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Studies on Bioethanol Production from Rice Stalk using Co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae*

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Abstract
Bioethanol production from lignocellulosics as an alternative automobile and engine fuelhas received a considerable attention from researchers worldwide. In the current work, Bioethanol was produced from sulfuric acid and alkaline hydrogen peroxide treated rice stalk employing simultaneous saccharification and fermentation process using co-cultures of isolated and characterized strains of A. niger and S. cerevisiae. The proximate composition of the substrate was determined following standard procedures described by Association of Official Analytical Chemist. The composition of the substrate treated with sulfuric acid was moisture (4.95%), ash (4.75%), fats (4.50%),protein (5.25%), fibre (50.90%),carbohydrates (80.55%), while that of alkaline peroxide-treatment was moisture (3.65%),ash (5.10%), fats (6.60%),protein (7.00%), fibre (38.65%) andcarbohydrates (77.65%). At optimal fermentation conditions of 35ºC temperature, 5.0 pH, 4% substrate concentration, 300rpm agitation rate and 4 days fermentation period and after determining the quantity of the ethanol produced using specific gravity method, a maximum of 5.06g/100ml and 3.91 g/100ml of ethanol was obtained from sulfuric acid and hydrogen peroxide treated rice stalk respectively. The qualitative analysis using FTIR-Spectrophotometry shows the absorbance peaks of the ethanol functional groups from all the ethanol samples produced and the functional groups had their absorption peaks within their normal ranges of 3100-3600cm^{-1}, 2800-3000cm^{-1} and 1600-1675cm^{-1} for hydroxyl, alkane and alkene functional groups respectively.
Keywords: *Aspergillus niger, Bioethanol, Hydrogen peroxide, Rice stalk, Saccharomyces cerevisiae and Sulfuric acid.*

INTRODUCTION

Global concern about climate change and the consequent need to diminish greenhouse gas emissions have encouraged the use of bioethanol as a gasoline replacement or additive.

Prelude to the prediction of the world energy consumption to increase by 54% between 2001 and 2025 as a consequence of increase in world population at an alarming rate that is associated with such global concerns as global warming, depletion of fossil fuel reserves and augmentations in prices of petroleum products have obliges the search for alternative energy sources with lesser greenhouse gas emissions, cost effectiveness and sustainable carbon neutral energy sources to meet future needs(Balat *et al.*, 2008).

Lignocellulosic cellulose is abundant in nature. Cellulosic biomass of forestry, agriculture and municipal origin serves best as afeedstock for the production of biofuels and other by-products (Carlo *et* al., 2008). However, such characteristics of these lignocellulosic biomass as its crystallinity, presence of lignin and hemicellulose, inaccessible surface area, degree of cellulose polymerization, and degree of acetylation of hemicelluloses roots its resistant to enzymatic degradation. In light of that, pretreatment step is thus of paramount importance to economically convert the lignocellulosic cellulose into fermentable sugars(Wyman, 1996).

Pretreatment is amongst the most costly steps in biochemical conversion of lignocellulosic biomass, accounting for up to 40% of the total processing cost (Zheng *et al.*, 2009). Thus, cost-effective pretreatment of lignocellulosic biomass is a major challenge of cellulosic biofuels and bioproducts technology research and development in recent years (Hamelinck*et al.*, 2005).

The goal of pretreatment is to solve the problems of lignocellulosics mentioned above (Taherzadeh and Niklasson, 2004).

Aspergillus niger can be used in the industry for biomass hydrolysis, production of citric and gluconic acids, in biotechnology industries and in enzyme glucose oxidase production used in design of glucose biosensors (Ojumu*et al.*, 2003).

Saccharomyces cerevisiae is a yeast whose cells are round to ovoid, 5–10 µm in diameter that reproduces by budding. It is well and intensively studied eukaryotic model organisms in molecular and cell biology widely used and instrumental in baking, brewing and employed for most common types of fermentation(Feldmann, 2010).

Simultaneous saccharification and fermentation (SSF), (a cellulose-based ethanol production technology), is empirical providing such numerous advantages as greater ethanol yield, sequestration of inhibitory compounds released during saccharification, reduced chances of contamination (Hader *et al*., 2013).

Bioethanol as a commodity can provide opportunities for non-oil-producing countries to be self-sufficient in fuel. It is of detrimental importance apart of been a feasible alternative to fossil fuels, putting into consideration its low toxicity, biodegradability and its adventurous ability to be effectively blended with gasoline requiring not any engine modifications (Carlo *et al*., 2008) and (Harun and Danquah, 2010).

The aim of this study was to investigate the effects of alkaline hydrogen peroxide (H_2O_2) and dilute sulfuric acid (H_2SO_4) pretreatmentson the final ethanol yieldof rice stalkas substrate using isolated and identified strains of *Aspergillus niger* and *Saccharomyces cerevisiae* as co-cultures in a simultaneous saccharification and fermentation process.

MATERIALS AND METHODS

Substrate sample collection

Rice stalk substrate was obtained from the local rice processing plants in Basawa of Sabongari Local Government Zaria, Kaduna State.

Substrate preparation and pretreatment

The substrate sample collected was dried at 105ºC for 48 hrs., it was later ground, sieved to particle size of 2-5mm and left for 48 hrs. at room temperature and then stored in plastic bags until further processing. The substrate (rice stalk) was pretreated with alkaline H_2O_2 according to the method of (Rivera *et al*., 2010) and dilute H_2SO_4 acid according to the method of (Saha*et al*., 2005).

After the pretreatment, the proximate/composition analysis of the substrates was as well carried out on the treated and non-treated substrate(A. O. A. C, 2010) at Department of Food Sciences, Faculty of Agricultural Sciences/I.A.R, A.B.U. Zaria.

Sources of Fungal Cultures

The *Aspergillus niger* culture was isolated from a garden soil while the *Saccharomyces cerevisiae* culture was obtained from palm wine, an ideal habitat to obtain a fungal strain that adapts well to fermentation environment for optimal ethanol yield. The samples of the soil and the palm wine are all serially diluted using a tenfold dilution and plated out on a Saboroud Dextrose agar in order to obtain a pure culture (Ameh *et al*., 1989) and (Ratnasri *et al*., 2014).

Media

The growth medium used for preparing the organisms and for the simultaneous saccharification and fermentation process is called the basal or liquid mineral medium and it constitutes the following (per 100ml); Peptone, 0.1g; Malt Extract, 0.1g; Yeast Extract, 0.2g; Magnesium Chloride, 0.1g; Calcium Carbonate, 0.2g; Ammonium Phosphate, 0.2g; and Ferrous Sulphate, 0.001g and the substrate that will serve as the sole source of carbon, 4.0g (Ado*et al.,* 2010).

Inocula preparation

The inocula for *A. niger* was prepared from the stored slant cultures of Saboroud Dextrose agar (oxoide) by washing with 10ml of 0.25% sterile tween 80 and counting under the microscope with a haemocytometer in order to obtain the cfu/ml of organism (Ado*et al.,* 2010).

S. cerevisiae inocula was prepared by re-growing the organism in a Yeast Peptone Glucose broth after which it was been centrifuged, washed with distilled water and compared with McFarland's standard scale one (Ado *et al.,* 2010).

The fermentation medium was inoculated with both of the inocula at a concentration of 5% v/v. *A. niger* corresponding to 2.5 x 10^6 spores/ml and *S. cerevisiae* corresponding to 3 x 10^8 cells/ml for Simultaneous Saccharification and Fermentation (SSF) (Ado *et al.,* 2010).

Experimental set-up and fermentation procedure

Simultaneous saccharification and fermentation was carried out in Erlenmeyer conical flasks containing mineral media. The media contains the compositions stated above and the 4% of the substrate pretreated with alkaline H_2O_2 and dilute H_2SO_4 acid. The solutions in the whole flaks were prepared and autoclaved and after autoclaving, the inocula were aseptically introduced and incubated (Ado *et al.,* 2010) and (Hader *et al.*,2013).

Quantitative analysis of the ethanol samples produced

Preparation of ethanol specific gravity standard curve

Eight stocks of pure ethanol of varying concentrations 2%, 4% to 16% were prepared. Five (5ml) of each of these concentrations was taken into specific gravity bottle and weighed against equal volume of distilled water.

The values obtained were then used to plot a standard curve against the eight known concentrations (Emeka, 2001).

Determination of specific gravity and concentration

This was done by removing 30ml from each fermentation flask and centrifuging it at 1500rpm for 3minutes after which the content has been distilled at 78.3^{0}C at 24hrs intervals and the distillate was obtained and its volume noted (Ado *et al.*, 2010). A 30mls capacity specific gravity bottle was filled with distilled water of equal volume with the distillate to be measured. The bottle was then emptied, dried and then filled with the produced sample and then reweighed and the specific gravity was measured as follows:

$$\text{Specific gravity} = \frac{\text{Weight of unit volume of the sample}}{\text{Weight of unit volume of water}}$$

The specific gravity values for the produced ethanol samples obtained using the formulae above were used to determine the ethanol concentration from a standard curve prepared using known concentrations of pure ethanol (Emeka, 2001).

Qualitative analysis of ethanol samples produced

The quality test of the produced ethanol sample was achieved by infrared determination that was carried out on all the produced ethanol samples using FTIR spectrophotometry. The infrared spectra of an absolute ethanol was used as a gold standard (Ado*et al.*, 2010).

RESULTS

Proximate Composition of the Substrate

Following the pretreatment of the substrate with dilute sulfuric acid and alkaline hydrogen peroxide, the proximate composition of the substrates was determined. Table 1is the proximate composition of rice stalk treated with both sulfuric acid and hydrogen peroxide.

Ethanol standard curve

Figure 1 is the ethanol standard specific gravity curve that was prepared by plotting the specific gravities of different known concentrations of ethanol against their concentrations.

Ethanol yield by co-cultures of *A. niger* and *S. cerevisiae* from sulfuric acid-treated rice stalk

The result of ethanol yield from sulfuric acid-treated rice stalk is presented in figure 2 below. The highest and the optimum ethanol yield of this research was obtained from sulfuric acid-treated rice stalk which was 5.06%.

Ethanol yield by co-cultures of *A. niger* and *S. cerevisiae* from peroxide-treated rice stalk

Ethanol yield from hydrogen peroxide-treated rice stalk is presented in Figure 3. The highest ethanol quantity obtained from this treatment option was 3.91% while the least was recorded to be 1.73%.

Table 1: Proximate composition of the treated and untreated rice stalk substrates

	Proximate Compositions (%)					
Sample Code	**Moisture**	**Ash**	**CF**	**CP**	**CFb**	**CHO**
N-T RS	5.00	6.80	6.90	5.25	33.90	76.05
H_2O_2-T RS	3.65	5.10	6.60	7.00	38.65	77.65
H_2SO_4-T RS	4.95	4.75	4.50	5.25	50.90	80.55

KEY:
CF: Crude fat.
CP: Crude protein.
CFb: Crude fibre.
CHO: Carbohydrates
N-T RS: Non-treated rice stalk.
H_2O_2-T RS: Hydrogen peroxide-treated rice stalk.
H_2SO_4-T RS: Sulphuric acid-treated rice stalk.

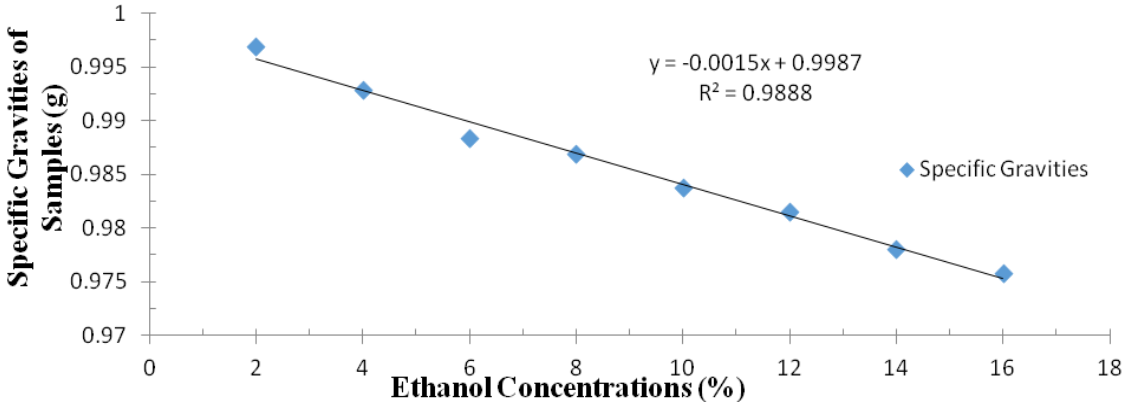
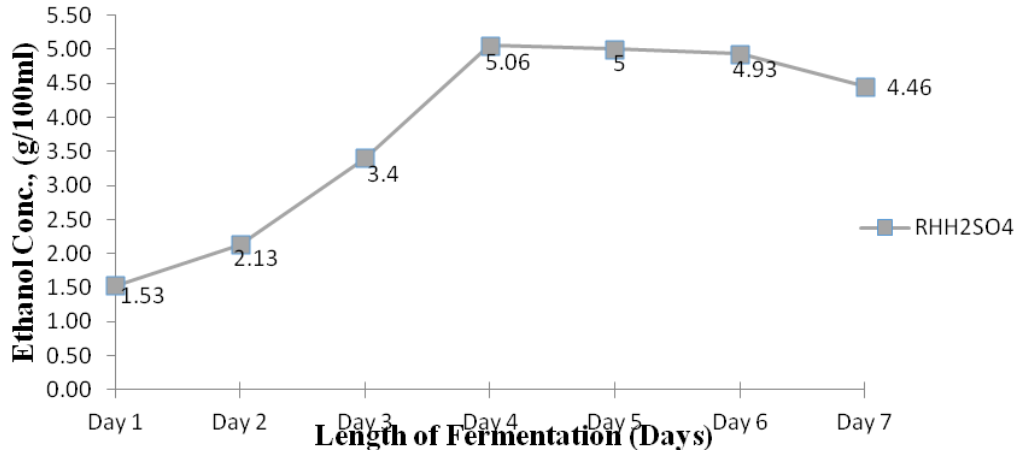


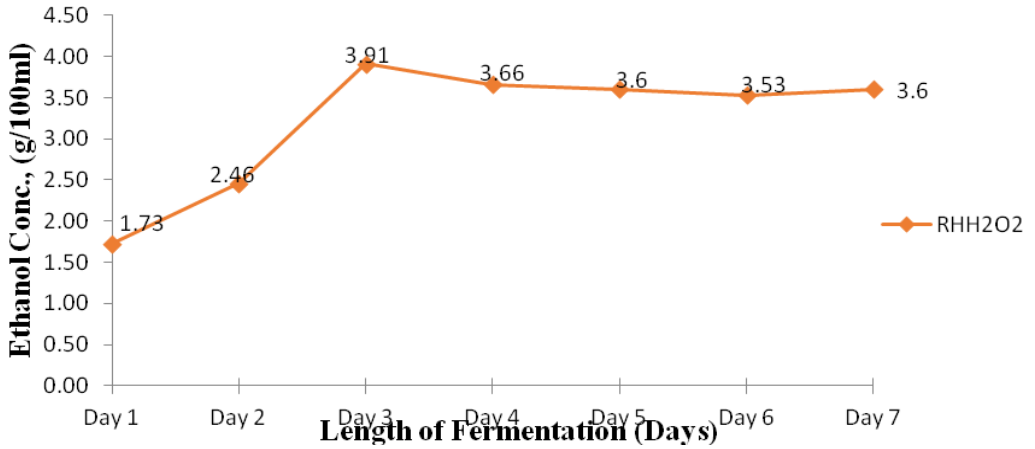
Figure 1: Ethanol standard specific gravity curve.



t = 1.6967 *df = 6* *p = 0.1407*

Figure 2: Ethanol Yield by co-cultures of *A. niger* and *S. cerevisiae* from Rice Stalk Treated with Sulphuric acid.

KEY: RS-H_2SO_4: Sulphuric acid-treated rice stalk.



t = 1.6967 *df = 6* *p = 0.1407*

Figure 3: Ethanol Yield by co-cultures of *A. niger* and *S. cerevisiae* from Rice Stalk Treated with Hydrogen Peroxide.

KEY: RS-H_2O_2: Hydrogen peroxide-treated rice stalk.

FTIR spectrophotometry of the absolute ethanol golden standard

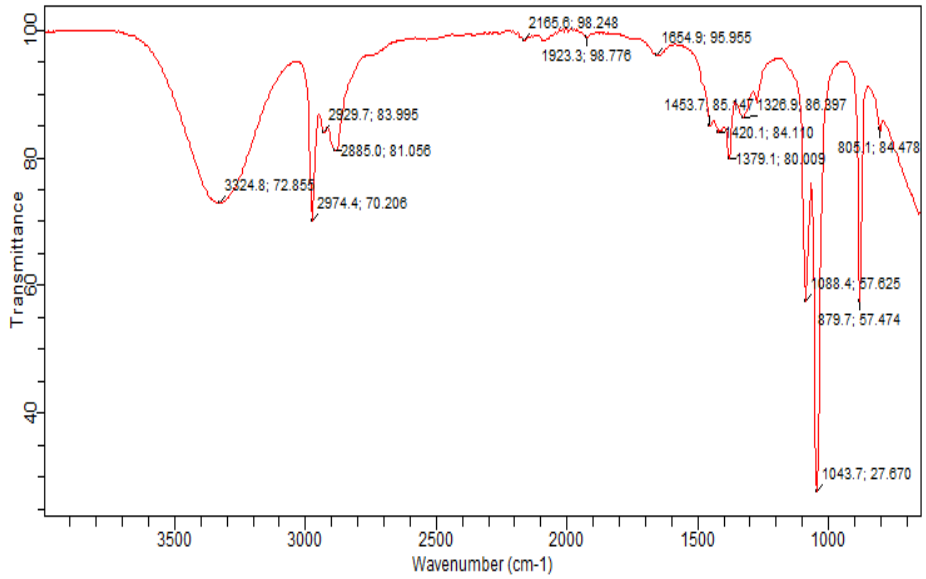
The result of the Fourier transform infrared spectrophotometric analysis of the gold standard was given in Figure 4. It explains the specific wavelengths or their ranges within which specific functional groups of the test samples can be detected.

FTIR spectrophotometry of the sulfuric acid-treated rice stalk ethanol sample

Figure 5 shows the result of FTIR O-H stretch and the H-C-H symmetric and asymmetric stretches of the sulfuric acid treated-rice stalk ethanol sample (RS-H_2SO_4). The absorbance of the O-H stretch was found at 3306.1cm^{-1} whereas it's (H-C-H) absorbance was at a wavelength of 2981.9cm^{-1}.

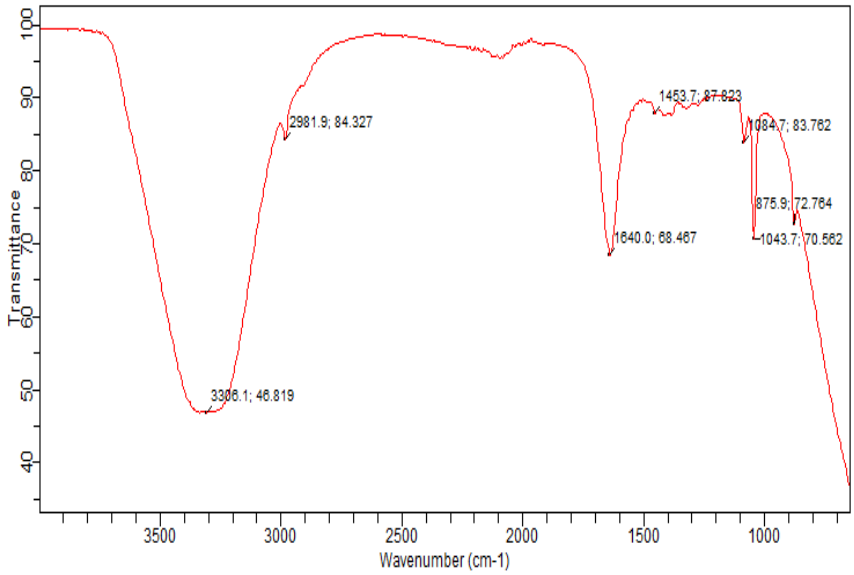
FTIR spectrophotometry of the hydrogen peroxide-treated rice stalk ethanol sample

The FTIR result of the hydrogen peroxide-treated rice stalk ethanol sample (RS-H_2O_2) is shown in Figure 6. The hydroxyl (OH) group of this sample had its absorbance at a wavelength of 3306.1 cm^{-1} but interestingly unlike the rest, there was no H-C-H stretches absorbance (the absorbance of saturated part of the compound for this sample).



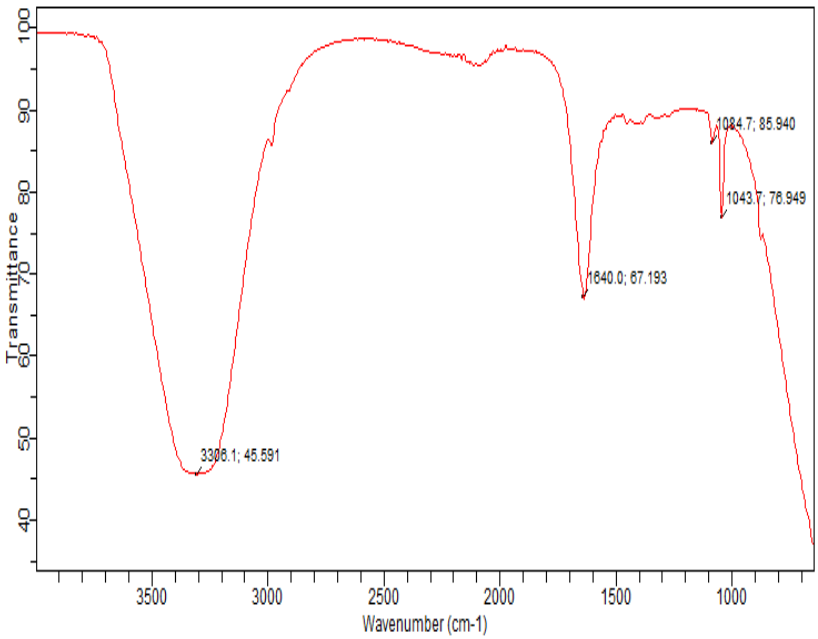
_____ : Ethanol gold standard.

Figure 4: FTIR spectrophotometry of the ethanol gold standard



_____ : RS-H_2SO_4 Ethanol sample.

Figure 5: FTIR spectrophotometry of the sulphuric acid-treated rice stalk (RS-H_2SO_4) ethanol sample.



: RS-H_2O_2 Ethanol sample.

Figure 6: FTIR spectrophotometry of the hydrogen peroxide-treated rice stalk (RS-H_2O_2) ethanol sample.

DISCUSSION

Proximate composition

Previous work reported (86%) carbohydrate content, 3.3% ash, 2.3% protein and 7.5% moisture contrary to the maximum carbohydrates content shown by this research 80.55%, the ash content is higher (5.10%), protein content is higher (7.00%) and moisture content is lower (4.95%) than that shown above by (Omoniyi and Olorunnisola, 2014). Saponification of the intermolecular ester bonds and the heterogeneous breakages of hydrogen bonds by both hydrolytic chemical reaction & physical factors are the mechanisms of lignocellulosic hydrolysis of peroxide and acid respectively (Xiang*et al.*,2003).

Ethanol standard curve

This standard curve was used to compare with the values of specific gravities obtained in this research work in order to ascertain the concentrations of the ethanol that has been produced.

Ethanol yield by co-cultures of *A. niger*and *S. cerevisiae* from sulfuric acid-treated rice stalk

The highest ethanol quantity of 5.06% obtained from this treatmentcan be the attributed to the influence of a complement of high percentages of ash and protein (6.30% and 3.05% respectively) contents as well as the relatively higher carbohydrate content of 84.15% observed with the sulfuric acid-treated cane bagasse after proximate composition analysis. This result is in partial contrast to 9.62g/100ml of (Lakkana*et al.*, 2012).

Ethanol yield by co-cultures of *A. niger* and *S. cerevisiae* from peroxide-treated rice stalk

The least quantity of ethanol of 3.91% obtained from this optioncan be justified by the percentages of convertible sugars present in the substrate as it was found to have the least carbohydrate content as illustrated in Table 1. This is comparably having the best and highest amalgamative and cumulative effect of ash and protein contents (5.10% and 7.00% respectively) representing high mineral salt and protein source respectively that are required by *S. cerevisiae* for enhanced cellular growth and bioethanol production, probably this may affect the speed of production and maximum yield to be obtained within the shortest period of time thus better in supporting biomass production. These results are in partial contrast to 9.62g/100ml, 7.68g/100ml demonstrated by (Lakkana *et al.*, 2012) and (Arifa *et al.*, 2010) respectivelyand in partial agreement with that of (Harun and Danquah, 2010) that realized a final ethanol quantity of 4.30g/100ml.

FTIR spectrophotometry of the absolute ethanolstandard, sulfuric acid and peroxide-treated rice stalk ethanol samples.

The result of the Fourier transform infrared spectrophotometric analysis of the gold standard explains the specific wavelengths or their ranges within which specific functional groups of the test samples can be detected.

The absorbance of the O-H stretch of sulfuric acid-treated rice stalk ethanol sample was found at 3306.1cm^{-1} whereas it's (H-C-H) absorbance was at a wavelength of 2981.9cm^{-1}.The hydroxyl (OH) group of peroxide-treated rice stalk ethanol sample had its absorbance at a wavelength of 3306.1 cm^{-1} but interestingly unlike the rest, there was no H-C-H stretches absorbance (the absorbance of saturated part of the compound for this sample).This may perhaps be largely due to either excess dilution or oxidation of the sample that is ought to have taken place. This is supported by the fact that the chemical agents (sulphuric acid and hydrogen peroxide) used for pretreatment are all oxidizing agents. This leads to formation of unsaturation because of the removal of hydrogen atom leading to realization of alkene with –C=C symmetric stretch whose absorbance was at a wavelength of 1640.0 cm^{-1}. Further oxidation will lead to formation of alkynes with –C≡C stretching that will be found at wavelength range of 2100-2200 cm^{-1}.The presence of the absorptionwavelengths of these functional groups in the ethanol samples produced consent to the quality of the product as buttressed by(Aiyejagbara, 2015)who also showed the absorptions of these functional groups within ranges similar to the once reported in the current study.

CONCLUSION

At the end of this study, dilute sulfuric acid treatment of bioethanol production substrate was advantageous over alkaline hydrogen peroxide treatment because it bequeath realization of higher percentages of convertible sugars following proximate analysis and thus, the highest ethanol yield. Likewise, simultaneoussaccharification and fermentation of rice stalk substrate in bioethanol production is more exploratory and courageously feasible than the previously employed techniques.

Recommendations

1. There is need for species improvement of the yeast in order for it to be able to ferment pentose and hexose at the same time for this will improve the overall production efficiency of ethanol even at commercial level.
2. Further research should be conducted for novel lignocellulosic biomass, determine a suitable pretreatment for their maximum sugar recovery and even attempt their genetic improvement such that lignocellulosic plants with increased yields of fermentable sugars, requiring less costly preprocessing will be available for bioethanol production.

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