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Mycotoxigenic Fungi in Fish Feeds With Reference To Seasonal Variations

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Abstract
Addition of artificial feeds is one of the most important aspect of fish farming which ensures healthy and early growth of fishes. In a tropical country like India. Where 26% of the total fungi have been reported to possess toxigenic potentiality. (Verma, 1989), there is ample chance of mycotoxin contamination of food and feeds. Hence the fish, specially the culturable species, in this region are also exposed to mycotoxins hazards through naturally contaminated feeds. An extensive survey of fish feeds was conducted during January to December, 2019 in different blocks of Darbhanga District. During present investigation maximum number of apparently moulded samples were encountered retailer or former level. Better sanitation prevents them to be affected from relatively hight atmosphereic humidity. Trenk and Hartman (1970) however, suggested that rehumified grain deteriorates much faster than grains harvested damp and this is reflected in the increased levels of various mycotoxins and specially aflatoxin in the former. In contrast, lack of ventilation, sanitation and gradual increase in moisture content of oil cakes (9-10%) at retailer or farmer level increase the pace of mycobial contamination, growth and cosequently results in high percent of moulded samples. The water content of the oil cackes and ground pulses mixture is relatively low as their production and marketing involve drying. During monsoon months the high humidity/moisture available from any source (increased relative humidity, storage in contact of moist soil without any protection barrier against moisture penetration and recurrent flood in the area) is trapped rapidly by these substrates (since they are dried products). A. niger appeared as most dominant mycoflora on present substrates followed by A. flaus and A. versicolor. The concentration of sterigmatocystin was found to be highest in MOC-II samples.

Keywords: *aflatoxin, Sterigmatocystin, A versicolor, fish feeds.*

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I. Introduction

Addition of artificial feeds is one of the most important aspect of fish farming which ensures healthy and early growth of fishes. The common supplementary feeds used in this geographical area (Darbhanga, Bihar) are mustard oil cake, mixed oil cake, ground cheap pulses mixture, fish meal, rice bran, wheat flour and ground maize. These agricultural byproducts that the bulk of the artificial dietary formulation, since growing to harvesting and subsequent storage are prone to fungal spoilage (Moreau, 1979; Goldblatt. 1969, Verma, 1989, Verma and Mukherjee, 2001, Prasad 2002, Manning 2010 and Bryden, 2012).

In a tropical country like India. Where 26% of the total fungi have been reported to possess toxigenic potentiality. (Verma, 1989), there is ample chance of mycotoxin contamination of food and feeds. Besides, the items incorporated in fish feeds have been reported to be contaminated with alarming levels of mycotoxins in various parts of India (Anonymous, RRI, Hyderabad, 1967; Amla *et. al.*, 1971; Krishnamachary *et. al.*, 1975 and 1975b, Mishra and Singh, 1978; Vora, 1978; Neelkanthan, 1979; Verma, 1989 and Prasad, 2002).

The excellent environmental conditions such as ambient temperature, high humidity, moisture and insanitory conditions in storage of fish feeds accelerates the pace of mycobial spoilage with high risks of mycotoxin contamination.

In addition to this, natural incidence of aflatoxins and other mycotoxins in oil cakes, which comprise the important constituents of fish feeds, have also been reported from Bihar (Daradhiyar *et. al.*, 1982; Verma and Pandey, 1982; Verma *et. al.*, 1985 and Verma *et. al.*, 1986a). Hence the fish, specially the culturable species, in this region are also exposed to mycotoxins hazards through naturally contaminated feeds.

II. Materials And Methods

An extensive survey of fish feeds was conducted during January to December, 2019 in different blocks of Darbhanga District viz. Jale, Singhwara, Keoti, Darbhanga Sadar, Bahadurpur, Hayaghat, Manigachi, Baheri, Benipur, Ghanshyampur, Biraul and Kusheshwarasthan.

Three types of fish feeds like mustard oil cake (MOC-I), mixed oil cake (MOC-II) and ground cheap pulses mixture (GPM); most commonly used in the district were collected during summer (S), Winter (W) and rainy (R) months from retail shops, whole sale depots and oil mills.

Minimum 500g of different types of fish feed was obtained from each lot by random sampling. Each sample was kept in polythene bags, serially numbered and brought to the laboratory. Samples brought to the laboratory were finely blended in Bajaj Blender. A 100g of subsample was separated from the homogenous sample and kept in polythene bags for further investigation. The original a lot of sampls were also preserved.

Moisture content of feed sample was determined by wet weight basis as suggested by Neergard (1977). 50g of each feed sample was subjected to culture in B.O.D. incubaton at 25 ± 2^0C for 5 to 6 days.

At the end of 5-6 days, the different fungal colonies appearing on the feeds were isolated by transferring a few conidial heads with sterilized inoculation needle to the sterilized culture tubes containing Asthana and Howker's "A" medium (Glucose, 59; KNO_3–3.5g; KH_2PO_4 –1.75g; $MgSO_4.7H_2O$–7.5g; Agar agar–30g; and distilled water 1 Lit.) Repeated sub-culturings were done to maintain the pure culture of different fungal isolates. All sub cultures were done in culture cabinet fitted with germicidal UV- tube.

Pure culture of identified strains obtained by inoculating conidial heads with sterilized inoculation needle to the culture tubes containing AH media slants respectively and incubated in B.O.D at 28 ± 2^0C for 7–10 days.

Different fungal isolates were identified at the generic or in some cases at the species level. The culture strains of *Aspergillus vericolor* isolates were properly numbered and maintained by repeated sub-culturing time to time on sterilized culture slants. Sub culturing was done every 45 day.

The frequency of occurrence of various fungal forms at the end of incubation period on moistened feed samples in petriplates was determined in terms of percentage incidence and percentage abundance using the following formula:

$$\text{Percent incidence} : \frac{\text{Number of observations in which a.species appeared}}{\text{Total number of observations}} \times 100$$

$$\text{Percentage abundance} : \frac{\text{Total number of colonies of a species in all observations taken together}}{\text{Total number of colonies of all species}}$$

III. Observations

Table-1 shows the numbers of apparently moulded samples among the fish feeds collected from whole-seller depots, retailer shops (fig. 1) farmer's godown and oil mills. The maximum percentage of moulded samples (74/66.2%) was recorded in case of MOC-II (mixed oil cakers) followed by mustard oil cake (111/35.1%) and ground pulses mixture (75/32%) respectively. The table also depicts that fungal infestation were more apparent on the samples collected from farmer's godown (15/45.4%) followed by retailer's shop (42.4%) and wholesale depots (25.7%). The samples collected from oil mills were relatively less contaminated (16.6%).

Incidence of various fungi on the fish feeds in three different seasons and their percentage colonization on the feed samples collected during present work has been presented in table 2. The fungi recorded from all the three types of fish feeds round the year are *Rhizopus sps*, *Penicillium* and *Aspergillus sps*. The *Sycephalastrum sps*. could be recorded only one MOC-II samples during rainy months. similarly, The *Alternaria sps*. And *A.ochraceous* were found only on oil cakes samples (MOC I and II) but not on GPM. (Table.2).

Out of the seven species of *Aspergillus*, *A. niger* (fig.2) was recorded as most dominant species on all the fish feed samples followed by *A.flavus* and *A.versicolor* (fig. 3). It was important to note the presence of *A. nidulanse* only on MOC-I and GPM samples. On MOC-I the species (*A. nidulanse*) were in trace but on GPM they showed a significant appearance (Table.2). Thus, the species uniformly recorded on all the feed samples included *A. niger, A.versicolor* and *A. flavus* (Table.2). The two species *A. terreus,* and *A. tamarii* were noted as infrequent invaders. Among penicillium group most commonly found strain included *Penicillium viradicatum.*

Table-3 decipts the overall infestation of different toxic fungi on all the samples studied presently and the percentage of the strains capable of producing sterigmatocystin as their secondary toxic metabolites. The

total number of moulded samples collected during all the three seasons (summer, Rainy and winter) was, recorded on MOC II (49) followed by MOC-I (42) and GPM (24). Out of which 13 samples of MOC-II, 8 samples of MOC-I and only 7 samples of GPM were found to positive to sterigmatocystin under UV light. This indicates that less than 18% of MOC–I samples, about 20% of MOC–II samples and 25% of GPM samples harboured toxigenic strain of *A. versicolor* producing sterigmatocystin.

The concentration of sterigmatocystin on TLC plates were estimated visually in comparison to standard spots. The result has been presented (Table. 4) It shows that out of the 8 strains chemically assayed for sterigmatocystin, 7 were found positive. Similarly 12 of the strains collected from MOC-II and all the seven strains chemically assayed from GPM samples were positive to sterigmatocystin in variable quantities. In two samples of MOC-I (3 and 6), 6 samples of MOC-II and one sample of GPM the sterigmatocystin concentration was recorded as high. Similarly, low concentration of sterigmatocystin was recorded in 2 of MOC-I (4 and 5), one of MOC-II (7) and three of GPM (4, 5 and 6). In others the concentration was simply in trace (Table. 4).

Table No.1
Survey of moulded samples in three types of fish feeds from different sources in Darbhanga district.

Type of feed.	Wholeseller		Retailer shops		Farmer Godown		Oil Mills		Total sample/ moulded samples (No/ %)
	No. of samples	Moulded (No./%)	No. of samples	Moulded (No./%)	No. of samples	Moulded (No./%)	No. of samples	Moulded (No./%)	
MOC-I	35	9/25.7	28	12/42.8	33	15/45.4	18	3/16.6	111/35.1
MOC-II	30	12/40	17	11/64.7	18	11/61.1	11	3/27.2	74/66.2
GPM	40	8/20	16	6/37	34	10/29.4	–	–	75/32

MOC-I, Mustard oil cakes; MOC-II -mixed oil cakes; GPM- ground pulses mixture.

Table No. 2
Details of mycoflora on three types of fish feeds and their percentage colonization during three different seasons in Darbhanga District.
(t indicates percent colonization less than 5%).

Common Mycoflora	MOC-I			MOC-II			GPM		
	S	R	W	S	R	W	S	R	W
Alterneria Sp.	–	T	t	T	t	t	–	–	–
Syncephalastrum Sp.	–	–	–	–	t	–	–	–	–
Curvularia Sp.	t	T	T	–	–	–	–	–	–
Penicillium Sp.	20	10	10	20	10	25	12	7	8
Rhisopus Sp.	20	25	15	–	20	12	8	25	20
Aspergillus Sp.	48	60	45	55	65	52	50	62	40
CD = 14.64									
A. terreus	t	–	–	T	t	t	t	t	–
A. tamarri	–	–	–	–	t	–	–	–	–
A. parasiticus	–	–	–	–	–	–	t	t	t
A. nidulanse	t	T	T	T	t	t	16	18	15
A. rubur	–	T	T	–	–	–	–	–	–
A. versicolor	32	39	40	51	44	40	18	40	46
A. niger	59	36	40	51	40	42	46	40	35
A. flavus	30	60	35	38	48	40	14	25	20
CD = 15.04									

MOC-I, mustard oil cake, MOC-II mixed oil cake, GPM- ground pulses mixture, S- Summer, R- Rainy, W- winter.

Table No. 3
Number of moulded samples of three types of fish feeds collected during three different seasons from Darbhanga distric.

Type of feed	Number of samples collected during different seasons/number of moulded samples.			Total number of moulded samples.	Positive to sterigmatocystin under UV light
	Summer (S)	Rainy (R)	Winter (W)		
MOC-I	37/11	38/23	36/8	42	8
MOC-II	24/17	25/21	25/11	49	13
GPM	25/6	25/13	25/5	24	7

MOC-I, mustered oil cake, MOC-II - mixed oil cake, GPM- ground pulses mixture.

Table No. 4
Visual estimation of the sterigmatocystin concentration in positive samples in three types of fish feeds.

Type of feed	Serial of samples assayed for sterigmatocystin	Instensity of sterigmatocystin spots on TLC plates under UV-light (365 nm) (Intensity graded as high (h), low (I) and traces (t).			
		High	Low	Trace	Not detected
MOC-I	1	–	–	t	–
	2	–	–	–	–
	3	H	–	–	–
	4	–	I	–	–
	5	–	I	–	–
	6	H	–	–	–
	7	–	t	–	–
	8	L	–	–	–
MOC-II	1	H	–	–	–
	2	–	–	t	–
	3	–	–	t	–
	4	H	–	–	–
	5	H	–	–	–
	6	H	–	–	–
	7	–	L	–	–
	8	H	–	–	–
	9	–	–	t	–
	10	–	–	–	–
	11	–	–	t	–
	12	H	–	_	–
	13	–	–	t	–
GPM	1	H	–	–	–
	2	–	–	t	–
	3	–	–	t	–
	4	–	L	–	–
	5	–	L	–	–
	6	–	L	–	–
	7	–	–	t	–

MOC-I, mustered oil cake, MOC-II - mixed oil cake, GPM- ground pulses mixture.

Fig.–1. Various types of agricultural products incorporated in fish feeds at retail shop

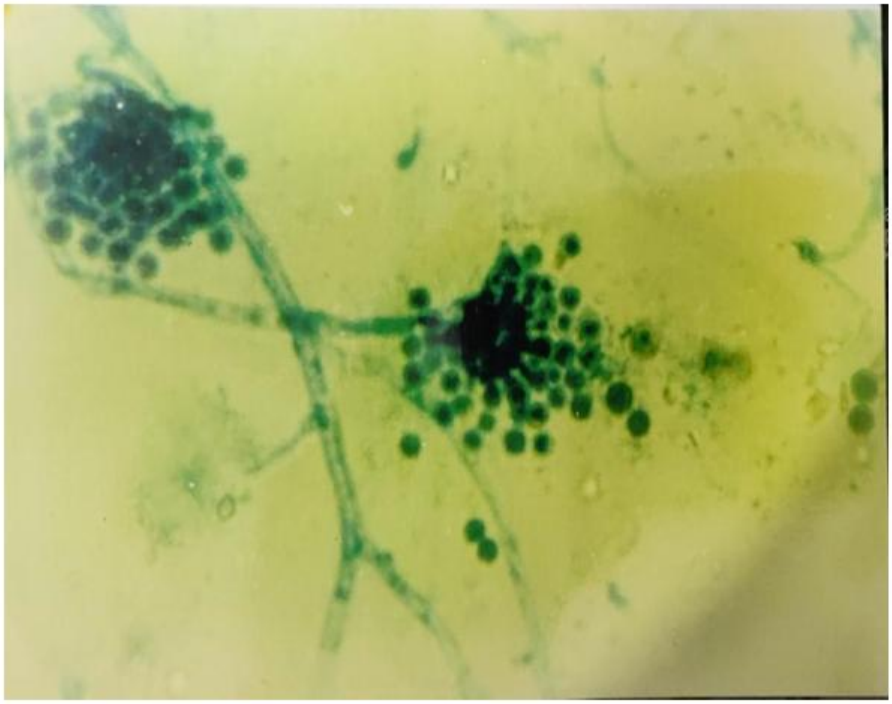

Fig.–2. Conidial head of *A. niger* ×600

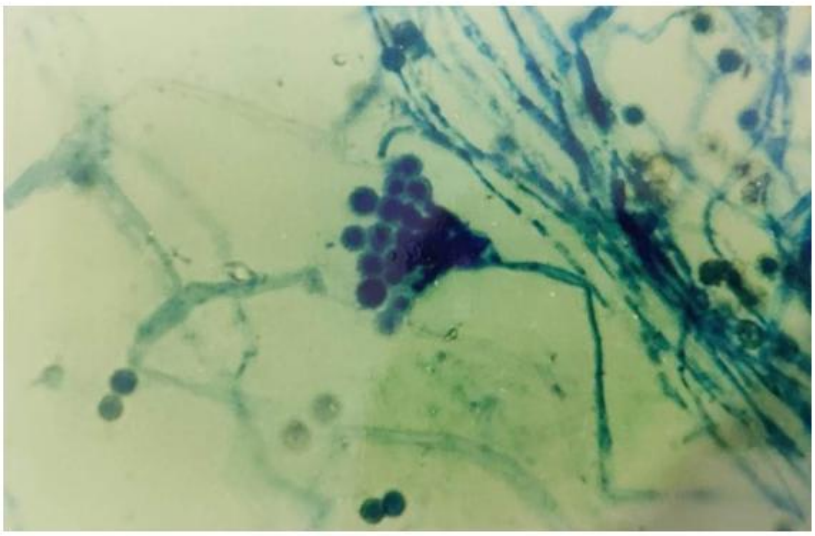

Fig.–3. Conidial head of A. Versicolor ×600.

IV. Discussion

All agricultural commodities and their byproducts are always susceptible to fungal spoilage in different pre-and post-harvest stages (Moreau, 1974, Kurata, 1978, Marijani, 2019; Kholife *et. al.*, 2019; Greco *et. al.*, 2015, Dalcero *et. al.*, 1998; Ariyo *et. al.*, 2013, D'Mello and Mac Donald, 1997). But the storage period has been regarded as most critical from mold contamination point of view (Morean, 1974) due to various factors operating simultaneously inside the system. These factors have been indentified as source of contamination, physical condition of the substrates and the type of storage. As a matter of factor the above factors decide the degree of mould spoilage (Verma, 1989).

During present investigation maximum number of apparently moulded samples were encountered retailer or former level. The samples collected from wholesaller depots or oil mills were comparatively less moulded. During field survey it was generally observed that the feed samples were stored in well aerated store houses with relatively better sanitation and ventillation. It has been opined that such factors prevent mould infestation in many ways (Bertuzzi *et. al.*, 2017, Ferre, 2016, Kumar *et. al*,, 2008 Surekha *et. al.*, 2011 and FSA, 2002). Schroeder and Sorenson (1961) have observed that ventillation of stored grains reduce the mould attack.

The fresh oil cakes, in general, contain 5-6% of water which is usually not favourable to mould contamination and growth of fungus so frequently (Austwick and Ayerest, 1963). Better sanitation prevents them to be affected from relatively hight atmosphereic humidity. Trenk and Hartman (1970) however, suggested that rehumified grain deteriorates much faster than grains harvested damp and this is reflected in the increased levels of various mycotoxins and specially aflatoxin in the former.

In contrast, lack of ventilation, sanitation and gradual increase in moisture content of oil cakes (9-10%) at retailer or farmer level increase the pace of mycobial contamination, growth and cosequently results in high percent of moulded samples. Similar findings have also been reported earlier by Verma, 1989; Takahashi *et. al.*, 1984; Tanaka, 2007 and Uhlig *et. al*., 2013). During collection trips, the walls and ceilings of retailer shops were frequently observed moist. Fusy and Nicot (1951) also held that walls and ceilings, floors and the jute sacs used for storage, as noted during present investigations (fig. 8 & 9), may be excellent source of mycobial contaminatin. The samples collected from regular fish farmers (fig. 6) and many of the retail shops (fig. 5) were found to be lying on KACHCHA (uncemented) ground floor in direct conact of soil in gunny bags or in contact with earthen walls. Such conditions provide opportunity for the moisture to penetrate the substrate, which consequently results in high fungal contamination/incidence (Verma, 1989; Tandon and chauhan, 1955; Trenk and Hartman, 1970),

Besides the various factors operating in environment of storage system, the climatic conditions also play a major role in deciding the fate of the substrate stored (Davis and Diener, 1970; Bertuzzi *et. al.*, 2017). Temperature is the most important environmental factor affecting germination of spore, growth and related metabolic activities of the fungus (Moreau, 1974). The temperature range of the present geographical area (area of investigation) varies from 4^0–30^0C but usually remain between 20^0 Cand 36^0C during major part of the year. The temperature favouring growth of *Apsergillus* group of fungi in general, and *A.flavus* and *A. versicolor* group of fungi is 6^0–54^0C (Jackson, 1965; Tandon and Chauhan, 1955) with an optimum of 20^0C (Tandon and Chauhan, 1955). Verma (1989) suggest a range of 20^0–28^0C temperature for the optimal growth of *Aspergilus* group of fungi. The survey of literatures suggest that majority of fungi grow between 15–30^0C with optimum growth rate at 20–25^0C (Moreau, 1974), (Wyllie and Morehause, 1977). Thus, the reasons of higher percentage of moulded samples during summer and still higher during the monsoon months may be attributed to the conjenial range of climatic temperature added with high relative humidity in the later case during the rains. The temperature range might be favouring the germination of spores and mycelial growth of a large number of fungi consequently accelerating the pace of mycobial spoilage.

The water content of the oil cackes and ground pulses mixture is relatively low as their production and marketing involve drying. During monsoon months the high humidity/moisture available from any source (increased relative humidity, storage in contact of moist soil without any protection barrier against moisture penetration and recurrent flood in the area) is trapped rapidly by these substrates (since they are dried products). This provides ample opportunity for better mycelial growth. Jemmali *et. al*. (1969) have suggested that the production of aflatoxin may be more important in substrates which have involved a drying stage. Effect of flood on aflatoxin contamination level in grains has been observed by Mishra and Singh (1978).

In contrast, during winter, the climatic factors like low atmospheric temperature, relatively lower humidity and moisture content of the substrate do not favour the fungal spore germination or growth and hence a low incidence is generally recorded, as found presently.

Among different groups of fungi recorded presently, the *Aspergillus* and *Penicillum* have been recognized as storage fungi taking over and dominating over the substrates (Christensen and Kaufman, 1965, Varga *et. al*., 2013). *Alternaria* and *Rhizopus* have been designated as field fungi, contaminating the substrates during post- harvest conditions (Christensen and Kaufman, 1965). *Rhizopus*, as second dominating mycoflora on oil seeds and pulses, has also been collected from fields and various purchase centres of Uttar Pradesh and Madhya Pradesh (Verma, 1978).

A. niger appeared as most dominant mycoflora on present substrates followed by *A. flaus* and *A. versicolor*. Earlier investigations suggest better mycelial growth of *A. niger* at 32^0C (Moreau, 1979), Verma, 1989, Bertuzzi, 2017) which exists during most part of the year, in particular during summer. The increased humidity during rainy season with congenical ranges of temperature help many of the fungi of *Apsergillus* group including *A. versicolor* (the present strain) to grow and often dominate over the substrate under given conditions. *A nidulanse* and *A. terreus* are mainly soil organisms but have been isolated from varities of substrates (Moreau, 1974; Wyllie and Morehause, 1977 and Verma, 1989). These fungi contaminate the substrates during storage in direct contact of soil, A. rubur, frequently occuring on ground nuts (Walt, 1965), has not earlier been recorded with mustard or its derivatives.

Natural contamination of substrates with any mycotoxin depends on the genotype of strain invading the substrate and the environment in which the mold is growing (Schroeder and Ashworth, 1966 and Wogan, 1975). The concentration of sterigmatocystin was found to be highest in MOC-II samples. This may be due to incorporation of various types of oil seeds as ingredient. Moreover, the MOC-II, being one of the cheapest items

of the lot, receives poor handling and longer storage period. On the other hand, MOC-I, a pure yellow mustard derivative, is a byproduct of oil prepared for human consumption. Similarly, GPM is a pulse by product prepared for human consumption. Besides, the GPM, is an item of relatively high commercial value, receives due attention. The oil seeds, in general, have been reported to harbour more toxigenic isolates than cereal products (Shotwell *et. al.*, 1969). In case of oil cakes the left out amount of oil also becomes an important factor affecting rate of mycotoxin production. The beneficial effects of fatty acids on biosynthesis of aflatoxin B_1 has already been recognized (Eldridge *et. al.*, 1965 and Patte *et. al.*, 1967) and in case of sterigmatocystin by JECFA , 2021; Shivamaruthi *et. al.*, 2019 and Tabata, 2002.

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