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Research Paper

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Isolation and molecular characterization of *Enterobacter* species ENKS2 and its phosphate solubilisation from rhizospheric soil

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***Abstract*-** In this present study, the potential of a phosphate solubilising bacterium (PSB) was isolated from rhizosphere soil. The phosphate solubilisation was grown in Pikovskaya's agar medium and the isolate was purified in AT salt minimal medium and further it was cultured in NBRIP liquid media for characterization, identification processes. Based on the morphological, biochemical characterization and 16S rRNA gene sequencing the phosphate solubilising bacterium was identified as *Enterobacter* species ENKS2. The maximum phosphate solubilisation was in 96h at pH 7.3 in NBRIP liquid media. The *Enterobacter* species ENKS2 optimized phosphate solubilisation was at pH 7 (281µg/cfuX10^6), temperature at 30°C (268µg/cfuX10^6) at pH 6.8, salt conditions was 0.5% (169µg/cfuX10^6) at pH 7.1, carbon source xylose (213µg/cfuX10^6) at pH 6.3 and nitrogen source Pottassium nitrate (212µg/cfuX10^6) at pH 6.1. The different time intervals were followed to maximum phosphate solubilisation in 96h (531µg/cfuX10^6) at pH 5.8. Hence, this study was useful to develop the biofertilizer for improves the soil, plant growth and reduce the chemical fertilizer usages.

***Keywords*:** Phosphate solubilisation, Rhizospheric soil, *Enterobacter* sp., 16S rRNA gene sequencing, acid phosphates

I. INTRODUCTION

Soil nutritional was important and most significant to agricultural fields. Many essential nutrients present soil and also important for crop growth. Phosphorus is an essential macronutrient and "Master key" for crops growth and not only a development; it's also involved in metabolic pathways like photosynthesis, biological oxidation, nutrient uptake and cell division [1]. In worldwide, 40%-60% of crop yield utilize the limited amounts of P only availability so they preferred inorganic P as chemical fertilizers to support crop production but repeated use of fertilizers deteriorates soil quality [2]. The soil quality which is helpful for the increases of soil salinity, pH. In agricultural fields, 400 - 1200 mg/kg soil Phosphorus present. In plant and animal cycle <10% only enter into phosphorous cycle, others were locked by insoluble forms [3]. Phosphate fertilizers are very expensive than other fertilizers. So the phosphorus solubilising microbes are significant and important for phosphate reserves in soil. Many bacteria such as *Pseudomonas, Bacillus, Enterobacter, Azotobacter, Agrobacterium,Achromobacter, Rhizobium, Burkholderia, Flavobacterium* and *Micrococcus* isolated from soil in various countries have been reported to solubilise phosphorous [4,5,6]. The application of the phosphate-solubilizing bacteria which is help to convert the insoluble P to soluble forms of P and by release the organic acids, metal chelating activity and ion exchange [7,8,9]. The physicochemical (sorption-desorption) and biological (immobilization-mineralization) of soil P was characterized by many processes. High amount of P applied in fertilizers, that immobile precipitate react with Al3+ and Fe3+ in acidic, and Ca2+ in calcareous or normal soils [10, 11]. Microbes were assimilates the soluble form of P and prevent from adsorption or fixation [12]. Since 1903, many researchers tried the rhizospheric phosphorus solubilizing microorganism (PSM) and achieve some results. Bacteria are more effective in phosphorus solubilisation than fungi [13]. Among the whole P solubilizers,1-50% were fungi and 1-0.5% were highly potential, in bacteria 88% PSB present in North Iranian soil [14]. The ecto-rhizospheric strains from *Pseudomonas* and *Bacilli*, and endosymbiotic *Rhizobia* have been described as effective phosphate solubilizers and *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B.sircalmous*, *Pseudomonas striata*, and *Enterobacter* could be referred as the most important strains for phosphorous solubilisation [15,16]. The research paper considered to moves the isolation of *Enterobacter sp*. as PSB at high temperature , salt and alkaline pH and it was identified by biochemical characterization and 16S rRNA gene sequencing [17,18].

II. MATERIALS AND METHODS

Sample collection and Analysis

The rhizospheric soil sample was collected from Trichy district. The soil sample was collected based on 12cm depth and 5cm diameter. The soil sample was stored at -80°C. The soil sample was analysed by some modification methods. After stored soil sample was homogenised and suspended with in double distilled water in 1:2 ratio and centrifuged at 8000 rpm for 5 min. The supernatant was collected and the pH was measured. Then the total phosphorus was measured in air dried soil and extracted with mixture of concentrated H2SO4:HClO4; 3:2 ratio digestion using V2O5 as a catalyst. Again the sample was mixed with acid contents and placed on hot plate at 80°C [19]. Then sample was making up to 25ml with warm double distilled water. Then added the 1:20 ratio of 250mg activated charcoal at soil/ 0.5mol/l of NaHCO3 (pH 8.5) [20]. Then it was shacked well and incubated at 25°C for 1 h. The extract was filtered in Whatman No.1 filter paper and extract was acidified with concentrated H2SO4 (pH 5.0). After the clear extract was helps to determine the phosphorus by molybdophosphoric acid method [21].

Isolation and identification of phosphorus solubilising bacteria

The soil sample was added with 0.85% sterile sodium chloride solution. 100µl of sample was taken serially diluted and it was spread on Pikovskaya's agar medium and to avoid the fungal contamination added 50 µg/ml cycloheximide for isolation of phosphorus solubilising bacteria [22]. After inoculation, the plate was incubated at 37°C for 3 days. The selected bacterial isolate was purified on AT salt minimal medium [23]. Finally, NBRIP liquid media was suitable for characterized the phosphorus solubilising bacteria isolate [24].

Morphological and biochemical characterization

The phosphorus solubilising bacterial morphological characterization and cell shape was determined by gram staining under light microscope. The biochemical tests catalase, oxidase, urease and acid gas formation, utilization of citrate, glycerol were tested. Starch hydrolysis, tween 80, gelatine, chitin and pectin were analysed. The antibiotic sensitivity assay was tested against gentamycin, chloramphenicol and ampicillin [25, 26, and 27]. The utilization of glycerol and sorbitol were also studied and followed to the addition of 50 mg/l of tetrazolium chloride as colour indicator to the basal growth medium [28].

Molecular identification of phosphorus solubilising bacteria

The DNA isolation method was followed by minor modification methods [29]. The PCR amplification was using the primers 8F AGAGTTTGATCCTGGCTCAG and 518R: ATTACCGCGGTGCTGG [30] were commercially synthesized from Sigma Chemical Co., USA. The 50µl of PCR reaction mixture was prepared and the cyclic temperatures as follows; initial denaturation at 95°C for 5 min, followed by 30 cycles of incubation each consisting of 1 min denaturation at 94°C, 1.5 min annealing at 58°C, 2 min extension at 72°C and a final extension of 10 min at 72°C. the Amplified PCR products were resolved on 1% agarose gel. The -PCR product was purified using QIAquick@ gel extraction kit, QIAGEN). After, gel extraction the PCR products was sequenced by Eurofins, Bangalore. The sequence was finally submitted in NCBI gene bank. After the phylogenetic tree was construct by using the MEGA 7.0 software using the neighbour- joining DNA distance algorithm with 1000 bootstrap replicates.

Estimation of phosphate solubilisation

The Phosphate solubilisation was estimated by this method [31]. The phosphate solubilisation was determined on NBRIP medium. The isolate was inoculated in NBRIP medium and incubated at 30°C with a temperature controlled incubator shaker at 100 rpm. After incubation completed, the 5ml of culture was taken at different time intervals and monitored the growth rate, pH drop and soluble phosphorus level from the supernatant. This standard method was followed in APHA. During the exponential phase the Phosphate solubilization
was measured at 880nm [32].

Determination of acid phosphatase production

The acid phosphatase enzyme activity was determined by slight modification of this method [33]. The supernatant of bacterial culture was taken for enzyme activity. 2ml of bacterial universal buffer pH 6.5. Then add 1 ml of 0.25 mM disodium p-nitrophenyl phosphate (tetrahydrate) was mixed and incubate at 37 °C for 1 h. After incubation 1 drop toluene was added and stops the microbial growth. And additionally add 4 ml of 0.5 M NaOH and 1 ml of 0.5 M $CaCl_2$. Mix well. Then it was filtered in Whatman filter paper. Then it was measured UV–Vis spectrophotometer at 420nm. p-nitrophenol was used as a standard.

Optimization of phosphate solubilisation

The phosphate solubilising isolate produced acid phosphatase was carried out after 48 h incubation in NBRIP broth. And finally, different carbon sources (sucrose, xylose, mannitol, fructose, maltose and glucose) and nitrogen sources (ammonium molybdate, potassium nitrate, urea and ammonium sulphate). The salt tolerance was tested by using different concentrations of salt ranges 0.5%-2%. Different time intervals were also followed in phosphate solubilisation (24hrs- 120hrs).The acid phosphatase determination and optimization was carried out by the slight modification of Tabatabai and Bremner methods.

Phosphate solubilization in pot experiment

12 cm diameter x 14 cm height was measured in earthen wares. 300 g of sterile and unsterile soil was taken and the unsterile soil was inoculated by potential phosphate

solubilising bacteria. Then additionally add 5g of tricalcium phosphate, incubate the soil at 30°C and keep the moisture conditions. After seven days, from the pots the phosphorus was extracted and quantified by APHA method such as molybdophosphoric acid method.

III. RESULTS AND DISCUSSION

Isolation and identification of bacteria

The rhizospheric soil sample from Trichy district, the pH ranges of soil sample was 7.21 and the electrical conductivity ranges 1.0 to 2.0, indicates alkaline nature. This soil had a high amount of available phosphorous. The Phosphate solubilising bacteria well grown in NBRIP medium and it was characterized by morphological and biochemical analysis. The bacterium was gram negative and positive result for ureaase prodcution, citrate, acid gas formation and catalyse. The utilization of glycerol and sorbitol was positive. Further, the hydrolysis of starch, gelatine, casein was positive. The bacterium was resistance to Gentamycin, Chloromphenicol and Amiphicillin. The results were showed in Table.1.

Molecular identification and phylogenetic analysis of bacteria

The efficient phosphate solubilising bacteria was molecular characterized and identified as *Enterobacter* species ENKS2 (KU243131). The comparisons of 16S rRNA gene sequence data in Genebank using BLAST homology search was used to identify the generic level. The phylogenetic analysis of (Figure.1) *Enterobacter* species ENKS2 was closely related to *Enterobacter hommaechei* (100%), *Enterobacter cloacae* (99%), *Enterobacter ludwigi* (98%) similarity respectively.

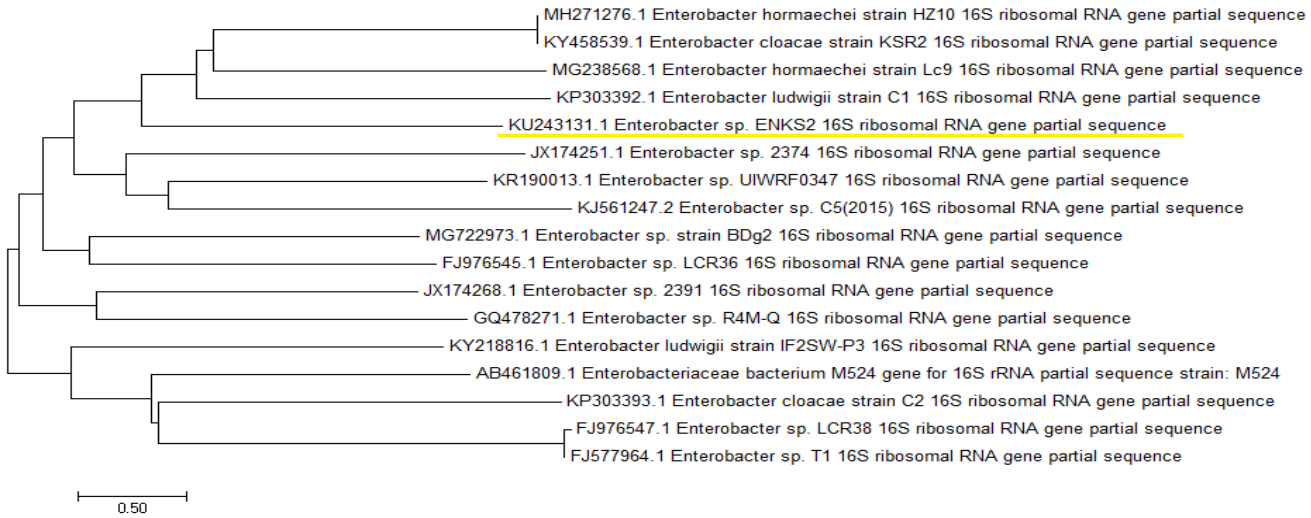

Figure.1 Phylogenetic analysis of *Enterobacter* species ENKS2 (KU243131).

Figure.2 shows the 16srrna secondary structure of *Enterobacter* species ENKS2 had 42 stems RNA folds. The free energy of the thermodynamic ensemble is -318.92 kcal/mol. The ensemble diversity is 101.75. Further molecular analysis was using the NEB cutter software 52 restriction enzymes were identified (Figure.3).

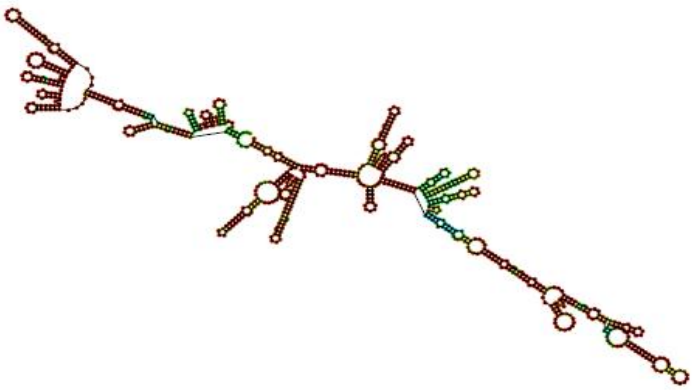

Figure.2 16srrna secondary structure of *Enterobacter* species ENKS2

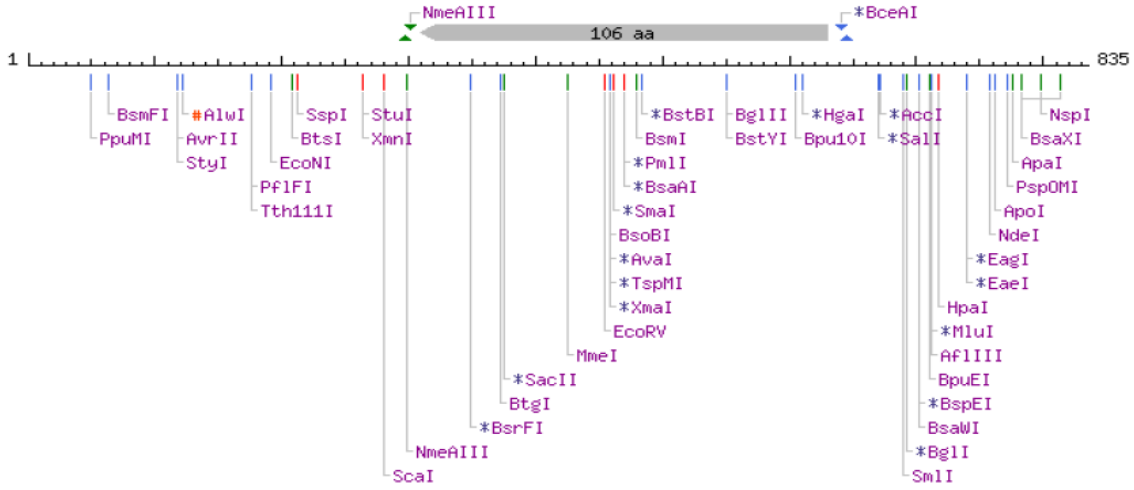

Figure.3 restriction enzymes analysis of *Enterobacter* species ENKS2

Phosphate solubilisation and Acid phosphatase estimation

Phosphate solubilisation isolate was determined on NBRIP medium. The maximum phosphorus solubilisation was estimated in 96h at pH 7.3. The acid phosphatase enzyme production was carried out on NBRIP medium at 96h at pH 7.1 (68.04U/ml). Further phosphate solubilisation was optimized by using various parameters. The phosphate solubilising bacteria was able grown in all temperatures, pH, all carbon sources, and all nitrogen sources and different time intervals. The maximum phosphate solubilisation effect was estimated at pH 7 (281µg/cfuX10^6) (Figure.4).

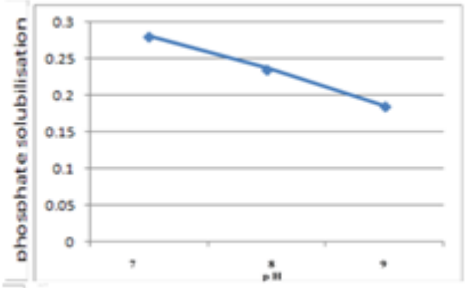

Figure.4 effect of phosphate solubilisation at alkaline pH

Figure.5 shows phosphate solubilisation on temperature at 30°C (268µg/cfuX10^6) at pH 6.8, a salt condition was 0.5% (169µg/cfuX10^6) at pH 7.1 (Figure.6).

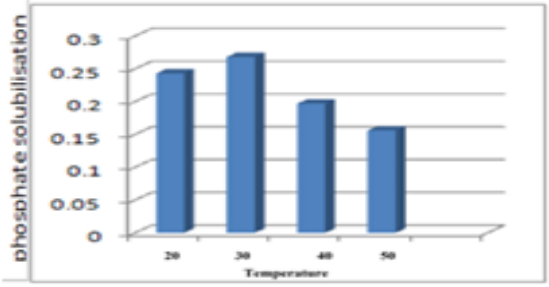

Figure.5 phosphate solubilisation on temperature at 30°C

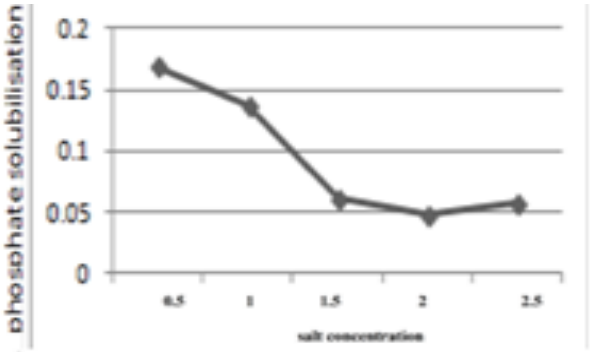

Figure.6 phosphate solubilisation on salt condition at pH 7.1

Figure.7 shows maximum phosphatase solubilisation was observed in xylose (213µg/cfuX10^6) at pH 6.3 and Pottassium nitrate (212µg/cfuX10^6) at pH 6.1 (Figure.8). Different time intervals are also followed to observe the phosphate solubilisation. Figure.9 shows the maximum solubilisation was observed in 96h (531µg/cfuX10^6) at pH 5.8.

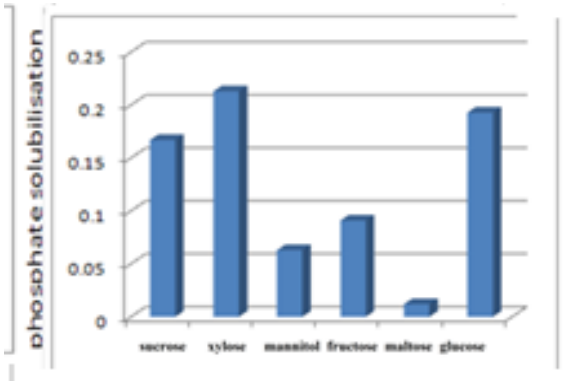

Figure.7 maximum phosphatase solubilisation in xylose

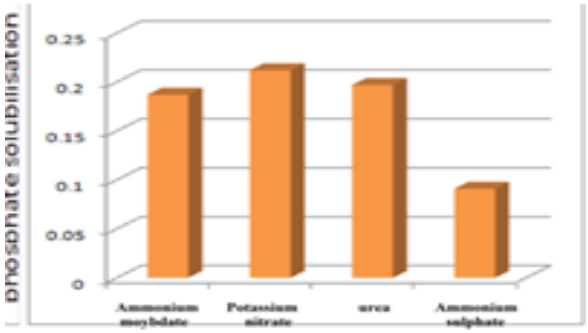

Figure.8 maximum phosphatase solubilisation in Pottassium nitrate

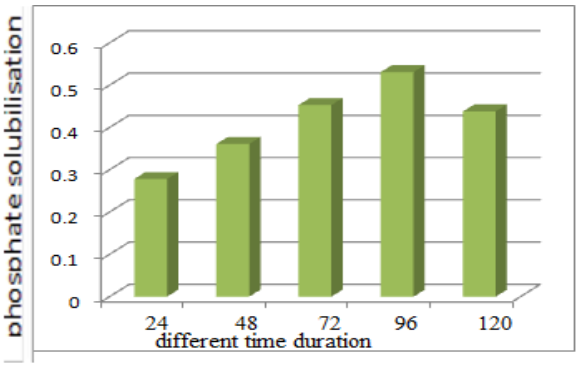

Figure.9 maximum phosphatase solubilisation in different time durations

Phosphate solubilisation in pot experiment

The *Enterobacter* species ENKS2 pot experiments was demonstrated the solubilisation of phosphate in sterile and non sterile soils. Figure.10 shows that (282µg/cfuX10^6) at pH 6.8.

of phosphate solubilisation was estimated in sterile soil and non sterile soil phosphate solubilisation was (308µg/cfuX10^6) at pH 7.3.

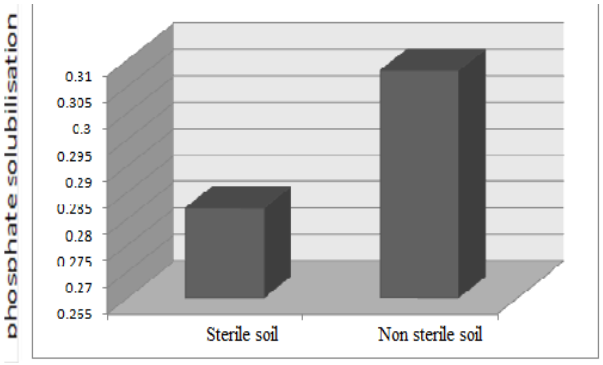

Figure.10 Phosphate solubilisation in pot experiment

IV. CONCLUSION

The present study investigated highly potential phosphate solubilising bacteria was isolated in rhizosphere soil. The phosphate solubilisation was important to plant growth and IAA production and reduce the chemical fertilizers and also improves the soil quality for further plant cultivations. Therefore in future the strain was *Enterobacter* species ENKS2 may consider as bio fertilizers.

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