EFFECT OF pH AND TEMPERATURE ON PHYTASE AND BIOMASS PRODUCTION BY SUBMERGED FERMENTATION WITH *Aspergillus niger* var. *phoenicis* URM 4924

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ABSTRACT - Phytase production and biomass was evaluated in present work by submerged fermentation with *Aspergillus niger* var. *phoenicis* URM 4924. Experimental assays were done under different conditions of pH (4.0 to 8.0) and temperature (25 to 35 ºC), and the influence of these variables on the responses was studied through a 2^2 central composite design and response surface methodology. Phytase and biomass production were affected by the pH and temperature used during submerged fermentation. Phytase activity was increased in up to 7.8-fold (from 1.04 to 8.09 U/mL) and the ergosterol content was increased in up to 38-fold (from 9.3 to 354.09 µg/mL). The maximum values of both responses were achieved when using pH 4.0 and 30 ºC.

1. INTRODUCTION

Approximately 60-80% of phosphorus (P) in vegetal feed ingredients is in the form of phytate. Due to the lack of phytate-degrading enzymes in the gastrointestinal tract of non-ruminant animals, phytate P is almost completely unavailable to poultry and pigs. Inorganic P is usually added to pig and poultry diets in order to meet nutritional requirements for optimizing growth performance and bone strength of the animals (Esmaeilipour et al., 2012).

Phytases belong to a sub-class of the family of histidine acid phosphatases with the in vitro capability to release at least one phosphate from phytate. The distribution of phytases is widespread among bacteria, yeast, fungi, plants, and also in animals. Fungal phytases are widely used in animal feed due to their acid tolerance and higher yield in comparison to the bacterial phytases (Zhang et al., 2010).

The biomass determination is many times a challenge in fermentative process, and is an essential parameter in kinetic studies and for characterization of the growth for different fungi (Augustine et al., 2006). Several indirect methods have been employed in order to determine fungal biomass in submerged fermentation conditions, which are based on measurements of the content of cell components like chitin, ergosterol and protein. Ergosterol, one of the most important components in fungal membranes, is involved in numerous biological functions and control of the cellular cycle (Alcazar-Fuoli et al., 2008). As a marker molecule for fungal biomass, ergosterol has several advantages since it is easy to extract and measure by HPLC.

Literature data on the production of phytase by submerged fermentation is scarce, and most of the published studies report the production of this enzyme by solid-state fermentation.

However, is well known that submerged fermentation systems present several advantages when compared to solid-state fermentation systems (Mussatto et al., 2012).

For all the above mentioned reasons, the present study consisted in evaluating the production of phytase and biomass (estimated by the ergosterol content) by submerged fermentation with *Aspergillus niger* var. *phoenicis* URM 4924.

2. MATERIAL AND METHODS

2.1. Fungal Strain and Fermentation Medium

Aspergillus niger var. *phoenicis* URM 4924 from the URM Culture Collection of the Department of Mycology, Federal University of Pernambuco (Brazil), was the fungal strain used in the present study. The culture was grown and maintained on potato-dextrose-agar slants at 4 °C. An eight-day-old sporulated culture was used for the inoculum preparation. Fermentation assays were carried out in 250-mL Erlenmeyers flaks containing 100 mL of medium with the following composition: 1.0% (*w/v*) rice bran, 3.0% (*v/v*) corn steep liquor, 0.5 g/L KCl, 1.5 g/L $MgSO_4.7H_2O$, 2.0 g/L $CaCl_2.2H_2O$, 1.5 g/L $Fe_2SO_4.7H_2O$ (salts). According to the pH to be studied, specific buffers were also added to the medium, namely: 0.2 M acetate buffer pH 4.0, 0.2 M acetate buffer pH 6.0, or 0.2 M TRIS-glycina buffer pH 8.0. Fermentation medium was sterilized at 121 °C for 15 min, and was inoculated with 70 µL of the spore suspension containing 10^6 spores/mL. The fermentation runs were carried out in orbital shaker at 90 rpm, for 72 h, under different conditions of pH and temperature (Table 1).

2.2. Phytase Activity and Ergosterol Analysis

Phytase activity was determined by using the method of ammonium molybdate. Enzyme activity was expressed in units per milliliter (U/mL). Ergosterol content was estimated according to Gourama and Bullerman (1995) with modifications. Ergosterol was measured by high performance liquid chromatography (HPLC) and Galaxie chromatography data system. The chromatographic separation was carried out with a 20 min isocratic run on a C_{18} reversed-phase YMC-Pack ODS-AQ analytical column (250 × 4.6 mm i.d., 5 µm).

2.3. Statistical Analysis

All the experimental conditions were carried out in triplicate leading to 27 sets of experiments, and mean values ± standard errors are presented (Table 1). Statistical significance of the variables was determined at 5% probability level (p<0.05). Statistica version 7.0 was the software used for regression and graphical analyses of the data.

3. RESULTS AND DISCUSSION

The experimental matrix with the real and coded levels of the process variables, as well as the results of phytase activity and ergosterol content obtained to each experimental assay, is presented in Table 1. As can be seen, the values of the responses were strongly affected by the conditions of pH and temperature used for fermentation. The phytase activity, for example, was increased in up to 7.8-fold (from 1.04 to 8.09 U/mL) while the ergosterol content was increased in up to 38-fold (from 9.3 to 354.09 µg/mL). For both responses, the

maximum values were achieved when using the lowest pH (4.0) and the intermediate value of temperature (30 ºC), conditions of the assay 6.

Table 1. Phytase activity and biomass (estimated by the ergosterol content) obtained by submerged fermentation with *Aspergillus niger* var. *phoenicis* URM 4924 under different conditions of pH and temperature, according to a 2^2 central composite design. All the experimental assays were carried out in triplicate and results are presented as mean values ± standard errors.

Experimental assays	Process variables - real and (coded) values		Responses	
	pH	Temperature (°C)	Phytase activity (U/mL)	Biomass (ergosterol content) (ppm)
1	4.0 (-1)	25 (-1)	3.03 ± 0.003	49.25 ± 2.16
2	8.0 (+1)	25 (-1)	1.97 ± 0.000	17.93 ± 1.10
3	4.0 (-1)	35 (+1)	5.69 ± 0.000	134.41 ± 4.80
4	8.0 (+1)	35 (+1)	1.04 ± 0.003	9.30 ± 0.43
5	6.0 (0)	30 (0)	4.82 ± 0.003	80.69 ± 3.19
6	4.0 (-1)	30 (0)	8.09 ± 0.000	354.09 ± 1.96
7	8.0 (+1)	30 (0)	1.97 ± 0.000	16.52 ± 0.80
8	6.0 (0)	25 (-1)	4.83 ± 0.003	79.10 ± 3.61
9	6.0 (0)	35 (+1)	4.16 ± 0.003	54.87 ± 0.37

An analysis for estimation of the effects of the variables on phytase production revealed that the pH was the variable with the highest influence on this response, with both linear (L) and quadratic (Q) terms being significant at $p<0.01$ and $p<0.05$, respectively. Fig. 1a clearly shows that the results of phytase activity were improved when the fermentation pH was decreased to 4.0, which is in agreement with the results presented in Table 1.

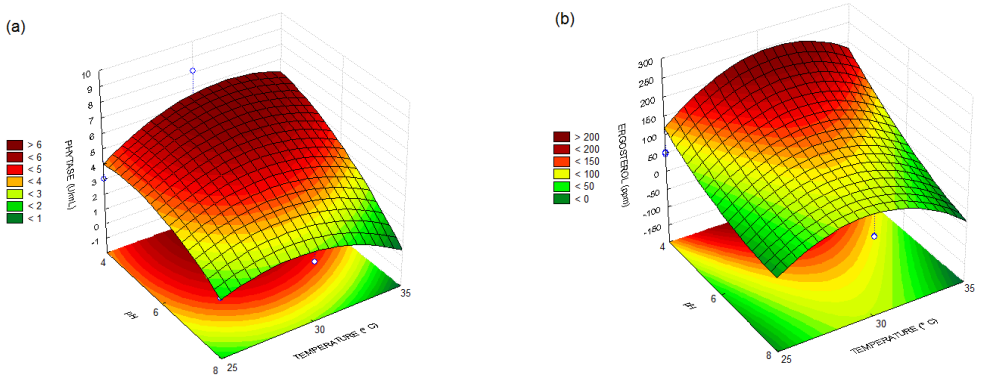

Fig. 1. Response surfaces representing the phytase activity (a) and ergosterol content (b) variations according to the pH and temperature used during the fermentation with *Aspergillus niger* var. *phoenicis* URM 4925.

In brief, pH and temperature were demonstrated to be variables of great influence on the production of phytase by submerged fermentation with *A. niger* var. *phoenicis* URM 4924. The pH was also an important variable affecting the production of phytase by other fungal

strains. For example, phytase production by *A. niger* 307 was maximized at pH 5.0 (Gargova and Sariyska, 2003), and, similar to the present study, pH 4.0 was the best for phytase production by *A. niger* NCIM 563 (Soni and Khire, 2007).

The ergosterol content was also influenced by the pH and temperature used during the fermentation; but in this case, only the linear (L) term of pH and the quadratic (Q) term of the temperature affected this response. In fact, when comparing the three-dimensional surfaces obtained for the two responses (Fig. 1a, b) it is clear that the effect of the variables was not the same for both the responses, although the results were maximized in a similar region (pH 4.0, 30 ºC), as previously discussed.

4. CONCLUSION

The production of phytase by *A. niger* var. *phoenicis* URM 4924 by submerged fermentation was dependent on the biomass formation, and the temperature and pH used during fermentation affected the phytase production as well as the biomass formation by this fungal strain. The best temperature and pH for phytase production was found to be 30 °C and pH 4.0. These results are of great relevance as they show that the cultivation conditions affect the production phytase by *Aspergillus niger* var. *phoenicis* URM 4924.

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