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PRELIMINARY STUDIES ON THE CAPACITY OF SOME MICROORGANISMS FOR THE SYNTHESIS OF PHYTASES

Andreea DOBRE[1,2], Iulian GROSU[1], Alina Elena BUȚU[2], Călina Petruța CORNEA[1]

[1]University of Agronomic Science and Veterinary Medicine – Bucharest, no. 59, Blvd. Bd. Mărăști, zip code 011464, Bucharest, Romania, phone +40 (21) 3182564, fax +40 (21) 3182567, dobre_mc_andreea@yahoo.com, suntalex@yahoo.com, pccornea@yahoo.com
[2]National Institute of Research and Development for Biological Sciences Bucharest, no. 296, Splaiul Independenței, zip code 060031, Bucharest, Romania, phone: 021-220.77.80; 021-220.79.09, fax: 021-220.76 95, dobre_mc_andreea@yahoo.com, alina_butu@yahoo.com

Corresponding author email: dobre_mc_andreea@yahoo.com

Abstract

Phosphorus is one of the major constituents of which are involved in metabolic processes, nucleic acid and cell membranes biosynthesis as well as in the regulation of a large number of enzymes. Phytate (myo-inositol hexakisphosphate 3-phosphorilase) is the main storage form of phosphorus in various crops (cereals, legumes, and oilseed crops) and its accumulation in natural ecosystems could reduce the availability of various metal ions such as Fe, Zn, Mg or Ca, and could cause environmental pollution effects. Phytases are enzymes that catalyze the hydrolytic phosphate cleavage of phytic acid to lour inositol phosphate esters and inorganic phosphate. In the case of monogastric animals which do not have microbial phytases in their digestive system, the formation of insoluble metal cation-phytate complexes at physiological pH values is regarded as the major reason for poor mineral availability, because these complexes are essentially nonabsorbable from the gastrointestinal tract. However, phosphorus from soil is largely unavailable due to its rapid immobilisation of the organic and inorganic soil constituents. Several studies are focused on the exogenous and endogenous microbial phytases (produced by fungi - Aspergillus niger, Aspergillus ficuum, yeasts - Saccharomyces cerevisiae, or bacteria - Leuconostoc mesenteroides, Bacillus amyloliquefaciens) and their influence in the phytic acid dephosphorylation. The identification and the characterization of new microbial strains able to produce phytase and possible other important compound continue to be of large interest both for fundamental studies and for practical applications. For this reason, the aim of the present work was the identification of new phytase producing microbial strains from soil samples of different origins and by using collection microbial strains. The phytase activity was detected by cultivation on phytase specific medium (PSM) [1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01%, $CaCl_2.2H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 6.5 with 0.5% sodium phytate). Six bacteria strains (BPA, OS15, OS17, B4, B5 and B6) and one fungal strains (A.niger An) capable of hydrolyzing sodium phytate were recognized by their surrounding clear halo on PSM containing plates. Preliminary experiments on the characterization of the new isolates were also realized.

Keywords*: phytase, phytate*

INTRODUCTION

One of the macroelements existing in the living cells is phosphorus in the form of ortho or pyrophosphoric acids. Phosphorus is a part of the structure of nucleic acids, phospholipids, enzymes, hormones, etc. In the agricultural sector, the biggest part of the phosphorus-containing fertilizers (>90%) becomes unavailable to plants. This is due the interaction with the soil structure, adsorption and precipitation, which includes phosphorus into organic conglomerates or insoluble inorganic minerals (Mukhametzyanova et al., 2012). Important proportion of the supplied phosphorus is immobilized in the soil as phosphates of several divalent and trivalent metals like iron, aluminium or calcium (Kaur and Reddy, 2013). Though, the lack of phosphorus bioavailability leads to the need of frequent application of phosphate fertilizers, its presence causing environmental pollution, specifically eutrophication of rivers, lakes and water basins (Haefner et al., 2005; Naves et al, 2012; Szara and Sosulski, 2012).

Phytic acid is a chemical derivate of a six fold alcohol, inositol, with six molecules of phosphoric acid residues bound to its six

hydroxyl groups. Phytic acid is a source of phosphorus which can be used by human, animals, plants and microorganisms. Phytic acid or its salt form, phytate, [myo-inositol(1,2,3,4,5,6) hexakisphosphate], is able to bind the ions of divalent or trivalent metals like sodium, calcium, copper, with aminoacid residues, proteins or carbohydrates.

Phytate is the major storage form of phosphorus in plants. During plants maturation, phytic acid is accumulated in plant seeds, mostly as salts of mono and bivalent cations. It is known that phytic acid acts as phosphorus, cations and myoinositol source. Phytic acid also acts as a natural antioxidant in seeds during dormancy (Graf et al 1987). Phytic acid was also found in roots and tubers, vegetables, fruits, nuts, and pollen of various plant species. In feeds, its concentration varies considerably. In most grains and oilseeds the concentration of pytate is around 0.7-2%. It was stated that about 14.4 mil of tones is annually produced by seeds and fruits production at global level (Kumar et al., 2011).

Its presence in the monogastric intestinal tract is being the primary reason for poor mineral availability because of its ability to form insoluble metal cation-phytate complexes at physiological pH values, causing the lack of nutrients absorbance by the organism (Lopez et al, 2002).

The interest in phytate-degrading enzymes and their application in nutrition, agriculture and environmental protection have recently advanced significantly (Hussin et al., 2010). Phytase (myo-inositol hexakisphosphate 3-phosphorilase) catalyses the sequential release of phosphate by the hydrolysis of phytic acid in a stepwise manner, releasing lour inositol phosphates and inorganic phosphate (Naves et al., 2012). Phytases are divided in to categories, 3-phytase (myo-inositol hexaphosphate 3-phosphohydrolase) and 6-phytase (myo-inositol hexaphosphate 6-phosphohydrolase). Based on their catalytic characteristic, phytases are classified as HAP (histidine acid phosphatase), BPP (ß-propeller phytase), CP (cysteine phosphatase) and PAP (purple acid phosphatase (Mullaney and Ullah, 2003).

Phytase reduces the antinutritional properties of phytic acid and eutrophication (Khumar and Batt, 2011). Also, lately, phytase has been used in aqua feed industries in order to enhance the growth performance, nutrient utilization and bioavailability of macro and micro minerals in fish and also to reduce the P pollution into the aquatic environment (Kumar et al., 2011).

They can be synthesized both by micro organisms (bacteria, fungi and yeast) including the rumen microbes in ruminants, and soil microbes, and plants like wheat, barley, rice, corn or rye (Paik, 2003). According to Zhang et al. (2013), phytases can be isolated even from mushrooms. They isolated a phytate-degrading enzyme from *Lentinus edode*, a shitake mushroom, which has been shown to be relatively tolerant to high pH and is thermostable. The activity of endogenous phytase is comparatively higher in cereals and cereal by-products than in legume seeds (Kumar et al., 2011). The microorganisms able to produce phytases can also be isolated from various plants. Marlida et al. (2010) isolated species of *Rhizoctonia* and *Fusarium* as phytases producers from stem and root fragments from soybean and (Dave and Modi, 2013) from mangroves rhyzosphere.

The most phytases which are currently available for application as feed enzymes have shortcoming such as a narrow pH optimum and lack of resistance in high temperatures. Because of the fact that most feed manufactured for non-ruminants are pelleted at temperatures between 65 and 80^{o}C, most of the phytase activity will be lost under the processing conditions. Moreover, the phytases which are commonly used are rather susceptible to proteolysis (Elkhalily et al., 2007). The plant phytase activity in mature seeds is very low, contrary to its activity at the level of germination stage. The second reason for which the phosphorus from seeds used as feeds doesn't reach to animals is the fact that the phytases are highly thermolabile. Moreover, it appears that in feedstuffs, the phytases activity present great variations due its dependency on the genetic and environmental factors (Steiner et al., 2007).

The phytases which are secreted into soil by the microorganisms are participating to decomposition of plant debris and also to releasing of phosphorus from organic compounds.

Among the fungi which are able to produce phytases, we remind the *Aspergillus spp.*, *Fusarium spp.*, *Mucor spp.*, *Penicillium spp.* and *Rhizoctonia spp.* Several bacteria genus are synthesizing as well phytases, like *Bacillus*, *Enterobacter*, *Klebsiella*, *Escherichia*, *Leuconostoc*, etc. Also yeasts which are capable to produce these enzymes are *Saccharomyces* spp. and *Candida* spp. Also, during the fermentation process of bread dough, according to Nuobariene et al (2012) study, all yeasts involved are synthesizing pytases.

It is still a controverse in the scientific community whether the lactic acid bacteria are synthesizing or not phytases. During the sourdough fermentation, the lactic acid bacteria are either involved in phytate degradation or they decrease the pH, caused by lactic acid produced, and the plant's intracellular phytases are activated due this pH level. Although it was demonstrated that some strains of *Lactobacillus san franciscensis* was one of the best lactic acid bacteria which are able to produce phytases.

The optimum pH levels of phytases vary from 2.2 to 8. Generally, fungal phytases are activated in the pH range between 4.5 and 5.6. The optimal pH levels for those synthesized by bacteria and especially by *Bacillus spp*., are 6.5 – 7.5. Elhadi et al. (2011) observed that some strains of *E. coli* and *Klebsiella* spp. are able to produce phytases which have optimal enyzimatic activity to neutral, respectively acid pH.

The optimum temperature of phytases varies from 45 to 77°C (Caipang et al., 2011). Dechavez et al. (2011) stated that most phytases from *Bacillus* strains shows maximum activity to 37^{o}C, but are maintaining its activity even until 80^{o}C. However, Yu and Chan (2013) were able to isolate a strain of *Bacillus nealsonii* which shows a strong thermal stability, reaching the highest enzymatic activity at 55^{o}C and neutral pH.

The main objective of the present study was to detect the ability of some microbial strains from different species of *Bacillus sp*. (*Bacillus subtilis, B.amyloliquefaciens, B.pumilus*) to synthesize phytases in *in vitro* conditions. *Bacillus sp.* in one of the most studied and used bacteria genus for phytate-degrading enzyme synthesizing (Fu et al., 2008). Currently, about 60 % of the worldwide industrially produced enzymes are due to *Bacillus sp* (Fu et al. 2007).

According to Maksimov et al. (2011), *Bacillus subtilis* is able to produce extracellular phytases. In 1992 Shimizu was able to obtain a 36 kDa phytase from a strain of *B.subtilis* with the activation energy of 9.87 kcal/mol for sodium phytate, at 60^{o}C and pH 6,0 – 6,5. Kim et al. (1998) has isolated some *Bacillus* species producing a thermostable phytase from the soil of Korean cattle shed. They were also able to isolate a phytase which demonstrated that it has optimal activity at 70^{o}C and pH 7. Hong et al. (2010) has succeeded to isolate a strain from cattle feces which produced a phytase of 46 kDa molecular weight and having an optimal activity at 60^{o}C, pH 7.

Bacillus amyloliquefaciens is another species intensively used as phytase producer (Mukhametzyanova et al., 2011). Oh et al. (2011) observed that a thermostable phytase from *B.amyloliquefaciens DS11* is Ca^{2+}-dependent in order to catalyse the sequential release of phosphate from phytic acid. Also it was observed that the thermal stability of *B.amyloliquefaciens* phytases is strongly dependent on calcium ions (Ha et al. 1998). Although *Bacillus pumilus* did not receive the same consideration as other *Bacillus* species as phytase synthesizing bacteria, it was brought into attention its ability to be efficiently genetically manageable in order to produce qualitative phytases because its high capacity to secrete extracellular enzymes and to exhibit a pronounced resistance against oxidative stress (Wemhoff and Meinhardt, 2013). One of the currently uses is the control of several soil fungal pathogens (Maksimov et al., 2011). According to Vassilev et al. (2012), there were discovered phytase-producing strains of *Bacillus* sp. which are resistant to salinity, high pH and high temperature, thus helping mitigate the problems caused by the stress factors in agriculture.

The aim of our work was the screening of some phytate degrading microorganisms, isolated from natural sources.

MATERIALS AND METHODS

Microorganisms and culture preservation

The microorganisms used in the present study were presented in table 1.#

Table 1. Microbial strains used in experiments

Strain designation	Species	Source
Bl	*Bacillus licheniformis ATCC 14580*	Fac. Biotechnology collection
7.1.	*B.amyloliquefaciens*	Soil (Israel et al., 2012)
BN7	*B.amyloliquefaciens*	
Omf	*B.amyloliquefaciens*	
BW	*B.amyloliquefaciens*	Soil (Sicuia et al, 2012)
OS17	*B.amyloliquefaciens*	Onion rhizosphere (Sicuia et al, 2012)
OS15	*B.amyloliquefaciens*	
B2Vio	*B.subtilis*	Oil contaminated soil (Olteanu et al., 2011)
BPA	*Bacillus subtilis*	Soil
BIR	*B.pumilus*	Soil
B1	Unindentified	Compost
B2	Unindentified	
B3	Unindentified	
B4	Unindentified	
B5	Unindentified	
B6	Unindentified	
An	*Apergillus niger*	Airborne sprores

The bacteria were routinely maintained on nutrient agar and the filamentous fungi were cultivated on Potato Dextrose Agar (PDA).

The screening method

In order to select the phosphate solubilising microorganisms, three type of culture media were used: phytase specific medium (PSM): 1,5% glucose, 0,5% $(NH_4)_2SO_4$, 0,05% KCl, 0,01% $MgSO_4.7H_2O$, 0,01% NaCl, 0,01% $CaCl_2.2H_2O$, 0,001% $FeSO_4$, 0,001% $MnSO_4$, pH 6,5 and 0,5% sodium phytate (Bae et al., 1999); Pikovskaya's agar plates (PKA) (g L-1): glucose: 10.0; $(NH_4)_2SO_4$: 0.50; KCl: 0.20; $MgSO_4.7H_2O$,: 0.010; MnSO4·H_2O: 0.0001; FeSO4·7H2O: 0.0001, yeast extract: 0.50; 0.5% tri-calcium phosphate as the sole P source, pH 7.0 (Kaur and Reddy, 2013); wheat bran extract agar containing $(NH_4)_2SO_4$-0.04%, $MgSO_4.7H_2O$-0.02%, casein-0.1%, KH_2PO_4-0.05%, K_2HPO_4-0.04% and agar-2% (Sreedevi and Reddy, 2012). The inoculated plates were incubated for 3-7 days at 30°C. The experiment was realized with three repetitions in order to obtain accurate results. The detection of clear zone surrounding the bacterial colonies is the sign of phosphate solubilising activity or phytase biosynthesis. The efficiency of hydrolysis was determined by the formula Z-C/C, were Z = halo diameter and C = colony diameter (Joseph and Jisha, 2008).

RESULTS AND DISCUSSIONS

The great industrial significance of phytases explains the interest in isolation of new microbial strains producing such enzymes and in optimization their biosynthesis. Moreover, the ability of phytase producing bacteria for phosphate solubilization could be of interest for agronomic applications (increase soil fertility in an organic fields) (Kaur and Reddy, 2013).

Among the 16 bacterial strains used in experiments differences were observed on the three culture media tested. When wheat bran extract agar was used, the majority of the bacterial strains tested presented clear halo around their colonies (fig.1)

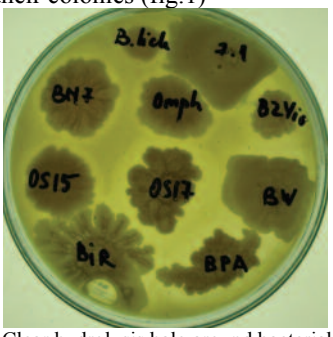

Figure 1. Clear hydrolysis halo around bacterial colonies on wheat bran agar

On PKA plates, small halo were detected only for B4, B6, BPA and OS15 bacterial strains.

Positive reaction on PSM medium was obtained for the strains 7.1, OS15, OS17, B4, B5, B6 BPA (fig.2) as well as for *A.niger* strain An.

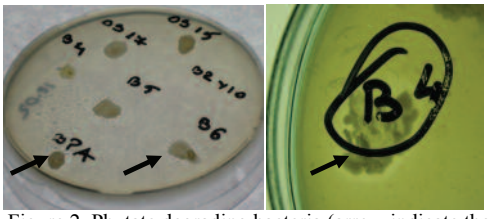

Figure 2. Phytate degrading bacteria (arrow indicate the hydrolysis area) on PSM medium

Best results, as hydrolysis efficiency were obtained with the strains BPA (over 81%), B6 (66%) and B5 (50%). Interesting aspect was observed for the *A.niger* strain: the hydrolysis area started after 48 h of incubation and its diameter continued to increase for the next 5 days (Fig. 4).

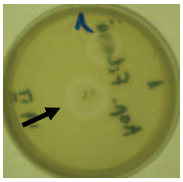

Figure 3.Hydrolysis area for *A.niger* strain on PSM mediu.

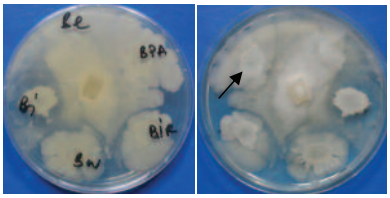

Figure 4. Growth inhibition of *Pythium debarianum* by bacteria. The arrow indicate the strain BPA

The results suggest that despite the positive reaction of bacteria on wheat bran agar, not all the strains are able to produce phytate. In this order, this medium could be used for a preliminary screening of possible hydrolysis of phytate from wheat bran but the confirmation of phytase biosynthesis need to be performed on PSM medium.

The results obtained in these experiments with soil derived bacteria are in accordance with the data of Gulati et al. (2007) and Singh et al. (2013) that isolated several strains of *B.subtilis* from rhizosphere, which were able to produce phytases.

Moreover, the strains designated B5 and B6 that presented large hydrolysis halo on PSM medium (fig.3) were isolated from compost, natural medium with increased phosphorus content, and they could be involved in the reduction of this compound from the environment (the presence of microbial phytases can reduce the environmental pollution of phosphorus in areas of intensive animal production)(Lei and Porres, 2003).

Regarding the strain designated as OS15, it was identified as *B.pumilus* (using Biolog GEN III system, data not shown); its ability for phytate hydrolysis is in accordance with the results of Watharkar et al. (2013).

Interesting results were obtained with *B.amyloliquefaciens* strains (described in literature for the thermostablitity of the phytases)(Haefner et al., 2005): clear phytate degrading area presented the strains OS17. It has to be noticed that the strains B6, BPA and OS15 presented also clear halo on PKA medium, suggesting their ability to hydrolyse both inorganic phosphorus (insoluble tri-calcium phosphate) and phytate.

Moreover, the strains BPA, BW, B5 and B6 presented antagonistic activities against several plant pathogenic fungi (like *Pythium debarianum*) (Fig. 4).

These results allow the conclusion that the selected bacterial strains could be of great interest not only for the phytate degrading activity but also for their biocontrol properties.

CONCLUSIONS

Several microbial strains were selected for phytate degrading abilities. The highest hydrolysis areas were detected for the strains BPA, OS15, OS17, B4, B5, B6 and *A.niger* An. The strains B4, B6, BPA and OS15 have inorganic phosphate solubilising properties.

It is obvious that the source of isolation (i.e. compost) is very important in the detection of the bacteria able of phytate degradation, but also the quality of nutritive media and parameters like temperature or pH.

The results of the present study suggest that the use for inoculation of such bacteria (with phytate degrading and antifungal abilities) is a good tool to improve the soil's available P level without the use of chemical phosphorous fertilizers and also to provide plant protection.

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