Production of Chloroflavonin, an Antifungal Metabolite of *Aspergillus candidus*

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Production of chloroflavonin, a new antifungal antibiotic, by strains of *Aspergillus candidus* is described. Two wild strains of the fungus had distinctly different chloroflavonin-producing capabilities. One strain produced 25 μg of chloroflavonin per ml per 4 to 5 days in a pilot scale fermentor with stirring, using a medium containing corn steep liquor and glucose. Production of antibiotic was favored by high rates of agitation-aeration. Crude chloroflavonin was extracted from the whole brew with a hydrocarbon solvent and then purified by recrystallization from benzene and petroleum ether. The overall yield from fermentation brew to pure product was 50%.

Recent publications (1, 2) reported the presence of chloroflavonin, a new antifungal antibiotic, in cultures of *Aspergillus candidus*. The structure has been determined (1) as 3'-chloro-2',5-dihydroxy-3,7,8-trimethoxy flavone (Fig. 1).

In this paper, results of production of chloroflavonin by two strains of *A. candidus* are given and procedures for assay, fermentation, extraction, and purification are described.

**MATERIALS AND METHODS**

**Assay procedures.** Two methods of estimating chloroflavonin were developed, one involving direct spectroscopy, and the other microbiological assay against the mold *Paecilomyces variotii*. The spectrum of chloroflavonin has absorption maxima at 266 and 350 nm (at 350 nm, log ε = 3.85). Thus absorption of chloroflavonin in a suitable solvent, such as ethanol, was used for assay purposes, and the results were in close agreement with the assay of antimicrobial activity.

Bioactivity was measured against *P. variotii* BRL 513 by using a paper disc agar diffusion method. The assay organism was cultured in 8-oz (240-ml) medicinal flat bottles on MYGP solid medium, containing (per liter): malt extract (Oxoid), 3 g; yeast extract (Difco), 3 g; glucose (Cerelose), 10 g; bacteriological peptone (Oxoid), 5 g; and agar (Oxoid no. 3), 20 g. The medium was adjusted to pH 6.8 before sterilization. Cultures were incubated for 1 week at 25°C, and a suspension was made by washing off the spores with 25 ml of sterile water containing 0.02% (v/v) Tween 80. This suspension (10 ml) was used to inoculate 400 ml of Sabouraud (glucose) medium, containing (per liter): bacteriological peptone (Oxoid), 10 g; yeast extract (Difco), 1 g; glucose (Cerelose), 40 g; and agar (Oxoid No. 3), 20 g. The medium was adjusted to pH 6.6 before sterilization. To suppress bacterial contamination, 2 ml of a solution of 5 mg of benzylpenicillin per ml and 10 mg of streptomycin per ml was added to the seeded liquid agar before pouring the solution onto assay plates (13 by 15 inch). Samples of whole fermentation brew were extracted with 5 volumes of toluene. After separating the phases by centrifugation, 13-mm assay discs were dipped into the toluene extract, dried at 90°C for 0.5 hr, and placed on the seeded plate. Incubation at 27°C for 18 to 22 hr gave clear zones which were best read by measuring the diameter of "surface" growth inhibition. Standard solutions containing 0.625, 1.25, 2.5, and 5.0 μg of chloroflavonin per ml in toluene were included on each assay plate, and a straight line was obtained when the zone diameter was plotted against the logarithm of chloroflavonin concentration. Solvents other than toluene could be used; however, it was essential to make up standard solutions in the same solvent since different solvents gave distinctly different response lines.

**Fermentation.** Spores of *A. candidus* strain ATCC 20022 and strain CMI 16046 were preserved in dry soil and stored in sealed jars at 5°C. Slant cultures were prepared by inoculating the surface of 40 ml of solid medium in an 8-oz medicinal flat bottle with a small amount of suspension of soil stock in sterile water. Crawford's medium, which was used routinely for slant cultures, contained per liter: meat extract (Oxoid, Lab Lemco), 10 g; bacteriological peptone (Oxoid), 10 g; glucose monohydrate, 10 g; and agar (Oxoid no. 3), 15 g. The medium was adjusted to pH 7.4 before sterilization. Inoculated slants were incubated at 25°C for 7 days and then stored at 5°C up to three months. One of these slants was used to inoculate similar slants for immediate use as starter cultures for fermentation.

The seed medium contained per liter: corn steep liquor (50% solids), 76 g; sucrose, 20 g; CaCO₃, 10 g; and corn oil, 2.5 g. The medium was adjusted to pH 5.8 before sterilization. The final fermentation medium contained per liter: corn steep liquor (50% solids),
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![Structure of chlorflavonin](image)

**Fermentation brew 1600 l.**

| 50.4 g. chlorflavonin |
|-----------------------|
| filter at pH 4 |
| Mycelial solids |
| **[49.3 g.]** |
| Filtrate 1425 l. |
| **[1.08 g.]** discard |
| extract with SBP6 |
| Solvent |
| **[48.8 g.]** |
| Spent solids |
| **[0.5 g.]** discard |
| crystallization stages |
| Pure chlorflavonin |
| **[26.5 g.]** |
| Mother liquors etc. |
| **[22.3 g.]** |

**FIG. 2. Outline of process for isolation of chlorflavonin by means of extraction with SBP6 hydrocarbon solvent. Values in brackets are amounts of chlorflavonin present in the various fractions.**

60 g; glucose monohydrate, 50 g; CaCO₃, 2 g; KCl, 5 g; and lard oil, 2.5 g; the final medium was adjusted to pH 5.8 before sterilization.

The temperature of incubation for seeds and final fermentations was 26°C.

Flask work on a shaker was carried out with 100-ml quantities of medium in 500-ml conical flasks closed with cotton wool plugs. Seed flasks were inoculated with spores from a slant culture and incubated on a rotary shaker for 48 hr. Final flasks were each inoculated with 5 ml of seed and shaken for 6 days.

For stirred tank fermentations, two seed stages were employed. For the first, 5 liters of seed medium was sterilized in a 10-liter stainless-steel fermentor, inoculated with an aqueous suspension of spores from one slant culture, and incubated for 48 hr (5 liter of air/min and 500 rev/min). The content of one of these fermentors was then used to inoculate 150 liters of seed medium in a 300-liter stainless-steel fermentor. After 30 hr of incubation with 150 liter of air/min and stirring at 210 rev/min, the mixture was used as inoculum for 1,500 liters of final fermentation medium in a 500-gal, stainless-steel fermenter. The final fermentation medium was incubated for 96 to 120 hr with aeration and stirring.

**Extraction.** The extraction procedure is outlined in Fig. 2. Harvest brew (1,600 liters) was acidified to pH 4.0 with 25% (v/v) H₂SO₄. After stirring for 30 min, the brew was filtered and the filtrate was discarded. Damp mycelial solids were extracted at 60°C for 3 hr with 200 liters of SBP6, a commercial hydrocarbon solvent produced by Shell-Mex and B.P. Ltd., Shell-Mex House, Strand, London. The extract was decanted, and the solids were again extracted with fresh solvent (200 liters). Solvent extracts were combined and concentrated under reduced pressure (30 mm of Hg) until the product began to crystallize (usually when the volume had been reduced to approximately 15 liters). After leaving the concentrate at 5°C for 2 hr, the crude product was filtered off and recrystallized from benzene-petroleum ether or dimethylformamide-water.

**RESULTS AND DISCUSSION**

The chlorflavonin-producing capabilities of two strains of *A. candidus* were compared in shaken flasks. Both cultures had been maintained in collections subsequent to their isolation from natural sources; neither had been subjected to artificial mutagenic treatment. Table 1, which shows the concentrations of chlorflavonin achieved in shaken flask fermentations of strains CMI 16046 and ATCC 20022 carried out under identical conditions, clearly indicates the superiority of CMI 16046.

Fermentations on a stirred-tank scale were first carried out with strain ATCC 20022. Of eight consecutive fermentations in a 500-gal fermentor, under otherwise identical conditions (airflow rate, 1,300 liters/min; 26°C), four were stirred at 166 rev/min and four at 182 rev/min. The results (Table 2) show an increase in chlorflavonin assay titer as a result of increase in agitator speed.

The higher stirrer speed (182 rev/min) was employed for similar stirred-tank fermentations with strain CMI 16046, which gave considerably higher yields than ATCC 20022. The progress of a typical tank fermentation with strain CMI 16046 is plotted in Fig. 3. Reducing sugar was rapidly metabolized in the initial growth phase during the first 40 hr, after which chlorflavonin biosynthesis, as estimated by assay, proceeded steadily until the maximal concentration was reached at 100 hr after inoculation. The pH level of the medium rose continuously to approximately pH 8.3 at 80 hr and then remained fairly constant.

Chlorflavonin, which has phenolic properties, is essentially insoluble in water at low pH levels and is soluble at high pH levels. It is soluble in most organic solvents except paraffins. Solubility
in water is 8 μg/ml; ethanol, 100 μg/ml; chloroform, 25 mg/ml; and dimethylacetamide, 35 mg/ml. The solid is stable up to 160°C for several hours. Aqueous solutions of chlorflavonin are stable for at least 1 hr from pH 2 to 13 at ambient temperatures; however, at higher temperatures, it is evident that stability is favored by low pH levels. After 120 hr at 65°C, a loss of 4.5% was recorded at pH 4, whereas the loss was 41.5% at pH 7.

In fermentation brew at harvest, chlorflavonin is distributed between culture fluid and mycelium. If the brew is acidified, most of the compound precipitates and can be collected with the mycelial solids by filtration; it can then be extracted with solvent.

The mass balance of chlorflavonin in a typical extraction is illustrated in Fig. 2. If the extraction time is extended or the temperature increased, a brown waxy material is extracted, making purification of the product more difficult.

The crude chlorflavonin obtained from the concentrated SBP6 extract may be recrystallized in the usual way from solvents such as isopro-

| TABLE 1. Production of chlorflavonin, as estimated by bioassay, in shaken flasks of two strains of A. candidus |
|---|---|---|
| Age of culture | Chlorflavonin concn* |  |
| days | Strain ATCC 20022 | Strain CMI 16046 |
|---|---|---|
| 4 | μg/ml | μg/ml |
| 6.0 | 15.9 |
| 6.5 | 14.1 |
| 7.0 | 15.1 |

* Each value is the mean of duplicate flasks.

| TABLE 2. Chlorflavonin bioassay titers obtained in a 500-gal fermentor with A. candidus ATCC 20022 by using two different stirrer speeds |
|---|---|---|
| Stirrer speed | Chlorflavonin titer |  |
|---|---|---|
| Mean of four fermentations | Standard deviation |
| rev/min | μg/ml | μg/ml |
| 166 | 8.1 | 0.03 |
| 182 | 11.0 | 0.91 |

Fig. 3. Changes in pH, glucose, growth (mycelial dry weight), and chlorflavonin production (bioassay) during fermentation of A. candidus (CMI 16046) in a pilot scale fermentor. See text for details.

panol or benzene; highest yields, however, were obtained by the procedures outlined in the following examples. (i) Chlorflavonin (5.8 g, 59% pure) was dissolved in hot (70°C) benzene (300 ml). After filtering, the solution was added to hot (100 to 120°C) petroleum ether (1 liter), and the mixture was cooled to 25°C over a period of 3 hr and then kept at 5°C for 12 hr. The product was recovered by filtration, washed with hexane, and dried at 50°C in vacuo. The product was characterized by a 3.35-g yield, a melting point of 212°C, and 97.2% purity, as determined by absorbance at 350 nm. (ii) Chlorflavonin (71 g, 87% pure) was dissolved at 60°C in dimethylformamide (1.4 liter), and the solution was filtered to remove white and brown waxy particles. The addition of water (5.6 liters) precipitated the product, which was then filtered, washed with water, and dried in vacuo (1 mm of Hg) at 60°C. The product was characterized by a 59-g yield, melting point of 212°C, and 93% purity as determined by absorbance at 350 nm. (iii) Chlorflavonin was also purified by fractional sublimation. At 130°C and 4 mm of Hg, the white impurity sublimed; when the temperature was increased to 160°C, pure chlorflavonin was collected.

LITERATURE CITED
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