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Article

Application of Immobilization Technology in Solubilization of Rock Phosphate

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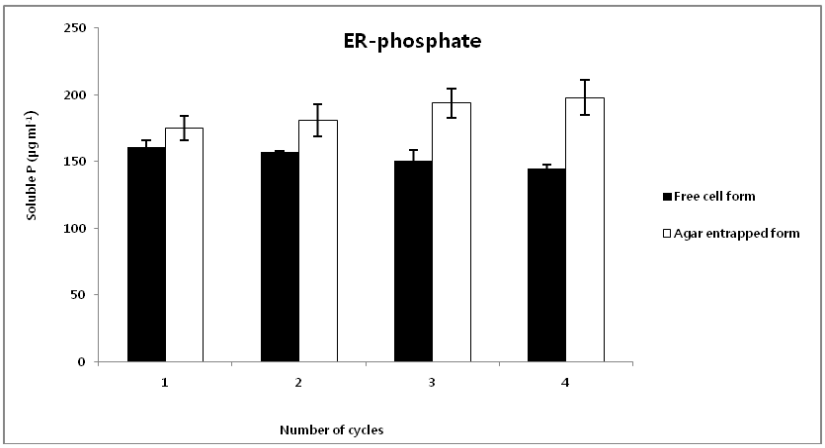
Phosphates solubilizing bacterial strains belong to *Pantoea*, *Burkholderia* and *Enterobacter* were isolated and employed in assessing their solubilization ability of Ca phosphate and ER phosphate (Eppawala Rock Phosphate). Among the bacterial strains used, PSB-13 (*Pantoea rodasii*) showed higher Ca-phosphate solubilization (1100 μg ml^{-1}) as well as rock phosphate solubilization (168 μg ml^{-1}). The strain was then immobilized in agar to further assess its phosphate solubilization ability. According to the results, agar encapsulated strain solubilized 0.3%, 7.31%, 20.24%, and 20.62% more Ca-phosphate and 11.53%, 15.29%, 28.48%, 36.55% (respectively in 4 cycles) more ER- phosphate than free cells. The reuse efficiency of agar entrapped bacterial cells for Ca-phosphate and ER-phosphate solubilization was greater than that by freely suspended bacterial cells. In conclusion, immobilization could enhance the phosphate solubilization capacity of the strains and thus could be used effectively in enhancing solubilization of ER phosphate.

Key words: *Pantoea rodasii*, Immobilization, Phosphate solubilization, Eppawala rock phosphate



Solubilization of ER phosphate by *Pantoea rodasii* free cells and encapsulated in agar during repeated batch fermentation. Values given here are the means (n = 3) ± standard deviation.

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Introduction

Phosphorus is an essential macro nutrient required by crop plants. As soil phosphorus deficiency may cause significant yield reduction, adequate supply of phosphorus is needed to ensure optimum growth and development of crop plants. Phosphorous supply depends exclusively on mined phosphates rocks (PR). As increased production cost is not affordable for farming communities in most of the developing countries, locally available RP deposits gain high recognition as economically viable source of phosphorous. However, due to diminishing resources, future supply of phosphate rock is predicted to be limited and more expensive to mine (MacDonald et al, 2011, Elser and Bennett, 2011, Cordell et al, 2011, Childers et al, 2011, Jasinski, 2010). Phosphorus is hard to be replaced by a synthetically produced fertilizer also, thus increasing scarcity of this finite resource is a matter of global concern.

An apatite bearing RP deposit has been discovered in Eppawala, Sri Lanka, (Jayawardena, 1989; Jayawardena, 1976). Total reserves are estimated as 60 million metric tons with an average grade of 33% P_2O_5 (Dinalankara, 1995). Solubility of this RP was however reported to be very low (6% P_2O_5 in 2% citric acid) making it difficult to convert into conventional water-soluble fertilizers. Several alternative methods have so far been proposed in making soluble phosphate fertilizer from this Eppawala RP (Tennakone, 1988; Tennakone et al., 1988). However, none of these procedures appeared to have been developed beyond the experimental stage. Therefore, usage of Eppawala RP is currently limited to the acid soils of the tea and rubber plantations in Sri Lanka along with imported RP from Egypt, Christmas Island and Israel.

Some microorganisms with varied potential to solubilize inorganic phosphates, also called phosphate solubilizing microorganisms (PSMs) have been found to exist in rhizosphere of plants. It has been demonstrated that the effectiveness, successfulness and safeness could be increased by applying phosphate solubilizing microorganisms as encapsulated cells (immobilized microbial cells) in biodegradable gel matrices (Vassilev et al., 2001). It releases respective microorganisms to the soil gradually and also helps survival by protecting them against extreme fluctuations of environmental conditions such as temperature, water content, pH, nutrient availability and potentially toxic pollutants (Jain et al., 2010). Therefore, application of PSMs as bio-inoculants in an immobilized state is received increasing attention recently (Jain et al., 2010; Vassilev et al., 2001). The present investigation was aimed at examine the effect of isolated PSMs on solubilization of inorganic phosphate (especially Ca phosphate and Eppawala Rock Phosphate-ER phosphate) and verifying the potential application of a selected strains in free and entrapped (agar) forms in *in vitro* condition.

Materials and Methods

Isolation of PSMs The phosphate solubilizing bacterial strains (*Pantoea* sp., *Burkholderia* sp., and *Enterobacter* sp.), which were isolated from tomato growing rhizosphere soil samples collected from green houses at Chungchugnam-do province, Gongju-Gun area in South Korea (Walpola and Yoon, 2013; Walpola et al, 2013) were used for preparing the immobilization cells. The pure culture of PSMs was maintained as a glycerol suspension (30% v/v) at -80°C and the colonies were obtained by restreaking on agar medium before use.

Culture medium for inorganic phosphate solubilization assay Ca phosphate (NBRIP-CaP) solubilization assay was performed using NBRIP liquid medium containing 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2.6H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of KCl and 0.1 g of $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 1999) while Eppawala RP (NBRIP − ERP) solubilization ability was assayed using rock phosphate having P_2O_5 content 33% instead of $Ca_3(PO_4)_2$ in NBRIP medium. This amount is equal to the amount of phosphorus in the standard NBRIP medium.

Inoculum preparation and inoculation For bacterial inoculum preparation, each single bacterial colony was transferred into 100 ml flasks containing 25 ml nutrient broth and grown aerobically on a rotating shaker (150 pm) for 48 hr at 30°C. The bacterial suspension was then diluted with sterile distilled water to a final concentration 10^8 CFU ml^{-1}, and resulting suspensions were used to inoculate sterilized 500 ml Erlenmeyer flasks (n = 3) containing 200 ml NBRIP medium. All inoculated flasks were incubated for 7 days with continuous shaking at 30°C. Sterilized un-inoculated medium served as a control.

Assay of inorganic phosphate solubilizing ability A 10 ml sample of each cultured and control were taken into centrifugation tube at 2, 5 and 8 days after inoculation and centrifuged for 10 min at 8,000 rpm. The clear supernatant was used to determine phosphorous release into the medium and to measure the medium pH. Phosphorous release into the medium was determined by phospho-molybdate blue color method (Murphy and Riley, 1962). The pH of the culture medium was also recorded with a pH meter equipped with glass electrode.

Culture medium for immobilization assay The growth medium contained 50 g of glucose, 5 g of $NaNO_3$, 1 g of KH_2PO_4, 0.1 g of $FeSO_4.7H_2O$, 0.5 g of KCl and 3 g of peptone in 1 L distilled water. The production medium used for the repeated batch cultivation contained 50 g of glucose, 3 g of $NaNO_3$, 0.5 g of KCl 0.01 g of $FeSO_4.7H_2O$, 2 g of peptone and tricalcium phosphate (Ca phosphate) or Eppawala rock phosphate (ERP) in 1 L distilled water. The amount of phosphate was

maintained equal to the earlier amount.

Inoculum preparation for immobilization Inoculum was prepared with 10^8 CFU ml^{-1} final cell concentrations as described earlier.

Immobilization of bacterial strain in agar For the immobilization of bacterial strain in agar, 3 ml of prepared inoculum having 10^8 CFU ml^{-1} was mixed with 3% nutrient agar and poured in a sterilized petri dish to form a 2 mm thin layer. After solidification, agar medium was cut into small pieces (3 mm X 2mm) using a sterilized sharp knife. The pieces were then dipped into olive oil for hardening. After 30 minutes, immobilized agar pieces were removed from olive oil and washed twice with sterilized distilled water before being used for further experiments. All the experiments were carried out under sterilized conditions.

Culture conditions and phosphate solubilization assay Agar blocks (150-160 agar blocks/5 ml) were transferred into 250 ml flasks containing 60 ml growth medium and was grown aerobically in flasks on a rotating shaker (150 pm) for 24 hr at 30°C. As free bacterial culture, 250 ml flasks containing 60 ml growth medium were inoculated with 150 µl of bacterial suspension (10^8 CFU ml^{-1} cell concentrations). Blocks were then separated by filtrations and washed with distilled water before being transferred to the production medium which consisted of different inorganic phosphate sources. Production medium was changed every 2 days and followed the same procedure as described above. Filtrate was used for the analysis of phosphorous release into the medium and pH change due to immobilized cells and free culture cells using phospho-molybdate blue color method (Murphy and Riley, 1962) and pH meter respectively.

Statistical analysis The data were subjected to analysis of variance (ANOVA) using SAS package (SAS, 1999). The Duncan's Multiple Range Test (DMRT) was applied to test the significance of treatment means at $P \leq 0.05$.

Results and Discussion

The phosphate solubilizing microorganisms used in the experiment were identified as *Panotea agglomerans* (PSB-1), *Burkholderia anthina* (PSB-5), *Enterobacter ludwigii* (PSB-8), *Enterobacter hormaechei* (PSB-10), *Pantoea rodasii* (PSB-13), *Burkholderia diffusa* (PSB-15) *and Burkholderia stabilis* (PSB-21) by Walpola and Yoon (2013).

By contrast, solubilization of phosphorous by the isolated bacterial strains was found to be relatively higher in tricalcium phosphate (Ca-phosphate) than that of rock phosphate (ER-phosphate). The highest phosphorous solubilization was recorded in NBRIP medium containing Ca-phosphate with PSB-13 (1100 µg ml^{-1}) followed by PSB-5 (1098 µg ml^{-1}) and PSB-15 (1092 µg ml^{-1}) isolates. In the case of ER-phosphate solubilization, the best performance was recorded from PSB-13 (168 µg ml^{-1}) followed by PSB-5 (140 µg ml^{-1}). As shown in Fig. 1 and 2, the solubilization was found to be high between day 2 and 5 of the inoculation.

Even though solubilization of Ca-phosphate was found to be higher than that of ER-phosphate, the reduction in pH of the medium was higher in ER-phosphate compared to Ca-phosphate (data not shown), which is analogous to Panhwar et al. (2009) who studied solubilization of Christmas Island RP (CIRP). The conversion reaction of glucose to gluconic acid is catalyzed by glucose oxidase enzyme under slightly acidic (pH above 4) conditions. The enzyme becomes inactivated at the pH below 2. Therefore, based on this, Panhwar et al. (2009) suggested that the lower solubilization of CIRP might be attributed to the lower pH of the medium during the incubation. In addition to adaptive nature of the glucose oxidase that is also responsible for their solubilization (Banik and Dey, 1983), Parasanna et al. (2011) have reported similar findings with isolated phosphate solubilizing microorganisms that solubilized Ca phosphate to a greater extent than rock phosphate, Al phosphate and Fe phosphate. Elaborating their findings, they have suggested that solubility of phosphorous might be associated with an activity of certain microbes in preferable phosphate sources or due to the activity of phosphatase enzyme.

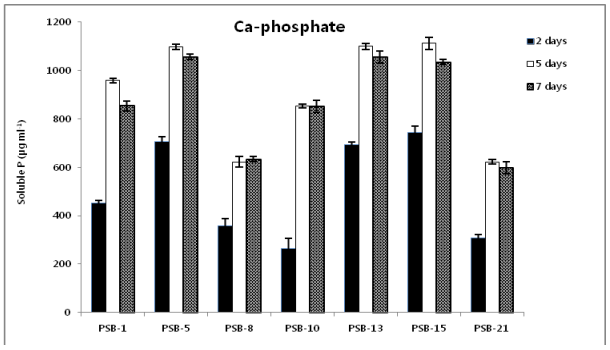

Fig. 1. Solubilization of Ca-phosphate by isolated phosphate solubilizing microorganisms during 7 days of incubation.

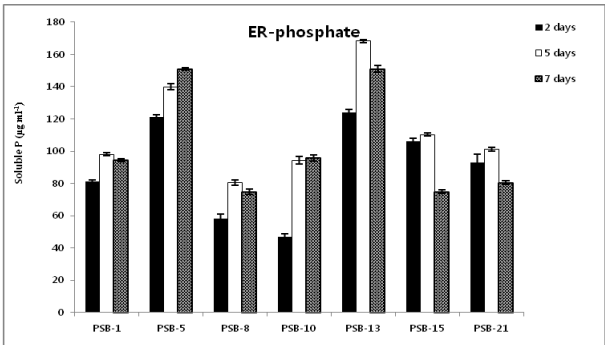

Fig. 2. Solubilization of ER-phosphate by isolated phosphate solubilizing microorganisms during 7 days of incubation.

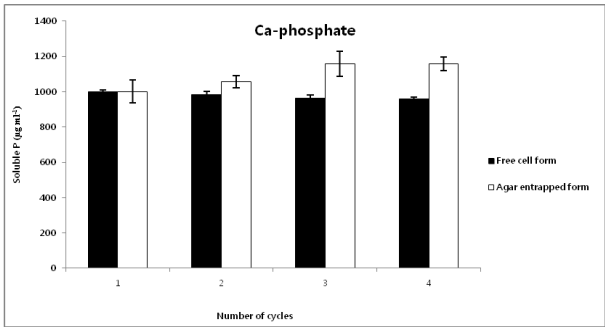

Fig. 3. Solubilization of Ca- phosphate by *Pantoea rodasii* free cells and encapsulated in agar during repeated batch fermentation. Values given here are the means (n = 3) ± standard deviation.

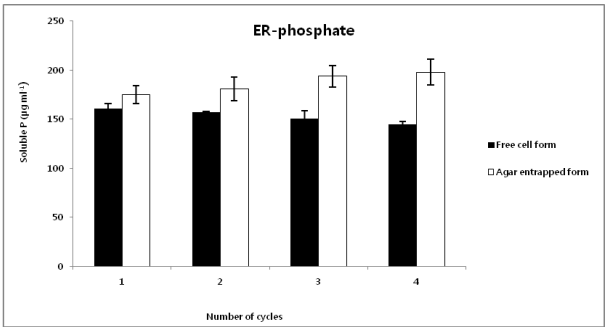

Fig. 4. Solubilization of ER phosphate by *Pantoea rodasii* free cells and encapsulated in agar during repeated batch fermentation. Values given here are the means (n = 3) ± standard deviation.

According to the present results, PSB-13 *(Pantoea rodasii)* released high amount of soluble phosphorous into the culture medium compared to other strains. Therefore PSB-13 was selected for further experiments. *Pantoea* sp. have previously also been identified as potential phosphate solubilizers (Deubel et al.2000; Son et al. 2006; Anandham et al. 2007).

As depicted in Fig. 3 and 4, phosphate solubilization of the bacterial strain immobilized with agar was found to be significantly higher ($P \leq 0.05$) than that of the free cells. Encapsulation of bacteria increased the Ca-phosphate and ER-phosphate solubilization during the 4 cycles implying that encapsulation also prolonged the durability of the bacterial in-oculums as previously reported by Saxena (2011).

Though no significant difference between free cell and agar encapsulated form was appeared in the 1st cycle, Ca-phosphate solubilization in free cell form started to decline significantly from the cycle 2 onwards. However, phosphate solubilization in agar entrapped form was continued to be increased significantly during the period. A similar trend of solubilization was observed with ER phosphate also. This may be attributed to the survival of bacterial cells from newly introduced environment and thereby reducing cell loss in immobilized form (Rekha et al., 2007). Immobilization provides protective environment with nutrient source (Saxena, 2011). Therefore it is obvious that an increased phosphate solubilization under such protective microenvironment is resulted. Furthermore this result could be explained by the fact that bacterial cells in inside the agar block did not exhibit any lag phase during the repeated batch cycles (Vassilev et al., 1997).

Respectively in 4 cycles, agar encapsulated bacteria solubilized 0.3%, 7.31%, 20.24%, and 20.62% more Ca-phosphate and 11.53%, 15.29%, 28.48%, 36.55% more ER- phosphate than free cells. The reuse efficiency of agar entrapped bacterial cells for Ca-phosphate and ER-phosphate solubilization was greater than that by freely suspended bacterial cells. Similar behavior of enhanced phosphate solubilization was reported with agar entrapped *Aspergillus awamori* (Jain et al., 2010), *Aspergillus niger* (Vassilev et al., 1996; Vassilev et al., 1997), *Penicillium variable* P16 (Fenice et al 2000), *Yarowia lipolytica* (Vassileva et al., 2000), *Enterobacter* sp. (Vassileva et al., 1999).

It is well known that phosphate solubilization mechanism is closely related with the production of low molecular weight organic acids such as gluconic acid, 2-ketogluconic acid, acetic acid, citric acid, lactic acid, propionic acid, glycolic acid, oxalic acid etc. (Ahmed and Shahab, 2011). Compared to freely suspended cells, phosphate solubilizing microorganism's immobilized cultures showed higher levels of acid production and phosphate solubilization (Vassilev et al., 2001). This was in agreement with earlier reports who observed similar close relationship between acid production and soluble phosphorous content in the medium for encapsulated *Yarowia lipolytica* (Vassileva et al., 2000), *Aspergillus niger* (Vassileva et al., 1998) and *Penicillium variable* (Vassile et al., 1996). As they stated, there may be direct mechanical effect (abrasion) of PR particles on the agar blocks and thus increase the acid production ((Vassileva et al., 1998). Similar abrasion effect was observed with Ca-phosphate on the surface growing fumaric acid producing *Rhizopus arrhizus* encapsulated in Ca-alginate by Petrucciolo and Angiani (1995).

Phosphate solubilizing immobilized bacteria possess a great potential to be used as bio-inoculants with insoluble PR sources to enhance the solubilization. Therefore PR deposit located in Sri Lanka (EPR) can be used as a source of insoluble phosphates for bio-inoculants. Some of the constraints associated with survival, stability, efficacy, storage and transportation etc could be arrested with the use of immobilized cells. However, further researches focusing at enhancing the immobilization procedure with use of various carriers are encouraged. Moreover, effectiveness of the introduction of immobilized PSMs as bio-inoculants into soil system needs to be assessed under field conditions.

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