Influence of Different Moisture Contents and Temperature on Growth and Production of Aflatoxin B₁ by a Toxigenic Aspergillus flavus Isolate in Wheat Flour

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Abstract: Experimental short time population and production of aflatoxin B₁ by Aspergillus flavus in wheat flour at 20, 25, 30, 35 and 40°C, as related to different moisture contents 5%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, 22% and 25%, was studied. Spore suspension of a toxigenic A. flavus containing ~ 3x10⁵ spore/ml was inoculated in each flask containing 50 g wheat flour and incubated for 15 days. Spore count was observed slightly in flour with a moisture content 5% at all incubation temperature, then increased gradually by rising the moisture content reaching the maximum at 30°C with moisture content 25%. High performance liquid chromatography analysis of AFB₁ showed the lowest quantification (482 ng/kg) at 20°C with 8% moisture content in comparison to uninoculated control. The higher quantity of AFB₁ (637835.4 ng/kg) was produced at 25°C with 25% moisture content. Accumulation of high amounts of AFB₁ was noticed at 20, 30 and 35°C with moisture content 25%. However, at 40°C with moisture contents 22% and 25%, high spore count was recorded, but traces of AFB₁ were produced. The significance of this investigation is that, in the event of wheat flour contamination with aflatoxigenic species, rise in moisture contents are hazardous in the storage of wheat flour around 25°C.

Keywords: Moisture contents, temperature, Aspergillus flavus, aflatoxin B₁, wheat flour.

1 Introduction

Aflatoxins (AFs) are secondary metabolites biosynthesized by numerous species of Aspergillus, especially A. flavus and A. parasiticus [1]. Not all A. flavus and A. parasiticus strains are aflatoxins producers, but about 60% of isolates were producer [2]. There are four major types of aflatoxins; B₁, B₂, G₁ and G₂ based on their fluorescence under UV light, and their two metabolites M₁ and M₂. However, the most harmful mycotoxin at present known is AFB₁. Naturally A. flavus produces aflatoxins AFB₁ and AFB₂, while A. parasiticus produces the four aflatoxins types [3]. Aflatoxins are highly toxic, mutagenic and carcinogenic compounds and chemically, they are a group of difuranocoumarin derivatives [4].

Aflatoxins biosynthesis is the outcome of a mix of species, substrate and environment. The factors influencing AFs production can be divided into three categories: physical, nutritional and biological factors. Physical factors include temperature, pH, relative humidity of the atmosphere, water activity, moisture, light, aeration and level of atmospheric gases [5]. The development of A. flavus and aflatoxin biosynthesis relies upon favorable conditions of temperature and humidity [6]. The conditions conducive to germination, growth and aflatoxin production by A. flavus and A. parasiticus demonstrate that germination happens over a wider range than that for growth, with the aflatoxin production range yet narrower than that for growth [7].

Optimal conditions of nutrition and physiological parameter changes for various strains [8]. There are numerous examinations about the best conditions for fungal development and toxin biosynthesis. Some contrasting...
results obtained are due to the diversity of strains, availability of supplements and structure of the utilized media [9, 10, 11, 12]. Generally, the ideal temperature for aflatoxin production is 28-30°C, and production reduces at temperature beneath 25°C and approaching 37°C. Better fungal growth and toxin production are observed at higher water activity values. Growth and spore germination rates slow at water activity (aw) below 0.85 and are completely inhibited at range between 0.70-0.75 aw [13, 14].

Wheat flour is a powder produced from the grinding of wheat used for human utilization and all its items can be contaminated by molds at all phases of the production chain [15]. Flour is a very hygroscopic material and its moisture content varies with the changes in temperature and humidity of the store environments [16]. Moisture content of flour is essential in regard to its shelf life, lower the flour moisture, the better its storage stability [17]. Wheat contamination with moulds and aflatoxins has been reported in several investigations [18]. When the storage conditions in grains storehouses are not standard, this toxin will be accumulated on them [19]. Conditioning wheat to increase the moisture content to a level appropriate for milling can also increase the counts of microbial contaminants into flour [20]. In the present research, population and production of aflatoxin B₁ by Aspergillus flavus in wheat flour at different temperature as related to different moisture content levels were studied.

2 Materials and Methods

2.1 Experiment Implementation

One isolate belonging to the genus Aspergillus: A. flavus was used for inoculation in these experiments. This toxigenic isolate was isolated from bakery's flour and maintained at 4°C on slants of Czapek's agar. Bakery's flour with moisture content 10.1% was used and kept at 4°C. About 50 g of wheat flour were put in 250-ml Erlenmeyer flasks, autoclaved for 30 minutes, then dried in a hot air oven at 105°C for 24 hrs to get a zero moisture content level. According to Rambo et al. [21] and Chang and Markakis [22] with some modification, the flour within flasks were rehydrated to achieve the required moisture content (MC) levels (5%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, 22% and 25%) by addition of sterile distilled water and inoculated with 1 ml toxigenic Aspergillus flavus spore suspension (~ 3x10⁸ spore/ml). Five sets of flasks were prepared as the same in addition to a sixth set of uninoculated flasks as a control. Each set of flasks with different moisture content levels were incubated at different temperature 20°C, 25°C, 30°C, 35°C and 40°C for 15 days. The control set of flasks was incubated at 30°C.

2.2 Spore Count

This was made by using "dilution-plate method" for the quantitative determination of fungi as described by Johnson and Curl [23]. Glucose-Czapek’s agar medium [24] was used in this study.

2.3 Detection of Aflatoxin B₁ Production

Each flask contents were defatted with n-hexane then extracted with chloroform. The extracts were dried over anhydrous sodium sulphate, filtered then concentrated under vacuum to near dryness [25]. Extracts were purified and examined for aflatoxins using the TLC technique as reported by Hans and Walter [26], El-Gohary [27], Bragulat et al. [28] and Samson et al. [29] with some modifications. The HPLC analysis of AFB₁ was done according to Bakirci [30] and Manetta et al. [31] using a UHPLC system (Shimadzu, Kyoto, Japan).

3 Results and Discussion

Experimental short time population and production of aflatoxin B₁ by Aspergillus flavus in wheat flour at 20, 25, 30, 35 and 40°C as related to 10 different levels of moisture contents (5%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, 22% and 25%) was studied. Spore suspension of A. flavus containing ~ 3x10³ spore/ml was inoculated in each flask containing 50 g wheat flour and incubated for 15 days, the initial population was ~ 60 CFUs/g. Spore count, TLC and HPLC analysis for the detection and quantification of AFB₁ were carried out. The results revealed that high populations of A. flavus were recorded at 30°C, while high levels of AFB₁ production were noticed at 25°C. Regarding to spore count, the data showed an increasing in spore counts under all temperature treatments by rising of the moisture contents and growth was observed at all levels of MC. Data were expressed log₁₀ CFUs g⁻¹ flour (Figure 1).

![Figure 1. Effect of different temperature and moisture contents on A. flavus spore count/g in wheat flour. Data](image-url)
were expressed (log_{10} CFUs/g flour).

At 20°C, initial spore count was 250 CFUs/g at 5% MC, while at 25% MC the spore count was 30×10^{6} CFUs/g. Spore count was highest (78.5×10^{7} CFUs/g) at 25°C and 25% MC, while the maximum spore count was 11×10^{10} CFUs/g under 30°C and 25% MC. At 35°C and 40°C and 25% MC, the A. flavus populations were 26.5×10^{7} and 20×10^{6} CFUs/g, respectively Table 1.

Our results concur with Borut and Joffe [32] who noticed a relationship between moisture and fungal growth and aflatoxins production in wheat flour. The conditions conducive to germination, growth and aflatoxin production by A. flavus demonstrated that germination occurs over a wider range than that for growth, with the aflatoxin production range yet narrower than that for growth [33]. Hill and Lacey [34] and Romo et al. [35] found that the lowest limit for growth of A. flavus happened at moisture contents ranging from 18 to 25%. Likewise, the data recorded by Park and Bullerman [36], indicated that growth of A. flavus occurs at 7.5 to 40°C with optimal growth around 25°C. Northolt et al. [37] stated that the water activity did not have an indistinguishable impact on aflatoxin production as it did on growth, and it is the most imperative factor for aflatoxin production but not for mycelial development. Slight growth is conceivable at low water activity without verifiable formation of aflatoxins [35].

Northolt et al. [37, 38] and Niles et al. [39] utilized water activity to express the MC of food as an index of water available for fungal growth, and have studied the development and aflatoxins accumulation in relation to this parameter. Water retaining properties of the substrate played an imperatively role, since the more hygroscopic the material, the lower the relative humidity at which the fungal development began [40]. Water is one of the end products of respiration (metabolism of microbes) which take in the moisture content inside the storage structure [41, 42]. Al-Defiery and Merjan [15] detected A. flavus in wheat flour samples after 3 months of storage at 10.7% humidity.

Similarly, Hussaini et al. [43] found that sorghum stored in the moist environment was more highly contaminated by A. flavus than that stored in the dry environment. Kusumaningrum et al. [44] exhibited that relative humidity can influence the growth of A. flavus in maize significantly. Moulds were observed on rice with more than 14.4% moisture content at 25°C, yet no form was observed on rice with less than 12.8% MC [45]. The constraining moisture level demonstrated by Lopez and Christensen [46] for growth of A. flavus was 17.5%. Al-Defiery and Merjan [15] indicated that storage at a temperature of 5°C diminished the population and sorts of molds on wheat flour. It is important to prevent molds growth by decreasing the temperature and moisture content [47]. The presence of moulds, however, does not really imply that the stored material is contaminated with mycotoxins. It is outstanding that mycotoxin production occurs just when the moulds are present under favorable conditions [48].

Concerning for AFB1, the results indicated that AFB1 levels in wheat flour at all levels of moisture contents was high at 25°C followed by 30°C, 20°C, 35°C and 40°C, respectively. As in spore count, AFB1 production was increased proportionally with moisture content. Data were expressed as log_{10} ng/kg flour Figure 2.

In non-inoculated wheat flour, AFB1 levels were around 73.18 ng/kg. Aflatoxin B1 production was higher at 25°C than at 30°C; however spore count at 30°C was more than at 25°C. Aspergillus flavus inoculated in wheat flour with 5% MC allowed AFB1 accumulation under 25°C, 30°C and 35°C, while AFB1 production at 20°C and 40°C started with 8% MC. Although AFB1 was detected in wheat flour under all temperature treatment as well as A. flavus populations, only at 40°C traces of AFB1 were detected in comparison to other temperature treatments. Thin layer chromatography analysis showed positive results starting at AFB1 concentration with 14% MC at 35°C. Below this corresponding concentration of AFB1, TLC analysis provided negative detection results. The lowest amounts of AFB1 detected by HPLC in inoculated wheat flour at 20, 25, 30, 35 and 40°C with 5% MC were 44.8, 1322.2, 416.4, 451.6 and 101.4 ng/kg, respectively, while the highest AFB1 levels at 25% MC were 217800, 637835.4, 514847.6, 152154.8 and 1563.2 ng/kg, respectively Table 2.

The same results were observed by Diener and Davis [49] who obtained maximal aflatoxin amount following 11 and 15 days of growing at the temperatures of 20°C and 30°C, respectively. Jarvis [50] demonstrated that aflatoxins will not be produced at temperatures below 13°C or over 42°C. Muntanola-Cvetkovic [51] stated that, in order to produce
aflatoxins, it is necessary to satisfy the following criteria: moulds should be genetically capable of production, relative humidity should be 85%, water content 30%, temperatures for the production of AF 35°C. Toxin production by A. flavus in inoculated wheat flour increased with increment in moisture content of the flour and incubation temperature. A combination of 30% moisture and incubation temperature of 30°C enhanced

Table 1. Effect of different temperature and moisture content on A. flavus spore count/g in wheat flour.

<table>
<thead>
<tr>
<th>MC Temperature</th>
<th>5%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>14%</th>
<th>16%</th>
<th>18%</th>
<th>20%</th>
<th>22%</th>
<th>25%</th>
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<td>20°</td>
<td>250</td>
<td>6x10^3</td>
<td>5x10^4</td>
<td>5x10^5</td>
<td>1x10^6</td>
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<td>500</td>
<td>5x10^5</td>
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<td>30°</td>
<td>1000</td>
<td>5.5x10^7</td>
<td>9x10^8</td>
<td>19.5x10^9</td>
<td>26x10^9</td>
<td>30.5x10^9</td>
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<td>7x10^4</td>
<td>1x10^10</td>
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<td>1000</td>
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<td>8.5x10^4</td>
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Table 2. Effect of different temperature and moisture content on aflatoxins B1 production by A. flavus in wheat flour; detection by TLC and HPLC ng/kg.

<table>
<thead>
<tr>
<th>MC Temperature</th>
<th>5%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>14%</th>
<th>16%</th>
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<tr>
<td>20°</td>
<td>TLC</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
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<td>+ve</td>
<td>+ve</td>
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<td></td>
<td>HPLC</td>
<td>44.8</td>
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<td>580</td>
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<td>98853.4</td>
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<td>4616</td>
<td>5015</td>
<td>15372.4</td>
<td>25775.4</td>
<td>46039.6</td>
<td>243049.2</td>
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<td>-ve</td>
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<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
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<td></td>
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<td>4244.6</td>
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<td>9369.4</td>
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<td>-ve</td>
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<td>43.8</td>
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temperature of 25°C, and a sufficient medium is fundamental. Schindler et al. [52] obtained best production of aflatoxins in corn between 25 and 35°C. High MC with mean of 22.43% (minimum = 11.2% and maximum = 31.5%) could provide conditions to aflatoxins biosynthesis [53]. Similarly, Schindler et al. [52], Diener and Davies [54] and Northolt et al. [37, 38] reported optimum temperatures for the production of AFB1 range from 24 to greatest aflatoxins biosynthesis [55]. High amount of aflatoxin B1 was produced even initial spore inoculum level was low [56]. Nandi and Haggblom [57] demonstrated that the range of aflatoxin concentrations in rice following 30 days of storage was 2430-10643 µg kg⁻¹ for aflatoxin B1, and 205-737 µg kg⁻¹ for aflatoxins B2. Aflatoxins biosynthesis was higher at 21% moisture than at 18%, and
more aflatoxins was produced at 30°C than at 25°C. Levels of aflatoxin produced by *A. flavus* in maize samples stored at 8°C were about 10 μg kg⁻¹ following 45 days of storage at all moisture contents. Generally, at 28°C levels of aflatoxin significantly increased with increment moisture content [58]. Low temperature of 4°C and relatively high temperature of 42°C demonstrated decreased production of aflatoxins [59]. Milani [33] stated that optimum conditions for aflatoxins biosynthesis by *A. flavus* is at 33°C and 0.99 aw; while that for growth is 35°C and 0.95 aw. Rabie and Smalley [60] stated that the optimal temperature for the development of *A. flavus* and the aflatoxin production ranges from 18 to 24°C. Das *et al.* [61] stated that *A. flavus* is a mesophilic fungus which grows well on a temperature of 30°C and accordingly the production of aflatoxins is expected to occur at the same temperature. Optimal aflatoxin production is observed at temperatures near 30°C. When temperature increases to above 36°C, aflatoxin production is almost or totally restrained [62].

Concerning the physical factors, the optimal temperature of aflatoxins production is situated in the vicinity of 28°C and 35°C. Above this temperature range, biosynthesis is repressed due to the attack of transcription genes aflR and aflS [62, 63], while under the states of dryness, the biosynthesis of the aflatoxins is high because of drought stress [64]. *Aspergillus flavus* usually contaminate food products and synthesize aflatoxins as metabolites in the presence of elevated levels of carbohydrates and low levels of protein [65]. Aflatoxins production is dependable firmly associated with high-carbohydrate and high-fat food because *Aspergillus Section Flavi* have a large diversity of enzymes able to degrade these substrates, including proteases, lipases, amylases and pectinases [66]. By varying the combination of the parameters involved in AF biosynthesis, toxin production can be completely inhibited or fully activated. It is therefore fundamental to know which combinations can control or be conducive to aflatoxins production in crops/substrates [67, 68].

5 Conclusions

Based on the results of the current study, an increase in spore counts under all temperature treatments by rising of the moisture contents and growth was observed at all levels of MC. Aflatoxin B₁ production was increased proportionally with moisture content. However, only at 40°C traces of AFB₁ were detected in comparison to other temperature treatments. In stored flour, temperature should be kept under 20°C and low moisture content as possible.

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