Study of Microbial Succession during Bioethanol Production from Waste Corn Cob using Indigenous Organisms

[1]Ifeanyi, V.O., [2]Obasi, N. P. and [3]Ayogu, C.V.
[1]Department of Microbiology, Michael Okpara University of Agriculture Umudike, Abia State.
[2]Aquatic Bioresources Training Centre, Adiabo, National Biotechnology Development Agency, Nigeria.
Corresponding author: chikagoodness@gmail.com; 08032108485

Abstract: The identity, diversity and dynamics of bacterial and fungal communities involved in the production of bioethanol from corn waste (corn cob) were studied. Corn cob was pretreated and hydrolyzed using concentrated H_2SO_4 then, neutralized with NaOH to pH6.5. The corn waste was introduced as source of indigenous organisms and fermentation was done for 7days. The fermented broth was distilled and ethanol yield was measured with a volumetric flask. Microbial succession was examined using standard microbiological procedures and the isolates were identified by molecular identification techniques. The physicochemical analysis such as pH, total suspended solids, total dissolved solids and total reducing sugars were tested during fermentation. Ethanol optimum yield was 10.00g/l and total quantity was 45.00g/l. The indigenous bacteria isolated and identified were *Klebsiella pneumoniae, Lactobacillus casei* and *Escherichia coli*. The indigenous fungi isolated and identified were *Saccharomyces cerevisiae* and *Mucor circinelloides*. There were variations in bacteria found having *Lactobacillus casei* dominating. Among these indigenous organisms, *Saccharomyces cerevisiae* had the ability to withstand the pH and ethanol content. The pH decreased from 5.0-3.0, total suspended solids decreased from 314.50ppm-104.00ppm, total dissolved solids increased from 0.40ppm-650.00ppm. Total reducing sugar was 38.90. This study reveals that indigenous bacteria during the production of bioethanol from corncob died as ethanol production increased while fungi survived in the medium until the sugar diminished. The death of bacteria shows that ethanol is an antimicrobial agent. Hence genetic modification of the bacteria to obtain strains that can withstand these toxic substances yet produce ethanol is recommended.
Key words: Corn cob, fermentation, bioethanol, bacterial succession, fungal succession.

INTRODUCTION

The increasing demand for renewable energy sources has led to the development of new technologies for biofuel production. Microbial biotechnology is one of the technologies that has being largely developed, allowing the development and production of several different biofuels using effluents and wastes as substrates. Through this, the cost of the process is greatly reduced, thereby improving their economic competitiveness and at the same time, reducing the environmental load for waste disposal (Zoppellari and Bardi, 2013).

Corn cob is a cellulosic plant material, source of fermentable sugar with emphasis on non-food lignocellulosic waste products. Production of bioethanol from corn waste can replace bioethanol production from edible food (corn). Corn is produced in large quantity every year in Nigeria. Based on this, large quantities of corn waste (corn cob) are generated yearly. Corn cob form about 30% of corn wastes (Zakpaa *et al.*, 2009). Most of these wastes end up in the environment thereby causing environmental pollution problem (Ado *et al.*, 2009). The corn cobs are burnt as fuel in order to reduce the waste but in the course of burning, greenhouse gases are released to the environment. However, researches have shown that these residues are made up of carbohydrates which could be processed into biofuel such as bioethanol and biogas, or combusted to produce electricity and heat (Soltes, 2000).

Bioethanol is a natural product that is produced by the fermentation of plants containing sugar and starch (Crop Energies AG, 2016).

Ethanol is commonly produced from biological substrates through fermentation processes. In the course of the process, monosaccharides are fermented to ethanol by yeast and bacteria. There are different carbohydrate containing feed stocks that yield monosaccharides for fermentation such as corn grain, sugarcane, wheat, sugar beet and other biomass (Stefan *et al.*, 2009). The fermentation is an anaerobic process in accordance with embden-meyerhoff pathway (EMP) that is catalyzed by enzymes produced by bacteria and fungi (Braide *et al*., 2016).

Microbial succession is a group of microorganisms that occur within a microhabitat. This type of succession is common in recently disturbed communities or newly available habitat. Microbial communities may also change due to products secreted by the bacteria and fungi present. Changes in pH in a habitat could provide ideal conditions for a new species to inhabit the area. In some cases the new species evolving may not out-compete the present ones for nutrients leading to the cell death (Franscisco *et* a*l.*, 2015).

Bacteria are microscopic and simple prokaryotic cells that are found in the environment. They contain a well-developed cell structure that is responsible for many of their unique biological properties. Under optimal growth conditions, bacteria can grow extremely rapidly and can double as quickly as every 10 minutes (Ogbulie and Nwakanma, 2015).

Fungi are well known lignocellulose degraders in toxic conditions due to their oxidative enzymes (Wang *et al.*, 2013). Some fungi such as dried yeast or *Saccharomyces cerevisiae* (Sheoran *et al.*, 1998) have been studied for ethanol production from sugar juices. *S. cerevisiae* is the most attractive choice in fermentation due to its greater efficiency in sugar conversion to alcohol. They also have the ability to produce flocs during growth, making it easier to settle or suspend on need (Kosaric and Velikonja, 1995), and high tolerance to ethanol (Olsson and Hahn-hagerdal, 1993).

The selection of strain for bioethanol production is made based on conditions such as their productivity, tolerance to ethanol, fermentation inhibitors and severe pH and temperature (Cao *et al*., 2014). Therefore, improvement programmes are required in order to obtain alcohol- tolerant strains for fermentation (Gunasekaran and Chandra, 2007). The aim of this research was to study bacterial and fungal succession in bioethanol production from corn cob.

MATERIALS AND METHODS

Source of sample and preparation

Four kilograms (4 kg) of corn cobs was collected using a sterile bag and transported to Cross River University of Technology (CRUTECH), Calabar, Microbiology laboratory for analysis. The corn cob was chopped, dried and milled to a powdered form using a mechanical grinder (Zheng, 2009). The ground corn cob waste was sieved into a bucket and properly covered. The sample was labeled and kept at room temperature.

Isolation, characterization and identification of indigenous organisms

Isolation of indigenous bacteria was carried out using the ground corn cob waste before fermentation, during fermentation and after fermentation for a period of 7 days. One gram of the ground corn cob waste was added into 9 ml of sterile distilled water in a test tube and mixed properly. Ten-fold serial dilutions were carried out and the desired dilutions were plated using pour plate technique. The poured plates were incubated at 30 ^{0}C for 24 hours. Nutrient agar, MacConkey agar and De Man Rogosa and Sharpe (MRS) agar were used. The three different media were prepared and used for pour plating.

Morphological and biochemical tests were carried out to characterize and identify the isolates (Holt, 1994). Molecular identification of the isolates was done using PCR-based 16S rRNA sequences. The 16S rRNA genes of bacterial isolates were

amplified by polymerase chain reaction (PCR) using 16S rRNA primers. Sequences were identified by aligning with sequences in GenBank (Sambrook and Russell, 2001).

Isolation and characterization of fungi

Isolation of indigenous fungi was carried out using the ground corn cob waste. Fungi were isolated before fermentation, during fermentation and after fermentation for a period of 7 days. One gram of the ground corn cob waste was added into 9 ml of sterile distilled water in a test tube and mixed properly. Ten-fold serial dilutions were carried out and the desired dilutions were plated using pour plate technique. Sabouraud Dextrose agar was used for pour plating. The poured plates were incubated at 28 ^{0}C for 72 hours. Fungi were characterized and identified based on macroscopic and microscopic examination (Holt, 1994). The fungal isolates were further identified using molecular identification techniques. Molecular identification of the isolates was done using PCR-based 16S rRNA sequences. The 16S genes of fungal isolates were amplified by polymerase chain reaction (PCR) using 16S primers. Sequences were identified by aligning with sequences in GenBank (Sambrook and Russell, 2001).

Determination of bacterial succession at 24 hours intervals

During isolation, the plates were observed and examined for changes in bacterial growth at 24 hours intervals during the fermentation periods. The different colonies in the media used were picked and sub cultured each day during the fermentation period of 7 days. The different colonies were labeled and examined for identification. The colonies that were seen during the production of ethanol were recorded. The microbial load was also recorded from the different media used.

Determination of Fungal succession at 24 hours intervals

The different colonies on the plate were examined based on shapes (colony form) and sizes. The margin, color and elevation of the colony were noted.

Physicochemical analysis

The solution were analyzed for pH, total suspended solids, total dissolved solids and reducing sugar using Association of Official and Analytical Chemists (1990) method.

The pH meter (JENWAY 4510) was used to check the pH of the fermented broth and also to check the pH of the broth before fermentation. Ten milliliters of the broth of the solution was put into a 50 ml flask and electrodes were dipped into the broth. The function selector was turned from standby to pH. The pH of the solution was read and recorded for each day (Abouzeid and Reddy, 1986). The pH was done in triplicates and the mean recorded. This was done before fermentation and each day during 7 days of fermentation.

Reducing sugar was estimated using a saccharometer (VLB Labo Tech D-13353 Berlin Grad Celsius). It works by determining the density of the fluid. The saccharometer was immersed into 10 ml of the fermented broth that was measured into a conical flask. Once the saccharometer stabilized and stopped bobbling, the reading was taken. The marks correspond to brix scales and there are expressions of the percentage of sugar in the fermented broth (Abouzeid and Reddy, 1986).

Total suspended solids (TSS) and Total dissolved solids (TDS) were estimated using 50 ml of the fermented broth. It was filtered with whatman No1 filter paper which was weighed into a beaker of known weight. The filtered paper and the content of the beaker were dried to at 105 ^{0}C using hot plate. The dried beaker and filtered paper were reweighed and recorded. This was done in triplicates and the mean calculated. The filter paper gave the result for TSS while the beaker gave the result for TDS. This was done each day during the 7 days of fermentation.

Production and quantity determination of bioethanol

The steps involved in the production of bioethanol were pretreatment, hydrolysis, fermentation and distillation. Dry milling was the pretreatment used for corn cob

(Zheng, 2009), 100 g of the ground corn cob was mixed with 1000 ml of water and hydrolyzed using 20 ml of 5% concentrated sulphuric acid (H_2SO_4) in a 2 litre container. Acid hydrolysis was done to remove lignin and breakdown cellulose to glucose (Wyman, 1996). The hydrolyzed samples were neutralized to pH6.5 using 1% of NaOH and the waste was reintroduced into the processed sample. It was covered properly and incubated at 30 ^{0}C (Kuhirun *et al.*, 2009).

After fermentation, the substrate was filtered. The filtrate was distilled at 78 ^{0}C and measured using a measuring cylinder. The quantity of ethanol produced was multiplied by the density and expressed in g/l (Humphrey and Okafoagu, 2007).

RESULTS AND DISCUSSION

Five (5) colonies isolated were labeled based on the media used A_N, D_N (nutrient agar), A_L (MRS), A_M and B_M (MacConkey agar) for identification and the biochemical tests as shown in Table 1. Three of the isolates were identified using molecular identification methods. The isolates were identified as *Lactobacillus casei*, *Klebsiella pneumoniae* and *Escherichia coli* as shown in tables 1 and 3. Two genera of fungi were seen in this research and they are *Saccharomyces cerevisiae* and *Mucor circinelloides* as shown in Tables 2 and 3.

The pH and reducing sugar decreased as fermentation time increased. The change in pH was from 5.0-4.0 and then decreased to 3.0 as shown in Fig 1. Total dissolved solids increased while total suspended solids decreased as fermentation time increased as shown in Fig 2. The production of bioethanol started at 48 hours of fermentation with a yield of 3.00 g/l. the production increased to an optimum of 10.00 g/l at 96 hours then decreased to 7.50 g/l at 168 hours as shown in Figure 3.

Table 1: Morphological and Biochemical characteristics of the bacterial isolates

Morphological and biochemical tests	Isolate A_N on Nutrient agar	Isolate D_N on Nutrient agar	Isolate A_L on MRS agar	Isolate A_M on MacConkey agar	Isolate B_M on MacConkey agar
Macroscopic features	Round and creamy white colony	Mucoid colony	Round creamy white colony	Pink colonies surrounded by bile precipitate	Light pink mucoid colony
Gram reaction	+	-	+	-	-
Shape of cell	Rods	Rods	Rods	Rods	Rods
Motility	-	+	-	+	-
Catalase	-	+	-	+	+
Oxidase	+/-	-	+/-	-	-
Urease	-	-	-	-	+
Coagulase	-	-	-	-	-
Nitrate	-	+	-	+	+
Methyl Red	+	+	+	+	-
Vogues proskauer	+	-	+	-	+
Indole	-	+	-	+	-
Citrate	-	-	-	-	+
Glucose	+	+	+	+	+
Sucrose	+	+/-	+	+/-	+
Lactose	-	+	-	+	+
Maltose	+	-	+	-	+
Probable isolate	A	D	A	D	C

Keynotes (+) positive, (-) negative, (+/-) variable

Table 2: Characterization and Identification of Fungal Isolates

Colony code	Macroscopic features	Microscopic features	Isolate identified
Isolate G	Dry spreading cells occurring singly	Spherical cells with starch grannules and an empty space	*Saccharomces cerevisiae*
Isolate H	White fluffy cotton-wool like mycelium or colonies	Non-septate hphae with sporangiophore. Colonies are raised	*Mucor* specie

Table 3: Molecular identification of bacterial and fungal isolates using DNA sequencing

Isolate Code	Description of Organisms	Accession	Ident
Isolate A	*Lactobacillus casei* strain M14SA5b1 16s ribosomal RNA gene, partial sequence	KY287779.1	92%
Isolate C	*Klebsiella pneumoniae* strain AR 0135 chromosome, complete genome	CP032178.1	99%
Isolate D	*Escherichia coli* BL21 (DE3), complete genome	NC012971.2	100%
Isolate G	*Saccharomyces cerevisiae* strain BY4742 chromosome 11, complete sequence	CP026296.1	100%
Isolate H	*Mucor circinelloides* f.lusitanicus ribosomal RNA gene, partial sequence	AF1134277.1	100%

Table 4: Total reducing sugar (brix level) of the final products

Time (hours)	Reducing sugar
0	8.70
24	7.10
48	6.90
72	5.32
96	4.54
120	2.90
144	2.42
168	1.02
Total	38.90
Mean	4.86

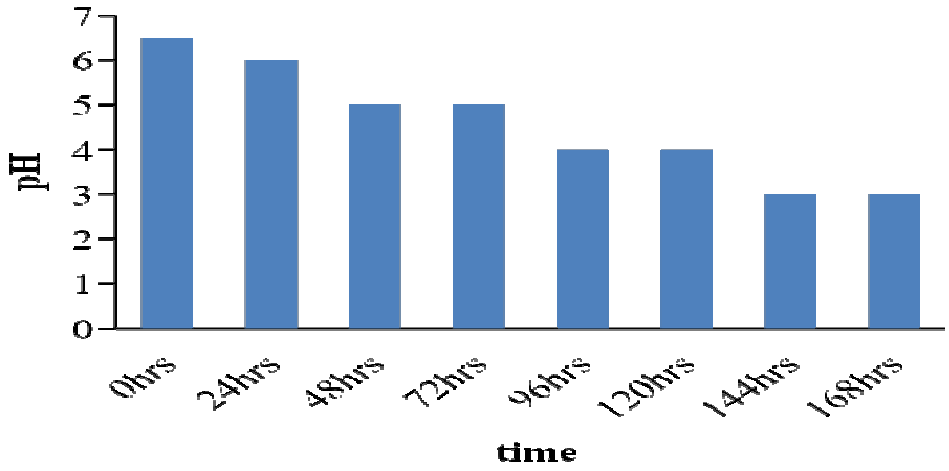

Figure 1: pH of the fermented broth

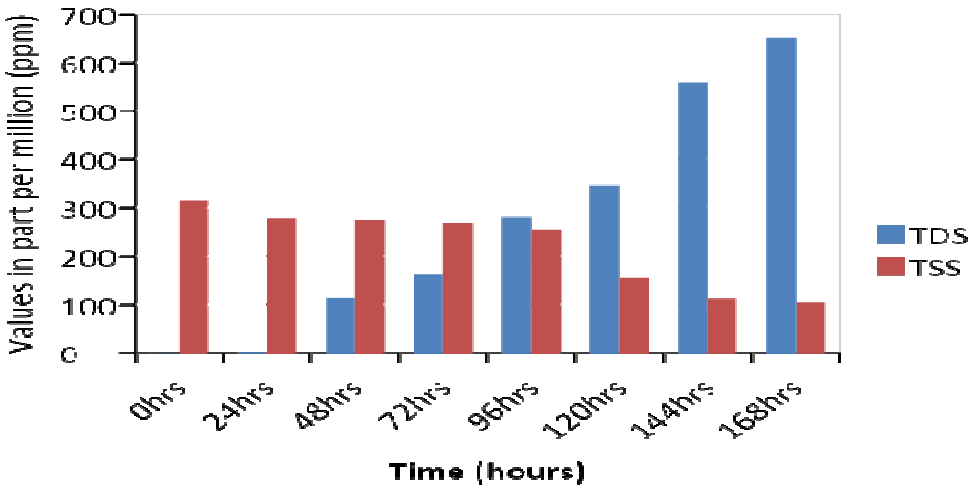

Figure 2: Total dissolved solids (TDS) and Total suspended solids (TSS) of fermented broth.

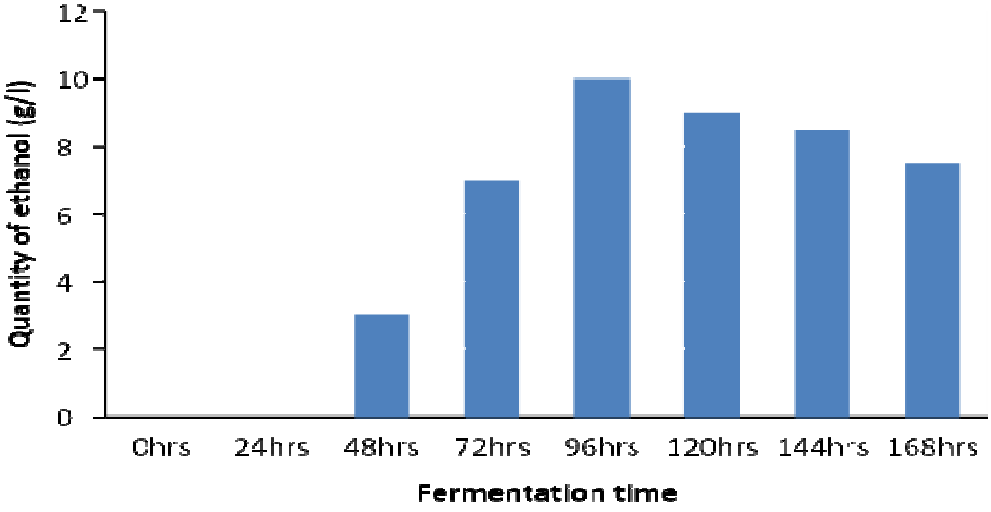

Figure 3: Quantity of ethanol produced

Table 5: Bacterial succession during bioethanol production

Time (hours)	Bacterial succession
0	*Lactobacillus casei, Escherichia coli* and *Klebsiella pneumoniae*
24	*Lactobacillus casei, Escherichia coli* and *Klebsiella pneumoniae*
48	*Lactobacillus casei* and *Escherichia coli*
72	*Lactobacillus casei* and *Escherichia coli*
96	*Lactobacillus casei* and *Escherichia coli*
120	*Lactobacillus casei*
144	-
168	-

Table 6: Fungal succession during bioethanol production

Time (hours)	Fungal succession
0	*Saccharomyces cerevisiae* and *Mucor circinelloides*
24	*Saccharomyces cerevisiae* and *Mucor circinelloides*
48	*Saccharomyces cerevisiae* and *Mucor circinelloides*
72	*Saccharomyces cerevisiae* and *Mucor circinelloides*
96	*Saccharomyces cerevisiae* and *Mucor circinelloides*
120	*Saccharomyces cerevisiae* and *Mucor circinelloides*
144	*Saccharomyces cerevisiae*
168	*Saccharomyces cerevisiae*

A decrease in pH was observed in the fermentation broth as fermentation proceeded for 7 days. This was as a result of fermentation by-products from incomplete oxidation of glucose residues to organic acids such as formic acid and acetic acid according to Michelle (2011). Noe *et al*. (2009) and Michelle (2011) reported pH 4.0 as the optimum pH for ethanol production. The report of Noe e*t al*. (2009) and Michelle (2011) co-relates with the results obtained in this study as the fermentation time increased then, the pH reduced to 3.0. The reduced pH provides an acidic environment that does not support bacterial growth during fermentation rather, it favors the growth of yeast (Braide and Nwaoguikpe, 2011).

Reducing sugar decreased with increase in fermentation time in this work. This shows that bacteria and fungi present are able to utilize the corn cob as energy source according to Uzochukwu *et al*. (1999). The decrease in residual sugar in the fermentation broth is due to the utilization of the sugar as carbon source for the growth, energy, metabolic activities of bacteria and fungi and subsequent ethanol production according to the report of Schugerl (1994).

Total dissolved solids (TDS) of the biodegrading corn cob increased with increase in fermentation time. Total suspended solids (TSS) of the biodegrading corn cob decreased as fermentation time increases. The decrease in total suspended solids observed could be attributed to the flocs formed by yeast cells as the population of fungi increased in this study. This co-relates with the work of Kosaric and Velikonja, 1995.

The bacterial growth observed in this work can be explained using bacterial growth kinetics which includes the lag phase, exponential phase, stationary phase and death phase. At 0 hours, the bacterial count was taken before fermentation. During fermentation, at 24 hours, bacteria took time to adjust in the new environment and this is the lag phase. There was an increase in the growth of bacteria cells indicating that the organisms have used up the nutrients contained in the medium and multiply very fast showing exponential phase. The decrease in bacterial growth observed from

the different media used reveals that nutrients were diminishing therefore growth rate is retarded leading to deceleration phase. Also, the death of bacterial cells observed in the work is as a result of continuous accumulation of toxic metabolites (Dubey, 2012).

The indigenous bacterial isolates are *Klebsiella pneumoniae*, *Lactobacillus casei* and *Escherichia coli*. These genera of bacteria were present since the substrate used is waste material. Among these three bacterial isolates, *Klebsiella pneumoniae* is a bacterium that can produce ethanol according to the report of Dubey, 2012. But because the population of this bacterium was low, they did not survive in the medium for a long period of time as observed in the result above. Also, the bacteria did not survive due to the production of toxic substances like organic acids and ethanol. These metabolites kill bacterial cells and this agrees with the report of Nadir *et al*. (2009) who also observed cell death caused by the toxic metabolites.

Ethanol production started with 2.0 g/l at 48 hours fermentation time. This is due to inadequate growth of bacteria and fungi present during fermentation thereby causing inefficient fermentation (Nadir, *et al*., 2009) leading to low ethanol yield. The results for ethanol yield had a maximum ethanol yield at 96 hours fermentation time. Then, a decrease was observed in this study. The decrease in ethanol yield with increasing fermentation time could be attributed to consumption of ethanol by yeast cells as time passed according to the report of Honsy *et al*. (2016).

Lactobacillus casei was the only species of Lactic acid bacteria in the corn waste that was present during the production of bioethanol. Jay *et al*., (2005) reported that during fermentation, lactic acid bacteria produce lactic acid. This is similar to the result in this study. It is this lactic acid that leads to the observed decrease in pH due to increased counts of lactic acid bacteria and their subsequent production of lactic acid as the end product of metabolism of sugar in the medium (Jay *et al*., 2005).

The slight increase in the population of bacteria can be as a result of an increase in the population of *Saccharomyces cerevisiae* and also due to the symbiotic relationship that exists between yeast and bacteria. This kind of yeast-bacteria interaction has been reported in other products such as kefir and koumis (Roostita and Fleet, 1996).

As ethanol production increased, the population growth of bacteria ceased due to the accumulation of toxic waste products. The reduced pH provides an acidic environment that does not support bacterial growth during fermentation as explained by Dubey, (2002)

Inhibition of enterobacteriaceae that was observed can be attributed to the low pH, high acid and the presence of ethanol. Early growth of yeast as seen in the result caused increase in the population of yeast that reduced the nutrients thereby making it less favorable for other organisms to grow. This could explain why bacteria growth decreased with fermentation time. Yeast also has the ability to hinder the growth of other organisms that exist in the same medium as they produce toxic metabolites (Dien *et al*., 2003).

CONCLUSION

Bioethanol production from corn cob has great potentials due to its widespread availability, abundance and relatively low cost of cellulosic materials. In this article, the indigenous organisms (bacteria and fungi) were able to produce bioethanol. Bacteria that might find themselves in the medium as contaminants, during the production of ethanol will be killed due to the production of toxic substances.

REFERENCES

Abouzeid, M.M. and Reddy, A. (1989). Direct fermentation of potato starch to ethanol by co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae*. *Journal of applied and environmental Microbiology*, 52: 1055-1059.

Ado, S.A., Kachalla, G.U., Tijjani, M.B. and Aliyu, M.S. (2009). Ethanol production from corn cobs by co-cultures of *Saccharomyces cerevisiae* and *Aspergillus niger*. *Journal of Pure and Applied Sciences* 2 (2): 99-101.

AOAC (1990). Official Methods of Analysis. Volume 1 of the AOAC. https://law.resource.org/pub/us/cfr/ibr/aoac.methods.1.1990.pdf

Braide, W. and Nwaoguikpe, R.N. (2011). Ethanol production from cocoyam. Journal of plant physiology and biochemistry, 3(3): 64-66

Braide, W., Kanu, I., Oranusi, U. and Adeleye, S. (2016). Production of Bioethanol from Agricultural Waste. *Journal of Fundamental and Applied Sciences,* p. 372-384.

Cao, L., Tang, X., Zhang, J., Tian, X., Wang, J., and Xiao, W. (2014). Two stage transcriptional reprogramming in *Saccharomyces cerevisiae* for optimizing ethanol production from Xylose. *Metabolic engineering,* 24: 150-159.

Dien, B., Cotta, M. and Jeffries, A. (2003). Bacteria engineered for fuel ethanol production; current status, *Applied Microbiology and Biotechnology,* 63 (3): 258-266.

Dubey, R.C. (1993). Microbial products In: A textbook of Biotechnology. First edition. S.chand and company Ltd, New Delhi, p. 386-397.

Franscisco, D., James, S., Elsas, D., Joana, F. (2015). Disentangling mechanisms that mediate the balance between balance stochastic and deterministic processes in microbial succession. PNAS 112 (11).

Gunasekaran, P. and Chandra K. (2007). Ethanol Fermentation Technology: *Z.mobilis*Madurai kamary University Madurai, India p. 1-22.

Holt, J., Krieg, N., Sneath, P., Stanley J. and Williams, S. (1994) Bergey's manual of systematic bacteriology. 9th ed. Williams and Wilkins Co. Baltimore, Maryland p. 786.

Honsy, M., Abo-state,M., El-Temtany, S. and El-Sheikh, H. (2016). Factors affecting Bioethanol Production from Hydrolysed Bagasse. *International Journal of Advanced Research in Biological Sciences,* 3 (9):130-138. http//www.fao.wanlos II.R IX 5490

Humphrey, C.N. and Okafoagu, U.C. (2007). Optimization of ethanol production from gracinia kola (bitter kola) pulp agro waste. *African Journal of Biotechnology*, 6 (17): 2033-2037.

Jay, M.J., Loessner, M.J., Golden, A.D. (2005). Modern food microbiology 7th edition, Springer science and Business media inc. New York, USA. P. 20.

Kosaric, N. and Velikonja, J. (1995). Liquid and gaseous fuels from biotechnology: challenge and opportunities. *FEMS microbiology Reviews*, 16 (2-3): 111-142.

Kuhirun, M., Punpayak, H., Dumronglot, S. and Kositrup, B. (2009). Report research guideline using cellulose from agricultural waste and agricultural industry for ethanol production.*Office of the National Research Council of Thailand*, p. 57.

Michelle, L.M. (2011). Factors affecting ethanol fermentation via simultaneous saccharification and fermentation. A study to determine the optimal operating conditions to convert cellulosic biomass into ethanol during enzymatic hydrolysis and microbial fermentations. Wolchester polytechnic institute. Retrieved from: https//web.wpi.edu/pubs/E-project/Available/E-project-042811-054328/

Nadir, N., Mel, M., Karim, I. and Yunus, R. (2009). Comparison of sweet sorghum and cassava for ethanol production by using *saccharomyces cerevisiae*. *Journal of Applied sciences,* 9 (17): 3068-3073.

Noe, A.L., Sandi, O., Amparol, Q. and Eladio, B. (2009). Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae*, and their interspecific hybrid. *International Journal of Food Microbiology* 131 (2):120-127.

Ogbulie, T.E. and Nwakanma, C. (2015). Microorganisms and man In Essential Analytical Methods in Biotechnology. WEBSmedia communication, Nigeria, p. 1-28.

Olsson, L. and Hahn-Hagerdal, B. (1993). Fermentative performance of bacteria and yeasts in lignocelluloses hydrolysate. *Process Biochemistry* 28 (4): 249-257.

Roostita, R. and Fleet, G. (1996). Growth of yeast in milk and associated changes to milk composition. *International Journal of Food Microbiology* 31, 205-219.

Sambrook, J., Maccallum, P. and Russell, D. (2001). Molecular Cloning: A Laboratory Manual 3rd. Cold Spring Harbor Press, New York.

Schugerl, K. (1994). Agricultural waste: A source of bulk products. *Journal of Chemical Engineering and Technology*, 17: 291.

Sheoran, A., Yadav, B., Nigam, P. and Singh, D. (1998). Continuous ethanol production from sugarcane molasses using a column reactor of immobilized *Saccharomyces cerevisiae*. *Journal of Basic microbiology*, 38 (20): 123-128.

Soltes, E. (2000). Thermo Chemical routes to chemical fuels and energy from forestry and agricultural residues.4th ed. Plenum Press, New York, pp. 23-47.

Stefan, S., Youngmi, K., Eduardo, X., Nathan, M. and Michael L. (2009). Ethanol Production from Maize. Molecular Genetic Approaches to maize improvement. *Biotechnology in Agriculture and Forestry,* 63: 347-361.

Uzochukwu, S., Balogh, E., Tucknot, O., Lewis, M., Ngoddy, P. (1999). Role of palm wine yeasts and bacteria in palm wine aroma. *Journal of Food Science Technology*, 36:301-304.

Wang, Y., Liu, Q., Yan, L., Gao, Y., Wang, W. (2013). A novel lignin degradation bacterial consortium for efficient pulping. *Bioresource Technology,* 139:113-119.

Wyman, C.E. (1996). Ethanol production from lignocellulosic biomass: overview. In: Wyman, C.E. (Ed.), Handbook on bioethanol: production and utilization. Taylor and Francis, Washington, DC, p. 11-12.

Zakpaa, H.D., Mak-Mensah, E.E. and Johnson, F.S. (2009). Production of bioethanol from corn cobs using *Aspergillus niger* and *Saccharomyces cerevisiae* in simultaneous saccharification and fermentation. *African Journal of Biotechnology*, 8 (13): 3018-3022.

Zheng, Y. (2009). Overview of Biomass Pretreatment for cellulosic ethanol production.*Journal of Agriculture and Biological Engineering,* p. 51-70.

Zheng, Y. (2009). Overview of Biomass Pretreatment for cellulosic ethanol production.*Journal of Agriculture and Biological Engineering,* p. 51-70.

Zoppellari, F. and Bardi, L. (2013). Production of bioethanol from effluents of the diary industry by *Kluyveromyce smarxianus. New Biotechnology,* 30: 607-613.