POTENTIAL FOR RELEASE OF ORGANIC AND INORGANIC PHOSPHORUS BY *Trichoderma harzianum* USING SOLID-STATE FERMENTATION

Natalia A. Rodrigues[1], Jana Font[2], Esther M. Peñate[2], Arnau Sala[2], Adriana Artola[2], Fernanda P. Casciatori[1], Cristiane S. Farinas[13]*

[1] *Graduate Program of Chemical Engineering, Department of Chemical Engineering, Federal University of São Carlos, São Carlos, Brazil.*
[2] *Composting Research Group, Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain.*
[3] *Embrapa Instrumentação, São Carlos, Brazil.*
** Corresponding author's email address: cristiane.farinas@embrapa.br*

ABSTRACT

This study aimed to investigate the release of organic and inorganic phosphorus (P) by *Trichoderma harzianum* cultivated under solid-state fermentation in agroindustrial residues. A 2^2 factorial design with 3 repetitions at the central point was conducted, considering the variables of ethanol and struvite contents in cultivation media. At the concentrations tested, struvite showed a negative effect on all evaluated responses, whereas ethanol exhibited a positive effect on acid phosphatase activity. The results revealed elevated final pH values in all evaluated conditions, indicating that the addition of ethanol did not stimulate greater production of organic acids. Consequently, P solubilization efficiencies in conditions containing struvite were less than 15%. On the other hand, ethanol addition increased acid phosphatase activity, reaching 4.6 U per $gram^{-1}$ of dry substrate (gds^{-1}), with significant biomass growth of $3x10^9$ spores gds^{-1}. Therefore, this condition holds promise for providing enzymes capable of releasing organic P, spores of a biocontrol agent, and readily available P for plant growth.

Keywords: Solid-state fermentation. *Trichoderma harzianum*. Phosphorous solubilization. Acid phosphatase.

1 INTRODUCTION

Phosphorus (P) is an essential macronutrient for plant development and is one of the most limiting elements for crop production due to its low mobility in the soil under natural conditions. Currently, commercial P fertilizers are primarily derived from phosphate rock reserves, which require an energy-intensive treatment involving strong acids for their production. However, this process generates undesirable contaminants, leading to significant environmental concerns[1]. Moreover, conventional P fertilizers result in substantial P losses through leaching, contributing to the eutrophication of local water bodies. With global phosphate ore reserves estimated to deplete within the next 50−100 years, it is mandatory to recover P from secondary resources such as agro-industrial wastes and wastewater, contributing to the circular economy[2].

In this context, solid-state fermentation (SSF) emerges as a promising alternative, offering the potential to mineralize P from agro-industrial waste through the activity of phosphate-solubilizing microorganisms, thus rendering it accessible to plants. These microorganisms can liberate organic P through enzyme production and inorganic P primarily through organic acid production. Struvite ($MgNH_4PO_4 \cdot 6H_2O$) is also an interesting source of phosphorus to be explored, as it is a low-solubility mineral that can be obtained from wastewater treatment. The addition of struvite to the solid matrix can provide nutrients for the development of the microorganisms and its solubility can be increased due to the production of organic acids[1]. In this context, the utilization of low molecular weight alcohols is also intriguing, as studies indicate that their inclusion can enhance the permeability of the cell membrane, thereby facilitating the excretion and accumulation of metabolites, such as organic acids[3,4].

Among phosphate-solubilizing microorganisms, the genera *Bacillus*, *Aspergillus*, *Penicillium*, and *Trichoderma* stand out[5]. *Trichoderma* strains are well-known as biocontrol agents, predominantly used as biostimulants; however, their potential for P release remains underexplored. Thus, prospecting environmentally friendly alternatives to improve the content of available phosphorus in soils, this study aimed to explore the release of organic and inorganic phosphorus by *Trichoderma harzianum* through SSF, along with examining the impact of ethanol addition. For this purpose, wheat straw and beer draff were used as solid substrates and struvite as a source of low solubility phosphorus.

2 MATERIAL & METHODS

2.1 Microorganisms and solid matrix

The fungal strain *Trichoderma harzianum* CECT 2929 was utilized, provided by the Spanish Type Culture Collection (CECT). The original strain was preserved at − 80°C in sterile cryovials containing 10% glycerol, as established by the provider of the strains. The fungal strain was cultured on plates with malt extract agar (MEA) at 25°C for 6–8 days before use. Subsequently, the agar surface was gently scraped with 10 mL of Tween 80 0.1% to obtain the spore suspension, and the spore concentration was determined using a Neubauer chamber.

As solid substrates, agro-industrial wastes wheat straw (from Universitat Autònoma de Barcelona (UAB experimental farms) and beer draff (Cervesa del Montseny S.L., Sant Miquel de Balenyà) were used. They were chosen for their low cost, high local availability and proven ability to promote high sporulation for this *Trichoderma* strain[6]. The beer draff was stored frozen (-20ºC), while the wheat straw was stored at room temperature (20-25°C). Before use, both substrates were autoclaved at 121°C for 30 minutes. Struvite was also added in some tests as a low solubility P source, kindly provided by Sostenipra Group (ICTA-UAB) from a WWTP in Denmark and ground into a powder before use. The substrates (beer draff and wheat straw) and the struvite were characterized for total P content using ICP-OES spectrometry by the chemical analysis service of the UAB, with the obtained values being 0.47%, 0.07%, and 13.90% (w w^{-1}) respectively.

2.2 Solid-state fermentation

Solid-state fermentation (SSF) was carried out in 0.5 L Erlenmeyer flaks. In each flask, 12.25 grams of dry substrate (gds) and the *Trichoderma* spore suspension (10^7 spores gds^{-1}) were added. The substrates proportion was 3:7 wheat straw:beer draff in dry matter basis, resulting in a humidity of 65%, which is adequate to promote good growth for this fungal strain[6]. The fermentations were conducted at 25°C for 5 days in BOD chamber, except for the time course, which was extended for 6 days.

A factorial design 2^2 with 3 replicates at the central point was employed, considering the variables struvite and ethanol. The low levels were set at 0% for both variables and the high levels were 20% (w w^{-1}) and 10% (v w^{-1}) for the struvite and ethanol, respectively.

2.3 Analytical procedures

At the end of the SSFs, the fermented solids (FS) were extracted with Tween 80 0.1% (9 mL g^{-1} of FS) to evaluate the spore production, distilled water (5 mL g^{-1} of FS) to assess pH and soluble P, and sodium acetate buffer pH 4.8 (15 mL g^{-1} of FS) to measure acid phosphatase activity. The mixtures were agitated for 30 minutes in an orbital shaking incubator.

To determine spore production, the extracts were diluted and plated on PDA agar containing 0.1 g/L of Rose Bengal. After incubation at 30°C for 2 days, the colonies were counted. Soluble P was measure using the 115 VAC PHOSPHAX sc, Hach-Lange, and the P solubilization efficiency was calculated considering the initial total P from struvite and the substrates in each condition. Acid phosphatase activity was measured as quantity of p-nitrophenol liberated from 5 mM p-nitrophenyl phosphate (p-NPP) in sodium acetate buffer (pH 4.8) at 37°C. After 10 minutes the reaction was stopped by adding 1 ml 0.1 M NaOH[7]. The amount of p-nitrophenol liberated was measured at 410 nm. One unit (U) of acid phosphatase activity was defined as 1 µmol of p-nitrophenol liberated per minute under the assay conditions.

3 RESULTS & DISCUSSION

Table 1 presents the results of the factorial design conducted, revealing a significant negative effect of struvite and the interaction of struvite with ethanol, while ethanol exhibited a significant positive effect on acid phosphatase production and P release efficiency. Both struvite and ethanol also showed a significant negative impact on spore production.

Table 1 Effects and p-values of the variables analyzed in the factorial design performed to evaluate the influence of ethanol and struvite addition on the phosphorus solubilization efficiency, acid phosphatase production and sporulation by *Trichoderma harzianum*.

Terms	Phosphorus solubilization efficiency		Acid phosphatase activity		Sporulation	
	Effect	p-value	Effect	p-value	Effect	p-value
Struvite (S)	-16.713	0.003	-0.153	0.049	-4.461×10^9	0.003
Ethanol (E)	5.362	0.026	2.692	0.000	-5.490×10^9	0.002
SxE*	-4.945	0.030	-1.257	0.001	1.967×10^9	0.015

*Interaction between S and E.

The negative effect of struvite on phosphorus solubilization efficiency can primarily be explained by the high final pH values, as depicted in Fig. 1a, indicating low production of organic acids, which are essential for solubilizing inorganic P sources. Although ethanol demonstrated a positive effect, its addition did not direct metabolism towards greater synthesis of organic acids, as observed in other cases such as *Aspergillus niger* for citric acid production[4].

On the other hand, the negative effect of struvite on phosphatase production may primarily result from the presence of soluble P, which can hinder the induction of phosphatase synthesis[8]. Analysis revealed that approximately 27% of the total initial P present, from both substrates and added struvite, existed in the form of P_2O_5. Additionally, the addition of struvite at the concentrations tested led to higher concentrations of N and Mg in the culture medium, potentially causing nutritional imbalances or osmotic stress, negatively affecting metabolite production. However, lower concentrations could be explored in order to avoid this effect. In contrast, the positive effect of ethanol is primarily related to its ability to increase cell membrane permeability[3,4].

According to Fig. 1a, the highest acid phosphatase production was achieved with the maximum addition of ethanol (10% v/v) and without struvite, resulting in a value of 4.6 U gds^{-1}. In this condition, 31% of the phosphorus present in the substrates was released, as shown in Fig. 1b. Despite being negatively affected by both variables, sporulation was expressive, reaching a value of 3×10^9 spores gds^{-1}. Sala et al. (2021)[6] reported a maximum sporulation of *T. harzianum* of 7.5×10^9 spores g^{-1} of dry matter through solid-state fermentation in 0.5 L packed bed bioreactors using beer draff as a substrate.

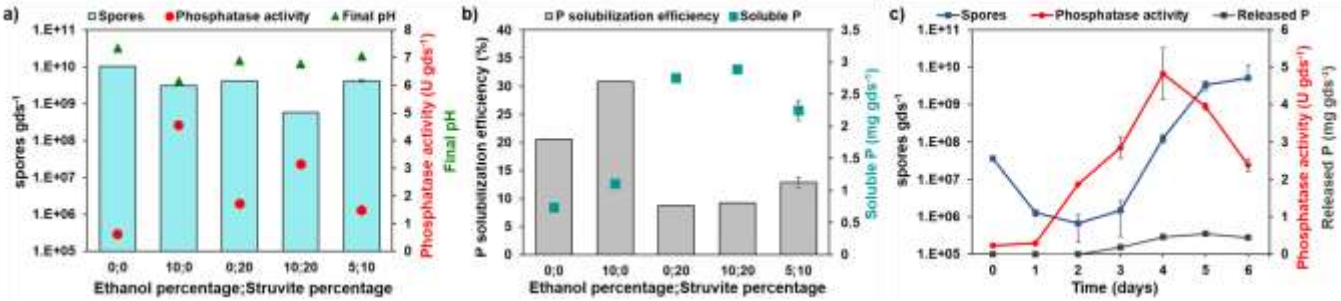

Figure 1 Factorial design results: a) spore production, acid phosphatase activity, final pH; b) soluble phosphorus and phosphorus solubilization efficiency. c) Temporal profiles of spore production, acid phosphatase activity, and phosphorus release with 8% ethanol addition and no struvite addition.

There are few studies in the literature on the production of phosphatases by *T. harzianum* through solid-state fermentation. Busato et al. (2020)[9] investigated acid phosphatase production during a vermicomposting process enriched with phosphate rock, observing a maximum production of 3 U g^{-1} of vermicompost when simultaneously inoculating *Trichoderma asperellum* and *Trichoderma virens*. Mercl et al. (2020)[10] studied soil phosphorus availability following inoculation with *Penicillium* sp. and *T. harzianum*, reporting an increase in acid phosphatase activity in the soil by *T. harzianum*, with a maximum value of 2.4 U g^{-1} dry matter. Lima et al. (2022)[11] evaluated acid phosphatase production by different *Trichoderma* species through submerged fermentation using soybean molasses as a culture medium, achieving a maximum activity of 2.5 U mL^{-1} with *T. harzianum* as inoculum. In this context, the production obtained in our work, 4.6 U gds^{-1}, demonstrates the significant potential of the studied strain for releasing organic P into the soil.

Thus, we aimed to evaluate the sporulation profile, organic phosphorus release, and phosphatase production under the most favorable condition identified. However, the addition of 10% ethanol proved to be critical, as slightly higher concentrations may be detrimental to the microorganism's development. Therefore, it was decided to evaluate an ethanol concentration of 8%, as depicted in Fig. 1c. Considering that high sporulation is desired, as well as high levels of enzyme and released phosphorus, the profiles indicate that a cultivation time of 5 days under the evaluated conditions is most suitable, resulting in a sporulation of $3x10^9$ spores gds^{-1}, a phosphatase activity of 4.0 U gds^{-1}, and a P release of 0.5 mg gds^{-1}, corresponding to a release efficiency of 31%.

4 CONCLUSION

In this study, we investigated the influence of ethanol and struvite addition on the release of organic and inorganic phosphorus by *Trichoderma harzianum* from agro-industrial waste through solid-state fermentation. Our findings revealed that ethanol does not stimulate the production of organic acids by *T. harzianum* and that its release of inorganic P is low. However, the addition of ethanol appears to delay sporulation and increase acid phosphatase excretion. Therefore, the evaluated strain proved to be effective in releasing organic P, and the addition of ethanol allowed to achieve a condition with high contents of enzyme, spores, and organic P released.

REFERENCES

1 VALLE, S. F. GIROTO, A. S. DOMBINOV, V. ROBLES-AGUILAR, A. A. JABLONOWSKI, N. D. RIBEIRO, C. 2022. Sci. Rep. 12 (1). 14176.
2 WANG, L. GUAN, H. HU, J. FENG, Y. LI, X. YUSEF, K. K. GAO, H. TIAN, D. 2022. J. Agric. Food Chem. 70 (35) 10738-10746.
3 USAMI, S. TAKETOMI, N. 1961. Hakko-Kyokaishi. 19 435–442.
4 ANWAR, S. ALI, S. SARDAR, A. A. 2009. SURJ (Science Series). 41 (1) 1–8.
5 RAWAT, P. DAS, S. SHANKHDHAR, D. SHANKHDHAR, S. C. 2021. JPNSS. 21 (1) 49-68.
6 SALA, A. VITTONE, S. BARRENA, R. SANCHEZ, A. ARTOLA, A. 2021. J. Environ. Manage. 295 113113.
7 TABATABAI, M. A. BREMNER, J. M. 1969. Soil Biol. Biochem. 1 (4) 301-307.
8 VASSILEV, N. VASSILEVA, M. BRAVO, V. FERNÁNDEZ-SERRANO, M. NIKOLAEVA, I. 2007. Ind. Crops Prod. 26 (3) 332-336.
9 BUSATO, J. G. FERRARI, L. H. CHAGAS JUNIOR, A. F. SILVA, D. B. PEREIRA, T. S. PAULA, A. M. 2021. J. Appl. Microbiol. 130 (4) 1208-1216.
10 MERCL, F. GARCÍA-SÁNCHEZ, M. KULHÁNEK, M. KOŠNÁŘ, Z. SZÁKOVÁ, J. TLUSTOŠ, P. 2020. Appl. Soil Ecol. 147 103360.
11 LIMA, F. A. ROLA, J. C. FREITAS, M. M. G. ALMEIDA AFONSO, J. M. M., RESENDE, M. M. 2022. Chem. Eng. Technol. 45 (5) 979-984.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support provided by the São Paulo State Research Foundation (FAPESP, grants #2019/25261-8 and #2016/10636-8), the Brazilian National Council for Scientific and Technological Development (CNPq, grants 441573/2023-1, 402713/2023-0, 303017/2022-8 and 401089/2022-3), the Coordination for the Improvement of Higher Education Personnel (CAPES, Finance Code 001), the Agronano Network, the Brazilian Agricultural Research Corporation (Embrapa) and the GICOM group at the Universitat Autònoma de Barcelona for the exchange opportunity.