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Original Article

Effect of various pretreatment conditions on enzymatic saccharification

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Abstract

The present work deals with the delignification of wheat straw and sugarcane bagasse with different pretreatment techniques followed by saccharification with commercial and indigenously produced cellulase enzymes. 100 g of sugar cane bagasse and whaet straw were treated with various concentrations of H_2O_2 (1-5% v/v) and its combination with 2% NaOH for one hour under steam at 30 psi. Sugarcane bagasse was 88% delignified with 5% H_2O_2 + 2% NaOH whereas wheat straw showed 51% delignification with 3% H_2O_2 + 2% NaOH. Moreover the saccharification with commercial cellulase enzyme was found to be 33.6% and 63.3% with pretreated wheat straw and sugarcane bagasse, respectively. However, very low levels of saccharification (6-14%) were found with indigenously produced cellulase enzyme.

Keywords: chemical pretreatment, lignocellulosic biomass, saccharification

1. Introduction

Various lignocellulosic materials such as agricultural residues (wheat straw, sugarcane bagasse, corn stover), forest products (hardwood and softwood), and dedicated crops (switchgrass, salix) are renewable sources of energy. Approximately 90% of the dry weight of most plant materials is composed of cellulose, hemicellulose, lignin, and pectin (Yat *et al.,* 2008). The presence of lignin in lignocelluloses leads to a protective barrier that prevents plant cell destruction by fungi and bacteria for conversion to fuel. For the conversion of biomass to fuel, the cellulose and hemicellulose must be broken. The digestibility of cellulose present in lignocellulosic biomass is hindered by many physicochemical, structural, and compositional factors. The lignocellulosic biomasses need to be treated prior to fuel production to expose cellulose. Pretreatment uses various techniques, including ammonia fiber explosion, chemical treatment, biological treatment, and steam explosion, to alter the structure of cellulosic biomass to make cellulose more accessible (Hsu *et al.,* 1980). The purpose of the pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials.

Both, bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. Fungi that have been reported to produce cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma, Aspergillus, Schizophyllum* and *Penicillium* (Sternberg, 1976; Fan *et al.,* 1987; Duff and Murray, 1996). Among all of these fungal genera, *Trichoderma* and *Aspergillus* has been most extensively used for cellulase production (Sun and Cheng, 2002).

There are three major groups of cellulases, which are involved in the hydrolysis process, (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.), which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends, (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91.), which degrades the molecule further by removing cellobiose units from the free chain-ends, and (3) β-glucosidase (EC 3.2.1.21), which hydrolyzes cellobiose to produce glucose (Sun and

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Cheng, 2002).

There are various factors, which affect the enzymatic hydrolysis rate such as substrate concentration, cellulase activity, and reaction conditions (temperature, pH, as well as other parameters). The enzymatic hydrolysis rate can be improved by optimizing these conditions (Sun and Cheng, 2002). Our major focus of this study is to optimize the pretreatment conditions, which maximize delignification process, thus enhancing the enzymatic hydrolysis rate.

2. Material and Methods

2.1 Lignocellulosic biomass

Sugar cane bagasse procured from Shakar Gunj Sugar mills (Pvt.) Limited, Jhang Road, Faisalabad, Pakistan, and wheat straw that was purchased from a local market 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.

2.2 Pretreatment

Sugar cane bagasse and wheat straw samples were pretreated as reported earlier (Irfan et al., 2010). The chopped sugar cane bagasse and wheat straw samples were soaked in different concentration of H_2O_2 and H_2O_2 + 2% NaOH ranging from 1-5% (v/v) solution at the ratio of 1: 10 (solid : liquid) for 2 hrs at room temperature. After that samples were steamed at 130°C for 60 minutes. After steaming the samples were filtered and solid residues were washed up to neutrality.

2.3 Estimation of sugars

Total sugars in the filtrate were determined by the method of Dubois *et al.* (1956) and reducing sugars in the filtrate were estimated by using 3, 5 dinitrosalysilic acid method (Miller, 1959).

2.4 Determination of cellulose

Cellulose content in treated and untreated samples was estimated by the method as described by Gopal and Ranjhan (1980). 1 g of oven dried sample was taken in round bottom digestion flask, 15 ml of 80% acetic acid and 1.5 ml of conc. HNO_3 was added to the flask and refluxed for 20 min. After refluxing the material was filtered through Whatmann filter paper #1 and washed with hot water. After washing the digested material was oven dried at 105°C overnight and weighed then incinerated at 550°C for 5 hrs in a muffle furnace and weighed again. The percentage of cellulose on dry matter basis can be calculated using the following eqaution:

$$\text{Cellulose (\%) Dry matter basis} = \frac{\text{Weight of Digested Material - Weight of Ash}}{\text{Weight of material on dry basis}} \cdot 100 \quad (1)$$

2.5 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 residues was filtered and washed with distilled water to remove sulphuric acid and oven dried at 105°C for constant weight. The lignin was expressed by using the following equation (Milagres 1994):

$$\text{Lignin (\%)} = \frac{\text{Lignin Weight (g)}}{\text{Bagass Weight (g)}} \cdot 100 \quad (2)$$

2.6 Determination of weight loss

Weight loss was determined by directly weighing substrates before and after treatments, after drying in an oven at 105°C to a constant weight.

2.7 Estimation of total phenolic compounds

Total phenolic compounds, which were released during pretreatment were estimated as described by Carralero *et al.* (2005) and Tsao and Deng (2004). In 2 ml of filtrate solution, add 10 ml of folin reagent (diluted 1,10 ratio) then add 8 ml of 75 g/L Na_2CO_3, make up the total volume of reaction mixture up to 20ml with distilled water. Place the reaction mixture at room temperature for 2 hrs and then measured the optical density at 765 nm. Vanillin was taken as standard to estimate total phenolic compounds in biomass.

2.8 Enzyme production

Solid state fermentation was carried out for enzyme production using *Trichoderma reesie*. About 25 g of ground raw sugarcane bagasse was moistened with 70 ml of tap water and sterilized at 121°C for 15 minutes. After that the media was inoculated with 10 mL of spore suspension and incubated at 30±1°C for four days of fermentation period.

2.9 Enzyme extraction

Enzymes from the fermented mash were extracted by simple contact method as reported by Krishna and Chandrasekaran (1996). In 25 g of fermented mash 250 mL of distilled water (1,10 solid to liquid ratio) was mixed and placed on shaker with the agitation speed of 150 rpm for 2 hrs. After complete mixing it was filtered through muslin cloth and the residues was discarded and the filtrate was centrifuged at 10,000 rpm for 10 min at 4°C to remove spores. The cell free extract was used for further analysis.

2.10 Estimation of enzyme activities

CMCase activity in the culture filtrate was determined

by incubating the 0.5 ml of crude enzyme sample with 0.5 ml of 1% CMC (0.05M Citrate buffer pH 5) at 50°C for 30 min. After incubation, the reaction was stopped by the addition of 1.5 ml of DNS and then boiled for 10 min in boiling water bath. The reaction mixture was allowed to cool and the reducing sugars released were estimated by Miller's method (1959).

For the estimation of FPase activity, 500 µL of culture filtrate was added to test tube containing Whatman No.1 filter paper strip (1x 6 cm) incubated at 50°C for 30 min. After that 1.5 ml DNS were added to test tube and boiled for 10 minutes and absorbance was taken spectrophotometrically at 550 nm. The reducing ends liberated were then measured with DNS (Miller, 1959).

2.11 Enzymatic hydrolysis

Cellulase enzyme indigenously produced from *Trichoderma reesei* with an activity of 96.1 filter paper unit (FPU/mL), CMCase activity of 180 IU/ml and 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 hrs. 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).

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugar formed} \cdot 0.9}{\text{Cellulose content in Pretreated Biomass}} \cdot 100 \quad (3)$$

2.12 Statistical analysis

Statistical analysis was done by ANOVA test using Graphpad instate software. The difference in values was indicated in the form of probability ($p \leq 0.05$) values.

3. Results and Discussion

Lignocellulosic biomass contains three major components (cellulose, hemicellulose and lignin), which made the plant body rigid and very hard. Among these three components lignin is the most important part which made the plants more hardened. To convert plant biomass into valuable products first we have to degrade the lignin component of the plant cell wall. Table 1 represents the compositional analysis of sugarcane bagasse and wheat straw. The difference in these main components might be due to the genetics, location and growth conditions.

3.1 Pretreatment study

Different pretreatment techniques were used to treat the sugarcane bagasse and wheat straw to optimize the suitable concentration of chemical which enhances the saccharification process. Two treatments were used; in first treatment the biomass was treated with different concentration of H_2O_2, and in the second treatment different concentrations of H_2O_2 + 2 % NaOH were used. Total sugars, reducing sugars, total phenolic compounds, lignin content, cellulose content, and weight loss at each treatment were observed. Figure 1 explains the release of sugars (total, reducing) and total phenolic compounds at each treatment process. Among various concentration of H_2O_2, 3% (v/v) was found best which released 6.77 mg/ml, 1.55 mg/ml and 20.4 mM/ml of total sugars, reducing sugars and total phenolic compounds, respectively. A decline in release of sugars was observed with further increase in concentration of H_2O_2. When sugarcane bagasse was treated with 5% (v/v) H_2O_2 6.6 mg/ml of total sugars, 0.6 mg/ml reducing sugar and 15 mM/ml of total phenolic compounds were released during treatment. Figure 1b shows the weight loss, cellulose and lignin content after each treatment. With increase in concentration of H_2O_2 decline in cellulose content and increased weight loss was observed. Maximum delignification (36%) was observed with 3% (v/v) H_2O_2.

Figure 2 represents the results of combined treatment of 2% NaOH and different concentration of H_2O_2 on sugar-

Table 1. Analysis of main components of sugarcane bagasse and wheat straw.

Biomass	Cellulose (%)	Lignin (%)	References
Sugarcane Bagasse	40	23	Present study
	40	20	Varhegyi *et al.* (1998)
	33.3	6.15	Aiello *et al.* (1996)
	35.8	21.1	Garcia-perez *et al.* (2002)
	43.6	18.1	Sun *et al.,* 2004; Xu *et al.* (2006)
Wheat Straw	39.4	17.3	Present study
	38.2	23.4	Wiselogel *et al.,* (1996)
	30	15	Reshamwala *et al.* (1995), Cheung and Anderson (1997), Boopathy (1998), Dewes and Hunsche (1998)

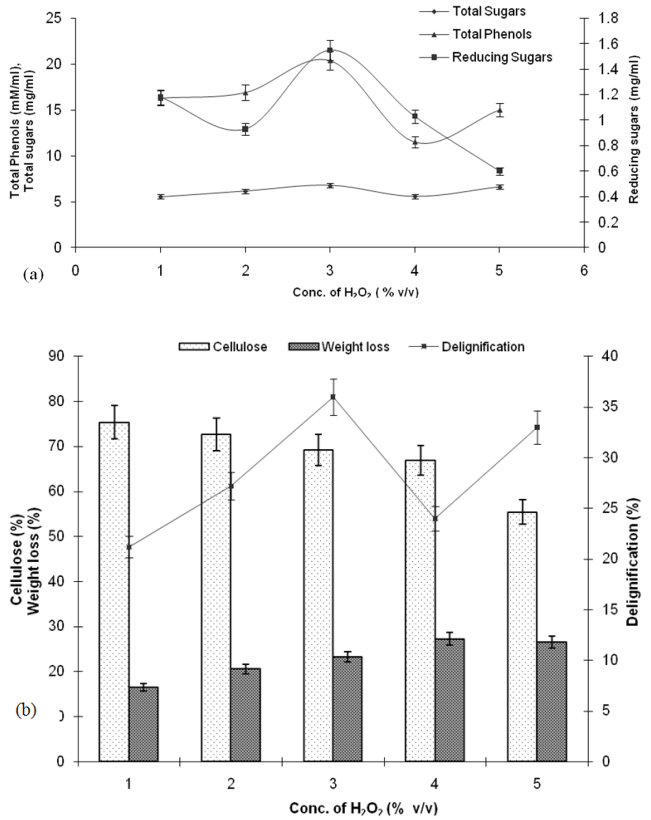

Figure 1. Effect of different concentrations of H_2O_2 on (a) release of total phenolic compounds, reducing sugars and total sugars (b) cellulose, delignification, and weight loss during pretreatment of sugarcane bagasse. The error bars represent the standard deviation of the means based on three replicas.

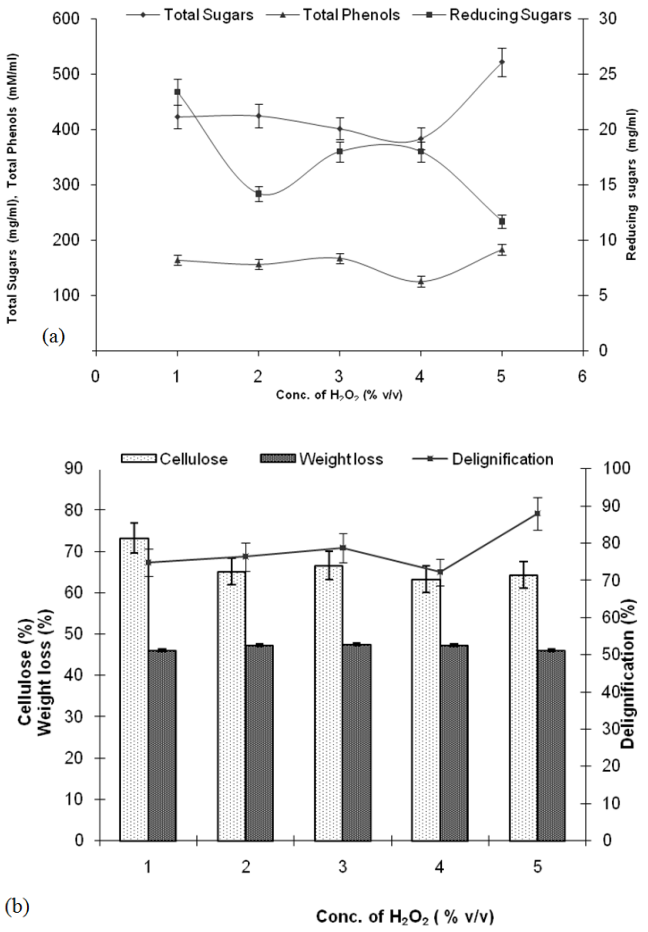

Figure 2. Effect of combined treatment of H_2O_2 + 2% NaOH on (a) release of total phenolic compounds, reducing sugars and total sugars (b) cellulose, delignification, and weight loss during pretreatment of sugarcane bagasse. The error bars represent the standard deviation of the means based on three replicas.

cane bagasse. In this treatment 423.2 mg/ml of total reducing sugar, 23.4 mg/ml of reducing sugar, 164.2 mM/ml of total phenolic compounds were released by using combination of 1% H_2O_2 + 2% NaOH respectively. By increasing the concentration of H_2O_2 greater destruction in cell wall component were observed. Highest delignification levels (78.8% and 88%) were observed with combination of 3% (v/v) H_2O_2 + 2% NaOH and 5% (v/v) H_2O_2 + 2% NaOH, respectively. Cellulose exposures and weight loss at these concentrations were 66.6%, 64.3%, and 47.5 % and 46% respectively. Weight loss during treatment represents the degradation of lignocellulosic biomass. According to Wyman (1996) the amount of weight loss during pretreatment process was due to lignin removal.

Figure 3 explains the results of different concentrations of H_2O_2 on wheat straw. Maximum breakdown in the cell wall components were noted with 3% and 5% (v/v) H_2O_2. At 3% (v/v) H_2O_2 128.5 mg/ml, 11.4 mg/ml and 19.8 mM/ml of total sugars, reducing sugars and total phenolic compounds were liberated. When wheat straw was treated with 5% (v/v) H_2O_2 138.5 mg/ml of total sugars, 6.23mg/ml of reducing sugars and 20.7 mM/ml of total phenolic compounds were released. Delignification of 48% was observed with 3% (v/v) H_2O_2, which was found more effective in this treatment process. Figure 4 described the combined treatment of different concentrations of H_2O_2 and 2% NaOH using wheat straw as substrate. The effective concentration for delignification was found 3% H_2O_2(v/v) + 2NaOH which degraded 51.7% lignin content from the biomass. In both substrates, i.e. sugarcane bagasse and wheat straw, the consecutive action of NaOH and H_2O_2 degrade maximum lignin content. The use of dilute NaOH treatment of lignocellulosic materials has been found to cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Sun and Cheng, 2002).

Azam (1989) reported that H_2O_2 degrade 50% lignin content in sugarcane bagasse. According to Misson *et al.* (2009) 99% degradation of lignin was achieved using combination of H_2O_2 + NaOH in case of empty palm fruit bunch.

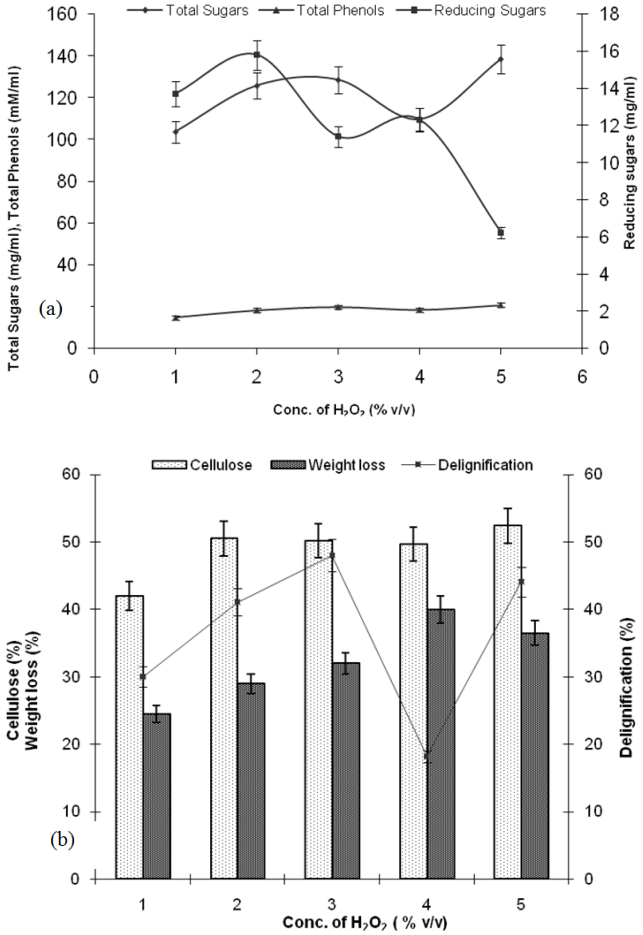

Figure 3. Effect of different concentrations of H_2O_2 on (a) release of total phenolic compounds, reducing sugars and total sugars (b) cellulose, delignification, and weight loss during pretreatment of wheat straw. The error bars represent the standard deviation of the means based on three replicas.

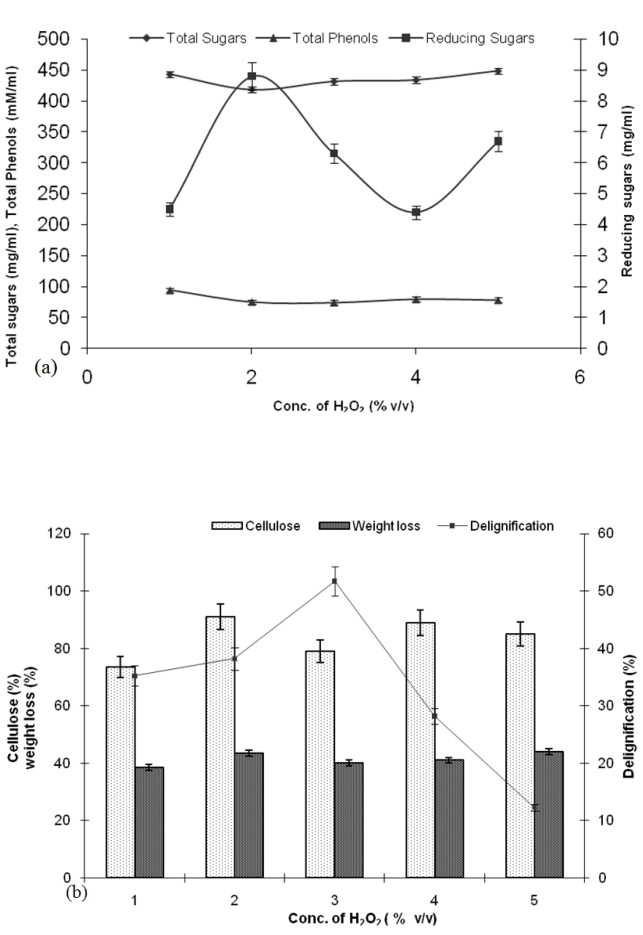

Figure 4. Effect of combined treatment of H_2O_2 + 2% NaOH on (a) release of total phenolic compounds, reducing sugars and total sugars (b) cellulose, delignification, and weight loss during pretreatment of wheat straw. The error bars represent the standard deviation of the means based on three replicas.

Treatment of wheat straw with wet oxidation and alkaline hydrolysis produced 85% conversion yield of cellulose to glucose at 170°C for 5–10 min (Bjerre *et al.,* 1996). Gould (1984) stated that pretreatment of wheat straw with suitable concentrations of H_2O_2 delignify to provide optimum cellulose for enzymatic hydrolysis. 1% H_2O_2 at pH 11.5 for several hours at room temperature releases more than one half of its lignin as a water soluble products using wheat straw as a substrate (Gould and Freer, 1984). During delignification process some water soluble aromatic compounds were formed which inhibits the cellulytic action of enzymes (Hattaka, 1983).

The major action of NaOH is to degrade the lignin by breaking ester bond cross linking, thus creating porosity in biomass (Rabeeca *et al.,* 2007) and addition of H_2O_2 is to detach and loosen the lignocellulosic matrix (Playne, 1984).

3.2 Saccharification of pretreated biomasses

Enzymatic hydrolysis of the pretreated biomasses (sugarcane bagasse and wheat straw) waere done with commercial enzymes and indigenously produced enzyme from *Trichoderma reesie* grown under solid state fermentation. Saccharification process increased with increase in H_2O_2 concentration and maximum saccharification (41.9%) rate was noted (Figure 5) with 5% (v/v) H_2O_2 in case of sugarcane bagasse. 63.3% and 54.7% saccharification was achieved with 5% (v/v) H_2O_2 + 2%NaOH and 2% (v/v) H_2O_2 + 2% NaOH using sugarcane bagasse as a substrate. When wheat straw was used as substrate saccharification rate of 33.6% and 27.1% was obtained with 3 %(v/v) H_2O_2 + 2% NaOH and 3% (v/v) H_2O_2 (Figure 6). Figure 7 and 8 represents the saccharification results of indigenous enzyme produced from *Trichoderma reesei* in solid state fermentation. Results showed that highest saccharification rate (14.1%) was observed by the combined treatment of 3 % (v/v) H_2O_2 + 2%NaOH in wheat straw. Akhtar *et al.* (2001) reported maximum saccharification rates of 33.0, 25.5, and 35.5% were obtained with 2% NaOH pretreated wheat straw, rice straw and bagasse, respectively. When 2% H_2O_2 was employed on sugarcane bagasse there was 50% degradation in lignin and

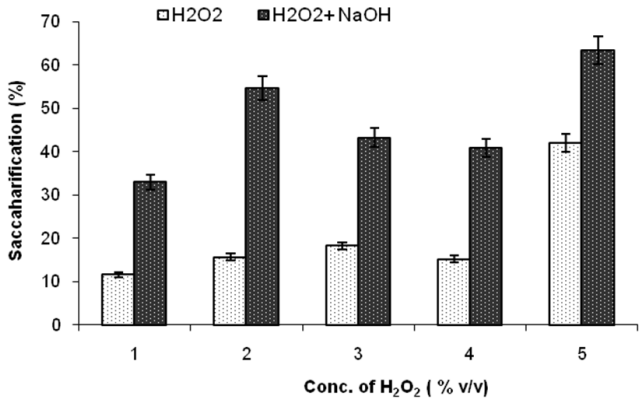

Figure 5. Effect of different pretreatment conditions on saccharification of sugarcane bagasse by commercial enzyme.

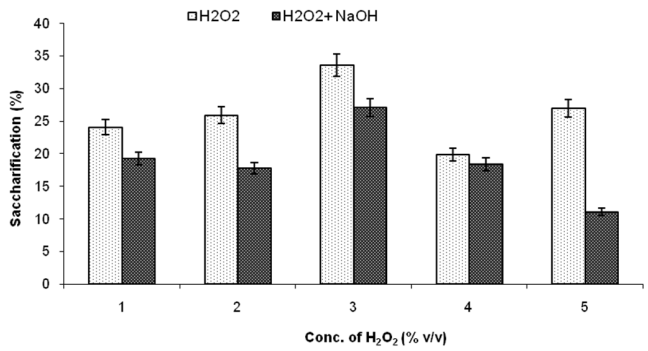

Figure 6. Effect of different pretreatment conditions on saccharification of wheat straw by commercial enzyme.

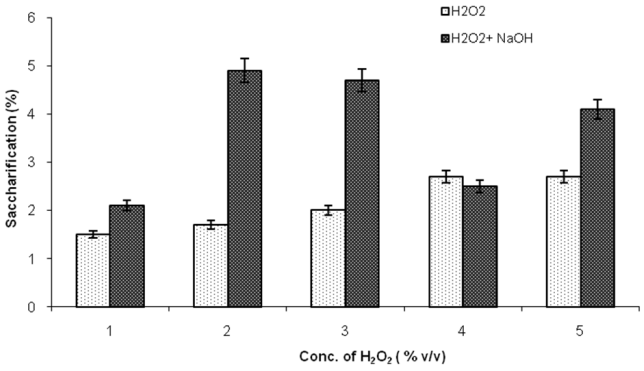

Figure 7. Effect of different pretreatment conditions on saccharification of sugarcane bagasse by enzymes of *Trichoderma reesei*.

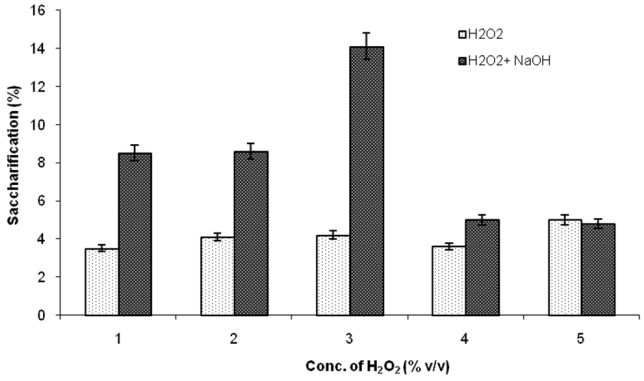

Figure 8. Effect of different pretreatment conditions on saccharification of wheat straw by enzymes of *Trichoderma reesei*.

hemicellulose was observed at 30°C within 8 hrs, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45°C for 24 hrs (Azzam, 1989). Saha and Cotta (2006) pretreated wheat straw with alkaline peroxide and obtained 97% fermentable sugars by enzymatic saccharification. In another study, Saha and Cotta (2007) reported that diluted alkaline peroxide treatment (7.5% H_2O_2(v/v), pH 11.5, 35°C, 24 hrs) is an efficient method for pretreatment of rice hulls, resulting in almost complete conversion (96%) of rice hulls to sugars after enzymatic hydrolysis. When mixture of cellulases (dose, 1.0 FPU/g substrate) from *A. ustus* and *T. viride* was used on 8% alkali-treated sugar-cane bagasse 90% enzymatic saccharification was achieved (Mononmani and Sreekantiah, 1987). Some structural features of biomass pretreated with lime affects the enzymatic hydrolysis (Kim and Holtzapple, 2006). Lignin content, crystallinity, and acetyl content of the biomass limits the enzyme digestibility (Chang and Holtzapple, 2000). Lignin content below 12% does not significantly affect the enzymatic digestibility (Chang *et al.,* 2001) and greater than 50% hemicellulose removal is required to increase the cellulose digestibility (Moiser *et al.,* 2005). The alkali pretreatment can result in a sharp increase in saccharification, with manifold yields (Kassim and El-Shahed, 1986).

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