E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2019; 7(6): 280-286

www.fisheriesjournal.com
Received: 21-09-2019
Accepted: 25-10-2019

Remisha Olokkaran
School of Industrial Fisheries, Cochin University of Science and Technology, Kochi, Kerala, India

Saleena Mathew
School of Industrial Fisheries, Cochin University of Science and Technology, Kochi, Kerala, India

Jubisha Alakkatt
Department of Marine Biology, Microbiology, and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala, India

Santu Kuzhikandathil Sunny
Department of Marine Biology, Microbiology, and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala, India

Corresponding Author:
Remisha Olokkaran
School of Industrial Fisheries, Cochin University of Science and Technology, Kochi, Kerala, India

Isolation and morphological identification of aflatoxigenic *Aspergillus flavus* in finished feed for farmed Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)

Remisha Olokkaran, Saleena Mathew, Jubisha Alakkatt and Santu Kuzhikandathil Sunny

Abstract
The most important problem facing the aquaculture sector today is the foodborne toxin exposure, such as aflatoxins. Aflatoxins are produced mainly by two fungal species, *Aspergillus flavus* and *Aspergillus parasiticus* that are normally; occur in hot and humid regions of the world. Practically, it is not possible to destroy the contaminated feed; therefore, to identify the fungal isolates is very important for taking remedial measures against aflatoxin contamination in fish. In this study, we isolated fungal isolates from five types of fish feed that prepared for farmed Nile tilapia. To know the characteristics features of these fungal isolates, three differential media including Potato dextrose (PD) broth, Czapek's yeast extract agar (CYA) and Yeast extract peptone dextrose broth (YEPD) were used for differentiation of *Aspergillus* species, colonizing in feeds comparing with standard cultures. According to the morphological features, all the isolates from the fish feeds were identified as *Aspergillus flavus* species in three of the culture media.

Keywords: Tilapia feed, *A. flavus*, macroscopic features, microscopic characteristics

1. Introduction
Contamination of food and agricultural commodities by various types of toxigenic fungi is an important and widely ignored problem [8]. The fungal contamination of the food and feedstuffs occur at different stages of production, harvesting, handling, processing, and storage [12]. The *Aspergillus spp* are filamentous and are among the most group of microorganisms that found in nature as in the soil, plant debris and indoor air environments [22]. Members of the fungal genus *Aspergillus* are most frequently been isolated from feed commodities kept under poor storage conditions i.e. aw of between 0.8 and 0.9 with a wide range temperature i.e. 24 to 30 °C [24]. Feeds and feed ingredients infected by *Aspergillus spp* and particularly presences of aflatoxins frequently recorded especially in livestock feeds formulated from cereals [26]. Aflatoxins are toxic carcinogenic secondary metabolites produced by *Aspergillus flavus, Aspergillus parasiticus and Aspergillus nomius* species of fungi. Whereas *Aspergillus flavus*, which produce aflatoxins B1 (AFB1), and B2 (AFB2), and *Aspergillus parasiticus* [2] which produce aflatoxins G1 (AFG1) and G2 (AFG2) [17]. In the case of the fungal species under study, identification and differentiation are important to understand the growth characteristics of these organisms at various environmental conditions [31]. In animal feeds, accurate as well as quick identification of contaminating fungal species are very significant. It is also important to distinguish if toxigenic fungi are present during pre- and post-production of feeds. Fungal growth causes weight loss, boosts local rises in temperature and moisture content, off-flavour and discolouration, and some common species produce aflatoxins, which recognized to be toxic and highly carcinogenic to a wide variety of animals, including some species of fish [16].
Generally, identification of the *Aspergillus* spp. based on the morphological characteristics of the colony by microscopic and macroscopic examinations [21]. Most *Aspergillus* spp. have described by using morphological features to differentiate species especially in earlier studies [27]. The important morphological character of *Aspergillus spp* is the spore-bearing structure called conidiophores,

which is the vertical hyphal branch, enlarges at its tips, making vesicles. The vesicles produce a fertile area called phialides that produce long chains of conidia of conidiospores. The shape and size of the vesicles, and the arrangement, colour and the size of the conidia are among the most important characteristics for identification, where most species have globose, sub-globose to pyrifom vesicles. Another characteristic is seriation, either uniseriate or biseriate. Vesicles with two cell layers, phialide and metulae are biseriate such as *A. flavus* and *A. terreus*, while vesicles that produce only phialide layers are uniseriate such as *A. fumigatus* and *A. clavatus* [18]. The vesicle shape, and also the conidia colour, arrangement and size are among the important morphological characteristics for identification of *Aspergillus* to species level [27, 21]. Other features used for identification are sclerotia, these are rounded masses of mycelium; sclerotia may function as resting structures to allow the species to survive in harsh conditions. They are generally round in shape and may scatter abundantly [18]. Other cultural features used in species identification are the colour of the colony and growth rate.

Isolation in culture and phenotypic identification of common isolates of *Aspergillus spp* is usually quick and easy. However, identification solely based on morphological characteristics is not sufficient, particularly to differentiate species within the same section or closely related species, which may show phenotypic variation and overlapping features. We used three differential culture media that enabled to conduct important *Aspergillus spp* in potato dextrose broth, Czapeks yeast extract agar, and Yeast extracts peptone dextrose broth as isolation media. However, molecular methods of identification continue to become more quickly available, microscopy and cultural methods remain essential tools for identification of *Aspergillus spp* [13]. Most of the *Aspergillus spp* are very close in their morphological characters and chances are to misidentify them. Therefore, correct identification of *Aspergillus spp* is important to develop proper management practices to control these toxigenic fungi and their toxins in food grains. In this context, we studied on morphological methods including macroscopic features of colonies and microscopic characteristics for identification of *Aspergillus spp* isolated from five different types of tilapia feed prepared in the laboratory.

2. Materials and Methods

2.1 Materials

2.1.1 *Aspergillus flavus* pure culture and maintenance medium

Aspergillus flavus (MTCC 2501) pure culture obtained from Marine biology microbiology department (School of Marine Sciences, CUSAT). The fungus subcultured on Potato dextrose agar (PDA) in slants and allowed to grow at 28 °C for 15 days [34]. Such slants kept in liquid paraffin and stored at 4 °C in a refrigerator. This pure culture used as standard *Aspergillus flavus* for future study.

2.1.2 Isolation and identification of fungal isolates from tilapia feed

The five-tilapia feed samples naming TF1, TF2, TF3, TF4 and TF5 weighing 20 g mixed with 180 ml of saline solution (0.85% Sodium chloride) on a horizontal shaker for 30 minutes. Then 1 ml of appropriate dilutions made up to 10 to 5 applied on identification media [26, 32]. To improve the sensitivity and specificity of routine culture approach for identification of *Aspergillus* in the level of species, we used three differential media including, Potato dextrose broth (PD), Czapeks yeast extract agar (CYA) and Yeast extract peptone dextrose broth (YEPD) [1].

2.1.3 Culture media and growth conditions

Weighed out dry ingredients into flask for preparing CYA agar (Czapek Concentrate 10 ml, Dipotassium hydrogen phosphate 1 g, Yeast extract 5 g, Sucrose 30 g, and Agar 15 g), YEPD broth (Peptone 20g, Yeast extract 10 g, and Dextrose 20 g) and PD broth (Dextrose 20 g, and Potato starch 4 g). Then after added distilled water to the flask until the volume is about 90% of the total volume. All three media were autoclaved at 121°C for 15 minutes. Then 1 ml of each fungal isolates inoculated in triplicates at the centre of Petri plates and a conical flask containing each of the culture media. The plates then incubated at 28°C for seven days in the dark [19, 25]. Growth and sporulation noted through macroscopic and microscopic method [38].

2.2 Methods

2.2.1 Morphological identification of fungal isolates from tilapia feed

Morphological characteristics of fungal isolates studied according to macro and microscopic features of the colonies using identification keys [6, 26, 18].

2.2.1.1 Macroscopic and microscopic method

Fungal isolates colonies from different media observed macroscopically for characteristic colonies of *Aspergillus spp* [15]. The major and significant macroscopic features in species identification were colony morphology such as the colony diameter, colony texture, size, color of the colony, sporulation and presence of exudates and pigments production studied and pictures were taken. Riddle's classic slide culture method done for the microscopic study of fungal isolates [13]. When the mould sporulated the coverslip carefully withdrawn from the media and mounted in a drop of lactophenol cotton blue stain on a microscope slide. Another drop placed on top of the small coverslip before completing the assembly with a coverslip. This pressed down slightly with the tip of the finger to expel any air bubble and additional disintegrate the hyphal growth to improve observation. The slides observed under 40-x magnification of a compound microscope (Olympus CX2LI bright field compound microscope). Microscopic features for the identification were the conidiophores, stipes colour and vesicles shape and seriation, metula covering, mycelia and shape, texture, and colour of conidia, conidial heads. Lastly, we compared the morphological characteristics of fungal isolates from the feeds with the standard species of *Aspergillus flavus* stored in liquid paraffin earlier.

3. Results

3.1 Signs of growth of fungal isolates in tilapia feed

Presence of fungal growth was clearly visible in all the feed. They were initially white in colour (Fig. 1) then turned into light green colour. Fungal growth affected the whole appearance of the feeds on which they were seen. Physical signs of fungal isolates in feeds were included dustiness, caking, poor flow out of grain bins, mouldy and musty smell and darkening of feed. Growth of fungal isolates on five tilapia feed were shown in Fig.1 (a, b, c, d and e).

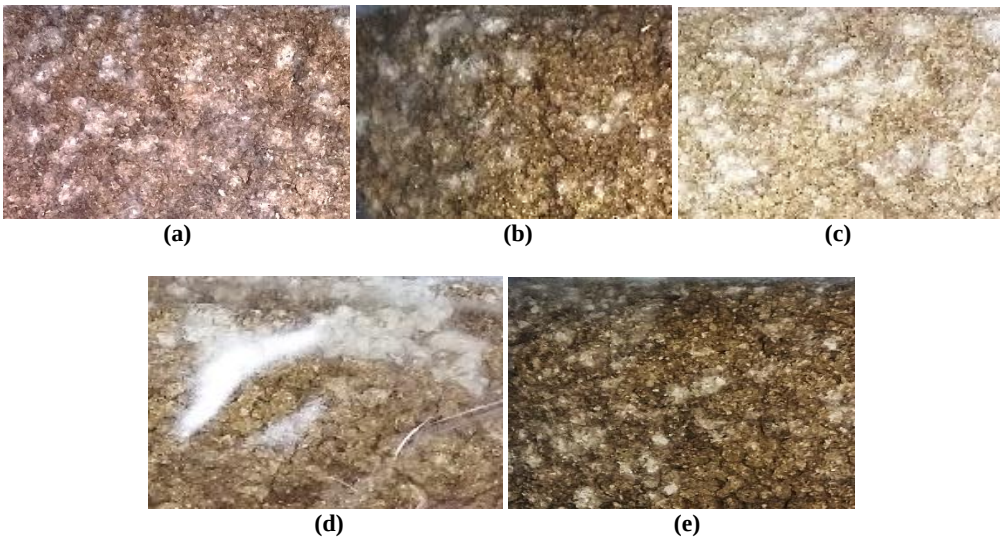


Fig 1: Growth of fungal isolates on Nile tilapia feed (a) TF1, (b) TF2, (c) TF3, (d) TF4, (e) TF5.

3.2 Macroscopic observations

Fungal isolates from feed grown in three media examined to determine their accurate identification and comparing the macroscopic characteristics such as colony color exudates, sclerotia and texture of the colony (Table 1 a, b, c).

3.2.1 Macroscopic Characteristics of the fungal isolates on PD broth

Colonies of fungal isolates from different feeds on PD broth at 28°C is yellowish green in colour in the front side and pale yellowish in reverse side with cottony texture (Fig. 2 a). The growth of the fungal isolates was rapid, initially the isolates acquired the white colour of the mycelia then it turned into green colour. Colony appeared with smooth margin in nature. The fungal colonies were plain and flat at the edges. The sclerotia, which are the compact masses of hardened fungal mycelia, seen in fungal isolates and they were brown in colour. Exudates produced and appeared as tiny uncoloured liquid droplets embedded within the mycelia. The isolates not produced any soluble pigments in the media.

3.2.2 Macroscopic Characteristics of fungal isolates on CYA agar

Colonies on CYA agar were flat and smooth margin and yellow colour at the beginning of growth but becoming bright to dark green with age. The undersides of the colonies were pale yellowish-orange in colour (Fig. 2 b). Colony showed velvety texture and the mycelia was white in colour. The growth of the fungal colony was moderate to rapid in nature. The colonies of all isolates appeared moist and exudate seen, but sclerotia not produced. No soluble pigments observed.

3.2.3 Macroscopic Characteristics of the fungal isolates on YEPD broth

The culture of the isolates on YEPD broth resulted in fungal colonies with green colour in the front side and colourless in reverse of the colony (Fig. 2 c). The growth of the fungal colony was moderate in nature. Colony pattern constricted in appearance and the colonies produced white mycelia which were very soft velvety on the surface. The isolates produced exudates, which were uncoloured. Sclerotia not produced any of the isolates and no soluble pigments seen.

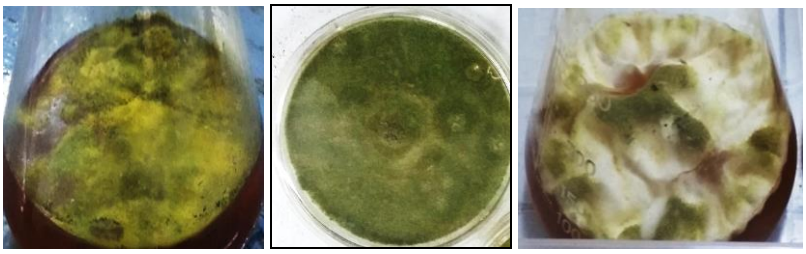


Fig 2: Growth of fungal isolates from Nile tilapia feed on different identification media: (a) PD broth, (b) CYA agar, (c) YEPD broth.

Table 1: Macroscopic characteristics of fungal isolates from Nile tilapia feed on three different growth media.

(a) Macroscopic characteristics used for the identification of fungal isolates from Nile tilapia feed on PD broth									
Feed types	**Colony pattern**	**Colony color**	**Texture**	**Mycelia colour**	**Sclerotia**	**Margins**	**Growth**	**Elevations**	**Exudate**
TF1-TF5	Smooth margin	Yellow green	Pale yellowish	Cottony	White	Present	Entire	Rapid	Umbonate
(b) Macroscopic characteristics used for the identification of fungal isolates from Nile tilapia feed on CYA agar									
TF1-TF5	Smooth margin	Dark green	Pale yellowish orange	Velvety	White	Absent	Entire	Moderate to rapid	Umbonate
(c) Macroscopic characteristics used for the identification of fungal isolates from Nile tilapia feed on YEPD broth									

TF1-TF5	Constricted margin	Green	Colourless	Velvety	White	Absent	Entire	Moderate	Umbonate

3.3 Microscopic observations

Fungal isolates from feed grown in three media examined to determine their accurate identification and comparing the microscopic characteristics such as conidiophores, vesicles, metulae, phialides, and conidia are shown in Table 2 (a, b, c).

3.3.1 Microscopic characteristics of fungal isolates on PD broth

Conidiophores (Fig. 3 a) surface appeared as spherical (Fig. 4.a) in shape and they were heavily walled, coarsely roughened and vesicle bearing. Vesicle elongated, globose in shape and conidial head appeared as yellow-green in colour (Fig. 5 a). Vesicle seriation was biseriate and the phialides were borne on the metuale, and, the metulae covered nearly the entire surface of the vesicles and radiated from the vesicles in all directions. Conidial masses radiate from conidia head. Conidial walls showed to be smooth to finely rough in nature (Fig. 6 a) which then dominated colony appearance.

3.3.2 Microscopic characteristics of fungal isolates on CYA agar

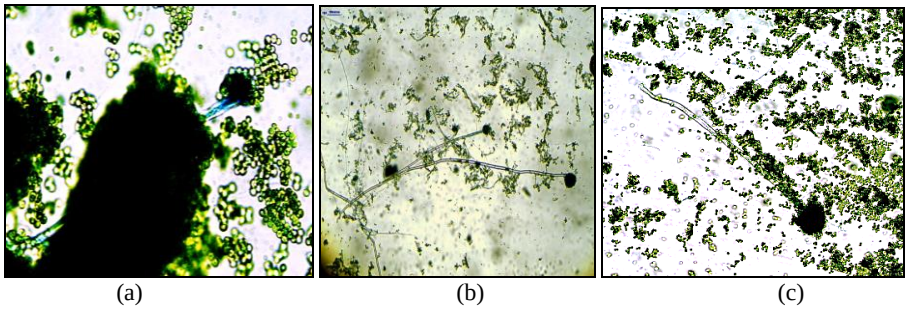
The conidiophores were, thick-walled, and coarsely roughened, spherical surface and were vesicle bearing (Fig. 4 b) and the vesicles were globose in shape and biseriate seriation was seen. Metulae covered almost the entire surface of the vesicles. Conidial heads are typically radiate and dark green in colour, later splitting to form loose columns (Fig. 5 b). Conidiophores were rough in appearance. The colonies produced olive green colour conidia with smooth to the rough surface (Fig. 6 b). The conidia covered the entire surface of the colonies except for the edges, where a white border produced. The white border then disappeared as the colonies became larger and produced more conidia (Fig. 3 b).

3.3.3 Microscopic characteristics of the fungal isolates on YEPD broth

Conidiophore appeared green in colour with a rough and spherical surface (Fig. 4 c). Vesicles were globose in shape and biseriate. Conidia were typically round, with smooth to finely roughened walls and appeared in chains (Fig. 6 c). Conidial heads were mostly radiate with conidial masses splitting into blocky columns (Fig. 5 c). During sporulation, the isolates produced dull light green conidia, which turned in to dark green after six days, which then dominated colony appearance (Fig. 3 c).

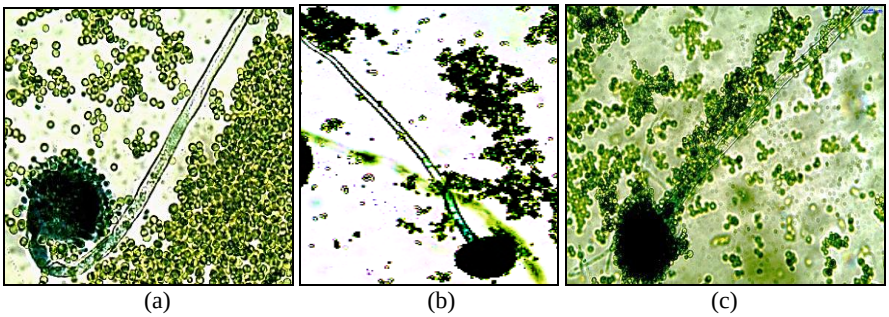
Table 2: Microscopic characteristics of fungal isolates from different Nile tilapia feed in three different growth media.

(a) Microscopic characteristics used for the identification of fungal isolates from Nile tilapia feed on PD broth								
Feed types	**conidiophore**			**vesicle**			**conidia**	
	Stipes colour	**Stipe walls**	**Surface**	**Vesicle shape**	**Vesicle seriation**	**Metula covering**	**Colour**	**Conidia surface**
TF1-TF5	Dark brown	Rough	Spherical	Globose	Biseriate	3/4	Yellow green	Smooth to rough
(b) Microscopic characteristics used for the identification of fungal isolates from Nile tilapia feed on CYA agar								
TF1-TF5	Greyish green	Rough	Spherical	Globose	Biseriate	3/4	Olive green	Smooth to rough
(c) Microscopic characteristics used for the identification of fungal isolates from Nile tilapia feed on YEPD broth								
TF1-TF5	Light green	Rough	Spherical	Globose	.Biseriate	3/4	Dark green	Smooth to rough



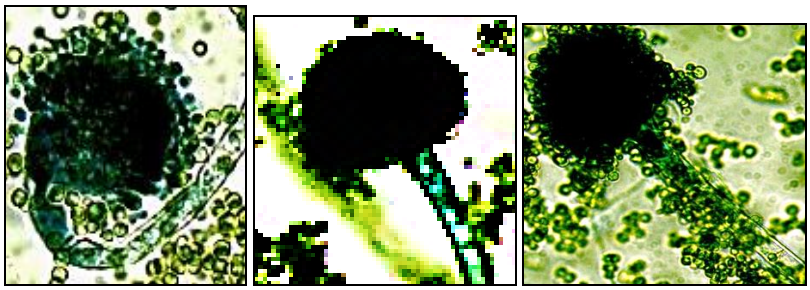
(a) (b) (c)

Fig 3: Isolated fungal isolates colony with spores from Nile tilapia feed on (a) PD broth, (b) CYA agar, (c) YEPD broth of 10 x magnification.



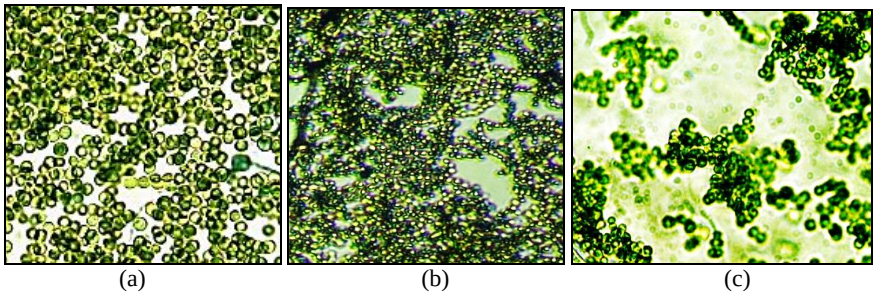
(a) (b) (c)

Fig 4: Isolated fungal isolates conidiophore with biseriate conidial head from Nile tilapia feed on (a) PD broth, (b) CYA agar, (c) YEPD broth of 40 x magnification.



(a) (b) (c)

Fig 5: Isolated fungal isolates conidial head from Nile tilapia feed on (a) - PD broth, (b) -CYA agar, (c)-YEPD broth of 40 x magnification



(a) (b) (c)

Fig 6: Isolated fungal isolates conidia from Nile tilapia feed on (a) - PD broth, (b) - CYA agar and (c)-YEPD broth of 40 x magnification.

4. Discussion

Aspergillus flavus is the most common species in section *Flavi* causing contamination of food and feed [18]. The highest dominance of *Aspergillus flavus* in the present study is similar with Magnoli *et al*. (2002) [20] in Serbia, Oliveira *et al*. (2006) [23] in Argentina and is similar to those published by Atehnkeng *et al*. (2008) [5], but differs to Saleemi *et al.* (2010) [33] in Pakistan who found that the most frequently *Aspergillus* were *Aspergillus niger* followed by *Aspergillus flavus*, because of high humidity and high temperature which responsible for higher frequency of *Aspergillus niger* in poultry feeds as a compared with other species of *Aspergillus*. Based on morphological studies, our finding showed that all the fungal isolates isolated from the five-tilapia feeds were closely similar to standard *Aspergillus flavus* species. Morphological identification of *Aspergillus* mostly followed the protocols of Raper and Fennell (1965) [27], Klich (2002) [18], Pitt and Hocking (2009) [26] and Samson (1989) [35]. Earlier studies on *Aspergillus* species with similar design reported by studies of Anaissie *et al*. (2003) [23], Curtis and Baker (2005) [11] and McClenny (2005) [21].

All the *A. flavus* isolates growth were moderate to rapid. Conidiophore, vesicles, and conidia evaluated in this study. In addition, the macroscopic characteristics were in harmony with the *Aspergillus flavus* characteristics previously described by Diba *et al*. (2007) [13] and Rodrigues *et al*. (2007) [30]. Sclerotia production has reported being a rare characteristic of *Aspergillus flavus*, although it is one of its identifying characteristics In accordance with the taxonomic descriptions by Klich (2002) [18] and Clayton (1977) [20]. Sclerotia in *Aspergillus flavus* contain aflatoxins and sclerotium production is associated with specific secondary metabolites, including indoloterpenes such as aflatrems, aflavazole, aflavinines, anominine, aspernomine, paspalines and polyketides such as aflavarins [10, 36, 37]. Therefore, this group of isolates from the test feed identified as *Aspergillus flavus* species. The basic microscopic morphology is the same for all isolates on three media. However, some other microscopic structures are distinctive to certain media and constitute the key features for species identification together with the surface color, growth and texture of the colony. Colour and texture of the *Aspergillus flavus* isolates were found to be a little variation in each media were yellowish-green, dark green, or olive colour colonies encircled by a white border, which ultimately overlapped by conidia. The conidia of isolates of this species are echinulate and conidiophore of this species are strictly rough as observed by Diba *et al*. (2007) [13]. The surfaces of the colonies were velvety to woolly in texture and often with a floccose centre. The isolates from the feed samples produced exudates on all media that were used and similar results were reported by Astoreca *et al*. (2011) [4], Doster *et al*. (2009) [14], Giorni *et al*. (2007) [15] and Rodrigues *et al*. (2009, 2011) [29, 30]. An important diagnostic feature for *Aspergillus flavus* was the globose vesicles and rough conidiophore walls as observed by Diba *et al*. (2007) [13].

Identification of *Aspergillus spp* by using differential media like PD broth CYA agar and YEPD broth demonstrated that it was a very easy and reliable technique for identification of *Aspergillus spp*. Culture time of 7 days or more on differential media is generally required for macroscopic and microscopic characteristics of fungal colonies to identify them. This study examined the effect of different media on the growth of *Aspergillus flavus* isolated from tilapia feed. Generally, PD Broth used for the isolation, enumeration, and identification of yeast and moulds, the Association of Public Health (APHA) recommends this medium. At temperature 28°C, *A. flavus* grew better on PD broth than CYA agar and YEPD broth. PD broth media showed a high affinity for the growth of mycelium and early spore formation than other media examined. These observations may suggest that the PD broth may influence the growth of *A. flavus* at 28°C. Nutrient components PD broth showed to play an important role in initiating mycelial growth and toxin production and this low - cost media can regularly use for the identification of *Aspergillus* spp.

5. Conclusions

In the present study different culture media and environmental factors affecting the growth and sporulation of the *Aspergillus*

flavus under different conditions. Using these three growth media, *A. flavus* isolated and successfully identified in the entire tilapia feeds. All the three culture media i.e. PD broth, CYA agar, and YEPD broth supported the growth of *Aspergillus flavus*, at optimal pH and temperature conditions, in which *Aspergillus flavus* showed excellent growth on Potato Dextrose broth as compared to CYA agar and YEPD broth after 9 days of the incubation period. The suitability of a growth medium depends upon the specificity of a fungus under study and the aim of the experiment. The nutrient media, temperature, and pH is a major factor that affects the growth and sporulation of fungi. In this study, the use of three media, which allowed sufficient assessment of the macroscopic and microscopic characteristics of *Aspergillus flavus* isolates from the feeds. Furthermore, it found that the PD broth supported a maximum growth rate of *Aspergillus flavus* of the three media tested. CYA and YEPD media showed a lower growth rate. Therefore, we recommend morphological identification in which macroscopic and microscopic studies of fungal isolates are a sensitive and reliable method for the identification of *Aspergillus spp*. No single method is perfect in recognizing species and a polyphasic approach recommended, where morphological examination and DNA sequence data considered together.

6. Acknowledgment

We are very much thankful to School of Industrial Fisheries Cochin, University of Science and Technology, Kerala, India for providing us necessary laboratory equipment's for research.

7. Conflict of interest

We declare that we have no conflict of interest.

8. References

1. Ahmed MM, Fakruddin M, Hossain MN, Mahbub KR, Chowdhury A. Growth response of *Aspergillus flavus* IMS1103 isolated from poultry feed. Asian Journal of Medical and Biological Research. 2016; 2(2):221-228.
2. Alwakeel SS. Bacterial and *Aspergillus spp*. contamination of domestic kitchens in Riyadh, Saudi Arabia. Saudi J. Biol. Sci, 2007; 14(1):1-6.
3. Anaissie EJ, Stratton SL, Dignani MC, Lee CK, Summerbell RC, Rex JH *et al*. Pathogenic molds (including *Aspergillus* species) in hospital water distribution systems: a 3-year prospective study and clinical implications for patients with hematologic malignancies. Blood. 2003; 101(7):2542-2546.
4. Astoreca AL, Dalcero AM, Pinto VF, Vaamonde G. A survey on distribution and toxigenicity of *Aspergillus* section *Flavi* in poultry feeds. International Journal of Food Microbiology. 2011; 146(1):38-43.
5. Atehnkeng J, Ojiambo PS, Donner M, Ikotun T, Sikora RA, Cotty PJ *et al*. Distribution and toxigenicity of *Aspergillus* species isolated from maize kernels from three agro-ecological zones in Nigeria. International Journal of Food Microbiology. 2008; 122(1, 2):74-84.
6. Barnett HL, Hunter BB. Illustrated genera of imperfect fungi. Mac. American Phytopathological Society Press. St. Paul, Minnesota, USA, 1998.
7. Bennett JW. An overview of the genus *Aspergillus*, *Aspergillus* molecular biology and genomics. Caister Academic Press, Norfolk, United Kingdom, 2010, 1-17.
8. Bhat R, Rai RV, Karim AA. Mycotoxins in food and feed: present status and future concerns. Comprehensive reviews in food science and food safety. 2010; 9(1):57-81.
9. Clayton YM. Medically Important Fungi: A Guide to Identification. Proceedings of the Royal Society of Medicine. 1977; 70(5):359.
10. Cole RJ, Dorner JW, Springer JP, Cox RH. Indole metabolites from a strain of *Aspergillus flavus*. Journal of Agricultural and food chemistry. 1981; 29(2):293-295.
11. Curtis L, Cali S, Conroy L, Baker K, Ou CH, Hershow R, Norlock-Cruz F *et al*. *Aspergillus* surveillance project at a large tertiary-care hospital. Journal of Hospital Infection. 2005; 59(3):188-196.
12. Devegowda G. Mycotoxins: Economic risks and their control. Handbook of Poultry Nutrition/G. Devegowda, VR Reddy, DT Bhosale//Published by American Soybean Association, 2001, 246-260.
13. Diba K, Kordbacheh P, Mirhendi SH, Rezaie S, Mahmoudi M. Identification of *Aspergillus* species using morphological characteristics. Pakistan journal of medical sciences. 2007; 23(6):867.
14. Doster MA, Cotty PJ, Michailides TJ. Description of a distinctive aflatoxin-producing strain of *Aspergillus nomius* that produces submerged sclerotia. Mycopathologia. 2009; 168(4):193.
15. Giorni P, Magan N, Pietri A, Bertuzzi T, Battilani P. Studies on *Aspergillus* section Flavi isolated from maize in northern Italy. International journal of food microbiology. 2007; 113(3):330-338.
16. Hasan M, New MB. On-farm feeding and feed management in aquaculture workshop. Manila, Philippines, 13-15 September 2010. FAO Fisheries and Aquaculture Technical Paper, 2013, 583.
17. Hesseltine CW, Shotwell OL, Ellis JJ, Stubblefield RD. Aflatoxin formation by *Aspergillus flavus*. Bacteriological reviews. 1966; 30(4):795.
18. Klich MA. Identification of common *Aspergillus* species. Centraal bureau voor schimmel cultures. AD Utrecht, Netherland, 2002, 116.
19. Kurtzman CP, Horn BW, Hesseltine CW. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie van leeuwenhoek, 1987; 53(3):147-158.
20. Magnoli C, Chiacchiera SM, Miazzo R, Palacio G, Angeletti A, Hallak C *et al*. The mycoflora and toxicity of feedstuffs from a production plant in Cordoba, Argentina. Mycotoxin Research. 2002; 18(1):7-22.
21. McClenny N. Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: the traditional approach. Medical mycology. 2005; 43(1):125-128.
22. Myatt TA, Minegishi T, Allen JG, MacIntosh DL. Control of asthma triggers in indoor air with air cleaners: a modeling analysis. Environmental health. 2008; 7(1):43.
23. Oliveira GR, Ribeiro JM, Fraga ME, Cavaglieri LR, Direito GM, Keller KM *et al*. Mycobiota in poultry feeds and natural occurrence of aflatoxins, fumonisins and zearalenone in the Rio de Janeiro State, Brazil. Mycopathologia. 2006; 162(5):355-362.
24. Parra R, Magan N. Modelling the effect of temperature and water activity on growth of *Aspergillus niger* strains and applications for food spoilage moulds. Journal of Applied Microbiology. 2004; 97(2):429-438.

25. Peterson SW, Ito Y, Horn BW, Goto T. *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. Mycologia. 2001; 93(4):689-703.
26. Pitt JI, Hocking AD. Fungi and food spoilage New York: Springer. 2009, 519.
27. Raper KB, Fennell DI. The genus *Aspergillus* Baltimore: The Williams and Wilkins Company. 1965, 686.
28. Rodrigues P, Santos C, Venancio A, Lima N. Species identification of *Aspergillus* section Flavi isolates from Portuguese almonds using phenotypic, including MALDI-TOF ICMS, and molecular approaches. Journal of applied microbiology. 2011; 111(4):877-892.
29. Rodrigues P, Venancio A, Kozakiewicz Z, Lima N. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* isolated from Portuguese almonds. International journal of food microbiology. 2009; 129(2):187-193.
30. Rodrigues P, Soares C, Kozakiewicz Z, Paterson R, Lima N, Venancio A. Identification and characterization of *Aspergillus flavus* and aflatoxins, 2007.
31. Rosso L, Robinson TP. A cardinal model to describe the effect of water activity on the growth of moulds. International journal of food microbiology. 2001; 63(3):265-273.
32. Saito M, Machida S. A rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor. Mycoscience. 1999; 40(2):205-208.
33. Saleemi MK, Khan MZ, Khan A, Javed I. Mycoflora of poultry feeds and mycotoxins producing potential of *Aspergillus* species. Pakistan journal of Botany. 2010; 42(1):427-434.
34. Samapundo S, Devlieghere F, De Meulenaer B, Debevere J. Growth kinetics of cultures from single spores of *Aspergillus flavus* and *Fusarium verticillioides* on yellow dent corn meal. Food microbiology. 2007; 24(4):336-345.
35. Samson RA. Filamentous fungi in food and feed. Journal of applied bacteriology. 1989; 67:27s-35s.
36. TePaske MR, Gloer JB, Wicklow DT, Dowd PF. Aflavazole: a new antiinsectan carbazole metabolite from the sclerotia of *Aspergillus flavus*. The Journal of Organic Chemistry. 1990; 55(18):5299-5301.
37. TePaske MR, Gloer JB, Wicklow DT, Dowd PF. Aflavarin and β-Aflatrem: new anti-insectan metabolites from the sclerotia of *Aspergillus flavus*. Journal of Natural Products. 1992; 55(8):1080-1086.
38. Thathana MG, Murage H, Abia ALK, Pillay M. Morphological characterization and determination of aflatoxin-production potentials of *Aspergillus flavus* isolated from maize and soil in Kenya. Agriculture. 2017; 7(80):1-14.