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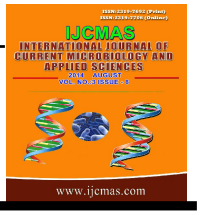

Original Research Article

Effect of *Azotobacter chroococcum* CL13 inoculation on growth and curcumin content of turmeric (*Curcuma longa* L.)

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A B S T R A C T

In the present study, *Azotobacter chroococcum* CL13 were isolated from the rhizospheric soil of turmeric and identified morphologically biochemically and through 16 S r RNA gene sequence analysis. The isolate significantly produced IAA, NH_3 HCN and solubilized tricalcium phosphate during PGP trait analysis. The isolate used as inoculants in rhizome prior to sowing following standard procedures. After inoculation plant growth parameters such as shoot height, shoot fresh biomass, rhizome fresh biomass enhanced in A.chroococcum CL13 inoculated turmeric than control (uninoculated). Curcumin is the most active and important constituents of turmeric having various pharmacological activity. The concentration of curcumin had been significantly enhanced by 6% in *A. chroococcum* CL13 inoculated turmeric.

K e y w o r d s

Turmeric, Curcumin, Plant growth Promotion, Bacterial inoculation, Yields

Introduction

The turmeric, a rhizotomous herb of family Zingibiraceae, is used as a spice, coloring agent and traditional medicine from the ancient time in South Asian and Middle Eastern countries. Plant is used as a herbal medicine for the treatment of asthma bronchial hyperactivity, rheumatism, diabetic wounds sinusitis, smallpox, skin cancer, menstrual difficulties and abdominal pain (Ammon and Wahl, 1990**).** The major curcuminoids, curcumin exhibited various biological effects such as anti-inflammatory, antioxidant, antimalerial, hypolipidemic activities. (Tonnesen, 1992; Reddy and Lokesh, 1992) and extensively studied as a chemo preventive agent in several cancer (Rao *et al.*, 1995). The rhizomes of turmeric contain numbers of biochemical ingredients like curcuminoids and sesquiterpenoids. For the separation and quantification of curcuminoids a variety of methods have been reported in the literature. He et al., (1988) reported online HPLC UV diode–array and electro spray mass spectrometer methods to analyze curcuminoids in fresh turmeric extracts. Jayaprakasha et al., (2002) reported the improved HPLC method for the separation and quantification of curcumin demethoxycurcumin and bisdemethoxycurcumin in the turmeric.

Plant growth promoting rhizobacteria (PGPR) is a group of free-living rhizosphere bacteria that enhances plant growth by performing activity of biofertilizers, biopesticides or bio control agents. The most commonly found PGPR *Pseudomonas fluorescence*, *Bacillus*, *Azotobacter*, *Azospirillum* and *Klebsiella*. PGPR facilitate the plant growth promotion directly by nutrient solubilization, nitrogen fixation and by producing growth regulators and antibiotics (Lucy et al., 2004), where as they indirectly involved in production of hydrogen cyanide, siderophores, competitive exclusion of pathogens and removal of phototoxic substances produced by deleterious microorganisms. PGPR contribute to sustainable agriculture by diminishing the use of chemical pesticides, chemical fertilizer and also by protecting human health (Adesemoye and Kloepper 2009). Recently PGPR have numerous biotechnological applications in agriculture, horticulture, forestry and environmental protection (Zahir et al., 2004). *Azotobacter* is regarded as free living aerobic N_2 fixer present in soil. Besides nitrogen fixation they also synthesize and secretes considerable amount of phytohormones to enhance the plants growth and pathogenic diseases tolerance (Van Loon, 2007).

In recently past *Azotobacter* broadly used as a soil or plant inoculants in agronomic field trails for the diseases management and growth enhancement (Amein et al. 2008; Maheshwari et al., 2012). In the present study, we evaluate the influence of PGPR strains *Azotobacter* inoculation on the morphological parameters as well as on the concentration of nutritional and medicinally important curcumin of turmeric.

Materials and Methods

Isolation and maintenance of bacterial isolates

Bacterial isolates were isolated from the rhizospheric soil using standard microbiological techniques from healthy and young turmeric (*curcuma longa*) growing in the Botanical Garden of Banaras Hindu University, India. (20^0 18' N and 80° 36' E, elevation 80.71m).

Powdered (1g) rhizospheric soil was suspended in 9.5 ml of sterilized distilled water and shaken on gravatory shaker for 1h. 0.1 ml of serially diluted soil suspension inoculated on N free glucose medium (Norris and Chapman, 1968) for the isolation of *Azotobacter* sp. Bacterial isolates were selected and identified according to Bergey's manual of systematic bacteriology (Garrity, 2005). On the basis of morphological, biochemical screening isolates were selected and further identified by 16s r RNA gene sequence analysis. (Kumar et al., 2006), the sequence was analyzed and queried with the BLAST

Plant growth promoting (PGP) traits of bacterial isolates

Azotobacter isolates were characterized for plant growth promoting properties including indole acetic acid production (IAA) (Brick et al., 1991), phosphate solubilization (Laslo et al., 2012), HCN production (Lorck, 1948), siderophore production (Schwyn and Neilands, 1987) NH_3 production (Cappuccino and Sherman 1992) as per standard protocols.

Rhizome bacterization

Young growing rhizotomus buds of turmeric collected from Botanical garden of Banaras Hindu University were bacteriazed by method of Weller and Cook (1983). Rhizotomus buds (7.5g) were surface sterilized with 1% $HgCl_2$ for 30 second and then washed with distilled water for 5–6 times, cell biomass of *A. chroococcum* CL13 were harvested through centrifugation (10,000g, 10 min) at 4°C from 72 h old culture.

The pellets resuspended in sterile distilled water (10^8 cfu ml^{-1}) and viable bacterial number was measured. The rhizomes were then coated with 20 ml bacterial inoculums (10^8cfu ml^{-1}) using 1% carboxymethyl cellulose (CMC) slurry as an adhesive. The rhizome coated with 1% slurry without bacterial strain served as control. The bacterial coated rhizome of turmeric sown in the experimental pots for (6 month) completion of life cycle under controlled natural condition.

Plant materials for curcumin quantification

For the quantification of curcumin in the bacterial inoculated turmeric, young and mature rhizomes of control (uninoculated) and *A. chroococcum* CL13 inoculated turmeric were collected from the experimental pot. The rhizomes of turmeric (1.0 g each) were treated with hexane (50 ml) by using a soxhlet extractor (30 min) for extraction. The hexane was removed through rotary evaporator, extracted samples were dissolved in 50 ml of methanol for 2 h. Before using, the extracted samples were filtered through 0.2 µm millipore filter.

Preparation of stock solution

The curcumin stock solution was prepared in methanol at a different concentration between 0.5 to 7mg/ml

Chemical and reference compounds

All the chemicals and solvents used were of analytical grade (E. Merck, Mumbai, India). Standard sample of curcumin obtained from Sigma Aldrich, (Bangalore, India) and before use, all solvents were filtered through 0.2 µm millipore membrane filter.

Equipment and chromatic condition

The HPLC analysis was performed on a system consisting of Hewlett-Packard quaternary HP1090 Series (Hewlett-Packard palo Atto CA, USA) with multi wave length Photodiode-Array detector set between 200 nm to 500 nm and managed by computer system HP 9000 workstation. The quantification of compounds was performed by using Luna RP-C18 prepacked column (150nm × 3m) with a particle size of 5 mm. Acetonitrile and 2% acetic acid 60:40 (v/v) used as mobile phase with the flow rate of 0.5 ml/min, the injected volume was 20 µl.

Calibration and linearity The linearity range of standards was determined by analyzing series of standard curcumin. Test solution ranging (0.5–7.0) mg/ml of curcumin was prepared and injected three times for linearity test. The linear regression curve was obtained by plotting the peak area count of curcumin at (y axis) separately against the concentration (x axis) of each injection separately. Linearity was found in the concentration range between 1–7mg with high reproducibility and accuracy. Regression analysis of experimental data showed linear relationship. The limit of detection (LOD) and limit of quantification (LOQ) were determined during HPLC by injecting series of standard solutions until

the signal to noise ratio (S/N) ratio for each compound was 3 for LOD and 10 for LOQ.

Results and Discussion

Isolation of bacterial isolates

Total 13 bacterial isolates were grown on N free agar specific plate, which were same in the morphology and biochemical tests. The isolates were Gram-negative, rod shaped, round, mucoid, smooth colonies. Morphological and biochemical characteristics of isolates have been presented in (table.1). One of the pure strain CL13 on 16s r RNA gene sequence analysis identified as *Azotobcter chroococcum* species, which show 100% similarity with the strain *A. chroococcum* GD5. The gene sequence were deposited in GeneBank under the accession number [KJ001770]

Plant growth promoting (PGP) traits of bacterial isolates

The PGP traits for the isolates *A. chroococcum* CL13 revealed that they produced IAA in the presence of different concentrations (50 to 500 µg/ml) of tryptophan, they did not produce IAA in absence of tryptophan. The content of IAA was produced by *A. chroococcum* CL13 (1.33 µg/ml) at 50 µg/ml of tryptophan and 10.97 µg/ml of IAA at 500 µg/ml IAA. Phosphate solubilization (zone of clearance 11 mm), production of siderophore and NH_3were detected positive during plant growth promoting properties.

Growth and yield of PGPR treated turmeric

The various morphological parameters like number of leaves shoot height, shoot and rhizome fresh biomass were measured in control and *A. chroococcum* CL13 inoculated turmeric up to 6 month (from June to November 2011). All these morphological parameters increased significantly (Multiple Tukey HSD, SPSS.16). The difference in leaves number (same in both) were not significant between control and *A. chroococcum* CL13 inoculated turmeric. Shoot height, shoot fresh biomass and rhizome fresh biomass increased significantly in *A. chroococcum* CL13 inoculated turmeric with the growth. Increase in morphological parameters, shoot height, shoots biomass and rhizome biomass was significant ($p<0.05$) with the growth periods (Table 2).

Peak identification

The HPLC chromatogram of control and *A. chroococcum* CL13 inoculated turmeric at the wavelength of 425 nm three peaks were observed at the different retention time (RT). The peak at the retention time of 8.50±0.55 (average of three replicates) was identified as curcumin. The purity of the compounds was checked by the standard compounds of curcumin (Rt. 8.90). In both the standard and sample peak, Peak purity was greater than 0.9899. The UV-spectral matching was found between 0.9899–0.9788.

Analysis of curcumin content in the sample of control and *A. chroococcum* CL13 turmeric were performed using HPLC-PDA detector. The variation in amount of curcumin was measured by comparing the area covered by the curcumin in the chromatogram of respective sample with the standard curve of curcumin (Fig.1). The standard curve were made by injecting different concentration 0.5 to 7 mg/ml of curcumin, which show linear relation between 1 to 7 mg/ml, The correlation Coefficient (R^2) of curcumin was 0.9980 and linear regression equation for the curve was 215e+004x-2.34e+004 achieved.

Table.1 Morphology and biochemical characterization of isolates

Biochemical characters	*Azotobacter isolate*
Gram stain, cell shape	Gram –ve, Rod
Colony morphology and Pigmentation	Watery, mucilaginous white with exuberant margin
Growth of N_2 free medium	+
Polysaccharides production	-
Catalase	+
citrate test	+
Carbohydrate utilization	
Glucose	+
Lactose	-
Sucrose	+
Mannitol	+
Hydrolysis	+
Starch	+
Lipid	+
Biotin	

Table.2 Various growth and yield parameters of *Azotobacter chroococcum* CL13 inoculated turmeric

Month	Shoot Height(cm)		Shoot Biomass(g)		Rhizome Biomass(g)	
	Control	*Azotobacter chroococcum* CL13	Control	*Azotobacter chroococcum* CL13	Control	*Azotobacter chroococcum* CL13
June	0	0	0	0	7.5	7.5
July	20 ± 0.55^{a}	22 ± 0.55^{b}	13 ± 0.92^{k}	14 ± 0.71^{l}	8 ± 0.63^{ab}	8 ± 0.66^{bc}
August	33 ± 0.63^{c}	37 ± 0.72^{d}	20 ± 0.66^{m}	25 ± 0.58^{n}	10 ± 0.62^{cd}	10 ± 0.58^{ef}
September	55 ± 0.46^{e}	59 ± 0.61^{f}	36 ± 0.61^{o}	39 ± 0.57^{p}	33 ± 0.61^{fg}	38 ± 0.31^{gh}
October	69 ± 0.66^{g}	73 ± 0.66^{h}	46 ± 0.60^{q}	51 ± 0.55^{r}	63 ± 0.39^{ij}	67 ± 0.57^{kl}
November	78 ± 0.81^{i}	81 ± 0.68^{j}	57 ± 0.60^{s}	61 ± 0.58^{t}	135 ± 0.70^{mn}	142 ± 0.60^{op}

Fig.1 HPLC chromatogram of control and *A. chroococcum* CL13inoculated turmeric at 425nm

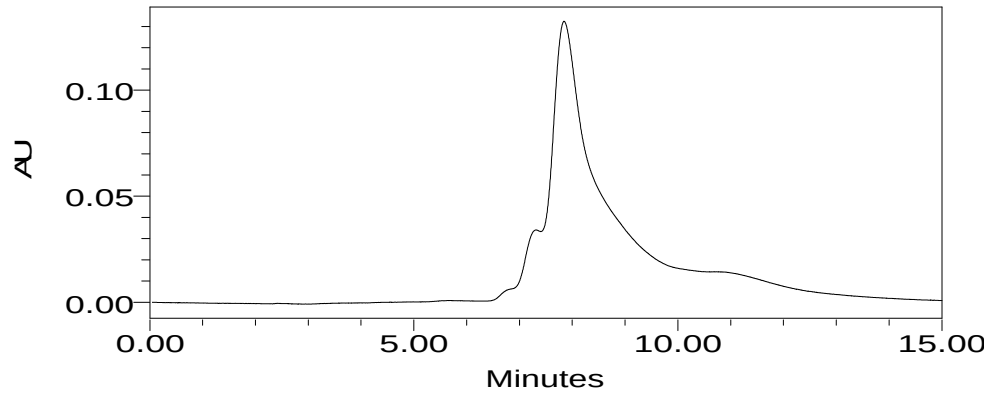


Fig.1a HPLC Chromatogram of standard curcumin at 425 nm

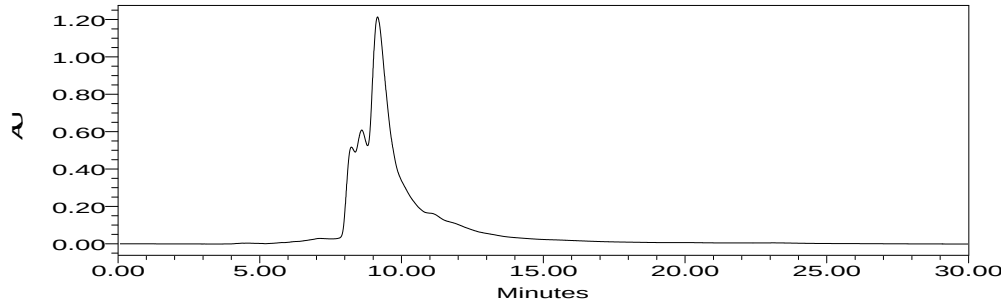


Fig1.b HPLC chromatogram of control turmeric (uninoculated) at 425 nm

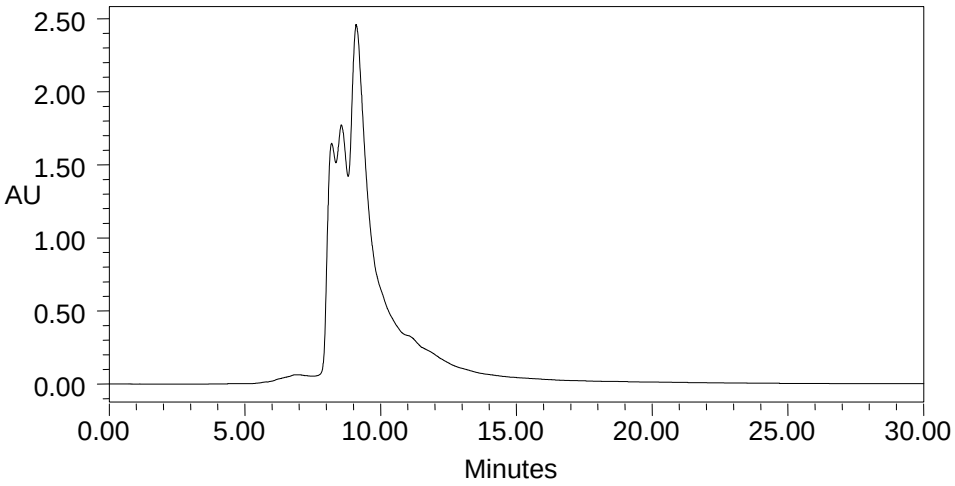
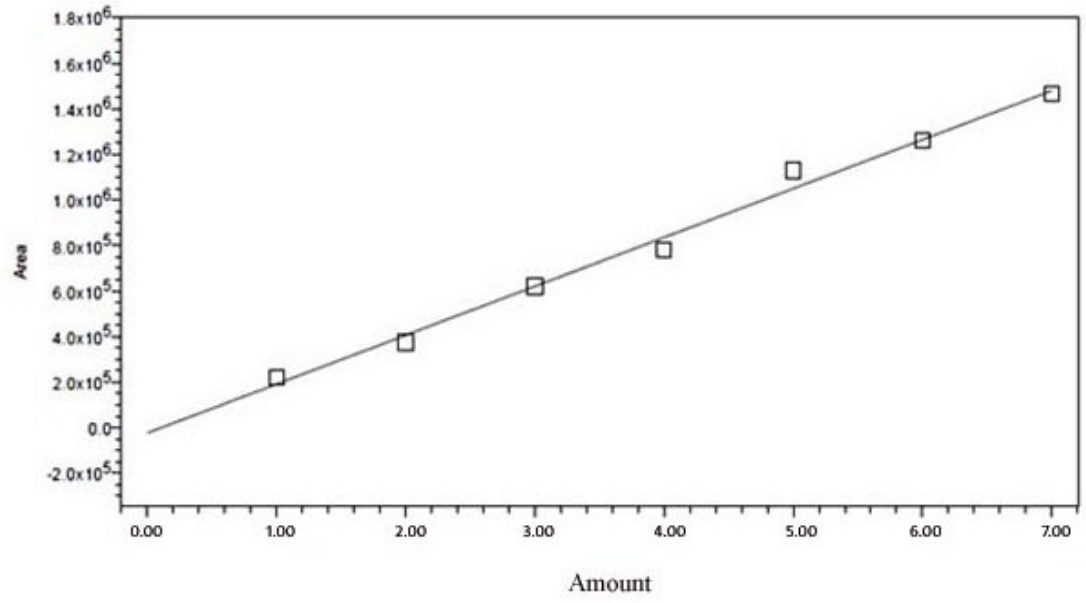


Fig1.c HPLC Chromatogram of *Azotobacter chroococcum* CL13 inoculated turmeric at 425 nm



Peak Name: Curcumin; RT: 1.593; Fit Type: Linear (1st Order); Cal Curve Id: 1994; R: 0.995759; R^2: 0.991536; Weighting: None; Equation: Y = 2.15e+004 X - 2.34e+004; Normalized Intercept/Slope: -0.027270; RSD(E): 5.619203

The concentration of curcumin in control (4.52±0.78 g) increased by 6% (4.79±0.45 g) in *A. chroococcum* CL13 inoculated turmeric (amount are the average of three replicates).

In the intensive agricultural system to obtain the maximum amount of product, huge amount of chemicals as fertilizers and pesticides are being applied for nutrient supplement and pest control. On the other hand continuous uses of chemicals adversely affect soil fertility, soil ecology, and environment as well as produce harmful effect on human health (Ayala and Rao, 2002). Hence, environment friendly sustainable agriculture is preferred to obtain the desirable yields.

The present study has shown that the growth of shoot and yield of rhizome biomass enhanced in *A. chroococcum* CL13 inoculation compared to control. This may be due to the production of IAA, NH_3 and phosphate solubilization by *A. chroococcum* CL13 which was observed during PGP trait analysis in this study. The plant growth promotion due to bacterial IAA production during root colonization is reported by other workers also. The IAA producing bacteria survive on root and proliferate by utilizing nutrients exuded by them and efficiently colonize the entire root system (Bloemberg and Lugtenberg, 2001). Reports of Rodríguez and Fraga (1999) stated that P. solubilizing bacteria are capable to increase the aviability of phosphorous in soil, which is beneficial for the plant growth. *A. chroococcum* CL13 is known as N_2 fixer in plant rhizosphere/soil and its inoculation increased the shoot height, shoot fresh biomass, rhizome fresh biomass.

Curcuminoids is an important component of turmeric. Amongst curcuminoids, curcumin is an important constituent. In this present study, *A. chroococcum* CL13 inoculation enhanced the concentration of curcumin. The exact mechanism through which *A. chroococcum* CL13 modulate the synthesis of curcumin had been not studied in this experiment but its chemotactic behavior were studied by workers in cotton and wheat seedlings. The results showed that this bacterium had strong chemotactic movements towards few amino acids like (Glutamic acid, Arginine, Threonine) and organic acids like (citric acid, succinic acid, Maleic acid and Malonic acid) (Kumar et al., 2007). The rhizome of turmeric contains number of phenolic compounds like curcuminoids and sesquiterpenoids. There exudates likely to attract *A. chroococcum* CL13 more suitably and effectively colonized roots resulting enhanced the production of curcumin. Hence positive response of *A. chroococcum* CL13 towards curcumin production was obtained. The *A. chroococcum* CL13 during PGP traits analysis produced IAA, solubilize P and produced NH_3, its inoculation might enhance the morphological yields and curcumin concentration.

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