Screening of Aflatoxigenicity of *Aspergillus flavus* Strains in Artificially Contaminated Yoghurt Samples

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**Abstract:** The aim of the present study was to experimentally investigate the toxicity of *Aspergillus flavus* (*A. flavus*) strains in yoghurt. The inoculated strains were isolated from cheese and yoghurt. The inoculated conidial count was adjusted at 2-5 x 10⁷ ml. Results revealed that all tested strains were toxigenic and produced aflatoxins with different concentrations as measured by HPLC. The highest toxin concentration was Aflatoxin B₁ (134.77 ppm). According to these findings it could considered that *A. flavus* at different concentrations was able to producing aflatoxins if yoghurt kept over 5°C. Therefore, yoghurt must be *A. flavus* free and kept in refrigerator from production till consumption.

**Key words:** Aflatoxins • *Aspergillus flavus* • Experimental contamination yoghurt

**INTRODUCTION**

Moulds are microscopic organisms that live on plant or animal matter. No one knows how many species of fungi exist, but estimates range from tens of thousands to perhaps 300,000 or more [1].

Mycotoxins are secondary toxic by-products (not essential for survive) of some moulds produced under certain conditions to achieve a competitive advantage for mould over other mould species and bacteria. It is cytotoxic, heat stable toxin for all living cells including higher organisms’ cells [2]. Among mycotoxins aflatoxins considered as the most harmful toxin to human body, these aflatoxins are produced mainly by *Aspergillus flavus* (*A. flavus*), *A. parasiticus* and some other species of *A. niger* or *A. nomius* [3].

Contamination of human food especially milk and dairy products with *A. flavus* even in trace counts must not pass for consumption, because *A. flavus* even in small counts can multiply and produce aflatoxins if food did not kept in refrigerator.

Therefore this work is adapted for screening of toxicity or toxogenicity of *A. flavus* in food samples as a one of most important species which has public health significance in toxin production.

**MATERIALS AND METHODS**

**Experimental Technique**

**Fungal Strains:** sixteen isolates of *A. flavus* were isolated from cheese and yoghurt samples from previous work [4].

**Media:** *Aspergillus flavus* Differentiation Medium (ADM) was used to select toxin producing isolates (orange colour at colony biases). Czakap Yeast agar (CYA) was for subculturring of *A. flavus* at three points for 7 days of incubation at 25°C.

**Inoculation:** Ten ml of distilled water containing 1cm² of toxogenic *A. flavus* colony were homogenized and inoculated (after counting of conidia in Thoma chamber and dilution was adjusted to have spores count range of 2:5x10⁷). Using sterile syringe, yoghurt samples were inoculated and then examined within the shelf-life. Total *A. flavus* count was done before inoculation of mould and 10 days after incubation [5].

**Aflatoxin Detection:** Preparations of samples were carried out according to Herzallah [6].
Extraction: A 20 g subsample was extracted with 40 ml acetonitrile and water with ratio (9: 1) using a mechanical shaker for 30 min.

Cleanup: A 2 ml portion of the filtrate was applied to an IMC (ISOLUTE TM Multimode Column, 3 ml capacity, 50 mg mass) that was passed through at a flow rate of 2 ml/min followed with 5 ml acetonitrile with or without water (9: 1) to completely elute the AF. The combined elute was evaporated to dryness and redissolved in 1 ml methanol by sonication and kept in an amber capped vial in a refrigerator at 4 °C until quantitated by HPLC.

Derivatization: An aliquot (300 µl) of sample solution was transferred directly into a HPLC vial and evaporated in a dryer block. n-Hexane (200 µl) and TFA (trifluoroacetic acid) (50 µl) were added to a dried extract, capped and mixed with a vortex mixer for 30 s. The mixture was kept for 5 min, after which 550 µl water± acetonitrile (9:1) was added and again mixed for 30 s with vortex. The layers were allowed to separate for 10 min. After pipetting out the upper hexane layer, the required volume of aqueous layer was auto injected on to the HPLC column.

Determination of Aflatoxin by HPLC: Aflatoxin was determined using an Agilent 1260 HPLC with a fluorescence detector, operated at an excitation and emission wavelength of 360 and 440 nm, respectively and a 250 x 4.6 mm id Zorbax C18 XDB column with 5 µm particle size. The flow rate was 1.0 mL/min with an oven temperate of 25°C and an injection volume of 20µL. The mobile phase consisted of acetonitrile: methanol: water (13:22:65 v/v/v). The residues in the real samples were tentatively identified by comparing the retention times (RTs) of the sample peaks with the RTs of the injected standards.

Experimental Conditions

Standard AFL: AFLS standard (B₁, B₂, G₁, and G₂) from Dr. Ehrenstorfer, Augsburg, Germany.

HPLC Conditions:
Column: Zorbax C18 XDB column with 5 µm particle size
Flow Rate: 1.0 mL/min
Temperature: Ambient (25 °C)
Detector: UV 360 and 440 nm
Injection volume: 20µL

RESULTS AND DISCUSSION

In referring to our previous work Khalifa et al. [4] sixteen isolates of A. flavus obtained from examined cheese and yoghurt samples were screened for their ability to produce aflatoxins on food substance. We selected 16 isolates from the 3 types of samples (feta, istanboli cheese and yoghurt) which shown toxon production on Aspergillus Differentiation Medium (ADM) to ensure its accuracy; the inoculated conidial count was adjusted at 2-5 x 10⁸ ml to examined the toxicity of A. flavus in yoghurt if it was contaminated by 2 x10⁷ ml and kept out of refrigerator till the end of shelf-life.

According to Table (1) all of the isolates were toxigenic and produced aflatoxins (B₁, B₂, G₁ and G₂) in different concentrations as assessed by HPLC method. Moradi et al. [7] detected 333 of 350 A. flavus isolates as aflatoxin producers, Dutta and Das [8] found 76% of 198 A. flavus strains isolates, were be toxigenic. The yoghurt before inoculation showed no detectable levels of aflatoxins (results not presented).

Isolates of A. flavus produced aflatoxin B₁ ranging from 6.59 to 134.78 ppb, AFB₂ was detected in 12 isolates ranging from 2.97 to 5.12 ppb, AFG₁ was positive in 14 isolates with average of 2.37 to 9.53 ppb while, AFG₂ was produced by all strains in level laying between 1.49 to 4.79 ppb.

Comparatively similar findings were detected in dairy and food products by Aycicek et al. [9] who detected AFB₁ contamination in samples in a range of <1 to 10 ppb and from <1 to 13 ppb, also, Youssefi et al. [10] found A. flavus isolets producing aflatoxin B₁, ranging from 0.32 to 12.18 ppb, while 2.3% of isolates produced 18.88 ppb and 0.36 ppb of aflatoxin B₁ and aflatoxin B₂ respectively.

However, higher results were obtained by Zerfridis [11], Hassanin [12] and Aaid [13] in cheese. El-Shanshoury et al. [14] detected AFB₁ in contents ranged between 427- 466 µg/kg in cereal grains and peanut and Arrus et al. [15] found 4483 ng/g of AFB₁ in Brazilian nuts kept at temperatures of 25-30°C.

It is cleared that not all isolates of A. flavus were capable of producing all types of aflatoxins, 10 of 16 isolates produced the 4 aflatoxins [16 and 14] while 2 isolates produced AFB₁ and AFG₂ [17] and AFB₂ not produced by 2 isolates only it is depends on the particular fungal strains and on a particular growth conditions. Wei and Jong [18] found 2 of 9 A. flavus strains were capable of producing 4 types of aflatoxins and 9 (100%) could form AFB₁. Moreover, Rodrigues et al. [19] able to detect AFBs and AFGs in 8% of A. flavus isolates.
Table 1: Screening of aflatoxigenicity of isolated A. flavus strains in artificially contaminated yoghurt samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Before inoculation</th>
<th>After inoculation</th>
<th>Total Aspergillus flavus count/ g</th>
<th>Aflatoxins concentration ppb</th>
</tr>
</thead>
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<td></td>
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<td></td>
<td>B&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>1</td>
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<td>1 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8.57</td>
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<td>7 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
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CONCLUSION

Increasing temperature during storage of yoghurt resulted in an increase in aflatoxin production ability of A. flavus especially with high relative humidity. Therefore yoghurt must be kept in refrigeration temperature till consumption.

REFERENCES


