Culture Medium for Amylase Production by Toxigenic Fungi

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ABSTRACT

Mycelial growth and amylase production by a mycotoxigenic strain of Fusarium moniliforme and Aspergillus flavus were evaluated in a culture medium containing starch, glycerol, wheat bran or corn. With emphasis on corn, different fractions composed by germ, degermed seed, starch, milky stage corn and the respective starch or supernatant fraction were analyzed for F. moniliforme growth . The medium composed of milky stage corn supernatant promoted the best mycelial growth ($p<0.05$), and it was used to prepare amylase production medium in the next step. The medium composed with 2% ground corn in milky stage corn supernatant (350g of milky stage corn blended with 250mL water and centrifuged) promoted the highest amylase production, which was at the 10^{th} day of fermentation, both for F. moniliforme (42.32U/mL) and A. flavus (4,745.54U/mL).

Key words: amylase, Fusarium moniliforme, Aspergillus flavus

INTRODUCTION

Corn is one of the most important commercial crops cultivated throughout the Brazilian territory. However, its rich nutritional profile makes it a target for phytopatogenic agents, mainly fungi and insects, that cause deterioration and subsequent mycotoxin contamination.

Fusarium, Aspergillus, Penicillium, Alternaria, Cladosporium, Epiccoccum, Nigrosporum and *Tricoderma* are the prevalent fungal genus in corn field (González et al., 1995), especially *Fusarium moniliforme* and *F. proliferatum* (Logrieco et al., 1995, González et al., 1995, Thiel et al., 1991 and Julian et al., 1995). These strain have been reported to produce fumonisins, a group of mycotoxins incriminated for toxicological damages, including leukoencephalomalacia in horses (Bezuidenhout et al., 1988), pulmonary edema in swine (Ross et al., 1991), weight loss in poultry (Weibking et al., 1993), along with human esophageal cancer risk in China and South Africa (Norred & Voss, 1994; Bullerman & Draughon, 1994).

Previous reports about natural contamination by fumonisins in Brazilian corn kernel stored at adequate humidity suggest that mycotoxin production is associated with pre harvested stage (Hirooka et al., 1996), which turns post harvested control ineffective. In this context, it is necessary to develop a new strategy in the field of pathogen control, produced as phenolic compounds, phytic acid, protease and amylase inhibitors (Gatehouse et al., 1992; Gomes & Xavier-Filho, 1994). The effect of plant amylase inhibitors on the fungal growth could be evaluated, since starch is one of the main components in grain (Watson & Ramstad, 1978).

In this article it is purposed an adequate culture medium for *F. moniliforme* and *Aspergillus flavus*

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amylase production, in a series of studies about amylase inhibitor activity for later introduction in mycotoxigenic fungal control using the natural plant defense mechanism.

MATERIALS AND METHODS

Materials

Microorganisms

F. moniliforme strain 113F, isolated from feed involved in animal intoxication, characterized by initial production of fumonisins reaching 54.2µg/g of FB_1 and 87.31µg/g of FB_2. In addition, one aflatoxin producing strain of *A. flavus*, isolated from animal feed, was included in the assay. The cultures were maintained at 4°C in Potato Dextrose Agar (PDA).

Culture media

Fungal growth and amylase production were carried out comparing culture mediums 1, 2 (Ohno et al., 1992), 3 (Santos et al., 1996), and formulations based on corn fractions to define the adequate substrate in medium 4.

Medium 1 (Ohno et al., 1992) was prepared with 2% glycerol, 0.125% peptone protease n.3, 0.003% yeast extract, 0.2% KH_2PO_4, 0.1% $MgSO_4.7H_2O$, 0.1% $CaCl_2.2H_2O$ in distilled water. **Medium 2** (modified Ohno et al., 1992) was composed of 0.5% starch (Reagen), 0.1% peptone protease n.3, 0.002% yeast extract, 0.01% KH_2PO_4 and 0.05% $MgSO_4.7H_2O$ in distilled water. **Medium 3** (Santos et al., 1996) was composed with 2% of wheat bran and Vogel minimum medium (Vogel, 1956.), which is a mixture of sodium citrate, mineral salts and biotin. **Medium 4** was determined after evaluating *F. moniliforme* 113F growth in 9 different formulations composed by corn fractions, such as mature and immature corn or corn germ.

Methods

Evaluation of corn based substrates

The corn (C) and respective starch (CS), degermed corn (DC), degermed and defatted corn (DDC), corn germ (CG) and defatted corn germ (DCG) were acquired in from a corn processing industry; milky stage corn supernatant (MSCS) and respective starch (MSCS) were prepared in the laboratory.

The milky stage corn supernatant was prepared by homogenizing 350g milky stage grain with 250mL water and centrifuged at 900 x g for 10 min. The milky stage corn starch was obtained homogenizing 250g milky stage grain with 100mL distilled water, followed by filtration and centrifugation at 900 x g for 10 min. The precipitate was washed with distilled water, centrifuged again and dried at room temperature.

Each culture medium formulation was prepared by weighing 0,2g (dry basis) of different corn fractions powdered at 48 granulometry mesh and added to 0.3g Bacto-agar in 20mL distilled water, except the milky stage corn supernatant medium. For this formulation, 0.3g Bacto-agar was added directly to 20mL milky stage corn supernatant. The media was autoclaved at 121°C for 15 min, distributed in Petri dishes (90/13mm) and a filter paper (Whatman n.1 sterile with 0.9mm diameter) was placed on the agar surface.

The assay in triplicate was carried out inoculating $3.3x10^7$spores/mL of *F. moniliforme* 113F on the agar surface and the plates were incubated at 25°C. Growth was assessed every day for 6 days and the result expressed in mm of colony diameter. The data was used to define medium 4 composition.

Evaluation of amylase production

The fungal spores activated in PDA media were transferred into 10mL of 0.1% Tween 80, counted in a Newbauer chamber, and 10^7 propagules inoculated in a 250mL Erlenmeyer flask containing 100mL of medium 1, 2, 3 or 4 (2% ground corn in milky stage corn supernatant). Each 5mL of culture incubated at 25°C for 2 days were transferred to 5 Erlenmeyers with 30mL of culture medium and again incubated at 25°C. The fermentation was interrupted on the 1^{st}, 3^{rd}, 5^{th} and 10^{th} day and centrifuged at 5,000 x g for 10 min at 4°C. The iodometric method was used to determine the amylase activity in the supernatant in buffer 0.05M KH_2PO_4-NaOH (pH6.0) for 10min at 40°C (Wilson & Ingledew, 1982). One unit of amylase activity (1U) was defined as the amount of enzyme that will hydrolyze 0.1mg starch in 10 min at 40°C when 5.0mg starch is present.

Statistical analysis

The data on colony diameter of *F. moniliforme* 113F growth in different corn based substrates was compiled, and analyzed by the Tukey test using SAS program (Statistical Analyses Systems, version 6.12) to formulate medium 4.

RESULTS AND DISCUSSION

Table 1 shows the performance of *F. moniliforme* 113F, where the mycelial growth was evaluated in nine corn based culture medium prepared with different fractions and grain maturation stages.

Macroscopic fungal growth started on the first day of incubation in all corn substrates. However, the formulation prepared with milky stage corn supernatant (MSCS) stood out from the other cultures since second day of assay, with colony diameter of 1.8mm. In the medium prepared with whole milky stage corn (WMSC), some degree of differentiation became visible after the 4th day of incubation (Table 1). After the 6th day of incubation, MSCS and WMSC formulations allowed colony growth of 8.1mm, showing a different performance from the others ($p<0.05$). No significant difference was noted compared to formulations composed with C, DC, DDC, CG, CS and MSCS1, which had colony diameter from 6.3 to 7.0 mm at the end of assay ($p<0.05$). Similar mycelial growth shown in such substrates suggests that their compositions did not affect *F. moniliforme* growth, i.e., nutrient components present only in immature corn supernatant (MSCS and WMSC) probably played important role in triggering mycelial overgrowth (Table 1). The result was evident, as the fungal growth diameter reached 6.9mm in culture medium prepared exclusively with milky stage corn starch (MSCS1), which is equivalent to that obtained with mature corn starch (CS, with 6.8mm diameter). The high sugar and amino acid content in the immature corn (Ingle et al, 1965) probably stimulated the fungal growth. Milky stage corn has a high level of glucose - 1.6mg/kernel, fructose -1.8mg/kernel - (Holder et al., 1974) and sucrose - 2 to 3mg/endosperm - (Tsai et al., 1970), while in mature corn these contents are low, due to their demand in polysaccharides and proteins biosynthesis (Ingle et al., 1965).

Table 1 - Mycelial growth of ***F. moniliforme*** in corn based culture media.

Substrate	**Colony diameter (mm)**					
	1st day	**2nd day**	**3rd day**	**4th day**	**5th day**	**6th day**
mature corn	0.3	1.4	2.2	3.9	4.5	7.0^{b}
degermed corn	0.2	1.5	2.4	4.1	5.1	6.7^{b}
degermed and defatted	0.3	1.5	2.9	3.6	4.8	6.3^{b}
corn germ	0.3	1.6	2.9	4.1	5.0	6.9^{b}
defatted corn germ	0.3	1.4	2.5	4.1	4.7	6.5^{b}
milky stage corn	0.4	1.5	2.9	4.8	6.2	8.1^{a}
corn starch	0.2	1.3	2.7	4.0	5.4	6.8^{b}
milky stage corn starch	0.3	1.6	2.8	4.4	5.6	6.9^{b}
milky stage supernatant	0.4	1.8	3.4	5.1	6.7	8.1^{a}

^{a} and ^{b}: indicators at 5% level significance statistically different.

Although the culture medium prepared with corn germ (6,9mm) caused *F. moniliforme* 113F growth when compared to degermed substrate (6,3mm), the protein composition did not significantly influence fungal growth ($p<0.05$). This results was expected, considering the high nutritional value of embryo albumins and globulins, when compared to the endosperm protein group of prolamins, albumins, globulins and glutelins (Watson & Ramstad, 1987).

The defatting process had no effective influence on *F. moniliforme* 113F growth ($p<0.05$). However, this data should be carefully interpreted along with fumonisin production, due to the

correlation between lipid and mycotoxin biosynthesis, as the conditions that decrease mycelial growth can induce a toxin pathway (Hirooka et al., 1993).

Data shown in Table 1 complements previous results (Hirooka et al., 1993), where fumonisins produced in mature corn substrate were 10^3 times higher then in milky stage corn. Probably the best nutritional content favored *F. moniliforme* growth, to the detriment of fumonisin biosynthesis. Starch content and lipid accumulation increase as the corn kernel matures (Ingle et al., 1965). Considering that fumonisins derive from a secondary metabolic pathway that resembles sphingolipid biosynthesis (Jackson et al., 1995), the corn maturation which increases lipid content can shunt the fungal metabolism toward fumonisin synthesis. On the other hand, fungal amylase would release glucose from stored starch, providing the glycolitic pathway and consequently, activating the lipids/fumonisins biosynthesis.

An ideal substrate to improve fungal amylase production should combine a biomass increasing property with enzyme synthesis induction. Data shown in Table 1 led to the establishment of a culture medium composed with mature corn starch supplemented with immature corn components to develop a further protocol for *F. moniliforme* and *A. flavus* amylase production assay. Medium 4 was formulated using both components, at a ratio of 2% ground corn in milky stage corn supernatant, prepared by blending 350g milky stage corn with 250mL water.

In addition to this medium, other three culture mediums described by Ohno et al. (1992) and Santos et al. (1996) were evaluated, where *F. moniliforme* 113F and *A. flavus* amylase activity was determined periodically in their crude extracts (Fig. 1 and 2).

F. moniliforme 113F had maximum amylase production in medium 4 (immature corn supernatant + ground corn), with 42.32U/ml at the 10^{th} day of fermentation, while in medium 3 (wheat bran added to the Vogel minimum medium) reached 7.64U/mL, using the same incubation time (Fig. 1). In the medium formulated with glycerol (medium 1) and starch (medium 2) as a carbon source, the maximum amylase activity was 2.16U/mL at the 8^{th} and 5.62 U/ml at the 5^{th} day of fermentation, respectively (Fig. 1).

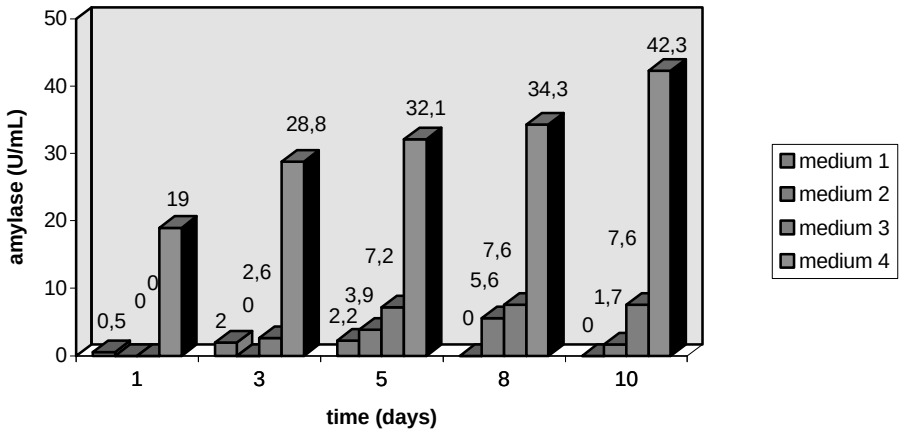

Figure 1 - Amylase production of *F. moniliforme* in different formulations.

Figure 2 clearly shows the high amylase production of *A. flavus* which reached a maximum activity of 4,745.54U/mL, in same condition observed with *F. moniliforme*, i.e. 10^{th} day fermentation in medium 4. In the medium 3 (wheat bran + minimum medium of Vogel), the maximum activity was 37.86U/mL at 8^{th} day. In the medium 1 and 2, the production decreased to 5.60U/mL at 10^{th} day and 4.65U/mL at 8^{th} day, respectively (Fig. 2).

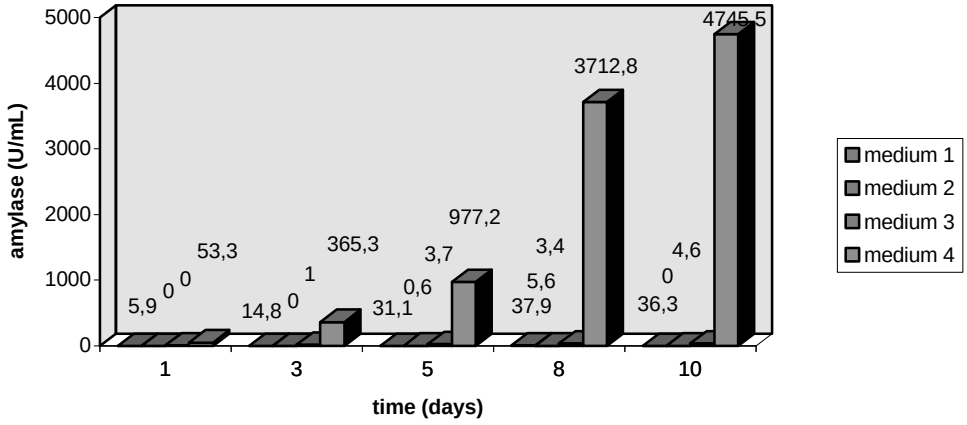

Figure 2 - Amylase production of *A. flavus* in different formulations.

The results indicated that medium 4 contains essential mono and disaccharides and free amino acids required for mycelial growth, which are provided by milky stage corn, while the amylase production is stimulated by starch of mature whole corn. The use of milky stage corn supernatant and starch supplemented with mineral salts was also reported for amylase production of *Penicillium expansum* and *Bacillus licheniformis* Doyle et al. (1989) and Takasaki et al. (1994), respectively.

The medium prepared with wheat bran added to minimum medium of Vogel (medium 3) showed lower amylase production than medium 4, but higher than the medium containing glycerol (medium 1) or starch (medium 2) as a carbon source. Glycerol induced amylase production in yeasts, which was better than starch (Ohno et al., 1992), but not for *F. moniliforme* and *A. flavus* amylase (Fig. 1 and 2). Probably the citrate, biotin and mineral salts used in the minimum medium Vogel complemented wheat bran and slightly stimulated amylase production mainly of *F. moniliforme* (Fig. 1 and 2).

Lachmund et al. (1993) reported *Aspergillus oryzae* and *A. nidulans* alpha-amylase induction by starch and maltose, but inhibited by glucose. High amylase production was expected in medium 2 containing starch; the low performance shown in Fig. 1 and 2 indicate that growth factors present as natural corn components play an important role in amylase production of mycotoxigenic fungi.

The amylase activity of *A. flavus* – 4,745.5U/mL - (Fig. 2) at 100 fold over *F. moniliforme* – 42.3U/mL - (Fig. 1) may partially explain the survival and gradual increase of this *Aspergillus* sp. during kernel storage. On the other hand, the intense *F. moniliforme* growth in milky stage corn supernatant (MSCS), which was significantly higher than in the starch medium (MSCS1) prepared with grain of stage $p<0.05$ (Tab. 1), reinforces the predominance of *F. moniliforme* in corn, whose natural contamination in grain analyzed by Blotter tests reached levels of 100% (Ono et al., 1996).

The amylase production of *F. moniliforme* and *A. flavus* requires complex interaction between natural corn components besides starch, which can maximize both growth and enzyme production. The culture medium formulated with milky stage corn supernatant and ground corn was recommended to *F. moniliforme* and *A. flavus* amylase production, and plant defense mechanisms based on amylase inhibitors should be studied further.

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RESUMO

No presente trabalho analisou-se a produção de amilase de *Fusarium moniliforme* 113F e de

Aspergillus flavus. Comparando-se o crescimento fúngico em 4 formulações constituídas por amido, glicerol, farelo de trigo ou milho; optou-se pela última formulação determinada a partir do crescimento de *F. moniliforme* 113F em diferentes frações de milho (germe, milho degerminado, amido de milho, milho verde e respectivo amido e caldo). O crescimento micelial máximo foi determinado no meio de cultura contendo caldo de milho verde ($p<0,05$), resultando na formulação de meio constituído por milho maduro e caldo de milho verde para avaliar produção de amilase. A maior produção de amilase ocorreu no 10º dia de fermentação, sendo que *F. moniliforme* atingiu produção de 42,32U/mL e *A. flavus* 4.745,54U/mL. Recomenda-se a formulação constituída por 2% de milho maduro triturado em caldo de milho verde (preparado pela homogeneização de 350g de milho verde + 250mL, seguido de centrifugação), para produção de amilase destes fungos.

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