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Phylogenetic Analysis for Molecular Characterization and Identification of *Rhodopseudomonas* Spp.

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ABSTRACT

The BLAST analysis is a fast method for identifying likely microbial species. However, it is not always able to definitively determine closely related genera or taxa because there may be numerous similar or identical scores from homologous sequences. In order to overcome this constraint, the utilization of phylogenetic tree construction and analysis is employed to get a more accurate molecular characterization of microorganisms. This study utilized the 16S rDNA sequence of BRSSOU1, which formed a cluster with a particular strain in the phylogenetic tree and exhibited a 99.92% alignment with Rhodopseudomonas sp. according to the NCBI-BLAST results. Therefore, BRSSOU1 was determined to be Rhodopseudomonas palustris. Our deduction is that BRSSOU1 is a variant of Rhodopseudomonas palustris. The strains were obtained from water samples collected at the source of the Jhelum River near Verinag, Kashmir. In addition, samples of wastewater were obtained from several rivers, namely Arpath, Lidder, Neelum, Sind, Kunhar, and Pohru. PNSB strains were isolated and identified using both traditional bacterial culture techniques and molecular approaches. The identification technique effectively uncovered these strains of Purple Non-sulfur Bacteria (PNSB).

Keywords: *BLAST, Biochemical, Rhodopseudomonas, Phylogenetic, Microorganisms.*

Introduction

A significant group of microorganisms known as anoxygenic phototrophic bacteria are those that share the bacteriochlorophyll-dependent photosynthesis metabolic process [1-5]. The phototrophic purple bacteria can be divided into two groups: purple Sulphur bacteria (*Chromatiaceae* and *Ectothiorhodospiraceae*) and purple non-sulfur bacteria [6]. The two main groups of anoxygenic phototrophs are. The phototrophic green bacteria belong to the families *Chlorobiceae* and *Chloroflexaceae* [7]. These are the two main groups of anoxygenic phototrophs. The phototrophic green bacteria include *Chlorobiceae and Chloroflexaceae* [8-10]. These species exhibit metabolic capacities that are generally representative, but not have all of the nutritional traits of other purple non-sulfur phototrophs [11-12]. *Rhodobacter capsulatus* is considered the most versatile among prokaryotes due to its ability to grow using various methods such as photoautotrophs, chemoautotrophy, photoheterotrophy, and chemoheterotrophs. It can also utilize a wide range of electron acceptors and ferment sugars [13-15]. It is not possible to include every metabolic activity in this overview due to their vast variety. As a result, the discussion will center on anaerobic respiration and photosynthesis. This study is relevant to previous works [16-20]. For the latest advancements in nitrogen fixation in phototrophic bacteria. The purple non-sulfur bacteria exhibit an impressive range of morphological, biochemical, and metabolic characteristics [21-22]. From a taxonomic perspective, there is also a wide range of diversity, with various genera falling under the Proteobacteria and its three subgroups [23-24]. The Marmur (1961) Extraction and purification of genomic DNA was performed using a specific method, while the G+C content of the DNA was measured using HPLC [25-26]. The cell material used for the 16S rRNA gene sequencing was obtained from a colony.

An extraction and purification of DNA was performed using a Qiagen genomic DNA extraction kit. PCR utilized recombinant Taq polymerase (Genei) for the experiment. The 16S rRNA gene sequence was obtained by sequencing with four primers: : F9-27 (59-GTTTGATCCTGGCTCAG 39), F9-372 (59-TACGGGAGGCAGCAG-39), F9-790 (59-GGTTACCTTGTTACGACT-39), and R9-1489 (59-TAC CTTGTTACGACTTCA-39). Positions 11–27 and 1489 1506 for F9-27 and R9-1489, respectively [27-28]. Obtaining the 16SrRNA gene sequences was done through cycle sequencing with a SequiTherm sequencing kit (Biozym) and chain termination reaction, without the need for gene cloning. (Sanger et al., 1977) Utilizing an automated laser fluorescence sequencer from Pharmacia. PCR amplification was conducted following the established protocol [29-30]. Using the EzTaxon server [31] and an NCBI BLAST search [32]. Calculating the pairwise 16S rRNA gene sequence similarities and identifying phylogenetic neighbours. MEGA4 [33]. Specialized software was employed to conduct the phylogenetic analysis of the individual sequences, while the MEGA4 CLUSTALW technique was applied for sequence alignments. The Kimura two-parameter model is widely used in the field of molecular evolution [34], was employed to calculate distances. Utilizing the MEGA4 programme, phylogenetic trees were reconstructed using neighbor-joining and minimum evolution techniques. A method based on bootstrap was employed to obtain support values (%). Genome DNA–DNA hybridization was employed to explore the taxonomic relationships among different strains of *Rhodopseudomonas*, just like a microbiologist would do. Utilizing a DIGG High Prime DNA labelling and detection starter kit II (Roche), a membrane filter method was employed to determine genomic similarity [35-36]. Hybridization was conducted for each sample with three replications, including a control where the strains used for binding and labelling were

reversed. The resulting mean values are expressed as DNA-DNA relatedness values. Even tiny differences in sequence can be enough to distinguish between organisms, as certain genes and genetic material are highly conserved among similar species. Working with bacteria at the molecular level allows for precise species characterization and the construction of taxonomic trees. There are approximately nine variable regions in the 16s rRNA gene, but for phylogenetic research, the V4, V5, and V6 areas prove to be particularly valuable. Figure 1-1 [37]. Similar to this, as shown in Figure 1-2 [38].

The Internal Transcribed Spacer (ITS) sections of the fungal 5.8s rRNA gene exhibit significant variation, making them effective for distinguishing between different species of fungi. For this study, we isolated microbial DNA and used universal primers specific to ribosomal DNA in a polymerase chain reaction (PCR) assay. The amplicons underwent purification and sequencing to identify the microorganisms at both the species and genus levels. The DNA from the bacteria sample was isolated and analyzed using agarose gel. Fragment of 16S rDNA was amplified using 27F and 1542R primers in PCR method and the resulting amplification was analyzed in an agarose gel. To eliminate contaminants that interfere with the sequencing reaction, the PCR product was filtered. Using the BDT v3.1 Cycle sequencing kit and the ABI 3730xl Genetic Analyzer, DNA sequencing was completed. BioLign/BioEdit or CodonCode Aligner software was used to create the consensus sequence of the PCR amplicon-based on forward and reverse sequence data. The non-redundant nucleotide (nr) database of the NCBI was searched for the consensus sequence using—tool for Basic Local Alignment Search (BLAST).

The top 10 organism sequences were obtained after the BLAST result was ranked according to a percentage of identity. Multiple sequence alignment of the top 10 BLAST hits and query sequences was carried out using the Clustal-W programme in MEGA-X. Using MEGA-X software, a phylogenetic tree was predicted [39].

Materials and Methodology

2.0. The 16S rRNA gene was amplified using universal primers according to the following procedure.

The PCR was programmed for amplification with the reaction mixture consisting of:

1. Primers: 2 microliters of each primer (10 µmol/µl)
2. DNA Template: 2 to 5 microliters (25 ng/µl)
3. DNase-free Water: 16-19 microliters
4. Master Mix: 25 microliters

The PCR steps were as follows

1. Initial denaturation: 5 minutes at 94°C
2. Denaturation: 1 minute at 94°C
3. Annealing: 1 minute at 55°C
4. Elongation: 1 minute at 72°C

These steps were repeated for 30 cycles. The final elongation was performed for 10 minutes at 72°C [42-45]

2.1. Agarose Gel Electrophoresis

- This procedure was performed to visualize the PCR products [46].
- A 0.8% agarose gel was prepared in 1x TAE buffer with Ethidium bromide (1 µg/ml). The mixture was heated until the agarose completely dissolved.
- The heated gel was cooled to 55°C and then poured into the casting tray of the electrophoresis unit.
- To equilibrate, the gel was run for 30 minutes at 50V. Subsequently, 10 µl of the sample (9 µl isolated DNA + 2 µl loading dye) was loaded into the wells and the gel was run at 80V for 30 minutes.
- The bands were visualized using a UV transilluminator under UV light [47].

2.2. 16S rRNA gene sequencing

The PCR product obtained using universal primers was outsourced to Primer Biotech Private Limited for sequencing [48].

2.3. Phylogenetic Analysis

The sequence that was acquired was sent to the National Centre for Biotechnology Information (NCBI) to determine phylogenetic relatives that were closely related [43]. The sequence, provided in FASTA format, underwent a BLAST search to identify organisms with high sequence similarity [49]. Subsequently, the identified sequences were deposited with an accession number [50]. To construct the phylogenetic tree, the 16S rRNA sequences were manually aligned with corresponding representatives of *Rhodopseudomonas palustris*. Data were retrieved from databases such as DDBJ, GenBank, and EMBL. Evolutionary analysis was conducted using MEGA X software [51-60].

3.0. Results

The strain of PNSB(BRSSOU1), the thermotolerant *Rhodopseudomonas sp*. identified from water samples collected at the origin of the Jhelum River in Verinag, Kashmir. Additional wastewater samples were collected from various rivers including the Arpath, Lidder, Neelum, Sind, Kunhar, and Pohru rivers. Traditional bacterial culture techniques and molecular methods were employed to isolate and identify these PNSB strains.

The identified strains of PNSB demonstrated significant potential in reducing water pollutants. Specifically, BRSSOU1 was identified as *Rhodopseudomonas palustris* and showed substantial reductions in chemical oxygen demand (COD), biological oxygen demand (BOD), and total dissolved solids (TDS). These reductions were quantitatively significant, highlighting the effectiveness of PNSB in wastewater treatment applications.

To further characterize these strains, a bioinformatics approach was utilized. The 16S rDNA sequence of BRSSOU1, another strain involved in the study, was analyzed using BLAST (Basic Local Alignment Search Tool) to rapidly identify probable microbial species. While BLAST analysis provided initial insights, it often fell short in conclusively determining closely related genera or taxa due to multiple homologous sequences yielding similar scores. To address this limitation, phylogenetic tree construction and analysis were employed for more precise molecular characterization see Figure 3.1 and Figure 3.2.

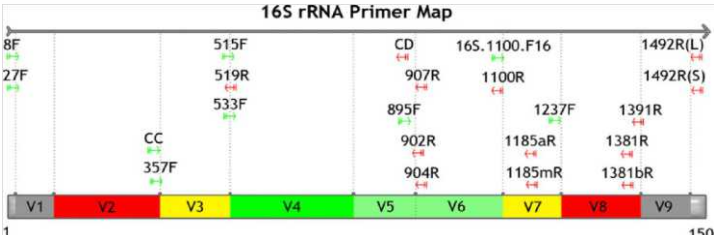


Figure 3-1: Variable regions of 16S rRNA gene in bacteria for phylogenetic-related analysis and species identification [58]

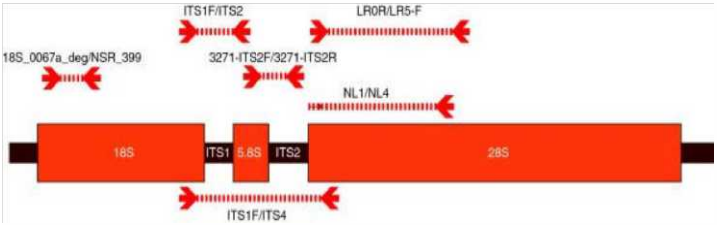


Figure 3-2: Fungal ribosomal gene cluster with ITS primer positions for phylogenetic classification and molecular characterization [12]

This current research underscores the importance of integrating bioinformatics tools with traditional molecular methods to enhance the accuracy of microbial identification and characterization. The findings reveal the significant potential of PNSB strains in wastewater treatment, contributing to sustainable environmental management practices. Further biochemical and biological investigations are recommended to confirm the exact genus and explore additional applications of these strains in improving wastewater treatment processes.

Overall, the integration of traditional culture techniques, molecular methods, and advanced bioinformatics analysis in this study provides a comprehensive approach to identifying and characterizing PNSB strains. This multi-faceted strategy not only enhances our understanding of these microorganisms but also paves the way for developing innovative solutions for wastewater treatment, promoting environmental sustainability. From the BRSSOU1 bacterium sample, DNA was separated. When genomic DNA quality was examined on an agarose gel, it was discovered to remain unaltered. The 16S rRNA gene was amplified using 27F and 1542R primers, and the amplicon length was discovered to be around 1500 bp for every sample (Figure 3-1).

3.1 Photos of Agarose gels

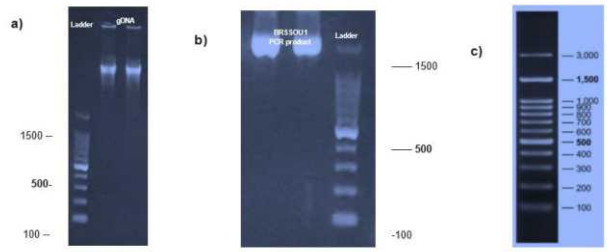


Figure 3-1: Genomic DNA and PCR products in 1% agarose gel: **a**) Lane1: 100bp DNA ladder; lane 2 ,3: genomic DNA; **b**) Lane 1,2: PCR product of ~1500bp BRSSOU1; lane 3: 100 bp DNA ladder **c**) 100bp DNA ladder, electus, Cat No. ELD100.

The sequences of the PCR products were discovered using DNA sequencing, and they are displayed here. The final sequence created by combining forward and reverse primer sequencing is known as the consensus sequence. BLAST result quickly makes us understand the probable microbial species. It is difficult to conclude the closely related genus or taxon based on BLAST results. Many of the times, BLAST result produces several similar/identical scores of homologous sequences. To circumvent the limitation in the BLAST analysis, phylogenetic tree construction and analysis is being used in molecular characterization of microorganisms. Phylogenetic analysis of the top hit sequences of the BLAST result along with query sequence enables to identification the closely related taxonomic group to the query sequence for BRSSOU1 sequence.

In this study, BRSSOU1 16S rDNA sequence is clustered with a specific strain in phylogenetic tree and 99.92% aligned with *Rhodopseudomonas sp.* in NCBI- BLAST result. BRSSOU1 has been identified as *Rhodopseudomonas palustris.* We conclude that BRSSOU1 is *Rhodopseudomonas palustris* strain. Biochemical and other biological investigations are recommended to identify the exact genus.

BRSSOU1 sequence

>BRSSOU1_27F

GGGGTGGGGCGGCCTTAACACANTGCAAGTCGAACGGGCGTAGCAG
TACATCAGTGGCAGACGGGTGAGTAACGCGTGGGA
ACGTACCTTTTGGTTCGGAACAACACAGGGAAACTTGTGCTAATAC
CGGAGAAGCCCTTACGGGGAAAGATTTATCGCCTA
AGGATCGGCCCGCGTCTGATTAGCTTGTTGGTGAGGTAATGGCTCA
CCAAGGCGACAATCATTAGCTGGTCTGAGAGGATG
ATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGG
CAGCAGTGGGGAATATTGGACAATGGGGGCAACCC
TGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAA
GCTCTTTTGTGCGGGAAGATAATGACGGTACCGCA
AGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGA
AGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAA
AGGGTGCGTAGGCGGGTTTCTAAGTCAGAGGTGAAAGCCTGGAGCT
CAACTCCAGAACTGCCTTTGATACTGGAAGTCTTG
AGTTCGGGAGAGGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTC
GTAGATATTCGCAAGAACACCAGTGGCGAAGGCGGC
TCACTGGCCCGATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAATGCCAGCCGTTAGT

>BRSSOU1_1542R

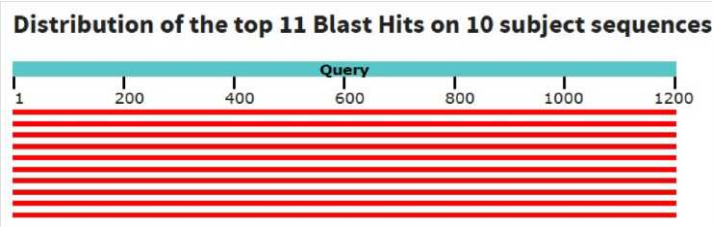
GGGTTTACTCACTAGTGGCGCAGCTAACGCTTTAAGCATTCCGCCTG
GGGAGTACGGTCGCAAGATTAAAACTCATAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAC
GCAACGCGCAGAACCTTACCAGCCCTTGACATGTC
CAGGACCGGTCGCAGAGACTTGACCTTCTCTTCGGAGCCTGGAGCA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCCGTCCTTAGT
TGCTACCATTTAGTTGAGCACTCTAAGGAAACTGC
CGGTGATAACCCCGCAGGAAAGGTGGGGATAACTTCNAGTCCTCAN
GGCCCTTACGGGGCTGGGCTNCACACGTGCNACAA
TGGGGGTTAACAATGGGGAAACCTAAGGGGGCAACCTTTCCAAAA
NTCAAAAAACCCTCCCCATTTTGGGANTGG
GCTGGCGGCAGGCTTAACACATGCAAGTCGAACGGGCGTAGCAATA
CGTCAGTGGCAGACGGGTGAGTAACGCGTGGGAAC
GTACCTTTTGGTTCGGAACAACACAGGGAAACTTGTGCTAATACCG
GATAAGCCCTTACGGGGAAAGATTTATCGCCGAAA
GATCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCA
AGGCGACGATCAGTAGCTGGTCTGAGAGGATGAT
CAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGGGCAACCCTG
ATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGC
TCTTTTGTGCGGGAAGATAATGACGGTACCGCAAG
AATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAG
GGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAG
GGTGCGTAGGCGGGTTTCTAAGTCAGAGGTGAAAGCCTGGAGCTCA
ACTCCAGAACTGCCTTTGATACTGGAAGTCTTGAG
TTCGGGAGAGGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTA
GATATTCGCAAGAACACCAGTGGCGAAGGCGGCTC
ACTGGCCCGATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAATGCCAGCCGTTAGTGGGTTTACTCACTAGTGGCGCAGCTAA
CGCTTTAAGCATTCCGCCTGGGGAGTACGGTCGCA
AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGACGCAACGCGCAGAACCT
TACCAGCCCTTGACATGTCCAGGACCGGTCGCAGAGACGTGACCTT

CTCTTCGGAGCCTGGAGCACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCCCGTCCTTAGTTGCTACCATTTAGTTG
AGCACTCTAAGGAGACTGCCGGTGATAACCCGCGAGGAAGGTGGGG
ATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGG
CTACACACGTGCTACAATGGCGGTGACAATGGGAAGCTAAGGGGCG
ACCCTTCGCAAATCTCAAAAA

3.4 BLAST analysis of BRSSOU1contig Query length: 1201bp

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Rhodopseudomonas palustris strain GJ-22 chromosome, complete genome	Rhodopseudo...	2213	4421	100%	0.0	99.92%	5042906	CP041387.1
Rhodopseudomonas palustris strain MP3 16S ribosomal RNA gene, partial sequence	Rhodopseudo...	2213	2213	100%	0.0	99.92%	1427	MK850206.1
Rhodopseudomonas palustris strain MP4 16S ribosomal RNA gene, partial sequence	Rhodopseudo...	2213	2213	100%	0.0	99.92%	1418	MK788359.1
Rhodopseudomonas palustris strain MP2 16S ribosomal RNA gene, partial sequence	Rhodopseudo...	2213	2213	100%	0.0	99.92%	1418	MK788358.1
Rhodopseudomonas harwoodiae KCTC 15604 gene for 16S ribosomal RNA, partial sequence	Rhodopseudo...	2213	2213	100%	0.0	99.92%	1446	LC221828.1
Rhodopseudomonas sp. JA253 partial 16S rRNA gene, isolate JA253	Rhodopseudo...	2213	2213	100%	0.0	99.92%	1449	FN995101.1
Rhodopseudomonas palustris rrnC operon 16S ribosomal RNA gene, partial sequence	Rhodopseudo...	2213	2213	100%	0.0	99.92%	1481	EU221585.1
Rhodopseudomonas sp. TUT3625 gene for 16S rRNA, partial sequence	Rhodopseudo...	2213	2213	100%	0.0	99.92%	1484	AB250616.1
Rhodopseudomonas palustris strain ATCC 17001 16S ribosomal RNA, partial sequence	Rhodopseudo...	2213	2213	100%	0.0	99.92%	1438	NR_114498.1
Rhodopseudomonas sp. DN62 gene for 16S rRNA, partial sequence	Rhodopseudo...	2211	2211	99%	0.0	99.92%	1380	LC757522.1

Description of the top 10 BLAST result hits



Graphic summary of the top 10 BLAST result hits

Organism	Blast Name	Score	Number of Hits	Description
Rhodopseudomonas	a-proteobacteria		11	
. Rhodopseudomonas palustris	a-proteobacteria	2213	6	Rhodopseudomonas palustris hits
. Rhodopseudomonas harwoodiae	a-proteobacteria	2213	1	Rhodopseudomonas harwoodiae h
. Rhodopseudomonas sp. JA253	a-proteobacteria	2213	1	Rhodopseudomonas sp. JA253 hits
. Rhodopseudomonas palustris DX-1	a-proteobacteria	2213	1	Rhodopseudomonas palustris DX-
. Rhodopseudomonas sp. TUT3625	a-proteobacteria	2213	1	Rhodopseudomonas sp. TUT3625
. Rhodopseudomonas sp.	a-proteobacteria	2211	1	Rhodopseudomonas sp. hits

3.4.1 Taxonomy of the top 10 BLAST result hits

The phylogenetic analysis of the top BLAST hit sequences alongside the query sequence enabled the identification of closely related taxonomic groups. In this study, the BRSSOU1 16S rDNA sequence clustered with a specific strain in the phylogenetic tree and exhibited a 99.92% alignment with *Rhodopseudomonas sp.* in the NCBI-BLAST results. Consequently, BRSSOU1 was identified as *Rhodopseudomonas palustris*.

3.4.2 Phylogenetic tree of BRSSOU1

Figure 3.4.2: Evolutionary analysis by Maximum Likelihood method

Evolutionary analysis by Maximum Likelihood method

The Tamura-Nei model and the Maximum Likelihood approach were used to infer the evolutionary history. [1].

The displayed tree has the highest log probability (-4993.20). By employing the Neighbor-Join and BioNJ algorithms on a matrix of pairwise distances calculated with the Tamura-Nei model, the initial tree or trees for the heuristic search were automatically generated. The topology with the highest log likelihood value was then chosen. There were twelve nucleotide sequences in this investigation. The resulting dataset contained 1201 locations in total. In MEGA11, evolutionary analyses were carried out.The potential microbial species is easily understood thanks to the BLAST result. Based on the BLAST result, it is challenging to determine the closely related genus or taxon. For the most part, the BLAST result yields many homologous sequence scores that are comparable or identical. Phylogenetic tree construction and analysis are being employed in the molecular characterization of microbes to get over the limitation in the BLAST analysis. The closest related taxonomic group to the query sequence can be found by performing a phylogenetic analysis of the top hit sequences from the BLAST result combined with the query sequence.

In this current study, the phylogenetic tree of the BRSSOU1 16S rDNA sequence clusters with a particular strain proves to be excellent, and the NCBI-BLAST result aligns 99.92% of the sequence with *Rhodopseudomonas sp., Rhodopseudomonas palustris* has been identified as the source of BRSSOU1. We deduce that *Rhodopseudomonas* is BRSSOU1. This study explores the identification and characterization of Purple Nonsulfur Bacteria (PNSB) for their potential in wastewater treatment.

Discussion and Conclusion

The BLAST analysis provided rapid identification of probable microbial species but often fell short in conclusively determining closely related genera or taxa due to multiple homologous sequences yielding similar scores. To overcome this limitation, phylogenetic tree construction and analysis were used for more precise molecular characterization. The 16S rDNA sequence of BRSSOU1 clustered with a specific strain in the phylogenetic tree and showed a 99.92% alignment with *Rhodopseudomonas sp.* in the NCBI-BLAST results.

The identified PNSB strains demonstrated significant potential in reducing water pollutants. Specifically, *Rhodopseudomonas palustris* (BRSSOU1) exhibited substantial reductions in chemical oxygen demand (COD), biological oxygen demand (BOD), and total dissolved solids (TDS). These findings highlight the effectiveness of PNSB in wastewater treatment and their role in promoting sustainable environmental management practices. Integrating traditional culture techniques with advanced molecular methods and bioinformatics analysis provides a comprehensive approach to identifying and characterizing PNSB strains. This study not only enhances our understanding of these microorganisms but also paves the way for developing innovative solutions for wastewater treatment, contributing to environmental sustainability. Further biochemical and biological investigations are recommended to confirm the exact genus and explore additional applications of these strains in improving wastewater treatment processes.

Declarations

Conflicting interests: The writers say they have none.

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