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Research Article
Bioethanol Production from Waste Cotton Lint Using *Saccharomyces cerevisiae*

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Abstract

Background and Objective: To reduce the cost of ethanol production while encouraging re-use and recycling of biomass, bioethanol production using secondary lignocellulose has been receiving attention. In this research, we aimed at using cotton lint waste, a waste product obtained from cotton processing industries, for bioethanol production. **Materials and Methods:** Ethanol production by *Saccharomyces cerevisiae* was monitored in a fermenter supplemented with the pre-hydrolysed cotton lint waste obtained by employing different pre-treatment methods (Concentration H_2SO_4, 3 and 5%) followed by enzymatic hydrolysis. **Results:** The exponential phase of growth started immediately with a steady increase in bioethanol production from 3.5-16.5 g L^{-1} for pre-hydrolysed cotton lint waste treated with 3% acid between 1 and 7 days, moreover, within experimental errors a 3.5-fold increase in bioethanol production was observed within the same time frame in the fermenter supplemented with pre-hydrolysed cotton lint waste treated with 5% acid. When grown in a fermenter supplemented with any of the pre-treatment methods, ^{1}H-NMR, DEPT-135 ^{13}C-NMR, GC-FID and UV-Vis spectroscopy revealed that *Saccharomyces cerevisiae* was able to convert substantial amount of sugars released from pre-hydrolysed cotton lint waste into bioethanol. **Conclusion:** Cotton lint waste is a suitable substrate for bioethanol production; however, the quantity of bioethanol produced depends on the pre-treatment method employed.

Key words: Bioethanol, cotton lint waste, pre-treatment, lignocellulosic, fermentation, pre-hydrolysed

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The rapid increase in energy demand and the consequences attached to fossil fuels has brought about a high interest in alternative energy sources. Alternative energy sources such as renewable sources of energy have been receiving attention recently due to the negative consequences attached to the fossil fuels including accumulation of atmospheric CO_2, inadequate energy security among others[1]. An alternative source of energy includes energy derived from solar, wind and biofuels. For the later, different forms of agricultural biomass has been used in the production of biofuels including bioethanol, biomethane and biodiesel[2]. Moreover, a waste product such as the corn stover, rice straw, grasses and wood chips have been utilised in the production of bioethanol. Their availability and also renewable nature make them a suitable raw material for biofuel generation[3].

Bioethanol production from biomass has been on the increased in most countries including United State of America (USA), Brazil, China among others. According to Renewable fuels association, the highest bioethanol production was observed in the USA with nearly 16 billion gallons in 2017[4]. Corn was primarily been used to produce ethanol in USA while Brazil mostly uses sugarcane. Depending on the nature of the substrate, the agricultural waste contains cellulose, hemicellulose, xylose among others[3]. Similarly, the microorganism used in bioethanol production requires simple sugar for fermentation. These sources are expensive and compete with food. Consequently, an alternative source of carbon is sought for[5]. The secondary source of carbon such as the lignocellulose has been implicated in bioethanol production[6]. These sources are mostly cheap and abundant and could be used to generate fuels.

Cellulosic materials obtained from wood and agricultural residues represent the most abundant global source of biomass. These facts have motivated extensive research towards making an efficient conversion of lignocellulosic materials into sugar monomers for further fermentation to ethanol. Unlike simple sugars which can be directly converted to ethanol, other biomasses such as starches, lignocelluloses and citrus waste need to be pre-treated to make sugars available for the subsequent fermentation step. Pre-treatment and hydrolysis release a mixture of fermentable sugars as well as other compounds. Hydrolysis using appropriate enzymes or chemically using acids represents the most effective method to liberate simple sugars from cellulosic materials that are used for the production of chemicals such as ethanol, glucose and furfural[7].

The worldwide desire to reduce greenhouse gas emission can be conveniently achieved by the use of bioethanol as a transport fuel. Domestic production and use of ethanol as fuel can decrease dependence on fossil fuel, create jobs in rural areas, reduce air pollution, and reduce global climate change and carbon dioxide build-up. Agricultural waste such as fibrous materials, root branches, straws, stalks, sticks, leaves etc and process-based residues such as rice husk, corn cobs, coconut shell, groundnut shells, sugarcane bagasse and sawdust are under-utilized[8,6]. On the other hand, many of these agricultural wastes could be used in bioethanol production. Study has shown that, a significant amount of bioethanol production could be achieved with lignocellulosic waste depending on the nature of the substrate and type of pre-treatment techniques employed[3,9].

Yeasts fermentation has for a very long time been used for the production of alcoholic beverages and bioethanol production. Yeasts from the genus *Saccharomyces* and from the specie *cerevisiae* for use as starter cultures have dominated these bioprocesses[10,11]. In this research, waste cotton lint off-cuts, a lignocellulosic material was investigated for use as a sugar substrate for bioethanol production by *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Study area: This study was carried out at the Microbiology Laboratory of Umaru Musa Yar'adua University, Nigeria between 3rd October, 2018 to 21 November, 2019. Part of the ethanol analysis including the NMR and GC-FID were carried out in the United Kingdom.

Chemicals: All media used were prepared according to standard manufacturers protocol, all general analytical reagents used for the purpose of this research were bought from Sigma-Aldrich Co. Ltd. (Gillingham, Dorset, UK) and Fisher Scientific (Loughborough, United Kingdom), unless otherwise stated.

Sample collection: Waste Cotton lint samples were collected from Marabar Kankara Ginning Company, Malumfashi Local Government, Katsina State, Nigeria. Samples were taking to the laboratory for further analysis.

Determination of proximate composition: The proximate compositions of the cotton lint waste were determined using standard analytical methods. Protocol reported by AOAC[12] was adopted while assessing the moisture content, ash content and crude lipid content. Similarly, Gabriel *et al.*[13] was adopted when studying the crude protein content and crude fibre content while de conto *et al.*[14] was used to assess the carbohydrate content of the cotton lint waste.

Autoclave assisted acid pretreatment: Unlike lactic acid bacteria that possess enzymes capable of cleaving the glycosidic linkages in polysaccharides such as starch for use as carbon source during fermentation, other microorganisms such as yeasts lack the enzymes responsible for the cleavage and as such require enzymatic or chemical pre-hydrolysis into monomeric units. Cotton lint waste contains mostly cellulosic material which are β-glucans and as such requires hydrolysis into glucose moieties for use as substrate by yeast in bioethanol production. However, incomplete hydrolysis liberates oligosaccharides that could not be utilized by yeast for fermented ethanol production. In this research, a combination of physico-chemical and enzymatic hydrolysis was employed in the hydrolysis of the cotton lint waste in view of optimizing the pre-hydrolysis procedure. A procedure reported by Dimos *et al.*[15] was adopted with slight modification when conducting the autoclave assisted Acid pretreatment. cotton lint waste (200 g) was completely immersed in 3 and 5% of concentrated H_2SO_4 solution respectively and the resulting mixtures were autoclaved at 121°C for 15 min. To neutralize the acid, the pH was adjusted to 7.0 after which the autoclaved cotton was washed thoroughly with ultrapure water and dried at 50°C in an air circulated oven to remove all residual moisture. The acid treated dried cotton lint waste was later ground into powder and used as the carbon source in the fermentation media.

Enzymatic hydrolysis: To completely hydrolyse the cotton lint waste into monomeric units of glucose, oligosaccharides liberated after acid hydrolysis (3 and 5%) were subjected to enzymatic hydrolysis. The acid treated dried cotton lint waste, 20% (w/v) was subjected to enzymatic hydrolysis using a mixture of commercial enzymes (enzyme complex, cellulase, xylanase, β-glucosidase (cellobiase) and hemicellulase) available in Novozymes Biomass Kit (Novozymes, Denmark). The enzymes were added to the mixture according to manufacturer's instruction and were incubated at 50°C for 24 h after White *et al.*[16] and Obata *et al.*[6]. Cellulase activity were inhibited by the hydrolyses of cellobiose by β-glucosidase.

Preparation of yeast Inoculum: Dried form of industrial yeast (*Saccharomyces cerevisiae*) was 1st prepared by mixing 0.5% (w/v) of the yeast into a sterile yeast peptone dextrose medium. This was prepared by dissolving 2 g of yeast extract, 4 g of dextrose, 4 g of peptone dissolve in 200 mL after Sebayang *et al.*[17]. The activated yeast was kept at 37°C shaken at 150 rpm before used for bioethanol production.

Fermentation: The hydrolysed product (3 and 5%) cotton lint waste obtained after acid treatment were fermented using *Saccharomyces cerevisiae* at 30°C and shaken at 150 rpm for 7 days. After every 24 h, 5 mL was taken out of the 2 fermenters (3 and 5%) and analyzed for yeast cell count using haemocytometer and the amount of ethanol produced quantified employing the method reported by Sayyad *et al.*[18]. At the end of the fermentation, the ethanol was distilled out using a procedure reported by Venkatramanan *et al.*[19]. The fermented cotton lint waste was centrifuged two times at 3000 rpm for 10 min, the supernatant was transferred into distillation setup where the heating temperature was set to 80°C and the ethanol being collected in a graduated conical flask for further analysis (the distillation was done 2 times to increase the chances of having only the ethanol).

Quantification of ethanol and reducing sugar: Samples collected after every 24 h from the fermenters were used to quantify the amount of ethanol present using Spectrophotometric method. This was done by solvent extraction followed by dichromate oxidation reaction after Sayyad *et al.*[18]. Tri-n-butyl phosphate (TBP, Sigma Aldrich, UK) was use to extract ethanol from the sample. This was performed by mixing equal amount of sample and TBP in a microtube, the mixture was vortexed for 3 min., after which it was centrifuged at 3000 rpm for 5 min to allow the formation of an aqueous and an organic layer. The upper organic (TBP) layer was transparent, while the lower aqueous phase turned turbid 500 µL of the upper phase (TBP) was mixed vigorously with 500 µL of dichromate reagent (10% w/v $K_2Cr_2O_7$ in 5 M H_2SO_4) for 1 min. The mixture was allowed to stand for 10 min under standard atmospheric temperature and pressure to allow oxidation product to develop at the lower phase resulting in blue green colouration. The 100 µL of the oxidation products were mixed with 900 µL of deionized water and the optical density was measured at 595 nm (A595) using spectrophotometer. the amount of ethanol in the sample was deducted using the ethanol standard curve derived from a relationship of absorbance (A595) and the concentrations of ethanol. Similarly, reducing sugar content was measured using protocol reported by Miller[20] and Sebayang *et al.*[17]. Using this method, 3, 5-dinitrosalicylic acid (DNS) was used to quantify the reducing sugar content by UV spectrophotometer.

NMR analysis: NMR experiments in this research were performed using a Bruker Avance (AVIII) 400 MHz. NMR spectra of the samples and ethanol standard were recorded in D_2O (0.30 mL in 0.65 mL) at room temperature. Bruker's Topspin 4.0.1 software was used for processing of the spectra obtained. The chemical shifts were recorded in part per million referenced to acetone.

GC analysis: The presence of ethanol in the samples was determined using a BP20 column on a Varian CP 3900 gas chromatography system equipped with a flame ionization detector (FID). A 100 ppm (v/v) of each sample and ethanol standard was prepared in ultra-pure water and 1.0 µL of the prepared sample was injected into the GC system at a split ratio 1:10 using He as the carrier gas at a flow rate of 1.0 mL min^{-1}. The column temperature was maintained at 80°C. The injector and detector temperatures were set at 200 and 280°C, respectively. The detector gases used were nitrogen at 60 psi, hydrogen at 40 psi and air at 60 psi which make up of flow at 30 mL min^{-1}, 30 mL min^{-1} and 300 mL min^{-1}, respectively. A Compass CDS software was used for processing of the chromatogram.

Statistical analysis: The data generated from this experiment were processed using different statistical package including Excel (for graphs), Standard error were deducted from experimental replicate and t-test was used to compare bioethanol production between data generated from 5 and 3% acid pre-treated samples using SPSS software.

RESULTS AND DISCUSSION

The sample of the grounded cotton lint waste was analysed for the different proximate composition including moisture content, ash content, crude lipid content, crude protein content, crude fiber content, carbohydrate and Fat content using standard techniques (Table 1). The cotton lint waste was determined to contain a high crude fibre content, Low content of moisture which conforms with the existing literature[21,19]. Low moisture content will enable the cotton waste to be stored for a longer time with less expose to microbial attack. While high amount of fibre and carbohydrate in cotton waste could be a good indicator of potential carbon source for bioethanol production[19]. To put the observed values (Table 1) into perspective, MacIntosh *et al.*[22] studied ethanol production from cotton gin trash and reported ash content of 10%, carbohydrate content of 17% and fibre content of 28%. Comparatively, the ash content in this study is lower than reported by MacIntosh *et al.*[22] perhaps due to nature of the material and physicochemical parameters of the cotton used. Generally, the results obtained in this study suggested that cotton lint waste has the potential to be used as substrate in bioethanol production

Ethanol fermentation of waste cotton lint waste using *Saccharomyces cerevisiae*: Ethanol production by *Saccharomyces cerevisiae* requires simple sugars[21], therefore cotton lint waste, a polymer of simple sugars, was pre-hydrolysed to break the glycosidic linkages in the cellulose and the fibre into simple sugars to make available for *Saccharomyces cerevisiae* to use during fermentation. Fermentation of the hydrolysis products has revealed steady ethanol production over seven days. From Fig. 1, it could be seen that there was a steady increase in the amount of ethanol produced over the period of seven days which conforms with other findings[23]. The same trend was reported by Malik *et al.*[21] and Cruz *et al.*[24] while evaluating alcohol production. The study also support report by Nikolić *et al.*[25] who observe steady increase in bioethanol production from cotton waste. However, lower production of ethanol observed when the cotton lint waste was pre-treated with 3% acid could be as a result of lower amount of monosaccharides liberated due to either low acid concentration (3%) or the time (24 h) left for hydrolysis as reported by Venkatramanan *et al.*[19].

Figure 2 shows the growth curve of *Saccharomyces cerevisiae* in the fermenter had an extremely short Lag phase and the exponential phase began almost immediately, which was because the yeast was taken from a dextrose peptone medium that is an exponentially growing culture. This could

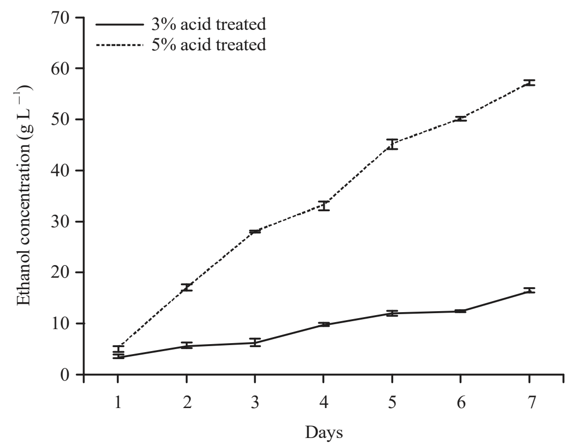

Fig. 1: Bioethanol production from cotton lint waste
Fermentation of the 3, 5% hydrolysed cotton lint waste using *Saccharomyces cerevisiae* shows a steady increase in bioethanol production over seven days, ethanol concentration for 3 and 5% acid hydrolysis deducted using the spectrometric methods, values represent Mean±SE, significant difference between the yield observe in 3% and that of 5% acid treated cotton lint waste (p<0.05)

Table 1: Proximate composition (%) of the cotton lint waste

Components	Mean value (%)
Moisture	2.50
Crude protein	6.65
Crude fibre	75.60
Crude lipid	2.00
Ash	1.51
Carbohydrate	11.75
Fixed carbon	3.00

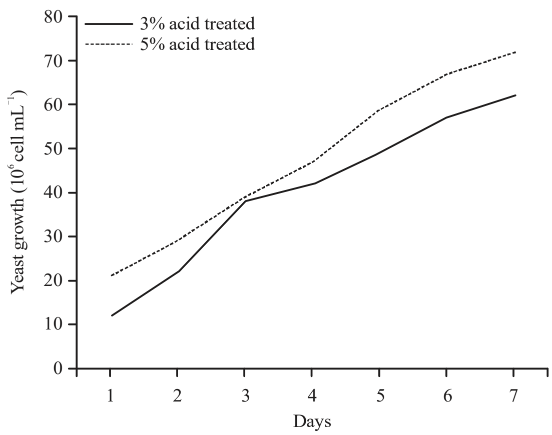

Fig. 2: Yeast growth curve
Sample obtained from bioreactor after 24 h interval was used to obtained the yeast cell count using haemocytometer, Presented in the table, is the number of yeast cell counted after every 24 h, values are those presented are evaluated in dilution factor 10^6 cell mL^{-1}

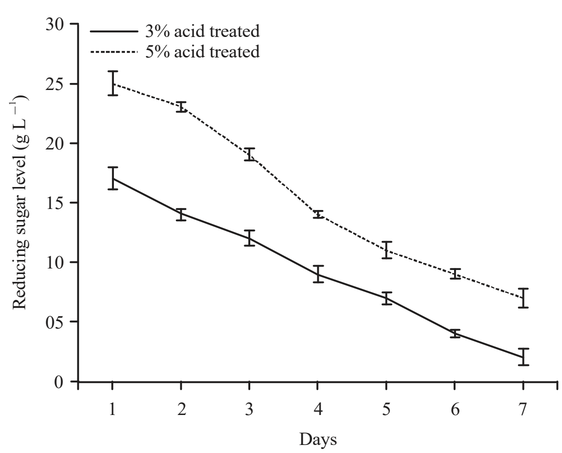

Fig. 3: Reducing sugar level
Reducing sugar level where quantified using UV spectrophotometry after 24 h during the fermentation experiment, level of reducing sugar in 3% (Orange) and 5% (blue), comparing the 2-sugar levels using t-test indicate a significant difference exist in the sugar level, values where computed in percentage and Means$\pm$SE

be observed by the increase in number of yeast cells in all the fermenters, indicating substantial utilisation of the carbon source available for fermentation and beginning of exponential growth after 1 day. Although small changes were observed in the cell counts of yeast after 72 h, this might be due to slow release of the sugar or accumulation of other sugars other than glucose[6]. However, this did not affect the quantity of ethanol produced.

Figure 3 shows the reducing sugar content of both the 3 and 5% acid treated fermenter has shown a decrease in reducing sugar level after 7 days, this explain the rate of sugar conversion to ethanol as reported by Malik *et al.*[21]. Comparing reducing sugar level between the 3 and 5% acid treated shows a significant different exist ($p<0.05$).

Ethanol identification in the samples: Analysis by NMR identified only ethanol present in the samples. The ^{1}H-NMR spectra obtained from the samples was inspected for the presence of the carbon source used in the fermentation process. Sugars have unique resonances split into 2 regions which are dependent on the chemical environment of the ^{1}H, the 1st is the anomeric region (situated downfield due to the electron withdrawing effects of the neighbouring ring oxygen atoms, 4.4-5.6 ppm) and the second is the bulk region (where the remaining ring protons are situated, 3.2-4.4 ppm).

The absence of signals in the anomeric region downfield indicated that the distilled ethanol is free from any carryover of the unused carbon source during fermentation. Uniquely for ethanol, the protons from the methyl group gives the strongest resonance upfield at 1.19 ppm due to the electron withdrawing effects of the neighbouring oxygen atom and as triplets due to the neighbouring effect of methylene group. Aless strong resonance from the methylene group is also observed at 3.66 ppm as multiplets due to the neighbouring effect of the methyl group. The absence of ^{1}H resonances associated with the secondary metabolite, glycerol, which is the largest concentration metabolite after the primary metabolites, ethanol and CO_2 during *Saccharomyces cerevisiae* fermentation suggests that pure ethanol was isolated from the fermentation media. In a similar manner, the ^{1}H NMR spectrum of all the samples contained the unique CH_3- and CH_2- resonances that is consistent with the presence of ethanol as displayed in the overlaid ^{1}H NMR of the samples and that of ethanol standard (Fig. 4).

A DEPT 135 ^{13}C-NMR spectrum performed on the samples and ethanol standard showed all the carbons present that are attached to a hydrogen (-CH_2 and -CH_3). CH_3 is shown as a positive peak (18 ppm), whereas, the CH_2 is shown as a negative peak (58 ppm) which is characteristics of those expected for ethanol (Fig. 5). The absence of any other carbon resonances confirms the purity of the distilled ethanol from secondary metabolites and pre-hydrolysed cotton lint waste.

Further analysis of the samples by GC-FID confirmed the presence of ethanol. The samples eluted as early peaks at RT = 2.47 min and the peak in each sample was identified by comparing the average retention times of the early eluting peak with that which is obtained by the average retention time of ethanol standard. All the samples were identified as having 100% ethanol by analysing their average peak areas and retention times (Fig. 6a-c).

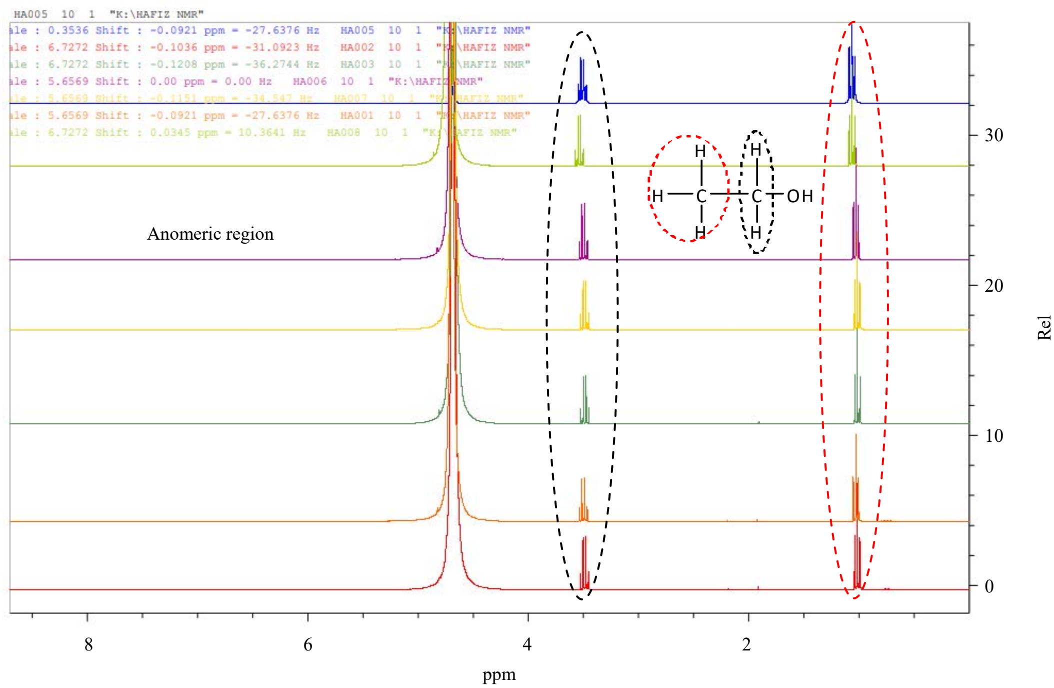

Fig. 4: Overlaid ^{1}H-NMR spectra of the samples

Ethanol showing the ^{1}H resonances of CH_3- and CH_2- in red and black dashed lines, respectively

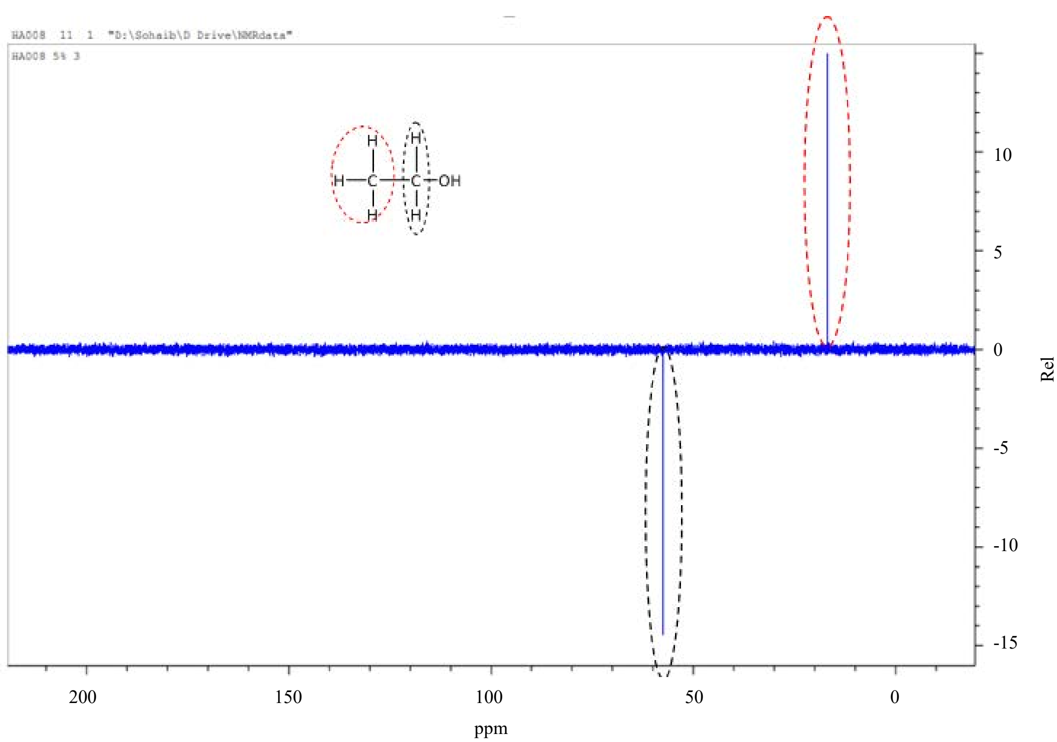

Fig. 5: A representative DEPT-135 ^{13}C-NMR spectrum recorded for one of the samples

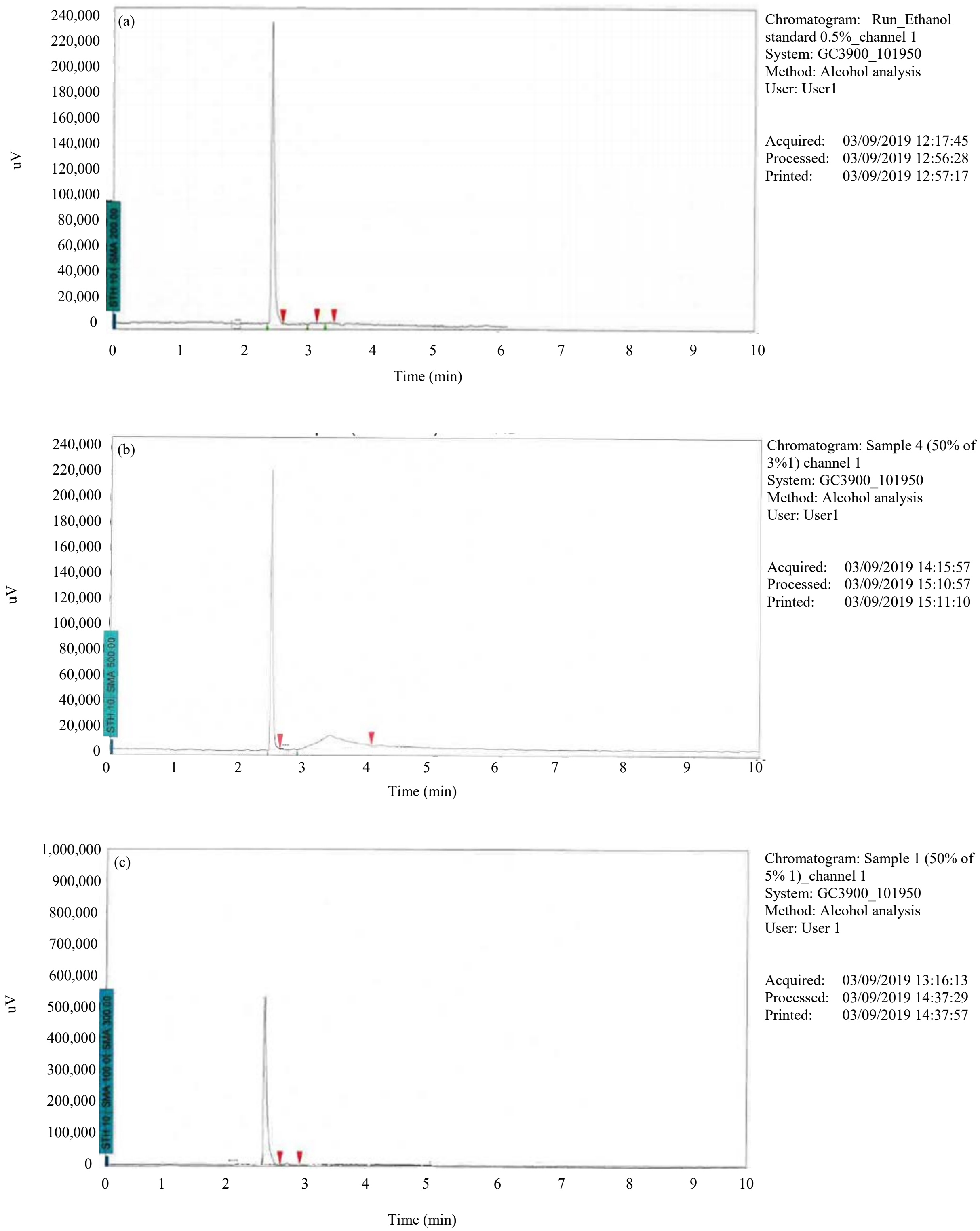

Fig. 6(a-c): Chromatogram of GC-FID analysis of fermented cotton lint waste, (a) Run_Ethanol standard 0.5% Data-FID, (b) Sample 4 (50% of 3% 1) Data-FID and (c) Sample 1 (50% of 5% 1) Data-FID

Analysis of fermented cotton lint waste with GC FID for presence of ethanol confirms the presence of bioethanol, this was done by comparing the retention time of standard ethanol and the peak generated as presented in the figure

Bioethanol is one of the renewable energy sources with so many advantages including reduces greenhouse gas emission and environmentally friendly. However, despite its numerous advantages, there are a number of shortcomings including quantity of ethanol produced, availability and nature of substrate used and cost of production. For the later, researchers are now employing the use of waste materials as substrate in bioethanol production. This will likely reduce the cost while encouraging reuse and recycle of materials. In this study, cotton lint waste that was previously been dumped as waste was used for bioethanol production. Although this study uses acid pre-treatment method combine with enzymes, future study will be to utilise a combination of alkaline and enzymes to further study production and to compare between the two pre-treatment methods.

CONCLUSION

The research presented the potential of cotton lint waste as substrate in bioethanol production. Although, slow production was observed during fermentation process, this could be due to the nature of the organisms involved and the conditions adopted. Cotton lint waste is rich in carbohydrate and could be better utilised in bioethanol production then the normal disposal system in Nigeria, similarly, the differences observed between the 3 and 5% acid pre-treatment indicate the need for improved pre-treatment method to hydrolyse the cotton and made available carbon for fermentation.

SIGNIFICANCE STATEMENT

This study further increases our understanding of waste recycling. It has been discovered that waste cotton lint could be used for bioethanol production; it was also observed that, pre-treatment strategy employ could affect production quantity. This study will assist researchers in understanding the best optimisation method to employ while using cotton lint for bioethanol production.

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