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Production and action of an *Aspergillus phoenicis* enzymatic pool using different carbon sources

Produção e ação de um pool enzimático de Aspergillus phoenicis com fontes de carbono diferentes

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Summary

Aspergillus phoenicis is an interesting heat tolerant fungus that can synthesize enzymes with several applications in the food industry due to its great hydrolytic potential. In this work, the fungus produced high enzymatic levels when cultivated on inexpensive culture media consisting of flakes from different origins such as cassava flour, wheat fibre, crushed soybean, agro-industrial wastes, starch, glucose or maltose. Several enzymatic systems were produced from these carbon sources, but amylase was the most evident, followed by pectinase and xylanase. Traces of CMCases, avicelase, lipase, β-xylosidase, β-glucosidase and α-glucosidase activities were also detected. Amylases were produced on rye flakes, starch, oat flakes, corn flakes, cassava flour and wheat fibre. Significant amylolytic levels were produced in the culture medium with glucose or when this sugar was exhausted, suggesting an enzyme in the constitutive form. Cassava flour, rye, oats, barley and corn flakes were also used as substrates in the hydrolytic reactions, aiming to verify the liberation potential of reducing sugars. Corn flakes induced greater liberation of reducing sugars as compared to the others. Thin layer chromatography of the reaction end products showed that the hydrolysis of cassava flour liberated maltooligosaccharides, but cassava flour and corn, rye, oats and barley flakes were hydrolyzed to glucose. These results suggested the presence of glucoamylase and α-amylase as part of the enzymatic pool of *A. phoencis*.

Key words: *Aspergillus phoenicis; Amylase; Rye flakes; Potato starch; Cassava flour.*

Resumo

Aspergillus phoenicis é um fungo termotolerante interessante, uma vez que pode sintetizar enzimas com diversas aplicações em indústrias alimentícias em função de seu grande potencial de hidrólise. Neste trabalho, verificou-se que esse fungo produziu níveis enzimáticos elevados, quando o mesmo foi cultivado em meio de cultura de baixo custo, constituído de flocos de diferentes origens, como farinha de mandioca, fibra de trigo, soja triturada, resíduos agroindustriais, amido e glicose ou maltose. Diversos sistemas enzimáticos foram produzidos a partir dessas fontes de carbono, mas a amilase foi a mais evidente, seguida de pectinase e xilanase. Traços de atividades de CMcases, avicelase, lipase, β-xilosidase, β-glicosidase e α-glicosidase também foram detectados. Amilases foram produzidas em flocos de centeio, amido, flocos de aveia, flocos de milho, farinha de mandioca e fibra de trigo. Níveis amilolíticos significantes foram produzidos em meio de cultura contendo glicose ou quando esse açúcar não estava mais presente, sugerindo uma forma constitutiva dessa enzima. Farinha de mandioca, flocos de centeio, aveia, cevada e milho também foram utilizados como substratos nas reações de hidrólise, com o objetivo de verificar a potencial liberação de açúcares redutores. Flocos de milho induziram maior formação de açúcares redutores, quando comparados com os outros substratos. A análise por cromatografia em camada delgada dos produtos finais da reação revelou que a hidrólise da farinha de mandioca liberou malto-oligossacarídeos, mas farinha de mandioca e flocos de milho, centeio, aveia e cevada foram hidrolisados até glicose. Esses resultados sugeriram a presença de glucoamilase e α-amilase como parte da mistura enzimática de *A. phoenicis*.

Palavras-chave: *Aspergillus phoenicis; Amilase; Flocos de centeio; Amido de batata; Farinha de mandioca.*

1 Introduction

Amylases are widely distributed in nature and play an important role in the starch-processing industry (GOTO et al., 1998). These enzymes are used in the food industry to improve final product quality, working to increase bread softness and volume. In addition, they promote the formation of a crispy crust on the bread surface and change its colour, thus attracting more consumers. Moreover, once bakery products are ready, they start to undergo changes leading to quality deterioration due to aging. These changes include shrinkage, less crispy skin, brownish colour and loss of flavour. One way to delay such process is to add various additives during bread production. Several types of de-branching and branching amylases have been suggested as anti-aging agents, for example: α-amylase, maltogenic-amylases, β-amylase and glucoamylases. During several types of bread manufacturing, amylases are added together with other enzymes, and thus the carbon sources, such as wheat fibre, rye flakes, barley flakes, cassava flour and others, are degrading more quickly. The sugars formed are promptly fermented by yeasts, releasing a considerable amount of carbon dioxide via anaerobic metabolism, consequently increasing bread softness and volume (GUPTA et al., 2003).

The main reasons for using enzymes in industrial processes are cost effectiveness, less time consuming processes, less space requirement and ease of modifying and optimizing the process (BANO et al., 2011). Microorganisms have been considered the best source of enzymes, since some of these microbes are able to grow at high temperatures and produce more heat stable enzymes, which are frequently more resistant and more efficient as catalysts than homologous proteins synthesized by mesophilic microorganisms (RIZZATTI et al., 2004). The fungi are frequently amongst the best enzyme producers, and *Aspergillus phoenicis* is an example of a heat tolerant fungus which grows well at 42 °C (RIZZATTI et al., 2001). This fungus was isolated from sugar cane bagasse from areas near sugar cane plantations, and has been shown to be an excellent producer of enzymes such as, for example, beta-xylosidase (RIZZATTI et al., 2001) and xylanase (RIZZATTI et al., 2004).

Trade and food industry waste produces substantial amounts of peel, seeds, kernel and other residues. These materials are rich sources of organic matter, such as protein, carbohydrate and essential oils, which can be used as substrates to produce enzymes by microbial fermentation (BAIG et al., 2004). Vandenberg et al. (2000) noted that the enrichment of a culture medium with cassava bagasse encouraged the growth of *Aspergillus niger*. Bravo et al. (2000) reported the use of sugar cane bagasse as a carbon source for microbial growth in the production of enzymes for applications in the fruit juice, wine, cocoa and coffee industries. Moreover, other researchers are using residues such as cassava root for the production of amylases (SILVA et al., 2009). It is important to remember that monomers, such as glucose, xylose, arabinose and others, can be used as inducers in the production of some enzymes, but this property varies for each type of fungus (ARO et al., 2005). In *Trichoderma reesei,* for example, the best yields of β-xylosidase were obtained when xylose was used as the inducer (KNOB et al., 2010). Arabinose induced the expression of a set of genes of *Aspergillus niger* that encoding for enzymes involved in the degradation of arabinoxylan (ARO et al., 2005), and xylose oligomers induced xylanase activity in *Trichoderma longibrachiatum* (ROYER and NAKAS, 1990). Thus, the aims of this study were to analyze the production of various enzymes from *A. phoenicis* using different carbon sources as inducers, and investigate the hydrolytic potential of the enzymatic systems produced and their end-products.

2 Material and methods

2.1 Microorganism and strain maintenance

A. phoenicis was isolated from sugar cane bagasse in decomposed material from the region of Ribeirão Preto - São Paulo, Brazil. The microorganism was identified as described by Raper and Fennell (1965) and Klich and Pitt (1988), using the nucleotide sequence analysis of ribosomal genes (rDNA), in particular the ITS (Internal Transcribed Spacer Sequence) spacer region. Species identification was based on similarity of over 96% and deposited in NCBI with the accession number: FJ810504. The microorganism was maintained on slants of complete Vogel medium (VOGEL, 1964). The inoculum consisted of a conidia solution prepared in sterile distilled water, which was added to the medium at a final concentration of 10^5 conidia/mL of culture medium.

2.2 Growth conditions

The most suitable conditions for amylase production were found to be in MB (Machado Benassi) medium, initial pH 6.0, standardized in the authors' laboratory. The fermentation process was carried out in conical flasks containing the following liquid medium (0.1 g NH_4NO_3; 0.065 g KH_2PO_4; 0.0181 g $MgSO_4$. 7 H_2O; 0.0049 g KCl; 0.00035 g $ZnSO_4$. 7 H_2O; 0.00069 g $MnSO_4$. H_2O; 0.0033 g $FeCl_3$. 6 H_2O; 0.000031 g $CuSO_4$. 5 H_2O; 0.025 g yeast extract; 0.25 g carbon source and 25 mL distilled water). Different carbon sources were tested at a concentration of 1%. Growth occurred statically in a bacteriological incubator for four days at 30 °C. The culture was then harvested by filtration through Whatman n° 1 filter paper, and the filtrate used as a source of extracellular amylolytic activity. The mycelial mass (g) was determined by weighing of the mycelium after it has been dried in

bacterial incubator. The mycelia pads obtained were ground in a mortar in an ice bath with 2 volumes of glass beads and suspended in 100 mM sodium acetate buffer, pH 5.0. The slurry was centrifuged at 12,000 g for 15 min at 4 °C and the supernatant was the source of intracellular enzyme.

2.3 Protein and enzymatic activity determinations

The protein content was determined according to Lowry et al. (1951), with bovine serum albumin as the standard. The enzymatic activities were estimated using different substrates and the formation of reducing sugars quantified using 3,5-dinitrosalicylic acid (DNS) (MILLER, 1959). For the determination of amylase activity, the assay was carried out at 65 °C with a mixture containing 250 μL of a solution of 1% potato starch in 100 mM sodium acetate buffer, pH 5.0 and 250 μL of diluted enzyme. Aliquots (100 μL) were withdrawn after different time intervals (15 and 30 min), and the assay tubes covered with parafilm and boiled for 5 min. The tubes were then chilled and 1 mL of distilled water added. The blanks consisted of 100 μL of reaction mixture with the immediate addition of 100 μL of DNS. The diastatic control was performed by incubating the substrates in 100 mM sodium acetate buffer, pH 5.0, for 30 min at 65 °C, and no reducing sugars were detected. The absorbance readings were made at 540 nm using a microplate. Glucose was used as the standard (0.1 to 1.0 $mg.mL^{-1}$) and one unit of enzyme activity (U) was the amount of enzyme required to produce one μmol of reducing sugar per minute per mL under the test conditions.

In order to determine the xylanase, CMCase, avicelase and polygalacturonase activities, 1% birchwood xylan, sodium carboxymethylcellulose – medium viscosity, avicel and sodium polypectate were used as substrates, respectively. The method was standardized using xylose (xylanase), glucose (CMCase and avicelase) and monogalacturonic acid (polygalacturonase) (0.1 to 1.0 $mg.mL^{-1}$), as previously described by Peixoto-Nogueira et al. (2009). The total activity was defined as the amount of enzyme required to produce one μmol of reducing sugar/min/ mL x total extracellular or intracellular filtrate volume. The specific activity was expressed as the total U/total mg protein of the mycelia extract ($U.mg^{-1}$ prot.).

2.4 Determination of enzymatic activity using synthetic substrates

The hydrolytic action of various enzymes was analyzed according to Somera et al. (2009) using 2.0 mM *p*-nitrophenyl-β-D-glucopyranoside (β-glucosidase), *p*-nitrophenyl-α-D-glucopyranoside (α-glucosidase), *p*-nitrophenyl-β-D-xyloside (β-xylosidase) and *p*-nitrophenyl-palmitate (lipase). The enzyme mixture consisted of 200 μL of 100 mM sodium acetate buffer, pH 5.0, 50 μL of synthetic substrate and 100 μL of enzyme and the assay was carried out at 60 °C at determined time intervals (10 and 20 min). The reaction was interrupted by adding 1 mL of a saturated solution of sodium tetraborate. A control was prepared by substituting the enzyme with distilled water. The absorbance was read at 540 nm using a microplate count and the method was standardized using *p*-nitrophenol (0.1 to 1.0 $mg.mL^{-1}$) as the standard. One unit of enzyme activity (U) was the amount of enzyme required to produce one μmol p-nitrophenol per minute per mL under the test conditions.

2.5 Chromatography of hydrolysis products

Thin layer chromatography (TLC) was used to analyze the hydrolysis products of the amylases. A volume of 5 μL of the reaction mixture was applied to silica gel plates (DC-Alufolien Kieselgel 60, Merck), and subjected to two consecutive ascending chromatographic runs using butanol/ethanol/water (5:3:2) as the solvent system. After air-drying the plates, the spots were developed by spraying with H_2SO_4 and methanol (1:9) containing 0.2% orcinol, and heating at 100 °C (FONTANA et al., 1988). A mixture of (1%) glucose, maltose, maltotriose, maltotetraose and maltopentaose was applied as the standard.

3 Results and discussion

3.1 Effect of carbon sources on the production of amylase

In order to evaluate the effects of different carbon sources on the production of amylase, the MB culture media were supplemented with different carbon sources. No carbon source was added to the control (Table 1). *A. phoenicis* exhibited variable mycelial protein and the best levels were noted in the medium supplemented with maltose, potato starch, glucose and cassava flour. With respect to the amylolytic activities (extracellular + intracellular), the carbon sources that resulted in the greatest amylase productions were: rye flakes (8.7-times), potato starch (8.6-times), oat flakes (7.7-times), as well as corn flakes, cassava flour, wheat fibre, glucose, maltose and barley flakes (average of 7.3-times in relation to the control). The majority of the sources contributed to the secretion of amylase activity, since the extracellular activities were approximately 90% of the sum of the extracellular and intracellular activities. The agro-industrial residues used were probably hydrolyzed in the culture medium to smaller compounds (maltodextrins) by constitutive amylases, which are located in the fungal cell wall. The intracellular transport of maltooligosacharides through the membrane is provided

Table 1. Effect of the supplementation of different carbon sources on the growth and production of amylase by *Aspergillus phoenicis*.

Carbon source (1%)	Amylolytic Activity			Protein (total mg)
	Extracellular (total U)	Intracellular (total U)	Total Extra + Intra	
No carbon source	124 ± 0.045	8 ± 0.02	132	0.5 ± 0.002
Glucose	794 ± 0.034	175 ± 0.01	969	6 ± 0.001
Starch	993 ± 0.018	140 ± 0.098	1133	6 ± 0.003
Maltose	763 ± 0.013	185 ± 0.014	949	6 ± 0.002
Rye flakes	1048 ± 0.013	102 ± 0.023	1150	2 ± 0.001
Cassava flour	890 ± 0.007	103 ± 0.067	992	6 ± 0.012
Oat flakes	889 ± 0.033	132 ± 0.023	1021	4 ± 0.022
Wheat fibre	881 ± 0.040	99 ± 0.014	980	5 ± 0.003
Barley flakes	880 ± 0.007	27 ± 0.039	907	0.2 ± 0.001
Corn flakes	861 ± 0.014	134 ± 0.049	995	0.7 ± 0.004
Crushed soybean	565 ± 0.028	110 ± 0.054	675	4 ± 0.002
Soy flakes	181 ± 0.010	27 ± 0.013	208	5 ± 0.027
Corn cob	102 ± 0.012	10 ± 0.009	112	1 ± 0.003
Sugar cane bagasse	72 ± 0.011	18 ± 0.020	90	0.5 ± 0.003

The samples were obtained from 25 mL of MB liquid culture medium after 4 days of incubation at 30 °C under static conditions. The measurements were carried out with 1% starch substrate in 100 mM sodium acetate buffer, pH 5.0, and the protein values were expressed as mg total protein of the mycelial sample. (All experiments were done in triplicate and the average values reported).

by permeases, which are specific dependent transporters. The maltooligosacharides may act as true inducers of the gene expression. According to Carlson (1987), fermentation of the oligosaccharides is controlled by repetitive gene families that are dispersed, such as SUC (Sucrose), MAL (Maltose) and MGL (α-Methylglucosides). Each family includes functionally equivalent multiple loci that control the fermentation ability. There are many reports of using food sources to produce enzymes. Balkan and Ertan (2010) used three carbon sources (wheat bran, rice husks and sunflower oil meal) to check for the production of α-amylase by *Penicillium brevicompactum,* and noted that all the substrates supported growth but that the sunflower oil meal did not support enzyme production. On the other hand, wheat bran showed the highest activity.

Table 1 also shows the production of amylase by *A. phoenicis* in a culture medium with no carbon source. This was probably due to salts, proteins and vitamins derived from the yeast extract. On the other hand, the description of glucose as a catabolic repressor acting on the regulation of gene expression at transcriptional levels is classic in the literature (CARLSON, 1987). The amylase of *A. phoenicis* is apparently a constitutive enzyme, as previously reported for the β-glucosidase from *Humicola grisea* var. *thermoidea* (POLIZELI et al., 1996).

3.2 Analyses of the hydrolysis of the different substrates

After observing the excellent levels of amylolytic activity, it was of interest to analyze the enzymatic hydrolysis potential with food sources. Thus extracellular extracts produced from the different carbon sources were incubated with solutions of 1% crushed soybean, cassava flour, corn flakes, oat flakes, soy flakes, wheat fibre, sugar cane bagasse, barley flakes, corn cob and rye flakes in 100 mM sodium acetate buffer, pH 5.0, as described in the methods section. The amount of reducing sugars formed after 30 min of reaction time was determined (Table 2), and the results obtained showed that the enzymes secreted when the fungus was grown on cassava flour were capable of hydrolyzing rye flakes, soy flakes, cassava flour and oat flakes (average activity of 141 $U.mL^{-1}$), and also when barley flakes were used as the source for fungal growth.

The amylolytic enzyme showed a high rate of hydrolysis with barley flakes, rye flakes, crushed soybean and oat flakes as substrate (average of 143 $U.mL^{-1}$). When rye flakes were used to produce the enzyme, the enzymatic pool hydrolyzed cassava flour and rye flakes as substrates (average activity of 151 $U.mL^{-1}$), whereas practically no activity was detected on wheat fibre. The enzymes produced on corn flakes led to excellent formation of reducing sugar when the same source of carbon was used (357 $U.mL^{-1}$), followed by hydrolysis on barley flakes (187 $U.mL^{-1}$), but wheat fibre was not efficiently hydrolyzed (4 $U.mL^{-1}$).

The hydrolysis of oat flakes, wheat fibre, crushed soybean and sugar cane bagasse produced low concentrations of reducing sugar. These results can be explained based on the different proximate compositions and different morphological structures, For instance, sugar cane bagasse, which produced low amounts of hydrolysis products, contains almost 70% of carbohydrates in its composition, and its morphological structure is complex (ROSSELL, 2006). On the other hand, the best carbon sources used in this study were rye flakes, corn flakes and cassava flour showed different

Table 2. Hydrolysis of the different substrates.

Substrates (1%)	Enzyme activity (total U) Inducing sources						
	Cassava flour	Barley flakes	Oat flakes	Rye flakes	Corn flakes	Wheat fibre	Crushed Soybean
Crushed soybean	32 ± 0.010	143 ± 0.013	34 ± 0.011	17 ± 0.004	11 ± 0.002	24 ± 0.002	34 ± 0.013
Cassava flour	114 ± 0.010	66 ± 0.041	56 ± 0.013	189 ± 0.010	75 ± 0.047	76 ± 0.014	92 ± 0.048
Corn flakes	69 ± 0.007	59 ± 0.012	62 ± 0.007	62 ± 0.014	357 ± 0.033	71 ± 0.010	45 ± 0.001
Oat flakes	112 ± 0.006	116 ± 0.084	112 ± 0.062	34 ± 0.004	73 ± 0.018	118 ± 0.004	77 ± 0.030
Soy flakes	145 ± 0.012	21 ± 0.003	27 ± 0.006	41 ± 0.0287	26 ± 0.012	24 ± 0.011	20 ± 0.011
Wheat fibre	49 ± 0.014	13 ± 0.004	11 ± 0.011	1 ± 0.001	4 ± 0.007	24 ± 0.004	34 ± 0.010
Sugar cane bagasse	11 ± 0.002	8 ± 0.002	4 ± 0.003	71 ± 0.034	19 ± 0.009	10 ± 0.006	10 ± 0.004
Barley flakes	40 ± 0.007	159 ± 0.013	164 ± 0.014	36 ± 0.009	187 ± 0.012	32 ± 0.008	93 ± 0.007
Corn cob	21 ± 0.008	33 ± 0.006	11 ± 0.008	24 ± 0.019	64 ± 0.048	15 ± 0.004	22 ± 0.007
Rye flakes	193 ± 0.094	153 ± 0.065	168 ± 0.062	113 ± 0.029	72 ± 0.005	101 ± 0.003	134 ± 0.032

The fungus was added to 25 mL of MB liquid culture medium and incubated for 4 days at 30 °C under static conditions. The measurements were carried out with 1% substrate in 100 mM sodium acetate buffer, pH 5.0. (All experiments were done in triplicate and the average values were reported).

values (76.7 g carbohydrates.100 g^{-1} food source and 1.07 g fibre.100 g^{-1} food source) (DUTRA-DE-OLIVEIRA and MARCHINI, 2008).This result shows that, in general, the nutritional source used had good potential for the induction of enzyme synthesis by *A. phoenicis*, and that the enzyme pool produced could hydrolyze several food sources, suggesting the possibility of application in industrial saccharification processes.

3.3 Evidence for the production of other enzyme activities from the carbon sources

The substrates added to the culture media had complex compositions, which could stimulate the production of other enzymes that would be able to take part in the hydrolysis of the food sources. With the objective of knowing more about the complex enzyme systems produced, three extracts with high hydrolytic action were selected: corn flakes, cassava flour and rye flakes. Several enzyme activities were determined (Table 3) and it was observed that *A. phoenicis* produced significant levels of amylase under all conditions, followed by polygalacturonase and xylanase in smaller proportions. Traces of other enzymes were also detected but probably did not contribute significantly to the hydrolysis of the sources analyzed.

The results indicated that the enzymes produced by A. phoenics using food sources from agro-industrial residues, had industrial potential, mainly for the food industry. The possibility of using these enzymes is very interesting, since it could significantly reduce the production costs. Also, it must be considered that due to the complex composition, these residues could induce multiple enzymes, which would lead to greater levels of hydrolysis.

It is known that the supply of enzyme mixtures (xylanases, phospholipases, amylases, glucose oxidases), together with emulsifiers (polysorbates) is a common practice to equilibrate the flour quality in mills that produce pre-mixes for breads, cakes and biscuits, especially French bread, replacing the old chemical oxidants such as bromate and azodicarbonamides (ESTELLER et al., 2004).

Table 3. Enzyme activities using medium supplemented with different carbon sources.

Enzymes	Enzyme activity (total U) Inducer sources		
	Corn flakes	Cassava flour	Rye flakes
Amylase	866.52	887.37	1017.64
Polygalacturonase	122.04	145.53	161.88
Xylanase	84.24	71.28	163.02
CMCase	2.16	5.28	7.60
Avicelase	0.36	0.33	7.60
Lipase*	0.36	0.33	0.38
β-xylosidase*	0.36	0.66	7.60
β-glucosidase*	1.08	1.32	1.52
α-glucosidase*	1.44	1.65	1.90

*Formation of μmols of *p*-nitrophenol/minute/mL. *A. phoenicis* was incubated in 40 mL of MB liquid culture medium with added corn flakes, cassava flour or rye flakes for 4 days at 30 °C under static conditions. (All the experiments were done in triplicate and the average values were reported).

3.4 Effect of glucose as carbon source on the production of amylase

An interesting result was obtained when glucose was used as the carbon source in the production of amylase (see Table 1). Relatively high levels of this enzyme were observed (total of 794 U), even though the carbohydrates were assimilated quickly by the microorganisms. This result is interesting because in most cases, glucose acts as a catabolic repressor.

Thus the time-course of the production of amylase was monitored using 1% glucose as the carbon source during four days of fungal growth. The protein content of the mycelial extract (intracellular) and of the culture filtrate (extracellular), and also the mycelial mass and final pH of the cultivation medium were also monmitored (Table 4).

The mycelial mass increased up to the third day, and remained stable thereafter. Concomitant to this result, it was found that on the first two days the final pH of the medium was acidic (2.4-3.6), whereas on the third and fourth days, it was close to neutrality (6.2). Regarding the levels of intracellular proteins, a decrease of about 17.5% in the amount of protein was observed from the third to the fourth days, followed by an increase in extracellular protein (97.6%) from the third to fourth days, suggesting enzymatic secretion.

The highest extracellular amylase activity occurred on the fourth day of cultivation. A decrease in the enzyme levels was observed from the second to third days with respect to the intracellular (15.3%) and extracellular (45.7%) activities, probably due to the accumulation of amylolytic hydrolysates of low molecular weight in the culture medium. The presence of high enzyme levels throughout the incubation period suggests constitutive amylolytic activity in the presence of glucose as the carbon source.

3.5 Hydrolysis products of starch saccharification

The enzymes produced when the fungus was grown on 1% corn flakes, rye flakes, oat flakes and cassava flour were incubated using several types of food source as substrates. The sugars formed after 2 and 24 h of saccharification were analyzed by thin layer chromatography (TLC). In these analyses, glucose was the only end product detected, suggesting the presence

Table 4. Effect of glucose as the carbon source on the growth and production of amylase by *A. phoenicis.*

Days	Mycelial mass (g)	Extracellular pH	Intracellular protein (total mg)	Intracellular activity (total U)	Extracellular protein (total mg)	Extracellular activity (total U)
1	1.48 ± 0.013	2.40	2.61 ± 0.008	237.14 ± 0.006	10.19 ± 0	1158.57 ± 0.012
2	1.88 ± 0.041	3.60	5.70 ± 0.008	172.86 ± 0.001	3.02 ± 0.006	1263.04 ± 0.001
3	2.01 ± 0.067	6.28	4.80 ± 0.001	146.43 ± 0.012	5.88 ± 0.002	685.54 ± 0.012
4	1.98 ± 0.020	6.25	3.96 ± 0.003	201.43 ± 0.011	11.62 ± 0.009	1268.75 ± 0.015

The fungus was grown in MB medium with added 1% glucose, under static conditions at 30 °C, for different numbers of days. The values for total amylase activity were expressed in μmols/minute/ mL x volume of filtrate, and the protein values were expressed as mg total protein of the mycelial sample (intracellular) or medium filtrate (extracellular).

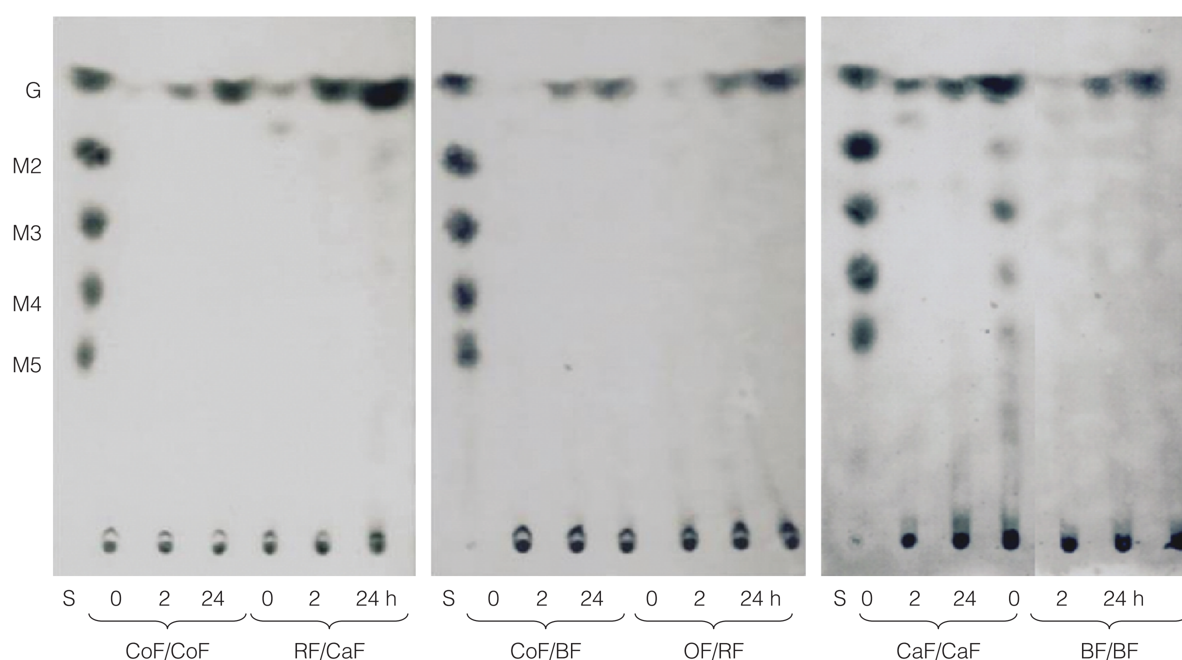

Figure 1. Thin layer chromatographic analysis. TLC of the hydrolysis products of amylases from *A. phoenicis* obtained with different carbon sources after 0 h (control), 2 and 24 h. S- standard (G- glucose, M2- maltose, M3- maltotriose, M4- maltotetraose, M5-maltopentose). The abbreviations represent the food sources used as carbon source and as substrates for the enzymatic reactions, respectively. CoF/CoF- Corn Flakes/Corn Flakes; RF/CaF- Rye Flakes/Cassava Flour; CoF/BF- Corn Flakes/Barley Flakes; OF/RF- Oat Flakes/Rye Flakes; CaF/CaF- Cassava Flour/Cassava Flour; BF/BF- Barley Flakes/Barley Flakes.

of glucoamylase. On the other hand, in the presence of cassava flour as carbon source, several sugars were detected by TLC as the hydrolysis products, such as glucose, maltose, maltotriose, maltotetraose and maltopentaose, suggesting, in this case, the induction of both α-amylase and glucoamylase (Figure 1).

4 Conclusions

The fungus *A. phoenicis* produced good amylase levels when the MB culture medium was supplemented with complex and inexpensive carbon sources. A close correlation was observed between the hydrolysis of certain substrates and the enzyme pool synthesized from inducers with complex compositions, the enzymes synthesized with major activities being amylase, polygalacturonase and xylanase.

Constitutive amylase was detected in the media containing glucose, as well as in non-inducing media supplemented with additional sources. The results suggest the application of the enzymes obtained from *A. phoenicis* in biotechnological processes, their use being of particular promise in the cereal area, especially in wheat milling, the processing of corn in the production of flours and the use of by-products (straw, bran) in the production of biofuel.

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