

Article

Isolation and Morphological Identification of the Bacterial Strain *Pseudomonas Chlororaphis*

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Abstract: In this article, the *Pseudomonas chlororaphis* bacterial strain was isolated by taking samples from the moderately saline soil of the Fergana Valley and identifying all microorganisms. The morphology of the obtained *Pseudomonas chlororaphis* bacterial strain was determined by Gram staining. As is known, combating plant diseases and increasing their productivity has always been an urgent issue in agriculture. It is no secret that such chemicals have a negative impact on crop quality and soil fertility. With the development of science, various biopreparations are replacing chemical drugs and fertilizers. Their use improves soil fertility and crop quality. The *Pseudomonas chlororaphis* strain is a bacterium that is widespread in nature in plant, soil and water ecosystems and has many positive properties.

Keywords: *Pseudomonas chlororaphis*, auxins, cytokinins, gibberellins, phytohormone, Ashby, Pikovskiy, Chapeka, MALDI TOF, Gram-negative.

Introduction

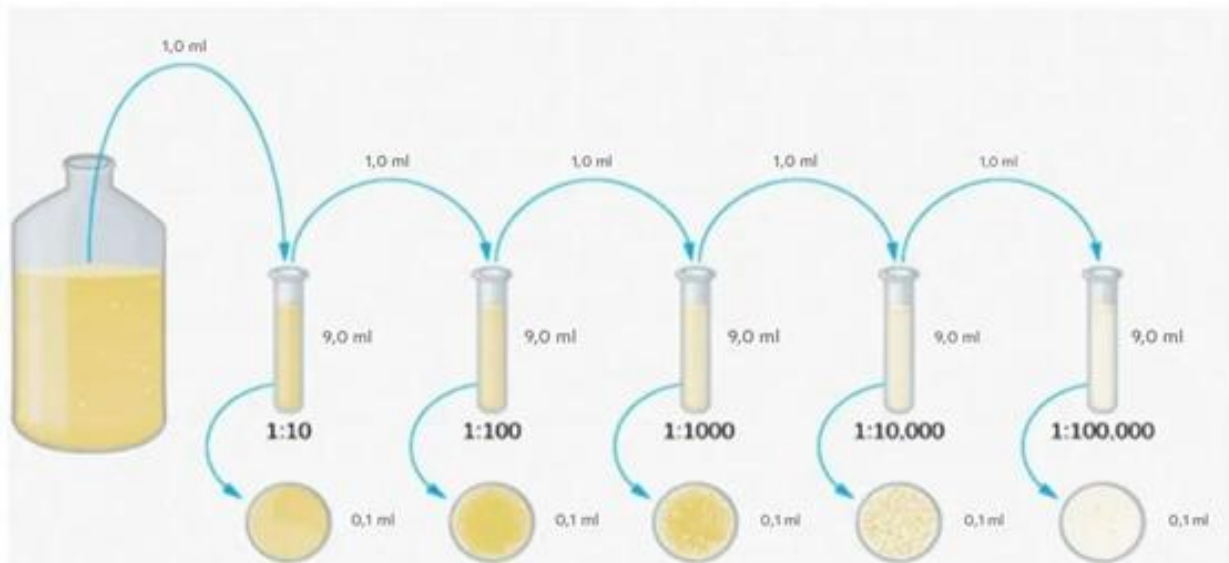
The agricultural sector is an important and integral part of our country's economy. Climate change and the decline in soil fertility due to various factors are detrimental to the quality and productivity of products in this sector. In such cases, the issue of increasing soil fertility and obtaining ecologically natural products remains a pressing issue[1]. Substances synthesized by microorganisms increase soil fertility and product quality. They are important in that they increase resistance to chemical factors[2]. A deep study of such properties of microorganisms, the creation and use of biopreparations based on them, can have a significant positive impact on the development of the agricultural sector[2,3]. Today, the main task in agriculture is to increase soil fertility and improve crop quality. Scientists now consider biological control as the solution to this problem. The strain *Pseudomonas chlororaphis* is especially widespread in the rhizosphere zone of the soil, and it changes the composition of the microbiota on the root surface, increasing the number of beneficial microorganisms and increasing resistance to various stress factors[4]. The genus *Pseudomonas* includes a very diverse group of bacteria found in soil, water and on plant surfaces[5]. This genus, which has more than 200 species, includes the known PGPRs, as well as opportunistic pathogens such as *P. aeruginosa* and obligate phytopathogens such as *P. syringae*[5,6].

The genus *Pseudomonas* is known for its rapid colonization and ability to engage in a wide range of interactions with plants, from beneficial to pathogenic. Known for their versatility in metabolic activities, *Pseudomonas* species produce phytohormones such as auxins, cytokinins, and gibberellins that directly stimulate plant growth[6]. In addition, they solubilize phosphorus, produce siderophores to increase iron availability, and contribute to the soil nitrogen cycle by facilitating colonization with AMF[7].

Methods

Plant growth promoting (PGP) microorganisms can be used as probiotics to increase the tolerance and resistance of plants to abiotic and biotic stresses, and in this regard, strains belonging to the *Pseudomonas chlororaphis* group have been shown to have such abilities as PGPR candidates [8,9]. Soil samples were collected from moderately saline soils in the Fergana Valley of the Republic (40°34'31.2"N 70°54'02.8"E). We collected samples from the top layer of soil (5–15 cm deep) using a sterile spoon, placed them in sterile tubes, and carefully transported to the microbiology laboratory. After sampling, they can be stored in the refrigerator for up to 24 hours. During this time, it is necessary to carry out microbiological culture, and it is advisable to carry out microbiological culture on the day of sampling.

If the soil is wet at the time of sampling and is stored for a long time, the number of bacteria in it may change. A decrease in humidity causes the growth of fungi in the sample, which can negatively affect the growth of other bacteria. To ensure the average homogeneity of the soil samples, they are thoroughly mixed, cleaned of plant roots and various impurities, observing the rules of aseptic technique. The soil samples were brought to the laboratory and placed in a refrigerator for storage at 40C. The standard "Soil dilution" method was used to dilute the soil samples. The soil solution was diluted from 10⁻¹ to 10⁻¹⁰ [10].



1-figure. Soil sample dilution process sequence.

The diluted soil samples were inoculated into 4 different nutrient media: Ashby, Pikovskiy, Chapeka and GPA. Each medium is designed for the growth of different microorganisms [122]. The inoculated samples were placed in a thermostat at 280C and incubated for 3 days. The grown colonies were collected using sterile microbiological rings in a laminar flow hood and transferred to new nutrient media.

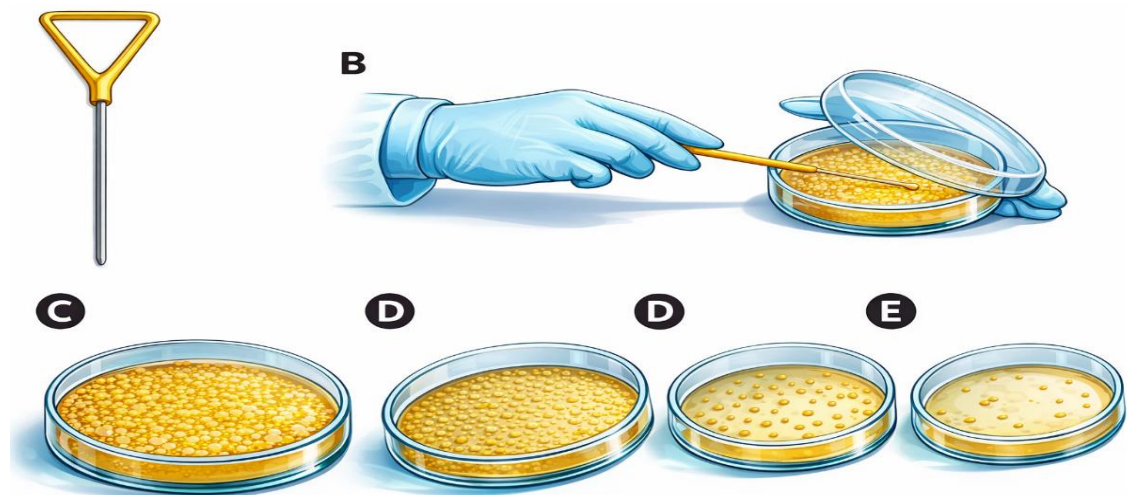


Figure 2. Drigalsky spatula and its use (A), cultures grown using the spatula (B,C,D,E).

The isolated pure cultures were identified by MALDI TOF and molecular methods. This process clearly establishes the taxonomic affiliation of the isolates, assesses their ecological or clinical significance, and provides a solid basis for further research. MALDI TOF mass spectrometry (Matrix-assisted laser desorption/ionization—time-of-flight) allows for rapid and high-resolution species identification by analyzing the whole-cell or protein mass fingerprint (PMF) of bacterial cells. Because each species is characterized by the mass spectra of its own ribosomal proteins and other stable proteins, this method, typically performed using systems such as Bruker Biotyper or VITEK MS, allows for identification to the family or species level within minutes[13].

Molecular methods, especially 16S rRNA gene sequencing, provide more precise information at the genetic level and are used as the “gold standard” in cases where MALDI TOF results are ambiguous or inconclusive. The combination of these two approaches provides a polyphasic strategy: proteomic and genetic data complement each other, minimizing identification errors and revealing more clearly the phylogenetic relationships of strains [14]. The MALDI-TOF MS method is based on the analysis of colonies of isolated bacterial strains, either directly or after simple preparation steps such as the addition of formic acid and matrix. This significantly reduces the time and labor required compared to traditional biochemical tests. The spectra are obtained in the mass range of 2000–20,000 Da and mainly reflect the characteristic features of ribosomal proteins and other constitutive proteins. The obtained spectra are then compared with reference spectra in Bruker or other databases. The results are evaluated by value: if this indicator is higher than 2.0, it indicates a reliable identification at the species level, and if in the range of 1.7–2.0, it indicates accuracy at the family level. This method is widely used in clinical, environmental and food microbiology, as it has high throughput and allows the simultaneous analysis of many strains [15,16]

However, for some rare or environmental isolates, the database may not be sufficient. In such cases, the accuracy of identification decreases, and confirmation by molecular methods is required. In a combined approach, MALDI-TOF is used as a rapid screening tool, while molecular methods are used for confirmation and further analysis [17].

The resulting sequences are compared using BLAST analysis in databases such as NCBI GenBank or EzTaxon. Typically, 98.65–99% similarity indicates species-level accuracy. This method is especially important for species that are not identified by MALDI-TOF or are very closely related (e.g., *Bacillus* or *Pseudomonas* groups), as it allows for the construction of phylogenetic trees and the characterization of new strains. However, 16S rRNA sequencing may be limited in some cases in distinguishing very closely related bacterial species. Therefore, additional genes (*gyrB*, *rpoB*) or whole genome sequencing (WGS) are used. The combination of both methods significantly increases the accuracy of identification. After isolation of pure cultures, the morphological characteristics of bacterial colonies were observed under an OLYMPUS BX 41 microscope (Japan) using the traditional Gram staining method [17,18].

The following main indicators are taken into account when characterizing a bacterial colony:

- colony shape;
- colony size;
- colony color;
- colony surface;
- colony edges;
- colony density and structure

Gram staining method

Gram staining uses a series of staining solutions to differentiate bacterial cells. Each solution has a specific chemical composition, and their concentration determines the efficiency of the process.

The first step is to use a crystal violet solution containing 1% crystal violet dye and 95-99% solvent (usually ethyl alcohol or water). This solution stains bacterial cells purple. A small amount of culture biomass is added to a drop of water placed on a microscope slide and mixed with a bacterial suspension. The excess biomass is burned off. The resulting weak turbidity is spread on the slide in a 2 cm diameter circle, air-dried, and a smear is prepared. In a properly prepared smear, the bacteria are scattered, forming a thin layer. Then a drop of crystal violet dye is added and held for one minute. It is held in running water until the color turns light and dried. In the next step, an iodine solution (gram iodine or lugol solution) is applied. Its composition consists of 1% iodine, 2% potassium iodide and 97% water. Iodine forms a complex with crystal violet and fixes the dye in the cell wall. After exposure, it is kept for one minute, rinsed in running water and dried. In the third step, 95-96% ethyl alcohol or acetone is used as a decolorizing solution. In this case, the solution washes the dye complex from gram-negative bacteria, as a result of which they become decolorized, while gram-positive bacteria retain their color. After using the solution, it is kept until the color fades and then washed in running water and dried [18].

In the fourth step, the sopphanim dye is applied, which consists of 0.5-1% sopphanim dye and 99-99.5% solvent. This dye solution stains the colorless gram-negative bacteria pink or red. After staining, they are washed in running water and dried [18,19].

Result

At the last stage, a drop of immersion oil is added and observed under a microscope. Microorganisms isolated from soil samples grew well on various selective nutrient media (Ashby, Pikovskaya, Chapek and GPA). After the incubation process, bacterial colonies with different morphological characteristics were formed. It was found that the colonies differed in shape, color, surface and structure, which indicates the diversity of soil microflora. The isolated pure cultures were analyzed microscopically using the Gram stain method. According to the results, all the studied isolates were classified as Gram-negative bacteria. Under the microscope, the cells appeared pink-red in color, which confirmed the presence of a thin peptidoglycan layer in their cell wall and the presence of an outer membrane. The predominance of Gram-negative bacteria may be related to the salinity level of the soil and environmental conditions. In such an environment, stress-resistant, metabolically adaptable bacteria are usually more common. The isolated colonies also differed from each other morphologically. Round and irregularly shaped colonies, smooth and rough surface species were observed among the colonies. This allows us to assume that the isolates belong to different taxonomic groups. The isolated pure bacterial cultures were subjected to preliminary identification using the MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) method. According to the results of the analysis, mass spectral profiles for the obtained isolates were successfully formed and compared with the reference database.

MALDI-TOF MS results allowed reliable identification of the majority of bacterial isolates to the genus level. Isolates with log(score) values above 1.7 were identified with certainty at the genus level, and isolates with log(score) values above 2.0 were identified with certainty at the species level. However, some isolates had low spectral similarity, which resulted in uncertainty in their identification at the exact species level. This is mainly explained by the insufficient representation of bacteria isolated from the environment in the database.

low spectral similarity, which resulted in uncertainty in their identification at the exact species level. This is mainly explained by the insufficient representation of bacteria isolated from the environment in the database.

Therefore, in order to confirm the identification results and increase the accuracy, 16S rRNA gene sequencing was performed. The obtained nucleotide sequences were compared in the NCBI GenBank and EzTaxon databases using the BLAST algorithm. According to the results of BLAST analysis, the majority of isolates showed 98.65–99.8% similarity, which confirmed their accurate identification at the species level.

Among the identified isolates, the bacterium *Pseudomonas chlororaphis* was selected for study.

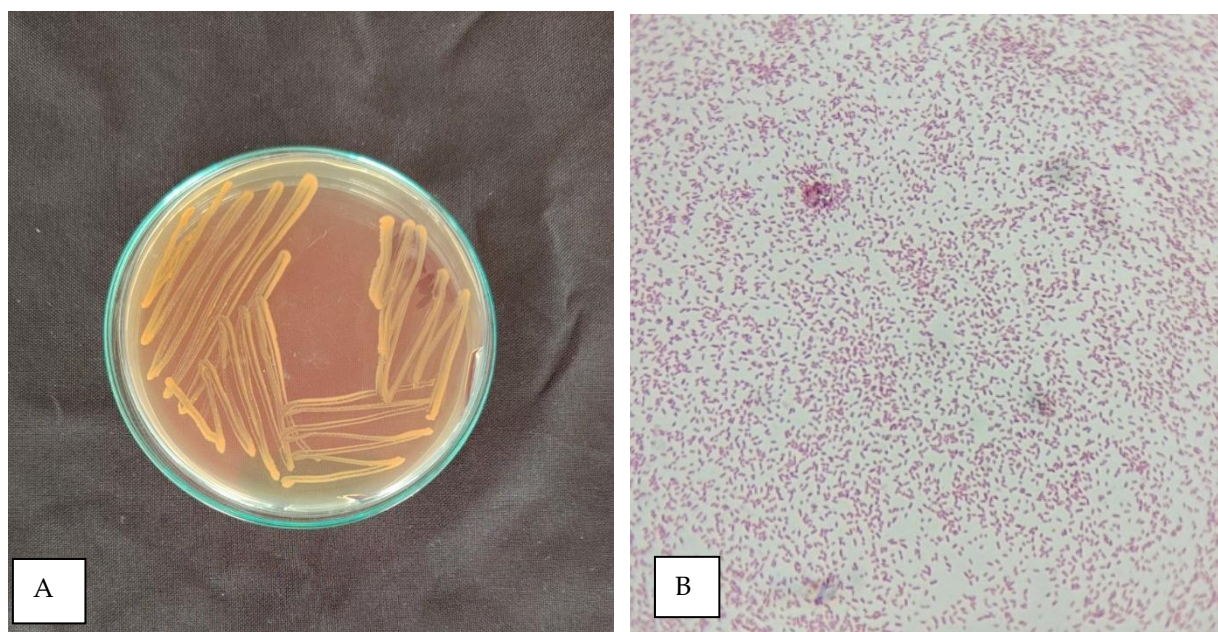


Figure 4. Colony of the bacterial strain *Pseudomonas chlororaphis* on MPA medium (A) and microscopic view (B).

The morphological characteristics of the colonies formed when the studied bacterium *Pseudomonas chlororaphis* was grown on MPA nutrient medium were analyzed. According to the observation results, the colonies grown in Petri dishes had a clear pigmentation and appeared in a light yellow-golden color. The shape of the colonies was mainly elongated along the lines of sowing, and in some places they were observed to merge with each other. The surface of the colony was smooth and shiny, which indicates that this is associated with the active metabolic processes of the bacteria. The consistency was soft and slightly sticky. The edge of the colonies was flat, and no uneven or tooth-like structures were observed. The colonies were relatively large in size, and in densely planted zones, cases of merging were also observed. When viewed under a microscope after Gram staining, gram-negative rod-shaped bacterial cells were clearly observed. These morphological characteristics correspond to the typical colonial characteristics of *Pseudomonas chlororaphis* and confirmed its ability to produce pigment and to grow actively in nutrient media.

In conclusion

Considering the many beneficial properties of *Pseudomonas chlororaphis*, by isolating this bacterial strain and determining its amount in the soil, we can use this bacterial strain in the cultivation of many useful plants and in increasing the yield. *Pseudomonas chlororaphis* was isolated and identified from the soils of the Fergana region and its morphological structure was determined to be a gram-negative bacterium.

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