Review Article

Bioethanol Production from Cotton Stalk through Simultaneous Saccharification and Fermentation System

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Abstract

The physicochemical analysis of the cotton stalk was carried out. Pretreatment of the cotton stalk was done with 7.5, 10, and 12.5 % of total solid load with the ortho-phosphoric acid concentration of 5, 7.5, and 10 % at 100˚C, and 121˚C for 1, 2, and 3 h of the time interval. With 12.5 % of total solid load and 7.5 % of ortho-phosphoric acid at 121˚C the sugar release and lignin reduction were highest after 3 h of pretreatment. Cotton millet stalk released about 38.96 g L^{-1} of total sugar while the lignin content was 11 % at the optimized condition. A lab-scale scale Simultaneous Saccharification and Fermentation (SSF) experiment was done two types of yeasts (*S.cerevisiae* and *P.stipitis*). The yield of ethanol was calculated as 24.96 g L^{-1} from cotton stalk after 96 h of fermentation with *S.cerevisiae* from the hydrolysate with added artificial sugar. The process parameters for SSF *viz.*, temperature and agitation speed were optimized. The SSF experiment with *S.cerevisiae* was done at 25, 30 and 35˚C. The three different agitation speed were used such as 75, 100 and 125 rpm for optimization. The highest ethanol concentration of was achieved from cotton stalk (32.65 g L^{-1}) at 30˚C with 100 rpm at 96 h compared to other temperatures and agitation speed. Hence, the optimized temperature and agitation speed selected were 30˚C and 100 rpm respectively for bioethanol production from cotton stalk.

Keywords: Cotton stalk, ethanol, pretreatment, *S.cerevisiae*, SSF

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Introduction

A lignocellulosic feedstock is considered an attractive raw material not only for liquid fuel but also for the production of chemicals and building materials [1]. Bioethanol can be produced by sugar, starch, etc. but it will compete with the limited agricultural and needed for food production and food production. The biomass conversion of lignocellulosic biomass to ethanol is severely restricted by the complexity of the structure and chemistry of biomass, making these materials a challenge to be used as feedstocks to produce cellulosic ethanol [2]. Cellulose and hemicellulose, when hydrolyzed into sugar, can be converted into ethanol by well-established fermentation technology. However, the sugar needed for fermentation is trapped inside the lignocellulose binding structure. Therefore, pre-treatment of biomass is always necessary to remove and/or modify the surrounding matrix of lignin and hemicellulose prior to enzymatic hydrolysis of polysaccharides of biomass, although 100 % of lignin from lignocellulosic biomass cannot be broken down [3]. Pre-treatment refers to a process that converts lignocellulosic biomass from its native state into another form in which cellulose hydrolysis is most effective using cellulase enzymes [4]. Advanced techniques should improve enzyme access by removing most of the lignin and/or hemicellulose, increasing porosity, and reducing cellulose crystallinity in biomass. Many technologies have been developed to improve the hydrolysis of lignocellulosic biomaterials. Lignocellulose can be converted to ethanol by saccharification and simultaneous fermentation (SSF) or separate enzymatic hydrolysis and fermentation (SHF) [5]. SSF is very popular because of its low potential cost. It leads to a higher yield of ethanol compared to SHF by reducing product inhibition important [6, 7]. In order to produce ethanol in the cotton stem, less expensive treatments should be chosen to remove lignin and increase glucose uptake, after the pretreatment process, enzyme and yeast selection are also important [8]. Therefore, this study was selected to perform an appropriate process by improving process parameters to obtain high ethanol production from biomass.

Materials and methods

The study was performed on a cotton stalk and the feedstock was selected based on local availability and low cost. The essential characteristics of biomass were determined using ASTM methods namely, moisture content (ASTM, E-871), ash content (ASTM, E-830), and NREL method for estimating cellulose, hemicellulose, and lignin content.

Pre-treatment of the selected feedstock

The purpose of previous lignocellulosic biomass treatment for bioethanol production is to break down the ligno-hemicellulose-pectin complex, disrupt/loosen the cellulose crystalline structure and increase the biomass porosity. These changes in lignocellulosic substances facilitate enzymatic saccharification (hydrolysis), result in higher levels of fermented sugars and will have a profound effect on the whole process [9]. Pretreatment of biomass can be physical, chemical and biological, etc. In this study, physical and chemical pretreatments were taken to pre-treat the cotton stalk.

Physical pretreatment (size reduction)

Reducing the particle size is intended to reduce the limit of weight transfer and temperature during the pretreatment and fermentation process. Selected substrates are dried at 45˚C to remove moisture and powdered in the milling machine. Powder samples were sieved to determine the size of 500 µ identical particles.

Chemical pretreatment

The different total solid content of feedstock viz., 7.5, 10, 12.5% were taken and transferred to a 250 ml Erlenmeyer flask. Substrates treated with 5, 7.5, and 10% ortho-phosphoric acid. These chemically pretreated substrates have been autoclaved at 100 and 121˚C at intervals of 1, 2, and 3 h respectively. After cooling, samples were taken each time and hydrolysates were collected. Reducing sugar in hydrolysates was measured using DNSA methods. Substrates were neutralized with sodium hydroxide and dried at 45˚C. Hydrolysate obtained from pretreated substrates was then incorporated into bioethanol fermentation.

Estimation of reducing sugars and lignin in pretreated biomass

The amount of sugar reduction was measured by the dinitro salicylic acid (DNSA) method [10]. 0.3 g of dried was taken and 3 ml of H_2SO_4 was added. It was incubated at room temperature for one hour. After 1 hour it was diluted with 84 ml of distilled water to reach 4% H_2SO_4. Autoclaved for one hour at 121˚C after which the sample was cooled and filtered on a pre-measured filter paper. The filter paper was dried and the weight of the dried residue was calculated. Acid Insoluble lignin can be measured using the following formula.

$$\text{Lignin, \%} = \frac{(\text{Final dry weight} - \text{Initial dry weight})}{\text{ODW}} \text{ x } 100$$

Laboratory scale experiment on SSF process

After the pretreatment, the glucose molecules are still imprisoned in long chains of cellulose ad hemicellulose and therefore not readily available for fermentation [11]. SSF is a method for producing bioethanol that utilizes enzymatic bond-breaking parallel with the enzymatic activity and simultaneously, sugar fermented into bioethanol by yeast. The SSF process offers benefits such as improved bioethanol yield by reducing product inhibition, resulting in reduced costs [12].

Measurement of cellulase enzyme activity (FPase assay)

The filter paper assay for commercial cellulase enzyme was performed. One ml of 0.05 M sodium citrate with pH 4.8 was added with 0.5 ml of the enzyme to the test tube. One strip of Whatman No. 1 filter paper (weighing 50 mg) was placed in a test tube. The tube was kept in a water bath at 50˚C for 60 minutes along with the test tube containing a blank. The DNSA method was used to measure the amount of sugar released by cellulase. One unit of enzyme activity is a one mole of reducing sugars in form of glucose-releasing molecule released in a single minute.

Measurement of xylanase enzyme activity

The endoxylanase activity xylanase enzyme was performed. 0.5 ml of 0.05 M sodium acetate trihydrate buffer diluted with 0.01 M of glacial acetic acid to maintain pH 4.8 was added with 0.5 ml of the enzymes to the test tube. 2% xylan (2 g per 100 ml of 0.05 M acetate buffer) was added to the test tube. This test tube and blank test tube were kept in a water bath at 50˚C for 60 minutes. At least two dilutions should be performed for each enzyme sample. One dilution should release a small amount and another slightly less than 0.5 mg of xylose in reaction conditions. The DNSA

method was employed to measure the amount of sugar released by xylanase. One unit of enzyme activity is one mole of reducing sugars in the form of xylose released in single minute

Selection of yeast for bioethanol production

The ethanogenic yeast *Saccharomyces cerevisiae* NCIM 3204 and *Pichia stipitis* NCIM 3298 has been selected for simultaneous saccharification and fermentation process. *Saccharomyces cerevisiae* is a yeast that is widely used in the production of bioethanol [13]. It converts glucose into ethanol. However, it cannot convert xylose into ethanol. For fermentation of both glucose and xylose sugar, various xylose fermentable bacteria include *Candida* shehatea, *Candida guilliermondi*, *Pichia stipitis*, *Zymomonas mobilis*, *Pachysolen tannophilus*, *Kluyveromyces marxianus*, *Mucor indicus* and *Rhizopus oryzae* have been used. In these yeast, *Pichia stipitis* is one of the most promising yeasts for fermentation of xylose into ethanol due to its low productivity [14]. Thus, two types of yeast viz., *Saccharomyces cerevisiae* and *Pichia stipitis* were selected to enhance the production of ethanol from hydrolysate.

Simultanous saccharification and fermentation process

The lab-scale SSF test was started with a working volume of 100 ml in a 250 ml of the flask. SSF process was carried out with the hydrolysate obtained after pretreatment with and without the addition of glucose (60 g L^{-1}) and yeast extract (10 %) as additional substrates. Other nutrient media was incorporated in the flask with the yeast extract (10 g L^{-1}), urea (6.4 g L^{-1}), KH_2PO_4 (2 g L^{-1}) and $MgSO_4.7H_2O$ (1 g L^{-1}) [11]. The flasks were autoclaved and added with enzyme (cellulase and xylanase) and inoculated with yeast *Saccharomyces cerevisiae* and *Pichia stipitis*. The dosages of enzymes and yeast were 40 FPU g^{-1} of cellulase, 25 U ml^{-1} of xylanase and 10 % (v/v) respectively. The inoculated flasks were placed under an anaerobic condition for 96 h. The ethanol was estimated by gas chromatography method and the residual reducing sugar was estimated by following DNSA method.

Results and Discussions

Physiochemical properties of raw materials

The physiochemical properties of the cotton stalk were determined as per the standard procedure and the water extractives, ethanol extractives, cellulose, hemicelluloses, and lignin were calculated and given in **Table 1**. To produce ethanol, biomass with high cellulose and hemicellulose content will produce high yields. (l/t). The effect of cellulose content of switchgrass and reported that ethanol yield 280 L t^{-1} compared to wood (205 L t^{-1}) due to an increased proportion of lignin in the wood [15].

Table 1 Physicochemical properties of cotton stalk

Cotton stalk	Moisture Content, %
Moisture Content, %	3.89 $\pm$ 0.34
Ash Content, %	5.31 $\pm$ 0.47
Bulk density, kg m^{-3}	119.62 $\pm$ 10.05
Water Extractives, %	4.88 $\pm$ 0.4
Ethanol Extractives, %	5.02 $\pm$ 0.4
Hemi cellulose, %	29.92 $\pm$ 2.52
Cellulose, %	38.33 $\pm$ 3.21
Lignin, %	22.11 $\pm$ 1.86

Pretreatment for selected feedstock

The different total solid concentration of the selected raw biomass was taken i.e., 7.5, 10 and 12.5 %. The raw biomass was pretreated with 5, 7.5 and 10 % ortho-phosphoric acid (H_3PO_4) at 100 and 121˚C at 1, 2 and 3 h time interval. The acid hydrolysis is of great interest because it is easy, cheap, and a faster process using dilute acids. Sulfuric acid (H_2SO_4) is a usual choice for acid hydrolysis, which causes problems such as extreme corrosion and chemical formation that prevent the fermentation process [16]. H_3PO_4 have lesser effect compared to sulfuric acid. H_3PO_4 is less aggressive than normal the acids used in biomass hydrolysis are more expensive than H_2SO_4. However, it is reported that the use of H_3PO_4 provides coproducts that can be used as plant fertilizer and other benefits such as showing a lower level of toxic production and decay. In addition, the use of H_3PO_4 at moderate temperatures (approximately 50 °C) make biomass amorphous by disrupting hydrogen bonds making it easier for enzymatic digestion by increasing the surface area [17]. The reducing sugar and lignin were estimated with different. From those

results, highest reducing sugar and lowest lignin content was observed at 12.5 % total solid content, 7.5 % acid concentration with 121˚C at 3 h. Hence, optimal pretreatment condition was fixed as 7.5 % ortho-phosphoric acid with 3 h at 121˚C. The acid pretreatment on lignocellulosic biomass was capable of solubilizing the lignin content and release some cellulose and hemicelluloses. During initial stages of pretreatment, 10 % acid pretreatment released higher quantities of total sugars than lower acid levels. However, over the time of treatment an increasing sugar release was evident from experimental results. Hence, highest amount of sugar release (32.66 g L^{-1}) with higher lignin loss was observed at 3 h of treatment with 7.5 % of acid concentration.

In cotton stalk, higher amount of sugar was obtained with 12.5 % total solids, 7.5 % acid (32.66 g L^{-1}). The lignin in cotton stalk after pretreatment was found to be the lowest (11 %) with 12.5 % of total solid and 7.5 % of acid concentration. The effect of pretreatment on lignocellulosic biomass improved digestion, modification of lignin structure and a small percentage of hemicellulose and provided improved access to cellulose to hydrolytic enzymes [18]. The effects of sugar release and lignin reduction on acid pretreatment across all substrates are presented in **Table 2**.

Table 2 Total sugar released and lignin content of cotton stalk with different of solid loading

Treatments	Sugar released, g L^{-1}		Lignin content, %		Treatments	Sugar released, g L^{-1}		Lignin content, %	
	121˚C	100˚C	121˚C	100˚C		121˚C	100˚C	121˚C	100˚C
7.5 % of total solid loading									
Water + 1 h	8.39	5.40	17.00	18.34	7.5 % + 2 h	23.38	19.04	14.00	16.00
5 % + 1 h	20.35	10.03	15.00	16.67	10 % + 2 h	24.62	21.42	13.34	15.67
7.5 % + 1 h	20.92	14.99	14.67	16.34	Water + 3 h	10.50	7.43	16.00	17.67
10 % + 1 h	21.26	18.02	14.00	16.00	5 % + 3 h	24.47	17.16	14.00	16.00
Water + 2 h	9.44	5.99	16.67	18.00	7.5 % + 3 h	25.60	20.42	13.67	15.34
5 % + 2 h	22.56	12.71	14.34	16.33	10 % + 3 h	26.70	21.77	12.67	15.33
10 % of total solid loading									
Water + 1 h	9.44	6.47	16.34	18.00	7.5 % + 2 h	28.72	22.86	13.34	15.34
5 % + 1 h	23.88	15.87	14.00	16.00	10 % + 2 h	28.93	23.44	12.67	14.67
7.5 % + 1 h	25.70	19.19	14.00	15.67	Water + 3 h	10.76	8.62	15.67	17.00
10 % + 1 h	28.68	20.33	13.34	15.34	5 % + 3 h	28.46	22.25	14.00	15.34
Water + 2 h	10.45	7.52	16.00	17.67	7.5 % + 3 h	30.27	24.65	12.67	15.00
5 % + 2 h	26.42	19.20	13.34	15.67	10 % + 3 h	30.87	26.68	12.00	14.33
12.5 % of total solid loading									
Water + 1 h	10.26	7.61	16.00	17.34	7.5 % + 2 h	30.89	24.44	11.34	14.67
5 % + 1 h	30.28	17.89	13.00	15.34	10 % + 2 h	31.05	25.62	11.67	14.00
7.5 % + 1 h	30.46	20.41	12.67	15.00	Water + 3 h	12.36	10.49	15.00	16.34
10 % + 1 h	30.77	21.36	12.34	14.67	5 % + 3 h	30.99	23.44	11.67	14.34
Water + 2 h	10.70	9.55	15.67	17.00	7.5 % + 3 h	32.81	25.61	11.00	14.34
5 % + 2 h	30.75	20.50	12.00	14.67	10 % + 3 h	32.66	27.57	11.33	13.67

The sugar concentration at 5, 7.5 and 10 % of ortho-phosphoric acid with 7.5, 10 and 12.5 % of total solids for 1, 2 and 3 h at 100˚C and 121˚C. Maximum sugar concentration of the selected feedstock varied from 32.81 to 5.40 g L^{-1}, while the lignin content varied from 11 to 17.67 %. Before acid pretreatment the lignin content of 22.11 + 1.86%.

Laboratory scale simultaneous saccharification and fermentation process

The lab scale SSF test was performed in three different conditions; pretreated hydrolysate without addition of artificial sugar, hydrolysate with addition of artificial sugar (sugar concentration up to 60 g L^{-1}), and pretreated hydrolysate with 10 % of yeast extractives (w/v). 100 ml of media was taken into the 250 ml of conical flask. The flasks were sterilized in autoclave and the essential amount of nutrients were added. After autoclaving, 40 FPU g^{-1} of cellulase enzyme, and 25 U ml^{-1} of xylanase were added to all the flasks. Two types of yeasts (10 % of *S.cerevisiae* and 10 % of *P.stipitis*) were used for the optimization of the fermentation. The samples were taken at 24, 48, 72, and

96 h time interval. Ethanol was recovered by the distillation method of fermented slurry. The effect of different types of yeasts, hydrolysates, and hydrolysates with different treatments on ethanol production and sugar consumption from cotton stalks were estimated at different time intervals and furnished in **Table 3**.

Table 3 Effect of yeast with different treatments and time on Ethanol yield and Sugar consumption

Yeast	Ethanol yield (g L^{-1})				Sugar consumption (g L^{-1})			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Hydrolysate alone								
S.cerevisiae	0.98	2.67	5.48	13.88	9.02	18.19	28.32	30.21
P.stipitis	0.75	1.54	4.67	11.96	5.4	10.01	16.45	22.67
Hydrolysate + glucose (60 g/l)								
S.cerevisiae	5.95	8.49	13.16	24.96	16.74	33.99	44.2	55.54
P.stipitis	3.03	7.06	11.47	15.43	9.48	19.05	28.77	41.91
Hydrolysate alone + yeast extract (10 %)								
S.cerevisiae	1.74	3.55	7.57	14.93	10.96	19.78	29.9	31.43
P.stipitis	1.23	2.14	5.26	12.39	6.55	11.23	17.62	23.89

The yeast *S.cerevisiae* was on par with *P.stipitis* and significantly different from *P.stipitis* for fermentation. The hydrolysate with added artificial glucose was the best performing treatment followed by the hydrolysate with 10 % of yeast extract and the poorest performing treatment was hydrolysate alone. Ethanol production increased significantly with an increase in time from 24 h to 96 h, while 96 h was the best time to produce ethanol. The interactions were found to be significant. *Saccharomyces cerevisiae* with hydrolysate and artificial glucose gave the highest amount of ethanol (24.96 g L^{-1}) than the other combinations. Hydrolysate with artificial sugar produced the highest ethanol at 96 h (20.19 g L^{-1}) compared to the other time intervals.

The highest amount of ethanol (24.96 g L^{-1}) was achieved with *S.cerevisiae*, hydrolysate with added artificial sugar at 96 h while the poorest ethanol production was observed at 24 h with other treatments (**Table 4**). The effect of sugar consumption from cotton stalk by using different yeasts, with different treatments of hydrolysate at different intervals of time. *S.cerevisiae* was significantly different than *P.stipitis* for the consumption of sugar. The hydrolysate with artificial sugar (total sugar concentrate on 60 g L^{-1}) was proved to be the best treatment than the hydrolysate alone and the hydrolysate with 10 % of yeast extract. This may be due to the presence of more sugar concentration than the hydrolysate without artificial sugar and hydrolysate with yeast extract. The best time for the fermentation was proved to be 96 h. The sugar consumption was increasing significantly with the increase of time in all the treatments. The *S.cerevisiae* with hydrolysate and artificial sugar consumed more sugar (37.61 g L^{-1}) than *P.stipitis* with the same treatment (24.80 g L^{-1}). The hydrolysate with artificial sugar was the best performing treatment at 96 h than other time intervals. The highest amount of sugar was consumed by *S.cerevisiae*; with hydrolysate and artificial sugar at 96 h (55.54 g L^{-1}) than other combinations of treatments.

Table 4 Ethanol yield from cotton stalk

Time, h	*Saccharomyces cerevisiae*			*Pichia stipitis*		
	Hydrolysate alone	**Hydrolysate with artificial glucose, 60 g L^{-1}**	**Hydrolysate with 10 % of yeast extract (w/v)**	**Hydrolysate alone**	**Hydrolysate with artificial glucose, 60 g L^{-1}**	**Hydrolysate with 10 % of yeast extract (w/v)**
24	0.98	5.95	1.74	0.75	3.03	1.23
48	2.67	8.49	3.55	1.54	7.06	2.14
72	5.48	13.16	7.57	4.67	11.47	5.26
96	13.88	24.96	14.93	11.96	15.43	12.39

The ethanol production with the sugar consumption from the cotton stalk with different hydrolysate treatments by using *S.cerevisiae* and *P.stipitis* were compared. Ethanol production increases with increased sugar consumption (**Figures 1** and **2**). Ethanol production and sugar consumption are almost identical to hydrolyzate alone and hydrolyzate with 10% yeast extract, whereas ethanol production and consumption of sugar were significantly higher than synthetic sugar hydrolyzate. Ethanol production and sugar consumption were higher using *S.cerevisiae* compared to *P.stipitis. The* hydrolysate alone the ethanol production was 5.75 g L^{-1} while the sugar consumption was 21.43 g L^{-1} by using *S.cerevisiae.* With the hydrolysate and artificial sugar, the ethanol production was 13.14 g L^{-1} and the sugar

consumption was 37.61 g L^{-1}. With the hydrolysate and 10 % of yeast extract, the ethanol production was 6.94 g L^{-1} while the sugar consumption was 23.01 g L^{-1} (Figure 1). The hydrolysate alone the ethanol production was 4.73 g L^{-1} while the sugar consumption was 13.63 g L^{-1} by using *P.stipitis.* With the hydrolysate and artificial sugar, the ethanol production was 9.24 g L^{-1} and the sugar consumption was 24.80 g L^{-1}. With the hydrolysate and 10 % of yeast extract, the ethanol production was 5.25 g L^{-1} while the sugar consumption was 14.82 g L^{-1} (Figure 2).

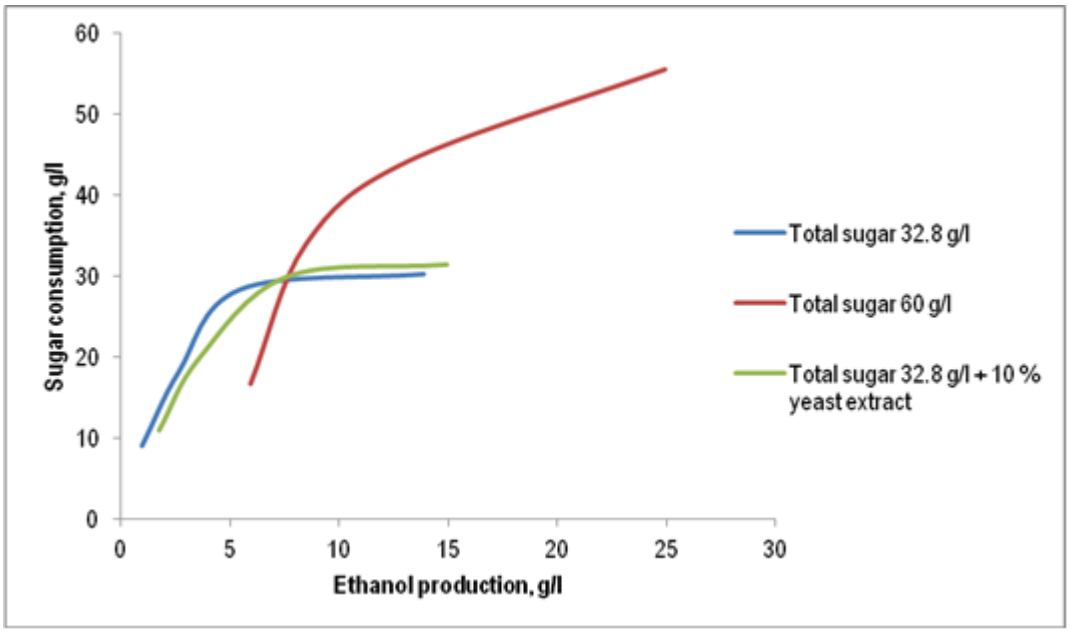

Figure 1 Ethanol production with sugar consumption with *S.cerevisiae*

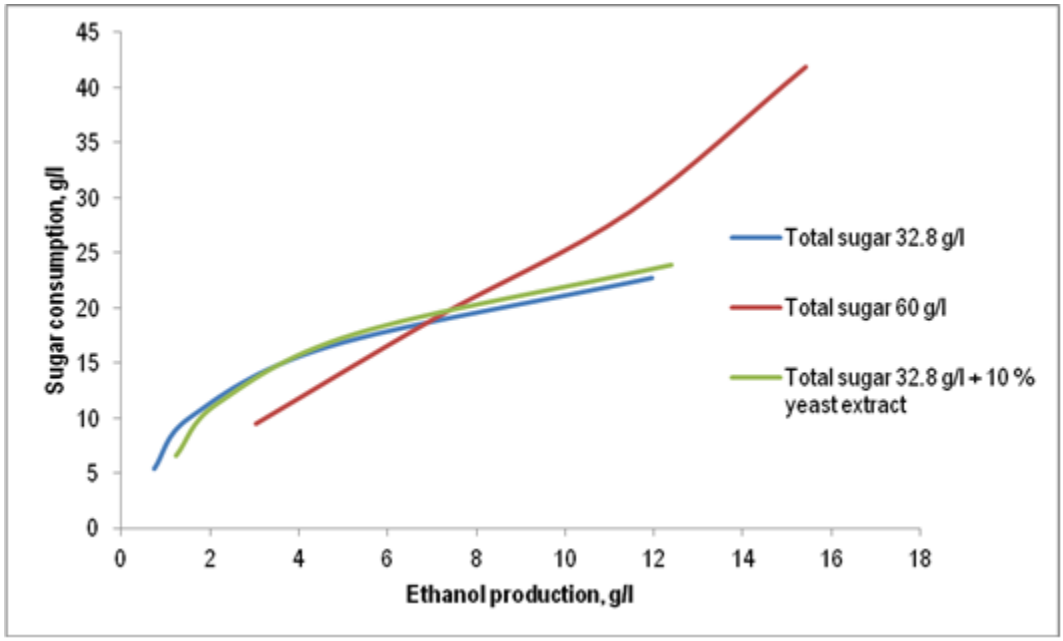

Figure 2 Ethanol production with sugar consumption with *P.stipitis*

Banana waste was used as a substrate, in which enzymatic hydrolysis was performed optimally to obtain approximately 100 g L^{-1} of glucose, using the enzyme glucan to 87 % ethanol yield [19]. Maize corn was investigated for hydrolysis and after 96 hours of hydrolysis, hydrolysate resulted in 62 g L^{-1} of total sugar, 51 g L^{-1} of sugar, glucose, 10 g L^{-1} of xylose, and 0.9 g L^{-1} of arabinose [20]. Maize stalk was investigated for saccharification and the fermentation process led to led to 17 g L^{-1} of ethanol, producing a 31% yield [21].

Process parameter optimization for ethanol production

The factors affecting bioethanol production are temperature and agitation speed. Hence, these parameters were optimized in the optimized treatments with *S.cerevisiae.*

Effect of temperature and agitation speed on sugar reduction and ethanol production

The sugar reduction of acid pretreated hydrolysate using the *S.cerevisiae* NCIM 3204 and commercial cellulase and xylanase enzymes at the temperatures of 25, 30, and 35˚C and mechanical agitator speeds of 75, 100, and 125 rpm during SSF. The sugar concentration of the broth was in the range of 37.29 to 40.56 g L^{-1} after 24 h which was reduced to 0.75 to 0.94 g L^{-1} at the end of 96 h fermentation (Table 5). The process temperature affected the reduction of sugar concentration significantly. The concentration reduced to 1.71 g L^{-1} from 40.56 g L^{-1} for cotton stalk when the temperature was 25˚C after 96 h which further reduced to 0.75 g L^{-1} respectively when the temperature was raised from 25 to 30˚C. The ethanol production was improved as the temperature raised from 25 to 30˚C while with a further rise in temperature from 30 to 35˚C the ethanol production was reduced. The optimal temperature for Enzymatic hydrolysis is 45 to 50 ° C, while fermentation is most effective at 28 to 35 °C [22]. The ethanol was recovered from the fermentation broth by a simple distillation method. The ethanol production was estimated at different temperatures with different speeds (Table 5). The ethanol production from the cotton stalk was maximum at 30˚C when incubated with *S.cerevisiae* for 96 h (32.65 g L^{-1}). The agitation speed affected ethanol production which was the most important factor for the growth of yeast cells. The agitator speed of 100 rpm was found to be optimum speed to produce high ethanol.

Table 5 Temperature and agitation speed effect on sugar reduction during fermentation

Temperature (˚C)	Fermentation time, h	Sugar reduction, g L^{-1}			Ethanol Production, g L^{-1}		
		75 rpm	100 rpm	125 rpm	75 rpm	100 rpm	125 rpm
25	24	40.56	39.39	39.53	6.02	9.69	7.29
	48	25.50	23.91	24.54	8.56	11.28	8.96
	72	11.88	9.48	10.50	13.55	16.03	14.77
	96	1.71	0.94	1.48	27.85	29.24	28.51
30	24	38.54	37.29	37.77	8.09	11.01	8.85
	48	24.04	22.57	23.64	10.67	13.61	11.42
	72	9.38	5.64	8.26	15.92	18.94	16.56
	96	1.16	0.75	0.95	29.37	32.65	30.71
35	24	39.66	38.28	38.50	6.97	10.52	7.58
	48	25.37	22.94	23.80	9.56	12.42	10.49
	72	10.12	6.98	9.39	14.54	17.80	15.67
	96	1.63	0.83	1.22	28.76	30.86	29.45

Glycerol was fermented using *Enterobacter aerogenes* and produced 25.4 g / l ethanol within 48 hours at high temperatures of 38 °C, below low oxygen levels [23]. It also revealed that the optimum temperature should be between 20 to 45 °C. This means that rising temperatures, in some cases, can increase ethanol production at a slower rate [24, 25]. A study with sugar maple revealed that with high agitation, high ethanol concentration reached 68.5 h of fermentation, and with low levels of fermentation, ethanol concentration appeared to increase after 139 hours of fermentation [26]. When more oxygen is supplied to cells, the ethanol yield will be reduced as more sugar is used to produce biomass; but when adequate oxygen is provided, an imbalance in fermentation may occur leading to the production of extra xylitol [27].

Conclusion

The experiment on the SSF process for bioethanol production was done from the cotton stalk. The pretreatment of the selected biomass was done with 5, 7.5, and 10 % of orthophosphoric acid. The total solid content of 7.5, 10, and 12.5 % were taken for acid pretreatment at 100˚C and 121˚C for 1, 2, and 3 h. The optimized process parameters were selected based on reducing sugars and lignin content in the pretreated biomass. The optimized condition for acid pretreatment was evaluated as 12.5 % of total solids, and 7.5 % of acid at 121˚C for 3 h. Cotton stalk released about 32.66 g L^{-1} of total sugar while the lignin content was 11 % at the optimized condition. The yield of ethanol was calculated as 32.65 g L^{-1} from cotton stalk after 96 h of fermentation. Ethanol concentration was increased over a period of time from 24 hours to 96 hours. The processes for ethanol production were optimized like temperature and agitation speed. With the increase in temperature, the ethanol concentration was increased and then reduced. Also, with an increase in speed, the ethanol concentration was increased and then decreased. The highest ethanol concentration was achieved from the cotton stalk (32.65 g L^{-1}) at 30˚C with 100 rpm at 96 h compared to other temperatures and agitation speeds. The sugar consumption was increasing with the increase in temperature and

agitation speed and then it reduced. The sugar reduction was highest at the temperature of 30˚C and 100 rpm from the cotton stalk (0.75 g L^{-1}). Hence, the optimized process parameters were 30˚C with 100 rpm.

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