Environmental and Nutritional Factors Affecting the Production of Rubratoxin B by Penicillium rubrum Stoll

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Rubratoxin B can be produced in a semisynthetic medium by Penicillium rubrum under varying environmental and nutritional conditions. Maximum production (552.0 mg/500 ml) was obtained with P. rubrum NRRL A-11785 grown in stationary cultures of Mosseray's simplified Raulin solution supplemented with 2.5% malt extract broth at ambient temperature. Zinc is required at levels of at least 0.4 mg per liter. In the absence of iron sulfate, there was a 50-fold reduction in rubratoxin B production but not in growth. No toxin was produced by this isolate in 5- or 7-liter fermentors.

Research on the mycotoxins has been extensively reviewed since the initial report in 1960 of "Turkey X" disease being caused by a toxin produced by Aspergillus flavus growing on peanuts (4, 18). However, few reports dealing with mycotoxins other than the aflatoxins have been published. The number of confirmed mycotoxicoses for which the responsible fungus has been identified and its toxin characterized is increasing. This paper deals with rubratoxin B, one of the toxic mold metabolites which has not received widespread publication.

The rubratoxins are secondary metabolites produced by Penicillium rubrum Stoll and P. purpurogenum Stoll and have been associated with toxicoses caused by moldy animal feeds (13, 15). Burnside et al. (1) originally isolated P. rubrum from feedstuffs involved in a mycotoxicosis occurring in the southeastern United States. Shortly after this investigation, Forgacs et al. (3) fed grain infected with P. rubrum and P. purpurogenum to chicks and observed a hemorrhagic syndrome. Wilson and Wilson (16) were able to extract an acidic material from corn cultures of P. rubrum P-13 (the original isolate of Burnside) that produced toxic hepatitis in laboratory animals similar to that seen by Burnside. Townsend et al. (14) isolated two toxic components, designated rubratoxin A and B, from another isolate of P. rubrum, MR 043, when grown in Raulin-Thom medium enriched with 2.5% malt extract. Hayes and Wilson (5) developed a method for extraction of large quantities of crystalline rubratoxin B from liquid cultures. The structures of rubratoxin A and B have been proposed (7-10) and are shown in Fig. 1. Natori et al. (11) have isolated rubratoxin B in crystalline form from P. purpurogenum and have reported pathological findings observed in HeLa cells and mice. The biochemical manifestations of rubratoxin B in mouse liver recently have been reported by Hayes and Wilson (6).

It is desirable to have these compounds available in large quantities if studies on their chemistry and toxicology are to be undertaken. Since a chemical synthesis has not yet been achieved, the rubratoxins must be produced by biological means. Bioproduction in a synthetic or a semisynthetic medium would be preferable to production on solid substrates because of ease in scale-up, simplicity of extraction, and the suitability of such media for studies on rubratoxin biosynthesis. The purpose of this report is to describe the effect of different growth and cultural conditions on the production of rubratoxin B in liquid culture.

MATERIALS AND METHODS

Organism. Unless otherwise stated, the P. rubrum used in this investigation is the original strain P-13 isolated by Burnside, received from the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., and designated NRRL A-11785. P. rubrum MR 043 and MR 180 were supplied by M. O. Moss of the Tropical Products Institute, London, England, and P. rubrum Stoll 2 was obtained from the Central Leather Research Institute.

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1 Presented in part at the 70th Annual Meeting of the American Society for Microbiology, Boston, Mass., April 1970.
Adyar, Madras, India. Stock cultures were maintained at 5 C on moistened, cracked corn.

Media. The basal medium employed in this investigation was Mosseray's simplified Ruailon solution (12): sucrose, 50 g; tartaric acid, 0.4 g; MgCO₃, 0.25 g; NH₄NO₃, 0.25 g; K₂CO₃, 0.4 g; (NH₄)₂HPO₄, 0.4 g; (NH₄)₂SO₄, 0.2 g; FeSO₄, 0.05 g; ZnSO₄, 0.05 g; deionized, distilled water, 1 liter, supplemented with malt extract broth (BBL). Any changes made in this medium for individual experiments are described below. In experiments with trace elements, glass-distilled deionized water was used. Sucrose was dissolved in this water and passed through an aluminum oxide column to remove mineral impurities (2). Stock solutions of minerals were prepared with CP chemicals and added to the chromatographed medium.

Culture. Low-form culture flasks (no. 4422; 2,500 ml; Corning Glass Works, Corning, N.Y.) containing 200 to 500 ml of semidefined medium were sterilized at 120 psi for 15 min. The medium was inoculated with a heavy conidial suspension from a corn slant and incubated for approximately 14 to 15 days at ambient temperature in a stationary environment unless otherwise stated. Previous experiments had demonstrated that rubratoxin B production approached a plateau in 14 to 15 days (5). At least two flasks were run for each of the variables investigated.

Fermentators (5- and 7-liters), containing 2 or 4 liters of semisynthetic medium, were sterilized 25 min at 120 psi. The medium was inoculated with a conidial suspension of P. rubrum and incubated for 14 days at 25 C with constant stirring (0, 10, 20, 40, and 80 rev/min) and aerating (0, 100, and 1,000 ml/min) in a Micro Ferm laboratory fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). Samples were taken periodically for measurement of rubratoxin concentration. The pH of the medium was monitored automatically.

Differences in rubratoxin B production between duplicate flasks were generally less than 20%, although occasional pairs differed by as much as 40%.

Assays. Dry weight of the filtered mycelium was determined after drying the mycelial mats at 70 C for 24 to 48 hr. Rubratoxin B was determined by the method of Hayes and Wilson (5) by extraction with diethyl ether. The diethyl ether extracts were either concentrated to dryness and redissolved in acetone or spotted directly onto Silica Gel HF₂₅₄ chromatographic plates. The plates were developed in an unlined tank saturated with glacial acetic acid-methanol-chloroform (2:20:80, v/v). Authentic rubratoxin B was spotted on these same plates, and, after development, the plates were observed under short-wave ultraviolet light (253 nm). Toxin was measured gravimetrically.

RESULTS

The effect of sucrose in the basal medium supplemented with 2.5% malt extract broth on rubratoxin B production by P. rubrum is shown in Table 1. This table indicates that a plateau for rubratoxin production is reached at 10 to 30 g sucrose per

![Figure 1. Structure of the rubratoxins, Rubratoxin A: C₉₅H₈₂O₁₁; R = H, OH. Rubratoxin B: C₉₅H₈₂O₁₁; R = O.](image)
liter, with maximum production occurring at 25 g per liter.

Table 2 indicates the effect of malt extract broth in the basal medium (50 g of sucrose/liter) on the production of rubratoxin B by P. rubrum. Relatively large quantities of rubratoxin were produced at all levels investigated with peak production occurring at 30 g per liter of malt extract broth. The production of the toxin was greatly reduced in the absence of malt extract broth.

Because a number of mineral elements have been implicated as being involved in production of other mycotoxins, the effects of trace metals on rubratoxin B production were investigated (Table 3). Since deletion of iron almost eliminated rubratoxin production, it appears that iron is needed for rubratoxin biosynthesis. The reduced yields of rubratoxin B obtained without iron were not due to an apparent reduction of growth. Zinc appears to be specifically required for production of the toxin, and this requirement is met by the addition of a minimum of 0.4 mg of zinc to the medium (Table 4). However, maximum

Table 3. Effect of mineral elements on rubratoxin B production by P. rubrum P-13 grown as stationary culture for 16 days at 25 C

| Elements removed from basal medium | Mycelial dry wt (g/500 ml) | Final pH | Rubratoxin B (g/500 ml) |
|-----------------------------------|-----------------------------|----------|-------------------------|
| None                              | 8.9                         | 3.2      | 212.40                  |
| Mg                                | 10.4                        | 2.8      | 197.27                  |
| K                                 | 10.7                        | 3.1      | 153.43                  |
| Fe                                | 8.2                         | 2.6      | 4.28                    |
| Zn                                | 4.0                         | 3.2      | None detected           |

Table 4. Effect of varying the zinc concentrationa on rubratoxin production by P. rubrum P-13 grown as stationary cultures for 14 days at 25 C

| Zinc concn (g/liter) | Mycelial dry wt (g/500 ml) | Final pH | Rubratoxin B (mg/500 ml) |
|----------------------|----------------------------|----------|--------------------------|
| 0                    | 3.01                       | 3.0      | None detected            |
| 4 \times 10^{-4}     | 9.2                        | 3.0      | 9.7                      |
| 4 \times 10^{-3}     | 11.3                       | 3.0      | 13.7                     |
| 4 \times 10^{-2}     | 7.4                        | 2.9      | 127.8                    |
| 4 \times 10^{-1}     | 7.5                        | 2.9      | 147.1                    |
| 4                   | 8.2                        | 2.9      | 126.2                    |
| 40                  | 10.5                       | 3.4      | 140.5                    |
| 5 \times 10^{-6}     | 11.1                       | 3.4      | None detected            |

*a As ZnSO4.

Table 5. Effect of temperature on rubratoxin B production by P. rubrum P-13 grown on stationary cultures for 20 days

| Temp | Mycelial dry wt (g/500 ml) | Final pH | Rubratoxin B (mg/500 ml) |
|------|---------------------------|----------|--------------------------|
| 45   | No growth                 | 6.9      | None detected            |
| 37   | 9.2                       | 2.5      | None detected            |
| 30   | 8.6                       | 3.2      | 117.1                    |
| 22   | 6.6                       | 3.2      | 240.0                    |
| 20   | 6.4                       | 3.4      | 74.2                     |
| 5    | No growth                 | 6.7      | None detected            |

* After 20 days placed at ambient temperature (22 to 25 C) for 14 days. Growth and toxin production in both cases: 5 C, 20 mg/500 ml; 45 C, 83.4 mg/500 ml.

Table 6. Effect of pH on rubratoxin B production by P. rubrum P-13 grown as stationary cultures for 14 days at 25 C

| Initial pHa | Mycelial dry wt (g/500 ml) | Final pH | Rubratoxin B (mg/500 ml) |
|-------------|---------------------------|----------|--------------------------|
| 1.0         | No growth                 | 1.0      | None detected            |
| 2.0         | 6.9                       | 1.7      | None detected            |
| 3.0         | 6.9                       | 2.0      | 0.60                     |
| 4.0         | 8.8                       | 2.1      | 155.78                   |
| 5.0         | 7.5                       | 2.2      | 220.00                   |
| 6.0         | 8.6                       | 2.6      | 260.10                   |
| 7.0         | 7.1                       | 3.1      | 230.06                   |
| 7.3b        | 7.7                       | 3.2      | 340.01                   |
| 8.0         | 6.5                       | 3.9      | 211.35                   |
| 9.0         | 6.7                       | 5.4      | 66.42                    |
| 10.0        | 5.2                       | 5.8      | 24.21                    |

*a Adjusted with either 1 N HCl or 1 N NaOH where necessary.

Table 7. Production of rubratoxin B by selected isolates of P. rubrum grown as stationary cultures for 14 days at 25 C in basal medium with 2.5% malt extract broth

| Isolate     | Mycelial dry wt (g/500 ml) | Final pH | Rubratoxin B (mg/500 ml) |
|-------------|---------------------------|----------|--------------------------|
| Stoll no. 2 | 4.5                       | 3.0      | None detected            |
| ATCC 10520  | 2.9                       | 6.8      | 12.21                    |
| MR 180      | 3.3                       | 6.4      | 6.43                     |
| MR 043      | 3.2                       | 6.5      | 6.43                     |
| P-13 (NNRL A11785) | 3.3 | 6.7 | 158.84 |
DISCUSSION

A medium composed of sucrose (25 g/liter), malt extract broth (20 g/liter), ZnSO₄ (0.5 g/liter) and FeSO₄ (0.5 g/liter) provided all the necessary ingredients for the production of rubratoxin B by P. rubrum P-13. Other chemical, cultural, and environmental conditions investigated had less effect on toxin production.

The requirement for zinc may explain the difficulty of obtaining rubratoxin production on un-supplemented media such as Czapek-Dox medium. This requirement for zinc may be a factor in the apparent loss of toxin-producing ability of the fungus when maintained for prolonged periods on Czapek Agar or on Czapek-Dox Agar (5). The reports of Natori et al. (11) on the production of rubratoxin B by P. purpurogenum in a Czapek-Dox medium modified with ZnSO₄ (0.01 g/liter) and by Wogan and Mateles (17) on submerged culture production by P. rubrum confirm this nutritional observation. The large reduction in toxin but not in fungal growth when iron sulfate was deleted from the medium also may help explain the loss of toxin biosynthesis by isolates maintained on artificial media. The Czapek-Dox medium used for production of rubratoxin B by P. purpurogenum (11) and the submerged culture medium (17) both contained FeSO₄. At present, however, there is insufficient evidence to speculate whether a lack of zinc or iron is selecting for nonrubratoxin-producing mutants.

During the past several years, fungi capable of producing aflatoxin, ochratoxin, and other mycotoxins have been isolated from a number of foodstuffs in this as well as other countries. These findings, along with the recent Japanese report of rubratoxin B production by P. purpurogenum (11), further indicate the importance of the mycotoxin problem throughout the world in food hygiene and in stored agricultural commodities. Such facts indicate a pressing need for screening of molds for detection of mycotoxins other than those commonly screened for at the present (i.e., aflatoxin and ochratoxin).

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LITERATURE CITED

1. Burnside, J. E., W. L. Sippell, J. Forgacs, W. T. Carll, M. B. Atwood, and E. R. Doll. 1957. A disease of swine and cattle caused by eating moldy corn. II. Experimental production with pure cultures of molds. Amer. J. Vet. Res. 18:817-824.
2. Donald, C., B. I. Passey, and R. J. Swaby. 1952. A comparison of methods for removing trace metals from microbial media. J. Gen. Microbiol. 5:211-220.
3. Forgacs, J., H. Koch, W. T. Carll, and R. H. White-Stevens. 1958. Additional studies on the relationship of mycotoxicoses to the poultry hemorrhagic syndrome. Amer. J. Vet. Res. 19:744-753.
4. Goldblatt, L. A. 1969. Aflatoxin. Academic Press Inc., New York.
5. Hayes, A. W., and B. J. Wilson. 1968. Bioproduction and purification of rubratoxin B. Appl. Microbiol. 16:1163-1167.
6. Hayes, A. W., and B. J. Wilson. 1970. Effect of crystalline rubratoxin B on liver composition and metabolism in mice. Toxicol. Appl. Pharmacol. 17:519-531.
7. Moss, M. O., F. V. Robinson, and A. B. Wood. 1968. Rubratoxin B, a toxic metabolite of Penicillium rubrum. Chem. Ind. (London), p. 587-588.
8. Moss, M. O., F. V. Robinson, A. B. Wood, and A. Morrison. 1967. Observations on the structure of the toxins from Penicillium rubrum. Chem. Ind. (London), p. 755-757.
9. Moss, M. O., F. V. Robinson, A. B. Wood, H. M. Paisley, and J. Peeney. 1968. Rubratoxin B, a proposed structure for a bis-anhydride from Penicillium rubrum Stoll. Nature (London) 220:767-770.
10. Moss, M. O., A. B. Wood, and F. V. Robinson. 1969. The structure of rubratoxin A, a toxic metabolite of Penicillium rubrum. Tetrahedron Lett., p. 367-370.
11. Natori, S., S. Sakaki, H. Kurata, S. Udagawa, M. Ichinoe, M. Saito, M. Umdea, and K. Ohtaubo. 1970. Production of rubratoxin B by Penicillium purpurogenum Stoll. Appl. Microbiol. 19:613-617.
12. Raper, K. B., and D. I. Fennell. 1965. Genus aspergillus, p. 37-38. The Williams & Wilkins Co., Baltimore.
13. Saito, M., M. Umdea, K. Ohtaubo, H. Kurata, S. Udagawa, and S. Natori. 1968. Studies on the detection of carcinogens in natural products. I. Toxic effects of fungi isolated
from foodstuffs, p. 59. Proc. Jap. Cancer Ass., 27th Annual Meeting, Tokyo.
14. Townsend, R. J., M. O. Moss, and H. M. Peck. 1966. Isolation and characterization of hepatotoxins from *Penicillium rubrum*. J. Pharm. Pharmacol. 18:471-473.
15. Wilson, B. J., P. A. Teer, G. H. Barney, and F. R. Blood. 1967. Relationship of aflatoxin to epizootics of toxic hepatitis among animals in southern United States. Amer. J. Vet. Res. 28:1217-1230.
16. Wilson, B. J., and C. H. Wilson. 1962. Extraction and preliminary characterization of a hepatotoxic substance from cultures of *Penicillium rubrum*. J. Bacteriol. 84:283-290.
17. Wogan, G. N., and R. I. Mateles. 1968. Mycotoxins. Progr. Ind. Microbiol. 7:149-175.
18. Wright, D. E. 1968. Toxins produced by fungi. Annu. Rev. Microbiol. 22:269-282.