

Production and Purification of Fumonisin From a Stirred Jar Fermenter

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ABSTRACT The production, isolation and purification of fumonisins from 10 litre liquid cultures are described. Measurements of sucrose, fructose and glucose consumption, oxygen demand, dry weight increase, CO₂, and fumonisin production were taken every 48 hours. The specific productivity of fumonisins was found to be similar to that reported for corn cultures but purification was much simpler, yielding an 89% recovery. The method developed for the purification of fumonisins from liquid culture was also applied to a corn culture, resulting in a 70.1% recovery. Published 1994 Wiley-Liss, Inc.

Key Words: Fumonisin, Liquid cultures, Isolation

INTRODUCTION

Discovered only in 1988, fumonisins are a family of compounds produced by the fungus *Fusarium moniliforme* Sheldon and related species. They have been found in corn-based food and feed wherever corn is grown, except in colder areas of the world [Bezuidenhout et al., 1988; Riley et al., 1993a; Scott, 1993]. These compounds have been demonstrated to cause a variety of diseases in domestic animals, including equine leucoencephalomalacia and porcine pulmonary edema [Colvin and Harrison, 1992; Kellerman et al., 1990]. Fumonisin B₁ produces liver tumors in male BD IX rats fed a cereal-based diet [Gelderblom et al., 1991]. However, these compounds are poor cancer-initiators and are not genotoxic [Gelderblom et al., 1992]. Fumonisin B₁ is a potent inhibitor of ceramide synthetase and this may explain their carcinogenicity [Riley et al., 1993b; Schroeder et al., 1993]. Human exposure to fumonisins may result in increased esophageal cancer. As has been the case with grain contaminated by other toxigenic fusaria, grain containing fumonisins is apparently more toxic than can be explained at present [Thiel et al., 1992].

The vast array of toxicological questions raised by the occurrence of fumonisins in food and feed have focused attention on the availability of radiolabelled material, pure fumonisins in quantity for toxicity tests, and the need for studies of cooccurring compounds. Fumonisin B₁ has been prepared by isolation from autoclaved corn on which *F. moniliforme* has been grown for ca. 6 weeks at 25°C. Approximately 40% of the fumonisin B₁ and B₂ (>90% purity) can be recovered from such fermentations [Cawood et al., 1991]. Yields at higher purities are much less.

The high hydrophilicity of fumonisins has made them very difficult to purify from corn. Unlike most known mycotoxins, fumonisins are not soluble in most organic solvents. The only satisfactory extraction methods use a combination of water with either methanol (1:3) [Cawood et al., 1991] or acetonitrile (1:1) [Plattner and Shackelford, 1992]. Even then, many polar impurities including salts, sugars and peptides are co-extracted. Separation of the fumonisins from most of these compounds is then achieved by ion-exchange chromatography, followed by silica gel and reversed-phase chromatography, usually with low recoveries. One way to avoid these contaminants is to produce the fumonisins in a medium that is less complex than corn. Liquid media are ideally suited for this purpose. From original reports of 74 mg of fumonisin B₁ (FB₁)/L [Jackson and Bennett, 1990], yields have now risen to over 500 mg FB₁/L of medium [Blackwell et al., 1993].

The use of a liquid medium greatly reduced the number of contaminants but also changed the nature of those found. For instance, a much larger amount of salt is present in liquid culture, while the amount of lipid found is much smaller than in corn culture. This prompted some changes in the purification method.

Most mycotoxins are secondary metabolites of filamentous fungi. Because of the industrial importance of such compounds (e.g., penicillin), the principles relating to their production in stirred-jar fermentation are fairly well under-

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stood. The synthesis of secondary metabolites requires specific nutrient limitations as well as certain O₂, pH, osmotic, and sometimes temperature conditions [Bu'Lock 1975; Miller and Greenhalgh, 1988]. The technology has been developed to produce satisfactory yields of several *Fusarium* metabolites in liquid fermentations. These include gibberellins [*F. moniliforme*, Borrow et al., 1961, 1964], zearalenone [*F. graminearum* Schwabe, Hidy et al., 1977], deoxynivalenol [*F. culmorum*, Greenhalgh et al., 1984; Miller and Blackwell, 1986], T-2 toxin [*F. sporotrichioides* Sherb., Greenhalgh et al., 1987], and enniatins [*F. avenaceum* (Corda) Fr., Blais et al., 1992]. In addition to the production of pure material, such fermentations have enabled the identification of numerous metabolites from the toxigenic fusaria, the production of radiolabelled toxins, and insight into the biosynthesis of several toxins [ApSimon, 1994; Miller and Arison 1986].

We have reported a method for the production of labelled fumonisin from experiments conducted in shake-flask liquid cultures [Blackwell et al., 1993]. This paper reports studies concerning the physiology of fumonisin biosynthesis and production at the 10 L scale.

MATERIALS AND METHODS

The rationale for the fermentation conditions used is provided in Blackwell et al. [1993]. Seed culture was prepared as follows. A 2% malt extract agar slant of *F. moniliforme* NRRL 13616 was macerated in 80 ml sterile distilled water. An aliquot (2.5 ml) was used to inoculate thirty-two 250 ml Erlenmeyer flasks each containing 50 ml of a medium containing glucose (20 g), malt extract (2 g), yeast extract (2 g), peptone (2 g), KH₂PO₄ (2 g), MgSO₄·7H₂O (2 g), FeSO₄·7H₂O (0.2 g) and ammonium chloride (3 g; amounts given per litre distilled water put through a Barnstead Nanopure II system, 18.3 MΩ/cm) and autoclaved at 121°C for 12 min. The cultures were put on a rotary shaker (3.81 cm throw, 220 rpm) at 28°C for 48 h. Production medium (9.3 litres) consisting of sucrose (40 g), glycerol (10 g), (NH₄)₂HPO₄ (1 g), KH₂PO₄ (3 g), MgSO₄·7H₂O (0.2 g) and NaCl (5 g) (amounts per litre ultrapure water as above) were added to a 15 L jar from a New Brunswick Scientific fermenter with pH (Ingold) and O₂ electrodes (N.B. Scientific 900 series) and autoclaved at 121°C for 20 min. Net CO₂ in the output air was measured with an ADC 225 MK3 instrument (ADC, Hoddeson, England). The seed culture was macerated and centrifuged in sterile 500 ml bottles at 10,400g. Fifty percent of the actual volume was removed and the macerate re-suspended under aseptic conditions and added to the fermenter. The culture was grown at 28°C and was stirred at 380 rpm. Air was pumped into the fermenter at 3.2 L/min. Foam was controlled with a mechanical foam breaker and the twice-daily addition of 1 ml Dow Corning antifoam B. The initial pH was 6.2, fell to <2 after ca. 24 h, and was not controlled. Experiments were conducted in

which pH was maintained at 4 for 24 h with NaOH followed by no control [low pH is required for production of fumonisins; Blackwell et al., 1993]. These resulted in inferior fumonisin yields.

Physical data on the fermentation (temperature, pH, O₂, CO₂, temperature) were acquired using a Hewlett-Packard 3421A data acquisition system, HP 9114B disc drive using custom software running on an HP41CX (developed by Dr. A. Taylor, NRC, Halifax).

Samples (100 ml) were withdrawn and filtered through a tared Whatman #1 filter (47 mm). The mycelium was washed and dried at 40°C under vacuum and the resulting dry weight determined. The culture filtrate was analysed in duplicate for fumonisin B₁ and B₂ using the method of Miller et al. [1993]. A 1 ml aliquot of filtrate was applied to a Bond Elut Certify II (200 mg, Varian) column preconditioned by aspirating methanol (6 ml) and water (6 ml) under vacuum. The minicolumns were then washed with water (6 ml) and methanol (6 ml). Fumonisins were then eluted with 0.1% trifluoroacetic acid (TFA) in methanol (2.5 mL). The TFA methanol fraction was concentrated to dryness and taken up in one ml of methanol. A 20 µl aliquot was transferred to a 2 ml vial and dried under a stream of nitrogen. The residue was redissolved in 100 µl of 0.05 M sodium borate buffer, pH 8.3 (adjusted with 1 N HCl). A freshly prepared solution of 4-fluoro-7-nitrobenzofurazan [100 µl of 22 mM NBD-F (Molecular Probes Inc.) in 95% ethanol] was added. After heating for 70 sec at 70°C, the solution was cooled and made up with 500 µl with a 1:1 mixture of HPLC mobile phases A (0.05 M NaH₂PO₄/methanol adjusted to pH 6.3 with 2N NaOH, 1:1) and B (acetonitrile/H₂O, 8:2). A 20 µl aliquot (corresponding to 8 ng of fumonisin B₁ and B₂ for standard solutions) was injected in duplicate onto a Lichrosorb 5 µm, RP-18, 250 mm × 4.6 mm column on a Varian Vista 5500 HPLC with a Varian Vista CDS-401 data system. An 11 min linear gradient of 0 to 100% B followed by a 2 min plateau was run at a flow rate of 1 ml/min. The derivatized fumonisins were then detected by their fluorescence at 490 nm after excitation at 450 nm (detector: Waters model 420-E).

Some of the remaining culture filtrate was analysed in triplicate for sucrose, glucose and fructose by HPLC using a Waters M-45 solvent delivery system (mobile phase 80% acetonitrile, 20% H₂O, 2 ml/min), a Waters carbohydrate column (PN 84038) and a Waters refractive index detector (R 401). The culture filtrate was analysed for ammonia by the alkaline distillation-Kjeldahl method in one run. The experiment was repeated twice.

To isolate large amounts of fumonisins, additional fermentations were run for 240 h without taking samples (a process that lowers yield by disturbing the geometry of the fermentation). After filtration as above, the pH of the resulting solution was adjusted to 6 with 1N NH₄OH and the solution was vacuum-filtered again through Celite 503 in a fine pore sintered glass funnel. The resulting culture filtrate was stored at -20°C until chromatographed.

Ion-Exchange Chromatography

For a better throughput, four gravity-fed columns were usually run simultaneously instead of one larger one. For each column, DEAE Sephadex A-25 (Pharmacia LKB) (20 g) was swollen overnight in distilled deionized (dd) water (200 ml). The next day, the gel was poured into a 100 ml column and charged with a formate counter-ion by washing with a 0.5 N formic acid solution (300 ml). The column was then rinsed with dd water until the pH of the eluent was above 3 (500–800 ml). The culture filtrate (500–1,250 ml) was then loaded onto the column and followed by a dd water wash (300 ml). Elution was then started with 0.1 N HCOOH (300–400 ml). A brown band moving from the top of the column was collected in 20 ml fractions. Analysis by tlc on silica gel, using a solvent consisting of 60:32:7:1 CHCl₃/MeOH/H₂O/AcOH, revealed which fractions contained fumonisins, usually the first three brown fractions after the start of the HCOOH elution. R_f's of 0.25 for FB₁ and 0.31 for both FB₂ and FB₃ were obtained.

Two solvent systems reported to resolve FB₂ and FB₃, 6:3:1 CHCl₃/MeOH/AcOH [Cawood et al., 1991] and 85:15 MeCN/H₂O [Plattner et al., 1992] did not work well for us, producing diffuse or streaky spots. Fumonisin spots were revealed with an anisaldehyde spray (0.5% in MeOH/sulfuric acid/acetic acid, 85/5/10) followed by heating on a hot plate. The fumonisin-containing fractions were combined, concentrated under vacuum and lyophilized. A typical yield of brownish solid was 1.45 g (total from 4 columns).

Silica Gel Chromatography

The crude solid (1.3 g) from above was dissolved in CH₃CN/H₂O (87:13) and chromatographed on an open silica gel 60 (63–200 μm, 30 g) column (20 mm i.d.) with the same solvent. Five fractions of 11 ml each, followed by 6 ml fractions were collected. The fractions were analyzed by tlc as above and the fumonisin-containing fractions (8–16) were combined. Typical yield of fumonisin-containing brown oil from such a column: 1.0 g.

Reversed-Phase Chromatography

Fractions containing similar combinations of fumonisins were combined (up to 2.0 g) and chromatographed on a reversed-phase column: 70 g LiChroprep RP-18 (40–60 μm) in a medium pressure 3 cm diameter column. The solvent was driven through the column with a Varian Vista 5500 HPLC system pump. The gradient shown in Table I was run at 1.5 ml/min to separate the fumonisins.

Approximately 1.0 g of fumonisin B₁ (at least 97% pure) was obtained from each run. In a typical fermentor run, 8 L of culture filtrate was obtained, containing 265.5 mg/L of FB₁ for a total of 2.12 g FB₁. After ion-exchange, 6.75 g of lyophilized material was obtained. After silica gel chromatography, the total mass was reduced to 5.71 g, and after a first chromatography on reversed-phase gel, 1.89 g of 97% pure FB₁ was obtained for a recovery of 89%.

TABLE I. Gradient Run at 1.5 ml/min to Separate the Fumonisin

| Time (min) | MeCN/0.1% TFA (%) | Water/0.1% TFA (%) |
|------------|-------------------|--------------------|
| 0 | 23 | 77 |
| 35 | 23 | 77 |
| 140 | 38 | 62 |
| 170 | 38 | 62 |
| 185 | 100 | 0 |

Isolation of more FB₁ as well as other fumonisins required multiple runs of similar gradients or the use of HPLC. Detection of fumonisins from these separations was done by TLC as above.

This purification technique was also applied to corn cultures. Extraction of a corn culture (400 g) with 1:1 acetonitrile/water yielded 27 g of freeze-dried extract. Because of the much lower salt content of corn cultures, the whole extract, dissolved in one liter of water, was loaded onto one 20 g DEAE Sephadex A-25 column. No overloading of the column was observed. Elution with 0.1 N HCOOH yielded 2.9 g of brown solid. Chromatography on silica gel proved more difficult as the crude fumonisin fraction dissolved very poorly in the 87:13 acetonitrile/water solvent. Because of this, the brown solid (1.4 g) was dissolved in 1:1 acetonitrile/water, combined with silica gel and concentrated to dryness. This dry mixture was then loaded onto the silica gel column and the column was eluted with 87:13 acetonitrile/water. Fractions containing fumonisins were lyophilized for a recovery of 476 mg. Reversed-phase chromatography was then performed on this material as for the liquid culture material and 280.2 mg of fumonisin B₁ was obtained for a recovery of 70.1%, based on analysis of the crude corn culture.

RESULTS AND DISCUSSION

Given the relatively large inoculum, growth in the first 48 h was rapid with dry weight increasing >6 times (Fig. 1). This rapid growth was reflected in demand for O₂ (evidenced by the dissolved O₂ values) and in the production of CO₂ per unit dry weight. As expected from the mass of cells produced, there was no more detectable ammonia by this time (or thereafter). Sucrose became limiting between 48 and 72 h. After 96 h, dry weight increased by ca. 5% every 48 h concomitant with slow rates of utilization of the fructose and glucose remaining and respiration rates <20% of peak values. The low standard errors for the replicate values for growth parameters (dry weight, CO₂) and sugars utilization indicated highly repeatable fermentation conditions.

Fumonisin production began near 48 h and the fastest rate had been achieved by 96 h. Production then doubled approximately every 48 hours as long as mycelial dry weight increased. This roughly accords with the calculated specific productivity values over the same time period (g fumonisins/g dry weight; 48 h-0.0002, 96 h-0.0086, 144 h-0.0185, 192

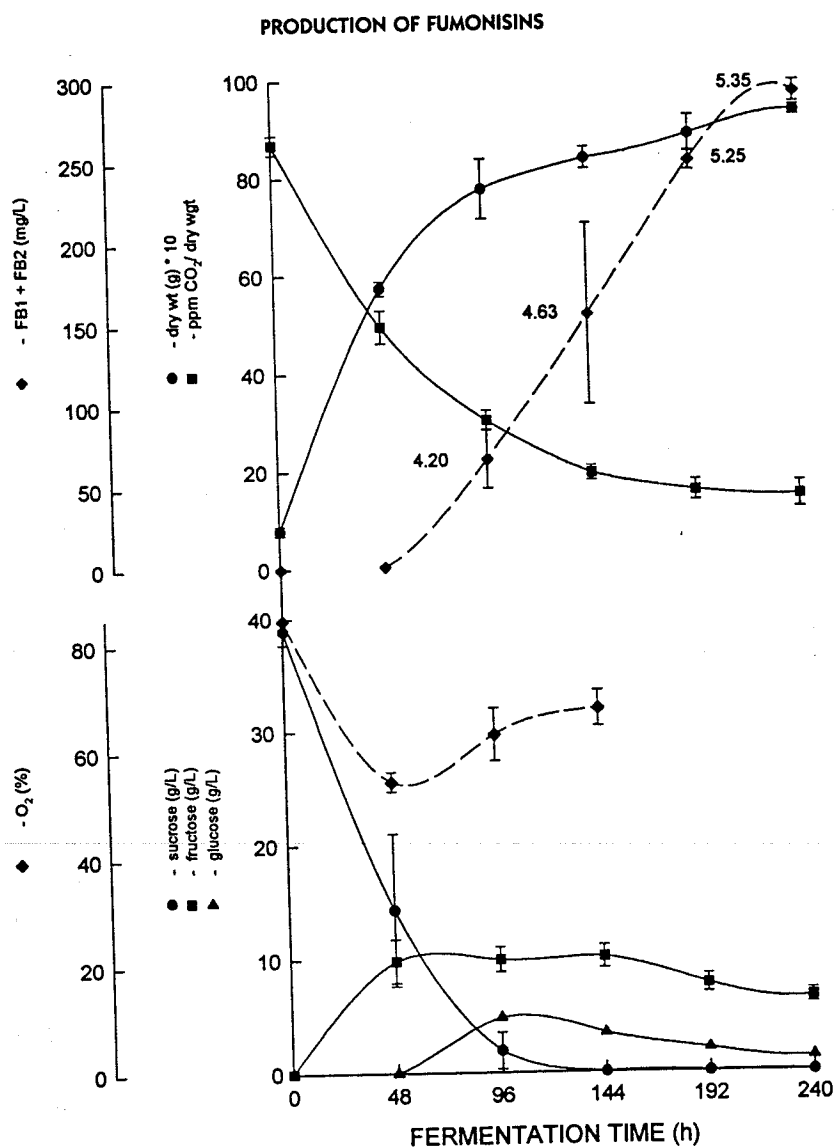


Fig. 1. Time course of fumonisin production including data on $FB_1 + FB_2$ production plus their ratio (numerical values on graph), dry weight, CO_2 production per g dry weight (top), dissolved O_2 and sucrose, fructose and glucose concentrations (bottom). Error bars represent the standard deviation as determined in triplicate analyses.

h-0.0281, 240 h-0.0312). The ratio of fumonisin B_1 to B_2 increased from ca. 4.2 to 5.4 from 48 to 240 h. The increase between each sampling was significant ($P < 0.001$). This reflects a faster decline in the rate of fumonisin B_2 production compared to B_1 . Stable isotope labelling studies of the biosynthesis of fumonisins indicated that fumonisin B_1 and B_2 are produced independently from different polyketide backbones [Blackwell et al., 1993]. These data are consistent with that view.

The results from this fermentation study indicate that fumonisins are produced under acid conditions after the cells are under nitrogen limitation but have the maximum amount of endogenous reserves. Cells in this phase can have 50–60% of their dry weight as reserves [see Martin and Demain, 1980]. The fastest rate of fumonisin production occurred

just as the sugars also became limiting, and dry weight production was greatly attenuated. The modest amount of residual fructose and glucose provide adequate amounts of ATP and reduced pyridine nucleotides for optimal metabolite production [Martin and Demain, 1980]. Fumonisins were ultimately produced at reasonable rates by cells with low respiration rates under acid conditions.

The broad features of this fermentation system are similar to those for trichothecenes (sesquiterpenes derived from mevalonic acid derived from acetate) and zearalenone [a polyketide derived from acetate; Hidy et al., 1977; Miller and Blackwell, 1986]. This further reinforces the view that the backbone of fumonisin is a polyketide. The striking difference is the apparent requirement for acid pH. Well-rotted kernels of corn infected by *F. moniliforme* are acid. Fumo-

nisins bind to various cations at neutral to alkaline pH values [Scott and Lawrence, 1993]. It appears that the natural environment where fumonisins occur has played an evolutionary role in their chemistry as well as their biology [Eschenmoser and Loewenthal, 1992; Wicklow, 1992].

Data provided by Alberts et al. [1990] on the formation of fumonisin in solid cultures appear to show a "stationary phase" during which fumonisin is produced. In their work, ergosterol was used to determine the fungal biomass present in the cultures [Schnurer, 1993]. Using the conversion factor proposed by Newell, ergosterol values were converted to fungal dry weight [see Gessner and Chauvet 1993]. The specific productivity can be estimated in the solid fermentations. For all but the first point in their fermentation where fumonisin was detected, the specific productivity values were similar. This demonstrates that the production of fumonisin was concomitant with the production of biomass. The cells comprising that biomass go through the same growth cycle outlined in the present work. This could not be detected because their measurements were taken every 14 days [as opposed to hours; see Miller and Greenhalgh, 1988]. The fact that the cells in solid cultures are not semi-synchronous provides a partial explanation for the observation that ^{14}C -methionine was not as efficiently incorporated into fumonisin as with liquid cultures [Alberts et al., 1993; Plattner and Shackelford, 1992]. The maximum apparent specific productivity value for MRC 826 grown on autoclaved corn kernels was estimated at 0.0302. This is very similar to the value in the present work.

Although the culture filtrates contained more salt and less lipid than solid cultures and required no extraction, broadly similar purification steps as for corn cultures could be employed. After filtration of the culture, four resins, XAD-2, XAD-16, SAX, and DEAE Sephadex A-25, were tested for the initial cleanup of fumonisins. Although it was originally thought that a weak anion exchange resin would not hold fumonisins, the best recovery and clean-up was obtained with the weak-anion exchange resin, DEAE Sephadex A-25. Our experience with XAD resins showed that, although they could purify fumonisins, a rather large volume of methanol was necessary for complete elution. To allow the fumonisins to bind to the DEAE column, the pH of the filtrate was brought from 2 to 6 with 1N NH_4OH before loading.

Chloride, the counter ion in new DEAE Sephadex, was too strong an anion to be displaced by fumonisins on the gel, and hydroxide led to hydrolysis of the fumonisins on the columns. These reasons and its high volatility made formate our anion of choice. The columns were thus equilibrated with 0.5 N formic acid before loading of the culture filtrate and eluted with 0.1 N formic acid. Although fumonisins do not absorb in the UV or visible ranges of light, they eluted with a brown band. Fractions of 20 ml were collected throughout the elution of this brown band from the column, and tlc analysis of the fractions usually showed fumonisins to be in the first three brown fractions.

Concentration and lyophilization of these fractions yielded a whitish/brown solid, which by NMR consisted mainly of fumonisins, with some fatty acids and other impurities. It has so far been impossible to get rid of all impurities with just one or two types of chromatography. Both silica gel and reversed-phase columns were also necessary to obtain fumonisin B₁ as a white solid. Furthermore, only reversed-phase chromatography separated the individual fumonisins.

Initial silica gel chromatography was performed with an 8:2:1 chloroform/methanol/acetic acid wash followed by a 6:3:1 elution. However, after further studies showed that acidic methanol induced esterification of the fumonisins, another solvent system was developed, an 85:15:1 mixture of acetonitrile/water/acetic acid. The presence of acid in the eluting solvent was found to be unnecessary, and the solvent was changed to 87:13 acetonitrile/water.

In the last step in the production of pure fumonisin B₁, a slow elution from a C-18 column using a stepped gradient allowed the collection of a large fraction of very pure fumonisin B₁, followed by a mixture of FB₁ and the other fumonisins. Repeated chromatography on the mixture of fumonisins eventually led to their separation.

These studies have provided information on the biology of fumonisin production by *F. moniliforme* as well as a basis for the further scale-up in larger stirred-jar fermenters.

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