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Biosolubilization of Mineral Insoluble Phosphates by Immobilized Fungi (*Aspergillus Niger*) in Fluidized Bed Bioreactor

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Abstract

Phosphorus (P) is one of the most essential macronutrients required for the growth of plants and is added to soil in the form of phosphatic fertilizers. However, because of mineral re-precipitation, large amount of applied phosphate fertilizer may become unavailable to the plant. The ability of soil microorganisms to transform insoluble forms of phosphorus to an accessible form is an important path in plant growth-promoting for increasing plant yields. In this study, *Aspergillus Niger*, a fungal strain isolated from agricultural soil samples, was tested for its ability to solubilize different phosphated matrixes (TCP, DCP, phosphates rock). The isolated fungus exhibits high capacities to solubilize all tested phosphates. The solubilization of insoluble phosphates was associated with a drop in the pH of the culture medium. The fungal biomass was entrapped in alginate and polyacrylamide gels and was used for solubilizing mineral phosphates in fluidized bed bioreactor. The highest specific solubilization rates were obtained when *A. Niger* was entrapped in alginate beads. The use of the bioreactor in consecutive cycles of solubilization showed the interest of the biomass immobilization in the stability of the bioreactor. Immobilized cells in alginate continuously solubilize phosphate even after 5 cycles of solubilization without loss of activity. The phosphorus biosolubilization performances of isolated strains may open new possibilities for their biotechnology application and allow the use of this fungus in the soil fertilization.

Keywords: Solubilization; *Aspergillus niger*; Phosphatic fertilizers

Introduction

Phosphorus is an essential macronutrient for plants and is added to soil in the form of phosphatic fertilizers. However, the applied quantity of soluble forms of phosphate fertilizers is easily precipitated into insoluble forms and is not efficiently taken up by the plants [1-4]. Use of phosphatic fertilizers has become a costly affair, also environmentally undesirable and there is need for alternative sources [5].

The ability of microorganisms to solubilize different forms of calcium phosphate has been reported [6-8]. The solubilization of inorganic phosphates by microorganisms supplies phosphates for plant nutrition and increases their growth [9,10]. The attractive approach of microbially mediated solubilization of phosphate has been successfully proved in soil conditions which resulted in agriculture production similar or better than achieved with soluble phosphate [11,12].

Phosphate solubilizing microorganisms convert insoluble phosphates into soluble forms through the processes of acidification, by the production of organic acids [13-15], production of acid and alkaline phosphatases [2] and to H^+ production [16]. These organic acids can either dissolve phosphates as a result of anion exchange or can chelate Ca, Fe or Al ions associated with the phosphates [17].

A substantial number of microorganism species, mostly those associated with the plant rhizosphere have been isolated and characterized for their ability to solubilize unavailable reduced phosphorus to available forms exerting a beneficial effect upon plant growth [6-8]. It was assumed that phosphate-solubilizing activity was greater for filamentous fungi than for bacteria [18].

Immobilization of living microorganisms has been described by several investigators [19,20] to be useful in several biotechnological applications. It is widely known that immobilized cells offer a lot of advantages: reusability of the same biocatalyst, control of reactions, and the no contamination of products [21].

The main objective of this study was to examine the abilities of *Aspergillus niger*, isolated from agricultural soil, to solubilize different insoluble phosphate substrates (DCP, TCP and Rock phosphates) at different culture conditions.

Materiel and Methods

Microorganisms

Phosphate-solubilizing microorganisms are isolated from rhizospheric samples by plating serial dilutions of rhizospheric soil extracts in NBRIP solid medium (glucose 10g, $MgCl_2.6H_2O$ 5g, $MgSO_4.7H_2O$ 0.25g, KCl 0.2 g and $(NH_4)_2SO_4$ 0.1g, agar 15g, H_2O 1L) supplemented with 5g/l of TCP [22]. That medium contains insoluble tri-calcium phosphate allowing the detection of phosphate solubilizer microorganisms by the formation of "halos" around their colonies.

All plates were incubated at 30°C for 1 week. Colonies surrounded by solubilized clear zone were picked and streaked onto NBRIP plates containing 5g/l of TCP. Plates were again incubated at the same conditions to confirm their abilities to solubilize insoluble phosphate. Stock cultures were routinely maintained on LB agar supplemented with 1% of glucose.

Among the isolated strains, *Aspergillus niger* showing higher

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phosphate solubilization ability (larger solubilization halo surrounding the colony) in NBRIP plate was selected for further study. It was identified based on the visual observation of isolates grown on PDA plates, micro-morphological studies in slide culture [23] at room temperature, and the taxonomic keys described by Hoog and Guarro [24] as well as the compendium of soil fungi [25].

Stock cultures of isolated strain were routinely maintained on a potato dextrose agar (PDA).

Phosphate solubilization in solid media

One fungal isolate with higher phosphate solubilization abilities was selected. Abilities of the isolated fungus to solubilize different kind of insoluble phosphates were investigated. For this, the NBRIP plates supplemented with DCP, TCP or natural rock phosphates PR1, PR2, PR3and PR4 at 5g/l were used. The plates were inoculated with the selected fungus and incubated at 30°C. The diameter of clear zone (halo) surrounding the fungal growth as well as diameter of the colony were measured after 10 days of incubation.

Phosphate solubilization in liquid media

Precultures of the fungus were prepared by inoculating plugs (diameter 0.5 cm) from the growing zone of fungus on agar plate to 50 ml of nutrient broth (NB). Then, cells were cultivated statically at 30°C for 3 days. Afterward, the precultures were homogenized aseptically using a homogenizer. Aliquots of 1.5 ml of homogenized precultures were used to inoculate volumes of 150 ml of NBRIP containing 5 g/l of tested insoluble phosphates (DCP, TCP, PR1, PR2, PR3 or PR4) in 250-ml Erlenmeyer flasks. The cultures were incubated aerobically at 30°C on a rotary shaker at 150 rpm for 7 days. At several time intervals 4 ml aliquots of fungal cultures were sampled and centrifuged at 15,000 rpm for 15 min.

The clear supernatant was used for determination of the pH and the soluble phosphorus released into the solution. Phosphorus was determined colorimetrically by using the vanado-molybdate method [26]. The pellets were washed with 0.5 N HCl solutions to dissolve the residual insoluble phosphate and then dried at 105°C for 24 h to determine the biomass dry weight. All experiments were performed in duplicates.

Fungal biomass preparation

Aliquots of 1.5 ml of homogenized preculture of isolated fungus, prepared as described above, were used to inoculate 250-ml Erlenmeyer flasks containing 150 ml of NB. The cultures were incubated aerobically at 30°C on a rotary shaker at 150 rpm for 5 days. After cultivation the fungal biomass was harvested by filtration and then rinsed with sterile sodium chloride water (0.9%).

Fungal biomass immobilization

Entrapment in calcium gel: 100 ml of sterile sodium alginate solution (2% w/v) was mixed, until homogenous, with 2 g of fungal biomass. The mixture was extruded into 150 mM $CaCl_2$, forming beads of 5 mm diameter. The beads were allowed to harden in the $CaCl_2$ solution at room temperature for 30 min, and rinsed with sterile sodium chloride water (0.9%).

Entrapment in polyacrylamide gel: 2 g of fungal biomass were mixed with 78 ml of Tris-HCl buffer (50 mM, pH 7), 20 ml acrylamid-bisacrylamide solution (30-0.8 % wt/vol), and 1 ml ammonium persulfate solution (10 % wt/vol.). The polymerization was initiated adding 100µl of N,N,N',N'- tetramethyl-ethylenediamine. The polyacrylamide gel was then divided into particles of 0.5 cm diameter and rinsed with sterile sodium chloride water (0.9%).

Solubilization of DCP in fluidized bed bioreactor using free and immobilized fungal biomass

The fluidized bed bioreactors are composed of 500 ml conical flasks containing the immobilized fungal biomass suspended in 200 ml of NBRIP liquid medium and supplemented by 5g/l of DCP. The bioreactors were placed in a rotary shaker at 25°C, and the fluidization was assured by stirring at a rate of 120 rpm. The phosphate solubilization rate was followed according to time in the bioreactor. The same bioreactor have been used for studying DCP solubilization with free cells; thus, 2 g of fungal biomass were suspended in 200 ml of NBRIP liquid medium and supplemented by 5g/l of DCP. Solubilization rate was followed according to time in the bioreactor placed in the same conditions previously cited. For each experiment, a control test without fungal biomass was conducted under the same conditions.

At several time intervals, 1-ml aliquots were collected from the bioreactors and centrifuged at 15,000 rpm for 15 min. The supernatants were analyzed spectrophotometrically to determine the amount of soluble phosphorus and to determine the pH of the reactional media.

Repeated batch operation of phosphate solubilization in fluidized bed bioreactor using free and immobilized fungal biomass

The longevity of solubilization activity of the immobilized fungal biomass was investigated in repeated batch solubilization tests. A fresh reactional medium (NBRIP containing 5 g/l of DCP) was first inoculated with immobilized fungal biomass in the fluidized bed bioreactor, described above, and placed at 30°C in a rotary shaker at 120 rpm. After one week, the reactional medium was discharged and the immobilized fungal biomass were collected, rinsed with sterile NBRIP, and transferred into a fresh reactional medium for the next cycle of solubilization experiment. Solubilization rates were monitored according to time in all bioreactors. For comparison, the repeated batch experiments were also conducted using free fungal biomass under identical experimental procedures.

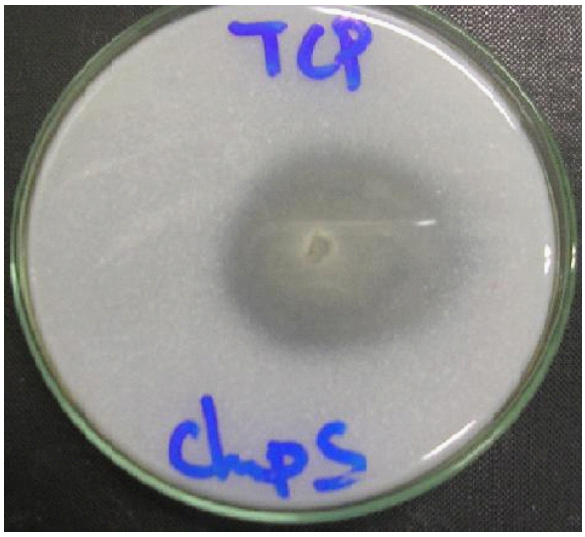

Figure 1: Phosphate solubilizer isolated fungal strain forming clear zone.

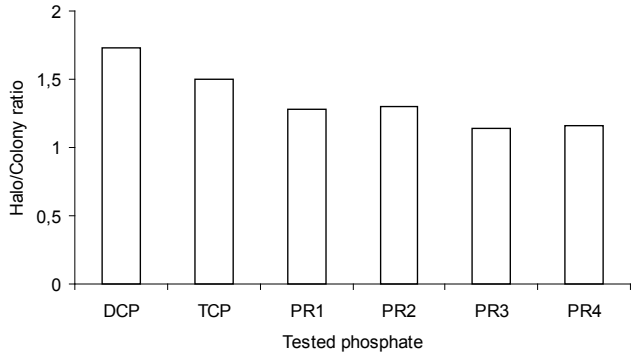

Figure 2: Solubilization tests on NBRIP plates containing tested phosphate matrixes using isolated fungal strain.

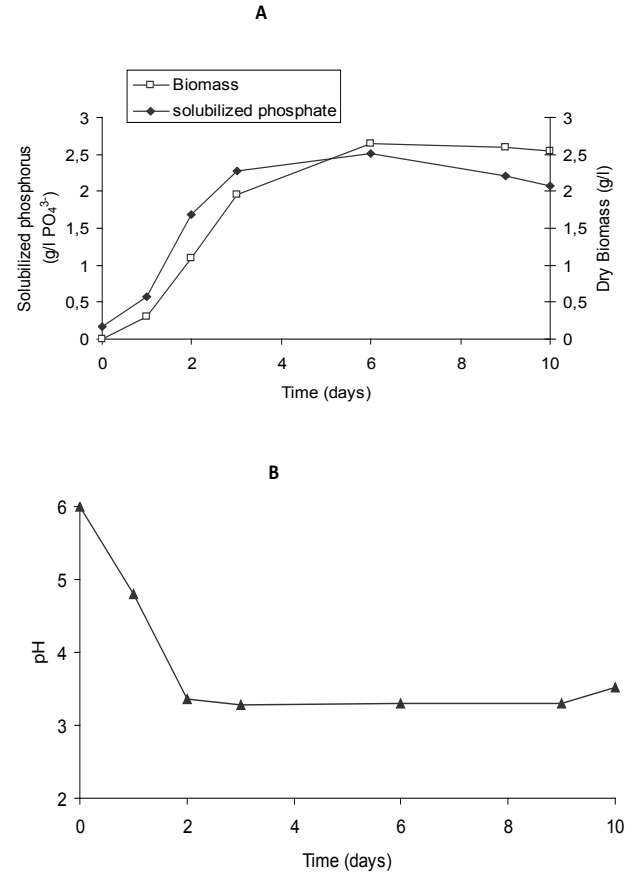

Figure 3: Evolution of pH, released orthophosphates concentration and fungal dry biomass in the culture media of isolated fungus cultivated in presence of DCP.

A: Evolution of released orthophosphates concentration and fungal dry biomass,
B: Evolution of pH

Results and Discussion

Five bacterial isolates and 2 fungal strains, which are solubilized TCP (forming clear halo surrounding colonies), were isolated from rhizospheric soil samples. Among these strains, one fungal isolate identified as *Aspergillus niger* with higher DCP solubilization ability (larger solubilization halo surrounding the colony) in NBRIP plate was selected for further study (Figure 1).

The selected isolate was cultivated, in first time, in NBRIP solid medium containing DCP, TCP or rock phosphates PR1, PR2, PR3 and PR4 as sole insoluble phosphate sources. The obtained results shown as the ratio of halo/colony diameters were represented in (Figure 2).

The obtained results show that the fungal strain *Aspergillus niger* manage to solubilize all tested phosphate substrates as indicated by the presence of solubilization halo on their culture media. The importance of the solubilization depends of the used phosphate. The highest solubilization halo/colony ratio around 1.73 was observed on plate containing DCP. It is followed by TCP, PR2, PR1, PR4 and PR3 with halo/colony ratios of 1.5, 1.28, 1.16 and 1.14 respectively.

It is generally accepted that phosphate solubilizing microorganisms convert insoluble phosphates into soluble forms through the process of acidification, by producing of organic acids, chelation and exchange reaction [15, 27-29]. However, the absence of the halo of solubilization or its reduced diameter can be explained by the diffusion limitation of secreted organic acids [30]. Consequently the phosphate solubilization abilities of isolated fungal strain were screened in NBRIP liquid medium containing the same phosphate substrates at 5g/l. The pH of the culture, the fungal biomass evolution and the concentration of the released orthophosphates were monitored according to time for each tested phosphate.

The obtained results, represented in (Figure 3A and 3B), concerning the solubilization of DCP during the cultivation of the isolated fungal strain indicated that this fungus has a high ability to solubilize DCP. The solubilization of DCP was accompanied by significant drop in the pH (to pH= 3.3). During the cultivation of *Aspergillus niger*, the solubilization rate of DCP was initially low. However more than 79% of insoluble phosphate was released as orthophosphates between the 3rd and the 6th day at which time the fungal biomass began to grow intensively. Conversely, the non-inoculated control presented no solubilization (data not shown).

The solubilization TCP and 4 different kinds of rock phosphates by isolated *Aspergillus niger* was investigated. The obtained results, plotted in (Table 1), testify of high ability of isolated fungus to solubilize mineral phosphates. Indeed, the use of *Aspergillus niger* enables a solubilization of all tested phosphates but with different performances that vary according to the used phosphate. *Aspergillus niger* solubilizes better and more quickly the DCP compared to others tested phosphates with solubilization efficiency and specific solubilization rate of 79.1% and 302.8 mg/g/d respectively. It is followed by TCP with solubilization efficiency and specific solubilization rate of 71.1% and 254.8 mg/g/d respectively. The lowest solubilization was recorded using PR3 with solubilization efficiency and specific solubilization rate of 30.8 and

	Final pH value	Solubilization efficiency (%)	Specific solubilization rate (mg/g/d)
DCP	3.3	79.1	302.8
TCP	3.5	71.1	254.8
PR1	3.3	34.4	73.5
PR2	3.2	36.1	92.5
PR3	3.3	30.8	58.0
PR4	3.2	31.7	55.1

Table 1: Final pH value in reactional medium, Solubilization efficiency and specific solubilization rate of tested phosphate matrixes recorded by isolated fungus in growth condition.

148mg/g/d respectively. The solubilization of all tested phosphate matrixes was accompanied by significant drop in the pH (inferior to 3.5).

The solubilization of DCP in fluidized bed bioreactor using free and entrapped fungal biomass in alginate and polyacrylamide gels was investigated. The concentration of released orthophosphates in reactional medium was measured at predefined interval time. Thus, free and immobilized fungal biomass exhibited similar solubilization patterns for DCP. The concentration of soluble phosphorus increased progressively in the reactional medium. The recorded solubilization efficiency for free fungal biomass reached 84.7%. Using immobilized fungal biomass, the obtained solubilization efficiencies were 73.4% and 66.1 respectively with alginate and polyacrylamide as entrapment matrixes (Table 2). The lower solubilization rates for immobilized biomass compared to free biomass can be attributed to mass transfer restriction arising from fungal biomass entrapment.

To investigate the possibility of the reusability of the same fungal biomass in successive cycles of solubilization, repeated batch experiments were performed. As shown in (Table 3), free biomass of *Aspergillus niger* remind active during all the 4 cycles of solubilization. However, a progressive decrease in solubilization efficiency was observed over the cycles of solubilization. After 4 Cycles of solubilization, the recorded solubilization efficiency and specific solubilization rate dropped from 84.7 to 70.2% and from 399 to 260.5mg/g/d respectively. The immobilization of *Aspergillus niger* in calcium alginate gel greatly stabilizes the fungal activity for more than 4 cycles. This stability returns to soft polymerization condition of the gel and to a direct role of the calcium in the cells conservation [31]. The use of *Aspergillus niger* entrapped in polyacrylamide gel in repeated batch fluidized bioreactor has proven to be not interesting. Indeed the phosphate solubilization system lost more than 54 % of its solubilization efficiency at the 4th cycle of treatment, and practically cancelled at the end of the 6th cycle (data not shown). This limitation of the biological activity is due to the existence of a favorable microenvironment inside the gel matrix and the presence of residual monomer that leads to a toxicity of microbial cell [32].

The obtained results indicate that isolated fungal strain is able to mobilize phosphorus from inorganic source and may serve as a good rock phosphate solubilizer when inoculated into soils where rock phosphate is used as phosphatic fertilizers. Encapsulated microbial systems could be adopted for different application: solubilization of inorganic phosphate in bioreactors; preparation of microbial inoculants for introduction in soils enriched with untreated rock phosphate and pre-treatment of ores.

Entrapment matrix	Solubilization efficiency (%)	Specific solubilization rate (mg/g/d)
Without (Free biomass)	84.7	399.0
Alginate	73.4	190.5
Polyacrylamide	66.1	179.0

Table 2: The Solubilization efficiency and specific solubilization rate of DCP recorded by free and immobilized biomass of isolated fungus.

	Free biomass		Alginate		Polyacrylamide	
	T_s (%)	V_s (mg/g/d)	T_s (%)	V_s (mg/g/d)	T_s (%)	V_s (mg/g/d)
Cycle 1	84.7	399.0	73.4	190.5	66.1	179.0
Cycle 2	82.2	354.5	73.2	182.5	40.5	103.5
Cycle 3	78.4	290.5	73.4	185.0	36.2	98.5
Cycle 4	70.2	260.5	73.3	180.0	35.7	96.5

Table 3: Solubilization efficiency (T_s) and specific solubilization rate (V_s) of DCP recorded by free and immobilized biomass of isolated fungus during repeated batch solubilization cycles.

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