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Influence of Different Moisture Contents and Temperature on Growth and Production of Aflatoxin B_1 by a Toxigenic *Aspergillus flavus* Isolate in Wheat Flour

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Abstract: Experimental short time population and production of aflatoxin B_1 by *Aspergillus flavus* in wheat flour at 20, 25, 30, 35 and 40ºC, 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 ~ 3×10^3 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 30ºC with moisture content 25%. High performance liquid chromatography analysis of AFB_1 showed the lowest quantification (482 ng/kg) at 20ºC with 8% moisture content in comparison to uninoculated control. The higher quantity of AFB_1 (637835.4 ng/kg) was produced at 25ºC with 25% moisture content. Accumulation of high amounts of AFB_1 was noticed at 20, 30 and 35ºC with moisture content 25%. However, at 40ºC with moisture contents 22% and 25%, high spore count was recorded, but traces of AFB_1 were produced. The significance of this investigation is that, in the event of wheat flour contamination with afltoxigenic species, rise in moisture contents are hazardous in the storage of wheat flour around 25ºC.

Keywords: Moisture contents, temperature, *Aspergillus flavus*, aflatoxin B_1, 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; B_1, B_2, G_1 and G_2 based on their fluorescence under UV light, and their two metabolites M_1 and M_2. However, the most harmful mycotoxin at present known is AFB_1. Naturally *A. flavus* produces aflatoxins AFB_1 and AFB_2, 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

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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 (*a*w) below 0.85 and are completely inhibited at range between 0.70-0.75 *a*w [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 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 B_1 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 (~ 3×10^3 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 B_1 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 AFB_1 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 B_1 by *Aspergillus flavus* in wheat flour at 20, 25, 30, 35 and 40ºC 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 ~ 3×10^3 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 AFB_1 were carried out. The results revealed that high populations of *A. flavus* were recorded at 30ºC, while high levels of AFB_1 production were noticed at 25ºC. 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 $\log_{10}$ CFUs g^{-1} flour (Figure 1).

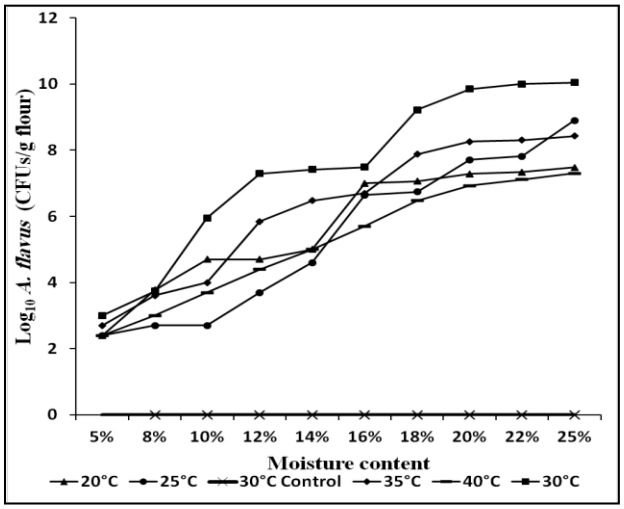

Figure 1. Effect of different temperature and moisture contents on *A. flavus* spore count/g in wheat flour. Data

were expressed (log_{10} CFUs/g flour).

At 20ºC, 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 25ºC and 25% MC, while the maximum spore count was 11×10^{10} CFUs/g under 30ºC and 25% MC. At 35 ºC and 40ºC and 25% MC, the *A. flavus* populations were 26.5×10^7 and 20×10^6 CFUs/g, respectively Table1.

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 imperative 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 AFB_1, the results indicated that AFB_1 levels in wheat flour at all levels of moisture contents was high at 25ºC followed by 30ºC, 20ºC, 35ºC and 40ºC, respectively. As in spore count, AFB_1 production was increased proportionally with moisture content. Data were expressed as log_{10} ng/kg flour Figure 2.

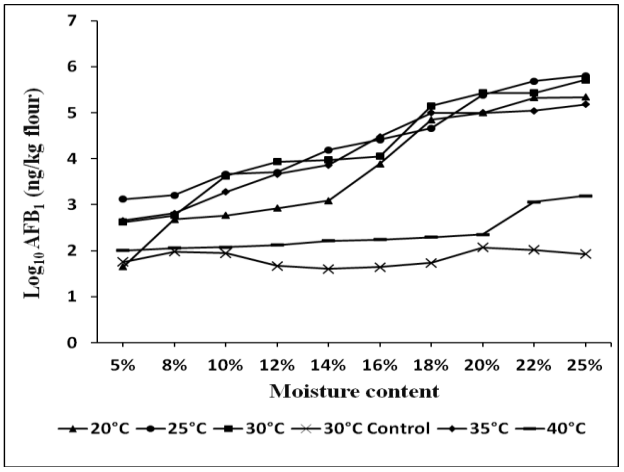

Figure 2. Effect of different temperature and moisture contents on aflatoxin B_1 production by *A. flavus* in wheat flour. Data were expressed as (log_{10} ng/kg flour).

In non-inoculated wheat flour, AFB_1 levels were around 73.18 ng/kg. Aflatoxin B_1 production was higher at 25ºC than at 30ºC, however spore count at 30ºC was more than at 25ºC. *Aspergillus flavus* inoculated in wheat flour with 5% MC allowed AFB_1 accumulation under 25ºC, 30ºC and 35ºC, while AFB_1 production at 20ºC and 40ºC started with 8% MC. Although AFB_1 was detected in wheat flour under all temperature treatment as well as *A. flavus* populations, only at 40ºC traces of AFB_1 were detected in comparison to other temperature treatments. Thin layer chromatography analysis showed positive results starting at AFB_1 concentration with 14% MC at 35°C. Below this corresponding concentration of AFB_1, TLC analysis provided negative detection results. The lowest amounts of AFB_1 detected by HPLC in inoculated wheat flour at 20, 25, 30, 35 and 40ºC with 5% MC were 44.8, 1322.2, 416.4, 451.6 and 101.4 ng/kg, respectively, while the highest AFB_1 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%, 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 AFB_1 range from 24 to 35ºC. 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 greatest aflatoxins biosynthesis [55]. High amount of aflatoxin B_1 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 B_1, and 205-737 µg $kg^{-1}$for aflatoxins B_2. Aflatoxins biosynthesis was higher at 21% moisture than at 18%, and

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

MC / Temperature	5%	8%	10%	12%	14%	16%	18%	20%	22%	25%
20°	250	6×10^3	5×10^4	5×10^4	1×10^5	10×10^6	11.5×10^6	19×10^6	21.5×10^6	30×10^6
25°	250	500	500	5×10^3	4×10^4	4.4×10^6	5.5×10^6	51.5×10^6	65.5×10^6	78.5×10^7
30°	1000	5.5×10^3	9×10^5	19.5×10^6	26×10^6	30.5×10^6	16.5×10^8	7×10^9	1×10^{10}	11×10^{10}
30° Control	0	0	0	0	0	0	0	0	0	0
35°	500	4×10^3	1×10^4	7×10^5	30×10^5	5×10^6	7.5×10^7	18×10^7	20×10^7	26.5×10^7
40°	250	1000	5×10^3	2.5×10^4	10×10^4	5×10^5	3×10^6	8.5×10^6	13×10^6	20×10^6

Table 2. Effect of different temperature and moisture content on aflatoxins B_1 production by *A. flavus* in wheat flour; detection by TLC and HPLC ng/kg.

MC / Temperature		5%	8%	10%	12%	14%	16%	18%	20%	22%	25%
20°	**TLC**	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
	HPLC	44.8	482	580	834.4	1227	7698.8	69747.4	98853.4	211436.8	217800
25°	**TLC**	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
	HPLC	1322.2	1608.6	4616	5015	15372.4	25775.4	46039.6	243049.2	487682.2	637835.4
30°	**TLC**	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	HPLC	416.4	581.8	4244.6	8512.8	9369.4	11187	140796.8	266909.8	267008.8	514847.6
30° Cont.	**TLC**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	HPLC	57.2	95.4	88.2	46.4	40.4	43.8	54.6	118.2	103.6	84
35°	**TLC**	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
	HPLC	451.6	643	1895.2	4582.6	7216.2	30237	99380	99528.2	110762.2	152154.8
40°	**TLC**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	HPLC	101.4	113.8	118.6	133.2	162.8	175	196.8	224.6	1153.8	1563.2

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 *a*w; while that for growth is 35°C and 0.95 *a*w. 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, aflatoxins 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]. *Asperigillus 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 dependably 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 B_1 production was increased proportionally with moisture content. However, only at 40ºC traces of AFB_1 were detected in comparison to other temperature treatments. In stored flour, temperature should be kept under 20ºC and low moisture content as possible.

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