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Original article

Identification, characterization and optimization of phosphate solubilizing rhizobacteria (PSRB) from rice rhizosphere

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ABSTRACT

Two billion people worldwide take rice (*Oryza sativa* L.) as a staple food. Phosphorus (P) and Nitrogen (N) are the major requirements of rice; although these are available in limited concentrations within rice growing regions. Among different types of Plant growth-promoting rhizobacteria (PGPR), Phosphate solubilizing rhizobacteria (PSRB) constitute an important class. These are known for plant growth promotion by enhancing P and N uptake. PSRB are nowadays used as biofertilizers to restore the soil health. Under the present investigation identification, characterization and optimization of phosphate solubilizing activity of these microbes at different pH, temperature and salt concentrations was carried out. Thirty-seven isolates were recovered from different regions of rice rhizosphere on Pikovskaya (PVK) agar among which 15 isolates were recovered from R.S. Pura, 12 isolates from Bishnah and 10 isolates were recovered from Akhnoor sector of Jammu, India. A prominent halo zone of clearance was developed around the colonies of 12 different isolates, indicating phosphate solubilization activity. Four distinct isolates were amplified, cloned and sequenced for taxonomic identification using 16S primers. The results indicated that PS 1, PS 2, PS 3, PS 4 were related to *Pseudomonas aeruginosa, Bacillus subtilis* strain 1, *B. subtilis* strain 2, *B. subtilis* strain 3, respectively. These strains when grown at a wide range of ecological factors showed maximum growth at pH between 6.8 and 8.8, temperature between 28 °C and 37 °C and salinity between 1% and 2%. Screening for phosphate solubilization activity revealed that the halo zone diameter formed by these isolates extended from 2.1 to 3.2 mm. The phosphate solubilizing efficiency (SE) ranged from 35.4 to 50.9 with highest value of 50.9 by PS4 and maximum P solubilization of 10.22 μg/ml was recorded by PS4 at 7th day. Phosphate solubilization activity of these identified PSRB strains can be utilized and explored in the rice growing belts of Jammu region which are deficient in phosphorus. MIC value for zinc sulphate heptahydrate in 12 isolates varied from 1 mg/ml to 6 mg/ml. Phosphate solubilization activity and MIC of these identified PSRB strains can be utilized and explored in the rice growing belts of Jammu region which are deficient in phosphorus.

1. Introduction

Interaction of microbes residing in the rhizosphere zone in soil with plants and roots occurs through root colonization. It harbors several plant beneficial microbes that have got an inherent ability to improve the soil fertility and increase growth leading to a fully developed plant body (Parray et al., 2016; Babu et al., 2017). These interactions play a crucial task in regulating different geochemical and biophysical processes occurring within the soil (Dutta and Podile, 2010). A broad canopy of rhizosphere harboring bacteria (PGPR) releases the growth enhancing substances that facilitate different developmental processes within a plant (Qiao et al., 2017). Sugars and amino acids in root exudates attract the colonizing rhizospheric microorganisms. They use these compounds to synthesize different growth promoting phytohormones (Bais et al., 2004; Ortíz-Castro et al., 2009). The root exudations help

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in the survival of the rhizosphere microorganisms in the nutrient limited conditions (Barnawal et al., 2019; Khan et al., 2020). PGPR enhance the developmental processes of the plants by producing different phytohormones such as auxins (IAA) and cytokinins, by dissolving phosphorous and potassium, and by synthesizing 1 –a minocyclopropane-1-carboxylate (ACC) deaminase (Saleem et al., 2007; Bhat et al., 2020) ultimately reducing environmental stresses by enhancing plant resistance (Pérez-Montaño et al., 2014; Khanna et al., 2019a,b,c; Yaseen et al., 2020).

The genera *Bacillus* and *Pseudomonas* are among the most abundant and phylogenetically diverse group of PGPR which show avid rhizosphere colonization and provide a considerable interest in enhancing crop production and yield (Podile and Kishore, 2007; Zhou et al., 2016; Sansinenea, 2019). Different bacterial strains such as *Bacillus*, *Rhizobium* and *Pseudomonas* are reported as the dominant solubilizers of phosphorus (Rodrıguez and Fraga, 1999). Diverse bacterial strains such as *Herbaspirillum*, *Azospirillum*, *Enterobacter*, *Azotobacter*, *Acetobacter* and *Pseudomonas* increase the developmental reactions within the plant by either increasing the bioavailability of both micro and macro nutrients (e.g. iron, zinc and phosphorus), production of the phytohormones or by fixing atmospheric nitrogen (Ahmad et al., 2008; Barea and Richardson, 2015; Arya et al., 2020; Ummara et al., 2021; Nawaz et al., 2020). PGPR associated with cereals has received a greater interest in recent years and different studies noticeably established the positive and valuable effects of PGPR on overall growth and development of various plant species (Mehnaz et al., 2010; Zhang et al., 2012; Baliah et al., 2016; Teng et al., 2018; Chawngthu et al., 2020; Khanna et al., 2019a,b,c). In order to understand the spread and diversity of bacterial colonies around the rhizosphere of different crop species, information regarding their identification, characterization and optimization is required (Chahboune et al., 2011; Thomas et al., 2018). Increasing knowledge regarding the adverse effects of various chemical fertilizers based agricultural practices, makes it imperative to find regionally specific microbial strains that are important for cultivation of a desired crops.

The disproportionate application of chemical fertilizers not only affects the economy, environment and soil health but also delimits the availability of essential nutrients like phosphorus despite its large amount present in soil. Inorganic fertilizers which are costly and deteriorate the soil health are used by the rice growing farmers in the region, since phosphorus deficiency is found to be prominent in the rice belt region of Jammu (Rai et al., 2018). Previous attempts were made to isolate and characterize PSB from the rhizosphere of rice (Gupta and Sharma, 2018). The present study was aimed with the objective of isolation of phosphate solubilizing rhizobacteria (PSRB) from rhizosphere of the rice grown at different regions of Jammu District. The study also aimed at identification of bacterial isolates through biochemical and molecular characterization (16 s rDNA). Tri-calcium phosphate solubilizing capacity of these isolates was optimized at different pH, temperature and salt concentrations. The isolates were subjected to *in vitro* optimization for growth at different pH, temperature and salt concentration in growth medium.

2. Materials and methods

2.1. Study area

The current study was conducted at RS Pura, Bishnah and Akhnoor sector in the Jammu division of Jammu and Kashmir UT of India. Study areas lies within the geographical coordinates of 32°36′17.50″; 74°43′25.56″ and 32°55′58.41″N; 74°44′33.20″E and altitudinal gradients of 273–333 m.a.s.l.(meters above sea level) The Jammu presents mainly mountainous and hilly landscape, with valleys stretching for few areas surrounding the Jammu district. Jammu receives humid and subtropical climate with temperatures ranging between 46 °C (June-July) and 4 °C (January-February).

2.2. Collection of samples

The rhizospheric soil of rice plants (*Oryza Sativa*) was collected from three different rice grown areas in Jammu (R.S.Pura, Bishnah and Akhnoor) following the protocols put forward by Son et al. (2006). Each soil sample was thoroughly mixed and homogenized. From a particular location about 50 g of soil were taken as representative sample. Soil samples were collected in polyethene bags, transferred to the Soil Microbiology Laboratory, SKUAST-Jammu where these samples were processed for the separating rhizobacteria that possess phosphate solubilizing ability (PSRB).

2.3. Isolation of phosphate solubilizing rhizobacteria

Plant growth-promoting rhizobacteria were isolated using serial dilution method and samples were serially diluted upto (10^{-5}). Pour plate and spread plate method were used for the enrichment, isolation, screening, and maintenance of PSRB using Pikovskaya's agar medium (Pikovskaya,1948) containing tri-calcium phosphate (TCP). In order to confirm the phosphate solubilizing activity, these plates were incubated at 37 °C for 4 days till the clear halo zones were formed around their colonies. The bacterial colonies producing a distinct tri-calcium phosphate solubilization zone around the colony with different morphology were further selected and sub-cultured to obtain pure cultures which were maintained in 30% glycerol at −20 °C for further use.

2.4. Genomic DNA isolation and molecular characterization

Genomic DNA of the PSRB isolates was isolated by following Wilson (1997). The electrophoresis of the Genomic DNA was carried out in 0.8% agarose gel stained with ethidium bromide (0.5 mg/ml), under submerged conditions using 1 × TAE buffer as tray buffer. To each 5 μl of genomic DNA sample, 2 μl of 1X loading dye was added. 100–1500 bp DNA ladder marker was used as standard and the gel was run at 100 V until the loading dye reached the gel front. The genomic DNA of the isolates were viewed under the gel documentation system in the form of bands and the image was taken and saved in computer and used for further molecular studies.

2.5. Amplification of genomic DNA

It was carried out using specific oligonucleotide primer sequences. The 25 μl PCR reaction mixture consisted of 50 ng genomic DNA, 15 mM Tris/HCl pH 8.5, 10 mM KCl, 0.1% (v/v) Triton X, 3 mM $Mgcl_2$, 0.25 mM each dNTP, 2 U *Taq* DNA polymerase and 0.5 μm each primer was subjected to amplification which was carried out in thermal cycler (Eppendorf Gradient) with a total of 35 cycles using a set of forward (*CCGAATTCGTCGACAACA GAGTTTGATCCTGGCTCA) and reverse primers (CCCGGATCCAAGCTT ACGGCTACCTTGTTACGACTT*) under cycling conditions that consisted of an initial denaturation at 94 °C for 5 min and then 33 cycles with denaturation at 94 °C for 40 s, annealing at 55 °C for 45 s and extension at 72 °C for 45 s. All the amplifications products were then electrophoresed in 1.0% agarose gel and the amplified DNA bands were viewed under the UV transilluminator and the image was taken using gel documentation system. The gel aliquots with amplified products were sent to agrigenome for sequencing and further analysis.

2.6. Characterization of isolated phosphate solubilizing rhizobacteria

2.6.1. Morphological and biochemical characterization

Morphological features of the bacterial isolates were studied on the basis of colour, texture and shape of bacterial colonies. The isolated bacteria were characterized on morphological and biochemical parameters performed by gram staining and different biochemical tests.

2.6.2. In-vitro optimization for growth at different pH, temperature and salt concentration in growth medium

In-vitro characterization of the selected isolates was checked for growth at diverse pH, temperature and salt concentration where broth culture of selected isolates was inoculated in four test tubes containing 10 ml of Pikovskaya's medium maintained at 5.5, 6.5, 7.5 and 8.5 pH with 1 N HCl and 1 N NaOH, incubated under shaking conditions for 48 h to check growth with optical density measured in spectrophotometer at different pH conditions in the growth medium. Similarly, broth culture inoculated in four test tubes containing 10 ml of Pikovskaya's medium was incubated at 28 °C, 33 °C and 37 °C under shaking conditions for 48 h with optical density measured in spectrophotometer to check growth at different temperature conditions in the growth medium. 10 ml of Pikovskaya's medium with TCP at different concentrations (1.0, 1.5, 2.0 and 2.5%) was inoculated in four test tubes with broth culture of selected isolates to check growth at different salt concentration in growth medium.

2.7. Screening for phosphate solubilization activity

Solubilization efficiency is used as an important parameter for assorting the possible isolates of PSB. It helps to determine the quantitative measurement of culture mediated solubilizing degree of insoluble organic phosphate (Chen et al., 2006). The PS capability of the isolated strains of rhizobacteria was determined by both qualitative and quantitative methods following the methodology of Srivastav et al. (2004).

2.8. Qualitative analysis

Pikovskaya's agar plates were prepared. Prior to agar plating, wells with 10 mm diameter were made with sterile cork borer. 10 μl cell free supernatant of 3 days old from each of the bacterial strain was poured in each well. Afterwards incubation of the plates was performed at 28 ± 2 °C for 1 day till yellow zone appears around the wells. The phosphate solubilizing efficiency was determined by formula put forward by Nguyen et al. (1992).

$$Solubilizing\ efficiency\ (SE) = \frac{Halo\ zone\ diameter}{Colony\ diameter} \times 100$$

2.9. Quantitative analysis

Available or soluble orthophosphate in the form of μg/ml in supernatant and calibrated from KH_2PO_4 at different days' interval following spectro vanadomolybdate phosphoric yellow color method (Jackson, 1973). To 10 ml supernatant 5 ml ammonium molybdate reagent was added followed by rigorous shaking. Then chlorostannous acid (1 ml) was supplemented from working solution making the total volume of the reaction mixture 50 ml with distilled water. O.D. was determined at 600 nm. Presence of the phosphorus present was obtained through curve of potassium dihydrogen phosphate.

2.10. Screening for zinc tolerance ability

The isolated phosphate solubilizing rhizobacteria (PSRB) were streaked on 1 mg/l of zinc sulphate heptahydrate supplemented nutrient agar containing to determine zinc tolerance ability. Incubation of the bacterial plates was performed at 37 °C for 2 days. MIC of the isolates was determined by agar plate dilution method (Luli et al., 1983). Streaking of the isolates was performed on the agar medium added with different concentrations (1–25 mg/ml) of zinc sulphate heptahydrate. Inoculated with the test culture, the petri plates were incubated at 37 °C for 3 days. Nutrient agar medium supplemented with zinc concentrations ranging from 2 to 10 mM was used to determine the MIC of bacterial isolates. These isolates were incubated for 3 days at 37 °C and their MIC was determined when they fail to show their growth on the plates.

3. Results

3.1. Isolation, identification and molecular characterization of phosphate solubilizing rhizobacteria

A total of 37 isolates were recovered using standard plating methods from 58 rhizosphere soil samples of rice. Out of 37, 15 isolates were recovered from rice rhizosphere of R.S. Pura, 12 isolates from Bishnah, and from the rhizospheric soil samples of Akhnoor, 10 isolates were recovered (Fig. 1).

Out of total 37 isolates recovered on PVK agar, only 12 produced maximum halo zone of clearance around their colonies. This indicates their greater phosphate solubilization activity. The isolates that were recovered were purified and cultured on PVK agar. They showed a significant difference in halo zone diameter which was developed upto 7 days of incubation. The isolated genomic DNA of the selected four phosphate solubilizing bacteria was screened regarding their ability to phosphate solubilizing activity. Distinct morphological and cultural characteristics were processed for 16SrDNA gene sequencing and the amplified fragment was obtained (Fig. 2).

BLAST homology search was used for identification of the bacterial isolates at species and generic levels with the construction of phylogram. Gene sequences were compared with existing Genbank

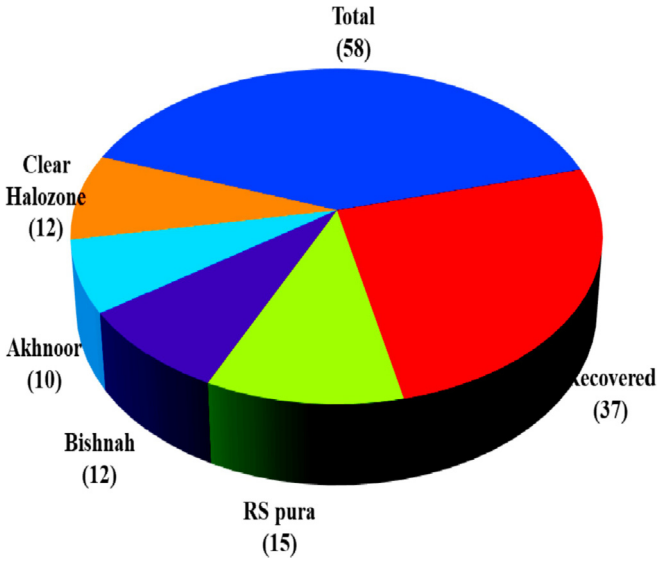

Fig. 1. Phosphate solubilizing bacteria isolated from the rhizosphere of rice (*Oryza sativa*) in Jammu.

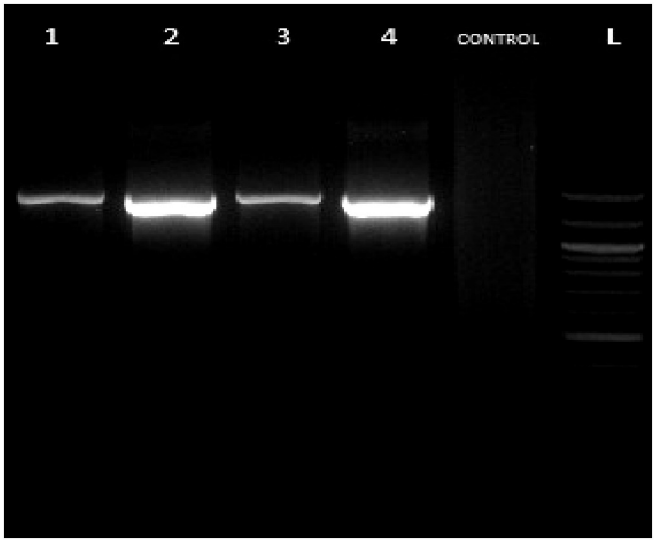

Fig. 2. Amplified PCR products of 4 isolates run in 1.0% agarose gel M-1400 bp marker.

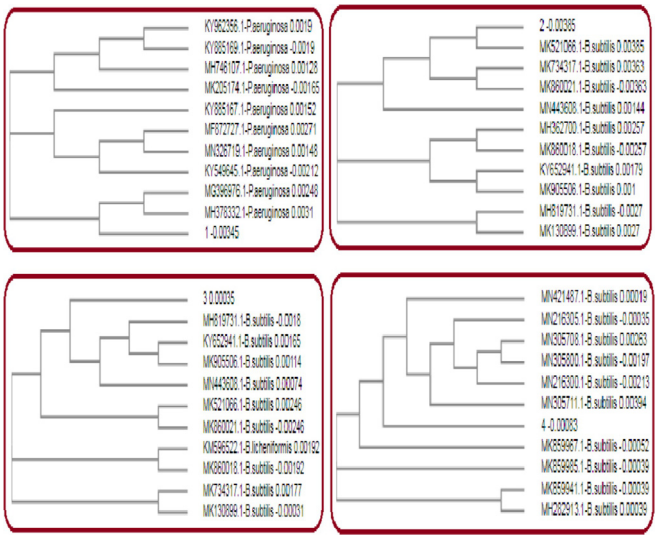

Fig. 3. Phylogram of the four identified species at generic and species level.

data (Fig. 3). The sequences were deposited in the NCBI Genbank under different accession numbers as MW406991, MW406992, MW406993, MW406994 as reported in (Table 1).

Table 1
Nomenclature and decoding of plant growth promoting rhizobacteria isolates at genbank-NCBI.

S.No	Isolate code	Genus and species	Accession number
1	PS1	Isolate 1 (*Pseudomonas aeruginosa*)	MW406991
2	PS2	Isolate 2 *(Bacillus subtilis strain 1*	MW406992
3	PS3	Isolate 3 (*Bacillus subtilis strain 2*)	MW406993
4	PS4	Isolate 4 (*Bacillus subtilis strain 3*)	MW406994

3.2. Characterization of isolated phosphate solubilizing rhizobacteria

3.2.1. Morphological and biochemical characterization

The morphological characteristics of the bacterial isolates showed that the color of the colonies developed was light yellow, creamish yellow and transparent. Colonies of the isolates were slimy in texture. Cell morphologies were scattered round, round, round with scattered margins and round with scattered margins and raised arrangements. Among four, the three strains PS1, PS2, PS4 were found to be gram negative, however, PS3 was found to be gram positive. All the selected strains were oxidase positive, Catalase positive, nitrate reductase positive except PS3 which was oxidase negative and all the strains exhibited the ability for citrate utilization (Table 2).

3.3. In-vitro optimization of growth conditions at different pH, temperature and salt concentration

Bacterial isolates were grown at different pH, temperature and salt concentrations and the growth of four isolates at optimum culture conditions was found at maximum OD i.e. 610 nm. Studies show that PS 1 (*Pseudomonas aeruginosa*) when grown at a wide range of pH 5.5 to 8.5 showed its maximum growth at 6.5 pH. Similarly, when grown at different temperature (28–37 °C) showed its maximum growth at 33 °C and when grown at differemt salinity from 1 to 2.5% showed its maximum growth at 1.5% and showed its minimum growth at 8.5 pH, 37 °C and 1% salinity. PS2 (*Bacillus subtilis strain 1*) PS2 (*Bacillus subtilis strain 1*) showed its maximum growth at 7.5 pH, temperature 37 °C and salinity 2% and its minimum growth at 6.5 pH, 28 °C, 1.5% salinity. PS 3(Bacillus *subtilis strain 2*) showed its maximum growth at 7.5 pH, temperature 37 °C and salinity 2% and its minimum growth at 6.5 pH, 28 °C, 1.5% salinity. PS 3 (*Bacillus subtilis strain 2*) showed its maximum growth at 7.5 pH, temperature 33 °C and salinity 2.5% and its minimum growth at 5.5 pH, 1% salinity and 37 °C PS 4 (*Bacillus subtilis strain 3)* showed its maximum growth at 7.5 pH, temperature 28 °C and 1.5% salinity and its minimum growth at 5.5 pH, 2% salinity and 33 °C (Fig. 4).

3.4. Screening for phosphate solubilization activity

The isolates which were separated from the rice rhizosphere soil were screened for production of phosphate solubilization activity by qualitative and quantitative method in Pikovskaya's agar and broth.

3.4.1. Qualitative method

Phosphate solubilization capability was determined as clear zone formed around the well at 28 °C after 48 h of incubation on PVK agar**.** All bacterial isolates (12) showed clear zone on PVK agar and selected four isolates exhibited maximum clearance zones of varying diameter. The halo zone diameter formed by the four isolates range from 2.1 to 3.2 mm. SI of PS1 was found to be highest 2.82 among all the isolates. The phosphate solubilizing efficiency (SE) was calculated for the selected isolates which ranged from 35.4 to 50.9 with highest value of 50.9 by PS4 followed by PS3 (41.2), PS2 (40.4) and PS1 (35.4) respectively (Fig. 5).

3.4.2. Quantitative analysis

The phosphate solubilization capability of the bacterial isolates varied and showed an increase with the increase in time interval but maximum P solubilization was recorded by PS4 at 7th day 10.22 μg/ml followed by PS3 −9.42 μg/ml, PS2 −8.26 μg/ml, PS1 −7.23 μg/ml respectively (Fig. 6).

Table 2
Different cultural characteristics of the isolates of phosphate solubilising rhizobacteria.

Isolate code	Colony morphology			Biochemical characterization				
	Colour	Texture	Shape	Gram staining	Oxidase	Catalase	Nitrate	Citrate utilization
PS1	Light yellow	Slimy	Scattered round	Gram negative	Positive	Positive	Positive	Positive
PS2	Off-white	Slimy	Round	Gram negative	Positive	Positive	Positive	Positive
PS3	Transparent	Slimy	Round with scattered margins and raised	Gram positive	Positive	Negative	Positive	Positive
PS4	Creamish yellow	Slimy	Round with scattered margins	Gram negative	Positive	Positive	Positive	Positive

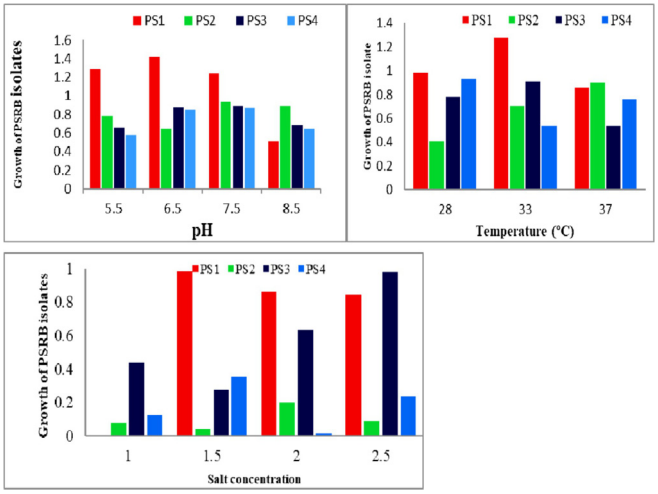

Fig. 4. Effect of different pH, temperature and salt concentration on growth of PSRB isolates.

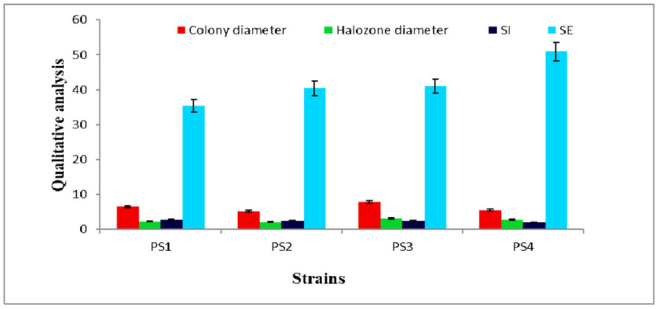

Fig. 5. Qualitative analysis of PSRB isolates.

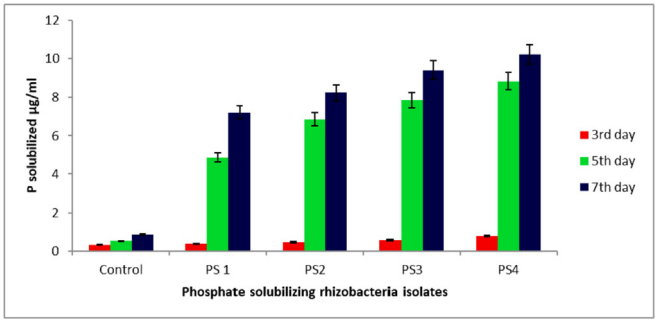

Fig. 6. Quantitative estimation of phosphate solubilization (KH_2 PO_4) in culture medium.

3.5. Screening for zinc tolerance ability

On the nutritional agar containing 1 mg/ml of heptahydrated zinc sulphates, 12 from 37 different isolates were able to grow. The other isolates were seen to be sensitive to zinc. Nutrient agar supplemented with different concentrations of zinc sulphate heptahydrate (2–10 mM) was used to estimate the minimum inhibitory concentration (MIC) for zinc sulphate heptahydrate. The MIC value ranged between 1 mg/ml to 6 mg/ml (Fig. 7). Highest MIC was seen for PSB- 1 (6 mg/ml) followed by PSB- 12 (4 mg/ml), PSB- 2 (3 mg/ml) and PSB-7 (2 mg/ml) (Fig. 7) and were further subcoded as: PS-2, PS-3, PS-1, PS-4 which were subjected to molecular identification through 16srDNA gene sequencing.

4. Discussion

Nature has provided the soil with ample faunal diversity that provides a significant function to maintain the fertility of soil and development of plant. Among the micro fauna, bacteria hold a promising significance as they actively invade the rhizosphere thereby enhancing the plant growth and development (Kumar et al., 2014; Zhu et al., 2017). The interaction of microbes with plant roots within a rhizosphere appears as detrimental factors for growth and yield of the crop species (Mumtaz et al., 2017; Yin et al., 2017; Maharajan et al., 2018). PGP potential of the rhizobacteria that are isolated from the rhizosphere of the rice field of Jammu Division was carried by polyphasic method. Molecular characterization of the isolated bacterial strains through 16S rDNA gene sequencing depicted that the four rice rhizosphere dwelling bacteria show greater degree of similarity with genus *Bacillus* (3) and *Pseudomonas* (1); *Bacillus* and *Pseudomonas* species are found to be efficient phosphate solubilizers (Maheswar and Sathiyavani, 2012; Aarab et al., 2015). Using 16S rRNA and BLAST analysis, Rekha et al. (2018) and Chawngthu et al. (2020) confirmed the identification of the bacterial isolates from rice rhizosphere. Our

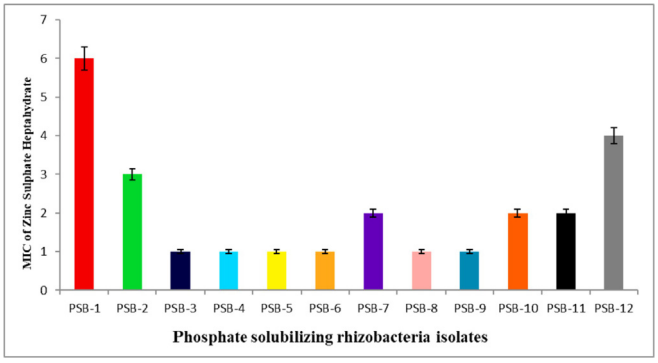

Fig. 7. Minimum inhibitory concentration (mg/ml) of $ZnSO_4{\cdot}7H_2O$ exhibited by Phosphate solubilizing rhizobacteria isolates.

study also corroborated to the results of Baliah et al. (2016); Teng et al. (2018) and Rfaki et al. (2020) that identified PSB strains using 16S primer sequencing.

The rhizosphere of several crop species show greater association with gram-negative rhizobacteria as compared to gram positive rhizobacteria (Muleta, 2009). The PSRB strains were morphologically and biochemically characterized, revealing that the three isolates (PS1, PS2, PS3) were found to be gram negative cocci or rods and PS4 was found to be gram positive rods. These findings are equally supported by the studies of Khan et al. (2010) & Mujahid et al. (2014) who found dominance of gram negative over gram positive PSRB strains. The rhizodeposition stimulates gram negative bacteria and makes them motile whereas this deposition inhibits the activity on gram positive bacteria (Johansen and Olsson, 2005). Moreover, gram negative bacteria are attracted by the root exudates which in turn increase their population around the roots and in turn release substances which can be absorbed by roots for plant growth promotion whereas gram positive bacteria are aerobic bacteria due to deficiency of oxygen around the roots their population around the roots decreases.

Plants are experiencing environmental stress all the time because of being sessile in nature (Ahmad et al., 2010, 2019; Kohli et al., 2019). In order to overcome stressful conditions and neutralize the effect of hydrogen peroxide, PGPR strains produce an enzyme catalase (Mumtaz et al., 2017). Among the selected four isolates, three isolates (PS2, PS3, PS4) were found to be catalase positive and PS1 was found to be catalase negative. The greater number of catalase positive PSRB is strongly supported by the findings of Bumunang and Babalola (2014); Shrivastava (2013); Shruti et al. (2013) who also reported similar findings for *Oryza sativa* from other countries. PSRB are known to be found in wide variety of soil types but their function is heavily impacted by different environmental variables that include organic matter, cation exchange capacity, temperature, salinity, pH and soil composition, (Mujahid et al., 2014; Zaidi et al., 2014; Rfaki et al., 2018). Thus, tolerance to intrinsic and extrinsic stress is highly significant for microorganism growth, survival and establishment in the rhizospheric soils which was tested in the current research at *ex situ* (laboratory) conditions by optimizing the developmental processes of the selected strains at different pH, temperature and salt concentrations. PS1 (*Pseudomonas aeruginosa*) when grown at a different pH concentration showed its maximum enlargement at 6.5 pH, 33 °C, 1.5% salinity and showed its minimum growth at 8.5 pH, 37 °C and 1% salinity. PS2 (*Bacillus subtilis* strain 1) showed its maximum growth at 7.5 pH, temperature 37 °C and salinity 2% and its minimum growth at 6.5 pH, 28 °C, 1.5% salinity. Maheswar and Sathiyavani (2012); Patil (2014) also found similar results for different bacterial strains grown at the somewhat similar environmental conditions. PS 3 (*Bacillus subtilis* strain 2) showed its maximum growth at 7.5 pH, temperature 33 °C and salinity 2.5% and its minimum growth at 5.5 pH, 1% salinity and 37 °C PS 4 (*Bacillus subtilis* strain 3*)* showed its maximum growth at 7.5 pH, temperature 28 °C and 1.5% salinity and its minimum growth at 5.5 pH, 2% salinity and 33 °C. The results of Shruti et al. (2013); Arindam et al. (2014) supported our findings in more parallel way as they also found maximum growth at of different bacterial strains at alkaline pH.

The qualitative as well as quantitative analysis of phosphate solubilization activity of the selected isolates was expressed in the present study. The diameter of halo zones produced by the four isolates range from 2.1 to 3.2 mm. These findings are in accordance with the results of Muleta et al. (2013); Sadiq et al. (2013) who found that solubilization index in different bacterial isolates varied between 2.56 and 4.50 including *Bacillus* spp. and further revealed that halo zones are formed by the bacterial because of the production of organic acids in the growth plates and hence were selected to be good solubilizers of phosphate.

The phosphate solubilizing efficiency (SE) of the selected isolates ranged from 35.4 to 50.9 with highest value of 50.9 by PS4 (*Bacillus subtilis* strain 3*)*. Panhwar et al. (2012) reported the phosphate solubilizing efficiency of the PSB isolates isolated from th**e** aerobic rice varying from 12% to 62.5%. Selvi et al. (2017) also reported the solubilization efficiency of the PSB isolates isolated from the different leguminous plants varying from 13.04% to 85% and the variation in solubilization efficiency is considered to be due to difference in the ability of the isolates to produce organic acids for solubilization of phosphates

The solubilization of tricalcium phosphate varied significantly and depends upon the phosphate solubilization capacity of the different isolates. The P solubilization capacity was increased by all the isolates with the increase in time interval but maximum P solubilization was recorded by PS4 (*Bacillus subtilis* strain 3*)* at 7th day 10.22 μg/ml due to organic acid production by the isolate which leads to insoluble phosphate solubilization within the medium. Mihalache et al. (2018) reported the phosphate solubilizing action of different strains in liquid media isolated from the runner bean rhizosphere. Greater concentrations of solubilized phosphorus was seen on 5th day (19.84 ± 1.89 μg P ml^{-1}) for strains *Pseudomonas lini* (R8 strain) and *Bacillus mycoides* (R3 strain) (17.51 ± 1 21 μg P ml^{-1}, 7th day (19.12 ± 0.35 μg P ml^{-1}) for *Bacillus pumilus* (R10 strain) and attributed to increase in secretion of organic acids and resulted in reduction of cation chelation and hydrogen potential, thus increases phosphate solubilization in liquid medium.

The MIC for zinc sulphate heptahydrate in 12 isolates varied from 1 mg/ml to 6 mg/ml. Similar findings were found by Bhojiya and Joshi (2012), who found that zinc ions had a very high MIC of 10 mM in *Pseudomonas aeruginosa* HMR1 and *Pseudomonas aeruginosa* HMR16. According to Sen and Joshi (2017), the MIC of 38 isolates for zinc sulphate heptahydrate varied from 2 mg/ml to 24 mg/ml, with PSB 16 having the highest MIC of 24 mg/ml, followed by PSB 11 with 21 mg/ml, and PSB 51 with 15 mg/ml. The capacity of the isolates to withstand high doses of zinc sulphate heptahydrate was indicated by their high MIC. According to Owolabi and Hekeu (2015), *Bacillus megaterium*, *Bacillus azotoformans*, *Citrobacter* spp. and *Proteus mirabilis* have considerable tolerance to Zn^{2+} at 4 to 6 mM concentrations. The *Aeromonas hydrophila* HM3 strain developed on solidified medium at a concentration of 1 mM Zn^{2+}. The maximum range for which *Aeromonas hydrophila* HM4, *Serratia marcescens* HM1, *Aeromonas caviae*, *Micrococcus varians*, and Citrobacter spp. tolerated different concentrations of Zn^{2+} metal are comparable to the minimum inhibitory concentrations of previously reported for *Pseudomonas aeruginosa* HMRI and *P. aureginosa* HMR2 (10 mM Zn2 + ions) by Bhojiya and Joshi (2012) and in *P. putida* strain 06909 by Lee et al. (2001). These reports have revealed the tendency of these bacterial isolates to tolerate high amounts of zinc sulphate heptahydrate.

5. Conclusion

From the current study it is concluded that out of total of 58 isolates, only 37 were able to recover on pikovskaya agar. From these isolates, only 12 isolates showed good phosphate solubilization capacity based on clear zones formed by the bacterial colonies. The genomic DNA of the selected four phosphate solubilizing bacteria was isolated and screened. Distinct morphological characteristics, phosphate solubilization activity and minimum inhibitory concentration of zinc sulphate heptahydrate were further processed and the amplified fragments obtained were further identified at generic and species level through 16s rDNA gene sequencing. The identified regionally specific microbial isolates from

site-specific rice growing regions, with the high phosphate solubilization capacity can be utilized to increase the available insoluble forms of phosphorus and augment zinc nutrition in the soil and further used as bioinoculants for sustainable integrated plant nutrient supply (IPNS) system in inceptisols.

Author Contribution

RG drafted the experimental design, performed the experiments and initial draft of manuscript text. RG, AK,SS helped in data collection and data analysis. AN, HD and OMA provide necessary funding for publishing manuscript. All authors read the manuscript before communication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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