British Biotechnology Journal
3(2): 213-220, 2013

SCIENCEDOMAIN *international*
www.sciencedomain.org

Diversity of Bacterial Community in Fermentation of African Oil Bean Seeds (*Pentaciethra macrophylla* Benth*)* by comparison of 16S rRNA Gene Fragments

C. N. Opara[1], P. I. Okolie[2*] and S. V. A. Uzochukwu[1]

[1]Department of Food Science and Technology, Federal University of Agriculture (FUNAAB), Abeokuta, Nigeria.
[2]Biotechnology Centre, Federal University of Agriculture (FUNAAB), Abeokuta, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author SVAU designed the study. Author CNO wrote the protocol, and wrote the first draft of the manuscript. Author CNO and PIO managed the analyses of the study. Author CNO managed the literature searches. All authors read and approved the final manuscript.

Research Article

Received 7th November 2012
Accepted 9th March 2013
Published 3rd April 2013

ABSTRACT

Aims: The microbial diversity, fermentation dynamic and the predominant micro-organisms involved in the fermentation of African oil bean (*Pentaciethra macrophylla Benth*) seeds to "Ugba" traditional African food in Eastern Nigeria were investigated by analyzing the microbial community DNA of the food using sequences of their 16S rRNA genes fragment analysis.
Study Design: Universal bacterial conserved 16S rRNA gene region was used to study bacterial dynamics as well as the diversity during fermentation stages. Predominant microorganisms were investigated with the view to establishing the best possible starter culture for the production of high flavoured "Ugba".
Place and Duration of Study: Biotechnology Centre of Federal University of Agriculture, Abeokuta, Ogun State, Nigeria, between January 2007 and May 2009.
Methodology: Raw seeds were boiled for two hours for easy removal of the seed coats. Peeled seed cotyledons were sliced, cooked for 4hrs until softened. Sliced cotyledons were washed, wrapped in local leafs for fermentation for a period of 96hrs. Sampling for analysis was performed, at every 24 hours interval. Bacterial Community of freshly fermenting "Ugba" was obtained by washing seeds at room temperature in 0.40% NaCl salt solution for 15 minutes. The supernatant was used for streaking on both Nutrient agar and "Ugba" agar plates and for Community DNA extraction. DNA

**Corresponding author: Email: piusokolie@yahoo.com;*

extraction was carried out from community DNA extracts and culture isolates grown in LB (Luria – Bertani) broth at 37°C for 24 hours using Promega DNA extraction kit. Partial 16S rRNA genes of isolates DNA and entire microbial community DNA were amplified using 16S rRNA primers. Amplified fragments were cloned using the PCR-TRAP. The transformed clones were sequenced and aligned with reference sequences in the NCBI data base for identification.

Results: This analysis indicated that from community DNA, seventeen clones were identified as *Bacillus subtilis*, Nine as *Bacillus pumilus*, four as *Bacillus licheniformis,* two as *Bacillaceae bacterium*, two as *Bacillus sp* Van 22, and two as *Staphylococcus spp*. Also, of the ten sequenced cloned isolates from the cultural technique, eight were identified as *Bacillus subtilis*, while two sequences were identified as *Bacillus pumilus*. The percentage abundance revealed that *Bacillus subtilis* had the highest abundance of 47.2% followed by *Bacillus pumilus* with 25%.

Conclusion: *Bacillus subtilis* is the predominant species in Ugba fermentation as it had high percentage abundance throughout the fermentation period. This study indicated that molecular analysis of community DNA provides a more accurate picture of diversity and dynamics of microbial communities.

Keywords: Microbial diversity; fermentation dynamic; 'Ugba'; 16S rRNA genes; Community DNA.

1. INTRODUCTION

African oil bean seed is one of the most widely eaten fermented foods in the eastern part of Nigeria. The condiment is taken as a delicacy or added to soups and sauces as flavouring agents, and also as a soup mix and in porridges [1]. Ugba" is a fermented product of African oil bean seed. Previous studies on "Ugba" have concentrated on the microorganisms involved, the biochemical changes that occurred during fermentation and the nutritional quality [2]. These previous studies used cultivation approaches to isolate microorganisms from the fermenting African oil bean seeds, and they identified them based on the physiological properties of the isolates. The lack of strongly informative morphology found in complex microorganisms has precluded the understanding of the community structure at the microbial level in most ecosystems [3].

Furthermore, because cultivation is a prerequisite for examining such properties, most microbes cannot be characterized in natural settings. It therefore becomes impossible to determine total bacterial diversity from an environmental sample. To overcome this problem the use of molecular tools, mainly the analysis of total or partial ribosomal genes (rDNA) or rRNA sequences has become popular [4]. Rapid molecular methods have been developed to analyze diversity within bacterial communities based on direct analysis of DNA in the environment, which avoid potential bias introduced by cultivation of microbes alone [5]. Culture independent techniques have suggested the existence of a vast undiscovered microbial diversity, both in nature and in the intestinal flora [6,7]. Food systems have been shown to be no exception to this diversity, and culture independent analyses have been applied to wine, sausage, cheese, sourdoughs and other foods as reviewed by [8]. DNA extraction methods have been used to detect specific microorganisms either by PCR amplification of partial 16S rDNA with specific primers or by hybridization with 16S or 23S rDNA probes [9]. The cultivation-dependent approaches do not necessarily provide reliable information on the composition of entire microbial communities [10]. It is therefore difficult to assess the significance of cultured microorganisms in microbial ecosystems because of the disparity between culturable and in-situ diversity. Indeed, microbial communities may contain viable but non-culturable bacteria and also bacteria that would be culturable if appropriate medium had been developed. Thus, in order to study interactions between microorganisms, it is

important to study the ecosystem without dissociating it. However, community structures have not been determined using this approach, microbial community diversity was restricted to those taxa that were culturable in vitro. PCR of 16S rDNA from environmental sample and subsequent sequence analysis has facilitated a greater understanding of microbial diversity [11]. Over the last few years, cultivation independent methods have proven to be powerful tool for providing an alternative view of the microbial diversity in food samples [10]. One of the major challenges for microbial ecology is to gain more information below community level, to describe community structures and diversity using molecular based methods [12].

1.1 Main Objective

The Objective of this work is to study microbial diversity and to determine the predominant microorganisms involved in the fermentation of African oil bean by analyzing the microbial Community DNA of the food using sequence analysis of their 16S rRNA gene fragments and to investigate the fermentation dynamic of “Ugba”.

2. MATERIALS AND METHODS

Samples of raw African oil bean seeds used in this research were obtained from local markets Lagos in western Nigeria. The extraction kits, PCR kit, PCR-TRAP cloning kits and chemical were imported from international Biotechnology companies in USA.

2.1 Methods

2.1.1 Preparation of samples

The fermented seed product ugba was traditionally prepared by the method of [13]. The seeds were boiled for two hours for easy removal of the seed coats. Slicing of the cotyledons, cooking until the sliced cotyledons became soft with reduced bitterness, washing and fermenting the cotyledons for a period of four days. Sampling was performed from freshly prepared oil bean (ugba) under aseptic preparation at 24 hours intervals during the fermentation periods.

2.1.2 Sample preparation for extraction of microorganism

The method of [14] was followed for microbial extraction from the African oil bean seeds (ugba). About 5.0grams of ugba was put into a beaker containing 10mls of 0.40% NaCl salt solution. The beaker was shaken with a vortex mixer at room temperature for 15 minutes and the supernatant was used for streaking on agar plates and for community DNA extraction.

2.2 Microbial Isolation

Microorganisms were isolated using streaking techniques. Saline solution containing microorganisms were used for streaking. A loopful of the sample was streaked on nutrient agar and “Ugba” agar plates. This was done in duplicate and the plates were inverted and incubated at 37ºC for 24hours. The plates were examined after incubation and the numbers of colonies per plate were noted.

2.2.1 Bacterial culture

A platinum loop was used to pick up pure colonies. The colony was transferred into sterile tubes containing 5mls of LB broth (Luria-Bertani). Then vortexed briefly to ensure that clump of colonies were dispersed and incubated at 37°C for 24 hours.

2.3 DNA Extraction from Microbial Communities

About 5gms of "Ugba" was washed with 10mls of 0.40% salt (sodium chloride) solution to obtain a suspension of the microorganisms on the "Ugba" slices. The microorganisms washed from "Ugba" were collected via centrifugation for 5 min at 10, 000xg from 5ml of the salt solution containing the microorganisms. DNA extraction was essentially performed in accordance with manufacturer's instruction (Promega DNA extraction kit). DNA yield was determined by measuring the concentration of the DNA in the sample by its absorbance at 260nm and 280nm with a T90 + UV/VIS Spectrophotometer (PG instrument limited). Deionized water was used as diluents and blank.

The concentration was calculated by the formula,

$$\text{Concentration} = \frac{\text{Optical Density}}{\text{Absorbance} \times \text{Conversion factor [15]}}$$

The ratio of the absorbance at 260nm and 280nm (A_{260} / A_{280}) gave the purity of DNA [16].

2.4 PCR Amplification of 16S rRNA Genes of Microbial Community

Forward primer used was 5' AGAGTTGATCCTGGCTCAG 3' and the reverse primer were 5' ACGGCTACCTTGTTACGACTT 3' to amplify the fragments of 16S ribosomal genes. The PCR conditions used were according to the manufacturers' protocol (Promega PCR Mix). The PCR conditions used were initial denaturation at 97°C for 3min followed annealing at 55ºC for 2min and a final extension of 72°C for 3 min. The PCR assay was performed in a PCR Techgen thermal cycler for 30 cycles.
A PCR reaction with all reaction components without DNA template and reaction with all reaction components without primers were used as negative control while DNA marker (1kb) was used as positive control.

2.5 Cloning of PCR products

2.5.1 Ligation

Amplicons derived from PCR of 16S rRNA gene of both community DNA and isolates DNA were used for ligation. 10µl ligation reactions were used, the following were added, dH_2O 5µl, 10x ligation buffer 2µl, Insert-ready PCR-TRAP vector 2µl, PCR product 2µl, T4 DNA ligase 1µl. They were mixed well by finger tipping, spun briefly and legated, allowed to progress overnight at 16°C.

2.5.2 Transformation of *E.Coli.*

The recombinant vectors obtained from ligation process were used to transform competent *E. coli* cells. Colonies containing 16S rRNA insert were screened by picking well separated single white colonies and inoculated onto a minimal medium supplemented with 20% sucrose. Only *E. coli* hosts expressing the clone dextranase gene grew on the medium.

2.6 Sequencing Analysis of Transformant

The 16S rRNA gene sequence of the isolated bacteria (from 151 community DNA and isolates) were determined by direct sequencing using primers Lgh forward 5'-ACAACACCGATAATC-3' and reverse primer Rgh 5'-CAACGAAGCAAGCGCAG-3'and ABI PRISM Big Dye Terminator cycle sequencing (Macrogen labolatory Korea) and an ABI PRISM genetic analyzer. The 16S rRNA gene sequences obtained from the transformed bacteria was compared with those from the NCBI nucleotide sequence database using the BLAST program, in order to identify the microorganism.

3. RESULTS

3.1 DNA Extraction

DNA extracted from microbial community and isolates were colour less and sufficiently pure for PCR amplification. Genomic DNA bands from bacteria community at different stages of fermentation of African oil bean seeds were obtained. DNA bands from agarose gel electrophoresis stained with Ethidium bromide were observed with the aid of UV light.

3.2 PCR Analysis of DNA

The amplification of 16S rRNA gene from community DNA using universal 16S rRNA gene primers resulted in a single well defined bands of about 1500bp when match with the band from the DNA Marker.

3.3 Identification of Bacterial Isolates by 16S rRNA Sequence Analysis

The microorganisms isolated based on the sequence analysis of 16S rRNA gene fragment analyzed from isolates indicated members of the gram-positive bacteria genus *Bacillus*. *Bacillus subtilis* was dominated both with Nutrient agar (NA) and Ugba. Also based on the sequence analysis of the 16S rRNA gene fragments analyzed sequences fell into three lineages of Bacterial domain - *Bacillus, Bacillales* and *Staphylococcaceae.* These organisms were *Bacillus subtilis* (17 clones), *Bacillus pumilus* (9 clones)*, Bacillus licheniformis* (4 clones)*, Bacillus spp VAN 22* (2 clones)*, Bacillaceae bacterium* and *Staphylococcus (*2 clones).There were variations in the clone identity of microorganisms at different stages of fermentation.

3.4 Microbial Profile of the Fermentation

Based on the identity of the most frequently isolated bacteria, microbiological profile of the fermentation was calculated as relative abundance of the different bacteria. Results clearly indicate that *Bacillus subtilis* is the predominant species during the fermentation. Other *Bacillus spp* such as *Bacillus licheniformis* and *Bacillus pimilus* had a much lower relative abundance. Furthermore, *Bacillaceae bacterium* was only detected at the end of the fermentation (72 hours and 96 hours). The *Bacillus VAN 22* was detected at the middle of fermentation and was later displaced. *Staphylococcus spp* was detected at the beginning of the fermentation (24 hours and 48 hours) but disappeared later (Table 1).

Table 1. Microbial identification of microorganisms involved in Ugba fermentation

Clone Identity	24hrs		48hrs		72hrs		96hrs		Gene bank accession number
	C DNA	Isolate	C DNA	Isolate	C DNA	Isolate	C DNA	Isolate	
Bacillus subtillis	2(33%)	1(50%)	4(40%)	1(100)	5(50%)	1(100%)	6(60%)	1(100%)	AY833569
Bacillus sp Van 22	-	-	1(10%)	-	1(10%)	-	-	-	AF286483
Staphylococcus spp	1(17%)	-	1(10%)	-	-	-	-	-	EM86592.1
Bacillus licheniformis	1(17%)	-	1(10%)	-	1(10%)	-	1(10%)	-	AY017347
Bacillus pumilus	2(33%)	1(50%)	2(20%)	-	3(30%)	-	3(30%)	-	AY456263
Bacillaceae bacterium	-	-	-	-	1(10%)	-	1(10%)	-	FM162988

4. DISCUSSION

The fermentation was initiated through natural inoculation. A total of 46 clones of spore forming bacteria were sequenced and BLAST analysis was carried out. The result showed that a low diversity of bacterial groups were present during fermentation of African oil bean seeds to ugba. The anaerobic organisms identified comprised mainly of Gram positive rods and cocci, which are probably facultative anaerobes. After comparison of community profiles and elimination of repeated identity coming from same day of fermentation, the follow organisms were identified *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus spp VAN 22, Bacillaceae Bacterium* and *Staphylococcus spp.* Species diversity was more pronounced in microbial community than in isolates. While from microbial community, six species were isolated (*Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus, Bacillus spp VAN 22, Bacillaceae bacterium* and *Staphylococcus spp.*), in isolates, the species found were only *Bacillus subtilis* and *Bacillus pumilus. Bacillus subtilis* was the most abundant species detected in the clone library of 16S rRNA obtained by PCR amplification of bacteria community DNA and isolates extracted from 24-96 hours fermented Ugba sample. This is in agreement with results using culture techniques where *Bacillus subtillis* had been found dominant during African oil bean fermentation [17]. The consistent dominance of *Bacillus subtillis* in the fermentation process of vegetable condiments in sub-Saharan Africa was confirmed in this study. Also in Nigeria, *Bacillus subtilis* have been reported to be predominantly associated with the fermentation of African locust bean (*parkia biglobosa*) for iru, cotton seed (*Gossypium hirsutum*) for owoh, and *prosopis Africana seeds* for okpehe [18]. Only in a few cases has the development of non culture-dependent molecular techniques been taken into account and exploited to study the microbial compositions of spontaneously fermented condiments, such as the diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soybeans “Kinema” and locust beans “Soumbala” [19]. The dominant microflora associated with “Hawaijar” a traditional fermented soybean, food of Manipur, India has been investigated [20]. In vitro fermentation studies and evaluation of *Bacillus* strains as starter culture for the production of “Okpehe”, traditional African fermented condiments [21] have been carried out. The results reported by these workers showed that *Bacillus subtilis* was detected in their fermentation and are the predominant organism. This is in agreement with the findings of this study with microbial community DNA. The use of the community DNA approach demonstrated the clear succession of microbial communities during the fermentation of African oil bean seeds. The main evidence found was the great impact of *Bacillus subtilis* populations, which were stably present throughout the fermentation period. This study employs for the first time 16S rRNA gene sequence analysis to detect the bacterial diversity present in a traditional fermented African oil

Bean seeds to Ugba. The use of a pair of 16S rRNA primers showed that *Bacillus subtilis* are the main microorganism in the analyzed sample.

5. CONCLUSION

Both community DNA analysis, and analysis of isolates, from fermented samples, indicated that *Bacillus subtilis* is the predominant species in Ugba fermentation as it had high percentage abundance throughout the fermentation period. The results from this study indicated that molecular analysis of community DNA provides a more accurate picture of diversity and dynamics in microbial communities than molecular analysis of isolates. They permit the identification of specific predominant microorganism which can be subsequently used as starter cultures to have a uniform product and to improve the sensory profile.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the support of Biotechnology Centre, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria for providing the enabling facilities for carrying out this research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Abiodun I, Sanni CM, Wilhm HH. Invitro Fermentation Studies for Selection and Evaluation of *Bacillus* Strains as Starter Cultures for the Production of Okpehe, a Traditional African Fermented Condiment. Int. J. Fd. Microbiol. 2006;22:109-123.
2. Ogbulie JN, Ogiehor SI, Isu NR, Njoku HO. Effect of Chemical and Physical Treatments on the Shelf Life for Ugba Fermented African Oil Bean Seed (*Pentaclethra macrophyla Benth*). J. Biotechn. 1988;11:168-178.
3. Garcia-Martinez JA, Agnas SG, Anton AI, Rodriguez-Valeral F. Use of the 16S – 23S Ribosomal Gene Spaces Region in Studies of Protoaryotic Diversity. J. Microbiol. Methods. 1999;36:55-64.
4. Escalante A, Wacher C, Farres A. Lactic Acid Bacterial Diversity in the Traditional Mexican Fermented Dough Pozol as Determined by 16S rDNA Sequence, Analysis. Intern J. Fd. Microbiol. 2001;64:21-31.
5. Randazzo CL, Vaugh EC, Caggia C. Artisanal and Experimental Pecorino Siciliano Cheese: Microbial Dynamics during Manufacture assessed by culturing and PCR DGGE Analysis. Inter. J. Fd Microbiol. 2005;109(1-8):734-740.
6. Amman RI, Ludwig W, Schieifer KH. Phylogenetic Identification and In situ Detection of Individual Microbial Cells without Cultivation. Microb. Rev. 1995;59:143-169.
7. Suau A. Molecular Tools to Investigate Intestinal Bacterial Communities. J. Pediatric Gas and Nutr. 2003;37:223-224.
8. Fleet GH. Microorganism in Food Ecosystem. Int. Fd. Microbiol. 1999;50:101-117.
9. Agerborg A, Reidun D, Martinez I. Saple preparation and DNA extraction Procedures for Polymarase Chain Reaction Identification of *Listeria Monocytogen* in Sea foods. Int. J. Fd. Microbiol. 1997;35:433-452.
10. Duthoit F, Gordon JJ, Montel MC. Bacterial Community Dynamics During Production of Registered Designation of Origin Salers Cheese as Evaluated by 16S rRNA Gene Single stand Conformation Polymorphisms Analysis. Appl. Environ Microbiol. 2003;69:3840-3848.

11. Baker GC, Cowan DA. 16S rDNA Primers and the Unbiased Assessment of Thermophile Diversity. Biochemical Society Transaction. 2004;32(2):218-221.
12. Giraffa G, Neviani E. DNA Based Culture–independent Strategies for Evaluating Microbial Communities in Food Associated Ecosystem. FEMS Microb. Rev. 2001;65:111-120.
13. Enujiugha VN. Development of Ugba Butter from African Oil Bean (*Pentaclethra macrophylla*). Appl. Trop. Agric. 2002;5:89-94.
14. Rolling WFM, Kerker J, Braster M, Apriyantono A, Stam H, Van Verseveld HW. Microorganisms with a Taste for Vanilla: Microbial Ecology of Traditional Indonesian Vanilla Curing. Appl and Environ Microbiol. 2001;1995-2003.
15. Wllfinger WW, Mackey M, Chomcynski. Effect of PH and Ionic Strength on the Spectrophotometer Assessment of Mideic Acid Puntry. Biotechn. 1997;22:474.
16. Brown TA. Gene cloning. Chapman and Hall: 2-6 Boundary Row, London SE1 8HN; 1993.
17. Ogueke CC, Aririatu LE. Microbial and Organoleptic Changes Associated with Ugba Stored at Ambient Temperature. Nig. Fd. J. 2004;22:133-140.
18. Achi OK. Traditional Fermented Protein Condiments in Nigeria. African J. Biotech 2005;13:1612-1614.
19. Sarkar PK, Hasenack B, Nout MJR. Diversity and Functionality of Bacillus and Related Genera Isolated From Spontaneously Fermented Soybeans (Indian Kinema) and Locust Beans (African Soumbala). Inter. J. Fd. Microbiol. 2002;(3):175-186.
20. Jayeram K, Mohendro Singh W, Premarani T, Ranjita Devi A, Selina Chanu K, Talukdar NC, et al. Molecular Identification of Dominant Microflora Associated with “Hawaijar” - A Traditional Fermented Soy-bean (*Glycine max* (L)) Food of Manipur, India. Fd Microbiol. 2008;122(3):259-268.
21. Oguntoyinbo FA, Sanni AI, Franz CM, Holzapfel WH. In vitro Fermentation Studies for Selection and Evaluation of Bacillus Strain as Starter Culture for the Production of “Okpehe”, A Traditional African Fermented Condiment. Inter. J. Fd. Microbiol. 2007;113(2):208-218.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=183&id=11&aid=1202