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Solubilization of Iron Phosphate by Free or Immobilized Spores and Pellets of *Aspergillus niger*

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Abstract: The production of pellets by *Aspergillus niger* and the $FePO_4$ (FeP) solubilization through the use of free or immobilized spores and pellets were studied. The media Sabouraud, MS-FeP, MS-K_2HPO_4, Czapek and Malt were used for pellets yield. Enhanced growth and pellets production were found in the Sabouraud and MS-FeP than those produced by the other media. Pellets produced in Sabouraud were larger in size and formed well-defined spheres than those produced in MS-FeP. Pellets amounts varying from 2 to 8 mg mL^{-1} were inoculated in the MS-FeP medium. The greatest quantity of solubilized FeP was found when 6 mg of pellets mL^{-1} were used. While the free spores were the worst form used for FeP solubilization in culture medium, free pellets allowed for the production of the greatest quantities of soluble phosphate, even after repeated use of the pellets. In the soil, pellets solubilized similar quantities of FeP compared to the immobilized *A. niger* spores and can be used with advantage since they are easily produced.

Key words: Immobilization, pellets reuse, phosphate solubilization, production of acids, soil inoculation

Introduction

Cell suspensions or fungi spores are commonly used to inoculate microorganisms in the soil (Wu *et al*., 2005; Peix *et al*., 2001). The inoculation of selected fungi or bacteria for the rock phosphates solubilization has been the object of study of innumerous authors (Nahas, 1996; Vassilev *et al*., 1996; Sundara *et al*., 2002). Their beneficial effect is providing soluble phosphates for plant nutrition, principally in countries with soils poor in phosphates or with limited reserves of phosphate fertilizers (Das *et al*., 2003). Moreover, a predominance of Fe-P and Al-P over Ca-P and low contents of available P was found in Brazilian soils (Barroso and Nahas, 2005).

Recently, considerable attention has been given to the production of metabolites through the immobilization of microorganisms (Angelova *et al*., 1998; Couto *et al*., 2004; Mandal and Banerjee, 2005). Intact vesiculae and hyphae of the fungus *Glomus intraradices* encapsulated in sodium alginate were shown to be viable in the inoculation of garlic plants even after long-term storage (Plenchette and Strullu, 2003). The immobilization of mycelial biomass has been used in the production of intracellular lipases by *Rhizomucor miehei* and *Yarrowia lipolytica* (Adamaczak and Bednarski, 2004). This technique has been used through spore encapsulation in studies of citric acid production by *Aspergillus niger* (Honecker *et al*., 1989; Demirel *et al*., 2005) and phosphate solubilization (Fenice *et al*., 2000). Nevertheless, the use of immobilized microorganisms in the soil with the aim of inorganic phosphate solubilization and the nutrition of plants requires further research (Vassilev *et al*., 2001).

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Another alternative has been the use of pellets of filamentous fungi. Infante Majolli and Aguirre (1999) observed the development of the fungus *Aspergillus wentii* in the form of small spheres or pellets, approximately 1.5 mm in diameter in shaken culture medium and that pellet numbers increased with an increase in citric acid production. Similar results were found by Iwahori *et al*. (1995) who observed the production of pellets with a diameter varying between 2 and 10 mm and the production of organic acids using the fungus *Aspergillus niger* during five days of incubation. A small round pellet was most effective form for mycelial biomass production of citric acid by *Aspergillus niger* (Haq *et al*., 2003). According to Xu *et al*. (2000), the production of fungi pellets constitutes a form of immobilization, aside from the advantage of not requiring any artificial technique. Besides, pellets exhibit some advantage in relation to filamentous fungi because can be reused in a continuous process.

The objective of this study was to examine the production of pellets by the fungus *Aspergillus niger* and compare the effect of these pellets with free and encapsulated spores in the solubilization of iron phosphate.

Materials and Methods

Microorganism

The fungus used was *Aspergillus niger* F_{111} isolated from a Alfisol soil among 401 isolates (Barroso and Nahas, 2005) and maintained on Sabouraud agar slants.

Effect of Culture Media on Pellet Production

Media of Malt (Dixon-Hardy *et al*., 1998), Sabouraud (Medvedeff *et al*., 2001), Czapek (Iwahori *et al*., 1995) or MS (Nahas *et al*., 1994) were used, with 0.4 g P L^{-1} as $FePO_4$ (MS-FeP) or K_2HPO_4. FeP was selected because *A. niger* has high solubilization capacity of this salt. Erlenmeyer flasks of 250 mL containing 50 mL of medium were inoculated with 1 mL of spore suspension (22.3×10^6 spores mL^{-1}) and incubated at 30°C with circular agitation (160 rpm) for 4 days. The pellets were separated from the culture medium by a 1 mm mesh filtration sieve, washed with distilled water, counted and dried for 24 h at 105°C to determine dry weight.

Influence of Increasing Concentrations of Pellets on the Solubilization of $FePO_4$

Pellets produced in Sabouraud medium were washed with distilled and sterilized water. The pellets were inoculated in quantities of 4, 6 or 8 g L^{-1} moist weight in 250 mL Erlenmeyer flasks containing 50 mL of MS-FeP medium with 2 g $FePO_4$ L^{-1} and incubated at 30°C with circular agitation (160 rpm) for up to 8 days. Three flasks were removed daily, and the culture medium was filtered through Whatman No. 40 filter paper. Soluble phosphate, titrable acidity, pH, growth and total number of pellets were determined.

Influence of Increasing Sizes of Pellets on the Solubilization of $FePO_4$

Pellets produced in Sabouraud medium were washed with sterilized water and sieved aseptically through sieves of 1, 2, 3 and 4 mm meshes. The pellets, separated by diameter, were inoculated in quantities of 6 g L^{-1} moist weight in 250 mL Erlenmeyer flasks containing 50 mL of MS-FeP liquid medium with 2 g $FePO_4$ L^{-1} and incubated at 30°C with circular agitation (160 rpm) for up to 8 days. The following procedures were as described previously earlier.

Immobilization of Pellets and Spores

Three milliliter of a suspension of 22.3×10^6 spores mL^{-1} was mixed in 100 mL of 3% (w/v) sodium alginate solution, the mixture was pipeted into 100 mL of 0.5 M $CaCl_2$ and shaken for 30 min (Vassileva *et al*., 1998). The pellets produced in 50 mL of Sabouraud medium (a quantity

corresponding to 3 mL of a suspension of 22.3×10^6 spores mL^{-1}) were also added to a 3% (w/v) sodium alginate solution and the mixture pipeted into 100 mL of 0.5 M $CaCl_2$ while shaken. After forming consistency, the capsules were washed several times with sterilized water.

Solubilization of $FePO_4$ in Liquid Medium by Free and Immobilized Spores and Pellets

An 0.5 mL of a suspension with 22.3×10^6 free or immobilized spores mL^{-} or equivalent quantities of free or immobilized pellets produced in Sabouraud medium was added to Erlenmeyer flasks of 250 mL containing 50 mL of MS medium and 2 g L^{-1} of $FePO_4$ and incubated at 30°C under circular agitation (160 rpm). Samples from the media were removed daily to determine solubilized phosphate. After 8 days of incubation, the culture medium was filtered and the fungus retained on filter paper was washed with sterilized water and transferred to 50 mL of fresh culture medium in aseptic conditions. This cycle was repeated again after a further 8 days of incubation, transferring the fungus retained in the filtration to a fresh culture medium.

Solubilization of $FePO_4$ in Soil by Free and Immobilized Spores and Pellets

An 200 g (dry weight) of a mixture consisting of Alfisol soil and sand (proportion 1:3) with 0.9 mg of $FePO_4$ g^{-1} of dry soil were added to 2500 mL flasks. Molasses were added at a concentration of 0.02 mL g^{-1} of dry soil as a carbon source in the previously sterilized rehydration water. The moisture content was equilibrated to 70% of the water retention capacity. The flasks were inoculated with 1 mL of a suspension with 45.0×10^6 free or immobilized spores mL^{-1} or corresponding quantities of free or immobilized pellets produced in Sabouraud medium. Daily, the respiratory activity was determined and 11 g of moist soil samples were removed from each flask. These samples were dried at 45°C and used to determine soluble phosphorus.

Respiratory Activity

The production of CO_2 in the inoculated flasks with free or immobilized spores or pellets was determined according to Rezende *et al*. (2004). The flasks were incubated for up to 15 days at 30°C and every 24 h the 1 M NaOH solution from each flask was titrated with 1 M HCl.

Analytical Methods

Soluble phosphate from the culture medium was determined by the molybdate-ascorbic acid method of Ames (1966). Titrable acidity and pH were determined by titrating 10 mL of culture medium with a standard solution of 0.02 M NaOH up to pH 7.0 using an automatic titrator, Titration Manager TIM 850 (Cerezine *et al*., 1988). Soluble phosphorus in the soil was determined by the Watanabe and Olsen (1965) method.

Statistical Analysies

All the results are sum means of three replicates. Data were subjected to analysis of variance and regression analysis using the SAS statistical package for ANOVA. F-test were calculated at the 0.01 level of probability. Maximum and minimum points were calculated by using Maple 8 software.

Results and Discussion

In shaken culture medium, the fungus *A. niger* grows forming small spheres (pellets) containing hyphae intertwining on the surface and a hollow center. According to Ryoo and Choi (1999), the formation of pellets by *A. niger* is of a coagulating type in which the spores are coagulated in the early cultivation phase and the hyphae formed overlap each other to form pellets. The number of pellets formed varied from 2.3 to 73.0 mL^{-1} of culture medium (Fig. 1a) and a mycelial dry mass

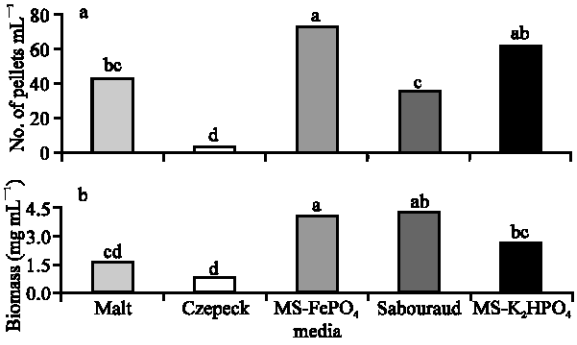

Fig. 1: Effect of culture on the production of pellets by *Aspergillus niger*. Number (a) and biomass (b) of pellets. Bars followed by the same letter, are not significantly different according to an LSD value ($p<0.05$)

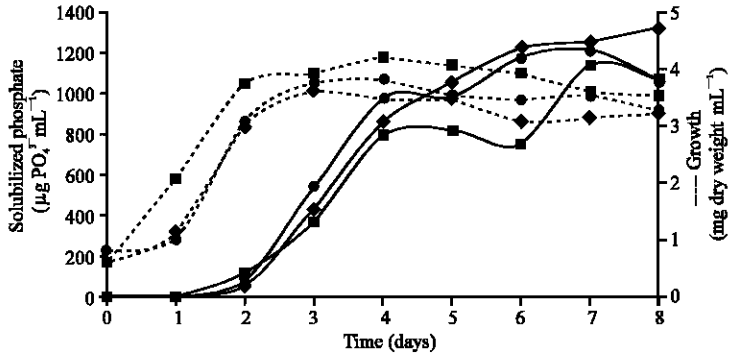

Fig. 2: Dependence of pellet amounts on the $FePO_4$ solubilization and growth of *A. niger*. (■) 4 mg; (♦) 6 mg and (●) 8 mg pellets mL^{-1} medium

of 0.8 to 4.2 mg mL^{-1} (Fig. 1b). Of the different culture media used, Sabouraud, MS-FeP, MS-K_2HPO_4, Czapek and Malt, the production of mycelial dry mass after 4 days of incubation in the first two media was greater than in the remainder. Although the number of pellets produced in the Sabouraud medium was less than MS-FeP, the production of dry mass was one of the largest. This is because the pellets produced in Sabouraud medium were larger in size and formed well-defined spheres compared to those produced in MS-FeP medium (<1 mm). It has been observed that depending on the culture medium used, the pellets do not always present well defined forms. In the Malt medium, the pellets showed a hairy aspect (hairy pellets), while in the remaining media, the spheres presented a smooth surface (smooth pellets).

Factors like the medium composition and pH, the quantity of inoculant and the presence of surfactants influenced the formation of the fungal pellets (Ryoo and Choi, 1999). Dynesen and Nielsen (2003) reported that pellet production by *Aspergillus nidulans* diminished with an increase in the quantity of acid in the culture medium, because in low pH the conidia carry highly positive charges which inhibit coagulation. In the MS medium, a dramatic drop in the pH was found on the first day of incubation and an increase in the acidification of the culture medium after 3 to 4 days of incubation (data not shown). This behavior could explain why the aggregation of the conidia and consequent pellet formation were compromised in the MS medium while in the Sabouraud medium the pellets were larger and better formed.

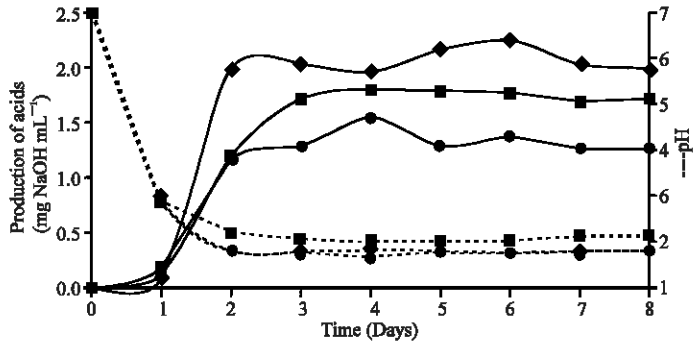

Fig. 3: Dependence of pellet amounts on the production of acids and final pH of culture medium added of $FePO_4$. (■) 4 mg; (♦) 6 mg and (●) 8 mg pellets mL^{-1} medium

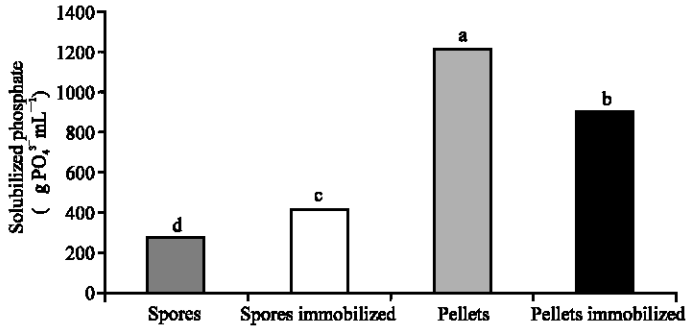

Fig. 4: $FePO_4$ solubilization by free and immobilized spores or pellets of *A. niger*. Bars followed by the same letter, are not significantly different according to an LSD value ($p<0.05$)

The greatest quantity of solubilized phosphate was found when 6 mg of pellets mL^{-1} were inoculated (Fig. 2). With this quantity of pellets, the greatest production of acid and pH drop were also found (Fig. 3). Factors like pH drop, greater production of acids and less fungal growth influenced these results. The influence of pH and acid production on the solubilization of inorganic phosphates has been reported by several authors (Cerezine *et al*., 1988; Illmer and Schinner, 1995; Nahas, 1996). Fungus growth is important for phosphate solubilization, nevertheless, the quantity of biomass produced should be sufficient so as not to uptake the solubilized phosphate from the culture medium. Therefore, the solubilization ability depends on the size of the inocula.

With the data of Fig. 2 and 3, the maximum and minimum points corresponding to solubilized phosphate, titratable acidity, pH and mycelial dry mass were determined (Table 1). Although all the equations were significant (F-test, $p<0.01$), the determination coefficients indicated that the equations of 3rd degree polynomial regression were those which most closely adjusted to the results obtained and were greater than the values obtained for 1st and 2nd degree regressions. The maximal theoretical values were obtained after 7 days of incubation for solubilization and pH drop, after 5 days for the acid production and after 4 days for fungus growth. These results suggest that maximal solubilization could be anticipated by the maximum values of fungal growth and acid production and the minimum values of pH lowering. Although fungal growth is unpredictable, at the maximal point of solubilization a reduction in growth must occur and, possibly, the uptake of solubilized phosphate by the fungus. This conclusion was verified when the culture medium was inoculated with increased pellet sizes (<2 to >4 mm): The greatest solubilization was found with 4 mm diameter pellets, even though fungal

Table 1: Maximum and minimum points corresponding to solubilized phosphate, titratable acidity, pH and mycelial dry mass

		Maximum		Minimum			
	Equations	Days	Values	Days	Values	R^2	F test
Phosphate	$Y = -51.75+177.47x$	-	-	-	-	0.88	570.51**
($\mu g\ PO_4^{3-}\ mL^{-1}$)	$Y = -151.62+263.07x-10.70x^2$	12.29	1465.39	-	-	0.89	331.58**
	$Y = -18.05-24.73x+84.70x^2-7.95x^3$	6.95	1232.55	0.15	-19.88	0.94	379.44**
Titratable acidity	$Y = 0.52+0.20x$	-	-	-	-	0.45	77.55**
(mg NaOH mL^{-1})	$Y = -0.09+0.72x - 0.06x^2$	5.54	1.90	-	-	0.76	126.21**
	$Y = -0.19+0.95x - 0.14x^2+ 0.01x^3$	5.16	1.81	9.65	1.53	0.78	88.81**
pH	$Y = 4.13-0.39x$	-	-	-	-	0.40	52.96**
	$Y = 5.66-1.70x + 0.16x^2$	5.19	1.24			0.77	127.29**
	$Y = 6.53-3.58x + 0.79x^2 - 0.05x^3$	6.65	2.25	3.46	1.41	0.93	364.71**
Mycelial dry mass	$Y = 2.29+0.33x$	-	-	-	-	0.42	58.03**
(mg mL^{-1})	$Y = 0.97+ 1.46x - 0.14x^2$	5.16	4.73	-	-	0.83	191.92**
	$Y = 0.50+2.46x - 0.48x^2+ 0.03x^3$	4.00	4.54	7.39	1.41	0.90	241.86**

**; Significant ($p<0.01$)

Table 2: Influence of increasing sizes of pellets on the solubilization of $FePO_4$

Pellets size (mm)	Solubilized phosphate ($\mu^{-1}g\ PO_4^{3-}\ mL^{-1}$)	Titratable acidity (mg NaOH mL^{-1})	pH	Mycelial dry mass (mg mL^{-1})
<2	650.52	1.14	1.88	4.79
2 a 3	1038.59	1.70	1.84	3.71
3 a 4	1184.98	2.07	1.80	3.51
> 4	1227.97	2.06	1.79	3.23

Table 3: $FePO_4$ solubilization by the reuses of free and immobilized spores or pellets of *A. niger*

	Time (days)							
	1	2	3	4	5	6	7	8
	($\mu g\ PO_4^{3-}\ mL^{-1}$)*							
1st reuse								
Spores	8.42c	8.83d	36.81c	48.16d	104.94d	109.93d	118.32d	156.03d
Spores immobilized	384.33b	425.29b	448.00b	685.23c	840.94c	1012.88a	1014.10a	916.37a
Pellets	696.18a	709.15a	724.97a	930.97a	1027.08a	800.91c	699.83c	689.28c
Pellets immobilized	386.36b	407.04c	575.58ab	775.66b	856.76b	901.77b	893.66b	901.37b
2nd reuse								
Spores	00.00	96.82d	95.61d	137.78d	159.68b	169.01d	158.06d	144.27d
Spores immobilized	00.00	179.96b	477.20a	500.72c	513.29ab	522.62b	393.66c	375.01c
Pellets	00.00	273.63a	450.03b	570.87a	477.60ab	434.21c	415.97b	407.85b
Pellets immobilized	00.00	116.29c	312.96c	541.67b	594.80a	596.01a	466.25a	422.86a

* Columns followed by the same letter, are not significantly different according to an LSD value ($p<0.05$)

Table 4: CO_2 production by free and immobilized spores or pellets of *A. niger*

	Spores	Spores immobilized	Pellets	Pellets immobilized
Time (days)	(mg CO_2 100 g dry soil)*			
1	81.14b	45.93c	81.44b	100.35a
2	156.44a	151.56ab	144.22c	149.11bc
3	150.12b	138.04c	164.78a	144.94b
4	134.29b	139.79b	167.75a	163.63a
5	114.40c	126.13b	176.98a	167.69a
6	68.10b	71.76b	99.52a	98.48a
7	40.62b	45.69b	81.23a	78.41a
8	26.28b	29.33b	48.89a	51.33a
9	18.67b	20.67b	35.33a	35.33a
10	12.47c	16.13bc	19.80ab	24.20a
11	169.48b	200.44a	201.26a	201.26a
12	192.50a	192.50a	168.67b	197.08a
13	184.38b	187.52ab	187.52ab	202.19a
14	157.67b	165.00b	167.44b	188.22a
15	118.80b	129.07b	137.87b	206.80a

* Columns followed by the same letter, are not significantly different according to an LSD value ($p<0.05$)

growth was less than that obtained with the remaining inocula (Table 2). Confirming these results, enhanced phosphate consumption due to the abundant growth of the fungus *A. niger* was reported by Vassileva *et al.* (1998). In the present study, pellets with a diameter greater than 4 mm did not limit the solubilization ability of the fungus. However, optimal conditions for destruxin B yield by *Metarhizium anisopliae* were dependent on pellet size (Feng *et al*., 2004). The control of pellet size is important in order to avoid oxygen and substrate limitation in large pellets (Ryoo and Choi, 1999). Oxygen transport to the center of the pellet depends on cultivation conditions and pellets density (Feng *et al*., 2004).

The reuse of microorganisms constitutes an important benefit in biological processes. After 8 days of incubation, free pellets solubilized more than 1200 μg PO_4^{3-} mL^{-1} of FeP and the amounts of phosphate produced decreased in the following order: free pellets > immobilized pellets > immobilized spores > free spores (Fig. 4). Pellets and spores were washed and transferred to a new sterile medium. During the first reuse, the greatest solubilization of FeP was found with pellet inoculation up to the 5th day and with immobilized spores from the 6th day (Table 3). In the second reuse (Table 3), solubilization was greatest with pellet inoculation than with spores. During the incubation of the fungus, some of the immobilized spores swelled, others agglomerated with each other forming a single pellet and in others the pellets broke open. The free pellets, when inoculated, increased in size maintaining their spherical form or produced other smaller pellets due to hyphae disintegration (Smith *et al*., 1990). Immobilized pellets maintained their original form, though some break open. The free spores formed small pellets (<1 mm) when used and reused.

While the free spores were the worst form used for FeP solubilization, inoculation with free pellets allow for the production of the greatest quantities of soluble phosphate, both in first use and reuse. Similar results were obtained with immobilized spores, though only on reuse. A drastic reduction in the quantity of solubilized phosphate was found, principally with the second reuse. Calcium alginate has been used by various authors in the immobilization of *A. niger* for citric acid production (Tsay and To, 1987; Honecker *et al*., 1989; Demirel *et al*., 2005). However, there are very few reports about the use of free or immobilized spores and pellets for inorganic phosphate solubilization. Vassileva *et al.* (1998) reported that a reduction in solubilization by *A. niger* when the spores were encapsulated in Ca-alginate but not in agar or k-carrageenan. This report explain the results found in this study with spores immobilized in Na-alginate.

The secretion of organic acids is one of the mechanisms which explains the solubilization of inorganic phosphates by *A. niger* (Cerezine *et al*., 1988). Demirel *et al.* (2005) reported a decreased production of citric acid by *A. niger* spores immobilized in Ca-alginate with increased reuse, resulting from the obstruction of the capsule pores. Consequently, decreased solubilization of Fe-P could be related to decreasing citric acid production by the fungus.

Free or immobilized spores and pellets were inoculated in soil to investigate their effect on the respiratory activity and solubilization of FeP. The production of CO_2 was greatest in soil inoculated with free or immobilized pellets than with spores (Table 4). With a second addition of molasses on the 10th day of incubation, the production of CO_2 by the pellets continued to be greater than that of the spores and increased more than two-fold in relation to the initial addition of molasses. Free or immobilized pellets solubilized the greatest quantities of FeP up to the 5th day of incubation, whereas the spores were more efficient for solubilizing FeP from the 4-6th day of incubation onward (Fig. 5). However, the average amount of phosphate produced was similar among the spores and pellets.

In conclusion, the production of pellets of *A. niger* could be of some importance, since they can be obtained in 3 to 4 days of incubation in Sabouraud culture medium. The greatest FeP solubilization was found using 6 mg ofpellets mL^{-1} and of a size > 4 mm. The inoculation of fungus in the soil with the objective of inorganic phosphate solubilization has been well studied, though few authors had used

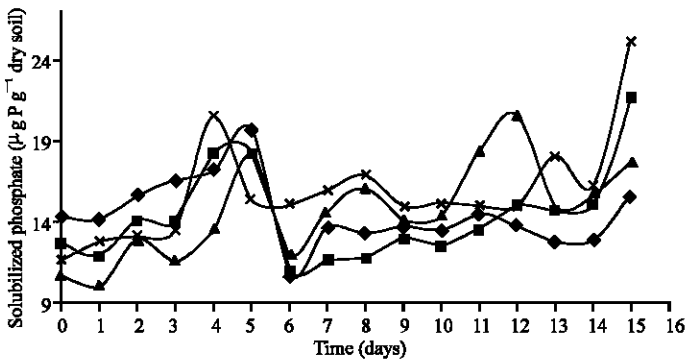

Fig. 5: $FePO_4$ solubilization by free and immobilized spores or pellets of *A. niger* inoculated in the soil. (×) spores; (▲) spores immobilized; (■) pellets and (◆) pellets immobilized. ↓Molasses added

immobilizing phosphate solubilizer microorganisms (Kucey and Leggett, 1989). In this study, the FeP solubilization by free or immobilized spores and pellets inoculated in the soil was compared. The results showed that the pellets solubilized similar quantities of FeP than immobilized *A. niger* spores, though with the advantage that pellets are more easily produced.

Acknowledgments

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