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BIOCONVERSION OF CASSAVA STARCH TO ETHANOL IN A SIMULTANEOUS SACCHARIFICATION AND FERMENTATION PROCESS BY CO-CULTURES OF *Aspergillus niger* AND *Saccharomyces cerevisiae.*

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ABSTRACT
Ethanol production by co-cultures of *A. niger* (GS4) and *S. cerevisiae* (BK6) was studied using cassava starch as substrate. At 1% substrate concentration ethanol yield was 0.35g/100ml while the ethanol concentration increased to a maximum of 3.60g/100ml at 8% substrate concentration. When the culture conditions were optimized, the ethanol yield further increased to 4.30g/100ml at a temperature of 35^{o}C, pH 5.0, 300rpm agitation rate and reduced fermentation period of 4 days.

Keywords: *A. niger*, *S. cerevisiae*, Fermentation, Ethanol, Cell dry weight, Residual sugar

INTRODUCTION
Cassava (*Manihot esculenta*) is one of the important roots crops that provide the major part of the daily calorie needs of people in the tropics and grown more widely than grains (Omemu *et al*., 2005). Unlike grains, however, cassava, like other root and tuber crops have high moisture content which accounts for their poor storage and utilisation potential with an estimated post-harvest life of less than 72 hrs and post-harvest loss of about 23% for freshly harvest roots (FIIRO, 2009). As such, a very large proportion of the harvested crops are lost yearly (Ihekoronye & Ngoddy, 1995). Since the starch of this crop is susceptible to hydrolysis, these could be converted to ethanol after hydrolysis by either chemical, enzymatic or by the combination of both (Seeman *et al*., 2007).

In recent years, a new technique was introduced that combines both hydrolysis and fermentation of starch in a single-step process reffered to as simultaneous saccharification and fermentation. In such a process, two organisms with synergistic relationships are co-cultured together in the same vessel. A study has shown that co-culturing of an efficient sugar fermenter such as *Saccharomyces cerevisiae* with an *Aspergillus* species in a starch medium would prevent accumulation of inhibitory concentrations of reducing sugar and hence, an enhancement of the amylolytic activity, the amount of starch metabolizable, and the total ethanol yield (Abouzied & Reddy, 1986).

The purpose of this study was to examine the effects of some environmental factors on the bioconversion of cassava starch into ethanol using co-cultures of *A. niger* and *S. cerevisiae* in a simultaneous saccharification and fermentation (SSF) process.

MATERIALS AND METHODS
Organisms : Amylase-producing *A. niger* (GS4) was isolated from the soil by burying starch containing grains in a moist, humus garden soil for five days. Aliquot of 0.1ml of 10^{-5} suspension of the burried grains in sterile distilled water was spread on sterile solidified isolation medium consisting of soluble starch (1% (w/v), peptone (1% (w/v), yeast extract (1% (w/v) and agar- agar (1.5% (w/v). After incubation of the plates at 30 ^{o}C for 5 days, the plates were flooded with iodine solution and observed for blue-black colour disappearance around colonies. The colony with the largest zone of clearance was subcultured on fresh, sterile potato dextrose agar (PDA) for further studies. Based on its starch hydrolysing ability, Isolate GS4 was selected for use and was maintained on PDA slants at 4 ^{o}C.

Saccharomyces cerevisiae used in the study was isolated from a local fermented beverage (*burukutu*). Aliquot of 0.1ml of 10^{-5} dilution of the beverage was spread on the surface of a solidified PDA plate and incubated for 72 hrs at 30^{o}C. Colonies suspected to be *S. cerevisiae* based on colonial characteristics were subcultured on PDA slants for subsequent identification. A smear of the isolate was examined microscopically after staining with methylene blue. Thermo tolerance, osmotoleranc, ethanol tolerance, sugar utilization tests as well as ethanol production potential of the yeast isolates were determined (Ameh *et al*., 1989). The isolate (BK6) found to be most suitable was selected and maintained on fresh PDA slants at 4 ^{o}C

Cassava Starch Preparation: The cassava starch used for this investigation was prepared into soluble starch by the modified method of Abouzied & Reddy (1986). Peeled and chopped portion of the fresh tuber was wrapped in a piece of muslin cloth, immersed in water and heated to extract the starch. The starch

extract was dried on layers of muslin cloth in an airy condition for 48 hrs. The dried starch was pulverized and stored in air-tight container for further use.

Media: The growth medium used for preparing the organisms contained (per 100ml); Soluble Starch, 1g; Glucose, 1g; Peptone, 0.1g; Malt Extract, 0.1g; Yeast Extract, 0.2g; Magnesium Chloride, 0.1g; Calcium Carbonate, 0.2g; Ammonium Phosphate, 0.2g; and Ferrous Sulphate, 0.001g. The fermentation medium used for ethanol production in this experiment was the same as the growth medium stated above except that starch concentrations ranging from 1 - 10 % was used when testing for the effect of different substrate concentrations. One normal HCl and one normal NaOH were used to adjust the pH of the medium to 6.0.

Inocula Preparation: *A. niger* and *S. cerevisiae* inocula were prepared by using slant cultures to inoculate 50ml of sterile growth medium contained in 200ml Erlenmeyer flasks. The flasks were incubated with shaking (200 rpm) at 30 ^{o}C for 5 days.

Experimental Set-Up and Fermentation Procedure: A starting batch of 400ml medium in 1 litre Erlenmeyer flask was used. Four grams (4g) of cassava substrate was utilised in the first batch. Varying amounts (1 to 10 % w/v) of the substrate was however used to test the effect of substrate concentration on ethanol yield. The flasks were sterilized by autoclaving at 121 ^{o}C for 15 min and *A. niger* corresponding to 2.5 x 10^{6} spores/ml and *S. cerevisiae* (3 x 10^{8} cells/ml) were used as the inocula and incubation was at 30^{o}C, 200 rpm for 7 days.

Optimization of Culture Conditions: To study the effect of pH on ethanol production, the fermentation was carried out with the initial pH adjusted to 3, 4, 5, 6, 7 and 8. The effect of temperature was studied by carrying out fermentation at temperature range of 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C and 50^{o}C. The effect of agitation rate was determined by carrying out fermentation at agitation rates of 300rpm, 400rpm and 500 rpm. The length of fermentation was extended to 14 days at the optimum culture parameters, 8% (w/v) substrate concentration, pH 5, 35 ^{o}C and agitation rate of 300 rpm to determine the effect of length of fermentation on the final ethanol yield.

Analytical Procedure: Thirty milliliter aliquots from each flask were taken after thorough shaking of the flasks in order to homogenize the contents at 24-hrs interval during fermentation. This was used for the analysis of cell biomass, ethanol concentration, and residual sugar concentration. Cell biomass was determined by centrifuging ten milliliters sample, drying the cells obtained to a constant weight at 60 ^{o}C, and expressing the dry weight as grams per 100 ml growth medium. Ethanol concentration was determined by measuring its specific gravity after distillation. The specific gravity value was used to determine ethanol concentration from a standard curve prepared using known concentrations of ethanol. The residual sugar in the fermentation medium was analyzed by the dinitrosalicylic (DNS) acid method of Miller (1959). A standard curve was previously prepared using varying concentration of glucose and the calibration curve was used to determine the residual sugar. Qualitative test (infrared determination) was carried out on all sample of ethanol produced using infrared spectrophotometer (Model: Nicolet IR 100 FT-IR). The infrared spectra of the liquid ethanol were compared with that of an absolute ethanol.

RESULTS

The result in Table 1 shows ethanol yield, reducing sugar, cell dry weight as well as pH and temperature variation obtained during the fermentation of 1% cassava starch. The spectra reading carried out on all samples also showed the presence of OH functional group at the appropriate position confirming its ethanol status. From this result there was a gradual increase in yield of ethanol from the first day (0.05g/100ml) through to the fifth day when a maximum of 0.35g/100ml was obtained. This represents 35% of the starch utilized and 68.6% of the theoretical yield. Five days was therefore selected as the optimum length of fermentation. The cell dry weight (growth) increased throughout the length of fermentation from 0.15g/100ml to 0.63g/100ml. The reducing sugar concentration also reduced from 0.24g/100ml obtained on the first day of fermentation to 0.01g/100ml on the 7th day. The pH of the medium decreased from 6.0 to 4.70 and the temperature increased gradually from 26.3 ^{o}C to 27.9 ^{o}C (Table 1).

TABLE 1. CO-CULTURE FERMENTATION OF 1% CASSAVA SOLUBLE STARCH FOR ETHANOL PRODUCTION

Fermentation time (Days)	Temp.(^{o}C)	pH	CDW (g/100ml)	Reducing sugar (g/100ml)	Ethanol yield (g/100ml)
1	26.3	6.0	0.15	0.24	0.05
2	26.4	5.03	0.21	0.19	0.12
3	27.4	4.95	0.30	0.16	0.18
4	27.2	4.66	0.43	0.13	0.28
5	27.2	4.59	0.49	0.09	0.35
6	27.7	4.82	0.50	0.03	0.33
7	27.9	4.70	0.63	0.01	0.34

CDW = Cell dry weight

The result of optimization of cassava substrate concentration shows increased ethanol yield from 1% (0.35g/100ml) to 8%, which gave the highest yield of 3.60g/100ml. Concentrations above 8% show reduction in ethanol yield. Substrate concentration with the highest ethanol yield (8%) was therefore selected for further studies. At 1% concentration, 0.46g/100ml cell dry weight was recorded while 1.68g/100ml was recorded at 8% concentration (Fig 1).

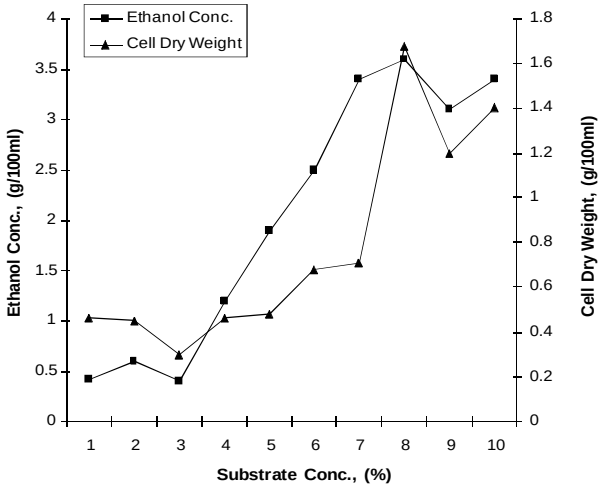

FIG. 1. EFFECT OF CASSAVA SUBSTRATE CONCENTRATION ON ETHANOL YIELD AND GROWTH (CELL DRY WEIGHT) BY CO-CULTURE OF *A. niger* AND *S. cerevisiae*

Fig. 2 shows the effect of pH variation on ethanol yield. There was a gradual increase in ethanol yield from pH 3 to 5 (1.50g/100ml to 3.80g/100ml respectively) and then declined. Cell dry weight also increased from 0.56g/100ml at pH 3 to 1.98g/100ml at pH 5.0 and then declined beyond this pH. pH 5.0 was then chosen for further studies.

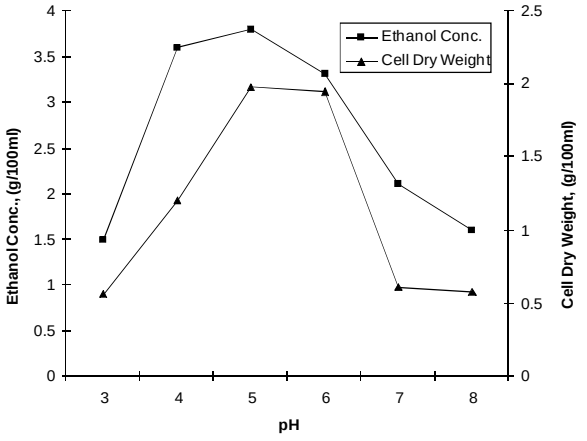

FIG. 2. EFFECT OF PH VALUES ON ETHANOL YIELD AND GROWTH (CELL DRY WEIGHT) BY CO-CULTURES OF *A. niger* AND *S. cerevisiae* GROWN ON 8% CASSAVA SUBSTRATE

The effect of temperatures on ethanol yield is shown in Fig. 3. The study revealed that ethanol yield increases from 3.40g/100ml at 30°C to a maximum of 3.80g/ml at 35°C. At temperatures beyond

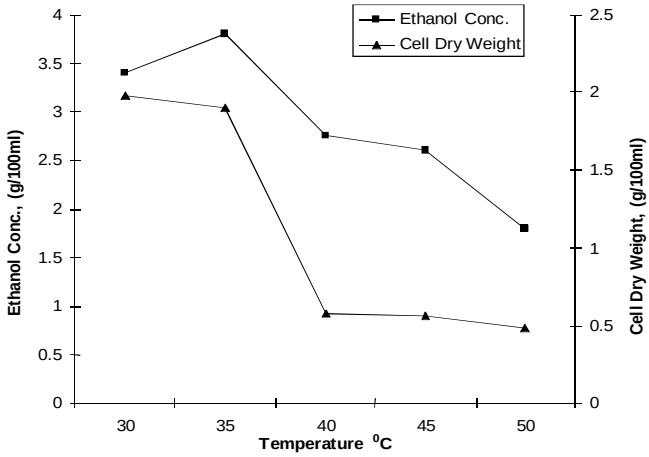

FIG. 3. EFFECT OF TEMPERATURE ON ETHANOL YIELD AND GROWTH (CELL DRY WEIGHT) BY CO-CULTURES OF *A. niger* AND *S. cerevisiae* GROWN ON 8% CASSAVA SUBSTRATE

35°C, there was a reduction in ethanol yield. Therefore, 35°C was chosen as the optimum temperature in subsequent fermentation. Growth of organisms was also maximal at temperatures between 30 and 35°C (1.98g/100ml). Fig. 4 shows the effect of agitation of culture medium during fermentation.

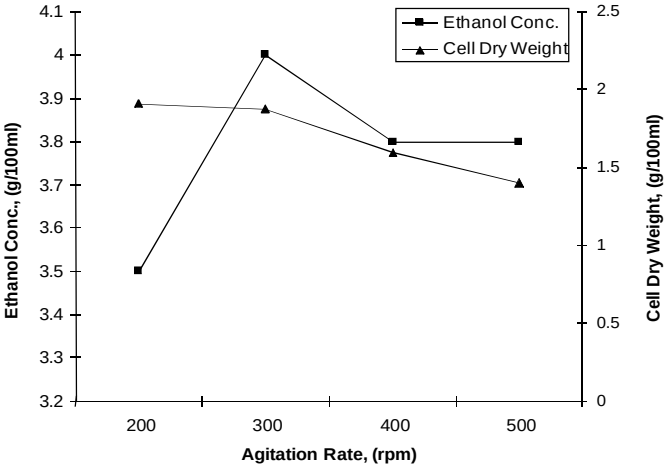

FIG. 4. EFFECT OF AGITATION ON ETHANOL YIELD AND GROWTH (CELL DRY WEIGHT) BY CO-CULTURES OF *A. niger* AND *S. cerevisiae* GROWN ON 8% CASSAVA SUBSTRATE

There was an increase in ethanol yield from 3.50g/100ml obtained at 200rmp to 4.00g/100ml at 300rpm. Agitation rate of 300rpm was then selected for further study. Cell dry weight decreased from 1.91g/100ml to 1.40g/100ml with increase in agitation rate.

Fig. 5 shows the result of the study carried out to determine the effect of length of fermentation on ethanol yield. The optimum fermentation conditions were brought to bear during this fermentation i.e. 8% cassava starch concentration, 35 °C, 300rmp and pH 5.0. Maximum ethanol concentration of 4.30g/100ml was obtained on the 4th day of fermentation. This yield represents 53.8% of the starch utilized and a theoretical yield of 105%.

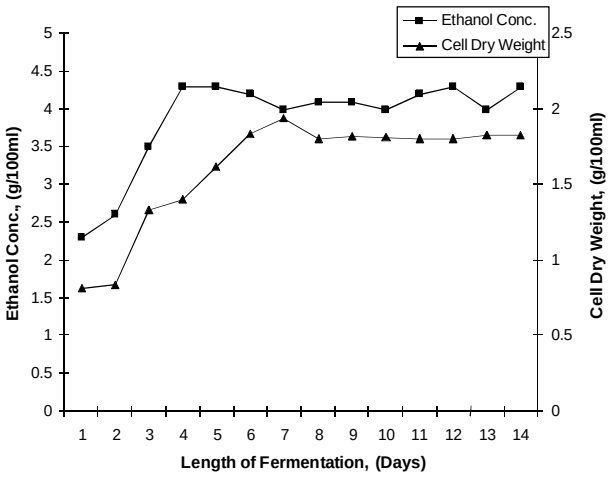

FIG. 5. ETHANOL YIELD AND GROWTH (CELL DRY WEIGHT) BY CO-CULTURES OF *A. niger* AND *S. cerevisiae* GROWN ON 8% CASSAVA SUBSTRATE AT Ph 5, 300rpm, 35°C And 14 Days

DISCUSSION

The result of this findings shows that soil is rich in amylase-producing organisms. This agrees with the study carried out by Adebiyi & Akinyanju (1998) that the soil is known to be a repository of amylase producing organisms. The result also show that simultaneous fermentation of cassava substrate to ethanol by a mixture of starch digesting fungus (*A. niger*) and non-starch-digesting, sugar-fermenting organism such as *Saccharomyces cerevisiae* is feasible (Abouzied & Reddy, 1986).

The efficiency of starch conversion to ethanol was enhanced by optimization of the culture conditions. This is obvious when the maximum theoretical yield was increased from 68.6% (using 1% substrate) to 105% (using 8% substrate). It clearly showed that the proposition for high substrate loading for industrial fermentation is feasible and hence always desired (Nagodawithana *et al*., 1974). It is noteworthy that when the culture condition was optimized, the length of fermentation decreased from 5 days to 4 days. Since decreasing length of fermentation is desirable for ethanol yield as it reduces time input, optimal culture conditions should therefore be maintained during industrial fermentation. As it was observed in the course of the study, increasing agitation rate beyond 300rpm did not increase ethanol yield. Also, an increase in the length of fermentation beyond five days neither enhanced ethanol yield nor cell dry weight.

In conclusion, the result of this study shows that simultaneous saccharification and fermentation of cassava substrate is feasible. It equally revealed the fact that optimization of culture condition could enhance ethanol production from cassava using co-culture technique, thereby increasing the economy, in terms of percentage of starch fermentation to ethanol. It also showed that increasing temperature, pH, agitation rate and length of fermentation beyond certain level neither increase ethanol yield nor cell dry weight (Aysun *et al*., 2007).

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