Asian J. Med. Biol. Res. 2016, 2 (2), 221-228; doi: 10.3329/ajmbr.v2i2.29064

Asian Journal of Medical and Biological Research
ISSN 2411-4472 (Print) 2412-5571 (Online)
www.ebupress.com/journal/ajmbr

Article

Growth response of *Aspergillus flavus* IMS1103 isolated from poultry feed

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Received: 7 June 2016/Accepted: 20 June 2016/ Published: 30 June 2016

Abstract: *Aspergillus flavus* strains were isolated from locally available poultry feeds. Effect of temperature, pH and culture media on growth of *Aspergillus flavus* was studied. Temperature ranged from 4-42°C (4, 10, 20, 25, 30, 37 and 42°C) was examined. Except for 4°C and 10°C, the isolate was able to grow for the whole temperature range. The growth was maximum at 25°C and was influenced with increasing or decreasing of temperature from 42°C to 20°C.The lag time was strongly influenced by the temperature at lower temperature level than at higher temperature range. Effect of pH on growth of *Aspergillus flavus* was also examined; from comparison of 3 different pH levels, it is concluded that at most temperatures pH 6.5 showed a higher growth rate and as a consequence required a shorter time to achieve maximum colony diameter. No significant variations in the lag time were observed. A natural poultry feed meal agar medium (FMAM) was developed in the laboratory and growth of *A. flavus* was compared with other 2 synthetic dehydrated media namely; Czapek'sdox Agar (CDA) and potato dextrose Agar (PDA). Poultry feed meal agar medium showed better growth response than Czapek'sdox agar and potato dextrose agar at all conditions. At 25°C and pH 6.5 found optimum for growth of *Aspergillus flavus* in feed meal agar medium whereas, temperature 30°C and pH 6.5 found optimum for growth for Czapek'sdox agar media and temperature 30°C and pH 6 showed high growth rate on potato dextrose agar. Poultry feed meal media showed high affinity for growth of mycelium and early spore formation than other media examined.

Keywords: *Aspergillus*; growth; response; temperature; pH

1. Introduction

Food and feed usually become contaminated and spoiled by moulds during their production and especially during storage. Moulds are particular concern to the food industry as spoilage organism and also as potential mycotoxin producers (Spadaro *et al*., 2010). In recent time, feed stuffs are also contaminated by mould growths and especially presences of aflatoxins are reported frequently in poultry and fish feeds. For this reason, it is important to understand the growth characteristics and kinetics of these organisms at various environmental conditions and influences of extrinsic and intrinsic factors on their growth (Rosso and Robinson, 2001).Food losses resulting from occurrences of moulds are worldwide economic problem. The Food and Agriculture Organization (FAO) of the United Nations estimated that due to mould infection approximately 25% of all food production worldwide is lost after harvesting (Zeinab *et al*., 1992).

Aspergillus flavus is an asexual filamentous fungus having agronomic and health importance. Under favorable environmental conditions, drought stress and high temperature, it can infect multiple crops, such as peanut, tree nut, corn and cotton (Payne, 1998). *Aspergillus flavus* is a potential aflatoxin producer and infect some crops at field level and also grows at post-harvest conditions. Aflatoxin is reported to carcinogenic and toxic both to human and animals (Bennett and Klich, 2003). Surveillance and/or control of aflatoxin contamination are

increasingly important and have been reported throughout the tropical and subtropical regions (Arim, 1995). Aflatoxin research in Philippines began with an aflatoxin survey of various foods in 1967. Uganda is an Eastern African country with tropical climate and was one of the countries where aflatoxin studies first started (Kaaya and Warren, 2005). Many previous studies have showed influence of environmental factors on production of aflatoxin by *Aspergillus flavus* (Northolt *et al*., 1977; Trucksess *et al*., 1988; Aldred *et al*., 1999; Astoreca *et al*., 2007; Giorni *et al*., 2008; Mehra and Jaitly, 1995). Mousa *et al*. (2013) stated that temperature is the most important factor in the physical environment affecting metabolic activities of the fungi.

Major objectives of the present study were to investigate the effect of different temperatures and pH on growth of *Aspergillus flavus* isolates, isolated from poultry feeds from different feed producer's stores. The study was sequenced in three main parts. Firstly, the effect of temperatures on the growth was studied at different temperature namely 4, 20, 25, 30, 37 and 42°C. To observe variation of growth kinetics, three different media; potato dextrose agar (PDA), Czapek'sdox agar and feed meal agar (FMA) and various level of pH e.g. 6.0, 6.5 and 7.0 were examined. The results are discussed in details in the results and discussions part of this thesis. A general conclusion is drawn on the basis of the results obtained.

2. Materials and Methods

2.1. Sample collection

15 poultry feed samples were collected from different farms and shops. Feed samples were collected during a 6 months-long period from December 2009 to May 2010. Samples were collected aseptically following standard microbiological protocol and transported to the laboratory as soon as possible.

2.2. Isolation of *Aspergillus* spp.

Dilute plate technique was used for isolation of fungi from the samples according to Pratiwi *et al*. (2015). A part of feed sample weighing 20 g was mixed with 180 ml of saline solution (0.85% sodium chloride) on a horizontal shaker for ca. 30 minutes. Then, 1 ml of appropriate dilutions made up to 10-5 was applied on Potato Dextrose Agar for *Aspergillus flavus*. After 5-7 days of incubation at 25°C in dark presumptive colonies were selected and transferred onto appropriate identification media.

2.3. Identification of *Aspergillus* spp.

Colony morphology of fungi in different artificial media alone can give a preliminary idea about identification of most fungal strains (Klich, 2002). Conidial suspensions of isolated *Aspergillus* spp. was inoculated on the Czapek's-dox- agar (CYA), Potato Dextrose agar (PDA) and Feed meal agar media (FMAM) for observation of colony characteristics and incubated in the dark at 25°C for 7-14 days. Species identification was carried out according to Balajee *et al*. (2007). The development of the colony over a period was observed considering the texture, size and color of the colony, sporulation and reverse color. Identification also includes microscopic observation of the presumptive *Aspergillus* spp.

2.4. Assessment of hyphal growth rate

The radial mycelial growth of each plate was measured in two directions at right angles to each other. Measurements were recorded on alternate days during the growth until the Petri plates were completely colonized. This was measurements by digital slide carlipus. Radial mycelial growth vs. time was plotted and radial growth rates were evaluated from the slopes by linear regression (Baxter *et al*. 1998; Aldred *et al*. 1999).The growth rate (μ) was expressed as the increase in colony radial growth per unit of time.

2.5. Specific growth rate determination

Maximum specific growth rate was determined from the growth curve of *Aspergillus flavus* at different temperature and different pH (Guinea *et al*., 2005).

2.6. Aflatoxin production assay

Colony margins together with adjacent surrounding zones were scraped into large test tube (32 × 200 mm) containing 10 ml chloroform: acetone (85:15 v/v). The suspension was incubated at room temperature (25°C) for 15 - 20 min and agitated every 5 min using a vortex stirrer. The extract was filtered through Whatman no.1 filter paper and the filtrate evaporated to dryness under 40°C in an air circulated oven dryer. The residue was resuspended in 500 μl of methanol and aseptically filtered using a 0.2 μm syringe filter. It was kept at 4°C before been used for analysis by HPLC (Yazdani *et al*., 2010).

3. Results

3.1. Isolation of *A. flavus* from poultry feed samples

Poultry feed samples were collected from different areas around Dhaka city. Sample was first pre-enriched in the potato dextrose broth for 24 hours at 25°C. Enriched cultures were then inoculated on potato dextrose agar plates for colony development. Plates were incubated at 25°C for 5-6 days. Based on morphological characteristics and microscopic observation, spores from suspected colonies were further inoculated on selective media for confirmation of *A. flavus* and confirmed according to Rath (2001).

3.2. Growth on potato dextrose agar (PDA)

Colonies on PDA at 25°C is olive to lime-green in color with whitish pigmentation (Figure 1). Texture is woolly, granular presentation. Sclerotia found dark brown to black. Pale brown to cream color pigmentation were present in some isolates. Effuse, lime-green colonies with rough conidiophores and smooth to slightly rough conidia distinguish *A. flavus* isolates.

3.3. Growth on Czapek'sdox agar (CDA)

Colonies on Czapek'sdox agar were granular, flat, often with radial grooves, yellow at beginning of growth but becoming bright to dark yellow-green with age (Figure 2). Conidial heads are typically radiate, mostly 300-400 µm in diameter, later splitting to form loose columns, bi-seriate but having some heads with phialides borne directly on the vesicle. Conidiophores are hyaline and coarsely roughened, the roughness often being more noticeable near the vesicle. Conidia are globose to sub-globose in shape (3-6 µm in diameter) and pale-green in color.

3.4. Microscopic observation

After observed the microscopic slide, conidiophores were heavy walled, uncolored, coarsely roughened, usually less than 1 mm in length. Vesicles were elongate. Phialides was uniseriate or biseriate. Conidia was typically round, globose to sub – globose, with smooth to finely roughened walls, appear in chains, and with diameter size of 3 - 6 µm. Conidial heads was mostly radiate with conidial masses splitting into blocky columns. Hyphae were septate, pellicle formations were also observed. The distinct colonies were stained on a slide using Lactophenol cotton blue (Figure 3).

3.5. Influence of temperature on growth of *Aspergillus flavus* IMS1103

The growth curves based on colony diameters are characterized by a lag phase followed by a period of linear growth. In the beginning of the growth, formation of white mycelium starts at the place of inoculation and this measurement corresponds with the first measurement point in the growth curve. At temperature 4°C and 10°C no growth was observed on FMAM (Figure 4). For all other temperatures (20, 25, 30, 37 and 42°C) the *A. flavus* isolate starts sporulation with light brown to pale-green spores and mycelium growth successively extended in atypical radial way.

In potato dextrose agar, at temperature 4°C, 10°C and 42°C no growth was observed (Figure 5). For all other temperatures (20, 25, 30, 37°C), the *A. flavus* isolate starts sporulation with olive to lime green spores and different mycelium growth was observed.

In Czapek'sdox agar media, no growth was observed at temperature 4°C, 10°C and 42°C (Figure 6). Rest of the temperature (20, 25, 30 and 37) °C the *A. flavus* isolate starts sporulation with yellow to dark yellow green spores.

3.6. Influence of pH on growth of *A. flavus*

To determine the influence of pH, 3 different level of pH (6.0, 6.5 and 7.0) were examined. At temperature 25°C, pH 6.5 showed maximum growth rate and shortest time to reach maximum colony diameter. For pH 6.0 & 7.0 the growth rate was almost similar but colony diameter was smaller for 7.0 and growth retarded earlier than 6.0 (Figure 7).

At temperature 30°C, pH 6.5 showed maximum growth rate for *A. flavus* isolate but at pH 6 the fungus also showed very close growth performance that indicating the optimum pH for the growth of the fungus is between and around 6 and 6.5 (Figures 8 and 9).

At temperature 30°C, pH 6 showed maximum growth rate for *A. flavus* isolate on potato dextrose agar media, but at pH 6.5 the fungus also showed very close growth performance that indicating the optimum pH for the growth of the fungus is between and around 6 and 6.5.

Table1. Growth features of *A. flavus* on potato dextrose agar & Czapek'sdox agar plates.

Media	Color	Conidiophores	Conidia
Potato dextrose agar	Olive to lime-green in colour with whitish pigmentation.	Effuse and rough conidiophores.	Smooth to very finely roughed conidia
Czapek'sdox agar	Yellow at first but quickly becoming bright to dark yellow-green with age.	Conidiophores are hyaline and coarsely roughened, the roughness often being more noticeable near the vesicle.	Conidia are globose to sub-globose in shape (3-6 µm in diameter) and pale-green in colour.

Table 2. Microscopic observation *of A. flavus* isolates.

Size	Stipes Color	Surface	Vesicle Serration	Metula covering	Shape	Conidia Surface
400-800	Pale brown roughened	Quietly spherical	Biseriate	3\4	Glubose ellipsoid	Smooth finely roughened

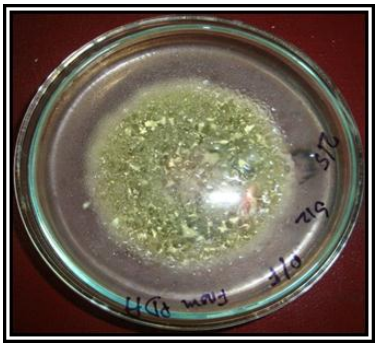

Figure 1. 4 day growth of *A. flavus* IMS1103 on potato dextrose agar at 30°C.

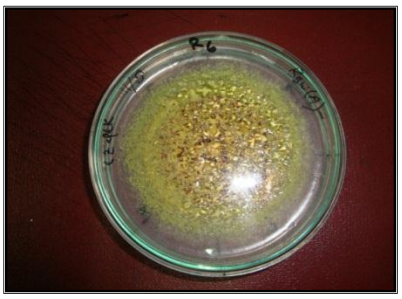

Figure 2. 4 day growth of *A. flavus* IMS1103 on Czapek'sdox agar at 30°C.

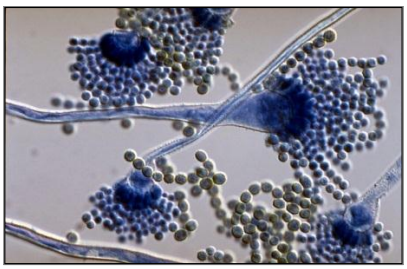

Figure 3. Photograph of isolated *Aspergillus flavus* strain in the laboratory.

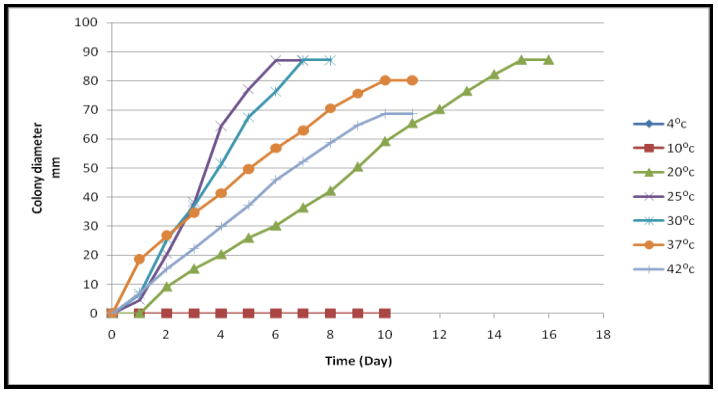

Figure 4. Growth of *A. flavus* isolate on FMAM at different temperatures.

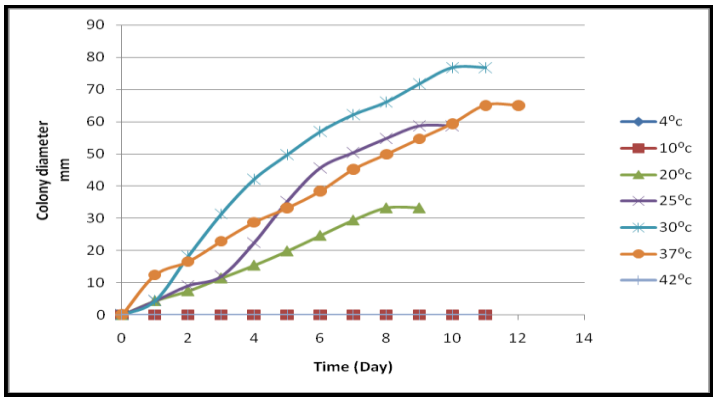

Figure 5. Growth of *Aspergillus flavus* at the different temperatures onCzapek'sdox agar media.

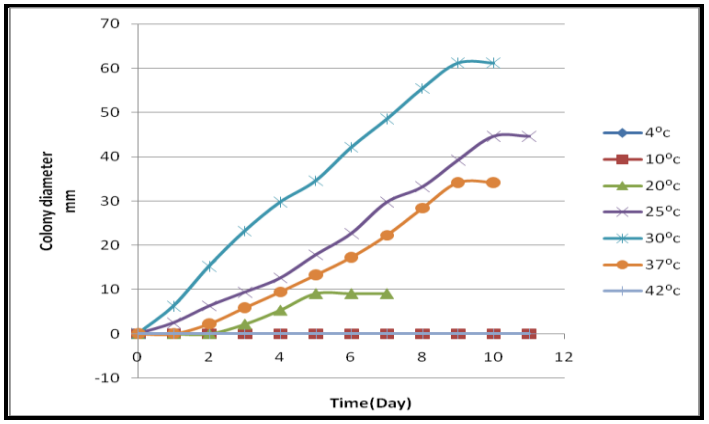

Figure 6. Growth of *Aspergillus flavus* at the different temperatures on potato dextrose agar media.

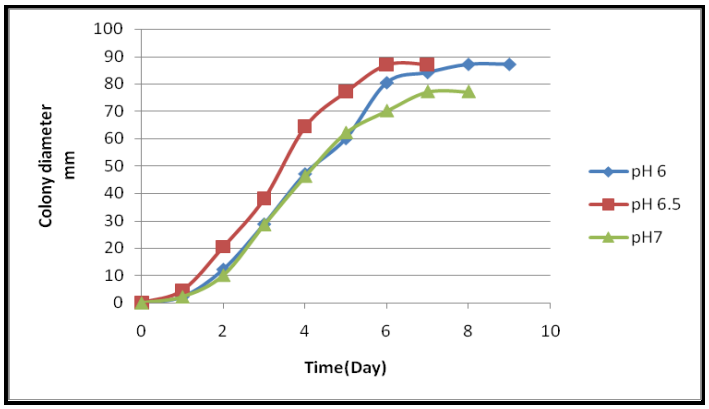

Figure 7. Growth of *Aspergillus flavus* at temperature 25°C for pH 6.0, 6.5 and 7.0 on FMAM.

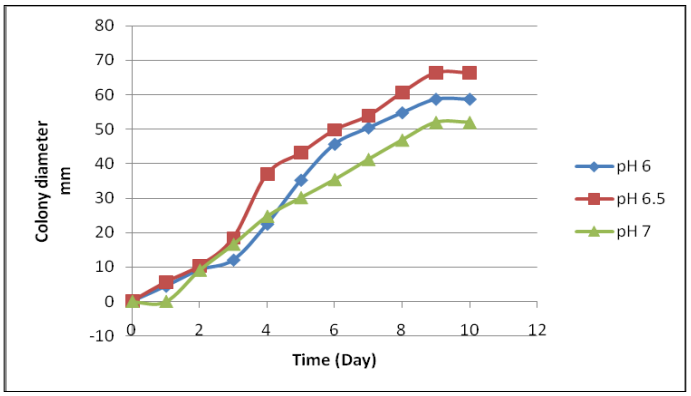

Figure 8. Growth of *Aspergillus flavus* on temperature 30 °Cat pH 6, 6.5 and 7 on Czapek'sdox agar media agar media.

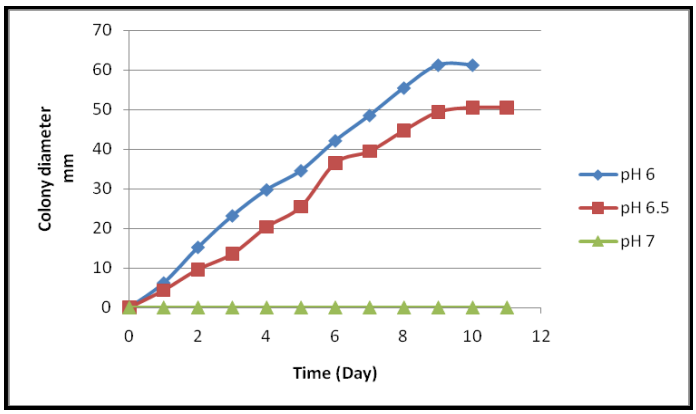

Figure 9. Growth of *Aspergillus flavus* on temperature 30°C at pH 6, 6.5 and 7 on potato dextrose agar media.

3.7. Comparisons of three media (Feed meal agar media, Czapek'sdox agar media and potato dextrose agar) on the growth of *A. flavus*

Tables 1 and 2 summarizes comparison of three media for growth of *Aspergillus flavus* IMS 1103. Feed meal agar media proved to be as suitable as Czapek'sdox agar and potato dextrose agar for growth of *Aspergillus*.

3.8. Aflatoxin production

Aspergillus flavus IMS1103 can produce aflatoxin B1, B2 and G1 but not aflatoxin G2. Feed meal agar media was found more suitable for aflatoxin production by *Aspergillus flavus*.

4. Discussion

The present study was carried out to investigate the effect of extrinsic factors namely temperature and pH on growth of *Aspergillus flavus* IMS1103 isolated from poultry feed and to evaluate the performance of a newly developed media (Feed Meal Agar Media) on growth and toxin production of *Aspergillus* spp.

Temperature showed to have influence on lag time of growth of A. *flavus*. In case of growth on FMAM, the lag time for 20°C was 24 hours while, for 25°C it was nearly 18 hours but for other higher temperatures the lag time was 6 to 12 hours. For 25°C, the time needed to reach the maximum colony diameter was the shortest (6 days) and a decrease or increase temperature resulted in an increase of time to reach the maximum colony diameter. Although 25°C showed maximum growth rate for *A. flavus* isolate but at 30°C the fungus also showed very close growth performance that indicating the optimum temperature for growth of the fungus is between and around 25°C and 30°C. At temperature 37°C, the fungus showed very fast starting of growth and the lag time for the temperature was the shortest among all the temperatures tested. It is also observed that after 24 hours of incubation the growth rate became slower for 37°C and time required to reach the maximum colony diameter was 10 days while for 25°C it was 6 days. Slower growth rate was also observed at 20°C and 42°C. It grew

poorly at 42°C and highest colony diameter was 68.59 mm and no more growth occurred after 9 days even the colony diameter was not completed. At 20°C, the lag time was not influenced compared to other temperatures but showed consistent growth at a slower rate. Based on the results discussed, 25°C can be considered as the optimum temperature for growth of the *A. flavus* strain (Figure 6). In case of growth on Czapek'sdox agar, except temperature 37°C the lag time for 20, 25 and 30°C temperatures was 20-24 hour but different growth kinetic was observed. At temperature 20°C showed consistent growth at a slower rate. This temperature the highest colony diameter was 33.25 mm and growth ceased after 9 days. Slower growth rate was also observed at temperature 25°C and no more growth occurred after 9 days even the colony diameter (58.78mm) was not completed. Only temperature 30°C showed maximum colony diameter (76.84mm) within 10 days in this media. But it was not highest colony diameter (87mm), which was observed at temperature 25°C on FMAM. At temperature 37°C the fungus showed very fast starting of growth and lag time for the temperature was the shortest among all the temperatures tested. But growth rate was slower than the temperature 30°C (Figure 5). So the lag time was not influenced the formation of highest colony diameter. From the above results, 30°C is the best temperature for the growth of *Aspergillus flavus* in Czapek'sdox agar media.

In case of growth on PDA, The lag time for 20°C was 72 hours while, for 25°C and 37 °C it was nearly 24 hours. Very slow growth kinetic was observed at temperature 20°C and highest colony diameter was 9.03 mm and growth was stopped after 6 days even the colony diameter was not completed. Slower growth rate was also observed at 25^{o}C and 37^{o}C and very close growth performance were (44.69 mm and 34.16 mm respectively) also showed but growth was completely ceased after 9 to 10 days. At temperature 30°C the *A. flavus* showed very fast starting of growth and lag time for the temperature was the shortest among all the temperature tested (Figures 5 and 6). In potato dextrose agar highest colony diameter (61.24 mm) was observed at the temperature 30°C This growth kinetic was so far different from Czapek'sdox Media and feed meal agar media. So from the above results 30°C can be considered as the optimum temperature for growth of the *A. flavus* on the potato dextrose agar media.

On FMAM, lower pH (6 & 6.5) showed better growth rate and colony diameter than pH 7.0 (Figure 7). Lag time at all pH is lower than that on other media (Czapek'sdox and PDA). On Czapek'sdox agar, for pH 7, the lag time was 24 hours, and highest colony diameter was 52 mm and no more growth occurred after 9 days even the colony diameter was not completed. At pH 7, the lag time was not influenced compared to other pH but showed consistent growth at a slower rate (Figure 8). Based on the results, pH 6.5 can be considered as the optimum pH for the growth of *Aspergillus flavus* on the Czapek'sdox agar. On potato dextrose agar, highest colony diameter showed 61.24 mm at pH 6 and growth was ceased after 10days. At pH 6.5 maximum colony diameters was 50.49 mm and no more growth was observed after 11days. No growth was observed at pH 7 (Figure 9). So, temperature 30^{o}C and pH 6 was the optimum for the growth of *Aspergillus flavus* on the potato dextrose agar media.

There was no significant growth of *A. flavus* at the temperatures of 4°C and 10°C on the three culture media. The temperatures of 25°C and 30°C favored fast colony diameter for the growth of *A. flavus* on the three culture media. On the other hand slower growth was observed at temperatures 37°C and 42°C on all the culture media. At temperature 25°C, *A. flavus* grew better on feed meal agar media while Czapek'sdox agar media and potato dextrose agar, it was 30°C. At the temperature of 42°C, *A. flavus* was significantly inhibited in the Czapek'sdox agar media and potato dextrose agar. These observations may suggest that the feed meal agar media may influence the growth of *A. flavus* at higher temperature. Furthermore, it was found that the feed meal agar media supported maximum growth rate of *A. flavus* of the three media tested. PDA and Czapek'sdox agar media showed lower growth kinetic than the feed meal agar media.

The isolate was found to be able to produce 3 types of aflatoxin (aflatoxin B1, B2 and G1). On FMAM and PDA, three types of aflatoxin were produced, but on Czapek'sdox agar, only aflatoxin B1 and G1 was produced. Toxin production was found to be better on FMAM than on other two medias (PDA and Czapek'sdox).

Growth performance and toxin production of *Aspergillus flavus* on newly developed FMAM media showed to be comparable to PDA and Czapek'sdox agar media. Nutrient components in feed meal agar medium showed to play an important role in triggering mycelial growth and toxin production and this low cost media can be routinely used for growth of *Aspergillus flavus*.

5. Conclusions

This study examined the effect of temperature and pH on growth of *Aspergillus flavus* IMS1103 isolated from poultry feed. Data obtained in this study is critical in building up a picture of the key factors which influence growth and sporulation of strains of this important mycotoxigenic species. Poultry feed meal agar media showed

high affinity for growth of mycelium and early spore formation than other media examined. This low cost media can be used in place of commercial media which are expensive.

Conflict of interest

None to declare.

References

Agnihotri VP, 1964. Studies on aspergilli. XVI. Effect of pH, temperature, and carbon and nitrogen interaction. Mycopathol. Mycol. Appl., 24: 305-314.

Aldred D, N Magan and BS Lane, 1999. Influence of water activity and nutrient on growth and production of squalestatin S1 by a Phoma sp. J. Appl. Microbiol., 87: 842–848.

Arim RH, 1995. Present status of the aflatoxin situation in the Phillippines. Food Add. Contamin., 12: 291-296.

Astoreca A, C Magnoli, ML Ramirez, M Combina and A Dalcero, 2007. Water activity and temperature effects on growth of *Aspergillus niger*, *A. awamori* and *A. carbonarius* isolated from different substrates in Argentina. Intl. J. Food Microbiol., 119: 314–318.

Balajee SA, J Houbraken, PE Verweij, S-B Hong, T Yaghuchi, J Varga and RA Samson, 2007. *Aspergillus* species identification in the clinical setting. Stud. Mycol., 59: 39-46.

Baxter CJ, N Magan, B Lane and HG Wildman, 1998. Influence of water activity and temperature on in vitro growth of surface cultures of *Phoma* sp. and production of pharmaceutical metabolites, squalestatins S1 and S2. Appl. Microbiol. Biotechnol. 49: 328–332.

Bennett JW and M Klich, 2003. Mycotoxins. Clin. Microbiol. Rev., 16: 497–516.

Cotty PJ, 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic to cotton. Phytopathol., 79: 808-814.

Giorni P, P Battilaniand N Magan, 2008. Effect of solute, matric potential and temperature on *in vitro* growth and sporulation of strains from a new population of *Aspergillus flavus* isolated in Italy. Fungal Ecol., 1: 102-106.

Guinea J, T Peláez, L Alcalá and E Bouza, 2005. Evaluation of Czapeck agar and Sabouraud dextrose agar for the culture of airborne *Aspergillus* conidia. Diag. Microbiol. Infect. Dis., 53: 333–334.

Kaaya AN and H Warren, 2005. A review of past and present research on aflatoxin in Uganda. Afr. J. Food Agri. Nutr. Dev., 5: 1-18.

Klich MA, 2002. Indentification of common *Aspergillus* species, CBS, Netherlands.

Mehra S and A Jaitly, 1995. pH and temperature optima for growth and sporulation in some common fungi from city waste. Mycosci., 36: 243–246.

Northolt MD, HP Van Egmond and WE Paulsch, 1977. Differences between *A. flavus* strains in growth and aflatoxin B l production in relation to water activity and temperature. J. Food Prot., 40: 778-781.

Payne GA, 1998. Process of contamination by aflatoxin-producing fungi and their impact on crops. In: Sinha K.K. and Bhatnagers D., editors. Mycotoxins in Agriculture and Food Supply.Marcel Dekker, New York, NY.

Rath PM, 2001. Phenotypic and genotypic characterization of reference strains of the genus *Aspergillus*. Mycoses, 44: 65–72.

Rosso L and TP Robinson, 2001. A cardinal model to describe the effect of water activity on the growth of moulds. Intl. J. Food Microbiol., 47: 141-146.

Spadaro D, S Patharajan, A Lore, Ml Gullino and A Garibaldi, 2010. Effect of pH, water activity and temperature on the growth and accumulation of ochratoxin A produced by three strains of *Aspergillus carbonarius* isolated from Italian vineyards. Phytopathol. Mediterr., 49: 65-73

Trucksess M, L Stoloff and P Mislivec, 1988. Effect of temperature, water activity and other toxigenic mold species on growth of *Aspergillus flavus* and aflatoxin production on corn, pinto beans and soybeans. J. Food Prot., 51: 361-363.

Yazdani D, MA Zainal Abidin, YH Tan and S Kamaruzaman, 2010. Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. Afr. J. Biotechnol., 9: 7654-7659.

Zeinab HK, IH Nagwa and HAmra, 1992. Effect of incubation time, temperature and substrate on growth and aflatoxin production. Intl. Biodeterior. Biodeg., 30: 17-27.