SUMMARY

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Solubilization of insoluble phosphate or silicate compounds by strains of Rhizobium and Bradyrhizobium were surveyed under laboratory conditions. Results indicated that root-nodule bacteria also are capable of solubilizing insoluble phosphate and silicate compounds like many other soil microorganisms. Initial screening carried out on solid medium containing insoluble hydroxyapatite or magnesium trisilicate exhibited transparent zone of solubilization around bacterial growth revealing the ability of phosphate and silicate solubilization by the strains. Assay of solubilizing principle following physical and chemical treatments of culture filtrates indicates that the process of solubilization is not mediated by enzymes but implicates the involvement of acidity developed in the cultures.

Quantitative estimation of soluble phosphate or silicate in culture filtrates after bacterial growth in ammonium sulphate yeast extract glucose (AYG) broth shows that most of the strains solubilize insoluble phosphate from hydroxyapatite or tricalcium phosphate and silicate from magnesium trisilicate, calcium silicate, silica gel or magnesite ore. Ability of solubilization varies among the strains and depending on the source of insoluble materials. The release of phosphoric acid or silicic acid in cultures was accompanied by a drop

of medium pH indicating production and/or generation of acids. High negative correlation coefficient values were calculated between the end pH of cultures and the amount of phosphate or silicate solubilized. However, quantitatively the strains released higher level of soluble phosphate than silicate in the cultures. In kinetic studies, measurements of soluble phosphate or silicate in cultures and of reduction of medium pH by Rhizobium and Bradyrhizobium also reveal a relationship between culture pH and phosphate or silicate solubilization. As the medium pH progressively decreased during bacterial growth there was a concomitant increase in the level of soluble phosphate or silicate in the culture.

Studies with representative strains, R. meliloti SU 47 and Bradyrhizobium sp. (Cicer arietinum) 27A15, reveal that glucose serves as the best carbon source for solubilization of phosphate or silicate while gluconate or tricarboxylic acid cycle intermediates, although, supported substantial growth of the organisms did not help in phosphate solubilization. Among the nitrogen sources, $(NH_4)_2SO_4$ caused highest reduction of medium pH vis-a-vis highest level of solubilization. Nitrate, tryptone, yeast extract or casein hydrolysate were highly effective nitrogen sources for phosphate solubilization by most of the strains.

Initial presence of soluble phosphate in the growth medium had no discernible influence on the extent of solubilization of phosphate by R. meliloti SU 47 and Bradyrhizobium sp. (Cicer arietinum) 27A15.

Addition of extra dosage of yeast extract or Mg to AYG medium enhanced phosphate solubilization. On the other hand, addition of calcium either as $CaCl_2$, $CaCO_3$ or $Ca(OH)_2$ caused reduced solubilization of phosphate when added initially to the growth medium or to stationary phase cultures growing on hydroxyapatite as source of phosphate. Fe and Mn although required in very low quantity as medium component, in higher concentrations inhibited phosphate solubilization. Presence of heavy metal ions also caused inhibition of bacterial growth and affected phosphate solubilization by R. meliloti SU 47.

Added EDTA and calcium specific chelator, EGTA, enhanced the extent of solubilization of phosphate from hydroxyapatite probably by the chelation of free calcium ion released during dissolution of hydroxyapatite.

In the yeast extract glucose (YG) broth also, the strains of Rhizobium and Bradyrhizobium, in the absence of $(NH_4)_2SO_4$, released soluble phosphate in the culture filtrate. Solubilization of phosphate was accompanied by a decrease in culture pH. A comparison of phosphate solubilization by the strains in AYG and YG media reveals that seven of the Rhizobium strains, BICC 635, CC 365, CC 511, BICC 630, BICC 631, BICC 632 and BICC 608 solubilized comparable amounts of phosphate from hydroxyapatite in both AYG and YG medium. But nine of the strains, BICC 601, SU 47, SU 216, CC 169, BICC 604, U 45, NZP 2213, BICC 620 and TAL 621, solubilized significantly higher level of phosphate in AYG medium than in YG medium while three

strains, SU 391, CC 224, and BICC 637, solubilized more phosphate in YG medium than in AYG medium. Similarly, 13 strains of Bradyrhizobium solubilized comparable amounts of phosphate in AYG and YG medium while three strains in AYG medium and three strains in YG medium solubilized phosphate more than in the counter medium. These indicate that separate mechanisms are involved for phosphate solubilization in the presence or absence of NH_4^+ in the two media and different strain has different capacity to use these mechanisms.

Glucose supports maximum phosphate solubilization also in YG medium as revealed in the cultures of R. meliloti CC 169 and R. leguminosarum biovar viceae BICC 635 but added mannitol, gluconate or succinate are of little value in respect of phosphate solubilization. NH_4^+ or NO_3^- supplement did not improve phosphate solubilization by the strains R. leguminosarum biovar viceae BICC 635, R. meliloti SU 47, SU 216 and BICC 604 but enhanced solubilization of phosphate from hydroxyapatite by the strain R. meliloti CC 169. Calcium as $CaCl_2$ adversely affected P solubilization in this medium while EDTA enhanced the release of phosphate from hydroxyapatite.

Most strains of Rhizobium and Bradyrhizobium solubilized phosphate from hydroxyapatite in the synthetic AG medium in absence of yeast extract using either $(NH_4)_2SO_4$ or KNO_3 as sole source of nitrogen. Presence of NH_4^+ than NO_3^- in the medium helped phosphate solubilization better except for the strain BICC 635 which solubilized comparable amount of phosphate in both the media. The differences in the solubilization of phosphate in presence of these nitrogen sources are

possibly due to involvement of different mechanisms in the development of acidity in the cultures.

Most of the strains solubilized phosphate also in soil extract-yeast extract broth and the strain BICC 635 was found to be superior to all the strains studied. Solubilization of phosphate was highly correlated with the drop of culture pH in this medium.

Chromatographic analyses revealed the presence of organic acids in the bacterial cultures. The nature of the organic acids produced varied among the strains. 2-ketogluconic acid and gluconic acid were identified to be present in the culture of Bradyrhizobium sp.(Cicer arietinum) 27A15 while in the culture of R. meliloti SU 47 citric and oxalic acids were also present along with 2-ketogluconic and gluconic acid in AYG medium containing hydroxyapatite. Phosphate solubilization by organic acids was evaluated in vitro and the results emphasized a major role of organic acids produced in culture in phosphate solubilization.

Ability of the strains of Rhizobium and Bradyrhizobium was tested for the solubilization of phosphate rocks. Mussoorie phosphate rock materials (P_1 and P_3) as models of low grade and Purulia phosphate rock materials (P_5 and P_6) as super grade variety were used for the purpose. Although phosphate rocks as compared to hydroxyapatite or tricalcium phosphate were less vulnerable to attack by the root-nodule bacteria the bacteria were able to release appreciable amounts of phosphate from phosphate rocks either in presence of $(NH_4)_2SO_4$ in AYG medium or in absence of it in YG medium. Among the strains of

Rhizobium and Bradyrhizobium the strain BICC 635 proved to have highest rock phosphate solubilizing ability. End pH of cultures and the amount of phosphate solubilized by the strains were statistically highly correlated and the correlation value was negative indicating that release of soluble phosphate from phosphate rocks is related to the acidity of cultures. Kinetic studies of the parameters of cultures pH and phosphate solubilization also supported the above findings. 2-ketogluconic acid was identified to be produced in the culture of R. leguminosarum biovar viceae BICC 635 grown on AYG medium containing Purulia phosphate rock-P_6 as source of phosphate. 2-ketogluconic acid efficiently solubilized rock phosphate in vitro. Extracted total organic acid from the cultures of R. leguminosarum biovar viceae BICC 635 in AYG and YG media containing Purulia phosphate rock-P_6 solubilized comparable amounts of rock phosphate in vitro, indicating that there is no large difference in the quantity of organic acid produced in the two media by the strain. The values were only slightly lower than that of bacterial solubilization of rock phosphate in the corresponding medium, indicating that organic acids play a major role in the bacterial solubilization of rock phosphate. In vitro studies with different concentrations of succinic acid to solubilize rock phosphate from the phosphatic rocks as well as from hydroxyapatite revealed that titratable acidity rather than pH alone was important for phosphate solubilization.

Initial presence of soluble phosphate did not affect dissolution of phosphate rock. Calcium in the form of $CaCl_2$, $CaCO_3$ or $Ca(OH)_2$

adversely affected the solubilization process while EDTA or EGTA enhanced the dissolution of phosphate rock.

Two strains of Rhizobium, BICC 635 and SU 47 selected arbitrarily for the purpose solubilized Purulia phosphate rock-P_6 in soil under laboratory condition.

Since increased crop productivity is as much dependent upon nitrogen availability as upon the availability of phosphate and nitrogen fixation has crucial relationship with available nitrate in the soil, the expression of the enzyme, nitrate reductase (NR), in R. meliloti was studied.

Initially a relationship between growth in yeast mannitol medium and the ability of cells of R. meliloti SU 47 at different growth phases to reduce nitrate to nitrite was established. NR activity, assayed using whole cells reached its peak during early stationary phase of bacterial growth. In YM medium nitrate was not required for appearance of NR activity which seems to be constitutive in nature, as such may be dissimilatory in function. Surprisingly, in YM medium supplemented with nitrate, NR activity of the cells was significantly lower than when the cells were grown in YM medium alone. Highest specific activity of nitrate reductase was ascertained when the YM medium grown cells were adjusted to a density of 2×10^9/ml. Addition of NH_4^+ or chloramphenicol to the assay medium containing nitrate as substrate did not affect the NR activity of the cells grown in YM medium confirming the constitutive nature of the enzyme.

When the cells grown in YM medium were assayed in potassium phosphate buffer, NR activity was comparatively lower than when assayed in YM medium. However, supplementation of carbon sources, viz., succinate, gluconate, mannitol, glucose or sucrose to the buffer caused enhanced NR activity.

Of the different carbon sources added to the growth medium, succinate was the best followed by sucrose, mannitol, gluconate and glucose for the enzyme activity. NH_4NO_3, $NaNO_3$ and KNO_3 tested as substrate for the enzyme were almost equally preferred for reduction while $PbNO_3$ resulted in diminished NR activity.

Presence of NH_4^+ or glutamine in YM medium caused decreased NR activity in comparison to control. In vitro studies with extracts of cells grown in YM medium supplemented with these fixed nitrogen also exhibited reduced activity as compared to that of the cells from control medium.

Cyanate enhanced NR activity when added either to the growth medium or to assay medium and probably acts as a modulator of NR activity. Addition of molybdenum to the growth medium promoted NR activity confirming the molybdoprotein nature of the enzyme while tungstate caused abolition of NR activity probably due to synthesis of tungstate analogue of NR. Supplementation of amino acids such as valine, aspartic acid, methionine and glutamic acid to YM growth medium did not affect NR activity appreciably, whereas, threonine inhibited the expression considerably.

During the growth in YM medium containing nitrate or nitrite under both aerobic and anaerobic conditions the strains R. meliloti CC 169, Rhizobium sp. (Cicer arietinum) BICC 632 and B. japonicum CC 709 exhibited gas production under both the conditions. The strains R. meliloti BICC 604 and Rhizobium sp. (Cicer arietinum) BICC 637 produced gas only during aerobic growth. Gas producing strains showed nitrite reductase activity even during aerobic growth in YM medium while R. meliloti SU 47 which does not produce any gas showed no nitrite reductase activity. However, the strain SU 47 exhibited both nitrate-and nitrite reductase activity when grown anaerobically in YM medium.

The expression of assimilatory nitrate reducing system in R. meliloti SU 47 was detectable in Sherwood's synthetic medium using nitrate or nitrite as sole source of nitrogen. Assimilatory NR activity of four strains of R. meliloti SU 47, SU 216, CC 169 and BICC 604 was exhibited in GTS/glutamate medium. The strains also exhibited nitrite reductase activity in the presence of nitrite. These follow that the enzymes were inducible in nature and required the presence of nitrate and nitrite respectively in the medium for the expression of assimilatory nitrate- and nitrite reductase activity. Both ammonium and chloramphenicol in the medium inhibited the expression of the enzymes confirming their inducible nature.