JASEM ISSN 1119-8362

Full-text Available Online at
http:// www.bioline.org.br/ja

J. Appl. Sci. Environ. Mgt. 2005
Vol. 9 (3) 53 - 57

Mycotoxigenic and Proteolytic Potential of Moulds Associated with Smoked Shark Fish (*Chlamydoselachus anguincus*)

*** ESSIEN, J P; EKPO, M A; BROOKS, A A**

Department of Microbiology, University of Uyo, P. M. B. 1017 Uyo, Nigeria.

ABSTRACT: Among the 33 moulds isolated from 20 samples of wood-smoked *Chlamydoselachus anguincus* (shark-fish) 20 isolates were capable of producing metabolites toxic to fertile Hubbard Golden Comet (Niger chick) eggs. *Aspergillus* and *Pencillium* isolates were the predominant moulds. Other toxigenic moulds isolated were *Eurotium*, *Fusarium* and *Cladosporium* species. The protease producing potential of the isolates varied among the genera and between isolates of the same species. The existence and growth of these moulds on smoked fish is a pointer to the potential health risk associated with the consumption of mouldy dry fish. @JASEM

Preservation of fish by smoking is carried out after catching, and smoked fish may be eaten without further cooking. From the processing centres to the market centres smoked fishes are often contaminated with microorganisms including moulds (Okafor and Nzeako 1985, Wu and Salunkhe 1978). Information on the effect of wood smoke on the properties of food and microflora associated with foods is gradually being generated (Wu and Sahunkhe 1978, Okafor and Nzeaka 1985, and Essien *et al.;* 1999). But reports on the role of moulds in the spoilage of smoked foods and their mycotoxigenicity is scanty. In view of the present day concern about mycotoxins and the kind and activities of moulds in proteinaceous foods; the mycotoxigenic and proteolytic potentials of moulds associated with smoked shark fish (Chlamydoselachus anguincus) was investigated.

MATERIALS AND METHODS:

Sample Collection: A total of 50 woodsmoked shark fish (*C. anguincus*) samples were obtained from Ine Okoi (Fish processing centre) and Uyo main market (Market centre) located respectively, in Oron and Uyo Local Government Areas of Akwa Ibom State, Nigeria.

Isolation of Moulds Associated with Smoked Shark Fish Samples: Dilution plate and direct isolation techniques were used for the isolation of moulds from the smoked fish samples. In the isolation process, malt extract agar (Difco) and potato dextrose agar (Difco) plates were used. To prevent bacterial growth 100 ppm oxytetracyline was added (Mossel *et al* 1975 and Pitt 1979).

Fish samples often surface sterilization in sodium hypochloride with 1000ppm available chlorine were aseptically milled and the fish powder were diluted in sterile distilled water with 0.05% Tween 80 at a ratio of 1/10 (v/w) (Jarvis 1978). During the isolation process 20g of the sample was diluted in 180ml sterile distilled water and homogenized for 3 minutes in a top drive homogenizer. Petri dishes were inoculated using the spread-plate technique and incubated in the dark at 25^0C for 7 days. Afterwards petridishes with visible colonies were selected and the mould contamination levels for 1g samples were determined. The samples were examined with the naked eye and with a stereomicroscope. The colonies with different morphological characters were repeatedly inoculated on appropriate media for purification.

Identification of the mould isolates was made for penicillia according to Pitt and Hocking (1985), aspergilli to Raper and Fennell (1965) and other Deuteronomycetes, Ascomycetes and Zygomycetes to Pitt and Hocking (1985) and Samson *et al* (1981).

Cultivation of Isolated Molds and Production of Mycotoxins: For the production of mycotoxins, spores of 10^6 (determined by means of a hamocytometer) per ml from the sample were added to 50ml of potato dextrose broth (Difco) in Erlenmeyer flasks (250ml) and incubated at 27^0C for one month. When moulds did not produce spores mycelia were used for inoculation. After incubation the content of the flasks were sterilized by autoclaving (121^0C for 15 minutes) and 10ml of culture filtrate was prepared from each of the isolates. The remaining material from the flasks was extracted with chloroform with the aid of a high speed mixer (Gallenkamp – 300 England). The extraction procedures was repeated 2 times using 100ml of chloroform for each extraction. The chloroform extracts were combined, concentrated in a flask evaporator and diluted to 5ml with chloroform.

Assay of Mycotoxigenicity of Mould Isolates
The mycotoxigenicity of the mould metabolite was assayed using the chicken embryo toxicity test (Horwitz *et al* 1975, Wu and Salunkhe 1978, Essien 2000). The air sacs of groups of ten fertile Hubbard Golden Comet (Niger chicks) eggs were loaded with 0.02ml of the culture and chloroform extracts before incubation. The rate of hatching was taken as the index of toxicity. Control groups of eggs were injected with chloroform and sterilized potato dextrose broth.

Determination of Proteolytic Activity of Mould Isolates: The Medium to detect protease produced by fungi contained nutrient agar (Difco) to which gelatin was added as the protein source (0.4% final concentration) (Anagnostakis and Hankin 1975). Inoculated plates were incubated for 7 days, afterwards a saturated solution of ammonium sulphate was poured over the agar surface to enhance visibility of the zones of proteolysis (SAB 1951).

The concentration of protease enzyme produced over time by the moulds was estimated based on teh ability of the isolates to produce coagulase enzymes that can clot milk (Elsie and Mansel 1987). In this analysis 1ml of the cell free extract from 4 day old mould cultures and 1ml of sterile cow milk were mixed in a test tube. Control experiments which consisted of 2ml of milk only and 2ml of extract only in separate test tubes were also prepared. The samples were then incubated at 27^0C and checked every 15 minutes interval until complete coagulation of the milk samples occurred. The rate of clotting was determined using the Folman equation (Berridge 1955 and Elsie and Mansel 1987) as follows:

$$T = \frac{K}{C} + t$$

where T = clotting time (minutes)

C = the concentration of enzyme

K and t are constants depending on the enzymes and milk substrate respectively.

The effect of mould growth metabolites on the hatchability of the eggs, is presented in Table 2. Toxicity was noticed in either the culture filtrate or chloroform extract of 20 cultures and in some cases in both fractions. The extent of toxicity depended on the species and strains of the moulds. Only 8 of the 15 *Aspergillus* cultures were toxic to chick embryo. Toxic aspergilli included 6 isolates of *A. flavus* and 2 isolates of *A. fumigatus.* None of *A. niger* isolates found on the smoked fish produced toxic methabolites. Among the mycotoxin producing aspergilli PC-31, MC-8, and MC-10 of *A. flavus* and MC-32 and MC-33 of *A. fumigatus* demonstrated the highest level of toxicity to chick embryos. Seven of the 9 *Penicillium* cultures from the fish samples were also found to be mycotoxigenic. These include 3 isolates of *P. expasum* (MC-23, MC-26 and MC-27) and 4 isolates of *P. viridicatum* (PC-30, MC-24, MC-25 and MC-28). Their toxicity was observed mostly in chloroform extracts of cultures and decreased with increase in dilution level. The culture filtrate of mycotoxigenic penicilli had little toxic effect on chick embryos. Similarly chloroform extract of *Cladosporium* isolate MC-3, as well as MC-9 and MC-12 of *Fusarium roseum* exhibited mycotoxigenicity.

The mycotoxigenic potentials of the mould genera implicated in the present study have earlier been reported by Davis (1981) and Heperken and Alperden (1988). The most significant member of the mould flora, *A. flavus*, is known to produce aflatoxins, while *P. expansum* and *P. viridicatum* produce penicillic acid and ochratoxin respectively when cultured on organic substrates (Joffe 1965). Of the remaining genera, *Eurotium repens* have been associated with the production of sterigmatocystin. Fusaria species produces trichothecenes and zearalenone (Davis and Diener 1978, Marasas *et al* 1979) while *Cladosporium* species are known to produce epicladosporic acid and fagicladosporic acid (Joffe 1965).

The protease enzyme concentration was calculated by extrapolating the formula above, where

$$T = \frac{K}{C+t}$$

RESULTS AND DISCUSSION

Mould contamination levels were calculated for 1g samples of the smoked fish from fish processing and market centres. The results are presented in Table 1. The mould counts were higher in fish samples from the market centre indicating a gradual loss in the preservative efficacy of wood-smoking over storage time. The higher mould counts of smoked fish from market centre may also be attributed to continuous contamination of the item through frequent handling and to the general poor sanitary condition of many Nigerian market centres. The fish samples from the market centre also harboured more mould genera and species.

Of the 33 moulds isolated from the 20 smoked fish samples. Only seven (21%) of the isolates were found in samples from the fish processing centre in Ine Okoi and 26 (79%) from the Uyo market centre. *Aspergillus flavus* was the dominant (27.2%) mould flora of the smoked fish. *Aspergillus niger*, *Penicillium expansum* and *P. viridicatum* were also frequently encountered in the smoked fish. These 4 mould species (*A. flavus, A. niger, P. expansum* and *P. viridicatum*) comprised 72.7% of the total fungal isolates, while *Cladoporium* sp, *Eurotium repens*, *Fusarium roseum* and *Paecilomyces* sp comprised the remaining 27.3%.

Results on the protease producing potential of the mould isolates (Table 3) showed that the moulds have weak proteolytic potential. Their proteolytic activities however varies among the isolates. Among the isolates, strains of *P. expansum* and *P. viridicatum* demonstrated the most noticeable proteolytic activity, folowed by strains of *A. niger* and *A. flavus.* Their ability to elaborate protease may be responsible for their preponderance in the smoked fish samples analysed. It is also an indication of their active role in the spoilage of smoked fish. On the other hand *Cladosporium* sp, *Eurotium repens* and *Fusarium roseum* exhibited a relatively low protease producing potential, and were not commonly encountered in the food item.

The frequent existence of mycotoxigenic moulds that can produce proteases in smoked fish, strengthened the possibility of high level of contamination by moulds. This is an indication of the possible health risk associated with the consumption of mouldy dry fishes. Therefore mouldiness of smoked proteinaceous foods should be viewed with serious concern because of the ability of the moulds to produce mycotoxins, some of which are very dangerous and lethal to humans and animals even in small doses (Purchase 1974). Proper storage of smoked fish is also necessary because poor storage methods and unhygienic handling of the items are known to predispose them to microbial contamination.

Table 1. Microbial Flora of smoked *Chlamydoselachus anguincus.*

Property	Processing Centre (PC) n=12	Market centre (MC) n = 8	Total No. of isolates	Isolate code
(a) Mould Count (10^2 cfu/g)	2.18	3.86		
(b) Mould Isolates				
Aspergillus flavus	+	+	9	PC-5,-15,-31,MC-6,-8,-10,-11,-13,-14. MC-32-33
Aspergillus fumigatus	-	+	2	PC-1,MC-4,-5,-16
Aspergillus niger	+	+	4	PC-17,MC-3,-19.
Cladosporium sp.	+	+	3	MC-2,-18
Eurotium repens	-	+	2	MC-9,-12,-20
Fusarium roseum	-	+	3	MC-22
Paecilomyces sp.	-	+	1	MC-21,-23,-26,-27
Penicillium expansum	-	+	4	PC-29,-30,MC-24,-25,-28.
Penicillium viridicatum	+	+	5	
Total	4	9	33	

n = Number of samples,
+ = Present,
- = Absent

Table 2: Percent hatchability of chick eggs loaded with mould growth metabolites.

Mould	Strain	Culture filtrate		Chloroform extract		
		Undil.	1:10	Con.	1:10	1:100
A. flavus	PC-5	10		0	0	40
	PC-31	0	20	0	10	
	MC-8	0	40	0	0	30
	MC-10	0	20	0	20	
	MC-11	60		0	0	60
	MC-14	80		10		
A. fumigatus	MC-32	0	30	0	0	10
	MC-33	0	50	0	0	20
Cladosporium sp	MC-3	60		0	20	
Eurotium repens	MC-18	60		0	0	40
Fusarium roseum	MC-9	60		0	0	20
	MC-12	40		0	20	
	MC-20	70		10		
P. expansum	MC-23	80		0	0	30
	MC-26	70		0	0	20
	MC-27	80		0	10	
P. viridicatum	PC-30	50		0	20	
	MC-24	40		0	10	
	MC-25	60		0	0	40
	MC-28	50		0	0	30

Mean of Hatchability of eggs loaded with chloroform was 95% and for sterile Potota dextose broth was 100% (controls).

Table 3: Proteolytic potential of moulds isolated from *Chlamydoselachus anguincus*

Mould	Isolate code	Milk clotting time (minutes)	Concentration of protease (ug/ml)
A. flavus	PC-5	555	0.0014
	-15	570	0.0016
	-31	615	0.0015
	MC-6	555	0.0015
	-8	615	0.0016
	-10	570	0.0013
	-11	570	0.0015
	-13	555	0.0014
	-14	555	0.0015
A. fumigatus	MC-32	705	0.0015
	-33	090	0.0014
A. niger	PC-1	600	0.0016
	MC-4	615	0.0015
	-5	630	0.0014
	-16	615	0.0016
Cladosporium sp	PC-17	690	0.0014
	MC-3	705	0.0013
	-19	690	0.0011
E. repens	MC-12	690	0.0014
	-18	690	0.0015
F. roseum	MC-9	615	0.0016
	-12	630	0.0014
	-20	615	0.0015
Paecilomyces sp	MC-22	690	0.0015
P. expansum	MC-21	615	0.0016
	-23	570	0.0017
	-26	540	0.0019
	-27	555	0.0018
P.viridicatum	PC-29	570	0.0017
	-30	555	0.0016
	MC-24	570	0.0018
	-25	615	0.0016
	-28	615	0.0015

REFERENCES

Anagnostakis S. L. And Hankin L. (1975). Use of selective media to detect enzyme production by microorganisms in food products. J. Milk Food Technol 38 (10): 570 – 572.

Berridge N. J. (1955). Methods in enzymology, Volume 2. Academic Press London. pp. 125 – 132.

Davis N. D. and Diener U. L. (1978). Mycotoxins. In Food and beverage mycology. R. Beuchat (ed.) Avi Publishing Co. West Port C. T.

Davis N. D. (1981). Sterigmatocystin and other mycotoxins produced by *Asperillus* species J. Food Prot. 9:711 – 719.

Elsie I. and Mansel W. G. (1987). Studies on milk coagulating enzymes. Nig. Food. J. 1, 157 – 162.

Essien J. P., Odoemena C. S., and Essien E. P. (1999). Effect of Traditional preservation on seedborne pathogens and seed viability of capsicum okra and tomato. Trop. Sci. 39, 1- 6.

Essien J. P. (2000). Mycotoxigenic moulds in maize stores in Nigerian Mud rhumbus. Trop. Sci. 40, 154 – 158.

Horwitz W., Senzel A., Reynolds H. and Park D. L. (1975). Natural Poisons. In Official methods of Analytical Chemist – Chapter 26. Washington D. C.

Heperken D., and Alperden I. (1988). Mycological survey of chicken feed and some feed ingredients in Turkey. J. of Food Prot. 51(10) 807 – 810.

Jarvis, B. (1978). Methods for detecting fungi in food and beverages. In Food and beverage mycology. L. R. Beuchat (ed.) Avi-Publishing Co. Westport C. T. pp. 471 – 504.

Joffe A. Z. (1965). Mycotoxins in Foodstuffs. MIT. Press Cambridge pp. 111 – 132.

Marasas W. F. O., Leistner L., Hijniann G. And Eckardt C. (1979). Occurrence of toxigenic strains of *Fusarium* in maize and barley in germany. European J. Appl. Microb. Bistech. 7, 289 – 305.

Mossel D. A. A., Vega C. L., and Put H. M. C. (1975). Further studies on suitability of various media containing antibacterial antibiotics for the enumeration of moulds in food and food environment, J. Appl. Bacteriol. 39:15-22.

Okafor N., and Nzeako B. C. (1985). Microbial flora of fresh and smoked fish from Nigerian Fresh Water. F. Microbiol. 2, 71 – 75.

Purchase I. F. (1974). Mycotoxins. Elsevier Scientific Publishing Company New York pp. 104 – 110.

Pitt J. I. (1979). The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Taloromyces.* Academic press New York.

Pitt J. I. and Hocking A. D. (1985). Fungi and Food Spoilage Academic press. New York.

Raper K. B. and Fennel D. I. (1965). The genus *Aspergillus* Williams and Wilkins Co. Baltimore. MD.

Raper K. B. and Fennel D. I. (1977). The genus *Aspergillus*. 686p. Robert G. Krieger Publishing Co. Hunting New York.

Samson R. A., Hoekstra E. S., and VanOoorschat C. A. N. (1981). Introduction to foodborne fungi. Centraalbureau Voor Schimmelcultures Baarn, Holland.

Society of American Bacteriologist (SAB) (1951). Manual of methods for pure culture study of bacteria. McGraw Hill New York.

Wu M. T., and Salunkhe D. K. (1978). Mycotoxin producing potential of fungi associated with dry shrimps Appl. Bacteriol. 45, 232 – 238.