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Research Article

Isolation and Characterization of Rhizobacteria Screened from Roots of *Limnobium laevigatum*

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Abstract: The quantity of wastewater is increasing globally due to accelerated urbanization, population growth, and economic development. This drives up the demand for methods to resolve the wastewater problem that has been in short supply. *Limnobium laevigatum* acts as a hyperaccumulator due to the high accumulation of heavy metals found in the roots of *L. laevigatum,* such as Zn, Cr, Pb, and Ni, thus showing potential for use in wastewater treatment. This study aims to identify the characteristics of rhizobacteria that screened from the roots of *L. laevigatum*. This study is randomized, in which 50 colonies are randomly selected from the origins of *L. laevigatum*. The roots of *L. laevigatum* are cultivated, and the isolation of the rhizobacteria strains is performed. The characterization of the rhizobacteria is determined by gram staining and biochemical testing. The biochemical testing is evaluated to determine the unidentified rhizobacteria species with catalase activity. 58% of the isolates are found in gram-positive, and 42% of them are gram-negative. The sphere-shaped rhizobacteria arrangements are found in single streptococcus and staphylococcus. In contrast, the rod-shaped rhizobacteria arrangements are observed as single, Diplo, and palisades. The biochemical test resulted in 23 colonies, of which 46% were catalase-positive, and 27 colonies, 54% were observed as catalase-negative. In this study, Fourier transform infrared (FTIR) spectroscopy is performed to identify the different characteristic peak values of various functional compounds in the roots of *L. laevigatum*. It analyzes the prominent peaks at 56.2454 in 696 cm^{-1} that showed strong C-Br stretching, indicating the presence of a halo compound, which can efficiently degrade certain specific aromatic compounds present in wastewater. The rhizobacteria play a vital role in wastewater treatment by decomposing organic matter and pollutants into less toxic or non-toxic substances, reducing biological oxygen demand (BOD), and promoting plant growth by the interaction between plant growth-promoting rhizobacteria and the aquatic plants. In short, this study is expected to advocate sustainable and eco-friendly wastewater treatments by using rhizobacteria that screened in the roots of *L. laevigatum*.

Keywords: *Limnobium laevigatum*, wastewater treatment, heavy metals, gram staining, biochemical test, Fourier transform infrared (FTIR)

1. Introduction

Heavy metals and persistent organic pollutants, including cadmium, lead, arsenic, chromium, mercury, and nickel, are significant global concerns due to increasing industrial and agricultural activities [1, 2]. These pollutants accumulate

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in soil, water bodies, and living organisms, often through factory wastewater, leading to harmful effects on both human health and the environment [3]. While advanced technologies have been developed to mitigate these contaminants, they often face challenges such as high energy requirements, incomplete metal removal, and the production of toxic sludge.

This study proposes an alternative solution by isolating rhizobacteria from the roots of *Limnobium laevigatum*. These naturally occurring bacteria offer a more sustainable and cost-effective approach to removing heavy metals and organic pollutants from wastewater, presenting a promising improvement over traditional physical and chemical treatments [4, 5] inefficient at low concentrations of metal (incomplete metal removal. Additionally, the study addresses the growing global concern of waste management and water shortages by focusing on an environmentally friendly method to treat contaminated water sources.

2. Materials and methodology

The research work was conducted in the laboratories of UCSI University, Kuala Lumpur, Malaysia. This study was performed to isolate the rhizobacteria screened from the roots of *Limnobium laevigatum* and identify the characteristics of rhizobacteria that screened from the roots of *L. laevigatum*.

2.1 *Preparation of materials and chemicals*

2.1.1 *Preparation of nutrient agar*

The nutrient agar was used to isolate the rhizobacteria screened from the roots of *L. laevigatum*. The ingredients and amount needed are listed in Table 1. A total of 28 g of nutrient agar powder was suspended in 1 L of distilled water and mixed completely. The liquid was then poured into the media bottle and sterilized by autoclaving at 121 °C for 15 minutes. After autoclaving, a total of 20 mL of liquid was measured and transferred into the petri dish, and then the medium was waited to solidify. This process was done in the laminar hood to prevent any contamination. The agar was ready to use once the agar was solidified.

Table 1. Composition of nutrient agar

Ingredient	Amount
Nutrient agar powder	28.00 g
Distilled water	1.00 L
pH	± 0.2

2.1.2 *Preparation of nutrient broth (NB)*

Table 2. Composition of nutrient broth

Ingredient	Amount
Nutrient broth powder	13.00 g
Distilled water	1.00 L
pH	7.4 ± 0.2

The nutrient broth was used throughout the experiments, especially in the identifying of the rhizobacteria from the roots of *L. laevigatum*. The ingredients needed for the preparation of nutrient broth are listed in Table 2. A total of 13 g of nutrient broth powder was dissolved in 1 L of distilled water and mixed completely. Then, pour them into the Scott bottle. The media bottle was then sterilized by autoclaving at 121 °C for 15 minutes.

2.1.3 *Preparation of 3% hydrogen peroxide*

The 30% hydrogen peroxide was prepared by the Microbiology Laboratory, UCSI University, Kuala Lumpur, Malaysia. Microbiology Laboratory, UCSI University, Kuala Lumpur, Malaysia prepared the 30% hydrogen peroxide. The dilution was done to prepare 3% hydrogen peroxide by adding 10 mL of 30% hydrogen peroxide solution then dissolved it in 90 mL of distilled water and mixed vigorously by vortex mixer. Then, the solution was transferred into a media bottle and ready to use as shown in Table 3.

Table 3. Composition of 3% hydrogen peroxide solution

Ingredient	Amount
30% hydrogen peroxide solution	10.00 mL
Distilled water	90.00 mL

2.1.4 *Preparation of Limnobium laevigatum*

The raw materials of *L. laevigatum* samples were collected at the Aquatic Science Laboratory, UCSI University, Kuala Lumpur, Malaysia, and the floating aquatic plants were placed in the tank to grow until mature. The growth of the *L. laevigatum* took around 1 month, with every increase 2 times within a week. *L. laevigatum* was then air-dried at room temperature for 2 days until thoroughly dried. The roots of *L. laevigatum* were collected for further isolation and characterization of rhizobacteria from the roots of *L. laevigatum*.

2.2 *Isolation of rhizobacteria strains screened from roots of Limnobium laevigatum*

A total of 1 g of root sample was weighed and crushed in a mortar and pestle. The root sample was then dissolved in 9 mL of distilled water, followed by a vortex. A total of 100 µL from a mixture of root sample and water was pipetted and plated onto the nutrient agar plate by a glass spreader and further incubated at 37 °C for 24 hours because it is the optimal temperature for bacterial growth, closely mimicking the natural environment of many bacteria, including those from the human body. This ensures efficient growth and accurate experimental results. After 24 hours of incubation, the colonies can be seen thoroughly on the nutrient agar plate. Then, the nutrient agar plates were stored in the chiller at 4 °C. There are 50 single colonies of pure cultures randomly selected and picked from the plate. Each single colony was transferred to the centrifuge tube with 5 mL of nutrient broth for incubation at 37 °C for 24 hours for further analysis. Each isolate was then tested for identifying and characterizing rhizobacteria with gram staining and biochemical testing [6, 7].

2.3 *Characterization of rhizobacteria from roots of Limnobium laevigatum*

2.3.1 *Gram staining*

A total of 20 µL of the sample was collected from the centrifuge tube. The sample was then placed on the microscope glass slide and heat-fixed. The slide was stained with crystal violet as the primary stain for 1 minute, then rinsed with water. The slide was then treated using the iodine solution as the mordant for 1 minute and rinsed with water. The slide was decolorized rapidly with the alcohol that contained acetone and rinsed with water. The slide was

then counterstained with safranin for 1 minute and then rinsed with water. Finally, the microscope slide was examined under a compound light microscope. The procedure was repeated for the remaining samples. The morphological characteristics were observed and recorded as the results [8, 9].

2.3.2 *Biochemical test*

The biochemical test conducted in this study focused on determining the catalase activity of the unidentified rhizobacteria species. A total of 20 µL of the sample was pipetted onto a glass slide, and a few drops of 3% hydrogen peroxide were added. The formation of bubbles indicated a catalase-positive result, while no bubbles indicated a catalase-negative result. Although only the catalase test was performed in this study, additional biochemical tests and the 16sRNA method will be considered in future research for a more detailed and precise identification of the bacterial isolates [8].

2.3.3 *Fourier transform infrared spectroscopy (FTIR) analysis*

Fourier-transform infrared spectroscopy (FTIR) analysis was carried out to determine and characterize organic and inorganic materials by an infrared spectrum of absorption or the emission of the plant sample which is *L. laevigatum.* The dried *L. laevigatum* was blended into fine powder form to ensure homogeneity and increase the surface area for analysis. A total of 1 mg sample was mixed with 50 mg of KBr in an FTIR grade to form the pellet. The pellet is then placed into the sample holder. The testing and analysis were performed at the Department of Chemistry, University of Malaya. The background signal is measured by scanning an empty sample holder. The baseline spectrum is then subtracted from the sample spectrum to remove interference caused by the instruments and environment. The sample holder is placed into an FTIR spectrometer with the pellet prepared. The infrared spectrum is recorded by scanning a range of wavelengths typically from 4,000 cm^{-1} to 500 cm^{-1}. The functional groups present in the plant sample are analyzed from the spectrum obtained.

3. Results

3.1 *Gram stain*

In this study, rhizobacteria were isolated from the roots of *L. laevigatum* and analyzed to identify different bacterial species. A total of 50 isolated single colonies of pure cultures were randomly selected for gram staining using a gram-staining kit to classify their Gram nature and arrangement. These results, along with other morphological characteristics, are detailed in Table 4. The selected colonies showed diverse morphologies, with shapes classified into rod-shaped and cocci-shaped, further organized by their arrangements, such as bacillus, dicloxacillin, chains, palisades, spiral, coccus, diplococci, and clusters [10]. This range of shapes and arrangements helps ensure a broad representation of bacterial diversity in the sample.

Beyond Gram nature, various morphological aspects, such as colony shape, size, and texture, were also considered when selecting colonies to represent the diversity of rhizobacteria. These criteria contribute to a more comprehensive understanding of the bacterial populations present.

Bacteria are complex microorganisms, varying significantly in characteristics and morphology [10]. In this study, the rhizobacteria from the roots of *L. laevigatum* were categorized based on their shapes including rod and cocci, and their arrangements, including coccus, diplococci, chains, palisades, and clusters for cocci-shaped bacteria. Rod-shaped bacteria were classified into bacillus, diplobacilli, chains, clusters, spiral, and palisade arrangements as shown in Figure 4. These morphological features can change depending on environmental conditions or the growth phase, which can complicate the characterization process.

The arrangement of bacterial cells after division plays a crucial role in identifying them. Common arrangements include monococci (single cocci), diplococci (pairs), tetrads (groups of four in a square pattern), and clusters (staphylococcal arrangement). Examples of bacteria in these arrangements include Staphylococcus aureus, Streptococcus pneumoniae, and others [11].

Table 4. Gram stain result and morphology of 50 colonies from each pure culture

Sample	Gram	Shapes	Arrangements
R-(1)	Negative (-)	Rod	Bacillus
R-(2)	Positive (+)	Cocci	Diplococci
R-(3)	Positive (+)	Rod	Diplobacilli
R-(4)	Positive (+)	Rod	Chains
R-(5)	Positive (+)	Rod	Diplobacilli
R-(6)	Positive (+)	Cocci	Chains
R-(7)	Positive (+)	Rod	Chains
R-(8)	Positive (+)	Rod	Palisades
R-(9)	Negative (-)	Rod	Diplobacilli
R-(10)	Positive (+)	Rod	Chains
R-(11)	Negative (-)	Rod	Chains
R-(12)	Positive (+)	Rod	Chains
R-(13)	Negative (-)	Cocci	Coccus
R-(14)	Positive (+)	Rod	Diplobacilli
R-(15)	Negative (-)	Cocci	Chains
R-(16)	Positive (+)	Cocci	Palisades
R-(17)	Positive (+)	Cocci	Chains
R-(18)	Negative (-)	Rod	Chains
R-(19)	Negative (-)	Rod	Palisades
R-(20)	Negative (-)	Cocci	Clusters
R-(21)	Negative (-)	Rod	Diplobacilli
R-(22)	Negative (-)	Rod	Spiral
R-(23)	Positive (+)	Rod	Chains
R-(24)	Negative (-)	Rod	Diplobacilli
R-(25)	Negative (-)	Cocci	Clusters
R-(26)	Positive (+)	Cocci	Diplococci
R-(27)	Negative (-)	Rod	Diplococci
R-(28)	Negative (-)	Cocci	Clusters
R-(29)	Positive (+)	Cocci	Coccus
R-(30)	Negative (-)	Rod	Palisades
R-(31)	Negative (-)	Rod	Chains

Table 4. (cont.)

Sample	Gram	Shapes	Arrangements
R-(32)	Positive (+)	Rod	Spiral
R-(33)	Negative (-)	Rod	Palisades
R-(34)	Negative (-)	Rod	Chains
R-(35)	Positive (+)	Rod	Diplobacilli
R-(36)	Negative (-)	Rod	Bacillus
R-(37)	Positive (+)	Rod	Palisades
R-(38)	Positive (+)	Rod	Chains
R-(39)	Positive (+)	Cocci	Clusters
R-(40)	Negative (-)	Rod	Chains
R-(41)	Positive (+)	Cocci	Chains
R-(42)	Positive (+)	Cocci	Chains
R-(43)	Positive (+)	Cocci	Palisades
R-(44)	Positive (+)	Rod	Diplobacilli
R-(45)	Negative (-)	Rod	Chains
R-(46)	Positive (+)	Rod	Bacillus
R-(47)	Positive (+)	Rod	Spiral
R-(48)	Positive (+)	Rod	Diplobacilli
R-(49)	Positive (+)	Cocci	Coccus
R-(50)	Positive (+)	Rod	Chains

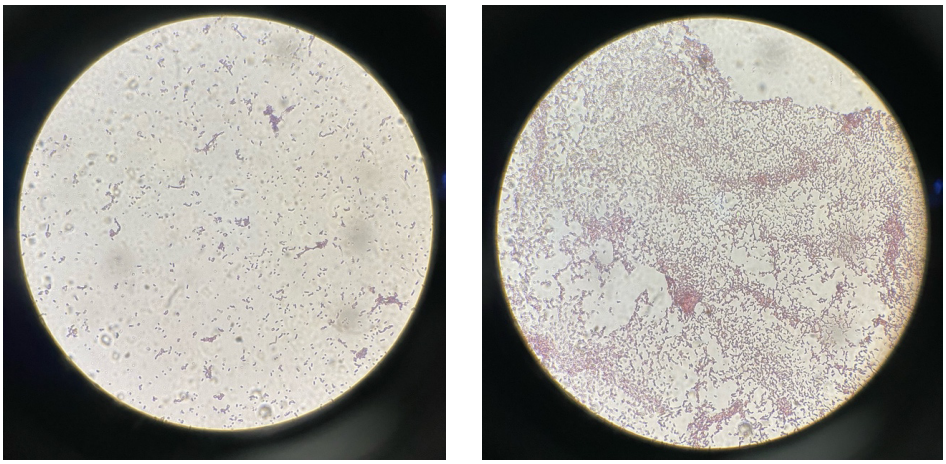

Figure 1. Gram-positive in left and gram-negative in right of isolated rhizobacteria screened from the roots of *L. laevigatum* under 40x magnification

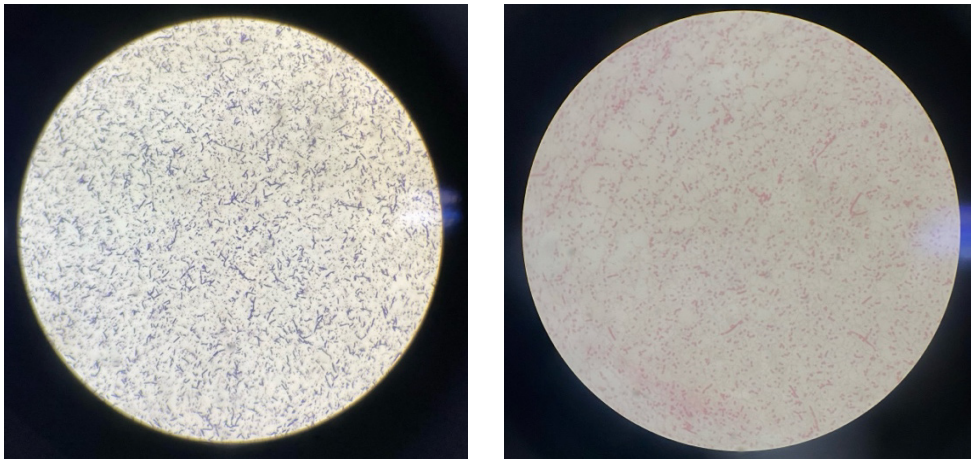

Figure 2. Gram-positive in left and gram-negative in right of isolated rhizobacteria screened from the roots of *L. laevigatum* under 100x magnification

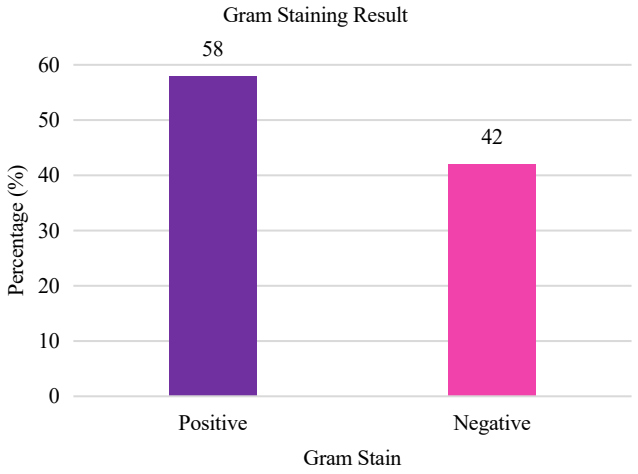

Figure 3. Gram staining of 50 isolates randomly selected from the roots of *L. laevigatum*

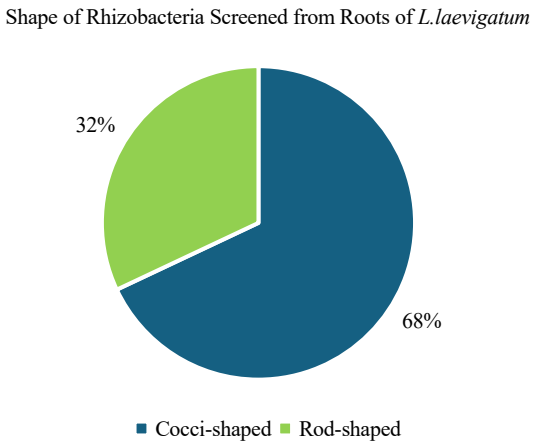

Figure 4. Shape of rhizobacteria screened from roots of *L. laevigatum*

3.2 *Biochemical test*

In addition to morphological analysis, biochemical testing was conducted to assess the catalase activity of the isolated rhizobacteria strains. The test checks for the catalase enzyme, which breaks down hydrogen peroxide (H_2O_2) into water and oxygen. A positive catalase reaction, indicated by the formation of gas bubbles, was observed in the tested isolates. The results of the biochemical tests for each pure culture are presented in Table 5 and Figure 5. This additional biochemical profiling complements the morphological and Gram stain results, providing a more holistic view of the bacterial characteristics.

Table 5. Result of biochemical test from 50 isolated single colonies from each pure culture

Sample	Biochemical test
R-(1)	Positive (+)
R-(2)	Positive (+)
R-(3)	Positive (+)
R-(4)	Positive (+)
R-(5)	Positive (+)
R-(6)	Positive (+)
R-(7)	Positive (+)
R-(8)	Negative (-)
R-(9)	Negative (-)
R-(10)	Positive (+)
R-(11)	Negative (-)
R-(12)	Negative (-)
R-(13)	Negative (-)
R-(14)	Negative (-)
R-(15)	Negative (-)
R-(16)	Negative (-)
R-(17)	Negative (-)
R-(18)	Negative (-)
R-(19)	Negative (-)
R-(20)	Positive (+)
R-(21)	Positive (+)
R-(22)	Negative (-)
R-(23)	Positive (+)
R-(24)	Positive (+)

Table 5. (cont.)

Sample	Biochemical test
R-(25)	Positive (+)
R-(26)	Negative (-)
R-(27)	Positive (+)
R-(28)	Negative (-)
R-(29)	Positive (+)
R-(30)	Negative (-)
R-(31)	Positive (+)
R-(32)	Negative (-)
R-(33)	Positive (+)
R-(34)	Positive (+)
R-(35)	Positive (+)
R-(36)	Positive (+)
R-(37)	Negative (-)
R-(38)	Negative (-)
R-(39)	Negative (-)
R-(40)	Positive (+)
R-(41)	Negative (-)
R-(42)	Positive (+)
R-(43)	Negative (-)
R-(44)	Negative (-)
R-(45)	Negative (-)
R-(46)	Negative (-)
R-(47)	Positive (+)
R-(48)	Negative (-)
R-(49)	Negative (-)
R-(50)	Negative (-)

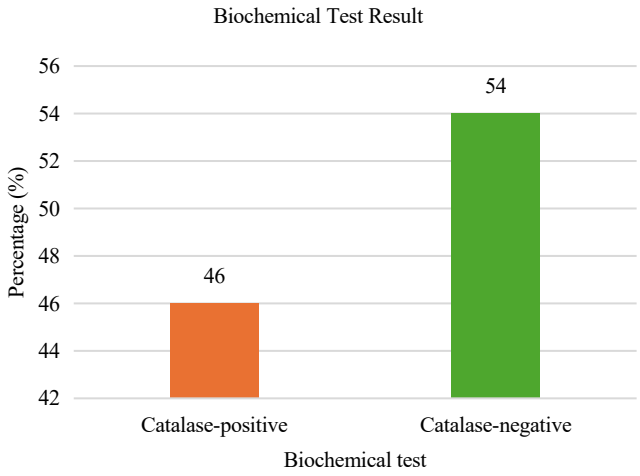

Figure 5. Biochemical test of 50 isolates randomly selected from roots of *L. laevigatum*

3.3 *Fourier transform infrared spectroscopy (FTIR) analysis*

Table 6. FTIR spectrum peak values and functional groups of roots of *L. laevigatum*

Spectrum No.	Wavenumber, cm^{-1}	Wavenumber, cm^{-1}	Functional group	Predicted compound
1	3,026	3,100-3,000	m, = C-H stretch	Alkenes
2	2,921	3,200-2,500	Br, O-H stretch	Carboxylic acids
3	2,850	2,900-2,800	s, C-H stretch	Aldehydes
4	1,636	1,700-1,630	s, C = O stretch	Amides
5	1,492	1,570-1,490	s, NO_2 stretch	Nitro compounds
6	1,451	1,625-1,440	m to w, C = C stretch	Aromatic compounds
7	1,412	1,350-1,410	w, C-H stretching vibration	Secondary alcohol-free
8	1,151	1,000-1,400	C-F stretch	Alkyl & aryl halides
9	1,067	1,000-1,400	C-F stretch	Alkyl & aryl halides
10	1,028	1,000-1,400	C-F stretch	Alkyl & aryl halides
11	875	820-920	w-m, C-C skeletal vibration	Alkane
12	876	820-920	w-m, C-C skeletal vibration	Alkane
13	749	< 600-840	C-Cl stretch	Alkyl & aryl halides
14	696	< 700	C-Br stretch	Alkyl & aryl halides

*Abbreviations: s = strong, m = medium, w = weak

In this research study, the Fourier- transform Infrared Spectroscopy (FTIR) was performed to analyze the chemical bonds or functional groups in the roots of *L. laevigatum*. The characteristics of IR absorption peaks of functional groups are shown in Table 6 above.

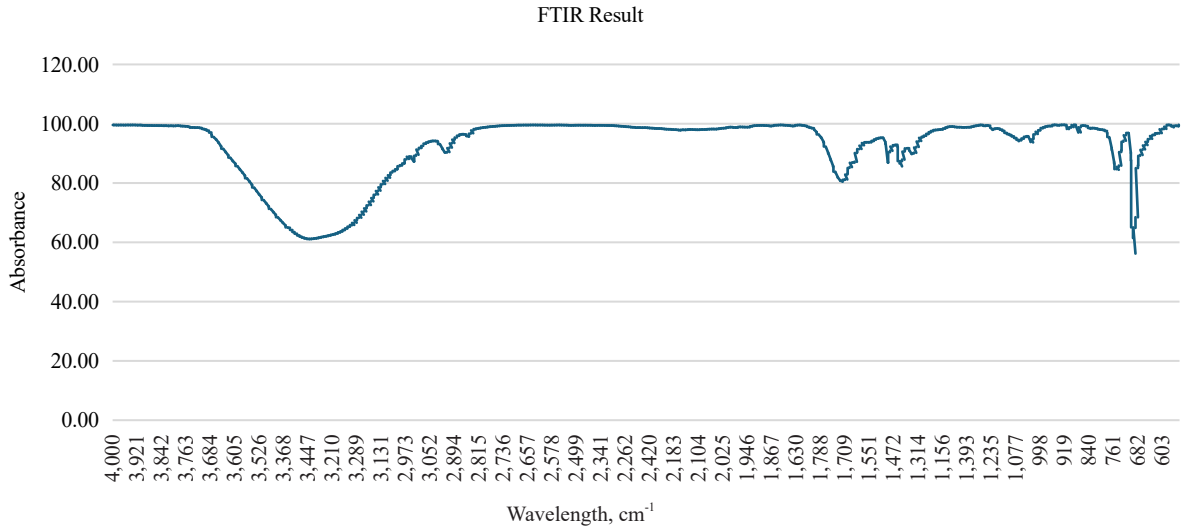

Figure 6. FTIR Spectrum of roots of *L. laevigatum*

4. Discussion

The characteristics and morphologies of rhizobacteria isolated from the roots of *Limnobium laevigatum* were identified through Gram staining, biochemical testing, and FTIR analysis. Our results revealed that 54% of the isolates were gram-positive and 46% were gram-negative in Figures 1-3. The rod-shaped rhizobacteria exhibited diverse arrangements, including diplobacilli, bacillus, spiral chains, and palisades, while the cocci-shaped rhizobacteria were found in arrangements such as coccus, diplococci, chains, clusters, and palisades as shown as Figure 4.

The classification of gram-negative rod-shaped rhizobacteria as catalase-positive suggests that these bacteria might include genera such as *Escherichia coli*, *Acinetobacter*, and *Vibrio cholerae* [12]. These genera are known for their robust metabolic pathways and ability to thrive in various environments. The presence of catalase enzyme in these bacteria facilitates their survival by neutralizing hydrogen peroxide, a toxic byproduct of metabolic processes. On the other hand, gram-negative rod-shaped rhizobacteria that were catalase-negative could potentially belong to genera like Salmonella, which do not produce catalase but have similar morphological traits.

Gram-positive rod-shaped rhizobacteria with positive catalase tests, such as those from the genera *Corynebacterium* and *Bacillus*, are known for their significant role in soil ecosystems and their ability to form endospores. These endospores provide resistance to extreme environmental conditions, such as high temperatures and desiccation, enhancing their survival and utility in wastewater treatment. Notably, species like *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus anthracis* are well-documented for their genetic adaptability and resilience [13, 14].

In contrast, gram-positive rod-shaped rhizobacteria that are catalase-negative, such as those from the genera *Clostridium* and *Erysipelothrix rhusiopathiae*, are typically associated with anaerobic environments and are less likely to withstand oxidative stress. Gram-positive cocci-shaped rhizobacteria with positive catalase tests are suspected to be from the genus *Staphylococcus*, including species like *Staphylococcus aureus* and *Staphylococcus epidermidis*. These bacteria are known for their ability to cause infections but can also play roles in environmental processes. Conversely, gram-positive cocci-shaped rhizobacteria that are catalase-negative, such as *Streptococcus pneumoniae*, are generally involved in different pathogenic mechanisms and environmental interactions [15].

FTIR analysis of the roots of *L. laevigatum* revealed strong peaks at 56.24 cm^{-1}, 84.64 cm^{-1}, 85.66 cm^{-1}, 86.89 cm^{-1},

and 80.62 cm^{-1}, which correspond to various functional groups including alkenes, carboxylic acids, aldehydes, amides, nitro compounds, and aromatic compounds as shown in Figure 6. Notably, a prominent peak at 696 cm^{-1} indicated strong C-Br stretching, suggesting the presence of halo compounds, particularly brominated compounds. In the context of wastewater treatment, halo compounds, especially those containing bromine, are significant due to their role in the degradation of specific aromatic pollutants. Halide ions, such as bromide (Br^-), are known to participate in advanced oxidation processes (AOPs) which are crucial for breaking down complex organic pollutants [16]. These processes generate reactive species that can attack and degrade aromatic compounds, which are often persistent and hazardous in wastewater [14].

The presence of C-Br stretching in the FTIR spectra indicates that these halo compounds could potentially contribute to the degradation of aromatic pollutants through halogenation reactions. Brominated compounds are effective in facilitating the breakdown of aromatic rings, which are common in synthetic dyes and other pollutants. This suggests that the rhizobacteria associated with *L. laevigatum* might enhance the bioremediation of wastewater by utilizing these halo compounds to degrade harmful aromatic substances [17]. Additionally, the FTIR spectra showed nitrogen and oxygen functional groups, which are also relevant for their role in nutrient absorption and interaction with environmental stressors [18]. These functional groups can support the overall effectiveness of the rhizobacteria in wastewater treatment by facilitating the breakdown of pollutants and enhancing nutrient availability.

The rhizobacteria isolated from *L. laevigatum* show promise in environmental remediation due to their potential roles in promoting plant growth and development [19]. While this study focused on preliminary characterization, further research could explore specific plant growth-promoting traits such as nitrogen fixation, phosphate solubilization, and phytohormone production [20, 21]. The rhizobacteria from *L. laevigatum* are known to interact symbiotically with plant roots, enhancing nutrient uptake and stress tolerance [22, 23]. This interaction is crucial for improving plant growth and soil health and supporting sustainable agricultural practices.

The potential of these rhizobacteria in wastewater treatment is also significant. For instance, *Acinetobacter baumannii* has been used in secondary wastewater treatment due to its resistance to certain antibiotics, which aids in its survival in treatment environments [24, 25]. *Bacillus* species, including *Bacillus subtilis* and *Bacillus amyloliquefaciens*, are effective in decomposing organic materials and reducing biochemical oxygen demand (BOD), contributing to the reduction of sludge and odor in wastewater treatment processes [26, 27].

Rhizobacteria in the rhizosphere are involved in various mechanisms that promote plant growth, including the production of phytohormones such as auxin, cytokinin, ethylene, gibberellins, and abscisic acid [21]. These hormones play critical roles in stress responses and plant development. For example, *Bacillus subtilis* has been shown to produce genes that enhance stress response and plant resistance during root colonization [28, 29].

In summary, while this study focused on the morphological and biochemical characterization of rhizobacteria, the observed attributes suggest their potential utility in wastewater treatment and plant growth promotion. Future research should include tests for specific plant growth-promoting activities to provide a more comprehensive understanding of these rhizobacteria's roles in environmental and agricultural applications [30].

Moreover, rhizobacteria have been utilized in wastewater treatment especially due to the pollution caused by the textile industry. The utilization of aerobic and anaerobic rhizobacteria has the potential and ability in the biodegradation of synthetic dyes including triphenylmethane, azo, anthraquinone, and heterocyclic dyes [28, 31, 32]. There are various rhizobacteria strains have been used such as *Pseudomonas putida*, *Staphylococcus hominis*, *Clostridium bifermenrans*, *Bacillus cohnii*, *Staphylococcus arlettae*, *Enterococcus casseliflavus*, *Clostridium sp.*, *Acinetobacter baumannii*, *Bacillus cereus*, *Bacillus subtilis*, *Rhizobum sp.*, and more [33, 34]. Based on the study, *B. subtilis* has been found a highly potent bacterial strain in the biodegradation of orang II dyes due to its ease of production of azoreductase at temperature up to 37 °C [35]. Azoreductase found in *B. subtilis* can breakdown azo colors and convert them into colorless substances. *B. subtilis* also able to breakdown the bond of azo dyes with the presence of glucose which acts as a carbon source, the p-phenylenediamine and aniline compounds were formed [27, 34].

L. laevigatum is an ideal alternative in phytoremediation treatment due to its high hyperaccumulation potential and abilities without disturbance and negative effects on survival [2, 36]. Other macrophytes, such as *Phragmites australis*, *Myriophyllum spicatum*, *Hydrilla sp.*, *Azolla sp.*, *Lemna sp.*, *Eichhornia crassipes*, *Ceratophyllum demersum*, *Potamogeton pectinatus*, *Salvinia sp.*, *Typha latifolia*, *Ludmigia stolonifera*, and *Vallisneria spiralis*, also play a role in phytoremediation treatment due to their high resistance towards contaminants [37-39].

The identified rhizobacteria contribute to the hyperaccumulation of heavy metals through several key mechanisms, including metal solubilization, immobilization, detoxification, and promoting [21, 40, 41]. Species such as *Bacillus, Pseudomonas*, and *Acinetobacter* are particularly efficient due to their ability to produce metal-chelating agents, form biofilms, and transform toxic metals into less harmful forms [42]. These bacteria enhance metal uptake by plants and protect them from toxicity, making them highly effective for bioremediation in contaminated environments [43, 44]. *Bacillus* species, in particular, are well-known for their ability to tolerate and detoxify heavy metals like lead, chromium, and cadmium [14, 45, 46].

While *Limnobium laevigatum* and its associated rhizobacteria offer promising benefits for wastewater treatment, there are potential environmental risks. The plant's rapid growth can make it invasive in non-native ecosystems, disrupting local biodiversity and outcompeting native species. Additionally, its fast growth may cause oxygen depletion in water bodies, creating hypoxic conditions that threaten aquatic life. The accumulation of toxic metals within the plant biomass poses another risk, as improper disposal could lead to the re-release of these metals into the environment [47]. Furthermore, certain rhizobacteria involved in bioremediation may produce harmful byproducts or metabolites, and there is a potential for these bacteria to harbor pathogens that could impact human health or wildlife [48, 49]. To mitigate these risks, careful management, regular monitoring, and proper disposal of the plant and its biomass are essential.

In conclusion, rhizobacteria plays a vital role in wastewater treatment by decomposing organic matter and pollutants into less toxic or non-toxic substances, reducing BOD, and promoting plant growth the interaction between plant growth-promoting rhizobacteria and aquatic plants [18, 50].

5. Conclusion

This study focused on identifying and characterizing rhizobacteria associated with the roots of *Limnobium laevigatum*, a floating aquatic plant. A total of 50 rhizobacterial isolates were obtained using gram staining and biochemical testing. The results revealed that 54% of the isolates were gram-positive, while 46% were gram-negative. Among the isolates, various morphological arrangements were observed, including rod-shaped rhizobacteria in diverse configurations and cocci-shaped rhizobacteria in coccus, diplococci, chains, clusters, and palisades.

The identification of gram-negative rod-shaped rhizobacteria with positive biochemical tests suggested possible genera such as *Escherichia coli*, *Acinetobacter*, and *Vibrio cholerae*. Other gram-negative rod-shaped isolates were suspected to belong to genera like *Salmonella*. Gram-positive rod-shaped isolates were linked to genera such as *Corynebacterium*, *Bacillus*, *Clostridium*, and *Erysipelothrix rhusiopathiae*. Additionally, cocci-shaped gram-positive isolates were associated with genera such as *Staphylococcus* and *Streptococcus* [15].

The study highlights the potential of *L. laevigatum* rhizobacteria for wastewater treatment; however, challenges such as rapid diffusion rates of signals and nutrients, coupled with the unique habitat of free-floating roots, may impact their effectiveness. Future research should explore advanced molecular analyses, such as metabolomics and metagenomics, to address these challenges and enhance the application of rhizobacteria in wastewater treatment and the ecological management of freshwater systems.

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Conflict of interest

The authors declare that they have no conflicts of interest regarding the publication.

References

[1] Arán DS, Harguinteguy CA, Fernandez-Cirelli A, Pignata ML. Phytoextraction of Pb, Cr, Ni, and Zn using the aquatic plant *Limnobium laevigatum* and its potential use in the treatment of wastewater. *Environmental Science and Pollution Research International*. 2017; 24(22): 18631-18639.

[2] Martino LJ, Fernández San Juan MR, Angelo CD. Potential phytoremediation system using macrophyte *Limnobium laevigatum* to remove in situ Cr from contaminated bottom sediments. *Environmental Technology*. 2023; 44(18): 2770-2780.

[3] Nnaji ND, Onyeaka H, Miri T, Ugwa C. Bioaccumulation for heavy metal removal: A review. *SN Applied Sciences*. 2023; 5(5): 125.

[4] Filote C, Roșca M, Hlihor RM, Cozma P, Simion IM, Apostol M, et al. Sustainable application of biosorption and bioaccumulation of persistent pollutants in wastewater treatment: Current practice. *Processes*. 2021; 9(10): 1696. Available from: https://doi.org/10.3390/pr9101696.

[5] Kanamarlapudi S, Chintalpudi VK, Muddada S. Application of biosorption for removal of heavy metals from wastewater. *Biosorption*. 2018; 18(69): 70-116. Available from: http://dx.doi.org/10.5772/intechopen.77315.

[6] Vejan P, Abdullah R, Khadiran T, Ismail S, Nasrulhaq Boyce A. Role of plant growth promoting rhizobacteria in agricultural sustainability: A review. *Molecules*. 2016; 21(5): 573.

[7] Makino A, Nakai R, Yoneda Y, Toyama T, Tanaka Y, Meng XY, et al. Isolation of aquatic plant growth-promoting bacteria for the floating plant duckweed (*Lemna minor*). *Microorganisms*. 2022; 10(8): 1564.

[8] Haider FU, Liqun C, Coulter JA, Cheema SA, Wu J, Zhang R, et al. Cadmium toxicity in plants: impacts and remediation strategies. *Ecotoxicology and Environmental Safety*. 2021; 211: 111887. Available from: https://doi.org/10.1016/j.ecoenv.2020.111887.

[9] Wong FH, Lim LH, Teo SS. Identification of bacteria species in aquaponics system using RapID system and 16S rRNA sequencing. *Research Journal of Biotechnology*. 2021; 16(7): 1-14.

[10] Sapkota A. Morphology of bacteria-sizes, shapes, arrangements, examples. *Microbe Notes*. 2020. Available from: https://microbenotes.com/bacterial-sizes-shapes-arrangement.

[11] Sood A, Uniyal PL, Prasanna R, Ahluwalia AS. Phytoremediation potential of aquatic macrophyte, Azolla. *Ambio*. 2012; 41(2): 122-137.

[12] Cabral JPS. Water microbiology: Bacterial pathogens and water. *International Journal of Environmental Research and Public Health*. 2010; 7(10): 3657.

[13] Vejan P, Abdullah R, Khadiran T, Ismail S, Nasrulhaq Boyce A. Role of plant growth promoting rhizobacteria in agricultural sustainability: A review. *Molecules*. 2016; 21(5): 573.

[14] Han JH, Shim H, Shin JH, Kim KS. Antagonistic activities of *Bacillus* spp. strains isolated from tidal flat sediment towards anthracnose pathogens *Colletotrichum acutatum* and *C. gloeosporioides* in South Korea. *The Plant Pathology Journal*. 2015; 31(2): 165. Available from: https://doi.org/10.5423/PPJ.OA.03.2015.0036.

[15] Traxler MF, Kolter R. Natural products in soil microbe interactions and evolution. *Natural Product Reports*. 2015; 32(7): 956-970.

[16] Xue Y, Wang Z, Naidu R, Bush R, Yang F, Liu J, et al. Role of halide ions on organic pollutants degradation by peroxygens-based advanced oxidation processes: A critical review. *Chemical Engineering Journal*. 2022; 433: 134546.

[17] Kumara UMA, Jayaprada NVT, Thiruchchelvan N. Bioremediation of polluted water. In: Soni R, Suyal DC, Morales-Oyervides L, Sungh Chauhan J. (eds.) *Current Status of Fresh Water Microbiology*. Singapore: Springer Nature; 2023. p.321-346. Available from: https://doi.org/10.1007/978-981-99-5018-8_14.

[18] Olanrewaju OS, Glick BR, Babalola OO. Mechanisms of action of plant growth promoting bacteria. *World Journal of Microbiology and Biotechnology*. 2017; 33(11): 197.

[19] Glick BR. The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology*. 1995; 41(2): 109-117. Available from: https://doi.org/10.1139/m95-015.

[20] Ahemad M, Kibret M. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *Journal of King Saud University-Science*. 2014; 26(1): 1-20.

[21] Glick BR, Todorovic B, Czarny J, Cheng Z, Duan J, McConkey B. Promotion of plant growth by bacterial ACC deaminase. *Critical Reviews in Plant Sciences*. 2007; 26(5-6): 227-242. Available from: https://doi.org/10.1080/07352680701572966.

[22] Khan N, Ali S, Shahid MA, Mustafa A, Sayyed RZ, Curá JA. Insights into the interactions among roots, rhizosphere, and rhizobacteria for improving plant growth and tolerance to abiotic stresses: A review. *Cells*. 2021; 10(6): 1551.

[23] Hansen BL, Pessotti RC, Fischer MS, Collins A, El-Hifnawi L, Liu MD, et al. Cooperation, competition, and specialized metabolism in a simplified root nodule microbiome. *MBio*. 2020; 11(4): e01917-20.
[24] Howard A, O'Donoghue M, Feeney A, Sleator RD. *Acinetobacter baumannii*: An emerging opportunistic pathogen. *Virulence*. 2012; 3(3): 243-250.
[25] Howard A, O'Donoghue M, Feeney A, Sleator RD. *Acinetobacter baumannii*: An emerging opportunistic pathogen. *Virulence*. 2012; 3(3): 243-250. Available from: https://pubmed.ncbi.nlm.nih.gov/22546906.
[26] EnBiorganic Technologies. *The power of Bacillus bacteria in wastewater treatment*. Available from: https://enbiorganic.com/blog-the-power-of-bacillus-bacteria-in-wastewater-treatment [Accessed 14th Oct 2024].
[27] Zalila-Kolsi I, Ben-Mahmoud A, Al-Barazie R. *Bacillus amyloliquefaciens*: Harnessing its potential for industrial, medical, and agricultural applications: A comprehensive review. *Microorganisms*. 2023; 11(9): 2215.
[28] Jin XC, Liu GQ, Xu ZH, Tao WY. Decolorization of a dye industry effluent by *Aspergillus fumigatus* XC6. *Applied Microbiology and Biotechnology*. 2007; 74(1): 239-243.
[29] Basu A, Prasad P, Das SN, Kalam S, Sayyed RZ, Reddy MS, et al. Plant growth promoting rhizobacteria (PGPR) as green bioinoculants: Recent developments, constraints, and prospects. *Sustainability*. 2021; 13(3): 1140.
[30] Pradhan S, Jena MK. In-vitro assessment of native plant growth promoting rhizobacterial isolates against diverse fungal phytopathogens. *International Journal of Plant & Soil Science*. 2023; 35(18): 1904-1921. Available from: https://doi.org/10.9734/ijpss/2023/v35i183473.
[31] Jerry A. *Water pollution. Definition, causes, effects, solutions, examples, types, & facts*. Encyclopedia Britannica; 2024. Available from: https://www.britannica.com/science/water-pollution [Accessed 14th Oct 2024].
[32] Vocciante M, Grifoni M, Fusini D, Petruzzelli G, Franchi E. The role of plant growth-promoting rhizobacteria (PGPR) in mitigating plant's environmental stresses. *Applied Sciences*. 2022; 12(3): 1231.
[33] Ikram M, Naeem M, Zahoor M, Rahim A, Hanafiah MM, Oyekanmi AA, et al. Biodegradation of azo dye methyl red by *Pseudomonas aeruginosa*: Optimization of process conditions. *International Journal of Environmental Research and Public Health*. 2022; 19(16): 9962. Available from: https://doi.org/10.3390/ijerph19169962.
[34] Ikram M, Naeem M, Zahoor M, Hanafiah MM, Oyekanmi AA, Islam NU, et al. *Bacillus subtilis*: As an efficient bacterial strain for the reclamation of water loaded with textile azo dye, orange II. *International Journal of Molecular Sciences*. 2022; 23(18): 10637. Available from: https://doi.org/10.3390/ijms231810637.
[35] Muliyadi M, Purwanto P, Sumiyati S, Mussadun M. Bioaccumulation of heavy metals using aquatic plants in wastewater. *International Journal of Public Health*. 2023; 12(2): 690-698.
[36] Jeevanantham S, Saravanan A, Hemavathy RV, Kumar PS, Yaashikaa PR, Yuvaraj D. Removal of toxic pollutants from water environment by phytoremediation: A survey on application and future prospects. *Environmental Technology & Innovation*. 2019; 13: 264-276.
[37] Abdelaal M, Mashaly IA, Srour DS, Dakhil MA, El-Liethy MA, El-Keblawy A, et al. Phytoremediation perspectives of seven aquatic macrophytes for removal of heavy metals from polluted drains in the Nile Delta of Egypt. *Biology*. 2021; 10(6): 560.
[38] Diaconu LI, Covaliu-Mierlă CI, Păunescu O, Covaliu LD, Iovu H, Paraschiv G. Phytoremediation of wastewater containing lead and manganese ions using algae. *Biology*. 2023; 12(6): 773. Available from: https://www.mdpi.com/2079-7737/12/6/773.
[39] Saeed Q, Xiukang W, Haider FU, Kučerik J, Mumtaz MZ, Holatko J, et al. Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: A comprehensive review of effects and mechanisms. *International Journal of Molecular Sciences*. 2021; 22(19): 10529. Available from: https://doi.org/10.3390/ijms221910529.
[40] Fan D, Smith DL. Characterization of selected plant growth-promoting rhizobacteria and their non-host growth promotion effects. *Microbiology Spectrum*. 2021; 9(1): 10-1128. Available from: https://doi.org/10.1128/spectrum.00279-21.
[41] Olaniran AO, Balgobind A, Pillay B. Bioavailability of heavy metals in soil: Impact on microbial biodegradation of organic compounds and possible improvement strategies. *International Journal of Molecular Sciences*. 2013; 14(5): 10197-10228.
[42] Wang T, Dai X, Zhang T, Xin C, Guo Z, Wang J. Formation and microbial composition of biofilms in drip irrigation system under three reclaimed water conditions. *Water*. 2022; 14(8): 1216. Available from: https://doi.org/10.3390/w14081216.
[43] Bose P. Bioremediation: An overview. *AZo Life Sciences*. 2021. Available from: https://www.azolifesciences.com/article/Bioremediation-An-Overview.aspx [Accessed 14th Oct 2024].
[44] Dixit R, Wasiullah X, Malaviya D, Pandiyan K, Singh UB, Sahu A, et al. Bioremediation of heavy metals from soil and aquatic environment: An overview of principles and criteria of fundamental processes. *Sustainability*. 2015;

7(2): 2189-2212.
[45] Kerur SS, Bandekar S, Hanagadakar MS, Nandi SS, Ratnamala GM, Hegde PG. Removal of hexavalent chromium-industry treated water and wastewater: A review. *Materials Today: Proceedings*. 2021; 42: 1112-1121.
[46] Haider FU, Liqun C, Coulter JA, Cheema SA, Wu J, Zhang R, et al. Cadmium toxicity in plants: impacts and remediation strategies. *Ecotoxicology and Environmental Safety*. 2021; 211: 111887. Available from: https://doi.org/10.1016/j.ecoenv.2020.111887.
[47] Rigoletto M, Calza P, Gaggero E, Malandrino M, Fabbri D. Bioremediation methods for the recovery of lead-contaminated soils: A review. *Applied Sciences*. 2020; 10(10): 3528. Available from: https://doi.org/10.3390/app10103528.
[48] Kinuthia GK, Ngure V, Beti D, Lugalia R, Wangila A, Kamau L. Levels of heavy metals in wastewater and soil samples from open drainage channels in Nairobi, Kenya: Community health implication. *Scientific Reports*. 2020; 10(1): 8434.
[49] Yan A, Wang Y, Tan SN, Mohd Yusof ML, Ghosh S, Chen Z. Phytoremediation: A promising approach for revegetation of heavy metal-polluted land. *Frontiers in Plant Science*. 2020; 11: 359. Available from: https://doi.org/10.3389/fpls.2020.00359.
[50] Ke X, Feng S, Wang J, Lu W, Zhang W, Chen M, et al. Effect of inoculation with nitrogen-fixing bacterium Pseudomonas stutzeri A1501 on maize plant growth and the microbiome indigenous to the rhizosphere. *Systematic and Applied Microbiology*. 2019; 42(2): 248-260.