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Original Article

Elucidating the Role of Plant Growth Promoting Bacteria for Nitrate and Phosphate Bioremediation: A Sustainable Approach Towards Crop Productivity and Environmental Protection

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Abstract

Introduction: The production of healthy food along with environmental sustenance is an everlasting mission for the coming ages. The utilization of chemical fertilizers may fulfil the requirement but it could also compromise the environment. The present study aimed to tap beneficial microbes that could not only harbour plant growth-promoting traits but could also remove environmental pollutants, nitrate and phosphate.

Materials and Methods: The culture-dependent approach was taken into account to isolate and characterize the bacterial population from dumping ground and mangrove soil. The selected isolates were further tested for remediating nitrate and phosphate by standard biochemical tests.

Results: Three isolates from dumping ground and four bacterial strains were proved to contain three out of four Plant Growth-Promoting (PGP) traits (HCN, IAA, Phosphate-solubilisation, and Nitrogen fixation). Two out of three selected bacterial strains were found to have the ability to remove nitrate and phosphate up to 74% and 62% ($p<0.05$) respectively. This is while all the other selected bacterial strains from mangrove soil could effectively remove nitrate and phosphate in the range of 62% to 79% and 24% to 100% ($p<0.05$) respectively. Two novel strains of *Streanomonas* sp., *Alkaligens* sp. and one each *Bacillus* sp., *Corynebacterium, Pseudomonas* and *Citrobacter* species isolates were found in the present case.

Conclusions: The study represents the dual ability of the novel bacterial strains in plant growth promotion as well as remediation of environmental pollutants. Thus, the study might aid in designing a microbe-based bio-fertilizer for plant growth promotion along with maintenance of soil and environmental health.

Keywords: Bacterial-Remediation, Sustenance, Crop Health, Environment, Mumbai, Mangrove

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Introduction

With the ever-increasing population rate along with, changing lifestyle patterns of the human population, it is of utmost importance to develop methods which can increase crop yield to meet the need of the population. In the 20th century, the employed strategies are to increase plant productivity by using chemical fertilizers. Chemical fertilizers including ammonium phosphate and ammonium nitrate were usually used to provide plant, keeping in mind that nitrate and phosphate are the essential nutrients for plants in order to maintain their physiological processes. Conversely, they have emerged as the most abundant pollutants in the environment with aberrant use in crop fields to increase production.[1]

Nitrate pollution, currently considered as a global concern and is the second most dangerous pollutant after pesticides. High levels of nitrate could cause "blue baby syndrome". It has carcinogenic effects and a concentration of greater than 100 ppm can cause stomach cancer in infants.[2] Also, the complications like nausea, headache, and respiratory problems[3] are also associated with nitrate pollution. The usage of phosphorous as a phosphate fertilizer in soil has proven to be responsible for causing some serious environmental problems like eutrophication in water bodies eventually causing significant amounts of loss of aquatic organisms.[4] To overcome the above-stated problem, an alternative could be the use of microbial bio-fertilizer. A few numbers of studies in the past five years have concentrated on designing the microbe-based fertilizer and testing its efficacy towards crop production.[5,6] The common goal of the above studies was to enhance the crop productivity in an eco-friendly manner, and to restore and enhance the fertility of the soil. These beneficial soil microorganisms are called 'Plant Growth Promoting Bacteria' (PGPB). An increase in the PGP activity can help us to increase plant growth. Hence isolation of bacterial strains with higher PGP abilities can be

extremely useful in the production of effective bio fertilizers.[7] The PGP rhizobacteria are free-living that can directly or indirectly facilitate nutritional uptake. They do so by various mechanisms like nitrogen fixation while phosphate and potassium are made obtainable by solubilisation of their insoluble forms. Another mechanism engaged in growth promotion includes PGP hormones.[8,9] Soil is a rich source of various kinds of the microbial population. According to a recent study, 1 cm^3 of productive soil contains 20 billion microbial cells.[10] The heterogeneity of the soil defines the total number of microorganisms and their activity, composition, and the amount of specific systemic groups present in it.[11] Soil microorganism plays a dynamic role in the remediation of several ranges of pollutants like hydrocarbons[12], phosphate, nitrate compounds[4] and pesticides.[13] Apart from the biotechnological advancement, we are still in search of an effective solution for the above mentioned problems.

There are fewer studies available regarding the microbes which might have dual activity on bioremediation of pollutants and plant growth promotion. Thus in the present study, we have tried to cultivate the PGP bacterial strains from the soil which could serve dually in both crop productivity and environmental remediation. The prime focus is to elucidate the ability of removing environmental pollutants like nitrate and phosphate using the above bacterial strains, which could be a unique mode of action towards environmental maintenance.

Materials and Methods

Sampling Sites

Two different sampling sites were selected for the collection of soil samples. The first one is the dumping ground, Turbhe, Navi Mumbai (19.076284N, 73.027305E) Maharashtra, India (Figure 1A). This site was one of the biggest dumping grounds in Mumbai (132 hectares). The domestic and agricultural wastes from all around the city are disposed to this site. Thus, the microorganisms from the soil are expected to expose varied pollutants and could have the ability to remediate them. The second soil sample was collected from the mangroves, Vashi, Navi Mumbai (19.077064N, 72.998992E) Maharashtra, India (Figure 1B). The mangroves mostly found in tropical and sub-tropical region, and form a transition between terrestrial and marine environments.[14] The mangrove usually provides a distinctive ecological site to different microbes because they are rich in carbon and other nutrients.[15] This site was also selected as it has remained undisturbed by manual intervention. As per our knowledge, the microbial exploration from the above-mentioned sites was not reported elsewhere. Thus these two sites were chosen to cultivate multifunctional microbes.

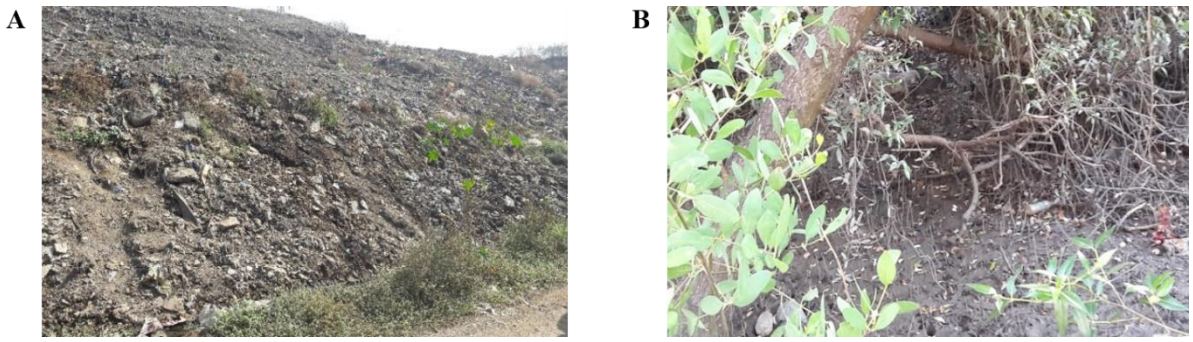

Figure 1. Sample Collection Sites: **A)** Dumping ground, Turbhe, Navi Mumbai (19.076284N, 73.027305E) Maharashtra, India. **B)** Mangroves areas, Vashi, Navi Mumbai (19.077064N, 72.998992E) Maharashtra, India.

The soil samples were collected aseptically in a zip-lock bag, from 10cm depth. The collected samples were then immediately transferred to the laboratory and processed within 24 hours of collection. The temperature of mangrove soil and dumping ground soil at the time of collection was found to be 19 °C and 21 °C respectively and the pH was around 6.8 to 7.2 for the samples. Other physical parameters were kept constant at the time of collection.

Cultivation of Bacteria

The cultivation of the bacteria from soil was performed in a culture-dependent approach. For this purpose, 1 g of soil sample was weighed to which 10 ml of saline (0.85% Nacl pH 6.8-7.2) was added and vortexed (Neo Labs, UK) at maximum speed for 15 minutes. The mixture was allowed to settle for 10 minutes. The upper suspension was taken and a finite volume (0.1 ml) spread on Luria Bertani (LB) agar Media plates (HiMedia M1151, India) after proper dilution. The experiments were performed thrice to get the different bacterial colonies. The plates were put into incubation at room temperature (25 °C - 28 °C) overnight. The different bacterial isolates were visually identified by their morphological characteristics (colour, shape) and they were further cultured on fresh LB agar plates (HiMedia M1151, India).

Screening for PGP Activity

The different bacterial colonies were screened for the PGP activity. For this purpose, four different PGPB traits were

used including Indole Acetic Acid (IAA) production, phosphate solubilisation, Hydrogen Cyanide (HCN) production, and Nitrogen fixation. The bacterial isolates were chosen for further tests which showed positive results towards at least three out of the five above mentioned traits. The details of the test procedures are described below.

Indole Acetic Acid (IAA) Production

The bacterial isolates were evaluated for IAA production by the method given by Bric, Bostock.[16] The bacterial isolates were inoculated in sterilized nutrient broth (HiMedia MM244, India) supplemented with tryptophan (0.005 M) (HiMedia, GRM067, India) and incubated at room temperature for 48 hours under shaker condition at 100 rpm (Orbit Shaker Incubator, Neolabs, UK). The cultures were centrifuged at 10000 rpm (29102 xg) for 10 minutes (Superspin R-V/FM, Plasto Crafts, India). Amounts of 2 ml of Salkowski reagent (50 ml 35% perchloric acid (Merck, 100514, Germany), 1 ml of 0.5 M FeCl3 (SRL, 7705-08-0, India) and 2 drops of orthophosphoric acid (HiMedia, AS010, India) were added to 2 ml of the supernatant and incubated for 20 minutes. The formation of pink to red colour indicates IAA production. The absorbance was measured at 535 nm after 30 minutes of incubation in dark. The concentration of the produced IAA was calculated from the standard graph prepared using different concentrations of IAA (HiMedia, PCT1404, India).

Phosphate Solubilisation

The ability of the bacterial isolates to solubilize the phosphate was evaluated by the method given by Rao.[17] The bacterial isolates were streaked on Pikovskaya's agar plates (HiMedia M520, India) and incubated at room temperature for five days. Clearance around the growth indicates phosphate solubilizing activity of the isolate due to the production of extracellular phosphatase enzyme.

Hydrogen Cyanide (HCN) Production

The bacterial isolates producing HCN were screened using the method given by Rijavec and Lapanje[18] with minor modifications. The bacterial isolates were streaked on Nutrient agar plates (HiMedia M001, India) supplemented with 0.4% glycine (SRL, 56-40-6, India) and were incubated at room temperature for 24 hours. Whatman No. 1 (Whatman, 1001-917) filter paper soaked in 0.5% picric acid solution (HiMedia, S026, India) (in 2% sodium carbonate) was placed on the Petri lid. The plates were then sealed tightly with para-film. A colour change from deep yellow to reddish-brown after incubation indicates the presence of HCN production.

Nitrogen fixation

The ability of the bacterial isolates to fix the nitrogen was evaluated by the method given by Gothwal, Nigam.[19] Nitrogen-fixing bacteria were screened using Nitrogen free Malate media, (DL-Malic acid 5.0 g/L, KOH 4.0 g/L, K_2HPO_4 0.5 g/L, $FeSO_4.7H_2O$ 0.05 g/L, $MnSO_4.H_2O$ 0.01 g/L, $MgSO_4.7H_2O$ 0.1 g/L, NaCl 0.02 g/L, $CaCl_2.2H_2O$ 0.01 g/L, $Na_2MoO_4.2H_2O$ 0.002 g/L, Bromothymol blue 2ml (0.5% alcoholic solution), Agar 1.75 g/L, pH 6.8). In this study, bromothymol blue was used as an indicator. The incubation was taken place for 48 hours at room temperature. Blue colour formation around the growth indicates nitrogen-fixing isolates.

Bioremediation Ability of Bacterial Isolates

As per our aim of the study, we have checked the remediation or removal ability of two environmental pollutants (nitrate & phosphate) by selected PGP bacteria. Nitrate quantification was determined by the method of Cataldo, Aarón.[20] The bacterial cultures were inoculated (1%) in nitrate broth (HiMedia M439S, India) and incubated at room temperature for 24 hours. The cultures were subjected to centrifugation at 10,000 rpm for 10 minutes to separate the cells. 40µl of the supernatant was added to 200 µl to salicylic acid (5% salicylic acid in H_2SO_4) (SRL, 69-72-7, India), vortexed (Neo Labs, UK), and was incubated for 30 minutes in dark. In order to stop the reaction, 2 ml of 4N sodium hydroxide (SRL, 1310-73-2, India). The optical density was measured at 420 nm using a double beam UV-Visible spectrophotometer (Shimadzu UV 1700, Japan). The optical density was then compared to the standard curve, prepared with known concentrations of sodium nitrate (SRL, 7631-99-4, India) to estimate the remaining concentration of nitrate in the medium. The data presented here as removal ability (in %) of nitrate by the bacterial isolates with respect to the control media (without bacteria). The following formula used here:

Removal ability (%) = [(C-T)/C] * 100

Here T = Concentration of nitrate present in media in presence of bacteria

C = Concentration of nitrate present in presence of control media (absence of bacteria).

Phosphate quantification was measured by the method of Krishnaswamy et al.[21] The bacterial isolates were inoculated (1%) in nitrate broth and incubated at room temperature for 24 hours. The cultures were centrifuged at 10,000 rpm (29102 xg) for 10 minutes (Superspin R-V/FM, Plasto Crafts, India). An amount of 1 ml of the supernatant was added to 97 ml of distilled water to which two millilitres of ammonium molybdate (SRL, 12054-85-2, India) was added followed by 80 µl of stannous chloride (HiMedia, India). The blue colour thus formed was quantified after 10 minutes at 660 nm using a double beam UV-VIS spectrophotometer (Shimadzu UV 1700, Japan). The concentration of phosphate remaining in the medium was determined by comparing it with a standard curve prepared using different concentrations of potassium

diphosphate (SRL, India). The data drafted as removal ability (in %) of Phosphate by the bacterial isolates with respect to the control media (without bacteria). The following formula used here:

Removal ability (%) = [(C-T)/C] * 100

Here T = Concentration of phosphate in the media in presence of bacteria

C = Concentration of phosphate in the control media (absence of bacteria)

Biofilm Formation Ability

Biofilm assay was performed according to the modified Crystal violet method of Ghosh et al.[22] For this purpose, 1% of overnight grown bacterial culture in LB medium was inoculated in sterile 1ml LB medium in a 24 well-coated microtitre plate (Tarsons, 980030, Korea) and incubated at room temperature for 24 hours. The next day, 250 µl of 1% Crystal Violet (HiMedia SO12, India) was given in the medium and was let to stand for 10 minutes to stain the grown biofilm. The medium was then carefully drained and washed with distilled water and allowed to dry for 10 minutes. After on, 1 ml of 95% ethanol (Merck, 107017, Germany) was added to extract the colour from the biofilm and the absorbance was measured at 620 nm after 10 minutes, using a double beam UV-VIS spectrophotometer (Shimadzu UV 1700, Japan). The LB medium without bacteria was used as a blank.

Identification of the Bacterial Isolates

The selected isolates were further characterized morphologically by their shape, size, colour, margin, elevation, secretion, and texture according to Bergey's Manual of Taxonomy 7th eds.[23] The Gram staining of the bacterial isolates was performed accordingly.

The production of the extracellular secreted enzymes like protease[24], lipase[25], amylase[26], cellulase[1] were checked as per the above-stated protocols. Production of catalase enzyme was checked by pouring 10% hydrogen peroxide solution to each bacterial colony.

The molecular identification of the above selected bacterial isolates was performed at NCMR, India (www.nccs.res.in) with a standard procedure. Phenol–chloroform method[27] was used to isolate genomic DNA and the purity of the same was checked by Nanodrop spectrophotometer. The 16S rRNA gene was amplified using universal primer set 16F27 [5'-CCAGAGTTTGATCMTGGCTCAG-3'] and 16R1492 [5'-TACGGYTACCTTGTTACGACTT-3']. Polyethylene glycol (PEG)-NaCl was used to purify the PCR amplified products. The purified product was then directly sequenced with the help of the ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit on a 3730xl Genetic Analyzer (Applied BioSystems). Sequencing was carried out from both ends using additional internal primers so that each position was read for at least twice. Lasergene SeqMan Pro (DNASTAR Inc.) was used to assemble the data followed by identification using the EzBioCloud database.[28] These sequences were further subjected to Multiple Sequence Alignment (MSA) using MUSCLE and the output data of MSA were used to construct a phylogenetic tree.[29] The phylogenetic tree was constructed using the Neighbour-Joining method with bootstrap using 1000 replicates.[30] The MSA and construction of the phylogenetic tree were done using MEGA 7.0.[29] The sequences were submitted to GenBank (https://www.ncbi.nlm.nih.gov/WebSub/) to obtain GenBank accession numbers.

Statistical Analysis

All the quantitative tests have been replicated at least five times and the results were documented as mean ± Standard Error (SE). The significant differences in the mean values of nitrate and phosphate removal from the control samples were validated through two samples one-tailed t-test at a 95% confidence interval. All the statistical procedures and graphical representations were performed using Microsoft Excel + Analyse It® (USA).

Results and Discussion

Cultivation of the Bacteria

There was a varied amount of cultivable organisms after cultivation on complex media. We found more numbers of bacterial counts in terms of Ln (Colony Forming Unit/gm) from waste dumping ground soil compared to mangrove soil (Table 1).

Table 1. The total Count of Cultivable Bacteria from Waste Dumping Ground Soil & Mangrove Soil

Source	(Cfu/gm of soil)*
Dumping ground soil	43.79 ± 3.03
Mangrove soil	32.85 ± 2.09

*the total number of bacterial count is expressed as logarithm with base 10

As a significant number of organic and inorganic pollutants were used to pile up on the dump yard, the above observations could be justified. Nutrient availability was one of the prime factors for bacterial colonization. On the other hand, mangrove soil was expected to be less exposed to pollutants, thus the nutrient variety as well as mobility was also expected to be less frequent. Actually, several inorganic and organic pollutants in the water could be contributing factors for microbial variety. The role of uncultivable bacteria in nutrient recycling was also evident in an earlier report.[31]

In the present study, we primarily obtained 18 different bacterial colonies. The obtained colonies were maintained as individual bacterial cultures on Nutrient agar slants at 4 °C. Each bacterial isolates (OD = 0.45 @ 600 nm) were further mixed with sterile 70% Glycerol (HiMedia GRM1027, India)

in a 1:1 ratio and stored at -80 °C for long term preservation.

Screening for Plant Growth Promoting Bacteria (PGPB)

The screening for PGB traits among the 18 bacterial isolates was performed qualitatively. The production ability of any three of five tested traits by the above bacteria was set as the selection criteria for screening as Plant Growth-Promoting Bacteria (PGPB). The summarized results are presented in Table 2. All the results were repeated three times to ensure the positive and negative responses of the bacteria in case of their different PGP traits. In the present study, 50% of the total bacterial isolates tested were found to produce HCN, where only 20% of the bacteria could produce phosphatase. About 75% of the total bacterial isolates could fix the nitrogen and be able to produce IAA. The quantitative estimation of ammonia production using the above eight isolates was also performed to check their ability to produce ammonia which could further be helpful for plant growth. It was observed that the bacterial isolates could produce 15 to 32 ppm of ammonia in the present experimental condition (Table 2).

Table 2. Screening of Bacterial Isolates for Plant Growth Promoting (PGP) Traits

Bacterial Isolates Designation	Plant Growth promoting (PGP) traits				
	IAA Production	Phosphatase Production	HCN Production	Nitrogen Fixation	Ammonia Production
Dumping ground Soil					
SG1	+				
SG2	+	+	+	+	
SG3	+				
SG4	+				
SG5	+	+		+	+
SG6			+	+	
SG7		+	+	+	+
SG8					
Mangrove Soil					
SM1	+		+	+	+
SM2					+
SM3	+				+
SM4	+	+		+	+
SM5	+	+			
SM6	+				+
SM7	+				+
SM8	+				+
SM9	+		+	+	+
SM10			+	+	+

+ indicates the positive production whereas – indicates for negative production

Indole acetic acid is one of the prime important hormones responsible for plant growth regulation. It was observed that mostly rhizospheric organisms usually produce the IAA for interaction with the root cells of the plants.[32] Heterotrophic bacterial community has been reported for the production of IAA. The consequence of IAA production was not only helpful for plant growth but the reduction of phytopathogen invasion was also observed in many cases.[33,34] The production of HCN was thought to be helpful for the plant as it was earlier hypothesized that HCN might inhibit phytopathogenic fungal communities.[35] Another view in 2016, disclosed that HCN helps to sequester Fe^{3+} ions and thus release the bound PO_4^{2-}ions which was previously bound with Fe^{3+} as salt form. Thus released PO_4^{2-} ions would readily be used by plant roots.[18] Phosphatase is another extracellular enzyme produced by certain kinds of bacteria which help to release free PO_4^{2-} ions and make available to the plants.[36] Ammonia is usually produced by bacteria in facultative anaerobic conditions, with the help of nitrate or any other nitrogen-containing products. There are two views on the role of ammonia on plant growth. It is mostly believed that ammonia help plants in protein synthesis pathways[37] but in recent reports, it was observed that due to the presence of ammonia, the pH of the available water becomes high which subsequently affects the growth of certain tested plants.[38] Overall, the above-mentioned PGP traits harboring bacteria was historically considered as a safe and sustainable approach towards plant health as well as crop production.

According to the criteria for selecting PGPB, we have selected a total of eight bacterial isolates (SG2, SG5, SG7, SM4, SM6, SM8, SM9 and SM10) among 18 bacterial isolates for further tests.

Bioremediation Ability of PGPB Isolates

The eight previously selected PGP bacterial isolates were further tested for their bioremediation ability of two environmental pollutants, nitrate and phosphate. The results exhibited effective remediation efficiency by the eight selected bacterial isolates (Figure 2). The primary concentration of nitrate and phosphate was set at 100 ppm and 50 ppm respectively and the removal of the pollutant's concentration by the bacteria was checked within 18 hours at room temperature (25 °C-30 °C). SM6 is the highest nitrate reducer (80% in just 18 hours) whereas SM8 reduces 62% (p<0.05, n = 10) of nitrate in 18 hours. The average nitrate removal efficiency

was found at 71%. In addition, 100% of phosphate removal efficiency was observed highest (100%) by SM3 and the lowest (14%) (p<0.05, n = 10) by SM1. The average removal efficiency was found to be 57%. The standard culture or phosphate removal *Acenetobactor jejuni* could remove phosphate up to 35% within 18 hours.[4]

Nitrate ions are highly soluble in water and are usually found in a lesser amount in water bodies. Runoff from the industrial area and agricultural field was primarily identified as the main source of nitrate contamination in water. However, the chance of nitrate contamination in drinking water sources was also remained by the above-stated sources. Prokaryotes generally utilize nitrate from environmental sources for three different purposes. First, to assimilate nitrogen for protein synthesis; next is respiration, to get energy for metabolic activity and dissimilation. The third is to release nitrogen gas for redox balancing.[39] Three bacterial enzymes, including the cytoplasmic assimilatory (Nas), membrane-bound respiratory (Nar), and periplasmic dissimilatory (Nap) nitrate reductase have a distinct role in nitrate reduction.[40] On the other hand, phosphorous is a macronutrient required by the plants in high quantities as it is involved in the transfer of energy as well as biosynthesis of nucleic acids. The organic phosphate is converted to an inorganic form with the help of an enzyme phosphatase, produced by the microorganism thus making it available for the plants. The bacteria usually accumulate phosphate in their vacuoles whereas in the aerobic condition they can release it into the nature. The presence of genes like *polyP and (p)ppGpp* has been proved to be responsible for phosphate accumulation in bacteria.[41] However, there has been a negative correlation between nitrate and phosphate removal by the bacterial species found in earlier studies.[4]

Bacterial isolates in the present study were primarily screened for their PGP traits. They have also exhibited the ability to remove environmental pollutants like nitrate and phosphate with an average of 50% and above which makes them important contenders to use as a biofertilizer. The dual activity of these isolates could serve the plant growth and productivity and could also decontaminate nature from environmental pollutants.

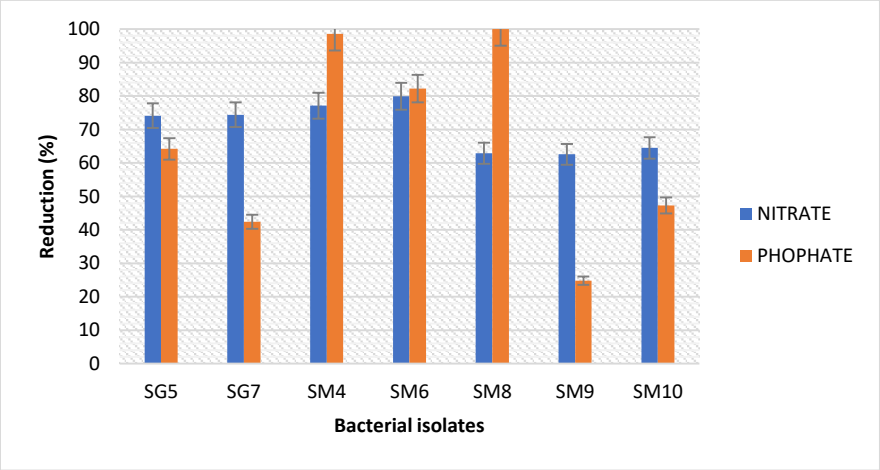

Figure 2. Represents the Nitrate Reduction and Phosphate Reduction of the Selected Bacterial. The data was given as mean ± SD (n = 5).

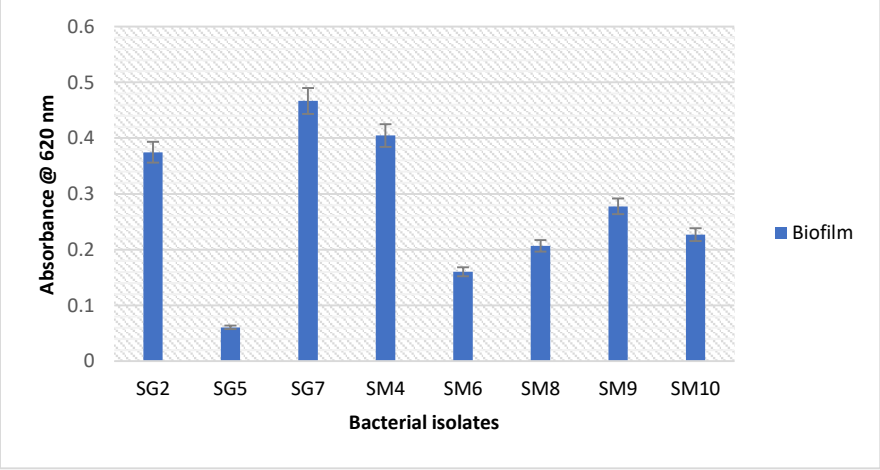

Figure 3. Represents the Absorbance of Biofilm Formed by the Bacterial Isolates from Soil. The test was performed at least thrice and the data was given as mean ± SD (n = 10).

Table 3. Morphological & Biochemical Identification of Bacterial Isolates

Isolates	Morphological Characteristics						Extracellular Enzyme Production				
	Shape	Elevation	Colour	Secretion	Margin	Gram Nature	Shape	Protease	Lipase	Amylase	Celullase	Catalase
SG2	Circular	Convex	Pale	Yes	Serrated	Negative	Bacilli	+	+	+	+	+
SG5	Circular	Convex	Pale yellow	No	Serrated	Negative	Rods	-	-	+	-	+
SG7	Irregular	Flat	White	Yes	Irregular	Positive	Rods in chains	+	+	+	-	+
SM4	Circular	Convex	Fluorescent yellow	No	Regular	Positive	Rods in singles	-	+	+	-	+
SM6	Circular	Flat	Pale	No	Regular	Negative	Rods	-	-	+	+	-
SM8	Circular	Flat	White	No	Regular	Negative	Cocci	+	+	+	-	-
SM9	Circular	Convex	Greyish	No	Regular	Negative	Cocci	-	+	+	-	+
SM10	Circular	Convex	Pale	No	Regular	Negative	Cocci	-	+	+	+	+

+ indicates the positive production whereas – indicates for negative production

Biofilm Formation Ability of the PGPB Isolates

Biofilm is defined as assemblages of related bacteria generally termed as aggregates, which are associated with similar or various kinds of micro-colonies.[42] Biofilm is formed by the bacteria as a survival mechanism to evade external stress. The property of biofilm formation would ensure a more stable association of the applied PGPB with plant roots.[22] Since biofilms are more resistant than planktonic cells, they would also aid in the defense of the plants against pathogens and make nutrition more readily available. The strong biofilm former is SG7 with SM4 and SG2 are moderate biofilm formers (Figure 3). The classification of strong, moderate, and weak biofilm formers is suggested by Ghosh et al.[22] Recent reports suggested that the biofilm formation of PGPB in the root region aid the plant system by trapping the mineral salts and make it available to the vicinity of the root system.[43] Also, the biofilm formation both on the root and leaf region protects the plant's system from pathogen invasion.[44] The extracellular polymeric substances associated with biofilm formation contain several essential micronutrients which also have a pronounced role in plant growth.[45] The recent report of Mallick et al.[46] reported the biofilm formation of rhizosphere bacteria in arsenic bioremediation. The adsorption of arsenic in the biofilm of the bacteria was noted as a significant amount in the above case. A similar finding has also been observed in the case of remediation of heavy metals like lead, zinc, cadmium, etc. reviewed by Gupta and Diwan.[47] Thus, the virtue of strong biofilm formation of the present studied bacterial isolates would aid in both bioremediation of environmental pollutants (nitrate and phosphate) and as a biofertilizer for plant growth.

Identification of the Bacteria

The eight PGP bacteria were identified by their morphological, biochemical, and molecular characteristics. The morphological characteristics varied among the eight isolates. Four out of eight of the bacteria were gram-positive and the rest were gram-negative. All the bacteria were bacilli in regards to their shape except for two of them which were found cocci. All the bacteria were found to produce different extracellular enzymes. Extracellular amylase enzyme was predominantly produced by all the tested bacterial isolates followed by lipase, cellulase, and protease. Oxidation-protecting enzymes like catalase were produced by all the bacterial isolates (Table 3). The 16S rDNA based molecular identification of the isolates along with the nearest neighbor are presented in Table 3. As per the NCBI-BLAST database, most of the bacterial strains belong to the alpha-proteobacterial group. The nearest neighbor of the bacterial strains is found by phylogenetic analysis using the neighbor-joining method (Supplement data 1). The accession number of GenBank-NCBI of the novel bacterial strains have been presented in Table 4.

Table 4. Molecular Identification of Bacterial Isolates

Bacteria Isolate Name	GenBank Accession Numbers Obtained from NCBI	Closest organism according to NCBI-BLAST	% of identity
SG2	MH100804.1	*Stenotrophomonas rhizophila*	99.5
SG5	MH107103.1	*Alcaligenes faecalis*	99.7
SG7	MH107112.1	*Bacillus subtilis*	99.8
SM4	MH108116.1	*Corynebacterium glutamicum*	99.2
SM6	MH107132.1	*Alcaligenes faecalis*	99.7
SM8	MH645795.1	*Stenotrophomonas rhizophila*	99.2
SM9	MH107117.1	*Pseudomonas guariconensis*	99.7
SM10	MH107105.1	*Citrobacter murliniae*	98.4

Among the identified bacterial groups, recent reviews on *Bacillus subtilis* and *Pseudomonas* sp. were identified to have different PGP activity in earlier reports.[48-50] The role of novel strains of *Bacillus* sp. in nitrate and phosphate remediation was elaborated by Debroy et al.[4] Any strains of *Strenotrophomonas* sp. and *Citrobactror* sp. not yet reported in remedial activity of pollutants, according to our knowledge. Apart from our current report, Wolf et al have observed

Strenotrophomonas rizophila as noble non-pathogenic strains.[51] The report showed that *Strenotrophomonas rizophila* exhibited antifungal activity which imparted immunity to the plant roots from the pathogenic fungus. The PGP traits harbored by several strains of *Strenotrophomonas rizophila* also support our observations.[52] Thus the present report could be considered as the first of its kind to explore the plant growth promotion as well as the bioremediation ability of the *Strenotrophomonas rizophila.* The presence of *Corynebacterium glutamicum* strains could be an added advantage for the current study. This is due to the fact that currently the present species of bacteria is industrially used as an L-glutamate producer. The whole-genome sequencing of the present bacterial strain revealed its non-pathogenicity with the presence of several PGP genes.[53] However, its remediation ability of pollutants is yet to be verified. Rhizosphere associated *Alcaligenes faecalis* was found to suppress plant pathogen and also has ACC (1-aminocyclopropane-1-carboxylic acid) de-aminase production ability.[49] The present study, therefore, claims its novelty based on elucidating the dual nature of the novel bacterial strains. This could be a cutting edge advance in pollution management as well as in the field of agricultural.

Conclusion

In the present study, we could screen eight novel bacterial strains based on their PGP ability. Their role in the remediation of environmental pollutants like nitrate and phosphate have also been revealed. Thus, the dual characteristics of the bacterial strains were eventually established. The biofilm formation ability of the present bacterial strains could also support their application as a bio-fertilizer. Therefore, in conclusion, the prospect of the present study is to formulate a microbe-based bio-fertilizer for plant health enhancement in an eco-friendly manner.

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Authors' Contributions

SK has performed all the experimental results. JS has compiled and format the manuscript. SG has written the manuscript, designed and supervised the full study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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