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Toxigenic Molds Infesting Fish Feed Ingredients In Parts Of North Bihar

Shambhu Prasad,
Directorate of Distance Education
L.N. Mithila University Darbhanga, 846008

***Abstract*--Altogether 1774 samples of fish feed ingredients like mustard oil cake (MOC), ground pulses mixture (GPM), wheat flour (WF), rice bran (RB), soya flour (SF), wheat bran (WB), Rice flour (RF) and commercial ration (CR) available in the local market, oil mills, farmers store room were collected during winter (569). summer (596) and monsoon (609). Of these, 894 samples appeared to be fresh. However, when these fresh samples were incubated at 30 + 2° C for 10 days following routine culture techniques, 45.8% of winter. 50.7% of summer and 72.1% of monsoon samples developed fungal colonies. *Aspergillus* group of fungi was recorded as dominant mycoflora represented by *A. flavus* isolated from 51.5% of the samples and others like *A. niger* (50.8%), *A. ochraceous* (28.1%) *A parasiticus* (21.1%) *A. versicolor* (12.2%) *A. rubur* (6.9%) and *A. fumigatus* (2.2%). Among the Penicillium group *P. viradicatum* and *P. islandicum* were isolated from 17.7 and 14.2% samples, respectively. The degree of infestation was maximum on the samples obtained from retail shops during monsoon followed by farmers' store room. Maximum number of aflatoxigenic strains of *A. flavus* and *A. parasiticus* were obtained from MOC while WB and WF appear to be substrates of choice for Ochratoxin A producing strains like *A ochraceous* and *P Viradicatum*. Findings suggest that 51.1% of *A. flavus*, 38.0% of *A. parasiticus* and 50% of *A. rubur* were capable of elaborating aflatoxins (B, and B.) in quantities ranging between 2 and 100 mg/kg). A versicolor (35,0%) bore capabilities of producing sterigmatocystin (>8 to <10 mg/kg).**

1. Introduction:

The gradual introduction of formulated rations based on agricultural products, their byproducts and various other agricultural and animal wastes for production of animal protein has given several new dimensions to the sector. One of them is certainly the "microfungi and their secondary toxic metabolites". Till date, whenever the food/feed is a subject, the microbiologists concentrate on bacteria giving a marginal attention to fungi. But, the last few decades have witnessed significant change in the state-of-the-art of the subject due to induction of toxic microfungi to the list of study as well as research.

Mold spoilage of food/feeds is specially associated with the countries, either developed or developing, where there is a surplus of production and subsequent storage. It has been amply indicated that higher the prevalence of storage practices, the higher is the risk of mycobial spoilage. Though peoples in ancient times also had some idea about the toxicity the fungi infesting food commodities but since the discovery of antibiotics studies in microfungi and their toxic metabolites have received greater attention (**Hayes, 1981**). Investigation made during 1960-80s could establish that approximately 30-40% of the microfungi growing on food commodities are toxic (**Moreau, 1979**). Thus, use of such food/feeds may be disastrous to those for whom they are destined.

A joint FAO/WHO/UNEP conference on mycotoxins held in Nairobi, Kenya (**September, 1977**) gave several general and specific recommendations at international level to reduce the problems posed by mycotoxins. The reasons, apparently, were attributed to the facts:

The molds are ubiquitous in occurrence with variable magnitude of incidence depending upon the climatic factors.

The mycotoxins problem is closely related to storage practices that provide opportunity for the spores to invade the substrates.

Improper pre- and post-harvest handling of the commodities accelerates the pace of mycobial infestation.

Less than optimum processing of the products, delay in drying after crop harvesting, prolonged drying in conditions of high humidity, re-wetting of the substrates are some factors that make the food/feed stuffs prone to mycobial spoilage.

2. Indian Climate versus Mycotoxin Problem:

Indian climate is mainly tropical but highly diversified and variable. The rainfall and temperature, the two most critical factors for mycobial infestation, vary greatly to the extent that one can speak of the regional average rather than national average. The land masses at plains (at sea level) to altitude up to 30.50 m are used for agriculture purposes and all the crops to a greater or lesser extent suffer from damages by various agents including fungi. About 70% of the produced grains are stored in small units. In urban areas staple food stuffs are often kept in mixed storage godowns in such locations which are difficult to keep free from infestation. Although national estimate of food spoilage due to fungi is not available but about 20% loss due to various factors has been estimated costing the nation more than thousand crores.

Mycotoxin contamination of agricultural commodities in India was started being viewed with serious concern following the reports (**Brook, 1966, Christensen 1965 and Feuell, 1966**) on toxicity syndromes resulting from ingestion of contaminated food stuffs. **Dr P.G. Tulpule (1964)** pioneered Mycotoxins Research in India with survey on occurrence of aflatoxins in groundnut and subsequent demonstration of hepatotoxic effects in young

monkeys. He also co-authored a monograph on health hazards of mycotoxins in India. Subsequent works of the same group of scientists (**Madhavan and Gopalan, 1965, Madhavan et al., 1965a and b; Gopalan et al., 1972, Tilak, 1975**) created significant awareness towards the mycotoxin problem in India. By the end of 1970s a few organised research programmes were taken up under the leadership of **Vora (1978)** at CDRI, Lucknow, **Neelkanthum (1979)** at Tamil Nadu Agriculture University, Coimbatore, **Mehan and McDonald (1980)** at ICRISAT, Pathancheru through Groundnut Improvement Programme and **Bilgrami (1981)** at Bhagalpur University, sponsored by FAO.

The investigations highlighted that mycotoxin contamaination is a serious problem because susceptibility of agricultural products in pre- and post-harvest mold spoilage due to congenial climatic conditions and practices of storage in vogue all through the country are far below from the standards that have been set forth by the application of modern researches in the field. Even the various warehousing and procurement agencies are unable to store the grains in ideal conditions.

Fungal spores are present everywhere and they multiply and spread fast on foods when the conditions are favourable. All they need to take the start is a little moisture. There are hundreds of fungi and isolated reports have confirmed the presence of many of the toxic strains in Indian environment (referred ahead) but no comprehensive list is available except the reports of the above cited FAO sponsored investigation programmes.

3. Toxic Fungi and Their Metabolites:

The major taxa that contain genera of fungi that may produce mycotxins are Ascomycetes, Phycomycetes (in part). Basidiomycetes, Deuteromycetes, Hyphomycetes, Coelomycetes (**Wyllie and Morehouse, 1977**). A comprehensive list of the fungi responsible for mycotoxicosis (**Moreau, 1979**) indicate that strains (number indicated in parentheses) of genera Absidia (2), Alternaria (3), Aspergillus (41), Byssochalmys (2), Cephalosporium (1), Chaetomium (2), Cladosporium (8), Curvularia Sp., Fusarium (19), Gibberella (3), Mucor (9), Penicillium (91) and many others elaborate secondary toxic metabolites like aflatoxins, ochratoxins, sterigmatocystin, penicillic acid, patulin, candulin, gliotoxin, fumagillin, oxalic acid, citreoviridin, luteoskyrin, palitantin, rubratoxins, rugulosin etc. A list of mycotoxins considered significant from aquaculture point of view (**Verma, 2001**) and a general account of molds responsible for toxicosis and the toxins associated with them may be referred (**Moreau, 1979**). However, it is essential to note that among several species, only certain isolates or strains may produce toxin only on specific substrates (**Wilson and Wilson, 1962**). With such an impressive number of toxic moulds it is better to conclude the subject in words of **Steyn (1933)** that in practice all moldy foods should be considered as suspect until proven to be safe.

4. Mycotoxins and Freshwater Aquaculture:

Recent technological advances making artificial feeding inevitable during aquaculture vis à-vis fish culture has increased the risk of mycotoxin exposure to fish. Possibilities of mycotoxin exposure to fish with special reference to aflatoxins has been schematically. Almost all fish feed ingredients either of plant or animal origin are potent substrates for mold invasion and subsequent toxic metabolite production. Isolated but numerous accounts are available (**Anonymous, RRL, Hyderabad, 1967, Amla et al., 1971, Krishnamachary et al., 1975a and b; Mishra and Singh, 1978; Vora, 1978; Neelkanthan, 1979; Verma, 1989; Verma, 1997: Das, 2002; Das et. al., 2002; Prasad 2002, Prasad and Verma, 2002; Verma 2002**) substantiating the fact Improper handling of these commodities, unsanitary storage, inadequate processing and reckless application are some factors that accelerate the pace of mycobial spoilage and thereby a cordial invitation to epidemic outbreaks. During survey programmes varieties of fish feed ingredients, even if they were aesthetically and organoleptically unacceptable, were found to be marketed for animal consumption. However, the extent of damage from toxicology point of view will depend chiefly on the toxigenic potentials of the infesting fungal strain. A lot of feed infested with the fungi having low toxigenic potentials may render the fish crop having no adequately operating immune system thus making them prone to all secondary infections. Unfortunately, the immunosuppressive potentials of mycotoxins have not yet attracted due scientific attention. This particular pathological aspect of mycotoxin problem, at least in inland context, appears to be a potent topic of research on account of the prevalent fish culture schedule being practiced. Most usually we practice annual crop harvesting system that provide no required time span for the appearance of characteristic chronic response of mycotoxins and acute exposure in field condition is a rare phenomenon.

5. Toxic Fungi on Fish Feeds and Their Toxins:

It appears to be difficult to present a national scenario on this score due to paucity of literature. The present study (Chapter 1), however, covers this aspect in detail. The findings suggest that fungi belonging to Aspergillus, Penicillium, Rhizopus, Curvularia, Mucor and Altemaria are the most common invaders on fish feed ingredients. In particular, the genus Aspergillus, that produce many of the toxins of significance e.g. aflatoxins, citrinin, sterigmatocystin, ochratoxin, paulin and penicillic acid, appear as the most dominant mycofiora as also reported earlier ny Verma et al., 1990. The present study has been made with special reference to aflatoxins (produced by A. flavus and A. parasiticus and A. rubur), ochratoxin A (produced by A. ochraceous) and sterigmatocystin (produced by A. versicolor). The climatic and nutritional factors affecting production of these toxic metabolites have been discussed in the ensuing chapters (I and II). However, an interesting aspect of mycotoxin problem in relation to fish/animal feeds deserves mention. Available data suggest that the germ hulls contained 90% of the toxin traced in the substrate. Similarly, when brown rice was milled, about 60-80% of the aflatoxins were separated with bran and polish (**Goldblatt, 1969**). About 85-90% of the aflatoxins present

in the kernels is left in the cake processed in the expeller mill. The protein isolated from peanut contained 60-70% of the toxin originally present in the cake (**Moreau, 1979**).

5. Materials and methods:

The fish feed (agricultural products and by-products) samples were collected following the guidelines suggested by US Food and Drug Administration and Tropical Products Institute, London. Before sampling history of each lot was depicted with special reference to:

I Time of processing/production
II Processing details
III Climatic condition during processing
IV Storage period
V Storage condition, and
VI Packaging materials used

Each lot, prior to sampling, was minutely inspected visually for fungal spoilage/extent of damage, if any. The samples, thus, registered were designated as Fresh (F), Partially infected (PI) and Infected (1) depending upon the physical condition of the substrates.

The samples (minimum 500 g) and maximum depending upon the size of the lot (upto 2.5 kg in 50 g randomly sampled units) were serially numbered, registered and brought to the laboratory.

6. Culture And Identification:

The samples (in bulk) brought to the laboratory were thoroughly mixed using pestle and mortar (for small sizes) or Electrical Blender and were reduced to working size of approximately 50 g by quartering following **Dickens and Whitaker (1982)**.

The sub-samples were subjected to culture using two routine techniques:

A Blotter technique and technique (Agar plate technique)
B Solid medium

All the ingredients were mixed in 1000 ml conical flask (Borosil) and allowed to boil by dipping in alluminium container half-filled with water (avoiding direct exposure to heat) till transparent (30 - 40 minutes) in appearance. The medium after cooling were poured in petriplates and autoclaved for 15 minutes at 15 lb pressure or 2 hrs in oven at 100°C. The samples were spread over the medium after cooling and incubated for 7- 10 days in B.O.D. at 28 + 2°C.

Fungal colonies in the petriplates can be seen appearing gradually from day 3 - 4 onwards but it assumes characteristic/diagnostic colour pattern only after 5 - 7 days. At the end of incubation (after 8 - 10 days) colonies are identified on the basis of characteristic colour appearance

Pure culture of the identified strains were obtained by innoculating conidial heads with sterilised innoculation needle to the cultures tubes containing AH and CZ media slants respectively and were incubated in B.O.D. at 28 ± 2°C for 7-10 days.

Microscopic identification of all the six strains under investigation were made on the following micro-morphological features with special reference to **Moreao (1979)** and **Wyllie and Morehouse (1977).**

Table 1: Characteristic Colony Colour Patterns of Some Toxic Fungal Strains

STRAIN	CHARACTERISTIC COLOUR OF THE COLONY	MEDIUM
A. flavus	- Heads yellow-green*	AH and CZ
A. ochraceous	- Heads yellow to ochraceous	AH
A. parasiticus	- Heads dark yellow green*	AH and CZ
A versicolor	- Heads light-yellow green	AH and CZ
A. rubur	- Heads blue-green to olive green	AH and CZ
P. viradicatum	- Green to yellow-green	CZ
[Sometimes colony of P. viradicatum appear pigmented if viewed from below (purple-brown), a strong obnoxious, musty odour is produced.]		
AH - Asthana Howker's A, CZ = Czapek's Agar medium, * Not easily distinguishable unless verified through microscopic features		

Table 2: Characteristic Micromorphological Features of the Toxic Fungal Strains

Strain	**MICROMORPHOLOGICAL FEATURES**
A. flavus Link (5a & b)	Sterigmata almost entirely biseriate
A. ochraceous Wilh.	Conidial heads globose or radiate, heads large, vesicles large and globose or nearly so, sclerotia sometimes occuring.
A. parasiticus Spear	Sterigmata uniseriate.
A versicolor (Vuil.) Tir.	Sterigmata uniseriate.
A rubur Thom. & Church	Phialides strictly uniseriate, conidiophone smooth.
P. viradicatum Westl.	Conidophore heads are assymetrical, consisting of sterigmata, metulae and branches (often rough walled), sterigmata flask shaped, condidia sub-globose and rough walled.

7. Observations and Discussion:

Records of the fungal strains isolated from present substrates during different seasons and preference for the substrates has been presented (Table 3). The table has also been supplemented with the type of toxin normally elaborated by them.

Data on the incidence of each of the present mycobial strains during different seasons suggest that the

prevalent climatic conditions suits them to invade and propagate further over the substrates. The climatic factors prevalent in the area and their range that has been found to support these strains have already been discussed.

Aspergillus flavus appeared as the most dominant mycoflora in general followed by Aspergillus niger almost all the year round. Both Aspergillus and Penicillium have been recognised as storage fungi taking over and dominating over the substrates (**Christensen and Kaufman, 1965; Verma and Pandey, 1990)**. However, there is now overwhelming evidence for preharvest invasion of the crops by these strains mainly carried out by the insects **(Lillehoj et al., 1976)**.

In many cases, it becomes difficult to locate A flavus and A. parasiticus infections on the natural substrates which are often indistinguishable. These strains, usually are discussed commonly on account of their taxonomic and physiological resemblances viz common colour appearance of the colony. preference for the substrates, nutritional requirements, congenial climatic factors and most significantly the similar secondary toxic metabolite production i.e. aflatoxins. The only micromorphological feature that allows one to identify the two strains is uniseriate phialides in case of A. parasiticus which is biseriate in A.

Table 3: Seasonal incidence and substrate preference of the fungal strains isolated from agricultural products and their by-products.

Fungi	Seasonal Incidence			Substrate Preference Shown Order of Abundance	Percent Samples Positive				Toxis Produced
	W	S	M		W	S	M	Avg.	
A. flavus	+++	+	+++	MOC,GPM,WF,RF,WB,RB,SF	51.0	40.06	63.0	51.5	Aflatoxins
A.ochraceous	++	++	+	WF,WB,RF,GPM,MOC	38.0	27.5	19.0	28.1	Ochratoxins
A. niger	++	++	++	GPM, MOC, RF, WF, WB, RB	52.2	42.0	58.4	50.8	Oxalic acid
A. paraciticus	+	+	++	GPM,MOC,WF,SF	42.0	12.4	23.5	25.9	Aflatoxins
A.rubur?	+	+	+	MOC,RB,WB,GPM	8.5	7.0	12.6	6.9	Aflatoxins
A. fumigatus	+	-	-	MOC,RF,GPM	6.8	-	-	2.2	Gliotoxin
A. versicolor	+	++	-	WF,RF,GPM	16.0	20.5	-	12.1	Sterigmatocystin
A. candidus	+	+	-	RF,GPM	-	5.4	-	0.8	Candidulin
A. nidulanse	+	+	–	GPM,MOC,WF,SF	12.0	12.4	-	8.1	Nidulin
P.citrinum	+	–	++	MOC,GPM,RB, WB, RF	32.5	-	52.0	28.1	Citrinim
P.viradicatum	+	+	++	RB, GPM, WB, MOC, RF, WF	20.6	-	32.5	17.7	Ochratoxins
P.islandicum	+	+	++	MOC,RB,GPM	6.6	10.0			Luteoskyrin
P.cyclopium	+	+	+	MOC,GPM,WF	7.0	11.0	16.0	26.0	Penicillic acid
Rhizopus sp.	-	-	+++	MOC,GPM,RB, WB, WF, RF	-	-	42.0	14.0	
Curvularia sp.	+	+	–	MOC,RB,WB,GPM	8.0	11.5	-	6.5	
Mucor sp.	+	–	++	MOC, RB, WB, GPM, RF, WF	24.0	-	47.0	23.6	

Alternaria sp.	-	+	-	RB,WB,GPM,MOC	-	9.0	-	3.0	
A= Aspergills, P= Penicillium, ? = not confirmed									

The growth of A flavus, A. parasiticus and A. niger as well is favoured by humidities in excess of 85% RH (**Diener and Davis, 1970**) corresponding to the substrate water content of 12 - 15%. It has been suggested that 32"C climatic temperature is optimum for the growth of A. flavus and 35°C for A. niger. The latter grow conveniently even at comparatively low RH of 77% **(Ayrest, 1969**). **Jackson (1965)** suggested that A flavus is capable of growth over the temperature range of 6 - 54C and optimum growth in different substrates as well as culture media occurs between 30 - 35°C.

The spores of these fungi retain their viability for longer periods, even several years under favourable conditions and undergo germination finding congenial climatic factors. In culture condition, a medium with high sucrose concentration (30 - 2000 g) and with nitrate as the source of nitrogen, is particularly favourable for growth.

According to **Raper and Fennel (1965**) the group A. ochraceous comprises about nine species but only a few of them are common invaders. The species is somewhat xerophytic (**Christensen, 1965**) growing at about 85% RH and has been commonly reported from heating grains (during storage), polished and unpolished rice. In laboratory cultures **Sansing et. al., (1973**) obtained good ochratoxin A at 25°C. In general 80 - 82% RH with 6% water content of the substrate favours the sporulation and growth of the fungi (Table 1.7) at 26 – 28°C. The maximum and minimum temperature for sporulation and growth has been reported to be 10 - 40°C. Almost all species of this group produces two important myotoxins like ochratoxins and/or penicillic acid. A few toxins of unknown significance (Secalonic acid) has also been reported.

During present work A. versicolor was selected on account of its capability to produce sterigmatocystin. The group includes 17 species (**Raper and Fennel, 1965**) but only A. versicolor and A. sydowi are known to be mycotoxigenic. The earlier reports on the occurrence of this species suggest it to be uncommon on cereals and grains but may invade the agricultural byproducts like wheat paste products and shredded grains etc. Presently too, its incidence on wheat and rice flour only deserve consideration. Our experience suggests that the species invades the substrates rich in free nitrogen content with onset of deterioration. No fresh samples, even after incubation, were found to be contaminated with A. versicolor but the same when left for 20 - 22 days were found contaminated with this species.

A. rubur belongs to A glacus group that comprises most commonly occurring fungi in stored products because of their ability to grow at low relative humidities of 75 to 85%. Kulik and **Holadey (1966)** have suggested that some strains of A. rubur may produce aflatoxin B1. Some of the other members of Aspergillus group invading the present substrates most commonly are A. candidus, A. nidulanse and A. ferreus. A. candidus group represented by single species is readily recognised by its white globose to radiate conidial heads and has been reported from a wide variety of substrates during storage. Graves and **Hesseltine (1966**) reported A candidus as predominant fungus in refrigerated dough products. A. nidulanse like A. versicolor is known to produce streigmatocystin and significant amounts of penicillin (**Ditchburn et al., 1974**). **Lafont and Lafont (1969)** isolated it frequently from corn, wheat, barley kernels and it was very common on animal feed in France. **Ward and Cowley (1972)** gave the cardinal temperature of isolates of A. nidulanse to be 20, 40 and 46°C. **Holzapfel et al. (1966)** obtained sterigmatocystin from A. nidulanse grown on com meal. A. terreus is small group of one species and two varieties, The pencil-shaped (cinnamon) conidial heads are diagnoistic. It has usually been reported from heating com often accompanied by A. flavus or P. viradicatum (producing ochratoxins) and P. islandicum (producing Luteoskyrin) appear as most common offenders on the present substrates Almost all of these require high relative humidity than Aspergillus justifying their abundance during monsoon months (Table 1.3). During winter and summer months they grow only on those substrates that have gained extra moisture from any source. Similarly, Rhizopus and Alternaria also require high RH (90 - 92%) with substrate content in the range of 17 - 18%, they mostly appear on the substrates during monsoon months. High RH or substrate water content, if prevail during other parts of year facilitate sporulation and growth of the fungi. The rate of growth of both these species is very high and the whole petriplate gets overcasted within 3 - 4 days of the culture.

The genus Penicillium are unquestionably important to man. Certain species are desirable. Other species are however undesirable. Penicillium species are seemingly always involved when food/feeds, raw materials or finished products in fields or storage are undergoing deterioration and spoilage. A number of Penicillium species are of added concern because of their ability to produce mycotoxins.

The present strain *Penicillium viradicatum* has become established as Ochratoxin A producer along with some other species of the group (*P. palitans, P. commune, P. variabile and P. cyclopium*).

Registry of cognizable growth in inland freshwater aquaculture sector reflects the efficacy and adaptability of recent technological advances by the fish growers. However, in a rush to raise production one vital aspect of mycotoxin contamination of supplementary feeds appears to have been largely ignored. The outcome of the present investigation and some isolated reports available on incidence of toxic micro-fungi and subsequent production of toxic metabolites on the fish feed ingredients are quite enough to make the issue a national problem. Mycotoxin contamination of fish feeds must be taken up with all gravity by the people concerned with aquaculture sector directly or indirectly because entry of the same in human food chain may be disastrous.

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