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Medium-Scale Production of Citrinin by *Penicillium citrinum* in a Semisynthetic Medium

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A convenient method is described for the production of up to 1.75 g of citrinin per liter by *Penicillium citrinum* growing in stationary culture in a 5-gallon (18.925 liters) carboy containing 4 liters of 4% sucrose and 2% yeast extract medium.

In 1951 *P. citrinum* was isolated from yellowed rice imported from Southeast Asia to Japan. Saito et al. (12) reported that in 3-week experiments with rats fed *P. citrinum*-contaminated rice Tsunoda (16) found enlarged kidneys to be the characteristic lesions caused by this fungus, which has been frequently isolated from peanuts (3), corn (9), rice (12), and other raw and processed agricultural commodities. Citrinin was first isolated by Hetherington and Raistrick (5) in 1931 from a culture filtrate of *Penicillium citrinum* Thom. It has since been produced by 10 or more species of *Penicillium* and *Aspergillus*, and is regarded as a potentially important mycotoxin that may be ingested by man and animals as a contaminant of wheat, barley, rye, and oats (4, 7, 13).

Hetherington and Raistrick (5) obtained yields of 525 to 700 mg of citrinin per 350 ml of medium with *P. citrinum* Ad. 23 in 12 to 16 days at 28 to 32 C. Timonin and Rouatt (15) obtained 500 to 700 mg of citrinin per 200 ml of mineral salts medium with *Aspergillus candidus* incubated for 20 days at 26 C. Highest yields of citrinin were produced by *A. candidus* on media containing honey (15) or maple syrup (17) rather than glucose as the carbon source. In 1966 Rodig (11) produced the equivalent of 2.6 g of citrinin per liter in 14 to 16 days at 40 C with *Aspergillus niveus* and 1.2 g of citrinin in 35 to 40 days at room temperature with *P. citrinum* using Timonin's medium containing twice as much glucose.

This paper reports production of citrinin in 5-gallon (18.925 liter) carboys containing 4 liters of 4% sucrose and 2% yeast extract nutrient solution. This medium was previously described for ochratoxin A production by *A. ochraceus* (1, 2). A strain of *P. citrinum*, isolated from corn associated with toxicity in horses in Alabama, proved to be an efficient producer of citrinin. Subsequently, we found that Timonin's isolate of *A. candidus* deposited in a culture collection had lost its ability to produce citrinin and he no longer had a viable culture (personal communication with M. I. Timonin). Increased demand for citrinin for mycotoxicological investigations made it desirable to investigate the conditions for production of the toxin by this high-yielding strain of *P. citrinum*.

P. citrinum AUA 532, used throughout this investigation, was identified by D. I. Fennell of the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., and accessioned there as NRRL 5927. After sporulation, cultures were stored at 4 to 5 C on slants of agar containing 2% dextrose, 0.7% yeast extract (Difco), 0.5% KH_2PO_4, and 2% agar, and also as lyophilized cultures stored at −30 C. Wide-mouthed, 5-gallon Pyrex carboys containing 4 liters of 4% sucrose and 2% yeast extract nutrient solution were stoppered with cotton plugs and autoclaved for 30 min at 121 C (2). These were inoculated with a suspension of spores from 10- to 14-day-old cultures of *P. citrinum* and incubated at room temperature (30 ± 3 C) as stationary cultures. Carboys were placed on their sides to give maximal surface-area-to-volume ratio. Two experiments with seven carboys per experiment were conducted with yields being measured as crude citrinin produced after 8, 10, 12, 14, 16, 18, and 21 days of incubation. A third experiment involved 10 carboys with data being taken on the above days and also after 25, 27, and 31 days of incubation. Streptomycin sulfate (0.2 g/liter) was added to the medium in the latter experiment to protect against bacterial contamination.

The procedure for extraction and isolation of citrinin was essentially as described by Hetherington and Raistrick (5). The mycelium and

culture solution were filtered through Whatman no. 1 filter paper and the filtrate was acidified to pH 1.5 by stirring in approximately 50 ml of concentrated HCl per 4 liters of medium to precipitate the crude citrinin. After precipitation was complete, the bright yellow mass of crude citrinin was filtered onto Whatman no. 42 filter paper, dried in vacuo at 50 C, weighed, and reported as total crude citrinin per liter of medium. The crude citrinin was dissolved in a minimal amount (150 ml) of chloroform and filtered to remove nonchloroform soluble impurities. The chloroform solution was evaporated to dryness, and the residue was taken up in hot ethanol, filtered, and recrystalized three times by the hot alcohol method of Hetherington and Raistrick (5). Comparison of the chemical and physical characteristics of the purified material with the ultraviolet, infrared, mass, and nuclear magnetic resonance spectra, melting point, and optical rotary power of citrinin as reported in the literature (6, 8, 10, 12) confirmed identification of the compound.

Production of crude citrinin by *P. citrinum* in sucrose-yeast extract medium for varying periods of incubation is illustrated by the regression curve in Fig. 1 (data for three experiments were averaged and the curve plotted by quadratic regression). Citrinin was detected on day 8 with the highest yield recorded on day 21. The addition of streptomycin sulfate to the medium in the third trial had no apparent effect on citrinin production. As much as 7.3 g of crude citrinin was produced per carboy (4 liters of medium). Yields seemed to plateau and did not decrease thereafter. Apparently citrinin is not reabsorbed and further metabolized. Thus, production runs may be terminated at the convenience of the researcher rather than according to a predetermined or rigid time schedule. Surprisingly, yields were comparable to those scaled up (by calculation) from smaller systems (5, 11, 15, 17) rather than diminished as often occurs when small systems are scaled up. About 45 to 50% of the crude citrinin was recoverable in pure form by the method used (5). However, Timonin and Rouatt (15) reported that they recovered 70 to 80% of the crude product as pure crystalline citrinin using the dioxane method of Tauber et al. (14). Timonin and Rouatt (15) conducted a very thorough study on factors influencing citrinin production by *A. candidus*. Results of their investigation on *A. candidus* seem directly applicable to citrinin production by our *P. citrinum* isolate AUA-532. This isolate is maintained by lyophil in our culture collection and is available for distribution. Small amounts of citrinin are also available as qualitative standards, or in larger amounts where circumstances warrant.

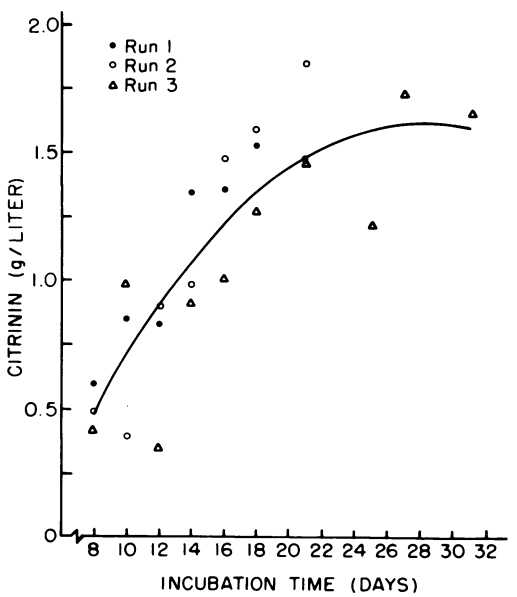

FIG. 1. *Production of total crude citrinin by P. citrinum growing in 4 liters of 4% sucrose-2% yeast extract medium at 30 C.*

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