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RESEARCH ARTICLES

Characterization of culture condition dependent, growth responses of phosphate solubilizing bacteria (*Bacillus subtilis* DR2) on plant growth promotion of *Hordeum vulgare*

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Abstract
Phosphorus (P) is one of the essential macro nutrients required for the growth and development of plant. Phosphate solubilising bacteria (PSB) are very effective in improving soil fertility by solubilising insoluble soil P, making them readily available to the plants. In the present work, PSB were isolated from the rhizosphere of *Eragrostis cynosuroides* on Pikovskaya's agar media at 30 °C and pH 7. Among positive isolates, the isolate DR2 was selected, based on highest zone of solubilization (15 mm), phosphate solubilization efficiency (150%) and solubilization index (4.5) on 4th day of incubation period. The isolated DR2 was identified as *Bacillus subtilis* on the basis 16 S rRNA gene sequence (Gene bank Accession Number KP455653). Optimized cultural conditions resulted in maximum P-solubilization after 96 h of incubation at 35 °C in Pikovsakaya's broth (having 1% TCP) of pH 7.0 with glucose and ammonium sulphate, used as carbon and nitrogen sources, respectively. *B. subtilis* DR2 significantly enhanced the growth of barley seedlings in terms of seed germination (60%) with percent enhancements in root length (93.71%), shoot length (41.30%) and biomass (22.44%), over control. The strain *B. subtilis* DR2 can be used as a biofertilizer for a sustainable integrated plant nutrient supply (IPNS) system.

Keywords Rhizosphere · Phosphate solubilization · 16S rRNA gene sequence · *Bacillus subtilis* · Barley seedlings

Introduction

Macronutrients play a pivotal role in the growth and development of plants, as they significantly participate in controlling and managing diseases, through three main elements, viz. phosphorus, nitrogen, and potassium (P, N, K). These must be readily available in abundance, either via soil medium or externally added fertilizers. Every macronutrient poses its own character, and accordingly, it is involved in various metabolic processes in plants (Zewdie and Melash 2021). After nitrogen, phosphorus is regarded as one of the most essential growth-limiting plant nutrients, which affects the overall plant growth by influencing various key metabolic processes in plants, such as the biosynthesis of macromolecules, cell division, photosynthesis, respiration, etc. (Wang et al. 2020; Kumar et al. 2020). In other words, it includes root development, stalk and stem strengthening, flower and seed formation, crop maturity and quality of production, nitrogen fixation in legumes, and disease resistance. Although, phosphorus naturally exists in diverse forms in the soil, it is broadly grouped into insoluble organic phosphorus and insoluble inorganic phosphorus. Approximately 95–99% of soil phosphorous is in the form of insoluble phosphates, so plants cannot use it (Kannapiran and Ramkumar 2011; Sanjotha and Manawadi 2016).

Numerous microorganisms, including bacteria present in the rhizosphere are known to solubilize the insoluble phosphorus into soluble form making it available to the plants in a more eco-friendly and sustainable manner are known as

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Phosphate solubilizing Bacteria (PSB) (Kumar et al. 2020). Among them the species of *Bacillus* having multiple plant growth promoting potentials are widely used as important PSB to boost up the plant growth and yield of various crops (Kumar et al. 2012; 2020; 2021).

After rice, wheat, and maize, barley, *i.e. Hordeum vulgare*, is a major cereal crop that ranks fourth in total world cereal production. It is a flexible crop that is utilised for both human nourishment and animal feed. It also serves as a crucial experimental model plant for advancements in plant genetics, physiology, pathology, biochemistry, and, more recently, plant biotechnology (Harwood, 2019). Being a whole grain, it is rich in fiber, vitamins, minerals, etc., and is widely used as food, fodder, base malt for beer, and other distilled beverages. However, barley production relies heavily on the application of synthetic fertilizers, especially P, which affects both the size and quality of barley grain. Phosphorous is an essential micronutrient and plays a significant role in increasing root ramification and strength, thereby imparting vitality and disease resistance capacity to plants (Sharma et al., 2013). P deficiency in barley reduces plant tillering and biomass, while exogenous application has been pointed out that, it is very poorly available, because P recovery by crops in the year it is applied, is often only 10–15% (Richardson et al., 2011). In this regard, the barley plant was chosen as a model plant to find an alternative fertilization method to enhance P uptake in soils that is sustainable as well as eco-friendly.

One of the key goals of barley production systems is to evaluate supplementary or alternative nutrition sources and ways for enhancing nutrient uptake efficiency. In terms of vigour, emergence, nutrient uptake, biomass, plant height, and root system development in early growth stages, experimental and field application of rhizobacteria has been demonstrated to considerably increase plant growth and yield of many cereal crops, including wheat, barley, and others (Sahin et al. 2004; Rosas et al. 2009). One of the most potent rhizospheric bacteria, *Bacillus,* viz., *B. subtilis, B. cereus, B. pumilus, B. thuringiensis, B. megaterium*, etc. has developed its own mechanism to promote plant growth by increasing the availability of the nutrient (Meena et al. 2016). PSB do show the presence of nutrients through the solubilization of insoluble phosphate into soluble phosphate in the soil (Minakshi et al. 2020). Inoculating soil or crops with PSB is thus a promising strategy for improving plant phosphorus absorption and, as a result, reducing the use of environmentally harmful chemical fertilizers (Bhadaniya et al. 2021).

Keeping the above environmental concerns in mind, a study is being carried out in order to investigate the phosphate solubilizing rhizospheric bacteria from *Eragrostis cynosuroides* and to identify the characteristics of the most efficient strain using its optimization properties with cell density at different media, incubation period, pH, temperature, carbon and nitrogen sources, and concentration of TCP (tri calcium phosphate), along with its assessment on the promotion of growth in *H. vulgare*, through glass house experiments. We have selected kush grass (*E. cynosuroides*) because grasses grow luxuriantly, achieving significant biomass in a short span of time and are also easily accessible on road sides or in arid places, and their development is independent of any exogenous source of synthetic fertilizers. Therefore, the rhizospheric soil and roots of grasses are the best choice to isolate potential PGPRs. Relatively small numbers of PGPR have been isolated from kush grass to date. This work may help to retain the fertility of the soil and discourage the use of fertilizers.

Materials and methods

Sampling

Rhizospheric soil sample of *E. cynosuroides* plant was collected from road side Danapur (devoid of any fertilizer), Patna, Bihar, India, (25º 34' 56.2" N, 85º 2' 37.06" E). The plants were uprooted, and rhizospheric soil was obtained by shaking and brushing the remaining root system soil. The samples were collected, placed in sterile polythene bags, and transported aseptically to the laboratory, and processed within 3 hours.

Bacterial isolation and purification

To isolate rhizospheric bacteria, the soil sample was taken in sterile distilled water and serially diluted up to 10^{-6} dilution, and inoculated on Nutrient Agar Medium (NAM) in triplicate and incubated at 30 °C for 24 h. Based on the dominancy of colonies, seven isolates were purified by repeated streaking in the same culture medium and were designated as DR1, DR2, DR3, DR4, DR5, DR6, and DR7.

Detection and efficiency estimation of PSB

Dominant colonies on Pikovskaya agar medium containing tricalcium phosphate, were chosen and evaluated for the development of halo zones to detect PSB after 48 h of incubation at 30 ± 2 °C (Kumar et al. 2012). Colonies showing halo zones were selected to test their phosphate solubilization index (PSI) and phosphate solubilizing efficiency (PSE). The colonies with the highest PSI and PSE values were selected for further experiments. PSI was determined by measuring both, the diameters of colony and halo zone, applying the formula (PSI = colony diameter + halo zone)/

colony diameter), while PSE was calculated by applying the formula (PSE % = SZ- CD / CD x 100); Where, PSE = Solubilization efficiency (%), SZ = Solubilization zone (mm), CD = Colony diameter.

Using Pikovskaya's broth, quantitative measurement of phosphate solubilization by isolates was carried out. Pikovskaya's broth inoculated with 10 µl of 48 h old bacterial cultures (10^7 cfu ml^{-1}) in triplicates, and incubated at 30 ± 2 °C for 4–5 days on in incubator shaker at 150 rpm. Cultures were centrifuged at 10,000 rpm for 10 min after regular intervals, and available soluble phosphate in supernatants was measured spectrophotometrically at 610 nm using the chlorostanous reduced molybdophosphoric acid blue method (Jackson 1967; Naik 2013). The phosphate released in the supernatant was measured as blue colour at 610 nm, using a UV-VIS spectrophotometer (Systronics), with potassium dihydrogen phosphate as a standard. The isolates showing the highest phosphate solubilizing activity were used for optimization.

pH change

An Estimation of the change in pH of the broth, due to the growth of PSB was measured with a pH meter at different incubation periods of 2, 3, and 4 days (Panda et al. 2015).

Morphological characterization

Bergeys Manual of Determinative Bacteriology was used to examine the morphological and biochemical properties (Holt et al. 1994). Potential isolates, were identified on the basis of phenotypic and genotypic characterization.

16 S rDNA sequencing and analysis

To identify the isolates, 16S rDNA sequencing (1339 bp) was performed using the universal and specific primers (F) 5'-AGA GTT TGA TCC TGG CTC AGT-3' primer (R) 5'-ACG GCT ACC TTG TTA CGA CTT-3'at Genomic Excelris Lab., Ahmedabad, Gujarat, India. Sequences were submitted to NCBI database to get an accession number.

Effect of various parameters on the efficiency of phosphate solubilization

All the isolates were screened for P-solubilization activities to select the potential isolate having maximum P-solubilization activities for optimization studies. In this study, one factor at a time was applied for the optimization of P-solubilization efficiency. Quantification of P-solubilization was performed using the standard plot.

Media and growth condition

The Phosphorus solubilizing ability of bacterial strains was tested in four different types of liquid media (NBRIY (National Botanical Research Institute's phosphate growth medium devoid of yeast extract), AYG (Ammonium sulphate yeast extract glucose medium), PVK (Pikovskaya's) and NBRIP (National Botanical Research Institute's phosphate growth medium) (Sagervanshi et al. 2012). Composition of different media is given in Table 1. The rest of the experiment was performed in PVK media with 0.5% tricalcium phosphate.

Incubation time

Effect of incubation time on P -solubilization was studied by growing the bacterial isolates at different incubation periods i.e. 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h at 30 ± 2 °C and growth was recorded at 24 h of incubation. The optimum incubation time for P-solubilization was maintained for further experiments.

Temperature

Temperature is also an important parameter for the growth of microorganisms, i.e., the growth of bacteria is affected by low or high temperature. P-solubilization was tested at 25 °C, 30 °C, 35 °C, and 40 °C for 4–5 days. The density and activity of the cells were measured.

Table 1 Calculation of phosphate solubilization efficiency and index in the PVK agar medium after 2, 3, and 4 days in all positive bacterial isolates

S. No.	PSB isolates	Solubilization zone diameter (mm)	Phosphate Solubilization Efficiency % (PSE)			Phosphate Solubilization Index (PSI)		
		4th day	2nd day	3th day	4th day	2nd day	3th day	4th day
1	DR1	4	18	22	25	2.3	2.5	3
2	DR2	15	50	120	150	3.5	4.2	4.5
3	DR4	8	20	25	33.33	2.75	2.8	3.3
4	DR6	6	15	23	26	2.3	3	3.2
5	DR7	10	33.33	75	100	3.3	3.5	4

pH

pH is one of the most important physicochemical parameters. A range of pH 4–9 was tested for its effect on P-solubilization and the optimum pH was maintained for all further experiments.

Carbon sources

The isolate was grown in PVK broth medium containing various carbon sources, i.e., glucose, fructose, mannitol, sucrose, and lactose, individually, at the concentration of 0.5 g/l to observe phosphate solubilising activities.

Nitrogen sources

The effects of different nitrogen sources on P-solubilization were evaluated by replacing ammonium chloride with five different nitrogen sources, i.e., ammonium sulphate, ammonium nitrate, sodium nitrate, calcium nitrate, potassium nitrate, and urea at a concentration of 0.5 g/l.

Various concentrations of TCP

To study the effect of various concentrations of tricalcium phosphate (TCP) at which, the bacteria solubilize it effectively, the PVK medium was amended with 0.1, 0.25, 0.5, 0.75, 1.0, and 1.5% of the same and the isolate was grown on that at 30 °C ± 2 °C for 5 days.

In vitro study of *B. subtilis* DR2 on plant growth promotion

Proficient isolate of *B. subtilis* DR2 was subjected to observe its plant growth promoting potential on barley (*H. vulgare*) seedlings, under glass house conditions for two weeks. Barley seed was sterilized with 2.5% bleach, rinsed five times in sterile water, and then incubated in the corresponding bacterial solutions for one hour with moderate shaking (Cardinale et al. 2015). A 48 hours old culture (10^8 cfu ml^{-1}) of *B. subtilis* DR2 grown in nutrient broth was used for the bacterization of seeds (30 seeds). Treated seeds were sown in each pot containing autoclaved mixture of sand and soil (1:1) having one third moistened saturation capacity. After 3–4 days of seedling emergence, thinning was done and five plants per pot were maintained. Three replicate pots per treatment were placed in completely randomized design under glass house conditions. Seeds dipped in autoclaved distilled water were kept as uninoculated controls. The Uninoculated control treatment was also kept in the same manner. The percent (%) germination, root-shoot length and root-shoot biomass were recorded.

Statistical analysis

Each experiment was carried out in triplicates. The data obtained were statistically analyzed for social science (SPSS 16.0) software, and graphically represented as the mean ± standard error (n = 3).

Results and discussion

Isolation and characterization of rhizospheric bacteria

The goal of this study was to isolate phosphate solubilizing bacteria from the rhizospheric soil of *E. cynosuroides* using NAM media. Seven isolates were selected on the basis of morphological characters, such as colony, color, elevation, texture etc. Since, the rhizosphere of selected plants has not been explored much more for phosphate solubilizing bacteria.

Screening of phosphate solubilizations

A total of seven bacterial isolates were screened for P-solubilization on PVK agar. Based on the clear zone surrounding the colonies on solid plates, the solubilization efficiency and index were calculated (Table 1). P-solubilization zones were developed in five bacterial isolates i.e. DR1, DR2, DR4, DR6, and DR7 ranging from 4 to 15 mm where DR3 and DR5 were unable to solubilize phosphate. similar findings were reported by Eramma et al. (2020), who found that isolate PPSB-21 of paddy rhizospheric soil had the highest halo zone diameter of 18.4 mm. Similarly, Mohamed et al. (2018) also observed three isolates (C2, T1 and T5), with the halo zone diameter 1.1 cm, 1 cm, and 0.9 cm respectively. There was a correlation between incubation time and zone size. It was observed that an increase in incubation time, resulted an increase in the zone size of each isolate. PSE% ranged from 15 to 150% from 2nd to 4th day of incubation. Similarly, the phosphate solubilization index was also enhanced with incubation period, which varied from 2.3 to 4.5 among the 5 efficient isolates. The DR2 showed the highest zone of PSI (4.5) in PVK agar media. According to Kongbrailatpam and Putatunda (2018), the isolates PSBN B4 and PSBN B5 showed a maximum phosphate solubilizing efficiency (PSE) of 100%. Similarly, Mazylyte et al. (2022) also recorded that the bacterial strain *Bacillus aryabhattai* B8W22 demonstrated a maximum phosphate solubilization index, ranged from 2.50 to 2.83 with an enhanced incubation period (2 to 14 days) respectively, which is in conformity with our result.

Table 2 Quantitative estimation of phosphate solubilization and effect of PSB isolates on change of pH in Pikovsakaya's broth. Values are expressed as mean ± standard error

S. No.	PSB isolates	Amount of P released (mg/l)			Change in pH in media		
		2nd day	3th day	4th day	2nd day	3th day	4th day
1	DR1	2.72 ± 0.45	6.02 ± 0.41	6.94 ± 0 0.29	6.72	6.36	6.13
2	DR2	21.21 ± 0.81	33.44 ± 0.21	40.36 ± 1.61	4.28	3.96	3.40
3	DR4	17.44 ± 0.43	25.33 ± 0.25	34.38 ± 0.53	5.95	5.64	4.87
4	DR6	11.94 ± 0.52	19.47 ± 0.57	23.87 ± 1.25	6.24	5.86	5.13
5	DR7	17.02 ± 1.19	21.34 ± 0.89	24.16 ± 0 0.71	5.65	5.30	4.96

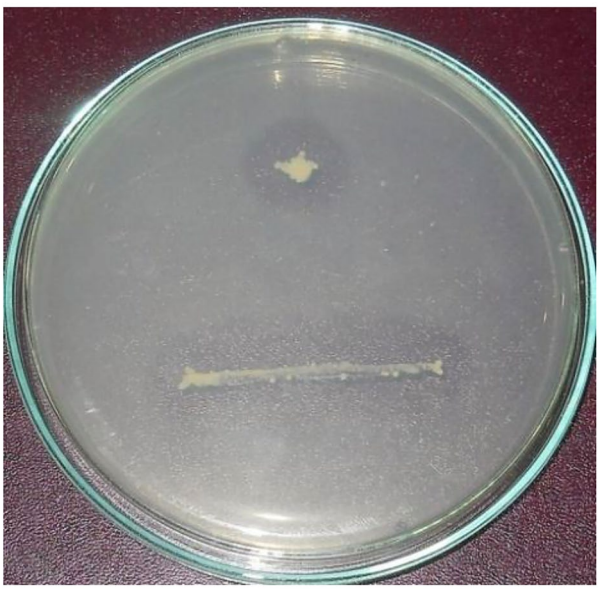

Fig. 1 Halo zone on PVK media of *Bacillus subtilis* DR2

Quantification of phosphate solubilization

All five isolates were able to solubilize the insoluble phosphorus in Pikovaskaya's broth at different incubation period of 2nd, 3th and 4th days (Table 2). The phosphate solubilization ability of the bacterial isolates varied and showed an increase with the increase in time interval but maximum P-solubilization was recorded by DR2 at 4th day, 40.36 mg/l followed by DR4 (34.38 mg/l), DR7 (24.16 mg/l), DR6 (23.87 mg/l) and DR1 (6.93 mg/l) respectively. In accordance with our findings, various reports have been observed. Among them, Yadav et al. (2016), observed maximum phosphate solubilization (8.2 mg/mL) on the sixth day of incubation in the wheat rhizosphere. Similarly, Eramma et al. (2020) reported available phosphorous content was ranged from 31.92 to 171.84 mg/L in all the ten isolates from paddy rhizospheric soil. Gupta et al. (2022) also found that *B. subtilis* 3 (PS4) had the maximum P solubilization of 10.22 µg/ml at the seventh day of incubation.

pH change

All the isolates were tested for decrease in pH in media (6.72 to 3.40) with increased incubation period. DR2 showed highest reduction in pH of 3.40 on 4th day. The drop in pH was due to the production of organic acids. Baliah and Begum (2015) observed a pH change of up to 4.6 by the *Bacillus* sp., whereas, Pande et al. (2017) reported a lowering of pH ranging from 3.08 ± 0.08 to 3.82 ± 0.12 by bacteria, which is in conformity with the present finding.

DR2 appeared to be the most efficient phosphate solubilizer (40.36 mg/l of phosphate). Therefore, on the basis of maximum zone formation (Fig. 1) and phosphate solubilization production, compared to the others, DR2 was found to be the most potent and hence, selected for further analysis.

Phenotypic characterization

The selected isolate, DR2 formed a round creamy white colony (1–2 mm diameter), with irregular margin. Microscopic examinations revealed that the isolate was a gram positive, motile, spore forming rod, measuring 3.1 to 3.5 µm long and 0.5 to 0.7 µm wide. The biochemical characteristics of the isolate showed that DR2 was positive for oxidase, citrate, ammonia, the Voges-Proskauer reaction and starch hydrolysis. Negative reactions were recorded for methyl red, gelatin, urease hydrolysis and H_2S production.

Genotypic characterization

Isolate DR2 was subjected to 16 S rRNA gene sequence analysis which showing 99% identity with *B. subtilis*, so it was identified as *B. subtilis* DR2 (Accession No.-KP455653) and 16 S rRNA sequences of different strains of *B. subtilis* (obtained from the NCBI Database: (http://www.ncbi.nlm.nih.gov/entrez) (Tamura et al. 2011).

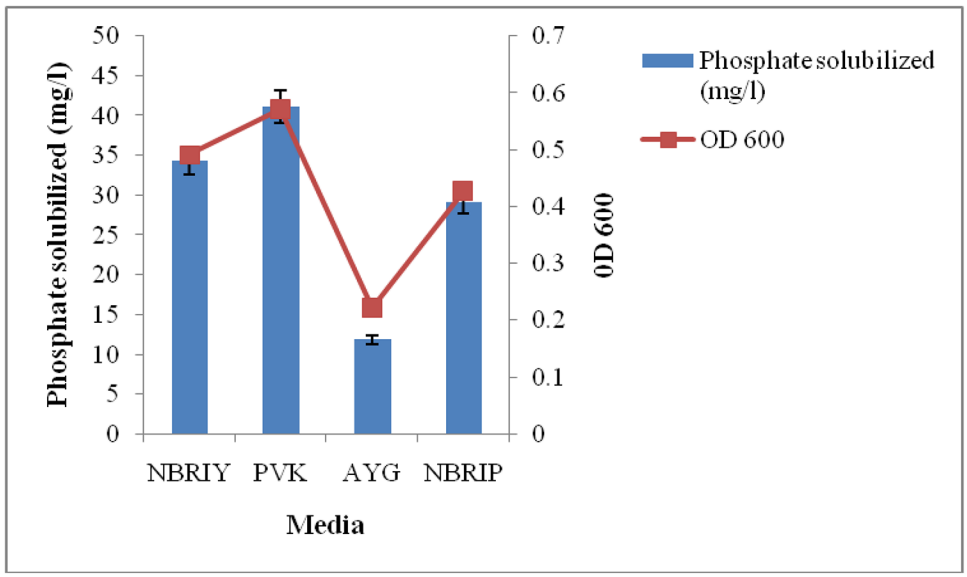

Fig. 2 Media optimization for phosphate solubilization and the correlation with OD value by B. subtilis DR2

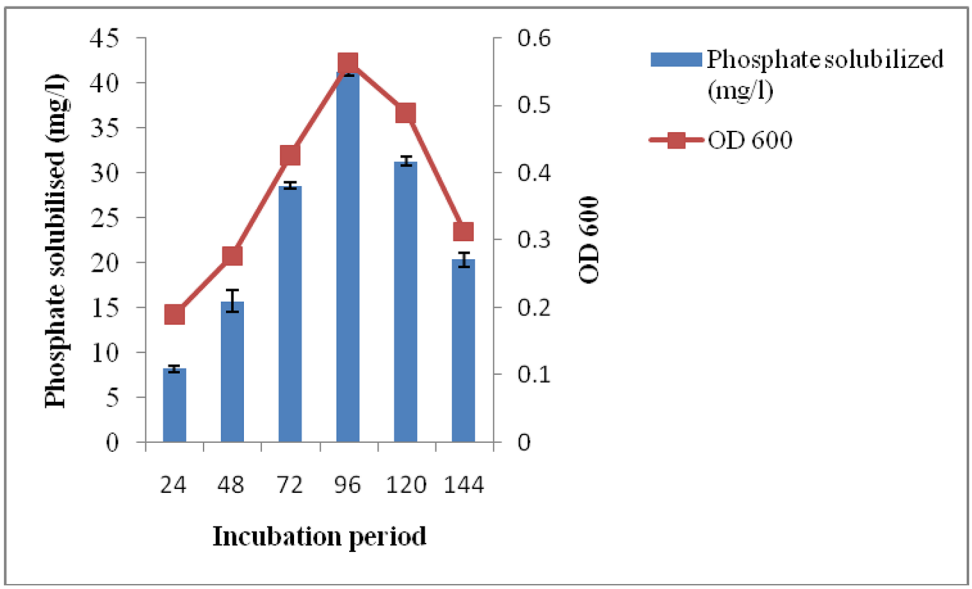

Fig. 3 Incubation period for phosphate solubilization and the correlation with OD value by *B. subtilis* DR2

Optimization of phosphate solubilization parameters

Optimization of media and growth conditions for phosphate solubilization

Phosphate solubilizing ability of *B. subtilis* DR2 was tested in four different types of broth media viz., PVK, AYG, NBRIY and NBRIP. The maximum phosphate solubilization was observed in PVK medium (41.10 mg/l) with a corresponding maximum OD value of 0.570 followed by NBRIY, NBRIP and AYG (Fig. 2). Hence, PVK medium was selected for the further studies. This observation was also in accordance with Sagervanshi et al. (2012) and Shaikh and Saraf (2017).

Growth responses of *B. subtilis* DR2 at different incubation time

The growth activity of the *B. subtilis* DR2 was minimum (8.12 mg/l) after 24 h and maximum (41.32 mg/l) after 96 h, of incubation with corresponding to a maximum OD value (0.563) and a minimum OD value (0.189), respectively. Phosphate solubilizing activity however, decreased with further increase in time (Fig. 3). Jena and Rath (2013) found maximum phosphate solubilizing activity in *Bacillus* and *Pseudomonas* after 72 h of incubation, while Banerjee et al. (2010) achieved a similar result in the bacterial isolate

TRSB10, after 72 h incubation. However, some workers reported more than 10 days (Sridevi and Mallaiah, 2009) and even up to 15 days (Sahu et al. 2007) for phosphate solubilization by various bacterial isolates. Narveer et al. (2014) reported that *Bacillus* sp. NPSBS3.2.2 has maximum solubilization after 96 h, which is in conformity with the present finding.

Growth responses of *B. subtilis* DR2 at different temperature

The effect of temperature was studied in the range of 25–45 °C, whereby maximum yield of P-solubilization (44.51 mg/l) was observed at 35 °C with subsequent increase in OD value (0.637) (Fig. 4). *B. subtilis* DR2 produced phosphorus in a linear relationship with temperature up to 35 °C, after which it declined. Present results are in line with Sagervansi et al. (2012); Malleshwari and Bhagyanarayana (2013) where the optimum temperature for P-solubilization was 35 °C. Different temperatures (10 °C, 25 °C, 28, and 45 °C) show bacterial adaptation to their indigenous environment and therefore, their metabolic activities are linked to the temperature of the environment (Shahab and Ahmad 2008). In our study, the average temperature fluctuated between 30 and 40 °C and the maximum enzyme activity was recorded at 35 °C, proving the adaptation of DR2 to its indigenous environment.

Growth responses of *B. subtilis* DR2 at different pH

One of the most important parameters for the growth of PSB and their metabolic activity is the pH of the phosphate solubilization medium. *B. subtilis* DR2 solubilized phosphate at pH 4 to 8. Very low solubilization was observed at pH 4 (0.41 mg/l), while optimum solubilization was recorded at pH 7.0 (45.86 mg/l), and decreased on subsequent increase in pH (Fig. 5). Gupta et al. (2022) found that PS2 (*B. subtilis* strain 1) grew best at a pH of 7.0 ± 0.5, which is consistent with our findings. Similar observations have been reported by Narveer et al. (2014) for *Bacillus* sp. NPSBS3.2.2, where maximum solubilization of calcium phosphate was obtained at pH 7 and almost negligible at pH 3, also Maheswar and Sathiyavani (2012) and Kongbrailatpam and Putatunda (2018) observed pH 7, as most suitable for phosphate solubilization *in B. subtilis*.

Growth responses of *B. subtilis* DR2 at different carbon sources

Carbon source provide energy for growth and various metabolic activities of microorganism. The Phosphate solubilization of *B. subtilis* is influenced by the nature of the carbon source. The isolate was then tweaked to see how other carbon sources, such as glucose, fructose, sucrose, lactose, and mannitol, affected phosphate solubilization by *B. subtilis* DR2 in broth. It was found that glucose was the best carbon source for the maximum growth of the isolate (OD = 0.592) and it solubilized maximum phosphate at 46.12 mg/l, followed by fructose, sucrose, lactose, and least in mannitol (Fig. 6). This could be due to the fact that glucose is the simplest sugar, making it easier for the microbe to take up, resulting in better phosphate solubilization. Many researchers have also reported that glucose is beneficial for bacterial phosphate solubilization (Selvi and Ravindran 2012; Kumari and Gupta 2013). In conformity with our finding, Wang et al. (2020) also observed good phosphate solubilizing ability using glucose (189.1 µg/ml) as a carbon source in *B. subtilis* BPM12.

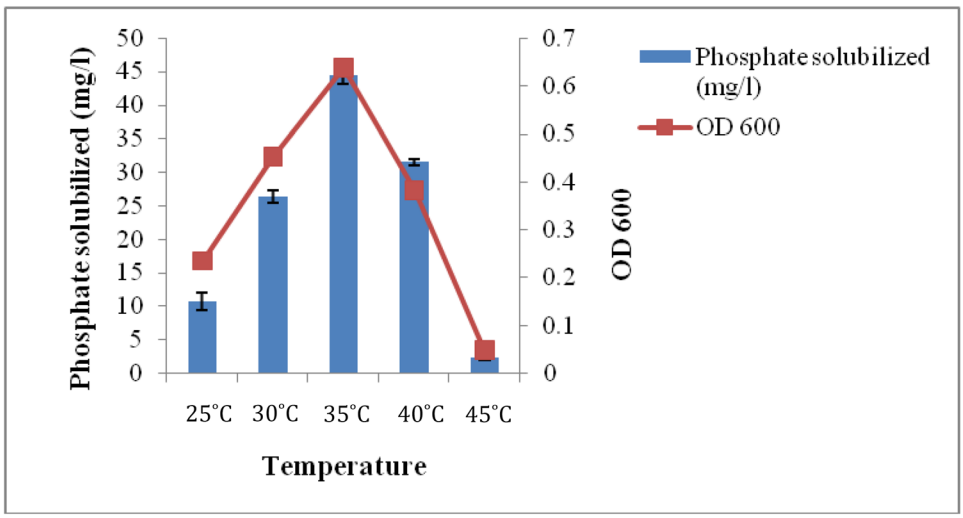

Fig. 4 Temperature for phosphate solubilization and the correlation with OD value by *B. subtilis* DR2

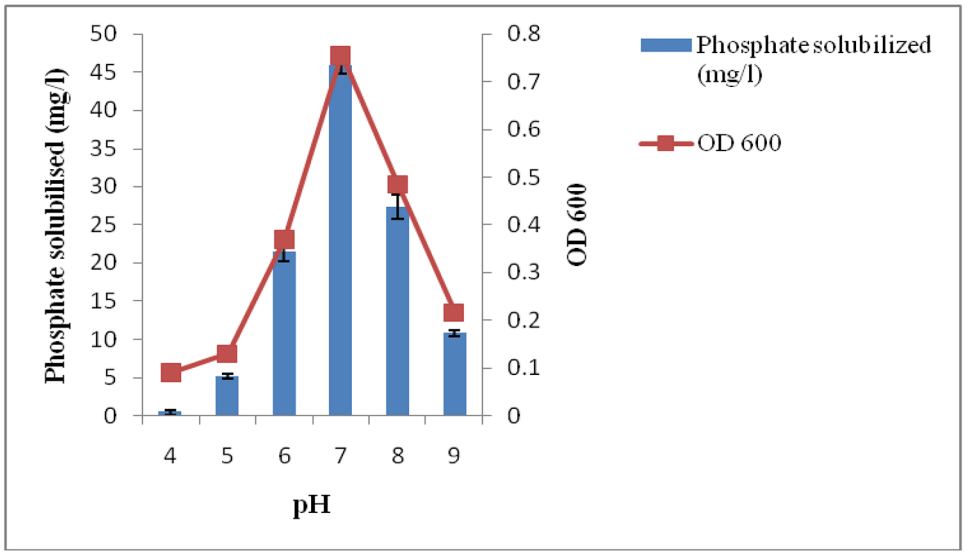

Fig. 5 pH for phosphate solubilization and the correlation with OD value by *B. subtilis* DR2

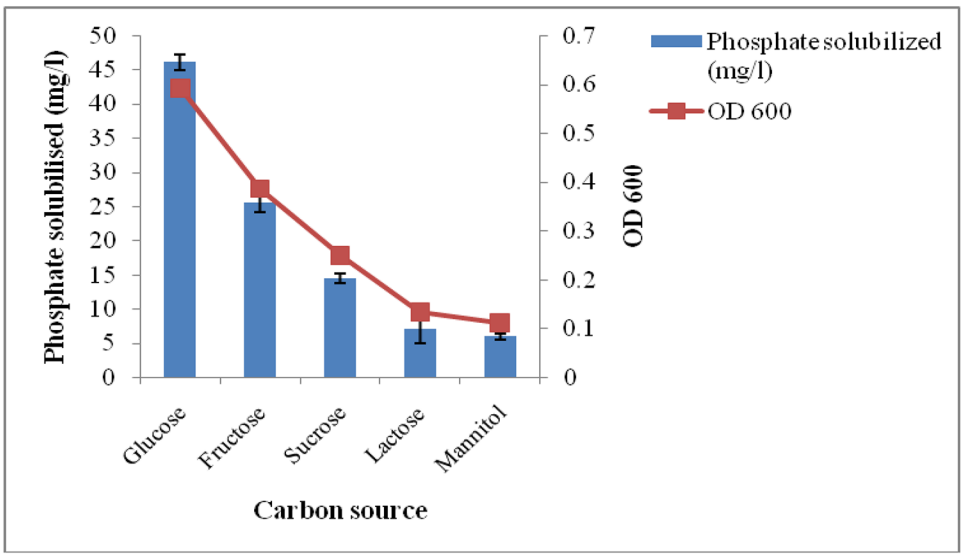

Fig. 6 Carbon source for phosphate solubilization and the correlation with OD value by *B. subtilis* DR2

Growth responses of*B. subtilis* DR2 at different nitrogen sources

Nitrogen, as one of the most important nutritional factor, serves as the building block material of organisms, so it will be the basal component of medium. Different nitrogen sources (ammonium sulphate, ammonium nitrate, calcium nitrate, potassium nitrate, sodium nitrate, and urea) were used in broth to see the optimum phosphate solubilizing activity of *B. subtilis* DR2. Although, the selected isolate showed comparable activity with all the nitrogen sources, among them ammonium sulphate appeared as the most suitable (48.16 mg/l), closely followed by ammonium nitrate, calcium nitrate, potassium nitrate, sodium nitrate and lowest activity in urea (Fig. 7). Similar results were recorded in the bacterial strains PSB22 and PSB37 (Balamurgan et al. 2010). Both the strains exhibited the best phosphate solubilizing activity, when ammonium sulphate was used, as a nitrogen source. A substantial number of bacteria are capable of solubilizing phosphate exclusively in the presence of ammonium as the nitrogen source (Kumari and Gupta 2013; Narveer et al. 2014).

Growth Responses of *B. subtilis* DR2 at different TCP

A gradual increase in P-solubilization by *B. subtilis* DR2 was also observed with the increase in TCP concentration (0.1, 0.25, 0.5, 0.75, 1.0, and 1.5%). At a concentration of 1% of TCP, maximum P-solubilization (51.80 mg/l) with a corresponding maximum OD (0.70) was recorded (Fig. 8). Similar results were reported by Minakshi et al. (2020) in the bacterial strain *B. subtilis* KA(1)5r, where 1% TCP

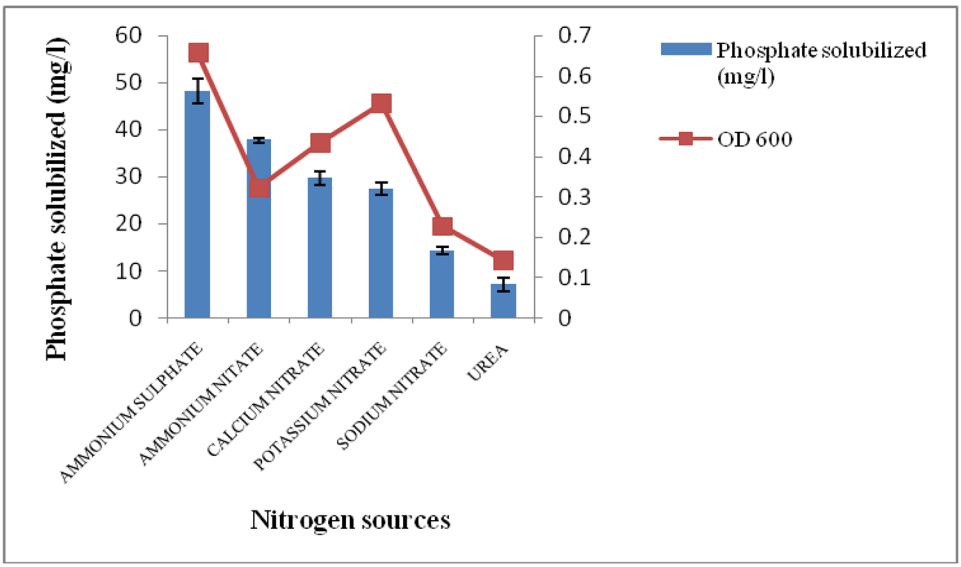

Fig. 7 Nitrogen source for phosphate solubilization and the correlation with OD value by *Bacillus subtilis* DR2

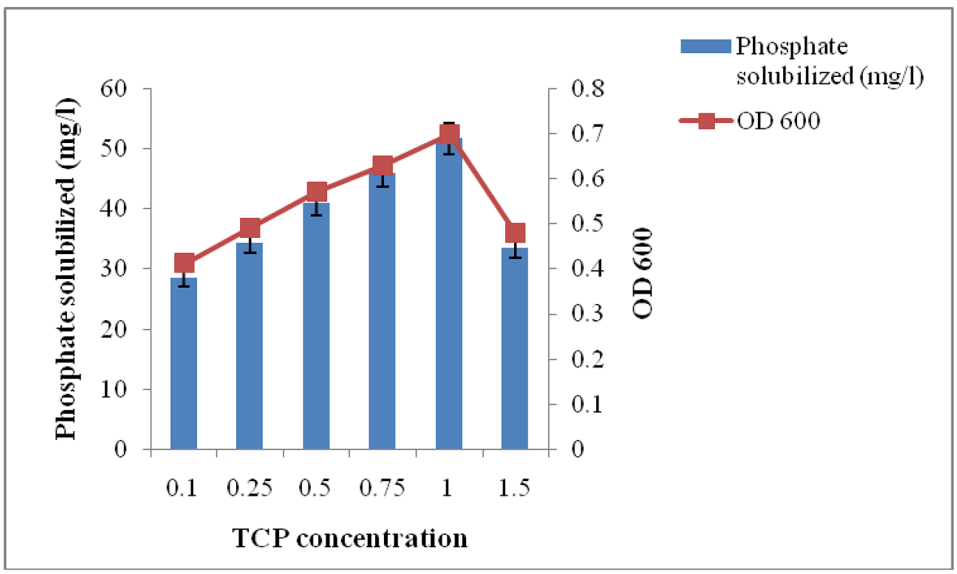

Fig. 8 Concentration of tricalcium phosphate for phosphate solubilization and the correlation with OD value by *B. subtilis* DR2

Table 3 Evaluation of the *B. subtilis* DR2 to exhibit growth parameters in in-vitro condition after 14 days. Values are expressed as mean ± standard error

Cereal	treatment	Length (cm)		Fresh weight (g)		Dry weight (g)	
		shoot	root	shoot	root	shoot	root
Barley	*B.subtilis* DR2	19.5 ± 1.67	15.73 ± 1.13	0.163 ± 0.015	0.08 ± 0.01	0.0683 ± 0.021	0.055 ± 0.018
	control	13.8 ± 1.7	8.12 ± 1.41	0.101 ± 0.02	0.049 ± 0.01	0.039 ± 0.01	0.013 ± 0.009

concentration was maximum for phosphate solubilization (192 µg/ ml).

In vitro plant growth promotion studies

It is a widely held scientific belief based on numerous evidences that PGPR improves plant growth, seed emergence, and crop yield, as well as contributing to plant protection against pathogens and pests (Dey et al. 2004). Therefore, in the present study, the inoculation effect of *B. subtilis* DR2 was evaluated on barley seedlings. The glass house experiment was carried out with DR2, where it demonstrated positive effects on all the growth parameters, in terms of seed germination, shoot length, root length, fresh weight, and dry weight in barley as compared to their controls (Table 3). Statistically significant enhancements in seed germination

(60%), root length (93.71%), shoot length (41.30%), and biomass (22.41%) were recorded in seeds treated with *B. subtilis* DR2 over uninoculated controls. In the similar pattern, Reetha et al., (2014) also reported *B. subtilis* as a better plant growth promoter in terms of shoot length, root length, fresh weight and dry weight. The enhancement in growth after inoculation with *B. subtilis* DR2 must be due to total nutrient uptake, different PGP mechanisms, such as phosphate solubilization, nitrogen fixation, etc.

Conclusion

Phosphate solubilizing *B. subtilis* strains, isolated from the rhizosphere of *E. cynosuroides*, may attract more attention in the field of bio-fertilization with their beneficial properties. These can effectively increase the available P, in the soil containing insoluble forms of this biogenic element, as well as improve the growth and yield of crop plants. The strain *B. subtilis* DR2 showing highest PSI and PSE, along with dramatically lowering the pH values over the days, was selected to assess the impact of various parameters to optimize phosphate solubilization. The most suitable conditions for optimum phosphate solubilization observed were 96 h incubation, pH 7 and 35 °C temperature with glucose and ammonium sulphate as carbon and nitrogen sources, respectively. The strain has the potential to be used as a plant biofertilizer, as well as for the betterment of soil fertility. Its application in pot experiments favours the integration of biological management for plant improvement. The reproducibility of the result needs to be further standardized, so that the bacterium could be recommended as bio fertilizer, because PSM are very effective for increasing available phosphorous to the plants for enhancing their growth and yield by increasing the soil fertility.

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Declarations

Conflict of interest All authors declare that there is no conflict of interest.

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