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A SIMPLE METHOD FOR PRODUCING AFLATOXIN B_1 ON RICE MEDIUM FOR USE IN EXPERIMENTAL ANIMAL FEEDS

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ABSTRACT

A simplification and standardisation of the existing methods to produce aflatoxin B_1 (AFB_1) on rice medium is described. As per conventional method, rice were first soaked in 1 ml water/2 g of rice for 2 hr, autoclaved, and then inoculated with *Aspergillus flavus*. During different stages of incubation, water was added according to different regimens. It was concluded that 1 ml water/100 g of rice at 24 to 36 hr of incubation is the maximum amount of water that could be added for optimal AFB_1 production.

Key words: Aflatoxin, Rice, *Aspergillus flavus*

INTRODUCTION

Use of commercially available pure mycotoxins in animal feeding trials is very costly. Furthermore, purified mycotoxins usually do not depict the natural conditions of mycotoxin production (Eriksen and Pettersson, 2004). Due to these reasons, the best approach in most cases is to produce the desired mycotoxin in the laboratory. Aflatoxin (AF) B_1 is a well studied mycotoxin for its effects on animal health (Bennett and Klich, 2003). However, in recent years there has been growing interest on the effects of AFB_1 on intestinal tissues (Gursoy *et al*., 2008; Yunus *et al*., 2010).

The laboratory scale production of AFB_1 on rice media by using *Aspergillus flavus* has been described previously by Shotwell *et al*. (1966). However, these authors did not mention the amount of water that should be added during different stages of incubation, which makes replication of their protocols difficult. Secondly, the specific equipment required for optimising the AFB_1 yield are sometimes not available in laboratories specialising only in nutritional analysis. Present experiments were therefore conducted to standardise and simplify the method of AFB_1 production so that all the inputs are known in exact amounts, and that the method can be run by using simple equipment.

MATERIALS AND METHODS

For production of AFB_1 *A. flavus* strain number NRRL 3357 (USDA/ARS Culture collection) was used. The strain's lyophilized cultures were a kind gift of Prof. Gary Payne (Department of Plant Pathology, Box 7567, North Carolina State University, Raleigh, North Carolina 27695-7567). The *A. flavus* was grown for 7 to 10 d at 28 °C as 3 colonies on a standard potato dextrose agar plate. The colonies from each plate were harvested by using 6 ml of sterile 0.1% Tween 80 and then vortexed for 30 sec. Then 1 ml of this solution was used to inoculate 125 g rice in a 1 L flask. Before inoculation, the rice in each flask were left soaked in tap water (1 ml water/2 g of rice) for 2 hr which was followed by autoclaving at 125°C for 15 min. After inoculation with A. *flavus*, each flask was shaken vigorously and placed in oven at 28 °C. In one attempt, 1.25 ml water/g of rice was used for soaking the rice before autoclaving. However, rice in this case clumped together so tightly after autoclaving that this experiment had to be stopped. The rice soaked in 1 ml water/2 g of rice also formed small clumps. However, these clumps could be broken by using sterile plastic pipettes.

In total 5 different regimens for addition of water during incubation were tested. These regimens included: 10 ml water/100 g rice once at 24 hr; 3.3 ml water/100 g rice twice at 24 and 48 hr; 1.5 ml water/100 g rice twice at 24 and 48 hr; 1 ml water/100 g rice once at 48 hr; and 1 ml water/100g rice once at 36 hr (Table 1). For each regimen, 3-7 flasks were taken and each flask was manually shaken 2 to 3 times a day so as to avoid clumping of rice together. After 5 d incubation, the contents from the flasks under same treatment were pooled. The AF contents were analysed in duplicate for each treatment. For determining the AF contents, the samples were cleaned up by using immunoaffinity columns (IAC) (Aflaclean; LCTech, Germany). The extraction procedures were according to the specifications of the manufacturer of IAC. The detection of AF contents was carried out by using high pressure liquid chromatography (HPLC) (LC-9A; Shimadzu, USA) with fluorescence detection (Waters 474; Waters, USA). The LOD (limit of detection) for AFB_1, AFB_2, AFG_1, and AFG_2 was 0.1, 0.1, 0.15 and 0.16 µg/kg,

respectively. The LOQ (limit of quantification) for these toxins was 0.44, 0.5, 0.52 and 0.6 µg/kg, respectively.

RESULTS AND DISCUSSION

Results regarding AFB_1 content of rice cultures are presented in table 1. It is apparent that the AFB_1 production was inversely related with the amount of water added during incubation. Highest amount of AFB_1 was observed when only 1 ml water was added to the incubation medium at either 48 or 36 hr. Originally, Shotwell *et al*. (1966) recommended addition of water at 24 and then at 48 hr. However, addition of water only once was sufficient in the present experiment.

Table 1. Amount of water and the AFB_1 content

Water before autoclaving (ml/100g of rice)	Water during incubation (ml/100g of rice)			Aflatoxins (µg/g)			
	24 hr	48 hr	36 hr	B_1	B_2	G_1	G_2
50	10	-	-	7.59	0.21	0.29	n.d
50	3.3	3.3	-	13.6	0.02	n.d.	n.d
50	1.5	1.5	-	27.43	1.12	n.d.	n.d
50	-	1	-	37.97	1.55	n.d.	n.d
50	-	-	1	32.69	1.12	n.d.	n.d

n.d., not detected.

As noted earlier by Shotwell *et al*. (1966), the appearance of green colour after incubation of rice is an indicator of low AFB_1 levels. During initial stages of the present experimentation, appearance of dark green colour and cloudy conditions were observed when flasks were not shaken during incubation. Such flasks were found to have low AFB_1 contents. However, this problem could be solved by manual shaking of flasks 2 times a day and by breaking any clumps of rice with the help of sterile plastic pipettes. It should be noted that addition of water during incubation tends to increase lumping of rice, and also darkens the existing colour of the medium. Therefore, if there is already an indication of light green colour in the flasks, then water should not be added. The right colour at the end of incubation should be off white to light brown.

Under the present experimental conditions, addition of 1 ml water/100 g of rice once after 24 hr of incubation was optimum. However, 24 to 36 hr after starting incubation can be recommended as the appropriate time to add water. This is because the right flask needing water can then be chosen from the colour of its contents.

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