

Isolation, Identification and Characterization of Antimicrobial Compound Producing Bacterium *Bacillus subtilis* from *Cucumis melo*

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Abstract

Cucumis melo is well known for its therapeutic potential and thereby represents a promising host system in which antimicrobial compound producing bacteria may harbour. The aim of the present study was to isolate, identify and characterize antimicrobial producing bacteria from *Cucumis melo*. Bacterial isolate was identified as *Bacillus subtilis* based on its cultural, biochemical characteristics and 16S rRNA sequencing. The disc diffusion method was performed to determine the antimicrobial activity of the cell free extract of *Bacillus subtilis* against Gram positive pathogenic organism *Staphylococcus aureus*. The cell free extract showed a zone of inhibition of 6 mm against *Staphylococcus aureus* after 24 hours of incubation at 37 °C. The isolate was then cultivated into liquid media containing different carbon sources such as fructose, sucrose, and soluble starch at the concentration of 5% and 10% (w/v). The growth was evaluated based on absorbance measured using a spectrophotometer. Among these carbon sources, 5% soluble starch showed the optimum growth, suggesting that it may be the most suitable carbon source for maximizing bacterial growth and/or antibiotic production. Thus, the study on *Bacillus subtilis* isolated from *Cucumis melo* brought out its potential as an antimicrobial producing bacterium. Further research work is underway to establish it as a promising cell factory for the production of antimicrobial compounds, and their characterization.

1. Introduction:

Plants are rich sources of compounds with antimicrobial properties against human pathogens. Muskmelon (*Cucumis melo*) is an excellent, succulent, scrumptious product of the family *Cucurbitaceae* and is an important crop worldwide [1]. Muskmelon is grown in the whole tropical and subtropical territories and is also grown in temperate regions under protected agricultural conditions. It is well-known for its nutrition and therapeutic potential [2].

A study has shown that it contains some antimicrobial compounds such as polyphenols, organic acids, lignans and other polar compounds that are beneficial to human health [3]. Even the waste from muskmelon contains rich sources of biologically active

compounds, such as polyphenols, vitamins, enzymes, and dietary fibres [4].

Antimicrobials are therapeutic substances used to prevent or treat infections. They include antiseptics, antibiotics, antivirals, antifungals and antiparasitics. Disinfectants are also a kind of antimicrobial agents applied to non-living surfaces. Antimicrobials can kill microorganisms and prevent their growth by targeting key steps in cellular metabolism such as the synthesis of biological macromolecules, the activity of cellular enzymes, or cellular structures such as the cell wall, cell membranes etc [5].

The presence of antimicrobial agents in an ecosystem, whether natural or man-made, always has an ecological impact [6]. Antimicrobials have been present in nature for much longer than their use by humans. The study of

interactions between different microorganisms, whether prokaryotes or eukaryotes, is an important source of new antimicrobial discovery [7-8].

Traditionally, melons have been used for medicinal reasons so as to relieve constipation due to their dietary fiber, water richness, and nutrient contents [8]. The dynamic fermentation in any food matrix is a complete microbiological process involving interactions between quite different microorganisms [8]. As per the previous studies on muskmelon it could be considered that muskmelons have beneficial impact on economy as they may help sustain a good medicinal market.

The present study aims to explore the antimicrobial producing potential of microbes residing in *Cucumis melo*. The objectives were as follows: a) To isolate the bacterium from *Cucumis melo*, b) Identify bacterium at genus and species level using cultural and biochemical characteristics as well as 16SrRNA sequencing. c) Obtain cell free extract from the liquid culture and to test its antimicrobial activity against pathogenic test organism by agar well diffusion method.

2. Materials and Methods:

2.1 Sample collection

Cucumis melo was bought from the local market and processed for bacterial isolation as described previously [7, 8]. The sample was brought and stored in refrigerator at 4°C. The samples were later transferred into the laboratory for the isolation of bacteria.



Figure 1: *Cucumis melon* sample (a) kept in the vessel in the form of small pieces (b) kept on a table in the form of two fruits and cut pieces.

2.2 Preparation of samples

The sample was prepared through tenfold serial dilution method [9]. 10ml of distilled water was added to 1 gram of homogenized sample in a test tube, and it was called the stock dilution. 1ml of solution was collected from the first test tube and transferred to the second test tube; then, 1 ml of solution was collected from the second test tube and transferred to the third test tube. The same procedure was repeated until the

seventh test tube.

2.3 Isolation of bacteria using pour plate method

Isolation of bacteria was performed using the pour plate technique on Nutrient agar according to standard microbiological procedure [10]. Nutrient agar was prepared as per the media composition (Hi Media Laboratories), then it was autoclaved for 15 minutes at 121°C. After this, the warm media was poured into sterile petri plates with 1 mL of the last three serial dilutions. This was done carefully then shaken slightly, to avoid contact with the cover, and incubated at 37 °C for 24 hours.

2.4 Streak Plate method

The streak plate method on nutrient agar was performed to obtain pure and isolated colonies of bacteria directly from the sample [11].

2.5 Identification of isolates: cultural characteristics

2.5.1 Gram staining

For preliminary identification of the bacterial isolate, Gram staining was performed as a standard procedure [12].

2.5.2 Starch Hydrolysis

Using aseptic technique, an inoculum from the nutrient agar plate was inoculated onto the starch agar plate which had the following composition in 100 ml distilled water: Soluble starch: 0.2g, peptone: 0.5g, yeast extract: 0.3g, bacteriological agar: 1.5g. The plate was incubated at 37°C for 24–48 hours. After incubation, a small amount of Gram's iodine was added to flood the starch growth. It was then observed for clear zone around the bacterial growth [13].

2.5.3 Oxidase test

It was performed using starch agar plate. After inoculation, the plates were incubated at 37°C for 24–48 hours. After incubation, drops of N,N,N',N'-tetramethyl-p-phenylene diamine dihydrochloride (TMPD) reagent was added to the bacterial colony and observed for color change from pink to purple [14].

2.5.4 Catalase test

A loopful of the bacterial colony was added to 1% hydrogen peroxide solution. It was then observed for the effervescences/bubble formation for the positive test or no effect for the negative test [15].

2.6 Biochemical Analysis

To identify and differentiate bacteria based on their biochemical activities and metabolic properties the following tests were performed [16].

2.6.1 Sugar Fermentation test

The bacterial isolate was tested for its ability to ferment a given sugar with the production of acid and gas or acid only. The growth medium used was peptone water which was prepared in a conical flask with indicators. The mixture was dispensed into test tubes containing Durham's tubes. The tubes with contents were sterilized by autoclave at 121°C for 15 minutes. A 1% solution of the sugar was prepared and sterilized separately at 115°C for 10 minutes.

Then, aseptically dispensed in 5ml aliquot volumes into the tubes containing the peptone water and indicator. The tubes were incubated at 37°C. Acid production was indicated by the change of the medium from light green to yellow colour while gas production was indicated by the presence of gas in the inverted Durham's tubes. Glucose, sucrose, and lactose sugars were used [16].

2.6.2 Methyl red test

Tubes of buffered glucose-peptone broth (reconstituted as per the instructions by media manufacturer) were lightly inoculated with the isolates. The tubes were incubated at 37°C for 48 hours. About 5 drops of methyl red reagent was added into 5ml of culture. Methyl red indicator consisted of 0.1g methyl Red dissolved in 300ml of 95% ethyl alcohol [17]. The production of bright red color immediately on the addition of reagent was observed for a positive test.

2.6.3 Voges-Proskauer test

A test tube with buffered glucose-peptone broth were lightly inoculated with a culture of the isolates. The tubes were incubated at 37°C for 48 hours. Barritt's reagent was used for the test. 0.6% of solution A and 0.2 ml of solution B were added into 1 ml of the culture in turns. Solution A contained 5 g of α -naphthol in 100 ml of absolute ethyl alcohol. Solution B contained 100 ml distilled water and 40 g potassium hydroxide. The mixtures were shaken well after each addition. A positive reaction was looked for by observing a pink color that appears immediately within 5 minutes at the top of the test tube [17].

2.6.4 Citrate utilization test

The citrate test was performed to identify the organism that can utilize citrate as the sole sources of carbon for metabolism. The medium used for this test was the Simon's citrate agar. Slant tubes of Simon's

citrate agar were inoculated with culture of the isolates, the inoculation was done by stabbing medium on the tubes using sterile straight inoculating wire loop containing the culture. The tubes were then incubated at 37°C for 24 hours. A change in colour from green to blue after 24 hours of incubation indicates positive result [18].

2.7 Identification by 16SrRNA Sequencing

Identification was performed by gathering genomic DNA, 16S rRNA gene fragments, and assessing the 16S rRNA gene sequence [19].

2.8 Effect of different carbon sources on growth and antimicrobial compound production

The carbon sources, for example, glucose, sucrose, and soluble starch were added to the nutrient broth medium in the concentrations of 5% and 10%. The mixed solutions were divided into 5ml portions and sterilized at 121°C for 15 minutes in various test tubes. Into the test tubes holding, the combined solution and the isolates were added. They were, then, cultured at 37°C for 72 hours [20].

2.9 Antimicrobial activity

The antimicrobial activity of the cell free extract, obtained after the growth of *B. subtilis*, was checked against *Staphylococcus aureus* as test organism using the disc diffusion method as described elsewhere [21].

3.Result:

3.1 Microbial isolation on Nutrient agar

The tenfold serial dilution method was performed to reduce the microbial population sufficiently to obtain separated colonies from a *Cucumis melo* (muskmelon) sample.

3.2 Streak Plate method

A saline suspension of a separated colony was streaked on Nutrient agar plate (Figure 2) and colony characteristics were observed as in table 1.

Table 1: Colony characteristics of a typical colony on nutrient agar after incubation at 37 °C for 24 hours.

Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram's Nature
1mm	circular	White	Entire	Flat	opaque	Butyrous	Gram Positive rods



Figure 2: Isolated Colonies on petri-plate.

3.3 Gram Staining

The image (figure 3) shows the microscopic appearance of a bacterial isolate following Gram staining. Numerous rod-shaped bacterial cells (bacilli) are visible throughout the microscopic field. The cells appear dark purple to violet in colour, indicating retention of the crystal violet stain, which is characteristic of Gram-positive bacteria.

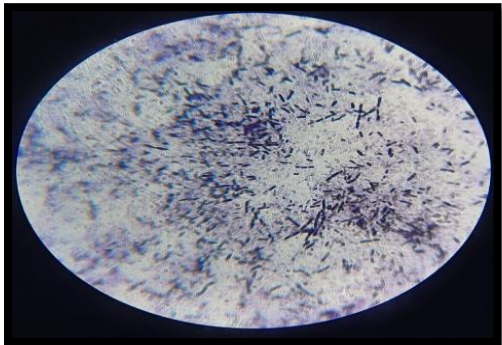


Figure 3: Gram Positive Rods

3.4 Starch Hydrolysis

A clear zone around the bacterial growth was observed indicating utilization of starch as a carbon source.



Figure 4: Starch hydrolysis test (positive)

3.5 Catalase Test

After the addition of loopful bacterial colony into the hydrogen peroxide solution the bubble formation was observed, which indicated that the result was positive for catalase.



Figure 5: Catalase Test Positive

3.6 Sugar Fermentation Tests



(A) Dextrose (B) Sucrose (C) Glucose

Figure 6: shows the positive result for the test tubes A, B, and C (from left) for sugars dextrose, sucrose and glucose respectively.

3.7 Methyl red test

The production of a bright red color appeared immediately on the addition of the reagent indicated a positive test result.



Figure 7: MR test (positive)

3.8 Voges-Proskauer (VP) test

A positive reaction is indicated by a pink color that appeared immediately within 5minutes at the top of the test tube. Since no pink colored was observed the VP test was negative.



Figure 8: VP Test negative

3.9 Citrate utilization test

A change in color from green to blue after about 24 hours of incubation indicated positive result.



Figure 9: Citrate Test Positive

Table 2: Summary of the results of biochemical tests

Biochemical Test	Results
Gram Staining	Gram positive rods
Starch Hydrolysis Test	Positive
Oxidase Test	Negative
Catalase Test	Positive
Methyl Red Test	Positive
Voges-Proskauer Test	Negative

Citrate Utilization Test	Positive
Sugar Fermentation Tests-	
Glucose	Positive
Sucrose	Positive
Dextrose	Positive

3.10 Effect of carbon sources of on the growth of the bacterial isolate *Bacillus subtilis*

Sucrose, Fructose, and soluble starch were tested as carbon sources at the concentration of 5 and 10 % (w/v). Soluble starch at the concentration of 5% (w/v) was found to be optimal followed by sucrose and fructose at the same concentration. A blank was used for comparison of growth observed in terms of turbidity.

Table 3: Effect of carbon sources on the growth (measured as turbidity) of *B. subtilis*

Carbon Sources	Absorbance at 620 nm	Absorbance at 620 nm
	5% sugar Concentration	10% Concentration
Sucrose	1.2981	0.7943
Fructose	1.2188	1.1243
Soluble Starch	1.5184	1.0625
Blank	0.6872	0.6872

3.11 Identification by 16S rRNA Sequencing

16S rRNA sequencing was performed as a molecular technique to identify, classify, and determine the evolutionary relationships of the bacterial isolate. This process involved genomic DNA extraction, amplification of 16S rRNA gene fragments, and sequence analysis of the 16S rRNA gene. Based on 16S rRNA sequencing results, the isolate was identified as *Bacillus subtilis*.

3.12 Antimicrobial activity

The antimicrobial activity was checked against, *Staphylococcus aureus*, as a test organism. A zone of inhibition of 6 mm was obtained as shown in figure 11.

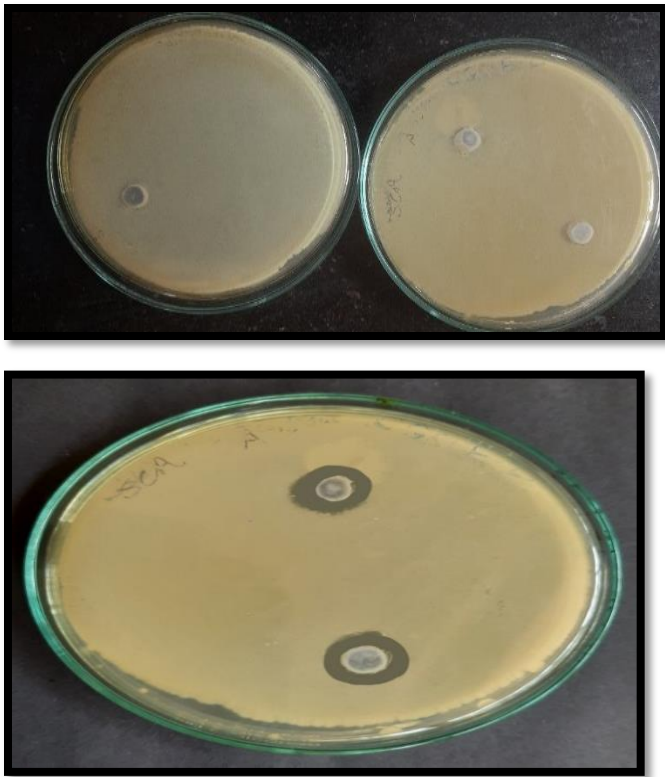


Figure 11. Antimicrobial activity control plates with no zone of clearance, and plates showing zone of inhibition in the cell free extract of *Bacillus Subtilis*.

4. Discussion

Isolation and identification of bacteria producing antimicrobial compounds are crucial steps in microbial research for discovering novel bioactive molecules and understanding pathogenic resistance [22]. In the present study, successful isolation, characterization, and molecular identification of a bacterium from samples of *Cucumis melo* were carried out, highlighting its biochemical potential and antimicrobial activity through the observation of a zone of inhibition.

Recent study [23] on *In vitro* antimicrobial activity of *Cucumis* L. and *Momordica* L. against human pathogens has shown the antimicrobial activity of *Cucumis melo* against the human pathogens. But there has been uncertain identification for the specific organism that has attributed antimicrobial activity.

Many *Bacillus* species are able to secrete large quantities of enzymes and antibiotics. *Bacillus amylo liquefaciens* is one such a source of a natural antibiotic protein and alpha amylase used in starch hydrolysis [24]. In our study starch hydrolysis was used to differentiate closely related *Bacillus* species and

determine whether the microorganism could produce enzymes, specifically α -amylase and oligo-glucosidase. In another study [25], it has been demonstrated that *Bacillus subtilis* concurrently produces antibiotics and spores.

The initial morphological examination and biochemical screening confirmed classic *Bacillus* traits: Gram-positive, endospore-forming, catalase-positive rods capable of utilizing citrate and demonstrating diverse carbohydrate fermentation pathways [26]. For the confirmation of species, sequential biochemical tests were conducted. Sugar fermentation tests were performed to evaluate the ability of the bacterium to ferment different sugars. Glucose, sucrose, and fructose were tested. The methyl red (MR) test was conducted and determined that *Bacillus subtilis* produced and maintained sufficiently stable acidic products from glucose fermentation. This test also helped characterize its glucose fermentation pathway and differentiate it from other *Bacillus* species [17].

Further, 16S rRNA gene sequencing was deployed to secure an accurate species designation. This molecular approach bypasses the pitfalls of biochemical mutability, confirming our isolate's identity as *B. subtilis* with a high sequence identity (>99%). The effect of different carbon sources on *Bacillus subtilis* demonstrated that, among the tested carbon sources, starch was utilized most efficiently by the bacterium. More carbon sources are needed to be tested. Antimicrobial activity was evaluated using the disc diffusion method. A clear zone of inhibition was observed against *Staphylococcus aureus* as a test organism. More pathogenic test organisms such as Gram negative bacteria and yeast like *Candida* are required to be tested for the antimicrobial activity.

5. Conclusion:

The present study brought out that *Cucumis melo* harbors beneficial microorganisms with antimicrobial potential. The bacterial isolate obtained from the sample was identified as *Bacillus subtilis* based on cultural, biochemical, and 16S rRNA sequencing analysis. The isolate demonstrated antimicrobial activity against *staphylococcus aureus*, indicating its potential as a source of antimicrobial compounds.

Among the tested carbon sources, soluble starch supported maximum bacterial growth, suggesting its

suitability for enhanced antimicrobial compound production. The findings of this study indicate that *Bacillus subtilis* isolated from *Cucumis melo* may serve as a promising candidate for future antimicrobial research.

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Competing interest: The authors declare that no conflict of interest exists.

Ethical Statement: This work does not violate ethical guidelines.

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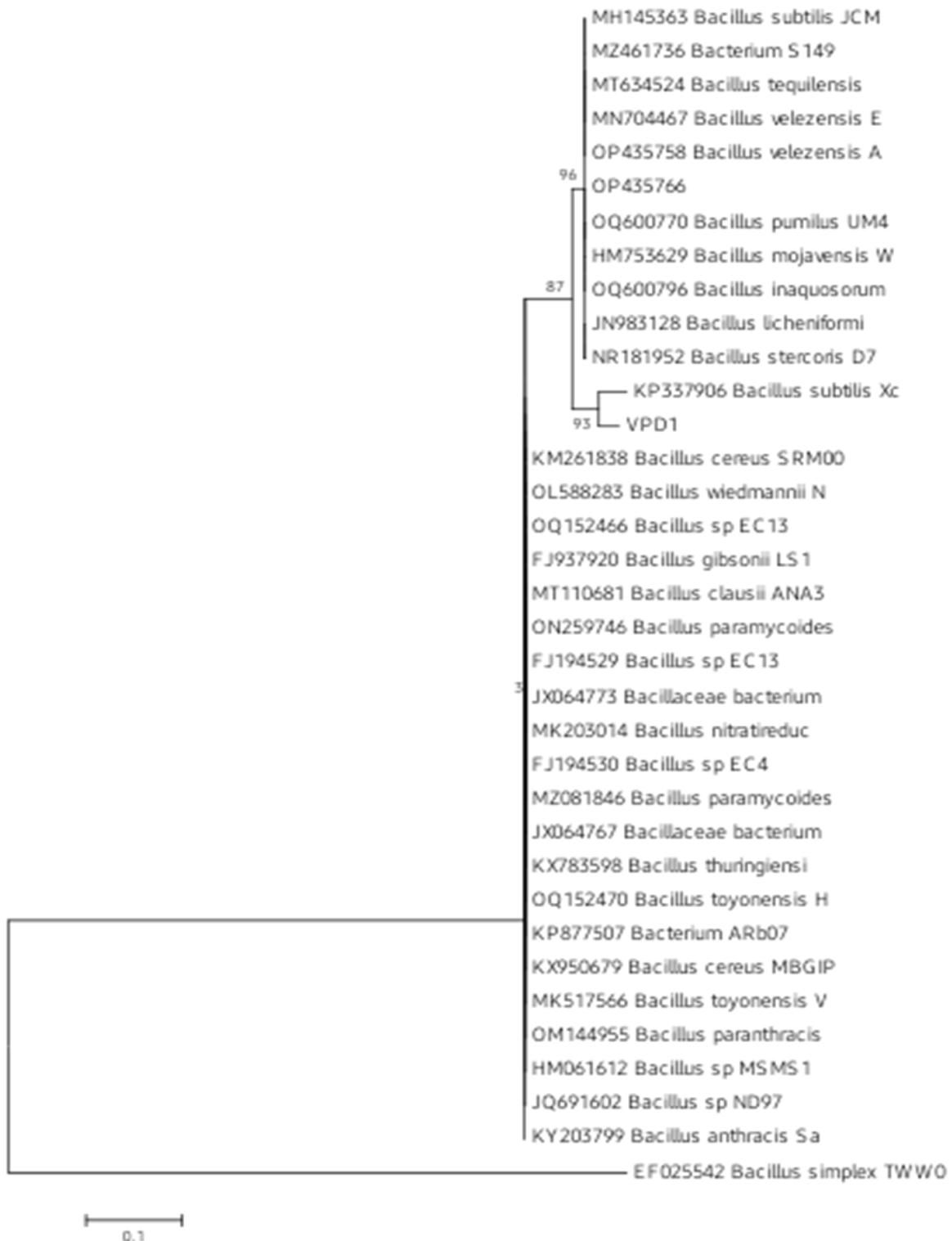
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Appendices:

Figure 11a: Dendrogram showing evolutionary relationship and identification of the isolate as *Bacillus subtilis*.

>*Bacillus subtilis* VPD1

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GTGCTATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTGAACCGCATGGTTCAAACA
TAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGCGCATTAGCTAGTTGGTGAGGTAAACGGCTACCAAGGC
AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTCCGAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGC
TCTGTTGTTAGGGAAGAACAAGTACCGTTTCAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTC
TTAAGTCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAACTGGGGAACCTGAGTGCAAGAGGAGA
GTGGAATTCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAAGTGGCGAAGGCGACTCTCTGGTCTGTAA
CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT
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AAACTCAAAGGAATTGACGGGGGCGCACAAAGCGGTGGAGCATGTGGTTAATTGGAAGCAACGCGAAGAACCTTACC
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GGCCTTGACACACCGCCGTCACACCAGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCG
CCGAAGGTGAC
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Figure 11b: 16s RNA sequencing for the identification of the bacterial isolate.

Abbreviations:

°C: Degree Celsius

H₂O₂: Hydrogen peroxide

(+ve), (-ve): Positive and Negative

mg/mL: Milligrams per Milliliter

1mm:1 Millimeter

TMPD:-p-phenylene diamine dihydrochloride