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RESEARCH ARTICLES

Stenotrophomonas: a versatile diazotrophic bacteria from the rhizospheric soils of Western Himalayas and development of its liquid biofertilizer formulation

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Abstract
Rhizosphere is a rich repository of plant growth promoting rhizobacteria (PGPR) which is a sustainable tool to increase crop productivity and maintain soil health. In this context, 43 isolates were obtained on Jensen's medium from the rhizosphere of *Triticum aestivum*, *Zea mays*, *Solanum tuberosum*, *Aloe barbadensis* and *Bacopa monnieri* grown in Palampur, (Himachal Pradesh) India. Out of these isolates, only six isolates (WT-A2, WT-A1, MZ-A2, PT-A1, PT-A3 and BM-A3) exhibited significantly higher nitrogenase activity (451.45, 441.58, 440.91, 444.02, 383.64 and 374.44 nmole C_2H_4 h^{-1} mg^{-1} protein) as compared to the reference strain of *Azotobacter chroococum* MTCC 446 (372.85 nmole C_2H_4 h^{-1} mg^{-1} protein). The isolate WT-A2 was the most efficient with respect to nitrogenase activity (451.45 nmole C_2H_4 h^{-1} mg^{-1} protein), indole acetic acid production (17.45 µg ml^{-1}), ammonia production and siderophore production. Isolate WT-A2 was identified as *Stenotrophomonas rhizophila* on the basis of morphological, biochemical and 16S rRNA sequence analysis. In order to prepare liquid bioinoculant formulation, survivability studies on *S. rhizophila* was carried out in four different liquid carriers (Compost Tea, Biogas slurry, Vermiwash and Minimal Growth Medium) at room temperature (average maximum temp. was 23.83 °C and average minimum temp. was 11.91 °C). The results showed that *S. rhizophila* survived better in different liquid carriers (9.873 log cfu ml^{-1} in biogas slurry; 9.843 log cfu ml^{-1} in vermiwash; 9.163 log cfu ml^{-1} in minimal growth medium), and Compost Tea was the best carrier to support higher bacterial load (9.907 log cfu ml^{-1}) on 180th day of storage. The results are of practical importance as this (compost tea) liquid carrier could be used to produce liquid biofertilizer formulation. Also, *S. rhizophila* could be a potential biofertilizer candidate as it posses multifarious plant growth promoting traits.

Keywords Liquid carriers · Nitrogenase · PGPR, Rhizosphere · 16S rRNA gene · *Stenotrophomonas rhizophila*

Introduction

The rhizosphere is a hub of biological and various other activities (chemical, and physical) around the plant roots. It is generally considered to be a narrow zone of soil where root exudates stimulate microbial populations and their activities (Igiehon and Babalola 2018; Tkacz and Poole 2015), and thus result in enormous microbial diversity (Imam et al. 2016; Tkacz and Poole 2015). The bacteria in rhizospheric zone promote plant growth either directly or indirectly and are referred to as plant growth promoting rhizobacteria (Gouda et al. 2018; Verma 2019).

Nitrogen is one of the most common nutrients required for plant growth and productivity. Since, nitrogenous chemical fertilizers have deleterious effect on soil health therefore biological nitrogen fixation is an excellent alternative replenish nitrogen in soil and also maintain soil health (Igiehon and Babalola 2018). Many genera like *Azotobacter, Azospirillum, Bacillus, Pseudomonas, Burkholderia, Rhizobium, Herbaspirillum* etc., have been shown to be associated with roots of many plants (Dubey and Fulekar 2013; Gouda et al. 2018; Karagoz et al. 2012;

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Kumar et al. 2014; Vejan et al. 2018) and now a days the rhizospheric microbial interactions are manipulated to achieve strong positive interaction between both (Imam et al. 2017; Kumar et al. 2016a, b). The plant growth promoting rhizobacteria (PGPR) facilitate plant growth directly by increasing the accessibility and uptake of nutrients (N, P, Indole acetic acid etc.) and indirectly by producing antagonistic metabolites (like HCN and various enzymes which lyse the pathogens), by competitive exclusion and by induction of systemic resistance (Basu et al. 2018; Sui et al. 2019; Tyagi et al. 2018). The literature showed that many rhizobacteria may have one or more than one of these beneficial attributes and therefore, the potential PGPR strains can be utilized for the development of bioinoculants (Gouda et al. 2018; Igiehon and Babalola 2018; Vejan et al. 2018).

Among various plant growth promoting rhizobacteria, *Stenotrophomonas* is one of the versatile PGPR candidate which have a multifarious traits (Denet et al. 2018; Ryan et al. 2009). *Stenotrophomonas* spp. prevalent in various ecological niches and species like *S. rhizophila* and *S. maltophilia* usually reported to isolated from endosphere (plant internal tissue) and rhizosphere soil which showed various plant growth promoting activities (Karagoz et al. 2012; Ryan et al. 2009). The genus *Stenotrophomonas* also plays a significant role in the biogeochemical cycles like sulphur and nitrogen (Banerjee and Yesmin 2002; Park et al. 2005; Ramos et al. 2011; Ryan et al. 2009). In contrast to the phylogenetically closely related pathogenic genera *Xanthomonas* and *Xylella*, no *Stenotrophomonas* species is known to be phytopathogenic (Ryan et al. 2009).

Bioinoculant formulation is an important aspect for the successful delivery and survival of PGPR isolate in the inoculated soil (Malusa et al. 2012). The formulation (carrier) provides a better microenvironment which prevents the rapid decline of the bacteria when inoculated in the soil (Mahanty et al. 2017). Generally, solid based carrier is used to produce bioinoculant formulation but this solid based formulation has many disadvantages like short shelf-life, high contamination rate etc., (Mahanty et al. 2017; Malusa et al. 2012). So, to overcome this problem liquid based formulations are introduced. Several liquid formulations available today sustain high viable microbial counts for extended periods of time (Hegde 2008; Kaur et al. 2018). Still, the selection of an economically viable and easily available carrier, capable of maintaining high viable count is an important area of research.

Keeping all this in view, the present study was designed to isolate and identify diazotrophic native bacteria from the rhizospheric soils of Western Himalayas and characterize them for plant growth promoting traits along with their survivable studies in liquid carriers to develop a formulation with native isolate.

Materials and methods

Collection of soil samples and isolation of diazotrophic bacteria

Rhizosphere soil samples of *Triticum aestivum* (wheat), *Zea mays* (Maize) and *Solanum tuberosum* (Potato), *Aloe barbadensis* (Aloevera) and *Bacopa monnieri* (Brahmi) grown in Palampur, India were collected carefully by uprooting the root system and ten grams of rhizospheric soil was collected and processed by making serial dilutions for isolation of diazotrophic bacteria. The diazotrophic microorganisms were isolated using Jensen's medium (HiMedia, India) throughout the study.

The reference strain of *Azotobacter chroococum* (MTCC 446) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Plant growth promoting tests

Nitrogen fixation was determined in nitrogen free medium (Jensen's medium) by the acetylene reduction assay (Hardy et al. 1968). Ethylene production was measured using a Hewlett Packard gas chromatograph (Model HP Series 5890, USA) fitted with flame ionization detector and a Porapak-N column. After completion of the ARA, the protein concentration in the cells was determined by the method of Lowry et al. (1951). The isolates showed more than 150 nmole C_2H_4 h^{-1} mg^{-1} protein activity were stocked for further study.

Indole-3-Acetic acid (IAA) was estimated by the method of Gordon and Weber (1951) in which Luria-Bartani medium (HiMedia, India) was supplemented with 5 mM L-tryptophan (Sigma, USA) and inoculated with bacterial cultures. The inoculated medium was incubated at 30 °C ± 1 °C in an orbital shaker at 100 rpm and cell free supernatant was used to estimate the IAA.

Siderophore was detected by the method of Schwyn and Neilands (1987) using agar plates containing the dye chrome azurol sulfate (Merck, India). Orange halos around the colonies were indicative of siderophore production.

To detect ammonia production the test isolate was grown in peptone water at 30 °C ± 1 °C for 4 days and 1 ml of Nessler's reagent was added. Production of ammonia was depicted by development of faint yellow to dark brown color (Bakker and Schippers 1987).

Phenotypic and genotypic characterization of bacterial isolates

Morphological and biochemical characteristics of the efficient bacterial isolates were studied by the methods described in Bergey's Manual of Systematic Bacteriology (Holt et al. 1994).

For genomic characterization 16S ribosomal gene was amplified by using the universal primers i.e. 27F (5′ AGAGTTTGATCATGGCTCAG 3′) and 1487R (5′ TACCTTGTTACGACTTCACC 3′) (Heddi et al. 1998). The sequence was aligned by using Clustal W software (Thompson et al. 1994) and phylogenetic analysis was performed with neighbor-joining method using program in Molecular Evolutionary Genetics Analysis (MEGA) version 7 (Kumar et al. 2016a, b).

Survivability studies in liquid carriers

Various liquid carriers i.e., Compost Tea, Biogas slurry, Vermiwash and Minimal Growth Medium (Peptone water) were used for the development of liquid biofertilizer with native strain WT-A2 and reference strain of *Azotobacter chroococum* (MTCC 446). The sterilized liquid substrates amended with glycerol (10 mM) were suspended with 1% mid-log phase inoculum and incubated at room temperature (average maximum temp. was 23.83 °C and average minimum temp. was 11.91 °C). The population of suspended bacteria in liquid carriers was enumerated at monthly intervals by serial dilution technique on Jensen's medium up to 6 months (180 days).

Statistical analysis

Results of the measurements were subjected to analysis of variance (ANOVA) and significance at the 1% level was tested by Least Significant Difference (LSD) using Windowstat package, Version 8.0. All treatments were in triplicate.

Gene submission and culture deposition

The nucleotide sequence of efficient PGPR isolate WT-A2 was deposited in Gen Bank under accession number GU371215. This isolate was deposited with the National Bureau of Agriculturally Important Microorganisms (Indian Council of Agricultural Research) Kusmaur, Mau Nath Bhanjan, Uttar Pradesh, India under the NBAIM accession number NAIMCC-B-00877.

Results

Isolation and screening of diazotrophic bacteria

In the present study, a total of 43 isolates were obtained on Jensen's medium from the rhizosphere of different rhizospheric soil samples. Out of 43 strains, only 18 isolates showed more than 150 nmole C_2H_4 h^{-1} mg^{-1} protein nitrogenase activity. Out of 18 isolates six strains i.e. WT-A1 (411.58 nmole C_2H_4 h^{-1} mg^{-1} protein), WT-A2 (451.45 nmole C_2H_4 h^{-1} mg^{-1} protein), MZ-A2 (440.91 nmole C_2H_4 h^{-1} mg^{-1} protein), PT-A1 (444.02 nmole C_2H_4 h^{-1} mg^{-1} protein), PT-A3 (383.64 nmole C_2H_4 h^{-1} mg^{-1} protein) and BM-A3 (374.44 nmole C_2H_4 h^{-1} mg^{-1} protein) showed significantly higher nitrogenase activity as compared to the reference strain of *A. chroococum* (372.85 nmole C_2H_4 h^{-1} mg^{-1} protein strain).

PGP characteristics of the diazotrophic bacteria

Out of 18 isolates, only 13, nine and eleven isolates were found to produce IAA, siderophore and ammonia, respectively (Table 1). WT-A2, the most efficient isolate with respect to nitrogenase activity, showed 17.45 µg ml^{-1} of IAA production which was significantly higher than the reference strain of *A. chroococcum* (15.51 µg ml^{-1}).

The isolate WT-A2 was also found positive for siderophore and ammonia production. PT-A1s most efficient isolate with respect to nitrogenase activity showed small amount of ammonia and siderophore production whereas showed no IAA production. The isolate MZ-A2 showed 10.96 µg ml^{-1} of IAA production, small amount of ammonia production and also showed siderophore production. The isolate WT-A1 showed ammonia production, produced 8.65 µg ml^{-1} of IAA but found negative for siderophore production. The isolate PT-A3 showed 15.14 µg ml^{-1} of IAA production, small amount of siderophore production whereas found negative for ammonia production. The isolate BM-A3 showed no production of IAA and siderophore whereas showed small amount of ammonia production.

So, based on these plant growth promoting characteristics the isolate WT-A2 which was isolated from wheat rhizosphere was found to be most efficient isolate. This isolate was further characterized and studied for liquid bioinoculant formulation development.

Biochemical and molecular characterization of efficient isolates

On morphological and biochemical characterization of WT-A2, it was found to be gram negative rods, motile, positive for catalase, oxidase, gelatin hydrolysis and negative

Table 1 Nitrogenase and other plant growth promoting activities showed by screened diazotrophs

S. no.	Isolate	Nitrogenase activity*	IAA production (μg ml^{-1})	Ammonia production	Siderophore production
1	WT-A1	441.58[a]	8.65[a]	+	–
2	WT-A2	451.45[b]	17.45[b]	+	++
3	WT-A3	225.48[c]	10.66[c]	+	+
4	WT-A4	256.29[d]	–	–	–
5	MZ-A1	287.52[e]	18.14[d]	–	+
6	MZ-A2	440.91[a]	10.96[e]	+	+++
7	MZ-A3	194.37[f]	–	–	–
8	PT-A1	444.02[g]	–	+	+
9	PT-A2	183.23[h]	12.51[f]	–	–
10	PT-A3	383.64[i]	15.14[g]	–	+
11	PT-A4	155.40[j]	16.06[h]	+	–
12	PT-A5	237.63[k]	12.05[f]	+	–
13	AV-A1	241.28[l]	20.35[i]	–	–
14	AV-A2	168.49[m]	12.82[j]	+	+
15	AV-A3	291.60[n]	16.37[h]	+	++
16	BM-A1	151.51[o]	–	+	–
17	BM-A2	207.41[p]	11.89[f]	–	++
18	BM-A3	374.44[q]	–	+	–
19	*A. chroococcum*	372.85[r]	15.51[g]	+	+

Each value represents mean of three replicates. In the same column, significant differences according to LSD at $P \leq 0.01$ levels are indicated by different letters. Same letters represent that their values are at par

*nmole C_2H_4 released h^{-1} mg^{-1} protein

for indole, methyl-red, urease and H_2S production. Strain WT-A2 utilized sugars like fructose, glucose, inositol, maltose, melibiose and sorbitol, while sugars like arabinose, galactose, lactose, galactose, rhamnose and xylose were not metabolized by this strain. The phenotypic characterization of the efficient isolate WT-A2 indicated broad similarity to the genus *Azotobacter*.

In the present study, indigenous diazotrophic isolate WT-A2 was identified as *S. rhizophila* on the basis of 16S rRNA gene sequencing. The nucleotide sequence analysis of isolate WT-A2 revealed maximum homology (99%) with *Stenotrophomonas rhizophila* (MH144280) which belongs to γ-*proteobacteria* (Fig. 1).

Survivable of bacterial isolates in liquid substrates

The results clearly showed that compost tea was superior then the other liquid carriers tested in supporting higher

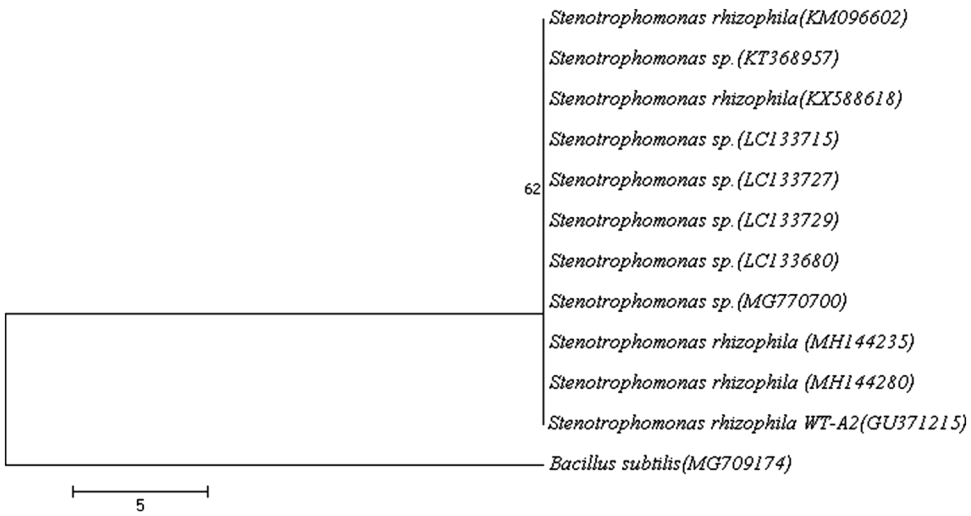

Fig. 1 Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 16S rRNA gene sequence of native isolate and related sequences obtained from NCBI

Table 2 Survivable (log cfu ml^{-1}) of *Stenotrophomonas rhizophila* in different liquid substrates at different days of storage

Substrates	Days of incubation						Mean
	30	60	90	120	150	180	
1. Compost tea	10.993	10.870	10.753	10.287	9.960	9.907	10.461
2. Biogas slurry	10.910	10.853	10.723	10.243	9.900	9.873	10.417
3. Vermiwash	10.903	10.833	10.680	10.213	9.873	9.843	10.390
4. Minimal growth medium	10.850	10.813	10.623	9.973	9.710	9.163	10.188
Mean	10.914	10.842	10.694	10.179	9.860	9.696	

CD at 5%: substrates (0.07)

Population of *S. rhizophila* at 0 day: 7. 491 log cfu ml^{-1}

microbial load (Table 2). It maintained 9.907 log cfu ml^{-1} of *S. rhizophila* (WT-A2) on 180th day of storage which was significantly higher than the other tested liquid carriers. After compost tea, biogas slurry was found to be the second efficient substrate to sustain the bacterial load of 9.873 log cfu ml^{-1} on 180th day of storage. Whereas, vermiwash was the third best carrier to support the bacterial growth of 9.843 log cfu ml^{-1} after 6 months of storage (Table 2). The minimal growth medium showed the least survival of inoculated bacterial culture on 180th days of storage.

Discussion

Soil biota is complex and heterogeneous in nature, which serves as a major reservoir of soil enzymes and nutrients for plant growth. Previous isolations of nitrogen fixing bacteria have revealed a broad diversity in the crop rhizosphere (Igiehon and Babalola 2018; Venieraki et al. 2011; Verma et al. 2019) and the present study surveyed the rhizosphere soil of different crop plants cultivated in Himachal Pradesh, India for the presence of nitrogen fixing bacterial isolates. Though the ability to reduce acetylene is an indirect measure of N_2-fixation, it is specific for monitoring functional nitrogenase activity, and is indicative of N_2-fixing potential (Andrade et al. 1997; Mehnaz et al. 2007). Hence, for screening and selection of prospective strains, ARA was used as a test for diazotrophy.

Phytohormones play an important role as regulators of growth and development of plants. There are different pathways for IAA biosynthesis like tryptophan-dependent and independent pathways in microorganisms. It has been reported by various workers that the precursor L-tryptophan is necessary for IAA production by microorganisms (Ahemad and Kibret 2014; Estenson et al. 2018; Park et al. 2005; Spaepen et al. 2007; Tsavkelova et al. 2007). In the present study, culture medium with tryptophan induced the microorganisms to produce IAA. In natural conditions, L-tryptophan may be available in root exudates as noticed by Benizri et al. (1998), which might be inducing these microorganisms to produce IAA in the rhizosphere.

Siderophores are low molecular weight iron chelating ligands synthesized by microorganisms (Gouda et al. 2018; Winkelmann 1991). Most bacteria and fungi produce siderophores that differ according to their functional groups. In the present study, N_2 fixers were found to produce siderophores. Microbial siderophore may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens by scavenging iron and making it less available to the pathogens (Ahemad and Kibret 2014; Khan et al. 2018; Marek-Kozaczuk et al. 1996).

Ammonia is considered as one of the plant growth promoting metabolites produced by various microbes inhabiting rhizosphere, and in the present study, the most of the native isolates produced ammonia. There are number of sources of ammonia secreted by rhizospheric microorganisms like amino acid degradation, nitrogen fixation, hydrolysis of 1-aminocyclopropane-1-carboxylic acid etc. (Asano and Lubbehusen 2000; Parnell et al. 2016; Saribay 2003). Some authors consider the production of ammonia as antagonistic in controlling the disease (Saraf et al. 2008).

An attempt was made to identify and decipher the phylogenetic affiliation of efficient bacterial isolate using 16S rRNA gene sequencing. In the present study, native diazotrophic isolate WT-A2 was identified as *S. rhizophila* on the basis of 16S rRNA gene sequencing. Previously this genus is reported to be nitrogen fixer and has been isolated from the rhizosphere of different crops/plants (Gulati et al. 2011; Martinez-Hidalgo et al. 2019; Park et al. 2005; Reinhardt et al. 2008; Singh and Jha 2017).

Carrier is an important component of biofertilizer technology and is defined as the vehicle carrying efficient microbial strains from the laboratory to the field with minimum damage to the viable cell population (Bashan 1998; Mahanty et al. 2017; Parnell et al. 2016). In the present study, compost tea supported higher survivability as compared to the other tested liquid carriers which might be attributed to its high nutritional status. Compost Tea provides soluble nutrients, humic substances, and bioactive substances that promote plant growth (Diver 2003). The minimal growth medium was least effective in supporting higher microbial load may

be due to presence of low amount of nutrients in comparison to other tested liquid carriers on longer storage. Further, the amendment of different liquid carriers with glycerol also improves the survivability of bacteria during storage period. Glycerol has a high water binding capacity and may protect cells from the effect of desiccation by slowing the rate of drying (Lorda and Balatti 1996). It often serves the function of an osmolyte, balancing external osmotic pressure (Brown 1978; Blomberg and Adler 1992; Sunder et al. 1996).

In the present study, an indigenous 43 diazotrophic strains were isolated from rhizospheric soils. The isolate WT-A2 showed better plant growth promoting traits as compared to the reference strain *A. chroococcum*. Therefore, WT-A2 could be a potential strain to be used as a bioinoculant under local agro-climatic condition to increase crop production. The molecular identification confirmed WT-A2 as *S. rhizophila*. Further, liquid bioinoculant formulation survival study showed that *S. rhizophila* survives better in different tested liquid formulations. Compost Tea was found best liquid carrier which showed longer shelf life of inoculated plant growth promoting rhizobacteria. So, overall *S. rhizophila* was found to be multifarious strain which survive longer in the tested liquid formulations.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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