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Bioethanol Production from Cotton Waste using Cellulase Extracted from *Fusarium* species

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Abstract: Cotton waste from textile mills were collected, processed and was subjected to acid and alkali pretreatment to expose the sugars for further enzymatic hydrolysis by the cellulase enzyme produced from *Fusarium* species.It was used as source for the production of bio-ethanol by microbial saccharification and fermentation process. The results of pretreatment showed that the acid pre-treated substrate enhanced enzyme action and released more amount of sugar compared to the alkali pre-treated substrates. The amount of sugar released was found to increase with the increasing concentration of acid (90 mg/ml) or alkali (63 mg/ml). The sugars were then fermented with *Saccharomyces cerevisiae* using simultaneous saccharification and fermentation. The amount of alcohol produced in batch fermentation was 11.8 mg/ml. Thus, the results of the present work clearly revealed that the cellulosic cotton wastes could be converted into bioethanol with enzymatic hydrolysis followed by fermentation.
Keywords: Bioethanol; Microbial saccharification; Fermentation; Cellulosic cotton waste; Enzymatic hydrolysis.

Introduction

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials[1]. The complete enzymatic hydrolysis of cellulosic materials needs different types of cellulase; endoglucanase (1,4-β-d-glucan- 4-glucanohydrolase; EC3.2.1.4),exocellobiohydrolase (1,4-β-d-glucanglucohydrolase; EC3.2.1.74) and β-glucosidase (β-d-glucosideglucohydrolase; EC 3.2.1.21). The endoglucanase randomly hydrolyzes the β-1,4 bonds in the cellulose molecules, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and, bacteria are the main natural agents of cellulose degradation [2]. The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa [3,4]. However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular. Cotton is typically composed of 88–96% cellulose, the remainder being protein, pectin materials and wax. Cotton must be scoured and usually bleached before use, whereby its cellulose content is enriched to about 99% [5]. It is therefore possible to hydrolyze the cotton by enzyme or acids to glucose and then ferment it to ethanol. Cellulose (40-60% of the dry biomass) is a linear polymer of glucose, the orientation of the linkages and additional hydrogen bonding make the polymer rigid and difficult to break. In hydrolysis the polysaccharide is broken down to free sugar molecules by the addition of water. This is also called

saccharification. The product, glucose is a six-carbon sugar or hexose. It provides the major source for hexose in woody biomass. Cellulose is believed to have a highly crystallized structure due to the existence of hydrogen bonds. In contrast to its amorphous region, the crystalline region of cellulose makes it hard to hydrolyze. Some scientist also explored ethanol production from cotton gin waste. Microorganisms are required to produce ethanol from lignocellulosic hydrolysates with a high yield from all sugars present using an economically feasible process. Different fermentation organisms among bacteria, yeasts, and fungi (natural as well as recombinant) have been reviewed with emphasis on their performance in lignocellulosic hydrolysates. The simultaneous saccharification and fermentation (SSF) process is a favored option for conversion of the lignocellulosic ecofriendly biomass into ethanol using different fungus because it provides enhanced rates, yields and concentrations of ethanol. There are mainly two processes involved in the conversion (i) hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars (hexoses as well as pentoses) and (ii) fermentation of the reducing sugars to ethanol. Ethanol can be used as a gasoline fuel additive and transportation fuel. This in turn helps to alleviate global warming and environmental pollution. The decreasing reserves and increasing value of petrochemicals have renewed the interest in the production of bioethanol and its use as fuel and chemical feedstock. Bio-ethanol used as a source of energy is a more welcoming alternative fuel [6-23].

Materials and Methods

Collection of *Fusarium* species

The fungal culture was obtained from the Department of Biotechnology, K.S.R.C.T. It was subcultured in PDA media.

Identification of culture

The culture was identified using Lacto phenol cotton blue.

Production of Cellulase Enzyme

Media composition (g/l)

KH_2PO4	- 2.0g
$(NH_4)_2PO_4$	- 1.4g
Urea	- 0.3g
$Cacl_2.2H_2O$	- 0.3g
$MgSo_4.7H_2O$	- 0.3g
Peptone	- 1.0g
Tween 80	- 0.2% (v/v)
$FeSo_4.7H_2O$	- 5.0mg
$MnSo_4.2H_2O$	- 1.6mg
$ZnSo_4.7H_2O$	- 1.4mg
$CoCl_2.6H_2O$	- 2mg
Carboxy methyl cellulose	- 10.0g
Distilled water	- 1000ml
pH	- 5.5

The flask containing the production media was inoculated with a 3% (v/v) of the fungal spore suspension.

Extraction of cellulase

Cell suspension was centrifuged at 3000 rev/min for 20 mins. Supernatant was used as a crude extract and it was used for further analysis.

Estimation of cellulase (DNS method)

An aqueous solution of glucose was prepared at a concentration of 0.5mg/ml. To a series of 10 test tubes, a glucose stock solution corresponding to the required sugar concentration (0.1-1ml) was added. The volume was made up to 3ml using double distilled water (use distilled water as a blank). 2ml of DNS was

added and heated in boiling water bath at 80°c for 15 minutes and cooled. The absorbance was readed at 580nm. The values were plotted on a graph. 0.05ml of supernatant was added to a test tube and the volume was made up to 3ml using distilled water. To this 2ml of DNS was added and heated in boiling water bath at 80°c for 15 minutes and cooled. The absorbance was readed at 580nm. The concentration of reducing sugar/ml is determined using the standard graph.

Collection of Sample

The cotton sample was collected from the nearby area of Devanankurichi (Tiruchengode) for the production of bioethanol.

Pretreatment of cotton waste

Acid pretreatment

About 200 ml of dilute sulphuric acid was prepared with a concentration range of 0%, 0.5%, 1.0% up to 5.0% in separate 500ml Erlenmeyer flasks. The flasks were added with 3g of processed cotton waste and autoclaved at 121°C for 30 minutes. The flasks containing the pre-treated cotton waste were then neutralized by washing with distilled water. The acid pre-treated samples were dried separately for further analysis.

Alkaline pretreatment

About 200 ml of dilute sodium hydroxide was prepared with a concentration range of 0%, 0.5%, 1.0% up to 5.0% in separate 500 ml Erlenmeyer flasks. The flasks were added with 3g of processed cotton waste and autoclaved at 121°C for 30 minutes. The flasks containing the pre-treated cotton waste were then neutralized by washing with distilled water. The alkaline pre-treated samples were dried separately for further analysis.

Compositional analysis of the cotton waste

The compositional analyses of the cotton wastes before and after pretreatment namely, moisture content, acid insoluble residues, ash content were performed using standard methods. The moisture content of the processed cotton waste was determined by the solid determination method of ASTM E 1754-95 (ASTM, 1995). The acid insoluble residue and ash fractions were also determined following the ASTM E1721-95 procedure (ASTM, 1995).

Moisture content

Moisture content of the raw material (untreated cotton gin waste) was determined by the solids determination methods of ASTM-95 (ASTM, 1995). Moisture in triplicate samples was driven off at 105^{o}C in the laboratory oven (Thelco lab oven, precision scientific Chicago, Illinois).The dried samples were cooled in a dessicator and weighted. The process was repeated until a constantvmass was obtained. The moisture content was then calculated.

$$\text{Moisture} = \frac{B-C}{B-A} \times 100\%$$

Where,

A = weight of empty dish
B =weight of dish with cotton
C = weight of dish with dried powder

Acid insoluble residue and ash analysis

Sulfuric acid (H_2SO_4) at a concentration of 72% was used to hydrolyze 0.3g of the substrate for 2 hours at 30^{o}C in a water bath. The hydrolyzed substrate was filtered using a medium porosity glass filtering crucible. The filtrate was collected and used as the stock sample for carbohydrate analysis. The remaining residue was dried in the lab oven at 105^{o}C overnight and weighted. The dried residue was then ashed in a muffle furnace at 575^{o}C for 3 hours and weighted.

The following equations were used to calculate % acid insoluble residue and %ash.

$$\text{Acid Insol} = \frac{\text{acidinsol- ash}}{\text{Raw material}} \times 100\%$$

Where,

Acid in sol = % acid insoluble residue on an oven- dried basis (%)
Acid in sol = oven dried weight of acid insoluble residue (g)
Ash = weight of residue following ashing at 575^{o}C (g) and,
Raw material = initial oven dried weight of substrate (g).

$$\text{Ash} = \frac{\text{Ash}}{\text{Raw material}} \times 100\%$$

Where,

Ash = % ash on an oven- dried basis (%)
ash = weight of residue following ashing at 575oC (g) and,
Raw material = initial oven dried weight of substrate (g).

Cultivation of Yeast

Dried-form of industrial *Saccharomyces cerevisiae* yeast was used in this research.For inoculum, 100ml of distilled water was heated to 40°c in a shake flask. After that, 0.5% (w/v) of *S. cerevisiae* yeast was added into the warmed water to activate the yeast. The mixture was left for 15 min at 150 rpm.The *Saccharomyces cerevisiae* was enriched in Saboraud's Dextrose broth.

SDB Media

Dextrose - 4g
Mycological peptone - 1g
Distilled water - 100ml
pH - 5.6±0.2 at 25^{o}C

To this media added 2.5ml of crude enzyme extract and 3g of untreated, acid pretreated and alkaline pretreated cotton sample and incubated at room temperature for 5 days.

Simultaneous saccharification and fermentation (SSF)

The SSF experiments were carried out in 250ml conical flasks added with 3% (w/v) solid substrates containing 50mM sodium citrate buffer (pH 4.8). The media was sterilized an autoclave at 121^{o}C. After that 2.0 ml of the crude enzyme were added for enzymatic hydrolysis at 45^{o}C for 5days. After 5 days of saccharification, 10% of *S. cerevesiae* culture were added to all the flasks and incubated at 35^{o}C for six days. During the fermentation process every 24 hours samples were taken for the estimation of bioethanol.

Extraction of ethanol by distillation method

Distillation apparatus was set up. The fermented mash was centrifuged at 3000 rpm for 10 mins and then the supernatant was transferred into the distillation apparatus. The first 10% of the liquid was distilled into the graduated cylinder (It was not distilled to dryness because ethanol evaporate first with the first 5ml that are distilled). The distillate was collected and used for further analysis.

Conformation of ethanol

Iodoform test

10 drops of distillate and 25 drops of iodine along with 10 drops of NaOH was added in the test tube. After few minutes cloudy formation in the test tube gives the conformation of the presence of ethanol and it also gives yellow precipitate and antiseptic smell.

Estimation of ethanol

Preparation of alcohol standard graph

Prepare different concentration of alcohol 1-10% in double distilled water. Starting with 1% of the alcoholic solution added 24 ml of distilled water in the conical flask. Pour 25ml of sample to distillation flask. Distill the contents. Collected 10-15 ml of distillate in beaker containing 25 ml of 3.4% chromic acid. Make up the volume to 50 ml using double distilled water and mixed thoroughly. Heat the contents up to 80°c for 15 minutes. The absorbance was read at 580nm. Plotted the values and prepared the standard graph. Analyze of sample for alcohol content . Add 1 ml of sample to 24 ml of distilled water. Pour 25 ml of sample to distillation flask. Distill the contents. Collect 10-15 ml of distillate in beaker containing 25 ml of 3.4% chromic acid. Make up the volume to 50 ml using double distilled water and mixed thoroughly. Heat the contents up to 80°c for 15 minutes. The absorbance was read at 580nm and plotted the values.

Results and Discussion

Identification of Culture

The organism was identified using Lacto Phenol Cotton blue staining. The result of staining was given in (Figure- 1) and it was identified as *Fusarium species*.

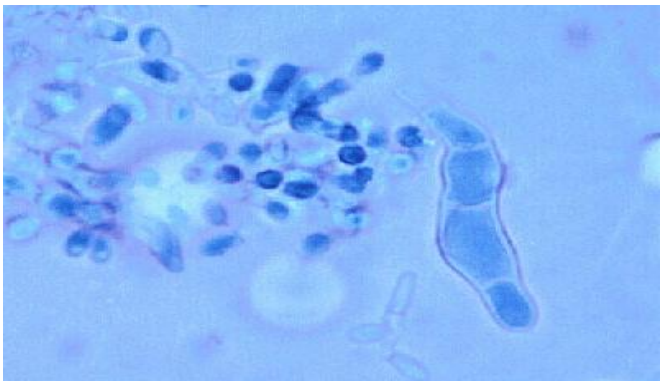


Figure 1: Microscopic view of *Fusarium*species

Production of Cellulase Enzyme

The isolate was checked for their ability to degrade carboxy methyl cellulose by the production of an enzyme cellulase. Isolate was capable of degrading CMC given in (Figure- 2).

Figure 2: cellulase enzyme production

Estimation of Cellulase (Dns Method)

Initial content of free reducing sugar in cellulase production broth was found to be 0.63mg/ml. There was a gradual increase in reducing sugar content with increased time of incubation(Table- 1),which correlates with the findings of Baig using banana agro waste (2004) as the substrate.

Table 1: Reducing sugar content in liquid state fermentation media inoculated with *Fusarium* speciessupplemented with CMC.

Culture period (days)	Reducing sugar (mg/ml)
2	0.63
4	0.90
6	1.15
8	1.23
10	1.08

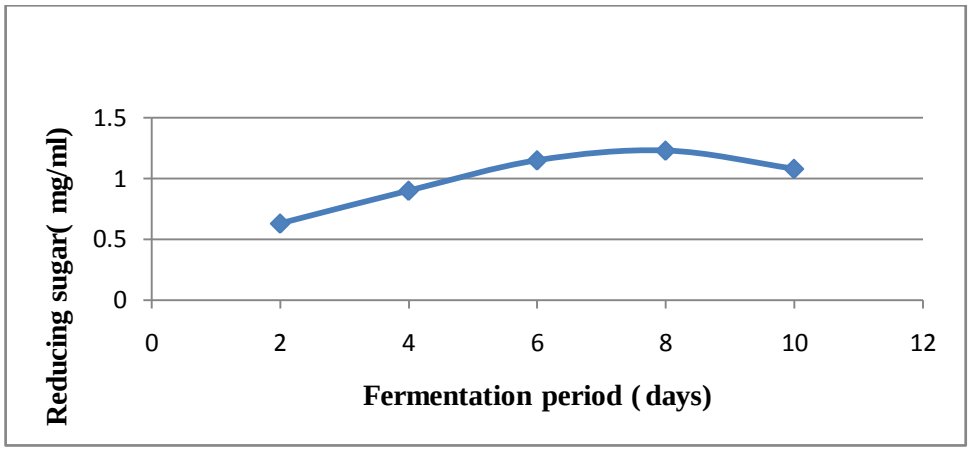


Figure 3: Reducing sugar content

Compositional Analysis of Cotton Waste

The cotton waste was collected, processed mechanically and the chemical composition of the cotton waste was determined according to the standard methods and the results were compared to the contents released after acid and alkali pretreatment (Table- 2).

Table 2: Compositional analysis of untreated and pretreated cotton waste

Composi-tion analysis	Before pretrea t-ment (%)	After pretreatment (%)									
		Acid treatment					Alkaline treatment				
		1	2	3	4	5	1	2	3	4	5
Moisture content	12.63	11.43	11.69	12.08	12.20	11.94	11.18	11.27	11.35	11.47	11.18
Acid insoluble residue	17.84	18.21	20.16	22.58	21.84	20.63	17.64	18.39	19.85	19.48	18.53
Ach content	7.75	6.32	7.54	8.86	7.36	6.79	6.17	6.74	7.26	6.94	6.41

The result showed that acid pre-treated cotton waste was found to possess relatively higher percentage of total sugars (23.98%) compared to alkali pre-treated cotton wastes (19.75%). Similarly, the percentage concentration of ash and acid insoluble residues was also found to be relatively higher for acid pre-treated 8.86 and 22.58(%) for alkali pre-treated cotton wastes 7.26 and 19.85 (%) respectively.

Effect of Pretreatment on the Cotton Waste

Acid and alkali pretreatment had great influence on the sugar release through enzymatic hydrolysis of

the cotton waste. (Figure- 4) (Table- 3) shows the amount of sugar released upon enzymatic hydrolysis of the acid / alkali pre-treated cotton waste. It was understood that the amount of sugar released upon enzymatic hydrolysis was greater for acid pre-treated cotton waste compared with alkali pre-treated cotton waste. As the percentage of acid or alkali used for pretreatment increased, the amount of sugar released also increased which correlates with the findings[20].

Table 3: Efficiency of pretreatment on enzymatic hydrolysis of cotton waste

Acid/ Alkaline treatment (%)	Sugar released upon enzymatic hydrolysis (mg/ml)	
	Acid pretreatment	Alkaline pretreatment
1	60	20
2	72	30
3	79	42
4	83	55
5	90	63

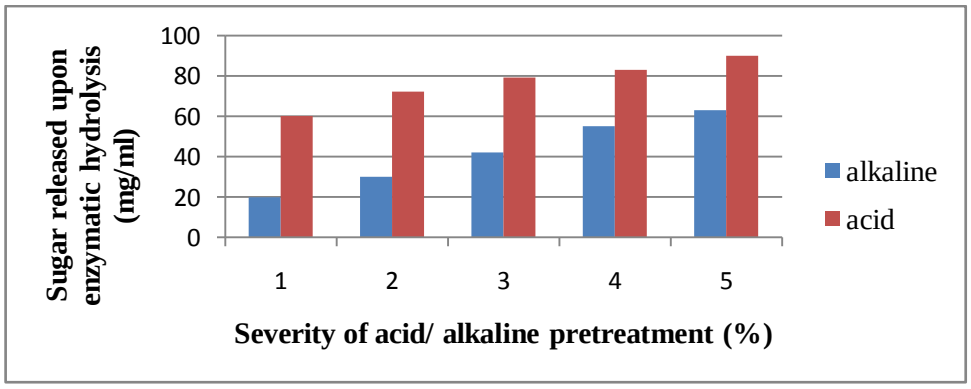
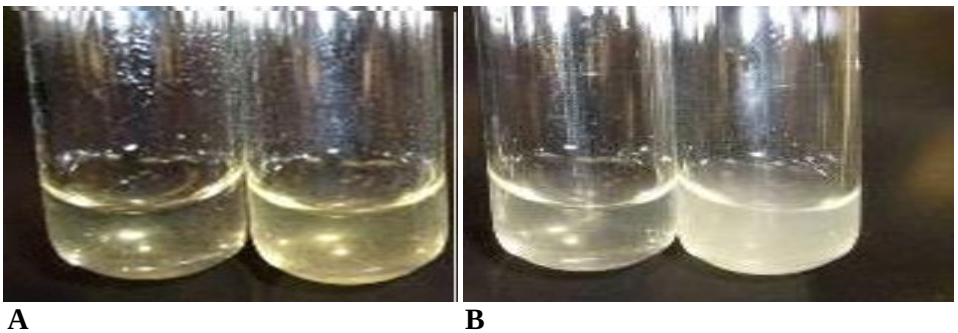


Figure 4: Efficiency of pretreatment on enzymatic hydrolysis of cotton waste

Iodoform Test

Yellow precipitation and cloudy formation shows the presence of ethanol in this sample which is shown in (Figure- 5).



A **B**

Figure 5: Iodoform Test: A. Yellow precipitation B. Cloudy formation

Fermentation of Released Sugar to Bioethanol

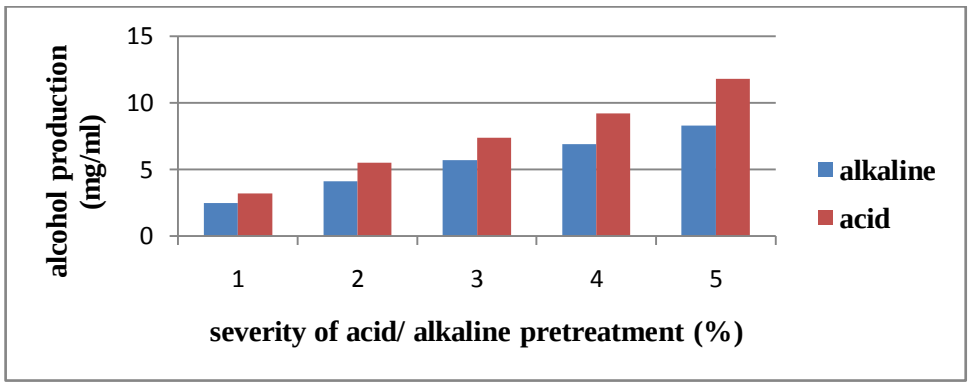
The acid and alkali pre-treated substrates were then subjected to alcohol production to release fuel ethanol. The amount of alcohol produced upon action of *Saccharomyces cerevisiae* on the pre-treated and hydrolyzed cotton waste was presented in (Table- 4) & (Figure- 6).

It was clear that the amount of alcohol produced increases with increasing concentration of acid or alkali pretreatment and was found that the acid pre-treated cotton waste was efficient in alcohol production compared to alkali pre-treated cotton waste which correlates with the findings of [20].

Table 4: Alcohol production from pretreated and hydrolyzed cotton waste

Acid/ Alkaline pretreatment samples(%)	Alcohol Produced (mg/ml)	
	Acid pretreated sample	Alkaline Pretreated sample
1	3.2	2.5
2	5.5	4.1
3	7.4	5.7
4	9.2	6.9
5	11.8	8.3

Figure 6: Alcohol production from pretreated and hydrolyzed cotton waste



Conclusion

The application of cellulolytic enzymes in degrading lignocellulosic materials needs the actions of different types of cellulases. The microorganism namely *Fusarium*species was screened for their ability to produce cellulase. In this present study cotton waste from textile mill was collected, processed and used a cheaper substrate for the production of Bioethanol. The enzyme cellulase obtained in this study was used for enzymatic hydrolysis of the cheaper substrate cotton by simultaneous saccharification and fermentation. Prior to enzymatic hydrolysis the cotton waste was subjected to acid and alkali treatment. The result showed that acid pretreated cotton waste yields more sugar when compared to alkaline pretreated cotton waste and hence the yield of ethanol was high in acid pretreated cotton compared with alkaline pretreated cotton waste.

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