



Pre-treatment, Physicochemical Properties and Production of Bioethanol from Rice Husk using Fungi Isolated from Waste Dumpsite in Kaduna, Nigeria

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ABSTRACT: This research is based on the pre-treatment and physicochemical characterization of rice husk and production of Bioethanol from rice husk using fungal isolates obtained from waste dump sites in Kaduna Nigeria. Standard procedures were followed. The results revealed a reduction in lignin (from 21.40 to 12.08%), hemicellulose (22.04-18.92%), protein (14.56-8.56%) and ash (1.31-1.04%) contents which resulted to an increase in the cellulosic fraction (49.55-58.29%) of the rice husk. A significant difference ($p < 0.05$) was observed in the reducing sugar concentrations of the 50g substrate concentration using *Aspergillus niger* SIF2 (0.60±0.30mg/ml), *Aspergillus flavus* CMXY22565 (0.56±0.29mg/ml) and a consortium (1.17±0.82mg/ml) of both organisms after 120 hours of enzymatic hydrolysis. The results also revealed a significant yield ($p < 0.05$) in bioethanol production using *Pichia kudriavzevii* IPBCC.y.161552 (4.91±2.51%), *Saccharomyces cerevisiae* FJI (5.39±2.87%) and a consortium (9.02±4.83%) of both organisms. There was an increase in temperature and titratable acidity and a decrease in pH and specific gravity. The FTIR revealed a high ethanol content of 68% from the bioethanol produced from the 50g substrate concentration hydrolysed and fermented using a consortium of molds and yeasts. The results therefore suggest that bioethanol can be produced from rice husk with maximum yields obtained using a consortium of *Aspergillus niger* SIF2 and *Aspergillus flavus* CMXY22565 for hydrolysis as well as a consortium of *Saccharomyces cerevisiae* FJI and *Pichia kudriavzevii* IPBCC.y.161552 for fermentation.

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The world needs substantial energy to maintain and improve the quality of life (Paul *et al.*, 2015). Thus in recent times, there has been increase in energy demand. However, the type of energy needed should not constitute health and ecological problems as evidenced from the use of fossil fuels (Samsudin and Mat-Don, 2014; Kim *et al.*, 2015; Amadia *et al.*, 2016). Lignocellulosic biomass as the most abundant biopolymer on earth consists of biological resources and plant waste residues that produce fuels by biological procedure (Pauly *et al.*, 2013). Ethanol production from lignocellulosic residues has therefore earned attention as many feedstocks can be used as resources with no competition with the food chain. It is also cheap, renewable and found almost everywhere on earth (Michael *et al.*, 2017). Numerous bacteria, yeasts, and fungi have been used for the production of

ethanol (Dogaris, 2013; Khare *et al.*, 2015; Kumar *et al.*, 2017) although, yeast (*Saccharomyces cerevisiae*) is the most frequently used microorganism for ethanol production by fermentation process (Cook *et al.*, 2012; Elfasakhany *et al.*, 2015). In nature, filamentous fungi are the major decomposers of lignocellulosic biomass due to their ability to develop hyphae, they also have an extremely efficient enzymatic system. However, mixed cultures can have more effective activity on substrate because of the higher production of enzymes and resistance to contaminant microbes compared to pure cultures (Paul *et al.*, 2015; Diana *et al.*, 2017). The most important factor in the use of mixed cultures is strain compatibility, which can influence the general performance of microbes, their organization, distribution, population size as well as the ecological balance of the communities (Paul *et al.*, 2015). Hence,

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a compatible consortium of lignocellulolytic fungi might play a central role in the speedy degradation of agricultural residues (Choudhary *et al.*, 2016). Rice is the agricultural commodity with the third highest worldwide production after sugarcane and maize, Nigeria being seventeenth in the world (Ogwo *et al.*, 2012; Onwuakor *et al.*, 2017). It is the most important grain with regard to human nutrition and caloric intake, providing more than one fifth of the calories consumed worldwide by humans. The production of bioethanol from food crops such as grains (first generation biofuels) has resulted in an undesirable direct competition with food supply (Ogwo *et al.*, 2012; Oso *et al.*, 2018). A switch to a more abundant inedible plant material should help to reduce pressure on the food crops. Thus, rice husk has several characteristics that make it a potential feedstock for biofuel production. It has high cellulose and hemicellulose contents that can be readily hydrolyzed into fermentable sugars (Onwuakor *et al.*, 2017). Ethanol-fermenting microorganism can therefore utilize these sugars and convert into ethanol. Hence, this research was therefore aimed at the pretreatment and physicochemical characterization of rice husk and production of bioethanol from the rice husk using fungal isolates obtained from waste dump sites in Kaduna Nigeria.

MATERIALS AND METHODS

Collection and processing of Samples: Rice husks were collected in clean polythene bags from two milling sites; Station market and Bakin Dogo markets respectively, within Kaduna metropolis, Kaduna State, Nigeria. The rice husks were washed in water, dried at 60°C for 30minutes in a hot air oven, ground to powder form using a blender (Kenwood) and sieved using a 2mm sieve to get uniform particles. The substrates were stored in plastic bags at room temperature until required.

Soil sample was collected into clean polythene bags from waste dump sites in Kaduna metropolis, Kaduna State, Nigeria. The soil samples were labelled and transported to the Microbiology Laboratory of Kaduna State University (KASU), Kaduna, Nigeria for the isolation of fungi.

Fungi used: The moulds used in the study were *Aspergillus niger* SIF2, *Aspergillus flavus* CMXY22565 while the yeast were *Pichia kudriavzevii* IPBCC.y.16.1552 and *Saccharomyces cerevisiae* FJ1. All fungal isolates were obtained and identified as stated in our previous study (Audu *et al.*, 2020).

Physicochemical Analysis of Rice Husk: The substrate (rice husk) was analyzed before and after pretreatment for the following parameters using standard methods as described by Association of Official Analytical Chemists, AOAC (2005):

pH of the substrate was determined using pH meter (Micro pH 2000) on 1:2.5 (w/v) substrate/water mixture. Moisture content was determined by oven dry weight method at 105°C for 3 hours while ash content was determined by ashing the substrate in a furnace at 550°C. Crude protein content of the substrate was determined using the macro Kjeldah method (AOAC, 2005; Logah, 2011). Lignin and alpha cellulose contents were determined gravimetrically (AOAC, 2005; Anwar *et al.*, 2012); Hemicellulose estimation was done by NaOH treatment and refluxing at 80°C for 4 hours; Cellulose estimation was done by subtracting from 100%, the sum of hemicelluloses, lignin, ash, alpha cellulose and moisture while holocellulose was estimated by the sum of cellulose and hemicelluloses (AOAC, 2005)

Bioethanol Production: The processes for bioethanol production were pretreatment, hydrolysis, fermentation and fractional distillation (Oyeleke *et al.*, 2012 and Onwuakor *et al.* 2017).

Pre-treatment of rice husk: The pretreatment of rice husk was done using the methods described by Oyeleke and Jibrin (2009), Anwar *et al.* (2012) and Arooj *et al.* (2017). Exactly 200g of the milled rice husk was weighed into four 500ml capacity conical flasks. Chemical pretreatment (Acid pretreatment) was then employed by soaking the substrate in each flask with a 5% (v/v) 1M H₂SO₄. The flasks were plugged with cotton wool wrapped in aluminum foil, heated at 98°C for 2 hours in a water bath and then sterilized by autoclaving at 121°C for 15 minutes. The flasks were allowed to cool to room temperature (28±°C). The pretreated rice husk was rinsed with distilled water to wash off the acid completely, it was filtered through No. 1 Whatman filter paper and dried at 45°C for 2 hours in the hot air oven.

Enzymatic hydrolysis of rice husk: Enzymatic hydrolysis of rice husk was carried out through the following stages:

Preparation of solid bed: The pre-treated rice husk was weighed in different quantities (30g, 40g and 50g) into three sets of four 250ml capacity conical flasks, giving a total of twelve conical flasks. The solid bed for the solid state fermentation was prepared using the method described by Zambare (2010) and Ghadi *et al.* (2011). Each 10g of the substrate was moistened with 9mL of water and sterilized by autoclaving at 121°C for 15minutes after which the contents of the flasks (i.e substrate) were brought to room temperature (28±2°C).

Preparation of fermentation culture: Spores from a five day old culture of each fungi isolates (i.e *Aspergillus flavus* CMXY22565 and *Aspergillus niger* SIF2) were aseptically dislodged using 50ml of normal saline containing 0.1% tween 80. The mixture was

aseptically filtered off after 30mins through sterile glass wool to get spores and the spore count determined by serial dilution and spread plating method (Zambare, 2010).

Fermentation of substrate: Two sets of each treatment i.e 2 sets of 30g, 2 sets of 40g and 2sets of 50g were separately inoculated with 1mL (9.2×10^6 cells/mL) of each fungi fermentation culture of *Aspergillus niger* SIF2 and *Aspergillus flavus* CMXY22565 respectively. Another 2 representative sets of 30g, 40g and 50g of the flask were inoculated with a consortium of both fungi isolates while the remaining treatments (flasks) were uninoculated and served as controls. The whole content was mixed thoroughly and incubated at room temperature for 5days in a stationary condition (Zambare, 2010; Oyeleke *et al.*, 2012).

Separation of enzyme solution: Exactly 200ml of distilled water was added to each flask and homogenised in a rotary shaker for 15minutes. The solution was passed through No. 1 Whatman filter paper to separate the fungal spores. The hydrolysate was then sterilized by autoclaving at 121°C for 15minutes and brought to room temperature (Zambare, 2010; Ghadi *et al.*, 2011).

Preparation of 0.5 McFarland Standard: A 0.5 Mcfarland was prepared by adding 1 ml of concentrated sulphuric acid (H_2SO_4) to 99ml of distilled water in a beaker and mixed well in order to attain a 1% (v/v) solution of H_2SO_4 . Exactly 0.5 g of dehydrate barium chloride salt ($BaCl_2 \cdot 2H_2O$) was also dissolved in 50ml of distilled water to obtain a 1% (w/v) of $BaCl_2$. 0.6ml of the $BaCl_2$ solution was added to 99.4 ml of H_2SO_4 solution to make up to 100 ml which served as the stock solution of the 0.5 McFarland turbidity standards. Exactly 2 ml of the solution was transferred into capped tubes and stored at room temperature until use (Damisa-Okorhi, 2015).

Inoculum preparation: The fermentation medium used for ethanol production had the following composition; glucose 150g, yeast extract 2g, peptone 2g, malt extract 3g, $MgSO_4 \cdot 7H_2O$ 1g, NH_2SO_4 4g, NaCl 1g, and $FeSO_4$ 0.01g in 1L of distilled water. The medium was sterilized by autoclaving at 121°C for 15mins and cooled to room temperature. A 24 hour old culture of each yeast isolate was inoculated into its respective flask containing the fermentation medium and incubated in an orbital shaker at an agitation rate of 150rpm at 30°C for 72 hours to enable organisms attain their log phase in growth. It was then diluted to match the turbidity standard which contained approximately 1.5×10^8 cfu/ml (Kale and Zanwar, 2016).

Fermentation of hydrolysate: Three millilitre (3mL) of the yeast isolate (*Pichia kudriavzevii* IPBCC.y.16.1552) was inoculated into a set of flask (30g, 40, and 50g respectively) containing the

hydrolysates that was hydrolysed by each mold isolate (*Aspergillus niger* SIF2 and *Aspergillus flavus* CMXY22565) using 0.5 Mc Farland standard. Another set of flask (30g, 40g and 50g) containing the hydrolysates hydrolysed by the mold isolates were inoculated with exactly 3mL of the yeast isolate (*Saccharomyces cerevisiae* FJI). A corsotium of both yeast isolate was inoculated into another set of flasks (30g, 40g and 50g) containing the hydrolysates hydrolysed by each mould isolate respectively while the last set of flask (30g, 40g and 50g) served as the control (Zambare, 2010; Oyeleke *et al.*, 2012). The flasks were incubated at room temperature for seven days during which it was observed and monitored daily for variations in pH, temperature, specific gravity and titratable acidity respectively.

Distillation of hydrolysate: This was carried out using a distillation apparatus. The fermented liquid was transferred into round bottom flasks and placed on a heating mantle fixed to a distillation column enclosed in running tap water. Another flask was fixed to the other end of the distillation column to collect the distillate at 78°C (standard temperature for ethanol production). This was done for each of the fermented broth (Humphrey and Okafoagu, 2007; Oyeleke and Jibrin, 2009).

Estimation of reducing sugars in hydrolysates: The reducing sugar contents released during hydrolysis and fermentation was determined using Dinotrosalicyclic acid (DNS) method as described by Miller *et al.* (1959). DNS reagent was prepared by dissolving 10g of 3, 5- dinitrosalicyclic acid (DNS) and 300g of sodium potassium tartarate in 500 ml of water followed by the addition of 200ml 2N NaOH solution and finally raising the volume to 1L with distilled water. Exactly 3.0 ml of DNS solution was added to 3.0 ml of the sample in a test tube. The test tubes were heated in a boiling water bath at 90°C for 5 mins. A blank containing 3.0 ml of the control and 3.0 ml of DNS was also run parallel. The tubes were allowed to cool at room temperature after which 8.0 ml of distilled was added to each and absorbance was noted at 540nm using UV/VIS Spectrophotometre (PEC MEDICAL USA). A standard graph of glucose solution (10mg/ml of working standard) was needed to calculate the glucose concentration in the samples. The concentrations of glucose standard used were 0.2 g/ml, 0.4 g/ml, 0.6 g/ml and 0.8 g/ml and 1.0 g/ml respectively.

Determination of ethanol yield from hydrolysate: Ethanol yield was determined volumetrically as the ratio of the volume of alcohol produced to the quality of fermented substrate distilled as described by Mbajiuka *et al.* (2010) and Onwuakor *et al.* (2017). A measured quantity (15mL) of each fermented rice husk hydrolysate was mixed with equal volume of distilled water and transferred into a large bottom distillation

flask. The flask was connected to the condenser and its thermometer was slotted into position while the flask stood in the heating mantle. As the mixture boiled, the temperature was noted and the thermometer was constantly observed for any change. Meanwhile, the recovery outlet tube through which the alcohol flows was put into a receiving flask in such a way that the tube passed through a stopper and a barrier. The receiver flask was corked to minimise the escape of received ethanol. The distillation was stopped when the temperature at the base of the condenser started rising and becoming misty. The volume of distilled ethanol was measured and the ethanol yield was estimated thus;

$$\% \text{yield} = \frac{VE \times 100}{VF}$$

Where VE = volume of alcohol distilled off; VF = volume of fermented hydrolysate distilled

Determination of titratable Acidity: Titratable acidity (TTA) of each hydrolysate was determined using the method described by Mbajiuka *et al.* (2010). Exactly 5ml of each hydrolysate was mixed with 45ml of distilled water and 3 drops of phenolphthalein was added to serve as indicator. It was titrated against 0.1M NaOH solution to an end point marked by a pink colouration which lasted for 15 seconds and beyond. Titratable acidity was estimated thus;

$$\% TTA = \frac{100 \times N \times \text{Titre}}{V}$$

Where N = normality of titrant; V = volume of sample used

Characterization of Bioethanol

Triodomethane test for qualitative detection of ethanol: Triodomethane test used for the qualitative detection of ethanol in the samples was carried out as described by Pandey *et al.* (2013). Exactly 10 drops of the sample was transferred to a clean dry test tube and 3mL of iodine solution was added. 0.1M sodium hydroxide was added to decolourise the iodine. It was mixed gently for 2 minutes and observed in the cold. It was then heated gently at 90°C for 5 minutes in a water bath and observed for the formation of a very pale yellow precipitate of triodomethane (iodoform).

Combustion test for the qualitative detection of ethanol: Combustion test for the detection of ethanol was carried out as described by the American Herbal Products (2017). Exactly 2ml of the ethanol was transferred into a test tube. A boiling chip was added, heated and the tube held with a test tube holder until the liquid was boiling. The open end of the test tube was held to the flame and the ethanol vapour ignited.

Determination of ethanol content using specific gravity: Specific gravity (SG) was determined using

the pycnometer gravimetric method (James, 1995). The pycnometer (specific gravity bottle) was weighed while it was clean and dry but with its stopper in place (Wa). It was then filled with water, up to the capillary of the stopper. The outside was wiped with a dry blotting paper, weighed and filled with water (Ww). The water was poured out and the bottle was allowed to dry without heating. It was then filled with the sample solution and weighed (Ws). The specific gravity was determined thus:

$$S.G = \frac{Ws - Wa}{Ww - Wa}$$

Where Wa = weight of empty specific gravity bottle; Ws = weight of bottle + sample; Ww = weight of bottle + water

Determination of ethanol content using FTIR spectroscopy: Ethanol content was determined using the methods described by Coldea *et al.* (2013) and Corsetti *et al.* (2015). The IR spectra of the solutions were collected over the range 1000-1200 cm^{-1} using a Shimadzu spectrometer. The nominal resolution was 6 cm^{-1} . In order to increase the signal to noise ratio, for every sample 10 scans were averaged. The instrument was equipped with an attenuated total reflection (ATR) module. During the measurement, the samples on the ATR crystal were covered with a small glass cap to avoid sample evaporation. Highly pure ethanol (Merck, 99.9% purity) was used to prepare series of ethanol concentrations (100, 80, 60, 40 and 20 %) and the corresponding absorbance recorded at 1044 cm^{-1} . The absorbance was used to plot a straight line calibration curve of percentage ethanol. Individual sample absorbance was measured based on peak areas from 1044 cm^{-1} , the percentage concentration of ethanol in samples was calculated from the equation of graph obtained from the calibration curve using Microsoft Excel software (MS Excel 10.0).

Statistical Analysis: One – way analysis of variance was used to analyze the results. The results of the experiment were presented as ($M \pm SD$), while the significant differences were determined at $p \leq 0.05$. Duncan's Multiple Range Test was used to test significance of means according to the Statistical Package for Social Sciences (SPSS) 2018 version 23.0 (Shukla, 2018).

RESULTS AND DISCUSSION

Physicochemical Properties of Rice Husk: Table 1 illustrates the percentage composition of both untreated and pretreated rice husk. The results revealed that the amount of cellulose, alpha cellulose, holocellulose and total carbohydrate present in the rice husk before pretreatment were 49.55%, 3.19% and 71.59% which increased to 58.29%, 8.18% and 77.21 respectively after pretreatment while nitrogen, protein, ash and moisture contents decreased from 2.33%, 14.56%, 1.31% to 1.37%, 8.56%, 1.31% and 1.49%

respectively. The pH of the substrate also decreased from 8.0 to 4.7 after pretreatment. The reduction of lignin (21.4% to 12.8%) and hemicellulose (22.04% to 18.92) ultimately led to an increase in the cellulosic fractions of the rice husk thereby making the pretreated sample easily degradable as well as increasing the surface area of the husk when exposed to fungal cellulose

Table 1: Physicochemical properties of untreated and pre-treated rice husk

Parameter	Untreated	Pretreated
pH	8.00	4.70
Moisture (%)	2.51	1.49
Ash (%)	1.31	1.04
Lignin (%)	21.40	12.08
Cellulose (%)	49.55	58.29
Hemicellulose (%)	22.04	18.92
Alpha cellulose (%)	3.19	8.18
Holo cellulose (%)	71.59	77.21
Carbohydrate (%)	71.59	77.21
Nitrogen (%)	2.33	1.37
Protein (%)	14.56	8.56

Also, the reduction in ash content is crucial because a high percentage of ash content retards enzymatic hydrolysis. Ash cations migrate into the solution and affect cellulase activity (Rambo *et al.*, 2015). Nyachaka *et al.* (2013) and Charanchi *et al.* (2018) reported a positive result in the sulphuric acid pretreatment of rice husk. Pretreatment of agricultural residues is an important instrument for realistic cellulose degradation processes. Pretreatment is necessary to alter the structures of lignocellulosic biomass thereby making it more accessible to the enzymes as well as cellulase producing microorganisms that convert the carbohydrate polymers into fermentable sugars. Besides cellulose, it also renders other carbohydrate fractions in plants more accessible for enzymatic hydrolysis and ethanol fermentation thereby improving ethanol yield.

Reducing Sugar Concentration in Hydrolysates during Hydrolysis and Fermentation: Enzymatic hydrolysis of the rice husk using *Aspergillus niger* SIF2, *Aspergillus flavus* CMXY22565 and a consortium of both organisms in solid state fermentation revealed that the reducing sugar concentration in the hydrolysates of the consortium was more than the reducing sugar present in the hydrolysates of the individual fungi isolate. This implies that the consortium of both organisms was more effective than the individual organism. Notwithstanding, the result (Fig. 1) showed that *Aspergillus niger* SIF2 performed better than *Aspergillus flavus* CMXY22565. In the production of bioethanol from sweet potatoes using a combination of acid and enzymatic hydrolysis, Putri *et al.* (2012) reported that the highest reducing sugar was obtained from a combination of the acid and *Aspergillus niger*. Thus, besides its none fastidious

nutritional requirement and its ability to produce various cell-wall hydrolysing enzymes, *Aspergillus niger* SIF2 may have been at more advantage than *Aspergillus flavus* CMXY22565 due to its ability to withstand certain inhibitors released during dilute acid pretreatment of the rice husk. In addition, research has shown that *Aspergillus niger* exhibits the potential to produce higher activity level of cellulase enzyme under solid state fermentation using mineral salt medium (MSM). However, Ajayi *et al.* (2007) reported both organisms to be among the best cellulase producing microbe. Nonetheless, a significant difference ($p < 0.05$) was observed in the reducing sugar concentrations of both organisms (consortium), this implies that both organisms can work in synergy to support cellulase production which ultimately resulted in the release of higher concentrations of reducing sugars. This corresponds to the findings of Noratiqah (2017) who reported that co-culture of *Aspergillus* species in solid state fermentation using sawdust as substrate produced the highest lignocellulolytic enzyme activity than pure cultures. Tan (2012) had earlier reported that co-cultures of *Aspergillus* species gave higher yields of reducing sugar compared to mixed cultures. The results (Fig. 1) also showed that the highest concentration of reducing sugar was attained after 120 hours. A high concentration of 0.91 ± 0.80 mg/ml was observed in the hydrolysates of the consortium as against 0.41 ± 0.24 mg/ml for *Aspergillus niger* SIF2 and 0.39 ± 0.20 mg/ml for *Aspergillus flavus* CMXY22565.

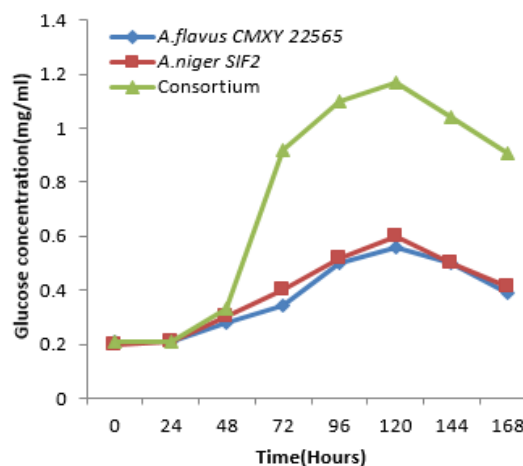


Fig 1: Reducing sugar concentration during hydrolysis of rice husk by *Aspergillus niger* SIF2 and *Aspergillus flavus* CMXY22565

This result agrees with the findings of Tan (2012) that the optimum incubation period, temperature and pH for maximum reducing sugar was achieved after 120 hours. Ahmad *et al.* (2017) also revealed that there was a significant difference ($p < 0.05$) in yields of the reducing sugar obtained from the substrate (50g) using *Aspergillus niger* and *Trichoderma harzianum* after 120 hours of hydrolysis. The decline in the reducing sugar concentration after 144 hours and 168 hours may be due to depletion of nutrients, exhaustion of cellulase

as well as accumulation of secondary metabolites that had adverse effects on cellulase production. Also at the beginning of the hydrolysis (0 - 24hours), the reducing sugar concentration in all hydrolysates was observed to be constant. This may be attributed to the fact that the organisms were observing a lag phase to enable them adapt to the medium, hence there was no activity that resulted in the production of reducing sugars. Concentration of the substrate was studied as an important factor for improved enzyme activity. The results (Fig. 2) showed that the activity of cellulases was significantly higher at high substrate concentrations (50g) than at low substrate concentrations. This may be as a result of adequate amount of nutrients in the substrate at that concentration (50g). This agrees with the findings of Oyeleke *et al.* (2012) in the production of bioethanol from cassava and sweet potato peels using *Gloeophyllum sepiarium* and *Pleurotus ostreatus* for hydrolysis and *Zymomonas mobilis* and *Saccharomyces cerevisiae* for fermentation. The highest percentage ethanol yield (26%) was derived from the hydrolysates of the fungal consortium containing 50g of the substrate concentration. *Saccharomyces cerevisiae* FJI utilised a little more of glucose than the isolate *Pichia kudriavzevii* IPBCC.y.16.1552 although there was no significant difference ($p>0.05$) observed in their capabilities (Fig. 3). However, the decrease in sugar concentration was more evident in the hydrolysates of the consortium and a significant difference was observed between the hydrolysates of the consortium and the hydrolysates of the respective individual hydrolysates of *P. kudriavzevii* and *S. cerevisiae* plus there was a significant difference in the utilization of glucose by the yeast isolates as $p<0.05$. This result is contradictory to the findings of Gidado *et al.* (2017) in the production of ethanol from different carbohydrate sources using alcohol tolerant yeast where *Pichia kudriavzevii* strain GY1 utilized more reducing sugars than *Saccharomyces cerevisiae* strain T.

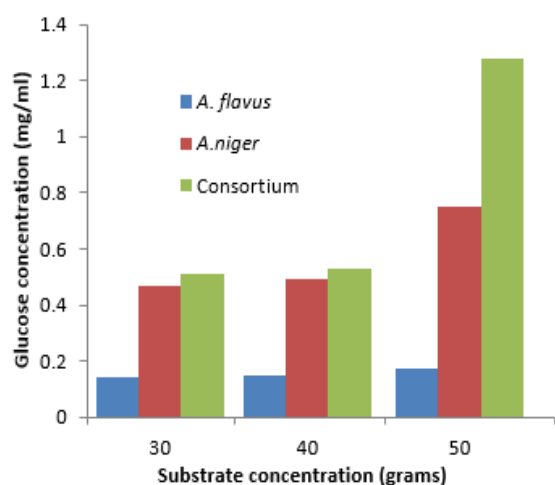


Fig 2: Reducing sugar concentration at varying substrate concentrations using *Aspergillus niger* SIF2 and *Aspergillus flavus* CMXY22565

It however agrees with the findings of Sulphari *et al.* (2019) who reported a significant decrease in reducing sugars in the production of bioethanol from algae *Spirogyra peipingensis* using a consortium of *Saccharomyces cerevisiae* and *Pichia kudriavzevii*. The result also depicts a steady increase in growth of organisms during the fermentation period (0 - 96 hours) which corresponds to a decrease in reducing sugar concentration thus, reflecting the fact that the organisms are catabolizing the glucose present in the media for growth via the Embden Meyerhof-Parnas (EMP) pathway to pyruvate which is further decarboxylated to an aldehyde (ethanol) with the release of carbon dioxide. The growth of the yeast isolates was steady throughout the fermentation period in the consortium especially in the hydrolysates of the moulds consortium containing 50g of substrate which means that reducing sugar utilization was in accordance with the concentration of sugars obtained during hydrolysis. The stagnant growth observed in the hydrolysate of the individual organisms could be as result of other by-products in the hydrolysates that might have caused the inhibition of the yeast.

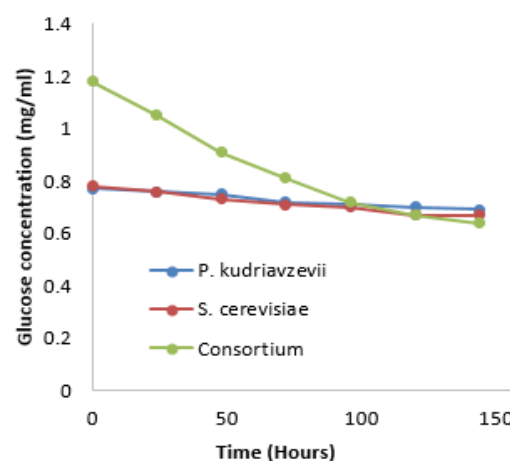


Fig. 3: Reducing sugar concentration during fermentation of hydrolysate by *Pichia kudriavzevii* IPBCC.y.16.1552 and *Saccharomyces cerevisiae* FJI

Additionally, the increase in ethanol concentrations of the fermentation cultures could also be the reason for stagnant fermentation process after 120 - 144 hours. It is also possible that beyond the fifth day all the sugars that could be fermented by the *S. cerevisiae* and *P. kudriavzevii* had been completely utilised.

Ethanol Yield during Fermentation: Fermentation of the various hydrolysates by the yeast isolates, *S. cerevisiae* FJI and *P. kudriavzevii* IPBCC.y.16.1552 yielded ethanol in accordance with the sugar concentration obtained. This therefore implies the yield of ethanol was directly proportional to concentration of sugar in the fermenting broth. The study revealed that *Saccharomyces cerevisiae* FJI produced more ethanol ($5.19\pm 2.87\%$) than *Pichia kudriavzevii* IPBCC.y.16.1552 ($4.61\pm 2.51\%$) although there was no significant difference ($p>0.05$) in the

yields (Fig. 3). *Saccharomyces cerevisiae* have been established as the most ethanol tolerant yeast of the eukaryotic organisms and are able to tolerate over 20% ethanol.

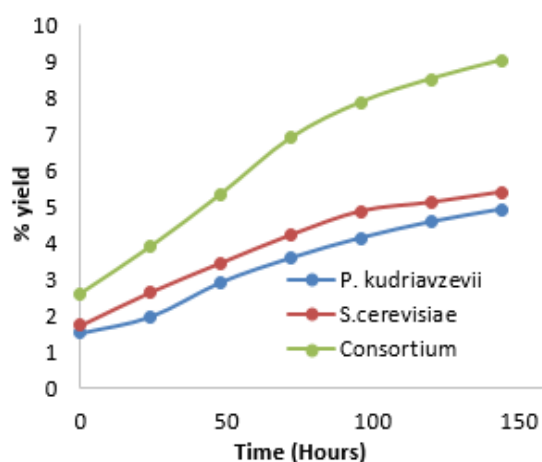


Fig. 4: Percentage yield of ethanol in hydrolysates during fermentation

However, the use of highly adaptable non *Saccharomyces* species to a variety of sugars in the pursuit of enhanced ethanol production creates a unique prospective for large scale industrial applications. *Pichia kudriavzevii* on its own could not ferment more glucose to ethanol compared to *Saccharomyces cerevisiae* even though certain strains are known to utilise a wide range of glucose including xylose than *Saccharomyces cerevisiae* (Oberoi *et al.*, 2012; Radecka *et al.*, 2015; Gidado *et al.*, 2017). This may be attributed to the fact that *Pichia kudriavzevii* performs or ferments better at elevated temperature of over 40°C (Hasunuma *et al.*, 2013). This temperature condition was however not provided in the study. This finding however does not agree with the findings of Gidado *et al.* (2017) who produced ethanol from different carbohydrate sources using alcohol tolerant yeast, *Pichia kudriavzevii* strain GY1. The organism produced a significantly higher yield of 10±0.2mg/ml than *Saccharomyces cerevisiae* strain T which yielded 5±0.2mg/ml at 96 hours. In a study carried out by Rhulani *et al.* (2017), *Pichia kudriavzevii* was found to emerge as the most effective thermotolerant strain for ethanol production at high temperature. It exhibited good growth and ethanol production capability at temperatures up to 45°C. The limited or slow yield derived from the hydrolysates of the respective pure cultures after 120 to 144 hours could be attributed to the fact that the yeasts were progressing to the stationary phase and could no longer utilize the limited sugar present in the sample. However, the consortium of both yeasts produced better yields of ethanol (9.02±4.83%) throughout the fermentation period and a significant difference ($p < 0.05$) was observed on the hydrolysates containing 50g substrate hydrolysed by the mould consortium. It is an established fact that certain strains of *P. kudriavzevii* possess the ability to ferment xylose, grow in the presence of inhibitors

(furan derivatives tolerance) released during fermentation as well as withstand higher temperatures and lower pH. Rhulani *et al.* (2017) isolated *Pichia kudriavzevii* from the dung of animal which produced the highest ethanol concentration (2.3±0.5g/l) from xylose. Thus the synergistic action of both isolates produced better ethanol yields. This is in agreement with the findings of Ado *et al.* (2009) and Oyeleke *et al.* (2012) where the highest yield of ethanol obtained was from the 50g substrate concentration hydrolysed by a consortium of moulds and also fermented by a consortium of yeast isolates.

Physicochemical Properties of the Hydrolysates during Fermentation: Temperature variations during fermentation: Fermentation temperatures ranged from 27°C to 30°C (Fig. 5) and there was no significant difference observed in all hydrolysates ($p < 0.05$). This temperature range is slightly below the temperature range observed in some studies probably because of the period the research was carried out (i.e. the cold season of the year). However, the result agrees with the temperature range encountered by Onwuakor *et al.* (2017) where a fall in temperature was observed and was attributed to environmental factors that influenced fermentation. If temperature is favourable, activities of microorganisms present during the conversion of the sugar will be high but if the temperature is low, the activities of microbes will be slow.

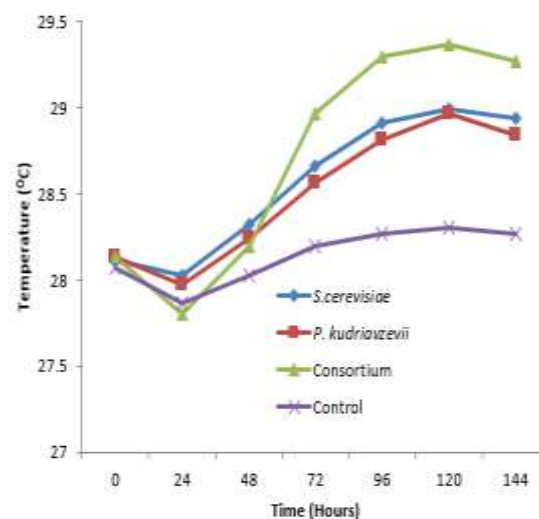


Fig. 5: Temperature variation during fermentation of hydrolysates by *Pichia kudriavzevii* IPBCC.y.16.1552 and *Saccharomyces cerevisiae* FJI

However, temperature range is important in regulating enzymatic activity of the yeast because lower temperature slows down metabolic activities while higher temperatures may denature the enzymes (Kwon *et al.*, 2011; Mimitsuka *et al.*, 2015; Gidado *et al.*, 2017). Although, many workers have reported different temperatures for maximum production either in flask or in fermentor studies suggesting that the optimal temperature for production also depends on the strain variation of the microorganism (Kang *et al.*,

2014; Nasir *et al.*, 2016; Rhulani *et al.*, 2017). The gradual rise in temperature observed in this study portrays a high release of ATP during the active growth of the yeast isolates (starter culture) present during fermentation as well as other biochemical changes that may have occurred during the conversion of glucose to carbon dioxide and ethanol. This can be confirmed in the very slow temperature variation observed in the controls which revealed that no biochemical activity was taking place in the medium. This agrees with the findings of Subhasree (2010) who reported that a rise in temperature occurred throughout the course of an ideal fermentation process. The results also showed that there was a decrease in temperature in all the hydrolysates after 24 hours. This may be attributed to the fact that the organisms might be going through a lag phase, thus there was no significant activity since the organisms were trying to adapt to the new environmental conditions.

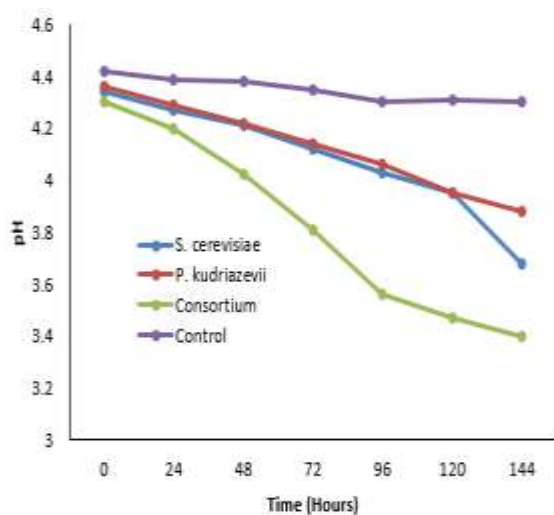


Fig. 6: pH Variation during fermentation of hydrolysates by *Pichia kudriavzevii* IPBCC.y.16.1552 and *Saccharomyces cerevisiae* FII

pH variations during fermentation: The pH observed throughout the fermentation period (0 to 144 hours) decreased from 4.34 ± 0.03 to 3.68 ± 0.16 in *P. kudriavzevii* IPBCC.y.16.1552 hydrolysate, 4.36 ± 0.02 to 3.88 ± 0.18 in *S. cerevisiae* FII hydrolysate, 4.30 ± 0.05 to 3.40 ± 0.51 in the consortium and 4.42 ± 0.02 to 4.30 ± 0.02 in the control (Fig. 6). Although the initial pH recorded was a little below the starting pH recorded in most research, however the pH range still lies within the range of pH changes during beer and wine production as reported by Idise (2011). Nasir *et al.* (2016) also reported optimum yields of bioethanol production at pH 4.0 and 6.0 respectively. The gradual decrease in pH was due to the release of CO_2 which also reflected the rate of biochemical activity in the respective hydrolysates. The lowest pH value was recorded in the consortium signifying a high biochemical reaction in the hydrolysate. The high pH value recorded in the consortium reflected low biochemical activities.

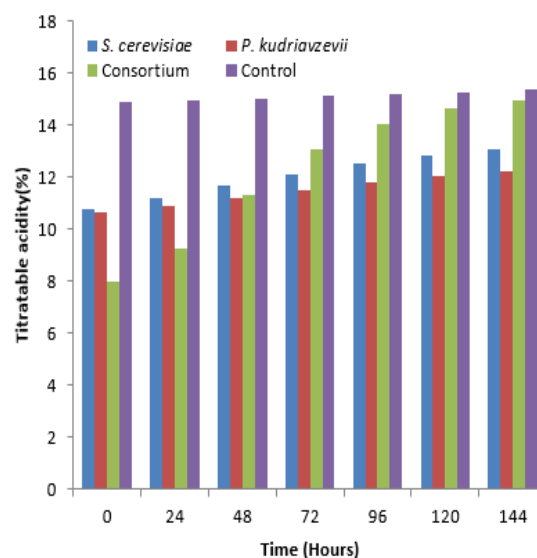


Fig. 7: Percentage titratable acidity of hydrolysates during fermentation by *Pichia kudriavzevii* IPBCC.y.16.1552 and *Saccharomyces cerevisiae* FII

Total titratable acidity (TTA) during fermentation: The total titratable acidity (TTA) of the hydrolysate increased as the fermentation time increased (Fig. 7) and there was no significant difference ($p > 0.05$) in the mean values. Increase in the acidic content of the hydrolysates is a function of microbial growth which brings about the release of carbondioxide via the conversion of reducing sugars to ethanol and other organic acids during fermentation (Samsudin and Mat-Don, 2014; Bhavser *et al.*, 2015; Nachaiwieng *et al.*, 2015). Findings from this research agree with the work of Onwuakor *et al.* (2017) on the production of ethanol and biomass using cultures of *Aspergillus flavus*, *Aspergillus eamarii* and *Saccharomyces cerevisiae* where titratable acidity was observed to increase gradually with increasing ethanol concentration in the hydrolysate.

Characterization of Bioethanol Produced: Iodoform test for detecting ethanol: Triodomethane test is one of the common techniques used for qualitative analysis of ethanol either alone or in mixtures. It is one of the tests used to distinguish ethanol from methanol. When ethanol is warmed with iodine in the presence of sodium hydroxide, it forms a yellow colour precipitate due to the formation of CHI_3 (iodoform) alongside a characteristic iodoform odour. The test gives positive results for other alcohols containing a methyl group and a hydrogen atom attached to carbon bearing the hydroxyl group. In this study, all the distillates gave positive results with the iodoform test which was proven by an arousing characteristic iodoform odour and the formation of coloured precipitate, hence it could be stated that there was alcohol compounds in the distillate. However, a high concentration of the triodomethane precipitate was observed in the samples containing 50g substrate concentration, this may be probably due to the high concentration of ethanol in the hydrolysates because the opposite was observed in

the 30g substrate concentration, where there was little or no precipitates in the sample signifying low or no concentrations of ethanol. The low concentration of ethanol may have been as a result of the low concentrations of reducing sugars during fermentation or the escape of some ethanol during distillation. On this basis, only the 50g substrate concentrations were subjected to quantitative determination of ethanol content. This implies that the iodoform test was more glaring in samples with high ethanol content. This agrees with the findings of Pandey *et al.* (2013) where samples with high reducing sugar concentration gave positive results. Of the seven samples subjected to combustion test, two samples; BE₃ and BE₅ (Table 3) burnt with a pale blue flame producing no smoke which indicated positive test for ethanol as described by the American Herbal Products Association (2017).

Specific gravity of bioethanol: Table 2 shows that the specific gravity of the hydrolysates decreased with time and a significant difference was observed among the hydrolysates with the exception of the control which showed no significant difference at $p < 0.05$. The specific gravity of the *Saccharomyces cerevisiae* FJI hydrolysate decreased from 1.04 ± 0.72 to 1.01 ± 0.72

while that of *Pichia kudriavzevii* IPBCC.y.16.1552 decreased from 1.08 ± 0.26 to 1.06 ± 0.27 . In the same vein, the specific gravity of the Consortium (Crs) decreased from 0.96 ± 0.80 to 0.92 ± 0.10 while that of the control decreased from 1.11 ± 0.00 to 1.08 ± 0.09 . Generally, the specific gravity of absolute ethanol is 0.789 while that of distilled water is 1.0. A sample with high ethanol content has a specific gravity that tends towards 0.789 while a sample having more water content has a specific gravity that tends towards 1.0 or more, Thus a decrease in specific gravity indicates high reducing sugar concentration as well as increased ethanol production. On this basis, it is evident that the 50g consortium contained a high percentage of reducing sugar which resulted to increased ethanol production, hence, had a low specific gravity (0.96 ± 0.80 to 0.92 ± 0.10) compared to the specific gravity recorded in other hydrolysates. Contrary to this was the specific gravity recorded in the controls which showed that there was a high percentage of water in the hydrolysate. This results agree with the findings of Mbajiuka *et al.* (2010) and Onwuakor *et al.* (2017) where specific gravity was observed to decrease in hydrolysates with high concentration of reducing sugars.

Table 2: Specific gravity of hydrolysates during fermentation of rice husk

Time (Hours)	<i>S. cerevisiae</i> FJI	<i>P. kudriavzevii</i> IPBCC.y.16.1552	Consortium	Control
0	1.04 ± 0.72^a	1.08 ± 0.26^a	0.96 ± 0.80^a	1.11 ± 0.00^a
24	1.03 ± 0.73^a	1.08 ± 0.25^a	0.95 ± 0.86^a	1.10 ± 0.001^{ab}
48	1.03 ± 0.75^a	1.07 ± 0.26^a	0.95 ± 0.90^a	1.10 ± 0.007^{bc}
72	1.02 ± 0.76^a	1.07 ± 0.27^a	0.94 ± 0.09^a	1.09 ± 0.07^{bcd}
96	1.02 ± 0.78^a	1.07 ± 0.27^a	0.93 ± 0.09^a	1.09 ± 0.07^{cd}
120	1.02 ± 0.76^a	1.06 ± 0.27^a	0.92 ± 0.10^a	1.08 ± 0.08^{cd}
144	1.01 ± 0.72^a	1.06 ± 0.27^a	0.92 ± 0.10^a	1.08 ± 0.09^a

Values are mean \pm SD. Means with the same letter are not significantly different ($p > 0.05$).

Fourier transform infra-red (FTIR) analysis of bioethanol: Usually, ethanol has an absorbance at $3005-2960$, $1200-950$ and $900-850\text{cm}^{-1}$.

This absorbance is due to C-H stretching and O-H bending variations respectively. However, the spectra at $1200-950$ and $950-850\text{cm}^{-1}$ are the most sensitive and exclusive absorbance region for ethanol (Debebe *et al.*, 2017). In this study, pure ethanol peak was found at 1044cm^{-1} and was used for the quantification (Table 3).

Table 3: Percentage ethanol content of hydrolysate

Sample code	Absorbance	% ethanol content
BE1 (50gCrsY ₁)	2.09	35.17
BE2 (50gB ₃ Y ₂)	1.47	29.63
BE3 (50gCrsCrs)	5.87	68.95
BE4 (50gB ₃ Y ₁)	0.70	22.74
BE5 (50gCrsY ₂)	3.30	45.98
BE6 (50gD ₂ Y ₁)	1.54	30.25
BE7 (50gD ₂ Y ₁)	0.70	22.83
BE8 (50gCon)	0.54	21.31

BE- bioethanol, Crs-consortium, B₃- *Aspergillus flavus* CMXY22565, D₂- *Aspergillus niger* SIF2, Y₁-*Pichia kudriavzevii* IPBCC.y.16.1552, Y₂-*Saccharomyces cerevisiae* FJI, Con- Control

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 $1044 - 1047\text{cm}^{-1}$. Coldea *et al.* (2013) obtained ethanol peak at 1047cm^{-1} which was used to determine ethanol in various fruits while Corsetti *et al.* (2015) utilized the ethanol peaks at 1047 1050cm^{-1} to determine the ethanol content in gasoline/alcohol blends.

The results (Table 3) also showed that BE3 which represents the ethanol hydrolysed by the mould consortium and fermented by the yeast consortium had the highest percentage ethanol content of 63.95% which clearly explains its low specific gravity. This may be attributed to the presence of a high concentration of reducing sugars present in the 50g substrate concentration. However, the lowest ethanol content was found in BE4 which corresponds to the ethanol hydrolysed by *Aspergillus flavus* CMXY22565 and fermented by *Pichia kudriavzevii* IPBCC.y.16.1552 which is clearly justified by the relative low amount of reducing sugar made available to these organisms during fermentation. This agrees

with the finding of Oyeleke *et al.* (2012) and Onwuakor *et al.* (2017) where the ethanol with the highest percentage ethanol contents were those produced via hydrolysis and fermentation with consortium of organisms respectively. Interestingly, a low ethanol content was noted in the control which was not inoculated with organisms, this implies that a biochemical reaction caused by certain organisms was taking place in the control, these organisms may have gained access into the medium either from the environment or from the substrate.

Conclusion: Bioethanol was produced using four fungal isolates from waste dumpsites. *Aspergillus niger* SIF2 was found to hydrolyse more cellulose to reducing sugars than *Aspergillus flavus* CMXY22565 while *Saccharomyces cerevisiae* FJI fermented more reducing sugar than *Pichia kudriavzevii* IPBCC.y.16.1552. However, maximum ethanol concentrations (68.95%) was produced from the 50g substrate concentration of the hydrolysate by the mould consortium and fermented by the yeast consortium. Thus, these organisms could be used at the industrial level for bioethanol production and other biodegradation studies. The moulds could further be genetically modified to enable them produce cellulose more efficiently, thus making them more useful in bioethanol production.

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