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Effect of ph and Temperature on the Activity of Phytase Products Used in Broiler Nutrition

■Author(s)

Naves L de P[1]
Corrêa AD[2]
Bertechini AG[3]
Gomide EM[4]
Santos CD dos[2]

[1]Graduate student in Monogastric Nutrition of the Animal Science Department – Federal University of Lavras (UFLA).
[2]Professor of the Chemistry Department/ UFLA.
[3]Professor of the Animal Science Department/ UFLA.
[4]Ph. D. student in Monogastric Nutrition of the Animal Science Department/UFLA.

■Mail Adress

Correponding author: Departamento de Zootecnia da Universidade Federal de Lavras
Campus Universitário – Caixa Postal 3037
37200-000 – Lavras, MG, Brasil
E-mail: luciana.naves@hotmail.com

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ABSTRACT

The activity of three commercial microbial phytase (*Aspergillus oryzae*, *A. niger*, and *Saccharomyces cerevisae*) products used in broiler nutrition was determined at different pH (2.0 to 9.0) and temperature (20 to 90°C) values. Enzymatic activity was determined according to the reaction of the phytase with its substrate (sodium phytate), in four replicates, and was expressed in units of phytase activity (FTU). *A. oryzae* phytase exhibited optimal activity at pH 4.0 and 40°C, but its absolute activity was the lowest of the three phytases evaluated. *A. niger* phytase exhibited maximal activity close to pH 5.0 and 45°C, whereas *S. cerevisae* phytase presented its highest activity at pH close to 4.5 and temperatures ranging between 50 and 60°C. It was concluded that *A. niger* and *S. cerevisae* phytase products exhibited the highest absolute activities *in vitro* at pH and temperature values (pH lower than 5.0 and 41°C) corresponding to the ideal physiological conditions of broilers, which would theoretically allow high hydrolysis rate of the phytate contained in the feed.

INTRODUCTION

Poultry production is an important economic activity in Brazil and one of the most developed agricultural industries. However, in addition to the increasing number of broilers produced per year, modern commercial broiler strains present rapid growth, and consequently, diet formulation needs to follow up the changes in their nutritional requirements (Brandão *et al.*, 2007). Phosphorus is nutritionally essential, as evidenced by the different roles it plays in metabolism, and it is particularly important for broiler growth (Assuena *et al.*, 2009).

Phytate (or phytic acid) is the main form of phosphorus (P) storage in seeds and grains commonly included in broiler diets; however, phytate P is poorly available to monogastrics because these animals present low phytase activity in their digestive tract. Furthermore, phytic acid can act as an anti-nutritional factor as it reduces the bioavailability of other nutrients, particularly divalent minerals (Lei & Porres, 2003). In addition, the excretion of non-absorbed phytate P in the environment causes environmental pollution, specifically eutrophication of rivers, lakes and water basins (Maenz, 2001).

One economical solution is to supplement broiler diets with the enzyme phytase, releasing P from phytate. Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) catalyzes the hydrolysis of phytic acid in a stepwise manner, releasing lower inositol phosphates and inorganic phosphate, which can be absorbed in the intestine (Vats & Banerjee, 2004). Consequently, there is less P excretion in the environment and less dicalcium phosphate needs to be added to the feed, reducing production costs (Gomide *et al.*, 2007).

Several studies have been conducted to evaluate the performance and other parameters of broilers fed diets supplemented with phytase (Singh, 2008; Assuena *et al.*, 2009; Nagata *et al.*, 2009). However, few scientific studies evaluating the effect of pH and temperature on the activity of different phytases were published (Wyss *et al.*, 1999; Igbasan *et al.*, 2000).

The objective of the present study was to determine the effect of pH and temperature on the activity of three commercial phytases used in broiler feeds to select which products present stronger activity at pH and temperature values corresponding to the physiological conditions of broilers.

MATERIALS AND METHODS

Location and phytase products

The present study was conducted at the Biochemistry Laboratory of the Chemistry Department of the Federal University of Lavras, Brazil. Three commercial phytase products, in powder, sold for use in broiler nutrition were used: a) 6-phytase EC 3.1.3.26 (synthesized by *Aspergillus oryzae*, genetically modified by the addition of a gene from *Peniophora lycii*); b) 3-phytase EC 3.1.3.8 (produced by *A. niger*, genetically modified by a phytase-encoding gene from *A. ficuum*); and c) 6-phytase EC 3.1.3.26 (synthesized by *Saccharomyces cerevisae,* genetically modified by the addition of genes from *Citrobacter brakii* and *Escherichia coli*).

Phytase extraction and determination of enzyme activity

Phytase was extracted in 0.25 mol L^{-1} acetate buffer, containing 0.05% Triton X-100 (w/v) and 0.05% bovine serum albumin (w/v), at pH 5.0 under horizontal shaking for 30 minutes in the presence of ice. After extraction, the sample was centrifuged (2,000 x g for 10 minutes at 4°C) and the supernatant was collected to determine enzyme activity.

Enzyme activity was determined in the supernatant based on the reaction of phytase with the substrate 7.5 mmol L^{-1} sodium phytate ($C_6H_6O_{24}P_6Na_{12}$ – Sigma-Aldrich) in buffered medium and at controlled temperature for four different incubation times. The reaction was ended by adding a mixture of three solutions (21.67% nitric acid; 0.081 mol L^{-1} ammonium molybdate; and 0.02 mol L^{-1} ammonium vanadate) at 2:1:1 ratio, respectively. The developed color was measured at 415 nm. The standard phosphorus curve (0.156 to 2.500 µmol) was used as reference (Engelen *et al.*, 1994). The activity was calculated using the slope of the straight line resulting from the assay with the sample (absorbance/minute) and the slope of the straight line arising from the standard curve (absorbance/µmol of P). One unit of phytase activity (FTU) was defined as the amount of enzyme that released one µmol of inorganic phosphate in one minute of reaction.

Effect of pH on phytase activity

Phytase was incubated with 7.5 mmol L^{-1} sodium phytate at 37°C and pH 2.0 to 9.0 for four different incubation times to determine its pH curve. Initially, the following buffers were used: HCl-KCl (pH 2.0); glycine-HCl (pH 2.0 and 3.0); citrate (pH 3.0, 4.0, 5.0, and 6.0); maleate (pH 6.0 and 7.0); and Tris-HCl (pH 7.0, 8.0, and 9.0). However, no enzyme activity of the three evaluated phytases was detected with citrate buffers (pH 3.0 to 6.0). Citrate buffers may possibly have chelated some co-factor and further studies are required to determine which molecule performs this function. Thus, the citrate buffers were replaced by acetate buffers (pH 3.0, 4.0, 5.0, and 6.0) while the other buffers were maintained. For pH assays, all buffers were prepared at the concentration of 0.1 mol L^{-1}. Four replicates were performed for each pH value.

Effect of temperature on phytase activity

Temperature assays were conducted at the optimal pH pre-established for each phytase product using a fixed substrate concentration (7.5 mmol L^{-1}) and incubated for four different times at the following temperatures: 20, 30, 40, 50, 60, 70, 80, and 90°C. Four replicates were performed for each temperature value.

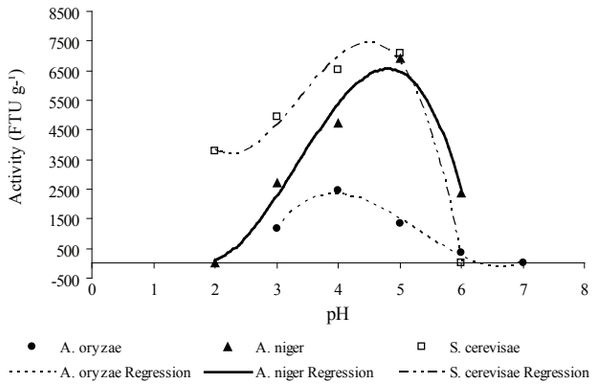
Statistical analysis

This experiment was conducted according to a completely randomized design, with eight treatments (pH and temperature values) and four replicates per phytase product. The obtained data were submitted to ANOVA using the SISVAR software (Ferreira, 2008), and regression models ($P<0.05$) were employed.

RESULTS AND DISCUSSION

Effect of pH on phytase activity

As expected, changes in pH values resulted in changes ($p<0.01$) in phytase activity of the evaluated products (Figure 1).



Regression equations:

A. oryzae

($y = 250.33x^3 - 3981.7x^2 + 19750x - 28982$; $R^2 = 0.9855$);

A. niger

($y = -501.23x^3 + 4994.6x^2 - 13296x + 10733$; $R^2 = 0.9684$);

S. cerevisae

($y = -670.25x^3 + 6791.4x^2 - 20421x + 22883$; $R^2 = 0.9887$).

Figure 1 – Activities of phytases derived from A. oryzae, A. niger, and S. cerevisae as a function of pH. Each point represents the mean of four replicates. The coefficients of variation of A. oryzae, A. niger, and S. cerevisae phytases were 4.37%, 5.07%, and 12.64%, respectively.

A. oryzae phytase showed activity in acetate (pH 3.0, 4.0, and 5.0) and maleate (pH 6.0) buffers; *A. niger* phytase was active in glycine (pH 3.0), acetate (pH 3.0, 4.0, and 5.0), and maleate (pH 6.0) buffers; and *S. cerevisae* phytase showed activity in glycine (pH 2.0 and 3.0) and acetate (pH 3.0, 4.0, and 5.0) buffers. As suggested by Gomori (1955), when the activity was determined in two different buffers (with the same pH value), the average activity value was used.

A. oryzae phytase showed maximum activity at pH 4.0, whereas both *A. niger* and *S. cerevisae* phytases displayed maximum activity at pH ranging between 4.5 and 5.0. These results agree with studies indicating that the optimal pH for most phytases ranges between 4.0 and 6.0 (Lei & Porres, 2003; Vats & Banerjee, 2004).

The optimal pH observed in our study for *A. niger* phytase (close to 5.0) is consistent with the results of several research studies (Maenz, 2001; Mullaney & Ullah, 2003). On the other hand, the optimal pH determined in the present study for *A. oryzae* phytase (pH 4.0) was lower than that of 5.5 reported in literature (Shimizu, 1993; Dvoráková, 1998). However, this difference is probably due to genetic changes (site-directed mutation, random mutagenesis) in the enzyme-producing microorganism and/or in the gene-donor microorganism, which may change phytase pH profile. The optimal pH for the phytase from *S. cerevisae* determined in this study (near 4.5) was similar to that reported for *Citrobacter braakii* phytase (pH 4.0) (Kim *et al.*, 2003) and for *E. coli* phytase (between 4.0 and 4.5) (Wyss *et al.*, 1999; Igbasan *et al.*, 2000). Accordingly, it should be recalled that genes from these two microorganisms were used to genetically modify *S. cerevisae* yeast used to produce the commercial phytase product.

According to Liebert *et al.* (1993), phytase is mostly active in the crop and proventriculus of broilers. In the crop, pH ranges between 4.0 and 5.0, and between 2.5 and 3.5 in the proventriculus. Furthermore, phytic acid phosphate groups are protonated at low pH, allowing their hydrolysis by phytases (Maenz, 2001). Therefore, higher *in-vitro* phytase activity at low pH (lower than 5.0) indicates higher phytase activity at those segments of the digestive tract of broilers. In the present experiment, phytases produced by *S. cerevisae* and *A. niger* showed higher absolute activity at pH values ranging between 2.0 and 5.0, whereas *A. oryzae* phytase presented the lowest absolute activity.

Effect of temperature on phytase activity

Temperature changes resulted in differences ($p<0.01$) in phytase activity. *A. oryzae* phytase showed increasing activity up to 40°C, and then became inactive at 50°C (Figure 2B). On the other hand, *A. niger* phytase showed increasing activity until almost 45°C, and then became inactive at 60°C (Figure 2A). *S. cerevisae* phytase showed highest activity between 50 and 60°C, and then became inactive at 70°C (Figure 2A). According to Vats & Banerjee (2004), phytases usually present high activity at temperatures ranging between 45 and 60°C.

Regression equations:

A. niger

($y = -1.2996x^3 + 130.6x^2 - 3785.2x + 37720$; $R^2 = 0.9959$);

S. cerevisae

($y = -0.5537x^3 + 60.587x^2 - 1789.9x + 18309$; $R^2 = 0.9938$).

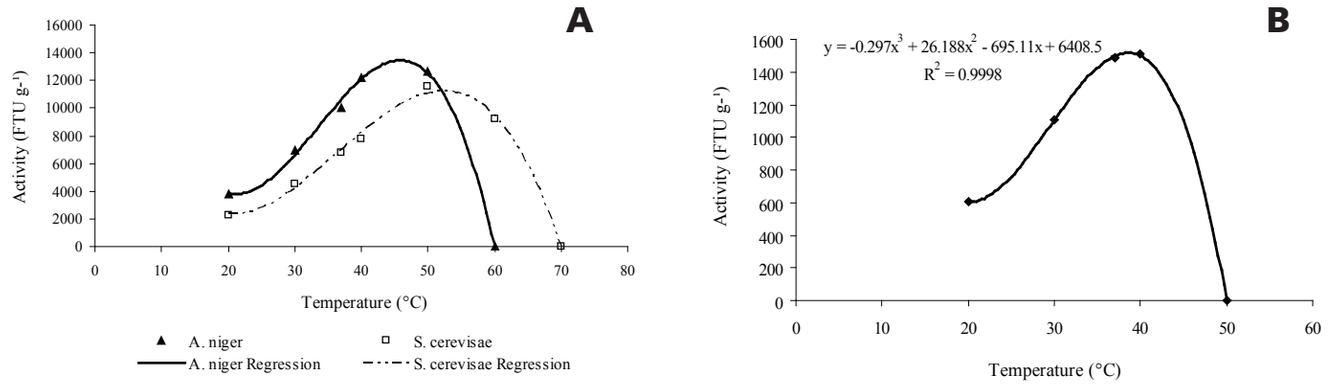


Figure 2 – Enzyme activity as a function of temperature. A) A. niger and S. cerevisae phytases and B) A. oryzae phytase. Each point represents the mean of four replicates. The coefficients of variation of A. oryzae, A. niger, and S. cerevisae phytases were 13.17%, 2.49%, and 4.25%, respectively.

Wyss *et al.* (1998) evaluated the influence of temperature on the activity of fungal phytases and reported that *A. fumigatus* and *A. niger* T213 phytases completely lost their catalytic activity after 65°C. However, in the present study, *Aspergillus* phytases lost their activity at a lower temperature.

According Kim *et al.* (2003), a phytase isolated from *C. braakii* showed higher activity at 50°C, and others researchers reported that the activity of *E. coli* phytase was highest between 55 and 60°C (Greiner *et al.*, 1993; Wyss *et al.*, 1999; Igbasan *et al.*, 2000). These temperatures are close to that determined for the *S. cerevisae* phytase in the present study (between 50 and 60°C). Again, it must be noted that specific genes of *C. braakii* and *E. coli* were transferred to the *S. cerevisae* that synthesized phytase in the present study.

The activity of the evaluated phytases also varied according temperature range. *S. cerevisae* phytase was active at the widest range of temperature, between 20°C and close to 70°C. On the other hand, *A. oryzae* phytase exhibited activity at the narrowest range of temperature, between 20°C and close to 50°C; whereas *A. niger* phytase showed an intermediate temperature range, between 20°C and close to 60°C.

According to Welker *et al.* (2008), body temperature of mature broilers ranges between 41 and 42°C. Considering the highest activity determined as 100%, *A. oryzae* phytase exhibited 96.44% of its maximum activity at 41°C. Accordingly, *A. niger* and *S. cerevisae* phytases presented 92.87 and 76.79% of their maximum activity at 41°C, respectively. However, the absolute activity of *A. oryzae* phytase was the lowest of the three evaluated enzymes and therefore, *A. niger* and *S. cerevisae* phytases were considered the most appropriate.

CONCLUSION

It was concluded that phytases produced by *Aspergillus niger* and *Saccharomyces cerevisae* exhibited the highest *in-vitro* absolute activities at pH and temperature values (pH lower than 5.0 and 41°C) that correspond to optimal physiological conditions of broilers, which would theoretically allow a higher rate of hydrolyses of the phytate contained in feed.

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