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Characterization of novel acidic and thermostable phytase secreting *Streptomyces* sp. (NCIM 5533) for plant growth promoting characteristics

Kumar Raja Puppala[a,b], Kavita Bhavsar[b], Vidya Sonalkar[b], Jayant M. Khire[a,b], Mahesh S. Dharne[a,b,*]

[a] Academy of Scientific and Innovative Research (AcSIR), Anusandhan Bhawan, New Delhi, India
[b] NCIM Resource Center, CSIR - National Chemical Laboratory, Pune, India

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ABSTRACT

Successful agricultural practices in acidic and arid region soils are still challenging worldwide. We investigated Actinomycete strain obtained from soil, which was found to produce extracellular phytase having the optimum pH, temperature of 2.5 and 70 °C, respectively and Plant growth promoting (PGP) attributes. These abilities of the phytase make it novel as compared to other Streptomyces derived phytases. Strain was also evaluated for physiological traits such as salinity, temperature, and pH followed by morphological and molecular identification. The *Streptomyces* sp. strain (NCIM 5533) displayed additional PGP traits such as indole-3-acetic acid (IAA), ammonia and phosphate solubilization activity. A significant increment in Tomato plant (*Solanum lycopersicum*) root, shoot and as well as total height in bioassays at laboratory scale and green house level and its ability to colonize roots substantiated its potency as a true plant growth promoting rhizobacteria (PGPR). To our knowledge this is the first report of *Streptomyces* sp. having the ability to produce acidic and thermostable phytase as well as PGP traits showing enhanced plant growth could have implications in soils having acidic nature and also in arid region.

1. Introduction

Current soil management strategies are mainly dependent on inorganic chemical-based fertilizers. Phosphorus (P) is one of them which is jeopardized to such an extent that we are comprising with food security of rapidly and exponentially growing world population. P is critical and the third important indispensable nutrient required for plant growth and development (Bhardwaj et al., 2014). Existing P reserves are a ticking time bomb as they are the finite, nonrenewable resources and there is no substitute to P. Mismanagement of P-bearing resources is quite far from the principles of sustainability and causing an alarming price increase of P mainly because of increases in rock phosphate processing costs (Vassileva et al., 2010).

Soil contains a wide range of organic substrates, which are the source of P for the growth of plants. But it must be hydrolyzed to inorganic P before its assimilation by the plants. The predominant form of organic P in soil is phytate nearly 60% (Singh and Satnarayana, 2011). Many of the plants cannot uptake the phytate-P and organic phosphorous, because the lack of sufficient level of phytase and phosphatase (Gujar et al., 2013). So in order to reduce the depletion of global P reserves, research should be oriented towards more effective utilization of phosphates. Problem of decreasing resources of phosphate rock for P fertilizers production can be mitigated by searching microbes producing phytase and plant biostimulant.

Plant biostimulant, or agricultural biostimulant, include diverse substances and microorganisms that enhance plant growth. The global market for biostimulant has been projected to reach $3.29 billion by 2022 and to have a compound annual growth rate of 10.43% from 2017 to 2022 (Wu, 2016). According to the same study, the largest market for biostimulant in 2012 was Europe. The European biostimulant industry council (EBIC) reported that in 2013 over 3 million hectares were treated with biostimulant in Europe (defined as the European Economic Area) (European Biostimulants Industry Council, 2013).

The present agronomists are majorly focusing on agriculturally beneficial microbes also called as plant biostimulants (Calvo et al., 2014), which are playing a key role in sustainable agriculture due to its PGP attributes, ability to improve soil ecosystem by enhancing the soil physicochemical properties and its biodiversity (Bhattacharyya and Jha, 2012). By using of such biostimulants, which is having the ability to enhance the P uptake in plants by releasing protons, organic acid

* Corresponding author. NCIM Resource Center, National Chemical Laboratory, Pune, 411 008, India.
E-mail address: ms.dharne@ncl.res.in (M.S. Dharne).

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anions or phenolics, P-mobilizing phytase, and phosphatase enzymes increase the availability of P in the soil (Gyaneshwar et al., 2002). Plants may change their root morphology, increase the affinity of nutrient transporters in the plasma membrane and exude organic compounds (carboxylates, phenolics, carbohydrates, enzymes, etc.) and protons. Plants can change their root morphology to increase the nutrients like P uptake in nutrient deficient soils. In such type of soils phytase producing organisms can play a major role to enhance the P uptake by plants by degrading the organic P (Hunter et al., 2014).

Plants are constantly involved in interactions with a wide range of bacteria. Soil microorganism's plays a crucial role in the biogeochemical cycling of organic and inorganic nutrients (Ahmad et al., 2008). The main role of these bacteria is to supply nutrients to plants, stimulate the growth, inhibit the activity of plant pathogens, and improve soil structure and bioaccumulation of inorganics (Spaepen et al., 2009). These bacteria also help in increase in biomass and modify rhizospheric communities of different plant species for better growth (Ji et al., 2019).

The rhizospheric soil bacteria may extent the plant growth by directly or indirectly. The direct promotion can be done by providing the useful nutrients by producing the plant growth promoting attributes like IAA, ACC deaminase activity, HCN, Siderophore and N_2 fixation, indirect promotion can be done by providing the antagonistic properties like inhibiting the phytopathogens (Saraf et al., 2014). Unknowingly, the interaction between plants and PGPR cannot be stable. The results which are produced under laboratory conditions which may not reproducible at field conditions. This variation may be due to several environmental factors like climate, weather conditions, and soil characteristics or the composition and activities of the indigenous microbial flora of the soil (Adesemoye and Kloepper, 2009). In PGPR, Actinomycetes has a major role, due to its antimicrobial producing capability and PGP traits, which are important in maintaining soil ecology and fertility (Flores-Gallegos and ErikaNava-Reyna, 2019).

The present work includes the screening of soil for a credible phytase producer and PGPR candidate i.e. *Streptomyces* sp. They are also known for the production of various types of secondary metabolites, antibiotics, and pharmaceutically important compounds. These bacteria are recognized by the characteristic, earthy smell in soil and they have important role in organic material conversion (Seipke et al., 2012). PGP potential of *Streptomyces* sp was reported on tomato, wheat, rice, bean and pea (Jog et al., 2012; Gopalakrishnan et al., 2014) wherein they promote plant growth either by producing siderophore (Aldesuquy et al., 1998), and/or indole-3-acetic acid (Tokala et al., 2002) or as biocontrol agent (Anelise et al., 2012). *Streptomyces* sp known for their antagonistic activity against different bacterial and fungal plant pathogens and these are effective colonizers of plant roots (Vurukonda et al., 2018). There is no single report on phytase and PGP from *Streptomyces longwoodensis*, so in the present work we have investigated the role of phytase along with additional PGP traits.

2. Material and methods

2.1. Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including Himedia, BDH, Sigma and Glaxo. Tomato seeds are collected from the Acharya NG Ranga University, Hyderabad, India.

2.2. Isolation and screening for phytase producing bacteria

Phytase producing bacteria were isolated from the soil collected from Sanjivani i-lands Maharashtra, India. The soil sample was serially diluted and plated on phytase screening media (PSM). PSM media consisting of (g l^{-1}): 15 D-glucose, 3 Ca-Phytate, 5 $NaNO_3$, 0.5 $MgSO_4.7H_2O$, 0.5 KCl, 0.01 $FeSO_4.7H_2O$, 0.01 $MnSO_4.6H_2O$, 20 agar, pH 5.5. Plates were incubated at 28 °C and checked for phytase production on the basis of the zone of hydrolysis around the colonies. Primary inoculum is developed in ISP2 media consisting of (g l^{-1}): 10 malt extract, 4 yeast extract, 4 glucose, 2 $CaCO_3$ and pH 7.4 for 48 h at 28 °C. This was transferred to phytase production media (MGA broth) containing (g l^{-1}): 1 L-arginine, 12.5 glycerol, 1 $CaCl_2$, 1 NaCl, 0.5 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$, 0.001 $CuSO_4$, 0.001 $MnSO_4.6H_2O$, and 0.001 $ZnSO_4$.

2.3. Characterization of selected isolates

The morphological and physiological characteristics were studied using scanning electron microscopy (SEM). Phylogenetic analysis was done using 16S rRNA sequence (Wilson et al., 1990). Amplification done using the universal primers 16R1525 – (5′TTCTGCAGTCTAGAAGGAGGTGWTCCAGCC 3′) and F27 – (5′ AGAGTTTGATCMTGGCTCAG 3′). The PCR condition included an initial denaturation at 94 °C for 3 min followed by 35 PCR cycles consisting denaturation at 94 °C for 1min, annealing temperature 55 °C for 1min, and elongation at 72 °C for 1.30 min and final elongation for 7 min. The PCR amplified products were run on 0.8% agarose gel and visualised using gel documentation (Protein Simple). The amplified PCR products were purified by EXOSAP method.

Purified products were sequenced in an automated DNA sequencer (Applied Biosystems 3730) at DNA sequencing facility, (National collection of industrially important microorganism) NCIM resource centre. The obtained sequence was compared with the sequences in the Gen Bank database from the National Centre for Biotechnology information (NCBI). The sequence has been submitted to NCBI Gen Bank under the accession number of KJ575561. The culture has been deposited in NCIM resource centre and will be referred further as *Streptomyces* sp NCIM 5533.

2.4. Phosphate solubilising and phytase activity assay

The culture was checked for P solubilization by inoculating on Pikovskaya (1948) agar medium consisting of (g l^{-1}) 0.5 Yeast extract, 10 Dextrose, 5 Ca-phosphate, 0.5 NH_4SO_4, 0.2 $MgSO_4.7H_2O$, 0.2 KCl, 0.001 $FeSO_4.7H_2O$, 0.0001 $MnSO_4.6H_2O$, 15 Agar, pH 6.8. Plates are incubated at 28 °C for 14 days and examined for zone of hydrolysis around the colony.

Phytase activity was measured at 70 °C in pH 2.5200 mM Glycine-HCl buffer with enzyme for 30 min. The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method as described by Heinhonen and Lathi (Heinohen and Lathi, 1981). A freshly prepared 4 ml solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v^{-1} v^{-1} v^{-1}) and 400 µl of 1 M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 µmol phosphorus per minute under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments.

2.5. Effect of pH and temperature on phytase

Phytase activity according to pH and temperature was measured as described above. The buffer used for pH measurement was 0.2 M glycine-HCl, 0.2 M Na-acetate, 0.2 M Tris-HCl in the ranges of pH 1.5–3.5, pH 4.5–6.5 and pH 7.5–9.5 respectively and optimum temperature was measured in the range of 40 °C–80 °C.

2.6. Partial identification of melanin production

The strain was grown in CSYP medium contains casein starch soluble yeast extract peptone bacteriological for 24 h. The cell free extract

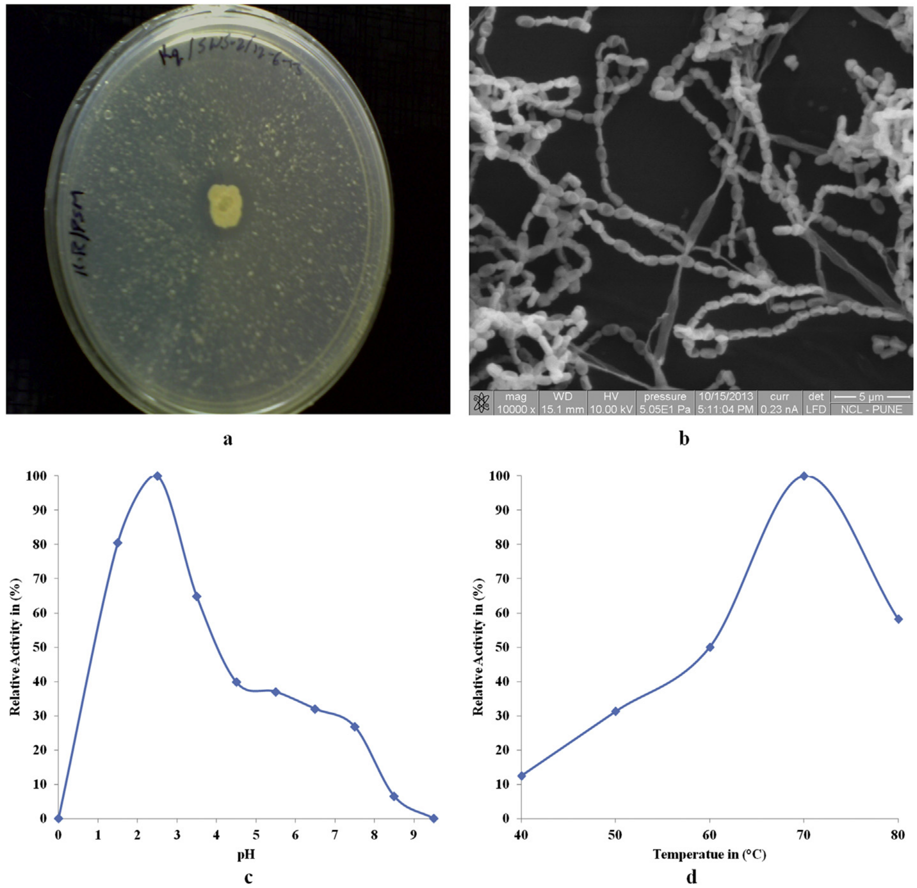

Fig. 1. Screening, morphology and characteristics of *Streptomyces* sp producing phytase. 1a- Hydrolysis of phytate on PSM plate, 1b -SEM image of *Streptomyces* sp, 1c- Optimum pH of phytase and 1d- Optimum temperature of phytase.

obtained after centrifugation is concentrated using Rotavapour. FTIR (Fourier transform infrared spectroscopy) was used to analyze the pigment, which accumulated in culture extract of the *Streptomyces* sp. strain, since it is regarded as the most informative method for structural analysis of melanin. The sample was made into pellets by KBr with 2 cm^{-1} resolution.

2.7. Plant growth promoting attributes and physiological traits

Production of IAA by *Streptomyces* sp. was determined in LB broth supplemented with L-tryptophan (500 μg ml^{-1}) at 72 h as described by Patten and Glick (1996). Cell free extract was mixed with Salkowski's reagent in the ratio of 1:4 and incubated at room temperature for 20 min. Development of a pink colour indicated indoles and its absorbance was measured at 530 nm and quantified using an IAA standard graph.

The isolates were grown in peptone water for 72 h at 30 °C and cell free extract obtained was checked for ammonia determination with Berthelot colour reaction method. The presence of faint and deep blue colour was denoted as + and + + for ammonia produced (Charles and Crouch, 1977).

Siderophore production was checked by growing isolate in blue indicator dye chrome azurol S agar plates using the method Schwyz and Neilands (Schwyn and Neilands, 1987). The organism is stabbed on CAS agar plates using sterile toothpicks and incubated at 28 °C for 2 weeks in the dark. The colonies with orange zones were considered as siderophore producing strains.

HCN production was inferred by the qualitative method of Bakker and Schipper (1987). The change in the colour of the filter paper previously dipped in 2% sodium carbonate prepared in 0.05% picric acid, from yellow to dark brown was rated visually depending on the intensity of the colour change.

The ability of isolated strain to grow in different temperature range (20 °C - 40 °C), salt concentration (0–25% W/V NaCl) and pH (5-13) was studied. All the experiments were done in triplicates. Observation were recorded as positive (growth) and negative (no growth). All experiments were done in triplicates and values of mean are presented.

2.8. Bioassay to assess PGP ability

Seeds of tomato (*Solanum lycopersicum*) (variety: *ARKA VIKAS*) were collected from Acharya NG Ranga Agriculture college, Hyderabad. Seeds were surface sterilized in laminar air flow with 70% alcohol and 2% sodium hypochlorite for 2 min with intermittent washing with sterile water 5–10 times. Surface sterilized Seeds were air dried in laminar air flow and infected with 24 h grown culture of *Streptomyces* sp (1.94 g wet wt biomass) for 45min on shaker, after infection seeds were air dried under aseptical condition and sowed in 0.6% agar tubes and 100 gm of soil pots. Control consisted of uninfected seeds. The tubes are kept at 28 °C for 20 days and pots are kept it at standard green house conditions and periodically observed for growth of plants. Seedlings were then analyzed for root, shoot and total heights of control and test (Dastager et al., 2010).

2.9. Scanning electron microscopy studies

For the removal of agar particles adhered to roots, seedlings are gently washed with sterile distill water aseptically. Small pieces of these roots (2 mm length) were mounted on aluminum stubs with the help of carbon conducting tape, coated with gold by sputter coater (Emitech)

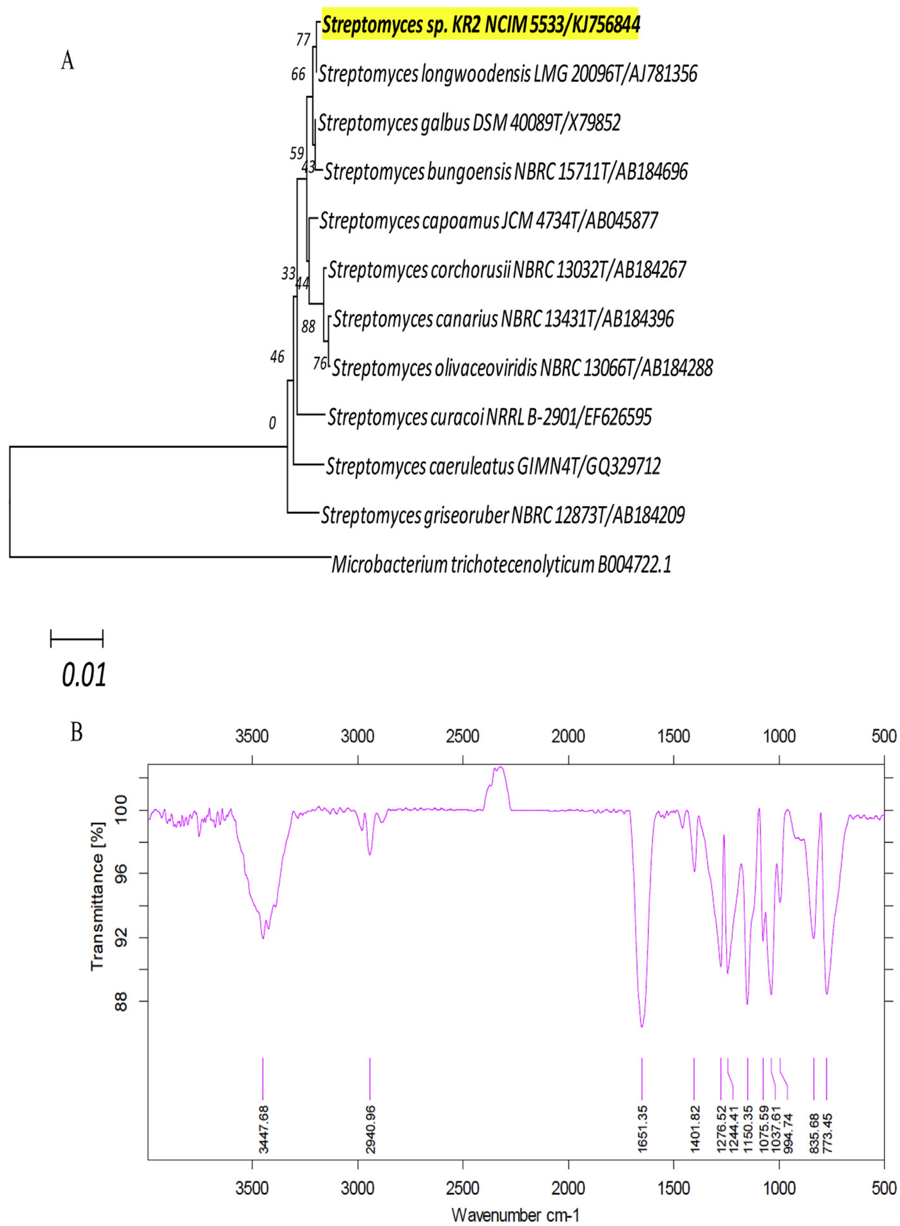

Fig. 2. Phylogenetic tree and melanin production by streptomyces sp.. Fig. 2A Neighbour-joining tree derived from 16S rRNA gene sequences showing the relationships between *Streptomyces* sp NCIM 5533 and related *Streptomyces* sp. Bootstrap percentages (based on 1000 replications) greater than 50% are given at branching points. Bar 0.01 substitutions per nucleotide position. Accession numbers are given after slash. Fig. 2B FTIR graph of melanin produced by *Streptomyces* sp.

and a FEI (Model: QUANTA 200 3D) SEM operated at 15 kv was used to observe root vascular systems and *Streptomyces* sp growth pattern.

3. Results and discussion

Soil P plays an important role in P cycle of agricultural soils. The predominant forms of P are phytates is a problem for agricultural system. Phytate are negatively charged molecules that bind to positively charged metal ions and forms insoluble complexes thereby limiting the availability of P. Because of its low mobility and high fixation in soil, low P availability is a worldwide constraint for crop productivity so there is need for microbial bioinoculant producing phytase which will target the solubilization of these fixed complexes and mitigate the mismanagement of P resources and its aftereffects. The present investigation aims at screening of phytase producer from soil followed by assessing its potentiality as PGPR.

3.1. Isolation and identification of phytase producing bacteria

Selection of phytase producer was done on the basis of zone of hydrolysis on PSM media as seen in Fig. 1a. However, the method is unable to differentiate between phytase activity and acid production. Hence, all the positive strains are quantified and confirmed for phytase production in submerged fermentation. Primary inoculums was prepared by inoculating a spore suspension in 5 ml ISP 2 and incubated at 28 °C for 48 h 10% inocula transferred to 50 ml GA broth and estimated for extracellular phytase production after removal of periodic samples. Among the four strains, one strain exhibited maximum phytase activity of 0.04 IU ml^{-1} in 24 h and that was selected for further studies. This phytase activity was found to be comparable with other reported values of *Streptomyces* sp. (Ghorbani-Nasrabadi et al., 2012) has mentioned one of the Streptomyces sp producing the phytase of 0.02 IU $ml^{-1}day^{-1}$. But our results showed that 0.04 IU $ml^{-1}day^{-1}$ have better productivity then the reported once. According to Grobelak et al.

Table 1
Physiological and PGPR traits of Streptomyces sp NCIM 5533.

Type	Trait	Observation
Physiological	Melanin	+
	Salinity (%)	
	0	+
	2	+
	4	+
	6	-
	pH	
	5	+
	7	+
	9	+
	11	+
	13	-
	Temperature (°C)	
	20	+
	30	+
	40	+
	50	+
Plant growth promoting	IAA	+ + 32 mg ml^{-1}
	HCN	-
	Siderophore	-
	Ammonia	+ +
	Phytase	+ .04 IU ml^{-1}
	Phosphate solubilization	+

(2018) PGPR can act as a alternative source for mineral fertilizers and helps in soil amendments and phytoremediation (Grobelak et al., 2018).

Morphological characterization was done by detecting the colony morphology, colour, size and shape was detected by using scanning electron microscopy as shown in Fig. 1b. The optimum temperature for phytase production is 28 °C. The optimum pH of 2.5 and temperature 70 °C was observed for Streptomyces phytase as seen in Fig. 1c and d respectively. The low pH and high temperature of phytase shows its ability for use in animal feed and nutrition. These features of the enzyme make it novel as compared with the Reza ghorbani nasrabadi (Ghorbani-Nasrabadi et al., 2012) *Streptomyces* phytase having pH-5 and temperature of 55 °C. Molecular level identification was done for phytase producing bacteria by using 16s rDNA method with the help of universal primers, the sequencing results shown a 99.93% similarity with *Streptomyces longwoodensis*. The phylogenetic tree was drawn by using the software EZ Taxon as shown in Fig. 2A. The single isolate obtained was characterized and identified on the basis of morphology and molecular techniques and designated as *Streptomyces* sp NCIM 5533. To the best of our knowledge, this is the first report of phytase from *S. longwoodensis.*

3.2. Plant growth promoting attributes and physiological traits

The isolate was able to grow in 4% NaCl, pH 5–11 and temperature 20 °C − 50 °C. The *Streptomyces* sp. forming the clear zone of hydrolysis on Pikovskaya agar plates indicating the solubilization of phosphate present in media (Table 1). A maximum of 32 mg ml^{-1} IAA on 3rd day was produced and ammonia production was confirmed by the deep blue obtained by Streptomyces. Partial determination of melanin production was conformed by FTIR (Fig. 2B). The isolate was found negative for HCN and siderophore production (Table 1).

PGP microbes stimulate plant growth directly by either solubilization of complex nutrients, nitrogen fixation and IAA production or indirectly by antagonizing plat pathogen by siderophore production, antibiotic and cyanide. It is known that the physiological status of the plant, the presence of microbes and the presence of products from rhizobacteria influence PGP ability. In the present investigation it was seen that *Streptomyces* sp promotes plant growth and this may be the mutual effect of all PGP traits produced by the strain like increased availability of P in rhizosphere region due to solubilization of phytate by phytase. Secondly the direct effect of IAA and ammonia produced in high quantities in mobilization of solubilized nutrients enhances its credibility as PGP.

Ammonia production is another important trait of PGPR where an organism can break down complex nitrogenous materials like peptones and release ammonia in soil. Released ammonia is taken up by plant as a nutrient source. Soils which are rich in nitrogen, there can be an accumulation of ammonia creating alkaline condition of the soil which suppresses the growth of certain fungi (Jha et al., 2012).

The positive physiological traits of *Streptomyces* sp growing in a wide range of pH, salt concentration (5%) and wide temperature range are the significant features that are an added advantage. Based on these results the enhanced plant tolerance when subjected to abiotic and biotic stress can be studied for PGP *Streptomyces* sp.

3.3. Bioassay and SEM analysis

The PGP potential of *Streptomyces* sp was determined by root colonization bioassay in tomato seeds. An increase in growth of the plant as compared to the uninoculated control seedlings was observed (Fig 4A and C). After 20 days seedlings are uprooted gently measured the root,

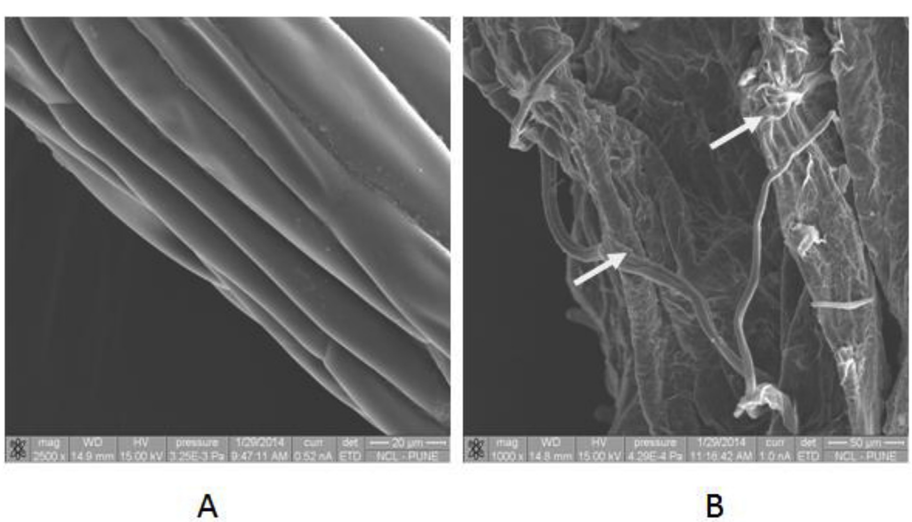

Fig. 3. Scanning electron micrograph of tomato seedlings 3A- Root surface of untreated seedlings free of bacteria 3B-Seedlings treated with *Streptomyces* sp NCIM 5533. Arrowhead show the mycelia growth.

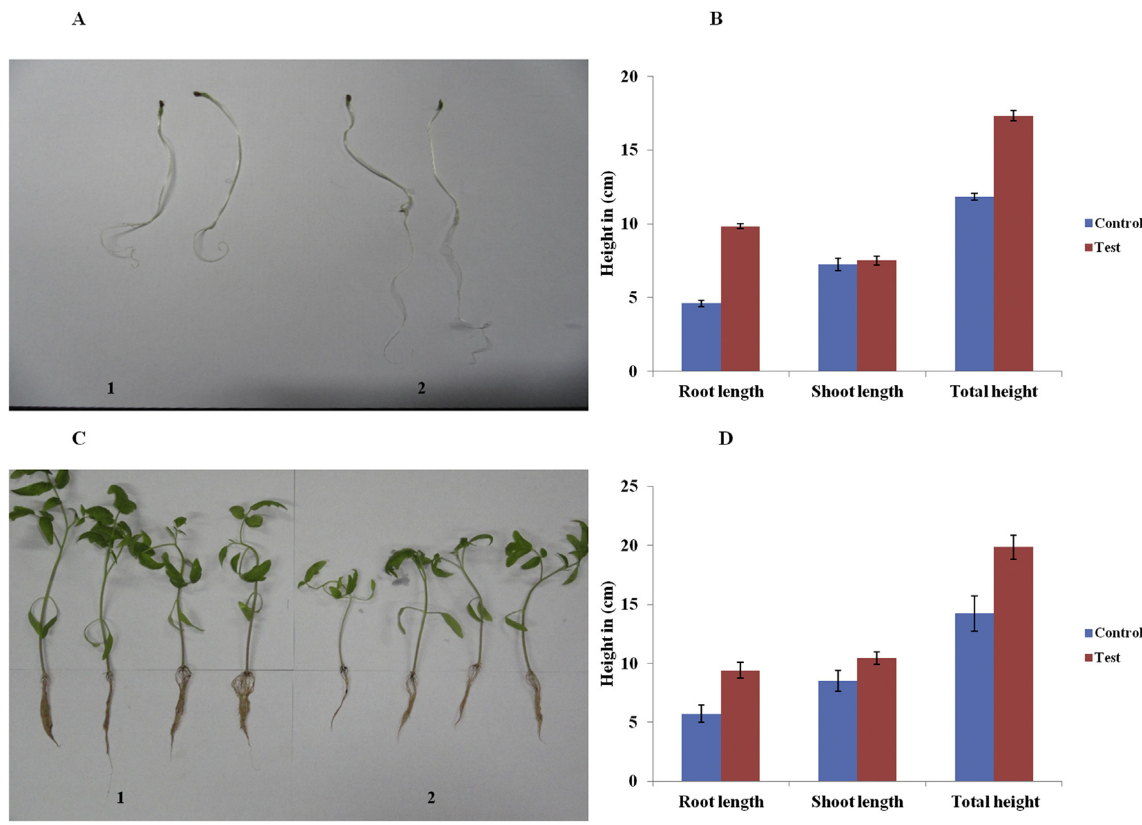

Fig. 4. Effect of inoculation of PGP *Streptomyces* sp NCIM 5533 on the growth of tomato seedlings over uninoculated in lab level and green house level. Lab level experiment results: Fig. 4A 1- treated with sterile distilled water (control) 2- treated with *Streptomyces* sp NCIM 5533 (test) Fig. 4B Height in (cm) Control-, *Streptomyces* sp –. Green house experiment results: Fig. 4C 1-treated with *Streptomyces* sp NCIM 5533 (test) 2- treated with sterile distilled water (control) Fig. 4D Increments in % Control-, *Streptomyces* sp –. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

shoot and total heights. The infected seedlings showed the increment of 113, 7 and 49% in lab level and in green house 63, 23 and 39% increment in root shoot and total height, respectively as compared with control seedlings, the difference in height of different parts of plant was showed in the form of bar graph with error bars (Fig. 4B and D). It was observed that seed bacterization resulted in greater enhancement of the root growth, as compared to the shoot growth.

Primary root sections of tomato bacterized with *Streptomyces* showing potential plant growth ability was examined by SEM. The results revealed that cells of isolates were consistently distributed on the surface of roots depicting effective colonization (Fig. 3B). Root seedlings free of inoculants typically revealed a smooth, undamaged epidermal root surface (Fig. 3A).

It is also observed that the root length is increased more as compared to shoot length. This phenomenon can be attributed to the ability of the isolate to produce high quantities of IAA, as IAA positively influences root growth and development, thereby enhancing nutrient uptake (Khalid et al., 2004). This may confer advantages to the host system with respect to its health and growth. Increased root length also improves the survival of young seedlings, especially at the initial stages of development. The root were gradually colonized, first by single cells that later grew out to microcolonies and now called biofilms usually consist of multiple layers of *Streptomyces* filaments in SEM images. Different actinobacteria and its potential PGP traits are given in Arumugam et al. (2017). It mentions that these bacteria are hidden repertoire for different bioactive and natural products. These are explored in agricultural sector in a great extent (Sathya et al., 2017).

The present study was successful in selecting and identifying the role of *Streptomyces* sp NCIM 5533 as PGPR. This is essential in reduced input and poor agro-systems wherein microbial activities will play a decisive role in reducing soil degradation and improving its functioning. The isolate possesses the ability to produce high IAA with tryptophan, ammonia and solubilize P in culture medium. This isolate therefore has a potential for use as bioenhancer for plant growth promotion and is confirmed by the bioassays results. However further research in ascertaining the credibility of this isolate in situ, plant microbe interaction is certainly needed. PSM and PGP give an added niche for this biostimulant as it has the potential to increase P availability while curtailing P fertilizer application after effects.

4. Conclusion

Unlocking of the phytate phosphorus from soil using microbial phytase has been shown recently very useful for increased plant growth promoting activity. If additionally organisms also possess other plant growth promoting activities (PGP) like phosphate solubilization, indole acetic acid (IAA), ammonia and sidophore production its application as bioinoculant in soil will be promising. We have isolated strain of *Streptomyces* sp. from soil which secretes acidic and thermostable extracellular phytase and showed PGP activities. So its application as bioinoculant will increase the available P in soil along with reduced requirement of P-fertilizers, will decrease environmental pollution and promote sustainable agriculture.

Declarations of interest

None.

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