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RESEARCH ARTICLE

Isolation and molecular characterization of microbial isolates from Saryu river water

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Abstract

This study was aimed at investigating the molecular characterization of microbial isolates like bacteria and fungi using a culture-dependent approach and analyzing the phylogenetic relationship between the isolates. The identification of bacteria and fungi was confirmed by DNA sequencing and polymerase chain reaction (PCR) amplification using 16S rRNA and internal transcribed spacer (ITS) genes, respectively. The DNA sequences of bacterial and fungal isolates were identified using nucleotide basic alignment search tools (nBLAST) against the data available in GenBank. On the basis of retrieved DNA sequences from the database, phylogenetic trees were constructed, and phylogenetic relationships among the bacterial and fungal isolates were computed.

Keywords: Bacteria, Fungi, Polymerase chain reaction, 16S rRNA, Internal transcribed spacer, Genbank, Phylogenetic tree.

Introduction

It is demonstrated that the natural water ecosystem is a complex system having various phases like suspended particulate matter, sediments, and biofilms (Battin *et al.*, 2016; Boulard *et al.*, 2020). The river ecosystem is characterized by the continuous flow of running water and temporary locations for storing and transporting a broad range of materials. It is explored that the microbial communities existing in solid phases are different due to the complex composition and structure of the substances coexisting in the water micro-environment. Microbial investigations of lotic systems have highlighted their critical involvement in the biogeochemical cycle for complex microbial river communities and generating primary production through photosynthetic activity (Bockelmann *et al.*, 2000; Hou *et al.*, 2017). Furthermore, due to their strong interactions, biofilms, suspended particles, and sediments in water are important for regulating global biogeochemical flux and pollutants (Li *et al.*, 2020; Desiante *et al.*, 2022). Microbes are widespread and massive, and they are also regarded as a vital component in all ecosystems since they perform a variety of ecological activities. The abundance of bacteria varies based on the type of ecosystem and is typically present in millions/mL. It is estimated earlier that there are approximately 3,000 and 11,000 microbial genomes per gram of soil, making them one of the world's most diverse resources of biodiversity (Curtis *et al.* 2002; Schmeisser *et al.* 2007).

High-throughput molecular biology technology has recently advanced, improving microbial community structure and function analysis. Additionally, various researchers encouraged the isolation of microorganisms in water, sediments, and biofilm (Lv *et al.*, 2017). It is demonstrated that culturing microbes is a challenging task that is critical for tapping the biotechnological potential of microbial life (Oberhardt *et al.*, 2015). Best practices for culturing new organisms have been investigated for a long time. However, despite these best practices, the typical procedure for culturing new microbe still warranted a great deal of experience for trial and error. It is depicted that reproducibility of results, control of external variables, and laboratory experimental design are advantageous properties that are greatly facilitated in pure culture studies (Garzah and Dutilh, 2015). In various habitats

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such as water, soil, or marine sediments, only about 0.01 to 1% of the microbes are yet cultivable on artificial media. These discrepancies are being named "great plate count anomaly" (Staley and Konopka, 1985).

Hence, it is explicit that more concerted efforts are still required to fully understand the microbial biodiversity in an ecosystem. The attempts made by researchers in the past decades to bring the uncultivated microbial "dark matter" into cultivation in the post-omics era are increasingly recognized. Conventional untargeted techniques and high-throughput methods of cultivation, like culturomics platforms, provided a powerful tool for culturing several new and uncultured lineages being brought into culture through the use of ranges of culture media and high-throughput screening approaches (Bilen *et al*., 2018; Zhang *et al*., 2021). It is essential to cultivate microbes because the stable pure cultures of microbes are valuable resources that can be used for the investigation of microbial traits, novel genes, and improvement in gene annotation (Liu *et al*., 2022).

Further, the cultures are also important to generate reference genomic data for the interpretation of functionality of microbes through metagenomics and meta-transcriptomics analyses (Zhang *et al*., 2020; Stewart *et al*., 2019). Moreover, the availability of cultures enables new possibilities for applications for the biological interpretation of microbial function in ecosystems, including health and industrial sectors. It is established that 16S rRNA is a housekeeping marker gene present in bacteria due to its presence in almost all bacteria and exists as a multigene family (Janda and Abbott, 2007). The presence of multiple conserved/hypervariable regions allows the design of a polymerase chain reaction (PCR) primer for amplification of genes. Further, in molecular characterization of fungi, it is reported that the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi (Schoch *et al*., 2012). The physicochemical and microbial characterization of Saryu river water is reported elsewhere (Maurya *et al*., 2023). In the present study, an attempt has been made to enrich and isolate the microbes from Saryu river water along with molecular tools and techniques used to detect and identify the bacteria and fungi through 16S rRNA and ITS genes, respectively, for sequencing and sequence analyses.

Materials and Methods

Sampling of River Water

Water sample was taken in a sterilized plastic bottle with proper labeling from the Saryu river (lotic water body). A total of 1-liter samples were taken from water of the Saryu river in the Ayodhya district ((27^{0}20'59.99" N 81^{0}22'59.99" E) of Uttar Pradesh, India, in order to enrich the microbial isolates (bacteria and fungi). Sampling details are described elsewhere (Maurya *et al*., 2023).

Enrichment and Isolation of Microbial (bacteria and fungi) Community

Enrichment of bacteria from the river water was done using the nutrient broth medium, prepared by dissolving 5.0 g peptone, beef extract 3.0 g, NaCl 5.0 g in distilled water of 1L with a pH 7.0 (Garcha *et al*., 2016). The medium was sterilized at 121˚C for 20 minutes. Further, the growth of bacteria was observed after incubation at 37°C for 12 hours.

The recipe for making potato dextrose broth (PDB) from fresh potatoes was performed as described by Griffith *et al*. (2007) in which 100 g peeled and sliced potatoes are homogenized with 300 mL distilled water using blender and allowed to steep overnight at 4˚C. Further, crude solution was filtered through muslin cloths to get extract. The whole solution preparations were sterilized by autoclaving at 121˚C for 20 minutes before dispensing into petri dishes. Repeated subculturing was made for the isolation of bacterial and fungal isolates.

DNA Extraction and Quantification

Genomic DNA was isolated from the bacterial and fungal isolates was done as per the protocol described by Shukla *et al.* (2009) and Ezeonuegbu *et al.* (2022). A single colony was inoculated in nutrient broth and grown at 37°C for 12 hours. The cells were harvested from 10 mL of culture and 1-mL of extraction buffer added and transferred to a 2 mL-microfuge tube. After the extraction equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tubes and mixed well by gently shaking the tubes. The tubes were centrifuged at room temperature for 15 minutes at 14,000 rpm. The upper aqueous phase obtained after centrifugation at room temperature for 10 minutes at 14,000 rpm was transferred to a new tube. The DNA was extracted from the aqueous layer by adding isopropanol. The dried DNA pellet was dissolved in 100 µL of 1X TE buffer. To remove RNA 5 µL of DNAse free RNAse A (10 mg/mL) was added to the DNA. DNA was quantified using Nanodrop spectrophotometer.

PCR Amplification

PCR amplification of 16S rRNA gene for bacterial isolates (SN1 and SN2) and ITS gene for fungal isolate was used for amplification along with 10 pm of each primer. The universal primer pairs for bacteria 16s forward GGATGAGCCCGCGGCCTA and 16S reverse CGGTGTGTACAAGGCCCGG were used, while for fungi ITS 4 forward GGAAGTAAAAGTCGTAACAAGG and ITS 5 reverse TCCTCCGCTTATTGATATGC (White *et al*., 1990). PCR mixture was subjected to initial denaturation at 94°C for 3 minutes, followed by denaturation for 1-minute at 94°C, annealing of 1-minute at 50°C, extension for 2 minutes at 72°C for 30 cycles and final extension of 7 minutes at 72°C.

PCR amplification for ITS gene was achieved in a vial containing 10x buffer, $MgCl_2$, dNTP 0.2 mmol, forward and reverse primer of 10 pm each, Taq DNA polymerase 2 µL,

DNA template of 1-µL and Milli Q water. The PCR reaction was carried out using a thermal cycler with conditions of initiation denaturation for 10 minutes at 94°C, denaturation for one minute at 94°C, annealing for 30 seconds at 55°C, and extension for 1-minute at 72°C. The thermal cycler was run for 35 cycles, then a final extension cycle was run at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis on 1.2% agarose.

Sequencing and Phylogenetic Analysis

DNA sequencing of PCR amplified sequences was performed on both strands in an ABI PRISM® 3100 Genetic Analyzer (ABI, USA) using BigDye Terminator Kit (Version 3.1). Details are described elsewhere (Shukla *et al.*, 2009). The 16s rRNA and ITS genes sequences were obtained and then aligned against known sequences present in the GenBank database of nucleotide Basic Alignment Search Tools (nBLAST) algorithms at National Centre for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/ search.

The sequences were initially aligned using Clustal W (Thompson *et al.*, 1994) available in MEGA 11 (Tamura *et al.*, 2021). The phylogenetic relationships were estimated using neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the sequences analyzed (Felsenstein, 1985).

Nucleotide Sequence Accession Numbers

The GenBank accession numbers for the 16S rRNA and ITS genes were described in this study are OR533825–OR533826 and OR575176, respectively.

Results and Discussion

PCR Amplification

PCR amplification of 16S rRNA gene for bacterial isolates SN1 and SN2 were found to be positive and an expected size of 1.5 Kb was observed. The intense single bands were observed on 1.2% agarose gel stained with ethidium bromide, as shown in Figure 1a. It is reported that 16S rRNA gene sequence analysis can discriminate among various bacterial strains compared to phenotypic methods (Clarridge, 2004). It can also help to allow a more precise identification of rarely or poorly described bacterial strains. In the last two decades, it is reported that in GenBank nucleotide databank has over 20 million deposited sequences, out of them, approximately over 90,000 are of 16S rRNA gene (Clarridge, 2004). Recently the database contains above 6.25 trillion base pairs from over 1.6 billion nucleotide sequences for 450 000 formally described species (Sayers *et al.*, 2020).

The PCR amplification of ITS region of the fungal isolate SN3 yielded the PCR amplicons of 600 bp (Figure 2a). It confirms that water sample is contaminated with fungi. A study reveals that only a fraction of the fungi inhabiting in any environment are metabolically active. They determined the soil-related study and suggested that relic DNA may account for approximately 40% of the recovered ITS sequences (Carini *et al.*, 2016). Further, it is suggested that the ITS is one of the most commonly used DNA markers in plant phylogenetic and DNA barcoding analyses (Cheng *et al.*, 2016).

DNA Sequencing

The 16S rRNA sequences of bacterial isolates SN1 and SN2 were performed using BLAST for species identification. The BLAST search showed that the sequence data of the isolates SN1 shared 100% similarity with *Bacillus subtilis* (OR533825)

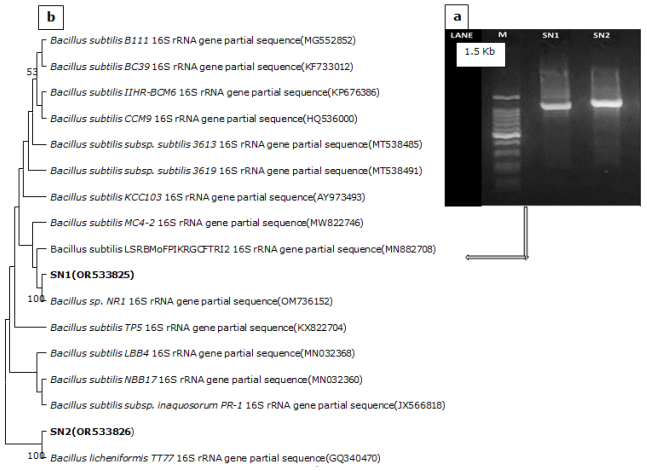

Figure 1: a. PCR amplification of 16S rRNA gene for bacterial isolates (SN1 and SN2); M: Markers b. Phylogenetic tree

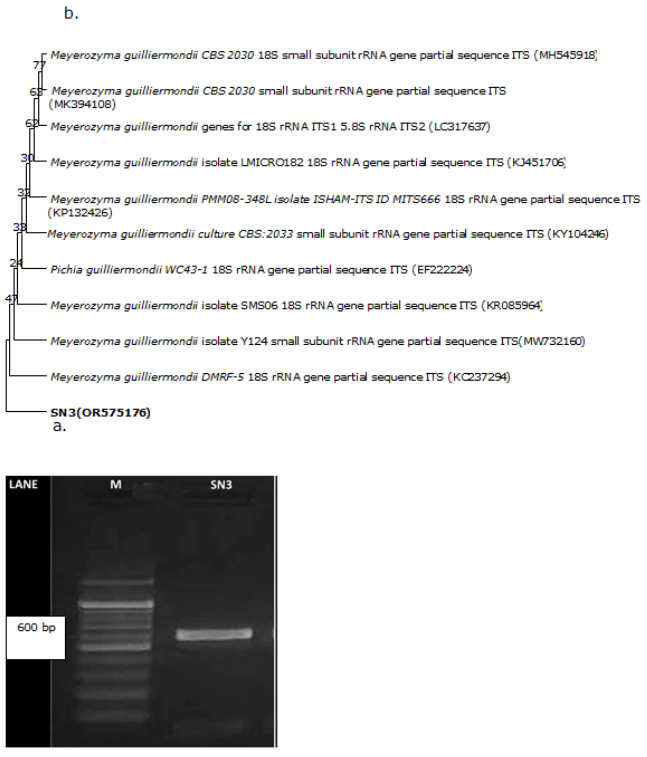

Figure 2: a. PCR amplification of ITS gene for fungal isolate (SN3); M:Markers b. Phylogenetic tree

Table 1: BLAST results for 16S rRNA gene sequenced bacterial isolate (SN1)

S. No.	*Bacterial strain/isolates*	*Percent identity*	*Genbank accession number*
1.	*B. subtilis* isolate SN1	100	OR533825
2.	*B. sp.* (in: Bacteria) strain NR1	98.48	OM736152
3.	*B. subtilis* strain LSRBMoFPIKRGCFTRI2	98.12	MN882708
4.	*B. subtilis* strain LBB4	98.12	MN032368
5.	*B. subtilis* strain NBB17	98.12	MN032360
6.	*B. subtilis* strain B111	97.40	MG552852
7.	*B. subtilis* strain IIHR-BCM6	97.83	KP676386
8.	*B. subtilis* strain MC4-2	98.12	MW822746
9.	*B. subtilis* strain TP5	98.04	KX822704
10.	*B. subtilis* strain M408f10-3MF9	98.04	OR574169

Table 2: BLAST results for 16S rRNA gene sequenced bacterial isolates (SN2)

S.No.	*Bacterial strain/isolates*	*Percent identity*	*Genbank accession number*
1.	*B. licheniformis* isolate SN 2	100	OR533826
2.	*B. licheniformis* strain TT77	96.72	GQ340470
3.	*B. licheniformis* strain SCC112027	96.72	JN998724
4.	*B. licheniformis* strain KD-1	96.72	MT032348
5.	*B. licheniformis* strain P8	96.72	CP045814
6.	*B. licheniformis* strain Ali5	96.72	MN629226
7.	*B. licheniformis* strain ATCC 14580	96.72	CP034569
8.	*B. licheniformis* strain FJAT-46196	96.72	MK859953
9.	*B. licheniformis* strain CC91	96.72	MK618601
10.	*B. licheniformis* strain HTI101-3	96.72	MG835970

Table 3: BLAST results for ITS gene sequenced fungal isolates (SN3)

S.No.	*Fungal strain/isolates*	*Percent identity*	*Genbank accession number*
1.	*Meyerozyma guilliermondii* SN3	100	OR575176
2.	*M. guilliermondii* strain CBS 2030	97.73	MH545918
3.	*M. guilliermondii* strain CBS 2030	97.73	MK394108
4.	*M. guilliermondii* strain: IFM 63277	97.73	LC317637
5.	*M. guilliermondii* culture CBS:2033	97.73	KY104246
6.	*M. guilliermondii* isolate SMS06	97.73	KR085964
7.	*M. guilliermondii* isolate LMICRO182	97.73	KJ451706
8.	*M. guilliermondii* strain PMM08-348L	97.73	KP132426
9.	*M. guilliermondii* isolate Y124	97.73	MW732160
10.	*M. guilliermondii* strain DMRF-5	97.73	KC237294

and SN2 shared 100% similarity with *Bacillus licheniformis* (OR533826) as described in Tables 1 and 2. The 16S rRNA sequences of the *B. licheniformis* and *B. subtilis* isolates were aligned, and nine representatives were selected for the analyses of genomic diversity. On the basis of sequence alignment, a phylogenetic tree was constructed as shown in Figure 1b. It is discussed earlier that sequencing of 16S rRNA gene is generally used as an important identification tool for bacterial classification (Clerck *et al.*, 2004). The plausible reason could be due to the presence in almost all bacteria and its functionality has changed over time (Janda and Abbott, 2007). It is reported earlier that *B. licheniformis* and *B. subtilis* regulate the immune response in the form of probiotics when added in water and show enhanced resistance against fungal infection (Monier *et al.*, 2023). Further, Korenblum *et al.* (2005) reported that bacterial strains like *Bacillus subtilis*

LFE-1, *B. firmus* H2O-1 and *B. licheniformis* T6-5 produces antimicrobial substances isolated from an oil reservoir in Brazil. In present study the availability of these bacterial isolates could be useful in Saryu river water.

The ITS gene sequences of fungal isolate SN3 were analysed using BLAST for species identification. The BLAST search showed that the sequence data of the isolate SN3 showed 100% similarity with *Meyerozyma guilliermondii* SN3 (OR575176) as described in Table 3. The fungal isolate *M. guilliermondii* SN3 sequences were aligned with nine representatives obtained from BLAST search and the phylogenetic tree was constructed (Figure 2b). It is investigated that the ITS genes were used for a more refined differentiation in fungi due to highly variable and recommended to be adequate for analyses with high phylogenetic resolution (Weber *et al.*, 2009). *M. guilliermondii* is well known for the production of flavoured compounds in fermented food products (Wah *et al.*, 2013).

Conclusion

The present study reveals the isolation and identification of bacterial isolates, namely *B. subtilis* isolate SN1, *B. licheniformis* isolate SN2, and fungal isolate, *M. guilliermondii* SN3, from Saryu river water. This study suggests that river water is a large reservoir of microbial communities, and therefore, it is imperative to understand the actual diversity of uncultured microbiota through high-throughput molecular techniques-based metagenomics approaches.

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