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Bioethanol Production from Cassava Peel Hydrolysate by *Saccharomyces cerevisiae*

Oshoma C. E.*, Okoye S. and Bello-Osagie I.

Department of Microbiology, Faculty of Life Sciences, University of Benin, P.M.B. 1154, Benin City, Nigeria.

*cyprian.oshoma@uniben.edu Tel. 08032015334

Abstract

Bioethanol production from renewable feedstock by yeast fermentation has proven to be a sustainable and promising source of energy need. Optimization of ethanol yield is through supplementation and the efficiency of converting available sugars in the hydrolysates to bioethanol. The influence of two nitrogen sources supplemented Cassava peels hydrolysates on ethanol fermentation by *Saccharomyces cerevisiae* was investigated. The formulated hydrolysate media for fermentation were glycine supplemented cassava peels, ammonium sulphate supplemented cassava peels and unsupplemented cassava peels (control). Parameters analyzed in all the hydrolysates were cell number, pH, ethanol, glycerol concentration, Sugars and inhibitory compounds. The, results revealed that ammonium sulphate supplemented cassava peels medium had highest cell number, glycerol and ethanol concentrations of 2.38±0.32 x 10^7 cells/mL, 9.23±0.37 mg/L and 23.16±3.00 mg/L respectively. There was significant difference in ethanol production when comparing the nitrogen sources to the control medium ($p< 0.05$). The highest inhibitor produced was acetic acid with value 9.40±1.12 mg/L from the ammonium sulphate supplemented medium while the least 7.46±0.65mg/L from glycine supplemented cassava peels medium. The conclusion was that ethanol production could be achieved when cassava peels hydrolysate is supplemented with nitrogen sources mostly ammonium sulphate.

Keywords: Cassava peel, inhibitors, *Saccharomyces cerevisiae*, fermentation, bioethanol.

Introduction

Bio-ethanol has found use in the pharmaceutical industry in preparations such as tonics, cough syrups and as solvent for hop constituents. Ethanol is used as co-surfactant in oil-water micro emulsions (Obueh *et al.*, 2016), and can be used in the production of antiseptic and disinfectant (Gutiérrez-Rivera et ., 2012). As a disinfectant, it is widely used in its raw form to decontaminate work benches and equipment in laboratories. The use of

ethanol as fuel and fuel additive in automobiles and its advantages over the use of fossil fuels is fast gaining global attention (Sun and Cheng, 2007).

Bio-ethanol can be obtained by the biological production through fermentation of sugar by microorganisms. Fermentation of simple sugars such as glucose and fructose by microorganisms or their enzyme requires direct conversion of the sugar into ethanol while fermentation of complex carbohydrates such as starch and glycogen requires saccharification of the carbohydrate to release the simple sugars which can then be utilized by the microorganisms and converted to ethanol (Tesfaw and Assefa 2014). Cellulosic and lignocellulosic plant materials require pretreatment of the complex polysaccharide probably by acid hydrolysis to release their components that can be converted to ethanol by the fermenting organism (Sun and Cheng, 2007).

Cassava (*Mannihot esculenta*) is one of the most extensively cultivated food crops in Africa (Muniafu *et al*., 2015). The cassava roots can be processed into food products such as fufu, tapioca and garri (Odunfa, 1987). During processing of cassava, the root residues, mostly peels, are termed as wastes and large amount are generated annually (Akponah and Akpomie, 2012) and constitute about 10 – 35 % weight of the tuber. The cassava peel, as a waste, is a thin brown outer covering and a thicker leathery parenchymatous inner covering (Obadina *et al*., 2006). The wastes are mostly discarded allowed to rot thereby causing offensive odour and pollution (Obueh *et al*., 2016). This cassava waste contains high organic matter content that can be hydrolyzed into fermentable sugars (Nuwamanya *et al*., 2012). Rather than allowing the cassava waste to become an environmental problem, it is necessary to convert it into useful product such as ethanol.

It is noteworthy that if sugar rich substrates such sugarcane juice and molasses are used for fermentation, then processes like milling, pretreatment, hydrolysis, and detoxification are not necessary due to presence of simple sugars in the substrates. However, the production of ethanol from starchy plant materials requires milling, liquefaction, and saccharification (Itelima *et al*., 2013). In the case of lignocellulosic material (cassava waste), milling, pretreatment, and hydrolysis are required (Nachaiwieng *et al*., 2015). Furthermore, a detoxification process is mostly required due to the presence of inhibitory compounds toxic to yeast cells during fermentation (Oshoma *et al*., 2015). The differences between the bio-ethanol production processes from simple sugar, starch, and lignocellulosic material are the steps involved prior to the main fermentation process which include milling, pretreatment, hydrolysis and detoxification (Mussatto *et al.*, 2010).

Acid hydrolysis of cassava peels is a process that involves splitting of the complex carbohydrate compounds into fermentable sugars. The economic potential of this process mainly depends on the low cost of the materials, energy, operating and investment cost (Zamora *et al*., 2010; Surendra *et al*., 2015). Lignocellulosic hydrolysates are known to be low in nitrogen nutrient (Greetnam *et al*., 2014) hence, supplementation of the hydrolysate with nitrogen sources will favour yeast fermentative ability for ethanol production (Yue *et al*., 2012). The aim of this study was to determine the influence of ammonium sulphate and

glycine as nitrogen supplementation on the production of ethanol from cassava peel by *Saccharomyces cerevisiae*.

Materials and methods

Isolation and propagation of yeast

Saccharomyces cerevisiae was isolated from cut pineapples left at room temperature to undergo spoilage. The yeast isolate was isolated based on cultural and microscopy characterization following standard methods Barnett and Hunter (1972) and Larone (1086), and maintained on potato dextrose agar (PDA) slant and stored at 4^{o}C. The broth medium used for propagation was yeast extract, peptone and D-glucose (YPD) medium and it was prepared using 20g D-glucose, 20g peptone and 10g yeast extract. Strain for inoculation was prepared according to the method of Oshoma *et al*. (2015).The number of cells was counted using a haemocytometer and inoculum size of 10^6 cells/mL was used to inoculate all the media.

Acid hydrolysis

Acid hydrolysis was carried out by weighing 50 g of milled cassava peel into 500 mL of water in 1000 mL conical flasks followed by transferring 100 mL of 1% H_2SO_4 and autoclaved at 121^{o}C for 15 minutes. The resulting autoclave mixture was cooled and subsequently neutralized using 5 drops of 10 M NaOH[6].The acid hydrolysates were designated as glycine supplemented cassava peel (GSCP) medium with composition glycine (1.0 g/L) made up to 1 L cassava peels medium (CPM), ammonium sulphate supplemented cassava peel (ASCP) medium with composition $(NH_4)_2SO_4$, (1.0 g/L) made up to 1 L CPM and the third, unsupplemented cassava peel (USCP) medium made up to 1 L CPM. The pH of the media was adjusted to 4.5 using phosphoric acid and/or NaOH. From the pH adjusted broth, 100 mL was transferred into mini fermentation vessels (FVs) and sterilized at 121^{o}C for 15 minutes.

Fermentation process

After the inoculum preparation of *S. cerevisiae* strains as stated above. Fermentation was carried out at room temperature of 28±2^{o}C on an orbital shaker at a speed of 120 rpm using three hydrolyzed Cassava peel media according to modified method of Oshoma *et al*. (2015). The prepared inoculum size of 10^6 cells/ mL of yeast suspension was aseptically transferred into each of the bottles. The bottles were sealed and equipped with a bubbling CO_2 outlet. Fermentation was carried out for the inoculated hydrolysate in all bottles at temperature of 28±2^{o}C on an orbital shaker at a speed of 120 rpm for 4 days. Samples were collected after 4 d of fermentation to determine the total cell number using methylene blue staining method and pH values. At the same time samples were withdrawn and centrifuged (4000 rpm for 5 minutes). The supernatant was transferred into a tube and frozen at -20 ^{o}C. These were analyzed for concentrations of glucose, xylose, hydroxymethyl furfural (HMF), furfural, acetic acid, glycerol and ethanol using gas chromatography. All fermentations were carried out in triplicate.

Total cell number of yeast analysis

The total cell number was determined with a haemocytometer according to the method of Sami *et al*. (1994). Methylene blue 0.01 % (w/v) was dissolved in sodium citrate 2% (w/v) solution. Yeast broth at various

sampling time point was diluted using sterile water. The cell suspension was mixed with methylene blue solution in a ratio 1:1. The solution was examined microscopically and total cells counted using Neubauer haemocytometer (depth 0.1 mm, area 0.0025 mm^2, Marienfield, Germany) and compound light microscope (Zeiss, Oberkocken, Germany) at ×40 objective lens.

Gas Chromatography analysis

The concentrations of glucose, xylose, HMF, furfural, acetic acid, glycerol and ethanol were analyzed using headspace gas chromatography according to the modified method of Wang *et al.* (2003).

Statistical analysis

All fermentation cultures were carried out in triplicate. Mean and standard deviation of triplicate samples were calculated using Excel (Microsoft, USA). The differences between samples were compared using t-test one-way analysis of variance (ANOVA) (Excel Microsoft, USA). Differences were considered statistically significant when $p<0.05$.

Results

The fermentation progress was measured routinely by the observation of yeast cell number (Fig.1A). The maximum cell number of 2.38±0.32 x 10^7 cells/mL was from the media supplemented with ammonium sulphate at day 4 of fermentation while control medium had the least (1.55± 0.07 x 10^7cells/mL). Statistically, there was significantly different when comparing the nitrogen sources media to the control medium ($p<0.05$).

The pH ranged from 4.50 on day 0 to 3.90 on day 4. The glycine supplemented cassava peel had higher pH values (4.23) at day 4 of fermentation (Fig. 1B).

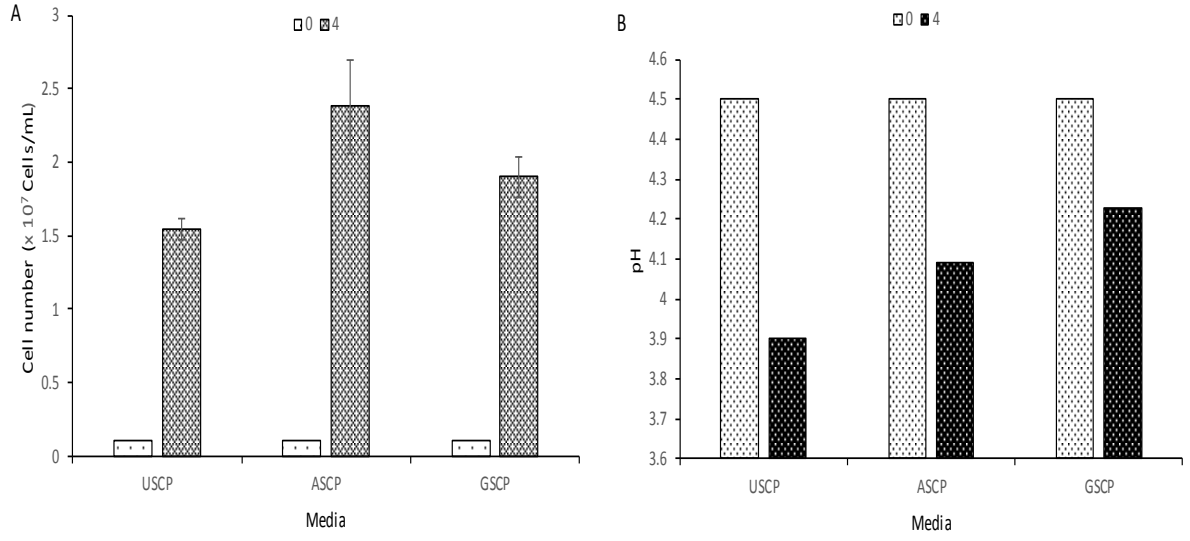

Figure 1: Cell number (A) and pH (B) of *Saccharomyces cerevisiae* during fermentation of Cassava peel hydrolysate at day 0 and 4. Values are the mean of three experiments and vertical error bars represent standard deviation. ASCP: Ammonium sulphate supplemented cassava peel medium. GSCP: Glycine supplemented cassava peel medium and USCP: Unsupplemented cassava peel medium.

After pretreatment sugars such as glucose and xylose were determined, also the concentration of these sugars after fermentation were analyzed at day 4. The values of glucose and xylose analyzed before and after fermentation are shown in Fig.2 A and B respectively. After pretreatment (day 0) glucose concentration was 34.04±3.44mg/L while at day 4 after fermentation, the highest concentration (17.473±2.48mg/L) was from the control medium. Ammonium sulphate supplemented cassava peel had the lowest glucose concentration of 12.56±1.77mg/L at 4 days of fermentation. Same trend was observed in the xylose concentrations in all the media. Glucose consumption rate was found to be significantly different comparing the control medium to the different nitrogen sources media ($p < 0.05$).

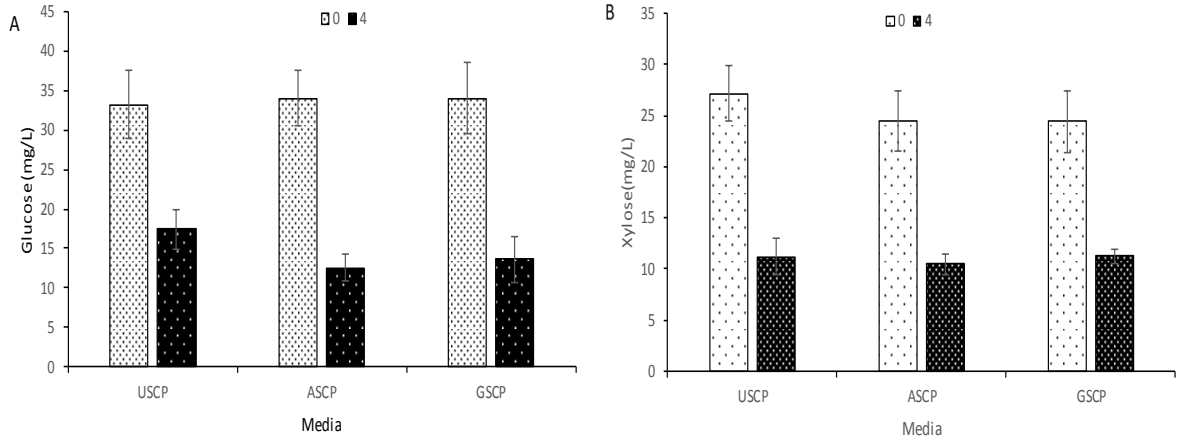

Figure 2: Production/presence of (A) Glucose and (B) Xylose (mg/L) in the fermentation of cassava peels hydrolysate using *Saccharomyces cerevisiae* at day 0 and 4. Values are the mean of three experiments and vertical error bars represent standard deviation. ASCP: Ammonium sulphate supplemented cassava peel medium. GSCP: Glycine supplemented cassava peel medium and USCP: Unsupplemented cassava peel medium.

The inhibitors measured at day 0 and 4 during fermentation were furfural and hydroxyl methyl furfural (HMF). The result from Fig 3A showed that on day 0 the highest furfural concentration was 1.72 ± 0.16 mg/L and on day 4 the highest was 1.56 ± 0.12 mg/L from the media ASCP and GSCP respectively The highest concentration of HMF produced (Fig. 3B) at day 4 of fermentation was from unsupplemented, cassava peel (USCP) medium while the least from ASCP medium values were 6.12 ± 0.91 and 4.88 ± 0.88 mg/L respectively.

At day 4 of fermentation, the highest acetic acid concentration produced was 9.40 ± 1.12 mg/L and the least was 7.46 ± 0.65 mg/L from ASCP and GSCP media respectively (Fig. 4A). The highest glycerol concentration at day 4 was from ammonium sulphate supplemented and the least was from glycine supplemented cassava peels with values 9.23±0.37 and 6.58±0.95 mg/L respectively (Fig. 4B).

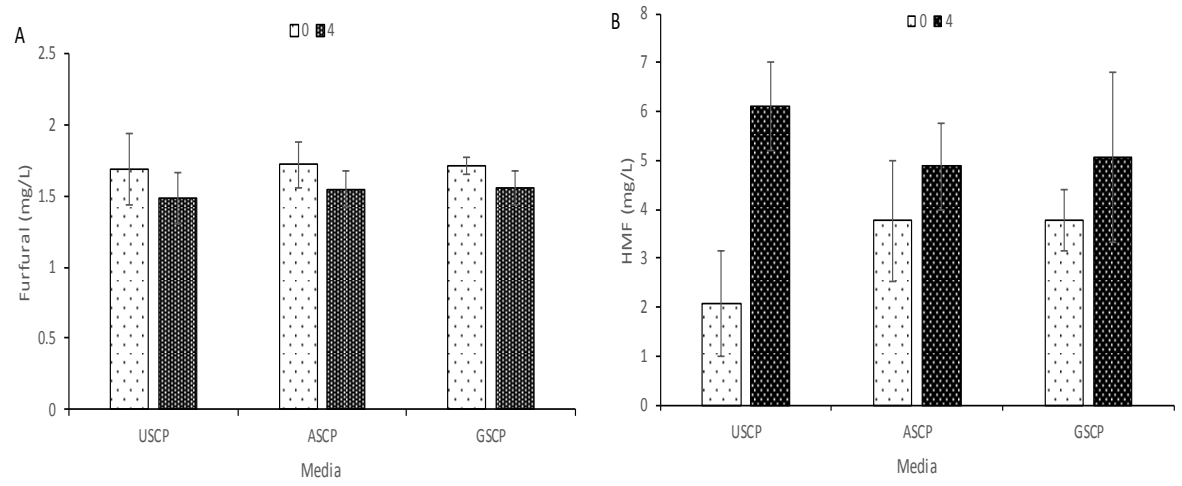

Figure 3: Production/presence of (A) Furfural and (B) Hydroxymethyl Furfural(HMF) (mg/L) before (0) and after (4) fermentation of cassava peels hydrolysate using *Saccharomyces cerevisiae*. Values are the mean of three experiments and vertical error bars represent standard deviation. ASCP: Ammonium sulphate supplemented cassava peel medium. GSCP: Glycine supplemented cassava peel medium and USCP: Unsupplemented cassava peel medium

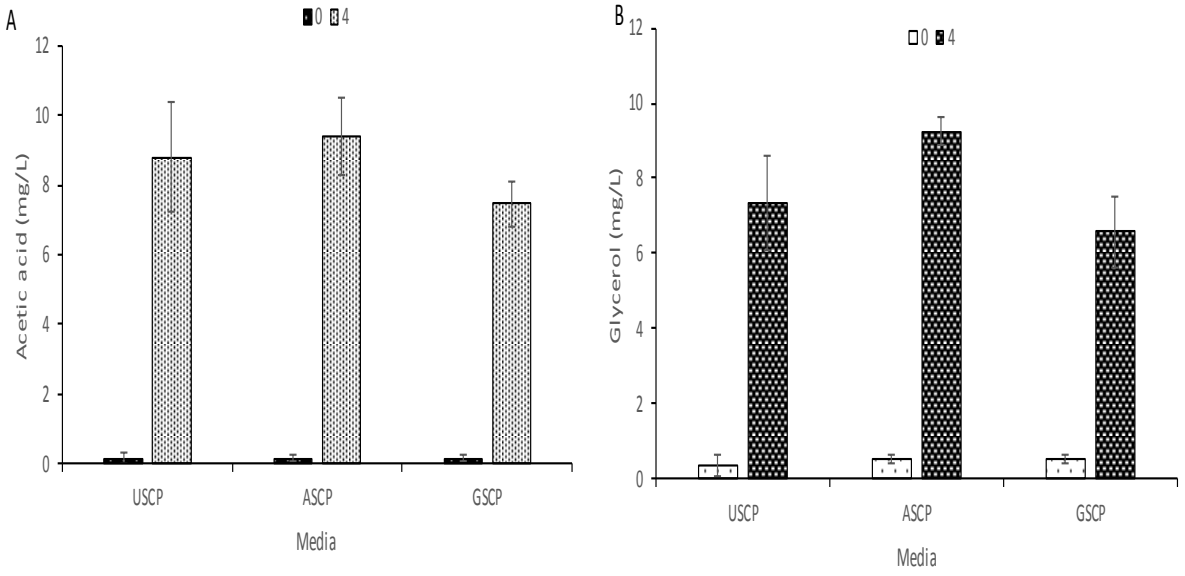

Figure 4: Production of (A) Acetic acid and (B) Glycerol (mg/L) before (0 d) and after (4 d) fermentation of cassava peels hydrolysate using *Saccharomyces cerevisiae*. Values are the mean of three experiments and vertical error bars represent standard deviation. ASCP: Ammonium sulphate supplemented cassava peel medium. GSCP: Glycine supplemented cassava peel medium and USCP: Unsupplemented cassava peel medium

The highest ethanol produced was from ammonium sulphate supplemented medium (23.16±5.00 mg/L) and least was the unsupplemented cassava peel (17.15 ±

1.53mg/L) as shown in Fig. 5. There was significant difference in ethanol production when comparing the nitrogen sources with the control media ($p < 0.05$).

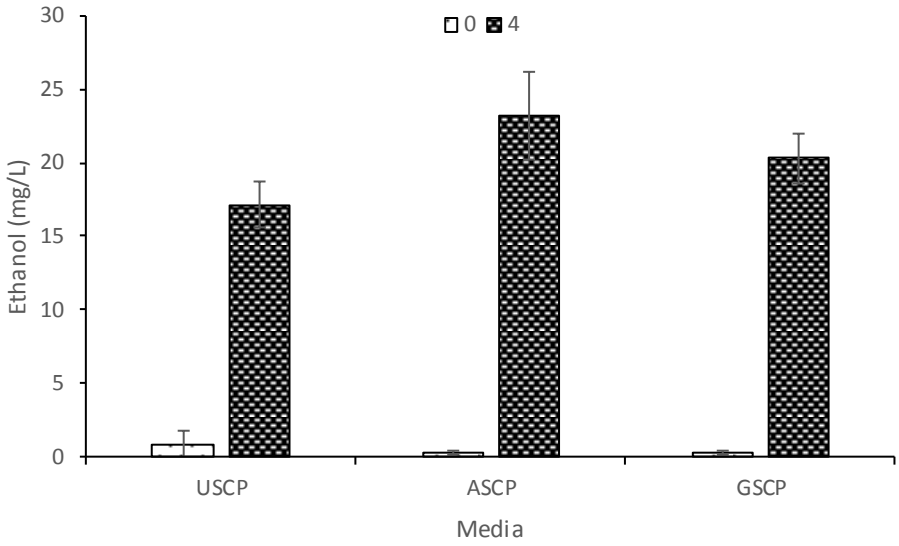

Figure 5: Concentration of Ethanol (mg/L) in the fermentation of cassava peels hydrolysate using *Saccharomyces cerevisiae*. Values are the mean of three experiments and vertical error bars represent standard deviation. ASCP: Ammonium sulphate supplemented cassava peel medium. GSCP: Glycine supplemented cassava peel medium and USCP: Unsupplemented cassava peel medium

Discussion

Yeast strains have the ability to utilizing a wide varieties of nitrogen sources for cell biomass increase but, not all nitrogen sources influence growth equally (Shure *et al*., 2000). Cell growth on nitrogen sources has been shown to be essential for cell biomass, thus influences yeast tolerance to fermentation stress (Schure *et al*., 2000; Yue *et al*., 2012). Ammonium compounds have been implicated as a good nitrogen source, reason been that yeast cells easily convert the molecule into glutamate and glutamine. Therefore, media supplementation with nitrogen source is necessary for yeast cell growth during fermentation, this in turn impart the cell tolerance to stresses such as ethanol and inhibitory compound present in the lignocellulose hydrolysate (Yue *et al*., 2012).

Evaluating the effect of ammonium sulphate and glycine on yeast tolerant during ethanol fermentation on the hydrolysate showed that ammonium sulphate improved yeast tolerance and produced more ethanol than glycine. Previous studies also collaborate our investigated that nitrogen supplementation influences yeast strains tolerance to fermentation stress (Albers *et al*., 1996; Greetham *et al*., 2014). It was observed that the nitrogen sources enable the yeast strains to utilize the glucose faster and increased ethanol production than the control medium. Probable reason may be that the strains were able to use the nitrogen sources to meet their protein and nucleic acid requirement (Akponah and Akpomie, 2012). The control medium was deficient of nitrogen source, hence lower

cell growth, poor glucose utilization and lower ethanol yield.

Yeast grows well in acidic conditions, this helps to control contamination from competing microbes while promoting relatively high ethanol productivity (Borrego *et al.,* 1988; Benerji *et al.,* 2010). With increase in pH yeast produces acid rather than alcohol in order to restore its adapted optimum pH. The rate of ethanol production by yeast cells is highly affected by the pH of the fermentation medium. More acidic and basic conditions, both retard the yeast metabolic pathways and hence the growth of cells (Willaert and Viktor, 2006). Productivity may decrease by increase and decrease in pH due to the lower metabolic rate of the yeast cells (Van Hanh and Kim, 2009). It may also be due to the growth of other microbes with the increase in pH, as in fermentations carried out without sterilization (Amutha and Paramasamy, 2001; Kourkoutas *et al*., 2004). According to Lin *et al.,* (2012) the optimum pH for *S. cerevisiae* ranged between 4.0–5.0. When the pH was lower than 4.0, the incubation period was prolonged thus reducing the ethanol concentration and when the pH was above 5.0, the concentration of ethanol diminished substantially. Formation of acetic acid was enhanced when the pH was below 4.0 and pH above 5.0 favored butyric acid productions (Lin *et al.,* 2012). These reports show that different acids produced by the yeast or added exogenously to the media created optimum pH or unfavorable pH range for the *S. cerevisiae.*

Based on the data presented, it is important to supplement media with nitrogen sources in order to overcome the stress caused by the inhibitors generated in the cassava peels hydrolysate. The presence of inhibitory compounds in the hydrolysate from lignocellulose biomass cannot be avoided (Palmqvist and Hahn-Hagerdal 2000; Almeida *et al*., 2007). During pretreatment and hydrolysis processes, fermentable sugars are liberated and generated weak acids, furan derivatives and phenolic compounds as inhibitors (Almeida *et al*., 2007). Increase in HMF concentration after fermentation indicates further degradation of hexose sugars to the inhibitor (Jonsson and Martin, 2016). This work demonstrated that nitrogen sources enable yeast cells to cope with inhibitors and fermentation stresses during ethanol production. We observed that in the control medium, higher concentration of glucose was recorded after 4 days of fermentation hence lower yield of ethanol.

Nitrogen supplementation was used in high sugar fermentation under controlled conditions that led to the contribution of assimilable nitrogen for yeast growth and metabolites production (Bely *et al.,* 2003). Therefore, lignocellulosic hydrolysates are found to be low in nutrient and nitrogen with some of the agricultural waste pretreatment contributed only about 0.056 % nitrogen content (Hamadi *et al*., 2014). The addition of nitrogen sources into the hydrolysate deficient of nitrogen may be economically useful during ethanol production from lignocellulose biomass. Greetham *et al*. (2014) observed that proline, a nitrogen source, its addition improved yeast tolerance to acetic acid and furfural stress during fermentation.

Glycerol concentration was found to be higher in nitrogen sources media than the

control media. Glycerol is one of the major metabolites produced by yeast cells during fermentation (Chen *et al* 2014). Glycerol production demonstrated that certain amount of carbon was diverted for other product formation instead of ethanol (Keating *et al*., 2006). The glycerol is acting as a compactible solute to the cells which enhances yeast tolerance to fermentation stresses (Chen *et al* 2014; Walker, 1998). The control medium produced lower amount of glycerol compared to the ammonium sulphate supplemented medium. Martin *et al*. (2002) suggested that production of lesser amount of glycerol could be the consequence of low cell number in the control medium due to poor availability of nitrogen.. This study agreed with the report of Tomas-Pejo *et al*. (2010) that increase in glycerol production contributed to yeast tolerance to inhibitory compounds found in lignocellulose hydrolysate which resulted to better cell growth.

Conclusion

The result of the investigation showed that inhibitory compounds in the lignocellulose hydrolysate negatively impact yeast fermentative performance. The inhibitory effect was seen in cell number, glucose consumption and ethanol production in the control (without nitrogen) medium. It can be concluded that nitrogen sources such as ammonium sulphate influences yeast cells tolerance to fermentation stress thus, producing higher amount of ethanol.

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