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Solubilization of Phosphorus by Isolated Fungus of Iron ore Tailings

Glalber Luiz da Rocha Ferreira[1]*, José DG Vieira[1], Lilian C Carneiro[1] and Geraldo Sadoyama[2]

[1]Laboratory of Environmental Microbiology and Biotechnology. Institute of Tropical Pathology and Public Health. Federal University of Goiás. Cep 74605-050, Goiânia, GO, Brazil

[2]Department of Biological Sciences CAC - UFG. Federal University of Goiás. Cep 75704-020, Catalão, GO, Brazil

ABSTRACT

In view of the high technological expectations in the mining sector today, an inability of miners to reach their totality in the use of minerals present in nature is verified, and this is caused primarily by the use of methods considered fallible in the mining process. In view of this condition, it is necessary to develop new technologies with innovative behavior, seeking more productive forms. Among the existing technologies, a study with a technological and promising view is proposed, which deals with the process of phosphorus biosolubilization from a synthetic source considered insoluble by a fungus isolated from iron ore. Genetic tests revealed that the microorganism belongs to the *Aspergillus* genus, that is, the *Aspergillus terreus* species. The analytical tests proposed in this study pointed to a solubilization of 90.93 % of phosphorus by fungal biomass, which corresponds to 296.56 mg L^{-1} of soluble phosphorus, occurring in a 168 hour process, accounting for an average solubilization of 1,76 mg h^{-1}. The biosolubilization process proved to be a great technological alternative in reducing production costs and establishing the sustainability of the mineral extraction system, being a viable alternative in purifying and adding value to iron ore, and also in obtaining another compound consisting of phosphorus, generating a new economic source, as an example in the production of fertilizers.

***Corresponding author**

Glalber Luiz da Rocha Ferreira, Department of Microbiology, Institute of Tropical Pathology and Public Health, Federal University of Goiás-UFG. Goiânia, Goiás / Brazil. Rua Delenda Resende de Melo Left / 1. Avenida S / N Laboratory of Environmental Microbiology and Biotechnology, University Sector, CEP 74605-050. Tel: (62)99113-9140; E-mail: glalberocha@gmail.com

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Introduction

Iron ore is considered the main raw material in the steel industry, acting directly in the manufacture and transformation of pig iron and steel [2]. Brazil, as well as Western Australia, South Africa and south-central China, are highlighted for having an enormous amount of iron resources, more with high phosphorus content, in many areas of iron ore production [3]. Iron ore is generally designated as having a high or low grade based on its iron content, with high quality ores, which are found to contain less than 0.07% phosphorus, being considered commercially as most desired, and for this reason they are running out very quickly, leaving in the future the exploration and processing of low-grade iron ores [4,5].

As it is not known exactly how phosphorus is found in iron ore, it is deduced that phosphorus is present in the ore in the form of phosphate, adsorbed on the surface of the particle or occluded in the micropores. Another possibility also suggested is that phosphorus is within the structure of oxyhydroxides or as a phosphate mineral. In Brazil, wavellite, $Al_3(PO_4)_2(OH)_3.5(H_2O)$; senegalite, $Al_3(PO_4)(OH)_3(H_2O)$; turquoise, $CuAl_6(PO_4)_4(OH)_8.0,5(H_2O)$, are the main phosphates found in iron ore [6].

In the iron ore steelmaking process, phosphorus is highlighted as being a harmful element in the steel production process, and thus significantly damaging the quality of steel products, such as increasing hardness, fragility and consecutively ductility [3]. In other industrial segments, phosphorus is seen as an essential element, such as in the production of fertilizers, automobiles, electronics, medicines, fuel cells and processed foods [1].

In order for us to have the continuous availability of iron ore, it is essential to develop new systems and methods for the exploitation of low quality iron ore, which are unusable and unexplored, and thus, contributing in the future with the supplementation of reserves that smaller in high-grade iron ore [7].

Iron ore dephosphorization is an important challenge for the sustainable development of the steel industry, and for this reason, efforts have been intensified in the search for new technologies to remove phosphorus from iron ore a acceptable levels [4]. There are several processes used to remove phosphorus from iron ores, including roasting magnetization, direct reduction based on coal, heat treatment, microwave treatment, ultrasonic treatment, acid leaching Cai, alkaline leaching, selective agglomeration, and flotation [4,8,9].

Considering the biosolubilization process, it is seen as a biological treatment carried out by microorganisms, which, through their metabolic reactions, generate chemicals such as mineral acid, organic acids, polymers and enzymes. These chemical by-products attack the tailings contained in the ore, promoting its dissolution and producing its selective removal [10]. There are several microorganisms that have been used with considerable efficiency

in phosphate solubilization, such as bacteria and fungi [8,11- 20].

As a general rule, edaphic microorganisms that are added to the ore, react with better efficiency, and this is due to a better adaptation and less ecological distortions of the environment. For this reason, this study will use a filamentous fungus, extracted from raw iron ore, aiming to dephosphorize iron ore and biosolubilize phosphorus in liquid medium, and thus, contributing to the viability of new biotechnological studies.

Experimental Part

The iron ore tailing sample obtained came from a mining company, located in the city of Catalão, Goiás. Approximately a 10 kg portion of the waste generated under the mining company's soil was separated for later isolation of biological material and conducting studies of phosphorus solubilization.

Means of growth and enrichment

The microorganism was obtained from the inoculation of the iron ore tailing sample in four different enrichment media, which are the following media: NBRIP, 9K-Glucose, 9K and general purpose, as shown in Table 1. All media were dissolved in distilled water, and sterilized at 121 ºC for 20 minutes.

Microbial growth and enrichment

To obtain the microorganism from the iron ore sample, 5 g of the iron ore tailing sample was inoculated into the four culture media, as shown in Table 1, in Erlenmeyers flasks containing 50 mL of the medium. The incubation occurred for a period of seven days in an incubator under the conditions of 30 ± 2 ºC and 130 rpm. After incubation, the inoculation was repeated by removing an aliquot of 1 ml of each medium for the same four new media in Erlenmeyers presented in Table 1, containing 5 g of iron ore sample and sterilized at 121 ºC for 20 minutes. He led them back to the incubator under the same conditions as described above. The same procedure was repeated two more times, completing three stages of enrichment.

Isolation of the microorganism

The microorganisms belonging to the third enrichment stage were transferred by inoculating 100 µL of the enrichment medium in Petri dishes containing the four culture media shown in Table 1, plus 1.7% agar and 0 , 5% calcium phosphate, $Ca_5(OH)(PO_4)_3$, as a phosphorus source, sterilized at 120 ºC for 20 minutes, and incubated at 30 ± 2 ºC, for 10 days. The microorganism considered predominant, morphologically distinct and phosphorus solubilizer, showing the formation of a clear halo at the edges, was selected and purified in Petri dishes with medium containing 1.7% agar and 0.5% agar. calcium phosphate, $Ca_5(OH)(PO_4)_3$, and incubated at 30 ± 2 ºC, for 7 days. This procedure was repeated for two more times, completing three stages of isolation of the microorganism. After incubation, the isolate was preserved by cultivation in Petri dishes with PDA medium, being incubated at 30 ± 2 ºC, for 7 days, and kept refrigerated at 4 ºC until its next use.

Fungal DNA extraction

DNA extraction was tested using the mycelium as biological material. A small fragment of the fungal mycelium was inoculated, grown in PDA medium, in a test tube containing 10 mL of Malt Extract Broth (MEB), which consists of: 2% malt extract, 2% glucose and 1% glucose. peptone. The inoculated MEB was incubated for 72 hours at 30ºC, in the dark and shaking at 130 rpm. The mycelium was collected by vacuum filtration on a sterile cellulose filter and then washed twice with 10 ml of 0.85% NaCl. The mycelium was collected and used for DNA extraction. DNA extraction was performed using the SDS Lysis Buffer solution, as described in [21]. The DNA extract was resuspended in 100 µL of ultra pure water and stored at -20ºC.

DNA amplification and purification

The extracted DNA was used for PCR amplification in the ITS1-18S-ITS2 region, using primers ITS1 (5'-GAACCWGCGGARGGATCA-3') and ITS2 (3'-GCTGCGTTCTTCATCGATGC-5') [22,23]. The amplifications were performed in a total volume of 20 µL containing 50 ng of extracted DNA, 4 µL of HOT MOLPol Blend Master Mix (Molegene, Germany), and 0.5 µM of each of the primers (F) forward and (R) reverse. The amplification was carried out in a thermocycler programmed for an initial denaturation of 15 min at 95 ºC, followed by 35 cycles from 30 s to 95 ºC, 30 s at 55ºC and 30 s at 72 ºC. The annealing was carried out with a temperature of 52 ºC for 30 s in triplicate. The final stretching was done at 72 ºC for 5 min. Purification was done using Agencourt AMPure XP SPRI magnetic spheres. The PCR products were normalized after quantifying them with a Qubit 2.0 fluoromer (Invitrogen), with the Qubit dsDNA HS test kit (Invitrogen).

DNA sequencing

The amplified DNA fragments were sequenced using the MiSeq Sequencing System (Illumina Inc., USA), using together the Kit V2, with 300 cycles and single-end sequencing. The sequences were analyzed using the Sentinel pipeline. In the Sentinel pipeline, the fastq files were evaluated for Phred quality (QP) using the FastQC v.0.11.8 program [24]. Then, the fastq files were subjected to the trimming of primers and sequences with low quality (QP & lt; 20). The proprietary software used for this purpose was built in Python v.3.6, which was inspired by the features of the BioPython project [25]. The BLAST software tool, available at: http://blast.ncbi.nlm.nih.gov/Blast. cgi, was used to compare the similarities of the nucleotide acid sequence with the sequences of the ITS1-18S-ITS2 region of fungal species deposited with GenBank.

Phosphorus solubilization in synthetic medium

For the biosolubilization process, calcium phosphate, $Ca_5(OH)(PO_4)_3$, was used as a known source of phosphorus. In view of this, twenty-two Erlenmeyers flasks containing 1.7631 g of $Ca_5(OH)(PO_4)_3$ were prepared in 100 mL of solution in the NBRIP medium, corresponding to 1000 mg L-1 of phosphate or 326.3 mg Phosphorus L-1, with pH 7.0 ± 0.2 sterilized at 121 ºC for 20 minutes. Then, 3 fragments of 4 mm of the inoculum grown in Petri dishes were inoculated in each Erlenmeyer flask, and incubated at 30 ± 2 ºC and 130 rpm for 7 days. One Erlenmeyer flask was removed on the day, corresponding to time zero, and three Erlenmeyer flasks within 24 hours. After the incubation, samples were collected from each Erlenmeyer flask, evaluating for the content of soluble phosphorus, using the Spectro kit (Alfakit); the acid phosphatase activity, using the commercial kit (Labtest®); biomass production by measuring dry mass content and hydrogen potential.

Results and Discussion

The microorganism isolated from the iron ore tailing sample, and grown in a medium enriched with a known source of phosphorus, $Ca_5(OH)(PO_4)_3$, considered predominant in solid medium, morphologically distinct and phosphorus solubilizer, being in the latter forming a clear halo at the edges of the colony, proved to be a fungus in terms of genetic analysis.

Through genome sequencing was obtained from a nucleotide sequence (TTACCGAGTGCGGGTCTTTATGGCCCAACCT CCCACCCGTGACTATTGTACCTTGTTGCTTCG GCGGGCCCGCCAGCGTTGCTGGCCGCCGGG GGGCGACTCGCCCCCGGGCCCGTGCCCGCCG GAGACCCCAACATGAACCCTGTTATGAAAGC TTGCAGTCTGAGTGTGATTCTTTGCAATCAG TTAAAACTTTCAACAATGGATCTCTTGGTTCCG) of the regions of interest (ITS) IT-ITS1-18S ITS2 region, the sequence being subjected to all the species level identification by means of comparison with sequences deposited in the database Genbank, which made it possible to identify the fungal species as Aspergillus terreus.

In a previous test, the fungus Aspergillus terreus was subjected to enrichment and growth in NBRIP media, general use, 9K-glucose and 9K, as shown in Figure 1. A. terreus isolated from the iron ore sample showed great growth potential in previous test in the NBRIP medium, and thus, being defined as the main means of growth and development of the phosphorus biosolublization process in this study, thus represented in Figure 1A. The ability to solubilize phosphorus by the fungal strain in the NBRIP medium remained at a high level over 7 days, favoring the use of the NBRIP medium in the process in an efficient way to solubilize phosphorus.

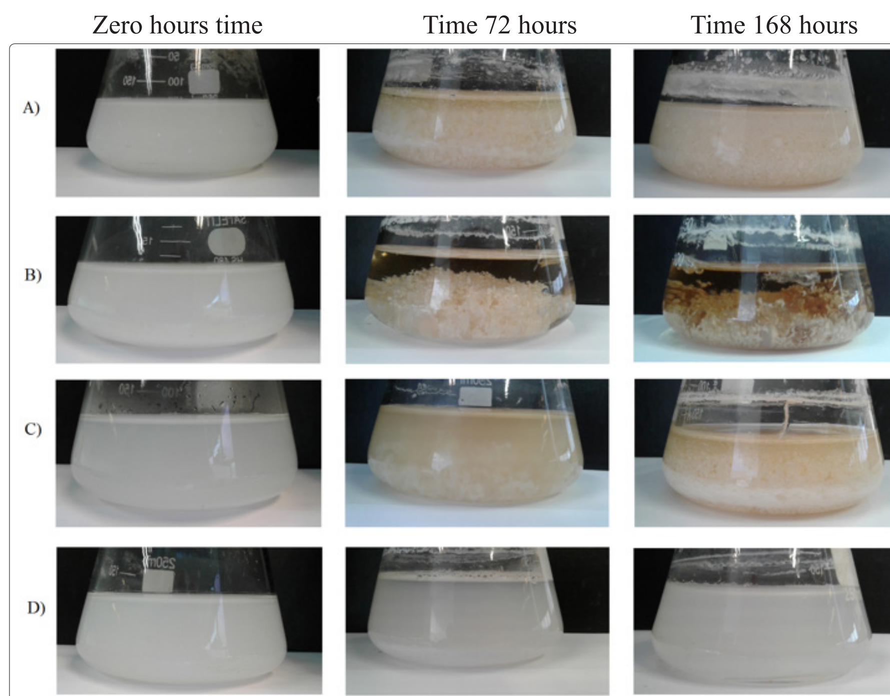

Figure 1: Evolution of fungal biomass in 168 hours in NBRIP (A), general use (B), 9K-glucose (C), and 9K (D) media

The Aspergillus terreus fungus isolated from the iron ore sample showed a potential for phosphorus solubilization capacity in a medium enriched with calcium phosphate, $Ca_5(OH)(PO_4)_3$, as shown in Table 2. A maximum biosolubilization of 908 was observed, 85 mg L^{-1} of phosphate or 296.56 mg L^{-1} of phosphorus by the fungus, contributing to the total solubilization of 90.93% of the phosphorus present in the medium enriched with calcium phosphate. Considering the maximum amount of phosphorus solubilized by the fungal biomass and the maximum process time, an average solubilization of 1.76 mg h^{-1} of phosphorus by the biosolubilization process. This allows us to conclude that approximately 182.13 mg of phosphorus per gram of calcium phosphate sample was solubilized.

From Figure 2A, an important interaction of Aspergillus terreus is observed in the process of converting insoluble phosphorus into its soluble form, with a marked solubilization of the mineral element phosphorus present in the iron ore sample, and thus, the stabilization is perceived. between 144 and 168 hours of phosphorus solubilization.

As shown in Table 2, the fungus Aspergillus terreus resulted in a final mycelial mass production of 1544.4 mg of biomass, being represented significantly by a progressive increase in the fungal mycelium mass. Figure 2B corroborates with the confirmation of the efficient capacity in the production of biomass tangentially to the solubilization of phosphorus, with an important jump observed in the first 48 hours in the biomass content. The speed of development of fungal biomass occurred similarly to the production of biomass in the first 48 hours, demonstrating an increase in the speed of up to 31.1 mg h^{-1}, after which there was a reduction in this speed, which is ascertained by the reduction of assimilation capacity of the source of insoluble phosphorus present in the medium. Paralleling the amount of solubilized phosphorus and the quantitative production of fungal biomass, a solubilization of approximately 0.19 mg of phosphorus per gram of biomass produced was verified.

Table 1: Elementary constitution of means of enrichment and growth

Growth Means	Concentration in g 100 mL^{-1}										Reference
	Glicose	$MgCl_2.6H_2O$	$MgSO_4.7H_2O$	$(NH_4)^2SO_4$	$Ca(NO_3)_2$	$FeSO_4.7H_2O$	KCl	NaCl	NH_4Cl	pH	
NBRIP	1,0	0,5	0,025	0,01	-	-	0,02	-	-	7.0 ± 0,2	Nautiyal, 1999.
9K – Glucose	1,0	-	0,05	0,3	0,0013	0,001	0,01	-	-	4,5 ± 0,2	Silverman e Lundgren, 1959.
9K	-	-	0,05	0,3	0,0013	0,001	0,01	-	-	4,5 ± 0,2	Silverman e Lundgren, 1959.
General use	1,0	-	0,1	-	-	-	-	0,1	0,5	7,2 ± 0,2	Verma et al., 2001.

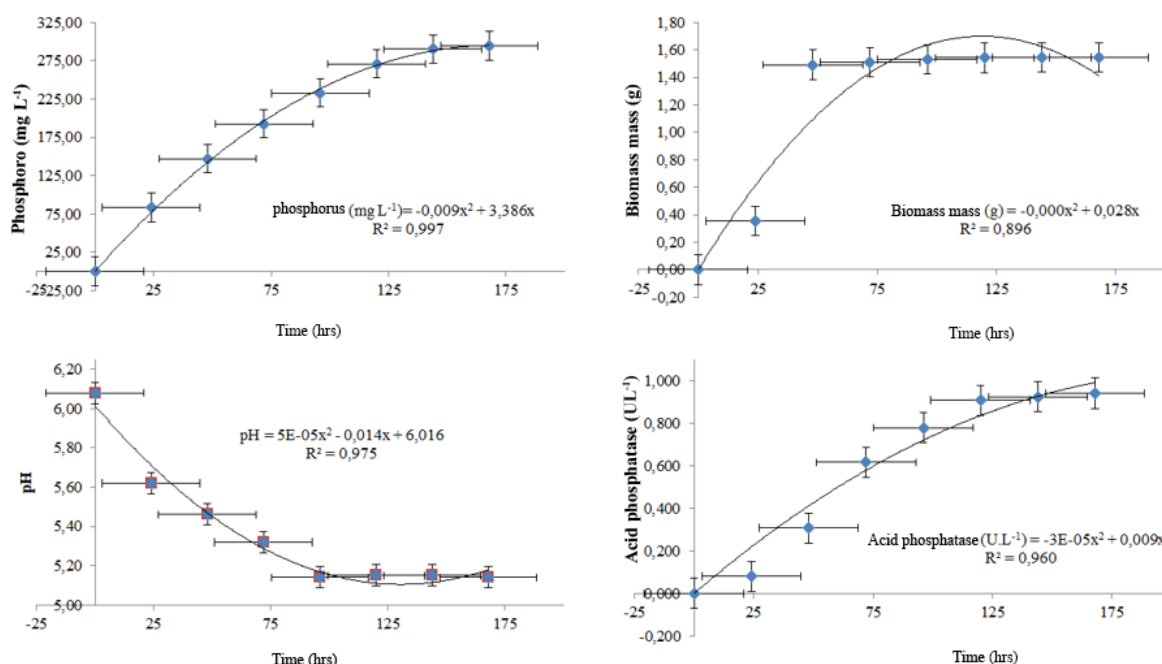

Figure 2: Graphical representation of physical-chemical tests to verify the phosphorus solubilization potential

Both pH and acid phosphatase contributed to the phosphorus solubilization process by the fungus Aspergillus terreus, representing an excellent quality marker of the biosolubilization process [26]. report that the primary mechanisms in phosphorus solubilization carried out by both plants and microorganisms are exercised through the excretion of H^+, the production of organic acids, and acid phosphatase biosynthesis. The results presented in Table 2, as also shown by Figure 2C and 2D, demonstrate exactly the correlation of the primary mechanisms of phosphorus solubilization, by decreasing the hydrogen potential (pH) and increasing the enzymatic activity of acid phosphatase, contributing to the dissolution efficiency of the insoluble phosphorus present in the calcium phosphate enriched medium. The increase in the enzymatic activity of acid phosphatase shown in Table 2, is discussed by Mónica et al., [27], As the main mechanism of phosphorus mineralization in the soil, which also has its optimal pH activity. Other works such as Govarthanan et al. and Adhikari and Pandey, argue that the release of acid phosphatase is directly related to the production of organic acids, and consequently by reducing the pH, through the release of protons, and so, being considered the main mechanism used in the solubilization of phosphorus in the soil [9,28].

According to Behera et al., organic acids can form complexes with cations (Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}), contributing to the release of phosphorus, either by chelation reaction or simple exchange [24]. It is evident that the increase in acidity contributes to the accessibility of phosphorus in the liquid medium, making the phosphorus that was previously insoluble in its soluble form, which also due to the identity of the fungus isolated from the iron ore tailings proved to be great development capacity in an inhospitable condition of acidity, more than contributing to an efficient mobilization and solubilization of insoluble phosphorus compounds.

Table 2: Evolution of the potential and verification of the mechanisms of phosphorus solubilization by the fungus strain

Time (hrs)	[PO43-] soluble (mg L^{-1}) ± SD	[P] soluble (mg L^{-1}) ± SD	P reduction in iron ore%	Biomass production (mg) ± SD	Growth rate (mg h^{-1})	pH ± DP	Acid phosphatase (U L^{-1}) ± SD
0	0,00 ± 0,00	0,00 ± 0,00	0,00	0,0 ± 0,0	0,0	6,08 ± 0,000	0,000 ± 0,000
24	255,16 ± 6,10	83,26 ± 1,99	25,53	354,7 ± 0,2	14,8	5,65 ± 0,035	0,086 ± 0,041
48	456,42 ± 4,72	148,93 ± 1,54	45,67	1491,6 ± 0,6	31,1	5,43 ± 0,025	0,395 ± 0,082
72	590,15 ± 3,50	192,57 ± 1,14	59,05	1511,8 ± 1,4	21,0	5,29 ± 0,025	0,719 ± 0,098
96	717,56 ± 3,99	234,14 ± 1,30	71,80	1538,7 ± 1,3	16,0	5,14 ± 0,020	0,751 ± 0,092
120	839,32 ± 8,98	273,87 ± 2,93	83,98	1542,1 ± 1,0	12,9	5,13 ± 0,015	0,811 ± 0,049
144	889,88 ± 3,05	290,37 ± 0,99	89,04	1544,0 ± 0,8	10,7	5,13 ± 0,021	0,822 ± 0,050
168	908,85 ± 6,02	296,56 ± 1,96	90,93	1544,4 ± 1,1	9,2	5,13 ± 0,006	0,832 ± 0,025

DP – Standard Deviation.

Conclusion

This study confirmed that the fungus Aspergillus terreus isolated from iron ore tailings showed great potential for solubilization of insoluble phosphorus, and thus, being proven through the results of physical-chemical attributes, such as pH and acid phosphatase, such as main mechanisms of phosphorus mineralization in the tailings. These attributes demonstrated an important correlation tool both in the development of the fungal biomass of Aspergillus terreus and in the efficiency of phosphorus solubilization in the reaction medium. It is suggestive that other works should be carried out aiming at the removal and or use of phosphorus contained in the reaction medium, in the application of different biotechnologies, as an example, in biofertilization.

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Conflict of Interest

The authors declare no conflict of interest.

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