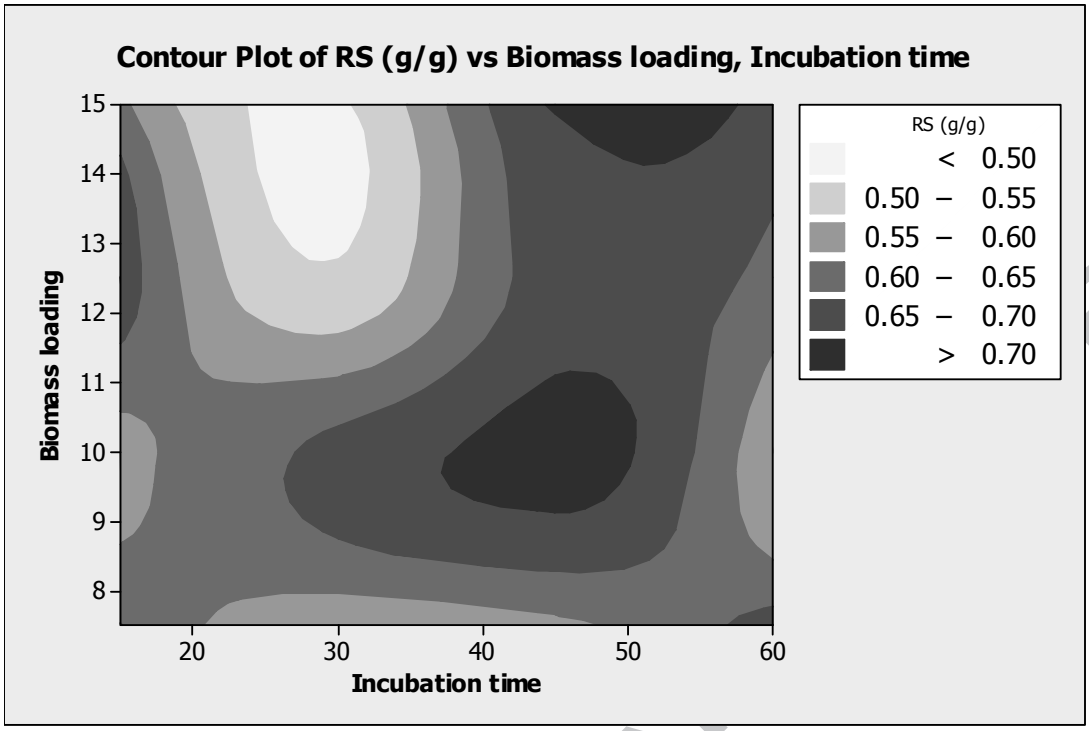
Figure Captions

Fig. 1. Contour plots showing interactions of various process parameters affecting sequential pretreatment of sugarcane trash (a) interactions between incubation time and biomass loading (b) interactions between biomass loading and ferric chloride concentration (c) interactions between ferric chloride concentration and sodium hydroxide concentration and (d) interactions between crude glycerol concentration and incubation time

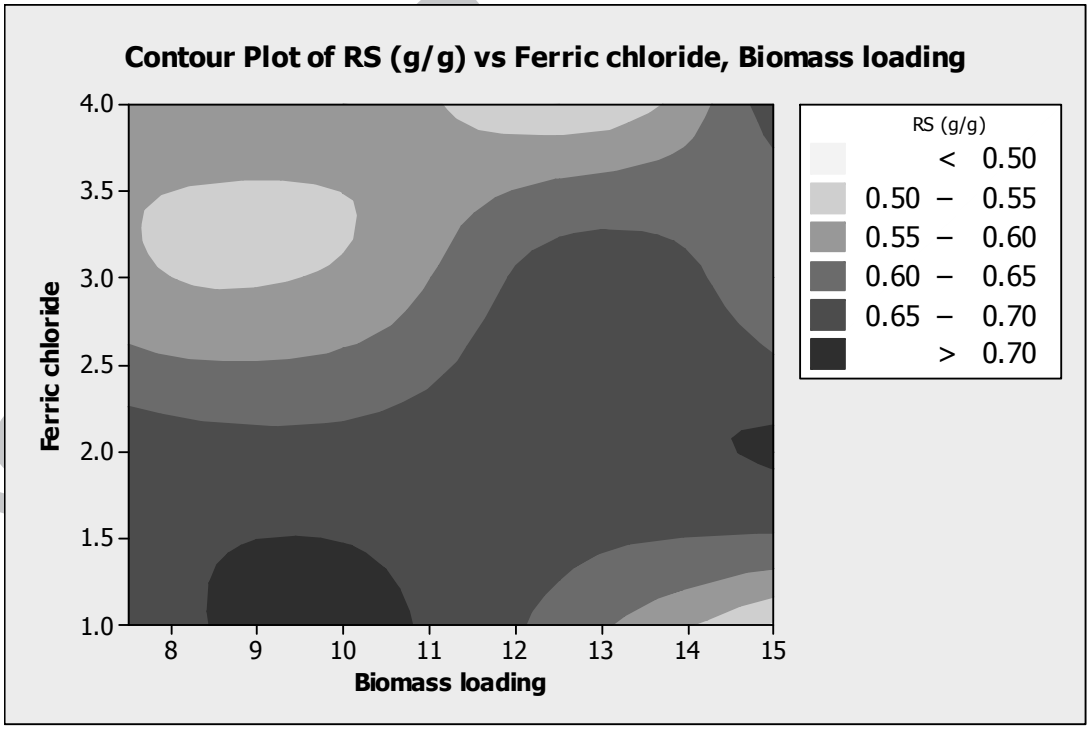
Fig. 2. Contour plots showing interactions of various process parameters affecting hydrolysis of sequential pretreated sugarcane trash (a) interactions between biomass loading and enzyme loading (b) interactions between biomass loading and incubation time (c) interactions between biomass loading and surfactant concentration (d) interactions between enzyme loading and incubation time (e) interactions between enzyme loading and surfactant concentration and (f) interactions between incubation time and surfactant concentration

Supplementary Fig. 1. X-ray diffractogram of native and pretreated sugarcane trash

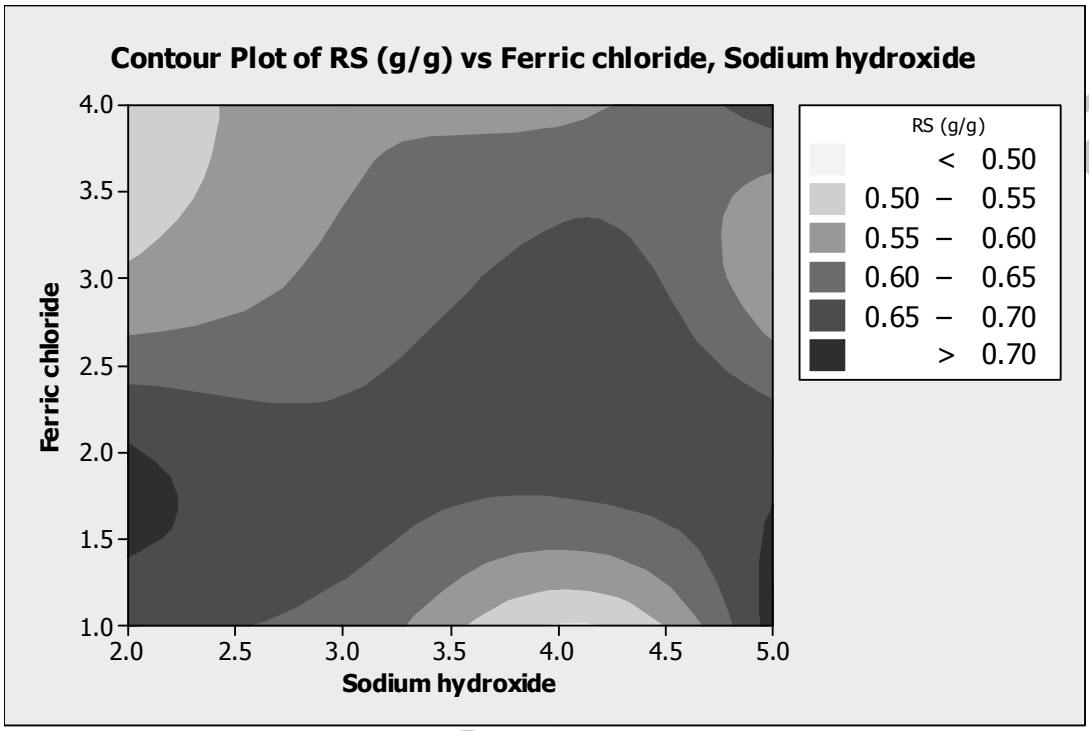
Supplementary Fig. 2. FTIR spectrum of native and pretreated sugarcane trash (A) Native SCT (B) $FeCl_3$ SCT (C) Crude glycerol SCT (D) NaOH SCT (E) Crude glycerol + $FeCl_3$ (F) Sequential SCT


Contour Plot of RS (g/g) vs Biomass loading, Incubation time
Biomass loading
Incubation time
15
14
13
12
11
10
9
8
20
30
40
50
60
RS (g/g)
< 0.50
0.50 – 0.55
0.55 – 0.60
0.60 – 0.65
0.65 – 0.70
> 0.70

a


Contour Plot of RS (g/g) vs Ferric chloride, Biomass loading
Ferric chloride
Biomass loading
4.0
3.5
3.0
2.5
2.0
1.5
1.0
8
9
10
11
12
13
14
15
RS (g/g)
< 0.50
0.50 – 0.55
0.55 – 0.60
0.60 – 0.65
0.65 – 0.70
> 0.70

b



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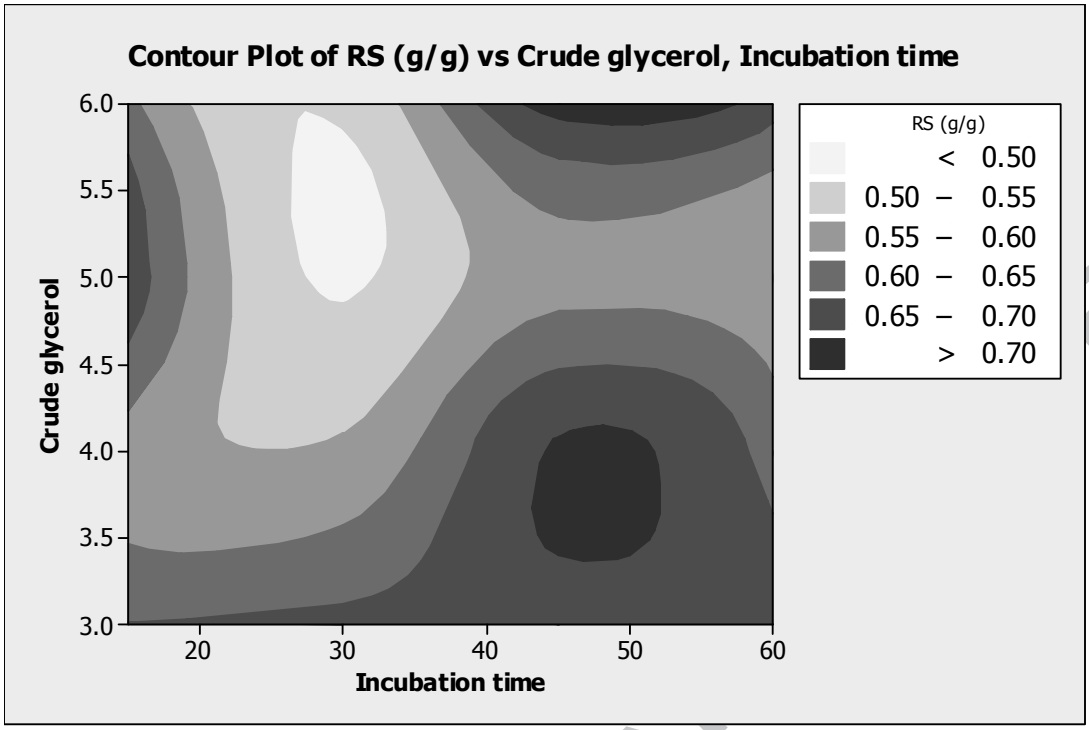
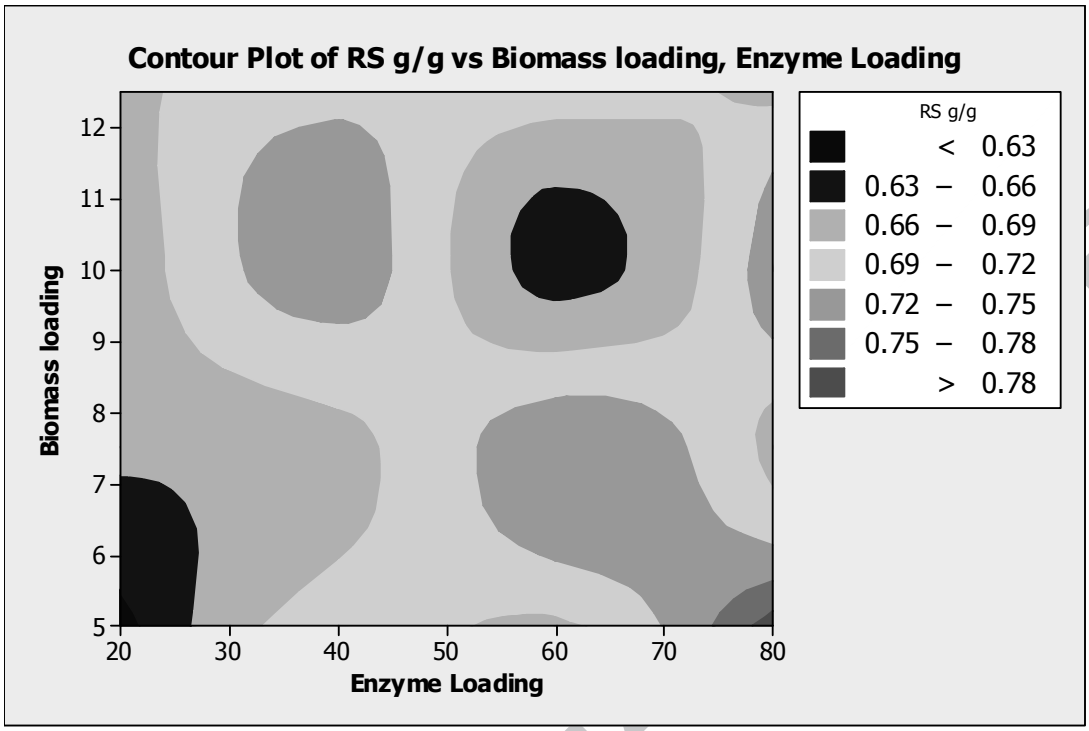
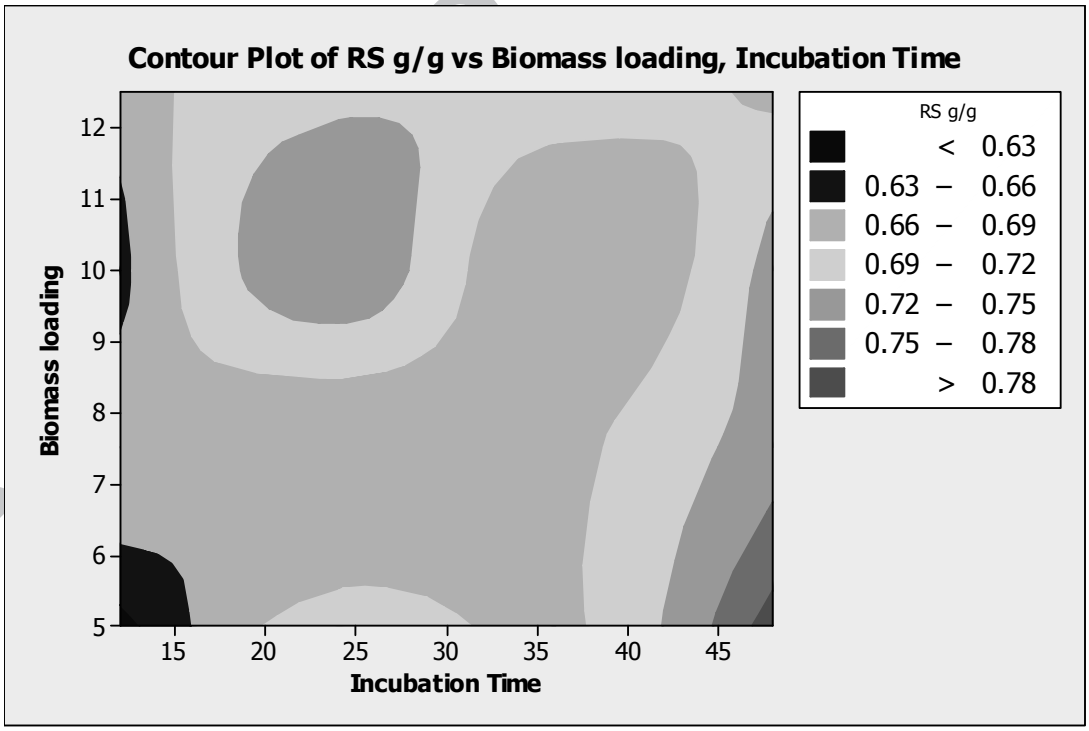


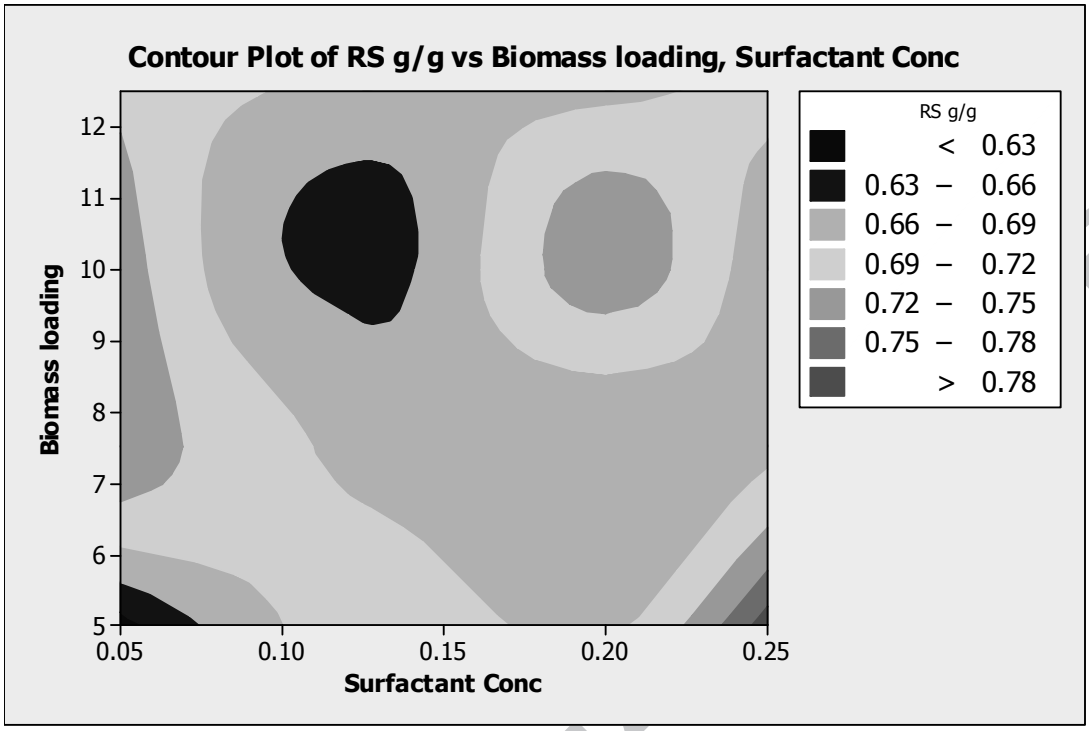
Fig. 1 a-d



a



b



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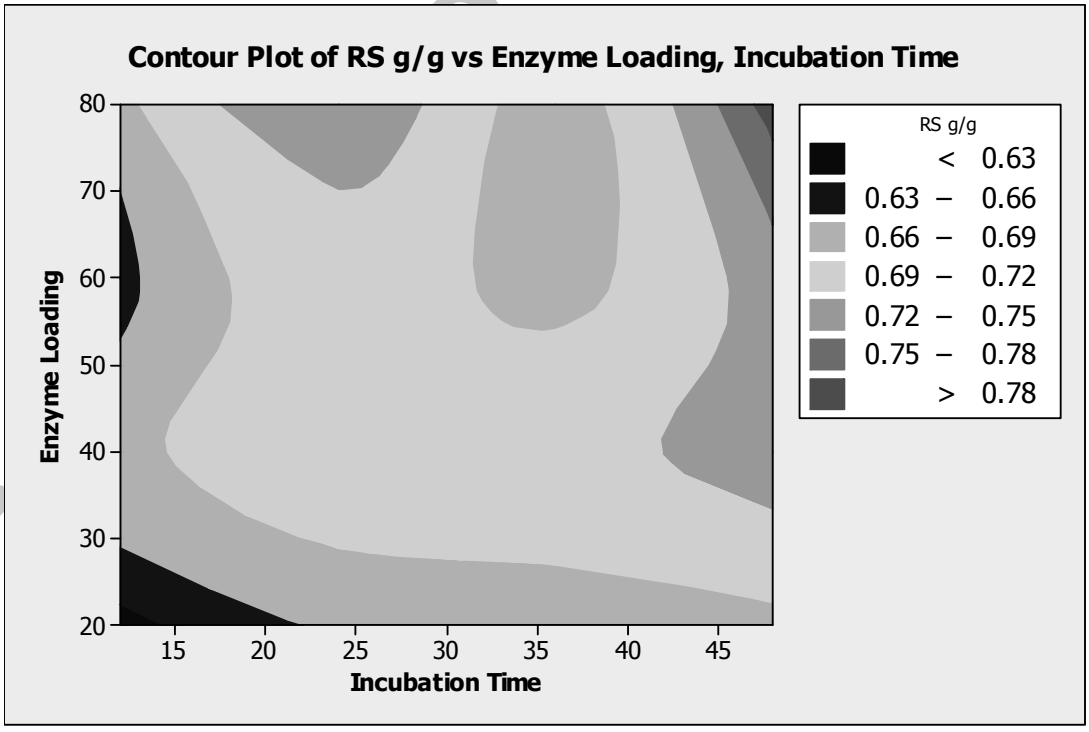
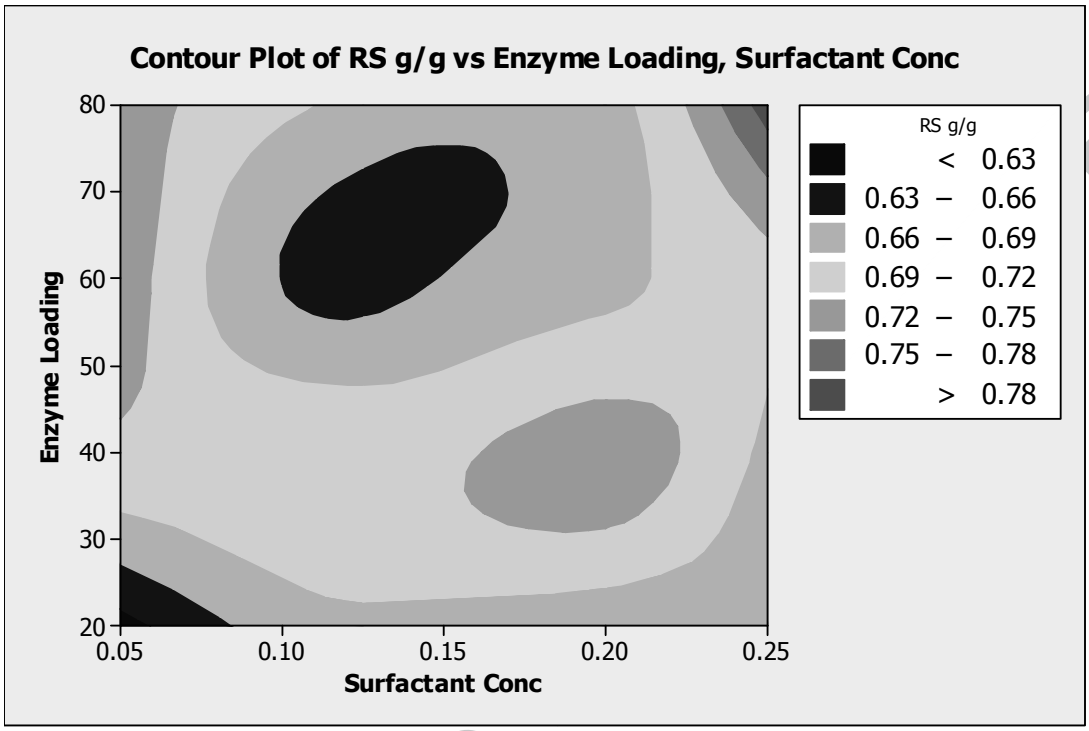
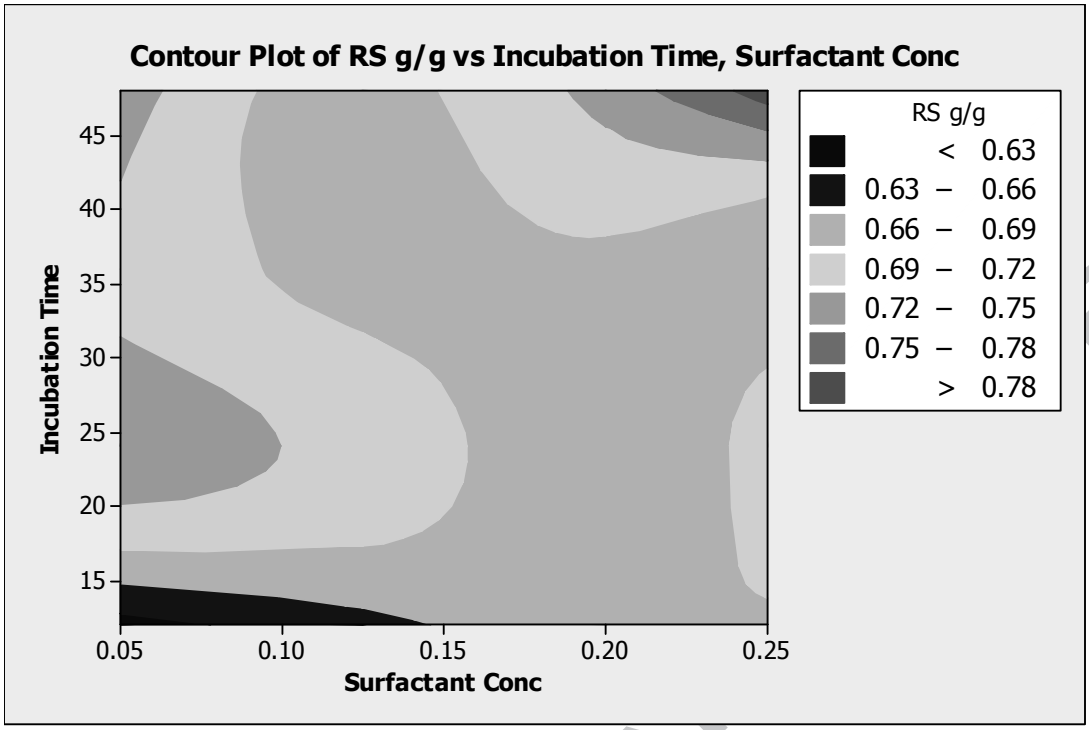


d



e



f

Fig. 2 a-f

Highlights

- First report on crude glycerol assisted transition metal and alkali pretreatment.
- Effectively removed hemicelluloses and lignin.
- Maximum reducing sugar yield is 0.796 g per g of dry biomass.
- Hydrolyzate is devoid of fermentation inhibitors like organic acids and furfurals.
- Fermentation yielded 31.928 g of ethanol with an efficiency of 78.89%.