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Isolation and Identification of Nitrogen-fixing Bacteria from the Banks of Meenachil River, Kottayam (District), Kerala

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Abstract

In plants, nitrogen is a critical limiting factor required for the proper maintenance of growth and production. Nitrogen forms about 78% of the total atmospheric gases, and plants are capable of utilising its reduced forms only. Certain microorganisms in the soil have the potential to build up atmospheric nitrogen into its reduced and plant-absorbable form, a process called nitrogen fixation. In conventional agricultural practices, farmers rely largely upon chemical fertilisers in nitrogen-deficient soils to counter the deficiency and increase the crop yield. Besides the benefit, more often, these chemicals are expensive and associated with several ecological problems. On the contrary, nitrogen-fixing bacteria (NFB) can effectively substitute chemical fertilisers and are eco-friendly too. But, research in the development of NFB into biofertilizers is in its infancy, and many areas in India are not properly studied for

the presence of novel NFB strains. In this context, an attempt has been made to isolate and identify nitrogen-fixing bacteria from the banks of the Meenachil River, Kottayam. Two of the isolates from the Meenachil River exhibited significant nitrogen-fixing efficiency in biochemical analysis. These isolates were identified as *Agrobacterium* strains by 16S rRNA sequence analysis and colony morphological study. Further studies are required for species-level identification of the two isolates.

Keywords: Nitrogen, Meenachil, 16S rRNA, *Agrobacterium,* PCR, Agriculture, Microbial culture.

1. Introduction

Nitrogen (N) is a crucial and critical factor for plant growth and production. Also, it is a major component of amino acids, proteins and enzymes. The overall development of a plant includes an increase in height, root collar diameter, chlorophyll content, physiological and biochemical metabolism etc., which depends on the proper availability of nitrogen [Shah et al., 2016; Razaq *et al*., 2017; Gojon. 2017]. The influence of nitrogen in the synthesis of chlorophyll content drives proper photosynthetic functional activity in leaves; it also plays a major role in biotic and abiotic stress management and maintains the balance in metabolic activities of plants [Zhang *et al*., 2015; Denis *et al*., 2018]. The deficiency of nitrogen in plants results in stunted growth, inhibition in leaf and shoot growth, lack of chlorophyll synthesis and yellowing of leaves [Manjula. [2016; Tang *et al*., 2019].

Nitrogen is one of the most abundant elements in the atmosphere and constitutes about 78%. In the atmosphere, nitrogen is present in the form of inert gases [Agatha, 2017]. Plants are capable of utilising only the reduced form of nitrogen through a process called biological nitrogen fixation [Monica 2018; Kishan *et al*., 2020]. Biological nitrogen fixation (BNF) is executed by some microorganisms present in the soil, like *Rhizobium*, *Azotobacter*, *Glucanobacter,* etc., and they take a vital role in the nitrogen uptake in plants [Florence *et al*., 2016]. The soil microbes can reduce atmospheric nitrogen into plant-absorbable form through the nitrogen cycle. They possess an enzyme complex called nitrogenase, which catalyses the conversion of gaseous nitrogen into its reduced and useful form called ammonia. Nitrogenase is the only family of enzymes which can accomplish this process [Dighe, 2010]. Population explosion and dwindling of agricultural lands have forced us to produce more crops from the available land. The application of NFB as a biofertilizer will be an effective agronomic management strategy to enhance crop performance [Zhao *et al*., 2018].

In conventional agricultural sectors, farmers largely rely on artificial and chemical fertilisers for the supply of soluble nitrogen to plants, which are highly costlier, and the use of these chemicals is associated with negative impacts on the environment, such as pollution, and adversely affects the overall quality of soil as well as the development of the plant [Randeep *et*

al., 2019]. Thus, the high cost of chemical fertilisers and associated pollution has warranted the use of eco-friendly microbes, which are capable of fixing atmospheric nitrogen in the soil as an alternative source to nullify these problems, and they can be released as bio-fertilisers. But, research in the development of NFB into biofertilisers is in its infancy, and many areas in India are not properly studied for the presence of novel NFB strains. In this context, as a preliminary study, this work has attempted to isolate and identify nitrogen-fixing bacteria from the banks of the Meenachil River, Kottayam.

2. Materials and Methods

2.1 Specimen collection: Soil samples were collected randomly from six different sites on the banks of the Meenachil River. Samples from each site were pooled together and labelled accordingly for further studies.

2.2 Culture medium preparation and primary culture: Serial dilution of each sample was carried out using sterilised water up to a dilution of 10^{-2}. Using 10^{-2} solution, a primary culture of bacteria was developed by pour plate method in Jensen's media contains sucrose -7g (350 ml), dipotassium phosphate -0.35g (350 ml), magnesium sulphate-0.175 g (350 ml), ferrous sulphate -0.035 g (350ml), calcium carbonate -0.7g (350ml), sodium chloride- 0.175g(350ml), sodium molybdate-0.00175 g(350ml) and agar-8.4 g (350ml) (Jensen, 1965).

2.3 Subculture: Morphologically distinctive colonies were identified and subjected to subculture in freshly prepared Jensen's medium. The subculture was done by the quadrant streaking method in an aseptic condition and kept for incubation.

2.4 Biochemical screening to determine nitrogen-fixing potential: Bacterial samples from the subculture were screened to determine the nitrogen-fixing potential by biochemical analysis, which includes ammonia and nitrate reduction tests (Blazevic *et al*., 1975). Separate medium for each test were prepared in a sterilized test tubes that include peptone water 0.6 g (100ml), NaCl -0.3 g (100ml), distilled water -0.6 g (100ml) that were used for preparing ammonia broth for the ammonia test and sodium nitrate-0.1 g(100ml), magnesium sulphate-0.05 g(100ml), ferrous sulphate-0.003 g(100ml), sodium chloride -0.03 g(100ml), sodium carbonate-0.1 g(100ml), dipotassium phosphate-0.1 g(100 ml) and distilled water-100 ml was used respectively for preparing nitrate broth for nitrate test and results were confirmed through counter staining method using Nessler's reagent and diphenylamine, to observe the colour change in the sample.

2.5 Establishment of pure culture: Samples which showed maximum nitrogen-fixing potential were subjected to pure culture in Jensen's medium (Jensen, 1965) by the quadrant streaking method.

2.6 Morphological study of the bacteria and colony: Gram staining method (Colco, 2005) and microscopic analysis (Mishra, 1996) of the bacterial colony were used for the morphological study.

2.7 DNA Barcoding using universal primers of 16srRNA

2.7.1 Genomic DNA isolation from Bacteria: The pure culture of bacteria was then subjected to 16s rRNA sequencing for identification. Isolation of genomic DNA was carried out using NucleoSpin® Tissue Kit (Macherey-Nagel) without modification of the manufacturer's instructions.

2.7.2 Agarose Gel Electrophoresis for DNA quality check: DNA quality check was carried out using 0.8% agarose gel electrophoresis (30% sucrose in TE buffer, pH 8.0). Gels were observed under UV light using a UV transilluminator (Genei), and the image was captured using a Gel documentation system (Bio-Rad).

2.7.3 PCR Analysis: A PCR thermal cycler (Gene Amp PCR system 9700 Applied Biosystems) was used for the process of amplification. Table 1 shows various components and their concentration used in amplification, whereas Table 2 shows the forward and reverse primer sequences used for targeting 16S rRNA.

Table 1. Concentration of each item:

2 X Phire Master Mix	5 µL
D/W	4 µL
Forward Primer	0.25 µL
Reverse Primer	0.25 µL
DNA	1 µL

Table 2. The sequence of the forward and reverse primers used for targeting 16S rRNA

Target	Primer name	Direction	Sequence
16S rRNA	16S rRNA-F	Forward	CAGGCCTAACACATGCAAGTC
	16S rRNA-R	Reverse	GGGCGGWGTGTACAAGGC

PCR amplification profile is shown below:

95°c - 5.00 min
95°c - 30 sec
60 °c - 40 sec 35 cycles

72 °c - 60 sec
72 °c - 7.00 min
4 °c - ∞

2.7.4 Agarose Gel Electrophoresis of PCR products:
4 µl of PCR product mixed with 1 µl of 6X loading dye were electrophoresed in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. Bands were compared with the molecular standard 2-log DNA ladder (NEB).

2.7.5 EXO SAP- IT treatment: Exo-SAP IT treatment was performed to remove unwanted primers from the PCR products. 5 µl of PCR products along with 0.5µl of ExoSAP-IT were incubated at 37°C for 15 minutes, followed by enzyme inactivation at 85 °C for 5 minutes.

2.7.6 Sequencing using Big Dye terminator v 3.1:
Exosap-treated PCR product was sequenced in a PCR thermal Cycler (Gene Amp PCR System 9700, Applied Biosystems), using Big Dye terminator V 3.1 cycle sequencing kit (Applied Biosystems, USA), following the manufacturer's protocol. The components of the sequence PCR mix are depicted in Table 3.

Table 3: Components in sequencing PCR mix and their concentration:

D/W	6.6 µL
5X Sequencing buffer	1.9 µL
Forward primer	0.3 µL
Reverse primer	0.3 µL
Sequencing Mix	0.2 µL
Exosap treated the PCR product	0.2 µL

Sequencing PCR amplification profile

96ºC - 2min
96ºC - 30sec
50ºC - 40sec 30 cycles
60 ºC - 4min
4 ºC - ∞

2.7.7 Post sequence PCR cleanup: Performed with a mix of D/W, 125mM EDTA, 3M sodium acetate, pH 4.6 and ethanol using standard protocol. The cleaned, air-dried PCR product was then sequenced using an ABI 3500 DNA Analyser (Applied Biosystems). Components used in post-sequencing PCR clean-up are shown in Table 4.

Table 4: Components used in post-sequencing PCR cleanup:

D/W	5 µL
3 M Sodium Acetate	1 µL
EDTA	0.1 µL
100% Ethanol	44 µL

2.7.8 Sequence Analysis: Sequence scanner software V1 (Applied Biosystems) was used for sequence quality check, while Geneious Pro V 5.16 software was used for sequence alignment and editing.

2.7.9 BLAST and phylogenetic tree construction: The quality of forward and reverse sequence was visually validated by analysing the Q value for each nucleotide base using Finch TV (https://finchtv.software.informer.com/1.4/). Sequences were edited and combined to produce a single continuous sequence using the software Bioedit Version 7.2 (https://bioedit.software. informer.com/download/). The sequence was then submitted to NCBI under the accession numbers MW 386626 and MZ 572228, respectively. Blastn was used for query sequence comparison with the known 16S rRNA gene sequences in the NCBI database. Programs such as CLUSTAL W for sequence alignment and MEGA X (https://www.megasoftware.net/) for phylogenetic tree construction were used. For the phylogenetic tree construction, the maximum likelihood method was followed with a minimum bootstrap value of 1000 and opting HKY+7 as the substitution model based on the lowest BIC value (Abhirami and Jayakumar, 2021).

3. Results

Soil samples from the banks of the Meenachil River produced a varying number of bacterial colonies in each agar plate containing 3.9% Jensen's medium (Table 5)

Table 5: Number of colonies counted on each plate obtained from soil samples collected from six different sites:

SL.NO	No. of Colonies		
	Plate 1	**Plate 2**	**Plate 3**
1	22	15	22
2	15	25	28
3	23	21	13
4	20	24	30
5	5	16	9
6	17	12	26

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Vol. 7 & Issue 3 (July 2025)

Morphological analysis of the bacteria subcultured after the primary culture in the pour plate method in Jensen's medium led to the identification of five distinct bacterial colonies, as shown in Figure 1.

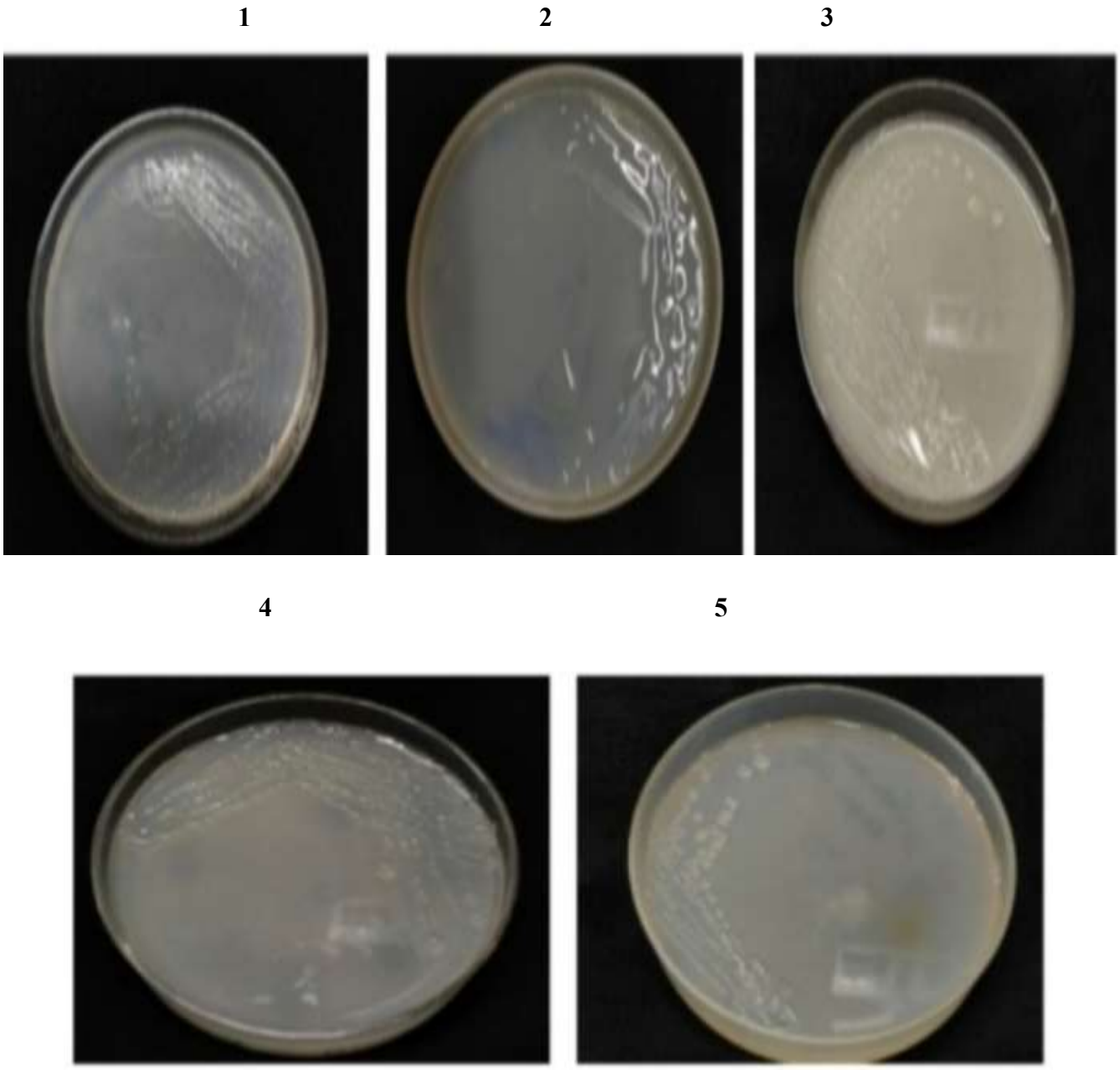

Fig 1: Subculture of five morphologically distinct bacterial colonies.

Further, these five distinct bacterial strains were evaluated for their nitrogen-fixing potential biochemically by the ammonium and nitrate reduction test. Among the five samples tested, sample 3 and 4 exhibited high potential in fixing nitrogen (fig.3and 4) and they were subjected

to pure culture. The results were confirmed by identifying the differences in colour gradient in both tests. In the ammonia test, the sample showing higher nitrogen fixing potential (S4) indicates intense brown colour in ammonia broth, and those with pale brown colour show less efficiency in nitrogen fixation (fig.2) Similarly in nitrate test, sample showing intense-blue colour (S3) indicates the maximum efficiency in nitrogen fixation and those showing pale-blue colour indicates lesser efficiency (fig.3)

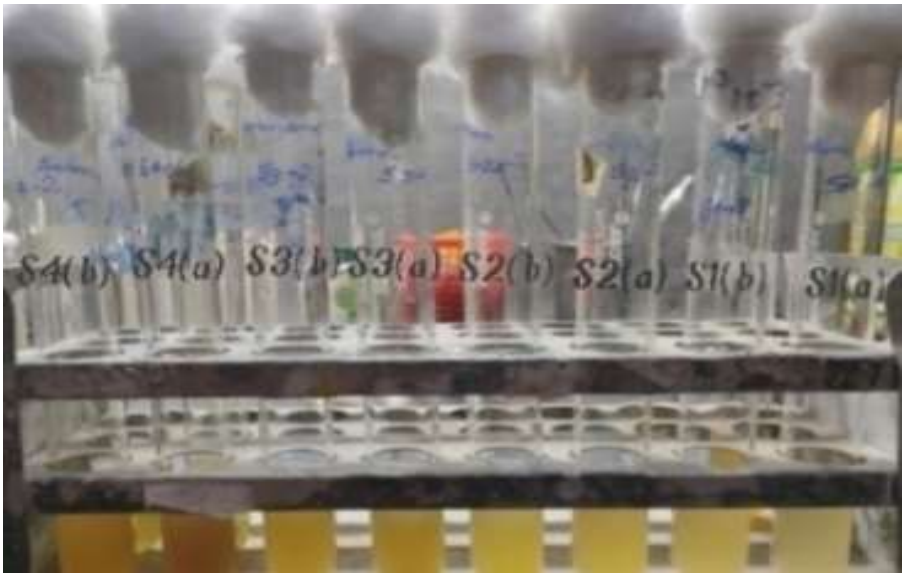

Fig 2: Nitrogen fixing potential exhibited by various bacterial strains in terms of variation in colour intensity produced during the ammonia test.

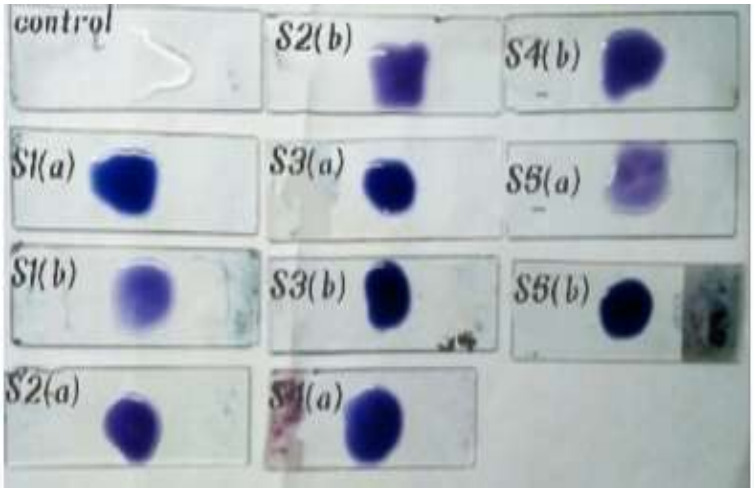

Fig 3: Colour gradient produced by different bacterial strains during nitrogen fixing potential analysis in the nitrate test

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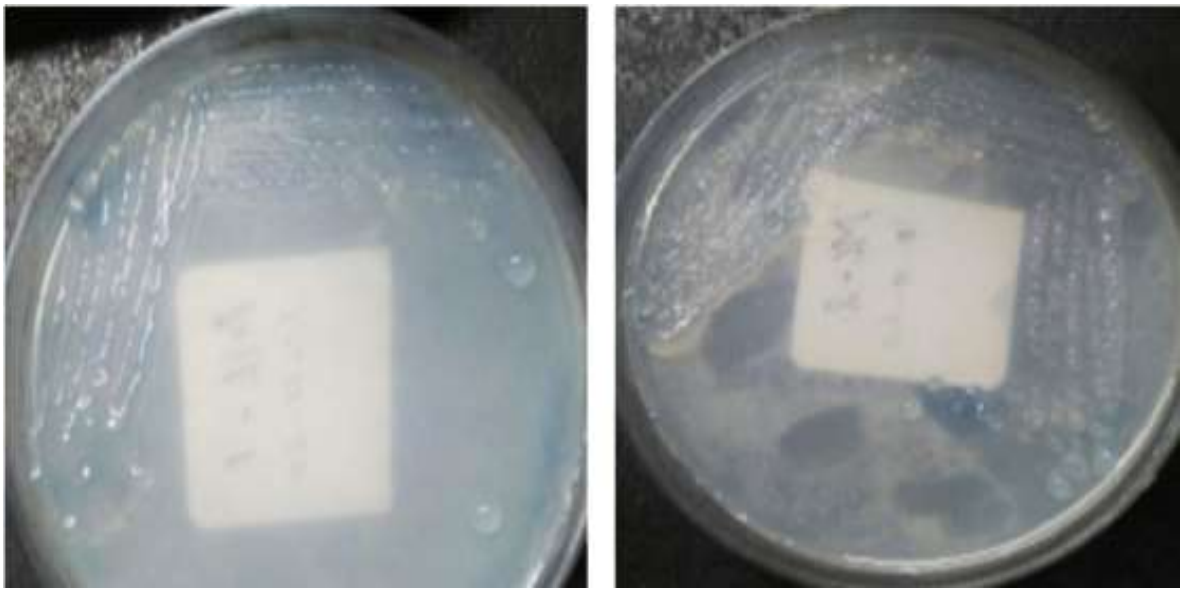
The Voice of Creative Research
Vol. 7 & Issue 3 (July 2025)

Morphology-based studies on colony characters of both the pure cultured samples revealed that sample 3(b) as transparent, convex, circular, smooth, non-pigmented and slimy and 4(a) as transparent, oval, convex, slimy and raised colonies (fig.4) and they were observed as rod-shaped structure under microscope and identified as gram-negative on gram staining (fig.5). Gram staining technique is widely used to categorise bacteria into gram positive and gram negative (Colco, 2005).



Sample 3(b)Sample 4(a)

Fig 4: Colony morphology of both samples

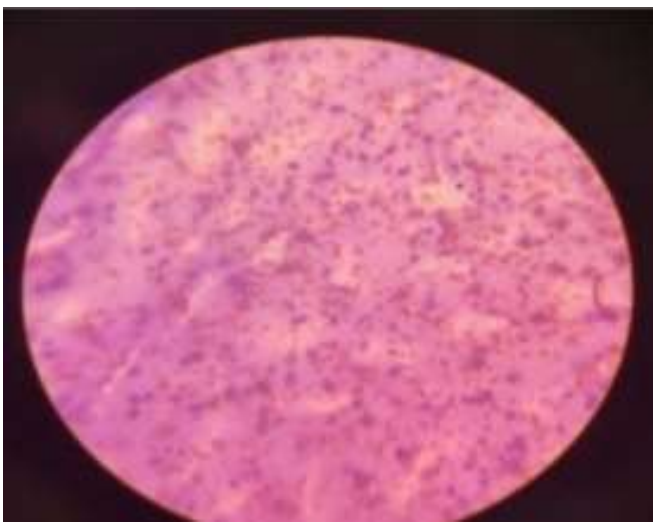


Fig 5: Rod-shaped bacterial strains exhibiting pink colour (Gram staining, microscope view).

Primary findings lead to the assumption of the identity of bacterial strains 3(b) and 4(a) as *Agrobacterium*. To confirm the findings, 16S rRNA sequence analysis of samples 3(b) and 4(a) was carried out using primer set 16S-RS-F and 16S-RS-R as forward and reverse primers, respectively.

The quality evaluation of the isolated DNA samples using agarose gel electrophoresis provided a single intact band which represents high quality and no contamination of the sample (Fig.6) Agarose gel electrophoresis is the technology to separate DNA samples based on their mass, charge, size, and movement of molecules depends on the potential differences in power supply through the channel in which it migrates (Yiran, 2020). The image of the DNA sample obtained from the UV trans illuminator indicates a DNA sample with a tight band, which represents the purity of the sample. If any sample is stuck in the well during electrophoresis, it is an indication of the presence of high molecular weight impurities in the sample (Abdel and Osman 2017)

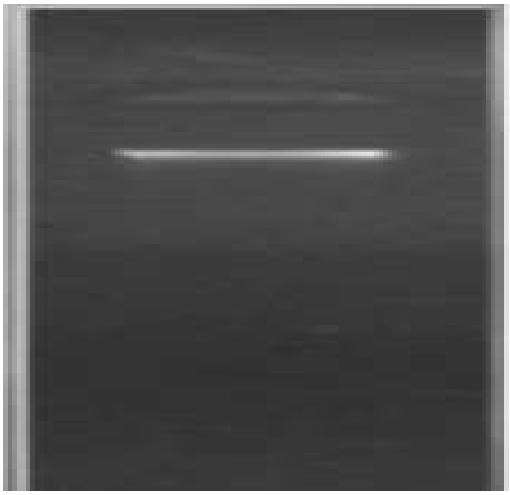
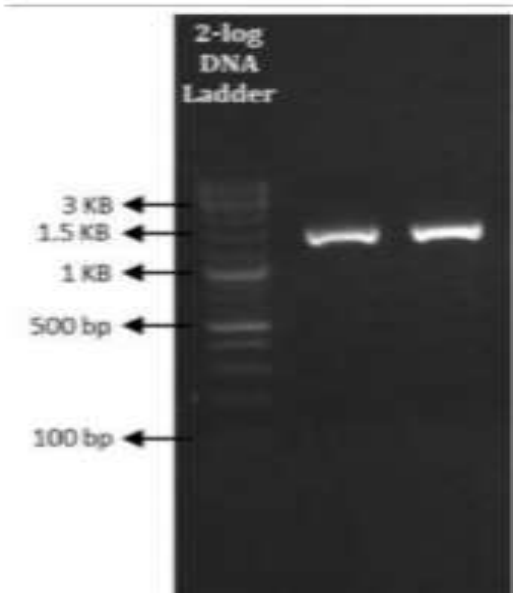


Fig 6: Agarose gel electrophoresis revealing a single intact band during quality check

35 cycles of PCR amplification using forward and reverse primers (16S-RS-F and 16S-RS-R) led to the effective amplification of the DNA sample. The agarose gel electrophoresis of this amplified sequence exhibited a bandwidth of 1.5 KB in the 2-log DNA ladder (Fig. 7), and the digested DNA included bands ranging from 100 bp to 3 kb. This DNA ladder is a molecular weight-size marker which is used as a standard to identify the approximate size of a molecule run by gel electrophoresis, which is based on the principle that the molecular weight

of the molecules is inversely proportional to their migration rate. Therefore, after gel



electrophoresis, markers will provide a logarithmic scale(Carlson *et al*., 2013).

Fig 7: PCR DNA against 2-log DNA ladder.

Figures 8 and 9 show the sequence chromatogram of *Agrobacterium* strains, and the type and quality of each sequence are represented by the features of individual peaks. Strong and uniform peaks indicate the high quality of the sequence obtained after PCR sequence amplification. Low-quality sequences, which are more common towards the ends of the sequence, were identified and discarded in FinchTV. The final sequence for NCBI submission resulted after combining the forward sequence and the reverse complement of the reverse sequence. *Agrobacterium sp*. Strain SRSJ3 and *Agrobacterium sp*. Strain SRSJ6 consisted of 1230 bases and 1229 bases and were submitted under the accession numbers MW386626 and MZ 572228, respectively.

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The Voice of Creative Research
Vol. 7 & Issue 3 (July 2025)

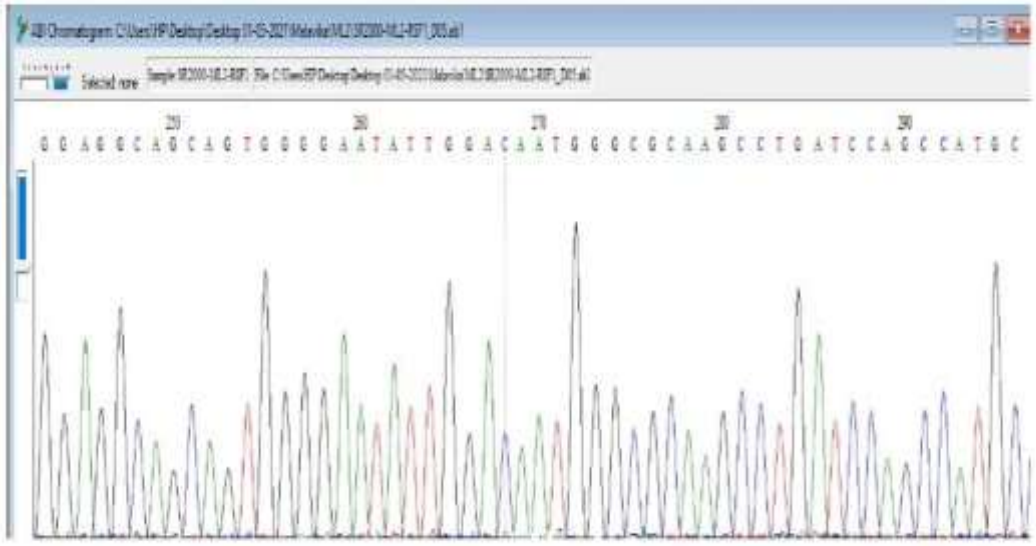

Fig 8: Chromatogram of *Agrobacterium* sp. Strain SRSJ3 shows the quality of each peak towards the respective sequences.

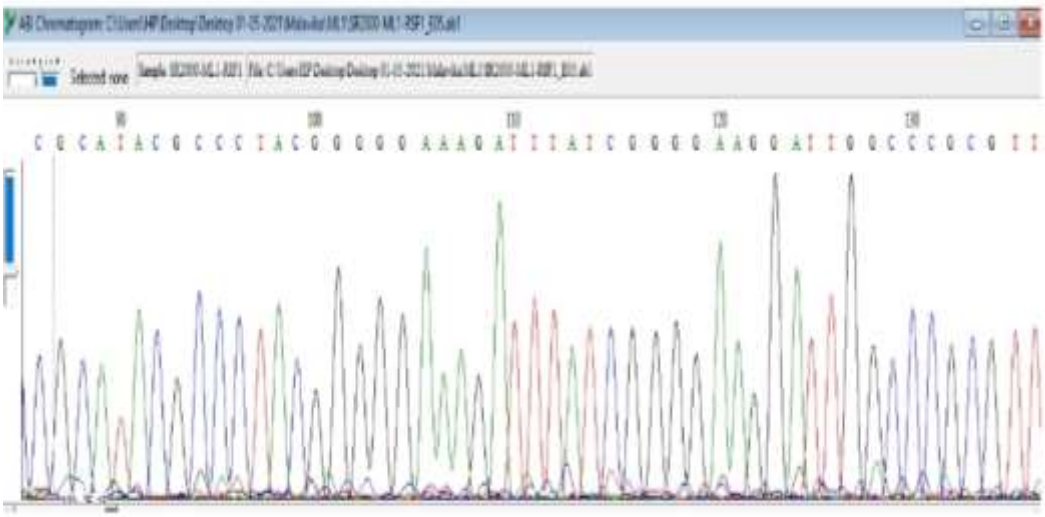

Fig 9: Sequence chromatogram of Strain SRSJ6.

A phylogenetic tree was constructed to infer the evolutionary relationship of the SRSJ3 and SRSJ6 sequences with that of other bacterial strains, as shown in fig. 10. *Rhizobium* strains were used as outgroups. Thus, BLAST results, when combined with morphological characteristics, validated the identity of the isolates SRSJ3 and SRSJ6 as *Agrobacterium* strains. The phylogenetic tree exhibited several clades and subclades. SRSJ3 and SRSJ6 formed a clad with *Agrobacterium salinitolerans* strain YIC 5082 with a node value of 100.

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The Voice of Creative Research
Vol. 7 & Issue 3 (July 2025)

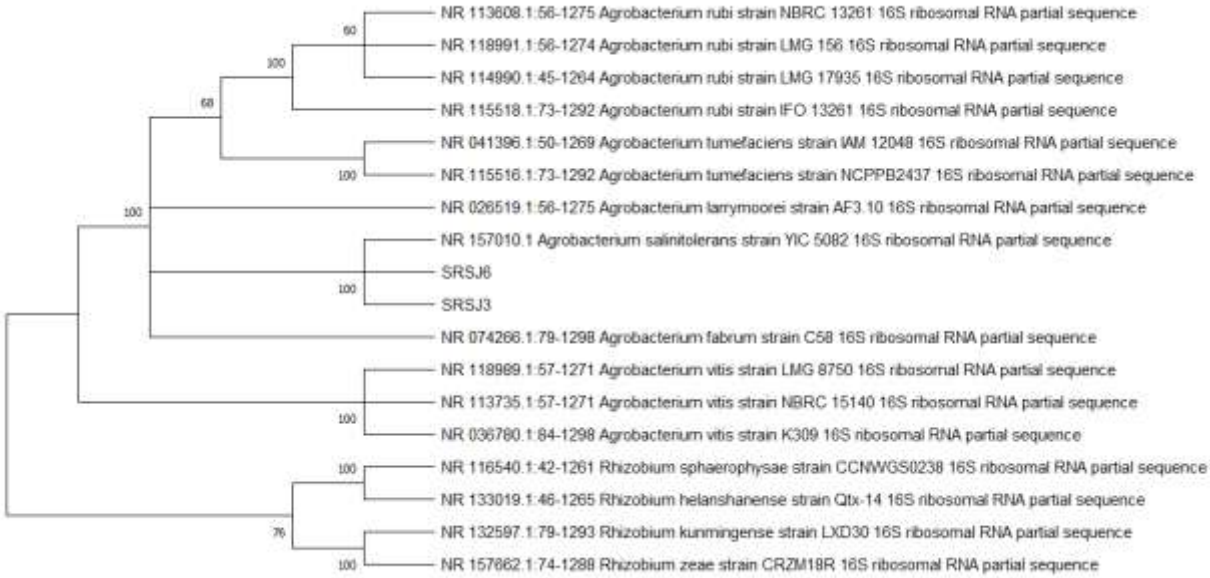

Fig 10: Phylogenetic tree showing the evolutionary relationship of SRSJ3 and SRSJ4 strains with other *Agrobacterium* species.

4. Discussion

Jensen's medium is an effective culture media which selectively enhances the growth of nitrogen-fixing bacteria from multiple bacterial mixtures. Jensen's medium is selectively used in the culture of free-living nitrogen-fixing bacteria because they thrive well in nitrogen-free mineral salt medium like Jensen's medium which does not contain any added organic nitrogen. Only the organisms which can convert the gaseous nitrogen into nitrogenous compounds will grow in this medium, so it is a useful strategy to isolate a diverse range of free-living nitrogen-fixing bacteria with a constant growth rate (Babur *et al*., 2012; Yendi *et al*., 2016). Jensen's media suppresses the growth of other microbes due to the lack of nitrogen, which is an important element for culturing other microorganisms. The nutrients supplied through this medium, like sodium molybdate, help in increasing the nitrogen fixation by bacteria because molybdenum acts as a cofactor for the nitrogenase enzyme to catalyse the redox reaction and to convert the elemental nitrogen into ammonium and nitrate (Zhou *et al*., 2017). Sucrose acts as an energy source while sodium chloride maintains the osmotic equilibrium in the medium (Navorro *et al*., 2016).

In vitro studies on nitrogen-fixing bacteria by Naganand and his colleagues were carried out to study the effect of these bacteria as a biofertilizer, and to carry out mass production of bacterial colonies in 500ml Jensen's medium and isolated more than 50 strains including different strains of *Rhizobium* and *Azotobactor* after an incubation period of 5 days, all strains were free- living nitrogen fixing (Naganand *et al*., 2010). In a comprehensive work by Madhu and her colleague on the isolation and characterisation of free-living nitrogen-fixing bacteria from alkaline soils, they isolated different strains of bacteria, like *Pseudomonas, Azotobacter,*

etc., on Jensen agar plates with bacteria exhibiting higher growth rates (Madhu and Dinesh, 2017). An authentic work by Devendra and his colleagues attempted to study the diversity of free-living nitrogen-fixing bacteria in soil samples collected from different semiarid regions of India, and they isolated 24 different bacterial strains when grown in Jensen's media, where a higher growth rate was observed within a short incubation period of about 5- 7 days (Devendra *et al.,* 2021.)

The nitrate and ammonium broth containing bacterial inoculum is effective in determining the ability of bacteria to reduce nitrogen into its simplest forms, like ammonia and nitrate (Buxton *et al*. 2011). The reduction of nitrogen took place by the catalytic activity of the nitrogenase enzyme complex present in the bacteria. Nitrogenase catalyses the ATP-dependent reduction of nitrogen in its most prevalent forms (Lance *et al*., 2020). It consists of two proteins, catalytic molybdenum-iron protein (Mofep) and reductase iron protein (fep), which drive the conformational changes in ATP and thus stimulate the transfer of electrons and protons from the substrate, which further leads to the substrate reduction (Brian *et al*.,2014; Wenyu *et al*., 2020). The nitrate broth containing nutrients and potassium nitrate is the source of nitrogen compounds, which are to be reduced by the bacterial inoculum present in the broth. These nutrients and potassium nitrate act as an oxidiser while adding the reagent di-phenyl amine, which is a solution of ammonium chloride and sulphuric acid which get oxidised and gives a blue colouration if nitrate ion is present in the broth by the reduction process (Scot, 2019). Similarly, the ammonium broth containing nutrients, which acts as a source of nitrogen compounds, reacts when adding Nessler's reagent and produces an intense brown colour, which indicates the reduction of nitrogen into ammonia. Nessler's reagent consists of potassium cations and tetraiodomercurate anion. Once these ions react with ammonia, they will produce a brown colour, and hence it is exhibited in the ammonium test (Yunxuan *et al*.,2019).

In an authentic work by Suraj and his colleagues for determining the nitrogen-fixing potential of bacterial isolates from the rhizosphere soil of *Crotalaria pallida*, six bacterial strains were isolated, and their nitrogen-fixing capacity was assessed through the nitrate reduction test. Five out of six bacterial isolates exhibited maximum potential in fixing nitrogen during the nitrate test (Suraj *et al*., 2013). In another work by Zakiah and his colleagues to determine the nitrogen-fixing potential of bacteria isolated from the rhizosphere soil of *Acacia mangium*, 24 bacterial colonies were isolated and subjected to ammonia and nitrate reduction tests. Most of the bacterial samples were found to be capable of fixing nitrogen on both tests (Zakiah, 2018).

16S rRNA is the RNA component of the 30S subunit of a prokaryotic ribosome (SSUrRNA), which binds to the Shine-Dalgarno sequence and thus provides most of the SSU structure. The gene coding for it is known as the 16S rRNA gene, and it is used in reconstructing phylogenies. Due to the complexity of hybridisation, 16S rRNA gene sequencing is widely used as a tool for identifying bacteria at the species level and finding out the difference between closely related bacterial species (Wang *et al*., 2015).

Belay and his colleagues conducted isolation and molecular identification studies on lactic acid bacteria using 16S rRNA sequencing. 24 purified bacterial isolates were isolated and subjected to 16S rRNA sequence analysis using primer sets rDI and fDI. The sequence analysis and comparison led to the identification of microbes into species and strain levels, including *Lactobacillus sparacasei. Lactobacillus brevis*, *Enterococcus durans*, *Enterococcus lirae*, *Enterococcus avium* and *Enterococcus fecium* (Belay *et al*., 2018). Huawei and co-workers isolated bacterial samples from seeds and stems of cereal crops and conducted molecular identification using 16S rRNA sequence analysis. 16S rRNA sequencing led to the identification of 10 strains of bacteria, which are nitrogen-fixing bacteria, including organisms from the genera *Paenibacillus*, *Enterobacter*, *Klebsiella* and *Pantoea* (Huawei *et al*., 2017).

Agrobacterium, which is included in *Rhizobiaceae,* can be used as a biofertilizer, acting as an associative nitrogen-fixing bacterium. In a study conducted by Natalia and colleagues on the nitrogen-fixing efficiency of *Agrobacterium*, they isolated 227 pure cultures collected from the root surface of vegetable plants and grown in nitrogen-free media. In biochemical studies, all cultures exhibited nitrogen-fixing ability and positive effects in model experiments (Natalia *et al*., 2015).

In another comprehensive work by Shaista and colleagues titled "Microbes as biofertilizers", they isolated *Agrobacterium* from free soil samples and conducted model experiments by inoculating bacteria on various crop plants like tomato, rice, etc.. The results of model experiments state that *Agrobacterium* promotes plant growth. They can be used as effective biofertilizers by promoting growth and development in plants, facilitating biotic and abiotic stress tolerance, helping in the mineralisation of soil by decomposing organic matter, and enhancing seed germination and soil fertility (Shaista *et al*., 2021).

The comprehensive work by Laxman entitled "Isolation and identification of endophytic nitrogen fixing bacteria from sugarcane and selection of efficient strains for their mass production as liquid state bioinoculant with formulations by fermentation-based biotechnology", He isolated *Agrobacterium* strains from the internodes of sugarcane grown with 10-20 % sugar at 25-degree °C. The biochemical screening and the screening of strains *in vitro* show that they have a higher potential in fixing nitrogen. On screening, *Agrobacterium diazotropicus* shows more efficiency. They are further subcultured and tested in crop plants for the confirmation of the study (Laxman, 2007).

Conclusion

The present study focused on the isolation and identification of nitrogen-fixing bacteria from the banks of the Meenachil River in the Kottayam district. Samples were collected randomly from six different sites and grown in a selective Jensen's medium. A further 5 morphologically distinct colonies were identified and subjected to biochemical analysis to determine the nitrogen-fixing potential of the isolates. Isolate SRSJ3 and isolate SRSJ6 exhibited maximum potential in nitrogen fixation during biochemical analysis. Morphological and microscopical analyses were carried out for primary confirmation, and 16S rRNA analysis of two strains, which exhibit maximum potential in nitrogen fixation, were found to be

Agrobacterium spp. Though the study succeeded in the genus-level identification of the isolate, more genetic and biochemical studies are warranted for species confirmation.

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