International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.4, No.4, pp 1497-1502, Oct-Dec 2012

Production Of Fermentable Sugars By Dilute Acid Pretreatment And Enzymatic Saccharification Of Three Different Lignocellulosic Materials

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Abstract: Lignocellulosic materials contain cellulose and hemicellulose which, upon suitable processing, can be converted into fermentable sugars such as glucose and xylose. The processing involves a physico-chemical pretreatment followed by enzymatic saccharification. In the present work, comparative fermentable sugar production was carried out from three different lignocellulosic materials- wheat straw (WS), pearl millet straw (PS) and sugarcane bagasse (SB). Dilute acid pretreatment was carried out using 2% (v/v) H_2SO_4 and subsequent enzymatic saccharification of biomass using enzymes- cellulase *Trichoderma reesei* and β-glucosidase. Maximum total reducing sugar (TRS) of pretreatment was obtained with sugarcane bagasse followed by almost similar TRS yield for wheat straw and pearl millet straw. Optimization of reaction conditions were carried out using pure cellulose powder. Temperature 50°C, pH 5.0 and agitation speed of 170 rpm were found to be the most suitable reaction conditions for saccharification. Maximum TRS yield of pretreatment and saccharification was obtained with sugarcane bagasse followed by pearl millet straw and wheat straw which was 53 to 57% of the total potential sugars in biomass.

Key words: Lignocellulosic feedstock; Fermentable sugars; Pretreatment; Saccharification.

Introduction and Experimental

Lignocellulosic materials serve as a cheap and abundant feedstock for production of bio energy[1]. Their conversion to fermentable sugars is an intensive process that involves a combination of pretreatment (chemical and/or mechanical) and hydrolysis (chemical or enzymatic). Pretreatment is required to increase the accessibility of lignocellulosic materials for hydrolysis. The most commonly used pretreatment method is dilute-acid pretreatment (0.5–2.0% H_2SO_4) with high-pressure steam explosion[2]. During this pretreatment, hemicellulose is readily hydrolyzed into monomeric sugars and a complex mixture of compounds that tend to inhibit the fermentation of sugars, while cellulose is essentially inert. Lignin does not contribute to ethanol production, but can be separated after pretreatment and may be used as a solid fuel for the generation of heat and/or electricity[3,4].

Enzymatic saccharification is the next step after pretreatment and it can be done by chemical or enzymatic method. Cellulases (EC 3.2.1.4), the most commonly used enzymes for hydrolysis of cellulose to glucose, mainly consist of three enzymes: endo-glucanases, exo-glucanases, and β-glucosidases. The enzymatic saccharification is affected by reaction conditions such as pH, temperature, agitation speed, substrate concentration, enzyme activity and its concentration. The goal of saccharification is to obtain maximum yield of glucose from complex

polysaccharide cellulose. Cellulose content of the feedstock is an important factor in saccharification. Cellulose in biomass is present in crystalline and amorphous forms. The ratio of both the forms is important factor as amorphous cellulose is more assessable to hydrolysis than crystalline cellulose[5]. In the next step, ethanol is produced by fermentation of sugars obtained by pretreatment and saccharification. The produced ethanol then can be recovered by various product recovery operations.

Agricultural residues, which are primarily composed of stalks, leaves, and straws contain higher amount of cellulose and hemicellulose. This makes them good candidates for bioethanol production through proper pretreatment, saccharification and fermentation. The choice of feedstock material depends on availability, cost and composition of feedstock; i.e. cellulose content and ratio of hexosans and pentosans. The overall chemical composition of biomass could slightly differ depending on species, soil, and climate conditions[5,6,7]. Wheat and pearl millet are major crops of India and this leads to generation of significant amount of straw after crop harvesting. The same is true with sugarcane bagasse. After juice extraction, the resultant bagasse is of low value. The straw and bagasse are mainly used as animal feed and also serve as the component of soil humus. However, utilization of this straw for fermentable sugar production is a promising alternative.

In the present work, lignocellulosic residues- wheat straw, pearl millet straw and sugarcane bagasse were subjected to dilute acid pretreatment and subsequent enzymatic saccharification for production of fermentable sugars and comparison was made in sugar yields. In addition, optimization of saccharification parameters was also carried out to obtain most suitable saccharification conditions and to obtain high yield of fermentable sugars.

Materials

Lignocellulosic biomass- wheat straw and pearl millet straws were collected from a farm near Jaipur, India, while sugarcane bagasse was collected from local market in Jaipur, India. Biomass was washed with water to remove dust and particles and dried at 40°C for two days. After washing and drying, lignocellulosic biomass was stored at room temperature till further processing. Enzymes- cellulase from *Trichoderma reesei*, (Sigma product code C8546) and *β*-glucosidase from *Aspergillus niger* (Sigma product code 49291) were purchased from Sigma-Aldrich Co. (India).

Characterization of biomass

Moisture, ash and lignin content of biomass were determined using NREL (National Renewable Energy Laboratory, CO, USA) laboratory analytical protocols[8, 9, 10]. Cellulose content was determined using method from literature [11].

Pretreatment of biomass

Pretreatment was carried out by soaking the biomass in dilute H_2SO_4 (2% v/v). This slurry was then subjected to high pressure steam at 121°C in a vertical autoclave for 120 minutes. After pretreatment, steam of autoclave was quickly released and solids and liquid were separated by filtration. Liquid hydrolysate obtained after pretreatment was detoxified as per the method from literature[12]. The filtered samples were analyzed for reducing sugars[13]. The solid fraction was dried and stored until further use.

Delignification of pretreated solids

Delignification of pretreated solid materials was carried out as per the method from literature[12]. The delignified material was filtered with a muslin cloth, washed with water till the neutral pH was obtained. The residues were dried overnight and then the dried mass was grinded and stored until further use.

Effect of reaction parameters on saccharification of pure cellulose

Effects of reaction parameters (temperature, pH and agitation speed) on saccharification of pure cellulose were determined using cellulase from *Trichoderma reesei* (CTR). For this, 1 g of pure cellulose powder was mixed in 20 ml citrate buffer and reaction was carried out for 2 h.

Enzymatic saccharification

Saccharification was carried out in 250 ml Erlenmeyer flasks at 50°C using sodium citrate buffer of pH 4.8. Cellulase from *Trichoderma reesei* (CTR) supplemented with β-glucosidase from *Aspergillus niger* was used for saccharification. Experiments were carried out in a shaking incubator at 150 rpm. Cellulase loading was 15 FPU/g biomass and reaction time of 96 h was used. A constant cellulase to β- glucosidase ratio of 1.5:1 was used. Sodium azide (2% w/v solution, 200 µl) was added to avoid microbial growth. For each sample, a duplicate run was carried out and average readings were reported. Saccharification percentage was calculated as follows;

$$\text{Saccharification percentage (\%)} = \frac{\text{glucose yield of saccharification in } \left(\frac{g}{kg}\right) X\, 0.9}{\text{cellulose content of biomass used for saccharifiaction } \left(\frac{g}{kg}\right)} \times 100$$

..... (1)

Analysis of total reducing sugars

Reducing sugars analysis (for both pretreatment and saccharification) was done using Dinitrosalicylic Acid (DNS) Method[13].

Results and discussion

Characterization of lignocellulosic biomass

The characterization results for cellulose, lignin, moisture, ash are presented in table 1. Cellulose content in biomass was found to be within a narrow range of 24.5-25.2% (w/w). Maximum lignin content was obtained with wheat straw while minimum was found with pearl millet straw. Ash content in biomass was found in the range of 3.5-4.2% (w/w). Total reducing sugar (TRS) content in biomass was found in the range of 578 g/kg to 640 g/kg biomass. TRS in sugarcane bagasse was slightly on the higher side compared to other biomass probably due to the presence of residual sugar in bagasse. Chen et al. have obtained 36% glucan, 26% total lignin and 3.6% ash in wheat straw which is on higher side compared to results obtained in present study[5]. While with pearl millet hays, 29% glucan, 22% total lignin and 11% ash was reported by Chen et al., which is similar to present results except lignin content. The difference in the results may be attributed to growing location, season and stage of harvest, harvesting methods, and analytical procedures[5].

Pretreatment and detoxification of lignocellulosic biomass

Pretreatment conditions of low temperature and longer reaction time generally tend to produce higher xylose yield due to less thermal degradation of sugar into inhibitors[14]. Total reducing sugar (TRS) yield after pretreatment is presented in table 2. TRS yield was found maximum with sugarcane bagasse (249 g/kg), while with wheat straw (227 g/kg) and pearl millet straw (231 g/kg), almost similar yield was obtained. The cellulose contents of biomass was also determined and was found to increased after pretreatment to almost twofold and ranged from 49-52.4% (w/w). Higher TRS yield (0.25 g/g) with sugarcane bagasse may be due to the presence of residual sugar (from sugarcane juice) in addition to structural sugars, which is difficult to remove completely even after several cycles of washing.

Lignocellulosic hydrolysate also contains biomass degradation products (such as 5- (Hydroxymethyl) furfural (HMF), acetic acid, furfural, and phenolics) in addition to sugars which tend to inhibit fermentation. To remove these inhibitors, the hydrolysates were neutralized and detoxified by overliming with $Ca(OH)_2$. This method of detoxification is reported to remove most of the fermentation inhibitors[12,15]. However, no quantitative measurement of inhibitors concentration could be taken in present work.

Table 1 Characterization of biomass

Component	Content % (w/w)		
	Wheat straw	Pearl millet straw	Sugarcane bagasse
Cellulose	25.2	24.5	25.0
Lignin	18.5	13.7	16.2
Moisture	5.6	8.7	4.8
Ash	4.2	3.8	3.5
*Others	*46.5*	*49.3*	*50.5*

*Others consists of hemicelluloses, extractives and other minor components

Table 2 Total reducing sugar yield in liquid hydrolysate after pretreatment

Feedstock	TRS after pretreatment (g/kg of dry biomass)	Cellulose content after pretreatment (% wt)
Wheat straw	227	49.0
Sugarcane bagasse	249	50.0
Pearl millet straw	231	52.4

TRS- Total reducing sugar yield; Weight of biomass: 4g,
Total liquid volume: 100 ml, Reaction temperature: 120°C,
Acid concentration: 2% (v/v) H_2SO_4; Reaction time: 2h

Effect of reaction condition on saccharification of pure cellulose with cellulase from *Trichoderma reesei* (CTR)

Effect of temperature

To study the effect of temperature, experiments were carried out in the temperature range of 40-60°C. The trend of TRS with different temperature is presented in **Fig. 1.** Total reducing sugar (TRS) yield increased from 24.7 g/kg to 32.8 g/kg with increase in temperature from 40-50°C, respectively and was found maximum at temperature of 50°C. Further increasing temperature from 50°C to 55°C decreased the sugar yield from 32.8 g/kg to only 12.0 g/kg. At 60°C, negligible sugar yield was obtained. Above a certain temperature, (in present case; 50°C) enzyme activity decreases with temperature because of enzyme denaturation which is also called temperature inactivation or thermal denaturation. High temperature tends to change the protein folding which leads to enzyme denaturation[16]. From the above data, 50°C appears to be the most suitable temperature for saccharification of cellulose powder with CTR.

Effect of pH

TRS yield increased from 24.9 g/kg to 31.7 g/kg with increase in pH from 4.0-5.0 respectively and was found maximum at pH 5.0. Slightly lower TRS yield was obtained at pH 4.8. Further increasing pH from 5.0 to 5.5, decreased the sugar yield from 31.7 g/kg to only 7.0 g/kg. At pH 6.0, lowest TRS yield of 3.4 g/kg was obtained. Variation of in the pH of medium results in changes in ionic form of active site which leads to denaturation of enzyme`s active sites. This results in decreased activity of enzyme and the rate of reaction[16]. From the above table, pH 4.8-5.0, appears to be most suitable pH for saccharification with CTR.

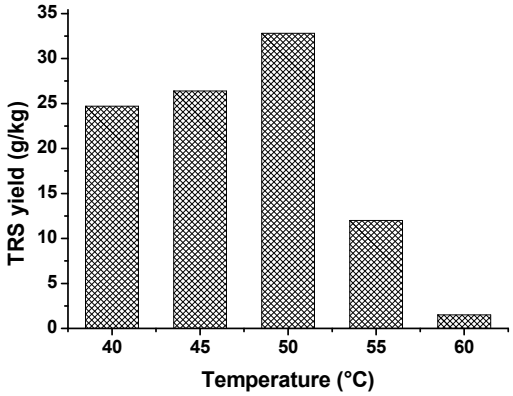

Fig. 1 Effect of temperature on saccharification of cellulose powder with cellulase *Trichoderma reesei*

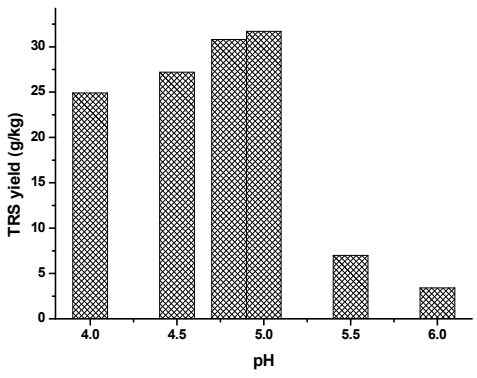

Fig. 2 Effect of pH on saccharification of cellulose powder with cellulase *Trichoderma reesei*

Effect of agitation speed

To study the effect of agitation speed on TRS yield of saccharification, agitation speed range of 100-250 was chosen. The trend of TRS with different agitation speeds is presented in Fig. 3. TRS yield increased from 21.5 g/kg to 33.0 g/kg with increase in RPM from 100 to 170 and was found maximum at 170 rpm. Further increasing agitation speed from 170 rpm to 200 rpm decreased the TRS yield from 33.0 g/kg to 24.7 g/kg. At 250 rpm, the TRS yield was found lowest probably due to instability of enzyme at high RPM. Unlike temperature and pH, agitation speed has moderate effect on saccharification. Agitation speed of 170 rpm can be considered most suitable for saccharification with CTR.

From the above observations, pH of 5.0; temperature of 50°C and agitation speed of 170 RPM were found to be most suitable for saccharification of cellulose powder with cellulase *Trichoderma reesei*. Others researchers have also obtained similar results for effects of reaction conditions on saccharification and they have reported pH 5.0, temperature 50°C and agitation speed of 150 rpm as the optimum values for saccharification with CTR[17,18].

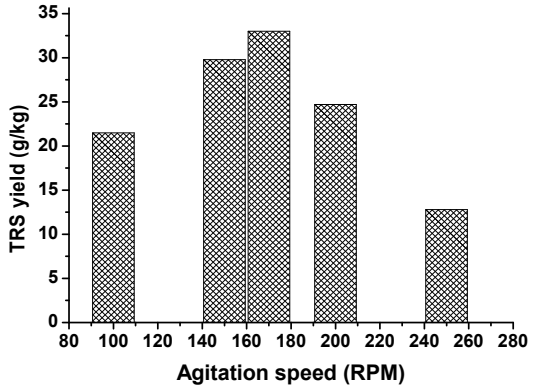

Fig. 3 Effect of agitation speed on saccharification of cellulose powder with cellulase *Trichoderma reesei*

Saccharification of pretreated biomass

The most suitable reaction conditions of temperature, pH and agitation speed obtained from saccharification of cellulose powder with CTR were used for the saccharification of pretreated biomass. The results of saccharification and total sugar yield of pretreatment and saccharification are presented in table 3. Almost similar sugar yield of saccharification was obtained with wheat straw and sugarcane bagasse (180 g/kg and 184 g/kg respectively) while maximum sugar yield (TRS) of 205 g/kg was obtained with pearl millet straw. Total sugar of pretreatment and saccharification (on initial weight basis) was found to be in the range of 53.3% to 57% of the total potential sugar in biomass. Maximum total sugar of pretreatment and saccharification was obtained with sugarcane bagasse (341 g/kg biomass) but the total sugar percentage yield (53.3%) was less due to high total potential sugar in bagasse (640 g/kg). Slightly lesser sugar yield was obtained with pearl millet straw (334 g/kg). Ballesteros et al., (2006) obtained sugar yield of 230 g/kg dry matter at a higher acid concentration of 0.9% (w/v) at the same temperature and time period[19], while a much higher yield of 378 g/kg dry matter was obtained by Linde et al., (2008) with pretreatment conditions of 190°C, 10 min, and 0.2% (v/v) acid concentration[20].

Conclusions

In this study, maximum sugar yield of pretreatment was obtained with sugarcane bagasse at 120°C, 120 min and 2% (v/v) acid concentration while maximum sugar yield of saccharification was obtained with pearl millet straw. These results show that in order to obtain maximum fermentable sugar yield from lignocellulosic biomass, a balance in pretreatment and saccharification should be maintained.

Table 3 Total sugar of pretreatment and saccharification (on initial weight basis)

Substrate	S^P	S^S	S^{PS}	TPS (g/kg)
Wheat straw	227	90	317	578
Sugarcane bagasse	249	92	341	587
Pearl millet straw	231	103	334	640

S^P -TRS yield of pretreatment (g/kg substrate); S^S - TRS yield of saccharification (g/kg substrate)
S^{PS} - Total sugar of pretreatment and saccharification (g/kg substrate)
TPS- Total potential sugar in biomass (g/kg)

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