International Journal of Applied and Natural Sciences (IJANS)
ISSN(P): 2319-4014; ISSN(E): 2319-4022
Vol. 3, Issue 4, July 2014, 103-108

International Academy of Science, Engineering and Technology
Connecting Researchers; Nurturing Innovations

CHELATION EFFECT ON PHOSPHATE SOLUBILIZING ACTIVITY BY *CITROBACTER FREUNDII* MTCC 6738

PRAGYA RATHORE

Department of Biotechnology, Sanghvi Institute of Management & Science, Indore, Madhya Pradesh, India

ABSTRACT

Chelators are long known to enhance the phosphate solubilizing efficiency in soil. Production of organic acids is not the only mechanism of phosphate solubilization. As, soil is a good buffer medium, phosphate solubilization cannot occur only because of the production of acid and lowering of pH. Since the organic acids are known to bring about the solubilization of phosphorus either by lowering the pH or by chelating with calcium, the amount of phosphorus released can be considered to be largely due to the production of these acids. Thus apart from acidic environment the production of chelators like α – ketogluconic acid seems to play a major role in P solubilization from Tricalcium phosphate (TCP) and Udaipur rock phosphate (URP). The previous experiments with lower initial pH values have failed to show efficient P solubilization. In the present study the solubilization efficiency of the organism *Citrobacter freundii* in presence of different concentrations of EDTA was studied in order to know the effect of chelators on solubilization. Two phosphate sources Udaipur Rock Phosphate and Tri-calcium phosphate were inoculated with the organism and different concentration of EDTA and the resulting solubilization was compared with the control un-inoculated with EDTA.

KEYWORDS: Citrobacter Freundii, Chelation, Phosphate Solubilization, TCP and URP

INTRODUCTION

Phosphate solubilizers have been found to produce monocarboxylic acid (acetic, formic); monocarboxylic hydroxy (lactic, gluconic, glycolic); monocarboxylic keto (2-keto gluconic); dicarboxylic (oxalic, succinic); dicarboxylic hydroxy (malic, maleic) and tricarboxylic hydroxy (citric) acids in liquid media from simple carbohydrates (Rose, 1957; Sperber, 1957; Louw and Webley, 1959; Taha *et al*., 1969; Nahas, 1996 and Maheshkumar *et al.*, 1999).

Sethi and Subbara Rao (1968); Pareek and Gaur (1973) and Salih *et al.* (1989) have showed the release of phosphate from TCP and RP (rock phosphate) by organic acids. In the present study efforts were made to identify the type of organic acids produced by the organism. The acids produced were identified as tartaric acid and α - ketogluconic acid. α – ketogluconic acid is known to be the powerful chelator of calcium (Katznelson *et al*., 1962; Halder *et al*., 1992).

Since the organic acids are known to bring about the solubilization of phosphorus either by lowering the pH or by chelating with calcium, the amount of phosphorus released can be considered to be largely due to the production of these acids. Thus apart from acidic environment the production of chelators like α – ketogluconic acid seems to play a major role in P solubilization from TCP and URP as the previous experiments with lower initial pH values failed to show efficient P solubilization. For efficient solubilization initial good growth and simultaneous production of these organic acid seems to be a major requirement (Gyaneshwar *et al.*, 1998).

Several workers have reported the production of organic acids by organisms like *Pseudomonas fluorescens* (Dave and Patel, 1999), *Bacillus megaterium, B.circulans* and *Escherichia freundii* (Bajpai and Sundara Rao, 1971).

α - ketogluconic acid can solubilize phosphorus without concomitant decrease in pH as observed by Duff *et al*. (1962). Since the given organism produces α - ketogluconic acid, this chelator might be helpful in bringing about solubilization of phosphorus even under buffered condition. As the organism is capable of solubilizing sufficient amount of phosphorus under buffered conditions, it can be used as an inoculant in soil for phosphorus solubilization.

MATERIALS AND METHODS

Inoculum Preparation

A flask containing 100 ml of nutrient broth was inoculated with culture from Pikovskaya agar slant and incubated at 30 ± 2 °C for 48 hr. The cells were separated from the medium by centrifugation at 10,000 rpm for 20 minutes, washed twice with distilled water and resuspended in sterile distilled water such that it gave 1.0 O.D. at 660 nm. 1.0 O.D. suspension contained 8.7 x 10^9cells/ml. This suspension was used as an inoculum. All the steps were carried out under aseptic conditions.

Inoculation and Growth Conditions

Culture Media

- **Pikovskaya's broth:** Pikovskaya's broth containing tricalcium phosphate (TCP) equivalent to 50 mg % P_2O_5 was used.
- Modified Pikovskaya's broth :
- EDTA 1 to 5 mg/ ml was added to the Pikovskaya's broth medium containing TCP equivalent to 50 mg % P_2O_5.
- EDTA at 1 to 5 mg / ml concentration was incorporated in the Pikovskaya's broth in which TCP was replaced by URP equivalent to 50 mg % P_2O_5.

Solution of Chelator: 50 mg EDTA was dissolved in 50 ml distilled water.

Nutrient Broth: This medium was used to prepare inoculum.

The reaction of organic acids with TCP is one of acid dissolution, the amount of phosphate released depending primarily on the strength of the acid. When dibasic and tribasic acids were used, a secondary effect appeared due to the ability of these acids to form unionized associated compounds with Ca^{2+}, thus removing Ca^{2+} from solution and soluble phosphate concentration was increased, the effect was chelation. Calcium was chelated to a small extent with alpha- hydroxy aliphatic monobasic acid (lactic), more strongly with dibasic acids (malic and tartaric) and most strongly with a tribasic acid (citric). Among the dibasic aliphatic acids, the hydroxy derivatives formed the strongest complexes and alpha- substitution by the hydroxyl group of an aliphatic acid exhibited a greater effect than beta- substitution of the same acid. Chelation also occurred with the dibasic aromatic acid but did not occur to any appreciable extent with mono- basic aromatic acids.

RESULTS AND DISCUSSIONS

EDTA is a powerful chelating agent and the effect of different concentrations of EDTA on TCP and URP solubilization was studied. The TCP and URP solubilizations were enhanced with the addition of EDTA in the medium as

presented in table.

From Table 1 it is evident that the maximum TCP solubilization occurred on 2nd or 3rd day with all concentrations of EDTA. The phosphate from TCP was solubilized maximally (93.21 mg% P_2O_5) with 1 mg concentration of EDTA on 3rd day. Table 2 reveals that addition of EDTA caused increase in the URP solubilization. The maximum URP solubilization (20.79 mg %) was observed on the 6th day with 1 mg concentration of EDTA. Enhanced TCP and URP solubilization indicate that the addition of EDTA, a chelator of divalent cations, to phosphate solubilizing cultures increase the level of soluble Phosphorus. The pH remained acidic with all concentrations of EDTA. The maximum acidity was observed during URP solubilization on the 15th day with 2 mg concentration of EDTA. The maximum fall in the pH was 3.31 in TCP and 3.3 in URP solubilization. In this study it was observed that addition of EDTA enhanced the extent of phosphate solubilization. Similar results were obtained by Halder *et al.* (1990) when they tested the effect of chelation on P solubilization on the strain of *Bradyrhizobium.* Addition of increasing concentration of EDTA caused a linear increase in the dissolution of hydroxyapatite till complete solubilization.

Table 1: Effect of Different Concentrations of EDTA on TCP Solubilization by *C.Freundii*

EDTA (Mg /Ml)	Days of Incubation						
	1	2	3	4	5	6	7
	Mg % P_2O_5 * (Ph)						
0	60.47± 0.84 (3.98± 0.06)	67.77± 0.71 (3.81± 0.21)	72.1 ±0.02 (3.48± 0.05)	68.35± 0.61 (3.41±0.16)	50.67 ±0.20 (3.75±0.10)	48.8 ±0.34 (3.59±0.21)	27.7 ±0.41 (3.51± 0.05)
1	53.0±0. 61 (5.0±0. 27)	79.1 ±0.98 (4.81±0.34)	93.21±2.99 (4.65 ±0.11)	13.22±0.27 (4.10±0.05)	31.32±0.35 (3.91±0.14)	11.1±0.22 (3.81±0.20)	6.17± 0.36 (3.65± 0.25)
2	60.16± 0.79 (4.95 ±0.04)	71.2±0.48 (4.65±0.06)	69.98±1.23 (4.51±0.65)	53.32±0.57 (3.99±0.01)	47.6±0.79 (3.82±0.65)	23.02 ±0.15 (3.61±0.05)	10.11± 0.28 (3.55± 0.66)
3	40.2±0. 30 (4.56±0 .19)	51.35± 0.65 (4.42± 0.02)	40.98± 1.76 (4.13±0.02)	36.72±0.48 (3.86±0.09)	39.16±0.30 (3.64±0.20)	20.61 ±1.33 (3.52 ±0.19)	12.11± 0.44 (3.44± 0.50)
4	39.82± 0.22 (4.39±0 .09)	40.77± 1.23 (4.13± 0.90)	38.16 ± 0.52 (3.92± 0.04)	36.71±0.25 (3.51±0.10)	24.21±0.32 (3.31±0.21)	12.10±0.43 (3.42±0.02)	6.89± 1.07 (3.43± 0.12)
5	39.79± 0.22 (4.37 ±0.26)	40.66± 1.36 (4.12±0.14)	38.21± 0.58 (3.98± 0.20)	35.67±0.58 (3.52±0.02)	12.61±0.53 (3.42±0.10)	10.12±0.30 (3.30±0.09)	5.76± 0.32 (3.31± 0.05)

*=net P_2O_5 solubilized after deduction of respective control

Table 2: Effect of Different Concentrations of EDTA on URP Solubilization by *C. Freundii.*

EDTA (mg /ml)	Days of Incubation				
	3	6	9	12	15
	Mg % P_2O_5 (Ph)				
0	10.78±0.51 (3.62 ± 0.05)	16.13 ± 0.37 (3.61 ± 0.20)	10.11±0.30 (3.5 ± 0.12)	18.8± 0.89 (3.57± 0.04)	10.20± 0.20 (3.98± 0.20)
1	15.56± 0.53 (4.51± 0.04)	20.79± 0.31 (3.65 ± 0.41)	21.54± 0.89 (3.53± 0.04)	10.11±0.33 (3.5 ± 0.09)	19.55± 0.61 (3.33± 0.19)

Table 2: Contd.,

2	15.56± 0.53 (4.51± 0.04)	20.56± 0.61 (3.71± 0.05)	19.55± 0.61 (3.62± 0.34)	17.5± 0.37 (3.5± 0.20)	6.4± 0.42 (3.3± 0.04)
3	16.76 ± 0.41 (4.43± 0.12)	19.31± 0.49 (4.02 ± 0.20)	17.5 ± 0.37 (3.71± 0.40)	16.13±0.37 (3.61± 0.04)	8.26± 0.29 (3.41± 0.05)
4	17.21 ± 0.45 (4.41 ± 0.61)	18.8 ± 0.23 (3.89 ± 0.05)	19.5 ± 0.64 (3.61± 0.20)	15.5± 0.52 (3.59± 0.10)	14.6± 0.72 (3.5± 0.60)
5	17.21 ± 0.45 (4.41 ± 0.61)	19.55 ± 0.61 (4.21± 0.02)	19.5±0.64 (3.61± 0.20)	13.33± 0.39 (3.65 ± 0.20)	10.20± 0.20 (3.51± 0.10)

CONCLUSIONS

The presence of low levels of $Ca^{2+}$and EDTA in the medium enhanced phosphate solubilization (Nautiyal *et al,* 2000). The principal underlying mechanism of action of chelators is formation of unionized association compounds with Ca++, Fe++, Al+++ and thus, increasing soluble phosphate concentration by scavenging phosphate from mineral phosphates. EDTA, in case of control treatments, was found to increase progressively soluble P levels in the medium.

REFERENCES

1. Rose, R. E. (1957).Techniques of determining the effect of microorganisms on insoluble inorganic phosphates. N. Z. J. Sci. Technol. 38: 773-780.
2. Sperber, J. I. (1957). Solution of mineral phosphates. Nature. 180: 994-995.
3. Louw, H. A. and Webley, D. M. (1959). A study of soil bacteria dissolving certain mineral phosphate fertilizers and related compounds. J. Appl. Bact. 22: 227- 233.
4. Taha, S. M., Mahmoud, S. A. Z. , Halim, El- Damaty, A. and Abd. El- Hafez A. M. (1969). Activity of phosphate dissolving bacteria in Egyptian Soils. Plant and Soil. 31: 149-160.
5. Nahas, E. (1996). Factors determining rock phosphate solubilization by microorganisms isolated from soil. W. J. Microbiol. Biotechnol. 12: 567-572.
6. Maheshkumar, K. S., Krishnaraj, P. V. and Algawadi, A. R. (1999). Mineral phosphate solubilizing activity of *Acetobacter diazotrophicus*: A bacterium associated with sugarcane. Curr. Sci. 76 (7): 874-875.
7. Sethi, R. P. and Subba Rao, N. S. (1968). Solubilization of tricalcium phosphates and calcium phosphate by soil fungi. J. Gen. Appl. Microbiol. 14: 329-331.
8. Pareek, R. P. and Gaur, A. C. (1973). Release of phosphate from tricalcium phosphates by organic acids. Curr. Sci. 42: 278-279.
9. Salih, H. M., Yahya, A. I., Abdul- Rahem, A. M. and Munam, B. H. (1989). Availability of phosphorus in a calcareous soil treated with rock phosphate or superphosphate as affected by phosphate dissolving fungi. Plant and Soil. 120: 181-185.
10. Katznelson, H., Peterson, E. A. and Rouatt, J. W. (1962). Phosphate dissolving microorganisms on seeds and in the root zone of plants. Can. J. Bot. 40: 1181-1186.

11. Halder, A. K., Banerjee, A., Mishra, A. K. and Chakrabartty, P. K. (1992). Microbial solubilization of phosphate. Everymen's Science. 38-41.
12. Gyaneshwar, P., Kumar, N. G. and Parekh, L. J. (1998). Effect of buffering on the phosphate solubilizing ability of microorganisms. World J. Microbiol. and Biotech. 14: 669-673.
13. Dave, A. and Patel, H. H. (1999). Inorganic phosphate solubilizing soil Pseudomonads. Indian J. Microbiol. 39: 161-164.
14. Bajpai, P. D. and Sundara Rao, W. V. B.(1971). Phosphate solubilizing bacteria. Part 2. Extracellular production of organic acids by selected bacteria solubilizing insoluble phosphate. Soil Sci. and Plant Nut. 17: 44-45.
15. Duff, R. B., Webley, D. M. and Scott, R. O. (1962). Solubilization of minerals and related materials by 2- ketogluconic acid producing bacteria. Soil Sci. 95: 105-114.
16. Halder, A. K., Mishra, A. K., Bhattacharya, P. and Chakrabartty, P. K. (1990). Solubilization of rock phosphate by *Rhizobium* and *Bradyrhizobium*. J. Gen. Appl. Microbiol. 36: 81-92.
17. Nautiyal S.C. , Kumar P., Lal H., Mondal R., Verma D. (2000). Stress induced phosphate solubilization in bacteria isolated from alkaline soils. FEMS Microbiology letters. 182 (2): 291-296.