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Research Article

Characterization and Determination of Aflatoxigenic and Non-aflatoxigenic *Aspergillus flavus* Isolated from Bakery Food Products from Frontier around Davangere District

Sowmya K. L.[1*] and Ramalingappa B.[2]

[1]Research Scholar, [2]Professor,

Department of Microbiology, Davangere University, Shivagangotri, Davangere-577007, Karnataka

*Corresponding Author E-mail: swomyakl456@gmail.com

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ABSTRACT

Mycotoxins are secondary metabolites produced by filamentous fungi in food and feed due to several conditions that affect fungal growth and mycotoxin production in different ways. Mycotoxin contamination in bakery food products is seriously dangerous to humans and animals. This study aimed to morphologically characterize and determine the aflatoxigenic and non-aflatoxigenic Aspergillus flavus isolates. Thirty three isolates of A.flavus were obtained from bakery food products collected from bakeries in rural areas like Bathi, Kodaganur, Bada, sasalu, Mayakonda, Avargere, Anaberu, Alur, Attigere, Echagatta around Davangere city. They were cultured on Potato Dextrose Agar medium (PDA), Rose Bengal Agar medium (RBA), Czapec(dox) agar medium (CZA) and Sabouraud's dextrose agar medium (SDA). By observing the colony color and texture macromorphological characteristics were determined and micromorphological characteristics were determined by observing the spore color, size, structure, conidiophore structure, conidia, vesical shape under microscope. The production of aflatoxin was determined by UV fluorescence of isolates on coconut cream agar (CCA) medium plates. In aflatoxin detection 20 (60.60%) isolates of A.flavus shows positive results and 13 (39.39%) isolates of A.flavus shows negative results. The highest incidence of A.flavus was recorded in samples collected from Attigere followed by Bathi, Alur, Echagatta, Kodaganur, Bada, Anaberu, Sasalu, Mayakonda, Avargere. The incidence of A.flavus that produces aflatoxin highlights the necessity of taking action to eliminate their presence in food. The non-aflatoxigenic strains are being used in a biological control strategy to outcompete the aflatoxigenic ones.

Keywords: *Aspergillus flavus, aflatoxin, Morphological Characterization, coconut agar medium.*

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INTRODUCTION

Bakery foods are popular staples in most parts of the country. Bread, buns, cakes, cookies, chips, pizza bases, toast, and so on are the most common products. Cereals used in bakery products are a valuable source of nutrients, providing with the majority of our food calories and roughly half of our protein requirements. Carbohydrates, proteins, lipids, vitamins, calcium, iron, minerals, starch, and energy are all found in bakery products (Patil & Kukade, 2020). Microorganisms and their toxins contaminate the majority of foods. Mycotoxicosis is caused by consuming food products contaminated with toxigenic fungi. Mycotoxicosis is a disease caused by eating foods contaminated with mycotoxins produced by toxigenic fungi. Mycotoxins are secondary metabolites produced by several fungi species that significantly impact food quality, causing serious problems for humans and animals. Food contamination with mycotoxin is an ongoing global concern that is both unavoidable and unpredictable (Alshannaq & yu, 2017). The most important group of mycotoxins [Aflatoxin (AF), zearalenone (ZEA), Patulin and deoxynivalenol (DON)] that are of major health concern for humans and animals (Daou et al., 2021; Chen et al., 2016; & Salem & Ahmad, 2020). They are mostly found as natural contaminants of food products sold by supermarket chains and grocery markets and are detrimental to human health. They induce negative effects on human health by making food unsafe for consumption (Chen et al., 2016). Molds of the genus Aspergillus, Fusarium and Penicillium are the most important in producing mycotoxins. Aflatoxins, produced mainly by A. parasiticus and A. flavus (Wogan & Pong, 1970), are recognized as the most hazardous mycotoxins. While the most common mycotoxins found in bakery food products are aflatoxins B1, B2, G1 and G2, Ochratoxin A, Fumonisins B1, B2 and B3, Zearalenone, Deoxynivalenol and Patulin (Streit et al., 2013). Aflatoxins are extremely carcinogenic, predominantly found in bakery food products.

Aflatoxins are produced only by a closely related group of *Aspergilli*: *A. flavus, A.parasiticus* and *A.nomius* strains.Other species such as *A.bombycis, A.pseudotamari* are also aflatoxin producing species, but they are found less frequently. Aflatoxins are a problem for many commodities. Aflatoxin B1 (AFB1) was also tested for carcinogenicity, mutagenicity, and acute toxicology and was found to be a human carcinogen. Blue fluorescence is a technique used in the development of qualitative cultural methods for detecting aflatoxigenic Aspergillus species grown on appropriate media (Jarvis et al). These methods employ either a solid medium, such as coconut agar medium (CAM) or potato dextrose agar (PDA), or a liquid medium, such as aflatoxin producing-ability medium (APA). Under long-wave UV light, the aflatoxins producer Aspergillus was detected (365nm). This rapid identification method distinguishes aflatoxigenic isolates from non-aflatoxigenic isolates by appearing blue to blue-green fluorescent to aflatoxigenic, and non-aflatoxigenic isolates do not produce fluorescent.

MATERIALS AND METHODS

2.1. Narration of the study area:

During July to December 2022, samples were collected from rural side bakeries in Bathi, Kodaganur, Bada, Sasalu, Mayakonda, Avargere, Anaberu, Alur, Attigere, and Echagatta around Davangere city.

2.2. Sample collection:

A total of 73 bakery samples were collected at random, stored in sterile plastic bags that were properly sealed, kept at room temperature, and examined the day after collection (Daou et al., 2021).

2.3. Isolation and Identification of *A. flavus*:

Aspergillus flavus were isolated by using Potato dextrose agar medium (PDA), Rose Bengal Agar medium (RBA), Czapec(dox)

agar medium (CZA) and Sabouraud's dextrose agar medium (SDA) by three isolation methods including spread plate method, serial dilution method and direct plate method (Al-kahtani, 2014).

2.3.1. Growth media preparation:

The growth media were prepared with chloramphenicol and autoclaved for 15 minutes at 121^0C. After allowing the sterilized media to cool for 15-20 minutes, it was transferred into sterile Petri plates under aseptic conditions. Plates were kept at 40°C after solidification until inoculation.

2.3.2. *Aspergillus flavus* isolation from bakery food samples:

In direct plate method, 1gm of bakery sample was directly placed/ sprinkled on solidified agar media (PDA+RBA+CZA+SDA) and incubated at 30-37^0C for 3-7 days. After incubation fungi were observed and identified based on microscopic observation and morphology characteristics (Al-kahtani, 2014). In spread plate method, 1 gm of sample was mixed with 9 ml of distilled water and a homogenate (0.5-1 ml) was added on the surface of the media (PDA+RBA+CZA+SDA) and spreader evenly over the surface using sterile L- shaped spreader. Then, the plates were incubated at 30-37^0C for 3-7 days. After incubation fungi were observed and identified based on microscopic observation and morphology characteristics (Patil & Kukade, 2020). In the serial dilution method, 1 gm of sample was mixed with 9 ml of sterile water and thoroughly mixed, yielding a 10^{-1} dilution that was serially diluted up to 10^{-7}. 0.1 ml of sample from each dilution was spread plate inoculated on solidified agar media (PDA+RBA+CZA+SDA). The plates were then incubated for 3-7 days at 30-37°C. Fungi were observed and identified after incubation based on microscopic observations and morphology characteristics. (Patil & Kukade, 2020).

2.3.3. *Aspergillus flavus* Characterization:

Aspergillus flavus were identified based on morphological and microscopic observation, after staining with lactophenol cotton blue stain followed by Domsch et al. 1980; Subramanian, 1983; Ellis & Ellis, 1997; Gilman, 2001 & Nagamani et al. 2006.

***2.4.* Aspergillus flavus Aflatoxigenic Potential Determination:**

To screen the ability of *A.flavus* to produce aflatoxins, coconut cream agar medium (CCAM) was used, thus it shows the ability of aflatoxigenic & non-aflatoxigenic isolates. According to Fente et al.; Davis et al. coconut cream agar medium was prepared, autoclaved and poured into the sterile petriplates (supplemented with antibiotic). Following solidification, a well is formed in the centre of the CCAM-containing plate. Spores from a 7-day-old A. flavus culture were suspended in distilled water containing 0.025% Tween 80. The Petri plate was then aseptically inoculated with 10µl of spore suspension. The inoculated plates were incubated for 7 days at 28°C. Following incubation, the inoculated plates were examined under UV light for aflatoxin fluorescence screening and the results were recorded.

RESULTS

3.1. *A.flavus* Detection*:*

Thirty-three *A. flavus* isolates were found in bakery food products (Figure 1). The *A. flavus* isolates were identified by observing their morphological characteristics in accordance with the key descriptions provided by Domsch et al. 1980; Subramanian, 1983; Ellis & Ellis, 1997; Gilman, 2001 & Nagamani et al. 2006. The % detection of *A. flavus* in Bathi, Kodaganur, Bada, sasalu, Mayakonda, Avargere, Anaberu, Alur, Attigere, Echagatta was 18.18% (6), 6.06% (2), 6.06% (2), 3.03% (1), 3.03% (1), 0% (0), 3.03% (1), 15.15% (5), 36.36% (12), 9.09% (3) respectively.

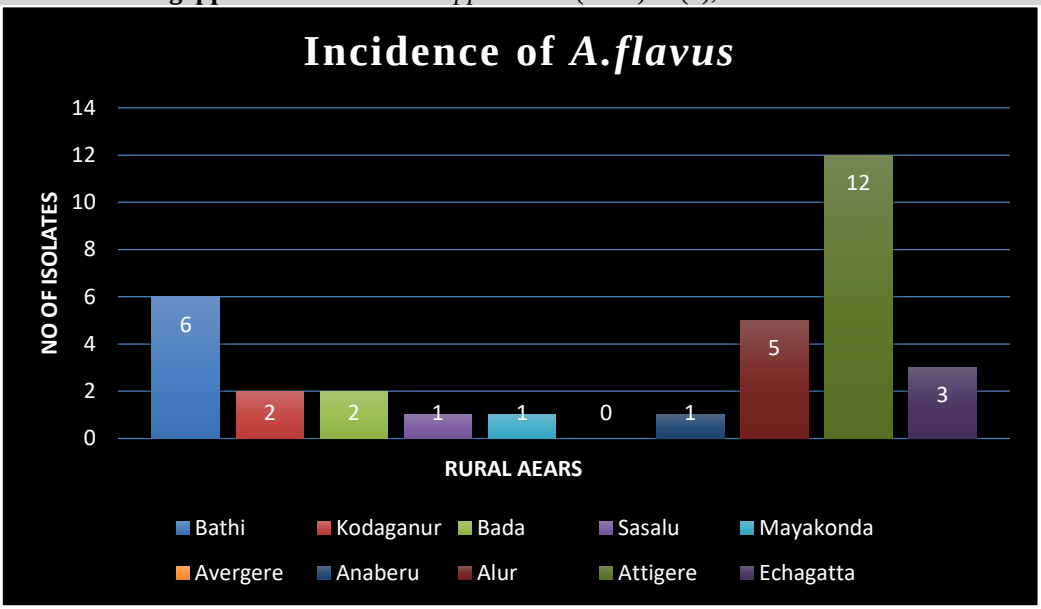

Figure.1. Prevalence of *A.flavus* isolates in Bathi, Kodaganur, Bada, sasalu, Mayakonda, Avargere, Anaberu, Alur, Attigere, Echagatta

2.5. Macroscopic characteristics of *A.flavus* on PDA and RBA:

The morphology of *A.flavus* colonies on PDA and RBA medium plates is presented in **Figure.2.** After 2 days of incubation, the mycelial colour of *A.flavus* was white and after 3rd day of incubation, the colonies on both plates turn olive green conidia that dominated the colonies appearance. The colony diameter was ranged from 60-70mm (PDA), 55-70mm (RBA) surrounded by white circle.

A **B** **C**

Figure.2. Colony morphology of *A.flavus* on PDA and RBA medium plates. A. PDA plates, B. RBA plates, C. Under micro lens (1.9 X)

2.6. Microscopic characteristic of *A.flavus*:

Figure 3, depicts the microscopic characteristics of *A.flavus* isolates. The conidiophores of *A.flavus* isolates were colourless, thick walled, roughed, and bearing vesicles. *A.flavus* isolates had globose to sub-globose vesicles. Cells were either uniseriate or biseriate. The conidia were globose, with thin walls that were slightly roughened.

A **B**

Figure.3. A. Macroscopic characteristics of *A.flavus* on PDA. B. Microscopic characteristics of *A.flavus* under 40X objective

2.7. Aflatoxin production Screening:

Figure 4, depicts the results of *A.flavus* UV light screening, displaying fluorescent and non-fluorescent isolates of *A.flavus* on CCA. It was discovered that 20 (60.60%) of the 33 isolates fluoresced blue on CCA medium plates when exposed to UV light at 365 nm. It indicates that 60.60% (20) of the isolates out of 33 isolates have the ability to produce aflatoxin **(Table 1)**, while the remaining 39.39% (13) of the isolates do not exhibit blue fluorescence under UV light, indicating non-aflatoxin production. **Figure 5**, depicts the distribution of aflatoxigenic and non-aflatoxigenic *A.flavus* isolates in ten (10) different locations. Four of the six isolates from the Bathi bakery tested positive for aflatoxin production, while the remaining two isolates tested negative. Aflatoxin production was detected in 1 isolate from Kodaganur bakery samples, while the remaining 1 isolate did not produce aflatoxin. Similarly, aflatoxin production was detected in one of the two isolates of Bada bakery samples, while the other isolate did not produce aflatoxin. Aflatoxin is produced by one isolate from Sasalu bakery samples. Four isolates from Alur bakery samples tested positive for aflatoxin production, while one isolate tested negative for aflatoxin production. Seven isolates from Attigere bakery samples tested positive for aflatoxin production, while the remaining five isolates tested negative. Finally, two isolates from Echagatta bakery samples tested positive for aflatoxin production, while one isolate tested negative for aflatoxin production.

A **B** **C**

Figure.4. Aflatoxigenic and non-aflatoxigenic isolates of *A.flavus* observed under UV light (365 nm). A. Growth of *A.flavus* on CCA medium plate. B. Aflatoxigenic production. C. Non-aflatoxigenic production

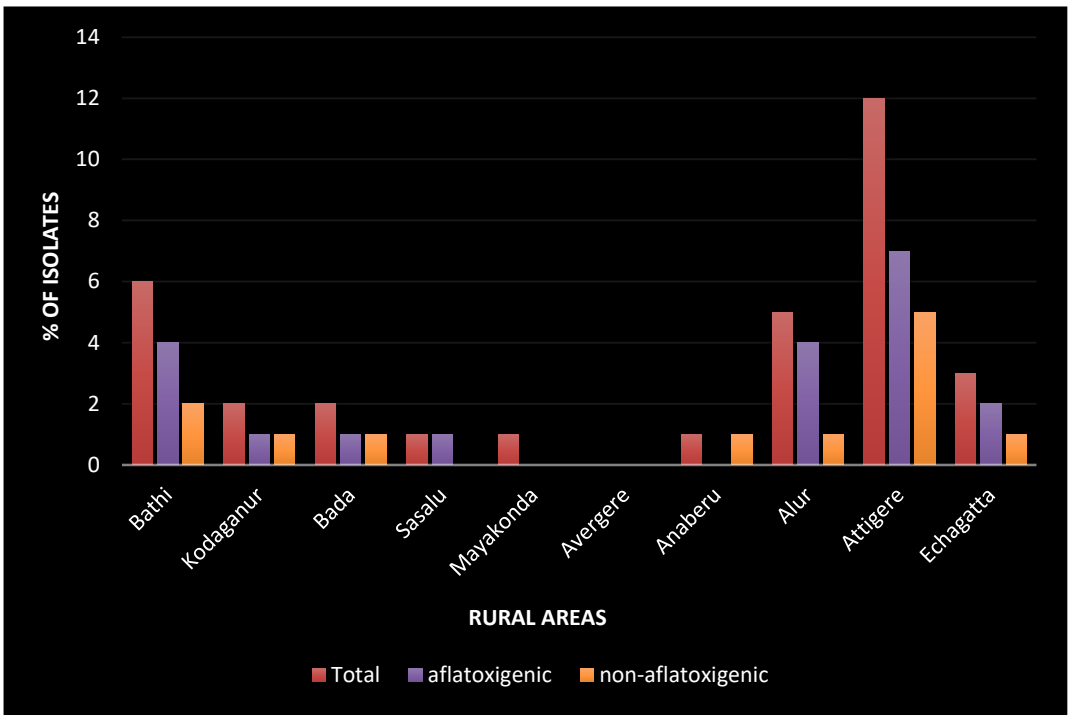

Figure.5. The distribution of the aflatoxigenic & non-aflatoxigenic isolates of *A.flavus* in ten bakery food samples collected from rural areas

Table 1. Identification of production of aflatoxin through screening by UV fluorescence

SL.NO	ISOLATES	RESULTS
1	RS 1	+
2	RS 2	+
3	RS 3	+
4	RS 4	+
5	RS 5	-
6	RS 6	+
7	RS 7	-
8	RS 8	-
9	RS 9	+
10	RS 10	-
11	RS 11	-
12	RS 12	-
13	RS 13	+
14	RS 14	+
15	RS 15	+
16	RS 16	-
17	RS 17	-
18	RS 18	-
19	RS 19	-
20	RS 20	-
21	RS 21	+
22	RS 22	+
23	RS 23	+
24	RS 24	+
25	RS 25	+
26	RS 26	+
27	RS 27	+
28	RS 28	+
29	RS 29	+
30	RS 30	+
31	RS 31	+
32	RS 32	-
33	RS 33	-

DISCUSSION

The highest incidence of *A.flavus* (18.18%) was found in Bathi bakery samples, while the lowest incidence (3.03%) was found in Sasalu and Mayakonda bakery samples, respectively. The difference in the occurrence of *A.flavus* in the different bakeries could be attributed to differences in environmental conditions.

The observation of the main macro and micromorphological features of fungi cultured

on various media is a widely used method for fungal identification. The morphological Characterization was performed in this study to emphasize the importance of such basic identification methodologies for the rapid screening of isolates in most developing countries, where access to unconventional tools is a significant challenge. Observing key morphological characteristics allowed for consistent identification of *A.flavus* isolates. The physical characteristics of *A.flavus*, such as colony colour, texture, and edges, among others. *A.flavus* had olive green, yellowish-green, or dark green colonies that were surrounded by a white circle that was eventually covered by conidia. The textures of the colonies were typically velvety or woolly with a floccose centre. According to Klich's interpretation of taxonomic descriptions, the colony morphology of the isolates in this study resembled *A.flavus*, as shown in Figures 2 and 3. *A.flavus* possessed rough and thick-walled conidiophores, globose vesicles with radiated sterigmata, and thin-walled finely rough conidia. The macroscopic and microscopic characteristics shown in Figures 2 and 3 are similar to those described by Rodrigues *et al.*, and Diba et al. for *A. flavus*. In this study, the use of Potato Dextrose Agar medium (PDA), Rose Bengal Agar medium (RBA), Czapec (dox) agar medium (CZA), and Sabouraud's dextrose agar medium (SDA) allowed for adequate growth and sporulation, allowing for a satisfactory examination of the macroscopic and microscopic features of *A.flavus.* After morphological Characterization on SZA, many *A.flavus* isolates isolated from corn in North-Eastern China were identified (Gao et al., 2007). Diba et al. observed the macroscopic and microscopic features of *Aspergillus* species in medical and environmental samples and concluded that the use of PDA, DRBC, AFPA, and other growth media may stimulate the growth and sporulation process of *Aspergillus* species. Morphological characterization approaches have also been used on *Aspergillus* species in Iran's Fars and Kerman provinces (Mohammadi et al., 2009) and Pakistan's Sindh province's Larkana district (Afzal et al., 2013). *A.flavus* isolates were screened based on their fluorescence characteristics under UV light at 365 nm. When aflatoxigenic *A.flavus* isolates were exposed to UV light, they produced a blue fluorescence on the reverse of the colonies, whereas non-aflatoxigenic *A.flavus* produced no fluorescence (Figure 4). The use of CCA medium in this study allowed for the rapid differentiation of aflatoxigenic *A.flavus* from non-aflatoxigenic *A.flavus*. When 20 (60.60%) of 33 isolates were exposed to UV light at 365 nm, they fluoresced blue on CCA medium plates, demonstrating their aflatoxin producing potential. In most developing countries, where aflatoxin determination may be delayed due to a lack of advanced detection instruments, culture-based techniques may be useful for rapid screening of isolates. For aflatoxin detection, culture-based techniques are relatively simple and inexpensive. These techniques, however, are typically used in conjunction with chromatographic methods.

CONCLUSION

Finally, in conclusion, both aflatoxigenic and non-aflatoxigenic strains of *A.flavus* were found in bakery food products from various regions. In this study, culture media and morphology are quick tools for *A.flavus* Characterization, and screening *A.flavus* isolates for aflatoxin detection under UV light (365nm) is a quick and reliable method for distinguishing aflatoxigenic from non-aflatoxigenic isolates.

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Author contribution: Prof. Ramalingappa., supervision, devised the work, the main

conceptual ideas and proof outline and planned the experiments and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

ORCID ID: 0000-0003-0471-7036

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