Bioethanol Production from Pineapple Waste by Solid State Fermentation Method

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ABSTRACT: Production of bioethanol from pineapple waste was investigated using the solid state fermentation method. *Aspergillus niger* was co-cultured with *Saccharomyces cerevisiae* on the substrate to produce saccharification and fermentation activities respectively. The microorganisms were isolated from natural sources; soil and palm-wine respectively. Two highest cellulose-hydrolyzing strains of *A. niger* were selected for the study. Two strains of *Saccharomyces cerevisiae* were isolated from the palm-wine and identified using both colonial morphological studies and various biochemical tests. The strain that showed tolerance to 15 % ethanol concentration was selected for the bio-ethanol production study by co-culturing it with each of the two *A. niger* strains selected. Results of the bioethanol production showed that the fermentation process terminated at 120hr with the highest bioethanol concentration of 11.3 % (v/v) in one combination (FB6 + SC) and 7.0 % (v/v) in the second combination (SW3 + SC). The pH of the substrate dropped from 4.1 to 3.3, and the initial total soluble solids of 9.4 mg/g decreased to 3.9 mg/g at the end of fermentation. In addition, production of reducing sugars peaked at 24 hr of fermentation with 57.8 mg/g, after which it declined steadily to 16.0 mg/g at the end of fermentation. The potential shown by solid state fermentation of pineapple waste for bioethanol production indicated that it can favourably compete with submerged fermentation method commonly used for bio-ethanol fermentation. The use of *A. niger* isolated from natural sources produced good saccharification results, favourably competitive with that observed in commercially sold enzymes used by several workers. This may present a cheaper alternative to the commercially sold enzymes. In commercial production of bioethanol from cellulosic/lignocellulosic materials, solid state fermentation method may require smaller area of space compared to submerged fermentation method.
Keywords: Bioethanol, fermentation, pineapple waste, saccharification, solid state.

INTRODUCTION

Bioethanol has gained its importance, not just as a chemical feedstock, an industrial solvent or a beverage, but in recent years, it is emerging as a fuel option for automobile particularly in countries like United States of America, Brazil and Canada (Qian *et. al*., 2014). Bio-ethanol is a renewable energy source produced mainly by sugar fermentation process.

Bioethanol production processes use energy from renewable sources and in its combustion; there is no net carbon dioxide emission to the atmosphere, thus making bioethanol an environmentally beneficial energy resource. One of the major drivers of bioethanol promotion worldwide is the concern about climate change and the potential of biofuels to reduce the Green House Gas (GHG) emissions (Micic and Jotanovic, 2015). There is a growing global interest in the utilization of bio-ethanol as an alternative to fossil fuels because of its property as the only liquid transportation fuel that does not contribute to the greenhouse gas effect (Anuj *et. al.*, 2007).

Cellulosic sources of bioethanol are mainly agricultural and industrial wastes such as pineapple waste. This is a renewable energy source because the crop/plant sources are regenerated through continuous cropping. Recent research is mainly focused on lignocellulosic materials, being considered the most promising feedstock due to availability and low cost (Qian *et. al*., 2014). The bioconversion of these agricultural and industrial wastes to beneficial products such as bio-ethanol, help to reduce the pollution effect of the wastes on the environment. Ethanol can be produced from several substrates such as starch, lignocelluloses, and different wastes. Lignocellulosic biomass is more preferred than starch or sugar-based crops for production of ethanol, since it does not compete with food and takes care of agricultural and plant residues in an environmentally sustainable process (Gutierrez-Rivera *et. al*., 2011; Ishola and Taherzedah, 2014).

The domestic and industrial processing of pineapple fruits generates a huge amount of 'unusable' waste materials which pose serious environmental problems. It is estimated that Nigeria produces about 1.42 million metric tonnes of pineapple (FAO, 2012), with about 50 % (w/w) total fruit weight reported to constitute the waste in peel, core, stem, crown and leaves (Saravanan *et. al*., 2013). Pineapple waste is reported to be composed as peel cellulose (19.8 %), peel hemi-cellulose (11.7 %), pulp cellulose (14.3 %), pulp hemi-cellulose (22.1 %), pulp lignin (2.3 %), all on dry weight basis (Atul *et. al.*, 2010). These materials show huge potentials in the composition of fermentable sugars.

Bioconversion of biomass into bioethanol is based on two major chemical processes of saccharification and fermentation. The most commonly used method is simultaneous saccharification and fermentation (SSF). *A. niger* is well known to produce enzymes that break down polysaccharides to simple sugars, particularly cellulose (Nekiunaite *et. al*., 2016). *S. cerevisiae* is the most exploited yeast in alcoholic fermentation due to its high ability to produce ethanol, while its ability to utilize simple sugars in solid state fermentation has been employed in dough leavening in baking. The yeast is known to possess high ethanol yielding property and high ethanol tolerance (Tesfaw and Assefa, 2014).

Bioconversion is considered to be a useful measure in the proper management of pineapple waste. The objectives of this study were to isolate *Aspergillus niger* and *Saccharomyces cerevisiae* from natural sources, screen the *A. niger* for hydrolytic ability on pineapple waste, and produce bioethanol from pineapple waste by co-culturing *A. niger* and *S. cerevisiae* in solid state.

MATERIALS AND METHODS

Sample collection and preparation

Fresh samples of pineapple waste were collected aseptically from the fruit processing plant of Fumman Agricultural Products Industries Plc., Ibadan, South West Nigeria. The waste consists of crushed peel and core of pineapple fruits. The waste sample was dried on sterile stainless steel trays inside a hot air oven (Shel Lab 1330FX) at 60 ^{0}C for 5 days, until a constant weight was obtained. The dried waste was blended into fine powder with a Moulinex house-hold blender. 15 g of the dry fine powder was analyzed to determine the pH, reducing sugars, total soluble solids and bio-ethanol content.

Isolation and characterization of microorganisms

The *Aspergillus niger* strains were isolated from soil samples collected within the premises of Fumman Agricultural Products Industries Plc, Ibadan, at three locations-flower beds, solid waste dump site and car park. The soil samples were serially diluted in sterile peptone water to 10^{-6}, and plated out on Potato Dextrose Agar (Lab M) by pour plate method. The petri dishes were incubated at 30 ^{0}C for five days. Colonies with morphological characteristics comparable to documented pictures and descriptions of *A. niger* were further inoculated on freshly prepared sterile plates of PDA for purification by streaking. The two-day old pure cultures were subjected to both macroscopic and microscopic examinations. The mold was identified in the Microbiology laboratory of Fumman Agricultural Products Industries Plc. by comparing the cultural and microscopic characteristics with those of known taxa using the schemes of Domsch and Gams (1970) and Kalwart *et. al*. (1991) as references.

The *Saccharomyces cerevisiae* used in this study was isolated from commercially sold palm-wine. Aliquot of 1 ml of 10^{-8} dilution of the palm-wine was plated on solidified plate of sterile Malt Extract Agar (Lab M) by pour plate method, incubated at 28 ^{0}C for two days. Colonies suspected to be *S. cerevisiae* based on colonial morphology were purified by streaking on fresh sterile plates of Malt Extract Agar.

The pure cultures with colonial and microscopic characteristics similar to described features of *S. cerevisiae* were selected for further identification by sub-culturing on MEA slants. A smear of isolate grown in standard broth containing 20 g glucose, 5 g yeast extract and 10 g peptone in 1000 ml distilled water, was examined microscopically after staining with 5 % Malachite green solution. Sporulation test (Barnett *et al*., 1984), fermentation of glucose test (Barnett *et. al*., 1990), assimilation of carbon sources (Sulieman *et al*., 2015), urea hydrolysis and ethanol tolerance tests (Guimaraes *et. al.,* 2006), were carried out to characterize the isolate. Identification was done using Barnett *et. al.* (2000) and Kurtzman *et. al.* (2011) as references.

Screening of *A. niger* for hydrolytic ability on the substrate

The method of Adenipekun and Fasidi (2005) was modified for this study. 15 g of dried pineapple waste was reconstituted with 45 ml distilled water in 250 ml flasks, sterilized in an autoclave at 121 ^{0}C for 15 mins and inoculated with 1 ml of harvested spore suspension (inoculum size 1.7×10^5 spores/ml) of 3-day old culture of the *A. niger* grown on PDA plates. The inoculation was done in duplicate flasks, incubated at 30 ^{0}C for 7 days. After incubation, 100 ml of sterile distilled water was added to each flask, shaken vigorously and allowed to stand for 1 hr. The content was filtered through No.1 Whatman filter paper, with the supernatant subjected to reducing sugar test using 3, 5- dinitrosalicylic acid (Itelima *et. al*., 2013). Two control flasks were incubated without inoculation and treated for reducing sugars test as well. The two best hydrolyzing isolates of *A. niger* were used for further studies.

Simultaneous Saccharification and Fermentation (SSF)

Fifteen grammes of the dried pineapple waste was weighed into conical flasks and moistened with 45 ml sterile distilled water. The flasks were prepared in duplicates and stoppered with cotton wool. All flasks were sterilized in an autoclave at 121 ^{0}C for 15 mins. Two-day old cultures of the two best hydrolyzing isolates of *A. niger* were used to inoculate the sterilized flasks separately. FB6 inoculum size of 1.64×10^5 spores/ml and SW3 at inoculum size 1.7×10^5 spores/ml were used respectively. The *A. niger* strains were grown in co-culture with the selected *S. cerevisiae* strain (inoculum size 1.9×10^8 cells/ml). One flask was left uninoculated as control experiment. All the flasks were incubated for 7 days at 30 ^{0}C, and samples of each duplicate flasks were taken at 24 hr intervals for analyses of reducing sugars, pH, total soluble solids and ethanol concentration.

Analytical Procedure

Whole sample flasks were taken at 24 hr intervals and 100 ml of sterile distilled water was added, shaken thoroughly, and allowed to stand for 1 hr. The supernatant was filtered through No. 1 Whatman filter paper and used to determine the percentage ethanol content, reducing sugars, pH and total soluble solids. Ethanol was distilled out of the filtrates using rotary evaporator heating at 78 ^{0}C from the method of Itelima *et. al.* (2013). 1 ml of 3, 5-dinitrosalicylic acid was added to 1 ml of the sample. The mixture was boiled for 5 minutes and 10 ml of distilled water added. The absorbance of the treated sample was measured at 540 nm in a Jenway 6400 spectrophotometer. The reducing sugar values were obtained from a glucose standard curve.

Determination of Quantity of Ethanol Produced

The method of Oyeleke and Jibrin (2009) was employed. The distillate was collected over a slow heat at 78 ^{0}C and was determined by measuring the quantity of ethanol produced using a 1000 ml measuring cylinder, and multiplying the volume by the density of ethanol. The quantity of ethanol is then expressed as % of the filtrate.

Determination of Total Soluble Solids

This was done by the refractometry method using a refractometer used in industrial processes.

Aliquots from the filtrates were put on the cell of an Atago Pal 1 digital refractometer, and the direct reading on the digital screen taken after pressing the "Start" button.

Determination of pH

This was done using a Horiba 200 V digital pH meter on a two-point calibration with pH buffers pH 4 and pH 7.

Determination of Reducing Sugars

This was done as reported by Itelima *et. al.* (2013), using the 3, 5-dinitrosalicylic acid method. 1 ml of 3, 5-dinitrosalicylic acid was added to 1 ml of the sample. The mixture was boiled for 5 minutes and 10 ml of distilled water added. Absorbance of samples was measured at 540 nm using Jenway 6400 spectrophotometer.

RESULTS

Cultural, Morphological and Biochemical Characteristics of Isolates

A. niger and *S. cerevisiae* isolated from natural sources were the organisms co-cultured for the fermentation procedure. *A. niger* grew as brownish/black spores producing mycelium on PDA plates. It had septate hyphae, smooth-walled conidiophores, hyaline stipe, and globose conidia with surface bearing phialides.

S. cerevisiae showed white-creamy smooth colonies on the MEA plates. The cells are spherical with visible budding state. The yeast was observed to be positive to sporulation and glucose fermentation tests, while being negative to urea hydrolysis. The strain tolerated 15 % ethanol concentration and assimilated glucose, fructose, sucrose, galactose and raffinose sugars, while it failed to utilize lactose and xylose sugars.

Table 1: *Aspergillus niger* Isolates Cellulose Hydrolytic Ability Test

Isolate Code	Inoculum Load (spores/ml)	Reducing Sugars (mg/g)
Control	0	4
FB1	3.4×10^5	13
FB2	3.4×10^5	12
FB3	3.5×10^5	18
FB4	3.4×10^5	17
FB5	2.9×10^5	16
FB6*	3.2×10^5	33
FB7	2.8×10^5	12
FB8	3.1×10^5	13
SWS1	3.2×10^5	20
SWS2	3.8×10^5	16
SWS3*	2.9×10^5	25
SWS4	3.1×10^5	16
SWS5	3.6×10^5	12
SWS6	3.2×10^5	12
SWS7	2.3×10^5	11
SWS8	2.7×10^5	21
SWS9	3.5×10^5	19
SWS10	3.9×10^5	18
SWS11	2.8×10^5	19
CP1	3.3×10^5	18
CP	2.5×10^5	10

FB – flower bed soil
SWS – solid waste dumpsite soil
CP – car park soil
*Samples selected for the study (with the highest hydrolytic abilities)

Table 2: pH measurement in SSF

ISOLATES	0HR	24HR	48HR	72HR	96HR	120HR	144HR	168HR
FB6 + Sc	4.1	4.0	3.9	3.9	3.7	3.6	3.4	3.3
SW3 + Sc	4.1	4.1	4.0	3.8	3.7	3.5	3.3	3.3

FB6 = *A. niger* obtained from flower bed soil
SW3 = *A. niger* obtained from solid waste dumpsite soil
Sc = *S. cerevisiae* screened for the study.

Table 3: Total Soluble Solids (mg/g) in SSF

ISOLATES COMBINATION	0HR	24HR	48HR	72HR	96HR	120HR	144HR	168HR
FB6 + Sc	9.4	9.2	8.8	6.8	5.3	3.9	3.9	3.9
SW3 + Sc	9.4	9.2	9.0	6.9	5.5	4.0	3.9	3.9

FB6 = *A. niger* obtained from flower bed soil
SW3 = *A. niger* obtained from solid waste dumpsite soil
Sc = *S. cerevisiae* screened for the study

Table 4: Reducing Sugars Measurement (mg/g) in SSF

ISOLATES COMBINATION	0HR	24HR	48HR	72HR	96HR	120HR	144HR	168HR
FB6 + Sc	4.0	57.8	48.4	33.7	23.2	16.0	16.0	16.0
SW3 + Sc	4.0	55.9	50.6	45.3	24.0	18.2	16.2	16.2

FB6 = *A. niger* obtained from flower bed soil
SW3 = *A. niger* obtained from solid waste dumpsite soil
Sc = *S. cerevisiae* screened for the study.

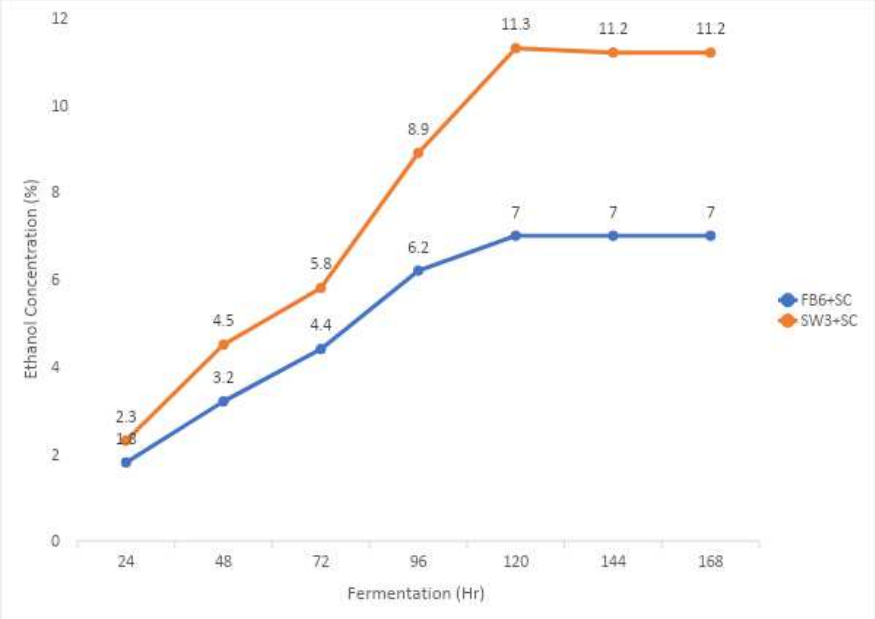

Figure 1: Ethanol Concentration (%) in SSF

Note: FB6+Sc is co-culture of FB6 and *Saccharomyces cerevisiae*
SW3+Sc is co-culture of SW3 and *Saccharomyces cerevisiae.*

DISCUSSION

The strains of the *A. niger* used for the work were isolated from soils from a flower bed and a solid-waste dumpsite. Both strains (FB6 and SW3) displayed good abilities to break down the complex cellulose and hemi-cellulose molecules in the pineapple waste into simple sugars which can be converted into the desired ethanol. The strain FB6 produced 33 mg/g and SW3 generated 25 mg/g of reducing sugars from the dried pineapple waste in the hydrolytic abilities test (Table 1).This might be due to the ability of *Aspergillus niger* to produce cellulase which breaks down the polysaccharide cellulose into simple sugars. The primary uses of *A. niger* are for the production of enzymes such as amylase, amyloglucosidase, cellulases, lactase, invertase and pectinase (Villena and Gutierrez-Correa, 2007; Suganthi *et . al*., 2011). This attribute could have informed the selection of the mold for saccharification purposes in the works of Ado *et. al*. (2009), Oyeleke and Jibrin (2009), and Itelima *et. al*. (2013). The result of this finding shows that soil is rich in cellulase-producing organisms. This agrees with the reports of Adebiyi and Akinyanju (1998) and Ado *et. al*. (2009).

The strain of *S. cerevisiae* used was isolated from palm-wine, which is a naturally fermented beverage. The strain showed strong abilities to ferment glucose which is an attribute that was strongly desirable for the study. Also, the strain showed ethanol tolerance at 15 % concentration. *S. cerevisiae* is the preferred organism for most ethanol fermentation as documented in similar ethanol production studies (Hossain and Fazliny, 2010; Akponah and Akpomie, 2012; and Itelima *et. al.*, 2013). *S. cerevisiae* is commonly used for ethanol fermentation due to its high ethanol yield, high productivity and high ethanol tolerance (Tanaka, 2006).

Decline observed in the pH of samples during fermentation from 4.1 to 3.3 (Table 2), was similarly reported by other workers. The significant drop in the pH may be due to production of yeast catabolites and the release of D-galacturonic acid from pectin in pineapple waste during saccharification process (Hossain and Fazliny, 2010: Tropea *et. al.*, 2014).

However, this observed similarity is regardless of the differences in the fermentation method between this work and those of the other workers.

Reduction in total soluble solids during fermentation was indicative of active utilization of the available sugars (which are the main soluble solids) in the fermenting substrates by the yeast. The initial total soluble solids dropped from 9.4 mg/g to 3.9 mg/g in both fermentation runs. This agrees with the trends reported in submerged fermentation studies by De Prados *et. al.* (2010), Hossain and Fazliny (2010) and Tropea *et. al*. (2014). However, De Prados *et. al*. (2010) and Hossain and Fazliny (2010) recorded higher initial values of total soluble solids (13.4 mg/g and 12.8 mg/g respectively) in the substrates used for fermentation. This could be as a result of presence of extracted juice in the substrates, which is richer in soluble solids.

The production of bio-ethanol in this study terminated on Day 5 (120 hr) in Figure 1, although, the fermentation process extended to Day 7 (168 hr). This agrees with report of Ohgren *et. al.* (2007). However, different fermentation periods have been reported by various workers on lignocellulosic materials (Hossain and Fazliny, 2010; Akponah and Akpomie, 2012; Itelima *et. al*, 2013). Ado *et. al*. (2009), in the study on cassava substrate recorded the highest ethanol yield on Day 5, likewise Akponah and Akpomie (2012) on cassava effluent. However, Hossain and Fazliny (2010), fermented rotten pineapple fruits in 3 days to produce ethanol. A reduced fermentation period could be desirable in considerations of industrial exploitation for production of bio-ethanol.

The trend in reducing sugars production indicated a peak of 57.8 mg/g and 55.9 mg/g at 24 hr of incubation for combinations FB6+Sc and SW3+Sc respectively, and steady drop to 16.0 mg/g until 168 hr (Table 4). This apparently was due to saccharification activities by the *A. niger* ahead of fermentation and led to quick accumulation of reducing sugars before progressive conversion into ethanol by the *S. cerevisiae*. The steady decline in the reducing sugars is in tandem with the report of Itelima *et. al*. (2013). The reduction trend indicated rapid continuous utilization of the reducing sugars evident by the coincidence with the sharp rise in ethanol production by the yeast in Figure 1.

Co-culture of *A. niger* strain FB6 and the *S. cerevisiae* (FB6+Sc) resulted in ethanol concentration of 11.3 % (v/v/). This figure is higher than the maximum ethanol concentration of 5% reported by De Prados *et. al.* (2010), 7.8 % by Hossain and Fazliny (2010), 8.4 % by Itelima *et. al*. (2013) and 3.9 % by Tropea *et. al*. (2014). However, the maximum ethanol concentration of 7.0 % obtained from the co-culture of *A. niger* strain SW3 and the *S.cerevisiae* (SW3+SC) falls within the range reported by these workers.

The findings in this study reveal that solid state fermentation of pineapple waste may produce as much as or more yield of ethanol as submerged state fermentation most commonly used in bio-ethanol production. In addition, a good saccharification activity on pineapple waste was obtained from the two strains of *Aspergillus niger* used, but strain FB6 tends to produce more reducing sugars than strain SW3. The combination of strain FB6 with the *S. cerevisiae* tends to favour higher production of bio-ethanol than the combination of strain SW3 with the *S. cerevisiae*. The encouraging saccharification activities showed by the two strains of *Aspergillus niger* may compete favourably with commercially sold enzymes. De Prados *et. al*. (2010) and Tropea *et. al.* (2014) made use of commercially sold enzymes. Ethanol yield in the entire process of bio-ethanol production depend on the efficiency of the saccharification procedure and fermentation dynamics. A reduced saccharification activity will affect ethanol production because of the limited supply of simple sugars available for conversion into ethanol. Solid state fermentation may reduce that effect on *A. niger* owing to the aerobic condition available for growth.

CONCLUSION

From this work, results revealed that bio-ethanol could be produced from pineapple waste in solid state fermentation by co-culturing *Aspergillus niger* and *Saccharomyces cerevisiae*. This could provide a renewable energy source as well as control environmental pollution, while reducing the large space/surface area required for submerged state fermentation method.

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