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# Production and some properties of extracellular phytase from *Thermomyces lanuginosus* IMI 096218 on rice flour as substrate

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**Abstract**

Thermophilic fungi *Thermomyces lanuginosus* IMI 096218 strain produces phytase enzyme on rice flour in submerged fermentation. The maximal activity was achieved on the 4$^{th}$ day of fermentation using 5 (w/v)% rice flour and at 220-rpm agitation speed. Additionally, the enzyme production was enhanced by supplementation of 0.1% Tween-80 detergent into fermentation medium. The phytase was purified about 9.1 fold with yield of 5.1%. The optimal pH and temperature of the purified phytase enzyme were pH 5.5 and 70°C, respectively. The half-life times of enzyme at temperature 54-58°C and pH range 5.0-7.5 were longer than 100 min. Kinetic parameters of phytase on sodium-phytate substrate were determined by linear Lineweaver-Burk plot: $K_M$=0.285 mM, $v_{max}$=0.126 mM/min, and Hanes-Woolf plot: $K_M$=0.312 mM, $v_{max}$=0.132 mM/min, respectively. The presence of 5 mM $Zn^{2+}$, $Ag^{+}$, $Co^{2+}$ and $Cu^{2+}$ ions strongly inhibited the enzyme reaction. The residual activities were 55%, 49%, 38% and 34% respectively. This phytase can be considered as a potential candidate in animal feeding as well as in the production of some intermediates for clinical applications.

**Key words** – characterisation – degradation of phytic acid – kinetic constants – submerged fermentation – natural substrate – purification

## Introduction

Phosphorous is one of the most essential elements in the energy metabolism and it plays an important role in the growth and health status of bone. Phosphorous is basic compound in nucleic acids, ATP and phospholipids. For animals and human phosphorus-containing water and plants are the main sources of phosphorus in their diet. In the plant kingdom, phytic acid is a storage form of phosphorus, which is presence in different amount depending on vegetable raw materials. Cereal grains, legumes and oil seeds contain 1.4-55.1 g/mg phytic acid (Konietzny & Greiner 2003). The feedstuffs originating from plant contain about 60-90% of their total phosphorus content in the form of phytate (Loewus 2002). Phytic acid and its salts are anti-nutritional factors, because they have the ability to chelate various essential divalent metal ions blocking or slowing down their absorption and utilization (Angel et al. 2002, Konietzny & Greiner 2003, Simpson & Wise 1990). Additionally, they are also able to form complexes with dietary proteins, starch and lipids as well as to inhibit a number of nutritionally important enzymes (De Rham & Jost 1979, Kumar et al. 2010). Due to lack of phytase activity, organic phosphorous may pass through the digestive tract of non-

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ruminant animals undigested (poultry, pig, fish) – same situation in the human digestion tract. Therefore, in intensive livestock farming the feed must be supplemented with inorganic phosphate, and the phosphorus bound in phytate is excreted in the manure (Lei & Porres 2003) causing numerous environmental problems. Furthermore, the increased emission of phosphorous often contributes to eutrophication of surface waters (Cao et al. 2007).

Phytase enzyme that is able to hydrolyse ester linkages in phytic acids, should serve as good catalists for release of organic phosphorous in feed or foodstuff, thus help to decrease environmental pollution problem. This enzyme may also have importance in human nutrition, and should help to decrease the risk of diseases in certain groups of people such as vegetarians or in the population of developing countries who usually consume high amount of plant-based foods.

It has been recognised that the sequential hydrolysis of phytate results different myo-inositol phosphate intermediates that have health and pharmaceutical effects. Some clinical studies proved that these intermediates reduce the risk of colon cancer, the level of serum cholesterol and triglycerides in test animals, and reduce lipid peroxidation, and act as antioxidants (Kumar et al. 2010, Phillippy & Graf 1997).

Phytase enzyme occurs widespread in the world, which results in the production of different source of enzyme. Although several strains of bacteria, yeasts and fungi have been used for production of phytase under different conditions, especially those originating from filamentous fungi have most commonly been employed for commercial production of this enzyme (Haefner et al. 2005). In submerged culture, different strains of *Thermomyces lanuginosus* produce some enzymes including amylolytic enzymes (Nguyen et al. 2000), α-galactosidase (Rezessy-Szabó et al. 2007), β-galactosidase (Fischer et al. 1995), lipase (Arima et al. 1972), phytase (Gulati et al. 2007), xylanase (Singh et al. 2003) and protease (Li et al. 1997). Technologically, phytases that are used as animal feed supplements should be able to withstand temperatures of 60°C to 90°C, which may be reached during the feed pelleting process. Furthermore, enzymes originated from thermophilic organisms have been much more heat tolerant than ones from mesophilic or plant sources, thus these organisms should be ideal sources for production of enzyme.

In this work, production and partial characterisation of phytase from thermophilic fungus *Thermomyces lanuginosus* strain IMI 096218 on rice flour as fermentation substrate were focused.

## Materials & Methods

### *Microorganisms and cultivation*

*Thermomyces lanuginosus* strains (ATCC 16455, ATCC 28083, ATCC 34626, ATCC 36350*, ATCC 38905, ATCC 44008*, ATCC 46882, ATCC 84400, CBS 218.34, CBS 288.54, CBS 395.62b*, DSM 5826*, IMI 096218*, IMI 158749*) were either provided by Dr. Bhat (marked with *, Institute of Food Research, Norwich, England) or purchased from different culture collections. Strains grew on Potato Dextrose Agar (PDA) at 47°C for 1-2 days. Then they were stored at 4°C until use.

### *Fermentation procedure for enzyme production*

Inoculum culture was prepared using 500mL flasks filled with 150 mL broth containing (g $L^{-1}$): glucose 20, L-asparagine 4.5, $KH_2PO_4$ 3.0, $K_2HPO_4$ 2.0, $MgSO_4$ x7$H_2O$ 0.5, Vogel's trace elements 1 mL (Vogel 1956). First, conidia were washed from the surface of PDA slant culture with 5mL of 0.01% (w/v) triton x-100 solution and then transfer into flasks. The cultures were incubated in an orbital shaker with 200 rpm at 47°C for 1-2 days.

Phytase production was carried out in 500 mL Erlenmeyer flask containing 150 mL of fermentation medium with the following compounds (g $L^{-1}$): $MgSO_4$ x7$H_2O$ 0.5; KCl 0.5; $FeSO_4$ 0.1 and $NaNO_3$ 8.6 as mineral salts. The medium was supplemented with phytate containing crop or rice flour base on the individual situation of experiments. Fermentations were initiated with 5 % (w/v) of 1-2-day old inoculum and then incubated in an orbital shaker at 47°C up to 2-7 days.

***Phytase activity assay***

Sample from fermentation broth was filtered to remove fungal mycelia and the phytase activity was assayed from the filtrate fraction using the method described by Engelen and co-workers (1994). The released phosphate develops yellow coloured complex with ammonium molibdate that was quantified spectrophotometrically at 415 nm using calibration curve prepared with inorganic phosphate under the same conditions. One unit of phytase activity was defined as the amount of enzyme capable of releasing one μmol phosphate per min under the reaction conditions (65°C, 10 min, pH 5.5).

***Enzyme purification***

Extracellular proteins were recovered from cell-free ferment broth and precipitated by ammonium sulphate up to 80% saturation at 4°C for a night. The precipitated protein was collected by centrifugation (17300g, 20 min, 4°C) and then dissolving in 50 mM sodium acetate buffer (pH 5.5). The crude enzyme was dialysed and concentrated by ultrafiltration using a 10 kDa membrane cut out. Enzyme purification was achieved by combination different chromatographic columns connected to FPLC system.

The first step hydrophobic interaction chromatography was applied. Sample was loaded onto a Phenyl Sepharose column pre-equilibrated with 50 mM sodium acetate buffer, pH 5.5, 1.3 M $(NH_4)_2SO_4$. The bounded protein was subsequently eluted with 50 mM Na-acetate buffer at 3 ml/min flow rate. Fractions with phytase activity were pooled and concentrated by ultrafiltration. The sample then was loaded onto a Phenyl Sepharose column pre-equilibrated with 50 mM sodium acetate buffer, pH 5.5 1.7 M $(NH_4)_2SO_4$. The elution was achieved as the same method. The active fractions were pooled, concentrated by ultrafiltration with an Amicon PM 10 membrane and loaded onto a Q-Sepharose ion-exchange column (1 cm x 25 cm) pre-equilibrated with 50 mM Tris/HCl buffer, pH 7.2. The column was washed with the same buffer and eluted with a linear gradient of 0-1 NaCl. Protein concentration was determined by Bradford assay (Bradford 1976).

***Characterization of purified phytase***

The effect of pH on phytase activity was assessed by using buffers between 1.5 and 9.0 of pH [sodium citrate/HCl buffer (pH 1.5 to 3.5), sodium acetate buffer (pH 3.5 to 6.5), Tris-maleate/NaOH (pH 5.5 to 9.0)], and performing assays at 65°C.

For determining optimum temperature for the activity of phytase, the enzyme assays were carried out in the temperature range between 35 and 90°C (sodium acetate buffer containing sodium phytate substrate, pH 5.5).

The thermostability of the phytase was achieved by incubating the enzyme at 55°C, 60°C, 65°C at pH 3.5-7.5 (sodium acetate and 0.1 M Tris–maleate/NaOH) buffers. The samples were withdrawn at regular intervals and residual phytase activity was measured at 65 °C (pH 5.5).

The effect of various metal ions on the enzyme activity by incorporating these into the reaction mixtures in concentration of 5 mM except $Fe^{2+}$ and $Fe^{3+}$ ions where 1 mM.

Determination of kinetic constants was achieved over a range of sodium phytate substrate concentrations (0.1–10 mM). The kinetic constants ($K_M$ and $V_{max}$) were computed from a Lineweaver–Burk and Hanes-Woolf plot.

***Electrophoretic analysis***

Denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 10 % polyacrylamide gels with 0.1 % SDS as described by Laemmli (1970). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

***Statistical analysis***

In order to enhance data evaluation several statistical methods were performed. All data are presented as the mean and standard deviation (SD). The correlation between independent variables was tested by Pearson correlation test. Also two- and three-dimensional linear models were

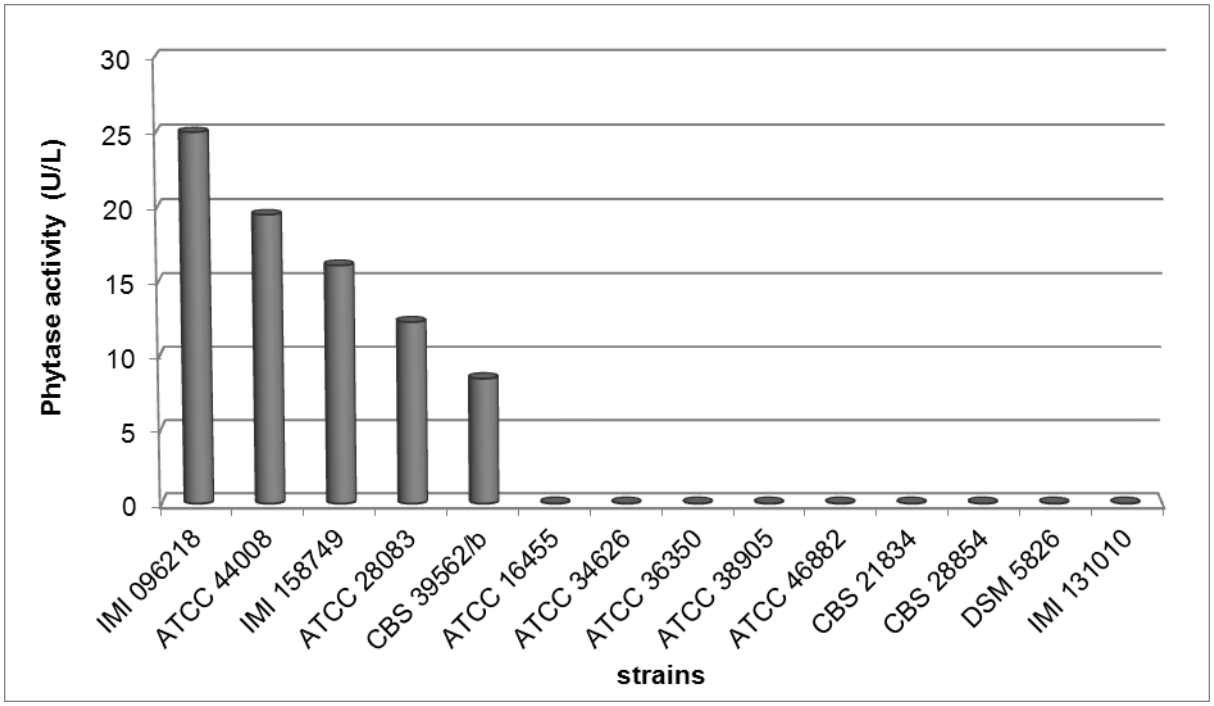


**Fig. 1** – Screening of T. lanuginosus strains based on phytase activity (47°C, 220 rpm, 3% corn flour, 48 h).

performed to mention the connections of the variables. One-way analysis of variance (ANOVA), unpaired and paired Student's t-tests were done using Statistica 10.0 software package (StatSoft, USA). Generally, only $p<0.05$ was accepted as the statistical significance level. The response surface method was applied to investigate the stability of enzyme (Rezessy-Szabó et al. 2007). The goodness of the fitted model was evaluated by regression analysis.

## Results & Discussion

### *Production of extracellular phytase*

Screening works were done using fourteen *Thermomyces lanuginosus* strains from different culture collections on culture media containing 30g $L^{-1}$ corn flour as main carbon source and inducer. Phytase activity of extracellular fraction was assayed at different time periods of the fermentation process. Fig. 1 showed the results at 48$^{th}$ hour of fermentation. Only 5 strains (IMI 096218, ATCC 44008, IMI 158749, ATCC 28083 and CBS 359.62/b) exhibited extracellular phytase activity and the *T. lanuginosus* IMI 096218 strain was proved to be the best one (25 U $L^{-1}$) on the corn flour substrate. The effect of other natural substrate (rice flour) on secretion of phytase was also investigated. Rice flour was proved to be good substrate for production of phytase by *Aspergillus niger* (Bujna et al. 2013). In the case of *T. lanuginosus* mould, the enzyme production was also enhanced (generally increase in 2-5 times comparing with ones used standard media), when the medium was prepared with TRIS-maleate/NaOH buffer (pH 7.5) and rice flour as natural substrates.

The effects of different agitation speed and concentration of rice flour on enzyme production were investigated. Based on our results, the optimal rice flour concentration was 5 (w/v)%. It can be observed that using 220 rpm agitation speed the maximal activity was achieved on the 4$^{th}$ day of fermentation (Fig. 2), whereas at 120 rpm agitation speed it should take 7 days. Application of 8 (w/v)% rice flour at 120 rpm resulted as same enzyme activity on the 7$^{th}$ day as at 220 rpm on the 4$^{th}$ day of fermentation. The medium containing 8 (w/v)% rice flour was rather viscous which makes agitation and aeration difficulties during submerged fermentation.

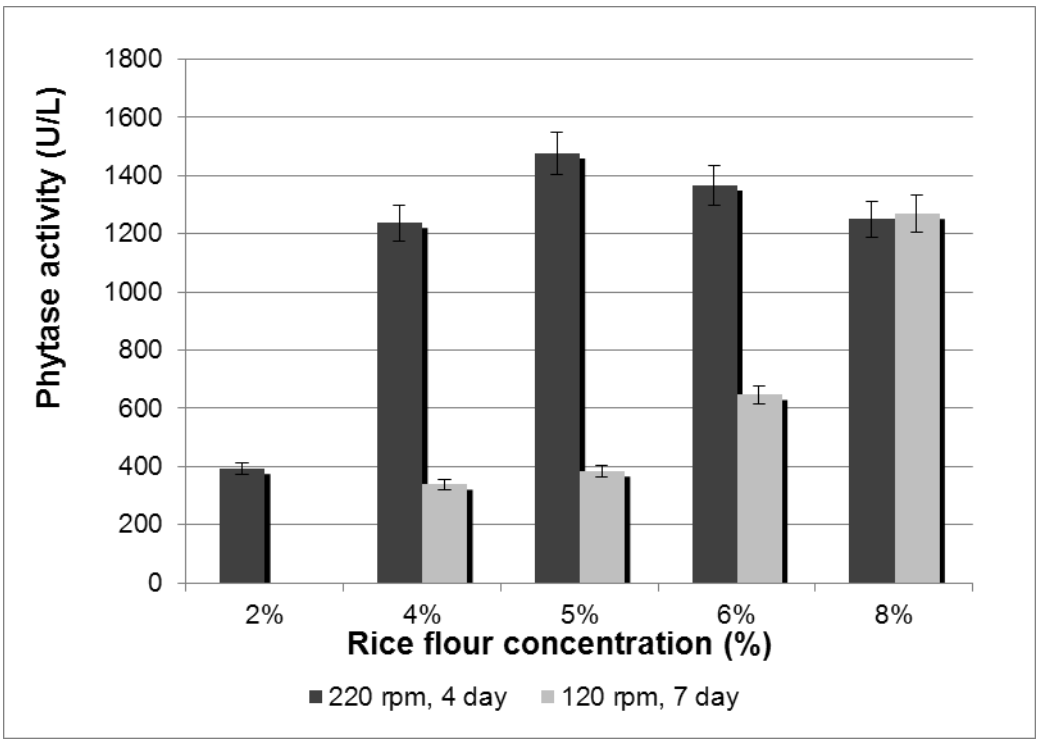


**Fig 2** – Comparison of maximal phytase activity at different agitation speed (120 rpm and 220 rpm) and rice flour concentration.

However the high temperature and moisture are appropriate for thermophilic fungi-like compost where the concentration of $CO_2$ inside composts can be as high as 10 to 15% (Maheshwari et al. 2000). Although $CO_2$ is not regarded as a nutritional requirement for fungi, growth of *T. lanuginosus* was severely affected if the gas phase in the culture flask was devoid of $CO_2$.

The effect of agitation speed on phytase production by *Sporotrichum thermophile* thermophilic fungi were investigated by Singh & Satyanarayana (2012). They found that continuous increase in agitation rates from static conditions to 300 rpm resulted gradually enzyme activity enhancement through the improved availability of oxygen and nutrients. The highest enzyme activity was measured at 250 rpm. Further increase in agitation rates caused a fall in the enzyme production, which could be due to disruption of fungal mycelium at very high agitation speeds, as well as shorter contact between cells and the medium, thus hindering the uptake of the nutrients. Our results were quietly closed to Singh & Satyanarayana (2012), thus in further experiments phytase fermentations with *Thermomyces lanuginosus* IMI 096218 strain were hold on 220 rpm during $7^{th}$ day fermentation

The effect of the fermentation medium supplemented with various additives such as Tween 80, citric acid and yeast extract in 0.1 % concentration both in themselves and in all possible combinations on the production of enzyme was investigated (Table 1). Yeast extract alone has no significant increasing effect on the phytase activity, but together with Tween resulted more than 25% higher enzyme activity. These results are very similar to those reported by Dharmsthiti et al. (2004) who were enhanced phytase production of *Pseudomonas putida* applying 0.1 % yeast extract, 0.1% citric acid and 0.1 % Tween 80 altogether. Citric acid in concentration of 0.1 % has strong inhibitory effect on phytase production of *T. lanuginosus* IMI 096218 strain. The activity was nearly 90 % lower than control. The best result was achieved using Tween 80. The presence of Tween 80 surfactant enhanced the secretion of extracellular phytase activity, and this effect was eliminated when combining with citric acid. This detergent was applied by Arnesen and co-workers (1998) for enhancing production and excretion of □-amylase by *Thermomyces lanuginosus* and 2.7 times higher activity was obtained. Effects of different natures and concentration of Tweens on

**Table 1** Effects of supplementation with different additives on phytase activity in fermentation medium.

| Experimental run | Concentration | | | Effect |
|---|---|---|---|---|
| | Yeast extract | Tween 80 | Citric acid | |
| 1 | 0.1% | - | - | |
| 2 | - | 0.1% | - | + |
| 3 | - | - | 0.1% | - |
| 4 | 0.1% | 0.1% | - | + |
| 5 | 0.1% | - | 0.1% | - |
| 6 | - | 0.1% | 0.1% | - |
| 7 | 0.1% | 0.1% | 0.1% | |
| 8 (control) | - | - | - | |

+ higher than 25 % increase in enzyme activity - higher than 25 % decrease in enzyme activity

**Table 2** Purification details of phytase from *T. lanuginosus* IMI 096218.

| Steps | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Fold |
|---|---|---|---|---|---|
| Culture broth | 1200 | 126.2 | 9,5 | 100 | 1 |
| After precipitation | 493.9 | 35.7 | 13.8 | 41.2 | 1.5 |
| Hydrophobic interaction chromatography | 121.8 | 4.5 | 27.0 | 10.2 | 2. 8 |
| Ion exchange chromatography | 61.5 | 0.7 | 86.7 | 5.1 | 9.1 |

secretion of phytase enzyme by *T. lanuginosus* (Bujna et al. 2011) were also studies and the best result was obtained in the case of the addition of 0.1% Tween 40 surfactant to the fermentation medium.

***Purification and characterization of phytase enzyme***

The extracellular phytase from *T. lanuginosus* IMI 096218 strain growing under the optimized fermentation conditions was purified by a combination of chromatographic procedures after precipitation with ammonium sulphate. The phytase was purified about 9.1 fold with yield of 5.1 % (Table 2). A phytase enzyme originated from *Thermomyces lanuginosus* isolated from soil completed with compost purified by Gulati and co-workers (2007) resulted 3.44 % yield and 40.75 fold. The reason of the higher purification fold may be due to the initial protein synthetized on wheat bran during solid state fermentation. The ratio of phytase is lower in the crude protein, which leads lower fold.

The purified enzyme was checked by polyacrylamide gel electrophoresis. The molecular mass of purified phytase was estimated to be approximately 60 kDa (data are not shown). This result is closed to one reported by Berka and co-workers (1998) as well as to one published by Gulati and co-workers (2007).

The purified phytase enzyme exhibited an optimal pH value at pH 5.5. Chadha and co-workers (2004) produced phytase from *Rhizomucor pusillus* by solid-state fermentation technology and partially purified. The optimal pH of the enzyme preparation was pH 5.4, but 80% of maximal activity was assayed in the wide range of pH (pH 3-7.8). Similar results were observed in the case of phytase originated from thermophilic fungi *Aspergillus fumigatus, Myceliophthora thermophile* and *Sporotrichum thermophile* (Singh & Satyanarayana 2011, Wang et al. 2007, Wyss et al. 1999) and optimal pH was 5.5. The pH optimum at phytase from *Thermomyces lanuginosus* TL-7 mutant strain and *Thermoascus aurantiacus* thermophilic fungi were determined at pH 5.0, respectively (Gulati et al. 2007, Nampoothiri et al. 2004). Another recombinant *Thermomyces lanuginosus* strain's phytase enzyme pH optimum was at pH 7 (Berka et al. 1998).

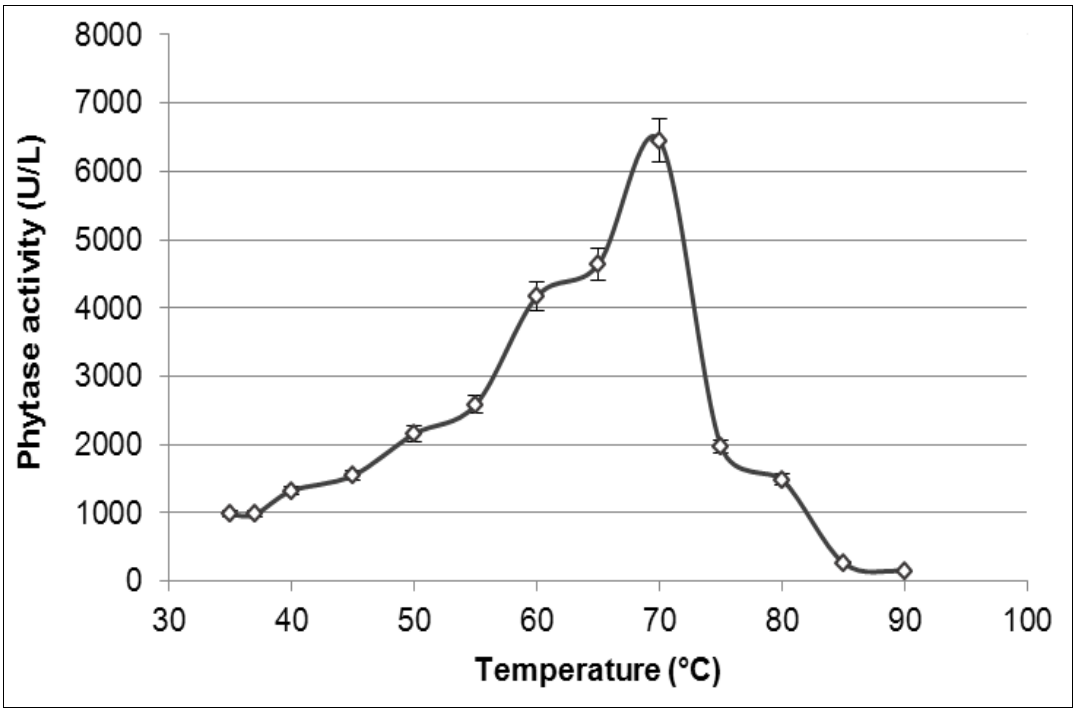


**Fig 3** – The effect of temperature on phytase from *T. lanuginosus* IMI 096218 (pH 5.5).

The optimum temperature of phytase from *T. lanuginosus* IMI 096218 was measured at 70°C (Fig 3). Similar results were determined in case of partially purified phytase from *Rhizomucor pusillus* (Chadha et al. 2004) and *T. lanuginosus* TL-7 mutant (Gulati et al. 2007). Among the other thermophilic fungi *Thermoascus aurantiacus, Sporotrichum thermophile* (Nampoothiri et al. 2004) and *Thermomyces lanuginosus* (Berka et al. 1998) reached the maximal activity at 55 °C, 60 °C, and 65 °C respectively.

The high temperature optimum and thermostability are particularly important features of phytases applied in livestock as feed pelleting, when it is commonly performed at temperatures between 65 °C and 95 °C (Vohra & Satyarayana 2003). The stability of purified phytase was investigated at different temperatures (55 °C, 60 °C, 65 °C) and pH (in range from pH 3.5 to pH 7.5 with stepwise pH 0.5). The results were evaluated using response surface method where independent factors were pH and temperature, as well as the half-life time and the initial inactivation slope of enzyme were selected as dependent variables. Full quadratic polynomial models were applied to describe the half-life time and initial inactivation slope, respectively.

It can be observed that among the tested temperatures the enzyme most stable at 55 °C (Fig 4). The half-life times of enzyme when incubation at pH range of pH 5.5 and pH 7.5, as well as at 55 °C varied from 135 min up to 148 min. The stability has decreased when the temperature increase or the pH change to more acidic. The half-life times were 106-107 min at 60 °C pH 6-6.5 and 45-46 min at 65°C pH 5.5-6.0, respectively. The purified enzyme loses all its activity at pH 3.5 or lower pH. The highest stability of phytase originated from filamentous fungi was determined by Pasamontes and co-workers (1997). They found that the phytase from a gene manipulated *Aspergillus fumigatus* strain retained 90 % activity at 100 °C during 20 minutes.

The initial inactivation velocity was also affected by temperature and pH. Incubation of enzyme at 56 $^{o}$C in the range of pH from 4.7 to 7.0 resulted slowly lose of activity. The purified phytase inactivates drastically in the first stage where incubation of it at temperature higher than 65 $^{o}$C or/and at pH < 3.

Our results were confirmed experimentally by carrying out some runs at pH 5.5 and 70 $^{o}$C, pH 6.0 and 60 $^{o}$C, pH 6.5 and 55 $^{o}$C, pH 6.5 and 60 $^{o}$C, pH 7.5 and 55 $^{o}$C, respectively.

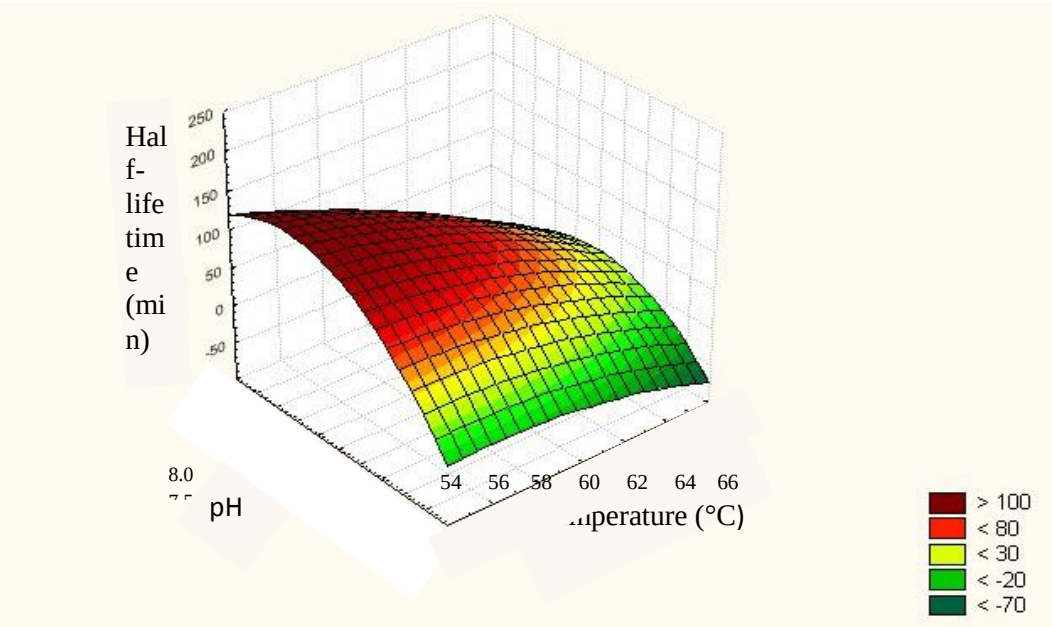


**Fig 4** – The half-life time (min) of phytase from *T. lanuginosus* IMI 096218 in function of pH and temperature.

**Table 3** Kinetic parameters of different source of phytase on sodium phytate substrate (with Lineweaver-Burk linearization method).

| Producing strain | $K_M$ (mM) | $v_{max}$ | References |
|---|---|---|---|
| *A. ficuum* | 0.295 | 55.9 nmol/min | Zhang et al. 2010 |
| *A. oryzae* | 0.33 | - | Shimizu et al. 1993 |
| *A. terreus* 9A1 | 0.0106 | - | Wyss et al. 1999 |
| *A. terreus* CBS | 0.0232 | - | Wyss et al. 1999 |
| *A. niger* ATCC 9142 | 0.1 | 7 nmol/min | Casey & Walsh 2003 |
| *T. lanuginosus* TL-7 | 0.00455 | 0.833 μM/min | Gulati et al. 2007 |
| *A. niger* NCIM 563 PhyI | 2.01 | 5.018 μmol/min | Soni et al. 2010 |
| PhyII | 0.145 | 1.671 μmol/min | |
| *T. lanuginosus* IMI 096218 | 0.285 | 0.126 μM/min | Our study |

Gulati and co-workers (2007) investigated the pH and thermostability of phytase from *T. lanuginosus* TL-7 mutant strain. It was observed that despite of optimum pH 5.0, the enzyme much more stable at pH 7.0. The thermostability of phytase from *Sporotrichum thermophile* determined by Singh & Satyanarayana (2009). The retained activity was 100 % during 5 hours incubation at 60 °C. The half-life time was 16 hour and 90 min at 60 °C and 80 °C, respectively. The gene (Ncphy) encoding a putative phytase in *Neurospora crassa* was cloned and expressed in *Pichia pastoris* by Zhou and co-workers (2006). Experiments on the thermostability of the purified rNcPhy revealed that the initial activity after exposure to 80 °C for 10, 20 or 60 min retained 76%, 58% or 43%, respectively. It means that the phytase from *T. lanuginosus* IMI 096218 strain is less thermostable as ones from originated mutant or genetically modified strains.

Kinetic parameters of phytase originating from *T. lanuginosus* IMI 096218 strain on sodium phytate substrate were determined by linear Lineweaver-Burk plot: $K_M$=0.285 mM, $v_{max}$=0.126 μM/min, and Hanes-Woolf plot: $K_M$=0.312 mM, $v_{max}$=0.132 μM/min. Kinetic parameters of phytases from different sources on sodium phytate substrate can be seen in Table 3. The $K_M$ value in our case was similar with the ones from *A. ficuum* (0.295 mM, Zhang et al. 2010) and *A. orzya*e (0.33 mM, Shimizu et al. 1993), meaning similar affinity to sodium phytate substrate. The phytase from *T. lanuginosus* TL-7 strain exhibited significantly higher affinity ($K_M$ 0.00455 mM, Gulati et al. 2007) to substrate than other ones from moulds.

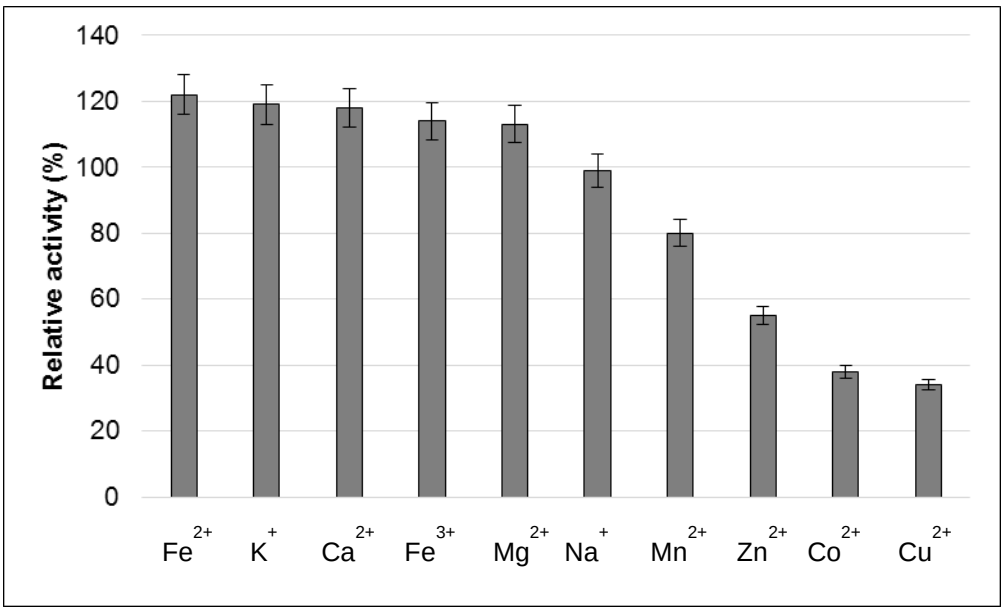


**Fig 5** – Effect of metal ions on purified phytase activity from *T. lanuginosus* IMI 096218. 100% phytase activity was obtained when phytase assay was undertaken in the absence of metal ions (Concentration: 5 mM, in the cases of $Fe^{2+}$ and $Fe^{3+}$:1 mM).

Effects of various metal ions on enzyme activity were investigated. Results in relative activity are on Figure 5, where the run without metal ions serve as control. The presence of 1 mM $Fe^{2+}$, $Fe^{3+}$ and 5 mM $K^{+}$, $Ca^{2+}$ and $Mg^{2+}$ ions resulted in 13-22% increase of activity, whereas the presence of 5 mM $Zn^{2+}$, $Ag^{+}$, $Co^{2+}$ and $Cu^{2+}$ ions strongly inhibited the enzyme reaction. In these cases, the residual activities were 55%, 49%, 38% and 34%, respectively. $Ca^{2+}$ ions have important role in catalytic property of phytase, mainly in beta-propeller phytases that are especially isolated from *Bacillus*. The thermostable phytase from *Bacillus amyloliquefaciens* DS11 hydrolyzes phytate to less phosphorylated myo-inositol phosphates in the presence of $Ca^{2+}$. Oh and co-workers (2001) were observed that *Bacillus amyloliquefaciens* DS11 thermostable phytase enzyme activity follows a rapid equilibrium ordered mechanism in which binding of $Ca^{2+}$ to the active site is necessary for the essential activation of the enzyme. $Ca^{2+}$ turned out to be also required for the substrate because the phytase is only able to hydrolyze the calcium-phytate complex. In fact, the excess of both $Ca^{2+}$ and free phytate that do not make complex with each other are competitive inhibitors (Oh et al. 2001). The influence of zinc, iron, copper, magnesium and calcium has potentially significances with regard to applicability in animal feed. In contrast of our results, Wyss et al. (1999) reported that the $Fe^{2+}$ and $Fe^{3+}$ ions have inhibitory effect on phytase (originated from different fungi *A. fumigatus, E. nidulans, A. niger, A. terreus*) activity in concentration of 1 mM. Just like $Cu^{2+}$ and $Mn^{2+}$ ions, which have inhibitory effect on phytase originated from *T. lanuginosus* IMI 096218 strain, where nearly 70% decrease in activity observed with $Cu^{2+}$ ions, and 20% in the presence of $Mn^{2+}$ in concentration of 5 mM. Addition of $Zn^{2+}$, $Ag^{+}$ and $Co^{2+}$ ions resulted inhibitory effect on phytase activity. Only 55%, 49% and 38 % activity, respectively, were retained when addition of these ions. $Na^{+}$ ions have no effect on phytase activity of *T. lanuginosus* IMI 096218.

$Cu^{2+}$ ions in concentration of 1mM inhibited phytase from *Neurospora crassa* expressed in *Pichia pastoris*, and $Ca^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Zn^{2+}$ ions have no significant effect on enzyme activity (Zhou et al. 2006). $Cu^{2+}$ ions have also inhibitory effect on phytase from *Thermomyces lanuginosus* TL-7 mutant strain, but each of $Ca^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $K^{+}$, $Mn^{2+}$, $Na^{+}$, $Ba^{+}$, $Zn^{2+}$ ions decrease the enzyme activity in concentration of 5 mM (Gulati et al. 2007). The enzyme activity of this mutant strain showed the greatest decrease in case of $Mg^{2+}$ ions, while during at our examined enzyme the use of $Mg^{2+}$ increased the activity by 13%. Singh & Satyanarayana (2009) reached enhanced phytase activity in case of *Sporotrichum thermophile*, in presence of magnesium ions,

and all of the tested ions have inhibitory effect in concentration of 5 mM. $Zn^{2+}$ and $Fe^{3+}$ ions had the greatest inhibitory effect on enzyme (40-60% decrease) similarly results in case of phytase from *T. lanuginosus* IMI 096218. The relative activity decreased to 55% in presence of $Zn^{2+}$.

Our findings suggest that the inhibitory effect is depends on not only the applied metal ion concentration, but also on nature of origin such as the genetic structure of microorganisms or the individual strains.

**Conclusion**

Thermophilic fungus *Thermomyces lanuginosus* IMI 096218 secretes high level of extracellular phytase on medium containing rice flour as main carbon source, reaching 25 U $L^{-1}$. Thus, this naturally available substrate may serve cheap raw material for phytase production in fermentation process. The characteristics of this phytase were much more closed to ones from *A. niger* or *A. oryzae* than on from genetically modified *T. lanuginosus* TL-7 strain. Since the phytase from *T. lanuginosus* IMI 096218 strain had high specific activity (87 U $mg^{-1}$) as well as is stable for 1-2 hours at about 58-60$^{o}$C, it can be considered as a potential commercially viable candidate in animal nutrition and/or production of some intermediates for medical applications.

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