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Research Paper

Ethanol production from agricultural wastes using *Sacchromyces cervisae*

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Abstract

The main objective of this study was production of ethanol from three lignocellulosic biomasses like sugarcane bagasse, rice straw and wheat straw by *Sacchromyces cervisae.* All the three substrates were ground to powder form (2 mm) and pretreated with 3%H_2O_2 + 2% NaOH followed by steaming at 130 °C for 60 min. These substrates were hydrolyzed by commercial cellulase enzyme. The whole fermentation process was carried out in 500 mL Erlenmeyer flask under anaerobic conditions in submerged fermentation at 30 °C for three days of incubation period. FTIR analysis of the substrates indicated significant changes in the alteration of the structure occurred after pretreatment which leads to efficient saccharification. After pretreatment the substrates were hydrolyzed by commercial cellulase enzyme and maximum hydrolysis was observed in sugarcane bagasse (64%) followed by rice straw (40%) and wheat straw (34%). Among all these tested substrates, sugarcane bagasse (77 g/L) produced more ethanol as compared to rice straw (62 g/L) and wheat straw (44 g/L) using medium composition of (%) 0.25 $(NH_4)_2SO_4$, 0.1 KH_2PO_4, 0.05 $MgSO_4$, 0.25 Yeast extract by *S. cervisae*.

Key words: lignoccellulosic biomass, Pretreatment, *Sacchromyces cervisae*, ethanol production.

Introduction

Lignocellulosic biomasses like wheat straw, sugarcane Bagasse and rice straw are the world renewable resource in the biosphere (Gruno *et al.*, 2004). Among this rice straw is to be considered as the largest available biomass inn the world which is about 7.31 x 10^{14} dry rice straw per year and Asia is the largest region in the world which is responsible for 90% of the annual global production (Kim and Dale, 2004). Sugarcane bagasse is the main by-product of sugarcane processing can also be used in fuel generation systems and produce more ethanol positively from corn. Lignocellulosic biomasses have only one problem is that enzymatic hydrolysis yield can not be greater than 20% of the theoretical maximum glucan conversion, even under a high level of enzyme loading or by employing longer reaction time (Kim and Lee, 2007). So to enhance this yield lignocellulosic biomasses need some pretreatment methods to alter the structure for greater enzyme accessibility for conversion of cellulose into glucose units. There are several pretreatment methods which can be employed on biomasses to alter their structure. Main pretreatment methods are; Milling and grinding, pyrolysis, high-energy radiation, high pressure steaming, alkaline or acid hydrolysis, gas treatment (chlorine dioxide, nitrogen dioxide, sulfur dioxide, ozone), hydrogen peroxide treatment, organic solvent treatment, hydrothermal treatment, steam explosion, wet oxidation and biological treatment (Mosier *et al.*, 2005). The cellulose content present in these substrates is hydrolyzed by mixture of enzymes which converts it into glucose which is important factor in ethanol production from lignocellulosic biomasses (Curreli *et al.*, 2002).

In Brazil and the United States, fuel ethanol is produced by fermentation of corn glucose in the US or sucrose in Brazil which are the largest ethanol producers in the world (MacDonald *et al.*, 2001) but this can also be prepared from agro-residues according to the agronomic-based economy of the country. There are some potential methods for low cost ethanol production by using agricultural wastes (Krishna *et al.*, 1999) and wheat straw is one of the most abundant agricultural wastes, has been extensively

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studied (Ballesteros *et al.*, 2004; Curreli *et al.*, 1997). Ethanol can be produced by variety of microorganisms. Cellulose-to-ethanol bioconversion can be conducted by various anaerobic thermophilic bacteria, such as *Clostridium thermocellum* (Ingram *et al.*, 1987), *Zymomonas* (Matthew *et al.*, 2005), Engineered *Escherichia coli* (Millichip and Doelle, 1989) as well as by some filamentous fungi, including *Monilia* sp. (Saddler and Chan, 1982), *Neurospora crassa* (Gong *et al.*, 1981), *Neurospora* sp. (Yamauchi *et al.*, 1989), *Zygosaccharomyces rouxii* (Pastore *et al.*, 1994), *Aspergillus* sp. (Sugawara *et al.*, 1994), *Trichoderma viride* (Ito *et al.*, 1990), and *Paecilomyces* sp. (Gervais and Sarrette, 1990). This study is focused on pretreatment of the lignocellulosic biomasses (sugarcane Bagasse, rice straw and wheat straw) and ethanol production from these biomasses using strain of *Sacchromyces cervisiae* in submerged fermentation.

Materials and Methods

Lignocellulosic biomass

Sugar cane bagasse procured from Shakar Gunj Sugar mills (Pvt.) Limited, Jhang Road, Faisalabad, Pakistan, and wheat straw and rice straw were purchased from a local market of Lahore city was used as a source of lignocellulosic biomass. The biomass was washed and dried to remove the unwanted particles and then milled into powder form (2 mm) with hammer beater mill.

Microorganism

Sacchromyces cervisae was obtained from microbiology laboratory, Food and Biotechnology Research Center (FBRC), PCSIR and maintained on PDA slants stored at 4 °C for further use.

Pretreatment of biomasses

Sugar cane bagasse and wheat straw samples were pretreated by method as reported earlier (Irfan *et al.*, 2011a). The chopped sugar cane bagasse and wheat straw samples were soaked in 3% H_2O_2 + 2% NaOH solution at the ratio of 1: 10 (solid : liquid) for 2 h at room temperature. After that samples were steamed at 130 °C for 60 min. After steaming the samples were filtered and solid residues were washed up to neutrality.

Determination of lignin

The lignin content in treated and untreated samples was measured being considered as lignin the remaining solid residue after hydrolysis with 1.25% H_2SO_4 for two hours and 72% H_2SO_4 hydrolysis for four hours. The residue was filtered and washed with distilled water to remove sulphuric acid and oven dried at 105 °C for constant weight. The lignin (%) and delignification (%) was expressed by using the following equations (Irfan *et al.*, 2011a):

$$\text{Lignin (\%)} = \frac{\text{Lignin weight (g)}}{\text{Substrate weight (g)}} \times 100 \quad (1)$$

$$\text{Delignification (\%)} = \frac{L_u - L_t}{L_u} \times 100 \quad (2)$$

where L_u = Lignin (untreated sample), and L_t = Lignin (treated sample).

Enzymatic hydrolysis

Enzymatic hydrolysis was done as described earlier (Irfan *et al.*, 2011a). Commercial enzyme with CMCase activity of 2900 IU/mL and filter paper activity of 1500 FPU/mL enzyme solution was used for the hydrolysis experiments. Pretreated substrates at 5% solids loading (grams dry weight per 100 mL) in distilled water were incubated in flasks in a shaking water bath at 50 °C and 140 rpm for 8 h. After termination of enzymatic hydrolysis the material was centrifuged at 10,000 rpm for 10 min. The supernatant was removed for sugar content analysis. Saccharification (%) was calculated as described by Uma *et al.* (2010).

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugars (g)} \times 0.9}{\text{Cellulo content in pretreated substrate}} \times 100 \quad (3)$$

Ethanol production

The medium used for ethanol fermentation composed of (%) 0.25 $(NH_4)_2SO_4$, 0.1 KH_2PO_4, 0.05 $MgSO_4$, 0.25 Yeast extract; These chemicals were added to the filtrate from saccharified biomasses (Bagasse, rice straw, wheat straw) and sterilized at 121 °C for 15 min. After sterilization the medium was allowed to cool at room temperature. After that one milliliter suspension of *Sacchromyces cervisea* were inoculated and incubated anaerobically at 30 °C for four days of fermentation period. After termination of the fermentation period, ethanol produced was estimated and the ethanol yield was calculated by using following formula as described in Yoswathana and Phuriphipat (2010).

$$\text{Ethanol yield} = \frac{\text{Measured ethanol in sample (g)}}{\text{*Theoretical ethanol (g)}} \quad (4)$$

*Theoratical ethanol = amount of initial sugar content (g) in fermentation solution x 0.5

Estimation of ethanol

The ethanol content was measured spectrophotometrically as described by Caputi *et al.* (1968). One milliliter of the fermented sample was taken in 500 mL Pyrex distillation flask containing 30 mL of distilled water. The distillate was collected in 50 mL flask containing 25 mL of potassium dichromate solution About 20 mL of distillate was

collected in each sample and the flasks were kept in a water bath maintained at 60 °C for 20 min. The flasks were cooled to room temperature and the volume raised to 50 mL. Five mL of this was diluted with 5 mL of distilled water for measuring the optical density at 600 nm using a spectrophotometer.

Estimation of sugars

Reducing sugars were estimated by the method of Miller (1959) and total sugars were measured by the method as described by Duboise *et al.* (1956).

Fourier transform infra red spectroscopy of substrates

FTIR was used to check the chemical changes in treated and untreated samples as described earlier (Irfan *et al.*, 2011b). Mixture of sample and KBr (5% sample : 95% KBr) were passed into a disk for Fourier Transform Infrared Spectroscopy measurement. The spectrum was recorded with 32 scans in the frequency range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1}.

Statistical analysis

Statistical analysis was done by ANOVA test using Microsoft Excel program. The difference in values was indicated in the form of probability ($p < 0.05$) values.

Results and Discussion

In this study three different substrates like sugarcane Bagasse, rice straw and wheat straw were used for ethanol production by *Sacchromyces cerisae* in 500 mL Erlenmyer flask at 30 °C for four days of fermentation period. For ethanol production, pretreatment of substrate is first step which disrupt the entire structure of the biomass and provides more surface area for enzymatic action. So, all these substrates *i.e.* sugarcane Bagasse, rice straw and wheat straw were first subjected to physical pretreatment which is size reduction and forming into powder form (2 mm) and then applied to chemical pretreatment (3% H_2O_2 + 2% NaOH) followed by pressurized heating at 130 °C for 60 min. In native form cellulose is present in crystalline form which affects the enzymatic hydrolysis (Kumar *et al.*, 2009). Table 1 summarizes the lignin content in untreated and treated substrates. Lignin removal in terms of delignification was found to be 77.8%, 52.2% and 56.1% in sugarcane bagasse, wheat straw and rice straw showing the effects of pretreatment respectively. After that the pretreated biomass was analyzed by Fourier transform infrared spectroscopy to check the structural changes created by this pretreatment technique. FT-IR spectroscopy is a powerful tool for studying the physico-chemical and conformational properties of polysaccharides (Sun *et al.*, 2000).

Figure 1 showed the FTIR spectroscopic analysis of wheat straw under three condition; untreated, pretreated and saccharified. The main bond between hemicellolose and lignin was C-O-C having a characterstic absorption peak at 1420.93 cm^{-1} and 1382.82 cm^{-1} which was shown in untreated wheat straw and these peaks were not as sharp or clear in treated wheat straw revealing the dislocation of hemicellulose and lignin components. The prominent peaks at 1021.21, 1252.93 and 1420.93 cm^{-1} represents the C H, O H, or CH_2 bending frequencies (Himmelsbach *et al.*, 2002) also showed the presence of hemicellulose (Mwaikambo and Ansell, 2002). Peaks in range of 3400 cm^{-1} represent the stretching vibration in OH (Xiao *et al.*, 2001; Sun *et al.*, 2000). Han *et al.* (2012) pretreated wheat straw with NaOH and reported that pretreatment produced a significant structural modification as evidenced by FTIR and SEM analysis.

Table 1 - Lignin content in treated and untreated substrates.

Substrate	Lignin in untreated (%)	Lignin in treated (%)	Delignification (%)
Sugarcane bagasse	23	5.1	77.8
Wheat straw	17.3	8.1	52.2
Rice straw	13	5.7	56.1

Figure 2 shows the spectrum of treated, untreated and saccharified sugarcane bagasse. The result indicated that there is degradation of fibriller structure of cellulose and lignin to greater extent. The absorbance at 3655, 2914, 1601, 1239, 1034, cm^{-1} in Figure 2a are associated with untreated sugarcane bagasse as reported by Sun *et al.* (2001). The band at 3334.2 cm^{-1} is depicting the stretching of hydroxyl group in treated bagasse (Figure 2b). The absorption at 2920.22 cm^{-1} in treated sugarcane bagasse arises from C-H stretching, moreover the absorbance at 1423.70, 1381.56, 1161.06 and 1022.55 cm^{-1} corresponds to the aromatic skeleton vibration and ring breathing in the C-O stretching in lignin (Sun *et al.*, 2000). The peak at 1381.56 cm^{-1} is attributed to absorption by C-H and C-O stretching in acetyl group in hemicellulose respectively. The strong band at 1161.06 cm^{-1} in pretreated sugarcane bagasse was assigned to C-O stretching in cellulose, hemicellulose and lignin or C-O-C stretching in cellulose and hemicelluloses. The band at 897.02 cm^{-1} is due to glucosidic linkage (Liu *et al.*, 2007).

Figure 3 illustrated the IR spectroscopic analysis of untreated, pretreated and saccharified rice straw. A dominant peak at 1043.48 cm^{-1} corresponds to C-O stretching in cellulose, hemicellulose and lignin, or C-O-C stretching in cellulose and hemicellulose (Williams and Nugranad, 2000) and this band was much expanded in pretreated rice straw which is showing the separation in some parts of cellulose, hemicellulose and lignin. Small peaks at 1378.90, 1422.76 and 1510.69 cm^{-1} communicate to the aromatic skeletal vibrations and ring breathing with C-O stretching in lignin. A small peak at 897.32 cm^{-1} represents the α-glucosilic linkages between the sugar units in cellulose and

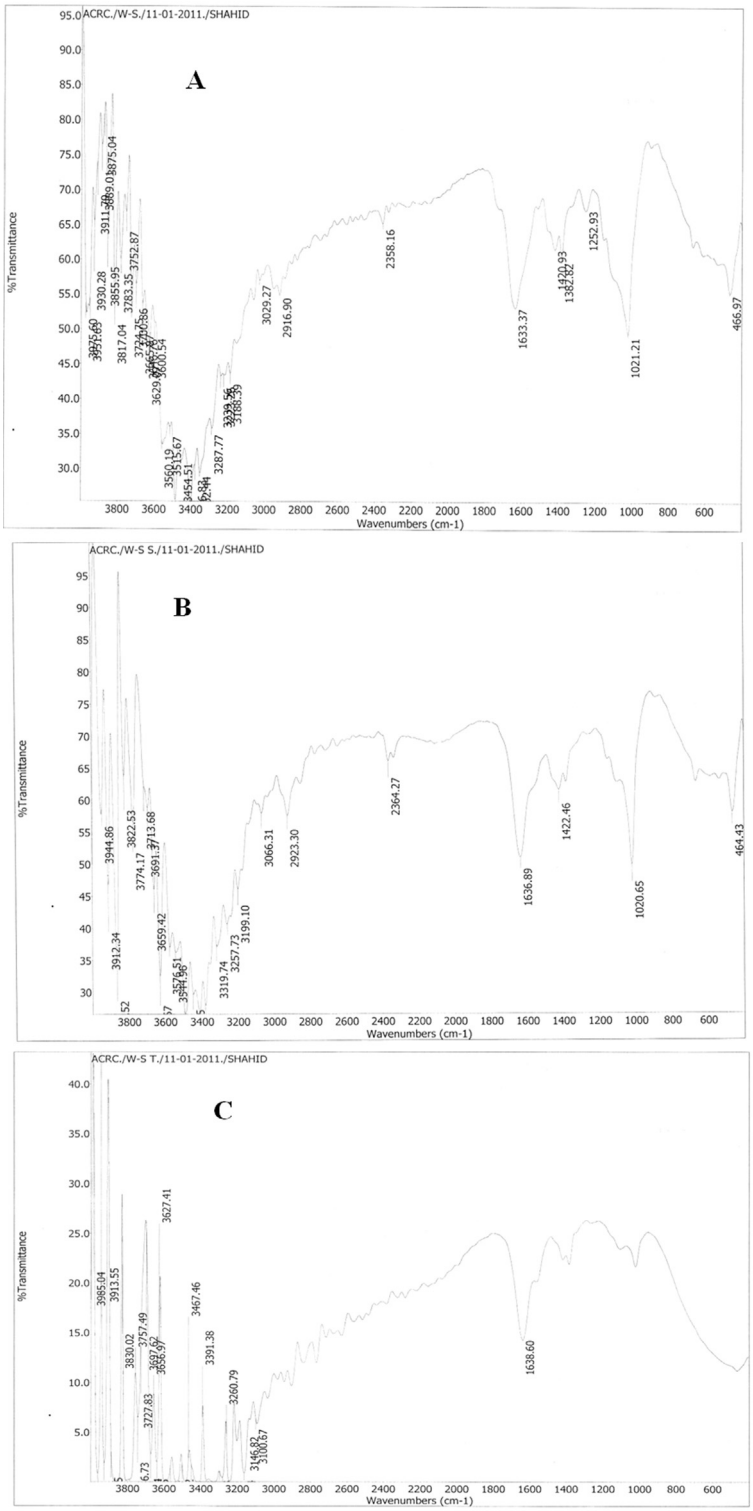

Figure 1 - FTIR spectra of (A) Untreated Wheat straw (B) Saccharified wheat straw (C) pretreated wheat straw.

hemicellulose. Sharp and large band at 1638.94 cm^{-1} and 2916.63 cm^{-1} denotes the stretching in C-O and C-H bonds. From this FTIR study of the substrates, it was observed that pretreatment had significantly alter the structures of the substrate thus loosing the cellulose, hemicelluose and lignin connections thus leading to efficient enzymatic attacks.

After pretreatment, each substrate was saccharified by using commercial enzyme at 50 °C for 8 h. Results (Figure 4) indicated that maximum saccharification was done

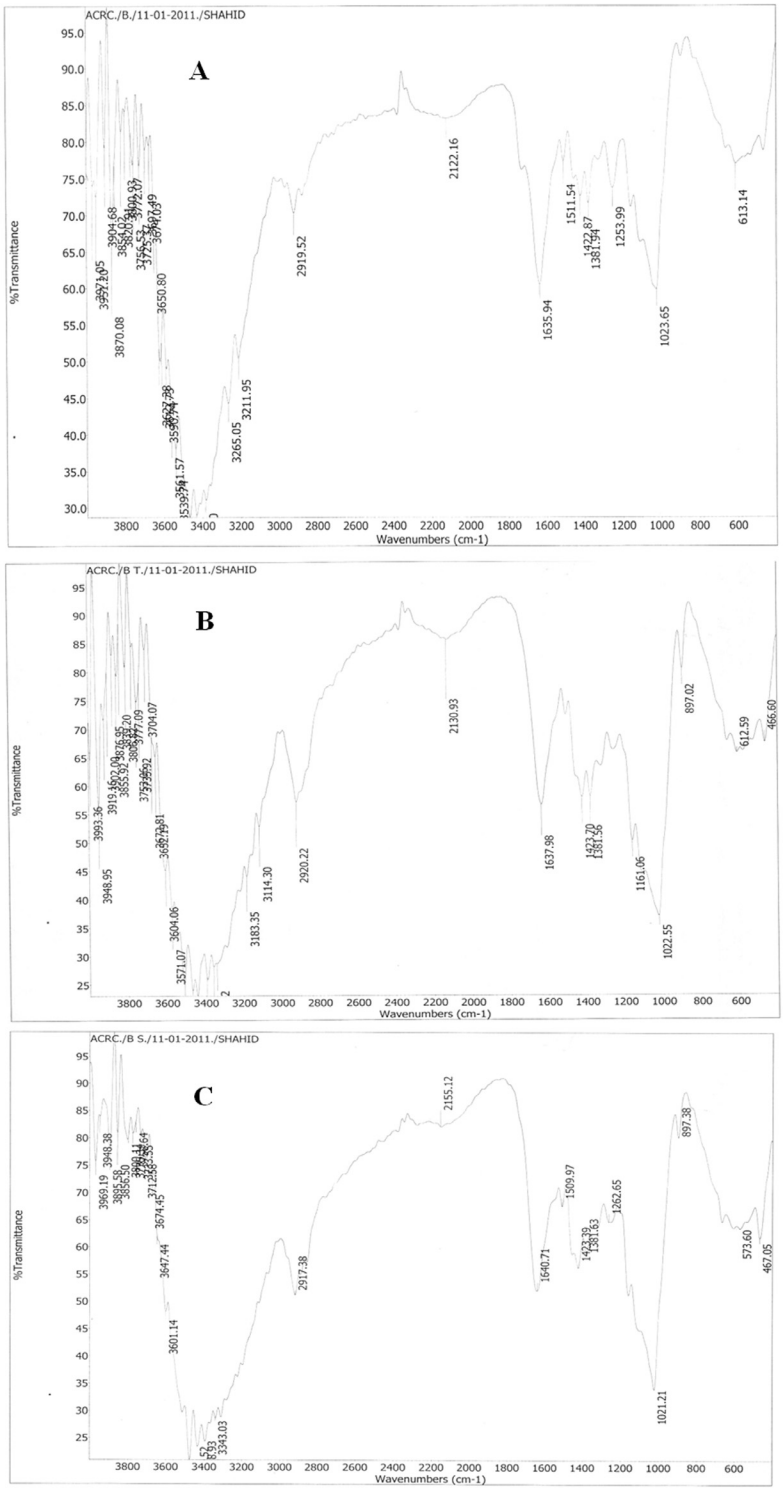

Figure 2 - FTIR spectra of (A) Untreated sugarcane Bagasse (B) pretreated sugarcane Bagasse (C) Saccharified sugarcane.

with sugarcane bagasse (64%) followed by rice straw (40%) and wheat straw (34%). Maximum saccharification of sugarcane bagasse is due to the presence of more cellulose and saccharification rate also vary with the presence of cellulosic content in biomasses. In another report maximum saccharification rate of 33.0, 25.5, and 35.5% were obtained with 2% NaOH pretreated wheat straw, rice straw and bagasse, respectively (Akhtar *et al.*, 2001).

Figure 5 illustrate the ethanol production from bagasse, rice straw and wheat straw by *Sacchromyces cervisiae* in submerged fermentation at 30 °C for four days of incubation. Results indicated that maximum ethanol pro-

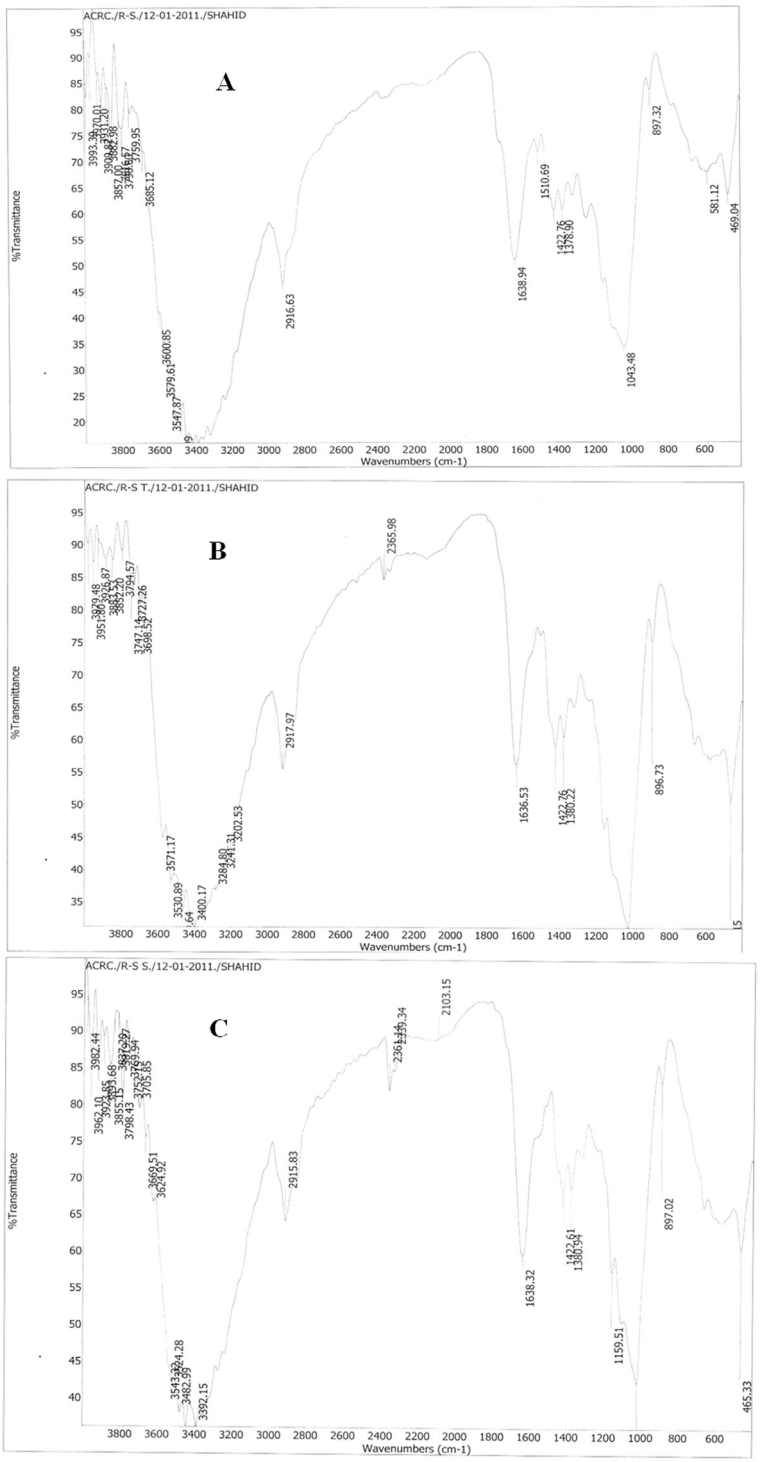

Figure 3 - FTIR spectra of (A) Untreated Rice straw (B) pretreated Rice straw (C) Saccharified Rice straw.

duction was observed by sugarcane bagasse (66 g/L) with ethanol yield of 0.41. Rice straw (49 g/L) and wheat straw (34 g/L) also produced ethanol but their production is not good as compared to sugarcane bagasse. This difference in ethanol production was due to the availability of fermentable sugars from cellulose present in biomasses. Jalil *et al.* (2010) used commercial enzyme for saccharification and reported that treated rice straw gave better ethanol production (85 g/L) as compared to untreated (70 g/L) rice straw. Uma *et al.* (2010) pretreated sugarcane bagasse with 1 N NaOH and obtained 48% ethanol production by *C. cladosporoides* after 48 h of fermentation under static condition. Sasikumar and Viruthagiri (2010) obtained maximum ethanol production (3.36 g/L) from pretreated

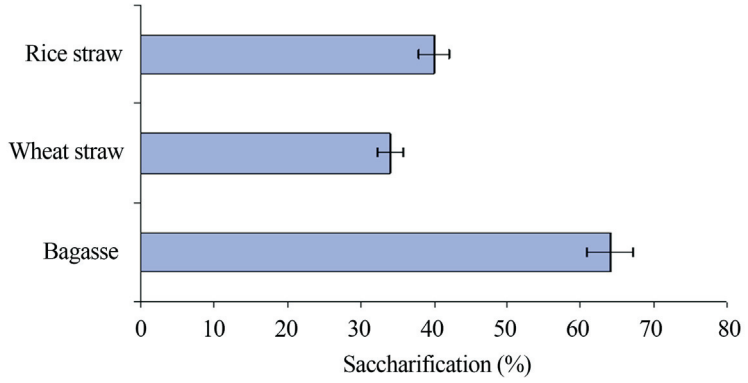

Figure 4 - Saccharification of different lignocellulosic biomasses.

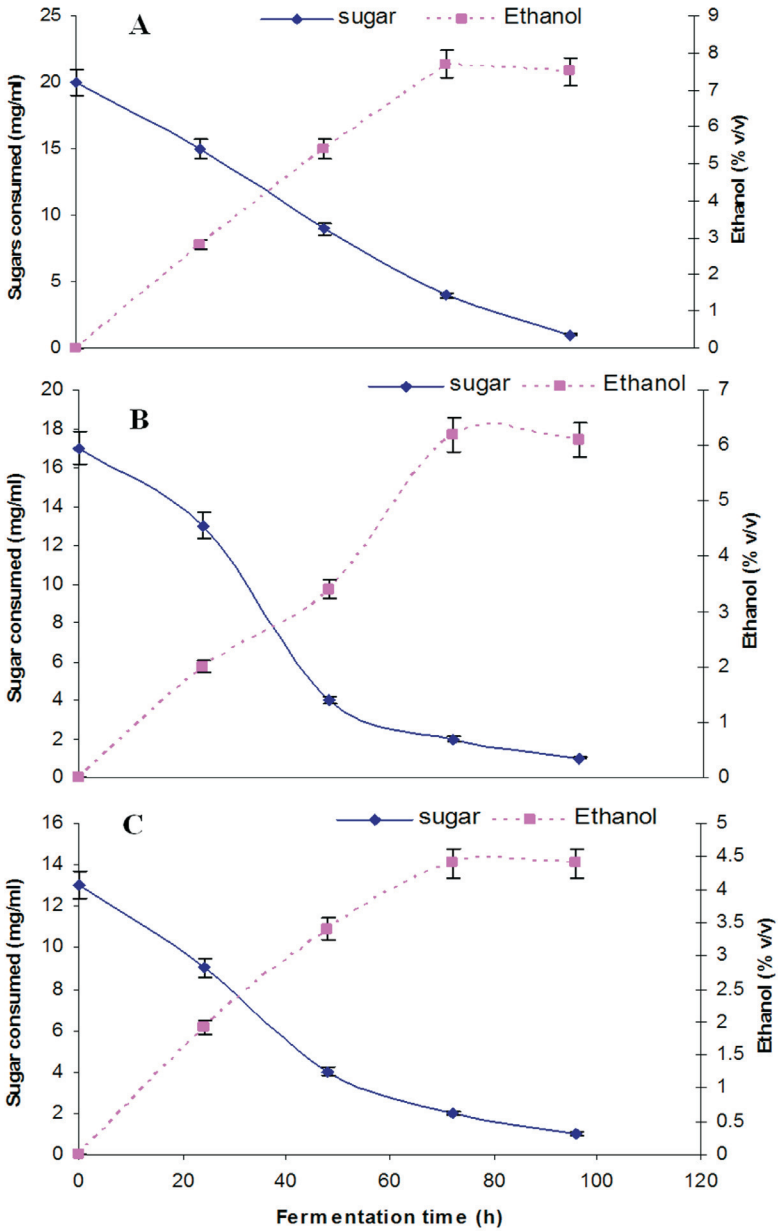

Figure 5 - Time course of ethanol production on various substrates (A) sugarcane bagasse (B) Rice straw and (C) Wheat straw.

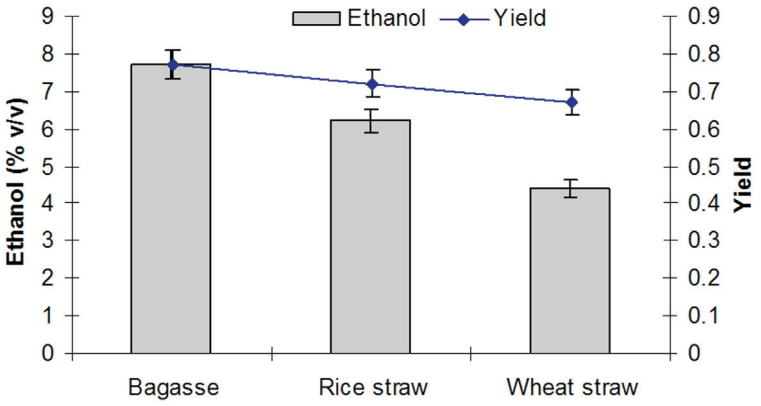

Figure 6 - Ethanol production from different lignocellulosic biomasses.

sugarcane bagasse under optimized process conditions in aerobic batch fermentation.

Conclusion

Results of this study revealed that pretreatment of the substrate are necessary for obtaining of more sugars as a result of enzymatic hydrolysis. The sugars produced by enzymatic hydrolysis were readily converted into ethanol by using *S.cervisae* in submerged fermentation at 30 °C for four days of incubation. Sugarcane bagasse is found to be very effective substrate for ethanol production and this might be helpful for scaling up and make the process cost effective.

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