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International Journal of Food Microbiology 83 (2003) 219–225
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Short communication

Development of a method for direct visual determination of aflatoxin production by colonies of the *Aspergillus flavus* group

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Received 28 September 2001; accepted 28 June 2002

Abstract

This report describes a simple, rapid and reliable method for screening the aflatoxin production by moulds of the *Aspergillus flavus* group. Strains were cultivated on yeast extract agar to which methylated β-cyclodextrin derivative plus sodium desoxycholate was added. Production of aflatoxins was readily detectable by direct visualisation of a beige ring surrounding colonies after an incubation time of 3 days at 28 °C. When this ring was examined under UV light, it exhibited blue fluorescence. The presence of aflatoxins was confirmed by extracting the medium with chloroform and examining the extracts by HPLC with fluorescence detection.

Keywords: Aflatoxin production; *Aspergillus flavus*; *Aspergillus parasiticus*; Screening method

1. Introduction

The aflatoxins are secondary fungal metabolites with highly toxic and carcinogenic properties produced by some strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Kurtzman et al., 1987). Since it was discovered that "turkey-X" disease was caused by aflatoxins, thousands of strains belonging to the *A. flavus* group have been isolated from foods and animal feeds to determine their ability to produce aflatoxins. However, since not all strains are able to produce aflatoxins, research has been focused to develop suitable methods to determine the aflatoxin-producing ability (APA) of a particular isolate. Methods using liquid and solid media as natural or semisynthetic substrates have been used for this purpose (Davis et al., 1966; Hara et al., 1974). However, with those methods, a later extraction of the medium and its analysis by chromatographic techniques is necessary to assure the presence of aflatoxin. Testing large numbers of isolates on a variety of substrates is expensive and time consuming. For this reason, several screening methods for direct visual determination of aflatoxin production have been developed. These methods use more or less complicated culture media containing a variety of salts (Adye and

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0168-1605/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0168-1605(02)00362-8

Mateles, 1964; De Vogel et al., 1965; Torrey and Marth, 1976) or additives such as corn steep liquor (Hara et al., 1974) and coconut (Lin and Dianese, 1976; Davis et al., 1987; Lemke et al., 1988, 1989; Dyer and McCammon, 1994) to enhance the production of aflatoxins to achieve direct visual determination of a bright fluorescent area surrounding colonies under UV radiation.

The cyclodextrins are relatively simple organic compounds formed by the action of the enzyme cyclodextrin transglycolase on dextrans. These compounds have the ability to form inclusion complexes with a variety of molecules and they are readily available (Li and Purdy, 1992). Their physical and chemical properties have been described in literature (Sozsef, 1988; Li and Purdy, 1992; Connors, 1997). The capacity of cyclodextrins, specially methylated β-cyclodextrin derivatives, to enhance the natural fluorescence of aflatoxins have been studied previously (Vázquez et al., 1991, 1999; Cepeda et al., 1996; Franco et al., 1998).

Recently, we have exploited the property of cyclodextrins to enhance the fluorescence of aflatoxins (Fente et al., 2001) to develop a method for the visual detection of aflatoxin production by *A. flavus* and *A. parasiticus* using Mβ-cyclodextrin as additive for a culture medium traditionally used for fungal isolation from foods (Sabouraud dextrose agar) and in a culture medium used for mycotoxin production in general (yeast extract sucrose (YES) medium). The results obtained demonstrate that Sabouraud dextrose and yeast extract sucrose media added with a methylated β-cyclodextrin derivative, the β-W7M 1.8-cyclodextrin are adequated to efficiently detect aflatoxin production through the observation of fluorescence surrounding colonies under UV light (365 nm) after 3 days of incubation at 28 °C. However, using those media, evaluation at fifth or sixth day of incubation became difficult because mycelial growth reached almost the margin of the Petri dish.

In this paper, we report results of experiments made using YES medium plus β-W7M 1.8-cyclodextrin containing an additional additive: sodium desoxycholate as an effective growth inhibitor. In medium containing sodium desoxycholate, it was possible to detect, without using UV light, a beige ring surrounding only aflatoxigenic colonies. The ring observed exhibited blue fluorescence under UV light.

2. Materials and methods

2.1. Microorganisms

The representative strains of *A. flavus* (NRRL 6538, NRRL 6540, NRRL 6541, NRRL A-3537), *A. parasiticus* (NRRL 2999, NRRL 3145), *Aspergillus ochraceus* (NRRL 3174, NRRL 405) and *Aspergillus versicolor* (NRRL 3499, NRRL 573) were supplied from The Spanish Type Culture Collection (Burjassot, Valencia, Spain). Five additional aflatoxigenic strains (1316, 876, 132, 42, 19) and 20 non-aflatoxigenic strains of the *A. flavus* group were also included. All were isolated from cheeses (Vázquez-Belda et al., 1995), spices and feed, and they belong to the culture collection of our laboratory. A total of 35 strains were used in our experiments.

2.2. Media

Culture media used were: Czapek agar (CZ), aflatoxin-producing ability medium (APA) and yeast extract sucrose agar (YES). All supplied by Difco (Detroit, USA). The additives used were β-W7M 1.8-cyclodextrin (Mβ-cyd) from Wacker (Munich, Germany) and sodium desoxycholate from Sigma (St. Louis, MO, USA).

The improved medium, called YCSD, consisted of commercial YES medium supplemented with 0.3% Mβ-cyd and 0.6% sodium desoxycholate. The basal agar added Mβ-cyd was sterilized at 121 °C for 15 min, cooled to 40 °C and the sodium desoxycholate added aseptically. Vigorous shaking was needed to get a complete incorporation of this compound into agar. Medium was settled 10 min in a warm water bath to eliminate air bubbles before plating. Poured plates were clear and yellow in color.

2.3. Cultivation

All strains were cultivated in Czapek agar at 25 °C in darkness during 7 days, then an inocula with a loopful of spores of each strain was placed at the center of the solidified agar assayed. The effect of the addition of sodium desoxycholate as growth inhibitor at three concentrations (0.6%, 0.8% and 1%), temperature of incubation (25, 28 and 30 °C) and time of incubation (1–10 days) were investigated in a basal

medium consisting of yeast extract sucrose agar added Mβ-cyd at 0.3% (YES + CYD). All experiments were made in duplicate.

2.4. Determination of the aflatoxin-producing ability

All strains tested were cultivated in APA medium and incubated during 10 days at 28 °C to determine their aflatoxin production abilities through the visualisation of a bright blue or greenish blue fluorescence zone surrounding colonies under UV light (365 nm). All experiments were made in duplicate. Results were reported as positive (+) or negative (−). In addition, these strains were also cultivated in YES medium (without additives) under the same conditions of APA. Chloroformic extracts of these cultures were analysed by the chromatographic procedure described below to confirm the presence of aflatoxin.

2.5. Extraction and confirmation of aflatoxin production in the new medium

The presence of aflatoxins in culture media with colonies showing the beige ring (fluorescent under UV light) was confirmed by HPLC with fluorescence detection. All strains cultivated in the new culture medium (YCSD) were extracted according to El-Banna et al. (1987) with some modifications: the strains and the surrounding agar were transferred to Stomacher bags. Extraction was carried out using 20 ml of chloroform (10 ml × 2) and homogenized during 4 min in a Stomacher. The chloroformic phase was filtered through anhydrous sodium sulfate and evaporated to dryness under nitrogen steam at 40 °C. The residue was redissolved in 200 μl of mobile phase. Volumes of 50 μl of extract were injected into the HPLC system. The methodology used was a modification of Cepeda et al. (1996) method. Apparatus and conditions used were: column, Tracer Inertsil ODS2 5 μm (150 × 4.6 mm I.D.) from Teknokroma (Barcelona, Spain); mobile phase, methanol–water (45:55, v/v); flow rate, 0.6 ml/min. The pump was a PU-l580 Jasco (Tokyo, Japan) and the injector was a Rheodyne Model 7125 equipped with a 50-μl loop (CA, USA). The fluorescence detector, a RF-535 Shimadzu (Kyoto, Japan), was set up at $\lambda_{ex} = 365$ nm, $\lambda_{em} = 418$ nm.

3. Results and discussion

3.1. Aflatoxin-producing abilities of strains assayed

As a first step, all the strains used in this experiment were examined for aflatoxin production in APA and YES media without additives. The aflatoxigenic *A. flavus* NRRL 6540, *A. parasiticus* NRRL 2999, *A. parasiticus* NRRL 3145 and the aflatoxigenic *A. flavus* group 1316, 876, 132, 42 and 19 strains showed fluorescence in APA medium after 10 days of incubation. The results obtained for these strains when cultivated in YES medium and analysed by the chromatographic procedure were in concordance with those observed in APA medium. As none of the non-aflatoxigenic strains showed fluorescence in the experiments carried out, we only report data from a

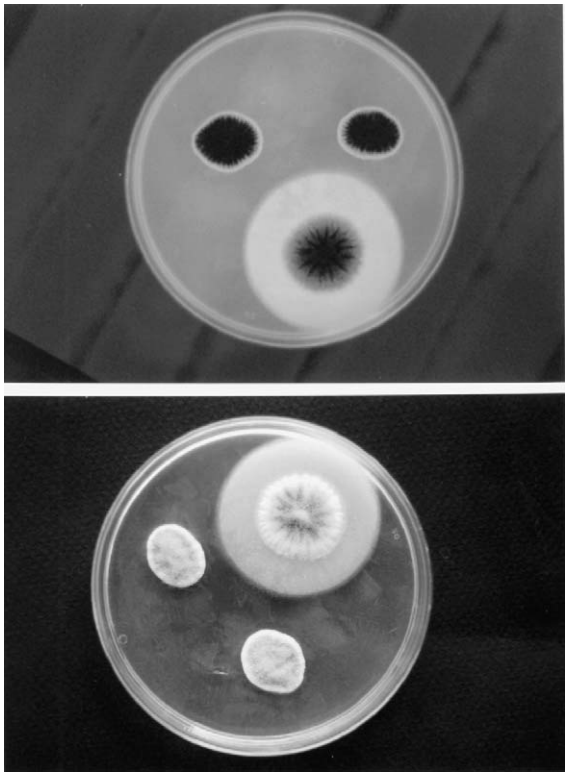

Fig. 1. Colonies of aflatoxigenic NRRL 2999 strain and two non-aflatoxigenic strains (NRRL 6538 and NRRL 6541) cultivated in YCSD medium at third incubation day at 28 °C, observed with (top) and without UV light (bottom).

representative negative strain, strain 893 belonging to the *A. flavus* group.

3.2. Effect of the addition of sodium desoxycholate

This experiment was carried out in YES + CYD medium that was developed recently (Fente et al., 2001). The addition of sodium desoxycholate to YES + CYD gave rise to a considerable reduction in the colonies size compared with colonies on YES + CYD without inhibitor, the formation of a beige ring surrounding all aflatoxigenic colonies, visible without UV light, which was not observed for any of the non-aflatoxigenic strains (Fig. 1). Visualisation of a blue fluorescent ring surrounding aflatoxigenic colonies under UV light was also possible. The fluorescent ring observed under UV was also the same beige ring observed through direct visualisation. In our previous work (Fente et al., 2001), we observed the presence of blue fluorescence surrounding aflatoxigenic strains, but by using sodium desoxycholate, this fluorescence is restricted to a well-delimited ring. This favours greatly the visualisation of fluorescence.

Lemke et al. (1989) used 0.8% sodium desoxycholate to inhibit the growth of strains of *Aspergillus* spp. in coconut extract agar. We assayed the effect of the addition of three different concentrations: 0.6%, 0.8% and 1%. Using 0.8% level, colonies were from 2

Table 1
Colony diameter of aflatoxigenic strains on media assayed

Strain	Incubation time [a] (days)	YES	YES + CYD	YCSD (0.6% of sodium desoxycholate)	YCSD (0.8% of sodium desoxycholate)
NRRL 2999	2	2.4 cm	2.9 cm	8 mm	6 mm
	3	4.2 cm	4.7 cm	1.2 cm	9 mm
	6	×	×	3.7 cm	3.2 cm
	10	×	×	5.1 cm	4.7 cm
NRRL 3145	2	2.2 cm	2.8 cm	7 mm	5 mm
	3	3.7 cm	4 cm	1 cm	8 mm
	6	×	×	3.5 cm	3 cm
	10	×	×	4.9 cm	4.4 cm
NRRL 6540	2	2.3 cm	2.9 cm	6 mm	4 mm
	3	3.7 cm	4 cm	9 mm	8 mm
	6	×	×	2.7 cm	2.3 cm
	10	×	×	4.1 cm	3.6 cm
1316	2	2.1 cm	2.5 cm	7 mm	5 mm
	3	3.9 cm	4.3 cm	1.2 cm	9 mm
	6	×	×	3 cm	2.7 cm
	10	×	×	4.7 cm	3.7 cm
876	2	2.3 cm	2.8 cm	6 mm	4 mm
	3	3.8 cm	4.3 cm	1 cm	7 mm
	6	×	×	2.5 cm	2.2 cm
	10	×	×	3.9 cm	3.1 cm
132	2	2.5 cm	3 cm	9 mm	7 mm
	3	4.4 cm	4.9 cm	1.3 cm	1.1 cm
	6	×	×	3.9 cm	3.4 cm
	10	×	×	5.4 cm	5.0 cm
42	2	2.2 cm	2.7 cm	6 mm	4 mm
	3	3.6 cm	4.1 cm	1 cm	8 mm
	6	×	×	2.5 cm	2.1 cm
	10	×	×	3.7 cm	3 cm
19	2	2 cm	2.5 cm	5 mm	4 mm
	3	3.4 cm	3.9 cm	9 mm	7 mm
	6	×	×	2.3 cm	2 cm
	10	×	×	3.5 cm	3 cm

× : Colony occupies the whole Petri dish.
[a] Incubation temperature: 28 °C.

to 5 mm smaller (Table 1), the beige ring was smaller and fluorescence under UV light was less intense with respect to those observed when 0.6% level was used. When 1% of sodium desoxycholate was used, inhibition growth was almost complete. All non-aflatoxigenic strains were negative using all concentrations assayed. As a result of these assays, YES + CYD medium containing 0.6% sodium desoxycholate (YCSD) is recommended for the detection of aflatoxigenic strains.

3.3. Effect of temperature of incubation

This experiment was carried out in the YCSD medium developed. When strains were incubated at 25 °C, there was a notable reduction on sporulation and size of the colonies and not all aflatoxigenic strains showed the beige ring and fluorescence under UV light compared to the higher temperature. No significant differences in the presence and size of the beige ring and in the intensity of fluorescence under UV were observed when the strains were incubated at 28 and 30 °C during 3 days. All subsequent tests were made incubating cultures at 28 °C.

3.4. Effect of incubation time

Using YCSD medium at 28 °C, we looked for the minimal incubation time necessary to detect the beige ring and fluorescence under UV light surrounding aflatoxigenic strains. Daily, during 10 days, a Petri dish of the medium assayed was examined for the presence of the beige ring, and then, under UV light to detect fluorescence. The chloroformic extract of this agar was analysed by HPLC. For all aflatoxigenic strains studied, the response was clearly conclusive through the direct visualisation of a beige ring, fluorescent under UV light, surrounding colonies after 3 days of incubation. Only two incubation days were needed for the stronger (unpublished data) aflatoxin-producing strains NRRL 2999, NRRL 3145 and 132. The diameter of the beige ring and the intensity of its fluorescence under UV increased with time and the maximum was observed at fourth day. After additional incubation days (7th to 10th day), visualisation of the beige ring and fluorescence under UV light was still possible because the maximum diameters of colonies reached were 4.9 and 5.1 cm for NRRL 2999 and NRRL 3145, respectively (Table 1). Other media

Table 2
Comparison of aflatoxigenic and non-aflatoxigenic strains responses after three incubation days at 28 °C in media assayed

Organism	Strain	APA response[a]	YES		YES + CYD		YCSD		
			Beige ring[b]	Fluorescence[c]	Beige ring[b]	Fluorescence[c]	Beige ring[b]	Fluorescence[c]	Aflatoxin-HPLC response
A. parasiticus	NRRL 2999	+	–	–	–	+	+	+	+
A. parasiticus	NRRL 3145	+	–	–	–	+	+	+	+
A. flavus	NRRL 6540	+	–	–	–	+	+	+	+
A. flavus group	1316	+	–	–	–	+	+	+	+
A. flavus group	876	+	–	–	–	+	+	+	+
A. flavus group	132	+	–	–	–	+	+	+	+
A. flavus group	42	+	–	–	–	+	+	+	+
A. flavus group	19	+	–	–	–	+	+	+	+
A. flavus group	893	–	–	–	–	–	–	–	–
A. flavus	NRRL 6538	–	–	–	–	–	–	–	–
A. flavus	NRRL 6541	–	–	–	–	–	–	–	–
A. flavus	NRRL A-3537	–	–	–	–	–	–	–	–
A. ochraceus	NRRL 3174	–	–	–	–	–	–	–	–
A. ochraceus	NRRL 405	–	–	–	–	–	–	–	–
A. versicolor	NRRL 3499	–	–	–	–	–	–	–	–
A. versicolor	NRRL 573	–	–	–	–	–	–	–	–

[a] Responses after 10 incubation days at 28 °C.
[b] Direct visual detection.
[c] Detection under UV light (365 nm).

currently in use for the screening of aflatoxin-producing strains (De Vogel et al., 1965; Hara et al., 1974; Torrey and Marth, 1976; Davis et al., 1987; Lemke et al., 1988; Dyer and McCammon, 1994) needs 2–10 days for the visualisation of fluorescence under UV light.

3.5. Correlation between the formation of a beige ring and aflatoxin-producing ability

During the development of this method, we observed that all aflatoxigenic strains produced conspicuously a beige ring visible without UV light surrounding colonies. Therefore, we decided to test again all 28 non-aflatoxigenic strains used in this work, three strong (unpublished data) aflatoxigenic strains (NRRL 2999, NRRL 3145, 132) two aflatoxigenic strains, (NRRL 6540 and 876) and 10 additional non-aflatoxigenic strains to determine if this ring could be used to identify aflatoxin-producing ability without using an UV lamp. None of the non-aflatoxigenic strains including *A. ochraceus* and *A. versicolor* strains produced the ring after 3–10 days of incubation at 28 °C in YCSD medium and the aflatoxin HPLC analysis were negative. However, in all cases where the beige ring was observed, aflatoxins were detected by the HPLC method (Table 2).

According to the results mentioned above, it seems evident that the presence of a beige ring visible at first sight along with fluorescence under UV surrounding colonies in the medium developed is strongly indicative of aflatoxin-producing ability. A yellow pigmentation has been associated previously with aflatoxin production by toxigenic strains (Wiseman et al., 1967; Bothast and Fennell, 1974; Lin and Dianese, 1976). Further investigations are needed to make clear the identity of the beige ring observed.

Acknowledgements

This study was supported by the Spanish Government Office for Education and Science as part of the research project CICYT-Ali-96/1163.

The authors wish to thank Encarnación González for the technical collaboration.

Judith Jaimez Ordaz is supported by a scholarship from CONACYT, México.

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