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Screening and identification of novel halotolerant bacterial strains and assessment for insoluble phosphate solubilization and IAA production

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Abstract

Background: Salinity is typical in seashore soils due to the interruption of seawater in the groundwater. Soil microbes of coastal regions play a vital role in increasing plant yields. Microbe-plant associated growth and its wide spectrum with soil environment remain one of the prime factors in agriculture for field application. Making such, in this study, very precise research work is outlined to serve microbial-based solution for solubilizing the insoluble phosphate under various harsh environmental conditions and IAA production. Salt-affected soils along the coast of Bay of Bengal, Sundarbans, India, have been collected.

Results: A total of five isolates effectively solubilize the considerable amount of Tri-calcium phosphate {TCP, $(Ca_3PO_4)_2$} ranging from 50.67 to 116.66 P_2O_5 parts per million (ppm) under optimized conditions, i.e., pH 8.0, 5 to 10% saline and 30 °C temperature. Out of five, three produced Indole Acetic Acid (IAA) ranging from 0.054 to 0.183 (g l^{-1}). Identification of isolates has been carried out by morphology, biochemical characterization and 16S rDNA sequencing. Among the sequenced isolates, 1 belonged to Firmicutes, 3 were Proteobacteria and 1 was Actinobacteria.

Conclusion: This is the first report which shows the presence of phosphate solubilizing activity by the member of the genus *Halomonas* and *Halobacillus* from the study site. These stress-tolerant bacteria will deliver reliable and cost-effective methods to overcome the existing scenario of saline-affected agriculture.

Keywords: Saline soil, Stress-tolerant bacteria, Phosphate solubilizing bacteria, IAA producing bacteria, 16S rDNA

Background

Soil salinity is one of the most prominent agricultural dilemmas that adversely influences crop productivity as salination inhibits plant progression and quality in the salt-accumulated area around the world. Salinity is a deep-rooted risk to crop harvests in countries where irrigation is a crucial aid to agriculture (Shrivastava and Kumar 2015). The costs associated with the enrichment of soil salinity are potentially enormous but salinity arises seriously in agriculture, biodiversity and also the environment. The influences of PGPR to plant health and their osmotolerance functions are vital. It is of thoughtful issue, as the saline regions under agriculture have been expanding every year globally (Paul and Nair 2008). In addition, the stressful environmental factors act to influence the growth and phosphate solubilizing activity of phosphate solubilizing bacteria (PSB). A high level of salinity interrupts plant progress through the osmatic results, the toxicity of salt ions and the modifications in the physio-chemical properties of soil. These conditions may result in poor growth and the existence of PSB. The accessible phosphorus (P) in these soils is

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lousy, and the most suitable solution to this situation is to use PSB as bio-inoculants. However, precise studies on PSB of alkali soils have not been reported.

The development and improvement of plants are subject to P availability which is the utmost essential macronutrient. Phosphates readily available in soils are generally less for crop development. The P-content in normal soils is approximately 0.05% (w/w) but only 0.1% of the overall P is available to plants (Zahir et al. 2004). Insufficiency of soil P is one of the most significant chemical aspects limiting plant growth in soils. Subsequently, massive quantities of soluble forms of P fertilizers are provided to attain maximum plant yield. However, the applied soluble forms of P fertilizers are effortlessly precipitated into insoluble forms $CaHPO_4$, $Ca_3(PO_4)_2$, $FePO_4$, and $AlPO_4$ and are not proficiently uptake by the plants, which lead to an additional application of P fertilizer to cropland (Sharma et al. 2013). Since P is an essential nutrient for plant growth, soils usually contain 1.32 to 15.5×10^{-2}% 'P' and insoluble phosphate (Pi) that is not straightly accessible to plant and/or microorganisms and contain around 95 to 99% of the total phosphate (Gyaneshwar et al. 2002). The adverse effects like the yields and profits of crop production in saline soil are reduced drastically because it suppresses the P uptake by plant roots and reduces the available P by sorption procedure and poor solubility of Ca-P minerals (Chookietwattana and Maneewan 2012). In this concern, the use of existing P in the chemical fertilizers is the most common method to recover soil fertility which is quickly stabled to the unavailable forms particularly in salt-affected soil, and accounts for less P use efficiency. PSBs are well known to improve the solubilization of fixed soil P by the production of organic acids and acid phosphatases and result in the increase of crop yields.

A cluster of biofertilizers comprising beneficial rhizobacteria identified as plant growth-promoting rhizobacteria (PGPR) strains from genera of *Pseudomonas, Azospirillum, Azotobacter, Bacillus, Burkholdaria, Enterobacter, Rhizobium, Erwinia,* and *Flavobacterium* (Vessey 2003). Free-living PGPRs have shown potential as effective biofertilizers (Mitra et al. 2020).

In general, a huge number of artificial fertilizers which are incorporated to restock the soil phosphate is expensive as well as cause a threat to the environment. Most of the phosphate in insoluble compounds is unavailable to plants. PSB plays a key role in agriculture as they enhance P uptake and perform a critical role as PGPR in bio-fertilization (Vyas et al. 2007). As a result, the utilization of aforesaid microbe-based ecofriendly biofertilizer may promote to curtail the use of costly phosphatic fertilizers. Phosphorus biofertilizers improve the accessibility of accumulated P (by solubilization), due to the development of plant growth-promoting ingredients.

Apart from phosphate solubilization, numerous phosphate solubilizing microorganisms (PSM) increase the mycorrhizal root colonization through the production of certain metabolites as vitamins, amino acids and Hormones (Kobae 2019; Hodge and Storer 2015; Revillini et al. 2016). A modification in plant physiology, experiencing salt stress unfavorably affects nutritional uptake, enzymatic reducing of plant ethylene levels and/or by the production of siderophores and as well crop growth (Kohler et al. 2006). PGPR expedite plant growth indirectly by diminishing plant pathogens, or straightly by solubilization of inorganic phosphates, facilitating the nutrient uptake through phytohormone production with the compounds synthesized by the bacterium (e.g., auxin, cytokinin and gibberellins), enriched iron nutrition by iron-chelating siderophores and the volatile compounds that disturb the plant signaling pathways (Glick 2020).

In this study, saline soil samples of the coastal region of Bay of Bengal, Sundarbans, from central soil salinity research institute (ICAR), canning, (West Bengal) has been examined to assess the profound effect of soil-associated microbial strains in a wide range of stress tolerance with relative Pi solubilization and IAA production capability.

Methods

Sample collection and soil analysis

Saline soil samples were collected from different experimental plots of Central Soil Salinity Research Institute (CSSRI), canning, South-24-PGs, attached to the coastal saline region of Bay of Bengal, Sundarbans, West Bengal, India. Three replicate soil samples (100 g) were taken from the horizon (0–20 cm soil depth) of the study plot. The samples were stored in aseptic conditions. The soil samples were analyzed for pH and electrical conductivity (EC). To measure pH and EC, air-dried soil samples were extracted with milli-Q water (soil: water = 1:5) for 1 h and were measured using Professional pH – EC Meter PP-20 (Sartorius).

Isolation and screening of stress-tolerant bacteria

To extract bacteria from saline soil, fields moist soils were suspended with sterile 0.2% (w/v) NaCl solution (soil: solution = 1:100) and shaken overnight at 150 rpm (Park et al. 2011). Suitable serial dilutions of the soil suspension were taken and individually spread as inoculums on Lysogeny broth (LB) agar medium containing (g l^{-1}) Tryptone, 10; Yeast extract, 5; NaCl, 5; agar, 15 and adjusted the pH at 7.0 ± 0.2. Petri plates were incubated at room temperature and bacterial growths were observed subsequently per day. After 24–48 h, colonies

were obtained and again individual isolates were purified by subcultured in LB agar medium. In order to select stress-tolerant strains which are having beneficial traits for plants, we adopted a screening procedure (Nautiyal et al. 2000; Attar et al. 2019). Effects of different environmental stress factors like salt, pH and temperature on isolated strains were studied. All pure cultures were tested with 1% (v/v) inoculum in LB broth medium for their stress-tolerance ability, containing different concentrations of NaCl ranging from 0.5 to 15% (w/v), pH ranging from 4 to 10 and an incubation temperature range from 10 to 50 °C. The absorbance at 660 nm was taken at 24 h intervals up to 7 days of individual isolates grown under different stress concentrations. The experiment was carried out in triplicate. Pure cultures with higher stress tolerance were selected and maintained in LB medium for further studies.

Phenotypic and biochemical characterization of the isolates

Bacterial isolates were performed for the identification of bacteria species based on the differences in the morphology and biochemical activities of different bacteria. Phenotypic characters of the isolates like cellular morphology, Gram reaction, shape and motility were carried out by standard procedures (Holtz 1993). Biochemical characteristics of the isolates like starch hydrolyzing activity, catalase activity, citrate, oxidase, MR, VP, Indole test, NO_3^- utilization, gelatinase activity, Carbohydrates Fermentation Test, etc. were examined by Rapid Biochemical Identification Test Kits (Himedia Laboratories Pvt., Mumbai).

Molecular characterization and phylogenetic analysis

Genomic DNA isolation has been carried out for all five isolates using the standard phenol–chloroform method. Cells were harvested from overnight grown culture by centrifugation at 6000 rpm for 6 min. and processed immediately for DNA isolation (Sambrook et al., 1989). After getting genomic DNA bands, partial 16S rRNA gene was amplified by GeneAmp PCR system 9700 (Applied Biosystem, US) using the universal bacterial forward primers 5′-TGGCTCAGAACGAACGCGGCGGC-3′ and reverse primer 5′ –CCCACTGCTGCCTCC CGT AGGAGT-3′. Amplification was performed in 25 µl reaction volumes containing 1 mM $MgCl_2$, 1.25 U Taq polymerase, 200 µM of each dNTP, 0.5 µM of each primer, 1 X Taq buffer, 2 µL template DNA and remaining nucleus free water. The thermal cycling was performed as initial denaturation at 95 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 45 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR-generated anticipated DNA fragment was analyzed on 1.2% agarose gel. The size(s) of PCR products was determined by comparison with a molecular weight marker, i.e., DNA ladder (Sigma-Aldrich PCR Low Ladder), which contained DNA fragments of known size, run on the gel alongside the PCR products. The amplified PCR product was sequenced using an automated sequencer and submitted to NCBI. BLAST tools were employed for the identification of bacterial isolates and strains were allotted accession numbers. The sequences were further aligned in the ClustalW tool, and a phylogenetic tree was generated from the evolutionary distance data by the neighbor-joining method with help of MEGA X software (Padmanaban et al. 2019; Kumar et al. 2018).

Isolation and estimation of phosphate solubilizing bacteria

Pikovskaya's medium (Srinivasan et al. 2012; Attar et al. 2019) containing (g l^{-1}) glucose, 10; tri-calcium phosphate {TCP, $(Ca_3PO_4)_2$}, 5; ammonium sulphate, 0.5; sodium chloride, 50 or 100 (5% (w/v) supplement for SB 2 and SB 3 while 10% (w/v) for SB 1, SB 4 and SB 5 of NaCl); $MgSO_4.7H_2O$, 0.1; potassium sulphate, 0.2; yeast extract, 0.5; $MnSO_4$, 0.002; $FeSO_4$, 0.002; agar, 15 and pH-adjusted to 8.0 ± 0.2 was used for Pi solubilization estimation under optimized growth conditions of individual isolates in stress environment. For the isolation of TCP dissolving micro-organisms, earlier method was adopted (Wang et al. 2017; Singh and Prakash 2012). The soil was incubated with 0.5% TCP as a substrate for 2 weeks at 25 °C and was placed on a TCP medium.

One gram of the soil was added to a flask containing 100 ml pikovskaya broth (tri-calcium) and three successive transfers were made at 2 days intervals to enrich the medium. Then, from the final flask, phosphate solubilizers were isolated by streaking a loopful of culture on above-mentioned pikovskaya medium. The colonies around which zones of Pi solubilization developed were transferred to agar slant on the specific medium and allowed to grow at 30 °C for overnight. All bacterial isolates were purified and maintained on LB medium by sub-culturing. Microbial solubilization of Pi in a liquid medium was done as described by Gaur (1990). Estimation of phosphorus solubilization, i.e., water-soluble phosphorus was determined by the chloromolybdic acid and stannous chloride method described by Jackson (1967). In brief, chloromolybdic acid and chlostannous acid were added and the solution was turned blue. This blue colour intensity of the solution was measured at 600 nm (UV 2700, Thermo Scientific) for quantification (Narsian and Patel 2000; Banerjee et al. 2010).

IAA production

Selective phosphorous solubilizing strains were analyzed for IAA production. For relative IAA production

of all isolates, 0.5 ml broth culture of each sample were inoculated in LB broth medium containing 5% of NaCl for SB 2 and SB 3 while 10% NaCl for SB 1, SB 4 and SB 5, pH 8.0 ± 0.2 and incubate for 7 days amended with and without filter-sterilized L-tryptophan ranging from 0 to 200 mg l^{-1} at 30 °C on a rotary shaker at 150 rpm. The estimation of IAA production was carried out in 24 h intervals up to 7 days. L-tryptophan serves as a physiological precursor for the biosynthesis of auxins in microbes. The experiments were carried out in triplicate with control. After 7 days, 1 ml broth culture of each sample was taken and centrifuged at 10,000 rpm for 5 min. Supernatant (1 ml) with additional 4 ml IAA reagent containing $FeCl_3$ (0.5 M) 1 ml, H_2SO_4 conc. sp. gr. (1.84) 30 ml and distilled water 50 ml was taken and a final volume of 5 mL was made up. Incubation for 30 min at room temperature showed pink colour development (Kumar et al. 2012). Absorbance at 530 nm (UV spectrophotometer 2700, Thermo Scientific) was taken and the concentration of IAA in the culture medium was determined using a standard curve prepared with various concentrations of analytical grade IAA.

Statistical analysis

To normalize the data set, all data were log-transformed before any statistical analysis. The statistical significance of results was analyzed by one-way ANOVA test for three independent experiments. Differences were considered significant at $p < 0.05$ and values are reported as mean ± SD.

Results

Saline soil increases the negative effect of nutrients uptake by plants. In this study, an attempt has been made to evaluate the adaption, phosphate solubilization and IAA production by microorganisms at different stress conditions isolated from soil samples collected from central soil salinity research institute (ICAR), canning, (West Bengal).

Soil characteristics

Seasonality had an important bearing on the soil. Table 1 shows pH variations and electrical conductivity of different CSSRI plots. Soil pH varies from neutral to slightly alkaline, i.e., 7.13 to 8.62 and EC was varied between 5.6 to 7.29. Five stress-tolerant strains SB 1, SB 2, SB 3, SB 4 and SB 5 isolated from different CSSRI plots have been screened for further study. A close estimation of the amount of salt in soil and the ability of an aqueous solution to carry an electric current has been measured by the electrical conductivity of the soil. The results indicate that electrical conductivity was found directly proportional to the pH which showed increment with increasing salinity.

Table 1 Physio-chemical characteristics of soil samples used for isolation of stress-tolerant bacteria

Collection site	Category of soil based on salt level	pH	Electrical conductivity (ds/m)	Isolated strains
Plot – I	Slightly	7.13	5.6	SB 2 and SB 5
Plot – II	Slightly	7.25	6.74	SB 4
Plot – III	Medium	8.62	7.29	SB 1 and SB 3

Effect of different environmental stress on isolates

Effect of different environmental stress factors like salt, pH and temperature on isolated strains of saline-alkaline soils collected from CSSRI, West Bengal, showed variance with increased sustainability. Salinity is seldom constant with time or uniform in space, and fluctuating trends in salinity levels generally occur in coastal saline soils. Growth of mostly all 5 strains was found highest with the increasing amount of NaCl of up to 10% (w/v) except strains SB 2 and SB 3 (Fig. 1a). SB 2 and SB 3 showed maximum growth in 5% (w/v) NaCl medium but beyond that drastic decline was observed in the growth behavior of these two isolates. The influence of pH on the growth behavior of isolates was investigated in the range of pH 4 to 10. The growth performance of all isolates was varied for all pH values tested, and they all were able to survive. Maximum growth of all isolates was observed at slightly alkaline conditions, i.e., pH 8 (Fig. 1b). All isolates were able to withstand a mildly acidic environment but showed better growth in basic conditions. Thus, all were alkaliphilic strains. To investigate the effect of temperature on isolated strains, cultures were incubated at a different temperature ranging from 10 to 50 °C. The isolated strains were able to grow at all temperatures tested and grew especially well in the range of 30 to 40 °C. The growth of isolates increased gradually with the increase of temperature up to 30 °C but beyond that drastically decline in growth performance was observed because of may be the cells aren't able to grow or due to metabolic inactivity above 30 °C. The highest growth of isolates was observed at 30 °C (Fig. 1c). The survival capacity of these isolates under different temperatures was significant.

Phenotype and biochemical characterization

To study the phenotypic and biochemical characterization of the isolates, 24 h incubated cultures were used. The cells of strains SB 1, SB 2 and SB 3 were rod-shaped, gram-negative and motile. The others SB 4 and SB 5 were rod and coccoid shaped, gram-positive and non-motile strain. After 24 h incubation, colony shape, margin,

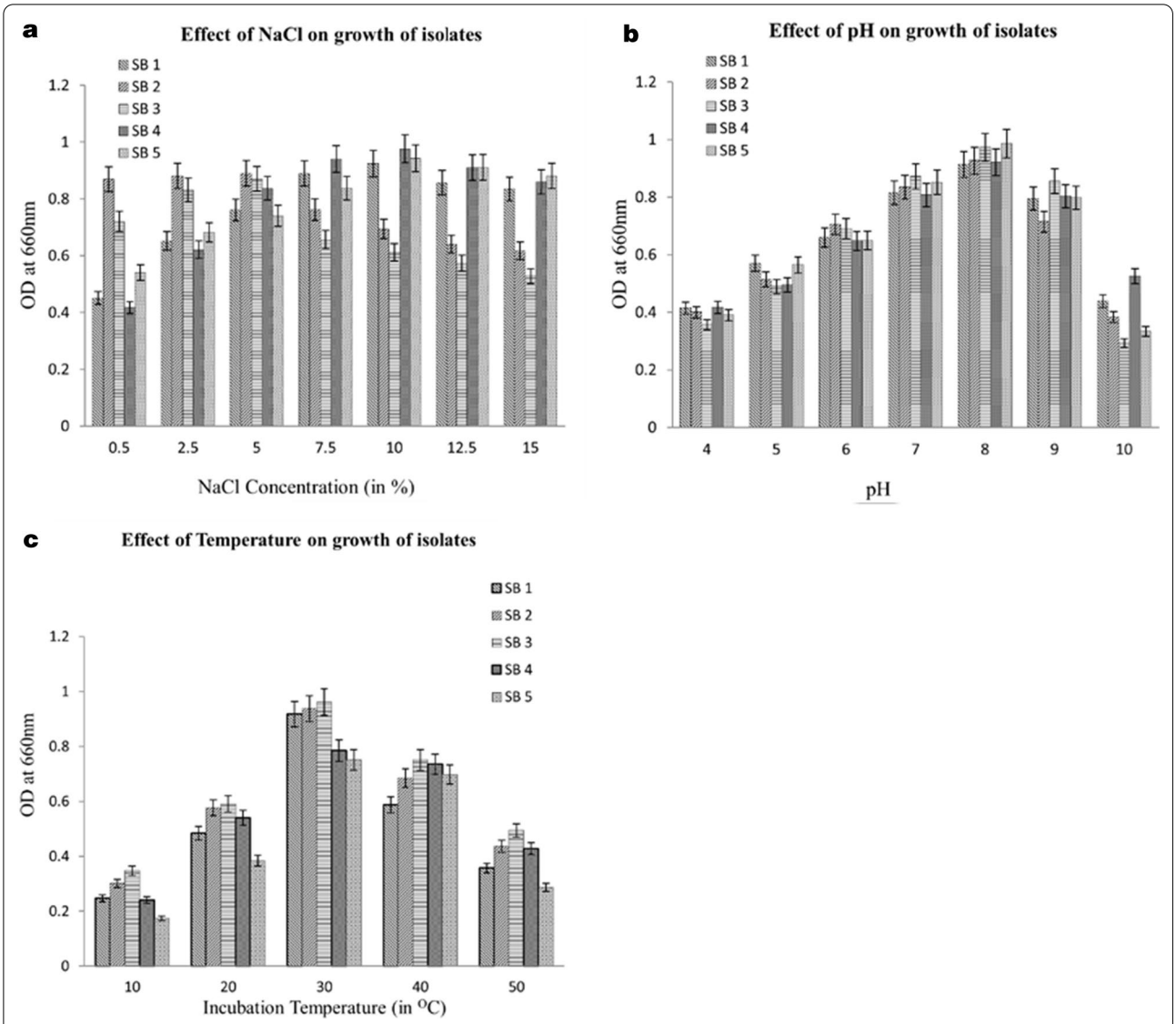

Fig. 1 Growth comparison of different isolated bacteria under different abiotic conditions **a** NaCl concentration 0.5–15%, **b** pH 4–10 and **c** temperature 10–50 °C. Results represented as mean ± SD ($n = 3$)

Table 2 Phenotypic characterization of stress-tolerant bacteria isolated from soil samples (CSSRI, 24 South Paragonas)

Isolates	Characteristics of colony					Characteristics of cells		
	Shape	Margin	Elevation	Colour	Size (mm)	Motility	Shape	Gram staining
SB 1	Cir	Ent	Flat	White	1–2	+ ive	Rod	− ive
SB 2	Cir	Ent	Cvx	Red	1–2	+ ive	Rod	− ive
SB 3	Irre	Und	Flat	Red	3–4	+ ive	Rod	− ive
SB 4	Irre	Und	Flat	Yellow	1–3	− ive	Rod	+ ive
SB 5	Cir	Ent	Cvx	OW	2–4	+ ive	Coccoid	− ive

Cir Circular, *Irre* Irregular, *Ent* Entire, *Und* undulate, *Cvx* convex, *OW* Off white, − ive Negative, + ive Positive

elevation, colour and size were also examined in LB agar medium for all isolates (Table 2). All the strains could use fructose, dextrose, trehalose, sodium gluconate, glycerol and ribose for acid production, but they were VP, melibiose, dulcitol and α-methyl-D-mannoside negative. The isolates showed a positive test for catalase and oxidase. Other carbohydrate fermentation tests were also carried out for all strains (Table 3).

Molecular characterization

The genomic DNA of all five isolates has been isolated and amplified through PCR. The amplified portions of the sample's 16S rDNA gene segments were sequenced and submitted to NCBI. The assigned NCBI accession numbers along with their specific details are provided in Table 4. Two isolates revealed the closest similarity with *Serratia* at the species level, and *Arthrobacter, Halomonas,* and *Halobacillus* at the genus level (Fig. 2). The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.89808372 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Kumar et al. 2004). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kumar et al. 2004) and are in the units of the number of base substitutions per site. This analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). Evolutionary analyses were conducted in MEGA X.

Potential of stress-tolerant bacteria to solubilize phosphate

On the basis of bacterial performance under saline stress environmental condition, all five selected cultures were tested for phosphate solubilization ability using the conditions optimized for respective isolates on Pikovskaya medium, i.e., 5% (w/v) NaCl supplement for SB 2 and SB 3 and 10% (w/v) NaCl supplement for SB 1, SB 4 and SB 5 at pH 8.0 and incubation temperature 30 °C. The establishment and performance of PSM are severally affected by environmental factors, especially under stressful conditions**.** Initial selections of isolates were done by visual analysis of solubilizing zones on the agar medium. Observations were taken for 2, 4 and 6 days after incubation. In broth medium, results showed variations in the amount of solubilization of TCP by the isolated bacterial strains. All the cultures solubilized considerable amounts of TCP ranging from 50.674 to 116.669 P_2O_5 (ppm) showed by SB 4 and SB 3 (Fig. 3). Solubilizations of TCP were in

Table 3 Biochemical characterization of stress-tolerant bacteria isolated from soil samples (CSSRI, 24 South Paragonas)

Biochemical tests	SB 1	SB 2	SB 3	SB 4	SB 5
Utilization of citrate	+	+	+	−	+
Methyl red	+	−	−	+	+
Voges Proskauer	−	−	−	−	−
Indole	−	−	+	+	+
Nitrate reduction	+	−	−	−	−
Hydrolysis of gelatin	+	+	−	+	−
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
ONPG	−	+	+	−	−
Esculin hydrolysis	+	+	+	−	+
Malonate utilization	+	+	+	+	−
Hydrolysis of urea	+	−	−	+	−
Hydrolysis of starch	+	−	−	+	−
Carbohydrates fermentation test (acid production from carbohydrates)					
Lactose	−	+	−	−	+
Xylose	−	+	−	+	+
Maltose	−	+	+	+	+
Fructose	+	+	+	+	+
Dextrose	+	+	+	+	+
Galactose	+	+	+	+	+
Raffinose	−	+	−	−	+
Trehalose	+	+	+	+	+
Melibiose	−	−	−	−	−
Sucrose	+	+	+	−	+
L-Arabinose	−	+	−	−	+
Mannose	−	+	+	+	+
Inulin	+	−	−	−	+
Sodium gluconate	+	+	+	+	+
Glycerol	+	+	+	+	+
Salicin	−	+	+	+	+
Glucosamine	−	+	+	−	+
Dulcitol	−	−	−	−	−
Inositol	−	+	+	+	−
Sorbitol	−	−	+	−	−
Adonitol	−	−	−	−	+
α-Methyl-D-glucoside	−	+	−	−	−
Ribose	+	+	+	+	+
Rhamnose	+	−	+	−	−
Cellobiose	−	+	−	−	−
Melezitose	+	+	−	−	+
α-Methyl-D-mannoside	−	−	−	−	−
Xylitol	−	−	−	+	+
D- Arabinose	−	−	−	−	+
Sorbose	+	−	−	−	+

+Tested positive/utilized as substrate, −Tested negative/not utilized as substrate

Table 4 Molecular characterization of the isolates

Isolated strains	NCBI accession number	Most closely related organism based on 16S rDNA sequence analysis along with their accession number	% Similarity
SB 1	FN563487	*Halomonas* sp., AM945678	100
SB 2	FN563488	*Serratia* sp., MK954166	100
SB 3	FN563489	*Serratia marcescens*, MN252011	100
SB 4	FN563490	*Arthrobacter* sp., MK016481	100
SB 5	FN563491	*Halobacillus* sp., MH708593	100

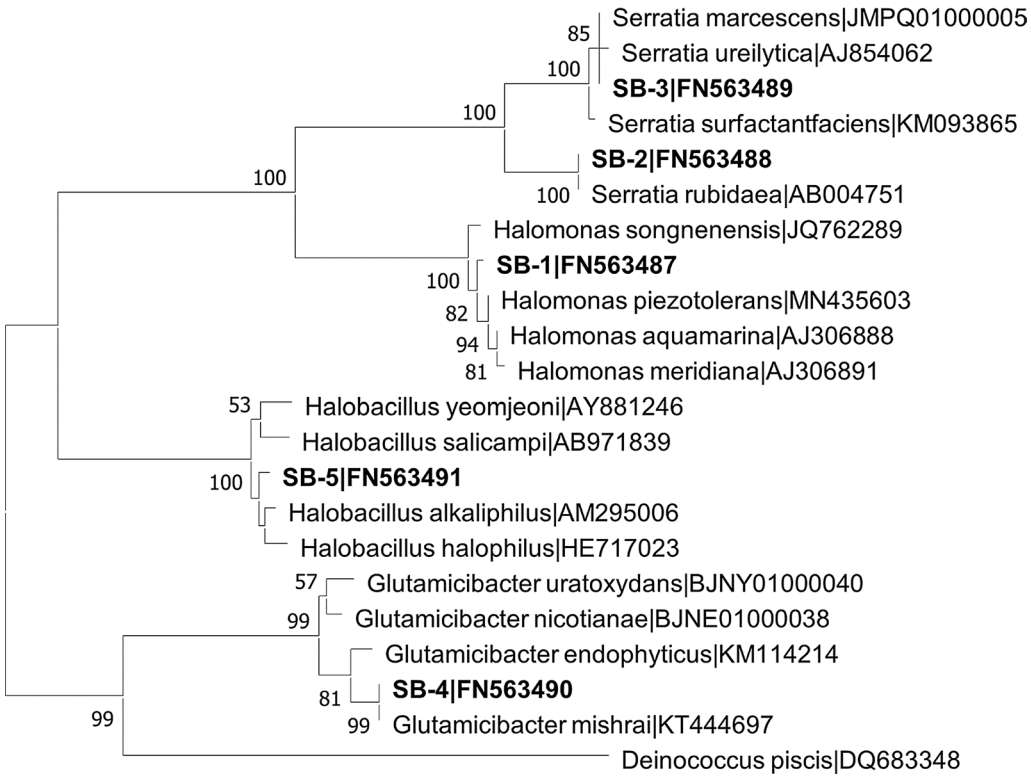

Fig. 2 16S rRNA gene sequence-based neighbor-joining phylogenetic tree, showing the position of halotolerant phosphate solubilizing strains isolated from saline soil among members of the related genera. Bar, 0.05 substitutions per nucleotide position

the increasing order of SB 3 followed by SB 2, SB 5, SB 4 and SB 1. All isolates showed maximum P solubilization ability within 4 days of incubation. A drastic decline was observed in the final pH of the medium after 6 days of inoculation. Initially, the pH of the medium was set at 8.0 but after 6 days it varied from 3.26 of SB 4 followed by 3.29 of SB 2, 3.31 of SB 5, 3.34 of SB 3 and 3.73 of SB 1.

Production of IAA by stress-tolerant phosphate solubilizing bacteria

IAA production by stress-tolerant phosphate solubilizing microorganisms affects increasing crop productivity in the agricultural environment. On the basis of stress tolerance and P solubilizing capacity, all five strains were analyzed for IAA production with and without L- tryptophan under optimized growth conditions. The strains were grown in LB media with optimized NaCl concentration, incubation temperature 30 °C and pH 8.0 ± 0.2 that could be important for plant growth in saline conditions so that the strains could be used as potential microbial inoculants for saline-soil agriculture. Out of five, only SB 3, SB 4 and SB 5 showed activity for production of IAA under stress conditions. SB 3 showed maximum production of IAA with and without L-tryptophan, i.e., 0.183 ± 0.036

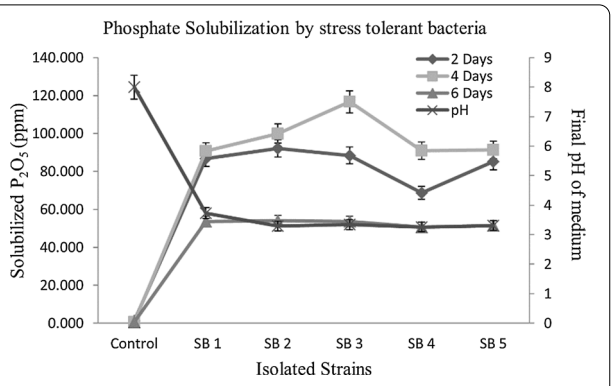

Fig. 3 Comparison of different types of stress-tolerant phosphate solubilizing bacteria by insoluble tri-calcium phosphate. Results represented as mean ± SD (n = 3)

and 0.062 ± 0.013 g l^{-1}, whereas SB 5 showed minimum production of IAA , i.e., 0.151 ± 0.043 g l^{-1} with L-tryptophan and 0.054 ± 0.015 g l^{-1} without L-tryptophan after 7 days of inoculation (Fig. 4). All three isolates revealed the ability to produce the best amount of IAA in presence of L- tryptophan under stress conditions, indicating that these isolates could utilize L- tryptophan as a precursor for growth. Statistical analysis showed a significant difference ($p < 0.05$) among all the bacteria. The production of IAA by SB 3 was significantly higher ($p < 0.05$) as compared to the control samples.

Discussion

Throughout limited rainfall or summer season, salinity is on the peak at the surface because a drought-kind situation prevails and water is lost from the system. The coastal region shallow groundwater is saline and because of quick vaporization during the summer times, the salts are deposited on the surface through capillary action. Thus, the salts form a layer on the surface of the soil, increasing the salinity of the horizon and resulting EC peaked in the summer season (0–20 cm) (Tripathi et al. 2006). These continuous processes of 'salinity increase followed by rapid decrease' go on cyclically. Thus, stress-tolerant ability of bacterial isolates is of great significance in high salt accumulated soils. It is also reported that calcium phosphates can be formed by phosphate precipitation, which includes rock phosphate (fluorapatite and francolite), which are insoluble in soil. Their solubility is indirectly proportional to soil pH, if solubility increases soil pH decreases. PSMs improve P availability by producing organic acids that drop the soil pH (Satyaprakash et al. 2017).

On the basis of stress-tolerant activity of all isolates, we optimized the environment growth parameters and maintained it throughout the studies for all isolates, i.e., 5% (w/v) supplement of NaCl for SB 2 and SB 3 while 10% (w/v) for SB 1, SB 4 and SB 5, pH 8 and temperature 30 °C. Recently finding showed that, in the alkaline soils of the tropics, concentrations of salt and pH may be as high as 2% and 10.5, respectively, and temperatures may range between 35 and 45 °C (Srinivasan et al. 2012). The interesting spatiality on the growth behavior of SB3 was observed with non-adaptability to withstand salinity (more than 5% NaCl concentration). SB 1, SB 4 and SB 5 actively pumped out salt, keeping the inside of the cell at a normal salt concentration with the increasing salinity and survive over a longer span of duration, while the others gradually stop growing as the limits of their salinity tolerance are reached. *Pseudomonas alcaligenes* PsA15, *Bacillus polymyxa*BcP26, and *Mycobacterium phlei* MbP18, three PGPR isolates were thermo and halotolerant and therefore confer on them prospective economical benefit to survive in arid and salt-affected soils such as calcisol (Egamberdiyeva 2007). Maybe halotolerant microbes isolated in this study adapt the interior protein chemistry of the cell to high salt concentration. High NaCl concentrations normally disrupt membrane transport systems and denature proteins, some microorganisms, possess unusual plasma membranes and many unusual enzymes to survive over a longer duration under such extreme stress conditions. It has been found that the osmo-tolerance reactions of MSP- 393 viz. de novo synthesis of osmolytes and excess production of salt stress proteins efficiently abolished the harmful influences of high osmolarity. *Pseudomonas fluorescens* MSP-393 can be used as a model bioinoculant for crop production in saline soils (Paul and Nair 2008). More research work is needed to evaluate their efficiency and mechanisms under salt stress conditions. The relative efficiency of plant inoculation was higher under extreme conditions of soil temperature in different trials. The seasonal bound salinity, the temperature in farm plots may be the most possible reason behind that depicts their true applicability of these stressful characteristics of isolates. However, their dominance in soil with season can't be stated affirmatively. Our results showed that bacteria-isolated from saline soil having a great potential to survive in the stress environment over a longer span of duration.

Among the sequenced isolates, 1 belonged to Firmicutes, 3 were proteobacteria and 1 was actinobacteria. The phylogenetic tree of nucleotide acid sequences of SB 1 showed the grouping of SB 1with *Halomonas piezotolerans* MN435603, *Halomonas aquamarina* AJ306888, *Halomonas meridiana* AJ306891 in a single cluster at the genus level. SB 2 revealed grouping with *Serratia rubidaea* AB004751 while SB 3 displayed the grouping with *Serratia ureilytica* AJ854062 and *Serratia marcescens*

JMPQ01000005 in a single cluster at the species level. Nucleotide analysis of SB 4 represents cluster together with *Glutamicibacter mishrai* KT444697, a member of *Arthrobacter,* and formed a single cluster pattern at the genus level. Nucleotide acid sequence-based phylogenetic tree analysis showed a single cluster pattern for SB 5, *Halobacillus alkaliphlius* AM295006 and *Halobacillus halophilus* HE717023 at the genus level. On the basis of phylogenetic analysis, *Serratia* sp. was found to have similarity at species level compared to the *Halomonas* sp., *Arthrobacter* sp. and *Hallobacillus* sp. (Fig. 2).

All the isolates were demonstrated maximum P solubilization within 4 days of incubation in the range of 50.674 to 116.669 P_2O_5 (ppm). A drastic drop in pH was also noticed throughout the experiment. These results are in agreement with previously reported results (Son et al. 2006), which identified bacteria capable of solubilizing P under up to 50 g l^{-1}. The reducing phosphate solubilization with improvement in the incubation time has been stated for few microorganisms, which could be attributed to the depletion of nutrients in the culture medium (Vyas et al. 2007). The P concentration in the culture broth as a signal of phosphate solubilizing ability should be carefully noticed, and a kinetic study of this parameter would suggest a more trustworthy picture of cellular behavior toward P. Soil phosphates mostly the apatites and metabolites of phosphatic fertilizers are fixed as calcium phosphate under alkaline condition. P solubilization by the response of microorganisms is the outcome of the combined effect of pH decline and organic acid production. The drop in pH clearly suggests acid production, which could be a possible reason for P solubilization. It is suggested that microorganisms, which decrease the medium pH during growth, hold a great promise to convert insoluble P into soluble (Selvi et al. 2017; Park et al. 2011). The mineralization of organic P in soil is the result of organic acid production and acid phosphatases. Production of low molecular weight organic acids results in acidification of the microbial cell and its surrounding. Consequently, may be proton substitution for Ca^{2+} is important to released Pi from a mineral phosphate. The excretion of H^+ to the external surface in interchange for cation uptake or with the support of H^+ translocation ATPase could form different ways for solubilization of mineral phosphates (Vessey 2003).

This is already reported that member of genus *Arthrobacter* and *Serratia* are good solubilizers of TCP. They solubilized 519.7 and 421.8 g l^{-1} of insoluble TCP, respectively. Hence, the presence of these members in the rhizosphere might be helpful to plant P nutrition and development (Chen et al. 2006). On the basis of phenotypic and molecular characterization, members of the genus *Halomonas* and *Halobacillus* are being reported first time as effective phosphate solubilizers from saline soil. The bacterial isolates were isolated from alkali soil, which was alkali and had high salinity levels. The soil was also subject to desiccation under periodic spells of drought.

These phosphate-solubilization microbial isolates from saline soils were capable of solubilizing a considerable amount of Pi and are likely to be more successful as microbial-based inoculants than the microorganisms isolates from other soils because of their capability to survive the stress factor that affects crops productivity. These phosphate solubilizing soil bacteria could use as effective bio-fertilizer applicants for improving the phosphate nutrition of crop plants helps to minimize the phosphate fertilizer application. The pH of the saline soil in CSSRI varies with seasonal bound alkalinity; such alkaline soils are rich in calcium phosphate. In another way, we can say that in alkaline soils, the activity of calcium is high and thus favors the formation of insoluble TCP. These alkaline soils containing free calcium carbonate phosphate ions coming with solid-phase $CaCO_3$ are precipitated on the surface of these particles. Therefore, widespread studies to analyze the effect of single and dual inoculations of saline-alkaline soil-based PSB species on yields of different crops are required immediately.

Regarding this characteristic of phosphate solubilizing capacity of the isolates in the bulk, soils have been studied, all the cultures had shown remarkably high ability to solubilize insoluble-phosphate, a character quite interesting and important from 'application in agriculture' point of view. These, after their field evaluation in saline soil, if found suitable, can be commercially used to substitute the application of costly 'Phosphate fertilizer' like Single Super Phosphate by much lesser costly and rather cheaply available 'Rock-Phosphate' and dolomite. Further studies on these kinds of stress-tolerant cultures are to be very interesting and important.

Stress-tolerant PSB isolates were capable to produce a remarkable amount of IAA with and without L-tryptophan. SB 3 showed the highest value of IAA production (0.183 g l^{-1}) followed by SB 4 (0.178 g l^{-1}) and SB 5 (0.151 g l^{-1}) after 4 days of inoculation in the presence of 100 mg l^{-1} tryptophan, while 0.062, 0.057 and 0.054 g l^{-1} IAA production were observed without L- tryptophan, respectively (Fig. 4). In a previous study, it is conveyed that the range of IAA productions in several PSB isolates was 57–288 µg/ml culture media (Shahab et al. 2009). Some of the PSM act as plant growth promoters due to their ability to produce IAA but there is a different IAA production potential among PSM (Souchie et al. 2007).

PSM isolates such as *Bacillus polymyxa, Bacillus pulvifaciens, Pseudomonas striata, Aspergillus awamori, Aspergillus niger* and *Penicillium digitatum* tested for

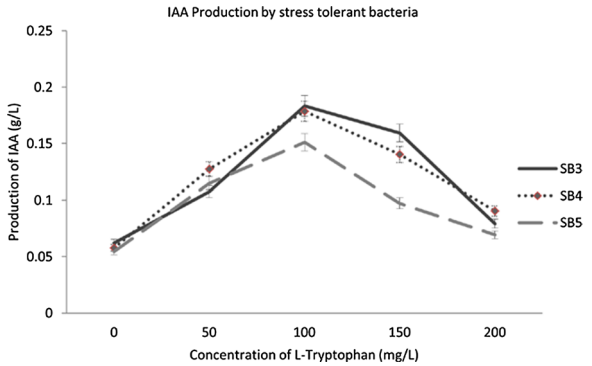

Fig. 4 Effect of L-Tryptophan on production of IAA by different types of stress-tolerant bacteria. Results represented as mean ± SD (n = 3)

the synthesis of auxin and gibberellins. Maximum auxin produced by *P.striata* and the least auxin activity was recorded by *A.awamori* (Vassilev et al. 2006). Production of IAA by isolated PSB ranged between 0.054 and 0.183 g l^{-1} indicating that the isolated PSB has a plant growth-promoting effect. The IAA production capability of PSB is remarkable in the application of the isolated PSB to phytostabilization (Park et al. 2011). The reactions most frequently invoked to describe the direct effects of plant growth-promoting bacteria on plants is the production of phytohormones, including auxins such as indole acetic acid or IAA (Patten and Glick 2002; Haque et al. 2020). *B. amyloliquefaciens,* a gram-positive bacterium is capable to produce and secrete a significant amount of IAA. Improved IAA production after supplementing of L-tryptophan and drastic decline of IAA production in engineered *trp* mutants indicate that the main pathway of IAA biosynthesis in this bacterium is dependent on L-tryptophan (Idris et al. 2007). To demonstrate that IAA synthesis in bacteria is dependent on tryptophan concentration, a mutant of the gram-negative plant-beneficial bacterium *Pseudomonas putida* was used, deficient in *ipdc* gene product (indole-3-pyruvate decarboxylase) (Patten and Glick 2002). IAA production by plant growth-promoting microbes is an important feature in the development of plant growth. There exist sufficient research studies that several soil microorganisms are keenly involved in the auxins synthesis. IAA was identified in around 80% of bacterial isolated of the rhizosphere.

In most cases, the production of IAA has been analyzed in saline-free conditions (Barua et al. 2012). This is the first evidence that PSM augments plant growth due to the biosynthesis of growth-promoting substances. As per our best knowledge, this is the first time report that saline soil-associated isolates could potentially promote plant growth under saline conditions because they are capable to produce plant growth promoters under such stress conditions. These isolates are very useful to saline soil agriculture point of view which enhances crop productivity.

Interaction studies on PGPR and other microbial community and their effect on the biochemical function of crop plants in the presence of soil salinity regimes are still in an incipient stage. Potent PGPR and other microbe's inoculations might work as the prospective mechanism for reducing the stress of salinity in salt-sensitive crops. Therefore, broad-spectrum investigations are required in this field, and the usage of PGPR and other symbiotic microorganisms can be beneficial in developing strategies to enable sustainable agriculture in saline soils.

Conclusion

On the basis of this study, we concluded that the strains isolated from alkaline soils have the potential to solubilize phosphate and produce IAA under different stress conditions like salt, pH and temperature, which make them good microbial inoculants for crop growth and can be used as strong phosphate solubilizers in a stressful agriculture environment. Two bacterial isolates namely *Halobacillus* sp. and *Halomonas* sp. are being conveyed for the first time as phosphate solubilizers. PSB caters to plant growth-promoting potential through IAA production. The preliminary finding of the present work is suggesting that the use of these stresses-tolerant PGPB will increase the available P and IAA in soil, helps to reduce environmental pollution and promote sustainable agriculture. However, it requires detailed characterization of plant growth promotion properties with an emphasis on saline soils.

Abbreviations

TCP: Tri-calcium phosphate; IAA: Indole acetic acid; PGPR: Plant growth-promoting rhizobacteria; PSB: Phosphate solubilizing bacteria; P: Phosphorus; Pi: Insoluble phosphate; PSM: Phosphate solubilizing microorganisms; CSSRI: Central Soil Salinity Research Institute; ICAR: Indian Council of Agricultural Research; EC: Electrical conductivity; w/v: Weight/volume; g: Gram; NaCl: Sodium chloride; LB: Lysogeny broth; g l^{-1}: Gram per liter; h: Hours; °C: Degree celsius; MR: Methyl red; VP: Voges-ProsKauer; NO_3^-: Nitrate; DNA: Deoxyribonucleic acid; rRNA: Ribosomal ribonucleic acid; PCR: Polymerase chain reaction; µL: Microliter; µM: Micromol; dNTP: Deoxynucleoside triphosphate; min: Minute; NCBI: National Centre for Biotechnology Information; BLAST: Basic local alignment search tool; MEGA: Molecular evolutionary genetics analysis; ml: Milliliter; mg l^{-1}: Milligram per liter; ANOVA: Analysis of variance.

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Authors' contributions
GJ: Conceptualization, Methodology, Formal analysis, Investigation, Writing—original draft, Writing—review & editing. VK: Conceptualization, Methodology, Formal analysis, Investigation, Writing—review & editing. SKB: Supervision, Project administration, Writing—review & editing. All authors have read and approved the manuscript.

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