INFLUENCE OF THE PHYSIOCHEMICAL PROPERTIES OF MANIHOT ESCULENTA (CASSAVA) ON THE HYDROLYSIS AND FERMENTATION YIELDS

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ABSTRACT

The present study focuses on utilizing four local varieties of the starch rich tropical tuber, Manihot esculenta Cranz (cassava), for the production of ethanol. Each variety of cassava was characterized. Moisture content, starch granule size, and gelatinization data were obtained. The reaction parameters that were varied during hydrolysis of starch included acid type (sulfuric acid, nitric acid) and acid concentration (0.2 %, 0.5 %, 0.8 %, 25 %, and 35 % v/v). The concentration of reducing sugars present in the hydrolyzate was determined using the colorimetric Dinitrosalicyclic acid method (DNS) at 540 nm. Conditions that were varied during fermentation included fermentation time (16 to 24 hrs) and substrate concentration under constant temperature (32 °C) and pH (4.0) using Saccharomyces cerevisiae. There was a significant ($p<0.05$) difference among the other varieties and unclemac when 35 % sulfuric acid was used for hydrolysis. It was observed that a concentration of three quarters the original hydrolyzate concentration fermented for 24 hrs gave significantly different ($p<0.05$) results when compared to the other three varieties (butterstick, rusta and brancha). This study also successfully outlined the significant influence of granule size and gelatinization temperature on the hydrolysis yield of sugars from the cassava varieties. This study provides a comprehensive characterization of raw material and optimization of reaction parameters for ethanol production from a cheap, readily available, and renewable source of sugar found in Guyana.

Keywords: *Acid hydrolysis, fermentation, cassava, starch characterization, gelatinization*.

1. INTRODUCTION

This research study focuses on examining the possibility of producing ethanol from the sugars obtain after the hydrolysis of the starch present in cassava. Cassava belongs to the family *Euphorbiaceae.* The main aim of this study was to characterize the physiochemical properties of cassava and determine whether these factors affected the hydrolysis of starch. This entailed investigating the effect of different conditions on starch hydrolysis and the fermentation process. Cassava is a subsistence crop valued because of its drought resistance, its ability to grow in poor soils and its relative resistance to weeds and pests. It is grown between 30 ^{o} north and south latitudes and at altitudes below 2000 m.Yields decrease vastly with latitude above 1000 m near the equator. Some of the more common varieties of cassava grown in Guyana include, *Brancha, Butterstick, Uncle Mack, Mex 52* and *Bad Woman.*
Given the global problems facing humans, inclusive of Global warming (as a result of emission of gases into the atmosphere) and climate change, new technologies are given importance in terms of migration from fossil fuels- the major contributor of pollutants in the air. As a result, extensive research is being done in areas such as biofuels and other aspects of alternative sources of energy. Biofuels, derived from plant sources, hold immense potential for meeting the global future energy needs. In this world of uncertainty of regular availability and constant supply of traditional fuels, it is a welcome sign that bio-fuels are being given serious consideration. This is so given that they are potential sources of energy in the future, particularly in countries with very tight foreign exchange positions and insufficiently availability of traditional fuels within their own geographical boundaries.
As a result, researchers have continuously searched for methods of producing fuels and drop in chemicals from cleaner and renewable sources. In order to supplement the use of fossil fuel for transportation, many countries have embarked on research pertaining to biofuels either as additive to gasoline or as 100 % fuel for flex vehicles. The production of ethanol from grains especially corn [1] and molasses [2] in the United States and Brazil, respectively has been revolutionized over the past decades and are now competitive with fossil fuels. Nevertheless, the debate always revolves around the food vs fuel aspect and these feedstocks are not seen as feasible/ideal sources for the future. Instead work has focused on lignocellulosic materials with new enzyme technologies and pretreatment

processes that are capable of degrading the resistant structure of the material. On the other hand, another platform involves the hydrolysis of feedstocks native to a given region. For example, in Guyana, there is a large scale production of starch rich crops such as cassava, yam, and sweet potato, with the potential to produce in excess of domestic consumption. Hence, for countries such as ours, the ideal platform would be to utilize resources that are abundant and inexpensive locally. As a result, hydrolysis of the starch present in tubers for the production of sugars which are then fermented to alcohols is an important study for developing countries.

The hydrolysis of starch can be accomplished in many ways including acid catalyzed, base catalyzed, enzymatic hydrolysis, and heat induced cooking. The methods that have found widespread application are those of enzymatic and acid catalyzed hydrolysis. Starch hydrolysis involves the processes of liquefaction and saccharification. Liquefaction entails the conversion of the starch granules into a low viscosity solution. On the other hand, saccharification involves breaking down starch hydrolysis products into glucose.

Numerous enzymes have been employed for the enzymatic pathway such as glucoamylase. This enzyme catalyzes the release of D-glucose from the non reducing ends of starch or related oligo- and polysaccharide molecules. Glucoamylases (1, 4-α-D-glucan glucohydrolase, EC 3.2.1.3) are produced by several filamentous fungi and yeasts, with those from *Aspergilli* being commercially most important [3]. But before we introduce this enzyme, amylase is usually employed to initiate the process. In fact, the partial hydrolysis of the precursor starch utilizing α-amylase provides the initial breakdown of the starch molecules by hydrolyzing internal α-(1- 4) linkages. It was found that the initial hydrolysis using α-amylase is run at a temperature of approximately 105 °C and very high starch concentration is processed, usually 30 % to 40 % solids. The initial hydrolysis is usually carried out for five minutes at this elevated temperature. The partially hydrolyzed starch can then be transferred to a second tank and incubated for approximately one hour at a temperature of 85 to 90 °C to derive a dextrose equivalent (D.E.) of 10 to 15. Next, this batch of hydrolysate is then exposed to the glucoamylase at around 55 °C to 60 °C with the pH maintained at 4 to 4.5 [3]. Analysis of the media should be determined given that factors such as amount of fibbre in media; physical form, cooking conditions, and the presence of a natural amylase inhibitor affect the results. Ratman et al. (2003) [4] found that amylase exhibits maximal activity on a variety of starch substrates when the temperature is maintained at 135 °C and at a pH of 6.5.

In brief, the enzyme most suited for liquefaction is α-amylase whilst glucoamylase carries out the saccharification role. It should be noted that the starch source needs to be gelatinized before proceeding with hydrolysis. Hence, the hydrolysis of starch can be achieved with a greater degree of efficiency when it is exposed to an internal enzyme such as α-amylase before stripping one glucose molecule off at a time (glucoamylase). This is done so as to facilitate the exposure of more non reducing ends of the chain for glucoamylase to work on. Based on the literature search, the optimum conditions for enzyme activity (both amylase and glucoamylase) include pH of 4.0 to 6.5 at a temperature of 55 to 80 °C [5].

Another method that has been used for starch hydrolysis is that of heat -induce cooking. Extrusion of starch granules is most effective when coupled with enzymes. The starch is treated under conditions of high temperature, pressure, and moisture. Extrusion facilitates the rupturing of starch granules and the breakdown of crystallinity of the granules. This effectively renders the amylose and amylopectin susceptible to gelatinization [5].

It has been found that starch can also be hydrolyzed in an alkaline medium. Brown in 1935 [6] investigated the capability of hydrogen peroxide and ferrous sulphate in hydrolyzing starch. The experimental conditions included temperatures about 37 °C and reagent concentrations of 0.01 M ferrous sulphate and 1 % hydrogen peroxide. It was found that the rate of splitting was directly proportional to amount of ferrous sulphate. The main concern with this process is that the hydrolysis does not stop, instead oxidation of the simple sugars to aldehydes and ketones occur. Further, Gatin– Gruzewska (1910) [7] and Gerber (1912) [8] demonstrated that starch hydrolysis may be brought about by using hydrogen peroxide and ferric chloride. Thus starch hydrolysis may be brought about if hydrogen peroxide (1 %) is utilized in conjunction with some metal catalyst such as iron.

The other method employed for starch hydrolysis consists of an acid medium. This method utilizes acid such as hydrochloric, sulfuric, phosphoric, and nitric acids. According to Miller and Muller in 1981 [9], hydrochloric acid can be employed under conditions of 126 °C to about 316 °C, in which range the pressure of the steam can vary from about 25 psig to about 250 psig. This is done over a period of 1 to 15 minutes. Additionally, Ya-Jane Wang, Van-Den Truong and Linfeng Wan (2003) [10] found that higher concentrations of hydrochloric acid more readily hydrolyzed both the amylose and amylopectin portions of corn starch. (i.e. 1.0 N was more active than 0.04 and 0.16 N). Similarly, Betancur and Chel (1997) [11] demonstrated that hydrochloric acid concentration of 0.5 − 1.5 % within a temperature range of 45−55 °C, and a reaction time of 3 to 6 hours had the most breakdown on different starch sources.

Choi and Kim in 2007 [12] investigated the conditions for the hydrolysis of starch and found that the best yields were obtained when sulfuric acid concentrations of 1 % to 5 % at a temperature range of 90 to 100 °C were utilized. Although, sulfuric acid has only 56 % conversion power as that of hydrochloric acid, it has several advantages.

Some of these include no change in specific rotation of the hydrolyzate solution and a better sugar yield. Some of the parameters investigated for sulfuric acid hydrolysis include utilizing acid concentration of 0.25 – 0.5 % and pressurized steam heating of 145 – 155 °C. Other patents that have recorded good conversion include using 32.2 % acid under 190 – 210 °C. The use of mild acid conditions such as 0.7 % sulfuric acid at 190 °C for 3 minutes can be employed for the recovery of the 5 carbon sugars whilst harsher conditions of 0.4 % acid at 213 °C for 3 minutes can be used for the six carbon sugars. The liquid hydrolyzates are subsequently recovered from this stage and neutralized and subsequently fermented to ethanol.

The method selected for acid hydrolysis depends on several factors. The concentrated acid method utilizes acid in a very high concentration of acid and relatively low reaction temperatures. It is associated with a very high sugar yield (approximately 90 %), rapid reaction time (10- 12 hours) and usually gives little or no degradation. Nevertheless, given that more acid is used, this method is much more expensive.

In summarizing, a method for starch hydrolysis by adding it to an acidified medium can be accomplished by using about 1 to about 5 percent by weight of mineral acid based on the weight of starch to be hydrolyzed, at a temperature above about 80 °C and over a period of from about 5 to about 30 minutes. Most often than not, either hydrochloric or sulfuric acid is used because of the greater sugar yield.

The basis of this research serves to investigate the optimum parameters for the conversion of cassava starch to simple sugars and the eventual production of ethanol. This was done on a four locally grown varieties of the cassava. A conclusive investigation was done to determine which variety of cassava and which reaction parameters are most efficient for this process. One should keep in mind that the study seeks to find a way of producing an alcohol from a possibly abundant source of renewable feedstock- cassava. Further, the research will seek to investigate the influence of different parameters such as acid strength and hydrolysis time on the hydrolysis process. Additionally, conditions such as fermentation time and substrate concentration will also be investigated so as to determine the optimum operation parameters for fermentation. From above, one can appreciate that this project will seek to find out whether this technology has the potential in terms of an alternative fuel on the basis of its efficiency of the optimum processing technique.

Currently, the use of cassava starch to produce fuel ethanol faces significant technical and economic challenges. Its success depends largely on the development of environmentally friendly pretreatment procedures, highly effective enzyme systems for conversion of pretreated cassava to fermentable sugars, and efficient microorganisms to convert multiple sugars to ethanol. The research will also focus on characterizing the starch found in the different varieties of cassava. As a result, parameters such as plasticity, granule size and rigidity of structure were studied. In the end, we present a conclusive section on whether these properties can affect the sugar yield after hydrolysis and the subsequent production of ethanol.

2. MATERIALS AND METHODS

2.1 Materials

The different varieties of cassava were retrieved from a farm down the Demerara River, Guyana. Sulfuric acid (95 – 98 %), nitric acid (> 70 %), phenol (> 98.5%), sodium hydroxide (> 98 %) were sourced from Sigma-Aldrich. *Saccharomyces cerevisiae* (yeast) was obtained from Demerara Distiller's Limited (Diamond, East Bank Demerara, Guyana).

2.2 Methods

2.2.1 Preparation of feedstock

The different varieties of cassava were washed, peeled, and then grated so as to increase the surface area for treatment. Approximately 200 g of feedstock was then placed into two crucibles and dried in under vacuum for 24 hours at 45 °C. Once dried, the samples were then comminuted using a pestle and mortar and the powder obtained was used to for the subsequent analyses.

2.2.2 Characterization of feedstock

A Brabender Amylograph-E was used to determine the moisture content of the different sample powders, the temperature at which gelatinization began, and ended. For this analysis, 80 g of sample was weighed into a 1000 ml flask and 360 ml of distilled water was added. The flask was plugged and inverted for 50 times in a 30 seconds periods (making sure no lumps were formed). The suspension formed was then poured into the amylograph bowl and placed into the instrument. The testing was initiated and a print out of the amylograph provided the necessary information.

2.2.3 Moisture content of samples

Given the uniqueness of the powder obtained (cooks at 100 °C), a method needed to be developed to estimate the moisture content. Each variety was done in triplicates; the crucibles were dried in oven at 70 °C prior to analysis. Samples were then analyzed at 55 °C and the mass of the samples monitored every hour until constant. The moisture content was then calculated using the difference of mass lost.

2.2.4 Starch granule size

The sizes of starch granules for the different cassava varieties were determined using a microscope. A small amount of powder was placed on a slide using a glass transfer pipette. The sample was then carefully covered with iodine. The slide was covered with a cover slip and then placed under the microscope. The sizes of the granules were estimated via averaging the size of 20 granules per sample using a magnification of a 100.

2.2.5 Hydrolysis of starch in feedstock (Mini-treatments)

The starch inherent in the powder samples for each variety was hydrolyzed to simple sugars that can be subsequently used for fermentation. Acid concentrations of 35, 25, 0.8, 0.5, and 0.2 % (v/v) were used for both acids (sulfuric and nitric acid) in triplicates. Approximately 5 g of cassava powder was placed into each labeled flask with 10 ml of distilled water. These flasks were then placed into a water bath at 80-85 °C to promote gelatinization of starch granules. 15 ml of acid of different concentration was added to the respective flasks and continuously stirred while being heated. This was done for 1 hour, after which the hydrolyzates were cooled to room temperature and the sugar concentration quantified using the phenol sulfuric acid method.

2.2.6 Determination of sugar concentration

The phenol-sulfuric acid method was used to determine the quantity of reducing sugars present in each flask after hydrolysis. This method utilizes concentrated sulfuric acid and 0.4 % phenol solution to produce a yellow orange colored that can be read spectrophotometrically at 490 nm.

The method entailed diluting 3 mL of hydrolyzate to 100 mL using distilled water (done in triplicates). Three 100 µL aliquots from each flask was placed into a test tube, 100 µL water was added to each tube along with 0.5 mL 0.4 % phenol. The blank for the method was prepared by placing 200 µL of distilled water into a tube. To each tube, 2.5 mL of concentrated sulfuric acid was added and immediately placed in the dark to allow color to develop. The absorbance for each tube was then read at 490 nm.

2.2.7 Bulk hydrolysis

Once the most efficient (most sugars produced) acid and acid concentration were selected for each variety of cassava, the next step was to proceed to the bulk hydrolysis. In each case, 100 g of cassava was weighed into a 2000 mL beaker, 240 mL distilled water was subsequently added to the beaker to facilitate gelatinization at 80-85 °C. Once gelatinization was evident (formation of as paste), 360 mL of the acid was added to initiate the hydrolysis. The mash was continuously stirred for one hour. After hydrolysis, the hydrolyzate was obtained via filtration, and the sugar concentration determined.

2.2.8 Fermentation

When the hydrolyzate was obtained it was prepared for fermentation. Before the actual fermentation, the very acidic media was neutralized to a pH of approximately 4.0. Fifteen 100 mL conical flask were labeled in triplicate as follows: 16 hrs, 20 hrs, 24 hrs, 0.75, and 0.5. All of the flasks labeled as time, 30 mL of the hydrolyzate was added. Likewise in the flasks labeled 0.5 and 0.75, 15 and 22.5 mL, respectively of hydrolyzate were added (distilled water was added so as to make the final volume 30 mL). The contents of these flasks were then neutralized using 30 % (w/v) of sodium hydroxide until a pH of 4.0 was obtained. A water bath was gradually used to heat the contents of the flask up to about 38 °C and approximately 1 g of yeast (*Saccharomyces cerevisiae*) was added to each flask. The flasks were then stirred and the temperature was allowed to gradually drop to about 32 °C. The time for fermentation was varied according to how the flasks were labeled. For the flask with different concentration of sugar, 24 hours of fermentation time was afforded. Once the time for fermentation had elapsed, the contents of the stoppered flask were heated to about 70 °C for 5 minutes so as to inactivate the yeast. The fermented mix was then measured and distilled.

2.2.9 Distillation and alcohol content determination

Once the samples were quenched, the liquid was distilled to remove the alcohols. The distillates were stored at 4 °C until ready to be analyzed. A 10 mL Pycnometer (Kimax) that was calibrated at 20 °C was used to determine the

amount of alcohol in the samples. The pycnometer was cleaned using distilled water. The samples were placed into cold storage until the temperature adjusted to 15 °C. A sample was then transferred to the pycnometer and covered using the thermometer. The outside of the pycnometer was cleaned using lint free tissue. The temperature was allowed to adjust to about 19 °C and then the weight of the pycnometer was taken. The same was done for all samples.

3. RESULTS AND DISCUSSION

3.1 Characterization of different varieties of cassava

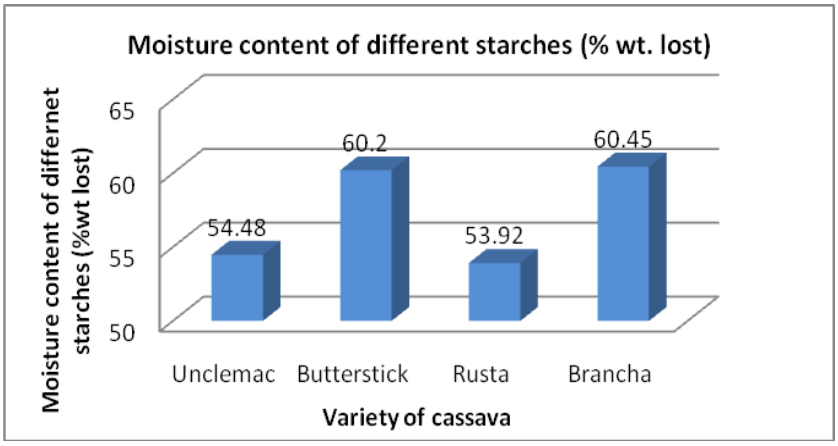

Figure 1. Moisture content of the different cassava varieties.

Based on the moisture content displayed above, assuming the other components such as lipids and proteins are very small it can be postulated that Rusta and Unclemac have the greater starch content. Greater starch content leads to more simple sugars once hydrolysis is efficient. Table 1 illustrates the main properties of the different varieties based on the Brabender Amylograph and the microscopy study of the granule size.

Table 1. Characteristics for each variety of cassava

Variety of cassava	Moisture content (% wt lost)	Granule Size (µm) ± 0.25 µm	Begin of Gelatinization (^{O}C)	Gelatinization temperature (^{O}C)	Gelatinization Maximum (AU)
Unclemac	54.48	4-28	68.5	78.8	2566
Butterstick	60.20	2-4	71.9	78.0	2789
Rusta	53.92	4-12	70.9	76.3	2851
Brancha	60.45	2-8	70.8	77.3	3253

Unclemac had the largest granule size whilst *butterstick* has the smallest granules. This property can influence the gelatinization temperature given that larger granules (greater surface area) are affected faster by heating in presence of water (gelatinization). This was supported by the evidence provided by the amylographs for the different starches, i.e., it was found that *Unclemac* began to gel faster than the other starches whilst *butterstick's* starch granules needed much more energy (in form of heat) to rupture. Based on this, the size of granules correlates to the heat of gelatinization needed for each variety. The gelatinization maximum gives an indication of the gelling property of the starch. A high AU value indicates a high gelling property, hence a stronger gel. A high value corresponds to more swelling and expansion of the gel. Hence, it should be appreciated that *brancha* with a high value should be the least susceptible to hydrolysis given that it forms a stronger gel. On the other hand, *unclemac* should be most susceptible to acid attack given that it does not expand that much and forms a not so strong gel.

3.2 Hydrolysis data

It should be noted that the research focused on determining the optimum hydrolysis conditions for this particular feedstock- cassava. Nitric and sulfuric acids were used at different concentrations {35 %, 25 %, 0.8 %, 0.5 %, 0.2 % (v/v)} at a fixed temperature. Figure 2 outlines the hydrolysis yield for each variety of cassava using the different acids at different concentrations. No data was possible for the dilute acid systems (0.5 and 0.2 %) because after

hydrolysis the mash formed was too viscous to remove any hydrolyzate. Nevertheless, sulfuric acid at 35 % for *unclemac* was significantly different ($p<0.05$) from the other systems. This is due to the larger starch granules associated with this variety (as mentioned earlier) and the low temperature at which gelatinzation began (relative to other varieties). The low gelatinization temperature allowed faster swelling of the granules and subsequent hydrolysis.

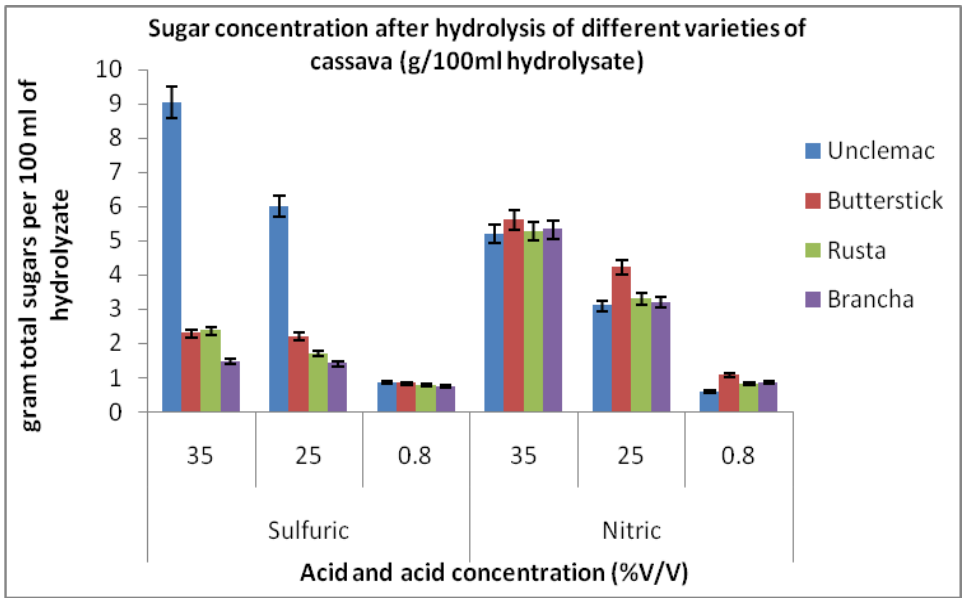

Figure 2. Hydrolysis yield (g sugar/100 mL hydrolysate) for different systems

Despite the good yield for *unclemac* with sulfuric acid, it was observed that nitric acid had a better overall result for the different varieties as reflected in Table 2. Table 2 lists the efficiency for each system, as pointed out, the *unclemac* system hydrolysed by sulfuric acid is the most efficient, but for the other varieties, nitric acid had a better yield.

Table 2. Hydrolysis efficiency for the two acids used at different concentration.

Variety of cassava	**Hydrolysis efficiency (% theoretical yield of sugar obtained)**					
	Acid					
	Sulfuric acid			**Nitric acid**		
Unclemac	89.37	59.25	8.38	51.46	30.59	5.81
Butterstick	25.88	24.98	9.41	63.48	47.96	11.99
Brancha	16.86	16.05	8.27	60.55	56.50	9.82
Rusta	23.16	16.60	7.50	51.45	32.23	7.89
	35	**25**	**0.8**	**35**	**25**	**0.8**
	Acid concentration (v/v)					

Uthumpon et al. (2010) [13] reported that the sugar yield after hydrolysis is dependent on the starch content that is accessible. Although they utilized enzymes to break down the polymer, the mechanism are quite similar. For acid hydrolysis, a proton is the initiator for the reaction, whereby the α-1, 4 glycosidic bonds are cleaved when the oxygen molecule forming the bond accepts a proton, resulting in an unstable oxygen which is released when a nucleophile such as HSO_4^- or NO_3^- acts the site. This reaction is promoted by an increase in temperature and acid concentration. More concentrated the acid, faster the rate of hydrolysis given the greater number of free protons in solution.

Once the hydrolysis step was optimized, the best system (optimum) was selected for the bulk hydrolysis so as to obtain enough hydrolyzate for optimizing fermentation parameter.

3.3 Fermentation data

Saccharomyces cerevisiae was used for the fermentations, conditions of pH ≈ 4.0 and temperature of ≈ 38 °C were maintained for each system. Fermentation time and concentration of sugars were varied to determine the effect of these two variables on the fermentation yield. These parameters were examined given that time and sugar concentration if not readily optimized can lead to losses industrially.

Figure 3 illustrates the effect of fermentation time on the alcohol yield for the different varieties. For the *unclemac* variety, apparently there was little change in yield which was rather strange given the high concentration of sugar in the hydrolyzate. Several reasons could have contributed to this, including that there was an excess enzyme in solution, the present of proteases in the fermentation broth may have inhibited the activity or the accumlation of ethanol may have lower the yeast activity in the system.

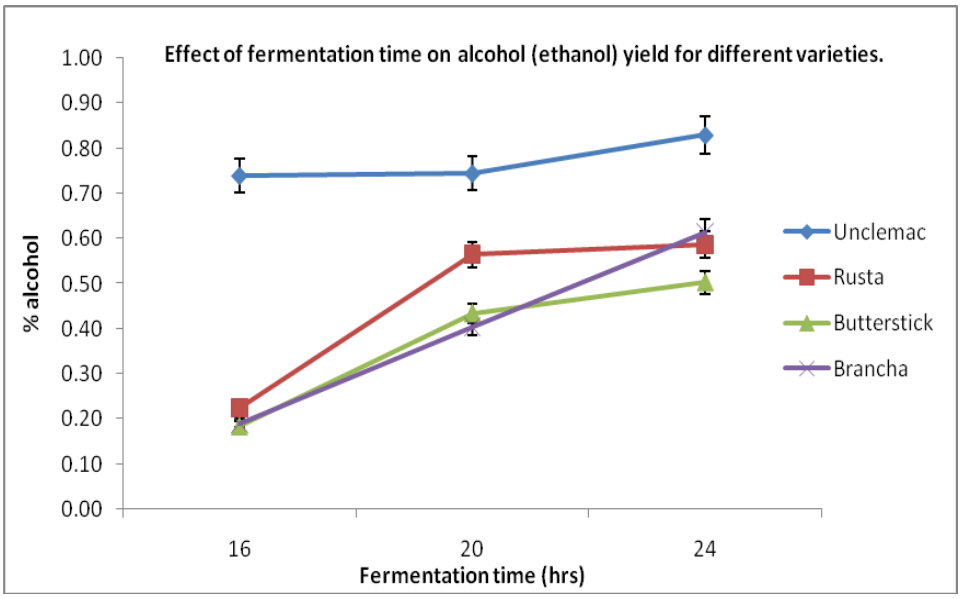

Figure 3. Fermentation rate represented as percentage alcohol produced per hour.

Additionally, the higher concentration of ethanol produced from unclemac can be attributed to two main reasons. The first being the initial high concentration of sugars (given the better hydrolysis rate) and it is known from the literature (Jones, 1987) [14] that nitrate ions tend to denature proteins when present in solution. Although the fermentation liquid was neutralized after hydrolysis, there may be residual nitrate ions that inhibit the action of *Saccharomyces cerevisiae.*

The second fermentation parameter that was investigated was the effect of the hydrolyzate sugar concentration on the alcohol yield. Figure 4 shows as the concentration of the initial sugar concentration is reduced, the yield of alcohol decreases correspondingly for *unclemac* variety. On the other hand, for the other three varieties, there is no significant difference ($p<0.05$) between the original and three quarters (0.75) the original concentration of sugar.

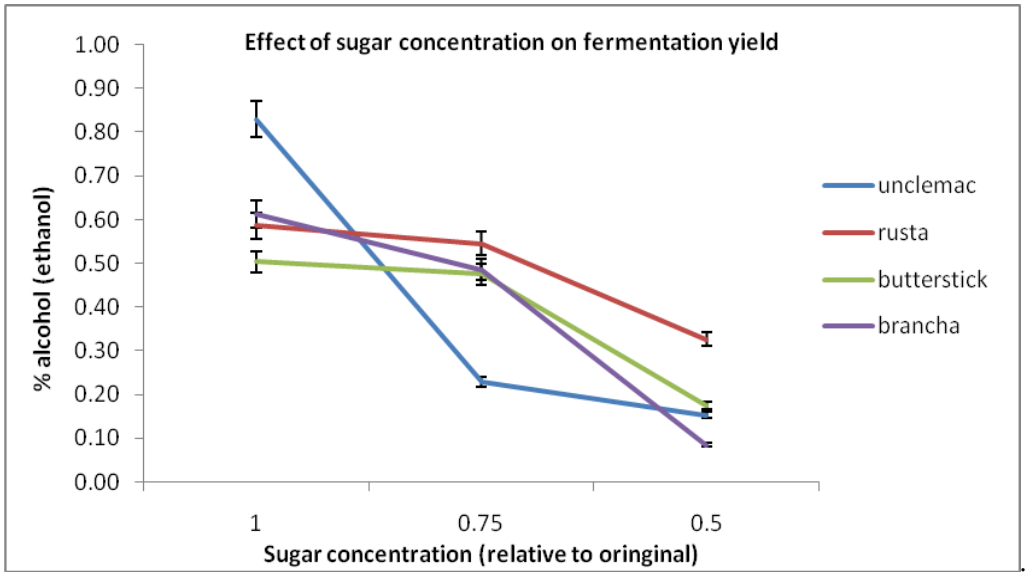

Figure 4. Effect of sugar concentration on alcohol yield.

4. CONCLUSION

Based on the research done, it can be concluded that *unclemac* is best suited for this type of application. The large granule size facilitates easy gelatinization whilst the low gelatinization maximum value indicates the formation of a weakly linked starch solution or gel. As a result, *unclemac* is most susceptible to acid attack at any concentration of acid.

At 95 % confidence level, it was found that all four varieties when compared at a given concentration of a particular acid gave major differences. Likewise, when the effects of the different acid concentrations were compared for a given variety, major difference was once again observed. Nevertheless, it was found that 35 % sulfuric acid gave the best yield of sugars for *unclemac* whilst 35 % nitric acid was the best system condition for the other three varieties.

It was observed that a concentration of sugar three quarters (0.75) the original hydrolyzate concentration fermented for 24 hours gave the best results for three varieties (*butterstick, rusta and brancha*). On the other hand, for *unclemac*, the original hydrolyzate concentration of sugars when fermented was more efficient that the other systems. Finally, alcohol was recovered under all reaction parameters.

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