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APPLICATION OF YEAST AS FEED ADDITIVES AND BIOCONTROL AGENTS FOR THE CONTROL OF MYCOTOXIGENIC *Aspergillus flavus*

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ABSTRACT

The contamination of animal feed with mycotoxigenic fungi continues to be a disastrous problem along the food chain. *Pichia anomala* was applied as a biocontrol yeast to prevent *Aspergillus flavus* growth in vitro and in feed corn. The yeast strain able to inhibit *A. flavus* growth and the enzymes assay indicated that *P. anomala* was able to produce β-1,3-glucanase and chitinase which could propose a mode of action for its antifungal activity. Scanning electron micrographs, of fungal hypha cultures with *P. anomala,* revealed that yeast cell could colonize the fungal hypha leading to their lysis and deterioration. The application of *P. anomala* as a protein supplement resulted in apparent increase in protein content in feed grain and animals' weight gain, in addition to a remarkable decrease in the mortality rate among fed animals after consumption of *P. anomala* -supplemented feed.

Keywords: Yeast killer toxin ,biological control , *pichia anomala*, *Aspergillus flavus*.

INTRODUCTION

Aflatoxins, the most well-known class of mycotoxins, are mainly produced by the fungus *Aspergillus flavus*. Aflatoxins are the most potent chemical liver carcinogens known. Moreover, the combination of aflatoxin with hepatitis B and C, which is prevalent in Asia and sub-Saharan Africa, is synergistic, increasing more than tenfold the risk of liver cancer compared with either exposure. Aflatoxins are also associated with stunting in children and possibly immune system disorders (Miller*,* 1994).

Aflatoxins contaminated feed-stuffs are a potential risk for the consumer because of their residues in meat- and milk-products. They may contaminate dairy products by molds growing on them, or by the carry-over of mycotoxins occurring in animal feedstuffs ingested by dairy cattle, aflatoxin M_1 is the hydroxylated metabolite of aflatoxin B_1 that may be found in milk or milk products obtained from livestock that have ingested contaminated feed (van Egmond, 1983). Acute toxic syndromes and even fatal poisoning of unknown etiology have been observed in beef, dairy and sheep that consumed moulded silage (Le Bars and Le Bars 1989; Driehuis et al., 2008).

Aspergilli are the most common fungal species that can produce mycotoxins in food and feedstuffs (Reddy et al., 2009)*. As. flavus* is the main producer of aflatoxins B1 and B2 which are hydroxylate metabolized to aflatoxins M1 and M2 in many ruminant bodies and may be found in their milk or milk products after ingestion of contaminated feed. Aflatoxin M1 is

cytotoxic, as demonstrated by the results of in vitro studies in human hepatocytes, and its acute toxicity in several species is similar to that of aflatoxin B1.

Contamination of various food and feed stuffs and agricultural commodities with mycotoxins is a major problem especially in the tropics and sub-tropics, where climatic conditions and agricultural and storage practices are favorable for fungal growth and toxin production (Fung and Clark, 2004).
Animal feed can become contaminated with a group of fungal-produced mycotoxins, which are harmful to humans and animals at low concentrations.
Aflatoxin contamination in corn (*Zea mays* L.) is, also, a chronic problem across the world that limits maize marketability and causes economic losses. The majority of these sources of resistance lack agronomic performance, which precludes their direct use in commercial hybrids. In addition, no competitive commercial hybrids are available that are resistant to aflatoxin (Chelkowski, 1991; Desjardins and Hohn, 1997)

Since the prevention is always better than cure, so that the general aim of this study is to prevent the contamination of milk, dairy and meat products with aflatoxins via the elimination of mycotoxigenic *A. flavus* growth in animal feed and consequently secretion of their aflatoxins.
Different yeasts are part of the naturally occurring flora on cereal grain (Fleet, 1992). The presence of yeast in feed is not always deteriorative; on the contrary, the yeast Pichia anomala has been shown to inhibit mold growth in malfunctioning airtight storage systems (Fredlund *et al*., 2002).

Pichia anomala is among the naturally occurring yeast on grain and is already favored by the conditions in the silo. Many studies have even seen that naturally occurring *P. anomala* in non-inoculated silos grows to the same levels as in inoculated systems (Murphy & Kavanagh, 1999; Fredlund et al., 2004). At this point, competitive displacement is certainly a mode of action of the biocontrol effect and the risk for non- target effects within the closed silo environment should be low. However, it is important to take precautions during production, formulation and inoculation to avoid overexposure of personnel.

However, the current study aimed to evaluate *Pichia anomala* as a biocontrol agent against *A. flavus* contamination in corn grains, for animal feeding, and to explore the antifungal mode of action of this antagonistic yeast as well as its value to increase animals' performance after feeding.

MATERIALS AND METHODS

Microorganisms

The yeast strain used in this study, *Pichia anomala* DBVPG-3003, was maintained in YEPD media (1% yeast extract, 2% glucose, 2% peptone, and 1.8% agar when required). Fungi were isolated using a protocol developed by Skaar and Steinwig (1996) with some modifications. Fifty grams of apparently molded maize grains were ground with a stein mill (Fred Stein Lab, Inc., Atchinson, KS) and 10 g portion of the sample were soaked in 50 mL of sterile 0.9% aqueous NaCl solution (w/v) for 30 minutes at room

temperature. After incubation, the mixture was manually shaken for 1 minute and filtered through four layers of cheese cloth. The resulting suspension was diluted to 10^{-3} with sterile 0.9% NaCl solution and 100 µL of the diluted solution was spread onto a PDA plates. Plates were incubated for 7 days at 25 °C. During this period plates were examined and individual fungal colonies were transferred at first observation to PDA slants. The selected fungi isolates were identified, according to Klich and Pitt, 1988; Klich, 2002.

***In vitro* yeast- fungi direct interaction**

The interaction of yeast with the fungal pathogen hyphae was assessed in Petri dishes containing yeast malt extract agar media (YMA). On the surface of agar, *A. flavus* was inoculated as a single streak in the middle of the plate, and then the yeast cells were inoculated as two spots at the margin of the plates and incubated for 3 days at 28 °C until the growth take place.

Mode of Action of the Antagonistic Yeast

Enzyme Assay

P. anomala was cultured in (YMB) with glucose as the sole carbon source. A 250 ml flask containing 100ml culture media was incubated on a rotary shaker at 200 rpm at 28°C for 3 days. Culture filtrate was harvested by centrifuging at 6000 x g for 5 min, and the supernatant was used for enzyme assays.

β-1,3-glucanase activity was assayed by measuring the ability of *P. anomala* secretions to degrade β-1,3-glucan. A reaction mixture was prepared by adding 1gm of β-1,3-glucan (Sigma) + 2gm of agar in 100ml distilled water and shaken well until completely melting, then media was poured in petri dishes and left to solidify. After solidification, pores were made on each plate by cork poorer and inoculated each pore by stable amount of filtrate and left the plates for 20-30 min. After that it was stained with congo-red (1%) (Which stain the polysaccharides with red color and does not react with monosaccharide) left for 30 sec, then wash thoroughly with a solution of Nacl (15gm of Nacl+500ml distilled water) until developing clear zone.

For the chitinase assay a reaction mixture was prepared by the same method of β -1,3-glucanase assay, but instead of glucan, chitin was used, (knowing that chitin is not soluble in water even after boiling).

Scanning Electron Microscopy

This method was carried out to further observe the direct interaction between fungi and yeast. Also antagonism was carried out in YMB by inoculating the broth firstly with *P. anomala*, and then *A. flavus* was inoculated. After shaking incubation for 24 h at 28°C, a touch was taken from the broth on magnetic slides and coated with gold-palladium for cell interaction assays. After that, samples were dehydrated in a graded ethanol series, critical-point dried with CO_2 and coated with gold-palladium for cell interaction assays. The tissues were then viewed using a Hitachi S-800 SEM, Japan.

Determination of protein content in yeast supplemented feed

To evaluate the efficiency of biocontrol yeast as feed additives to increase the protein content in corn based feed, 3 days old *P. anomala*

cultures in YMB were added at rates of 2, 3 and 4 mg / g of experimentally contaminated corn grain with *A. flavus*.

The total protein, calculated from amine nitrogen percentage, in corn grains, either in control group or after addition of yeast culture, was determined using Kjeldahl protocol according to AOAC official methods (2000).

Animals and experimental diets

The experiment was made using eight weeks old male CD-1 mice weighing 18 ± 1.5 g. The animals were maintained at 23 °C in polypropylene cages (six individuals per cage) in a 12 h were fed according to the indicated experimental diets. Iterations (mice) 12.and Tretment groups 4.

Preparation of diets: the diets of the studied animals were prepared with corn (90%) and the recommended mixture complex of vitamins, minerals, and proteins (National Research Council, 1978); then, the selected concentration of *A .flavus* and/or biocontrol yeast (*P. anomala*) were added to the diet.

The experimentally contaminated corn (ECC) was formulated by inoculating ~1 X $10^{1°}$ from *A .flavus* spores / kg of corn and keeping them at 25°C in loosely closed polypropylene pages(one kg per page) in a 70 ± 10% humidity for 21 days before mice experiment.

Mice were fed 6 weeks and after that, the average weight gain was calculated for each group as well as the mortality percentage. Weight gain was calculated as (Wf - Wo / Wo) X 100, where Wf is the mean final weight at the end of the trial and Wo is the mean beginning weight of each group.

*Mortality rate, for each group was calculated at the end of the trials as:

$$\frac{\text{Number of dead animals}}{\text{Total number of the group}} \times 100$$

RESULTS AND DISCUSSION

Examination of the slide culture under light microscope showed a typical conidial heads and the shape of colony (Fig. 1). On the PDA medium, it was observed a colorless mycelium at the periphery of the colony and a green central part of the colony which referred to the presence of conidia. All these features, in addition to physiological examinations, indicated that the isolated fungus is "*Aspergillus flavus".*

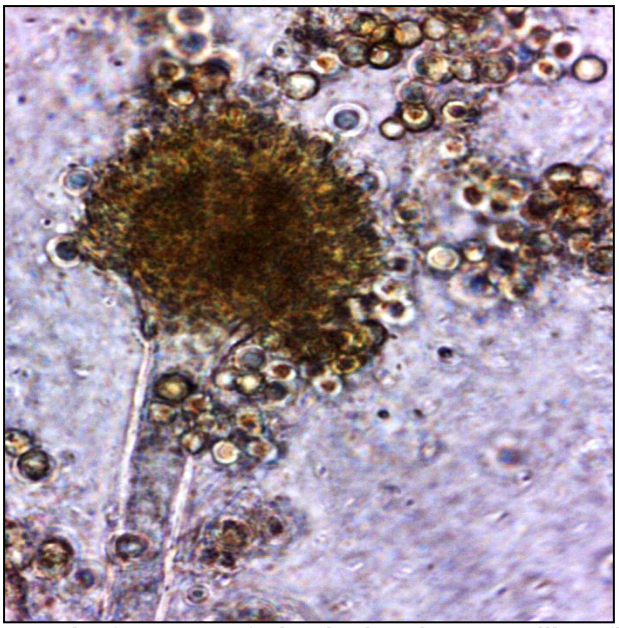

Fig.1: Microscopic features of the isolated *Aspergillus flavus* using slide culture technique under light microscope.

The antagonism experiment between the biocontrol yeast *P. anomala* and the pathogenic fungus *A. flavus*, on Petri dish containing YMA, revealed that *P. anomala* could effectively prevent the fungal growth as proved by the empty zone surrounding yeast culture (Fig. 2)

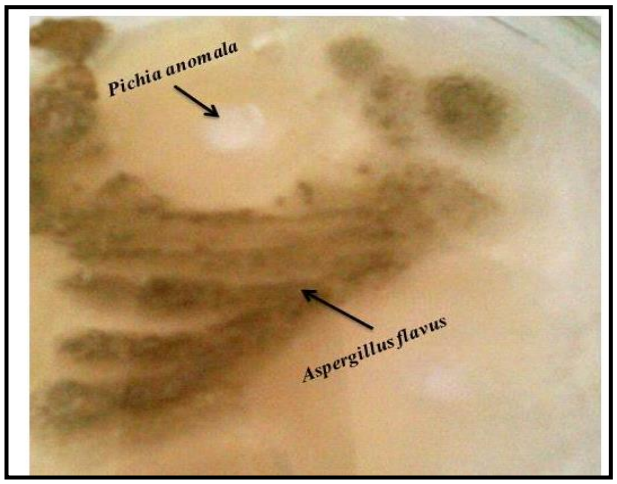

Fig. 2: The antagonism between the biocontrol yeast *P. anomala* and the pathogenic fungus *A. flavus*

P. anomala could produce β-1, 3-glucanase in medium supplemented with β-1,3- glucan, as a sole carbon source. The appeared clear zone increased with incubation time and when the concentration of stain was very low (1%) (Fig. 3a).

Chitinase activity could be, also, detected for *P. anomala* cultured on medium containing chitin (Fig.3.b). The appeared clear zones could be easily visualized under day light and confirmed the yeast production of both β-1, 3-glucanase and chitinase enzymes.

It was reported that mycoparasitism; destruction or alteration of the hyphae of the pathogen, involving physical contact and predation, followed by enzymatic lysis. Extracellular glucanases and chitinases possess antifungal activity and are involved in mycoparasitism (Castoria *et al.,* 1997; Jijakli & Lepoivre, 1998).

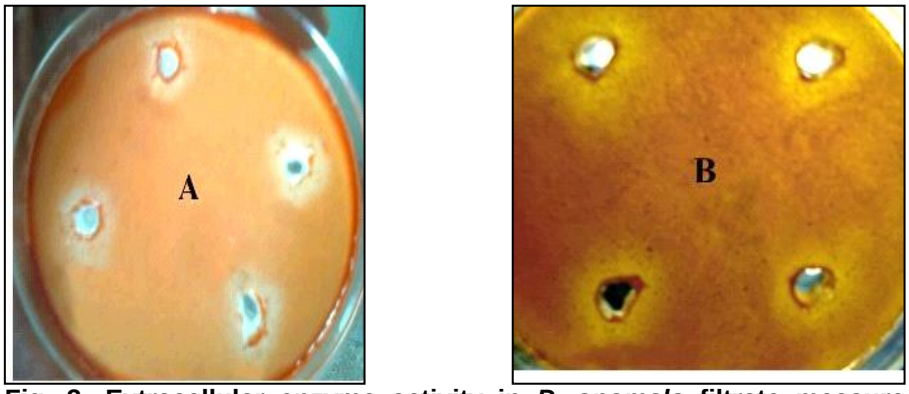

Fig. 3: Extracellular enzyme activity in *P. anomala* filtrate measured using Congo red staining. a: β-1,3-glucanase activity , **b:** Chitinase activity

For further clarification of *P. anomala* mode of action as biocontrol agent against *A. flavus,* the SEM imaging was applied. The strong hyphal colonization produced by *P. anomala* was appeared using an SEM to obtain their attachment in depth and to understand the possible mode of action of this yeast in suppressing the pathogen. The SEM examination showed adherence between hyphae of *A. flavus* and *P. anomala*. In some areas, *A. flavus* hyphae were totally surrounded by the yeast cells (Fig.4b). In other regions, the hyphae of *A. flavus* were totally penetrated and destroyed by the action of the antagonistic yeast (Fig.4c). In captured micrographs, healthy fungal hypha was appeared with normal shape and size (Fig.4a).

Harmonized results were obtained by Chan and Tian (2005); they indicated that *P. anomala* had a stronger capability for attachment to the fungal hyphae of *Monilinia fructicola, Penicillium expansum* and *Rhizopus stolonifer* than did *Candida albidus*.

The determination of protein content in corn-based feed fortified with *P. anomala,* as protein supplement, indicated a gradual increase in content from 7.11%, for the unfortified corn, to 9.25%, for the corn fortified with *P.*

anomala at supplementation rate of 4mg/kg (Table 1). The calculated percentages of protein increasing in the feed were 16.74, 21.65 and 30.10% for the corn feed supplemented with 2, 3 and 4mg/kg, respectively.

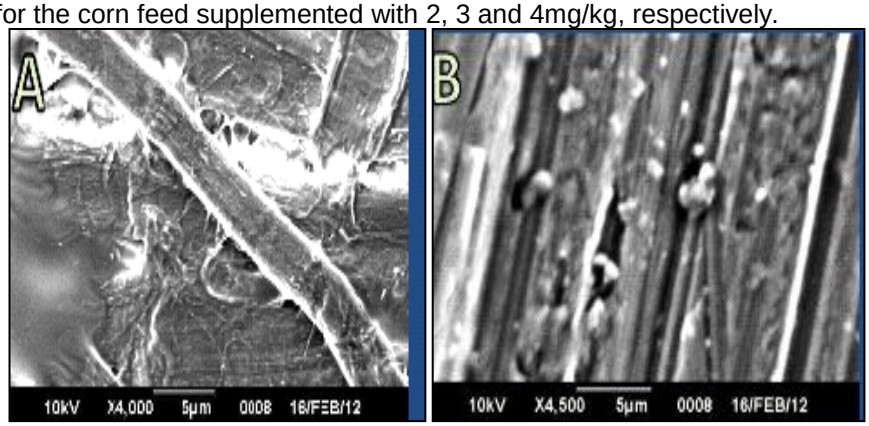

Fig. 4: Scanning electron micrographs of interacted *P. anomala cells* with *A. flavus* after incubation together for 24 h at 28°C.
A: Control A. flavus mycelium B and C: Cultured fungal mycelia with *P. anomala*

Table 1: Protein content in supplemented feed with *Pichia anomala*

Treatment	Protein content %	Protein increase %
Control Corn without yeast	7.11±0.17	0.0
Corn + *P. anomala* (2mg/kg)	8.30±0.20	16.74
Corn + *P. anomala* (3mg/kg)	8.65±0.21	21.65
Corn + *P. anomala* (4mg/kg)	9.25±0.23	30.10

In addition to its growth promotion capability, *P. anomala* could efficiently prevent animals from mortality after feeding with contaminated feed. Results in Table 2 indicated that the mortality rate was lower, in uncontaminated corn, than in experimentally contaminated corn (ECC). Feed supplementation with *P. anomala* resulted in an apparent increase in weight gain and decrease in mortality rate. This could be considered, especially in ECC, as an indication of *P. anomala* effect on controlling fungal growth and its harmful consequences in animals. It was reported that *P. anomala* as a biocontrol yeast was able to prevent growth of spoilage molds (Björnberg & Schnürer, 1993) and *Enterobacteriaceae* in stored feed grain. *P. anomala* has, also, a considerable phytase activity and that could enhance the biological value of feed grains (Vohra and Satyanarayana, 2001).

Table 2: Influence of feed supplement with Pichia anomala on the performance of treated animal

Treatment groups	Treatment designation	Weight gain %	Mortality rate%
A	Uncontaminated corn (control).	64.8	8.33
B	Experimentally contaminated corn (ECC)	43.7	41.67
C	Uncontaminated corn and 0.3 % of *P. anomala* cells (~1*10⁸ live cells/g).	65.1	0.0
D	ECC+ 0.3 % of *P. anomala* cells.	53.6	16.67

*** Results are the mean values in each group.**

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تطبيق الخميرة كأضافات غذائية وعوامل المكافحة البيولوجية للسموم الفطرية

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تلوث العلف الحيواني بالفطريات لا تزال مشكلة قائمة ووخيمة على مر السلسلة الغذائية ولقد تم تطبيق الخميرة *Pichia anomla* للمكافحة الحيوية في التجارب المختبرية لمنع نمو الفطر *Asp.Flavus* . و أوضحت سلالة هذه الخميرة أنها قادرة على انتاج انزيمين هما: β-1,3-glucanase and chitinase.

هذان الانزيمان أقترحا كطريقة عمل لنشاط الخميرة كمضادات للفطر.

وبالفحص الميكروسكوبي الالكترونى تم الكشف على الهايفات الناتجة من الفطر وأيضا الكشف على تثبيط الخميرة للفطر . هذا التثبيط يؤدى بدوره الى تحلل وتدهور تلك الهايفات.

وبتطبيق هذه الخميرة أوضحت النتائج أنها مكملة وتزيد نسبة البروتين لحبوب العلف كما تعمل على زيادة وزن حيوانات التجارب بالإضافة إلى انخفاض ملحوظ فى معدل الوفيات لتلك الحيوانات عند تغذيتها بالخميرة .

قام بتحكيم البحث

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