BIOINFORMATIVE TOOL FOR ACURATE IDENTIFICATION AND PHYLOGENETIC RELATIONSHIP OF BACTERIA COMMUNITY OF FERMENTING AFRICAN OIL BEAN

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ABSTRACT

Alkaline fermentation of African oil bean seed (*Pentaclethra macrophylla*) results into a product called *ugba* which is consumed by over 20 million people in Nigeria. Most works on the fermentation of African oil bean seed into *ugba* have focused on isolation and identification of desirable microorganisms involved in the fermentation process. These organisms were detected using the routine culture-based method with its inherent weaknesses in detecting and resolving the identity of some organisms, especially the viable but unculturable ones. There is no information on the application of molecular and bio-informative tools in the study of microbial community associated with fermenting African oil bean seed. In this study, molecular (cloned library) technique and bio informative tools were deployed to evaluate the microbial ecology of fermenting African oil bean seed. The application of these molecular tools revealed a wider bacterial diversity in the fermented mash of the African oil bean seed. New bacterial species such as *Arthrobacter, Empedobacter, Providencia, Brevibacterium, Elizabethkingia, Acinetobacter, Burkholderiales, Proteobacterium, Watersiella, Dysgomonas, Zymomonas* and *Flavobacterium* species were uniquely identified by the clone library technique. The phylogenetic relationship of these organisms was established in this study.

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Key Words: Bio informative, phylogenetic, bacteria, African oil bean.

Introduction:

Ugba is a food condiment that is derived from alkaline fermentation of African oil bean seed (*Pentaclethra macrophylla*) (Obeta, 1983). It is consumed by the Ibos and other ethnic groups in southern Nigeria as native salad and soup condiment. The microbiota in any fermenting food matrix is a function of the hygienic status of the production environment, the utensil and the raw materials used and the handlers. The traditional fermentation method employed in the processing of *ugba* is by chanced inoculation (Isu and Ofuya, 2000; Sanni *et al*., 2002; Ogueke *et al.*, 2015). The microbial interaction during its production is, therefore, determined by the microbiological status of the raw materials, utensils, handlers and the production environment. These factors vary from one community to the other and from one processor to another. The microbial interplay in the fermenting mash, therefore, may also vary from one processing community to the other and from one processor to another and even from one batch to another.

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The major fermenting microorganisms involved in the fermentation process of the African oil bean seed were identified as proteolytic *Bacillus* species e.g. *B. subtilis* (which is the most predominant), *B. licheniformis, B. megaterium, B. macerans*, and *B. circulans* (Obeta, 1983; Sanni, 1993; Isu and Ofuya, 2000; Sanni *et al*., 2002). The endospores of these bacilli are believed to be associated with the cotyledons of the seed from the beginning of the fermentation process.

Other groups of organisms that have been found to be associated with the fermentation of this condiment include *Escherichia, Proteus, Pediococcus, Micrococcus, Staphylococcus, Streptococcus, Alcaligenes, Pseudomonas, Corynebacterium* and *Enterococcus* species (Odunfa, 1981; Antai and Ibrahim, 1986; Ogbadu and Okagbue, 1988; Njoku and Okemadu, 1989; Ogueke and Aririatu, 2004; Eze *et al*., 2014; Ogbulie *et al*., 2014; Anyanwu *et al*., 2016). *Staphylococcus* and *Micrococcus* species are very active at the early stage of the fermentation process (Ogueke and Aririatu, 2004).

They rapidly multiply in number within 24 h of fermentation and then decrease as fermentation progresses. *Escherichia* species, *Proteus* and *Pediococcus* are generally observed to play a minor role in the fermentation process (Odunfa, 1985a) while *Staphylococcus* and *Micrococcus* species play subsidiary role in the production process (Obeta, 1983; Odunfa and Komolafe, 1989).

Members of the *Enterobacteriaceae* have also been associated with the ecology of fermenting plant proteins (*ugba* inclusive) especially at the early stages of production (Mulyowidarso *et al*., 1989; Achi, 1992). These species do not survive until the end of the fermentation, presumably because of the modified environment (Ogueke and Aririatu, 2004). It is evident that production of this fermented condiment is initially mediated by a diverse microbial flora, which eventually becomes Gram-positive flora (a reflection of many African fermented foods) (Odunfa, 1985b).

The traditional microbiological methods for evaluating the microbial ecology of most food products rely on growth in culture media, followed by isolation and biochemical and serological identification. These routine methods are time consuming and may bias the real species composition of the bacteria population (Fratamico and Bayles, 2005). They often result in disappointing identification results and misidentification as they lack adequate discriminatory power. They are slow and incomplete release of data which, as a consequence, can be used for retrospective evaluation only (Vossen *et al*., 1996). Another significant disadvantage of traditional culture method is their inability to detect viable but non-culturable organisms in food matrix (Davey and Kell, 1996; Gunasekera *et al*., 2002). The deployment of other techniques like DNA sequencing tools in the study of microbiological quality of the fermenting African oil bean seed might be needed to reveal the real extent of microbial diversity associated with its fermentation process.

MATERIALS AND METHODS

Sample collection

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Six market samples of fermented African oil been seed (each of about 500 g), and the unfermented raw seeds were collected from different localities within Lagos, Imo and Abia states in Nigeria.

Microbiological quality assessment of fermented *ugba*

Laboratory preparation of *ugba*

The traditional method of preparing *ugba* as reported by Odunfa and Oyeyiola (1985) was employed in the laboratory preparation of *ugba*, though with slight modification (boiling was done in an autoclave under pressure). The process involved the following; one kilogramme (1kg) of the oil bean seeds were boiled in an autoclave at a temperature of 121^0 C and a pressure of 15 pounds per square inch (psi) for 45 min to soften the hard brown testa (shell). The shells were removed and the kernels washed, drained and rewashed with cold water three (3) times. The washed cotyledons were cut into long thin slices. The slices were soaked in boiled water and allowed to stand overnight. They were washed three (3) times with cold water. These slices were wrapped in small packets with leaves and lightly tied. The small packets were placed in a basket to ferment at room temperature for 3 days to yield '*Ugba*'. *Ugba* purchased from open market served as control.

Microbiological counts

Total viable count, coliform count, *Lactobacillus*, yeast and mould counts were carried out on both the market and laboratory prepared samples. Five grams (5 g) of each sample (OK1-OK6) (OK1 to OK5 were market sample, while OK6 was laboratory sample) were agitated vigorously with 10 ml of phosphate buffered saline for 5 min and centrifuged at 7,500 rpm for 4 min. The supernatants were used as stock solutions. Serial dilutions of stock samples were prepared and cultured on different media. Enumeration of the viable count was on standard plate count agar, coliforms count was on MacConkey agar, *Lactobacillus* count was done using MRS agar while yeast and mould

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count was done using potato dextrose agar**.** Preparation of these media was according to the instructions of the manufacturers (Oxoid). These tests were done following standard microbiological procedure; using poured plate method.

ISOLATION AND IDENTIFICATION OF ISOLATES

Culture-based method

The same stock solutions (OK1-OK6) as used for the enumeration of the various microbiological counts above were used. These stock solutions were serially diluted and plated onto an all-purpose medium - nutrient agar and four different selective media - MacConkey agar, violet red bile agar, *Salmonella/Shigella* agar and *Staphylococcus* agar using the spread plate method. All media were prepared according to manufacturer's (Oxoid) protocol. Colonies with different cultural characteristics were selected and purified on same medium used for isolation, they were characterized and identified.

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Characterization and identification of isolates

Isolates from each sample were characterized based on cultural, morphological and biochemical tests including sugar fermentation. For fermentation tests, 9 sugars were tested at 1 % concentration. Five milliliters of each sugar solution (glucose, sucrose, maltose, mannitol, lactose, fructose, sorbitol, xylose and arabinose) were filter sterilized and inoculated with the isolates individually. Other biochemical tests performed were gram reaction, catalase, oxidase, indole production, methyl red, Voges-Proskauer, urea hydrolysis, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, nitrate reduction, coagulase and citrate utilization. These tests were performed according to standard microbiology protocols as described by Collins and Lyne (1989). Members of *Enterobacteriaceae* were further characterized using the analytical profile index kit (API 20E kit, Biomereux, France), according to manufacturer's specifications.

Molecular (Culture-independent) method

The same samples as used for culture-based isolation and identification of organisms were used. Part of the stock solutions (OK1-OK6) in the culture-based isolation process were dried down to pellets and stored under -20^{0} C until when used for DNA extraction

DNA Extraction

Genomic DNA was extracted from dried supernatants (pellets) of the 6 *ugba* samples (OK1-OK6). Prior to use, pellets were separately suspended in 100 µl of nuclease free water. Genomic DNA was isolated from each sample using QIAmp DNA Mini Kit (Qiagen, USA) according to the manufacturer's protocol. Sixty (60) µl of 20 % SDS was added to each of the sample to lyse the bacteria cells. Five (5) µl of proteinase K was added, votexed briefly and incubated at 60^{0} C in a water bath to precipitate out protein. Five (5) µl of RNase was added and incubated at 37^{0} C for 15 min to digest RNA. Six hundred and sixty (660) µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the mixture centrifuged at 11,500 rpm for 20 min. Four hundred and fifty microliter (450 µl) of supernatant was transferred into another tube and equal volume (450 µl) of chloroform: isoamyl alcohol (24:1) added, the mixture was centrifuged at 11,500 rpm for15 min. Two hundred and ten microliter (210 µl) of ice cold 100 % isopropanol was added to the supernatant and centrifuged at 12,000 rpm for 10 min. Supernatant was decanted and 300 µl of ice cold 70 % ethanol was added and centrifuged at 12,000 rpm for 5 min. Supernatant was decanted, spinned down and 40 µl of 0.1 m of TE buffer was added and isolated DNA was stored at -20^{0} C until used. Deoxyribonucleic acid (DNA) concentration and purity were determined using Nanodrop (ND 1000) Spectrophotometer

PCR Amplification of 16S rDNA

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The 16s rDNA fragment of genomic DNA contains highly conserved primer binding sites and gene sequences with hyper-variable regions that provide specific signature sequences that are useful for identification of bacteria. PCR were carried out on genomic DNA from each sample to amplify genes coding for bacterial 16s rDNA using 16s primer sequence; Forward: 5'CCTACGGGAGGCAGCAG-3'and Reverse 5'-CCGTCAATTCCTTTRAGTTT-3'. .Amplification was performed in a thermal cycler (Eppendorf, Germany) in 25 µl reaction volume consisting of 12.5 µl GoTaq Green Master Mix (Promega), 1.25 µl each of forward and reverse primers (Eurofins genomics), 9 µl water and 1 µl DNA template under the following conditions: denaturation 94^0 C 35 sec, annealing 49^0 C 35 seconds, and extension 72^0 C 1 min for 30 cycles.

Gel electrophoresis

The PCR product was stained with a dye: SYBR green and loaded into 0.8 % agarose gel to check the success of the PCR protocol and to separate the amplicons by size. One hundred (100) bp DNA ladder was loaded as a size marker. Amplified DNA fragments were viewed with UVP Gel Doc-It Imaging system.

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Cloning of amplified 16S fragment of isolated DNA materials

PCR products from each sample were cloned onto pGEM-T Easy Vector (Promega, USA), following the protocol of the manufacturer. This was followed by transformation into JM109 competent *Escherichia coli* cells (Promega). The ligation process is the inserting of the amplified 16S fragment of the isolated DNA material into a vector (pGEM-T Easy Vector). It was done according to the protocol of the manufacturer of the kit and the reaction mixture was incubated at 4^0 C overnight.

The second step in the cloning experiment is the transformation of the DNA insert in a competent *E. coli* cells (JM109 Promega). This was done following the instructions of the manufacturer of the reagents. LB/ampicillin/IPTG/X-Gal plates were prepared following the instruction of the

manufacturer (Promega, USA) The ligation reaction mixture was briefly centrifuged and 2 μl of the mixture was put into a sterile 1.5 ml tube on ice. A control with uncut plasmid was prepared. Competent *E. coli* cells were thawed on ice (~ 5 min) and mixed by gently flicking the tubes. Fifty (50) μl of cells were added to ligation reaction tubes while 100 μl of cells were used for the uncut DNA control tube. The tubes were gently flicked and incubated on ice for 20 min, heat shocked cells for 45-50 sec in a water bath at exactly 42^0 C and immediately returned to ice for 2 min. Nine hundred and fifty (950) μl of SOC medium was added to the transformation reaction tubes while 900 μl was added to uncut control tube and the tubes were incubated for 1.5 h at 37^0 C with shaking (~150 rpm). Eighty-one hundred (80-100) μl of each transformation culture was plated in triplicate on Amp Blue medium. A 1:10 dilution with SOC was used for the uncut DNA control. The plates were incubated at 37^0 C overnight. White colonies indicated positive cloning, while blue colonies indicated negative cloning. White colonies were therefore selected and used for colony PCR

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Colony PCR

This protocol is designed to quickly screen for plasmid inserts directly from *E. coli* colonies. White colonies at the end of the cloning experiment indicated positive result. A total of 384 positive clones were selected (64 clones x 6 samples). Colony PCR was carried out on each clone with same primer pairs used for initial PCR. Each of the 384 white colonies was put into a 50 µl tube containing 30 µl of nuclease free water, 5 µl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1.0 % Triton X 100), 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs (10 mM each dATP, dTTP, dGTP. dCTP), 1 µl of 20 µM forward primer, 1 µl of 20 µM reverse primer and 1 µl of *Taq* polymerase (to

constitute the colony PCR reaction mixture) which was mixed thoroughly. The 64 clones from each sample constituted a clone library for each sample. PCR was performed using 1 μl of each clone mixture (total of 64 clones for each sample). The primers used were same as used in the amplification of the 16s fragment of isolated DNA materials Amplification conditions were also same as stated in the amplification of 16s fragment earlier and were confirmed by gel electrophoresis.

Sequencing of 16S fragments from Colony PCR

Amplified fragments from colony PCR were purified with ExoSAP-IT reagent (Affymetrix, USA). When PCR amplification is completed, any unconsumed dNTPs and primers remaining in the PCR product mixture will interfere with the downstream processing (i.e. sequencing). Post colony PCR reaction product was then purified using exonuclease 1 – shrimp alkaline phosphotase (EXOSAP reagent). The exonuclease 1 removes excess primers while the shrimp alkaline phosphatase removes excess dNTPs. For the purification process, 10 μL of post colony PCR reaction product was mixed with 2 μL of ExoSAP-IT reagent. The mixture was incubated at 37^0 C for 15 min, and further incubation was at 80^0 C for 15 min. The amplicons were diluted to a concentration of between 20-40 ng/μL. Sequencing of forward and reverse strands was performed by Sanger method at Eurofins Genomics (Kentucky, USA). Sequencing primers were same as used for PCR amplification of the DNA fragments

Sequence analysis and bacteria identification

Sequencing data were assembled, trimmed, and quality-checked by using DNA Sequence Assembler v4 (2013), Heracle BioSoft, (www.DnaBaser.com). The resulting sequences were subsequently compared with deposited sequence information in the genebank using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi/). 16S rRNA identification at the genus level was done at ≥97 % identity, and species level, ≥99 % identity. Scores due to poor sequence quality were considered

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as non-interpretable results. Bioedit was used to conduct multiple alignment of derived sequences from above, after which MEGA6 was used to construct phylogenetic trees from sequences derived from each *ugba* sample. The phylogenetic trees were used to infer diversity and abundance of different bacteria in the *ugba* samples.

RESULTS

Microbiological counts

The result of various microbiological counts on fermented African oil bean seed is shown in Table 1. The dominant group of organisms present in the fermented African oil bean seed is bacteria (6.0×10^8 cfu/g for market samples and 5.7×10^7 cfu/g for laboratory prepared sample). *Lactobacillus* count, yeasts and moulds counts and coliform counts were very low (4.2×10^2 cfu/g, 3.4×10^2 cfu/g and 2.1×10^2 cfu/g respectively for the market samples and 3.1×10^2 cfu/g, 2.3×10^2 cfu/g and 1.3×10^2 cfu/g respectively for the laboratory samples).

Isolation and identification of organisms

Culture-based method

Culture-dependent and PCR-clone-based sequence methods were used to evaluate the microbial community of fermented African oil bean seeds. The cultural method revealed diverse but limited number of bacteria species (Table 2).

DNA extraction and PCR

The purity and concentration of extracted DNA materials was determined using Nanodrop spectrophotometer ND 1000 (Table 3). The success of the amplification of the 16S rRNA segment of the isolated DNA materials and the size of amplicons were verified by gel electrophoresis (using

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0.8% agarose, TBE buffer pH 8), as shown in Figure 1. The bands seen indicated positive amplification of the targeted 16S rRNA segment of the isolated DNA materials.

Cloning and Colony PCR

The amplified 16s rRNA segment of the DNA materials were cloned on to (PGMT) vector and transformed in competent *E. coli* cells. Three hundred and sixty-four (364) clones were selected using blue/white cloning technique (Figure 2). White bacteria colonies indicated positive cloning of the target 16s rRNA fragment while blue colonies indicated negative cloning. More than 50 % of cloned 16s fragment were positive. Colony PCR of the cloned target fragment using the 16s rRNA primers confirmed that cloned inserts were target fragments (Figure 3). The bands indicate positive amplification of the cloned segment of' the DNA materials.

Sequence analysis and identification

The base sequences of the 16S rRNA fragments of isolated DNA materials from the 6 *ugba* samples (OK1 – OK6) are shown in Figure3. Phylogenetic tree of identified bacterial species was constructed using the generated DNA sequences from samples (Figures 4). These phylogenetic tree revealed the bacterial diversity and the evolutionary trend among the bacterial isolates in the samples. Organisms in the same clade on the phylogenic trees are closely related. They are assumed to have evolved from the same parent and could even be the same organism or different strains of a particular species. The relative abundance of identified organisms in each sample is shown in (Figures 5, 6, 7, 8, 9 and 10) respectively. The microbial interplay in the samples as revealed by these figures varied from one sample to another.

Table 1:Microbiological counts of market and laboratory samples of *ugba*

Count	Samples from market (cfu/g)	Laboratory samples (cfu/g)

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Total viable count	6.0×10^8	5.7×10^7
Coliform count	2.1×10^4	1.3×10^2
Lactobacillus count	4.2×10^2	3.1×10^2
Yeast & mould count	3.4×10^2	2.2×10^2

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Table 2: Microbial succession during fermentation of African oil bean seed

Isolate	Growth rate (cfu/g)					
	0 h	24 h	48 h	72 h	96 h	120 h
Bacillus subtilis	1.0×10^{3}	1.8×10^{4}	2.6×10^{5}	3.3×10^{6}	3.6×10^{8}	3.2×10^{8}
Bacillus pumilus	8.0×10^{3}	1.2×10^{4}	1.5×10^{5}	2.0×10^{7}	2.5×10^{7}	2.3×10^{8}
Bacillus mycoides	0.3×10^{3}	0.2×10^{4}	0.2×10^{5}	0.3×10^{6}	0.4×10^{8}	0.2×10^{8}
Bacillus megaterium	0.4×10^{3}	0.6×10^{4}	1.1×10^{5}	1.6×10^{6}	2.0×10^{8}	1.8×10^{8}
Bacillus macereus	0.3×10^{3}	0.8×10^{4}	1.0×10^{5}	1.4×10^{6}	1.1×10^{8}	0.9×10^{8}
Bacillus coaglans	0.7×10^{3}	1.0×10^{4}	1.5×10^{5}	2.1×10^{6}	2.4×10^{8}	2.0×10^{8}
Bacillus licheniformis	0.8×10^{3}	0.9×10^{4}	1.6×10^{5}	2.0×10^{6}	2.1×10^{8}	1.5×10^{8}
Bacillus polymaxa	0.8×10^{3}	1.2×10^{4}	1.5×10^{5}	1.9×10^{6}	2.2×10^{8}	1.8×10^{8}
Bacillus circulans	0.2×10^{3}	0.5×10^{4}	0.8×10^{5}	1.2×10^{6}	1.8×10^{8}	1.5×10^{8}
Bacillus laterosporus	0.4×10^{3}	0.3×10^{4}	0.6×10^{5}	o.8 x 10^{6}	0.7×10^{8}	0.7×10^{8}
Bacillus brevis	0.6×10^{3}	0.8×10^{4}	1.4×10^{5}	1.5×10^{6}	1.2×10^{8}	1.0×10^{8}
Corynebacterium sp.	0.5×10^{3}	0.3×10^{3}	0.2×10^{3}	0.2×10^{3}	-	-
Micrococcus sp.	0.6×10^{3}	0.4×10^{3}	0.3×10^{3}	0.2×10^{3}	-	-
Staphylococcus sp.	0.4×10^{3}	0.5×10^{4}	0.4×10^{3}	0.3×10^{3}	-	-
Alcaligenes sp.	0.3×10^{3}	0.2×10^{3}	-	-	-	-
Pseudomonas sp.	0.2×10^{3}	0.2×10^{3}	-	-	-	-

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Table 3: Purity and concentration of DNA extracts from *ugba* samples (260/280 ng/μL)

Sample ID	DNA concentration (ng/ml)	Purity (ng/ μL)
OK 1	26.4	2.01
OK2	22.4	2.12
OK3	22.7	2.19
OK4	21.9	2.17
OK5	61.9	2.15
OK6	117.8	2.16

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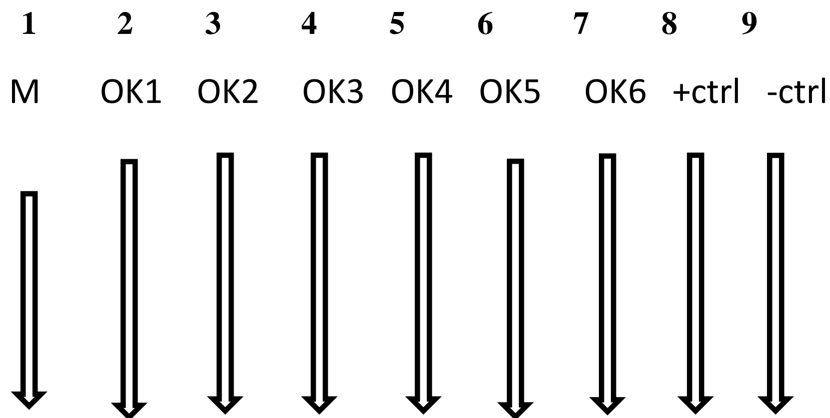

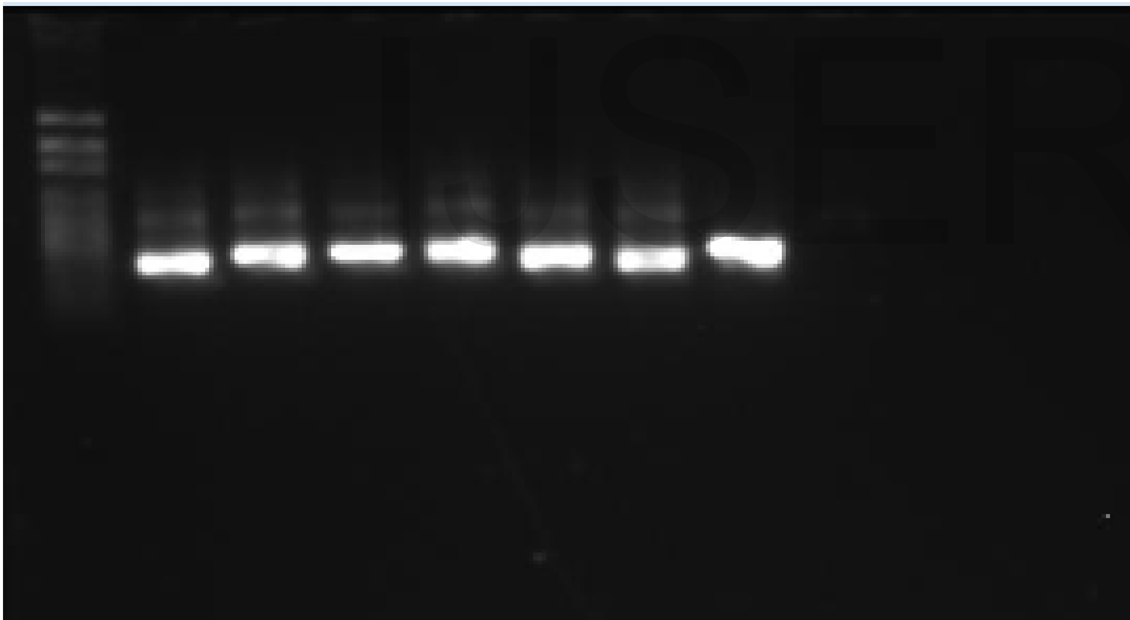

Figure 1: Gel electrophoretogram of PCR amplicons of 6 *ugba* samples (OK1-OK6) using 16S rRNA primers. Lane 1 = Marker, Lanes 2-7 = OK1-OK6 samples, lane 8 = +ve control, Lane 9 = -ve control

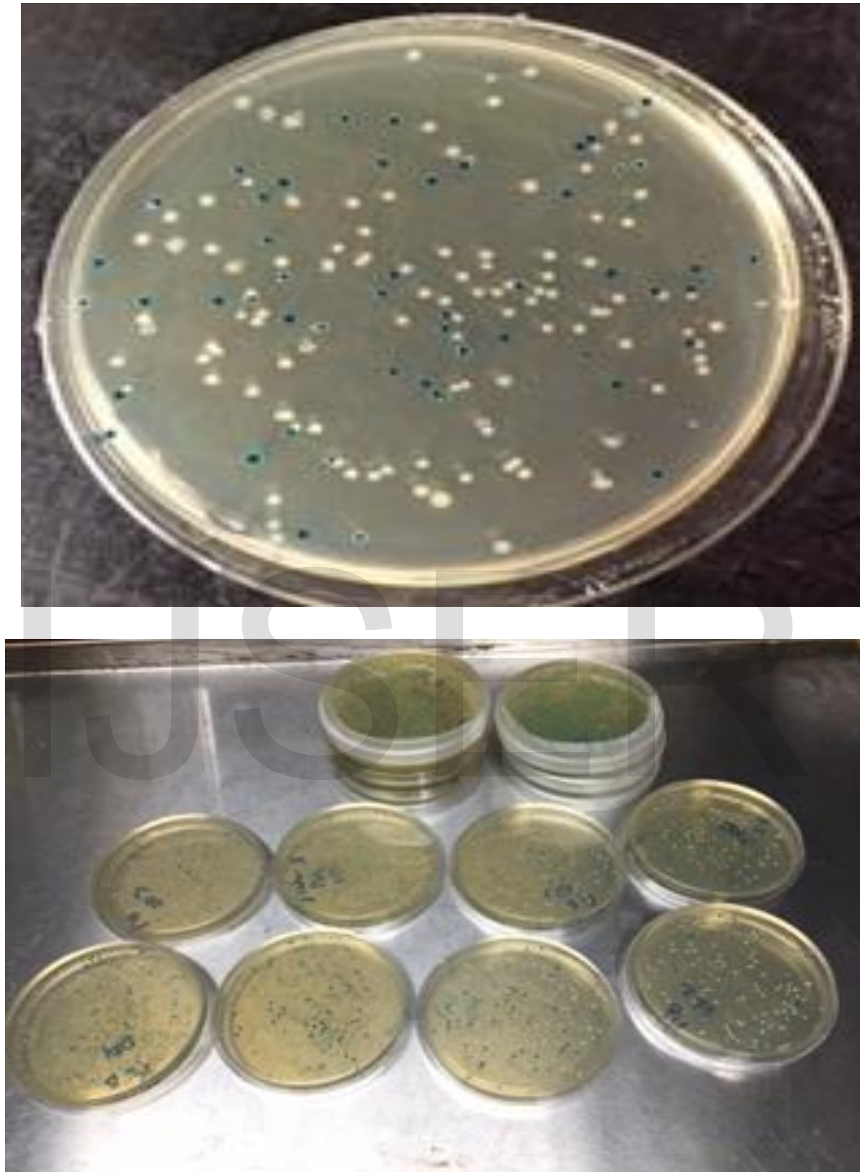

Figure 2: Blue/white cloning of PCR products from six *ugba* samples (OK1-OK6) transformation of *E. coli* cell. White colonies indicate positive cloning, while blue colonies indicate negative cloning

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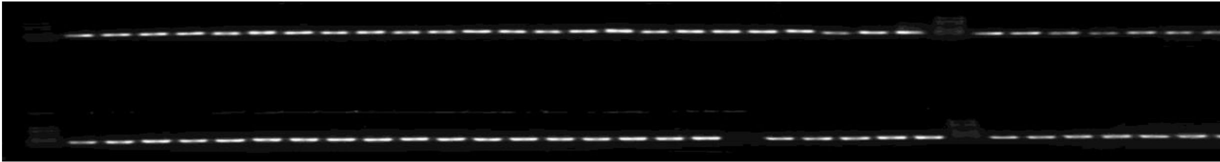
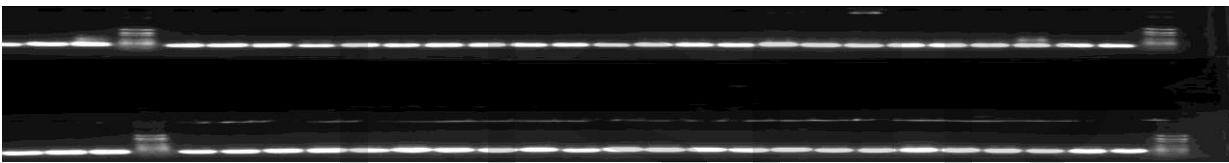
M M



OK1



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OK3

OK4

OK5

OK6

Figure 3: Gel pictures of colony PCR of 364 cloned DNA fragments from 6 *ugba* samples (OK1-OK6). Letters M represents the marker, while the bands in-between represent positive amplification of the cloned 16S fragments of isolated DNA.

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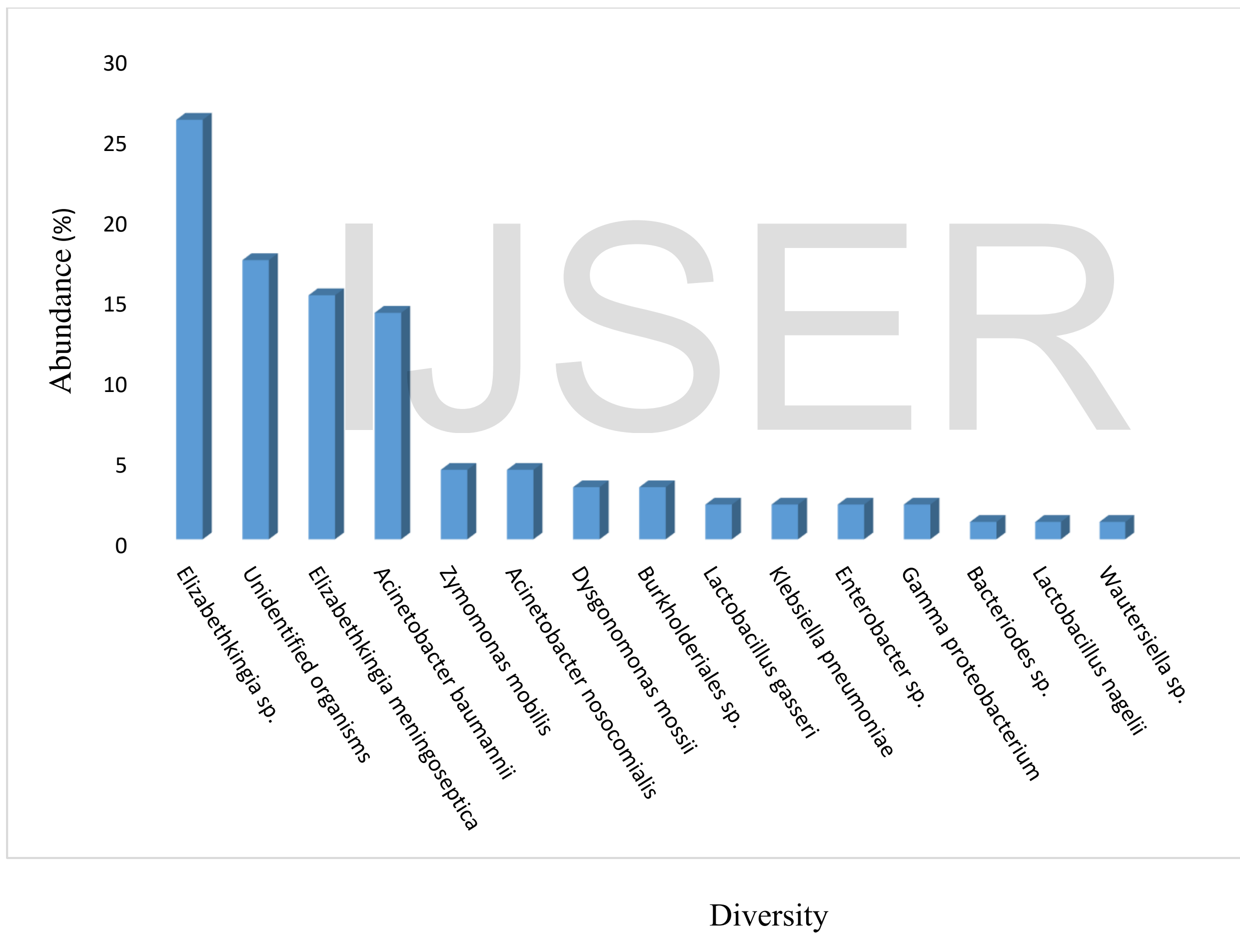
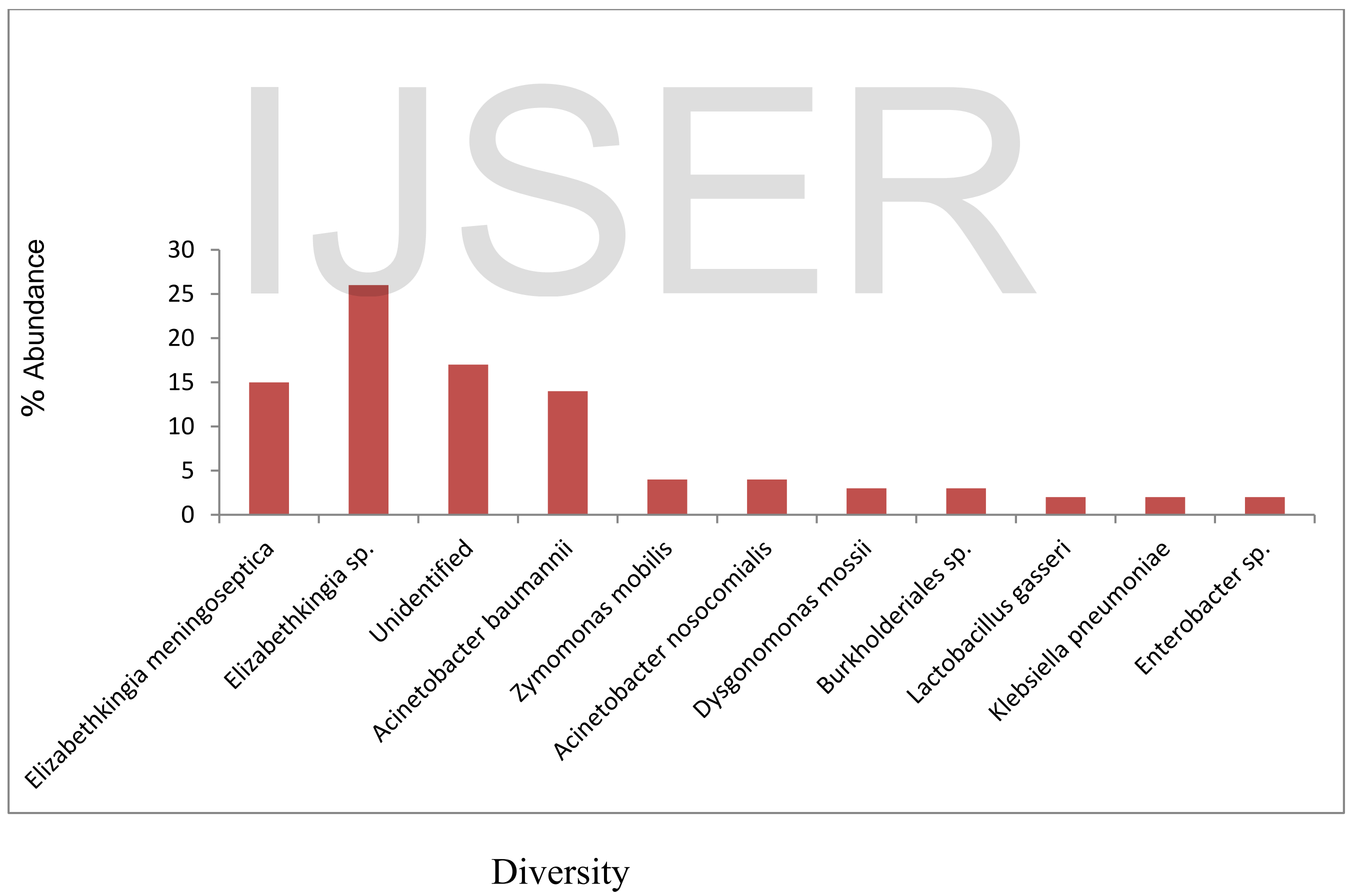
Figure 4: Phylogenetic relationship of identified organisms from fermenting African oil bean seed.



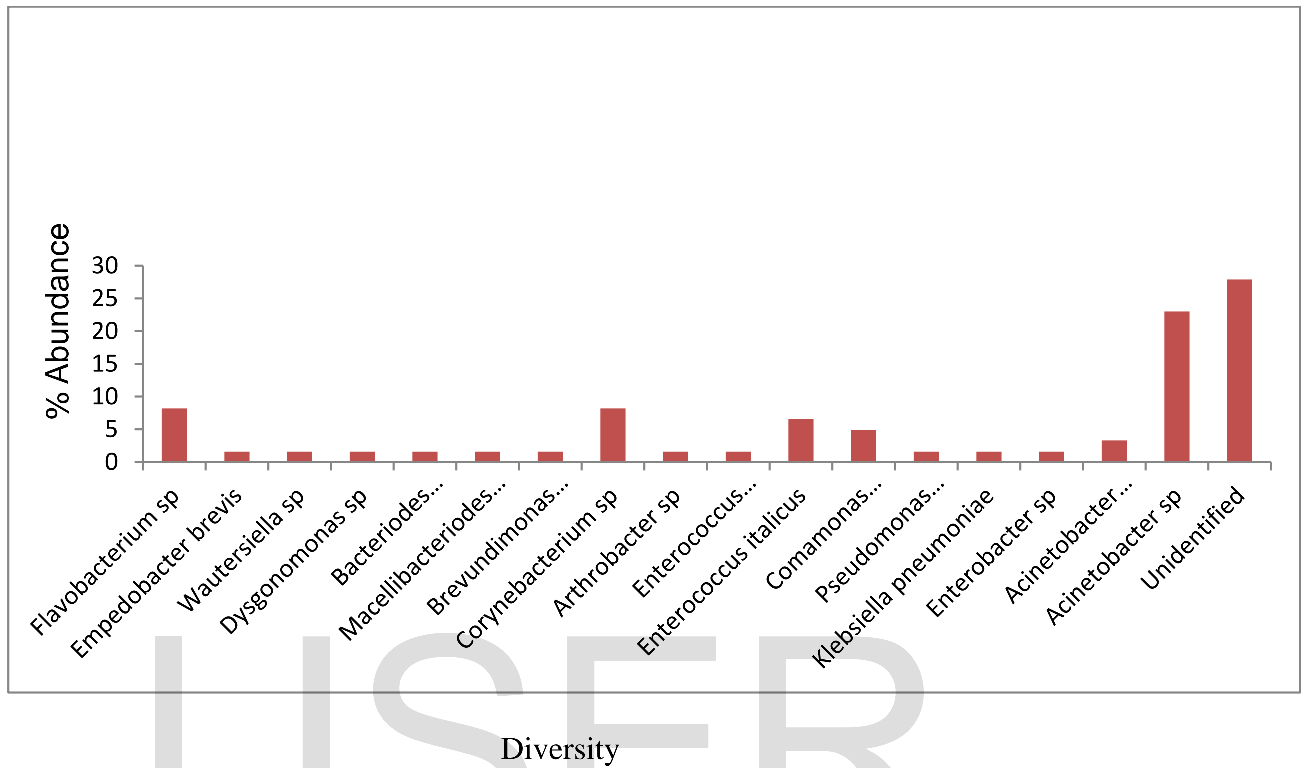
Diversity

Figure 9: Diversity and % abundance of organisms present in OK1



Diversity

Figure 10: Diversity and % abundance of organisms present in OK2 sample



Diversity

Figure 11: Diversity and % abundance of organisms present in OK3 sample

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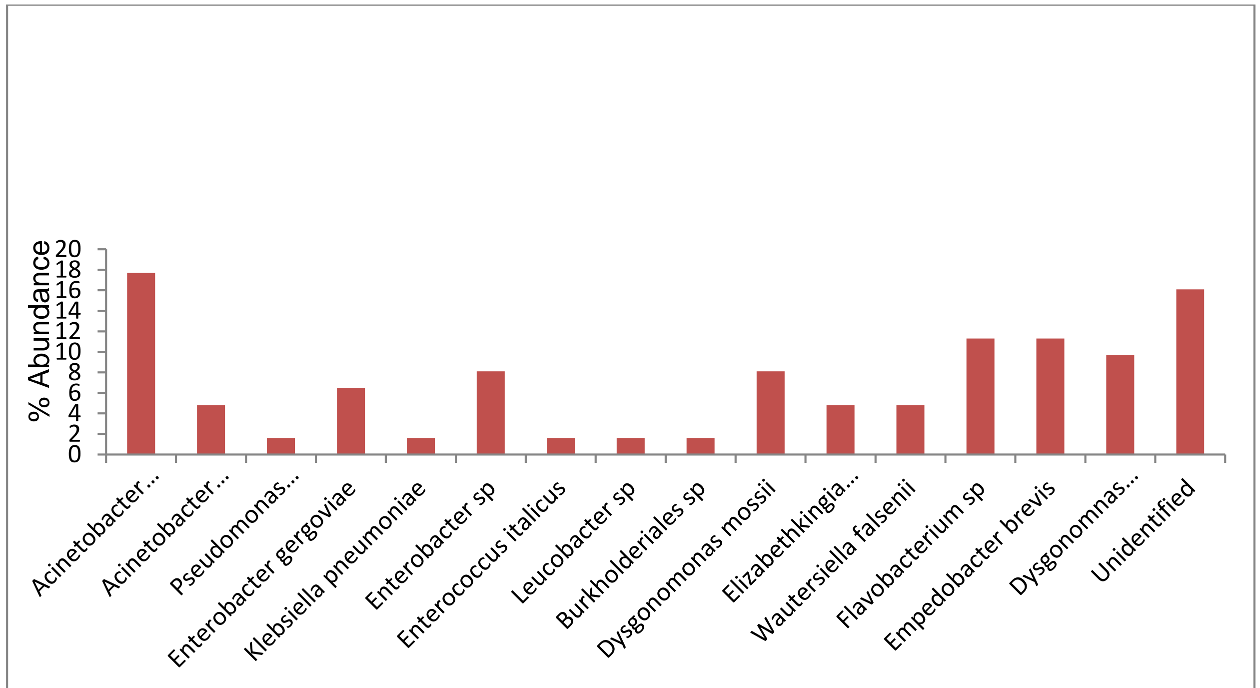

Diversity

Figure 12: Diversity and percentage abundance of organisms present in OK4 sample

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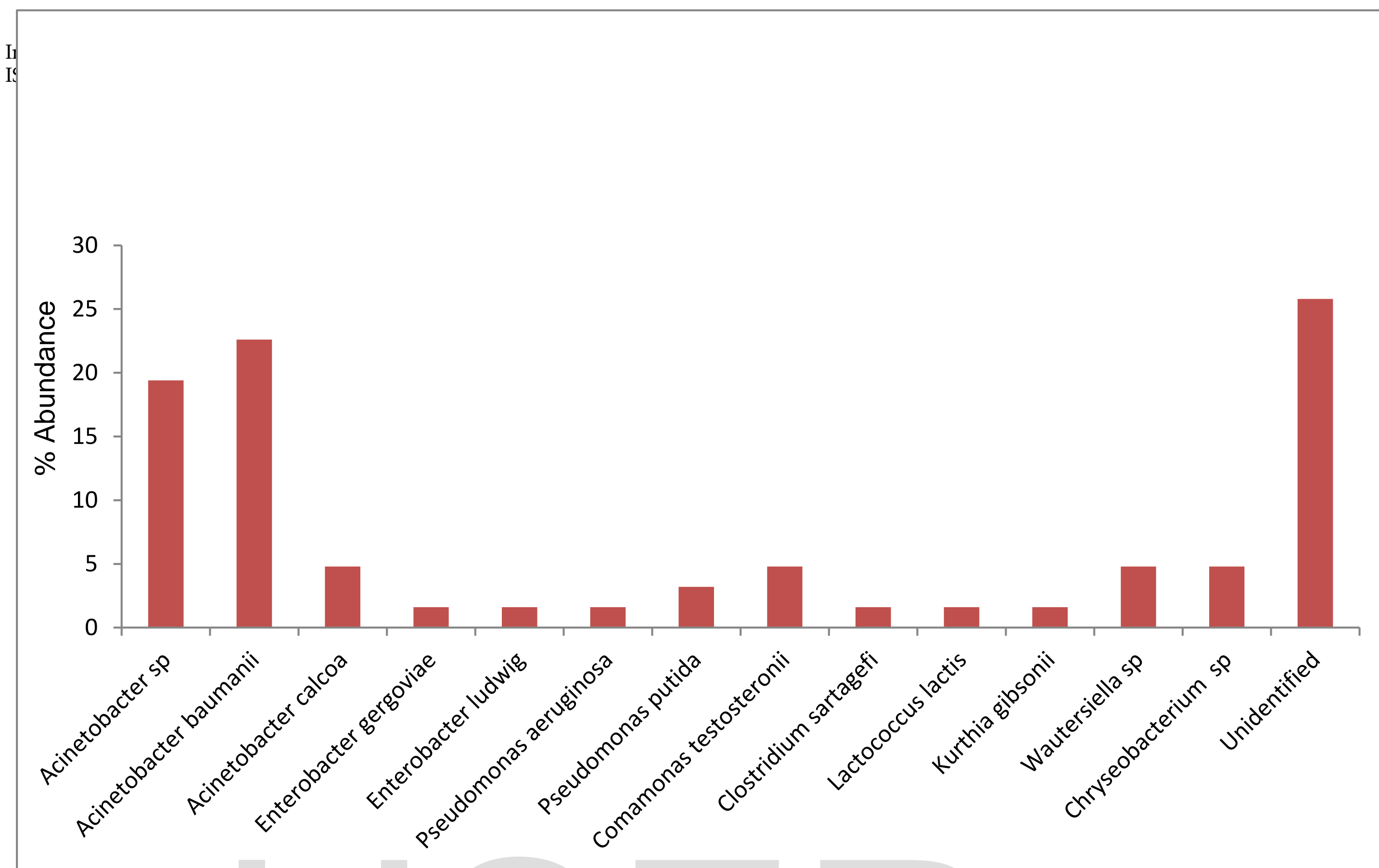

Diversity

Figure 13: Diversity and % abundance of organisms present in OK5 sample

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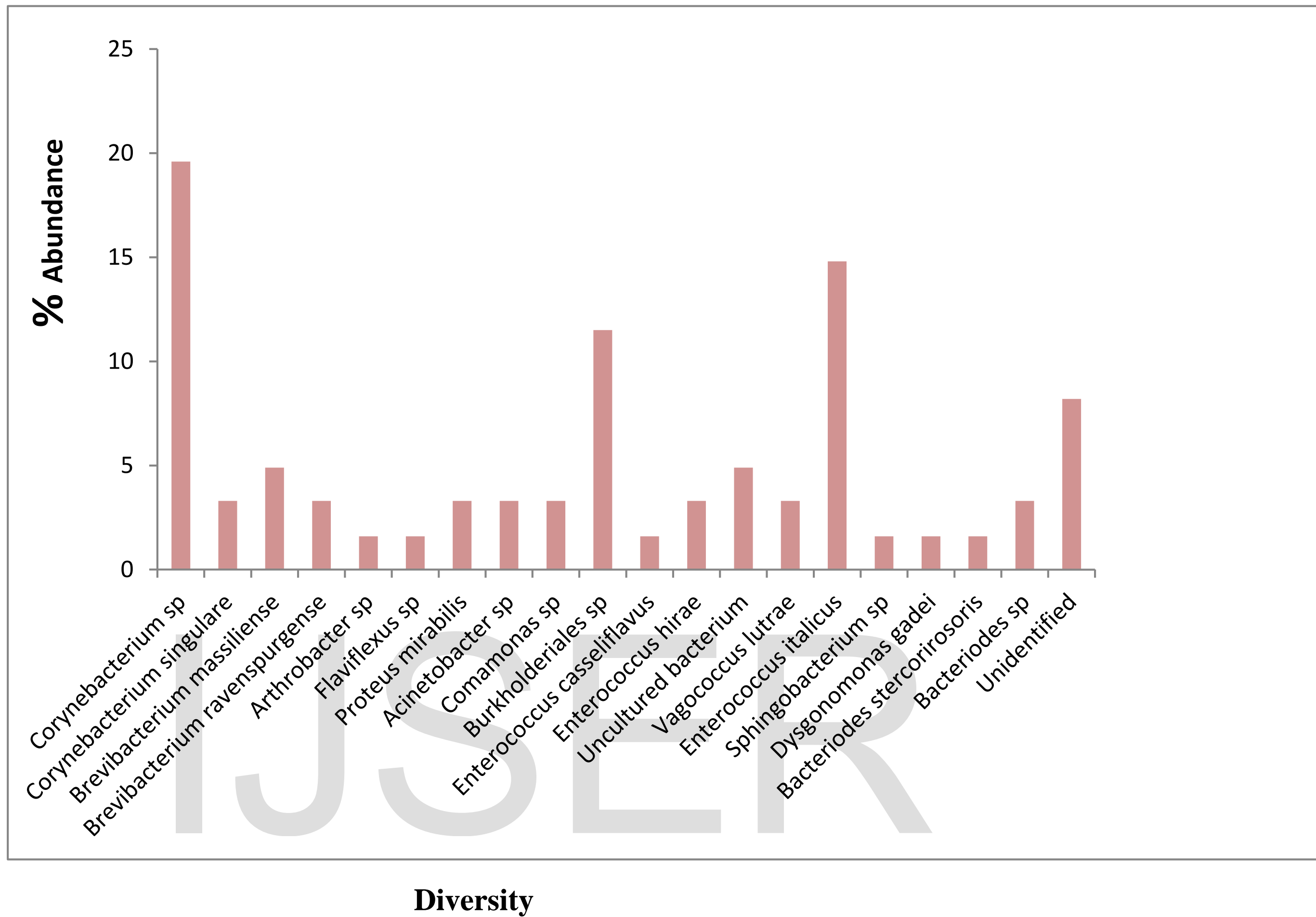

Figure 14: Diversity and % abundance of organisms present in OK6 *ugba* sample

DISCUSSION

Microbial ecology involves the study of the relationship of microorganisms to their surroundings and to each other. It is very important to understand the dynamics of microbial interplay in the fermenting mash of the oil bean seed to be able to select appropriate starters and effectively deploy them for control fermentation of this product. Evaluation of the microbial ecology of the fermenting mash of African oil bean seeds using phenotypic approach revealed the presence of diverse groups of microorganisms which include the following: *Bacillus*, *Proteus*, *Micrococcus*, *Staphylococcus, Corynebacterium, Alcalegen, Pseudomonas* species and some members of enterobacteriaceae. These species of bacteria have been associated in the past with the fermentation of *ugba* and other fermented food condiments (Odunfa, 1981; Antai and Ibrahim, 1986; Odunfa and Oyewole, 1986; Diawara *et al*., 1998; Ogueke and Aririatu, 2004). The application of molecular tools (clone libraray technique) however, revealed a wider bacterial diversity in the fermented mash of the African oil bean seed. New bacterial species such as *Arthrobacter, Empedobacter, Providencia, Brevibacterium, Elizabethkingia, Acinetobacter, Burkholderiales, Proteobacterium, Watersiella, Dysgomonas, Zymomonas* and *Flavobacterium* species were uniquely identified by the clone libraray technique. The presence of these organisms was, however, not consistent in all the samples studied and their numbers were in some cases very low. It is therefore, possible that they could have been contaminants which may not have had any active role to play in the fermentation of this

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product (Ogueke *et al*., 2010). Many of these organisms that were uniquely detected by the clone library technique were very low in numbers, this could therefore explain why they were not detected by the culture-based method, but were detected using molecular tools (clone library technique) which has a higher identification and resolution power.

It is believed that molecular methods have a superior ability to detect and identify organisms that are viable but may not be culturable or are in very low numbers and could not be detected by pure culture isolation and phenotypic characterization. This conclusion that molecular techniques have superior resolution power compared to culture-based method as expressed in this study is supported by similar assertions made by other authors. According to Gao and Moore (1996), Schloss and Handelsman (2005) and Cecchini *et al*. (2011), molecular techniques, such as representational difference analysis, consensus sequence–based PCR, and complementary DNA library screening, have led to the identification of several previously unculturable infectious agents. Rhoads *et al*. (2012), in a study evaluating culturing versus 16S ribosomal DNA sequencing as tools for identifying bacterial species in human chronic wound infection, identified 145 unique genera using molecular methods while only 14 unique genera were identified using aerobic culture methods.

Davey and Kell (1996) and Gunasekera *et al*. (2002) noted that a major disadvantage of using culture-based methods in the analysis of food samples is their failure to detect viable but non-culturable organisms. They raised

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doubts about the effectiveness of culture-based methods in the recovery of sub-lethally injured cells that may occur in heat treated products such as pasteurized milk. Since the fermentation process of *ugba* is usually terminated by boiling the final product in water, it is possible that the boiling process could have injured but not eliminated some of the bacteria and rendered them unculturable. However, because the molecular methods are culture independent, such cells are detected and identified. The inability of the culture dependent method to detect such microbial cells and post fermentation handling and processing could, therefore, account for the wider range of bacterial genera/species detected by molecular techniques against what was detected by cultural methods in *ugba* in this study.

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