In Vitro Biochemical Characterization of Plant Growth Promoting Attributes of *Bacillus megaterium* (TRS 7)- A Potent Plant Growth Promoting Rhizobacterium (PGPR)

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Abstract- *Bacillus megaterium (TRS 7) was isolated from the rhizosphere soil of tea bushes from Nagrakata Tea estate. The culture was preliminarily identified on the basis of morphological, microscopic and biochemical characterization and finally identity of the strain was confirmed from the Plant Diagnostic and Identification Services, UK and also by 16S rDNA sequencing. The sequence was deposited in NCBI, GenBank database under the accession Nos. JX 312687.1 for Bacillus megaterium (TRS 7). The bacterium was characterized in vitro for their plant growth promoting activities. B. megaterium was non- chitinase producing strain which was also able to produce IAA, volatiles, siderophores and solubilised phosphates in vitro but did not produce HCN. B. megaterium grew best at pH 6.0 with log cfu values of 22.8 log cfu/ml and at around 35ºC. The present study will focus on the in vitro PGPR attributes of Bacillus megaterium (TRS 7) which may lead towards the commercial use of this bacterium as PGPR that could be utilized in suitable formulations commercially which would benefit the tea industry where use of biological products to replace or supplement chemical use is the need of the hour.*

Keywords- *Bacillus megaterium* (TRS 7), PGPR, *in vitro* PGPR attributes, IAA, Siderophore, Phosphate solubilisation.

I. INTRODUCTION

Rhizosphere bacteria can have a profound effect on plant health. Since rhizosphere colonization is important not only as the first step in pathogenesis of soil borne micro-organisms, but also is crucial in the application of microorganisms for beneficial purposes (Lugtenberg *et al.* 2001). Colonising microorganisms can be detected attached to the roots, as free organisms in the rhizosphere or as endophytes. The interactions between plants and microorganisms are immensely complex and very little is known about the sum of factors that lead to reliable biocontrol and biofertiliser applications. Plant growth promoting rhizobacteria (PGPR) are a common group of bacteria that can actively colonize plant roots and increase plant growth (Kloepper and Schroth 1978). These PGPR can prevent the deleterious effects of phytopathogenic organisms from the environment. The mechanisms by which PGPR can influence plant growth may differ from species to species as well as from strain to strain. Growth promotion mechanism may be direct i.e. production of growth hormones, phosphate solubilization, nitrogen fixation or indirect viz, suppression of deleterious microorganisms by siderophore production or secretion of antifungal metabolites (Kloepper 1993). But, in field conditions, the above traits may not be sufficient to account for the observed growth promotion. The biochemical or physiological changes induced in the host that are activated by the PGPR, also lead to plant growth promotion and develop resistance capacity in the host against pathogens. Thus, though hundreds of candidate PGPR strains have been screened and evaluated in laboratory, greenhouse and field studies across the world, implementation of this technology has been hindered by the lack of consistency and variation in responses that are obtained in field trials from site to site, year to year, for different crops (Martinez-Viveros *et al.* 2010). Successful establishment of the introducing bacteria depends on proper selection that must tailored to the soil and crop combination. Besides, understanding of the interactions between PGPR and their plant hosts and the resident microflora are still limited, and there is also paucity of information on how environmental factors influence processes that contribute to plant growth promotion.

Ability of bacteria to solubilise phosphate is an important criterion when considering their use as biofertiliser. Out of 37 *Acenetobacter* sp. isolated from rhizosphere of wheat, 36 were able to solubilize phosphates under different experimental conditions (Chopade 2003). Siderophore production has also long been considered as one of the mechanisms of suppression of fungal growth in the rhizosphere. Jagadeesh and Kulkarni (2003) reported that of 38 rhizobacterial strains isolated from tomato which showed antagonism to *Alstonia solanacearum*, 23 were siderophore producers. Arkhipova *et al.* (2005) isolated many PGPR from the rhizosphere that could produce cytokinins that exerted a pronounced growth stimulatory effect in different crop plants.

In order to effectively reduce the excessive use of chemicals in agriculture, currently, much emphasis is being laid on use of eco-friendly biological materials for use in sustainable agriculture. One of the worst affected crops is tea, where, in order to overcome any loss of productivity, fungicides and insecticides, along with chemical fertilizers have been regularly applied. Tea plantations of North Bengal region, including Darjeeling hills have been facing this problem and keeping this in mind, the present study has been undertaken to characterize biochemically a potent PGPR that could be used as potent plant growth promoter on the basis of *in vitro* PGPR attributes to reduce the use of pesticides in tea industry.

II. MATERIAL AND METHODS

SOURCE OF BACTERIAL CULTURE

Initially several microorganisms were isolated from different rhizospheric soil of tea gardens and screened for *in vitro* PGPR activities. One bacterial strain, isolated from the rhizosphere soil of tea bushes from Nagrakata Tea estate showed positive response in *in vitro* PGPR tests. The culture was preliminarily identified on the basis of morphological, microscopic and biochemical characterization and finally identity of the strain was confirmed from the Plant Diagnostic and Identification Services, UK and also by 16S rDNA sequencing.

ASSESSMENT OF GROWTH IN LIQUID MEDIUM

The isolate was cultured in Nutrient Broth medium (Himedia, M002-100G, ingredients- peptic digest of animal tissue- 5.00g/litre, sodium chloride- 5.00g/l, beef extract- 1.50 g/l, yeast extract- 1.50 g/l, final pH 25°C -7.4±0.2) and was allowed to grow properly, with shaking at 37^{o}C at 120 r.p.m for 24, 48, 72, 96 and 120 h. At the interval of 24 hr, optical density of bacterial suspension was measured by colorimeter and that O.D. value was converted to log cfu/ml broth. Then the standard curves for optimization of growth of the isolate was also prepared.

In vitro CHARACTERIZATION OF PLANT GROWTH PROMOTING ACTIVITIES

IAA PRODUCTION

For detection and quantification of IAA, the selected bacterial cells were grown for 24 h to 48 h in high C/N ratio medium. Tryptophane (0.1 mM) was added in order 65 to enhance acetic acid (IAA) production by the bacteria (Prinsen *et al.* 1993). Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere *et al.* (1999). For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per litre in 7.9 M H_2SO_4 was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm.

PHOSPHATE SOLUBILISATION

Primary phosphate solubilizing activity of the isolate was carried out by allowing the bacterium to grow in selective medium i.e., Pikovskaya's agar (Himedia- M520; ingredients- yeast extract-0.50 g/l, dextrose- 10.00 g/l, calcium phosphate- 5.00 g/l, ammonium sulphate- 0.50 g/l, potassium chloride- 0.20 g/l, magnesium sulphate- 0.10 g/l, manganese sulphate- 0.0001 g/l, ferrous sulphate- 0.0001 g/l and agar- 15.00 g/l) for 7 to 10 days at 37°C (Pikovskaya 1948). The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

SIDEROPHORE PRODUCTION

The bacterial isolate was characterized for siderophore production following the method of Schwyn and Neiland (1987) using blue indicator dye, chrome azurol S (CAS). For preparing CAS agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl) and volume made up to 1L. With constant stirring this solution was added to 72.9 mg hexadecytrimethyl ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile petriplates (20 ml per plate). The plates were inoculated with the bacteria and incubated for 10-15 days till any change in the color of the medium was observed.

HCN PRODUCTION

Production of hydrocyanic acid was determined using the procedure described by Reddy *et al.* (2010) with slight modification. The selected bacterial isolate was grown at room temperature (37ºC) on a rotary shaker in nutrient broth(NB) media. Filter paper (Whatman no.1) was cut into uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed in side the conical flasks in a hanging position. After incubation at 37ºC for 48 hr, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The color was eluted by placing the filter paper in a clean test tube containing 10 ml distilled water and the absorbance was measured at 625 nm.

CHITINASE PRODUCTION

For detecting the chitinolytic behavior of the bacterium chitinase detection agar (CDA) plates were prepared by mixing 1.0% (w/v) colloidal chitin with 15 g of agar in a medium consisted of (Na_2HPO_4 6.0 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, yeast extract 0.05g and distilled water 1 L; pH 6.5). The CDA plate was spot inoculated with organism followed by incubation at 30°C for 7-10 days. Colonies showing zones of clearance against the creamy background were regarded as chitinase producing strains (Kamil *et al.* 2007). The colloidal chitin was prepared by following the method described by Roberts and Selitrennikoff (1988). 5 g of chitin powder was slowly added to 60 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 2 L of ice cold 95 % ethanol with rapid stirring and kept overnight at 25°C.

The precipitation formed was collected by centrifugation at 7000 rpm for 20 min at 4°C and washed with sterile distilled water until the colloidal solution became neutral (pH 7). The prepared colloidal chitin solution (5 %) was stored at 4°C until further use.

LIPASE PRODUCTION

Lipolytic activity of the strain was performed by allowing them to grow on spirit blue agar media. Lipase production by bacterium was assessed by the method of Marshall (1992).

PROTEASE PRODUCTION

Protease activity was detected on 3% (wt/vol) powdered milk-agar plates according to Walsh *et al.* (1995).

III. RESULT AND DISCUSSION

CHARACTERISTICS OF B. megaterium MICROSCOPIC OBSERVATION AND IDENTIFICATION

Bacillus megaterium (TRS 7) was studied under both light and scanning electron microscope. The BLAST query of 16S r DNA sequence of the isolate against GenBank database confirmed identity. The sequence was deposited in NCBI, GenBank database under the accession Nos. JX 312687.1 for *B. megaterium.*

LIGHT MICROSCOPY

Morphological observation of *B. megaterium* showed that *B. megaterium* was rod shaped, Gram (ve), with wavy cell margin, rough surface and opaque nature in density. *B. megaterium* also produced endospores (**Fig.1 A-D**).

SCANNING ELECTRON MICROSCOPY

Scanning electron micrograph also confirmed the structure of *B. megaterium*- larger rod shaped (size-2µm and width-8.5mm; **Fig.1 E-H**).

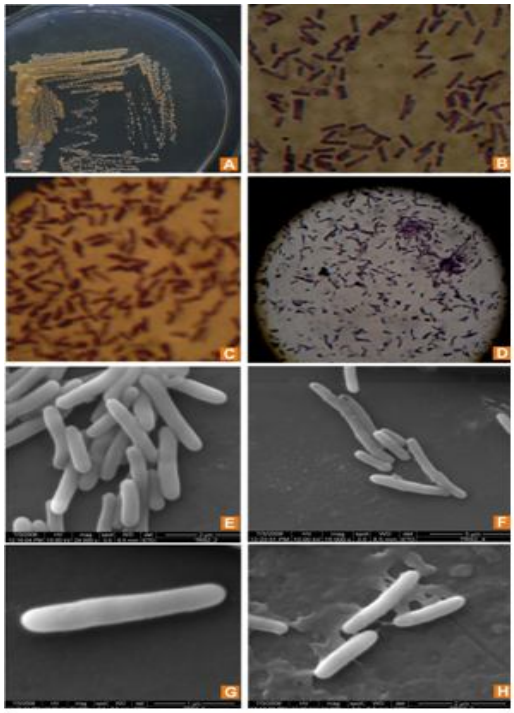

Figure 1: Light microscopic view (A-D) and scanning electron micrograph (E-H) of *Bacillus megaterium*

CULTURAL CHARACTERISTICS

INCUBATION PERIOD

The growth of *B. megaterium* was found to be best at 48 h. At 48 h (log phase) log cfu value of *B. megaterium* was found to be highest- 17.2 log cfu/ml broth.

pH

B. megaterium grew best at pH 6.0 with log cfu value of 22.8 log cfu/ml.

TEMPERATURE

The growth of the isolate was observed at different temperatures ranging from 20 to 50ºC. The isolate grew best at 35ºC with 18.81 log cfu/ml in case of *B. megaterium*.

Kobayashi *et al.* (2000) observed that *Bacillus cereus* isolate 96 and *Bacillus* pumilus isolate 235 have an optimal temperature for growth at 30ºC but survived even at 40 ºC and 50ºC respectively. Umamaheswari *et al.* (2003) also assessed the growth of different strains of fluorescent pseudomonads under different pH and temperature conditions. Optimum pH for the different strains ranged between 6 & 7.

***In vitro* PGPR ACTIVITIES**

SIDEROPHORE PRODUCTION

Siderophore production by bacterial strain was detected by growing the bacterium in chrome azurol S agar plate. The plate was observed for 10- 15 days after inoculation with bacterium. The appearance of yellow halo region was observed around *B. megaterium* which indicated that the bacterial isolate was able to chelate Fe^{3+} from chrome azurol S agar. The diameter of halo region was 1.8 cm after 12 days of incubation (**Table 1; Fig. 2A**).

PHOSPHATE SOLUBILISATION

Formation of clear zone around the colony grown in Pikovskaya's medium is an indication of phosphate solubilisation by rhizobacterium. In Pikovskaya's medium *B. megaterium* produced clear zone of 1.7cm diameter after 5-7 days of incubation, indicating that the isolate could solubilise insoluble phosphate (**Table 1; Fig. 2B**).

PROTEASE PRODUCTION

The bacterium was spot inoculated in skim milk agar medium and incubated at 30ºC for 5-7 days. The appearance of clear region was observed around *B. megaterium* which indicated that the bacterial isolate was able to produce protease. The diameter of clear zone was 2.8 cm (**Table 1; Fig. 2C**).

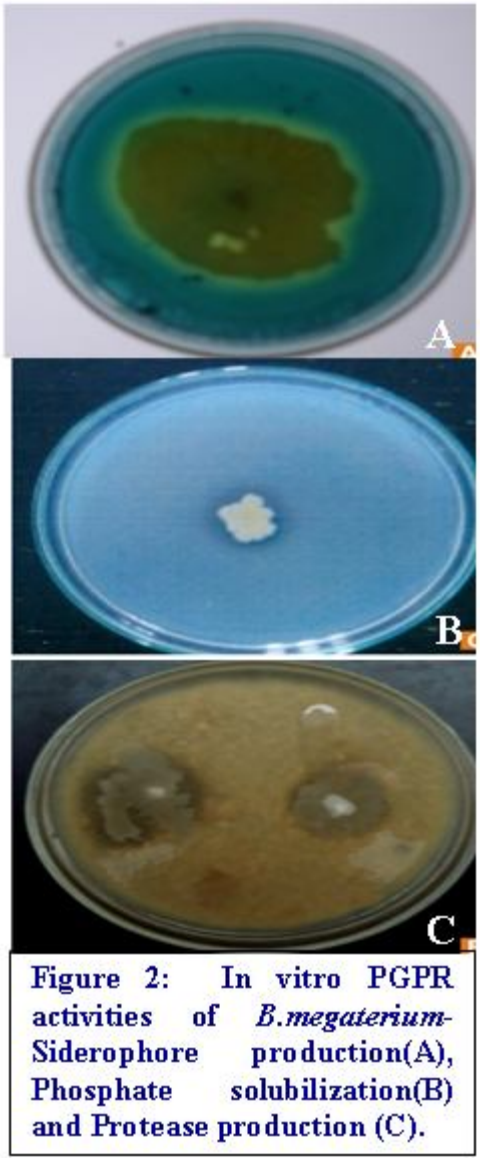

Figure 2: In vitro PGPR activities of *B.megaterium*- Siderophore production(A), Phosphate solubilization(B) and Protease production (C).

IAA PRODUCTION

The bacterial strain was assessed for the ability to produce indole acetic acid by growing in Nutrient Broth/ Luria Bertani Broth supplemented with tryptophane (0.1 mM). For quantification, HPLC analysis of IAA from *B. megaterium* was done by injecting 10µl of the filtered extracts onto a (C18,5µm 25×0.46 cm) in a chromatograph equipped with a differential ultraviolet detector absorbing at 280 nm. Mobile phase was methanol: H_2O- 80:20 (vol:vol), flow rate- 1.5ml/min. Retention times for peaks were compared to IAA standard (peak at retention time of 2.5 min for IAA standard) and quantified. *B. megaterium* recorded IAA production of 0.05 mg/ml (**Fig. 3**). Khalid *et al.* (2004) also evaluated thirty isolates from the rhizosphere soil of wheat plants for their

potential to produce auxins *in vitro*. They designated four isolates as plant growth promoting rhizobacteria (PGPR) based upon auxin production and growth promoting activity. Huang *et al.* (2010) reported that volatiles produced from *Bacillus megaterium* YFM3.25 were characterized and had nematicidal activity against *Meloidogyne incognita*. It has also been reported by Ortiz-Castro *et al.* (2008) that plant growth promotion by *Bacillus megaterium* involves cytokinin signaling in *Arabidiopsis thaliana* and *P. vulgaris* seedlings. The role of cytokinin signaling in mediating the plant responses to bacterial inoculation was investigated using *A. thaliana* mutants lacking one, two or three of the putative cytokinin receptors CRE1, AHK2, AHK3 and RPN12- a gene involved in cytokinin signaling. Cytokinin receptors play a complimentary role in plant growth promotion by *B. megaterium.*

HCN PRODUCTION

To determine the ability of *B. megaterium* to produce hydrocyanic acid (HCN), the bacterium was grown in medium amended with glycine. *B. megaterium* was found to be non-cyanogenic in nature (**Table 1)**. Hydrogen cyanide (HCN) production by different bacterial species including *Pseudomonas aeruginosa*, *P. fluorescens* and *Chromobacterium violaceum* was observed (Siddiqui *et al.* 2003).

LIPASE PRODUCTION

B. megaterium showed negative response for lipolytic activity (**Table 1)**.

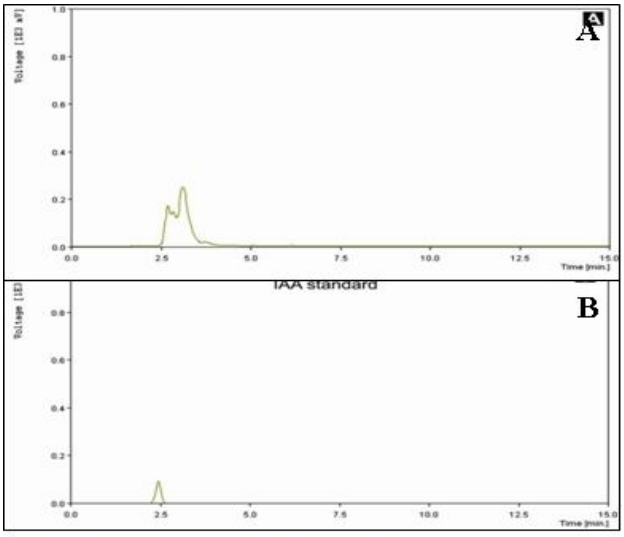

Figure 3: HPLC Profile of IAA from *B. megaterium* (A) with comparison to IAA standard (B)

Table 1: *In vitro* PGPR characteristics of *B. megaterium*

Characteristics	*B. megaterium*
Phosphate solubilization	+
Siderophore production	+
Protease production	+
Chitinase production	-
HCN production	-
Volatile production	+
IAA production	+

+= activity present; - = activity absent

IV. CONCLUSION

The major aim of this study was to study whether *Bacillus megaterium* (TRS 7) showed positive response in *in vitro* plant growth promoting attributes. Thus this bacterium as potent phosphate solubilizer (PSB), siderophore, IAA and protease producer isolated from tea rhizosphere, could be used as potent PGPR for increasing crop productivity in order to give much emphasis on use of eco-friendly biological materials for use in sustainable agriculture.

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REFERENCES

[1] Arkhipova TN, Veselov SU, Melentiev AI, Mertynenko EV and Kudoyarova GR. Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. *Pl. Soil.* **272**: 201-209, 2005.

[2] Chopade BA. Phosphate solubilisation by *Acinetobacter* spp. isolated from rhizosphere of wheat. *In: Proceedings of 6th International Workshop on Plant Growth Promoting Rhizobacteria,* Calicut, India. pp. 409, 2003.

[3] Dobbelaere S, Croonenberghs A, Thys A, Vande Broek A and Vanderleyden J. Photostimulatory effects of *Azospirillum brasilense* wild type and mutant strain altered in IAA production on wheat. *Pl. Soil.* **212**: 155-164, 1999.

[4] Huang Y, Xu CK, Ma L, Zhang KQ, Duan CQ and Mo MH. Characterisation of volatiles produced from *Bacillus megaterium* YFM3.25 and their nematicidal activity against Meloidogyne *incognita*. *Eur. J. Plant. Pathol.* **126**:417–422, 2010.

[5] Jagadeesh KS and Kulkarni JH. Mechanisms of biocontrol in the rhizobacteria of tomato, antagonisted to *Ralstonia solanacearum* E. F. Smith causing bacterial wilt in tomato. *In: Proceedings of 6th International Workshop*

on Plant Growth Promoting Rhizobacteria, Calicut, India. pp. 475, 2003.

[6] Kamil Z, Rizk M, Saleh M and Moustafa S. Isolation and identification of rhizosphere soil chitinolytic bacteria and their potential in antifungal biocontrol. *Glob. J. Mol. Sci.* **2**: 57-66, 2007.

[7] Khalid A, Arshad M and Zahir ZA. Screening plant growth promoting rhizobacteria for improving growth and yield of wheat. *J. Appl. Microbiol.* **96**:473, 2004.

[8] Kloepper JW and Schroth MN. Plant growth promoting rhizobacteria on radishes. IV. *International Conference on Plant Pathogenic Bacteria.* Angers. France. **2**:879-882, 1978.

[9] Kloepper JW. Plant growth promoting rhizobacterial as biological control agents. *In:* Soil *Microbial Ecology*-Applications in Agricultural and Environmental Management. Eds. Metting F.B.Jr., Marcel Dekker Inc., NY, USA, pp. 255- 274, 1993.

[10] Kobayashi K, Laura G, Ana V and Jorge, y Cozzi. Suppressive effects of antagonistic bacteria and metabolites on a pathogenic *Rhizoctonia solani* strain increased production in a specific medium. *Rev. Invest. Agrop*. **29**: 63-76, 2000.

[11] Lugtenberg BJJ, Dekkers L and Bloemberg GV. Molecular determinants of rhizosphere colonization by *Pseudomonas. Ann. Rev. Phytopathol*. **39**: 461-490, 2001.

[12] Marshall RT. Standard methods for the Examination of Diary products. 16th ed. *Am. Publ. Health Assoc*, Washington, DC, 1992.

[13] Martinez-Viveros O, Jorquera MA, Crowley DE, Gajardo G and Mora ML. Mechanisms and practical considerations involved in plant growth promotion by rhizobaceria. *J. Soil Sci. Plant Nutr.* **10**: 293 – 319, 2010.

[14] Ortiz-Castro R, Valencia-Cantero E and Lopez-bucio J. Plant growth promotion by *Bacillus megaterium* involves cytokinin signaling. *Pl. signal. Behav*.**3**: 263-265, 2008.

[15] Pikovskaya RE. Solubilisation of phosphorous in soil in connection with vital activity of some microbial species. *Microbiologia*. **17**:362-370, 1948.

[16] Prinsen E, Costacutra A, Michielis K, Vanderleyden J and Van Onckelen H. *Azospirillum brasilense* indole-3-acetic acid biosynthesis: evidence for a nontryptophan dependent pathway. *Mol. Plant-microbe. Interact*. **6**: 609-615, 1993.

[17] Reddy DM, Mohan BK, Nataraja S, Krishnappa M and Abhilash M. Isolation and molecular characterization of *Bacillus megaterium* isolated from different agro climatic zones of Karnataka and its effect on seed germination and plant growth of *Sesamum indicum. Res. J. Pharmaceu, Biolog.Chem.Sci.* **1**:614- 625, 2010.

[18] Roberts WK and Selitrennikoff CP. Plant and bacterial chitinases differ in antifungal activity. *J. Gen. Microbiol.* **134**: 169-176, 1988.

[19] Schwyn B and Neiland JB. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **160**: 47-56, 1987.

[20] Siddiqui IA, Shaukat SS, Khan GH and Ali NI. Suppression of *Meloidogyne javanica* by *Pseudomonas aeruginosa* I.E.-6S+ in tomato: the influence of NaCl, oxygen and iron level. *Soil. Biol. Biochem.* **35**:1625–1634, 2003.

[21] Umamaheswari C, Nallathathambi P and Dhandar DG. Factors influencing growth and fluorescent pseudomonads. *In: Proceedings of 6th International Workshop on Plant Growth Promoting Rhizobacteria*, 5-10 Oct. 2003. Calicut, India. pp. 455, 2003.

[22] Walsh GA, Murphy RA, Killen GF and Headon Power DR RF. Technical note: detection and quantification of supplemental fungal β-glucanase activity in animal feed. *J. Animal. Sci*. **73**: 1074-1076, 1995.