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Effect of Zinc, Copper, and Iron on Ochratoxin A Production

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The effect of zinc, copper, and iron levels on production of ochratoxin A by *Aspergillus ochraceus* Wilhelm in a synthetic medium in a shake culture was investigated. Optimal concentrations of $ZnSO_4$, $CuSO_4$, and $FeCl_3$ for ochratoxin A production were 0.055 to 2.2 mg/liter, 0.004 to 0.04 mg/liter, and 1.2 to 24 mg/liter, respectively. Zinc and copper levels greater than optimum reduced the rate of ochratoxin accumulation without altering either glutamate or sucrose utilization. Ochratoxin A production was correlated with rapid utilization of sucrose by the fungus and decreasing pH of the medium. Most of the glutamic acid was removed from the medium prior to ochratoxin production. There was no correlation between mycelial dry weight and ochratoxin A production.

Seven species of the *Aspergillus ochraceus* group have been found to produce ochratoxins A and B (7). Research on the production of ochratoxin A in synthetic media has been reported by several investigators (2, 3, 5, 6), but only Lai et al. (8) studied the effect of trace elements. Maximum yields of ochratoxin A (100 mg/liter) were obtained by Ferreira (5, 6), whereas Lai et al. (8) obtained 98 mg of ochratoxin per liter on a different synthetic medium. This paper reports the effect of zinc, copper, and iron on ochratoxin A production by *A. ochraceus* when grown in a shake culture in a medium similar to that used by Ferreira (5, 6).

The culture used throughout this investigation was *A. ochraceus* NRRL 3174 obtained from C. W. Hesseltine, Northern Regional Research Laboratory, USDA, Peoria, Ill. Cultures were maintained at 5 C on Czapek agar with 20% sucrose supplemented with 0.7% yeast extract (Difco). Flasks (125 ml) containing 50 ml of medium were stoppered with foam plugs and autoclaved for 15 min at 121 C. Each culture was inoculated with 1 ml of a spore suspension (1×10^7 spores/ml). Flasks were shaken in a model G-25 gyratory incubator shaker (New Brunswick Scientific) at 28 C at 150 counts/min through a 3-inch (approximately 7.5-cm) circle. The medium was that of Ferreira (5) supplemented with 0.3% L-glutamic acid. Sucrose was purified by the aluminum oxide method of Donald et al. (4); L-glutamic acid and mineral salts were purified by repeated recrystallization from glass-distilled water. Concentrations of $ZnSO_4$, $CuSO_4$, and $FeCl_3$ were varied in the experiments.

After the final pH of the medium was determined, the cultures were filtered, mycelial pellets were dried for 12 h at 70 C, and the mycelial weights were measured. The mycelial pellets were stirred into 50 ml of chloroform for 10 min, and the chloroform was collected by filtration. Culture filtrates were adjusted to 50-ml volumes. Five ml of chloroform extract and 5 ml of culture filtrate were combined with 50 ml of chloroform in a 500-ml separatory funnel and shaken together for 1 min. The chloroform was evaporated to dryness over a steam bath. Ochratoxin A was then assayed as previously described (3).

Glutamic acid was determined by the ninhydrin method of Clark (1), and sucrose was determined by the anthrone method of Morse (10) as modified by Loewus (9).

Effects of various concentrations of $CuSO_4$, $ZnSO_4$, and $FeCl_3$ on ochratoxin production, mycelial dry weight, and final medium pH were evaluated by incubating the several treatments for 7 days at 28 C and by other experiments in which cultures were assayed periodically over an 8-day period. Flasks containing media not supplemented with zinc, copper, or iron were inoculated and assayed with each experiment as a check on the efficiency of the purification procedures. Experiments were run in duplicate.

The effect of $ZnSO_4$ concentrations on ochratoxin A production by *A. ochraceus* after incubation at 28 C in shaken culture for 7 days

is shown in Fig. 1. Maximal ochratoxin A production (4 mg/50 ml) was obtained with 1.1 mg of $ZnSO_4$/liter. Concentrations of $ZnSO_4$ greater than 2.2 mg/liter markedly reduced ochratoxin A production. Mycelial dry weight was essentially constant, but 110 mg of $ZnSO_4$ per liter resulted in 35 to 40% greater mycelial growth. Final pH increased gradually from 6.3 to 7.2 as the $ZnSO_4$ concentration in the medium increased from 0 to 110 mg/liter. Little growth of *A. ochraceus* occurred in a medium with $ZnSO_4$ omitted.

The effect of $CuSO_4$ concentration on ochratoxin A production by *A. ochraceus* is shown in Fig. 2. Maximal production (2 mg/50 ml) was obtained with 0.04 to 0.2 mg of $CuSO_4$ per liter. Concentrations of 0.39 to 20 mg/liter reduced ochratoxin production to 1 mg/50 ml of medium. Mycelial dry weight was constant at $CuSO_4$ concentrations of 0.04 to 2.0 mg/liter, but decreased about 25% at 3.9 to 20 mg/liter levels. There was little change in medium pH values ranging from 6.6 to 7.0 with increased $CuSO_4$. Medium with no copper added produced about one-third the mycelial growth of media containing copper.

Maximal ochratoxin production (2 to 2.5 mg/50 ml) in $FeCl_3$ treatments was obtained in the range of 1.2 to 24 mg/liter (Fig. 3). Mycelial dry weight was constant at $FeCl_3$ concentrations of 0.24 to 24 mg/liter. At a $FeCl_3$ concentration of 120 mg/liter, a precipitate formed when the medium was autoclaved; ochratoxin A production was only 0.1 mg/liter, and mycelial dry weight was reduced about 10%. Final pH was essentially constant near 7.0 ± 0.3, with the highest pH at 12 mg of $FeCl_3$ per liter. Cultures in a medium with $FeCl_3$ omitted produced little growth and no ochratoxin A.

The relation of time to ochratoxin A production by *A. ochraceus* was investigated at three concentrations of $ZnSO_4$. Maximal ochratoxin (7.5 mg/50 ml) was obtained in 5 days with $ZnSO_4$ concentrations of 0.55 and 2.2 mg/liter, whereas ochratoxin A production was inhibited by a $ZnSO_4$ concentration of 22 mg/liter. Production remained relatively constant between 5 and 8 days. Glutamic acid was rapidly removed from the medium by *A. ochraceus* during the first 3 days after inoculation, and sucrose was most rapidly utilized by the fungus between 3.5 and 5 days after inoculation. Rates of utilization

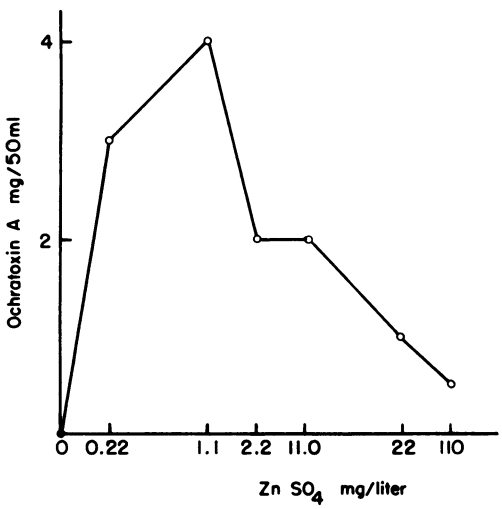

FIG. 1. *Effect of $ZnSO_4$ concentration on ochratoxin A production by Aspergillus ochraceus in a shaken culture at 28 C for 7 days.*

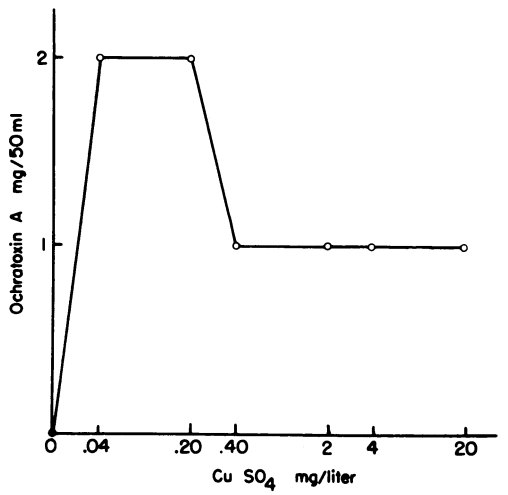

FIG. 2. *Effect of $CuSO_4$ concentration on ochratoxin A production by Aspergillus ochraceus in a shaken culture at 28 C for 7 days.*

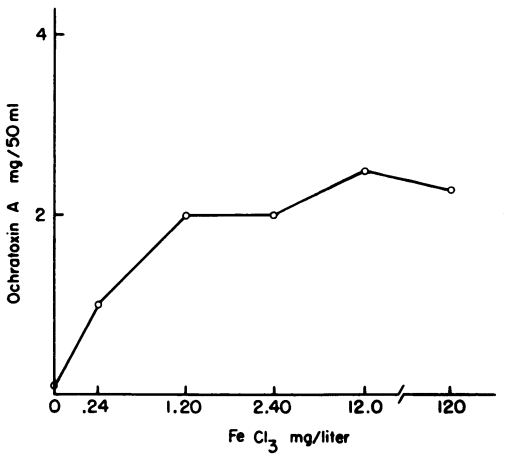

FIG. 3. *Effect of $FeCl_3$ concentration on ochratoxin A production by Aspergillus ochraceus in a shaken culture at 28 C for 7 days.*

of glutamic acid and sucrose were similar at the several $ZnSO_4$ concentrations tested.

In the $CuSO_4$ tests, maximal ochratoxin production was obtained in 6 days with 0.04 mg of $CuSO_4$ per liter; higher levels in the medium (0.4 to 4.0 mg/liter) reduced ochratoxin A production. As in $ZnSO_4$ treatments, glutamic acid was removed rapidly from the medium by *A. ochraceus* during the first 3 days after inoculation, whereas sucrose was most rapidly utilized between 3.5 and 5 days after inoculation. Rates of glutamic acid and sucrose utilization were similar at the three concentrations of $CuSO_4$ investigated.

The effect of three concentrations of $FeCl_3$ (0.12, 1.2, and 12.0 mg/liter) on ochratoxin A production by *A. ochraceus* in relation to time was also investigated. No ochratoxin A was produced in a medium with 0.12 mg of $FeCl_3$ per liter. Levels of 1.2 and 12 mg of $FeCl_3$ per liter resulted in ochratoxin yields of 0.5, 1.5, and 2.5 mg/50 ml after 3.5, 4, and 6 days of incubation, respectively. Mycelial dry weight increased about 10 to 20% with time after 6 days in comparison with mycelial weights after 3.5 and 4 days. No ochratoxin and very little mycelial growth occurred in check cultures without $FeCl_3$.

The optimal concentration of $ZnSO_4$ for ochratoxin A production by *A. ochraceus* NRRL 3174 was between 0.55 and 2.2 mg/liter, whereas the optimal concentration of $CuSO_4$ appeared to be between 0.004 and 0.04 mg/liter. Higher concentrations of zinc (22 to 110 mg/liter) or copper (0.4 mg/liter) reduced ochratoxin A production. Thus, the $ZnSO_4$ and $CuSO_4$ concentrations of 21.90 and 3.93 mg/liter, respectively, used by Ferreira (5, 6) may have been far from optimal for ochratoxin A production and possibly reduced toxin production. The $ZnSO_4$ and $CuSO_4$ concentrations of 17.6 and 0.3 mg/liter, respectively, of Lai et al. (8) were lower, but also were outside of the optimal range for ochratoxin production.

Concentrations of zinc and copper optimal for ochratoxin A production did not result in alterations of either sucrose or glutamic acid utilization from those obtained at concentrations of zinc and copper that inhibited ochratoxin production. Nor was mycelial growth of the fungus different at optimal or inhibitory concentrations of zinc and copper. Thus, the efficiency of carbohydrate utilization was the same at optimal and inhibitory concentrations of zinc and copper, and this mechanism does not seem to provide an explanation for the inhibition of ochratoxin production by high concentrations of zinc and copper. It appeared that high levels of zinc and copper inhibited the final concentration of ochratoxin A by reducing the net rate of its accumulation.

Ochratoxin A was produced during a period of rapid sucrose utilization and after more than 98% of the glutamic acid had been removed from the medium. This indicates that glutamate exerts its stimulatory effect on ochratoxin A production indirectly.

The optimal concentration of $FeCl_3$ for ochratoxin A production, as determined in this investigation, was 1.2 to 2.4 mg/liter; this range includes the $FeCl_3$ concentrations utilized by Ferreira (5, 6) and Lai et al. (8) in their studies. The iron requirement for maximal ochratoxin A production (12 mg of $FeCl_3$) was greater than that required for maximal mycelial growth (1.2 mg/liter). The maximal concentration of iron that could be incorporated into the medium without precipitates being formed (24 mg/liter of $FeCl_3$) was within the optimal concentration range for ochratoxin A production.

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