Effect of pH and temperature on phytase and biomass production by submerged fermentation with *Aspergillus niger var. phoenicis* URM 4924

Efeito do pH e da temperatura na produção e biomassa de fitase por fermentação submersa com *Aspergillus niger var. phoenicis* URM 4924

Efecto del pH y la temperatura sobre la producción de fitasa y biomasa por fermentaciónsumergida con Aspergillus niger var. *Phoenicis* URM 4924

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Abstract
The production of phytase and biomass (estimated by the ergosterol content) by submerged fermentation with Aspergillus niger var. phoenicis URM 4924 was studied. Experimental assays were carried 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 22 central composite design and response surface methodology. Phytase and biomass production were strongly affected by the pH and temperature used during 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. Good correlation (second-order fit, R2 = 0.9875) was found between the data obtained for phytase activity and ergosterol content, suggesting that the phytase production depends on the biomass formation. These results are of interest since they contribute for the development of an industrial process for phytase production with elevated yields by submerged fermentation.
Keywords: *Aspergillus niger*; Ergosterol; Phytase; Submerged fermentation.

Resumo

A produção de fitase e biomassa (estimada pelo teor de ergosterol) por fermentação submersa com Aspergillus niger var. phoenicis URM 4924 foi estudada. Ensaios experimentais foram realizados sob diferentes condições de pH (4,0 a 8,0) e temperatura (25 a 35 ºC), e a influência dessas variáveis nas respostas foi estudada por meio de um planejamento estatístico composto e metodologia de superfície de resposta. A produção de fitase e biomassa foi afetada pelo pH e temperatura utilizados durante a fermentação. A atividade da fitase aumentou em até 7,8 vezes (de 1,04 a 8,09 U/mL) e a concentração de ergosterol foi aumentada em até 38 vezes (de 9,3 a 354,09 µg/mL). Os valores máximos de ambas as respostas foram alcançados com pH 4,0 e 30 ºC. Boa correlação (ajuste de segunda ordem, R2 = 0,9875) foi encontrada entre os dados obtidos para atividade de fitase e teor de ergosterol, sugerindo que a produção de fitase é depende da formação de biomassa. Esses resultados são interessantes, pois contribuem para o desenvolvimento de um processo industrial de produção de fitase com elevados rendimentos por fermentação submersa.

Palavras-chave: *Aspergillus niger*; Ergosterol; Fitase; Fermentação submersa.

Resumen

La producción de fitasa y biomasa (estimada por el contenido de ergosterol) por fermentación sumergida con Aspergillus niger var. phoenicis URM 4924 fue estudiado. Se realizaron ensayos experimentales en diferentes condiciones de pH (4,0 a 8,0) y temperatura (25 a 35 ºC), y se estudió la influencia de estas variables en las respuestas mediante un diseño estadístico compuesto y metodología de superficie de respuesta. La producción de fitasa y biomasa se vio afectada por el pH y la temperatura utilizados durante la fermentación. La actividad de fitasa aumentó hasta 7,8 veces (de 1,04 a 8,09 U/ml) y la concentración de ergosterol aumentó hasta 38 veces (de 9,3 a 354,09 µg/ml). Los valores máximos de ambas respuestas se alcanzaron a pH 4,0 y 30 ºC. Se encontró una buena correlación (ajuste de segundo orden, R2 = 0,9875) entre los datos obtenidos para la actividad de fitasa y el contenido de ergosterol, lo que sugiere que la producción de fitasa depende de la formación de biomasa. Estos resultados son interesantes, ya que contribuyen al desarrollo de un proceso industrial para la producción de fitasa con altos rendimientos por fermentación sumergida.

Palabras clave: *Aspergillus niger*; Ergosterol; Fitasa; Fermentación sumergida.

1. Introduction

Approximately 60-80% of phosphorus (P) in vegetal feed ingredients is in the form of phytate (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) (Diarra et al., 2010). Due to the lack of phytate-degrading enzymes in the gastrointestinal tract (GIT) 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. If phytate P could be available for absorption in monogastric animals, this might reduce or eliminate the addition of inorganic P to the diet (Esmaeilipour et al., 2012).

Phytases, myo-inositol hexakisphosphate phosphorhydrolase (EC 3.1.3.8), 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 (Haefner et al., 2005; Zhang et al., 2010). The distribution of phytases is widespread among bacteria, yeast, fungi, plants, and also in animals (Mullaney et al., 2000; Haefner et al., 2005). The importance of microbial phytases is to release phytate bound phosphorous

and improve phosphorous bioavailability of plant ingredients for non-ruminants animals and to reduce the phosphorous levels in effluent from intensive animal units (Khan et al., 2004). Recently, the production, properties, and structure of phytase have been reviewed (Mullaney et al., 2000; Choi et al., 2001). Extensive studies made on microbial phytases have proved their efficacy that could be tapped for animal nutrition, environment protection, and also for human health (Pandey et al., 2001; Vohra and Satyanarayana, 2003).

Although large number of microrganisms including bacteria, yeast and fungi are reported to produce phytases (Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004), fungal phytases are widely used in animal feed due to their acid tolerance and higher yield in comparison to the bacterial phytases. Among fungi, many *Aspergilli* are known to be active phytase producers (Soni & Khire, 2007). As *Aspergillus niger* is Generally Recognised as Safe (GRAS) it is frequently used in food and feed applications. To enhance plant phosphorus utilization and to circumvent the deleterious effect of phytic acid in animal nutrition, phytases from *Aspergillus* species have been fed to non-ruminants animals (Vats and Banerjee, 2002).

The biomass determination is many times a challenge in fermentative process, and is an essential parameter in kinetic studies and for characterization of the optimum growth and sporulation conditions 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 certain cell components like chitin, ergosterol and protein (Desgranges et al., 1991). Such methods include glucosamine estimation, ergosterol accumulation, protein (Kjeldahl) content, DNA estimation, dry weight changes and CO_2 evolution; however, all of these methods have their own weaknesses (Mussatto et al., 2012). The content of the different cell components can markedly change in fungi according to the specie, growth conditions and culture age (Oojikas et al., 1998).

Ergosterol, one of the most important components in fungal membranes, is involved in numerous biological functions, such as, membrane fluidity regulation, activity and distribution of integral proteins and control of the cellular cycle (Alcazar- Fuoli et al., 2008). These facts make ergosterol and its biosynthetic pathway essential for fungal growth. As a marker molecule for fungal biomass, ergosterol has several advantages since it is easy to extract and measure by HPLC (Gessner and Chauvet, 1997b), and in many cases it is also the dominating sterol in fungi. Additionally, ergosterol is a more sensitive and reliable indicator of viable fungal biomass than other biochemical molecules, such as chitin (Newell, 1996). The determination of ergosterol is also valuable in correlating fungal activity to synthesis of fungal secondary metabolites (Marín et al., 2005).

Although phytase shows great potential for phytate bioconversion, the enzyme activities and yields need to be increased to make them possible for industrial application. Therefore, there is ongoing interest in identifying novel phytases, as well as establishing a process able to produce phytase with elevated yields (Haefner et al., 2005; Luo et al., 2006). 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, which includes shorter cultivation times, better aeration, agitation and mass transfer conditions, and others (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. Experimental assays were carried out under different conditions of pH and temperature, and the influence of these variables on the responses was studied through a 2^2 central composite design and response surface methodology.

2. Methodology

2.1 Location of the study

This present research was conducted at the Laboratory of Fermentation. Department of Biological Engineering, University of Minho.

2.2 Fungal strain and inoculum preparation

Aspergillus niger var. *phoenicis* URM 4924 (non-OTA producer) 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 (PDA) slants at 4 °C. An eight-day-old sporulated culture was used for the inoculum preparation. The conidial suspensions were obtained by washing the stock tube cultures with sterile solution of 0.1% Tween 80. The spores' concentration was adjusted to 10^6 spores/mL by counting in a Neubauer chamber.

2.3 Fermentation medium and conditions

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 (carbon source), 3.0% (*v/v*) corn steep liquor (nitrogen source), 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). After fermentation, mycelium was separated by filtration followed by centrifugation (10,000 g, 30 min, 4 °C), and was used to estimate the ergosterol content. The supernatant was collected and used to estimate phytase activity (U/mL).

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

Source: Authors.

2.4 Analytical methods

2.4.1 Phytase activity

Phytase activity was determined by using the method of ammonium molybdate (Heinonen & Lathi, 1981) with modifications. Sodium acetate buffer (350 µL at 0.2 M, pH 4.8) containing sodium phytate (875 nmol) was used as substrate. After pre-incubation at 37 °C for 10 min, the enzymatic reaction was initiated by the addition of enzyme extract (50 µL). The homogenized solution was incubated for 30 min at 37 °C. In order to estimate the liberated inorganic phosphate, 1.5 mL of a freshly prepared solution of acetone: 2.5 M H_2SO_4: 10 mM ammonium molybdate (2:1:1 *v/v/v*) and 100 µL of 1M citric acid were added to the assay mixture. The absorbance at 355 nm was then read after 10 min of reaction. One unit of phytase was defined as the amount of enzyme that releases 1 µmol of inorganic phosphorus per min under standard assay conditions. Enzyme activity was expressed in units per milliliter (U/mL). The standard curve was made with dibasic potassium phosphate with 10 to 600 µM phosphate per mL. The amount of inorganic phosphate present in the medium before inoculation of the microorganism was also determined in order to minimize interference residues of P present in the culture medium.

2.4.2 Ergosterol analysis

Ergosterol content was estimated according to Gourama and Bullerman (1995a) with modifications. The total biomass

was filtered and then immediately extracted with 40 mL of 10% KOH in methanol, under magnetically stirring for 30 min. A 10-mL aliquot obtained by suspension was then transferred to a screw-cap tube and placed in a hot water bath at 55-60 °C for 20 min, being subsequently allowed to cool to room temperature. Then, 3 mL of water and 2 mL of hexane were added to the tube and the mixture was agitated in a vortex mixer for 1 min. After separation of the layers, the upper layer (hexane) was transferred to a 10-mL vial. Hexane extraction was repeated twice using 2 mL each time. The extracts were combined and evaporated to dryness under a stream of nitrogen. The dried extracts were dissolved in 2 mL of methanol, and filtered through 0.45 μm acetate filters. Ergosterol was measured by high performance liquid chromatography (HPLC) using a system composed of a UV detector (Waters 2487) at 282 nm, a pump (Varian 9002), a Marathon Basic autosampler with loop of 50 μL, a fluorescence detector (Jasco FP-920), and a 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), fitted with a pre-column of the same stationary phase. The mobile phase was composed of methanol filtered in 0.45 μm membrane (GHP, Gelman) and degassed. Ergosterol was quantified taking as reference an ergosterol standard calibration curve prepared from a standard solution (1000 μg/mL (1 ppm), purchased from Sigma Chemicals (St. Louis, MO). A linear relationship was observed between peak heights measured on HPLC chromatograms and ergosterol concentration ($R^2 = 0.998$). All the analytical determinations were carried out in triplicate.

2.4.3 Correlation between dry weight and ergosterol content

In order to verify a possible correlation between dry weight (g) and ergosterol content (ppm) in the samples, fermentation assays were carried out using the following substrates, instead of rice bran: phytic acid 1% (w/v), glucose 4 % (w/v), sucrose 4% (w/v), starch 4% (w/v), and corn steep liquor 2% (v/v). These substrates were used as rice bran particles cannot be separated from the biomass present in the culture medium. This would make impossible to study a possible correlation between dry weight and ergosterol content in the samples under submerged fermentation. The fermentation runs were carried out in orbital shaker at 90 rpm for 120 h. During the fermentations, samples were withdrawn each 24 h, being filtrated with filter paper. The supernatant was used for phytase activity (U/mL) determination. The fungal biomass was dried in an oven at 80 °C for 2 h. The dried biomass was used to estimate the ergosterol content (ppm).

2.5 Statistical analysis

The influence of the variables pH and temperature on the phytase and biomass production (expressed by ergosterol content) by *A. niger* URM 4924 under submerged fermentation conditions was evaluated through a 2^2 central composite design with three coded levels. For statistical analysis, the variables were coded according to Eq. 1, where each independent variable is represented by x_i (coded value), X_i (real value), X_0 (real value at the center point), and ΔX_i (step change value). The range and levels of the variables, which selected based on literature data, are given in Table 1.

$$x_i = \frac{(X_i - X_0)}{\Delta X_i}$$

Eq. 1

All the experimental conditions were carried out in triplicate leading to 27 sets of experiments, and mean values ± standard errors are presented (Table 1). Repetitions were carried out in order to estimate the experimental error and to examine the presence

of curvature in the response surface. Statistical significance of the variables was determined at 5% probability level ($p<0.05$). Data obtained from the design were fitted to a second order polynomial equation. Statistical significance of the regression coefficients was determined by Student's t-test, and the proportion of variance explained by the model was given by the multiple coefficient of determination, R^2. Statistica version 7.0 was the software used for regression and graphical analyses of the data.

3   Results and Discussion

3.1   Phytase production

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.

An analysis for estimation of the effects of the variables on phytase production (Table 2) 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. The temperature used for fermentation presented also important influence on the production of phytase, with individual and interaction effects significant at $p<0.01$ (Table 2). However, for the individual effects, only the quadratic term of this variable was significant on this response, suggesting that the phytase activity was improved when the temperature was increased, but there was a maximum value of temperature after which the results were not improved. Such behavior can be easily visualized in Figure 1a. Under the lowest pH value (4.0), which improved the results of phytase activity, the temperature increase improved the results of enzyme activity but until a certain value, after which no more significant improvements on phytase production were observed.

Table 2 - Effect estimates (EE), standard errors (SE) and level of significance (p) of the variables pH (x_1) and temperature (x_2), on the responses phytase activity and biomass (estimated by the ergosterol content) obtained by submerged fermentation with *Aspergillus niger* var. *phoenicis* URM 4924, according to a 2^2 central composite design.

Factors [a]	Phytase activity			Biomass (ergosterol content)		
	EE	SE	p	EE	SE	p
x_1 (L)	-3.94	± 0.43	0.00***	-164.67	± 30.92	0.00***
x_1 (Q)	-1.94	± 0.74	0.02**	50.73	± 53.56	0.35
x_2 (L)	0.36	± 0.43	0.41	17.43	± 30.92	0.58
x_2 (Q)	-3.01	± 0.74	0.00***	-185.92	± 53.56	0.00***
x_1x_2	-1.79	± 0.52	0.00***	-46.89	± 37.87	0.23

[a] (L) linear and (Q) quadratic levels. *** significant at 99% confidence level; ** significant at 95% confidence level. Source: Authors.

Figure 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
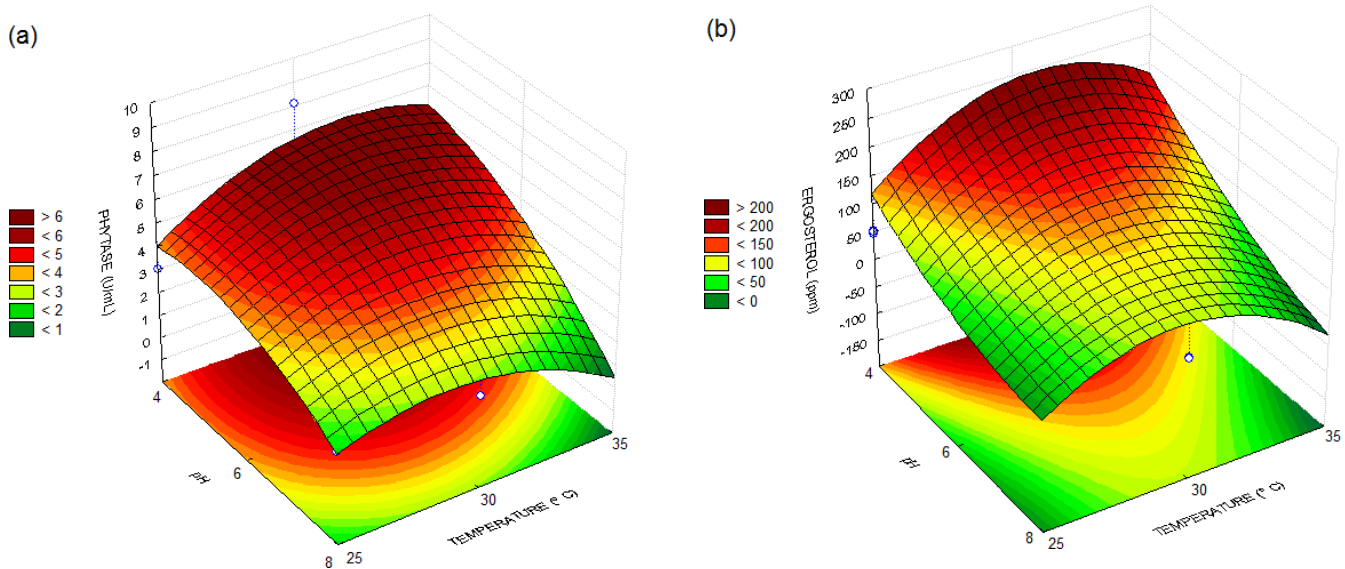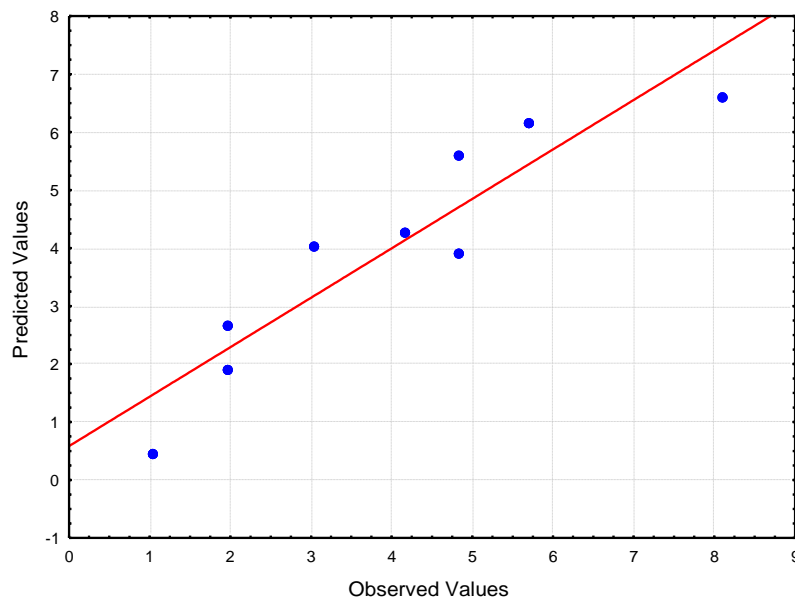

Source: Authors.

The statistical significance of the quadratic terms of the variables suggests that a second-order polynomial equation is more suitable than a linear equation to describe the variations of phytase activity as a function of the pH and temperature used for fermentation. The coefficients of the second-order model equation able to estimate the phytase activity variations as a function of the process variables variations were then obtained by multiple regression analysis, and the mathematical model obtained was expressed by the Eq. 2, where the variables pH (x_1) and temperature (x_2) assumed their coded values. This model is very useful for a fast prediction of the phytase activity value to be obtained when using pH and temperature in the range of values here studied. This model presented high coefficient of determination ($R^2 \geq 0.85$), explaining more than 85.0% of the variability in the response. Such a fact can also be verified through Figure 2, which represents the observed values of phytase activity versus the values predicted by the model equation. As can be seen in this figure, the results are distributed close the medium values, revealing a good agreement between the predicted and observed values of phytase activity when using the established second-order model equation.

Phytase activity (U/mL) = $5.61 - 1.97x_1 - 0.97x_1^2 + 0.18x_2 - 1.51x_2^2 - 0.90x_1x_2$
$R^2 = 0.853$

Eq. 2

Figure 2 - Values predict by the second order model as a function of the values observed for the response phytase activity (U/mL)during the fermentation with *Aspergillus niger* var. *phoenicis* URM 4925

Source: Authors.

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 presenting more significant influence than the temperature in the studied range of values. The pH was also an important variable affecting the production of phytase by other fungal strains. For example, the phytase production by *A. niger* 307 was maximized at pH 5.0 (Gargova & Sariyska, 2003), while the production of this enzyme by *A. ficuum* was improved when using pH values between 4.0 and 6.0 (Han et al., 1987). Similar to the present study, a pH value of 4.0 was the best for phytase production by *A. niger* NCIM 563 (Soni and Khire, 2007). Vats and Banerjee (2002) analyzed the phytase production by a newly isolated strain of *A. niger* var. *teigham* and found that the optimum growth temperature was 37 °C while the maximum enzyme activity was obtained when the microorganism was grown at 30 °C and at an initial pH of 6.5. Among the various carbon sources used, a combination of glucose and starch (3.0 and 1.0%, respectively) was found as being optimum for phytase production.

It merits emphasizing that, according to the statistical analysis, a pH value of 3.6 and temperature of 32 °C were estimated as being the best conditions to maximize the results of phytase activity by *A. niger* URM 4924. However, increasing the temperature to 32 °C and decreasing the pH to 3.6 will result in a higher cost for the enzyme production, and probably will not justify the little increment that will be obtained in the enzyme activity. Therefore 30 °C and pH 4.0 were considered as optimum values for phytase production by *A. niger* var. *phoenicis* URM 4924.

The results obtained in the present work can be well compared to other studies. For example, Shah et al. (2009) obtained 6.18 U/mL of extracellular phytase by *A. niger* NCIM 563 after 144 h of submerged fermentation using rice bran (1%

w/w). In the present study, higher phytase production (8.09 U/mL) was obtained in a shorter cultivation time (72 h). The phytase production by *Rhizoctonia* sp. and *Fusarium verticillioides* at 28° C for 48 h was also lower than in the present study, with values of 2.72 and 6.11 U/mL respectively (Marlida et al., 2010).

On the other hand, in a study performed by Khan et al. (2004) the synthesis of phytase by *Aspergillus niger*-23 reached 84 U/mL when using ammonium nitrate as nitrogen source, and rice bran (1% *w/w*) as carbon source, after 72 h at 30

°C. Although the lower results, it is noteworthy that the nitrogen source used in the present study is significantly cheaper than that used by Khan et al. (2004), since corn steep liquor is a byproduct of the corn industry. Corn steep liquor is composed of (%) water (46), phytic acid (7.8), non-protein nitrogen (7.5), lactic acid (26), fat (0.4) protein (47) and ash (17) (White and Johnson, 2003); and according to Spier et al. (2010), substrates with considerable content of phytic acid and inorganic reduced phosphorus content (like corn steep liquor) have potential for inducing the production of phytases.

3.2 Biomass content

Besides the enzyme production, the biomass determination is also a fundamental parameter when studying microbial cultures, since metabolic activities such as substrate consumption and product formation are strongly related to the growth rate and the biomass content. Therefore, the biomass formation in the fermentation medium was also taken into account in the present study. Many studies have described the relationship between ergosterol and biomass in filamentous fungi (among which *A. niger* was quoted) (Bindler et al., 1988). However, the physiological status of mycelium changes with age, and this may have effects on ergosterol content (Bermingham et al., 1995). Different researchers proposed a relationship between ergosterol content of grains and the presence of moulds (Schwadorf & Muller, 1989; Torres et al., 1992). The determination of ergosterol has also been valuable in correlating fungal activity and the synthesis of fungal secondary metabolites, such as enzymes, aflatoxins and antimicrobial metabolites (Gourama & Bullerman, 1995b; Saxena et al., 2001). Ergosterol analysis has been successfully applied to cereals for estimation of fungal spoilage (Olsson et al., 2002; Saxena et al., 2001). According to Taniwaki et al. (2006), the ergosterol content per colony varied widely between genus, medium and even within genus, reflecting differences in growth density and membrane composition. For all the above mentioned reasons, the biomass in the present study was estimated by measurement of the ergosterol content.

Statistical analysis of the results obtained in the present study revealed that 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 (Table 2). Although the effects of these variables were closing similar, and both significant at $p<0.01$, the effect of the temperature was slightly higher than the effect of the pH. Such behavior is opposite of that observed for the phytase activity, where the pH showed more significant effects than the temperature. In fact, when comparing the three-dimensional surfaces obtained for these 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 °C), as previously discussed.

Gougouli and Koutsoumanis (2010) reported that *A. niger* AN-YV7 presented faster mycelium growth and shorter lag time at 32.8 °C. Nevertheless, there are evidences suggesting optimum temperature for *A. niger* growth at 30 °C (Vats and Banerjee, 2002), 33 °C (Parra and Magan, 2004), 35 °C (Leong et al., 2006), 37 °C (Marín et al., 1998), 35-37 °C (Pitt & Hocking, 1997) and 30-37 °C (Bellí et al., 2004). Among these evidences, the results obtained by Vats and Banerjee (2002) are the most similar to those found in the current study.

Zeng et al. (2007) evaluated the effect of the culture medium composition in the lanosterol and ergosterol contents of the filamentous fungus *Inonotus obliquus* by submerged fermentation at 26 °C for 192 h. According to these authors, the highest ergosterol concentration occurred at pH 6.5 using rice bran (0.5% *w/w*) as carbon source, and yeast extract (0.4%) as nitrogen source. These results are different of the present study where the greater ergosterol accumulation was found when using pH 4.0 and 1.0% rice bran (*w/w*) in the culture medium.

Finally, a correlation analysis of the data obtained in the present study for phytase activity and ergosterol content (Figure 3) revealed good correlation (second-order fit - $R^2 = 0.9875$) between these two responses, suggesting a close relationship between them. Other studies with different fungal strains reported also correlation between the phytase production and

microorganism growth (Krishna & Nokes, 2001; Spier et al., 2009; Spier et al., 2010). According to these studies, the ergosterol content can be considered a good indicator for biomass.

Figure 3 - Correlation between the responses phytase activity and ergosterol content obtained according to the 2^2 central composite design. Values were correlated through a second order polynomial.
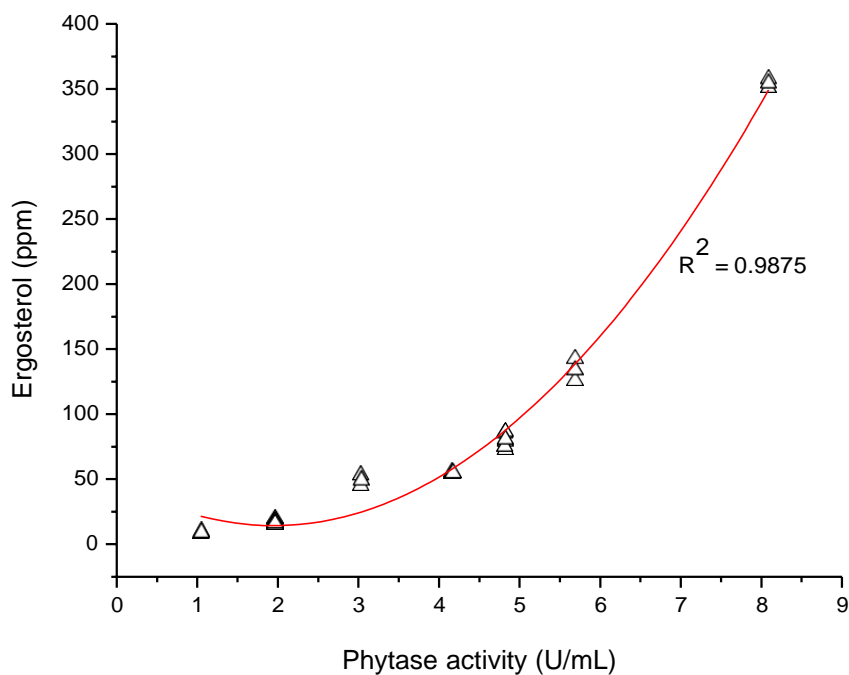
Source: Authors.

3.3 Correlation between dry weight and ergosterol content

Ergosterol is a constituent of membranes in mycelia, spores, and vegetative cells (Newell, 1992). Ergosterol content has been widely used as an estimate of fungal biomass in various environments, e.g., in soil and aquatic systems, because a strong correlation has been found between ergosterol content and fungal dry mass (Pasanen et al., 1999). However, the amount of ergosterol in fungal tissue is not constant. There are interactions between the amount of ergosterol and fungal species, age of the culture, developmental stage (growth phase, hyphal formation, and sporulation), and growth conditions (growth media, pH, and temperature), although no clear trend for the ergosterol content in any of these factors has yet been detected (Pasanen et al., 1999).

Figure 4 shows the results obtained in order to correlate the fungal biomass dry weight with the ergosterol content. These experiments clearly shown correlation between these two responses, for all the substrates tested ($R^2 > 0.9936$). Similar results have also been reported by other authors using different fungal strains. Spier et al. (2009) observed good correlation ($R^2 = 0.9863$) between these variables during the phytase production by solid state fermentation with *A. niger* FS3. Ng et al (2008) found also good correlation between dry biomass and ergosterol content ($R^2 = 0.9645$) during the cultivation of *A. niger* ATCC 24126 in grains and feeds. According to Carvalho et al. (2006), this correlation should be used with care, since it probably depends on the microorganism used and on the cultivation conditions.

In hyphomycetes and ascomycetes, ergosterol concentrations ranging from 2.3 to 11.9 mg of ergosterol/mg of dry mycelium have been reported (Gessner and Chauvet. 1997a), and the ergosterol content for *Aspergillus*, *Penicillium*, *Fusarium*,

Rhizopus, *Cladosporium*, *Candida*, and *Alternaria* species has ranged from 0.4 to 14.3 mg/mg (Schnurer, 1993).

Pasanen et al. (1999) analyzed the ergosterol content in various fungal species and biocontaminated building materials and concluded that there is a good agreement between the ergosterol concentration and viable fungal concentrations was detected in the wood chip ($r > 0.66$, $P < 0.009$) and gypsum board samples ($r > 0.48$, $P < 0.059$), whereas no relationship between these factors was observed in the glass wool samples. The authors emphasized that ergosterol concentration could be a suitable marker for estimation of fungal concentrations in contaminated building materials with certain reservations. Ergosterol analysis has recently been suggested for quantitative monitoring of fungi in solid substrates because of the good agreement between hyphal length and ergosterol contente and between total ergosterol concentration and mycelial mass, even with more than one fungal species present (Newell, 1996; Schnürer, 1993). In this work was found a good correlation between ergosterol content and dry weigth in *Aspergillus niger* var. *phoenicis* URM 4924 under submerged fermentation. This result supports the previous contention that ergosterol is a good indicator of fungal concentrations, particularly for *Aspergillus niger* var. *phoenicis* URM 4924.

However, as discussed by Schnürer (1993), vacuole formation and autolysis of cell contents may occur in aging cultures which would lead to a reduction in weight per unit of length and, consequently, to an increased ergosterol to dry weight ratio. Differences in degree and type of sporulation by the species studied here also clearly play a major part in the variations in the ratios of mycelial dry weight to hyphal length and ergosterol contente to hyphal length observed here. For example, *Aspergillus flavus* and the *Penicillium* species produce relatively little vegetative mycelium relative to conidiophores and conidia, increasing mycelial dry weight and probably decreasing ergosterol in relation to hyphal length. (Nout et al., 1987; Torres et al., 1992).

Biomass dry weight is considered the target value to be indirectly estimated by the diferente methods (Schnürer, 1993; Bermingham et al., 1995; Reeslev et al., 2003). Marín et al., (2005) found a good correlation between colony diameters and biomass dry weight for the different species (Pearson correlation coefficients=0.67–0.99). This suggests that the widespread use of colony diameters as fungal growth estimator for research purposes in single cultures is a good choice as it is easier to be obtained (Reeslev & Kjoller, 1995).

A further explanation for variation in the ergosterol content of fungi is that sterols other than ergosterol can be produced by some species. For example, ergosterol and 22-dihydroergosterol have been reported as the predominant sterol in *A. flavus* (Vacheron and Michel, 1968; Weete, 1973). Other sterols identified as products of deuteromycetous fungi include cerevisterol, ergosterol peroxide, lanosterol, 24-methylenelophenol and 14-dehydroergosterol (Weete, 1973).

Ergosterol concentrations have been reported as 2 to 14 µg mg $^{-1}$ for *Candida albicans*, *Aspergillus fumigatus*, *A. flavus*, *Aspergillus niger*, *Alternaria spp.*, *Cladosporium spp.*, and *Penicillium* spp. cultured on Sabouraud dextrose agar for 2to 4 days (Axelsson et al., 1995). Nevertheless, *Aspergillus niger* var. *phoenicis* URM 4924 presented a higher ergosterol content which was described by the authors cited above (354.09 µg mL^{-1}). This fact can be explained by the use of corn steep liquor as a nitrogen source in the fermentation medium submerged. Several authors studied the effect of the inclusion of corn steep liquor on steroid production by filamentous fungi (Ghanem et al., 1990; Naim et al., 1985; Al-Refai ,1964).

Ghanem et al. (1990) studied the development of cheap culture medium to ergosterol production under optimized conditions by *Penicillium crustosum*. A cheep and economic fermentation medium that favoured maximum streol and ergostrerol yileds was found to contain 3% of corn steep solid, with the formation of ergosterol (62.1%), stigmasterol (17.2%), fungisterol (16.4%), and lanosterol (4.3%). It was also found that a cheap culture medium consisting of 8% treated molasses and 1% corn steep liquor favoured maximum growth and total lipids yields of *Fusarium oxysporum* (Naim et al., 1985).

Al-Refai (1964) demostrated the stimulatory effect of the phenylalanine present in corn steep liquor on mycelial growth, total proteins and sterols produced by *Aspergillus fumigatus*. However, Michalin (1958) and Osman et al. (1969) have explained

stimulatory effects of corn steep liquor on growth and lipid production by fungi as due to the presence of certain sugars, amino acids, keto acids and vitamins.

The production of sterols, more particularly ergosterol; the precursor of vitamin D, from was the aim of many investigators since the isolation of ergosterol from ergot (Ghanem et al., 1990). Several workers (Evans and Gealt, 1985; Shapiro and Geatl, 1982; Ruppol, 1949) reported that the sterol contents of a number of *aspergilli* and *penicilla* varied with different species and with the growth medium employed. Usually two or three different sterols form about 90% of the total sterols and the remaining 10% is represented by intermediate structures formed during synthesis of the predominant species (Ghanem et al., 1990).

Figure 4 - Correlation between dry weight (g) and ergosterol content (ppm) in submerged fermentation with *Aspergillus niger* var. *phoenicis* URM 4924 using phytic acid 1% (w/v), glucose 4 % (w/v), sucrose 4% (w/v), starch 4% (w/v), and corn steep liquor 2% (v/v) as substrate.
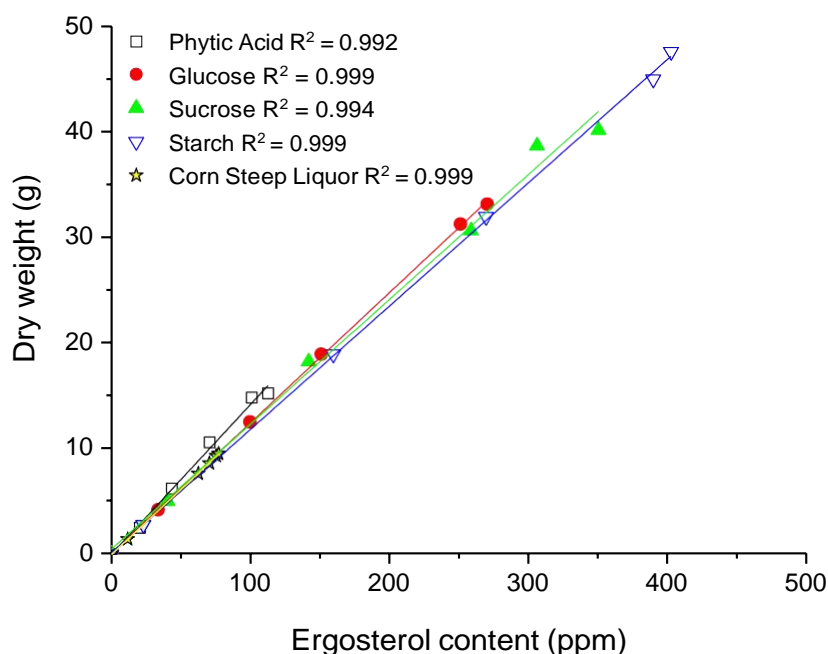
Source: Authors.

Figure 5 reveals that phytase production only occurred when using carbon sources that contained phytic acid in their composition, suggesting that this enzyme behaves as an inductive enzyme or it is only secreted to the culture medium in the presence of phytic acid. In fact, substrates with considerable content of phytic acid and reduced content of inorganic phosphorus have been reported to have potential for inducing the phytases production (Spier et al., 2011). Although thepresence of inorganic phosphorus in the culture media is essential for phytase synthesis, the amount of component must be carefully controlled, since low inorganic-P substrate stimulates phytase synthesis, but an excess of inorganic-P causes a well- known repression of phytase synthesis in many fungi. Phytase synthesis begins when the phosphate in the culture medium is consumed by the cells and then, the repression of phytase synthesis by phosphate is eliminated (Spier et al., 2010).

Figure 5 - Phytase production (U /mL) after 120 h of submerged fermentation with *Aspergillus niger* var. *phoenicis* URM 4924 using different substrates: phytic acid 1% (w/v), glucose 4 % (w/v), sucrose 4% (w/v), starch 4% (w/v), and corn steep liquor 2% (v/v).
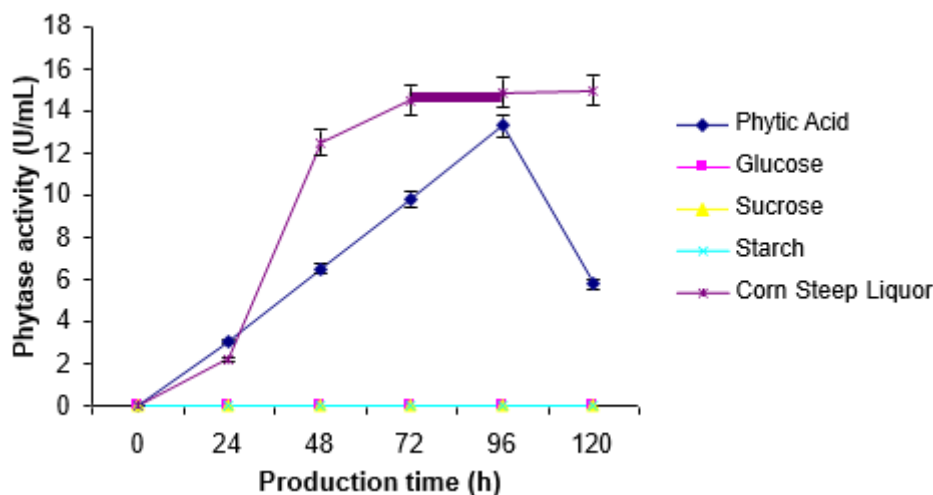
Source: Authors.

4 Conclusion

This study demonstrated the possibility of producing phytase enzyme with elevated values of enzymatic activity under submerged fermentation conditions. Moreover, the use of corn steep liquor brings important advantages for this process, because it is a low cost agro-industrial waste and contains phytic acid in the chemical composition, which contributes in the induction of the fungal phytase production.

The production of phytase by *A. niger* var. *phoenicis* URM 4924 by submerged fermentation was demonstrated to be 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. Establishing the fermentation conditions are therefore of great importance in order to maximize the enzyme production by submerged fermentation, which is desirable for the development an industrial process for phytase production with elevated yields. The phytase produced and secreted into the extracellular medium by *A. niger* var. URM *phoenicis* 4924 is only produced in culture media containing phytic acid in their composition. Ergosterol is a useful indicator of *A. niger* var. *phoenicis* URM 4924 growth in submerged fermentation.

Acknowledgments

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