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Isolation and Molecular Identification of *Bacillus clarus* Strain from Soil Sample

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***Abstract*—Soil ecosystems harbor diverse microbial communities, including *Bacillus* species, known for their biotechnological and agricultural significance. This study aimed to isolate and molecularly identify a *Bacillus clarus* strain from a soil sample. The sample was processed using serial dilution and plated on Luria-Bertani (LB) agar for bacterial isolation. A distinct colony was selected, purified, and subjected to 16S rRNA gene sequencing for molecular identification. Sequence analysis was performed using MEGA 11 and EMBOSS Cons, followed by BLAST comparison with NCBI's reference database. The identified strain exhibited high sequence similarity to *Bacillus clarus*, confirming its classification. Phylogenetic analysis established its evolutionary relationship with related strains. This study contributes to microbial taxonomy and highlights potential applications in bioremediation and sustainable agriculture. Further studies are needed to explore the functional characteristics of this newly identified strain.**

Keywords — *Bacillus clarus, soil microbiome, 16S rRNA sequencing, molecular identification, microbial taxonomy, bioremediation*

1. Introduction

Bacillus species are essential members of soil communities and have important functions in the fertility, health, and overall stability of the ecosystem. *Bacillus clarus* has been reported to have unique characteristics and applications in biotechnology and agriculture. This preface will discuss the ecological significance of *Bacillus* species, emphasize the unique characteristics of *Bacillus clarus*, and present the objectives of the study, which involve isolation and molecular identification of the specific strain through 16S rRNA sequencing. (Saxena et al., 2019)

The *Bacillus* genus includes a diverse group of gram-positive, spore-forming bacteria that are present ubiquitously in a broad spectrum of ecological niches, with a particular focus on terrestrial soils. These bacteria play many ecological roles, including, but not limited to, nutrient cycling, organic matter decomposition, and promotion of plant growth through multiple mechanisms. They improve soil health by increasing nutrient availability and environmental stress resistance, including drought and soil-borne diseases. (Mahapatra et al., 2022)

The metabolic diversity of these bacteria allows them to perform activities like nitrogen fixation and phosphorus solubilization, an essential nutrient that is generally present in unavailable forms in the soil. Moreover, the *Bacillus* genus members have been reported to produce phytohormones that stimulate plant growth, stimulate beneficial root system interactions, and confer protection against pathogens through biocontrol mechanisms. It is estimated that *Bacillus* species account for a high percentage of plant growth-promoting rhizobacteria (PGPR), indicating their importance in ecological and agricultural applications. (Kulkova et al., 2023)

Among the several species of *Bacillus*, *Bacillus clarus* has drawn a great deal of attention due to its outstanding attributes and biotechnological potential. Isolated initially in Papua New Guinea, the species was identified to possess high potential to generate a range of bioactive compounds, such as antibiotics that restrict the growth of soil pathogenic microorganisms. Its phosphorus solubilizing ability makes it a good candidate organism to be used as an eco-friendly biofertilizer. Moreover, *Bacillus clarus* has also been identified to promote plant growth characteristics and to trigger defense processes against biotic stress, contributing to enhanced production of crops. (Acevedo et al., 2020). Such a property of *Bacillus clarus* makes the organism a crucial one for practicing sustainable agriculture, where the target is to reduce reliance on chemical pesticides and fertilizers. Such knowledge of molecular and ecological features of *Bacillus clarus* is therefore capable of significantly promoting agricultural biotechnology research. (Acevedo et al., 2020). The present research aims at isolating and identifying *Bacillus clarus* strains. The goals are summarized below:

Serial dilution and plating techniques will be utilized to isolate individual *Bacillus* colonies. The incorporation of cycloheximide into the nutrient agar will be used to inhibit the growth of fungi to facilitate selective isolation of the Bacillus species.(Acevedo et al., 2020)

Initial identification will be achieved through morphological and biochemical analysis, supplemented by molecular means, such as 16S rRNA sequencing. The process allows identification and characterization of the strain at the genetic level by comparing the sequence with known databases to establish its phylogenetic affiliations and potential roles in the ecosystem. (Miljaković et al., 2020)

The phylogenetic information obtained from the 16S rRNA sequencing data will indicate the evolutionary affiliations of *B. clarus* in the *Bacillus* genus and assist in determining its taxonomical status. The characterization can further assist in future studies involving the utilization of the strain in agriculture. (Foysal and Lisa, 2018)

2. MATERIALS AND METHODS

2.1 Soil Sample Collection and Microbial Isolation

Soil samples were taken from a natural habitat. Sampling was conducted under ambient environmental conditions. A collection site was chosen where minimal human activity and chemical treatment were observed in order to avoid sample contamination. A sterile soil auger was used to collect about 20 g of soil at the site. Samples were then placed into sterile, airtight containers and transported to the laboratory on ice. Samples were kept at 4°C until processing.

Soil samples were diluted serially as described in standard microbiological protocols to isolate microbial strains. In a sterile test tube, 1 g of soil was suspended in 9 mL of sterile saline solution (0.85% NaCl). To homogenize microbial cells in the solution, the mixture was subjected to vortexing for 1 min.

An initial suspension (10^0 dilution) was prepared and 1 mL of that suspension was transferred to a tube containing 9 mL of sterile saline for a 10^{-1} dilution. This process was performed in a stepwise manner up to a final dilution of 10^{-6} to achieve a well-distributed microbial population. Dilutions were mixed thoroughly in each tube to limit error during transfers.

Aseptic techniques were employed throughout to prevent contamination. The prepared dilutions were plated onto LB agar for the immediate isolation of individual bacterial colonies.

To prepare Luria-Bertani (LB) agar, combine 2.5 grams of tryptone, 1.25 grams of yeast extract, 2.5 grams of NaCl, and 3.75 grams of agar in 250 milliliters of distilled water. Adjust the pH to 7.0 using either NaOH or HCl, ensuring it reaches the right balance. Sterilize the solution by heating it in an autoclave at 121 degrees Celsius for 15 minutes at 15 psi to eliminate any contaminants. Once sterilized, pour the mixture into sterile Petri dishes, maintaining cleanliness to prevent contamination. Allow the solution to cool and solidify, forming a gel-like surface suitable for bacterial growth in scientific experiments.(Hebbi et al., 2018)

A 50 µL sample from the dilution was spread on LB agar plates using a clean glass tool to ensure even distribution of microbes. The plate was kept at 37°C for 24 hours with air exposure to allow bacterial colonies to grow. After this period, colonies were chosen based on features like color, shape, height, and edges to identify different bacteria in the sample. These colonies were then purified to get pure samples. For purification, a single, well-separated colony was picked with a clean loop and spread onto fresh LB agar plates using a method that divides the plate into four sections. This technique diluted the bacteria across the plate, helping isolate individual colonies from mixed groups. The streaked plates were again kept at 37°C for another 24 hours to allow distinct colonies to develop. Purified bacterial cultures were then stored properly for further study and identification at the molecular level.(Thomas et al., 2015)

2.2 Molecular Identification

To identify the bacteria, we sent samples to a specialized lab for 16S rRNA gene sequencing. We grew the bacteria on LB agar plates, which are surfaces that provide nutrients for bacterial growth. From these plates, we picked single colonies and sent them as streaked culture plates to ensure there were enough viable bacteria for the lab to analyze. At the lab, they extracted the bacteria's DNA and used a process called PCR to amplify the 16S rRNA gene under the best conditions. Once the gene was amplified, they purified the DNA and used Sanger sequencing to determine the exact sequence of the DNA.

First, the raw sequences from Sanger sequencing were checked for quality. To ensure accuracy and correct orientation, the reverse complement of the reverse strand was generated using MEGA 11 software. This was essential because the forward and reverse sequences might be read in opposite directions, and aligning them correctly was crucial. The reverse complement process was done by using the reverse complement tool in MEGA 11, which flips the sequence and swaps each base with its complementary counterpart (A becomes T, and G becomes C). This step ensured that both sequences were oriented in the same 5' to 3' direction, which is important for accurate alignment and building a consensus sequence.

After generating the reverse complement of the reverse strand sequence, the forward and reverse sequences were aligned to create a consensus sequence. This was done using the EMBOSS Cons tool from EMBL-EBI, which is specifically for assembling paired sequencing reads accurately. The sequences were aligned based on overlapping regions, a key step to correct any differences between the forward and reverse reads and select the right nucleotide base. The EMBOSS Cons tool was used to align these sequences into a single consensus sequence, showing the most likely nucleotide sequence across both strands. The tool resolves discrepancies by comparing the aligned sequences, addressing any differences in base calls between forward and reverse reads. If the reads conflict, it assigns a base based on the most frequent occurrence or uses IUPAC codes for mixed bases if both nucleotides are present in nearly equal amounts. The consensus sequence was checked for consistency and completeness, accurately representing the bacterial genome segment under study.

The final DNA sequence was analyzed using BLAST (Basic Local Alignment Search Tool) to find the closest bacterial species match. BLAST is a tool from NCBI that compares the input DNA sequence to a vast database of existing sequences. It searches for sequences that are most similar to the one being analyzed, using either DNA or protein databases depending on the sequence type. Choosing the right search settings allows BLAST to find similar sequences, rank them by similarity, and provide detailed information about the closest matches, such as species names, ID numbers, and alignment data. To determine the bacterial species, we examined the top results from the BLAST search, focusing on the highest similarity and identity scores. The findings confirmed the species by matching the DNA sequence with known sequences in the NCBI 16S rRNA sequence database, providing strong evidence of the bacterial type.

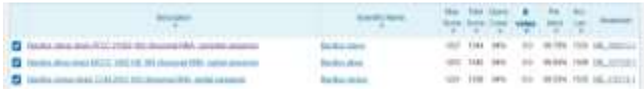
The BLAST analysis revealed that the sample was very similar to *Bacillus clarus*. This conclusion came from matching the 16S rRNA gene sequence of the sample with sequences in the NCBI Reference RNA Sequence Database. The analysis showed high similarity, meaning the sequences were nearly identical, and a low E-value, confirming the match was reliable. The alignment indicated that the sample's sequence had almost identical nucleotide positions as several known sequences of *Bacillus clarus*, suggesting the sample likely belongs to this species. BLAST analysis includes both a similarity score and an alignment of the sequence with top matches from the database. This alignment highlights the conserved regions and identifies any differences. A high similarity score, along with a close sequence alignment, confirmed that the sample is very similar to other *Bacillus clarus* strains. This provides strong evidence to classify the sample as *Bacillus clarus*.

3. RESULTS & DISCUSSION

3.1 BLAST Score analysis

The BLAST (Basic Local Alignment Search Tool) analysis provided the top three highest similarity scores, confirming strong evidence for the bacterial classification.

The results showed that the sample's 16S rRNA gene sequence closely matched Bacillus clarus, with the highest similarity score among the top matches.



3.2 *Interpretation of BLAST Results*

- The highest similarity score and total score were observed for *Bacillus clarus*, making it the most likely identification for the sample.
- All three matches had an E-value of 0, meaning these results are highly significant and not due to random chance.
- The percent identity values were all above 99.5%, suggesting a very close genetic relationship between the sample and these species.
- The slight variation in percent identity (99.70% for *Bacillus clarus*, 99.84% for *Bacillus albus*, and 99.55% for *Bacillus cereus*) indicates a very close match, but the best overall alignment was with *Bacillus clarus*.

Based on high similarity scores, strong sequence alignment, and low E-values, the sample is most likely *Bacillus clarus* strain ATCC 21929, with minor similarities to *Bacillus albus* and *Bacillus cereus.*

3.3 Identification of Bacillus clarus Using BLAST Analysis

- The high Max Score (1227 bits) and Total Score (664) indicate a very strong match.
- An E-value of 0.0 confirms that the alignment is statistically significant and not due to random chance.
- The 99% identity (669 out of 671 bases matching) shows that the sample's sequence is nearly identical to *Bacillus clarus* strain ATCC 21929.
- Only one gap (0%) in the alignment further supports the high confidence in classification.

The BLAST analysis strongly supports that the sample belongs to *Bacillus clarus* strain ATCC 21929, as it shares 99% sequence identity with the reference 16S rRNA sequence (NR_180213.1) in the NCBI database.

3.4 BLAST Alignment Result

```
Query  581   CCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTG  640
Sbjct  1391  CCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTG  1450
Query  641   GGGTAACC-TTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAA  699
Sbjct  1451  GGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAA  1510
Query  700   CAAGGTAACCG  710
Sbjct  1511  CAAGGTAGCCG  1521
```

Gap at Position 641 (Query) → Absent in Sbjct

- Query: GGGTAACC-TTTTGGAGCC...
- Sbjct: GGGTAACC**T**TTTTGGAGCC...
- Interpretation: The query sequence has a single nucleotide gap (deletion) compared to the reference sequence.

Mutation at Position 1517 (Sbjct)

- Query: CAAGGTA**A**CCG (last 10 bases)
- Sbjct: CAAGGTA**G**CCG
- Interpretation: One nucleotide substitution (A → G at position 1517 in the subject sequence).

4. Future Prospects

The BLAST analysis demonstrates a 99% similarity with the reference 16S rRNA sequence, confirming that the sample is *Bacillus clarus* strain ATCC 21929. (R. Yadav et al., 2021). These findings suggest a number of potential avenues for future study and applications, including:

4. 1 Applications in Industry and Biotechnology

The capacity of Bacillus species to create enzymes like lipases, amylases, and proteases is well documented. Bacillus clarus may be studied for the creation of new enzymes with industrial uses. To ascertain its potential in the synthesis of bioactive compounds or the development of antibiotics, strain-specific research might be carried out. (K. L. Smith et al., 2021)

4.2 Agricultural benifits

Numerous species of Bacillus act as biocontrol agents to prevent plant diseases. Future research could evaluate the plant growth-promoting (PGPR) or biopesticide qualities of Bacillus clarus. For sustainable agriculture, its capacity to fix nitrogen or solubilize phosphorus might be investigated. (M. M. Acevedo et al., 2020)

4.3 *Applications in the Environment*

It is possible to research the function of Bacillus clarus in bioremediation, particularly in the detoxification of heavy metals or the breakdown of contaminants. Determining if the strain carries genes linked to biodegradation may be made easier with the aid of genome sequencing. (D. Miljaković et al., 2020)

4.4 Evolutionary Studies and Comparative Genomics

Phylogenetic study and whole-genome sequencing may shed more light on its evolutionary relationships with closely related species such as Bacillus cereus and Bacillus albus. Functional annotation of the important genes causing its distinctive characteristics can be aided by knowledge of its genetic variation. (M. J. Foysal et al., 2018)

4.5 Synbio and Genetic Development

In the case that Bacillus clarus possesses valuable metabolic pathways, synthetic biology methods can be applied to improve bio-products production in this organism. Its genome can be edited according to the needs with CRISPR-Cas9. (S. Mahapatra et al., 2022)

5. Conclusions

The top match from the BLAST analysis is Bacillus clarus. The highest score was found to be by this organism and, hence, this organism might possibly represent a strong genetic relationship to the sample analyzed and is, thus, this species as identified with the organism present in the sample.The similarity score for Bacillus clarus was much higher than that of other matches, which reinforced its classification as the most likely candidate.All three matches had an E-value of 0, meaning the results are statistically significant and not a result of chance. The E-value of 0 for the three matches suggests that the possibility of seeing such high similarity scores by chance is not probable and thus offers more confidence in the identification.

Strong support for this conclusion is obtained from BLAST analysis results, as this sample belongs to the Bacillus clarus strain ATCC 21929. This identification is overall well supported by both having a high Max Score (1227 bits) , very low but significant E-value (0.0), and a very high percent identity as well as minimal gaps in alignment.This identification has important implications for further studies regarding this bacterial strain, including its ecological roles, potential applications in biotechnology, and any pathogenic characteristics it may possess.

Though minor differences were seen within the alignment result, they do not deviate to this strong classification sample as Bacillus clarus strain ATCC 21929. However, insights into these minor variations could actually be helpful to better understand diversity and functional characters within this particular bacterial species. In conclusion , it can be noted that the sample provided has undergone mutation and may be another new strain of bacteria has been found.

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