



Isolation and Characterization of Acetic Acid Bacteria from Orange and Watermelon Peel Wastes in Ilorin, Nigeria

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Abstract

Fruit peel wastes may serve as inexpensive sources of acetic acid bacteria (AAB) for biotechnological applications. This study investigated the isolation and characterization of AAB from orange (*Citrus sinensis*) and watermelon (*Citrullus lanatus*) peels obtained from Ipata Market, Ilorin, Kwara State, Nigeria. The samples were subjected to enrichment and serial dilution, followed by isolation on Glucose Yeast Calcium Carbonate Agar (GYC). Presumptive isolates were characterized using cultural, morphological and biochemical tests and screened for oxidizing activity on Carr's medium. Total heterotrophic bacterial counts were $1.60 \pm 0.20 \times 10^5$ CFU/g for watermelon peel and $1.50 \pm 0.30 \times 10^5$ CFU/g for orange peel, while presumptive AAB counts were $2.20 \pm 0.20 \times 10^3$ and $4.80 \pm 1.40 \times 10^2$ CFU/g, respectively. Two presumptive AAB isolates were recovered. Based on phenotypic and biochemical characteristics, the isolates were assigned to *Acetobacter* sp. and *Gluconobacter* sp. 16S rRNA gene sequencing identified representative isolates as *Acetobacter aceti* and *Gluconobacter oxydans*. The findings demonstrate that orange and watermelon peels harbour AAB and may serve as accessible sources of potentially useful strains for further investigation of acetic acid fermentation and other biotechnological applications.

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1.0 Introduction

The increasing generation of fruit waste has become an important environmental and economic concern due to the rapid growth of fruit processing, marketing and consumption. Fruit wastes, including peels, pulp and seeds are often disposed of through uncontrolled dumping

or other inappropriate methods. Such practices may contribute to environmental pollution, unpleasant odours, greenhouse gas emissions, the proliferation of microorganisms and disease vectors (Sarker *et al.*, 2024). However, fruit waste contains considerable amounts of fermentable sugars, organic acids, minerals, and

other nutrients that can support microbial growth, serving as substrates for the production of valuable bioproducts. Consequently, the conversion of fruit waste into useful products represents an important approach to waste valorization and the development of sustainable biotechnological processes (Galanakis, 2021).

Acetic acid bacteria (AAB) are a group of strictly aerobic, Gram-negative bacteria belonging primarily to the family Acetobacteraceae. The genera *Acetobacter* and *Gluconobacter* are among the most important members of this group because of their ability to oxidize ethanol into acetic acid through oxidative fermentation. In addition to ethanol oxidation, AAB can oxidize a wide range of sugars and alcohols to their corresponding organic acids, making them valuable microorganisms in food and industrial biotechnology. They are extensively used in vinegar production and the biosynthesis of commercially important compounds such as gluconic acid, dihydroxyacetone, and bacterial cellulose (Román-Camacho *et al.*, 2023; Han *et al.*, 2024).

Acetic acid bacteria are widely distributed in natural environments where sugars and ethanol are available. They are commonly isolated from fruits, flowers, fermented foods, alcoholic beverages, and fruit processing residues. Damaged fruits and fruit peels provide favourable ecological niches for these organisms because of their high moisture content, abundance of carbohydrates, and naturally occurring microbial populations. Indigenous AAB isolated from local fruit wastes may possess physiological characteristics that enhance their adaptation to specific environmental conditions, including tolerance to acid stress, temperature fluctuations, and variable substrate composition, which are important attributes for industrial fermentation processes (YassunakaHata *et al.*, 2023; Hua *et al.*, 2024). The metabolism of AAB is characterized by membrane-bound dehydrogenase systems that catalyse the oxidation of ethanol to acetaldehyde

and subsequently to acetic acid. During vinegar fermentation, these bacteria are exposed to high concentrations of ethanol and acetic acid, conditions that can inhibit microbial growth and fermentation performance. Understanding the physiological properties of indigenous AAB strains is therefore important for selecting organisms with improved fermentation efficiency and acid tolerance (Román-Camacho *et al.*, 2023). Recent studies have highlighted the importance of microbial diversity and stress adaptation mechanisms in improving industrial acetification processes (Hua *et al.*, 2024).

Orange (*Citrus sinensis*) and watermelon (*Citrullus lanatus*) are widely consumed fruits that generate substantial quantities of waste during processing and consumption. Their wastes contain varying concentrations of fermentable sugars and other nutrients that may support the growth of microorganisms (YassunakaHata *et al.*, 2023). Differences in the chemical composition of these fruit wastes may also influence the occurrence, diversity, and abundance of AAB associated with each substrate. Consequently, the isolation of AAB from different fruit wastes may provide an opportunity to recover organisms with varying physiological and metabolic properties (Han *et al.*, 2024).

Despite increasing interest in fruit-waste valorization and the established association of AAB with fruit materials, information remains limited on the occurrence and molecular identity of AAB associated specifically with discarded orange and watermelon peels in Ilorin, Nigeria. In addition, local studies have largely emphasized isolation and preliminary phenotypic characterization, with limited integration of phenotypic screening and 16S rRNA-based identification.

Therefore, this study investigates AAB associated with these two locally available fruit wastes and evaluates their preliminary ethanol-oxidizing activity as a basis for subsequent quantitative fermentation studies.

2.0 Materials and Methods

2.1 Sample Collection

Fruits wastes of orange and watermelon were randomly procured from fruit marketers at Ipata market, within Ilorin city, Kwara State. Collected samples were each placed in ziploc polythene bags, and immediately transported to the laboratory for analysis.

2.2 Sterilization of Materials

The workbench was disinfected with 70% ethanol-soaked cotton wool before laboratory procedures. Culture media were sterilized by autoclaving at 121 °C and 15 psi for 15 min. Sterile pipette tips, sterile diluents, and sterile disposable Petri dishes were utilized for containment of cultures.

2.3 Media Preparation

The general-purpose medium Nutrient Agar (ReadyMED RDM-NA-01, India) was used for the enumeration of total heterotrophic bacteria associated with the samples; 28grams of the dehydrated NA was dissolved in 1000 mL distilled water; the mixture was homogenized on a magnetic stirrer hot plate, sterilized, and cooled before pouring. The Glucose-Yeast Extract-Calcium carbonate (GYC) agar was the constituted medium used for the isolation and the presumptive screening for targeted acetic acid bacteria. The medium was composed of 10 % D-glucose, 1.0% yeast extract, 2.0% calcium carbonate (dissolved in distilled water at 80±10 °C), and 1.5% agar-agar.

Carr's medium was the constituted medium used as a confirmatory assay for potential bacteria with acetic acid production. The medium was made of yeast extract (30 g/L), bromocresol green (0.02 g/L), agar-agar (20 g/L), ethanol 2% (v/v); 100 mg/L pimarin was inhibitory supplement to the growth of yeast and molds, and pH was at 6.7 (Martani *et al.*, 2025).

2.4 Acetic Acid Bacteria Isolation

For enumeration of total heterotrophic bacteria, 10 g of each raw fruit waste sample was aseptically homogenized in 90 mL of sterile

distilled water to obtain the initial 10⁻¹ dilution. Serial ten-fold dilutions were subsequently prepared up to 10⁻⁶. Aliquots of 1 mL from appropriate dilutions were pour-plated on nutrient agar and incubated in an inverted position at 37 °C for 24 h. After incubation, colonies were counted and the total heterotrophic bacterial counts were expressed as CFU/g.

Five grams (5 g) of each fruit sample were homogenized and separately submerged in 25 mL of enrichment medium containing 3% (v/v) acetic acid and 4% (v/v) ethanol as final concentrations, as described by Dienye *et al.* (2025). The samples were incubated at 30 °C for 72 hours. Following enrichment, 1 mL of the enriched fruit sample was transferred into 9 mL of sterile saline to obtain a 10⁻¹ dilution. Serial ten-fold dilutions were subsequently prepared up to 10⁻⁶. Aliquots (0.5 mL) from appropriate dilutions were spread-plated onto GYC agar containing 10% glucose, 1.0% yeast extract, 2.0% calcium carbonate, and 1.5% agar. The plates were incubated at 30 °C for 48 h. After incubation, the plates were examined for colonies surrounded by clear halos, indicating acid production (Dienye *et al.*, 2025).

The total heterotrophic bacterial load was calculated as follows:

$$\text{Microbial Load in CFU/g} = \frac{N \times D}{V}$$

where: N = number of colonies counted; D = dilution factor; and V = volume of inoculum plated (mL).

2.5 Acetic Acid Bacteria Screening

Purified presumptive acetic acid bacterial isolates obtained from GYC agar were qualitatively screened for ethanol-oxidizing activity using Carr's medium. The medium consisted of yeast extract (30 g/L), ethanol (2% v/v), bromocresol green (0.02 g/L), and agar (20 g/L), adjusted to pH 6.8. Pimaricin was added at 100 mg/L as an antifungal supplement to inhibit the growth of yeasts and moulds. Each isolate was inoculated into the medium by stabbing and

incubated at 30 °C for 48 h. The isolates were differentiated based on changes in the colour of the medium associated with ethanol oxidation. A change from green to yellow was recorded as evidence of acidification. *Gluconobacter* isolates generally produced a yellow colour, whereas *Acetobacter* isolates could initially produce a yellow colour followed by a change back to green during prolonged incubation (Rahman *et al.*, 2024).

2.6 Stock Culture Maintenance

Purified colonies of the selected isolates were maintained in GYC broth, GYC agar slants growth was ensured at 30 °C incubation compared with un-inoculated medium. Stock cultures were kept in the refrigerator at 4°C for further utilizations such as microscopy, biochemical characterizations, and molecular characterization; these stocks were refreshed every fortnight over the duration of experiment (Dienye *et al.*, 2025).

2.7 Gram Staining

Smears of the bacteria isolates were first prepared: A drop of sterile distilled water was mid-placed on a grease-free slide. An inoculating loop was sterilized by flaming red-hot and then allowed to cool; bacteria colony was picked with the loop and emulsified in the drop of distilled water on the slide, the smear was heat fixed by passing quickly through flame thrice. The smear was flooded with crystal violet solution (primary dye) for 60 seconds and water rinsed, excess water was drained to avoid diluting the mordant, the smear was flooded again with iodine solution (mordant) for another 60 seconds, water rinsed and then decolorized with 70% alcohol and rinsed with water. The smear was flooded again with safranin (counter stain) for 60 seconds, rinsed with water and then air dried. The slide was observed under the microscope using the oil immersion (x100) objective lens. Gram positive cells appear purple while Gram negative cells appear pink (Tripathi *et al.*, 2025).

2.8 Biochemical Tests

2.8.1 Oxygen Relationship Test

Nutrient agar was prepared and dispensed into McCartney bottles and slanted; upon solidification,

the test organism was streaked, stabbed into the media and incubated at 37°C for 48 hours. Growth concentrated at the surface was interpreted as consistent with an obligately aerobic organism, while growth throughout the medium indicated facultative anaerobic growth and growth restricted to the lower region indicated anaerobic growth (Narimatsu *et al.*, 2025).

2.8.2 Catalase Test

Each bacterial isolate was taken from a pure culture and emulsified in a drop of distilled water on a slide. Two drops of hydrogen peroxide were added. The occurrence of effervescence was recorded as a positive catalase reaction, while the absence of effervescence was recorded as negative. The reaction results from the breakdown of hydrogen peroxide into water and oxygen gas (Dienye *et al.*, 2025).

2.8.3 Oxidase Test

Oxidase reagent was prepared as a 1% solution of tetramethyl-p-phenylenediamine dihydrochloride. Filter-paper discs were soaked in the reagent and used immediately. A portion of a purified colony was rubbed onto the reagent disc. Development of a purple colour was interpreted as a positive oxidase reaction (Dienye *et al.*, 2025).

2.8.4 Citrate Utilization Test

Simon's citrate agar was prepared according to the manufacturer's instructions and dispensed into sterile Petri dishes at approximately 20–25 mL per plate. The test organisms were inoculated onto the medium and incubated at 35 ± 2 °C for 3 days. A colour change from green to blue was interpreted as a positive citrate utilization reaction, whereas the absence of colour change was recorded as negative (Dienye *et al.*, 2025).

2.8.5 Motility Test

Motility was examined using the wet-mount method. A loopful of bacteria from a fresh 24h culture was placed at the centre of a clean microscope slide and gently emulsified to form a thin suspension. A grease-free coverslip was

carefully placed over the suspension to avoid trapping air bubbles. The preparation was examined microscopically, initially using the $\times 10$ objective and subsequently using the $\times 40$ objective to observe bacterial movement (Fawole & Oso, 2007)

2.8.6 Sugar Fermentation Test

A 10 % solution of each of three sugars (sucrose, fructose and maltose) was prepared in ordinary peptone water containing phenol red as the indicator. Ten millilitres of the prepared medium was dispensed into test tubes containing inverted Durham tubes. The media were sterilized and subsequently inoculated with the respective bacterial isolates. One uninoculated tube for each sugar served as the control. The tubes were incubated at 35 ± 2 °C for 3 days. A change in colour was interpreted as evidence of acid production, while gas production was indicated by the accumulation of gas in the Durham tube (Obumneme *et al.*, 2019).

2.9 Molecular Identification

Selected bacterial isolates were subjected to molecular identification based on 16S rRNA gene sequencing. Genomic DNA was extracted from pure cultures, followed by PCR amplification and sequencing of the 16S rRNA gene. The resulting sequences were compared with reference sequences in the NCBI GenBank database using BLAST. Isolate identification was based on sequence similarity and query coverage. Phylogenetic analysis was subsequently performed using closely related reference sequences (Li *et al.*, 2024).

3.0 Results

3.1 Total Heterotrophic Bacteria and Presumptive Acetic Acid Bacteria Counts

The total heterotrophic bacterial (THB) counts and presumptive acetic acid bacteria (AAB) counts obtained from the orange and watermelon peel samples are presented in Table 1. Watermelon peel recorded a THB count of $1.60 \pm 0.20 \times 10^5$ CFU/g, while orange peel recorded $1.50 \pm 0.30 \times 10^5$ CFU/g. The presumptive AAB

count was $2.20 \pm 0.20 \times 10^3$ CFU/g for watermelon peel and $4.80 \pm 1.40 \times 10^2$ CFU/g for orange peel. No growth was observed in the uninoculated experimental control.

3.2 Colonial Morphology of Presumptive Acetic Acid Bacteria

Two presumptive AAB isolates were recovered from the fruit peel samples, comprising one isolate from orange peel (O10) and one isolate from watermelon peel (W10). The cultural, morphological, and biochemical characteristics of the isolates are presented in Table 2. Both isolates were Gram-negative and showed catalase-positive, citrate-positive, and oxidase-negative reactions. The isolates also showed motility and an aerobic growth pattern. Based on their cultural, morphological, and biochemical characteristics, O10 was presumptively assigned to *Gluconobacter* sp., while W10 was presumptively assigned to *Acetobacter* sp.

3.3 Screening of Acetic Acid Bacteria on Carr's Medium

The selected presumptive AAB isolates were screened qualitatively on Carr's medium. Both isolates initially produced a colour change from green to yellow (Table 3). These observations indicate differences in the colour response of the two isolates on Carr's medium. The *Gluconobacter oxydans* isolate maintained the yellow colouration, whereas *Acetobacter aceti* subsequently changed from yellow back to green after prolonged incubation.

3.4 Molecular Identification of Acetic Acid Producing Bacteria

The selected isolates were identified by 16S rRNA gene sequencing. The watermelon isolate was identified as *Acetobacter aceti*, showing 100% sequence identity and 100% query coverage with *Acetobacter aceti* strain W1. The orange isolate was identified as *Gluconobacter oxydans*, showing 100% sequence identity and 100% query coverage with *Gluconobacter oxydans* strain KACC 22393. The phylogenetic analysis revealed the placement of the *Acetobacter aceti* and *Gluconobacter oxydans* isolates among closely related reference strains (Figure 1).

Table 1: Total Heterotrophic Bacteria and Presumptive Acetic Acid Bacteria Counts

Sample	Total Heterotrophic Bacteria count (10 ⁵ CFU/g)	Acetic Acid Bacteria Count (CFU/g)
Orange	1.50 ± 0.3	4.80 ± 1.4 × 10 ²
Watermelon	1.60 ± 0.2	2.20 ± 0.2 × 10 ³
Experimental control (uninoculated medium)	NG	NG
NG – No Growth		

Table 2: Morphological Characteristics, Gram Reaction and Biochemical Characteristics of Bacterial Isolates

Bac	Shape	Col	Ele	Sur	Mar	Gro	Opt	Gr	Cell shape	Cat	Cit	Oxi	Mot	Oxy	Suc	Fru	Mal	Organism
O10	Irregular	White	Flat	Moist	Filiform	Slow	Translucent	-	Bacilli	+	+	-	+	Ae	A	A	A	<i>Gluconobacter</i> sp.
W10	Rhizoid	Cream	Flat	Rough	Rhizoid	Slow	Translucent	-	Bacilli	+	+	-	+	Ae	A	AG	A	<i>Acetobacter</i> sp.

Bac - Bacterial Isolates, Col- Colour, Ele – Elevation, Sur – Surface, Mar – Margin, Gro – Growth, Opt – Optical, Gr- Growth, Cat – Catalase, Cit – Citrate, Oxi – Oxidase, Mot – Motility, Oxy - Oxygen Relationship, Suc – Sucrose, Fru – Fructose, Mal – Maltose, +: Positive, -: Negative Isolates

4.0 Discussion

The present study identified two representative acetic acid bacterial isolates from the fruit peel wastes. The watermelon peel isolate was identified as *Acetobacter aceti*, while the orange peel isolate was identified as *Gluconobacter oxydans* based on 16S rRNA gene sequence analysis. Acetic acid bacteria are commonly associated with fruits, fermented foods, and other substrates containing sugars and ethanol, where they contribute to oxidative fermentation and the production of organic acids (Gomes *et al.*, 2018; Han *et al.*, 2024).

The watermelon peel isolate was identified as *Acetobacter aceti*, showing 100% sequence identity and 100% query coverage with the reference sequence of *A. aceti* strain W1. *Acetobacter* species are important members of the acetic acid bacteria because of their ability to oxidize ethanol to acetic acid and their established role in vinegar fermentation (Gomes *et al.*, 2018; Román-Camacho *et al.*, 2023). In the present study, the *A. aceti* isolate initially changed Carr's medium from green to yellow and subsequently changed from yellow back to green after prolonged incubation. The initial colour change provides qualitative evidence of acidification associated with ethanol oxidation under the screening conditions. However, the

Carr's medium assay used in this study was qualitative and therefore cannot be used to determine the actual concentration, yield, or productivity of acetic acid.

The orange peel isolate was identified as *Gluconobacter oxydans*, also showing 100% sequence identity and 100% query coverage with the reference sequence of *G. oxydans* strain KACC 22393. *Gluconobacter* species are recognized for their oxidative metabolism and their ability to oxidize sugars and alcohols to organic acids and other oxidation products (Gomes *et al.*, 2018; Han *et al.*, 2024). In this study, the *G. oxydans* isolate produced an initial green-to-yellow colour change on Carr's medium and maintained the yellow colouration during the observation period. This reaction indicates oxidative activity under the conditions of the qualitative screening assay but does not by itself establish the amount of acetic acid produced.

The different Carr's-medium reactions observed for the two identified isolates may reflect differences in their oxidative characteristics. The *G. oxydans* isolate maintained the yellow colouration, whereas the *A. aceti* isolate subsequently showed a change from yellow back to green. Carr's medium has been used as a

qualitative approach for observing oxidative reactions among presumptive AAB, including differences between *Acetobacter* and *Gluconobacter* isolates (Dienye *et al.*, 2025). The present findings therefore provide preliminary information on the oxidative behaviour of the two identified isolates.

The molecular identification of the two selected isolates provides stronger taxonomic evidence than the preliminary phenotypic characterization alone. The use of 16S rRNA gene sequencing is an established approach for bacterial identification and taxonomic analysis (Li *et al.*, 2024). The 100% sequence identity and complete query coverage obtained for both isolates support their identification as *A. aceti* and *G. oxydans*, respectively.

The recovery of *A. aceti* from watermelon peel and *G. oxydans* from orange peel demonstrates the occurrence of recognized AAB species in the fruit wastes examined. Similar studies have reported the isolation of AAB from fruit and other agro-food substrates, supporting the association of these microorganisms with plant-derived materials (Dienye *et al.*, 2025; Han *et al.*, 2024). The findings therefore provide baseline information on the occurrence and molecular identity of AAB associated with orange and watermelon peel wastes in the study area.

Although the two isolates demonstrated oxidative reactions on Carr's medium, the present study did not quantify acetic acid

production or determine fermentation yield, productivity, ethanol conversion, or tolerance to acetic acid and other fermentation stresses. These parameters are important when evaluating AAB for controlled vinegar or acetic acid fermentation (Román-Camacho *et al.*, 2023; Hua *et al.*, 2024). Further studies should therefore evaluate the two identified isolates under controlled fermentation conditions to determine their actual acetic acid-producing performance.

5.0 Conclusion

Orange and watermelon peel wastes collected from Ipata Market, Ilorin, Kwara State, Nigeria, harboured acetic acid bacteria. The representative isolates selected for molecular identification were identified by 16S rRNA gene sequencing as *Acetobacter aceti* from watermelon peel and *Gluconobacter oxydans* from orange peel. Both isolates showed ethanol-associated acidification reactions on Carr's medium, providing qualitative evidence of oxidative activity under the screening conditions. These findings support the potential of fruit peel wastes as accessible sources of AAB. However, quantitative acetic acid production, fermentation efficiency, substrate utilization and tolerance to ethanol and acetic acid were not determined in the present study. Further fermentation and physiological studies are therefore required to establish the suitability of the isolates for acetic acid production and other biotechnological application.

Table 3: Screening of Acetic Acid Bacteria on Carr's Medium

Bacterial Isolates	Initial colour	Observed Colour Change	Organism
O10	Green	Yellow	<i>Gluconobacter oxydans</i>
W10	Green	Yellow then Green	<i>Acetobacter aceti</i>

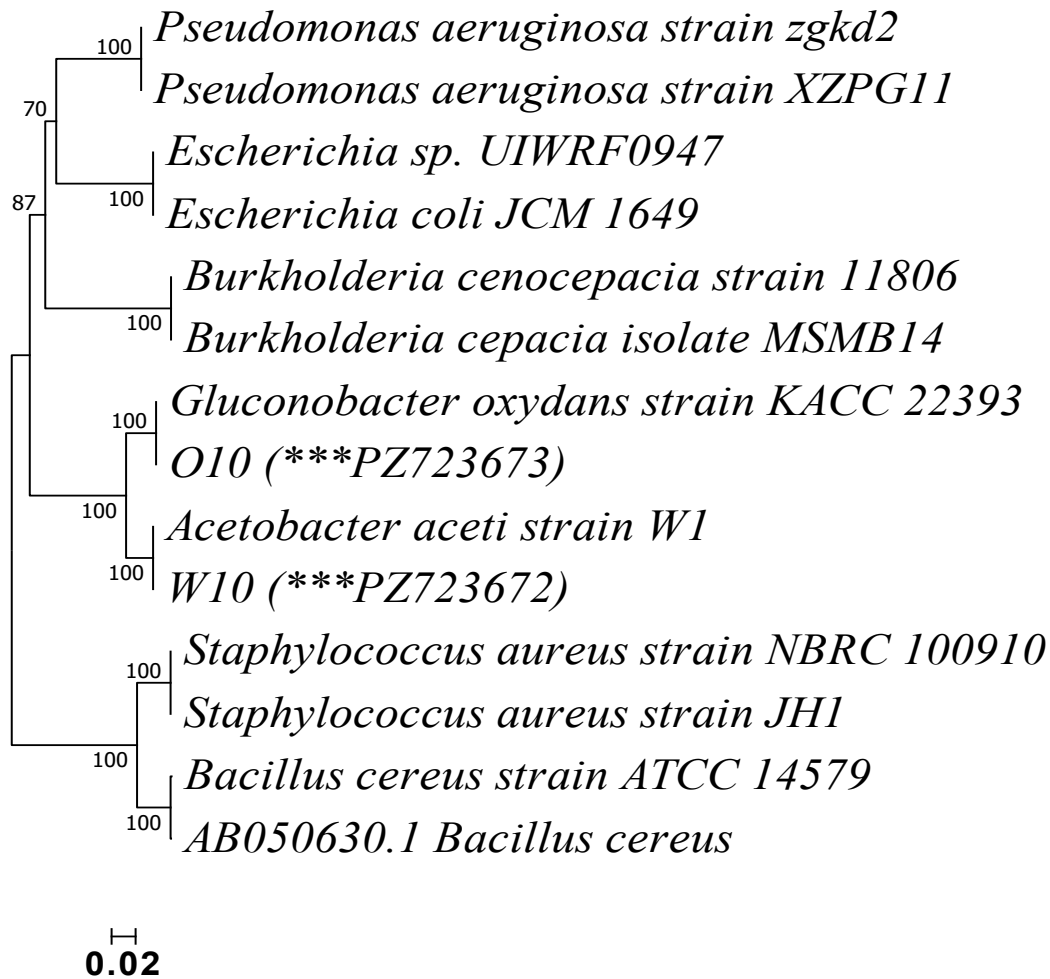


Figure 1: Phylogenetic relationship of *Acetobacter aceti* and *Gluconobacter oxydans* isolates based on 16S rRNA gene sequences

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