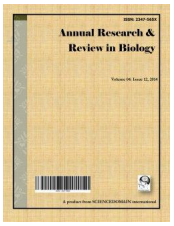

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Bioethanol Production by Enzymatic Hydrolysis and Fermentation of Cocoyam

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Bioethanol, produced by anaerobic fermentation of carbohydrates with microorganism is a liquid fuel used either as energy source or as an additive/enhancer for fossil petrol. This research was carried out to explore the potential of cocoyam starch as an alternative feedstock for bioethanol production. Cocoyam corms and cormels were peeled, dried and milled to flour, the slurries were then mashed with different enzyme cocktails comprising of amylase, glucoamylase and protease enzymes. The saccharified wort obtained was fermented with yeast; Saccharomyces cerevisiae without exogenous nutrient supplementation. Two fermentation processes were employed. Simultaneous Saccharification and Fermentation (SSF) and Separate Hydrolysis and Fermentation (SHF).

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Glucose liberated during mashing was determined by glucose oxidase method and it was found that enzymatic hydrolysis of cocoyam flour was effective in yielding favourable levels of fermentable glucose up to 86g glucose/100g substrate with batch 1 of enzymes. Ethanol production was measured from the cocoyam mash and it was found that S. cerevisiae produced ethanol levels equating to 398 L/ton which compares favourably with yields from cassava 280 L/ton and corn 420 L/ton. These observations indicated that cocoyam can serve as a very cheap alternative biomass for bioconversion to bioethanol with minimal inputs.

Keywords: Bioethanol; fermentation; cocoyam and hydrolysis.

1. INTRODUCTION

The issue of energy security is a major problem globally particularly in developing countries that largely relies on the importation of refined products such as petrol, diesel, kerosene, and even liquefied petroleum gas. Nigeria planned to reverse this trend by partly investing in bioenergy development. Nigeria biofuel policy and incentives was released in 2007 with the aim of spurring a vibrant bioenergy sector development [1]. Consequently, biofuels are attracting interest as transport fuels to mitigate energy security concerns in many countries [2]. Biofuels apply to solid, liquid or gaseous fuel produced from biological materials (biomass), which can be used for the generation of power, heat or fuel for automobiles [3,4]. Some of the liquid biofuels are bioethanol, Biodiesel and biogas. Bioethanol produced from plant biomass has been reported to be more environmentally friendly and sustainable for use as an additive or substitute to petroleum [5]. Majority of energy used in Nigeria are of fossils type, leading to the large burden of carbon dioxide produced during the combustion of fossil fuels and an increase in global warming [6]. Bioethanol, a plant-based liquid fuel is used as an additive or substitute to petroleum. It has been established that ethanol reduces tailpipe carbon monoxide emissions by as much as 30%, toxics content by 13% (mass) and 21% (potency), and tailpipe fine particulate matter (PM) emissions by 50% [7]. Bioethanol, therefore, helps in reducing green house gas emissions and contributes to sustainable development. Furthermore, bioethanol offers the opportunity to improve access to modern energy services for the poorest members of the society [8].

Cocoyam is one of the stable root crop in Nigeria, with over 20 million tonnes lost yearly due to inadequate storage and processing facilities [9]. It thrives in infertile and difficult terrains that are not well suited for large-scale growing of most conventional staple crops [10]. Cocoyam corms and tuber crops are well known for their high carbohydrate contents. Cocoyam has been reported to contain 70 – 80% starch with small size granules [11]. The corm and cormel contain 34.04% and 34.26% amylose and invariably about 66% amylopectin respectively. Cocoyam possesses high nutritional values when compared with other tubers like cassava and yam, with substantial vitamin, and mineral contents.

2. MATERIALS AND METHODS

2.1 Plant Sample Preparation

Freshly harvested cocoyam, *Colocasia esculenta* corms and cormels were obtained from Yankaba Market, in the metropolis of Kano, northwest Nigeria. The sample was taken to Biological Sciences Department of Bayero University, Kano for identification; an Accession Number **BUKHAN0354** was assigned. The cocoyam corms were cleaned, washed, peeled, sliced into chips of 2–2.5 cm thick, and then open dried under shade for 7 days. The dried sample was milled to flour using an electric blender, oven dried at 75ºC for 4 hours, then stored at 4ºC in an airtight polythene bags throughout the period of the experiments.

2.2 Glucose Determination Using Glucose Oxidase Method

Glucose was determined after enzymatic oxidation using glucose oxidase method. The principle is that glucose react with glucose oxidase enzyme to produce glucuronic acid and hydrogen peroxide. The Hydrogen peroxide librated subsequently reacts with aminophenazone and phenol in the presence of peroxidase to produce quinoneimine, which is a chromogen, and the intensity of the red-violet colour formed is directly proportional to the concentration of the glucose [12].

$$\text{Glucose} + O_2 + H_2O \longrightarrow \text{Glucoronic acid} + H_2O_2$$

$$2\ H_2O_2 + \text{4- aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4H_2O$$

Into three test tubes, labeled blank (B), standard (S) and test (T), 1000 µl of Glucose reagent (GOD-PAPand buffer) was added to each of the test tubes. Distilled water (10 µl), standard solution (10 µl) and serum (10 µl) were added respectively. The tubes were mixed and incubated for 5 min at 37°C. The absorbance of the test (A test) was measured against the reagent blank at 540 nm.

2.3 Mashing

2.3.1 Mashing without exogenous enzyme supplement

Mass of 15g of the cocoyam flour was measured in triplicates for each group of selected mashing temperatures, i. e. 50ºC, 60ºC, and 80ºC. A volume of 100mL of distilled water were added to form slurry. The slurries were then autoclaved at 121ºC for 15 minutes, samples were allowed to cool and 100mL of distilled water were added, stirred to homogenize. The samples were then cooked for 60 minutes, 90 minutes, and 150 minutes respectively. After mashing, glucose and protein concentration were determined and recorded.

2.3.2 Mashing with enzyme supplement

Exactly 15g of the cocoyam flour were measured in triplicates for each group of selected cooking temperature; 50ºC, 60ºC, and 80ºC. A volume of 100mL of distilled water were added to form slurry; the slurries were then autoclaved at 121ºC for 15 minutes, samples were allowed to cool and 100mL of distilled water was added and stirred in order to homogenize the whole mixture. The freshly prepared enzyme cocktail (120µL each containing 60µL protease, 30µL alpha amylase and 30µL glucoamylase) was added. The three cooking styles employed were as follows:

At steady temperatures of 60ºC, the slurries were cooked for 90 minutes straight and enzyme cocktail 120µL each containing 60µL protease; 30µL alpha-amylase and 30µL glucoamylase were added at the beginning, while glucose and protein were determined after the mashing.

At a higher temperature of 80ºC, enzyme supplement added were 40µL protease 30µL alpha amylase and 20µL glucoamylase the slurries were cooked for 60 minutes and the temperature was adjusted to 60ºC followed by the addition of 20µL protease and 10µL glucoamylase which completed the enzyme cocktail of 120 µL. it was then cooked for 30 minutes to complete the cooking time of 90 minutes.

At a lower temperature of 50ºC, enzyme supplementation were 45µL, 20µL, and 20µL of protease, amylase and glucoamylase respectively. The slurry was cooked for 60 minutes and the temperature was adjusted higher to 80ºC.

2.4 Yeast Culture

Saccharomyces cerevisiae was inoculated in a 400mL flask media. This media comprised 4% (w/v) bacteriological peptone, 2% (w/v) yeast extract and 4 %(w/v) glucose. The YEPD culture was inoculated at 32ºC for 24 hours a 150rpm in the shaking incubator [13].

2.4.1 Viability of the yeast cells

The cells were tested for viability after the culture in the population using Bromo Phenol Blue method. The principle was that, the living cells only would absorb the dye. The cells were observed under the microscope when reacted with the dye. Over 80% of the population was found to absorb the dye.

2.5 Fermentation Using Incubator with Orbital Shaking

The fermentation protocols were adopted from the work [14] with little modifications Fermentations were set to run at the same

Table 1. Enzymes cocktail preparation

Enzymes	Batch 1	Batch 2	Batch 3
Protease (Alcalse)	60µL	45µL + 15µL	40µL + 20µL
Alpha amylase	30µL	20µL+10µL	20µL + 10µL
Glucoamylase	30µL	20µL + 10µL	15µL + 15µL
Total	120µL	120µL	120µL

All three enzyme cocktail (batch 1, 2 and 3) contained same volume of enzymes 120µL.

conditions, mashed sample replicates were prepared for two methods of fermentation; Simultaneous Saccharification and fermentation, (SSF) and separate hydrolysis and fermentation (SHF).

A volume of 100ml of the wort each were placed in 250mL conical flask and 1mL of the cultured yeast cell were added. With the use of pH meter, the pH of each was adjusted using H_2SO_4/NaOH to lower pH of 6.0 for yeast's growth. The conditions for the incubator shaker were set to be 32ºC, rpm 150 and 72 hours for the temperature, rotations, respectively. At 24 hour, 48 hour and 72, hour intervals, samples were drawn to monitor the progress of the fermentation.

2.5.1 Ethanol quantification using UV/visible spectroscopy LAMDA 35

Absolute ethanol (pure ethanol) sample was first scanned in the rage of 190-1100nm of the wavelength, it was found out that 192nm was the Lambda max for the ethanol, and this wavelength fall in the far Ultra-violet regions. Literature has shown that pure ethanol absorbs at ultraviolet regions. Serial dilutions were made of the working standard, 2, 4, 6, and 8 %, these were measured in the spectrophotometer and a calibration curve of 0.9783 r^2 value was attained. The samples were run against the curve, and then concentrations of the test sample were obtained by multiplying with dilution factor [15].

2.5.2 Fourier transform infrared spectroscopy method

The method of [16] was adopted for this analysis. Serial dilution of standard ethanol sample was made as follows: 6, 12, 24 and 48%. The samples of the two fermentation processes SSF and SHF were measured and compared with the standard ethanol samples at 1087 and 1047 cm^{-1}. Since ethanol has a specific frequency of vibration at 1087 and 1047 cm^{-1}. The concentrations of the samples were evaluated by extrapolating with standards absorbance against cocentration.

3. RESULTS AND DISCUSSION

3.1 Glucose and Protein Yield after Mashing

3.1.1 Mashing without enzymes supplementation

Fig. 1 presents the concentrations of the released/hydrolyzed glucose from mashed samples without exogenous enzyme supplementation at varying temperatures of 50^0C, 60ºC and 80ºC within time frames that ranges from 0 to 150 minutes. Readings were recorded at the 0^{th}, 60^{th}, 90^{th} and 150^{th} minutes. The concentration of glucose increases with rise in temperature. However, the highest concentration of up to 22.3g/100g was obtained in the samples mashed for 90 minutes at 80ºC. Lowest concentration of 9.7g/100g was recorded in samples mashed for 60 minutes at 50ºC. There was a recorded significant difference at $p>0.05$ level of significance between samples mashed for 90 and 150 minutes at all the three temperatures.

3.1.2 Mashing with enzymes supplementation

Fig. 3 presents glucose concentrations after mashing with the different enzymes supplementations. The cocktails are named as batches 1, 2 and 3. The highest concentration of up to 86.8g/100g glucose was obtained when the sample was mashed with batch 1 enzyme cocktail. The value was found to be significantly higher ($p>0.05$), than the values obtained from batch 2 and 3 (75.7g/100g and 76.2g/100g) respectively.

3.1.3 Effect of protease on release of FAN (Free Amino Nitrogen)

FAN concentrations shown in figure 5 suggests nutrient content of the hydrolysate especially the nitrogen content of the wort before fermentation. The values are expressed, as *mean* ± SD. There was a drastic fall in the concentrations of FAN after fermentation. In SSF1, for example, the initial FAN concentration was 258.5mg/L and 67.1mgL after the fermentation (final). The highest initial FAN concentration of up to 269.9mgL was recorded in SSF3 while the final concentration dropped to 19.66mg/L.

3.2 Rate of Glucose Utilization during Fermentation

During fermentation, yeast consumes glucose initially for energy and development. It can be observed from the figure below that about 50% of the initial sugar concentration has been consumed in the first few hours. This may be attributed to multiplication of yeast through reproduction in the lag phase. The rate of glucose utilization continues to progress drastically up to 72 hours fermentation time. However, fermentation stops finally with very little residual glucose concentrations.

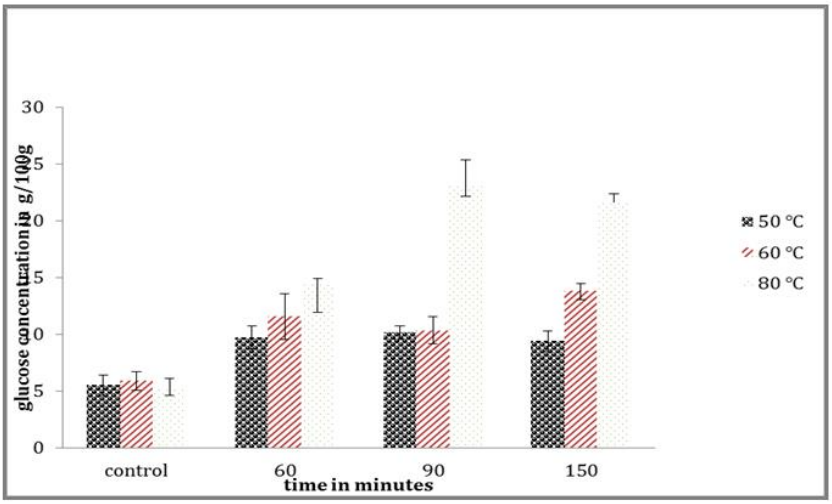

Fig. 1. Glucose yields at different mashing temperatures and time without enzyme supplementations. Values are expressed as mean ±SD. Values analyzed using ANOVA under Duncan Posthoc analysis at p>0.05 level of significance

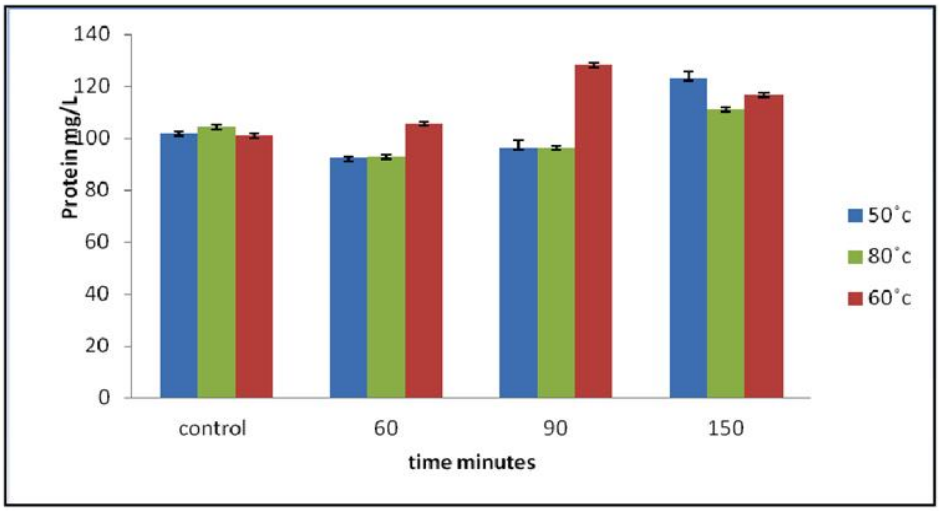

Fig. 2. Protein yields (in mg/L) at different mashing temperatures and time without enzyme supplementations. Values are expressed as mean ±SD. Values under the same group with different letter superscripts are significantly different at p>0.05 level of significance

3.3 Fermentation

Following the hydrolysis of the biomass, the samples subjected to fermentation were considered for the two different fermentation protocols. The ethanol yields during separate hydrolysis and fermentations (SHF) and simultaneous hydrolysis and fermentation (SSF) were measured and expressed in L/ton. Fig. 7 presents the results with SSF having significantly higher values (398 L/ton) compared to the SHF at p>0.05 level of significance. Replicates of the two different fermentations were read and presented as *mean* ± SD. Surprisingly, Separate Hydrolysis and Fermentation (SHF) yielded 180.5 L/ton. The difference in the yield may be attributed to the fact that SSF process has advantage of continues release of free sugar and subsequent fermentation while the SHF method released all free glucose and commences fermentation.

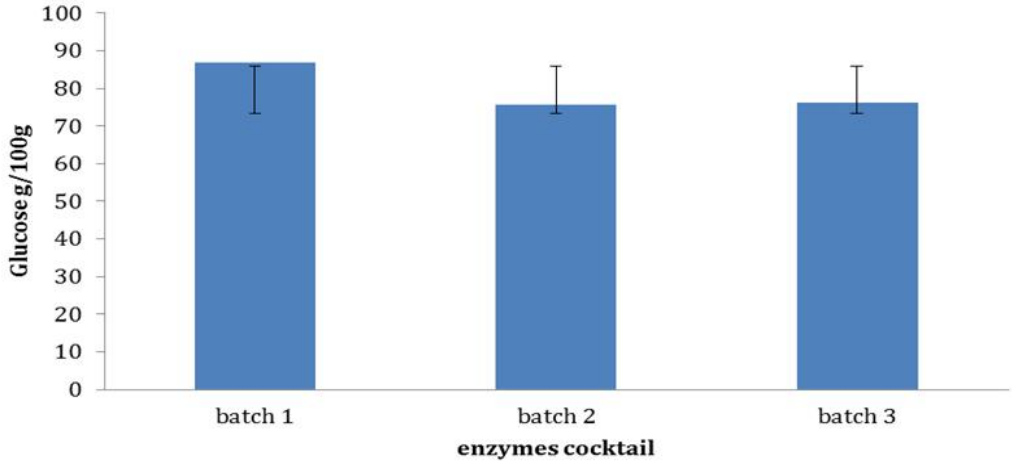

Fig. 3. Glucose released after mashing with enzyme supplementations. Three replicates of the results are presented as mean ±SD. Batch 1 was mashed at 60°C for 90 minutes; batch 2 was mashed at 60°C for 60 minutes and then 80°C for 30 minutes; and batch 3 was mashed at 80C for 60 minutes and 60°c for 30 minutes

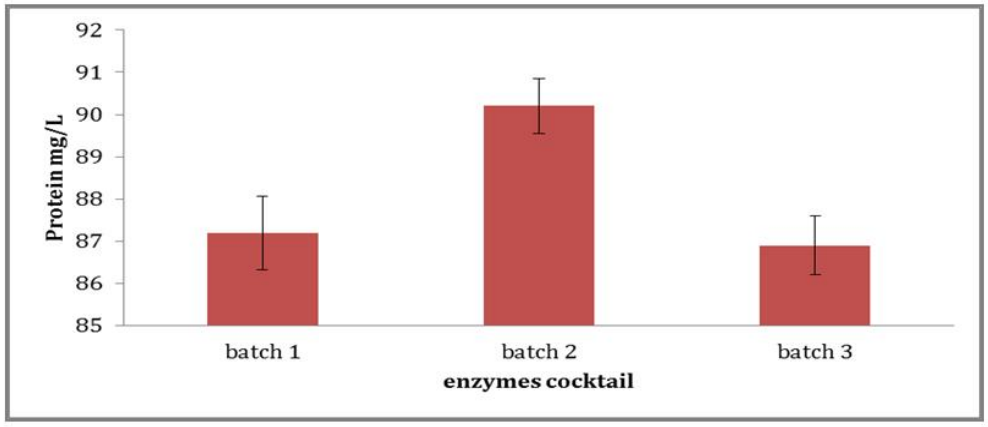

Fig. 4. Protein concentrations in mg/L after mashing with enzymes supplementations. Batch 1 was mashed at 60ºC for 90 minutes; batch 2 was mashed at 60ºC for 60 minutes and then 80ºC for 30 minutes; and batch 3 was mashed at 80ºC for 60 minutes and 60ºC for 30 minutes

4. DISCUSSION

The presence of high carbohydrate (starch) content indicates potential of cocoyam as a very good source of starch for bioconversion to ethanol. Previous researches have reported 76-80% starch content in cocoyam [17,18]. During mashing without enzyme supplementation, a progressive increase in glucose concentrations was observed with a corresponding increase in temperatures and time. This may be attributed to latent increase in activity of the enzymes present in the substrate at higher temperature. However, protein concentration decreases with rise in temperature as shown in Fig. 2. Proteins are mostly known to be denatured at higher temperatures. The varying temperature in this research showed that proteins compositions at higher temperature are lower compared to those at lower temperatures. During mashing at different temperature and time, lower temperatures recorded lower glucose and higher protein yield while high glucose yield were recorded at a higher temperature (P>0.05). This indicated that the latent amylases were more active at higher temperatures. P. Three different batches of the enzyme cocktail treatments at different temperatures and time were used in order to optimise the most appropriate conditions for the hydrolysis of cocoyam starch for an

optimum release of the reducing sugars for better ethanol yields. Varying these temperatures was aimed at establishing the suitable temperature for cocoyam hydrolysis. To attain optimal starch to glucose hydrolysis, exogenous enzymes supplementation are required, from the results above less than 30% of the starch has been hydrolysed.

The highest glucose released (Fig. 3) was obtained following mashing with batch 1 enzyme. The other two optimised mashing temperatures also released high glucose. Batch1 liberated this highest glucose at lower temperature compared to the other 2 batches, which were mashed at the 2 combined temperatures. These results revealed that exogenous enzyme supplementation increases starch hydrolysis by over 60%. For optimal release of glucose from cocoyam starch, enzyme supplementation is highly recommended due to the observed significantly increased glucose release rate. These enzymes batches showed that while amylases degraded starches, complex proteins are equally being hydrolysed to simple ones by the protease (*Alcalase*). Ethanol stress or ethanol tolerance by yeast cell is usually considered to be genetically based and also dependent on the physiological status of the cell and physicochemical nature (process parameters and bioavailability of essential nutrients) of the fermentation medium [19].

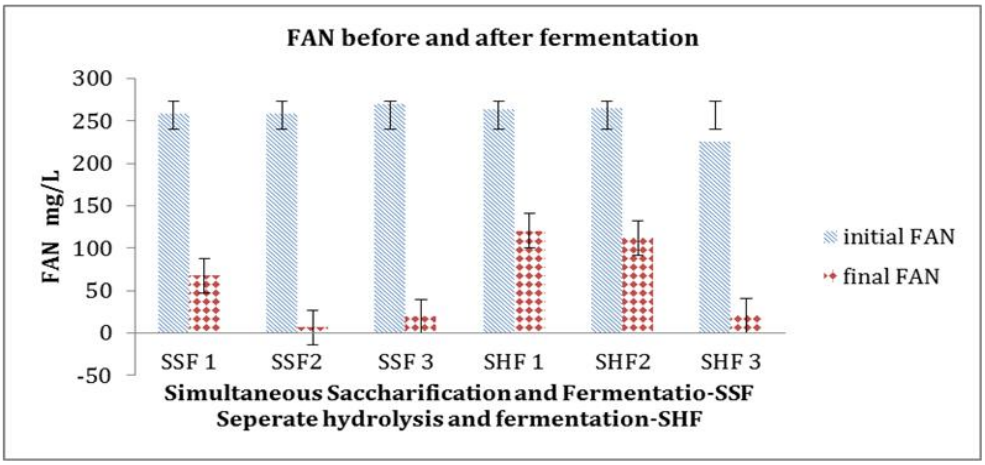

Fig. 5. Free Amino Nitrogen concentration before and after fermentation for the simultaneous scarification and fermentation and the separate scarification and fermentation

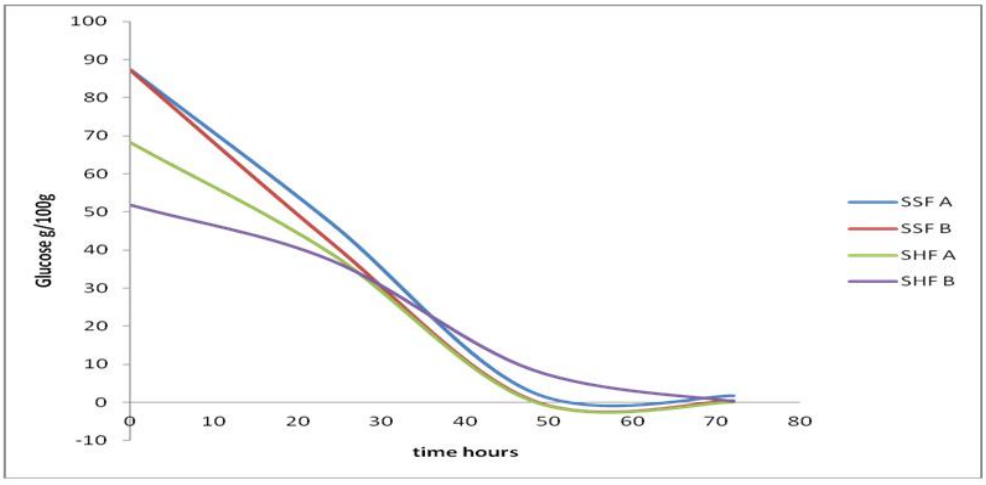

Fig. 6. Rate of glucose utilization during fermentation glucose concentration declining with time of fermentation.

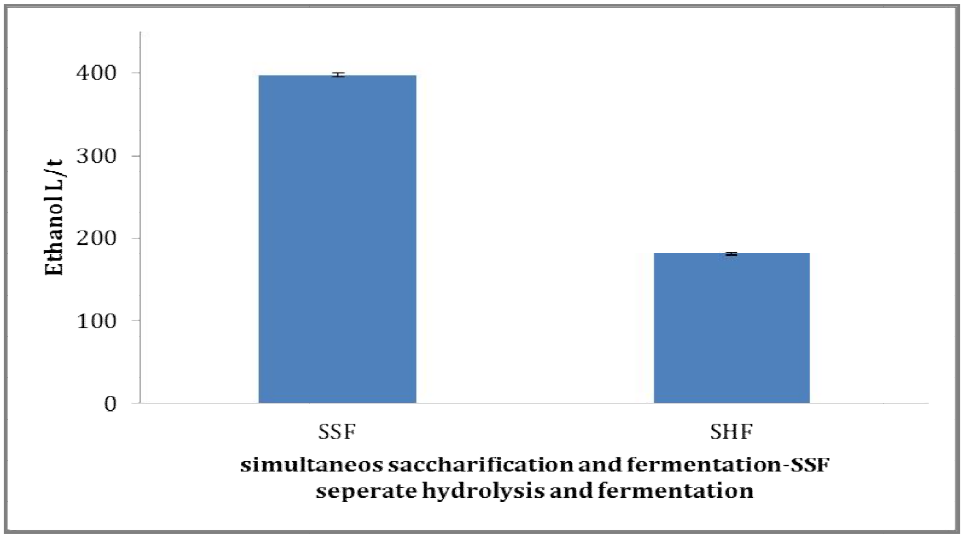

Fig. 7. Ethanol yield expressed in L/ton "SSF (Simultaneous Saccharification and Fermentation), SHF (Separate Hydrolysis and Fermentation)

FAN is required for efficient yeast metabolism during fermentation; its high initial concentration determines how long the yeasts can survive in the fermentation medium. Complex proteins are hydrolyzed to simple proteins or peptides for yeast easy assimilation. This affects growth of yeast and ethanol production.FAN concentrations were also influenced by the hydrolysis of the complex protein by the action of protease in the enzymes cocktail. FAN released were high when it was analyzed. These concentrations indicate that no exogenous nitrogen source is required for effective bioconversion [20].

The higher ethanol yield of 398 L/ton fir SSF is attributed to the fact that more sugars were liberated as fermentation is taking place. Other required nutrients might be present in the residue, and contributed to effective start to completion of most sugars to ethanol. Higher investment costs for two separate reactors for both hydrolysis and fermentation will make the process more expensive compared to SSF. Inhibition of the high glucose concentration to fermenting organisms is one major disadvantage of SSF [21].

The observed ethanol concentration of SSF (398L/tone) is slightly higher compared to previously reported ethanol concentrations of cassava 280 L/tone [20] and husk sorghum grain [14] 379 L/tone.

4. CONCLUSION

Cocoyam (*Colocasia esculenta*) has promising potential as a feedstock for bioethanol production. Its ethanol yield is higher comparable to most of the established feedstock. Therefore, our findings indicate that cocoyam could be utilized as a low-cost feedstock source for bioethanol production. Interestingly, the results suggest that cocoyam sourced from fields or storage facilities could be utilized directly for bioethanol production without prior investment in pre-treatment processes and no addition of nitrogenous supplement is needed. Exclusion of these processes prior to fermentation of substrates would not only save costs, it will reduce energy consumption that might otherwise be needed to carry out such processes. Less energy consumption is beneficial in both costs and green house gas emission reductions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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