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EFFECT OF CULTURE MEDIA AND FERMENTATION PARAMETERS ON PHYTASE PRODUCTION BY THE THERMOPHILIC FUNGUS *MYCELIOPHTHORA THERMOPHILA* IN SOLID STATE FERMENTATION

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ABSTRACT

Phytase production by thermophilic filamentous fungi was evaluated in solid state fermentation. A total of twenty strains belonging to *Rhizomucor* spp. (9), *Myceliophthora thermophila* (6), *Thermoascus aurantiacus* (4), and *Paecilomyces variotii* (1) were screened. Sugar cane bagasse was used as support for assessing the effect of different carbon and nitrogen sources, as well as mineral salts. Solid state fermentation parameters (initial moisture, initial pH, aeration rate) were also studied. *Myceliophthora thermophila* showed higher phytase activity and was selected for further studies. Synthetic culture media containing different carbon and nitrogen sources were tested for phytase production by this species in solid state fermentation. *M. thermophila* showed the highest phytase activity after 36 h of fermentation, when grown on a synthetic culture medium (glucose 10 g/L; phytic acid 2 g/L). $(NH_4)_2HPO_4$, $CaCl_2$, and $MgSO_4$ favored high phytase activity in *M. thermophila*. Optimal initial culture conditions for phytase production were determined (pH, 6.0; moisture, 75%; aeration rate, 25 ml/min/column). In comparison with *Aspergillus ficuum*, the phytase activity of *M. thermophila* was 2.5 times higher. Phytase was most active at pH 5.5 and 45-50 C, but it was also active at 70 C. Optimization studies resulted in a 4-fold increase of phytase activity.

Key words: Phytase, thermophilic fungi, solid state fermentation, *Myceliophthora thermophila*, carbon source, nitrogen source, $CaCl_2$, $MgSO_4$.

INTRODUCTION

Thermophilic filamentous fungi play several important roles in nature, human health and industrial applications. Regarding the latter, thermophilic fungi are able to produce various enzymes by submerged fermentation (SmF) or solid sate fermentation (SSF). SSF can be defined as the growth of microorganisms on solid substrates in the absence or near absence of liquid water. In comparison with SmF, SSF is simple, does not require drastic controls, and shows high productivity. Among supports used in SSF, sugar cane bagasse is widely used for protein enrichment or for producing either spores, enzymes or secondary metabolites[19].

Phytic acid is the major storage form of phosphorus in plants, legumes and oil plant seeds. However, it has high capacity to chelate essential minerals (*e.g.*, Ca, Mg, Fe), to bind to amino acids, proteins and sugars, and to inhibit digestive enzymes. Phytic acid is accordingly an antinutritional compound and must be previously hydrolysed by phytases before incorporation in diets used for animal feeding[17].

Phytases (myo-inositol-hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8) are enzymes capable of catalysing the hydrolysis of phytate (myo-inositol-hexakisphosphate) to myo-inositol and phosphoric acid[15]. This is important for many metabolic processes in plants, animals and microorganisms. Phytases are involved in seed germination to liberate phosphorus, myo-inositol, and cations from phytic acid[7]. Filamentous fungi, such as *Aspergillus fumigatus*[10], *Rhizopus* spp.[16], *Mucor racemosus*[18], *Thermoascus aurantiacus*[13], and *Myceliophthora thermophila*[11], can produce phytases in SSF systems. In general, fungal phytases are inducible and extracellular. They have recently received more attention due to their high yields and tolerance to acid conditions for feed production[15, 18]. The addition of phytases to animal feed increases digestibility and animal growth[1]. In fact, nutritional quality of animal feed is upgraded by liberating phosphorus, proteins, and minerals, while phosphorus supplementation, which can cause water pollution, is avoided[16].

In this work, we screened several thermophilic filamentous fungi for phytase production in SSF using sugar cane bagasse as support. Optimal culture conditions and fermentation parameters for phytase production were assessed in a selected strain of *Myceliophthora thermophila*. Enzyme stability under different high temperature and pH conditions was also analysed.

MATERIALS AND METHODS

Microoorganisms. All strains used are deposited at the IAV-IRD collection of thermophilic fungi. Nine strains of *Rhizomucor* spp., six strains of *Myceliophthora thermophila* (Apinis) Oorschot, four strains of *Thermoascus aurantiacus* Miehe, and one strain of *Paecilomyces variotii* Bainier were selected for SSF studies. *Aspergillus ficuum* (Reichardt) Thom & Currie (TECT-66876) was used as a reference strain for comparative analysis. Strains were maintained on potato-dextrose-agar (PDA) slants and stored at 4 C.

Inoculum preparation. Each strain was inoculated in sterile flasks containing PDA and phytic acid (0.25 g/L). Inoculation was made in bulk in order to produce a high quantity of synchronous spores of about the same age. PDA flasks were inoculated at 45 C (before solidification of PDA) with 0.2 ml of spore suspension (10^7 spores/ml). After 7 days incubation at 30 C, 100 ml of sterile distilled water containing 0.01% Tween-80 were added to flasks, which were

shaken vigorously under aseptic conditions. Appropriate dilutions were carried out for optimal inoculum concentration.

Solid state fermentation conditions. SSF was carried out in column fermentors using sugar cane bagasse as solid support. The column bioreactor used was composed of a small glass column, 4 cm in diameter and 20 cm in length, with an effective reaction volume of 250 ml. Sugar cane bagasse was sieved to reach a particle size of 0.70-2 mm, washed 2-3 times with distilled water, adjusted to 50% moisture with distilled water, and sterilized at 121 C for 20 min. For 100 g of sugar cane bagasse (dry weight), 100 ml of nutritive solution (glucose, 10 g/L; phytic acid, 2 g/L; KH_2PO_4, 0.1 g/L; NH_4NO_3, 2 g/L; $CaCl_2$, 1 g/L; $MgSO_4.7H_2O$, 1 g/L; pH= 6) were mixed with 100 ml of 2 x 10^8 spore suspension (equivalent to 2 x10^7 spores/g of initial dry matter of support). Final moisture was 77%. Fermentation was carried out at 45 C for 5 days. Aeration of columns was performed by injecting humid air at 25 ml/min in each column**.** The concentration of KH_2PO_4 (0.1 g/L) had been previously optimized for high phytase activity.

Dry weight determination. Two grams of fermented product were dried in an oven at 105 C for 24 h to assess moisture, before and after SSF.

Estimation of glucose and soluble proteins. Proteins were determined by the Bradford method[2] using bovine serum albumin as a standard. Residual glucose was determined according to Dubois *et al*.[4] using glucose as a standard.

Enzyme extraction. The fermented solid material was compacted using a laboratory manual press. All samples obtained were stored at -18 C for enzyme assays. Assays were carried out in triplicate.

Table 1. Culture media used for studying the effect of substrate composition on phytase production by *Myceliophthora thermophila* growing at 45 C.

Culture media (g/L)	A	B	C	D	E	F	G	H	I	J	K	L	M
Carbon sources													
Glucose	10	0	0	0	0	10	10	10	10	10	10	10	10
Sucrose	0	10	0	0	0	0	0	0	0	0	0	0	0
Starch	0	0	10	0	0	0	0	0	0	0	0	0	0
Phytic acid	2	2	2	10	2	2	2	2	2	2	2	2	2
Myo-inositol	0	0	0	0	10	0	0	0	0	0	0	0	0
Nitrogen sources													
Peptone	0	0	0	0	0	2	0	0	0	0	0	0	0
$(NH_4)_2HPO_4$	0	0	0	0	0	0	2	0	0	0	0	0	0
KNO_3	0	0	0	0	0	0	0	2	0	0	0	0	0
NH_4NO_3	2	2	2	2	2	0	0	0	2	2	2	2	2
Mineral salts													
$CaCl_2$	1	1	1	1	1	1	1	1	1	1	0	0	0
NaH_2PO_4	0	0	0	0	0	0	0	0	0	0	1	0	0
$MgSO_4 \cdot 7H_2O$	1	1	1	1	1	1	1	1	1	0	0	1	0
NaCl	0	0	0	0	0	0	0	0	0	0	0	0	1
KH_2PO_4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table 2. Phytase production by seven, out of 20, thermophilic filamentous fungi studied, growing at 45 C, 77% initial moisture, initial pH= 5.5, and 25-30 ml/min aeration rate.

Thermophilic fungi	Strain code	Phytase (EU/ml)	
		24 h	48 h
Rhizomucor spp.	Lomy 586	15	14
Rhizomucor spp.	Lomy 587	24	31
Rhizomucor spp.	Lomy 594	33	24
Myceliophthora thermophila	Lomy 603	32	37
Myceliophthora thermophila	Lomy 633	46	38
Myceliophthora thermophila	Lomy 710	29	38
Myceliophthora thermophila	Lomy 713	50	42

EU= Enzymatic units.

Phytase assay. Enzyme activity was assessed according to the method of Engelen *et al.*[5]. One unit of phytase is defined as the amount of enzyme capable of releasing one micromole of phosphorus per min and per ml of fermented juice under assay conditions.

Effect of substrate composition on phytase production of M. thermophila. Several culture media were tested as shown in **Table 1**, containing different standard carbon sources (A, B, C, D, E), nitrogen sources (F, G, H, I), and mineral salts (J, K, L, M). The effect of every constituent on phytase production was studied after 36 h of SSF. Incubation temperature was 45 C.

Effect of fermentation parameters on phytase production. The influence of solid material, initial moisture (60%, 65%, 70%, 75%, 80%), see SSF conditions above, initial pH of the culture medium (4.0, 5.0, 6.0, 7.0), and culture aeration rate (ml/min per column: 20, 25, 30, 35) on phytase production was studied**.** After optimization of phytase production by *M. thermophila*, enzyme production was compared with that of *Aspergillus ficuum* (reference strain) cultivated under the same fermentation conditions. *A. ficuum* was incubated at 28 C. Stability of phytase activity was tested in enzymatic assays at different pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5), and at various temperatures (35 C, 40 C, 45 C, 50 C, 55 C, 60 C, 65 C, 70 C, 75 C, 80 C, 85 C).

Table 3. Phytase production by *Myceliophthora thermophila* on different culture media studied growing in SSF at 45 C, 77% initial moisture, initial pH= 5.5, and 25-30 ml/min aeration rate.

Notation*	Culture media	Phytase (EU/ml)
Carbon sources		
A	Glucose	165 ± 6.3
B	Sucrose	28 ± 2.2
C	Starch	73 ± 4.1
D	Phytic acid	0
E	Myo-inositol	0
Nitrogen sources		
F	Peptone	106 ± 4.8
G	$(NH_4)_2HPO_4$	156 ±6.1
H	KNO_3	142 ±11.4
I	NH_4NO_3	79 ± 3.7
Mineral salts		
J	$CaCl_2$	163 ± 1.9
K	NaH_2PO_4	11 ± 0.2
L	$MgSO_4 \cdot 7H_2O$	116 ± 6.8
M	NaCl	19 ± 0.9

* Codes correspond to those shown in Table 1. EU= Enzymatic units.

RESULTS AND DISCUSSION

Phytase production by thermophilic filamentous fungi. Strain selection was carried out according to their mycelial growth in solid culture media containing phytic acid as a sole carbon source. The screening for phytase production showed that seven strains tested produced phytase (**Table 2**). These strains belonged to *Myceliophthora thermophila* (4) and *Rhizomucor* spp. (3). *M. thermophila* Lomy 713 was selected for optimization studies

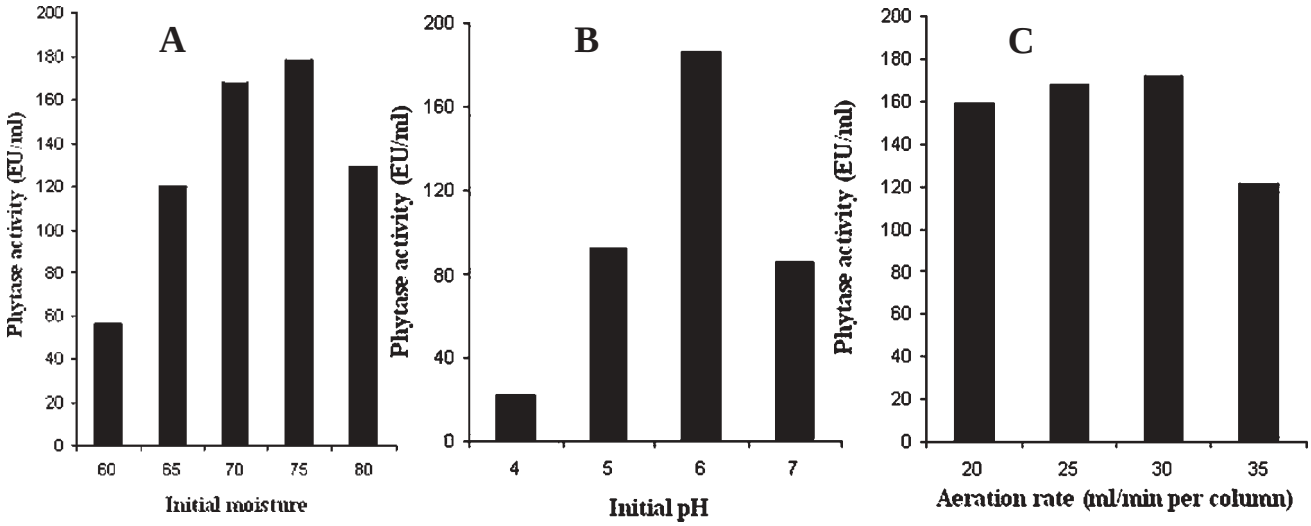

Fig. 1A-C. Influence of initial moisture (**A**), initial pH (**B**), and aeration rate (**C**) of culture media on phytase production by *Myceliophthora thermophila* growing at 45 C, 75% initial moisture, initial pH= 6, and 25 ml/min aeration rate. EU= Enzymatic units.

of phytase production on the basis of higher enzyme production, and high production of conidia.

Effect of culture media on phytase production by M. thermophila. A preliminary study (data not shown) showed that the concentration 0.1 g/L of KH_2PO_4 gave a maximum of phytase activity, although it has previously been reported that the presence of phosphate has a repressive effect on phytase production[20]. The addition of phytic acid (0.25 g/L) to PDA medium to produce inoculum, also had a positive effect on phytase production. Glucose as a sole carbon source in culture media showed the highest phytase activity (**Table 3**), as it is simple and easily consumed by microorganisms. Glucose allowed a sufficient quantity of biomass for phytic acid dephosphorylation. Culture media with starch and sucrose showed phytase activity, but significantly lower than media with glucose. Glucose, starch and sucrose are favorable carbon sources for phytase production by *M. thermophila*[4] and *Rhizopus oligosporus*[16]. The phytic acid and myo-inositol when used as carbon sources did not promote phytase production by *M. thermophila*.

As reported by others, nitrogen sources influenced phytase production[16, 18]. Phytase activity was the greatest in media with $(NH_4)_2HPO_4$, followed by KNO_3, peptone, and NH_4NO_3 (**Table 3**). Phosphate may be stimulating the growth of *M. thermophila* which in turn is then capable of attacking phytic acid for phophorus needs.

$CaCl_2$ was an important salt for phytase production by *M. thermophila*. $MgSO_4$ also had a positive effect on phytase activity (**Table 3**). These salts also effect the pH[9]. The addition of NaH_2PO_4 and NaCl showed low values of phytase activity. Oh *et al.*[14] showed a Ca-dependent catalytic activity in the phytase produced by a strain of *Bacillus amyloliquefaciens*. Salts containing $Fe^{2,3+}$, Zn^{2+}, Mg^{2+} and Ca^{2+} also affected phytase activity by *Rhizopus oligosporus*. *R.*

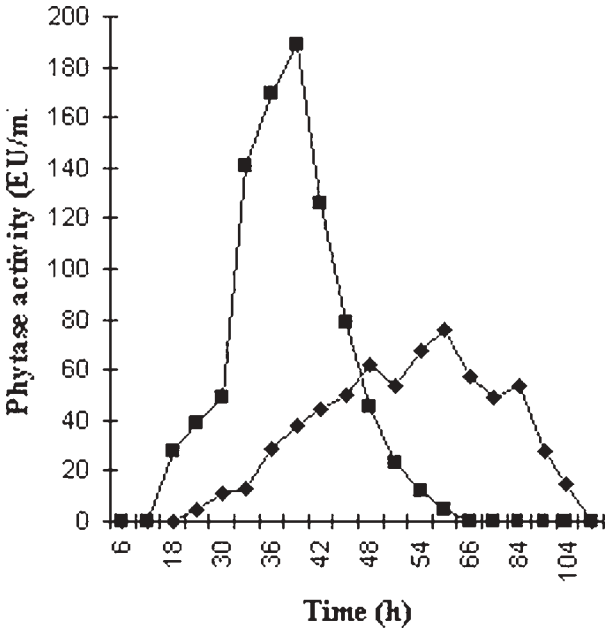

Fig. 2. Phytase activity by fungi studied. ■ = *Myceliophthora thermophila*; ◆ = *Aspergillus ficuum*. EU= Enzymatic units.

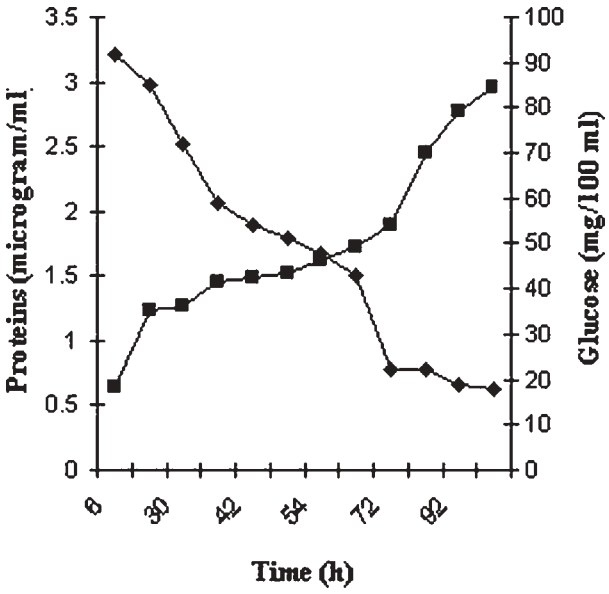

Fig. 3. Evolution of intracellular proteins and glucose during phytase production by *Myceliophthora thermophila*. ■ = Proteins; ◆ = Glucose. EU= Enzymatic units.

oligosporus phytase activity was highest in the presence of Mg^{++}. However, in the presence of Ca^{++}, *R. oligosporus* phytase activity was lower than with the other ions[3].

Effect of fermentation parameters. Optimal initial moisture was important for maximizing phytase production. A moisture content of 70-75% resulted in the highest enzyme production, reaching up to 178 enzymatic units (EU/ml) at 75% (**Fig. 1A**).

Initial pH was also a key parameter for phytase production. In this study, the optimal initial pH to produce phytase is shown in **Fig. 1B**. An initial pH 6 resulted in the highest phytase activity. Gargova and Sariyska[6] and Kim *et al.*[8] also found that the initial pH of the culture media had influence on phytase production by *A. niger* 307 and *Aspergillus* sp. 5990. Phytase was active from pH 3 to 8; it was optimum for *A. niger* phytase at pH 5 and for *Aspergillus* sp. phytase from pH 6 to 7.

Aeration allowed the removal of heat to regulate optimal temperature of growth. An aeration rate of 25 ml/min per column permitted to obtain enough quantity of experimental fermented juice for enzyme assay (**Fig. 1C**). However, when the aeration rate was high (35 ml/min), *M. thermophila* growth was affected negatively which influenced phytase production.

Characterization of phytase activity by M. thermophila. This species showed high phytase activity with glucose as sole carbon source after 36 h at 45 C (**Fig. 2**). Effects of pH and temperature on enzyme stability and thermostability were studied. *M. thermophila* showed higher phytase activity than *Aspergillus ficuum* (**Fig. 2**). *M. thermophila* phytase activity was 4 times higher than the reference strain of *A. ficuum*. Likewise, peak values of phytases production were reached in shorter time by *M. thermophila* (36 h) in comparison with *A. ficuum* (60 h). Enzyme production was correlated with consumption of glucose and

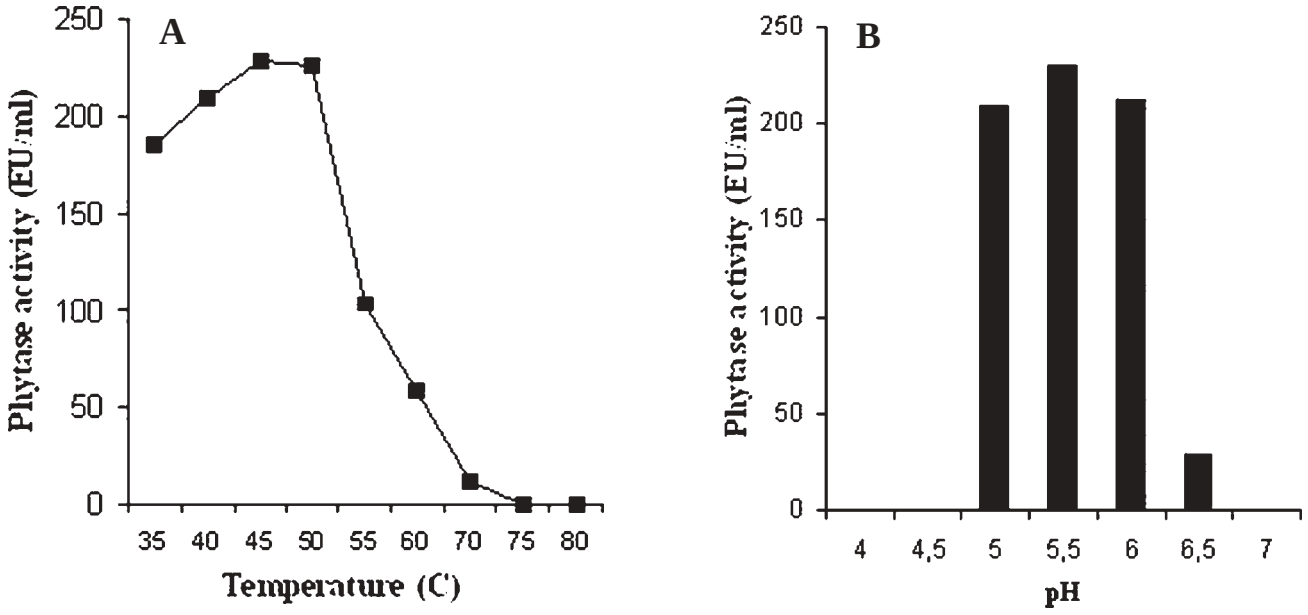

Fig. 4. Stability of phytase extracts from *Myceliophthora thermophila* growing at 75% initial moisture, and 25 ml/min aeration rate, and at different temperature (**A**) and pH (**B**) conditions. EU= Enzymatic units.

the increase of intracellular proteins by *M. thermophila* (**Fig. 3**). The highest phytase activity was reached at 45 C and 50 C (**Fig. 4A**). Thereafter, phytase activity decreased with increasing temperatures. The lowest enzyme activity was recorded at 70 C (12 EU/ml). There was no phytase activity at temperatures above 70 C. It has been reported that *A. fumigatus* produces phytase at an optimum stability temperature of 70 C, keeping enzyme activity up to 85 C[12].

Phytase activity was significantly influenced by pH (**Fig. 4B**). Maximum enzyme activity occurred at pH 5.5, while no enzymatic activity was recorded at pH 4, 4.5, and 7. Phytase was active at pH 5, 5.5, 6 and 6.5. These results are in agreement with those from Mitchell *et al.*[11] who showed that *M. thermophila* had maximum relative activity at a pH between 5.5 and 6.5. The effect of pH and temperature on phytase activity has previously been studied in several filamentous fungi showing wide variation in optimum values of strains[15].

In conclusion, *Myceliophthora thermophila* and *Rhizomucor* produced phytase when grown in SSF, using sugar cane bagasse as support. *M. thermophila* was the best phytase producer and was selected for further studies. *M. thermophila* produced the highest phytase activity after 36 h at 45 C, when grown on a synthetic culture medium (glucose 10 g/L; phytic acid 2 g/L). The best initial conditions found for phytase production were: pH 6.0, moisture of 75%, and an aeration rate of 25 ml/min/column. Carbon and nitrogen sources significantly influenced phytase activity by *M. thermophila*. Glucose was better than other carbon sources studied showing the highest phytase activity. Starch and sucrose also resulted in phytase activity. $(NH_4)_2HPO_4$, along with glucose, $CaCl_2$ and $MgSO_4$, increased phytases activity up to 165 enzymatic units (EU/ml) at optimum temperature and pH. Optimization studies

resulted in a 4-fold increase of phytases activity. This fungus is particularly interesting for phytases production with high activity in comparison with the reference strain and with other fungi cited in the literature. Phytase had an optimum temperature of 50 C, and a pH of 5.5. These conditions will be used for scaling up phytase production.

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