Submerged Fermentation of *Penicillium paxilli* Biosynthesizing Paxilline, a Process Inhibited by Calcium-induced Sporulation

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A submerged fermentation process for the production of the tremorgenic mycotoxin paxilline by *Penicillium paxilli* has been developed. The fungus did not sporulate and accumulated paxilline to 1.5% (w/w) in freeze-dried cells within 6 d in a 60 l stirred fermenter. Induction of extensive differentiation of conidiophores and profuse sporulation by adding 2% (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to the medium at batching reduced paxilline yield by 97%. Paxilline biosynthesis occurred when the glucose in the medium had been exhausted, implying that carbon catabolite repression may be involved in the biosynthesis of this alkaloid, even when calcium-induced sporulation inhibits or delays the onset of paxilline biosynthesis. Sporulation-induced inhibition of indole-terpenoid alkaloid biosynthesis in *P. paxilli* contrasts with the situation in some other penicillia elaborating indole alkaloids and allows disassociation of aspects of secondary metabolite biosynthesis from growth-associated differentiation, which formerly seemed to be linked.

INTRODUCTION

The biosynthesis of secondary metabolic products of fungi is sometimes associated with specific cellular differentiation, such as occurs during asexual sporulation, so that, for example, Bu'lock (1975) concluded that secondary metabolism is an aspect of the differentiation which limited growth usually implies. Representatives of two structurally distinct groups of tremorgenic mycotoxins, verruculogen representing the diketopiperazines and the penitrems representing the indole-terpenoids, illustrate this principle, since penicillia which readily elaborate these molecules while growing as sporulating mats in stationary liquid culture apparently produce them in submerged culture only when penicillus differentiation is induced by Ca^{2+} (Day *et al.*, 1980; Mantle *et al.*, 1984).

Another indole-isoprenoid tremorgen, paxilline, has recently commanded renewed interest since, in addition to being a metabolite of *Penicillium paxilli* (Cole *et al.*, 1974), it has been shown to be synthesized by the endophytic fungus *Acremonium loliae* (Weedon & Mantle, 1987). The intimate association of *A. loliae* with its ryegrass (*Lolium perenne*) host has been correlated with the occurrence of the indole-terpenoid lolitrems (Gallagher *et al.*, 1984) which cause the important spasmodic neurological disorder of ruminants known as ryegrass staggers. The mechanism of regulation of indole-terpenoid biosynthesis in *A. loliae*, whether concerning paxilline only or also concerning lolitrem B (of which paxilline may be a precursor), is therefore of agricultural importance in the context of the endophyte/host interaction. A formal model of paxilline biosynthesis *per se* is potentially available in the isolate of *P. paxilli* which has been shown to produce it in stationary liquid cultures (Cole *et al.*, 1974; Cockrum *et al.*, 1979) and has thus been studied to provide some mechanistic understanding as a background against which to explore putative regulatory mechanisms in *A. loliae*. Development of a large-scale submerged process has provided an effective source of paxilline for animal studies since this neurotoxin also occurs in endophyte-infected ryegrass (Weedon & Mantle, 1987). From the previous studies on developing submerged-culture processes for tremorgenic mycotoxin production (Day *et al.*,

1980; Mantle *et al.*, 1984) it was expected that calcium-induced sporulation would be a prerequisite for success. In finding otherwise, the present investigation seeks also to identify some of the fundamental mechanisms in the initiation of paxilline biosynthesis and to discuss the wider topic of tremorgenic indole alkaloid production.

METHODS

Organism. The strain of *Penicillium paxilli* Bain. used in this study (ATCC 26601) was supplied by J. I. Pitt, CSIRO, Division of Food Research, North Ryde, Australia, where the organism is catalogued as FRR 1900.

Media. The liquid media used were based on Czapek Dox broth supplemented with yeast extract (CDYE broth), pH 7.3, containing (g l⁻¹ in distilled water): sucrose, 30; NaNO₃, 3; K₂HPO₄, 1; MgSO₄·7H₂O, 0.5; KCl, 0.5; FeSO₄·7H₂O, 0.01; yeast extract (Difco), 5. Addition of Ca²⁺, where required, was achieved with CaCl₂·2H₂O (20 g l⁻¹).

Inoculum development. Cultures were grown on potato dextrose agar for 7 d at 27 °C. Sporing mycelium from 15 ml potato dextrose agar was macerated in 100 ml CDYE broth in a baffled 500 ml Erlenmeyer flask and incubated for 24 h on a rotary shaker (200 r.p.m.; 10 cm eccentric throw), then 10 ml samples of this primary-stage culture were transferred to production-stage (unbaffled) flasks containing 100 ml medium. For pilot-plant fermentations 100 ml of a 24 h primary-stage culture was transferred to 1 l CDYE broth in a 4 l conical flask with side arm and incubated for a further 24 h at 27 °C; 2 l of secondary-stage inoculum was used to inoculate a 60 l stirred fermenter.

Shaken flask fermentations. Four-litre flasks containing 1 l CDYE broth were inoculated with 100 ml primary-stage culture and incubated for 12 d.

In the first experiment in 500 ml flasks, 100 ml CDYE broth was inoculated with 1 ml 24 h primary-stage culture. In the later experiments on delayed addition of CaCl₂, 500 ml flasks containing 100 ml CDYE broth were inoculated with 10 ml primary-stage culture and supplemented with 10 ml sterile 20% (w/v) CaCl₂ solution immediately after inoculation or after 8, 15 or 24 h fermentation, and harvested 10 d after inoculation.

Stirred fermenter fermentations. The vessels used 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 (60 l min⁻¹) through a ring sparger into the culture fluid, which was agitated by means of a single disc turbine impeller rotating at 367 r.p.m. Polypropylene glycol antifoam (P 2000) was routinely batched in the medium (0.1 ml l⁻¹). Medium (55 l) was prepared *in situ* and the pH value adjusted to 6.0 by addition of NaOH. Medium and fermenter were sterilized at 121 °C for 20 min by live-steam injection. Allowance was made for condensate subsequently formed during sterilization bringing the post-sterilization volume to 60 l. After inoculation, fermenters were monitored for contamination daily throughout the fermentation by microscopic examination of samples, and by culture at 24 °C and 37 °C on media favourable for bacterial or fungal contaminants.

Samples for analysis were taken from the fermenter as follows: post-inoculation, 2 l; at 6, 12 and 18 h, 1 l; thereafter 0.5 l samples at intervals.

Analytical procedures. Fermenter samples were filtered through Whatman no. 1 paper and cells or filtrate analysed as appropriate.

(i) *Mycelial dry weight.* The mycelium from at least 100 ml culture was freeze-dried to constant weight.

(ii) *Sporulation.* Whole culture was examined microscopically to assess differentiation of sporophores. A few ml of culture were also coarsely filtered through cotton wool to remove hyphae and the spore content of the broth, suitably diluted where necessary, was measured using a haemocytometer.

(iii) *Reducing sugars.* The concentration of reducing sugars in the culture filtrate was determined by the procedure of Schoorl (1929). To Fehling's solution (10 ml) was added 10 ml of culture filtrate (diluted as appropriate) and the mixture was boiled for 3 min. After cooling, 10 ml 2 M-H₂SO₄ was added, followed by 5 ml 30% (w/v) KI solution. The mixture was titrated against 0.025 M-Na₂S₂O₃ solution, using starch indicator, and compared against a standard curve for glucose which was linear over the range 0–0.3%. Total sugars, expressed as free reducing sugars after hydrolysis, were measured as above (although against a standard curve for sucrose), but after boiling 5 ml culture filtrate with 5 ml 1 M-HCl and neutralizing, after cooling, with 5 ml 1 M-NaOH.

(iv) *Glucose.* Glucose concentration in culture filtrate was determined by a glucose oxidase assay processed automatically using a Beckman Glucose Analyser.

(v) *pH.* The pH value of samples was measured by a pH meter standardized against phthalate and phosphate buffers over the range pH 4–7.

(vi) *Phosphate.* Phosphate in the culture filtrate was measured by a method based on that of Fiske & Subbarow (1925). The modification substituted 4-amino-3-hydroxy-naphthalene-1-sulphonic acid as a reducing agent in place of hydroquinone.

(vii) *α-Amino nitrogen.* A ninhydrin reagent (0.5 g ninhydrin, 6 g KH₂PO₄, 4 g Na₂HPO₄, 0.3 g fructose, distilled H₂O to 100 ml) and a diluting solution (0.2 g potassium iodate in 60 ml H₂O and 40 ml 96% v/v, ethanol)

was prepared. Culture filtrate (2 ml) and ninhydrin reagent (1 ml) were incubated together at 100 °C for 16 min and transferred to another waterbath at 20 °C for 20 min. Diluting solution (5 ml) was added and the absorbance measured at 540 nm. A standard curve for glycine was linear over the range 0–10 mg l⁻¹.

(viii) *Nitrate*. Nitrate was estimated in culture filtrate by a method based on that of Nicholas & Nason (1957). Filtered fermentation broth was diluted 500-fold and 0.5 ml was mixed with 0.5 ml 2 M-NaOH in a 5 ml conical flask. Powdered zinc (25 mg) was added and the flask shaken continuously. After 4, 6 and 8 min a 0.2 ml sample was removed and centrifuged (MSE Centaur microcentrifuge) for 30 s to pellet the zinc and stop the reduction. Then 0.1 ml supernatant was taken from each of the three time-course samples and mixed with 0.9 ml distilled H₂O, 0.5 ml 1% sulphanilamide in 3 M-HCl, and 0.5 ml 0.02% *N*-(1-naphthyl)ethylenediamine hydrochloride in H₂O. After 10 min the absorbance was read at 540 nm. From each group of these time-course samples the value of maximum absorbance was taken as indicating maximum generation of nitrite.

(ix) *Paxilline*. A sample of spores was obtained from the fermenter containing CaCl₂, at 48 h. Five litres of culture suspension was poured into five 1 l measuring cylinders. After several minutes the pelleted mycelium had largely sedimented, leaving a supernatant suspension of spores which was decanted and centrifuged to form a spore pellet. The pellets were resuspended in a little distilled water and freeze-dried.

Spores prepared as above, and other samples of freeze-dried *P. paxilli*, were treated with acetone for 24 h. The solvent was filtered off and the cells were re-extracted in acetone for a further 24 h. Combined acetone extracts were taken to dryness under reduced pressure.

Acetone extracts were dissolved in an appropriate volume of CHCl₃ and 20 µl injected into an HPLC system using a Waters Z-module containing a µ Bondapak-NH₂ cartridge (8 mm × 10 cm). The mobile phase was dichloromethane/isopropanol (100:1, v/v) pumped at 4 ml min⁻¹. Spectrophotometric detection (281 nm) linked to a chart recorder facilitated quantitation of injected paxilline over the range 0–2.5 µg.

Preparative isolation of paxilline from a 60 l fermentation. At the end of the 60 l fermentation without CaCl₂, the culture (40 l), together with fermenter washings, was filtered through Whatman no. 50 paper to retain the cells. The filter cake was treated with acetone (10 l) for 65 h, filtered and the mycelium re-extracted in acetone (10 l) for 48 h. Combined acetone extracts were taken to dryness in a rotary evaporator and residual water was removed by freeze-drying. The acetone extract was dissolved in chloroform (200 ml), and silica gel (100 g, Kieselgel 60, 230–400 mesh, Merck) was added. The solvent was evaporated and the remaining brown powder applied to the top of a silica gel column (1 kg) prepared as a slurry using 4 l chloroform/acetone (15:1, v/v) and settled for 15 h in a sintered column (8 × 82 cm). The same chloroform/acetone solvent was used for elution under gravity until brown solute components had reached the bottom and 24 samples of 300 ml had been collected. Each fraction was examined by TLC (Camlab polygram SIL G/UV254) in chloroform/acetone (15:1). Spraying with Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in concentrated HCl) followed by heating with a hairdryer for 1 min revealed paxilline by its colour, yellow becoming green. Complementary indication of paxilline (Cole *et al.*, 1974) used toluene/ethyl acetate/formic acid (5:4:1, by vol.) as developing solvent and visualization (*R_F* ~0.75) by spraying with 50% ethanolic H₂SO₄ and heating at 100 °C for 5 min.

Paxilline eluted from the silica gel column in fractions 11–21 inclusive. The solutes from these were combined and resolved by flash chromatography (Still *et al.*, 1978) in a 5 × 29 cm column, using 75 g silica and 400 ml chloroform/acetone (15:1), which was also used for column elution. A series of 35 ml samples was collected and TLC showed that paxilline appeared first in fraction 6, though apparently purest in fractions 13–31, from which paxilline was crystallized (1.9 g).

RESULTS

Shaken flask fermentations

In exploratory 500 ml shaken flask cultures the courses of biomass accumulation and paxilline yields were as represented in Fig. 1. The rate of paxilline biosynthesis declined 2–3 d after maximum biomass accumulation and paxilline then reached a concentration of ~1.6% (w/w), which was subsequently sustained. This demonstration, for the first time, of submerged culture production of paxilline in significant yield justified extension of the study to stirred fermenters. Preliminary evidence of an inhibitory effect of CaCl₂ on paxilline biosynthesis (Fig. 1) pointed to a need to include study of this aspect also in stirred fermenters.

60 l fermentation in CDYE broth

Biomass accumulation was complete within 36 h of inoculating the fermenter (Fig. 2). The mycelium was in the form of branched septate hyphae, some of which were small fragments; others were organized into small granular pellets. No spores were produced and no branching characteristic of the differentiation of penicilli was evident.

Fig. 1. Progress of 500 ml shaken flask fermentation of *P. paxilli* in CDYE broth. ●, Biomass; ○, paxilline yield. The relative biomass (●) and paxilline yield (○) in a flask supplemented with CaCl_2 are also shown.

Fig. 2. Progress of stirred 601 fermentation of *P. paxilli* in CDYE broth. ●, Biomass; ○, paxilline yield; ■, broth pH; □, broth phosphate.

The uptake of phosphate from the medium (Fig. 2) was concomitant with growth during the first 36 h, but was not complete and therefore growth had not been limited at this stage by the availability of this nutrient. Nitrogen sources, batched mainly in the form of nitrate but also including a range of amino acids, provided by the yeast extract, were detected only in low concentration as growth ceased (Fig. 3). In the case of α -amino nitrogen this may have represented amino acids and peptides that were not utilizable. The assay of nitrate, while clearly demonstrating considerable uptake during the 36 h period of biomass accumulation, was insufficiently sensitive to be reliable in determining small concentrations in culture broth.

Fig. 3

Fig. 3. Progress of stirred 60 l fermentation of *P. paxilli* in CDYE broth. ●, Biomass; ▼, broth nitrate; ▽, broth α -amino nitrogen.

Fig. 4

Fig. 4. Progress of stirred 60 l fermentation of *P. paxilli* in CDYE broth. ●, Biomass; ○, paxilline yield; ■, broth total sugars; □, broth glucose.

However, it was concluded that replicatory growth was probably limited by exhaustion of utilizable nitrogen sources. Approximately half of the carbon source, batched as sucrose, was hydrolysed during sterilization so that approximately 0.75 g glucose was detected in 100 ml medium in the post-sterilization sample (Fig. 4), and a corresponding amount of fructose would also have been generated. Glucose had disappeared within 36 h when total reducing sugars, as measured after hydrolysis, were at about 20% of the amount batched and were probably all in the form of fructose. This had been, in effect, exhausted by 72 h but seems to have been sufficient to contribute carbon skeletons for the secondary metabolite accumulated after cessation of growth.

Significant paxilline biosynthesis commenced while biomass was still accumulating, but occurred mostly after growth was complete, reaching a maximum value equivalent to 1.5% of the dry weight of cells by 6 d (Fig. 2). A small amount of paxilline was detected in the mycelial inoculum, some cells of which had been already 1 or 2 d in the seed stages of the inoculum development. The measured decrease in paxilline content of mycelium during the first 12 h in the fermenter is consistent with mycelial proliferation without any biosynthesis of paxilline in the newly formed hyphae.

An initial decline in pH value (Fig. 2) may reflect some organic acid production in the presence of excess sugar. Thereafter pH increased to neutrality and became progressively alkaline after 4 d, approximately concomitant with a decrease in biomass and possibly reflecting some hyphal autolysis.

60 l fermentation in CDYE broth supplemented with 2% CaCl₂

The fermenter batched with additional CaCl₂ supported different growth dynamics in which asexual sporulation was a prominent feature.

The seed mycelium inoculated into the fermenter was, as usual, in the form of long, infrequently branched hyphae. By 6 h the incidence of branching had greatly increased and by

Fig. 5. Progress of stirred 60 l fermentation of *P. paxilli* in CDYE broth supplemented with CaCl_2 . ●, Biomass; ○, paxilline yield; △, sporulation; ▼, broth total sugars; □, broth phosphate (the graph has been extrapolated back to the batched concentration on account of the unreliability of assays during the first day); ■, broth pH.

12 h simple penicillus differentiation was evident. At 18 h many sporophores were seen and at 24 h they bore chains of conidia; some spores were already free in the broth. At this stage the culture became grey-green, the green becoming more intense over the next 24 h. The broth, yellow up to 24 h, became pink by 36 h and red by 48 h. A finely divided precipitate of calcium phosphate, which was evident in the fermenter broth before and immediately after inoculation, gradually became associated with the fungal hyphae but by 48 h had disappeared. Some of the mycelium became aggregated in small pellets which became more obvious as free hyphal fragments autolysed after 6 d.

Biomass achieved a higher value, presumably including a significant amount of the added Ca^{2+} , and it accumulated over a longer period (60 h) (Fig. 5) than in the fermentation without CaCl_2 (36 h). The rate of uptake of reducing sugars was correspondingly decreased (Fig. 6) and the availability of glucose similarly protracted (Fig. 7) but the diauxic utilization of glucose and fructose was clearly evident. The α -amino nitrogen and pH profiles showed a similar pattern but phosphate was more completely taken up from the medium after growth had ceased.

The prominent feature of sporulation, which occurred during the median part of the trophophase, preceded biosynthesis of paxilline. Paxilline biosynthesis occurred only after glucose had been exhausted from the medium, accentuated in Fig. 7 by plotting both the glucose and paxilline dynamics on larger scales. Nevertheless, paxilline biosynthesis amounted to only about 3% of that in the fermentation without Ca^{2+} . Two other 60 l fermentations, similarly supplemented with Ca^{2+} , showed the same trend, giving maximum yields of 0.5 and 4% of that in the supplemented fermentation, reached at 60 h and 72 h, respectively.

Surprisingly, the spore fraction (61 mg), separated from 5 l of culture (60 g total biomass) at the 48 h stage, contained 50 μg paxilline. The spores were therefore about 50 times richer (w/w) in paxilline than was the total biomass, of which they were a part, at that time. Since the proportion of the paxilline in the inoculum carry-over attributed to the 5 l sample, assuming no catabolism, is ~ 3 mg, the ~ 1 mg paxilline measured in the 5 l sample implies that the 50 μg measured in freshly produced (<30 h) spores is at least most, if not all, of the paxilline synthesized in the fermentation during the first 48 h.

Fig. 6

Fig. 6. Progress of stirred 60 l fermentation of *P. paxilli* in CDYE broth supplemented with CaCl_2 . ●, Broth total sugars; ○, broth free reducing sugars; ■, broth sucrose; □, broth glucose.

Fig. 7

Fig. 7. Progress of stirred 60 l fermentation of *P. paxilli* in CDYE broth supplemented with CaCl_2 . ●, Biomass; ○, paxilline yield; ▽, broth α -amino nitrogen; ▼, broth nitrate; □, broth glucose (scale exaggerated to emphasize temporal relationship with the course of paxilline production).

A shaken flask fermentation of two 1 l amounts of CaCl_2 -supplemented culture in 4 l conical flasks inoculated with the same batch of inoculum as the complementary fermenter yielded cells containing $3.8 \text{ mg paxilline g}^{-1}$. This was only about one-quarter of the paxilline yield in the fermenter and may have been a reflection of differences in aeration and agitation in the two types of vessel.

An experiment in 500 ml conical flasks, on the effect of delayed addition of Ca^{2+} , showed that, whether added at the beginning of the fermentation or added 8 or 15 h later, the fungus yielded $2\text{--}3 \times 10^8$ spores ml^{-1} and $\sim 250 \mu\text{g paxilline (g biomass)}^{-1}$. Ca^{2+} added at 24 h produced 8×10^7 spores ml^{-1} and $750 \mu\text{g paxilline g}^{-1}$. Mycelium in flasks to which Ca^{2+} was not added did not sporulate and yielded $3.9 \text{ mg paxilline g}^{-1}$.

DISCUSSION

Comparison of the pattern of growth expressed in submerged fermentations with and without a CaCl_2 supplement shows a slower rate of maximum biomass accumulation in the presence of Ca^{2+} . The first dry weight values recorded for the CaCl_2 -supplemented fermentation were biomass contaminated with a particulate suspension of phosphate, but by 36 h this contamination was negligible. The apparent lag phase, lasting about 6 h, is therefore accounted for by this artefact. However, the maximum biomass value achieved was much greater in the presence of Ca^{2+} (140 mM). This must be attributed to uptake of Ca^{2+} , together with any corresponding adjustment in the intracellular concentration of other ionic species, since Pitt &

Poole (1981) showed in a strain of *P. notatum* [= *P. griseoroseum* (Pitt, 1979)] that the rapid uptake of Ca^{2+} added (10 mM) to submerged cultures to give a mycelial concentration of $12 \mu\text{mol (g dry wt)}^{-1}$ was matched by a corresponding molar efflux of Na^+ . Mycelial Na^+ concentrations decreased from the normal value of approximately $35 \mu\text{mol (g dry wt)}^{-1}$ to about $25 \mu\text{mol (g dry wt)}^{-1}$, thus effecting a nett gain in the biomass equivalent to the difference between the relative atomic masses of Ca and Na. Intracellular Mg^{2+} and K^+ were not affected. Furthermore, the associated nett increase in cations may require additional uptake of anions.

The losses of biomass (per unit volume of culture) after the trophophase are probably due to respiration of assimilated carbohydrate, hyphal autolysis and/or biomass deposition on fermenter walls as the working volume decreased with sampling and evaporation. The different morphologies in the sporing and filamentous cultures will have caused variation in the relative contributions of these three factors.

Discussion of the present fermentations in relation to the series of studies by D. Pitt and co-workers on conidiation in submerged fermentations of *P. griseoroseum* and *P. cyclopium* seems appropriate. In spite of the approximately 15-fold greater amount of Ca^{2+} added in the present studies with *P. paxilli* and the use of a two-stage fermentation process involving a more nutritious (although undefined) medium including yeast extract there are many features in common; for example, temperature and aeration conditions were similar to those used by Pitt & Poole (1981). The present use of a relatively large addition of CaCl_2 was simply repetition of the method found advantageous in effecting efficient submerged sporulation and associated maximum production of the characteristic tremorgenic mycotoxins of *P. simplicissimum* (Day *et al.*, 1980), *P. raistrickii* (Mantle & Wertheim, 1982) and *P. janczewskii* (Mantle *et al.*, 1984). Pitt & Poole (1981) and Ugalde & Pitt (1983) found that calcium-induced conidiation was associated with a diminution in biomass accumulation, particularly in *P. cyclopium*. In the present studies a similar general effect on growth rate was evident but the difference was eventually obscured and then reversed by the overall greater yield of biomass in the presence of Ca^{2+} .

The replicatory component of biomass reflects sugar metabolism. Thus, since Pitt *et al.* (1983) concluded that the mode of sucrose utilization was similar whether or not calcium-induced conidiation occurred, the present findings of the same rapid inversion of sucrose and a loosely diauxic utilization of the released glucose and fructose is indicative, in each case, of patterns of general metabolic activity in common. The slower uptake of monosaccharides is clearly evident in the sporing fermentation, as is nitrogen source uptake. The two fermentations differed somewhat in the disaccharide/monosaccharide composition at inoculation. In the absence of CaCl_2 there was approximately 50% hydrolysis of the batched sucrose during sterilization. With CaCl_2 less than 20% hydrolysis occurred, but thereafter invertase transformed all remaining sucrose within the first 24 h of fermentation. Pitt & Poole (1981) implied that invertase was not a constitutive enzyme and that it disappeared from culture filtrates after sucrose inversion was complete. Thus in the present fermentation with CaCl_2 the sudden temporary rise in the concentration of α -amino nitrogen associated with the disappearance of sucrose could reflect the products of autolysis of the considerable amount of induced invertase necessary to have hydrolysed more than 1 kg sucrose in the fermenter.

Gluconate, which Pitt *et al.* (1983) found accumulating in *P. griseoroseum* grown on glucose without Ca^{2+} , but which was negligible in calcium-induced sporulation, was probably not a significant metabolite in the present *P. paxilli* fermentations. The small decrease in pH value, occurring in both *P. paxilli* fermentations during the first 12 h, might be attributable to organic acid production before the maximum respiratory demands were imposed on dissolved oxygen. An instance in which organic acid, probably gluconate, is obviously produced is evident during calcium-induced sporulation by *P. simplicissimum* in the trophophase before it then accumulates verruculogen (Day & Mantle, 1985); a decrease of 1.5 units occurred during the first 48 h, coincident with sugar uptake.

The present studies have demonstrated submerged culture production of paxilline for the first time. The maximum yield of paxilline in the fermenter without CaCl_2 compares very favourably with that achieved for penitrems in similar vessels (Mantle *et al.*, 1984). Paxilline was produced more quickly and in greater amount than in a 4 l shaken flask, although in a similar yield and

with dynamics similar to that in 500 ml shaken flasks. Thus no particular constraint on scale-up was apparent. However, the amount of paxilline measured is only the mathematical product of anabolic and catabolic processes; the extent to which paxilline is turned over during the idiophase is unknown. Indeed, since paxilline is only known to be produced by one isolate of *Penicillium*, the question arises as to whether, in other fungi, it is actually an intermediate in the biosynthesis of some other indole-terpenoid metabolite which fails to accumulate in this organism owing to a defective enzymic step beyond paxilline. In this case typical regulatory mechanisms might not operate properly. Paxilline may even form part of a metabolic grid of indole-terpenoid secondary metabolites such as has been shown in *P. griseofulvum* for a group of tetraketide metabolites (Bu'Lock, 1975).

The poor yield of paxilline when *P. paxilli* is induced to sporulate by CaCl_2 may be due either to diminished biosynthesis or to increased metabolic transformation of paxilline to another product. If diminished biosynthesis is responsible, the finding of most of the paxilline in the spores not only indicates that it is not just the product of enzymes already synthesized in the filamentous inoculum. It also suggests that it is not the actual process of sporulation which inhibits paxilline biosynthesis but rather some changes in the hyphae consequent on their differentiation into spore-forming structures. If increased metabolic transformation is occurring it may be an oxidative process. The expected higher dissolved oxygen content of the broth, resulting from slower sugar uptake and the pelleted sporulating culture's rheological properties being more favourable to oxygen transfer, may promote oxidative catabolism of paxilline. However, there were no qualitative differences between chromatograms of extracts of mycelia grown with or without CaCl_2 .

In both vegetative and sporulating fermentations the first appearance of paxilline in the cells coincides closely with completion of glucose uptake, whether this occurs early in the second day of solely vegetative fermentation or at the end of the second day in a sporulating culture. A simple conclusion is that regulation of paxilline biosynthesis, at least with respect to the last step, involves release from carbon catabolite repression or inhibition, a classical example of which is the penicillin fermentation.

In the present studies the partial precipitation of calcium phosphate after sterilization and its subsequent gradual dissolution would provide a continuous supply of Ca^{2+} during the period of sporophore differentiation. It is remarkable that, within only a few hours of formation, spores contained paxilline, while no nett increase in paxilline content of biomass was yet apparent. This apparent incongruity could be rationalized by regarding spores as no different from the hyphae subtending them, in terms of secondary biosynthetic potential. It is possible that Ca^{2+} taken up into the mycelium is not distributed evenly to spores produced several hours later and that, therefore, their secondary biosynthetic potential is less affected than is that of hyphae.

It is notable also that paxilline seems only to be formed in spores after they have been released into suspension, i.e. after 48 h into the batch fermentation. Ugalde & Pitt (1986) showed that as little as 30 s exposure of *P. cyclopium* to 9 mM- Ca^{2+} stimulated subsequent differentiation of conidiophores as efficiently as if the fungus was left in the Ca^{2+} environment. The Ca^{2+} uptake was biphasic, initial binding to hyphae being followed by active transport of the cation to the subcellular sites described earlier (Ugalde & Pitt, 1984). However, the study did not extend to include redistribution into spores. Thus these findings may allow discrimination between the effect of calcium on sporulation and its adverse effect on paxilline biosynthesis. The extent to which bound calcium is subsequently absorbed, or absorbed calcium located on membranes or in organelles is transported to new domains in new cells, is not clear. Whether or not spores show the same dynamics as vegetative hyphae with respect to calcium binding and uptake is also unknown.

Paxilline was the only prominent secondary metabolite extracted from the mycelium with organic solvents. However, at about the 24 h stage the sporulating mycelia developed a green pigment and at about 36 h a red water-soluble pigment was evident in the broth. This implied anthraquinone biosynthesis suggests some analogy with the gibberellin fermentation (Bu'Lock *et al.*, 1974), in which differential biosynthetic demand on acetate is displayed, a polyketide metabolite (bikaverin) being formed first and the terpenoid gibberellin somewhat later.

The present findings with *P. paxilli* add a further permutation of apparently contradictory instances concerning the importance of sporulation in submerged culture for the production of tremorgenic mycotoxins. The fact that these compounds have potent tremorgenic activity in animals is probably irrelevant; they simply represent a fairly well explored group of secondary metabolites. Thus *P. simplicissimum* produces the diketopiperazine verruculogen in submerged culture only if induced to sporulate by CaCl_2 (Day *et al.*, 1980), but persistently vegetative mycelium of *P. raistrickii* will produce the same secondary metabolite, in at least as good yield, in the presence of 2% CaCl_2 (Mantle & Wertheim, 1982). Within the indole-terpenoid group of tremorgens, penitrems are produced in high yield in submerged culture only by a strain of *P. janczewskii* given CaCl_2 (Mantle *et al.*, 1984); other penicillia producing penitrems in stationary liquid culture fail to elaborate more than a trace of penitrems. Conversely, *P. paxilli* producing paxilline, the structure of which is virtually identical to a large part of the penitrem molecule, does so in submerged culture without sporulating and production is severely impaired if the fungus is forced to sporulate.

Exploring a hypothesis that secondary metabolites might act as morphogens in conidiogenesis by *P. urticae* [= *P. griseofulvum* (Pitt, 1979)], Sekiguchi & Gaucher (1977) studied the fungus in submerged culture during sporulation induced by Ca^{2+} . Sporulation occurred during the trophophase and the polyketide patulin was produced only after sporulation, negating a role in differentiation. However, Sekiguchi & Gaucher (1977) also recorded a 75% reduction in the yield of patulin when the fungus was forced to sporulate by addition of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1%, w/v) to the medium at batching. This is therefore qualitatively similar to the present findings with *P. paxilli*.

Thus, while Bu'Lock's (1975) perceived link between secondary metabolism and differentiation may well be valid in certain fungi, the process of sporulation in penicillia, which involves the most complex asexual differentiation that they can achieve, does not seem to be very closely connected with the biosynthesis of secondary metabolites. Nevertheless, calcium-induced conidiogenesis in submerged culture does seem to have consequences for the operation of secondary biosynthetic pathways but it is not possible to recognize a consistent pattern of expression. It should be noted that Bu'Lock's conclusion assumed that differentiation is closely associated with growth limitation by a nutrient becoming scarce. Submerged calcium-induced sporulation by penicillia is very much a mid-trophophase event in its fullest expression of large numbers of spores released into suspension. Moreover, the first evidence of the divergent morphology which is a prelude to sporulation is seen only a few hours into a batch fermentation, when all nutrients are abundant. Thus the dynamics of submerged culture sporulation in penicillia may place these rather prominent differentiations outside the scope of Bu'Lock's conclusion.

In any case, the penicillia exhibit such a diverse array of secondary metabolites that further study of the neglected area of biosynthetic regulation in the context of growth dynamics will assist in revealing the extent of commonality of underlying, and perhaps exploitable, principles.

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