

## Production of Fumonisin B1 by *Fusarium moniliforme* NRRL 13616 in Submerged Culture

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**Fumonisin B1, a recently discovered mycotoxin, was synthesized by submerged cultures of *Fusarium moniliforme* NRRL 13616 grown for 29 days at 28°C and 220 rpm in a basal salts medium (pH 5.0) supplemented with 90 g of glucose per liter and 3.5 g of ammonium sulfate per liter. Under these culture conditions,  $74 \pm 23$   $\mu\text{g}$  of fumonisin B1 per ml was produced by 29-day-old *F. moniliforme* NRRL 13616 cultures. Fumonisin B1 was detected in liquid culture extracts by high-performance thin-layer chromatography. Fumonisin B1 was confirmed and quantitated by gas chromatography and gas chromatography-mass spectral analysis of the trimethylsilyl derivative. The use of a defined medium for producing fumonisin B1 in a submerged culture facilitates its isolation and provides an excellent method for conducting biosynthetic studies.**

The association of *Fusarium moniliforme* with grains and feeds implicated in leukoencephalomalacia in horses, hepatotoxicity in rats, and esophageal cancer in humans is under intense investigation (1, 5, 7). Characterization of the fungal secondary metabolites produced by these strains has shown that at least two compounds with potential to cause disease are produced, fusarin C and fumonisin B1 (2, 8). Fusarin C has been shown to be mutagenic, but attempts to confirm carcinogenicity have been unsuccessful (3). Recent studies have shown that intravenous injection of fumonisin B1 in a horse produced symptoms characteristic of leukoencephalomalacia (6). Fumonisin B1 has also been shown to be a cancer-promoting factor in rats (2).

Fumonisin B1 is 2-amino-12,16-dimethyl-3,5,10-trihydroxy-14,15-propane-1,2,3-tricarboxy icosane. Although it can be produced by growing various strains of *F. moniliforme* on corn (G. A. Bennett, J. J. Ellis, and O. L. Shotwell, Proc. 103rd AOAC Annu. Int. Meet. 1989, p. 32), purification is difficult owing to the coextraction of numerous interfering compounds. Our previous studies on the nutritional regulation of fusarin C synthesis by *F. moniliforme* NRRL 13616 (4) provided basic information on how nutrients effect primary and secondary metabolism in this organism. We report here on a method for producing fumonisin B1 with *F. moniliforme* NRRL 13616 by submerged culture growth in a defined liquid medium.

### MATERIALS AND METHODS

**Culture inoculum.** Spore inocula were produced on a V8 agar plate from a single-spore isolate of *F. moniliforme* NRRL 13616. V8 agar consisted of 200 ml of V8 juice per liter, 3.0 g of calcium carbonate per liter, and 20 g of agar per liter. The V8 agar plate containing spores was rinsed with deionized water, and the spores were dried on silica beads for long-term storage at 4°C.

For submerged-culture inocula, silica-dried spores were grown and sporulated on V8 agar in petri plates at room temperature. Spores were harvested by rinsing the agar surface of V8 agar plates with sterile-filtered, deionized

water. A concentration of  $5 \times 10^6$  spores per ml was used in all submerged-culture experiments.

**Submerged culture.** The medium for submerged-culture synthesis of fumonisin B1 consisted of glucose (Difco Laboratories, Detroit, Mich.), 90 g/liter; ammonium sulfate (Fisher Scientific Co., Pittsburgh, Pa.), 3.5 g/liter; potassium phosphate monobasic (Mallinckrodt, Inc., St. Louis, Mo.), 2.0 g/liter; magnesium sulfate heptahydrate (Fisher), 0.3 g/liter; calcium chloride dihydrate (J. T. Baker Chemical Co., Phillipsburg, N.J.), 0.4 g/liter; manganese sulfate monohydrate (Mallinckrodt), 16 mg/liter; thiamine, riboflavine, pantothenate, niacin, pyridoxamine, and thiotic acid (Sigma Chemical Co., St. Louis, Mo.), 500  $\mu\text{g}$  of each per liter; and folic acid, biotin, and vitamin B<sub>12</sub> (Sigma), 50  $\mu\text{g}$  of each per liter. Glucose stock solutions (300 g/liter) were autoclaved separately. Deionized water (Milli-Q ultrapure water system; Millipore Corp., Bedford, Mass.) was used in all media.

Liquid culture experiments were carried out in duplicate 1-liter baffled Erlenmeyer flasks (no. 2543-01000; Bellco Glass, Inc., Vineland, N.J.) at a 500-ml volume. The cultures were incubated for 29 days at 28°C and 220 rpm in a rotary-shaker incubator. A pH of 5.0 was maintained by daily adjustment with 2 N HCl or 2 N NaOH.

**Sample preparation.** A 50-ml portion of filtered liquid culture broth was applied to a 25-g XAD-2 column (Sigma) which had been packed and equilibrated with water. The column was washed with water (250 ml), and the fumonisin B1 was eluted with methanol (250 ml). The methanol was removed by rotoevaporation, and the residue was reconstituted in 50 ml of water. The sample was partitioned twice with 50 ml of ethyl acetate in a 250-ml separatory funnel, and the ethyl acetate fractions were discarded. The water fraction was evaporated to dryness in a rotoevaporator, and the residue was dissolved in methanol and transferred to a 2-ml vial. The methanol was evaporated at 60°C under a stream of nitrogen, and the residue reconstituted in 500  $\mu\text{l}$  of methanol for analyses.

**Analytical methods.** Fumonisin B1 was detected by high-performance thin-layer chromatography (HPTLC). Pre-coated silica gel 60 plates (10 by 10 cm; E. Merck AG, Darmstadt, Federal Republic of Germany) were used for all

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TABLE 1. Production of fumonisin B1 and fusarin C by submerged cultures of *F. moniliforme* NRRL 13616 in a defined medium

Culture age (days)	Amt ( $\mu\text{g/ml}$ ) of:	
	Fumonisin B1	Fusarin C
3	TR <sup>a</sup>	40
8	40	177
29	74 $\pm$ 23 <sup>b</sup>	374

<sup>a</sup> TR, Trace.

<sup>b</sup> The amounts of fumonisin B1 measured in culture extracts from three separate experimental trials were 44, 78, and 100  $\mu\text{g/ml}$ .

assays. Fumonisin B1 standard was kindly provided by R. D. Plattner, U.S. Department of Agriculture, Northern Regional Research Center. Samples (1- $\mu\text{l}$ ) of partially purified culture extract (0.1 ml eq) and fumonisin B1 reference standard (1  $\mu\text{g}/\mu\text{l}$ ) were applied to the HPTLC plates, developed with chloroform-methanol-acetic acid (6:3:1), and air dried. Fumonisin B1 was visualized by evenly spraying the plates with 0.5% *p*-anisaldehyde solution (2) and drying them at 150°C for 3 min. The presence of fumonisin B1 in liquid culture extracts was determined by comparing the

color and  $R_f$  value of sample spots with those of fumonisin B1 standard.

Fumonisin B1 in liquid culture extracts was confirmed and quantitated by gas chromatography (GC) and gas chromatography-mass spectrometry analysis (GC-MS) of the trimethylsilyl (TMS) derivative of fumonisin B1. Samples (50  $\mu\text{l}$ ) of culture extracts (5 ml eq) and fumonisin B1 standard (50  $\mu\text{g}$ ) were dried under nitrogen and hydrolyzed with 1.8 N KOH (500  $\mu\text{l}$ ) at 60°C for 1 h. The hydrolysate was neutralized with 1 N HCl and applied to a 2.5-g XAD-2 column. The column was washed with water (30 ml), and the hydrolysis product was eluted with methanol (30 ml). The 30-ml methanol fraction was dried in a rotoevaporator, and the hydrolysis product was dissolved in methanol and transferred to a 2-ml vial. The samples were dried under a stream of nitrogen, and the residue was derivatized in 100  $\mu\text{l}$  of Tri-Sil TBT (Pierce Chemical Co., Rockford, Ill.). The Tri-Sil TBT sample mixture was allowed to react for 45 min at 60°C.

GC of derivatized standard and samples (1 or 2  $\mu\text{l}$ ) was performed on a gas chromatograph (model 5890; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a DB-5 capillary column (5 m by 0.53 mm) and a flame ionization detector. The carrier gas was hydrogen (flow rate, 2 ml/min),

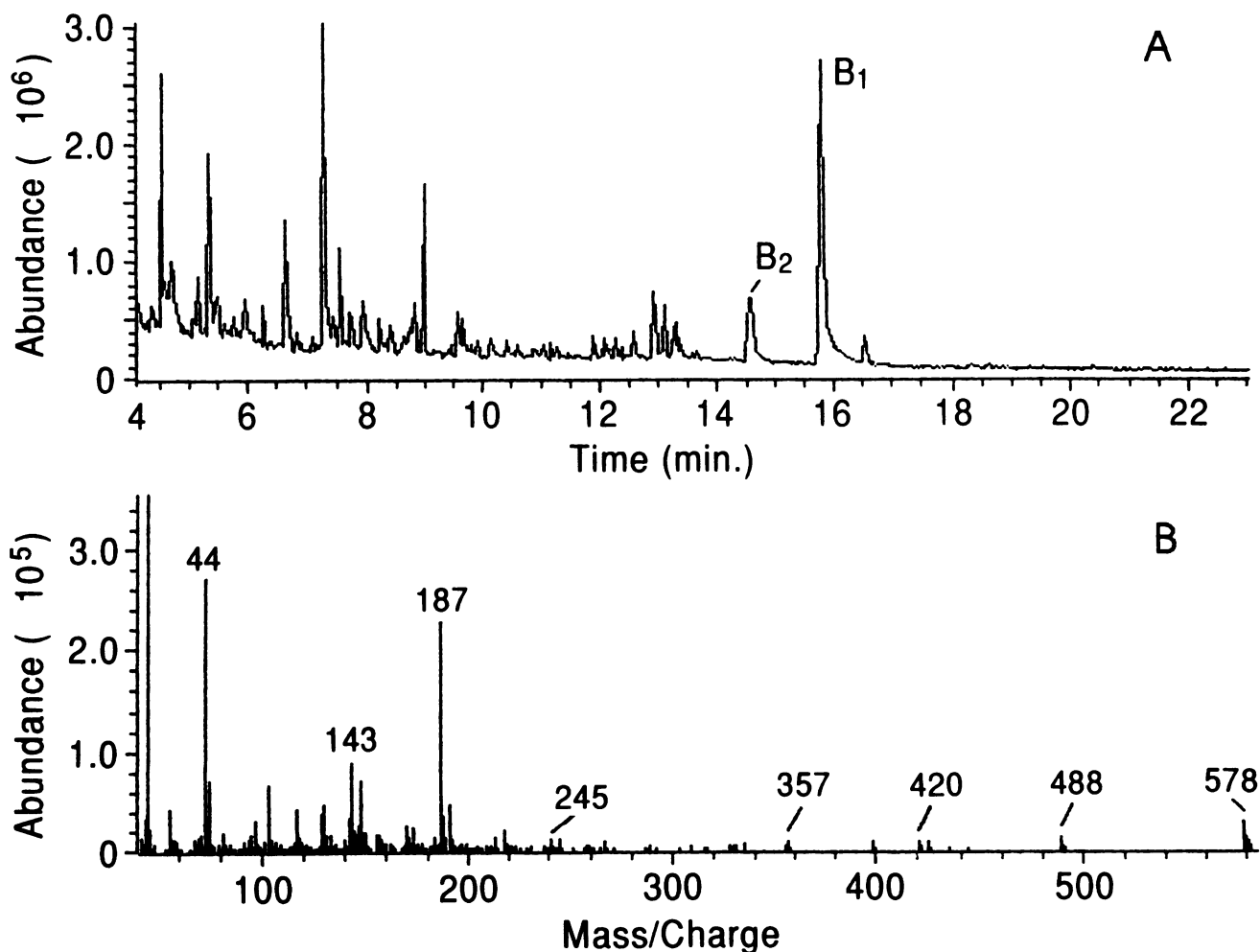


FIG. 1. GC-MS of a TMS derivative of hydrolyzed fumonisin B1 extracted from *F. moniliforme* NRRL 13616 liquid culture broth. The total ion chromatogram (A) and mass spectrum (B) are identical to those obtained with standard fumonisin B1.

and the column oven temperature was programmed as follows: 100°C for 2 min, 100 to 200°C in 5 min and then 200 to 270°C in 14 min. The TMS derivative of fumonisin B1 elutes at 19.5 to 20.2 min under these conditions. Levels of fumonisin B1 produced in liquid cultures were determined by comparing peak areas of samples with peak areas of authentic fumonisin B1 standards. When required, samples were diluted with hexane so that sample peak areas approximated the peak area produced by 1 µg of fumonisin B1 reference standard. Fumonisin B1 identity was confirmed by GC-MS of the TMS derivative of samples and fumonisin B1 standard as previously described (R. D. Plattner, W. P. Norred, C. W. Bacon, K. A. Voss, R. Petterson, D. D. Shackelford, and D. Weisleder, submitted for publication), using a Hewlett-Packard 5890 gas chromatograph in tandem with a Hewlett-Packard selective ion detector.

Fusarin C and glucose analyses were performed by high-performance liquid chromatography, using previously described methods (4).

## RESULTS AND DISCUSSION

Under the submerged-culture conditions described above, *F. moniliforme* NRRL 13616 cultures grown in a defined liquid medium produced detectable levels of fumonisin B1 by day 3 of growth (Table 1) and the highest levels ( $74 \pm 23$  µg/ml) in the terminal, 29 day-old sample. The presence of abundant glucose (24.8 mg/ml) in the supernatant of 29-day-old cultures suggests that an extended incubation period may increase fumonisin B1 production under these growth conditions. Additional work is needed to optimize the incubation time for *F. moniliforme* NRRL 13616 submerged-culture synthesis of fumonisin B1.

The nutritional composition of the defined medium used for fumonisin B1 production is also suitable for fusarin C synthesis, and significant levels of fusarin C were produced by *F. moniliforme* NRRL 13616 cultures producing fumonisin B1 (Table 1). We are currently investigating the possibility that nutritional factors that influence fusarin C synthesis are also important in regulating fumonisin B1 synthesis.

The isolation and detection of fumonisins derived from liquid culture broths is much simpler than for solid substrate fermentations. Since fumonisins are water soluble, culture extracts can be directly applied to the XAD cleanup column. Some compounds do remain with the fumonisins after methanol elution of the XAD column, but the number and quantity of these interfering compounds are greatly reduced when compared with corn-grown culture extracts. Fumonisin B1 is detected by normal-phase HPTLC when its concentration in the culture medium exceeds 5 µg/ml. Both color (blue-violet) and  $R_f$  value (0.24 to 0.26) must match with those of the reference standard, since similarly colored zones were detected just above and below fumonisin B1 on HPTLC plates. At present, we can easily detect 0.2 µg of fumonisin B1 standard with the *p*-anisaldehyde spray reagent.

The TMS derivative of hydrolyzed fumonisin B1 was readily measured by capillary GC with flame ionization detection. Temperature programming resulted in excellent separation of the fumonisin B1 hydrolysis product from other peaks. Fumonisin B1 was confirmed by GC-MS of the TMS derivative. Figure 1 shows the total ion chromatograph

(A) and the mass spectrum (B) of fumonisin B1. The intense ions at  $m/z$  578, 187, and 44 (base peak) correspond to those produced by standard fumonisin B1 and arise from cleavage between C-14 and C-15 ( $m/z$  578 and 187) and between C-2 and C-3 (R. D. Plattner, personal communication). The retention time and mass spectrum of derivatized fumonisin B1 from our liquid cultures were identical to those of authentic fumonisin B1 standard.

GC of the TMS-derivative of fumonisin B1 isolated by the procedure described above indicates that *F. moniliforme* NRRL 13616 can produce up to 100 mg of fumonisin B1 per liter in 29 days. Recovery of fumonisin B1 from liquid cultures was demonstrated to be quantitative (92%) by spiking 50 ml of liquid culture medium with 100 µg of fumonisin B1 standard and analyzing the sample by the procedure described above. These data demonstrate for the first time that fumonisin B1 can be produced in liquid culture at levels suitable for preparation of laboratory-scale quantities of this metabolite. The defined nature of this medium makes it well suited for biosynthetic and nutritional studies on fumonisin B1 synthesis by *F. moniliforme* NRRL 13616 as well as for use as a screening medium for fumonisin B1 production by other toxigenic strains of *F. moniliforme*. The use of this defined medium also provides a method for producing radiolabeled fumonisin B1.

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## LITERATURE CITED

- Cheng, S. J., Y. Z. Jiang, M. H. Li, and H. Z. Lo. 1985. A mutagenic metabolite produced by *Fusarium moniliforme* isolated from Linxian County, China. *Carcinogenesis* 6:903-905.
- Gelderblom, W. C. A., K. Jaskiewicz, W. F. O. Marasas, P. G. Thiel, R. M. Horak, R. Vlegaar, and N. P. J. Kriek. 1988. Fumonisin—novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl. Environ. Microbiol.* 54:1806-1811.
- Gelderblom, W. C. A., P. G. Thiel, K. Jaskiewicz, and W. F. O. Marasas. 1986. Investigations on the carcinogenicity of fusarin C—a mutagenic metabolite of *Fusarium moniliforme*. *Carcinogenesis* 7:1899-1901.
- Jackson, M. A., P. J. Slininger, and R. J. Bothast. 1989. Effect of zinc, iron, cobalt, and manganese on *Fusarium moniliforme* NRRL 13616 growth and fusarin C biosynthesis in submerged cultures. *Appl. Environ. Microbiol.* 55:649-655.
- Kriek, N. P. J., T. S. Kellerman, and W. F. O. Marasas. 1981. A comparative study of the toxicity of *Fusarium verticillioides* (= *F. moniliforme*) to horses, primates, pigs, sheep, and rats. *Onderstepoort J. Vet. Res.* 48:129-131.
- Marasas, W. F. O., T. S. Kellerman, W. C. A. Gelderblom, J. A. W. Coetzer, P. G. Thiel, and J. J. Van Der Lugt. 1988. Leukoencephalomalacia in horse induced by *Fusarium moniliforme* isolated from *Fusarium moniliforme*. *Onderstepoort J. Vet. Res.* 55:197-203.
- Marasas, W. F. O., N. P. J. Kriek, J. E. Fincham, and S. J. Van Rensburg. 1984. Primary liver cancer and oesophageal basal cell hyperplasia in rats caused by *Fusarium moniliforme*. *Int. J. Cancer* 34:383-387.
- Wiebe, L. A., and L. F. Bjeldanes. 1981. Fusarin C, a mutagen from *Fusarium moniliforme* grown on corn. *J. Food Sci.* 46:1424-1426.