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Full Length Research Paper

Optimization of bio-ethanol production from cassava effluent using *Saccharomyces cerevisiae*

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The ethanol producing capability of *Saccharomyces cerevisiae* from cassava waste water was enhanced. Enhancement was obtained by varying pH, culture age and nutrient supplementation during fermentation until optimum conditions were attained. Optimum fermentation duration for ethanol production in acid and enzyme hydrolysates was 24 h. The start-up fermentation experiment process with *S. cerevisiae* resulted in 1.47 and 1.00% (v/w) ethanol in acid and enzyme hydrolysates, respectively. Appropriate adjustment in pH however, yielded 3.60 and 1.88% (v/w) ethanol in both acid and enzyme hydrolysates of cassava waste water. The pH value that resulted in optimum ethanol production by the test isolate was 5.5. Furthermore, culture age that resulted in highest ethanol yield was 3 days old culture. Consequently, when 3 days old cultures were employed in fermentation of acid and enzyme cassava waste water hydrolysates, ethanol generated increased to 3.61 and 2.91% (v/w), respectively. The addition of NPK fertilizer, $NaNO_3$ and K_2HPO_4 salts to each fermentor further enhanced the glucose utilization capacity of *S. cerevisiae* and concomitant ethanol generation such that ethanol produced increased to 14.5, 8.15 and 5.74 (%v/w) in acid hydrolysate containing NPK fertilizer, $NaNO_3$ and K_2HPO_4 salt supplements, respectively. The corresponding ethanol yield in enzyme hydrolysates were 8.30, 5.10 and 3.00 (%v/w) respectively. All the three nutrient supplements proved suitable for enhancing the fermentative capability of *S. cerevisiae* in a decreasing order NPK > K_2HPO < $NaNO_3$. The results reveal that optimum combinations of pH, nutrient concentrations and cultural status play a major role in getting maximum bioconversion of cassava waste water to ethanol.

Key words: *Saccharomyces cerevisae*, cassava wastewater, hydrolysates, enhancement, nutrient supplement.

INTRODUCTION

With industrial development growing rapidly, there is need for environmentally sustainable energy sources (Zaldivar et al., 2001). Bio-ethanol is an effective sustainable energy source. Based on the premise that fuel bio-ethanol can contribute to a cleaner environment and with the implementation of environmental protection laws in many countries, demand for efficient bio-ethanol production processes may increase.

One important requirement is to have efficient micro-organisms that are able to ferment a variety of sugars as well as to tolerate stress condition (Manikandan and Viruthagiri, 2010). Bacterial and yeasts strains having traits for ethanol production have been constructed through metabolic engineering. After several rounds of modification/evaluation, three main microbial platforms, *Saccharomyces cerevisiae, Zymomonas morabilis* and *Escherichia coli* have emerged and they function well in pilot studies (Manikandan and Virutagiri, 2010). While there are on-going efforts to further enhance their properties, improvement of fermentation process is just one of the second factors that need to be fully emphasized and integrated to generate competitive fuel bio-ethanol.

During batch fermentation, the rate of ethanol per milligram of cell protein is maximal for a short period and declines progressively as ethanol accumulates in the

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surrounding broth (Moulin et al., 1984; Assien et al., 2010). However, studies including that of Millar et al. (1982) and Dombek and Ingram (1983) demonstrated that the removal of accumulated ethanol during fermentation, does not immediately restore fermentative activity and provided evidence that decline in metabolic rate is not exclusively due to the presence of ethanol. In their work, viability remained at 90% and the specific activities of certain enzyme (glycolytic and alcohologenic enzyme) in *S. cerevisiae* remained high throughout batch fermentation. None of these factors appear to be related to fall in fermentative activity during batch fermentation explained by Casey and Ingledew (1986), Ingram and Buttke (1984) and Moulin et al. (1984). Therefore, identification of the constraints represents an important step toward the development of improved process conditions for more rapid ethanol production.

In this study, we aimed to examine some physiological and nutritional factors (pH, cultural status, nitrogen and phosphorus sources) as possible cause of changes in fermentative activity during batch fermentation.

MATERIALS AND METHODS

Source of sample/collection

Cassava slurry used in this study was obtained from a cassava processing mill located in Abraka, Delta State using a clean wide mouth 10-L plastic container. The effluent was allowed to settle for a period of 24 h after which the supernatant was carefully decanted leaving a solid residue at the bottom of the container for further analysis.

Physico-chemical analysis of sample

The Association of Official Analytical Chemist (AOAC) (1990) methods were adopted in the determination of cyanide, starch, pH and glucose contents of the cassava slurry. Ethanol content was determined using alcohol meter.

Sample hydrolysis

Two methods were used for the conversion of the cassava effluent starch residue to fermentable sugar viz: acid hydrolysis and enzyme hydrolysis.

Acid hydrolysis involved the use of dilute trioxonitrate (v) acid, while a combination of α-amylase and gluco-amylase was used in enzymatic hydrolysis. Each hydrolysate was dispensed in equal amounts into ten 1000 ml Erlenmeyer flasks and the volume was made up to 500 ml with sterile distilled water for various experiments. Also, pH of each hydrolysate was adjusted to initial pH of effluent.

Activation/standardization of test isolate

Brewers' yeast (*S. cerevisiae*) used in this study was obtained from Nigeria Breweries PLC, Benin City, Edo State. The dehydrated test organism was weighed in 1 g amount and inoculated aseptically into ten milliliter of distilled water containing a cube of table sugar. This was allowed to stand for 24 h and then centrifuged using 800D centrifuge at 4000 rpm for 15 min. Sediment obtained served as standard inocula for various experiments.

Effect of fermentation duration on ethanol yield

This experiment was done to determine the residence time and the dynamics for optimum ethanol yield by the test isolate. The standardized inoculum was added to 500 ml of each hydrolysate contained in 1000 ml Erlenmeyer flask. These were then incubated at 27°C ± 2 for 168 h. However, at intervals of 0, 6, 24, 72 and 168 h, ethanol and reducing sugar concentrations were determined. The time at which maximum ethanol yield was obtained was noted and subsequent experiments on pH, cultural status and supplements were therefore used as the duration.

Effect of pH on ethanol yield

To evaluate the effect of pH on ethanol yield, the pH of each hydrolysate was varied ranging from 4.0, 4.5, 5.0, 5.5 to 6.5. The standardized inocula were then introduced into various flasks and fermented for a duration that resulted in maximum ethanol production. During fermentation, the pH of each set-up was monitored at intervals of 30 min for stabilization with appropriate buffer when necessary. At the expiration of the fermentation duration, ethanol and glucose concentrations were respectively determined and the pH that gave highest yield in ethanol was chosen for further experiments.

Effect of culture age on ethanol yield

The standard inoculum obtained as previously described was aseptically introduced into 10 ml malt extract broth contained in a test-tube and then incubated immediately at 27°C ± 2. At incubation periods of 1, 3, 7, 14 and 21 (days), cells were harvested, washed in sterile phosphate buffered saline to yield 1, 3, 7, 14 and 21 days old cultures, respectively. The harvested cells were separately introduced into each hydrolysate with pH appropriately adjusted. Also, fermentation was done immediately at a temperature of 27°C ± 2 for duration as earlier determined, at the end of which the values of ethanol produced and residual glucose concentrations were evaluated. Culture age that produced highest ethanol concentration was selected for further studies.

Effects of inorganic nutrient supplementation on ethanol yield

Logarithmic concentrations of 0.001, 0.01, 0.1 and 1.0 (mg/l) of $NaNO_3$, K_2HPO_4 and NPK, respectively were prepared with each hydrolysate. The pH of various hydrolysates was adjusted appropriately and 3 days old standard inocula were introduced and fermentation was done for 24 h (choice based on results of experiments described previously). At the end of fermentation duration, ethanol yield and glucose concentration were determined.

RESULTS

The result of the physico-chemical analysis of the cassava slurry is as presented in Table 1. Initial starch, glucose, pH and cyanide concentrations were 20.81,

Table 1. Chemical composition of cassava effluent residue.

Component	Composition
Starch	20.81 mg/l
Glucose	5.62 mg/l
Cyanide	64.0 mg/l
pH	4.64

Mean value of three determinations.

5.62, 4.64 and 64.9 mg/l, respectively. Acid and enzyme hydrolyses resulted in glucose concentrations of 60.71 and 46.14 mg/l, respectively.

The results obtained on the effect of fermentation duration on the rate of ethanol yield when *S. cerevisiae* was exposed to each hydrolysate showed that ethanol yield reduced progressively with increase in fermentation duration. Optimum ethanol yield obtained in acid hydrolysate (1.47 %v/w) and enzyme hydrolysate (1.0 %v/w) was at the 24th hour of fermentation as shown in Figure 1. Analysis of variance at 95% confidence limit indicates that there was a significant difference in ethanol produced at the various fermentation periods, therefore, 24 h was chosen as duration for optimal yield. Also, it was noticed that reducing sugar (glucose), decreased progressively as fermentation duration decreased. High amounts of glucose were consumed within 24 h of fermentation after which, utilization rate reduced.

The R-square value of the ethanol yield when considering the fermentation duration was significant for acid hydrolysate ethanol (0.8%) but was not significant for the other trials. Statistical analysis at $p > 0.05$ showed that there was a significant difference in the amounts of ethanol produced at the various pH tested. Maximum ethanol yield were 3.60 and 1.88 (%v/w) in acid and enzyme hydrolysates, respectively, and both were achieved at pH 5.5 as shown in Figure 2. These values were also significantly higher (t-test) than those obtained from initial experiment of effect of fermentation duration on ethanol production. Sugar consumption was also observed to increase as pH increased. pH at which highest utilizations occurred corresponded to pH at which optimal ethanol yield was recorded (Figure 2). Generally, the amounts of ethanol produced in acid hydrolysate at all given pH values were higher than those produced in enzyme hydrolysate.

Furthermore, the experiments of effect of culture age on ethanol yield by the test isolate at the selected pH (5.5) and fermentation duration (24 h) indicate that younger cultures had higher capabilities of ethanol production than older cultures as shown in Figure 3. Ethanol yield and glucose consumption in both hydolysates decreased as culture age of isolate increased although, there was an initial increase between fermentation by 1 day old culture and 3 days old culture of *S. cerevisiae* and as such 3 days old culture resulted in the highest yield in ethanol (5.61 and 2.91% v/v in acid and enzyme hydrolysates, respectively). A sharp drop in ethanol concentration produced in each hydrolysate was observed when 7 days old cultures were introduced for the fermentation. Overall, ethanol produced during fermentation by cultures beyond 3 days was insignificant. The P-value for the effect of culture age on ethanol yield for acid hydrolysate and enzyme hydrolysate were 0.096 and 0.093 respectively, hence there was no significant difference. The P-value for the acid hydrolysate was 0.027, hence culture age has a significant effect on enzyme hydrolysate-ethanol yield. Glucose utilized at the end of fermentation duration in fermentors that contained culture ages of 1, 3, 7, 14 and 21 were 33.34, 43.63, 21.13, 4.76 and 1.46 (mg/l), respectively in acid hydrolysate and the respective values obtained in enzyme hydrolysate were 22.94, 36.07, 12.33, 5.94 and 0.14 (mg/l).

The results of the effects of $NaNO_3$, K_2HPO_4 and NPK on the generation of ethanol and glucose utilization are presented in Figures 4, 5 and 6, respectively. Supplementation with various sources of nitrogen and phosphorus resulted in an additional increase in the amount of ethanol generated in each hydrolysate. However, results obtained reveal that additional ethanol generation was nutrient concentration dependent. Nutrient concentrations that resulted in maximal ethanol production were 0.001, 1.0 and 0.01 for $NaNO_3$, K_2HPO_4 and NPK, respectively. Ethanol produced at these respective concentrations were 5.74, 8.15 and 14.5 (%v/v) in acid hydrolysate, while corresponding values recorded in enzyme hydrolysate were 3.0, 5.1 and 8.3 (%v/v). In acid hydrolysate, supplementation with optimum concentrations of $NaNO_3$, K_2HPO_4 and NPK, respectively, led to an ethanol increase of 0, 45.5 and 150.5%. Similarly, the respective percent increase in ethanol generated in enzyme hydrolysate fermentors that received optimum concentrations of $NaNO_3$, K_2HPO_4 and NPK were 3.1, 75.3 and 185.2%, respectively. Thus, nutrients in form of NPK facilitated ethanol production than K_2HPO_4 and the least was $NaNO_3$.

DISCUSSION

The start-up fermentation experiment carried out in this study revealed that the time course of ethanol production followed an initial upward trend till the 24th hour, thereafter, there was no significant increase in ethanol produced with time. The limitation in ethanol production might be suggestive of build-up of toxic by-products in the fermentation medium and inhibitory effects of accumulating ethanol as previously reported by Zakpaa et al. (2009). Contrarily, further results obtained in this work indicated that the limitation in ethanol production

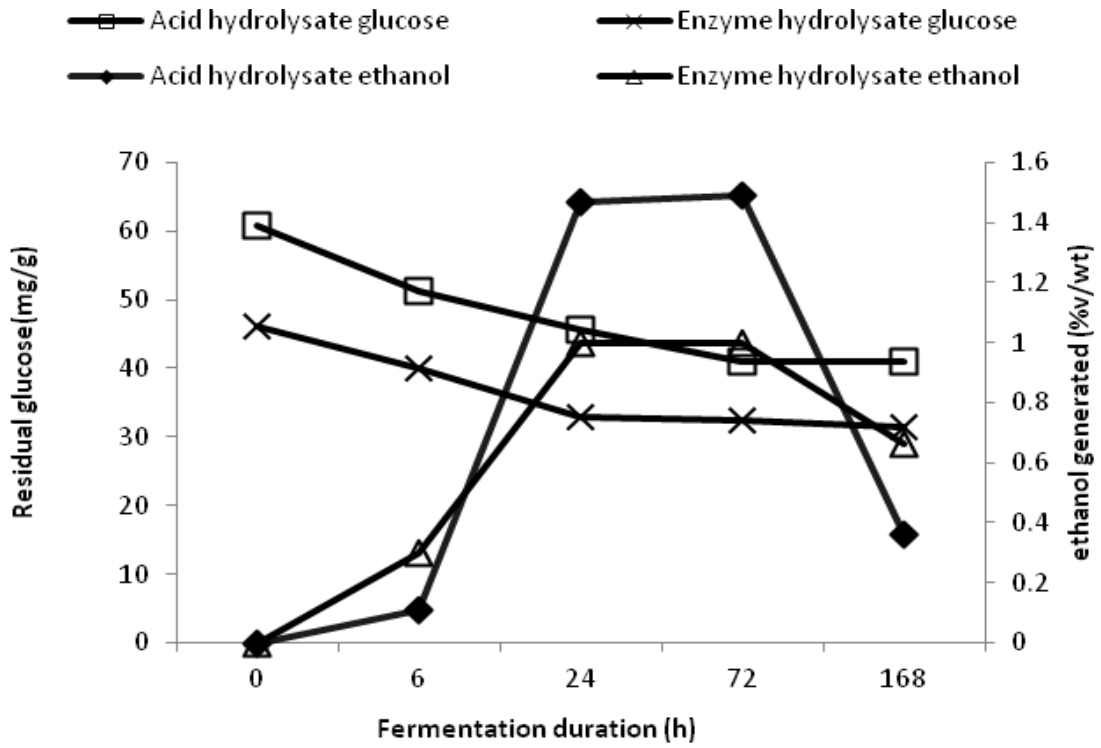

Figure 1. Effect of fermentation duration on glucose utilization and ethanol generation.

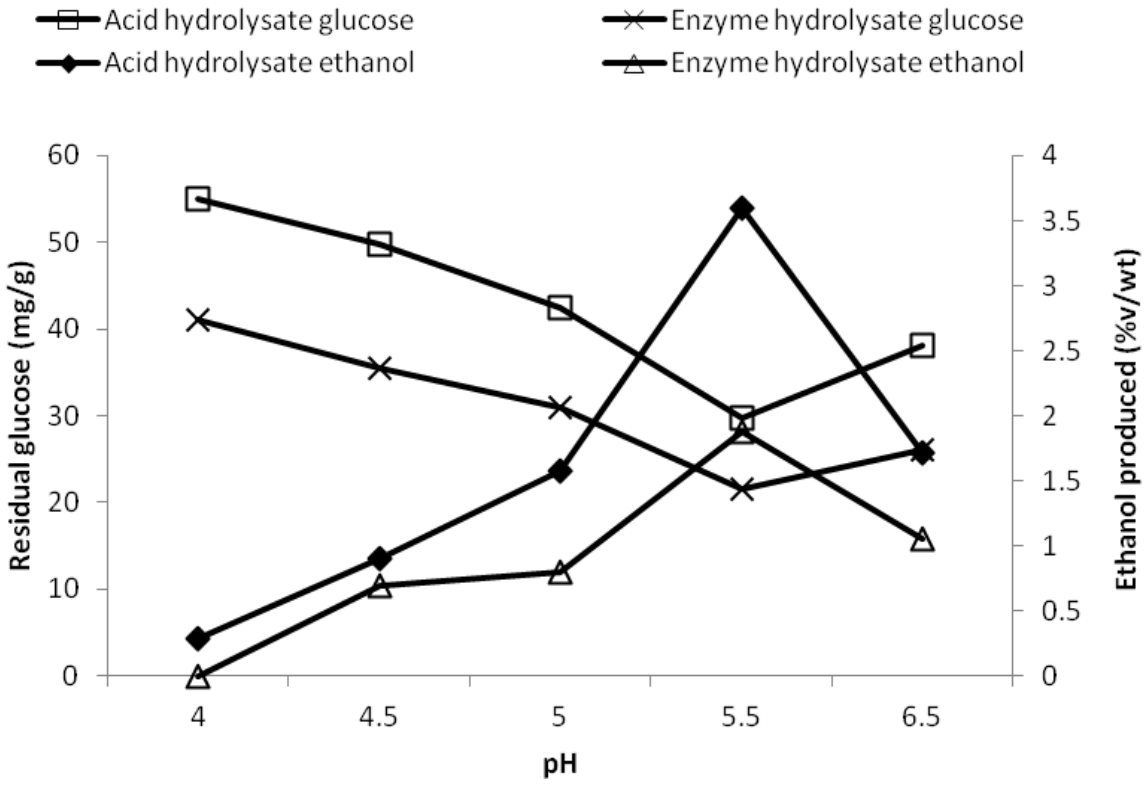

Figure 2. Effect of pH on glucose utilization and ethanol generation.

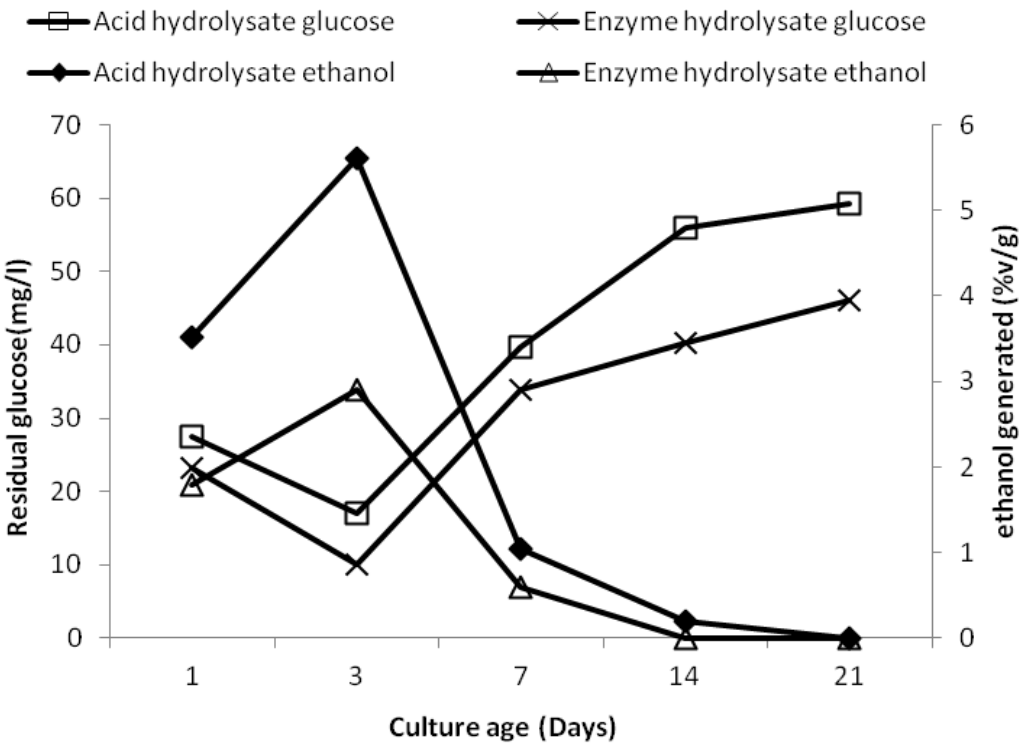

Figure 3. Effect of culture age on glucose utilization and ethanol generation at 5.5.

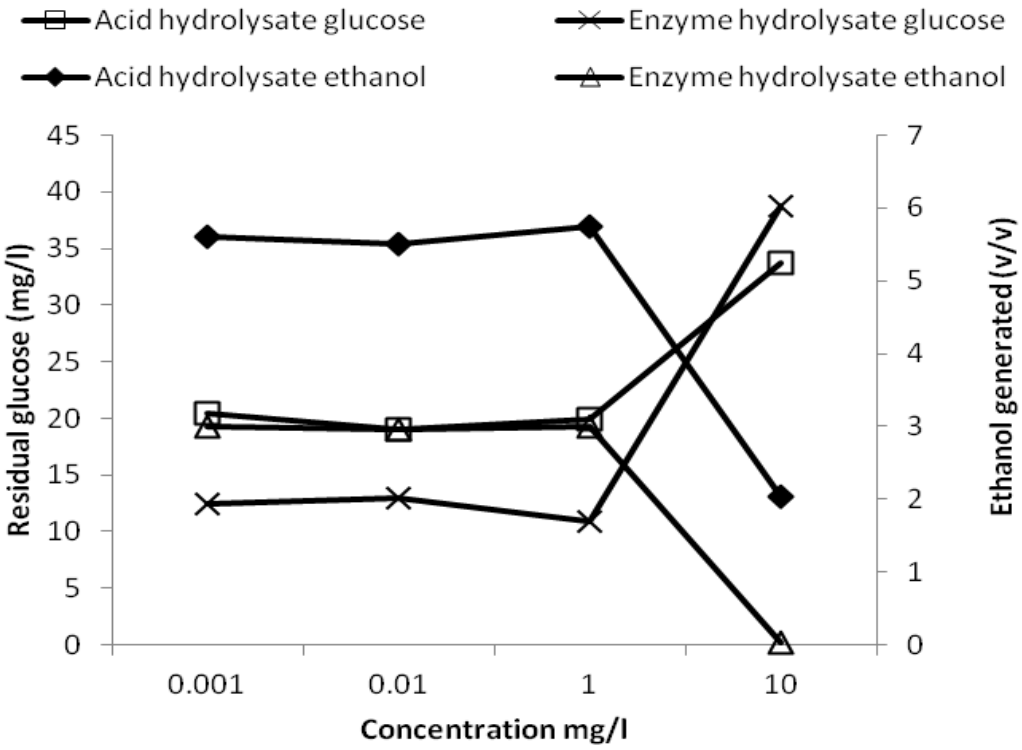

Figure 4. Effect of $NaNO_3$ on glucose utilization and ethanol generation at pH 5.5 using 3 days old cultures.

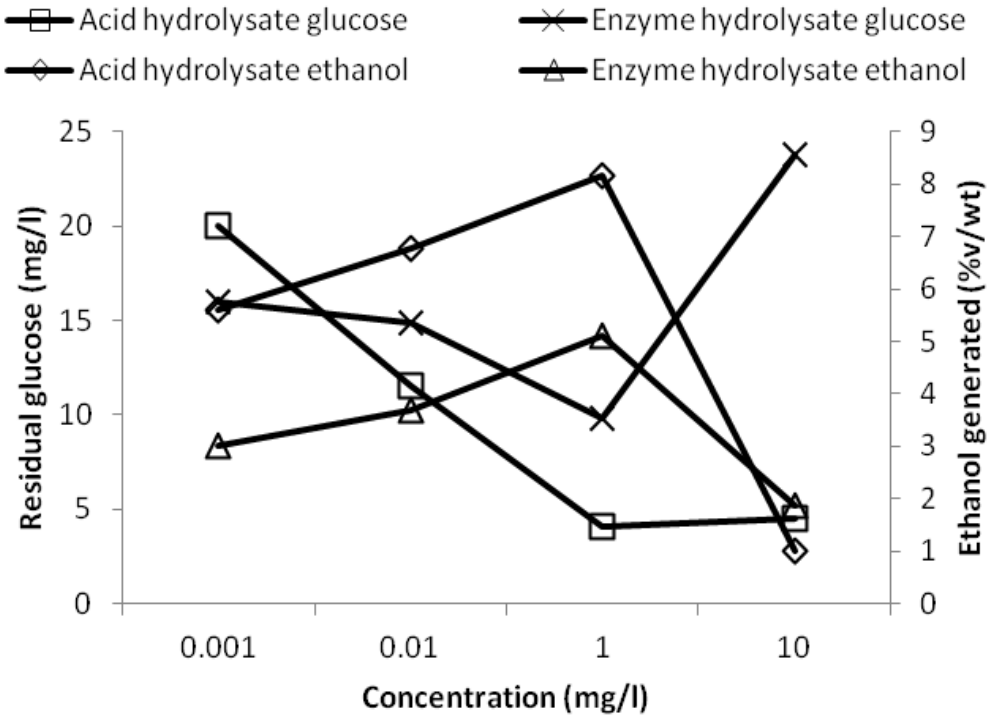

Figure 5. Effect of KH_2PO_4 supplementation on glucose utilization and ethanol generation at pH 5.5 using 3 days old cultures.

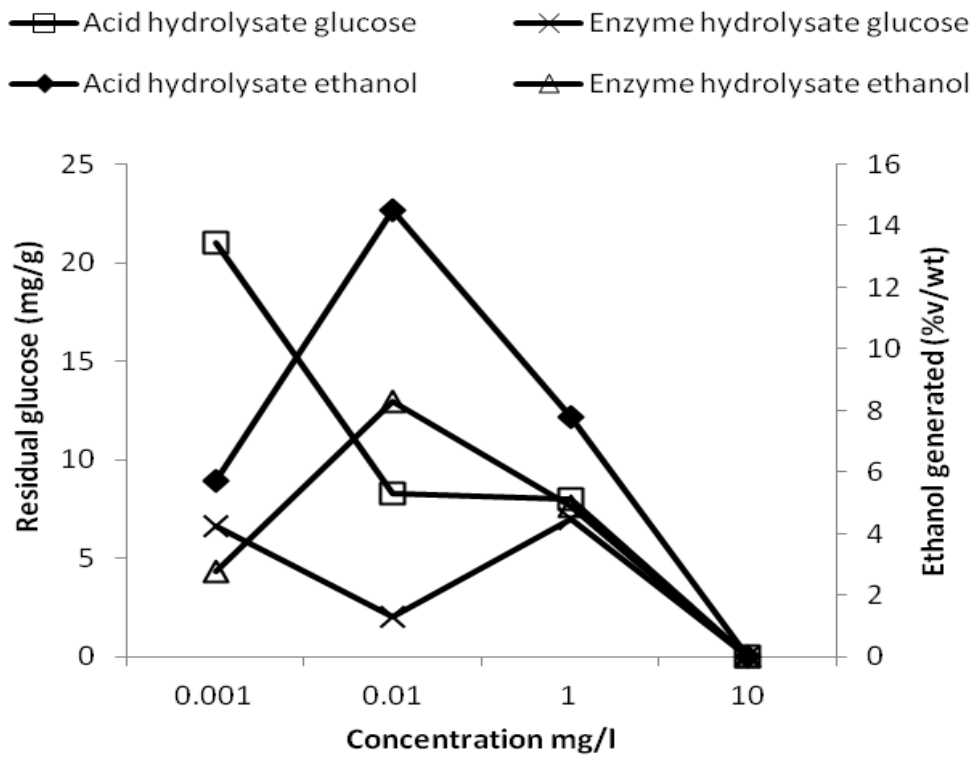

Figure 6. Effect of NPK on glucose utilization and ethanol generation at pH 5.5 using 3 days old cultures.

beyond the 24th hour of fermentation was more or less due to unfavourable operating or cultural conditions for the test isolate. The mode of fermentation determined the concentration of ethanol produced. Just as Olofsson et al. (2008) reported that temperature of fermentation affected ethanol production, results obtained in this study, showed significant variations in ethanol produced at various pH, cultural and supplied nutrient conditions or status.

The prevailing pH was applicable in influencing fermentation rates and pH 5.5 was noticed to be optimal for ethanol production. This might be the pH at which the test isolate *S. cerevisiae* grew best and probably was most favourable for its fermentative activities. Benerji et al. (2010) reported that *S. cerevisiae* grows best at slightly acidic pH. Also, similar result was obtained by Akponah and Akpomie (2011). In this study, ethanol yield obtained at optimal pH (5.5) were 59.17 and 47% higher than ethanol concentrations obtained from initial experiment of effect of fermentation duration in acid and enzyme hydrolysates, respectively.

Evaluating the effect of culture age on ethanol production ability of test isolate showed that 3 days old culture had the highest capability for ethanol production. This seems to correspond with the exponential growth phase of the organism. Although, this result is in contrast with the reports of Roukas (1996) and Irfana et al. (2009) that cultures at stationary phase were most efficient in ethanol production. The decline in ethanol yield with age observed might be due to the fact that younger cultures have a higher metabolic rate than older ones. It is also possible that the older cultures had impaired ability to tolerate ethanol inhibition. Low ethanol productivity could as well be attributed to lengthened lag phase on introduction of the stationary cultures into the environment or maybe low metabolic rates, fragility as well as to increased permeability to ethanol generated.

Also, results obtained reveal that the addition of nitrate and phosphate from exogenous sources improved substrate utilization and ethanol yield. Generally, it was observed that low concentrations of each nutrient source stimulated higher fermentation rates and ethanol production than higher nutrient concentration. Probably, this might be as a result of toxic effect of high concentration of the nutrient. The tolerable nutrient concentration might be useful in synthesis/repair of damaged DNA and cell membrane in addition to enhancing cell growth. Introduction of NPK must have corrected the nutritional imbalance by supplying the limiting nitrogen and phosphorus. Microorganisms require phosphorus as phospholipids in synthesizing cell membranes, as components of nucleic acid and for sugar phosphorylation (Andrew and Jackson, 1996). They also exploit nitrate sources to meet their protein and nucleic acid requirement. NPK fertilizer contains these two inorganic nutrients and hence when it was added to the cassava effluent, it was better in establishing the nutritional balance than $NaNO_3$ and K_2HPO_4. Thus, it is important in all ethanol fermentation programmes to monitor the nutritional variables that could influence resultant ethanol production and to adjust same appropriately. The abrupt final decline in activity reflects the near complete exhaustion of the substrate glucose. Thus, optimum combinations of pH, nutrient concentrations and cultural status play a major role in getting maximum bio-conversion of cassava effluent to ethanol.

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