



Microbial Diversity of Acetic Acid Producing Bacteria from Protein-Rich Residues

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Authors' contributions

This work was carried out in collaboration among all authors. Author GCO conceived and designed study, performed research and analyzed data. Authors OKA and GCO conceived and contributed new reagents/experimental apparatus. Author FSI contributed new methods. Author GCO wrote manuscript. Authors GCO, FSI and OKA read, edited and approved the manuscript for publication. All authors read and approved the final manuscript.

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ABSTRACT

Background: Acetic acid bacteria (AAB) are concrete sets of organism which act as precursor for acetic acid production. Acetic acid is a colorless liquid with strong pungent and sour smell. It is synthesized from oxidation of ethanol by AAB. Vast studies have been made from sugary sources in the isolation of AAB.

Aim: The needs to study and utilize our protein-rich residues (PRR) for AAB presence spurn this study.

Place and Duration of Study: Department of Microbiology, University of Port Harcourt, between June and December 2018.

Methodology: The samples (beans, groundnut and powdered milk) used in this study were surface-sterilized, homogenized, pre-enriched (in balsam medium) and serially diluted with inoculum size (0.1ml) inoculated on sterilized glucose yeast peptone agar, Mannitol agar and low glycemic index (LGI) media and incubated at 30°C for 48 h using the spread plate technique. A total of 11 bacterial isolates were obtained and screened for acetic acid production in brain heart infusion and yeast

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glucose ethanol acetic acid broth at 30°C for 14 days and positive isolates were identified by titration method. AAB isolates with the highest acetic acid concentration were selected for molecular identification and further studies.

Results: Two Acetic acid bacteria identified in this study were *Acetobacter* and *Gluconobacter*. The result of this study indicated that *Acetobacter* had acetic acid concentration of 3.6g/100ml while *Gluconobacter* had 1.8g/100 ml. However, molecular identification highlighted *Acetobacter* as *Bacillus cereus* with Genbank accession number MK 332142; whereas *Gluconobacter* was *Stenotrophomonas maltophilia* MK 332143. The neighbor-joining phylogenetic tree and bioinformatics revealed *B. cereus* and *S. maltophilia* as 97% and 96% similarity index, 854 and 883 nucleotide sequencing letters as well as 450 and 410 base pairs.

Conclusion: This finding implied that “*S. maltophilia*” and “*B. cereus*” are predominant Acetic acid bacteria in spoilt beans and groundnut; and can act as potential strains with industrial importance to man and environment.

Keywords: Protein-rich residues; acetic acid bacteria; *Stenotrophomonas maltophilia*; *Bacillus cereus*; genbank accession numbers.

ABBREVIATIONS

AAB : Acetic acid bacteria,
 PRR : Protein-rich residues,
 LGI : low glycemic index,
 CO₂ : Carbondioxide,
 H₂O : Water.

1. INTRODUCTION

Acetic acid bacteria (AAB) are concrete sets of organism [1] and are of industrial importance [2]. Different species have been used in the production of bioactive compounds like acetic acid, extracellular polymers like bacterial cellulose, Vinegar, gluconic acid, sorbose and ascorbic acid, ethyl acetate etc. [3].

With respect to their naming and taxonomy, AAB belong to the large group of bacteria known as “the Proteobacteria” which has over 500 known genera, hence are said to be remarkably diverse and includes a wide or broad variety of pathogens which are; *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia*, *Legionellales* etc as well as free living bacteria (responsible for nitrogen fixation) [4]. Furthermore, following the phylum Proteobacteria, there are five classes which are: “Alphaproteobacteria (α)”, “Betaproteobacteria (β)”, “Gammaproteobacteria (γ)”, “Deltaproteobacteria (δ)” and “Epsilonproteobacteria (ε)”. The alphaproteobacteria which is also written as α-proteobacteria has about 7 orders and 20 families where AAB falls into [4]. They were stratified into two classes on the basis of their capacities to over oxidize acetate or lactate into CO₂ and H₂O and the placement of their flagella

[2]. These two classifications were known to fall into two core genera namely: *Acetobacter* and *Gluconobacter* which are the only bacteria of recent research focus and is of severe industrial importance because of their ability to produce acetic acid [5].

AAB are commonly present in food substances mainly at their deteriorated form and rarely in their good state. They have been isolated from some fruits like grape, orange, cherry, mango, apple, pawpaw etc [6]. These bacteria have been isolated from alcoholic beverages, vinegar, flowers, honey, bees, sugar cane juices, soil, and water [2]. They require nutrients like carbon source such as d-glucose, d-mannitol, cassava flour, yam flour, chlorella biomass etc.; nitrogen sources such as peptone, yeast extract, corn-steep liquor; minerals like KH₂PO₄, Na₂HPO₄ and MgSO₄; Phosphorus source such as unripe plantain peel extract and other nutrients like 90% ethanol and acetic acid are also often added. All these aid in the recovery of the microorganisms during any fermentation process [5].

Studies have revealed the diversity of AAB in different fruits, vegetables, soil, water, carbohydrate sources [2, 6]. But has not been reported in any protein source, thus spurn the need for this research as a means of waste management/recycling and conservation in the society at large. The aim of this study was to isolate, characterize, identify AAB from Beans, Groundnut and powdered milk and estimate its ability to produce acetic acid. The identity of the isolated acetic acid bacteria was obtained through the 16S rRNA analysis.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Protein-rich agro residues (raw beans, groundnuts and powdered milk) were purchased at Kaduna state, Rumuokoro and Choba market Port Harcourt, Rivers state, Nigeria. They were taken to the University of Port Harcourt Herbarium, Department of Plant Science and Biotechnology for proper identification, authenticated and with subsequent analysis performed in Food and Industrial Microbiology Laboratory, Faculty of Science under standard microbiological conditions.

2.2 Pretreatment of Samples

The samples were prepared and processed in the laboratory, partitioned separately as Portion A (Good protein rich substrates) stored in the refrigerator and Portion B (Spoilt protein rich residues) induced to spoilage by storing under a humid environment at low temperature of 4°C for 7-10 days. They were surface sterilized by disinfecting with 70% alcohol (v/v) for 30 seconds and washed with distilled water from a water distiller.

2.3 Pre-enrichment, Serial dilution and Isolation of AAB

Portion A and B samples were pre-enriched for microbiological enumeration by aseptically homogenizing in a stomacher laboratory blender using a stomacher bag containing the basal medium (5% glucose, 1% yeast extract, 100 ppm cycloheximide, 1000ml distilled water) and LGI broth (K_2HPO_4 , KH_2PO_4 , $CaCl_2 \cdot 2H_2O$ 0.02g, $MgSO_4 \cdot 7H_2O$ 0.2g, $Na_2MoO_4 \cdot 2H_2O$, $FeCl_3$, Bromothymol blue (0.5% in 0.2 N KOH), Sucrose

5.0g and pH 6.0) and samples. They were stored for 18 to 24 h and diluted serially (10^{-1} to 10^{-6}); and cultured onto Glucose Yeast Peptone (GYP) Agar (Glucose 2% m/v, Na-acetate.3H₂O 0.5% m/v, Tryptone 0.5% m/v, Yeast extract 0.5% m/v, Potassium phosphate 0.1 m/v, Tween 80 0.5% v/v, Agar 1.7% m/v) Distilled H₂O 1000ml), Mannitol (MA) Agar (mannitol 2.5% m/v, Yeast extract 1% m/v, Agar 1.5% m/v, Distilled H₂O 1000ml) and Low Glycemic Index (LGI) medium (K_2HPO_4 , KH_2PO_4 , $CaCl_2 \cdot 2H_2O$ 0.02g, $MgSO_4 \cdot 7H_2O$ 0.2g, $Na_2MoO_4 \cdot 2H_2O$, $FeCl_3$, Bromothymol blue (0.5% in 0.2 N KOH), Sucrose and agar), respectively employing the spread plate technique [7] and incubated at 30°C for 24 - 48 h. Plate 1-6 shows the different portions of the samples.

2.4 Cultural Morphology, Microscopic Characterization and Biochemical Test

Sequel to isolation of the acetic acid bacteria from these protein-rich residues, macroscopic examination (with respect to cultural characteristics such as colony size, pigmentation, elevation, margin, light transmission etc.) were studied after incubation. Microscopic examination and the following biochemical test: oxidase, catalase, motility, methyl red/vogues proskauer, indole, sugar fermentation test (glucose, sucrose, lactose etc.), gelatin liquefaction, oxidation of ethanol and ketogenesis from glycerol were carried out on the AAB isolates following the method of [8]. The ninth edition of Bergey's Manual of Determinative Bacteriology was used to identify the AAB present in the protein-rich residues [8, 9]. The results from biochemical test were further used to validate the data obtained through molecular identification.



Plate 1-6. From left to right: good powdered milk, spoilt powdered milk, good raw beans, spoilt raw beans, good raw groundnut, and spoilt raw groundnut. Source: Researcher

2.5 Screening and Estimation for Acetic Acid Production

The tentatively identified AAB isolates were transferred into brain heart infusion broth (calf brain infusion 200g, beef heart infusion 250g, protease peptone 10g, dextrose 2g, Sodium Chloride (NaCl) 5g, Disodium phosphate 2.5g, distilled water 1000ml, pH 7.4 ± 2) and incubated at 30°C for 2 days. Thereafter, about 4% of the prepared cultures i.e. the inocula size from the preceding BHI broth was cultured in yeast glucose ethanol acetic acid (YEGA) media (Yeast extract 3%, D-glucose 2%, ethanol 3%, acetic acid 3%) for acetic acid production. Fermentation was carried out at 30°C for 14 days at 150 rpm and sample were collected at 48 h intervals for optical density monitoring at (OD_{600nm} = 0.5N) [10]. Acetic acid content was determined by titration method with 0.5 N NaOH using phenolphthalein as an indicator [10, 11]. This was done by dispensing 5 ml of the supernatant culture solution into a conical flask with addition of 20 ml distilled water and 5 drops phenolphthalein, with gradual introduction of the NaOH until the point at which a pink color change in was observed. The amount (g) of acetic acid produced in 100 ml of the medium was titrated and calculated using the titrimetric formular:

$$\text{Acetic acid (g/100ml)} = \text{volume of NaOH (ml) used in the titration} \times 0.03 \text{ ml (volume of Phenolphthalein used)} \times 20 \text{ ml (volume of distilled water used)}.$$

AAB Isolates that gave the highest acetic acid concentration were selected for further studies.

2.6 Molecular Identification of the AAB Isolates

2.6.1 Purification of Tentative AAB Isolates

The preserved isolates were purified by sub-culturing on Luria-betani agar, incubated at 30°C for 18 to 24 h for molecular identification at the Regional Centre for Bio-fuels and Bioresource.

2.6.2 Extraction of Bacterial DNA

The total genomic DNA was extracted from pure bacterial cultures following the protocol of Quick-DNA™ Fungal/Bacterial MiniPrepKit (Zymo Research Group, California, USA) as described by the manufacturer, with modifications.

2.6.3 Quantification/ Concentration of DNA Material

After the extraction of the bacterial DNA, they were quantified for their respective concentrations using the Thermo-scientific Nanodrop 2000C Spectrophotometer which read through the DNA for 3 consecutive times and revealed their Nucleic acid concentration, the maximum and minimum levels, its purity level as well as its unit.

2.6.4 Phylogenetic analysis

The 16S rRNA products of 2 isolates were amplified from the genome through PCR, purified and sequenced for strain identification [2]. The

forward and reverse sequences were assembled using the BigDye Terminator Kit on a 3510 ABI sequencer by Inquaba Biotechnological, Pretoria South Africa; they were compared to the GenBank database of the National Centre of Biotechnology Information (NCBI) using basic local alignment search tool (BLASTN) [12]. In order to obtain their close phylogenetic relatives, BLAST was prepared against NCBI reference sequence database. The nucleotide sequences obtained were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-joining method in MEGA 6.0 [13, 14]. The evolutionary distances were computed using the Jukes-Cantor method [15]. The maximum likelihood method was adopted to contrast the phylogenetic tree and the reliability of inferred trees was tested from 500 replicates with bootstraps consensus tree test.

3. RESULTS AND DISCUSSION

3.1 Mean Bacterial Count

The quantitative estimation of the mean bacterial colony count is presented in Fig. 1. This revealed that in Portion A (the good state) of these protein rich substrates, there was a form of equivalence of acetic acid bacterial count in both milk (7.9), groundnut (7.9) (Log₁₀Cfu/ml) substrates, respectively but a higher increase in acetic acid bacterial was shown with beans (8.1) Log₁₀Cfu/ml). However in Portion B, (the spoilt state) of these protein rich substrates, an increase occurred in the microbial load of beans substrate having a high bacterial load of (8.2), followed by groundnut (8.1) and milk (8.11) Log₁₀Cfu/ml respectively.

Table 1. Morphological characterization of Acetic acid bacterial isolates

S/N	PARAMETERS TESTED	ACETIC ACID BACTERIAL ISOLATES										
		LGI.GGI	LGI.GG2	LGI.SG1	LGI.SG2	MA.SG1	LGI.GM1	LGI.GM2	LGI.SM1	GYP.SM1	LGI.SB1	MA.SB1
MORPHOLOGICAL CHARACTERIZATION												
1.	Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
2.	Size (mm)	3	3	2	1	1	1	2	2	2	2	2
3.	Pigmentation	Yellow	Yellow	Cream	Cream	Cream	Yellow	Yellow	Yellow	Yellow	Yellow	Cream
4.	Elevation	Convex	Raised	Convex	Convex	Convex	Convex	Raised	Convex	Raised	Convex	Convex
5.	Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
6.	Appearance	Shinny	Shinny	Shinny	Shinny	Shinny	Shinny	Shinny	Shinny	Shinny	Shinny	Shinny
7.	Light transmission	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
8.	Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire

Key: LGI = Low glycemic index, GG = Good Groundnut, SG = Spoilt Groundnut, MA= Mannitol agar, GM = Good Milk, SM = Spoilt Milk, GYP = Glucose yeast peptone, SB = Spoilt beans

Table 2. Microscopic and Biochemical characterization of Acetic acid bacterial isolates

S/N	PARAMETERS TESTED	ACETIC ACID BACTERIAL ISOLATES										
		LGI.GGI	LGI.GG2	LGI.SG1	LGI.SG2	MA.SG1	LGI.GM1	LGI.GM2	LGI.SM1	GYP.SM1	LGI.SB1	MA.SB1
MICROSCOPIC EXAMINATION												
1.	Gram reaction	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
2.	Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
3.	Arrangement pattern	Clusters	Clusters	Clusters	Clusters	Clusters	Clusters	Clusters	Clusters	Clusters	Clusters	Clusters
BIOCHEMICAL CHARACTERIZATION												
1.	Catalase	+	+	+	+	+	+	+	+	+	+	+
2.	Oxidase	-	-	-	-	-	-	-	-	-	-	-
3.	Indole	-	-	-	-	-	-	-	-	-	-	-
4.	Motility	+	+	+	+	+	+	+	+	+	+	+
5.	Oxidation of ethanol	+	+	+	+	+	+	+	+	+	+	+
6.	Overoxidation of ethanol	-	-	+	-	-	-	-	+	-	-	+
7.	Sugar fermentation											
	i. Glucose	+	+	+	+	+	+	+	+	+	+	+
	o. Sucrose	-	-	-	-	-	-	-	-	-	-	-
	o. Lactose	-	-	-	-	-	-	-	-	-	-	-
	i. Maltose	-	-	-	-	-	-	-	-	-	-	-

S/ N	PARA- METERS TESTED	ACETIC ACID BACTERIAL ISOLATES										
		LGI.GGI	LGI.GG2	LGI.SG1	LGI.SG2	MA.SG1	LGI.GM1	LGI.GM2	LGI.SM1	GYP.SM1	LGI.SB1	MA.SB1
MICROSCOPIC EXAMINATION												
	1. Mannitol	-	-	-	-	-	-	-	-	-	-	-
	2. Fructose	-	-	+	-	-	-	-	+	-	-	+
	3. Xylose	+	+	+	+	+	+	+	+	+	+	+
	4. Galactose	+	+	+	+	+	+	+	+	+	+	+
	5. Mannose	+	+	-	+	+	+	+	-	+	+	-
8.	Growth in Gelatin	+	+	+	+	+	+	+	+	+	+	+
9.	Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-
10.	Ketogenesis from glycerol	+	+	+	+	+	+	+	+	+	+	+
11.	Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-
12.	Growth in peptone	-	-	-	-	-	-	-	-	-	-	-
PROBABLE		<i>Acetobacter</i>	<i>Acetobacter</i>	<i>Gluconobacter</i>	<i>Acetobacter</i>	<i>Acetobacter</i>	<i>Acetobacter</i>	<i>Acetobacter</i>	<i>Gluconobacter</i>	<i>Acetobacter</i>	<i>Acetobacter</i>	<i>Gluconobacter</i>
GENERA		<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>	<i>er sp.</i>

Key: LGI = Low glycemic index, GG = Good Groundnut, SG = Spoilt Groundnut, MA= Mannitol agar, GM = Good Milk, SM = Spoilt Milk, GYP = Glucose yeast peptone, SB = Spoilt beans

Table 3. Titrimetric analysis for estimation of acetic acid production in AAB isolates

S/N	Isolates Code	AAB Isolate Equivalent Code	Volume of 0.5N NaOH (ml)	Volume of Phenolphthalein (ml)	Volume of distilled H ₂ O (ml)	Acetic acid (g/100ml)
1.	1	GYP.SM1	1.98	0.03	20	1.188
2.	2	LGI.SG1	1.96	0.03	20	1.176
3.	3	LGI.SG2	3	0.03	20	1.83
4.	5	LGI.SB2	2.5	0.03	20	1.5
5.	8	LGI.GG2	1.02	0.03	20	0.612
6.	9	LGI.SM1	2	0.03	20	1.2
7.	11	MA.SB1	3	0.03	20	1.8
8.	12	LGI.GM1	3.95	0.03	20	2.37
9.	16	LGI.GM2	2	0.03	20	1.2
10.	17	LGI.GG1	1.02	0.03	20	0.612
11.	18	MA.SG1	5.97	0.03	20	3.582

Key: LGI = Low glycemic index, GG = Good Groundnut, SG = Spoilt Groundnut, MA= Mannitol agar, GM = Good Milk, SM = Spoilt Milk, GYP = Glucose yeast peptone, SB = Spoilt beans, NaOH: Sodium Hydroxide, H₂O: Water

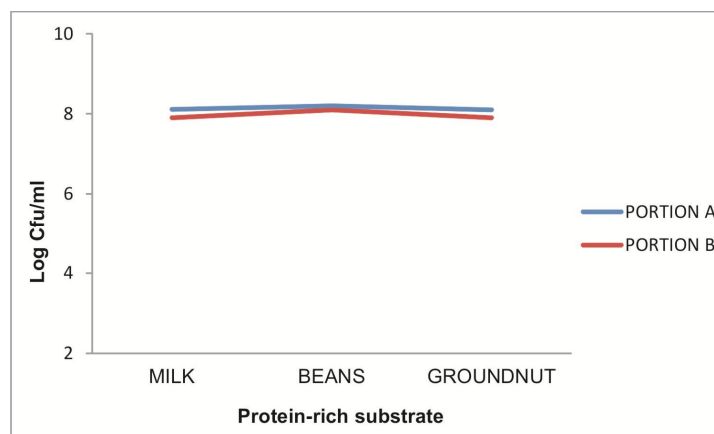


Fig. 1. Quantitative estimation of the mean aerobic heterotrophic bacterial colony count from the Protein-rich substrates (milk, beans and groundnut)

Key: Portion A = (Good protein-rich substrate), Portion B = (Spoilt protein rich substrate)

3.2 Morphological Characterization of the AAB

The cultural and morphological characteristics of the colonies on the different media were observed and characterized using the following colonial characteristics which ranged from: size, shape, texture, color, etc. About 21 colonies were initially picked; but a total of 11 isolates were tentatively identified and distinguished from each other. The findings revealed that all the isolates were opaque, smooth and circular, some colonies were yellow and others cream as presented in Table 1.

Some had little zones of clearance round the colony; whereas there were changes in color from blue to yellow thick pellicles on the LGI broth and yellow clearance colonies on LGI agar respectively, thus indicating the presence of AAB as shown in Plates 7- 8.

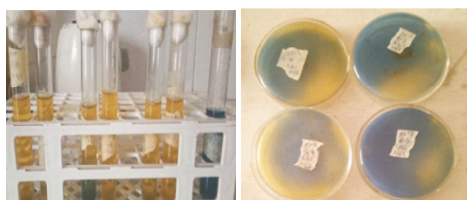


Plate 7-8. From left to right: Test tubes of LGI broth culture, LGI agar plates with yellow colonies of AAB after incubation. Source: Researcher

3.3 Biochemical Characterization of AAB

This study revealed that the AAB isolates from the good and spoilt protein-rich residues were

catalase positive, oxidase negative, indole negative, all motile, same gram reaction, shape and arrangement pattern with some able to ferment some sugars with the production of acid and gas. However, a total of eleven isolates were tentatively identified and distinguished as *Acetobacter sp.* and *Gluconobacter sp.* respectively. Three of these isolates (from spoilt groundnut, milk and beans respectively) were identified as *Gluconobacter sp* while the other eight isolates (from good groundnut, spoilt groundnut, good milk, spoilt milk and spoilt beans) were identified as *Acetobacter sp.* as shown in Table 2.

3.4 Acetic Acid Production from Titrimetric Analysis

Acetic acid production abilities were examined and estimated by measuring the acidity of the culture medium released by the acetic acid bacteria isolates where isolate 18 - MA.SG1 (*Acetobacter sp.*) had acetic acid concentration of 3.6g/100 ml, Isolate 11- MA.SB1 (*Gluconobacter sp.*) had 1.8g/100 ml, Isolate 17 - LGI.GG1 (*Acetobacter sp.*) had 0.612g/100 ml, Isolate 8 - LGI.GG2 (*Acetobacter sp.*) had 0.612g/100 ml, isolate 2 - LGI.SG1 (*Gluconobacter sp.*) gave 1.2g/100 ml, Isolate 3 - LGI.SG2 (*Acetobacter sp*) had 1.83g/100 ml, Isolate 12 - LGI.GM1 (*Acetobacter sp*) had 2.4g/100ml, Isolate 9 - LGI.SM1 (*Gluconobacter sp.*) had 1.2g/100 ml, Isolate 5 - LGI.SB1 (*Gluconobacter sp.*) had 1.5g/100 ml, Isolate 16 - LGI.GM2 and Isolate 1- GYP.SM1 (*Acetobacter sp*) had 1.2g/100 ml respectively as shown in Table 3.

However, isolate 11 and Isolate 18 were selected since they had the highest production capability as shown in Fig. 2.

3.5 Molecular Identification of AAB Isolates

The molecular screening processes revealed that the tentatively identified *Acetobacter* sp and *Gluconobacter* sp were *Bacillus cereus* and *Stenotrophomonas maltophilia* respectively. *S. maltophilia* which is designated as AEAG2 has a total of 883 letters and molecular weight of 0.45Kbp; while *B. cereus* which is designated as AEAG3 has a total of 854 letters and 0.410Kbp using a Ladder of 1.0Kbp with two bands as presented in Fig. 3.

3.6 Phylogenetic Information of AAB Isolates

Phylogenetic tree was constructed based on 16SrRNA sequences for evolutionary relatedness. The phylogenetic tree in Fig. 4 revealed that isolate 11 had 3 closely related genera and species (*Stenotrophomonas* sp strain MRC2-1 (KX214768.1), *Stenotrophomonas maltophilia* (KY606632.1) and *S. maltophilia* (MH141455.1)) with differences in their percentage similarity index, matrix integrity as well as accession numbers. The first "*Stenotrophomonas* sp. strain MRC2-1 (KX214768.1) has a similarity index of 100%, the next is "*Stenotrophomonas maltophilia* (KY606632.1)" with similarity index of 100% and lastly, "*Stenotrophomonas maltophilia*

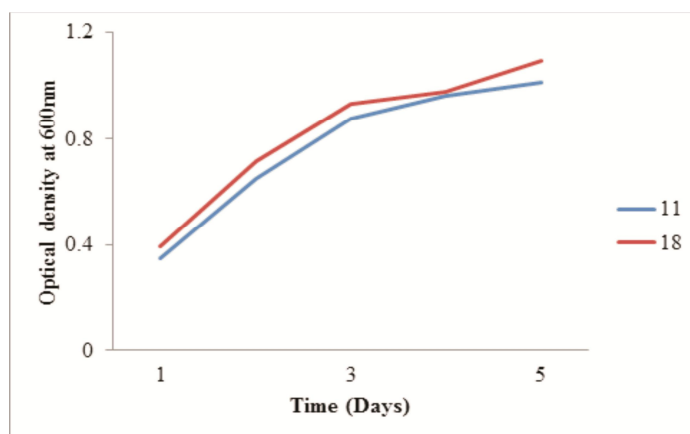


Fig. 2. Acetic acid production in Yeast Glucose Ethanol Acetic Acid (YEGA) media
Acetic acid production rate was estimated by titration method where isolate 18 - MA.SG1 (*Acetobacter* sp.) had acetic acid concentration of 3.6g/100ml and Isolate 11 - MA.SB1 (*Gluconobacter* sp.) had 1.8g/100ml

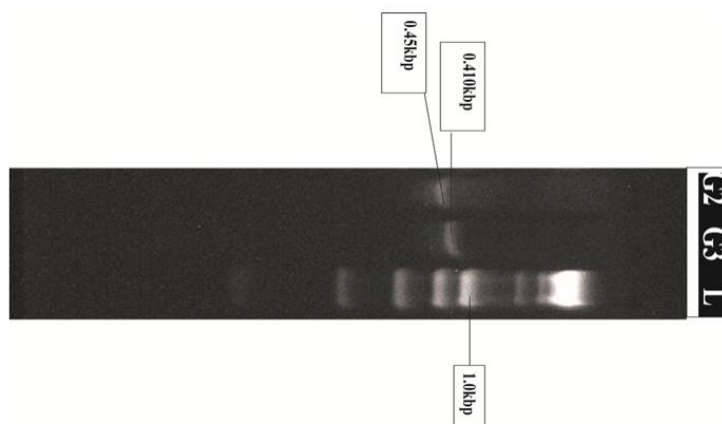


Fig. 3. Agarose gel electrophoresis showing the amplified bands for *Stenotrophomonas maltophilia* and *Bacillus cereus*

(MH141455.1) with similarity index of 62%. But molecular identification does not stop at the genera level, hence this implied that isolate 11 was *Stenotrophomonas maltophilia*. For isolate 18, it was distinguished to have 3 firmly related genera and species yet different percentage similarity index, matrix percentage as well as accession numbers. The first is seen as *Bacillus* sp FO-011 (AF2348421) without a similarity index, trailed by *Bacillus cereus* with the highest similarity index of 55% and an accession number of MG027666.1. The last is *Bacillus thuringiensis* with similarity index of 37% and an accession number of KJ722439.1. Thus, this implied that isolate 18 is *Bacillus cereus*.

There is a worldwide interest in the use of acetic acid (in oil and gas segment, food and beverage industries, road construction companies as well as medical settings) on a daily basis. This is possible by fermentation process performed by acetic acid bacteria. Acetic acid bacteria are diverse in nature and have been confined from various sources. *Acetobacter* and *Gluconobacter* are two main strain genera useful to the food and beverage industries responsible for the industrial production of vinegar. They are diverse in their species namely: *Acetobacter aceti*, *Acetobacter pastuerianus*, *Acetobacter cerevisiae*, *Gluconobacter oxydans* (flore of palm tree) and so forth. [16] reported that they have been isolated from several natural origins for example, grapes, dates, coconut toddy (mnazi), palm resources and so on; and have been utilized for the production of a few vinegar types from different substrates like sugarcane, rice [17] and amber [6,18]. Aside from acetic acid/Vinegar, these strains have been accounted for to create

3.7 Bioinformatics Studies of AAB Isolates

After the BlastN process and securing of the bacterial accession numbers, the bioinformatics result was presented in the Table 4.

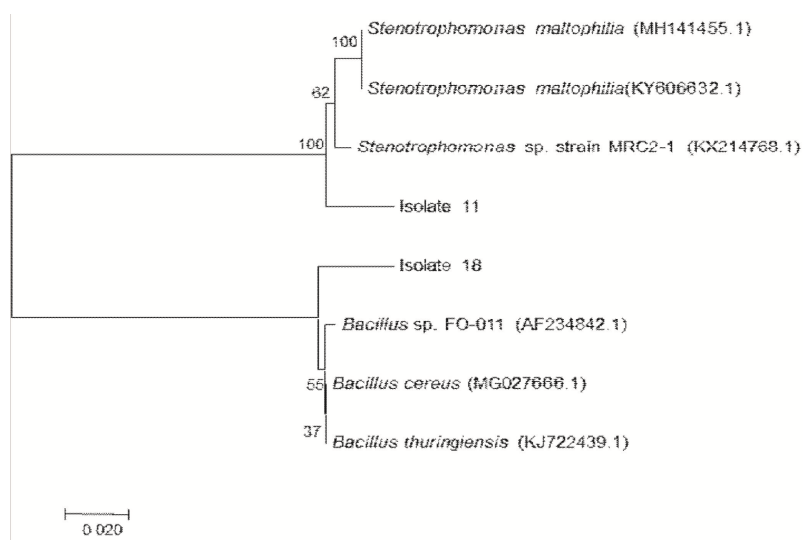


Fig. 4. Neighbor-joining phylogenetic tree of the bacterial isolates showing their relatedness. Phylogenetic tree was predicted using 16S ribosomal Ribonucleic acid sequences of acetic acid producing microorganisms. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The bootstrap considered 2000 replicates

Table 4. Bioinformatics Results

Isolate Code	Tentative AAB Name	Percentage similarity	Blasting identity	Sequence Read	Accession number
Isolate 11-AEAG2	<i>Gluconobacter</i> sp.	96%	<i>Stenotrophomonas maltophilia</i>	883 letters	MK332143
Isolate 18-AEAG3	<i>Acetobacter</i> sp.	97%	<i>Bacillus cereus</i>	854 letters	MK332142

AAB: Acetic acid bacteria

different other compounds that influence the nature of wine [16]. They can produce some polysaccharides like cellulose, levan, dextran [16]. *Acetobacter xylinum* KJ-1 has the ability of producing bacterial cellulose in shaking cultures [19]. Other AAB are *Acidomonas*, *Gluconoacetobacter*, *Fratueria*, *Asaia*, *Neoasaia*, *Kozakia* etc. It can thus be deduced that various kinds of deteriorated fruits are reservoir for AAB [20].

Fruits and crops produce are presently available at all seasons due to the developing standard of Nigeria's agricultural system and thus huge amount of them are disposed daily by local fruit markets (e.g. oil mill market in Port Harcourt); thus constituting nuisance for air pollution in the environment. Some of these fruits are utilized for microbiological analysis and other viable purposes thereby managing waste. They act as starter cultures and are kept in culture collection centers (thus saving time, energy, resources, labor), for biodegradation during oil drilling and simulation, production of vinegar (an important ingredient for making pickles, jams, jellies and other vinegar-based products), cellulose, acetic acid etc.; thus contributing a lot in the economy of the country.

This present study revealed that the microbial quality of protein-rich food crops (groundnut, beans and milk) harbors an amazing microflora of bacteria. They were higher in Portion B (beans 8.2, groundnut 8.1 and milk 8.11 Log₁₀cfu/g, respectively) compared to Portion A (beans 8.1, groundnut and milk 7.9 Log₁₀cfu/g, respectively). This observation is consistent with the report by [2,6] that producers of acetic acid are predominantly occurring in deteriorating food crops and substrates. [20] inferred that biomining of high throughput strains of AAB could be accomplished from transitory organic products as in perishable or rotten fruits. In this manner, producers of acetic acid could be isolated from these perishable fruits for the creation of acetic acid to such an extent that in lieu of outright disposal, waste to wealth which is a sustainable venture has now turned into a potential pattern for all researchers and science specialists [21].

The biochemical characterization revealed that the AAB isolates were catalase positive, oxidase negative, indole negative, all motile, Gram-negative, shape and arrangement pattern with some able to ferment some sugars with the production of acid and gas. These results are in agreement with report of [16] where acetic acid

bacteria were identified to be gram-negative, motile, rods. In addition, [22] also opined that acetic acid producers are Gram-negative, rods, motile, polarly flagellated, and have the ability to produce acetic acid. The technique depicted by [20] was utilized for distinguishing the genera *Acetobacter* from *Gluconobacter*. The over-oxidation of ethanol to CO₂ and H₂O was also utilized to distinguish between *Acetobacter* and *Gluconobacter*. The results obtained from these evaluations showed that the 8 isolates fell under the genera *Acetobacter* while the other 3 isolates belong to "the genera *Gluconobacter*". The tentatively identified AAB were *Acetobacter* and *Gluconobacter* from Portion B (Beans and Groundnut individually) with respect to the characterization pattern in the ninth edition of Bergey's manual of systematic bacteriology [9]. The occurrence of *Acetobacter* and *Gluconobacter* in Portion A and B was in the ratio of 8:3, respectively which was almost similar with the report of [23] for fermented juices and fruits.

The results from titrimetric examination revealed that they are fit for the production of acetic acid where *Acetobacter* had 3.6g/100ml and *Gluconobacter* had 1.8g/100ml. [24] in his report revealed that *Acetobacter* sp. produced least amount of acetic acid (7.0g/100ml) in the presence of 2% ethanol in the medium which was higher than the findings of this study.

From this study, *Bacillus cereus* and *Stenotrophomonas maltophilia* are the two unique bacterial isolates characterized using 16S DNA sequencing approach with accession number MK 332142 and MK 332143, respectively. In addition, [25] had a stern agreement with the outcomes of this study having documented that his routinely identified *Acetobacter* sp., which was later determined and identified through molecular analysis had attributes similar to that of our *Stenotrophomonas maltophilia*. Furthermore, this study thus corroborates with the findings of [25] since he ascertained the presence of both *Stenotrophomonas maltophilia* and *Bacillus cereus* from lipase rich substrates. In addition, this study concurred with the findings of [26] and [27] in whose report archived the presence of *B. cereus*, *B. thuriengensis* and *B. anthracis* from raw milk tests. This finding is also in line with [24, 28] who reported the nearness of *Bacillus* sp. F-3 (KF668639) from fruits as a potential maker of acetic acid or its subsidiaries.

However, the PCR-molecular weights of the isolates were within the range of 410 and 450 base pairs. There was no recognizable alteration with the report of [27], who recorded that PCR-molecular weight of *Bacillus cereus* was 475 base pairs which were also similar to [25] whose report documented molecular weight of about 500 base pairs. The findings of [40] differed with the result of this investigation as the PCR-molecular weight was 900 base pairs. The incongruities with these reports could emerge from the situations where their isolates were acquired.

S. maltophilia is a gram negative, obligate, aerobic, rod shaped, non-fermentative bacteria with a slightly smaller size of $0.7-1.8 \times 0.4 - 0.7\mu\text{m}$ and a few polar-flagella for motility. It has the ability of surviving or striving in nutrient-depleted aqueous environments. They are catalase *positive*, oxidase *variable*, motile, indole *negative*, citrate *variable*, methyl red *negative*, Voges-Prokauer *negative*, positive utilizers of most carbon sources like glucose, lactose, maltose and trehalose, negative to starch and urea hydrolysis as reported by [29]. They have been confined from soil, banana-stem waste, some draining machines and so forth [22]. Being aborigines of the soil, *S. maltophilia* are widely found in aqueous environment, the root nodules, stems and in rhizoids region; animals [30, 31]. It is an endophyte which aids in the promotion of agricultural produces especially as a plant growth promoting bacterium [32]. It also plays a vital role as a nitrogen fixing bacteria and is used as a biofertilizers [33]. Endophytic bacteria are aborigines of the roots and pods of most leguminous crops and have the strength of producing acetic acid [29]. They also offer beneficial effect on crop growth and yield. However, they are plant growth promoting rhizobacteria serving as resilient bioenhancer and biofertilizer for banana cultivation [34]. It aids in the formation of fluid-bioinoculant for economically convenient and sustainable agriculture. Further studies by [35] reported this bacterium to be a chitinolytic bacteria having been gotten from different samples of dead termites, termites affected trees, termite's mounds, termites affected gardens, fields and crabs. It can produce the enzyme chitinases by utilizing extracted crab chitin and is a helpful economic biological control agent of some insect pest like mosquitoes, termites, bedbugs etc [35]. More so, as chitinolytic bacteria they are capable of controlling insects under optimal conditions as reported by [36].

B. cereus has a place with the low guanine and cytosine Gram positive bacteria. [37] opined that it is a Gram positive, spore forming, motile, aerobic or facultative anaerobic rod-shaped, beta-haemolytic bacterium with large vegetative cells. They are catalase positive, oxidase negative, positive to Voges-proskauer, nitrate reduction and starch hydrolysis test, citrate positive and are equipped for delivering acid and gas for some sugar fermentation [38]. *B. cereus* are widespread in nature hence are said to be ubiquitous; and are readily found in soil, where it acts saprophytically. It germinates and grows as well as sporulates in its environment massively [39]. It is found in diverse kinds of food, raw plant foods like rice, potatoes, peas, beans and spices; they also dominate most of the processed or cooked foods which is caused by contamination of raw materials prior to the cooking and handling process, the subsequent resistance of spores to thermal and other manufacturing processes. Generally, the genera *Bacillus* has gained massive uses and applications in the industries, research and environmental sectors etc. *Bacillus* sp. keeps on being predominant bacterial workhorses in microbial fermentations [40]. The striking microbial member in the continuous generation of soya-based conventional natto fermentation is *Bacillus subtilis* (natto), though *Bacillus* species are put on the Food and Drug Administration's GRAS (generally regarded as safe) list. They have been put among the most considerable industrial enzyme producers. They are used for making some preservatives, behaving like probiotics etc. Some strains of *B. cereus* produce bacteriocins active against different *B. cereus* strains or other Gram-positive bacteria [40].

4. CONCLUSION

Protein-rich residues are reservoir for the diversity of AAB useful in the production of acetic acid. It sheds light on the diversity of bacteria within the Nigeria environment. The gammaproteobacteria (*S. maltophilia*) and Firmicutes (*B. cereus*) are AAB widely distributed with great potential to produce acetic acid like other organic acid producing bacteria for industrial purposes. The titrimetric analysis revealed the level of acetic acid produced by *B. cereus* as 3.6g/100ml whereas *S. maltophilia* as 1.8g/100ml. Further studies on the fermentation kinetics and modeling of *S. maltophilia* and *B. cereus* could be performed and their subsequent applicability in the industries.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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