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APPLICATION OF DIFFERENT IMMOBILIZED FORMS OF *Aspergillus Awamori* IN INORGANIC PHOSPHATE SOLUBILIZATION

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ABSTRACT

***Aspergillus awamori*, a phosphate solubilizing filamentous fungas, isolated from waste mushroom bed of *Agaricus bisporus* in South Korea was immobilized using various immobilized forms such as calcium alginate, agar, vermiculite and zeolite to assess the phosphate solubilizing potential in free and immobilized forms. Phosphate solubilization was carried out in repeated 48 hrs batch fermentation using the fungal strain. Among the four immobilized forms used, fungi immobilized with agar were shown to release the highest content of soluble phosphorus into the medium, followed vermiculite and sodium alginate. However phosphate solubilization of the fungal strain immobilized with zeolite was found to be very low. Thus, it can be concluded that a culture technique with immobilization especially with agar is more suitable for solubilization of inorganic phosphate by *Aspergillus awamori* than other forms.**

***Key words*:** *Aspergillus awamori,* calcium alginate, immobilization, vermiculite, Zeolite

INTRODUCTION

Phosphorus (P) is considered to be the second most important macro-nutrient for crops thus plays a key role in sustainable agricultural production systems. Compared to the other essential macro-nutrients (with exception of nitrogen), P is one of the less-abundant (0.1% of total) elements in the lithosphere, thus often regarded to be a limiting nutrient in agricultural soils (Jones and Oburger, 2011). Majority of the soluble phosphorus applied to soil as chemical fertilizers is rapidly converted into complexes such as calcium phosphate, aluminum phosphate and iron phosphate making them unavailable to plants. Crop plants can therefore utilize only a fraction of applied phosphorus, which ultimately results in poor crop performance. In rectifying this and maintaining soil fertility status, application of chemical fertilizers is frequently practiced, though it is a costly affair and also environmentally undesirable.

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Diverse group of soil microorganisms was reported to be involved in solubilizing insoluble phosphates (Phosphate Solubilizing Microorganisms–PSMs) making them available for plants uptake, particularly in soils with limited phosphorus supply. Though PSMs are naturally found in soils, their growth and phosphate solubilization highly vary with environmental conditions such as temperature, moisture content, pH, nutrient availability, salt concentration and availability of potentially toxic pollutants in the soils. Inadequate abundance of such organisms also makes difficulties in competing with other microbial species in the rhizosphere (Jain *et al.,* 2012). Therefore, inoculation of target microorganism at higher concentrations is needed to ensure successful solubilization of insoluble phosphates (Jain *et al.,* 2010). Furthermore, in protecting the inoculants from harsh environments, it may be desirable to use a carrier also.

Immobilization of microbial cells using various techniques such as covalent binding,

physical adsorption and entrapment etc., could increase the effectiveness, successfulness and safeness of the applying microorganisms (Vassilev *et al.,* 2001). It helps survival of microorganisms by protecting them against extreme fluctuations of environmental conditions. The present investigation was aimed at assessing the phosphate solubilizing potential of a filamentous fungus, *Aspergillus awamori* in various immobilized forms (free, entrapped and adsorbed forms) in *in vitro* condition.

MATERIALS AND METHODS

Isolation of phosphate solubilizing strain

The phosphate solubilizing fungal strain (*Aspergillus awamori* bxq33110) was isolated from waste mushroom bed of *Agaricus bisporus* in South Korea. The fungus was maintained on potato dextrose agar (PDA) slants at 4°C, sub cultured every month and its stability was checked periodically.

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 5 g tricalcium phosphate (Ca phosphate) in 1 L distilled water.

Inoculum preparation for immobilization

Spores from a 5-day old culture of the isolate on PDA were suspended in 0.1% solution of Tween-80 to a final spore concentration of 5×10^8 spores ml^{-1}.

Immobilization in sodium alginate

Three ml of spore suspension having 5 $x10^8$ spores ml^{-1} was mixed in 100 ml of 3% sodium alginate solution prepared in distilled water and then extruded in 250 ml of 0.5M calcium chloride solution with the help of syringe (syringe size 5 ml; needle size 0.56mm × 22 mm). Beads of 2–3 mm were allowed to harden for 30 min under gentle stirring at 30°C. These beads were further used for *in vitro* experiments.

Immobilization in agar

For the immobilization of fungal strain in agar, 3 ml of prepared spore suspension having 5 x 10^8 spores ml^{-1} strength 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 2 mm) 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.

Immobilization in vermiculite

Three ml of spore suspension having 5 $x10^8$ spores ml^{-1} was mixed in 5 g of washed autoclaved vermiculite.

Immobilization in Zeolite

Zeolite was twice washed with sterile 0.8% (w/v) NaCl solution and used for the immobilization of 3 ml of fungal spore suspension having 5 $x10^8$ spores ml^{-1}.

All the experiments were carried out under sterilized conditions.

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 conditions and phosphate solubilization assay

Each 5 ml sodium alginate beads (5 ml: 250-

260 beads) and agar blocks (5 ml: 150-160 agar blocks) were transferred into 250 ml flasks containing 60 ml growth medium and was grown aerobically in flasks on a rotating shaker (150 rpm) for 24 hr at 30°C. As immobilization on vermiculite, 75 mg of vermiculite and Zeolite were separately transferred into 250 ml flasks each containing 60 ml growth medium. Free mycelium cultivation was carried out in 250 ml flasks containing 60 ml growth medium were inoculated with 150 µl of spore suspension (5 x 10^8 spores ml^{-1}). Immobilized cells were then separated by filtrations and washed with distilled water before being transferred to the production medium which consisted of inorganic phosphate source. 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.

Statistical analysis

Data generated were subjected to analysis of variance procedure (ANOVA) for a Completely Randomized Design (CRD) with three replicates using SAS 9.1 software. Duncan's Multiple Range Test and Least Significant Difference at $P \leq 0.05$ was used to separate the means.

RESULTS AND DISCUSSION

Previous studies have demonstrated that some *Aspergillus* species posses the ability to solubilize inorganic phosphates, which subsequently results in increased availability of P for plant uptake (Yadav *et al.,* 2011; Srividya *et al.,* 2009; Relwani *et al.,* 2008). In the present study, we isolated *Aspergillus awamori* bxq33110 from waste mushroom bed of *Agaricus bisporus* and phosphate solubilizing potential of the strain was assessed in various immobilized forms. This fungal strain has been entrapped in both calcium alginate and agar, and immobilized by physical adsorption on vermiculite and zeolite.

As shown in figure 1-4, the fungal strain *Aspergillus awamori* was able to solubilize inorganic phosphate by free as well as immobilized state (except immobilized in zeolite) show the results of inorganic phosphate solubilization and pH change in the medium inoculated with the fungal strain both as free cells and immobilized forms. The results showed that though the highest phosphate solubilization was recorded from free cell form in the 1st cycle (Figure 1), it was started to decline significantly from the cycle 2 (Figure 2) onwards.

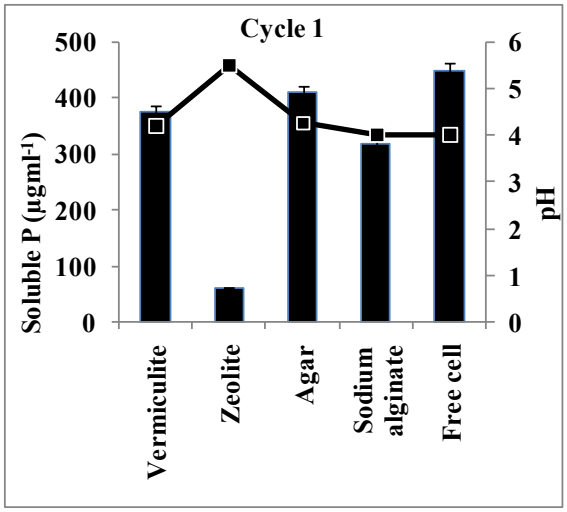

Figure 1: Changes in pH and soluble phosphorus content by immobilized fungal strain in cycle 1

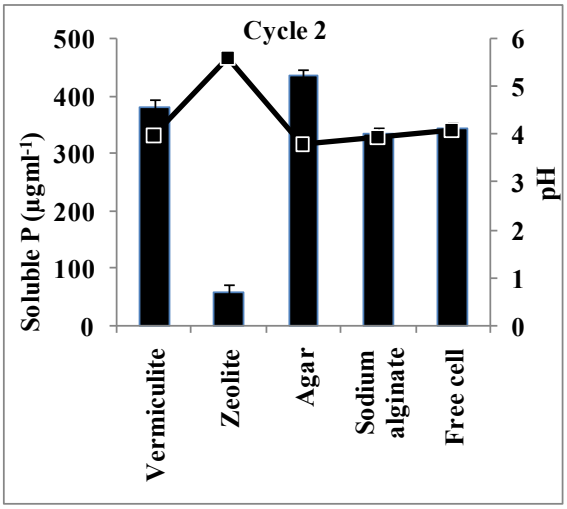

Figure 2. Changes in pH and soluble phosphorus content by immobilized fungal strain in cycle 2

However, phosphate solubilization in immobilized forms (except immobilized on zeolite) were continued to be increased significantly after the 1st cycle (Figures 2, 3 and 4). One of the reasons for this may be attributed to the survival in the newly introduced environment and thereby reducing cell loss in immobilized form (Rekha *et al.,* 2007). Immobilization provides protective environment with nutrient source (Saxena, 2011). As reported by Kim *et al.* (1996) immobilization provides less exposed to adverse environmental factors since encapsulation tends to stabilize cells, potentially enhancing their viability and stability in the production, storage and handling of cultures and also confers additional protection during rehydration and lyophilization. Additionally it helps to slow release of cells (Rekha *et al.,* 2007). Therefore it is obvious that an increased phosphate solubilization under such protective microenvironment is resulted.

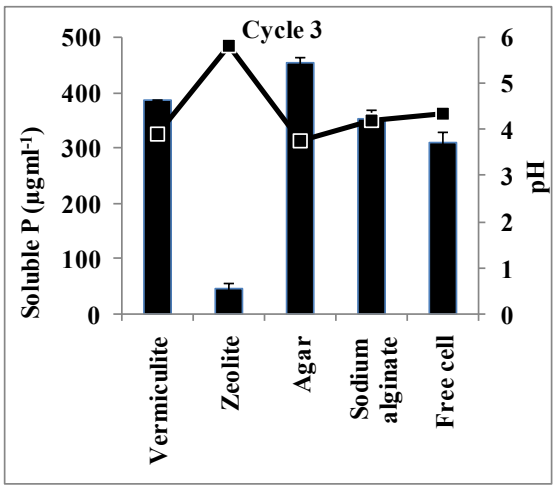

Figure 3. Changes in pH and soluble phosphorus content by immobilized fungal strain in cycle 3

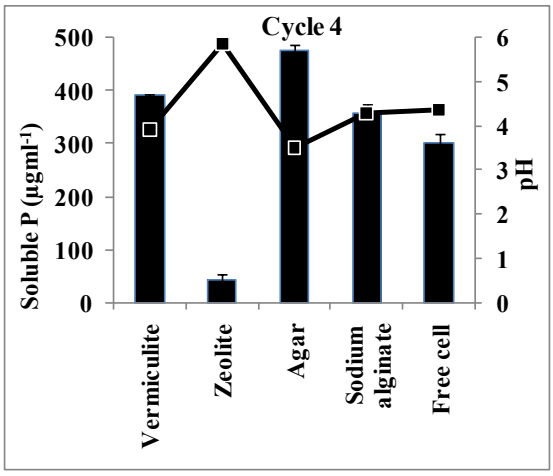

Figure 4. Changes in pH and soluble phosphorus content by immobilized fungal strain in cycle 4

Among the four immobilized forms used, fungi immobilized with agar were shown to release the highest content of soluble phosphorus into the medium, followed by vermiculite and sodium alginate. Agar encapsulated strain solubilized 28.8%, 44.0%, and 50.5% more soluble phosphorus (respectively in 2nd, 3rd and 4th cycles) than free 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).

In this study, pH values showed negative relation with phosphate solubilization throughout the experiment period suggesting that organic acids production plays major role in inorganic phosphate solubilization. Present results are in line with the Vassileva *et al.* (1998) who observed similar close relationship between the rate of acid production by the encapsulated *Aspergillus niger* and level of soluble P in the medium.

It was noticed that alginate beads become weak and breakable (fragile) when it used repeatedly. This finding was also evident from the results that increased phosphate solubilization up to 3rd cycle and a significant drop in soluble phosphorus level in the 4th cycle. This may be due to release of more cells by fragile beads to the medium and enhance the phosphate solubilization. The degradation of beads has been reported to be due to the presence of

free phosphate ions in the medium acting as calcium ion trapping, thus affecting the stability of the gel (Kennedy and Cabral, 1985) In order to overcome this problem, aluminum ion, strontium ion, or several other divalent metal ions can be used instead of calcium ion (El-Komy, 2005).

As demonstrated by pervious studies, application of immobilized, mainly entrapped and encapsulated, cells of microorganisms has more advantages over traditionally used free cell inoculation processes such as phosphate solubilization, IAA (indole-3-acetic acid) production and nitrogen fixation (Vassilev *et al.,* 2007; Bashan *et al.,* 2002; Fenice *et al.,* 2000).

CONCLUSION

It could be concluded that immobilization, in particular with agar is more effective for solubilization of inorganic phosphate by *Aspergillus awamori* than other microbially-based techniques. However, further investigations are needed in verifying the optimum culture conditions and evaluating economically viable carbohydrate sources. Moreover application of continuously-operating column bioreactors to scale up of immobilized cell solubilization would also worth investigating.

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