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Factors Affecting Territrem B Production by *Aspergillus terreus* CCRC 32111 in Potato-dextrose Medium

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ABSTRACT

The effects of incubation time, temperature, medium volume, and initial pH on the production of territrem B (TRB) by *Aspergillus terreus* CCRC 32111 were investigated. At 28˚C, maximal TRB production and mycelial growth were seen after 12 days' incubation in potato-dextrose (PD) liquid medium. Greatest TRB production occurred after rapid depletion of available carbohydrate at the stationary phase of fungal growth. TRB production decreased when the broth volume/culture container volume ratio increased, but increased if initial pH was alkaline. These results show that the TRB production is affected by factors including incubation time, temperature, medium volume, and the initial pH of medium.

Key words: incubation time, temperature, medium volume, initial pH, territrem B, *Aspergillus terreus* CCRC 32111, stationary phase, carbohydrate

INTRODUCTION

A cause-and-effect relationship between adverse effects in residents in Shuang-Chih Township in Taipei County and their intake of moldy rice contaminated by toxic strains of *Aspergillus* was reported in 1967[(1)]. This incident prompted us to study fungal contaminants of stored unhulled rice in Taiwan. We found that, of the 206 isolates of the *Aspergillus spp.* examined, only *Aspergillus terreus* 23-1 was capable of producing tremorgenic mycotoxins, designated as territrem A, B, and C (TRA, TRB and TRC)[(2-6)], TRB being the major product. These compounds induce whole-body tremors in mice and rats following intraperitoneal injection and show anti-acetylcholinesterase (AChE, E.C.3.1.1.7) activity[(7)]. Among 17 strains of *Aspergillus terreus* obtained from the Culture Collection and Research Center, Hsin Chu, Taiwan, only the CCRC 32111 and 32664 strains produced territrems in rice media[(8)]. These two strain, were isolated from stored paddy rice, collected in the area of Hsinchu and Changhua, Taiwan.

Fungi, a major cause of spoilage of stored grains and seeds, rank second only to insects as a cause of deterioration and loss[(9)]. Spoilage of stored grain is often accompanied by the formation of mycotoxins produced by toxigenic fungi. It has been postulated that fungal growth and toxin formation are influenced by environmental factors, such as moisture, temperature, incubation time, and substrate type[(10-11)]. In a previous investigation[(12)], *Aspergillus terreus* 23-1 was found to produce territrems when grown on food commodities such as peanut, unpolished rice, millet, wheat, and soybean, but not dried corn. Furthermore, territrems can be produced in potato-dextrose (PD) liquid medium, but not in Czapek's-Dox medium[(13)]. However, the effects of environmental factors on territrem production have not been studied. In the present work, the effect of incubation time and temperature, initial pH of medium, and the inoculum volume of the medium on TRB production by *Aspergillus terreus* CCRC 32111 were investigated.

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MATERIALS AND METHODS

I. *Strain and Culture Condition*

(I) *Organism*

The territrem B-producing strain, *Aspergillus terreus* CCRC 32111, was obtained from the Culture Collection and Research Center, Hsin Chu, Taiwan.

(II) *Inoculum preparation*

Aspergillus terreus CCRC 32111 was cultured and maintained at 28˚C on PD agar (Merck No. 10130). Conidiospores obtained from the edge of 7-day-old colonies were used as the inoculum source. Before use, the PD disks were washed with sterilized 0.01% Triton X-100 in distilled water. Spore suspensions (10^8 spores per mL) were prepared using a hematocytometer.

(III) *Culture conditions*

Unless otherwise specified, 50 mL of sterilized PD broth (initial pH 5.0) in a 125 mL Erlenmeyer flask was inoculated with about 10^8 spores.

II. *Experimental Design*

To study the time-course of territrem B production, Erlenmeyer flasks containing 50 mL of PD borth were inoculated with spore suspension and incubated at 28˚C, then cultures were harvested at 0, 4, 8, 12, 16 days. To study the effect of temperature, Erlenmeyer flasks containing 50 ml of inoculated PD broth were incubated at various temperatures ranging from 18 to 38˚C and the cells harvested as stationary cultures on day 12. To study the effect of medium volume, six different volumes of PD broth ranging from 30 to 80 mL were added to an Erlenmeyer flask, then, after spore incubation, the cultures were incubated at 28˚C and harvested on day 12. To study the effect of initial pH, the initial pH of the media was adjusted over the range from 3 to 11 by addition of 1 N NaOH or 2 N HCl after medium sterilization. All experiments were performed in triplicate and repeated twice. The amount of TRB, mycelial dry weight, residual carbohydrate, residual nitrogen, and the final pH of the medium were determined at the end of incubation.

III. *Measurement of Mold Growth*

Mycelial dry weight was determined by filtering the culture broth through a dry pre-weighed filter paper (Whatman 1, Whatman International, U. K.), which was then washed with distilled water, dried in an oven at 105˚C for 24 hrs and weighed. Mycelial dry weight was determined by subtracting the weight of the pre-weighed filter paper from that of the filter plus mycelium.

IV. *TRB Determination*

To extract TRB, the dry mycelium was mixed with 30 mL of chloroform and the mixture shaken mechanically for 30 mins at room temperature. The extract was filtered and evaporated to dryness, then redissolved in 2 mL of chloroform and subjected to thin layer chromatography analysis[8]. After development, the TRB was eluted from TLC plates and determined by HPLC[8].

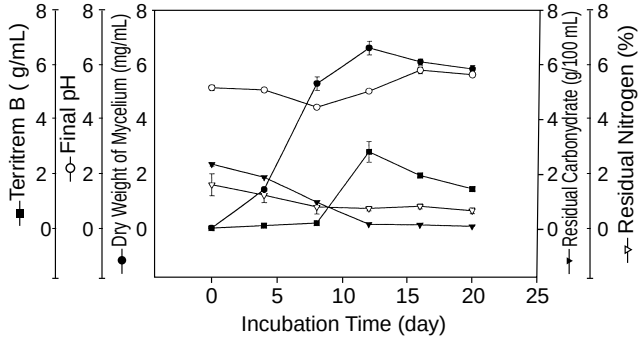

Figure 1. Fermentation time course of territrem B production in a 125 mL of flasks. All flasks, containing 50 mL of potato-dextrose broth at pH 5.0, were incubated at 28˚C.

V. *Determination of Carbohydrate, Nitrogen, and pH*

Carbohydrate was measured by the Anthrone method[14] and nitrogen by the Kjeldahl method[14]. The final pH of the medium was determined by using a pH meter.

VI. *Statiatical Analysis*

Unless otherwise stated, each value is the mean and SEM of three individual measurements. The data were analyzed using the SAS program for one-way analysis of variance, and the significance of differences between means was analyzed using Duncan's multiple range test.

RESULTS AND DISCUSSION

I. *The Time Course of TRB Production*

In the first series of experiments, *Aspergillus terreus* CCRC 32111 was grown for 4 – 20 days at 28˚C in 50 ml of PD broth at an initial pH of 5. Figure 1 shows that TRB production started after 4 days' incubation. As incubation continued, TRB production increased gradually and reached a maximum after 12 days, at which time fungal growth entered a stationary phase. TRB production ceased when glucose in the medium was completely used up. An incubation time of 12 days was used in the subsequent experiments.

The above results showed that, using an initial pH of 5, a temperature of 28˚C, and 50 mL of medium in a 125 mL of flask, TRB production reached a maximum after carbohydrate depletion and that highest mold growth was seen after12 days. Shin and Marth[15] also reported that maximal amount of aflatoxin appeared after rapid consumption of glucose and when the logarithmic phase of growth ceased. We also found that TRB production was first on day 4 during the exponential growth phase of the fungus, in agreement with the results of Bu'Lock[16-17] and Buchanan and Lewis[18], who found that aflatoxin, the secondary metabolite, first appears during the exponential phase of mold growth due to the expression of enzymes involved in secondary metabolism and glucose catabolism. Righelato[19] postulated that the decrease in fungal growth and toxin production were, respectively, due to autolysis of the fungal cells and toxin degradation by fungal enzymes. In the present study, fungal growth and TRB production began to decrease after 12 days.

II. *Effect of Incubation Temperature*

Using 50 mL of medium in a 125 flask and at an initial pH of 5, *Aspergillus terreus* CCRC 32111 grew at all five temperatures from 18 to 38˚C (Figure 2). The yield of TRB was maximal when the cultures were incubated for 12 days at 28˚C ($p < 0.05$ compared to all other temperatures tested). However, the yield of TRB was much lower at 18, 23, and 38˚C .

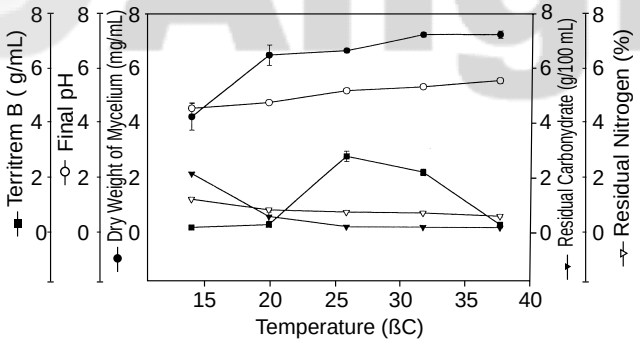

Figure 2. Production of territrem B by *A. terreus* CCRC 32111 at various temperature. All flasks, containing 50 mL of potato-dextrose broth at pH 5.0, were incubated at different temperature for 12 days.

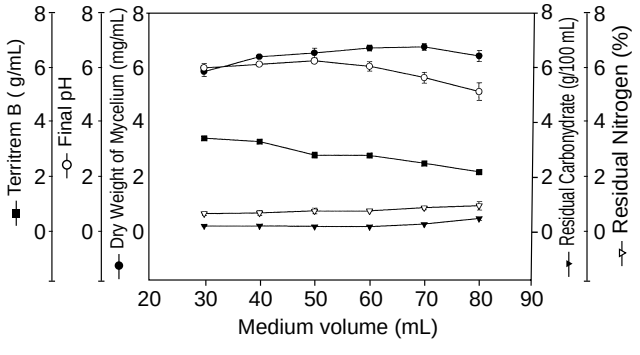

Figure 3. Effect of the medium volume size on the production of territrem B by *A. terreus* CCRC 32111. All flasks, containing different volume size of medium at pH 5.0, were incubated at 28˚C for 12 days.

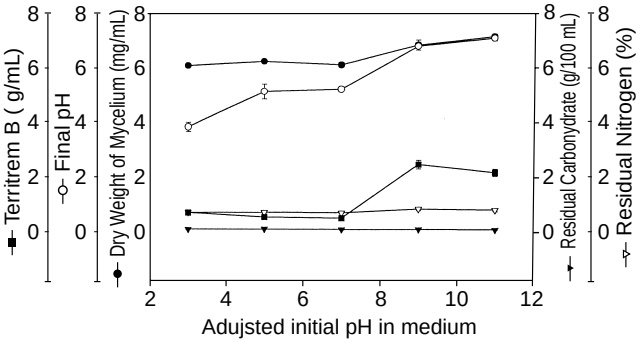

Figure 4. Effect of initial pH of culture medium on the production of territrem B by *A. terreus* CCRC 32111. All flasks, containing 50 mL of potato-dextrose broth, were incubated at 28˚C for 12 days.

Temperature is one of the most important environmental factors influencing the growth and toxin production of toxigenic fungi[20-26]. TRB production by *Aspergillus terreus* CCRC 3211 increased with increasing temperature up to 28˚C, then decreased at 33˚C and 38˚C. These data clearly show that temperature is an important factor affecting TRB production. Schrindler et al.[21] reported that different enzymes were responsible for growth and toxin production. Our data also show that only a small amount of TRB production and low *Aspergillus terreus* growth were seen when the culture was incubated at 18˚C, suggesting that the TRB contamination could be avoided by storing the commodities at low temperatures.

III. *Effect of Medium Volume Size*

Using culture conditions of 28˚C, an initial pH of 5, and an incubation time of 12 days, when the medium volume in the flask increased over 80 mL, mycelial growth was not affected, but the yield of TRB declined at volumes greater than 50 mL, and the final pH of the culture became more acidic at medium volume of 60 mL or higher (Figure 3). At medium volumes ranging from 30 to 60 mL, the carbohydrate and nitrogen were almost entirely consumed by the microorganism, whereas a further increase in medium volume resulted in an increase in residual carbohydrate and a decrease in TRB production. El-Enshasy et al.[27] reported that increasing the volume of the culture medium resulted in a decrease in the amount of glucose consumed and in natamycin production, due to a shortage of oxygen supply. Aflatoxin production is reported to decrease with an increase in the carbon dioxide concentration and a decrease in the oxygen concentration[28-30]. Our data show that TRB production decreased with an increase in the volume of medium, suggesting that oxygen was important for TRB production.

IV. *Effect of Initial pH*

Using culture conditions of 28˚C, 12 days' incubation, and 50 mL of medium in a 125 mL flask, the initial pH was varied from 3 to 11. At an initial pH of 9, TRB production was 4-fold higher than at pH 3-7 (Figure 4). In general, most fungi can grow at pH values ranging from 2 to 8.5[31]. Our data show that *Aspergillus terreus* CCRC 32111 can grow at pH values in the range of 3 to 11. Lie and Marth[32] reported that maximal aflatoxin production occurred at extreme alkaline pHs. The present study showed that TRB production and mold growth were maximal with an initial pH of 9-11.

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