

Original Research Article

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Exploration of Novel Plant Growth Promoting Bacteria *Stenotrophomonas maltophilia* MTP42 Isolated from the Rhizospheric Soil of *Coleus forskohlii*

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ABSTRACT

The current exploration is anticipated for isolation and characterization of diversified plant growth promoting activities of coleus rhizobacteria and augmentation of coleus plant growth. An incongruous group of bacteria known as the plant growth promoting bacteria are found in the rhizosphere, they are seen at the root surfaces and in union with roots. Thereafter, they directly or indirectly improve the scope and quality in the growth of the plant. Though the mechanism of them whether directly or indirectly is not well understood, but its production of plant growth regulators viz., indole 3-acetic acid, gibberellic acid, indole butyric acid and their enmity against plant pathogenic microorganisms by bearing siderophores, antibiotics, phosphates and other nutrients. In this paper we reported the isolation of *Stenotrophomonas maltophilia* from rhizosphere of *coleus forskohlii* Briq. Procured from the district of Anand, Gujarat. The isolate was characterized for morphological and biochemical attributes and was identified as *Stenotrophomonas maltophilia* MTP42 on the basis of 16S-rRNA partial sequence analysis. The bacterial isolate MTP42 (KT428130) showed of inorganic phosphate solubilization (818ppm), Acid phosphatase activity (1.62 IU/ml), IAA production (93µg/ml), Ammonia production (80 µg/ml) and able to produce siderophore and HCN under optimized growth conditions and Trehalose, as carbon source. The following research revealed that *Stenotrophomonas maltophilia* MTP42 is a propitious plant growth promoting rhizobacteria with wide array of components. Leading report of using *Stenotrophomonas maltophilia* MTP42 as plant growth promoting rhizobacteria from *Coleus* rhizosphere and use of this isolate MTP42 as Plant growth promoting rhizobacteria attempts attractive way to replace chemical fertilizers and pesticides.

Keywords

Coleus, *Stenotrophomonas maltophilia*, Plant growth promoting rhizobacteria, MTP42, Rhizosphere.

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Introduction

The appellation Rhizobacteria represents a batch of rhizosphere bacteria capable of colonizing the root environment. The beneficial and plant growth promoting and root colonizing rhizobacteria are exemplified by diacritic traits: (1) root can be inhabited (2) they can grow in the habitats associated with the surface of the root and they can live

in competition with other microbes, and (3) plant development must be supported (Adesemoye, 2013). The soil or seeds when coated with PGPR invigorate the growth of the plant directly/indirectly by supplying nutrients or by decreasing the destruction caused by soil-borne plant pathogens (Ramadan, 2016). The foreboding of

rhizosphere was an perilous zone of soil surrounding the roots where microbe populations are fervent by root activities has been extended to include the soil surrounding a root in which physical, chemical and biological properties have been changed by root growth and activity (Halder and Sengupta, 2015). The bacteria are found to affect the plant physiology to a great extent, as they are the most abundant microbes in the rhizosphere and majorly causing changes in the root colonizing characteristics (Krogan, 2006). Those microbes are selected by the plant whose contribution is found most in releasing the organic compounds by creating a selective environment (Davey and O'toole, 2000). They are found to implant a positive impact on the plants by inhabiting the plant roots from unambiguous control mechanisms to an indirect effect (Ptak, 2015). Thus, plant growth promoting bacteria can be epitomized as the ones which are beneficial to the plants and the ones who inhabit the rhizosphere (Pielach, 2008). In recent times, plant growth promoting rhizobacteria have gained great importance as they are a bionetwork of bacteria serving as a crucifier in the biosphere (Kumar, 2007).

A diversity of bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to increase the plant growth (Tilak, 2005). The important traits of plant promoting bacteria are; Biocontrol (control of plant diseases), Biofertilizer (enhanced nutrient uptake), Bio stimulants (production of phytohormone), systemic resistance enhancement and production of antibiotics or siderophore (Saharan and Nehra, 2011). PGPB serves as a defence role by releasing heavy metal in the rhizosphere which suppresses the growth of pathogens as they lack nutrition (Siderophore), solubilise phosphate to increase the intake of

phosphorus, nitrogen fixation and antibiotic released by them reduces the growth of pathogens (Ramakrishna, 2014). Certain hormones like Auxin (IAA), gibberellins, cytokinins and ethylene are released by plant growth promoting bacteria which helps in promoting their growth. Indole acetic acid (IAA) microbially is enhanced by tryptophan which are released from the roots (Glick, 2012). *Stenotrophomonas maltophilia* is a known bacteria in the rhizosphere of corn, beets, cereal crops, cruciferous plants and are also found in the internal tissue of potato plants (Rania, 2016). *S. maltophilia* though does not control potato brown rot but it has been proven as an important biocontrol against fungal and oomycetous pathogens (Kumar and Audipudi, 2015). *Stenotrophomonas maltophilia* constrains the growth of *Rhizoctonia solani* and *Verticillium dahliae* in vitro, apparently because of antibiosis and provision of lytic enzymes (Narayanasamy, 2013). In the present research heterogeneous bacteria *Stenotrophomonas maltophilia* screened from the *C. forskohlii* rhizosphere has been characterized in terms of its plant growth promoting traits and Indole acetic acid production (Berg, 1999). The aim of this study was to assess the auxin production through chromatography and to investigate its plant growth promoting traits.

Materials and Methods

Site description of soil sampling and collection

Soil samples were taken in April 2014 from the rhizosphere of *Coleus forskohlii* which was procured from the irrigated fields of Anand district of Gujarat at latitude of 22.5645° N, 72.9289° E. The samples were taken in polythene bags and preserved at 4⁰ C and it was processed within 48 hours.

Enrichment and isolation of *Stenotrophomonas maltophilia*

A bacterium (MTP 42) was isolated from the rhizosphere of a medicinal plant *Coleus forskohlii*, on normal nutrient agar medium with glucose at pH 7, temperature 37⁰ C and with incubation period 48 hrs. The bacterial isolate was further characterized by its cultural conditions, morphological (Gram's staining) and biochemical characteristics (Bergey's Manual of Systematic Bacteriology).

Growth profile study

To examine growth profile of the isolate under liquid culture conditions, 100 ml broth in tryptic soybean broth (TSB), Hi media laboratories, Mumbai, India was used to inoculate the organism at 1% inoculum level. Viable cell count method was used to study the growth curve of the organism. 50 ml of Tryptic soybean broth was inoculated by early exponential phase culture to study the growth profile. The Mean growth rate constant (K) was calculated by withdrawing the isolate every 2 hrs:

$$K=3.322(\log Z_t - \log Z_0)/Dt$$

Where Z₀ and Z_t are the initial and final cell populations

While, Dt is the difference in culture time. (Pandey and Maheshwari 2007)

Plant growth promoting attributes

Phosphate solubilization activity

P solubilisation was checked using tricalcium phosphate as insoluble phosphate. Spot inoculation of the isolate was done in the centre of the Pikovskaya's medium amended with bromophenyl blue. The plates

were then incubated at 37⁰ C for 48 to 72 hrs. Phosphate solubilisation was checked in the form of a clear halo formed around the colony representing the production of organic acids as a possible mechanism of the phosphate solubilisation.

Quantitative P solubilisation was carried out in liquid Pikovskaya's medium in 250 ml flasks for 14 d. The concentration of the soluble phosphate in the supernatant was estimated every 7 d by stannous chloride (SnCl₂, 2H₂O) method (Gaur, 1990).

A simultaneous change in the pH was recorded. Phosphate solubilization activities were screened by measuring the clearing zone surrounding the developed bacterial colony via calculation of phosphate solubilization index:

$$\text{Phosphate Solubilization Index} = A/B \times 100$$

A= total diameter (colony+ halo zone)

B= diameter of colony

Assay for siderophore production

According to the methodology described by Gopalakrishnan, S., (2012), bacteria were streaked on the centre of Chrome Azurol S (CAS) agar media and incubated at 30°C for 48h. When the bacteria consume iron, present in the blue-colored CAS media, yellow-orange halos around the colonies indicate the incidence of siderophores. Percent decolorization (Siderophore Units) was calculated by using following formula

$$\% \text{ decolorization} = Ar - As \times 100$$

Where,

Ar = Absorbance of reference at 630 nm and

As = Absorbance of sample at 630 nm.

Quantitative estimation

One ml actively growing isolates with 0.5 O.D. at 600 nm inoculated in 50 ml of MM9 medium in 250 ml EM flasks. All flasks were incubated at 30^o C for 30h on orbital shaker. After 30 h, all cultures were centrifuged at 5,000 rpm for 20min. Supernatant was collected and tested for pH, fluorescence and siderophore production.

A simultaneous change in growth pattern of the isolate was also carried out. Catecholate types of siderophores were checked by Arnov's method (Arnows 1937) and for hydroxymate type of siderophores Csaky's method (Csakya, 1948) was used.

Hydrogen cyanide production

The production of Hydrocyanic acid (HCN) was detected by spreading 1 ml of 24 h old broth culture on nutrient agar slants and incubation of the slants with the Whatman filter paper flooded with the solution containing 0.5% picric acid in 2% sodium carbonate inserted in the tubes.

After 24-48 HCN production was checked on the basis of changes in colour from yellow to light brown, moderate brown or strong brown of the yellow filter paper strips.

Ammonia production

Ammonia production was estimated by Nessler's reagent. Freshly grown culture was inoculated into 4ml of peptone water and incubated for 48 h at 37^oC.

Broth was collected, centrifuged and 1ml Nessler's reagent was added to 1ml of supernatant and the volume of this mixture was made up to 10ml by addition of ammonia free distilled water. Development of brown to yellow color was a positive test for ammonia

production and optical density was measured by spectrophotometer at 450nm. The concentration of ammonia was estimated based on a standard curve of ammonium sulfate ranging from 0.1 to 1 μ mol ml.

Exopolysaccharide (EPS) production by the isolate

EPS production was studied in medium containing 5% sucrose as carbohydrate source (Modi *et al.*, 1989) 10 ml of culture suspension was collected after 5-6 days and centrifuged at 30,000 rpm for 45 mins. Add thrice the volume of chilled acetone.

EPS will be separated from the mixture in the form of a slimy precipitates. It was then collected on a predried filter paper. They were then dried overnight at 50^o C. Weigh the filter paper again after overnight drying.

The EPS produced will be shown in the increased weight of the filter paper.

Molecular Identification of Bacterial Isolate

Pure culture of MTP42 bacterial isolate was grown until log phase achieved and genomic DNA was isolated (Bazzicalupo, 1995). The amplification of 16S rRNA gene was done by using universal bacterial primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and 27F (5' - AGAGTTTGATCMTGGCTCAG-3') (Pandey, *et al.*, 2005). The PCR product was sequenced at Chromous Biotech Pvt Ltd. The sequences obtained were compared with those from the GenBank using the BLAST program and Phylogenetic trees reconstructions were obtained by the Neighbor joining method 1000 bootstrap replicates were performed to assess the statistical support for each branch in the tree (Tamura *et al.*, 2007).

Results and Discussion

Isolation and characterization

Out of the 72 bacteria isolated from the coleus rhizosphere, one of the colonies showed mucoid and water bubble morphology (plate 1A). On the basis of culture, morphological, biochemical and molecular characteristics, the bacterial isolate was identified as *Stenotrophomonas maltophilia* MTP 42 (Table 1; Fig. 1).

Growth profile study

Growth curve of isolated colony was determined by spectrophotometric method. Growth profile (Figure 2) of the isolate was determined by inoculating early exponential phase culture in 50ml of nutrient broth under aseptic conditions. Samples were withdrawn every 2 hours. Mean growth rate constant (K) was calculated using the formula: $K=3.322(\log Z_t-Z_0)/Dt$; Where Z_0 and Z_t are the initial and final cell populations, While Dt is the difference in culture time. It was a fast growing isolate. K value of MTP42 $0.87\pm 0.04\text{ h}^{-1}$. According to the result MTP42 is found to be the fastest grower and on the basis of its growth profile other PGPR parameters were designed.

Phosphate solubilising activity

Results shows that the isolate is a good Psolubilizer and it showed zone of phosphate solubilisation on solid Pikovskaya's medium after 4 days of incubation at $30\pm 2^{\circ}\text{C}$ (Plate 1B). In liquid medium the phosphate solubilization was observed as (40 $\mu\text{g/ml}$). The pH of the medium also showed a decrease from 7.1 to a maximum of 3.4 after 21 d in MTP 42. However, from the observation it is clear that no correlation could be established between the degree of P- solubilization and the final pH of the medium (Tank and Saraf 2003) (Table 2; Figs. 3 and 4).

Assay for siderophore production

Siderophore production by the isolate carried out on solid CAS blue agar showed a clear zone of decolorization representing iron chelation by the isolate in the medium. The zone of dye decolorization observed was 23 mm after 120 h and the siderophore production was 28 $\mu\text{g/ml}$ after 96 h respectively. Siderophore production reduced thereafter on further incubation up to 144 h. Hydroxamate type of siderophore production was seen from qualitative and quantitative estimation (Fig. 5).

Chandra *et al.*, (2007) reported production of 32 $\mu\text{g/ml}$ of hydroxamate type of siderophore by *M.loti* after 48 h of incubation. Production of siderophore results in siderophore mediated competition among the bacteria which further results into exclusion of siderophore non producer pathogens from the rhizosphere due to lack of iron depletion for sclerotia germination and hyphal growth. This was supported by Singh *et al.*, (2008) who showed that rhizosphere isolate *Bacillus subtilis* BN1 inhibited the growth of *M. phaseolina* upto 60%.

Hydrogen cyanide (HCN) and ammonia production by the isolate

Ammonia production was studied from 10th to 13th days of incubation as per metho given by dye (1968). Maximum concentration of ammonia production was observed in the isolate MTP42 and it was 43 $\mu\text{g/ml}$ (10th day) and 45 $\mu\text{g/ml}$ (11th day). Consecutive reading after 11th days of incubation showed that there was a decrease in ammonia production in all isolates. This continued till 14 days. Maximum ammonia production was seen on the 11th day after which there is a decrease in the ammonia production. Ammonia released by diazotrophs is one of the most important traits of PGPR's which benefits the crop (Kundu, 1987). This accumulation of

ammonia in soil may increase in pH creating alkaline condition of soil at pH 9-9.4. It suppresses the growth of certain fungi and nitrobacteria due to its potent inhibition effect. It also upsets the microbial community and inhibits germination of spores of many fungi (Martin 1982). Christiansen *et al.*, (1991)

have reported that level of oxygen limiting conditions. However, Joseph *et al.*, (2007) reported ammonia production in 95% of isolates of bacillus followed by pseudomonas (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45%).

Fig.1 The isolated and pure colonies of *Stenotrophomonas maltophilia*

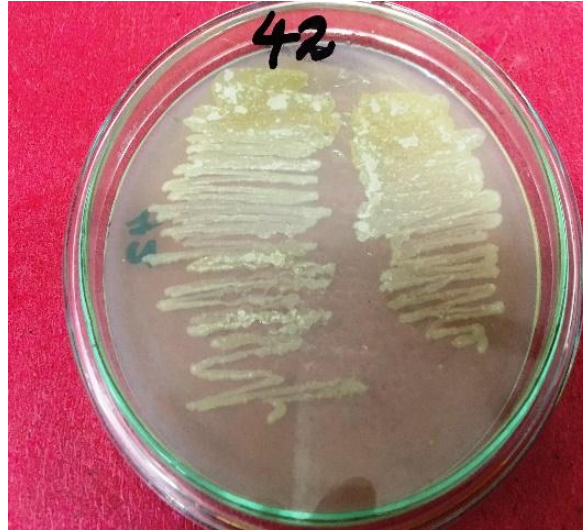


Fig.2 Logarithmic growth studies of MTP 42 isolate

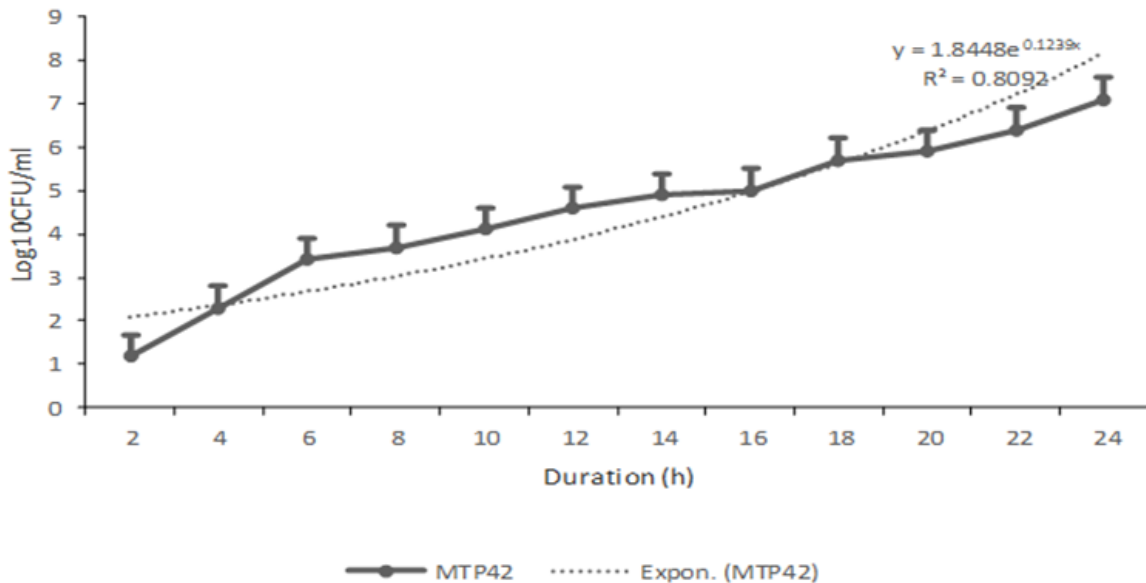


Fig.3 Phosphate solubilisation by *Stenotrophomonas maltophilia* MTP42

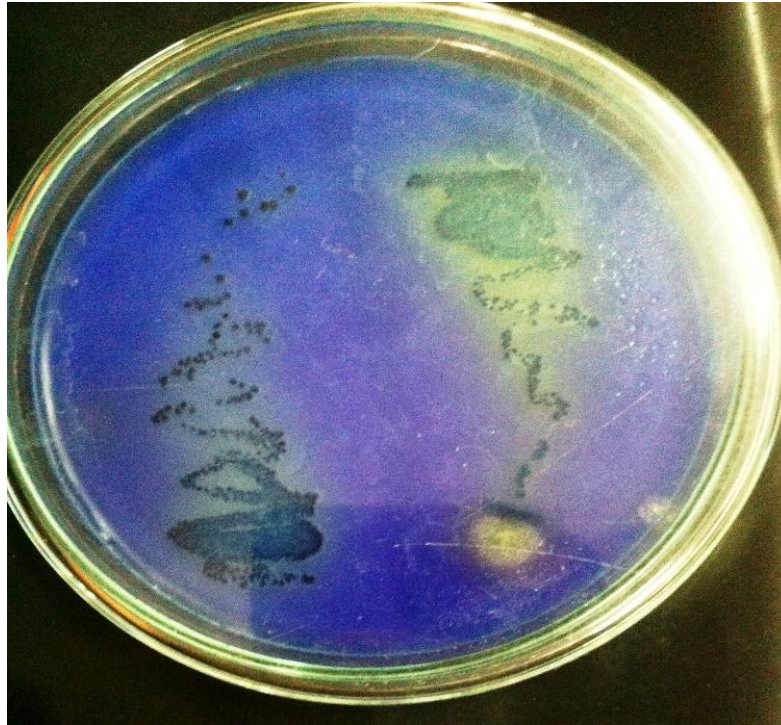


Fig.4 Phosphate solubilisation by *Stenotrophomonas maltophilia* MTP 42

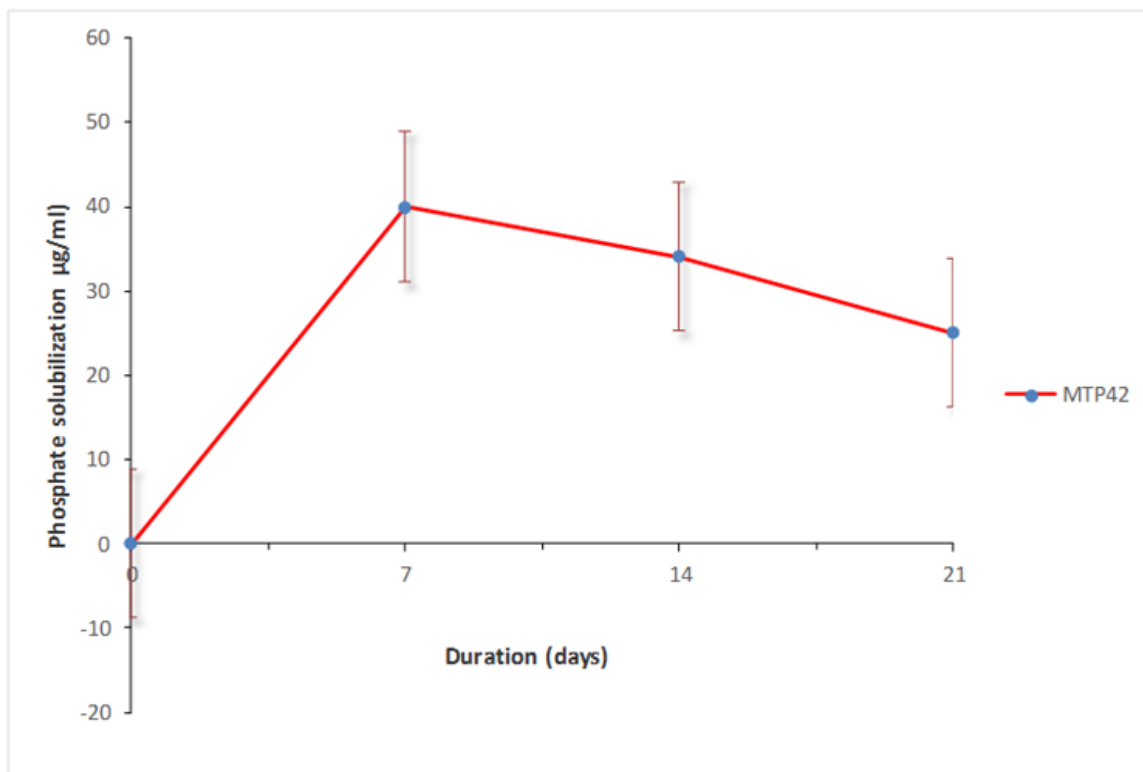


Fig.5 The siderophore production of the isolate MTP 42

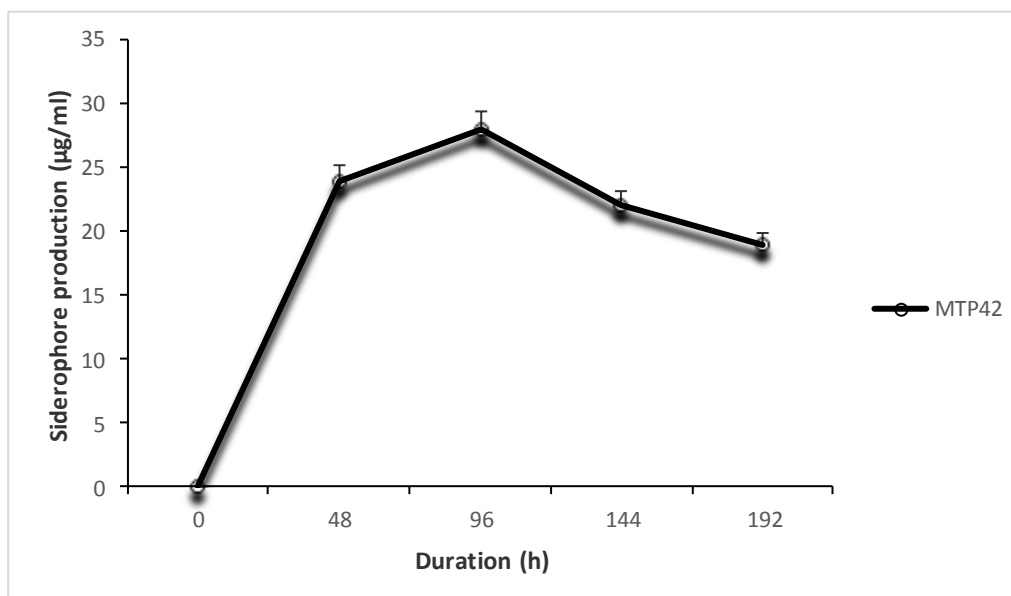


Table.1 Morphological, physiological and biochemical characterization of MTP42

Morphological and physiological characteristics								
Test	Morphology arrangement	Gram staining/Pigmentation	Motile	urease	starch	Glucose	lactose	sucrose
MTP 42	Rod and single	Pink and no pigmentation	-ve	-ve	-ve	+ve	+ve	+ve
Biochemical characteristics								
Test	oxidase	catalase	H ₂ S	Nitrate reduction	Indole	Methyl red	Voges proskuaer	Citrate utilization
MTP 42	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve

Table.2 Change in pH during P solubilisation upto 21st day after incubation

Isolate	0 day	7 th day	14 th day	21 st day
MTP42	7.1	4.54	4.24	3.4

HCN production was checked in all isolates which showed significant results in phosphate solubilisation and IAA production potential.

It showed HCN production after 42 and 72 h of incubation. It shows a significant potential against phytopathogens. Cattelan *et al.*, (2007) reported that production of cyanide

was an important trait in controlling fungal diseases in wheat seedlings under in-vitro conditions. Chandra *et al.*, (2007) reported production of HCN by the PGPR which was inhibitory to the growth of *S. sclerotium*. Kumar *et al.*, (2008) also reported in vitro antagonism by HCN producing PGPR against sclerotia germination of *M. phaseolina*.

Exopolysaccharide (EPS) production

The EPS produced by the isolate MTP 42 was observed to be (31 mg/ml) after five days of incubation. Sucrose was found to give better production of EPS as compared to other carbon sources. EPS production was higher during the early stationary phase compared to the late stationary of the isolate (Modi *et al.*,). Borgio *et al.*, (2009) reported three bacterial strains, *bacillus subtilis* NCIM 2063

After seven days of incubation the isolate MTP 42 showed high phosphate solubilization. 37°C temperature, 0.7%, NaCl (salinity), pH 7 and Glucose were identified as influencing factors for optimization of growth and maximum phosphate solubilization. In the present study MTP 42 showed significant production of ammonia and strong phosphate solubilization. This infers that MTP 42 isolate in the rhizosphere makes ammonia and phosphorus available to the plant by which nutritional needs of the plant can be fulfilled.

The morphological and biochemical analysis indicated highest (98%) similarity of the isolate with the genus *Stenotrophomonas* when compared with Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). In addition the phenotypic characteristic of this species correlate well with the molecular analyses based on 16s rRNA partial sequence analyses. Naz *et al.*, (2010) identified phosphate solubilizing bacteria belonging to genera *Stenotrophomonas maltophilia* by 16s rRNA.

Microbial PGPR has been implicated in the stimulation of growth or pathogenesis of plants. A diverse group of microbes, including soil, epiphytic and tissue colonizing bacteria have been found to synthesize IAA (Patten, 1996). In this study bacterial strains produced considerable amount of phosphate,

ammonia and HCN, which is comparable with earlier studies on various bacteria including *Pseudomonas* and *Stenotrophomonas* (Malik *et al.*, 1997). This study reports the isolation and characterization of the strain *S. Maltophilia* MTP 42 from the rich rhizosphere soils of *Coleus* plant confirming their plant growth potential. Bacteria from this genus was generally regarded as good phosphate solubilizers and as biofertilizers (deFreitas *et al.*, 1997). So it clearly reveals that apart from the normally encountered rhizosphere microflora: *Azospirillum*, *Azotobacter*, *Herbaspirillum*, *Klebsiella*, etc., other species may also possess diazotrophy. *S. Maltophilia* has an ambivalent character, first as a biocontrol and bioremediation agent and second as a multiresistant pathogen in nosocomial infections. There are numerous reports on the isolations from diverse rhizospheres. The clinical isolates are separated from the rhizosphere isolates by 16s rDNA analysis (Minkwitz, 2001). However it requires further studies on the virulence of the rhizosphere isolates before recommending it as a bioinoculant.

Conventionally, insoluble phosphates are chemically processed by reacting with sulphuric acid or phosphoric acid into soluble P. However, this process increases P fertilizer cost, and has environmental implications. In view of environmental concerns and current developments in sustainability, research efforts are concentrated on the development of a technique that uses phosphate solubilizing microorganisms to solubilize insoluble phosphates (Biswas, 2006). However, the mechanism of phosphate solubilization by microorganisms is also a subject of controversy today. Therefore, it needs further studies to understand the characteristics and mechanisms of phosphate solubilization by phosphate solubilizing microorganisms. Moreover, the role of phosphate solubilizing microorganisms on

plant growth under field conditions is also important and necessary to be studied.

It is expected that this report will prompt further screenings of phosphate solubilizing microorganisms so as to enhance agronomic value of soils and benefit crop growth. Evaluation of this isolate under the field condition and thorough investigation of *Stenotrophomonas maltophilia* MTP 42 use as a plant growth promoting rhizobacterial agent constitute future research. This shows that multiple potential of MTP 42 can help in plant protection and enhance plant growth.

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