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Research paper

Isolation and characterization of free-living nitrogen fixing bacteria from alkaline soils

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Abstract

Free living nitrogen fixing bacteria were isolated from soil on Jensen agar plates and were characterized phylogenetically by 16S rDNA sequence analysis. All the isolates (VS1, VS2, VS3, VS4) were Gram –ve, rod shaped. Antibiotic test revealed VS2 to be resistant to ampicillin and VS4 was resistant to both ampicillin and kanamycin; otherwise all the isolates were sensitive to chloramphenicol. Nitrogen fixation was studied by the estimation of total nitrogen and available nitrogen fixed by cultures in the medium and compared with the control culture of *Azotobacter* CBD15 (Azo) procured from IARI. VS2 and VS3 fixed 12.02 ppm/ml and 10.635 ppm/ml as available nitrogen content and 14.44 ppm/ml and 18.73 ppm/ml as total nitrogen content. 16S rDNA studies revealed identification of the isolates- *Pseudomonas* sp. (VS2) and *Paenibacillus* sp. (VS3 and VS4). VS3 and VS4 showed 98% similarity with *P. borealis*. Soils from which these microbes isolated were also characterized to understand the environment of these microbes. The pH and chemical characterization (Organic Carbon, Phosphorus, Sulphur, heavy metal analysis of different metals e.g. Zn, Cu, Cr, Pb, Ni and water holding capacity) of the soils showed them to be slightly alkaline and clayey loamy. Cloning of VS2 was done successfully with plasmid pMMB277 isolated from *E. coli* 2842.The significance of this study lies in the isolation of those bacteria which are comparable in their nitrogen fixing potential to *Azotobacter*.

Keywords: *16S rDNA; Nitrogen Fixing Bacteria; Paenibacillus; Pseudomonas; Soil.*

1. Introduction

Nitrogen, the most important nutrient required for the growth, is the main component of proteins, nucleic acids and other cellular constituents. But, in order for nitrogen to be used for growth it must be converted to ammonium (NH_4^+) or nitrate (NO_3^-) ions [1], [2]. Only certain prokaryotic organisms are capable of utilizing dinitrogen [3]. These are the only organisms, capable of taking gaseous nitrogen and combining it with hydrogen to make ammonia [4]. So, nitrogen-fixing bacteria are an essential part of all the ecosystems. Generally biological nitrogen fixing bacteria are free-living soil organisms, but some have symbiotic association with plants, where they infect their roots for food and fix nitrogen which can be used by the plant for growth. It can also be fixed by industrial methods. However, concern over the environmental impact of agricultural chemicals has led to peculiar efforts to develop biological control agents [5]. In some cases efforts are being hampered by the lack of a full understanding of the interactions between microorganisms, plants and environmental factors. Bacterial functioning is critical to soil and plant health. So, enhancing knowledge of soil bacterial functioning and diversity will aid in the development of sustainable agro ecosystems [6]. The objective of this study was to explore bacterial diversity by isolation and characterization of free-living nitrogen fixing bacteria from soil, check out their nitrogen fixing potential, to reveal their identity by 16s rDNA and physico- chemical analysis of soil. It was *Paenibacillus borealis* in this study. The genus *Paenibacillus* represents a new phylum that encompasses several species described as nitrogen-fixing bacilli [7], [8].

2. Materials and methods

2.1. Chemicals and reagents

All the Chemicals and reagents were of Analytical grade purchased from Merck Ltd., India; Himedia Pvt. Ltd., Mumbai, India, SD fine chemical Ltd., Ambala, India.

2.2. Collection and soil sampling

For the study, the soil samples were collected from different field areas of Thapar University campus, Patiala, Punjab, India. The samples were collected from 0-30 cm depth, processed and analyzed for various physical, chemical and microbiological characteristics. After collection, the soil was divided into two portions, one of which was immediately refrigerated and was later used for microbiological analysis. It was gently broken up for clumps and macro aggregates followed by removal of plant residues, gravel and other debris. Another portion was spread on polythene sheets, air-dried and sieved and used for physical and chemical analysis.

2.3. Isolation and strain maintenance of bacteria

In the study, bacteria from soil were isolated by Serial dilution method at 28 °C. Four bacterial cultures: - VS1, VS2, VS3 and VS4 were isolated and were streaked on the Jensen's media plates and observed the plates for 24, 48 h. Standard culture of *Azotobacter* (Azo) CBD15 procured from IARI (Indian Agricultural Re-

search Institute, New Delhi, India) was also used in the study. Two different media were used to grow bacterial cultures: (a) Jensen's Media [9] was used as selective media to grow nitrogen fixing bacteria. (b) Luria HiVeg^{TM} media was used to grow bacterial isolates. The strains were maintained as stocks in 50% v/v glycerol and stored at -20 ˚C. The bacterial isolates were propagated on Jensen's media. For activation prior to experimental use bacterial strains were individually subcultured from glycerol stocks at -20 ˚C in 10 ml Luria broth and were incubated at 28 ˚C for their optimum growth.

2.4. Biochemical characterization

Different Biochemical tests were performed to characterize the bacteria. To manifest the Gram character, Gram staining of the bacterial isolates was done as per [10]. Antibiotic sensitivity profile of the bacterial isolates was carried out by using standard antibiotics Kanamycin, Ampicillin and Chloroamphenicol. The Stock solutions of antibiotics were of 10 mg ml^{-1}conc., whereas the working conc. was 10µg ml^{-1}.

2.5. Nitrogen potential

Available and total nitrogen potential were estimated. Available nitrogen was estimated as per given by [11] in the culture filtrate. For this, 5 ml of bacterial culture was taken in a Kjeldahl flask. A blank was run without culture and calculation was done as following:

Calculation

Available N in culture (in ppm) = (X) x 0.00028 x 100/5

where, X stands for the titre value of 0.02 N H_2SO_4 consumed. Blank run value in ppm/ml was subtracted while calculating the nitrogen content of the bacterial cultures.

While, Total nitrogen in culture filtrate was estimated as per the Kjeldahl method given by [12]. The bacterial cultures were first digested with digestion mixture (K_2SO_4:$FeSO_4.7H_2O$:$CuSO_4$ (10:1:0.5) and conc. H_2SO_4 in the digestion chamber at 100°C for two hours. The color change was monitored from dark brown to greenish white and the contents were cooled and 100 ml volume make-up was done with distilled water. 10 ml of above digested sample was taken in the Kjeldahl flask and in this case calculation was done as following:

Calculation

1 ml of N/50 H_2SO_4 = 0.0028 of nitrogen

2.6. Physico-chemical analysis of soil

The physico-chemical properties of the soils were analyzed for different parameters. Water holding capacity was measured as per the method given by [13, 14]. Determination of soil texture was done as per method given by [15]. The final result was calculated by multiplying the volume percentage with factors to convert it into weight percentage. For the chemical analysis of soil, pH and Electrical conductivity were determined as per the method given by [16]. Method given by [17] was used to estimate mineral composition such as total organic carbon. Available phosphorus was estimated by the standard procedure given by [18] for the alkaline soils as manifested by pH determination of soils. Estimation of available sulphur was done by the method given by [19]. Heavy metal analysis of Zn, Cu, Cr, Pb and Ni was done as per the modified method given by [20]. For this soil samples were first digested by adding HNO_3 and $HClO_4$ in 3:1 ratio. The samples were digested on a hot plate at 100°C for 3-4 hours until a whitish brown dry mass was obtained. The samples after digestion were dissolved in 20 ml, acid water mixture containing HCl and water in a 1:1 ratio and filtered through Whatman No.42 filter paper. The filtrate was analyzed for total Zn, Cu, Cr, Pb and Ni in both soil and fly ash samples using an atomic absorption spectrophotometer (GBC 932AA, Australia).

2.7. Phylogentic analysis of 16S rDNA sequences

Bacterial genomic DNA was isolated as per standard method given by [21]. The colonies were characterized by amplifying and sequencing the 16S rDNA by using universal primers. It included two steps - PCR reaction and then sequencing.

2.7.1. PCR amplification of 16S rDNA

2 µl genomic DNA was used for the amplification of 16S rDNA gene sequence by Polymerase Chain Reaction (PCR). Taq DNA polymerase and primers - 27F (AGAGTTTGATCCTGGCTC AG) and 1492R (GGTTACCT TGTTACGACATT) as described by [22] used for amplification were supplied by MBI Fermentas. Amplification reaction mixture (25 µl) consisted of 2 µl of template DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 200 nM primers, and 1.0 U of Taq polymerase. Amplification was performed in automated thermal cycler (Applied Biosystems) at 92°C for 2:10 min followed by 30 cycles of 92 °C for 1:10 min, 48 °C for 30s, and 72 °C for 2:10 min, and a final elongation step at 72°C for 6:10 min. To ensure that no contaminating DNAs would give false positive results, one sample lacking a template was included as negative control and one as positive control (containing DNA template whose PCR had been done previously under that reaction conditions). Aliquots of the amplification products were analysed by agarose gel electrophoresis.

2.7.2. Sequencing of the PCR products

For Sequencing the PCR amplified samples were sent to Lab India, Gurgaon; where Sanger method was used to do sequencing. Assembled sequences were compared with those in the public domain through a BLASTn search and a phylogenetic tree was constructed by MEGA4 and multalin [23].

3. Results and discussion

The aim of present study was to screen and characterize nitrogen fixing bacteria from soil, so that they could be introduced in the soil to enhance soil fertility by increasing nitrogen content of the soil. The bacterial strains were screened for various morphological, biochemical and molecular characteristics. Out of the four isolates, three isolates - VS2, VS3 and VS4 with efficient nitrogen fixing ability were selected for phylogenetic analysis by 16S rDNA sequencing. The following text presents the results on physico-chemical characterization of soil, morphological, biochemical and molecular characterization of selected nitrogen fixing bacteria.

3.1. Characterization of microbes

3.1.1. Morphological characterization of bacterial isolates

Bacterial isolates were characterized morphologically for the colony shape, colony color, colony elevation and for Gram character as per the standard protocol [24]. The colonies of isolates VS1, VS2, VS3 and VS4 were circular and flat, whereas colonies of Azo were circular but domed in shape . VS1, VS2, VS3 and VS4 were of off-white color and small in shape as comparison to Azo, which were transparent and large sized. Microscopic studies showed that bacterial strains VS1, VS2, VS3, VS4 [25] were Gram -ve, and rod shaped stained red colored with counter stain saffranin.

3.1.2. Biochemical characterization

Different biochemical test performed on VS1, VS2, VS3 and VS4 are given in Table 1. Bacteria were checked for their sensitivity with ampicillin, kanamycin and chloramphenicol. The activity profile of bacterial strains showed them to be sensitive to chloramphenicol, while VS4 was resistant to ampicillin and kanamycin and VS2 was resistant to ampicillin.

Table 1: Biochemical tests of Nitrogen fixing bacterial cultures

Isolates-	VS1	VS2	VS3	VS4	Azo
MacConkey's Agar test	+	+	+	+	+
Catalase test	+	+	+	+	+
KOH test	-	-	-	-	-
Simmons Citrate Agar test	+	+	-	-	-
Urease test	-	-	-	-	-
Phenylalanine Deaminase test	-	-	-	-	-
Casein hydrolysis test	-	-	-	-	-
Starch hydrolysis test	+	+	+	+	+
SIM Medium test	-	-	-	-	-
Carbohydrate test					
(i)Glucose	+	+	+	+	+
(ii)Maltose	+	+	-	-	+
(iii)Sucrose	+	+	+	+	+
(iv)Lactose	+	+	+	-	+
Gelatin hydrolysis test	-	-	+	+	+
MR test	-	-	+	+	-
VP test	+	-	-	-	-

*Biochemical characterization showed similarity between the biochemical properties of VS3, VS4 and Azo

3.2. Physico-chemical characterization of soil

Different soil samples S1, S2, S3, S4 from which the bacterial isolates (VS1, VS2, VS3 and VS4) were isolated respectively, were analyzed for their physico-chemical properties which include pH, EC, organic carbon, available sulphur, available phosphorus and heavy metal analysis. Water holding capacity, particle size distribution and soil texture analysis were also studied. Water holding capacity of soils varied from 34.24 - 51.41 where, S1 had the lowest water holding capacity while S4 had the highest (Table 2). Water holding capacity reveals the relationship between the soil and its water and important for the organisms live in it. The water holding capacity of soils also depends on their particle size distribution and texture [26], [27].

In the various soil samples collected, the percentage sand, silt and clay was found to vary from 51.3- 77.23 %, 3.31- 15.0 % and 24.72 to 38.0 % respectively. The texture of soils S2, S3, S4 was sandy clay, while S1 was sandy clay loamy (Table 2). The pH of soils S1, S2, S3 and S4 were slightly alkaline (Table 2). The electrical conductivity range was between 0.16 mS/cm to 0.22 mS/cm (Table 2) and thus soluble salt content of the soils was also in most favorable range. Increase in electrical conductivity of soil by any factor, thereby increases the availability of soluble salts [28], which may have detrimental effects on microbial respiration, enzyme activity and soil nitrogen cycling etc. Organic carbon varied from 0.225-1.1%.

Table 2: Physico-Chemical Characterization of Different Soils

Soil Sample Parameter	S1	S2	S3	S4
pH	7.45	7.71	8.19	8.22
EC(µS / cm)	157.7	208	220	217
Organic C %	0.225	0.975	1.1475	1.1
P (ppm)	0.143	0.297	0.271	0.171
S (ppm)	104.04	1.288	9.5904	23.714
Heavy metal analysis (ppm)				
Zn	41.32	BDL	28.06	25.2
Cu	33.8	BDL	BDL	BDL
Cr	40.74	31.52	73.74	59
Pb	23.14	38.7	33.66	25.52
Ni	13.98	11.68	27.84	22.96
Water holding Capacity (%)	34.24	39.42	47.3	51.41
Sand %	77.23	51.3	66.4	63.54
Silt %	7.63	15.0	3.31	7.5
Clay %	24.72	37.32	38.0	35.7
Texture	Sandy clay loam	Sandy clay	Sandy clay	Sandy clay

* BDL- Below detection limit

*pH showed the slightly alkaline nature of different soil samples from which bacterial isolates were taken. The soils were sandy clayey (loamy) in texture and different elements and heavy metal presence gave the fair idea about the chemical characteristics of Punjab soils

Available phosphorus for S2 was highest, while for S1 it is lowest and diverged from 0.143 to 0.297 ppm. Available sulphur was analysed for soil samples S1, S2, S3, S4 and it ranged from 1.228-104.04 ppm. Plants use inorganic sulphate as sulphur nutrition; Sulphate esters as organic sulphur or any other carbon bonded sulphur also get convert into inorganic sulphate to be available for the plants [29]. The above results were blend of both forms. Heavy metal analysis was done for Zn, Cu, Cr, Pb and Ni of S1, S2, S3 and S4. Zn was found to be below detection limit in S2, while it was highest in S1 (2.066 ppm). Cu was below detection limit in three soil samples S2, S3 and S4. It was only found in S1 (1.690 ppm). Cr was highest in S3 (73.74 ppm), while it is lowest for S2. Pb was detected in all the four soil samples, with its detection highest in S2 and lowest in S1. Ni was lowest in S2 (11.68) soil sample, while it was highest in S3 (27.84 ppm) sample (Table 2).

3.3. Nitrogen potential

This analysis showed that *Azotobacter* CBD 15 was able to fix available and total nitrogen in the range of 7.975-9.225 ppm/ml and 10.96 to 12.89 ppm/ml respectively.VS2 was able to fix 12.02 ppm/ml nitrogen in the available form as its maxima after 12 days of incubation (Fig. 1-4) while VS3 fixed 10.635 ppm/ml after 9 days of incubation. Although total nitrogen content gave a more precise account of the nitrogen fixing potential of the organisms. Available nitrogen is more important than total nitrogen content due to its readily availability.

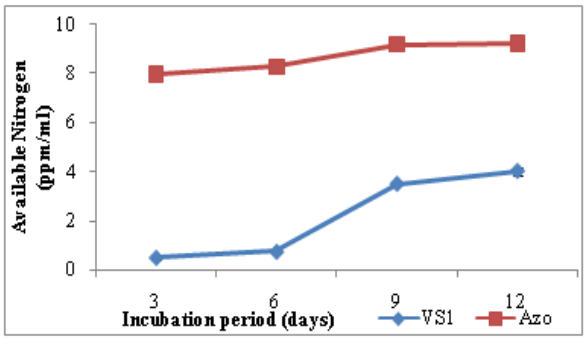

Fig. 1: Available nitrogen content of VS1 vs Azo after 3-12 days of incubation in Jensen's media with stirring at 120 rpm at 28 ˚C.

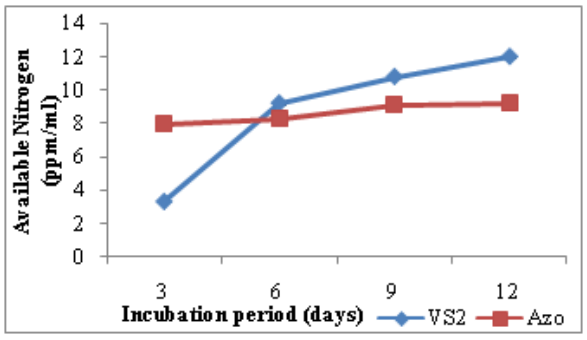

Fig. 2: Available nitrogen content of VS2 revealed it to be better in its potential than Azo after 6th day of 3 -12 days of incubation in Jensen's media with stirring at 120 rpm at 28 ˚C.

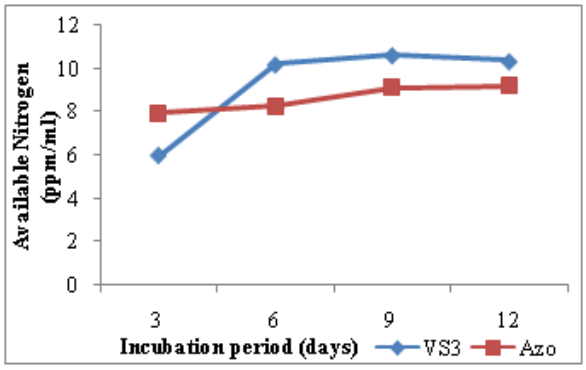

Fig. 3: Available nitrogen content of VS3 showed it to be comparable to Azo; with its maxima as 10.635 ppm/ml on 9th day of 3-12 days of incubation in Jensen's media with stirring at 120 rpm at 28 ˚C.

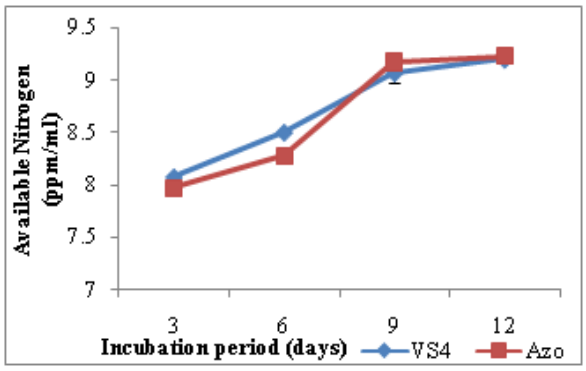

Fig. 4: Available nitrogen content of VS4 showed its potential quiet similar to Azo in 3-12 days of incubation in Jensen's media with stirring at 120 rpm at 28 ˚C.

Total nitrogen content is available only after treated with harsh conditions. VS2, VS3 and VS4 had good nitrogen fixing ability (Fig. 5-8) as their cultures have more fixed nitrogen in Jensen's media in ppm in all 15 days in comparison to Azo.

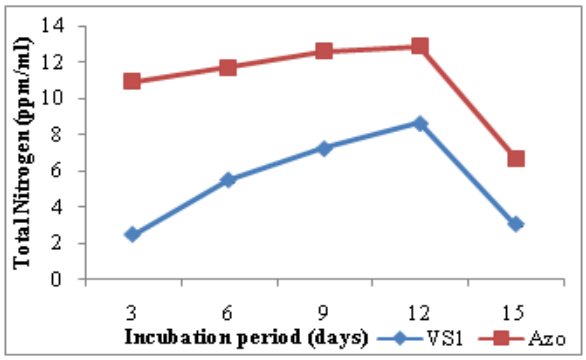

Fig. 5: Total nitrogen content of VS1 showed that it's potential to fix nitrogen is not comparable to Azo in Jensen's media.

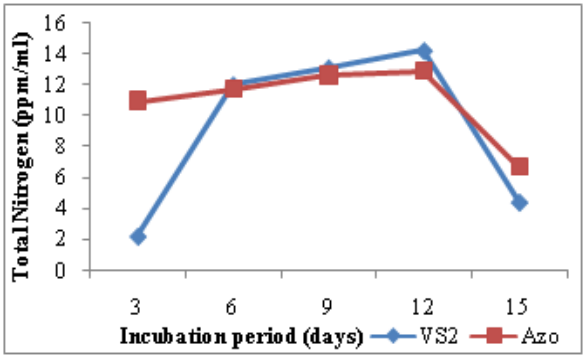

Fig. 6: Total nitrogen content of VS2 showed it to be similar to Azo after 3-12 days of incubation in Jensen's media with stirring at 120 rpm at 28 ˚C.

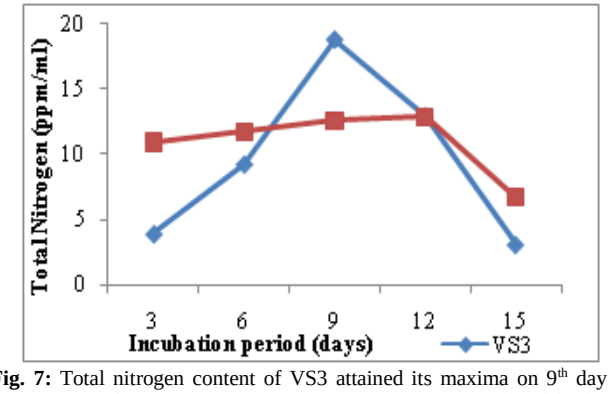

Fig. 7: Total nitrogen content of VS3 attained its maxima on 9th day of incubation by fixing 18.73 ppm/ml nitrogen in Jensen's media with stirring at 120 rpm at 28 ˚C.

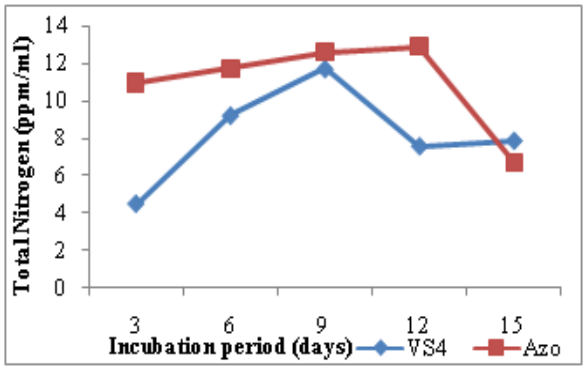

Fig. 8: Total nitrogen content of VS4 showed its maxima with 11.73 ppm/ml nitrogen fixation on the 9th day of incubation in Jensen's media with stirring at 120 rpm at 28 ˚C.

3.4. 16S rDNA analysis

A phylogenetic analysis of the genes of VS3 and VS4 were related to *Paenibacillus* sp. (Fig. 9) The sequences were compared with sequences obtained from on comparison of sequences in multalin was constructed using the neighbour-joining GenBank and aligned using the Blastn having maximum identity and first five sequences on this basis was taken. A phylogenetic tree was prepared using MEGA4 software [23].

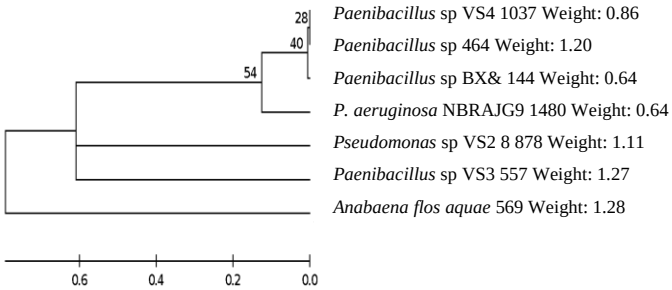

Fig. 9: 16S rDNA gene Sequence-based Phylogenetic Tree generated by using the Neighbour-Joining method showing the relationships between the strains studied and their representatives

4. Conclusions

VS2, VS3 and VS4 have a substantial nitrogen fixing ability, when compared to *Azotobacter* CBD15. These cultures can be used for plants to fix nitrogen in soil. Efficient monitoring systems to monitor their influence on plants and the influence of physico-chemical parameters of soil on these microorganisms can enhance their usage as nitrogen fixers.

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