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Bacterial Succession Studies during Fermentation of African Locust Bean (*Parkia biglobosa*) to Iru Using Molecular Methods

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Research Article

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ABSTRACT

Aims: The identity, diversity and dynamics of the bacterial community involved in the fermentation of African Locust Bean (*Parkia biglobosa)* to "Iru", a protein-rich condiment in Western Nigeria; was studied using the 16S rRNA gene sequence analysis.
Study design: 16S rRNA gene was used to study bacterial succession and diversity in this solid-state fermentation with a view to develop a framework for improving quality control of this important and nutritious solid-state fermentation product and possibly develop starter cultures for commercializing the product.
Place and Duration of Study: Biotechnology Centre of University of Agriculture, Abeokuta, Ogun State, Nigeria and Biological Sciences Department, Florida Atlantic University, Davie Campus, Florida U.S.A., between July 2008 and October 2009.
Methodology: Raw seeds were prepared in the traditional African way by boiling them for 6hr to soften the seed coat; and for another 1hr to soften the cotyledon. The boiled seeds were immediately transferred into a jute-bag and wrapped tightly to prevent heat loss. They were left at ambient temperature to ferment for 72hr. Total Bacterial Community of the seed was obtained by vigorously rinsing seeds in phosphate buffered saline, before boiling and immediately after boiling (0hr), and subsequently at intervals of 24hrs for three days. To compare cultivable phyllotypes and possible non-cultivable bacteria, subsamples of the extracted bacteria were cultured on Tryptic Soy Agar. Total community small subunit (SSU) rRNA was amplified from extracted genomic DNA by Plate wash polymerase chain reaction (for cultured bacteria) and classic PCR directly from seed-buffer extract (uncultured

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bacteria). Genomic DNA was extracted employing a modified protocol of the freeze-thaw and Qiagen DNA extraction methods. Extracted genomic DNA was run on 1% agarose gel to rule out shearing before PCR amplification of the 16S rRNA gene with the 27F and 1492R primer pair. The amplified samples were cloned using TOPO cloning vector and transformed samples were sequenced. Identity of samples were done by aligning samples in Ribosomal database Project and close relatives was identified.

Results: The process was found to be a classic alkaline fermentation (pH 6 – 8.39). Cultivable bacterial populations changed from 120CFU/g at start of fermentation to 1630000000 CFU/g on day 3. The most abundant organism present in the raw African Locust Beans isolates (Clone 1A) had 97% match to *Acinetobacter sp.* Cooked Locust beans isolates (Clone 2A) shared 100% identity to *Bacillus subtilis.* Organisms present *a*t 0 hr, 24 hr and 48 hr of fermentation (Clones 3A, 4A and 5A) proved to have 100% match to *Bacillus anthracis* relatives; *Bacillus cereus; and Bacillus sp*. respectively. *Enterobacter sp*. (99% similarity to Clone 6A) was only detected after 72 hrs; amidst the bacilli. Even less abundant clones were identified as various *Bacillus* phyllotypes. Cultured and non-cultured bacterial phyllotypes in this system clustered similarly and appear to be the same; confirming that *Bacillus* species were primarily responsible for the fermentation products of iru. While the bacterial identity and low diversity index reported here, is not surprising given the resilience of bacterial endospores to boiling; it provides convincing evidence to explore the use of endospores from these cultivable non-pathogenic bacillus strains as starter cultures for the solid state fermentation.

Conclusion: It is concluded from this study that the use of molecular method in the identification of organisms present during African locust beans fermentation to Iru, revealed varieties of organisms that had not been previously reported by culture- dependent methods. The application of these techniques in the field of microbiology allows a better understanding of the ecology of food fermentation.

Keywords: African locust beans; fermentation; bacterial succession; 16S SSU rRNA; plate wash PCR; molecular methods.

1. INTRODUCTION

Fermented foods have a long tradition and historically they have been produced in order to extend the shelf life of raw materials and increase their safety. In their production, microorganisms play a vital and essential role contributing to the improvement of the physiochemical, sensory and safety characteristics of the final products (Cocolin and Ercolini, 2009). The production of fermented African locust beans (Iru) condiments is largely on a traditional small-scale, household basis under highly variable conditions (Odunfa, 1985). In addition, the fermentation is usually carried out in a moist solid state, involving contact with appropriate inocula of assorted microorganisms and is accomplished by the natural temperatures of the tropics. The desired state of fermentation of the condiments is indicated by the formation of mucilage and overtones of ammonia produced as a result of the breakdown of amino acids during the fermentation (Omafuvbe, 1998). The characteristic ammoniacal odour and flavour of condiments enhance the taste of traditional soups and sauces especially the various soups used as accompaniment to the starchy root and tuber diets. Condiments are also known to contribute to the calorie and protein intake (Simmons, 1976; Umoh and Oke, 1974) and are generously added to soups as low-cost meat substitute

by low-income families in parts of Nigeria (Odunfa, 1985). Knowledge of the microorganisms contributing to the characteristic properties of foods during fermentation process is essential in order to allow control of the process through selection of appropriate conditions for the technology and by the use of defined starter culture. The last twenty years have seen a renewed interest in microbial ecology due to advances made in molecular biology such as advent of polymerase chain reaction and DNA sequencing (Cocolin and Ercolini, 2009). These methods help to identify microbial populations; including those that grow in laboratory media, those that did not grow, as well as those that are both active and dormant in the community. This modern tactic comprises the use of molecular methods on isolated strains during the fermentation for their identification and characterization (culture-dependent methods), as well as direct application of molecular biology techniques in order to profile the microbial diversity without the need of cultivation (culture-independent methods). Their application in the field of the microbial ecology of fermented foods has been recently reviewed (Cocolin et al., 2001). The use of culture-independent methods can avoid the biases related to the cultivation of the microorganisms. Since the microorganisms are detected by targeting the DNA and/or the RNA extracted directly from the food sample, problems relating to the lack of growth of some populations, or the need of selective/elective enrichments for the recovery of stressed or injured cells, do not jeopardize the outcome of the investigation

The reason for this project is that DNA technology has shown that major organisms classified by previous researchers are not the true representation of these organisms; they might belong to other groups apart from those specified. It is widely accepted that plate culturing techniques reveal a little portion of the true microbial population in natural ecosystems. The reasons essentially are the inability of detecting novel microorganisms, which might not be cultivable with known existing media, and the inability of recovering known microorganisms which are viable but enter a non-cultivable state (Roszak and Colwell, 1987; Fleet, 1999; Del Mar Lleo et al., 1998, 1999). Some researches have been carried out on the production of fermented condiments-*iru*-from African locust bean (Eka, 1980; Odunfa, 1986). Succession of microorganisms involved during the fermentation of African locust bean seeds using the culture dependent techniques have been studied by many researchers and it has shown that the major microorganisms present are *Bacillus spp.* Oil seeds are generally processed to yield condiments and the microorganisms involved in the fermentation of African locust bean (*Parkia biglobosa*) have been extensively studied but so far, no investigation has been carried out on the diversity studies of fermenting iru using 16S rRNA gene analysis and genetic profiling of the microbial community.

1.1 Main Objective

The main objective of the present study is to evaluate the microbial community of fermented African locust bean seeds (Iru) using 16S rRNA gene analysis and to study bacterial succession and diversity in this solid-state fermentation.

2. MATERIALS AND METHODS

The African locust bean seed (*Parkia biglobosa*) needed for this research was purchased in a local market in Abeokuta and transported to Biological Sciences Laboratory, Florida Atlantic University, Davie campus, Florida, U.S.A.

All the chemicals and enzymes used for the research were supplied by Biotechnology Centre of University of Agriculture, Abeokuta, Ogun State, Nigeria and Biological Sciences Laboratory, Florida Atlantic University.

2.1 Methods

2.1.1 Processing of African locust beans 'Iru'

Traditional method of processing African locust beans to 'Iru' was used and their unit operations were closely monitored especially at the critical control points. The method used by local processor in Abeokuta was employed.

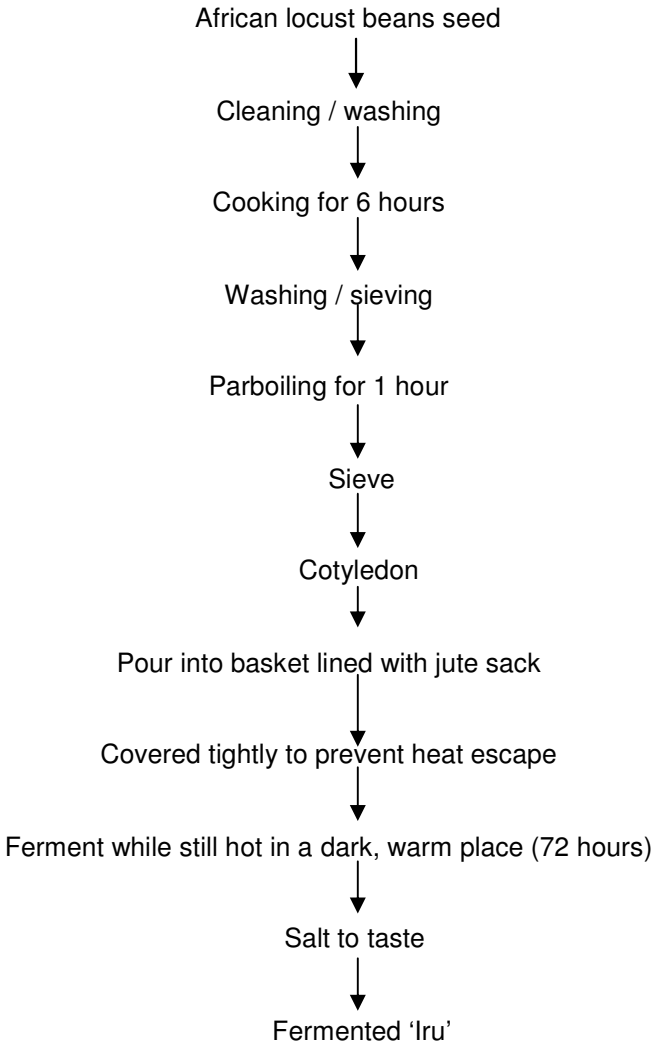

Fig. 1: Traditional processing of fermented African locust beans (Odunfa and Oyewole, 1986)

The raw seeds were cleaned to remove any impurities like shafts and stones, washed in water and pour into a large cooking pot. The seeds were then cooked for about 6 hours until over 50% of the seed coat cracked. The uncracked seeds were pounded gently in a cyclical manner in a mortar to separate the seed coat from the cotyledons. The cotyledons were washed severally and thoroughly in water and then sieved to remove the non cotyledon materials. The cotyledons were parboiled for 45 minutes to one hour. The colour of the cotyledons changed from whitish yellow to light brown at this stage.

Metal sieve was used to remove the cotyledons and the water was allowed to drain before pouring into a basket lined with jute sack and covered tightly immediately to prevent the heat from escaping. The basket was wrapped with several thick clothes and then fermented in a dark, warm and moisture free environment for three days.

At the end of fermentation, the colour of the cotyledons changed to ash white. Salt was added to enhance the taste.

2.2 Isolation of Microorganisms

The African locust beans seed was fermented for three days. At 0h and 24h intervals, 1.5g of fermenting seed was mixed with 13.5ml Phosphate Buffered Saline (PBS) buffer (pH 7.58) to bring out the micro flora. Three fold dilutions were carried out on the mixture, from each of the three samples; 100µl of sample was poured on Tryptic Soy Agar (TSA) and spread out properly on the agar surface. The plates were incubated overnight at 30°C. Representative colonies growing on the plates were streaked on fresh agar to purify the isolates.

2.3 pH Determination

The pH of the raw, cooked seed and at 0h and 24h intervals, pH of fermenting seeds were measured by mixing 1g of sample with 9ml of distilled water.

2.4 DNA Extraction

The Qiagen DNeasy Tissue protocol with slight modification was used for the DNA extraction. The community of all the colonies on each agar plate was taken by rinsing each agar plate with 1000µl of 1 x PBS buffer. The culture from each plate was transferred into a clean micro centrifuge tube and centrifuged for 10 minutes at 7500 rpm. The supernatant was discarded. Pellet in the tubes were resuspended in 180µl Buffer ATL, and five cycles of freezing-thawing was done to bring out the DNA from the cell. Each cycle consist of 5 minutes of freezing and 5 minutes of thawing in a 65^{o}C water bath with gentle agitation and 10 seconds of vortexing at highest setting. First cycle of freezing and thawing was the longest which includes 40 minutes of freezing and 5 minutes of thawing. After the cycles, 20µl proteinase K was added, mixed by vortexing and incubated at 55°C for minimum of 1 hr, until the tissue was completely lysed. After incubation, the tubes were vortex for 15 seconds, and 200µl Buffer AL was added to the samples, mixed thoroughly by vortexing and incubated at 70°C for 10 minutes. It is important that the sample and Buffer AL are mixed immediately and thoroughly to yield a homogeneous solution. 200µl of ethanol was added to the samples and mixed thoroughly by vortexing.

The mixture was pipette into the DNeasy spin column placed in 2ml collection tube and centrifuged at 8000 rpm for 1 minute, flow through was discarded. The spin column was

placed in a new 2ml collection tube and 500µl buffer AW1 was added and centrifuged for 1 minute at 8000 rpm, flow through was discarded. The spin column was placed in a new 2ml collection tube and 500µl buffer AW2 was added and centrifuged for 3 minutes at full speed to dry the DNeasy membrane, flow through and collection tubes were discarded. This centrifugation ensures that no residual ethanol was carried over during the elution stage.

The DNeasy spin column was placed in a clean 1.5 or 2ml micro centrifuge tube and 200µl buffer AE (or nano pure water) was pipette directly into the DNeasy membrane, incubated at room temperature for 1 minute and centrifuged for 1 minute at 8000 rpm to elute. Elution with 100µl increases the final DNA concentration in the eluent, but decreases the overall DNA yield. The extracted DNA in the tubes was kept in the fridge till further analyses.

2.5 Agarose Gel Electrophoresis

The success of the DNA extraction was investigated by running a DNA agarose gel.

2.5.1 Procedure

1g of agarose was added to 100ml 1xTBE buffer in a 250-500ml conical flask. The flask was placed in a microwave for 1 minute to dissolve the agarose and then cooled to about 50℃ (or safe enough to be hand-held). 30µl of 2.5 mg/ml Ethidium bromide (EtBr) solution was added and the cooled agarose was poured into the gel caster with the comb properly placed in the case. The gel was allowed to set at room temperature.

5µl of the gel loading buffer was added to the DNA extract and then mixed. The sample was vortex briefly and the new mixture was placed in the wells of the gel. Marker DNA containing DNA fragments of known sizes was loaded alongside the samples. The order in which they are loaded was recorded. The electrodes were connected to the electric power source and the gel run at 100V for 1 hour. The gel was checked under UV light and recorded accordingly.

2.6 Photographing the Gel

EtBr molecules intercalate with DNA and as the dye fluoresces on exposure to UV, the record of the experiments was kept by taking a picture of the fluorescent bands observed in the agarose gel in the camera that has been fitted with a red filter for UV.

2.6.1 Procedure

The gel was removed from the gel holder and placed on the UV box. The gel was observed from the UV box using protective eyeglasses. Polaroid camera cone was placed over the box and the gel photographed.

2.7 Amplification by Polymerase Chain Reaction

All the culture isolates were amplified using universal bacterial primer. 27F and 1492R with Qiagen Master Mix (Qiagen Inc, Valencia, CA). 20µl of PCR reaction consists of 2 x master mix at concentration of 2.5 units Taq DNA polymerase, 1 x PCR buffer, 200µM dNTP's, and primers at concentration of 0.5µM. The PCR was run for 35 cycles at conditions: 94^0C for 1 min Denaturation; 44℃ for 30 sec annealing; 72℃ for 2 min extension; final extension step

72℃ for 4 min. To validate PCR results, each PCR reaction batch was always carried out with positive and negative controls. Positive control had all PCR reagents and *E. coli* with known genomic DNA conc. Negative control had all reagents except in place of DNA template, nano pure water was used. PCR was done in triplicates. All PCR products were verified by 1% agarose gel made with 1 x TBE (Tris boric-EDTA) stained with ethidium bromide. The gel was run using 10 x loading dye and with Lambda marker at 100 volts for 30 min to verify the presence of DNA.

2.8 Sequencing

All the 96 samples of cloned DNA fragments were sent for sequencing at Macrogen Inc., South Korea.

2.9 Phylogenetic Analysis

All the sequences were submitted to Sequence Match Program and Classifier tool of Ribosomal Database Project (RDP) Version 9.0 (Cole et al., 2007) to find close relatives and taxonomical classification based on Bayesian rRNA classifier. Species which had less than 90% sequence identity with known representatives of the database were classified as unknown species and sequences which had more than 90% sequence identity were classified as known species.

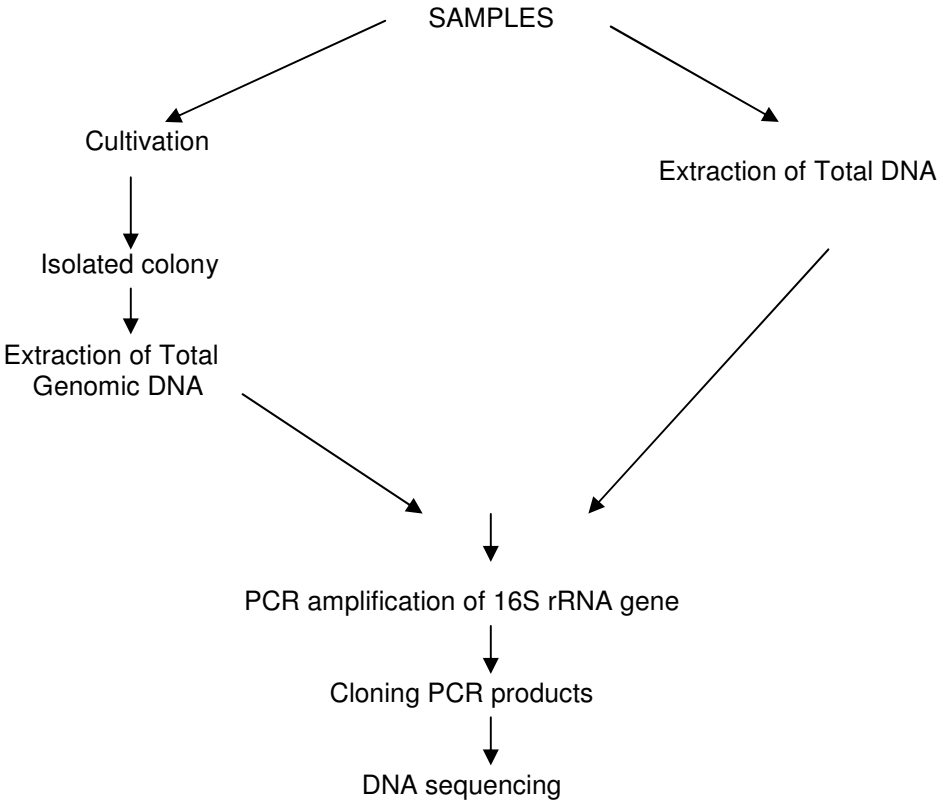

Fig. 2: Molecular diagnosis for bacterial identification

3. RESULTS AND DISCUSSION

3.1 Determination of pH of Fermented Iru Samples

The pH of the raw sample was 6.00, and it increased to 8.39 after 72hr fermentation as shown in Table 1. Also, the colony count of the raw seed was 4.0 x 10^3 CFU/g, and it increased throughout the fermentation to 1.63 x 10^9 CFU/g after 72hr fermentation. The process was found to be a classic alkaline fermentation (pH 6 – 8.39). Bacterial populations changed from 120CFU/g at start of fermentation to 1630000000 CFU/g on day 3. The diversity index also increased dramatically from an apparent monoculture to six operational taxonomic units whose DNA sequence is being analyzed

Table 1: The pH values and microbial counts of Iru samples

Samples	pH	CFU/g
Raw seed	6.00	4.0 x 10^3
0hr fermentation	6.46	1.2 x 10^1
24hr fermentation	6.63	3.59 x 10^4
48hr fermentation	7.57	1.28 x 10^5
72hr fermentation	8.39	1.63 x 10^9

3.2 Genomic DNA and 16s Amplicons from Uncultured Bacterial Community and Cultured Isolates

Amplification of the genomic DNA samples using the F27 and R1492 (F27 Bacteria (8–27) AGAGTTTGATC(A/C)TGGCTCAG & R1492 Bacteria (1492–1513) TACGG(C/T)TACCTTGTTACGACTT) resulted in well defined single bands of about 1450 bp when checked in 1% agarose gel electrophoresis stained with ethidium bromide and viewed with Ultra violet transilluminator (Fig. 3).

3.3 Transformation of *E. coli* with Recombinant Vector

The amplified PCR product was ligated with pCR Blunt TOPO cloning vector as per manufacturer's instructions. Non-transformed cells did not grow on media due to presence of kanamycin. Colonies which grew on the plate were assumed to be transformed. Two to five colonies were picked from each plate.

3.4 Sequencing of the 16s rRNA Gene Fragment

The 16S rRNA gene product for each of the 70 clones of individually Isolated DNA and 26 clones from amplified community DNA was expectedly about 1380 nucleotides long on a gel containing a DNA marker ranging from 350 - 1000 bases. The sequence data was received in a FASTA format.

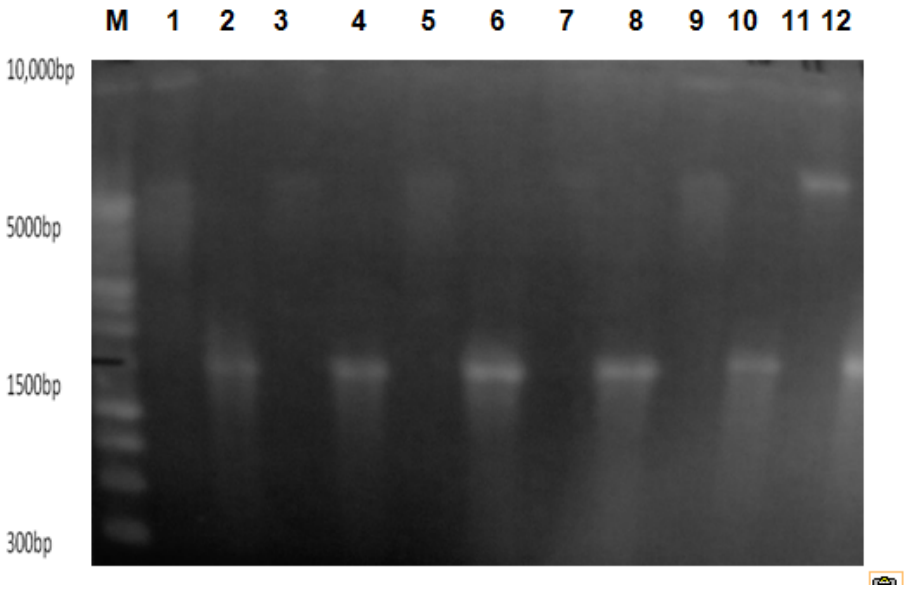

Fig. 3: Bands of genomic DNA and their corresponding 16S amplicons, from total bacterial communities from locust beans at various stages of fermentation.

Legends:
M=Marker (exaACTGene, Fisher BioReagents)
Lane 1= Genomic DNA of Raw Seed; Lane 2= 16S Amplicon of Raw Seed; Lane 3= Genomic DNA at 0hr; Lane 4= 16S Amplicon at 0hr; Lane 5= Genomic DNA at 24hr; Lane 6= 16S Amplicon at 24hr; Lane 7=Genomic DNA at 48hr; Lane 8= 16S Amplicon at 48hr; Lane 9= Genomic DNA at 72hr; Lane 10= 16S Amplicon at 72hr; Lane 11=Genomic DNA of Dawadawa; Lane 12= 16S Amplicon of Dawadawa.

3.5 Identity of Organisms and Closest Match of Community DNA of Iru Based on rRNA Database

All the sequences were aligned and subjected to a BLAST. The first isolate organism present in the raw African Locust Beans (Clone 1A) had 97% match to *Acinetobacter sp.* Cooked Locust beans isolate (Clone 2A) shared 100% identity to *Bacillus subtilis.* At 0 hr fermentation, 24 hr fermentation and 48 hr fermentation (Clones 3A, 4A and 5A) organisms present proved to have 100% match to *Bacillus anthracis relatives; Bacillus cereus; Bacillus sp*. respectively. At 72 hr fermentation (Clone 6A), the organisms were found to have 99% identity to *Enterobacter sp.* The organisms present in the community bacteria DNA of Iru at 24 hr fermentation (clones 4B) had 96% identity to *Bacillus subtilis*. Clone 5B (48 hr fermentation) was found to have 99% identity with *Bacillus sp*.

The diversity and dynamics of the microbial communities during the fermentation of African locust beans (*Parkia biglobosa*) to Iru, protein-rich condiments in Western Nigeria was investigated. Culture independent approach (molecular methods) using 16S rRNA gene was used to study bacterial succession and diversity in this solid-state fermentation. The findings of this study, however, are similar to the report of many researchers that used the biochemical methods in the identification of the microbial community. Some studies have been carried out on the production of fermented condiments -*iru*, from African locust bean

(Eka, 1980; Odunfa, 1986), and soybean (daddawa*)* (Omafuvbe et al., 2000, 2002). Succession of microorganisms involved during the fermentation of African locust bean seeds have been studied by many researchers (Odunfa and Oyewole, 1986; Ogbadu and Okagbue, 1988, Ouoba, 2003a; Oguntoyinbo et al., 2007) and it has been reported that the major microorganism present is *Bacillus subtilis.*

Phylogenetic analysis of the 16S rRNA sequence placed all of the organisms (from cultured and uncultured communities) under the Phyllum Firmicutes, and order Bacillales. The spore-forming species *Bacillus spp*. was identified as the main bacteria present. The amplification of the 16S rRNA gene of fermenting African locust beans (Iru) has proven to be an effective tool for the identification of the microbial community associated with their fermentation. The sequence analysis however revealed that there may be other *Bacillus* species present since all the sequences varied in terms of their similarity coefficients.

4. CONCLUSION

It is concluded from this study that the use of molecular method in the identification of organisms present during African locust beans fermentation to Iru, revealed varieties of *bacilli* that had not been previously reported by culture- dependent methods. Of particular significance was the presence of *Bacillus cereus* which is capable of secreting enterotoxins in these fermented condiments which may indeed constitute consumer hazard by possibly causing flatulence and diarrheal disease in some individuals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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