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Evaluation of Plant Growth Promoting Rhizobacteria (PGPR) for their Iron and Zinc solubilizing attributes

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ABSTRACT
Micronutrient insufficiency is common, and it is known as malnutrition. Iron (Fe) and zinc (Zn) are key micronutrients that must be consumed in the proper amounts through a regular diet in order to support many life processes. Various PGPR produce low molecular weight compound known as siderophore that chelates iron from insoluble iron complex present in the soil. These microbes also secrete organic acids or decrease soil pH that leads to increase in zinc concentration. This study aims to determine iron and zinc solubilising properties of PGPR. Total 7 isolates were able to solubilise different zinc complexes. The isolates gave zone of solubilisation ranging from 7mm to 22mm. The zinc solubility index was maximum for the 4 isolates identified as Pseudomonas aeruginosa, Serratia marcescens, ZSM1 and ZSM2. Siderophore was produced by Pseudomonas aeruginos, and Staphylococcus aureus as detected on CAS-agar plate. Siderophore activity of P. aeruginosa and S. aureus was found to be 89% and 68.46% respectively. Preliminary identification was done for the bacteria. Plant growth promoting traits of the selected isolates was determined. All the isolates were able to produce IAA, exopolysaccharides and ammonia. Phosphate solubilisation was detected by Pseudomonas aeruginosa, ZSM1, ZSM2, Staphylococcus aureus. HCN production was given by ZSM2 only.
Keywords: micronutrients, iron, zinc, PGPR, siderophore

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INTRODUCTION

The main diet of humans is largely comprised of plant-based foods. Micronutrients and macronutrients must be consumed in a balanced diet for both plants and people to grow and develop properly. Agriculture has benefited quantitatively but not qualitatively from the green revolution. The green revolution prioritized increasing yields over producing high-quality food. This decreased the land's productivity, which led to the production of less nutrient-dense edible cereals. It is necessary to consume trace levels of micronutrients every day. Malnutrition is brought on by meals lacking essential micronutrients such iron, zinc, selenium, copper, and vitamins. Malnutrition generally exhibits deficient symptoms that are not visible, hence the term "hidden hunger." Reduced consumption of a high-quality food loaded with vitamins, minerals, and proteins is to blame for this. Malnutrition has a disproportionately negative impact on developing nations and the poor. People with limited resources typically eat cereal-based diets because they cannot regularly afford to include a variety of meals or nutritional supplements in their diets. Urban regions with a low awareness of a balanced diet are equally prone to malnutrition. Malnutrition can result in serious health issues and even death [26]. Inadequate intake of vitamins and minerals in the diet accounts for about 7.3% of disease-related mortality worldwide. 38 percent of children under the age of five are underweight in India, while about 18 percent of infants are born with birth weights that are below average [5]. India is ranked 101st out of 116 countries in the Global Hunger Index 2021, with a score of 27.5. (Index, 2021). Deficits in iron, zinc, and iodine affect more than two billion individuals worldwide.

Micronutrient malnutrition, is more common in women and young children and is primarily caused by low dietary intake of micronutrients, especially zinc (Zn) and iron (Fe). Due to their high requirement for iron for metabolic processes, pregnant women and young children are disproportionately affected by iron insufficiency, which affects between 2.5 and 5 billion people worldwide [10]. A national survey conducted by All India Coordinated Research Project (AICRP) for micronutrients and secondary nutrients has stated that almost half of the Indian soil is deficient in metals like zinc.

Zinc is one of the essential micronutrients that is required in low quantity but it's deficiency can lead to retarded growth and development in living organisms. Zinc is required as co-factor in many prominent enzymes that catalyses metabolic reactions. Enzymes like RNA polymerase, carbonic anhydrase, superoxide dismutase, alcohol dehydrogenase and tryptophan synthetase and many more enzymes require zinc for their functioning. In addition, zinc also helps in metabolism of biomolecules, chloroplast development, metabolism of hormones like auxin [9]. Zinc is involved in internode enlargement and increase in yield also. It also plays a crucial role in controlling the gene expression required for plants to tolerate environmental stresses. Several factors contribute to zinc deficiency in plants such as non-availability of soluble forms of zinc, low zinc content in the soil, high pH of the soil like calcareous soil, higher content of Magnesium, sodium or phosphate in the soil [8]. In animals, hair loss, skin rashes, wasting, and recurrent diarrhoea are symptoms of severe zinc deficiency. Inadequate intake throughout childhood and adolescence can hinder growth, sexual development, and psychomotor development. The mineral seems to be especially crucial during times of rapid growth.

Since iron can operate as both an electron donor and acceptor, it serves a variety of crucial roles in the human body. The body needs iron for energy metabolism and oxygen transport, and it also helps a variety of nonheme enzymes like ribonuclease reductase to catalyse reactions. One of the biggest health issues brought on by an iron deficiency is anaemia. According to a World Health Organization assessment, anaemia affects around one-fourth of the global population. According to the Global Nutrition Report 2021, anaemia affects one in three women worldwide between the ages of 15 and 49. In addition to causing anaemia, iron deficiency in humans also impairs immune system performance and has an impact on the brain system. If humans are iron deficient, it harms the development of their neurocognitive abilities. Chlorosis and decreased agricultural output are the results of iron deficiency in plants [26]. Growth retardation, delayed bone and sexual development, skin lesions, diarrhoea, baldness, reduced appetite, increased susceptibility to infections caused by immune system deficiencies, and the emergence of behavioural disorders are clinical signs of severe zinc deficiency in humans.

Several strategies have been employed to resolve the issue of micronutrient deficiency. Use of micronutrient rich chemical fertilizers, nano fertilizers, supplements, dietary diversification, food fortification, biofortification, etc is known to be done to alleviate malnutrition. There has been a renaissance of interest in developing environmentally friendly ways of crop production and protection as a result of the rising expense of pesticides and fertilisers and concern over environmental degradation [7]. In order to offer more micronutrients over the long term, sustainably, and at a reasonable cost, food crops are fortified through a process known as biofortification. Biofortification is the process of increasing micronutrient concentration in the edible portions of the food crop. Various strategies such as mineral fertilizers, conventional and molecular breeding, genetic modifications, agronomic biofortification, microbe-mediated biofortification are among prominent methods employed for biofortification [23] [25]. Microbe- associated biofortification is the cost effective and sustainable approach to defy malnutrition. Rhizospheric bacteria present in the rhizosphere of the soil interacts with the plants and promotes their growth and development either through direct or indirect mechanisms. This plant growth promoting rhizobacteria (PGPR) are also known to solubilize prominent micronutrients present in the soil as complexes. Thus, PGPR enhances micronutrients content in the plants and helps in reducing micronutrient deficiency. Most prominent PGPR include the genera belonging to *Acetobacter, Acinetobacter, Alcaligenes, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Derxia, Enterobacter, Gluconacetobacter, Klebsiella, Pantoae, Pseudomonas, Rhodococcus, Serratia* and *Stenotrophomonas* [14].

In order to achieve iron biofortification of food crops, various methods are used. It is common to apply iron fertilisers to the soil and to the leaves. Low molecular weight chemical compounds called siderophores that have a strong affinity for iron are releases by PGPR. The four major families of siderophores produced and secreted by bacteria are hydroxamate, catecholates, carboxylates, and pyoverdines [16]. By coordinating with an iron atom at the surface of the mineral, siderophores create extremely stable iron complexes that speed up the dissolution of iron-bearing minerals. Zinc becomes less soluble with increasing pH, organic matter and bicarbonate content, magnesium to calcium ratio, and P and Fe availability. When applied to agricultural fields in soluble forms, the metal transforms into a variety of insoluble forms, including $Zn(OH)_2$ in high pH soils, $ZnCO_3$ in calcium-rich alkali soils, zinc phosphate in near-neutral to alkali soils

with heavy P fertiliser applications, and ZnS under reducing conditions, particularly during flooding [6]. PGPR converts insoluble form of zinc to soluble forms by decreasing the pH of the soil. This is done by production of citric acids or poly-carboxylic acids that results in proton extrusion. Apart from this, PGPR also produces various organic acids such as gluconic acid, butyric acid, lactic & oxalic acid that helps in zinc solubilization. Among this, α- ketogluconic acid is produced by majority of rhizobacteria. Zinc chelation by siderophores produced by bacteria have also been reported.

MATERIAL AND METHODS

Screening of siderophore producing and zinc solubilising bacteria

Total 19 isolates were procured from the Department of Microbiology and Biotechnology, Gujarat University. For qualitative estimation of siderophore producing bacteria, CAS-agar plate was used [19]. Zinc solubilizing bacteria were spot inoculated on Bunt and Roviera agar media supplemented with 0.1% insoluble zinc source. Different zinc sources used were zinc carbonate ($ZnCO_3$), zinc oxide (ZnO), zinc sulphide (ZnS) and zinc phosphate ($ZnPO_4$). Bacterial colonies showing halo zone of solubility were chosen, and measurement of solubility area diameters was done. The zinc solubility index (SI) was calculated as described by the following formula.

$$Solubility\ index\ (SI) = \frac{Bacterial\ colony\ diameter\ +\ diameter\ of\ halo\ zone}{bacterial\ colony\ diameter}$$

Quantitative estimation of siderophore production

Isolates that produced yellow-orange zone on CAS agar plates were selected for siderophore quantification study. CAS-shuttle assay was done for detection of siderophore activity [18]. Briefly, equal volumes of culture supernatant and CAS reagent was mixed and absorbance was measured at 630nm. Absorbance of uninoculated broth used as control was also measured. Presence of siderophore was determined using the following formula:

$$\%siderophore\ units = \frac{Ar-As}{Ar} \times 100$$

Two distinct siderophore production assays, namely Arnow's assay for catecholate type [1] and Csaky's assay for hydroxamate type [24] by spectrophotometric method, further confirmed it.

Determination of plant growth promoting traits of the selected isolates

Selected isolates that were able to solubilize iron and zinc were further tested for plant growth promoting attributes such as Indole acetic acid (IAA) production, phosphate solubilization, exopolysaccharide (EPS) production, production of ammonia and siderophore.

Indole acetic acid production: Salkowski reagent was used to evaluate the indole production by the bacterial isolates in tryptone yeast medium containing 50mg/L of L-Tryptophan. The intensity of the pink colour at 530 nm was measured spectrophotometrically. The standard graph of pure indole acetic acid was used to determine how much IAA was synthesized.

Solubilization of insoluble phosphate: In Pikovskaya's medium containing 0.1% tricalcium phosphate, a qualitative assay for phosphate solubilization was investigated by spot inoculation. Afterwards, for the following 48–72 hours, these plates were incubated at 37° C. A visible halo zone that formed around the colony, which could have been caused by the colony producing organic acids, served as a visual indicator of the phosphate solubilization process. The Olsen and Sommers (Penrose) method was tested for the quantification of phosphate solubilization, and analysed up to 7 days after incubation [20]. By using the Stannous Chloride ($SnCl_2.\ 2H_2O$) method, the concentration of the soluble phosphate in the supernatant was determined every 7 days.

Production of Ammonia, Hydrogen cyanide (HCN) and Exopolysaccharide (EPS): Production of ammonia was tested peptone water for each isolate. Nessler's reagent is added to determine the colour change to yellow or orange indicating positive test. Quantification of the ammonia produced was done after 10 days by measuring absorbance at 540nm (Patel et al., 2018). Picrate test was used to measure HCN generation on nutritional agar slant and filter paper impregnated strip for 24-48 hours with 0.5% picric acid and 2% sodium carbonate [14]. EPS production was determined in basal media with 10% sucrose. After 5 days, EPS was extracted from culture supernatant by adding chilled acetone in 1:3 ratio. The wet weight and dry weight of the crude extract was measured

Characterization of the selected rhizobacteria

Morphological & Cultural characterization: The gram staining method was used to investigate morphological characteristics. After gram staining, cells were examined under a microscope (oil immersion, 100 X). On solidified nutrient agar plates, isolated colonies of purified strains were observed,

and information was gathered about the colonies' shape, size, texture, elevation, margin, consistency, pigmentation and optical feature [21] (modified).
Biochemical characterization: Once it was observed that amongst 19 different bacteria, total five bacteria were favourable PGPR for iron and zinc solubilization, thus these isolates were utilized for their preliminary characterization. Biochemical test was performed for the primary identification of selected five rhizobacteria.

RESULTS AND DISCUSSION

Results for screening of zinc solubilising and siderophore producing bacteria

Results show that among all the isolates, seven isolates gave zone of solubilization in media containing ZnO containing insoluble zinc source. These isolates were *Serratia marcescens*, *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis*, MGP10, ZSM1 and ZSM2. The isolates gave zone of solubilization ranging from 5mm to 27mm. The maximum zone of solubilization was given by *P. aeruginosa* followed by ZSM1 and ZSM2. Zinc solubility index (SI) was detected in the range of 100- 500%. All the isolates mentioned above (except MGP10) and isolate R1 were also able to solubilize media containing $ZnCO_3$ as Zinc source. Total 5 isolates were able to solubilize $ZnPO_4$ as insoluble zinc source. These are R1, ZSM1, ZSM2, *P. aeruginosa* and *Serratia marcescens*. The solubility index (SI) of all the isolates in medium containing different zinc sources are summarized in Table 1. Khan et al., 2022 [11] also investigated zinc solubilising isolates for plant growth promotion using the same method and $ZnCO_3$ as zinc source. The maximum zinc solubility index was reported to be 233% by isolates HRM29 and PAWR 28 which is similar to findings in present study. Different *Pseudomonas sp.* have been reported to solubilize zinc [8]. Kushwaha et al., 2021 [15] worked with *Bacillus sp.* that were capable of zinc solubilization. When included in solid and liquid growth medium, a strain of *P. aeruginosa* (CMG 823) isolated from a tannery air environment solubilized both ZnO and $Zn_3(PO_4)_2$ [3]. *P. aeruginosa* and *S. aureus* showed orange halo zone on CAS-agar media indicating positive result for siderophore production (Figure 1). These isolates were selected for further siderophore tests. The halo zone produced by *P. aeruginosa* and *S. aureus* were 16mm and 11mm respectively after 72 hours of incubation. Kumar et al., 2021 [13] reported siderophore production by *Pseudomonas sp*. RP21.

Results for detection of siderophore activity and Arnow's assay

For detection of siderophore activity, both the isolates were cultured on liquid succinic acid medium. Siderophore generation was monitored by CAS assay after 48 hours of incubation. Both the isolates produced positive siderophore assay results. The maximum siderophore activity of 89% was given by *P. aeruginosa* and *S. aureus* gave 68.46% siderophore units. The observed colour change is given in Figure 2. Patel et al., 2018 [18] reported siderophore activity of *P. dispersa* MPJ9 and *P. putida* MPJ6 as 89.9% and 85.3% respectively. This is approximately similar to the siderophore activity given by isolates in present study. It was found that MPJ9 was producing catecholate type of siderophore. The present study also found that *P. aeruginosa* was able to produce catecholate and hydroxamate type of siderophore as detected by Arnow's assay and Csaky's assay respectively. *S. aureus* was able to produce hydroxamate siderophore but not the catecholate type of siderophore.

Results for Plant growth promoting traits of the selected isolates

Auxin Production: Five isolates *P. aeruginosa*, *S. aureus,* ZSM1, ZSM2 *and S. marcescens* were selected for determining Plant growth promoting attributes. All the isolates showed positive auxin production. Maximum auxin production was given by *S. marcescens* (60 µg/mL) and ZSM1 produced minimum auxin (20 µg/mL). Figure 3 (I) depicts the quantitative estimation of auxin production by the selected isolates. Maximum IAA production was given by HRM29 i.e., 49.21 µg/mL followed by PAWR28 with 46.20 µg/mL which are similar with the present study. According to the findings of Donate-Correa et al.,2005 [2], the greatest levels of observed IAA and HCN production were seen in *Pseudomonas* strains.

Phosphate solubilization: *P. aeruginosa*, ZSM1 and ZSM2 gave zone of solubilization of 17mm, 13mm and 17mm respectively. On liquid Pikovskaya's broth medium, maximum phosphate solubilization was found by ZSM2. All the three isolates were studied for quantitative estimation of phosphate solubilization (Figure 3 (II)). *Pseudomonas fluorescens* and *Burkholderia spp*. were also capable of phosphate solubilization. Phosphate solubilization was also reported by different Bacillus spp. on pikovskaya's medium that showed halo zones around their colonies [17].

EPS Production: *P. aeruginosa, S. aureus* and *S. marcescens* were able to produce EPS. ZSM1 and ZSM2 showed negative result for EPS production. Maximum EPS was produced by *S. marcescens* showing 8mg/mL of dry weight production followed by *P. aeruginosa* that produced 6mg/mL of EPS. *S. aureus* produced 2mg/mL EPS. EPS production by all the isolates are compared in Figure 3 (III).This is approximately similar to the EPS production by *Pseudomonas entomophila* PE3 that produced

0.54g/100mL under non-saline condition [4]. *Rhizobium radiobacter* (LB2) produced 70 µg/mL of EPS that is less than the number of EPS recorded in present study [22].
Production of Ammonia and HCN: All 5 bacteria developed yellow to orange colour indicating positive for ammonia production. Maximum ammonia was produced by *S. marcescens*. Figure 3 (IV) shows the result for quantitative estimation of ammonia production. Kumar et al., 2014 [14] found that isolates produced ammonia that helped in growth and development of wheat. The synthesis of ammonia from peptone water, which is another crucial characteristic of PGPR and is utilised by plants as a source of nitrogen for their growth, was also strongly demonstrated by Bacillus isolates including *Bacillus licheniformis* (BGBA-1), *Bacillus coagulans* (BGBA-2), *Bacillus circulans* (BRBA-1), *Bacillus niacin* (BRBA-2)(Pahari et al., 2017). For HCN production by picrate test, only ZSM2 was able to turn the colour of the filter paper to brown from yellow indicating HCN production. All other 4 bacteria showed negative result. Donate-Correa et al.,2005 [2] also reported that while most of the PGPR were not able to produce HCN, only three strains of *Pseudomonas fluorescence* turned the colour of the filter paper from brown from yellow.
Results for characterization of the selected rhizobacteria
Morphological characteristics as observed by the gram staining method is given in Table 2. Cultural and Biochemical characteristics of *P. aeruginosa, S. aureus, S. marcescens,* ZSM1 and ZSM2 are summarized in Table 3 and Table 4 respectively.

CONCLUSION

Bacteria having capability to produce siderophores and solubilization of different insoluble zinc sources were screened in the present study. In addition, these bacteria also showed plant growth promoting attributes such as Phytohormone production, Ammonia, EPS and secondary metabolites (HCN) production as well as solubilization of insoluble phosphates. Thus, these bacteria constitute a group known as plant growth promoting rhizobacteria (PGPR). These properties help in plant growth and development and increased micronutrient concentration in edible portions of the plants.

Zinc Source	***P. aeruginosa***	***S. marcescens***	***S. aureus***	**ZSM1**	**ZSM2**	**MGP10**	***B. Subtilis***	**R1**
ZnO	180	160	140	160	144	118	200	-
$ZnCO_3$	147	104	500	118	120	-	100	118
$ZnPO_4$	183	150	154	178	-	-	-	125
ZnS	-	-	-	-	-	70	-	-

Table 1: Zinc solubility index (SI) (in %) of different rhizobacteria in various Bunt- Roviera agar media amended with various insoluble zinc sources.

Characters	***P. aeruginosa***	***S. marcescens***	***S. aureus***	**ZSM1**	**ZSM2**
shape	Rod	Rod	Round	Rod	Rod
size	Small	intermediate	Small	Intermediate	Big
arrangement	Single/in pair	Single/in chain	In clusters	Single	Single/chain
Gram's reaction	Gram negative	Gram negative	Gram positive	Gram negative	Gram positive

Table 2: Results for morphological characteristics for rhizobacteria

Table 3: Results for cultural characteristics by the means of visualized observation from the rhizobacteria grown on nutrient agar medium

Colony characters	***P. aeruginosa***	***S. marcescens***	***S. aureus***	**ZSM1**	**ZSM2**
Shape	Irregular	Round	Punctiform	Round	Round
Size	small	big	Intermediate	Small	Big
Surface	Smooth	Smooth	Vesicular	Smooth	Smooth
Elevation	Low convex	umbilicate	Convex	Flat	Convex
Margin	Entire	Entire	Entire	Entire	Entire
consistency	Moist	Moist	Butyrous	Moist	Moist
Opacity	opaque	opaque	Opaque	Translucent	Opaque
pigmentation	Green	Red	Yellow	white	white

SR. NO.	Name of the test	*P. aeruginosa*	*S. marcescens*	*S. aureus*	ZSM1	ZSM2
1	Methyl red test	-	-	+	+	+
2	Voges-Proskauer test	-	-	+	+	+
3	Sugar fermentation test					
	1.glucose	-	+	+	⊥	⊥
	2.mannitol	-	+	+	-	-
	3.maltose	+	+	+	⊥	+
4	Citrate utilization test	+	+	+	+	+
5	Starch utilization	+	+		-	-
6	Lipid utilization	-	+	+	+	+
7	Urea utilization	-	-	+	-	+
8	Indole production	-	-	-	-	-
9	H_2S production	-	-	-	-	-
10	Gelatine hydrolysis	+	+	+	-	-
11	Phenylalanine test	-	-	+	-	-
12	Dehydrogenase test	+	+	-	-	-
13	Triple sugar ion test	Alkaline; -H_2S	Alkaline; -H_2S	-	-	-
NOTE: (-) Negative, (+) Positive, (⊥) presence of only acid						

Table 4: Results for biochemical characterization for rhizobacteria through biochemical tests

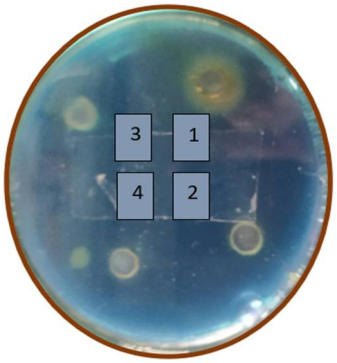

Figure 1: (1) *P. aeruginosa,* (3) *S. aureus* showing zone on CAS-agar plate while isolate *S. marcescens* (2) and *Bacillus subtilis* (4) showing negative result.

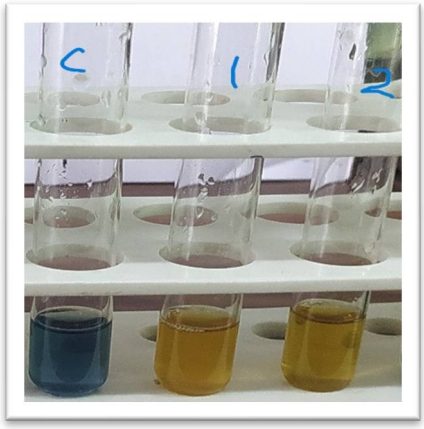

Figure 2: Results for determination of siderophore activity by *P. aeruginosa* (1) and *S. aureus* (2) compared to control (C).

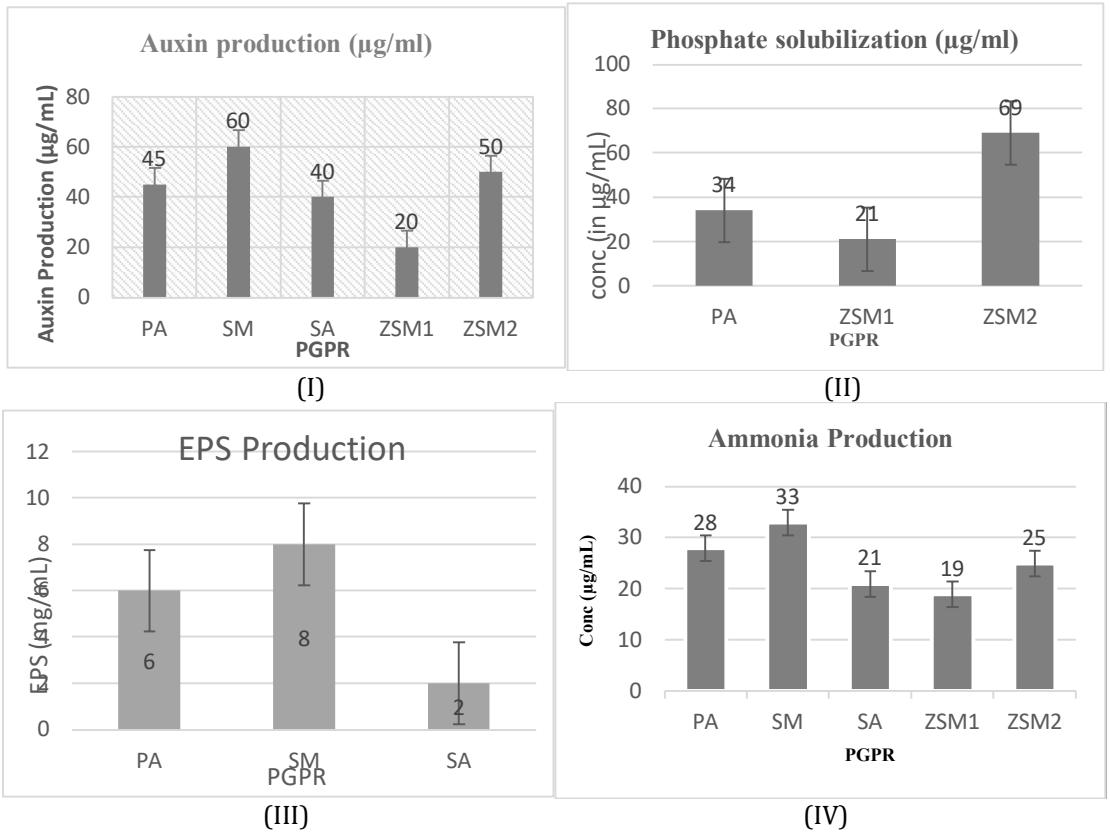

Figure 3: Results for the quantitative estimation of Plant growth promoting traits of selected rhizobacteria (I) Auxin Production (II) Phosphate solubilization (III) EPS production (IV) Ammonia production.

COMPETING INTEREST

The authors have declared that no competing interest exists.

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