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BIO-ETHANOL PRODUCTION THROUGH SIMULTANEOUS SACCHARIFICATION AND CO-FERMENTATION (SSCF) OF OXIDATIVE DELIGNIFICATION PRETREATED COTTON BIOMASS

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ABSTRACT

The practice of producing ethanol from cotton waste is an eco-friendly process in biofuel production since it makes use of renewable sources. This research focused on the pretreatment processes and the enzyme applications on cotton waste with the goal of enhancing sugar release from hard waste and grinding waste obtained from textiles in Coimbatore, India. Several pretreatment techniques, such as wet oxidation and liquid hot water pretreatment, were tested for cellulose, hemicellulose, and lignin solubilization. Then cellulase and laccase were utilized in enzymatic hydrolysis in varying ratios, and it was found out that the order of cellulose and then laccase showed the best sugar yields, and the process was performed with *Zymomonas mobilis* and *Candida sp.* yeast. Significant ethanol production was obtained from grinding waste compared to hard waste, with a maximum yield of 0.2 grams of ethanol per gram of sample obtained. Ethanol production from the biomass was further characterized by FTIR, where the presence of alcoholic functional groups in the distilled ethanol confirmed the fermentation process. This paper outlines the potential of utilizing cotton waste as a suitable substrate for bioethanol production.

INTRODUCTION

One of the most popular biofuels with industrial applications is ethanol, sometimes referred to as ethyl alcohol. This is largely used because of the use of ethanol in transportation fuels, where it acts as an oxygenate to lower emissions and improve octane ratings. Ethanol is abundantly produced in major countries, especially the USA and Brazil, and the USA dominates with more than 16 billion gallons of ethanol for fuel and other uses annually. On the basis of many national studies, ethanol definitely gives a promise in the aspects of greenhouse gas emissions control, energy self-sufficiency, and consequently generation of income through job opportunities in the agricultural and manufacturing sectors. It also finds application within the pharmaceutical industry, in the food industry, for preservation and flavouring, and also as a medical antiseptic. Therefore, as countries seek to achieve the renewable energy targets and spare the penury of fossil fuels, the role of ethanol is elevating, hence stimulating a rise in the industry's investment in the R&D of more productive processes of production and different feedstock [1].

Ethanol is a biofuel that can be made from plant materials that are grown on purpose for energy use as well as from structural waste materials. India, with its third highest production of cotton in the world, accounts for a large percentage of wastes that are high in cellulose content, comprising about 50% of that volume. In chemical terms, cellulose is a linear crystalline polymer that contributes to multi-dimensional structural rigidity and crystallinity high in value as it exists together with enzyme hydrolysable hemicelluloses. Additionally, Zack composite structure is built from units of lingo-sulphonates that are structural polysaccharides embedded in treated airy dry cotton sludge as feedstock for bio-ethanol production. All these developmental processes require cellulosic nanofibers and purified cellulose to be firstly degraded into cellobiose or glucose through appropriate treatment prior to usage.

There are different forms of pre-treatment, such as physical, chemical, physiochemical, and biological treatments, which are all aimed at lowering the degree of cellulose crystallinity and enhancing the exposure area. An advanced oxidation method, wet oxidation, aims to oxidize the organic compounds into CO_2, water, and other non-toxic organic substances. After hydrolyzing hemicellulose and extracting lignin using liquid hot water pre-treatment, cellulose is produced, which can then be further broken down at high temperatures.

Cellulose digestion is an enzymatic process that can be carried out by a range of microorganisms, such as fungi and bacteria, which synthesize cellulase and hydrolase. The cellulolytic enzymes include three parts: i) Endoglucanase(s) that attack the glycosidic bonds of cellulose chains at random locations ii) Cellobiohydrolase that removes cellobiose units from the reducing ends of cellulose chains iii) ß 3-glucosidase, which converts cellobiose into glucose [2]. Laccase, as an enzyme, catalyzes the oxidation of phenolic compounds and many other deteriorating substances, besides aiding the growth of microorganisms and fermentation of substrates [3].

Hydrolysate originated after pre-treatment and also enzymatic treatment is highly rich in monomeric sugars. Simultaneous saccharification and co-fermentation (SSCF) improves the saccharification followed by fermentation of glucose/pentose sugars in one operation stage [4]. Co-fermentation is done using *Candida sp.,* and *Zymomonas mobilis* to make use of the monomeric units generated by the hydrolysate (cotton biomass) produced. This study proposes optimization of pre-treatment and enzymatic methods for increased sugar release, which also assists in co-fermentation for alcohol production.

MATERIALS AND METHODS

Collection of cotton wastes

Cotton wastes were collected from spinning mills in the Coimbatore district. Two types of cotton wastes were used for the study: hard waste and grinding waste, obtained through the carding process and the settling of dust particles in the blow room, as well as waste accumulating on the floor and chimneys.

Compositional analysis (ASTM, 2015)

The compositional analyses were performed on each cotton waste type before and after pretreatment processes such as moisture content, acid and reducing sugar content following standard operating procedures. The samples were milled prior to conducting the analyses in order to improve the analytical outcome.

Moisture content (ASTM, 2012)

Cotton waste moisture content was analyzed before and after pretreatment processes. Samples were dried in a hot air oven at 105°C and allowed to reach room temperature.

The moisture content was later determined by using the following formula:

Moisture% = [B – C / B – A] X 100 ------------------ Equation 1

The weight of the empty petri dish is labelled as A, the weight of the petri dish filled with cotton is B, and the weight of the petri dish with the dried cotton is C.

Detection of reducing sugar by dinitrosalicylic acid (DNSA)

Dinitrosalicylic acid reagent contains 1% DNSA, 0.2% phenol, 1% NaOH, and 0.05% sodium sulfite. Rochelle's salt solution is 40% sodium potassium tartrate. Standard glucose solutions were made at the concentration of 10-100 µg/ml glucose and 100-1000 µg/ml of distilled water. To this, 0.3 ml of Rochelle salt solution was added and mixed with the same volume of solution treated with DNSA reagent, heated for 10 minutes, and cooled to room temperature. Optical density was measured at 540 nm using an ELICO SL 244UV-VIS spectrophotometer, and a graph of OD values plotted against glucose concentration was made. [5]

Pretreatment of cotton wastes

The cotton waste was cutted into thin fibers measuring approximately between 0.5 cm and 1 cm and kept in a dry environment at normal room temperature [6]. After that, the wastes went through different pre-treatment processes in a bid to determine the best suitable process for every cotton waste type in enhancing hydrolysis.

Wet oxidation by hydrogen peroxide

20 ml of hydrogen peroxide (H_2O_2) solution, ranging in concentration from 5% to 30%, were added to a 100 ml conical flask containing 1 g of cotton waste. The flask was then left undisturbed for 5 to 30 minutes. The cotton wastes were evenly soaked in the solution for even chemical treatment and left for shade drying. After shade drying, the samples were taken, and reducing sugar content was determined using the DNSA method.

Liquid hot water pre-treatment (LHW)

100 ml conical flask holding 15 ml of sterile distilled water was filled with 1 g of sterile cotton waste. Additionally, this flask was kept in a water bath with temperatures adjusted to vary between 80 to 110°C for 5, 10, 15, and 20 minutes. The DNSA method was used to determine the degree of sugar reduction.

Enzymatic hydrolysis

The enzymes (laccase and cellulase) were tested at proportions of 25:75, 50:50, and 75:25. Then 100 ml of carboxymethyl cellulose (CMC) medium was added in a sterile 250 ml conical flask containing 0.5 gram of pretreated cotton. Depending on the percentage, laccase was added, and the sample was then put in an orbital shaker incubator for 3 days followed by the addition of cellulase for another 3 days. A simple experimental arrangement was set up and incubated with no enzymes added. Upon the completion of the incubation period, the reducing sugar concentration was measured following the DNSA method [7].

Simultaneous saccharification and co-fermentation

100 ml of fermentation medium were prepared and sterilized in a 250 ml flask at 121° C for 15 minutes. Then 1 g of pre-treated cotton waste was added, along with 7.5 ml of cellulase, 2.5 ml of laccase, and 1 ml of *Candida sp*. The mixture was then placed in a shaking incubator at 38° C for 3 days. After that, a 1 ml of *Z. mobilis* was added to the mixture and kept at room temperature for 3 days. The amount of ethanol produced was then determined according to the method of William Reese with the use of dichromate [8].

Brix - Refractometer

The reducing sugar content was determined using a Brix pocket refractometer (PAL-1: SUS). Brix measures the sugar concentration in an aqueous solution. Approximately 0.3 ml of the sample was placed on the prism to obtain the sugar concentration [9].

Distillation of bioethanol from fermented sample

100 ml fermented sample was taken after 6 days of fermentation and distilled using an alcohol distillation unit at a fixed temperature of 78.4 °C (the boiling point of ethanol). The evaporated steam was condensed using a condenser, and the condensed sample was collected through the outlet and stored at 4°C for further analysis [10].

Quantitative analysis by using the acid dichromate method

Acidic dichromate solution (0.01 Mol/L) was prepared by taking 70 ml of conc. H_2SO_4 and adding it to 125 ml of distilled water with stirring, cooling, and later 0.75 g of Potassium dichromate was introduced. A starch solution (1%) was made by adding 1 g of soluble starch to boiling water. Dissolving 20 g of Potassium iodide in 100 ml of distilled water gives a concentration of 1.2 Mol/L, and sodium thiosulphate (0.03 Mol/L) was prepared by taking 7.44 g in 100 ml of distilled water. 10 ml of acid dichromate was measured out with a 250 ml conical flask. 1 ml of the diluted sample was placed into a 5 ml glass vial, closed tightly with thread, and lowered into the acid dichromate and sealed with the rubber cork. The arrangement was then kept stationary for a period of 16-24 hours at 25-30 ° C. The pouch was then removed, and 100 ml of distilled water and 1 ml potassium iodide were added, forming a brown colour. The solution was then subjected to sodium thiosulphate titration until the brown solution turned into light yellow. 1 ml of starch solution was then added, and the mixture turned blue. Titration continued until the blue colour disappeared. The volume of sodium thiosulphate consumed was noted for the determination of ethanol concentration. Blanks were similarly prepared in triplicate and then titrated. The total amount of acid dichromate was evaluated using blanks in relation to samples with known amounts of dichromate. The volume of sodium thiosulphate employed in the sample was deducted from the blank to obtain the moles of sodium thiosulphate. Ethanol concentration was found out from the molecular relation. If the sample was diluted, the finding was adjusted by the dilution factor and computed in grams per 100 ml and later on in litres [11].

Fourier Transform Infra-Red (FTIR) Spectroscopy

FTIR was used to determine functional groups, with the spectrum analyzed using IR solution software. The sample's transmittance and reflectance of infrared rays at various frequencies were translated into an IR absorption plot, matched with known material signatures from the FTIR library. The wave numbers in the spectrum represent the sample's functional groups, particularly the alcoholic group. In this study, the fermented ethanol product was subjected to FTIR characterization to identify the alcoholic group, confirming the presence of ethanol in the sample [12].

RESULTS AND DISCUSSION

Collection of cotton wastes

Two types of waste, hard waste and grinding waste were selected for the study, both generated during various processes in the textile industry.

Moisture analysis

The moisture content of the wastes was measured both before and after pretreatment, and the results are presented in Table 1. After pretreatment, wet oxidation-pretreated hard waste had a higher moisture content (2.54%) compared to liquid hot water-pretreated hard waste (0.93%). Similarly, wet oxidation pre-treated grinding waste had 6.90% moisture, while liquid hot water pre-treated grinding waste had 3.60%. The results showed higher moisture content in grinding waste than in hard waste.

Table 1: Moisture content of the cotton waste before and after pretreatment

Pretreatment		Moisture Content (%)	
		Hard waste	Grinding waste
Before pretreatment		11.18	9.58
After pretreatment	Wet oxidation	2.54	6.90
	Liquid hot water	0.93	3.60

Pre-treatment

Wet oxidation by hydrogen peroxide

The performance of the wet oxidative pretreatment was done under conditions of hydrogen peroxide concentration ranging between 5% and 30% and time range from 5 to 30-minute intervals. Comparing the hard waste with the control, it showed a medium level of sugar release. The optimized concentration was found to be 25% for 10 minutes, which was more effective than the control. As time exposure increased, the chemical's effectiveness decreased, showing a limited impact on the hard waste based on the obtained results (Figure 1).

The production of acids through hydrolytic processes and oxidative reactions are the primary reactions in wet oxidation pretreatment. Hemicelluloses are broken down into monomeric sugars by large amounts; lignin is broken down and oxidized; and cellulose is partially broken down, which makes it sensitive to enzymatic hydrolysis [13]. The cleavage of lignin leads to partial cellulose degradation, enhancing its susceptibility to enzymatic action for better saccharification. Ayeni *et al.*, conducted pretreatment with hydrogen peroxide and noted that at lower concentrations, it was an effective oxidative agent for biomass pretreatment [14].

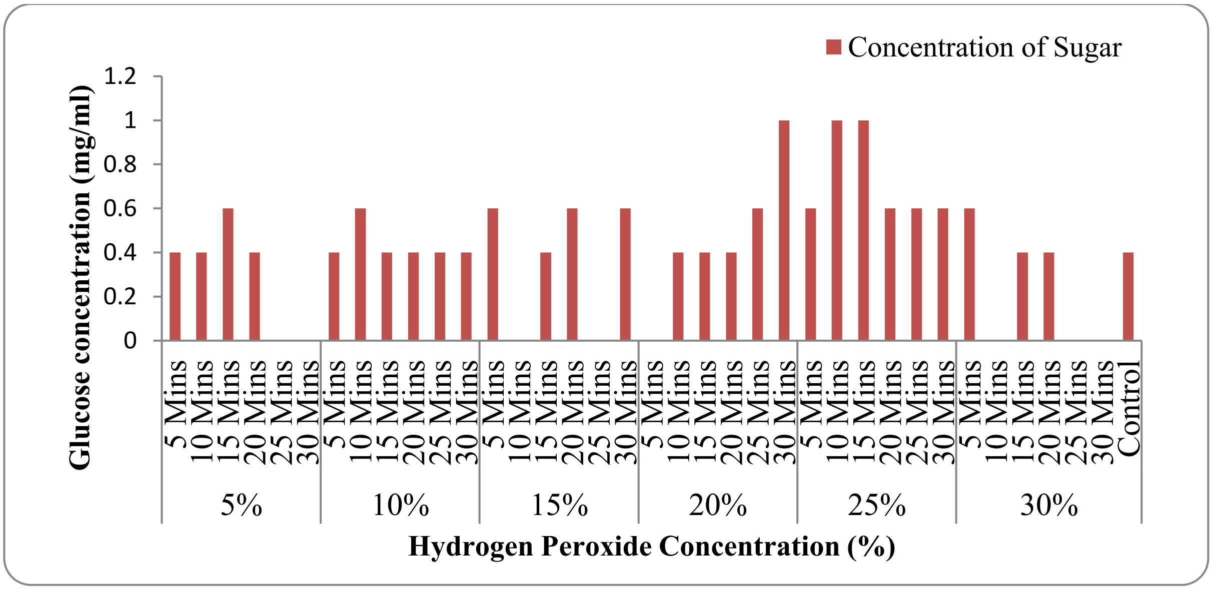

Figure 1: Pretreatment of cotton hard waste by wet oxidation method

The wet oxidative pretreatment was performed using concentrations between 5% to 30% for time ranges of 5 to 30 minutes. There was a noticeable effect in terms of the chemical reaction for grinding waste, in contrast to hard waste. The optimized value for the grinding waste grade turned out to be 20% for 10 min duration. In this case, as time exposure increases, the chemical action also increases, yielding a higher sugar release as obtained, as interpreted (Figure 2).

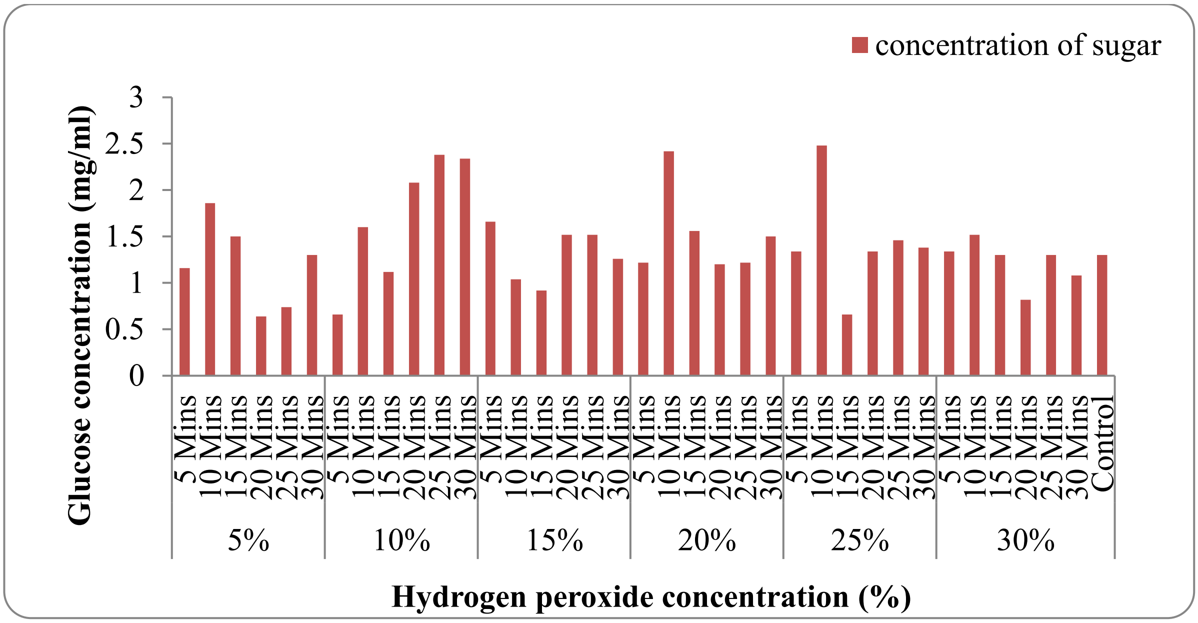

Figure 2: Pretreatment of grinding cotton waste by wet oxidation method

Wet oxidation oxidizes hemicellulose fractions into intermediates such as carboxylic acids, acetaldehydes, alcohols, and eventually CO_2 and H_2O. The degree of fractionation is influenced more by temperature than by time and oxygen dose [15]. Silverstein *et al.*, found that hydrogen peroxide at lower concentrations, aided by different temperatures, enhanced saccharification from cotton stalks [16]. Wet oxidation has been shown to produce fewer by-products like furfural and hydroxymethyl furfural and it is expected that the sugar degradation products associated with the inhibition of microorganisms will not be produced in excessive amounts under such conditions [17]. Therefore, the sugar concentration in the grinding waste, even after oxidation, is rather low.

Liquid hot water pretreatment (LHW)

The liquid hot water pretreatment was carried out at various temperatures ranging from 80°C to 110°C with different time exposures between 5 minutes to 20 minutes. The temperature range of 80°C and 90°C showed minimal sugar release on the hard waste, even at higher time intervals. The optimized condition was 100°C for 5 minutes for the liquid hot water pretreated grinding waste. Other temperatures yielded a comparatively equal amount of sugar. As time exposure increased, the sugar release also increased (Figure 3).

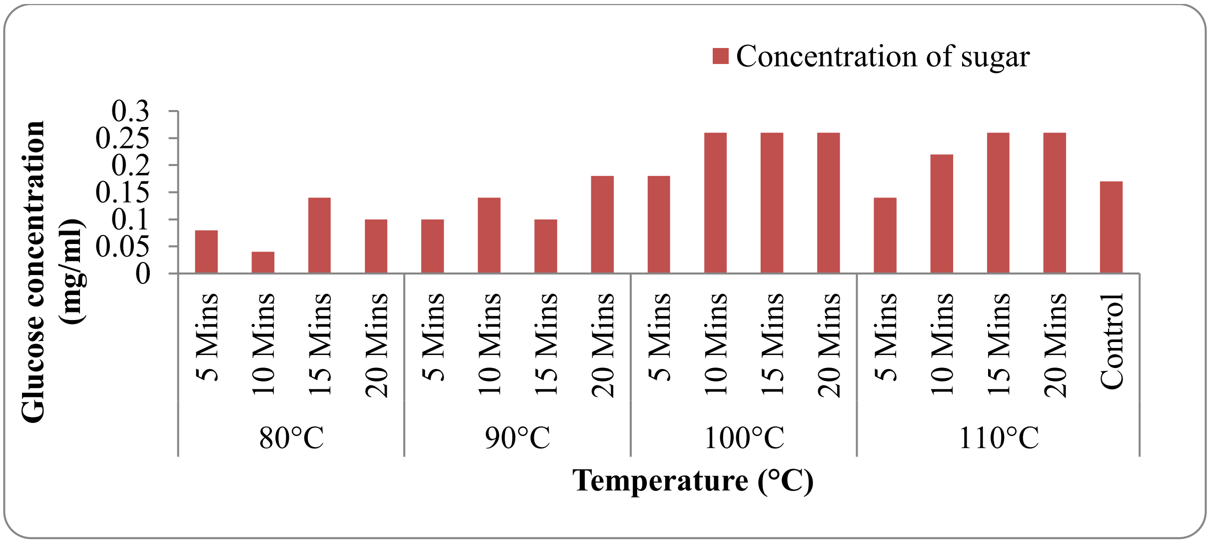

Figure 3: Pretreatment of cotton hard waste by liquid hot water method

In liquid hot water pretreatment (LHW), pressure aids in maintaining the water in a liquid state at higher-than-normal temperatures, removing up to 80% of hemicellulose and improving the enzymatic digestibility of pretreated biomass like corn fiber and sugarcane bagasse. Lignin is typically removed after pretreatment [17], enhancing higher sugar releases and better saccharification.

The same liquid hot water pre-treatment process was carried out for grinding cotton waste. The efficient condition for the process of hot liquid water pretreatment grinding waste was 100ºC for five minutes. In this stage of pretreatment, sugar release yield was comparable with the control. Time variations with other concentrations and intermediate results are provided for reference (Figure 4).

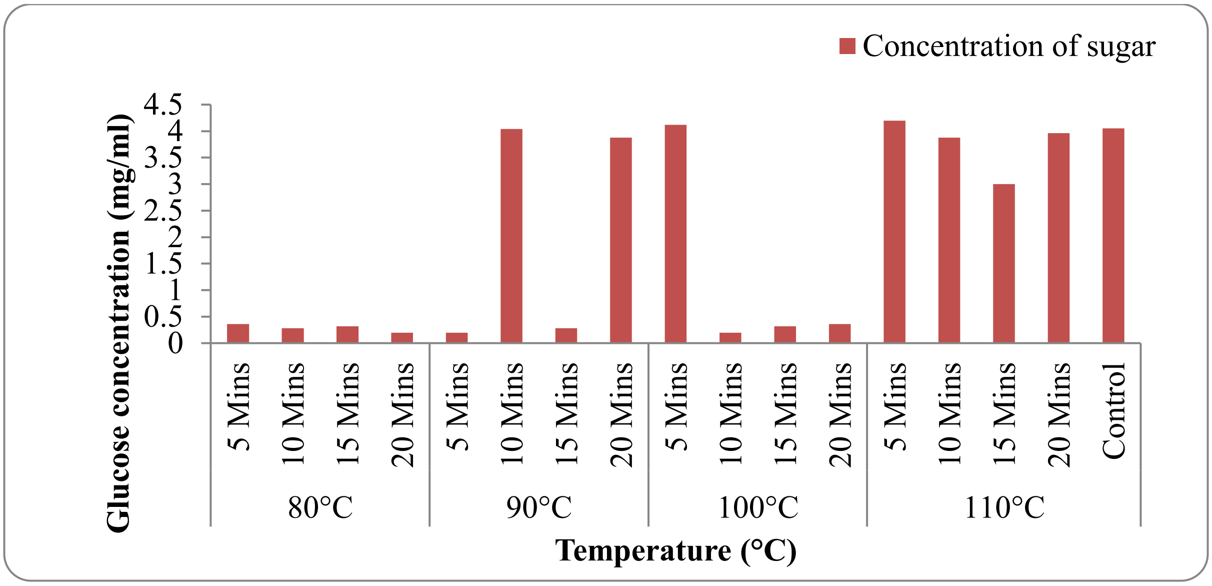

Figure 4: Pretreatment of grinding cotton waste by liquid hot water method

LHW employs heated water under pressure (less than 5 Mpa) for various time intervals at a temperature of between 170˚C and 230˚C and then decompresses, the solubilized hemicelluloses and lignin are present in low concentrations [18]. Pressurized hot water degrades hemicellulose and lignin, decreasing cellulose crystallinity and yielding higher sugar release, aiding enzymatic treatment. Wan *et al.*, stated that liquid hot water enhances the cellulose digestibility of lignocellulosic biomass compared to other treatment methods [19].

Enzymatic treatment

The enzymatic treatment for pretreated cotton waste was undertaken with the use of cellulase and laccase at three ratios, which are 25:75, 50:50, and 75:25, respectively. The treatment comprised two criteria, namely cellulase treatment in advance of laccase treatment and laccase treatment in advance of cellulase treatment. The enzymatic treatment had better sugar release on the hard waste compared to the grinding waste. Cellulase followed by laccase had higher amount of sugar release than other. The optimum enzyme ratio was 75:25 in both cases for pretreated hard waste, as shown in (Figure 5).

Ethanol production is enhanced when biomass is treated with cellulose, as cellulase facilitates the hydrolysis of cellulose to glucose, which is then utilized for the production of bioethanol. Laccase serves the purpose of oxidizing the phenolic units present in lignin, which helps in breaking that down [20]. Sanchez *et al.* stated that laccases disrupt the crystalline structure by oxidizing phenolic compounds [3]. Percentage of sugar yield from grinding waste was higher than that from hard waste, although cellulase followed by laccase was still the effective treatment (Figure 6). Sukumaran *et al.*, found out that the concentration of cellulase proved to be an important factor affecting the yield of the reducing sugar [8]. The amount of sugar release was estimated using Brix refractometer, which showed that cellulase followed by laccase recorded higher sugar release than the reverse. Treated samples had higher sugar release than the control (Figure 7 and Figure 8).

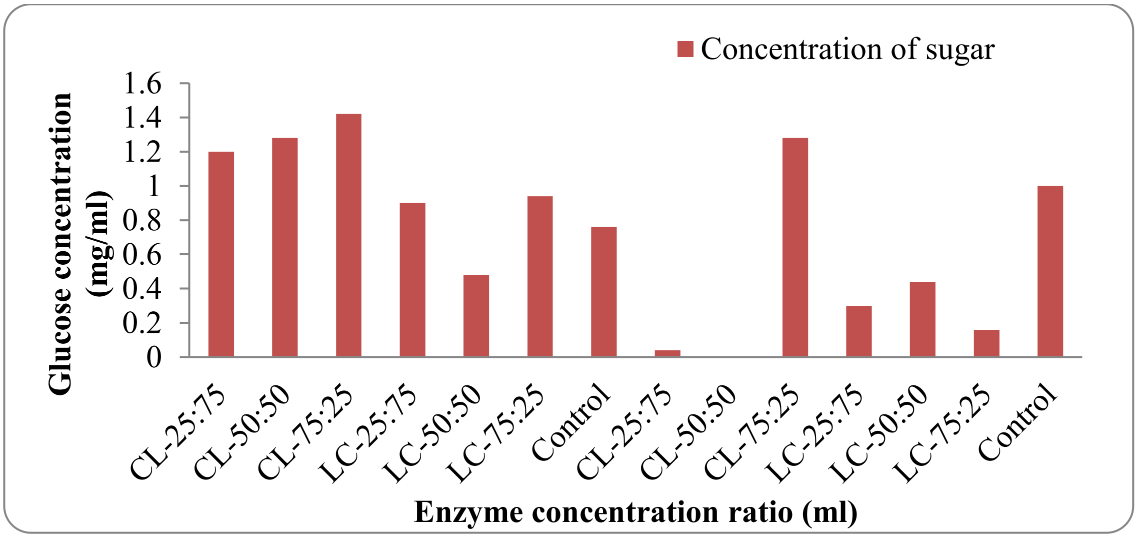

Figure 5: Enzymatic treatment of cotton hard waste

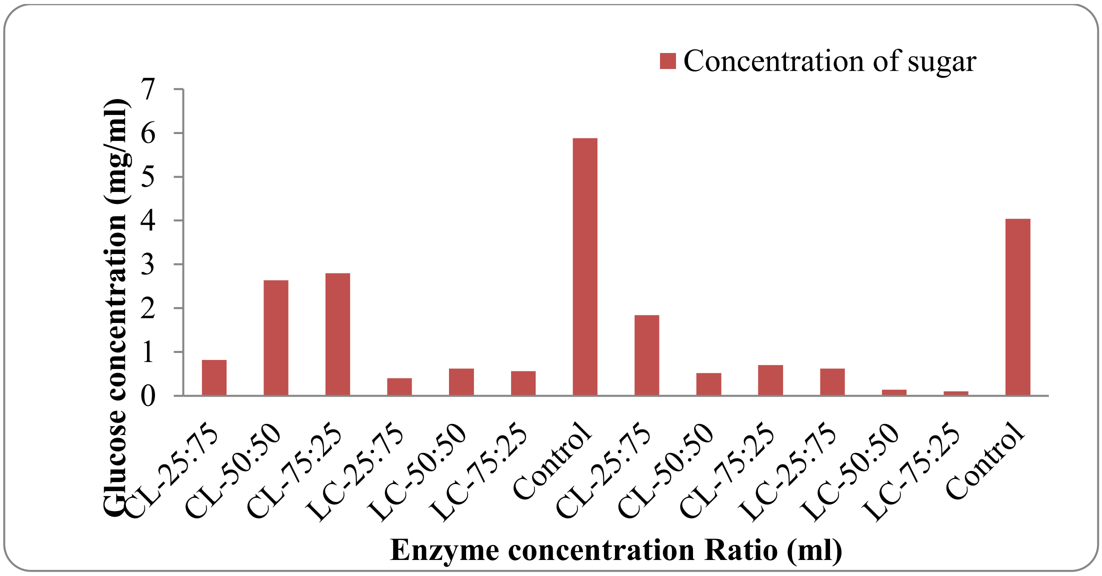

Figure 6: Enzymatic treatment of grinding cotton waste

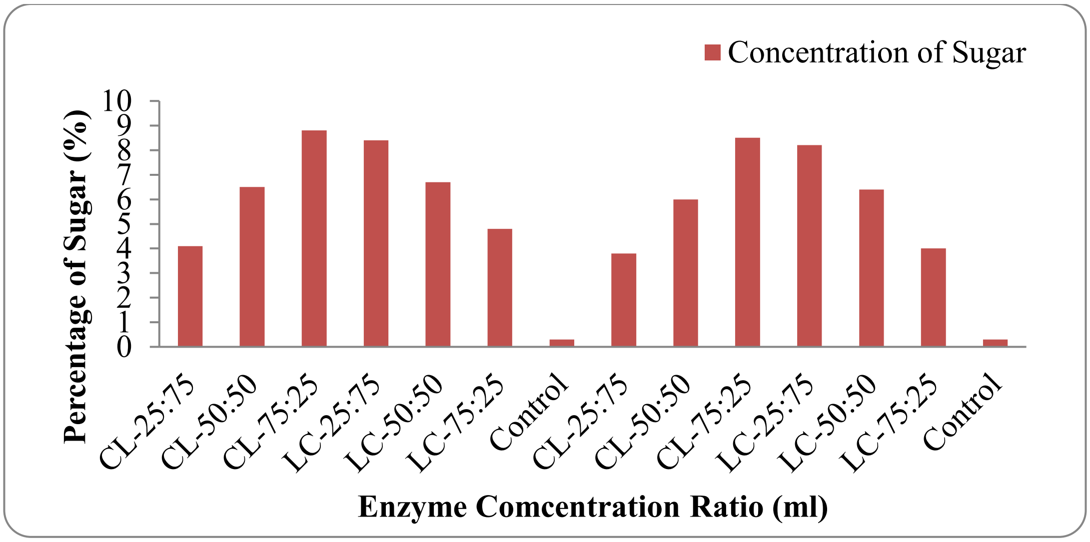

Figure 7: Percentage of sugar release from hard waste - Refractometer

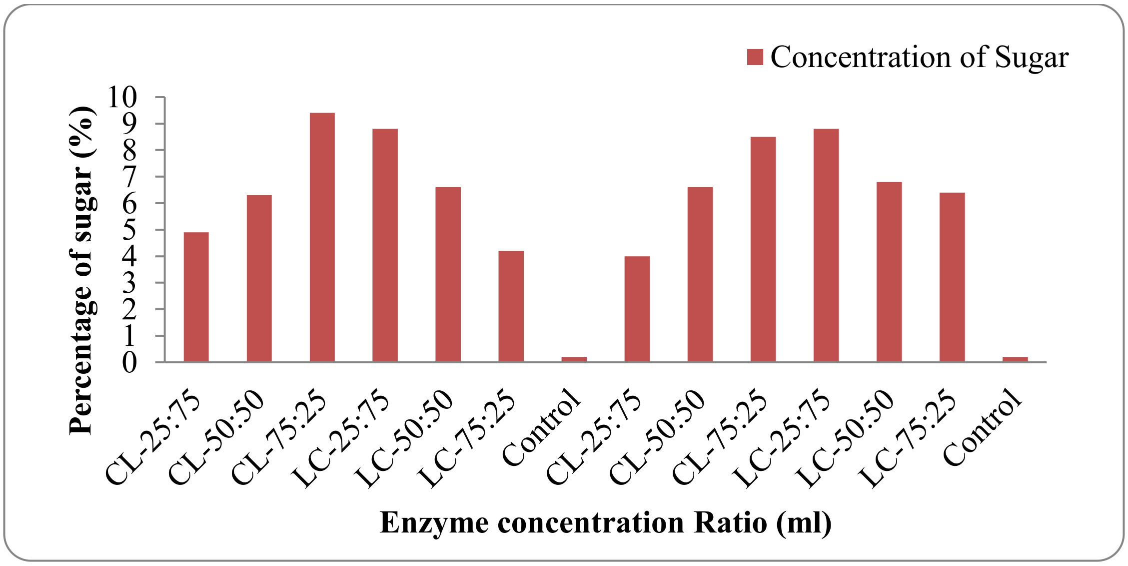

Figure 8: Percentage of sugar release from grinding waste - Refractometer

Simultaneous saccharification and co-fermentation (SSCF)

SSCF was conducted using *Candida sp.* and *Z. mobilis*, added to the fermentation medium at 3-day intervals. The percentage of sugar release from both pretreated cotton wastes was estimated using a Brix refractometer. Cellulase followed by laccase-treated hard and grinding waste had equal sugar yield compared to laccase followed by cellulase, as shown in (Figure 9). Kim et al., used *S. cerevisiae* for co-fermentation of sugars and stated that non-glucose derivatives like cellobiose and xylose with β-glucosidase produced higher ethanol yields from marine plant biomass [21].

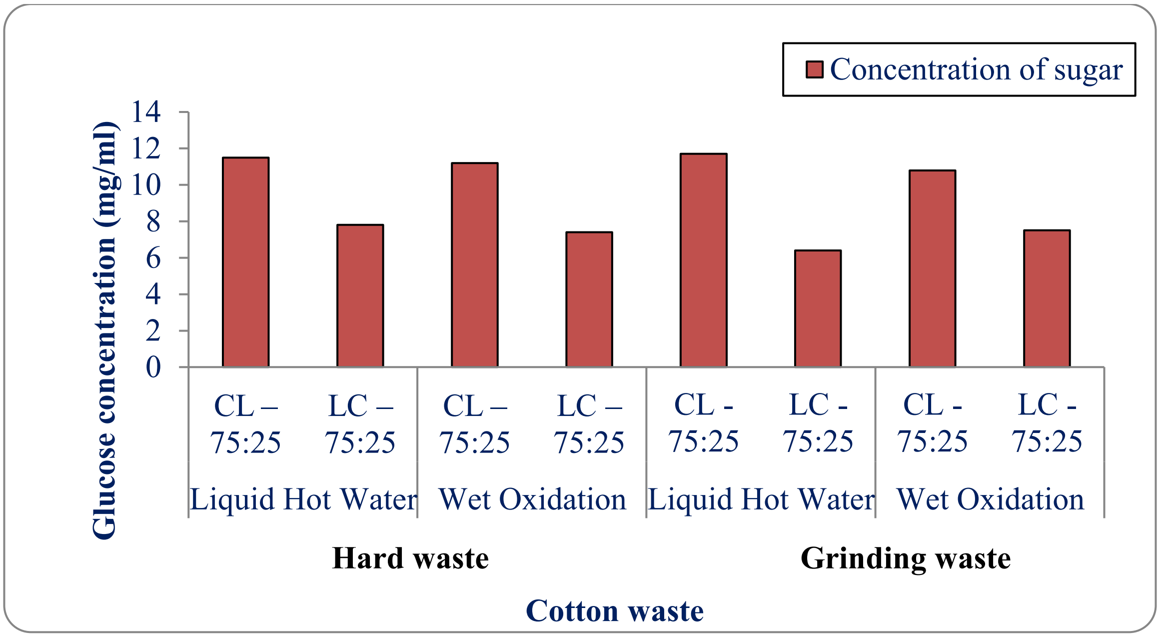

Figure 9: Percentage of sugar release in the fermentation medium

Percentage of ethanol produced after the co-fermentation

Grinding waste had a higher ethanol yield compared to hard waste, with the highest ethanol percentage being 0.2 per gram of sample. Hard waste had a comparatively lower ethanol yield than grinding waste. The results are shown in (Figure 10).

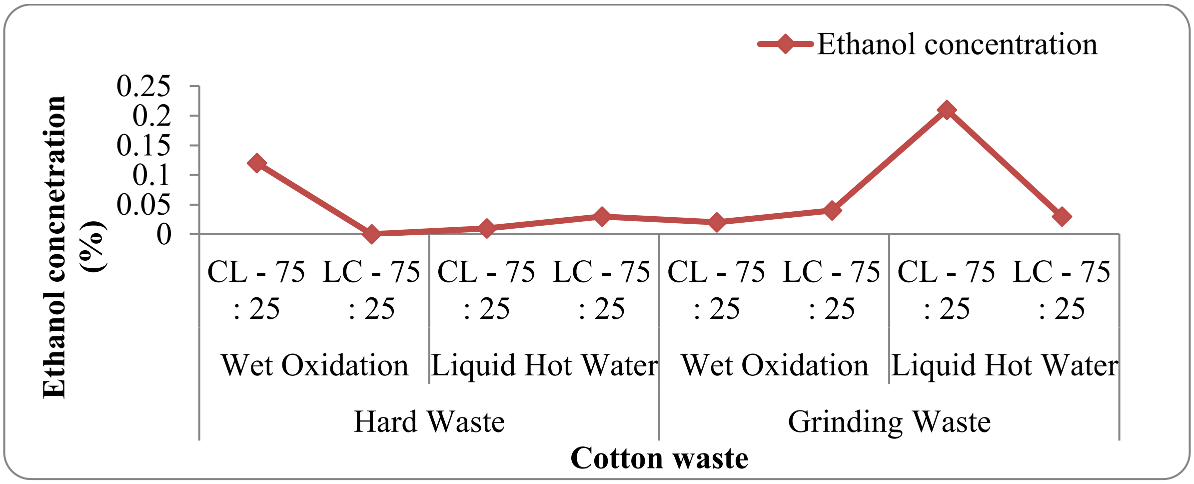

Figure 10: Percentage of ethanol production from the cotton wastes

Fourier Transform Infra-Red spectroscopy

FTIR analysis of the distilled sample

The three peak values 1280.73 cm^{-1}, 1211.30 cm^{-1}, and 1041.56 cm^{-1} in (Figure 11), represent C-O stretch, and peaks at 3317 cm^{-1} of OH stretch indicating the presence of alcoholic compounds in the sample. Additionally, the presence of other peak values indicates alkenes, alkynes, and aldehyde compounds. Thus, purification of the sample aids in obtaining pure ethanol (Table 2).

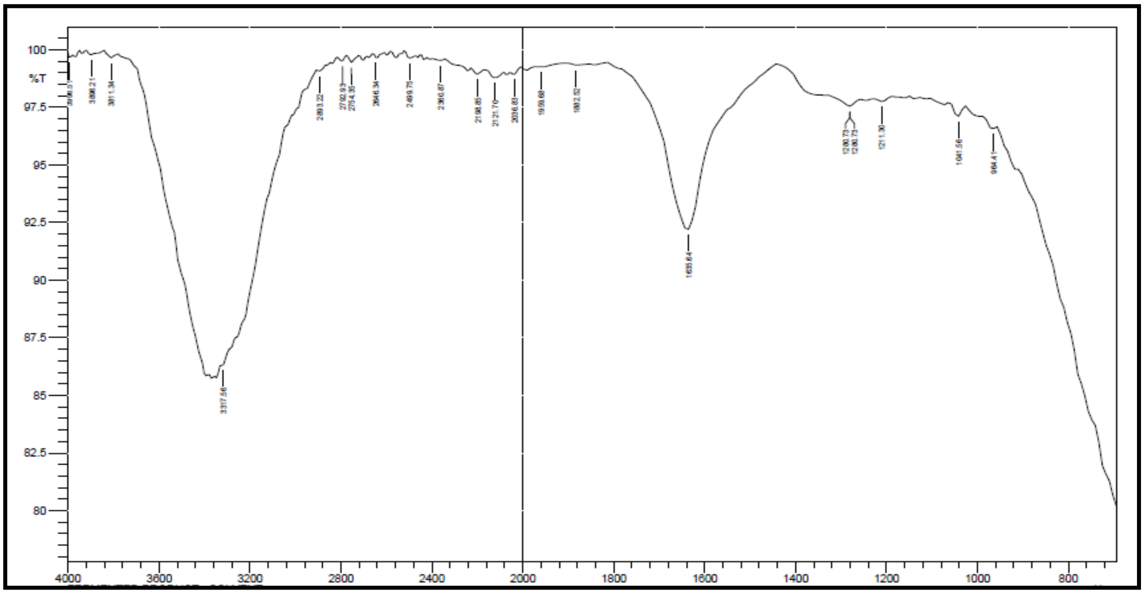

Figure 11: FTIR analysis of the distilled sample

Table 2: FTIR analysis of the distilled sample

Obtained Frequency/cm^{-1}	Bond	Functional group
3317	O-H Stretch	Alcohol
2792.93, 2754.35. 2646.34	H-C=O: C-H Stretch	Aldehydes
2121.70, 2198.85	C (triple bond) C Stretch	Alkynes
1280.73, 1211.30, 1041.56	**C-O Stretch**	**Alcohols, carboxylic acids, esters, ethers**
964.41	=CH-H Bend	Alkenes

Standard FTIR analysis of ethanol

The highest peak value in the distilled sample was 3317.56 cm^{-1}, indicating the presence of O-H stretch and the functional group of alcohols. Thus, the fermented product was found to be alcohol compared to standard ethanol (Figure 12 and Table 3).

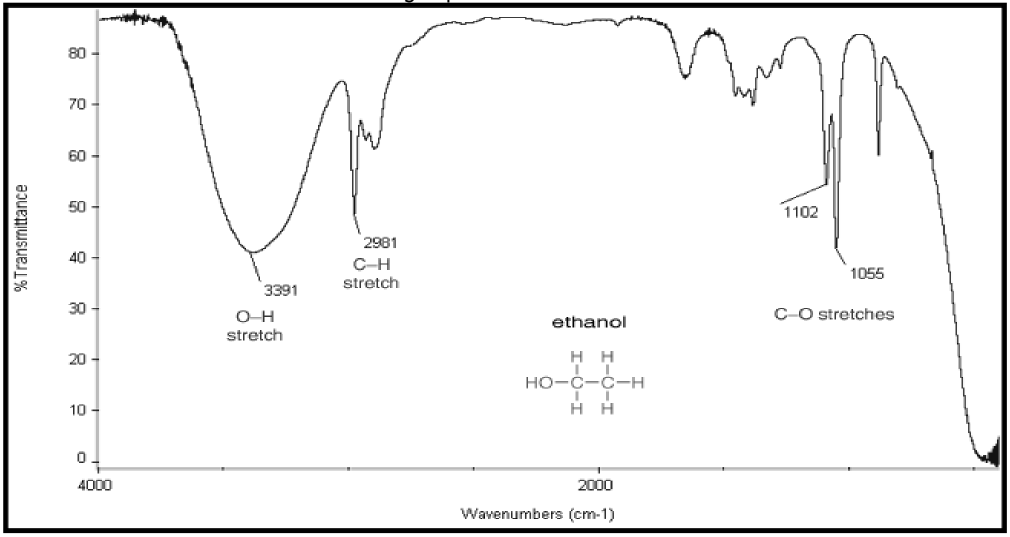

Figure 12: Standard FTIR chart for ethanol

Table 3: Standard FTIR characteristics of ethanol

Obtained Frequency/cm	Bond	Functional group
3391	O-H Stretch	Alcohols

2981	C-H Stretch	Alkanes
1102 & 1055	C-O Stretch	Alcohols

CONCLUSION

Among the other renewable resources, ethanol stands out since it is available in plenty and can be produced from energy crops as well as lignocellulosic biomass. The use of cotton biomass is considered an effective technology for ethanol production due to the high amount of cellulose in the raw material. Enzymatic hydrolysis was done on two wastes: hard waste and grinding waste in order to assess the yield of bioethanol production. The primary focus of the pretreatment is to enhance surface area and minimize the degree of crystallinity of accessible cellulose. Both wet oxidation and liquid hot water pretreatment procedures were chosen and optimized in order to achieve maximum sugar release from the cotton biomass. The optimal conditions were determined to be 25% –10 minutes for wet oxidative treatment and 100°C–5 minutes for liquid hot water treatment. Cellulase and laccase enzymes were used to treat these parameters in different orders of cellulase-laccase and laccase-cellulase in three ratios of 25:75, 50:50, and 75:25. For both treatment options, the best enzymatic ratio was found to be 75:25. This ratio was incorporated for simultaneous saccharification and co-fermentation using *Candida sp.* and *Zymomonas mobilis*, for producing bioethanol, and the potassium dichromate estimation method of bioethanol quantification was used. Following fermentation, the distilled sample was shelved, from the two wastes used, the grinding waste provided a greater number of sugars, obtained bioethanol yield of 0.2% which was higher compared to that of the hard waste.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors for publishing this manuscript.

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