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Production of fuel ethanol from *Vitellaria paradoxa* fruit pulp by simultaneous saccharification and fermentation

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Abstract

Biofuels have become the world's greatest, safest, cleanest and cheapest alternative to fossil fuels. Transport sector continues to receive influx of cars and general machinery every year worldwide leading to an overwhelming use of fossil fuel. The fossil fuel sources are gradually becoming depleted. Sustainable bioethanol recovered from inexhaustible sources are constantly been used to replace fossil fuels in the transport sector. The shea nut pulp (SNP) is a cheap source of carbon, nitrogen and calcium for the production of bioethanol. This study has employed the shea nut pulp as substrate, Saccharomyces cerevisiae-the Baker's Yeast strain-as the fermentation microbe, Alpha Amylase enzyme for hydrolysis of the substrate and supported the simultaneous fermentation process with nutrient supplements. The data were subjected to statistical analysis. Approximately 37.8 and 40.0 g/L of ethanol after 16 and 20 h, were produced from without-α-amylase and with-α-amylase treatments, respectively. Fermentation efficiencies of 58.6 and 65.0 % were observed ethanol yields for without-α-amylase and with-α-amylase, respectively when compared to the theoretical yield. This research is useful in harnessing the potentials of the shea nut pulp as industrially relevant substrate for use independently or in combination with other substrates in microbial fermentation processes for ethanol production. An assessment of the fermentation process has revealed that the SNP is a good source of bioethanol going into the future. The research therefore recommends further work on scale-up of the bioethanol production process from the shea nut pulp.

Keywords: Fermentation, Bioethanol, *Saccharomyces cerevisiae, Aspergillus niger,* Amylase

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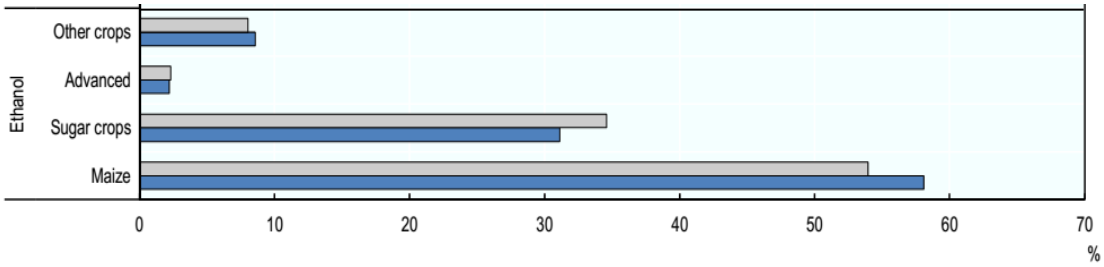
INTRODUCTION

The over dependence of modern societies on fossil fuel as primary energy source has become a serious concern (IPCC 2007). The growing concern has been the impact of fossil fuel usage on natural resource depletion and climate change stresses. Most air pollution comes from the extensive use of fossil fuels (US EPA, 2017) and that remains the cause of destruction of natural landscapes and habitats in addition to the cause of most environmental catastrophes (National Research Council, 1999). Unborn babies and young children are more vulnerable to the many adverse effects of toxic air pollutants (Perera, 2018) and climate change from fossil-fuel combustion than any other individual (US EPA, 2017).

There is the urgent need to switch from these fossil fuels to clean energy, and that remains a challenge and at same time an opportunity (Watts *et al*., 2015). Many countries such as Brazil, the USA, and the European Union have reached varying milestones in an alternative and safe fuel production for use in automobiles. Clean, safe and sustainable energy sources include hydroelectricity, nuclear power and other renewable energies such as biohydrogen, biodiesel and bioethanol (Tomas, 2013). Bioethanol is noted to be the most produced biofuel in the world (RFA, 2012) and the production from first generation source (Klanarong *et al.,* 2012, Yuwa-Amompitak, 2010), second generation source (Zakpaa *et al*., 2010; Suhas *et al.,* 2013) and third generation material sources (Abdul-Mumeen *et al*., 2016; Wadi *et al.,* 2019; Kostas *et al.,* 2020) have received the greatest attention worldwide. Much of Ghana's local research has assessed the production of bioethanol from industrial waste (Bensah *et al.,* 2012), agricultural waste (Zakpaa *et al*., 2010) and seaweeds (Abdul-Mumeen *et al*., 2016).

Ethanol produced by microbial fermentation or their enzymes is used blended or alone, primarily as a substitute for gasoline (Tomas, 2013). Global ethanol usage is expected to increase by 17 billion litters by 2026 and 90% of this increase will take place in developing countries (OCED/FAO, 2017) although bioethanol usage is driven primarily by policies mandating usage levels (FAPRI-MU, 2018). Currently ethanol is being used minimally industrially and as rocket fuel and in most developing countries, it is used to replace kerosene for cooking and in lanterns (Tomas, 2013). The increasing use of ethanol is due to its renewability as an energy source and the numerous advantages it has over fossil fuels. These include low CO_2 production during combustion, CO_2 uptake from biomass, non-production of toxic gases and rapid biodegradation in the environment, higher octane number than gasoline, lower energy required to produce ethanol compared to the equivalent amount of gasoline, which allows for higher thermal efficiency. (Bailey, 1996).

Almost 60% of all commercial bioethanol is produced from maize in the USA and about 30% from sugar crops (Figure 1) in Brazil (OECD/FAO, 2017). Sugar crops include sugar cane and suagr beets. Other sources of biomass for bioethanol production encompass starch from potatoes, wheat, cassava, rye and barley; or cellulose obtained from most fruit rinds or directly from sugar beet. Starch is a polysaccharide but can be hydrolysed into monomeric glucose; cellulose, a disaccharide, is composed of both glucose and fructose which are easily fermented to bioethanol by the Baker's yeast, *Saccharomyces cerevisiae*.



Source: OECD/FAO, 2017; 2014-2016 2026

Figure 1: Sources and percentage quantity of bioethanol production between 2014 and 2016 and estimated projections into 2026

Higher ethanol yield is still generated from first generation crops or food crops or energy crops such as maize, cassava and sugar cane and beet. This phenomenon has shown numerous benefits but has always done so with myriad of concerns. The large acreage of arable land required for first generation crop production to meet the requisite quantities of ethanol demand is a concern which poses a huge toll of competition with food and animal feed. Other criticisms highlight the raw material processing cost which can take up to 40 % of the total production cost.

The use of industrial, agricultural, household and municipal waste or second generation source materials for ethanol production has become the immediate solution to the concerns of using food crops. Residual biomass can contain high carbohydrates content that can be converted to bioethanol. Fruit rinds remain one of the most abundant and affordable raw material source for second generation bioethanol production.

Achieving a sustainable economy and avoiding the future dependence on fossil fuel usage lies on sustainable and efficient production of bioethanol from these residual biomass. Fermentation is the process by which ethanol is made from sugars (Thomsen *et al*., 2003). Ethanol used for fuel, alcoholic drinks and for industrial purposes is made by the process of fermentation (Alzeer and Khaled, 2016). All ethanol fermentation is still based, practically, on the use of microbes such as the Baker's yeast or *Saccharomyces cerevisiae*, which requires monomeric sugars as the raw material. Fermentation using microrganisms produces 0.51 kg of ethanol from 1 kg of any of the C6 sugars: glucose, mannose and sucrose (Thomsen *et al.,* 2003) although *Saccharomyces cerevisiae* can also be used to produce ethanol from C5 sugars such as xylose. But to commercialize the bioethanol production from residual biomass, several challenges have to be overcome. The major challenge is how to effectively convert all the polysaccharides and disaccharides into monomeric sugars for the fermentation with microorganisms to proceed. There has been several conversion methods of hydrolysing complex polysacharrides into reducing sugars and these have been mainly grouped into chemical; acid and alkaline (Van der Wal *et al.*, 2013; Abd Rahim et al., 2014) and steam explosion (Tan *et al.,* 2014), thermal/hydrothermal (Schultz-Jensen *et al.,* 2013), physical and enzymatic (Trivedi *et al.,* 2013; Puspawati *et al.,* 2015; Yazdani *et al*., 2015; Karray *et al*., 2015) or microbial/biological (Lee *et al.,* 2015; Gao *et al.,* 2015) treatments for bioethanol production. The various specific treatments have been mentioned by many researchers to include: thermal, ultrasound, dilute acid thermal, dilute alkaline thermal, hydrothermal (HTT), ball milling, and enzymatic. For the myriad of challenges bedevilling the acid and alkaline pretreatments ranging from rust of containment to extreme rapture of substrates for bioethanol generation, research using enzymes or microbial consortia in simultaneous processes seems to lead the way. Haruki *et al*. (2014) stating the significance of saccharification and fermentation (SSF) is a technique through which enzymatic hydrolysis and fermentation are achieved simultaneously in a single unit reactor. The combined process can reduce the number of vessels needed and thereby reducing the initial costs. Using the SSF process qlso eliminates the inhibition of saccharifying enzyme by sugars because the resulting sugars are immediately converted to ethanol by fermentation microorganisms.

In the enzymatic process, the hemicellulose portion is hydrolyzed using bases or acids, whereas cellulase enzymes convert the cellulose. Working with enzymes enables the cellulose hydrolysis and ethanol fermentation to be combined, despite the process being less advantageous than technologies using acid catalysts due to the market price of cellulases and the substantially softer processing conditions used (Kádár *et al.,* 2004). The ability to reduce the enzyme loading and, consequently, the production cost, which is the primary drawback of enzymatic hydrolysis, is made possible by the simultaneous saccharification and fermentation process (Kádár *et al.,* 2004). The main issue with simultaneous saccharification and fermentation is the difference in temperature optima of the cellulases and the fermenting microbe, notwithstanding the economic advantage of simultaneous saccharification and fermentation over separate hydrolysis and fermentation. Although Saccharomyces strains are well known for being efficient ethanol producers, they need a temperature of 35 °C to function (Kádár *et al*., 2004).

The ideal temperature for fungal cellulases, which are most usually used in the hydrolysis of cellulose, is 50 °C (Kádár *et al*., 2004). The significantly slower hydrolysis rates at lower

temperatures would be detrimental in terms of longer processing times. Using thermotolerant yeast strains rather than saccharomyces strains could help solve this issue because they allow for higher processing temperatures and higher hydrolysis rates. The current research employed alpha amylase enzymes from *Aspergillus oryzae* to selectively hydrolysed shea nut pulp to glucose and fructose monomer to produce bioethanol by fermentation using the versatile *Saccharomyces cerevisiae*. Ojo and Adebayo (2013) have reported the *Aspergillus oryzae* as one of the microorganisms that cause rot to the shea nut fruit when it falls under the influence of gravity. The Alpha Amylase and the *Saccharomyces cerevisiae* for the respective saccharification and fermentation were carried out in simultaneous processes.

MATERIALS AND METHODS

The SNP derived from shea nut fruits (SNF) was the main raw material used as substrate. The SNFs were obtained from five regions of Ghana: Northern (NR), Upper East (UER), Upper West (UWR), Volta (VR) and Brong-Ahafo (BA), representing the main shea tree zones of Ghana (Table 1). Table 1 shows the specific locations where the shea nut fruit samples were sourced from May 2018 to July 2018.

Table 1: The distribution of shea nut fruits sampling points

Region	Local Name	Harvest date	Location	Coordinates
Brong-Ahafo	Nku (Ngu)	2018 - May - 20	Dawadawa	8.44N;1.56W
Volta	Ku (yɔkuti)	2018 - May - 28	Sibi	8.41N;0.04W
Northern	Tama	2018- June - 12	Tolon	9.43N;1.06W
Upper East	Taama	2018 - June -19	Navrongo	10.89N;1.09W
Upper West	Taama	2018 - July - 03	Wa	10.06N;2.50W

Monomeric Sugar determination

Air-dried shea nut pulp samples which were stored at -20 °C were retrieved and approximately 3 g each of the air-dried material was weighed and ground in a blender to 0.2 mm. About 0.5 g was weighed into a sterile 50 mL polypropylene centrifuge tube and 20 mL of distilled water added. This was homogenized with an Ultra-Turrax blender for 1 min, centrifuge at 4 °C and 15000 rpm for 10 min. The supernatant was recovered and the crude sample frozen for further analysis. The sample was purified by filtration through a cloth filter to remove debris. The C18 cartridge phase, most effective for monomeric sugar detection, was activated with methanol and filled up with 2 g of Duolite resin, then the sample filtered through it and later through a glass pre-filter and a 0.45-µm nylon filter. The concentrations of sucrose, glucose and fructose were measured by modification from Abdel-Hameed *et al.* (2011). Analysis of carbohydrates (glucose, sucrose and fructose) were carried out with a high-performance liquid chromatography system (Waters 1525 binary pump) equipped with a refractive index (RI) detector (waters 2414). An Aminex HPX 87 H columns (300 mm x 7.8 mm) operated at a temperature of 80 °C was used to achieve chromatographic separation. Sugars (fructose, glucose and sucrose), and ethanol were eluted with 0.0065 M sulphuric acid at a flow rate of 0.8 mL/min. Individual sugars and ethanol were identified and their concentrations determined by comparison with retention times and amounts of authentic standards. Prior to injection of 20 µl, samples were filtered through a Millex-HV 0.45 µm filter unit.

Simultaneous saccharification and fermentation

Enzymes for Substrate Saccharification

Alpha Amylase (E8A01727) 200 g, an Aspergillus oryzae amylase preparation from the Philip Harris, UK was sourced for the saccharification of the substrate into its monomeric units. A pre-sterilized substrate was subjected to 2 % enzyme treatment at optimal conditions for 30 min prior to fermentation (Table 2).

Fermentation microorganism

Saccharomyces cerevisiae (Baker's yeast strain) was obtained from Lesaffre, France for this study. The microbe was first stored at -20 °C prior to the fermentation process and during the fermentation; 0.2 g/100 ml concentration of the yeast was used for the conversion of the monomeric sugars to ethanol in fermentation tanks.

Nutrient supplement during fermentation

The fermentation medium was supplemented with the following nutrients prior to fermentation: magnesium sulphate heptahydrate, bacteriological peptone, anhydrous ammonium sulphate, anhydrous potassium hydrogen phosphate and glucose (Thygesen *et al.,* 2011). The concentrations of the various nutrients are shown in Table 2 below.

Table 2: Nutrient supplement for the growth and performance of *Saccharomyces cerevisiae* used during the fermentation

Nutrient Component	Concentration in g/200 ml	%Concentration
$MgSO_4.7H_2O$	0.2	0.1
Bacteriological peptone	1.0	0.5
$(NH_4)_2SO_4$	0.6	0.3
KH_2PO_4	0.6	0.3
Glucose	4.0	2.0

Substrate pretreatments

Shea nut fruits after collection were immediately de-pulped and the pulp sun-dried for seven consecutive days. The dried samples were collected into sample containers appropriately labelled and kept at a temperature of - 4 °C for further analysis. Prior to clinical analysis, the samples were milled into fine powder of 0.5 mm – homogenous form. The total solids, moisture and ash contents were investigated and the right dry matter quantities for other analysis were calculated prior to chemical analysis.

Fermentation

Shea nut pulp was prepared into homogenous form (0.5 mm) and 20 g (DM) each of five treatments was weighed into two hundred and fifty millilitres (250 ml) duplicate blue cap flasks containing 200 ml of distilled water. Another duplicate set of five treatments was prepared to run the fermentation without hydrolytic enzymes (Alpha amylase). The first set of duplicate tanks was subjected to 2 % amylase treatment (Table 3). Nutrient supplement consisting of 0.5 % bacteriological peptone, 0.3 % ammonium sulphate, 0.3 % potassium hydrogen phosphate, 0.2 % magnesium sulphate and 2 % glucose was added to each flask. Baker's yeast (Saccharomyces cerevisiae) of 0.400 g was added to the content of each flask after which the yeast locks filled with 2 ml glycerol were put on each flask. The pH of the flasks was adjusted to 4.8, the optical density and the glucose level were noted before they were put in an incubator (SI-900R, JEIO Tech) at 30 °C, with shaking at 150 rpm. The samples were examined for microbial growth rate by measuring optical density, glucose concentration, pH and ethanol concentration. This was done after aseptically drawing off 5 ml of the sample mast every 4 h from 0 to 24 h. fermentation efficiency (FE) of the fermentation process was estimated using the theoretical and actual concentration of the ethanol produced. The calculation was done based on the formula below:

$$TE = SK \times V \times 0.511$$

$$EY = \frac{Total\ Ethanol - Initial\ Ethanol}{End\ Sugar - Initial\ Sugar}$$

Thus,

$$FE = \frac{Ethanol\ Yield\ in\ grams}{Theoretical\ Ethanol\ Yield} \times 100\ \%$$

Where: TE = Theoretical ethanol; SK = Sugar Concentration; V = Volume of the fermentation medium; EY = Ethanol Yield; FE = Fermentation Efficiency

Microbial Cell (Yeast) Growth Rate

Microbial cell (*Saccharomyces cerevisiae)* growth rate *was* monitored by measuring the optical density of the fermentation process at 4 h intervals for 24 h. The optical density was measured at 600 nm (OD600) against a blank using the UV spectrophotometer (Jen Way 6405 UV). Dilutions were made where necessary and the measurements taken using the 1 ml cuvettes (plastic).

Table 3: Optimal fermentation conditions for enzyme and microbe during this study

Fermentation parameter	**Alpha Amylase (**E8A01727)	**(Baker's Yeast)**	**Optimal fermentation condition used**
Working Concentration (%)	0.1 - 1.0	0.2 - 0.5	2/0.2
pH Range	3.0 - 6.0	1.0 - 6.5	4.8
Temperature (°C)	20.0 - 60.0	20 - 35	32
% Substrate	2.0	0.2	2/0.2
Product	Maltose	Ethanol	Ethanol
Source	*Aspergillus oryzae*	*S. cerevisiae*	SNP

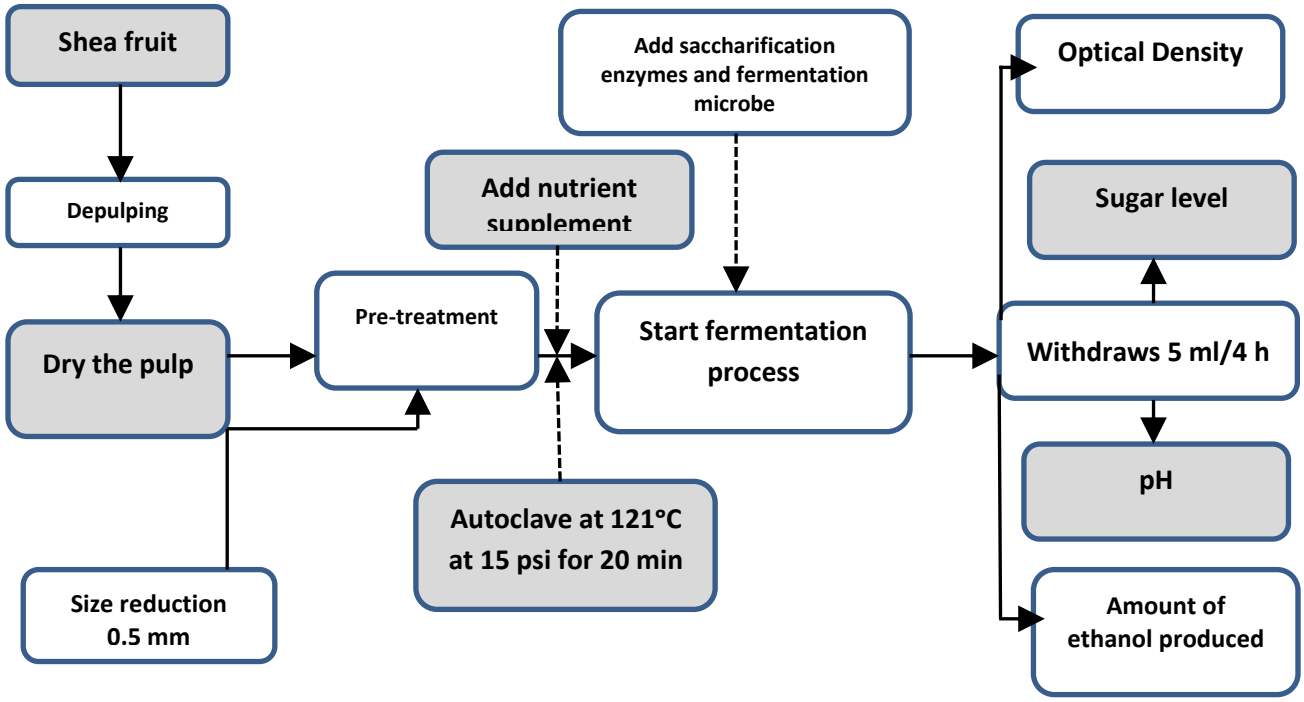


Figure 2: Flow chart of simultaneous saccharification and fermentation process

pH of the fermentation process

The pH of the fermentation flasks was slightly adjusted to 4.8 (Table 3) prior to the start of the fermentation process using 0.1 M H_2SO_4. At 4 h interval, the pH was monitored using a pre-calibrated pH meter (Mettler Toledo).

Total sugar determination

The total reducing sugar levels of the mast was monitored from 0 h to 24 h. Approximately 5 ml of the solution was withdrawn after every 4 h for reducing sugar level determination using a diabetic kit (Nocoding One Plus, i-SENS, Inc.).

Ethanol content determination

The ethanol content was determined according to Gladis *et al.* (2015). HPLC was used for the analysis of ethanol using a chromatographic system equipped with a differential refractive index detector (RID-10A) (both from Shimadzu, Kyoto, Japan). All samples were passed through a filter with a pore diameter of 0.20 lm prior to analysis to remove particles. The filtered samples were stored at - 20°C before analysis. The samples from SSF were diluted if necessary, and analyzed using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 50 °C with 5 mmol/L H_2SO_4 as eluent, at a flow rate of 0.5 mL/min, to separate ethanol from the other substance such as lactic acid, acetic acid, formic acid, levulinic acid, HMF and furfural.

Statistical analysis

All measurements involving chemical compositional analysis of SNP biomass were carried out in triplicates. Hydrolysis and fermentation were measured in replicates. All reported values in this study were means of replicate values. For data generated by HPLC, TLC and GC-MS, linear standard curves of glucose and other monomeric sugars were drawn and the amounts estimated thereafter.

RESULTS AND DISCUSSION

These results are records of the bioethanol yield from both with-α-amylase and without α-amylase hydrolysis by HPLC determination. The bioethanol yield was compared to the theoretical yield and the fermentation efficiency was thereafter calculated. To have a better understanding of the bioethanol production dynamics, after every 4 h, the concentration of reducing sugar, the microbial cell density and the changes in pH were documented and appropriately discussed.

Monosaccharide composition of SNP

According to table 4, the amount of sucrose found in shea nut pulp across some five regional shea zones of Ghana ranged from 49.49 ± 2.12 mg/g in the Volta Region to 105.75 ± 1.06 mg/g in the Upper West Region. Shea nut fruits from the Upper West Region recorded the highest reducing sugar concentration and the most useful for bioethanol production due to its high sucrose (1105.75 ± 1.06 mg/g) levels. Sucrose is a disaccharide consisting of 50% each of glucose and fructose. Maltose consist of two glucose units and maltose was not detected in the shea nut pulp.

Table 4: The ratio of reducing sugars composition of shea nut pulp

Sugar	BA	NR	VR	UER	UWR
	Concentration (mg/g)				
Sucrose	77.24±5.30^{a}	50.25±3.18^{b}	49.49±2.12^{b}	76.50±4.24^{a}	105.75±1.06^{c}
Maltose	-	-	-	-	-
Glucose	20.94±0.12^{d}	21.12±0.04^{d}	18.88±0.21^{d}	20.83±0.13^{d}	21.09±0.24^{d}
Fructose	98.02±0.65^{e}	71.26±1.96^{f}	27.76±0.49^{g}	63.88±0.98^{h}	103.80±1.31^{i}
G/F ratio	0.2	0.3	0.7	0.3	0.2

Glucose is present in the shea nut pulp as one of its monomeric sugars (Table 4). The lowest amount of glucose (18.88 ± 0.21 mg/g) was found in the Volta Region while the highest of 21.12 ± 0.04 mg/g was found with Northern Region. The amount of glucose across the regions was examined statistically and found that the difference across the regions was not significant ($p \geq 0.5$) as shown in Table 5. The glucose concentration levels were generally lower than the concentration levels of fructose. Fructose concentration in the shea nut pulp was in larger amounts compare to glucose and sucrose. The largest concentration of fructose was in the UWR with 103.80±1.31 mg/g. The least amount of fructose was found at the VR, 27.76 ± 0.49 mg/g. Fructose concentration levels was significantly different ($p < 0.05$) across the shea regional zones of Ghana (Table 5). The glucose-fructose ratio was competed and the two reducing sugars in the shea nut pulp were closest at VR with a ratio of 0.7. The highest disparity between glucose and fructose (with a ratio of 0.2) was recorded in samples from the UWR and the BA.

Saccharification and fermentation results

Total sugars, ethanol yield and fermentation efficiency

Bioethanol was produced from SNP by fermentation using the Baker's yeast strain, *Saccharomyces cerevisiae.* The maximum ethanol content (40.0 ± 1.4 g/L) after 16 h fermentation was produced from α-amylase treated samples at an average of 54.9 ± 0.5 % fermentation efficiency (Table 6). The least amount of ethanol (29.0 g/L) was generated from both with α-amylase and without-α-amylase treated samples at about 65.5 and 63.2 % fermentation efficiency, respectively. The most efficient fermentation process (65.5 %) was α-amylase treated samples but 29.0 ± 0.3 g/L ethanol was recovered, 11.0 g/L lower than the maximum ethanol production (4.0 ± 1.4 g/L). Results are means of 2 × 5 determinations. No ethanol (0.0 g/L) was detected before the introduction of the fermentation microbe. The amount of ethanol measured for both with α-amylase and without-α-amylase treated samples generally increased over time, from 0.0 to 40.0 ± 1.4 and from 0.0 to 38.7 ± 0.7 g/L respectively. Alpha-amylase treated samples did not produce ethanol significantly different from without-α-amylase treated samples (Table 6). The α-amylases, on the bases of the results generated, did not unlock the substrate to release more of the soluble sugars in solution for higher ethanol production.

Reducing sugars can be obtained from micro-algae and fruit pulps including the shea nut pulp by various methods (Abdul-Mumeen *et al*., 2016). In the present research, α-amylases were used to hydrolyze shea nut pulp to release the reducing sugars for fermentation to proceed. The amount of reducing sugar content of the Shea Nut Pulp was not higher than 65.7 g/L across both with-α-amylase and without-α-amylase treatments. Sugar levels were generally higher in all Shea Nut Samples in which 1 % α-amylase was introduced to aid the saccharification process.

Table 5: Mean reducing sugar concentrations and significance of the difference in concentration

SNP	Mean ± SD / Range		P - Value
	Concentration (mg/g)		
Sucrose	71.85 ± 3.30	49.49 -105.75	P ≤ 0.05
Maltose	-	-	-
Glucose	20.57 ± 0.23	18.88 - 21.12	P › 0,05
Fructose	72.94 ± 1.06	27.76 - 103.80	P ≤ 0.05

Table 6: Ethanol yield, fermentation efficiency and sugar concentration of with α-amylase treated and without-α-amylase treated shea nut pulp using the Baker's yeast strain incubated at 32 °C, pH 4.8 for 24 h at 1 % α-amylase concentration

Time/h	Soluble Sugar Concentration (g/L)		Ethanol Yield (g/L)		Theoretical Yield (g/L)		Fermentation Efficiency (%)	
	With A-amylase	Without A-amylase	With A-amylase	Without A-amylase	With A-amylase	Without A-amylase	With A-amylase	Without A-amylase
0	63.6±2.4	65.7±2.5	0.0±0.0	0.0±0.0	32.1±0.0	33.4±0.0	0.0±0.0	0.0±0.0
4	39.0±1.8	41.5±1.7	29.3±0.3	29.0±0.3	44.7±0.2	45.9±0.3	65.5±0.2	63.2±0.3
8	21.3±1.7	15.7±2.8	34.2±0.8	30.1±0.4	53.0±0.3	58.3±0.4	64.5±0.4	51.6±0.3
12	8.2±0.7	6.7±1.1	34.3±0.7	31.7±0.4	60.4±0.2	63.3±0.4	56.8±0.3	50.1±0.2
16	5.9±0.9	4.7±0.7	40.0±1.4	35.4±0.7	61.5±0.7	64.5±0.7	65.0±0.5	54.9±0.6
20	4.2±0.9	4.6±0.7	37.2±1.3	37.8±0.7	62.4±0.6	64.5±0.7	57.9±0.7	58.6±0.7
24	3.5±1.1	3.0±0.9	39.1±1.4	38.7±0.9	62.9±0.5	64.4±0.8	62.2±0.8	60.1±0.4

As was unexpected, the difference between the sugar levels of the hydrolysate solution from both with A-amylase and without-α-amylase hydrolysates were not significant (Table 4). However, both treatments, enzymatic and without-enzymatic, were significantly different from the blanks throughout the fermentation (Figure 3). Since the results of the two treatments were found to be statistically the same, the release of the sugars could have been aided by the thermal pretreatment of the substrate at 121 °C and 15 psi for 20 minutes during the sterilization. It is also possible that in the shea nut pulp, once ripe, the glucose and the fructose are directly released in aqueous medium and sucrose is hydrolyzed during autoclave of the substrate for sterilization purposes only. The reducing sugar concentration consistently reduced in both with-α-amylase (63.6 g/L to 3.5 g/L) and without-α-amylase (65.7 g/L to 3.0 g/L) during the fermentation processes, from 0 h to 24 h, respectively (Figure 3 and Table 4).

Primarily, the yeast *S. cerevisiae* breaks down glucose to ethanol under anaerobic conditions following Embden-Meyerhof pathway (Borines *et al.*, 2013). This pathway is denoted by the following reaction:

$$C_6H_{12}O_6 \xrightarrow{yields} 2C_2H_5OH + 2CO_2$$

The reaction stoichiometry indicates that for every mole of glucose consumed by the Yeast, 2 moles of ethanol is generated. That is, every 0.5 moles of glucose will give 1 mole of ethanol and that proportion is about 51 % (w/w) ethanol yield under ideal conditions. From Table 4, 40.0 g/L of ethanol was generated from α-amylase hydrolyzed fermentation process whereas a maximum of 37.8 g/L ethanol was obtained from without-α-amylase fermentation. On average ethanol produced by α-amylase catalyzed fermentation was higher than without-α-amylase catalyzed fermentation (Figure 8) but the difference was not significant ($P < 0.5$). The highest theoretical ethanol yield, 64.5 g/L for without-enzymatic treatment and 61.5 g/L for with-α-amylase treatment resulted in 54.9 and 65 % fermentation efficiency respectively after 16 h of simultaneous saccharification and fermentation of the SNP. However, the best fermentation efficiency of 63,2 and 65.5 % respectively was achieved after 4 h fermentation when 29.0 and 29.3 g/L were accordingly produced (table 4). Thus, α-amylase hydrolysis did not show any significant effect on the reducing sugar concentration and the eventual quantity of bioethanol produced.

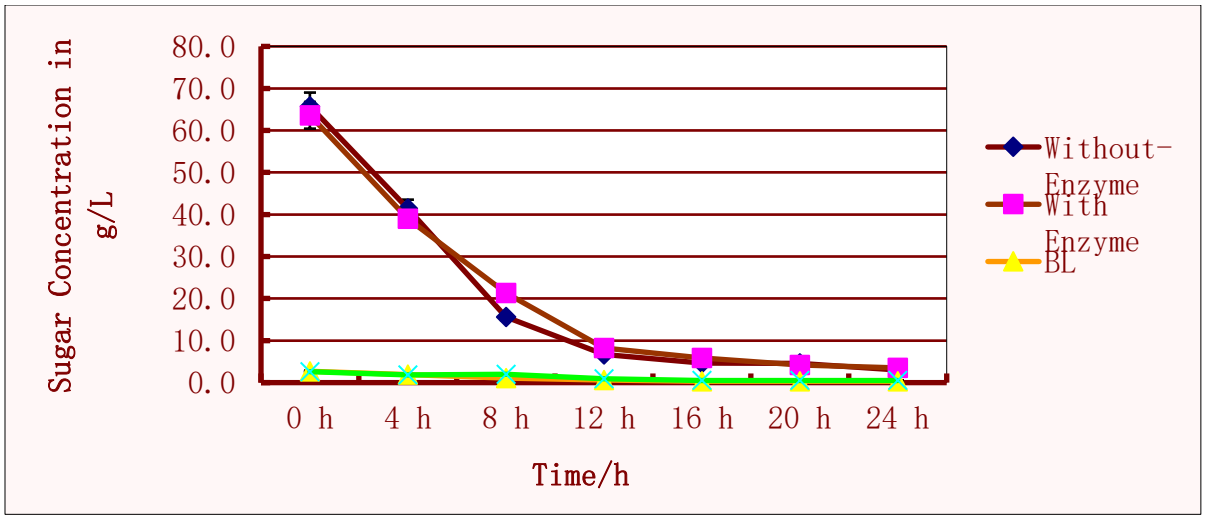


Figure 3: Graph showing the trend of sugar consumption in g/L for hydrolysates from α-amylase and without α-amylase hydrolysis as fermentation proceeds from 0 h to 24 h. BL (Nutrients and *S. cerevisiae only)* and BLE (Nutrients, Alpha amylase enzymes and *S. cerevisiae only)* are without-α-amylase and with-α-amylase blanks respectively.

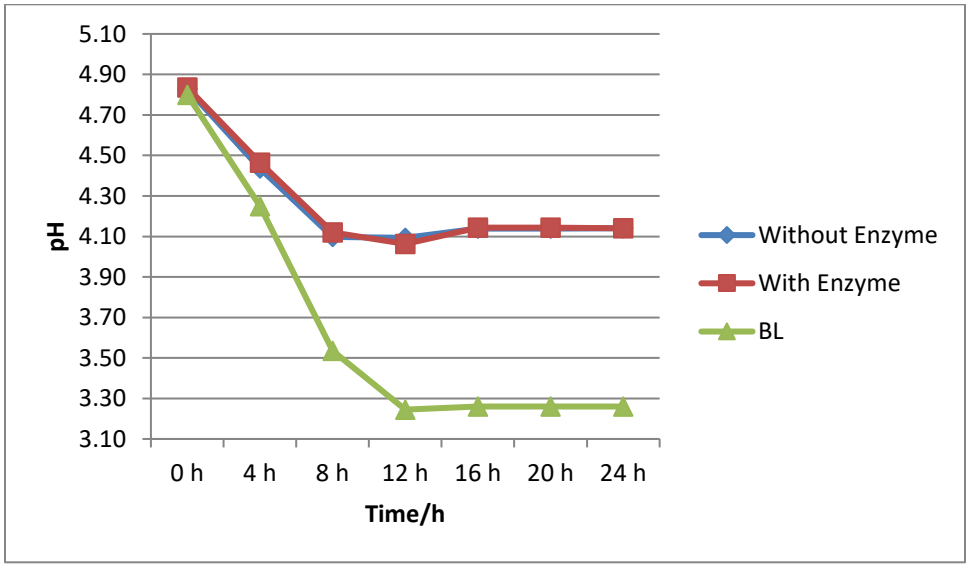


Figure 4: The pH of fermentation of with A-amylase hydrolysate and without-α-amylase hydrolysate of Shea Nut Pulp, monitored over a period of 24 h. BL (Nutrients and *S. cerevisiae* only).

Monitoring the pH of the fermentation process

Prior to fermentation the pH of hydrolysates was adjusted to 4.8 and this dropped to pH of about 4.2 for both without-α-amylase and with- α-amylase fermentation process (Figure 4). A blank sample was monitored for change in pH along with the 2 treatments. The blank produced the most acidic solution (pH = 3.2) at the end of the fermentation. The decrease in pH from the start of microbial activity can be attributed to the formation of ethanol which protonates in solution and that increase the acidity of the fermentation must. pH decreases with increasing acidity and

increase with decreasing acidity and thus acidity and pH were observed to be inversely proportional. The increasing acidity had no effect on the growth and performance of Saccharomyces cerevisiae since the microbe grows and performs well over a wide range of pH although it is best at pH range of 4.5 to 4.8 (Arino *et al.,* 2010; Chen *et al.,* 2009).

The difference between the pH of two treatments (pH 4.2) and the pH of the blank (pH 3.2) is quite remarkable. The blank contains refined glucose as component of the nutrient supplement (4.0 g/L) and its conversion to ethanol and carbon dioxide is quite straightforward. This could explain why the pH is low the hydrogen ion concentration will be very high.

The pH did not seem to have any effect on the growth of the microbe. The growth of the microorganism was uniform (Figure 5) throughout the fermentation process except that after 8 h fermentation the density of the fermentation microbe in which the α-amylase was contained markedly reduced from about 1.5 to 1.1 absorbance unit. This may not be attributed to pH effect but possibly a readjustment of the microbe due to a rundown of the carbon source. Shea nut pulp contains glucose and fructose and S. cerevisiae is glucophilic and so the microbe could be readjusting for fructose metabolism.

Monitoring the performance of the fermentation microbe

The growth of Saccharomyces cerevisiae detected by measuring the cell growth or by monitoring cell density during fermentation is a determinant of how the fermentation process is performing. There are usually four notable phases the microbe passes through: lag, log, stationery and the death phase. At the lag phase, the microbe is dull and actively and gratuitously breaks down the carbon source, glucose and fructose in this case, at the log phase when cell mass increases as a result. The S. cerevisiae reaches the stationary phase when the carbon source and the supplemented nutrients are exhausted and competition for the limited carbon source builds up. The lack of, or reduced nutrients, leads to the death of yeast in the death phase giving lower absorbance readings.

The cell growth density of Saccharomyces cerevisiae of both treatments; with-α-amylase and without-α-amylase, were monitored and recorded as shown in figure 5. The optical density of the must containing α-amylase and without-α-amylase during the log phase of the fermentation were statistically the same ($P \geq 0.5$) during the first 8 h. A near-stationary state growth of the microorganism was observed after 8 h and before 16 h as shown in Figure 5. It is noteworthy that while the without-α-amylase process achieved the highest viable cell growth at 8 h, microbial activity within the with-α-amylase saccharified hydrolysate slowed after the first 4 h, became inactive after 8 h before rising and reaching a stationary phase at 16 h till the end of the process. The without-α-amylase process showed its highest cell viability at 8 h before declining and reaching the stationary phase at 16 h. The slow activity of the microbe at 16 h reaching the stationary phase corresponded with a marked decrease in total sugar content, lower than 1 g/L and may not be attributed to a run-down in nutrient supplement. Saccharomyces cerevisiae is a natural evolution designed to efficiently consume sugars (sucrose, glucose and fructose). It remains an essential cell factory because by it action it's vigorous, it has the capacity to endure stress when subjected to it continuously, it can be genetically accessed and modified its survival, growth and reproduction needs are simple and finally it has the track record of successful use in industrial processes. However, recent findings (Berthels *et al*., 2004; Fleet, 1998) suggest that during fermentation, yeasts show a higher inclination to glucose than to fructose. Several factors could have hindered the uniform growth of the Saccharomyces cerevisiae in the with-α-amylase saccharified hydrolysate. The nutrient supplement could have run out and the microorganism runs out of nitrogen source, or the glucophilic *S. cerevisiae* was slowing for a swap to fructose metabolism after exhausting the glucose. It is important to note that there was linearity between maximum cell growth and ethanol production with S. cerevisiae strains (Henderson *et al*., 2013). But cell growth and ethanol production, as was expected, were of reverse proportions to the rate of reducing sugar consumption. Yet the rate at which the reducing sugar is consumed is not proportionate to the amount of ethanol produced. The low ethanol conversion efficiency by S. cerevisiae might be due to a portion of the substrate converting to cell mass and other products (Ofosu-Appiah *et al*., 2016).

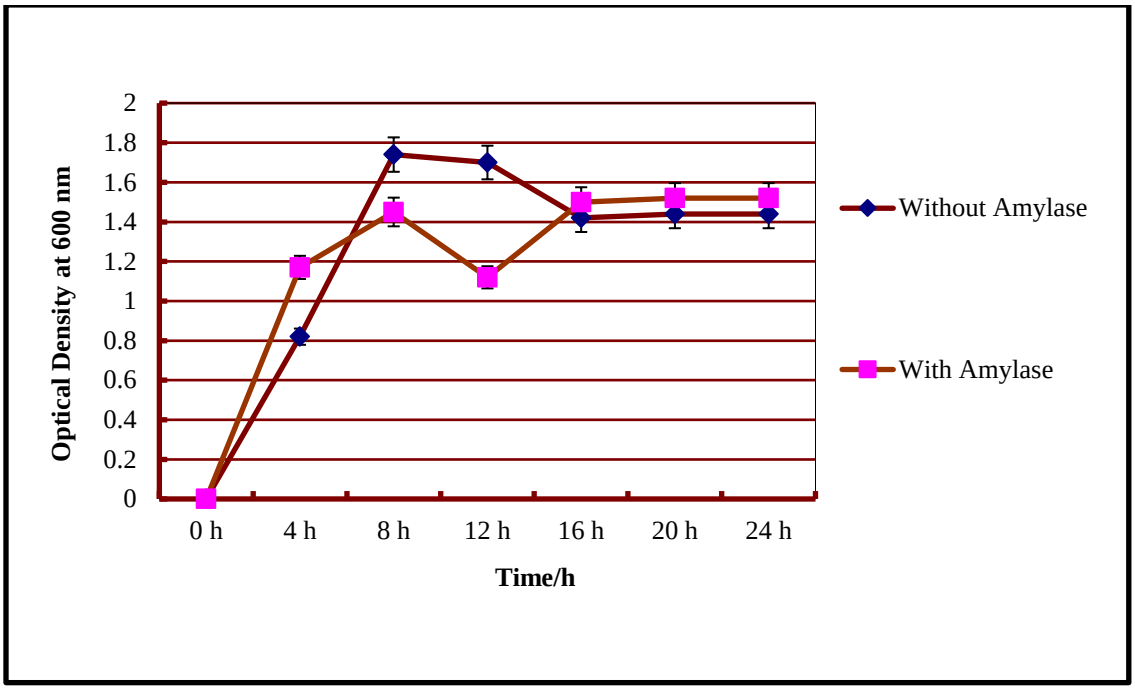


Figure 5: The microbial cell Growth curves of *Saccharomyces cerevisiae* based on optical density at 600 nm during the fermentation of SNP to bioethanol for hydrolysates with α-amylases and without α-amylases.

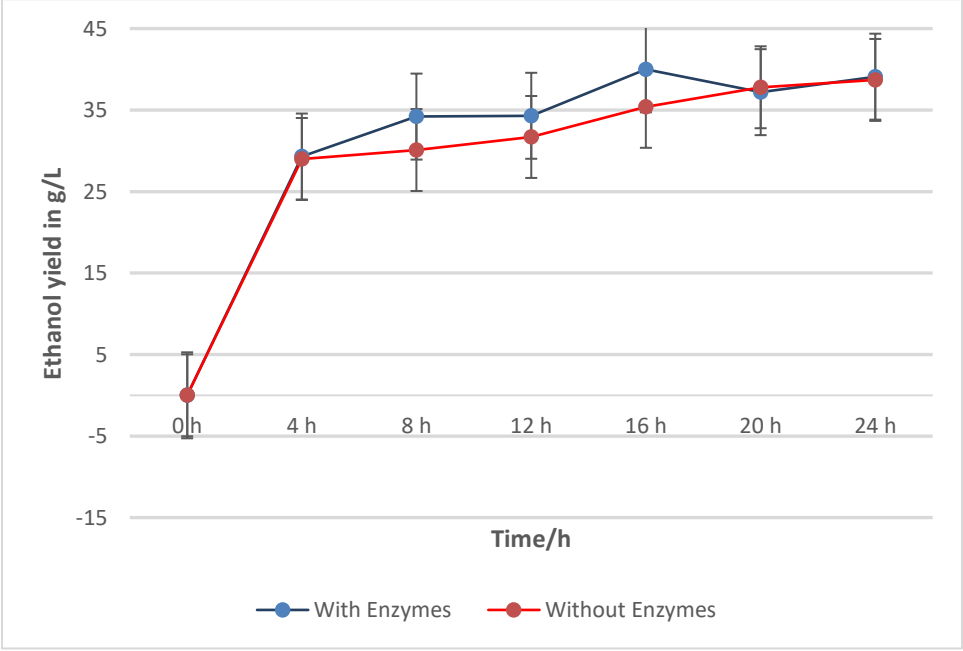


Figure 6: The rate of bioethanol fermentation of SNP enzymatic and without-enzymatic hydrolysates using *Saccharomyces cerevisiae* as fermentation microbe

Monitoring Glucose and fructose metabolism

The metabolism of fructose in α-amylase and without-α-amylase treatments is presented in figure 6. The rate of metabolism of fructose in α-amylase treatment was lower in relation to without-α-amylase treatment. The rate of fructose metabolism was quite erratic in both with α-amylase and without-α-amylase treatments. In the without-α-amylase treatment, the metabolism of fructose was very effective after the first 4 h and slowing after 8 h of fermentation to reach lowest concentration of about 15 g/L from about 4 g/L. The metabolism of glucose in α-amylase and without-α-amylase treatments is presented in figure 7. The rate of glucose metabolism in α-amylase treatment was the same as in without-α-amylase treatment. The rate of glucose metabolism was uniform in both with α-amylase and without-α-amylase treatments for the first 4 h fermentation during which time the concentration of glucose reduced from 16 and 18 g/l to 2 g/L for both treatments respectively. In the without-α-amylase treatment, the metabolism of glucose was undetected after the first 4 h when the concentration reached 1.4 g/L till the end of the fermentation process. The concentration of glucose slightly rose in the α-amylase treated samples and levelled off at 1.4 g/L after 16 h and became undetected till the end of the fermentation process.

In general, the metabolism of fructose was far lower than the metabolism of glucose (**Figures 6 and 7**). The yeast Saccharomyces cerevisiae showed affinity for glucose. However, both fructose and glucose were consumed by the Yeast from the very start of the fermentation process. This may explain why Tronchoni *et al*. (2009) suggests that some species of yeasts *Saccharomyces cerevisiae* at low temperatures, exhibit some fructophilic character especially during the few hours of start of fermentation.

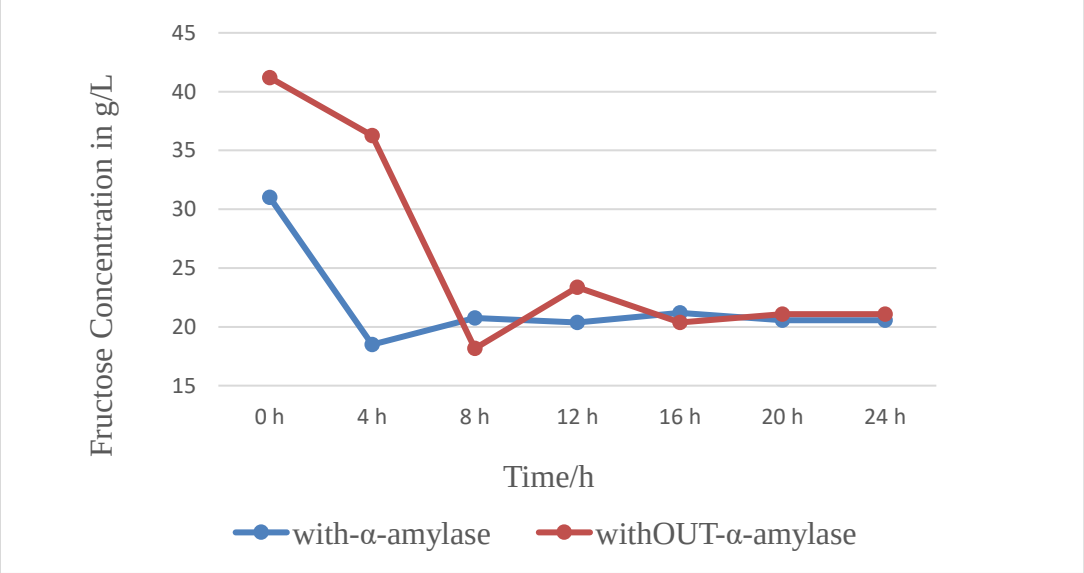


Figure 7: The trend in g/L of residual fructose during the fermentation of SNP hydrolysate at 32 °C, pH 4.8 for 24 h duration using Saccharomyces cerevisiae. The g/L concentrations are mean values of duplicate determinations of five treatments

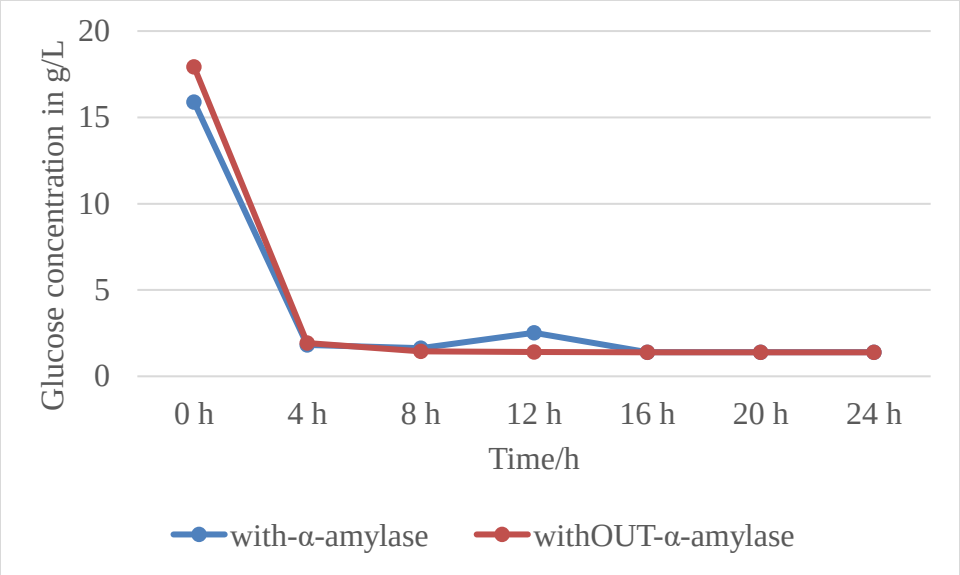


Figure 8: The rate of glucose consumption by Saccharomyces cerevisiae during the fermentation of SNP hydrolysate

Glucose metabolism was highest during the first four hours of fermentation and this was uniform for both with-α-amylase and without-α-amylase treatment. The metabolism of glucose was far greater than the rate of fructose metabolism after 4 h. This observation is supported by previous research when researchers such as Fleet (1998) and Berthels *et al.* (2004) noted that the yeasts *S. cerevisiae species* have a slightly higher preference for glucose than for fructose during fermentations. Thus, in most fermentation processes, there is marked difference between the metabolism of both sugars, glucose and fructose, resulting in a considerable amount of fructose left in the fermentation must. *Saccharomyces cerevisiae,* the Baker's strain possibly preferred glucose over fructose since the microbe is more glucophilic than fructophilic (Amos, 2018). The affinity of *S. cerevisiae* for glucose breakdown during fermentation has been well documented (Ofosu-Appiah *et al.,* 2016; Paul, 2010).

There are usually equal amounts of fructose and glucose in most fruit pulps and grapes according to Fleet and Heard (1993) with a concentration range of 160 to 300 g/L of total sugars. Jones *et al.* (2005) however believe that climatic change increases the proportion of fructose to glucose and Ojo and Adebayo (2013) suggest that the general low total sugar levels (lower than 160 – 300 g/L) was as a result of the rapid deterioration of the SNP during air-drying.

Wiratno *et al.* (2014) asserts that when reducing sugars in some given treatments decrease slightly during fermentation so that a reducing sugar still remains in a high concentration then the fermentation process is not running at optimal conditions. At 3.5 and 3.0 g/L reducing sugar concentration for both with-α-amylase and without-α-amylase treatments respectively (Table 4), the fermentation process reached the stationary phase. Several factors could have caused the slow down. The one unique factor is that the fermentation process possibly run out of nitrogen supplement. Nitrogen has been reported to deeply affect the performance of *S. cerevisiae.* Roca-Mesa *et al.* (2020) stipulates that nitrogen compounds are the nutrients mostly assimilated by yeasts, after carbon compounds, during alcoholic fermentation.

Figures 6 and 8 show that the rate of ethanol production was dependent on glucose metabolism by *S. cerevisiae.* It can be deduced from the two figures that whiles the glucose concentration dropped steadily from about 18.0 or 16.0 g/L to about 2.0 g/L during the first four hours of fermentation, the ethanol concentration rose rapidly from 0.0 to about 30.0 g/L for both treatments within the same period of time. This phenomenon indicates that the ethanol production efficiency was nothing more than 65.5 % during the first 4 h. Towards the end of the fermentation a little more ethanol was generated from the hydrolysate solution from α-amylase treated fermentation than the without-α-amylase

treatment, reaching 63.2 % ethanol conversion efficiency. The difference in ethanol production between α-amylase and without-α-amylase musts was however not significant.

The maximum theoretical ethanol yield was 64.5 g/L and this estimate is made from without-α-amylase treatment. The maximal ethanol concentration was reached after 16 h, at 40.0 ± 1.4 g/L from shea nut pulp and was at 64.5 % of the theoretical yield. The findings in this study compares well with other research works that examine the production of ethanol from fruits and fruit rinds using *S. cerevisiae*. Ofosu-Appiah *et al.* (2016) using a 500 mL fermentation flask at pH 6 and 30 ºC, obtained 3.03 g/L ethanol from sorghum pito mash using *S. cerevisiae* in a fermentation process that lasted 4 days. Suhas *et al.* (2014) got 4.64 g/L ethanol from Jack fruit rind and 4.38 g/L from Pineapple rind at 25 ºC in a 250 mL flask after 4 days fermentation process. This study found 40.0 g/L ethanol form shea nut pulp and this is higher than the findings of most previous works on fruit rinds.

CONCLUSION

This research focused on bioethanol production from shea nut pulp by simultaneous processes at optimal conditions. Enzymatic saccharification during an SSF yielded simple sugars directly from the SNP pretreated by size reduction and standard autoclave conditions for 20 min. but did not show significant difference from the aqueous saccharification. The SNP is a cheap source of Nitrogen which is very useful to bioethanol generation using *Saccharomyces cerevisiae,* the Baker's Yeast strain, at favorable conditions. The highest ethanol titer from this substrate, 40.0 g/L, was observed at after 16 h fermentation at optimal conditions. The process requires scale-up research to quantify the bioethanol concentration. It is important to conclude strongly on the following, that:

Enzymatic and non-enzymatic hydrolysis of SNP yielded statistically insignificant difference of sugar concentration

S. cerevisiae gratuitously converted SNP to bioethanol at optimal fermentation conditions when a single-step (simultaneous) process was tested for ethanol production from SNP where Alpha Amylase converts the substrate to hexose sugars,

The highest ethanol titer from SNP, 40.0 g/L, was observed after 16 h of fermentation of SNP hydrolysate

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Author Contributions

Abdul-Mumeen Iddrisu designed the study, carried out the research work and wrote the first draft of the manuscript. Zakpaa Hilary D, Felix Charles Mills-Robertson and Samuel Tetteh Lowor reviewed the manuscript. All authors performed data analysis and interpretation and approved the final draft of the manuscript.

Conflict of Interest

The authors declare no actual or potential conflict of interest.

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