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BIODECOMPOSITION OF JORDAN PHOSPHORITE BY PHOSPHATE-SOLUBILIZING FUNGI

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Abstract - The bio-solubilization of Jordan phosphorite by the phosphate-solubilizing fungus *Aspergillus niger* has been investigated. The effect of the phosphate concentration in the liquid medium, the duration of biodecomposition, titratable acidity and the effect of preliminary mechanical activation on the process of dissolution have been studied. The investigations indicate that almost complete extraction of P_2O_5 from Jordan Phosphorite in a form utilizable by plants can be achieved. A maximum degree of P_2O_5 extraction 99.10% was obtained on the 15^{-th} day in a medium containing 0.5% w/v non-activated Jordan phosphorite. The preliminary mechanical activation of the phosphate facilitates the dissolution until a definite period of the bioconversion. Investigations with mechanically-activated Jordan phosphorite showed that a maximum extent of 92.40% of phosphate solubilization was observed on the 10^{-th} day at a phosphorite concentration of 0.5% w/v.
Keywords: Rock phosphate; Solubilization; Biodecomposition; *Aspergillus niger*.

INTRODUCTION

Phosphorus is second only to nitrogen in mineral nutrients that most commonly limit the growth of crops. A deficiency in soluble P for many agricultural soils is one of the major factors hampering crop production worldwide. Only 1 to 5% of the total soil P is in a soluble plant–available form (Arcand and Schneider, 2006). Usually it is introduced through the traditional phosphoric fertilizers- the super phosphates. It is known that the utilization of phosphorus from these fertilizers is about 15-20%, because a large portion of soluble inorganic phosphate applied to soil is rapidly immobilized soon after application and becomes unavailable to plants (Bojinova *et al*., 1997; Rodriguez and Fraga, 1999; Kang *et al*., 2007; Kang *et al*., 2008). This phenomenon occurs as a result of complex chemical and biochemical processes in the soil, resulting in fixation and precipitation of P in the soil. This generally depends on pH and the soil type. The fixed forms of P in acidic soils are aluminum and iron phosphates, while in alkaline soils they are calcium phosphates (Rfaki *et al*., 2014). According to Lindsay (1979) super phosphate contains a sufficient amount of calcium for the precipitation of its own P as dicalcium phosphate ($CaHPO_4$) or dicalcium phosphate dihydrate ($CaHPO_4.2H_2O$).

The second major component of soil P is organic matter (nucleic acids, phospholipids, phosphotriesters, etc.). The organic forms of P may constitute up to 30-50% of the total phosphorus in most soils. Many of these P compounds are materials with high molecular mass. They can be assimilated by the cell (Goldstein, 1994) after their bioconversion to either soluble ionic phosphate (Pi, HPO_4^{2-}, $H_2PO_4^-$), or low molecular-mass organic phosphate. These Ca-P compounds are generally resistant to chemical hydrolysis and biodegradation, but recently several reports documented microbial release from these sources (Rodriguez and Fraga, 1999).

Based on the current rate of use, it is expected that the worlds, known reserves of high quality rock

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phosphate (RP) will be depleted within the current century. Consequently, the production of phosphate-based fertilizers will require the processing of low-grade rock phosphates at significantly higher cost and the production costs of P-fertilizers will rise (Mendes *et al*., 2013).

The development of new nonacid methods, applicable to both high-quality and low-quality raw materials, is important for solving technological and ecological effectiveness of phosphorus fertilizers production. One of the available non-acid methods of rock phosphate processing is direct application of phosphates as a source of phosphorous in the soil. The phosphorus released from directly applied ground phosphate rock is often too low to provide sufficient P for crop uptake (Vassilev *et al*., 2001). The direct application of the phosphate as a fertilizer is limited due to its structure, resulting in low solubility. The improvement of phosphate structure through mechanical activation changes the phosphate chemistry and increases its solubility (Ibrahim *et al*., 2010).

The usage of phosphate-solubilizing microorganisms (PSM) as a biotechnological alternative for producing soluble P fertilizers from rock phosphate (RP) is the other alternative. Microorganisms are an important component in the soil. The ability of PSM to mobilize P from sparingly soluble sources can be a useful tool in P fertilization management. Some studies have shown that the product obtained after the treatment of RP with PSM or even the direct application of PSM to soil can improve plant growth and P uptake (Mendes *et al*., 2013). Therefore, an efficient process including microbial mediated ones able to exploit lower-grade RP and/or after native P sources (Vassilev, *et al*., 2013) at low cost has to be developed in the near future.

Soil bacteria and fungi mediate soil process such as decomposition, nutrient mobilization and mineralization, storage release of nutrients and water (Rashid *et al*., 2004). Several reports have indicated that some microorganisms are able to solubilize rock phosphates and to release soluble P (Sharma *et al*., 2012; Sanjotha *et al*., 2011; Yadav *et al*., 2011; Deepa *et al*., 2010; Pradhan and Sukla, 2005; Kang *et al*., 2002). A wide range of microorganisms able to solubilize inorganic P have been cultivated from soil, including bacteria (e.g *Actinomycetes, Pseudomonas* and *Bacillus* spp.) and fungi (e.g. *Aspergillus* and *Peniciluus* spp.). It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. The production of organic acids such as citric, oxalic, gluconic, malonic, succinic, etc. by phosphate-solubilizing microorganisms has been well documented and seems to be most frequent agent for mineral phosphate solubilization (Khan *et al*., 2007). Such organic acids can either directly dissolve the mineral phosphate as a result of anion exchange of PO_4^{3-} by acid anion or can chelate both iron and aluminum ions associated with phosphate (Omar, 1998). Some elements that may be released during RP solubilization could affect these mechanisms by promoting changes in microbial metabolism (Gadd, 1993). The amount of P solubilized is dependent on the form of inorganic P precipitate used (including various sources of RP, pure Ca, iron and aluminum phosphates) along with culture and sampling procedures (Whitelaw *et al*. 1999; Barroso *et al*., 2006; Richardson and Simpson, 2011). In general, phosphate-solubilizing fungi produce more acids and consequently exhibit greater phosphate-solubilizing activity than bacteria in both liquid and solid media (Venkateswarlu *et al*., 1984). The fact that all identified P-solubilizing fungi belong to the *Aspergillus* or *Penecillium* genus agrees with reports of several authors (Nahas *et al*. 1994; Ghosh and Banik, 1998; Rashid *et al*. 2004; Seshadri *et al*., 2004; Deepa *et al*., 2010). Fungal diversity affects soil agglomeration, thereby increasing the soil quality and fertility (Tallapragada and Seshachala, 2012). Species of fungi, particularly *Aspergillus*, are capable to produce citric acid and form non-ionizable association with calcium. *Aspergillus niger*, used in the industrial production of citric acid, has been reported as one of the most effective organisms for rock phosphate solubilization (Arcand and Schneider, 2006).

The dissolution of different types of P-contained resources (including Ca, iron and aluminum phosphates and various sources of rock phosphate) by *Aspergillus niger* has been demonstrated earlier (Vassilev *et al*. 2005; Nahas, 1996; Bojinova *et al*., 1997; Goenadi *et al*. 2000; Barroso *et al*., 2006; Bojinova *et al*., 2008; Mendes *et al*., 2013). The amount of P solubilized in culture is also depend on the composition of the medium (carbon and nitrogen composition), medium volume, pulp density, particle size, initial pH of the medium, temperature, inoculum concentration, along with culture and sampling procedures for the solubilization of the phosphates.

Solubilization of inorganic phosphate by microorganisms involves a wide range of processes concerning the secretion of organic acids, lowering of the pH as a result of acid production, ion chelating and exchange reactions which are a part of the phosphorus cycle (Akuntokin *et al*., 2007).

The aim of the present study is to investigate the biodecomposition of Jordan phosphorite using the phosphate-solubilizing fungus *Aspergillus niger*. We

use in our investigation Jordan phosphorite, which has not been studied with this objective. Jordan phosphorite is imported into Bulgaria for the phosphoric fertilizer industry. The effects of the phosphate concentration in the liquid medium, the duration of biodecomposition, the concentration of citric acid generated from the fungus and results with/without preliminary mechanical activation of the phosphorite, were studied.

MATERIALS AND METHODS

Natural Phosphate

The chemical composition of the initial Jordan phosphorite (JP) is shown in Table 1. The fraction below 0.2 mm was used. The total content of phosphorus was determined by dissolving in 25% HCl, the citric-soluble and water-soluble phosphorus was determined after extracting with 2% citric acid or water followed by spectrophotometric analysis as a vanadate-molybdate complex (Jackson, 1967). Ca was determined complexometrically, Si by weight and the other elements using Atomic Absorption Spectrophotometry (AAS).

The mechanical activation was performed for 4 hour using a "Pulverisite 5" planetary mill. Metal balls of 20 mm diameter were used and the rotation applied was 320 rpm. The weight ratio of phosphorite to milling bodies was 1:20.

The phosphorus content was determined in the non-activated Jordan phosphorite (NAJP) as well as after its mechanical activation (MAJP). It was analyzed as total P_2O_5 ($P_2O_{5\ t.}$), citric-soluble ($P_2O_{5\ c.s.}$) and water-soluble ($P_2O_{5\ w.s}$). The phosphate $P_2O_{5\ t}$ value was 35.37% for the both examined phosphate types (NAJP and MAJP). The values of $P_2O_{5\ c.s.}$ were 11.75% (NAJP) and 16.99% (MAJP) and those of $P_2O_{5\ w.s.}$ were 0.01% and 0.03%, respectively.

Microorganisms and Nutritive Medium

Investigations were performed using the *Aspergillus niger* strain obtained from the Institute for Microbiology, Bulgarian Academy of Sciences. The bioconversion was studied through deep incubation of the microorganisms in a liquid nutritive medium containing (in g/L): Glucose - 120; $(NH_4)_2SO_4$ - 3; KH_2PO_4 - 1; K_2HPO_4 - 1; $MgSO_4.10H_2O$ - 0.5; $MnSO_4.5H_2O$ - 0.02; $FeCl_3.6H_2O$ - 0.01.

The initial pH value of the nutritive medium was 6.8.

Experimental Methods

Incubation with *Aspergillus niger* was carried out in 300 ml Erlenmeyer flasks containing 100 ml of sterilized nutritive medium. After cooling to 30 ºC, 1 mL inoculums with a concentration of spores of 1×10^7/mL and JP (MAJP and NAJP) were introduced into the reaction medium. The flasks were incubated in a shaking water bath at 31±1 ºC for different period of time with a rotational speed of 150 rpm. The concentrations of NAJP and MAJP in the nutrient medium were 0.5, 1 and 2% w/v. After different time intervals, the samples were filtered and pH, sugar content (Bernfeld, 1959), titratable acidity through titration with 0.1 N NaOH and the content of water-soluble $P_2O_{5\ w.s.}$ (c_1) in this first filtrate were determined. The precipitate (biomass and remaining mineral mass) was treated for 2 hours with 2% citric acid at room temperature. After filtration the solution obtained was analyzed for citrate-soluble $P_2O_{5\ c.s.}$(c_2). The precipitate, which contained residual mineral mass and biomass was dried to a constant weight at 60 ºC and was ashed to a constant weight at 500 ºC. The loss of weight during heating is equal to the biomass produced during cultivation. The P_2O_5 content in the residue of mineral mass after thermal treatment was also determined - $P_2O_{5\ m.m}$. The P_2O_5 content in the biomass ($P_2O_{5\ b.}$) was determined using material balance for P_2O_5 based on the quantity of P_2O_5 input in the system (with phosphorite and nutritive medium) and, at the exit, after biodecomposition, ($P_2O_{5\ w.s.}$, $P_2O_{5\ c.s.}$, and $P_2O_{5\ m.m}$).

The process was analyzed by using two parallel samples at various times of incubation and the results obtained were averaged.

Investigations of solubilization of Jordan phosphorite in the nutritive medium without microorganisms were made for the longest incubation period of 15 days at the studied concentrations of Jordan phosphorite. The obtained α value for 0.5, 1.0 and 2.0% JP was nearly 2%.

Table 1: Chemical composition of Jordan phosphorite.

Al_2O_3	CaO	Fe_2O_3	SiO_2	TiO_2	MgO	P_2O_5	Pb	Cu	Zn	Cd	Ni	Ag	As	Mo
%							mg/kg							
0.26	53.69	0.27	2.60	0.02	0.26	35.37	18	32	183	4	10	1	7	4

Calculation of the Conversion of Jordan Phosphorite

On the basis of the results obtained, the extent of the JP solubilization and conversion of P_2O_5 to water-soluble (α_1), citrate-soluble (α_2) forms and P_2O_5 in biomass (α_3) were determined and expressed as follows:

$$\alpha_1 = \frac{P_2O_{5w.s.}}{P_2O_{5t.}} 100 \text{ , \% w/w} \quad (1)$$

$$\alpha_2 = \frac{P_2O_{5c.s.}}{P_2O_{5t.}} 100 \text{ , \% w/w} \quad (2)$$

$$\alpha_3 = \frac{P_2O_{5b.}}{P_2O_{5t.}} 100 \text{ , \% w/w} \quad (3)$$

The total degree of extraction is:

$$\alpha = \alpha_1 + \alpha_2 + \alpha_3 \text{ , \% w/w} \quad (4)$$

where:

$P_2O_{5\ w.s.}$ – P_2O_5 content in the first filtrate (g)
$P_2O_{5\ c.s}$ – P_2O_5 content in the second filtrate (g)
$P_2O_{5\ b}$ – P_2O_5 content in the dry biomass (g)
$P_2O_{5\ t.}$ – total P_2O_5 content in the system, with the phosphorite and nutritive medium (g).

A simple correlation was run to determine correlation coefficients (r) by the method of Ordinary Least Squares (OLS).

RESULTS AND DISCUSSION

Figure 1 presents the change in the concentration of glucose and citric acid (A) and the extents of P_2O_5 extraction α_1, α_2, α_3, α (B) in nutritive medium containing 0.5% w/v NAJP.

Microorganisms consume glucose as a carbohydrate source and its concentration decreased from 120 g/L at the beginning of the experiment to 11.5 g/L on the 15th day. The titratable acidity slowly increased up to the 12th day of incubation when it reached a maximum of 10.24 μE.mL^{-1}; it then decreased to the end of the experiment, when its value was 7.5 μE.mL^{-1}. As seen from the data, the extent of P_2O_5 extraction in water-soluble form (α_1) increased with time and achieved 73.70% on the 15th day. The extent of P_2O_5 extraction in citric-soluble form (α_2) slowly decreased from 20.80% on the 3rd day to 12.20% at the end of the period. The total extent of P_2O_5 extraction (α) achieved a maximum of 99.10% on the 15th day.

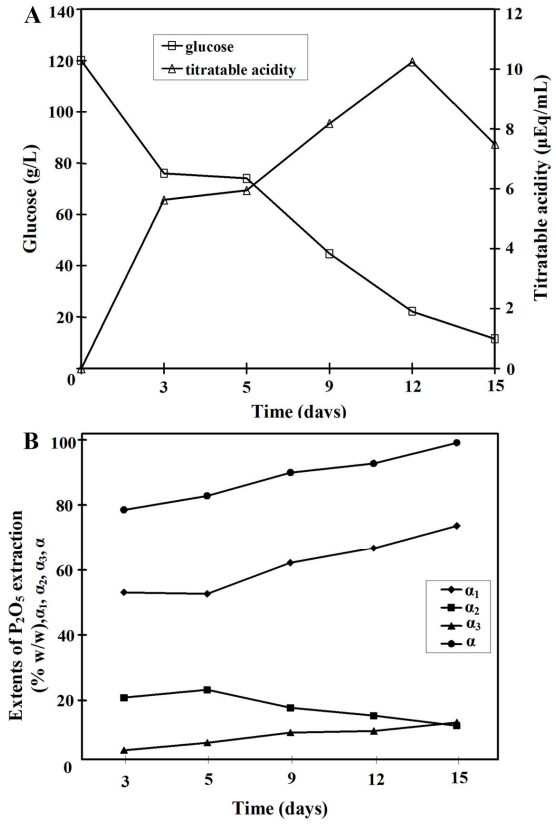

Figure 1: Change in the concentration of glucose and the titratable acidity (A) in the culture medium containing 0.5% w/v NAJP, and extents of P_2O_5 extraction, α_1, α_2, α_3, α (B), after 3, 5, 9, 12 and 15 days of incubation. The values are the average ± SD ($p < 0.05$) from duplicate experiments.

When 0.5% w/v MAJP was added to the nutritive medium the titratable acidity increased and reached a value of 10.66 μE.mL^{-1} on the 12th day (Figure 2A).

The extents of P_2O_5 extraction are insignificantly lower than those in the investigation with 0.5% w/v NAJP, but these values were achieved for a shorter period of incubation (Figure 2B). For example, using 0.5% NAJP the Σ (α_1+ α_2) and α reached values of 82% and 92.7% on the 12th day. If MAJP was used under the same conditions the value for the Σ (α_1+ α_2) and α were 84% and 92.4% on the 10th day. α_2 decreased in the range of 23.4 to 21.25 at the end of the incubation period. The quantity of P_2O_5, separated together with biomass (α_3) increased from the first day up to the 15th day for (NAJP) and 12th day for (MAJP) with the values in the range from 4.7 to 13.2% and from 1.29 to 10%, respectively.

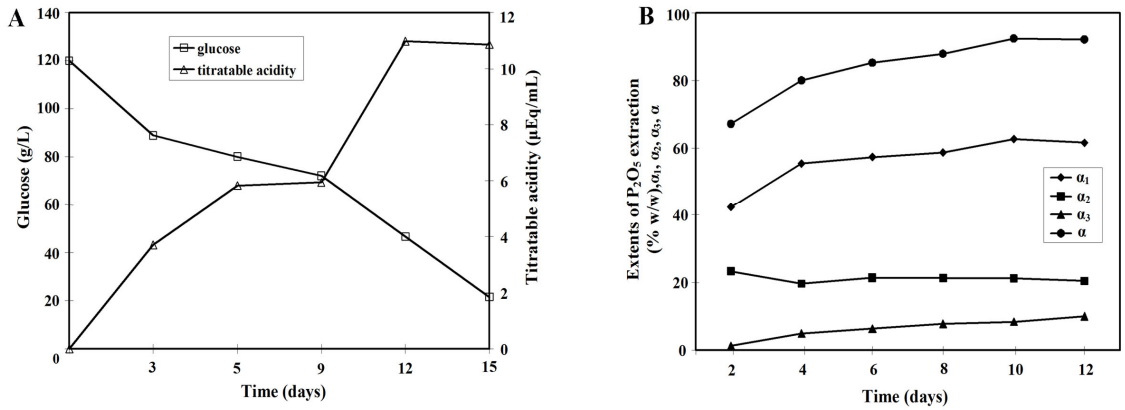

Figure 2: Change in the concentration of glucose and the titratable acidity (A) in the culture medium containing 0.5% w/v MAJP, and extents of P_2O_5 extraction, α_1, α_2, α_3, α (B), after 2, 4, 6, 8, 10 and 12 days of incubation. The values are the average ± SD ($p < 0.05$) from duplicate experiments.

With increasing concentration of JP (NAJP and MAJP) in the nutritive medium the extents of P_2O_5 extraction fall (Figure 3 and Figure 4). The result shows that the higher rate of phosphate dissolution was achieved in the investigations with 1% w/v NAJP - α reached a maximum of 80.80% on the 15th day (Figure 3B).

The maximum of α in nutritive medium containing 1% w/v MAJP was 69.29% on the 12th day (Figure 4B). If we compare the results for Σ (α_1+ α_2) and α, the same tendency can be seen. In the investigations with NAJP Σ (α_1+ α_2) had a value of 56% on the 9th day, but for MAJP this value was 59% on the 8th day. In the same conditions, the α values were 61.5% and 64%. The reason is the higher values of α_3 for MAJP compared with those for NAJP. The titratable acidity is higher in the experiments with MAJP – 13.88 μE.mL^{-1} on the 10th day (Figure 4A) compared with 11 μE.ml^{-1} on the 12th day for NAJP (Figure 3A). The results show that there is no correlation between citric acid produced and the phosphate solubilization after the 12th day (NAJP) and 10th day (MAJP).

Increasing the JP concentration to 2% w/v in liquid medium the titratable acidity increases (Figure 5 and Figure 6). In the experiment with 2% w/v NAJP it reached a maximum of 13 μE.mL^{-1} on the 12th day and slowly decreased to the end of the period when its value was 11.2 μE.mL^{-1} (Figure 5A). In the nutritive medium containing 2% w/v MAJP the titratable acidity achieved a value of 15.7 μE.mL^{-1} on the 12th day (Figure 6A).

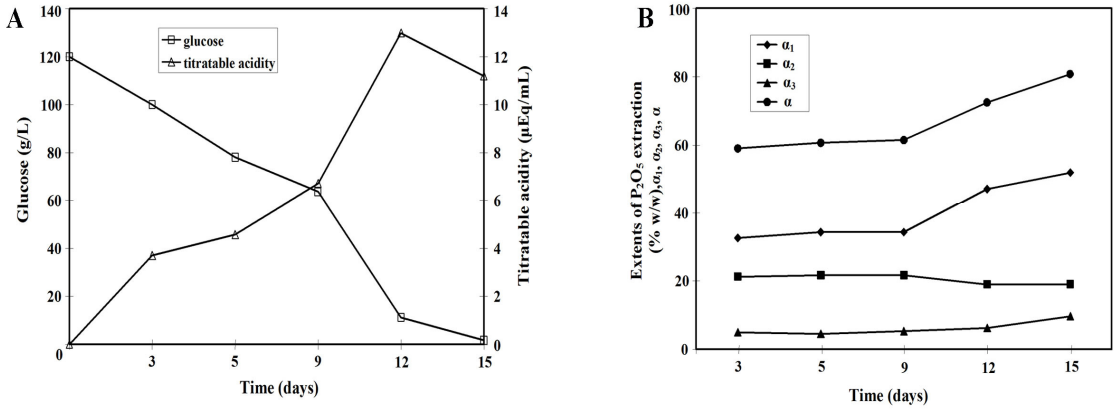

Figure 3: Change in the concentration of the glucose and the titratable acidity (A) in the culture medium containing 1.0% w/v NAJP, and extents of P_2O_5 extraction, α_1, α_2, α_3, α (B), after 3, 5, 9, 12 and 15 days of incubation. The values are the average ± SD ($p < 0.05$) from duplicate experiments.

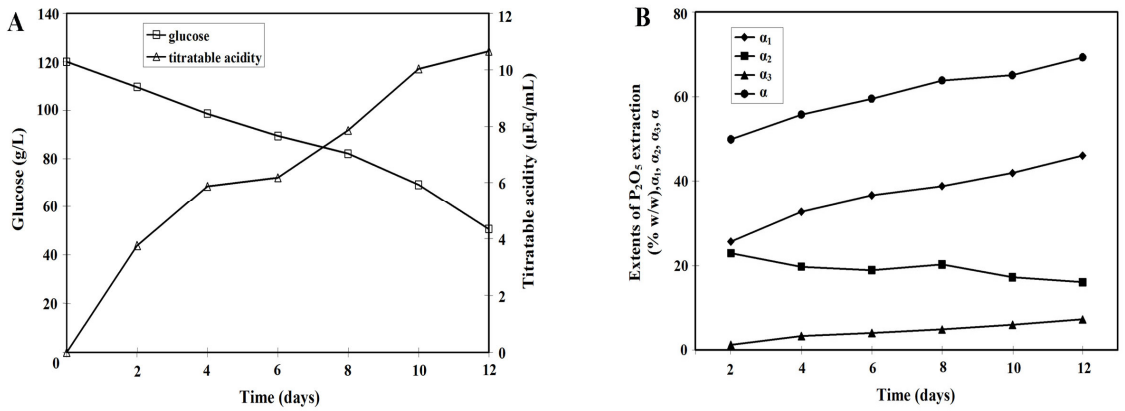

Figure 4: Change in the concentration of the glucose and the titratable acidity (A) in the culture medium containing 1.0% w/v MAJP, and extents of P_2O_5 extraction, α_1, α_2, α_3, α (B), after 2, 4, 6, 8, 10 and 12 days of incubation. The values are the average ± SD ($p < 0.05$) from duplicate experiments.

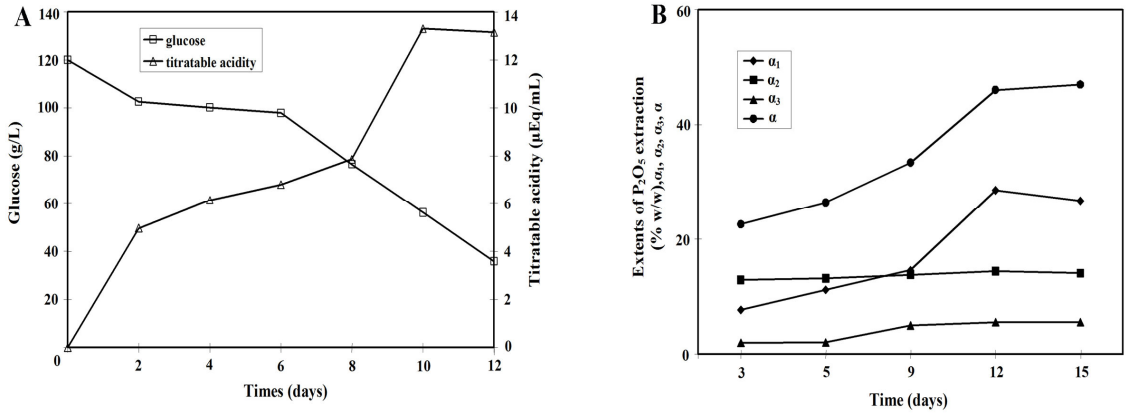

Figure 5: Change in the concentration of the glucose and the titratable acidity (A) in the culture medium containing 2.0% w/v NAJP, and extents of P_2O_5 extraction, α_1, α_2, α_3, α (B), after 3, 5, 9, 12 and 15 days of incubation. The values are the average ± SD ($p < 0.05$) from duplicate experiments.

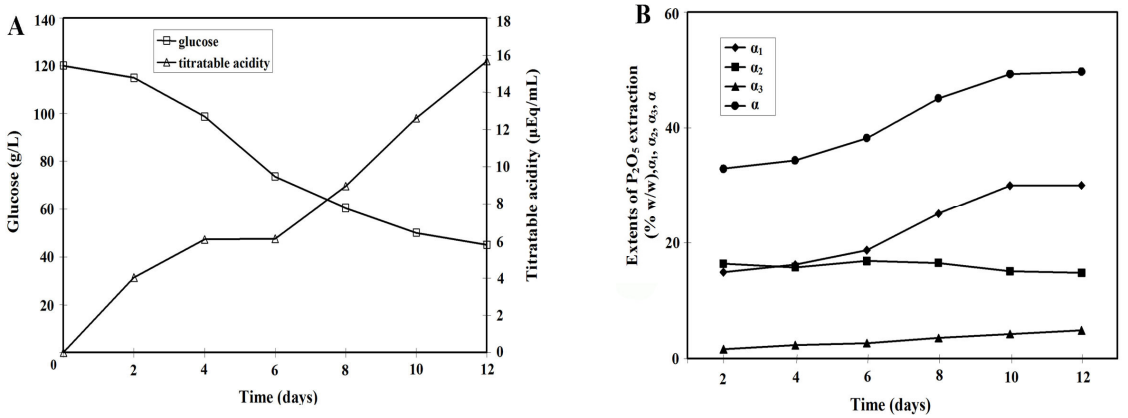

Figure 6: Change in the concentration of glucose and the titratable acidity (A) in the culture medium containing 2.0% w/v MAJP, and extents of P_2O_5 extraction, α_1, α_2, α_3, α (B), after 2, 4, 6, 8, 10 and 12 days of incubation. The values are the average ± SD ($p < 0.05$) from duplicate experiments.

There is no significant difference between the extent of P_2O_5 extraction in both experiments (2% w/v NAJP and 2% w/v MAJP). The total degree of phosphate solubilization (α) had a maximum value of 46.02% in the experiments with NAJP on the 12th day (Figure 5B) and 49.735% in the investigations with MAJP on the 10th day (Figure 6B), respectively, and these values were preserved to the end of the incubation period. The same tendency was observed for the lower concentrations connected with higher α value and shorter time: for the study with 2% NAJP, α had a value of 33.4% on the 9th day, but for MAJP a value of 39.5% was obtained on the 8th day.

The culture pH and the concentration of P_2O_5 extracted from the phosphate detected in the first filtrate, the second filtrate and in biomass expressed as P_2O_5 (C_1, C_2, C_3) and their sum (C) in the investigation with NAJP in the culture are presented in Table 2.

Table 2: Change in culture pH and P concentrations (C, g/L) in different forms, after 3, 5, 9, 12 and 15 days of incubation in nutritive medium containing 0.5, 1.0 and 2.0% NAJP.

Day	pH	Concentration of P_2O_5 (g/L)			
		C_1	C_2	C_3	C
0. 5% NAJP					
3	3.30	1.34	0.52	0.12	1.98
5	3.30	1.32	0.58	0.17	2.07
9	3.30	1.57	0.45	0.25	2.27
12	3.30	1.66	0.39	0.27	2.32
15	3.31	1.86	0.31	0.33	2.50
1% NAJP					
3	3.30	1.38	0.88	0.19	2.45
5	3.30	1.45	0.90	0.18	2.53
9	3.30	1.45	0.90	0.23	2.58
12	3.30	1.94	0.80	0.26	3.00
15	3.31	2.18	0.81	0.41	3.40
2% NAJP					
3	3.90	0.58	0.98	0.15	1.71
5	3.30	0.85	1.00	0.16	2.01
9	3.30	1.10	1.05	0.38	2.53
12	3.30	2.14	1.09	0.43	3.66
15	3.30	1.97	1.06	0.41	3.44

* c_1 - $P_2O_{5\,w.s.}$; c_2 - $P_2O_{5\,c.s.}$; c_3 - $P_2O_{5\,b}$; $c - \Sigma\,(c_1+c_2+c_3)$
The values are the average ± SD ($p < 0.05$) from duplicate experiments

The culture pH of the initial nutritive medium decreased from 6.8 to 3.3 immediately on the 3rd day and preserved this value to the end of the period for all the experiments. The concentration of the P_2O_5 extracted from the phosphate (C) increased with increasing concentration of NAJP and had a constant value of 3.66 g/L from the 12th day to the end of the incubation period at the highest concentration of 2% w/v NAJP in the liquid.

The result from the investigation with MAJP showed that the culture pH also decreased insignificantly with the incubation period (Table 3). The concentrations of P_2O_5 in the filtrates are about two times higher than those obtained when the culture contained NAJP. The concentration of P_2O_5 released (C) achieved a value of 6.31 g/L on the 15th in the experiment with 2% w/v MAJP.

Table 3: Change in culture pH and P concentrations (C, g/L) in different forms, after 2, 4, 6, 8, 10 and 12 days of incubation in nutritive medium containing 0.5, 1.0 and 2% MAJP.

Day	pH	Concentration of P_2O_5 (g/L)			
		C_1	C_2	C_3	C
0. 5% MAJP					
2	3.22	1.22	0.67	0.05	1.94
4	3.02	1.64	0.58	0.15	2.37
6	3.00	1.77	0.66	0.20	2.63
8	2.83	1.96	0.71	0.26	2.93
10	2.61	2.28	0.77	0.29	3.34
12	2.74	2.31	0.77	0.38	3.46
1% MAJP					
2	3.28	1.33	1.19	0.06	2.58
4	3.14	1.66	1.00	0.17	2.83
6	3.14	1.94	1.00	0.21	3.15
8	3.00	2.09	1.09	0.26	3.44
10	2.86	2.55	1.05	0.36	3.96
12	2.79	2.61	0.91	0.41	3.93
2% MAJP					
2	3.37	1.39	1.53	0.15	3.07
4	3.22	1.72	1.67	0.25	3.64
6	3.22	1.83	1.65	0.26	3.74
8	2.96	2.92	1.59	0.47	4.98
10	2.89	3.27	1.50	0.52	5.29
12	2.91	3.86	1.89	0.56	6.31

* c_1 - $P_2O_{5\,w.s.}$; c_2 - $P_2O_{5\,c.s.}$; c_3 - $P_2O_{5\,b}$; $c - \Sigma\,(c_1+c_2+c_3)$
The values are the average ± SD ($p < 0.05$) from duplicate experiments

The results from the present as well as earlier (Ivanova *et al*., 2006; Bojinova *et al*., 2008a) studies indicate that the lower the quantity of phosphate applicable, the greater is the conversion percentage, independent of the type of JP used (NAJP or MAJP). These results are in conformity with those reported by others (Nahas *et al*., 1990; Ghosh and Banic, 1998; Reddy *et al*., 2002).

In our earlier study we also documented that preliminary mechanical activation facilitated phosphate dissolution during bioconversion of Morocco phosphorite (MP) by *Aspergillus niger* up to the 3rd day, when the total P_2O_5 extraction had higher values using AMP (Bojinova *et al*., 2008b). In conformity with these investigations a positive correlation between the solubility of nonactivated (NMP) and activated Morocco phosphorite (AMP) and titratable acidity was observed to the 12th day of the incubation period with 1% w/v in the liquid culture. When the incubation time was prolonged to the 15th day under the same conditions this correlation was negative. If

the concentration of NMP and AMP was 2% the total P_2O_5 extraction increased with increasing production of citric acid for all investigated incubation periods of time. According to the present studies the data showed that in the investigations with NAJP (0.5%, 1% and 2%) the tendency is similar. The titratable acidity achieved a maximum on the 12th day of incubation and decreased to the 15th day (Figure 1, Figure 3 and Figure 5), but the extent of P_2O_5 extraction into water-soluble forms (α_1) and the total degree of extraction (α) increased for all periods of time investigated. The decrease of the titratable acidity after the 12th day (Figure 3), 10th day (Figure 4) and 12thday (Figure 5), respectively, can be explained by the partial neutralization as Ca-citrate of the citric acid produced with the Ca^{2+} ions liberated as a result of decomposition of the phosphate structure. As seen from the data (Figure 2, Figure 4 and Figure 6) for experiments with MAJP, α_1 and α increased together with increasing titratable acidity, independent of the concentration of MAJP used. The correlation coefficients (r) between the quantities of extracted P (C, g/L) and the titratable acidity for different concentrations of NAJP were: (0.64 at 0.5% w/v; 0.92 at 1% w/v and 1.0 at 2% w/v, $p< 0.001$), and: (1.0 at 0.5% w/v; 0.96 at 1% w/v and 0.98 at 2% w/v, $p< 0.001$) for MAJP. This is in accordance with the results obtained by others (Nahas *et al*., 1996; Goldstein, 2000).

The results show a negative correlation between the final pH value and titratable acidity. However, the results with MAJP showed values for r (-0.96; -0.96; -0.90, $p< 0.001$) distinct from the results with NAJP (r = -0.001; -0.60; -0.60, $p< 0.001$), respectively for 0.5, 1.0 and 2% w/v RP.

The result obtained by Rashid *et al*. (Rashid *et al*., 2004; Deepa *et al*., 2010) show a positive correlation between organic acid excretion and P solubilization and a negative correlation between pH and P solubilization. Our results indicate a negative correlation between the quantity of P extracted from the phosphate (C, g/L) and the change of pH. The values of the correlation coefficients (r) were (-0.74; -0.85 and -0.65, $p< 0.001$) for NAJP and (-0.96; -0.97 and -0.94, $p< 0.001$) for MAJP at similar conditions.

Many of the calcium phosphates, including rock phosphate ores (fluorapatite, francolite), are insoluble in soil with respect to the release of inorganic P at rates necessary to support agronomic levels of plant growth (Goldstein, 2000). Gerretsen (1948) first showed that pure cultures of soil bacteria could increase the P nutrition of plants through increased solubility of Ca phosphates. Their solubility increases with a decrease of soil pH. Phosphate solubilization is the result of the combined effect of pH decrease and organic acid production (Fankem *et al*., 2006). Obviously, various chemical elements contained in phosphates are liberated concurrently with P during microbial solubilization. The fungus *Aspergillus niger* used in our investigations produced mainly citric acid and low concentrations of other organic acids. The dynamic variations of the medium conditions due to changes in *Aspergillus niger* metabolism and in chemical equilibria are probably the reason for the variations in the solubilized P concentrations ($P_2O_{5\ c.s.}$ and $P_2O_{5\ b.}$) observed throughout the incubation (Mendez *et al*., 2013). Vassilev *et al*. (1995) observed that decreases in soluble P in the fermentation medium were accompanied by decreases in titratable acidity and suggested that this resulted from the consumption of organic acid by fungus under conditions of C depletion. The data obtained in our work support this hypothesis since the decreases in titratable acidity apparently occurred after the 12th day of incubation (Figures 1, 2 and 3) in response to the beginning of a new growth cycle, when the fungus may have used part of the organic acid in the metabolism. The fact that the concentration of biomass increased constantly from the 1st to the 15th day (NAJP) or from the 1st to the 12th day (MAJP) may be a proof for this. At the same time the organic acids form complexes with metal ions like Ca, Fe and Al, liberating soluble phosphate. This fact is the other reason for the increasing titratable acidity.

The extent of P_2O_5 extraction in citric soluble form (α_2) decreased from 20.8% to 12.2% (NAJP) and from 23.4% to 20.5% (MAJP), from the 3rd to the 15th day and from the 2nd to the 12th day, respectively, when the concentration was 0.5% (Figure 1 and Figure 2). The same tendency can be seen for the other concentrations of RP used in the study (Figure 3 – Figure 6). Obviously, the bioconversion was performed in a complex heterogeneous system with simultaneously occurring biosynthetic and chemical reactions, with different velocities depending on the continuously changing concentrations of macro- and microelements, diffusion velocity, etc. Phosphorus-containing compounds and other ions (Ca^{2+}, Mg^{2+}, Fe^{2+}, Al^{3+}, K^+, Na^+, etc.) move to the liquid medium face after a defined period of bioconversion. As a result of the phosphate solubilization and a high Ca^{2+} ion concentration, a process of partial reprecipitation of P as slowly soluble and insoluble phosphates in citric acid together with biomass may possibly occur.

Gyaneshwar *et al*. (2002) suggested that the organic acid secreted can either directly dissolve the mineral phosphate as a result of anion exchange of PO_4^{3-} by the acid anion or can chelate both Fe^{3+} and Al^{3+} ions associated with phosphate. Complexing of

cations is an important mechanism in P solubilization if the organic acid structure favors complexation (Fox *et al*., 1990). Organic acids may form soluble complexes with metal ions associated with insoluble P and thus P is released (Rashid *et al*., 2004; Kim *et al*., 2005).

Some authors indicate that acid production is not the only reason for phosphate release into the medium (Pradhan and Sukla, 2005; Gyaneshwar *et al*., 2002). In certain cases phosphate solubilization is induced by phosphate starvation (Gyaneshwar *et al*., 1999). Buffering capacity of the medium reduces the effectiveness of PSB in releasing P from tricalcium phosphates (Stephen and Jisha, 2009).

CONCLUSIONS

The production of chemical phosphoric fertilizers is a highly energy-intensive process. On the other hand most of these fertilizers are transformed into insoluble compounds in the soil, unavailable for plants,. Thus, the dependence of fertilizer production on fossil energy, and the prospects of diminishing availability of costly input material for fertilizer production in years to come have obviously brought the subject of mineral phosphate solubilization to the forefront (Khan *et al*., 2007). It is well known that the high-grade rock phosphate reserves deplete continuously. Hence, the exploration of an alternative phosphate source like low-grade phosphate at low cost should be developed in the near future. These are important reasons to use phosphate–solubilizing organisms in agronomic practice as advocated by several researchers.

According to our investigations and the arguments mentioned above, the study with MAJP can be considered to be an alternative for intensification of P-utilization. The investigations indicate that almost complete extraction of P_2O_5 from Jordan Phosphorite in a form utilizable by plant can be achieved by using the phosphorus-solubilizing fungus *Aspergillus niger*. The results obtained can be employed to reduce the fertilizer used if a half dose of P-fertilizer mixed with biofertilizer is introduced into the soil. It is possible to achieve a double effect: the production cost is minimized and the net return maximized.

NOMENCLATURE

C	total concentration of soluble P_2O_5 (g/L)
C_1	concentration of soluble P_2O_5 in the first filtrate (g/L)
C_2	concentration of soluble P_2O_5 in the second filtrate (g/L)
C_3	concentration of soluble P_2O_5 in the biomass (g/L)
$P_2O_{5\ b}$	content of P_2O_5 in the dry biomass (g)
$P_2O_{5\ c.s.}$	content of citric-soluble P_2O_5 (g)
$P_2O_{5\ m.m.}$	content of P_2O_5 in the residue of mineral mass (g)
$P_2O5_{\ t.}$	content of total P_2O_5 (g)
$P_2O_{5\ w.s.}$	content of water-soluble P_2O_5 (g)
rpm	rotational speed (revolutions per minute)

Greek Letters

α	total extent of P_2O_5 extraction (% w/w)
α_1	extent of P_2O_5 extraction in water-soluble forms (% w/w)
α_2	extent of P_2O_5 extraction in citric-soluble forms (% w/w)
α_3	extent of P_2O_5 extraction in biomass (% w/w)

Abbreviations

JP	Jordan Phosphorite
MAJP	Mechanically Activated Jordan Phosphorite
NAJP	Non-Activated Jordan Phosphorite
OLS	Ordinary Least Squares
RP	Rock Phosphate
PSM	Phosphate-Solubilizing Microorganisms
PSB	Phosphorus-Solubilizing Bacteria

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