

# Expression of *Bacillus ginsengihumi* M2.11 bacterial phytase by recombinant *Pichia pastoris* strains

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**Abstract.** Phytic acid is the main storage form of organic phosphorus. Due to its structural features, phosphorus in phytate is inaccessible for assimilation by animals. Moreover, remaining inaccessible reservoir of phosphorus for animal nutrition, phytic acid is capable of forming insoluble complex salts, which lead to soil and water pollution. Microbial enzymes - phytases, capable of decomposing phytic acid to organic phosphorus are being used as feed additives in animal nutrition to solve this problem. Thus, search and development of technologies for the production of enzymes on an industrial scale are the most urgent. Methylotrophic yeast *P. pastoris* are widely used in biotechnology, as an efficient system for the recombinant proteins expression. They have many advantages, including rapid growth on inexpensive media, a wide range of molecular tools for genetic manipulation in optimizing production processes, they are safe for humans and animals, carry-out many post-translational modifications and produce recombinant proteins intracellularly or extracellularly within a short period of time. It was found that the recombinant *P. pastoris* strains pPINK-LC- $\alpha$ -MF -phyC, pPINK-HC- $\alpha$ -amyl -phyC, pPINK-LC- $\alpha$ -amyl -phyC, pPINK-HC- $\alpha$ -MF -phyC are able to produce and to secrete *B. ginsengihumi* bacterial phytase M 2.11 phyC. The maximum activity was observed in the pPINK-LC- $\alpha$ -MF strain – 2.6 (U / mg). Recombinant *B. ginsengihumi* M 2.11 phytases exhibited high activity in a wide pH range from 2.5 to 9.0. The MF-phyC-HC construction is pH stable. The temperature optimum of all recombinant phytases corresponds to 37 ° C; recombinant phytases retain their activity in the range from -80 to 90C.

## 1 Introduction

Plant foods contain high levels of phytic acid and related salts called phytates. Phytates represent the main form of phosphorus storage in mature grains and seeds. The phytate content of cereals, legumes and oilseeds varies widely depending on botanical diversity and environment. Phytic acid chelates cations, forming insoluble complexes with minerals in the upper gastrointestinal tract and cannot be digested or absorbed by animals and humans due to the absence of intestinal phytase enzymes [1].

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Phytases - are a class of phosphatases that sequentially hydrolyze phytic acid to less phosphorylated myo-inositol, with the release of inorganic phosphate [2]. Monogastric animals such as pigs, poultry and fish cannot effectively use phytate-bound phosphorus in plant-based feeds due to the limited activity of endogenous intestinal phytases. Poor absorption of phytate, in turn, increases the cost of animal husbandry, since an additional source of phosphorus is needed as a feed additive to meet the nutritional needs of the animal. In addition, phytic acid can cause environmental pollution. It forms stable complexes with minerals and proteins in the digestive tract, reducing their bioavailability for animals, and excreted with feces, they disrupts the balance of ecosystems, causing eutrophication of water [3].

One of the important tasks in biotechnology is the optimization of pathways for the industrial production of enzymes, including phytases. One of the ways to solve this problem is to create a stable phytase expression system based on the yeast – *Pichia pastoris*. *P. pastoris* carry-out many post-translational modifications and produce recombinant proteins intracellularly or extracellularly within a short period of time. Due to these properties, the *P. pastoris* yeast has become the most preferred host organism for various types of biotechnology. The aim of this work was to evaluate the activity of *Bacillus ginsengihumi* M 2.11 phytase in a heterologous expression system based on the *P. pastoris* yeast.

## 2 Materials and methods

We used a recombinant *E. coli* DH5 $\alpha$  bacterial strain carrying the pUC57 plasmid containing the *B. ginsengihumi* M 2.11 phyC phytase gene sequence optimized for yeast expression. The sequence of own signal peptide was excluded. C-terminal histidine (His-tag) tag was added to the structural region of the gene in order to detect the protein during immunoblotting and purify it from the culture liquid of yeast by affinity chromatography. The integrative yeast vectors pPINK-HC (high copy vector) and pPINK-LC (low copy vector) (Invitrogen) were used to clone the optimized bacillary phytase gene into the yeast genome. Signal peptide sequences of  $\alpha$ -amylase ( $\alpha$ -amyl) and  $\alpha$ -matting factor ( $\alpha$ -MF) (Invitrogen) were used for extracellular expression. Methylophilic yeast *P. pastoris* protease-deficient strains were used to transform the genetic (invitrogen).

BMGY medium (Buffered Glycerol-complex Medium, Invitrogen) was used for biomass accumulation of the recombinant *P. pastoris*: 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB (Yeast Nitrogen Base), 0.00004% biotin, 1% glycerol.) BMMY (Buffered Methanol-complex Medium, Invitrogen) was used for the induction of protein expression by the recombinant *P. pastoris*: 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 0.00004% biotin, 0.5 % methanol. One colony of yeast transformants was transferred into 10 ml of BMGY medium and grown in 150 ml flasks for 1-2 days at 28 ° C and 300 rpm. Then the cells were pelleted at 1500 rcf for 5 min, the supernatant was discarded. To induce protein expression, the cells were resuspended in 2 ml of BMMY medium and grown at 28 ° C and 300 rpm for 24 hours. Then, 40% methanol was added to the medium in a volume of 100  $\mu$ l per 1 ml of the medium and grown for another 24 hours. After that, the culture was centrifuged at 1500 rcf for 10 min, the resulting supernatant was frozen in liquid nitrogen and stored at - 20 ° C.

Protein electrophoresis. Separation of proteins by weight was carried out under denaturing conditions in the presence of SDS in 12.5% polyacrylamide gel (PAGE) according to Laemmli's method.

Determination of phytase assay. The enzyme activity was determined by the Greiner method by the amount of released phosphorus during hydrolysis of the sodium phytate substrate (Sigma Aldrich, United States) [4Greiner, 2004]. The reaction mixture of the experimental sample contained 55  $\mu$ l of 10 mM sodium phytate substrate, 125  $\mu$ l of 100

mM M Tris-HCl buffer with pH 8.0, 35  $\mu$ l of enzyme, 20  $\mu$ l of 10 mM CaCl<sub>2</sub> solution. The mixture was incubated at 37 ° C for 1 hour. In the control sample containing 50  $\mu$ l of substrate 10 mM sodium phytate, 20  $\mu$ l of 10 mM CaCl<sub>2</sub>, and 125  $\mu$ l of 100 mM M Tris-HCl buffer with pH 8.0, the enzyme was added after incubation at 37 ° C. The reaction was stopped by adding 15% TCA, then 900  $\mu$ l of a freshly prepared AAM solution ( 10 mM ammonium molybdate, 5N H<sub>2</sub>SO<sub>4</sub> solution and acetone in a ratio of 1: 1: 2) was added. After that, 60  $\mu$ l of 1 M citric acid was added to the mixture. The absorption of the experimental and control samples was measured on a spectrophotometer at 355 nm in a 1 cm cuvette. A unit of activity was defined as the amount of enzyme that cleaved sodium phytate to form 1  $\mu$ M inorganic phosphate in one minute. Phytase assay was calculated using the formula:

$U = (EA-EB) * F * \text{dilution}$ , where EA - values of Dopt experience, EB - values of Dopt control,

$F = 1 / t * 1 / (d * \epsilon) * (V \text{ total}) / (V \text{ farms})$ , where d = 1 cm (cuvette),  $\epsilon = 8.7 \text{ cm}^2$  (extinction coefficient of sodium phytate substrate).

To study the pH stability, the enzyme was incubated at 37 ° C for 1 h in buffer at different pH values (from 2.5 to 9.0). The enzyme activity was determined according to the standard method, the following buffers were used: 100 mm glycine-HCl (pH 2.5 to 3.5), 100 mm sodium acetate (pH 3.5 to 5.5), 100 mm Tris-acetic acid (pH 5.5 to 7.5), 100 mm Tris -HCl (pH 7.5 to 9.0).

The pH optimum of the enzyme activity was determined by the hydrolysis of sodium phytate at 37 ° C in the pH range from 2.5 to 9.0 with a step of 0.5 pH units. The temperature optimum of the enzyme action was determined by hydrolysis of sodium phytate in 100 mM Tris-HCl buffer, pH 9.0, in the temperature range from 25 to 80 ° C.

Determination of thermal stability was carried out by preliminary incubation of the enzyme for 1 h at temperatures from -80 ° C to 90 ° C, after which the activity of phytase was determined by hydrolysis of phytate according to the standard method.

The results are presented as the mean of two independent experiments, taking into account the standard deviation.

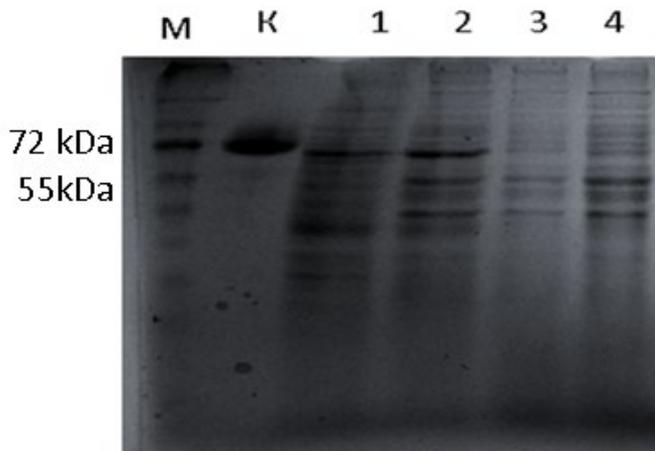
### 3 Results and discussion

The most optimal eukaryotic expression systems for the production of recombinant proteins are yeast systems, in particular, *P. pastoris*. Yeasts provide a high level of expression of heterologous proteins, which in *P. pastoris* is achieved due to the presence of a strong AOX1 promoter. Recombinant strains of *P. pastoris* pPINK-HC / LC -phyC were obtained by integration into the genome of genetic constructs based on integrative yeast plasmids pPINK-LC- $\alpha$ -MF-phyC, pPINK-HC- $\alpha$ -MF-phyC, pPINK-LC- $\alpha$  -amyl-phyC and pPINK-HC- $\alpha$ -amyl-phyC containing the *B. ginsengihumi* M 2.11 alkaline phytase gene optimized for expression in yeast and the  $\alpha$ -amylase and  $\alpha$ -mating factor signal peptide sequences for protein extracellular expression. It was of interest to study and compare the properties of the recombinant phytases  $\alpha$ -MF-phyC-LC,  $\alpha$ -MF-phyC-HC,  $\alpha$ -amyl-phyC-LC, and  $\alpha$ -amyl-phyC-HC expressed by the yeast *P. pastoris*.

The extracellular accumulation of the expressed protein in the culture medium by the recombinant *P. pastoris* strains was checked by protein electrophoresis in PAGE and phytase assay. The culture liquid of yeasts, secreting recombinant phytase AgpP from *Pantoea brenneri* with a molecular weight of 70 kDa, was used as a positive control. After 24 hours of methanol induction, recombinant protein was detected in the PAGE of culture liquid of *P.pastoris* - specific band with apparent molecular mass of 70 kDa was observed.

In their study, J. M. Viader-Salvadó et al compared the properties of recombinant beta-propeller phytases of the Bacillus secreted in *P. pastoris* yeast expression systems. Three

recombinant phytases (FTE, FTEII and FBA) showed a smear on SDS-PAGE gel ranging from 45 to 66 kDa. [5]

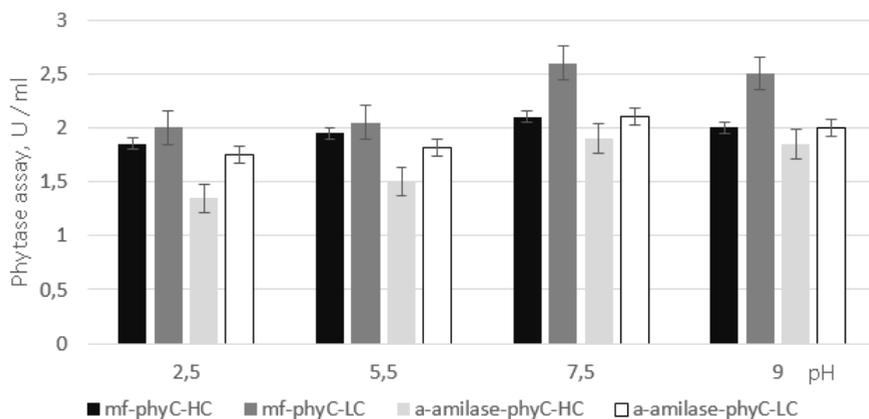


**Fig. 1.** Protein electrophoresis of the culture fluid (CL) of the *P. pastoris* yeast. M - protein marker; 1- MF-phyC-HC, 2-MF-phyC-LC, 3-a-amylase-phyC-LC, 4- a-amylase-phyC-HC. K - positive control (QOL of yeast with the pPINK-HC / agpP construct).

The phytase activity of the secreted enzyme was measured by the hydrolysis of the substrate - sodium phytate. CL samples of the recombinant *P. pastoris* were taken 24 h after induction with methanol. The strains carrying the pPINK-LC- $\alpha$ -MF-phyC construct showed a maximum activity of 2.6 (U / mg ) at 24 h after induction. The strains with the pPINK-HC- $\alpha$ -MF-phyC construct had an activity of 2.1 U / mg. The strains carrying the pPINK-LC- $\alpha$ -amyl-phyC and pPINK-HC- $\alpha$ -amyl-phyC constructs had their maximum activity of 2.1 and 1.9 (U / mg), respectively. Our results are consistent with the data obtained for recombinant *Bacillus* phytases expressed in *P. pastoris*. The *B. subtilis* VTT E-68013 strain showed a 50% greater extracellular enzyme activity (1.2 U / mg) than the GS115-PhyC strain at 24 h after induction [6]. The *B. amyloliquefaciens* KM71FBA strain by 48 h of induction showed phytase activity in the range of 0.8 to 3.4 (U / mg) [5].

In the digestive tract of poultry, enzyme will pass through the digestive sections, the pH of which is significantly differ from each other. To show its maximum activity, the enzyme should not be inactivated under the influence of different pH values in the digestive tract. Thus, an increase in stability, both at acidic and alkaline values of the medium, is of practical importance, since it is necessary to take into account the low pH level (4-5) in the anterior (cardiac) part of the stomach, as well as in the glandular and muscular stomach (pH 2-5) of the gastrointestinal tract of chicken [8].

The pH stability of the recombinant PhyC phytase was determined in the pH range from 2.5 to 9.0. It was found that all enzymes retained about 90% of their activity at pH values from 7.5 to 9.0, while at acidic values the activity of phytases was not more than 80% (Figure 2).



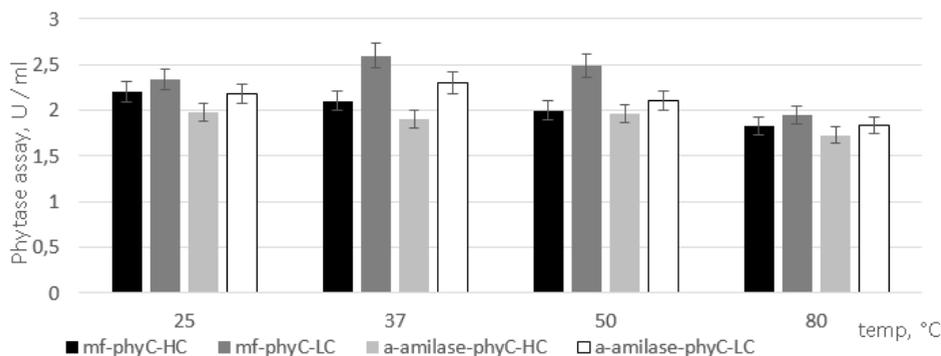
**Fig. 2.** pH stability of recombinant phytases. The activity of enzymes incubated at pH 9.0 was taken as 100%.

All enzymes containing the a-amylase signal peptide exhibited lower activity compared to enzymes with the  $\alpha$ -mating factor signal peptide. At the same time, the phytases MF-phyC-HC and a-amylase-phyC-LC were more stable than the phytases MF-phyC-LC and a-amylase-phyC-HC, as they retained approximately the same activity at all pH values.

Thus, it was found that the greatest activity of phytase exhibited at alkaline pH. *Bacillus* phytases are beta propeller phytases that require calcium ions for their activity and stability, exhibit an optimal pH of 6.0 to 9.0 and tend to be suitable for using as feed additives in animals with a neutral pH of digestive tract [6]. Our data are consistent with the data obtained by J. M. Viader-Salvadó et al – three recombinant phytases showed maximum activity at pH 7.5 (FTE and FBA) and 9.0 (FTEII). [5].

Each enzyme is stable at its corresponding pH values, since changes in the pH of the medium change the conformation of the protein molecule, its active center and, consequently, the ability to carry out catalysis [8]. Thus, all enzymes showed the greatest activity at alkaline values. Based on their pH profiles, phytases can be said to be suitable as feed additives for animals with a neutral-basic digestive tract as well as for animals with acidic pH values. Thus, the enzymes will be active during the dephosphorylation of phytate throughout the entire gastrointestinal tract of chickens, which makes it possible to use them as a feed additive, since they will not be inactivated while passing through the gastrointestinal tract of chickens.

Temperature optimum. The internal body temperature of a chicken ranges from 40-42 ° C. An important feature for enzymes effectiveness as feed additives is their ability to exhibit high activity at a given temperature. In this regard, it is necessary to study the temperature optimum of enzymes [9]. It was found that their maximum activity phytases exhibited at 37 ° C. The level of phytase activity at 25 ° C and 50 ° C was similar to that at the optimum temperature, only slightly decreasing. With an increase in temperature to 80 ° C, the activity of recombinant phytases decreased by 15%.



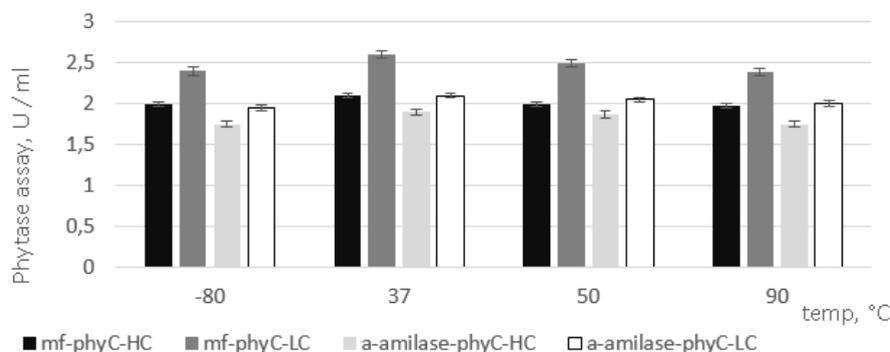
**Fig. 3.** Temperature optimum of recombinant phytases. The enzyme activity at 37 °C was taken as 100%.

In their study, M. Guerrero-Olazarán et al, studying the properties of the native and recombinant enzymes of *B. Subtilis* VTT E-68013, found that the optimal temperature for both phytases was from 55 to 70 °C [6].

All recombinant enzymes were highly active over a wide temperature range, which means they will be active at body temperature in chickens and will be capable of efficiently hydrolyze phytates in poultry.

The thermal stability of *Bacillus* phytases in the high temperature range, from 60 to 95 °C, is another important and useful feature for their use as feed additives for animals, since steam and high temperatures are used at the pelletizing process [6]. Despite the fact that alternative technologies allow phytases to be sprayed onto feed after pelleting, which avoids thermal denaturation of the enzyme, thermostable phytases are still more preferable candidates for use as feed additives [10]. We studied the effect of temperature on the stability of enzymes in the range from -80 °C to 90 °C, incubated for 1 hour. It was found that all phytases retained their activity in the range from -80 to 50 °C. When the temperature was raised to 90 °C, the enzymes retained more than 90% of their activity (Figure 4). Thus, it can be concluded that the enzymes are stable over a wide temperature range.

The MF-phyC-LC enzyme exhibited the highest activity at all temperatures, while a-amylase-phyC-HC exhibited the least activity. The enzymes MF-phyC-HC and a-amylase-phyC-LC exhibited the same activity.



**Fig. 4.** Thermostability of recombinant phytases, the activity of enzymes incubated at 37 °C was taken as 100%.

Guerrero-Olazarán compared the efficiency of FTEII phytase with three commercial phytases in terms of thermal stability at 99 ° C. The FTEII enzyme showed the highest thermal stability with a residual activity of 82 ± 3 after 1.5 min of treatment at 90 ° C [6].

Based on the foregoing, it can be concluded that recombinant enzymes have high thermal stability both at negative and positive temperatures, which will allow them to undergo the feed pelletizing process without significant loss of enzymatic activity.

Thus, in the course of this work, such characteristics of four recombinant phytases as pH stability, temperature optimum and thermal stability were investigated and compared (Table 1).

**Table 1.** Enzymatic properties of phytases.

Properties	MF- <i>phyC</i> - HC	MF- <i>phyC</i> - LC	<i>a-amylase</i> - <i>phyC</i> -HC	<i>a-amylase</i> - <i>phyC</i> -LC
pH stability (≥80% activity)	2.5-9.0	2.5-9.0	5.5-9.0	2.5-9.0
Temperature optimum	37	37	37	37
Thermal stability in% (1 h) at:				
-80°C	95	92	92	93
37°C	100	100	100	100
90°C	94	92	92	95

## 4 Conclusions

Based on the analysis of all the characteristics of proteins, it can be concluded that all the obtained enzymes have similar properties. The MF-*phyC*-LC enzyme is the most stable in a wide pH range, has the highest activity and stability in the temperature range from -80 to 90 ° C compared to other phytases, which makes it the most preferred as a feed additive. An increase in the thermostability of the recombinant enzymes in comparison with the native protein was found, which is probably associated with the process of glycosylation. This is a significant advantage in the industrial production of enzymes. Considering that all phytases were obtained in the same *P. pastoris* expression system, it can be assumed that the differences in the activity and stability of phytases are due to the choice of the vector or signal sequence. Probably, the signal peptide  $\alpha$ -mating factor promotes better secretion of the enzyme into the extracellular environment.

It was found that the recombinant strains pPINK-LC- $\alpha$ -MF -*phyC*, pPINK-HC- $\alpha$ -*amyl* -*phyC*, pPINK-LC- $\alpha$ -*amyl* -*phyC*, pPINK-HC- $\alpha$ -MF -*phyC* of the *P. pastoris* yeast are able to synthesize and to secrete *B. ginsengihumi* M 2.11 bacterial phytase *phyC*. The maximum activity was observed in the pPINK-LC- $\alpha$ -MF strain. Recombinant phytases from *B. ginsengihumi* M 2.11 exhibited high activity in a wide pH range from 2.5 to 9.0. The MF-*phyC*-HC construction is pH stable. The temperature optimum corresponds to 37 ° C, the recombinant phytases retain their activity in the range from -80 to 90 ° C.

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