

Original Research Article

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Assessment of Native Pink Pigmented Facultative Methylo-trophs of Chilli (*Capsicum annuum* L.) for their Plant Growth Promotional Abilities

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ABSTRACT

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Investigations were carried out to study the plant growth promotional ability of native pink pigmented facultative methylo-trophs (PPFMs) of major chilli (*Capsicum annuum* L.) growing areas of North Karnataka. Selected isolates were screened for beneficial characters like production of phytohormones, phosphate solubilisation and siderophore production. Highest indole 3 acetic acid and gibberellic acid production was recorded in PPFM6, 19.77 and 128.28 µg/ml of culture filtrate, respectively. The strain PPFM170 recorded highest cytokinin production (2.54 µg/ml). Mineral phosphate solubilisation index was in the range of 0.31 to 0.97 and the isolate PPFM6 produced higher amount of inorganic phosphorous at 5 days after incubation (6.5%) and even at 10 days after incubation (8.99 %). The production of catechol type of siderophores was observed among PPFM isolates which ranged from 0.2 to 0.61 µmoles of Di Hydroxy Benzoic Acid. The present study has identified potential native PPFM strains from major chilli growing districts of North Karnataka for their exploration in improving production and productivity of chilli.

Introduction

Methylo-trophs are those microorganisms which are able to grow utilizing the reduced carbon compounds, like methanol (released during plant metabolism), containing one or more carbon atoms having no carbon-carbon bonds. Obligate methylo-trophs grow only on such compounds whereas, facultative methylo-trophs thrive on a variety of other organic multi-carbon compounds (Anthony,

1982). Different species of methylo-trophs are distributed in a diverse variety of natural and manmade environments, including soil, air, dust, fresh water, marine water, water supplies, polluted soil, bathrooms, air conditioning systems, masonry, etc. (Trotsenko *et al.*, 2001). Several species of methylo-trophic bacteria are found growing in association with terrestrial and aquatic plants, colonizing roots, leaf surfaces and growing buds (Lidstrom and Chistoserdova, 2002). The

structural and functional diversity of microorganisms on the plant surface differ among the plant species due to differences in their exudates. The pink-pigmented facultative methylotrophs (PPFMs) are widely distributed in nature and are particularly known for their close association with plants (Lidstrom and Christoserdova, 2002; Lodewyckx *et al.*, 2002).

The natural occurrence of PPFM with varied population intensities among different vegetable crops *viz.*, tomato, chilli, eggplant, bitter gourd, bhendi, coccinia, cucumber, cauliflower, radish and mint at flowering stage has been reported (Anurajan, 2003). The spatial distribution of PPFM on various vegetable leaves like eggplant, green perilla, small green pepper, pumpkin, bitter melon, okra, and tomato has also been studied (Mizuno *et al.*, 2012). Several beneficial aspects such as stimulation of seed germination, plant growth promotion, production of phytohormones and induction of defense responses in rice and peanut against *Rhizoctonia solani*, *Aspergillus niger* and *Sclerotium rolfsi* have been reported for *Methylobacterium* (Omer *et al.*, 2004; Madhaiyan *et al.*, 2004; 2006a). They influence plant growth by producing auxins (Doronina *et al.*, 2002) and cytokinins (Koenig *et al.*, 2002). In addition, they can fix atmospheric nitrogen (Jourand *et al.*, 2004), bring about mineral phosphate solubilisation (Jones *et al.*, 2007), regulate the ethylene level in rhizosphere by 1-aminocyclopropane-1-carboxylate deaminase (Madhaiyan *et al.*, 2006b) and stimulate the resistance against plant pathogens (Madhaiyan *et al.*, 2006a).

Often, the bio inoculants used for crop plants as plant growth promoters are isolated generally from rhizosphere soil and deserving attention has not been paid to the phyllosphere microorganisms and very few studies have focussed on studies on bio inoculants in chilli.

Hence, by considering the importance of PPFM as plant growth promoting bacteria, we isolated and identified the native isolates of PPFM from chilli fields of major chilli growing districts of north Karnataka in order to assess their growth promotional ability through phytohormones production, phosphate solubilisation and siderophores production so that they can be further utilized as potential bioinoculants to improve the growth, yield and quality of chilli.

Materials and Methods

The samples of rhizosphere soils, roots and phyllosphere were collected from chilli fields of major chilli growing districts of North Karnataka *viz.*, Dharwad, Gadag and Haveri (Table 1) for isolation of PPFM. They were isolated by leaf imprinting and serial dilution technique (Savitha *et al.*, 2013) and putatively identified as PPFM based on pink pigmented colonies on ammonium mineral salts (AMS) agar media with methanol as sole source of carbon and energy. All the isolates were initially screened for qualitative production of indole 3 acetic acid (John *et al.*, 1991) and their ability to inhibit the growth of *Colletotrichum capsici* on agar plates by dual culture technique (Ganesan and Gnanamanikyam, 1987). The positive isolates were further subjected for quantitative estimation of phytohormones, P - solubilization and siderophores production.

Production of phytohormones by PPFM isolates

The production of phytohormones was estimated by extracting the cell free culture filtrate of PPFM twice with equal amount of ethyl acetate (Tien *et al.*, 1979) and used for quantification of indole-3 acetic acid (IAA) and cytokinin through high performance liquid chromatography (HPLC) (Tien *et al.*, 1979; Omer *et al.*, 2004). Gibberellic acid (GA)

production was estimated through Spectrophotometry (Mahadevan and Sridhar, 1986).

Qualitative and quantitative estimation of siderophores

PPFM isolates were grown on iron deficient AMS medium and culture filtrate was separated from cells by centrifugation at 7000 rpm for 20 min. Both catechol and hydroxamate type of siderophores produced were extracted (Modi *et al.*, 1985). Unit volume of the Hathway's reagent (Reeves *et al.*, 1983) was added to same volume of the sample and development of wine or orange colour was noted as the presence of catechol and hydroxamate type of siderophores, respectively. The development of wine colour was read in spectrophotometer at 700nm with 2, 3-dihydroxy benzoic acid as standard for quantification of catechol type of siderophores. While hydroxamate siderophores were measured according to Csaky (1948).

Screening of PPFM isolates for mineral phosphate solubilisation (MPS) activity

The isolates were subjected for preliminary phosphate solubilization on Pikovskaya's medium with tricalcium phosphate (TCP) as insoluble source of phosphorous and the pH of the media was adjusted to 7.0. The zone of phosphate solubilization was observed after 10 days after incubation (DAI) and solubilization index was calculated (Seshadri *et al.*, 2002). The quantification of inorganic phosphate (*P_i*) released from TCP in broth was estimated on 5th and 10th DAI by phosphomolybdic blue colour method (Jackson, 1973) and change in pH was also recorded.

Results and Discussion

A total 200 PPFM strains were isolated on AMS agar media from more than 250 samples

collected from chilli fields of major chilli growing districts of North Karnataka and coded as PPFM series. Of the total 200 isolates obtained, 30 isolates were found positive for IAA production and 9 revealed antagonistic activity against *C. capsici*. Morphological characterization of these isolates showed differential pink pigmentation when grown on AMS agar media (Table 1). These 30 shortlisted isolates were further evaluated for quantitative production of IAA, GA and cytokinins, P-solubilisation ability and siderophore production. Among 30 isolates, all of them showed differential phytohormones production, eight were showing MPS activity and nine isolates were positive for siderophores production. So these nine best PPFM isolates which were having characteristic of PGPR were further shortlisted and presented here.

Production of phytohormones by PPFM isolates

The production of IAA significantly varied with different isolates (Fig. 1A) and the highest IAA production of 19.77 μ g/ml of culture filtrate was recorded for PPFM6 and lowest was recorded for PPFM85 (1.27 μ g/ml). HPLC chromatogram for retention time of IAA is presented in Figure 2. For GA production, highest value was recorded for PPFM6 (128.28 μ g/ml) whereas, lowest was observed in PPFM85 (30.10 μ g/ml) (Fig 1B). PPFM170 produced significantly higher Cytokinin (2.54 μ g/ml) compared to other isolates (Fig. 1C). Production of cytokinin was confirmed through HPLC (Fig. 3).

Siderophores production

Out of 30 isolates tested, nine were positive for siderophore production and development of wine color in all isolates indicated production of catechol type of siderophores by PPFM isolates. The absence of orange color conveyed isolates inability to produce

hydroxymate type of siderophores. The PPFM isolates which were found to produce the catechol type of siderophore were further quantified for the production of siderophores and was expressed as μ moles of Di Hydroxy

Benzoic Acid (DHBA). The amount of siderophore production ranged from 0.2 to 0.61 μ moles of DHBA (Fig. 4).

Table.1 Morphological characteristics of selected PPFM isolate

Isolates	Habitat	Place	GPS Location	Pigmentation
PPFM 6	Phyllosphere	Annigeri	15.434833 N, 75.440369 E	Dark pink
PPFM 35	Root endophyte	Amminabhavi	15.545342 N, 75.049431 E	Pale pink
PPFM 38	Root endophyte	Sulla	15.451612 N, 75.173586 E	Medium pink
PPFM 65	Root endophyte	Navalgund	15.548526 N, 75.344917 E	Medium pink
PPFM 85	Root endophyte	Lakshmeshwar	15.137514 N, 75.469916 E	Pale pink
PPFM99	Phyllosphere	Belavanki	15.667781 N, 75.566872 E	Dark pink
PPFM 140	Phyllosphere	Devaragudda	14.666920 N, 75.572186 E	Pale pink
PPFM 155	Root endophyte	Masangi	14.668410 N, 75.427936 E	Medium pink
PPFM170	Phyllosphere	Savanur	14.967365 N, 75.327599 E	Dark pink

Table.2 Phosphate solubilisation of shortlisted PPFM isolates on Pikovskaya's media

Isolates code No.	Phosphate Solubilisation Index	Final pH		Per cent <i>Pi</i> released	
		5 ays	10 Days	5 Days	10 Days
PPFM 6	0.97	4.20	3.20	6.50 (14.77)	8.99 (17.45)
PPFM 35	0.55	4.50	3.60	6.10 (14.30)	7.50 (15.89)
PPFM 38	0.47	5.40	5.00	4.20 (11.83)	4.52 (12.27)
PPFM 65	0.33	5.30	4.80	4.70 (12.52)	5.00 (12.92)
PPFM 99	0.42	4.80	4.50	5.40 (13.44)	5.80 (13.94)
PPFM 140	0.70	5.00	4.65	5.10 (13.05)	5.45 (13.50)
PPFM 155	0.31	5.60	5.20	3.80 (11.24)	4.00 (11.54)
PPFM 170	0.92	4.30	3.40	6.40 (14.65)	8.20 (16.64)
SEm \pm	0.01	0.01	0.02	0.07	0.09
CD (0.01)	0.03	0.05	0.07	0.31	0.40

Note: Arc sine transformed values are represented in parentheses

Fig.1 Production of phytohormones (A) IAA (B) GA and (C) Zeatin by PPFM isolates

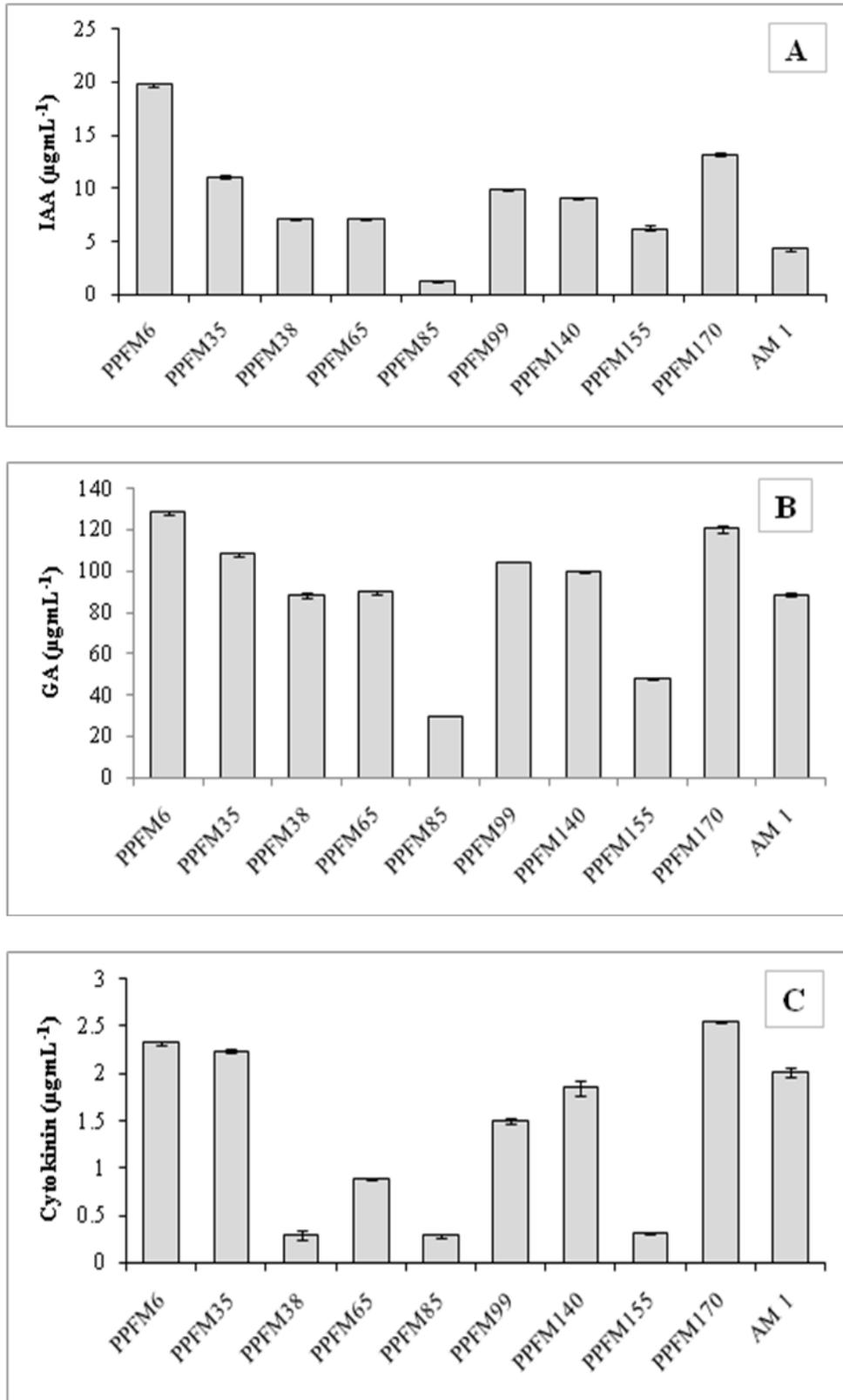


Fig.2 HPLC chromatogram of IAA produced by native PPFM isolate

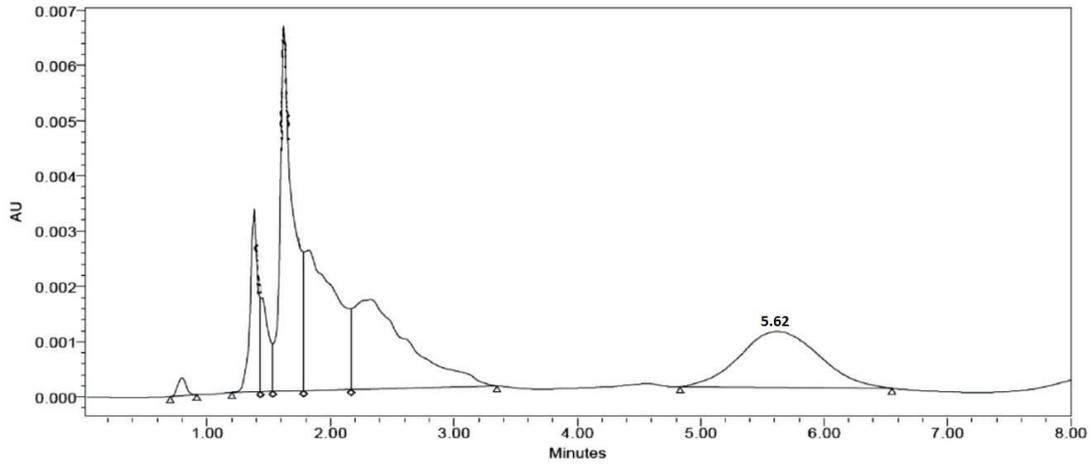


Fig.3 HPLC chromatogram of cytokinin (zeatin) produced by native PPFM isolate

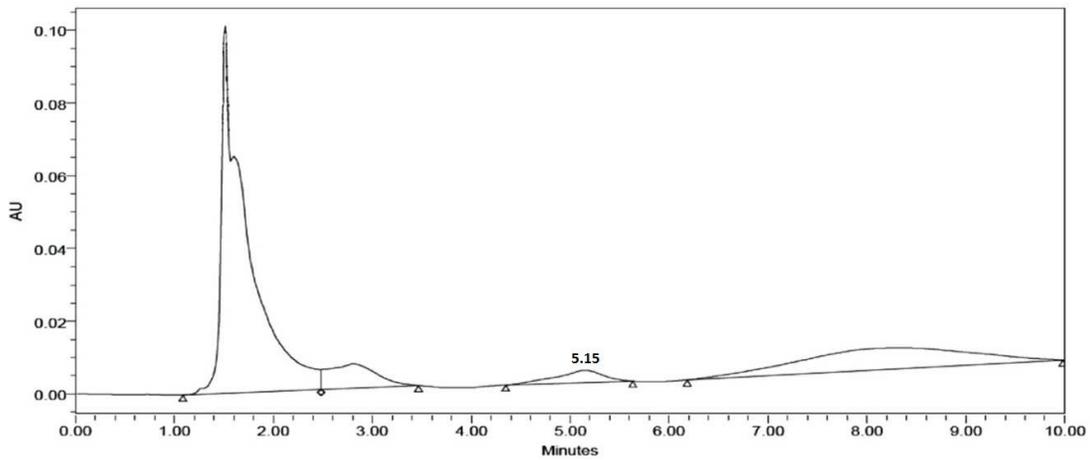
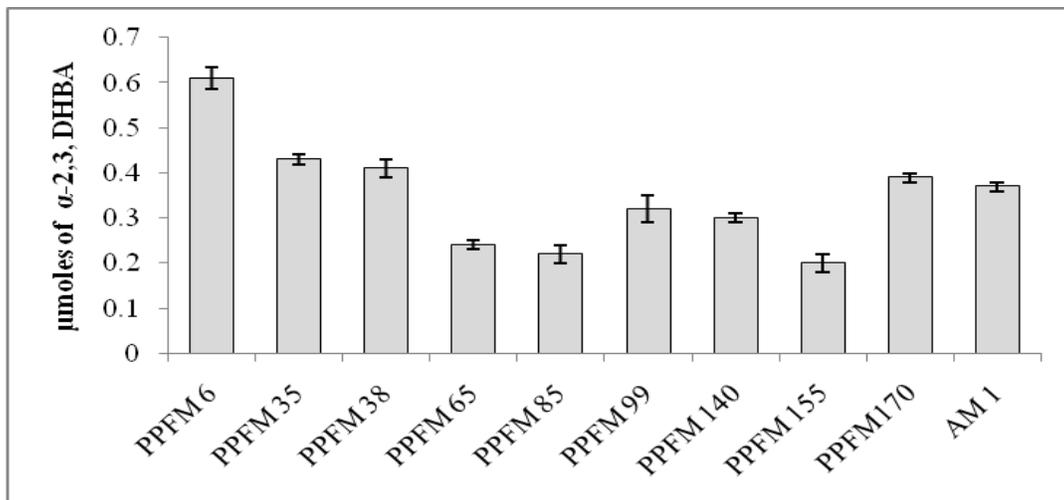


Fig.4 Quantitative estimation of siderophore produced by PPFM isolates



Phosphate solubilisation by PPFM isolates

Out of 30 isolates tested for P solubilisation ability, eight isolates revealed MPS activity. Phosphate solubilisation ability of PPFM isolates is expressed as phosphate solubilisation index (PSI), which ranged from 0.31 to 0.97. Gradual increase in *Pi* release from 5th to 10th DAI as observed. The isolate PPFM6 produced higher amount of *Pi* both at 5th DAI (6.5%) and 10 DAI (8.99%). While reference strain *Methylobacterium extorquens* AM1 didn't show any MPS activity. Further decrease in pH was observed with higher amount of *Pi* released in all isolates. The drastic reduction in pH from 7 to 4.2 and 3.2 was found at 5DAI and 10DAI respectively, was observed in isolate PPFM6 (Table 2).

In the present study PPFMs isolated from chilli rhizosphere soil, roots and phyllosphere were screened for ability to produce phytohormones like IAA, GA and cytokinins. The isolates tested produced phytohormones in varying quantities. The amount of IAA produced was ranging from 1.27 to 19.77 μ g/ml of culture filtrate, GA was in the range of 30.10 to 128.28 μ g/ml of culture filtrate and cytokinin produced was ranging from 0.29 to 2.54 μ g/ml of culture filtrate.

This varied production of phytohormones is strain-dependent and therefore a "species-specific" characteristic. The isolate which is able to produce significantly higher amount of phytohormones known to perform better with respect to improving growth and yield of crop plants.

The IAA is produced and secreted by different strains of *Methylobacterium* (Doronina *et al.*, 2002; Ivanova *et al.*, 2001; Hornscluh *et al.*, 2006 and Kutschera, 2007). However, the first report on the production IAA in significant amount by methylotrophs was given by Ivanova *et al.*, (2001).

Omer *et al.*, (2004) unambiguously confirmed that PPFM produced plant hormone IAA through HPLC and Nuclear magnetic resonance. Production of GA was varying among different isolates as reported by Anurajan (2003) (10.9 to 106.97 μ g/ml), Thangamani (2005) (28.86 μ g/ml to 98.26 μ g/ml), Radha (2007) (24.11 to 70.30 μ g/ml) and Jones (2010) (53.20 to 273.20 μ g/ml). The GA production was estimated by spectrophotometry by Sheela *et al.*, (2013) in PPFM isolates, where PPFM 14 (59.13 μ g/ml) isolate produced higher amount. Traditionally, the study of cytokinin production by plant-associated bacteria has been associated with microbes known to cause plant disease or to enter into an intimate symbiosis with a plant host. Koenig *et al.*, (2002) sought to rectify the omission of plant commensal bacteria from this field of study by making a detailed examination of cytokinin production by PPFMs. Studies by Reddy (2002) revealed that the cytokinin production of PPFM leaf isolates ranged from 21.46 to 124.32 ng/l of culture filtrate.

Thangamani (2005) and Jones (2010) observed cytokinin production ranging from 0.147 ng/l to 11.27 ng/l and 0.07 to 1.84 μ g/ml, respectively. Bacterial siderophores are low-molecular weight compounds with high Fe^{3+} chelating affinity (Sharma and Johri, 2003) responsible for the solubilization and transport of Fe^{3+} element into bacterial cells. Some bacteria produce hydroxamate-type siderophores while others produce catecholate-types (Neilands and Nakamura, 1991). The production of siderophores by microorganisms is beneficial to plants, because it can inhibit the growth of plant pathogens (Sharma and Johri, 2003). In the present study, PPFM isolates tested produced only catechol type of siderophore ranging from 0.20 μ moles of DHBA to 0.61 μ moles of DHBA. Similarly, Anurajan (2003), Senthilkumar (2003) and Vaidehi and Sekar

(2012) observed catechol type of siderophore production by PPFMs.

In the present study, it was observed that longer incubation period increased the soluble P concentration in broth indicating the slow action of the PPFM strains under controlled conditions. Microbial growth associated with decrease in pH of the medium has been reported to be efficient for P-solubilization (Khan *et al.*, 2006). *In vitro* studies have shown that P solubilisation can be associated with a marked drop in pH, production of phosphatases and organic acids. In the present study significant decline in pH level indicates medium acidification responsible for P solubilisation (Whitelaw *et al.*, 1999; Achal *et al.*, 2007). A pH regime of 3.4-4.6 was reported to be enough for significant solubilization of the Ca-phosphate in the presence of various carbon sources (Whitelaw *et al.*, 1999).

In conclusion, this study showed varied amount of phytohormones production, phosphate solubilisation and siderophores production by PPFM isolates isolated from chilli crop. So these PPFM isolates exhibit characteristics of plant growth promoting microorganisms. Based on our study, we propose further exploration of these identified potential PPFM isolates as bioinoculants in improvement of production and productivity of chilli crop.

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