February 20th, 2020

Ref: MS No.: soil-2019-50. Title: Adsorption to soils and biochemical characterization of purified phytases.Maria Marta Caffaro et al.

Dear Topical Editor
Dr Jeanette Whitaker
SOIL Journal

We would like you and the anonymous reviewers for their valuable suggestions which helped us to greatly improve our ms. We have followed each one of the suggestions and the detailed responses and changes made to the original manuscript are given below and included in the new manuscript. If additional modifications are required, please let us know.

We are uploading a Word file with all the modifications included and a second file with the annotated version.

Regards,

Dr. Gerardo Rubio
School of Agriculture - University of Buenos Aires

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Detailed response to the Editor and the reviewers:

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Topical Editor

1. Thank you for your detailed responses to the reviewers comments. There are quite a number of substantive changes and improvements which the reviewers recommend to improve the clarity and presentation of the manuscript. If you would amend the manuscript as you have described in your responses then the manuscript will be reconsidered for publication. On one specific issue, reviewer 1 comment 23 recommends the table and figure titles need to be more precise. Your suggested changes result in titles which are too long and contain methodological information which does not need to be included. I would encourage the authors to construct specific titles which enable the reader to understand the table or figure without reference to the text but in a more concise way. Where a figure has related data in a table this can be simply referenced to the respective table.

R: We incorporated the comments made by the two reviewers into the new version of the manuscript. Legends of the tables and figures were rewritten to make them more concise.

……..

Anonymous Referee #2

1. The paper adsorption to soils and biochemical characterization of purified phytases, by Caffaro et al, uses conventional techniques for the evaluation of known commercial phytases. They have some success trying to prove that phytases have the potential to be used as complement for soil fertilizers. There are many issues that need to be clarified before publication: The title itself is ambiguous and misleading. Recently it has been a discussion about the term phytase. Certainly, one definition is that all enzymes which area use phytate as substrate are phytases. However, several authors i.e Greiner have pointed out that many of those are actually phytate degrading enzymes particularly the ones in E coli. Therefore it might be that those are not true phytases, The main reason is that their function is not related to processing phytate, different from some other's "real" phytases in plants i.e. PAP phy.

R: Yes, definitely, not all enzymes that are capable of degrading phytates are "real" phytases. According Misset (2003), the "real" phytases are those enzymes capable to degrade completely the phytate molecules and release all the phosphates contained in it. However, the term phytases is a topic of debate as pointed out by the reviewer and one paper of the author cited by the reviewer includes the specific and general term in the tile (" Molecular and catalytic properties of phytate-degrading enzymes (phytases)").

Enzymes used in this work are commercially sold under the name "phytases" but we did not perform tests to evaluate the three-dimensional structure of the enzyme. However, many authors refers a phytate degrading enzymes from *E. coli* as phytases (e.g. Menezes-Blackburn et al. 2011, doi:10.1016/j.biortech.2011.07.054; Derjsant-Li and Kwakernaak 2019, doi: 10.1016/j.anifeedsci.2019.05.018). Then, we believe that it is correct to use the term phytase to describe enzymes that degrade phytates from *E. coli* and *A. niger*.

Taking into account this explanation, we enlarge the introduction to clarify this issue. Now read as:

"Phytases are enzymes released by bacteria, fungi, plants and animals (Jorquera et al., 2008) and are able to catalyze the release of P from phytates. Phytases have the ability to release the 6 Pi molecules that are contained in phytate (Misset 2003)."

An alternative title may be: "Adsorption to soils and biochemical characterization of phytate-degrading enzymes (phytases)" (following Konietzny and Greiner 2002). We are ready to move for this title if the editor and reviewer consider this as a better option. Anyway, and we propose to maintain the original title "Adsorption to soils and biochemical characterization of purified phytases", since "a priori" we did not know the real capacity of the purified enzymes to release P.

…

2. The authors refer to the work of Misset 2003 as a reference of E coli phytases and their relevance in the industry. There are a couple of issues here. First I'm not really sure of the relevance of all strains of E coli phytases for the industry. If any which ones?.

R: Commercial phytases available in Argentina are purified from *A. niger* and E.coli, so we considered necessary to cite previous reports about E.coli as a source of phytase. Our E coli enzymes came from different commercialized products, mainly used for animal feed application. The strains are called with the same name as the product (TS Smizyme phytase, by Quimtia EDF, and Ronozyme, by DSM Nutritional Products Argentina S.A.). We used the same approach than Menezes-Blackburn et al. 2015 doi: 10.1021/acs.jafc.5b01996, who mention three *E. coli* strains that are isolated and commercialized. See below the table extracted from this paper

…

3. Many strains of *E. coli* possess an active phosphatase A gene witch can provide a certain level of phytate degradation but a real level of commercial degradation, I'm not sure about it

R: We agree. For this reason, we decided to perform these experiments to verify the actual activity of commercial enzymes purified from *E. coli* and *A. niger*.

…

4. Only until the lines 60 to 70, the really important point of the work was revealed. The main point in my perspective is the usage of phytases as biological fertilizers to re-lease inorganic P from organic P sources. But so far the whole history sounded more focused on something else. The major problem of the paper starts with the first hypothesis: Phytases have the ability to release P from different organic P sources, with a preference for phytic acid. In that way is redacted that is not a hypothesis that contributes at all with new knowledge in the field. It is already known that some phytases are highly specific and others are not but have preferences for phytate. Similar to the other two. Many references for that just two examples: doi:10.1128/AEM.01384-15 doi:10.1128/mBio.01966-18

R: OK, we understand that our hypothesis in the original version can lead to misunderstandings. We are not talking about phytases in general, but specifically about commercial enzymes of our work.

First hypothesis now read as … "four commercially available phytase products tested in this work have the ability to release P from different organic P sources, with preference for phytic acid".

…

5. Is the norm of the journal to include only some of the line numbers? That makes it more difficult for review.

R: Yes, we use the journal template for submitting the paper.

…

6. The abstract is very misleading because implies that the authors isolated the phytases from the fungi by themselves. That is not the case.

Line 18-19: The proportion of phytases found in the solid phase of the soil 60 minutes after addition was lower than that found in the liquid phase (23-34% vs.66-77%). This result is not well connected in the abstract, is coming out of nowhere.

R: OK, we rearranged the paragraph.

Abstract now read as …" Four purified phytases isolated from *Aspergillus niger* and *Escherichia coli* were characterized biochemically and in terms of their adsorption to soils belonging to the Mollisol order. Three different organic P substrates were used to measure enzyme activity in a wide range of pH (2.3 to 9) and temperatures (-10º to 70ºC): p-nitrophenyl-phosphate (pNP), glyceraldehyde-3-phosphate (G3Phospahte) and phytic acid. Phytases have low affinity for solid phase (23.34% of adsorption after one hour of incubation. Phytases from *A. niger* showed a higher capacity to release P (13% on average), than phytases from *E. coli*. All phytases were active throughout the pH and temperature ranges for optimum crop production. The amount of P that *A. niger* phytases release at pH that is commonly found in agricultural soils (5.5-7) is as follows: pNP > phytic acid > G3Phosphate, whereas in *E. coli* phytases the order was pNP / phytic acid > G3phosphate. Obtained results are 20 promising in terms of the use of phytases as a complement to P fertilization in agricultural settings and encourages further studies under field conditions.."

…

7. Lines 38-39: There are different forms of inositol-phosphates and the most abundant from phytate (refers only to the salt form). But what exactly is the meaning of phytates in these lines and in the subsequent text in general?.

R: In our work, we want to test the ability of commercial products to release P from different P organic sources, so in this paragraph we introduce the different forms of organic P found in the soil and in what proportion they are found. Please take into account our reply to comment 1, in which we explain that the definition of phytases is enlarged in the new version.

…

8. Line 48. E coli and the rest of the text please italicize where required.

R: OK: Done

…

9. The hypotheses are not real hypotheses in the way their current state. It is already known that phytases can use different substrates. The number two was proved by a paper that the authors cite https://doi.org/10.1002/jpln.201600421. Finally, the hypothesis number 3 is way too basic for being a good work hypothesis.

R: We understand the point raised by the reviewer. The original hypotheses may appear as basic for ultra-purified enzymes or recombinant proteins produced for academic or related activities. Previous studies about phytases mainly come from ultrapurified enzymes like the ones provided by lab products retailers such as Sigma and are used in academic labs. It is clear that this high quality but extremely expensive products cannot be used in real agricultural settings by farmers. In this report we tried to test at which extent commercial phytases have comparable performance than the ultrapurified materials. We think that it is not correct to extrapolate results from both type of products. In such sense, in the new version we will modify

the text highlighting the commercial nature of our evaluated phytases.. Anyway, we will modify the hypotheses by clarifying that we refer to these commercial enzymes.

Hypothesis now read as… " i) the four commercially available phytase products tested in this work have the ability to release P from different organic P sources, with preference for phytic acid, but differ in the pH and temperature levels to reach their optimum activity ii) the retention of commercial phytases in the soil solid phase is associated to the soil clay content.

…

10. The biochemical characterization needs to include the catalytic efficiency of the reactions.

R: Done. Information was added to fig. 4

…

11. It has been demonstrated recently by the works of Tan et al (doi:10.1007/s00253-015-7097-9) and others in 2019 using metagenomes that phytases are also present in metagenomes of soils. In fact, their presence is underestimated. Where is the experiment which proves that the used soils have low phytase activity?. The control reactions of the initial experiments are missing.

R: Very good observation. In all experiments, we use blank reactions to ensure that the results presented in this work are those observed by the interaction of the enzymes with the soil. We measure soil phytase activity without the addition of the enzyme and observe soil phytase activity less than 1nkat g soil $^{-1}$.

This topic was not clear enough in the previous version of the ms. Materials and methods now read as… "Phytase activity of the soil suspension was calculated as the difference between the soil suspension with enzyme minus the soil suspension without enzyme."

…

12. Line 134 I don't think is a good idea to use a demo or student versions of any software for statistical analysis in a publication.

R: Table Curve Demo version gives a limited period of software use (30 days), but it has the same mathematical functions as the full version. Anyway, we agree to remove the "demo version" if required. Statistical analysis were performed with the Statistix student version which is the software that we used in our lab since long time ago and was tested several times. For this particular paper and following this comment, we performed again all our analysis with INFOSTAT software (https://www.infostat.com.ar/) and we did not obtain different results. This software is cited in the new version.

…

13. Were the buffers set at the optimal temperature?

To evaluate the performance of the enzymes along a pH range (2.3-9.0), 200 μl of each enzyme solution was diluted with 400 μl of 50 mM glycine-HCl buffer (pH 2.3-4.4),

R: The buffers were prepared at normal room temperature (20-24 ºC) and the incubation experiments were performed at 25 ºC (for evaluating optimum pH and kinetic parameters) and along a temperature range (-10-70ºC) for evaluating optimum temperature. In this experiment pH was set as 5.5. For these experiments we followed the approach proposed by George et al. (2005) and Hayes et al. (1999). The original text was somewhat unclear at this point and we reworded the ms accordingly.

…

14. The authors refer at the begging of the manuscript to the type of enzymes as 3-phytases. But they do not mention what type of enzymes are from the structural point of view. Are they acid phytases? Maybe that's is why need pH relatively low to act. But nothing of this is mentioned in the text. Is the optimal pH was determined before that the temperature is obvious that they did not set the buffer for the pH test at the right temperature. Therefore the pH characterization is not trustworthy.

R:

Commercial phytases used as a complement to poultry nutrition must be active at the stomach pH of the animals (about pH 3). Phytases that are active at that pH value are acid phytases, so in the introduction we mention the 3-phytases and 6- phytases that are by definition acid phytases (lines 41-50 of new paper version, "*A. niger* phytases are mainly extrinsic (Azeem et al., 2015), and are classified as 3-phytases, because they primarily dephosphorylate the phosphate group located at 3-position. *E. coli* phytases are mainly membrane-associated proteins and were classified as 6-phytase (Azeem et al., 2015). The classification as 3- or 6-phytases is related to which phosphate group is attacked first and would be determined by conformational differences in the β–domain of each phytase (Konietzny and Greiner, 2002)"). This is not a paper on poultry nutrition so we made no mention of this topic in the introduction section. It is possible that when we did the pH assay, the buffers were not set at the optimal temperature of the enzyme, but we decided to perform the assays according to Hayes et al. (1999) in order to make our results comparable with the literature. Anyway, as it can be seen in the results section, optimal temperature of function of the enzymes is close to the values worked in the experiments (20-29ºC).

…

15. It seems that the authors did not review any literature about phytases in 2019.

R: We checked all papers on phytases published in the top journals before the submission date of our SOIL ms. To the best of our knowledge we cited the most relevant paper but probably we missed some.

……..

Interactive comment on “Adsorption to soils and biochemical characterization of purified phytases” by Maria Marta Caffaro et al.

Anonymous Referee #1

1. Comments for editor. The research work carried out under the theme “Adsorption to soils and biochemical characterization of purified phytases” is of scientific significance and has practical application for release of Pi from native or exogenously added organic P. Though, the study conducted is well organized but certain points need due attention. The Accession no of microbial strains used in the study is missing.

R: Thanks for your comment; in our experiment we used four commercial phytases. Two came from two different batches of *A. niger* commercially sold under the name “Habio phytases”, which were obtained from Sichuan Habio Bioengineering Co.Ltd (Sichuan, China). The other two, came from two strains of *E. coli*. One is sold under the name “TS Smizyme phytases”, obtained from Quimtia EDF (Buenos Aires Argentina) and the other is sold under the name “Ronozyme”, obtained from DSM Nutritional Products Argentina S.A. Unfortunately, and as usual for commercial strains, no accession number was provided by the supplier. Anyway, we rearranged the paragraph to provide all available information.

Materials and methods now read as (New ms lines 68-73):

“Two phytases isolated from *A. niger* and two from *E. coli* were used in our experiments. In the first case, here named *A. niger* 1 and 2, phytases came from two different batches of the fungus which are commercially sold under the name “Habio phytases” by Sichuan Habio Bioengineering Co.Ltd (Sichuan, China), In the E.coli group, the first selected enzyme (here called *E. coli* 1) is sold under the name “TS Smizyme phytase”, by Quimtia EDF (Buenos Aires Argentina), and the second (here called *E. coli* 2) is sold under the name “Ronozyme”, by DSM Nutritional Products Argentina S.A.”

….

2. The cost incurred on purchase of purified phytases and their availability needs mention.

R: Enzymes were provided for free by the different companies producing and / or importing enzymes in the country. This information is included in the new version.

Materials and methods now read as (New ms line 73):

“These enzymes are in powder format at a concentration of 5000 U g-1 and was provided free of charge by the companies that produce or import them.”

….

3. A comparative study with crude phytase obtained from wild strains of *A. niger* and *E. coli* could have also been conducted alongside.

R: This would be a good option for the next phase of this investigation. Although it was was not the main objective of the present study. Anyway, this comment is highly appreciated and will be taken into account in our next phase.

….

4. Technical Comments for authors

Abstract needs some modification indicating the % increase in P release with *A. niger* over *E. coli*.

R: Done.

Abstract now read as (New ms line 12):

"Phytases from *A. niger* showed a higher capacity to release P, than phytases from *E. coli* (+13% on average)."

….

5. L-13-14 Please shift substrates pNP, G3phosphate and phytic acid after substrates

R: Done.

Abstract now read as (New ms line 11):

"….. p-nitrophenyl-phosphate (pNP), glyceraldehyde-3-phosphate (G3Phospahte) and phytic acid."

…

6. L-16 Please write that the order of P release from different substrates by *A. niger* and *E. coli* followed this trend (mention the trend).

R. OK. Iif we understood the meaning of the comment, the order is mentioned a couple of lines above.

Abstract now read as (New ms lines 13-15):

"All phytases were active throughout the pH and temperature ranges for optimum crop production. The amount of P that *A. niger* phytases release at pH that is commonly found in agricultural soils (5.5-7) is as follows: pNP > phytic acid > G3Phosphate, whereas in *E. coli* phytases the order was pNP / phytic acid > G3phosphate".

…

7. Introduction

L-24 Delete appropriate

R: OK.

Introduction now read as (New ms line 20):

"Most strategies for enhancing P nutrition of agricultural crops aim to maintain soils at the convenient P critical level so that yields…"

….

8. There are approximately 38 references in introduction. The no. can be reduced.

R: OK. In the new version, the number of references was reduced to 22.

….

9. L-45-48. The first phytase was discovered—– delete this paragraph.

R: OK, deleted.

….

10. L-74 instead of level write pH and temperature optima.

R: OK.

Introduction now read as (New ms line 65):

… " the two evaluated phytases differ in their optimum pH and temperature to reach their maximum activity .."

….

11. Material and methods

L-77 *A. niger* in italics

L-79 powder form and not format

L-81 Superscript g^{-1}

R. OK, all these editing issues were arranged as suggested.

….

12. L-87 If one g soil was mixed with 20 ml phytase solution how can you take a sub sample of 500 ml. Please check the unit

R: We apologize because it was our mistake. The subsample volume is 500 microliters (not ml). Arranged in the new version.

Materials and methods now read as (New ms line 84):

"…sub-samples of soil slurry (500 μl) were taken for phytase activity measurements…"

….

13. L-92 150 ml or 150 microliter

R: Correct, same as above, it is 150 microliter. Arranged in the new version.

Materials and methods now read as (New ms line 86):

"An aliquot (150 μl) of the soil slurry was used to measure the enzyme activity…"

….

14. L-105 total protein (Lowry et al.)

R. OK.

Materials and methods now read as (New ms line 100):

"Biochemical characterization of the phytases included: total protein (Lowry et al., 1951)"

….

15. Phytase activity was measured with 3 substrates

R: OK, arranged as suggested

Materials and methods now read as (New ms line 102):

“Phytase activity was measured with 3 substrates containing…”

….

16. L-119 Blanks for measuring enzyme activity included (i) (ii) (iii)

R: OK.

Materials and methods now read as (New ms lines 112- 113):

“The activities were tested against three blanks: (i) reaction buffer without enzyme or substrate; (ii) reaction buffer with enzyme without substrate; and (iii) reaction buffer without enzyme with substrate.”

….

17. L123-126 Please rewrite this portion

R: OK.

Materials and methods now read as (New ms line 114):

“For the pNP substrate, the enzymatic activity was measured at 412 nm which is the absorbance value of p-nitrophenol (Hayes et al., 1999). The concentration of 3 substrates was determined as the concentration of the whole sample minus the concentration of the reaction blank.”

Please check that the sentence: “Phytase activity with phytic acid and glyceraldehyde-3-phosphate as substrates was measured as P release measured by the 125 Murphy-Riley method (Murphy and Riley, 1962)” was eliminated because this procedure is provided in the previous sentence.

….

18. Please mention the amount of TCA added to stop the reaction

R: OK.

Materials and methods now read as (New ms line 91):

“Reactions were stopped with an equal volume of 10% TCA (300 μl in soil slurry experiments and 700 μl in soil solution experiments).”

….

19. L-192 Modify the sentence

R: OK.

Results now read as (New ms line 180):

“All four enzymes were effective in releasing P from phytic acid throughout the entire pH range analyzed”

….

20. L-200 pH 7.8 was detrimental for release of Pi from pNP by *A. niger*

R: OK, arranged as suggested.

Results now read as (New ms line 186):

“pH 7.8 was detrimental for release of Pi from pNP by *A. niger*, probably because the hydrolysis of the substrate

….

21. L-216 Change offered to tested substrates

R: OK.

Results now read as (New ms line 202):

“*A. niger* showed maximum activity at 24 °C (Fig. 3a), releasing 33% of the original P contained in the substrate.”

….

22. Results and discussion

Discussion part is totally missing and needs to be written properly

R: OK, results and discussion sections were now written separately so as not to create confusion for the reader.

….

23. No explanation for findings is given Conclusion needs to rewritten.

R: OK, conclusion section was reworded accordingly. After reorganizing the ms and split the Results and Discussion section into to separate parts, the conclusion was incuded in the last paragraph of the discussion. In this paragraph the test of hypothesis is specifically considered

Conclusion now read as (New ms lines 281- 295):

"Obtained results partially support our first hypothesis since the selected phytases showed a great ability to release P from different organic P sources, but *A. niger* 1, 2 and *E. coli* 1 release more P from pNP than phytic acid while *E. coli* 2 has no preference for any particular substrate. Regarding to activity of phytases at different pH and temperature levels, phytases exhibited some differences in their pH and temperature levels to reach their optimum activity In contrast, our results did not support the second proposed hypothesis, since the retention of phytases by the soil solid phase did not have a clear association with the analyzed soil properties. In this regard, it must be taken into account that the seven selected soils belonged to the Mollisol order. After being added to the soil, tested phytases showed an adsorption to soil solid phase ranging from 20 to 40%. Those phytases that remain in the solution could release Pi from the organic P of the soil, whereas phytases that remain adsorbed to the soil solid phase could be released later. All studied phytases remained active at the optimum soil pH range of the most productive agricultural soils (5-7). In the same line, optimal temperatures for phytase activity were also within the temperature range more suitable for most agricultural crops (20-30ºC). Our results suggest that purified phytases may constitute a feasible tool to be used as a complement to P fertilization. In such sense, further experiments should be performed to evaluate the enzyme performance under field conditions to evaluate the ability of phytases to release from organic soil P sources, their interaction with soil microorganisms and to test if crops can capitalize the eventual provision of inorganic P released."

….

23.Tables and Fig titles need to be precise

R: OK, all titles were rewritten

Tables and figures now read as:

"Table 1. Characteristics of seven representative soils of the Argentina's Pampa Region used for testing phytases adsorption. Samples were taken at 0- 20 cm, air dried and sieved at 1 mm prior to the analysis.

Table 2. Coefficients of the adjusted Lorentizian-peak functions for phytase activity (see graphs in Fig. 2) at different pH levels with phytic acid, p-nitrophenyl-phosphate and glyceraldehyde-3-phosphate as substrates. The equations were adjusted from the observed results of the release of P from each substrate used. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve were fitted.

Different letters correspond to significant differences between treatments (P <0.05, LSD procedure) Coefficient a is the maximum percentage of P released; b is the pH value where the enzyme has maximum activity (a P release peak); c estimates the standard deviation of the distribution and x is the pH value.

Table 3. Coefficients of the adjusted Lorentizian-peak functions for phytase activity (see graphs in Fig. 2) at different temperature levels with phytic acid, p-nitrophenyl-phosphate and glyceraldehyde-3-phosphate as substrates. The equations were adjusted from the observed results of the release of P from each substrate used. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve were fitted. Different letters correspond to significant differences between treatments (P <0.05, LSD procedure) Coefficient a is the maximum percentage of P released; b is the temperature value where the enzyme has maximum activity (a P release peak); c estimates the standard deviation of the distribution and x is the temperature value.

FIGURE 1. Phytase activity distributed in the liquid and solid phases for the phytase soil adsorption experiment. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. Experiments were performed with the seven soils described in Table 1. For *A. niger* 1 and 2 and *E. coli* 1 phytases, a unique curve decay (Eq. 2), and exponential increase (Eq. 3) involving the seven soils was fitted because no significant differences (after F tests) were found between them. For *E. coli* 2, no function could be adjusted because a 37% binding to the soil solid phase was observed at 5 minutes and remained stable throughout the incubation period. Each point represents the average of three observations. Bars represent standard error of the mean.

FIGURE 2. Phytase activity measured at different pH levels with phytic acid, pNP and G3Phosphate as substrates. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve was fitted. Each point represents the average of three observations. Bars represent standard error of the mean. Coefficients of each adjusted model are shown in Table 2.

FIGURE 3. Phytase activity measured at different temperature levels with phytic acid, pNP and G3Pphosphate as substrates. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve was fitted. Each point represents the average of three observations. Bars represent standard error of the mean. Coefficients of each adjusted model are shown in Table 3.

FIGURE 4. Kinetic parameters for phytic acid, pNP and G3Pphosphate as substrates of purified phytases (two isolated from *A. niger* and two from *E. coli*). The activity was determined at different P concentrations (0 to 100 mM) contained in each substrate. Each point represents the average of three observations. Bars represent standard error of the mean. Data were fitted to a Michaelis-Menten curve and the estimated Vmax and Km values obtained by the Lineaweaver-Burk method are shown."

….

24. Table 1 Mg+2 and Ca+2 and not Ca+1

25. Provide space between C total, P total and P inorganic

26. Fig.1. spelling for enzymatic

R: OK, all these editing issues were arranged as suggested.

Adsorption to soils and biochemical characterization of purified phytases

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Abstract. ~~Abstract.~~ Four purified phytases isolated from *Aspergillus niger* and *Escherichia coli* were characterized biochemically and in terms of their adsorption to soils belonging to the Mollisol order. Three different organic P substrates were used to measure enzyme activity in a wide range of pH (2.3 to 9) and temperatures (-10º to 70ºC): phytic acid, p-nitrophenyl-phosphate (pNP)~~,~~ and glyceraldehyde-3-phosphate (G3Phosphate) ~~and phytic acid~~.. Phytases ha~~ved~~ low affinity for the solid phase~~,~~: 23-34% of the added amount was absorbed ~~of~~ adsor~~ption~~bed after one hour of incubation. Phytases from *A. niger* showed a higher capacity to release P (13% on average~~36 to 50% of P contained in the substrates, 44 to 62 µg P~~), than phytases from *E. coli* ~~(24 to 15 36%, 20 to 44 µg P)~~. All phytases were active throughout the pH and temperature ranges for optimum crop production. At pH values commonly found in agricultural soils (5.5-7) *A. niger* phytases released P following the ranking of substrates ~~The amount of P that *A. niger* phytases release at pH that is commonly found in agricultural soils (5.5-7) is as follows~~ : pNP > phytic acid > G3Phosphate, ~~The amount of P released from organic P substrates by *A. niger* phytases followed the following range: p-nitrophenyl-phosphate > glyceraldehyde-3-phosphate > phytic acid~~ whereas ~~in~~ *E. coli* phytases released P following the ranking: ~~the order was~~ pNP / phytic acid > G3~~p~~Phosphate~~ p-nitrophenyl-phosphate/glyceraldehyde-3-phosphate > phytic acid~~. ~~All phytases were active throughout the pH and temperature ranges for optimum crop production.~~ ~~The proportion of phytases found in the solid phase of the soil 60 minutes after addition was lower than that found in the liquid phase (23-34% vs. 66-77%).~~ Obtained results are ~~20~~ promising in terms of the use of phytases as a complement to P fertilization in agricultural settings and encourages further studies under field conditions.

1 Introduction

Phosphorus (P) is the second nutrient that limits agricultural crop productivity worldwide. Most strategies for enhancing P nutrition of agricultural crops aim to maintain soils at the convenient ~~appropriate~~ P critical level so that yields are not constrained by this nutrient and environmental pollution risks are avoided or minimized (Simpson et al. 2011).

The most widely used practice to overcome soil P deficiencies is the application of inorganic P fertilizers produced from phosphate rock (PR). However, world PR reserves that can economically be extracted are estimated to be depleted in the next 50-100 years (Cordell et al., 2009). Several strategies have been suggested to increase P utilization efficiency and reduce PR-derived fertilizers consumption (~~Cornish, 2009; Richardson et al., 2009; Veneklaas et al., 2012;~~ Fernandez and Rubio, 2015). Richardson et al. (2011) summarizes these strategies in three groups: i) root-foraging strategies that reduce the critical P requirements for plant growth; ii) P-mining strategies that enhance the P availability from sparingly-available sources in soil, and iii) physiological strategies that lead to higher yields per unit of P uptake.

Soil P is comprised of inorganic and organic forms. Phosphates present in the soil solution are the main source of P for higher plants. Due to the strong interaction with the soil matrix, concentration of phosphates in the soil solution is very low (<10µM) (Dalal, 1977). In general, soil organic P content varies in a wide range (between 30-70% of total soil P; ~~Condron et al., 2005; Nash et al., 2014;~~ Cabello et al., 2016~~; and even on a wider range, Harrison 1987~~). The predominant soil organic P fractions are usually phytates (Harvey et al., 2009; Steffens et al., 2010), followed by nucleic acids,

phospholipids and sugar-phosphates (Tiessen, 2008). Phytates and other organic P forms cannot be directly utilized by plants and need to be mineralized before being ready for plant uptake. The discovery of phytate-degrading compounds changed the conventional perception that phytate was a recalcitrant molecule in the environment (~~Condron et al., 2005;~~ Harvey et al., 2009).

Phytases are enzymes released by bacteria, fungi, plants and animals (Jorquera et al., 2008) and are able to catalyze the release of P from phytates. Phytases have the ability to release the ~~6~~six P_i molecules that are contained in phytate (Misset 2003). Although ~~Although~~ phytases are distributed throughout the soils, the higher concentrations are found in the rhizosphere (Li et al., 2008). ~~The first phytase was discovered in the early 20th century (Hill and Richardson, 2006), but their precise identification was not made until the mid-60s (Tabatabai and Bremmer, 1969). Phytases are proteins widely distributed in soil microorganisms (Mullaney and Ullah, 2007).~~ The high capacity of *A. niger* and *E. coli* to secrete phytases has promoted their use as a source of these enzymes in commercial production by the industry (Misset, 2003). *A. niger* phytases are mainly extrinsic (Azeem et al., 2015), and are classified as 3-phytases, because they primarily dephosphorylate the phosphate group located at 3-position. *E. coli* phytases are mainly membrane-associated proteins and were classified as 6-phytase (Azeem et al., 2015). The classification as 3- or 6-phytases is related to which phosphate group is attacked first and would be determined by conformational differences in the β-domain of each phytase (Konietzny and Greiner, 2002).

Besides being a key fraction of soil organic P, phytates are present in other nature components. For example, 60-90% of P in cereal and oil grains is present under phytic acid forms (63% in soybean, 77% in wheat, 83% in maize; Lott et al., 2000). One of the most common uses of these grains is for livestock feed (Misset, 2003). However, the microbial population of the digestive tract of monogastric animals (e.g. poultry) is unable to utilize phytate as a P source. The benefit of adding phytases to poultry diet to enhance phytic acid P utilization was demonstrated some time ago and nowadays is a widespread practice in poultry nutrition management (El-Sherbiny et al., 2010). ~~It was demonstrated that~~ U~~u~~sing phytases from different microorganisms (i.e. *Aspergillus* spp and *E.~~scherichia~~ coli*) ~~for this practice~~ may also entail environmental benefits by reducing the P content of poultry manure.

Extensive use of phytases in livestock and aquaculture production contrasts with the practically null use in agriculture. There are very few reports in which phytases were studied to enhance soil P availability (e.g. Findenegg and Nelemans, 1993; ~~Gaind and Nain, 2015,~~ Liu et al., 2018). Adding phytases to poor P soils increased biomass accumulation of maize by around 32% (Findenegg and Nelemans, 1993). ~~-~~Undoubtedly, phytase research appears to be a promising path to increase soil P use efficiency (~~Büneman, 2008; Gaind and Nain, 2015;~~ Menezes-Blackburn et al., 2016; Liu et al., 2018). Some reports indicate that the adsorption of phytases to the soil matrix may reduce their affinity for substrates containing P (George et al., 2005; ~~Giaveno et al., 2010;~~ Yang and Chen, 2017). When pH increases, clay charge changes, decreasing the phytase affinity (Ruyter-Hooley et al., 2015).

In this work we evaluated the performance of four commercially available phytases, two extracted from *A.~~spergillus~~ niger* and two from *E.~~scherichia~~ coli* as candidates to be used as a biological fertilizer to release inorganic P from organic P sources. Our working hypotheses were: i) the four commercially available phytase products ~~tested in this work~~ have the ability to release P from different organic P sources, with preference for phytic acid, but differ in the pH and temperature levels to reach their optimum activity, and ii) the retention of commercial phytases in the soil solid phase is associated to the soil clay content.~~i) phytases have the ability to release P from different organic P sources, with preference for phytic acid; ii) the retention of phytases in the soil solid phase is associated to the soil clay content; iii) the two evaluated phytases differ in the pH and temperature levels to reach their optimum activity~~.

2 Materials and methods

2.1 Enzyme preparation

Four phytases were used in our experiments; two isolated from *A. niger* of two different batches of Habio (Sichuan Habio Bioengineering Co., Ltd, Sichuan PRC) here named *A. niger* 1 and 2 and two isolated from *E. coli* (TS Smizyme phytase, Quimtia EDF, Buenos Aires, Argentina and Ronozyme, DSM Nutritional Products Argentina S.A) named *E. coli* 1 and 2, respectively. These enzymes are in powder format at a concentration of 5000 U g^{-1} and was provided free of charge by the companies that produce or import them. Two hundred mg of each phytase were suspended in a solution composed by 20 ml of 360 mM $CaCl_2$, 1 mM buffer pH 5.5 sodium acetate, and 100 mg g^{-1} Tween 20. The solution was mixed 30 min at 4 °C and subsequently centrifuged at 6900 g for 30 min at the same temperature. Final concentration of enzymes in the solution was 10 mg enzyme ml^{-1}.

2.2 Phytase adsorption on soils

Soil samples (0-20 cm) were taken from seven representative soils of the Pampean Region, the most productive area of Argentina (Table 1). All soils belong to the Mollisol order (Rubio et al. 2019). One gram of each soil and 20 ml of phytase solution (17.6 nKat g^{-1} of soil, specific activity 8.3 nKat mg^{-1} protein) was placed in 50 ml screw-capped polyethylene tubes at room temperature (22 ºC). After shaking the tubes on a flat bed shaker (75 oscillations min^{-1}) sub-samples of soil slurry (500 µl) were taken for phytase activity measurements at 5, 10, 15, 30 and 60 min. To obtain a representative sample of the suspension, aliquots of soil slurry were taken using a pipette tip after vigorously mixing the soil suspension. An aliquot (150 µl) of the soil slurry was used to measure the enzyme activity (here called soil suspension). The remainder portion of the sample was centrifuged at 15,000 g for 5 min and the supernatant was taken for measuring the phytase activity (called soil solution).

Phytase activities in aliquots of soil solutions and suspensions were measured at a 1:1 sample to buffer ratio. Assays were performed against phytic acid substrate for 60 min at 37°C at a final concentration of 2 mM and buffered to pH 5.5 with 15 mM MES (George et al., 2005). Reactions were stopped with an equal volume of 10% TCA (300 µl in soil slurry experiments and 700 µl in soil solution experiments). Samples were centrifuged at 3800g for 5 min prior to determination of P concentration in the supernatant using Murphy-Rilley method (Murphy and Riley, 1962). Phytase activity retained in the solid phase was determined by calculating the difference between the phytase activity of the soil suspension and activity of the soil solution. Phytase activity of the soil suspension was calculated as the difference between the soil suspension with enzyme minus the soil suspension without enzyme. To determine which soil characteristics (Table 1) affected phytase distribution between soil solid and liquid phases, a linear regression and correlation analysis between y_{max} (maximum distribution of the enzyme in the soil solid phase) and k (rate at which distribution peaks) with soil characteristics were performed.

2.3 Biochemical characterization, pH and temperature optimum levels

Biochemical characterization of the phytases included: total protein ~~by Lowry method~~ (Lowry et al., 1951), enzymatic activity as a function of pH and temperature, kinetic parameters V_{max} and K_m and adsorption to seven selected soils.

Phytase activity was measured with 3~~three~~ substrates containing 10 mM P: 2 mM phytic acid, 10 mM p-nitrophenyl-phosphate and 10 mM glyceraldehyde-3-phosphate. In this experiment incubation temperature was 25 °C according to Hayes et al. (1999).

To evaluate the performance of the enzymes along a pH range (2.3-9.0), 200 µl of each enzyme solution was diluted with 400 µl of 50 mM glycine-HCl ~~buffer~~ (pH 2.3-4.4), 50 mM Na-acetate (pH 3.6-5.8), 50mM MES-KOH (pH 5.2-7.3) and 50 mM Tris-HCl (pH 6.1-9.0), as a reaction buffer. To evaluate the performance of the enzymes along a temperature range (-10-70ºC), 200 µl of each enzyme solution was diluted with 400 µl MES (pH 5.5) buffer. For both pH and temperature studies, incubation time was 1 h and the reaction was terminated by the addition of 10% trichloroacetic acid (TCA). In the temperature studies, the buffer containing the substrates is heated until the desired temperature is reached. At this point the enzyme is added and the incubation time starts Measurements were performed in triplicate. The activities were tested against three blanks: (i)~~blank 1:~~ reaction buffer without enzyme or substrate; (ii)~~blank 2:~~ reaction buffer with enzyme without substrate; and (iii)~~blank 3:~~ reaction buffer without enzyme with substrate. When the substrates were phytic acid and glyceraldehyde-3-phosphate, phytase activity was measured by the Murphy-Riley method (Murphy and Riley, 1962). For the p-Np substrate, the enzymatic activity was measured at 412 nm which is the absorbance value of p-nitrophenol (Hayes et al., 1999). The concentration of 3 substrates was determined as the concentration of the whole sample minus the concentration of the reaction blank.

~~For p-nitrophenyl phosphate, the enzymatic activity was measured at 412 nm which is the absorbance range of p-nitrophenol (Hayes et al., 1999). The concentration of P or p-nitrophenol was determined as the subtraction of sample concentration and blank of reaction concentration Phytase activity with phytic acid and glyceraldehyde-3-phosphate as substrates was measured as P release measured by the Murphy-Riley method (Murphy and Riley, 1962).~~

To estimate V_{max} and K_m, 200 mg of each phytase were suspended for 1h in solutions containing 0, 6.25, 12.5, 25, 50, 100 mM of P using the three substrates mentioned in the previous section (phytic acid, glyceraldehyde-3-phosphate and p-nitrophenyl phosphate). The reaction was stopped by the addition of 10% TCA. The kinetic parameters were determined by the graphical method of Lineweaver-Burk.

2.4 Statistical analysis

In order to find the pH and temperature value at which phytases show the maximum activity, different peak functions were adjusted with 2D Table Curve demo version. Experimental data of enzyme activity at different pH or temperatures were expressed as percentage of P released from each substrate and fitted to Lorentzian peak model for each treatment calculated following Eq. (1):

$$\%\ P\ released = \frac{a}{1+(\frac{x-b}{c})^2}, \quad (1)$$

Where a is the maximum percentage of P released; b is the pH value where the enzyme has maximum activity (a P release peak); c estimates the standard deviation of the distribution and x is the pH value. Parameters of each Lorentzian distribution for each enzyme and substrate were compared using F tests (Mead et al., 1993). In those cases where non-significant differences between enzymes (analyzed by F tests, analyzed by Statistix 9, student version) were found, a unified curve was fitted. The parameters and the obtained functions were compared by t-tests.

Results obtained from the experiments of phytase distribution between soil solid and liquid phases were expressed as enzyme activity per soil gram (nkat g soil^{-1}). Exponential decay equations for enzyme distribution in liquid phase were fitted according to the Eq. (2):

$$y = (y_0 - b) * be^{-kx}, \quad (2)$$

where y_0 is the minimum enzyme activity in soil liquid phase, k is the relative exchange rate between the liquid phase and the solid phase and x is the time considered.

Exponential increase equations for enzyme distribution in the solid phase were fitted according to the Eq. (3):

$$y = y_{max} * (1 - e^{-kx}), \quad (3)$$

where y_{max} is the maximum enzymatic activity in the solid phase of the soil, k is the relative exchange rate between the liquid phase and the solid phase and x is the reaction time. All functions where fitted by Table Curve 2D software.

In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve was fitted. To determine the soil property effect on enzyme adsorption, the distribution of the enzymes between the solid and liquid soil phases were adjusted with linear functions between the enzyme activity and each analyzed soil property (Table 1).

3 Results and discussion

3.1 Phytase adsorption on soils

Figure 1 shows the distribution of phytases between liquid and solid phases in seven different soils of the Pampean Region (Mollisol order, Table 1). *A. niger* 1 showed the lowest adsorption to the solid phase, around 19% of the original substrate P content (Fig. 1e). This value remained stable after 30 minutes of incubation. *A. niger* 2 showed the greatest adsorption to the solid phase (40%, at 10 min Fig. 1f). *E. coli* 1 (Fig. 1g) presented 39% of binding to solid phase at 60 minutes whereas *E. coli* 2 presented a 37% binding to the soil solid phase at 5 minutes (Fig. 1h). This early maximum fixation prevented the fitting of a consistent and representative function.

No linear relationship was observed between the parameter k and the analyzed soil characteristics for any of the four enzymes. In the case of y_{max}, we observed no linear relationship between soil characteristics for *A. niger* 1, 2 and *E. coli* 2. For *E. coli* 1, we found a significant correlation between the calcium content and y_{max} (data not shown). Our results contrast with those reported by Yang and Chen (2017), who observed that soils showed a great variation in their capacity to retain phytases to the solid phase of the soil (19-40% observed in our work vs 17-93% in Yang and Chen (2017) work) and that sandy soils had the lowest phytase fixation. The soils used in this work did not have a wide range of texture. There is a tradeoff between phytase retention to the soil matrix and phytase activity, whose outcome would determine the real contribution of the enzyme to soil P availability. A low retention of phytases implies more enzyme in the soil solution and eventually a faster release of soil organic P. On the other side, phytases in soil solution could be denatured by soil microorganisms (Yang and Chen, 2017), whereas retained phytases would be released gradually, providing additional available P at later stages (Mezeli et al., 2017).

3.2 Biochemical characterization

Protein analysis indicated that *A. niger* 1, *A. niger* 2, *E. coli* 1 and *E. coli* 2 phytases had 4.2, 5.4, 8.2 and 2, 13.01 μg enzyme per mg of product, respectively. ~~All four enzymes were effective at releasing inorganic P from the three offered organic P source. The four phytases released inorganic P from phytic acid along the whole range of pH following functions from which optimum and suboptimal pH values could be identified (Fig. 2).~~ In both pH and temperature experiments, no significant differences were observed between *A. niger* 1 and 2 in terms of released inorganic P, thus data from both were pooled for performing the analyses.

3.2.1 Effect of pH on enzyme activity

All four enzymes were effective in releasing P from phytic acid throughout the entire pH range ~~analys~~tested. ~~All enzymes were effective to release P from phytic acid throughout the analyzed pH range.~~ *A. niger* optimum activity was observed at pH 5.9, value slightly higher than those reported earlier (5-5.5) (Konietzny and Greiner, 2002; Menezes- Blackburn et al., 2015; Sariyska et al., 2005). A 37% release of the original P contained in the substrate was observed at the peak activity section of the pH range (Fig. 2a). In *E. coli* 1 and *E. coli* 2 phytases (Fig. 2b and c) the peak activity was observed at pH 5.5 and 4.7, with 30% and 24% release of the initial P, respectively. Optimum pH values for *E. coli* were in line with previous reports (4.5-5) (Konietzny and Greiner, 2002; Menezes-Blackburn et al., 2015). The maximum value of P_i released differed between P_o sources (see coefficient *a* of Table 2), while the optimum pH for enzyme activity only differed between *A. niger* 1 + 2 and *E. coli* 2 (coefficient b of Table 2).

Probably because the hydrolysis of the substrate, pH values higher than 7.8 w~~as~~ere detrimental for the release of Pi from pNP ~~by~~in *A.niger* ~~Phytase activity with p-nitrophenyl phosphate as substrate was notoriously diminished at pH values higher than 7.8, probably because the hydrolysis of the substrate~~. The peak activity of *A. niger* phytases was verified at pH 6.2 (Fig. 2g), with 50% P release. For *E. coli* 1 the maximum release of P was 37% at pH 5.8 (Fig. 2h), whereas for *E. coli* 2 the values were 24% and pH 5.9 (Fig. 2i). The comparison of the functions for the four enzymes revealed that they only differed in the *a* coefficient (Table 2), which represents the maximum P release. We did not find previous reports determining the optimum pH for enzyme activity with p-nitrophenyl-phosphate as organic P source.

For the substrate glyceraldehyde-3-phosphate, P release sharply decreased at pH values higher than 6 in *A. niger* and *E. coli* 1 enzymes, and at pH values higher than 8 in E. coli 2. *A. niger*, *E. coli* 1 and *E. coli* 2 enzymes showed a peak of activity at pH 3.9, 4 and 6, with a P release of 42 % (Fig. 2d), 37% (Fig. 2e) and 24% (Fig 2f), respectively. No statistical differences were observed on fitted coefficients between *A. niger* 1 + 2 and *E. coli* 1 functions, but these coefficients differed with the ones found for *E. coli* 2, revealing the particular shape of the function (Fig. 2i) (coefficients *a*, *b* and *c*, Table 2). As mentioned for p-nitrophenyl-phosphate, we did not find previous reports determining the optimum pH for enzyme activity with glyceraldehyde-3-phosphate as organic P source.

3.2.2 Effect of temperature on enzyme activity

The four enzymes remained active and could release Pi from the offered substrates throughout the whole temperature range evaluated (Fig. 3). When the substrate was phytic acid, both species of *A. niger* (1+2) showed the same response to temperature and consequently their functions were unified. The same occurred with *E. coli* 1 and 2 phytases. *A. niger* showed maximum activity at 24 °C (Fig. 3a), releasing 33% of the original P contained in the substrate. For *E. coli* enzymes (Fig. 3b), the peak was detected at 29 °C, with a 25% P release. The three coefficients of the function fitted for each pair of enzymes showed significant differences (Table 3), which reflects that *A. niger* had maximum release of P, but at a lower temperature than in *E. coli*. No difference between the four tested enzymes was found in the amount of P released. Obtained data on enzyme activity with phytic acid as substate agrees with Hayes et al. (1999), who found maximum activities in the 0-40ºC range. Other authors (Azeem et al., 2014; Sariyska et al., 2005) found maximum activities between 55ºC and 65ºC.

When the substrate was p-nitrophenyl-phosphate, the four enzymes showed a somewhat equivalent range of optimum temperatures than those found for phytic acid. For this substrate, the two *A. niger* enzymes showed the peak activity at 29 °C, releasing 17% of the substrate P (Fig. 3f). *E. coli* 1 phytase (Fig. 3g) released 22% of P at 29 °C and *E. coli 2* (Fig.

3h) also had the peak activity at 29 °C but lower P release: 13%. When comparing the coefficients of the fitted curves, they only differed in a coefficient (Table 3), representing the maximum P released.

When glyceraldehyde-3-phosphate was the substrate, the two *A. niger* enzymes had a similar behavior (Fig. 3c) with a peak activity at 24 °C and 10% release of the P contained in the substrate. *E. coli* 1 enzyme released 7% of the substrate P at 30 °C (Fig. 3d) and *E.coli* 2 (Fig. 3e) showed maximum activity at 20 °C, releasing 13% of the original P. No difference between adjusted coefficients of *A. niger* 1+2 and *E. coli* 1 functions was observed, but they differed with *E. coli* 2 coefficients (coefficients a, b and c in Table 3). We did not find previous reports determining the optimum temperature for phytase activity with p-nitrophenyl-phosphate and glyceraldehyde-3-phosphate as organic P sources.

3.2.3 Kinetic parameters

The response of the four enzymes to increasing concentrations of phytic acid is shown in Fig. 4 a-d. *A. niger* 2, *E. coli* 1 and 2 did not differ in the V_{max} value (0.7 nkat mg^{-1}), while *A. niger* 1 showed a slightly lower value (0.6 nkat mg^{-1}). K_m values of the four enzymes covered a narrow range (48 mM to 59 mM). *A. niger* 1 had the highest affinity (48.2 mM) followed by *E. coli* 1 (50.4 mM), *E. coli* 2 (54.3 mM) and *A. niger* 2 (59.2 mM). The K_m values for phytic acid observed in our experiments were somewhat lower than those found by Konietzny and Greiner (2002) and Menezes-Blackburn et al., (2015). These differences could be related to the methodological approach, i.e. buffer and temperature conditions. For example, some inhibitory effects of the Ca^{2+} concentration of the buffer on the enzyme activity can affect the kinetic parameters (Vohra and Satyanarayana, 2003; Nannipieri et al., 2012). However, despite the relatively low enzyme affinity for phytic acid, the proportion of P released at optimum conditions was high (24% to 41% in one hour of incubation, Fig. 2).

Phytase activity of the purified enzymes in response to increasing concentrations of p-nitrophenyl phosphate showed a very narrow range of V_{max} values (0.2 to 0.4 nkat mg^{-1}) (Fig. 4). *E.coli* 2 had the lowest V_{max} and the highest substrate affinity (0.2 nkat mg^{-1} and 22.8 mM), *E. coli* 1 (0.2 nkat mg^{-1} and 25.8 mM), then by *A. niger* 1 (0.4 nkat mg^{-1} and 51.7 mM) and finally *A. niger* 2 (0.4 nkat mg^{-1} and 66.7 mM). These K_m values are higher than those found by Soni et al. (2010) for *A. niger* phytases.

Finally, when the substrate was glyceraldehyde-3-phosphate (Fig. 4 e-h), a wide range of V_{max} (4.2-60.7 nkat mg^{-1}) was observed for the four enzymes. *A. niger* 1 showed the lowest value (4.2 nkat mg^{-1}), followed by *A. niger* 2 (12.1 nkat mg^{-1}), *E. coli 2* (14.3 nkat mg^{-1}) and *E. coli* 1 (60.7 nkat mg^{-1}). K_m values of the enzymes also had a wide range (2.4 mM to 34.1 mM). *A. niger* 1 showed the the highest affinity for this substrate (2.5 mM) followed by *E. coli* 2 (4.6 mM), *A. niger* 2 (5.2 mM) and *E. coli* 1 (34.1 mM). We did not find reports in the literature where the kinetic parameters of phytases were evaluated using glyceraldehyde-3-phosphate as substrate.

4 Conclusions

Obtained results partially support our first hypothesis since the selected phytases showed a great ability to release P from different organic P sources, but *A. niger* 1, 2 and *E. coli* 1 release more P from p-nitrophenyl phosphate~~pNP~~ than phytic acid while *E. coli* 2 has no preference for any particular substrate. Regarding to activity of phytases at different pH and temperature levels, phytases exhibited some differences in their pH and temperature levels to reach their optimum activity In contrast, our results did not support the second proposed hypothesis, since the retention of phytases by the soil solid phase did not have a clear association with the analysed soil properties. In this regard, it must be taken into account that the seven selected soils belonged to the Mollisol order. After being added to the soil, tested phytases showed an adsorption to soil solid phase ranging from 20 to 40%. Those phytases that remain in the solution could release P_i from the organic

P of the soil, whereas phytases that remain adsorbed to the soil solid phase could be released later. All studied phytases remained active at the optimum soil pH range of the most productive agricultural soils (5-7). In the same line, optimal temperatures for phytase activity were also within the temperature range more suitable for most agricultural crops (20-30ºC). Our results suggest that purified phytases may constitute a feasible tool to be used as a complement to P fertilization. In such sense, further experiments should be performed to evaluate the enzyme performance under field conditions to evaluate the ability of phytases to release from organic soil P sources, their interaction with soil microorganisms and to test if crops can capitalize the eventual provision of inorganic P released.~~The prospects of using phytases as biofertilizers were evaluated in experiments performed under controlled conditions. The maximum enzyme activities were observed at pH values ranging from 3.9 to 6.2. All studied phytases remained active at the optimum soil pH range of the most productive agricultural soils. Optimal temperatures for phytase activity were also within the temperature range more suitable for most agricultural crops (20-30ºC). After being added to the soil, tested phytases showed a low adsorption to soil solid phase (20-40%). Phytases that remain in the solution could release Pi from the organic P of the soil, whereas phytases that remain adsorbed to the soil solid phase could be released later, providing an additional release of P. Our results suggest that purified phytases may constitute a feasible tool to be used as a complement to P fertilization. In such sense, further experiments should be performed to evaluate the enzyme performance under field conditions to evaluate the ability of phytases to release from organic soil P sources, their interaction with soil microorganisms and to test if crops can capitalize the eventual provision of inorganic P released.~~

Author contributions

M. M. Caffaro, K. Balestrasse and G. Rubio designed the experiments and the method of data analysis. M. M. Caffaro performed the experiments and analyzed the data with G. Rubio. Finally, M. M. Caffaro prepared the manuscript with the contribution of all co-authors.

Conflicts of interest

Authors declare no conflict of interest regarding this research.

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Tables

Table 1. Characteristics of seven representative soils of the Argentina's Pampa Region that were used for to test phytases adsorption tests of phytases to soils. The samples were taken at a depth of 20 cm, air dried and screened at 1 mm prior to the analysis. Characteristics of the seven soils (0–20 cm) used in the phytase adsorption experiment.

Soil		Alberti	Adelia María	Lincoln	Oliveros	San Antonio de Areco	Balcarce	Balcarce
Soil type		Typic Argiudoll	Entic Haplustoll	Typic Argiudoll	Typic Argiudoll	Typic Argiudoll	Typic Argiudoll	Typic Argiudoll
pH		5.9	6.3	6.0	5.7	6.1	6.5	5.9
Ca^{+2+}	$cmol_c$	3.6	3.0	3.0	2.5	9.1	6.5	5.2
$Ca^{+2+} + Mg^{+2+}$	kg^{-1}	4.5	4.0	4.0	3.2	6.1	7.1	5.6
C_T total	g kg^{-1}	26.0	11.5	14.2	14.0	20.2	38.6	36.9
Clay		16.3	16.7	8.8	28.8	30.0	27.6	36.4
Sand	%	44.0	51.3	68.0	8.3	19.4	34.6	23.8
Silt		39.8	32.0	23.3	63.0	50.6	36.5	48.6
$P_{Bray\ 1}$		14.9	16.2	3.4	14.9	3.4	24.6	35.6

$P_{Mehlich\ 3}$		20.3	19.3	12.9	20.8	6.9	36.1	48.6
P_{T} ~~total~~	mg kg^{-1}	351	308	284	290	228	441	453
P_{O} ~~org~~		208	148	150	181	163	339	325
P_{i} ~~inorg~~		142	159	134	109	64	102	129
Al^{+3+}	$mmol_c$	1.0	0.7	0.5	0.7	0.8	1.3	1.8
Fe^{+3+}	kg^{-1} ~~$mmol_e$ kg^{-1}~~	1.3	1.1	1.3	1.1	1.4	1.9	2.3
$Clay_{ssa\text{-}BET}$	$m^2\ g^{-1}$	12.6	9.8	3.5	13.7	31.4	20.5	32.5

Table 2. Coefficients of the adjusted Lorentizian peak ~~adjusted~~ functions for phytase activity at different pH levels (see graphs in Fig. 2). ~~The function was adjusted from the observed results of the release of P from each of the substrates used at different pH levels.~~ The substrates used w~~as~~ere~~measured at different pH levels with~~ phytic acid, ~~pNP~~pNP-~~nitrophenyl~~Nitrophenyl ~~phosphate~~ and G3P~~G3Phosphate~~~~glyceraldehyde 3 p~~hosphate as substrates. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve were fitted. Different letters correspond to significant differences between treatments (P <0.05, LSD procedure).

Adjusted function: $y = \frac{a}{1+(\frac{x-b}{c})^2}$

Enzyme	Coefficients			R^2
Substrate: Phytic acid				
	a	b	c	
A. niger 1 + *A. niger* 2	36.6a	5.9a	2.7a	0.73
E. coli 1	30.1b	5.5ab	4.2a	0.55
E. coli 2	24.2c	4.7b	3.8a	0.66
Substrate: p-Nitrophenyl phosphate				
A. niger 1 + *A. niger* 2	49.96a	6.2a	1.03a	0.79
E. coli 1	36.88b	5.8a	1.96a	0.70
E. coli 2	24.16c	6.0a	1.54a	0.77
Substrate: Glyceraldehyde-3-phosphate				
A. niger 1 + A. niger 2	44a	3.9b	0.7b	0.94
E. coli 1	36.6b	4.1b	0.8b	0.89
E. coli 2	24.2c	6.0a	1.5a	0.77

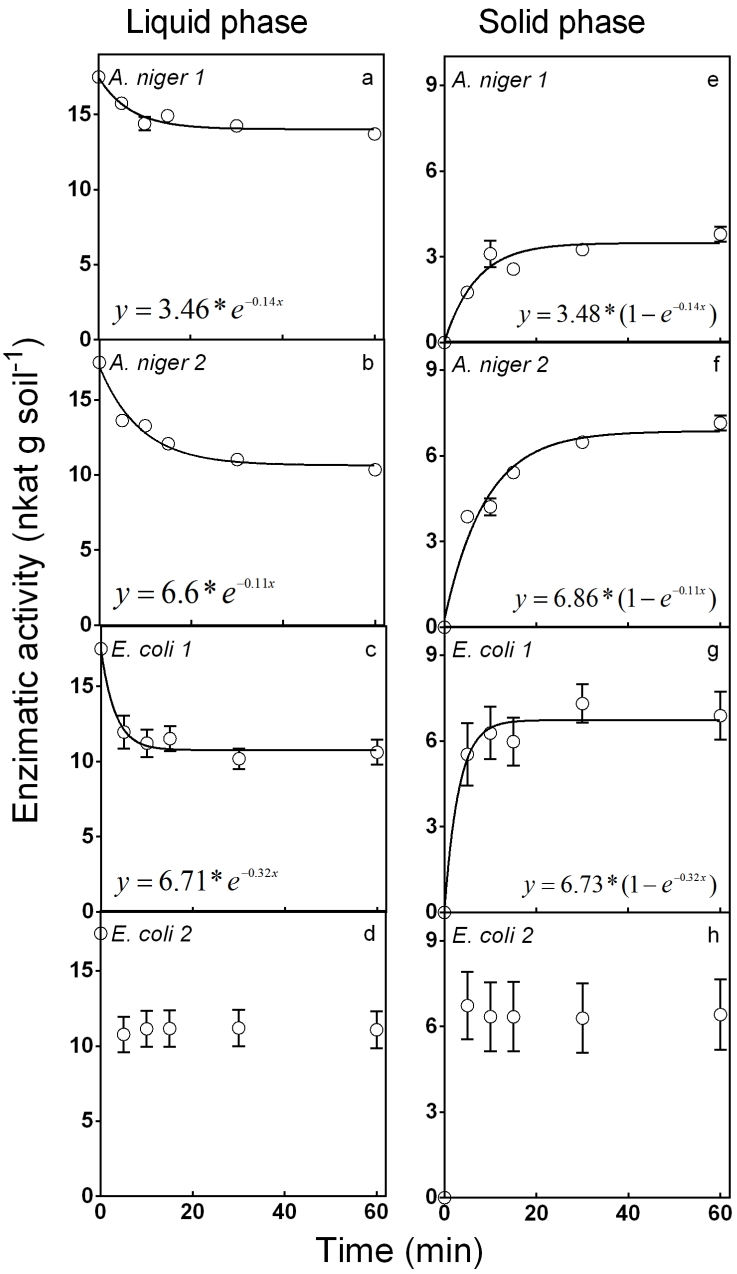
Table 3. Coefficients of the adjusted Lorentizian peak functions for phytase activity at different temperature levels (see graphs in Fig. 3). ~~The function was adjusted from the observed results of the release of P from each of the substrates used at different temperature levels.~~ The substrates used were~~as levels with~~ phytic acid, ~~pNP, G3Phosphate~~pNP ~~nNitrophenyl phosphate~~ and G3P~~glyceraldehyde-3-p~~hosphate ~~as substrates~~. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve were fitted. Different letters correspond to significant differences between treatments (P <0.05, LSD procedure)

~~Coefficients of the adjusted functions for phytase activity (see graphs in Fig. 3) measured at different temperature levels with phytic acid, p nitrophenyl phosphate and glyceraldehyde 3 phosphate as substrates. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve were fitted. Different letters correspond to significant differences between treatments (P <0.05, LSD procedure).~~

Adjusted function: $y = \frac{a}{1+(\frac{x-b}{c})^2}$				
Enzyme	Coefficients			R^2
Substrate: Phytic acid				
	a	b	c	
A. niger 1 + *A. niger* 2	33.47a	24a	13.12b	0.94
E. coli 1+ E. coli 2	24.53b	29a	21.61a	0.86
Substrate: p-Nitrophenyl phosphate				
A. niger 1 + *A. niger* 2	17.74b	29a	20.78a	0.97
E. coli 1	22.18a	29a	19.49a	0.96
E. coli 2	13.22c	29a	19.5a	0.95
Substrate: Glyceraldehyde-3-phosphate				
A. niger 1 + *A. niger 2*	10.05a	24b	42.03b	0.80
E. coli 1	6.62a	30b	36.34b	0.84
E. coli 2	12.61b	20a	53.4a	0.43

Figures

FIGURE 1. Phytase activity distributed in the liquid and solid phases for the phytase soil adsorption experiment. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. Experiments were performed with the seven soils described in Table 1. ~~For *A. niger* 1 and 2 and *E. coli* 1 phytases, a unique curve decay (Eq. (2)) and exponential increase (Eq. (3)) involving the seven soils was fitted because no significant differences (after F tests) were found between them. For *E. coli* 2, no function could be adjusted because a 37% binding to the soil solid phase was observed at 5 minutes and remained stable throughout the incubation period.~~ Each point represent the average of three observations minus the controls described in Materials and Method section. Bars represent standard error of the mean.


Liquid phase
Solid phase
A. niger 1
a
$y = 3.46 * e^{-0.14x}$
A. niger 2
b
$y = 6.6 * e^{-0.11x}$
E. coli 1
c
$y = 6.71 * e^{-0.32x}$
E. coli 2
d
A. niger 1
e
$y = 3.48 * (1 - e^{-0.14x})$
A. niger 2
f
$y = 6.86 * (1 - e^{-0.11x})$
E. coli 1
g
$y = 6.73 * (1 - e^{-0.32x})$
E. coli 2
h
Enzimatic activity (nkat g soil^{-1})
Time (min)

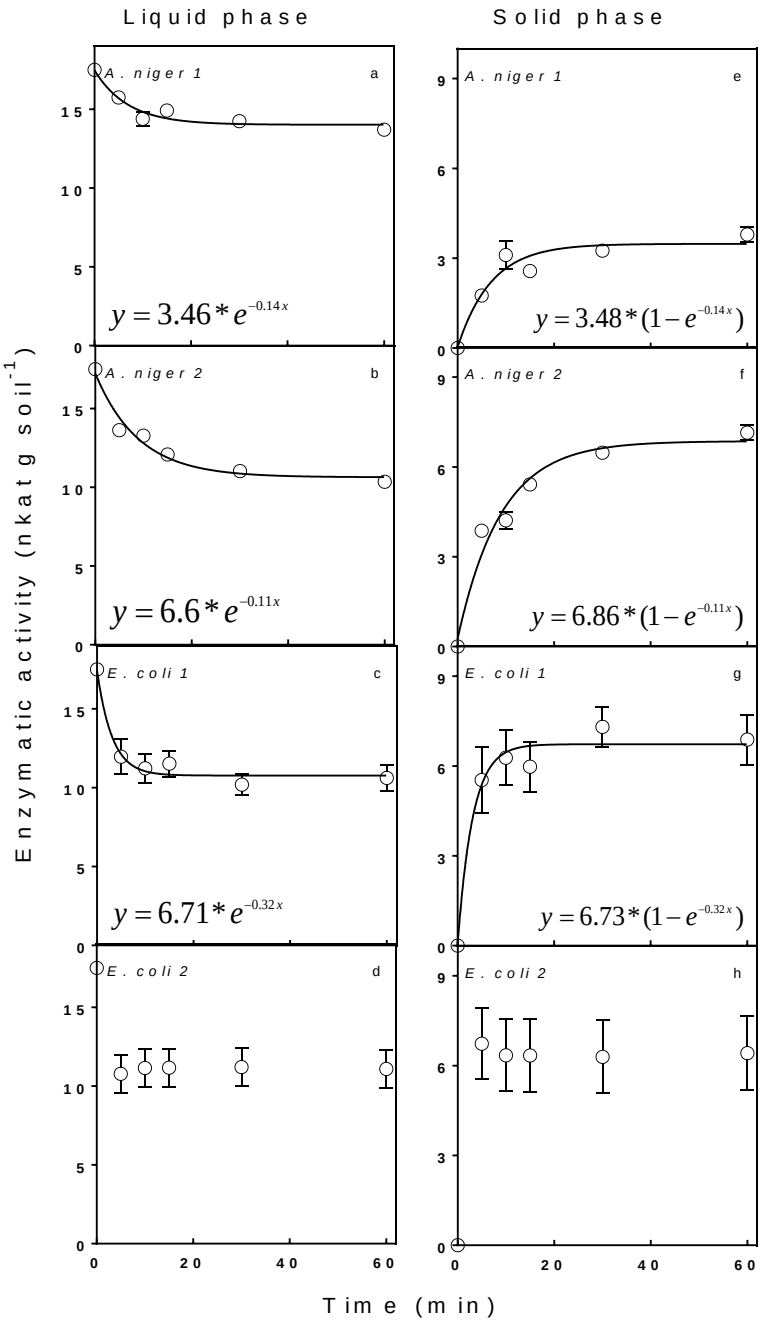

Liquid phase
Solid phase
A. niger 1
a
y = 3.46 * e^-0.14x
A. niger 1
e
y = 3.48 * (1 - e^-0.14x)
A. niger 2
b
y = 6.6 * e^-0.11x
A. niger 2
f
y = 6.86 * (1 - e^-0.11x)
E. coli 1
c
y = 6.71 * e^-0.32x
E. coli 1
g
y = 6.73 * (1 - e^-0.32x)
E. coli 2
d
E. coli 2
h
Enzymatic activity (nkat g soil-1)
Time (min)

FIGURE 2. Phytase activity measured at different pH levels with phytic acid, ~~pNP~~p-~~nitrophenyl~~NP~~itrophenyl phosphate~~ and G3P~~G3Phosphate glyceraldehyde 3 phosphate~~hosphate as substrates. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve was fitted. Each point represent the average of three observations minus the controls described in Materials and Method section. Bars represent standard error of the mean. Coefficients of each adjusted model are observed in table 2.

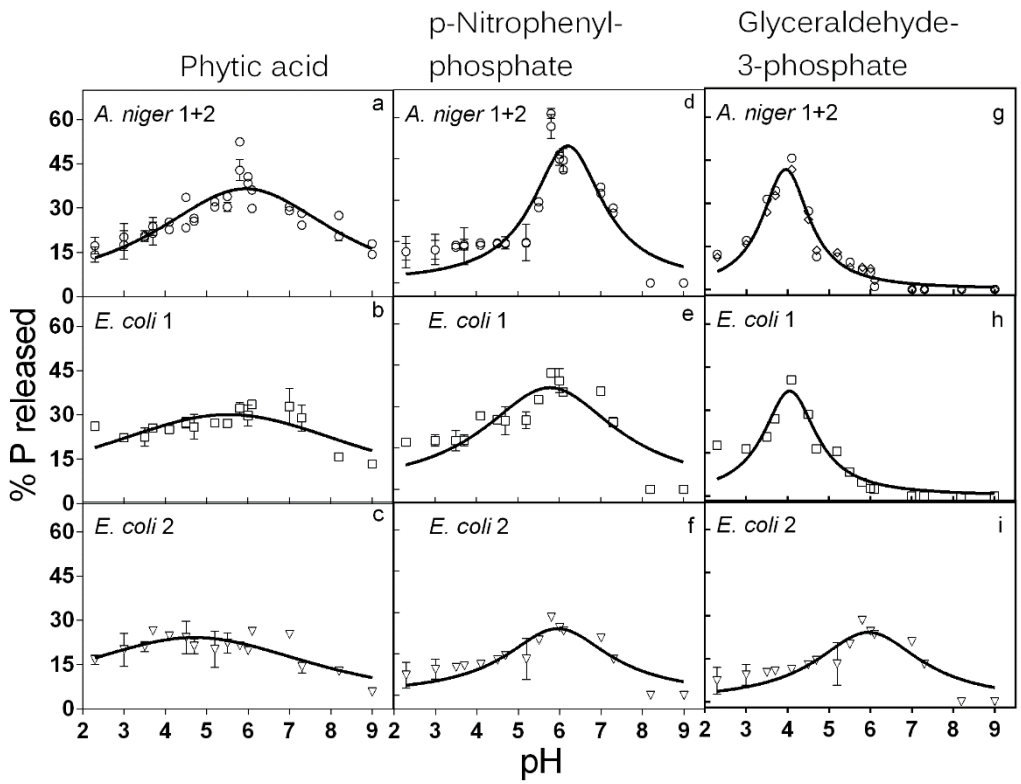


FIGURE 3. Phytase activity measured at different temperature levels with phytic acid, ~~pNP and G3Phosphate~~ pNP-~~nitrophenyl~~Nitrophenyl ~~phosphate~~ and G3P~~glyceraldehyde 3 p~~hosphate as substrates. Four purified phytases (two isolated from *A. niger* and two from *E. coli*) were evaluated. In those cases where significant differences between enzymes (analyzed by F tests) were not found, a unique curve was fitted. Each point represent the average of three observations minus the controls as described in the Materials and Method section. Bars represent standard error of the mean. Coefficients of each adjusted model are observed in table 3.

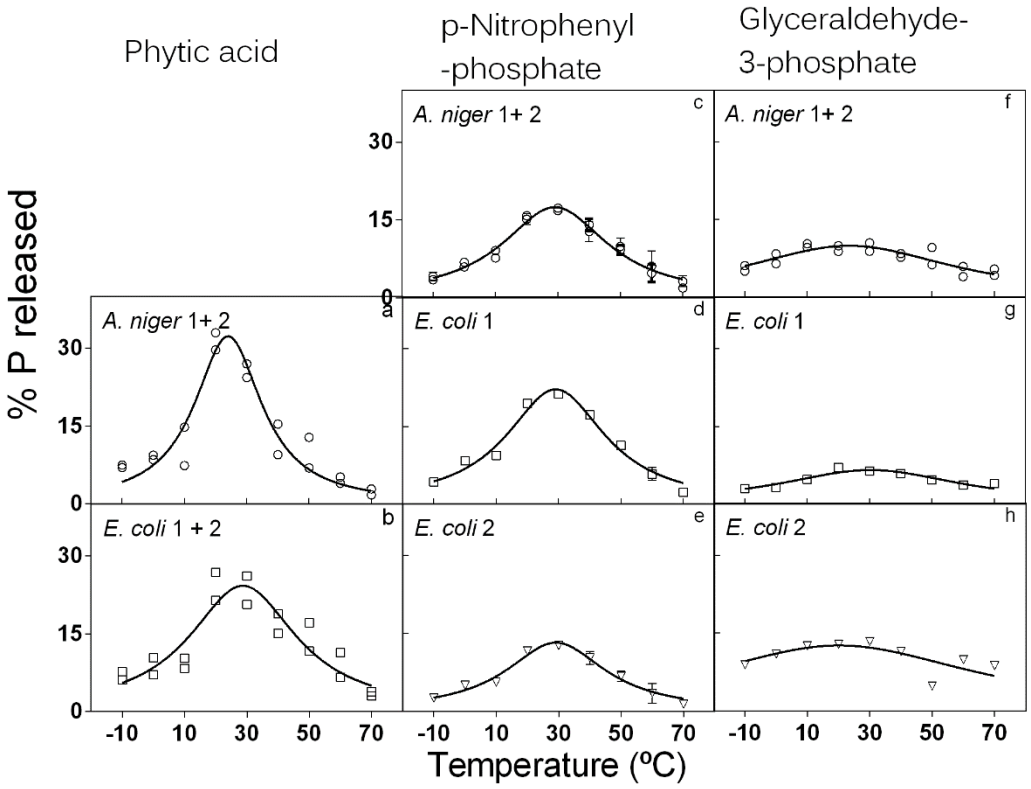
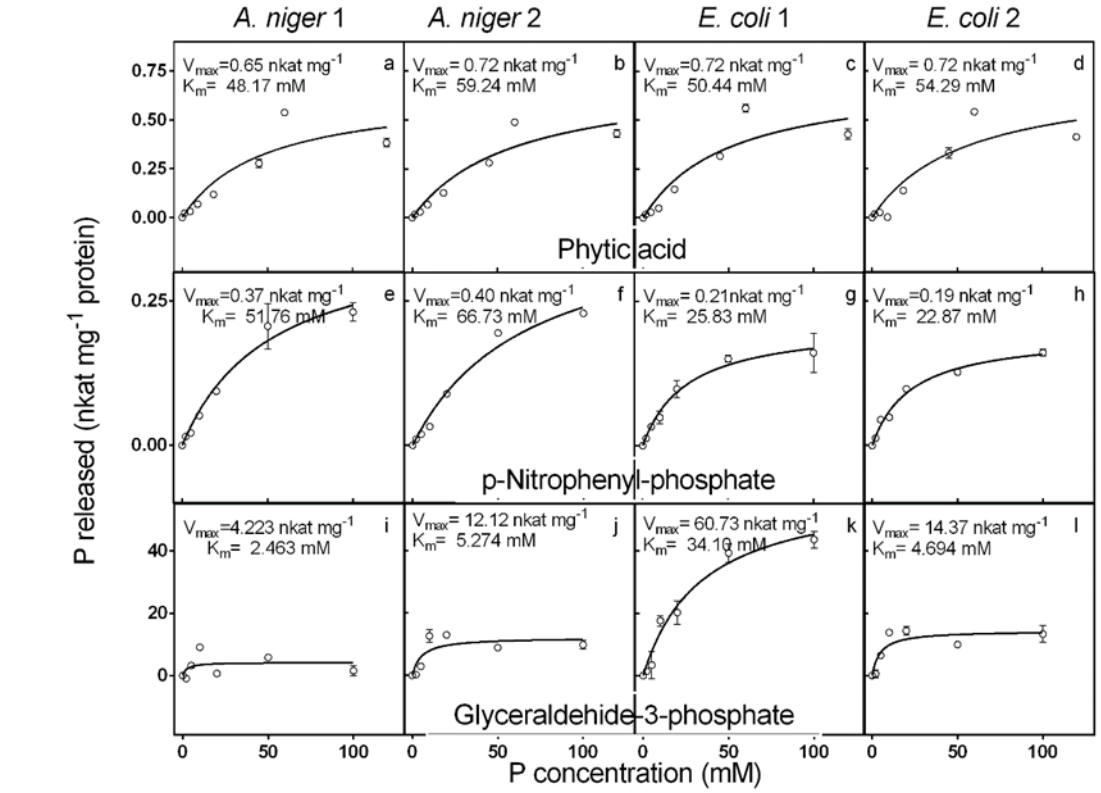
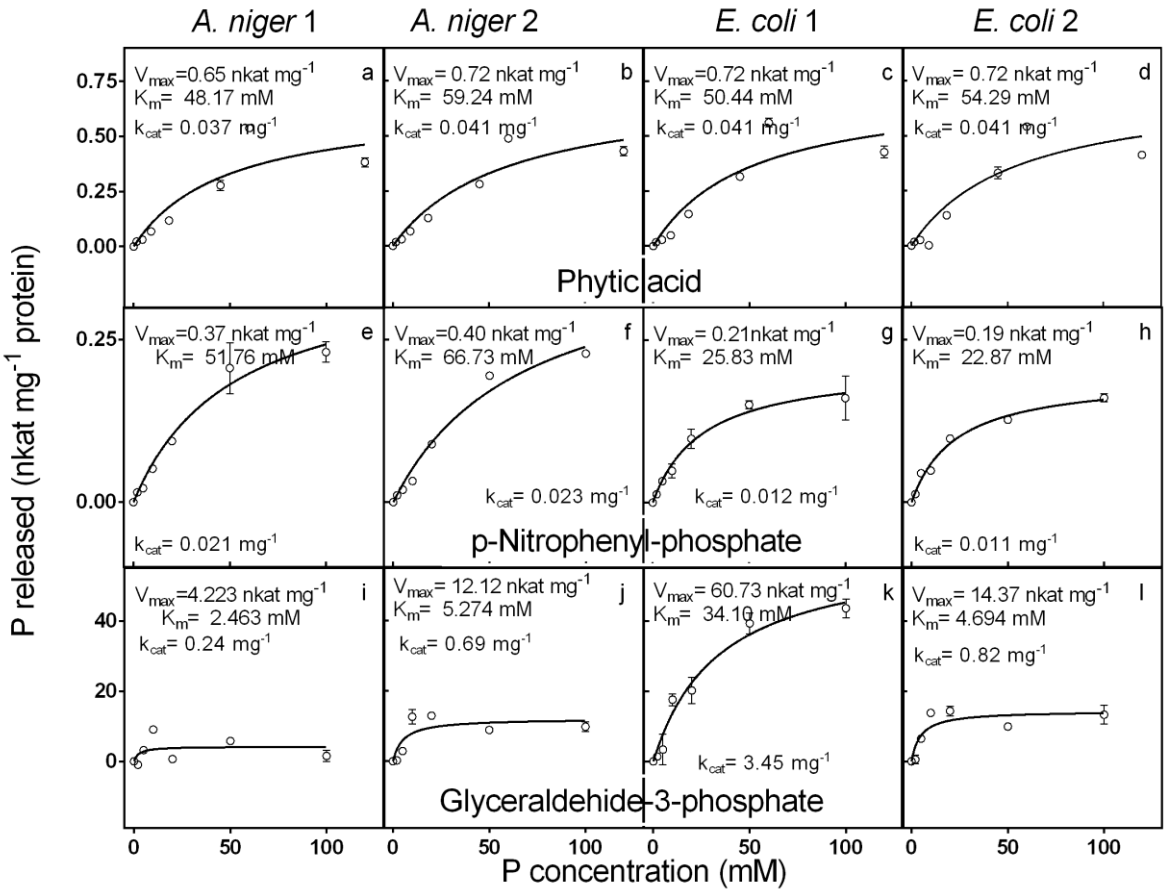


FIGURE 4. Kinetic parameters for phytic acid, pNPpNP- ang- G3Phosphatep- nitrophenylNitrophenyl- phosphate and G3Pglyceraldehyde- 3- phosphate as substrates of purified phytases (two isolated from *A. niger* and two from *E. coli*). The activity was determined at different concentrations of P (0 to 100 mM) contained in phytic acid as substrate. Each point represent represents the average of three observations minus the controls described in Materials and Method section. Bars represent standard error of the mean. Data were fitted to a Michaelis- Menten curve and the estimated V_{max} and K_m values obtained by the Lineaweaver- Burk method are shown.


A. niger 1
A. niger 2
E. coli 1
E. coli 2
Vmax=0.65 nkat mg-1
Km= 48.17 mM
Vmax= 0.72 nkat mg-1
Km= 59.24 mM
Vmax=0.72 nkat mg-1
Km= 50.44 mM
Vmax= 0.72 nkat mg-1
Km= 54.29 mM
Phytic acid
Vmax=0.37 nkat mg-1
Km= 51.76 mM
Vmax=0.40 nkat mg-1
Km= 66.73 mM
Vmax= 0.21nkat mg-1
Km= 25.83 mM
Vmax=0.19 nkat mg-1
Km= 22.87 mM
p-Nitrophenyl-phosphate
Vmax=4.223 nkat mg-1
Km= 2.463 mM
Vmax= 12.12 nkat mg-1
Km= 5.274 mM
Vmax= 60.73 nkat mg-1
Km= 34.10 mM
Vmax= 14.37 nkat mg-1
Km= 4.694 mM
Glyceraldehide-3-phosphate
P released (nkat mg-1 protein)
P concentration (mM)


A. niger 1
A. niger 2
E. coli 1
E. coli 2
Vmax=0.65 nkat mg-1
Km= 48.17 mM
kcat= 0.037 mg-1
Vmax= 0.72 nkat mg-1
Km= 59.24 mM
kcat= 0.041 mg-1
Vmax=0.72 nkat mg-1
Km= 50.44 mM
kcat= 0.041 mg-1
Vmax= 0.72 nkat mg-1
Km= 54.29 mM
kcat= 0.041 mg-1
Phytic acid
Vmax=0.37 nkat mg-1
Km= 51.76 mM
kcat= 0.021 mg-1
Vmax=0.40 nkat mg-1
Km= 66.73 mM
kcat= 0.023 mg-1
Vmax= 0.21nkat mg-1
Km= 25.83 mM
kcat= 0.012 mg-1
Vmax=0.19 nkat mg-1
Km= 22.87 mM
kcat= 0.011 mg-1
p-Nitrophenyl-phosphate
Vmax=4.223 nkat mg-1
Km= 2.463 mM
kcat= 0.24 mg-1
Vmax= 12.12 nkat mg-1
Km= 5.274 mM
kcat= 0.69 mg-1
Vmax= 60.73 nkat mg-1
Km= 34.10 mM
kcat= 3.45 mg-1
Vmax= 14.37 nkat mg-1
Km= 4.694 mM
kcat= 0.82 mg-1
Glyceraldehide-3-phosphate
P released (nkat mg-1 protein)
P concentration (mM)