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ISOLATION AND FUNCTIONAL CHARACTERISATION OF ENDOPHYTIC BACTERIAL ISOLATES FROM *CURCUMA LONGA*

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ABSTRACT

Endophytes are unique group of microorganisms residing within the specific chemical environment of host plants. Medicinal plants can have potential and diverse microbial association. The rhizome of turmeric is very remarkable due to its metabolite richness and the physiological processes associated with these tissues. But the functional role of endophytic microorganisms in this tissues remain totally unexplored. In the present study two endophytic bacterial isolates, *Alcaligenes faecalis* and *Enterobacter* sp were found to have the ability to produce IAA, ACC deaminase, phosphate solubilisation and to fix atmospheric nitrogen. By considering these plant growth promoting properties, *Alcaligenes faecalis* and *Enterobacter* sp can expect to have considerable effect on the growth of turmeric.

KEYWORDS: ***Endophytic bacteria, 16S rDNA sequensing, Alcaligenes faecalis, plant growth promotion, Enterobacter sp.***

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INTRODUCTION

Endophytes live in the internal tissues of plants without affecting the normal functioning[1]. Rich biodiversity of endophytic bacteria with unexplored biosynthetic potential have been reported [2,3]. Most of these endophytic bacteria have the molecular machinery for the synthesis of host plant specific bioactive compounds and are thus considered as an untapped source of natural products. These endophytic bacteria produce a plethora of bioactive metabolites and hydrolytic enzymes to survive in the unique chemical environment of the host plant[1]. Moreover their metabolic activities can contribute to the growth, health, and development of plants. Endophytes showed high growth promoting activities than rhizospheric microbes[4,5]. Bacterial endophytes promote plant growth through diverse process like production of phytohormones[6]; phosphate solubilisation[7], nitrogen fixation[8], ACC deaminase production[9], iron chelation and by inhibiting growth of pathogens through the production of antimicrobial secondary metabolites or by siderophores to inhibit pathogenic microorganisms[10,11] Endophytic microbes can have significant effect on plants but their functional roles are known only with limited number of isolates. Production of auxin like molecules are the most important contribution of these endophytes[12]. Many root associated bacteria including *Pseudomonas* sp., *Enterobacter* sp., and *Azospirillium* sp. has been shown to have produced Indole-3- acetic acid. IAA produced by endophytic bacteria has gained a great deal of attention due to its high relevance in plants[13]. In addition to the IAA production endophytic bacteria also perform phosphate solubilization, NH_3, ACC deaminase production, nitrogen fixation etc. Microbial population perform phosphate solubilization by the secretion of organic acids that convert the insoluble phosphates into soluble dibasic and monobasic ions and thus making it available to plants[14,15]. Plant associated endophytic bacteria can also produce ACC deaminase which promotes plant growth by regulating the synthesis of ethylene. ACC deaminase causes the degradation of immediate precursor of ethylene such as 1-aminocyclopropane-1-carboxylic acid in to α-ketobutyrate and ammonia, thus reduces the inhibitory effect of elevated level of ethylene and thereby reduces its harmful effects[11]. Thus isolation and characterization of endophytes from unexplored sources with diverse properties will have several applications in plant growth promotion[16]. In order to explore the potential of endophytes, various communities of endophytes should be isolated from different tissues of metabolically distinct and taxonomically diverse plants. Plants of *Curcuma longa* and its rhizome is well known for its medicinal properties: antitumor, anti-inflammatory and antiulcerogenic activities. The active constituents of turmeric rhizome include curcumin and various volatile oils, including atlantone, tumerone and zingiberone[17]. Hence several interesting groups of microorganisms can be expected to be present in the rhizome with diverse roles. However the functional traits of endophytic bacteria associated with turmeric has not yet been well investigated. So the isolation and characterization of endophytic bacteria associated with turmeric is very significant. The present study is mainly based on two endophytic bacteria isolated from turmeric rhizome and these isolates were found to have the ability to produce ACC deaminase, IAA, to solubilise inorganic phosphate and to fix atmospheric nitrogen. The present study was focused to identify the plant growth promoting properties of endophytes associated with rhizomes of turmeric.

MATERIALS AND METHODS

1. *Endophytic bacteria: Isolation and characterisation*

Turmeric rhizomes were collected from different places of Kerala, India. These rhizomes were first washed with tap water to remove soil and cut into small pieces. Then it was washed with tween 80 for 10 min with vigorous shaking followed by washing with distilled water. The samples were further washed with 70% ethanol for 1 min followed by 1% sodium hypochlorite for 15 min. The samples were then washed several times with sterile distilled water and the final washed water was spread onto a nutrient agar plate (g/L; beef extract 2, peptone 5, sodium chloride 5, yeast extract 3, and agar 18, pH 7.0) as control.

2. *Isolation of endophytes*

Isolation of endophytes from Turmeric rhizomes were carried out according to methods described by M A Surette *et al*., 2003 with minor modifications36. The rhizome was peeled with a sterilized knife. The outer, middle, inner tissues of the turmeric were cut and scraped using sterilized knife. The pieces were macerated with sterile Ringer solution using sterilized mortar and pestle. It was then transferred to 100 ml Ringer solution (215 mg NaCl + 7.5 mg KCl+ 12 mg $CaCl_2$ +50 mg sodium thiosulphate in 100 ml distilled water, P^H 6.6) and kept in shaker for 45 minutes. Then serial dilution was carried out and 1 ml from 10^{-5}, 10^{-6} and 10^{-7} dilution of each tissue layer were pour plated in nutrient agar (g/L; agar 18, peptone 5, yeast extract 3, sodium chloride 5, beef extract 2 and, p^H 7.0) and incubated at room temperature for 2-3 days. From each plate bacterial colonies were randomly selected and purely isolated by streaking on TSA and maintained at 4^0C.

3. *Detection of plant growth promoting properties of isolates*

3.1 Indole-3-acetic acid (IAA) production

Nutrient broth (10ml) containing 0.2% (v/v) of L-tryptophan used for IAA production. Bacterial isolates were inoculated in to nutrient agar and incubated for 10 days at 28^0C. After incubation, the culture was centrifuged at 3,000rpm for 20 min and the supernatant was screened for the presence of IAA as per the method described by Rahman *et al*., 2010[18]. Initially the culture is centrifuged and 1 ml of culture supernatant was then mixed with Salkowski reagent (2 ml 98ml 35% $HClO_4$ + 0.5 mol L^{-1} $FeCl_3$). After 20 minutes, the sample was

observed for red color. Uninoculated growth medium was used as negative control.

3.2 Ammonia production

The isolates were inoculated into peptone water (peptic digest 10 g/l, NaCl 5g/l, distilled water 1000 ml, p^H 7.2) and incubated for 2 days at 27± 3^0C. After incubation, 2-3 drops of Nessler's reagent was added in each tube and tubes were observed for brown to yellow colour formation. Uninoculated growth medium was used as negative control [19]

3.3 Phosphate solubilization

Pikovskaya medium (glucose 10 g/L, $Ca_3(PO_4)_2$ 5 g/L, $MgSO_4.7H_2O$ 0.1 g/L, NaCl 0.2 g/L, $(NH_4)_2SO_4$ 0.5 g/L, $FeSO_4.7H_2O$ 0.002 g/L, KCl 0.2 g/L, Yeast extract 0.5 g/L, $MnSO_4$ 0.002 g/L, agar 20 g/L P^H-7.0) with 2.4 mg/ml bromophenol blue was used for phosphate solubilization[20]. The media inoculated with the isolates were incubated for 48 hours and observed for yellow colour change.

3.4 ACC deaminase production

Endophytic bacterial isolates were further screened for ACC deaminase production using the method described by Dworkin and Foster [21]. For this, the isolates were inoculated onto DF salts minimal medium(KH_2PO_4 4g/L, Na_2HPO_4 6g/L, $MgSO_4$. $7H_2O$ 1 mg/L, H_3BO_3 10µg/L, $MnSO_4$ 10µg/L, $ZnSO_4$ 70µg/L, $CuSO_4$ 50µg/L, Mo O_3 10µg/L, glucose 2 g/L, Citric acid 2 g/L, agar 12 g/L) amended with 0.2 %(w/v) ($(NH_4)_2SO_4$ and incubated for 4 days.

3.5 Nitrogen fixation

Nitrogen fixation was further screened by inoculating the bacterial isolates on Jensen's medium[22]. Components of Jensen's medum were sucrose 20 g/L, K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, NaCl 0.5 g/L, $FeSO_4$ 0.1 g/L, Na_2MoO_4 0.005 g/L $CaCO_3$ 2g/L and agar 15 g/L. Bacterial colonies were inoculated on the medium and incubated for 4 days.

3.6 Hydrogen cyanide (HCN) production

Nutrient agar containing 4.4 g/l of glycine was used for the production of HCN. A filter paper soaked with 0.5% picric acid in 2% Na_2CO_3 was layered in the upper lid of the culture plate. Plates were then sealed and kept for incubation at 30^0C for 4 days. Yellow filter papers changed to cream, light brown, dark brown and eventually turned into reddish-brown when HCN produced[23]

4. Identification and characterization of isolates

The isolates were identified by studying the morphological, cultural and biochemical properties as per the Bergey's Manual of Systematic Bacteriology[24]

4.1 Molecular Identification of isolates

The molecular identification of isolate was carried out by 16s rDNA sequence based method. The genomic DNA from isolates were used as template for PCR amplification of 16S rDNA using primers 16SR (50-AAg gAg gTg WTC CAR CC-30) and 16SF (50-AgA gTT TgA TCM Tgg CTC-30) as per the previous reports of Chunand Goodfellow (1995). PCR was performed at a final volume of 50 µL reaction mixture containing 20 pmol of each primer, 50 ng of genomic DNA, 1.25 units of Taq DNA polymerase (Bangalore Genei), 1X PCR buffer and 200 IM of each dNTPs. PCR was performed for 35 cycles in a Mycycler TM (Bio-Rad, USA). Initial denaturation at 94^0C for 3 mins, cyclic denaturation at 94^0C for 30 seconds, annealing at 58^0C for 30s and extension at 72^0C for 2 min with a final extension at 72^0C for 7 min. Then the PCR product was confirmed by agarose gel electrophoresis. Reaction product was further purified and used as the template by using Big Dye Terminator Sequence Reaction Ready Mix, Applied Biosystems. The sequences were further subjected to BLAST analysis[25]

RESULTS

The standardized surface sterilization procedure used for the isolation of endophytic bacteria in the experiment was satisfactory. Also, adequate number of colonies were obtained in the nutrient agar plates inoculated with macerated plant samples. A total of 50 strains were obtained. 35 isolates were selected on the basis of their plant growth promoting properties Fig 1. These endophytic isolates were further screened for the production of various plant growth promoting traits. The endophytes produced clearing around the colonies after 48 hours of incubation on solidified Pikovskaya medium supplemented with ammonium sulfate indicated phosphate solubilizing ability of the test strain Fig 3. The culture supernatant of the bacterial isolates were checked for indole-3-acetic acid production calorimetrically. The endophytes produced red colour after the addition of Salkowski reagent were taken as positive. The result was compared with positive control, which had pure indole-3-acetic acid Fig 4. Concentration of IAA was then quantified using a standard curve by spectrophotometry. ACC deaminase production was indicated by the growth of the strains on DF salt minimal medium with 2% ammonium sulfate Fig 5. It was also found that some endophytes could also produce ammonia Fig 6. The list of growth promoting traits possessed by endophytes isolated from *Curcuma longa* are listed in Fig 2. These endophytes which showed atleast one growth promotion property were further identified and genus wise breakup of the positive isolates is shown in Table I. Of the 35 isolates MTM 3 and MPD 1 exhibited all the tested plant growth promoting properties such as phosphate solubilization, IAA production, ammonia production and ACC deaminase production. So it was further screened for more properties such as HCN production and nitrogen fixation. MTM 3 and MPD 1 were not produced HCN as it did not change the colour of the filter paper from yellow to red. The bacterial isolates were also screened for nitrogen fixation using Jensen's media. Both MTM 3 and MPD 1 were positive for nitrogen fixation.The plant growth promoting traits of MTM 3 and MPD 1 were shown in Table II. MTM 3 and MPD 1 which showed all the growth promoting characteristics were

further identified by 16S rDNA sequencing and the sequences were subjected to BLAST analysis Fig 7. BLAST result showed the isolates MTM 3 and MPD 1 were 100 and 99 percent similar to *Alcaligenes faecalis* and *Enterobacter sp* respectively with the NCBI accession number KJ716500 and KF923841.1.

Table I
Elucidation of growth promoting properties of endophytes isolated from Curcuma longa

Endophytes	Sample Name	Phosphate Solubilization	ACC Deaminase Production	NH3 Production	IAA Production	HCN Production	Nitrogen fixation
Klebsiella	OPD 1	+	+	+	-	-	-
Citrobacter	OPD 2	+	+	-	+	-	-
B. cereus	OPD 3	-	+	+	-	-	-
Micrococcus	OTM 1	+	+	-	-	-	-
Micrococcus	OTM 3	-	+	-	+	-	-
Citrobacter	OTM 5	-	+	+	+	-	-
Serretia	OTM 6	+	+	-	-	-	-
B. cereus	OTM 7	+	+	-	-	-	-
Unidentified	OTM2	+	+	+	-	-	-
Citrobacter	OKLM 1	+	+	-	+	-	-
Citrobacter	OKLM 2	+	+	+	+	-	-
Unidentified	OKTM 2	+	+	+	-	-	-
Unidentified	OIDKI 3	+	+	+	-	-	-
Citrobacter	OIDKI 4	+	+	-	-	-	-
Citrobacter	OKAL 5	+	+	-	-	-	-
Enterobacter	**MPD 1**	+	+	+	+	-	+
Pseudomonas	MPD 2	+	+	+	+	-	-
Unidentified	MPD 3	+	+	+	-	-	-
B. cereus	MPD 4	+	+	+	-	-	-
A. faecalis	**MTM 3**	+	+	+	+	-	+
B. cereus	MTM 5	+	+	-	-	-	-
Klebsiella	MTM 2	+	+	-	+	-	-
Unidentified	MTM 1	+	+	-	-	-	-
Unidentified	MTM 2	+	+	+	-	-	-
Pseudomonas	MKLM	-	-	+	-	-	-
Unidentified	MIDKI 1	+	+	+	-	-	-
Unidentified	MIDKI 2	+	+	+	-	-	-
Citrobacter	MKAL 3	+	+	-	-	-	-
Unidentified	IPD 1	+	+	+	-	-	-
Klebsiella	IPD 2	+	+	+	-	-	-
Unidentified	ITM 2	+	+	+	-	-	-
Micrococcus	IKLM 1	+	+	+	-	-	-
Citrobacter	IKTM 2	+	+	+	-	-	-
Klebsiella	IKAL 1	+	+	+	+	-	-
Others	-	-	-	-	-	-	-

Table II
Functional traits of endophytic bacteria MTM 3 and MPD 1

Plant growth promotion properties	MTM 3	MPD 1
Phosphate solubilization	Positive	Positive
ACC deaminase production	Positive	Positive
Ammonia production	Positive	Positive
Indole-3 acetic acid production	Positive	Positive
Hydrogen cyanide production	Negative	Negative
Nitrogen fixation	Positive	Positive

Figure 1
Percentage of endophytes isolated from curcuma longa n=35

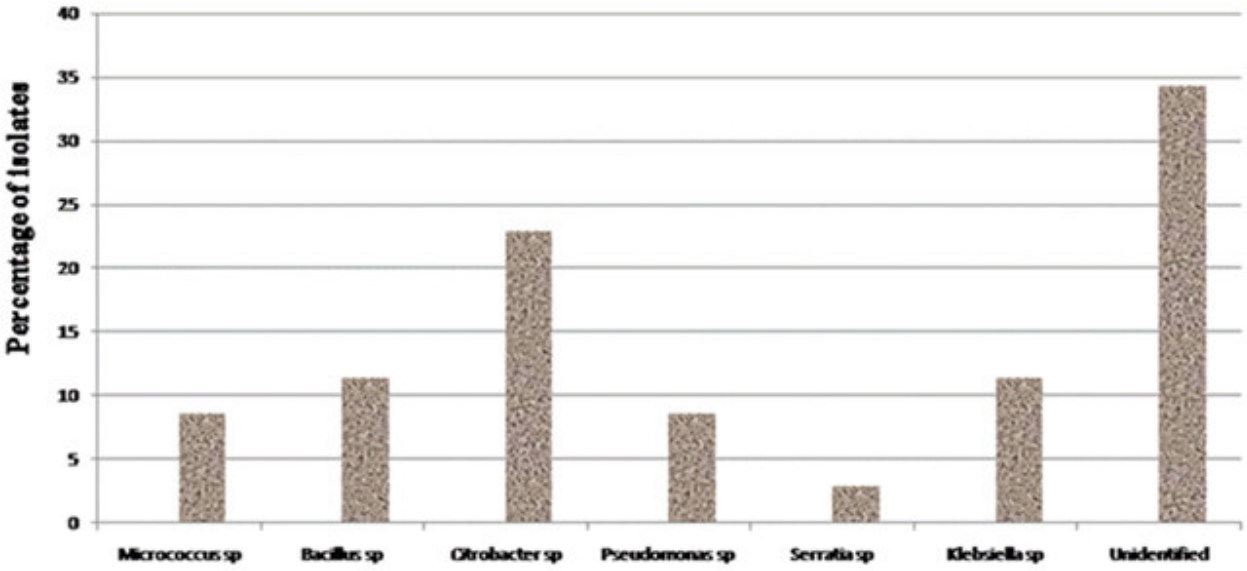
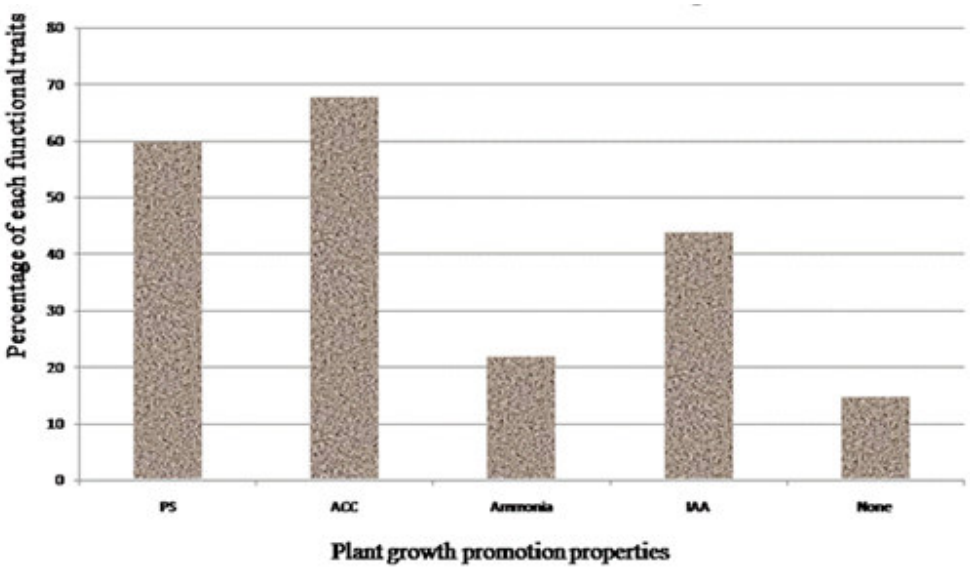


Figure 2
Growth promoting properties of endophytic bacteria isolated from curcuma longa N=35



PS: Phosphate solubilisation; ACC: ACC deaminase; IAA: Indole-3-acetic acid

Figure 3
Phosphate solubilisation shown by endophytic isolates

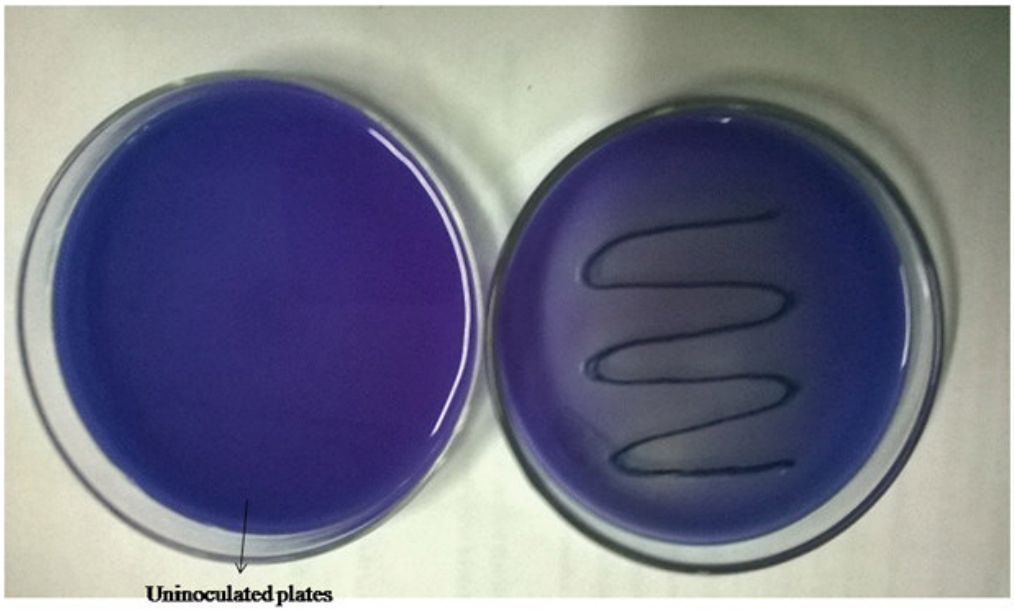


Figure 4
IAA production shown by endophytic bacterial isolates

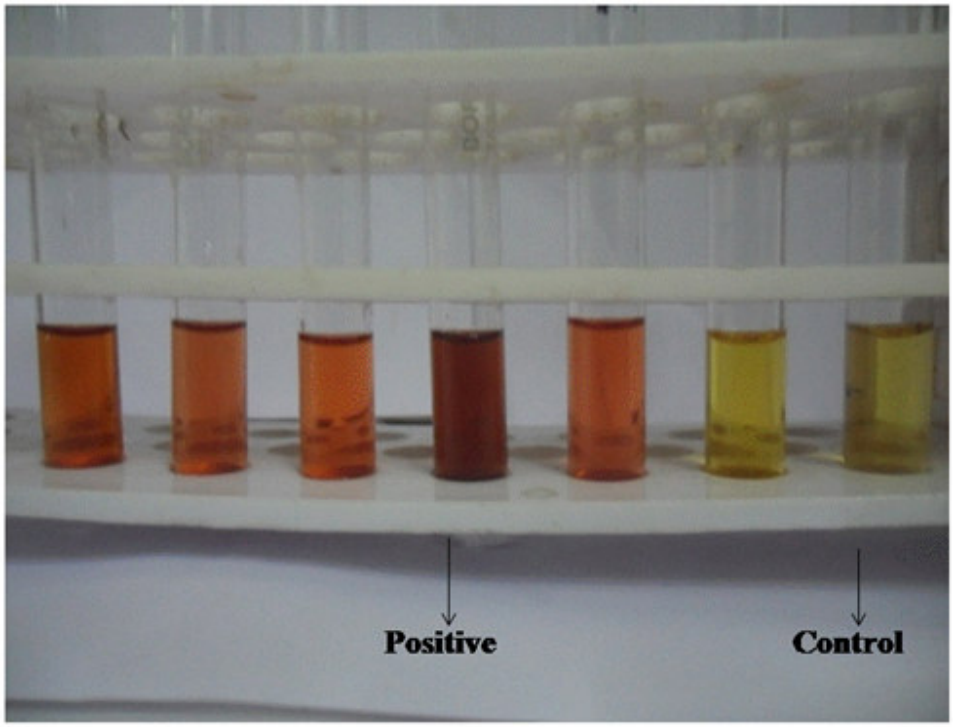


Figure 5
ACC deaminase production shown by endophytic isolates

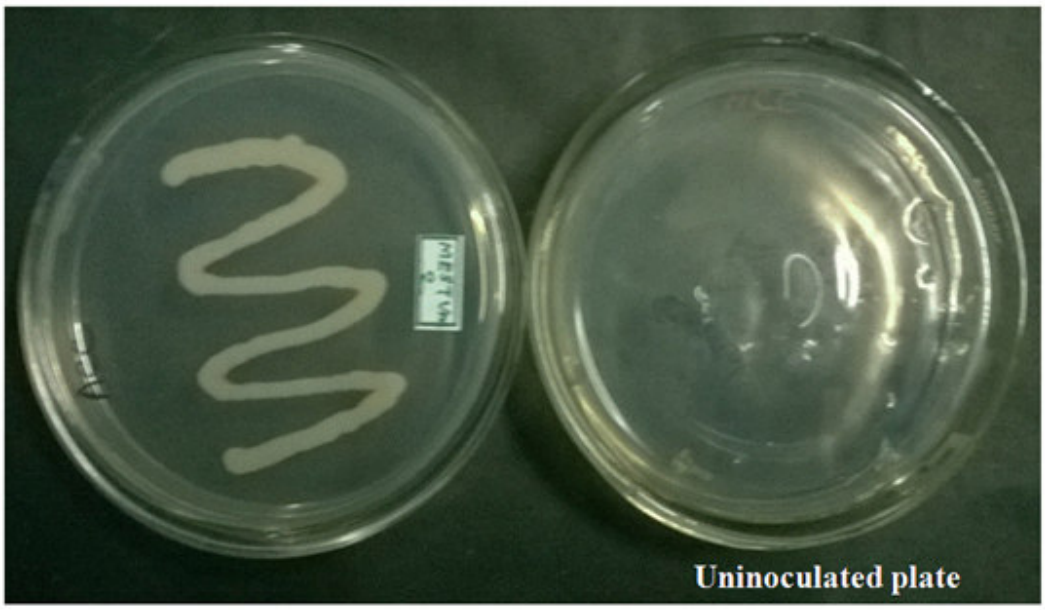


Figure 6
Ammonia production

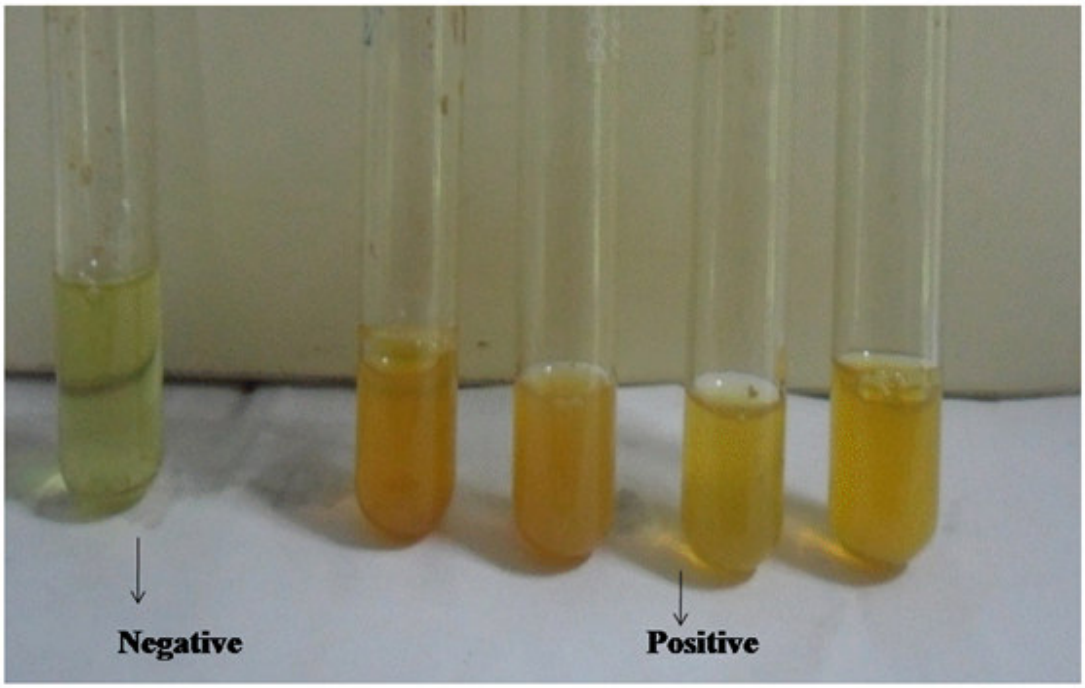
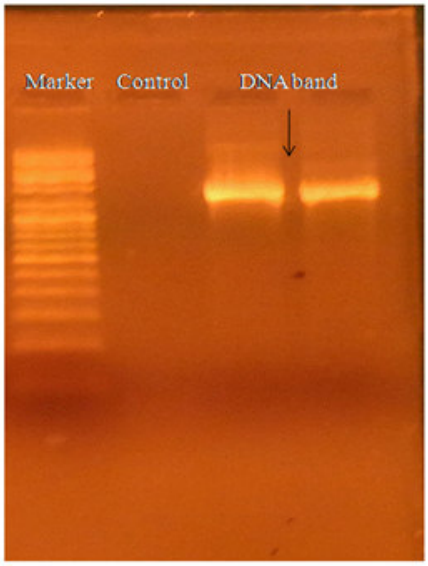


Figure 7
The PCR amplification of 16S rDNA of the selected strains



DISCUSSION

Endophytic bacteria have been found virtually in most of the plants, where they colonize the internal tissues of their host plant and form bacteria-host relationship which may be symbiotic, mutualistic, commensalistic and also trophobiotic. It was demonstrated that endophytes entered the plant tissue primarily through the root zone; however, aerial parts of plants, such as stems, cotyledons and flowers, are also used for entry. It was demonstrated that endophytic bacteria can promote plant growth and can be used as biocontrol agents[26]. Bacterial endophytes could enhance plant growth through various process like ACC deaminase production[27], production of phytohormones[28], nitrogen fixation[29], phosphate solubilisation[8], iron chelation[30] and also by inhibiting growth of pathogens. In the present study endophytic bacteria have been isolated from outer, middle and inner tissue layers of turmeric. The current report focuses on two bacterial endophytes MTM 3 and MPD1 which were found to be promising in its initial studies. According to Kloepper and Schroth (1981), PGPR (plant growth promoting bacteria) mediated plant growth promotion caused by the alteration of the entire microbial community in rhizosphere niche through the production of various substances[31]. Usually, PGPR could promote plant growth directly by either modulating plant hormone levels or facilitating resource acquisition (phosphorus, nitrogen and essential minerals) or indirectly by decreasing the inhibitory action of various pathogens on plant growth. The solubilization of inorganic phosphorus occurs by the action of low molecular weight organic acids synthesized by various soil bacteria[32]. Phosphate-solubilizing bacteria (PSB) has been considered as good biofertilizers because they can supply phosphorus from sources otherwise poorly available by various mechanisms. Both of the selected strains were showed phosphate solubilisation on Pikovskaya medium. Generally, ethylene is essential for the normal growth and development of plants[33]. For instance, the high concentration of ethylene may cause defoliation and other related cellular processes which will lead to reduced crop performance [34].The endophytic bacteria possess the enzyme, ACC (1-amino cyclopropane -1-carboxylate) deaminase, enhance plant growth by decreasing the level of ethylene, inducing salt tolerance and reducing drought stress in plants. MTM 3 and MPD 1 were produced ACC deaminase enzyme. Microbial synthesis of the phytohormone auxin (indole acetic acid/indole-3-acetic acid /IAA) is well known. It has been demonstrated that 80% of microorganisms isolated from the rhizosphere have the ability to synthesize auxins as secondary metabolites[17]. Generally, IAA affects plant cell division, differentiation and extension; increases the rate of xylem and root development; stimulates seed and tuber germination; biosynthesis of various metabolites; controls processes of vegetative growth; mediates responses to gravity and light; initiates lateral and adventitious root formation; affects photosynthesis; pigment formation; and resistance to stressful conditions. Of the 50 isolates 11 strains shows IAA production. MTM 3 and MPD 1 developed orange red colour after 30 minutes of incubation indicating the production of IAA. It was also found out that MTM 3 and MPD 1 were also able to produce NH_3 and thus perform nitrogen fixation. Howell *et al*., (1988) reported that volatile compounds such as NH_3 produced by *Enterobacter cloacae* was involved in the suppression of *Pythium ultimum* induced damping off of cotton[35]. Since the indiscriminate and excessive application of chemical fertilizers has lead to health and environmental hazards, farmers are desperate to find alternative strategies that could provide better yield while protecting soil health. In this condition investigation of noval endophytic bacteria with multiple growth promoting properties is quite promising in the growth of plant and regeneration of rhizomes.

CONCLUSION

The results from the study showed that diverse community of endohytic bacteria are associated with the rhizome of curcumin. Among these endohytic bacteria *Enterobacter* sp and *Alcaligenes faecalis* were found to have the ability to form ACC deaminase, Indole-3-acetic acid and ammonia. These isolates were also found have the capacity to perform phosphate solubilisation which have a high effect on plant growth. Thus these isolates can be considered to have growth promoting effect in turmeric.

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