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Ethanol production from *Sorghum bicolor* using both separate and simultaneous saccharification and fermentation in batch and fed batch systems

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The objective of this work was to find the best combination of different experimental conditions during pre-treatment, enzymatic saccharification, detoxification of inhibitors and fermentation of *Sorghum bicolor* straw for ethanol production. The optimization of pre-treatment using different concentrations of dilute sulfuric acid, various temperatures and residence times was achieved at 121°C, 1% acid concentration, 60 min residence time and enzyme saccharification using cellulase (celluclast 1.5 L) and β-glucosidase (Novozyme 188) at 50°C and pH 4.8 for 48 h. Different surfactants were used in order to increase the monomeric sugar during enzymatic hydrolysis and it has been observed that the addition of these surfactants contributed significantly in cellulosic conversion but no effect was shown on hemicellulosic hydrolysis. Fermentability of hydrolyzate was tested using *Saccharomyces cerevisiae* Ethanol Red[TM] and it was observed that simultaneous saccharification and fermentation (SSF) with both batch and fed batch resulted in better ethanol yield as compared to separate hydrolysis and fermentation (SHF). Detoxification of furan during SHF facilitated reduction in fermentation time from 96 to 48 h. 98.5% theoretical yield was achieved in SHF with detoxification experiment attaining an ethanol concentration and yield of 23.01 gL^{-1} and 0.115 gg^{-1} DM respectively. During the SSF batch and fed batch fermentation, the maximum yields of ethanol per gram of dry matter were 0.1257 and 0.1332 g respectively.

Key words: *Sorghum bicolor*, ethanol, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), furfural and hydroxymethyl furfural, surfactant, batch and fed batch fermentation.

INTRODUCTION

Liquid fuels are used as energy sources throughout the world and there have been progressive increases in their utilization. In the year 2004, average liquid fuel consumption was 83 million barrels per day which is projected will be 97 million barrels per day in the year 2015 and 118 million barrels per day in 2030 (EIA, 2007). Ever increasing demand for liquid fuel will deplete currently available fuel resources and there is increased interest in exploring alternative sources for the production of this energy resource. Several recent studies have shown that lignocellulosic biomass can be utilized to produce ethanol (liquid fuel). Lignocellulosic materials are composed of sugars polymerised to cellulose and hemicellulose that can be liberated by hydrolysis, and subsequently fermented to ethanol by microorganisms (Palmqvist and

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Hahn-Hagerdal, 2000b). Ethanol is one of the preferable liquid fuel due to its combustion properties and its use as an additive with gasoline (Galbe and Zacchi, 2002). Of importance is that a mix of ethanol and gasoline reduces green house gas emissions at certain levels but also minimizes dependence on fossil fuel.

Utilization of lignocellulosic materials for the production of ethanol as one of the liquid fuels has generated inte-rest to explore several available lignocellulosic materials. Pakistan is an agro-based country and produces a number of cellulosic materials that could be used to produce good quality ethanol. Among the routine cellu-losic biomass materials, wheat, rice, cotton, sugar cane and maize are major crops and share 24% in the national economy. Sorghum, barley and millet are considered as minor crops, generally used as fodder and grown in the arid zones of country. These minor crops are drought resistant and need only limited water and conditions to grow (Mehmood et al., 2008a). Among these minor crops, sorghum is the most promising particularly for ethanol production, due to its high biomass and carbohy-drate contents (Mehmood et al., 2008b). Using sorghum stalk for ethanol production may lead to incur-poration in total energy production. The bioconversion of lignocellu-losic material comprises hydrolysis of cellulose to reduce-ing sugar followed by fermentation by fungus or bacteria. Different methodologies have been applied for the separation of cellulose, hemicellulose and lignin com-ponents to enhance monomeric sugar yields. Pretreat-ment process helps to remove hemicellulose, reduce cellulose recrystallinity and increase the porosity of the material (Sun and Cheng, 2005). Among known pretreat-ment technologies, dilute acid pretreatment has been widely used because it is inexpensive and effective. This method can effectively solubilize hemicellulose into monomeric sugars (glucose, xylose, etc) and soluble oligomers, thus improving cellulose conversion. The enzymatic conversion of glucose depends on the syner-gism of three enzymes in the cellulase system which includes β-1,4-endoglucanase, β-1,4-exoglucanase and cellobiase. The higher final yield of cellobiose, glucose or xylose will cause severe feed back inhibition to cellulase and hemicellulase reactions, as the enzyme is more sus-ceptible to end product inhibition caused by cellobiose then glucose (Duff and Murray, 1996; Wen et al., 2004). Surfactant can be used to overcome feed back inhibition problems. Surfactant adsorption to lignin is believed to prevent unproductive binding of enzymes to lignin, there-by producing higher yields and better recycling of enzymes (Kristensen et al., 2007).

Sorghum bicolor is one of the major crops that could yield high quality ethanol. We evaluated various para-meters like dilute acid pretreatment, time, temperature and catalyst concentration on the production of mono-meric sugars from sorghum. Production of monomeric sugars is an intermediary step for fermenting micro-orga-

nisms to produce ethanol. To enhance monomeric sugar yields and to detoxify the inhibitor(s) produced during pretreatment, surfactant and calcium hydroxide were used respectively. Fermentation was carried out by separate and simultaneous saccharification and fermen-tation.

MATERIALS AND METHODS

Sorghum straw

Sorghum straw (Variety YSS-9) was obtained from the Millet Re-search Station Rawalpindi, Pakistan under the National Uniform Sorghum Yield Trial (NUSYT) program. This variety was grown during July - October, 2006. After harvesting, the biomass was air dried in the field for one week and prepared according to the methodology described by the laboratory analytical procedure (Hames, 2004) and stored at -20°C for further analysis.

Dilute acid pretreatment

The biomass at a solid loading of 20% (w/w) was mixed with dilute sulfuric acid (final concentration: 0.5, 1 and 2% (w/w) and pretrea-ted in an autoclave at two different temperatures (121 and 140°C) with the residence times of 10, 30 and 60 min. The pretreated wheat straw was adjusted to pH 5.0 with 10 M NaOH before enzy-matic saccharification.

Enzymatic hydrolysis

After dilute acid pre-treatment, enzymatic hydrolysis at the solid loading of 5% DM (w/w) was performed using cellulases and β-glucosidase at 50°C and 160 rpm for 48 h in a water bath shaker. 50 mM sodium citrate buffer (pH 4.8) was used in the mixture to maintain the pH at 4.8, while ampicilin (100 ug/ml) and chloro-mphenicol (100 ug/ml) was added to inhibit microbial infection. Cellulases (celluloclast 1.5 L) from *Trichoderma reesei* and Novo-zyme 188 was provided by Novozyme A/S, Bagsvaerd, Denmark with the declared activity of 700 EGU/g (60 FPU g^{-1}) for cellulases and 250 CBU g^{-1} for Novozyme 188. The enzyme loading in the reaction mixture was 3:1 ratio of celluloclast (10 FPU g^{-1} cellulose) and Novozyme 188, respectively.

Enzymatic hydrolysis with surface active additives

The tested additives (surfactants) were non ionic detergent (Tween 20 and Tween 80), polyethylene glycol (PEG 3350) and bovine serum albumin (BSA). The surfactant concentration was 0.2 % (w/w) g^{-1} DM. The conditions for the enzymatic hydrolysis with surfactants were the same as in the hydrolysis without surfactants. All experiments were performed in duplicate.

Detoxification of dilute sulfuric acid hydrolyzate with Ca(OH)₂

Detoxification of dilute sulfuric acid hydrolyzate was carried out in a

Table 1. Composition of *S. bicolor* straw (YSS-9).

Component	Dry solid (%, w/w)
Cellulose	35.01± 0.71
Hemicellulose	24.40 ± 1.06
Lignin	19 ± 2.07
Ash	7.02 ± 1.93
Moisture	4.98 ± 0.11

250 ml flask in an incubated water bath shaker according to the methodology described by Purwadi et al. (2004). In brief, highly concentrated slurry of calcium hydroxide was added to 200 ml hydrolyzate until the desired pH (10) was achieved. The flasks were then placed in a shaking water bath at different temperatures (30, 40, 50 and 60°C). Samples were taken after 30, 60 and 90 min, neutralized until pH 7, centrifuged to remove the solid material and kept below 4°C for further analyses.

Yeast strain and preparation of inoculum

The yeast strain *Saccharomyces cerevisiae* Ethanol Red[TM] was used in this study. The strain was maintained in glycerol vials at - 20°C for use as working stock. This stock solution was incubated in defined yeast medium gL^{-1} ((NH_4)$_2$$SO_4$, 5.0; $MgSO_4.7H_2O$, 0.5 and KH_2PO_4, 3.0) with addition of ergosterol/Tween 80 solution, vitamins gL^{-1} (Ca-Pantothenate, 1.0; Nicotinic acid, 1.0; myo-Inositol, 25; Thiamin-HCl, 1.0; Pyridoxin-HCl, 1.0 and; p-aminobenzoic acid, 0.2), trace metals gL^{-1} ($ZnSO_4 \cdot 7H_2O$, 4.5; $MnCl_2. 2H_2O$, 0.84; $CoCl_2.6H_2O$, 0.3; $CuSO_4.5H_2O$, 0.3; $Na_2MoO_4.2H_2O$, 0.4; $CaCl_2.2H_2O$, 4.5; $FeSO_4.7H_2O$, 3.0; H_3BO_3, 1.0 and; KI, 0.1) and glucose 200 gL^{-1}. Cultures were incubated for 24 h at 32°C and 120 rpm and used as seed cultures for fermentation experiment.

Fermentation experiments

Separate hydrolysis and fermentation (SHF)

For SHF experiments, fermentation was performed at pH 5.0 using the liquid portion of the hydrolyzate after separation from solid, supplemented with 5 gL^{-1} yeast extract. The hydrolyzate was obtained from dilute acid pre-treatment (20% w/w) and enzymatic hydrolysis of biomass. Ethanol fermentation was carried out at 32°C under anaerobic conditions, with the final seed culture of 1.0 OD (0.5 gL^{-1} of inoculum). A 5 M NaOH solution was used for pH control. Samples were withdrawn periodically to determine ethanol content and residual sugars and stored at -20°C prior to analysis. Detoxification of enzymatic hydrolyzate was done by adding Ca(OH)$_2$ until the pH 10 was obtained. The whole mixture was stirred for 90 min at 60°C. After detoxification, the slurry was allowed to cool slowly to room temperature and then adjusted to pH 5.0 with HCl. It was then centrifuged (14000 rpm, 10 min) to remove any precipitate formed before using as substrate for fermentation.

Simultaneous saccharification and fermentation (SSF):

For SSF experiments dilute acid pretreated sorghum straw hydroly-

zate was used after adjusting the pH 5.0 with 10 M NaOH before adding enzyme and inoculum. Enzymes were added to this hydrolyzate and allowed for pre-saccharification at 60°C for 2 h. After 2 h, the inoculum was added with the supplementation of 5 gL^{-1} yeast extract. The fed batch SSF was started under the optimal batch SSF conditions except for enzyme loading and the concentration of the pretreated sorghum straw. The feeding of pre-treated sorghum straw was four times after every 24 h. All experiments were performed in duplicate and the average standard deviation was calculated.

Analytical procedure

The composition of the straw was analyzed by strong acid hydrolysis method. The dried sample was treated with 1.5 ml of 72% H_2SO_4 in four different Pyrex tubes and placed in a water bath with a temperature of 30°C. After 1 h, samples were diluted with 42 ml Milli-Q water for the first two tubes and 43 ml with other tubes. 1 ml spiked solution contains 33 gL^{-1} of glucose and 30 gL^{-1} of xylose. The samples were then autoclaved for 1 h at 121°C. After cooling, samples were taken and analyzed by HPLC.

Sugars (glucose and xylose), end-fermentation products (ethanol, lactate and acetate), furfural and Hydroxymethyl furfural (HMF) were determined by HPLC (Agilent Technologies, 1200 system) equipped with an Aminex HPX-87H organic acid analysis column (Bio-Rad) at 60°C. The eluent was 4 mM H_2SO_4 at a flow rate of 0.6 ml/min with detection on a refractive index detector. Prior to HPLC analysis, 1 ml samples were acidified with 10 µl of 20% H_2SO_4 and centrifuged at 14 000 rev/min for 10 min, followed by filtration through a 0.45 µm membrane filter.

RESULTS

Dilute acid pretreatment and enzymatic saccharification

We carried out dilute acid pre-treatment (various concentrations) and saccharification enzymes effects on the production of monomeric sugars. As these sugars are hydrolytic products of complex cellulosic materials, it is essential to know the initial levels of these constituent in the starting material. Our analyses revealed that sorghum straw is composed/constituted of cellulose (35.01 ± 0.71%) and hemicellulose (24.40 ± 1.06%) (Table 1) and total carbohydrate contents 59.41% on a dry solid basis. Initial experiments were performed with the solid loading of 10% (w/w) and after initial procedural optimization, higher solid loading (15, 20 and 25%, w/w) was also evaluated. Our data revealed that solid loading rate between 10 to 20% (w/w) had similar yield of the product but with 25% solid loading, the yield dropped (data not shown). Onward, all experiments were performed with a solid loading of 20% (w/w). The effect of dilute sulphuric acid doses (0.5, 1 and 2%, v/v) at 2 different temperatures (121 and 140°C) with retention times of 10, 30 and 60 min and enzymatic saccharification using cellulase (Celluclast 1.5 L) and β-glucosidase (Novozyme

188) at 50°C and pH 4.8 for 48 h on sorghum straw are shown in Figure 1. Pretreatment condition (121°C, 1% acid concentration and 60 min) gives a maximum carbohydrate yield (0.51 gg^{-1} DM). 71.98% (0.28 gg^{-1} DM) of cellulose and 84.84% (0.23 gg^{-1} DM) of hemicellulose was converted into their respective monomer sugars. It was also noted that with increased temperature, acid concentration and retention time the yield of glucose was increased but at the same time the xylose yield was increased up to a certain level and then suddenly decreased with increased temperature, acid concentration and residence time. The highest yield of xylose was achieved with a temperature of 121°C having acid concentration of 2% and residence time of 10 min but these conditions were not used due to several reasons: first glucose yield was not up to its maximum and secondly higher levels of acid concentration will take part in total economy of the process and erosion of the equipment. Under the same conditions, the concentrations of lactate, acetate, furfural and hydroxymethyl furfural were also evaluated and during optimized conditions; the concentrations of these compounds were 0.0061, 0.0217, 0.00119 and 0.0231 gg^{-1} DM, respectively.

Effect of surface active additives in enzymatic hydrolysis

The effect of surfactant on pretreated sorghum straw during enzymatic hydrolysis (solid loading 5%, w/w) was also assessed to enhance the production of monomer sugars and results are summarized in Figure 2. Pretreatment conditions and enzyme dosage was the same as without surfactant. Cellulose and hemicellulose conversion were compared by quantifying the amount of released glucose and xylose with and without surface active additives. With surfactant added, glucose concentration increased in all experiments and significant increase was achieved with PEG 3350, 0.32 gg^{-1} DM (82.26%) whereas Tween 20 resulted in the lowest conversion of cellulose (0.291 gg^{-1} DM). 0.30 and 0.293 gg^{-1} DM of glucose was achieved with Tween 80 and BSA, respectively, during the hydrolysis of cellulose. Interestingly, no effect of surfactant was seen in hemicellulosic hydrolysis to xylose (data not shown).

Detoxification of hydrolyzate

The effect of detoxification of hydrolyzate by overliming on the fermentable sugars, furfural and hydroxymethyl furfural (HMF) was examined and results are shown in Figure 3. The results revealed that at all temperatures, degradation of HMF and furfural occurred. Increasing the temperature and time resulted in more effective degradation of these toxic substances. At 30°C, degradation of furans occurred rapidly in the first 30 min but it became stable for the remaining time but at temperatures 40, 50 and 60°C, almost a linear behaviour was observed. Intriguingly, no significant variation in the concentration of glucose and xylose was found during the cultivation of hydrolyzate at all examined temperatures.

## Fermentation of dilute acid pretreated hydrolyzate

The results of fermentation of dilute sulphuric acid pretreated and enzymatically saccharified sorghum straw by *S. cerevisiae* Ethanol Red[TM] are summarized in Table 2. It is observed that simultaneous saccharification and fermentation (SSF) with both batch and fed batch resulted in better ethanol yield as compared to separated hydrolysis and fermentation (SHF). In SHF, detoxification of furan facilitated trimming down fermentation time from 96 to 48 h. 98.5% theoretical yield (based on the theoretical yield of 0.51 g ethanol/g glucose) was achieved in SHF with the detoxification experiment attaining an ethanol concentration and yield of 23.01 and 0.115 gg^{-1} DM, respectively. During the SSF batch and fed batch fermentation, the maximum yields of ethanol per gram of dry matter were 0.1257 and 0.1332, respectively. Fermentation pattern of sorghum hydrolyzate during SHF and SSF (batch and fed batch) with *S. cerevisiae* Ethanol Red[TM] is shown in Figure 4. In all experiments ethanol production rate was different. During the SHF without detoxification, *S. cerevisiae* took a much longer time to habituate in the environment and to tolerate the inhibitors. Fermentation was carried out gradually and maximum yield was obtained at 96 h but in SHF with detoxification of inhibitors and SSF batch experiments, ethanol production rates were much higher at the initial stages. The final ethanol yields did not differ significantly between batch and fed batch.

DISCUSSION

Production of ethanol through fermentation process from lignocellulosic biomass is dependent on its quality. Several studies in the past have described that chemical composition vary in different lignocellulosic biomasses and is also associated with environmental and genetic factors. Primarily, the major constituents of lignocellulosic material are carbohydrates (cellulose and hemicellulose) and lignin polymers. Carbohydrate contents of lignocellulosic materials are directly proportional to the commercial yield of ethanol. For producing high quality ethanol, pretreatment of biomass is essential and this leads to better enzymatic hydrolysis fractionate, solubilize, hydrolyze
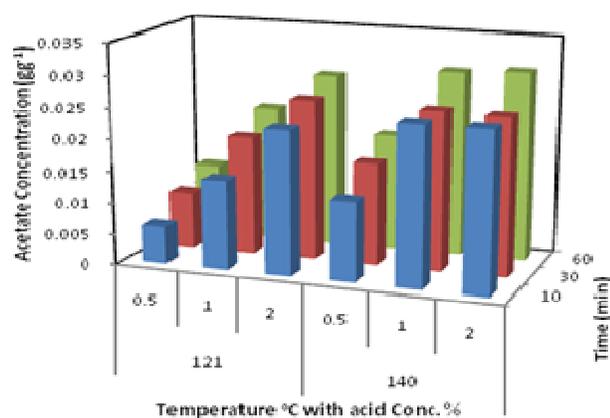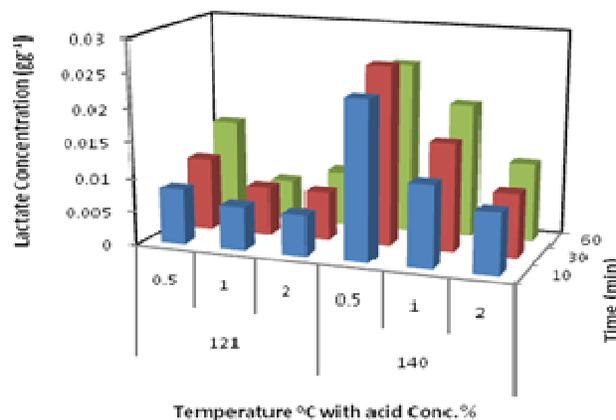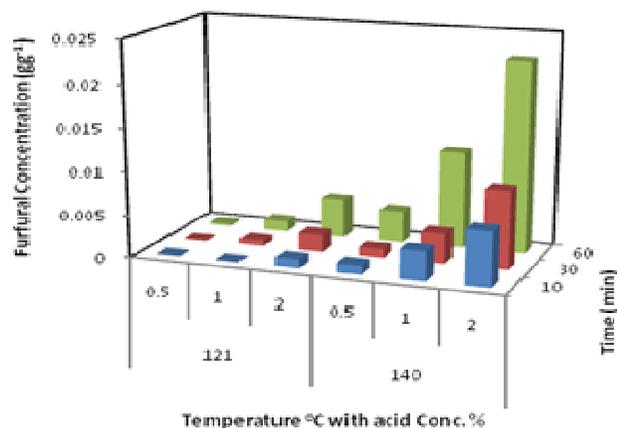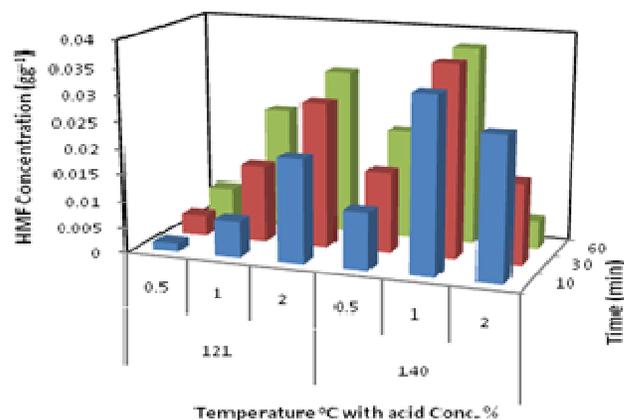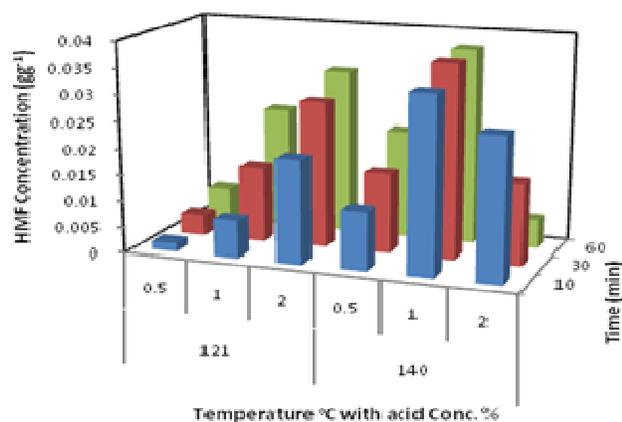
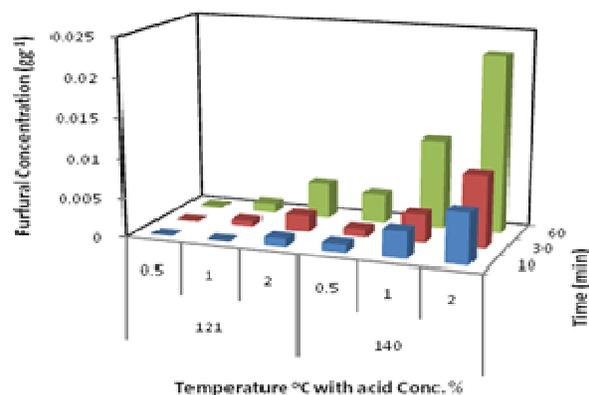**Figure 1.** Effect of pretreatment on the production of monomeric sugars, by products and inhibitors by dilute sulfuric acid and enzymatic saccharification of *S. bicolor* variety YSS 9. Pretreatment conditions: Temperature (121 and 140°C), acid concentration (0.5, 1 and 2%) and residence time (10 ■, 30 ■ and 60 min ■) and enzymatic saccharification conditions 50°C, pH 4.8, 48). Monomeric sugars glucose (a) and xylose (b); by-products acetate (c) and lactate (d) and inhibitors HMF (e) and furfural (f).
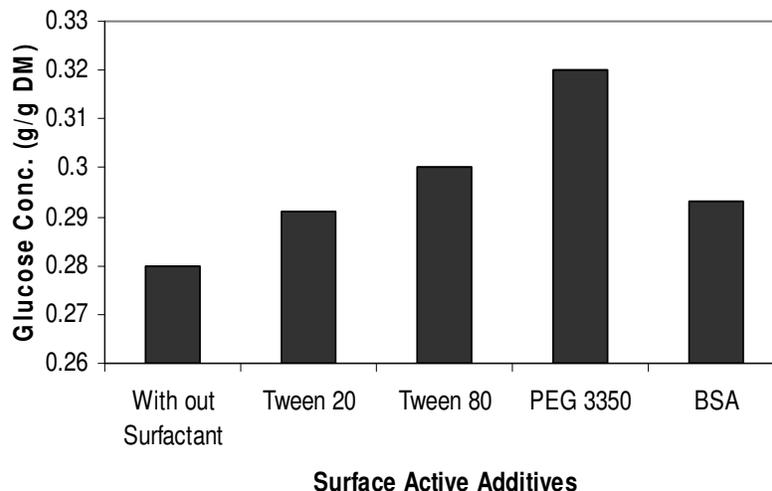
Figure 2. Effect of surfactant (0.2%, w/w of DM) on the enzymatic hydrolysis of sorghum straw pretreated with dilute sulfuric acid (121°C, 1% acid, 1 h) during 48 h of enzymatic hydrolysis. Substrate concentration was 20% (w/w).

lyze and separate cellulose, hemicellulose and lignin components (Saha, 2003). Several treatment technologies include concentrated acid (Badger, 2002), dilute acid, alkaline, steam explosion, wet oxidation and liquid hot water. Among these methods, acid hydrolysis is frequently used as a pretreatment because it can be tailored to a wide variety of feedstocks. This method not only exposes cellulose for enzymatic saccharification but also solubilizes hemicellulose and converts it into a fermentable sugar, xylose (Saha and Bothast, 1999). However, rapid and efficient fermentation of fermentable sugars is limited because of toxic compounds such as furfural and HMF which are generated during high temperature pretreatment (Palmqvist and Hahn-Hagerdal, 2000a) and ultimately inhibits microbial growth. The rationale behind this study is the dilute sulfuric acid pretreatment of sorghum straw to obtain a higher yield of monomeric fermentable sugars with an aim to minimize inhibitor generation.

Optimum conditions for the dilute acid pretreatment of sorghum straw are 1% (w/w) sulfuric acid, at 121°C for 1h. It has been noted that glucose concentration was very high at 2% (w/w) H_2SO_4, at 140°C for 30 min; but at these conditions, the concentration of xylose was very low. The selected conditions gave the maximum overall yield of monomeric sugars (glucose and xylose). Another reason for the selection of these conditions was that it generated fewer amounts of lactate, acetate, furfural and HMF. The data in Figure 1c, d, e and f revealed that the yield of these compounds is dependent upon H_2SO_4 concentration, time and temperature of pretreatment. Furthermore, several factors also obstruct cellulose conversion to higher yields of monomeric sugars during enzymatic hydrolysis (Kristensen et al., 2007) and ethanol during fermentation (Palmiqvist and Hahn-Hagerdal,

2000b). One important limiting factor is the lignin fraction of lignocellulose that has been proved to be responsible for unspecific adsorption of celluloses (Eriksson et al., 2002) and ultimately lowers the monomeric yield. Addition of surfactants such as non ionic detergents (Park et al., 1992) and protein (Ooshima et al., 1986) significantly increase enzymatic conversion of cellulose into soluble sugars. In the present study, surfactants were found to increase cellulose hydrolysis significantly. Among all surfactants, PEG3350 has the tendency to perform best in sorghum straw enzymatic hydrolysis. No effect was seen in hemicellulose conver-sion. It may be because hemicellulose was converted during pretreatment and enzymatic hydrolysis into xylose and other degradation products. Same results were also reported by Kristensen et al. (2007) during enzymatic hydrolysis of wheat straw lignocellulose using surface active additives.

Another factor that hampers ethanol production during fermentation is sugar degradation products such as weak acids, furans and phenolic compounds released during pretreatment. Furfural and HMF are two furan derivatives which are formed by the further hydrolysis of sugars, pentoses and hexoses, respectively (Purwadi et al., 2004). Biological, physical and chemical methods have been employed for detoxification (that is, the specific removal of inhibitors prior to fermentation) of lignocellulosic hydrolyzates (Plamqvist and Hahn-Hagerdal, 2000a). The most economical method of detoxification involves treatment of hydrolyzates with solid calcium hydroxide (Ranatunga et al., 2000). This method is pH dependent and affected by the concentration of $Ca(OH)_2$. The drawback of this method is the loss of sugar during detoxification. Using mild conditions (not too high pH and
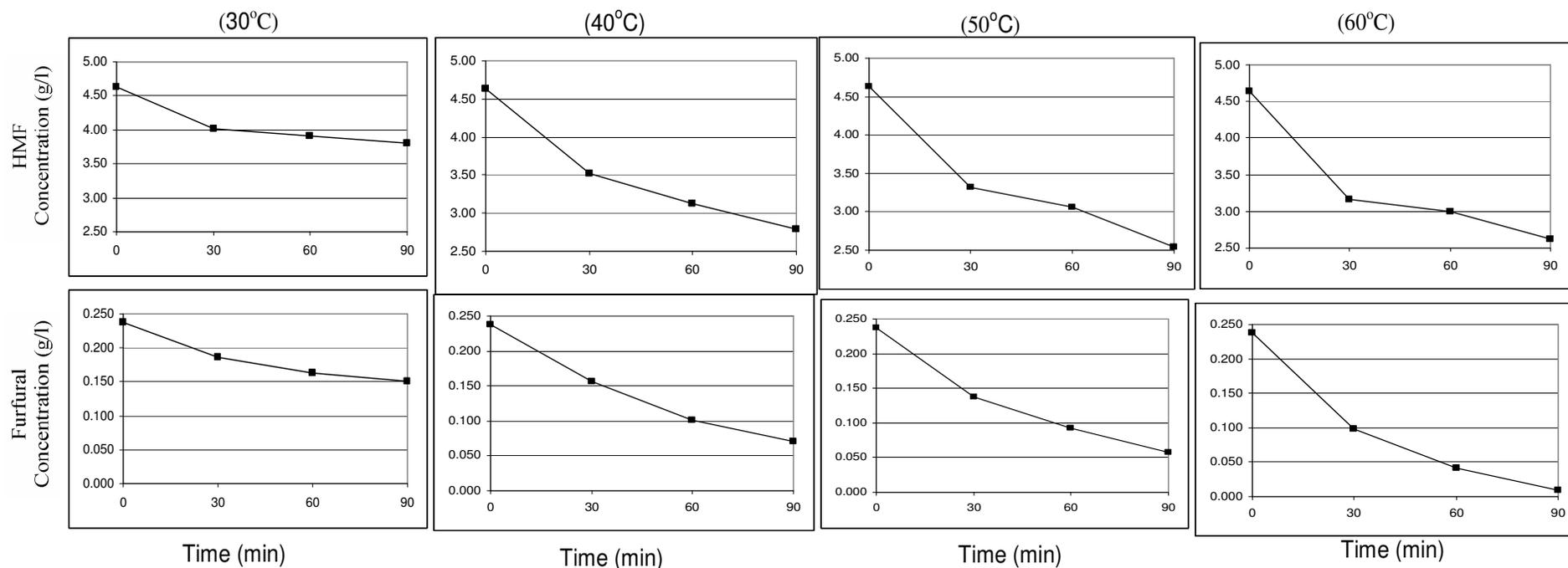
Figure 3. Effect of temperature and time on the concentration profile of HMF and furfural during detoxification with Ca(OH)$_2$. Data is the mean of two experiments.

Table 2. Ethanol production from *S. bicolor* straw (YSS-9) hydrolyzate by *S. cerevisae* Ethanol Red[TM].

Hydrolyzate	Fermentation time*	Glucose (gL^{-1})	Theoretical yield (gL^{-1})	Ethanol concentration (gL^{-1})	Ethanol yield (gg^{-1} DM)
SHF					
With out detoxification	96	46.077±0.66	23.5	22.97±1.36 (97.78)[a]	0.1148
With detoxification	48	45.8±0.24	23.36	23.01±0.20 (98.50)[a]	0.115
SSF					
Batch	96	-	-	25.1547±1.68	0.1257
Fed batch	120	-	-	26.25±1.95	0.1332

*Time (maximum ethanol production achieved); [a]percentage of theoretical yield (based on the theoretical yield of 0.51 g ethanol /g glucose)
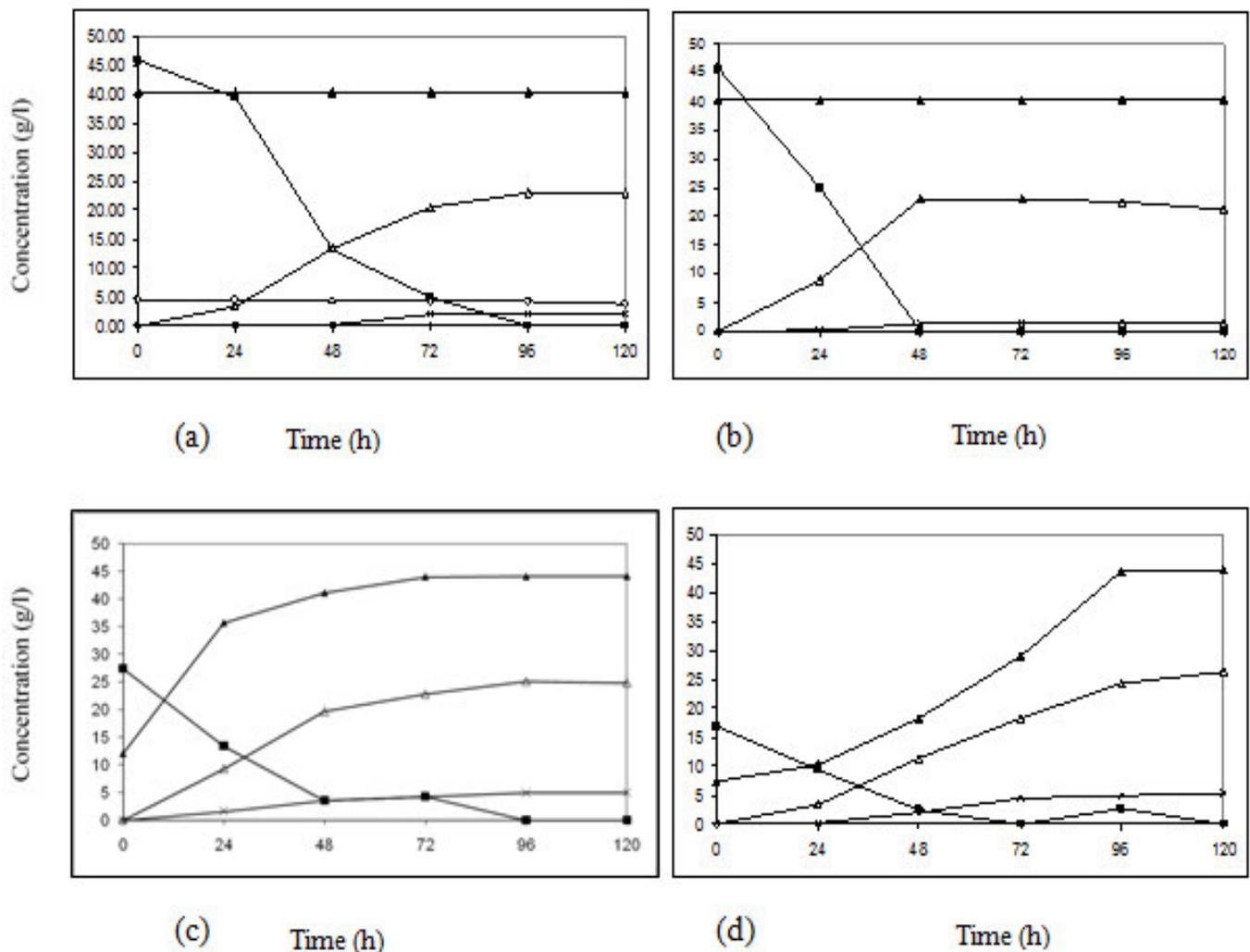
Figure 4. Glucose fermentation by *S. cerevisae* Ethanol Red™ from *S. bicolor straw* (20% w/w pre-treated with 1% sulfuric acid at 121°C for 1 h). (a) SHF without detoxification, (b) SHF after detoxification, (c) SSF Batch and (d) SSF with fed batch. Symbols: (■) Glucose, (▲) xylose, (x) glycerol, (Δ) ethanol, (◊) HMF and (+) furfural.

temperature) may lead to detoxification of the inhibitors without any loss of sugars. Interestingly, there was no sugar loss at pH 10 with all temperatures used in this study. Fermentation was performed in batch (SHF with and without detoxification and SSF) and fed batch modes. It was clear from the result that SSF performed better both in batch and fed batch, when compared with SHF. This lower ethanol yields in SHF experiments could be due to feed back inhibition of enzymes by the end product(s). In SSF experiment, fed batch fermentation proved to be slightly superior to batch fermentation. Another advantage of SSF is that when combining the two process (saccharification and fermentation) steps, it results in a lower capital cost and risk of contamination (Wyman et al., 1992). However mixing the lignin residue with yeast makes yeast recirculation very difficult (Ohgren et al., 2007).

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