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# Stability of New Macromycetes Phytases under Room, Cooling and Freezing Temperatures of Storage

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***Abstract*—**Phytases are enzymes used as an important component in monogastric animals feeds in order to improve phosphorous availability, since it is not readily assimilated by these animals in the form of the phytate presented in plants and grains. As these enzymes are used in industrial activities, they must retain its catalytic activities during a certain storage period. This study presents information about the stability of 4 different phytases, produced by four macromycetes fungi through solid-state fermentation (SSF). There is a lack of data in literature concerning phytase from macromycetes shelf-life in storage conditions at room, cooling and freezing temperatures. The 4 phytases from macromycetes still had enzymatic activities around 100 days of storage at room temperature. At cooling temperature in 146 days of studies, the phytase from *G. stipitatum* was the most stable with 44% of the initial activity, in U.gds (units per gram of dried fermented substrate). The freezing temperature was the best condition storage for phytases from *G. stipitatum* and *T. versicolor*. Each condition provided a study for each mushroom phytase, totalizing 12 studies. The phytases showed to be stable for a long period without the addition of additives.

***Keywords*—**macromycetes, phytase, solid-state fermentation, wheat bran, stability

## I. Introduction

PHYTASES are phosphomonoesterases that catalyze the stepwise release of orthophosphate from myo-inositol hexakisphosphate (phytate) [1], [2]. They hydrolyze phosphate from phytic acid to inorganic phosphate and myo-inositol phosphate derivatives [3]. Since 85-90% of total plant phosphorous is bound in phytate and cereal-based feedstuffs offer phosphorous mostly in this form. However, phytate chelates vital ions, reducing their solubility and bioavailability. It also forms complexes with some proteins, which might inhibit digestive enzymes. Therefore, phytate is considered to be an anti-nutritional factor [4], [5], [6], [7]. As monogastric animals lack intestinal enzymes at the level needed to hydrolyze phytate [8], to use phytases as an ingredient in swine and poultry feeds, greatly improves the bioavailability of phytate-bound phosphorous. The addition of phytase to feeds also results in a reduction of the phosphorus outlet, avoiding nutrient over-enrichment [1]. Phytase activity usually decreases during feed pelleting (85-90ºC) by 30% or more and many studies have been made to improve its thermostability [9]. The low heat stability of commercially available phytase products (after pelleting) has also been of concern, since the enzyme would not only need to resist the feed pelleting but also be relatively stable during feed distribution and storage [10].

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However, the stability of phytase in vitro, before feed pelleting, remains a critical and less studied issue to the implementation of this process. Storage stability, or shelf life, refers to an enzyme's maintaining its catalytic abilities in the period between manufacture and eventual use [11]. Once the enzyme has been purified, a main objective for commercial interest is to retain the activity during storage, distribution and application. It is of primary importance to the enzyme producer and customer that the enzymes retain the activity during storage and use. Enzyme stability may be increased during storage by using stabilizers, preservatives, salts and diluents which allow standardization between production batches of different specific activities [12]. In this study we present phytases storage stabilities at room temperature, cooling temperature and freezing temperature without any additives. Phytase activity over time has been extensively tested and found that it can be negatively affected by different storage temperatures. The phytases studied were produced by *Schizophyllum commune*, *Trametes vervicolor*, *Ganoderma applanatum* and *Ganoderma stipitatum* through solid-state fermentation process. The use of fungi in the production of commercially important products through solid-state fermentation has gained much research interest during recent years [3, 13, 14] and allows the usage of agroindustrial wastes, such as the wheat bran, as the solid substrate-support [15]. Wheat bran is an important by-product of the flour industry, containing starch, protein and hemicelluloses. It also contains many phenolic acids, such as ferulic and vanillic acids [16]. Solid-state fermentation has also reduced energy requirements, simpler fermentation media, easier aeration and reduced bacterial contamination [3, 17].

## II. Materials and Methods

### A. Enzyme production

Four macromycetes species were selected from a screening in search for phytase producers, being named by the following codes: DRL-01 *Schizophyllum commune*, DRL-18 *Trametes versicolor*, DRL-56 *Ganoderma applanatum* and DRL-70 *Ganoderma stipitatum*. The cultures were provided from the culture collection of UFPR Biotechnological Processes Laboratory. These were used in the preparation of inoculum using mycelial blocks (5x5 mm) from an agar plate culture. After, they were inoculated in 100 mL of liquid Czapek media (30g glucose; 6g yeast extract; 1g $KH_2PO_4$; 0.5g $MgSO_4$; 0.01g $FeSO_4$; in 1L of deionized water), pH 6.0, incubated in shaker at 30ºC, 120 rpm for 3 days [18]. Wheat bran was used as substrate for solid-state fermentation, due to its large availability and nutritional factors.

The bran was submitted to wash and thermal pre-treatment to reduce contamination levels and phosphorus content. The fermentation was carried out in 250 mL Erlenmeyer flasks, each one containing 10g of treated wheat bran and 0.4% w/w of wet pellets per dried wheat bran from the pre-inoculum. The moisture content was adjusted to 50% with sterilized water (pH 6.0). All flasks were then incubated at 30ºC for 72h [18].

*B. Phytase extraction*

After the incubation period, the content of the fermented flasks was extracted with ultra pure water (pH 6.0) at a ratio of 1:10 of fermented media to ultra pure water. This extraction consisted by maceration for 3 minutes followed by filtration at 4ºC to prevent enzyme denaturation. This primary extract was also centrifuged for 15 minutes at 4500 rpm to remove biomass and solid particles; then the supernatant was stored in microtubes [19].

*C. Stability assays*

Microtubes containing the extracts were stored in: room temperature, which varied between 25±2ºC, cooling temperature (4ºC) and freezing temperature (-18ºC). The stability study of phytases stored in different conditions was conducted for 5 months and phytase assays were made following Heinonen and Lahti (1981) method [20], with some modifications by Spier et al (2008) [7]. The assays detected the presence of inorganic phosphates released during enzymatic reaction. A standard curve with $KH_2PO_4$ was prepared and the results were expressed by units per mL (U/mL).

At first, the phytase extracts stored at room temperature were used in assays 3 times a week. However, the enzyme activity remained stable during various days, so the assays were conducted weekly.

## III. Results And Discussion

*A. Stability at Room Temperature*

Samples containing phytases stored at room temperature presented phytase activity apparently stable in the first weeks of storage and decreasing during the time. Figure 1 presents phytase from *S. commune* (DRL-01) and *Trametes versicolor* (DRL-18) lost their activities after 126 days of storage at room temperature. *Ganoderma applanatum* (DRL-56) phytase lost its activity after 112 days of storage, while *Ganoderma stipitatum* (DRL-70) phytase showed the highest initial activity value of 62.81 $U.gds^{-1}$ (31.5% of remained activity) and also the longest shelf-life period: it was still active with approximately 9.15 $U.gds^{-1}$ 5% of remained activity of its initial activity 140 days after extraction stored at room temperature (25ºC±2ºC).

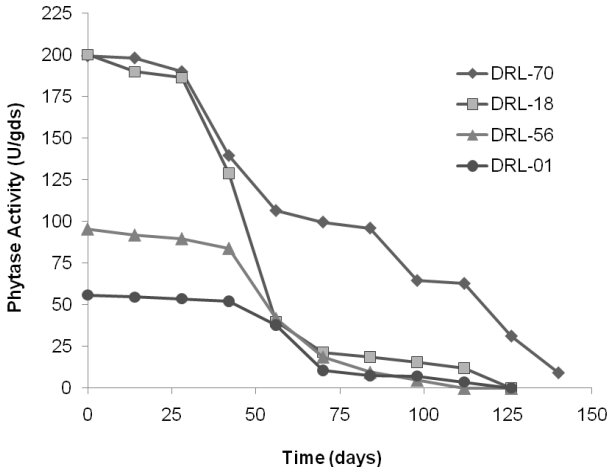


Fig. 1 Stability of *S. commune* (DRL-01), *G. applanatum* (DRL-56), *G. stipitatum* (DRL-70) and *T. versicolor* (DRL-18) phytases stored at room temperature

Table I shows relative activity and loss of activities during storage at room temperature from 0 to 140 days. In practically 30 days of storage, *G. stipitatum* DRL-70 phytase lost only 5%, while S. commune DRL-01 phytase activity lost 4%, G. applanatum DRL-56 6% and T. versicolor DRL-18 7%. After 70 days of storage *G. stipitatum* DRL-70 showed high stability among the phytases studied (from 199.42 to 99.1 $U.gds^{-1}$), losing just 50% against 81% in *S. commune* DRL-01 phytase activity, 80% in *G. applanatum* DRL-56 and 89% in *T. versicolor* DRL-18 phytase activity. After almost 100 days *G. stipitatum* DRL-70 still retained 32% of activity, showing as a good phytase for commercial interest. *G. stipitatum* DRL-70 phytase not only shows great stability behavior at room temperature. In 0 days of storage the activity was 199.42 $U.gds^{-1}$ and after 112 days it still have 62.81 $U.gds^{-1}$.

TABLE I
Loss Of Activities Of DRL-18, DRL-56, DRL-70 And DRL-01 Phytases At Room Temperature

| Storage time (days) | *S. commune* DRL-01 | | *G. applanatum* DRL-56 | | *T. versicolor* DRL-18 | | *G. stipitatum* DRL-70 | |
|---|---|---|---|---|---|---|---|---|
| | RA (%) | LA (%) | RA (%) | LA (%) | RA (%) | LA (%) | RA (%) | LA (%) |
| 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| 14 | 97.7 | 2.2 | 96.3 | 3.7 | 94.9 | 5.1 | 99.3 | 0.6 |
| 28 | 96.1 | 3.9 | 94.0 | 5.9 | 93.1 | 6.9 | 95.2 | 4.8 |
| 42 | 93.2 | 6.8 | 87.9 | 12.1 | 64.5 | 35.5 | 69.9 | 30.0 |
| 56 | 67.8 | 32.2 | 43.9 | 56.1 | 19.9 | 80.1 | 53.4 | 46.6 |
| 70 | 19.2 | 80.8 | 19.6 | 80.4 | 10.7 | 89.3 | 49.9 | 50.0 |
| 84 | 13.4 | 86.6 | 10.1 | 89.9 | 9.2 | 90.7 | 48.1 | 51.8 |
| 98 | 12.8 | 87.2 | 5.0 | 94.9 | 7.7 | 92.2 | 32.3 | 67.6 |
| 112 | 6.4 | 93.6 | 0 | 100 | 5.9 | 94.0 | 31.5 | 68.5 |
| 126 | 0 | 100 | 0 | 100 | 0 | 100 | 15.6 | 84.4 |

RA means relative activity (%); LA means loss of activity (%)

According to Koegel et al (1996), a transgenic alfalfa phytase also demonstrated no appreciable loss of activity over 3 weeks (21 days) of storage at room temperature (22°C).

Considering this study, our results are better because the room temperature in this study was higher (25°C), and the phytases were not GMO origins.

There was no loss of enzyme activity for 3 months at room temperature of phytase from *Pichia anomala* [24]. Quantum™ Phytase 5000 L is a preparation of 6-phytase (EC 3.1.3.26), produced by the genetically modified yeast *Pichia pastoris* (DSM 15927) commercialized by Syngenta Ltd. It retained an average activity of at least 80% after 25 weeks of storage at 21°C, falling to approximately 69% at 21°C and 49% at 37°C after 48 weeks of storage. The data supports a shelf life of six months when stored at 21°C or below [21], showing that this commercial feed additive must has stabilizers present in its formulation. Transgenic rice phytase was stored for up to 12 weeks, with no decrease in the activity of the heterologous phytase [22].

### *B. Stability at Cooling Temperature*

At 4°C storage, the four macromycetes phytases were able to maintain their activities for more than 4 months (Table II). It is possible to observe that, after 70 days of storage, the activity decreased from 95.3 to 60.8 U.gds$^{-1}$), retained 63.8% of its initial activity. After 140 days after extraction, *G. applanatum* DRL-56 phytase still held 10% of its initial activity (9.96 U.gds$^{-1}$).

*S. commune* DRL-01 phytase showed 20% of its initial activity, *T. versicolor* DRL-18 phytase held 30% of its original activity and *G. stipitatum* DRL-70 phytase was again the most stable at cooling temperature, maintaining its activity at 44% of the initial value (Fig. 2). This is in accordance with data reported by Aggabao (1997) [23], in which the crude phytase of some molds can be stored under refrigeration or freezing for 33 days without significant loss in its activity. Vohra and Satyanarayana (2002) [24] studied a purified phytase from *Pichia anomala* which retained its complete activity after six months at 4ºC.

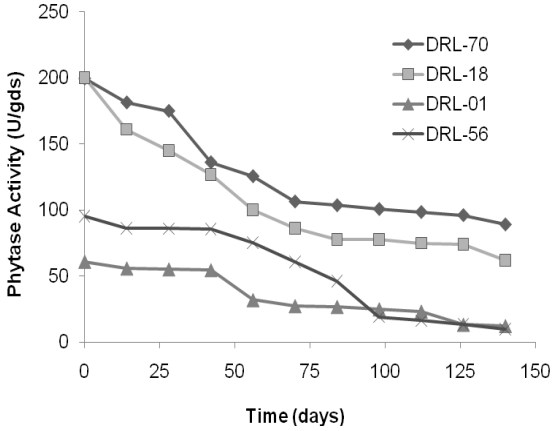


Fig. 2 Stability of *G. stipitatum* DRL-70, *T. versicolor* DRL-18, *S. commune* DRL-01 and *G. applanatum* DRL-56 phytases stored at cooling temperature

TABLE II
LOSS OF ACTIVITIES OF *T. VERSICOLOR* DRL-18, *G. APPLANATUM* DRL-56, *G. STIPITATUM* DRL-70 AND *S. COMMUNE* DRL-01 PHYTASES AT COOLING TEMPERATURE

| Storage time (days) | *S. commune* DRL-01 | | *G. applanatum* DRL-56 | | *T. versicolor* DRL-18 | | *S. stipitatum* DRL-70 | |
|---|---|---|---|---|---|---|---|---|
| | RA (%) | LA (%) | RA (%) | LA (%) | RA (%) | LA (%) | RA (%) | LA (%) |
| 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| 14 | 91.7 | 8.3 | 90.3 | 9.6 | 80.4 | 19.6 | 90.9 | 9.1 |
| 28 | 90.6 | 9.4 | 90.2 | 9.8 | 72.4 | 27.6 | 87.7 | 12.3 |
| 42 | 89.5 | 10.4 | 90.0 | 9.9 | 63.2 | 36.7 | 68.2 | 31.8 |
| 56 | 52.5 | 47.5 | 79.1 | 20.9 | 49.9 | 50.0 | 62.9 | 37.0 |
| 70 | 45.0 | 54.9 | 63.8 | 36.2 | 43.0 | 56.9 | 53.3 | 46.6 |
| 84 | 44.0 | 55.9 | 48.6 | 51.4 | 38.8 | 61.2 | 51.9 | 48.0 |
| 98 | 41.1 | 58.9 | 20.1 | 79.9 | 38.8 | 61.2 | 50.5 | 49.5 |
| 112 | 38.1 | 61.9 | 17.1 | 82.9 | 37.4 | 62.5 | 49.3 | 50.6 |
| 126 | 21.9 | 78.1 | 14.1 | 85.9 | 36.9 | 63.0 | 48.2 | 51.8 |
| 140 | 20.2 | 79.7 | 10.4 | 89.5 | 30.9 | 69.0 | 44.6 | 55.3 |

*G. stipitatum* DRL-70 phytase activity presented similar stability at 4°C compared to several phytases reported in the literature. For example, an *A. niger* FS3 phytase reported by Spier et al (2011) [12] retained 88.9% of its activity after 30 days of storage at 4°C. *G. stipitatum* DRL-70 retained 87% in the same storage time. After 140 days of storage, DRL-70 phytase also retained more than 50% of its activity at room temperature, as well as results of *A. niger* FS3 phytase reported.

### *C. Stability at Freezing Temperature*

The enzymatic extracts stored at -18°C showed the following activity losses: *G. stipitatum* DRL-70 phytase was again significantly stable, maintaining 70% of its initial activity (from 156.3 to 109.4 U.gds$^{-1}$) after 98 days of freezing storage, while *S. commune* DRL 01 presented 39% of its initial activity after the same period (from 46.5 to 18.3 U.gds$^{-1}$) . *T. versicolor* DRL-18 phytase showed 62% of its initial activity (from 172.1 to 107.1 U.gds$^{-1}$) 112 days after its extraction and *G. applanatum* DRL-56 was the least stable, with an activity of 38% only 14 days after extraction (Fig. 3).

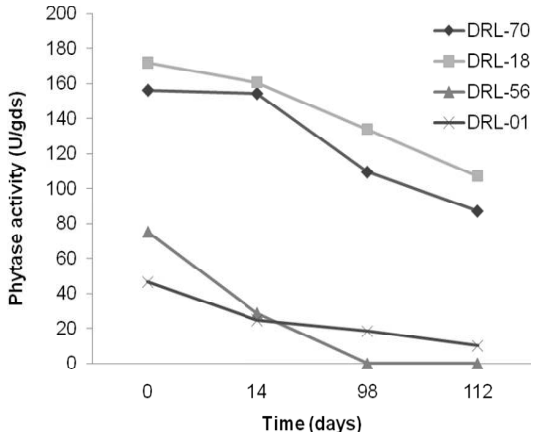


Fig. 3 Stability of *G. stipitatum* DRL-70, *T. versicolor* DRL-18, *S. commune* DRL-01 and *G. applanatum* DRL-56 phytases stored at freezing temperature

Phytase produced by DRL-70 was the most stable throughout the period of this study, under all conditions (room temperature, cooling and freezing temperature). DRL-18 phytase was also stable, although with smaller remained activities compared to DRL-70 phytase. When comparing DRL-70 phytase in three storage conditions studied, the lower activity lost was observed at cooling temperature (4°C), equivalent to 50% in 112 days. The lost of activity at room and freezing storage was similar: 67% and 68.5% at -18°C and room temperature, respectively.

In literature, phytase from *Pseudomonas sp.* retained 75% of activity for 100 hours at freezing temperature [25]. Another stability study was conducted by Powar and Agannathan [26], with a phytase from *Bacillus subtilis* very stable and that could be stored at -20°C for at least a year without significant loss in activity.

Others researches with higher temperatures has shown phytase enzyme activity can decrease rapidly over time when subjected to heat. Stability of phytase in base mixes or premixes is another key area of concern given the adverse affects of trace minerals and hygroscopic substances, such as sodium chloride. Natuphos is a commercial phytase with recommended storage conditions of 23ºC and 50% relative humidity. It was reported that Natuphos® retained 75% of its activity after three months under these conditions. After two months of storage at 30°C, only 65% of original activity was maintained.

Pelleting can also be stressful to enzymes such as phytase. Natuphos phytase does not withstand temperatures required for pelleting and thus requires post-pelleting liquid application when used in pelleted diets. Ronozyme® is a commercial phytase is reported to survive the pelleting process better than Natuphos phytase, indicates phytase retention rate was approximately 40% higher for Ronozyme than Natuphos at 85° C. Therefore, it is suggested to use Ronozyme in pelleted diets [27,28,29].

## IV. Conclusions

Different storage conditions affect phytases stability from 4 macromycetes. The best storage condition for the liquid extracts, concerning the maintenance of phytase activity, was the freezing temperature. Although room temperature and cooling temperature also permitted stable for phytases tested, mainly for DRL-70 and DRL-18 phytases, which showed higher phytase activities at -18°C in comparison to those observed after the same periods of storage in cooling and room temperatures. *Ganoderma stipitatum* DRL-70 is the higher phytase producer among the four macromycetes tested in this work, achieving almost 200 $U.gds^{-1}$. Besides, it showed a good stability at freezing and cooling temperature and 95% of its initial activity after almost 30 days of storage at room temperature.

The development of a new phytase formulations with the addition of additives such as stabilizers to improve shelf life and compare to phytases commercially available are been studied by this research group (data not shown but it will presented in the ICCABBE 2012).

Based on these studies, it is obvious that storage conditions can play an important role in maintaining the shelf life of the enzymes.

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