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Green ethanol production from cotton stalk

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Green ethanol production from cotton stalk

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Abstract. This study was initiated to produce green ethanol from cotton stalk lignocelluloses using separate hydrolysis and fermentation (SHF) process. The influence of sodium hydroxide (2%) pretreatment at different residence times was also investigated. The effect of incubation period, enzyme concentration, pH and substrate concentration during enzymatic saccharification on sugar yield and ultimately on the ethanol production was studied. The results show that pretreatment with 2% NaOH for 90 min at 121°C was most effective and gave the highest percentage of cellulose (78.2±1.64%) along with highest delignification rate (63.9%). During the enzymatic saccharification, under the optimized conditions, maximum concentration of reducing sugars of the hydrolyzate was 67.83 ± 0.35 g/L with the maximum saccharification efficiency of 78.06%. The total sugars were 77.48 ± 2.37 g/L and total phenols were found to be 1.6 mm. Fermentation was carried out by using the enzymatically saccharified hydrolyzate and monoculture of *Saccharomyces cerevisiae*. Highest ethanol concentration of 22.93 ± 1.74 g/L with 0.36 g/g ethanol yield was achieved after 48 h of incubation at 30°C, pH 5 and 3% inoculum size.

1. Introduction

Energy demand is on the rise with escalating population of the world. According to the latest statistics, Global energy demand will rise to about 30% by 2035 with an average growth of 1.3% annually, while in developing countries it is estimated that the energy demand will increase by almost 30% by 2040 [1]. Presently, the renewable energy sources like solar, wind, biomass etc provide an attractive alternative to meet the growing energy demands as well as protecting the environment. In many countries, government policies and incentives are promoting use of renewable energy sources. Renewable energy is the world's fastest growing source of energy, at an average rate of 2.6 % annually. According to an estimate, by 2040, \$44 trillion is going to be invested in the energy supply and there is a decline in the share going to the fossil fuels. It is believed that by year 2040 the renewable power generation capacity rise to 60% [1].

Globally, biomass fuel is turning out to be an attractive and reasonable alternate for conventional fossil fuels because of growing interest for green energy approaches and declining fuel reserves. It not only decreases reliance on oil import from other countries but also sustains rural economies by creating employment opportunities and provides resources for income [1]. The most common renewable fuel today is bioethanol; being the most widely used biofuel in over 64 countries [2]. There is a large variety of biomass sources which can be used for bioethanol production including some aquatic plants, woody plants and herbaceous plants/grasses [3]. Agricultural residues are considered to be one of the most suitable choices for ethanol production worldwide as these residues can serve as a replacement of grains for ethanol production with no threat of food scarcity [4].

Corn has been extensively used for bioethanol production; almost all of the ethanol in USA is produced from corn [5, 6]. Maize, corn stover, wheat and rice straw have been investigated for high ethanol yield by many researchers [7-13]. The lignocellulosic biomass is a favorable resource among all for bio-fuels production [14, 15]. These renewable raw materials have the potential to create green products [16, 17].



Many different technologies are being developed and many are underway/developmental stage to produce bioethanol from lignocellulosic biomass applicable to different stages of production process of bio-ethanol; pretreatment, hydrolysis/saccharification and fermentation [18].

Cotton stalk is one such lignocellulosic biomass. It is the residue of plant after harvesting of cotton. Cotton is produced in over 80 states in the World, which generates the huge quantity of cotton stalk [19]. Cotton stalk is a potential raw material for conversion to ethanol because it is rich in cellulose (32–46%) and hemicellulose (20–28%) [19]. Bio-ethanol from cotton stalk was produced utilizing two-stage dilute acid hydrolysis and fermentation of detoxified hydrolysate a [20]. In Pakistan, during 2010-2011, approximately 1,474,693 metric tons of cotton residue was produced. This residue of the cotton crop has the potential of power generation around 3,071 GWh [21].

Cotton stalk has high holocellulose content which makes it a potential feedstock for bio-ethanol production [17]. In a study done on cotton stalk, cellulose in the range of $40.10 \pm 1.55\%$, hemicelluloses $13.60 \pm 0.64\%$ and lignin $29.40 \pm 0.95\%$ was found [22]. As cotton stalk/residue is extensively produced biomass in Pakistan, the present study was designed to study the potential of producing bioethanol from cotton stalk which not only offers a cleaner fuel but also will help in recycling waste and help in waste management. Hence, this study was initiated to produce bioethanol from cotton stalk and also to optimize its process to achieve maximum ethanol production.

2. Materials and method

In practical work of the current study, analytical grade chemicals and reagents were used. The Cotton stalk (*Gossypium arboreum*) biomass was acquired from the local market. After the careful inspection of the biomass, the undesirable dust particles were removed and after that it was dried in an oven for 48 hours (h) at 100°C . Physical pretreatment was carried out by reducing the size of cotton stalk to 2 mm by crushing in a hammer beater mill, followed by chemical pretreatment using sodium hydroxide (NaOH). Cotton stalk (100 g) were soaked for one hour in 1000 ml (2%) NaOH solution in 3 flasks and autoclaved at the residence times of 30, 60 and 90 min at constant temperature of 121°C with 15 psi pressure. The solid liquid ratio was taken as 1:10.

2.1. Enzymatic saccharification

Enzymatic saccharification of pretreated cotton stalk was performed with 0.05M citrate buffer (100 ml) with 0.02% sodium azide and added in pretreated substrate for incubation in saccharification process. The commercial enzyme cellulase was added in it. It was then incubated at 50°C , 120 rpm for 92 h and was removed from the incubator at 24 h intervals for sugar analysis. The enzymatic conversion of cotton stalk was enhanced by optimizing the concern parameters.

2.2. Fermentation

The microorganism strains of *Saccharomyces cerevisiae* was used during experimental work. The strains were maintained on PDA (Potato Dextrose Agar) slants. pH of medium was maintained at 6.0 and sterilized at 121°C for 15 mins. The inoculum was prepared by adding YPD (Yeast, Peptone and Dextrose) media and strains of *Saccharomyces cerevisiae* for 48 h at 30°C . The prepared cultures of *Saccharomyces cerevisiae* were used as inoculum in fermentation.

Fermentation was the 3rd step in bioethanol production from cotton stalk. First, the inoculum size was optimized followed by fermentation by using all pre-optimized parameters and inoculum size to achieve the maximum ethanol concentration. To determine optimum inoculum size of *Saccharomyces cerevisiae* for fermentation, different inoculum sizes (1-4%) were studied. The enzymatically saccharified hydrolysate (100ml) with the highest reducing sugars were added in the fermentation medium consisting of 1.5 g yeast extract, 1 g each of peptone and $(\text{NH}_4)_2\text{SO}_4$, 0.5 g each of K_2HPO_4 , $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ and

MnSO₄ at pH 5. The medium was sterilized for 15 min at 121°C. After cooling the media, different inoculum sizes of 1-4% was added into 4 flasks containing sterilized media. Fermentation was carried out for 96 hours at 30°C. Initially, aerobic conditions were provided for 24 hours followed by anaerobic conditions for 48 hours. The samples were collected at 24h intervals throughout the fermentation process and analyzed for ethanol content and reducing sugars.

2.3. Analytical methods

The percentage of cellulose, hemicellulose and lignin in cotton stalk samples was estimated by method of [23-25]. The total phenol and ethanol estimation was carried out by using method adopted [26,27] respectively. The ethanol yield was calculated according to the equation given in a study done by [28].

3. Results

3.1. Chemical composition of raw cotton stalk

The results revealed that the cellulose content in the raw (untreated) cotton stalk was 43.7 ± 0.45 % and hemicellulose was 12.53 ± 0.40 %; whereas, the lignin content was found to be 28.6 ± 0.45 %. Cellulose and hemicelluloses content in a defined combination makes the holocellulose, which was found to be 55.8% in the present study on dry weight basis.

3.2. Results of NaOH pretreatment of cotton stalk

High lignin content ($28.6 \pm 0.45\%$) was found in the present study, so delignification with NaOH pretreatment constituted an important step. The results after physiochemical treatment indicated that when cotton stalk was soaked in 2% NaOH at 121°C in an autoclave with the residence times of 30, 60, and 90 min, the cellulose exposure increased with time. Table 1 summarizes the results of chemical composition of cotton stalk before and after pretreatment, along with the holocellulose and delignification percentages. Cotton stalks autoclaved for 90 min gave the highest percentage of cellulose (78.2 ± 1.64) and also highest delignification rate (63.9%). 30 min residence time in an autoclave was not very effective and the cellulose was increased by only 8% from the untreated cotton stalks.

3.3. Enzymatic saccharification of NaOH treated cotton stalk

Table 1. Chemical Composition of Cotton Stalk

Autoclave residence time (min)	Cellulose (%)	Hemicellulose (%)	Holocellulose (%) (cellulose + hemicellulose)	Lignin (%)	Delignification (%)
Untreated	43.7 ± 0.45	12.5 ± 0.40	56.2	28.6 ± 0.45	-----
30 min	52.5 ± 1.64	11.2 ± 0.43	63.7	19.8 ± 0.83	30.7
60 min	65.4 ± 1.63	6.3 ± 1.52	71.7	13.4 ± 0.95	53.1
90 min	78.2 ± 1.64	4.9 ± 0.32	83.1	10.3 ± 1.23	63.9

All results are represented as mean value \pm standard deviation among triplicates

In enzymatic saccharification, the pretreated cotton stalk with 78.2 ± 1.64 % cellulose and 63% delignification was selected for enzymatic saccharification. Pretreated cotton stalk was incubated with 0.05M citrate buffer containing 0.02% sodium azide (100 ml) as the production medium for 96 h and were

removed from the incubator for sugar analysis at 24 h intervals. Figure 1 shows the optimization of incubation period. It was found out that hydrolysis effect increased at first 72 h but started to decrease after 96 h.

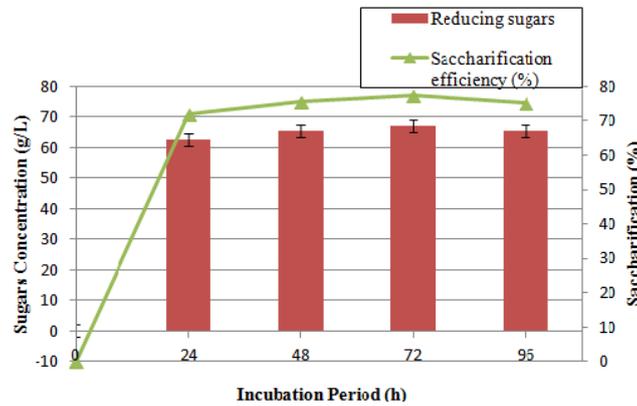


Figure 1. The Effect of Incubation Period on Reducing Sugar Concentration and Saccharification Efficiency

The effect of cellulase concentration on the enzymatic saccharification was carried out by adding cellulase enzyme at different concentrations of 200 IU/ml, 400 IU/ml, 600 IU/ml and 800 IU/ml. According to the results of the effect of enzyme concentration on the reducing sugar yield, the reducing sugars increased at

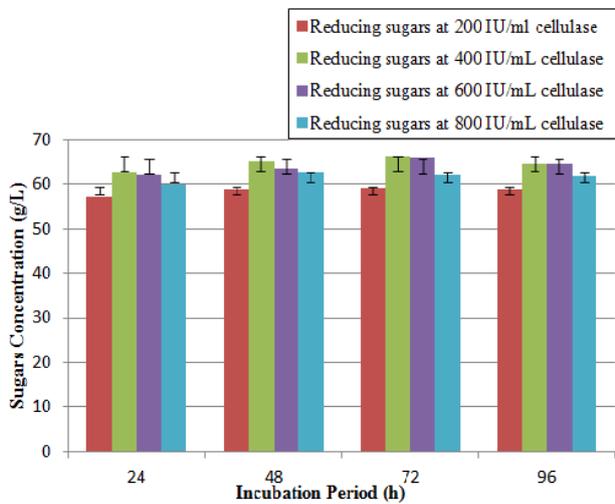


Figure 2. Effect of Incubation Period and Enzyme Concentration on Reducing Sugar Concentration

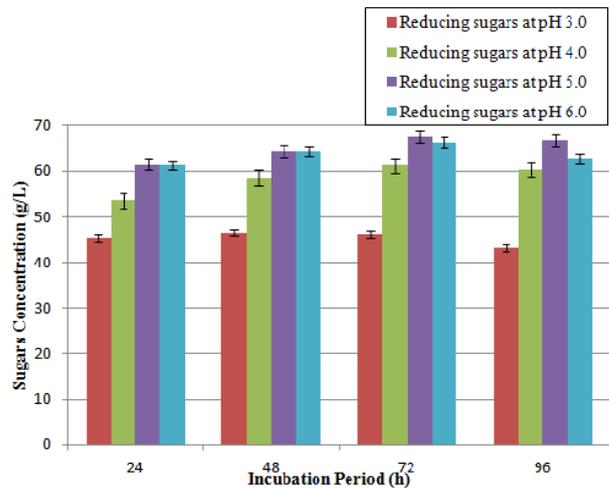


Figure 3. Effect of Incubation Period and pH on Reducing Sugar Concentration

first 72 h of saccharification but started to decrease after 96 h at all enzyme concentrations. The results of enzyme optimization at all concentrations are shown in figure 2. The effect of pH on the enzymatic saccharification was studied at various loadings of pH ranging from 3.0 to 7.0. Figure 3 shows the results

of pH optimization. Initially, the reducing sugars increased with the increase in pH till 5.0. Further increase in pH decreased the sugars.

The substrate concentration on the enzymatic saccharification was studied by using 0.5 g, 2.5g, 5g and 7.5g of substrate per 100mL of citrate buffer. The effect of substrate concentration on reducing sugars (figure 4) shows that saccharification efficiency decreased when the substrate taken was increased from 5g/100mL buffer. When all conditions of the enzymatic saccharification were optimized, an experiment was conducted again with all the pre-optimized conditions i.e. 400 IU/ml cellulase enzyme was used, which was added in 5g substrate in 100mL citrate buffer with pH 5 at 50°C temperature for 72 h incubation period. The total sugars, reducing sugars and total phenols of the hydrolysate obtained after 72 h of optimized conditions were taken.

The reducing sugars of the hydrolysate were found to be 67.83 ± 0.35 g/L with the maximum saccharification efficiency of 78.06%. The total sugars of the enzymatically saccharified hydrolysate were 77.48 ± 2.37 g/L and the total phenols were found to be 1.6 mm.

3.4. Fermentation of enzymatically saccharified cotton stalk

After the enzymatic saccharification, the next task was to determine optimum inoculum (*Saccharomyces cerevisiae*) size for maximum ethanol yield. The enzymatically saccharified hydrolysate attained after all the optimized parameters with the highest reducing sugars (67.83 ± 0.35 g/L) were utilized in the optimization of inoculum (*Saccharomyces cerevisiae*) size for fermentation. The experiment was conducted for 96 h at 30°C with pH adjusted at 5.0 and varying the inoculum size from 1% to 4%.

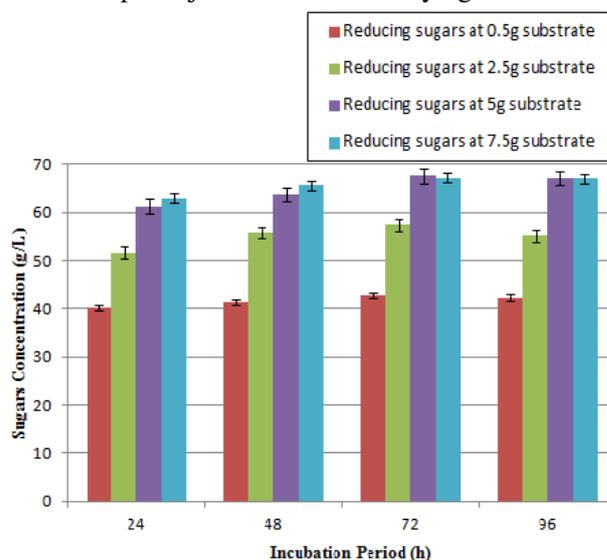


Figure 4. The Effect of Incubation Period and Substrate Concentration on Reducing Sugar Concentration

Maximum ethanol concentration and reducing sugar consumption rate was found at 48 h of incubation period in fermentation with ethanol concentration increasing with increase in inoculum size till 3% (v/v), after which it started to decrease with further increase in inoculum size. The reducing sugars also decreased with the increase in inoculum size as shown in Table 2. The inoculum size of 3% showed the maximum ethanol concentration of 22.79 ± 0.75 g/L with the ethanol yield of 0.36 g/g after 48 h of incubation at 30°C with pH 5.

Table 2. The Effect of Inoculum Size of *Saccharomyces cerevisiae* on Reducing Sugars, Ethanol Concentration and Ethanol Yield after 48 h of Fermentation

Inoculum size % (v/v)	Reducing sugars g/L	Ethanol concentration g/L	Ethanol yield g/g
1	7.33 ± 2.35	17.85 ± 1.52	0.29
2	6.26 ± 1.76	20.58 ± 0.38	0.33
3	5.47 ± 1.84	22.79 ± 0.75	0.36
4	5.10 ± 2.61	21.21 ± 0.53	0.33

All results are represented as mean value ± standard deviation among triplicates

After inoculum size optimization, fermentation of enzymatically saccharified hydrolysate was conducted at 30°C at pH 5 by using inoculum size (3%) of *Saccharomyces cerevisiae*. The reducing sugars in the hydrolysate were 67.39 ± 0.35 g/L before inoculum addition (at 0 h). According to the results given in Table 3 and Figure 5, ethanol concentration and yield increased at first 48 h and started to decrease after 48 h. The highest ethanol concentration was 22.93 ± 1.74 g/L with 0.36 g/g ethanol yield at 62.2 % reducing sugars consumption rate.

Table 3. The Effect of Incubation Period on Reducing sugars, Ethanol concentration and Ethanol yield

Incubation Period (h)	Reducing sugars g/L	Ethanol concentration g/L	Ethanol yield g/g
24 h	18.85 ± 0.59	17.23 ± 1.32	0.35
48 h	5.23 ± 1.61	22.93 ± 1.74	0.36
72 h	5.11 ± 1.58	21.64 ± 1.35	0.34
96 h	4.62 ± 1.74	21.43 ± 1.86	0.33

All results are represented as mean value ± standard deviation among triplicates

The highest ethanol concentration of 22.93 ± 1.74 g/L with 0.36 g/g ethanol yield was achieved after 48 h of incubation at 30°C, pH 5 and 3% inoculum size.

4. Discussion

The similar results of high delignification with 2% NaOH treatment was found in another

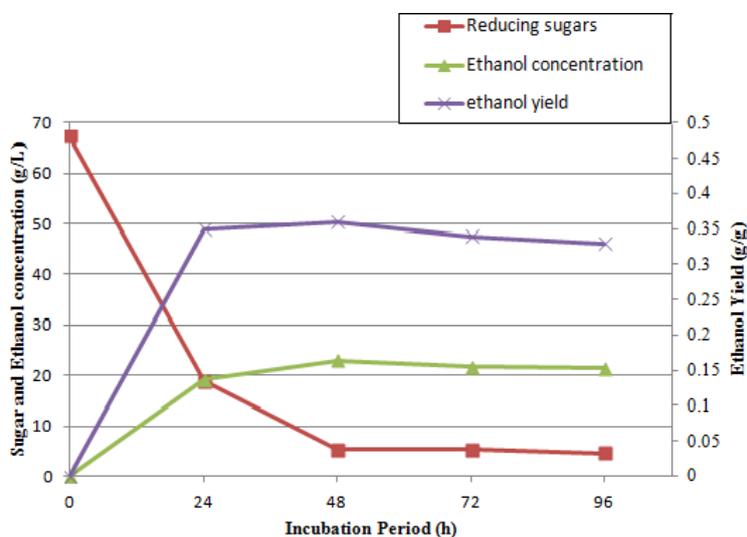


Figure 5: The Effect of Time on Reducing Sugars, Ethanol Concentration and Ethanol Yield

study done by [29]. Alkali pretreatment, especially with NaOH is very effective for delignification as it breaks the crosslinking ester bonds of lignin, also breaks the heteromatrix of carbohydrate fraction. This effect causes the porosity of biomass to increase and decrease the crystallinity of cellulose [30]. The maximum holocellulose content also changed significantly from 55.8% (untreated) to 83% (2% NaOH, 90 min) on dry weight basis. In a current study, the maximum holocellulose content conversion is slightly higher than the holocellulose conversion found in a study done by [22] which is $53.70 \pm 0.58\%$ (untreated) to $76.20 \pm 1.21\%$ (3% NaOH treatment) on dry weight basis.

In enzymatic saccharification, the pretreated cotton stalk with $78.2 \pm 1.64\%$ cellulose and 63% delignification was selected for enzymatic saccharification. Citrate buffer (0.05 M) was used as the production medium for enzymatic saccharification. Citrate buffer (0.05 M) for the hydrolysis of cotton stalk was reported by [31] in a study done on cotton stalks and also in another study done on sorghum straw by [32]. Cellulase enzyme was added during saccharification and one factor was kept constant at a time to optimize the activity of cellulase enzyme. Temperature was kept constant at 50°C during all experiments. The optimum temperature for the cellulase enzyme was reported to be $40\text{--}50^\circ\text{C}$ [33]. The optimum temperature range provided by *Trichoderma koningii* is also in the range of $40\text{--}55^\circ\text{C}$. According to a study, the parameters which were optimized during the enzymatic saccharification played an important role in the reducing sugar yields during enzymatic hydrolysis. It was reported that for the enzymatic hydrolysis of the lignocellulosic biomass, substrate concentration, cellulase activity and conditions such as temperature and pH are the main factors for the hydrolysis rate [33].

During the optimization of incubation period, the hydrolysis effect increased at first 72 h but started to decrease after 96 h. The highest reducing sugar values were $67.25 \pm 1.62\text{ g/L}$ obtained after 72 h of hydrolysis with the saccharification efficiency of 77.39%. The maximum released sugars were obtained at short period of 72 hours as the commercial cellulase enzyme was used in the current study. The maximum saccharification efficiency after 72 h of cotton stalk saccharification was also found in another study done [20].

The ratio of enzyme to substrate used is an important factor in the efficient hydrolysis rate [33]. Optimization of the commercial cellulase enzyme is important to find its optimum range, as the increase in cellulase levels would also increase the cost of the process. The effect of enzyme concentration

on the reducing sugar yield showed that the reducing sugars increased at first 72 hours of saccharification but started to decrease after 96 hours at all enzyme concentrations. The maximum sugars were obtained at 400 IU/ml (2g) of enzyme concentration at 72 h with 66.17 ± 0.68 g/L of reducing sugars with the saccharification efficiency of 76.15%. At higher concentrations of cellulase i.e. 600 IU/ml and 800 IU/ml, the sugars and hydrolysis efficiency started to decrease. It was found in another study that the increase in enzyme loading limit the hydrolysis yields. This happens due to the improper bindings and product inhibition effect [32].

Enzymatic saccharification of cellulose by the cellulase enzymes works best under specific, mild conditions i.e. pH 4.5-5 and temperature 40-50°C. This reduces the corrosion problems; utility consumption is low and also the low toxicity of the hydrolyzates [33]. In the present study, the maximum sugars were obtained at pH 5.0 after 72 h. Initially, the reducing sugars increased with the increase in pH till 5.0. Further increase in pH decreased the sugars.

The saccharification efficiency decreased when the substrate taken was increased from 5g/100mL buffer. It was reported that substrate concentration is one of the main factors in the yield of sugars in the initial rate of enzymatic hydrolysis. Inhibition can be caused by high substrate concentration which can cause low hydrolysis rate [33]. The maximum sugars of 67.63 ± 0.35 g/L were obtained with 5g substrate with the saccharification efficiency of 78.06% after 72 h. Further increase in the substrate level resulted in the downfall of sugars, which indicates that 5g substrate is the optimum concentration of substrate for the maximum hydrolysis rate. The increase in the substrate from the optimum concentration of substrate results in the decrease of sugars shows the fact that the hydrolysis of biomass is linked with the characteristics of the biomass feedstock [32]. The highest reducing sugars of the hydrolysate after the enzymatic saccharification at all optimized conditions had the reducing sugars of 67.83 ± 0.35 g/L with the maximum saccharification efficiency of 78.06%. The total sugars of the enzymatically saccharified hydrolysate were 77.48 ± 2.37 g/L and the total phenols were found to be 1.6 mm. Similar results of 68.20 ± 1.16 g/L of reducing sugars with different conditions were obtained after the hydrolysis of alkaline extraction of steam exploded cotton stalk in another study done by [20]. The maximum ethanol concentration and reducing sugar consumption rate was found at 48 h of incubation period in fermentation. The inoculum size of 3% showed the maximum ethanol concentration of 22.79 ± 0.75 g/L with the ethanol yield of 0.36 g/g after 48 h of incubation at 30°C with pH 5. The inoculum size (v/v) of *Saccharomyces cerevisiae* at 3% was also optimized in another study done by [28].

The ethanol concentration and yield increased at first 48 h and started to decrease after 48 h. The highest ethanol concentration was 22.93 ± 1.74 g/L with 0.36 g/g ethanol yield at 62.2 % reducing sugars consumption rate. It was reported in a study that the decline in the ethanol production after 48 h can be due to the simultaneous consumption of accumulated ethanol and sugars in the medium by the adapted yeast. The results of the present study are in harmony with the results found in a study done by [22]; they reported the ethanol concentration of 23.17 ± 0.84 g/L with a yield of 0.44 g/g with the monoculture of *Saccharomyces cerevisiae* with different conditions. The results obtained by [34] were slightly higher than the present study. The ethanol concentration was 24.4 g/L with 0.44 g/g ethanol yield [34].

5. Conclusions

Cotton stalk is a promising feedstock for bioethanol production as it has high cellulose content. The pretreatment with 2% NaOH solution at 121°C for 90 min had the most significant effect on cellulose exposure and delignification. Sugar yield was affected by the variety of factors, including the incubation period, enzyme concentration, pH and substrate concentration during enzymatic saccharification. By using the enzymatically saccharified hydrolysate and monoculture of *Saccharomyces cerevisiae*, the highest ethanol concentration of 22.93 ± 1.74 g/L with 0.36 g/g ethanol yield was achieved after 48 h of incubation at 30°C, pH 5 and 3% inoculum size. However, further research is needed on making the

process more feasible and improving the ethanol yields to make the bio-ethanol from cotton stalk more economically viable, before making it on the commercial-scale.

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