Original Research Article

COMPARATIVE STUDIES ON PRODUCTION OF BIOETHANOL FROM RICE STRAW USING *Bacillus subtilis* AND *Trichoderma viride* AS HYDROLYZING AGENTS.

ABSTRACT: Ethanol production by S. cerevisiae was carried out using rice straw as substrate and B. subtilis and T. viride as hydrolyzing agents. The aim of this research is to compare the potential of rice straw (non-edible waste material) for bioethanol production using Bacillus subtilis and Trichoderma viride as cellulose hydrolyzing agents. The sample was dried and ground; and was subjected to chemical pretreatment and microbial hydrolysis to maximize sugar production. Standard methods were used to carry out isolation, identification and analysis of sample which includes proximate, mineral and physicochemical analysis. The sample was fermented for seven days during which ethanol yield was determined. Cellulose hydrolysis screening carried out on each of the two organisms revealed T. viride having the higher clearance zone of 1.8cm, while B. subtilis had 1.5cm. Proximate analysis obtained from the samples showed that the pretreatment method was relatively effective giving an increase in the cellulose and decrease in the hemicellulose and lignin contents of the samples. This showed rice straw having a cellulose content of 51.33 ± 0.17% after pretreatment. Potassium content was relatively high (17.96mg/g), Hydrolysis using T. viride gave higher reducing sugar yield than that obtained using B. subtilis with 26.6g and 12.21g respectively. The pH was observed to decrease during fermentation while total titratable acidity observed showed an increase. Highest ethanol yield of 16.21g/100g was obtained using T. viride as hydrolyzing agent.

Keywords: Bioethanol, Rice straw, Bacillus subtillis, Trichoderma viride, Saccharomyces cerevisiae

INTRODUCTION

Ethanol, also called ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol, is a volatile, flammable, colorless liquid. (Ocloo and Ayenor, 2010).

The environmental crisis and the shortage of fossil fuels have turned public attention to the utilization of other forms of energy, which are environmentally friendly and renewable, such as bio-ethanol. During recent years research has focused on the so-called second-generation biofuels, where wastes or by-products are utilized as raw material, compared to the first-generation biofuels where sugars and starch were utilized, as the utilization of either sugars or corn for the production of biofuels have contributed to the increase of their price worldwide, resulting in severe problems for the poorer countries (Matsakas *et al.,* 2014). Lignocellulosic biomass represents great potential to be utilized as raw material due to the high amounts produced every year, and their wide availability.

Rice straw is one of the abundant lignocellulosic waste materials in the world. It is annually produced in large quantity reaching about 731 million tons distributed in Africa (20.9 million tons), Asia (667.6 million tons), Europe (3.9 million tons) and America (37.2 million tons). This

amount of rice straw can potentially produce 205 billion liters of bioethanol per year, which is the largest amount from a single biomass feedstock.

MATERIALS AND METHOD

Source for Samples

One thousand (1,000) grams of fresh rice straws were collected from Ekiti state (Igbimo). The sample was washed thoroughly; sun dried and powdered using Tiger ES250 Engine food processor. The sample was divided into two portions; the first portion was used for analysis while the second was used for fermentation.

Pretreatment of samples

A two-stage process which combines the Dilute Acid Pre-hydrolysis (DAPH-100-121) and alkaline delignification using NaOH as described by Olugbenga and Ibileke (2011) was used. Dry sample was treated with dilute sulfuric acid which involved the use of 1.25% (w/v) H_2SO_4 solution in a 1:8 g: g solid: liquid ratio. The One step Dilute Acid Pre-hydrolysis (DAPH-121) was performed in an autoclave at 121°C for 17 min, after which the solids were collected and drained. The solids were then treated with 2% (w/v) sodium hydroxide solution in a solid: liquid ratio of 1:20 g: g, at 120°C for 90 min. After that, the residual solid material (cellulose pulp) separated by filtration was washed with water to remove the residual alkali, and was dried at 50 ± 5°C for 24 h.

Microorganisms for Fermentation

Saccharomyces cerevisiae was obtained from the stock culture of Microbiology Laboratory Federal University of Technology, Akure, while *Bacillus subtilis* and *Trichoderma viride* was isolated from rice straw.

Cellulose Hydrolysis Test on Isolated Strains of *Bacillus subtilis* and *Trichoderma viride*

A modified method of Van Tuan and Dinh Thi (2010) was used. Cellulase activity was examined relatively by measurement of the diameter of clearance on agar plates containing substrate (carboxy methyl cellulose). Bacterial and fungi isolates were grown in appropriate liquid medium containing 0.5% (w/v) CMC for 48 and 96 hours respectively.
After incubation, centrifugation was carried out, after which 0.5ml of each culture supernatant was dropped into wells made on appropriate agar plates containing 0.5 % (w/v) CMC and incubated for 24 hours at 4°C to diffuse enzyme. After that, the agar plates were incubated for further 24 hours at 37°C, after which the plates were stained with 1% (w/v) lugol iodine, the diameter of clearance of each plate was then measured.

Preparation of Inoculum

*Trichoderma viride, Bacillus subtili*s and *Saccharomyces cereviceae* inocula were prepared by introducing slant cultures to 150ml of sterile growth media contained in 500ml conical flasks. The flasks were incubated on a rotary shaker at 30^0C for 96 hours (Ado *et al.,* 2009).

Ethanol Fermentation Medium

Rice straw was used as the base medium supplemented with all of the other ingredients of growth medium.

Microbial hydrolysis

One hundred (100) grams of the pretreated substrate was weighed in duplicates into 1000ml conical flasks and made up to mark with distilled water, corked and sterilized at 121^0C for 15 min. Sterile distilled water was added to the flasks to a final volume of 1 liter and the flasks plugged with sterile cotton wool. After cooling, the medium was inoculated with 50ml of 36hours culture of *Trichoderma viride* and *Bacillus subtilis* separately; the pH of the medium was then adjusted to 5.5. Hydrolysis was carried out at ambient temperature for five days on a rotary shaker. A second un-inoculated flask served as control. Samples were taken at the end of Five days for reducing sugar determination (Abdullahi, 2013).

Fermentation

Each of the medium from the above was inoculated with 50ml culture of *Saccharomyces cerevisiae* to carryout fermentation for seven (7) days. The fermentation was then monitored from day 1, the pH of the hydrolysate containing *Saccharomyces cerevisiae* was adjusted to 5.5 and fermentation carried out at ambient temperature on a rotary shaker. The ethanol yield was determined at 48 hours interval during fermentation. The fermentate was separated by centrifugation at 9000 rpm to separate the waste from the supernatant (Abdullahi, 2013). All procedures were carried out in triplicates.

Centrifugation and Distillation

After fermentation, the broth was centrifuged at 6000rpm for 10 minutes. The supernatant was collected and fed into a simple distillation column. The boiling temperature of ethanol is 78°C hence distillation was carried out around that temperature to facilitate the evaporation of ethanol. The vapour was collected and got condensed by means of the circulation of cold water around the column. The distillate having ethanol was recovered in a conical flask at the other end of the column.

Determination of Reducing Sugar Produced

The reducing sugar was determined by titrimetric methods. 30ml of hydrolysis broth was weighed into the burette. 10ml of mixed Fehlings solution was pipetted into a conical flask and 4 drops of 1% methylene blue indicator was added. The solution was heated and while boiling the broth on the burette was titrated against the solution on the conical flask until the colour disappears (Srichuwong and Jane, 2011). The reducing sugar was calculated as follows:

$$\%\text{Reducing Sugar} = \frac{47.5 \times 300}{T \times W}$$

T represents the titre value

W represents the weight of the peel sample

Determination of Ethanol Yield

The distillate collected was weighed and its mass obtained, ethanol yield was the expressed as weight of ethanol in grams/weight of sample in grams. (Abdullahi 2013).

Determination of Ethanol Concentration

Ethanol concentration was determined by comparing the density of the ethanol produced with the standard ethanol density curve. Standard ethanol curve would be obtained by taking series of percentage (v/v) ethanol (10%, 20%, 30%, 40% and 50%) solution which was prepared in a 100ml volumetric flask and the weights was measured as described by Oyeleke and Jibril (2009). The density of each of the prepared ethanol solution was calculated and a standard curve of density against percentage ethanol (w/v) was plotted.

Determination of pH.

The pH of each fermenting substrate was measured at 24 hours interval for seven days using a digital pH meter, standardized with buffer of 7.0. The pH was then determined by inserting the electrode bulb into a sample from each fermenting substrate.

Total titratable acid

This was determined using the method of Lyumugabe *et al.* (2010) 10ml of the fermenting medium was transferred into a beaker, followed by the addition of 3 drops of phenolphthalein indicator. The sample was then titrated against 0.1M NaOH to an end point of a definite pink color. The volume of NaOH used was noted and the titratable acid percentage was calculated using the following formula:

$$TTA(\%) = V \times 0.15$$

Were; V = Volume of NaOH used

Comparative analysis of the properties of bioethanol produced from rice straw and commercial ethanol

Comparative analysis of properties (such as appearance, relative density, melting point, boiling point, viscosity, refractive index, burning characteristics and flash point) of bioethanol produced from cocoyam and sweet potato peels and conventional ethanol was determined using standard methods (A.O.A.C, 2012).

Statistical Analysis

The data obtained from various treatments was expressed as the mean ± standard error. Computer software SPSS 16 was used to determine the differences between each parameter using one-way analysis of variance (ANOVA). Duncan multiple range tests was used to compared the means at probability level of 0.05%

RESULTS

The cellulose hydrolyzing ability of each of the isolated organism is shown on table 1. The two isolated organisms tested positive for cellulose activity. *Trichoderma viride* showed the higher cellulose hydrolyzing ability with clearance diameter of 1.8cm. *Bacillus subtilis* on the other hand showed a clearance diameter of 1.5cm.

The proximate composition of Rice straw is giving in figure 1. Rice straw had a moisture content of 7.86%, protein content of 3.10%, fiber content of 20.83% and lipid content of 1.43%. Rice straw was however observed to contain high cellulose content of 33.50.

The mineral composition Rice straw is shown in table 2. Rice straw showed high Potassium content of 17.96mg/g, likewise the same was observed for Calcium (3.00mg/g). It was also shown that rice straw was relatively low in sodium content. Nitrogen content gave 0.80mg/g. It can however be observed that rice straw had high mineral composition.

Table 1: Diameter of clearance for cellulose hydrolysis (cm) by the microbial isolates

Microorganisms	Cell diameter	Diameter of clearance
Bacillus subtillis	0.80 ± 0.00^{a}	1.50 ± 0.00^{e}
Trichoderma viride	0.90 ± 0.17^{a}	1.80 ± 0.10^{e}

Data are represented as mean ± standard deviation, n=3 with the same superscript down the row are not significantly different ($p<0.05$)

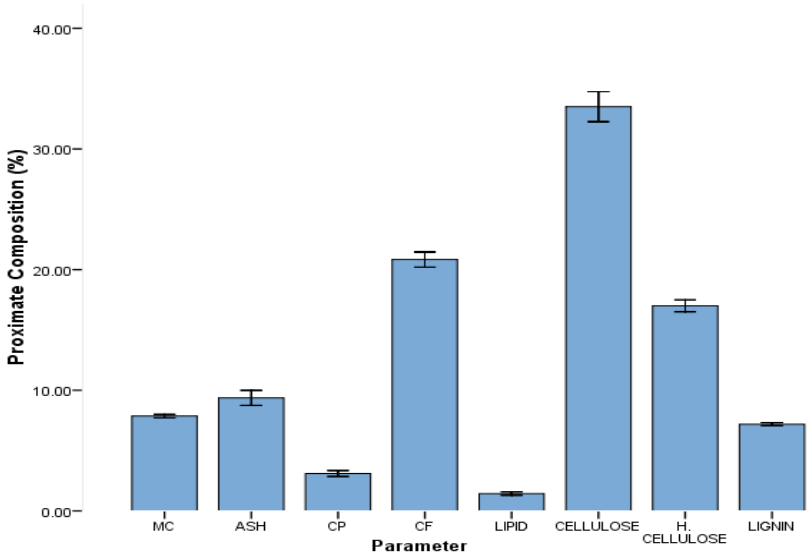

Figure 1: Proximate composition of rice straw before pretreatment (%)

Key; MC=Moisture content, CP=Crude protein, CF= Crude fibre

Table 2: Mineral composition rice straw

Parameter	Rice straw
Na	2.30 ± 0.40^{b}
K	17.96 ± 0.15^{c}
P	0.90 ± 0.03^{c}
Ca	3.02 ± 0.07^{c}
C	48.90 ± 0.05^{d}
Mg	1.87 ± 0.03^{b}
N	0.80 ± 0.01^{a}
C:N Ratio	61.12 ± 0.11^{e}

Key: Na=sodium, K= potassium, P=phosphorous, Ca=calcium, C=carbon Mg=magnesium, N=nitrogen

Data are represented as mean ± standard deviation, n=3 with the same superscript across the column are not significantly different ($p<0.05$)

Table 3 shows the effect of pretreatment on the cellulose, hemicellulose and lignin components of rice straw. There was a relatively high increase in cellulose from 33.50% to 51.30%. It was also observed that hemi-cellulose decreased from 17.00% to 10.67%. Similar decrease is shown for lignin content as well, with a decrease from 7.19% to 5.68%.

The reducing sugar produced after five days of hydrolysis using *T. viride* and *B. subtilis* is giving in figure 2. *T. viride* showed the higher reducing sugar yield of 26.60g/100g. *B. subtilis* on the other hand gave relatively lower yield of 12.21g/100g.

Table 3: Effect of Acid pretreatment on the cellulose, hemi-cellulose and lignin components of rice straw.

Parameter	Before Pretreatment	After Pretreatment
Cellulose	33.50 ± 0.17^{a}	51.33 ± 0.17^{a}
Hemi-cellulose	17.00 ± 0.17^{a}	10.67 ± 0.17^{a}
Lignin	7.19 ± 0.17^{a}	5.68 ± 0.17^{a}

Data are represented as mean ± standard deviation, n=3 with the same superscript down the row are not significantly different ($p<0.05$)

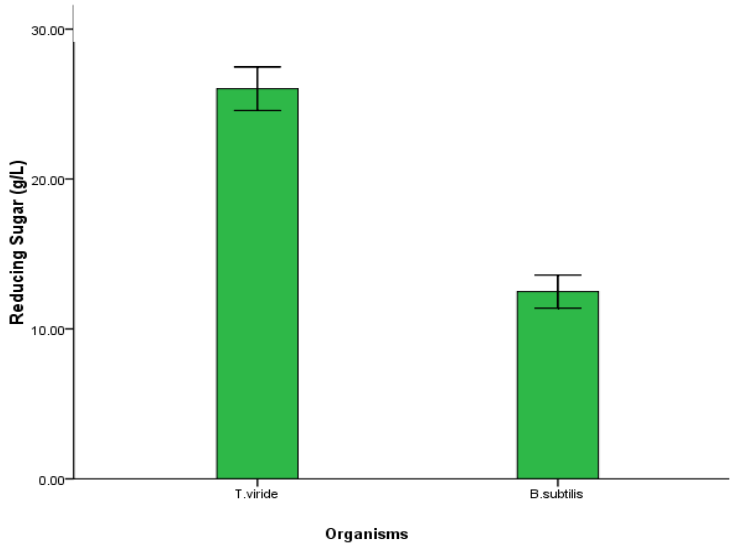

Figure 2: Reducing Sugar produced by rice straw after 3 days hydrolysis using *Trichoderma viride*

The changes in pH during fermentation rice straw using *T. viride* and *S. cerevisiae and B. subtilis* and *S. cerevisiae* is shown in figure 3. A general decrease in the pH was observed from the initial standardized pH of 5.5 as fermentation proceeded. Fermentation showed a decrease, with a final pH of 3.8 after 7 days when using *T. viride* and *S. cerevisiae,* while a final pH of 4.5 after 7 days was observed using *B. subtilis* and *S. cerevisiae.*

The total titratable acidity during fermentation of rice straw using *T. viride* and *S. cerevisiae* and *B. subtilis* and *S. cerevisiae* is shown in figure 4. An increase in the TTA was observed from the initial TTA as fermentation proceeded. Result showed an increase in TTA, from an initial TTA of 0.06% to 0.15% after 7 days when using *T. viride* and *S. cerevisiae,* while a relatively high increase in TTA, from an initial TTA of 0.09% to 1.66% after 7 days was observed while using *B. subtilis* and *S. cerevisiae.*

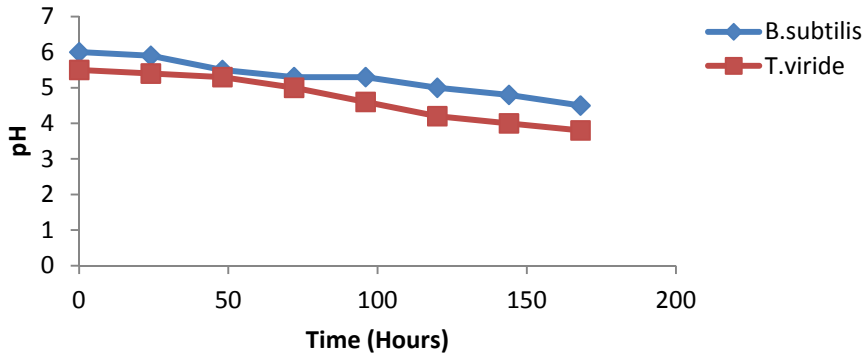

Figure 3: pH of rice straw during fermentation using *T. viride* and *S. cerevisiae* and *B. subtilis* and *S. cerevisiae*

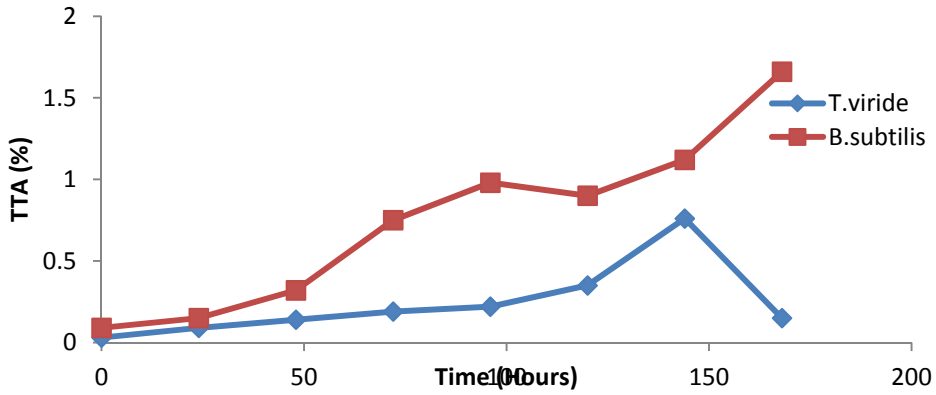

Figure 4: Total titratable acidity (%) of rice straw during fermentation using *T. viride* and *S. cerevisiae* and *B. subtilis* and *S. cerevisiae*

Figure 5: shows the ethanol yield of the rice straw during seven days fermentation using *T. viride* and *S. cerevisiae* and *B. subtilis* and *S. cerevisiae*. The ethanol yield was observed to increase as the fermentation proceeded. The combination of *T. viride* and *S. cerevisiae* had an initial yield of 1.92g/100g while *B. subtilis* and *S. cerevisiae* had an initial yield of 1.90g/100g after 24 hours. However after 7 days of fermentation, *T. viride* and *S. cerevisiae* showed a final yield of 16.21g/100g while *B. subtilis* and *S. cerevisiae* showed a final yield of 7.59g/100g.
The percentage (v/v) of final ethanol produced by rice straw using *T. viride* and *S. cerevisiae* and *B. subtilis* and *S. cerevisiae* is given in figure 6. The highest percentage of 2.08% was givin by the combination of *T. viride* and *S. cerevisiae*, while *B. subtilis* and *S. cerevisiae* gave a final volume of 0.96%.

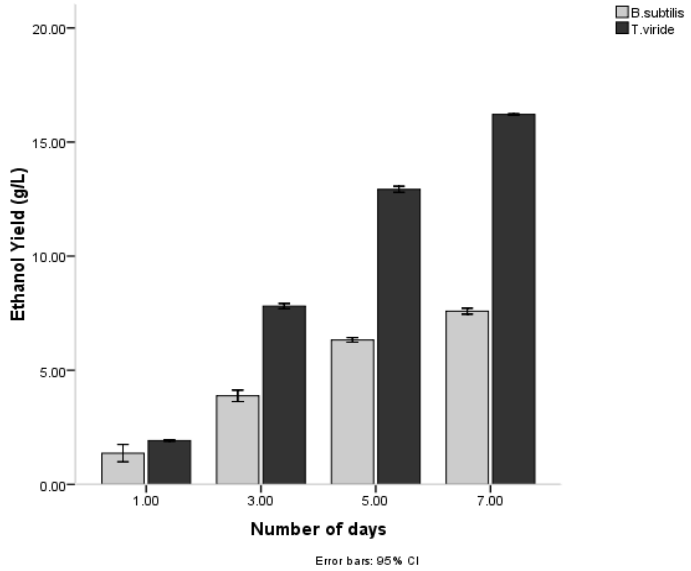

Figure 5: Ethanol yield from rice straw using *T. viride* and *S. cerevisiae* and *B. subtilis* and *S. cerevisiae*

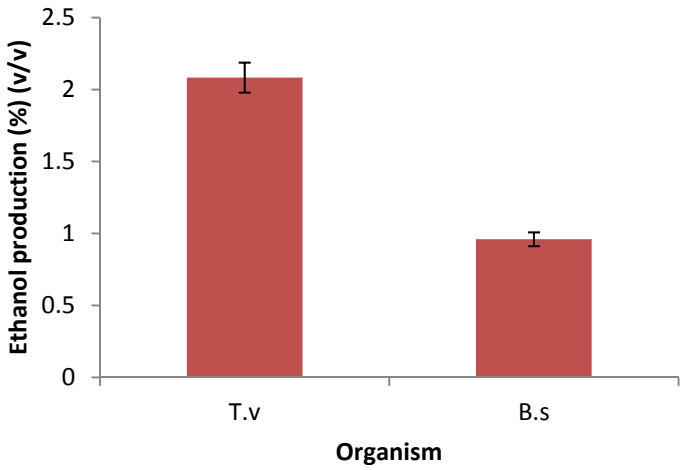

Figure 6: Percentage (v/v) ethanol produced rice straw using *T. viride* and *S. cerevisiae* and *B. subtilis* and *S. cerevisiae*

Key: T.v= *Trichoderma viride,* B.s= *Bacillus subtilis*

Table 4 reveals the result of comparative analysis of the bioethanol produce from rice straw and the conventional ethanol commercially available. Both the ethanol produced and commercial ethanol appeared colourless, burns with blue flame and have refractive index of I.36. Other properties of the bioethanol produced from rice straw and commercial ethanol such as relative density (0.77g/cm3, 0.75g/cm3 and 0.789g/cm3 respectively), boiling point(-112ºC, -113ºC and -114ºC respectively), melting point(78.5ºC, 78.5ºC and 78.37ºC respectively), viscosity (0.0119, 0.0122 and 0.0012 respectively) and flash point(11ºC, 12ºC, and 13-14ºC respectively) showed little discrepancies.

Table 4: Comparative analysis of commercial ethanol and bioethanol produced from rice straw

Ethanol properties	**Bioethanol from Rice straw using *T. viride* and *S. cerevisiae***	**Bioethanol from Rice straw using *B. subtilis* and *S. cerevisiae***	**Commercial Ethanol**
Appearance	Colourless	Colourless	Colourless
Relative density	0.77g/cm^3	0.75g/cm^3	0.789g/cm^3
Melting point	-112ºC	-113ºC	-114ºC
Boiling point	78.5ºC	78.5ºC	78.37ºC
Viscosity	0.0119	0.0122	0.0012 pa s at 20ºC
Burning characteristics	Burns with blue flame	Burns with blue flame	Burns with blue flame
Refractive index	1.36	1.36	1.36
Flash point	11ºC	12ºC	13-14ºC

DISCUSSION

The screening for the production of cellulase (Table 1) showed that the two organisms screened, tested positive for cellulase activity with zones of inhibition ranging from 0.5cm to 1.8 cm. Zones of clearance ranging from 0.4cm to 2.9 cm by *Aspergillus sp*., using same method had earlier been reported, indicating that the zone of clearance was a direct result of the amount of cellulase enzyme produced by the organism. Both *Bacillus subtilis,* and *Trichoderma viride* showed high cellulase activity of 1.5cm and 1.8cm respectively. This was supported by the work of Sadhu and Tushar, (2012), which demonstrated cellulase activity in similar organisms. Elsayed, (2013) also reported *T. viride* isolated from rice straw having a relatively high cellulase activity.

The proximate analysis of Rice straw (Fig 1), Showed the cellulose and lignin content of rice straw was 33.5% and 7.18% respectively which was relatively high, this was somewhat lower than 40.8% and 7.8% obtained by Hashem *et al.* (2013), and this is probable the result of varying environmental, soil and growth conditions in which the plant were obtained. Moisture content of 7.86% was recorded, Okoye *et al.* (1998) reported a range of moisture content of 8.5 – 9.1% (dm) as well as Pothiraj *et al.* (2014). The results from the proximate analysis shows that the digestibility of rice straw is hindered by many physicochemical, structural, and compositional factors which require a suitable pretreatment in order to enhance the susceptibility of biomass for hydrolysis.

The mineral analysis (Table 2) showed a relative variation in composition. Rice straw had a relatively high level of Potassium (17.96mg/g) which was considerably higher than 16.79 reported by Wannapeera *et al.,* (2008). Nitrogen content was found to be relatively low.

The result for the acid pretreatment of Rice Straw was highly effective especially after the application of NaOH (Table 3). The result showed a drastic increase in the cellulose composition of rice straw and a subsequent decrease in the hemicellulose and lignin content. This is due to the fact that the first treatment with sulfuric acid solubilized the hemi cellulosic fraction and increased the diffusion of sodium hydroxide into the lignocellulosic structure, thus enhancing soda pulping and liberating the cellulose fibers from lignin there by causing the washing away of hemicellulose and lignin during filtration hence obtaining a solid residue with high cellulose content (Musato *et al.,* 2007; Clayton *et al.,* 1989). The results obtained are in agreement with the finding of Liguori *et al.* (2015), who reported similar increase and decrease in the cellulose, hemi-cellulose and lignin contents of acid pretreated BSG, and in contrast to that of Abo-State *et al.,* (2014) who reported a decrease in all three components, probably due to simultaneous pretreatment and hydrolysis. The high cellulose content and decreased hemicellulose and lignin contents would allow for the enhancement of microbial saccharification (Jeya *et al*., 2009).

The reducing sugar yield obtained after three days hydrolysis of pretreated substrate with *T. viride* and *B. subtilis* (figure 2) showed that of *T. viride* relatively higher than *B. subtilis.* This was in agreement with Elsayed, (2013), who showed a great difference between the cellulose activity of *Trichodema sp.* and *Bacillus sp*. this can be attributed to the ability of *Trichodema sp.* to produce all components of cellulase complex, endocellulase, exocellulase, and β-glucosidase in good proportions as well as production of other enzymes such as xylanases or laccases in comparison to other enzyme producers (Arantes and Saddler, 2010). Rice straw had high reducing sugar yield of 26.6g using *T. viride* and 13.21g using *B. subtilis,*

There was significant decrease in the pH of the fermenting medium (Fig 3), this is due to the release of various organic acids from the utilization of the substrates (Omezurike, 2008). The medium gave a final pH of 3.8 using *T. viride*, 4.5 using *B. subtilis* and this could be the result of high nutrient composition of rice straw which favoured the growth of the organisms and hence the production of metabolites. The result of the total titratable acidity (Fig 4) showed a general increase in the TTA, this can however be attributed to the utilization of free sugars by yeast and lactobacillus (Akinleye, *et al.,* 2014).

The fermentation of the substrate using *Saccharomyces cerevisiae* (Figure 5) showed that the yield of ethanol is proportional to fermentation time where the yield increases with increase in time, this was due to the continuous utilization of the sugar by yeast, and this is in agreement with Yoswathana *et al.,* (2010). It was also revealed that the combination of *T. viride* and *S. cerevisiae* gave considerably higher ethanol yield, 100g of rice straw for instance gave an ethanol yield of 16.21g using *T. viride* and *S. cerevisiae* and 7.59g using *B. subtilis* and *S. cereviceae*. This was due to the efficiency of the organism during the hydrolysis stage, this was however lower than 17.31 reported by Harismah *et al.,* (2012), and higher than 15g/l by Yoswathana *et al.,* (2010) and 11g/l by Elsayad, (2013), who stated that the ethanol yield of the substrate is directly proportional to its cellulose content.

The percentage yield which was in direct relation to the initial yield observed (Fig 6), Rice straw having a percentage of 2.96 with *T. viride* and 0.08 with *B. subtilis.* Ethanol generated from acid pretreated rice straw compared favorably to commercially available ethanol and was found to poses similar properties. From the findings it can be said that Rice straw is a relatively efficient substrate for ethanol production and acid being an effective pretreatment method.

CONCLUSION

This study established the efficacy of Rice straw for bioethanol production, as well as the efficiency of selected microorganisms in the production process. The results therefore confirmed the use of acid as an important means of pre-treating lignocellulosic biomers, giving a high increase in cellulose generated for fermentation.

The proximate analysis showed rice straw to have high cellulose content, mineral content equally showed rice straw to be rich in mineral content, hence making it nutritious for microorganisms. However, *Trichodema viride* was found to be more effective in cellulose hydrolysis than *Bacillus subtilis,* thereby generated higher reducing sugar yield. Furthermore Rice straw was found to be an efficient substrate for bioethanol production with a maximum ethanol yield of 16.21g and a percentage yield of 2.08.

RECOMMENDATION

This research has successfully shown Rice straw to be an effective substrate for bioethanol production via the cellulose utilization pathway and can thus be employed as an alternative source for biofuel production, which would pose no environmental and food security problem. There is therefore the need for further research to improve the productivity and quality of ethanol

generated from this substrate. Furthermore, more research should be carried out to improve the cellulose hydrolyzing ability of promising microorganisms in a bid to enhance the bioethanol yield from cellulolytic biomers. Finally more attention should equally be given to the pretreatment method of Rice straw in other to improve its efficiency as well as produce alternative chemical-free methods in other to rid the environments of these toxic chemicals currently employed.

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