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Factors affecting the growth of *Fusarium proliferatum* and the production of fumonisin B_1: oxygen and pH

SE Keller[1], TM Sullivan[1] and S Chirtel[2]

[1]*National Center for Food Safety and Technology, US Food and Drug Administration, 6502 S Archer Rd, Summit-Argo, IL 60501;* [2]*US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Division of Mathematics, 200 C Street, SW Washington, DC 20204, USA*

Fumonisins are mycotoxins produced primarily by *Fusarium moniliforme* and *Fusarium proliferatum* in corn. In liquid culture, production of fumonisin B_1 (FB_1), the most common moiety of the family of fumonisins, can be obtained using a defined medium that is nitrogen-limited. Under nitrogen-limited conditions both growth and the production of FB_1 were greatly influenced by pH and aeration. At pH above 5.0, *F. proliferatum* grew normally but produced little FB_1 (<100 μg m^{-1}). At pH below 5.0, there was less growth but substantially more FB_1. Below a pH of 2.5, both growth and metabolism were slower with very little FB_1 produced. When the optimal pH range of between 3.0 and 4.0 under well-aerated conditions was used, both growth and FB_1 production were high. However, under oxygen-limited conditions, less growth occurred, glucose consumption was increased, and no FB_1 was produced.

Keywords: *Fusarium proliferatum*; fumonisin; mycotoxin

Introduction

Fumonisins are a group of mycotoxins produced predominately by *Fusarium* species [22]. Of the seven different fumonisins which have been characterized, fumonisin B_1 (FB_1) appears to be the most prevalent [14,16]. Fumonisins have been found to cause leukoencephalomalacia (LEM) in horses and pulmonary edema syndrome (PES) in pigs [9,12,19,25,26]. In addition, the presence of fumonisins has been linked to a high incidence of esophageal cancer in humans [4,5,7,18,21]. Fumonisins were first isolated and characterized from cultures of *F. moniliforme* and subsequently shown to occur in corn world wide [6,8,14]. Recent reports have suggested fumonisins may occur naturally in a variety of food crops other than corn [23,24].

Despite the importance of its toxicity to animals and its possible role in human esophageal cancer, little information is available regarding the production of fumonisins. *Fusarium* species are ubiquitous on corn and yet, even in visibly moldy corn, fumonisins are not always present. In contrast, visibly healthy corn may contain high levels of fumonisins [2]. Most studies on the production of fumonisins have been conducted with solid corn-based media [17]. Important factors related to production on solid media were reported to be temperature, with an optimal temperature range of 20–25°C [1,13], moisture, and aeration. Marin *et al* [15] found that increased water activity resulted in both increased growth and fumonisin production. Although Nelson *et al* [17] indicated good aeration to be an important factor in the production of FB_1, no data were provided concerning the effect of aeration. In contrast, Blackwell *et al* [3] indicated a low oxygen tension was required for optimal FB_1 production in liquid culture since higher levels of FB_1 were produced by increasing inoculum size or volume of media in flasks. Blackwell *et al* [3] also indicated that a pH below 3.0 was required for optimal production. Fumonisin production may be controlled in part by nitrogen levels. FB_1 was successfully produced in stirred bioreactors using nitrogen-limited medium [11]. This production was not affected by either high phosphate or high glucose levels. Variable aeration rates and pH were not examined. In the current study, the effects of aeration and pH were examined under conditions previously shown to result in high levels of FB_1 production.

Correspondence: SE Keller, FDA, National Center for Food Safety and Technology, 6502 S Archer Rd, Summit-Argo, IL 60501, USA
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Materials and methods

Defined media used for growth of *Fusarium* and production of fumonisin in shake flasks were modified from that of Jackson and Bennett [10] as follows: 90 g L^{-1} glucose, 2.0 g L^{-1} glycine, 1.0 g L^{-1} KH_2PO_4, 1.0 g L^{-1} K_2HPO_4, 0.3 g L^{-1} $MgSO_4$, 0.4 g L^{-1} $CaCl_2$, 16 mg L^{-1} $MnSO_4$, 32 mg ml^{-1} $ZnSO_4$ and 100 mg ml^{-1} $FeSO_4$ with vitamins filter sterilized and added after autoclaving as follows: 1 mg L^{-1} each of thiamine, riboflavin, pantothenoate, niacin, pyridoxamine, thiotic acid and 100 μg L^{-1} each of folic acid, biotin, and B_{12}. Initial pH was adjusted as required with 1 N HCl. For growth and production in stirred bioreactors 3.5 g $L^{-1}(NH_4)_2SO_4$ was used in place of glycine, and the pH was controlled throughout the fermentation by the addition of 1 N HCl. In both shake flasks and bioreactors, a fed-batch method was used. Glucose levels were maintained above 20 g L^{-1} by adding sterile glucose (50% w/w, in H_2O). In shake flask studies, volumes added to each flask were equalized by the addition of sterile H_2O to those not requiring additional glucose.

Fusarium proliferatum M5991 was originally obtained from the Fusarium Research Center at Pennsylvania State University. Frozen stock cultures were inoculated into 100 ml of defined medium in baffled flasks, placed at 25°C

on a rotary shaker set at 200 rpm and incubated for 5–7 days. These cultures were then used as inoculum for growth and production studies. For growth and production studies in shake flasks, a 1% inoculum was used in 100 ml of defined medium in a 500-ml baffled flask. For growth and production studies in bioreactors, a 5–10% inoculum was used. Shake flask grown culture was used to inoculate 2-L glass stirred-jar bioreactors (Braun Biotech, Allentown, PA, USA). The culture from the 2-liter glass stirred-jar bioreactors was then used to inoculate 10-L stainless steel stirred bioreactors (Braun Biotech) in which the growth studies took place.

Cell mass of cultures was determined by filtering 1 ml of culture through a pre-weighed 0.45-μm filter (Gelman Science, Ann Arbor, MI, USA) and washing with approximately 3 ml H_2O. The filter was then air dried overnight and weighed as an indication of growth.

Glycine concentration was determined by using ninhydrin reagent (Sigma, St Louis, MO, USA). Standards were prepared from 2.5–10 μg ml^{-1} glycine. For each assay, 0.1 ml reagent was added to 0.1 ml standard or culture filtrate, mixed and then boiled for 5 min. After heating, the reaction mixture was diluted to 5 ml with deionized H_2O and the absorbance read at 570 nm. The glycine was quantified by comparison to a standard curve.

For analysis of FB_1, cultures were first filtered using a 0.45-μm syringe filter (Gelman Science) to remove cells. No other purification of broth samples was required prior to analysis. FB_1 standard was kindly provided by Dr R Eppley, FDA, CFSAN, Washington, DC. Fumonisin concentrations were determined using the HPLC method of Shephard *et al* [20].

All statistical analyses were performed using SAS version 6.11 (Cary, NC, USA). The data in Table 1 were analyzed using Proc Mixed. The Satterwaite option was used with the glucose and FB_1 analysis in order to adjust for variance heterogeneity betwen pH groups. Dry weight was analyzed assuming a constant error variance. Individual group means were compared using two-tailed 1sd-*t*-tests where the overall *P*-values were less than 0.05. The data in Tables 2 and 3 were analyzed using two-tailed *t*-tests using Proc T-test with the Satterwaite adjustment for unequal variance. All comparisons were significant at the $P<0.05$ level.

Table 1 Effect of initial pH on FB_1 production and growth in shake flasks at 20 days

Initial pH	Glucose consumed (g L^{-1})	Max. dry wt. (g L^{-1})	FB_1 (ppm)
2.2	27.5 ±1.2[a]	11.7 ± 2.7[a]	9.4 ± 4.5[a]
2.6	39.2 ± 2.6[b]	11.1 ± 1.1[a]	33.3 ± 10.2[b]
3.0	44.7 ± 13.2[a,b]	12.0 ± 2.6[a]	261.6 ± 338.1[a,b,c]
3.7	89.3 ± 6.4[c]	13.8 ± 1.4[a]	436.7 ± 118.0[c]
4.2	90.1 ± 9.7[c]	16.7 ± 1.6[b]	432.3 ± 66.9[c]
5.6	94.1 ± 9.8[c]	24.4 ± 2.0[c]	16.9 ± 9.2[a,b]

[a,b,c]Groups within a column not sharing a common letter are different at the $P<0.05$ level.

Table 2 Effect of aeration on growth of *F. proliferatum* and FB_1 production in shake flasks at 20 days. Initial pH was adjusted to 3.5. Initial glycine concentration was 2.0 g L^{-1}

	Glucose consumed (g L^{-1})	Max. dry wt. (g L^{-1})	FB_1 (ppm)
Baffled 500-ml flasks	90.2 ± 4.2[a]	13.2 ± 0.6[a]	201 ± 135[a]
Un-baffled 500-ml flasks	121 ± 9.2[b]	11.2 ± 1.1[b]	10.5 ± 2.3[b]

[a,b]Groups within a column not sharing a common letter are different at the $P<0.05$ level.

Table 3 Effect of restricted oxygen on growth of *F. proliferatum* and FB_1 production in shake flasks at 20 days

	Glucose consumed (g L^{-1})	Max. dry wt. (g L^{-1})	FB_1 (ppm)
Un-restricted oxygen shake flasks	68.0 ± 6.4[a]	14.1 ± 0.5[a]	533 ± 88.4[*]
Restricted oxygen shake flasks	100.9 ± 11.8[b]	4.3 ± 0.3[b]	ND

[a,b]Groups within a column not sharing a common letter are different at the $P<0.05$ level.
[*]FB_1 was significantly greater than 0 at the $P<0.05$ level.
ND = none detected.

Results and discussion

The pH effects on culture growth and production were examined under shake flask conditions, with three flasks at each pH. Glycine was used as the nitrogen source to avoid the extreme drop in pH that occurs when $(NH_4)_2SO_4$ is used in shake flasks. With glycine as the nitrogen source, some drop in pH also occurs as shown in Figure 1. The changes

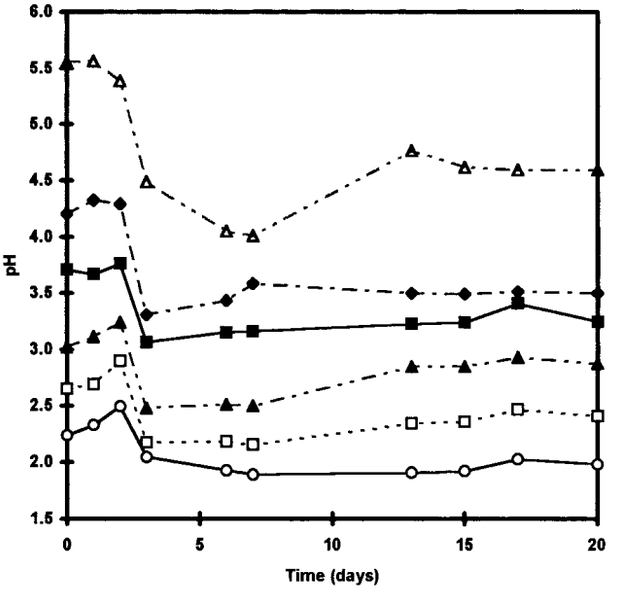

Figure 1 Changes in pH during growth of *F. proliferatum* in medium with variable initial pH. Initial pH as indicated. ○ pH = 2.2, □ pH = 2.6, ▲ pH = 3.0, ■ pH = 3.7, ◆ pH = 4.2, △ pH = 5.6.

that occurred were larger at higher initial pH values, but still small enough to allow substantial differences in the stationary phase pH values. As a result, it was possible to evaluate the most appropriate pH range for growth and toxin production by *F. proliferatum* regardless of the initial pH set. Figure 2 follows the disappearance of glycine over time at each pH tested. Lower pH ranges clearly slowed the rate of disappearance of glycine. However, with the exception of the lowest pH tested (initial pH 2.2), all glycine was removed after 3 days. At pH 2.2 glycine was gone after 5 days.

Table 1 shows the final estimated dry weights, glucose used, and FB_1 accumulated after 20 days at each initial pH tested. Cell mass accumulation, as indicated by dry weight estimates, is greatest at the highest initial pH tested, and decreases with decreasing initial pH. However, although growth and metabolism may be faster and more extensive at an initial pH above 4.0, optimal FB_1 production apparently requires a pH of less than 4.0. The largest amounts of FB_1 were produced when the initial pH of the flasks was adjusted to 4.2 or 3.7. At both of these initial conditions, pH dropped after the first 3 days and stabilized between pH 3.0 and 3.5. Greater variability was produced in flasks starting at pH 3.0. At 3.0, two out of three flasks produced almost no FB_1 whereas the third flask produced over 1000 ppm. It is possible that a sharp break point exists close to pH 3.0. below which FB_1 will not be produced. Flasks with an initial pH of 2.6 produced very little FB_1.

The effect of aeration on the growth of *F. proliferatum* and the production of FB_1 was examined using two different types of experiments with shake flasks. In the first experiment, aeration was modified in shake flasks by using both baffled and unbaffled 500-ml flasks. Five replicates of each type of flask were used and each flask was sampled once each day to determine dry weight, glucose concentration, and FB_1 accumulation. The results of this experiment are shown in Table 2. The flasks with more aeration (baffled) averaged higher dry weight estimates and higher FB_1 produced than did those with less aeration (unbaffled). However, the total amount of glucose used by the culture grown in the unbaffled flasks was higher than that used by the cultures in the baffled flasks.

In the second experiment, aeration was restricted by covering the cap of 500-ml baffled flasks with a layer of parafilm. Controls had no parafilm cover. Each flask was sampled daily. For those flasks with parafilm covering the caps, the parafilm was removed, the culture was sampled and the parafilm replaced prior to returning the culture to the incubator. The results of this experiment are shown in Table 3. The dry weight and FB_1 levels were higher with unrestricted aeration than when aeration was restricted. Also, glucose use was substantially higher in cultures with less aeration despite the lower biomass accumulation.

The effect of aeration on the growth of *F. proliferatum* and production of FB_1 was confirmed by growing cultures in 'twin' 10-L Braun bioreactors (pH 3.5, 25°C, 500 rpm, identical media, identical inoculum) with air flow rates set at 0.5 and 5.0 standard liters per minute (SLPM). The results are shown in Figures 3–6. As with the shake flask studies, biomass accumulation and FB_1 production were higher with higher air flow (Figures 3 and 4). Also as in shake flask studies, glucose utilizatiion was greater with lower air flow rates (Figure 5). Glucose utilization rates were determined by determining the amount of glucose used per day divided by the biomass measured as dry weight.

Use of the bioreactors allowed measurement of oxygen levels in the tanks as the culture developed (Figure 6). From the dissolved oxygen levels in the two tanks, greatest oxygen demand occurred in the first 2 days of growth. After the first 2 days, nitrogen was exhausted and biomass accumulation slowed. At high air flow rates, the dissolved oxygen dropped but quickly returned to approximately 80% after the growth phase, then began a slow decline to

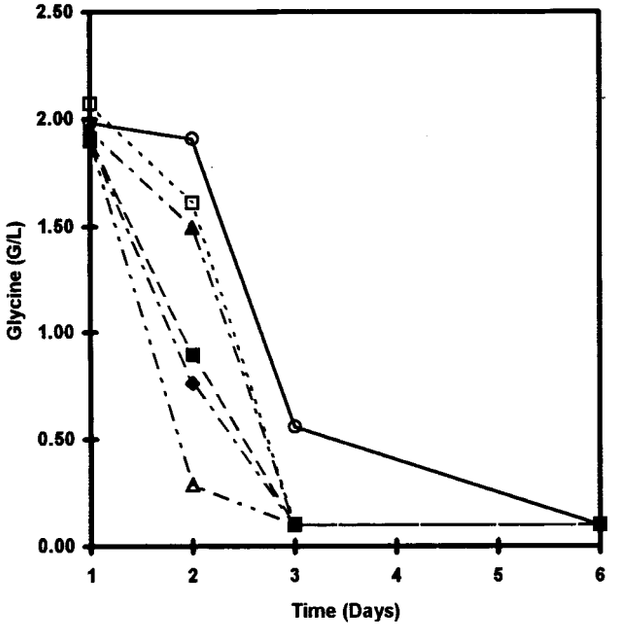

Figure 2 Glycine concentration in medium with variable initial pH. Initial pH as indicated. ○ pH = 2.2, □ pH = 2.6, ▲ pH = 3.0, ■ pH = 3.7, ▲ pH = 3.0, ■ pH = 3.7, ◆ pH = 4.2, △ pH = 5.6.

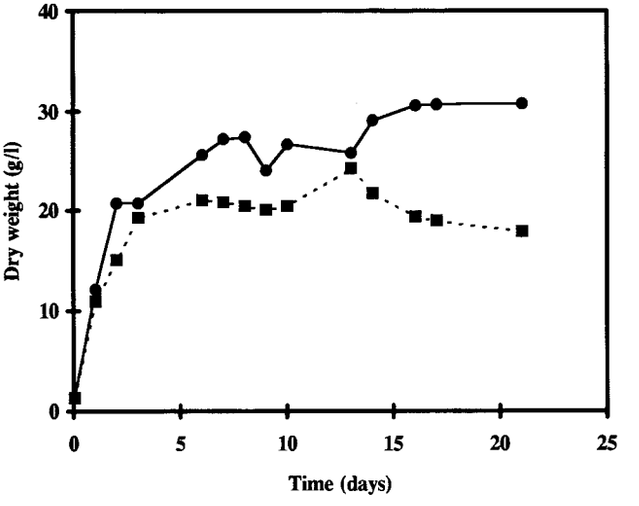

Figure 3 Biomass accumulation in bioreactors with two different air flow rates. —●— 5.0 SLPM, ----■---- 0.5 SLPM.

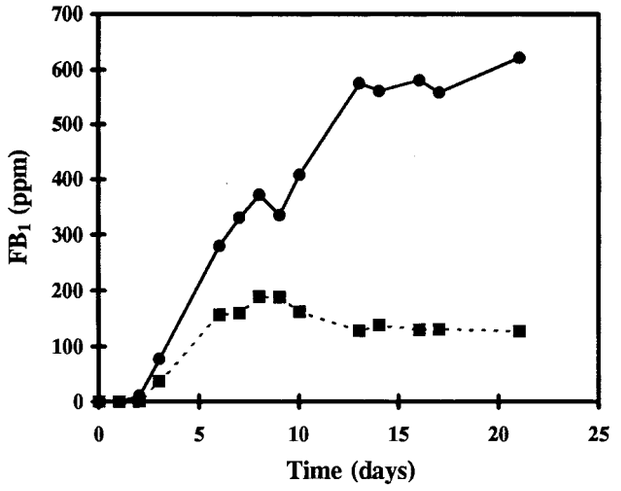

Figure 4 Fumonisin concentration in bioreactors with two different air flow rates. —●— 5.0 SLPM, ----■---- 0.5 SLPM.

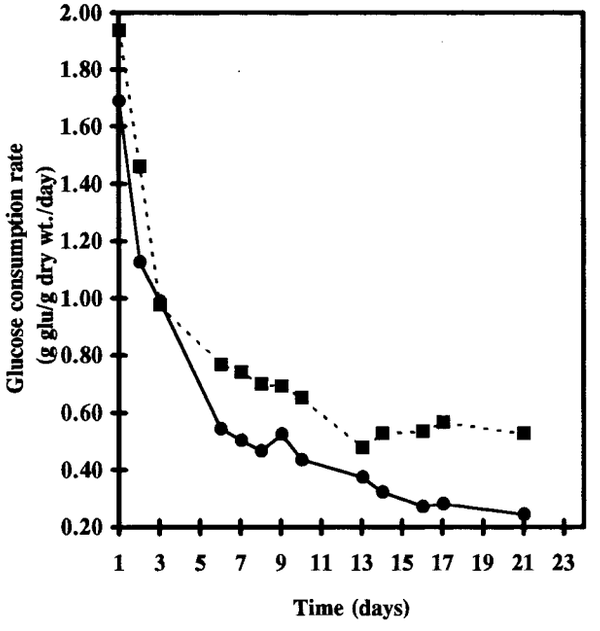

Figure 5 Rate of glucose consumption bioreactors with two different air flow rates. —●— 5.0 SLPM, ----■---- 0.5 SLPM.

approximately 50% after 20 days. In the low air flow tank, the initial dissolved oxygen dropped during growth but showed a slower increase to approximately 55%, after which the dissolved oxygen continued to increase slowly to approximately 80% after 20 days.

Since both the high and low air flow bioreactors began with the same nutrient content and inoculum, the differences observed in biomass, glucose consumption rates, and FB_1 produced would appear to be the result of oxygen supplied to the culture. Since dissolved oxygen levels after approximately 8 days were similar in both bioreactors, the critical period would appear to be during the log phase of growth where oxygen demand is highest. Examination of other successful bioreactor experiments in which cultures were produced in excess of 1000 ppm FB_1 in as little as 15

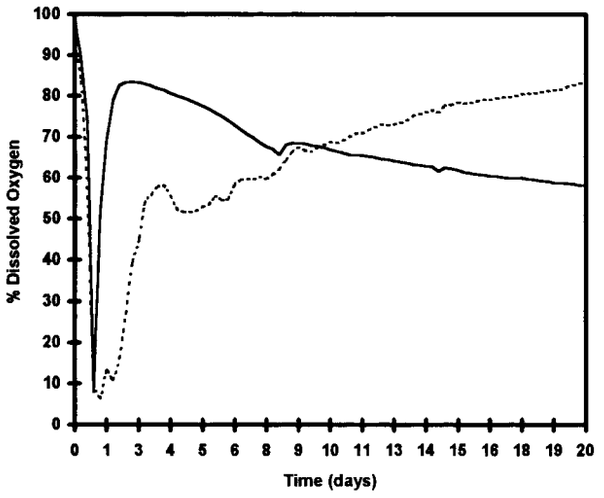

Figure 6 Oxygen level in bioreactors with two different air flow rates. —— 5.0 SLPM, -------- 0.5 SLPM concentration.

days, shows high levels of dissolved oxygen during the log growth phase (Figure 7). In this example, dissolved oxygen remained above 40% during the entire fermentation.

Conclusion

In this study, the effects of both pH and aeration have been examined. Both factors appear to have a profound effect on growth and the production of fumonisin. The effects of pH appear to coincide somewhat with that found by Blackwell *et al* [3] in that a pH lower than 4.0 appears to be required for good fumonisin production. Here, we have determined the best pH range for FB_1 production to be between pH 3.0 and 4.0. Within this pH range FB_1 production can exceed 1000 ppm provided there is sufficient aeration during the early growth period. The effect of oxygen on fumonisin production supports observations made on solid media [17]. The amount of aeration provided during shake flask growth studies affected not only FB_1 production, but the total amount of cell mass accumulated and the amount of glucose consumed. The increase in glucose consumed in cultures with reduced aeration indicates

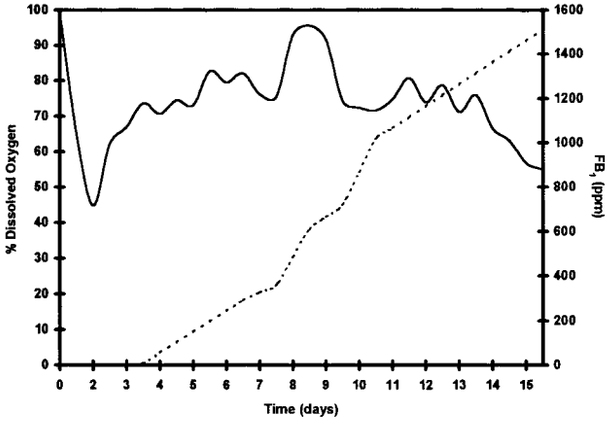

Figure 7 FB_1 production under optimal bioreactor conditions. —— Dissolved oxygen, -------- FB_1 concentration.

metabolism is altered but not stopped with reduced oxygen levels. This observation is consistent with changes attributed to the Pasteur effect. Under limited oxygen conditions, it is possible that respiration is limited and glucose catabolism via glycolysis is increased. This shift would result in less ATP produced, alter energy charge within the cell and consequently effect enzyme activity. The increase in glucose consumption is less likely to be a result of the Crabtree effect since glucose levels were the same in both well oxygenated and oxygen-limited conditions. In any case, as the culture reached stationary phase, and production of FB_1 progressed, oxygen levels do not appear as important. This conclusion is supported by studies in bioreactors, where measured oxygen levels increase during stationary phase yet FB_1 production remains unchanged. This clearly indicates the importance of the early stages of the fermentation to its final outcome.

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