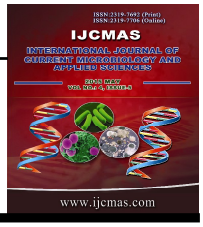
Int.J.Curr.Microbiol.App.Sci (2015) 4(5): 310-317

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 5 (2015) pp. 310-317**
http://www.ijcmas.com



Original Research Article

Aflatoxin Binding and Detoxification by Non-Saccharomyces Yeast – A New Vista for Decontamination

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Keywords

Aflatoxin binding, *Candida tropicalis, Clavispora lusitaniae, Pichiaanomala*

A B S T R A C T

Aflatoxin is known to be highly carcinogenic polyketide metabolites. *Saccharomyces cerevisiae* are known to bind to these toxins and help in decontamination.Yeasts other than *Saccharomyces* predominate in most of the fermented foods. However their binding capacities to these toxins are not known. On screening 3 promising isolates which had high binding affinity were observed. The bound toxins were characterised through LC-MS which indicated the formation of sodium adducts. The isolates were identified as *Pichia anomala, Clavispora lusitaniae* and *Candida tropicalis*. Among these strains *Candida tropicalis* was found to be efficient in aflatoxin binding. These forms are common residents of most of the fermented foods, which pave a new way for the decontamination of aflatoxin.

Introduction

Aflatoxins are produced by certain fungal species including *Aspergillus flavus* and *Aspergillus parasiticus.* These are highly carcinogenic polyketide metabolites (Jiang *et al.*, 2005). Dietary exposure to aflatoxins in the developing countries is extensively reviewed Williams *et al.* (2004). Aflatoxin B1 (AFB1) is a potent carcinogen and can be metabolised *in vivo* to Aflatoxin M1 9AFM1) which, in turn, can be found in milk and other animal products Prandini *et al.* (2009). Biological decontamination of mycotoxins using microorganisms is one of the well-known strategies for its management in foods and feeds. Among the different potential by decontaminating microorganisms, yeast represent a unique group. Feeding of *Saccharomyces cerevisiae* to poultry showed beneficial effects against aflatoxin induced toxicities Stanly *et al.* (1993). There are many reports on use of physically separated yeast cell walls obtained from brewery as feed additive in poultry diet resulting in amelioration of toxic effects of aflatoxins Santin *et al.* (2003). When dried yeast and yeast cell walls were added to rat-ration along with aflatoxin B1, a significant reduction in the

toxicity was observed Baptista *et al.* (2004). There are many studies on the fate of mycotoxins during the fermentation of beer and wines Scott *et al.* (1995). Scott (1996) reported that added toxin remained in the spent grains containing yeast cells indicating possible binding to the cells. But all these reports are on *Saccharomyces.*

Yeasts other than *Saccharomyces predominate* in most of the fermented foods. When yeasts are abundant, alone or in stable mixed populations with mycelial fungi or with bacteria; they have a significant impact on food quality parameters Aidoo *et al.* (2006). One of the most common characteristics of wild or indigenous yeasts is their low resistance to alcohol. Gilliland (1967) defined "Wild Yeast" as "any yeast which is not deliberately used and under full control". Wild yeasts are the naturally existing yeasts in air, on vegetation or fermenting vegetable matter. Not much work is reported on the aflatoxin binding capabilities of these yeasts. The present investigations were carried to screen different species of wild yeast isolated from various sources like fermented foods and beverages for their binding ability.

Materials and Methods

Aflatoxin production

Production of aflatoxin was carried out by partially crushing10 g of groundnut, moistened and inoculated with 2ml of fungal spore suspension (*A. flavus*). The inoculated samples were incubated for 10 days at 35º C. The infected groundnuts were crushed using warner blender and extracted with water and chloroform (1:10) on a rotary shaker for 30minutes and filtered. The filtrate was concentrated under vacuum using rotavapour. The residue was dissolved in 100µl of Chloroform Davis *et al.* (1980).

Aflatoxin was purified using a silica column. The column was eluted with 20ml hexane followed by 20ml di-ethylether. Elute was discarded. Aflatoxin was eluted with 20ml methanol-chloroform mixture in a ratio of 3:97, elute was evaporated to dryness using nitrogen gas.

Yeast collection

Various environmental samples were collected in sterile vials and transported to the laboratory at 8^0C. Appropriate quantity of samples were serially diluted and plated on Yeast Extract Peptone Dextrose Agar (YEPD) with chloromphinicol to control bacterial growth. Morphologically distinct colonies were isolated and purified by serial streaking. Individual colonies were sub cultured on YEPD slants and stored at 8^0C. The cultures were sub-cultured once in a month.

Aflatoxin binding to yeast cells

Cells for binding studies were prepared by growing the isolates in (YEPD) broth for 24 hrs at $25^0 \pm 2^0$C. The cells were centrifuged and washed twice with PBS buffer. Samples collected from various places were bought under aseptic condition at 8^0C. Yeast isolation was performed by plating the samples on SDA containing chloramphenicol. Isolated colonies were further purified by serial streaking using the same media and maintained on SDA. Finally the cells were suspended in PBS buffer and used. Yeast cells suspended in PBS were adjusted to 0.5 OD at 600 nm. 100 uL of culture suspension was mixed with 5uLcontaining 20ug of Aflatoxin and 895uL of PBS buffer. The mixture was incubated for various time intervals at ambient conditions. Pellets (cells) and supernatant were separated by centrifugation. Aflatoxin was extracted from the pellet by vortexing

with100uL of distilled water and 100uL of chloroform. The pellet was extracted twice with chloroform, all the chloroform fraction were pooled and concentrated. The concentrated samples were used for Aflatoxin analysis.

Supernatants were subjected to analysis without any extraction

The concentration of aflatoxins was determined by HPLC (SCL-10 AVP Shimadzu with fluorescence detector). A reverse phase C-18 column (15 cm and 150mm) was used with a flow rate of 1ml min^{-1}. Water: Acetonitrile: Methanol (6:3:1) were used as eluent. Samples dissolved in known quantity of Methanol were used for analysis. Fluorescence was detected at excitation wavelength of 350nm and emission wavelength 460nm Walter *et al.* (1980)

Aflatoxin which was analyzed by liquid chromatography were further subjected to mass spectrometry single quadruple using an electro spray ionization (ESI) source (LC-MS) in order to avoid derivatization. It was performed in electrospray ionization source (ESI) operating in the positive mode. Nitrogen was used as the collision gas. Capillary voltage was 35kv, cone voltage 100kv, source block temperature 80º C evaporation temperature 180ºC solvent gas 475L/h, cone gas 50L/h Ventura *et al.* (2004).

Yeast identification

Isolation of yeast DNA was carried out based on Hoffman and Wiston (1987). Yeast cells grown overnight in YEPD broth at 30ºC was centrifuged for 10 minutes at 8000rpm and was suspended in 0.5 ml of sterile distilled water, cell lysis was carried out using 200µl yeast lysis buffer and 20µl of proteinase k and incubated in water bath at 55 ºC for 2-3 hours. After incubation, phenol: chloroform: Isoamylalcohol was added in a ratio of 25:24:1, the mixture was centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected (250µl), to the above solution, 750µl of ice cold isopropyl alcohol along with, 20µl of 3M sodium acetate was added. The mixture was incubated overnight under refrigerated condition. The pellets were collected by centrifugation. The pellet was washed with 500µl of 70% ice cold ethanol, the pellet was air dried and resuspended in 20µl sterile water. DNA was visualized using Agarose Gel Electrophoresis.

PCR was carried out to amplify the targeted kb 28s r RNA gene of selected isolates. The reaction was carried out in the thermocycler gene Amp PCR system 9700 (Perkin Elmer) using standard protocol Sambrook and Russel (2001) primers used were BSF5' GCATATCAATAAGCGGAGGAAAAG 3' BSR 5' GGTCCGTGTTTCAAGACGG 3'. PCR cycle parameters: initial denaturation 95°C 3min35 cycles, denaturation 94°C 40sec,annealing 50°C 40sec, synthesis 72°C 1 min 20 sec, final extension 72°C 5 min**.** The size of the yeast amplicon was confirmed by comparing with 2kb ladder, which was used as a molecular size marker PCR amplification product was sent to sequencing then the sequence was analyzed using online software BLAST.

Results and Discussions

A total of 40 isolates were purified from various samples and used for study. Isolated yeast cultures grown on YEPD broth for 24 hr were harvested. The cells were washed twice and treated with aflatoxin. The supernatant and pellet samples were subjected to HPLC analysis. Samples with high concentration of toxin in cell pellets

were selected for further analysis. Based on the initial results from 40 isolates, 5 isolates were selected (Table 1). A standard *Saccharomyces cerevisiae* was included in the study as control.

The selected cultures were treated with aflatoxin and incubated for different intervals of time. The supernatant and cell pellets were subjected to HPLC separation with fluorescence detector. The retention times of the various toxins were compared with the standards. Concentration of the toxin was calculated based on the standard curve. On further analysis, the concentration of B1 at 0 hour of incubation was high in the pellet for isolates 5, 16, 18 and 29, indicating immediate binding of the toxin to cells (Table 1). High binding immediately to the cell wall was observed earlier by Shetty *et al.* (2007). However, in the supernatants of isolate 16 and 18, substantial concentrations of B1 were observed**.** After 2 hours of incubation, G2 was found to be bound in all the cultures tested, except in culture 16 and 29 where it was bound at 0 hour. By 4 hours of incubation majority of aflatoxin was bound to the cells. However, in isolate 29, G1 and G2 were absent. G1 was absent in both culture and supernatant at 2 hours of incubation, which needs further investigation. Earlier, four hours of incubation time was recorded for binding test using lactic acid bacteria by Haskard *et al.* (2001).

Concentration of B2 adsorbed by Yeast cultures were calculated based on HPLC analysis. It was observed that in culture number 16, initial adhesion was high (Table 2). After 2 hours of incubation, all the B2 had adhered to culture number 5, 16 and 30. At the end of 4 hours of incubation, the concentration of B2 adhered to the cell was high for all the yeast cultures except culture number 18, where equal concentrations in cell pellet and supernatant was observed.

Further the results were analyzed using mass spectra. This method is useful for the simultaneous determination of aflatoxin B1, B2 G1 and G2. The distribution of B1 and B2 were more in pellet, indicating the efficiency of adhesion by the yeast cells (Table 3). Some of the bound forms were found to be adducts of sodium. The molecular mass indicated that, the derivations were basically of B1. Such derivations were earlier observed by Ventura *et al.* (2004).

A few unknown compounds were present with molecular weight of about 324,340 and 327. These may be either the degraded products or cell free extracts, which needs to be further investigated.

On comparison of the binding capacity, B1 was found to adhere more than B2 in the first two hours of experiment. But by 4 hours of incubation B2 was found to adhere more than B1.

The 18s rRNA gene analysis was carried out for the amplification of the isolates specific to the primers. This was carried out using the BSF and BSR primers. The approximate size of amplified rDNA of isolates varied considerably.

Restriction fragmentation was performed using the enzymes Alu I and Hae III. They were used separately and simultaneously. Results indicate that the Yeast of culture code 16 and 30 had similar restriction pattern, to that of *Saccharomyces cerevisiae*, hence, concluded to be the same species. Further sequencing of culture 5, 18, and 29 was performed.

Table.1 Cultures selected for aflatoxin binding test

SL NO	CULTURE CODE	SAMPLES
1	115 WL iii	9Idli batter
2	706	Fermented vegetables
3	SL1 (6) 63	Tomato juice
4	571	Spoilt beans
5	H	Water hesianth
6	560	Carrot juice
7	706	Water sample (Madikeri)
8	CPI (2) 23	Coffee pulp
9	563	Water sample (Madikeri)
10	CF10	Jackfruit
11	CL(I) 32	Coffee pulp
12	530	Water sample (Puttur)
13	CF22	Jackfruit
14	C – P (1) V Pa	fermented rice
15	140	Water sample (Virajpet)
16	CF 20	Jackfruit (seed)
17	CF 17	Jackfruit (seed)
18	528	Ficus fruit
19	157	Ficus fruit (wild)
20	525	Ficus fruit
21	523	Ficus fruit
22	chI (2)38	Leaf Surface Microflora
23	575	Ficus fruit
24	217	*Garciniaindica*
25	546	Ficus fruit
26	6	Surface Microflora Of Beans
27	519	Litchi fruit
28	500	Litchi fruit (juice)
29	509	Litchi fruit (juice)
30	146	Ficus fruit
31	607	Fermented Milk
32	527	Industrial effluent
33	CF9	Jackfruit
34	SLI72 (8)	Toddy sample (Sullia)
35	GW_1	*Garciniagummigutta*
36	151	Domestic effluent
37	514	Industrial effluent
38	CF23	Jackfruit
39	CLII (4) VS	Coffee pulp
40	421	Rice wine

Table.2 Aflatoxin binding of selected yeast isolates at various time intervals (ug) Initial treatment 20ug

Isolate number	10 min incubation	2 hour incubation	4 hour incubation
5 cells	12	14	17
5supernatent	5	4	2
16 cells	14	14	15
16supernatent	3	3	4
18 cells	9	12	11
18supernatent	9	6	10
29 cells	8	11	16
29 supernatent	10	7	2
30 cells	11	15	16
30 supernatent	7	3	2
Standard cells	8	15	15
Standard sup	10	3	2

Saccharomyces was used as standard.

Table.3 Kinetics of aflatoxin bounding to yeast isolates

Isolate No.	0 hour			2hour			4 hour		
5p	B1	B2	B1+Na	B1	B2		B1	B2	B1+Na
5s	B2	UK^1	UK^2	B2	UK^1	UK^2	B1	B2	B1+Na
16p	B1	B2	B1+Na	B2	B1+Na		B2	UK^1	B1+Na
16s	B1	B2	B1+Na	B2	G2	B1+Na			
18p	B1	B2	B1+Na	B2	G2		B2	G2	UK^2
18s	B2	B1+Na	UK^2	B2	UK^2		UK^1	G_2	UK^3
29P	B1	B2	B1+Na	B2	B1+Na	UK^2	B2	B1+Na	UK^2
29S	B2	G1	UK^2	B2	G2	B1+Na			
30p	B1	B2	B1+Na	B2	UK1	UK3	B2	UK1	UK2
30s	B2	G1	B1+Na	B2	B1+Na	UK2	UK^1		

UK= Un Known, UK1=324.39, UK2 =340.35,UK3=327.23 S= supernatant, P=pellet

The nucleotide sequence analyzed by BLAST searches performed with the nucleotide data base at the National Center for Biotechnology Information (NCBI) Gene Bank Data Library. The isolate 5 was identified as *Pichia anomala.* The gene sequence was submitted to NCBI under account number FJ649613. Similarly 18 showed similarity with *Clavispora lusitaniae* (FJ649614) and Culture 29 was identified as *Candida tropicalis* (FJ649615)

To conclude of the total 40 yeast isolates, 5 cultures had the potential for aflatoxin binding. Isolates like *Pichiaanomala*, *Clavispora lusitaniae* and *Candida tropicalis* are common resident organisms of most of fermented foods. Their ability to

bind aflatoxin indicates its utility in decontamination of toxin from cereals and pulses during fermentation. Earlier, such work was carried only with *Saccharomyces cerevisiae* and this is the first report of its kind where other yeast forms have been observed for their aflatoxin binding capacity. This finding, we hope will open up a new field in food fermentation and aflatoxin management.

Acknowledgment

The authors thank the Director of the Central Food Technological Research Institute (CFTRI) and the Head of Food Microbiology for the facilities and their encouragement to do this work. Our thanks to ICMR for providing financial grant to carry out this work.

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