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Control of amylase production and growth characteristics of *Aspergillus ochraceus*

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ABSTRACT. The growth and the extracellular amylase production by *Aspergillus ochraceus* were studied in a stationary culture medium. Maximum growth rate of this fungus was found after 5 days of incubation at 30º C, but maximum amylase production was obtained after 2 days. The highest amylase production were attained with lactose, maltose, xylose and starch as carbon sources. The extracellular amylase production and mycelial growth were influenced by the concentration of starch. Other carbohydrates supported growth but did not induce amylase synthesis and glucose repressed it, indicating catabolite repression in this microorganism. The presence of both mechanisms of induction and repression suggests that at least these multiple forms of regulation are present in *A. ochraceus*. Of the nitrogen sources tested, casaminoacids, ammonium nitrate and sodium nitrate stimulated the highest yield of amylase. Optimal amylase production was obtained at pH 5.0, but enzyme activity was found only in the 4.0 - 6.0 pH range. These results were probably due to the inhibitory effect of NH_4^+-N in the culture medium.

Key words: α-amylase, *Aspergillus ochraceus*, carbon source, nitrogen source.

RESUMEN. El crecimiento y la producción de amilasa extracelular por *Aspergillus ochraceus* fueron estudiados en un medio de cultura estacionario. El máximo crecimiento del fungo fue encontrado después de 5 días de incubación a 30º C, pero la máxima producción de amilasa fue obtenida después de 2 días. La mayor producción de amilasa se obtuvo con lactosa, maltose, xilose y almidón como fuentes de carbono. La producción de amilase extracelular y el crecimiento micelial fueron influenciados por la concentración de almidón. Otros carbohidratos favorecieron el crecimiento pero no indujeron la síntesis de amilase y glucosa la reprimió, indicando represión catabólica en este microorganismo. La presencia de ambos mecanismos de inducción y represión sugieren que por lo menos estas formas múltiples de regulación están presentes en *A. ochraceus*. De las fuentes de nitrógeno probadas, casaminoácidos, nitrato de amonio y nitrato de sodio estimularon la mayor producción de amilasa. La producción óptima de amilasa se obtuvo a pH 5.0, pero la actividad de la enzima sólo se encontró en el rango de pH 4.0-6.0. Estos resultados fueron probablemente debidos al efecto inhibitorio de NH_4^+-N en el medio de cultura.

Palabras clave: α-amilasa, *Aspergillus ochraceus*, fuente de carbono, fuente de nitrógeno.

INTRODUCTION

Amylases are a group of enzymes that have been found in several microorganisms like bacteria[8,19,22,34] and fungi.[16,32] However, there have been few reports about the control of extracellular α-amylase production by fungi. Also, conflicting results have been reported with respect to the suggested mechanism of amylases synthesis control in fungi.

The induction of amylase requires a substrate having α-1,4 glucoside bond, including maltose, dextrin and starch.[13] Glucose, as a final product of the enzymatic reaction of substrate hydrolysis, represses enzyme synthesis by a well-known mechanism of catabolite repression.[7] However, it was found in several microorganisms that the carbohydrate used as carbon source did not induce amylase production according to the proposed models, showing that it is a constitutive enzyme.[23] Schmidell *et al.*[29] have reported that factors like composition of the culture medium, initial concentration of the polysaccharide, cultivation conditions and the microorganism itself may control the mechanism of enzymatic regulation. Facciotti *et al.*[15] observed that the glucoamylase activity of *Aspergillus awamori* decreased when the substrate concentration was increased up to 2%. Besides the carbon source, it has been suggested that the nitrogen source can also control amylase activity.[18]

The fungus *Aspergillus ochraceus* has been investigated by a number of workers,[1,9,12,14,26,30] but no study has been reported on its amylase activities. The study of growth and amylase production in this fungus may contribute to understand the mechanism of action in the environment. Thus, the present study deals with the control of the production of α-amylase (a-1,4 glucan glucanohydrolase E.C.3.2.1.1) by *A. ochraceus* in terms of the effect of the carbon and nitrogen sources and of the pH in the culture medium.

MATERIAL AND METHODS

Microorganism and inoculum preparation. *A. ochraceus* was isolated from soil.[27] For inoculum preparation, the organism was grown on Sabouraud agar slants for 7 days at 30ºC. The number of spores was determined with a Neubauer counting chamber and the inoculum was adjusted to 1.6 x 10^7 spores/ml.

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Growth medium and cultivation. The fungus was grown in liquid standing cultures. A citrate medium was employed (11) with soluble starch 2.0% (w/v) as carbon source and without the solution of trace elements and biotin. Unless otherwise stated, the pH of the medium was adjusted to 5.5 with 1 M HCl. The production of amylase was determined at several pH values (3.0-9.0) of the growth medium and the pH was adjusted with HCl or NaOH to various values in the 3.0 to 9.0 pH range.

The medium was inoculated with 0.5 ml of spore suspension and incubated for 72 hours (unless otherwise stated) at 30ºC. The mycelium was separated from the culture medium by filtration and the filtrate was used to determine the enzymatic activity. Dry weight was determined by drying the mycelium at 105° C for 24 hours. The residual NH_4^+-N of the medium was determined by the colorimetric indophenol blue method.[25]

Amylase assay. Amylase was assayed by the iodine method described by Jones and Varner[20] with slight modifications. Activity was estimated in a reaction mixture containing 1.0 ml of 0.15 % soluble starch in 0.1 M acetate buffer, pH 5.5, 200 mM $CaCl_2$ and 0.1 ml enzyme and incubated at 65º C for 10 minutes. After incubation, 1.0 ml of 1 M acetic acid, 1.0 ml of 0.2% iodine-2.0% KI solution and 15 ml of distilled water were added. The absorbance of the diluted solution was measured at 620 nm. A blank was prepared under the same conditions by adding enzyme solution after the reaction had been stopped by the addition of 1.0 M acetic acid. The optimum conditions of time, temperature and enzyme concentration in the reaction mixture have been previously reported.[31] One unit of dextrinizing activity was defined as the amount of enzyme which hydrolyzes 1 mg of starch/10 minutes under the above conditions. The specific activity was expressed as units per mg dry mycelium per hour. Starch concentration was determined from a standard curve under the same assay conditions using soluble starch.

Statistical analysis. The study was carried out in a completely randomized design and the data analyzed using the SAS for ANOVA. When a significant F value was detected, least significance difference (Tukey LSD) was used to compare treatments.

RESULTS

Highest amylase activities (56.7 U h^{-1} mg^{-1} mycelium dry weight) were detected on the 2nd day of cultivation, decreasing thereafter (Fig. 1). On the 9th day, the enzyme activity corresponded to 19 % of that detected on the 2nd day. The mycelial production increased until the 5th day of cultivation and the pH values decreased to 4.2 on the 4th day of cultivation, increasing thereafter (insert Fig. 1).

Although highest amylase activities were found on the 2nd day of cultivation, the time of incubation of 72 hours was chosen for the following assays because the fungus didn't grow with some carbon sources after 48 hours.

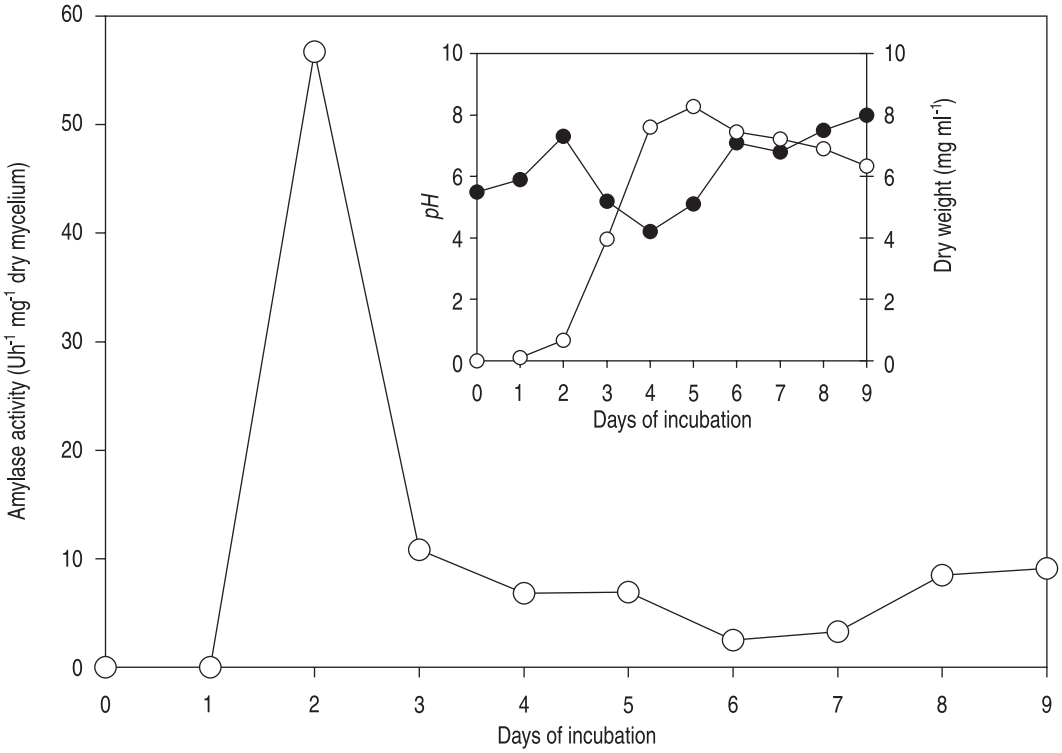

Figure 1. *Time course of amylase production by A. ochraceus in citrate medium pH 5.5 and soluble starch 2% (w/v) as carbon source. Insert: (●) pH; (○) dry weight.*

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Among the carbon sources tested, the maximal activities were produced on lactose > maltose > xylose > starch. The specific amylase activity in glucose-grown cultures was 6.1 times lower than that produced in starch-grown cultures, although total growth was similar (Table 1). When glucose (0.25%) and starch (1%) were simultaneously present in the culture medium, enzyme activity was stimulated, but at glucose concentration higher than that the specific amylase activity decreased (Table 2).

Increasing the starch concentrations in the culture medium enhanced fungal growth from 0.50 mg mL^{-1} (no starch) to 2.96 mg mL^{-1} (2%, w/v) (Fig. 2). The level of enzyme activity was markedly dependent upon the concentration of starch in the medium. The specific activity increased from

Table 1. Effect of the carbon source on growth and amylase production by *Aspergillus ochraceus.*

Carbon source (2% w/v)	Final pH	Mycelium dry weight (mg mL^{-1})	Amylase (U h^{-1} mg^{-1} dry mycelium)
Xylose	5.7	2.00	12.37
Glucose	4.3	5.23	1.67
Mannose	7.3	0.87	0.12
Rhamnose	6.3	0.79	<0.01
Sorbose	5.9	1.31	0.91
Sorbitol	5.3	1.77	1.03
Maltose	4.5	3.43	13.50
Cellobiose	5.4	1.72	0.71
Lactose	8.0	0.33	49.44
Starch	4.5	4.56	10.14
Cellulose	7.9	ND	2.41
LSD (Tukey)(*)	0.3	0.03	0.03

LSD - Least significant difference
ND - non determined
(*) P = 0.05

Table 2. Effect of glucose on growth and amylase production by *Aspergillus ochraceus.*

Glucose(+) (% w/v)	Final pH	Mycelium dry weight (mg mL^{-1})	Amylase (U h^{-1} mg^{-1} dry mycelium)
Control	4.2	1.62	9.98
0.25	5.1	3.71	12.24
0.5	4.8	4.00	4.72
1.0	4.5	4.29	3.85
1.5	4.4	4.19	3.12
2.0	4.1	6.38	2.02
LSD (Tukey)(*)	0.2	0.17	0.02

(+)Starch 1% (w/v) as carbon source
(*) P = 0.05

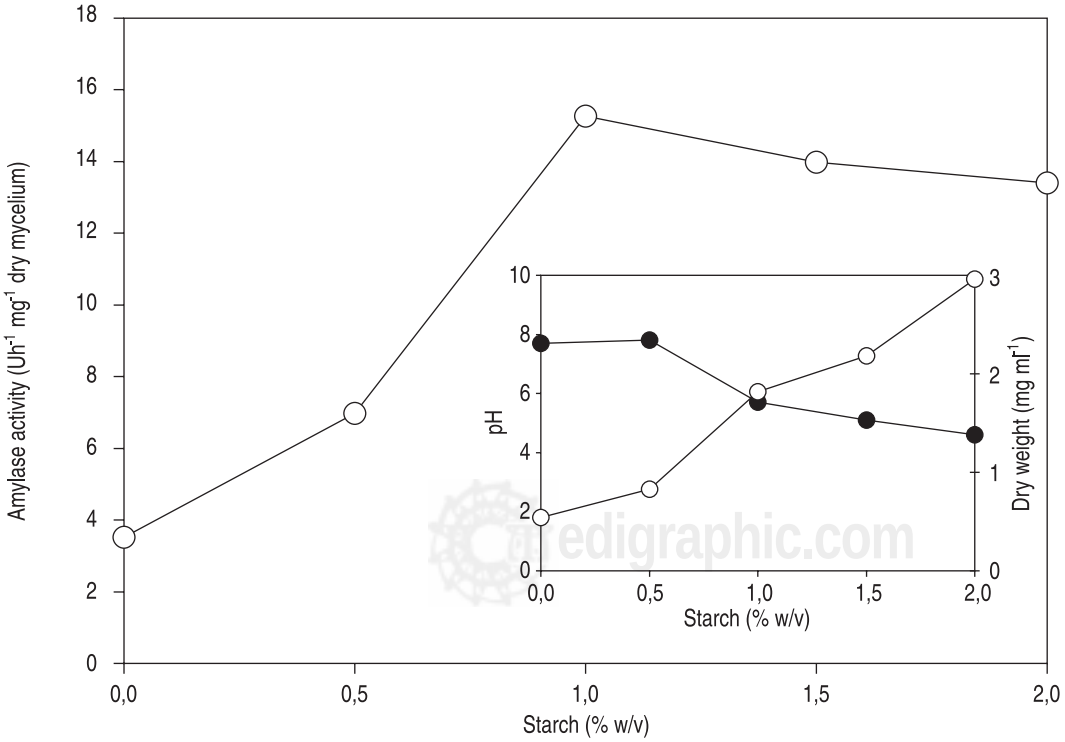

Figure 2. *Effect of starch concentration on amylase production and mycelial growth in* A. ochraceus. *Culture medium as Figure 1. Insert: (●) pH; (○) dry weight.*

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3.2 U h^{-1} mg^{-1} mycelium in the absence of starch to 15.27 U h^{-1} mg^{-1} mycelium when the starch concentration was 1.0 % (w/v). At starch concentrations higher than 1% the activity decreased. The final pH enhanced to 8.0 in the culture medium without starch.

The nitrogen source supplemented to the culture medium was also important to amylase production. The amylolytic activity with casaminoacids was 4.7 times higher compared to that of the yeast extract. The highest levels of enzyme activities were detected on casaminoacids, ammonium nitrate and sodium nitrate in this order (Table 3). The initial pH of the culture medium decreased to 3.2. Fungal growth was considered good on all N sources.

The optimum initial pH of the culture medium for enzyme production was 5.0, although activity was observed in the 3.0 to 6.0 pH range (Table 4). Maximum growth of *A. ochraceus* was found at pH 6.0, with a yield of 4.5 mg mL^{-1} dry weight. For pH below 4.0 or above 6.0, fungal growth was reduced by 67 to 99 % in relation to the optimum. The concentration of residual NH_4^+-N decreased with increasing fungal growth.

Table 3. Effect of the nitrogen source on growth and amylase production by *Aspergillus ochraceus*.

Nitrogen Sources(+) (0.35 g N L^{-1})	Final pH	Mycelium dry weight (mg mL^{-1})	Amylase (U h^{-1} mg^{-1} dry mycelium)
NH_4NO_3	4.2	6.06	14.02
$NaNO_3$	4.3	5.91	11.00
$(NH_4)_2SO_4$	3.2	4.92	7.77
Alanine	3.7	6.71	6.41
Asparagine	3.8	8.77	4.67
Casaminoacids	3.4	6.68	14.14
Peptone	4.2	4.61	9.84
Tryptone	3.9	4.57	6.36
Casein	4.0	4.88	4.25
Yeast extract	5.7	9.60	2.99
LSD (Tukey)(*)	0.2	0.09	0.03

(+) Starch 2% (w/v) as carbon source
(*) P = 0.05

Table 4. Effect of the pH on growth and amylase production by *Aspergillus ochraceus*.

Initial pH	Final pH	Mycelium dry weight (mg mL^{-1})	Amylase (U h^{-1} mg^{-1} dry mycelium)	NH_4^+-N (mg mL^{-1})
3.0	2.7	1.46	0.77	138.97
4.0	3.6	3.23	5.79	45.45
5.0	4.3	2.94	9.53	9.55
6.0	4.8	4.47	6.73	18.67
7.0	7.6	0.42	<0.01	126.55
8.0	7.7	0.55	<0.01	113.62
9.0	8.4	0.06	<0.01	75.40
LSD (Tukey)(*)	0.3	0.02	0.02	0.11

(*) P = 0.05

DISCUSSION

The results showed that the amylase activity of the fungus *A. ochraceus* was regulated by carbohydrate supply. While the mycelial growth on starch reached a maximum after 5 days, maximum amylase activity was produced after 2 days of cultivation. The decreased activity in the later phase of growth was probably due to catabolite repression by glucose released from starch hydrolysis, in agreement with the results reported in *Humicola grisea*[3] and *H. Brevis*,[6] but different from *Papulasporia thermofilia*[2, 10] in which the maximum amylase activity was recorded during the period of fungus autolysis.

Lactose, xylose, maltose and starch enhanced the amylase activity while sorbitol, mannose, cellobiose, sorbose, carboxymethylcellulose, cellulose, ramnose and, specially, glucose repressed amylase production. These results suggest that amylase production in *A. ochraceus* is induced by some carbon sources. While maltose and starch present an α-1,4 glucoside bond, xylose is a monosaccharide and lactose a disaccharide composed of a D-galactose and a D-glucose subunits with a β-1,4 bond. It was reported that maltose was the best inducer of α-amylase of *Aspergillus oryzae* among the carbon sources tested.[33] In *Humicola* sp, the extracellular amylase activity was induced by maltose and cellobiose, suggesting that the mechanisms of induction of amylase were not specific for α-1,4 glucoside bonds.[27] Accordingly Oso[28], amylase activity was induced by starch, and at a lower proportion, by cellobiose, glucose, fructose, galactose or maltose. Attia and Ali[5] showed that maltose, starch, dextrin and glucose were effective inducers in *A. awamori*. Soluble starch[17] and lignocelluloses[13] were considered to be inducers of amylase activity. Adams and Deploey[4] obtained higher activity with starch, but they claimed that other carbohydrates such as glucose, fructose and lactose acted as inductors too.

In *A. ochraceus*, it was clearly demonstrated that the production of amylase was repressed by glucose. This result contrasts with α-amylase from *A. oryzae* in which no repression was observed in glucose presence.[33] The decrease of the enzyme activity after 2 days of fungal growth (Fig. 1) and in the presence of starch concentrations above 1% (w/v) (Fig. 2) support this statement. In addition, when starch was associated with increasing glucose concentrations (Table 2), strong inhibition of the activity was observed. The small increase in amylase activity at 0.25%

glucose concentration (w/v) was probably due to a ready uptake and metabolism of this sugar, mimicking its absence in the medium. The induction by starch combined with the repression by glucose suggests that the *A. ochraceus* α-amylase is subject to multiple regulation forms.

The effect of nitrogen source on amylase activity was less pronounced than carbon source, with a range from 2.99 to 14.14 U h^{-1} mg^{-1} dry mycelium. Mahmoud[24] reported that glycine and yeast extract were the best sources of nitrogen for *A. flavus* and *A. fumigatus*, respectively. Consistent with the data reported here, Kundu *et al.*[21] showed that sodium nitrate and ammonium nitrate were the best nitrogen sources for maximum amylase production. It is important to point out that the most of organic sources of nitrogen led to higher *A. ochraceus* growth, although lower activities were obtained than with the inorganic sources, except for casaminoacids.

The best mycelial growth and amylase activity were detected at pH from 4.0-6.0, decreasing drastically at values outside this range, probably due to the inhibitory effect of NH_4^+-N (Table 4). This result was also observed when lactose was used as carbon source (data not shown).

Some fungi can produce amylase at both alkaline and acid pH range, while others can grow and synthesize amylase within a larger range of pH than that observed in the present study. In *A. oryzae*, maximum amylase production was obtained at pH 7.0-7.5 although activity was also observed at pH 5.0-10.0.[21] Maximum growth and activity were obtained at pH 6.0 and 8.0, respectively, with *A. fumigatus* and *A. flavus*.[1,24] Amylases from *Aspergillus niger* were produced in the range of pH 3.0 to 7.0.[16] Oso[28] reported that amylase activity in *Talaromyces emersonii* was found in the 4.0 to 8.0 pH range, with a maximum at pH 7.0.

Overall, the data imply that a-amylase from *A. ochraceus* was induced by lactose, maltose, xylose, starch, and repressed by glucose. Enzyme synthesis was affected by nitrogen sources, and maximal activity was shown attained with inorganic than organic nitrogen sources. Fungal growth and amylase production were found only within a narrow pH range (4.0-6.0).

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