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RESEARCH ARTICLE

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Biosynthesis of phytohormones from novel rhizobacterial isolates and their in vitro plant growth-promoting efficacy

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ABSTRACT

The health of the plant and soil fertility is dependent on the plant–microbes interaction in the rhizosphere. Microbial life tends to endure various rhizosphere plant–microbe interactions. Phytohormones such as auxins, cytokinins, gibberellic acid, ethylene and abscisic acid are termed as the classical group of hormones. Out of the 70 rhizobacterial strains isolated from the *Coleus* rhizosphere, three different rhizobacterial strains *Pseudomonas stutzeri* MTP40, *Stenotrophomonas maltophilia* MTP42 and *Pseudomonas putida* MTP50 having plant growth-promoting attributes were isolated and characterized for its phytohormone-producing ability. The phytohormones such as indole 3-acetic acid (IAA), gibberellic acid and cytokinin (kinetin and 6-benzyladenosine) were affirmed in culture supernatant of the above isolates. IAA was detected in all the three isolates, where in highest production was found in *S. maltophilia* MTP42 (240 µg/mL) followed by *P. stutzeri* MTP40 (250 µg/mL) and *P. putida* MTP50 (233 µg/mL). Gibberellic acid production was found maximum in MTP40 (34 µg/mL), followed by MTP42 (31 µg/mL) and MTP50 (27 µg/mL). The cytokinin production from the isolates, namely, MTP40, MTP42 and MTP50 were 13, 11 and 7.5 µg/mL, respectively. The isolates showing the production of plant growth enhancing phytohormones can be commercialized as potent bioformulations.

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Introduction

Soil is an essential portion of the connatural surrounding and is necessary for the sustainance of life. Soil is composed of minerals and organic matter which holds the nutrients, while soil and water makes it available to plants (Patel et al. 2015). Microscopic life containing bacteria, fungi, protozoa, actinomycetes and algae are present in the soil covering in plenteous form (Glick 2012). Rhizobacteria present in in vitro have the capability to produce phytohormones such as gibberellic acid, auxins, cytokinins, ethylene and abscisic acid (Zahir et al. 2003).

Gibberellins enhance plant growth by deconjugation of gibberellin-glucosyl conjugates secreted by the roots (Piccoli et al. 1997). Inactive 3-deoxy gibberellins present in the roots are converted to its active forms such as GA_1, GA_3 and GA_4 (Cassan et al. 2001) which are identified from the bacteria and fungi. Kobayashi et al. (1994) had concluded that 136 gases from 128 species, 28 gases from 7 fungi species and 4 gases were identified from bacterial species. Rhizobacteria like *Bacillus pumilus* and *Bacillus licheniformis* synthesizes gibberellins GA_1, GA_3, GA_4 and GA_{20}. *Rhizobium* strains produce indole 3-acetic acid (IAA) and GA_7 tentatively from its pure culture (Yanni et al. 2001). Auxins, gibberellins and cytokinins plant hormones are synthesized by the bacteria that inhabit the rhizosphere and are considered as the major factor responsible for the interactions with the host plant (Ahmad et al. 2008). Plant regulators exert their influences on the physiology and morphology of plants, but in very less amount, and they are also synthesized exogenously by natural and synthetic means (Vejan et al. 2016).

In the present investigation, three rhizobacterial strains *S. maltophilia*, *P. putida* and *P. stutzeri* have been used for the production and extraction of phytohormones on their respective medium and that it is a promising alternative strategy to inseminate enhancement in the development and growth of plants by eliminating the usage of synthetic hormones in in vitro by plant tissue culture technique

Materials and methods

Site description of soil sampling and collection

Soil samples were taken in between April and May 2014 from the rhizosphere of *Coleus forskohlii* at a depth of around 5 cm from the surface. The plant had been procured from the irrigated fields of Anand district of Gujarat (ICAR-Directorate of Medicinal and aromatic plants research station) at a latitude of 22.5645°N, 72.9289°E. The samples were taken in polythene bags and preserved at 4°C and it was processed within 48 h.

Plant growth-promoting rhizobacterial attributes

To prepare a soil suspension, 1 g of soil was suspended in distilled water and vortexed. The rhizobacteria isolates were obtained on the nutrient agar medium (28 ± 2°C, 3 days) by serial dilution method (10^{-1} to 10^{-7}). Well isolated colonies were selected, purified and preserved on the nutrient agar slants at 4°C.They were further tested for their plant growth-promoting rhizobacterial (PGPR) efficacy, that is, P solubilization, Ammonia production, HCN production, siderophore production, Exopolysaccharide production, catalase

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activity and phosphatase activity. IAA-, cytokinin- and gibberellic acid-producing isolates were selected by growing them on IAA production medium, M9 medium and gibberellic acid medium, respectively.

Molecular identification of the bacterial isolates

The isolates (MTP40, MTP42 and MTP50) were characterized based on the 16S r-RNA partial sequencing using universal primer set 16F (5′-AGAGTTTGATCCTGGCTCAG-3′) (5′-GCTGGTTAATACCTGGTTGGGA-3′) (5′-CAACTCG GGCGTTGGCATTCTGCT-3′). Amplification was performed in thermocycler with the following PCR conditions: 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min with initial denaturation at 95°C for 5 min and final extension at 72°C for 10 min. The isolates MTP40, MTP42 and MTP50 were identified as *P. stutzeri*, *S. maltophilia* and *P. putida*, respectively. The isolates were identified by 16S r-RNA gene analysis. Sequences obtained were aligned from the sequences obtained with forward primers using Basic local alignment search tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The database sequences were determined of having maximum homology.

Quantification of phytohormones-producing isolates

IAA production

For IAA production, the culture medium was inoculated with 24 h active cultures MTP40, MTP42 and MTP50 (1 OD cells/100 mL).The IAA production medium consisted of peptone 10 g/L, yeast extract 3 g/L, tryptone 0.5%,tryptophan 5 g/L, Agar 15 g/L and the pH was adjusted between 6.8 and 7.0 pH. The cultures were inoculated in 50 mL of above medium in 100 mL flasks and were covered with black paper and were incubated at 30°C, 160 rpm for 72 h. A sample of 5 mL medium was withdrawn after 72 h and was centrifuged at 1000 rpm for 20 min. The experiment was conducted in triplicates. The medium was assayed using Salkovasky's (50 mL of 35% perchloric acid and 1 mL 0.5 M FeCl3 solution) reagent by incubating it in dark for 1 h (Sarwar and Kremer 1995). Absorbance was measured at 536 nm using visible spectrophotometer and development of pink coloration was checked.

Cytokinin production

For cytokinin production, M9 medium supplemented with 0.2% Casamino Acids, 0.01% thiamine, and 2 pg of biotin per liter (Akiyoshi et al. 1987) was used; 250 mL flasks supplemented with 100 mL. M9 medium was inoculated with 1 mL of culture and incubated at 28 ± 2°C, 160 rpm for five days. The experiment was conducted in triplicates. For control, M9 medium was used. They were quantified spectrophotometrically at 665 nm on 72, 96 and 120 h, respectively.

Gibberellic acid production

Gibberellic acid production was carried out in the nutrient medium. Culture suspension was added to the nutrient medium and was incubated at 30 ± 2°C for 5 days. After that the medium was centrifuged at 10,000 rpm for 20 min. The pH of the supernatant was adjusted at 2.5 by using 15% HCL. The filtrate was extracted with ethyl acetate (1:3 of filtrate is to solvent ratio) and the extract was used for its gibberellic acid determination. The experiment was conducted in triplicates. In this assay gibberellic acid is converted into gibberellenic acid and is estimated at 254 nm absorbance (Pandya and Desai 2014).

Extraction, purification and characterization of phytohormones by TLC and HPLC

Extraction of IAA and its characterization

The supernatant obtained after centrifugation was acidified to pH 2.5 using 1 N HCL and is extracted thrice by using ethyl acetate solvent. This solvent mixture is then evaporated using rotary vaccum evaporator and the remaining substance is redissolved in 70% high performance liquid chromatography (HPLC) grade methanol. The extracts are spotted on to the silica plate and allowed to develop using mobile phase isopropanol: ammonia: water (16:3:1) (Mohite 2013). Rf value of the sample corresponding to the standard IAA were calculated and presence of pink spots were seen. HPLC was used to analyze the samples equipped with UV detector and C_{18} column. 100 µL of sample was filtered through 0.45 millipore filter and injected into the column. The solvent system used was acetic acid: methanol: water (1:30:70 v/v) and the run time of 20 min/sample was read at 280 nm absorbance. IAA (Sigma Chemicals, USA) was used as a standard.

Extraction of cytokinin and its characterization

For cytokinin, M9 broth with the inoculum is centrifuged at 10,000 rpm for 20 min at 4°C.The cell free supernatant is filtered through millipore filter and is lyophilized to dryness. Extraction was carried out three times using ethyl acetate and stored in methanol at −20°C. Thin layer chromatography (TLC) chromatograms were spotted with the samples and standard cytokinin (Kinetin and 6-benzyladenosine) using *n*-butanol: acetic acid: water (12:3:5 v/v/v) as mobile phase and was observed under the UV light (254 nm). The HPLC system with Kn and 6-BA standards, solvent system 70% methanol, flow rate 0.5 mL/min, absorbance 270 nm and 8.6 MPa pressure was run for cytokinin production (Hussain and Hasnain 2009; Ahmed and Hasnain 2010).

Extraction of gibberellic acid and its characterization

The cultures grown in the nutrient medium for gibberellic acid was centrifuged at 10,000 rpm for 20 min after 5 days of incubation time. The supernatant was extracted thrice with ethyl acetate and preserved in 70% methanol at −20°C. TLC chromatography using isopropanol: 25% ammonium hydroxide: water (10:1:1 v/v) and spot detection done by spraying 3% H2SO4 in methanol containing 50 mg FeCl3; after heating at 80°C for 10 min, plates generated greenish spots under UV light (Rangaswamy 2012). They were injected into a C18 HPLC column and were eluted at 0.5 mL/min flow rate, absorbance 206 nm and fractions eluting at the retention time corresponding to each pure standard were collected (Boiero et al. 2007).

Results

Plant growth-promoting attributes

In this study 70 rhizobacterial strains were screened on the basis of its plant growth-promoting attributes of which three

Table 1. Plant growth-promoting traits of the phytohormone-producing strains.

Isolates codes	P solubilization (µg/mL)	Ammonia production (µg/mL)	HCN production	Siderophore production (µg/mL)	EPS production	Catalase activity	Phosphatase activity
MTP40	400	42	+	30	+	+	+
MTP42	362	45	+	32	+	+	+
MTP50	396	39	–	26	+	+	+

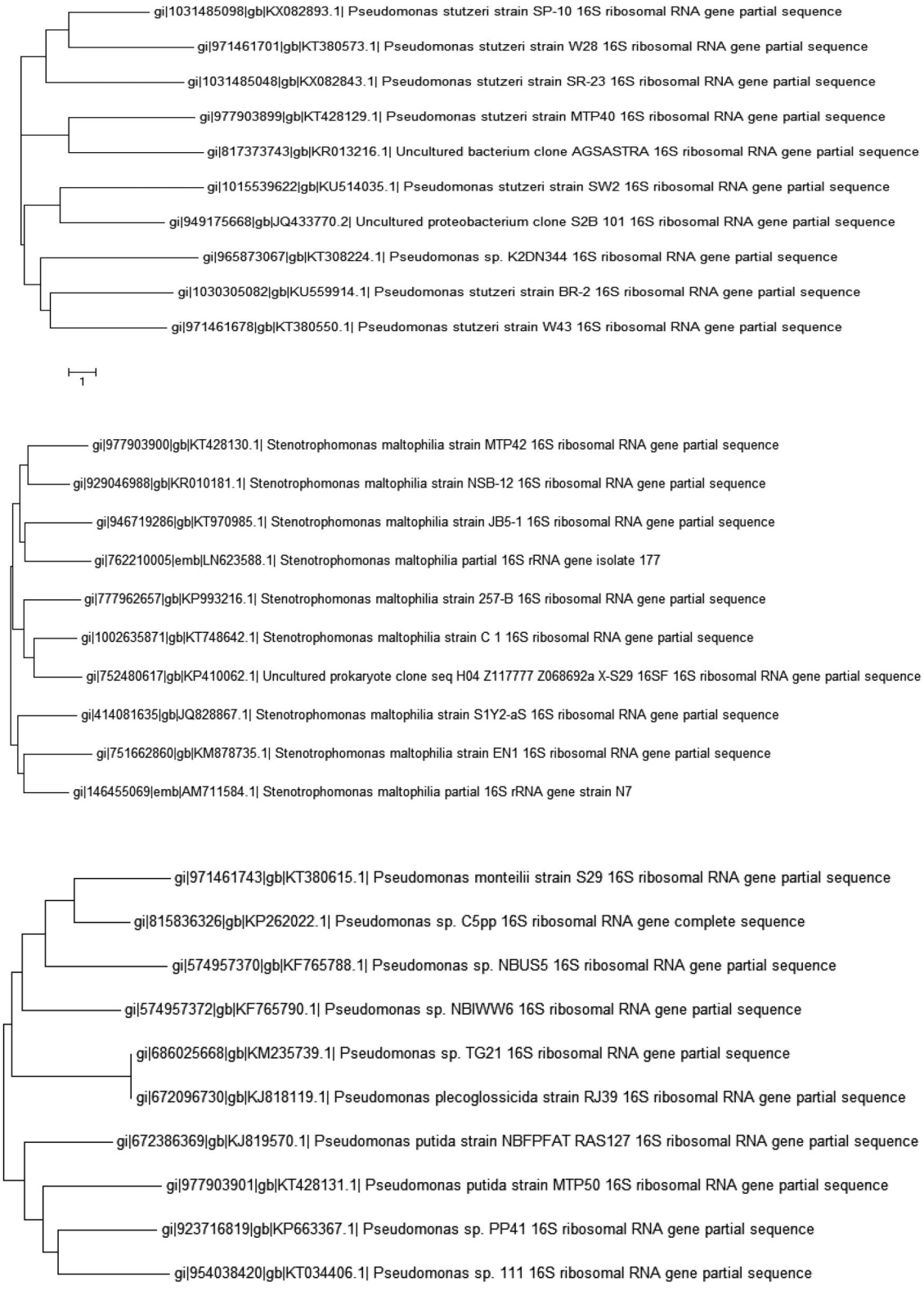

Figure 1. Phylogenetic analysis based on 16S r-RNA gene sequences available from National Centre for Biotechnology Information data library constructed after multiple alignment of data by ClustalX. Neighbor-joining method was used to study the evolutionary history. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA software version 6.0

Table 2. The concentration of IAA, cytokinin and gibberellic acid quantified spectrophotometrically at the time interval of 72, 96 and 120 h, respectively, using analysis of variance.

	After 72 h of time interval			After 96 h of time interval			After 120 h of time interval		
Phytohormones (µg/mL)	MTP40	MTP42	MTP50	MTP40	MTP42	MTP50	MTP40	MTP42	MTP50
IAA	238 ± 0.1	230 ± 0.2	231 ± 0.2	250 ± 0.2	240 ± 0.1	233 ± 0.1	244 ± 0.6	236 ± 0.5	230 ± 0.3
Cytokinin	7 ± 0.5	6.3 ± 0.13	4.5 ± 0.4	13 ± 0.4	11 ± 0.1	7.5 ± 0.2	12.7 ± 0.13	10.5 ± 0.2	7.0 ± 0.1
Gibberellic acid	32 ± 0.2	28.9 ± 0.2	25.6 ± 0.3	34 ± 0.1	31 ± 0.1	27 ± 0.15	34 ± 0.12	27.6 ± 0.4	26.7 ± 0.14

isolates having maximum phosphate and IAA production were evaluated further for its molecular identification (Table 1). *P. Stutzeri* MTP40 was found to be the most efficient P-solubilizer. Ammonia production is another important trait of PGPR that indirectly influence the plant growth and all the three efficient isolates were able to produce ammonia. Production of HCN was detected in two isolates, that is, MTP42 and MTP40. Catalase and phosphatase activity was detected in the isolates that may be potentially very advantageous.

Molecular identification of the rhizobacterial isolate

The isolates MTP40, MTP42 and MTP50 were screened on the basis of its maximum ability in the production of IAA, cytokinin and gibberellic acid. The isolates were identified by 16S r-RNA gene analysis. The 16S r-RNA NCBI BLAST analysis showed that there was 99% similarity of the isolates MTP40, MTP42 and MTP50 with the strain *P. stutzeri*, *S. maltophilia* and *P. putida*, respectively. Further phylogenetic analysis was used to confirm that these isolates were closely related to their respective strains through MEGA 6.0 software and were hence confirmed as *P. stutzeri*, *S. maltophilia* and *P. putida* (Figure 1) (NCBI bank accession number: KT428129, KT428130 and KT428131).

Quantitative analysis of IAA-, cytokinin- and gibberellic acid-producing isolates spectrophotometric method

IAA was produced by all plant growth promoting bacteria's (PGPB) isolates in liquid tryptophan yeast medium. Maximum amount of IAA was produced by MTP42, followed by MTP40 and MTP50 (Table 2). These isolates produced IAA in the range of 230–250 µg/mL. The production of IAA seems to be increased till 96 h, and after 120 h of incubation, there was a continuous decrease. Cytokinin production was read at 665 nm (Table 2). Gibberellic acid produced by the isolates was read at 254 nm (Table 2).

Identification of phytohormones by TLC and HPLC method

Screening of IAA-, cytokinin- and gibberellic acid-producing rhizobes by TLC

All the 3 isolates were then screened for phytohormone synthesis. TLC detection method showed that the isolates gave positive results. Amongst these two positive strains, MTP40 and MTP42 strain showed IAA (pink), cytokinin and gibberellic acid (green) spots (Figure 2(A–C)). MTP50 showed the presence of IAA and cytokinin but not gibberellic acid. Rf

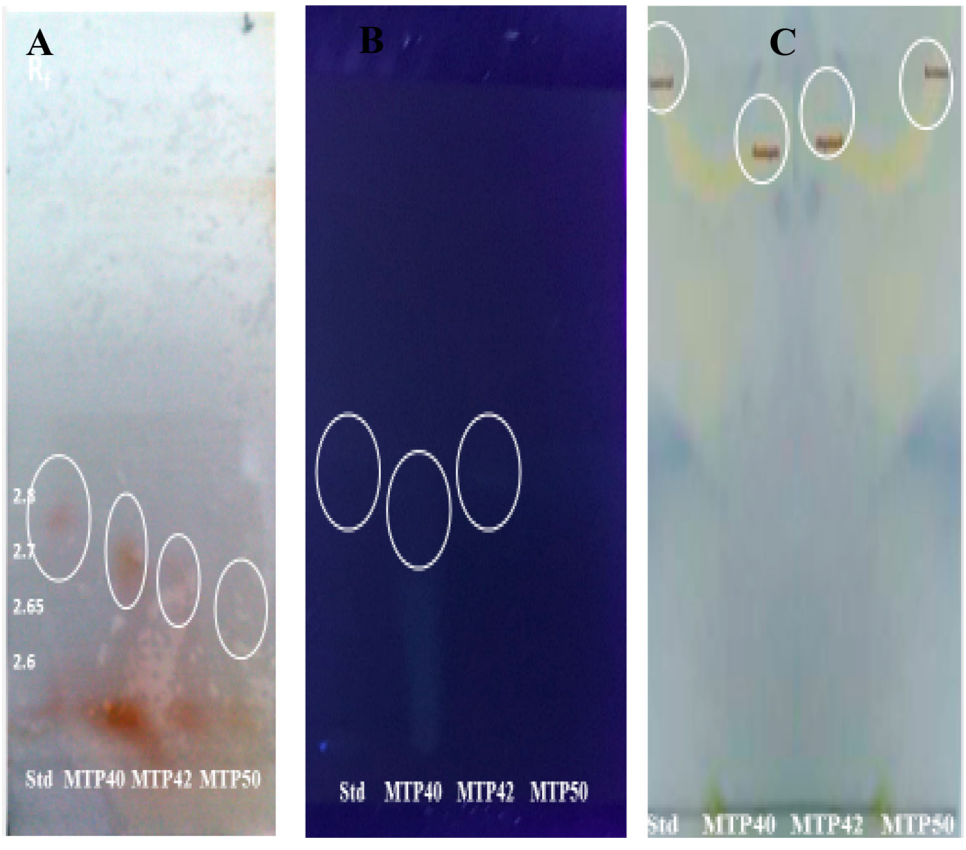

Figure 2. (A) Indole compound and isolates spots on the TLC. (B) Cytokinin and isolates spots observed under UV light. (C) Gibberellic acid and isolates give green color spots.

Table 3. Rf value of the phytohormone standards and their relative isolates determined using analysis of variance.

Sr.no.	Phytohormone	Spots	Rf value			
			STD	MTP 40	MTP 42	MTP 50
1	IAA	Pink	2.8 ± 0.2	2.7 ± 0.1	2.65 ± 0.15	2.6 ± 0.2
2	Cytokinin	Fluorescent	1.7 ± 0.14	1.65 ± 0.3	1.8 ± 0.15	–
3	Gibberellic acid	Green	2.5 ± 0.2	2.3 ± 0.3	2.4 ± 0.1	2.5 ± 0.0

value of the standard IAA, cytokinin, gibberellic acid and that of the isolates are mentioned in Table 3.

Discussion

In this study, out of 70 rhizobacterial isolates from *Coleus* rhizosphere (procured from ICAR, Bangalore, India), three isolates having potential benefits were screened for its phytohormone production, namely, *P. stutzeri* MTP40, *S. maltophilia* MTP42 and *P. putida* MTP50. They were identified by biochemical tests and 16S r-RNA sequence analysis. The efficacy of the phytohormones (hormone production) were justified by using HPLC and their recovery was found to be nearly 70% from isolates MTP42 and MTP50, while MTP40 was found to giving nearly 80% recovery (Figures 3–5).

From the above results, *S. maltophilia* is found to give maximum hormones recovery. Kumar and Audipudi (2015) reported that *S. maltophila* has found to have an ambivalent character, as a biocontrol and bioremediation agent as well as a multi-resistant pathogen in nosocomial infections. Halda-Alija (2003) studied that *P. stutzeri* produces IAA even if L-tryptophan is not added to the medium which can either be that IAA synthesis can occur independently of L-tryptophan or bacteria itself produces it.

The IAA production in the above gram negative isolates was found in between 230 and 260 µg/mL. Earlier studies

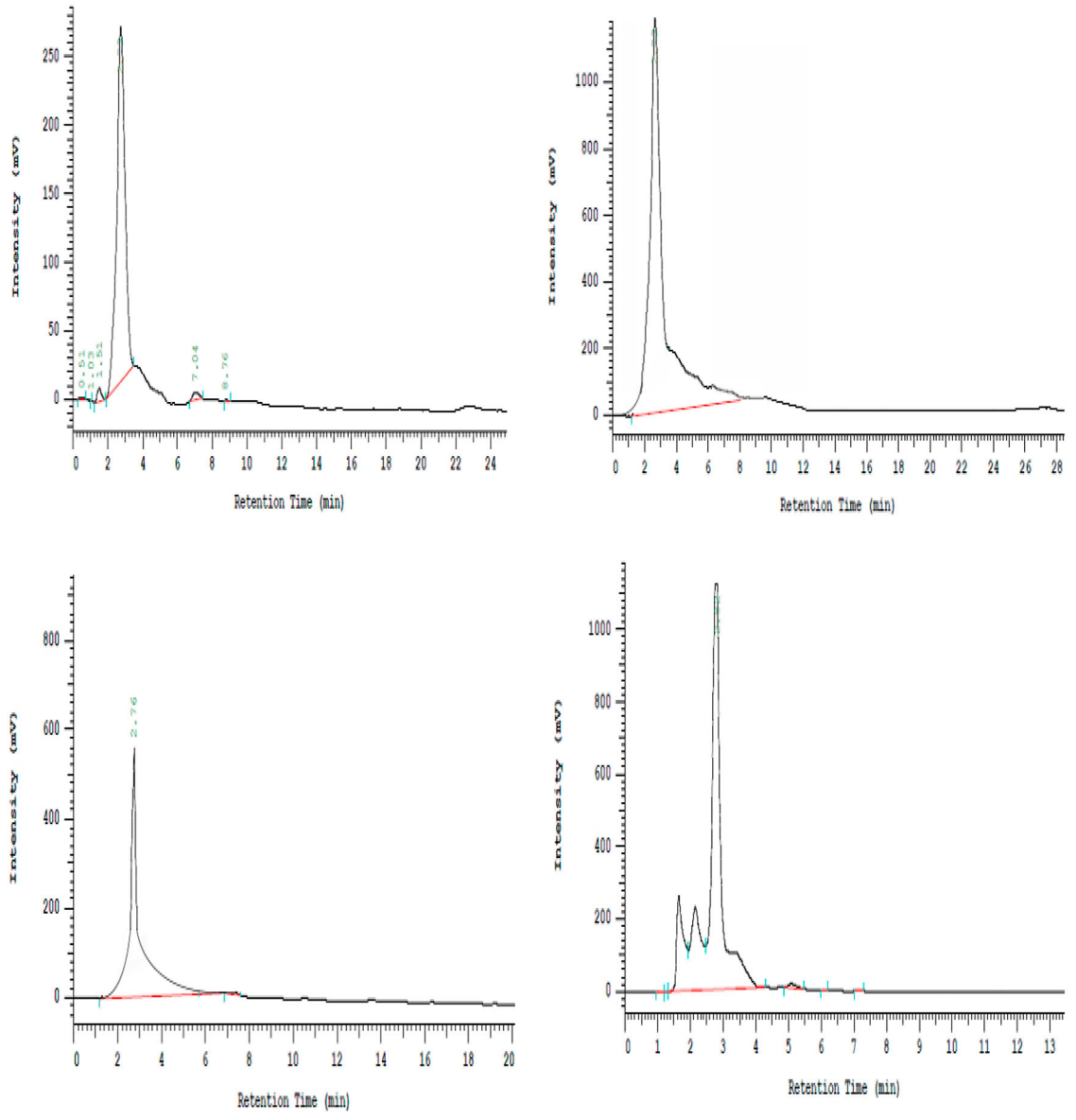

Figure 3. UV–HPLC profiles. (A) Authentic indolic compound standards. Retention times: IAA 2.73 min. (B) Indolic compounds produced by *P. stutzeri* at retention time 2.81 min. (C) Indolic compounds produced by *S. maltophilia* at retention time 2.76 min. (D) Indolic compounds produced by *P. putida* at retention time 2.80 min. The column was eluted in mobile phase acetic acid:methanol: water (1:30:70 v/v) at 280 nm absorbance.

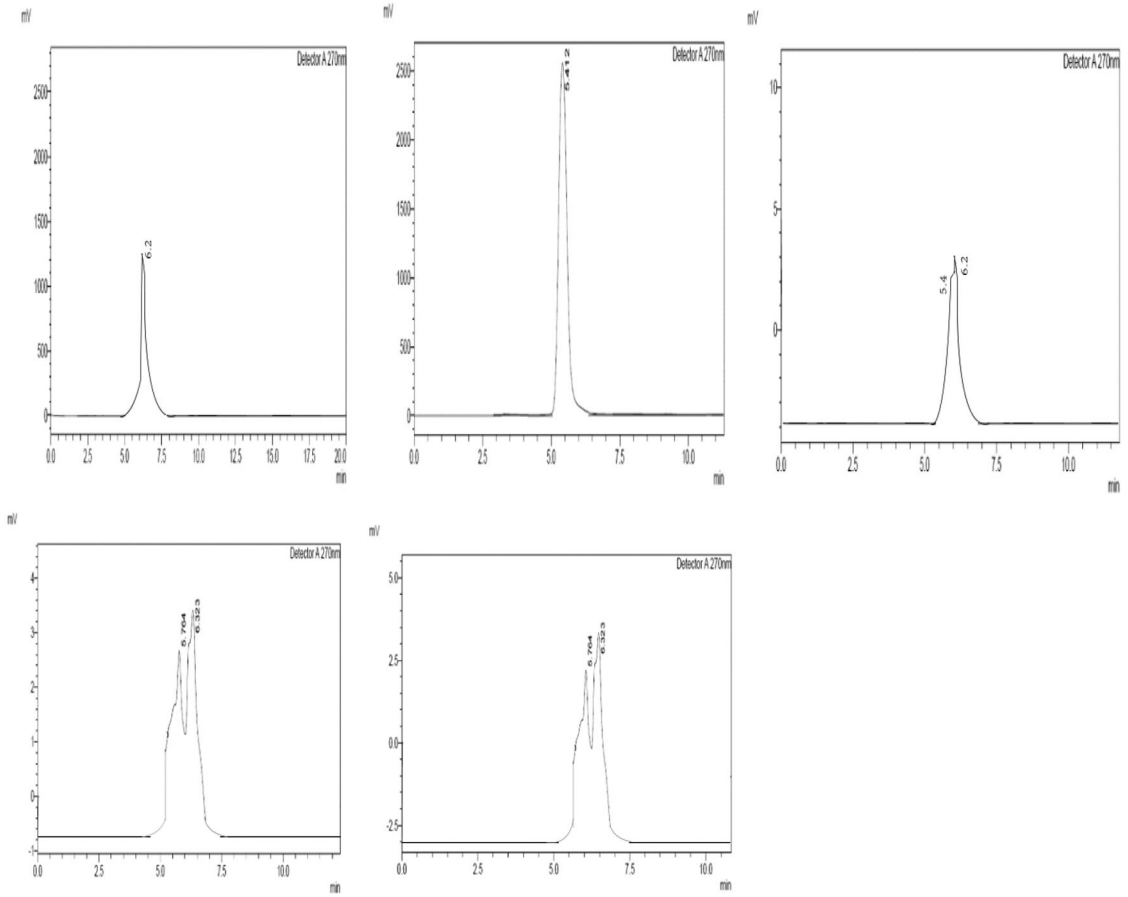

Figure 4. UV–HPLC profiles. (A) Authentic cytokinin (6-benzyladenosine) compound standards. Retention time: 6BA 6.2 min. (B) Authentic cytokinin (Kinetin) compound standards retention time: 5.4 min. (C) Cytokinin compounds produced by *P. stutzeri* at retention time 6.2 min. (D) Cytokinin compounds produced by *S. maltophilia* at retention time 5.4 and 6.2 min. (E) Cytokinin compounds produced by *P. putida* at retention time 5.4 and 6.3 min. The column was eluted in mobile phase 70% methanol, at 270 nm absorbance.

showed that IAA producers are mostly gram negative organisms. (Datta and Basu 2000). Such IAA-producing effect of *P. putida* has already been reported on cucumber seeds and canola seeds (Amer and Utkhede 2000). Most of the organisms are found to synthesize auxin found in the rhizosphere and the tomato plant naturally secretes tryptophan (Kamilova et al. 2006). IAA production increased linearly up to 96 h and decreased after 120 days in 0.5% L-trytophan supplemented medium due to the IAA degrading enzymes. Ivanova et al. (2001) in his study showed that certain obligate and facultative methylotropic bacteria were able to produce IAA in the range of 3–100 µg/mL. Certain nitrogen-fixing bacteria synthesize IAA, among which *Azospirillum* strain synthesize higher amount of IAA whereas *gluconacetobacter* and *pseudomonas* strain produce lower concentration of IAA, which also promote growth in the plant (Pedraza et al. 2004).

In the above report, the cytokinin and gibberellic acid in the isolates was found to be in the range of 4.5–13 and 25–34 µg/mL, respectively. Certain researches in cytokinin and gibberellic acid synthesized from *Azospirillum* species approximated in the range of 0.75 µg/mL kinetin and 20–40 pg/mL, respectively (Bottini et al. 1989). Garcia de Salamone (2000) showed that the growth on the lateral roots can be due to the hormonal balance in the secretion of IAA and cytokinin by strains GR12–2, G20–18, CNT2.

Alvarez et al. showed that HPLC used for the determination of IAA were like IBA determination present in the apple shoots cultured in vitro. In this study, the fraction time in HPLC was chosen to be 2 min in order to separate the IAA and IBA peaks. Alvarez used the acetonitrile/water solvent system to separate the IAA and IBA in his study.

Conclusion

The present investigation was focused on the production of various phytohormones such as IAA, cytokinin and gibberellic acid by the rhizobacterial strains. Three isolates were screened to produce phytohormones based on the quantitative and qualitative estimations by HPLC and TLC, respectively. MTP40 (*P. stutzeri*) amongst the three strains shows the highest production of phytohormones using HPLC which was nearly 80% during 72–96 hs of incubation. This study shows that the stain MTP42 *S. maltophilia* can be used for plant protection and for the enhancement of its growth. Hence this strain can be used to enhance the agronomic value of soils and benefit the crop growth. The evaluation of this isolate under the field condition and its investigation, MTP42 can be used as a PGPR agent constitute future research. Furthermore, *Stenotrophomonas* spp. should continue to be a source of usefulness or novel enzymatic capabilities, reflecting their metabolic versatility.

Disclosure statement

No potential conflict of interest was reported by the authors.

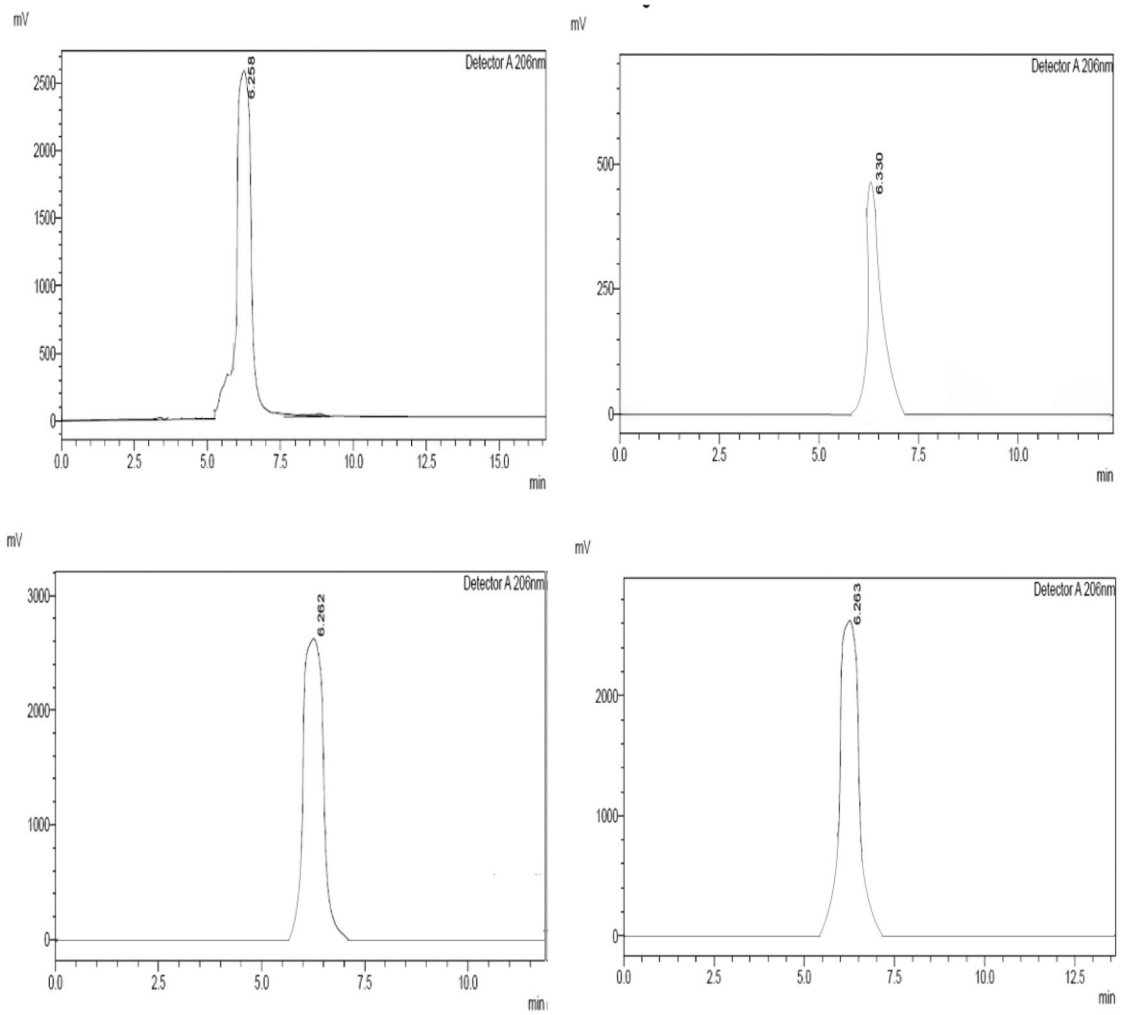

Figure 5. UV- HPLC profiles. (A): Authentic gibberellic acid compound standards. Retention times: GA 6.25 min. (B): Indolic compounds produced by *P.stutzeriat* retention time 6.3 min. (C): Indolic compounds produced by *S. maltophilia* at retention time 6.26. (D): Indolic compounds produced by *P. putida* at retention time 6.26 min. The column was eluted in mobile phase 70% methanol at 206 nm absorbance.

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