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Microbiological and Some Physico-Chemical Changes During Fermentation of Selected Cereals for Bioethanol Production

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Abstract

The use of Bioethanol produced from agricultural products is increasingly becoming a clean source of renewable fuel that can be used to complement petroleum and other fuel sources worldwide. Cereal substrates such as maize, millet and sorghum were fermented for bioethanol production purposes. Bacterial and fungal load for maize range from 4.6 x 10^4 cfu/mL and 1.5 x 10^4 sfu/mL in 0 hr to numerous counts and 9.6 x 10^4 sfu/mL after 96 hours respectively. The bacterial and fungal count for fermented millet substrate in 0 hr is 5.2 x 10^4 cfu/mL and 3.6 x10^6 sfu/mL to too numerous counts (TNC) respectively after 72 hours. Similarly, microbial load for sorghum varies from too numerous counts in 0 hour for both bacterial and fungal organisms to 1.13 x 10^5 cfu/mL and 9.1 x10^6 sfu/mL after 24hrs. Some physiochemical parameters during these processes shows that the pH of the fermented maize substrates decreases gradually from pH 7 in 0 hour to pH 4.1 after 96 hours. The total titratable acidity (TTA) in this regard ranges from low of 1.60 in 0 hour to 11.90 after 96 hours. Similarly, the temperature varies from 26^{0}C in 0 hour and rise gradually to 33^{0}C. This study shows variations in pH, TTA, and temperature of the fermented millet mash and fermented sorghum mash as the time of fermentation increases. The volume of Ethanol produced (mL) from fermented substrate sources determined varied to show the viability of this substrates for biofuel purposes and other clinical uses.

Introduction

Renewable fuels (biofuels) are becoming increasingly important due to heightened concern for the greenhouse effect, depleting oil reserves and rising oil prices (Ohgren, 2007). Bioethanol is one of many types of biofuels. Biofuel is a hydrocarbon that is made from living organisms that humans can use. Fermentation of corn, millet and sorghum harvested from different farms were investigated in this study with regards to pH, bacterial concentration, temperature and titratable acidity. Biofuels are broadly classed into three generations namely; First generation biofuels, Second generation biofuels, and Third generation biofuels (Lin, 2006).

Ethanol is a clear liquid alcohol that is made by the fermentation of different biological materials dated to the ancient age. Energy and environmental issues are the major concerns facing the global community today (Hu *et al*., 2008). Ethanol, chemically known as ethyl alcohol, is a clear, colourless liquid, with an agreeable odor (Bugaje, 2008). Bioethanol can be utilized as oxygenator of gasoline, elevating its, oxygen content, allowing a best oxidation of hydrocarbons and reducing the amount of aromatic compounds and carbon monoxide released into the atmosphere (Cardona and Sanchez, 2007).

Bioethanol is obtained from bioenergy crops and biomass which distinguishes it from that which is produced synthetically from petroleum (Ranola *et al.*, 2009). Different countries use different *et al.,* bioenergy crops such as corn, cassava and sugarcane for bioethanol production. Cassava and sugarcane are used mainly in Nigeria and Brazil (Naylor, 2007). Experts have pointed out that, cassava is the best energy crop for bioethanol production due to its comparative advantages overall known energy crops (Wang, 2002). The molecular conformations of the microbial strains involved were also determined.

This study therefore helps to determine, the production of ethanol from low grade food substrates such as infected maize, sorghum and millet. The bacterial and fungal isolates from food substrates used were also determined.

Materials and Methods

Sample source and materials used

Three different low-grade grains (corn, millet and sorghum) were bought from Ibaka Market, Akungba-Akoko. These were used separately for Ethanol production. Akungba-Akoko is located in Akoko South West Local Government, Ondo State, Nigeria Within longitude 5.44°E and 5.45°E and latitude 7.24°N and 7.28°N (Ologungbede, 2003). Materials and culture media such as nutrient Agar, Eosin Methylene Blue agar (EMB) and Potato dextrose agar used for this study were adequately sterilized.

Procedure for fermentation

Corn, millet and sorghum were washed in sterile distilled water. Three hundred grams of the grains were washed and placed in a clean bowl containing 2 litres of distilled water separately and then grinded. The grinded mash was allowed to ferment for 3 days at room temperature 30 ± 20C (Ojokoh, 2007). Determination of titratable value of fermented grains (Corn, Millet and Sorghum) mash. Twenty milliliter of the sample was dispensed into a conical flask and 2 drops of phenolphthalein indicator was added. The content of the flask was thoroughly mixed in the flask and titrated against 0.1M NAOH. The appearance of a pink colour marked the end point of the reaction (Iland *et al*., 2000). The titrable acidity (TTA) was then calculated using the formula below:

TTA = Average base titre (ml) x molarity of base (mol) x 100

Volume of sample (ml)

Determination of pH of fermented grains [Corn, Millet and Sorghum] mash pH meter was used to measure the pH of fermented samples. This was carried out by first standardizing the pH meter (Hanna multi –parameter –H1-9828) fitted with glass electrodes with buffer solutions and then the pH reading of the sample at 0 hour, 24 hours, 48 hours, and 72 hours (Iland *et al.*, 2000). Determination of the temperature of the fermented grains (Corn, Millet and Sorghum) mash 6. The temperature of each mash was measured with thermometer at 0 hour, 24 hours, 48 hours, and 72 hours (Iland *et al.*, 2000).

Preparation of culture media

Nutrient Agar (NA), which is a general purpose media, Eosin Methylene Blue agar (EMB) was used for the isolation and enumeration of bacteria. Potato Dextrose Agar (PDA) was used for Isolation and cultivation of fungi. All the culture media were prepared according to Manufacturer's action, brought to homogenous solution by allowing the soaking of the agar and was swirled and autoclaving was determined at 121°C for 15 minutes.

Isolation of organisms

Serial dilution was made for each mashed grains during fermentation. From the fermented mash, 1ml was taken aseptically with the aid of sterile syringe and dispensed into 9ml of distilled water in the first tube. Diluents provided in subsequent test tubes were used for the serial dilution. One millimeter from this dilution was drawn aseptically using a sterile into some already labeled Petri dishes. The already prepared media were then poured on the sample in the Petri dish and was swirled gently in circular motion to allow uniform growth and allowed to solidify.

Plates containing the nutrient agar were incubated in an inverted position at 37°C for 24 hours. While plates containing potato dextrose agar was incubated at 27°C for 72 hours. The analyses were done at 0 hours, 24 hours, 48 hours and 72 hours the mashes. All the colonies found were counted manually and then multiplied by the corresponding dilution factor. The colonies formed on the PDA plates were counted as spore forming units per gram of sample (Cowan and Steel, 2014; Buchanan and Gibbons, 2007).

Sub culturing

Microorganisms grow in mixed population with so many species. In order to obtain a pure culture there is need for sub culturing. A pure culture is a population of cell that arises from a single cell. A loop full of a colony that is farther from the other colonies is taken aseptically and spread over the surface of a prepared nutrient agar with a sterile inoculating loop. This reduces the density of the microbial cells. The plates were incubated at 37°C for 24 h on inverted position. The pure isolates were then transferred to agar slant and kept in the refrigerator at 4°C for subsequent observation, test and identification.

Colony Morphology

Colonial examination of all the various isolates were carried out by observing their characteristic growth patterns on the plates which were incubated at 37°C for 24 h and those that were incubated at the temperature of 25°C were examined at 72 h interval (Cowan and Steel, 2014).

Ethanol production

Approach used for production of bioethanol includes enzymes hydrolysis, fermentation and distillation process. In this context the substrates used was washed to remove dirt, dust and other impurities. Furthermore, it was sundried for three days to remove or extract moisture, thus leading to hydrolysis. The resultant sample was milled to enhance enzymatic activities. 20 grams of each substrate were weighed in two separates 500 cm conical flask. One of each set of conical flask acted as control. Sterile distilled water was added to make up the mark and covered aseptically to avoid contamination. The samples were adequately sterilized in an autoclave at 121°C for 15 minutes, allowed to cool and sterile distilled water was aseptically added to make up to mark again. Freshly harvested cells of was inoculated into a set of each substrates mixture *Aspergillus niger* under aseptic condition, while the other set served as control for the three substrates. The flasks were covered and were then incubated at room temperature (28°C) for seven days. The flasks were shaken at interval of 24 h to produce a homogenous solution with organisms in the substrates mixture. The mixtures were separately filtered using no 1 Whatman filter paper after seven days. Supernatant from this hydrolytic sources were transferred into another sets of conical flasks autoclaved at 121°C for 15 minutes and allowed to cool. Freshly harvested cells of was added into the set of *Saccharomyces cerevisiae* hydrolyzed supernatant (20g) for fermentation process. The flasks were corked using cotton wool, shaken and incubated at room temperature for seven days. The flasks were shaken at intervals to produce a homogenous solution and even distribution of the organisms in the substrates mixture.

The fermented liquid was transferred into round bottom flask and placed on a heating mantle fixed to a distillation column enclosed in running tap water. Another flask was foxed to the other end of distillation column to collect the distillate at 78°C (standard temperature for ethanol production). This task was repeated for each of the fermented broth. The distillate collected was measured with the aid of measuring cylinder and quantified as the amount of ethanol produced in g/L.

RESULTS

This study shows some variations in the microbial load of cereal substrates used for bioethanol production purposes. In Table 1, Bacterial and fungal load for maize range from 4.6 x 10^4cfu/ml and 1.5 x 10^4sful/mL in 0 hr to numerous counts and 9.6 x 10^6cfu/ml after 96 hours respectively. The bacterial and fungal count range for fermented millet substrate in 0 hr is 5.2 x 10^4cfu/ml and 3.6 x10^6cfu/ml to too numerous count (TNC) respectively after 72 hours (Table 2). Similarly, microbial load for sorghum during this fermentation processes varies from too numerous counts in 0 hour for both bacterial and fungal organisms. But after 24 hours, the counts are 1.13 x 10^5cfu/ml and 9.1 x10^6 sfu/mL. These decreases gradually till the 72 hour and increase again in 96 hour for bacterial and fungal species respectively (Table 3). The biochemical characterization and identification of these microorganisms are as shown in Table 4.

Some physiochemical parameters during these processes are shown in Tables 5, 6 and 7. In Table 5, the pH of the fermented maize substrates decreases gradually from pH 7 in 0 hour to

pH 4.1 after 96 hours. The total titratable acidity (TTA) ranges from low of 1.60 in 0 hour to 11.90 after 96 hours. Similarly, the temperature varies from 26^{0}C in 0 hour and rise gradually to 33^{0}C.

Table 6 also shows some variations in pH, TTA, and temperature of the fermented millet mash as the time of fermentation increases. At 0 hour of fermentation the pH decreases from 6.87 to 3.51 after 96 hours. There was substantial increase in TTA of the millet mash from 4.8 in 0 hour to 45.7 after 48 hours and later decreases to 20.7 after 96 hours. Temperature also increases from 26^{0}C to 31.6^{0}C in 0 hour and 96 hours respectively. Physiochemical properties determined from fermented sorghum mash were also shown in Table 7, which shows some variations in pH, TTA and temperature as the fermentation time increases. The volume of Ethanol produced (ml) from fermented substrate sources were also determined (Table 8). The physical appearance of sample substrates used is shown in Plates 1 to 3.

FERMENTATION TIME (HOUR)	Microbial load / medium		
	Total bacterial count (cfu/ml)	Fungal count (sfu/ml)	Coliform count (cfu/ml)
0	4.6 x 10^4	1.5 x 10^4	6.0 x 10^3
24	1.21 x 10^5	3.7 x 10^4	3.3 x 10^4
48	TNC	TNC	2.2 x 10^4
72	5.7 x 10^4	1.0 x 10^4	9.0 x 10^3
96	TNC	9.6 x 10^4	1.5 x 10^4

Table 1: Microbial load during fermentation of maize (corn)

KEY: TNC –Too numerous to count

FERMENTATION TIME (HOUR)	Microbial load (cfu/ml) / medium		
	Total bacterial count (cfu/ml)	Fungal count (sfu/ml)	Coliform count (cfu/ml)
0	5.2 x10^4	3.6 x10^6	1.7 x10^4
24	8.9 x10^4	4.8 x10^6	2.2 x10^4
48	TNC	1.7x10^6	9.0 x10^4
72	TNC	TNC	TNC
96	TNC	TNC	TNC

Table 2: Microbial load during fermentation of millet

KEY: TNC---- Too numerous to count**.**

FERMENTATION TIME (HOURS)	Microbial load (cfu/ml) / medium		
	Total bacterial count (cfu/ml)	Fungal count (sfu/ml)	Coliform count (cfu/ml)
0	TNC	TNC	1.4 x10^4
24	1.13 x10^5	9.1 x10^6	1.7 x10^4
48	TNC	1.1 x10^6	TNC
72	9.7 x10^4	1.5 x10^6	7.0 x10^3
96	1.01 x10^5	TNC	5.0 x10^3

Table 3: Microbial load during fermentation of sorghum

KEY TNC—Too numerous to count

Catalase	Motility	Fructose	Mannitol	Sucrose	Glucose	Lactose	H_2S	Starchhydrolysis	Gram's staining	Probable organism
+	-	A	AG	A	A	-	+	-	Positive	*Corynebacterium* spp.
-	-	A	-	-	A	-	-	+	Positive	*Leuconostoc* spp.
+	+	A	-	A	A	-	-	-	Positive	*Bacillus* spp.
+	-	A	A	A	A	-	-	-	Positive	*Lactobacillus* spp.
+	+	A	AG	AG	AG	AG	-	+	Positive	*Lactobacillus plantarum*
+	-	AG	AG	AG	AG	A	+	+	Positive	*Staphylococcus aureus*
+	-	A	-	AG	AG	-	-	+	Positive	*Micrococcus* spp
-	-	AG	-	A	A	-	-	+	Positive	*Leuconostoc* spp

Table 4: Biochemical characteristics of bacterial isolated from the fermentation of sorghum.

KEY + = Positive, - = Negative

AG= Acid and Gas production, A= Acid production without Gas.

FERMENTATION TIME (HOUR)	pH	TOTAL TITRATABLE ACIDITY (g/l)	TEMPERATURE (^{o}C)
0	7.00	1.60	26.00
24	6.10	35.90	28.00
48	6.80	37.80	30.40
72	4.60	34.80	31.20
96	4.10	11.90	33.00

Table 5: pH, Total Titratable Acidity (TTA) and temperature of maize mash during fermentation

FERMENTATION TIME (HOURS)	pH	TOTAL TITRATABLE ACIDITY (g/l)	TEMPERATURE (^{0}C)
0	6.87	4.80	26.00
24	6.25	36.10	28.00
48	5.40	45.70	30.10
72	3.51	45.50	31.00
96	3.51	20.70	31.60

Table 6: pH, Total Titratable Acidity (TTA) and temperature of millet mash during fermentation

FERMENTATION TIME (HOURS)	pH	TOTAL TITRATABLE ACIDITY (g/l)	TEMPERATURE (^{0}C)
0	6.52	1.30	26.00
24	5.58	33.80	28.00
48	5.00	42.60	30.40
72	4.28	35.00	31.20
96	4.01	22.40	31.30

Table 7: pH, Titratable Acidity (TTA) and temperature of sorghum mash during fermentation`

Distillation of substrates

The substrates were distillation after 72 hours of fermentation and the result were gotten as follow:

Substrate sources/Vol (ml)

Period

(Hours)	maize	millet	sorghum
24	20	12	14
48	24	15	18
72	28	20	25
96	30	28	32

Table 8: Volume of Ethanol produced (ml) from fermented substrate sources

Plate 1: Infected sorghum sample source used

Plate 2: Infected millet sample source used

Plate 3: Infected maize (corn) sample source used

DISCUSSION

This study shows the dynamics of various types of bacterial and fungal isolates involved in production of ethanol through fermentation processes (Tables 1 to 3). In Tables 4 to 6, the pH and the total titratable acidity of the fermenting substrates obtained at different fermentation time gives more insight into the fermentative conditions that can enhance ethanol production. There were changes in the pH of the fermenting substrates at different fermenting time. The highest value of pH 7.0, 6.8 and 6.52 were observed at 0hr for maize, millet and sorghum respectively. It reduced within 96hrs to give the least pH of 4.1, 3.51 and 4.01. A further increase and decrease in titratable acidity during fermentation stages were observed. Such a decrease in pH and increase in acidity may be due to microbial activity, this corroborates with the study of Aliya and Geervani, (1981) and Achi, (1990).

Effective inhibition of competing microorganisms appears to depend on achieving numbers of lactic acid bacteria sufficient to decrease the pH rapidly to levels where the growth of the pathogen is prevented. The varying pH and TTA may be attributed to the various types of microorganisms present in the fermented samples (Odunfa and Adeyele, 1985; Odunfa and Oyewole, 1998). This result is correlates with the work of Omemu *et al*., (2007) who found pH during the souring period of *ogi* ranging from 3.69 to 4.62. The low pH observed in the samples studied is also consistent with the report of Adebolu *et al., (*2007) who reported that the low pH confirms the presence of organic acids like lactic acid in cereal products.

The biochemical characterization tests carried out on the isolated microorganisms from the spontaneously fermenting substrates using API 20E kit revealed their probable identity Table 4 as *Corynebacterium* spp., *Leuconostoc* spp., *Lactobacillus plantarum*, *Micrococcus* spp., *Bacillus* spp., *Staphylococcus* spp., *Lactobacillus* spp., *Lactobacillus fermentum* and *Escherichia coli* and fungi species encountered were *Aspergillus niger*, *penicillum* spp., *Aspergillus fumigatus*, *Saccharomyces cerevisiae*, *Aspergillus flavus*, *Fusarium* spp. and *Cladosporium* spp. These organisms have been implicated in the fermentation of grains and tubers as reported by (Ganzle, 2004). The few species of microorganisms in the sample might be due to the antimicrobial properties of *Lactobacillus* spp.

The most predominant organism is *Lactobacillus plantarum* which helps in the souring of the maize. *Corynebacterium* hydrolyses the corn starch to form organic acids while *Saccharomyces cerevisiae* and *Candida* spp. contribute to the flavor development which is in line with the work done by Odunfa and Oyewole, (1998) and Caplice and Fitzgerald, (1999). The presence of these organisms in fermentation of cereals has been reported in previous investigation of (Akinrele, 1970, Odunfa and Oyewole, 1998; Ohenhen, 2002 and Ogunbanwo *et al,.* 2003). The microbial load (cfu/ml) during fermentation processes shows an increase in growth rate of the isolates within 0 hr to 48 hrs of fermentation and later decreases at the later hours of fermentation, this might be as a result of progressive decrease in pH and other microbial activities which create an inhibitory effects on the survival of the microorganisms. This study collaborates with the work of Adegoke and Babalola (2014) who found that low pH decreases microbial growth rate.

The results also showed that the bioethanol produced from maize was more than that produced from millet and sorghum, indicating that there was higher concentration of starch content in maize than millet and sorghum respectively. The quantity of bioethanol produced from maize, corroborates with the study of Oyeleke and Jubrin, (2009) who reported a peak production of bioethanol from maize to be higher than that of sorghum in his experiment when *Aspergillus niger* was used as the enzyme of fermentation. Wang and Liu (2014) study also show related result using *Saccharomyses cerevisiae* on food substrates.

The use of low grade cereal products is a worthwhile venture for bioethanol production; considering their low cost, lesser need in food production and as means of controlling environmental pollution thus making bioethanol production economical and environmentally friendly.

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