PRODUCTION OF ETHANOL FROM CASSAVA (*Mannihot esculenta*) WASTE WATER USING *Saccharomyces cerevisiae* and *Escherichia coli*

Akponah, E.
Department of Microbiology, Delta State University, Abraka

ABSTRACT

The ethanol producing capabilities of axenic cultures of *Saccharomyces cerevisiae* and *Escherichia coli* from cassava waste water was investigated. Analysis of the initial composition of the substrate showed that it contained 71.97%, 1.2%, 0.5% and 23% (w/v) of starch, protein, fat and cyanide respectively. The cassava waste water pre-treatment approach for converting starch into reducing sugar involved inoculation of a consortium of indigenous amylolytic fungi (which include *Geotrichum candidum*, *Aspergillus fumigatus* and *Aspergillus niger*) and heating at 80^oC for 30minutes which yielded 41.67% and 11.62% (w/v) glucose respectively. The glucose values decreased progressively with increasing duration of fermentation. At the end of fermentation period (7days), glucose levels recorded were 5.03% and 7.3% in amylolytic fungi pre-treated set-ups containing *S. cerevisiae* and *E. coli* respectively. In heat treated set-ups containing *S. cerevisiae* and *E. coli*, glucose levels at the end of fermentation were 1.01% and 1.82% (w/v) respectively. Also, the pH dropped with increasing duration of incubation such that pH values decreased from 4.48 to 4.1. A decrease in pH from 5.2 to 4.7 and 4.0 were observed in heat pre-treated set-ups containing *S. cerevisiae* and *E. coli* respectively while control pH dropped from 5.2 to 4.9. However, ethanol produced seemed to be limited by fermentation duration. Highest yield were obtained after 72h of fermentation. Further increases in fermentation duration did not result in any significant yield in ethanol production. Also, fungal hydrolysis method resulted in higher yield in ethanol production than heat hydrolysis of cassava waste water. Maximum ethanol produced were 13.33% and 5.6% (w/v) by *S. cerevisiae* and *E. coli* inoculated into cassava waste water hydrolyzed by amylolytic fungi. Control flask yielded 0.03% w/v ethanol. Heat pre-treatment of cassava waste water resulted in the production of 2.61% and 0.72% (w/v) ethanol by *S. cerevisiae* and *E. coli* respectively. The study revealed that the isolates had the ability of ethanol production from cassava waste water. Also, it demonstrates that cassava waste water is a suitable substrate which can be harnessed for ethanol production, thus curbing the pollution nuisance it poses in cassava processing areas.

Keywords: Cassava, ethanol, fungal hydrolysis, heat hydrolysis, *S. cerevisiae* and *E. coli*.

INTRODUCTION

Cassava (*Mannihot esculenta*) is cultivated extensively as a food crop in Africa. It is the third largest source of carbohydrate in food for human consumption in the world (Onabolu, 1999). Cassava roots play an important role in the African diet and they can be processed using simple traditional methods into products such as garri, fufu, tapioca and lafun flour (Odunfa, 1987).

In the processing of cassava into garri, it is milled and pressed to dry and consequently, a large amount of liquid waste is generated annually (Obadina *et al.,* 2006). In developing countries, there is a growing interest regarding the utilization of organic waste generated by the food processing sector and through other human endeavors (Adesayan *et al.,* 2008). This has led to a new policy of complete utilization of raw materials so that there will be little or no residue left that could pose pollution problems (Ofuya and Nwajuiba, 1990).Rather than allowing waste generated from agriculture-based industry to become municipal waste, it is necessary to convert them into useful products (Adesayan *et al.,* 2008).

It is now realized that these wastes may be utilized as cheap raw materials for some industries or as cheap substrate by microbiological processes (Nwabueze and Ugochinyere, 2006). Cassava waste has the potential to be utilized to produce ethanol due to its cellulose, hemi-cellulose and starch levels which are hydrolysable carbohydrates that can be converted into fermentable sugar. The use of cassava waste as raw material in ethanol production does not only reduce waste material created from the cassava starch industry, but also lowers the cost of production (Adesayan *et al.,* 2008).

Ethanol has been described as one of the most exotic chemicals because of its unique combination of properties as a solvent, a germicide, a beverage, a depressant and especially as a fuel (Moreira *et al.,* 2009). Thus, this has necessitated the increased production of this alcohol. Fermentation processes from any material that contain sugar can derive ethanol and as such it has been made from a variety of agricultural products such as grain, molasses, fruit, whey and sulfite waste liquor. However, the prices of these agricultural products like oil prices are on the increase. Therefore, this study was initiated to explore the possibility of using cassava waste water as a suitable substrate for the production of ethanol.

MATERIALS AND METHODS

Sample Collection

Cassava waste water was collected from a garri processing industry or mill located at Abraka, Delta State. A clean four liter plastic container was used for sample collection. The container was mounted under the pressing machine and the liquid waste was allowed to drop into it through a clean funnel. The sample collected was then transported to the laboratory for further analyses which were carried out within 60minutes of collection. Stock sample was however, maintained at 4°C in a refrigerator.

Physiochemical Analysis of Sample

The protein, lipid, starch, reducing sugaer (glucose) and cyanide composition of the cassava waste water sample were determined by various methods as described by AOAC (1990).

Test Isolates

Saccharomyces cerevisiae and *Escherichia coli* were the test isolates used in this study. *Saccharomyces cerevisiae* (Baker's yeast) was obtained from Abraka main market, Abraka Delta State. It was reconstituted in malt extract broth. *Escherichia coli isolated* from soil sample obtained from waste water dumpsite surrounding the cassava processing mill, was maintained in lactose broth.

Development / Standardization of Inoculum

One milliliter of the broth cultures of *Saccharomyces cerevisiae* and *Escherichia coli* were aseptically transferred into 200ml of appropriate freshly prepared broth and incubation followed immediately at room temperature under shaken conditions (120rpm) using HY-4 vibrator for 18-48hours. After which, cells were harvested by centrifugation at 4500rpm for 30minutes using an 800D model centrifuge (Techmel and Techmel, Texas, USA). Harvested cells were washed thrice in sterile phosphate buffer saline and reconstituted in 10ml sterile deionized water producing 1.20 x 10^{6}cfu/ml *Saccharomyces cerevisiae* and 2.57 x10^{6} cfu/ml *Escherichia coli.* These served as standard inocula for various fermentation procedures.

Starch hydrolysis

Two pretreatment methods which include amylolytic fungal hydrolysis and heat hydrolysis were employed for the hydrolysis of initial starch concentration present in the waste water to fermentable sugar (glucose).

Amylolytic fungal hydrolysis

Amylolytic fungal hydrolysis entailed, pretreatment of the cassava waste water with amylase producing fungi which were indigenous to the cassava waste water. These amylolytic fungi comprised *Aspergillus niger, Aspergillus fumigatus* and *Geotrichum candidum.*They were isolated and identified using the method of Barnet and Hunter, 1972. Subsequent screening for amylase production followed using the method of Arotupin (2007). A consortium of these amylolytic fungi was then obtained and inoculated into 500ml cassava

waste water contained in a 500ml Erlenmeyer flask. This was allowed to stand for 48hours at room temperature for starch hydrolysis to take place. At the end of the incubation period, the reducing sugar concentration and pH were determined by the AOAC (1990) method.

Heat hydrolysis

The cassava waste water was dispensed in 500ml amounts into 500ml Erlenmeyer flasks and sample pre-treatment was by heating at 80°C in a water bath for 30mins.The reducing sugar concentration and pH of pre-treated sample were determined by the AOAC (1990) method and recorded as initial values.

Fermentation procedure

The respective standardized inocula (10ml) were inoculated into 500ml of the various pre-treated cassava waste water contained in 500ml Erlenmeyer flask as follows:

i *Saccharomyces cerevisiae* + 500ml of amylolytic fungal pre-treated cassava waste water
ii *Escherichia coli* + 500ml of amylolytic fungal pre-treated cassava waste water
iii 500ml of amylolytic fungal pre-treated cassava waste water only (Control)
iv *Saccharomyces cerevisiae* + 500ml of heat pre-treated cassava waste water
v *Escherichia coli* + 500ml of heat pre-treated cassava waste water
vi 500ml of heat pre-treated cassava waste water only (Control).

The content of each flask was allowed to ferment for 7days. However, samples were withdrawn intermittently at intervals of Day 0, 1, 3, 5 and 7 from various flask for the determination of reducing sugar (glucose) levels, pH and ethanol production.

pH Determination

pH was determined using a pH meter (Jenway 3020). After standardization with appropriate buffers, the electrode sensor of the pH meter was inserted directly into 20ml of sample contained in a clean 50ml beaker. The stable value on the recorder was read as the pH value.

Ethanol Production/Estimation

Distillation method was used to obtain the ethanol produced or to separate ethanol produced from the fermentation mixture. The distillate obtained was re-distilled and percent ethanol was estimated thus:

$$\% \text{ ethanol (v/v)} = \frac{\text{Volume of distillate}}{\text{Volume of fermentation mixture}} \times 100$$

RESULTS AND DISCUSSION

The result of the initial chemical composition of the cassava waste water sample obtained is as shown in Table 1.The values of protein, lipid, starch, reducing sugar (glucose) and cyanide contents were 1.2, 0.5, 71.9, 1.48 and 23 (%w/v) respectively.

Table 1: Chemical composition of cassava waste water sample

Component	Concentration (mg/l)
Protein	1.2
Lipid	0.5
Starch	71.9
Reducing sugar(glucose)	1.48
Cyanide	23

The amylolytic fungal hydrolysis and heat hydrolysis methods utilized to convert the initial starch concentration into fermentable sugar, yielded, 41.67 and 11.02 (%w/v) glucose respectively, as presented in Table 2. Also, the resultant pH values were 4.48 and 5.2 in fermentation flask pre-treated with amylolytic fungal and heat respectively.

Table 2: Effect of pre-treatments on reducing sugar concentration and pH of cassava waste water

Pre-treatment	Reducing sugar (%w/v)		pH	
	Initial	Final	Initial	Final
Amylolytic fungal hydrolysis	1.48	41.67	5.2	4.48
Heat hydrolysis	1.48	11.02	5.2	5.2

Changes in residual reducing sugar concentration in both fungal and heat hydrolysate fermentors are presented in figure 1. It was observed that the residual reducing sugar concentration dropped progressively with increase in fermentation duration. However, results obtained revealed that the rate of sugar consumption or utilization by *Saccharomyces cerevisiae* and *Escherichia coli* in both fungal hydrolysate and heat hydrolysate fermentors decreased significantly with increases in the fermentation duration. In fungal hydrolysate fermentor containing *Saccharomyces cerevisiae*, the rate of glucose consumption at Day 1, 3, 5 and 7 were 0.94, 0.23, 0.04 and 0.02 (mgh^{-1}) respectively. While In fungal hydrolysate fermentor, containing *Escherichia coli* the respective rate of sugar utilization at Day 1, 3, 5 and 7 were 1.14, 0.05, 0.08 and 0.02 (mgh^{-1}). Similarly, the rate of sugar utilization in heat hydrolysate fermentors which contained *Saccharomyces cerevisiae* and *Escherichia coli*, were 0.34 mgh^{-1} (Day1), 0.05 mgh^{-1} (Day3), 0.001 mgh^{-1} (Day5) & 0.0006 mgh^{-1} (Day7) and 0.2 mgh^{-1} (Day1), 0.09 mgh^{-1} (Day3), 0.002 mgh^{-1} (Day5 & 7) respectively. This trend noticed, suggest that rates of sugar utilization by the isolates, increased with increase in sugar availability. On the contrary fungal hydrolysate fermentor that contained neither of the test isolates (control) showed slight increases in residual sugar concentration which is indicative of further starch hydrolysis by amylolytic fungal present. This can also be substantiated by the fact that the residual sugar concentration remained constant throughout the fermentation period in heat hydrolysate fermentor that did not receive any organism (control flask).

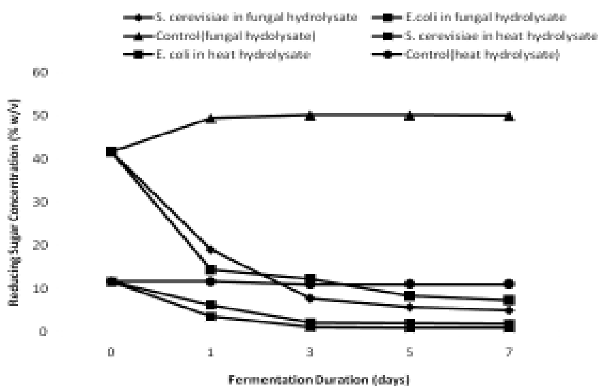

Fig.1: Changes in reducing sugar concentration in various hydrolysate fermentor

Also, data obtained as presented in figure 2 revealed that sugar utilization were higher in fungal hydrolysate fermentors than in heat hydrolysate fermentors irrespective of the test isolate introduced. At the end of the fermentation duration in fungal hydrolysate fermentors, *Saccharomyces cerevisiae* and *Escherichia coli* consumed 36.64 and 34.37% (w/v) glucose respectively. While the amounts consumed in various heat hydrolysate fermentors were 10.01 and 9.2 (%w/v) by *Saccharomyces cerevisiae* and *Escherichia coli* respectively. This probably may be attributable to the initial sugar concentration which was higher in the fungal hydrolysate.

Progressive drops in pH were observed in all experimental set-ups as shown in figs. 3 and 4. A drop in the pH from 4.48 to 3.9 and 3.2 were recorded in fungal hydrolysate fermentors containing *Saccharomyces cerevisiae* and *Escherichia coli*. The control fermentor showed a reduction from 4.48 to 4.1. Similarly, a gradual reduction in pH was observed in heat hydrolysate fermentors. At the end of the fermentation duration, the pH values had dropped from 5.2 to 4.7, 4.0 and 4.9 in heat hydrolysate fermentors containing *Saccharomyces cerevisiae*, *Escherichia coli* and control respectively. Generally, pH values recorded on each analysis day were significantly, lower in fungal hydrolysate fermentors than those pre-treated with heat. Also, fermentors that contained *Escherichia coli* had lower pH than those containing *Saccharomyces cerevisiae*. This may be consequent upon the type of fermentation carried out by *Escherichia coli* which is mixed acid fermentation that leads to the production of acetic acid, lactic acid and succinic acid which in turn influenced the pH of the medium.

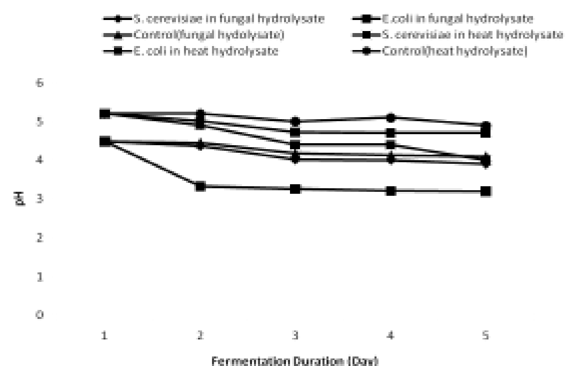

Fig.2: Changes in pH of various hydrolysate fermentors

The results of changes in ethanol produced in various fermentative vessels are presented in figure 3. Data obtained showed that ethanol was produced in all fermentors except in the control fermentor that was heat treated and did not receive any inoculum. Fermentative activities of *Saccharomyces cerevisiae* and *Escherichia coli* in fungal hydrolysate fermentors yielded 13.3% (v/v) and 5.6% (v/v) ethanol respectively while ethanol yield in the control was 0.03% (v/v).

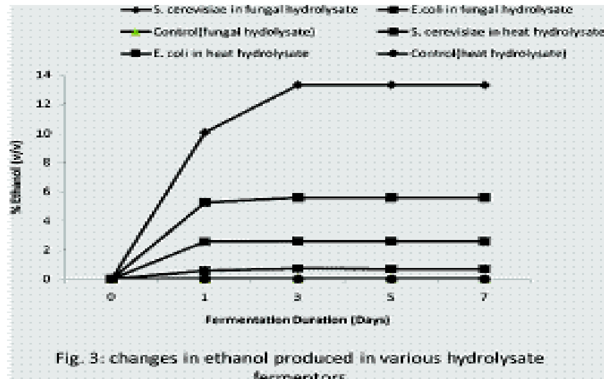

Fig. 3: changes in ethanol produced in various hydrolysate fermentors

These were significantly higher at $P < 0.05$ than values obtained from the corresponding fermentation of heat pre-treated cassava waste water by *Saccharomyces cerevisiae* (2.6% v/v) and *Escherichia coli* (0.7% v/v). Perhaps, this may be due to the fact that pre-treatment with amylolytic fungi resulted in higher reducing sugar concentration than heat pre-treatment. Additionally, there, there were positive correlations between sugar utilization and ethanol production. Ethanol was not produced in the control flask containing cassava effluent that was heat treated. These observations implied a direct proportionality between ethanol production and reducing sugar consumption. Again the production of ethanol in control flask that was neither inoculated with *Saccharomyces cerevisiae* nor *Escherichia coli* though pre-treated with amylolytic fungi is suggestive of the fact that some organisms indigenous to cassava waste water have the potential for ethanol production. Therefore, cassava waste water should be screened for ethanol producing strains and methods be devised to improve their abilities.

Furthermore, the results obtained in this study revealed that *Saccharomyces cerevisiae* had a higher ethanol producing ability than *Escherichia coli* in both pre-treated options. Various workers (Ergun and Freda, 2000; Amutha and Paramasamy, 2001, fregonesi *et al.,* 2007 and Moreira *et al*., 2009) reported that some organisms or species have been found more successful for ethanol production than others due to the fact that the different species adopt different metabolic pathways by having special genes or special enzymes for the conversion of sugar to ethanol or other metabolites.

Also, results obtained as shown in figs. 5 and 6, demonstrates that ethanol production decreased considerably with increase in fermentation duration. Although, there was an initial increase between Day 0 and 3, thereafter, there was little or no significant ethanol yield. The maximum yield at Day3, may be attributable to the cultural status of the isolates as previously reported by Irfana *et al* (2009) who showed that optimal ethanol production by various species of *Saccharomyces* was by cultures at their stationary growth phase. Aside this, retardation in ethanol yield beyond Day 3 in this experiment could also be due to the drop in pH observed in the various experimental set-ups. The pH condition might have affected the survival of the test isolates especially, *Escherichia coli* hence affecting fermentation and subsequently, ethanol yield. Importantly, this reason may also explain why ethanol yield by *Saccharomyces cerevisiae* was significantly higher than that by *Escherichia coli* since it can tolerate acid environment better. However, Willaert and Viktor (2006) noted that the pH of surrounding medium changes the configuration and permeability of the cell membrane thus reducing the rate of sugar fermenting enzymes or retarding metabolic pathway and microbial growth. Probably this might be responsible for the reduced rate of ethanol production with increased duration of fermentation. Another factor may be the competitive utilization of available glucose and growth of indigenous/ inoculated organisms.

Nevertheless, the study demonstrates that *Saccharomyces cerevisiae* and *Escherichia coli* have the ability of ethanol production and that

cassava waste water is a suitable substrate which can therefore be harnessed for ethanol production, thus curbing the pollution nuisance it poses especially in cassava producing areas. Additionally, the amylolytic fungal hydrolysis method can be recommended in the pursuit for fuel ethanol production as it is simple, inexpensive, environment friendly and reliable.

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