Original Research Article

Isolation and Molecular Characterization of Industrially Significant Bacteria Obtained from Rice-Husks Dumping Sites

ABSTRACT

Microorganisms are widely spread in our environment and rice husk is not an exception. Exploring the microbial communities associated with rice husk dumping sites could lead to the discovery of novel bacteria bacteria with potential industrial and environmental applications.This study was design to isolate and characterize bacteria from rice processing sites for possible industrial applications.A total of 9 soil samples were collected from Kalambaina area ofSokoto State. The heterotrophic plate count (CFU/g) revealed that soil Sample C (Diga-Tsallake, Kalambaina) had the highest bacterial population, indicating it as a promising source for industrially uses. Biochemical tests confirmed that all isolates were Gram-positive, rod-shaped, and spore-forming, consistent with the Bacillus genus. Out of 15 isolates screened for cellulose utilization using the Congo red assay, five exhibited significant activities for cellulose utilization, including *Bacillus subtilis,Bacillus sp. AT-b3,Bacillus sp. CMF 12,Bacillus lentus,* and *Paenibacillusnanensis*withMolecular identification through 16S rRNA sequencing confirmed *Bacillussp. AT-b3* and *Bacillus sp. CMF12*, respectively. This study provides valuable insights into the bacterial diversity inhabiting rice husk dumpling sites and sets the stage for further exploration of these microbial resources. These findings suggest that the strains characterize have potentials for industrial applications.

Keywords: Bacterial isolations, Rice husk, Molecular identification on novel bacteria.

1 INTRODUCTION

Rice husk is an agricultural waste that farmers have long experienced difficulties in disposing. The husk is the outer shell of the grain obtained during the first processing phase of parboiled rice, commonly called ''husking''[1][2]. The annual output of rice husks worldwide is about 120 million tons [3].

Rice husk's potential as a low cost substrate for microbial fermentation has been extensively explored, highlighting its suitability for bioactive compounds and other biotechnological applications[4]. Microorganisms have always been central in basic food processing techniques, for instance converting fibres into edible food when fermenting dough to produce bread, or milk into cheese, allowing its long-term preservation[5]. They have been often used as direct food source, as it is the case for yeast or algae. The latter, together with bacteria, constitute the microbial actors involved in processing food. They can also be used directly as feed or food source [6][7].

Rice husk is a waste product from rice milling that is abundant in Nigeria and generated during rice processing, posing environmental concerns and harbors diverse microbial communities which offers unique opportunities for the discovery of novel microorganisms. But it is underutilized due to the fact that it contains complex polysaccharide in form of lignin that is not edible for animal feed. Therefore, the need to isolate and characterize bacteria that can utilize rice husk for possible biotechnological applications.

2 Materials and Methods

2.1 Sample Collection

A total of 9 soil samples were collected from rice husk dumping sites in Kalambaina area of Sokoto State at three different locations in multiples of three (surface, 10cm depth). Ten (10g) of the samples are collected aseptically and transferred in to sterile plastic bags and transported to Microbiology laboratory of UsmanuDanfodiyo University Sokoto.

3.1 Preparation of Solutions, Reagents and Media

3.2 Nutrient Agar (NA)

NA was prepared according to manufacturer's instructions. 2.80 g of the nutrient agar powder was weighed and dissolved into 100ml of distilled water. The preparation was then heated using a hot plate to dissolve completely. A cotton wool was placed on the top of conical flask and wrapped with aluminum foil, the solution was then autoclaved at 121°C for 15min, it was then allowed to cool and was dispensed into sterile Petri-dishes [8].

3.2.1 Minimal Salt Media

Minimal salt media was prepared by dissolving 1.8g K_2HPO_4, 1.3g $KH_2\ PO_4$, 1.3g NH_4Cl, 1.03g $MgSO_4.7H_2O$, and 1.05g NaCl in 1L of distilled water. The pH was adjusted using pH meter; the medium was autoclaved at 121 °C for 15 min.

3.2.2 Starch Agar

Starch agar was prepared by dissolving 2.8 g in 100 ml of distilled water, the mixtures was placed on a hot plate and allowed to dissolve was prepared and autoclaved at 121 °C for 15 min. The agar was allowed to cool to about 45 °C before pouring into the plates and allowed to gel.

3.2.3 Urea Agar Base

Urea Agar Base (UAB) wasprepared by dissolving 2.1g of the powder in 100ml of distilled water. The mixture was heated to boiling on a hot plate. The boiled UAB was poured into various McCartney bottles and autoclaved at 121 °C for 15min. The bottles were then slanted and solidified.

3.3 Isolation and Identification of Bacteria

The samples were 6-fold serially diluted by weighing 1.0g of the soil and adding in 9ml of distilled water of the first test-tube (10^{-1}) and subsequently transferring 1ml to next test-tube until the last (10^{-6}). Afterwards, 0.1 ml of dilutions from the 5^{th}(10^{-5}) and 6^{th}(10^{-6}) test tube were transferred in to sterilized nutrient agar plates and incubated at 37 °C for 48 hours.

3.4 Inoculation:

After serial dilution was conducted, 1 ml of the 6^{th} and 7^{th} dilution fold was taken using a sterile syringe and inoculated into already prepared nutrient agar plates by spread plating method. This was done by putting the water sample on the solidified nutrient agar contained in the petri dishes, using a sterile bent glass rod, the inoculants was spread clockwise and anticlockwise, forward and backward in order to obtain the right distribution. The plates were then incubated for 24 hours at 37°C[9].

3.5 Microbial Count and Pure Culture Isolation

Total viable counts of bacteria were determined by enumerating the colony forming units (CFU) after incubation for 24 h. Here the numbers of colony forming units (CFU) was counted by dividing each of the Petri-dish into four quadrants, counting the number of colonies in one quadrant and multiply by four [6]. The average colony obtained from the countable quadrant plates, was expressed as colony forming unit per gram (Cfu/g) using the formula:

$N = A \times D$

Where N = Number of colonies, (cfu/g), A = Average count of colonies in the Petri plates and D = Dilution factor.

3.6 Sub-Culture

The various colonies observed were sub-cultured on nutrient agar using streak plate method until pure colonies were obtained. The morphologies of the colonies were observed; those with similar morphologies were considered the same and further sub-cultured into slant bottles and kept at 4°C for further analysis.

Morphological identification was performed based on visual appearance and Gram staining using the method described by [10].

3.7 Qualitative Screening of the Isolated Bacteria for Cellulose Utilization

Qualitative screening for cellulose utilization was conducted on solid state media according to the method described by [11]. The media used contained 1.7% tryptone, 0.3%peptone, 0.5% Carboxymethyl Cellulose (CMC), 0.25% dipotassium phosphate (KH_2PO_4), 1.8%agar, and 0.5% sodium chloride (NaCl). The media was prepared as follows.

After weighing the above constituents in a conical flask, the media was allowed to dissolved using a hot plate**.** The media was autoclaved at 121°C for 15 min, poured in to Petri dishes and allowed to cool at room temperature**.** The bacteria isolated was inoculated by streaking and incubated at 37°C for 48 hours**.** After incubation for 48 hours, the plates were flooded with 1% Congo red and allowed to stand for 15 minutes at room temperature. The plates were counterstained using 1.0 M NaCl**.** Clear zones appearing around bacterial colonies indicate cellulose hydrolysis. The bacteria having distinct and measurable zones will be selected for identification and characterization.

3.8 Biochemical Identification

3.8.1 Gram staining

The procedure for Gram staining was done according to the protocol of [12]. A drop of water was dispensed on a clean grease free glass slide, and a colony of young bacteria culture growth overnight were touched with sterile inoculating loop and spread evenly in the drop of water to make a thin smear. The smear was allowed to air dry and heat fixed by passing the slide through flame two to three times. Then the smear was flooded with crystal violet (primary stain) for 60 seconds and rinsed with running water. Lugol's iodine was added and allowed for 60 seconds before rinsing with running water. The reaction result was decolorized with 95% ethanol for 15 seconds. Safranin (counter stain) was added and allowed for 60 seconds and then washed with water. The slides were allowed to air dry and viewed with oil immersion objective (100X) of the microscope. Gram positive cellsretained the primary stain and appeared purple while gram negative cells appeared pink to red stain.

3.8.2 Urease test

Urease test was carried out to test for the ability of bacteria to produce urease. Bacterial isolates were inoculated in a medium which contains urea for one week at 37 ^{0}C using a sterile straight wire and indicator phenol red by stabbing through the center of the medium. It was then incubated at 37°C for 24 hours. A change in colour of the indicator to pink-red indicates a urease positive test while the initial yellow colour indicates a negative test [13].

3.8.3 Indole test

This test was conducted as described by [12]. A little portion of each isolate was inoculated into 5ml of peptone water enriched with 1% tryptophan and incubated at 37 °C for 48 hours. To the culture 0.5ml of Kovac`s reagent was added and gently shaken. In the positive test, indole (present in culture) dissolved in the reagent became red and form a layer at the surface of the medium, while yellow layer at the surface of the medium denotes a negative result.

3.8.4 Methyl Red (MR) AndVoges-Proskauer (VP) Test

This test was carried out as described by Ochei and Kolhatkar [14]. A little portion of each isolate was inoculated into a glucose phosphate peptone water medium and incubated at 37 °C for 48 hours. Few drops of methyl red were added to the culture, MR positive test indicates red colour formation

at the surface of the medium while no change denotes negative. MR test is usually done in conjunction with VP test. Retaining the initial colour in the medium during MR test indicates a positive VP test. Therefore, an organism is either MR or VP positive.

3.8.5 Motility Test

A sterile needle was used to pick a small loop of 24h old culture and stabbed onto nutrient agar (i.e. triple sugar iron agar) in glass vials. The vials was be incubated at 37 °C for 48hrs. Non-motile bacteria have a growth confined to the stab line with definite margins without spreading to surroundings area while motile bacteria gave diffused column extending from the surface [15].

3.8.6 Triple Sugar Ion Test

Triple sugar ion was prepared with a sterile needle, the culture from the solid medium was streaked on the surface of the slant and butt was stabbed 3 times and incubated at 37°C for 24h. Gas formation was indicated by the appearance of bubbles. TSI glucose fermenters were indicated by the butt becoming yellow. Yellowing of both the butt and slope indicates fermentation of glucose and sucrose, fermentation of lactose and sucrose but not that of glucose was observed by red butt and yellow slope [15].

3.8.7 Citrate Test

A little portion of the isolate was inoculated into Koser-Citrate medium and incubated at 37 °C for 72 hours. A positive citrate was confirmed by formation of bright blue colour while the initial green colour of the medium donates negative test [15].

3.8.8 Catalase Test

Catalase test was performed according to the procedure of [12].Catalase is an enzyme found in most bacteria. It catalyzes the breakdown of hydrogen peroxide with the release of free oxygen water. The test was used to determine whether the bacteria can produce catalase enzyme. A loop full of 24 hours old culture of each isolate was kept on a clean microscope slide. A drop of 3 % hydrogen peroxide (H_2O_2)was be added to it. The production of bubbles shows the presence of catalase enzyme while absence of bubbles indicates absence of catalase enzyme and was recorded negative.

3.8.9 Starch Hydrolysis Test

This is used to check for the ability of bacterial isolates to utilize starch. Bacteria isolates was inoculated on the agar and incubated at 37 °C for 24 hours after which Lugol's iodine was spread on the agar. The colour around the bacterial colonies was then be observed. The colonies with blue-black indicate absence of starch hydrolysis, while those that turned green or yellow coloration indicate starch hydrolysis.

3.10Molecular Identification

3.10.1 Identification of The Bacterial Strain Using 16S rRNA Gene Sequencing

3.10.2 DNA Extraction

The presumptiveisolates were sub-cultured on nutrient agar and incubated for 24h at 37 °C. [16]. Part of the bacterial colony was picked with a sterile wire loop and suspended in 200 µl of TE buffer in order to prevent the DNA from degradation, at pH 8 containing RNase (50 ng /ml), then 400 ml of lysis buffer was added followed by mixing well and incubation for 15 min at 37°C with intermittent shaking for every 5 min. Immediately chloroform and isoamyl-alcohol in the ratio (24:1) was taken

and mixed by inversion. Tubes was centrifuged at 10000 rpm for 5 min, supernatant was transferred carefully to another micro-centrifuge tube. To the supernatant, 0.1 µl 3 M sodium acetate (pH = 5.2) and 0.6 µlisopropanol was added, mixed well by inversion and kept in the ice for 10 min followed by centrifugation at 1000 rpm for 10 min. The pellet was washed with 70% ethanol with gentle shaking and centrifuged at 10000 rpm for 3 min. Supernatant was removed and pellet was air dried. Extracted DNA was visualized in 0.8% agarose gel electrophoresis and images were documented [17].

3.10.3 Amplification of the Bacteria 16S rRNA gene

The reaction master-mix for each sample was preparedusing 16S ribosomal RNA gene specific universal primers 27F 50-AGA GTT TGA TCC TGG CTC AG-30 and 1492R 50-GGT TAC CTT GTT ACG ACT T-30 (Sigma), the 16S rRNA gene was amplified with 2.5 µl of PCR master mix (Biolabs), 5.5 µl of Nuclease free water (Biolabs©), 5µl of DNA template and 1µL of each 16S forward primer and reverse primer 926R. The amplification was carried out in an Appliedbiosystem 9700 thermo-cycler using the following protocol:

Initial denaturation at 95 °C for 5 minutes, followed by a 36 cycles of Denaturationat 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C 1 minute 30 seconds and final extension 72 °C for 10 minutes.

3.10.4 Agarose Gel Electrophoresis

The electrophoresis was done using 1.5% agarose gel and stained with ethidium bromide DNA Gel stain (Sigma Aldrich Missouri USA) and 1X TBE buffer according to standard (85V, 3.00A, 300W) for 45 minutes. The cast and comb was set, the gel was poured and allowed to solidify. Positive bands were checked on the gel under UV-light using Bio-Rad gel imager. Amplicons of the positive samples were sent to Inqaba Biotechnical Industries Ibadan, which was further transported to the industry's main branch in Pretoria, South Africa for sequencing.

3.10.5 Sequencing

PCR products was purified using a Monarch PCR and DNA clean up kit (Biolabs) following the instructions of the manufacturer.DNA (10–100 ng) was sequenced in only forward directionwith a Big-Dyeterminator version 3.1 cycle sequencing kit (AppliedBiosystems). Sequence studio genetic analyzer, (AppliedBiosystems) using the PCR primers (IDT) as sequencingprimers.

3.10.6 Bioinformatics Analysis

Basic Local Alignment Search Tool (BLAST) was used to determine sequence identities and query cover. The obtained sense and anti-sense sequences was submitted for quality evaluation using Phil's Read Editor (Phred) online application [18]. The sequences were assembled together with the Cap-Conting application in Bio-edit 7.0.9.0 software. Multiple Sequence Comparison with Log Expectation (MUSCLE) was used to align the sequences with the reference sequences downloaded from National Center for Biotechnology Information (NCBI) database as recommended by [19] using MEGA 7 software [20].The software was used to construct a nucleotide Phylogenetic tree (Neighbor-joining, 1,000 bootstrap replications).

16S rRNA gene sequences from all relevant reference strains available in GenBank (NCBI) were used for comparison. The tree was used to determine the genotype of the sequenced 16S rRNA strains. The evolutionary distances were computed using the Maximum Composite Likelihood method [21].

All positions containing gaps and missing data were eliminated so that the total positions in the final dataset will be indicated.

3.10.7 Phylogenetic Analysis

The Phylogenetic tree of the BLAST sequences and the evolutionary history were inferred using the Neighbor-Joining method [22]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [23]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [21] and are in the units of the number of base substitutions per site [20]

3. RESULTS AND DISCUSSION

Bacteriological Analysis

Table 1 shows theheterotrophic bacterial count (CFU/g) from soil of rice husk dumping site in Kalambaina Area. The result shows thatSample C "from Digan TsallakeKalambaina" had the highest colony forming units as compared with that from Sample A "Wamakko Road" and Sample B "ShiyarArkilla".

Screening for Cellulose Utilization

The screening for cellulose utilization was presented in Table 2 the result indicated that five (5) isolates were positive for cellulose utilization out of which two of them shows the clearest and sharpest zone of hydrolysis.

Biochemical Identification Tests

Table 3 presents thebiochemical identification of the bacterial isolates. Five (5) bacterial isolates were identified in this study. The biochemical tests for all the isolates showed that all isolates were gram-positive rod and motile species as shown in (Table 3).

Table 1: Heterotrophic mean of bacteria count (CFU/g) of bacteria isolated from Rice husk contaminated soil in KalambainaSokoto.

Sample	Mean of Bacterial colony count (CFU/g)
A	$4.50x10^6$
B	$3.91 x10^6$
C	$5.00 x10^6$

CFU = Colony forming unit.

Table 2 Screening for Cellulose Utilization of bacteria Isolated from rice husk dumping sites in Kalambaina area, of Sokoto.

Isolate Code	KLB1A	KLB1B	KLB1C	KLB1C	KLB1D	KLB1E	KLB2A	KLB2B	KLB2C	KLB2D	KLB2E	KLB2F	KLB3A	KLB3B	KLB3C	KLB3D
Cellulose Utilization	+	-	-	-	-	-	-	+++	-	-	++	-	+	-	+	-

Table 3: Biochemical Identification of the Bacterial Isolates from rice husk dumping sites in Kalambaina area, of Sokoto.

Test	KLB1A	KLB2B	KLB2E	KLB3A	KLB3C
Gram	+Rod	+Rod	+Rod	+Rod	+Rod
Motility	+ve	+ve	+ve	+ve	+ve
Spores	+ve	+ve	-ve	-ve	-ve
Citrate	-ve	-ve	+ve	-ve	-ve
Catalase	+ve	+ve	+ve	-ve	-ve
V.P	+ve	-ve	-ve	-ve	+ve
MR	-ve	+ve	+ve	+ve	-ve
Indole	-ve	-ve	-ve	-ve	-ve
Starch	+ve	+ve	+ve	+ve	+ve
Glucose	+ve	+ve	+ve	+ve	+ve
Lactose	-ve	+ve	+ve	+ve	-ve
Sucrose	+ve	-ve	-ve	+ve	-ve
Urease	+ve	+ve	-ve	+ve	+ve
organism	*Bacillus subtilis*	*Bacillussp.AT-b3*	*Bacillus sp. CMF 12*	*Bacillus lentus*	*Paenibacillusnanensis*

3.4 Molecular Identification

The electrophoretogram of 16S rRNA primer products indicatesthe amplification based on polymerase chain reaction (PCR) which was presented in Plate 1. The amplification produced 16S ribosomal RNA gene amplicons of approximately 400bpon agarose gel electrophoresis.The sequences identified by NCBI BLAST indicate that the two isolates belonged to genera *Bacillussp.*with variation at species level. Their percentage hit similarityis above 90% as shown in Table 4.

3.5Phylogenetic Analysis of the Bacterial Isolates

Figure 1 and 2 shows the phylogenetic tree indicating the evolutionary relationship among the identified species and other species based on similarities and differences in their evolutionary genetic characteristics as compared with their related species from the database of the GenBank NCBI.

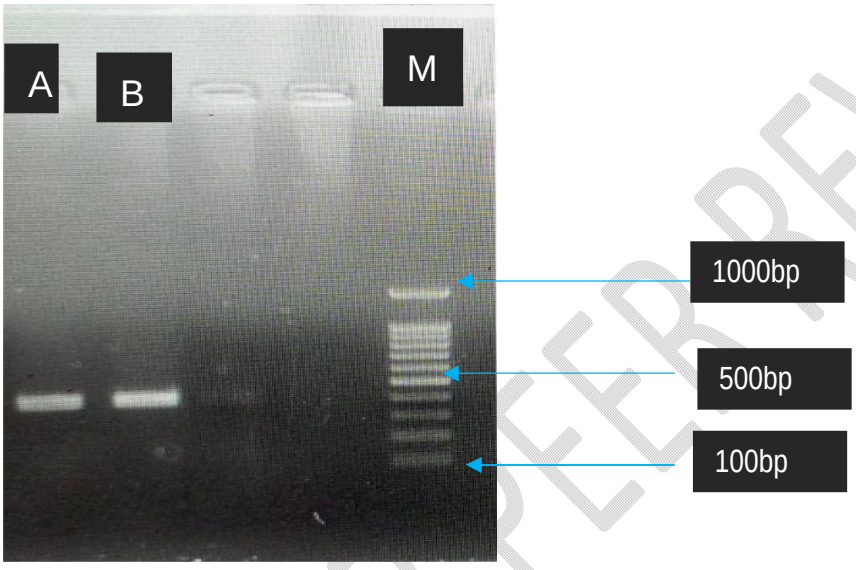

Plate 1: Gel image of the isolates by Agarose gel electrophoresis.

A=*Bacillus sp*. CMF 12

B= *Bacillus sp.*Atb3

M=DNA ladder

Table 4. Molecular identification of Single cell production bacteria isolated from soil of rice husk dumping site at Kalambaina Area of Sokoto state.

Isolates	Organism	Strain code	Percentage Identity (%)	NCBI Accession Number
KLB 2B	*Bacillus sp.*	Strain AT-b3	91.46	MH348970
KLB 2E	*Bacillus sp.*	Strain CMF 12	99.73	CP085392

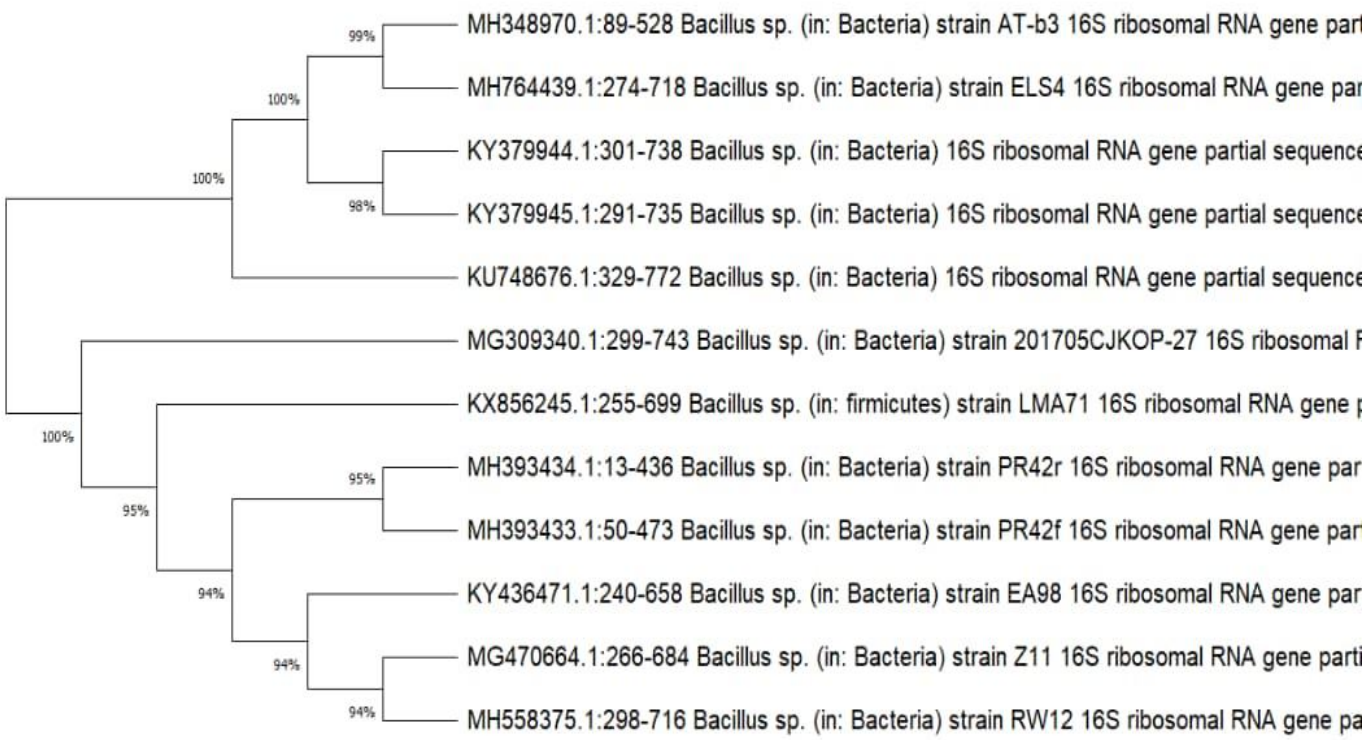

Figure 1: Phylogenetic Tree for *Bacillus spp. AT-b3*based on 16S rRNA Sequence using Neighbor Joining Method.

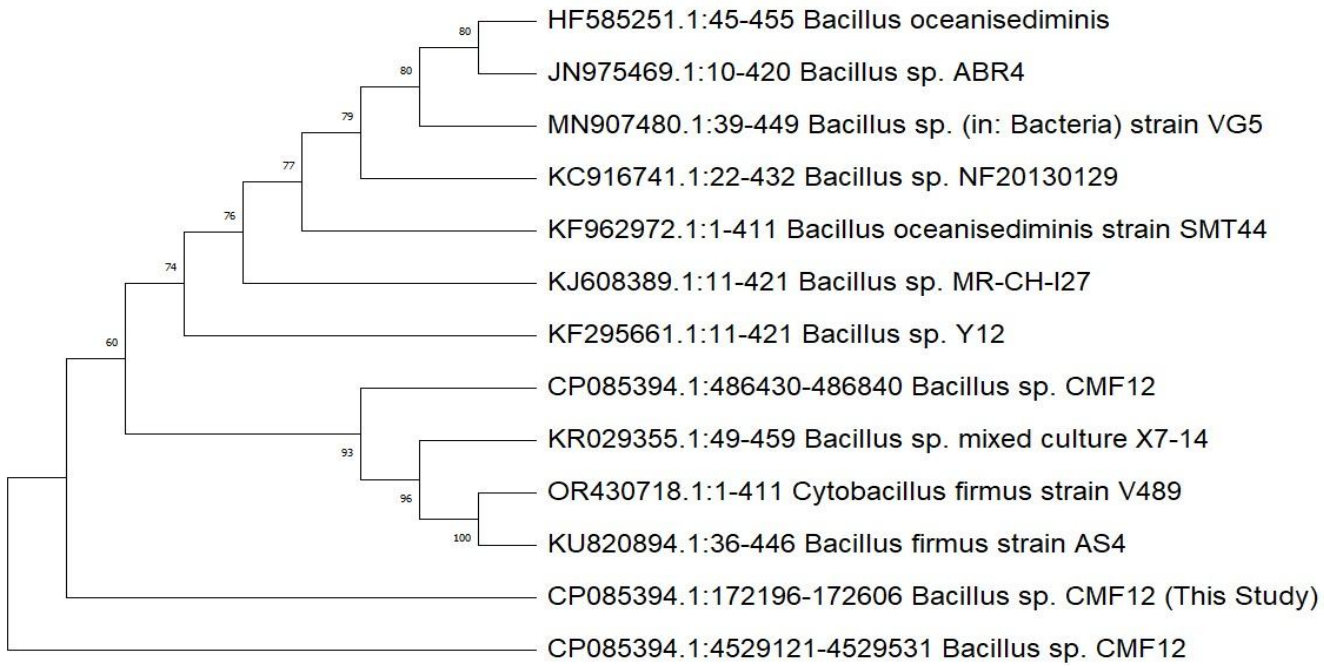

Figure 2: Phylogenetic Tree for *Bacillus sp.CMF12* based on 16SrRNA Sequence using Neighbor Joining Method.

The heterotrophic bacteria count (CFU/g) indicates a highest bacterial count in soil Sample C as compared to Sample A and sample B. This shows that there are more bacteria isolates from thesoil sample C from "Diga-TsallakeKalambaina" when compared to others. Therefore, when bio-prospecting for the bacterial colonies, soils from sample C should be considered as site for sample collection.Biochemical characterization indicated that all the bacterial isolates were Gram positive, rod shaped and spore forming bacteria.

Out of the fifteen (15) bacterial isolates isolated, five (5) isolates showed cellulose utilizing activity indicated by the zone of clearance due to change of the colour of Congo-red dye to white. The isolates were identified as *Bacillus subtilis, Bacillus sp. AT-b3, Bacillus sp. CMF 12, Bacillus Lentus and Paenibacillusnanensis*. Molecular characterization of the most potent bacterial isolates revealed the two isolates as *Bacillus sp. AT-b3* and *Bacillus sp. CMF 12*. Molecular identification of *Bacillus spp*. strain atb3 isolated from soil was identified [24] China. The occurrence of all these isolates in rice husk contaminated soil could be attributed to the abundance of microorganisms found in the soil and their ability to utilize or degrade the carbon source present in that soil.

4 CONCLUSION

The study isolated and characterized various bacterial strains from rice husk dumping sites in Kalambaina area of Sokoto State. The isolated bacteria were found to belong to Bacillus species, particularly *Bacillus sp. AT-b*3 and *Bacillus sp. CMF 12* based on 16S rRNA gene sequencing. These bacteria exhibited unique morphological, physiological and biochemical properties indicating their capacity for adaptationto rice husk environment. This capabilities for utilization of cellulosecan be used for environmental bioremediation and therefore suggest that these strains exhibited potential for industrial applications.

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