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ISOLATION AND MOLECULAR IDENTIFICATION OF *WEISSELLA CIBARIA* FROM CABBAGE

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ABSTRACT
Weissella is a genus of lactic acid bacteria that is gram positive, catalase negative, coccoid shaped. *Weissella cibaria* can be found in the initial stage of lactic acid fermentation in cabbage in the presence of salt. This study was conducted to isolate and identify *Weissella cibaria* to molecular level from cabbage. Freshly harvested cabbage was shredded and brined in 2.5 % salt (NaCl) for 48 h, it was inoculated aseptically into deMan, Rogosa and Sharpe agar (MRS) and incubated for three (3) days at 37 ^{o}C. Conventional tests carried out on isolate were grams reaction, catalase production, sugar fermentation tests using glucose, sucrose, maltose, lactose and fructose. Isolate was extracted using AccuPrep Genomic DNA Extraction kit, amplified, agarose gel electrophoresis was carried out and viewed under UV light, sequence was obtained and compared to those in the GenBank database using the BLAST program at the NCBI-GenBank database. Isolate was gram positive, cocci, catalase negative, could utilize certain sugars such as glucose, fructose, sucrose, maltose. Isolate showed bands at 789 base pair. Sequence submitted to the basic local alignment search tool (BLAST) resulted to *Weissella ciberia* strain PON10032 with 94 % identity, a score of 897 bits and a sequence ID of KC416978.1.

Keywords: *Weissella cibaria*, cabbage, Polymerase chain reaction, BLAST.

INTRODUCTION

Weissella is a genus named after a German microbiologist Norbert Weiss due to his contributions in lactic acid bacteria research. The Weissella genus is made up of lactic acid bacteria that are Gram positive, catalase negative, coccoid or rod shaped depending on medium of growth, they are obligate heterofermenters that produce carbon (iv) oxide (CO_2) from carbohydrate metabolism (Bjorkroth *et al.*, 2011). Lactic acid bacteria (LAB) are adept in producing substances with inhibitory activity against microorganisms present in foods (Das, 2019). Lactic acid bacteria are of industrial importance because they are generally regarded as safe (GRAS) and have ubiquitous appearance in food and contribute to the mucosal surface of the microflora of a healthy human (Das, 2019). Weissella belong to the firmicutes, class Bacilli, order Lactobacillales and members of the Leuconostocaceae family. Weissella species are grouped into five phylogenetic branches and 19 species based on 16S phylogeny (Bjorkroth *et al*., 2011). *Weissella cibaria* is the target organism which is drawing the attention of researchers because of their high probiotic potential and production of copious amounts of novel, non-digestible oligosaccharides and extracellular polysaccharides especially dextran (Thiyagarajan *et al*., 2017). Due to the large number of species molecular identification is need ascertain the exact specie that is isolated (Bjorkroth *et al*., 2011). *Weissella cibaria* grows rapidly in vegetables at various temperatures and salt concentrations. *Weissella cibaria* is associated with sauerkraut (fermented cabbage) and pickle fermentations by initiating the lactic acid fermentation required. This organism is used because of its unique difference from other lactic acid species due to its tolerance to high concentration of salt and sugar (Thiyagarajan *et al*., 2017).

MATERIALS AND METHODS

Collection of Sample

Freshly harvested cabbage herd were purchased after undergoing physical inspection to ensure maturity, size and firmness, placed in a sample bag and transported to the food and industrial laboratory in Kaduna State University (Thiyagarajan *et al*., 2017).

Isolation of *Weissella cibaria* from Fermented Cabbage

Cabbage was stored at room temperature for 24 h to bring to facilitate shredding. It was washed thoroughly and cut into thin slices using sterile scalpel. 5.0 g of shredded cabbage was placed in a sterile beaker with 2.5 % salt (NaCl), aluminium foil was used to cover the mouth of beaker providing holes for air. It was stored at 25 ^{o}C for 48 h to ferment. The fermented cabbage was subjected to mechanical pressure producing juice containing organism and fermented sugar (Thiyagarajan *et al*., 2017).

Isolation and Preparation of Inoculum

According to manufacturer's instruction de Man, Rogosa and Sharpe agar (MRS) was prepared and poured into petri dishes, exactly 0.1 mL of vancomycin was added to medium before solidification to inhibit the growth of unwanted organisms and allowed to solidify. Exactly 1.0 mL of cabbage juice was placed on the centre of sterile petri dishes using a sterile syringe. About 15 mL molten cooled MRS agar was poured into the petri dish containing inoculum and swirled to mix and incubated for 3days at 37 °C for growth of the organism. The culture with characteristic morphology was subjected to microscopic and biochemical tests (Thiyagarajan *et al*., 2017).

Conventional Identification of Lactic Acid Bacteria Isolated from Fermented Cabbage

Several conventional methods of identification were carried out there are grams reaction, catalase production, sugar fermentation tests using glucose, sucrose, maltose, lactose and fructose.

Grams Reaction

A fresh sample of 24 h of growth was used for this test. A glass slide was cleaned thoroughly, flamed and allowed to cool on work bench. Holding the edge of the slide a drop of water was placed on the centre. A sterile wire loop was used to transfer a loopful of bacterial sample from media plate to centre of the slide were water was added lightly mixed until a turbid suspension was formed. This was fixed by passing the slide over flame three times. The smear was stained with crystal violet solution for 60 sec which was the primary dye. It was washed with runny water. It was then flooded with Lugol's iodine for 30 sec and washed off under runny water. It was decolourised with alcohol by tilting the slide and adding it drop wise until all colour was removed. It was also washed off with water. The slide was then counter stained with diluted carbol-fuchsin for 60 sec which was also washed off with water and allowed to air dry. The smear was viewed under a microscope after adding oil immersion. This process was repeated for both samples (Oyeleke and Manga, 2008).

Catalase Test

One drop of 3 % hydrogen peroxide was placed on a sterile glass slide a loop full of the isolate was mixed on the slide and observed for bubbling or frothing (Oyeleke and Manga, 2008).

Sugar Fermentation Test

Sugar fermentation was determined using the following sugars sucrose, glucose, lactose, fructose, and maltose. The Kligler iron agar (KI) and the triple sugar iron agar (TSI) were prepared according to manufacturer's instruction in a bijou bottle making a slant before gelling. A needle full of isolate was aseptically streaked the top and stabbed to the bottom of the slant three times. Cap was loosely closed and incubated for 24 h. Gas formation was determined by was appearance of bubbles or crack at the bottom. Hydrogen sulphide was determined by blackening of the bottom. Glucose fermentation was determined by the bottom becoming yellow. For lactose and sucrose fermentation both the bottom and the slant will become yellow (Oyeleke and Manga, 2008).

Molecular Identification of *Weissella cibaria*

DNA Extraction of *Weissella cibaria*

The AccuPrep Genomic DNA Extraction kit was used for DNA extraction. Firstly Proteinase K was completely dissolved in 1.250 µL of nuclease-free water, RNase A was also dissolved in 600 µl of nuclease free water and absolute ethanol was corrected to WA1 Buffer and kept to be used. Cultured cell was centrifuged for 5 min, supernatant was carefully discarded without disturbing the pellet. 200 µl of phosphate buffer saline (PBS) was suspended into pellet. 20 µl of prepared proteinase K and 10 µl of RNaseA were added to pellet mixed thoroughly and incubated for 2 min at 25 °C. 200 µl of GBbuffer was added and mixed using a vortex mixer and incubated for 10 min at 60 °C. Exactly 400 µl of absolute ethanol was added and mixed by pipetting. Lysate was transferred into reservoir of binding column tube, closed and centrifuged at 8000 rpm for 1 min. Solution in collection tube was discarded and reused. 500 µl of W_2 buffer was added, closed and centrifuged at 8000 rpm for 1 min. Collection tube was discarded and reused. It was centrifuged at 13,000 rpm for 1 min to remove ethanol completely. Binding column tube was transferred to a 1.5 ml tube for elution, 50 µl of EA buffer was added into binding column tube and allowed for 1 min at 15 °C centrifuged at 8000 rpm for 1 min to elute (BIONEER, 2020).

DNA Amplification of *Weissella ciberia*

Primer pairs for DNA amplication were 27F (5`-AGAGTTTGATCCTGGCTCAG-3`) and 1492R (5`-GGTTACCTTGTTACGACTT-3`) (Angel *et al*., 2013). The optimized PCR conditions used was: PCR cocktail was pre-denatured at 94 °C for 5 min, denatured at 94 °C for 6 min and 30 cycles with each denaturation at 94 °C for 30 sec and annealing at 52 °C for 30 sec. Extension for 1 min at 72 °C and 35cycles, final extension was done for 5 min at 72 °C (BIONEER, 2020).

Gel Electrophoresis of DNA of *Weissella ciberia*

Exactly 1.5 g of agarose powder was dissolved into 100 ml of tris acetate EDTA (TAE), it was heated in the microwave and placed in water bath to cool at 55 °C. Gel casting tray was prepared using a casting system and the appropriate number of combs in gel tray. 5 µl of ethidium bromide was added to cooled gel and allowed for 30 min at 25°C. Comb was removed gel was placed in electrophoresis chamber and covered with TAE. The DNA and standard (ladder) were pipetted into wells and allowed to run for 30 min. It was viewed under UV light for the presence of bands. The large reaction was prepared using the same process. The bands were cut, buffer GB was added and incubated for 10 min to dissolve gel. It was transferred to a binding column which was placed in a collection tube before centrifuging for 1 min. Solution in collection tube was discarded, washing buffer was added and allowed to sit for 5 min before centrifuging. Collection tube was discarded, washing buffer was added again and centrifuged for 5 min at 12000 rpm. The PCR water was added to binding column and incubated for 3 min. Binding column was removed and send for sequencing.

Sequencing of DNA of *Weissella ciberia*

A fresh stop solution of glycogen mixture consisting of 2 ul of 3 M Sodium acetate, 2 ul of 100 mM Na2-EDTA and 1 ul of 20 mg/ml of glycogen (provided in the kit) was prepared. To each of the tubes 5 ul of the stop solution/glycogen mixture was added. Sequencing reaction was transferred to each of the tubes and mix thoroughly. 60 µl of cold 95 %(v/v) ethanol from -20 freezer was added and mix thoroughly. It was immediately centrifuged at 14,000 rpm at 4 °C for 15 min. carefully removed the supernatant with a micropipette. It was rinsed with 200 µl of 70 % (v/v) ethanol from -20 freezer and centrifuged at 14,000 rpm at 4 °C for 2 min carefully all the supernatant was removed with a micropipette and dried using a vacuum. Sample was resuspended into 40 µl of sample loading solution. The resuspended samples were transferred to the appropriate wells of the sample plate each was overlaid with one drop of mineral oil from the kit and loaded into the instrument to run.

Sequence Alignment and Basic Local Alignment Search Tool (BLAST) of Query

The obtained sequence was compared to those in the GenBank database using the BLAST program at the NCBI-GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Isolates was allocated to species based on percentage of sequence identity and manual inspection of sequence at key variable positions (Angel *et al*., 2013).

RESULTS AND DISCUSSION

Isolation from fermented cabbage

Shredded cabbage brined in 2.5 % salt (NaCl) after 48 h of fermentation created a distinct odour and flavour was produced.

The juice stood on the surface with a pale white colour while the shredded cabbage was at the bottom and the natural colour remained.

Lactic Acid Bacteria Isolated From Fermented Cabbage
Lactic acid bacteria were isolated from fermented cabbage and identified by microscopic characterisation and biochemical screening. The Table 1 shows two (2) lactic acid bacteria. The microscopic and biochemical characterization confirmed the species to be *Leuconostoc mesenteroides* and *Weissella cibaria.*

Molecular Confirmation of Isolated Lactic Acid Bacteria
Agarose Gel Electrophoresis of Isolated Lactic Acid Bacteria
Gel electrophoresis for both isolate 1 and 2 was viewed and photographed using a UV transilluminator as presented in Plate 1. Bands were seen on both samples.

Basic Local Alignment Search Tool (BLAST) for the Identified Isolates
The sequence alignment of the resulted query was compared to data available in NCBI GenBank data base by BLAST. This revealed that isolate 2 showed 94% identity to *Weissella ciberia* strain PON10032 16S ribosomal RNA gene and a sequence ID KC416978.1 with a score of 897 bits.

Table 1: Microscopic and Biochemical Characteristics of Isolated Lactic Acid Bacteria

Biochemical test/Isolate code	1	2
Grams Rxn	+	+
Shape	Cocci	Cocci
Catalase	–	–
Citrate utilization	+	+
H_2S production	+	+
Growth at 4°C	+	–
Growth at 37°C	+	+
Glucose	+	+
Fructose	+	+
Sucrose	+	+
Maltose	+	+
Lactose	+	–
Probable organism	*Leuconostic mesenteroides*	*Weissella cibaria*

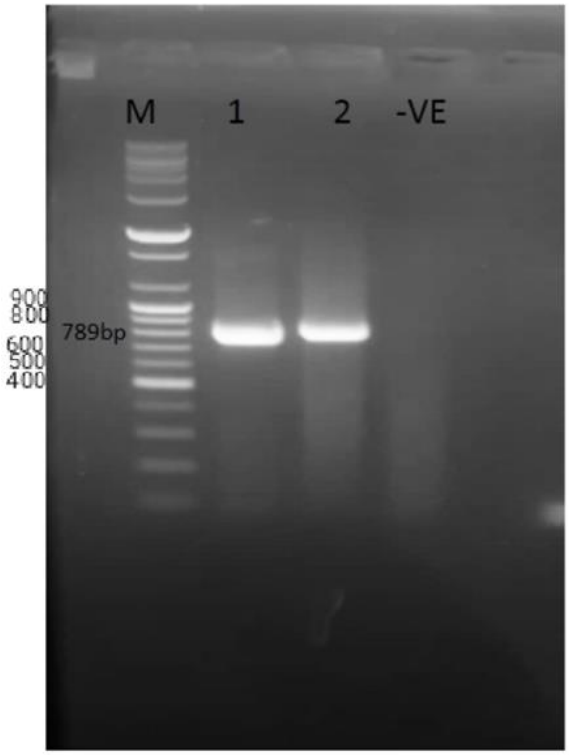

Fig.1: Gel electrophoresis of the amplicon

KEY
M: PCR Marker (789bp)
Lane 1: Positive PCR result
Lane 2: Positive PCR result

DISCUSSION
The source of culture which was the shredded fermented cabbage was squeezed out producing a pale white juice. The cabbage shred remained at the bottom of the beaker maintaining its natural colour. The 2.5 % salt (NaCl) enhanced the extraction of the vegetable juice, nutrients and sugars. As recorded by Thiyagarajan *et al*. (2017) salt (NaCl) inhibits the growth of microorganisms while enhancing the growth of lactic acid bacteria. The extracted brined vegetable juice was inoculated into deMan Rogosa and Sharpe agar supplemented with antibiotics vancomycin. Vancomycin resistance is a unique characteristic of lactic acid bacteria. After three (3) days of incubation at 37 °C growth was observed on media plate. The whitish, smooth edge, circular colonies observed on plates was in agreement with the characteristics of *Weissella ciberia* as listed by Collins in 1993. These finding agrees with Thiyagarajan *et al*. (2017) who reported to have gotten very tiny greyish, entire white, smooth, round colonies with a diameter of 1.0mm diameter on media plate after four (4) days of incubation. These two distinct colonies were subcultured for further identification. The colonies were both identified to be gram positive, cocci, catalase negative, citrate positive. Isolate 1 grew at temperatures 4°C and 37°C while isolate 2 did not grow at 4°C but grew at 37°C. This result agrees with Bjorkroth *et al*. (2012) who reported *Weissella ciberia* grew at 15°C, 37°C and 45°C but did not grow at 4°C. Sugar fermentation was carried out with the following

sugars glucose, sucrose, fructose, lactose and maltose. The results were same in some sugars but varied in some. In glucose, fructose, sucrose and maltose tested positive in both isolate 1 and 2 while lactose tested positive in isolate 1 while isolate 2 tested negative. Safika *et al*. (2019) and Thiyagarajan *et al*. (2017) recorded similar results as isolate B where the isolate tested negative for catalase, maltose and lactose, it tested positive for glucose, sucrose and fructose. *Weissella cibaria* hydrolyses sucrose, glucose, fructose as an obligatory substrate in extracellular synthesis homopolysaccharides such as dextran. Glucan-sucrose and fructan-sucrose are highly specific enzymes used to hydrolyse sucrose and fructose. They cleave the glycosidic linkage of sucrose, glucose and fructose unit to a glucan/fructan chain, water, sucrose and other acceptors (Meng *et al*. 2016).

Isolates were having characteristic properties of *Leuconostoc mesenteriodes* for isolate 1 and *Weissella ciberia* for isolate 1 they were further identified by carrying out molecular identification.

Agarose gel electrophoresis (0.8%) was carried out and viewed under ultraviolet light which showed bands on both isolates at 789 basepair. After sequencing of amplified DNA the result was compared in Genbank database using basic local alignment search tool (BLAST) where isolate 2 sequence result was submitted to the basic local alignment search tool (BLAST) which resulted to *Weissella ciberia* strain PON10032 with 94% identity, a score of 897 bits and a sequence ID of KC416978.1. *Weissella ciberia* is among the generally regarded as safe (GRAS) microorganisms used in dextran production by various industries such as food, pharmaceuticals and clothing industries.

Conclusion

This study shows that a lactic acid bacterium identified to be *Weissella ciberia* can be isolated from fermented cabbage with 94% identity.

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