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SCREENING OF FILAMENTOUS FUNGI FOR PRODUCTION OF ENZYMES OF BIOTECHNOLOGICAL INTEREST

Luis Henrique S. Guimarães[1]; Simone C. Peixoto-Nogueira[2]; Michele Michelin[1]; Ana Carolina S. Rizzatti[1]; Valéria C. Sandrim[3]; Fabiana F. Zanoelo[1]; Ana Carla M.M. Aquino[1]; Altino B. Junior[2]; Maria de Lourdes T.M. Polizeli[1]*

[1]Departamento de Biologia - Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - USP; [2]Departamento de Bioquímica e Imunologia, Pós Graduação em Bioquímica - FMRP/USP; [3]Instituto de Química, Pós Graduação em Biotecnologia, Araraquara - UNESP

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ABSTRACT

Many enzymes produced by fungi have relevant biotechnological applications in several industrial areas. The purpose of this study was to collect and isolate filamentous fungi from soil and humus, plants and sugar cane bagasse of different regions of the São Paulo state. Forty isolates were examined for their ability to produce xylanase, glucose-oxidase, alkaline phosphatase, acid phosphatase, phytase, pectinase and amylase. Among these, twenty three isolates exhibited enzymatic potential. The xylanases produced by two of these isolates (*Aspergillus caespitosus* and *A. phoenicis)* showed good potential for pulp bleaching. Among seventeen isolates, at least three produced high levels of glucose-oxidase, being *Rhizopus stolonifer* and *A. versicolor* the best producer strains. *A. caespitosus*, *Mucor rouxii*, and nine others still not identified were the best producers of phosphatases in submerged fermentation. Pectinase was best produced by IF II and C-8 belong *R. stolonifer*. Significant levels of amylase were produced by *Paecilomyces variotii* and *A. phoenicis.* A remarkable enzyme producer was *Rhizopus microsporus* var. *rhizopodiformis* that produced high levels of amylase, alkaline and acid phosphatases, and pectinase. Some morphological structures of this fungus were illustrated using light microscopy (LM) and scanning electron microscopy (SEM). This study contributes to catalogue soil fungi isolated in the state of São Paulo, and provides additional information to support future research about the industrial potential of these microorganisms that may produce enzymes and, eventually, also secondary metabolites with anti-microbial or anti-parasitic activities.

Key words: phosphatase, xylanase, glucose oxidase, pectinase, amylase, *Rhizopus microsporus* var. *rhizopodiformis*, fungi

INTRODUCTION

Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential. These enzymes are applied in the industrialization of detergents, starch, drinks, food, textile, animal feed, baking, pulp and paper, leather, chemical and biomedical products. The use of starch degrading enzymes was the first large-scale application of microbial enzymes in the food industry (1,2). Two enzymes carry out the conversion of starch to glucose, namely: (i) α-amylase, that cuts the large α-1,4-linked glucose polymers into shorter oligomers and (ii) glucoamylase that hydrolyses the oligomers to glucose. Amylases have applications in food, detergents, drinks, animal feed and baking (3,4). Pectinases are used in the preparation of wine and fruit juices (5,6). Fruit cell wall needs to be broken down to improve juice liberation. Pectins are polymeric substances in the fruit lamella and cell walls. Pectinases were classified according to its specificity to attack pectin, pectic acid and oligo-D-galacturonate. Pectin pectinesterases, depolymerizing enzymes and protopectinase are the three major types of pectinases. Presently, pectinases have been used in textile industries (5,6).

*Corresponding Author. Mailing address: Departamento de Biologia - FFCLRP - USP - Av. Bandeirantes, 3900 Monte Alegre cep: 14040-901 Ribeirão Preto, SP - Brasil. Tel.: (16) 3602-4680 ou (16) 602-3666. E-mail: polizeli@ffclrp.usp.br

Phosphatases hydrolyze esters and anhydrides of phosphoric acid (7). These enzymes are involved in various biological processes, e.g. in cell cycle, differentiation and others. These enzymes are classified as: i) alkaline phosphatases; ii) high molecular mass acid phosphatases; iii) low molecular mass acid phosphatase; iv) purple acid phosphatases and v) protein phosphatases (8). Alkaline phosphatases are used in enzyme-linked immunoabsorbent assays (ELISA), nonisotopic probing, blotting and sequencing systems (9). Industrial application of acid phosphatase is limited, but phytase, a type of acid phosphatase (EC 3.1.3.8) is used in animal feed (10). These enzymes catalyze the release of phosphate from phytic acid, the major phosphorus storage form in cereal grains, legumes and oilseeds. Pigs and poultry lack phytase and excrete large amounts of phosphorus in the form of phytate into the environment, resulting in pollution by this element.

Xylanases have potential application in food, feed, paper, pulp and textile industries (11). These enzymes degrade plant fibers made of xylan hemicellulose producing xylose monomers. One of the most important xylanase applications is the pretreatment of pulps, prior to bleaching, in pulp and paper industries (12). These enzymes release lignin fragments by hydrolyzing residual xylan and the pretreatment with xylanase reduces the usage of chlorine as the bleaching agent. Not less important is the use of xylanases for bread-making and beer production (13,14). On the other hand, in the industry of animal feed, the xylanases can be added to the ration increasing the intestinal absorption of nutrients (15).

Glucose oxidase (GOD), catalyzes the oxidation of D-glucose to D-gluconic acid and hydrogen peroxide in the presence of oxygen and water. Its major use is in the quantification of free glucose (16) but it has also been used for labeling antibodies used in the detection of tumor marker and viral antigens (17). In this study we have explored the biopotential of several filamentous fungi, isolated from soil samples, for the production of some of the above referred enzymes. This study also contributes to cataloguing soil fungi isolated in the state of São Paulo, in order to support future research about industrial application enzymes and eventual secondary metabolites with potential anti-microbial and anti-parasitic activities.

MATERIALS AND METHODS

Microorganisms and maintenance

The fungi used in this study were collected from samples of soil or decomposing organic material from several regions of the State of São Paulo (Brazil), as established by the SinBiota – FAPESP program. The best enzyme producer fungi were identified by the André Tosello Foundation, Campinas, SP. The organisms were maintained on slants of solid 4% oatmeal baby food (Quaker) medium or in slants of Vogel complete solid medium (18) with 2% glucose as the carbon source.

Culture conditions

The enzymes were obtained from cultures in liquid medium of composition adequate to the type of enzymatic activity of interest. To obtain phosphatases the fungi were grown in Adams media (19) with 2% glucose for 48 h. For amylases was used the CP (Carvalho Peixoto) medium (20) with 1% starch, and the fungi were grown for 120 h. For GOD (Glucose oxidase) production the fungi were incubated in medium supplemented with 8% glucose for 72 h (21). Xylanase was obtained in SR (Segato Rizzatti) medium (22) supplemented with 1% birchwood xylan for 72 h. All flasks were inoculated with a spore suspension to give a final concentration of 4×10^5 spores/ml of liquid medium, pH 6.0, at 40ºC, under orbital agitation (100 rpm). Pectinase activity was determined using solid Vogel medium supplemented with 1% pectin (pH adjusted to 8.0). After 5 days of incubation at 40ºC the activities were determined as described.

Production of enzymes by culturing in liquid medium

Mycelia obtained in liquid media were harvested by filtration, rinsed with distilled water, blotted on filter paper and stored at -15ºC until use. The mycelial mass was homogenized by grinding in a mortar with acid-washed sand (sea sand washed with sulfochromic solution and neutralized with water), at 4ºC, resuspended in distilled water and utilized for intracellular activity measurement. The filtrate was dialyzed against the buffer appropriate for each enzyme, in order to measure the extracellular activity.

Enzymatic assays and protein determination

Acid and alkaline phosphatase activities were assayed with *p*-nitrophenylphosphate as substrate as previously described (23,24). One unit was defined as the amount of enzyme that releases 1 nmol of *p*-nitrophenolate per min under the assay conditions. Phytase activity was determined using phytic acid as substrate and the inorganic phosphate (Pi) liberated was quantified as Heinonen and Lahti method (25). One unit was defined as the amount of enzyme that releases 1 nmol of inorganic phosphate per min under the assay conditions. Xylanase and amylase activities were assayed by measuring the reducing groups released Miller (26). For amylase, the reaction mixture consisted of 250 µl of 1% (m/v) starch in sodium acetate buffer 100 mM, pH 5.0 and 250 µl of enzyme. The reaction mixture was incubated at 60ºC for 15 min and the reducing sugars formed were quantified by spectrophotometer at 540 nm. One unit was defined as the amount of enzyme that releases 1 µmol of glucose per min under the assay conditions. Xylanase activity was determined according to Rizzatti *et al.* (22) using 1% birchwood xylan as substrate. The reaction mixture was incubated at 50ºC for 15 min. One unit was defined as the amount of enzyme that releases 1 µmol of xylose per min under the assay conditions. Pectinase activity was determined according to Paterson and Bridge (27), utilizing agar medium supplemented with 0.1% citric pectin as

substrate in Petri plates, as described previously. After microorganism growth, a solution of malic acid 0.1M was added and incubated for 1 hour, at room temperature. The solution was removed and 0.01% ruthenium red was added and incubated by 2 days, at 4ºC. The plates were washed with distilled water for 1 hour and the halos revealing activity were measured. For GOD activity assay the reaction mixture was composed of 0.01 M D-glucose, 0.02% o-dianisidine in phosphate buffer pH 7.0, 25 µg of peroxidase and 20 µl of enzyme solution (GOD). It was incubated for 30 min at 40ºC. The reaction was stopped by adding 0.5 ml of HCl 4N, and quantified at 490 nm (28). One unit was defined as the amount of enzyme that liberates one mmol of glucose per minute. Protein was determined according to Lowry (29), using bovine serum albumin as standard, and measured at 620 nm.

Reproducibility of results: All results are the means of at least three (n=3) independent experiments.

RESULTS AND DISCUSSION

Approximately forty filamentous fungi were isolated from various regions of São Paulo State, such as Ilha Solteira, Pereira Barreto, Marília, Ribeirão Preto, Luiz Antônio and Pirassununga, from soil, humus, mushroom compost and several plants from the cerrado, in order to carry out a screen of potential biotechnological enzyme producers. Some of these fungi have been identified, but others are reported here just by the isolation number.

Among sixteen fungi tested the IF-2, *Paecilomyces variotii*, *Rhizopus microsporus* var. *rhizopodiformis*, *Aspergillus phoenicis* and C-9A were the best amylase producers (Table 1). The production of glucoamylase and α-amylase from *Rhizopus microsporus* has been recently reported (20). Seventeen isolates were tested for glucose oxidase (GOD) production (Table 1).

Table 1. Amylase and glucose oxidase production by filamentous fungi.

Fungus	Amylase		Glucose oxidase	
	Protein (total mg)	Extra Activity (total U)	Protein (total mg)	Intra Activity (total U)
A-1	2.6	32.5	19.4	536
A. caespitosus	1.3	2.2	7.1	0
A. flavus	4.7	54.8	n.d.	n.d.
A. niger	n.d.	n.d.	7.1	0
A. phoenicis	2.2	118.8	10.5	0
A. versicolor	5.1	6.3	3	7523.8
Chaetomium thermophilum	4.6	97	n.d.	n.d.
C. lindemuthianum	n.d.	n.d.	11.1	143
C-7B	4.8	91	8.7	2297
C-8	n.d.	n.d.	5	0
C-9A	1.4	102.5	n.d.	n.d.
C-9D	1.2	97.5	n.d.	n.d.
H. grisea var. *thermoidea*	2.9	57.3	9.5	607
IF I	4.1	86.8	8.7	274
IF II	5.3	160.8	2.7	95.2
JII-1	n.d.	n.d.	19.4	95.2
A. niveus	2.2	88.2	n.d.	n.d.
M. rouxii	n.d.	n.d.	1.5	n.d.
N. crassa	n.d.	n.d.	7.4	95.2
P. variotii	1	130.0	6.3	310
R. microsporus var. *rhizopodiformis*	0.8	110.8	3.8	714.3
R. stolonifer	n.d.	n.d.	5.43	8964
S-1	1.6	8.8	9.2	274

n.d. not determined. Three fungi were collected from Ilha Solteira, SP: A-1 (*Arachis sp)*, IF I and IF II (*Inga heterophyla).* Ten fungi were collected from Ribeirão Preto, SP: *A. caespitosus (Solanium tuberosum), A. niveus (Mangifera hispida* L.), *A. phoenicis (Sacharum officinarum* bagasse*), A. versicolor* (collection), *Chaetomium thermophilum* (collection), *H. grisea* var. *thermoidea* (collection), JII-1 (soil and humus), *R. stolonifer (*soil and humus), *M. rouxii* (collection) and *N. crassa* (collection). Two fungi were isolate from Luis Antônio, SP: *A. flavus* (mushroom compost) and *A. niger* (*Eucaliptus*). Five fungi were collected from Marilia: *C. lindemuthianum* (*Phaseolus vulgaris L.),* C-7B (soil), C-8 (soil), C-9A (soil) and C-9D (soil). Two fungi were collected from Pereira Barreto, SP: *P. variotii* (*Psidium guajava* L.) and S-1 (*Glycine indica* L.). *R. microsporus* var. *rhizopodiformis* was collected from *Kielmeyera sp* in Pirassununga . The type of colleted sample was described between parentheses.

Among these strains, *A. versicolor*, C7-B and *Rhizopus stolonifer* produced de highest levels of intracellular glucose oxidase. In the literature there is no information about species from the *Rhizopus* genus as GOD producers, but the genus *Aspergillus* is considered one of the best producers of GOD (30), and the gene sequence and expression has been studied by Malherbe (31).

Alkaline phosphatases from thermophilic and thermotolerant fungi, such as *Scytalidium thermophilum* (24), *Aspergillus caespitosus* (23,32) and *Humicola grisea* var. *thermoidea* (33) have been characterized in our laboratory. These phosphatases are in general more termostable than those from mesophilic species. Table 2 shows the production of intra- and extracellular alkaline phosphatases, intracellular acid phosphatase and phytase, by various filamentous fungi. Some strains produced high levels of intracellular alkaline phosphatase, as for instance *A. caespitosus*, C-9A, JI-2, JII-1, JII-2, *R. microsporus* var. *rhizopodiformis* and T-I. But only A-2, *Mucor rouxii* and P-1 secreted high levels of alkaline phosphatase into the medium. Acid phosphatase was present only in intracellular materials and some fungi were more effective, such as *R. microsporus* var. *rhizopodiformis*, JII-2, JI-P, or C-9D. Phytase activity was significantly detected in cultures of *A. caespitosus*, *A. niger*, C-7A, JI-P, JII-2, P-1 and *R. microsporus* var. *rhizopodiformis.*

Table 2. Production of phytases, alkaline and acid phosphatases by filamentous fungi.

Fungus	Protein (total mg)	Alkaline Phosphatase		Acid phosphatase	Phytase
		Intra (total U)	Extra (total U)	Intra (total U)	Intra (total U)
A-1	8.01	1540	90	n.d.	n.d.
A-2	14.35	2110	3900	200	n.d.
A. caespitosus	29.40	6270	470	580	1612
A. niger	16.80	660	450	1130	2340
C-7A	14.15	780	500	320	2028
C-7B	31.40	3150	370	360	0
C-8	33.40	460	220	1500	1170
C-9A	17.50	4390	230	240	455
C-9C	11.00	1270	40	1260	0
C-9D	32.40	230	250	3760	0
C. lindemuthianum	8.40	860	120	n.d.	n.d.
F2P	1.13	90	60	2720	0
JI-2	11.80	4290	20	810	325
JI-3	7.50	1720	0	590	325
JI-P	10.05	2000	0	3830	2054
JII-1	13.30	3920	20	2740	n.d.
JII-2	9.45	3920	0	3510	2626
IF I	10.30	1510	0	2640	676
IF II	7.07	2015	200	0	0
ISA I	9.66	180	340	2510	806
LH-4	13.57	1830	160	1380	n.d.
Mucor rouxii	5.29	3000	1350	880	n.d.
P-1	13.17	2110	1930	80	2314
R. stolonifer	13.30	920	720	240	n.d.
R. microsporus var. rhizopodiformis	11.80	7410	330	4390	4654
S-1	8.84	1740	600	0	949
T-I	10.60	5680	100	500	0

n.d. = not determined. Seven fungi were collected in Ribeirão Preto, SP: A-2 (soil), F2P (*Ficus carica),* JI-2 (soil and humus), JI-3 (soil and humus), JI-P (soil and humus), JII-2 (soil and humus), LH-4 (soil and humus). C-9C was collected from soil of Marilia, SP. ISA I was collected from soil and humus of Ilha Solteira, SP. P-1 was collected from soil Pirassununga, SP. T-I was collected from *Triparis* sp of Pereira Barreto, SP. The type of colleted sample was described between parentheses.

Acid phosphatase with phytase activity from *A. caespitosus* has been previously characterized in our laboratory (34), moreover, an alkaline phosphatase from the same fungus has been successfully used in cloning protocols (32).

The screening for xylanase activity was performed in eighteen isolates. All fungi tested produced intra- and extracellular xylanases. However, the extracellular levels were highest than intracellular levels. ISA-1 and IF I, among others, were good producers of extracellular xylanases (Table 3). Two thermotolerant *Aspergillus* strains, identified as *A. caespitosus* and *A. phoenicis*, grown at 40ºC, produced the highest xylanase activity thermostable at 50-55ºC. The optimum pH was 6.5 and 3.5 for *A. caespitosus* and *A. phoenicis*, respectively. The xylanases produced by *A. caespitosus* showed good performance for paper pulp bleaching (35).

The screening for pectinase production was carried out with ten isolates. Among them, C-8, IF II, *R. microsporus* var. *rhizopodiformis* and *R. stolonifer* were classified as the best producers, producing clear halos in qualitative cup-plate assay corresponding at 19, 18, 17 and 16 mm, respectively. Strains with halos between and 13-9 mm, as *T. reesei*, *N. crassa*, *A. niger*, and *Colletotrichum* were classified as good producers. *Humicola grisea* var. *thermoidea* and *Mucor rouxii* produced halos smaller than 10 mm, indicating weak pectinase production (not shown).

Among the isolates, the morphological structures of *Rhizopus microsporus* var. *rhizopodiformis* were observed using light microscopy (LM), and scanning electron microscopy (SEM) (Fig. 1). There is no information or illustrations about this fungus in the literature, only some description is reported by Schipper and Stalpers (36). This species supports growth up to 45ºC, and it shows dark, gray brownish colonies. The spores (Fig. 1A) are (sub-)globose, with maximum size 5.7 µm of diameter/length, homogeneous, with slight spinulose ornamentation with ridges. Simple rhizoids (Fig. 1A), slightly or not pigmented, are a characteristic of this species. The ornamentation of sporangiophores (Fig. 1A, B, D) is a constant, with sporangia (Fig. 1B, D) (sub-)globoses and apparently homogeneous. The sporangiophore shows stolons (Fig. 1A) up to 500 µm in length, brownish, some times grouped. Zigospore (Fig. 1C) is a structure that denotes sexual reproduction, main characteristic of the zygomycetae.

In conclusion, forty isolates of filamentous fungi were obtained from soil and humus, plants and sugar cane bagasse of different regions of São Paulo state. Among these fungi, twenty three exhibited enzymatic potential for industrial uses. Remarkable production was verified for *R. microsporus* var. *rhizopodiformis*, but *A. caespitosus*, *A. phoenicis* and *R. stolonifer* also were excellent producers of the enzymes studied. Some studies have already been carried out with fungi from our collection, but we consider that these new isolates may have more potential for industrial uses, for instance IF II and C-9A.

Table 3. Xylanase production by filamentous fungi.

Fungus	Protein (total mg)	Xylanase	
		Intra (total U)	Extra (total U)
A. versicolor	0.12	5.55	n.d.
A. niveus	3.55	24.55	420.47
A. caespitosus	3.15	39.35	490.77
A. phoenicis	4.36	208.8	907.4
A. niger	3.51	129.15	753.8
P. variotii	3.98	38.45	100
T. reesei	3.09	5.1	109.06
T- II	2.32	56.95	348.3
A-1	2.65	63.9	473.4
ISA I	3.05	108.8	1407.33
C-10 gray	2.35	31	247.18
JI-2	3.86	49.05	381.71
JII-1	5.48	128.7	743.4
IF I	4.33	143.5	1255.6
IF II	2.06	7.4	81.4
V-1	3.01	16.2	83.4
V-2	2.69	56.5	416.6
N. crassa	2.24	15.3	101.8

n.d. = not determined. Four fungi were collected from Ribeirão Preto: *T. reesei* (soil and humus), V-1 (soil and humus), V-2 (soil and humus), C-10 gray (soil). T- II was collected from *Triparis* in Pereira Barreto, SP. The type of colleted sample was described between parentheses.

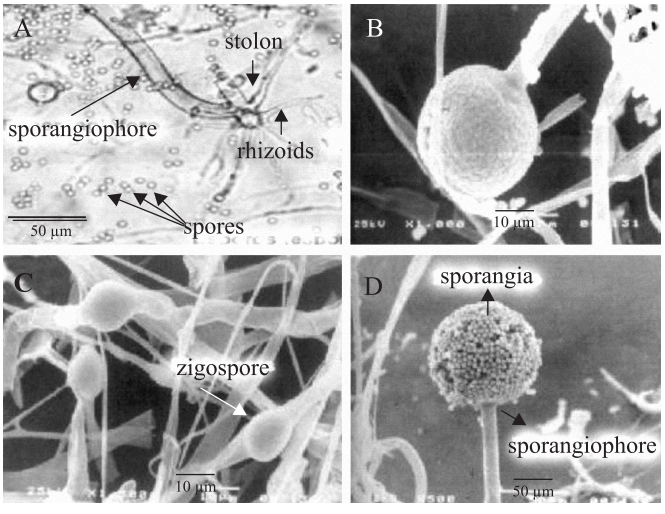

Figure 1. Guimarães *et al*. (2006).

All these isolates are available to those interested in secondary metabolites studies to screen for anti- microbial and anti-parasitic activities.

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RESUMO

Seleção de fungos filamentosos para a produção de enzimas de interesse biotecnológico

Muitas enzimas produzidas por fungos têm relevantes aplicações em diferentes áreas industriais. O objetivo desse trabalho foi coletar e isolar fungos filamentosos do solo e humus, plantas e bagaço de cana de açúcar de diferentes regiões do Estado de São Paulo. Quarenta isolados foram examinados quanto à sua capacidade de produzir xilanase, glicose-oxidase, fosfatase alcalina, fosfatase ácida, fitase, pectinase e amilase. Entre estes, vinte e três isolados exibiram potencial enzimático. Xilanases produzidas por dois destes isolados (*Aspergillus caespitosus* e *A. phoenicis)* mostraram bons resultados no biobranqueamento da polpa de celulose. Entre dezessete isolados, pelo menos três produziram altos níveis de glicose oxidase, sendo *Rhizopus stolonifer* e *Aspergillus. versicolor* os melhores produtores. *Aspergillus caespitosus*, *Mucor rouxii* e nove outros ainda não identificados foram os melhores produtores de fosfatases em fermentação submersa. Pectinase foi produzida preferencialmente por IF II e C-8 seguida por *Rhizopus stolonifer*. Níveis significantes de amilases foram produzidos por *Paecilomyces variotii* e *Aspergillus phoenicis.* Um notável produtor de diversas enzimas foi *Rhizopus microsporus* var. *rhizopodiformis*, que produziu altos níveis de amilase, fosfatase alcalina e ácida e pectinase. Algumas estruturas morfológicas deste fungo estão sendo ilustradas por microscopia de luz e microscopia eletrônica de varredura. Esse estudo contribui para catalogar fungos isolados do estado de São Paulo e fornece informações adicionais para pesquisas futuras sobre o potencial industrial destes microrganismos produtores de enzima e eventualmente também metabólitos secundários com atividade antimicrobiana e antiparasitária.

Palavras-chave: fosfatase, xilanase, glicose oxidase, pectinase, amilase, *Rhizopus microsporus* var. *rhizopodiformis*, fungos

REFERENCES

1. Bennett, J.W. Mycotechnology: the role of fungi in biotechnology. *J. Biotechnol*., 66, 101-107, 1998.
2. Dekker, M. *Handbook of Fungal Biotechnology*. Dilip K. Arora ed., 2003, New York, 600p.
3. James, J.A.; Lee, B.H. Glucoamylases: microbial sources, industrial applications and molecular biology - a review. *J. Food. Biochem.*, 21, 1-52, 1997.
4. Pandey, A.; Nigam, P.; Socol, C.R.; Socol, V.T.; Singh, D.; Mohan, R. Advances in microbial amylases - review. *Biotechnol. Appl. Biochem.*, 31, 135-152, 2000.
5. Kashyap, D.R.; Vohra, P.K.; Chopra, S.; Tewari. Applications of pectinases in the commercial sector: a review. *Bioresour. Technol*., 77, 215-227, 2001.
6. Sathyanarayana, N.; Panda, G.T. Purification and biochemical properties of microbial pectinases- a review. *Process Biochem*., 38, 987-996, 2003.
7. Wyckoff HW. Structure of *Escherichia coli* alkaline phosphatase determined by X-ray diffraction. *In*: Torriani-Gorini A, FG Rothman, S. Silver, A. Wright and E.Yagil (eds), *Phosphatase metabolism and Cellular Regulation in Microorganisms*. America Society for Microbiology, Washington, DC, 1987, p.118-126.
8. Vincent, J.B.; Crowder, M.W.; Averill, B.A. Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions. *Trends Biochem. Sci*., 17, 105-110, 1992.
9. Dong, G.; Zeikus, J.G. Purification and characterization of alkaline phosphatase from *Thermotoga neapolitana*. *Enzyme Microbial. Technol*., 21, 335-340, 1997.
10. Pandey, A.; Szakacs, G.; Soccol, C.R.; Rodriguez-Leon, J.A.; Soccol, V.T. Production, purification and properties of microbial phytases. *Bioresour. Technol*., 77, 203-214, 2001.
11. Polizeli, M.L.T.M.; Rizzatti, A.C.S.; Monti, R.; Terenzi, H.F.; Jorge, J.A.; Amorim, D.S. Xylanases from fungi: properties and industrial applications. Review. *Appl. Microbiol. Biotechnol.*, 67, 577-591, 2005.
12. Subramaniyan, S.; Prema, P. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Crit. Rev. Biotechnol*., 22(1), 33-64, 2002.
13. Harbak, L.; Thygesen, H.V. Safety evaluation of a xylanase expressed in *Bacillus subtilis*. *Food Chem. Toxicol.*, 40, 1, 2002.
14. Dervilly, G.; Leclercq, C.; Zimmerman, D.; Roue, C.; Thibault, J.F.; Sauliner, L. Isolation and characterization of high molecular mass water-soluble ararabinoxylans from barley malt. *Carbohydr. Polym.*, 47, 143, 2002.
15. Twomey, L.N.; Pluske, J.R.; Rowe, J.B.; Choct, M.; Brown, W.; Mcconnell, M.F.; Pethick, D.W. The effects of increasing levels of soluble non-starch polysaccharides and inclusion of feed enzymes in dog diets on faecal quality and digestibility. *Anim. Feed Sci. Technol.*, 108(1-4), 71-82, 2003.
16. Marks, V. An improved glucose oxidase method for determining blood, CS F and urine glucose levels. *Clin. Chim. Acta*, 251, 19-24, 1996.
17. Porter, D.D.; Poter, H.G. A glucose oxidase immunoenzyme stain for the detection of viral antigen or antibody on nitrocellulose transfer blot. *J. Immunol. Methods*, 72, 1-9, 1984.
18. Vogel, H.F. Distribution of lysine pathways among fungi: evolutionary implications. *Amer. Naturalist*, 98, 435-446, 1964.
19. Adams, P.R. Mycelial amylase activities of thermophilic species of *Rhizomucor*, *Humicola* and *Papulospora*. *Mycopatologia*, 112, 35-37, 1990.
20. Peixoto, S.C.; Jorge, J.A.; Terenzi, H.F.; Polizeli, M.L.T.M. *Rhizopus microsporus* var. *rhizopodiformis*: a thermotolerant fungus with potential for production of thermostable amylases. *Int. Microbiol.*, 6, 269-273, 2003.

21. Barbosa-Tessman, J.P.; Silva, D.A.; Peralta, R.M.; Kemmelmeier, C. A new species of *Fusarium* producer of galactose oxidase. *J. Basic Microbiol*., 41(3-4), 143-148, 2001.
22. Rizzatti, A.C.S.; Jorge, J.A.; Terenzi, H.F.; Rechia, C.G.V., Polizeli, M.L.T.M. Purification and properties of a thermostable extracellular β-D-xylosidase produced by thermotolerant *Aspergillus phoenicis*. *J. Ind. Microbiol. Biotechnol.*, 259, 156-160, 2001.
23. Guimarães, L.H.S.; Jorge, J.A.; Terenzi, H.F.; Jamur, M.C.; Oliver, C.; Polizeli, M.L.T.M. Effect of carbon source on alkaline phosphatase production and excretion in *Aspergillus caespitosus*. *J. Basic Microbiol.*, 43, 210-217, 2003.
24. Guimarães, L.H.S.; Terenzi, H.F.; Jorge, J.A.; Polizeli, M.L.T.M. Termostable conidial and mycelial alkaline phosphatases from the thermophilic fungus *Scytalidium thermophilum*. *J. Ind. Microbiol. Biotechnol.*, 27, 265-270, 2001.
25. Heinonen, J.; Lahti, R.J. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Anal. Biochem.*, 113, 313-317, 1981.
26. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31, 426-428, 1959.
27. Paterson, R.R.M.; Bridge, P.D. *Biochemical techniques for filamentous fungi*. CAB I Publishing International, 1994, Wallingford, United Kingdom, 144.
28. Tsuge, H.; Mitsuda, H. Studies on molecular complex of flavins IV. Activity and FAD-fluorescence change caused by the chemical modification of tryptophyl and tyrosyl residues in glucose oxidase. *J. Biochem.*, 73, 199-206, 1973.
29. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randal, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., 193, 267-275, 1951.
30. Kona, R.P.; Oureshi, N.; Pai, J.P. Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. *Bioresour. Technol.*, 78, 123-126, 2001.
31. Malherbe, D.F.; du Toit, M.; Cordero Otero, R.R.; van Rensburg, P.; Pretorius, I.S. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production. *Appl. Microbiol. Biotechnol*., 61(5-6), 502-11, 2003.
32. Guimarães, L.H.S.; Terenzi, H.F.; Jorge, J.A.; Leone, F.A.; Polizeli, M.L.T.M. Extracellular alkaline phosphatase from the filamentous fungus *Aspergillus caespitosus*: purification and biochemical characterization. *Folia Microbiol.*, 48(5), 627-632, 2003.
33. Buainain, L.B.; Kadowaki, M.K.; Polizeli, M.L.T.M.; Terenzi, H.F.; Jorge, J.A. Characterization of a conidial alkaline phosphatase from thermophilic fungus *Humicola grisea* var. *thermoidea*. *J. Basic Microbiol.*, 38, 85-94, 1998.
34. Guimarães, L.H.S.; Terenzi, H.F.; Jorge, J.A.; Leone, F.A.; Polizeli, M.L.T.M. Characterization and properties of acid phosphatases with phytase activity produced by *Aspergillus caespitosus*. *Biotechnol. Appl. Biochem*., 40, 201-107, 2004.
35. Sandrim, V.C.; Rizzatti, A.C.S.; Terenzi, H.F.; Jorge, J.A.; Milagres, A.M.F.; Polizeli, M.L.T.M. Purification and biochemical characterization of two xylanases produced by *Aspergillus caespitosus* and their potential for kraft pulp bleaching. *Process Biochem.*, 40(5), 1823-1828, 2005.
36. Schipper, M.A.A.; Stalpers, J.A. A revision of the genus *Rhizopus*. II. The *Rhizopus* microsporus-group. *Stud. Mycol*., 25, 20-34.