Production of Verruculogen by *Penicillium estinogenum* in Stirred Fermenters

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A spectrofluorometric assay for the estimation of the tremorgenic mycotoxin verruculogen in crude mycelial extract has been devised and used to determine concentrations as low as $0.2 \mu g$ ml^{-1}. Verruculogen production by *Penicillium estinogenum* has been extended from surface culture to submerged culture in 60 l stirred fermenters, in which the maximum cell-associated mycotoxin yield [5 mg (100 ml culture)$^{-1}$] was obtained within 7 d. It was found necessary to supplement the medium (Czapek Dox broth plus 0.5% yeast extract) with calcium chloride (2%) to induce profuse sporulation (2×10^7 conidia ml^{-1}).

INTRODUCTION

Verruculogen is a tremorgenic mycotoxin. It was first isolated from a culture of *Penicillium verruculosum* (Cole *et al.*, 1972) and its structure has been determined (Cole & Kirksey, 1973; Fayos *et al.*, 1974). This mycotoxin has also been identified as a metabolite of *Aspergillus caespitosus* (Schroeder *et al.*, 1975) and *P. piscarium* (Gallagher & Latch, 1977) growing on solid substrates and *A. fumigatus* and *P. estinogenum* growing in liquid media (Mantle *et al.*, 1978a). Apart from the isolation by Schroeder *et al.* (1975) of 172 mg verruculogen from 1 kg mouldy corn, yields of this mycotoxin have not been given, presumably because no quantitative assay has been available.

The mode of action of tremorgenic mycotoxins is poorly understood although recent experiments (Norris *et al.*, 1980) have extended earlier pharmacological studies (Hotujac & Stern, 1974; Hotujac *et al.*, 1976; Sobotka *et al.*, 1978) to define effects on amino acid neurotransmitters. Such effects are consistent with the proposal (Mantle *et al.*, 1978b; Di Menna & Mantle, 1978; Penny *et al.*, 1979) that soil-borne moulds producing tremorgens, of which verruculogen is probably the most potent, could be involved in the aetiology of certain reversible neurological disorders (staggers) of farm animals.

The requirement of pure tremorgen for continuing studies on the mode of action has prompted the present design of a fermentation process for verruculogen production in submerged culture, the deficiency in quantitative analysis of this mycotoxin having first been solved.

METHODS

Organism. The strain of *Penicillium estinogenum* Komatsu & Abe ex G. Sm. used in this study was originally isolated from the faeces of sheep affected with ryegrass staggers in New Zealand (Di Menna & Mantle, 1978). Stocks of the fungus were maintained as lyophilized cultures of conidia in skimmed milk solids (Onions, 1971).

Media. The medium used throughout was Czapek Dox/yeast extract (CD/YE) broth (Difco) pH 6·8,

containing (g l^{-1} in distilled water): sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0·5; KCl, 0·5; $FeSO_4.7H_2O$, 0·01; yeast extract, 5. For submerged culture the nutrient medium was supplemented with either $CaSO_4.2H_2O$ (20 g l^{-1}), $CaHPO_4$ (20 g l^{-1}) or $CaCl_2.2H_2O$ (1 to 50 g l^{-1}) to induce sporulation.

Culture conditions. Cultures were prepared by adding sterile distilled water to an ampoule of freeze-dried conidia and inoculating slopes of potato dextrose agar (PDA). These cultures were grown at 271 C for 7 d to provide a spore inoculum for fermentations.

Static flask cultures. Erlenmeyer flasks (1 l) containing 400 ml medium were sterilized at 121 'C for 30 min. Flasks were seeded with dry conidia (approximately 10^5) taken from the PDA slopes and incubated at 27 °C for up to 14 d.

Shaken flask cultures. Erlenmeyer flasks (500 ml, including some with baffles) containing 100 ml medium were sterilized at 121 °C for 20 min. The baffled flasks contained unsupplemented CD/YE broth and were used for the seed stage; they were inoculated with dry conidia (approximately 10^5) and incubated at 27 'C for 18 h on a rotary shaker (200 rev. min^{-1}, 10 cm eccentric throw). Production stage flasks contained CD/YE supplemented with calcium salts at various concentrations (Table 1) and, after inoculation with a 5 % (v/v) transfer from seed stage flasks, were similarly incubated for up to 14 d.

Stirred fermenters. The vessels employed were stainless-steel 60 l fermenters of conventional design (Banks *et al.*, 1974). Temperature control was achieved by the automatically regulated flow of cooling water through the fermenter jacket. Culture aeration was provided by sparging air through a ring sparger into the culture fluid, which was agitated by means of a single disc turbine impeller.

CD/YE medium (55 l) containing $CaCl_2.2H_2O$ (2 %, w/v) plus polypropylene glycol antifoam (0·1 %, v/v) was prepared *in situ*. Medium and fermenter were sterilized at 121 °C for 20 min by live-steam injection. Allowance was made for condensate subsequently formed during sterilization, the final post-sterilization volume of the medium being 60 l.

The fermenter was inoculated with 3 l of seed stage culture (two 4 l baffled flasks each containing 1·5 l CD/YE medium were each inoculated with 10^9 conidia and incubated for 18 h at 27 °C on a rotary shaker) and incubated at 27 °C for 8 d. The agitator shaft speed was 183 rev. min^{-1} during the first 24 h, then raised to 367 rev. min^{-1}, and subsequently reduced to 183 rev. min^{-1} from 72 h onwards to minimize hyphal damage. Polypropylene glycol antifoam was added as necessary to control foaming. The vessel was monitored for contamination throughout the fermentation.

Analytical procedures. Samples consisted of the mycelium filtered from flask cultures, or from 300 ml volumes taken from the fermenter at intervals. Prior to filtration the pH value of the whole culture was measured.

(*i*) *Mycelial dry weight.* Mycelium from at least 100 ml culture (two replicates) was dried to constant weight at 50 °C.

(*ii*) *Sporulation.* A few millilitres of culture were coarsely filtered to remove hyphae and the spore content of the broth, suitably diluted, was measured using a haemocytometer.

(*iii*) *Phosphate and reducing sugars.* Phosphate in the filtered broth was measured by the method of Fiske & Subbarow (1925). Reducing sugars were measured by the Fehlings method described by Banks *et al.* (1974).

(*iv*) *Verruculogen.* Verruculogen was separated from large-scale [20 g dried mycelium plus 1 l chloroform/ acetone (1:1, v/v)] extracts by chromatography, initially on preparative layer (1 mm) plates coated with silica gel GF_{254} (Merck), and then on thin-layer (0·25 mm) plates developed in chloroform/acetone (93:7, v/v). Although evidence for the presence of verruculogen could be obtained by the quenching of the fluorescent dye in the region R_F 0·3 to 0·4, the toxin was visualized by spraying the edge of a plate with 50 % (v/v) ethanolic sulphuric acid, and was seen, after heating, as a brown band in daylight and as a mustard fluorescence under long wavelength u.v. light. The verruculogen band was eluted from the silica with chloroform, concentrated *in vacuo* and rechromatographed as necessary to obtain a chromatographically homogeneous product. Purity was best assessed by chromatography on pre-coated silica gel GF_{254} plates (Camlab, Polygram, 0·25 mm), on which 0·1 µg verruculogen could be clearly seen by its fluorescence after spraying. The product was crystallized from chloroform/ethanol (3:2, v/v) and its identity was confirmed by electron impact mass spectrometry (Cole *et al.*, 1972). Such material was used to construct a standard curve for the following fluorimetric assay.

Samples of dried mycelium (approx. 1 g) were finely crushed in a pestle and mortar and extracted for 24 h at room temperature in 80 ml chloroform/acetone (1:1, v/v). The extract was filtered through absorbent cotton wool, the extracted mycelium was washed with fresh solvent and the combined extracts were made up to 100 ml. A 10 ml sample was taken to dryness *in vacuo* and the residue was taken up in absolute ethanol (10 ml) from which a series of four 10-fold dilutions was prepared. Two ml of each dilution was mixed with 0·13 ml of concentrated H_2SO_4 and incubated at 70 °C for 40 min, with the test tube mouth covered with a glass ball. The solution was immediately cooled to approximately 20 °C and losses due to

evaporation were corrected. The fluorescence intensity was measured (λ_{ex} 370 nm, λ_{em} 450 nm) in a Farrand fluorimeter, using 1 cm cuvettes, and compared with that of standard verruculogen solutions, similarly treated, in the linear range 0·2 to 1·0 μg ml^{-1}.

Bulk extraction of verruculogen. Fermenter mycelium was harvested at 7 d, filtered, freeze-dried and exhaustively extracted in chloroform/acetone (93 : 7, v/v). The extract was concentrated and the toxin was purified by chromatography (see above).

RESULTS

Preliminary studies in static and shaken flasks

Penicillium estinogenum incubated for 14 d in surface culture developed, during the first 4 d, a heavy sporulating mycelial mat in which verruculogen was produced during growth, although the concentration of toxin continued to rise after cultures had entered the stationary phase. Verruculogen yields were consistently obtained in the range 20 to 30 mg (g dry wt)$^{-1}$. There was negligible extracellular release of toxin into the culture broth.

In submerged culture, using the same medium, the fungus made good vegetative growth but failed to sporulate; the mycelium was cream-coloured and lacked the characteristic green pigmentation normally conferred by spores. Since no verruculogen was produced, toxin production appeared to be linked with sporulation and it seemed necessary to induce sporulation in submerged culture to obtain verruculogen.

Foster *et al.* (1945) reported that $CaCl_2$ stimulated sporulation in submerged cultures of *Penicillium notatum.* Addition of $CaCl_2.2H_2O$ (0·5%, w/v) to the present medium also effected sporulation in *P. estinogenum* and verruculogen was produced [10 mg (100 ml)$^{-1}$]. $CaHPO_4$ and $CaSO_4.2H_2O$ (2%, w/v) also induced conidium formation and similar toxin yields, but formed crystalline precipitates which severely interfered with dry weight determinations. $CaCl_2$ appeared to form precipitates to a lesser extent and was thus selected as the medium supplement for shaken cultures.

The optimum level of $CaCl_2$ supplement was investigated in the range 0·1 to 5·0% (w/v) (Table 1). The stimulatory effect on sporulation was clearly displayed not only by the number of conidia but also by the green colour imparted to the culture, which was evident at about 1·5% (w/v) $CaCl_2.2H_2O$ and became progressively darker with increasing concentrations of $CaCl_2$. Although the best verruculogen yield in shaken flasks at 5 d was obtained with 0·5% (w/v) $CaCl_2.2H_2O$, 2% (w/v) $CaCl_2.2H_2O$ gave a superior yield

Table 1. *Effect of $CaCl_2$ on sporulation and verruculogen production by*
Penicillium estinogenum in shaken flask culture

Shaken flask cultures of *P. estinogenum* were grown in Czapek Dox/yeast extract medium, supplemented with $CaCl_2.2H_2O$, at 27 °C. Sporulation, mycelial dry weight and verruculogen content were analysed after 5 d (mean of two cultures) and 10 d (mean of two cultures).

|	After 5 d incubation			After 10 d incubation		
$CaCl_2.2H_2O$ concn (%, w/v)	Spores (ml^{-1})	Mycelial dry wt [g (100 ml culture)$^{-1}$]	Verruculogen [mg (100 ml culture)$^{-1}$]	Spores (ml^{-1})	Mycelial dry wt [g (100 ml culture)$^{-1}$]	Verruculogen [mg (100 ml culture)$^{-1}$]
0	$2·77 \times 10^4$	1·16	0·52	$2·77 \times 10^4$	0·84	0·1
0·1	$5·5 \times 10^4$	1·25	1·13	$3·12 \times 10^5$	0·87	0·41
0·25	$5·0 \times 10^5$	1·44	0·86	$3·18 \times 10^5$	1·02	0·59
0·5	$2·5 \times 10^5$	1·65	6·38	$4·52 \times 10^5$	1·25	9·19
0·75	$8·75 \times 10^5$	1·62	4·11	$3·51 \times 10^6$	1·26	6·45
1·0	$1·37 \times 10^6$	1·7	3·74	$9·35 \times 10^6$	1·3	8·49
1·5	$3·75 \times 10^6$	1·66	2·88	$2·2 \times 10^7$	1·31	6·56
2·0	$1·15 \times 10^7$	1·92	1·7	$3·1 \times 10^7$	1·24	12·48
3·0	$1·4 \times 10^7$	1·76	1·13	$3·58 \times 10^7$	1·29	10·45
5·0	$3·75 \times 10^7$	1·7	0·46	$6·55 \times 10^7$	1·22	10·67
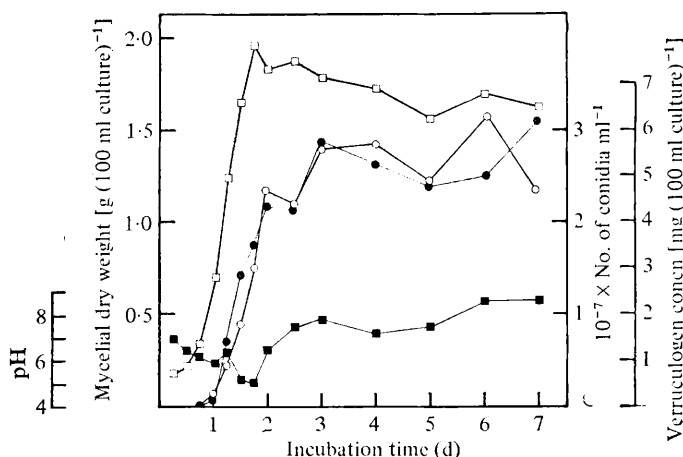
Fig. 1. Growth of *Penicillium estinogenum* in a 60 l fermenter containing Czapek Dox/yeast extract medium plus $CaCl_2.2H_2O$ (2 %, w/v) at 27 °C. Verruculogen yield (○), sporulation (●), mycelial dry weight (□) and broth pH (■).

at 10 d. Consequently, verruculogen production in stirred fermenters was investigated using a 2% (w/v) supplement of $CaCl_2.2H_2O$.

Studies in fermenters

In a preliminary 60 l fermentation lasting 13 d, maximum mycelial dry weight was obtained within the first 2 d, concomitant with the uptake of sucrose and phosphate from the medium. The culture became green as conidia were produced and reached a concentration of about $10^7 \, ml^{-1}$. During the first 2 d the pH value declined by approximately two units but became alkaline as the fermentation entered the stationary phase. An addition of antifoam (30 ml) was found to be necessary after 57 h. The maximum yield of verruculogen was achieved within the first week but thereafter decreased.

A second 60 l fermentation was performed similarly, but the incubation period was restricted to 7 d to ensure that the mycelial product retained the highest yield of toxin. Frequent sampling allowed the course of fermentation to be followed closely (Fig. 1). The familiar pattern of dry weight accumulation occurred, conidia becoming evident within 18 h with about 75% of the conidia being produced during the exponential growth phase. During the first few hours some fragmentation of inoculum occurred so that, by 12 h, the fermenter contained a dense suspension of branched hyphae showing differentiation of conidiophores. The first conidia were released between 12 and 18 h. Thereafter, during the second and third days, when a high impeller shaft speed was employed, hyphal fragmentation resulted in a finely divided branched mycelial form which contrasted with the smooth ovoid (about 1×2 to 3 mm) pellets characteristic of shaken flask cultures.

A notable feature of this fermentation process was that the rate of increase in the concentration of verruculogen in the cells closely followed the rate of sporulation. Although biosynthesis of verruculogen was therefore also closely coincident with growth, this metabolite continued to accumulate in the remaining 5 d of fermentation (the stationary phase) to give a yield of approximately 5·5 mg $(100 \, ml)^{-1}$. Since small amounts of precipitate unavoidably contaminated mycelial dry weight determinations, it was considered more meaningful to express verruculogen yield per unit volume of culture. Bulk toxin extraction of harvested mycelium and subsequent purification confirmed analysis of verruculogen content by fluorimetric assay.

The decline from the initial pH 6·9 to pH 5·0 coincided exactly with the period of growth. This acidity was neutralized during the first 1·5 d of the stationary phase, and the culture remained alkaline when sporulation and verruculogen production had ceased.

DISCUSSION

The fluorescence assay of verruculogen proved sensitive in estimating the mycotoxin in crude mycelial extracts at a minimal concentration of 0·2 μg ml^{-1} and there did not appear to be significant interference from other components. This method not only facilitated the present fermentation studies but may also be directly applicable to measuring verruculogen in other biological extracts.

The submerged culture production of verruculogen appears to be closely associated with sporulation, though this does not imply that the toxin is specifically located in the spores, as in the production of the mycotoxin sporidesmin by *Pithomyces chartarum* (Di Menna *et al.*, 1977). Although verruculogen production is more immediately coincident with growth and sporulation than is usual for fungal secondary metabolites (Bu'Lock, 1974), a similar close association with the trophophase has been reported for ochratoxin A production by *Aspergillus ochraceous* in submerged culture (Ciegler, 1972).

Although many mycotoxins are apparently best produced in solid substrate fermentations (Wyllie & Morehouse, 1977), the yields of verruculogen in surface and shaken liquid culture, reported here, are of the same order of magnitude as those readily obtained for aflatoxin B$_1$ (Ciegler *et al.*, 1966; Reddy *et al.*, 1971) and roquefortine (Scott *et al.*, 1976) in liquid media. It is anticipated that similar yields can be achieved in stirred fermenters after further development of the present fermenter process. Economy in the degree of culture agitation within the fermenter may optimize retention of verruculogen in the cells, which is a particularly convenient attribute of the production of this tremorgen.

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