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RESEARCH ARTICLE

BIOCHEMICAL AND MOLECULAR IDENTIFICATION OF OXALATE-OXIDIZING BACTERIA ISOLATED FROM RHIZOSPHERE OF BIOMENRALIZING TREE, *TERMINALIA ALATA* FROM KUMAUN HIMALAYA, INDIA

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ABSTRACT

Oxalate oxidation, which has environmental implications, is performed by oxalotrophic bacteria. In this study, forest survey was conducted in Bhujighat (BHU), Nainital district of Uttarakhand state in India. Biomineralizing tree, *Terminalia alata* was identified with the help of 10 % HCl treatment which showed effervesce. Soil samples were collected at different depths of soil profile. The Schlegel's agar medium was used to obtained microbial diversity. Microbial analysis of each soil sample was done by dilution plating method. Morphologically distinct colonies were examined by morphologically and biochemically by menace of oxalate assay, siderophore production and phosphate solubilization. Total 112 bacteria were isolated, out of these 9 potential isolates were amplified with 16S rDNA primer (Gm3f and Gm4r). Amplified pcr product was sent for DNA sequencing. The sequence results obtained after DNA sequencing, sequence similarity search was performed on NCBI-BLAST tool. The similarities of 2 most potent strains BHU A4 and BHU X1 were almost 99% with *Rhizobium sp.* The nucleotide sequence of both strains were submitted to NCBI gene bank database, *Rhizobium sp. strain* BHU A4 *(*KY021745*) and Rhizobium sp. strain* BHU X1 (KY021756). Both strains are being used in glasshouse experiment for plant growth promotion related activities under different pH level of soil, which is a basic supplement for plant health. This indicates that oxalotrophic bacteria are numerous and widespread in soils and that a relationship exists between the presence of the oxalogenic trees. *Terminalia alata* tree having abundance of oxalotrophic guilds in the total bacterial communities which explains the biomenralization and calcium carbonate accumulation below these trees, which act as long term carbon sink.

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INTRODUCTION

Terminalia alata from Kumaun Himalaya (Uttarakhand) having biominralizing properties in which oxalic acid play important roles. Calcium oxalate in plants has many different functions. Oxalic acid is a low molecular weight organic acid, which play a key role in calcium ions regulation in cytoplasm (Robert & Roland, 1989). Accumulation of oxalate in biomenralizing tree having a significant carbon source for oxalotrophic bacteria, which are abundance in the tree biomass and the surrounding litter. According to Braissant *et al*. (2002) aerobic degradation of calcium oxalate with the help of oxalotrophic bacteria leads to the calcium carbonate accumulation. This biologically induced accumulation of carbonate represents the atmospheric CO_2 sequestration which act as a long term carbon sink.(Cailleau *et al*., 2004). Due to this biological process increase in carbonate ion production through the oxalate carbonate pathway occurring around the rhizosphere (Cailleau *et al*., 2011). The pH of soil below plant upper layer was found to be alkaline while in case of soil 15m away from the biominralizing plant was acidic (upper layer) because increased amount of calcium carbonate was found below the tree in study in comparison to the tree which is away. In a study Cailleau *et al*. (2005). Presence of oxalotrophic bacteria in the rhizosphere of biominralizing tree creates favorable environment for $CaCO_3$ accumulation. During oxalate oxidation into carbonate by oxalotrophic bacteria, the pH increases, allowing the reaction of carbonate species with free Ca^{2+} ion, followed by the enhancement of calcium carbonate precipitation. Moreover, wood decomposing

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microbes also influence the oxalate carbonate pathway as a result of their production of large amounts of oxalic acid and other organic acids during the decay of litter (Cochrane, 1958; Dutton & Evans, 1996; Gadd, 1999). The aim of this study is to investigate the role of oxalotrophic bacteria in carbonate biomineralization within *Terminalia alata* Biomineralization.

MATERIALS AND METHODS

Collection of samples

In this investigation, a survey was conducted to find out biominralizing tree at Bhujiaghat (BHU) forest site located at Nainital district in Uttarakhand, India. The selection of the biomenralizing tree was done by 10% HCL treatment on bark and soil of the tree. The process was repeated almost on 20 to 25 trees in forest sites and finally a tree was obtained. A square shaped pit of size 1m x1m was prepared below the Biominralizing tree. Soil samples were collected from every 10 cm starting from top to the bottom. Another pit 15m away from the Biominralizing tree was also selected for sampling. Sampling was made in the same way as it was done for Biominralizing tree. The collected soil samples were kept in sterile bags and stored at 4^{o}C in cooling kit.

Isolation of Oxalotrophic (oxalate-oxidizing) bacteria

Two (2) gram of soil was weighed and mixed into 20ml freshly prepared Schlegel's basal mineral broth medium transferred into conical flask (250ml) and kept on rotating shaker at 120rpm for 5 days of incubation period at 28±2 ^{o}C. After 5 days of incubation 1 ml of enriched culture was transferred to newly prepared 19 ml broth medium to make final volume upto 20ml was again incubated for 5 days. This process was repeated for three times. Oxalotrophic bacterial isolation from each soil sample was done by dilution plating method on Schlegel's basal mineral medium (Aragno and Schlegel, 1992). Dilution was made with 10^{-1} to 10^{-9}, 100µl of enriched suspension from 10^{-4} and 10^{-6} dilution was placed on agar medium supplemented with 4 g potassium oxalate ($K_2C_2O_4$) and 15 g agar per liter. Before pouring the plates, 80 ml sterile $CaCl_2$ solution (0.1 mol l^{-1}) were added in 920 ml l^{-1} medium to convert part of the oxalate present to calcium oxalate ($Ca_2C_2O_4$). The plates were incubated for 72 h at 28±2 ^{o}C.

Morphological identification of bacteria

Bacterial colonies obtained after dilution plating was subjected to screening their morphology. Colonies were identified by their shape, size, colour, elevation etc. (Christopher, K. and E. Bruno. 2003) and purified by streak plate method. Schlegel's medium supplemented with potassium oxalate as sole energy source for oxalotrophic bacteria, was screened out by their zone formation around bacterial colonies. Morphologically distinct and zone forming on medium was isolated and purified on Schlegel's agar medium.

Biochemical identification of bacteria

Siderophore production

Overnight grown bacterial culture was spot inoculated on CAS (Chrome Azurol S) plates according to Schwan and Neilands (1987). Plates were incubated at 28±2 ^{o}C for 48-72 h. Appearance of yellow-orange hollow zone around the bacterial colonies indicates siderophore production activity.

Phosphate Solubilization

Pikovaskaya's agar plates were spot inoculated with overnight grown bacterial culture and incubated at 28±2 ^{o}C for 48-72 h. Formation of clear zone around the colonies indicates a positive test of phosphate solubilization (Pikovaskaya, 1948).

Molecular identification of bacteria

Genomic DNA isolation from oxalotrophic bacteria was performed with CTAB method (Jaufeerally-Fakim and Dookun, 2000). Overnight (24 hour) grown bacterial culture in NB medium was used to pellet out cells. A little modification was made in CTAB method while isolating the DNA. Genomic DNA was used in PCR amplification. The 16S rRNA genes of bacteria was amplified by 16S rDNA universal primers Gm 3f (5' AGA GTT PGA TCMTGGC 3') as a forward primer and Gm 4r (5' TAC CTT GTT ACG ACT T 3') as a reverse primer (Muyzer *et al.,* 1995). The PCR amplification was performed in a final volume of 50µl. The reaction mixtures were subjected to 35 amplification cycles in a thermocycler (Biorad). The first step was performed at 94^{0}C for 7 min, second at 94 ^{0}C for 1 min, third at 56 ^{0}C for 1 min, fourth at 72^{0}C for 1 min, fifth at 72 ^{0}C for 10 min and last at 4^{0}C.

RESULTS AND DISCUSSION

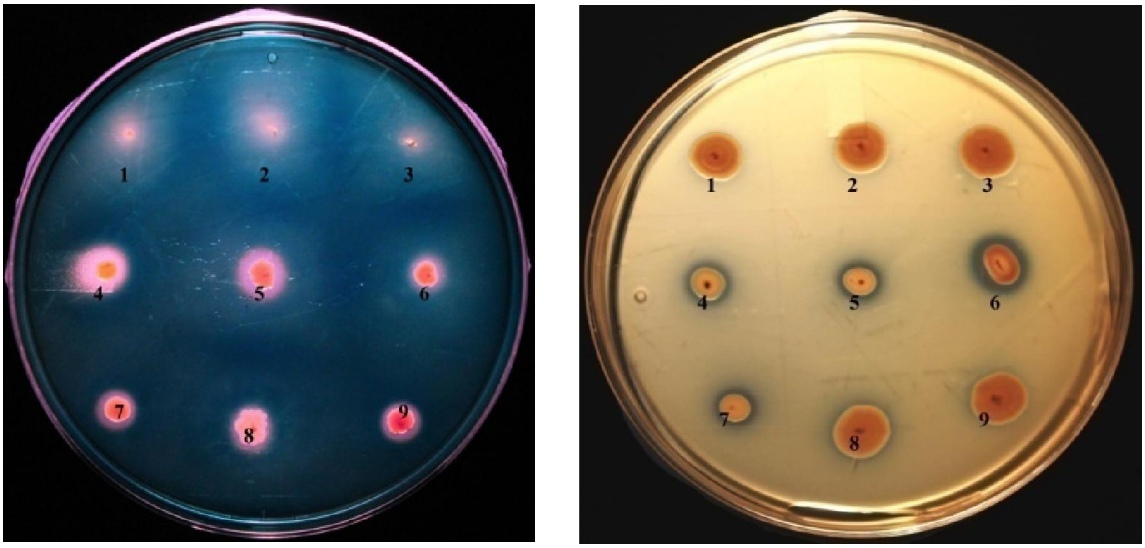
After the extensive survey at Bhujiaghat forest, biomineralizing tree was identified with the help of 10 % HCl treatment, effervescence was observed. The tree was identified as *Terminalia alata,* Commonly known as Asna in Uttarakhand, India, which belongs to *Combretaceae* family. The tree was large up to 35 meter tall, width up to 270 cm in diameter, bark surface with deep vertical fissures and transverse cracks, dark grey to whitish (commonly known as crocodile bark), inner bark reddish in colour. Whitish colour of bark due to calcium carbonate deposition. Leaves ovate-oblong, 10-30 cm x 6-10 cm, base obtuse, apex rounded to acute, petiole 1-2 cm long.

Morphological, biochemical and molicular characterization of oxalotripic bacteria

Pour plating method was used to isolate bacterial population. Wide range of bacterial colonies was obtained on schlegal solid medium. Morphologically distinct colonies that developed clear zones on the schlegal's medium after 10 to 15 days incubation (28±2ºC) were selected. Clear zones around colonies measured as reported previously (Aragno and Schlegel, 1992). Bacterial colony was picked up from schlegal's medium and purified by streaking and rechecked again in Schlegel's medium (Fig.1). After rechecking of selective isolates, were examined morphological basis. Both *Rhizobium* species showed circular shape, opaque colour, raised elevation, smooth surface, smooth edge and amorphous structure. After morphological identification of selective isolates, were examined with biochemical test Both *Rhizobium* species showed gram negative rod shaped structure and both showed siderophore and phosphate positive (Fig.2). Carson *et al*. 2000 reported that some PGPRs (*Bradyrhizobium japonicum* and *Rhizobium leguminosarum*) play an important role in Siderophore production for rhizosphere colonization with plant growth promotion related activity.

(A) Rhizobium sp. strain BHU.A4 (KY021745) **(B) Rhizobium sp. strain BHU.X1 (KY021756)**

Fig.1. *Rhizobium* sp. showing the clear zone in the Schlegel's basal mineral agar medium amended with potassium oxalate



(A) Sidrophore production **(B) Phosphate Solublization**

Fig.2. Potential Bacterial isolates1 to 9 showing the clear zone on biochemical tests

Fig.3. 16S rRNA gene amplified M-1kb DNA ladder. Lane 1 to 9 showing amplification of 16S rRNA gene (1600 bp size)

According to (Sturz and Nowak, 2000; Sudhakar *et al*., 2000; Mehnaz and Lazarovits 2006). Phosphorous solubilization in soil is shown by Bacterial genera like Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium and Serratia are reported as the most significant phosphate solubilizing bacteria. Total 9 potential isolates were amplified with 16S rDNA primer Gm3f and Gm4r. (Muyzer *et al.,* 1995). Band size of ~1600bp was observed on agarose gel (Fig.3). Amplification of same size in case of the oxalate utilising isolates confirms the presence of this region. Amplified pcr product was sent for DNA sequencing. The sequence results obtained after DNA sequencing, sequence similarity search was performed on NCBI-BLAST tool. The similarities of 2 most potent strains BHU A4 and BHU X1 were almost 99% with *Rhizobium sp.* The nucleotide sequence of both strains were submitted to NCBI gene bank database, *Rhizobium sp. strain* BHU A4

*(*KY021745*) and Rhizobium sp. strain* BHU X1 (KY021756). Both strains are being used in glasshouse experiment for plant growth promotion related activities under different pH level of soil, which is a basic supplement for plant health.

Conclusion

Looking to the recent secenario and increased demand of carbon sequestration, there is a possibility of the use of oxalogenic tree in forestry or agrogorestry programme. *Terminalia alata* reported to be the leading ones in serving this purpose. Using calcium from the plant and combining it with carbon dioxide inhaled by photosynthesis, calcium carbonate is made as a resultant product. In the present study, deposition of calcium carbonate was observed in the bark of the *Terminalia alata*. After death and decomposition of tree, oxalate is added as source through litter renewal and secretion from roots to the soil. However, efforts are still on way to find out if the inoculation of oxalotrophic bacteria could be successful in the existence of phenomenon in situ at an early time. To summarize, three conditions are necessary for calcium carbonate accumulations in soils: (i) Oxlate-biominralizing tree, (ii) appropriate oxalotrophic bacteria for oxalate oxidation into carbonate and (iii) acidic soil. These conditions exist in many areas of tropical India. Consequently, carbon storage as inert calcium carbonate in soils from atmospheric CO_2 through oxlate carbonate pathway identified and act as a carbon sink.

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