SUMMARY

ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBILIZING MICROORGANISMS SUITABLE AS BIOFERTILIZERS IN ALKALINE SOILS

Nitrogenous and phosphatic fertilizers have played a major role in the green revolution that India has seen in recent years. However, the excess use of chemical fertilizers has adversely affected soil composition leading to diminishing soil fertility. Additionally up to 90% of the added phosphatic fertilizer gets precipitated and thus is not available for plants. This has resulted in the accumulation of insoluble phosphates in soils. Therefore there is an urgent need to direct efforts towards harvesting the accumulated phosphate resources, by means of biofertilizers, to supplement, if not substitute, the use of phosphatic fertilizers. In recent times there has been a growing awareness of employing the microbial plant growth promoting activity in the rhizosphere to reduce the chemical applications for increasing the crop yields. As compared to the work done in understanding the biological nitrogen fixation and using nitrogen fixing bacteria as biofertilizers, the understanding of microbial phosphate solubilization is very meager.

Even though phosphorus is present in significant amounts in soils, it is one of the major limiting plant nutrient because of its insolubility. Alkaline soils contain inorganic and organic phosphates in unavailable form complexed with calcium (CaP), ferric (FeP) and aluminum (AlP). While the soil solution contain, on an average, most

mineral nutrients in millimolar amounts, phosphorus is present only in micromolar or lesser quantities.

Microbial phosphate solubilization has been long envisaged as an alternative means of providing these unavailable phosphates to the plants. Many Phosphate Solubilizing Microorganisms (PSMs) were isolated from soils, which were able to solubilize CaP complexes under laboratory conditions mainly by decreasing the pH of the medium of inoculation. PSMs are abundant in the rhizosphere of many plants, their numbers were estimated in the range of 10^7. It is paradoxical that P availability is a major factor limiting the plant growth in spite of high abundance of PSMs in the rhizosphere.

Surprisingly, PSMs isolated thus far have not shown consistent results in enhancing P uptake and plant growth in field trials. These variations are attributed to the differences in the composition and properties of soils, the nature and distribution of microflora, and the type of the crop. Since there is also no direct evidence of P release from soil by the PSMs, doubts have been expressed regarding the P solubilizing abilities of these PSMs under soil conditions. Additionally, not much is known about the biochemical and genetic basis of acid secretion and P solubilization. The PSMs have been screened, so far, based on their ability to solubilize CaP complexes, which are acid soluble using unbuffered media conditions. On the contrary, soils rich in calcium phosphate complexes have a good buffering capacities. Hence it could be possible that all the PSMs may not solubilize P under soil conditions.

In view of the above facts, the objectives of this work were (i) to find out the effectiveness of PSMs isolated under unbuffered media conditions to release P from CaP rich vertisol soil; (ii) to find out the effect of buffering of the media on the P

solubilizing property of the PSMs; (iii) to determine the efficacy of various organic acids in releasing P from alkaline soils; (iv) to isolate and characterize PSMs which could be effective in solubilizing P from alkaline soils and (v) to clone *mps* genes which would result in high organic acid secretion.

Two Phosphate Solubilizing (PS) bacteria were selected to determine their ability to release P from alkaline soil, (1) *viz. Citrobacter koseri*, a commercially supplied PS bacteria and *Bacillus coagulans*, a PSM isolated by Dr. T.K.S. Gowda, GKVK, Bangalore. These PS bacteria were grown with soil as the sole P source in the presence of 100mM glucose and 10mM ammonium chloride as carbon and nitrogen sources respectively. Both these PSMs could grow to high numbers but failed to decrease the pH as well as release Pi from soil. This indicated that either these PSMs are ineffective in releasing P or the solubilized P was getting reprecipitated. To avoid reprecipitation of the released P, these PSM were added to soil at 10^9-10^{10} cfu/ml and the pH drop and Pi released was monitored at shorter time intervals. Both the PSMs could neither release P nor reduce the pH of the soil. This showed that these PSMs were ineffective because of their inability to reduce the soil pH even when the carbon and nitrogen source were not limiting.

We further monitored the ability of these PSMs to grow on media containing hydroxyapatite or rock phosphate as sole P sources and reduce the pH of the medium in unbuffered and buffered media condition. Both PSMs could reduce the pH under unbuffered condition leading to complete solubilization of mineral phosphate, but failed to do so when the media was buffered with 100mM Tris-HCl pH 8.0. The growth of these PSMs was very poor in buffered media condition containing insoluble rock phosphate as sole P source but became normal when 1mM Pi was supplemented

in the medium suggesting that under buffered condition the growth was limited because of the inability of the PSM to solubilize P.

The result suggested that buffering capacity of soil could play an important role in determining the ability of PSMs to solubilize P for the plants and gave credence to the doubts expressed earlier in this regard. The organic acid secreted by these PSMs were analyzed and the amount of various organic acids required to release P from the same soil were estimated. The results revealed that the concentration of the acids secreted were not sufficient to release P from alkaline soil. The soil required about 10-50 times more of these acids to solubilize P. For eg. the PSMs were secreting about 1mM citric acid but the soil required 20mM of citric acid to drop the pH less than 5. Similarly acetic acid and lactic acids were required at 100mM concentration to reduce the pH of the soil whereas it is known that 1mM lactic acid is sufficient to solubilize hydroxyapatite *in vitro* under unbuffered conditions. Citric acid and oxalic acids were found to be the most effective at 10mM concentration whereas 100mM lactic acid was required to release the same amount of P. Citric acid could also release Fe from soil indicating that PSM secreting citric acid could also help in alleviating Fe deficiency related chlorosis which is prevalent in some parts of India and is known to result in huge losses in crop yields. These results show the quantitative and qualitative differences in the ability of various organic acids to release P from alkaline soils. This indicates that effective PSMs in soils could be much less in number than previously estimated and, if so, provides an explanation for the apparent paradox that the plant growth is limited by P despite the abundance of PSMs in soils and rhizosphere. These results show the limitations of the normal screening procedure for isolating PSM and suggest the importance of employing buffered media condition for the screening of "effective" PSM.

In view of the above results, the "effective" PSM could either be screened from soils by employing buffered media conditions or the phosphate solubilizing property can be incorporated into any desired microorganism by genetic engineering. To test the efficacy of employing buffered media conditions for isolating "effective" PSMs, local soils were screened for PSMs which could grow on rock phosphate as sole P source and reduce the media pH under buffered condition. The buffering was done by adding 100 mM Tris-HCl pH 8.0 in the medium. Three bacteria were isolated which were able to grow on rock phosphate plates and reduce the pH of the media with either ammonium or nitrate as the nitrogen source. The pH reduction was monitored by the change in the color of methyl red from yellow to red. The acidification produced as a result of ammonia utilization was a contributing factor in unbuffered media but not in the buffered media as demonstrated by the diameter of the pH reduction zone produced by these PS bacteria. The acid secretion property in all the isolates was found to be repressed by the presence of (1mM) free P in the medium. These PSM, in contrast to the PSMs isolated using unbuffered media *viz C. koseri* and *B. coagulans*, were able to reduce the pH of vertisol thereby releasing Pi. All the three isolates have been provisionally identified as *Rhizobium* sp. These PSMs were found to secrete mainly gluconic acid at different concentration. Isolate 1 could secrete 100mM gluconic acid in 12 hr., isolate 2 could secrete 50 mM gluconic and isolate 3 was found to secrete 70mM gluconic acid. Succinic acid was also secreted in small amounts by all isolates. Di-calcium phosphate, precipitated *in situ* (traditional way) does not seem to be a good indicator of P solubilizing potential of the isolates as it is easier to solubilize as compared to rock phosphate. Also since precipitating DCP *in situ* results in availability of residual free P, the organisms which show phosphorus-starvation induced acid secretion, like the one isolated, here will give smaller zone of

pH reduction. Thus, this study demonstrated that buffering of the media while screening for PSMs can allow the selection of "effective" PSM.

Glucose dehydrogenase (GDH) of these isolates was induced 5 fold upon P starvation. Comparison of GDH activity with other plant growth promoting rhizobacteria (PGPR) indicated that these isolates were able to increase both the apo-protein and the cofactor PQQ in P deficient conditions which could be part of *pho* regulon of these bacteria. Mutants in Mineral Phosphate Solubilization (*mps*-) were obtained by transposon mutagenesis and were biochemically characterized. The mutations were in the gene of GDH, PQQ biosynthesis and regulation.

In an effort to understand the genetic basis of organic acid secretion a novel method of *phenotypic screening* was employed. *E. coli* was transformed with the genomic DNA library of *Synechocystis* PCC 6803 (present in high copy number plasmid and the transformants were selected based on their ability to solubilize mineral phosphate. Five independent clones were obtained which could solubilize rock phosphate with various carbon sources like glucose, mannitol and glycerol.

In conclusion, the results obtained in this work indicate that PSMs isolated thus far may not be effective in soils because of the buffering capacity of the alkaline soils. PSMs capable of releasing P from alkaline soils are less prevalent than earlier estimates. Three PS bacteria secreting high concentration of gluconic acid and capable of releasing P from alkaline soils were isolated using buffered media conditions and were biochemically characterized. Mutants in P solubilization of one of these isolates were isolated using transposon mutagenesis. 5 *mps* clones were obtained by a novel method of phenotypic screening in *E. coli*.