

Jan 29th 2020

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

Dear Executive Editors
Soil Journal

We would like thank you and the anonymous reviewer (II) for the 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. This modifications will be added to the response to reviewer I.

Regards,

Dr. Gerardo Rubio (corresponding author)
School of Agriculture - University of Buenos Aires

.....

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

Anonymous Referee #2 Received and published: 10 January 2020

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 to Misset (2003), "real" phytases are those enzymes capable to degrade completely phytate molecules and to release all phosphates contained in them. However, the term phytases is a topic of debate as pointed out by the reviewer. Moreover, one paper of the author cited by the reviewer includes the specific and general term in the title (Konietzny, U., & Greiner, R. 2002. Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int. J. Food Sci. Tech., 37, 791-812.").

Enzymes used in this work are commercially sold under the generic name "phytases". We did not perform tests to evaluate the three-dimensional structure of the enzyme because

that issue was beyond the scope and objectives in this stage of our research. However, many authors refer the 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.anifeeds.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)."

Regarding the title, an alternative one 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, 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 generally 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 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 as an example of how they describe the strain.

SEE TABLE IN THE SUPPLEMENT FILE "soil-2019-50-supplement.pdf"

Table 1. General Information about the Phytase Products Used in the in Vitro Digestion Model

	name in this work						
	EC1	EC2	EC3	BSP	CB	PL	AN
trademark	Quantum	Quantum Blue	PhyzymeXP	AxtraPHY	Ronozyme Hiphos	Ronozyme NP	Natuphos
supplier	AB Vista	AB Vista	Danisco	Danisco	Novozymes/DSM	Novozymes/DSM	BASF
donor organism	<i>Escherichia coli</i>	<i>Escherichia coli</i>	<i>Escherichia coli</i>	<i>Buttiauxella</i> sp.	<i>Citrobacter braakii</i>	<i>Peniophora lycii</i>	<i>Aspergillus niger</i>
production organism	<i>Trichoderma reesei</i>	<i>Trichoderma reesei</i>	<i>Schizosaccharomyces pombe</i>	<i>Schizosaccharomyces pombe</i>	<i>Aspergillus oryzae</i>	<i>Aspergillus oryzae</i>	<i>Aspergillus niger</i>
formulation	liquid	liquid	liquid	liquid	liquid	liquid	solid

..

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

R: We agree. That`s why 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 con-tributes 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 agree that our hypothesis can lead to misunderstandings. We are not talking about phytases in general, but specifically about the 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”. See our reply to comment 7 for more explanations on this topic.

...

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 (-10° to 70°C): p-nitrophenyl-phosphate (pNP), glyceraldehyde-3-phosphate (G3Phospahte) and phytic acid. Phytases had low affinity for the solid phase (23-34%of adsorption after one hour of incubation). Phytases from *A. niger* showed a higher capacity to release P, than phytases from *E. coli* (+13% on average). All phytases were active throughout the pH and temperature ranges for optimum crop production under field conditions. The amount of P that *A. niger* phytases release at pH values commonly found in agricultural soils (5.5-7) was as follows: pNP > phytic acid > G3Phosphate, whereas in *E. coli* phytases the order was pNP / phytic acid > G3phosphate. Obtained results are 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.

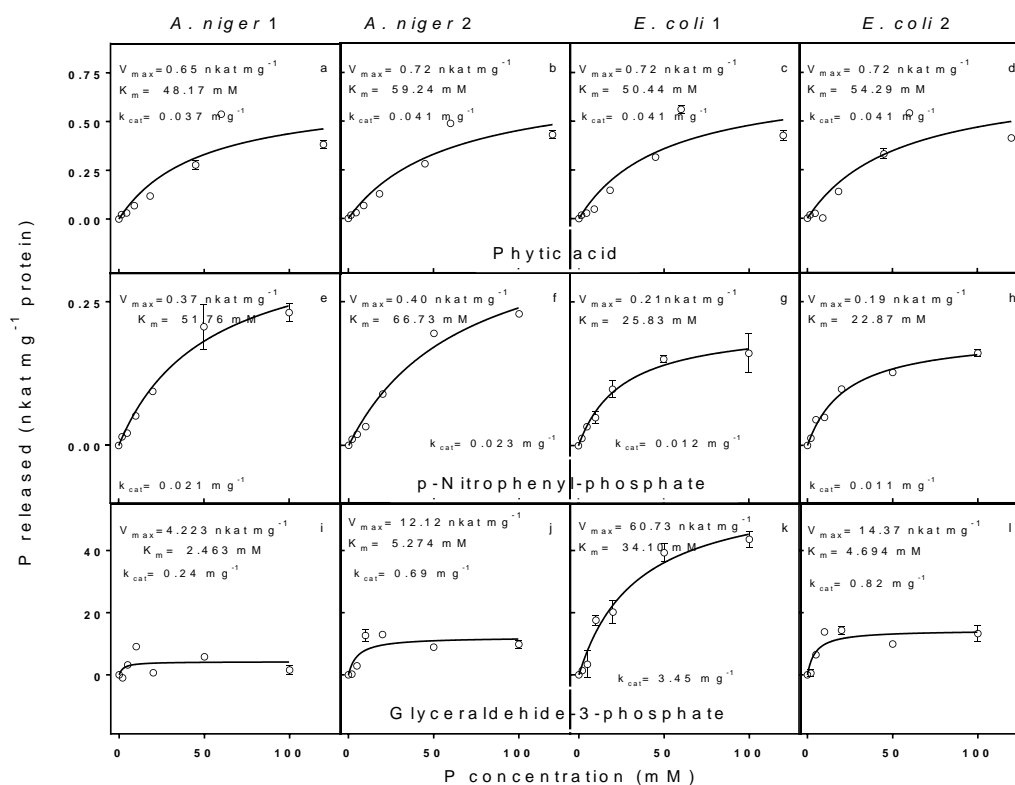
R: We understand the point raised by the reviewer. The original hypotheses may appear as basic for ultra-purified enzymes or recombinant proteins produced for academic or related activities. Previous studies about phytases mainly come from ultrapurified enzymes like the ones provided by lab-supplies companies such as Sigma and used in academic labs. It is clear that this high quality but extremely expensive products cannot be used in real agricultural by farmers. In this report we tested at which extent commercial phytases have comparable performance than the ultrapurified enzymes. 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



SEE FIGURE IN THE SUPPLEMENT FILE “soil-2019-50-supplement.pdf”

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. The sentence in Materials and methods was reworded 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” as required. Statistical analysis was 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 the results were exactly the same. This software is cited in the new version.

...

13. Were the buffers set at the optimal temperature?

R: The buffers were prepared at normal room temperature (20-24 °C) and the incubation experiments were performed at 25 °C (for evaluating optimum pH and kinetic parameters) and along a temperature range (-10-70°C) for evaluating optimum temperature for enzyme activity. In this last 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 sentence 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 α -domain of each phytase (Konietzny and Greiner, 2002)"). The temperature during the pH experiment was set at 25 °C. This temperature is within the optimal range found for the 4 tested enzymes (20 to 29°C). On the other hand, 25 °C is also within the optimal range of crops growing under field conditions, which are the final context of our line of work on phytases in future experiments. These 25 °C are also clearly within the optimal range of the set of buffers used to generate the pH range (e.g. Hayes et al. 1999; cited in the ms).

...

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

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