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Plant growth promoting and antifungal asset of indigenous rhizobacteria secluded from saffron (*Crocus sativus* L.) rhizosphere

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ABSTRACT

Saffron (*Crocus sativus* L.) is an important plant in medicine. The Kashmir Valley (J&K, India) is one of the world's largest and finest saffron producing regions. However, over the past decade, there has been a strong declining trend in saffron production in this area. Plant Growth Promoting Rhizobacteria (PGPR) are free living soil bacteria that have ability to colonize the surfaces of the roots and ability to boost plant growth and development either directly or indirectly. Using the efficient PGPR as a bio-inoculant is another sustainable agricultural practice to improve soil health, grain yield quality, and biodiversity conservation. In the present study, a total of 13 bacterial strains were isolated from rhizospheric soil of saffron during the flowering stage of the tubers and were evaluated for various plant growth promoting characteristics under *in vitro* conditions such as the solubilization of phosphate, production of indole acetic acid, siderophore, hydrocyanic acid, and ammonia production and antagonism by dual culture test against *Sclerotium rolfsii and Fusarium oxysporum*. All the isolates were further tested for the production of hydrolytic enzymes such as protease, lipase, amylase, cellulase, and chitinase. The maximum proportions of bacterial isolates were gram-negative bacilli. About 77% of the bacterial isolates showed IAA production, 46% exhibited phosphate solubilization, 46% siderophore, 61% HCN, 100% ammonia production, 69% isolates showed protease activity, 62% lipase, 46% amylase, 85% cellulase, and 39% showed chitinase activity. Three isolates viz., AIS-3, AIS-8 and AIS-10 were found to have the most plant growth properties and effectively control the growth of *Sclerotium rolfsii* and *Fusarium oxysporum*. The bacterial isolates were identified as *Brevibacterium frigoritolerans* (AIS-3), *Alcaligenes faecalis* subsp. *Phenolicus* (AIS-8) and *Bacillus aryabhattai* (AIS-10) respectively by 16S rRNA sequence analysis. Therefore, these isolated rhizobacterial strains could be a promising source of plant growth stimulants to increase cormlets growth and increase saffron production.

1. Introduction

Saffron, commonly referred to as Zafran, and scientifically classified as *Crocus sativus*, belongs to the Iridaceae family of the order asparagales [31]. It is a small perennial plant with flowers that are purple in colour. The corm, which is an underground component of the plant, raises the flower stalks and leaves [22]. Saffron is commercially important because it is the highest-priced aromatic medicinal plant in the world and is referred to as the 'Golden spice.' Besides being used as a flavoring and coloring, the spice has certain medicinal properties [31]. Traditionally, plant stigmas have been used to treat various human diseases. Some of the documented pharmacological properties of saffron extracts include anti-cancer, anti-diabetic and neurological diseases. [10,56], anti-nociceptive and anti-inflammatory [62], antioxidant [41]; anticonvulsant, antidepressant [1,44] immunomodulatory, antifungal [33], hypolipidemic, antimicrobial [7], and several other bioactivities. The main saffron producing countries in the world are Spain, Iran and India. The only state in India where saffron is grown is Jammu and Kashmir.

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Pampore, a town in Kashmir, is renowned worldwide for its high quality saffron [35].

Unfortunately, Kashmir's total saffron cultivation area decreased from 5707 ha to 2667 ha from the year 1997–2007, whereas the yield has observed a dip from 15.95 to 5.61 tonnes in the decade [54]. The key causes of the declining trend in saffron yield are biological stress induced by different pathogens. *Macrophomina phaseolina*, *Sclerotium rolfsii*, *Fusarium moniliforme*, *F. pallidoroseum, F. equiseti, Phoma crocoplila, Mucor* sp., *Penicillium* sp. and *Rhizoctonia crocorum* are some of the common fungal pathogens that invade the saffron and produce corm rot [26]. Inherently adapted to low input farming systems, saffron demonstrates a good response to biofertilizers [29]. Chemical fertilisers are widely used across the globe to provide the soil-plant system with vital nutrients. In today, s agriculture, however the costs, availability, and environmental problems of chemical fertilizers are real issues. Cultivation of saffron only in specific belts in world, its economic importance and corm root cycle makes it an attractive candidate for studying its rhizosphere. The most dynamic ecosystem on earth is the rhizosphere, first described by Hiltner (1904). Diverse and complex contact occurs in the rhizosphere between plant roots, soil micro biota and the soil that has developed between plants and microbes due to mutual benefits. The plant partner supplies the rhizosphere with substrate and energy flow and in return receives nutrients and minerals, necessary for its growth and development [24]. Risphosphere has been the subject of agricultural research for many years, due to its significance in productivity of crops, soil health and sustainable agriculture [4,63,64]. Plant growth-promoting rhizobacteria are free-living soil bacteria that have the ability colonize the rhizosphere/or the surfaces of the plant roots, and improve the growth, and yield of plants when used on seeds or crops (Kumar et al., 2014). The effect of PGPR on plant growth is mostly explained by the release of metabolites that directly stimulate growth. Numerous mechanisms have been proposed to explain how PGPR supports the host plant. These include: (a) the ability to generate plant growth phytohormones such as indole acetic acid (IAA), gibberellins and cytokinins [23,40]; (b) the ability to enhance asymbiotic N2 fixation (Sahin et al., 2004 [32]; (c) the solubilization of inorganic phosphates and organic phosphate and/or other nutrient mineralization [2,23] and (d) antagonistic action against phytopathogenic microorganisms due to the development of siderophore, the production of antibiotics and enzymes that degrade the cell wall of pathogenic fungi, as well as competition with detrimental microorganisms [6,50].

A large number of bacteria have been isolated and identified from the rhizosphere soil over the past few decades, including common species of *Bacillus, Alcaligens, Azospirillum, Acinetobacter, Azotobacter, Rhizobium, Pseudomonas, Burkholderia, Acinetobacter, Klebsiella, Arthobacter, Enterobacter, Burkholderia,* and *Serratia* [23,34]. Knowing the interactions between PGPR and plants would be useful in designing strategies for improving plant growth. Chemical fertilizers being highly costly and extremely harmful to the environment may pose a significant threat to human health. The use of PGPR in agriculture continues to grow as nutrient supplements for the soil and as bio-control agents. They offer substitute to chemical fertilizers, herbicides, antibiotics, and pesticides [4,49]. The significance of the production of saffron stigma in our state's economy encouraged us to isolate and screen new PGPRs. Identifying beneficial soil bacteria and using them as biofertilizers or biopesticides is a sustainable way to increase saffron growth and yield with lowest possible environmental risks. Some bacteria have been shown to increase fresh and dry stigma weight [3,8,59], leaf numbers, stigma length, corm weight, and saffron stigma yield [51]. In this article, the information on the isolation and identification of bacteria isolated from the rhizospheric soil of saffron with the potential to be used both for the management of plant pathogens and for achieving good crop yields was provided.

2. Materials and methods

2.1. Sampling process and bacterial isolation

Rhizosphere soil samples were taken during the flowering period of saffron corms from the fields in Pampore area of Pulwama district (34.02°N; 74.93°E; 5164 feet high), Jammu and Kashmir, India, for the isolation of rhizobacterial strains. The corms were uprooted and the soil was collected in sterilized plastic bags, adhering to the roots reflecting rhizosphere soil. The soil samples were then transported to the microbiology laboratory of the Osmania University, Hyderabad, for immediate processing. Around 10 g of rhizospheric soil was mixed in a flask with 90 mL of sterile distilled water and shaken on a rotary shaker for 30 min. Subsequently 1 mL of flask suspension was applied to the 10 mL vial and up to 10^{-7} dilution was made in successive dilutions. On nutrient agar media plates amended with 50 μg/mL cycloheximide to prevent the growth of fungi, approximately 0.1 mL of this suspension was spreaded. For typical bacterial colonies, the plates were observed and well-isolated single colonies were selected for streaking on fresh nutrient agar plates to get the pure culture. The most prominent isolates were stored on nutrient agar slants at 4 °C in the refrigerator for further examinations.

2.2. Morphological and biochemical characterization of isolated bacteria

The isolated bacterial cultures were characterized phenotypically to the genus-level based on its colony morphology (colour, shape, margin, elevation, appearance and pigmentation), microscopic observation as described by Vincent 1970 (cell arrangement and Gram staining), and biochemical tests (starch, catalase, oxidase, nitrate reduction, motility, citrate utilization, indole, methyl red, Voges Proskauer, H_2S, gelatinase and urease) were performed according to the standard procedures (Aneja, 2003 [18];). The 8th edition (Buchanan and Gibbons, 1974) and 9th edition (Bergey and Holt, 2000) of the Bergey's Determinative Bacteriology Manual were referred for confirmation of the identity of the bacterial isolates.

2.3. In vitro plant growth promoting traits of the isolated bacteria

The isolated rhizobacteria were evaluated *in vitro* for plant growth promoting activities such as solubilization of phosphate, production of siderophore and indole acetic acid, ammonia and hydrocyanic acid production. Phosphate solubilizing capacity was performed by spot inoculating the bacterial cultures on Pikovskaya medium to determine their ability to solubilize tri-calcium phosphate according to the method by Ref. [46]. The ability to solubilize phosphate was observed by the development of a halo zone around the colony (10 μL inoculum, 1×10^8 CFU mL^{-1}) after 72 h of incubation. The isolated bacteria were qualitatively analyzed for the siderophore production by spot inoculating (5 μL inoculum, 1×10^8 CFU mL^{-1}) on chrome Azurol S agar plates and incubated for 4–5 days in dark at 30 °C. The formation of yellow to orange halo around the bacterial colony against a blue background indicated the production of siderophore [53]. Isolates has been streaked on nutrient agar plates amended with 4% glycine for qualitative estimation of HCN. Whatman filter paper was immersed in a 2% Na_2CO_3 solution containing 0.5% picric acid was placed between base and lid of Petri dish and incubated for 96 h at 28 ± 2 °C in the inverted position and observed for a colour change from yellow to brown as defined by Ref. [11]. Bacterial isolates were examined for the development of ammonia in peptone water. 10 mL of peptone water broth were inoculated with 100 μL of actively growing selected bacterial cultures and incubated for 48 at 30 °C and 120 rpm in an incubator shaker. After incubation, 1 mL of Nesslers reagent was added to each tube and observed for color change. Development of brown to yellow colour was considered a positive test for the production of ammonia [18]. The isolated bacteria were qualitatively assayed for the production of indole

acetic acid by spot inoculation on nutrient agar medium supplemented with 5 mM L-tryptophan and after incubation for 24–48 h; the inoculated points were superimposed with a nitrocellulose membrane (NCM) disk, 10 mm in diameter, pre-saturated with few drops of Salkowski reagent (1 mL 0.5 M $FeCL_3$, 50 mL H_2SO_4). The appearance of pink color was observed after few minutes, which was an indicator of IAA production [42].

2.4. Screening for hydrolyzing extracellular enzymes

The isolated rhizobacteria were screened *in vitro* by plate assay methods for extracellular enzyme activity. A qualitative assay for protease production was carried on skim milk agar plates. Actively grown isolates were spot inoculated, incubated for 24–48 h at 30 °C, and then the plates were analyzed for the clearance zone around the colonies suggesting enzymatic protease degradation [15]. Amylase activity was performed by inoculating the bacterial isolates on starch agar medium [(w/v) soluble starch 0.2%; yeast extract 0.15%; peptone 0.5%; NaCl 0.5%; beef extract 0.15%; agar 1.5%, pH 7.0 ± 0.2] and incubated for 24–48 h at 30 °C. The plates were flooded with iodine solution at the end of incubation period, stored for a minute and the poured out. Iodine reacts with starch to form a compound of blue colour. The colour less zones surrounding colonies shows the production of amylase [17]. Chitinase activity was performed by inoculating rhizobacterial strains on plates containing colloidal chitin agar and incubated for 5–6 days at 30 ± 2 °C. The plates were stained with 0.1% congored solution for 1 h after incubation, and destained with 0.1% sodium chloride. The evidence of clear zone around the colony refers the production of chitinase [55]. Cellulase activity was performed by inoculating the actively grown cultures on carboxy methyl cellulose congo red media. The plates were incubated at 28 ± 2 °C for 2–3 days. Halo zone formation around the bacterial colonies shows the production of the cellulase enzyme [27]. Lipase activity was determined by inoculating the actively grown culture on Tween 80 agar media (peptone 10 g, $CaCl_2$ 2 g, tween80 4.7 mL, agar 20 g, distilled water 1 L, pH 7) and the plates were incubated at 28 ± 2 °C for 3–4 days. The formation of halo area around the bacterial colonies demonstrates the production of lipase enzyme [12].

2.5. In vitro antagonistic activity against Sclerotium rolfsii and Fusarium oxysporum

Antagonistic activity against *Sclerotium rolfsii* and *Fusarium oxysporum* was evaluated by the dual culture method. Soil borne plant pathogenic fungi *Sclerotium rolfsii* and *Fusarium oxysporum* were grown on potato dextrose agar (PDA) media. From an actively growing fungal culture, a 5 mm diameter plug of fungal mycelium was cut and mounted in the centre of the Petri dish containing fresh potato dextrose agar. On one edge of a 90 mm diameter Petri dish, a loop of exponentially grown culture of each isolate was streaked in a straight line and the distance between the fungus and the test culture was held at 2 cm, and the plates were incubated for 4–7 days at 28 °C. Radial growth inhibition of test fungus has been observed regularly. Plates with cultures of the studied fungus only served as control. Three replicates have been taken in each case. After five days, the diameters of the colonies were measured and average values compared with control were taken as a measure of fungitoxicity. Growth inhibition (%) of test fungus was determined according to the following formula cited by Pani and Patra (1997), [48].

$$\text{Percent inhibition (I)} = \text{C-T/C} \times 100$$

Where, C- is radial growth of pathogen in control and T-is radial growth of pathogen in treatment.

2.6. Identification of bacterial isolates

Bacterial isolates were identified morphologically by Gram's staining and biochemical tests according to the Bergey's manual (1994). To molecularly identifify the isolated bacteria, their pure cultures were grown in nutrient broth until the log phase. Genomic DNA was extracted in accordance with the [13] procedure. Amplification of 16S rDNA regions was performed with forward 27f (5-AGATTTGACMTGGCTAG-3) and reverse primer 1492R (5- GGTTACCTTGTTACGCTT-3) as per the method described by Ref. [38]. PCR amplifications were carried out in thermal cycler (Eppendorf PCR thermal cycler, Hamburg, Germany) programmed for 30 cycles, an initial denaturation step of 3 min at 94 °C, then 30 s at 94 °C for denaturation, 30 s at 55 °C for primer annealing and primer extension of 2 min at 72 °C, followed by a final extension of 10 min at 72 °C. The PCR products were checked by electrophoresis on 1.2% agarose gel in TBE buffer for 1 h at a constant of 70-V. The image of the resulting gel was obtained using a Document Gel system after dying with 1% of DNA safe stain. All amplified PCR products have been purified and sequenced at Eurofins Genomics Pvt. Ltd, Bangalore, India. Further, the identification of isolates was done by BLAST using Ez-BioCloud server. On the basis of analysis outcome, similarity of bacterial isolates was detected; sequences were deposited at EMBL to obtain accession numbers. The phylogenetic tree of newly isolated strains and their associated members were developed using the software MEGA6 [60].

3. Results and discussion

3.1. Isolation, morphological and biochemical characterization of isolated bacteria

During the flowering season, a total of 13 bacteria were isolated from the rhizosphere soil of saffron on nutrient agar, as the roots are fully grown during this stage. All the isolates were labelled as AIS followed by numeric digit. The isolates are designated as AIS-1, AIS-2, AIS-3, AIS-4, AIS-5, AIS-6, AIS-7, AIS-8, AIS-9, AIS-10, AIS-11, AIS-12 and AIS-13. Each isolate was characterized by their colonial features such as size, shape, color, margin, elevation, opacity and were also characterized for cellular morphology by light microscopy (Table 1, Fig. 1 A). All isolates were motile, rod shaped. The highest percentage occurrence of bacterial isolates of the saffron rhizosphere on culture media 69.23% (9) were gram negative and 30.76% (4) were gram positive (Fig. 1B). The results of the biochemical propertie of the isolated bacteria are represented in Table 2. All the isolated bacteria were positive for catalase and oxidase as indicated by release of gas bubble around the bacterial colonies and change in colour of oxidase discs (Fig. 1C). Most of isolates exhibit positive results for starch hydrolysis except the isolates AIS-11, AIS-12, and AIS-13 which showed negative results with starch hydrolysis. Iodine and starch produce a blue color complex, while iodine does not react with any starch degradation product, so no color is produced in such cases. Clear zones around the bacterial colonies were observed when flooding the inoculated plates with iodine solution, while blue colour occurs on no growth areas (Fig. 1D). In the nitrate reduction test, a positive test is shown by a red color change when sulphanilic acid and α-naphthylamine are incorporated. All the isolates were found to be positive for nitrate reduction during the *in vitro* examination (Fig. 1E). In the Methy Red (MR) test, acid produced from the glucose, the isolates AIS-1 and AIS-10 were positive, whereas remaining isolates were negative. Most of the isolates exhibit positive result for gelatinase enzyme except AIS-7, AIS-8 and AIS-9 which showed negative results for gelatinase enzyme (Fig. 1F). All the isolates exhibit negative results with urease except AIS-3 and AIS-4. Based on the morphological, microscopy, and biochemical reactions, the isolated rhizobacterial strains were tentatively identified as genus *Bacillus* spp, *Alcaligenes* spp, *Pseudomonas* spp, and *Pantoea* ssp.

3.2. In vitro plant growth promoting traits of the isolated bacteria

The bacterial isolates were tested for various plant growth promoting

Table 1
Morphological characteristics of the isolates obtained from the rhizosphere soil of saffron.

Isolate	CS	CC	CE	CM	Appearance	Cell arrangement	Gram reaction
AIS-1	Irr	CW	Raised	Entire	Opaque	Short rods	Positive
AIS-2	Cir	LG	Flat	Convex	Opaque	Short rods	Negative
AIS-3	Cir	CW	Flat	Convex	Opaque	Short Rods	Positive
AIS-4	Cir	CW	Flat	Convex	Opaque	Short Rods	Positive
AIS-5	Irr	W	Flat	Entire	Opaque	Rod shaped	Negative
AIS-6	Irr	W	Flat	Entire	Translucent	Rod shaped	Negative
AIS-7	Irr	W	Flat	Entire	Translucent	Rod shaped	Negative
AIS-8	Irr	W	Flat	Entire	Translucent	Rod shaped	Negative
AIS-9	Irr	MW	Flat	Entire	Opaque	Rod shaped	Negative
AIS-10	Irr	CW	Raised	Entire	Opaque	Short rods	Positive
AIS-11	Cir	Y	Flat	Convex	Opaque	Short rods	Negative
AIS-12	Cir	LG	Flat	Convex	Opaque	Short rods	Negative
AIS-13	Cir	W	Flat	Entire	Opaque	Short rods	Negative

CS: colony shape, CC: colony color, CE: colony elevation, CM: colony margin, Irr: irregular, Cir: circular, CW: creamish white, LG: light green, W: white, MW: milky white, Y: yellowish.

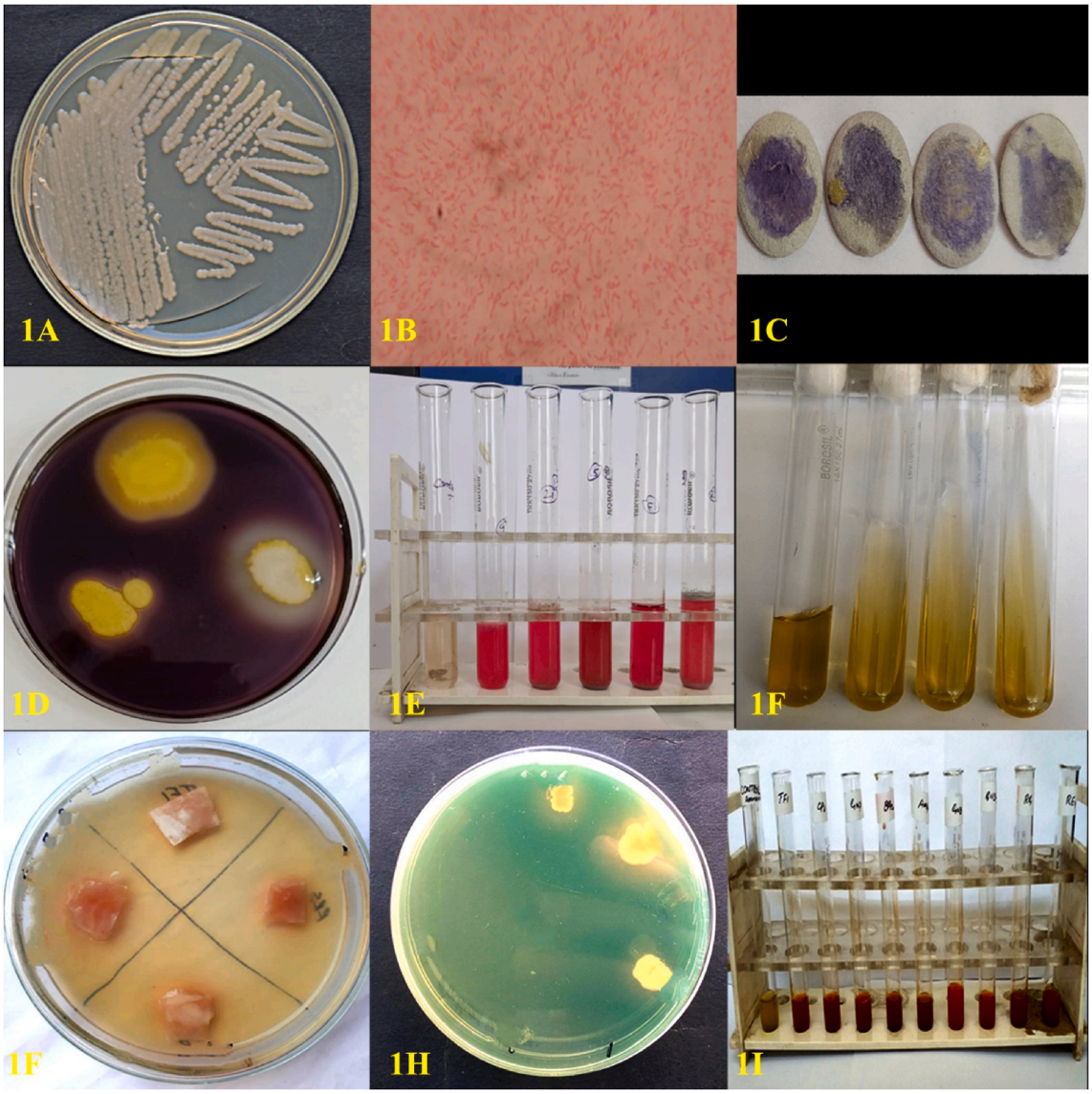

Fig. 1. A) Bacterial cultures on nutrient agar media, B) Gram staining, C) oxidase discs, D) starch hydrolysis; E) nitrate reduction, F) gelatinase activity; G) IAA production, H) siderophore production, and I) ammonia production.

activities which are shown in Table 3. The isolates showed varying levels of PGPR characteristics such as solubilization of phosphate, IAA, ammonia, siderophore and HCN production. Out of 13 bacterial isolates, 10 were able to produce IAA in this study. The isolates AIS-3, AIS-8, and AIS-10 showed high intensity (+++) of pink color indicating higher production of plant growth promoting hormone indole acetic acid.. The

Table 2
Biochemical characteristics of the isolates obtained from the rhizosphere soil of saffron.

Isolate	Star	Cat	Oxid	Nitrate	Mot	Indole	Cit	MR	VP	Gel	H_2S	Urease
AIS-1	+	+	+	+	+	–	+	+	–	+	–	–
AIS-2	+	+	+	+	+	-	+	–	–	+	-	-
AIS-3	+	+	+	+	+	+	+	–	–	+	+	+
AIS-4	+	+	+	+	+	+	+	–	–	+	+	+
AIS-5	+	+	+	+	+	–	+	–	–	+	–	–
AIS-6	+	+	+	+	+	–	+	–	+	+	–	–
AIS-7	+	+	+	+	+	–	+	–	v	–	–	–
AIS-8	+	+	+	+	+	–	+	–	v	–	–	–
AIS-9	+	+	+	+	+	–	+	–	v	–	–	–
AIS-10	+	+	+	+	+	–	+	+	–	+	–	–
AIS-11	–	+	+	+	+	–	+	–	–	+	–	–
AIS-12	–	+	+	+	+	–	+	–	–	+	–	–
AIS-13	–	+	+	+	+	–	+	–	–	+	–	–

Note: (+) positive, (−) negative, Star: starch, Cat: catalase, Oxid: oxidase, Mot: motility, Cit: citrate, MR: methyl red, VP: Voges- Proskauer, Gel: gelatin, H_2S: hydrogen sulphide gas. V: variable.

Table 3
In vitro screening of bacterial isolates for PGPR traits.

Isolate	P solubilization (zone in mm)	HCN	Ammonia	IAA	Siderophore
AIS-1	0	+	++	++	–
AIS-2	0	_	++	+	–
AIS-3	16	++	+++	+++	+++
AIS-4	0	–	++	+	
AIS-5	10	++	+++	–	–
AIS-6	11	+	+	+	+
AIS-7	0	–	+	+	–
AIS-8	19	+++	+++	+++	+++
AIS-9	–	–	+	–	–
AIS-10	18	++	+++	+++	+++
AIS-11	0	+	++	++	–
AIS-12	9	++	+	+	+
AIS-13	0	–	+++	–	+

Note: good activity: +++; moderate activity: ++; slight activity: +; no activity:

isolates AIS-1 and AIS-11 showed moderate intensity (++) of pink color. The isolates of AIS-2, AIS-4, AIS-6, AIS-7, and AIS-12 showed slight intensity (+) of the pink color indicating lower production of plant growth promoting, indole acetic acid (Fig. 1G). Out of 13 bacterial isolates, 6 isolates were capable to produce siderophore and this was verified by the appearance of orange halos around the colonies. Further, out of 6 isolates, the AIS-3, AIS-8 and AIS-10 showed strong (+++) siderophore production, and AIS-6, AIS-12 and AIS-13 exhibited slight activity (+) for siderophore production (Fig. 1H). All the isolates were capable of producing ammonia. Furthermore, out of 13 isolates, the AIS-3, AIS-5, AIS-8, AIS-10 and AIS-13 showed strong activity (+++) for ammonia production, while the AIS-1, AIS-2, AIS-4, and AIS-11 exhibited moderate activity (++) whereas the remaining 4 isolates viz., AIS-6, AIS-7, AIS-9 and AIS-12 showed mild activity (+) for ammonia production (Fig. 1I). Among the 13 isolates, eight isolates were reported positive for the hydrocyanic acid (HCN) production. Further, out of eight isolates, AIS-8 showing highest HCN production by changing the colour of filter paper from orange to brown. The isolates AIS-3, AIS-5, AIS-10, and AIS-12 showed moderate activity (++) whereas the three isolates namely AIS-1, AIS-6 and AIS-11 showed mild activity (+) for HCN production (Fig. 2A). After nitrogen, the most limiting nutrient for plant growth is phosphorus (P). Out of the 13 bacterial isolates, six isolates were found to solubilize tricalcium phosphate in Pikovskayas agar. Further, out of six isolates, the AIS-3, AIS-8, and AIS-10 exhibited the maximum solubilization zone (+++) whereas the AIS-5, AIS-6 and AIS-12 showed slight (+) solubilization zone (Fig. 2B).

3.3. Screening for hydrolyzing extracellular enzymes

In the rhizosphere, the microbial population produces a number of hydrolytic enzymes that are responsible for the breakdown of different components of fungal pathogens. All the thirteen bacterial isolates were qualitatively checked utilizing carboxy methyl cellulose (CMC), starch, colloidal chitin, tween 80 and skimmed milk agar media for production of hydrolytic enzymes like cellulase, amylase, chitinase, lipase and protease enzyme production. Proteolytic enzyme production was identified as the creation of a clear zone around the colony on skim milk agar medium. Out of 13 bacterial isolates, nine isolates were positive for protease production (Fig. 2C). Further, out of nine isolates, the AIS-3, AIS-8, and AIS-10 showed strong (+++) protease production. Eight isolates were positive for lipase, five isolates for chitinase enzyme production, eleven isolates for cellulase, and six isolates for amylase (Fig. 2D, E, 2F). Qualitatively, the hydrolysis zone depends on the amount of enzyme produced by the bacterial isolates. The results of the hydrolytic enzymatic activity of bacterial isolates are represented in Table 4.

3.4. In vitro antagonistic activity against Sclerotium rolfsii and Fusarium oxysporum

All 13 bacterial isolates were screened by dual culture method to determine their antagonistic activity against 2 phytopathogenic fungi (*Sclerotium rolfsii* and *Fusarium oxysporum*) on PDA. *Sclerotium rolfsii* mycelium growth was decreased by more than 50% by 5 isolates, with the highest inhibition by isolate AIS-10 (72%), followed by AIS-8 (70%), AIS-3 (65%) and AIS-4 (52%). Four isolates showed more than 50% of inhibition against *Fusarium oxysporum,* among them isolate AIS-8 displayed the highest inhibition of 71% followed by AIS-10 (69%), AIS-3 (66%) and AIS-11 (51%). Out of thirteen isolates 3 isolates viz., AIS-3, AIS-8 and AIS-10 exhibited inhibition potential against both *Sclerotium rolfsii* and *Fusarium oxysporum* (Table 5, Fig. 2G and H).

3.5. Identification of bacterial isolates

Thirteen bacteria were isolated from the rhizosphere soil of saffron and qualitatively screened for PGPR traits. Three potential isolates labelled as AIS-3, AIS-8 and AIS-10 showing significant phosphate solubilization, IAA, HCN, ammonia, siderophore, hydrolytic enzyme production and antagonistic activity against phytopathogenic fungi (*Sclerotium rolfsii* and *Fusarium oxysporum*) were selected for further evaluation. Isolates AIS-3, AIS-10, were primarily identified as Gram positive, rod shaped, and isolate AIS-8 was identified as gram negative, rod shaped bacteria based on Bergey's manual of determinative bacteriology (2010). For molecular identification, the sequences provided from Eurofins Genomics Pvt. Ltd, Bangalore, India (843 bp for AIS-3,

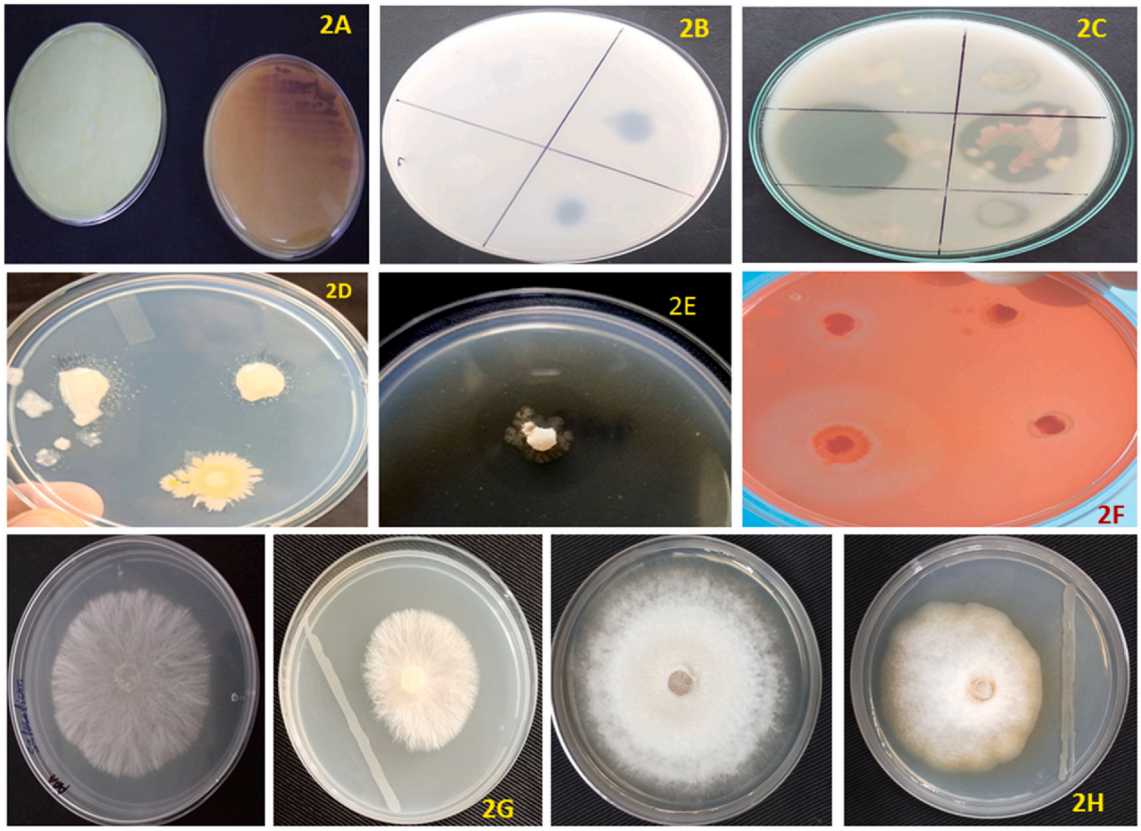

Fig. 2. A) Hydrocyanic acid production, B) TCP solubilization, C) protease production, D) lipase production, E) chitinase production, F) cellulase production, G) antifungal activity of isolates against *Sclerotium rolfsii*, and H) antifungal activity of isolates against *Fusarium oxysporum*.

Table 4
Hydrolytic enzymatic activity of isolated bacterial strains.

Isolate	Protease	Lipase	Amylase	Cellulase	Chitinase
AIS-1	+	++	–	+++	+
AIS-2	+	++	-	++	-
AIS-3	+++	++	++	+++	++
AIS-4	++	++	++	+++	+
AIS-5	+	-	++	–	–
AIS-6	–	-	–	+	–
AIS-7	–	-	–	–	–
AIS-8	+++	-	+++	++	+++
AIS-9	++	-	+	++	–
AIS-10	+++	+++	++	+++	++
AIS-11	–	++	-	++	-
AIS-12	–	++	-	++	-
AIS-13	++	++	-	++	-

Note: (+++: good activity, ++: moderate activity, +: slight activity, -: no activity).

Table 5
Antagonistic activities against plant pathogenic fungi.

Isolates	% growth inhibition of plant pathogenic fungi	
	Sclerotium rolfsii	Fusarium oxysporum
AIS-1	0	0
AIS-2	0	0
AIS-3	65	66
AIS-4	52	0
AIS-5	0	0
AIS-6	0	0
AIS-7	52	0
AIS-8	70	71
AIS-9	0	0
AIS-10	72	69
AIS-11	0	51
AIS-12	0	0
AIS-13	0	0

776 bp for AIS-8 and 1086 bp for AIS-10) were compared with related sequences from GenBank, aligned and the dendrogram inferred (Figs. 3 and 4). The sequences of 16 S rRNA gene of the isolated bacteria of AIS-3, AIS-8, and AIS-10 were found maximum identity with *Brevibacterium frigoritolerans, Alcaligenes faecalis* subsp. *Phenolicus,* and *Bacillus aryabhattai* respectively. The nucleotide sequences of the 3 isolated bacteria were submitted to EMBL (European Molecular Biology Laboratory) and NCBI (National Centre for Biotechnology Information) accession numbers were obtained as follows: AIS-3: LR828512; AIS-8: LR828513 and AIS-10: LR861809 (Table 6).

It is advisable to use novel PGP bacteria as biofertilizers, biopesticides and phytostimulator in the agricultural industry to increase yield production, quality, preserve soil fertility and prevents against pathogens. Thus agriculture-based research therefore focuses primarily on the rhizosphere, as this area is rich in microbial diversity [57]. In the present study, beneficial rhizobacteria were isolated from the rhizosphere soil of *Crocus sativus* during the flowering phase, as the roots are fully mature during this stage and were evaluated for different plant growth promoting traits and bio-control properties. A total of 13 bacteria were isolated on nutrient agar from the rhizosphere soil of saffron. All the isolates were characterized for their colonial traits such as size, shape, color, margin, elevation, opacity and also characterized for cellular morphology using light microscopy. According to Gram reaction, 9 isolates (69.23%) were found Gram negative and 4 isolates (30.76%) were Gram-positive. The large abundance of gram negative rod shaped bacteria is consistent with previous studies by Refs. [3,59] which reported higher level of gram negative bacteria in the rhizosphere soil of saffron relative to gram positive bacteria. Various workers have

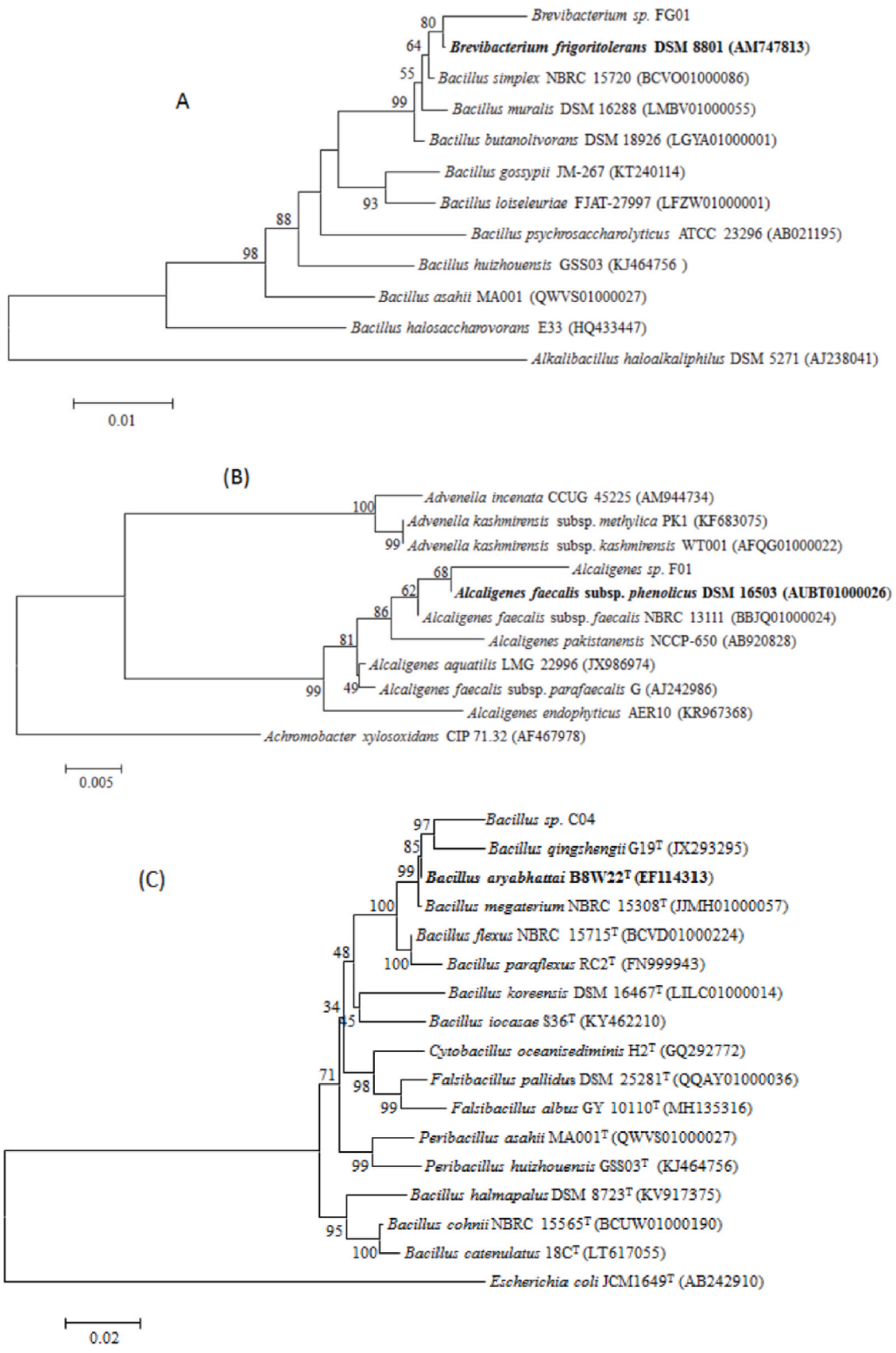

Fig. 3. Phylogenetic tree of isolates of A) AIS-3, B) AIS-8, and C) AIS-10.

reported similar findings [21,30,58].

Plant growth-promoting rhizobacteria are beneficial soil bacteria that compete for the rhizosphere and stimulate plant growth and productivity through various mechanisms. PGPR directly affect plant growth by enhancing nutrient intake by fixing atmospheric nitrogen [32,52], the ability to produce plant growth regulators or phytohormones such as indole acetic acid (IAA), cytokinins, and gibberellins [20], the solubility of insoluble phosphorus, the formation of siderophores and other inaccessible nutrients and indirectly promote plant growth through the production of lytic enzymes, antibiotics, and secondary metabolites. IAA is one of the most significant phytohormones for plant growth as it improves the elongation, division and differentiation of plant cells. In this study, out of 13 bacterial isolates tested, ten isolates were positive for IAA production (Table 3). Rhizobacterial

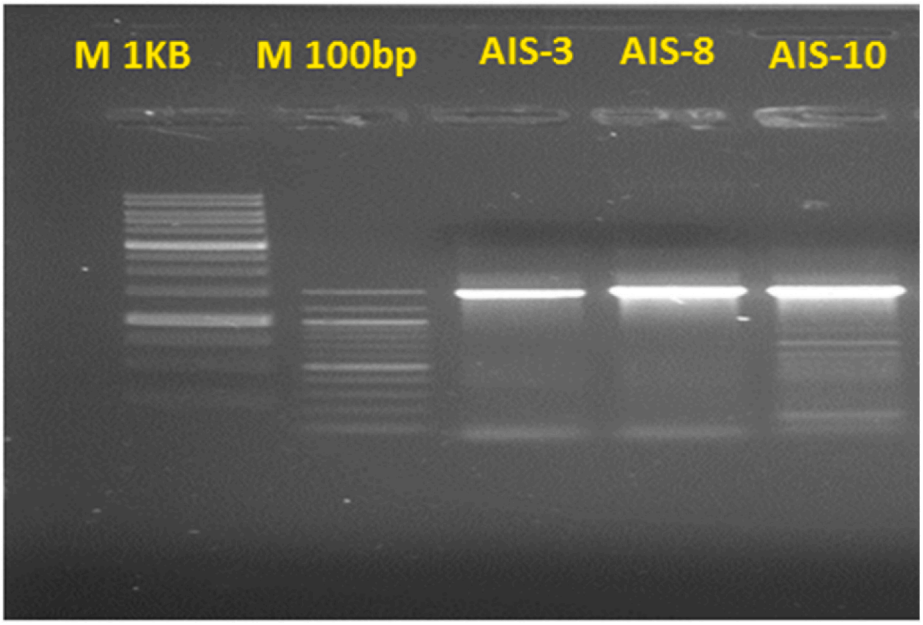

Fig. 4. Electrophoretic profile of 16S rDNA primers runs on 1% agarose gel.

Table 6
Molecular identification of potential plant growth-promoting bacterial isolates based on 16S rRNA sequence.

Isolate label	Source	Cell morphology	16S rRNA Sequence Length	Hit strain with accession number	Similarity (%)	GenBank accession number [EMBL]
AIS-3	Rhizosphere	Gram + ve rods	843bp	*Brevibacterium frigoritolerans* DSM 8801^T	99.13%	LR828512
AIS-8	Rhizosphere	Gram -ve rods	776bp	*Alcaligenes faecalis* subsp. *Phenolicus* DSM 16503^T	99%	LR828513
AIS-10	Rhizosphere	Gram + ve rods	1086bp	*Bacillus aryabhattai* B8W22^T	99%	LR861809

species identified from the rhizosphere are more potent auxin producers than those isolated from the non-rhizosphere soil [47]. This is an important mechanism for promoting plant growth because IAA facilitates root development and absorption of nutrients [14]. The examination of IAA production by the bacterial isolates showed that about 77% of the studied strains were able to produce auxin that matches with previous literature [36] which has validated the existence of large proportions of microorganisms capable of producing this hormone in the rhizosphere. The ability of rhizobacteria to solubilize insoluble phosphates has been of interest to agricultural microbiologist as it can increase the supply of phosphorus for the plant to boost plant growth and yield [39]. It has been reported that higher concentrations of phosphate-solubilizing bacteria are often found in the rhizosphere than in bulk soil [19]. These bacteria produce low molecular organic acids which acts on inorganic phosphorous. The utilization of phosphate solubilizing PGPR as inoculants is one of the substitute biotechnological solutions in sustainable agriculture to meet the phosphate needs of plants. In this study, out of 13 isolates, six isolates were able to solubilize insoluble phosphate. Highest zone of solubilization (19 mm) was recorded for isolate AIS-8 followed by AIS-10 (18 mm). The capacity of the isolated bacteria to solubilize insoluble phosphates is in line with the previous study by Ref. [43] who documented *Bacillus* sp., *Providencia* sp., *Brevundimonas* and *Alcaligenes* as phosphate solubilizers. Microorganisms also increases plant growth through scavenging available iron (Fe^{3+}), which involves release of high affinity, low molecular weight iron chelating ligands called siderophores [5]. Siderophores also play vital role in the biological control of certain soil-borne plant diseases caused by various pathogens. Since siderophores sequester the limited supply of iron in the rhizosphere, they reduce its availability to pathogens and eventually suppress their growth [37]. In this study, out of 13 isolates, six isolates were able to produce siderophore and it is verified by the production of orange halo zones around the colonies. The production of HCN by rhizobacteria is said to play vital role in the biological control of pathogens [16]. In this study, 61% of the bacterial isolates (AIS-8 AIS-3, AIS-5, AIS-10, AIS-12 AIS-1, AIS-6 and AIS-11) were positive for the production of HCN that acts as a plant resistance inducer. A number of factors have been identified to influence the rate of HCN production. Glycine has been found to be the direct precursor of microbial hydrocyanic acid formation and it has been found in root exudates [25]. Another significant feature of PGPR is the production of ammonia that indirectly affects the growth of plants. In this study, 100% of the isolates were able to produce ammonia, which is an inorganic volatile substance. This can be useful in biological control as Howell pointed out [28] when the ammonia-producing PGP bacterial appear to be one of the many mechanisms used by bacteria in the biological control of pre-emergence damping-off caused by pathogenic fungi.

Hydrolytic enzymes serve as agents for the prevention of plant diseases by inducing lysis of pathogenic microbes in close proximity to the plant, as they secrete increased levels of cell wall lytic enzymes (proteases, lipase, amylase, cellulase and chitinase) [61]. In this study, out of 13 bacterial isolates, 69% isolates were positive for the production of protease, 62% isolates were positive for lipase production, 46% isolates were positive for the production of amylase, 85% isolates were positive for cellulase production and 39% isolates were positive for chitinase production. It has been found that PGPR, which synthesizes one or more of these lytic enzymes, has bio-control capacity against a variety of phytopathogenic fungi and bacteria and improves crop yield. Soil borne phytopathogens are responsible for major plant diseases leading to loss of productivity and ultimately affecting economic values. Use of (PGPR) Plant Growth Promoting Rhizobacteria to manage the phytopathogens is

great natural bio-control approach; bio-control can help control the growth of phytopathogens and decrease the use of chemical fungicides, which are one of the main factors causing soil infertility. Antifungal activity of Plant growth promoting bacteria is an important feature for bacterial inoculants. It has been found that present isolates are good in inhibiting the growth of soil borne phytopathogens *S. rolfsii,* and *F. oxysporum* and *S. rolfsii* was inhibited more by AIS-10 (72%) followed by AIS-8 (70%), AIS-3 (65%) and AIS-4 (52%). Inhibition of *F. oxysporum* was high for AIS-8 (71%) followed by AIS-10 (69%), AIS-3 (66%) and AIS-11 (51%). The sequences of 16 S rRNA gene of the isolated bacteria of AIS-3, AIS-8, and AIS-10 were found maximum identity with *Brevibacterium frigoritolerans, Alcaligenes faecalis* subsp. *Phenolicus,* and *Bacillus aryabhattai* respectively. The same results were observed by Refs. [3,59] who isolated various species of *Bacillus* and *Brevibacterium* from the rhizospheric soil of saffron. [9,45]; also reported the isolation of various *Bacillus* spp. from the rhizospheric soil of saffron [56]. reported various endophytic *Bacillus* spp. from the saffron plants.

4. Conclusions

Globally, the consequences of the continuous and intensive use of agrochemicals to increase agricultural productivity can seriously damage soil fertility, the life of living organisms and their environment. In order to increase crop production on a sustainable basis, the use of plant growth-promoting rhizobacteria strains as agricultural crop bioinoculums is cheap and environmentally friendly solution. The plant-beneficial bioinoculum of PGP rhizobacteria can reduce global reliance on hazardous agrochemicals that threaten ecosystems. The present study was focused on the isolation, screening, and biochemical characterization of PGPR inhabiting saffron rhizosphere having excellent PGP properties such as phosphate solubilization, Indole acetic acid and ammonia production, bio-control properties such as hydrogen cyanide production, siderophore production and lytic enzymes production. Isolates with good plant growth promoting potentialities were characterized and the best three efficient isolates among them were identified. The isolates namely AIS-3, AIS-8 and AIS-10 have been identified as potential PGPR with best activities and they were further characterized by 16S rRNA sequencing as *Brevibacterium frigoritolerans, Alcaligenes faecalis* subsp. *Phenolicus,* and *Bacillus aryabhattai* respectively. With the success story of this primary screening protocol, we can further move on to their evaluation under field conditions which might be useful for the development of potential inoculants/biofertilizers for increasing the growth and productivity of *Crocus sativus.*

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication.

Akhtar Rasool: Methodology, Conceptualization, Investigation, Resources, Software, Formal analysis, Writing – original draft. Mohammad Imran Mir: Writing – original draft, Investigation, Software, Data curation. Muhammad Zulfajri: Writing – review & editing, Visualization. Marlia Mohd Hanafiah: Writing – review & editing. Syeda Azeem Unnisa: Writing – review & editing. Mohammed Mahboob: Supervision, Resources.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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